Role of CD40 and Zeb1 in Shaping Normal and Leukemic Bone Marrow Niche

Thesis

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Role of CD40 and Zeb1 in Shaping Normal and Leukemic Bone Marrow niche

Thesis presented for the degree of Doctor of Philosophy
The Open University, Milton Keynes (UK)

School of Life, Health and Chemical Sciences

Barbara Bassani
Personal Identifier: F8731025

April 2021
DECLARATION

The data presented in this thesis are original, were not previously used for any other PhD degree and were originated by me and by the cited below collaborators. During my PhD I worked in the Molecular Immunology Unit, Department of Research, at the Fondazione IRCCS Istituto Nazionale dei Tumori. My director of studies was Dr. Sangaletti Sabina (PhD) and my Supervisor was Dr. Colombo Mario Paolo (PhD).

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Immunohistochemistry included in this thesis (Figures 14, 26A,C, 27B, 29C, 31D) were performed by Prof. Tripodo Claudio (MD, PhD) and Dr. Alessandro Gulino, Tumor Immunology Unit, Department of Health Sciences, University of Palermo.

Bioinformatic analyses included in this thesis (Figures 13, 24 D, E) were performed by Dr. Simonetti Giorgia, Immuno-Hematology Unit, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS.

Real time analysis of human bone marrow mesenchymal cells (Figure 27A) was performed by Dr. Marilena Ciciarello (PhD), Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna.

All in vivo experiments were performed with the support of Laura Botti, the technician specialized in the animal manipulation and surgery.

The results reported in the 4.2 chapter of this thesis are included in a manuscript submitted and published on bioRxiv as a pre-print (doi: https://doi.org/10.1101/2020.08.10.243691) and published in Frontiers in Immunology1.
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<table>
<thead>
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<th>Definition</th>
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<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiogenin-1</td>
</tr>
<tr>
<td>APL</td>
<td>acute promyelocytic leukemia</td>
</tr>
<tr>
<td>arg1</td>
<td>arginase-1</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>BMT</td>
<td>BM transplantation</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine</td>
</tr>
<tr>
<td>CAR</td>
<td>CXCL12-abundant reticular cells</td>
</tr>
<tr>
<td>CBD</td>
<td>p300-P/CAF binding domain</td>
</tr>
<tr>
<td>cDC1</td>
<td>conventional Type-I DCs</td>
</tr>
<tr>
<td>CDKL2</td>
<td>cyclin-dependent protein kinase-like 2</td>
</tr>
<tr>
<td>CEBPA</td>
<td>CCAAT/enhancer-binding protein alpha</td>
</tr>
<tr>
<td>CID</td>
<td>CtBP interaction domain</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin kinase inhibitor</td>
</tr>
<tr>
<td>CLPs</td>
<td>common lymphoid progenitors</td>
</tr>
<tr>
<td>CM</td>
<td>central memory</td>
</tr>
<tr>
<td>CN-AML</td>
<td>Cytogenetically normal AML</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CR</td>
<td>complete remission</td>
</tr>
<tr>
<td>CSCs</td>
<td>cancer stem cells</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DLBCL</td>
<td>diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyl transferase</td>
</tr>
<tr>
<td>EBF1</td>
<td>early B-cell factor 1</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>EFS</td>
<td>event-free survival</td>
</tr>
<tr>
<td>EM</td>
<td>effector memory</td>
</tr>
<tr>
<td>eMDSC</td>
<td>early stage myeloid derived suppressor cells</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelia-to-mesenchymal transcription</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>EVs</td>
<td>extracellular vesicles</td>
</tr>
<tr>
<td>FAB</td>
<td>Franco- American-British</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FLT-3</td>
<td>fms like tyrosine kinase 3</td>
</tr>
<tr>
<td>FOG1</td>
<td>friend of GATA-1</td>
</tr>
<tr>
<td>GEO</td>
<td>gene expression omnibus</td>
</tr>
<tr>
<td>GEP</td>
<td>gene expression profiling</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
</tbody>
</table>
GMPs - granulocyte-macrophage progenitors
GVHD - graft-versus-host disease
HC - healthy controls
HDACi - HDAC inhibitors
HiDAC - high-dose cytarabine
HIF-1 - hypoxia-inducible factor-1
HMs - Haematological malignancies
hMSCs - human MSCs
HRP - horseradish peroxidase
HS - heavy smokers
HSCs - hematopoietic stem cells
HSPC - Hematopoietic stem and progenitor cell
i.v. - intravenous
ICAM - intercellular adhesion molecule
IDH - isocitrate dehydrogenase
IDO - indoleamine 2,3-dioxygenase
IFNs - interferons
IGF - insulin growth factor
IHC - Immunohistochemistry
IL - interleukin
ISCT - International Society of Cellular Therapy
JAKs - Janus kinases
JNK - c-Jun-NH2-kinase
KD - knock-down
LepR - leptin receptor
Lin - Lineage negative cells
LPS - lipopolysaccharide
LSD1 - lysine demethylase1
LSK - Lin−Sca-1+c-Kit+
LT-HSC - long-term-hematopoietic stem cells
LTHSC - long-term HSC
M-CSF - macrophage colony-stimulating factor
MCL - mantle cell lymphoma
MCP-1 - monocyte chemotactic protein 1
MEM - Minimum Essential Medium
MHC - major histocompatibility complex
MLPs - myelo-lymphoid progenitors
mMSCs - Mouse MSCs
MOI - multiplicity of infection
MPPs - multipotent progenitors
MSCs - Mesenchymal stem cells
Nes - Nestin
NG2 - neural/gial antigen 2
NK - natural killer
NPM - nucleophosmin
OPN - osteopontin
OS - overall survival
PanINs - pancreatic intraepithelial lesions
PARP - poly ADP ribose polymerase
PBMC - peripheral blood mononuclear cell
PCAF - P300/CBP-associated factor
PGE2 - prostaglandin E2
PI3K - phosphatidylinositol 3-kinase
qPCR - quantitative polymerase chain reaction
r/r - relapsed or refractory
s.c. - subcutaneously
SCF - Stem cell factor
Scr - Scramble
shRNA - short hairpin RNA
SID - Smad interaction domain
SMZL - splenic marginal zone lymphoma
SOCS - suppressor of cytokine signalling
STAT - signal transducer and activator of transcription
T-ALL - T-cell acute lymphoblastic leukemia
TAMs - tumor-associated macrophages
TBS - tris-buffered saline
TCGA - Cancer Genome Atlas
Teff - Tefector
TET2 - Tet Methylcytosine Dioxygenase 2
TFs - transcription factors
TGFβ - transforming growth factor-β
THPO - Thrombopoietin
TKI - tyrosine kinase inhibitors
TLR - toll like receptor
TNF - tumor necrosis factor
TNFR - TNF receptor
TRAFs - TNFR-associated factors
Treg - Tregulatory
VCAM-1 - vascular cell adhesion molecule 1
VEGF - vascular endothelial growth factor
VEGFR2 - vascular endothelial growth factor receptor 2
WHO - world health organization
YAP - Yes-associated protein
Zeb - Zinc finger E-box binding homeobox
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ABSTRACT

Acute myeloid leukaemia (AML) is an aggressive disease impairing normal haematopoiesis. Beyond the genomic landscape and the mutational profile of AML cells, increasing evidence demonstrated that bone marrow (BM) microenvironment contributes to leukemia development and, in particular, the immunologic landscape is emerging as a crucial component of leukemic microenvironment correlating with clinical parameters. Leukemic cells express different antigens that are able to activate T-cells triggering an anti-leukemia immune response. Thus, we hypothesized that immunogenic leukemia cells could set up mechanisms for immune escape including the establishment of immunosuppression. To escape from immune recognition leukemic cells could instruct neighbouring mesenchymal (MSCs) cells to trigger inhibitory T cell pathways through the up-regulation of CD40 or autonomously gain immunosuppressive properties activating epithelial to mesenchymal transition-related programs (i.e. ZEB-1). Hence, the aim of this study is to evaluate the impact of ZEB-1 expression on AML cell ability to mold an immunosuppressive microenvironment. Moreover, we also evaluated the role of CD40 on BM-MSCs in shaping the BM tolerogenic niche during leukemia development and in bone marrow transplantation.

To this aim we firstly analysed the gene expression profile of 61 AML cases that were divided in “ZEB1low” and “ZEB1high”. The two groups showed difference between the karyotypic features and expressed different levels of immunomodulatory markers. Analysis performed using public datasets, showed that ZEB1high patients displayed a worse overall survival compared with ZEB1low patients. Immunohistochemistry analysis performed on archival BM biopsy showed that ZEB1high cases also showed an expansion of CD3+IL-17A+ cells. To unveil the real contribution of ZEB-1 in shaping a BM microenvironment, we moved from humans to mouse models. Zeb-1 expressing or silenced C1498 murine AML cells were injected intra-bone in immunocompetent mice. Mice injected with Zeb-1+ cells showed a higher engraftment compared with mice injected with silenced cells. This phenotype correlated with an expansion of Th17 and an increase of TIM3+PD1+CD8+ cells within the BM of Zeb-1+ cell injected mice. Molecular studies highlighted that Zeb-1 silencing in C1498 induced a significant reduction of Il-6, Tgfβ and Il-23, cytokines involved in Th17 differentiation and maintenance. In turn, IL-17A promoted AML cell proliferation and aggressiveness in-vitro. Moreover, Zeb-1 silenced cells showed a reduced expression of Arg1 that has been reported to negatively affect the
activation of CD8+ T cells leading to the increase of cytotoxic CD8+ cells in mice injected with silenced C1498. In addition, Zeb-1 was able to up-regulate the expression of Cd40 and Ido-1 on BM-MSCs. Given the importance of stromal Cd40 expression in sustaining the progression of other haematological diseases, we hypothesized that its expression can be able to affect AML development. To this end, we injected C1498 cells in Cd40-KO mice and we showed that Cd40 deficiency was associated with graft failure. To evaluate the immune-regulatory role of stromal Cd40, we performed BM transplantation experiments. We observed that Cd40 deficiency leads to the persistent T-cell activation with the expansion of CD4+ INF-γ/TNF-α effector T cells and with the parallel depletion of regulatory T cells due to an increase production of Ox40l. Our results suggest that ZEB1 expression could characterize a group of particularly aggressive and chemotherapy resistant AML whose poor outcome is the result of both intrinsic clone aggressiveness and the immunosuppressive microenvironment initiated by ZEB1.
1. INTRODUCTION

1.1 Normal haematopoiesis

The blood system consists of more than ten different types of cells (lineages), each having peculiar functions. Among these cells, leukocytes are involved in immune response, red blood cells provide oxygen/carbon dioxide transport and platelets, derived from megakaryocytes are crucial players of coagulation and wound healing. The lifetimes of the several mature blood cell types range from hours to years. In emergency situations, including infections or anemia, blood cell counts rapidly increase and, only after recovery, declines back to normal. The process allowing the development of the different blood components is termed “hematopoiesis”. Doulatov et al. reported that hematopoiesis was postulated for the first time by Maximow in 1909 as a hierarchic process where blood cells at the basis derived from common stem cell progenitors that, during differentiation, loose their self-renewal and differentiation potential generating terminally differentiated mature cells.

In particular, blood cell repertoire arises from hematopoietic stem cells (HSCs) within the bone marrow (BM) that give rise to multipotent progenitors (MPPs) retaining multi-lineage potential. Increasing evidence demonstrated that MPPs first lose their erythroid/platelet/megakaryocytic potential and differentiate into myelo-lymphoid progenitors (MLPs) characterized by the ability to generate both myeloid and lymphoid lineage cells. Downstream of MLPs, we can find the granulocyte-macrophage progenitors (GMPs) that give rise to granulocytes, monocytes and myeloid dendritic cells and the common lymphoid progenitors (CLPs) that can differentiate into B lymphocytes, T lymphocytes and natural killer (NK) cells.

Thus, hematopoiesis represents a complex process that is finely tuned by both cell-intrinsic factors, including transcription factors (TFs) or epigenetics modifier and extrinsic factors, i.e. cytokines or molecules released by stromal cells within BM microenvironment.

1.1.1 Cell-intrinsic factors

The differentiation of HSCs into lineage-committed cells is associated to the expression of lineage-specific genes and concomitantly to the repression of those peculiar of another
lineage, pointing out that during hematopoiesis cells display relevant changes in their gene expression repertoire. These modifications are regulated by TFs, co-factors, chromatin modifiers, microRNAs, and other regulatory RNAs. A classic example of these alterations is represented by the “GATA switch” that occurs before erythropoiesis from HSCs. During the differentiation of HSCs into erythroid cells, GATA-1 in a complex with friend of GATA-1 (FOG1) directly represses GATA-2 transcription. GATA-1 – FOG1 complex induces the displacement of GATA-2 at the c-Kit locus, resulting in a chromatin rearrangement and in turn, in the down-regulation of c-Kit expression in HSCs. Laurenti et al using transgenic mouse models demonstrated that c-myc and N-myc are two crucial TFs regulating HSC proliferation, survival and differentiation, indeed, their combined deficiency resulted in a pancytopenia due to the induction of HSC apoptosis. Using an in vitro culture system, it has been demonstrated that E2A instructs lymphoid cell-fate activating the early B-cell factor 1 (EBF1) that antagonize the expression of myeloid-inducing factors such as pu.1, c/ebpa, and Id2 and parallely enhancing Pax5 activity, in turn promoting B-lineage commitment.

Regarding the epigenetic modifications, it has been shown that DNA methylation is associated generally to the up-regulation of differentiation genes, highlighting that DNA methyl transferase (DNMT) loss promotes the HSC expansion. Indeed, DNMT3a down-regulation has been demonstrated to decrease self-renewal genes, including Runx1 and Gata3. Moreover, the concomitant loss of both DNMT3a and DNMT3b in HSCs induces the activation of β-catenin pathway leading to the expansion of HSCs.

Polycomb complex protein BMI-1 expression, that is conventionally associated to chromatin compaction and decreasing nucleosomal turnover, regulates HSC self-renewal in mice through the repression of senescence-related gens, i.e. p16Ink4a, p19Arf, and p53. Another epigenetic modifier, the lysine demethylase1 (LSD1), which belong to the histone demethylase family has been reported to promote erythroid differentiation by inducing alterations in the GATA-2 locus. Importantly, all these intrinsic factors, either TFs or epigenetic regulators play a crucial role in maintaining the balance between quiescence and proliferation, self-renewal or differentiation, survival versus death. Potential alteration of their interplay lead to haematological malignancies.
1.1.2 Cell-extrinsic factors

The hematopoietic fate is also regulated by several extrinsic stimuli (i.e. membrane-bound, soluble cytokines, or extracellular matrix-associated ligands) that are released by the BM microenvironment.

BM is a very heterogeneous tissue that includes different cell types, such as blood cells, mesenchymal cells, osteoblasts, osteoclasts, endothelial cells, reticular cells, fat cells, and many other less-defined types. These cells provide numerous signals sustaining HSC self-renewal and functions. In particular, it has been shown that a multitude of cytokines, along with their receptors are important regulators of haematopoiesis. As reviewed by Mirantes et al, pro-inflammatory cytokines are able to regulate HSC or progenitor cells proliferation and differentiation. In particular interferons (IFNs) can exert either an anti-proliferative activity or promote HSC expansion. In this context, in vitro studies showed that IFN-I suppresses HSC quiescence by affecting the transcription of cell cycle regulators, such cyclin kinase inhibitor (CKI), Cdkn1c (p57) and Foxo3a and inhibit Notch and TGFβ pathways. On the contrary, it has been demonstrated that IFN-γ is able to negatively regulate HSC proliferation in mice, mainly by inducing the expression of suppressor of cytokine signalling (SOCS) 1 in HSCs that reduce thrombopoietin-induced phosphorylation of signal transducer and activator of transcription (STAT) 5, one of the main positive regulators of HSC self-renwal. Moreover, Reynaud et al., using a mouse model recapitulating the main features of human chronic myelogenous leukemia demonstrated that mmps, expressing high levels of interleukin (IL)-6 receptor, are sensitive to IL-6 stimulation, that, in vitro promote myeloid lineage differentiation abrogating lymphoid lineage output.

Other cytokines, including IL-1, IL-17 and tumor necrosis factor (TNF) play an important role in haematopoiesis. Indeed, IL-1 has been reported to induce myelopoiesis during infections and IL-17 positively regulates granulopoiesis and megakaryopoiesis. Finally, TNF has been shown to suppress HSC activity in vivo.

1.2 Myeloid malignancies

Haematological malignancies (HMs) are a heterogeneous group of neoplastic diseases involving hematopoietic and lymphoid tissues, with clinical presentation as leukaemia, lymphoma, or myeloma. During the last decades, several classifications have been
Introduction

proposed (as reviewed in 23-28). The World Health Organization (WHO) in 2001 developed the first worldwide consensus classification on hematological tumors based on their clinical, morphologic, biologic, immunophenotypic and genetic features 29. Thereafter, in 2008 a new classification of HMs was published by WHO in conjunction with the Society for Hematopathology and the European Association of Hematopathology 27. The last update was in 2016, when the WHO classification was ameliorated based on novel information, including genetic mutations recently discovered (as reviewed in 28,30). During the last edition of the International Classification of Diseases for Oncology, two distinct groups of HMs have been recognized depending on cell lineage myeloid and lymphoid 24-26. Within each of these groups, a further sub-classification exists according to cell origin, levels of maturation, morphology, molecular markers, pathophysiological features, and clinical behaviour 31.

In particular, myeloid malignancies comprise a heterogenous group of clonal hematopoietic disorders that arise from the abnormal expansion and impaired differentiation affecting myeloid lineages These neoplasms are the result of a multistep process involving the accumulation of genetic and epigenetic lesions in HSCs and myeloid progenitors 27. Myeloid malignancies can be divided into acute and chronic myeloid neoplasms, that can be further classified on the basis of morphology and percentage of immature myeloid cells (blasts) in peripheral blood or BM according to Swerdlow et al. 32. In particular, 5 categories have been described: acute myeloid leukemia, myeloproliferative neoplasms, myelodysplastic syndrome, myelodysplastic/myeloproliferative neoplasms, and un-known myeloid neoplasms.

1.3 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a clonal disorder resulting from the neoplastic transformation of HSCs or progenitor cells that lose the ability to differentiate leading to the aberrant accumulation of immature myeloid cells within the BM. It is the most common form of leukemia accounting for 80% of all cases in the category of adult acute leukemia 33. GLOBOCAN estimates that the worldwide incidence of AML for 2012 was 4.7 new cases per 100,000 inhabitants per year in Europe and the United States, with a 5-year prevalence of 1.5% and a Male:Female ratio of ~1.4 34. Clinically, it is a heterogenous disease with an unclear etiology. It has been reported that several events contribute to the AML development, including environmental, socioeconomic, epigenetic and genetic factors 35. Moreover, even if in the majority of
cases appears as a de novo neoplasm, in some cases, AML can be the consequence of prior cytotoxic therapies with intercalating drugs or radiation. The diagnosis of AML is defined by the presence of more than 20% of myeloid blasts in the bone marrow.

### 1.3.1 Pathophysiology and classification

Chromosomal abnormalities can be observed in the 55% of the adult and at least in the 70% of childhood AML cases. Well-characterized chromosomal translocations, including the t(8:21) in core-binding factor AML (CBF-AML) or the t(15:17) in acute promyelocytic leukemia (APL) lead to the generation of chimeric proteins, the RUNX1-RUNX1T1 and the PML-RARA, respectively. In addition to chromosomal rearrangements, also genetic mutations have been recognized in more than 97% of cases.

In vivo studies contributed to the development of the “two-hit” model of leukemogenesis. Accordingly, class I mutations, i.e. internal tandem duplications (fms like tyrosine kinase 3 (FLT-3) FLT3-ITD), K/NRAS, TP53 and c-KIT occur with class II mutations, including nucleophosmin (NPM)1 and CCAAT/enhancer-binding protein alpha (CEBPA).

Recently, also alterations in epigenetic regulators (DNMT3A, Tet Methylcytosine Dioxygenase 2 (TET2), and isocitrate dehydrogenase (IDH) 1 and 2, have been proposed as a third class of mutations able to effect cellular differentiation and proliferation.

Previously, AMLs classification was mainly based on the morphological and immunohistochemical characteristic of leukemic cells observed in the patients’ BM and in their peripheral blood. The first comprehensive classification system of AML was developed in 1976 by the Cooperative Group Franco-American-British (FAB) (reviewed in). According to these directives, AML were divided into eight subtypes (M0-M7) based on AML cell type from which AML originates (Figure 1).
Therefore, in 2001 the WHO introduced a new classification system that was further improved in 2008. The last revision of 2016 takes also into account the genetic landscape in addition to morphology, immunophenotype and clinical presentation, thus defining six major groups: AML with recurrent genetic abnormalities; AML with myelodysplasia-related features; therapy-related AML; AML not otherwise specified; myeloid sarcoma; and myeloid proliferation related to Down syndrome. Moreover, further 11 subtypes were identified among those patients characterized by recurrent genetic abnormalities based on the peculiar chromosomal translocations. AML carrying NPM1 and CEBPA mutations were introduced as part of the 2008 revision. Nonetheless, only in 2016 revision AML with BCR-ABL1 translocation and RUNX1 mutated AML were considered in the classification (Figure 2).

**Figure 1 - FAB classification of AML** (adapted from [40]).

<table>
<thead>
<tr>
<th>M1</th>
<th>Acute myeloid leukaemia without maturation</th>
<th>Acute myeloid leukaemia without maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium-sized blasts with high nuclear/cytoplasmic ratio; eosinophilic, basophilic, or azurophilic granulation; blasts with or without perinuclear halo. Blasts can be seen in less than 10% of bone marrow aspirate.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunophenotype:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45+, CD33+, CD13+, CD117+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figure 1</td>
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<table>
<thead>
<tr>
<th>M2</th>
<th>Acute myeloid leukaemia with maturation</th>
<th>Acute myeloid leukaemia with maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small to medium-sized blasts with high nuclear/cytoplasmic ratio; eosinophilic, basophilic, or azurophilic granulation; blasts with or without perinuclear halo. The cytoplasm is basophilic and can contain Auer rods.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunophenotype:</td>
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<tbody>
<tr>
<td>Morphology:</td>
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<tr>
<td>Acute promyelocytic leukaemia with minimal differentiation. The nucleus is usually round or oval. The cytoplasm is generally more basophilic than in normal promyelocytes and contains azurophilic granulation. Some atypical Auer rods are seen in 5% to 10% of cases.</td>
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<tr>
<td>Morphology:</td>
<td></td>
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<tr>
<td>Highly mature, polymorphic blasts. The nucleus is usually round or oval. Promyelocytes and blasts have a round to oval nucleus with a prominent nucleolus and a thin chromatin net. The cytoplasm contains basophilic stippling, eosinophilic granulation, and fragments of megakaryoblasts.</td>
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<td>Morphology:</td>
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<tr>
<td>Monoblastic blasts with high nuclear/cytoplasmic ratio; eosinophilic, basophilic, or azurophilic granulation; blasts with or without perinuclear halo. The cytoplasm is basophilic and can contain Auer rods.</td>
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<tr>
<td>Immunophenotype:</td>
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<td>Morphology:</td>
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<tr>
<td>Medium-sized blasts with high nuclear/cytoplasmic ratio; eosinophilic, basophilic, or azurophilic granulation; blasts with or without perinuclear halo. The cytoplasm is basophilic and can contain Auer rods.</td>
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<tr>
<td>Immunophenotype:</td>
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<tr>
<td>Medium-sized blasts with high nuclear/cytoplasmic ratio; eosinophilic, basophilic, or azurophilic granulation; blasts with or without perinuclear halo. The cytoplasm is basophilic and can contain Auer rods.</td>
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<tr>
<td>Immunophenotype:</td>
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<tr>
<td>CD45+, CD33+, CD13+, CD68+, CD14+, CD64+, CD105+</td>
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The relevance of genetic alterations was recognized also for the prognosis of AML patients, thus strongly influencing the complete remission (CR) and patients’ overall survival (OS). Accordingly, AML were stratified into favorable, intermediate or adverse prognostic risk groups based on their mutational profile alone. In this context, t(8;21), t(15;17) or inv(16) chromosomal alterations grant a favorable prognosis, while three or more chromosomal abnormalities in the absence of any of the recurrent genetic abnormalities (complex karyotype) have all been associated with a significantly poor prognosis. Cytogenetically normal AML (CN-AML) representing the group of patients without any recognizable translocation or cytogenetic alteration, have been reported to display an intermediate risk with a 5-year survival rates between 35% and 45% (Figure 3).
1.3.2 Standard therapy

During the last decade, significant improvements in the treatment of AML have been obtained with the introduction of target agents. However, the standard therapy is still represented by chemotherapy that is conventionally administered to patients into two different phases, the so term “induction” and the further “consolidation” 37.

During the first phase, patients undergo to a short but intensive period of treatment aimed at reducing the number of blasts within the BM, while the consolidation phase is administered to eliminate the any remaining leukemia cells. In the second phase, chemotherapy is given in cycles, with each period of treatment followed by a rest period allowing patients’ recovery 37. The mainstay of induction therapy in younger patients (called “7 + 3 regimen”) consists in the administration of Cytarabine (cytosine arabinoside or ara-C) for 7 days along with an anthracycline (replaced by fludarabine or etoposide in cardiopathic patients) for 3 days 43. For some particular subtype of AML, a third drug could be added to implement the treatment. For instance, in the case of CN-AML carrying the FLT3 mutation, the multi-kinase inhibitor midostaurin might be given along with chemotherapy. For some patients having a CD33-positive AML, the gemtuzumab ozogamicin, a humanized IgG4 anti-CD33 could represent a combined approach 43.

Following the first cycle of chemotherapy, a second round could be given whether in the BM are detected leukemic cells. Importantly, the induction phase usually does not induce the complete elimination of blast in patients, but patients achieve the remission, meaning that within the BM, fewer than 5% blast cells are observed, upon chemotherapy treatment. Further treatments in the consolidation phase are administered depending on patients’ status. In younger patients from 3 to 4 cycles with high-dose cytarabine (HiDAC) are usually employed 44. This treatment might be followed by an allogeneic stem cell transplant or an autologous stem cell transplant. Older patients or patients who developed severe side effects during the induction treatment receive low-intensity chemotherapy with a single agent or a combination of drugs (chemotherapy and target drugs) or a non-myeloablative stem cell transplant 45. It has been reported that after the induction therapy 60%-80% of younger and 40%-60% of older patients can reach a CR 46,47. However, despite the intensive consolidation therapy approximately half of younger and 80%-90% of older patients relapse 46,47.
1.3. 3 Novel drugs

Besides to conventional therapies, recently, some novel drugs have been approved given the increase understanding in AML molecular underpinnings. These agents include FLT3-ITD inhibitors, such as sorafenib, quizartinib and midostaurin, signal transducer and activator of transcription (STAT) inhibitors, i.e. STAT-3 inhibitors C188-9 and MM-206 and the antipsychotics pimozide, and IDH1/IDH2 small molecule inhibitors, including BAY-1436032, Enasidenib (AG-221) and the Ivosidenib (AG-120).

1.4 The BM microenvironment in normal hematopoiesis

As previously discussed in 1.1-chapter, haematopoiesis is a complex process that is finely tuned by cell-intrinsic mechanisms, but also, and to large extent by cell-extrinsic signals provided by the BM microenvironment, also termed “BM niches”. The concept of BM niches, described as an anatomical and functional unit of physiology that integrates endocrine, autocrine, and paracrine signaling sustaining HSC was introduced for the first time by Schofield in the 1978. In this context, he pointed out the close link and the dependence between the microenvironment and the fate of HSCs. Indeed, he stated that HSC self-renewing, differentiation, mobilization, quiescence and proliferation are deeply influenced by the surrounding cells constituting the BM microenvironment. During the last decades, most of the cellular components of the BM niche have been identified including mesenchymal stem cells, osteoblasts, endothelial cells, adipocytes, sympathetic nervous system, megakaryocytes, and macrophages. (Figure 4). Within the BM, two major niches are described and are represented by the “endosteal niche” and the “vascular (also termed “central”) niche”, which are strictly anatomically and functionally interconnected inside the trabecular bone.
1.4.1 Osteoblastic niche (endosteal niche)

The endosteal niche is found closer to the bone surface and it is involved in HSC quiescence maintenance. This niche is mainly formed by osteoblasts, osteoblastic progenitors, and osteoclasts lining. The endosteal surface of the bone in nearly closed to the vessels. One of the first demonstration of the relevance of osteoblasts in supporting HSC growth was obtained by Taichman, that using in vitro approaches exploiting human osteoblasts and human hematopoietic progenitor cells highlighted the importance of cytokines produced by endosteal niche in hematopoiesis regulation. In addition, it has been shown that in transgenic mice characterized by the constitutive activation of the parathyroid hormone receptor, the increase of osteoblasts producing high levels of the Notch ligand jagged 1 was associated with a parallel enhancement of HSC frequency in the BM. In line with these findings, the conditional ablation of Col2.3-labeled osteoblast lineage in mice leaded to a reversible BM hypocellularity with a decreased HSC number accompanied with an increased extramedullary hematopoiesis. Accordingly, Bowers et al demonstrated that osteoblast ablation resulted in the increase of the LSK Flt3−CD150−CD48− long-term HSC (LTHSC) phenotype and with the decrease of LSK Flt3−CD34−CD49b−CD229− LTHSC subpopulation. Moreover, osteoblasts depleted mice were characterized by the loss of quiescence and reduced long-term engraftment and self-renewal ability. Moreover, the depletion of Osterix+ cells thus inhibiting osteolineage
differentiation resulted in the loss of hematopoiesis in the BM of adult mice \(^{60}\). Several molecules released by osteoblasts are able to influence HSC fate. Stier et al demonstrated that among all, osteopontin (OPN) deletion in mice induced an increase of HSC number in the BM, showing that OPN can be considered a negative regulator of HSCs \(^{61}\). Another important factor expressed by differentiated osteoblasts is represented by agrin, a proteoglycan involved in the neuromuscular junction. Indeed, apoptosis of CD34\(^-\)CD135\(^-\) Lin\(^-\)Sca-1\(+\)c-Kit\(+\) (LSK) cells and impaired hematopoiesis was observed in agrin-deficient mice, conditions that were reverted by an agrin-sufficient stroma \(^{62}\).

Regarding osteoclasts, Mansour et al demonstrated that the impaired osteoclast activity resulted in a reduced osteoblastic differentiation that in turn leaded to the compromised HSC homing to the BM \(^{63}\). It has also been reported that the use of bisphosphonates, a group of drugs that inhibit bone resorption mediated by osteoclasts reduced the frequency of primitive HSCs, while supporting the expansion of the hematopoietic progenitors \(^{64}\). Although all these evidence, the real contribution of osteogenic lineage cells has been questioned \(^{65}\). Recent studies performed in mouse models, including 3D imaging studies \(^{66,67}\) have shown that HSCs are not significantly associated with osteoblasts. Moreover, it has been reported that the specific deletion of CXCL12 \(^{68,69}\) or stem cell factor (SCF) \(^{70}\) from osteoblasts didn’t influence HSC proliferation or their frequency. In this line, hepatocytes and not osteoblasts, has been reported as the major functional source of thrombopoietin (THPO) required for HSC maintenance in mice \(^{71}\). Given this evidence, even if osteoblasts were one of the first cell population that was reported to be involved in HSC regulation, nowadays the most supported hypothesis is that osteolineage cells sustain the maintenance of more committed hematopoietic progenitors, in particular, the lymphoid lineage \(^{72}\).

1.4.2 Vascular niche (central niche)

Vascular niche is found closer to the central zone of the BM and is mainly formed by endothelial cells (ECs), which line the lumen of blood vessels. It has been reported that periarteriolar vascular niche as far as sinusoids strongly regulate the proliferation and differentiation of HSCs. Small arterioles seem to be more resistant to damages compared to sinusoids, suggesting that periarteriolar vessels might play an important role in hematopoiesis recovery after irradiation. Moreover, arterioles are characterized by a population of pericytes expressing neural/glial antigen 2 (NG2), that are involved in HSC quiescence maintenance. The depletion of NG2\(^+\) cells resulted in the reduction of HSCs
with long-term repopulating ability and expansion of cycling HSCs \textsuperscript{67}. Moreover, pharmacological, or genetic activation of HSC proliferation affect their distribution with cycling HSCs that moved from NG2\textsuperscript{+} peri-arteriolar niches to leptin receptor positive (LepR\textsuperscript{+}) peri-sinusoidal niches in mice \textsuperscript{67}. In this context, deletion of CXCL12 \textsuperscript{67} or SCF \textsuperscript{73} from NG2\textsuperscript{+} pericytes leaded to the mobilization of quiescent HSCs to the circulation, and therefore results in a reduction of HSC numbers in the BM of transgenic mice. Hooper et al. demonstrated that the conditional deletion of vascular endothelial growth factor receptor 2 (Vegfr2) in adult mice impairs sinusoidal regeneration and HSC reconstitution after myelosuppression \textsuperscript{74}.

In vitro studies demonstrated that ECs could induce HSC expansion through Notch ligands \textsuperscript{75} and Akt-mTOR-activated endothelial cells regulates HSC self-renewal by releasing CXCL12 and SCF \textsuperscript{76}. Similarly, EC-specific deletion of Notch ligand, Jagged-1, decreased HSC frequency and inhibited hematopoietic regeneration after irradiation \textsuperscript{77}. Furthermore, EC-specific inhibition of NF-κB induced HSC self-renewal and reduced the radiotherapy-related damage to the hematopoietic system \textsuperscript{78}. Other EC-related important factors that support BM hematopoiesis are represented by specific surface markers, including E-selectins, P-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule (ICAM) 1 as demonstrated using in vivo approaches and selective knockout mice \textsuperscript{79}.

1.4.3 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are rare elements within the BM, counting the 0.01\% of total BM cells \textsuperscript{80}. They were firstly described in 1867 as non-hematopoietic cells within the BM displaying self-renewal abilities \textsuperscript{49}. It is only in the 1970 that Dr. Friedenstein reported the isolation of plastic-adherent stromal cells that were able to form fibroblastic-like colonies and can differentiate into osteoblasts and reconstitute the hematopoietic niche in vivo after subcutaneous transplantation \textsuperscript{81,82}. Nowadays, the International Society of Cellular Therapy (ISCT) recognizes MSCs on the basis of their capacity of adhesion to plastic; their ability to differentiate into other cell types, such osteoblasts, adipocytes or chondrocytes and their specific phenotype \textsuperscript{83}. Mouse MSCs (mMSCs) were described as CD45\textsuperscript{−}Ter119\textsuperscript{−}PDGFR\textalpha\textsuperscript{−}Sca-1\textsuperscript{+/−} cells \textsuperscript{84}. Moreover, they were found to secrete CXCL12, a crucial factor that regulates HSC homeostasis \textsuperscript{84}. Regarding humans, the minimum criteria to define human MSCs (hMSCs) are the expression of CD105, CD73, and CD90, and the lack of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR expression. Other
surface antigens commonly expressed by hMSCs include CD13, CD29, CD44, and CD10.

Even if generally recognized as MSCs, more recently, different studies have demonstrated the heterogeneity of MSCs, identifying different sub-population (i.e. LepR⁺) cells, CXCL12-abundant reticular cells (CAR) cells, nestin⁺ cells and CD146⁺ cells) able to differentially regulate HSC biology and characterized by the expression of peculiar markers. In particular, in humans, CD146⁺ cells are usually found in the vascular niche and are characterized by the expression of Angiogenin-1 (Ang-1) and CXCL12 that allow their interaction with Tie-2⁺ and CXCR4⁺ HSC and endothelial cells. Nestin⁺ (Nes⁻) cells are mostly perivascular and support the homing and reduces the mobility of HSCs. They regulate the quiescence of HSCs through the release of several factors, such as CXCL12, SCF, Ang-1, IL-7, VCAM-1 and OPN. CAR cells are mainly found in the endosteal region of the BM and are characterized by the high expression of CXCL12, in turn regulating HSC cell cycle and self-renewing and promoting HSC quiescence. They are also a source of SCF and IL-7 that is crucial for the lymphoid progenitor and mature B cell maintenance.

As reviewed by Pinho and Frenette, the use of transgenic mouse models suggested that Nes–GFP⁺ cells overlap substantially with LepR⁺ cells in the BM. In detail, whole-mount 3D imaging of the BM has shown that LepR⁺ cells overlap with Nes-GFPlow cells and CAR cells that are scattered near sinusoids while Nes–GFPhigh cells expressing the pericyte marker NG2 or the smooth-muscle marker MYH11 are associated with arterioles (Figure 4).

1.5 The BM microenvironment in myeloid malignancies

Increasing evidence demonstrated that BM microenvironment contributes to leukaemia development and progression and several works suggested that it is also influenced by leukemic cells. Advances in BM imaging technologies clearly showed that various cell types are involved in leukemogenesis, including osteoblasts, perivascular stromal cells, endothelial cells, macrophages, CXCL12-abundant reticular cells.
1.5.1 Crosstalk between leukemic cells and BM niche

BM cell and blast interactions play a crucial role in the development of AML and can mediate the onset of resistance to therapy. In this context, niche stromal cells have been reported to prevent cytarabine-induced apoptosis. This phenomenon seemed to be associated with the release of soluble factors that stimulated the up-regulation of BCL-2 in human leukemia cells and occurred only when blasts were co-cultured with stromal cells. Moreover, the expression of the integrin α4β1 and its binding to the VCAM1 or fibronectin induced chemoresistance of AML cells. Regarding leukemia development, several are the BM cells/blasts-mediated mechanisms that have been demonstrated. The CXCR4/CXCL12 axis has been reported as a major regulator of the crosstalk between leukemia cells and BM microenvironment. Generally, this signalling is involved in the retention of blasts in the BM, thus, higher expression of CXCR4 on AML cells has been considered as a negative prognostic factor. The expression of CD44, a protein involved in cell–cell and cell–matrix interactions, on AML cells was shown to favour blast homing, motility and invasive properties and its higher expression along with mmp9 overexpression is associated with aggressiveness and relapse. In vivo studies performed by Miller et al showed that integrin β3 was crucial for MLL-AF9 AML development while it was dispensable for normal hematopoietic stem and progenitor cells engraftment. In addition, glycoprotein CD98 expressed on AML cells have been reported to bind the β1 and β3 integrins on BM niche cells. This interaction promoted murine as far as human cells spreading, migration, survival, and growth. Nishioka et al demonstrated that CD82 mediates adhesion of human AML leukemic stem/progenitor cells to the extracellular matrix, supporting the engraftment and the progression of the disease and speculated that this signalling might provide an advantage over normal hematopoietic stem and progenitor cell (HSPCs). Moreover, Moschoi et al by using co-culture and in vivo experiments observed that stromal cells were able to transfer functional mitochondria to AML cells influencing their survival and resistance to chemotherapy. Accordingly, Marlein et al showed that the inhibition of mitochondrial transfer enhanced AML cell death and prolonged survival of mice transplanted with patient-derived AML cells. Moreover, Von der Heide et demonstrated that BM-MSC derived from AML patients display transcriptional and epigenetic alterations that involved proteoglycans, adhesion molecules, endocytosis pathways, cytokine–cytokine receptor interactions, chemokine signaling and the impairment of crucial metabolic pathways. Increasing evidence have
shown a crucial role of exosomes released by AML cells that can mold the microenvironment supporting AML survival and development. In particular, it has been shown that AML-derived exosomes enhanced AML growth in vivo, while the inhibition of their secretion through the targeting of Rab27a results in a significantly delay of leukemia development. Indeed, AML exosomes are able on one hand to induce the expression of the Dickkopf-related protein 1, a protein involved in the osteogenesis and on the other, downregulate CXCL12, KITL and insulin growth factor (IGF) 1 that represent important factors supporting normal hematopoiesis.

1.5.2 BM stromal cells contribution to AML development

Different studies during the last decades have clearly shown that MSCs are able to affect leukemia growth in vitro and in vivo (Figure 5).

<table>
<thead>
<tr>
<th>Author</th>
<th>MSCs type</th>
<th>Tumor model</th>
<th>Findings</th>
<th>Proposed mechanism</th>
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<tr>
<td>Manabe et al.</td>
<td>Human bone marrow-MSCs</td>
<td>B-Acute lymphoblastic leukemia (patient cells)</td>
<td>Prevent of leukemic cells apoptosis</td>
<td>Prevention of apoptosis by secreting soluble factors and cytokines</td>
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<td>Garrido et al.</td>
<td>Human stromal cell line</td>
<td>AML (patient cells)</td>
<td>Improvement of leukemic cell survival</td>
<td>Inhibition of drug-induced apoptosis of AML cells in direct cell-to-cell contact</td>
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<td>Ramazamy et al.</td>
<td>Human bone marrow-MSCs</td>
<td>Chronic myeloid leukemia (K562 cell line)</td>
<td>Induce leukemic cell growth via reducing apoptosis</td>
<td>Formation of a leukemic stem cell niche to preserve the self-renewal ability of cancer cells</td>
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<td>Liang et al.</td>
<td>Human bone marrow-strom al cell line</td>
<td>Acute myeloid leukemia (HL-60, and HL-60/VCR cell lines)</td>
<td>Induction of specific gene expression, leading to cell cycle arrest</td>
<td>Induction of apoptosis via modulation of Blc-2 and active Caspase-3</td>
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<td>Zhu et al.</td>
<td>Human adipose tissue-MSCs</td>
<td>Acute myeloid leukemia (HL-60 cell line)</td>
<td>Inhibit cancer cell proliferation</td>
<td>Induction of cell cycle arrest through cytokine secretion such as DOK1</td>
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<td>Wei et al.</td>
<td>Leukemia patient's bone marrow-MSCs</td>
<td>Chronic myeloid leukemia (K562 cell line)</td>
<td>Inhibit leukemic cell growth and apoptosis</td>
<td>Induction of apoptosis via phosphorylation of the Akt and bad proteins</td>
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<td>Tian et al.</td>
<td>Human umbilical cord blood-MSCs</td>
<td>Acute myeloid leukemia (HL-60 cell line)</td>
<td>Inhibit leukemic cell growth</td>
<td>Potent proliferation inhibition of leukemic cells via activation of p38 MAPK signaling pathway</td>
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<td>Secchiero et al.</td>
<td>Human bone marrow-MSCs</td>
<td>Lymphoma (BJAB and SKW6.4 cell lines)</td>
<td>Inhibit lymphoma cell growth</td>
<td>Modulation of the lymphomas stromal network by inducing an increase of intra-tumor necrosis</td>
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<td>Han et al.</td>
<td>Human bone marrow-MSCs and CML patient's bone marrow-MSCs</td>
<td>Chronic myeloid leukemia (K562 and patient cells)</td>
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<td>Regulation of apoptosis-related protein expression and activation of the Wnt signaling pathway</td>
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<td>Yuan et al.</td>
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<td>T-Acute lymphoblastic leukemia (Jurkat cell line)</td>
<td>Inhibit Jurkat cell proliferation</td>
<td>Potential shielding effect of MSCs on leukemia cells by activating notch signaling</td>
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<td>Naderi et al.</td>
<td>Human bone marrow-MSCs</td>
<td>Acute lymphoblastic leukemia (patient cells)</td>
<td>Protect of leukemic cells from apoptotic cell death</td>
<td>Inhibition of tumor suppressive activity by PGE2 secretion and activation of cAMP-PKA signaling pathway</td>
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<td>Song et al.</td>
<td>Mouse bone marrow-MSCs</td>
<td>B-lymphoma (A20 cell line)</td>
<td>Inhibit leukemia/lymphoma cell growth</td>
<td>Cell cycle arresting of lymphoma cells due to reduction of interleukin (IL)-10 secretion</td>
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<td>Lee et al.</td>
<td>Human adipose tissue-MSCs</td>
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<td>Induce leukemia cell growth</td>
<td>Induction of cancer cell growth by increasing the luciferase activity</td>
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<td>Fathi et al.</td>
<td>Rat bone marrow-MSCs</td>
<td>Chronic myeloid leukemia (K562 cell line)</td>
<td>Promote apoptosis and change cell cycle distribution of leukemic cells</td>
<td>Induction of apoptosis by secreting the TIMP-1 and CINC-1 cytokines and via BAX and caspase-3 cascade pathways</td>
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Figure 5 – Studies demonstrating that MSCs support leukemia development and progression (adapted from)
In this context, several factors that are potentially released by MSCs (i.e. adhesion molecules, growth factors, cytokines, pro-angiogenic or immunomodulatory molecules) along with molecular alterations in the stromal cells within the BM have been reported to contribute to AML development. It has been demonstrated that osteoblast depletion enhanced MLL-AF9 AML cell proliferation and inhibited normal hematopoiesis in mice. Likewise, a reduced number of NG2+ pericytes and a parallel increase of Nestin-GFP+ MSCs were observed within the BM of MLL-AF9 AML mice. Pasquier et al. have shown that MSCs or ECs were able to transfer their cytoplasmic content (i.e. cytosolic molecules and organelles, including mitochondria) to AML cells by using filamentous actin-based structures forming tunneling nanotubes and in turn promoting the acquisition of chemoresistance. Recently, an increasing attention has been addressed to the role of extracellular vesicles (EVs) in leukemia development and progression. In particular, it has been demonstrated that MSC-EV may exert both pro- and antiangiogenic activities. Indeed, EVs derived from umbilical MSC have been shown to promote MSC migration and proliferation in vitro by activating the Wnt/beta-catenin and the NF-κB pathway. Paradoxically, murine BM-MSC-derived exosomes, that are found to be enrich in miR-16, downmodulated VEGF in breast cancer cells.

1.6 Immunomodulatory properties of MSCs

Several in vitro and in vivo studies have demonstrated that MSCs can acquire immunomodulatory properties playing a crucial role in the maintenance of peripheral tolerance, in transplantation conditions, in autoimmunity and in tumor evasion (reviewed in). Two major actors involved in the regulation of immunomodulatory properties of MSCs are represented by IFN-γ and TNF-α that are crucial orchestrators of the immunosuppressive microenvironment. In vitro studies have shown that MSCs are able to negatively affect lymphocyte activation and proliferation. This effect required cell-cell contacts; indeed, it has been demonstrated that tranwell co-cultures of MSCs and peripheral blood mononuclear cell (PBMCs) did not prevent lymphocytes proliferation. Among the different soluble factors, a role for transforming growth factor-β (TGFβ) and hepatocyte growth factors has been demonstrated in suppressing the T cell proliferation. Moreover, also prostaglandin E2 (PGE2) and the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) have been reported to contribute to negatively regulate lymphocyte activities.
Regarding NKs, MSCs inhibit their proliferation and impair IFN-γ production, dampening their cytotoxic abilities and promoting anergy against target cells. Human NK proliferation along with CD56 expression and cytotoxicity were demonstrated to be partially reduced in vitro by hMSC PGE2 secretion, that conversely did not alter the expression of activatory receptors. Recently it has been shown that hMSCs deeply impact on T cell differentiation, promoting Treg expansion. In this context, Zhang et al stated that MSCs-derived exosomes induce Treg differentiation. In line with this, Court et al demonstrated that the artificial transfer of MSC-derived mitochondria induces Treg differentiation by up-regulating FOXP3, IL2RA, CTLA4, and TGF-β1 in T-cells.

Moreover, MSCs interfere with dendritic cell (DC) differentiation, maturation, and function by releasing soluble factors such as IL-6, PGE2 and macrophage colony-stimulating factor (M-CSF) that inhibit the secretion of IFN-γ and TNF-α by DCs and promote the release of IL-10.

In vivo, outstanding results that functionally prove the immunosuppressive activity of MSCs was achieved in the treatment of graft-versus-host disease (GVHD) after allogeneic stem cell transplantation. Indeed, the systemic infusion of ex-vivo expanded MSCs was able to control lethal GVHD, although the mechanisms involved the immuno-modulatory effects of MSCs remain a critical and unresolved question.

### 1.7 AML and immune system

The immunologic landscape is emerging as a crucial component of the leukemic BM microenvironment correlating with clinical parameters influencing not only the disease progression, but also the response to therapy. Although an increasing attention has been given to the immune infiltrate within the BM of AML patients, the alterations in frequency of functional T cells, that represents a pivotal requirement for the successful application of immunotherapies, has not been fully elucidated. Recently, some studies have demonstrated that the AML BM milieu differs from healthy BM as well as from other hematological malignancies.

In particular, the AML microenvironment is characterized by immune cell dysfunction, an aberrant cytokine production and an increased ratio of suppressive cells. Indeed, since it has been reported that in patients the immune system is able to recognize and kill AML blasts, it is also well known that leukemic cells can set up different mechanisms to escape from host immune responses and bypass immune mediated elimination.
The major mechanisms of immunosuppression are represented by the up-regulation of negative checkpoint molecules including PD-L1 and TIM-3, an increase of arginase-2, the up-regulation of IDO1 and the expansion of MDSC and Treg cells or Th17 cells that are able to inhibit Th1 IFN-γ production. In this context, Sun et al described an increase of the MDSC frequency in the BM of AML patients and demonstrated that only in those patients achieving the complete remission, MDSC levels were significantly decreased after chemotherapy, while no differences were appreciated in the partial remission and non-remission groups. Pyzer et al have shown that AML blasts are able to induce MDSCs via exosomal transfer. Indeed, in vitro, exosomes induce leukemic cells proliferation positively regulating cell cycle proteins. Regarding the T-cell compartment, it is widely known that a high frequency of Tregs in AML patients is associated with a poor disease prognosis. Tregs within the bone marrow of AML patients have been reported to suppress adoptively transferred cytotoxic T cell proliferation, limiting their in vivo expansion, thus contributing to AML progression. CTLA-4+ Tregs have been shown to contribute to tumor immune escape by dampening anti-tumor immune responses and down-regulating CD80/CD86 expression on antigen presenting cells. On the contrary, the role of PD-1 on Treg cells have remained to be clearly understood. Some studies have demonstrated that PD-1 deficiency induces proliferation and suppressive activity of Tregs, suggesting that PD-1 block may need to be applied carefully since it can interfere with Treg activities. Concerning Th17, their frequency has been found significantly higher in the peripheral blood and bone marrow mononuclear cells from AML patients compared with healthy donors. This phenomenon was associated with an increase of (IL)-17, IL-22, IL-23, IL-1β, IL-6, and transforming growth factor (TGF)-β1 in the plasma of patients, identifying these cytokines as major regulator of Th17 differentiation, as also confirmed by in vitro experiments. IL-17A, a signature cytokine secreted by Th17 cells was shown to induce the proliferation of AML cells that express IL-17 receptor positively regulating the activation of PI3K/Akt and Jak/Stat3 signaling pathway. In addition, the combination of IL-17A and IL-22 negatively impact on the generation of Th1 cells and the production of interferon (IFN)-γ from healthy donor or AML patient peripheral blood mononuclear cells. The increase of IL17 producing T cells also correlates with a poor prognosis, whereas patients with high Th1 cell frequency had prolonged survival. Sanchez-Correa et al. have also investigated the role of natural killer cells in AML progression demonstrating that the decrease of their frequency in the BM of patients correlates with a decreased overall survival. NK cell anti-tumor activity is regulated by
the interaction of MHC-I expressed on leukemia cells with inhibitory receptors that have been reported to be up-regulated in AML patients, resulting in an impairment of NK cytotoxic functions\textsuperscript{135}. NK cells are one of the major sources of IFN\textgamma, that is traditionally associated with anti-tumor/immune-activating responses\textsuperscript{136,137}. However, increased expression of IFN-\textgamma and its associated genes have been unveiled in cancer cells and positively correlated with tumor invasion and with an unfavorable response due to the possible activation of immunosuppressive pathways, including the up-regulation of IDO1 and the PD-1/PD-L1 axis\textsuperscript{124,138}, suggesting that IFN-\textgamma may be involved also in tolerogenic and pro-tumoral pathways.

1.8 Zeb-1

Zinc finger E-box binding homeobox 1 (Zeb-1) is a zinc finger and homeodomain transcription factor that is also known as dEF1, ZFHX1A, Nil-2-a, TCF8, AREB6, or BZP. It belongs to the ZEB family that also accounts its homologous protein termed Zeb-2. ZEB factors display a high conservative structure across species\textsuperscript{139} characterized by two C2H2-type flanking zinc finger clusters that allow the interaction with paired CACCT(G)\textsuperscript{140,141} or CAGGT(G)\textsuperscript{142} E-box-like promoter elements on DNA. Zeb-1 also contains binding sites for other proteins, such Smad interaction domain (SID), CtBP interaction domain (CID) and p300-P/CAF binding domain (CBD) through which it has been demonstrated to interact with co-repressors or co-activators\textsuperscript{140,141}(Figure 6).

![Figure 6 - The schematic structure of ZEB1/2](image)

Its expression can be accomplished either at transcriptional or post-transcriptional levels. One of the most known mechanism of regulation is represented by the feedback loop
between Zeb-1 and miR-200 family which represses both Zeb-1 and Zeb-2 to maintain epithelial states $^{143}$. On the contrary, well-described positive regulators are represented by TGF-β, Wnt/β-catenin, NF-κB, PI3K/Akt and Ras/Erk pathways $^{144-146}$. In this context, ZEB1 represents the direct downstream target of the Wnt canonical signalling in lung cancer and its activation leads to the decrease of epithelial genes, including E-cadherin $^{147}$. In parallel, TGF-β induces Smad2 phosphorylation and activates ZEB1-dependent signaling, resulting in an enhanced invasiveness of glioblastoma cells $^{148}$. In addition, TGF-β is able to induce epithelia-to-mesenchymal transcription (EMT) of breast cancer cell lines on one hand up-regulating Zeb-1 levels and concomitantly suppressing miR-200a, miR-200b, miR-200c, miR-141 and miR-429 expression $^{149}$. Moreover, the activation of ERK/MAPK-ZEB1 axis by the hepatocyte growth factor in a model of prostate cancer increases the metastatic potential of tumor cells $^{150}$. ZEB1 has been firstly characterized as a major regulator of cell differentiation given its role during embryogenesis, cell differentiation in cartilage, bone and the regulation of hematopoietic compartment homeostasis and only later recognized for its role in cancer progression $^{141}$. In this context, several studies performed in mouse models and in humans demonstrated that ZEB1 expression can drive EMT by the simultaneous repression and activation of epithelial and mesenchymal genes $^{151,152}$, inducing a more aggressive phenotype $^{153}$. More recently, accumulating evidences suggest that besides being a key contributor of cancer cell metastasis, ZEB-1 is also involved in the regulation of immune cell function, immune escape and in the modulation of tumor chemoresistance (Figure 7) $^{153}$.
Although its role in cell differentiation, immune cell polarization and its pro-tumoral properties in solid cancers, the activity of this transcription factor in hematological malignancies has been largely overlooked because of the perceived lack of relevance in non-EMT contexts. In particular, the mechanisms through which ZEB1 can contribute to leukemia development and pathogenesis remain to be elucidated.

Figure 7 – Zeb-1 pleiotropic functions

1.8.1 Zeb-1 in solid tumours

ZEB family members have been firstly recognized as key factors regulating embryonic development and cell differentiation. Indeed, Zeb-1-/- mice die close to the birth due to widespread skeletal abnormalities. Based on these observations, in the past decades an increasing interest has been addressed to the possible role of Zeb-1 in determining cellular functions and states. In this context, several studies demonstrated a crucial role for Zeb proteins in regulating cell proliferation, response to DNA damage, and cell survival, with a
Introduction

dramatic impact on tumor development, from early steps of tumorigenesis to cancer progression. In particular, in solid tumors, it has been demonstrated that Zeb-1 expression is induced by multiple pathways that can be involved in its activation, including WNT, NF-kB, cyclooxygenase-2 (COX-2), and hypoxia-inducible factor-1 (HIF-1) signalling, stressing the correlation between Zeb-1 expression and an enhanced cellular motility and with the acquisition of stemness and proliferative properties. Moreover, in different solid malignancies, including breast cancer, lung cancer and pancreatic cancer, colon cancer and osteosarcomas, Zeb-1 aberrant expression is involved in the induction of EMT related programs that are associated with an increased aggressiveness, metastatic behavior, onset of resistance to therapy and poor outcome.

High levels of Zeb-1 are observed in a significant fraction of human in situ pancreas adenocarcinoma. These results are also corroborated by in vivo studies, that showed Zeb1 over-expression in noninvasive pancreatic lesions. In line with this, Zeb-1 decreased expression in K-ras mutant mice is associated with a reduction in both the number and grading of acinar ductal metaplasia and pancreatic intraepithelial lesions (PanINs), highlighting the role of Zeb-1 in the early steps of pancreatic tumorigenesis. In lung cancer, Zeb-1 activates phosphatidylinositol 3-kinase (PI3K) by derepressing miR-200 targets inducing an EGFR/ERBB2 autocrine loop and enhancing GATA3-induced expression of the p110α catalytic subunit of PI3K in turn promoting the invasive and metastatic propensities of tumor cells. In a model of breast cancer, Zeb-1 silencing suppresses the ubiquitin ligase CUL4A-driven proliferation also reducing EMT, tumorigenesis, and metastasis. In addition, in human breast cancer cell lines, it has been demonstrated that Zeb-1 silencing leads to the up-regulation of more than 200 genes and to the down-regulation of 30 genes involved in cell adhesion and epithelial differentiation. Lehmann et al have recently pointed out the clinical relevance of Zeb-1 levels, describing a direct interaction between Zeb-1 and Yes-associated protein (YAP), a protein effector of the Hippo pathway. In particular, in a cohort of hormone receptor-negative breast cancers, they showed a positive correlation between Zeb-1/YAP1 expression with a peculiar gene set that has been correlated with worse survival, therapy resistance and increased metastatic risk.

Several studies have also suggested a close correlation between EMT and stemness; indeed, after EMT, human mammary epithelial cells acquire stem-like features. In line with this, Weinberg et al demonstrated that the release of TGFβ by tumor
Introduction

microenvironment activated Zeb-1 that inhibits the expression of stemness-repressing miRNAs, including miR-200, but also of miR-183 and miR-203, which together target BMI1 and in turn promoted the transition from non-cancer stem cells (CSCs) to CSCs\textsuperscript{169}. Accordingly, it has been shown that Zeb-1 expression in normal human mammary stem cells is able to trigger antioxidant programs mainly driven by the methionine sulfoxide reductase MSRB3 that protects stem cells against the oxidative stress\textsuperscript{170}. In breast cancer, Zeb-1 expression is up-regulated by cyclin-dependent protein kinase-like 2 (CDKL2) that is able to activate a positive feedback circuit promoting β-catenin signaling pathway enhancing the mesenchymal characteristics and stem cell-like properties\textsuperscript{171,172}.

Moreover, Zeb-1 expression levels has been associated with an increased resistance to therapy. Indeed Wang et al. found that high ZEB1 levels are associated with a poor response to epirubicin in breast cancer patients. Likewise, in a large cohort of human breast cancer subjects, high levels of Zeb-1 correlate with an increase of Bcl-x1 and cyclin D1, predicting a poor response to chemotherapy\textsuperscript{173}. Molecularly, chemotherapy resistance is mainly due to an activation of the DNA repair system due to the activation of ZEB1/p300/ PCAF (P300/CPB-associated factor, PCAF) complex that activated ATM in turn promoting homologous recombination-mediated DNA damage response to clean up the chemotherapy-induced DNA fragments\textsuperscript{173}. Zeb-1 expression also confers anti-estrogen resistance in breast cancer. Indeed, Zeb-1 is reported to form a complex with DNMT3B and HDAC1 that silences the estrogen receptor (ER)-α promoter, subsequently attenuating the responsiveness of breast cancer cells to antioestrogen treatment\textsuperscript{174}. Bai et al. demonstrated that miR-200c suppressed the transcription factor ZNF217, a positive regulator of TGF-β/Zeb-1, increasing trastuzumab sensitivity and suppressed the invasive abilities of breast cancer cells\textsuperscript{175}.

1.8.2 Zeb-1 in the immune system

Even if Zeb-1 expression has been primarily studied in cancer cells due to its role in the promotion of survival, proliferation and stemness, in recent years several studies have focused on the hematopoietic compartment, and it is now clear that most of the cells of myeloid as (Figure 8) well as lymphoid origin (Figure 9) express Zeb-1 or Zeb-2. In these cells (including dendritic cells, macrophages, monocytes, B, T, and NK cells) Zeb factors regulate key transcriptional networks involved in cell differentiation, maintenance, and function\textsuperscript{176}.
In particular, concerning Zeb-1 expression in myeloid cell lineage, it has been reported that it is primarily expressed by DCs, macrophages and neutrophils\textsuperscript{176} even if its function in granulocytes has yet to be understood. In DCs, Zeb-1 expression is not confined to a particular subset as demonstrated by RNA seq results\textsuperscript{176}. Smita et al demonstrated that toll like receptor (TLR) 9 stimulation increases Zeb-1 levels and subsequently the production of \textit{il-6}, \textit{il-10} and \textit{il-12}, in turn controlling immunogenic responses of CD8\textalpha+ conventional Type-I DCs (cDC1)\textsuperscript{177}. Indeed, co-culture of Zeb1-deficient MutuDCs with CD4+ T helper cells skewed their differentiation toward Th2 subtype rather than a Th1\textsuperscript{177}.

Importantly, a recent study by Cortes \textit{at al} investigated the expression of Zeb-1 in normal and tumor-associated macrophages (TAMs) showing that its expression is crucial for their polarization toward a pro-tumor phenotype\textsuperscript{178}. Indeed, Zeb-1 directly induces ccr2 and positively regulates the expression of Cxcl15, Cd163, Mrc1, mmp9, il-10, Nfkb1/p50, vegf and Retnla in TAMs\textsuperscript{178}. 
Regarding T lymphocytes, ImmGen dataset analysis showed dynamic expression of Zeb-1 and Zeb-2 in T cells. It has been demonstrated that Zeb-1 plays a crucial role in regulating T cell development primarily repressing the E box-containing genes, including Cd4, Il2, Gata3, and Itga4 \(^{179-182}\). In addition, Guan et al showed that naïve CD8+ T cells express high levels of Zeb-1 that decrease upon activation to increase again in of memory CD8+ T cells \(^{143}\). Despite in tumor cells Zeb-1 and Zeb-2 have been demonstrated to cooperate in inducing the more aggressive EMT phenotype, it has been shown that in CD8+ cells their expression is mutually exclusive and, notably, they seem to repress each other \(^{143}\). Indeed, Zeb-1 expression increased in Zeb-2-deficient effector CD8+ T cells while Zeb-2
expression was enhanced in Zeb-1 knock-down (KD) memory CD8+ T cells\textsuperscript{143}. In this context, ChIP-qPCR studies showed that Zeb-1 directly binds the murine Zeb-2 promoter in naïve CD8+ T cells repressing its expression\textsuperscript{143}.

\begin{figure}
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\includegraphics[width=\textwidth]{figure9.png}
\caption{Zeb-1 and Zeb-2 expression in murine lymphoid cells (adapted from\textsuperscript{176}).}
\end{figure}
1.8.3 Zeb-1 in leukemia

Over the past decades, a growing interest has been addressed to the role of Zeb-1 in the control and promotion of hematologic malignancies. Indeed, even if the role of EMT-related proteins has been largely overlooked, recently it has been shown that the deregulation of such factors deeply influences leukemia development and pathological features. In particular, in vitro and in vivo studies suggest that Zeb-1 and Zeb-2 transcription factors are able to exploit an oncogenic or oncosuppressive function depending on the lineage (Figure 10) (reviewed in 142).

In T-cell acute lymphoblastic leukemia (T-ALL) ZEB1 seems to act as a tumor suppressor. Indeed, mutant mice displaying Zeb-1 impaired functions showed defects in lymphocyte maturation developing T-cell lymphoma/leukemia with a median onset of 6 months. Hidaka et al have demonstrated that in T-ALL cells Zeb-1 expression is often dysregulated by several mechanisms, including chromosomal translocations with heterozygous deletion, intragenic mutations or epigenetic mechanisms and its downregulation is associated with a
resistance to TGFβ-mediated growth arrest \(^{187,188}\). In this context, Zeb-1 forms a complex with Smad3 and Smad7 that is activated by TGFβ. This complex binds Smad-responsive elements of the p21(CDKN1A) promoter inducing its transcription \(^{188}\).

In B-cell malignancies, an altered Zeb-1 expression is observed in mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLBCL). In particular, the constitutive activation of Wnt/β-catenin signaling in MCL with is associated with the concomitant high expression of Zeb-1 that supports tumor growth capacity, promotes chemotherapy-resistance by increasing the expression of drug influx/efflux transporters \(^{189}\). In DLBCL, a strong Zeb-1 activation was associated with an adverse 3-year overall survival of patients compared to those with low Zeb-1 levels \(^{190}\). Accordingly, patients expressing high mir-200 levels that, as discussed before represents a negative regulator of Zeb-1, results in a less aggressive behaviour of this disease \(^{191}\).

Concerning myeloid malignancies, recently, Stavropoulou et al have highlighted the key role of Zeb-1 in the regulation of adhesion and invasion of long-term-hematopoietic stem cells (LT-HSC)-Early-AML blasts. By using an inducible MLL-AF9-driven AML mouse model they showed that the knockdown of Zeb-1 in leukemic cells significantly impaired their migration and invasion abilities in-vitro and compromised cell infiltration in the bone marrow and other organs in in-vivo studies \(^{184}\).

### 1.9 CD40

CD40 receptor is a 48-kDa type I transmembrane protein, belonging to the TNF receptor (TNFR) superfamily and is encoded by the gene located on the \(q\) arm of chromosome 20 \(^{192}\). It is formed by 193 amino acid extracellular domain, 21 aa leader sequence, 22 aa transmembrane domain, and a 62 aa intracellular domain in human (90 aa in mouse) \(^{193}\). Of note, in the extracellular domain of CD40, there are 22 cysteine residues that are conserved between the members of the TNFR superfamily \(^{193}\).

CD40 expression has been primarily associated to B cells and professional antigen-presenting cells, such as DCs. CD40 binds its ligand CD40L, which is transiently expressed on T cells and other non-immune cells under inflammatory conditions \(^{194}\). The activation of CD40/CD40L on the surface of dendritic cells promotes cytokine production, the induction of costimulatory molecules on their surface facilitating the cross-presentation of antigen \(^{194}\).

Molecularly, CD40 triggering by CD40L leads to the trimeric clustering of CD40 and the recruitment of TNFR-associated factors (TRAFs) adapter proteins. In particular, the
cytoplasmic domain of CD40 contains two binding sites, a proximal one for TRAF6 and two distal sites that bind TRAF1, TRAF2, TRAF3 and, indirectly, TRAF5. TRAFs interaction activates a wide number of well-characterized signal transduction pathways, including the nuclear factor-κB, p38 mitogen-activated protein kinase, c-Jun-NH2-kinase (JNK), Janus kinases (JAKs) and PI3K pathways. Moreover, CD40 can also activate directly JAK3 in a TRAF-independent manner inducing the phosphorylation of signal transducer and activator of transcription 5 (STAT5). 194.

1.9.1 CD40 expression by stromal cells

More recently it has been demonstrated that CD40 can also be expressed by non-immune cells, including stromal cells (fibroblasts, endothelial cells and MSCs) and tumors. Fries et al were the first to demonstrate that human fibroblasts up-regulated CD40 expression in response to IFN-γ stimulation 195. Accordingly, Gelbmann et al showed that primary colonic lamina propria fibroblasts increased the expression of CD40 and the CD40/CD40L binding induce IL-8, IL-6, or monocyte chemotactic protein 1 (MCP-1) secretion due to the activation of NFκB pathway 196. Moreover, it has been reported that in endothelial cell CD40 expression can be modulated by the proinflammatory cytokines such, TNF-α, IL-1 and IFN-γ, and by bacterial lipopolysaccharide (LPS) 197. Déchanet et al have also demonstrated that CD40 triggering leads to the release of pro-inflammatory cytokines (IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) involved in leukocyte recruitment and enhanced the expression of several adhesion molecules, i.e. CD54, CD62E and CD106 198,199.

CD40 engagement by CD40L on human aortic endothelial cells can increase CX3CL1 protein levels and induces TNF-α production 200.

In line with these findings, the relevance of CD40 expressed by MSCs in haematological malignancies has been highlighted by Franco G et al 201 who demonstrated that also BM MSCs are able to up-regulates CD40 expression in response to particular conditions 201. Indeed, in this study performed in mouse models and human samples it has been previously shown that MSCs derived from splenic marginal zone lymphoma patients expressed CD40 that promotes a pro-inflammatory loop with mast cells in turn sustaining tumor cell growth, suggesting that CD40 expression by MSCs can regulate immune cells within the microenvironment sustaining the progression of haematological neoplasms 201. Furthermore, it has been pointed out that the CD40 expression by MSCs can be used as an independent predictor of PFS in splenic marginal zone lymphoma (SMZL) cases 201.
Indeed, patients expressing high stromal levels of CD40 showed a mean PFS of 16.9 months (compared with 41.2 months for CD40<sub>low</sub> patients) and unfavorable clinical features such as low hemoglobin levels and elevated β2 microglobulin<sup>201</sup>. All these studies suggested that CD40 expression on stromal cells influences their immune-regulatory properties and play a crucial role in the regulation of inflammatory responses and immune landscape, shedding a new light on its possible involvement in the promotion of the tolerogenic environment that can sustain the development and progression of haematological malignancies including leukemia.
2 AIM OF THE STUDY

This thesis evaluated the contribution of clone- and microenvironment-intrinsic mechanisms that are involved in the induction of immune tolerance/suppression in the context of AML. In particular we focused our attention on two molecular drivers expressed by leukemic cells or BM-MSC respectively represented by Zeb-1 and CD40 and we aimed at unveiling their role in AML development and progression.

We foresee two possible scenarios (Figure 11):

• Leukaemia cells autonomously create an immunosuppressive environment via clone-intrinsic mechanisms including the activation of EMT-related programs (i.e. Zeb-1). Herein, Zeb-1 can serve as the key molecular link between EMT- and immunosuppression-related programs and may functionally regulate mesenchymal and AML clone suppressive activity.

• BM-mesenchymal cells can promote active tolerance by expressing co-stimulatory molecules, such CD40, influencing the T-cell status and creating an immunosuppressive environment that support AML progression.

Figure 11 - Hypothesis of the study
3. MATERIALS AND METHODS

3.1 Global gene expression profiling (GEP) and bioinformatics

61 AML and 7 healthy donors (HD) samples were analysed, and their characteristics are summarized in Table 1A.

Samples were obtained from AML patients and healthy donors after written informed consent, as approved by the institutional ethics committees (Comitato Etico Indipendente di Area Vasta Emilia Centro, protocol 112/2014/U/Tess and Comitato Etico della Romagna, protocol 5805/2019), in accordance with the Declaration of Helsinki.

Array data from 61 AML bone marrow samples have been generated by the Next Generation Sequencing platform for targeted Personalized Therapy of Leukemia (NGS-PTL) project.

Data quality control and normalization (signal space transformation robust multi-array average) of NGS-PTL data (Affymetrix Human Transcriptome Array 2.0) were carried out by Expression Console software (version 1.4.1, Affymetrix, Thermo Fisher Scientific). Expression profiles of NGS-PTL data have been recently deposited in the Gene Expression Omnibus (GEO) repository with accession number GSE161532.

For public gene expression datasets, including GSE6891 (N= 461 blood or bone marrow samples of acute myeloid leukemia patients; Affymetrix U133 Plus 2.0 array) and GSE37642 (N=562 of adult patients with acute myeloid leukemia; Affymetrix 140 HGU-133plus2; 422 HGU-133A; 422 HGU-133B), data were normalized by Transcriptome Analysis Console Software (version 4.0.1) using robust multi-array average normalization. In addition, a public dataset with 11 paired peripheral blood samples obtained at the times of diagnosis and relapse with cytogenetically normal AML was analysed (GSE66525) 202.

The Beat AML 203 and The Cancer Genome Atlas (TCGA) project on AML 204 transcriptomic cohorts were obtained from https://portal.gdc.cancer.gov (projects BEATAML1.0-COHORT and TCGA-LAML), respectively. The datasets used in the thesis are described in Table 1B.
NA= not available; t-AML=therapy related AML; sec= secondary

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**Table 1A. Patients’ characteristics NGS-PTL cohort**

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**Table 1A. Healthy donors’ characteristics**
### Materials and Methods

#### Table 1B. Datasets used in the manuscript

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BM: bone marrow; CPM: Counts Per Million; MNCs: mononuclear cells; PB: peripheral blood; RMA: robust multi-array average; sst-RMA: signal space transformation RMA; TMM: Trimmed Mean of M values

#### 3.2 Cell cultures and chemicals

The C1498 cell line, a murine AML cell line isolated from a leukemic 10-month-old C57BL/6 (H-2b) female mouse in 1941 205 was purchased from ATCC, while WEHI-3B murine myelomonocyte cell line syngeneic in BALB/c mice was from Sigma Aldrich. Cells were respectively cultured in DMEM ((Dulbecco's modified Eagle's medium) and RPMI-1640 (Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum (FBS; Thermo Fisher Scientific), 1 % antibiotics (Thermo Fisher Scientific), 2 mM glutamine, 1 mM sodium pyruvate, 1 mM HEPES and 1X Minimum Essential Medium (MEM) Non-Essential Amino Acids Solution, in a humidified atmosphere containing 5 % CO₂ at 37°C.
3.3 Stable gene-silencing

Lentiviral Particles (OriGene Technologies, cat number TL501051V) were purchased from OriGene Technologies. To knockdown Zeb-1 in C1498, $10^4$ cells were treated overnight with lentiviral particles containing plasmid (multiplicity of infection (MOI) of 100) expressing a short hairpin RNA (shRNA) sequences and green fluorescence protein (GFP). Four specific constructs (seq A, seq B, seq C and seq D) were tested for efficiency compared to a negative control construct (Scr). Two weeks after infection, the percentage of GFP+ cells were measured by flow cytometry using the LSRFortessa cell analyser (Becton Dickinson) and GFP+ cells were then isolated using a FACS Aria cell sorter (Becton Dickinson). These cells were used for bone engraftment studies.

3.4 Total RNA extraction, reverse transcription, and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using the Quick RNA micro prep kit (Zymo Research) and subsequently quantified by NanoDrop 2000c Spectrophotometer (Thermo Scientific). 1 µg of total RNA was reverse transcribed into cDNA using the High capacity Reverse Transcriptase kit (Applied Biosystems). 20 ng of cDNA were used and quantitative PCR was performed using the following Taqman Probes: Arg1 (Mm00475988_m1), tnf (Mm00443258_m1), Il23a (Mm00518984_m1), Tgfb1 (Mm01178820_m1), il6 (Mm00446190_m1), Sparc (Mm00486332_m1), Ido1 (Mm00492586_m1), Cd40 (Mm00441891_m1), Pd-l1 (Mm00452054_m1), Nos2 (Mm00440502_m1), Il-2 (Mm00434256_m1), Soxs2 (Mm00850544_g1), Mef2c (and beta-actin (Mm02619580_g1) and the Taqman Universal PCR Master Mix (Applied Biosystems). For human studies, the following Taqman probes were used: ZEB1 (Hs00232783_m1), ARG1 (Hs00163660_m1), PD-L1 (Hs00204257_m1), BETA-ACTIN (Hs03023943_g1). qPCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems). Values were normalized to internal control (beta-actin) using the ΔCT method.
3.5 *In vivo* murine models and bone marrow analysis

Animal studies were approved by Institutional Committee for Animal Welfare and by the Italian Ministry of Health and performed in accordance with national law D.lgs 26/2014 (authorization n. 781/2018-PR and 601/2019-PR). For our experiments, 10-15-week-old female C57BL/6 mice were obtained from Charles River Italia (Calco, Italy). *Tnfrsf5* (Cd40)-KO mice on a C57BL/6 (B6) background, were already available in our lab. For the experiments involving C1498 intra-bone (i.b.) injection, at day 0, 2x10^5 Scr-C1498 or shZeb-1-C1498 cells were injected into the tibia of immunocompetent mice. After 34 days, mice were sacrificed, and bone marrow cells were collected from tibias by flushing to evaluate the AML cell engraftment, measured as frequency of GFP+ cells. BM samples were also stained to assess the immune landscape with flow cytometry analysis. For subcutaneous (s.c.) and intravenous (i.v.) injection, 1x10^6 Scr-C1498 or shZeb-1-C1498 cells were injected in immunocompetent mice that were sacrificed after 30 days.

Canonical bone marrow transplantation (BMT) experiments were performed using Lineage negative (Lin-) cells, that include all stem and progenitor cells negative for mature cell lineage markers (i.e. Mac-1 for myeloid cells, CD4 and CD8 for T-cells, CD19 or B220 for B-cells and Ter-119 for erythrocytes). 2 × 10^5 Lin- cells were transplanted from donors into lethally irradiated WT and *Cd40*-KO mice as previously described. Lin- cells were isolated from the BM of B6 mice using the Lineage Cell Depletion Kit (Miltenyi Biotec) according to manufacturer instructions.

BM cells of mice injected with leukemic cells were stained with the following anti-mouse monoclonal antibodies: PE conjugated anti-PD-L1 (clone), BB700 conjugated anti-CD11b (clone M1/70), APC conjugated anti-B220 (clone RA36B2), BV421 conjugated anti-Ly6G (clone 1A8), BV605 conjugated anti-Ly6C (clone AL-21), BV786 conjugated anti-CD3 (clone 14S-2C11), PE conjugated anti-CD4 (clone GK1.5), APC conjugated anti-OX40 (clone OX-86), APC-Cy7 conjugated anti-CD8 (clone 53-6.7), BV421 conjugated anti-PD1 (clone RMP1-30), BV421 conjugated anti-TIM3 (clone SD12/TIM3) and BV650 conjugated anti-CD25 (clone PC61). For the intracellular staining, the Foxp3/Transcription Factor Staining Buffer Kit (Tonbo Biosciences) was used. Briefly, after surface marker staining, cells were washed with FACS buffer (PBS 1 X, 0.5 % EDTA, 3 % FBS) and fixed and permeabilized for 30
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minutes using Foxp3 / Transcription Factor Fix/Perm buffer provided by the kit. The following antibodies were used: PerCp-Cy5.5 conjugated anti-Foxp3 (clone FJK-16S), FITC conjugated anti-IL17A (clone TC11-18H10), Alexa 647 conjugated anti-Ki-67 (B56), BV510 conjugated anti-TNFα (clone MP6-XT22) and PE-CF594 conjugated anti-IFNγ (clone XMG1.2). All antibodies were purchased from Becton Dickinson.

Samples were analysed with the FACSCelesta flow cytometer equipped with FACSDiva software (v 6.0) (Becton Dickinson). Flow cytometry data analyses were performed using FlowJo software (v10.2).

3.6 Immunohistochemistry (IHC)

For IHC analysis on humans, BM biopsies from 36 AML patients were used. No clinical informations concerning disease features and patients outcome are currently available.

For IHC, Human and Murine BM samples were fixed in 10% buffered formalin, decalcified using an EDTA-based buffer, and paraffin-embedded. 4 µm-tissue sections were deparaffinized and rehydrated. Novocastra Epitope Retrieval Solution (pH 9) was used to unmask antigens in a PT Link Dako pre-treatment module at 98°C for 30 min. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidases with 3% H2O2 and Fc-blocking by a specific protein block, samples were incubated overnight at 4°C with primary antibodies: anti-mouse and human Zeb-1 (Novus Biological – 1:100), anti-human IL-17 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), anti-human PD-1 (1:50, Biocare), anti-human HLA-DR anti-human CD3 (1:400, Dako), anti-human Osteonectin/Sparc, (1:500, Life technologies) and anti-human CD40 (abcam – 1:100). The immunostaining was revealed by either a polymer detection method (Novolink Polymer Detection Systems Novocastra Leica Biosystems Newcastle Ltd Product No: RE7280-K) and following specific secondary antibodies: horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (H+L) (A16035, Invitrogen) and 3,3’-diaminobenzidine (DAB) substrate-chromogens.

For immunofluorescence analysis on BM derived from C1498-Scr and C1498 silenced injected mice, samples were included in OCT. 8 µm cryostat sections from the frozen tissue samples were fixed with cold acetone for 10 min and then washed with PBS1X. Samples were blocked using a 10%FBS solution for 1h and stained using Alexa Fluor
Materials and Methods

488 Rat Anti-Mouse CD146 (Clone ME-9F1) and PE Rat Anti-Mouse CD40 (Clone 3/23) for 2hs. Sections were washed, stained with DAPI for 5 min and then mounted using ProLong Gold Antifade Mountant (Thermo Scientific).

3.7 Metastases quantification

Livers from i.b. injected mice either with Zeb-1 expressing or silenced C1498 were explanted after 34 days, washed in PBS and fixed in 10% neutral buffered formalin overnight. Fixed samples were then embedded in paraffin. Four-micrometers-thick tissue sections were deparaffinized using xylol and firstly rehydrated in 100% ethanol for 5 min. Then, sections were incubated in 95%, 80%, 50% ethanol for 5 min and finally washed in distilled water. Sections were incubated with hematoxylin for 8 min and then washed. Eosin was added to the tissue sections for 3 min and then washed. The stained sections were dehydrated in 70% ethanol for 2 min, 100% ethanol for 2 min and finally in xylol twice for 5 min. Sections were mounted using Eukitt (Biosigma). Metastatic areas were then quantified using ImageJ.

3.8 Immunoblotting

30 µg of the total protein was separated on 8 or 12 % SDS–polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes (Amersham, Biosciences). Following blocking with 5 % bovine serum albumin (BSA) and 0.1 % Tween-20, the membranes were incubated with the following antibodies: Polyclonal Anti-ZEB-1 antibody (NBP1-05987-Novus Biologicals – 1:1000), and β-Actin (Cell Signaling– 1:1000) overnight at 4°C. After rinsing in tris-buffered saline (TBS) 0.1 % Tween-20, membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (Thermo Scientific; 1:2000) and reactions were visualized with the Pierce ECL Plus Western Blotting Substrate (Thermo Scientific).

3.9 Invasion assay

2.5x10^5 C1498 cells either silenced or expressing Zeb-1 were resuspended in 200 µL of serum-free high glucose DMEM and placed onto the upper chamber of a 24-well transwell plate (5-µm pore size) coated with growth factor reduced matrigel (1 mg/mL).
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750 µL of high glucose DMEM containing 10 % FBS was added into the lower transwell chamber. After 24 h at 37°C and 5% CO₂, top chambers, containing non-migrated cells were removed, while cells that migrated into the lower chamber were counted using the all-in-one digital inverted fluorescence microscope (EVOS fl – advance microscopy group). 5 randomly selected fields per well were counted.

3.10 Proliferation assay
To assess the proliferation of Zeb-1 expressing or silenced cells, we used the colorimetric Xtt assay. This test is based on the cleavage of tetrazolium salts added to the culture medium and allow the evaluation of cell viability and proliferation. Briefly, 10⁴ cells were seeded in a 96-well plate in 100 µl of DMEM 10% FBS for four different time point, termed t₀, t₂₄, t₄₈ and t₇₂. For each time point, plated cells were incubated with 50 µl XTT labelling mixture per well and incubate for 4 h at 37°C and 5 % CO₂. Absorbance of the formazan products was measured at 450 nm Tecan's Spark Mircoplate reader, while the reference wavelength was read at 670 nm.

3.11 In vitro suppression assay
4 ×10⁵ naïve C57Bl/6 splenocytes have been labeled with CFSE (Carboxyfluorescein Succinimidyl ester; 10mM, SIGMA Aldrich) and co-cultured with irradiated (3Gy) C1498 expressing or Zeb-1 silenced cells at different ratio in presence of 2 µg/ml of soluble anti-CD3 and 1 µg/ml of anti-CD28 to activate lymphocytes. Each sample was seeded in triplicate. Proliferation of CD4 and CD8 T cells has been assessed after 48h by flow cytometry evaluating CFSE dilution in the CD4+ and CD8+ gated populations.

3.12 MDSCs and neutrophil isolation from human and mice
G-MDSCs and anti-tumor neutrophils (N1) share common surface markers (CD11b, Ly-6G) but differently suppress T cell proliferation. MDSC subsets (CD11b, Ly-6G+ G-MDSC and CD11b, Ly6C+ M-MDSC) were isolated from mice bearing SN25ASP mammary tumors using the Myeloid-Derived Suppressor Cell Isolation Kit, mouse (Miltenyi Biotec) performing a magnetic beads separation. Anti-tumor N1-neutrophils were isolated from agar plugs as previously described by Sangaletti S et al 206. Briefly, neutrophils were collected from blocks of 2% agarose and 0.2% gelatin in saline
subcutaneously implanted in immunocompetent BALB/c mice. 5 days after the injection, agar blocks were explanted and washed 3 times with IMDM supplemented with 10% of FCS (GIBCO, Life Technologies). A total of 70%-75% of these cells are neutrophils that are enriched up to the 95% after 30 minutes of adherence on plastics.

Regarding MDSC and neutrophil isolated from human samples, either from lung cancer patients and healthy donors, whole blood samples were diluted 1:4 with PBS 1X and subsequently subjected to a density gradient stratification. Briefly, diluted whole blood samples were carefully layered onto Ficoll-Paque PLUS (GE Healthcare) and centrifuged at 1800 rpm for 20 minutes at room temperature without brake. For MDSC, the lymphocyte-enriched ring at the interface was transferred into a new collection tube and washed with PBS 1X by centrifugation at 1500 rpm for 5 minutes. Samples were stained using the following antibodies: Lin1 (FITC); HLA-DR (APC eFl780), CD11b (BB700) and CD33 (PE). Total MDSC were sorted according to HLA-DR, CD33 and CD11b expression using a FACSARia BD Instrument. Regarding neutrophils, we isolated normal density neutrophils (ND) from the bottom layer generated after Ficoll stratification. Bottom layers from human samples were lysed using a 0.15M NH4Cl, 10mM KHCO3 and 0.1mM EDTA solution. The 90% of cells are represented by neutrophils.

3.13 Transient silencing of human neutrophils

To achieve transient knock-down of ZEB-1 in human neutrophils, cells were transfected using a reverse transfection protocol in which siRNAs (Stealth siRNAs MSS210695, MSS210696, MSS210697, Thermo Fisher Scientific) and RNAiMAX (Thermo Fisher Scientific) have been used. A mix containing 3.25 µl of RNAiMAX in 200 µl Optimem (Gibco) and another one with 3.25 µl of siRNA (20 µM) stock were prepared, and after 5 min at RT were combined and incubated at RT for 30-40 min. 4.5 x 10^5 neutrophils were seeded and after the incubation, the siRNA/RNAiMAX mix was added. Cells were incubated for 48 h and ZEB-1 knock-down and the modulation of selected immunosuppressive genes were assessed using qPCR.
3.14 Isolation and culture of murine BM-MSCs
Murine BM-MSC cultures were obtained from the trabecular fraction of femurs and tibias of WT and CD40-KO mice. Briefly, the cellular fraction of the femurs and tibias was washed out and the compact bone was incubated with collagenase I (1 mg/ml) for 1 h at 37°C. After enzyme digestion, the bone suspension was passed through a 70-mm filter mesh to remove any bone spicules and large tissues. Cells were seeded in complete medium at a density of 25 × 10^6 cells/ml. Floating cells were removed every 3-4 days. Adherent cells were phenotypically characterized for the expression of the following markers: CD31, CD45, CD34, Ter119, CD44, Sca, and c-Kit. In vitro and in vivo experiments involving murine BM-MSCs were performed using cells between the 2nd and 5th passages.

3.15 Isolation of murine BM-MSCs from lethally irradiated mice
WT and CD40-KO 8-weeks-old mice were lethally irradiated (5 Gy) for 4 or 7 days. Then, MSCs were isolated as previously described in 3.14 by immunomagnetic separation using MACS Columns (Miltenyi) and recovered cells were lysed in RNA lysis buffer (Zymo Research). MSCs isolated from WT and CD40-KO mice were used as control.

3.16 MSC stimulation and treatment with anti-CD40 monoclonal antibody
To evaluate Cd40 modulation upon cytokine stimulation, MSCs isolated from wt or Cd40-KO mice were treated for 24h, 4 days or 7 days with IFN-γ (10 ng/ml), TNF-α (50 ng/ml), IL-1β (10 ng/ml), GMCSF (40 ng/ml), IL-6 (40 ng/ml), IL-17 (100 ng/ml) and TGF-β (5 ng/ml). To evaluate Ox40l expression upon cytokine stimulation, MSCs isolated from WT or Cd40-KO mice were treated with IFN-γ (10 ng/ml) and TNF-α (50 ng/ml). For the experiment using agonistic CD40 monoclonal antibody, MSCs or DCs isolated from wt mice were stimulated for 2h with IFN-γ (10 ng/ml) and TNF-α (50 ng/ml) and then treated for 24h with anti-mouse CD40 (Clone: FGK45.2) monoclonal antibody or isotype control (5µg/ml). All the cytokines were obtained from Peprotech.
3.17 Statistical analysis

Differences between groups were assessed using student’s t-test or one-way analysis of variance (ANOVA), followed by Tukey’s post-hoc test. Data analyses were performed using GraphPad Prism software (version 6.0). Differences were considered statistically significant at p <0.05. *In vivo* experiments were performed in duplicate. *In vitro* experiments were performed at least in duplicate.
4. RESULTS

4.1 Role of ZEB1 in stroma- and clone-related mechanisms of immunosuppression and aggressiveness in acute myeloid leukemia (AML).

4.1.1 Zeb-1 is expressed by human MDSC and neutrophils and correlates with immunosuppressive markers

In my hosting lab it has been previously shown that human immature myeloid derived suppressor cells (eMDSC) express the EMT-related protein SPARC. In these cells SPARC exerts immune suppressive activities acting on NF-kB signalling pathway. Extending the analysis to other EMT markers, we were able to show that murine MDSC along with mature neutrophils isolated from breast tumor bearing mice expressed Zeb-1 (Figure 12A). Notably, MDSCs isolated from tumor bearing mice expressed higher levels of Zeb-1 than anti-tumor N1 neutrophils derived from agar plugs (Figure 12A). Moreover, we observed and enhanced expression of ZEB-1 levels in the MDSC or neutrophils isolated from lung cancer patients compared with those derived from heavy smoker volunteers (HS) (Figure 12B). Interestingly, ZEB-1 levels correlated with the expression of important immune-related genes, such PD-L1 and ARG-1 (Figure 12C), suggesting that neutrophils isolated from lung cancer patients can acquire a pro-tumoral phenotype, resembling MDSC functions. To evaluate the potential role of ZEB-1 in neutrophils, CD15+CD11b+CD66b+ cells were sorted from PBMCs of lung cancer patients and transiently knock-down for ZEB-1 expression using three specific siRNA sequences. Among the three sequences, only one was able to downregulate ZEB-1 expression in human neutrophils as shown in Figure 12. Moreover, qPCR analysis showed that the reduction in ZEB-1 expression in interfered cells was associated with a reduced expression of the immune suppressive markers PD-L1, ARGINASE-I and TGFβ (Figure 12D).

Immature MDSC phenocopy myeloblasts thanks to their clear immature morphology representing de facto the “normal counterpart” of leukemia blasts.

The observation that ZEB-1 decreased is associated with reduced levels of immune suppressive markers in neutrophils, generated the hypothesis that when expressed AML
cells, ZEB-1 could be both a marker of tumour aggressiveness but also an immune regulatory mediator helping leukemia cells in molding its associated immune suppressive milieu.

**Figure 1.**

**Results**

Figure 12 – **ZEB-1 expression is correlated with immune-modulatory marker levels in human MDSCs and PMN** – **A.** ZEB-1 expression levels in FACS sorted neutrophils and MDSC (n=3) (Granulocytic (G)-MDSC and Monocytic (M)-MDSC subsets) isolated from tumor bearing mice. **B.** ZEB-1 expression levels in FACS sorted neutrophils (CD66b+CD11b+CD15+CD10+) normal density (ND) and eMDSC (n=3) (CD11b+ CD33^{high}) isolated from healthy volunteers (heavy smokers – HS; n=5 for neutrophils and n=3 for eMDSC) and Lung Cancer (LC) patients (n=5 for neutrophils and n=3 for eMDSC). **C.** PD-L1 and ARG-1 expression levels in FACS sorted neutrophils (CD66b+CD11b+CD15+CD10+) normal density (ND) and eMDSC (CD11b+ CD33^{high}) isolated from healthy volunteers (heavy smokers – HS; n=3) and Lung Cancer (LC; n=3) patients. **D.** ZEB-1 silencing is associated with the downregulation of ARG-1, PD-L1 and TGF-β (n=3).
4.1.2 Zeb-1 high levels identify a subgroup of Cytogenetically normal (CN)-AML patients with peculiar immune regulatory features.

Considering the above hypothesis, we established an *ad hoc* collaboration with Dr. Curti/Dr. Simonetti from the University of Bologna, and performed a GEP analysis on a cohort of 61 AML patients’ BM samples (>= 80% blasts) and 7 healthy controls (HC) showing that ZEB-1 subdivided AML patients in two groups according to ZEB-1 expression. We defined “ZEB-1\(^\text{low}\)” (86.9% (53/61)) those patients who displayed ZEB-1 expression levels below the average of HC and “ZEB-1\(^\text{high}\)” (13.1% (8/61)) those patients expressing higher levels of ZEB-1 than the average of HC (Figure 13A). We analyzed karyotypic features of ZEB-1\(^\text{low}\) and ZEB-1\(^\text{high}\) AML and showed significant differences between them (p=0.0394, Monte Carlo simulated Fisher’s exact test). In particular, ZEB1\(^\text{high}\) AML associated with a normal karyotype (67% of ZEB-1\(^\text{high}\) vs 36% of ZEB-1\(^\text{low}\) cases, Figure 13B). We therefore extended the analysis to public datasets from the Cancer Genome Atlas (TCGA)\(^{204}\) and Beat AML\(^{203}\). For in silico data, to define ZEB-1\(^\text{low}\) and ZEB-1\(^\text{high}\) AML, we used the median value of ZEB-1 expression among patients. We observed that ZEB-1\(^\text{high}\) and ZEB-1\(^\text{low}\) patients showed differences in term of FAB classification (Monte Carlo simulated Fisher’s exact test, p<0.0001); indeed, Zeb-1\(^\text{high}\) patients were enriched in the undifferentiated M0 (18% vs 3%) and poorly differentiated M1 (29% vs 17%) subtypes, while Zeb-1\(^\text{low}\) were largely represented by M4 (27% vs 20%) and M5/7 (32% vs 7%) (Figure 13C). Moreover, in the same public available datasets, we assessed the association between ZEB-1 and the mutational profile, and we found significant differences in the combination of FLT3-ITD and NPM1 mutations (p=0.005). In particular, ZEB-1\(^\text{high}\) patients were enriched for FLT3-ITD/NPM1-mutant cases (16% vs 6%; p=0.0407) (Figure 13C).

Focusing on our cohort (NGS-PTL), analysing the genes differentially expressed between ZEB-1\(^\text{high}\) and ZEB-1\(^\text{low}\) patients of our cohort, we found that, among the others, ZEB-1\(^\text{high}\) patients were also characterized by a higher expression of SPARC (857.189 vs 311.137; p=0.015), PD-L1 (CD274) (27.144 vs 19.415; p<0.001) and CD40 (28.045 vs 22.527; p=0.045) compared to ZEB-1\(^\text{low}\) AML (Figure 13D).

To test the clinical relevance of Zeb-1 as a prognostic biomarker, we evaluated whether ZEB-1 expression in AML patients can influence patients’ overall survival. To address this point, we analyzed public datasets reporting the percentage of survival of AML patients.
Notably, in 2 independent cohorts (GSE37642 and GSE6891) we found differences in terms of overall survival among treated ZEB-1\textsuperscript{high} and ZEB-1\textsuperscript{low} patients (Figure 13E).

Figure 13 - ZEB-1 high levels identify a specific group of AML patients. A. ZEB-1 expression levels obtained by GEP analysis in primary AML samples (n=61) and healthy controls (HCs, n=7; in green). The blue line indicates the mean value of normal controls that was used to discriminate ZEB-1\textsuperscript{high} (n=8; in red) and ZEB-1\textsuperscript{low} (n=53; in grey) patients. B. Cytogenetic characteristics of AML patients included in our cohort showed a difference
between the karyotypic features of ZEB-1\textsuperscript{high} and ZEB-1\textsuperscript{low} AML cases. C. Transcriptomic profile of AML samples from public datasets showed differences in term of FAB classification between ZEB-1\textsuperscript{high} and ZEB-1\textsuperscript{low} patients, being ZEB-1\textsuperscript{high} patients enriched in the undifferentiated and poorly differentiated subtypes (M0 and M1). Evaluating the differences in term of mutations using the same datasets, we also found that ZEB-1\textsuperscript{high} patients are associated with FLT3-ITD mutant cases. D. ZEB-1\textsuperscript{high} AML patients were characterized by the higher expression of immune-regulatory molecules and EMT-related genes including PD-L1, SPARC and CD40 compared to ZEB1\textsuperscript{low} AML. E. Kaplan-Meier curves showing that AML patients expressing high levels of ZEB-1 were associated with a poor prognosis and with a reduced overall survival after receiving conventional treatments compared with Zeb-1\textsuperscript{low} patients (Log-rank p-value <0.0001 (GSE37642) and p=0.0003 (GSE6891)).

4.1.3 In situ expression of Zeb1 and its correlation with immune regulatory markers in human AMLs

To investigate the expression of ZEB-1 at protein level we performed an immunohistochemical (IHC) analysis on 36 archival BM biopsies from AML patients, which were obtained from the University of Palermo (Dr. Tripodo Claudio) or the IRCCS Ospedale San Raffaele (Dr. Maurilio Ponzoni & Prof. Fabio Ciceri). We found that ZEB-1 was variably expressed in AML patients ranging from 5% up to 90% of blasts (Figure 14A).

In these patients ZEB-1 showed a positive correlation with the expression of PD-L1 and IL-17. AML cases with slight ZEB-1 expression were almost completely devoid of PD-L1 and showed a lower expression of IL-17. Notably, we found that Zeb-1\textsuperscript{high} cases showed increased IL-17A expression if compared to Zeb-1\textsuperscript{low} cases (Figure 14A). Double marker IF analysis showing a co-localization between IL17 and CD3 indicates that ZEB-1+ AML could be enriched in Th17 cells, that can sustain AML progression (Figura 14B).
Figure 14 – Zeb-1 levels in human AMLs is associated with the expression of immune regulatory markers – A. IHC analysis performed on archival BM biopsy (n=36) showed that in AML in which Zeb-1 is significantly expressed by the leukemic blasts, a parallel increased expression of PD-L1 and IL-17A was observed. B. IHC analysis performed on archival BM biopsy stratified according to Zeb-1 expression levels revealed that Zeb-1\textsuperscript{high} patients were characterized by an increase of CD3+IL-17+ cells compared with Zeb-1\textsuperscript{low} patients.
4.1.4 Zeb-1 expression in murine model of AML and its association with immune markers

To model ZEB1+ AML we first evaluated ZEB-1 expression in available and transplantable murine models of AML: the C1498 cells, syngeneic in C57BL/6 mice and the WEHI-3B cells, syngeneic in BALB/c mice. As positive control for Zeb-1 protein levels we used BM-MSCs. Western blot analysis and qPCR showed that C1498 cells display high levels of Zeb-1 compared with WEHI-3B that were almost negative for Zeb-1 expression (Figure 15A).

Interestingly, the different expression of Zeb-1 in C1498 and WEHI-3B cell lines was associated to a different expression of immune related molecules. We observed higher levels of Pd-l1, Arg-1, Il-2 and Tgf-β in Zeb-1+C1498 AML cells compared with Zeb-1neg-WEHI-3B, confirming the results obtained by GEP analysis and IHC. On the contrary, WEHI-3B expressed a different pattern of immune-regulatory genes, that included Nos2, Il-6, Il-23 and Ido-1 (Figure 15B), suggesting that either Zeb-1^{high} or Zeb-1^{low} leukemic cells may be able to promote immunosuppression even if activating alternative pathways.
Results

Figure 15 – C1498 murine AML cell line express high levels of Zeb-1 – A. Western blot analysis and qPCR showed the expression of Zeb-1 in WEHI-3B and C1498 murine AML cell lines either at protein or mRNA levels. BM-derived mesenchymal stem cells (MSC) isolated from BALB/c (B/c) mice were used as Zeb-1 positive control. β-actin was used as internal control. B. qPCR showed that C1498 and WEHI-3B cell lines were characterized by the expression of a peculiar pattern of immune-regulatory genes. Results are expressed as mean ± SD of at least two experiments (*p< 0.05; **p<0.01; ***p<0.001; ****p<0.0001)

4.1.5 Zeb-1 silencing impairs cell-intrinsic immunosuppressive properties of AML blasts

To study the interaction between ZEB-1+AML and the immune microenvironment we used a lentiviral vector-based approach to silence ZEB1 expression in C1498 cells (Figure 16A). To this end we used four target-specific shRNA (seq A, seq B, seq C and seq D) and a negative control (scramble control). The presence of the GFP tag, allowed the selection of GFP positive cells through FACS sorting. We obtained four different silenced cells that were called C1498 Zeb-A (C1498 shZeb A), Zeb-B (C1498 shZeb B), Zeb-C (C1498 shZeb C) and Zeb-D (C1498 shZeb D) and a negative control “C1498-Scr”. We found that all target-specific shRNA decreased Zeb-1 expression, both at RNA and protein levels compared with the negative control (scramble control) even if displaying a different efficiency (Figure 16B, C).

We first assessed whether Zeb-1 downregulation affected the immunosuppressive repertoire of C1498 cells. To perform this analysis and in vivo experiments we selected the two clones showing the best efficiency in ZEB-1 silencing. Comparing the two selected clones (ZEB-C and ZEB-D) with the control (C1498–Scr), we observed that Zeb-1 downregulation decreased the expression of Il-6, Tgfβ, and Arginase-1 (Arg1) but increased the expression of Il-2 levels (Figure 16D). Notably, the last represent a sort of positive control as ZEB-1 was initially discovered as IL2 negative regulator 208. Somewhat surprisingly, in the C1498 model, no alterations in Pd-ll expression were observed comparing the Scr and the silenced cells (Figure 16E) and, of note, only seqC was able to efficiently downregulate Sparc compared with negative control (Figure 16F).
Figure 16 – Zeb-1 knock-down using lentiviral particles in C1498 – A. C1498 were infected using different lentiviral particles containing plasmid expressing a short hairpin RNA (shRNA) sequence and green fluorescence protein (GFP). Four specific constructs (seqA, seqB, seqC and seqD) were tested for efficiency compared to a negative control construct (Scr). Flow cytometry was used to evaluate the percentage of infection and GFP+ cells were FACS sorted. B. qPCR and C. Western blot analysis showed the expression of Zeb-1 in C1498 Scr compared with
silenced cells. β-actin was used as internal control. Results are expressed as mean ± SEM (**p<0.01). D. qPCR showed that silenced cells expressed altered levels of crucial immune regulatory genes including Arg-1, Tgfb, Il-6 and Il-2 E. no changes in the expression levels of Pd-Il were observed in the silenced cells compared with Scr. F. Sparse expression was reduced only in seqC silenced cells, while no alterations in its expression were observed in shZeb-1 seq D cells. Results are expressed as mean ± SD of at least three independent experiments (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

4.1.6. C1498 cells suppress T-cell proliferation via ZEB-1

The strong modulation of Arg-1 and Il-2 expression upon Zeb-1 silencing, two key molecules able to negatively or positively interfere with T cell activation, respectively, suggested to evaluate whether the similarities between AML cells and MDSCs were rather than morphological also functional. We therefore evaluated whether C1498 AML cells were able to directly suppress T cell proliferation and if ZEB-1 silencing was able to interfere with this activity.

To perform this experiment C1498 cells were irradiated, to block their proliferation, and co-cultured with αCD3-αCD28 stimulated CFSE-labelled T-cells (total splenocytes) at different ratio. Proliferation was assessed 48h after. C1498 cells efficiently suppress proliferation of T-cells if compared with ZEB-D silenced cells in particular at higher doses of C1498 cells, which represents a more physiological condition as the microenvironment of AML is basically devoid of T-cells (Figure 17).
Results are expressed as mean ± SD of at least two independent experiments (*p< 0.05; **p< 0.01; ***p<0.001; ****p<0.0001)

4.1.7 Zeb-1 downregulation is associated with the decreased C1498 engraftment within the BM

To evaluate the impact of stable ZEB-1 silencing on tumour immune microenvironment in vivo we set up a murine model in which Zeb-1 expressing C1498 (Scr) or silenced (ZEB-D) cells were injected in the tibias of syngeneic immunocompetent C57BL/6 mice.

Mice were sacrificed after 34 days from injection and, at first, we evaluated the differences in cell engraftment in term of GFP positive cells within the mice BM. We observed that the percentage of GFP+ leukemic cells was higher in the bone marrow of mice injected with Scr compared with silenced ZEB-D cells (Figure 18A), confirming that Zeb-1 expression in leukemic cells support and improve AML cell engraftment. To further confirm this finding, we use other two widely used route of administration. Thus, parental and silenced cells were also injected subcutaneously (s.c.) (Figure 18B) and intravenous (i.v) (Figure 18C) in immune competent mice. For subcutaneous tumors explanted from mice, we observed the formation of a fibrotic capsule around tumors. Given this, since the tumor dimensions cannot be considered informative due to the formation of fibrotic tissues, to better characterize the frequency of tumor cells, we assessed the percentage of GFP+ cells within the tumors, confirming the impaired ability of Zeb-1 silenced cells to growth in vivo. Indeed, only 3 out of 10 mice injected subcutaneously with silenced cells were able to develop tumours and the percentage of GFP+ cells in explanted tumours were lower than in tumours derived from Scr injected mice. Accordingly, also the frequency of GFP+ leukemic cells was higher in mice injected i.v. with Scr cells compared with those injected with the shZeb-1 counterpart, clearly demonstrating that the downregulation of Zeb-1 significantly impacts on leukemic cell take, regardless of the route of administration.
Figure 18 – Zeb-1 silencing impairs the engraftment abilities of C1498 – A. C1498-Scr (n=13) and shZeb-1 (n=12) were injected in the tibia of immunocompetent C57BL/6 mice and the percentage of GFP+ cells within the BM was evaluated with flow cytometry 34 days post injection. Naive mice were used as controls. Representative dot plots showing the percentage of GFP within the BM of injected mice are shown B. C1498-Scr (n=10) and shZeb-1 (n=10) were injected subcutaneously in C57BL/6 mice. 31 days after injection, the percentage of mice engrafted with the two lines was shown and the frequency of GFP+ cells within the tumours was reported C. C1498-Scr (n=9) and shZeb-1 (n=8) were injected intravenously in C57BL/6 mice.
mice and the frequency of GFP+ cells within the BM was reported after 31 days from the injection.

4.1.8 Decreased AML engraftment in mice injected intra-bone with shZEB1 cells is associated with the expansion of cytotoxic CD8+ cells

We next evaluated the impact of ZEB-1 in instructing the BM immune microenvironment and its effect on specific BM immune populations. To this end we performed a multiparameter flow cytometry analysis on BM of transplanted mice that allow the evaluation of many different immune populations in the same sample. FACS analysis shows an increase in CD3+ cell frequency, while no significant changes were observed on total CD11b+ cells (Figure 19A). However, within the CD11b gate, we found a lower frequency of Ly6G\text{high}Ly6C\text{low} (G-MDSC) cells in shZeb-1 injected mice (Figure 19B). Moreover, no alterations were found in the percentage of B220+ B cells (Figure 19C). The increase in CD3 frequency was associated with changes in CD4/CD8 ratio that was in favour of CD8 T cell in mice receiving ZEB-1 silenced cells. No significant changes were appreciated in the Teff/Treg ratio (Figure 19C).
Figure 19 – Zeb-1 expression in C1498 is able to shape the BM immune microenvironment

A. Frequencies of CD3^+ and CD11b^+ cells within the BM of control mice (CTRL B6, n=5) or mice injected with Zeb-1-expressing (Scr, n=13) or silenced cells (shZeb-1, n=12) 34 days post injection. B. Frequencies of G-MDSC (Ly6G^highLy6C^low) and M-MDSC (Ly6C^highLy6G^low) cells within the gate of CD11b cells 34 days post injection. C. Frequencies of B220 lymphocytes and alterations of CD4/CD8 ratio and Teff/Treg ratio within the BM of injected mice (CTRL B6, n=5, Scr, n=13, shZeb-1, n=12) 34 days post injection. Results are expressed as mean ± SD of two independent experiments ( *p< 0.05; **p< 0.01; ***p<0.001; ****p<0.0001). Representative dot plots are shown.
Focusing on CD8 T cell subpopulation. FACS analysis showed a higher frequency of IFNγ+ CD8 T cell infiltrating the BM of mice injected with the silenced (ZEB-D) clone. (Figure 20A). CD8 T cells were also more proliferating showing an increased frequency of Ki-67 staining. Moreover, we also observed an increase in CD8+ PD-1+ cells (Figure 20B). Since PD1 is expressed on activated CD8 T-cells but is also a marker of exhaustion to better characterize this subpopulation we assessed the expression of TIM3, another marker of exhaustion and evaluated the ability of CD8+PD1+ to produce IFNγ. We found the increase of CD8+PD-1+Ki67+IFNγ+ cell fraction and the concomitant reduction of PD1+TIM3+ frequency (Figure 20C) in the BM of mice injected with the silenced cell line, confirming that Zeb-1 downregulation in leukemic cells is associated with the expansion of activated T cells. In addition, we observed an expansion of CD8+OX40+ lymphocytes (Figure 20D). OX40 triggering in Teff and CD8 T cells is associated both with the activation and cytokine production but also with the generation of a stable memory response.
Figure 20 – Zeb-1 down-regulation in C1498 is associated with the expansion of CD8 activated T-cells – A. Frequencies of total and IFN-γ producing CD8+ within the BM of control mice (CTRL B6, n=5) or mice injected with Zeb-1-expressing (Scr, n=13) or silenced cells (shZeb-1, n=12) 34days post injection. A representative dot plot showing the frequency of IFNγ+ CD8+ cells is displayed B. Frequencies of proliferating CD8+Ki67+ and PD1 expressing CD8 lymphocytes 34days post injection C. Frequencies of exhausted (PD1+TIM3+) and activated (PD-1+Ki67+IFNγ+) CD8 lymphocytes 34days post injection D. Percentage of CD8+OX40+ lymphocytes within the BM of injected mice (CTRL B6, n=5, Scr, n=13, shZeb-1, n=12) 34days post injection. Results are expressed as mean ± SD of two independent experiments, *p< 0.05; **p< 0.01; ***p<0.001; ****p<0.0001). Representative dot plots are shown.
The strong activation of CD8 T cell in the BM of mice receiving ZEB1-silenced C1498 cells, was further supported by qPCR analysis performed on total BM cells showing an overall increase in Ifn-γ, Perforin and Ox40l within the BM of ZEB-D injected mice compared to Scr (Figure 21).

In line with this, a trend toward an increased production of cytotoxic factors, such as Granzyme B and Tnf was also observed with the concomitant decrease of immunosuppressive molecules, including Arginase-1, Il-1β and Il-10 (Figure 21).

Figure 21 – Zeb-1 down-regulation was associated with an increase of cytotoxic cytokine release and with the concomitant decrease of immunosuppressive factors within the BM – mRNA levels of selected cytokines produced within the BM of mice injected with C1498 expressing or silenced for Zeb-1. BM of naïve mice was used as control. Beta-actin was used as internal control. Results are expressed as mean ± SD, *p< 0.05; **p< 0.01
The main findings showing that the downregulation of Zeb-1 in C1498 is associated with the T cell activation were also confirmed in the BM of mice injected i.v. (**Figure 22A**) and in the tumours derived from mice injected subcutaneously (**Figure 22B**). In particular we observed an increased frequency of cytotoxic CD8+ cells producing type I cytokines such IFN-γ and TNF-α in the BM of mice injected with shZeb-1 cells along with the expansion of CD8+PD1+ cells compared to mice that receive Scr counterpart, enforcing the results obtained in intra-bone experiments.

**Figure 22** – Zeb-1 downregulation is associated with the expansion of activated CD8+ cells regardless the route of administration – A. Frequencies of CD8+ IFN-γ+, CD8+PD1+ proliferating and activated T-cells within the BM of mice injected i.v. with Zeb-1-expressing (Scr, n=10) or silenced cells (shZeb-1, n=8) 30days post injection. B. Frequencies of CD8+ TNF-α+, CD8+Ki67+ and CD8+Ki67+PD1+ cells in mice (Scr n=7; shZeb1 n=3) 30days post s.c. injection. Results are expressed as mean ± SD, *p< 0.05; **p< 0.01; ***p<0.001.
4.1.9 Reduced expansion of IL17+ CD3 cells in the BM of mice receiving shZEB1 C1498 cells

Evaluating the CD4 population the BM of mice injected with shZEB1 C1498 cells showed an increased frequency of CD4+ cells producing IFN-γ and TNF (Figure 23A). Interestingly, in the same group of mice, we found decreased production of IL-17 by CD3+ cells and particularly by Treg cells (Figure 23B). IL17A+ Treg represents a particular subset of Treg that have been involved in the maintenance of the immune suppressive environment in cancer patients.209-212

The differentiation of Th17 cells relies on known factors including TGFβ, IL-6 and IL-23. According to the reduction in IL-17+ cells, silenced cells showed a significant down-modulation of il-6, Tgf-β (Figure 16D) and il-23 (Figure 23C).
Figure 23 – Zeb-1 expression promote the expansion of CD4+IL-17+ suppressive cells – A. Frequency of activated CD4 lymphocytes producing IFN-γ and TNF type I cytokines and Teff TNFα+ cells within the BM of injected mice (CTRL B6, n=5, Scr, n=13, shZeb-1, n=12) 34 days post injection. Representative dot plots showing percentage of CD4+TNFα+ cells are shown B. Frequency of IL-17+ CD3+ cells, IL-17+ CD4+ cells and Treg cells producing IL-17 within the BM of injected mice (CTRL B6, n=5, Scr, n=13, shZeb-1, n=12) 34 days post injection. Representative dot plots showing percentage of IL-17+ Treg C. qPCR showing the
expression of IL-23 in expressing or Zeb-1 silenced cells. Results are expressed as mean± SD of two independent experiments, *p<0.05; **p<0.01. Representative dot plots are shown.

4.1.10. IL17 promotes C1498 cells proliferation and the expression of stemness markers

To evaluate the biological significance of IL-17 in our context, C1498 cells expressing or silenced for Zeb-1 were firstly investigated for Il17 receptor a (Il17ra) expression by FACS. We did not observe any difference in term of frequency of cells expressing the selected marker as far as in term of intensity (mean fluorescence intensity – MFI) among silenced or Zeb-1 expressing cells (Figure 24A). Thus, we decided to treat C1498 cells for 24 h, 48h, 72 h, 4 days and 7 days with rIL-17. As a read out we evaluated cell proliferation and the expression of stemness markers. We found that rIL17 promoted C1498 cell proliferation (Figure 24B) and the expression of Soxs2, Mef2c and Mmp9 (Figure 24C), two key factors involved in controlling proliferation and stemness of hematopoietic cells also defining unfavourable leukemia.\textsuperscript{213,214}

Interestingly the treatment of C1498 cells with rIL-17 for 7 days induced the expression of Tgfβ and Il-23 (Figure 24C), thus promoting a Th17 self-maintaining loop. No differences in Arg1 expression were observed. Overall, these data suggest that the induction of Th17 cells in the leukemia microenvironment by ZEB1 could sustain tumour aggressiveness through the induction of stemness factors that could be involved for example in mediating tumour relapse after chemotherapy. In line, a GEP analysis performed onto matched AML blasts obtained at diagnosis and at relapse showed the enrichment in ZEB1 expression in relapsed sample along with an enrichment in the Th17 pathway (Figure 24D-E).
**Figure 24 – IL-17A promote and sustain AML aggressiveness** – A. FACS analysis showing IL-17RA levels among the Zeb-1 expressing or silenced cells. B. Cell proliferation was assessed by Xtt assay after 24h, 48h and 72h of IL17A stimulation 50 ng/mL. C. qPCR showing the induction of Socs2 and Mef2c and immune-regulatory markers upon stimulation (7 days) with IL17A 50 ng/mL. D. ZEB-1 expression levels at diagnosis and relapse. E. IL-17 enrichment pathway comparing patients at diagnosis and relapse.
4.1.11 Zeb-1 silencing impaired the invasive ability of C1498 without affecting cell proliferation

To assess *in vitro* the invasive ability of Zeb-1 expressing or silenced C1498 we use a transwell-based assay. We demonstrated that all the sequences used to downregulate Zeb-1 reduced the invasion of C1498 compared to negative control (*Figure 25A*). This evidence was also confirmed *in vivo*.

According to this experiment, *in vivo* we found a reduction of the liver metastatic areas in mice injected i.b. with shZeb-1 compared to Scr injected mice (*Figure 25B*). Moreover, we noticed diffuse and nodular infiltration in the liver parenchyma of Scr C1498-GFP injected mice, while in shZeb-1 C1498-GFP injected mice leukaemic cells were found mostly around blood vessels (*Figure 25B*), pointing out the limited invasive ability of silenced cells compared with controls. Molecularly, this phenomenon is associated with a significant reduction of *Mmp9* expression in silenced cells compared to Scr-C1498 (*Figure 25C*). Finally, to assess whether Zeb-1 silencing could negatively impact on cell growth, we performed an XTT assay showing that Zeb-1 downregulation had no influence on cell viability or proliferation (*Figure 25D*).
Results

Figure 25 – Zeb-1 expression is associated with an increased invasiveness in leukemic cells

A. *In-vitro* invasion assay using 24-well transwell plates (5 μm pore size) coated with 1 mg/ml Matrigel® growth factor reduced basement membrane matrix. Five consecutive fields per transwell were counted. Representative fields are shown B. Hematoxylin and eosin staining of liver explanted from Scr-injected and shZeb-1 injected mice. Metastases area quantification was assessed using ImageJ software C. mmp9 expression levels in Zeb-1 expressing (Scr) and
silenced cell lines (shZeb seq C and shZeb seq D); beta-actin was used as internal control D. XTT proliferation assay performed on Zeb-1 expressing (Scr) and silenced cell lines (shZeb seq C and shZeb seq D). Cell proliferation for each time point was calculated as the (Absorbance (Abs) at 450 nm – Abs at 670 nm) t24/48/72/(Abs at 450 nm – Abs at 670 nm) t0 *100. Results are expressed as mean± SD of two independent experiments, **p<0.01; ***p<0.001.

4.1.12 Zeb-1 silencing influences the immune-modulatory properties of the stromal compartment

So far, we demonstrated that Zeb-1 expression in leukemic cells skew the BM immune microenvironment toward Th17 differentiation to sustain AML development and progression. We next challenged whether leukemia cells could also influence the activity of BM-MSCs, which through the induction of soluble factors like IDO-1 and NOS2, are known to exert suppressive activity in the BM.

To address this point, we decided to investigate the expression of key immune regulatory molecules on BM-MSCs from mice injected with silenced or Zeb-1 expressing C1498.

Given the relevance of CD40 expression in lymphoma patients221, we firstly verified whether MSCs from mice injected with leukemic cells were able to express Cd40. Therefore, we performed an immunofluorescence analysis showing the presence of double positive Cd146+ Cd40+ elements within the BM of injected mice (Figure 26A).

Then, we evaluated the expression of key immune regulatory molecules, including Ido-1, Cd40, Il-6 and Tgfb at mRNA levels isolating BM-MSC from mice injected with silenced or Zeb-1 expressing C1498 (Figure 26 B, D). We observed that BM-MSCs derived from mice injected with C1498-Scr cells up-regulated the expression of Cd40 (Figure 26B) non only compared with naïve control mice, but, more importantly, compared with shZeb-1 C1498 injected animals.

In addition, an increased expression of Il-6 (Figure 26C) and Ido-1 (Figure 26D) was found in BM-MSC isolated from mice injected with C1498-Scr compared with mice injected with silenced cells. Interestingly, no changes were observed in the Tgf-β expression (Figure 26C).

The induction of Cd40 and Ido-1 in BM-MSC was further corroborated by IHC analysis highlighting the overall reduction of selected marker expression in the BM of mice
injected with silenced cells if compared to mice injected with C1498-Scr, reduction that was particularly evident in BM-MSCs (Figure 26 B, D).

Interestingly the treatment of ex-vivo isolated BM-MSCs with rIL17 induced an increase in Cd40 expression. Notably, we also found the up-regulation of *il-6* and *il-1β* (Figure 26 E) upon IL-17a stimulation, highlighting a possible positive feedback loop between BM-MSC and Th17.
Results

Figure 26 – Zeb-1 downregulation impacts on immune-modulatory MSC abilities. A. immunofluorescence showing CD146+ (green) CD40+ (red) cells within the BM of C1498 injected mice. B. qPCR showing Cd40 expression on BM-MSCs isolated from mice injected with Scr or shZeb1 cells and IHC confirming stromal Cd40 up-regulation in Scr injected mice. C. qPCR showing Il-6 and Tgf-β expression on BM-MSCs isolated from mice injected with Scr or shZeb1 cells. D. qPCR showing Ido-1 expression on BM-MSCs isolated from mice injected with Scr or shZeb1 cells and IHC confirming stromal Ido-1 up-regulation in Scr injected mice. E. qPCR showing Cd40, Il-6 and Il-1β expression on ex-vivo IL-17A (50 ng/ml – 24h) stimulated BM-MSCs. Results are expressed as mean± SD*p<0.05

4.1.13 CD40 is expressed by AML patient-derived MSCs

It is now widely accepted that BM-MSCs can be endowed of immune-regulatory activities as reviewed by Nauta et al 112, by suppressing the release of inflammatory cytokines such as IFN-γ and IL-12 and concomitantly promoting the secretion of immunosuppressive IL-10 by dendritic cells 121 or regulating T-cell functions 215. Moreover, we have previously reported (1.9.1 chapter) that in particular pathological condition, such as during lymphomagenesis and progression, MSCs stromal cells that in physiological conditions express very low levels of CD40, are able to up-regulate its expression and sustain tumor growth 201. Thus, we hypothesized that also in AML patients CD40 expression on stromal cells can be relevant in shaping BM tolerance during leukemia cell growth. For this reason, we at first investigated the expression of CD40 on human MSCs following co-culture with AML patients’ leukemic cells. We observed that CD40 expression, along with other crucial markers, was variably induced in MSC cells by blasts, suggesting that, as occurred in lymphoma patients, the expression levels of CD40 can also be consistently different among AML patients (Figure 27A).

To better study the expression of CD40 within the BM of AML patients, we used an independent cohort of 9 archival BM biopsy samples from the Tumor Immunology Unit of the University of Palermo. We observed that CD40 was induced on stromal cells of AML patients compared with the healthy subject. Moreover, as expected, we found significant difference in term of CD40 expression among AML patients (Figure 27B).
Figure 27 – CD40 is induced on MSCs after co-culture with blasts isolated from AML patients – A. qPCR showing the expression of Cd40, Pd-l1, Zeb-l and Sparc selected markers upon the co-culture with AML patients derived blasts (n=8) and healthy donors (BM88 and BM82). Gapdh was used as internal control. B. IHC staining performed on 9 AML patients showed that only some patients expressed CD40 on mesenchymal elements. BM of healthy subject was used as negative control. Magnification: 20X

4.1.14 CD40 stromal expression allow AML take and dissemination

To assess the relevance of stromal Cd40 expression on AML development, we injected $10^5$ GFP-tagged C1498 cells i.v. in WT and Cd40-KO mice. Following 30 days, mice were sacrificed and the percentage of GFP+ cells within the BM, the spleen and the
liver was evaluated. We found that the engraftment of WT mice was higher compared with Cd40-KO mice and, notably, we also observed an increased percentage of GFP+ cells within the spleen and the liver of WT mice compared with Cd40-KO mice (Figure 28).

**Figure 28 – CD40 expression support AML engraftment and dissemination** – Frequency of C1498-GFP+ cells in C57BL/6 mice (n=5) and Cd40-KO mice (n=5). Results are expressed as mean± SD, *p< 0.05

Overall, these data highlighted the relevance of CD40 in the BM microenvironment and in leukemia cell take. To study and better appreciate the relevance of CD40 in regulating BM immune homeostasis we moved to an hematopoietic stem cell transplantation (HSCT) model.
4.2 Stromal-derived CD40 regulates bone marrow immune homeostasis

4.2.1 CD40 stromal expression is induced by pro-inflammatory cytokines

In physiological conditions BM-MSCs do not express CD40, while we hypothesized that lethal radiation, that is the pre-transplantation regimen that resemble a stress condition, could be able to enhance CD40 expression on MSCs. To assess whether lethal radiation was able to induce CD40, we performed a qPCR on ex-vivo isolated and purified BM-MSCs showing an increased expression of CD40 at day 7 post-radiation compared to basal levels (Figure 29A). FACS analysis also showed the increase of CD40+ MSCs in both BALB/c and C57BL/6 lethally irradiated compared with non-irradiated mice (Figure 29B). As expected, no differences were observed in Cd40-Ko mice that were used as a negative control (Figure 29B). Histological analysis and IHC showed a visible upregulation of CD40 expression in the stroma of irradiated mice comparing to control non-irradiated mice (Figure 29C). Upon radiation, we also showed the up-regulation of IL-2 expression levels, a cytokine required for Treg survival and proliferation, only in CD40 wt MSC (Figure 29D). Moreover, we observed an increase of other immunomodulatory molecules in CD45.1 MSC, including IDO, PD-L1 and NOS2 while no changes have been found in Cd40-KO MSC (Figure 29E). These results clearly demonstrated the potential crucial role of CD40 in the promotion of the immune-suppressive environment and in particular, pointed out the potential effects on T cell compartment.
**Results**

Figure 29 – CD40 was up-regulated on BM-MSCs upon lethal radiation and its expression was associated with the increase of immuno-modulatory molecules – A. qPCR showing the increase of Cd40 expression 4 and 7 days post radiation B. FACS analysis showing the up-modulation of CD40 on BM-MSC isolated from irradiated (RAD) and non-irradiated (CTRL) BALB/c (CTRL n=6; RAD n=5), C57BL/6 (CTRL n=6; RAD n=6) and CD40-KO mice (CTRL n=6; RAD n=4) and its expression in BM post-irradiation. C. Double staining of CD40+ and CD169+ cells in WT mice harvested 7 days post-radiation showing the up-modulation of CD40 on BM-MSC isolated from irradiated (RAD) and non-irradiated (CTRL) BALB/c (CTRL n=5; RAD n=2). *p < 0.05, Mann-Whitney test. D. FACS analysis showing the up-modulation of CD40 on BM-MSC isolated from irradiated (RAD) and non-irradiated (CTRL) BALB/c (CTRL n=5; RAD n=2). *p < 0.05, Mann-Whitney test. E. RT-PCR analysis showing the expression of CD40 on primary BM-MSCs (not expanded).
n=6; RAD n=2) **C.** IHC showing the increase of CD40 on stromal elements within the BM of irradiated mice compared with controls **D.** qPCR showing the increase of il-2 expression in C57BL/6 (CD45.1) irradiated mice (n=3) **E.** up-regulation of Pd-1, Ido-1 and Nos2 expression levels after radiation (n=3). Results are expressed as mean± SD, ****p<0.0001

Since we assumed that the acquisition of CD40 by stromal BM-MSCs is able to influence their ability to promote immunosuppression, we then analysed the alterations within the BM microenvironment occurred in irradiated mice, focusing on the radioresistant T-cells, including both Teffs and Tregs. Indeed, for our hypothesis, it is expected that T cells can be influenced by soluble factors released by MSCs. However, we speculated that some cytokines, such as IFNg and TNFa produced by T-cells can modulate the expression of CD40 on MSCs in turn regulating their immune-regulatory properties.

Compared to the controls, the lethally irradiated mice showed an overall increased frequency of CD4+ T-cells, whereas the number of CD8+ T-cells was not significantly different (**Figure 30A**). Among the CD4+ T-cells, we observed an increased percentage of Teffs and the concomitant decreased frequency of Tregs (**Figure 30B**). Moreover, we also found that Teffs were activated and released TNF and IFN-γ, two cytokines that have been reported to regulate the immune-modulatory properties of MSCs 113 (**Figure 30C**).

Thus, we performed *in vitro* studies evaluating the potential ability of these cytokines to induce CD40 on BM-MSC (**Figure 30D**) and we found that TNF and IFN-γ were able to up-regulate cd40 expression, while no effects were exerted by other stimuli that were also released in the conditioned BM (i.e., G-CSF, GM-CSF+IL-6, and IL-17) or are conventionally used to stimulate mesenchymal cells (i.e., TGFβ) (**Figure 30D**). Notably, to test whether the gain of immune regulatory features in BM-MSCs occurred at the expense of their differentiation program we performed a qPCR evaluating the expression of well-recognized differentiation markers such as Osterix, Sparc, Spp1, Bglap, Runx2 and Ppar-g on TNF and IFN-γ stimulated cells. We demonstrated that TNF reduced Osterix, Sparc, Spp1, Bglap and Ppar-g and IFN-γ down-modulated the expression of Osterix, Runx2, Sparc and Spp1 but not Bglap and Ppar-g (**Figure 30E**). The combination of TNF and IFN-γ was additive in decreasing the expression of Spp1 (**Figure 30E**).
Figure 30 – CD40 expression is induced by pro-inflammatory cytokines and is associated with the acquisition of immune regulatory features

A. Frequency of CD4+ and CD8+ in WT irradiated mice
B. Frequency of Teff (CD4+Foxp3-) and Treg (CD4+Foxp3+) within the BM of WT irradiated mice
C. Frequency of CD4+ cells producing TNF and IFN-γ within the BM of irradiated mice
D. CD40 expression on BM-isolated MSCs (24h stimulation: IFN-γ 10 ng/ml, TNF 50 ng/ml; IL-1β 10 ng/ml; GCSF 40 ng/ml; GMCSF 40 ng/ml; IL-6 40 ng/ml;
4day stimulation: GMCSF 40 ng/ml; IL-6 40 ng/ml; 7 day stimulation: IL-17 100 ng/ml; TGFβ 5ng/ml); E. qPCR evaluation the expression of osteogenic and adipogenic lineage in MSCs stimulated with IFN-γ (10 ng/ml) and TNF (50 ng/ml) for 24h. Results are expressed as mean± SD, *p<0.05; **p< 0.01; ***p<0.001; ****p<0.0001

4.2.2 CD40 stromal expression instructs BM microenvironment via OX40L

Since we have demonstrated that CD40 expression can be induced by soluble factors release within the BM-microenvironment, we therefore verified whether the lack of CD40 expression could generate a pro-inflammatory microenvironment due to the impairment of Treg differentiation.

To this end, we analysed BM T-cell status in WT> Cd40-KO and WT>WT chimeras, 4 weeks after BMT showing that Tregs were reduced in the BM of WT> Cd40-KO chimeras compared to the WT>WT counterpart (Figure 31A), and IFNγ-producing Teff cells were significantly increased (Figure 31B,C).

Considering the strong T-cell activation at the expense of Treg observed in the CD40-KO recipients we evaluated the expression of OX40L, a co-stimulatory molecule involved in cytokine production, T-cell expansion, survival and memory development that has also been demonstrated to inhibit Treg differentiation as far as their suppressive activity.216,217

We evaluated Ox40l expression in Cd40-KO and wt BM-MSCs. IHC analysis performed on BM biopsies comparing the different mouse chimeras showed an overall increase in ox40l expression in Cd40-KO recipient mice (Figure 31D).

The mechanisms that are able to modulate the expression of OX40L are particularly studied in B cells and DCs were only CD40 triggering is able to induce OX40L, while little is known for MSCs. To unveil the mechanisms underscoring ox40l expression in BM-MSCs, we stimulated BM-derived DCs and BM-MSCs with the CD40-agonist mAb CD40 (to trigger CD40) but also with pro-inflammatory cytokine TNF and IFN-γ.

We demonstrated that, accordingly to the literature, DC up-regulates Ox40l only upon CD40 triggering, while pro-inflammatory cytokines did not induce any changes. (Figure 31E). Conversely in BM-MSCs also TNF was able to up-regulates Ox40l, independently from CD40 triggering (Figure 31E). Most notably this induction was reduced by CD40 triggering, indicating an opposite effect of CD40 triggering on Ox40l in BM-MSCs and DCs.
In line RT-PCR analysis performed onto ex-vivo stimulated BM-MSCs showed that Cd40-KO BM-MSCs had higher Ox40l levels than their WT counterparts in response to TNF (Figure 31F).

Figure 31 – CD40 deficiency on BM-MSC is associated with a decreased frequency of Treg in transplanted mice – A. Frequency of Treg (CD4+Foxp3+) within the BM of transplanted
**Results**

mice B. Frequency of CD4+ cells producing IFN-γ within the BM of transplanted mice C. Representative dot plot showing IFN-γ production by CD4+ cells comparing wt and CD40-ko recipient mice. D. IHC analysis showing Ox40l expression in the BM of transplanted wt and Cd40-KO. Upper panels 10X magnification; Panels below 20X magnification E. ox40l expression in BM-MSCs stimulated with IFN-γ 10 ng/ml, TNF 50 ng/ml and aCD40 mAb or isotype control (5µg/ml) for 24h F. Ox40l expression in wt and Cd40-KO BM-MSCs stimulated with IFN-γ 10 ng/ml, TNF 50 ng/ml for 24h. Results are expressed as mean± SD, *p< 0.05; **p<0.01; ***p<0.001; ****p<0.0001

**4.2.3 Altered B-cell lymphopoiesis in the BM is a characteristic of Cd40-KO recipient chimeric mice**

To assess whether Cd40 deficiency can impact on long term reconstitution we set up BMT experiments in which recipient mice, either WT or Cd40-KO were lethally irradiated and transplanted with HSCs from congenic WT donors. Twenty-one days post-BMT, FACS analysis on PB, BM, and spleens of recipient mice was performed. In the PB (Figure 32A), Cd40-KO recipients compared with WT mice showed a significant reduction in the frequency of B220+ B-cells and of CD3+ T-cells. In line with the PB analysis, we also observed a reduction of B220+ cells in the BM of Cd40-KO recipients (Figure 32B). Particularly, B-cell development was largely defective, as shown by the reduced B220+CD43+ and almost absent B220+CD43- fractions (Figure 32B). B-cell development was arrested at the A and B fractions, which correspond to the pre-pro-B and early pro-B phases, respectively, with reduced development of the fraction of C'-C precursors (late pro-B and large pre-B) (Figure 32C). Interestingly, also in the spleens of Cd40-KO recipients we noted an overall decrease of B220+frequency compared with WT mice (Figure 32D) suggesting that CD40 expression in radio-resistant cells is necessary to allow proper BM B-cell development, when CD40-competent HSC are infused.
Results

Figure 32 – Analysis of B-cell development in WT>WT and WT>Cd40-KO BM chimeras.
A. Cumulative data of PB FACS analysis showing the frequencies of B220+ and CD3+ cells in the PB of WT>Cd40-KO compared to WT>WT BM chimeras. B. Cumulative FACS analysis of the BM showing the overall decrease in B220+, B220+CD43+ and B220+CD43- B-cell subsets in Cd40-KO recipients C. Cumulative data showing the frequency of pre-pro-B and early pro-B precursors (A and B fractions), and of late pro-B and large pre-B precursors (C'-C) in chimeric mice; D. Frequency of B220+ B-cells in the spleen of Cd40-KO compared with WT mice.

Results are expressed as mean± SD, *p< 0.05; **p< 0.01; ***p<0.001; ****p<0.0001
5. DISCUSSION

This study demonstrates the role of ZEB-1, when expressed by leukemic blasts, and of CD40, when expressed by BM-MSCs, in promoting the immunosuppressive environment sustaining AML development and more in general immune suppression in the BM niche.

The establishment of immune suppression have been extensively characterized in solid tumors and depends on the recruitment of specific immune cell types (Treg and MDSCs), the engagement of inhibitory T-cells pathways (i.e. PD-L1/PD-1) and the interference with specific metabolic pathways such as IDO-1. All these mechanisms, although less characterized, could be potentially involved in AML immune escape, either through a blast-autonomous mechanism or through the engagement of bystander cells, like MSCs.

Here, we unveiled for the first time the role of ZEB-1 not only as a regulator of AML invasiveness, but also as a key orchestrator of the AML immunosuppressive environment.

Our data are in line with the literature and also extend previous results obtained in AML with complex Kariotype, to CN-AML and pointed out that the poor outcome of ZEB1+ AML could be the consequence of the immunosuppressive and self-maintaining microenvironment initiated by ZEB1.

Even if the majority of the studies agree in recognize ZEB1 as an oncogene in AML, a recent study performed by Almotiri A et al postulated that Zeb1 acts as a transcriptional regulator in haematopoiesis and its expression is required to suppress leukemic potential in AML. Indeed, analysing the overall expression of ZEB-1 in public datasets the authors found that ZEB-1 expression was lower in AML patients compared with healthy controls, thus implicating Zeb-1 as a tumor suppressor in AML.

We explained these apparent paradoxical results performing immunohistochemistry analysis and evaluating not only the expression of ZEB-1 at transcript level, but also its protein expression using AML patients BM biopsies. Notably, we found that CN AML patients display an intra-patient variable expression of ZEB1 and are characterized by the presence of ZEB1+ and ZEB1neg blasts. Although this finding deserves further investigations, our result seems to suggest that in CN AML, where the expression of ZEB1 is not triggered by a chromosomal aberration, the acquisition of ZEB1 can define
those AML clones displaying more aggressive features that could be responsible for patients relapse and can impact on chemotherapy response. Given these data, for the analysis of public datasets, to better describe the complex scenario occurring within the BM of AML patients, we decided to divide patients without considering the overall expression of ZEB1, while separating AML patients in two groups and identifying those patients expressing high levels of ZEB1 and those cases characterized by a low/negative expression.

We found that ZEB-1 expression defined a subgroup of AML patients (termed Zeb-1high) with a peculiar T-cell microenvironment enriched in IL-17+ T-cells. GEP analysis associated ZEB-1 expression to those of other immune regulatory genes, including PD-L1 and CD40.

To unveil the real contribution of ZEB-1 in shaping the BM T cell microenvironment, we designed ad hoc murine models knocking down ZEB-1 in the C1498 leukemia cell line. The injection of C1498 cell stable silenced for ZEB-1 expression, was associated with a significant reduction in tumour take, which was paralleled by a strong modification of BM T-cells. We found a reduction of exhausted lymphocytes along with an expansion of cytotoxic CD8+ T cells. Notably, these changes in the CD8 cells were associated to the decrease of IL-17A+ CD4+ and, more interestingly, IL-17A+ Tregs. Since it has been shown that TGF-β, IL-6, IL-23, and IL-1β are required for Th17 development and for the differentiation of Treg cells into IL-17A-producing Th17-like Treg cells (as reviewed in 221,222), we evaluated whether our AML cells were able to produce these cytokines. Interestingly, in C1498 cell lines, Zeb-1 downregulation was associated with the reduced expression of Tgf-β, Il-6 and Il-23, suggesting that the decrease production of such molecules by leukemic cells within the BM of mice injected with silenced C1498 could be responsible for the reduction in Th17 cells.

Backing to humans, the analysis of AML patients before and after chemotherapy showed and increased expression of ZEB-1 at relapse and this was associated with an enrichment of Th17 pathways, highlighting that the increase of IL-17 production can be considered as a relevant feature directly associated with ZEB-1 levels.

The relevance of IL-17A produced by Th17 cells in AML progression has not been fully elucidated. Indeed, only few studies have shown the effects of the reduced IL-17A levels in patients, mainly highlighting its impact on the prognosis. In particular, high levels of IL-17A in patients have been correlated with a poor outcome and with an
increased proliferation of leukemic cells. In line with these evidence coming from the literature, we also showed that IL-17A treatment was able to limit the proliferation of Zeb-1 silenced C1498, while the stimulation of parental cells was associated with an enhanced growth. More interestingly, IL-17A treatment of C1498-Ser cells up-regulated the expression of Socs2 and Mef2c, two molecules the have been reported to be associated with stemness and with a more aggressive phenotype.

Overall, our data support that ZEB1 could be a biomarker of AML aggressiveness able to identify patients with a worse prognosis.

Strengthening the role of ZEB-1 in immune suppression we showed for the first that AML blasts can directly suppress T-cell proliferation and that knocking down ZEB-1 reduced this capacity.

Reasons for such a reduction can be found in the capacity of ZEB-1 to directly regulate the expression of Arg-1 an enzyme crucial in suppressing CD4 and CD8 T-cell proliferation. In the AML context, Mussai et al have demonstrated that arg-2 expressed by blasts is able to inhibit T-cell proliferation in vitro creating the immunosuppressive microenvironment that allow leukemia progression. The possibility that ZEB1 directly regulate the immune suppressive capacity of blasts was supported our additional findings showing that ZEB1 silencing in neutrophils isolated from lung cancer patients was associated with ARG-1 reduction and decreased levels of PD-L1 and TGF-β.

Noteworthy, as demonstrated by Stavropoulou et al using an MLL/AF9-driven mouse model, also in our study Zeb-1 expression in leukemic cells negatively impacts on patients’ overall survival, with Zeb-1 expression clearly associated with poor outcome. Accordingly, in mouse models, Zeb-1 expression was associated with enhanced invasive abilities in vitro and in vivo. Mice injected with Zeb-1 expressing cells displayed increased hepatic metastatic areas compared with those injected with silenced cells, a phenotype that might depends on the capacity of ZEB-1 to directly regulate Mmp-9 expression.

Overall, these data unveiled new activities of ZEB1 in conferring tumor aggressiveness along leukemia onset and progression. These activities include the induction of Th17 cells that directly support tumour proliferation and aggressiveness, via IL17. Our results should promote ZEB1 as biomarker useful to identify CN-AML patients with most aggressive disease and offer new therapeutic options, also including immunotherapy, targeting the ZEB1-induced immunosuppression.
Along with immune cells, also MSCs play a crucial role in supporting AML development. In this context, BM-MSCs are able to inhibit the T-cells and B-cells proliferation or to impair NK cell functions and DC maturation and induce a tolerogenic environment\(^\text{225}\). Moreover, in vivo administration of MSCs is able to treat acute GVHD, and/or to prevent its occurrence in the human bone marrow transplant setting, highlighting the immunomodulatory properties of MSCs\(^\text{226,227}\). Our lab demonstrated that CD40 expressed by MSCs of splenic marginal zone lymphoma patients play a crucial role in sustaining tumor cell growth establishing a cross-talk with mast cells\(^\text{201}\). We therefore hypothesized that also in AML patients CD40 expression on stromal cells can be relevant in shaping BM tolerance during leukemia.

GEP analysis showed a variable expression of CD40 in AML patients. This result was also confirmed at protein level in a small cohort of AML patients, where we found that CD40 levels were overall increased in AML patients compared with a healthy control, The expression was again variable, either on stromal meshwork or confined at leukemic cells, suggesting that leukemic blast intrinsic features could influence CD40 levels on MSCs.

Accordingly, MSCs isolated from mice injected with C1498-Scr expressed higher levels of Cd40 compared not only with control mice, but also with mice injected with silenced cells, pointing out a possible relationship between Zeb-1 and Cd40 that deserves more studies. Moreover, Cd40 expression was also associated with enhanced levels of *Ido-1* and *Il-6*, confirming that the expression of Zeb-1 in leukemic blasts influences the immune-modulatory properties of BM-MSCs, in turn promoting a favourable microenvironment for AML growth.

Given this preliminary evidence, we hypothesized that the induction of an immunosuppressive microenvironment could be driven or by cell-autonomous mechanism (mainly regulated by Zeb-1 expression on leukemic cells) or by the engagement of mesenchymal bystander cells, that, through the up-regulation of CD40 could generate a tolerogenic microenvironment influencing T-cell status. To disclose the immunomodulatory role of CD40 expressed by MSCs, we set-up hematopoietic stem cells transplantation experiments, since we considered this setting the most representative condition that could force the need of homeostatic signals to prevent a pathological and unwanted T cell activation. We demonstrated that *Cd40* expression on MSCs is finely regulated within the bone marrow and in particular it is induced by pro-inflammatory conditions, including radiation and, importantly, by two significant
cytokines such TNF-α and IFN-γ. Along with Cd40, we also observed the increase of other immune-modulatory molecules (Pd-l1, Il-2, Nos2) and the concomitant down-regulation of genes involved in the differentiation towards osteoblast or adipocytes. These results suggest that the acquisition of Cd40 could mirror the acquisition of a peculiar immunosuppressive MSC phenotype and the gain of immune-regulatory features at the expense of the canonical structural properties by MSCs that, in particular conditions, are crucial for the maintenance and also the retrieval of the BM-proper homeostasis influencing T-cell activation. Indeed, for our hypothesis, it is expected that T cells can be influenced by MSCs but, we can also assume that some cytokines released by T cells can influence MSCs and, in particular, CD40 induction. In this context, our results underline that upon irradiation, the up-regulation of Cd40 on BM-MSC is associated with a decreased frequency of Treg and with a concomitant increase of Teffector cells producing type I cytokines, including TNF-α and IFN-γ, that, as discussed before, are the two major cytokines responsible for Cd40 induction on MSCs. Hence, the increased of Cd40 levels in radio-resistant BM-MSCs is required to restore the BM homeostatic conditions preventing the persistent activation of T-cells. Indeed, in the absence of stromal CD40, Tregs were reduced in favour of pro-inflammatory Teff. Molecularly, this was associated with an increased production of Ox40l by MSCs upon pro-inflammatory cytokine stimulation. Ox40l has been reported to suppress Treg activity and inhibits the generation of new Tregs, being crucial for the increase of activated T-cells. In this context, it has been shown that, since OX40 receptor is transiently induced on activated naive CD4 and CD8 T cells, OX40L can represent an additional mechanism to further amplify T cell responsiveness (reviewed in). The same mechanisms described above could be also in place during leukemia development. Indeed, a preliminary experiment showed a higher percentage of C1498-GFP+ cells not only in the BM, but also in the spleen and liver of WT mice compared with the Cd40-Ko counterpart, pointing out the relevance of CD40 in the induction of an immunosuppressive microenvironment needed for AML take and dissemination.

Taken together, our results shed a new light on the contribution of BM microenvironment in the AML development and progression pointing out the crucial role of two molecules, CD40 and Zeb-1 whose role was poorly understood in
hematological malignancies. These findings suggested that Zeb-1 can be considered as a biomarker able to identify a particular subset of aggressive AML that is characterized by a peculiar immunosuppressive bone marrow microenvironment. Novel approaches able to down-regulate Zeb-1 levels are needed. Moreover, a better characterization of the bone marrow microenvironment and the molecular drivers that are involved in AML development and progression could help to identify novel personalized therapeutic strategies that can not only target leukemic cells but can also interfere with the immunosuppressive mechanisms in place that sustain and promote AML.
6. REFERENCES


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