PhD Program in Translational and Molecular Medicine

(XXXI cycle, academic year 2017/2018)

University of Milano-Bicocca
Department of Medicine and Surgery

Role of bone marrow-mesenchymal stromal cells and inflammation in the pre-leukemic phase of ETV6-RUNX1-positive childhood acute lymphoblastic leukemia

Candidate: Linda Beneforti

Registration number: 772701

Tutor: Dott. Giovanni Cazzaniga

Co-Tutor: Dott.ssa Chiara Palmi

Coordinator: Prof. Andrea Biondi

Lo dedico a me perché ce l'ho fatta.

Lo dedico alla mia mamma e al mio babbo,
perché in loro ho sempre avuto l'esempio
del come si fa.
Alla mia sorellaccia,
che è gli occhi le gambe la bocca
e gli orecchi diversi
che non ho,
e che mi sono essenziali.

A nonna Tamara,
la sovrabbondanza dell'amore.
A nonna Edda e a nonno Elio,
per la forza misteriosa delle loro vite.
A zio Fabrizio e zio Fabio,
che sono i miei tatoni
(e alle mie zie, che li sopportano).

Alle altre zie, zii, cugine e cugini
grandi e piccini,
per l'immancabile eco delle loro risate.

Lo dedico ai pochi amici veri,
pazienti con le mie assenze,
e sempre presenti.
Agli amori
che mi hanno segnata
e ridisegnata.
L'importante è mettere sotto la propria firma.

Lo dedico alla Scienza,
e non perché debba tener conto di questo lavoro,
ma perché mi garba questa nobile Signora
che non fa difficoltà a palesare,
oltre alla qualità dei dati,
quella degli uomini e delle donne che la frequentano.

Ah,
ovviamente lo dedico a Chicca e Fulvio!
…che tante volte mi hanno salvato
dall'essere noiosamente Sapiens.

TABLE OF CONTENTS

CHAPTER 1:
GENERAL INTRODUCTION

Childhood Acute Lymphoblastic Leukemia (ALL)

An overview

Acute Lymphoblastic Leukemia (ALL) is the most frequent cancer in children in developed society, with a cumulative risk of 1:2000 up to the age of 15 years, a peak incidence in children aged 2-5 year and about 60 new cases per million per year in Italy (http://www.euro.who.int/__data/assets/pdf_file/0005/97016/4.1.-Incidence-of-childhood-leukaemia-EDITED_layouted.pdf). On the other hand, ALL occurs in adults with lower frequency but worse outcome. In addition, different genetic subtypes greatly differs in proportion compared to childhood disease, suggesting that a critical developmental window and peculiar biological mechanisms operate in ALL pathogenesis in children and adults (Greaves, 2018). From a clinical point of view, disease is characterized by uncontrolled proliferation of immature lymphoid progenitors (lymphoblasts) in the Bone Marrow (BM), followed by their dissemination into both hematopoietic (blood, spleen, liver) and non-hematopoietic organs (primarily central nervous system – CNS – and skin). Principal clinical signs and symptoms depend on the percentage of leukemic cell infiltration in the BM, which in turns determines the gravity of normal hematopoiesis impairment. Anemia, thrombocytopenia and neutropenia lead to pallor, fatigue, petechiae, bruising, bleeding, and fever. Lymphadenopathy, hepatomegaly and splenomegaly can also occur as consequent of extramedullary infiltration, but they are

usually asymptomatic. Headache, vomiting, and lethargy are indicators of central nervous system (CNS) metastasis, which occur in about 5% of patients (Larson, 2008). ALL diagnosis is based on morphological and histological analysis, flow cytometry immunophenotyping and identification of cytogenetic-molecular abnormalities in patients' BM and peripheral blood (PB) samples. Immunophenotype is performed by flowcytometry basing on CD (Cluster Differentiation) molecules surface expression. Specific panels of CD define the lineage commitment and the differentiative state of leukemic blasts, allowing to discriminate between different ALL subgroups. As shown in Fig.3, B-ALL is the most frequent pediatric ALL, accounting for about 75% of cases and, within B-ALL, B-Cell Precursor (BCP-) or common ALL (cALL) represents the major subgroup.

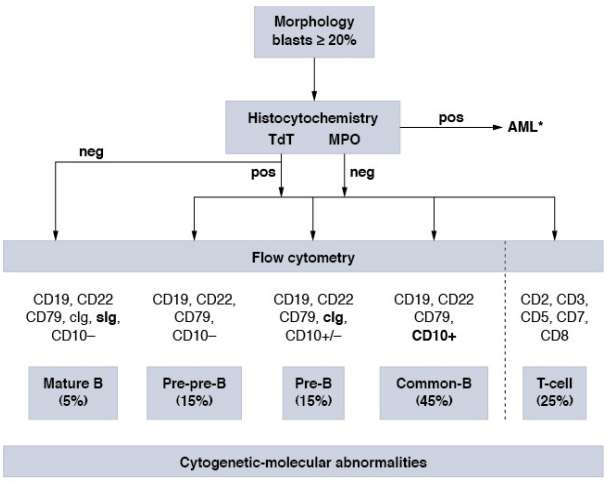


Figure 1. Diagnostic immunophenotyping of childhood ALL. From Kantarjian HM, *et al*. The MD Anderson Manual of Medical Oncology, 2011.

Biologically, expansion of undifferentiated lymphoid clones results from the accumulation of progressive genetic damages in hematopoietic stem and progenitors cells during normal hematopoiesis (Corces-Zimmerman and Majeti, 2014). Hematopoiesis is a hierarchic process of tune-fined differentiative stages regulated by both cellular and microenvironmental stimuli. During normal hematopoiesis, hematopoietic stem cells (HSC) give rise to multipotent progenitor (MPP), which in turn restrict their potential into common lymphoid or common myeloid progenitors (CLP, CMP); these latter further differentiate into lineage-committed progenitors that finally produce mature blood cells (erythrocytes, granulocytes, lymphocytes, platelets). While multipotency, or the potential to generate diverse cell types, is shared between HSC and MMP, self-renewal (the capacity to maintain stemness throughout the whole life) and quiescence (the ability to persist in a “dormant” state for very long periods until reactivation by local and systemic stimuli) only belong to HSC, at least in physiological conditions. Coexistence of self-renewal and differentiation in HSC is ensured by the so-called asymmetric division, a peculiar mitosis by which a daughter cell can initiates maturation process while the other maintains stem cell characteristics (Orkin and Zon, 2008).

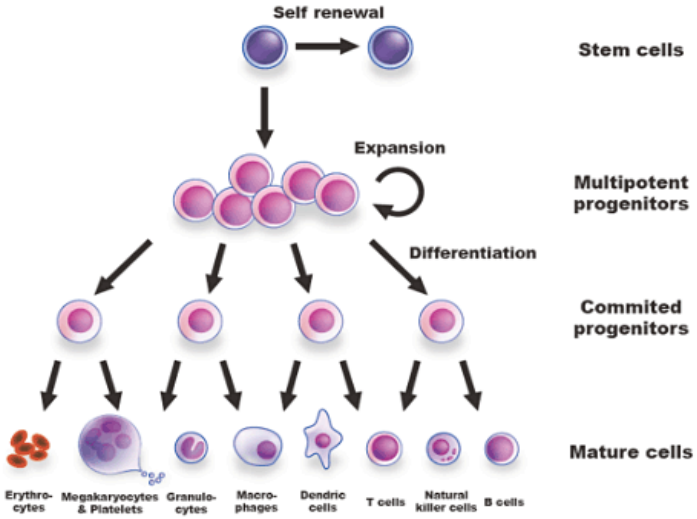


Figure 2. Schematic representation of hematopoiesis. From http://stemcell-u-tokyo.org/en/sc-se/.

Mutations normally occurs during hematopoiesis but the majority of them are molecularly corrected or cells eliminated by several systems (i.e.: programmed cell death, immunosurveillance, cellular senescence); in addition, mutations occurring into mature cells can't be extensively propagated due to limited cell lifespan. For this reason, the classical hypothesis for initiating leukemogenic mutations postulates that 1) they must occur in HSC, as they possess the necessary long-term survival and proliferation capability to acquire multiple lesions and go through clonal selection; 2) they must alter crucial pathways involved into cell proliferation and survival to provide clonal advantage to mutated cells. However, it is now established that also MMP and committed precursors could initiate disease if early genetic lesions confer them an "ectopic" self-renewal capability. Moreover, if first genetic lesions occur during fetal hematopoiesis, as

it happens in childhood ALL (see pag.10), a net distinction in these terms loses its meaning, as fetal precursors possess more "promiscuous" grades of potential. Independently of its precise nature, however, what is certain is that the initiating cells in ALL must possess self-renewal for DNA damages accumulation and overtime disease propagation (Fig.2) (Corces-Zimmerman and Majeti, 2014).

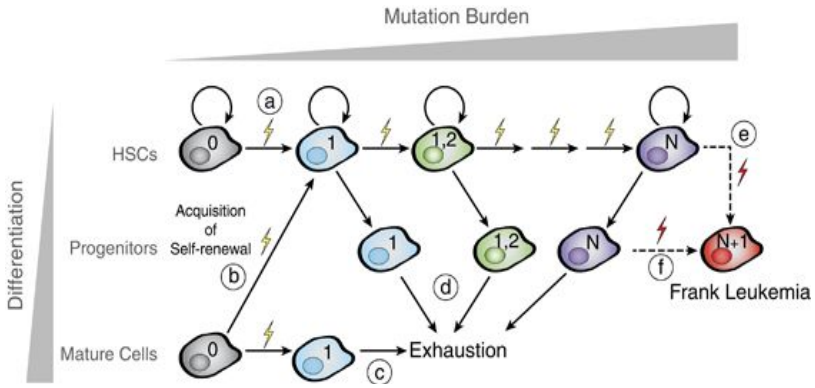


Figure 3. Model for leukemic evolution. Round arrows indicate self-renewal capability; numbers indicate sequential mutation. From Zimmerman *et al.*, Leukemia 2014.

Like every cancer, probability to develop childhood ALL results from cumulative combination of several factors: inherited susceptibility, exposure to internal/external environmental factors and chance. Inherited susceptibility includes genetic syndromes presenting an intrinsic genomic instability (i.e. Li-Fraumeni Syndrome, associated with inherited TP53 mutations, Fanconi's anemia), Down Syndrome, in which overexpression of HMGN1 gene, located on chromosome 21, leads to enhanced B-progenitors self-renewal, rare

but high-penetrant inherited mutations (i.e. mutations in *PAX5 and ETV6 genes)* and common low-risk allelic variants in various genes (i.e. IKZF1, ARID5B, CEBPE, CDKN2A/B). These latter, when co-inherited in the same subject, determine an additive effect on hematopoietic stem/progenitor cells that renders them vulnerable to malignant transformation. However, although genetic susceptibility is an effective contribution to ALL development, its impact remains modest compared to other cancers, as sibling show only a threefold risk of ALL against a background risk of 1:2000 (Greaves, 2018). Regarding environmental risk-factors, only ionizing radiations have been clearly established, as sadly documented by Hiroshima atomic bomb effects; for the other candidates (i.e. dietary bioflavonoids, pesticides, dipyrone, non-ionizing electromagnetic fields) only mild associations have been demonstrated and large multinational studies are still needed to address the question (Greaves, 2018). in addition, the role of infections and inflammation as ALL risk factors will be discussed later in this introduction.

From a genetic point of view, about 75% of ALL pediatric cases present gross chromosomal alterations detectable by karyotyping, Fluorescence In Situ Hybridation (FISH) or Polymerase Chain Reaction (PCR), which include: translocations with consequent fusion genes (i.e. t(12;21) originating ETV6-RUNX1, 22% of cases; t(1;19) originating TCF3-PBX1, 4% of cases; t(9;22) originating BCR-ABL1, 2% of cases), non-random hyperdiploidy (>50 chromosomes with gain in chromosomes X, 4, 6, 10, 14, 17, 18 and 21, 20% of cases), various

rearrangements of MLL gene at 11q23 locus (6% of cases) and hypodiploidy (<44 chromosomes, 1% of cases).

Besides being the most frequent lesions, chromosomal alterations are also the earliest mutational events in ALL occurring *in utero*; this is a peculiarity of childhood ALL, even if it is supposed that other pediatric tumors could have a pre-natal origin. More recently, innovative approaches of high resolution genetic profiling (i.e. microarrays for loss/gain of DNA regions, gene expression profiling – GEP, next generation sequencing – NGS) have allowed to identify recurrent sub-chromosomal lesions that not only clarify the leukemogenic process (especially in those cases that are negative for chromosomal variations), but also represent important tools for novel ALL subtypes definition, better prognostic stratification and innovative therapeutic targeting. Copy number variations (CNV) have been found in more than 50 genomic recurrent regions, with deletions occurring more frequently than duplications, but intra-chromosomic gene rearrangements and point mutations are also common. Both translocations and sub-microscopical lesions destroy functions of fundamental genes regulating hematopoietic cell proliferation, survival and differentiation. In particular, master transcriptional regulators of HSC homeostasis (i.e. ETV6, ETV6, MLL) and lymphoid progenitors differentiation (i.e. PAX5, IKZF1, EBF1), oncogenes (i.e. MYC), oncosuppressor (i.e. CDNK2A/B), cytokine receptors (i.e. EPOR, CRLF2, PDGFRB) and kinases (i.e. JAK1/2) are involved. Notably, some genetic lesion occurs in adults, children and infants (<12 months of age) but at very different frequency, revealing peculiar pathogenetic

mechanisms underlying childhood and adult leukemias (Fig.4) (Greaves, 2002).

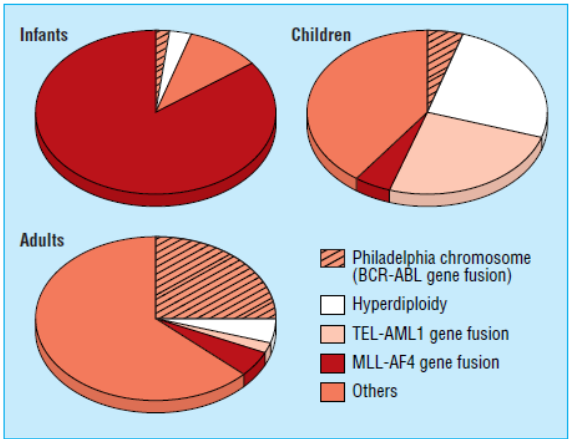


Figure 4. Major molecular subgroups in infant, childhood and adult ALL. From Greaves, BMJ 2002.

Despite the crucial role of implicated genes, a single alteration is insufficient to overt ALL and additional lesions are necessary to complete malignant transformation. Number of required mutations depends on the oncogenic potential of the first pre-natal lesion, which also determines the age of onset and the aggressiveness of the disease. In MLL-rearranged ALL, for example, one additional lesion is sufficient to complete leukemogenesis, as perturbations in MLL activity in the fetus lead to a potent and broad spectrum transforming effect; that's the reason why MLL^{+} ALL precociously arises (before 1 year of age), its progression is rapid and prognosis unfortunately poor. On the other hand, ETV6-RUNX1 is a weaker oncogene that requires several cooperating mutations to clinically manifest into leukemia,

explaining the longer period of latency of this specific ALL subtype (up to 15 years) (J L Wiemels *et al.*, 1999). Considering this, genetic molecular characterization represents a crucial issue for childhood ALL management. Indeed, in addition to other clinical and biological criteria, it represents a strong risk indicator guiding patients' assignment into risk-based therapeutic protocols (Fig.5).

Table 1 Prognosis Features in Childhood ALL

Risk Factor	Favorable	Unfavorable
Age	1-10 years	<1 or ≥ 10 years
Gender	Female	Male
Race/ethnicity	Caucasian, Asian	Black, Hispanic
White blood cell count at diagnosis	<50,000/μl	≥50,000/μl
Immunophenotype	B-precursor	T-cell, mature B-cell
Genetic features	Hyperdiploidy *ETV6-RUNX1*/t (12;21)	*BCR-ABL1*/t (9;22) *MLL* rearrangement Hypodiploidy iAmp(21) *CRLF2* overexpression
Extramedullary involvement (CNS, testicular)	No	Yes
Early treatment response		
Predisone window	Peripheral absolute blast count <1,0000/μl	Peripheral absolute blast count >1,000/μl
Induction day 8 peripheral blood MRD	<0.01-1%	>1%
Induction day 8 and day 15 bone marrow morphology	M1	M2 or M3
Later treatment response		
End-induction bone marrow MRD	<0.01%	≥0.01%

ALL = acute lymphoblastic leukemia; CNS = central nervous system; MRD = minimal residual disease.

Figure 5. Prognostic factors in childhood ALL. From Rabin KR & Poplack DG, Cancer Net., 2011.

Risk-based stratification, together with the introduction of combination chemotherapy, has allowed the achievement of around 90% of cure rate in childhood ALL, undoubtedly representing one of

the most success in oncology provided by basic and translational research. However, a percentage of patients still don't have therapeutic options due to intrinsic chemotherapy resistance of specific genetic subtypes; in addition, patients that achieve remission with conventional therapies could face ALL relapses, which remain the major cause of morbidity and mortality in pediatric age. To reach the 100% of cure, dose increasing of actual chemotherapies is not a viable strategy. Rather, a deeper comprehension of ALL etiopathogenesis is necessary, not only to offer alternative therapies but also to explore the hypothesis of a potential prevention of the disease (Greaves & Wiemels, 2003).

The two-steps model for childhood ALL development: the pre-leukemic phase

As anticipated before, a single lesion is insufficient for childhood ALL, as well as for cancers in general. What is particular in this case is the pattern of mutations acquisition, which is strictly defined in terms of time mutation type and frequency. Epidemiological and experimental data clearly prove a model for ALL development in which leukemogenesis occurs within at least two time-spaced windows of mutational susceptibility, consisting of the pre-natal origin of the disease and the latent pre-leukemic phase.

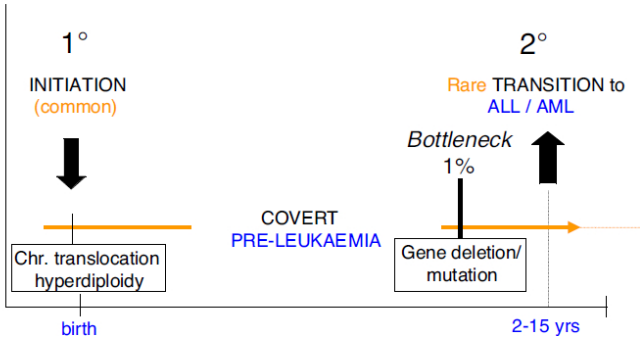


Figure 6. Minimal model of the natural history of childhood ALL. From Zelent *et al.*, Oncogene 2004.

The initiating event, or *first hit*, in childhood ALL arises *in utero* in the fetal Hematopoietic Stem and Progenitor Cells (hereafter indicated as HSPC) and consists in numerical or structural chromosomal alterations, in particular hyperdiploidy and balanced translocations (Ford *et al.*, 1998) (Greaves and Wiemels, 2003). These are anything but uncommon events, as some of them are estimated to occur in up 1-5% of healthy newborns (i.e. ETV6-RUNX1) (Wiemels *et al.*, 1999) (Mori *et al.*, 2002) (Schäfer *et al.*, 2018). This scenario describes the first window of leukemia susceptibility; however, although chromosomal alterations are necessary for disease initiation, they are insufficient for overt leukemia. Mutations occurring in the second exposure window, which is collocated generally between 2-5 years of age, are necessary to complete malignant transformation. This is true for the most frequent childhood ALL subtypes, while infant cases represent an exception in this sense, as leukemogenesis is mostly completed before birth in these patients, associated with a

very short latency (Greaves, 2018). Compared to the relative high frequency of *in utero* chromosomal aberrations, post-natal leukemia transition is a rare event, meaning that acquisition of favoring secondary hits represents a bottleneck in the leukemogenic process. A limiting step for disease onset, however, derives also from functional implications of the first hit: gene recombinations, in fact, must occur *in frame* and in the appropriate target cell to be effectively productive (Paashuis-Lew and Heddle, 1998) (Zelent, 2004). When this happens, they give rise to a clinically silent pre-leukemic clone which can persist in the individual for many years (up to 15) (Ford *et al.*, 2001) representing a susceptible cellular target for additional lesions accumulation. The period between the first hit and ALL diagnosis is defined as the pre-leukemic phase, a key aspect in childhood ALL (and AML) pathogenesis which not only represents the basis of the disease but also provides the cellular reservoir for relapses. Chemotherapies, in fact, while efficiently eradicating the leukemic burden, are often ineffective against covert pre-leukemic cells (Shlush *et al.*, 2014). Most of the evidences supporting the two-steps model have been obtained from studies on ETV6-RUNX1^{+} and MLL-rearranged BCP-ALL (Greaves, 2018). Translocated cases are particularly informative in this sense, since each patient gene fusion is characterized by a unique breakpoint sequence whose analysis provides a stable and sensitive marker for molecular tracking strategies. In addition, clone-specific rearrangements of immunoglobulin genes represent a solid indicator of cell clonality. In particular, the pre-natal origin of BCP-ALL is supported by three different approaches based on fusion sequences

analysis. The first one is represented by international studies on monozygotic twins with concordant disease. Twin pairs with ETV6-RUNX1^{+} and MLL fusions showed, in fact, a perfect homology in breakpoints and V(D)J clone-specific immunoglobulin heavy chain (IGH) rearrangements (Alpar *et al.*, 2015) (Bateman *et al.*, 2015). The most reasonable hypothesis for these acquired, non-germinal clonal mutations is that a unique leukemic funder clone, originated *in utero*, had migrated from one twin to the other through the vascular anastomosis in the monochorionic placenta (Greaves *et al.*, 2003). The second and more direct proof consists in the retrospective screening of Guthrie's blood spots (peripheral blood samples collected at birth to detect pathological genetic conditions) of ALL patients: these samples, in fact, showed identical MLL- or ETV6-RUNX1 translocation breakpoints found at diagnosis (Gale *et al.*, 1997) (J L Wiemels *et al.*, 1999), clearly indicating their pre-natal origin. Notably, while pre-leukemic founder lesions are shared between twins, secondary post-natal mutation are sibling-specific, indicating a divergent clonal evolution of the disease. It is noteworthy that concordance rate in monozygotic twins varies according to ALL subtype: in infants with MLL-rearranged ALL, in fact, concordance almost reaches 100%, as very few additional mutations are necessary to overt leukemia. An opposite scenario is typical of ETV6-RUNX1^{+} ALL instead, where concordant twins are rare due to the higher number of additional mutations required. Finally, large-scale screening of healthy cord blood revealed that leukemia-initiating chromosomic abnormalities

are commonly produced during fetal hematopoiesis, although they can be present in a very limited percentage of cells (Mori *et al.*, 2002). If concordant ALL twins demonstrate the fetal initiation of disease, necessity of a second hit is evident in *discordant* ALL twins. In these pairs, in fact, one sibling is affected by the pathology while the healthy individual only show the pre-leukemic clone, corroborating the transplacental migration hypothesis and demonstrating inability of a single translocations to induce leukemia. Moreover, healthy twins represent a very rare but precious source for *in vitro* and *in vivo* explorations of pre-leukemic cells. Studies on one healthy ETV6-RUNX1^{+} sibling, for example, demonstrated that the pre-leukemic clone possesses enhanced self-renewal and partial B-cell differentiation at the same time (Gale *et al.*, 1997). However, the experimental support to two-steps hypothesis for ALL development derive from mouse experimental models: despite differences in approaches (i.e. xenografts *versus* transgenic mice), in fact, they substantially concord in demonstrating the asymptomatic phenotype of chromosomal translocations and the requirement of additional genetic damages (i.e. chemicals-inducted, gene knock out, infectious exposure) for leukemia induction (Tsuzuki *et al.*, 2004) (Morrow *et al.*, 2004) (Schindler *et al.*, 2009) (Kantner *et al.*, 2013) (Rodríguez-Hernández *et al.*, 2017). Regarding possible causes of pre-natal lesions, no solid indications on pregnancy exposures have been produced up to date. The main hypothetical factor remains the spontaneous mutational rate during embryonic development. Strong proliferative signals, oxidative stress and massive apoptosis in early lymphopoiesis

could reasonably linked to DNA damages in hematopoietic precursors. It has been estimated that Double Strand Breaks (DSBs), which are the basis of chromosomal rearrangements, occur at about 50 per cell cycle in humans (Vilenchik and Knudson, 2003). In particular, cleavage activity of topoisomerases II and apoptotic endonucleases mediate chromosomal breaks; for this kind of damage, Non-Homologous End Joining (NHEJ) repair is engaged and, being an error-prone mechanism, could provoke altered sequence rearrangements (J L Wiemels *et al.*, 1999). It is quite impressive that chromosome translocations occur in normal blood at a rate that exceeds about the corresponding leukemia rate; recalling the concept illustrated in Fig.7, events that complete the oncogenic potential of chromosomal abnormalities thus represent the real limiting step for leukemia onset and, for this reason, they deserve a particular attention and great efforts for comprehension.

The "delayed infectious hypothesis" as specific causal mechanism for childhood B-Cell Precursor (BCP)-ALL

Identification of triggering causes of childhood leukemia is maybe one of the most compelling aim in the field. Excluding the case of massive exposure to ionizing radiations (i.e. Hiroshima's atomic bomb), the idea of a unique cause appears simplistic. In general, candidate causal mechanisms should satisfy at least these criteria: explain the peak incidence at 2-5 years as well as the preferential geographic area (the Western world) and provide biological explanations for the natural history and molecular aspects of ALL. In

1922 F. J. Poynton, H. Thursfield and D. Paterson suggested that *"…the solution of the problem of leukemia lies rather in some peculiar reaction to infection than in the existence of some specific infective agent"* (Poynton, F. J., Thursfield, H. & Paterson, 1922). The first rationale for their supposition was the coincidence in the age distributions between ALL and infective childhood disease (i.e. measles, varicella, upper-respiratory tract infections); at that time, however, no empirical data sustaining the hypothesis were produced. The idea was revived by Professor Mel Greaves in 1988, who proposed the so-called *"delayed infection hypothesis"*: according to this model, infections and inflammation promote secondary hits acquisition in pre-leukemic cells through a dysregulated immune response to common infections. The immune system has been programmed by evolutionary forces to anticipate infectious exposures and to be "educated" by these latter precociously in life. This serves to organize an effective but regulated immune response that protects the individual against exogenous agents without damaging "self" cellular components. Modern societies are characterized by population mixing and hyper-hygienic habits at the same time; in these conditions, children's delayed exposure to infective microenvironments avoid the correct education of their immune system and could predispose to aberrant immune responses which, in turns, could provoke proliferative and/or apoptotic stress in immune cells. A similar scenario appears extremely dangerous in presence of vulnerable pre-leukemic cells and of predisposing genetic backgrounds that altered immune signals. Although many efforts have been made to identify a

responsible infectious agent, none of them have provided evidences. For example, leukemic cells are negative for the most common viral sequences, although a missing-virus in the list or a "*hit-and-run*" mechanism can't be excluded (Greaves, 2018). Regarding this, another infectious hypothesis has been proposed; in the "*population-mixing model*", in fact, the cause of leukemia development resides in a defective immune response of susceptible individuals to a non-endogenous virus in the context of population mixing (Kinlen, Clarke and Hudson, 1990). Besides differences, however, the two models agree in defining leukemia as a rare response to one or more infections acquired by genetically and socially predisposed individuals. Several social arguments support the delayed infection hypothesis: day care attendance in infancy, birth order, mode of delivery, breast feeding, vaccinations and time-space clusters. It has been reported that day care attendance in the first 12 months of life is associated with decreased risk of ALL (Gilham *et al.*, 2005); day care attendance, in fact, can be considered as a surrogate of infections exposure and, consequently, a surrogate of the precocious education of the immune system. In the same way birth order can influence ALL risk as the firstborns, not being able to benefit from the exposure of sibling, are more susceptible to the disease than laterborns (Dockerty *et al.*, 2001). Babies born through a caesarian delivery have been deprived of vaginal benign microbiota and show a significant increase in ALL cases (Marcotte *et al.*, 2016); prolonged breast feeding (at least 6 months) also reduced risk of ALL (10–20%), likely due to the transfer of immunomodulatory factors (i.e. maternal antibodies, anti-

inflammatory molecules, ancillary maternal cells, lactobacilli and oligosaccharides that nourish the infant's intestinal microbiome (Penders *et al.*, 2006). Vaccinations by facilitating the early education of the immune response in children could be protective against ALL; although inconsistent results have been produced for the majority of vaccines, there is one exception: immunization against *Haemophilus influenzae* type B in infancy seems to provide protection against ALL (Ma *et al.*, 2005). Finally, time-space clusters of ALL cases have been found to be associated with a specific infection or microorganism species spread in particular social circumstances (Heath and Hasterlik, 1963) (Francis *et al.*, 2012) (Cazzaniga *et al.*, 2017). A key aspect of the Greaves' hypothesis is that it seems to be valid only for BCP-ALL, while is not applied to other pediatric cancer (i.e. childhood AML) nor to adult leukemias (Greaves, 2018). From a molecular point of view, the most plausible mechanism for a dysregulated immune response could be represented by altered T-cell functions and cytokine/chemokine profile (Ford *et al.*, 2009) (Blank and Karlsson, 2015) (Wang *et al.*, 2018). In this context, physiological hematopoiesis could be negatively affected in terms of proliferation and/or survival; on the contrary, pre-leukemic cells could show a different behavior, possibly due to alterations in pathways involved into immune/inflammatory responses, gaining a transient advantage in the bone marrow against the normal counterpart. Rounds of expansions of pre-leukemic cells within an inflammatory genotoxic microenvironment could finally results in secondary mutations acquisition and malignant transformation (Greaves, 2018). For that reason, genetic variations

influencing the strength of the immune response could play a role in the pre-leukemia to leukemia transition. It has been shown, for example, that genes polymorphisms cooperate with infectious exposure in infancy to define the cytokine release profile (Parker and Ollerton, 2013): candidate loci could likely include MHC, for which an association between HLA-DPB1*0201 allele and risk of childhood cALL has been demonstrated (Cazzaniga *et al.*, 2017), and those encoding cytokine/chemokine and their receptors. Concluding, the causal mechanism for childhood ALL proposed by the delayed infection hypothesis is necessarily multifactorial as it involves infection patterns, genetic predispositions, social habits and chance. The main concept, however, is that childhood ALL may arise from a *bias* between the evolutionary programming of the immune system and the contemporary hyper-hygienic society and the comprehension of molecular mechanisms underlying this *bias* could provide possibility for a better management of ALL risk in infancy.

Translocation t(12;21) and ETV6-RUNX1^{+} (TEL-AML1^{+}) BCP-ALL

Transcription factors (TF) that are critical to stem-progenitor cells development or lineage specification in haematopoiesis, such as ETV6 (ETV6), ETV6 (RUNX1), RARα, and MLL, are major targets for initiating alterations in childhood leukemias. TF alterations are classified as "*class II mutations*" and result in the generation of aberrant transcription factors which block differentiation and/or apoptosis by interfering with their wild-type counterparts functions[23]. Although required, class II mutations are insufficient for tumorigenesis and cooperation with "*class I mutations*" (i.e. growth-promoting tyrosine kinase mutations, onco-suppressor loss of function) are necessary for the development of a fully malignant disease[123]. The *ETV6-RUNX1* chimeric gene, originated from the chromosome translocation t(12;21)(p13;q22), is a class II mutation resulting in an aberrant transcription factor with constitutive repressive activity. Translocation occurs *in utero* and, as said in the previous chapters, is the most frequent initiating hit in the pathogenesis of childhood ALL (about 22% of cases); however, it is detected in *healthy* neonatal at a relative high frequency (1-5%), indicating that its generation is a common event during fetal hematopoiesis. On the contrary, ETV6-RUNX1 is rare in adult ALL, indicating that its pathological impact is determined and restricted by a precocious developmental step (Böiers *et al.*, 2018). Since only 1% of ETV6-RUNX1 carriers face childhood ALL

(average ALL incidence: 1:10.000-1:5000), this indicates that ETV6-RUNX1 itself is insufficient for driving leukemogenesis. Its activity, in fact, is limited to the generation of clinically covert pre-leukemic progenitors characterized by enhanced self-renewal and partial B-cell differentiation. Post-natal secondary hits in pre-leukemic cells are required to complete malignant transformation and their acquisition represents a limiting bottleneck in ALL pathogenesis. Deletion of the non-translocated ETV6 gene is often present at leukemic transformation (Takeuchi *et al.*, 1997), while additional genomic alterations have also been uncovered, including 12p aberrations, chromosome 21 gains, ETV6/RUNX1 duplications, KMT2A aberrations, deletions of 6q and 9p (including the CDKN2A gene) and numerous trisomies (chromosomes 21,4,10,16) (Mullighan, 2013).

Epidemiological and experimental evidence now supports the view that a plausible mechanism for this critical conversion step could involve an abnormal immune response to common infection, as postulated by the delayed infection hypothesis (Greaves, 2006).

The wild-type ETV6 (TEL) and RUNX1 (AML1) genes

ETV6 gene (Ets Translocation Variant gene 6), also known as TEL gene (translocation ets leukemia), is a member of the ETS family of transcription factors that essentially act as transcriptional repressors (Lopez *et al.*, 1999). In particular, accumulating genetic and functional evidences, both in vitro and in vivo, have induced some

authors to consider ETV6 as a tumor suppressing gene. The complete loss of function of this gene, in fact, is often a secondary promoting event in ALL (Takeuchi *et al.*, 1997). Human ETV6 gene is located on chromosomal 12p13 and spans 240kb. The presence of alternative polyadenylation sites at the 3' UTR of ETV6 generates three transcripts of 2.4, 4.3 and 6.2 kb. cDNA sequence is long 1356 kb and contains two alternative translational start codons (position 1 and position 43) leading to the expression of two isoforms of ETV6, originating two proteins of 57 kDa and 50 kDa respectively (Poirel *et al.*, 1997). ETV6 protein possesses a N-terminal PD domain, which mediates homo/heterodimerization and co-repressor binding, a central repression (CR) domain and a C-terminal ETS domain responsible for nuclear localization, protein-protein interactions and DNA-binding. By means of PD and CR repressor domains, ETV6 interacts with the corepressors N-Cor, mSin3 and SMRT, that recruit histone deacetylases (HDACs). So far, only few targets of the repressor activity of ETV6 have been described: monocyte colony stimulating factor receptor (MCSFR), megakaryocytic promoters GPIb and GPIX, the matrix metalloproteinase stromyelisin/MMP3, the oncogene T-cell lymphoma 1 (TCL1) and the anti-apoptotic factor Bcl-XL (Lopez *et al.*, 1999). A more comprehensive study identified identified 87 ETV6-modulated genes, 10 of which (AKR1C1, AKR1C3, IL18, LUM, PHLDA1, PTGER4, PTGS2, SPHK1, TP53 and VEGF) were validated by real-time quantitative reverse transcription-polymerase chain reaction. These genes are associated with cancer, in particular with cell adhesion, cell growth/proliferation, apoptosis and angiogenesis (Boily *et al.*, 2007).

The activity of ETV6 is modulated at various levels and particularly through protein localization: ubiquitination, phosphorylation and sumoylation at specific aminoacidic residues lead to increased nuclear export of ETV6, with consequent inhibition of ETV6 repressor activity (Bohlander, 2005). It has been shown that ETV6 is not directly required for yolk sac haematopoiesis, but it is essential for maintaining the integrity of the surrounding vascular network and for the survival of neural and mesenchymal cells within the embryo (Wang *et al.*, 1997). On the contrary, ETV6 is essential for the establishment of all hematopoietic lineages in the bone marrow as demonstrated by the inability of $ETV6^{-/-}$ haematopoietic HSPC transplanted into a wild-type blastocyst to repopulate the adult bone marrow. This could lead to a defect in migration toward the bone marrow or, more likely, in the failure of ETV6 deficient cells in responding appropriately and/or survive within the bone marrow microenvironment (Wang *et al.*, 1998). Thus, ETV6 is a transcription factor specifically required for bone marrow but not fetal hematopoiesis, at least not directly. ETV6 has been found rearranged with more than 40 chromosome bands, making it one of the most "promiscuous" gene involved in human leukemia. In many cases in which ETV6 is rearranged there is a concomitant deletion of the non-rearranged ETV6 allele, supporting its onco-suppressor activity and demonstrating the very important role for ETV6 loss of function as a secondary event in leukaemia. In addition, point mutations in the ETS domain of ETV6 have also been demonstrated and studies of single nucleotide polymorphisms (SNP) arrays technique identified ETV6 deletions as one of the three genetic

abnormalities more frequently detectable in paediatric ALL patients (on 399 patients: 29% hyperdiploidy, 29% $p16^{INK4A}$ deletions and 22% ETV6 deletions) (Kawamata *et al.*, 2008).

RUNX1 (Runt-related transcription factor 1), also named AML1 (acute myeloid leukemia 1), PEBP2α or CBFα, belongs to the Runt domain family of transcription factors which act as master regulators of gene expression in major developmental pathways. In mammals the family includes three genes: RUNX1/AML1, RUNX2/AML3 and RUNX3/AML2. AML1 represent the α subunit of the heterodimeric transcription factor PEBP2 (or CBF)48 and works as either a transcriptional activator or repressor in a context-dependent manner by regulating a large number of haematopoietic-specific genes49 50. RUNX1 gene is located on chromosome 21q22.12, spans about 260kb and it is organized in 12 exons. Transcription is initiated at two distinct promoters and both these promoters generate a large number of alternatively spliced mRNAs (Levanon *et al.*, 2001). These alternative mRNAs encode three different RUNX1 protein isoforms: the two major ones, RUNX1b and RUNX1c, own two large functional domains: a proximal DNA-binding region, the Runt homology domain (RHD) and a distal transactivation domain (TD) . The RHD domain is essential for both DNA binding to a specific DNA consensus sequence (T/cGT/cGGT) and for the heterodimerization with the PEBP2β/CBFβ. The heterodimeric RUNX1/PEBP2β complex regulates transcription of a large number of hematopoietic-specific genes: receptors such as the subunits of T-cell antigen receptor (TCR) and macrophage colony-stimulating factor (M-CSF) receptor, enzymes such as

myeloperoxidase (MPO), neutrophil elastase and granzyme B, cytokines such as IL3 and granulocyte macrophage colony-stimulating factor (GM-CSF) (Asou, 2003). As RUNX1/PEBP2β possess a weak intrinsic transactivation potential for the expression of these genes, it mainly functions as a transcriptional organizer that recruits tissue-specific transcription factors via interactions with transcriptional coactivators such as p300, CBP and P/CAF. During mouse embryonic development, high levels of RUNX1 can be detected in haematopoietic stem cells, endothelial cells of the aorta–gonad–mesonephros (AGM) region and other tissues. However, after organogenesis, high expression of RUNX1 is restricted primarily to cells of the haematopoietic lineages, including B and T lymphoid cells, granulocytic and monocytic cells, megakaryocytes, and CD34+ marrow cells (Gunji *et al.*, 2004). Null mutations in either RUNX1 or PEBP2β gene result in embryonic lethal phenotype at E12.5 with a complete lack of fetal liver hematopoiesis and central nervous hemorrhages, although primitive yolk sac erythropoiesis appears normal. These defects are intrinsic in the hematopoietic cells and not a result of a defect in the fetal liver microenvironment (Okuda *et al.*, 1996). In particular, several studies have revealed that RUNX1 and PEBP2β are required for the generation of hematopoietic stem cells (HSC) from haematogenic endothelium. In addition to the spatial-temporal pattern expression, the dosage of RUNX1 during fetal hematopoiesis is also critical. A hemizygous dose of RUNX1 results in a dramatic change in distribution of HSCs, leading to their earlier appearance in the yolk sac on E10 and their premature extinction in the AGM

(Yamagata, Maki and Mitani, 2005). On the contrary, adult murine HSCs do not require RUNX1 for their maintenance; rather, the absence of this gene seems to provide a proliferation advantage to haematopoietic progenitors. At the same time, AML is protective against genotoxic insults in adult HSC (Motoda *et al.*, 2007); to this regard, RUNX1 aploinsufficiency, which characterizes the familial platelet disorder with propensity to AML (FPD/AML), predisposes to acquisition of additional mutations and induce differentiation block thus favoring the transition to AML. Finally, RUNX1 is crucial for megakaryocytic maturation and for T and B lymphocyte differentiation (Ichikawa *et al.*, 2004). In contrast to haploinsufficiency and point mutations, RUNX1 overexpression or its amplification by polysomy of chromosome 21 or gene duplication have been reported mainly in pediatric ALL and less often in AML . In conclusion, RUNX1 is a master regulator of gene transcription that can work as either a transcriptional activator or repressor in a context-dependent manner by heterodimerizing with the PEBP2β subunit to form the CBF complex; its activity is regulated by the balance of cellular coactivators and corepressors as well as AML transcriptional and translational modifications. RUNX1 is specifically required for embryonic development of definitive haematopoiesis and regulation of lymphoid differentiation and megakaryocytic maturation, while it is dispensable for the maintenance of adult HSC.

Molecular pathogenesis of translocation t(12;21)

As anticipate above, early lymphopoiesis is characterized by high proliferation rate and massive oxidative/apoptotic stress that commonly induced spontaneous mutations (Paashuis-Lew and Heddle, 1998). Endogenously-driven DNA double-strand breaks (DSB), that are required for fusion gene recombinations, occur at ~50 per cell cycle in human cells (Vilenchik and Knudson, 2003). As no evidences have been reported up top date for exogenous risk factors, ALL-initiating chromosomal translocations can be considered as "developmental errors". Although early reports indicated the RAG1/2 and topoisomerase II enzyme as the mediator of chromosomal translocations in T-ALL, the wide-spread pattern of breakpoints cluster regions (BCR) in B-ALL likely excludes this mechanism, rather suggesting that the chromatin functional status could play a more important role. Chromosome-scaffold attachment regions and open chromatin regions, in fact, have been found to be associated with BCR in B-ALL; these regions are particularly susceptible to nucleases, which are exogenous in case of experimental demonstration but could be endogenous in the physiological context (Greaves, 2006). Effectively, implication of apoptotic endonucleases has been demonstrated for the ETV6-RUNX1 translocation: short-term culture of B-progenitor cell lines in the presence of apoptotic stimuli (i.e. serum starvation), in fact, induced DSB in intron 5 of the ETV6 gene and intron 1 of the AML gene originating a ETV6-RUNX1 fusion transcript (Eguchi-Ishimae *et al.*, 2001).

At the molecular level, ETV6-RUNX1 fusion gene results from the balanced translocation t(12;21)(p12;q22) which fuses the 5' portion of the ETV6 gene (encoding the PD and the CR domains) to nearly the entire sequence of RUNX1 gene maintaining the expression of the chimeric transcript under the ETV6 promoter. Most of the breakpoints occur in the intron 5 of ETV6 and in the intron 1 of AML, causing the joining of ETV6 exon 5 (nucleotide 1033) to RUNX1 exon 2 (nucleotide 503). ETV6-RUNX1 chimer protein retains the PD ant the CR domains of ETV6 while gains the RHD, the transactivation and the inhibitory domains of RUNX1 (Fig.7).

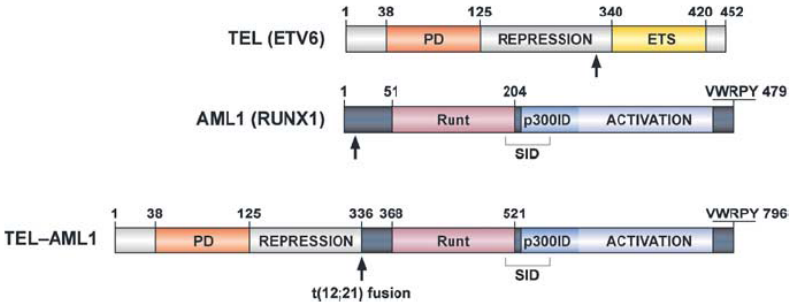


Figure 7. Schematic representation of ETV6, RUNX1 and ETV6-RUNX1 proteins. From Zelent *et al.*, Oncogene 2004.

While normal RUNX1, in association with PEBP2β (CBFβ), can function as a transcriptional activator or repressor depending on post-translational modifications and interaction with different co-factors, in ETV6-RUNX1 the ability of ETV6 moiety to dimerize and to bind co-repressors allows for the formation of a stable constitutive repressor

complex that renders the fusion protein resistant to the regulation described for RUNX1 (Fig.8).

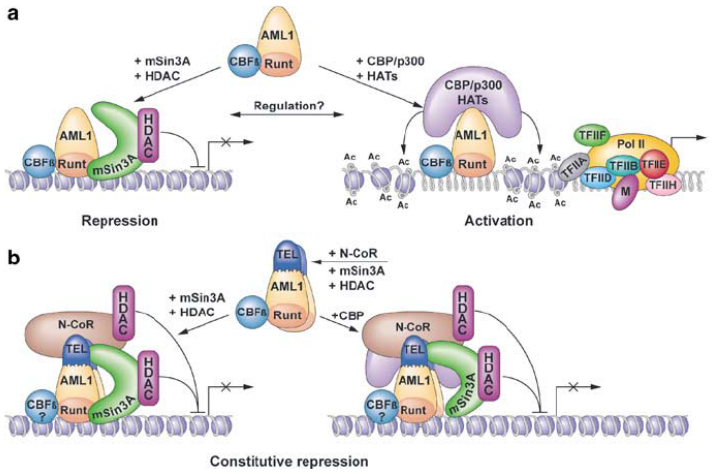


Figure 8. A molecular model for ETV6-RUNX1 constitutive repression activity: a) co-factors binding and post-translational modifications regulate RUNX1-CBFβ complex transcription; b) PD and CR domain of ETV6 abrogate the physiological RUNX1 transcriptional regulation lead to constitutive transcriptional repression of target genes. From Zelent *et al.*, Oncogene 2004.

Therefore, since ETV6-RUNX1 retains the ability to bind RUNX1 target sequences by RHD domain, it has the potential to function as a constitutive repressor of RUNX1 target-genes. In addition to act as a repressor domain *per se*, the PD and the CR domains mediate the ETV6-RUNX1 association with co-repressors; finally, ETV6-RUNX1 exerts dominant-negative effects also over ETV6 and RUNX1 proteins by heterodimerizing with them (Gunji *et al.*, 2004). While it is not clear if ETV6-RUNX1 requires PEBP2β heterodimerization for efficient DNA binding, it has been established that RHD domain of RUNX1 and PD

and CR domains of ETV6 are necessary for ETV6-RUNX1 transcriptional activity (Morrow *et al.*, 2007). Recent studies have individuated several *de novo* targets for ETV6-RUNX1 transcriptional regulation, demonstrating the complex and broad-spectrum activity of this aberrant transcription factor on cell expression profile, as discussed in detail in the next paragraph.

Concluding, ETV6-RUNX1 is a constitutive repressive transcription factor mainly acting on RUNX1-regulated genes but also binding *de novo* targets; in addition, it possesses dominant-negative effects over the RUNX1 and ETV6 proteins by binding the normal counterparts (Gunji *et al.*, 2004).

Exploring the ETV6-RUNX1 pre-leukemic activity: in vitro and in vivo models

While ETV6-RUNX1$^+$ fully leukemia is very common in pediatric patients facilitating its biological study, ETV6-RUNX1$^+$ pre-leukemia investigations are limited by the scarcity of available material. In fact, although 1-5% of healthy newborns presents the translocation, the silent nature of ETV6-RUNX1 prevents the possibility to individualize them prior an eventual fully disease. For this reason, efforts for the comprehension of ETV6-RUNX1 pre-leukemic role have been made by deducing ETV6-RUNX1$^+$ functions from primary blasts or leukemic cell lines and by setting *in vitro* and *in vivo* pre-leukemic experimental models.

ETV6-RUNX1 in vitro activities

By comparing gene expression profile (GEP) of ETV6-RUNX1^{+} and untranslocated BCP-ALL patients, Gandemer and colleagues (Gandemer *et al.*, 2007) identified five gene ontology (GO) categories that were enriched in the ETV6-RUNX1 subgroup and consisted in *cell differentiation, cell proliferation, apoptosis, cell motility and response to wounding*. Such categories were associated with fourteen differentially expressed genes that were able to cluster the ETV6/RUNX1 subgroup; interestingly, among these, overexpressed RUNX1 was frequently found in ETV6-RUNX1^{+} patients. Although this description didn't directly prove the ETV6-RUNX1 functional activity, particularly at the early stage of the disease, it gave a general view of the pathways impacted by the fusion gene. A major contribution in this sense has been provided by Linka and colleagues (Linka *et al.*, 2013): taking advantage from an inducible ETV6-RUNX1 system derived from the murine pro B-cell line Ba/F3 (Ford *et al.*, 2009) and integrating three different genome wide screening methods, authors were able to identify both direct and indirect ETV6-RUNX1-target genes; as expected, directly regulated promoters were enriched in RUNX1-binding sites. Moreover, five ETV6-RUNX1 direct targets (PDLIM5, INTS2, RSL1D1, METAP2 and GNB2L1) described in the Ba/F3 model were dysregulated also in ETV6-RUNX1^{+} patients, suggesting their importance through the ETV6-RUNX1-driven leukemogenesis. Very interesting, the majority of promoters were *de novo* targets of the fusion proteins and they didn't bind native ETV6 or RUNX1 proteins. In

line with the previous study, dysregulated genes identified here correlated with negative effects on proliferation and cellular transport mechanisms while they positively affected cellular migration, cellular stress responses and immunological responses. The negative impact of ETV6-RUNX1 on cell proliferation, at least in B-progenitor *in vitro,* has been reported by Ford et al.: through their inducible ETV6-RUNX1 Ba/F3 model, authors demonstrated that percentage of cells in the G0/G1 phase significantly increased in pre-leukemic cells compared to controls. In addition, the work highlighted a feature of ETV6-RUNX1$^+$ cells that appeared very interested in the context of a putative infection-driven leukemogenesis: ETV6-RUNX1-expressing Ba/F3, in fact, showed an intrinsic resistance to the anti-proliferative effect of TGFβ, a pleiotropic cytokine produced during inflammation by several stromal and immune cells (Pietras, 2017). The effect depended on the binding of ETV6-RUNX1 to SMAD2/3 proteins which avoided their correct TGFβ signal transduction; this led to the selective emergence of preleukemic cells against the normal counterpart in presence of the cytokine. More interestingly, TGFβ selected putative Leukemic Stem Cells (LSC) within ETV6-RUNX1-transduced human CD34$^+$ cells, providing a possible explanation for the promoting role of infection/inflammation in ETV6-RUNX1$^+$ leukemia. TGFβ signaling is not the only inflammation-related pathway that is affected by ETV6-RUNX1 expression: another work of global gene expression profiling in control and ETV6-RUNX1$^+$ murine and human hematopoietic progenitors, in fact, identified the interferon (IFN)α/β signaling as a primary target of ETV6-RUNX1 repression. In this case, ETV6-RUNX1

blocked the IL-7-induced B-cell differentiation by decreasing interferon-regulatory factor 3 (IRF3) expression and phosphorylation confirming the partial differentiation block observed in numerous mouse models of ETV6-RUNX1 pre-leukemia (De Laurentiis *et al.,* 2015). As documented by various studies, apoptosis is variously affected by ETV6-RUNX1 in both pre-leukemic and leukemic cells. ETV6-RUNX1 silencing by RNA interference (iRNA) in the REH cell line, for example, resulted in a downregulation of HSP70/HSP90 and survivin (BIRC5) proteins leading to increased apoptosis in silenced cells (Diakos *et al.*, 2007). Torrano et. al (Torrano *et al.*, 2011) showed that ETV6-RUNX1 directly binds the EPOR promoter inducing the expression of a functional receptor in both murine and human pre-leukemic cells; this correlated with increased cell survival through the activation of the JAK2- STAT5 pathway and the upregulation of anti-apoptotic BCL-XL. By upregulating RAC1 activity, ETV6-RUNX1 also increased STAT3 signaling with consequent MYC and surviving transcription; inhibition of this pathway caused cell cycle arrest and apoptosis in leukemic cell lines as well as in primary blasts (Mangolini *et al.*, 2013). If on one hand ETV6-RUNX1 decreases the cell capacity to proliferate, differentiate and execute apoptosis, on the other hand it positively affects pathways involved in oncogenic signaling and DNA damaging: on this regard, ETV6-RUNX1-induced upregulation of MYC represented a potential mechanism for oncogenic addition in ETV6-RUNX1^{+} cells (Mangolini *et al.*, 2013). Another contribution in this sense came from Kantner and colleagues: having observed, both *in vitro* and *in vivo,* elevated levels of reactive oxygen species (ROS) and

intracellular phospho-histone H2AX (a marker of DNA double strand breaks) in ETV6-RUNX1^{+} murine pro-B cells, the authors proposed ROS-mediated genotoxicity as a putative ETV6-RUNX1-driven mechanism for leukemia progression (Kantner *et al.*, 2013). Proliferation, apoptosis and differentiation are the crucial mechanism for cell homeostasis and represent critical targets of leukemic genes' transforming activity; in addition to cell-intrinsic regulative pathways, these cell functions are also regulated by cell-extrinsic signals which, in case of HSPC, derived from the surrounding bone marrow niche. BM niches, in fact, are specialized microenvironments composed by several cell types with different hematopoietic regulating functions explicated by cell-cell contacts and molecules secretion; in addition, cell/matrix interactions and physical/chemical gradients contribute to define the hierarchical, lineage-specific spatial organization in the niche (Lo Celso and Scadden, 2011). Cell adhesion and migration, as well as responsiveness to cytokines and growth factors are thus critical for HSPC cell fate in terms of retention, proliferation/survival and differentiation (Forsberg and Smith-Berdan, 2009). Interestingly, our previous data demonstrated that ETV6-RUNX1 induction in Ba/F3 cells dysregulated the expression of several adhesion molecules (CD44, CD11a, CD54, CD18, CD29) and impaired migration towards CXCL12 although increasing cellular basal motility (Palmi *et al.*, 2014). These alterations could lead to a dysregulated interaction of the pre-leukemic clone within the niche and to important functional consequences, not only in the normal context but also under inflammatory conditions.

The "real cell target" of ETV6-RUNX1

As functional studies on ETV6-RUNX1 activity progressed, it became more and more evident that experimental observations were strongly impacted by the cellular background and the levels of ETV6-RUNX1 expression. The question about the "real cell target" of ETV6-RUNX1 pre-leukemic activity has been widely debated and a crucial aspect in the issue is how to reproduce the early ETV6-RUNX1 pathogenesis given that the translocation occurs *in utero*. Primary fetal samples could represent a source for the purpose but there are obviously practical and ethical limits. Induced Pluripotent Stem (iPS) cells can provide a powerful opportunity in this sense as they permit to follow, starting from an "embryonic-like" status, the physiological development of a specific cell type *in vitro* through appropriate manipulations. In addition to the childhood affiliation, the natural history of ETV6-RUNX1^{+} ALL is characterized by other features that must be taken into consideration: 1) common ancestral clones in monochorionic twins present partial and monoclonal IgH DJ or even TCR rearrangements (Alpar *et al.*, 2015), strongly indicating that leukemic transformation occurs within an early RAG-expressing multipotent lymphoid progenitor and excluding a more primitive RAG^{-} HSC population (Alpar *et al.*, 2015) (Hong *et al.*, 2008); 2) aberrant expression of myeloid antigens are commonly seen in ETV6-RUNX1^{+} patients suggesting that disease may arise in multipotent progenitors that physiologically harbors myeloid as well as lymphoid potential (Gerr *et al.*, 2010) (Abdelhaleem, 2007); 3) a recent study individuated

murine fetal interleukin-7 receptor/Rag1 positive (IL-7R$^+$/Rag1$^+$) lymphoid progenitor that contributed to both myeloid and lymphoid lineages in the embryo, differently form adult Rag1$^+$ progenitors that contributed almost exclusively to the lymphoid lineage (Böiers *et al.*, 2013). Starting from these considerations, Böiers et al. have supposed that ETV6-RUNX1 pathogenesis must occur in a progenitor uniquely emerging in the embryonic life. In order to demonstrate that, authors have firstly characterized emerging B cells in first trimester human embryos identifying a developmentally restricted CD19$^-$/IL-7R$^+$ progenitor compartment which transits from a myeloid to lymphoid program during ontogeny, a developmental stage that has been recapitulated also in B-cell differentiating iPS. Interestingly, iPS expressing ETV6-RUNX1 from the endogenous ETV6 locus expand the CD19$^-$/IL-7R$^+$ compartment, showed a partial block in B lineage commitment and produce pro-B cells with an aberrant myeloid gene expression and potential, thus recapitulating features of the human pre-leukemic state (Böiers *et al.*, 2018).

ETV6-RUNX1 in vivo activities

If the previously described *in vitro* models provide direct insights into the molecular mechanisms of ETV6-RUNX1 activity, a "systemic" pre-leukemic ETV6-RUNX1-induced phenotype can be observed only *in vivo*. Both transplanted mice and transgenic mouse models have been widely used to explore ETV6-RUNX1 pre-leukemic

activity in absence of other confounding mutations (that are present, for example, in patients blasts or leukemic cell lines). Despite differences in the approach, the majority of them agree in demonstrating that the fusion gene is insufficient to over disease in absence of secondary genetic insults; its effect, in fact, is limited to induce a pre-leukemic status that persist with a clinical silent phenotype. This experimental observation was observed directly in humans in a milestone study of Hong and colleagues that characterized the pre-leukemic cellular phenotype in discordant ETV6-RUNX1^{+} ALL twins (Hong *et al.*, 2008). By analyzing the bone marrow and the peripheral blood of the diseased and the healthy sibling respectively, authors were able to identify a putative ETV6-RUNX1^{+} pre-leukemic "stem" cells (pre-LSC) with an aberrant stem/B-progenitor phenotype (CD34^{+}/CD38$^{-/low}$/CD19^{+}) in both. Although fully leukemic cells carried additional mutation (i.e wild-type Etv6 loss) compared to those in the pre-leukemic individual, IgH gene sequence analysis clearly demonstrated their clonal origin from a unique clone spreading via transplacental migration. The percentage of CD34^{+}/CD38$^{-/low}$/CD19^{+} in the "pre-leukemic" twin was very low but cells stably persisted over 18 months of observation, indicating their conserved self-renewal potential; on the contrary, a similar cell phenotype was completely absent in age-matched healthy children. Interestingly, while ETV6-RUNX1 transcript was expressed in the putative pre-LSC compartment, it wasn't found in committed B-progenitors, thus suggesting a precise cellular context restriction for its expression and activity. In order to further explore the CD34^{+}/CD38^{-}

$^{/low}$/CD19^{+} compartment, the same authors set up a pre-leukemic xenograft mouse model by transplanting into NOD/SCID mice human umbilical cord blood-derived CD34^{+} cells retrovirally transduced with ETV6-RUNX1. Effectively, the model reproduced the aberrant CD34^{+}/CD38$^{-/low}$/CD19 phenotype observed in twins. Of note, pre-LSC recovered from mice possessed a mixed pro-B and stem-cell gene expression profile and displayed both self-renewal and B-cell differentiation capacity *in vitro;* on the contrary, ETV6-RUNX1^{+} pro-B cells did not engraft despite the presence of the oncogene.

Before Hong's model of transplanted human ETV6-RUNX1^{+} UCB-CD34, several studies using murine HSPC have been proposed. One of the first attempts in the field was made by Tsuzuki and colleagues (Tsuzuki *et al.*, 2004): in their model, ETV6-RUNX1 was retrovirally delivered into murine bone marrow HSPC and cells transplanted into irradiated syngeneic mice. Mice didn't develop leukemia in the four months of the experiment and only showed a pre-leukemic phenotype: analysis of B lymphopoiesis, in fact, indicated an increase in the pro-B compartment accompanied by a reduction in mature B220^{+} B-cell in the bone marrow. This probably depended on reduced surrogate light-chain component λ5 and IL-7 receptor expression on ETV6-RUNX1^{+} pro-B cells which altered their differentiation. On the contrary, no significant alterations were observed in the myeloid and T-cell lineages, except a slightly increase in myeloid colonies formation *in vitro*. Importantly, ETV6-RUNX1^{+} mice showed a 6-fold increase in bone marrow primitive c-kit^{+}/Lin^{-} multipotent progenitors compared to controls. In another model, fetal

liver HSPC transduced with ETV6-RUNX1 produced a comparable phenotype after *in vivo* transplantation. In this case, however, ETV6-RUNX1 enhanced the reconstitution of both B- and myeloid lineages suggesting that, at earlier developmental stage (fetal liver *vs* bone marrow), its impact likely involved a bi-potential B/myeloid progenitor (Morrow *et al.*, 2004), as recently confirmed by Böiers et al. (Böiers *et al.*, 2018).

Transplantation of ETV6-RUNX1-expressing HSPC in mice has been one of the first approaches for modelling ETV6-RUNX1 functions *in vivo;* however, more reliable contributions derive from transgenic (TG) mice as virus-based transplanting models present some limitations in terms of inter-laboratory reproducibility and homogeneity. These animals are genetically engineered to express the human fusion protein in various hematopoietic compartments (Schindler *et al.*, 2009) (Kantner *et al.*, 2013) (Rodríguez-Hernández *et al.*, 2017), through various transgenic approaches. Schindler et al., for example, set up a TG mice in which an ETV6-RUNX1 "hybrid" protein was induced by targeting the human RUNX1 cDNA to the exon VI of murine ETV6; the fusion protein was than expressed in HSC or common lymphoid progenitors (CLP) through different Cre-mediated conditional expression strategies (Schindler *et al.*, 2009). The model presented three main advantages: ETV6-RUNX1 expression was under the ETV6 promoter, both wild type ETV6 and RUNX1 were in hemizygous status and the transgenic approach permitted to observe the phenotype during development. While mice expressing ETV6-RUNX1 at the HSPC level (Gata1-Cre ETV6-RUNX1 mice) didn't showed

significant hematopoietic alterations during the embryonic life, a marked reduction in pro-B, pre-B and IgM^+ cells was observed in adulty suggesting that the bone marrow microenvironment could play a role in mediating the ETV6-RUNX1 pathogenesis. Such reduction didn't occur if ETV6-RUNX1 was expressed under a pan-lymphoid promoter active in CLP (CD2-Cre ETV6-RUNX1 mice), indicating that the differentiation impairment implicated a multipotent background to occur and that it was still possible in those CLP that eventually escaped from the earlier block. Notably, ETV6-RUNX1 expanded the $Lin^-/Sca1^+/cKit^-$ (HSC) fraction although increasing the proportion of cells in the G0 phase, indicating that the oncogene enhancing self-renewal without compromising quiescence of the stem cell pool. At the same time, while expression of ETV6-RUNX1 in HSC promoted the development of T-ALL malignancies after chemical challenge in mice, its induction in CLP failed in causing leukemia. These lasts two observations demonstrated that ETV6-RUNX1 expanded a ''corrupted'' stem cell reservoir with enhanced self-renewal and quiescence that rendered it prone to accumulate damages and develop lymphoid leukemia (although specifically limited to T-ALL in this model).

Concluding, both mice transplanted with pre-leukemic cells and transgenic mouse models have been successfully used to reproduce the ETV6-RUNX1 first hit *in vivo* and, despite differences in the approaches, the majority of them agree in reproducing the main pre-leukemic features and the lack of disease in absence of secondary cooperating lesions. The next crucial step is to *in vivo* model the ETV6-

RUNX1^{+} pre-leukemia to leukemia transition possibly according to the natural history of the disease and its infective causal mechanism. The pending challenge, in fact, remains the identification of the relevant exposures contributing to the multistep ETV6-RUNX1 pathogenesis and the comprehension of the timing and mechanisms by which they operate, in order to explore the possibility of prophylactic interventions.

Role of infections/inflammation in ETV6-RUNX1^{+} pre-leukemia to leukemia transition

The first attempts to model ETV6-RUNX1^{+} pre-leukemia to leukemia transition *in vivo* used genetical or chemical triggers to induce additional lesions in an established pre-leukemic phenotype. Bernardin et al., for example, observed a great increase in leukemia incidence (both B-and T-ALL) in mice transplanted with ETV6-RUNX1-expressing BM-cells and deleted for p16^{INK4a} and p19^{ARF} oncosuppressors (Bernardin *et al.*, 2002). This condition effectively reflects a frequent clinical observation, as about 20% of B-ALL cases presents p16^{INK4a} and p19^{ARF} deletion or aploisufficiency, demonstrating that their loss of function cooperate with ETV6-RUNX1 in malignant progression (Takeuchi *et al.*, 1997) (Mullighan *et al.*, 2008) (Mullighan, 2013). On the other hand, Schindler et al. induced aggressive T-leukemia in their ETV6-RUNX1 conditional knock-in mice by treating them with N-ethyl-N-nitrosourea (ENU); interestingly, disease appeared only if the oncogene was expressed under a stem-

cell promoter, while no signs of leukemia was observed in case of CLP-specific one (Schindler *et al.*, 2009). A step forward was made by van der Weyden et al.: in order to identify novel genes favoring ETV6-RUNX1 leukemogenesis, they set up a TG mouse model in which the fusion transcript and the Sleeping Beauty (SB) transposase were co-expressed at the endogenous Etv6 locus. These mice were intercrossed with animals carrying a SB transposon array and screened for insertional mutagenesis. In contrast to previous ETV6-RUNX1 mouse models, this was the first model that specifically developed BCP-ALL although with a higher frequency compared to humans (20% of the offspring). Isolation and characterization of the transposon insertion sites in diseased mice revealed genes involved in ETV6-RUNX1 leukemogenesis, which were both known genes (i.e. EBF1 and EPOR) and novel candidates (Van Der Weyden *et al.*, 2011).

Despite these models provided important insights in the field, none of them had considered the specificity of childhood BCP-ALL etiopathogenesis. In fact, as herewith previously described, the infective/inflammatory hypothesis is the most accredited causal and specific mechanism for the disease, in addition to represent an its prerogative since no correlations between infections and solid pediatric tumors or childhood AML have been demonstrated up to date (Greaves, 2018). According to the delayed infection hypothesis, a dysregulated immune response to common infections could derive from a deficient “educating” exposures to infective agents during infancy. In particular, it is known that a deregulated T-cell response to infection leads to a potent and specific cytokine/chemokine release

profile that, at least transiently, suppresses growth and/or induces apoptosis in hematopoietic cells (i.e. TGFβ) (Ford *et al.*, 2009) (Blank and Karlsson, 2015) (Wang *et al.*, 2018) (Fig.9a).

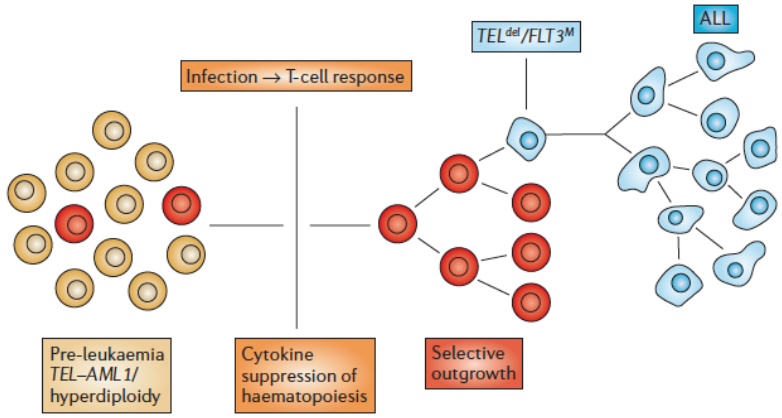


Figure 9a. Hypothetical model for infection-derived selective emergence in of pre-leukemic cells in childhood BCP-ALL. From Greaves, 2006.

In this context, the pre-leukemic clone could possess a survival and/or proliferative advantage compared to the normal counterpart; a subsequent wave of expansion of these cells might result in secondary mutations by chance (through the oxidative stress of the inflammatory microenvironment) or by the improper activation of AID and RAG1/2 (Greaves, 2006).These enzymes are required for the somatic recombination and diversification of immunoglobulin-encoding genes in B lymphocytes: in particular, RAGs mediates the homologous recombination of the V(D)J regions of immunoglobulins (IG) genes, while AID deaminates cytosine residues in the V and switch regions mediating IG somatic hypermutation (SHM) and class-switch

recombination (CSR) (Oettinger *et al.*, 1990) (Muramatsu *et al.*, 2000). However, their early expression in developing B cells can induce copy number alterations (CNV) or single nucleotide variations (SNV) in non-IG coding genes, leading to secondary off-targets genetic alterations (Mijušković *et al.*, 2015) (Papaemmanuil *et al.*, 2014) (Swaminathan *et al.*, 2015) (Fig.9b).

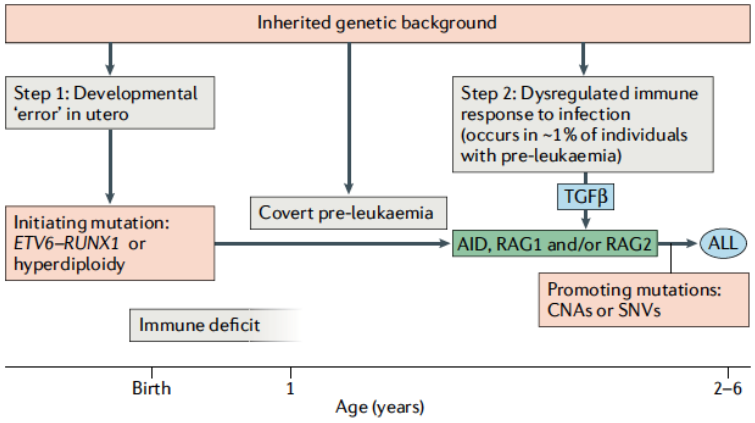


Figure 9b. Schematic representation of the two-hit model for role of infections in B cell precursor ALL. From Greaves, 2018.

A study published by Swaminathan et al. in 2015 was particularly informative on this regard: the authors, in fact, demonstrated that repetitive infective exposure cooperated with ETV6-RUNX1 in inducing BCP-ALL in mice in a RAG1/2 and AID-dependent manner (Swaminathan *et al.*, 2015). The same group previously showed that aberrant RAG-mediated V(D)J recombination at no-IG genes caused genetic lesions that drive the clonal evolution of pre-B ALL, especially in the ETV6-RUNX1^{+} subtype, where the fusion

oncogene directly upregulated RAG expression (Papaemmanuil *et al.*, 2014). They demonstrated that 34 out of the 40 most frequently mutated genes in ETV6-RUNX1^{+} ALL patients were targets of AID activity in murine B cells; moreover, higher-than-median AID mRNA expression indicated poor overall survival in patients, while AID mRNA abundance resulted increased at relapse in most cases. It has been shown that chromosomal translocation in human B cell malignancies are caused by the cooperation between RAG1/2 and AID enzymes at methylated cytosines (i.e. CpG sites). AID-mediated deamination of methylated cytosine, in fact, results in the conversion of cytosine to thymine and in the stable T:G mismatch. These mismatches generate "bubbles structures" that are recognized by the RAG recombinase which introduces a single-strand nick (Tsai *et al.*, 2008). Effectively, an enrichment for cytosine deamination of CpG in pediatric patients with ALL was observed in Swaminathan's study.

Starting from these premises, authors hypothesized that AID could contribute to RAG1/2 mediated deletions and gene rearrangements in pediatric BCP-ALL, particularly in those carrying the t(12;21). AID is normally induced by antigen presentation in mature B lymphocytes in the germinal centers; however, authors asked if a premature AID expression could verify also at early stages of the B-lymphopoiesis. Effectively, they found that a fraction of fetal liver and bone marrow pre-B cells showed a greater frequency of somatic hypermutation in the IGH V_H region, clearly indicating an ongoing AID activity. By studying RAG and AID expression in developing murine B-cells, they individuated a particular cellular stage of early B-ontogeny,

corresponding to the small pre-BII, which was characterized by a premature expression of both enzymes; in particular, AID upregulation was due to IL7R downregulation. As mature B-lymphocyte respond to infections by upregulating AID levels, authors question if this coul happen also in pre-B cells. By treating pre-B cells with LPS stimulation and IL7 withdrawal authors effectively observed a dramatic increase in AID levels and genetic instability in pre-BII subset. On that basis, they supposed that RAG1/2, AID and inflammation could operate together in accelerating leukemogenesis in ETV6-RUNX1^{+} ALL by acting on the pre-BII cells. Actually, when ETV6-RUNX1^{+} murine pre-B cells were treated with five cycles of IL7 withdrawal and LPS prior being transplanted in mice, they induced BCP-ALL in a RAG1/2 and AID-dependent manner.

The second interesting approach has been proposed by Rodríguez-Hernández and colleagues. Simply by moving immunocompetent ETV6-RUNX1^{+} transgenic mice from pathogen-free to conventional facility, authors demonstrated that the oncogene specifically predispose to BCP-ALL in a context of delayed infection exposure (Rodríguez-Hernández *et al.*, 2017). More in detail, the human ETV6-RUNX1 cDNA was expressed under the endogenous murine Sca1 promoter, considering that the ETV6-RUNX1 transcript is not detected in B-progenitor of *healthy* children carrying the translocation t(12;21) but only into multipotent progenitors (Hong *et al.*, 2008). ETV6-RUNX1^{+} mice maintained in specific pathogen free (SPF) conditions didn't develop leukemia during the two years of observation but only showed a tardive pre-leukemic phenotype

(increased of B precursors in the BM and normal count of mature B cells in the peripheral blood). However, if ETV6-RUNX1^{+} mice were moved from SPF to conventional facility at one moth of age, at the final read out of 2-years about 10% of mice specifically developed BCP-ALL. An increase in an aberrant pro/preB compartment (B220 ^{low}IgM^{-}), however, could be detected in pre-leukemic mice few months after the infective exposure, although the maturation of PB B cells was not impaired. The effect wasn't observed in the 90% of exposed but leukemia-free ETV6-RUNX1^{+} mice, indicating that the of the aberrant pro/preB phenotype was a pathogenetic step that specifically anticipated ALL onset. In order to comprehend the molecular mechanisms underlying pre-leukemia to leukemia progression, authors performed gene expression profile on pro/preB cells of ETV6-RUNX1^{+} and WT young mice maintained in common facility. A significant higher RAG1/2 expression was observed in ETV6-RUNX1^{+} cells, confirming what observed in human t(12;21)$^{+}$ fully leukemia (MILE study; http://r2.amc.n). Moreover, pre-leukemic pro/preB cells showed differential expression of several epigenetic regulators of the KDM family. Interestingly, authors found that sequence mutations and deletions frequently affected KDM genes in ETV6-RUNX1^{+} patients, confirming what previously observed by Papaemmanuil et al. (Papaemmanuil *et al.*, 2014). In particular, H3K4me2/3 demethylation was the most reduced function in both cohorts; according to this, KDM2B and KDM5C genes, which catalyzed H3K4me3 demethylation, showed *in silico* predicted loss of function mutations. As trimethylation of H3K4 is the necessary requirement for RAG1/2

binding and initiation of recombination activity (Matthews *et al.*, 2007) authors investigated if KDM5C loss of function in a human BCP-ALL cell line could mediate off-target RAG activity by increasing methylated H3K4 binding-sites. As predicted, higher levels of H3K4me3 coprecipitated with RAG2 in the KDM5C-deficient cells, laying the basis for RAG1 binding and potential dysregulated cleavage activity. Taken together, these findings suggested that exposure to infection promote ETV6-RUNX1^{+} leukemogenesis by increasing RAG1/2 expression and misregulating histone modification, thus facilitating RAG recruitment to cryptic recombination signal sequences (RSS) and its consequent off-target activity.

To conclude, *in vivo* models that accurately reproduce the natural history and the infective prerogative of ETV6-RUNX1^{+} BCP-ALL could provide fundamental insights on both extrinsic and intrinsic risk factors driving the pre-leukemia to leukemia transition. Their individuation will allow to provide strategies for minimizing risk exposures or prevent leukemic transformation in healthy ETV6-RUNX1 carriers.

ETV6-RUNX1^{+} relapses and the pre-leukemic clone: a matter of quiescence?

Studying mechanisms sustaining the ETV6-RUNX1^{+} pre-leukemic phase and its transition to fully disease is an important issue not only to a futurist prevention of the first ALL onset, but mostly for avoiding relapses. Although ETV6-RUNX1^{+} ALL is considered a

low/medium-risk subgroup, in fact, about 20% of patients face relapses after treatment cessation or many years after diagnosis (up to 20), at least in some protocols (Ford *et al.*, 2001). Relapses can have multiple biologic explanations: they can be chemotherapy-induced secondary leukemias clonally unrelated with the first leukemic event (in this case most of them have a myeloid phenotype) or they could represent an intra-clonal evolution of the original disease (Levasseur *et al.*, 1994) (Lo Nigro *et al.*, 1999). In this case, relapses could be derived from a minor sub-clone that was already present at diagnosis or could originate from *de novo* mutations occurring in therapy-resistant pre-leukemic cells. The last hypothesis was initially proposed in 2001 by Ford et al. which observed in two late-relapsed patients that cells at diagnosis and relapse possessed the same clonal IGH/TCR rearrangements but different alterations in the wild type ETV6 gene (Ford *et al.*, 2001). Although they were not able at that time to demonstrate the same clonotypic ETV6-RUNX1 breakpoint in diagnosis and relapsed samples, the study suggested that late relapse might represent a *de novo* transformation of the initial preleukemic clone that survived chemotherapy. Few years after, Konrad *et al*. were able to match the genomic ETV6-RUNX1 breakpoints of initial and relapse clones in 2 patients. The analysis confirmed that the initial and the relapse leukemias derived independently from a common ETV6-RUNX1^{+} precursor cell (Konrad *et al.*, 2003). Despite the small sizes of relapsed clones in the two patients ($5x10^3$ and $1x10^4$), authors identified them as initially slow-responding clones that were already present at diagnosis; interestingly, after they had developed into the

relapse leukemia, they were rapidly eradicated by the relapse treatment, underlining their different biology at the two time points. For that reason, they hypothesized that the minor clone was not fully malignant at the initial leukemic manifestation but possessed a preleukemic phenotype (Konrad *et al.*, 2003). The idea was confirmed, at least in some patients, by Van Delft and colleagues, which hypothesized that, if some very late relapses in ALL derive from pre-leukemic clones (defined by the unique presence of ETV6-RUNX1 fusion), no individual relapse CNA should be identifiable at diagnosis. By comparing genome-wide copy number alterations (CNA) at presentation and relapse (12–119 months) in 21 ETV6-RUNX1^{+} patients, authors found that in 14 out of 21 cases at least one 'driver' CNA was shared. However, in 4 patients the relapse clone had lost all CNA present initially and had acquired a completely new set of alterations (excluding ETV6/RUNX1 fusion) (Type 4 In Fig.10). This was possible only supposing that relapses were originated from pre-leukemic clones that resisted to induction chemotherapy, persisted during and after maintenance chemotherapy and gave rise to another ALL through the acquisition of *de novo* CNA (Van Delft *et al.*, 2011a). Using an analogue approach, Kuster *et al*. have more recently found that 78.6% of early relapses derived from a common ancestor and which subsequently acquired distinct CNA (Kuster *et al.*, 2018).

Overall, these observations suggested that, at least in some cases, very late relapses might originate from the ancestral pre-leukemic clone that resists to initial chemotherapy, possibly via quiescence, and subsequently acquired *de novo* secondary mutations.

	Presentation	Relapse	Possible clonal origins of relapse	Patients[3] Remission duration (years) < 2	2-5	> 5
Type 1	ABC	ABC	Dominant clone at diagnosis[1].	9		13
Type 2	ABC	ABC + DE	Dominant clone at diagnosis with further CNA or derivative[2] minor clone selected with or without further CNA.	20	10 26 21 22 23	2
Type 3	ABC	AB + DE	Minor clone at diagnosis selected with or without further CNA.	8 15	11 16	17 18
Type 4	ABC	DEF	Minor clone at diagnosis selected either: (i) pre-leukemia/ancestral with all CNA gained (ii) leukemic clone with or without further CNA.		6 7 14	19

Figure 10. Classification of clonal origin in relapsed ETV6-RUNX1 ALL. ABCDEF indicate 'driver' only copy number alterations (CNA). **From W. van Delft *et al.*, 2011.**

Bone Marrow Mesenchymal Stromal Cells (BM-MSC): a bridge between hematopoiesis, inflammation and leukemia

Mesenchymal Stromal Cells (MSC) are non-hematopoietic, fibroblast-like cells with the multipotent capability to differentiate, under appropriate stimuli, into cells of the three mesodermal lineages: osteocytes, adipocytes and chondrocytes. MSC specifically express the cell surface markers CD44, CD73, CD90 and lack the expression of CD14, CD34, CD45 and HLA (human leucocyte antigen)-DR. They can be isolated in adhesive culture from several tissues like bone marrow,

adipose tissue, amniotic fluid, endometrium, dental tissues and umbilical cord (Dominici *et al.*, 2006). Initially explored for their immunomodulatory and plastic properties in the context of chronic inflammatory diseases and regenerative medicine, in the last years they have been widely investigated for their role within the BM niche in both normal and pathological conditions. Up to date, a growing body of studies provides solid demonstrations that MSC play non-redundant functions in HSPC fate regulation and that they are actively involved in the induction and maintenance of several hematological malignancies.

Role of BM-MSC in the physiological BM niche

The stem cell niche represents the functional and anatomical 'node' in which signals from the periphery are integrated and elaborated to drive the appropriate HSPC behavior in term of localization, quiescence/proliferation and self-renewal/differentiation (Lo Celso and Scadden, 2011). In addition to hematopoietic cells, numerous stromal cells belonging to different cell lineages (i.e. cells of the sympathetic nervous system, adipocytes, osteoblasts, reticular cells, macrophages, endothelial cells, smooth muscle cells and MSC) compose the stem cell niche. Stromal cells not only physically surround blood cells but actively regulate hematopoietic processes through the secretion of cytokines, hormones and growth factors and the expression of receptors and adhesion molecules. Osteoblasts (OB)

localized at the endosteal surface of the niche were among the first identified stromal cells involved in HSPC regulation, in particular in HSPC quiescence maintenance, and were indicated as the key orchestrators of the "osteoblastic niche" (Calvi *et al.*, 2003) (Grassinger *et al.*, 2010) (Visnjic *et al.*, 2004); however, their role has been progressively resized and other cells allocated in proximity of BM vessels have attracted great interest. The so-called "vascular niche" was at first indicated as the "active niche" which induced proliferation, mobilization and differentiation in HSPC thus acting in opposition to the "quiescent" osteoblastic niche (Tong and Linheng, 2006). However, recent data depicts a more complicated picture, with functional overlaps between these two regions.

In the entire body, MSC are generally part of a larger population, the perycytes, that is located in close proximity of the vascular system and this is also the case of the BM stem cell niche (Crisan *et al.*, 2008). An elegant work of Mendez-Ferrer et al. individuated a rare population, characterized by the expression of the cytoskeletal protein nestin, that was spatially associated with sinusoids, adrenergic nerve fibres and HSPC. This population highly expressed HSPC maintenance genes, such as CXCL12, c-KIT ligand, angiopoietin-1, IL-7, VCAM-1 and osteopontin (Méndez-Ferrer *et al.*, 2010); of note, purified HSPC engrafted near nestin+ cells when transplanted in the BM of lethally irradiated mice, whereas *in vivo* nestin+ cells depletion significantly reduces the homing of hematopoietic progenitors and rapidly reduced HSPC content. Such nestin+ population was bona fide recognized as a mesenchymal sub-

population on the basis of phenotypical and functional properties. Previous studies found that nestin$^+$ MSC tightly associated with adrenergic nerve fibers of the sympathetic nervous system (SNS), which regulate HSPC mobilization through the circadian oscillations, and responded to parathormone (Katayama et al., 2006; Méndez-Ferrer et al., 2008). Effectively, authors found that homeostatic neural and hormonal mechanisms in tandem regulate HSPC maintenance and MSC proliferation and differentiation, demonstrated that HSPC and MSC formed a unique, co-regulated, bone marrow niche. The same intimate relationship between HSPC and MSC was later confirmed by Greenbaum et al: in order to better define crucial components regulating HSPC cell fate, they performed an *in vivo* conditional deletion of CXCL12, a key chemokine for HSPC localization and maintenance, in different cell types of the HSPC niche. Thanks to this approach, authors demonstrated that, while CXCL12-depletion from OB didn't altered HSPC function, loss of CXCL12 produced by more immature MSC significantly impaired HSPC maintenance and self-renewal (Greenbaum *et al.*, 2013). In addition to CXCL12, MSC secrete a variety of molecules regulating the proliferation and maturation of HSC precursors, such as jagged 1, trombopoietin (TPO), stem cell factor (SCF), transforming growth factor (TGF)-β, which is known to maintain HSC quiescence and IL6, a key cytokine for the growth and maturation of B lymphocytes (Nauta *et al.*, 2012).

More directly, MSC capability to support HSPC has been demonstrated by studies that recreated a functional humanized niche ectopically in mice. In particular, it was found that a specific CD146$^+$

mesenchymal fraction, which localized in the subendothelial layers of BM, was able to recapitulate a complex and complete hematopoietic microenvironment when subcutaneously transplanted in mice: thanks to their chondrogenic and osteogenic potential, these cells originated ectopic organoids which were vascularized and repopulated by murine hematopoietic cells (Sacchetti *et al.*, 2007) (Serafini *et al.*, 2014). Due to the abundant CXCL12 expression, multipotency, cell morphology and location, sub-endothelial CD146-expressing MSC can be considered the human counterpart of murine CXCL12-abundant reticular (CAR) cells (Sacchetti *et al.*, 2007); these are mesenchymal progenitors that create a physical network of cellular bodies and processes along which HSC, earliest B cell precursors, plasma cells, plasmatic dendritic (pDC) cells and natural killer (NK) cells are functionally regulated (Kohara *et al.*, 2007) (Noda *et al.*, 2011). Regarding this aspect, Tokoyoda et al. identified stage-specific niches for B lymphopoiesis that were defined by their localization and interactions with specific components of CAR cells. In particular, they found that earliest precursors and pre-pro-B cells, as well as end-stage B and plasma cells, required CXCL12 cells but, while multipotent hematopoietic progenitors were attached to CAR processes, pre-pro-B cells adjoined their cell bodies; on the contrary, maturing pro-B cells, that are IL-7-dependent, moved from CXCL12 to adjoin distant IL-7-expressing cells (Tokoyoda *et al.*, 2004) (Fig.11).

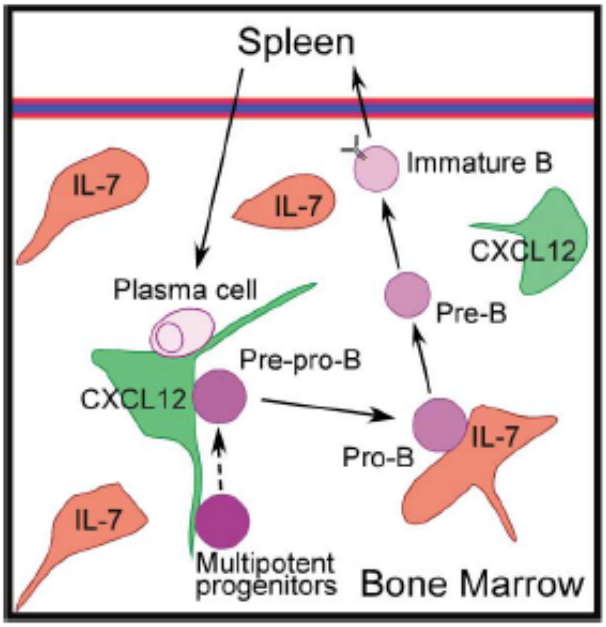


Figure 11. Regulation of B-lymphopoieisis in the bone marrow. Role of the CXCL12-expressing mesenchymal population. From Tokoyoda *et al*., 2004.

Analogously to Sacchetti and Serafini's studies, Reinisch *et al*. showed that BM-MSC exhibiting a specific chondrogenic transcriptional program, with hypomethylation and increased expression of RUNX3, RUNX2, BGLAP, MMP13 and ITGA10 genes, were able establish a subcutaneous humanized niche permitting homing, maintenance and differentiation not only of murine LT-HSC but also of human umbilical cord blood-derived $CD34^+/CD38^-/CD90^+/CD45RA^+$ HSC (Reinisch *et al.*, 2014).

Concluding, MSC are fundamental components of the stem niche as they participate to the vascular network of regulator stromal cells; MSC-mediated HSCP regulation and maintenance occurs through

the expression of HSPC-sustaining genes encoding cytokines, growth factors and adhesion molecules.

BM-MSC as mediators of inflammatory responses

Prior to their pro-HSPC functions, MSC have been widely investigated for their capacity of sensing inflammation, thanks to the expression of toll-like receptors (TLR) and cytokine/chemokine receptors, and to regulate both the innate and adoptive immune response (Keating, 2012) (Le Blanc and Mougiakakos, 2012). In particular, their strong immunosuppressive capability have provided the rationale for the use of MSC-based cell therapies in the context of chronic inflammatory diseases, first of all the graft versus host disease (GvHD) and autoimmune disorders (Le Blanc *et al.*, 2008) (Duijvestein *et al.*, 2010). However, MSC could mediate not only immunosuppression but also inflammation, depending on the type of TLR stimulation and the duration and intensity of the infection/inflammatory stimulus. TLR4-primed MSC, in fact, exerted proinflammatory functions while TLR3-primed MSC provided anti-inflammatory responses, in both cases by acting on macrophages and T cells polarization (Waterman *et al.*, 2010). Moreover, MSC cultivated under hypoxic culture conditions and stimulated with the proinflammatory cytokines IFN-γ, TNF, IFN-α, and IL-1β, upregulated the expression of TLR, HLA-class I, ICAM-1 and VCAM-1, thus increasing their sensitiveness and responsiveness to the inflammatory

microenvironment (Bernardo and Fibbe, 2013). On the contrary, prolonged stimulation with TLR ligands caused downregulation of TLR2 and TLR4, suggesting the existence in MSC of an auto-regulatory loop (Mo *et al.*, 2008). In mice, the recognition of microbial molecules by tissue-resident MSC results in increased production of molecules that recruit neutrophils and enhance their proinflammatory activity: IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage migration inhibitory factor (MIF) (Brandau *et al.*, 2014). In the bone marrow, nestin^{+} MSC are able to respond to TLR4 ligation by upregulating monocyte chemotactic protein-1 (MCP1) expression, which induces CCR2-dependent migration of monocytes from the BM into the circulation (Shi *et al.*, 2011). Thus, pro-inflammatory activation of MSC is beneficial in the early phase of inflammation as it helps in mounting a proper immune response mainly by promoting neutrophil migration and activation; on the contrary, when chronically exposed to pro-inflammatory cytokines, MSC adopt an immunosuppressive phenotype to protect tissues and promote regeneration through the secretion of several factors such as TGF-β, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO) and hemoxygenase (HO), which favor the differentiation of anti-inflammatory macrophages (M2) and regulatory T lymphocytes (Eggenhofer and Hoogduijn, 2012) (Melief *et al.*, 2013).

Collectively, these data indicate that MSC actively participate to the immune response by sensing infections and inflammation in general, thanks to the expression of toll-like and cytokine receptors. In

particular, they show a peculiar immunomodulatory plasticity that allows them to acquire a pro- or an anti-inflammatory phenotype.

BM-MSC and leukemia: a dangerous interplay

Considering the role of BM-MSC in regulating HSCP fate, as well as their capability to promote inflammation or immunotolerance in the niche, their contribution in the leukemic context is questioned. Indeed, it has been widely demonstrated that MSC and leukemic blasts establish an intensive cross-talk which eventually results into the constitution of a protective leukemic "sanctuary". In this process, leukemic cells induce alterations in MSC which in turn support leukemia, by both direct or indirect mechanisms, at the expense of normal hematopoiesis.

Despite the biological peculiarities of acute myeloid and lymphoblastic leukemia, a crucial interplay between MSC and blasts has been observed in both diseases. Applying dynamic *in vivo* imaging to a xenograft mouse model of BCP-ALL, Colmone *et al*. provided one of the first evidences in the field. Authors showed that malignant cells engrafted to a specific stromal cell–derived factor–1 (SDF-1) positive niche which overlapped with the perivascular HSPC niche, where MSC are a key component. Once established, disease started to alter the normal niche by decreasing SDF-1 secretion by the perivascular stroma and impeding the subsequent engraftment of healthy $CD34^+$ cells. In addition, expanding leukemic blasts were able to secrete SCF and

create alternative “trap” niches that initially attracted normal HSPC but then failed to sustain them (Colmone *et al.*, 2008). In line with these findings, Balandrán et al. showed that CXCL12 and SCF secretion was aberrantly decreased in the supernatants of MSC derived from ALL patients, while those of pro-inflammatory cytokines (i.e. IFNγ, IL1β, TNFα) resulted upregulated (Balandrán *et al.*, 2017). More recently, however, it has been shown that BCP-ALL cells reinforced the leukemic niche by acting on alternative chemokine pathways in place of the CXCR4/CXCL12 axis. De Rooji et al., in fact, observed that BCP-ALL blasts co-cultivated with BM-MSC increased the chemoattractive properties of these latter against leukemia. Such increased migration was CXCL12-indipendent, as demonstrated by CXCR4 inhibition. Moreover, while MSC/blasts co-cultures highly attracted leukemic cells, they strongly reduced migration of both healthy HSPC and primary MSC. The alternative chemokine axis which guided blasts toward the leukemic niche whle excluding normal hematopoiesis have been identified in chemokines MCP-1 and MDC, both ligands for the CCR4 receptor, and CXCL8/IL-8 and CXCL1/GRO, which bind to CXCR1 and CXCR2 receptors (De Rooij *et al.*, 2017).

These data suggest that alterations in the chemoattracting properties of the BM stroma are likely the initial step for the creation of the leukemic niche; however, other mechanisms must intervene to permit a stable advantage of the disease, both in terms of proliferation and survival. On this regard, it has been shown that MSC could provide anti-apoptotic effects to BCP-ALL cells by both direct contacts and soluble molecules. Tunneling nanotubes (TNT) have been identified as

a novel mechanism by which BCP-ALL cells communicated to primary MSC to induce the secretion of pro-survival cytokines, such as CXC chemokine ligand 10, (CXCL10), interleukin (IL8) and monocyte chemotactic protein-1 (MCP-1), which are responsible for stroma-mediated prednisolone resistance (Polak *et al.*, 2015). BM-MSC-derived PGE2 is also involved in the protection of primary BCP-ALL cells from apoptotic cell death by impeding p53 accumulation (Naderi *et al.*, 2015).

An intensive cross-talk between leukemic blasts and MSC have been reported also in the context of acute myeloid leukemia (AML): co-culturing MSC and AML blasts, in fact, profoundly altered the global gene expression profile of healthy MSC, particularly affecting genes involved in TLR and NFκB signaling and CCL/CXCL chemokine release (Reikvam *et al.*, 2015) (Binato *et al.*, 2015). Accordingly, BM-MSC derived from AML patients had altered gene expression profile compared to healthy MSC, particularly in genes related to proteoglycans, adhesion molecules and cytokines (Von Der Heide *et al.*, 2017). Moreover, AML-MSC were more efficient in sustaining AML blasts proliferation and chemoresistance compared to healthy MSC, in particular by activating Notch signaling in AML cells (Takam Kamga *et al.*, 2016). Finally, AML-MSC with different proteomic profile could affect the response to therapy and the clinical outcome of patients, as demonstrated by Kornblau and collegues (Kornblau *et al.*, 2018).

To conclude, leukemic cells (both ALL and AML) corrupt BM-MSC in order to create a protective microenvironment that favors

disease and excludes normal hematopoiesis in terms of migration, proliferation and survival.

BM-MSC contribution to leukemic progression in myeloid predisposing syndrome

As explained in the previous paragraph, mesenchymal contribution to leukemia has been mainly investigate in the context of the covert disease, while very less is known about the role of BM-MSC during the latent pre-leukemic phase. An insight in the field has been provided by two studies, conducted by the same group, on two different myeloid pre-leukemic conditions: myelodysplasia and Schwachman–Bodian–Diamond syndrome (SDS). The first one was published in 2010 and demonstrated that the specific perturbation of BM mesenchymal progenitors was sufficient to induce a pre-leukemic state which could potentially evolve into secondary leukemia, supporting the concept of niche-induced oncogenesis. More in details, authors specifically deleted Dicer1 in osterix (osx)-expressing mesenchymal progenitors as a means of altering several gene products and general cell homeostasis. Dicer1, in fact, is an RNase III endonuclease which regulates cell fate by ensuring the correct processing of miRNA; for that reason, Dicer1 deletion has been associated with tumorigenesis in a cancer-cell-autonomous manner. Interestingly, mice deficient for Osx-Dicer manifested a complex hematopoietic phenotype with similarities to human myelodysplasia; furthermore, an evolution to myelogenous leukemia

was observed in some animals following the accumulation of multiple genetic abnormalities in hematopoietic progenitors. Notably, Dicer$^{-/-}$ osteoprogenitors had reduced expression of Sbds, the gene mutated in Schwachman–Bodian–Diamond syndrome (SDS), known to be a human bone marrow failure syndrome characterized by the propensity to develop AML. Concordantly, also the deletion of Sbds in mouse osx^{+} mesenchymal progenitors induced bone marrow dysfunction and myelodysplasia (Raaijmakers *et al.*, 2010). Six years later, Raaijmakers' lab demonstrated that Sbds-deleted BM-MSC induced mitochondrial dysfunction, oxidative stress, and activation of DNA damage responses in HSPC. RNA sequencing of the specific CD271^{+} MSC population in SDS mice, as well as in several human pre-leukemic syndromes, identified a transcriptional activation of the p53-S100A8/9-TLR4 inflammatory signaling. In particular, it was found that damage-associated molecular pattern (DAMP) molecules S100A8 and S100A9 were strongly secreted from Sbds$^{-/-}$ MSC following p53 activation and were responsible, through the binding to TLR4, of the genotoxic stress in both murine and human HSPC. Moreover, overexpression of S100A8/9 in the CD271^{+} mesenchymal compartment predicted leukemic evolution and clinical outcome in human low-risk MDS (Zambetti *et al.*, 2016).

Collectively, these two studies provide evidences that primary alterations in the mesenchymal compartment of the BM niche are able to induce hematopoietic dysfunctions and myelodysplasia, in addition to predicted AML evolution, through the activation of specific inflammatory signaling in the BM niche.

Scope of the thesis

Aim of the study was to better investigate the relationship between childhood ETV6-RUNX1$^+$ pre-leukemia and BM-MSC under inflammatory conditions: in particular, we questioned if this interaction could have a role in the leukemogenic process by providing advantages to ETV6-RUNX1$^+$ cells in terms of persistence and malignant progression. While biology of ETV6-RUNX1$^+$ leukemia has been widely investigated, less is known about pre-leukemia. However, we think that a fully comprehension and a better management of disease must include elucidation of pre-leukemic mechanisms, as they are responsible not only for the onset but also for relapses of disease. *Chapter 1* provides a wide introduction on the general basis of childhood acute lymphoblastic leukemia (ALL), details on the natural history of the ETV6-RUNX1$^+$ subtype, especially focusing on the role of infection/inflammation, and an excursus on mesenchymal stromal cells (BM-MSC) functions in the bone marrow, both under physiological and pathological conditions. Briefly, it has been demonstrated by epidemiological and experimental data that infections and inflammation promote ETV6-RUNX1$^+$ pre-leukemia to leukemia transition, through various mechanisms. On the other hand, dysfunctions in BM-MSC can induce hematopoietic myelodisplasia and secondary myeloid leukemia, while mesenchymal inflammation cause genotoxicity in HSPC predicting Acute Myeloid Leukemia (AML) evolution in predisposing syndromes. Taking advantage from two pre-

leukemic cellular models (ETV6-RUNX1-expressing Ba/F3, a murine proB cell line, and human umbilical cord blood (UCB)-derived $CD34^+$ cells), we demonstrate in *Chapter 2* that emergence of the ETV6-$RUNX1^+$ cells against the normal counterpart is favored in an *in vitro* model of competitive inflamed BM-MSC niche. Moreover, ETV6-RUNX1 enhances migration toward inflamed BM-MSC, potentially favoring the establishment of the pre-leukemic clone in the sustaining niche. In addition, by persisting in a genotoxic microenvironment, pre-leukemic cells accumulate DNA damages, thus increasing their chance for malignant transformation. Finally, *Chapter 3* illustrates how we intend to proceed in the next future basing on presented results. In particular, we will be interested to *in vivo* inhibit key mechanisms sustaining the persistence of ETV6-RUNX1+ pre-leukemic cells within the inflamed BM niche in order to eradicate them and prevent relapses.

References

Abarrategi, A. *et al.* (2017) 'Versatile humanized niche model for studying human normal and malignant haematopoieisis', *Journal of Clinical Investigation*, 127(2), p. 543-. doi: 10.1172/JCI89364.

Abdelhaleem, M. (2007) 'Frequent but nonrandom expression of lymphoid markers on de novo childhood acute myeloid leukemia', *Experimental and Molecular Pathology*. doi: 10.1016/j.yexmp.2007.05.007.

Alpar, D. *et al.* (2015) 'Clonal origins of ETV6-RUNX1 + acute lymphoblastic leukemia: Studies in monozygotic twins', *Leukemia*. Nature Publishing Group, 29(4), pp. 839–846. doi: 10.1038/leu.2014.322.

André, V. *et al.* (2012) 'Mesenchymal stem cells from Shwachman-Diamond syndrome patients display normal functions and do not contribute to hematological defects', *Blood Cancer Journal*. doi: 10.1038/bcj.2012.40.

Andreasson, P. *et al.* (2001) 'The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo', *Cancer Genetics*, 130(2).

Asou, N. (2003) 'The role of a Runt domain transcription factor AML1/RUNX1 in leukemogenesis and its clinical implications', *Critical Reviews in Oncology/Hematology*. doi: 10.1016/S1040-8428(02)00003-3.

Auer, F. *et al.* (2017) 'Activation-induced cytidine deaminase prevents pro-B cell acute lymphoblastic leukemia by functioning as a negative regulator in Rag1 deficient pro-B cells', *Oncotarget*. doi: 10.18632/oncotarget.20563.

Balandrán, J. C. *et al.* (2017) 'Pro-inflammatory-related loss of CXCL12 niche promotes acute lymphoblastic leukemic progression at the expense of normal lymphopoiesis', *Frontiers in Immunology*, 7(JAN), pp. 1–14. doi: 10.3389/fimmu.2016.00666.

Bateman, C. M. *et al.* (2015) 'Evolutionary trajectories of hyperdiploid ALL in monozygotic twins', *Leukemia*. doi: 10.1038/leu.2014.177.

Bernardin, F. *et al.* (2002) 'Advances in Brief TEL-AML1 , Expressed from t (12 ; 21) in Human Acute Lymphocytic Leukemia , Induces Acute Leukemia in Mice 1', pp. 3904–3908.

Bernardo, M. E. and Fibbe, W. E. (2013) 'Mesenchymal stromal cells: Sensors and switchers of inflammation', *Cell Stem Cell*. Elsevier Inc., 13(4), pp. 392–402. doi: 10.1016/j.stem.2013.09.006.

Binato, R. *et al.* (2015) 'The molecular signature of AML mesenchymal stromal cells reveals candidate genes related to the leukemogenic process', *Cancer Letters*. Elsevier Ireland Ltd, 369(1), pp. 134–143. doi: 10.1016/j.canlet.2015.08.006.

Le Blanc, K. *et al.* (2008) 'Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study', *The Lancet*. doi: 10.1016/S0140-

6736(08)60690-X.

Le Blanc, K. and Mougiakakos, D. (2012) 'Multipotent mesenchymal stromal cells and the innate immune system', *Nature Reviews Immunology*. doi: 10.1038/nri3209.

Blank, U. and Karlsson, S. (2015) 'TGF-β signaling in the control of hematopoietic stem cells', *Blood*. doi: 10.1182/blood-2014-12-618090.

Bohlander, S. K. (2005) 'ETV6: A versatile player in leukemogenesis', *Seminars in Cancer Biology*. doi: 10.1016/j.semcancer.2005.01.008.

Böiers, C. *et al.* (2013) 'Lymphomyeloid Contribution of an Immune-Restricted Progenitor Emerging Prior to Definitive Hematopoietic Stem Cells', *Cell Stem Cell*. doi: 10.1016/j.stem.2013.08.012.

Böiers, C. *et al.* (2018) 'A Human IPS Model Implicates Embryonic B-Myeloid Fate Restriction as Developmental Susceptibility to B Acute Lymphoblastic Leukemia-Associated ETV6-RUNX1', *Developmental Cell*, 44(3), p. 362–377.e7. doi: 10.1016/j.devcel.2017.12.005.

Boily, G. *et al.* (2007) 'Identification of transcripts modulated by ETV6 expression', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.2006.06377.x.

Brandau, S. *et al.* (2014) 'Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes', *PLoS ONE*. doi: 10.1371/journal.pone.0106903.

Calvi, L. M. *et al.* (2003) 'Osteoblastic cells regulate the haematopoietic stem cell niche', *Nature*. doi: 10.1038/nature02040.

Cappuzzello, C. *et al.* (2016) 'Mesenchymal stromal cell-derived PTX3 promotes wound healing via fibrin remodeling', *Journal of Investigative Dermatology*. doi: 10.1038/JID.2015.346.

Carey, A. *et al.* (2017) 'Identification of Interleukin-1 by Functional Screening as a Key Mediator of Cellular Expansion and Disease Progression in Acute Myeloid Leukemia', *Cell Reports*. doi: 10.1016/j.celrep.2017.03.018.

Cazzaniga, G. *et al.* (2017) 'Possible role of pandemic AH1N1 swine flu virus in a childhood leukemia cluster', *Leukemia*, 31(8), pp. 1819–1821. doi: 10.1038/leu.2017.127.

Lo Celso, C. and Scadden, D. T. (2011) 'The haematopoietic stem cell niche at a glance', *Journal of Cell Science*, 124(21), pp. 3529–3535. doi: 10.1242/jcs.074112.

Colmone, A. *et al.* (2008) 'Normal Hematopoietic Progenitor Cells', *Science,* 322(December), pp. 1861–1865.

Corces-Zimmerman, M. R. and Majeti, R. (2014) 'Pre-leukemic evolution of hematopoietic stem cells: The importance of early mutations in leukemogenesis', *Leukemia*. Nature Publishing Group, 28(12), pp. 2276–2282. doi: 10.1038/leu.2014.211.

Corcione, A. *et al.* (2006) 'Human mesenchymal stem cells modulate B-cell functions', *Blood*. doi: 10.1182/blood-2005-07-2657.

Crisan, M. *et al.* (2008) 'A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human

Organs', *Cell Stem Cell*, 3(3), pp. 301–313. doi: 10.1016/j.stem.2008.07.003.

Van Delft, F. W. *et al.* (2011a) 'Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia', *Blood*, 117(23), pp. 6247–6254. doi: 10.1182/blood-2010-10-314674.

Van Delft, F. W. *et al.* (2011b) 'Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia', *Blood*. doi: 10.1182/blood-2010-10-314674.

Devapatla, B., Sharma, A. and Woo, S. (2015) 'CXCR2 inhibition combined with sorafenib improved antitumor and antiangiogenic response in preclinical models of ovarian cancer', *PLoS ONE*, 10(9), pp. 1–16. doi: 10.1371/journal.pone.0139237.

Diakos, C. *et al.* (2007) 'RNAi-mediated silencing of TEL/AML1 reveals a heat-shock protein- and survivin-dependent mechanism for survival', *Blood*. doi: 10.1182/blood-2006-04-019612.

Dockerty, J. D. *et al.* (2001) 'Case-control study of parental age, parity and socioeconomic level in relation to childhood cancers', *International Journal of Epidemiology*. doi: 10.1093/ije/30.6.1428.

Dominici, M. *et al.* (2006) 'Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement', *Cytotherapy*, 8(4), pp. 315–317. doi: 10.1080/14653240600855905.

Duijvestein, M. *et al.* (2010) 'Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: Results of a phase I study', *Gut*. doi: 10.1136/gut.2010.215152.

Eggenhofer, E. and Hoogduijn, M. J. (2012) 'Mesenchymal stem cell-educated macrophages', *Transplantation Research*. doi: 10.1186/2047-1440-1-12.

Eguchi-Ishimae, M. *et al.* (2001) 'Breakage and fusion of the TEL (ETV6) gene immature B lymphocytes induced by apoptogenic signals', *Blood*, 97(3), pp. 737–743. doi: 10.1182/blood.V97.3.737.

Enciso, J. *et al.* (2016) 'Modeling the pro-inflammatory tumor microenvironment in acute lymphoblastic leukemia predicts a breakdown of hematopoietic-mesenchymal communication networks', *Frontiers in Physiology*. doi: 10.3389/fphys.2016.00349.

Fan, X. *et al.* (2007) 'Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M607705200.

Ford, a M. *et al.* (1998) 'Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(8), pp. 4584–4588. doi: 10.1073/pnas.95.8.4584.

Ford, A. M. *et al.* (2001) 'Origins of "late" relapse in childhood acute lymphoblastic leukemia with TEL-AML1 fusion genes.', *Blood*, 98(3), pp. 558–64. doi: 10.1182/blood.V98.3.558.

Ford, A. M. *et al.* (2009) 'The TEL-AML1 leukemai fusion gene dysregulates the TGF-beta pathway in early B lineage progenitor cells', *J. Clin. Invest.*, 119(4). doi: 10.1172/jci36428ds1.

Forsberg, E. C. and Smith-Berdan, S. (2009) 'Parsing the niche code: The molecular mechanisms governing hematopoietic stem cell adhesion and differentiation', *Haematologica*, 94(11), pp. 1477–1481. doi: 10.3324/haematol.2009.013730.

Francis, S. S. *et al.* (2012) 'Unusual space-time patterning of the Fallon, Nevada leukemia cluster: Evidence of an infectious etiology', *Chemico-Biological Interactions*. doi: 10.1016/j.cbi.2011.02.019.

Fuka, G. *et al.* (2011) 'The leukemia-specific fusion gene etv6/runx1 perturbs distinct key biological functions primarily by gene repression', *PLoS ONE*. doi: 10.1371/journal.pone.0026348.

Gale, K. B. *et al.* (1997) 'Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 94(25), pp. 13950–4. doi: 10.1073/PNAS.94.25.13950.

Gandemer, V. *et al.* (2007) 'Five distinct biological processes and 14 differentially expressed genes characterize TEL/AML1-positive leukemia', *BMC Genomics*, 8, pp. 1–15. doi: 10.1186/1471-2164-8-385.

Gerr, H. *et al.* (2010) 'Acute leukaemias of ambiguous lineage in children: Characterization, prognosis and therapy recommendations', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.2009.08058.x.

Gilham, C. *et al.* (2005) 'Day care in infancy and risk of childhood acute lymphoblastic leukaemia: Findings from UK case-control study', *British Medical Journal*. doi: 10.1136/bmj.38428.521042.8F.

Golub, T. R. *et al.* (1995) 'Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia.', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.92.11.4917.

Grassinger, J. *et al.* (2010) 'Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential', *Blood*. doi: 10.1182/blood-2009-12-260703.

Greaves, M. (2002) 'Childhood leukemia', *Bmj*, 324, pp. 283–287.

Greaves, M. (2006) 'Infection, immune responses and the aetiology of childhood leukaemia', *Nature Reviews Cancer*, 6(3), pp. 193–203. doi: 10.1038/nrc1816.

Greaves, M. (2018) 'A causal mechanism for childhood acute lymphoblastic leukaemia', *Nature Reviews Cancer*. Springer US, 18(8), pp. 471–484. doi: 10.1038/s41568-018-0015-6.

Greaves, M. F. *et al.* (2003) 'Leukemia in twins: Lessons in natural history', *Blood*. doi: 10.1182/blood-2002-12-3817.

Greaves, M. F. and Wiemels, J. (2003) 'Origins of chromosome translocations in childhood leukaemia', *Nature Reviews Cancer*, 3(9), pp. 639–649. doi: 10.1038/nrc1164.

Greenbaum, A. *et al.* (2013) 'CXCL12 Production by Early Mesenchymal Progenitors is Required for Hematopoietic Stem Cell Maintenance', *Nature*, 495(7440), pp. 227–230. doi:

10.1038/nature11926.CXCL12.

Gunji, H. *et al.* (2004) 'TEL/AML1 shows dominant-negative effects over TEL as well as AML1', *Biochemical and Biophysical Research Communications*. doi: 10.1016/j.bbrc.2004.07.169.

Ha, H., Debnath, B. and Neamati, N. (2017) 'Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases', *Theranostics*, 7(6), pp. 1543–1588. doi: 10.7150/thno.15625.

Heath, C. W. and Hasterlik, R. J. (1963) 'Leukemia among children in a suburban community', *The American Journal of Medicine*. doi: 10.1016/0002-9343(63)90088-3.

Von Der Heide, E. K. *et al.* (2017) 'Molecular alterations in bone marrow mesenchymal stromal cells derived from acute myeloid leukemia patients', *Leukemia*, 31(5), pp. 1069–1078. doi: 10.1038/leu.2016.324.

Hol, J., Wilhelmsen, L. and Haraldsen, G. (2010) 'The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies', *Journal of Leukocyte Biology*. doi: 10.1189/jlb.0809532.

Hong, D. *et al.* (2008) 'Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia', *Science*, 319(5861), pp. 336–339. doi: 10.1126/science.1150648.

Ichikawa, M. *et al.* (2004) 'Runx1/AML-1 ranks as a master regulator of adult hematopoiesis', *Cell Cycle*. doi: 10.4161/cc.3.6.951.

Ijichi, H. *et al.* (2011) 'Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma', *Journal of Clinical Investigation*, 121(10), pp. 4106–4117. doi: 10.1172/JCI42754.

Jaffer, T. and Ma, D. (2016) 'The emerging role of chemokine receptor CXCR2 in cancer progression', *Translational Cancer Research*, 5(S4), pp. S616–S628. doi: 10.21037/tcr.2016.10.06.

Joseph, J. P. *et al.* (2017) 'CXCR2 Inhibition - a novel approach to treating CoronAry heart DiseAse (CICADA): Study protocol for a randomised controlled trial', *Trials*. Trials, 18(1), pp. 1–11. doi: 10.1186/s13063-017-2210-2.

Kaindl, U. *et al.* (2014) 'Blocking ETV6/RUNX1-induced MDM2 overexpression by Nutlin-3 reactivates p53 signaling in childhood leukemia', *Leukemia*. doi: 10.1038/leu.2013.345.

Kantner, H.-P. *et al.* (2013) 'ETV6/RUNX1 Induces Reactive Oxygen Species and Drives the Accumulation of DNA Damage in B Cells', *Neoplasia*. doi: 10.1593/neo.131310.

Kawamata, N. *et al.* (2008) 'Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray', *Blood*. doi: 10.1182/blood-2007-05-088310.

Keating, A. (2012) 'Mesenchymal stromal cells: New directions', *Cell Stem Cell*. doi: 10.1016/j.stem.2012.05.015.

Kfoury, Y. and Scadden, D. T. (2015) 'Mesenchymal cell contributions to the stem cell niche',

Cell Stem Cell. Elsevier Inc., 16(3), pp. 239–253. doi: 10.1016/j.stem.2015.02.019.

Kinlen, L. J., Clarke, K. and Hudson, C. (1990) 'Evidence from population mixing in British New Towns 1946-85 of an infective basis for childhood leukaemia', *The Lancet*. doi: 10.1016/0140-6736(90)93389-7.

Kohara, H. *et al.* (2007) 'Development of plasmacytoid dendritic cells in bone marrow stromal cell niches requires CXCL12-CXCR4 chemokine signaling', *Blood*. doi: 10.1182/blood-2007-04-084210.

Konrad, M. *et al.* (2003) 'Late relapses evolve from slow-responding subclones acute lymphoblastic leukemia : for the persistence evidence of a preleukemic clone', *Blood,* 101, pp. 3635–3640. doi: 10.1182/blood-2002-10-3252.

Kornblau, S. M. *et al.* (2018) 'Distinct protein signatures of acute myeloid leukemia bone marrow-derived stromal cells are prognostic for patient survival', *Haematologica*, 103(5), pp. 810–821. doi: 10.3324/haematol.2017.172429.

Krampera, M. *et al.* (2006) 'Role for Interferon-γ in the Immunomodulatory Activity of Human Bone Marrow Mesenchymal Stem Cells', *Stem Cells*. doi: 10.1634/stemcells.2005-0008.

Kuster, L. *et al.* (2018) 'ETV6_RUNX1-positive relapses evolve from an ancestral clone and frequently acquire deletions of genes implicated in glucocorticoid signaling', 117(9), pp. 2658–2668. doi: 10.1182/blood-2010-03-275347.The.

Kyurkchiev, D. (2014) 'Secretion of immunoregulatory cytokines by mesenchymal stem cells', *World Journal of Stem Cells*. doi: 10.4252/wjsc.v6.i5.552.

Larson, R.A; Anastasi, J. (2008) *Acute Lymphoblastic Leukemia: clinical presentation, diagnosis and classification.* Edited by Springer. Berlin.

De Laurentiis, A., Hiscott, J. and Alcalay, M. (2015) 'The TEL-AML1 fusion protein of acute lymphoblastic leukemia modulates IRF3 activity during early B-cell differentiation', *Oncogene*. Nature Publishing Group, 34(49), pp. 6018–6028. doi: 10.1038/onc.2015.50.

Levanon, D. *et al.* (2001) 'Architecture and anatomy of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1', *Gene*. doi: 10.1016/S0378-1119(00)00532-1.

Levasseur, M. *et al.* (1994) 'Relapse of acute lymphoblastic leukaemia 14 years after presentation: Use of molecular techniques to confirm true re-emergence', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.1994.tb04942.x.

Linka, Y. *et al.* (2013) 'The impact of TEL-AML1 (ETV6-RUNX1) expression in precursor B cells and implications for leukaemia using three different genome-wide screening methods', *Blood Cancer Journal*. doi: 10.1038/bcj.2013.48.

Liu, Q. *et al.* (2016) 'The CXCL8-CXCR1/2 pathways in cancer', *Cytokine and Growth Factor Reviews*. Elsevier Ltd, 31, pp. 61–71. doi: 10.1016/j.cytogfr.2016.08.002.

Lopez, R. G. *et al.* (1999) 'TEL is a sequence-specific transcriptional repressor', *Journal of*

Biological Chemistry. doi: 10.1074/jbc.274.42.30132.

Ma, X. *et al.* (2005) 'Vaccination history and risk of childhood leukaemia', *International Journal of Epidemiology*. doi: 10.1093/ije/dyi113.

Mangolini, M. *et al.* (2013) 'STAT3 mediates oncogenic addiction to TEL-AML1 in t(12;21) acute lymphoblastic leukemia', *Blood*. doi: 10.1182/blood-2012-11-465252.

Marcotte, E. L. *et al.* (2016) 'Caesarean delivery and risk of childhood leukaemia: A pooled analysis from the Childhood Leukemia International Consortium (CLIC)', *The Lancet Haematology*. doi: 10.1016/S2352-3026(16)00002-8.

Martz, L. (2012) 'CXCR2 antagonists in breast cancer', *Science-Business eXchange*, 5(31), pp. 1–2. doi: 10.1038/scibx.2012.807.

Matthews, A. G. W. *et al.* (2007) 'RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination', *Nature*. doi: 10.1038/nature06431.

Melief, S. M. *et al.* (2013) 'Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages', *Stem Cells*. doi: 10.1002/stem.1432.

Méndez-Ferrer, S. *et al.* (2010) 'Mesenchymal and haematopoietic stem cells form a unique bone marrow niche', *Nature*, 466(7308), pp. 829–834. doi: 10.1038/nature09262.

Miedema, K. G. E. *et al.* (2012) 'Polymorphisms in the TLR6 gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease', *Leukemia*. doi: 10.1038/leu.2011.341.

Mijušković, M. *et al.* (2015) 'Off-Target V(D)J Recombination Drives Lymphomagenesis and Is Escalated by Loss of the Rag2 C Terminus', *Cell Reports*. doi: 10.1016/j.celrep.2015.08.034.

Mo, I. F. Y. *et al.* (2008) 'Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors', *BMC Cell Biology*. doi: 10.1186/1471-2121-9-52.

Mori, H. *et al.* (2002) 'Chromosome translocations and covert leukemic clones are generated during normal fetal development', *Proceedings of the National Academy of Sciences*, 99(12), pp. 8242–8247. doi: 10.1073/pnas.112218799.

Morrow, M. *et al.* (2004) 'TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity', *Blood*, 103(10), pp. 3890–3896. doi: 10.1182/blood-2003-10-3695.

Morrow, M. *et al.* (2007) 'TEL-AML1 preleukemic activity requires the DNA binding domain of AML1 and the dimerization and corepressor binding domains of TEL', *Oncogene*, 26(30), pp. 4404–4414. doi: 10.1038/sj.onc.1210227.

Motoda, L. *et al.* (2007) '*Runx1* Protects Hematopoietic Stem/Progenitor Cells from Oncogenic Insult', *Stem Cells*. doi: 10.1634/stemcells.2007-0061.

Mullighan, C. G. *et al.* (2008) 'Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia', *Science*. doi: 10.1126/science.1164266.

Mullighan, C. G. (2013) 'Genomic characterization of childhood acute lymphoblastic leukemia', *Semin Hematol*, 50(4), pp. 314–324. doi: 10.1053/j.seminhematol.2013.10.001.

Muramatsu, M. *et al.* (2000) 'Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme', *Cell*. doi: 10.1016/S0092-8674(00)00078-7.

Naderi, E. H. *et al.* (2015) 'Bone marrow stroma-derived PGE2 protects BCP-ALL cells from DNA damage-induced p53 accumulation and cell death', *Molecular Cancer*, 14(1). doi: 10.1186/s12943-014-0278-9.

Nauta, A. J. *et al.* (2012) 'Immunomodulatory properties of mesenchymal stromal cells Review in translational hematology Immunomodulatory properties of mesenchymal stromal cells', 110(10), pp. 3499–3506. doi: 10.1182/blood-2007-02-069716.

Lo Nigro, L. *et al.* (1999) 'Clonal stability in children with acute lymphoblastic leukemia (ALL) who relapsed five or more years after diagnosis', *Leukemia*.

Noda, M. *et al.* (2011) 'CXCL12-CXCR4 chemokine signaling is essential for NK-cell development in adult mice', *Blood*. doi: 10.1182/blood-2010-04-277897.

Oettinger, M. A. *et al.* (1990) 'RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination', *Science*. doi: 10.1126/science.2360047.

Okuda, T. *et al.* (1996) 'AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis', *Cell*. doi: 10.1016/S0092-8674(00)80986-1.

Orkin, S. H. and Zon, L. I. (2008) 'Hematopoiesis: An Evolving Paradigm for Stem Cell Biology', *Cell*, 132(4), pp. 631–644. doi: 10.1016/j.cell.2008.01.025.

Paashuis-Lew, Y. R. and Heddle, J. A. (1998) 'Spontaneous mutation during fetal development and post-natal growth', *Mutagenesis*, 13(6), pp. 613–617.

Palmi, C. *et al.* (2014) 'Cytoskeletal Regulatory Gene Expression and Migratory Properties of B-cell Progenitors Are Affected by the ETV6-RUNX1 Rearrangement.', *Molecular cancer research : MCR*, 12(12), pp. 1796–806. doi: 10.1158/1541-7786.MCR-14-0056-T.

Panzer-Grümayer, E. R. *et al.* (2005) 'Immunogenotype changes prevail in relapses of young children with TEL-AML1-positive acute lymphoblastic leukemia and derive mainly from clonal selection', *Clinical Cancer Research*, 11(21), pp. 7720–7727. doi: 10.1158/1078-0432.CCR-05-1239.

Papaemmanuil, E. *et al.* (2014) 'RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia', *Nature Genetics*. Nature Publishing Group, 46(2), pp. 116–125. doi: 10.1038/ng.2874.

Parker, W. and Ollerton, J. (2013) 'Evolutionary biology and anthropology suggest biome reconstitution as a necessary approach toward dealing with immune disorders', *Evolution, Medicine, and Public Health*. doi: 10.1093/emph/eot008.

Penders, J. *et al.* (2006) 'Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy', *PEDIATRICS*. doi: 10.1542/peds.2005-2824.

Pietras, E. M. (2017) 'Inflammation: A key regulator of hematopoietic stem cell fate in health and disease', *Blood*, 130(15), pp. 1693–1698. doi: 10.1182/blood-2017-06-780882.

Poirel, H. *et al.* (1997) 'The TEL gene products: Nuclear phosphoproteins with DNA binding properties', *Oncogene*. doi: 10.1038/sj.onc.1200829.

Poynton, F. J., Thursfield, H. & Paterson, D. (1922) 'The severe blood diseases of childhood: a series of observations from the Hospital for Sick Children, Great Ormond Street.', *Br. J. Child. Dis.*, XIX.

Polak, R. *et al.* (2015) 'B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment', *Blood*, 126(21), pp. 2404–2414. doi: http://dx.doi.org/10.1182/blood-2015-03-634238.

Raaijmakers, M. H. G. P. *et al.* (2010) 'Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia', *Nature*. Nature Publishing Group, 464(7290), pp. 852–857. doi: 10.1038/nature08851.

Raghuwanshi, S. K. *et al.* (2012) 'The Chemokine Receptors CXCR1 and CXCR2 Couple to Distinct G Protein-Coupled Receptor Kinases To Mediate and Regulate Leukocyte Functions', *The Journal of Immunology*. doi: 10.4049/jimmunol.1201114.

Reikvam, H. *et al.* (2015) 'The cytokine-mediated crosstalk between primary human acute myeloid cells and mesenchymal stem cells alters the local cytokine network and the global gene expression profile of the mesenchymal cells', *Stem Cell Research*, 15(3), pp. 530–541. doi: 10.1016/j.scr.2015.09.008.

Reinisch, A. *et al.* (2014) 'Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation, *Blood*, 125(2), pp. 249–261. doi: 10.1182/blood-2014-04-572255.

Riether, C., Schürch, C. M. and Ochsenbein, A. F. (2015) 'Regulation of hematopoietic and leukemic stem cells by the immune system', *Cell Death and Differentiation*, 22(2), pp. 187–198. doi: 10.1038/cdd.2014.89.

Rodríguez-hernández, G. *et al.* (2017) 'Modeling the process of childhood ETV6-RUNX1 B-cell leukemias', 8(60), pp. 102674–102680. doi: 10.18632/oncotarget.21281.

Rodríguez-Hernández, G. *et al.* (2017) 'Infection exposure promotes ETV6-RUNX1 precursor B-cell leukemia via impaired H3K4 demethylases', *Cancer Research*, 77(16), pp. 4365–4377. doi: 10.1158/0008-5472.CAN-17-0701.

Romana, S. P. *et al.* (1995) 'High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia.', *Blood*.

De Rooij, B. *et al.* (2017) 'Acute lymphoblastic leukemia cells create a leukemic niche without affecting the CXCR4/CXCL12 axis', *Haematologica*, 102(10), pp. e389–e393. doi: 10.3324/haematol.2016.159517.

Rosu-Myles, M. *et al.* (2000) 'Characterization of chemokine receptors expressed in primitive blood cells during human hematopoietic ontogeny.', *Stem cells (Dayton, Ohio)*. doi: 10.1634/stemcells.18-5-374.

Sacchetti, B. *et al.* (2007) 'Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment', *Cell*. doi: 10.1016/j.cell.2007.08.025.

Schäfer, D. *et al.* (2018) 'Five percent of healthy newborns have an ETV6-RUNX1 fusion as revealed by DNA-based GIPFEL screening', *Blood*. doi: 10.1182/blood-2017-09-808402.

Schindler, J. W. *et al.* (2009) 'TEL-AML1 Corrupts Hematopoietic Stem Cells to Persist in the Bone Marrow and Initiate Leukemia', *Cell Stem Cell*. Elsevier Ltd, 5(1), pp. 43–53. doi: 10.1016/j.stem.2009.04.019.

Schinke, C. *et al.* (2015) 'IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells', *Blood*, 125(20), pp. 3144–3152. doi: 10.1182/blood-2015-01-621631.

Schraufstatter, I. U., Chung, J. and Burger, M. (2001) 'IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways', *Am J of Physiol. Lung Cellular and Molecular Physiology*. doi: 10.1080/01418618208236928.

Serafini, M. *et al.* (2014) 'Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells', *Stem Cell Research*. doi: 10.1016/j.scr.2014.01.006.

Shi, C. *et al.* (2011) 'Bone Marrow Mesenchymal Stem and Progenitor Cells Induce Monocyte Emigration in Response to Circulating Toll-like Receptor Ligands', *Immunity*. doi: 10.1016/j.immuni.2011.02.016.

Shlush, L. I. *et al.* (2014) 'Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia', *Nature*. Nature Publishing Group, 506(7488), pp. 328–333. doi: 10.1038/nature13038.

Sinclair, A. *et al.* (2016) 'CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells', *Blood*, 128(3), pp. 371–383. doi: 10.1182/blood-2015-08-661785.

Steele, C. W. *et al.* (2016) 'CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma', *Cancer Cell*. The Authors, 29(6), pp. 832–845. doi: 10.1016/j.ccell.2016.04.014.

Swaminathan, S. *et al.* (2015) 'Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia', *Nature Immunology*, 16(7), pp. 766–774. doi: 10.1038/ni.3160.

Swiergiel, A. H. and Dunn, A. J. (1999) 'The roles of IL-1, IL-6, and TNFα in the feeding responses to endotoxin and influenza virus infection in mice', *Brain, Behavior, and Immunity*. doi: 10.1006/brbi.1999.0565.

Takam Kamga, P. *et al.* (2016) 'Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia.', *Oncotarget*, 7(16), pp. 21713–27. doi: 10.18632/oncotarget.7964.

Takeuchi, S. *et al.* (1997) 'TEL is one of the targets for deletion on 12p in many cases of childhood B-lineage acute lymphoblastic leukemia', *Leukemia*. doi:

10.1038/sj.leu.2400743.

Taylor, G. M. *et al.* (2009) 'Strong association of the HLA-DP6 supertype with childhood leukaemia is due to a single allele, DPB1*0601', *Leukemia*. doi: 10.1038/leu.2008.374.

Tokoyoda, K. *et al.* (2004) 'Cellular niches controlling B lymphocyte behavior within bone marrow during development', *Immunity*. doi: 10.1016/j.immuni.2004.05.001.

Tong, Y. and Linheng, L. (2006) 'The stem cell niches in bone', *The Journal of Clinical Investigations*, 116(5), pp. 1195–1201. doi: 10.1172/JCI28568.the.

Torrano, V. *et al.* (2011) 'ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor', 118(18), pp. 4910–4919. doi: 10.1182/blood-2011-05-354266.

Tsai, A. G. *et al.* (2008) 'Human Chromosomal Translocations at CpG Sites and a Theoretical Basis for Their Lineage and Stage Specificity', *Cell*. doi: 10.1016/j.cell.2008.10.035.

Tsuzuki, S. *et al.* (2004) 'Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice.', 101(22), pp. 8443–8448.

Tsuzuki, S. and Seto, M. (2013) 'TEL (ETV6)-AML1 (RUNX1) initiates self-renewing fetal pro-b cells in association with a transcriptional program shared with embryonic stem cells in mice', *Stem Cells*, 31(2), pp. 236–247. doi: 10.1002/stem.1277.

Vilchis-Ordoñez, A. *et al.* (2015) 'Bone marrow cells in acute lymphoblastic leukemia create a proinflammatory microenvironment influencing normal hematopoietic differentiation fates', *BioMed Research International*. doi: 10.1155/2015/386165.

Vilenchik, M. M. and Knudson, A. G. (2003) 'Endogenous DNA double-strand breaks: Production, fidelity of repair, and induction of cancer', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.2135498100.

Visnjic, D. *et al.* (2004) 'Hematopoiesis is severely altered in mice with an induced osteoblast deficiency', *Blood*. doi: 10.1182/blood-2003-11-4011.

Wang, L. C. *et al.* (1997) 'Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL', *EMBO Journal*. doi: 10.1093/emboj/16.14.4374.

Wang, L. C. *et al.* (1998) 'The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow', *Genes and Development*. doi: 10.1101/gad.12.15.2392.

Wang, X. *et al.* (2018) 'TGF-β1 Negatively Regulates the Number and Function of Hematopoietic Stem Cells', *Stem Cell Reports*. doi: 10.1016/j.stemcr.2018.05.017.

Waterman, R. S. *et al.* (2010) 'A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype', *PLoS ONE*. doi: 10.1371/journal.pone.0010088.

Van Der Weyden, L. *et al.* (2011) *Modeling the evolution of ETV6-RUNX1-induced B-cell precursor acute lymphoblastic leukemia in mice*, *Blood*. doi: 10.1182/blood-2011-02-338848.

Wiemels, J. L. *et al.* (1999) 'Prenatal origin of acute lymphoblastic leukaemia in children', *Lancet*. doi: 10.1016/S0140-6736(99)09403-9.

Wiemels, J. L. *et al.* (1999) 'Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero.', *Blood*, 94(3), pp. 1057–62.

Yamagata, T., Maki, K. and Mitani, K. (2005) 'Runx1/AML1 in Normal and Abnormal Hematopoiesis', *International Journal of Hematology*. doi: 10.1532/IJH97.05075.

Zambetti, N. A. *et al.* (2016) 'Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia', *Cell Stem Cell*. doi: 10.1016/j.stem.2016.08.021.

Zelent, A., Greaves, M. and Enver, T. (2004) 'Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia', *Oncogene*, 23(24), pp. 4275–4283. doi: 10.1038/sj.onc.1207672.

CHAPTER 2:

INFLAMED BONE MARROW MESENCHYMAL STROMAL CELLS PROVIDE ADVANTAGE TO ETV6-RUNX1^{+} PRE-LEUKEMIC CELLS

Beneforti L.[1], *Dander E.*[1], *Bresolin S.*[2], *Bueno C.*[3], *Te Kronnie G.*[2], *Ford A.*[4], *Menendez P.*[3], *D'Amico G.*[1], *Biondi A.*[5], *Palmi C.*[1] *and Cazzaniga G.*[1]

1Tettamanti Research Centre, Department of Pediatrics, University of Milano-Bicocca, Fondazione MBBM, Monza, Italy;
2Department of Women's and Children's Health, University of Padova, Padova, Italy;
3Department of Biomedicine, Josep Carreras Leukemia Research Institute, School of Medicine, University of Barcelona, Barcelona, Spain;
4Centre for Evolution and Cancer, The Institute of Cancer Research, Sutton, GBR;
5University of Milano-Bicocca, Department of Pediatrics, San Gerardo Hospital/Fondazione MBBM, Tettamanti Research Center, Monza (MB), Italy

Manuscript in preparation

Abstract

ETV6-RUNX1 (E/R), generated by translocation t (12;21), is the most frequent fusion gene in pediatric cancers, exclusively leading to B-Cell Precursor Acute Lymphoblastic Leukemia (BCP-ALL). Translocation occurs *in utero* and leads to the expansion of a clinically silent pre-leukemic clone; post-natal secondary hits are thus required to complete malignant transformation. Up to date, a dysregulated inflammatory/immune response to common infections is the main candidate for the pre-leukemia to leukemia transition. Both normal and leukemic stem-progenitor cells are strictly regulated by bone marrow niche-derived signals. Within the niche, mesenchymal stromal cells possess regulative functions against both hematopoiesis and inflammatory processes, representing an interesting bridge between the two processes. Here, we demonstrate that ETV6-RUNX1-expressing Ba/F3 cells are favored in terms of proliferation and survival against the normal counterpart in an *in vitro* model of inflamed mesenchymal niche. Importantly, by persisting in a genotoxic microenvironment, pre-leukemic Ba/F3 accumulate DNA damages and upregulate AID expression, thus increasing chance for their malignant transformation. Finally, inflamed BM-MSC highly attract ETV6-RUNX1^{+} Ba/F3 in a CXCR2-dependent manner. Preferential migration toward inflamed MSC has been confirmed also in ETV6-RUNX1-expressing

human UCB-CD34^{+} progenitors. In this model, mesenchymal inflammation specifically preserves the pre-leukemic CD34^{+}IL7R^{+} compartment, recently individuated as the developmental stage that is susceptible to ETV6-RUNX1 activity, while negatively affecting controls. Overall, our data demonstrate that BM-MSC and inflammation cooperate in favoring the ETV6-RUNX1^{+} pre-leukemic clone against the normal counterpart.

Introduction

ETV6-RUNX1 (E/R), generated from translocation t(12;21)(p13;q22), is the most frequent fusion gene in pediatric cancer, exclusively leading to B-Cell Precursors Acute Lymphoblastic Leukemia (BCP-ALL) (Romana *et al.*, 1995) (Golub *et al.*, 1995). Translocation occurs *in utero* (Gale *et al.*, 1997) with a relatively high frequency in healthy newborns (1-5%) (Wiemels *et al.*, 1999) (Mori *et al.*, 2002) (Schäfer *et al.*, 2018); however, only 1% of E/R carriers develops BCP-ALL, indicating that additional post-natal mutations must occur to complete malignant transformation (Wiemels *et al.*, 1999) (Hong *et al.*, 2008) (Tsuzuki *et al.*, 2004) (Morrow *et al.*, 2007) (Andreasson *et al.*, 2001). E/R, in fact, is a weak oncogene that established a clinically silent pre-leukemic clone with the characteristics of early lymphoid progenitor partially blocked in B-cell differentiation and enhanced in quiescence and self-renewal (Hong *et al.*, 2008).

While biology of E/R$^+$ leukemia has been widely investigated, less is known about pre-leukemia. However, we think that a fully comprehension and a better management of disease must include elucidation of pre-leukemic mechanisms, as they are responsible not only for disease onset but also for relapses (Ford *et al.*, 2001) (Van Delft *et al.*, 2011a) (Kuster *et al.*, 2018). *In vitro* experiments indicate that E/R profoundly impacts the transcription profile of the target cell by downregulating pathways involved in cell proliferation and

enhancing those implicated in cell migration, DNA-damage response, apoptosis and immune response (Fuka *et al.*, 2011) (Tsuzuki and Seto, 2013) (Linka *et al.*, 2013) (Palmi *et al.*, 2014). More recently, it has been shown that E/R expression at early developmental stages of B-ontogeny produces a specific subset of $CD34^{+}CD19^{-}IL7R^{+}$ pro-B cells with an aberrant myeloid transcriptional signatures and potential (Böiers *et al.*, 2018). Next to *in vitro* studies, several animal models have been developed to explore E/R activity *in vivo*. Despite the different approaches, the majority of them concordantly reproduce the main features of E/R pre-leukemic activity, confirming lack of disease in absence of additional genotoxic stimuli (Andreasson *et al.*, 2001) (Tsuzuki *et al.*, 2004) (Morrow *et al.*, 2004) (Schindler *et al.*, 2009). Epidemiological and experimental data demonstrate that infections and inflammation play a crucial role in the pre-leukemia to leukemia transition. Regarding this, we previously showed that TGFβ, a pleiotropic cytokine produced under inflammation, favored the persistence of E/R-expressing Ba/F3, a murine pro-B cell line, in competitive co-culture with control cells; likewise, TGFβ selected a candidate population of pre-leukemic stem cells (pre-LSC) within the E/R-transduced human cord-blood (UCB) $CD34^{+}$ population (Ford *et al.*, 2009). More recently, it has been demonstrated that expression of AID and RAG1/2 enzymes cooperated with LPS exposure to induce E/R-driven leukemia in mice (Papaemmanuil *et al.*, 2014) (Swaminathan *et al.*, 2015), while Sca1-ETV6-RUNX1 transgenic mice

developed BCP-ALL at low penetrance only when exposed to common murine infective agents (Rodríguez-Hernández *et al.*, 2017).

It is now well established that both normal and malignant hematopoietic cells are tightly regulated by cell-intrinsic (i.e. transcription and epigenetic regulators) and cell-extrinsic signals, these latter mainly provided by Bone Marrow (BM) niches. BM niches are specialized microenvironments composed by several cell types with different hematopoietic regulating functions, which are explicated by cell-cell contacts and molecules secretion; in addition, cell/matrix interactions and physical/chemical gradients contribute to define the hierarchical, lineage-specific spatial organization in the niche (Lo Celso and Scadden, 2011). To this regard, cell adhesion and migration represent critical mechanisms for HSPC cell fate in terms of retention, proliferation/survival and differentiation (Forsberg and Smith-Berdan, 2009). Interestingly, we showed that E/R induction in Ba/F3 cells dysregulated the expression of adhesion molecules and impaired migration towards CXCL12 (Palmi *et al.*, 2014), possibly suggesting a non-physiological behavior of the pre-leukemic clone in the BM niche; moreover, CXCR4-indipendence concomitantly with the establishment of CXCR4-alternative migratory axis (i.e. CXCR1/2-mediated) favor the creation of a BCP-ALL niche (Balandrán *et al.*, 2017) (De Rooij *et al.*, 2017). BM-Mesenchymal Stromal Cells (MSC) are key determinants of the BM niche homeostasis, as they possess non-redundant regulative functions against HSPC and B-lineage cells (Corcione *et al.*, 2006) (Méndez-Ferrer *et al.*, 2010) (Kfoury and

Scadden, 2015). However, they can turn face in the leukemic context, where they actively cross-talk with blasts to favor leukemia and exclude normal hemopoiesis (de Rooij, et al., 2015) (Enciso *et al.*, 2016) (De Rooij *et al.*, 2017). At the same time, they possess both pro- and anti-inflammatory properties (Bernardo and Fibbe, 2013), representing an interesting bridge between hematopoiesis and inflammation. Moreover, it has been demonstrated that dysfunctions in BM-MSC can induce hematopoietic myelodisplasia and secondary myeloid leukemia (Raaijmakers *et al.*, 2010), while mesenchymal inflammation cause genotoxicity in HSPC predicting Acute Myeloid Leukemia (AML) evolution in predisposing syndromes (Zambetti *et al.*, 2016).

On that basis, we wanted to better investigate the relationship between E/R^+ pre-leukemia and BM-MSC, especially under inflammatory conditions: in particular, we questioned if this interaction could have a role in the leukemogenic process by providing advantages to E/R^+ cells in terms of persistence and/or malignant progression. Taking advantage from two E/R-expressing cellular models, the murine pro-B cell line Ba/F3 and human umbilical cord blood (UCB)-derived $CD34^+$ cells, we demonstrate that emergence of E/R^+ progenitors is favored in the context of BM mesenchymal inflammation. Moreover, the inflamed BM-MSC niche represents, at the same time, an attractive and genotoxic microenvironment for pre-leukemic cells.

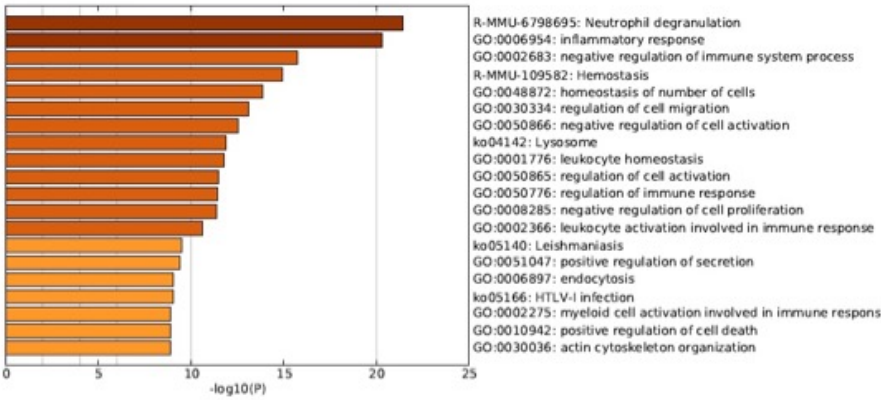
Results

ETV6/RUNX1-expressing Ba/F3 show a pro-inflammatory gene expression profile characterized by enhanced migration, myeloid signature and CXCL8-signalling activation

In order to explore potential pathways linking pre-leukemia, bone marrow microenvironment and inflammation, we performed gene expression profile (GEP) and gene ontology (GO) analysis on ETV6-RUNX1-expressing and control Ba/F3, a murine pro-B cell line that we and others had successfully used for modelling ETV6-RUNX1^{+} pre-leukemia *in vitro* (Ford *et al.*, 2009)(Palmi *et al.*, 2014)(Linka *et al.*, 2013)(Diakos *et al.*, 2007). Accordingly to our published data (Ford *et al.*, 2009), pre-leukemic cells were restrained in the cell proliferation program and overactivated genes involved in pathways like *negative regulation of cell proliferation, homeostasis of number of cells, negative regulation of cell activation and positive regulation of cell death.* Interestingly, ETV6-RUNX1^{+} Ba/F3 were characterized by a significant pro-inflammatory gene expression profile characterized by pathways involved in migration (*regulation of cell migration, actin cytoskeleton organization*) and myeloid cell activation (*neutrophils degranulation, myeloid cell activation involved in immune response*) (**Fig.S1-A**). The impact of ETV6-RUNX1 on cell migration was previously demonstrated by our group (Palmi *et al.*, 2014), while a pro-myeloid gene expression signature and clonogenic potential have been

recently observed in a developmental iPS model of ETV6-RUNX1 activity (Böiers *et al.*, 2018). We next analysed GEP data with Ingenuity Pathway Analysis and, among the predicted activated pathways with a significant *p-value*, the *IL8 signalling* (*p-value*=8.15E-06; 33,6% overlapped genes) emerged, representing a novel indication to our knowledge in ETV6-RUNX1^{+} cells (**Fig.S1-B**). CXCL8/IL8 is a pro-inflammatory cytokine binding to CXCR1 and CXCR2 receptors which induces migration and functional activation of neutrophils, macrophages and endothelial cells during inflammation (Schraufstatter *et al.,* 2001) (Raghuwanshi *et al.*, 2012). However, while CXCR1/2 are expressed in both humans and mice, CXCL8 is absent in the latter (Fan *et al.*, 2007). Murine CXCL1, CXCL2, CXCL3 (also named GROα, GROβ, GROγ) and CXCL5, CXCL6 (or ENA-78, GCP-2) chemokines bind CXCR1/2 and are considered functional analogues of the human CXCL8 (Hol *et al.,* 2010).

A


R-MMU-6798695: Neutrophil degranulation
GO:0006954: inflammatory response
GO:0002683: negative regulation of immune system process
R-MMU-109582: Hemostasis
GO:0048872: homeostasis of number of cells
GO:0030334: regulation of cell migration
GO:0050866: negative regulation of cell activation
ko04142: Lysosome
GO:0001776: leukocyte homeostasis
GO:0050865: regulation of cell activation
GO:0050776: regulation of immune response
GO:0008285: negative regulation of cell proliferation
GO:0002366: leukocyte activation involved in immune response
ko05140: Leishmaniasis
GO:0051047: positive regulation of secretion
GO:0006897: endocytosis
ko05166: HTLV-I infection
GO:0002275: myeloid cell activation involved in immune respons
GO:0010942: positive regulation of cell death
GO:0030036: actin cytoskeleton organization
0
5
10
15
20
25
-log10(P)

B

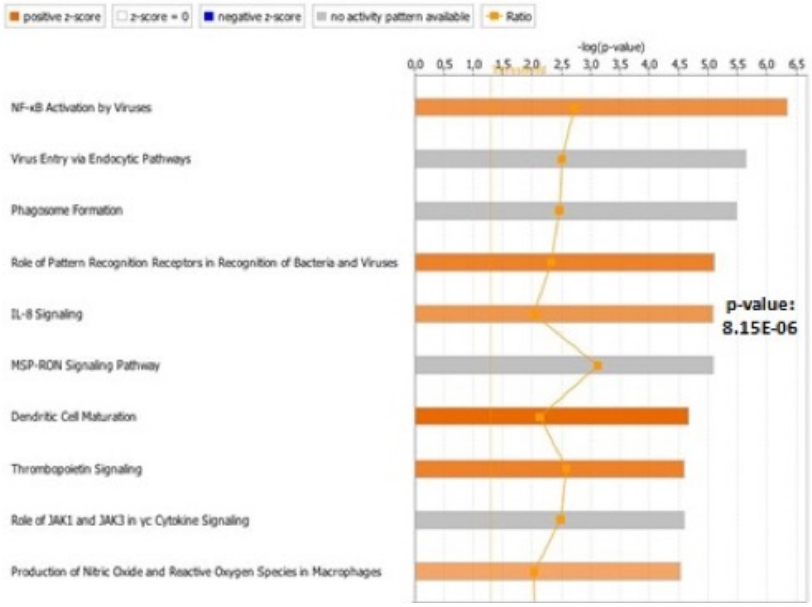

positive z-score
z-score = 0
negative z-score
no activity pattern available
Ratio
-log(p-value)
0,0 0,5 1,0 1,5 2,0 2,5 3,0 3,5 4,0 4,5 5,0 5,5 6,0 6,5
NF-κB Activation by Viruses
Virus Entry via Endocytic Pathways
Phagosome Formation
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
IL-8 Signaling
MSP-RON Signaling Pathway
Dendritic Cell Maturation
Thrombopoietin Signaling
Role of JAK1 and JAK3 in γc Cytokine Signaling
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
p-value:
8.15E-06

Figure S1. E/R+ Ba/F3 cells show a typical pro-inflammatory gene expression profile characterized by enhanced migration, myeloid signature and CXCL8 signalling activation. **A)** gene expression profile performed on Gene Chip Mouse 2.0 Arrays (Thermofisher) revealed an upregulation in pre-leukemic cells in the following pathways: regulation of cell proliferation, homeostasis of number of cells, negative regulation of cell activation, positive regulation of cell death, regulation of cell migration, actin cytoskeleton organization, neutrophils degranulation, myeloid cell activation involved in immune response. **B)** Top ten canonical pathways that were predicted to be activated (orange) in E/R+ Ba/F3 compared with controls, as shown by IPA pathway analysis. Pathways identified are represented on the y-axis. The x-axis corresponds to the –log of the P-value (Fisher's exact test) and the orange points on each pathway bar represent the ratio of the number of genes in a given pathway that meet the cut-off criteria, divided by the total number of genes that map to that pathway. GEP experiment was performed on 4 experimental replicates of ETV6-RUNX1 induction.

In addition to promote progression in solid tumors (Jaffer and Ma, 2016), the CXCL8/CXCR1-2 axis is involved in leukemogenic processes. CXCL8 and CXCR2 overexpression, in fact, represent a poor prognostic factor in Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) (Schinke *et al.*, 2015). Interestingly, BCP-ALL blasts stimulate BM-MSC to secrete CXCR1/2 ligands which attract blasts while excluding normal hematopoiesis, thus favoring the establishment of a self-reinforcing leukemic niche (De Rooij *et al.*, 2017). On that basis, we decided to investigate the role of CXCR1/2 in the context of ETV6-RUNX1^{+} pre-leukemia and mesenchymal inflammation. Control Ba/F3 transcriptionally express both receptors at very low levels and were negative for their surface immunostaining, likely suggesting that CXCR1/2 don't play a physiological role in normal pro-B cells. On the contrary, ETV6-RUNX1 strongly upregulated CXCR1 and CXCR2 mRNA (CXCR1 mRNA fold increase=55.3±7.9, *p=<0.001*; CXCR2 mRNA fold increase=140.2±45.2, *p=<0.001*) (**Fig.1A)** but

expressed only CXCR2 on the cell membrane (MFI: ER=1378±807; ctr=284±167, *p<0.05*) (**Fig.1B**).

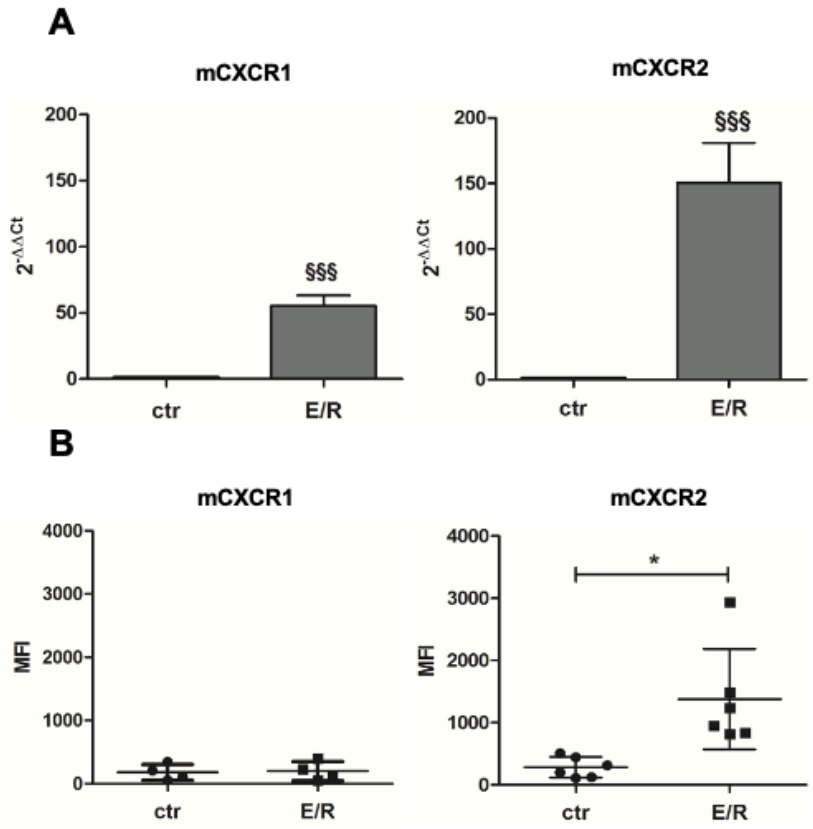


Figure 1. E/R$^+$ Ba/F3 overexpressed CXCR2 at both mRNA and membrane protein level. **A)** RQ-PCR analyses of CXCR1 and CXCR2 expression in control and E/R$^+$ Ba/F3 cells. Ba/F3 cells were incubated for 3 days in the presence of the inducer. cDNA was subjected to TaqMan RQ-PCR and normalized to Hprt expression. Levels of control Ba/F3 were used as refer value for Fold Increase calculation. One sample test: §§§, p<0.001. **B)** Analyses of the cell-surface expression of CXCR1 and CXCR2 measured as MFI levels by FACS. Student's t-test: *, p<0.05. Analysis was performed on 6 experimental replicates of ETV6-RUNX1 induction.

BM-MSC increase the secretion of CXCR1/2 ligands after stimulation with IL6/TNFα/IL1β pro-inflammatory cytokines

BM-MSC exert many of their functions by secreting molecules (i.e: growth factors, chemokines, interleukins, enzymes and metalloproteases) (Kyurkchiev, 2014). As we were interested in the relationship between BM-MSC and inflammation, we explored the cytokine secretion profile of human BM-MSC before and after stimulation with IL6/TNFα/IL1β. IL6, TNFα and IL1β are related pro-inflammatory cytokines precociously produced by pathogen recognition receptors (PRR)-expressing immune cells after viral and bacterial stimuli (Swiergiel and Dunn, 1999); moreover, contributions of IL6, TNFα and IL1β in the tumor microenvironment, including the leukemic niche, have been demonstrated (Vilchis-Ordoñez *et al.*, 2015) (Carey *et al.*, 2017) (Pietras, 2017). We screened unstimulated and inflamed BM-MSC supernatants of three healthy donors through 120 human cytokines-detecting protein arrays: interestingly, 5/10 of the most upregulated proteins were CXCR1/2 ligands (grey rows in **Fig.2A**); in particular, CXCL1 was the first in the list. CXCL1 levels dramatically increased also in inflamed murine BM-MSC supernatants compared to basal conditions, as indicated by the ELISA quantification (pg/mL: basal=78±28 *vs* +infl.ck=30162±4760, *p<0.01*) (**Fig.2B**).

A

	FC	*p-value*
CXCL1 (GROalpha)	**304,99**	***0,018***
GRO-family	**14,30**	***0,003***
CXCL8	**12,02**	***0,001***
GM-CSF	10,33	*0,061*
betaFGF	9,32	*0,011*
CXCL6	**8,77**	***0,045***
CCL5	6,29	*0,064*
betaNGF	5,65	*0,051*
CXCL5	**4,77**	***0,007***
HGF	4,00	*0,025*

B

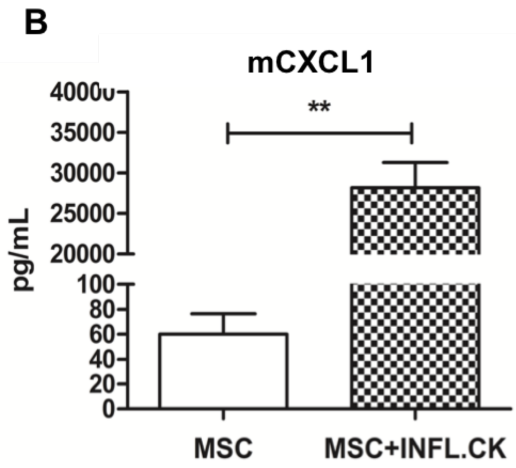


Figure 2. BM-MSC secrete high amounts of CXCR1/2 ligands after stimulation with IL6/aTNFα/IL1β pro-inflammatory cytokines. A) Unstimulated and inflamed BM-MSC supernatants of three healthy donors were analysed by the protein arrays Human Cytokines Array C1000 (RayBio) and data acquired through UVITEC Cambridge® instrument. Densitometry analysis was performed with ImageJ® software. The table shows the significative differentially expressed proteins in inflamed BM-MSC supernatants vs unstimulated. FC= fold change. **B)** Enzyme-linked immunosorbent assay (ELISA) of CXCL1 chemokine in supernatants of murine BM-MSC cultured in basal condition (MSC) or in presence of IL6/TNFα/IL1β (MSC+INFL.CK) for 48h. Results were depicted as mean ± SD of 3 experiments. Student's t-test: **, p<0.01.

ETV6-RUNX1^{+} Ba/F3 HIGLY MIGRATED TOWARDS INFLAMED BM-MSC CONDITIONED MEDIA IN A CXCR2-DEPENDENT MANNER

One of the primary functions of CXCR1/2 in leukocytes is to mediate chemotaxis towards sites of inflammation. In order to verify if the CXCR2 overexpression was functional in the pre-leukemic clone, we performed migration toward increasing doses of CXCL1. As shown in **Fig.S2**, ETV6-RUNX1^{+} Ba/F3 showed a strong, dose-dependent chemotaxis toward the chemokine. This observation confirmed that migratory pathways are important targets of the ETV6-RUNX1 transforming activity (Palmi *et al.*, 2014).

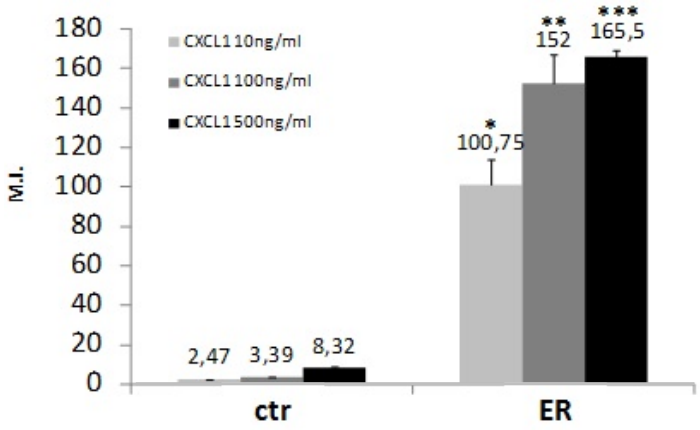


Figure S2. Migration towards CXCL1 was increased in E/R+ Ba/F3 cells. Transwell migration assays towards different concentration of CXCL1. Migration index (M.I.) =ratio between the number of cells migrated in 3h in response to chemokine and in its absence. Student's t-test: *, p<0.05: **, p<0.01; ***, p<0.001.

We then hypothesized that ETV6-RUNX1^{+} Ba/F3 could be attracted by inflamed BM-MSC supernatants in a CXCR2-dependent manner. To assess this, we tested control and ETV6-RUNX1^{+} Ba/F3 for migration toward unstimulated and inflamed BM-MSC conditioned media in presence or absence of the specific CXCR2 inhibitor SB265610. As shown in **Fig.3**, both control and pre-leukemic cells migrated more toward inflamed compared to unstimulated BM-MSC supernatants; however, the percentage of migrated cells in ETV6-RUNX1^{+} Ba/F3 was 2-folds higher than controls (% migrated cell/input toward INFL.MSC-CM: E/R=30.2±9.1; ctr=14.3±9.6, *p<0.01*). On the contrary, no differences in migration were observed between control and pre-leukemic cells in case of unstimulated BM-MSC conditioned media (% migrated cell/input toward MSC-CM: E/R=4.6±3.3; ctr=5.9±2.9). Blocking of CXCR2 significantly diminished ETV6-RUNX1^{+} Ba/F3 migration towards inflamed supernatants (% E/R migrated cells/input: +SB=7.7±4.3; -SB=30.2±9, *p<0.01*) and, to a less extend and without a statistical significance, toward unstimulated BM-MSC (% E/R migrated cells/input: +SB=4.6±3.3; -SB=2.2±1.3, *p>0.05*). As expected, treating control Ba/F3 with SB265610 didn't affect migration at all (% ctr migrated cells/input: +SB=7.5±6.9; -SB=5.9±2.9) (% ctr migrated cells/input: +SB=11.1±9.3; -SB=14.3±9.6). We thus demonstrate that ETV6-RUNX1 expression induced CXCR2-mediated migration in pro-B cells providing a specific mechanism for pre-leukemic clone attraction towards inflamed BM-MSC and, more in general, within inflammatory sites.

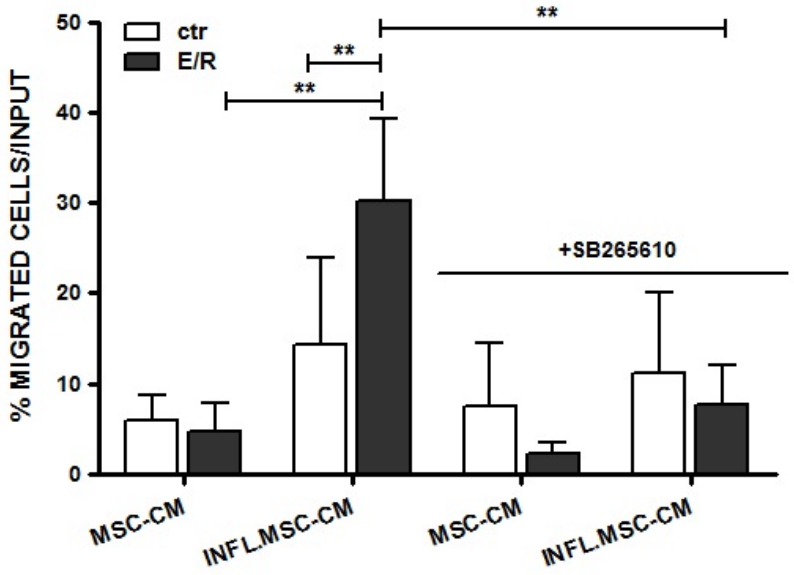


Figure 3. ETV6-RUNX1+ Ba/F3 highly migrated towards inflamed BM-MSC conditioned media in a CXCR2 dependent-manner. Transwell (8µm pores) migration assays towards MSC supernatant in basal (MSC-CM) or inflamed condition (INFL.MSC-CM) in absence or presence of CXCR2 inhibitor (SB265610). Student's t-test: **, p<0.01.

Inflammation favors ETV6-RUNX1^{+} Ba/F3 emergence against the normal counterpart in an *in vitro* model of competitive mesenchymal niche

Since we observed a preferential migration of ETV6-RUNX1^{+} Ba/F3 toward inflamed BM-MSC, we asked if such microenvironment could also provide advantages to the pre-leukemic clone in terms of persistence and/or progression. To address this, we set up an *in vitro* model of competitive mesenchymal niche by mixing ETV6-RUNX1^{+} and

control Ba/F3 at a starting ctr:E/R ratio of 20%:80% and co-culturing them directly on murine BM-MSC in presence or absence of IL6/IL1β/TNFα. The initial advantage of pre-leukemic cells was necessary since ETV6-RUNX1 negatively affected the cell-cycle in our inducible Ba/F3 model (Ford *et al.*, 2009). As internal experimental controls, mixed Ba/F3 were plated alone in standard medium without cytokines (basal condition), with IL6/IL1β/TNFα or with TGFβ; this latter represented a positive control, as we previously showed that ETV6-RUNX1^{+} HSPC were intrinsically resistant to its antiproliferative effect and gained a proliferative parity with the normal counterpart in mix assays (Ford *et al.*, 2009). After 4 days of culture, percentage of ETV6-RUNX1^{+} cells in the mix was analyzed: as expected, pre-leukemic cells lose the competition against control cells in basal conditions (% E/R: basal=28±14), while they maintained a number parity in presence of TGFβ (% E/R: + TGFβ =50±20). Interestingly, while unstimulated BM-MSC did not provide advantage to the pre-leukemic clone (% E/R: +MSC=21±12; basal=28±14), addition of pro-inflammatory cytokines within the competitive mesenchymal niche significantly increased the ETV6-RUNX1^{+} Ba/F3 fraction (% E/R: +MSC+INFL.CK=47±17). Of note, no advantaging effect was observed by stimulating Ba/F3 with IL6/IL1β/TNFα alone, indicating that the pro-inflammatory cytokines did not favor pre-leukemic pro-B cells *per sé* (**Fig.4**).

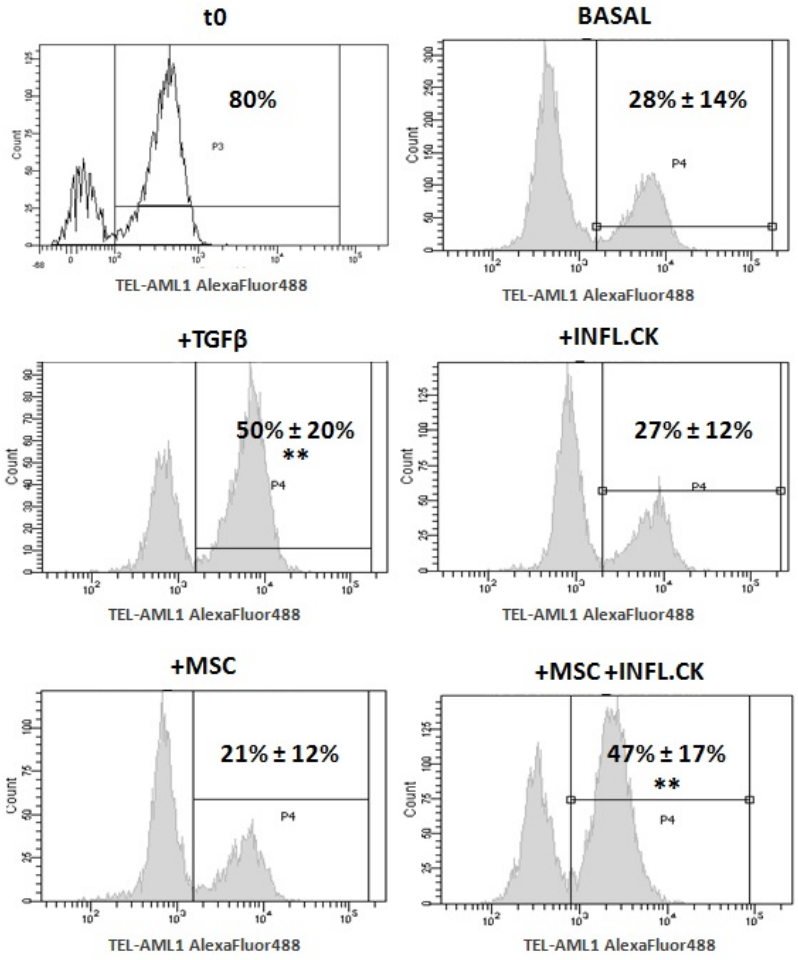


Figure 4. Inflammation favors ETV6-RUNX1^{+} Ba/F3 emergence against the normal counterpart in an *in vitro* model of competitive mesenchymal niche. A mixture of ETV6-RUNX1–expressing and non-expressing cells (80%:20%) was grown at the indicated conditions for 4 days. The percentage of E/R+ cells in the mix was analyzed by flow cytometry using an anti-V5 tag antibody. The figure shows a representative experiment. The numbers indicate the mean ± SD of the percentage of E/R+ cells in 6 experiments. T test analysis was applied to compare percentages of E/R+ cells in the mix grown for 4 days in the different conditions compared with the basal condition. Student's t-test: **, $p<0.01$.

In addition to inflammatory cytokines, we also performed the competitive niche assay in presence of lipopolisaccaryde (LPS), which has been reported to be promoting factor for ETV6-RUNX1-driven leukemogenesis *in vivo* (Swaminathan *et al.*, 2015). However, LPS stimulation didn't favored the emergence of the pre-leukemic clone compared to unstimulated BM-MSC (**Fig.S3**).

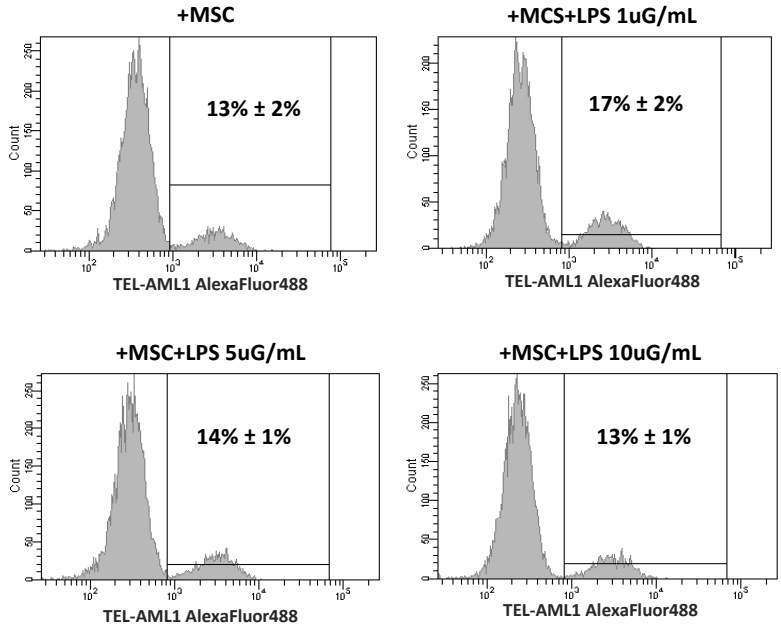


Figure S3. LPS stimulation doesn't favor ETV6-RUNX1^{+} Ba/F3 emergence against the normal counterpart in an *in vitro* model of competitive mesenchymal niche. A mixture of ETV6-RUNX1–expressing and non-expressing cells (80%:20%) was grown at the indicated conditions for 4 days. The percentage of E/R+ cells in the mix was analyzed by flow cytometry using an anti-V5 tag antibody. The figure shows a representative experiment. The numbers indicate the mean ± SD of the percentage of E/R+ cells in 3 experiments.

The inflamed mesenchymal niche strongly decreases normal pro-B cells proliferation and survival while mildly or no affecting the ETV6-RUNX1$^+$ clone

The emergence of the pre-leukemic clone within the inflamed mesenchymal niche could result from different mechanisms affecting both ETV6-RUNX1$^+$ and control Ba/F3. In order to better elucidate this aspect, we analyzed proliferation and apoptosis in the two fractions through CFSE, a cell-division tracker, and ANNEXIN-V staining, respectively. As shown in **Fig.5A**, proliferation diminished in both groups within the inflamed niche; however, decrease was more evident in control (CFSE MFI fold increase +MSC *vs* +MSC+INFL.CK: ctr=4.4±1.8, *p<0.05*) compared to pre-leukemic Ba/F3 (CFSE MFI fold increase +MSC *vs* +MSC+INFL.CK: E/R=2.2±0.6, *p<0.001*), resulting in an attenuation of E/R$^+$ Ba/F3 proliferative disadvantage compared to controls. Very interesting, a more evident discrepancy was observed in terms of survival: in presence of BM-MSC and inflammation, in fact, apoptosis was strongly induced in control cells (% ANN-V negative cells: +MSC=68.4±5.7 *vs* +MSC+infl.ck=48.2±1.3, *p<0.05*) while ETV6-RUNX1$^+$ Ba/F3 were completely unaffected (**Fig.5B**).

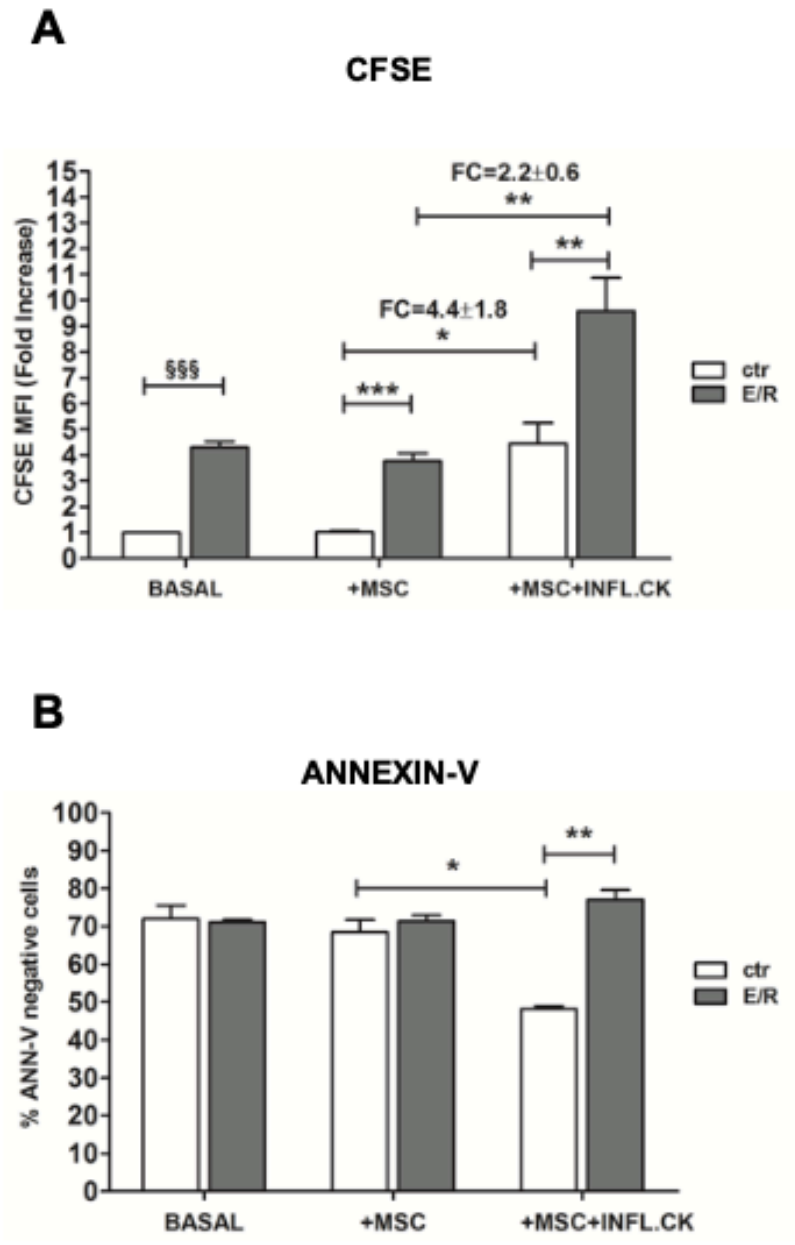

A
CFSE
CFSE MFI (Fold Increase)
FC=2.2±0.6
**
**
FC=4.4±1.8
*
§§§
***
ctr
E/R
BASAL
+MSC
+MSC+INFL.CK
B
ANNEXIN-V
% ANN-V negative cells
**
*
ctr
E/R
BASAL
+MSC
+MSC+INFL.CK

Figure 5. The inflamed mesenchymal niche decreases control Ba/F3 cell proliferation and survival.

A) A mix of ETV6-RUNX1+ and control Ba/F3 (80%:20%) were stained with CFSE and cultured for 4 days in basal condition or on a murine MSC layer in presence or absence of inflammatory cytokines. At the end of the culture, cells were permeabilized and stained with a mCD45 antibody (to identify the Ba/F3 cells) and with a specific anti-ETV6-RUNX1 fusion antibody (to identify the E/R+ Ba/F3 cells). Cells were then analyzed by flow cytometry to determine CFSE MFI. The CFSE MFI value of control cells grown in basal condition was considered as referrer value for fold increase calculations. The numbers indicate the CFSE MFI fold increase +MSC vs +MSC+infl.ck of control and E/R+ Ba/F3 cells. **B)** Control and ETV6-RUNX1+ Ba/F3 cultured for 4 days in basal condition or on a murine MSC layer in presence or absence of inflammatory cytokines. At the end of the culture, cells were stained with a mCD45 antibody and with the PE-conjugated Annexin V. The cells were then analyzed by flow cytometry. Percentages of Annexin V-negative cells are shown. Analysis was performed on 4 experimental replicates of ETV6-RUNX1 induction. One sample t-test: §§§, $p<0.001$. Student's t-test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

The pre-leukemic advantaging effect within the inflamed mesenchymal niche is mediated by soluble factors

We then investigated if soluble factors rather than cell-cell contacts mediated the pre-leukemic clone advantage within the inflamed mesenchymal niche. Co-culture of control and E/R Ba/F3 mix in Transwell® (0.4µM pores) in presence of BM-MSC and IL6/IL1β/TNFα provided identical results of the direct-contact niche model (**Fig. S4**), demonstrating that the pre-leukemia advantaging effect was predominantly mediated by secreted molecules. Considering our published data (Ford *et al.*, 2009), we wanted to verify if TGFβ was involved in the observation. However, as its concentration

in BM-MSC supernatants strongly decreased after IL6/IL1β/TNFα stimulation at every time point considered, we likely excluded its contribution in this context (**Fig. 6**).

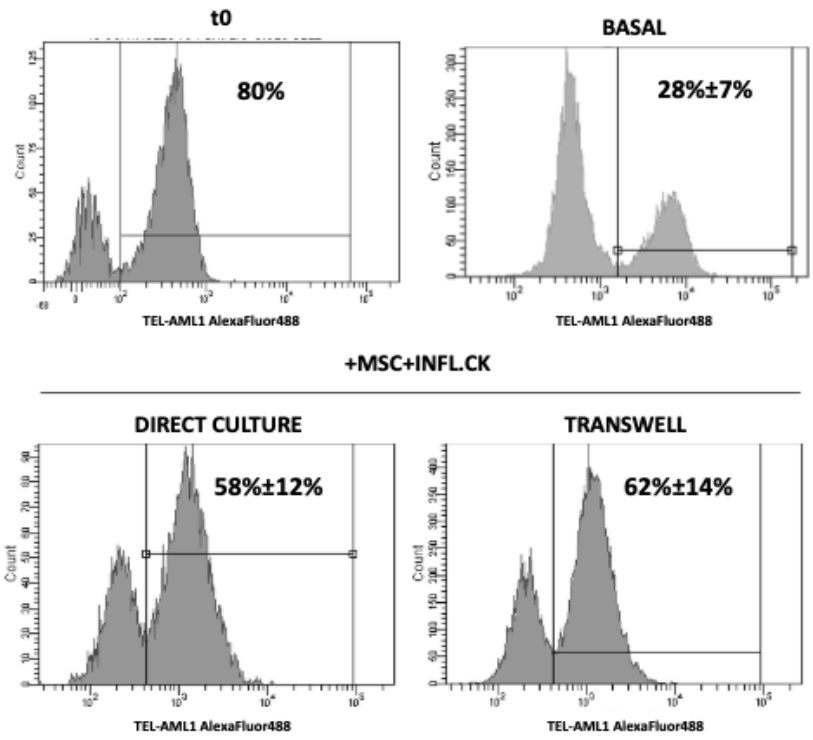


Figure S4. The pre-leukemic advantaging effect within the inflamed mesenchymal niche is mediated by soluble factors. A mixture of ETV6-RUNX1–expressing and control cells (80%:20%) was grown in basal conditions or in the presence of murine BM-MSC stimulated with IL6/TNFα/IL1β cytokines (MSC+INFL.CK) for 4 days. The mix was plated directly on the MSC layer (direct culture) or, to avoid the cell-cell contact, in a Transwell insert (0.4µm pores) with MSCs plated in the lower compartment. The percentage of E/R+ cells in the mix was analyzed by flow cytometry using an anti-V5 tag antibody. The figure shows a representative experiment. The numbers indicate the mean ± SD of the percentage of E/R+ cells in 3 experiments.

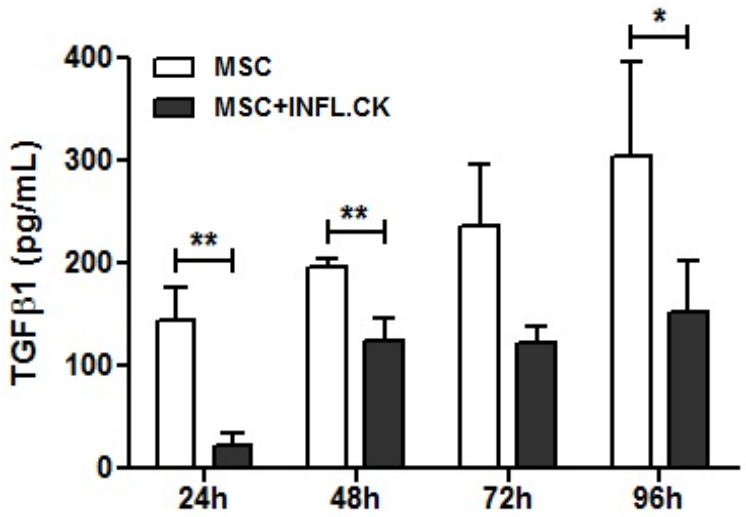


Figure 6. TGFβ1 concentration in murine BM-MSC supernatants strongly decreased after IL6/IL1β/TNFα stimulation. Supernatants of murine BM-MSC cultured in basal condition (MSC) or in presence of IL6/TNFα/IL1β (MSC+INFL.CK) and collected at the indicated time points were analyzed for TGFβ1 secreted cytokine. Results were depicted as mean ± SD of 3 experiments. Student's t-test: *, $p<0.05$; **, $p<0.01$.

CXCL1 was not implicated as well, since co-cultivating control and ETV6-RUNX1^{+} Ba/F3 with increasing doses of the chemokine did not provide advantage to the pre-leukemic clone (**Fig. S5**).

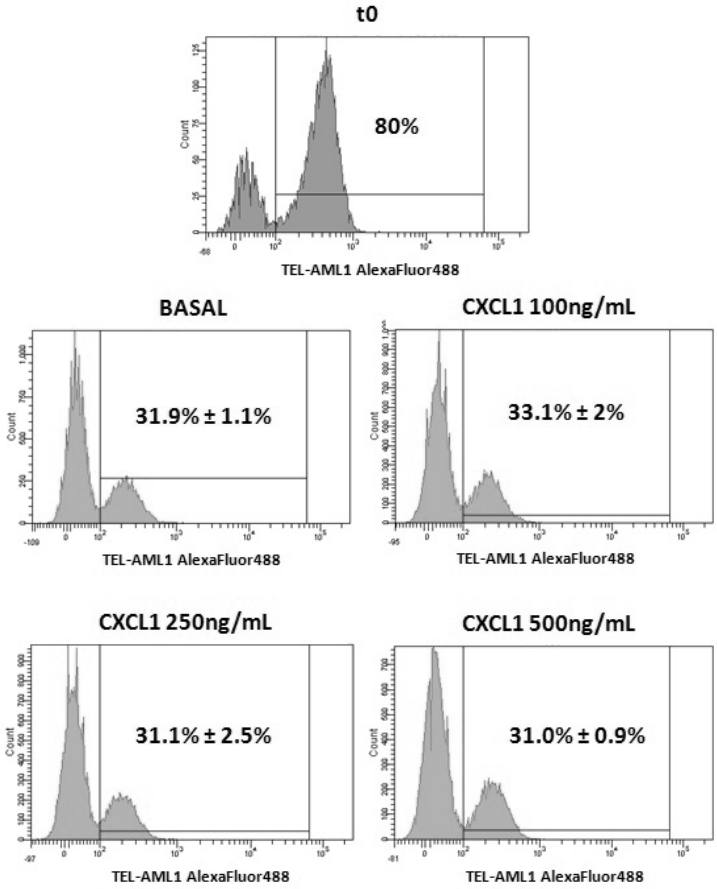


Figure S5. CXCL1 did not favor ETV6-RUNX1+ Ba/F3 emergence against the normal counterpart A mixture of ETV6-RUNX1–expressing and non-expressing cells (80%:20%) was grown in basal conditions or in the presence of the indicated concentrations of CXCL1 for 4 days. The percentage of E/R+ cells in the mix was analyzed by flow cytometry using an anti-V5 tag antibody. The figure shows a representative experiment. The numbers indicate the mean ± SD of the percentage of E/R+ cells in 3 experiments.

The inflamed mesenchymal niche increases DNA double strand breaks and AID expression in murine B-progenitors

ETV6-RUNX1 represents a weak oncogene insufficient for malignant transformation and additional genetic alterations must occur in pre-leukemic cells to overt disease (Wiemels *et al.*, 1999). It has been recently demonstrated that mesenchymal inflammation provides genotoxic stress in HSPC favoring the transition to secondary myeloid leukemia in genetic predisposing syndromes (Zambetti *et al.*, 2016). Thus, we question if genetic damage also increased in Ba/F3 after co-culture on inflamed BM-MSC. To address this, we evaluated phosphorylation levels of histone 2AX at Ser239 (γH2AX, a marker of DNA double strand breaks (DSB)) in control and ETV6-RUNX1^{+} Ba/F3 co-cultured alone, upon unstimulated BM-MSC or inflamed BM-MSC. As shown in **Fig.7**, pre-leukemic cells were characterized by higher basal levels of γH2AX compared to controls, indicating an intrinsic genetic instability of pre-leukemic cells (γH2AX MFI fold increase: E/R=1.86±0.65, *p<0.05*). Interestingly, while culturing Ba/F3 on unstimulated BM-MSC didn't affect results compared to the basal condition, addition of inflammatory cytokines to the mesenchymal niche significantly increased DNA DSB levels in both control and ETV6-RUNX1^{+} Ba/F3 (γH2AX MFI fold increase +MSC *vs* +MSC+infl.ck: E/R=2.28±1.56 *p<0.05*; ctr=4.36±1.61, *p<0.01*).

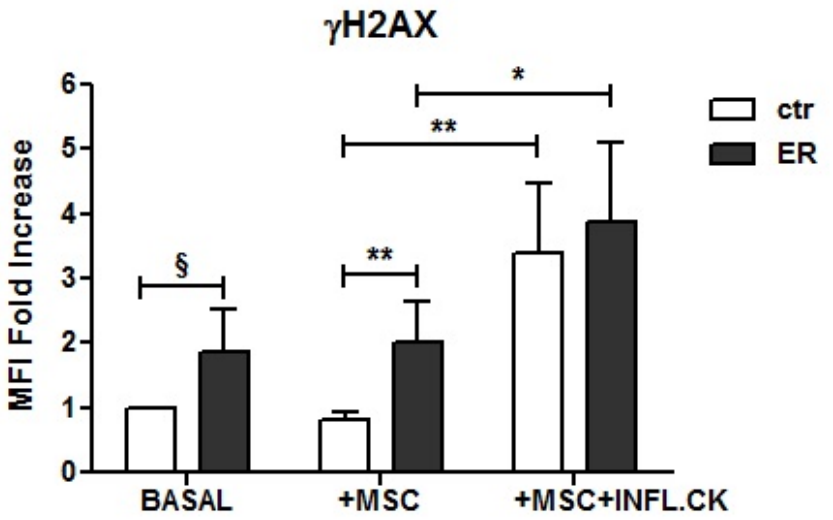


Figure 7. The inflamed mesenchymal niche increases γH2AX expression in control and E/R Ba/F3 cells. The graph shows the fold increase of the γH2AX expression measured as MFI levels by FACS considering the γH2AX expression level in control cells grown in basal condition such as 1. Analysis was performed on 5 experimental replicates of ETV6-RUNX1 induction. One sample t-test: §, $p<0.05$. Student's t-test: *, $p<0.05$; **, $p<0.01$.

It has been demonstrated that deregulated expression of activation-induced cytidine deaminase (AID) enzyme, which mediates Ig class switch recombination and V(H) gene somatic hypermutation in B cells, is a mutagenic mechanism driving ETV6-RUNX1$^+$ pre-leukemia to leukemia transition *in vivo* (Swaminathan *et al.*, 2015)(Rodríguez-Hernández *et al.*, 2017). We analyzed AID mRNA levels in control and ETV6-RUNX1$^+$ Ba/F3 before and after exposure to inflamed BM-MSC secretome. Even in this case, pre-leukemic cells showed higher basal levels of the enzyme compared to controls (AID mRNA fold increase E/R *vs* ctr: 4.9±2.1). Interestingly, mesenchymal inflammation induced

AID mRNA upregualtion in both groups (AID mRNA fold increase basal *vs* +MSC+INFL.CK: E/R=6.3±1.6, *p<0.05*; ctr=14.7±10.9) (**Fig.8**). Of note, if normal pro-B cells go through apoptosis within the inflamed niche, possibly due to increase DNA DSB and AID expression, pre-leukemic cells still persisted although accumulating genetic damages (**Fig.5**).

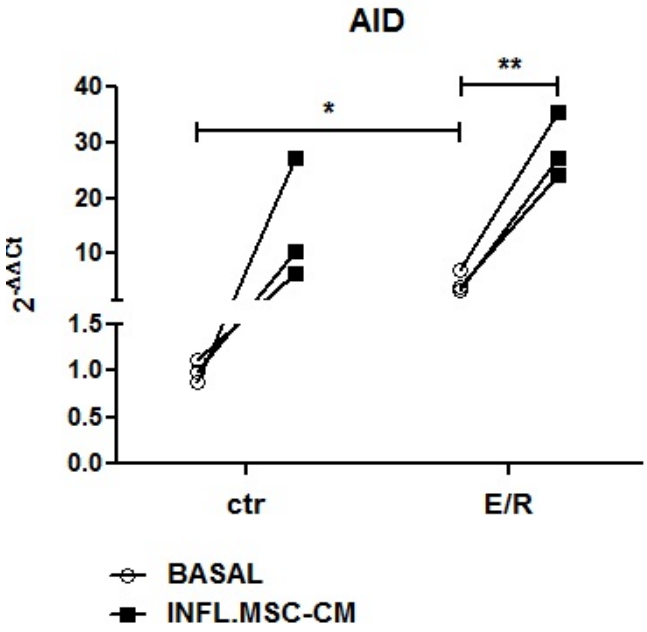


Figure 8. The inflamed mesenchymal niche increases AID expression in control and E/R Ba/F3 cells. RQ-PCR analyses of AID expression in control and E/R+ Ba/F3 cells. Ba/F3 cells were incubated for 3 days in the presence of the inducer and grown in basal condition or in a transwell insert of 0.4µm with inflamed MSCs plated in the lower compartment (INFL.MSC-CM) to avoid RNA MSC contamination in the RQ-PCR analysis. cDNA was subjected to TaqMan RQ-PCR and normalized to Hprt expression. Analysis was performed on 3 experimental replicates of ETV6-RUNX1 induction. Student's t-test: *, p<0.05; **, p<0.01.

ETV6-RUNX1-expressing human UCB-CD34$^+$ cells highly migrate toward inflamed BM-MSC conditioned media

Although inducible ETV6-RUNX1-expressing Ba/F3 represents a confident pre-leukemic model (Diakos *et al.*, 2007) (Ford *et al.*, 2009) (Linka *et al.*, 2013) (Palmi *et al.*, 2014), we wanted to confirm data in a more physiological cellular context. To this purpose, a pool of several healthy donor-derived umbilical cord blood (UCB) CD34$^+$ progenitors were lentivirally transfected with pRRL-GFP or pRLL-ETV6-RUNX1-GFP constructs. We therefore analyzed the migration of empty-vector (pRRL-GFP) and ETV6-RUNX1$^+$ (pRRL-E/R-GFP) CD34$^+$ cells toward unstimulated and inflamed BM-MSC conditioned media. As shown in **Fig.9**, pRRL-GFP HPCS migrated did not show differences between basal and inflamed BM-MSC conditioned media (% migrated pRRL-GFP cells: MSC-CM=4.42±0.34, INFL.MSC-CM=5.19±0.62). On the contrary, ETV6-RUNX1-expressing CD34$^+$ cells possessed enhanced migration toward both supernatants; however, the increase was higher in case of inflamed BM-MSC (% migrated pRRL-E/R-GFP cells: MSC-CM=12.63±1.24, INFL.MSC-CM=21.18±2.43, $p<0.01$; % migrated pRRL-GFP cells: MSC-CM=4.4±0.3, INFL.MSC-CM=5.2±0.6) (**Fig.9**).

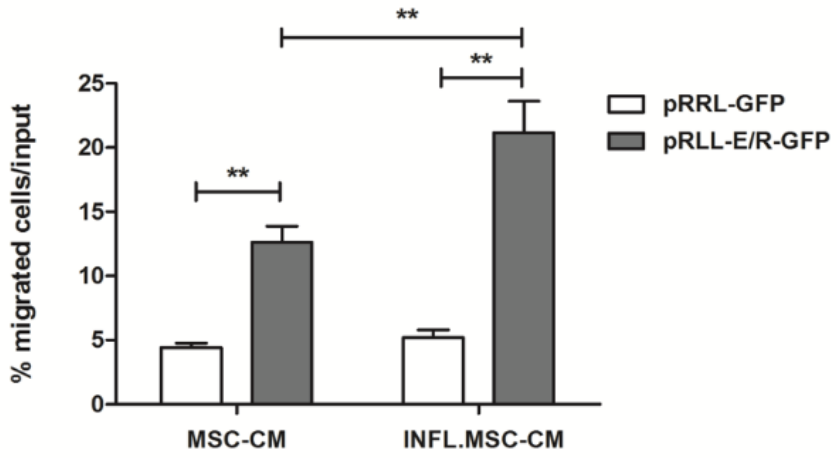


Figure 9. ETV6-RUNX1-expressing human UCB-CD34+ cells highly migrate toward inflamed BM-MSC conditioned media. Transwell migration (5µM pores) assays towards MSC supernatant in basal (MSC-CM) or inflamed condition (INFL.MSC-CM). The figure shows the mean ± SD of UCB-CD34+ cells subjected at one transduction experiment and tested against the supernatants of three different healthy donor derived-MSC. Student's t-test: **, p<0.01.

Mesenchymal inflammation preserves the $CD34^{+}IL7R^{+}$ pre-leukemic population while negatively affecting the normal counterpart

We previously demonstrated that TGF-β, a cytokine produced during inflammation, increased the absolute number of putative pre-LSC among ETV6-RUNX1-expressing UCB-$CD34^{+}$ while diminishing that of more mature cells; of note, the preferential expansion didn't occur in wild-type cells (Ford *et al.*, 2009). Taking advantage from a human iPS-derived model of developmental hematopoiesis, Böiers et al. have

demonstrated that fetal $CD34^+CD19^-IL7R^+$ progenitors are particularly susceptible to the ETV6-RUNX1 pre-leukemic activity during early development. Despite the particular affiliation to early hematopoiesis, authors detected this phenotype also in cord-blood samples. On that basis, we wanted to investigate if $CD34^+CD19^-IL7R^+$ cells could be advantaged by the presence of BM-MSC and inflammation in our transfected UCB-$CD34^+$ model. To verify this, we separately cultivated pRRL-GFP and pRRL-E/R-GFP $CD34^+$ cells on BM-MSC (with the addition of TPO, FLT3L, IL6, SCF and IL3) in presence or absence of IL6/IL1β/TNFα for 72h. In these culture conditions, no CD19 expression was observed while a $CD34^+IL7R^+$ phenotype was present in both experimental groups. We then analyzed the percentages of $CD34^+IL7R^+$, $CD34^+IL7R^-$ and $CD34^-$ subpopulations, as well as the total number of GFP^+ cells, in pRRL-GFP and pRRL-E/R-GFP progenitors after culture on unstimulated or inflamed BM-MSC. Interestingly, we found that mesenchymal inflammation differentially affected the numerosity and the composition within control and pre-leukemic populations. In particular, the percentage of control $CD34^-$ cells increased on inflamed compared to unstimulated BM-MSC (68.4±1.6 *vs* 59.8±1.7, *p<0.05*), while that of $CD34^+ILR^-$ progenitors slightly diminished 34.1±1.5 *vs* 28.5±1.3, *p<0.01*). Of note, the pRRL-GFP $CD34^+IL7R^+$ compartment was the most strongly affected, as it decreased by about 50% (6.1±0.5 *vs* 3.1±0.3, *p<0.01*) (**Fig.10A**). Overall, however, the total number of control GFP^+ cells was higher within the inflamed compared to the unstimulated niche (tot pRRL-

GFP$^+$: +MSC = 6117±1646; MSC+INFL.CK: 8077±1087, *p<0.05*), possibly due to the expansion of the more mature CD34$^-$ elements. An opposite scenario was observed within the pre-leukemic population: in fact, the CD34$^+$IL7R$^+$ subpopulation was perfectly maintained, if not increased 8.8±0.9 *vs* 11.4±0.5, *p=0.055*), CD34$^+$ILR$^-$ cells were unaltered (31±1.5 *vs* 31.9±0.4), while percentage of CD34$^-$ cells diminished (60.2±0.8 *vs* 56.8±0.4, *p<0.01*). In this case, the total number of GFP$^+$ cells was also decreased (tot pRRL-E/R-GFP$^+$: +MSC = 6727±1300; MSC+INFL.CK: 6374±997; *p<0.05*). Since a CD34^{high}IL7R$^+$ phenotype was recognizable in both experimental groups (**Fig. S6-A**), we decided to extend the analysis also to this fraction. As shown in **Fig. S6-B**, pRLL-GFP CD34^{high}IL7R$^+$ cells showed a consistent and significant decrease after the exposure to the inflammatory niche (fold-change pRRL-GFP +MSC+INFL.CK *vs* +MSC = 0.63±0.07; *p<0.05*), while the number of CD34^{high}IL7R$^+$ pre-leukemic cells slightly increased compared to the unstimulated condition (fold-change pRLL-E/R-GFP +MSC+INFL.CK *vs* +MSC =1.09±0.03; *p<0.05*). Thus, ETV6-RUNX1 expression specifically preserved the CD34$^+$IL7R$^+$ compartment by the negative effects of mesenchymal inflammation, possibly leading to the emergence of the pre-leukemic population against the normal counterpart under infective/inflammatory events.

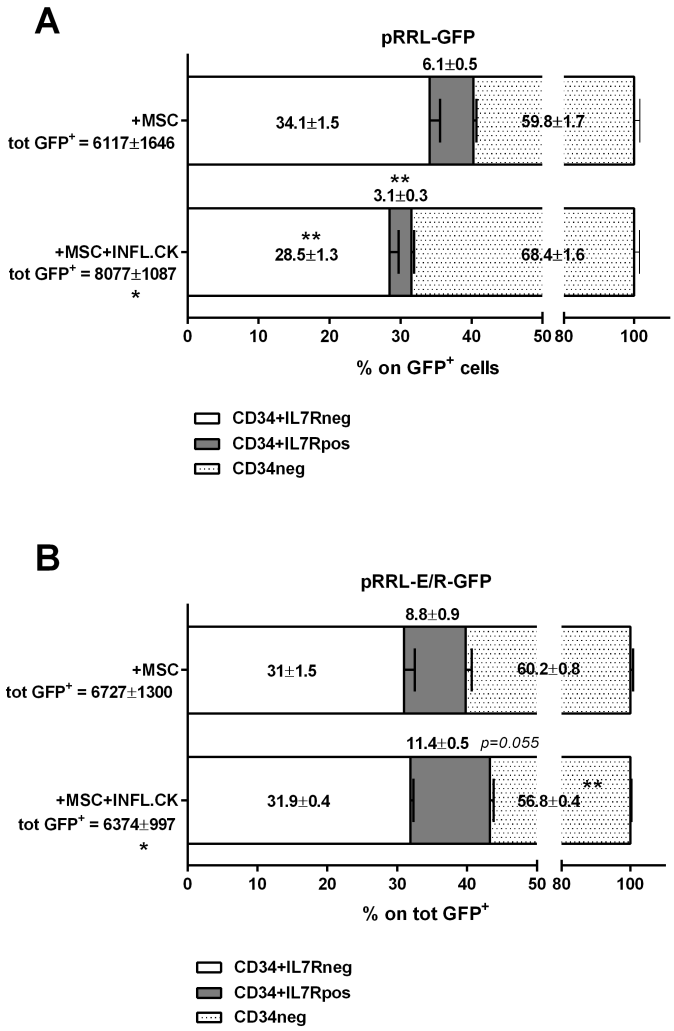


Figure 10. Mesenchymal inflammation selectively preserves the CD34⁺IL7R⁺ pre-leukemic compartment. pRRL-E/R-GFP and control pRRL-GFP CD34+ cells were cultured separately on BM-MSC (with the addition of SCF, FLT3L, TPO, IL3) in absence (+MSC) or presence of IL6/IL1β/TNFα pro-inflammatory cytokines (+MSC+INFL.CK) for 72h. At the end of the culture, cells were stained with anti-CD34 and anti-IL7R antibodies. Samples were quantitatively analyzed at FACS by bead-based normalization. Graphs show the total number of GFP+ cells and the percentages of distinct subpopulation, gated on GFP⁺, within control **(A)** and pre-leukemic **(B)** cells at the end of the indicated culture conditions. Student's t-test: *, $p<0.05$.

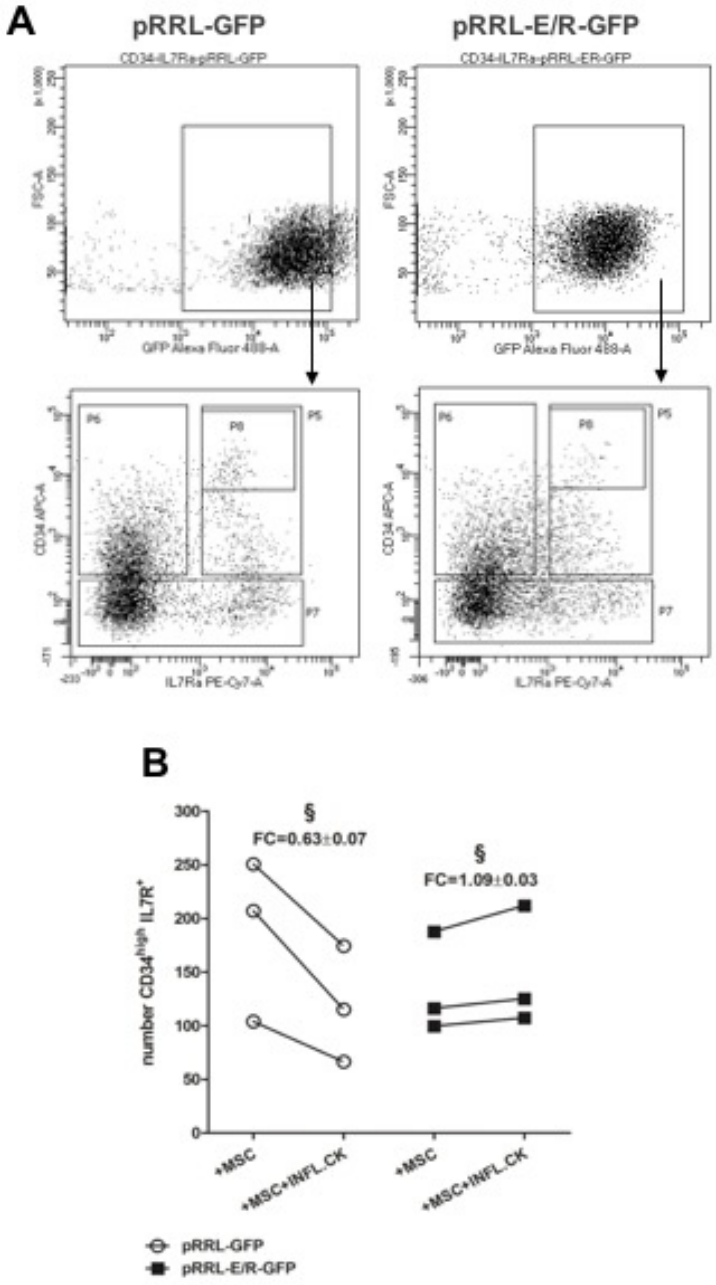

A
pRRL-GFP
pRRL-E/R-GFP
CD34-IL7Ra-pRRL-GFP
CD34-IL7Ra-pRRL-ER-GFP
FSC-A
GFP Alexa Fluor 488-A
P6
P8
P5
P7
CD34 APC-A
IL7Ra PE-Cy7-A
B
§
FC=0.63±0.07
§
FC=1.09±0.03
number CD34high IL7R+
300
250
200
150
100
50
0
+MSC
+MSC+INFL_CK
+MSC
+MSC+INFL_CK
pRRL-GFP
pRRL-E/R-GFP

Figure S6. A) Gating strategy of $CD34^-$, $CD34^+IL7R^-$, $CD34^+IL7R^+$ and $CD34^{high}IL7R^+$ subpopulations. Representative dot plots of pRRL-GFP and pRRL-E/R-GFP cells after 72h of culture on unstimulated BM-MSC in presence of SCF, FLT3L, TPO, IL3 for 72h. Cells were stained with anti- human CD34 and anti-human CD127 (IL7Rα) antibodies. Percentages of the different subpopulation were calculated on GFP^+ gated cells while absolute numbers obtained after the normalization on TrueCount beads. **B) Pre-leukemic $CD34^{high}IL7R^+$ cells are preserved under mesenchymal inflammation compared to the normal counterpart**. Graphs show the number of $CD34^{high}IL7R^+$ cells at the end of the indicated culture conditions. Transduced cells were gated for GFP positivity. The numbers indicate the fold-change +MSC+INFL.CK vs +MSC of pRRL-GFP and pRRL-E/R-GFP CD34+ cells. One sample t-test: *, $p<0.05$; **, $p<0.01$. The figure shows the results of UCB-CD34+ cells subjected at one transduction experiment and tested against the supernatants of three different healthy donor derived-MSC.

Discussion

We provide evidences in both murine and human hematopoietic progenitors that E/R expression leads to the selective advantages of pre-leukemic cells in the context of bone marrow mesenchymal inflammation. The advantage is mediated by several mechanisms, including proliferation, survival and migration, which differentially act on E/R^+ and control cells.

In the murine pro-B cell line Ba/F3, we found that E/R induction was associated with an inflammatory gene expression profile and a specific migratory/myeloid signature. A dysregulation of inflammatory and migratory pathways in the same model have been previously shown by other studies (Linka *et al.*, 2013) (Palmi *et al.*, 2014), while a recent iPS-based model of early hematopoietic development evidenced how E/R expression facilitates the co-expression of lympho-myelo genes in developing pro-B cells (Böiers *et al.*, 2018). Notably, E/R^+ blasts frequently show aberrant myeloid genes and surface antigens (Gerr *et al.*, 2010) (Abdelhaleem *et al.*, 2007). These observations reinforced our experimental hypothesis, that is a potential altered response of the pre-leukemic clone to niche-derived signals, especially during infective/inflammatory events. Actually, we observed in both Ba/F3 and human umbilical cord blood-derived $CD34^+$ (UCB-$CD34^+$) progenitors that E/R increased migration toward supernatants of inflamed bone marrow mesenchymal stromal cells (BM-MSC) compared to controls. Such preferential chemotaxis was, at

least in the Ba/F3 model, CXCR2-dependent: in fact, pre-leukemic Ba/F3 highly expressed the receptor, which is absent in normal pro-B cells instead. Enhanced intracellular signaling could be also involved, as effectively indicated by gene expression profile analysis.

Taking advantage from an *in vitro* competitive mesenchymal niche model, we demonstrated that the pre-leukemic/control Ba/F3 ratio increased within the inflamed niche compared to basal and unstimulated niche conditions. This advantage was mediated by the local secretome, which exerted a strong antiproliferative and pro-apoptotic effect against normal Ba/F3, while mildly or no affecting the pre-leukemic clone. An anti-proliferative effect of BM-MSC on mature activated B cells, mediated by secreted factors such as transforming growth factor (TGF)-β, indoleamine-2,3-dioxygenase (IDO), prostaglandin-E2, nitric oxide, have been widely demonstrated (Corcione *et al.*, 2006) (Krampera *et al.*, 2006). In our case, however, BM-MSC interacted with immature pro-B cells and in presence of IL6/IL1β/TNFα pro-inflammatory cytokines. After having excluded the involvement of TFGβ, on the basis of our published data (Ford *et al.*, 2009), and a novel role for CXCR2-ligands, a possible microenvironmental explanation could reside in the abundance of pre-myeloid factors that are secreted by BM-MSC under IL6/IL1β/TNFα stimulation (**Fig.2-A**). Those factors, in fact, could limit B-lymphopoiesis in favor of a physiological "emergency myelopoiesis" response (Riether, Schürch and Ochsenbein, 2015) which could preferentially sustain the "lympho-myelo" E/R^+ clone. Pre-leukemic

Ba/F3 increase the expression of cyclin-dependent kinase (Cdk) inhibitor proteins $p27^{KIP1}$ (CDKN1B) and $p21^{WAF}$ (CDKN1A). It is possible that basal induction of cell-cycle arrest could limit further anti-proliferative effects of external agents (Ford *et al.*, 2009), resembling a sort of intrinsic quiescent status. Similar considerations could be valid for apoptosis. Although pro-myeloid factors could exert a sort of protection against apoptosis in pre-leukemic cells, an intrinsic resistance of E/R^+ Ba/F3 to DNA damage is conceivable. In basal conditions, in fact, we observed higher levels of γH2AX in E/R^+ Ba/F3 compared to controls, indicating that the oncogene induced a genetic stress but, at the same time, provided mechanism to tolerate it. Kantner et al. demonstrated that ETV6-RUNX1 induced intracellular reactive oxygen species (ROS) accumulation driving the accumulation of DNA damage in B cells (Kantner *et al.*, 2013). On the other hand, it has been shown that ETV6-RUNX1 expression affected genes involved in the DNA damage repair (DDR) system (Linka *et al.*, 2013) (Fuka *et al.*, 2011), particularly impairing the p53 pathway (Kaindl *et al.*, 2014). On the other hand, mesenchymal inflammation further increased γH2AX levels in pre-leukemic as well as control Ba/F3. Regarding this, it has been shown that BM-MSC deleted for the causative gene of Shwachman-Diamond syndrome (SDS), a pre-leukemic disorder, acquired an inflamed status that induce a genotoxic stress in HSPC through the activation of p53-S100A8/9-TLR4 pathway. Moreover, transcriptional activation of this axis within the BM mesenchymal population predicted leukemic evolution in patients affected by

myelodysplastic syndrome (MDS) (Zambetti *et al.*, 2016). Our preliminary investigations, however, did not detected a clear surface expression of TLR4 in Ba/F3 cells, although the pre-leukemic clone modestly upregulated its transcriptional levels compared to controls. On the other hand, we had indications that human BM-MSC stimulated with IL6/IL1β/TNFα secreted the danger-associated molecular pattern S100A8/9 (data not shown). In the next future, it will be intriguing to explore the possibility that TLR4 membrane expression could be induced in Ba/F3 within the inflamed mesenchymal niche, leading to their increased genotoxic damage though the S100A8/9 pathway. An aspect that deserves a particular discussion is the overexpression of activation-induced cytidine deaminase (AID) enzyme. It has been shown that coordinate AID and RAG activity subverted genetic integrity in small preB-II cells, both in humans and mice, and that their activation in consequence of strong inflammatory stimuli drove ETV6-RUNX1^{+} pre-leukemia to leukemia transition *in vivo* (Swaminathan *et al.*, 2015). Ba/F3 are proB cells that don't express RAG enzyme; however, also in this cellular context a functional role for AID expression and activation have recently proposed. In a p19$^{ARF-/-}$Rag1$^{-/-}$ pro-B ALL-prone mice Auer and colleagues found that AID deletion surprisingly accelerated leukemia development. For that reason, they suggested that AID could act as negative regulator in Rag1$^{-/-}$ proB through the elimination of leukemia-prone cells, through a mechanism that resembles the AID-mediated clearance of alloreactive mature B lymphocytes (Auer *et al.*, 2017).

Such mechanism could be a valid explanation for the increased AID levels in both control and E/R^{+} Ba/F3 due to their persistence within the inflammatory mesenchymal niche. However, if this pro-apoptotic mechanism successfully acted in control cells, it likely failed in ETV6-RUNX1^{+} Ba/F3. At the same time, increased levels of the enzyme could possibly induce off-target mutations in apoptosis-resistant pre-leukemic cells, thus contributing to their malignant transformation. On this regard, it is noteworthy that the few proB ALL tumors developed by AID-competent p19$^{ARF-/-}$Rag1$^{-/-}$ mice displayed significant AID upregulation, indicating the plausibility of this hypothesis (Auer *et al.*, 2017).

Despite inducible ETV6-RUNX1 Ba/F3 can be reasonably considered a valid experimental context for modelling ETV6-RUNX1^{+} pre-leukemia, we were interested in investigating such dynamics also in a human pre-leukemic cellular model. Although the limit of a lentiviral expression and the use of post-natal hematopoietic progenitors, pRRL-ETV6-RUNX1-GFP (pRRL-E/R-GFP) expressing umbilical cord blood (UCB) CD34^{+} cells provided some interesting insights into the biological effects of E/R activity. First of all, we demonstrated that pre-leukemic UCB-CD34^{+} cells migrated more toward inflamed BM-MSC supernatants compared to controls and to unstimulated MSC conditioned media, confirming what observed in Ba/F3 cells. Although preliminary experiments evidenced a tendency to upregulate CXCR2 mRNA in cultivated pRRL-E/R-GFP compared to pRRL-GFP cells (data not shown), we didn't observe an increase in the

CXCR1/2 membrane expression in the general pRRL-E/R-GFP $CD34^+$ population. Human HSPC derived from primary blood samples (bone marrow, fetal blood, peripheral blood and umbilical cord blood) express CXCR1/2 in a very small percentage of cells (2-15%) (Rosu-Myles *et al.*, 2000); thus, it is possible that we lost a potential increase of the CXCR1/2 receptors in a specific subpopulation, especially considering the phenotypical and functional heterogeneity of $CD34^+$ cells cultivated *in vitro*. On this regard, the increased migration toward inflamed BM-MSC supernatants could derived from different chemokine axis acting in different subpopulation. On the other hand, it cannot be excluded that CXCR1/2 signaling could be overstimulated in pre-leukemic cells due to transactivation of downstream effectors mediated by ETV6-RUNX1, independently on the expression of the membrane receptors.

A part the detailed mechanism driving the pre-leukemic HSPC migration toward inflamed BM-MSC, it is noteworthy that these latter specifically preserve ETV6-RUNX1-expressing $CD34^+IL7R^+$ cells, which have been recently individuated as the fetal pre-leukemic population (Böiers *et al.*, 2018), while negatively affecting the normal counterpart. Mechanisms underlaying this effect have to be elucidated but a possible explanation is that a lympho-myelo identity, which characterizes the pre-leukemic $CD34^+IL7R^+$ compartment in the iPS model (Böiers *et al.*, 2018), could be maintained also in our ETV6-$RUNX1^+CD34^+IL7R^+$ cells, thus providing advantages in the context of IL6/IL1β/TNFα-stimulate BM-MSC. On this regard, it would be

intriguingly to investigate if genetic polymorphisms in inflammatory/immune genes that are relevant in our experimental setting (i.e. those regulating production and/or response to IL6/IL1β/TNFα, CXCR1/2 and their ligands, GM-CSF) could have a role in making difference between health and disease in ETV6-RUNX1^{+} carriers. Significant associations of leukemic risk with particular allelic variants has been already observed, for example, for MHC (Taylor *et al.*, 2009), interferon-γ (Krampera *et al.*, 2006), Toll-like receptor 6 (Miedema *et al.*, 2012) genes.

As future perspectives, a transcriptomic analysis of immunophenotypically-sorted subpopulations in ETV6-RUNX1^{+} and control cells will be performed before and after the exposure to the inflamed niche, in order to highlight mechanisms sustaining the pre-leukemia advantage. Targeting such mechanisms could represent a therapeutic strategy to selectively prevent the pre-leukemic clone malignant transformation. If this goal is less feasible in the prospective of prophylactic interventions against ALL diagnosis, it could be important to avoid the pre-leukemic clone-related relapses (Konrad *et al.*, 2003) (Van Delft *et al.*, 2011) (Panzer-Grümayer *et al.*, 2005). On this regard, we will be initially interested in testing the efficacy of CXCR1/2 inhibitors on the ETV6-RUNX1 pre-leukemia to leukemia transition *in vivo*. CXCR1/2 inhibitors have been used in several pre-clinical (Schinke *et al.*, 2015) (Devapatla *et al*., 2015) (Ijichi *et al.*, 2011) and clinical settings (Martz, 2012) (Steele *et al.*, 2016) (Joseph *et al.*, 2017), both against chronic inflammatory diseases and cancer. In our

case, immunocompetent ETV6-RUNX1-transgenic mice will be initially preferred as the involvement of CXCR1/2 receptors in the immune response could significantly impact on the experimental read out. In particular, we will be interested in evaluated if CXCR2 blocking can prevent pre-leukemic cells accumulation within inflamed bone marrow sites and, possibly, their emergence against the normal counterpart (**Fig.4**). Inhibitors will be also tested against ETV6-RUNX1^{+} CD34^{+} cells *in vitro*: migration but also apoptosis in presence of the mesenchymal niche will be evaluated. It has been shown, in fact, that CXCR2 mediate survival and self-renewal in human hematopoietic stem/progenitor cells (Sinclair *et al.*, 2016), while its inhibition decreases the leukemogenic potential of AML stem cells *in vivo* (Schinke *et al.*, 2015). It is possible that, although CXCR2 didn't mediate pro-survival effects in pre-leukemic Ba/F3, it could possess different functions in a very diverse cellular context which is the CD34^{+} multipotent progenitor. If *in vitro* experiments will be positive in this sense, a xenograft mouse model could be considered, even if with the limitation of a compromised immune response.

Material and methods

- **BM-MSC derivation and culture**

Murine bone marrow mesenchymal stromal cells (BM-MSC) were isolated and characterized as previously described (Cappuzzello *et al.*, 2016). A well-established primary line was cultured in DMEM low glucose, 20% tested FBS (Hyclone), 1% L-Glut, 1% P/S and used for experiments between passage 9 (P9) and 11 (P11). Human BM-MSC were isolated from healthy donors-derived BM aspirates, characterized and cultured as previously described (André *et al.*, 2012); cells between passages 3 (P3) and 5 (P5) were used.

- **ETV6-RUNX1-inducible Ba/F3 model**

The mifepristone-inducible GeneSwitch system (Life Technologies) was used to express ETV6-RUNX1 in the IL3-dependent murine pro-B cell line Ba/F3, as previously described (Ford *et al.*, 2009). Briefly, cells were transfected with the pSwitch plasmid expressing a GAL4 regulatory fusion protein (control cells); GAL4-positive clones were then transfected with pGene plasmid carrying the ETV6-RUNX1-V5epitope construct under the GAL4-regulated promoter (ETV6-RUNX1^{+} inducible cells). Propagating cultures were maintained in RPMI Advanced supplemented with 10% FBS, 1% L-Glut, 10ng/ml rm-IL3 (Immunotools), 10mmol/L 2-mercaptoethanol and 0.2 mg/mL

Hygromycin B (Invitrogen). In addition, ETV6-RUNX1^{+} inducible cells were cultured in presence of 0.05 mg/mL Zeocin (Invitrogen). Before performing experiments, both control and ETV6-RUNX1^{+} inducible cells were treated with optimized concentration of mifepristone (Invitrogen) for 72h. Cell viability and efficiency of ETV6-RUNX1 induction were verified by flow cytometry using a FITC-conjugated anti-V5epitope antibody (Abcam). Experiments were performed with cells showing >85% viability and >80% FITC positivity; during experiments, ETV6-RUNX1 expression was maintained adding mifepristone to media at the same induction concentration.

- **Human UCB-CD34^{+} cells isolation and transfection**

Human umbilical cord blood (UCB) derived CD34^{+} cells were obtained from volunteer mothers receiving informed consent and in accordance with ethical standards of the San Gerardo Hospital (Monza) ethical committee. Briefly, UCB-MNC were separated by density gradient centrifugation through Ficoll-Paque PLUS (GE Healthcare) within 24 hours from collection. Immunomagnetic on column separation was performed using the CD34 MicroBead Kit (MACS Miltenyi Biotec) and following the manufacturer's protocol. The previously described ETV6-RUNX1 myc-tag fusion construct (Tsuzuki *et al.*, 2004) was subcloned into the PmeI site of pRRL-EF1α-PGK-GFP dual-promoter plasmid. Vesicular stomatitis virus-G-pseudotyped viral particles were generated on 293T cells by polyethylenimine (PEI) transfection and

concentrated by ultracentrifugation. In details, 293T cells were plated into 6-well plates ($4x10^6$) in DMEM high glucose supplemented with pyruvate (BioWest) and added with 10% tested FBS (HyClone), 1%L-Glut, 1% P/S, 1% non-essential amminoacids (SIGMA) until reaching about 80% confluence after 24h. Transfection mixes were prepared as follows: 500μL DMEM high glucose, 4μg psPAX2 packaging vector, 2μg VSV-G envelope vector, 8μg pRRL-GFP (empty vector, EV) or pRRL-ETV6-RUNX1-GFP (ER), 35μg 25kDa linear PEI (Polysciences). After 48h and 72h, cell supernatants were collected, 0.45μm filtered and ultracentrifuged 26.000rpm, 2h30' at +4°C. UCB-CD34+ cells ($2x10^6$) were infected for 72h with concentrated viruses in StemSpam SFEM-II (StemCell) supplemented with SCF (100 ng/mL), FLT3 ligand (100 ng/mL), and IL-3 (10 ng/mL) (all from PeproTech) and polybrene (μ1g/mL; Sigma-Aldrich). After 72h, GFP^+ cells within EV and E/R populations were sorted (purity >95%) and maintained in stem culture medium (StemSpam SFEM-II (StemCell) supplemented with SCF (100 ng/mL), FLT3-ligand (100 ng/mL), IL-3 (10 ng/mL), IL6 (20ng/mL) and TPO (50ng/mL), all from Peprotech) for downstream experiments.

- **Ba/F3 gene expression profile**

Gene Expression Profile (GEP) of control and ETV6-$RUNX1^+$ Ba/F3 was performed on total RNA samples of four independent experiments of ETV6-RUNX1 induction. After 72h of mifepristone treatment, cells were checked for vitality (>90%) and ETV6-RUNX1 expression by

flowcytometry (>90%) as described above. Total RNA was extracted by using RNeasy Mini Kit (QIAGEN) following manufacturer's instructions. After having assessed the RNA quality by 2100 Agilent Bioanalyzer, RNA samples were used for microarray experiments. Samples were in vitro transcripted and biotin labeled according to Affymetrix 3' IVT Plus protocol and hybridized in Gene Chip Mouse 2.0 Arrays (Thermofisher). Microarray data (.CEL files) were generated using default Affymetrix microarray analysis parameters (Command Console suite software, Affymetrix). CEL files were normalized using the robust multiarray averaging expression measure of Affy-R package (www.bioconductor.org). Differentially expressed genes were identified using Significance Analysis of Microarray algorithm (SAM) coded in the *samr* R package (Tusher et al., 2001). In SAM, we estimated the percentage of false positive predictions (i.e., False Discovery Rate, FDR) with 100 permutations. Gene ontology analysis were performed using Ingenuity Pathway Analysis (IPA, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) by expressing raw data in linear fold change and Metascape (http://metascape.org). Enriched terms, hypergeometric p-value and enrichment factors were calculated and used for filtering from the Software. Remaining significant terms were then hierarchically clustered in a tree based on kappa-statistical similarities among their gene membership. Then 0.3 kappa score was applied as the threshold to cast the tree into the term clusters. In the dendogram the 20 best *p-value* were used in the representation. Heatmap cells

were colored according to their p-value. Grey cells indicate the lack of enrichment for that term in the corresponding gene list.

- **Murine CXCR1/CXCR2 immunostaining**

Surface expression of CXCR1/CXCR2 in Ba/F3 cells was evaluated by staining cells with PE-conjugated anti-CXCR2 (BioLegend) and anti-CXCR1 (R&D) for 30' at +4°C. Cells were then acquired through BD Bioscience FACSCanto-II and data analyzed by FACSDiva software (BD Bioscience).

- **Migration assay**

Control and ETV6-RUNX1^{+} Ba/F3 cells ($3x10^{5}$) were resuspended into 100µL of migration medium (Advanced RMPI, 2% FBS, 1% L-glutamine, mifepristone) and loaded into the upper chamber of 8.0µm Transwells© (Corning). Recombinant mCXCL1 (100ng/mL) (Peprotech) was added to migration medium (600uL) in the lower chamber. Murine BM-MSC were maintained in migration medium for 48h, in presence or not of rmIL6 (20ng/mL), rmIL1β (25ng/mL) and rmTNFα (25ng/mL) (Immunotools) and conditioned media (MSC-CM) collected, centrifuged 1260rcf for 10' and frozen at -80°C. SB-265610 1µM (Calbiochem) was used for the specific inhibition of CXCR2; in this case, cells were pretreated for 10' at 37°C prior loading into Transwells®; inhibitor was added also into the upper and lower chambers. After 3 hours of migration at 37°C, 20% O_2, 5% CO_2, Transwells® were removed

and migrated cells recovered. BD Calibration Beads were added into FACS tube prior counting cells at the FACSAria cytometer. Cells were counted in technical triplicates for 30". Number of migrated cells were normalized on counted beads and percentage calculated on cells loaded into the upper chamber (input). For $CD34^+$ cells migration assay, $7x10^4$ pRRL-GFP or pRLL-E/R-GFP $CD34^+$ cells were resuspended into 100 uL of migration medium (RPMI Advanced, 2% FBS, 1% L-Glut, 1% P/S) and loaded into the upper chamber of 5.0µm Transwells© (Corning). Human BM-MSC were maintained in migration medium for 48h, in presence or not of rhIL6 (40ng/mL), rhIL1β (50ng/mL) and rhTNFα (100ng/mL) (Immunotools); conditioned media (MSC-CM) were collected, centrifuged 1260rcf for 10' and frozen at -80°C. After 1 hour of migration at 37°C, 20% O_2, 5% CO_2, Transwells® were removed and migrated cells recovered. BD Calibration Beads were added into FACS tube prior counting cells at the FACSAria cytometer. Cells were counted in technical triplicates for 30". Number of migrated cells were normalized on counted beads and percentage calculated on cells loaded into the upper chamber (input). Data shown are the mediated results of a unique infection experiment in which cells were tested against the supernatants of three healthy donor derived-MSC.

- **Competitive mesenchymal niche model**

Control and ETV6-$RUNX1^+$ Ba/F3 were mixed ($2.5x10^4$ total cells) at a starting ratio of 20%:80% and plated directly on MSC or into 0.4µm

Transwells® (Corning) upon MSC. Inflammatory conditions were reproduced by adding rmIL6 (20ng/mL), rmIL1β (25ng/mL) and rmTNFα (25ng/mL) (Immunotools). As internal experimental controls, Ba/F3 were plated alone in absence (basal condition) or presence of rhTGFβ1 (10ng/mL) (R&D System). After 4 days of cultures, cells were harvested, kindly syringed with a 18G needle and stained with Horizon Fixable Viability Stain 450 (BD Bioscience) and the PE-conjugated anti-mCD45 antibody. Cells were then fixed and permeabilized (BD Bioscience) and stained with the FITC-conjugated anti-v5tag antibody. Percentage of $FITC^+$ cells was evaluated gating on $Horizon480^-$ /$mCD45^+$ cells. Stained cells were acquired through BD Bioscience FACSCanto-II and data analyzed by FACSDiva software (BD Bioscience).

- **Transfected UCB-$CD34^+$ and BM-MSC co-cultures**

Sorted pRRL-GFP and pRLL-E/R-GFP $CD34^+$ cells ($7x10^4$) were resuspended in stem culture medium and plated on BM-MSC monolayers (80% confluence) in presence or absence of rhIL6 (40ng/mL), rhIL1β (50ng/mL) and rhTNFα (100ng/mL) (Immunotools) for 72h. At the end of the co-culture, cells were collected and trypsinized to recover every $CD34^+$ adherent to BM-MSC. $CD34^+$ cells and BM-MSC were further separated by accurately pipetting in cold PBS. Cells were than stained for 30’ at +4°C with APC-conjugated anti-human CD34 (BD Biosciences) and Pe-Cy7-conjugated anti-human CD127 (IL7R) (BioLegend) antibodies. Stained cells were acquired

through BD Bioscience Fortessa and data analyzed by FACSDiva software (BD Bioscience). BD Calibration Beads were added into FACS tube prior counting cells at the FACSAria cytometer. Cells were counted in technical duplicates for 60". Number of cells were normalized on counted beads. Data shown are the mediated results of a unique infection experiment in which cells were tested against the supernatants of three healthy donor derived BM-MSC.

- **Cell cycle analysis**

Control and ETV6-RUNX1^{+} Ba/F3 ($0.5x10^{6}$) were stained with 2.5uM CSFE in 500ul PBS for 10' in the dark, while constantly shaking. Staining was blocked by incubating cells for 3' in cold FBS. After washing in RPMI 10% FBS, cells were incubated for additional 5' in RPMI 10% FBS and counted; $2.5x10^{4}$ stained cells were mixed (20%ctr:80%EV^{+}) and seeded into 6-well plates alone (basal condition) or upon MSC in presence or absence of inflammatory cytokines for 4 days. At the end of the culture, cells were harvested, kindly syringed with a 18G needle and stained with the PE anti-mCD45 antibody. Cells were then fixed/permeabilized (BD Bioscience) and stained with the primary anti-ETV6-RUNX1 fusion antibody (clone 6F2) (Millipore) in association with the secondary BD Horizon BV421 goat anti-rat IgG (BD Bioscience) in place of the FITC-conjugated anti-V5 antibody. CSFE MFI of control Ba/F3 in basal condition was considered as referrer value for fold increase calculations.

- **Apoptosis assay**

Control and ETV6-RUNX1^{+} Ba/F3 ($2.5x10^{4}$) were seeded into 6-well plates alone (basal condition) and upon MSC in presence or absence of inflammatory cytokines for 4 days. At the end of the culture, cells were harvested, kindly syringed with a 18G needle and stained with BV421-conjugated anti-murine CD45 antibody, prior being stained with GFP-certified PE-conjugated Annexin-V (Enzo). For each condition, percentage of BV451^{+}/PE^{-} cells was evaluated.

- **γH2AX staining**

Control and ETV6-RUNX1^{+} Ba/F3 ($2.5x10^{4}$) were seeded into 6-well plates alone (basal condition) and upon MSC in presence or absence of inflammatory cytokines for 4 days. At the end of the culture, cells were harvested, kindly syringed with a 18G needle and stained (0.5-$1x10^{6}$) with Horizon Fixable Viability Stain 450 (BD Bioscience) and fixed 10' at 37°C in 4% formaldehyde. Cells were chilled 1' on ice prior being permeabilized in 90% methanol. After 30' incubation on ice, cells were firstly stained with PE anti-CD45, then with Alexa Fluor® 647-conjugated anti-Phospho-Histone H2A.X Ser139 (20E3) (Cell Signalling) and FITC anti-V5. Alexa Fluor® 647-conjugate Rabbit (DA1E) mAb IgG XP® was used as isotype control (Cell Signalling).

- **Quantitative Reverse Transcription PCR (qRT-PCR)**

Real-time quantification of gene transcripts was performed on the Light Cycler 480II instrument using the Universal Probe Master System (Roche Diagnostics). Optimal primers and probe for cDNA amplification were selected by the Roche ProbeFinder software (https://www.roche-appliedscience.com/sis/rtpcr/upl). Data were expressed using the comparative 2_DDCt method (Csanaky G, Matutes E, Vass JA, Morilla R, Catovsky D. Leukemia 1997) using *hprt* as reference gene. Transcript level in pre-leukemic cells were always referred to those of control cells.

- **Enzyme-linked immunosorbent assays (ELISA) and protein arrays**

Supernatants of BM-MSC and Ba/F3 cells, treated or not with pro-inflammatory cytokines, were collected, centrifuged 10' at 1260rcf and immediately frozen at -80°C. Murine KC and murine TGFβ DuoSet Enzyme-Linked Immunosorbent Assays (R&D Systems) were used following the manufacturer's protocols and plates acquired through TECAN GENios® instrument. Human Cytokines Array C1000 (RayBio) was used following manufacturer's protocol and data acquired through UVITEC Cambridge® instrument; densitometry analysis was performed with ImageJ® software.

- **Statistical Analysis**

Student's *t*-test with $p<0.05$ was used to defined results statistically significant. Where indicated, one-sample *t*-test was used with the same significance threshold. Raw data were elaborated with Microsoft Excel or GraphPad programs.

References

Abarrategi, A. *et al.* (2017) 'Versatile humanized niche model for studying human normal and malignant haematopoieisis', *Journal of Clinical Investigation*, 127(2), p. 543-. doi: 10.1172/JCI89364.

Abdelhaleem, M. (2007) 'Frequent but nonrandom expression of lymphoid markers on de novo childhood acute myeloid leukemia', *Experimental and Molecular Pathology*. doi: 10.1016/j.yexmp.2007.05.007.

Alpar, D. *et al.* (2015) 'Clonal origins of ETV6-RUNX1 + acute lymphoblastic leukemia: Studies in monozygotic twins', *Leukemia*. Nature Publishing Group, 29(4), pp. 839–846. doi: 10.1038/leu.2014.322.

André, V. *et al.* (2012) 'Mesenchymal stem cells from Shwachman-Diamond syndrome patients display normal functions and do not contribute to hematological defects', *Blood Cancer Journal*. doi: 10.1038/bcj.2012.40.

Andreasson, P. *et al.* (2001) 'The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo', *Cancer Genetics*, 130(2).

Asou, N. (2003) 'The role of a Runt domain transcription factor AML1/RUNX1 in leukemogenesis and its clinical implications', *Critical Reviews in Oncology/Hematology*. doi: 10.1016/S1040-8428(02)00003-3.

Auer, F. *et al.* (2017) 'Activation-induced cytidine deaminase prevents pro-B cell acute lymphoblastic leukemia by functioning as a negative regulator in Rag1 deficient pro-B cells', *Oncotarget*. doi: 10.18632/oncotarget.20563.

Balandán, J. C. *et al.* (2017) 'Pro-inflammatory-related loss of CXCL12 niche promotes acute lymphoblastic leukemic progression at the expense of normal lymphopoiesis', *Frontiers in Immunology*, 7(JAN), pp. 1–14. doi: 10.3389/fimmu.2016.00666.

Bateman, C. M. *et al.* (2015) 'Evolutionary trajectories of hyperdiploid ALL in monozygotic twins', *Leukemia*. doi: 10.1038/leu.2014.177.

Bernardin, F. *et al.* (2002) 'Advances in Brief TEL-AML1 , Expressed from t (12 ; 21) in Human Acute Lymphocytic Leukemia , Induces Acute Leukemia in Mice 1', pp. 3904–3908.

Bernardo, M. E. and Fibbe, W. E. (2013) 'Mesenchymal stromal cells: Sensors and switchers of inflammation', *Cell Stem Cell*. Elsevier Inc., 13(4), pp. 392–402. doi:

10.1016/j.stem.2013.09.006.

Binato, R. *et al.* (2015) 'The molecular signature of AML mesenchymal stromal cells reveals candidate genes related to the leukemogenic process', *Cancer Letters*. Elsevier Ireland Ltd, 369(1), pp. 134–143. doi: 10.1016/j.canlet.2015.08.006.

Le Blanc, K. *et al.* (2008) 'Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study', *The Lancet*. doi: 10.1016/S0140-6736(08)60690-X.

Le Blanc, K. and Mougiakakos, D. (2012) 'Multipotent mesenchymal stromal cells and the innate immune system', *Nature Reviews Immunology*. doi: 10.1038/nri3209.

Blank, U. and Karlsson, S. (2015) 'TGF-β signaling in the control of hematopoietic stem cells', *Blood*. doi: 10.1182/blood-2014-12-618090.

Bohlander, S. K. (2005) 'ETV6: A versatile player in leukemogenesis', *Seminars in Cancer Biology*. doi: 10.1016/j.semcancer.2005.01.008.

Böiers, C. *et al.* (2013) 'Lymphomyeloid Contribution of an Immune-Restricted Progenitor Emerging Prior to Definitive Hematopoietic Stem Cells', *Cell Stem Cell*. doi: 10.1016/j.stem.2013.08.012.

Böiers, C. *et al.* (2018) 'A Human IPS Model Implicates Embryonic B-Myeloid Fate Restriction as Developmental Susceptibility to B Acute Lymphoblastic Leukemia-Associated ETV6-RUNX1', *Developmental Cell*, 44(3), p. 362–377.e7. doi: 10.1016/j.devcel.2017.12.005.

Boily, G. *et al.* (2007) 'Identification of transcripts modulated by ETV6 expression', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.2006.06377.x.

Brandau, S. *et al.* (2014) 'Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes', *PLoS ONE*. doi: 10.1371/journal.pone.0106903.

Calvi, L. M. *et al.* (2003) 'Osteoblastic cells regulate the haematopoietic stem cell niche', *Nature*. doi: 10.1038/nature02040.

Cappuzzello, C. *et al.* (2016) 'Mesenchymal stromal cell-derived PTX3 promotes wound healing via fibrin remodeling', *Journal of Investigative Dermatology*. doi: 10.1038/JID.2015.346.

Carey, A. *et al.* (2017) 'Identification of Interleukin-1 by Functional Screening as a Key Mediator of Cellular Expansion and Disease Progression in Acute Myeloid Leukemia', *Cell*

Reports. doi: 10.1016/j.celrep.2017.03.018.

Cazzaniga, G. *et al.* (2017) 'Possible role of pandemic AH1N1 swine flu virus in a childhood leukemia cluster', *Leukemia*, 31(8), pp. 1819–1821. doi: 10.1038/leu.2017.127.

Lo Celso, C. and Scadden, D. T. (2011) 'The haematopoietic stem cell niche at a glance', *Journal of Cell Science*, 124(21), pp. 3529–3535. doi: 10.1242/jcs.074112.

Colmone, A. *et al.* (2008) 'Normal Hematopoietic Progenitor Cells', *Science,* 322(December), pp. 1861–1865.

Corces-Zimmerman, M. R. and Majeti, R. (2014) 'Pre-leukemic evolution of hematopoietic stem cells: The importance of early mutations in leukemogenesis', *Leukemia*. Nature Publishing Group, 28(12), pp. 2276–2282. doi: 10.1038/leu.2014.211.

Corcione, A. *et al.* (2006) 'Human mesenchymal stem cells modulate B-cell functions', *Blood*. doi: 10.1182/blood-2005-07-2657.

Crisan, M. *et al.* (2008) 'A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs', *Cell Stem Cell*, 3(3), pp. 301–313. doi: 10.1016/j.stem.2008.07.003.

Van Delft, F. W. *et al.* (2011a) 'Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia', *Blood*, 117(23), pp. 6247–6254. doi: 10.1182/blood-2010-10-314674.

Van Delft, F. W. *et al.* (2011b) 'Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia', *Blood*. doi: 10.1182/blood-2010-10-314674.

Devapatla, B., Sharma, A. and Woo, S. (2015) 'CXCR2 inhibition combined with sorafenib improved antitumor and antiangiogenic response in preclinical models of ovarian cancer', *PLoS ONE*, 10(9), pp. 1–16. doi: 10.1371/journal.pone.0139237.

Diakos, C. *et al.* (2007) 'RNAi-mediated silencing of TEL/AML1 reveals a heat-shock protein- and survivin-dependent mechanism for survival', *Blood*. doi: 10.1182/blood-2006-04-019612.

Dockerty, J. D. *et al.* (2001) 'Case-control study of parental age, parity and socioeconomic level in relation to childhood cancers', *International Journal of Epidemiology*. doi: 10.1093/ije/30.6.1428.

Dominici, M. *et al.* (2006) 'Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement', *Cytotherapy*, 8(4), pp. 315–317. doi: 10.1080/14653240600855905.

Duijvestein, M. *et al.* (2010) 'Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: Results of a phase I study', *Gut*. doi: 10.1136/gut.2010.215152.

Eggenhofer, E. and Hoogduijn, M. J. (2012) 'Mesenchymal stem cell-educated macrophages', *Transplantation Research*. doi: 10.1186/2047-1440-1-12.

Eguchi-Ishimae, M. *et al.* (2001) 'Breakage and fusion of the TEL (ETV6) gene immature B lymphocytes induced by apoptogenic signals', *Blood*, 97(3), pp. 737–743. doi: 10.1182/blood.V97.3.737.

Enciso, J. *et al.* (2016) 'Modeling the pro-inflammatory tumor microenvironment in acute lymphoblastic leukemia predicts a breakdown of hematopoietic-mesenchymal communication networks', *Frontiers in Physiology*. doi: 10.3389/fphys.2016.00349.

Fan, X. *et al.* (2007) 'Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M607705200.

Ford, a M. *et al.* (1998) 'Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(8), pp. 4584–4588. doi: 10.1073/pnas.95.8.4584.

Ford, A. M. *et al.* (2001) 'Origins of "late" relapse in childhood acute lymphoblastic leukemia with TEL-AML1 fusion genes.', *Blood*, 98(3), pp. 558–64. doi: 10.1182/blood.V98.3.558.

Ford, A. M. *et al.* (2009) 'The TEL-AML1 leukemai fusion gene dysregulates the TGF-beta pathway in early B lineage progenitor cells', *J. Clin. Invest.*, 119(4). doi: 10.1172/jci36428ds1.

Forsberg, E. C. and Smith-Berdan, S. (2009) 'Parsing the niche code: The molecular mechanisms governing hematopoietic stem cell adhesion and differentiation', *Haematologica*, 94(11), pp. 1477–1481. doi: 10.3324/haematol.2009.013730.

Francis, S. S. *et al.* (2012) 'Unusual space-time patterning of the Fallon, Nevada leukemia cluster: Evidence of an infectious etiology', *Chemico-Biological Interactions*. doi: 10.1016/j.cbi.2011.02.019.

Fuka, G. *et al.* (2011) 'The leukemia-specific fusion gene etv6/runx1 perturbs distinct key biological functions primarily by gene repression', *PLoS ONE*. doi: 10.1371/journal.pone.0026348.

Gale, K. B. *et al.* (1997) 'Backtracking leukemia to birth: identification of clonotypic gene fusion

sequences in neonatal blood spots.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 94(25), pp. 13950–4. doi: 10.1073/PNAS.94.25.13950.

Gandemer, V. *et al.* (2007) 'Five distinct biological processes and 14 differentially expressed genes characterize TEL/AML1-positive leukemia', *BMC Genomics*, 8, pp. 1–15. doi: 10.1186/1471-2164-8-385.

Gerr, H. *et al.* (2010) 'Acute leukaemias of ambiguous lineage in children: Characterization, prognosis and therapy recommendations', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.2009.08058.x.

Gilham, C. *et al.* (2005) 'Day care in infancy and risk of childhood acute lymphoblastic leukaemia: Findings from UK case-control study', *British Medical Journal*. doi: 10.1136/bmj.38428.521042.8F.

Golub, T. R. *et al.* (1995) 'Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia.', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.92.11.4917.

Grassinger, J. *et al.* (2010) 'Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential', *Blood*. doi: 10.1182/blood-2009-12-260703.

Greaves, M. (2002) 'Childhood leukemia', *Bmj*, 324, pp. 283–287.

Greaves, M. (2006) 'Infection, immune responses and the aetiology of childhood leukaemia', *Nature Reviews Cancer*, 6(3), pp. 193–203. doi: 10.1038/nrc1816.

Greaves, M. (2018) 'A causal mechanism for childhood acute lymphoblastic leukaemia', *Nature Reviews Cancer*. Springer US, 18(8), pp. 471–484. doi: 10.1038/s41568-018-0015-6.

Greaves, M. F. *et al.* (2003) 'Leukemia in twins: Lessons in natural history', *Blood*. doi: 10.1182/blood-2002-12-3817.

Greaves, M. F. and Wiemels, J. (2003) 'Origins of chromosome translocations in childhood leukaemia', *Nature Reviews Cancer*, 3(9), pp. 639–649. doi: 10.1038/nrc1164.

Greenbaum, A. *et al.* (2013) 'CXCL12 Production by Early Mesenchymal Progenitors is Required for Hematopoietic Stem Cell Maintenance', *Nature*, 495(7440), pp. 227–230. doi: 10.1038/nature11926.CXCL12.

Gunji, H. *et al.* (2004) 'TEL/AML1 shows dominant-negative effects over TEL as well as AML1',

Biochemical and Biophysical Research Communications. doi: 10.1016/j.bbrc.2004.07.169.

Ha, H., Debnath, B. and Neamati, N. (2017) 'Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases', *Theranostics*, 7(6), pp. 1543–1588. doi: 10.7150/thno.15625.

Heath, C. W. and Hasterlik, R. J. (1963) 'Leukemia among children in a suburban community', *The American Journal of Medicine*. doi: 10.1016/0002-9343(63)90088-3.

Von Der Heide, E. K. *et al.* (2017) 'Molecular alterations in bone marrow mesenchymal stromal cells derived from acute myeloid leukemia patients', *Leukemia*, 31(5), pp. 1069–1078. doi: 10.1038/leu.2016.324.

Hol, J., Wilhelmsen, L. and Haraldsen, G. (2010) 'The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies', *Journal of Leukocyte Biology*. doi: 10.1189/jlb.0809532.

Hong, D. *et al.* (2008) 'Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia', *Science*, 319(5861), pp. 336–339. doi: 10.1126/science.1150648.

Ichikawa, M. *et al.* (2004) 'Runx1/AML-1 ranks as a master regulator of adult hematopoiesis', *Cell Cycle*. doi: 10.4161/cc.3.6.951.

Ijichi, H. *et al.* (2011) 'Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma', *Journal of Clinical Investigation*, 121(10), pp. 4106–4117. doi: 10.1172/JCI42754.

Jaffer, T. and Ma, D. (2016) 'The emerging role of chemokine receptor CXCR2 in cancer progression', *Translational Cancer Research*, 5(S4), pp. S616–S628. doi: 10.21037/tcr.2016.10.06.

Joseph, J. P. *et al.* (2017) 'CXCR2 Inhibition - a novel approach to treating CoronAry heart DiseAse (CICADA): Study protocol for a randomised controlled trial', *Trials*. Trials, 18(1), pp. 1–11. doi: 10.1186/s13063-017-2210-2.

Kaindl, U. *et al.* (2014) 'Blocking ETV6/RUNX1-induced MDM2 overexpression by Nutlin-3 reactivates p53 signaling in childhood leukemia', *Leukemia*. doi: 10.1038/leu.2013.345.

Kantner, H.-P. *et al.* (2013) 'ETV6/RUNX1 Induces Reactive Oxygen Species and Drives the Accumulation of DNA Damage in B Cells', *Neoplasia*. doi: 10.1593/neo.131310.

Kawamata, N. *et al.* (2008) 'Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray', *Blood*. doi: 10.1182/blood-2007-05-088310.

Keating, A. (2012) 'Mesenchymal stromal cells: New directions', *Cell Stem Cell*. doi: 10.1016/j.stem.2012.05.015.

Kfoury, Y. and Scadden, D. T. (2015) 'Mesenchymal cell contributions to the stem cell niche', *Cell Stem Cell*. Elsevier Inc., 16(3), pp. 239–253. doi: 10.1016/j.stem.2015.02.019.

Kinlen, L. J., Clarke, K. and Hudson, C. (1990) 'Evidence from population mixing in British New Towns 1946-85 of an infective basis for childhood leukaemia', *The Lancet*. doi: 10.1016/0140-6736(90)93389-7.

Kohara, H. *et al.* (2007) 'Development of plasmacytoid dendritic cells in bone marrow stromal cell niches requires CXCL12-CXCR4 chemokine signaling', *Blood*. doi: 10.1182/blood-2007-04-084210.

Konrad, M. *et al.* (2003a) 'Late relapses evolve from slow-responding subclones acute lymphoblastic leukemia : for the persistence evidence of a preleukemic clone', *Blood,* 101, pp. 3635–3640. doi: 10.1182/blood-2002-10-3252.

Kornblau, S. M. *et al.* (2018) 'Distinct protein signatures of acute myeloid leukemia bone marrow-derived stromal cells are prognostic for patient survival', *Haematologica*, 103(5), pp. 810–821. doi: 10.3324/haematol.2017.172429.

Krampera, M. *et al.* (2006) 'Role for Interferon-γ in the Immunomodulatory Activity of Human Bone Marrow Mesenchymal Stem Cells', *Stem Cells*. doi: 10.1634/stemcells.2005-0008.

Kuster, L. *et al.* (2018) 'ETV6_RUNX1-positive relapses evolve from an ancestral clone and frequently acquire deletions of genes implicated in glucocorticoid signaling', 117(9), pp. 2658–2668. doi: 10.1182/blood-2010-03-275347.The.

Kyurkchiev, D. (2014) 'Secretion of immunoregulatory cytokines by mesenchymal stem cells', *World Journal of Stem Cells*. doi: 10.4252/wjsc.v6.i5.552.

Larson, R.A; Anastasi, J. (2008) *Acute Lymphoblastic Leukemia: clinical presentation, diagnosis and classification.* Edited by Springer. Berlin.

De Laurentiis, A., Hiscott, J. and Alcalay, M. (2015) 'The TEL-AML1 fusion protein of acute lymphoblastic leukemia modulates IRF3 activity during early B-cell differentiation', *Oncogene*. Nature Publishing Group, 34(49), pp. 6018–6028. doi:

10.1038/onc.2015.50.

Levanon, D. *et al.* (2001) 'Architecture and anatomy of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1', *Gene*. doi: 10.1016/S0378-1119(00)00532-1.

Levasseur, M. *et al.* (1994) 'Relapse of acute lymphoblastic leukaemia 14 years after presentation: Use of molecular techniques to confirm true re-emergence', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.1994.tb04942.x.

Linka, Y. *et al.* (2013) 'The impact of TEL-AML1 (ETV6-RUNX1) expression in precursor B cells and implications for leukaemia using three different genome-wide screening methods', *Blood Cancer Journal*. doi: 10.1038/bcj.2013.48.

Liu, Q. *et al.* (2016) 'The CXCL8-CXCR1/2 pathways in cancer', *Cytokine and Growth Factor Reviews*. Elsevier Ltd, 31, pp. 61–71. doi: 10.1016/j.cytogfr.2016.08.002.

Lopez, R. G. *et al.* (1999) 'TEL is a sequence-specific transcriptional repressor', *Journal of Biological Chemistry*. doi: 10.1074/jbc.274.42.30132.

Ma, X. *et al.* (2005) 'Vaccination history and risk of childhood leukaemia', *International Journal of Epidemiology*. doi: 10.1093/ije/dyi113.

Mangolini, M. *et al.* (2013) 'STAT3 mediates oncogenic addiction to TEL-AML1 in t(12;21) acute lymphoblastic leukemia', *Blood*. doi: 10.1182/blood-2012-11-465252.

Marcotte, E. L. *et al.* (2016) 'Caesarean delivery and risk of childhood leukaemia: A pooled analysis from the Childhood Leukemia International Consortium (CLIC)', *The Lancet Haematology*. doi: 10.1016/S2352-3026(16)00002-8.

Martz, L. (2012) 'CXCR2 antagonists in breast cancer', *Science-Business eXchange*, 5(31), pp. 1–2. doi: 10.1038/scibx.2012.807.

Matthews, A. G. W. *et al.* (2007) 'RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination', *Nature*. doi: 10.1038/nature06431.

Melief, S. M. *et al.* (2013) 'Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages', *Stem Cells*. doi: 10.1002/stem.1432.

Méndez-Ferrer, S. *et al.* (2010) 'Mesenchymal and haematopoietic stem cells form a unique bone marrow niche', *Nature*, 466(7308), pp. 829–834. doi: 10.1038/nature09262.

Miedema, K. G. E. *et al.* (2012) 'Polymorphisms in the TLR6 gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease', *Leukemia*. doi: 10.1038/leu.2011.341.

Mijušković, M. *et al.* (2015) 'Off-Target V(D)J Recombination Drives Lymphomagenesis and Is Escalated by Loss of the Rag2 C Terminus', *Cell Reports*. doi: 10.1016/j.celrep.2015.08.034.

Mo, I. F. Y. *et al.* (2008) 'Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors', *BMC Cell Biology*. doi: 10.1186/1471-2121-9-52.

Mori, H. *et al.* (2002) 'Chromosome translocations and covert leukemic clones are generated during normal fetal development', *Proceedings of the National Academy of Sciences*, 99(12), pp. 8242–8247. doi: 10.1073/pnas.112218799.

Morrow, M. *et al.* (2004) 'TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity', *Blood*, 103(10), pp. 3890–3896. doi: 10.1182/blood-2003-10-3695.

Morrow, M. *et al.* (2007) 'TEL-AML1 preleukemic activity requires the DNA binding domain of AML1 and the dimerization and corepressor binding domains of TEL', *Oncogene*, 26(30), pp. 4404–4414. doi: 10.1038/sj.onc.1210227.

Motoda, L. *et al.* (2007) '*Runx1* Protects Hematopoietic Stem/Progenitor Cells from Oncogenic Insult', *Stem Cells*. doi: 10.1634/stemcells.2007-0061.

Mullighan, C. G. *et al.* (2008) 'Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia', *Science*. doi: 10.1126/science.1164266.

Mullighan, C. G. (2013) 'Genomic characterization of childhood acute lymphoblastic leukemia', *Semin Hematol*, 50(4), pp. 314–324. doi: 10.1053/j.seminhematol.2013.10.001.

Muramatsu, M. *et al.* (2000) 'Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme', *Cell*. doi: 10.1016/S0092-8674(00)00078-7.

Naderi, E. H. *et al.* (2015) 'Bone marrow stroma-derived PGE2 protects BCP-ALL cells from DNA damage-induced p53 accumulation and cell death', *Molecular Cancer*, 14(1). doi: 10.1186/s12943-014-0278-9.

Nauta, A. J. *et al.* (2012) 'Immunomodulatory properties of mesenchymal stromal cells Review in translational hematology Immunomodulatory properties of mesenchymal stromal

cells', 110(10), pp. 3499–3506. doi: 10.1182/blood-2007-02-069716.

Lo Nigro, L. *et al.* (1999) 'Clonal stability in children with acute lymphoblastic leukemia (ALL) who relapsed five or more years after diagnosis', *Leukemia*.

Noda, M. *et al.* (2011) 'CXCL12-CXCR4 chemokine signaling is essential for NK-cell development in adult mice', *Blood*. doi: 10.1182/blood-2010-04-277897.

Oettinger, M. A. *et al.* (1990) 'RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination', *Science*. doi: 10.1126/science.2360047.

Okuda, T. *et al.* (1996) 'AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis', *Cell*. doi: 10.1016/S0092-8674(00)80986-1.

Orkin, S. H. and Zon, L. I. (2008) 'Hematopoiesis: An Evolving Paradigm for Stem Cell Biology', *Cell*, 132(4), pp. 631–644. doi: 10.1016/j.cell.2008.01.025.

Paashuis-Lew, Y. R. and Heddle, J. A. (1998) 'Spontaneous mutation during fetal development and post-natal growth', *Mutagenesis*, 13(6), pp. 613–617.

Palmi, C. *et al.* (2014) 'Cytoskeletal Regulatory Gene Expression and Migratory Properties of B-cell Progenitors Are Affected by the ETV6-RUNX1 Rearrangement.', *Molecular cancer research : MCR*, 12(12), pp. 1796–806. doi: 10.1158/1541-7786.MCR-14-0056-T.

Panzer-Grümayer, E. R. *et al.* (2005) 'Immunogenotype changes prevail in relapses of young children with TEL-AML1-positive acute lymphoblastic leukemia and derive mainly from clonal selection', *Clinical Cancer Research*, 11(21), pp. 7720–7727. doi: 10.1158/1078-0432.CCR-05-1239.

Papaemmanuil, E. *et al.* (2014) 'RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia', *Nature Genetics*. Nature Publishing Group, 46(2), pp. 116–125. doi: 10.1038/ng.2874.

Parker, W. and Ollerton, J. (2013) 'Evolutionary biology and anthropology suggest biome reconstitution as a necessary approach toward dealing with immune disorders', *Evolution, Medicine, and Public Health*. doi: 10.1093/emph/eot008.

Penders, J. *et al.* (2006) 'Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy', *PEDIATRICS*. doi: 10.1542/peds.2005-2824.

Pietras, E. M. (2017) 'Inflammation: A key regulator of hematopoietic stem cell fate in health and disease', *Blood*, 130(15), pp. 1693–1698. doi: 10.1182/blood-2017-06-780882.

Poirel, H. *et al.* (1997) 'The TEL gene products: Nuclear phosphoproteins with DNA binding properties', *Oncogene*. doi: 10.1038/sj.onc.1200829.

Poynton, F. J., Thursfield, H. & Paterson, D. (1922) 'The severe blood diseases of childhood: a series of observations from the Hospital for Sick Children, Great Ormond Street.', *Br. J. Child. Dis.*, XIX.

Polak, R. *et al.* (2015) 'B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment', *Blood*, 126(21), pp. 2404–2414. doi: http://dx.doi.org/10.1182/blood-2015-03-634238.

Raaijmakers, M. H. G. P. *et al.* (2010) 'Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia', *Nature*. Nature Publishing Group, 464(7290), pp. 852–857. doi: 10.1038/nature08851.

Raghuwanshi, S. K. *et al.* (2012) 'The Chemokine Receptors CXCR1 and CXCR2 Couple to Distinct G Protein-Coupled Receptor Kinases To Mediate and Regulate Leukocyte Functions', *The Journal of Immunology*. doi: 10.4049/jimmunol.1201114.

Reikvam, H. *et al.* (2015) 'The cytokine-mediated crosstalk between primary human acute myeloid cells and mesenchymal stem cells alters the local cytokine network and the global gene expression profile of the mesenchymal cells', *Stem Cell Research*, 15(3), pp. 530–541. doi: 10.1016/j.scr.2015.09.008.

Reinisch, A. *et al.* (2014) 'Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation, *Blood*, 125(2), pp. 249–261. doi: 10.1182/blood-2014-04-572255.

Riether, C., Schürch, C. M. and Ochsenbein, A. F. (2015) 'Regulation of hematopoietic and leukemic stem cells by the immune system', *Cell Death and Differentiation*, 22(2), pp. 187–198. doi: 10.1038/cdd.2014.89.

Rodríguez-hernández, G. *et al.* (2017) 'Modeling the process of childhood ETV6-RUNX1 B-cell leukemias', 8(60), pp. 102674–102680. doi: 10.18632/oncotarget.21281.

Rodríguez-Hernández, G. *et al.* (2017) 'Infection exposure promotes ETV6-RUNX1 precursor B-cell leukemia via impaired H3K4 demethylases', *Cancer Research*, 77(16), pp. 4365–4377. doi: 10.1158/0008-5472.CAN-17-0701.

Romana, S. P. *et al.* (1995) 'High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia.', *Blood*.

De Rooij, B. *et al.* (2017) 'Acute lymphoblastic leukemia cells create a leukemic niche without

affecting the CXCR4/CXCL12 axis', *Haematologica*, 102(10), pp. e389–e393. doi: 10.3324/haematol.2016.159517.

Rosu-Myles, M. *et al.* (2000) 'Characterization of chemokine receptors expressed in primitive blood cells during human hematopoietic ontogeny.', *Stem cells (Dayton, Ohio)*. doi: 10.1634/stemcells.18-5-374.

Sacchetti, B. *et al.* (2007) 'Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment', *Cell*. doi: 10.1016/j.cell.2007.08.025.

Schäfer, D. *et al.* (2018) 'Five percent of healthy newborns have an ETV6-RUNX1 fusion as revealed by DNA-based GIPFEL screening', *Blood*. doi: 10.1182/blood-2017-09-808402.

Schindler, J. W. *et al.* (2009) 'TEL-AML1 Corrupts Hematopoietic Stem Cells to Persist in the Bone Marrow and Initiate Leukemia', *Cell Stem Cell*. Elsevier Ltd, 5(1), pp. 43–53. doi: 10.1016/j.stem.2009.04.019.

Schinke, C. *et al.* (2015) 'IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells', *Blood*, 125(20), pp. 3144–3152. doi: 10.1182/blood-2015-01-621631.

Schraufstatter, I. U., Chung, J. and Burger, M. (2001) 'IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways', *Am J of Physiol. Lung Cellular and Molecular Physiology*. doi: 10.1080/01418618208236928.

Serafini, M. *et al.* (2014) 'Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells', *Stem Cell Research*. doi: 10.1016/j.scr.2014.01.006.

Shi, C. *et al.* (2011) 'Bone Marrow Mesenchymal Stem and Progenitor Cells Induce Monocyte Emigration in Response to Circulating Toll-like Receptor Ligands', *Immunity*. doi: 10.1016/j.immuni.2011.02.016.

Shlush, L. I. *et al.* (2014) 'Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia', *Nature*. Nature Publishing Group, 506(7488), pp. 328–333. doi: 10.1038/nature13038.

Sinclair, A. *et al.* (2016) 'CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells', *Blood*, 128(3), pp. 371–383. doi: 10.1182/blood-2015-08-661785.

Steele, C. W. *et al.* (2016) 'CXCR2 Inhibition Profoundly Suppresses Metastases and Augments

Immunotherapy in Pancreatic Ductal Adenocarcinoma', *Cancer Cell*. The Authors, 29(6), pp. 832–845. doi: 10.1016/j.ccell.2016.04.014.

Swaminathan, S. *et al.* (2015) 'Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia', *Nature Immunology*, 16(7), pp. 766–774. doi: 10.1038/ni.3160.

Swiergiel, A. H. and Dunn, A. J. (1999) 'The roles of IL-1, IL-6, and TNFα in the feeding responses to endotoxin and influenza virus infection in mice', *Brain, Behavior, and Immunity*. doi: 10.1006/brbi.1999.0565.

Takam Kamga, P. *et al.* (2016) 'Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia.', *Oncotarget*, 7(16), pp. 21713–27. doi: 10.18632/oncotarget.7964.

Takeuchi, S. *et al.* (1997) 'TEL is one of the targets for deletion on 12p in many cases of childhood B-lineage acute lymphoblastic leukemia', *Leukemia*. doi: 10.1038/sj.leu.2400743.

Taylor, G. M. *et al.* (2009) 'Strong association of the HLA-DP6 supertype with childhood leukaemia is due to a single allele, DPB1*0601', *Leukemia*. doi: 10.1038/leu.2008.374.

Tokoyoda, K. *et al.* (2004) 'Cellular niches controlling B lymphocyte behavior within bone marrow during development', *Immunity*. doi: 10.1016/j.immuni.2004.05.001.

Tong, Y. and Linheng, L. (2006) 'The stem cell niches in bone', *The Journal of Clinical Investigations*, 116(5), pp. 1195–1201. doi: 10.1172/JCI28568.the.

Torrano, V. *et al.* (2011) 'ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor', 118(18), pp. 4910–4919. doi: 10.1182/blood-2011-05-354266.

Tsai, A. G. *et al.* (2008) 'Human Chromosomal Translocations at CpG Sites and a Theoretical Basis for Their Lineage and Stage Specificity', *Cell*. doi: 10.1016/j.cell.2008.10.035.

Tsuzuki, S. *et al.* (2004) 'Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice.', 101(22), pp. 8443–8448.

Tsuzuki, S. and Seto, M. (2013) 'TEL (ETV6)-AML1 (RUNX1) initiates self-renewing fetal pro-b cells in association with a transcriptional program shared with embryonic stem cells in mice', *Stem Cells*, 31(2), pp. 236–247. doi: 10.1002/stem.1277.

Vilchis-Ordoñez, A. *et al.* (2015) 'Bone marrow cells in acute lymphoblastic leukemia create a proinflammatory microenvironment influencing normal hematopoietic differentiation fates', *BioMed Research International*. doi: 10.1155/2015/386165.

Vilenchik, M. M. and Knudson, A. G. (2003) 'Endogenous DNA double-strand breaks: Production, fidelity of repair, and induction of cancer', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.2135498100.

Visnjic, D. *et al.* (2004) 'Hematopoiesis is severely altered in mice with an induced osteoblast deficiency', *Blood*. doi: 10.1182/blood-2003-11-4011.

Wang, L. C. *et al.* (1997) 'Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL', *EMBO Journal*. doi: 10.1093/emboj/16.14.4374.

Wang, L. C. *et al.* (1998) 'The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow', *Genes and Development*. doi: 10.1101/gad.12.15.2392.

Wang, X. *et al.* (2018) 'TGF-β1 Negatively Regulates the Number and Function of Hematopoietic Stem Cells', *Stem Cell Reports*. doi: 10.1016/j.stemcr.2018.05.017.

Waterman, R. S. *et al.* (2010) 'A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype', *PLoS ONE*. doi: 10.1371/journal.pone.0010088.

Van Der Weyden, L. *et al.* (2011) *Modeling the evolution of ETV6-RUNX1-induced B-cell precursor acute lymphoblastic leukemia in mice*, *Blood*. doi: 10.1182/blood-2011-02-338848.

Wiemels, J. L. *et al.* (1999) 'Prenatal origin of acute lymphoblastic leukaemia in children', *Lancet*. doi: 10.1016/S0140-6736(99)09403-9.

Wiemels, J. L. *et al.* (1999) 'Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero.', *Blood*, 94(3), pp. 1057–62.

Yamagata, T., Maki, K. and Mitani, K. (2005) 'Runx1/AML1 in Normal and Abnormal Hematopoiesis', *International Journal of Hematology*. doi: 10.1532/IJH97.05075.

Zambetti, N. A. *et al.* (2016) 'Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia', *Cell Stem Cell*. doi: 10.1016/j.stem.2016.08.021.

Zelent, A., Greaves, M. and Enver, T. (2004) 'Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia', *Oncogene*, 23(24), pp. 4275–4283. doi: 10.1038/sj.onc.1207672.

CHAPTER 3:

SUMMARY, CONCLUSION AND FUTURE PERSPECTIVES

The present study laid out to investigate the role of bone marrow mesenchymal stromal cells (BM-MSC) and inflammation in the pre-leukemic phase of ETV6-RUNX1-positive childhood acute lymphoblastic leukemia (ALL). In particular, we asked if an inflamed mesenchymal niche could provide selective advantages to pre-leukemic cells in terms of persistence and, possibly, malignant progression. Both epidemiological (Heath and Hasterlik, 1963; Francis *et al.*, 2012; Cazzaniga *et al.*, 2017) and experimental data (Swaminathan *et al.*, 2015) (Rodríguez-Hernández *et al.*, 2017), in fact, demonstrate that infections and inflammation promote ETV6-RUNX1^{+} pre-leukemia to leukemia transition, while dysfunctional BM-MSC are able to induce myelodysplasia and acute myeloid leukemia (AML) in genetic predisposing syndromes (Raaijmakers *et al.*, 2010) (Zambetti *et al.*, 2016).

ETV6-RUNX1^{+} ALL is the most frequent cancer in pediatric age. The first pathogenetic event consists in the chromosomal translocation t(12;21)(p13;q22) which generates the ETV6(TEL)-RUNX1(AML1) fusion gene (Romana *et al.*, 1995) (Golub *et al.*, 1995). Translocation occurs *in utero* (Gale *et al.*, 1997) with a relatively high frequency in healthy newborns (1-5%); however, only 1% of ETV6-RUNX1 carriers develops leukemia (Wiemels *et al.*, 1999) (Mori *et al.*, 2002) (Schäfer *et al.*, 2018). ETV6-RUNX1, in fact, is a weak oncogene which expands a clinically silent pre-leukemic clone: additional mutations are thus necessary to complete malignant transformation (Wiemels *et al.*, 1999) (Andreasson *et al.*, 2001) (Tsuzuki *et al.*, 2004)

(Morrow *et al.*, 2007) (Hong *et al.*, 2008). Secondary hits occur in the post-natal life likely in consequence of a dysregulated immune response to common infections/inflammation due to a delayed exposure to immunostimulating microenvironments (i.e. children with no day-care attendance) (Greaves, 2006). Despite the good prognosis, relapses can occur in about 20% of patients, sometimes with a very long latency (up to 15 years after remission) (Ford *et al.*, 2001). At least for some of these cases, the persistence of a quiescent, chemoresistant pre-leukemic clone and the acquisition of *de novo* secondary mutations is one of the most plausible causal explanation (Konrad *et al.*, 2003) (Van Delft *et al.*, 2011b) (Kuster *et al.*, 2018). For that reason, we think that a better management of ETV6-RUNX1$^+$ ALL must include the elucidation of mechanisms that sustain silent pre-leukemia in addition to the clinical disease. Although prophylactic interventions to avoid the potential ALL onset in ETV6-RUNX1$^+$ healthy carriers may arise ethical issues, prevention of late relapses in patients could be a desirable goal.

In order to study ETV6-RUNX1$^+$ pre-leukemia features and, in particular, its interaction with BM-MSC and inflammation, we took advantage from two ETV6-RUNX1-expressing cellular models (the murine pro-B cell line Ba/F3 and human umbilical cord blood (UCB)-derived CD34$^+$ cells) in addition to an *in vitro* system of mesenchymal niche.

The study demonstrated that bone marrow mesenchymal stromal cells (BM-MSC) and inflammation cooperated in favoring the emergence of ETV6-RUNX1$^+$ pre-leukemic cells against the normal

counterpart. In particular, we found that the inflamed MSC niche diminished proliferation and survival of control Ba/F3 while minimally or no affecting the pre-leukemic clone. We have evidence that BM-MSC stimulated with IL6/IL1β/TNFα pro-inflammatory cytokines (the condition of our inflamed mesenchymal niche model) highly secrete pro-myeloid factors, possibly in order to induce a physiological "emergency myelopoiesis" in response to inflammatory stimuli (Riether, Schürch and Ochsenbein, 2015). This observation, in addition to the ectopic expression of myeloid features in ETV6-RUNX1^{+} pre-leukemic cells (Böiers *et al.*, 2013) and blasts (Abdelhaleem, 2007) (Gerr *et al.*, 2010), could in part explain the advantage of the pre-leukemic clone within the inflamed MSC niche. On the other hand, we observed that ETV6-RUNX1^{+} Ba/F3 are characterized by an intrinsic resistance to DNA damage-induced apoptosis. Molecular investigations clarifying both these aspects will provide insights into the biology of ETV6-RUNX1^{+} pre-leukemic cells as well as their interaction with the surrounding microenvironment.

According to the literature, we also found that an inflamed mesenchymal niche represented a genotoxic microenvironment for hematopoietic cells (Zambetti *et al.*, 2016). When exposed to the inflamed niche, in fact, both control and pre-leukemic Ba/F3 accumulated DNA double strand breaks and increased expression of activation-induced cytidine deaminase (AID) enzyme, which has been demonstrated to mediate genetic instability in ETV6-RUNX1^{+} pre-leukemia and promote its progression *in vivo* (Swaminathan *et al.*, 2015). As we have evidences that ETV6-RUNX1^{+} Ba/F3 high express

onco-suppressor genes in basal conditions (data not shown), it is plausible that these *loci* could be the most affected by mutagenesis, thus representing sensible targets for malignant progression. Thus, it will be interesting in the future to characterized eventual mutations occurring in pre-leukemic Ba/F3 in terms of type, frequency and genomic localization.

Within the ETV6-RUNX1 $CD34^+$ pre-leukemic population, the inflamed MSC niche specifically expanded the compartment $CD34^+IL7R^+$, which has been recently indicated as the cellular target of ETV6-RUNX1 pre-leukemic activity during fetal hematopoiesis (Böiers *et al.*, 2018). This observation could be particularly relevant to explain the intimate association of disease onset with inflammatory/infective events. One could speculate that genetic polymorphisms in inflammatory/immune genes that are relevant in our experimental setting (i.e. those regulating production and/or response to IL6/IL1β/TNFα, CXCR1/2 and their ligands, GM-CSF) could have a role in making difference between health and disease in ETV6-$RUNX1^+$ carriers. On this regard, significant associations of leukemic risk with particular allelic variants has been observed for MHC (Taylor *et al.*, 2009), interferon-γ (Krampera *et al.*, 2006) and Toll-like receptor 6 (Miedema *et al.*, 2012) genes.

Importantly, we also found that both ETV6-$RUNX1^+$ Ba/F3 and $CD34^+$ cells were highly attracted by inflamed MSC conditioned-media; the preferential migration was, at least in the murine pro-B cell line, CXCR2-dependent. Effectively, ETV6-$RUNX1^+$ Ba/F3 ectopically upregulated CXCR2 expression, while gene expression profile

predicted its signaling activation. CXCR2 is a chemokine receptor involved in the trafficking and activation of neutrophils, monocytes and macrophages in response to infective/inflammatory stimuli. However, it is also implicated in chronic inflammatory diseases and cancer progression (Jaffer and Ma, 2016) (Liu *et al.*, 2016) (Ha, Debnath and Neamati, 2017). In particular, its overexpression represents a poor prognostic factor in myelodysplastic syndrome and acute myeloid leukemia (Schinke *et al.*, 2015) while BCP-ALL cells exploit a CXCR1/2-mediated migration to create a malignant MSC niche (De Rooij *et al.*, 2017). Although we weren't able to detect a membrane upregulation of CXCR2 and CXCR1 in ETV6-RUNX1^{+} CD34^{+} cells (data not shown), it cannot be excluded a functional activation of their pathway (De Rooij *et al.*, 2017) (Schinke *et al.*, 2015). CXCR1/2 downstream effectors, for example, could be transactivated by ETV6-RUNX1 despite unaltered receptors levels. A transcriptomic analysis, as well as a functional analysis of the CXCR1/2 signalling, on immunophenotypically-sorted subpopulations in ETV6-RUNX1^{+} and control cells will address the question.

CXCR1/2 inhibitors have been used in several pre-clinical (Schinke *et al.*, 2015) (Devapatla *et al*., 2015) (Ijichi *et al.*, 2011) and clinical settings (Martz, 2012) (Steele *et al.*, 2016) (Joseph *et al.*, 2017), both against chronic inflammatory diseases and cancer. In our case, immunocompetent ETV6-RUNX1-transgenic mice will be initially preferred as the involvement of CXCR1/2 receptors in the immune response could significantly impact on the experimental read out. In particular, we will be interested in evaluated if CXCR2 blocking can

prevent pre-leukemic cells accumulation within inflamed bone marrow sites and, possibly, their emergence against the normal counterpart (**Fig.4**). Inhibitors will be also tested *in vitro* on control and ETV6-RUNX1^{+} CD34^{+} cells cultured upon unstimulated or inflamed BM-MSC*:* migration, as well as apoptosis, will be then evaluated. It has been shown, in fact, that CXCR2 mediate survival and self-renewal in human hematopoietic stem/progenitor cells (Sinclair *et al.*, 2016), while its inhibition decreases the leukemogenic potential of AML stem cells *in vivo* (Schinke *et al.*, 2015). It is possible that, although CXCR2 didn't mediate pro-survival effects in pre-leukemic Ba/F3, it could possess different functions in a very diverse cellular context which is the CD34^{+} multipotent progenitor. If *in vitro* experiments will be positive in this sense, a xenograft mouse model could be considered, even if with the limitation of a compromised immune response.

In this case, we could take advantage from models of ectopic humanized bone marrow niche, which exploit the capacity of BM-MSC to recreate a functional niche and recapitulate human hematopoiesis. Such systems will allow us to specifically study the interaction between ETV6-RUNX1^{+} CD34^{+} cells and human BM-MSC (Reinisch *et al.*, 2014) (Abarrategi *et al.*, 2017). Finally, a gene expression profile of sorted, specific subpopulations within ETV6-RUNX1-positive and negative CD34^{+} cells will be performed before and after the exposure to the inflamed niche to highlight potentially targetable mechanisms, in addition to CXCR1/2 axes, to eradicate the pre-leukemic clone.

References

Abarrategi, A. *et al.* (2017) 'Versatile humanized niche model for studying human normal and malignant haematopoieisis', *Journal of Clinical Investigation*, 127(2), p. 543-. doi: 10.1172/JCI89364.

Abdelhaleem, M. (2007) 'Frequent but nonrandom expression of lymphoid markers on de novo childhood acute myeloid leukemia', *Experimental and Molecular Pathology*. doi: 10.1016/j.yexmp.2007.05.007.

Alpar, D. *et al.* (2015) 'Clonal origins of ETV6-RUNX1 + acute lymphoblastic leukemia: Studies in monozygotic twins', *Leukemia*. Nature Publishing Group, 29(4), pp. 839–846. doi: 10.1038/leu.2014.322.

André, V. *et al.* (2012) 'Mesenchymal stem cells from Shwachman-Diamond syndrome patients display normal functions and do not contribute to hematological defects', *Blood Cancer Journal*. doi: 10.1038/bcj.2012.40.

Andreasson, P. *et al.* (2001) 'The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo', *Cancer Genetics*, 130(2).

Asou, N. (2003) 'The role of a Runt domain transcription factor AML1/RUNX1 in leukemogenesis and its clinical implications', *Critical Reviews in Oncology/Hematology*. doi: 10.1016/S1040-8428(02)00003-3.

Auer, F. *et al.* (2017) 'Activation-induced cytidine deaminase prevents pro-B cell acute lymphoblastic leukemia by functioning as a negative regulator in Rag1 deficient pro-B cells', *Oncotarget*. doi: 10.18632/oncotarget.20563.

Balandrán, J. C. *et al.* (2017) 'Pro-inflammatory-related loss of CXCL12 niche promotes acute lymphoblastic leukemic progression at the expense of normal lymphopoiesis', *Frontiers in Immunology*, 7(JAN), pp. 1–14. doi: 10.3389/fimmu.2016.00666.

Bateman, C. M. *et al.* (2015) 'Evolutionary trajectories of hyperdiploid ALL in monozygotic twins', *Leukemia*. doi: 10.1038/leu.2014.177.

Bernardin, F. *et al.* (2002) 'Advances in Brief TEL-AML1 , Expressed from t (12 ; 21) in Human Acute Lymphocytic Leukemia , Induces Acute Leukemia in Mice 1', pp. 3904–3908.

Bernardo, M. E. and Fibbe, W. E. (2013) 'Mesenchymal stromal cells: Sensors and switchers of inflammation', *Cell Stem Cell*. Elsevier Inc., 13(4), pp. 392–402. doi: 10.1016/j.stem.2013.09.006.

Binato, R. *et al.* (2015) 'The molecular signature of AML mesenchymal stromal cells reveals candidate genes related to the leukemogenic process', *Cancer Letters*. Elsevier Ireland Ltd, 369(1), pp. 134–143. doi: 10.1016/j.canlet.2015.08.006.

Le Blanc, K. *et al.* (2008) 'Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study', *The Lancet*. doi: 10.1016/S0140-

6736(08)60690-X.

Le Blanc, K. and Mougiakakos, D. (2012) 'Multipotent mesenchymal stromal cells and the innate immune system', *Nature Reviews Immunology*. doi: 10.1038/nri3209.

Blank, U. and Karlsson, S. (2015) 'TGF-β signaling in the control of hematopoietic stem cells', *Blood*. doi: 10.1182/blood-2014-12-618090.

Bohlander, S. K. (2005) 'ETV6: A versatile player in leukemogenesis', *Seminars in Cancer Biology*. doi: 10.1016/j.semcancer.2005.01.008.

Böiers, C. *et al.* (2013) 'Lymphomyeloid Contribution of an Immune-Restricted Progenitor Emerging Prior to Definitive Hematopoietic Stem Cells', *Cell Stem Cell*. doi: 10.1016/j.stem.2013.08.012.

Böiers, C. *et al.* (2018) 'A Human IPS Model Implicates Embryonic B-Myeloid Fate Restriction as Developmental Susceptibility to B Acute Lymphoblastic Leukemia-Associated ETV6-RUNX1', *Developmental Cell*, 44(3), p. 362–377.e7. doi: 10.1016/j.devcel.2017.12.005.

Boily, G. *et al.* (2007) 'Identification of transcripts modulated by ETV6 expression', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.2006.06377.x.

Brandau, S. *et al.* (2014) 'Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes', *PLoS ONE*. doi: 10.1371/journal.pone.0106903.

Calvi, L. M. *et al.* (2003) 'Osteoblastic cells regulate the haematopoietic stem cell niche', *Nature*. doi: 10.1038/nature02040.

Cappuzzello, C. *et al.* (2016) 'Mesenchymal stromal cell-derived PTX3 promotes wound healing via fibrin remodeling', *Journal of Investigative Dermatology*. doi: 10.1038/JID.2015.346.

Carey, A. *et al.* (2017) 'Identification of Interleukin-1 by Functional Screening as a Key Mediator of Cellular Expansion and Disease Progression in Acute Myeloid Leukemia', *Cell Reports*. doi: 10.1016/j.celrep.2017.03.018.

Cazzaniga, G. *et al.* (2017) 'Possible role of pandemic AH1N1 swine flu virus in a childhood leukemia cluster', *Leukemia*, 31(8), pp. 1819–1821. doi: 10.1038/leu.2017.127.

Lo Celso, C. and Scadden, D. T. (2011) 'The haematopoietic stem cell niche at a glance', *Journal of Cell Science*, 124(21), pp. 3529–3535. doi: 10.1242/jcs.074112.

Colmone, A. *et al.* (2008) 'Normal Hematopoietic Progenitor Cells', *Science,* 322(December), pp. 1861–1865.

Corces-Zimmerman, M. R. and Majeti, R. (2014) 'Pre-leukemic evolution of hematopoietic stem cells: The importance of early mutations in leukemogenesis', *Leukemia*. Nature Publishing Group, 28(12), pp. 2276–2282. doi: 10.1038/leu.2014.211.

Corcione, A. *et al.* (2006) 'Human mesenchymal stem cells modulate B-cell functions', *Blood*. doi: 10.1182/blood-2005-07-2657.

Crisan, M. *et al.* (2008) 'A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human

Organs', *Cell Stem Cell*, 3(3), pp. 301–313. doi: 10.1016/j.stem.2008.07.003.

Van Delft, F. W. *et al.* (2011a) 'Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia', *Blood*, 117(23), pp. 6247–6254. doi: 10.1182/blood-2010-10-314674.

Van Delft, F. W. *et al.* (2011b) 'Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia', *Blood*. doi: 10.1182/blood-2010-10-314674.

Devapatla, B., Sharma, A. and Woo, S. (2015) 'CXCR2 inhibition combined with sorafenib improved antitumor and antiangiogenic response in preclinical models of ovarian cancer', *PLoS ONE*, 10(9), pp. 1–16. doi: 10.1371/journal.pone.0139237.

Diakos, C. *et al.* (2007) 'RNAi-mediated silencing of TEL/AML1 reveals a heat-shock protein- and survivin-dependent mechanism for survival', *Blood*. doi: 10.1182/blood-2006-04-019612.

Dockerty, J. D. *et al.* (2001) 'Case-control study of parental age, parity and socioeconomic level in relation to childhood cancers', *International Journal of Epidemiology*. doi: 10.1093/ije/30.6.1428.

Dominici, M. *et al.* (2006) 'Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement', *Cytotherapy*, 8(4), pp. 315–317. doi: 10.1080/14653240600855905.

Duijvestein, M. *et al.* (2010) 'Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: Results of a phase I study', *Gut*. doi: 10.1136/gut.2010.215152.

Eggenhofer, E. and Hoogduijn, M. J. (2012) 'Mesenchymal stem cell-educated macrophages', *Transplantation Research*. doi: 10.1186/2047-1440-1-12.

Eguchi-Ishimae, M. *et al.* (2001) 'Breakage and fusion of the TEL (ETV6) gene immature B lymphocytes induced by apoptogenic signals', *Blood*, 97(3), pp. 737–743. doi: 10.1182/blood.V97.3.737.

Enciso, J. *et al.* (2016) 'Modeling the pro-inflammatory tumor microenvironment in acute lymphoblastic leukemia predicts a breakdown of hematopoietic-mesenchymal communication networks', *Frontiers in Physiology*. doi: 10.3389/fphys.2016.00349.

Fan, X. *et al.* (2007) 'Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M607705200.

Ford, a M. *et al.* (1998) 'Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(8), pp. 4584–4588. doi: 10.1073/pnas.95.8.4584.

Ford, A. M. *et al.* (2001) 'Origins of "late" relapse in childhood acute lymphoblastic leukemia with TEL-AML1 fusion genes.', *Blood*, 98(3), pp. 558–64. doi: 10.1182/blood.V98.3.558.

Ford, A. M. *et al.* (2009) 'The TEL-AML1 leukemai fusion gene dysregulates the TGF-beta pathway in early B lineage progenitor cells', *J. Clin. Invest.*, 119(4). doi: 10.1172/jci36428ds1.

Forsberg, E. C. and Smith-Berdan, S. (2009) 'Parsing the niche code: The molecular mechanisms governing hematopoietic stem cell adhesion and differentiation', *Haematologica*, 94(11), pp. 1477–1481. doi: 10.3324/haematol.2009.013730.

Francis, S. S. *et al.* (2012) 'Unusual space-time patterning of the Fallon, Nevada leukemia cluster: Evidence of an infectious etiology', *Chemico-Biological Interactions*. doi: 10.1016/j.cbi.2011.02.019.

Fuka, G. *et al.* (2011) 'The leukemia-specific fusion gene etv6/runx1 perturbs distinct key biological functions primarily by gene repression', *PLoS ONE*. doi: 10.1371/journal.pone.0026348.

Gale, K. B. *et al.* (1997) 'Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 94(25), pp. 13950–4. doi: 10.1073/PNAS.94.25.13950.

Gandemer, V. *et al.* (2007) 'Five distinct biological processes and 14 differentially expressed genes characterize TEL/AML1-positive leukemia', *BMC Genomics*, 8, pp. 1–15. doi: 10.1186/1471-2164-8-385.

Gerr, H. *et al.* (2010) 'Acute leukaemias of ambiguous lineage in children: Characterization, prognosis and therapy recommendations', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.2009.08058.x.

Gilham, C. *et al.* (2005) 'Day care in infancy and risk of childhood acute lymphoblastic leukaemia: Findings from UK case-control study', *British Medical Journal*. doi: 10.1136/bmj.38428.521042.8F.

Golub, T. R. *et al.* (1995) 'Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia.', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.92.11.4917.

Grassinger, J. *et al.* (2010) 'Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential', *Blood*. doi: 10.1182/blood-2009-12-260703.

Greaves, M. (2002) 'Childhood leukemia', *Bmj*, 324, pp. 283–287.

Greaves, M. (2006) 'Infection, immune responses and the aetiology of childhood leukaemia', *Nature Reviews Cancer*, 6(3), pp. 193–203. doi: 10.1038/nrc1816.

Greaves, M. (2018) 'A causal mechanism for childhood acute lymphoblastic leukaemia', *Nature Reviews Cancer*. Springer US, 18(8), pp. 471–484. doi: 10.1038/s41568-018-0015-6.

Greaves, M. F. *et al.* (2003) 'Leukemia in twins: Lessons in natural history', *Blood*. doi: 10.1182/blood-2002-12-3817.

Greaves, M. F. and Wiemels, J. (2003) 'Origins of chromosome translocations in childhood leukaemia', *Nature Reviews Cancer*, 3(9), pp. 639–649. doi: 10.1038/nrc1164.

Greenbaum, A. *et al.* (2013) 'CXCL12 Production by Early Mesenchymal Progenitors is Required for Hematopoietic Stem Cell Maintenance', *Nature*, 495(7440), pp. 227–230. doi:

10.1038/nature11926.CXCL12.

Gunji, H. *et al.* (2004) 'TEL/AML1 shows dominant-negative effects over TEL as well as AML1', *Biochemical and Biophysical Research Communications*. doi: 10.1016/j.bbrc.2004.07.169.

Ha, H., Debnath, B. and Neamati, N. (2017) 'Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases', *Theranostics*, 7(6), pp. 1543–1588. doi: 10.7150/thno.15625.

Heath, C. W. and Hasterlik, R. J. (1963) 'Leukemia among children in a suburban community', *The American Journal of Medicine*. doi: 10.1016/0002-9343(63)90088-3.

Von Der Heide, E. K. *et al.* (2017) 'Molecular alterations in bone marrow mesenchymal stromal cells derived from acute myeloid leukemia patients', *Leukemia*, 31(5), pp. 1069–1078. doi: 10.1038/leu.2016.324.

Hol, J., Wilhelmsen, L. and Haraldsen, G. (2010) 'The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies', *Journal of Leukocyte Biology*. doi: 10.1189/jlb.0809532.

Hong, D. *et al.* (2008) 'Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia', *Science*, 319(5861), pp. 336–339. doi: 10.1126/science.1150648.

Ichikawa, M. *et al.* (2004) 'Runx1/AML-1 ranks as a master regulator of adult hematopoiesis', *Cell Cycle*. doi: 10.4161/cc.3.6.951.

Ijichi, H. *et al.* (2011) 'Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma', *Journal of Clinical Investigation*, 121(10), pp. 4106–4117. doi: 10.1172/JCI42754.

Jaffer, T. and Ma, D. (2016) 'The emerging role of chemokine receptor CXCR2 in cancer progression', *Translational Cancer Research*, 5(S4), pp. S616–S628. doi: 10.21037/tcr.2016.10.06.

Joseph, J. P. *et al.* (2017) 'CXCR2 Inhibition - a novel approach to treating CoronAry heart DiseAse (CICADA): Study protocol for a randomised controlled trial', *Trials*. Trials, 18(1), pp. 1–11. doi: 10.1186/s13063-017-2210-2.

Kaindl, U. *et al.* (2014) 'Blocking ETV6/RUNX1-induced MDM2 overexpression by Nutlin-3 reactivates p53 signaling in childhood leukemia', *Leukemia*. doi: 10.1038/leu.2013.345.

Kantner, H.-P. *et al.* (2013) 'ETV6/RUNX1 Induces Reactive Oxygen Species and Drives the Accumulation of DNA Damage in B Cells', *Neoplasia*. doi: 10.1593/neo.131310.

Kawamata, N. *et al.* (2008) 'Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray', *Blood*. doi: 10.1182/blood-2007-05-088310.

Keating, A. (2012) 'Mesenchymal stromal cells: New directions', *Cell Stem Cell*. doi: 10.1016/j.stem.2012.05.015.

Kfoury, Y. and Scadden, D. T. (2015) 'Mesenchymal cell contributions to the stem cell niche',

Cell Stem Cell. Elsevier Inc., 16(3), pp. 239–253. doi: 10.1016/j.stem.2015.02.019.

Kinlen, L. J., Clarke, K. and Hudson, C. (1990) 'Evidence from population mixing in British New Towns 1946-85 of an infective basis for childhood leukaemia', *The Lancet*. doi: 10.1016/0140-6736(90)93389-7.

Kohara, H. *et al.* (2007) 'Development of plasmacytoid dendritic cells in bone marrow stromal cell niches requires CXCL12-CXCR4 chemokine signaling', *Blood*. doi: 10.1182/blood-2007-04-084210.

Konrad, M. *et al.* (2003) 'Late relapses evolve from slow-responding subclones acute lymphoblastic leukemia : for the persistence evidence of a preleukemic clone', *Blood,* 101, pp. 3635–3640. doi: 10.1182/blood-2002-10-3252.Supported.

Kornblau, S. M. *et al.* (2018) 'Distinct protein signatures of acute myeloid leukemia bone marrow-derived stromal cells are prognostic for patient survival', *Haematologica*, 103(5), pp. 810–821. doi: 10.3324/haematol.2017.172429.

Krampera, M. *et al.* (2006) 'Role for Interferon-γ in the Immunomodulatory Activity of Human Bone Marrow Mesenchymal Stem Cells', *Stem Cells*. doi: 10.1634/stemcells.2005-0008.

Kuster, L. *et al.* (2018) 'ETV6_RUNX1-positive relapses evolve from an ancestral clone and frequently acquire deletions of genes implicated in glucocorticoid signaling', 117(9), pp. 2658–2668. doi: 10.1182/blood-2010-03-275347.The.

Kyurkchiev, D. (2014) 'Secretion of immunoregulatory cytokines by mesenchymal stem cells', *World Journal of Stem Cells*. doi: 10.4252/wjsc.v6.i5.552.

Larson, R.A; Anastasi, J. (2008) *Acute Lymphoblastic Leukemia: clinical presentation, diagnosis and classification.* Edited by Springer. Berlin.

De Laurentiis, A., Hiscott, J. and Alcalay, M. (2015) 'The TEL-AML1 fusion protein of acute lymphoblastic leukemia modulates IRF3 activity during early B-cell differentiation', *Oncogene*. Nature Publishing Group, 34(49), pp. 6018–6028. doi: 10.1038/onc.2015.50.

Levanon, D. *et al.* (2001) 'Architecture and anatomy of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1', *Gene*. doi: 10.1016/S0378-1119(00)00532-1.

Levasseur, M. *et al.* (1994) 'Relapse of acute lymphoblastic leukaemia 14 years after presentation: Use of molecular techniques to confirm true re-emergence', *British Journal of Haematology*. doi: 10.1111/j.1365-2141.1994.tb04942.x.

Linka, Y. *et al.* (2013) 'The impact of TEL-AML1 (ETV6-RUNX1) expression in precursor B cells and implications for leukaemia using three different genome-wide screening methods', *Blood Cancer Journal*. doi: 10.1038/bcj.2013.48.

Liu, Q. *et al.* (2016) 'The CXCL8-CXCR1/2 pathways in cancer', *Cytokine and Growth Factor Reviews*. Elsevier Ltd, 31, pp. 61–71. doi: 10.1016/j.cytogfr.2016.08.002.

Lopez, R. G. *et al.* (1999) 'TEL is a sequence-specific transcriptional repressor', *Journal of*

Biological Chemistry. doi: 10.1074/jbc.274.42.30132.

Ma, X. *et al.* (2005) 'Vaccination history and risk of childhood leukaemia', *International Journal of Epidemiology*. doi: 10.1093/ije/dyi113.

Mangolini, M. *et al.* (2013) 'STAT3 mediates oncogenic addiction to TEL-AML1 in t(12;21) acute lymphoblastic leukemia', *Blood*. doi: 10.1182/blood-2012-11-465252.

Marcotte, E. L. *et al.* (2016) 'Caesarean delivery and risk of childhood leukaemia: A pooled analysis from the Childhood Leukemia International Consortium (CLIC)', *The Lancet Haematology*. doi: 10.1016/S2352-3026(16)00002-8.

Martz, L. (2012) 'CXCR2 antagonists in breast cancer', *Science-Business eXchange*, 5(31), pp. 1–2. doi: 10.1038/scibx.2012.807.

Matthews, A. G. W. *et al.* (2007) 'RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination', *Nature*. doi: 10.1038/nature06431.

Melief, S. M. *et al.* (2013) 'Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages', *Stem Cells*. doi: 10.1002/stem.1432.

Méndez-Ferrer, S. *et al.* (2010) 'Mesenchymal and haematopoietic stem cells form a unique bone marrow niche', *Nature*, 466(7308), pp. 829–834. doi: 10.1038/nature09262.

Miedema, K. G. E. *et al.* (2012) 'Polymorphisms in the TLR6 gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease', *Leukemia*. doi: 10.1038/leu.2011.341.

Mijušković, M. *et al.* (2015) 'Off-Target V(D)J Recombination Drives Lymphomagenesis and Is Escalated by Loss of the Rag2 C Terminus', *Cell Reports*. doi: 10.1016/j.celrep.2015.08.034.

Mo, I. F. Y. *et al.* (2008) 'Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors', *BMC Cell Biology*. doi: 10.1186/1471-2121-9-52.

Mori, H. *et al.* (2002) 'Chromosome translocations and covert leukemic clones are generated during normal fetal development', *Proceedings of the National Academy of Sciences*, 99(12), pp. 8242–8247. doi: 10.1073/pnas.112218799.

Morrow, M. *et al.* (2004) 'TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity', *Blood*, 103(10), pp. 3890–3896. doi: 10.1182/blood-2003-10-3695.

Morrow, M. *et al.* (2007) 'TEL-AML1 preleukemic activity requires the DNA binding domain of AML1 and the dimerization and corepressor binding domains of TEL', *Oncogene*, 26(30), pp. 4404–4414. doi: 10.1038/sj.onc.1210227.

Motoda, L. *et al.* (2007) '*Runx1* Protects Hematopoietic Stem/Progenitor Cells from Oncogenic Insult', *Stem Cells*. doi: 10.1634/stemcells.2007-0061.

Mullighan, C. G. *et al.* (2008) 'Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia', *Science*. doi: 10.1126/science.1164266.

Mullighan, C. G. (2013) 'Genomic characterization of childhood acute lymphoblastic leukemia', *Semin Hematol*, 50(4), pp. 314–324. doi: 10.1053/j.seminhematol.2013.10.001.

Muramatsu, M. *et al.* (2000) 'Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme', *Cell*. doi: 10.1016/S0092-8674(00)00078-7.

Naderi, E. H. *et al.* (2015) 'Bone marrow stroma-derived PGE2 protects BCP-ALL cells from DNA damage-induced p53 accumulation and cell death', *Molecular Cancer*, 14(1). doi: 10.1186/s12943-014-0278-9.

Nauta, A. J. *et al.* (2012) 'Immunomodulatory properties of mesenchymal stromal cells Review in translational hematology Immunomodulatory properties of mesenchymal stromal cells', 110(10), pp. 3499–3506. doi: 10.1182/blood-2007-02-069716.

Lo Nigro, L. *et al.* (1999) 'Clonal stability in children with acute lymphoblastic leukemia (ALL) who relapsed five or more years after diagnosis', *Leukemia*.

Noda, M. *et al.* (2011) 'CXCL12-CXCR4 chemokine signaling is essential for NK-cell development in adult mice', *Blood*. doi: 10.1182/blood-2010-04-277897.

Oettinger, M. A. *et al.* (1990) 'RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination', *Science*. doi: 10.1126/science.2360047.

Okuda, T. *et al.* (1996) 'AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis', *Cell*. doi: 10.1016/S0092-8674(00)80986-1.

Orkin, S. H. and Zon, L. I. (2008) 'Hematopoiesis: An Evolving Paradigm for Stem Cell Biology', *Cell*, 132(4), pp. 631–644. doi: 10.1016/j.cell.2008.01.025.

Paashuis-Lew, Y. R. and Heddle, J. A. (1998) 'Spontaneous mutation during fetal development and post-natal growth', *Mutagenesis*, 13(6), pp. 613–617.

Palmi, C. *et al.* (2014) 'Cytoskeletal Regulatory Gene Expression and Migratory Properties of B-cell Progenitors Are Affected by the ETV6-RUNX1 Rearrangement.', *Molecular cancer research : MCR*, 12(12), pp. 1796–806. doi: 10.1158/1541-7786.MCR-14-0056-T.

Panzer-Grümayer, E. R. *et al.* (2005) 'Immunogenotype changes prevail in relapses of young children with TEL-AML1-positive acute lymphoblastic leukemia and derive mainly from clonal selection', *Clinical Cancer Research*, 11(21), pp. 7720–7727. doi: 10.1158/1078-0432.CCR-05-1239.

Papaemmanuil, E. *et al.* (2014) 'RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia', *Nature Genetics*. Nature Publishing Group, 46(2), pp. 116–125. doi: 10.1038/ng.2874.

Parker, W. and Ollerton, J. (2013) 'Evolutionary biology and anthropology suggest biome reconstitution as a necessary approach toward dealing with immune disorders', *Evolution, Medicine, and Public Health*. doi: 10.1093/emph/eot008.

Penders, J. *et al.* (2006) 'Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy', *PEDIATRICS*. doi: 10.1542/peds.2005-2824.

Pietras, E. M. (2017) 'Inflammation: A key regulator of hematopoietic stem cell fate in health and disease', *Blood*, 130(15), pp. 1693–1698. doi: 10.1182/blood-2017-06-780882.

Poirel, H. *et al.* (1997) 'The TEL gene products: Nuclear phosphoproteins with DNA binding properties', *Oncogene*. doi: 10.1038/sj.onc.1200829.

Poynton, F. J., Thursfield, H. & Paterson, D. (1922) 'The severe blood diseases of childhood: a series of observations from the Hospital for Sick Children, Great Ormond Street.', *Br. J. Child. Dis.*, XIX.

Polak, R. *et al.* (2015) 'B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment', *Blood*, 126(21), pp. 2404–2414. doi: http://dx.doi.org/10.1182/blood-2015-03-634238.

Raaijmakers, M. H. G. P. *et al.* (2010) 'Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia', *Nature*. Nature Publishing Group, 464(7290), pp. 852–857. doi: 10.1038/nature08851.

Raghuwanshi, S. K. *et al.* (2012) 'The Chemokine Receptors CXCR1 and CXCR2 Couple to Distinct G Protein-Coupled Receptor Kinases To Mediate and Regulate Leukocyte Functions', *The Journal of Immunology*. doi: 10.4049/jimmunol.1201114.

Reikvam, H. *et al.* (2015) 'The cytokine-mediated crosstalk between primary human acute myeloid cells and mesenchymal stem cells alters the local cytokine network and the global gene expression profile of the mesenchymal cells', *Stem Cell Research*, 15(3), pp. 530–541. doi: 10.1016/j.scr.2015.09.008.

Reinisch, A. *et al.* (2014) 'Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation, *Blood*, 125(2), pp. 249–261. doi: 10.1182/blood-2014-04-572255.

Riether, C., Schürch, C. M. and Ochsenbein, A. F. (2015) 'Regulation of hematopoietic and leukemic stem cells by the immune system', *Cell Death and Differentiation*, 22(2), pp. 187–198. doi: 10.1038/cdd.2014.89.

Rodríguez-hernández, G. *et al.* (2017) 'Modeling the process of childhood ETV6-RUNX1 B-cell leukemias', 8(60), pp. 102674–102680. doi: 10.18632/oncotarget.21281.

Rodríguez-Hernández, G. *et al.* (2017) 'Infection exposure promotes ETV6-RUNX1 precursor B-cell leukemia via impaired H3K4 demethylases', *Cancer Research*, 77(16), pp. 4365–4377. doi: 10.1158/0008-5472.CAN-17-0701.

Romana, S. P. *et al.* (1995) 'High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia.', *Blood*.

De Rooij, B. *et al.* (2017) 'Acute lymphoblastic leukemia cells create a leukemic niche without affecting the CXCR4/CXCL12 axis', *Haematologica*, 102(10), pp. e389–e393. doi: 10.3324/haematol.2016.159517.

Rosu-Myles, M. *et al.* (2000) 'Characterization of chemokine receptors expressed in primitive blood cells during human hematopoietic ontogeny.', *Stem cells (Dayton, Ohio)*. doi: 10.1634/stemcells.18-5-374.

Sacchetti, B. *et al.* (2007) 'Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment', *Cell*. doi: 10.1016/j.cell.2007.08.025.

Schäfer, D. *et al.* (2018) 'Five percent of healthy newborns have an ETV6-RUNX1 fusion as revealed by DNA-based GIPFEL screening', *Blood*. doi: 10.1182/blood-2017-09-808402.

Schindler, J. W. *et al.* (2009) 'TEL-AML1 Corrupts Hematopoietic Stem Cells to Persist in the Bone Marrow and Initiate Leukemia', *Cell Stem Cell*. Elsevier Ltd, 5(1), pp. 43–53. doi: 10.1016/j.stem.2009.04.019.

Schinke, C. *et al.* (2015) 'IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells', *Blood*, 125(20), pp. 3144–3152. doi: 10.1182/blood-2015-01-621631.

Schraufstatter, I. U., Chung, J. and Burger, M. (2001) 'IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways', *Am J of Physiol. Lung Cellular and Molecular Physiology*. doi: 10.1080/01418618208236928.

Serafini, M. *et al.* (2014) 'Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells', *Stem Cell Research*. doi: 10.1016/j.scr.2014.01.006.

Shi, C. *et al.* (2011) 'Bone Marrow Mesenchymal Stem and Progenitor Cells Induce Monocyte Emigration in Response to Circulating Toll-like Receptor Ligands', *Immunity*. doi: 10.1016/j.immuni.2011.02.016.

Shlush, L. I. *et al.* (2014) 'Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia', *Nature*. Nature Publishing Group, 506(7488), pp. 328–333. doi: 10.1038/nature13038.

Sinclair, A. *et al.* (2016) 'CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells', *Blood*, 128(3), pp. 371–383. doi: 10.1182/blood-2015-08-661785.

Steele, C. W. *et al.* (2016) 'CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma', *Cancer Cell*. The Authors, 29(6), pp. 832–845. doi: 10.1016/j.ccell.2016.04.014.

Swaminathan, S. *et al.* (2015) 'Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia', *Nature Immunology*, 16(7), pp. 766–774. doi: 10.1038/ni.3160.

Swiergiel, A. H. and Dunn, A. J. (1999) 'The roles of IL-1, IL-6, and TNFα in the feeding responses to endotoxin and influenza virus infection in mice', *Brain, Behavior, and Immunity*. doi: 10.1006/brbi.1999.0565.

Takam Kamga, P. *et al.* (2016) 'Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia.', *Oncotarget*, 7(16), pp. 21713–27. doi: 10.18632/oncotarget.7964.

Takeuchi, S. *et al.* (1997) 'TEL is one of the targets for deletion on 12p in many cases of childhood B-lineage acute lymphoblastic leukemia', *Leukemia*. doi:

10.1038/sj.leu.2400743.

Taylor, G. M. *et al.* (2009) 'Strong association of the HLA-DP6 supertype with childhood leukaemia is due to a single allele, DPB1*0601', *Leukemia*. doi: 10.1038/leu.2008.374.

Tokoyoda, K. *et al.* (2004) 'Cellular niches controlling B lymphocyte behavior within bone marrow during development', *Immunity*. doi: 10.1016/j.immuni.2004.05.001.

Tong, Y. and Linheng, L. (2006) 'The stem cell niches in bone', *The Journal of Clinical Investigations*, 116(5), pp. 1195–1201. doi: 10.1172/JCI28568.the.

Torrano, V. *et al.* (2011) 'ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor', 118(18), pp. 4910–4919. doi: 10.1182/blood-2011-05-354266.

Tsai, A. G. *et al.* (2008) 'Human Chromosomal Translocations at CpG Sites and a Theoretical Basis for Their Lineage and Stage Specificity', *Cell*. doi: 10.1016/j.cell.2008.10.035.

Tsuzuki, S. *et al.* (2004) 'Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice.', 101(22), pp. 8443–8448.

Tsuzuki, S. and Seto, M. (2013) 'TEL (ETV6)-AML1 (RUNX1) initiates self-renewing fetal pro-b cells in association with a transcriptional program shared with embryonic stem cells in mice', *Stem Cells*, 31(2), pp. 236–247. doi: 10.1002/stem.1277.

Vilchis-Ordoñez, A. *et al.* (2015) 'Bone marrow cells in acute lymphoblastic leukemia create a proinflammatory microenvironment influencing normal hematopoietic differentiation fates', *BioMed Research International*. doi: 10.1155/2015/386165.

Vilenchik, M. M. and Knudson, A. G. (2003) 'Endogenous DNA double-strand breaks: Production, fidelity of repair, and induction of cancer', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.2135498100.

Visnjic, D. *et al.* (2004) 'Hematopoiesis is severely altered in mice with an induced osteoblast deficiency', *Blood*. doi: 10.1182/blood-2003-11-4011.

Wang, L. C. *et al.* (1997) 'Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL', *EMBO Journal*. doi: 10.1093/emboj/16.14.4374.

Wang, L. C. *et al.* (1998) 'The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow', *Genes and Development*. doi: 10.1101/gad.12.15.2392.

Wang, X. *et al.* (2018) 'TGF-β1 Negatively Regulates the Number and Function of Hematopoietic Stem Cells', *Stem Cell Reports*. doi: 10.1016/j.stemcr.2018.05.017.

Waterman, R. S. *et al.* (2010) 'A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype', *PLoS ONE*. doi: 10.1371/journal.pone.0010088.

Van Der Weyden, L. *et al.* (2011) *Modeling the evolution of ETV6-RUNX1-induced B-cell precursor acute lymphoblastic leukemia in mice*, *Blood*. doi: 10.1182/blood-2011-02-338848.

Wiemels, J. L. *et al.* (1999) 'Prenatal origin of acute lymphoblastic leukaemia in children', *Lancet*. doi: 10.1016/S0140-6736(99)09403-9.

Wiemels, J. L. *et al.* (1999) 'Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero.', *Blood*, 94(3), pp. 1057–62.

Yamagata, T., Maki, K. and Mitani, K. (2005) 'Runx1/AML1 in Normal and Abnormal Hematopoiesis', *International Journal of Hematology*. doi: 10.1532/IJH97.05075.

Zambetti, N. A. *et al.* (2016) 'Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia', *Cell Stem Cell*. doi: 10.1016/j.stem.2016.08.021.

Zelent, A., Greaves, M. and Enver, T. (2004) 'Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia', *Oncogene*, 23(24), pp. 4275–4283. doi: 10.1038/sj.onc.1207672.

Acknowledgments

I firstly thank my tutor, Chiara Palmi, for her constant and discreet help. She left me free to realize my ideas and grow up without never making me feel alone. I think that this is a very rare human quality...
I wish her all the best for her career!
I also want to thank the hidden driving forces of the Tettamanti Centre, that are all the senior researchers (each of these women, with their own personal characteristics, has taught me something precious… in addition to be precious friends), our secretary Jenny (for her multitasking superpower!) and the diagnostic team (people really dedicate to work… and fun!).
I also thank Dott. Giovanni Cazzaniga and Prof. Andrea Biondi for giving me this challenging opportunity.
Finally, I thank Mary and Clara, companions of misfortune in the PhD adventure: with them, no obstacle was so great that it could not be overcome. It is an honor to share the goal with these tenacious women!
Of course, I thank my beautiful and crazy family, that is my greatest treasure.