UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of TSLP pathway in the development of B-Cell Acute Lymphoblastic Leukemia

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Biology

by

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The dissertation of Ifat Geron is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Dedication

To my beloved daughter Thalia and to my dedicated and supportive parents.

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ABSTRACT OF THE DISSERTATION

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by

Ifat Geron

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Professor Catriona HM Jamieson, Chair

Professor Cornelis Murre, Co-Chair

B-Cell precursor acute lymphoblastic leukemia (B-ALL) is the most common malignancy in children. Recently, we and others described a new subtype of the disease, affecting 60% of children with Down Syndrome (DS) and about 10% of patients with sporadic ALL, in which chromosomal rearrangements result in over-expression of the cytokine receptor-like factor 2 (CRLF2) receptor[1-4]. This over-expression is often accompanied by mutations in additional proteins in the CRLF2 pathway, such as JAK2, a downstream effector

in the pathway[5-9], and IL7RA, the second subunit in the TSLP receptor[10]. Based on mutation analyses, aberrant CRLF2 expression was thought to play a causal role in the development of B-ALL. While some data obtained in mouse systems support this assertion[2,3,6], no studies have been performed in human cells to ascertain whether or not CRLF2 contributes to B-ALL pathogenesis. Due to the prominent difference between mouse and human B lymphoid development, particularly in the TSLP/IL7 pathways, it is important to study the contribution of activation of the TSLP pathway to the development of B-ALL in human cells. In the research described here, I hypothesized that **aberrant expression of CRLF2 in cooperation with secondary mutations in the TSLP pathways contributes to B-ALL initiation.**

This hypothesis was tested primarily by utilizing cord-blood (CB) hematopoietic-progenitors transduced with a set of lentiviral vectors carrying CRLF2 alone or in combination with JAK2 or IL7RA mutations. Outcome of forced TSLP pathway activation was cell context specific.

Expression of CRLF2 in CB hematopoietic-progenitors from a ubiquitous promoter resulted in skewed differentiation towards the myeloid lineage while transcription of the same genes from a B-cell-specific promoter accelerated B-lymphoid differentiation *in vitro*, underscoring the importance of expressing the genes of interest in the right cellular context for B-ALL pathogenesis.

Transduced CB cells were transplanted in NOD/LtSz-*scid IL2Rγ*^{null} (NSG) mice, which are known to support human B-Cell differentiation. Transplanted cells expressing CRLF2 with mutant IL7RA exhibited population

expansion, enhanced B-cell differentiation, and a significant block in differentiation at the pro-pre B-cell stage, resembling the stage of differentiation of leukemic blast cells.

Chapter 1 Introduction

1.1 Introduction

While acute lymphoblastic leukemia (ALL) is the most common childhood malignancy, it also affects adults. Currently, more than 80% of children with ALL are cured by combination chemotherapy protocols[11]. However, up to 20 percent of children and fifty percent of adults relapse. It is important to study this poor-prognosis group of patients[12]. Like many other malignancies, B-ALL develops following the accumulation of several somatic mutations, leading to the formation of malignant clones. Pediatric B-ALL is highly heterogeneous and, while several chromosomal translocations (e.g., ETV6-RUNX1, TCF3-PBX1, BCR-ABL1, and rearrangement of MLL) are known to be primary acquired aberrations, approximately one third of patients lack chromosomal alterations[13]. The origin of the leukemia-initiating cell in B-ALL is unclear but it is believed that the primary event occurs in a committed lymphoid progenitor[14,15]. Understanding the underlying genetic background of the disease is crucial to the development of more effective targeted therapies that induce less systemic toxicity.

1.1.1 Normal hematopoiesis

To support maintenance of the hematopoietic system, mature differentiated cells must constantly be replenished. The whole array of hematopoietic cells originates from one precursor, namely the hematopoietic stem cell. A process of symmetric and asymmetric division[16,17] allows for maintenance of a stem cell pool as well as commitment of some daughter cells to more differentiated progenitor cells and, thereafter, to fully differentiated cells with reduced proliferative capacity. These processes are tightly regulated during normal hematopoiesis. The ability to self- renew, or in other words to divide without differentiating, is regulated by several pathways, including the WNT[18]and NOTCH[19] pathways, and is lost over the course of differentiation as transcription factors orchestrate the differentiation of lineage-committed cells.

Differentiation of hematopoietic cells was traditionally postulated to involve a two-fork differentiation tree with a myeloid lineage branch and a lymphoid lineage branch[20,21], as illustrated in Figure 1–1 A. However a vast body of evidence implies that there is much greater complexity to differentiation (reviewed in[22]) and plasticity, in terms of variance in cell commitment to one lineage or the other[23-26] under certain conditions and differences in hematopoiesis based on developmental stage and organismal age (reviewed in [27-29]). This was recently refined in the research by the Dick group that screened the differentiation potential of single cells from fetal liver, cord blood and adults and reached the conclusion that the stem cell/progenitor hierarchy changes throughout life. In fetal liver many stem and progenitor cell types are multipotent

and in the adult, the "two-tier" model suggests that multipotent stem cells are followed by uni-lineage progenitors without multipotent progenitors (Figure 1–1B). While these findings will require further in vivo functional validation, they underscore the importance of cell type and context-specific transcriptional regulation of hematopoietic stem and progenitor cell fate commitment. Transcription factors complexes and the balance between them govern differentiation along the course of hematopoiesis[30]. Early hematopoietic stem cells as well as hematopoietic progenitors express baseline quantities of multi-lineage factors that enables "lineage priming" [31]. During differentiation, the signature of lineage-specific transcription factors determines cells fate. The expression of transcription factors is regulated by non-cell autonomous cues via cell surface receptors and internal regulators, which include protein networks and regulatory RNAs[32,33] and by epigenetic alterations ([34]and reviewed in [35,36]).

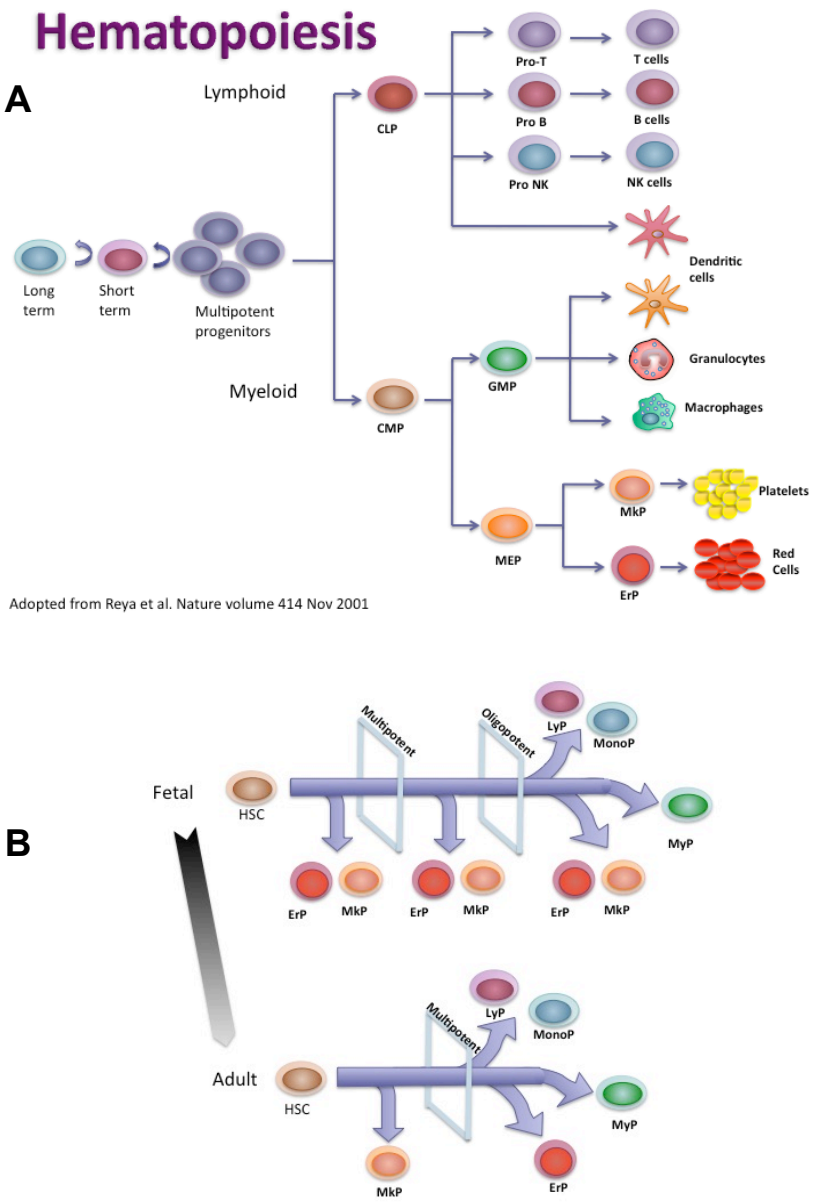

Figure 1–1 Normal hematopoiesis.
Schemes of normal hematopoiesis. A) "Classical" Hematopoiesis model describing a fork differentiation pattern. In each fork the cells further commit to a specific lineage (adopted from[37]). B) Changing differentiation patterns across ontogeny. In fetal liver the hematopoietic differentiation flows through stages of multipotent and oligopotent progenitors from which all lineages can emerge and adult differentiation in which multipotent cells commit to unilineage progenitors (adopted from[38]. Abbreviations: MPP=multipotent progenitor; CLP=common lymphoid progenitor; CMP=common myeloid progenitor; MEP=megakaryocyte erythrocyte progenitor; GMP=granulocyte-macrophage progenitor; Pro-T=T-cell progenitor; Pro-B=B-cell progenitor; Pro-NK=NK-cell progenitor; LyP=Lymphoid progenitor; MonoP=Monocyte progenitor; MyP=Myeloid progenitor;

1.1.2 Early B-cell differentiation

B-cells are an important part of the adaptive humoral immune system. Their most obvious roles are production of pathogen-specific antibodies at the time of disease (a role that is served by plasma cells (PC)) and maintaining in a memory B-cell pool that may respond to previously encountered pathogens for extended periods. The site of B-cell emergence through lymphopoiesis is dynamic throughout embryogenesis. In early gestation (7-8 weeks), B-cell lymphopoiesis occurs in the liver, while by 18-20 weeks, B-cell development occurs mainly in bone marrow, though B-cell progenitors can be found in the bone marrow, liver, spleen, lungs, and kidneys[39]. In adults, B-cells emerge strictly in the bone marrow from the B-cell progenitor population. The early lymphoid progenitor cascade prior to differentiation to pre-pro B-cells is controversial. Several progenitor hierarchies have been suggested but due to the plasticity of progenitor cells and terminal differentiation assays (as summarized in[40]), the existence of a conclusive order with strict progenitor potential for the differentiation of lymphoid progenitors to B-cells, T cells, natural killer (NK) cells, and dendritic cells has yet to fully elucidated. However, the expression of terminal deoxynucleotidyl transferase (TdT) and recombination activating genes (Rag1 and Rag2) that are required for immunoglobulin receptor editing is an accepted marker for committed early lymphoid progenitors (ELPs)[41,42]. Early B-cell differentiation is marked by initiation of chromosomal changes, following expression of B-cell receptor (BCR), which defines the

specificity of a given B-cell to a particular antigen. The receptor is composed of two similar cell-specific heavy chains, two similar cell-specific light chains, and an Igα/Igβ signal transducing heterodimer (CD79). To achieve receptor diversity, a complex process of genetic rearrangements in the B-cell receptor locus and sequence manipulations occurs during pre-pro B-cell differentiation. The recombination events occur in an orderly fashion and can be briefly summarized as follows: (1) joining of a D_H (diversity) segment to a J_H (joining) segment and subsequent addition of a V_H (variable) segment to form a heavy chain; (2) joining a V_L segment to a J_L segment to form a light chain, during which successful rearrangement of a κ light chain will prevent rearrangement in a λ light chain[43,44]. Each segment is randomly chosen from several available segments in the genome. Upon successful heavy chain rearrangement, the μ chain associates with a surrogate light chain composed of a λ5 chain and a VpreB chain, to form the pre-B-cell receptor (pre-BCR), which marks the transition from the pro-B to the pre-B stage. Signals from the pre-BCR prevent the rearrangement of the μ chain and allow for successive light chain rearrangement in the pre-B-cell. Additionally, pre-BCR signals through Bruton's tyrosine kinase (BTK) and B-cell linker protein (BLNK) are essential for continued normal differentiation (in human but not in mouse, as reviewed in[45]). Finally, after successive light chain rearrangements, a functional BCR complex is presented on the immature B-cell. Immature cells with autoreactive BCR are subject to secondary rearrangements or deletion by anergy or apoptosis. The receptor is responsible for proliferation cues and activation of the cell, as well as

for survival and mobilization of the antigen for presentation to T-cells. The expression of IgM in the immature BCR changes the expression profile and causes egress of the cells into circulation and into the spleen, where they differentiate into naive, follicular, or marginal zone (MZ) B-cells. Furthermore, cells circulate through the bloodstream to tonsils, Peyer's patches, and mucosal tissues, and form germinal centers (GCs) in spleen and lymph nodes. In GCs, B-cells undergo somatic hypermutation, a process that increases antibody affinity to antigens and requires activation-induced cytidine deaminase (AID) and enzymes of the non-homologous end-joining complex, as well as class switches to IgG, IgA, or IgE, and production of long-lived memory B-cells and plasma cells. The changing balance in transcription factors and signaling factors regulates differentiation along the course of the B-cell lineage. A summary of key transcriptional regulators and their roles in early B-cell differentiation is provided below.

PU.1 is important in early myelopoiesis. It is necessary for the emergence of early B-cell progenitor as well of myeloid progenitors since the expression of several cytokine receptors including granulocyte colony-stimulating factor-receptor (G-CSF-R) macro- phage colony-stimulating factor (M-CSF) receptor and interleukin 7 receptor A (IL7RA) is controlled by PU.1[46]. Additionally the expression of the B-cell-specific transcription factor EBF1 is directly regulated by PU.1. Examination of PU.1−/− fetal liver progenitors revealed the role of PU.1 in the development of Flk2/Flt3+, IL-7R+ lymphoid progenitors and in the expression of CD45R/B220[47].

IKAROS, a Kruppel zink finger family member, is required in early lymphopoiesis to direct nucleosome remodeling Deacetilase complex (NuRD) and Mi2β and targets them to lymphoid lineage genes [48,49]. In the switch from pro-to pre-B-cells where IKAROS expression together with the withdrawal of IL7 leads to vast transcriptional pattern alterations thus promoting the cascade of cell cycle exit, pre-BCR down regulation induction of Rag1,2 light chain rearrangements and B-cell differentiation [50]. IKAROS plays a role in the transition from stroma-dependence to aggression from the BM by repressing focal adhesion molecules (FAK) and integrin deregulation[51].

The E2A gene products E47 and E12 are basic helix-loop-helix DNA-binding proteins with specificity for E-box[52] that are essential to B-cell initiation. Although the products of the E2A gene are ubiquitously expressed in tissues, unlike other tissues where the E47 heterodimerizes with a tissue-specific HLH protein, in B-cells, homodimers of E2A gene products activate a range of B-cell-specific genes (reviewed in [53]); E2A is required for heavy chain rearrangements[54]. It induces the expression of surrogate light chain[55] and the B-cell-specific factors early B-cell factor (EBF), and Pax5[56]. Both the E2A gene products E12 and E47 are necessary for differentiation of B-cells and the obstruction of expression in each of them will blocks B-cell differentiation[57]. However two other HLH factors, E2-2 and HEB play an important role in normal B-cell production. In fact the total level of B-cell factors seems to be important in the differentiation process[58].

EBF is a homodimer transcription factor that is expressed in the hematopoietic system solely by pro, pre and immature-B-cells. It's deletion in mice confer a pro-B differentiation block phenotype[59]. E2A genes and EBF synergize to induce expression of the pre-BCR surrogate light chain and for IgH gene rearrangement[55,60] forced expression of EBF in EBF null mouse fetal liver progenitors restores expression of Pax5 Igα Igβ and surrogate light chain[47]

Pax5 (paired box protein 5) or B-cell-specific activator protein (BSAP) is a transcription factor that can be found in the mesencephalon and the spinal cord during embryogenesis. Therefore its absence causes irregularities in the posterior midbrain. Thereafter, it is can be found primarily in the B-cell lineage ([61] and reviewed in[62]). Pax5 acts by activating chromatin at promoters and enhancers of target genes[63] or repressing target genes by recruiting Groucho family repressor factors[64]. It is required for V_H rearrangements[61,65] and for the expression of CD19, Igα, N-myc and lymphoid-enhancer-binding Factor 1 (LEF1)[66]. Another crucial role of Pax5 is the maintenance of the B-cell phenotype and prevention of differentiation to other hematopoietic lines (particularly T-cells, by blocking Notch1[67]): $Pax5^{-/-}$ pro-B-cells are devoid of B-cell commitment and exhibit high plasticity[68]. This role persists beyond early B-cell differentiation to immature B-cells[69].

In summary, IKAROS and PU.1 are essential in early lymphopoiesis. PU.1 and E2A act to initiate B-cell differentiation and initiate B-cell receptor chain rearrangement and to induce expression of B-cell-specific factor EBF. The latter collaborates with E2A genes in programmed early B-lineage

differentiation and, in turn, induces PAX5, which maintains B-cell phenotype. Another IKAROS pulse is required to allow for egression of cells from the BM.

Fine balance between transcription factors in B-cell differentiation is vital. Alterations in B-cell regulators of differentiation affect more than two-thirds of B-cell ALL cases[70]. Moreover, B-cells are prone to malignant transformations due to their B-cell receptor rearrangement and editing machinery, which induces genetic instability, as demonstrated previously by Papaemmanuil et al. who showed that ETV6-RUNX1 rearrangements are the result of RAG activity[71]

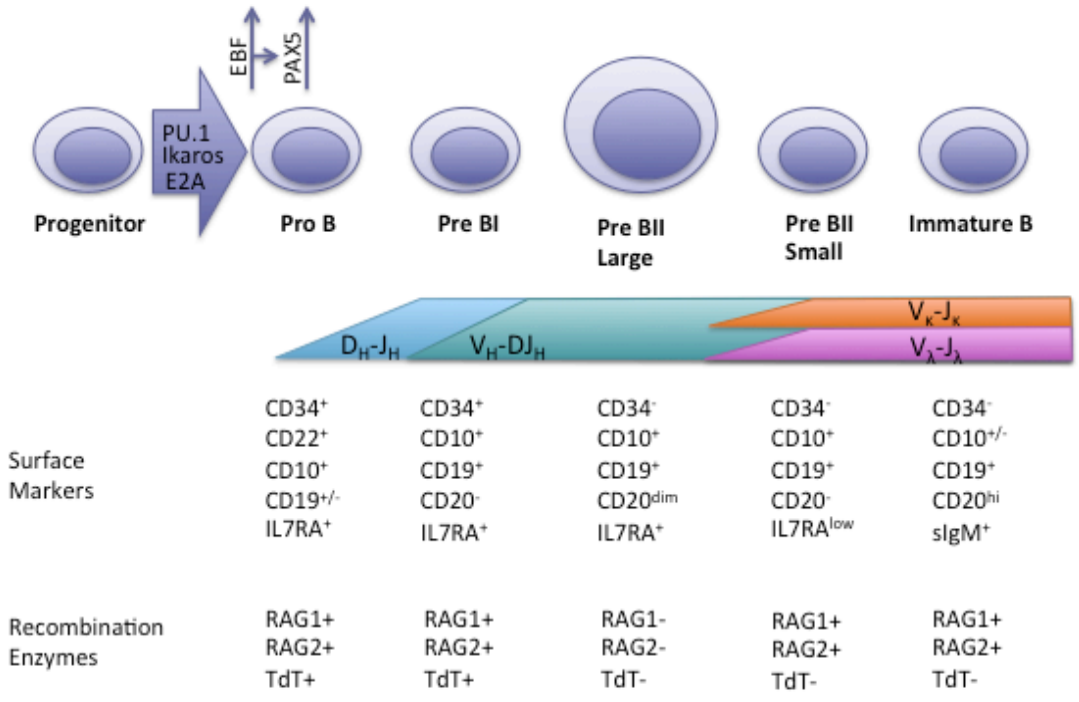

Figure 1–2 Early B-cell differentiation.
Scheme of B-cell differentiation depicting stages of B-cell receptor recombination, , surface markers and recombination enzymes in each stage. +/- indicates debated between several sources. Based on[47,72,73]

1.1.3 Acute Lymphoblastic Leukemia Pathogenesis

Acute lymphoblastic leukemia is a malignancy of progenitor lymphoid cells, predominantly of B-cell origin. The disease affects children as well as adolescents and adults. It is the leading malignancy in children and although under current treatment regimes cure rates are up to 90% (reviewed in[74]) in childhood B-ALL, better understanding of the disease is needed in order to brtter treat adults and infant malignancy as well as to reduce treatment related toxicity. According to the latest World Health Organization guidlines[75] the disease can be classified according to recurrent chromosomal translocations or aneuploidy.

The most common translocation in childhood B-ALL (15-25% of B ALL[76]) is the **ETV6-RUNX1** (t(12;21)(p13;q22) also known as TEL-AML1)[77] this re arrangement brings together ETV-6 – an ETS family transcription factor (specify), with RUNX.1 a Runt-related transcription factors (RUNXs) family member that functions in definitive hematopoiesis, hematopoietic differentiation and HSC sternness (reviewed in [78]). Unlike the wild type RUNX1, the fused protein functions as an inhibitor of transcription[79]. Furthermore the fussion product cause activation the JAK-STAT pathway by upregulating erythropoietin receptor and thus spurs survival [80] the translocation can be found in pre-birth blood cards at frequency that is much higher than the frequency of the ALL transformation in the population indicating that the development of overt leukemia is contingent on acquiring additional mutations.[81,82]

TCF3(E2A) translocations correspond to about 2-6% of childhood B-ALL incidents[76]. The common translocation is TCF3-PBX1 (t(1;19)(q23;p13) also known as E2A-PBX1) in which N-terminal transcriptional activation domain of E2A fused to the homeodomain from PBX1 that is normally not expressed in lymphocytes. Thus, in addition to disrupting one allele of E2A that is important for lymphocytes differentiation, it acts as a putative transcription factor that upregulates genes in an untimely manner and out of their cellular context [76,83]. The second and less common translocation, that affects mostly adult cases of B-ALL, is TCF3-HLF t(17;19)(q22;p13). In this translocation TCF3 amino terminal is fused with the DNA-binding region and the leucine zipper dimerization domain of HLF– a PAR family member. This fusion is sufficient for immortalization of mouse fetal liver progenitors via the activation of LMO2 and BCL-2[84].

BCR-ABL1 translocation also known as Philadelphia Chromosome (t(9;22)(q34;q11)) was originally identified in Chronic Myelogenous Leukemia (CML) and it is known to predominantly elicit this malignancy. Philadelphia Chromosome translocations are grouped according to the breakpoint in BCR and usually result in either a 210-kDa fusion protein (p210) that is generally associated with the myeloid malignancy or a 190-kDa (p190) protein mostly associated with B-ALL and an additional p230 protein that usually associated with chronic neutrophilic leukemia. The translocations results in a constitutively active ABL1(an Abelson family of non-receptor tyrosine kinases family member) capable of abruption variety of signaling pathways, resulting in proliferation,

deregulated differentiation and adhesion. BCR-ABL translocations are more prevalent in adult B-ALL (20%) than in the pediatric (~3%) disease[85].

Mixed-lineage leukemia Gene (**MLL**) located on 11q23 encodes for a DNA binding protein that up regulates his target genes due to its histone methyltransferase activity. MLL rearrangements frequently occur in utero, and require minimal additional mutations to develop overt leukemia, hence the majority of infant ALL incidents carrying MLL rearrangements. Infants with MLL rearrangements ALL have poor prognosis[86]. MLL rearranges with a vast array of genes, the most frequent rearrangements are MLL-AFF1(AF4) (t(4;11)(q21;q23)) then MLL-MLLT3(AF9), (t(9;11)(p22;q23))[87] MLL rearranged ALL exhibit a unique gene expression pattern and surface identifiers (they lack the early lymphoid marker CD10 but express myeloid markers as CD15) and therefore was suggested to be classified as a distinct leukemia[88].

In addition to major translocations aneuploidy is among the common abnormalities in B-ALL. Hyperdiploidy accounts for ~30% of B-ALL. Common recurrent duplications in chromosomes 4, 6, 10, 14, 17, 18, 21 and X lead to the accumulation of typically 51–67 chromosomes in the malignant cells. Hyperdiploidy as generally associated with good prognosis[89]. In contrast, hypodiploidy characterized with karyotype of 45 or less chromosomes, is associated with worse prognosis[90].

High resolution genomic profiling revealed that gross chromosomal abnormalities are commonly joined with small genetic lesions and micro deletions in genes that regulate cell cycle and B- cell differentiation[91].

Furthermore, recent technological advances, presented us with the ability to analyze expression profile thus opening a new prospect for characterizing and further categorize B-ALL. One such new class of B-ALL is the Philadelphia like B-ALL that exhibit similar gene expression profile as the BCR-ABL ALL (reviewed in[85]). The most prevalent event in this class is the aberrant expression of CRLF2 – a subunit of the receptor for TSLP. Our group and others reported the association of high expression of CRLF2 with B-ALL[1-4]. This abnormal expression is a consequence of either a juxtaposing CRLF2 to the IgH enhancer, or interstitial deletion in the pseudoautosomal region 1 (PAR1) of chromosome X, resulting in a P2RY8-CRLF2 fusion. The aberration is more prevalent in cases of Down syndrome ALL (~60%) but can also be found in sporadic ALL where it is mostly associated with Hispanic background.

1.1.4 TSLP pathway, its components and involvement in malignancy

1.1.4.1 TSLP pathway

In 1994 Friend et al. described the ability of thymic stromal cell line to support the differentiation of fetal liver cells into $SIgM^+$. The addition of anti IL7 to the media did not block this differentiation and as conditional media from the culture retained this ability, it was concluded that another cytokine was secreted from the cells which support the B- cell differentiation[92].

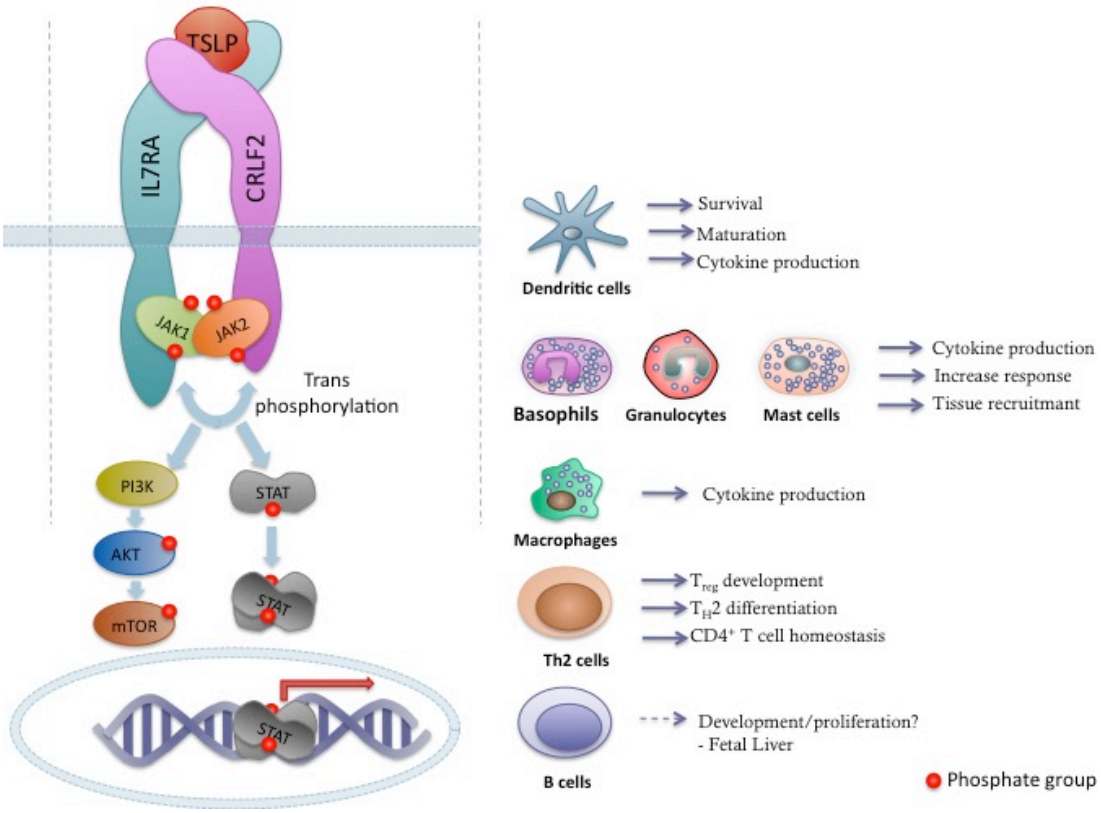

Figure 1–3 TSLP pathway.
Left: Binding of the TSLP to the receptor induces cross phosphorylation of JAK proteins and subsequent activation of JAK/STAT pathway and mTOR pathway. Right: TSLP effects on hematopoietic cells. Adopted from[93]

Thymic stromal lymphopoietin (TSLP) is a cytokine, closely related to IL-7, which is secreted by epithelial cells in the gut, skin and lungs in a response to trauma and is constantly produced in the thymus (reviewed in[94]) It was first discovered as a new growth factor, secreted by a thymic stromal cell line that can support the development of lymphocytes[92]. Nevertheless, its role in lymphoid development is unclear. TSLP is associated with inflammation and allergic reaction and is important for the development of T regulatory cells, and the differentiation to Th2 cells after interaction with stimulated dendritic cells[95]. In addition to its role in T cell differentiation, TSLP promotes T cell survival,

mostly by induction of Bcl2[96,97]. There are many contradictive evidences about the necessity of TSLP in the development of B-cells, some suggesting a differential role of TSLP in fetal verses adult B-cells[98-101].Despite the fact that TSLP was found as a lymphoid cytokine, it mainly affects dendritic cells, macrophages mast cells and basophils (reviewed in[94]). The receptor for TSLP is composed of two subunits; the first is the IL7RA that will be described in detail in the next section and the second, cytokine receptor-like factor 2 (CRLF2), is closely related to the IL-2Rγ. CRLF2 is an atypical type 1 non-tyrosine kinase cytokine receptor that is encoded on the pseudo-autosomal region (PAR) of the sex chromosomes Xp22.3 and Yp11.3. The homology between the human and the mouse form of the TSLPR is only 35%[102]. CRLF2 is lacking several of the conserved motifs found on other type 1cytokine receptors: The highly conserved WSXWS motif is replaced with WAS- like motif PSDWS. Additionally, Its intracellular domain has a proximal box1 motif that is important for its interaction with JAK2 but the distal box2 domain is either missing or replaced by a non-conserved sequence[102,103]. Binding of TSLP to the heterodimeric receptor, cause the cross phosphorylation of JAK2 bound to the CRLF2 subunit and of the IL7RA bound JAK1. This result in the activation of STAT5, Akt/mTOR signaling, and RPS6 (Figure 1–3) CRLF2-/- mice do not have any apparent lymphoid defect[104]. Yet, in mice lacking both IL2Rγ and CRLF2 have extended lymphoid defects when compared to mice lacking only IL2Rγ indicating that TSLP may have some redundancy with IL7. Similarly it was reported that IL2Rγ KO mice that lack the ability to react to IL7, presented with a transient B-cell

population at four weeks that disappeared by eight weeks while IL7RA KO mice that do not react to both IL7 and TSLP were devoid of this fetal "B1" population. The latter combined with the finding that CRLF2 is expressed on B-cell progenitors in human fetal liver and can induce their proliferation suggest a role of TSLP in fetal B-cell development[105]. Nevertheless, CRLF2 is not expressed on adult B-cells.

CRLF2 over expression in B-ALL was discovered by four different groups including ours, using different methodologies[1-4]. The aberrant expression is a result of two genomic rearrangements: internal micro-deletion in the PAR region of the sex chromosome resulting in fusion between the first non-coding exon of an upstream gene P2RY8 and CRLF2 and translocation of CRLF2 into the IgH enhancer locus on chromosome 14. The junctions of the IgH locus rearrangements were adjacent to putative V(D)J recombinase recognition signal[4]. Additionally, partly or fully conserved heptamer recombination signal sequences were found next to the deletion breakpoints in the PAR region [1] implying that both kinds of rearrangements are carried by the RAG enzymes, hence, the rearrangements occur in a lymphoid/B-progenitor. The translocations are found in high incidents in DS related B-ALL (50-60%) and in 5-10% of sporadic B-ALL, and is associated with poor prognosis[1-3,106-110]. Moreover, P2YR8-CRLF2 are commonly found (~30%) in B-ALL cases with somatic amplifications of a segment on the long arm of chromosome 21 (iAMP21)[111]. Indicating of a possible of collaboration between one or more genes within the amplified region on chromosome 21 to CRLF2 in inducing malignancy. Mouse

studies were conducted after the discovery of CRLF2 over expression and included enforced expression of CRLF2 alone or in conjugation of additional mutations in the TSLP pathway. The experiments resulted in enhanced proliferation in primary fetal liver cells or cytokine independent growth in pre leukemic mouse cells and suggested that CRLF2 expression contribute to the initiation of B-ALL[2-4]. Yet, these experiments showed that the expression of CRLF2 alone does not sufficiently activate the TSLP pathway and cannot confer transformation.

In addition to CRLF2 over expression, a point mutation in the receptor was reported in 10-15% of $CRLF2^{+}$ B-ALL [112]. CRLF2 F232C is a gain-of-function mutation in which phenylalanine adjacent to the trans-membrane domain is replaced by cysteine. It is thought that the mutation facilitates the dimerization of two subunits and that activation of down stream JAK-STAT pathway without cytokine binding[113].

The TSLP receptor transduces the signal through the binding of JAK2 to the CRLF2 subunit and JAK1 to the IL7RA subunit. Activating somatic mutations in all of these four proteins were found in B-ALL with aberrant expression of CRLF2. The most common mutation is a lymphoid-specific mutation in JAK2.

1.1.4.2 JAKs

Janus kinases (JAK1, JAK2, JAK3 and tyrosine kinase-2 (TYK2)) are a family of non-receptor tyrosine kinases and as such, they pair with non-tyrosine kinases receptors to transmit signals in the cells. The signal transduction

cascade starts with binding of the ligand, usually a cytokine hormone or growth factor, to the receptor, which leads to its oligomerization. The receptor then recruits JAK proteins that phosphorylate the receptor and undergo reciprocal phosphorylation. The phosphorylated receptor serves as loading dock to signal transducers and activators of transcription (STATs). JAK phosphorylates the bound STATs which in turn dimerize and enter the nucleus to facilitate transcription of downstream genes[114]. Hematopoiesis depends on several cytokines and growth factors that are signaling through the JAK/STAT pathways. Deletion of the JAK2 gene in mice resulted in embryonic lethality, due to failure of erythropoiesis and inability of the hematopoietic progenitors to respond to Epo, Tpo, IL-3 and GM-CSF, all essential factors for the differentiation and maintenance of hematopoiesis signaling through JAK2. However, response to IFN-γ IL-6 and G-CSF, which can also signal through JAK2 was not abolished[115]. This is probably due to JAK1 being the primary signal transducer for these cytokines as seen in the case of mice which lack the JAK1 gene[116].

In the lymphoid lineage JAK3 has an important role due to its part in mediating signaling through IL-2R common γ chain (γc), a common subunit for key lymphoid cytokine receptors, including the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15[117] . Mice lacking JAK3 were reported to have lymphoid deficiencies ranging from sever B-cell and T cell lymphopenia similar to severe combined immunodeficiency disease (SCID)[118] to block in B-cell development at the pre-B to pro-B stage and impaired T cell response to stimulation[119].

JAK2 is mostly activated in lymphocytes through IL-3 signaling during development, however other cytokine receptors such as prolactin receptor[120], IFN-γ and more relevant to the current work – CRLF2, transduce their signal in lymphocytes through JAK2.

JAK2 involvement in hematological malignancies was first noted after the discovery of Tel-JAK2 fusions in ALL and CML in which the kinase domain of the JAK2 is fused to the helix-loop-helix oligomerization domain of the Tel (ETV6) transcription factor[121,122]. In 2005, several groups reported a somatic activating mutation in JAK2, replacing valine 617 with phenylalanine (V617F) in the pseudokinase domain[123-127] This mutation was subsequently found to be the driving force for a substantial proportion of myeloproliferative neoplasms (MPNs), including polycythemia vera (PV) (98% of the cases) essential thrombocythemia (ET; 50% of the cases) and primary myelofibrosis (PMF; 50% of the cases) (reviewed in [128]). Additional mutations in JAK2 exon 12 were found in a portion of PV patients lacking the JAK2V617F mutation[129]. JAK2V617F is sufficient to recapitulate MPN phenotype when expressed in mice bone marrow [123-127,130-132]. In recent research, we discovered that lentiviral transduction of human cord blood progenitors with JAK2V617F was sufficient to induce a PV like phenotype in vitro and in vivo[133]. Additionally we and others demonstrated the beneficial effect of treating with JAK2 specific inhibitor TG101348 in JAK2V617F positive clones. These results formed the basis for selective JAK2 inhibitor clinical trials in MPNs[133,134].

While the role of JAK2 signaling in myeloid differentiation and MPN diagnosis and treatment has been intensively studied, its role in the pathogenesis of B-cell precursor acute lymphoblastic leukemia (B-ALL) is yet to be elucidated. Lately, our lab and others, reported of a new group of activating mutations in the pseudokinase domain of JAK2, surrounding R683[5-7,9]. These cases of B-ALL may also be potential candidates for JAK inhibitor therapy, and indeed clinical trials with JAK inhibitors are ongoing. Although both V617F and R683 JAK2 mutations are activating mutations capable of inducing a growth advantage of the myeloid lineage in mouse progenitor cell assays[5], thus far, no cases of R683 were found in MPNs just as no reports of V617F have been evident in lymphoid disorders[6]. This association between two different specific somatic point mutations in the same domain (pseudokinase domain) resulting in the same biochemical squeal (ligand independent activation) with two hematopoietic malignancies derived from distinct lineages is unprecedented.

JAK2 mutations in B-ALL are almost exclusively reported in the context of CRLF2 aberrant expression[135]. Although aberrant expression and mutations in the JAK2 and CRLF2 were found to predict poor outcome in B-ALL patients, the importance of the JAK2-STAT pathway in initiation of B-ALL has not been elucidated.

In Addition to JAK2, mutations in JAK1 were reported in a very small subset of CRLF2^{+} B-ALL. Unlike the JAK2R683 mutations that are restricted to B-ALL, the JAK1 mutations (JAK1 V658F) reported correspond, to the myeloid JAK2 V617F mutation[1]. Although mutations in JAK1 are rare in CRLF2+ B-ALL

mutations in its receptor skeleton – the IL7RA subunit are more commonly found.

1.1.4.3 IL7RA

In addition to its part in the TSLP receptor that was described above, IL7RA is primarily recognized, as its name implies, as the unique subunit of the IL7 receptor. IL7R is a heterodimer of IL7RA and the common IL2Rγ subunit that is shared with IL2, IL4, IL9, IL15, and IL21[136]. IL7RA is a typical non-tyrosine kinase, encoded by 8 exons on chromosome 5p13 with both soluble and membrane bound forms that arise from alternative splicing of exon 6. While the membrane bound form transmits the Il7 signaling, the soluble form is thought to attenuate the signal by binding circulating IL7[137,138] IL7RA binds JAK1 through its SRC homology domain while the IL2Rγ binds JAK3. Upon initial cytokine interaction with IL7RA the heterodimer is formed and the two subunits undergo a conformational change, followed by JAK kinases cross phosphorylation and initiation of JAK/STAT and PI3K/AKT signaling (Figure 1–4). IL7RA- IL7Rγ heterodimers as well as IL7RA homodimers can to form spontaneously on T-cell surface. However, without the cytokine stimulation there is no conformational changes of the subunits and no activation of the downstream pathway. Although IL7 binds preferentially to the IL7RA- IL2Rγ heterodimers, it is capable of binding IL7RA homodimer low affinity[136,139-142].

IL7 was originally isolated by Namen et al.[143] when searching after a growth factor that would stimulate lymphoid cells. Further in vivo studies

confirmed its ability to induce proliferation as well as survival in B and T lymphoid cells[144-146]. This 25kD IL7 glycoprotein protein is a type I cytokine produced by a range of cells, including thymic and bone marrow stroma, intestinal epithelium, keratinocytes and hepatic cells[143,147-151]. The mouse IL7 shares 81% homology in amino acids sequence with the human protein[147]. IL7 has a pivotal role in early lymphoid differentiation. Deletion of IL7 or its receptor in mice results in near to complete omission of B and T cells[152-155]. It was later demonstrated that IL-7 is necessary for differentiation of mouse lymphoid progenitors to B-cell progenitors[156]. Yet, blocking mutations/deletions in the IL7 signaling in human, result in autosomal recessive severe combined immune deficiency with a complete loss of T-cells but not of B and natural killer cells (SCID, T^-, B^+, NK^+) [157]. Pointing out a principal difference between the role of IL-7 in murine and human lymphocyte development. Nevertheless, IL7 was demonstrated to have a gross effect on B-cell directed differentiation and proliferation from human hematopoietic stem and progenitors in vitro (from CB and to further extent from BM) though its absence could partially be compensated by Flt3 ligand[158]. This is also supported by the marked expression of IL7RA on human b-cell precursors. Thus suggesting that IL7 has an important role in human B-cell development however some redundancy in the human system allows for B-cells to develop in its absence. IL7 induces the expression of the B-cell-specific transcription factors EBF and PAX5 in B-cell progenitors [159]. It was also shown to regulate early T and B receptor recombination[160,161]. Additionally, IL7 represses pro-death factors [10,162] such as

BAD and BAX and, activates survival factors such as MCL-1, BCL2, and BCL-XL, thus protecting early T and B-cells from DNA damage induced apoptosis due to receptor editing [163,164]. Furthermore, in high concentrations, IL7 induces proliferation[165]. Its ability to enhance survival and proliferation makes IL7 a feasible pro-oncogenic candidate. IL7 was found to have autocrine effects in solid tumors that aberrantly express its receptor and secrete the cytokine (reviewed in[166]).

Recently, our lab and others described IL7RA somatic activating mutations in both T-ALL and B-ALL cases. These mutations are either point mutations or – for the most part insertion deletion mutations (INDELs) [10,162,167-169]. A mouse syngeneic transplantation model, in which hematopoietic stem and progenitors were transduced with mutated IL7RA receptor, instated the leukemogenic potential of such mutations; the expression mutated IL7RA BM hematopoietic stem and progenitors, led to the development of myeloproliferative disorder, while its expression in common lymphoid progenitors resulted in development of mature B-cell ALL/lymphoma. Finally combined expression of the mutated IL7RA with Notch1 mutation enhanced a T-ALL progress[170].

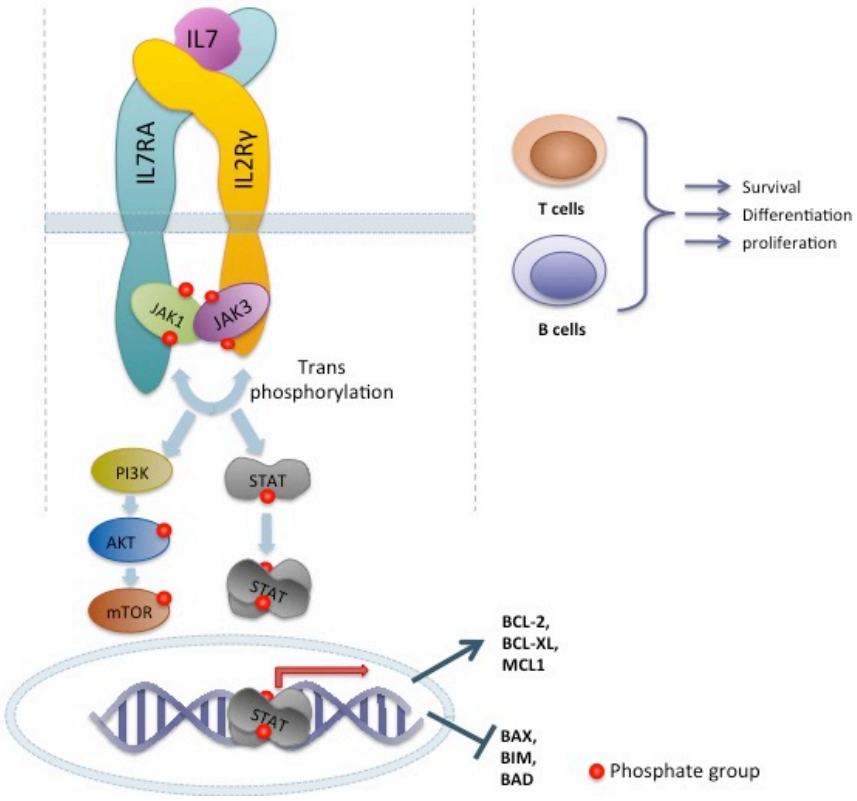

Figure 1–4 IL7 pathway.
Left: Binding of IL7 to the receptor induces cross phosphorylation of JAK proteins and subsequent activation of JAK/STAT pathway and mTOR pathway. Right: IL7 effects on lymphoid cells. Adopted from[93]

1.1.4.4 Summary findings of TSLP receptor aberrations in B-ALL

Normal hematopoiesis is contingent of strictly regulated network of transcription factors, epigenetic modifiers, cell cycle and self-renewal regulators. Activation of signal transduction pathways out of place or in high magnitude may perturb the balance between several of these factors and lead to the development of malignancy.

Aberrant activation of the TSLP pathway by over expression of CRLF2 in conjunction with numerous activating mutations in additional effector proteins in the pathway were reported (see Figure 1–5). The first and most common are

lymphoid unique mutations in JAK2 around arginine 683 the downstream effector of the TSLP pathway[5-7,9]. Then, mutations in two of the receptor subunits: CRLF2 and IL7RA that allows for dimerization and activation of down stream signal with no need for cytokine binding[3,112,113,167-169,10,162]. Moreover, loss of function mutations, were reported in negative regulators of the signal. One such mutation that was not described above was found in LNK -adaptor protein encoded by the SH2B3 gene that inhibits JAK2[167]. Additionally, samples from CRLF2^{+} B-ALL have activated JAK/SATA and mTOR signaling, and treatment with JAK inhibitors and mTor inhibitors were reported to inhibit their growth and survival both *in vitro* and *in vivo* [171,172]. Finally forced expression of CRLF2 together with other activating mutations of TSLP pathway proteins in mouse cells induced pre-leukemic phenotype[2-4]. Thus, vast body of evidence was accumulated suggesting that the aberrant activation of the TSLP pathway has a potential to drive leukemogenesis. Yet, no experiments were done in normal human system to support this theory.

Here, I hypothesis that CRLF2 together with secondary mutation in the TSLP pathway contributes to the initiation/development of BCP-ALL, and thus have set out to test it in primary human hematopoietc progenitors.

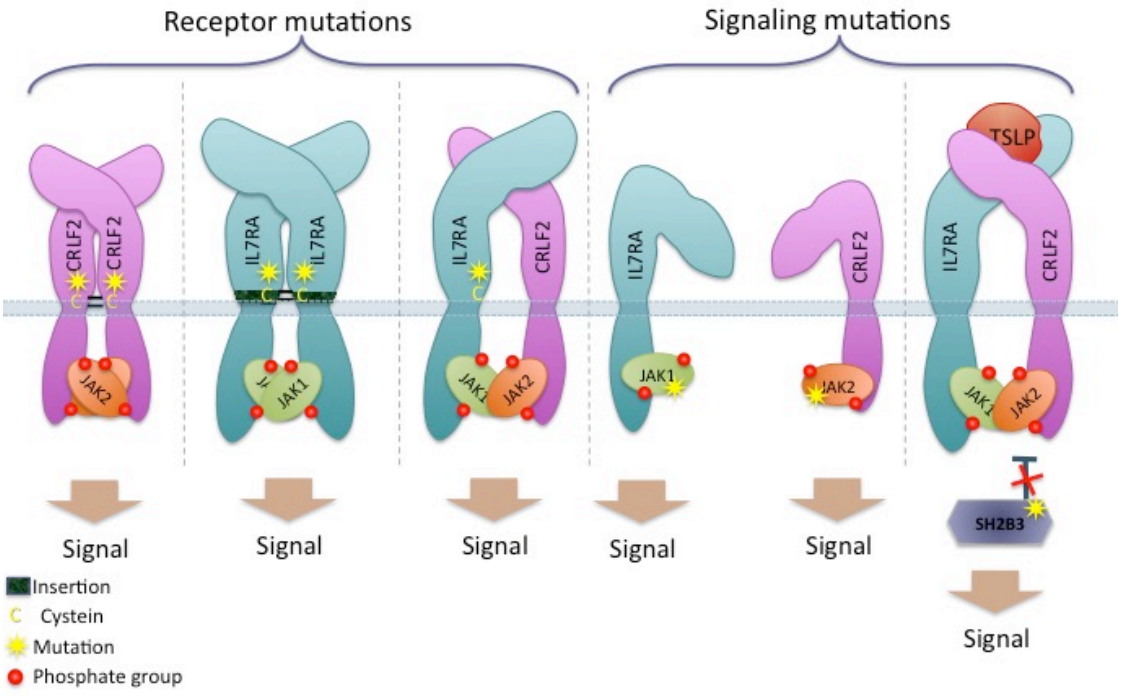

Figure 1–5 TSLP pathway mutations in B-ALL.
Illustration of TSLP pathway activating mutations in B-ALL. Mutations in the receptor result in spontaneous dimerization and cytokine independent activation of down stream pathway. Mutations in down stream signaling pathway result in constitutive activation of the pathway[93]

1.2 Materials and Methods

1.2.1 Cell cultures

1.2.1.1 Isolation of CB CD34^{+} hematopoietic progenitors

Fresh human cord blood samples were obtained from Sheba Medical Center public cord blood bank in compliance with Helsinki approvals (anonymized units that are otherwise discarded due insufficient volume for public storage). The blood was diluted 1:3 in HBSS (Sigma) and ficolled (ficoll-paque GE healthcare) according to manufacturer protocol. In Brief, 40ml diluted

blood was layered on 10ml ficoll and span @400g, 20°C Max acceleration, brakes off for 25min. PBMCs fraction was then collected, washed and counted.

$CD34^+$ cells were isolated from PBMCs by magnetic beads separation using CD34 MicroBead kit (Miltenyi Biotech, Bergisch Gladbach, Germany). 10^8 PBMCs were eluted in 300µl staining media (HBSS + 2%FBS) 100µl blocking solution and 100µl CD34 coated magnetic beads. After 15 minutes incubation at 4^0C the cells were washed and loaded onto pre washed LS magnetic column (Miltenyi Biotech). The column was washed 3 times with 5 ml of staining media and cells were eluted and reloaded on new column. After 3 washes the cells were eluted, counted and analyzed for purity by flow cytometer, using APC conjugated CD34 antibody (BD biosciences, New Jersy, USA). Average purity was ~80-90%

1.2.1.2 MS5 cultures.

MS5 cell line is a stromal cell line isolated from irradiated mouse BM[173] and was later found to support B-cell differentiation from human CB cells[174,175].

MS5 stromal cells were grown in α-MEM medium supplemented with 10% FBS, L glutamine 2mM, Non essential amino acids (0.1mM Glycine, L-Alanine, L-Asparagine, L-Aspartic Acid, L- Glutamic Acid, L-Proline, L-Serine) (all from Gibco-BRL, Paisley, United Kingdom) and Sodium pyruvate 1mM (Sigma USA), Cells were split 1:3-1:7 every 2-3 days when ~70% confluent. For co-culture experiments, 24 hours prior to CB seeding, plates were coated with Collagen Solution (Sigma USA) and MS5 cells were plated at ~60-70%

confluence. After seeding CB cells, antibiotics [penicillin/streptomycin 100 units/ml and

100 g/ml, respectively (Gibco-BRL, Paisley, United Kingdom)] and cytokines [hSCF (100ng/ml) hFLT3 ligand (100ng/ml) TPO (20ng/µl) and IL-6 (20ng/µl) /G-CSF (10ng/ml)] were added to the above α MEM medium.

1.2.1.3 Ba/F3 Cultures

The Ba/F3 cell line is a mouse interleukin 3 (IL3)-dependent pro-B cell line. Upon alternative activation of JAK/STAT pathway the cells loose their IL3 dependency. IL7RA expressing Ba/F3 cells[10] were used to validate the activity of CRLF2- JAK R683G that were cloned in the lentiviral vector.

Ba/F3-IL7RA cells, were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and the cytokine mouse IL3 (10ng/ml, PeproTech) with additional penicillin/streptomycin antibiotics (100 units/ml and 100 g/ml, respectively), and 2 mM L-glutamine (all from Gibco-BRL, Paisley, United Kingdom).

1.2.1.4 Differentiation of CB cells in co-cultures.

In order to allow B-cell differentiation of human hematopoietic progenitors $CD34^+$ CB cells were plated on previously prepared MS5 stoma (see above) in low number (200-600 CD34+ cells/well in 24-6 well plates respectively). Either by counting cells or by sorting transduced cells directly into wells with FACS ARIA I (BD biosciences, New Jersy, USA). Half of the media volume was replaced with fresh media weekly. When supernatant cell density was high, the media on each well was carefully mixed to allow for ~50% dilution of the cells

and analysis of the collected cells. Supernatant cells were analyzed from ongoing culture when wells were fed. Whole cell cultures (Adherent and supernatant cells) were harvested for analysis 4-5 weeks after seeding as following: supernatant cells were collected separately. Then adherent cells were carefully detached by pipetting. To reduce the amount of MS5 in the analysis, the collected adherent cells were seeded for 15-30 minutes on new plate to allow for MS5 cells to re-attach. Human cells were then gently collected from the supernatant. And analyzed by FACS for B-cell and Myeloid differentiation

1.2.1.5 018Z

018Z is a childhood B-ALL cell line with CNS phenotype that was established by passaging as a xenograft (karyotype: 47, XY, +8, del(9)(p13).

018Z are readily transduced and grown and therefore were chosen for routine work. Growth media: RPMI supplemented with 20% FBS, L glutamine 2mM, Non essential amino acids (0.1mM Glycine, L-Alanine, L-Aspargine, L-Aspartic Acid, L- Glutamic Acid, L-Proline, L-Serine) (all from Gibco-BRL, Paisley, United Kingdom) and Sodium pyruvate 1mM (Sigma USA)

1.2.1.6 HEK 293T cells

HEK 293T cells are human embryonic kidney cell line used routinely for virus preparations. The cells were grown in DMEM supplemented with 10% FBS, L glutamine 2mM, Non essential amino acids (0.1mM Glycine, L-Alanine, L-Asparagine, L-Aspartic Acid, L- Glutamic Acid, L-Proline, L-Serine) (all from

Gibco-BRL, Paisley, United Kingdom) and Sodium pyruvate 1mM (Sigma USA), Cells were split 1:10-1:20 every 2-3 days when ~80-100% confluent.

1.2.2 Viral work

1.2.2.1 Construction of lentiviral vectors

Cloning was preformed with standard cloning protocols. All fragments amplifications were carried out using Phusion High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland) in touchdown PCR program to account for different melting temperature of primers. Amplified fragments were purified directly from PCR reaction using the Wizard SV Gel and PCR Clean-up System (Promega). Fragments and vectors were digested using restriction enzymes according to manufacturer's protocols (NEB Ipswich, MA) and purified after gel electrophoresis using the Wizard SV Gel and PCR Clean-up System (Promega). Quick ligation kit (NEB Ipswich, MA) was used to ligate the vectors and inserts. The ligations were transformed to NEB 5-alpha F' Iq Competent E. coli suitable for toxic genes (NEB Ipswich, MA) according to manufactures' protocol.

1.2.2.1.1 Cloning CRLF2 into pCDH-EF1α-MCS-T2A-copGFP vector:

pCDH-EF1α-MCS-T2A-copGFP vector was purchased from SBI system biosciences (Mountain View, CA) to allow for efficient bi-cistronic expression[176] . CRLF2 that was previously cloned from cDNA [3] was amplified with primers carrying restriction enzymes sequences as following: EcoRI for 5' amplification (5'-ATATGAATTCGAGGGCATGGGGCGGCTGGT-3') and NotI for 3'

amplification (5'-AAGCGGCCGCCACAACGCCACGTA-3'). Purified inserts and pCDH-EF1α-MCS-T2A-copGFP vector were digested and with EcoRI and NotI restriction enzymes and ligated to form pCDH-EF1α-CRLF2-T2A-copGFP (Figure 1–6)

1.2.2.1.2 Cloning CRLF2 into B-cell-specific vector:

pRRL Eμ B29 GFP WPRE vector was kindly provided by Rawlings lab[177]

In order to clone the CRLF2 T2A GFP cassette to the above vector, the original GFP was excised and an additional restriction site (NheI) was added in the following way: The B29 promoter was re-amplified with forward primer: 5' TCGATGATACCCTGATGAAGC 3' and reverse primer carrying NheI and Kozak sequence in the 3' end

5' TATATGTCGACGCTAGCGGTGGCGGTCCACTGCTCTGTCTC 3'. This PCR product and pRRL-Eμ-B29-GFP-WPRE vector were digested with SalI and XcmI. Purified products were ligated. The new pRRL-Eμ-B29-NheI-salI-WPRE vector was then digested alongside pCDH-EF1α-CRLF2-T2A-copGFP vector with NheI and SalI. CRLF2-T2A-copGFP insert and digested pRRL Eμ B29 NheI salI WPRE were ligated to form pRRL-Eμ-B29-CRLF2-T2A-GFP (Figure 1–6).

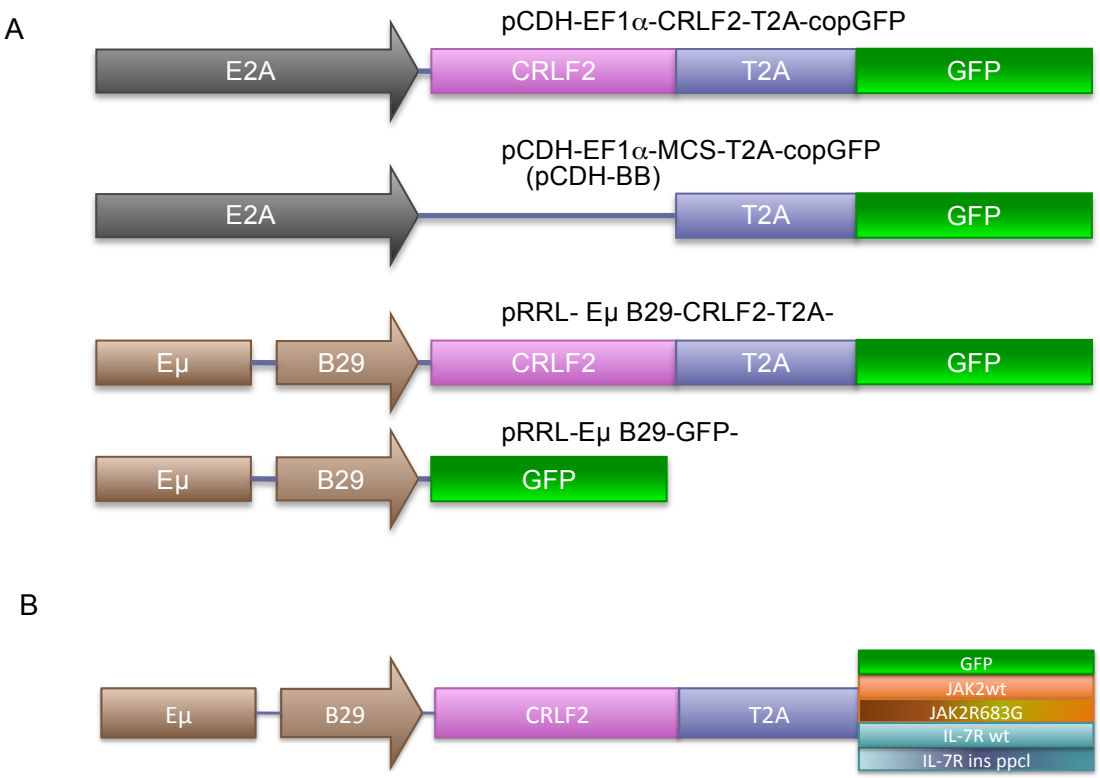

Figure 1–6 Scheme of the lentiviral vectors used in the research.
A) GFP and CRLF2 T2A GFP bicistronic cassette are expressed from E2A ubiquitous promoter or from EμB29 B-cell-specific promoter/enhancer B) Bicistronic expression of CRLF2 with GFP, JAKwt, JAKR683, IL7RAwt, IL7RAins ppcl

1.2.2.1.3 Cloning JAK2 wild type and JAK2 R683G

Human wild type JAK2 was amplified from human cDNA and was cloned in three stages into pCDH-EF1α-MCS-T2A-copGFP vector utilizing the fact that JAK2 has EcoRI recognition site on position 884 and BamHI on 2517 (positions are counted from ATG). First, the JAK2 N terminal was amplified using left primer containing NheI and KOZAK sequences (5'-ATATAAGCTAGCGCCACCATGGGAATGGCCTGCC-3') and right primer starting at base pair position 956 (5'-TCCTGTTCTGTCAGTGTCTCAC-3'). The amplified fragment was digested with NheI ad EcoRI alongside pCDH vector

and ligated. Next, the mid part of JAK2 was amplified using left primer from position 822 (5'-AACCTGGAAGTGGTCCTTCA-3') and right primer from position 2589 (5'-CACATCTCCACACTCCCAAA-3') this fragment as well as the vector from the previous step were digested with EcoRI and BamHI and ligated. Last, the C terminus was amplified with left primer from position 2492 (5'-GGGTTTTCTGGTGCCTTTG-3') and right primer containing a NotI site (5'-ATAGTTTAGCGGCCGCTCCAGCCATGTTATCCC-3'). This fragment as well as the plasmid from stage 2 were digested with BamHI and NotI and ligated together.

JAK2 R683G mutation was amplified from MUTZ5 B-ALL cell line cDNA carrying the desired mutation: The fragment within base-pairs 1654 and 2589 in JAK2 was amplified using left primer (5'- TGGCCAAGGCACTTTTACA-3') And right primer (5'-CACATCTCCACACTCCCAAA-3') the amplified segment and a pCDH JAK2wt T2A-copGFP were digested BsiWI (cuts at position 1686 in JAK2) and BamHI (cuts at position 2517 in JAK2) and ligated together thus inserting the mutated site. JAK2 was then moved to 2nd Gene position (after the T2A) by amplifying it with primers carrying BspEI (5'-ATATTCCGGAATGGGAATGGCCTGCC-3') and SalI (5'-CAGCATGTCGACTCATCCAGCCATGTTATC-3') from the cloned vectors.

The GFP in the pCDH-EF1α-CRLF2-T2A-copGFP vector was replaced with the amplified fragment above by digesting both vector and insert with BspEI and SalI and ligation to form pCDH-EF1α-CRLF2-T2A-JAK2wt and pCDH-EF1α-CRLF2-T2A-JAK2 R683G. CRLF2-T2A-JAK2 cassette was then

subcloned to pRRL-Eμ-B29 vector using the pRRL-Eμ-B29-NheI-salI-WPRE vector described in section 1.2.2.1.2 by digestion of the pCDH-EF1α-CRLF2-T2A-JAK2wt and the above vector with NheI SalI and ligation. To form pRRL-Eμ-B29-CRLF2-T2A-JAK2wt and pRRL-Eμ-B29-CRLF2-T2A-JAK2ins (Figure 1–6 B)

1.2.2.1.4 Cloning IL7RA wild type and IL7RA ins ppcl mutation

IL7RA was amplified from previously cloned vectors[10] using left primer carrying BspEI recognition site (5'- ATATTCCGGAATGACAATTCTAGG 3' and right primer carrying SalI recognition site (5'-CAGCATGTCGACCTACTGGTTTTGGTAGAAGCTGGA-3'). The amplified insert was digested alongside pRRL-Eμ-B29-CRLF2-T2A-GFP with BspEI and SalI. Purified insert and plasmid were ligated to form the pRRL-Eμ-B29-CRLF2-T2A-IL7RAwt and pRRL-Eμ-B29-CRLF2-T2A-IL7RAins (Figure 1–6 B)

1.2.2.2 Production and titration of lentivirus

Production of lenti vector was done as described in[178]. In short: 3rd generation lenti vector packaging plasmids were co transfected into 15 plates of 293T cells in the ratio of (15:10:5:4) (Lenti vector:pMDL:pVSVG:pREV) using ProFection Calcium Phosphate mammalian transfection system (Promega) according to manufacturer's protocol. Transfection medium was replaced 6-15 hour after transfection with 7ml fresh 5% serum DMEM serum and virus-containing supernatant was collected 24 and 48 hours after replacement. Supernatant was then filtered with 0.45um PVDF filters (Millipore,

Massachusetts, USA) aliquoted to round bottom ultracentrifugation tubes (Beckman-Coulter, California, USA) and centrifuged in ultra centrifuge using SW28 rotor for 2 hours in 19,400r.p.m (70,000g). The pellets from all tubes were combined and eluted/reconstituted in 300-600 SFEM medium (STEMCELL technologies, Vancouver, British Columbia, Canada). Concentrated virus was frozen in -80^{0}C until use. An aliquot of frozen virus was used for titer as following: 5X10^4 018Z cells were plated in 24 well plate with 400µl RPMI medium supplemented with 20% FBS 8µg/ml polybrene (SIGMA Chemical, MO, USA). 1µl, 0.2µl, 0.04µl, 0.008 µl of concentrated virus were added to each well by serial dilutions induplicates, and span 45 min in 800g@ 32^{0}C. 48 hour after transduction, percentage of transduced cells in each well was evaluated by flow cytometry using GFP or CRLF2 antibodies (Biolegend California, USA) Titer (infectious units/ml) was calculated according to the following equation:

$$\frac{\%transduced\ cells X \#cells\ at\ day\ of\ transduction}{total\ \mu l\ of\ virus/well} X1000 = virus\ IU/ml$$

1.2.2.3 Transduction of CB CD34$^+$ progenitors

For each lentiviral construct, 5x10^3 – 7.5x10^4 CB CD34$^+$ cells were plated in 96 U bottom-well plate (Corning Incorporated, NY, USA) in 50-100µl SFEM (STEMCELL Technologies Vancouver, British Columbia, Canada) supplemented with hSCF (100ng/ml) hFLT3 ligand (100ng/ml) TPO (20ng/µl) and IL-6 (20ng/µl). Cells are transduced twice in consecutive days by addition of virus in MOI of 50-200 and spin (800g 32^{0}C 45 min no break). 4-8 hours after

spin the wells are supplemented with fresh media. Prior to the second transduction, old media containing virus is discarded. Transduction efficiency is evaluated by flow cytometry either by GFP expression or CRLF2 expression after staining with PE conjugated CRLF2 antibody (Biolegend California USA)

1.2.3 Flow cytometry

1.2.3.1 Transduction purity and differentiation analysis

Standard staining protocols were used for analysis of cells. In brief, cells were washed in staining media (2%FBS in PBS) and re-suspended in 50ul of staining media containing fluorochrome-conjugated antibodies (see Table 1–1) and 7AAD for 30 min. Following staining, cells were washed twice, re-suspended in 100-200µl STM containing 7AAD and analyzed on Gallios flow cytometer (Beckman-Coulter, California, USA). Single stains and FMOs (Full minus one staining) of each fluorophore were used for cytometer setup and gating. Counting beads [countbright ThermoFisher Scientific Waltham, MA USA (molecular probes brand)] were added when cell numbers were of importance. Analysis was preformed using Kaluza software (Beckman-Coulter, California, USA) or FlowJo software (FlowJo LLC. Orlando, USA) on live cells after exclusion of 7AAD positive stained cells.

1.2.3.2 Phospho-flow assays

For phosphorylation assays, cells (from sub confluent culture) were first washed and starved for four hours (in media with no cytokines). Cells were then incubated with cytokines (hIL7, hTSLP) for 20 minutes, washed and stained with LIVE/DEAD Fixable staining antibody according to manufacturer’s protocol

[ThermoFisher Scientific Waltham, MA USA (molecular probes brand)] , cells were then stained for cell surface markers, fixed with 1.5% formaldehyde for 10 minutes, porated with ice-cold MeOH while vigorously vortexing and incubated at 4°C for at least 10 min. cells were then stored over night or more (up to 2 weeks) in -20. Fixed porated cells were then washed twice in staining media then resuspended in staining media containing pSTAT antibodies and re stained for surface markers. Stained cells were analyzed on Gallios™ Flow Cytometer (Beckman-Coulter, California, USA).

1.2.3.3 Cell cycle analysis

For cell cycle analysis, cells were harvested and stained for cell surface antigens as described in 1.2.3.1 and were then stained with LIVE/DEAD fixable cell staining following manufacturer's protocol [ThermoFisher Scientific Waltham, MA USA (molecular probes brand)]. Stained cells were fixed with 70% ice cold EtOH and incubated at 4^0C Over Night. After incubation, cells were washed twice with STM containing 0.16% saponin and stained with Ki67 in STM containing 0.16% saponin for 45 minutes in 4^0C. Cells were washed again and incubated with 10ng/ml 7AAD in NASS media (0.1M Sodium Citrate, 5mM EDTA, 0.15M NaCl, 0.5% W/V BSA, 0.02%W/V Saponin) 30 min. at room temperature. Cells were then washed with STM and analyzed on Gallios™ Flow Cytometer (Beckman-Coulter, California, USA).

Table 1–1 Antibodies and markers for flow cytometry.

Antigen	Marker of	Fluorochrome	Dilution	Reactivity	Species	Manufacturer
CD45	Human hematopoietic	Vio Green	1:50	Human	Mouse	Miltenyi
Ki67	proliferation	Alexa 488	1:100	Human	Mouse	Dako
pSTAT5	Phospho STAT5	APC	1:50	Human/ Mouse/ Rat/ Sheep	Mouse	BD
CRLF2	TSLP Receptor	PE	3:100	Human	Mouse	Biolegend
	Lymphoid:					
CD10	Early lymphoid	PC7	1:50	Human	Mouse	Beckman Coulter
CD19	Pan B-Cell	ECD	1:50	Human	Mouse	Beckman Coulter
	B- cell differentiation					
IgD	Immature B-cell	APC Cy7	1:100	Human	Mouse	Biolegend
IgM	Naïve B-cell	APC	1:100	Human	Mouse	eBioscience
	Myeloid					
CD14	Monocytes: Macrophages Dendritic cells Neutrophils	APC Cy7	1:100	Human	Mouse	Biolegend
CD15	Myelomonocytes : Neutrophils Eosenophils	Pacific Blue	1:50	Human	Mouse	Biolegend
	Progenitor					
CD34	Progenitor	APC	1:100	Human	Mouse	BD
	Non-antibody markers					
Nucleic acid	Dead cells	7AAD	1:50	No specie specificity	NA	BD
LIVE/DEAD fixable Dead cells staining	Dead cells	Near IR/Violet	1:1000	No specie specificity	NA	ThermoFisher Scientific (Molecular probes)
Annexin V	Apoptosis	APC	1:50	No specie specificity	NA	eBioscience

1.2.3.4 Apoptosis analysis

Apoptosis was assessed by annexin binding as follows: 018Z cells were harvested and stained for CRLF2 to allow for gating on transduced cells. Cells were washed and resuspended in 100µl Annexin binding buffer (eBioscience San Diego California), supplemented with 7AAD (1: 50) and Annexin (eBioscience San Diego California) (1:50). After 30 minutes incubation, volume was increased to 300µl and the cells were analyzed on Gallios™ Flow Cytometer (Beckman-Coulter, California, USA).

1.2.3.5 Sorting transduced CB cells

Transduced cord blood cells (minimum 48 hours after second transduction) were washed 3 times with 10X culture volume to reduce the risk of live virus in the sort. Cells were then stained as described in 1.2.3.1. under sterile conditions. Stained cells were sorted using ARIA I FACS sorter (BD Biosciences, San Jose, CA USA) after aseptic sort clean. 200-600 live CRLF2-positive/ GFP positive cells were sorted directly to 24 well plates seeded with MS5 stroma with growth medium (see 1.2.1.4.)

1.2.3.6 Flow cytometry *and sorting* of xenograft tissue

Hematopoietic tissues (Spleen, Bone marrow (BM) and liver) and peripheral blood (PB) were harvested from mice at sacrifice time (see 1.2.4) and kept throughout the processing time on ice. BM cells were collected from the hind leg bones by flashing cold STM with a 27Gx1/2 needle through the bones. Collected BM cells were then suspended and strained through a 70µm mesh cell strainer and pelleted. Spleen and liver were mashed on a 70µm mesh

cell strainer by applying force with a plunger and washing with cold STM, cells were collected into a 50ml conical tube and pelleted. PB and spleen were subjected to red blood cell lysis (Biolegend, San Diego, CA, USA) according to manufacturer's protocol and pelleted. All cells were eluted in STM soon after they were pelleted (BM eluted in 300µl STM, Spleen in 400-500µl STM, Liver in 2ml STM and PB in 50ulSTM).

For cell surface marker analysis, 5-10µl of eluted cells were blocked with FCγR blocking antibody (Biolegend, San Diego, CA, USA) for 15 minutes in 25µl. Volume was then adjusted to 50ul with antibody mix (see table) and cells were incubated for 30-45 minutes in 4^0C. After incubation cells were washed and eluted with STM containing 7AAD (BD Biosciences, San Jose, CA USA) and CountBright beads [ThermoFisher Scientific Waltham, MA USA (molecular probes brand)] and analyzed on Gallios™ Flow Cytometer (Beckman-Coulter, California, USA).

For RNAseq analysis, 100-200µl of the cell suspension was stained for 30-45 minutes, eluted in 300-500µl of 7AAD containing STM. 5000-20000 Live $CD45^+$ $CD3^-$ CRLF2/GFP^+ cells were sorted directly into mini-centrifuge tubes containing 800µl cold TRIzol (ThermoFisher Scientific Waltham, MA USA) using ARIA I FACS sorter (BD Biosciences, San Jose, CA USA). Tubes were vortexed immediately after sort and flash froze in liquid nitrogen for further RNA purification.

1.2.4 NOD/LtSz-scid IL2Rγnull (NSG) xenografts

NOD mice were purchased from Jackson laboratories (Mount Desert Island, Maine, USA). Mice were bred and housed in an SPF room at Tel Hashomer animal facility. 5-8 week old NSG females were sub lethally irradiated (1-1.5 Gy X-ray irradiation) 24 hours prior to transplantation. For each mouse, 10^5-$1.5x10^5$ cells were transduced [in two wells of 96 well plates (see 1.2.2.3)] 78-96 hours prior to transplantation. On the day of transplant, transduced cells were collected form the plates and washed 3 times with 10X culture volume of STM to prevent carryover of virus. Cells were then eluted in 150-200µl of cold STM and transplanted via tail vain injection to mice. 2-3µl of eluted cells were sampled prior to transplant to assess transduction efficiency.

Transplanted mice were screened for human engraftment (%human $CD45^+$ cells) in peripheral blood every 2-3 weeks starting week 15 post transplant. Upon detection of visible (>2%) human CD45 engraftment or after 23-30 weeks, mice were euthanized and hematopoietic tissues (spleen, bone marrow from femurs, liver and peripheral blood) were harvested. Single cell suspensions were prepared as described in 1.2.3.6.

1.2.5 Statistical analysis

Data was analyzed with the aid of Microsoft Excel and GraphPad Prism 5 software. Data is either graphed as mean ± SE or as scatter plot with mean ± SE. Comparisons between groups were performed by unpaired student t tests in two groups analysis or by ANOVA tests when more than two groups were compared. P values < 0.05 were considered statistically significant.

Figures 1-3 - 1-5 were published in a review authored by Noa Tal, Chen Shochat, Ifat Geron Dani Bercovich and Shai Israeli. “Interleukin 7 and thymic stromal lymphopoietin: from immunity to leukemia”. Cell Mol Life Sci. 2014 Feb;71(3):365-78. The authors and publisher granted permission for the use of the figures in the dissertation.

Chapter 2 The context counts - CRLF2 activation phenotype is cell-type and context-specific

2.1 Introduction

The finding of CRLF2 overexpression in B-ALL was reported by four different groups in 2009-2010[1-4,106]. CRLF2 translocations were found in patients with no other known sentinel translocation, thus raising the question whether this translocation has a causal nature. Primary studies in these reports suggested that aberrant CRLF2 expression is involved in leukemogenesis via activation of JAK-STAT signaling[1-3]. These studies however, were all preformed in mice using either cell lines, primary tissue or transgenic models. TSLP signaling and closely related IL-7 signaling differs greatly between mouse and human cells; while B-cell levels in IL-7R knockout mice are depressed[179], B-cell levels in humans who lack the IL-7 signaling (XSCID) pathway are unaffected[180]. *In vitro* addition of IL-7 to mouse hematopoietic progenitors will lead to B-cell differentiation, but this is not the case in human hematopoietic progenitors. Finally, both TSLP and CRLF2 show only ~50% similarity in nucleic acid and amino acid sequences between mouse and human cells. Therefore, there is a great importance in testing the effects of CRLF2 overexpression in human systems.

Hematopoietic stem and progenitor cells hold the potential to differentiate into cells of the myeloid and lymphoid lineages. To test whether CRLF2 can commence pre-leukemic transformation, we set out to express CRLF2 in normal human hematopoietic progenitor cells and assess its effects on their differentiation profile, proliferation and survival. The default differentiation of

human hematopoietic progenitors *in vitro* in liquid media or semi-solid media (methylcellulose) with the addition of growth factors is into the myeloid lineages. However, several groups reported feasibility of B-cell differentiation *in vitro* by growing human hematopoietic stem/progenitor cells on stromal cells with the addition of cytokines[173,174]. Since our research goal is to examine the effects of CRLF2 in B-ALL, we used the lymphoid culture conditions in our research.

2.2 Results

2.2.1 CRLF2 is expressed after transduction with lentiviral vector

To investigate whether untimely activation of CRLF2 contributes to the development of lymphoid malignancy in human primary cells, we set up a viral system suitable for gene expression in human $CD34^+$ progenitors. The CRLF2 gene was cloned downstream to the ubiquitous promoter EF1α in pCDH-EF1α-MCS-T2A-copGFP vector (SBI System Biosciences, Mountain View, CA) a vector with a 2A peptide (T2A) serving as a SKIP peptide that allows dual protein expression from one promoter. In this vector, a GFP protein is expressed after the cloned gene of interest. Transducing 293T cell line with the vector resulted in expression of both CRLF2 and the reported GFP, as detected in flow analysis (Figure 2–1).

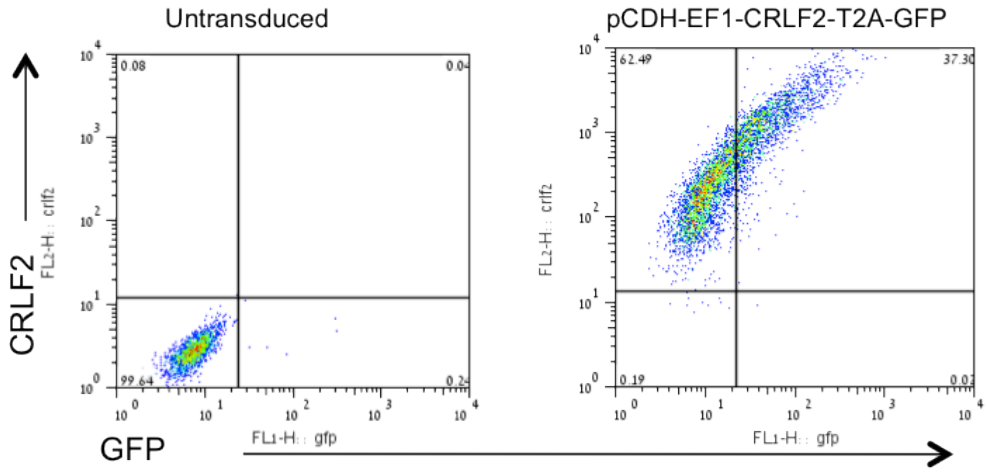

Figure 2–1 CRLF2-GFP is robustly expressed after lentiviral transduction. Flow cytometer plot of CRLF2 and GFP expression in transduced 293T 72hrs after lentiviral transduction with pCDH-EF1-CRLF2-T2A GFP.

2.2.2 High expression of CRLF2 in hematopoietic progenitors skews differentiation toward the myeloid lineage

B-ALL is a B-cell progenitor disease. Previously, it was reported that overexpression of CRLF2 transformed mouse fetal liver progenitors[2]. As our goal is to delineate the effects of CRLF2 in human system we decided to use human cord blood, which is a rich source for progenitor cells. However, the default differentiation path of these cells when grown *in vitro* is along the myeloid lineage. To test the effect of CRLF2 overexpression in a human B-cell progenitor context, we used a co-culture system of mouse stromal cells and human cytokines that support differentiation of cord blood hematopoietic progenitors along the B-cell lineage, as described in[174,175]. Briefly: co-culture of $CD34^+$ progenitors with MS5 stroma for 4-8 weeks will give rise to a large population of $CD19^+CD10^+$ cells. Cord blood $CD34^+$ progenitors were isolated

using magnetic beads and transduced with the lentivector described above. Analysis of the experimental co-cultures revealed that the relative CD10+CD19+ population among the CRLF2 transduced cells was dramatically lower than in the untransduced CRLF2neg population (Figure 2-2), suggesting that activation of CRLF2 under these condition either inhibits B-cell differentiation or induces myeloid differentiation. Further analysis of the transduced cells with myeloid markers confirmed a higher myeloid population of CRLF2^{+} cells (Figure 2–3). This result is not surprising, considering the myeloid nature of the TSLP pathway and the normal expression pattern of the receptor on myeloid cells[181].

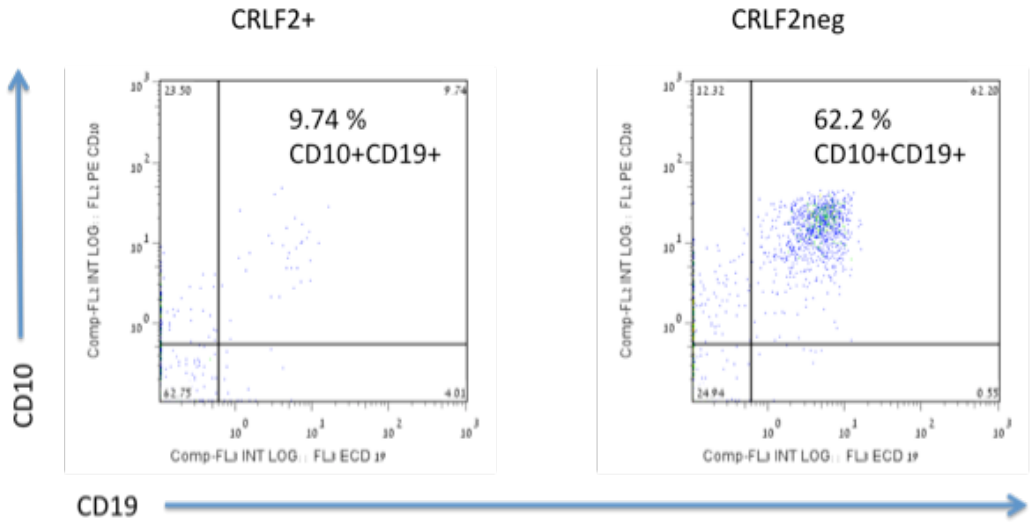

Figure 2–2 CRLF2 expression in CD34+ progenitors inhibits differentiation along the B-cell lineage.
Representative flow cytometer scatter plots of transduced CB CD34^{+} differentiation after transduction with CRLF2 expressing vector and culturing on MS5. The left scatter plot represents the differentiation of live human CRLF2^{+} gated cells and the right one depicts the CRLF2^{-} gated cells.

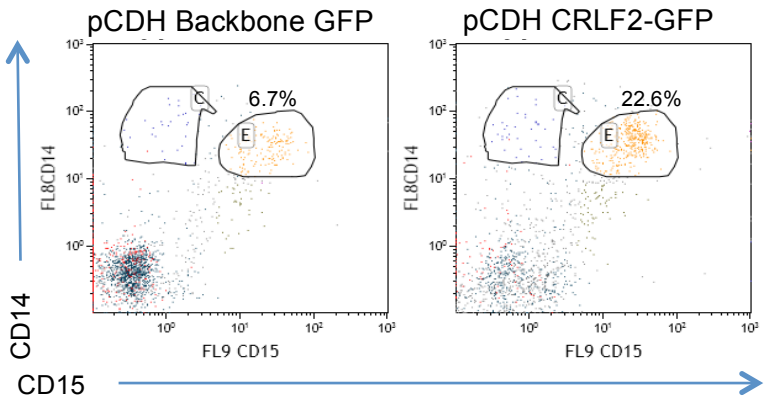

Figure 2–3 Myeloid differentiation on MS5 stroma.
Flow cytometer scatter plot of pCDH backbone and pCDH-CRLF2-T2A-GFP transduced CD34$^+$ CB after 4 weeks culture on MS5. Live human GFP$^+$ or CRLF2$^+$ cells were analyzed for the expression of CD14 and CD15.

The CD34$^+$ population isolated from CB is diverse, consisting of myeloid and lymphoid progenitors in variable differentiation stages. Elevated expression of CRLF2 in B-ALL is typically a consequence of juxtaposing CRLF2 to the IgH enhancer, or interstitial deletion in the pseudoautosomal region 1 (PAR1) of chromosome X, resulting in a P2RY8-CRLF2 fusion. These aberrations carry the footprint of RAG and AID activation and are therefore believed to occur in lymphoid progenitors or in early B-cell progenitors[182,183]. To better mimic the cellular context of CRLF2 overexpression, we sought to transduce more committed cells and started with CD10$^+$ lymphoid progenitors. CD34$^+$ CD10$^+$ cells were sorted from CB. The sorted cells were transduced and cultured on MS5 stroma. As depicted in Figure 2–4, similarly to the results that were obtained with whole CD34$^+$ progenitors, overexpression of CRLF2 in CD34$^+$CD10$^+$ reduced the differentiation of the transduced cells to B-cell progenitors.

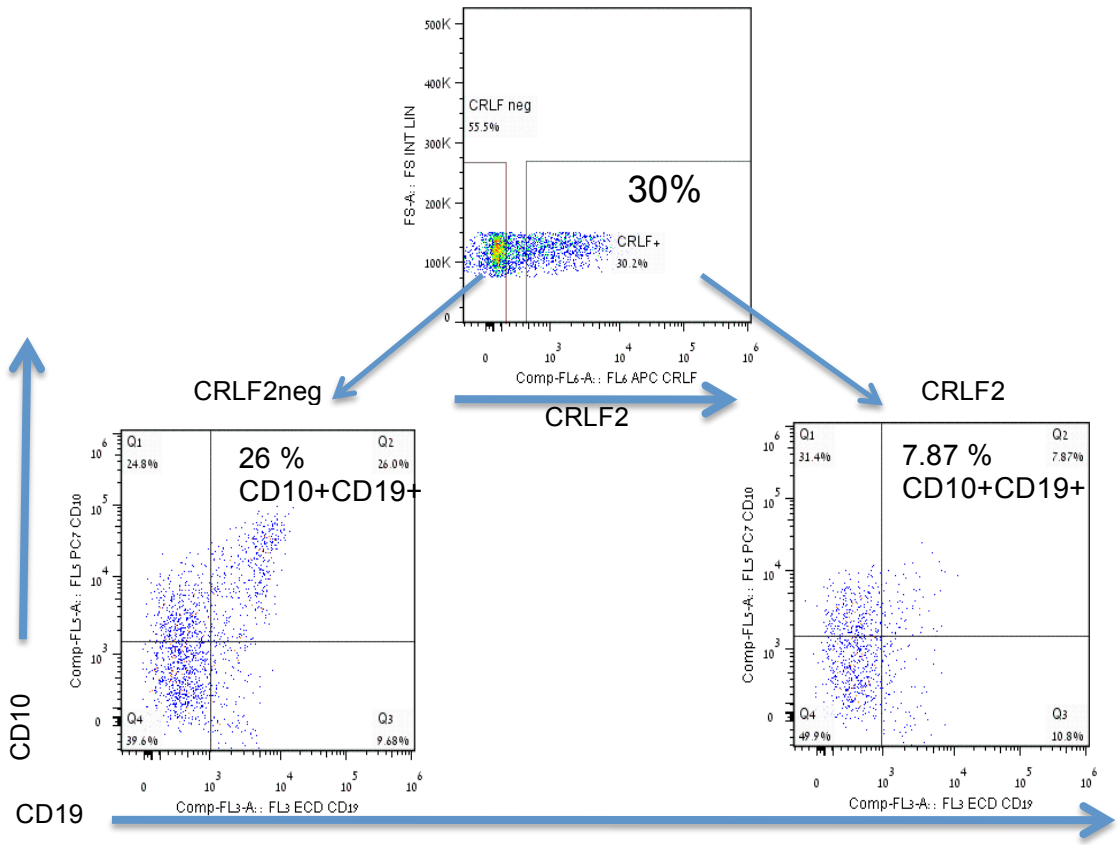

Figure 2–4 CRLF2 expression in CB CD34$^+$ progenitors inhibits differentiation of CD10$^+$ lymphoid progenitors to B-cell progenitors.
Flow cytometry differentiation analysis of pCDH-EF1-CRLF2-T2A GFP lentivector transduced sorted CD34$^+$CD10$^+$ CB cells after 5-week culture on MS5.

2.2.3 Expression of CRLF2 in a B-lineage-specific vector

Although CD10 is a known common lymphoid marker, CD34$^+$CD10$^+$ cells retain their ability to differentiate to myeloid cells. In lieu of the results above, we sought a better way to overexpress CRLF2 in B-cell committed progenitors. One possibility was to use CD19$^+$ progenitors. However, the CD34$^+$ CD19$^+$ population in CB is extremely rare and hard to isolate while isolating whole CD19$^+$ population from CB yielded a mostly differentiated population with poor *in vitro* survival and inconsistent results (data not shown). Therefore, we chose

to alter the lentiviral vector and modify it to over express the transgene preferably in $CD19^+$ cells. We received a vector that harbors a B-cell-specific enhancer and minimal promoter Eμ B29 [177] and switched the EF1α promoter in the pCDH-EF1α-MCS-T2A-copGFP vector with an Eμ B29 promoter/enhancer. To verify targeted overexpression of the vector in B-cells, B-cell-ALL cell line (018Z) and myeloid (megakaryocytic) leukemia cell line (CMK) were transduced with pRRL-Eμ-B29-GFP vector and expression level of GFP was measured. Expression of the GFP from the pRRL-Eμ-B29 vector was logs higher in the B-cell line than in the myeloid cells, consistent with the reported specificity of the element. We therefore decided to use this vector for the experiments hereafter.

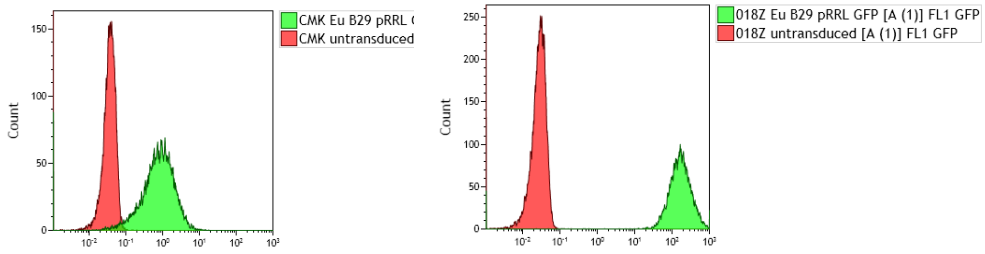

Figure 2–5 High expression of transgene from pRRL Eu B29 vector in B-ALL line. Flow cytometry histogram of GFP expression in A) CMK, an AML cell line and B) 018Z, a B-cell precursor ALL cell line. Untransduced vector (red), transduced with Eμ B29 pRRL vector (green).

2.2.4 B lineage CRLF2 expression skews $CD34^+$ differentiation to B-cell lineage

When transducing CB $CD34^+$ progenitor cells with pRRL-Eμ-B29-CRLF2-T2A-GFP, as soon as 2.5 weeks post-transduction an enhanced early lymphoid differentiation (measured as $CD10^+$ cells) and initial B-cell differentiation (measured by CD19) combined with reduced myeloid differentiation (measured

by CD14 and CD15) was observed in CRLF2 transduced cells, as compared to the control GFP transduced cells (Figure 2–6). This trend was further augmented in a 4-5 week-long culture, where CRLF2 expressing cells presented enhanced differentiation towards $CD10^{+}CD19^{+}$ B-cell progenitor when compared to the control GFP expressing cells (Figure 2–7). This data suggests that when overexpressed in potential B-cell progenitor cells, CRLF2 further increases B-cell differentiation. To test a possible augmentation of the phenotype by addition to TSLP to the culture, MS5 cells were transduced with lentiviral vector encoding TSLP (a kind gift from Dr. Payne [184]). The TSLP concentration in the media was 10-15ng/ml as measured by ELISA. No differences were observed between transduced CB progenitors that were growing on untransduced MS5 and those that were growing on TSLP expression MS5 (Figure 2–8).

Together, these data suggest that the outcome of CRLF2 overexpression is cell context specific – when expressed in cells with B-cell differentiation potential, CRLF2 will boost the B-cell differentiation whereas its expression in crude progenitor populations will block B-cell differentiation.

No other conclusions aside from the clear deviation in differentiation pattern could be deduced from our experiments. Although growth was evaluated visually, by counting beads in flow analysis, and in cell cycle analysis, great variation between replicates and technical difficulties due to low cell numbers limited our ability to collect conclusive data. Additionally, replating

assays for self-renewal evaluation were preformed but same limitations prevented us from decisively determine the effect of CRLF2 transduction.

2.2.5 Summary

CRLF2 aberrations in B-ALL are associated with poor prognosis and frequent relapses [106,107,185]. Characterizing the cellular context in which these aberrations occur is essential to the understanding of the disease. By analyzing the growth and differentiation of transduced human cord blood $CD34^+$ progenitors in a lymphoid-supporting co-culture system, we sought to evaluate the effect of CRLF2 on expression. Choosing the human system to model over-expression of CRLF2 was critical, due to the known difference between human and mouse CRLF2 and IL7RA signaling (reviewed in [45]).

We show here that the B-cell lineage directed over-expression of CRLF2, potentiated B-cell lineage differentiation, and inhibited myeloid differentiation. The directed B-lineage expression is biologically relevant as the translocations of CRLF2 are thought to occur in B-cell progenitors via B-cell promoter gene rearrangement machinery. Since we analyzed only transduced cells, and to rule out the possibility that the differentiation results were biased due to our use of B-directed promoter, we used a control GFP expression under the same promoter. Comparison between the expressions of CRLF2 and the control GFP confirmed that the expanded $CD19^+$ population that was observed in the CRLF2 group was due to the gene's specific expression and not a result of promoter-biased selection.

We further show that expression of CRLF2 with ubiquitous promoter inhibited differentiation to the lymphoid lineage. In a single analysis, increased myeloid differentiation ($CD14^+$ $CD15^+$) was observed (Figure 2–3) These results are not surprising as CRLF2 is originally a myeloid receptor and can be endogenously found in high levels on granulocytes and macrophages. The findings emphasize that the milieu in which the receptor is activated is critical to the outcome. A probable explanation for the cellular-context-dependent outcome is that CRLF2 activates common signaling pathways (JAK-STAT, PI3K), which activate available target genes in accordance with the other transcription factors activated in the cell.

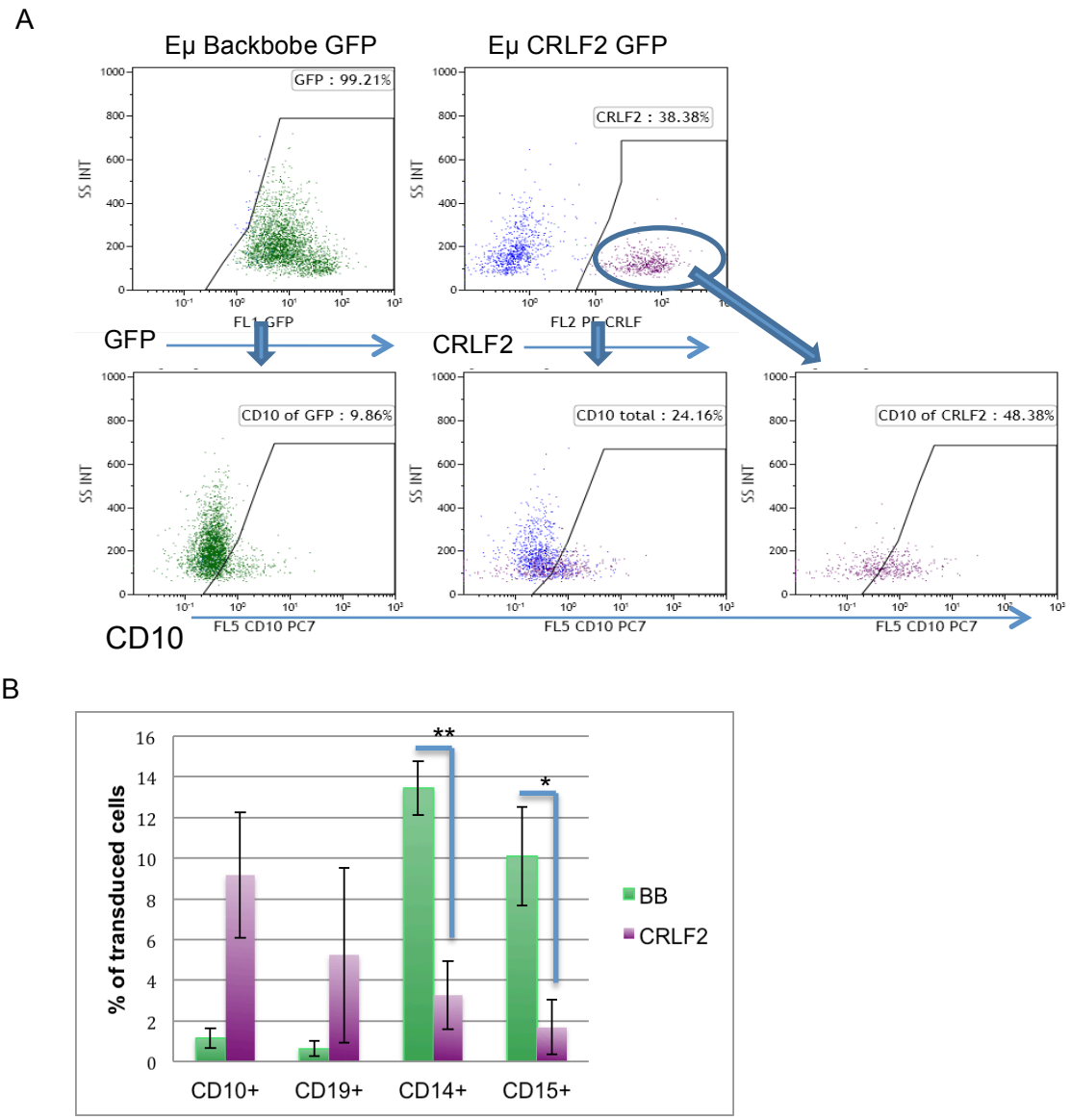

Figure 2–6 Enhanced lymphoid differentiation and reduced myeloid differentiation of CB progenitors expressing CRLF2 from B-cell promoter in short term MS5 culture.

Representative flow cytometry scatter plot (A) and quantitative differentiation analysis (B) of pRRL-Eμ-B29 backbone (Eμ backbone) and pRRL-Eμ-B29-CRLF2-T2A-GFP (Eμ-CRLF2-GFP) transduced CD34^{+}after short (2-2.5 weeks) culture on MS5. A) Human live cells were gated for transduced positive cells (upper). Whole wells of Eμ backbone or CRLF2-T2A-GFP transduced (lower left and middle respectively) and CRLF2 gated cells (lower right) were analyzed for the expression of CD10. B) Representative quantitative analysis of CD10, CD19, CD14 and CD15 of MS5 culture (n = 3 for each virus) supernatant cells. Graph shows mean +/- SEM; *p < 0.05, **p < 0.01 (unpaired t-test).

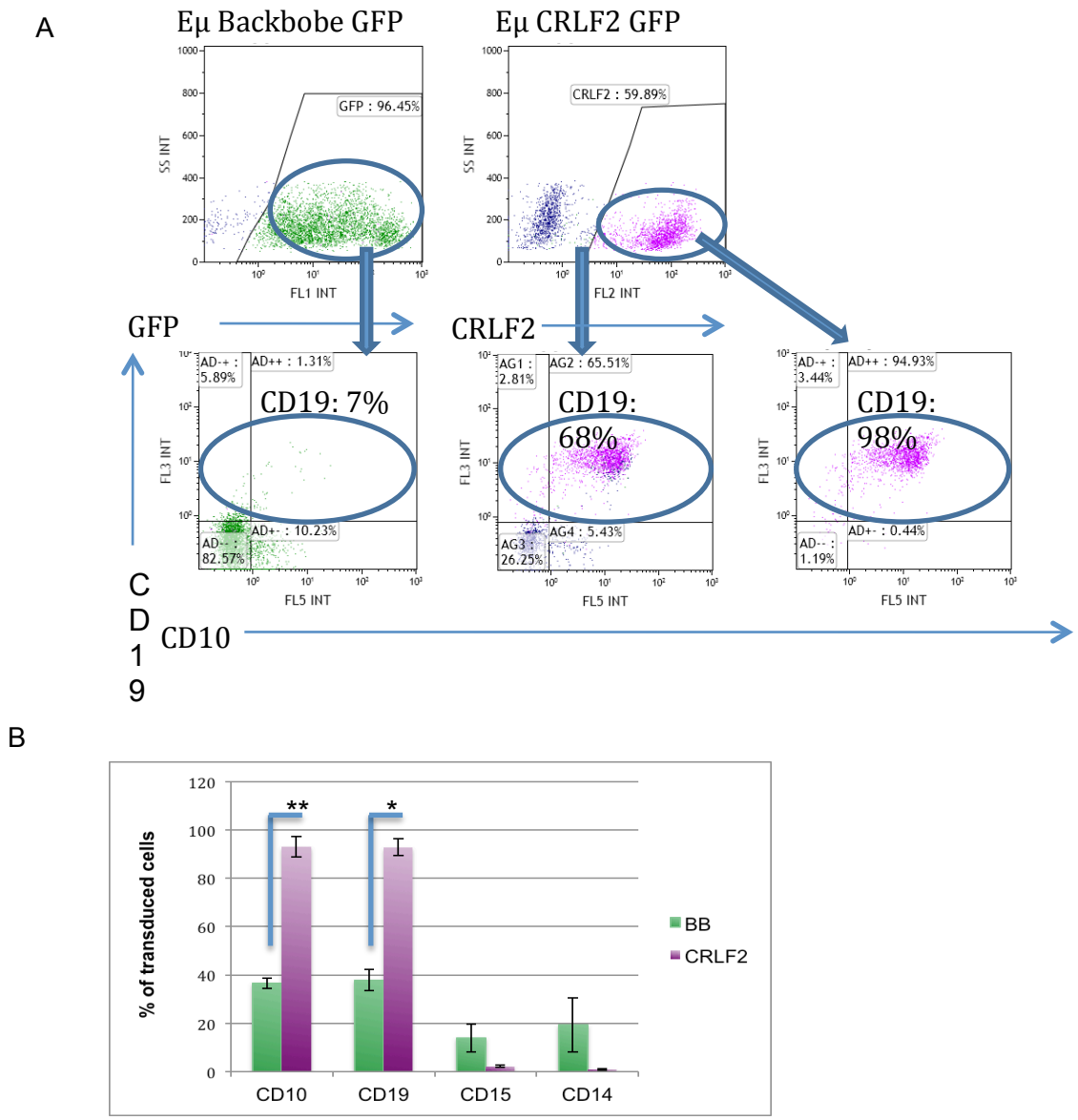

Figure 2–7 B-cell-directed overexpression of CRLF2 in CB CD34$^+$ progenitors enhances B-cell lineage differentiation.
Representative flow cytometry scatter plot (A) and quantitative differentiation analysis (B) of pRRL-Eμ-B29 backbone (Eμ backbone) and pRRL-Eμ-B29-CRLF2-T2A-GFP (Eμ-CRLF2-GFP) transduced CD34$^+$after long (4-5 weeks) culture on MS5. A) Human live cells were gated for transduced positive cells (upper). Whole wells of Eμ backbone or CRLF2-T2A-GFP transduced (lower left and middle respectively) and CRLF2 gated cells (lower right) were analyzed for the expression of CD10 and CD19. B) Representative quantitative analysis of CD10, CD19, CD14 and CD15 of MS5 culture (n = 3 for each virus) supernatant cells. Graph shows mean +/- SEM; *p < 0.05, **p < 0.01 (unpaired t-test).

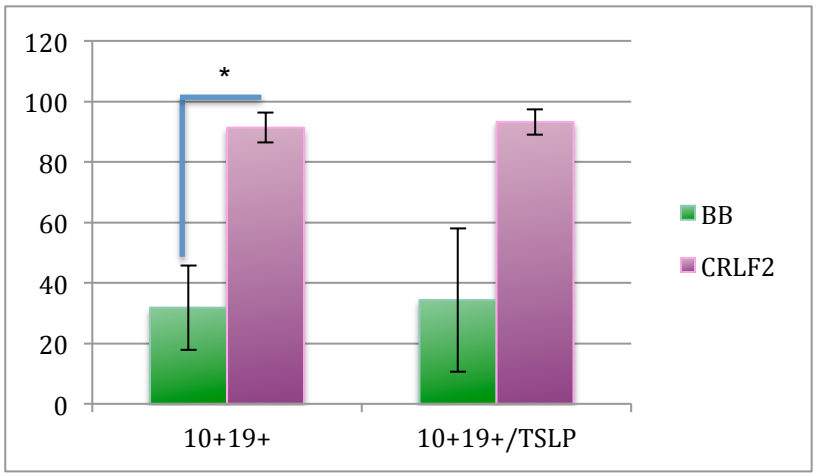

Figure 2–8 Lymphoid differentiation on TSLP expression MS5 stroma.
Quantitative $CD10^{+}CD19^{+}$ expression analysis of transduced cells after 4 weeks on regular MS5 or on TSLP expressing MS5 (/TSLP) (n = 3 for each of the transducing vectors pRRL-Eμ-B29-backbone, pRRL-Eμ-B29-CRLF2-T2A-GFP. Graphs shows mean +/- SEM *$p \leq 0.05$. B) Representative flow cytometer plot of CD10 CD19 analysis of gated transduced cells after 4 weeks in MS5 culture.

Parts of chapter 2 may be used in the future for publication. Ifat Geron, Angela Savino, Nava Gershman, Noa Tal and Shai Izraeli. “The role of CRLF2/IL7RA signaling in the development of B-ALL”. The dissertation author is expected to be the primary author and researcher of the manuscript.

Chapter 3 Aberrant activation of the TSLP pathway promotes a pre-leukemic differentiation pattern

3.1 Introduction

It is believed that ALL is a disease in which a chain of mutations acquired by a clone reaches a critical event load, bidding transformation to an overt disease. This paradigm is supported by the fact that in studies of neonatal blood spots (Guthrie cards), as many as 1% of newborns harbored ETV6-RUNX1 transformation, and yet the incidence of an ETV6-RUNX1 leukemia is 1:10000[186]. It is further strengthened by reports of twins baring the same sentinel translocations, with only one twin developing full-blown leukemia despite the similarity in genetic background[14,187,188]. Aberrant expression of the cytokine receptor CRLF2 was found in 60% of DS-ALLs and in up to 10% of sporadic B-cell precursor ALLs. Although transduction of mouse fetal liver cells with CRLF2 enhances proliferation and maintains a less differentiated state[2], expression of CRLF2 is not enough to wean BaF3 from cytokines[3]. This suggests that CRLF aberrations alone are not sufficient to activate the TSLP pathway and initiate transformation. Overexpression of CRLF2 is commonly associated with mutations in other genes in the pathway, most prominently in JAK2, but other mutations in the pathway like IL7RA JAK1 and SH2B3 have also been reported[1-4,106] and reviewed in[85,93]. This led us to the hypothesize that mutations in JAK2 or other pathway genes follow high CRLF2 expression, thus aberrantly activating the TSLP pathway and leading to pre-malignant behavior. To gain insight into the role of secondary mutations in the TSLP pathway in

leukemogenesis, we used the B-cell expression system described in the previous chapter.

3.2 Results

3.2.1 Addition of mutated JAK2

JAK2 is a signal transducer in the TSLP pathway. It binds the CRLF2 unit in the receptor and phosphorylates both the CRLF2 and the JAK1 bound to the IL7RA receptor subunit to start the STAT and mTOR signal transduction. Mutations in JAK2, particularly around Arginine 683 are the most common mutations associated with CRLF2 translocations[5-9]. These mutations are unique to B-ALL and found almost exclusively in CRLF2 cases[135]. Expression of CRLF2 together with JAK2R683 mutation allowed cytokine-free survival of the cytokine-dependent BaF3 cell line. In all current B-ALL research in which JAK2 overexpression was studied, mouse JAK2 was utilized. The use of human JAK2 was avoided due to difficulties in cloning and maintenance of the human gene in viral vectors (personal communications).

Since this research focuses on human cells, we determined that the use of the human gene is important and therefore set out to clone mutated human JAK2. To do so, we replaced the relevant segment in the wild type gene I previously cloned with an amplified mutated segment (see Materials and Methods). The clone was sequenced (Figure 3–1) and JAK2 expression was verified by flow cytometry in cell lines (Figure 3–4).

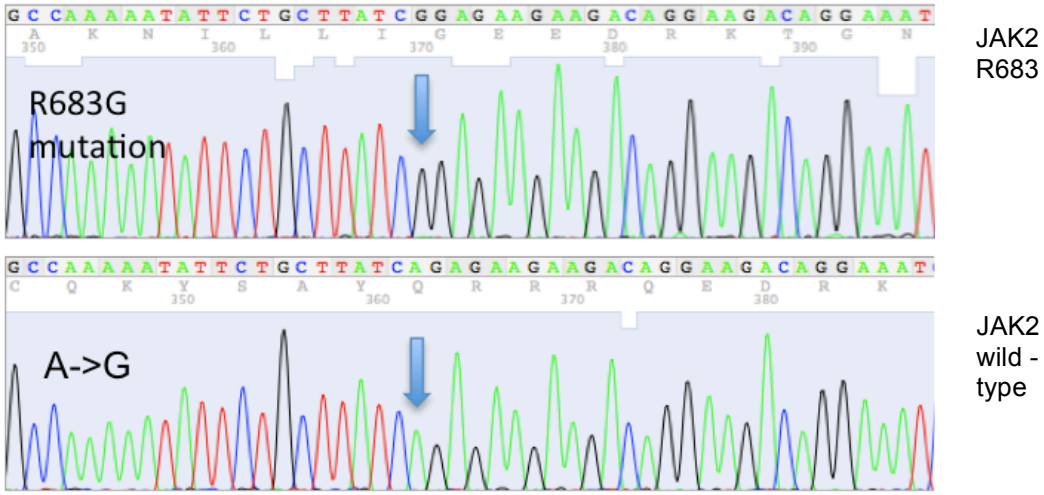

Figure 3–1 Sequence of cloned mutated JAK2.
Electropherogram of cloned JAK2. Arrows pointing at the mutation.

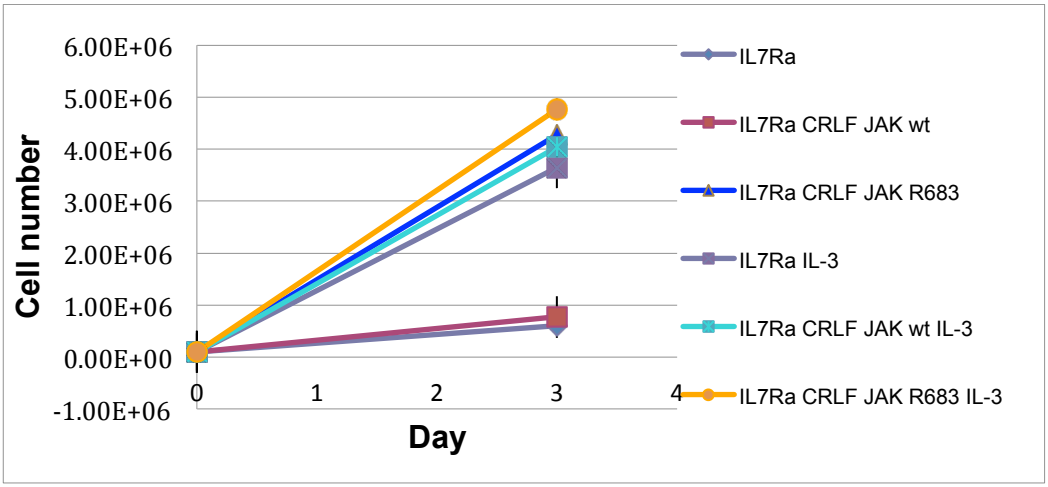

Figure 3–2 CRLF2-JAK2 R683 renders BaF3 cytokine independent.
Growth curve of IL7RA expressing Ba/F3. The cells were transduced with Lenti vectors expressing CRLF2 JAKwt or CRLF2 JAKR683G and grown with or without IL3 (10ng/ml).

3.2.2 JAK2 overexpression inhibits growth of transduced CB CD34$^+$ progenitors

The virus expressing CRLF2 JAK2 was tested and its ability to induce expression of JAK2 and CRLF2 was verified on 018Z and 293 cell lines. Additionally, as depicted in the activity of the genes expressed form the virus was confirmed by testing the reported ability of the CRLF2-JAKR683 combination to rescue Ba/F3 in cytokine withdrawal assay[1,3,4].However, while transducing CB CD34$^+$ progenitors, the CRLF2- JAK2 R683G transduced population was minimal when compared to CRLF2-GFP and control GFP viruses (Figure 3–3). Although the control GFP virus titer was high (~5X10^8 IU/ml) CRLF2-GFP and CRLF2-JAKR683G virus titers were comparable (~7X10^7-1X10^{8}IU/ml). Diminished CRLF2 JAK2 R683G population was also observed in transduced cell lines, albeit in reduced severity. Two possible explanations for this result are A) cell death or B) halted proliferation of the transduced cells.

The first option was rejected after similar cell death was measured by annexin/7AAD assay in the CRLF2-JAKR683 group and CRLF alone/GFP control group of transduced 018Z cell line (Figure 3–4).

Growth measurements revealed deceleration in CRLF2 JAK R683 transduced cell proliferation compared to the control CRLF2 and GFP transduced cells (Figure 3-5). Additionally, cells that were grown after sorting partially lost their phenotype – probably due to faster proliferation of the residual untransduced cells (Figure 3–4 upper panel).

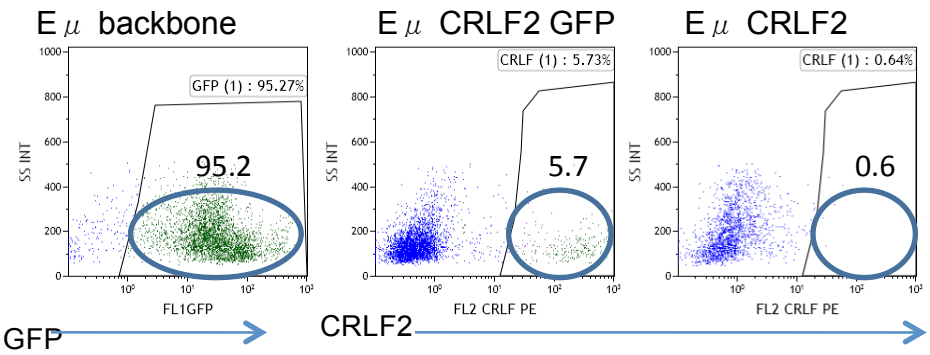

Figure 3–3 CRLF2-JAK2 R683 transduced population in culture.
Representative flow cytometry analysis of transduced CD34$^+$ CB **CD34$^+$** progenitors cultured on MS5 (comparable titer of pRRL Eμ B29 CRLF2-GFP and pRRL Eμ B29 CRLF2-JAKR683 viruses were used (~7X10^7-1X10^8 IU/ml), the titer of the pRRL Eμ B29 backbone was higher (~5X10^8 IU/ml)).

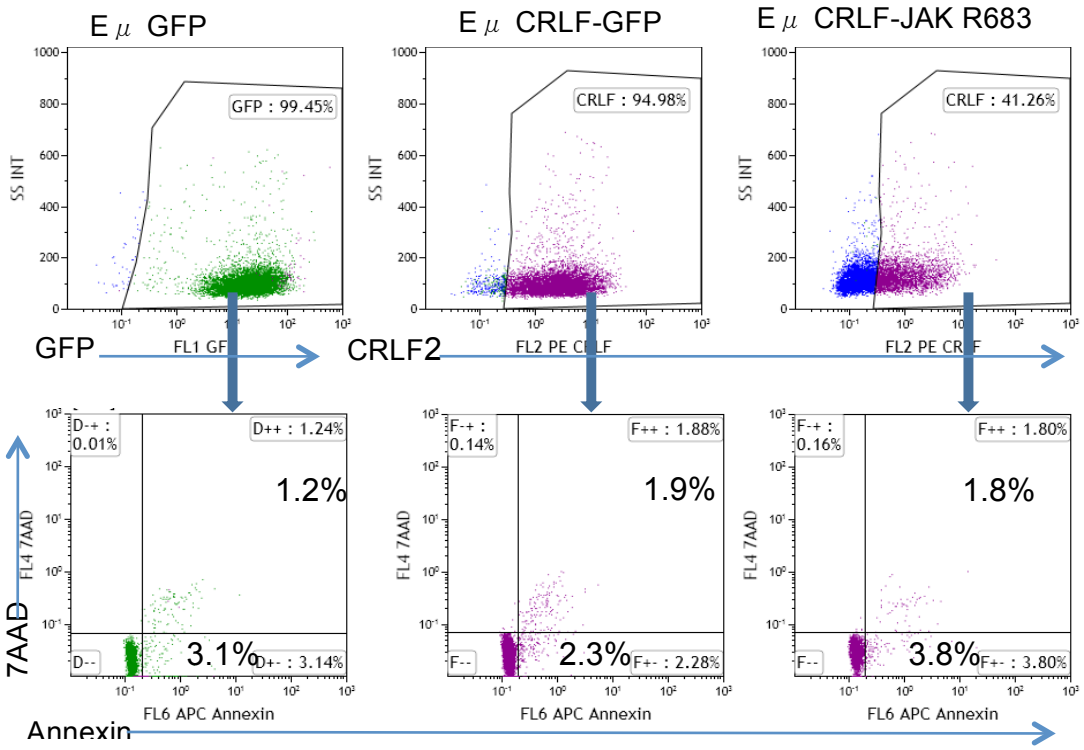

Figure 3–4 Comparable viability between CRLF2-JAK2 R683 expressing population and control populations.
Annexin analysis of 018Z cells after transduction with pRRL Eμ B29 backbone, pRRL Eμ B29 CRLF2-GFP and pRRL Eμ B29 CRLF2-JAKR683. Cells were sorted and grown in culture for 8 days before analysis. Viable cells were gated for transduction (top) and transduced cells were analyzed for Annexin and 7AAD staining.

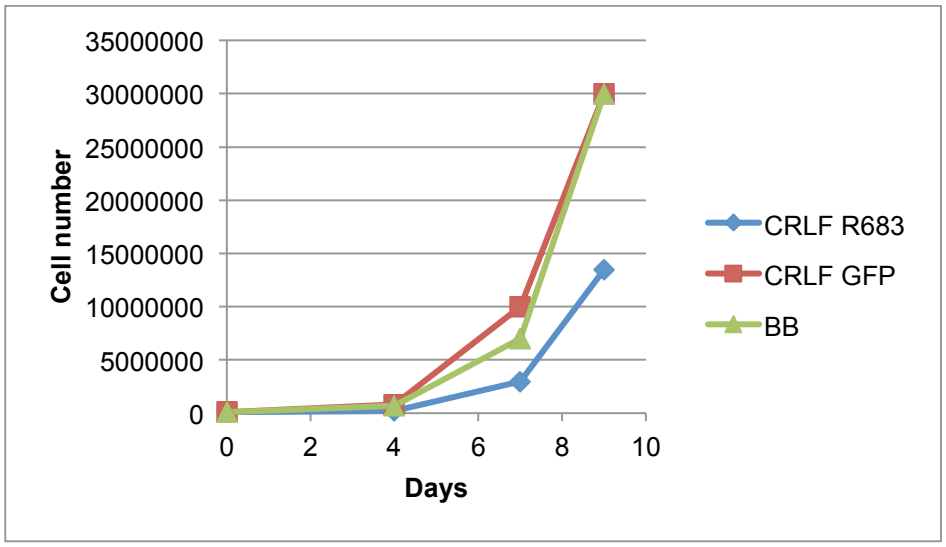

Figure 3–5 CRLF2 JAK2 R683 inhibits growth of 018Z-transduced cells.
Growth curve of 018Z transduced with either pRRL Eμ B29 backbone, pRRL Eμ B29 CRLF2-GFP or pRRL Eμ B29 CRLF2-JAKR683 and sorted. The count was continuous for 8 days (4 passages). Number of cells was extrapolated for passage dilution adjustment.

Quantitatively, JAK2 expression in B-ALL cells is similar to normal cells even though it is mutated, whereas in transduced cells the JAK mutation is overexpressed. Non-physiological general hyper-activation of the JAK STAT pathway might cause proliferation arrest.

3.2.3 Using IL7RA as second TSLP gene

Given our inability to maintain JAK2 transduced cells in culture, another TSLP pathway partner was tested as a secondary mutation. IL7RA, the second subunit of the TSLP receptor, was found in our lab to be mutated in several cases of B-ALL patients, as an additional mutation in CRLF2 overexpression[10,162].

We chose the insertion mutation IL7RA 243ins PPCL as a secondary mutation in CRLF2 overexpression as this mutation displayed a strong activation of STAT5 and RPS6[10]. IL7RA 243ins PPCL (IL7RAins) and IL7RA wild type (IL7RAwt) were cloned as a second gene in the Eμ vector after CRLF2 to form pRRL-Eμ-B29-CRLF2-T2A-IL7RAins and pRRL-Eμ-B29-CRLF2-T2A-IL7RAwt vectors (see Materials and Methods). The vectors were sequenced and the expression of IL7RA was verified by flow cytometry (Figure 3–6). To verify the activity of the expressed receptor, phosphorylation assay of activated downstream STAT5 was performed. As depicted in Figure 3–7, upon stimulation of the cells with IL-7 and TSLP cytokines, STAT5 was phosphorylated, confirming that the overexpressed receptor was active and delivered the signal. Interestingly, maximal activity of the receptor formed after CRLF2-IL7RAins transduction was reached following stimulation with IL-7 rather than with TSLP, as was in the case for CRLF2-IL7RAwt. This can be explained by the strong affinity of the IL7RAins unit to itself, resulting in a homodimer receptor, and is supported by the ability of IL7RAins alone to confer cytokine independence phenotype in the IL3 growth defendant BaF3 cell line[10]. The homodimer receptor is susceptible to IL7 binding and stimulation.

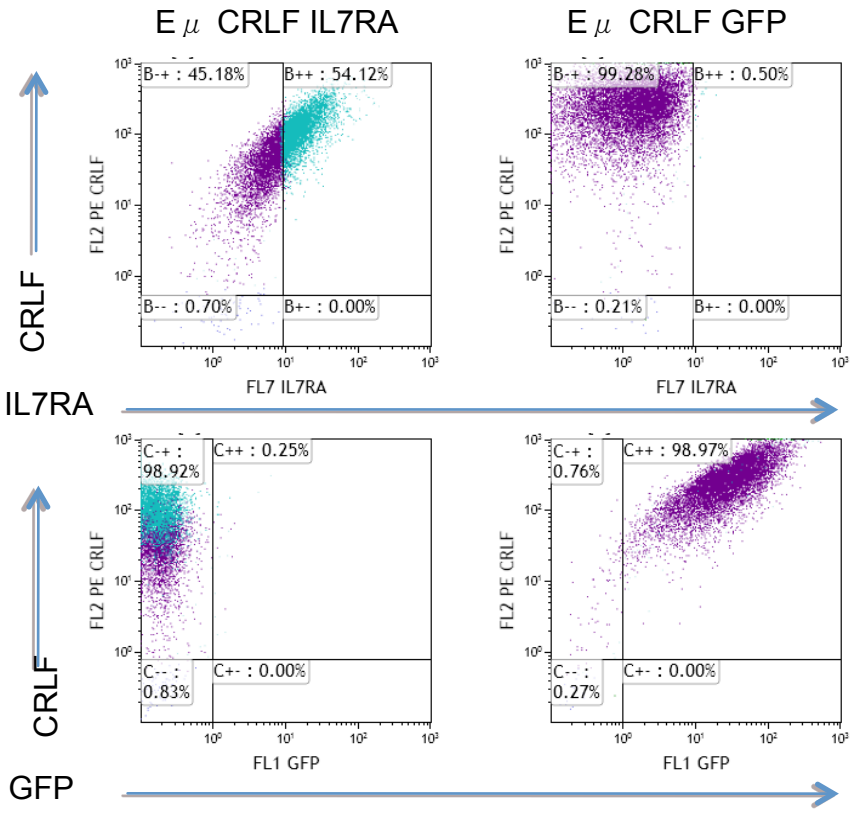

Figure 3–6 IL7RA is expressed in transduced cells.
Flow cytometer scatter plot of 018Z cells. Cells were transduced with lentivirus encoding CRLF2 GFP or CRLF2 IL7RA, cultured for 72hrs, stained and analyzed by flow cytometer for the expression of GFP, CRLF2 and IL7RA.

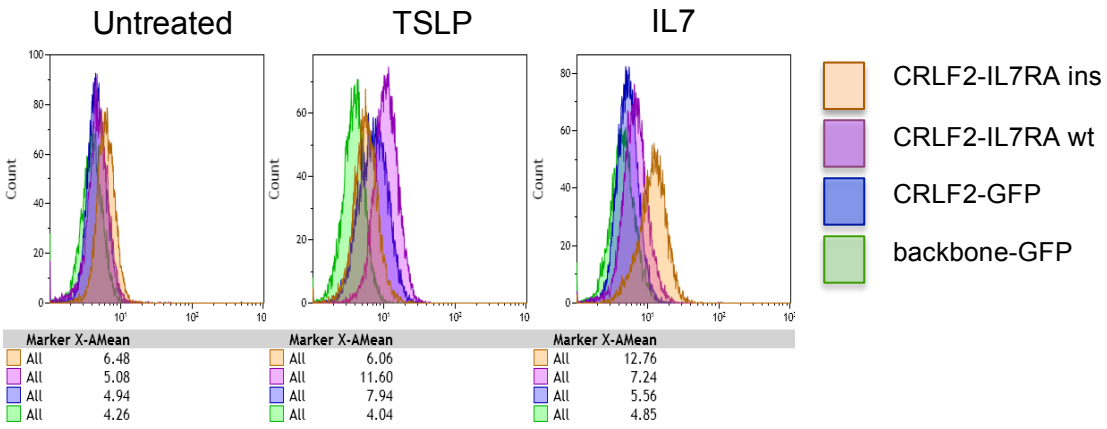

Figure 3–7 Transduction with CRLF2 IL7RA lentivector results in expression of active receptor.
Flow cytometer histogram of phospho STAT5 Transduced 018Z were starved and activated with TSLP (2ng/ml) or IL7 (2ng/ml) and subjected to phospho STAT5 staining and flow cytometer analysis. Mean fluorescent intensities are portrayed in the histogram and the values are listed below.

3.2.4 IL7RA overexpression in combination with CRLF2 supports B-cell differentiation *in vitro*

After confirming that the cloned receptors were active, we proceeded to CB transduction. As soon as two weeks after culturing CB $CD34^+$ that were transduced with pRRL-Eμ-B29-CRLF2-T2A-IL7RAins and pRRL-Eμ-B29-CRLF2-T2A-IL7RAwt vectors, a large population of the CRLF2-IL7RAins transduced cells had differentiated to $CD10^+$ cells while the BB control cells had only a minor pro-lymphoid population (Figure 3–8). Analysis of a longer (4 week) culture revealed a trend toward greater $CD19^+$ differentiation in the IL7RA transduced groups. However, at this stage, the variability within the groups was high and differences between the groups were not statistically significant.

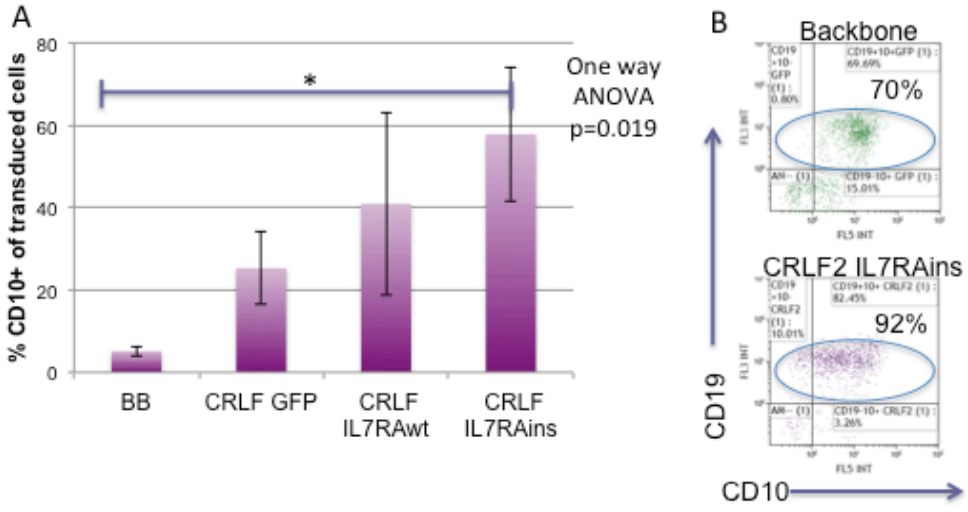

Figure 3–8 CRLF2/IL7RAins expression accelerates differentiation to lymphoid lineage.
A) Quantitative $CD10^+$ expression analysis of transduced cells after 2.5 weeks on MS5 (n = 3 for each of the transducing vectors pRRL-Eμ-B29-backbone, pRRL-Eμ-B29-CRLF2-T2A-GFP, pRRL-Eμ-B29-CRLF2-T2A-IL7RAins and pRRL-Eμ-B29-CRLF2-T2A-IL7RAwt). Graphs shows mean +/- SEM $*p \leq 0.05$. B) Representative flow cytometer plot of CD10 CD19 analysis of gated transduced cells after 4 weeks in MS5 culture.

3.2.5 Aberrant activation of the TSLP pathway in CB CD34$^+$ progenitors *in vivo* alters the differentiation in the Pro-Pre B-cell stage

In vitro experiments can contribute to the understanding of some of the transgene effects on normal cells. Nevertheless, the MS5 co-culture system is limited in its ability to support full differentiation of B-cells; the cell's differentiation on the stroma is blocked in the pro/pre B-cell stage, and rarely allows for differentiation into an immature cell, which is the critical stage in distinguishing between normal and leukemic differentiation. Additionally, the transduced CD34$^+$ population is variable and complex and the in vitro system requires a low number of cells to be plated on each well of the experimental plate, thus the composition of each experimental well is not a representative sample of the population As a result, lack of consistency between replicates often compromises the data. Engrafting cells in a xenograft setting allows near-optimal examination of transgene effects on the cell's differentiation capacity. Moreover, since the initial number of transduced CD34$^+$ progenitors engrafted in a mouse is large and represent the diverse progenitor population, the technical problem that arises *in vitro* due to initial difference in the small replicates population is avoided. NOD/LtSz-*scid IL2Rγ*^{null} (NSG) mice are immune deficient mice known to support human B-cell differentiation[189,190]. CD34$^+$ CB cells that are transplanted intravenously in NSG mice will home to the BM and start differentiating. The majority (up to ~80%) of the human cells in NSG mice differentiate to CD19$^+$ cells, unlike the expected normal lineage distribution in humans. This is thought to be due to cross-reactivity between mouse and

human IL7 – IL7RA in contrast to lack of cross-reactivity of other myeloid cytokines. Nonetheless, this trait is favorable for B-ALL research.

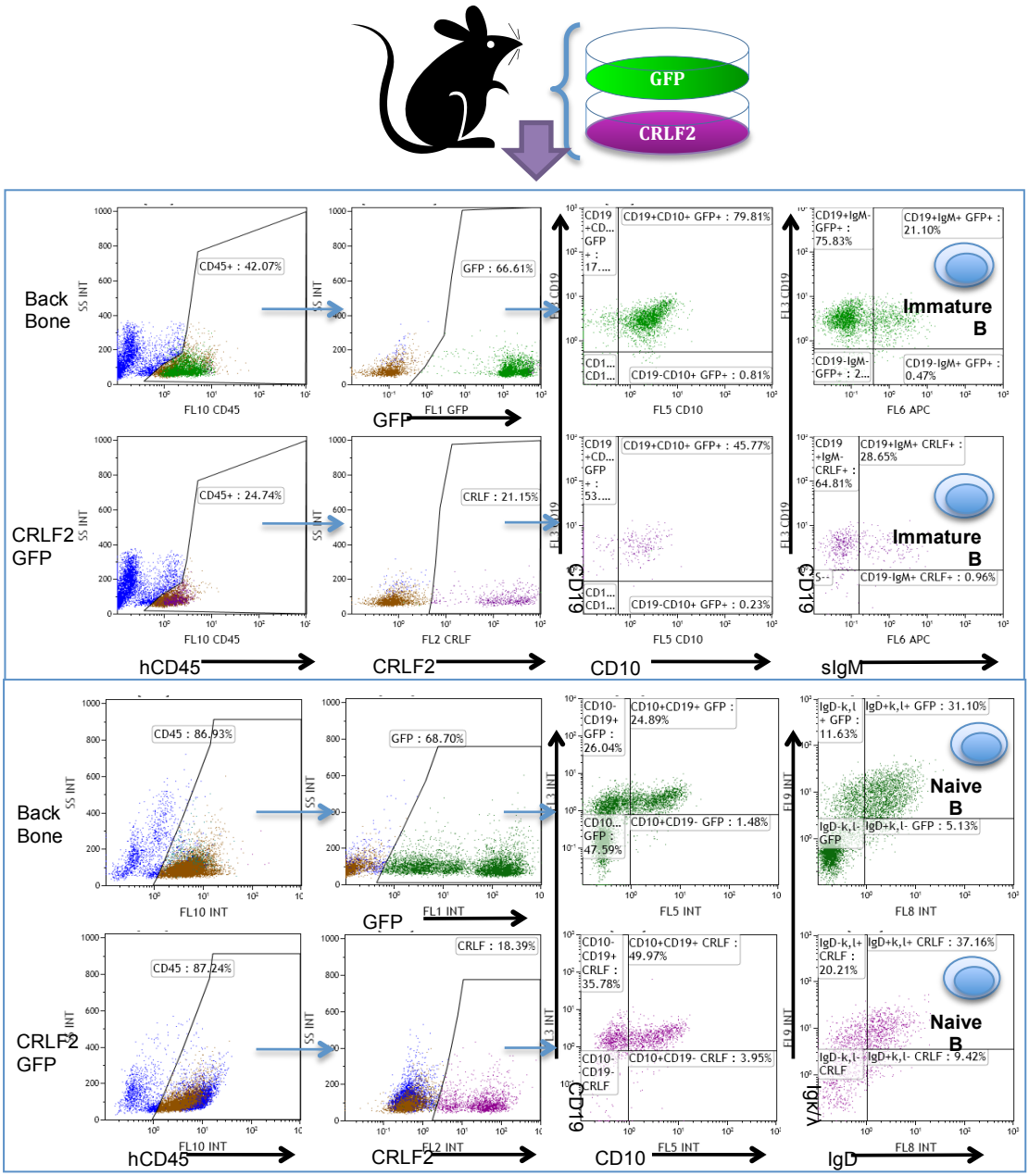

Figure 3–9 Transduced human CB cells support multi-lineage engraftment. Flow cytometry analysis and gating strategy of hematopoietic organs from xenografts. Upper panel: representative analysis of two BM samples from 20 week xenografts. Lower panel: representative analysis of two spleen samples from 26 week xenografts. Blue arrows indicate that the gated population was analyzed in the following scatters.

We set up a preliminary xenograft model and tested whether we could detect $CD19^+$ transduced CB cells in a long-term engraftment. As seen in Figure 3–9, 24-30 weeks post-engraftment, transduced cells could be detected in the hematopoietic tissues of the engrafted mice. These cells reached their full differentiation potential, as evidenced by expression of sIgM, IgD and Igκ/λ light chains. One of the markers of leukemic transformation is the block of differentiation in the pro-pre stage. This preliminary experiment demonstrated that CRLF2 alone is not sufficient to block differentiation, as the differentiation of $CRLF2^+$ cells was similar to the control BB group.

We next set out to evaluate whether the addition of IL7RA would convey any pro-leukemic transformation on transduced CB *in vivo*. A total of 56 mice were transplanted with transduced CB cells (see Materials and Methods) in a total of 16 transduction/transplant experiments, with 5 experimental conditions: untransduced, backbone (BB), CRLF2-GFP, CRLF2-IL7RAwt and CRLF2-IL7RAins denoting the virus that was used to transduce the cord blood. Seventeen to thirty-one weeks after engraftment, the mice were sacrificed and hematopoietic tissues were harvested. Rather than a true biological control, the untransduced group was used for staining control as it was not treated with virus like the other experimental groups and therefore was not included in the analysis. The differentiation state of human B lineage cells in the spleen and BM was determined by flow cytometer using the gating strategy described in figure 3-10. We first inquired whether the *in vitro* effect of increased B-cell population of cells expressing CRLF2 alone or in combination with IL7RA, will

be verified in the *in vivo* model. Transduction and engraftment efficiencies differed between groups and between experiments; only mice with clear population of transduced human cells (over 2%/50$CD45^+$cells and over 3%/30transduced cells –as set by untransduced group background) were analyzed. As portrayed in figure 3-11, There was a marked change in the B-cell relative population between the transduced cells groups with a clear trend of enlarged B-cell population in CRLF2 transduced cells (one way ANOVA spleen [F(3,24)=6.099,p=0.0031], BM [F(3,29)=3.060, p=0.0438]). These changes were especially significant in the spleen of mice transplanted with CRLF2-IL7RAwt/ins transduced CB when compared to the BB group (Tukey post hoc analysis p≤0.05).

We then analyzed the differentiation pattern of B-lineage transduced cells. For this analysis only mice with clear (over background) transduced $CD45^+CD19^+$ population at sacrifice were analyzed A one way ANOVA was conducted to compare the effect of enforced expression of the transgenes on differentiation of B-cells in the BM and spleen of transplanted mice, focusing on the precursor B-cell population, which encompasses the leukemic cells in B-ALL. Activating mutations in IL7RA blocked B-cell differentiation in spleens of transplanted mice on the expense of immature/naive B cells as evident by increased B-cell precursors (Figure 3-12 panels A,B) ([$F(3,30) = 8.123$, $p = 0.0004$] for Pro/Pre B-cells and [$F(3,33) = 5.599$, $p = 0.0032$] for immature/naïve B-cells) Post hoc analysis verified the statistical differences between the CRLF2 IL7RAins group to all of the other groups and not in

between the other groups. Similarly, the differentiation of the CRLF2 IL7RAins transduced cells to $sIgM^+$ immature and naïve cells in the BM of transplanted mice was blocked, [$F(3,33) = 5.449$, $p = 0.0037$)] as compared to the BB group and the CRLF2 IL7RAwt group ($p \leq 0.01$ and $p \leq 0.05$, respectively, using Tukey's post hoc analysis) Figure 3-12 panel D). As most of the $CD19^+$ cells in the BM were in an undifferentiated state, there was no significant increase in the pro-pre population in the transplanted BM between the transgenes Figure 3-12 panel C).

Together, these results demonstrate that overexpression of CRLF2 IL7RAins in human B-cell lineage progenitors might contribute to a pre-leukemic state by halting differentiation of B-cell progenitors to naïve B cells.

Halting the differentiation in progenitor state preconditions B-cells for the development of ALL. Providing a growth or survival advantage exerts an additional oncogenic effect towards the development of full-blown leukemia. We tested whether transduced cells had a survival/growth advantage by measuring the expansion of these cells *in vivo*, as portrayed by the relative size in the total human population post-transplant compared to the percentage on the day of the transplant. For this analysis all mice with visible (above untransduced background) transduction on the day of transplant and at the day of sacrifice were included. As depicted in Figure 3–13, there was a significant increase in the percentage CRLF2 IL7RAins transduced population in BM of transplanted mice (one way ANOVA [$F(3,30) = 3.583$, $p = 0.0252$)]. The same trend, though not statistically significant, was observed in the spleen.

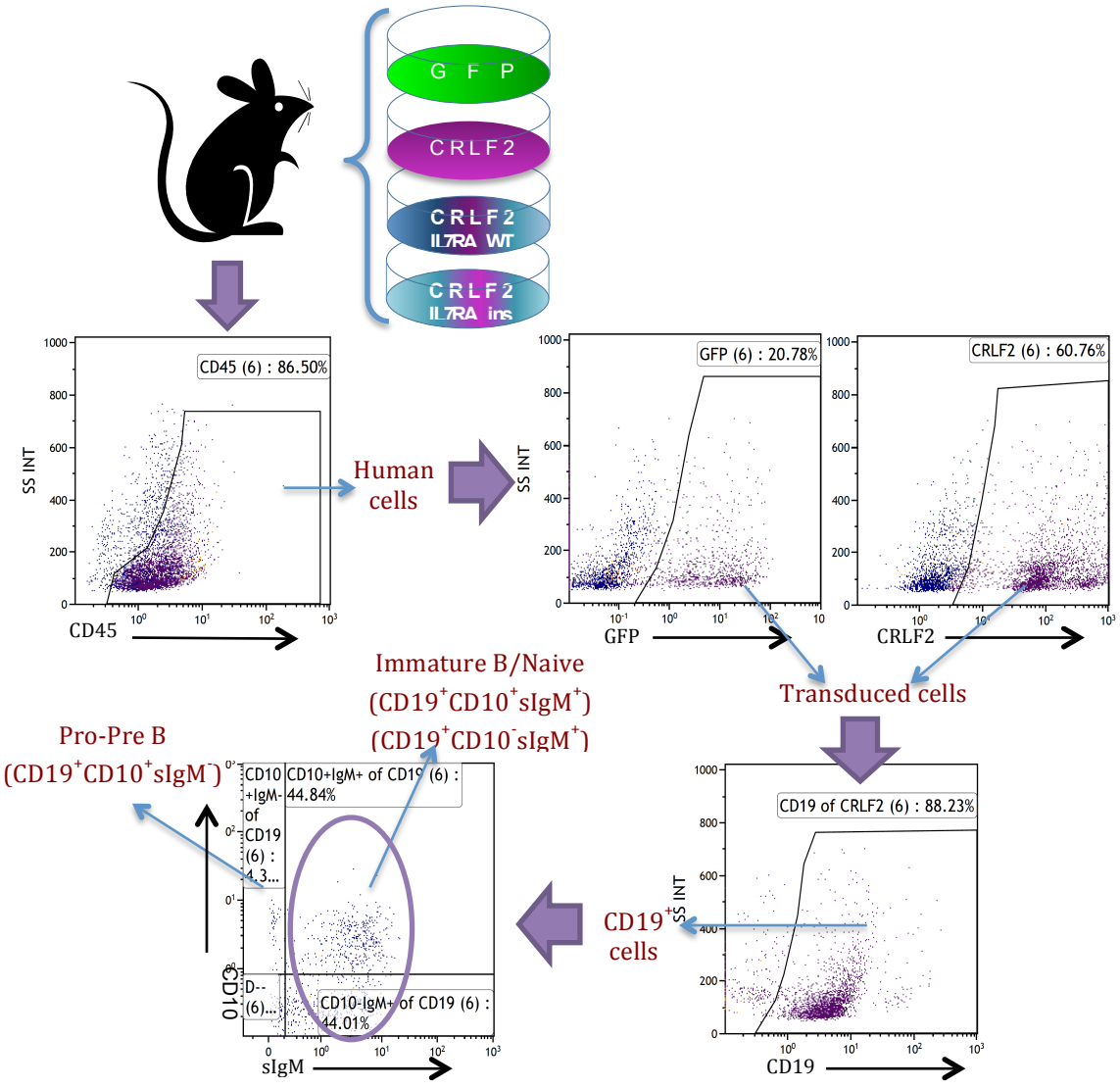

Figure 3–10 Experimental design for measuring the effects of overexpressing TSLP pathway genes on the differentiation of CD34$^+$ CB *in vivo.*

Flow cytometry analysis and gating strategy for analysis of early B-cell differentiation. Blue arrows indicate that the gated population was analyzed in the following scatters.

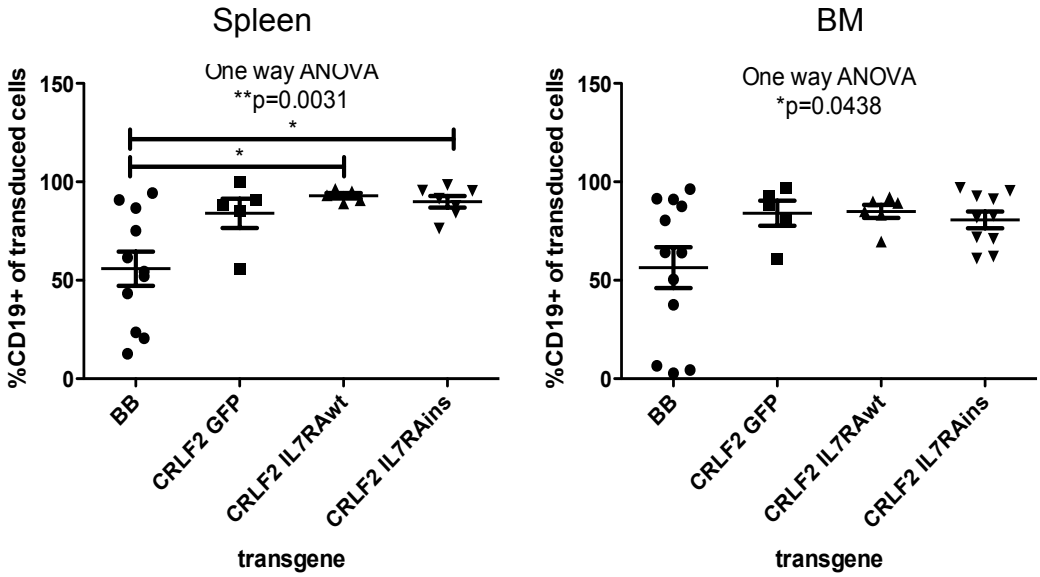

Figure 3–11 Enhanced B-cell differentiation of CRLF2 transduced CD34$^+$ CB cells *in vivo.*

Relative CD19$^+$ population of engrafted transduced CB in spleen and BM. Cells expressing GFP BB (spleen n = 11, BM n=12), CRLF GFP (n = 5), CRLF2 IL7RAwt (spleen n = 5, BM n=6) and CRLF2 IL7RAins (spleen n = 7, BM n=10) in spleen (left) and BM (right) of engrafted mice. Plots show sample scatter dot with mean +/- SEM. Statistical analyses were performed using one way ANOVA. *p < 0.05, using Tukey's post-hoc analysis.

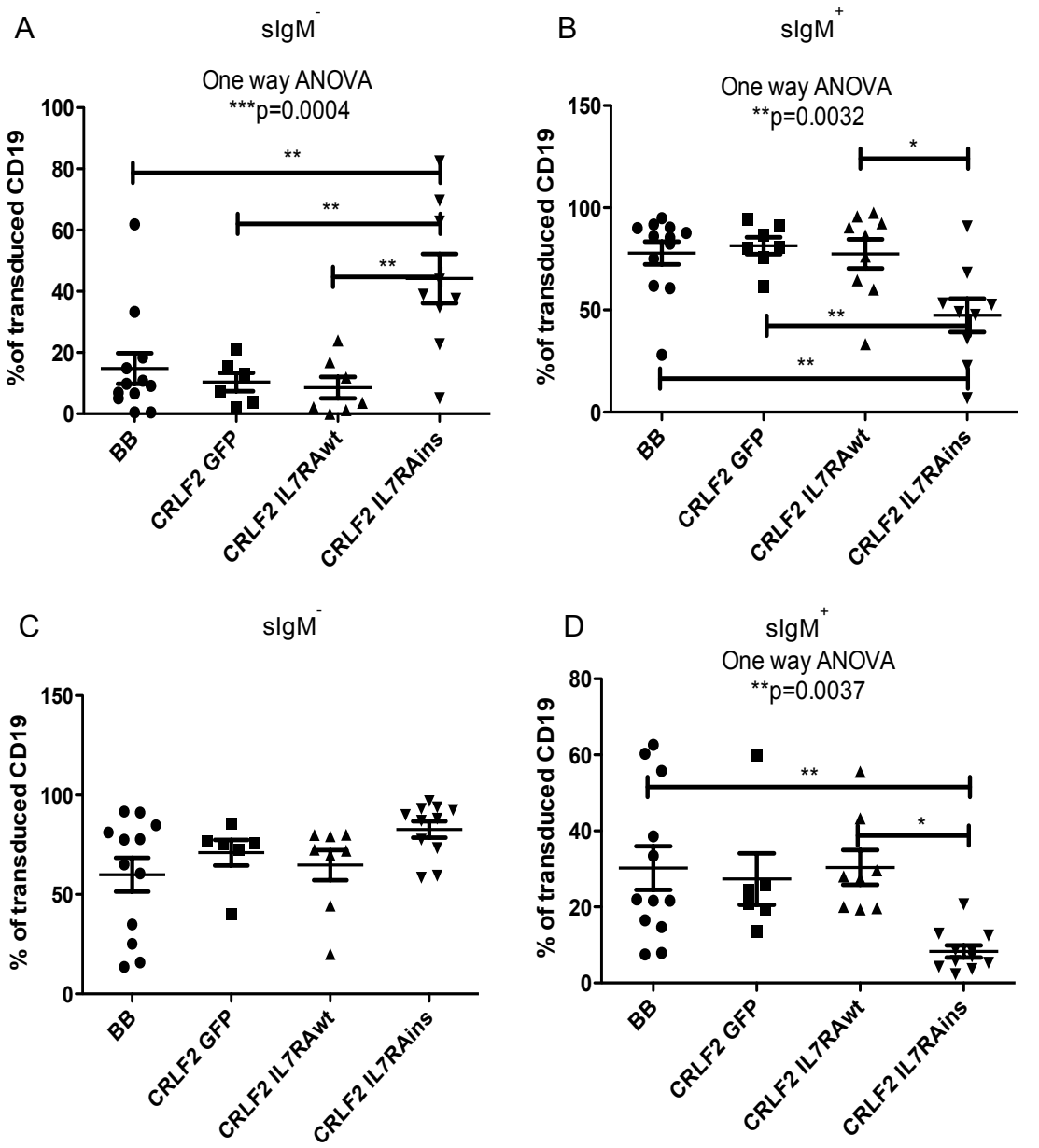

Figure 3–12 CRLF2 IL7RA transduction of CB CD34$^+$ progenitors alters B lineage differentiation in spleen and BM of transplanted mice.
Relative differentiation of human CB CD34$^+$ cells expressing GFP (BB, n = 12), CRLF GFP (n = 6), CRLF2 IL7RAwt (spleen n = 7; BM n = 8) and CRLF2 IL7RAins (spleen n = 9; BM n = 11) in spleen (A, B) and BM (C,D) of engrafted mice. A+C – differentiation to pro-pre B-cells (sIgM-); B+D – differentiation to immature/naïve (sIgM+) B-cells. Plots show sample scatter dot with mean +/- SEM. Statistical analyses were performed using one way ANOVA. *p < 0.05, **p < 0.01 using Tukey's post-hoc analysis.

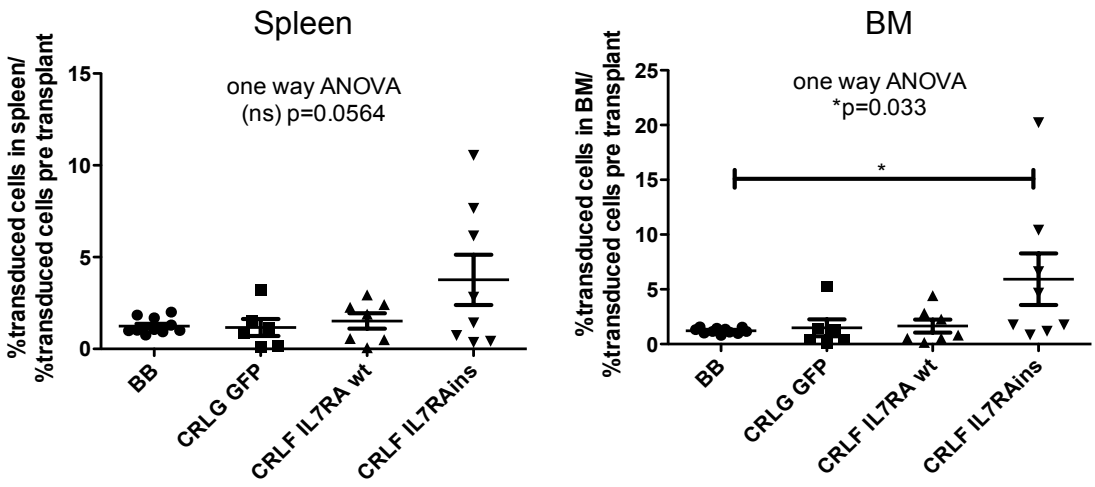

Figure 3–13 Overexpression of CRLF2 IL7RAins results in expansion of transduced cells.
Relative change in size of transduced CB population in xenografts. Cells expressing GFP (BB n = 11), CRLF GFP (n = 6), CRLF2 IL7RAwt (n = 7) and CRLF2 IL7RAins (n = 8) in spleen (left) and BM (right) of engrafted mice. Plots show sample scatter dot with mean +/- SEM. Statistical analyses were performed using one way ANOVA. *p < 0.05, using Tukey's post-hoc analysis.

3.2.6 Summary

The two hit model by Kelly and Gilliland[191] suggested that the development of overt leukemia requires both block in differentiation, usually acquired by perturbation of transcription factor and events that will give proliferative/survival advantage. Aberrant activation of kinase pathway such as RAS/FLT3/KIT/JAK-STAT may act as a single hit, grant the cell proliferative/survival advantage and instill a pre leukemic state. Here, we tested the hypothesis that acquisition of TSLP/CRLF2/IL7RA pathway aberrations may spur/incite pre-leukemic trait.

Over expression of CRLF2 alone is not sufficient for cytokine free activation of the TSLP pathway as evident by its inability to confer cytokine free

growth phenotype to Ba/F3 cells [1,3]. Indeed a second activating mutation in the TSLP pathway is often detected in CRLF2 aberrant B-ALL. We first attempted over expressing CRLF2 and mutated JAK2, as the majority of additional TSLP pathway mutations are JAK2 activating mutations. Surprisingly, over expression of JAK2 and in particular its mutated form diminished the population of transduced cells (Figure 3–3) probably due to growth inhibition as was evident upon transducing 018Z B-ALL cells with the same CRLF2-JAK2 expressing vectors (Figure 3-5).

Our group previously reported the finding of activated IL7RA mutants in CRLF2-positive B-ALL[10]. One of these IL7RA mutants (ins PPCL) was then used after activity verification (Figure 3–7) to complete the downstream pathway activation in our experiments. We found that the additional expression of IL7RAins in transduced cord blood $CD34^+$ progenitors significantly accelerated lymphoid differentiation *in vitro* (Figure 3–8), This finding may suggest a capacity of the mutation to affect normal differentiation. Nevertheless, over expression of the wild type IL7RA alongside CRLF2, resulted in a similar though diminished trend and did not reach statistical significant. The latter could imply that over expression and not just mutation could activate the pathway and thus circumspection must be applied when analyzing the results.

Setting up a xenograft model assisted us in further evaluating the effect of the TSLP pathway genes aberrant expression. As observed in the *in vitro* system, there was a trend, however not statistically significant, of relative increase in the B-cell lineage population of the CRLF2 GFP transduced cells *in*

vivo. The addition of both IL7RAwt and IL7RAins further enhanced the skewed differentiation to the B-lineage when compared to the BB transduced cells, particularly in the spleen of transplanted animals (Figure 3-11). Closer analysis of the Differentiation profile of transduced $CD19^{+}$ cells, revealed that the addition of IL7RA and in particular the IL7RAins mutant, significantly impede the transplanted CB cells in spleens and bone marrows of transplanted animals (Figure 3-12). Moreover, expansion of CRLF2-IL7RAins transduced population was observed in BM of transplanted animals (Figure 3–13) suggesting that as expected the activation of the pathway confers enhanced proliferation/survival of transduced cells.

Parts of chapter 3 may be used in the future for publication. Ifat Geron, Angela Savino, Nava Gershman, Noa Tal and Shai Izraeli. “The role of CRLF2/IL7RA signaling in the development of B-ALL”. The dissertation author is expected to be the primary author and researcher of the manuscript.

Chapter 4 Discussion

4.1 Discussion

B-ALL is a B-cell precursor malignancy with high prevalence in children. Although cure rates have increased dramatically from less than 10% in the 1960s to nearly 90% in the 2000s[74], it is still a major cause of cancer mortality in children[192]. Furthermore, the treatment effectiveness in adults and adolescents is insufficient. Great advancements in our understanding of the genetics foundations underlying the development of B-ALL have been made over the past years. However, much still remains to be learned about the biological mechanisms of the disease in order to find effective therapies that are targeted and less toxic. The latest technological advances in sequencing have allowed for vast and precise genetic analyses of malignant cells. The immense volume of information gathered from these studies includes a barrage of proposed mutations and genetic alterations thus raising the necessity to discriminate between events that participate in the development of the malignancies to events that bare no critical role.

One recently described alteration that was reported in subgroup of B-ALL, is the aberrant expression of CRLF2, which, in many of the cases, was accompanied with additional mutations in the TSLP pathway. CRLF2 is a subunit of the TSLP receptor that pairs with the IL7RA subunit to create the active receptor. The receptor transduces its signal via activation of the JAK/STAT and mTOR/PI3K pathways. Under the premise that B-ALL is a multi-factorial disease and several aberrant events are required to induce malignancy, I have aimed to illuminate the role of the TSLP/IL7RA pathway in

the development of CRLF2-positive B-ALL. The working hypothesis was that CRLF2, together with other mutations in the TSLP pathway contributes to the development of B-ALL. The rationale for this hypothesis was based on previous indications to the causative nature of CRLF2 in the malignancy:

i CRLF2 aberrations were found only in B-ALL cases that had no other known B-ALL chromosomal abnormality (ie. hyperdiploidy, ETV6-RUNX1, TCF3-PBX-1, MLL-AF4)[1-4]. Although circumstantial, the fact that the aberration is recurrent and associated with no other major known driver mutation suggests that the CRLF2 is not a mere passenger mutation.

ii Transduction of murine fetal liver progenitors with CRLF2 resulted in excess proliferation and reduced B-cell differentiation[2]. Additionally, transduction of the IL-3-dependent pre-leukemic mouse Ba/F3 cells with CRLF2[4] or a combination of CRLF2 with JAK2[3] allows them to grow in IL-3 free media. These findings suggest that over-expression of CRLF2 can induce cytokine-free growth/survival pathways.

iii Treating primary CRLF2-positive B-ALL samples with JAK/mTOR inhibitors both *in vitro* and *in vivo* resulted in growth inhibition [171,193]. The same was observed following blockage of CRLF2 by RNA silencing in MUTZ5 (a CRLF2-positive ALL cell line), and treatment with JAK and mTOR inhibitors[2]. These results demonstrate the dependence of CRLF2-positive ALL on the pathway's activity.

Together, the data described above strongly implies that CRLF2 is a driver event in B-ALL. Nevertheless, although its ability to induce proliferation in

mouse fetal liver progenitors has been demonstrated, and the dependence of already transformed cells has been corroborated, its tumorogenicity in human hematopoietic progenitors cells had never been tested. Thus the novelty in the current research is the use of normal hematopoietic progenitors to illuminate the contribution of CRLF2/ILTRA aberrations in the evolvement of leukemogenesis from normal human cells. Overall our results suggest that activation of the CRLF2/IL7RA pathway may have a role both in proliferation/survival of cells that acquire the mutation and in halted differentiation. Meanwhile, activation of the pathway in normal cord blood $CD34^+$ hematopoietic progenitors was not sufficient to transform the cells.

The results raise several questions about the mechanism by which activation of the TSLP pathway induces the changes that were observed. Moreover, the additional events collaborate with CRLF2/IL7RA during the progression to overt leukemia remain to be determined

4.1.1 The importance of the cellular context of CRLF2 aberrant expression

Our initial *in vitro* studies in which CRLF2 alone was ubiquitously overexpressed in all $CD34^+$ hematopoietic progenitor cells, suggest that the default differentiation of TSLP-induced non-committed/myeloid-primed progenitors inhibits B-cell differentiation in B-cell differentiation supporting conditions (Figure 2-2, Figure 2–4 and Figure 2–4). This outcome might be explained by the fact that CRLF2 is typically highly expressed in myeloid monocytes and dendritic cells[181]. The previous finding that transduction of

mouse fetal liver progenitors inhibits CD19 differentiation[2] also supports this result. A preliminary analysis of the CRLF2$^+$ cells that were not differentiated to B-cells revealed increased expression of the myeloid differentiation markers CD14 and CD15 (Figure 2–3). However, repetition of analysis with more markers is required for better characterization of the above cells.

In contrast to the effect of undirected expression of CRLF2 in human cord blood progenitor cells, the focused overexpression of CRLF2 in primed B-cell progenitors both accelerated early lymphoid differentiation and extended B-cell differentiation. This skewed differentiation pattern might be due to the activation of JAK/STAT signaling in cells that are primed for B-cell differentiation as the CRLF2 was under the control of a specific B-cell promoter/enhancer. (See further discussion below).

JAK2/STAT5 signaling can bestow self-renewal proliferation survival or differentiation depending on the cell in which it is activated and on the levels of activation[194-196]. As we recognized an effect of the CRLF2 expression on differentiation, we wanted to test whether proliferation would also be affected by CRLF2 expression. We performed cell cycle analysis on transduced cells and compared growth by counting cells on the day of analysis (data not shown). In contrast to previous reports of the ability of CRLF2 to induce proliferation[2] and in contrast to the expected outcome of activation of JAK/STAT signaling, no increase in proliferation was observed in the CRLF2-transduced cells as compared to the control GFP-transduced control cells. This being said, our ability to find such differences might have been compromised due to vast

variability between replicates (in the case of cell growth) and changing transduction efficiencies combined with overall low cell number which technically incapacitated our ability to acquire accurate and reproducible cell cycle data. Hence, we established that B-cell directed expression of CRLF2 enhances differentiation towards the B-cell lineage. However on its own, it does not confer advantageous qualities sufficient for pre-leukemic development, possibly due to insufficient activation of the pathway.

Two possibilities for further enhancement of the pathway's activation were tested. The first is the addition of another mutation in the pathway (discussed later), and the second is the activation the receptor by addition of TSLP. To test whether the addition of TSLP to the media would further activate the pathway and enhance the effect of CRLF2 on transduced cells, we added human TSLP to the media either exogenously (2ng/ml – data not shown) or by growing the cells on MS5 stroma that was transduced with hTSLP-expressing lentivector. No difference was observed in the differentiation pattern (Figure 2–8) or in cell numbers (data not shown) between the cultures. The CRLF2 subunit requires pairing with an IL7RA subunit for optimal TSLP activation. As differentiating cells in the culture express endogenous IL7RA that is available for pairing with the exogenous CRLF2, the induction with TSLP was expected to enhance or somewhat change the phenotype. Possible explanations for the lack of difference in the phenotype after the addition of TSLP are that the endogenous IL7 preferentially bound to the IL2Rγ and that the CRLF2 had a

TSLP-independent effect in the cells. This should be further tested by phosphorylation assays of the transduced cord blood cells.

Whether this enhanced differentiation to B-lineage gives any advantage to the cells is yet to be determined and will be discussed further in this work. An implication of these results is that CRLF2 expression might occur in concert with other mutations in order to induce transformation of normal human hematopoietic progenitors.

4.1.2 Additional mutations in the TSLP pathway adding to in vitro phenotype

4.1.2.1 Human JAK2 over-expression perturbs transduced cells

The data described above did not give indications for the ability of CRLF2 alone to induce leukemogenesis. This result might be expected as aberrant expression of CRLF2 is commonly accompanied by secondary mutations in the TSLP pathway. The most common mutations are in JAK2, frequently around arginine 683[1-3,106]. Human JAK2 was not routinely used in research when we started the study. The only group that used human rather than mouse JAK2 expressed JAK2 under the control of its endogenous promoter in a BAC system. The expression was restricted to the hematologic system, since general expression of human JAK2 was lethal [197]. While cloning and producing the JAK2 virus, we dealt with viability problems in both bacterial and human cells, probably, due to the potency of its kinase activity. This predicament led to low viral titers, and thus low transduction efficiencies (data not shown). Additionally, transduced CB $CD34^+$ cells did not persist in culture.

To understand the reason for the low number of transduced cells post-transduction, we transduced the B-ALL cell line 018Z, which also presented a similar reduction in CRLF2-JAK2-expressing cells after transduction. We sorted the cells and checked for the viability of the transduced cells. No difference was observed in the viability of the transduced cells (Figure 3–4). However, when tracking the cells in culture, a marked growth inhibition was visible (Figure 3-5). Additional tests are required to evaluate this phenomenon. However these are beyond the scope of the current work. A conditional low expression of JAK2 might be necessary in order to study the effects of the CRLF2-mutant JAK2 combination. Due to the technical limitations of our work, we chose to examine another mutation in the pathway.

4.1.2.2 The role of combined CRLF2 IL7RA in altering CB CD34^{+} progenitor differentiation

In a previous screen performed in our lab for additional TSLP pathway mutations that collaborate with CRLF2 over expression, IL7RA mutations were found in about 6% of the cases[10]. Two kinds of mutations were initially described: a replacement of serine 185 positioned at the extracellular domain with cysteine, and a set of in-frame insertions and deletions (INDELS) of 3-7 amino acids at the transmembrane domain of the receptor. The common feature of the mutations was the addition of cysteine, which was required for sovereign dimerization and basal activation of the receptor without cytokine binding[10]. Later, cysteine-independent activation mutations in IL7RA as well as in CRLF2 were reported[162]. Although these mutations were more frequent in

CRLF2 over-expression cases, they could be found in B-ALL cases without CRLF2 aberrations as well as in T-ALL[4,10,168]. In the current study, we cloned the IL7RAins PPCL activating mutation that showed strong STAT5 and RPS6 activation in Ba/F3 grown in cytokine-free media[10]. The expression of the two transgenes from the lentiviral vector was verified (Figure 3–6) and the activity of the expressed receptor in response to cytokine stimulation was evaluated using a STAT5 phosphorylation assay (Figure 3–7). As expected, CRLF2-IL7RAins had the highest basal activity, though this activity was much lower than after cytokine stimulation. Surprisingly, and unlike the previous report[10], no additional phosphorylation of STAT5 was evident after TSLP stimulation of the CRLF2-IL7RAins-transduced cells, suggesting that most of the IL7RAins homodimerizes and thus does not bind TSLP. However, this discrepancy can be explained by differences in cell lines and expression systems, sensitivity of assay methods, and, to a greater extent, cytokine concentrations that were used for stimulation (Ba/F3 separately transduced with CRLF2 and IL7RAins stimulated with100ng/ml TSLP and assayed by Western blot in the original report compared with 018Z transduced with bicistronic vector stimulated with 2ng/ml TSLP and assayed by flow cytometry in the current experiment). Thus, our test does not rule out the plausibility of CRLF2-IL7RAins dimerization in cells. Nevertheless, higher cytokine concentrations might be needed to induce the signal. IL7 highly stimulated STAT5 phosphorylation in the CRLF2-IL7RAins-transduced 018z cells, due to IL7RAins' demonstrated ability to homodimerize[10] and, to a lesser extent, in the CRLF2-IL7RAwt-transduced

cells. This might be due to low affinity of IL7 to the TSLP receptor or to some homodimerization of IL7RAwt in the cells. The second option appears credible, as the binding sites of IL7RAwt and IL7RAins are the same. Thus, the ability of IL7 to activate IL7RAins implies that it can do the same for homodimers of IL7RAwt. These results insinuate retrospectively that choosing the S185C rather than or in addition to the IL7RAins mutation would have been more appropriate for evaluating the activation of TSLP pathway, as the IL7RAins mutation preferentially homodimerizes and is thus activated by IL7, while the S185C mutation preferentially dimerizes with CRLF2[10]. However, the most common mutations are the INDELs and, since we confirmed that the expression of CRLF2-IL7RAins results in the formation of an active receptor that leads to basal activation of STAT5, we proceeded to test its effect on $CD34^+$ CB progenitors. Henceforth, in the absence of an IL7RAins-alone control, we refer to the studied pathway as the TSLP/IL7RA pathway.

We showed that the addition of IL7RAins to the CRLF2 vector significantly accelerates lymphoid differentiation of transduced $CD34^+$ CB progenitors; this was apparent by early differentiation to $CD10^+$ (Figure 3–8 A). A similar yet not statistically significant trend was observed in the IL7RAwt addition. The latter emphasizes that the excess expression, though not sufficient in itself, plays a role in activation of the pathway and that the phenotype is not solely a consequence of the mutation. Additionally, the mIL-7 secreted from the MS5 could contribute to both the observed mutant and wild type phenotypes. Though not completely cross-reactive, mouse IL7 is capable

of residual activation of human IL7RA[158]. Furthermore, a trend towards overall higher B-cell differentiation was evident, although not statistically significant. As in the case of CRLF2 alone, changes in cell numbers could not be appropriately quantified due to high variability in proliferation between replicates.

The *in vitro* system can provide an indication of the ability of the tested genes to alter normal cell behavior; nevertheless, as in all *in vitro* systems, the cell's growth conditions are dramatically different from their natural environment in terms of oxidative stress, growth media, environmental interactions with the niche, and more. Our *in vitro* system was further limited both in its capacity to induce differentiation beyond B-cell progenitors and particularly in the inconsistent growth/ proliferation results it produced. This is most likely due to the limitation on starting number of cells, which resulted in sampling of a small cell number in each replicate, such that the general diverse population of transduced cells was not represented. Therefore, in order to properly investigate the gross ramification of the aberrant activation of TSLP/IL7RA on normal CB $CD34^+$ progenitors, we proceeded to *in vivo* experiments.

4.2 The role of activated TSLP/IL7RA pathway in leukemogenesis studied in a xenograft model

Mouse disease models are an essential tool for understanding human diseases. Many mouse models have been generated to recapitulate leukemic progression, elucidate the pathogenetic mechanisms of the disease, and to search for inhibitory drugs[198-204]. These studies contributed greatly to our understanding of the disease and to advanced treatment studies. Yet, there are

gross differences between mouse models and humans, particularly with respect to the development of the B-lymphoid system (reviewed in [45]) and the relative ease of transforming mouse cells as compared to human cells[205]. One mouse disease model, recapitulating the SCID immunodeficiency[206], has paved the way for humanized mouse disease models[207]. Human in mouse xenografts have prominently advanced the modeling of human hematopoiesis, leukemogenesis, and drug testing[133,208-214]. Human CB $CD34^+$ repopulation was compared in several mouse strains and the overall $CD45^+CD19^+$ engraftment was found to be superior in NSG mice[189]. Since the $CD19^+$ population is the most relevant for B-ALL research, we chose to work with this mouse model.

We started by testing our ability to obtain a long-term engraftment of transduced cells with sustained expression of the transgene. Mice were sacrificed 24-30 weeks after transplantation of transduced CB $CD34^+$ cells and hematopoietic tissues were harvested. As portrayed in Figure 3–9, we successfully grafted CB-transduced $CD34^+$ progenitors that expressed the transgenes, were readily differentiated into immature and naïve B-cells, and were detectable 30 weeks post engraftment (no later time point was checked). We then set up several experiments with four $CD34^+$-transduced groups: BB, CRLF2-GFP, CRLF2-IL7RAwt, and CRLF2-IL7RAins. First, the overall differentiation of the transduced cells to the B-cell lineage was evaluated. Over-expression of CRLF2 alone had a modest effect, expanding the relative proportion of B-cell population within the transduced cells as compared to the BB control *in vivo,* resembling (though not as significant) the *in vitro* cultures

(Figures 2-6 and 3-11). Since the mice were sacrificed at the point when full differentiation was reached, it is possible that an early sacrifice would have revealed a more pronounced phenotype. The addition of IL7RAwt and IL7RAins to the transduction further emphasized the phenotypic increase of B-cell lineage differentiation, particularly in the spleens of transplanted mice (Figure 3-11). Detailed examination of the transduced B-cell population differentiation pattern established that CRLF2-IL7RAins blocks the differentiation of $CD19^+$ cells in the pro/pre-B ($CD10^+CD19^+sIgM^-$) stage (Figure 3-12), which corresponds to the differentiation stage of the blasts in the majority of B-ALL cases[75]. Finally, in addition to its effect on the differentiation of transduced CB $CD34^+$ progenitors, we showed that CRLF2-IL7RAins transduction resulted in the expansion of transduced cells *in vivo.*

In conclusion, we found that the aberrant expression of CRLF2-IL7RAins in CB $CD34^+$ progenitors skewed differentiation of transplanted cells *in vivo* towards the B-cell lineage, while halting the differentiation past B-cell precursors and inducing expansion of the transduced cells. These findings are profoundly significant in understanding of the role of CRLF2/IL7RA in B-ALL. However, they raise some additional questions, primarily about the mechanism underlying the phenotype induction.

First and foremost, it is essential to evaluate the contribution of CRLF2 expression to the observed *in vivo* phenotype: As can be deduced from the high signal transduction activation of transduced 018Z after IL7 stimulation, which was not observed with TSLP in the CRLF2-IL7RAins, IL7RAins subunit

preferentially homodimerizes (Figure 3–7). Hence, an additional IL7RAins-GFP-transduced group is required in order to resolve the question of CRLF2 contribution.

A related question is whether the phenotype observed is due to cytokine-independent basal activation of the downstream pathway. According to previous publications, mTSLP[184] and mIL7[144] do not cross-react with human receptors. However, indications of the species cross-reactivity of mIL7 [although in very low affinity (personal communications)] are backed by the finding of human T-cells, whose differentiation is dependent on IL7, in CB $CD34^+$ progenitors in xenograft models[189] and in *in vitro* mouse-human cultures, in which blockage of mIL7 inhibited the growth and differentiation of human B-cells from hematopoietic progenitors[158]. This raises the possibility that the phenotype found with CRLF2-IL7RAins is a result of the activation of IL7RAins homodimer by mIL7 and not due to the intrinsic activation of the mutation. In this case, we would expect that using the xenograft model proposed by the Payne group[184], in which human TSLP is constantly secreted, would yield a similar phenotype in the CRLF2-IL7RAwt group. Alternatively, treating mice with antibodies that block mIL7 would reverse the phenotype. In any case, we can state that the constitutive activation of TSLP/IL7 pathways in human B-cells contributes to a pre-leukemic phenotype.

While our results present circumstantial evidence for the activation of downstream TSLP/IL7RA effectors, we did not check *ex vivo* what factors are activated. STAT5, ribosomal protein S6, and 4E-BP1[215] are the obvious

phosphorylation targets downstream of both IL7RA and CRLF2 activation. It would also be intriguing to see whether a noticeable JAK2 phosphorylation is evident, which would imply that CRLF2 plays a role in the observed cellular changes, as JAK2 is not activated by IL7RA. (IL7RA binds and activates JAK1). Additionally, expression analysis of the transduced cells harvested from the mice is currently being performed. Thus, future experiments will focus on acquiring data and on analysis of downstream activated genes.

Finally, self-renewal is an important trait of ALL cells *in vivo*. It was interesting to test whether CRLF2-IL7RAins enhances the self-renewal capacity of transduced cells. In order to do so, preliminary experiments with serial transplantations were carried out. Cells from the spleens or BM of CRLF2-IL7RAins and backbone-transplanted mice were re-transplanted to irradiated NSG recipients. To date (25-30 weeks post-transplant), no engraftment has been detected in the peripheral blood of secondary recipients, while human $CD45^+$ was visible in all primary engrafted mice at 15-20 weeks after transplant. This suggests that CRLF2-IL7RAins over-expression does not enhance self-renewal. However, these results are not conclusive, as the mice had not yet been sacrificed and BM or spleen engraftment cannot be ruled out. Additional secondary transplants with different cell numbers and a more complete analysis must be performed before a final conclusion is reached.

4.3 Possible mechanisms of the transformation – How does it work?

Philadelphia-like ALL is a subgroup of ALL exhibiting an expression profile similar to that of BCR-ABL-positive ALL without carrying the translocation or any other major chromosomal aberration (reviewed in[85]). It is characterized by activation of either JAK or ABL kinases. This activation is caused by genomic aberrations in the leukemic cells, involving cytokine receptors or kinases. Aberrant CRLF2-expressing ALL represents a large portion of Philadelphia-like ALL with JAK2 activation.

In normal mouse early B-cell differentiation, JAK/STAT and PI3K signaling is activated by IL7, which has been found to be involved in differentiation via activation of EBF and PAX5[216-218]. Additionally, IL7RA induces proliferation and survival signals (needed to protect the cell from DNA damage-induced apoptosis caused by the V(D)J immune gene recombination) before and in parallel to signals from the pre-BCR[219] until the proliferation stage of large pre-B is completed and IL7 signaling is down-regulated by IKAROS[50]. At this stage, BCL6 survival signaling is activated by the pre-BCR[220]. Nevertheless, it appears that although IL7 pathway activation in early B-cell differentiation is compulsory, its withdrawal is necessary for the maturation of the cells in mouse system[50,221]. IL7 activity in human B-cell differentiation does not completely mirror its role in mice, as evidenced by the full differentiation of B-cells in X-linked severe combined immunodeficiency (SCID) resulting from IL2Rγ deficiencies and in SCID with IL7RA deficiencies[180]. However, its importance in human early B-cell differentiation and proliferation was

demonstrated in *in vitro* differentiation assays.[158]. Here, we enforced expression of CRLF2/IL7RA in CB $CD34^+$ progenitors under the control of B-lineage-specific promoter-enhancer, and saw a relative B-cell differentiation arrest at the pro-pre-B stage accompanied by an enlarged population (Figure 3-11, Figure 3-12 and Figure 3–13). Thus, we demonstrated for the first time that constitutive activation of the TSLP/IL7 pathway in B-cell progenitors inhibits human B-cell differentiation past pro/pre-B-cell stage and induces their growth *in vivo*. Further studies testing downstream activation patterns are needed to advance our understanding of this phenotype.

A recent study has shown that STAT5 has a role as a nuclear repressor for differentiation when not activated[222]. The study describes differentiation inhibition by repression of lineage-specific genes; in the studied case, the genes were of megakaryocytic lineage and repression was achieved through the association of unphosphorylated STAT5 (uSTAT) with CTCF, a Zink finger transcriptional repressor that modulates DNA structure, can block enhancer-promoter interactions, and mediates intradomain interactions in transcriptionally permissive compartments[223]. Although inhibition was specifically of differentiation to megakaryocytes, where CTCF was associated with ERG, it raises the possibility that a similar mechanism exists in lymphoid lineage-primed progenitors, where CTCF is known to be associated with the Igh locus and to mediate its rearrangement[224,225]. It is conceivable that uSTAT might bind with CTCF next to the recognition sites of lymphoid lineage-specific factors, thus blocking the access of the latter to variable lymphoid/B-cell gene targets. If so,

enforced activation of IL7 under a B-lineage promoter should elevate its expression in B-lymphoid primed progenitors, and accelerate the cell's differentiation towards the B-cell lineage by activating STAT5.

Early onset of ALL combined with the finding of clone-specific ETV6-RUNX1 translocation in twins previously raised the idea that some initiating leukemic events arise prenatally. This idea was verified when ETV6-RUNX1 translocations were found in the neonatal blood of children who were later diagnosed with B-ALL[81]. An initial translocation event might give a relative advantage to the cell in which it appears, thus priming the cell for acquisition of additional mutations. In our experiments, the addition of CRLF2 alone somewhat altered the differentiation pattern along the B-lineage. Whether and how this effect confers an advantage to the cell should be resolved in a system that can best simulate the right cell of origin. We attempted to do so by transducing CB cells. However, as was recently reported in [26,38], hematopoietic progenitor populations differ substantially between fetal liver and CB. In agreement with this, experiments performed in mice that lack IL7RA have demonstrated that fetal liver cells, but not BM cells, were able to differentiate to B-cells[226]. Additionally, as reported by the Roberts lab[227], there is a large $CD34^{+}CD19^{+}$ population in human fetal liver (~10%) that is not matched in human CB based on our experience (less than 1% to undetectable). Scheerenet al. reported that B-cell progenitors from fetal liver display higher expression of CRLF2 and proliferate upon addition of TSLP[105]. Taken together, these findings suggest that rearrangements of CRLF in utero in B-cell

progenitors might provide a growth advantage on top of the enhancement of B-lineage differentiation that was demonstrated in this work.

CRLF2^{+} B-ALL is classified as bad prognosis B-ALL, and the incidence of relapse in this group is high. In several cases, the CRLF^{+} clone disappears in relapse, raising doubts about the necessity of CRLF2 for disease maintenance and even for its contribution to B-ALL initiation. Swaminathan et al. described a mechanism of clonal evolution in B-ALL in which the enzymes that are responsible for BCR diversity, RAG1-RAG2 and AID, act together to initiate clonal evolution under inflammatory conditions[183]. Additionally, it was proposed in their paper that IL7 signaling protects the cells from this genetic vulnerability though a previously described inhibition of RAG1 and RAG2 that was found to be shared at least partially with AID repression; IL7 activates STAT5, which recruits the polycomb repressor EZH2 to Rag1 and Rag2 genes and in parallel activates Akt, which in turn phosphorylates and inactivates Foxo1, a transcriptional activator of Rag1 Rag2 and AID. Hence, the activation of STAT5 and Akt in CRLF2^{+} ALL might prevent the evolution of CRLF2^{+} clones in relapse and explain its absence in several relapse samples. This scenario, if proven to be accurate, can help us find a common initiating event in CRLF2^{+} B-ALL by comparing clones pre/post remission and finding common aberrations.

P2RY8-CRLF2 fusion results in the deletion of CSF2RA, IL3RA, and ASMTL[2,3,1] in between P2RY8 and CRLF2. Although present in the un-rearranged chromosome, haploinsufficiency of one or more of these genes can cooperate with the induction of CRLF2 to induce leukemogenesis. CSF2RA

encodes the GM-CSF receptor alpha subunit. A recent report from the Zhang lab suggests that GM CSFRA, but not IL3RA, acts as a tumor suppressor in RUNX1-ETO AML[228]. Additionally, its expression level in 59% of t(8;21) AML patients was found to be low due to deletion of the sex chromosome. The latter resembles the expression reduction expected in P2RY8-CRLF clones, suggesting that a similar tumor suppressor decrease could affect CRLF2$^+$ B-ALL. However, this can only account for some of the cases, as in a subset of CRLF2$^+$ DS B-ALL there is further addition of chromosome X, which compensates for this deletion.

Lane et al. recently generated the only available mouse model for CRLF2$^+$ B-ALL[229]. CRLF2 and JAKR683 were expressed in B-lineage cells under Eμ enhancer control, on the background of Pax5+/- mice. BM from these mice was further transduced with the dominant negative IKAROS splice variant IK6 and transplanted to recipient wild type mice. All mice developed ALL within 120 days. Interestingly, neither the combination of CRLF2-JAKR683 on Pax5+/- background without IK6 transduction nor the transduction of CRLF2-JAKR683-expressing BM from Pax5+/+ mice with IK6 was sufficient to induce ALL, suggesting that CRLF+ B-ALL requires a combination of at least four mutations for initiation and explaining the lack of leukemic phenotype in our experiments.

4.4 Down syndrome genetic background effect on the development of B-ALL with TSLP pathway mutations

Children with trisomy 21 have a 20-fold increased risk for B-ALL and a 150-fold risk for Acute megakaryoblastic leukemia (AMKL)[230]. However, DS is

not a classic genomic instability syndrome, as the probability of developing a solid tissue malignancy is reduced in DS[231]. Expression analysis of DS B-ALL revealed vast heterogeneity, implying that though trisomy 21 predisposes DS to B-ALL it does not act as initiating event, but rather a predisposing condition to multiple genetic subtypes[3]. Nevertheless, there is a significantly higher incidence of CRLF2$^+$ cases in DS B-ALL than in sporadic B-ALL (~60% compared with <10% respectively)[232]. Although CRLF2 does not correlate with any major B-ALL chromosomal translocations[2-4] (i.e., ETV6-RUNX1, MLL rearrangements, BCR-ABL, or TCF3-PBX1), P2YR8-CRLF2 translocations are found in ~30% of intrachromosomal amplification of chromosome 21 (iAMP21) B-ALL[111]. The iAMP21 common region of amplification overlaps with the Down syndrome-critical region (DSCR) on chromosome 21[233]. This further raises the plausibility of collaboration between one or more genes within DSCR and aberrant CRLF2 expression. Ts1Rhr mice carry triplications of 31 genes and 1 non-coding RNA corresponding to minimal human DSCR. When using these mice as a background for the previously described CRLF2$^+$ mouse ALL model employed by Lane et al., the penetrance of B-ALL was greater and the latency reduced compared to wild type mice with the same B-ALL gene inducing combination[229]. This further strengthens the hypothesis that DS-enhanced genes collaborate with genes inducing CRLF2$^+$ B-ALL. HMGN1 was proposed to be the DS gene that is responsible for the enhanced phenotype by suppressing global H3K27me3.

Transplanting human Down syndrome CB is a challenging task. In addition to low sample availability, the viability of the cells is low, as is their proliferation rate. According to personal communication with labs that routinely work with Down syndrome samples (Enver T. and Roberts I.) the prospects of engrafting CB $CD34^+$ progenitors are poor. We combined three Down syndrome CB samples and transduced them with BB, CRLF2-IL7RAwt, and CRLF2-IL7RAins. Transduction efficiency in CRLF2-IL7RA was too low to analyze (<2%). Interestingly, CD19+ CRLF2-IL7RAins cells were only observed in the BM and unlike what was observed with the normal CB $CD34^+$ progenitors, transduction with CRLF2 IL7RAins did not yield a high percentage of $CD19^+$ cells (61% $CD19^+$ of total transduced cells compared with 85% $CD19^+$ of the backbone). No difference was observed in the $CD19^+$ differentiation pattern. However, as only a single mouse was transplanted, further studies will be required to reach a conclusion.

To conclude, we hypothesized that CRLF2 in conjugation with additional mutations in TSLP pathway proteins plays a role in the development of B-ALL. We tested the effect of CRLF2 over-expression in human CB hematopoietic progenitor. Ubiquitous forced expression of CRLF2 under the control of EF1α promoter resulted in reduced B-cell differentiation. However, CRLF2 expression under a B-lineage specific promoter enhanced differentiation towards the B-lineage. Additional expression of IL7RA with an activation mutation augmented

the phenotype. Xenograft transplantations of CRLF2-IL7RAins-transduced hematopoietic B-cell-primed progenitors resulted in expansion of the transduced population, enhanced development towards the B-lineage, and halted differentiation in the pro/pre B-cell stage. Thus, we concluded that CRLF2/IL7RA participates in leukemogenesis.

This work differs from previous work conducted in the field, as it is the first to evaluate the contribution of the TSLP/IL7 pathway to the development of B-ALL in a primary **human** system. Additionally, this is the first work to show that enforced expression of activated IL-7RA halts B-cell progenitor differentiation in the pro/pre B-cell stage *in vivo*.

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