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MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE INTERPLAY BETWEEN MALIGNANT AND STROMAL CELLS IN ACUTE MYELOID LEUKEMIA AND MYELODYSPLASTIC SYNDROME

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Abstract

In this thesis, we studied the cross-talk between malignant cells and stromal cells, with the aim to elucidate the respective contribution to myeloid neoplasm onset and progression. First, we characterized and compared mesenchymal stromal cells (MSCs) isolated from myelodysplastic syndrome (MDS-MSCs) and acute myeloid leukemia (AML-MSCs) patients. We demonstrated that, despite some unaltered functions, patient-derived MSCs show also intrinsic, distinct functional abnormalities, which could all potentially favor a leukemia-protective bone marrow (BM) niche *in vivo*. Second, we investigated the ability of AML cells to modulate the AML-MSC functions. In a GEP-screening, we found that 40% of BM-derived AML samples show a higher IFN-γ expression, compared to the mean IFN-γ expression in healthy BM-derived cells. We demonstrated that in co-culture experiments, IFN-γ^+ AML cells modify AML-MSC gene expression and function, inducing the up-regulation of IDO1, and consequently the generation of T regulatory cells. Finally, we wondered if the transcriptome of stromal cells could be influenced by the hematopoietic-specific alterations, i.e. *Dnmt3a* and *Asxl1* mutations, which occur early in MDS/AML patients. We found that *Dnmt3a*- and *Asxl1*-null BM cells, when transplanted in wild-type mice, induce profound and deletion-specific modifications in the transcriptome of wild-type BM stromal cells, suggesting the ability of *Dnmt3a*- and *Asxl1*-null BM cells to shape the niche. Furthermore, we compared the transcriptome of wild-type BM stromal cells, obtained from transplantation experiments, with that of MSCs isolated from low-risk MDS patients with *DNMT3A* and *ASXL1* mutations, and we highlighted some common modifications, which could be potentially relevant for human disease and specific for *DNMT3A*/*ASXL1* mutations. In conclusion, this thesis pointed out that there is a bi-directional cross-talk, in which stromal cells can influence malignant cells, and in turn malignant/pre-malignant cells can alter stromal cell gene expression and function. Both mechanisms could potentially contribute to the pathogenesis of myeloid malignancies.

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INTRODUCTION

INTRODUCTION

Myeloid malignancies are characterized by stem-cell–derived clonal myelopoiesis. The abnormal self-renewal and/or differentiation defects of malignant clones result in the disruption of normal hematopoiesis. The group of myeloid malignancies includes Myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). In the last years, a growing body of evidence has been accumulated pointing out that epigenetic/genetic changes, unbalanced cytokine production, activation of immune system and BM stroma, all donate contribution to the pathogenesis of the diseases[1].

1. MDS AND AML GENERAL CHARACTERISTICS, ETIOLOGY, INCIDENCE AND CLASSIFICATION

1.1 MDS general characteristics

MDS are a heterogeneous group of BM neoplasms, characterized by altered and ineffective hematopoiesis leading to the peripheral reduction of mature blood cells (cytopenia), abnormal myeloid maturation (dysplasia) and a propensity to evolve into AML[2]. By definition, in the BM of MDS patients the percentage of immature blood cells, called blast, is lower than 20% otherwise the disease is AML[1]. It is thought that MDS arise from primitive hematopoietic stem cells which acquire several genetic and or cytogenetic alterations giving growth advantage and clonal dominance[2]. In particular, the ineffective hematopoiesis, leading to BM failure, is due to maturation defects and increased susceptibility to apoptosis of hematopoietic cells, which show also a reduced responsiveness to growth factors[3]. In the early stages, MDS is often asymptomatic, and the diagnosis could arrive after a routine blood test. Then, the clinical manifestations include a higher incidence of infections, hemorrhages (stroke), sepsis, cardio-respiratory/renal failure in MDS patients[4].

1.2 MDS etiology and incidence

MDS is considered to be a disease of aging, in fact, the mean age at diagnosis is 70 years[5]. In Europe, the incidence of MDS is around 8 new cases every 100000 habitants. This incidence quadruplicates in the elderly population[6]. In the United States, the annual incidence is between 5,3 to 13,1 cases every 100000 persons[7].

Based on the underlying causes of the disease, is possible to distinguish:

- *de novo* or primary MDS, when the specific causes leading to MDS are unknown, this subgroup represents the most common type of MDS;
- secondary or therapy-related MDS, this subgroup refers to MDS with a defined cause.

There are several risk factors for MDS. A class of risk is represented by congenital diseases, including inherited bone marrow-failure syndromes such as Shwachman-Diamond syndrome (SDS), severe congenital neutropenia or Fanconi anemia. For SDS patients the risk to develop MDS is 19% around 20 years old and increases with aging[8]. In addition, chemotherapy and ionizing radiation, used to treat a previous malignancy, are other risk factors (therapy-related MDS). Environmental factors are also involved: exposure to solvents and to pesticides or to benzene seems to increase the risk of MDS, although there are several studies which are not consistent[9]. In 2009, Ma and collaborators showed a positive correlation between the onset of MDS and the body mass index (BMI), studying a cohort of patients in US[10].

1.3 MDS Classification

There are different types of classification for hematological disorders, all of them are useful to define the best treatment for the patient.

In 1982 the French-American-British (FAB) group identified 5 subgroups of MDS, based on dysplasia and percentage of blasts in BM and peripheral blood. The FAB classification includes:

- refractory anemia (RA);
- refractory anemia with ring sideroblasts (RARS);
- refractory anemia with excess blasts (RAEB);
- refractory anemia with excess blasts in transformation (RAEB-T);
- chronic myelomonocytic leukemia (CMML)[11].

The FAB classification is the backbone of another more detailed classification, made by the World Health Organization (WHO). In 2016, WHO published the revised classification of MDS which includes:

- MDS with single lineage dysplasia (MDS-MSD)
- MDS with ring sideroblasts (MDS-RS)
    - MDS-RS and single lineage dysplasia
    - MDS-RS and multilineage dysplasia
- MDS with multilineage dysplasia (MDS-MLD)
- MDS with excess blasts (MDS-EB)
- MDS with isolated del(5q)
- MDS, unclassifiable (MDS-U)
- *Provisional entity:* Refractory cytopenia of childhood
- Myeloid neoplasms with germline predisposition

Despite the cytopenia is a typical feature of MDS patients, the WHO classification is mainly based on the level of dysplasia and blast percentage. According to this classification, the most common MDS is the MDS-MLD[12].

With the aim to evaluate clinical outcome for MDS patients, an international effort performed a prognostic risk analysis, combining cytogenetic, percentage of BM blasts, morphological features, and cytopenia. So far, an International Prognostic Scoring System (IPSS) has been generated, which identified 4 risk groups[13]. Recently, a revised International Prognostic Scoring System (IPSS-R) has been published, in which it is possible to distinguish the following risk categories, with different survival rates and propensity to develop into AML:

- very low risk (score 0-1,5);
- low risk (score 1,5-3);
- intermediate risk (score >3-4,5);
- high risk (score 4,5-6);
- very high risk (score >6).

The system is mainly based on BM cytogenetics, cytopenia and blast percentage but there are also other features like hemoglobin levels. For each factor is assigned a score, the final score that is obtained matches the patient in one of the 5 risk category[14].

1.4 AML General characteristics

Acute myeloid leukemia is a heterogeneous clonal BM neoplasm, characterized by an uncontrolled proliferation and a block of differentiation of hematopoietic cells, which accumulate in BM or peripheral blood (PB) and in other organs. AML diagnosis is established when the percentage of blasts, with myeloid characteristics, is 20% or more in BM or PB[15]. The term acute refers to an aggressive neoplasm, with a rapid progression. It is thought that AML evolves from a line of hematopoietic precursors, which acquires genetic alterations during years[16]. Despite malignant clones are immature cells, due to the block of differentiation, they still show some morphologic and immunophenotypic features of the normal counterpart, which allow physicians to recognize the normal precursor they belong to. Clinical manifestations of AML are mainly due to the accumulation of poorly differentiated cells. Patients show leukocytosis and signs of BM failure including thrombocytopenia and anemia. If untreated, AML induces death within a few months after diagnosis, because of infections or bleedings.

1.5 AML etiology and incidence

In adults, AML is the most common acute leukemia. In Europe, the incidence of AML is around 3 cases per 100000 person[17], whereas in the United States, the incidence is 3 to 5 cases per 100000 habitants[18] and the incidence profoundly increases with age (> 65 years old). However, a childhood AML can also arise in children aged <15 years.

For AML, it is possible to distinguish 3 groups, based on the etiology of the disease:

- *de novo* AML, when the cause of AML is unknown;
- secondary AML, when the patients have been exposed to known leukemogenic agents;
- secondary AML to an MDS, when AML represents the evolution of MDS in patients[4].

In particular, AML development has been correlated to different risk factors including:

- genetic/congenital disorders such as Down syndrome, SDS or Fanconi anemia;
- radiation exposure therapeutic or not. For example, it has been proved a correlation with ionizing exposure during flight operation and AML onset[19]. In addition, the atomic bomb survivors of Hiroshima and Nagasaki showed a higher incidence of AML[20];

- physical and chemical exposure such as to pesticides or benzene, an established leukemogen. Cigarette smoking also increases AML incidence, because of the high content of benzene[21];
- chemotherapy. Cytotoxic drugs, such as alkylating agents, administered to reduce a previous tumor mass, can influence AML incidence[22].

1.6 AML classification

AML is a very heterogeneous disease and different classifications have been proposed in the last years. From the first attempt of classification derives the French-American-British (FAB) classification in 1976, which distinguishes eight subtypes (M0 through M7) based on the cytochemical and morphological characteristics of the leukemic cells. The subgroups include:

- M0: Undifferentiated acute myeloblastic leukemia;
- M1: Acute myeloblastic leukemia with minimal maturation;
- M2: Acute myeloblastic leukemia with maturation;
- M3: Acute promyelocytic leukemia (APL);
- M4: Acute myelomonocytic leukemia;
- M5: Acute monocytic leukemia;
- M6: Acute erythroid leukemia;
- M7: Acute megakaryoblastic leukemia[23].

In 2001, the World Health Organization (WHO) proposed a new classification system, that was revised in 2008 and 2016. The latest version identifies six major AML groups, according to genetic information, immunophenotype, blasts morphology and clinical presentation. The groups are:

- AML with recurrent genetic abnormalities;
    - AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1,*
    - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11,*
    - APL with *PML-RARA,*
    - AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A,*
    - AML with t(6;9)(p23;q34.1); *DEK-NUP214* ,
    - AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM,*
    - AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1,*
    - AML with *BCR-ABL1,*

Provisional entity: AML with *BCR-ABL1,*

AML with mutated *NPM1,*

AML with biallelic mutations of *CEBPA,*

Provisional entity: AML with mutated *RUNX1,*

- AML with myelodysplasia-related features;
- therapy-related AML;
- AML not otherwise specified:
  - AML with minimal differentiation,
  - AML without maturation,
  - AML with maturation,
  - Acute myelomonocytic leukemia,
  - Acute monoblastic/monocytic leukemia,
  - Pure erythroid leukemia,
  - Acute megakaryoblastic leukemia,
  - Acute basophilic leukemia,
  - Acute panmyelosis with myelofibrosis;
- myeloid sarcoma;
- myeloid proliferation related to Down syndrome:
  - Transient abnormal myelopoiesis (TAM).
  - Myeloid leukemia associated with Down syndrome[24].

2. MDS AND AML PATHOGENESIS

A well accepted-concept is that MDS and AML originate from alterations of hematopoietic stem and progenitor cells (HSPCs)[25–28]. Due to an unknown trigger or potentially due to the aging, HSPCs acquire genetic and/or cytogenetic aberrations resulting in survival advantage on normal HSPCs. The subsequent clonal expansion of the mutated HSPCs leads to the acquisition of new alterations[29]. These cell autonomous/intrinsic mechanisms have been extensively investigated. Furthermore, for AML it is well accepted that the mutated clone, able to perpetuate and maintain leukemia, is the leukemia stem cell (LSC) population[30].

HSPCs reside physiologically within multiple stem cell niches in the BM microenvironment, which influences hematopoietic cell functions. In the last years, several components of the hematopoietic niche have been found to regulate HSCs, including osteoblast (OBs), osteoclasts, sympathetic nerve fibers, adipocytes, endothelial cells and mesenchymal stromal cells (MSCs). In particular, MSCs have been extensively investigated for their potential role in the pathogenesis of myeloid neoplasm. This cell-extrinsic concept challenges the evidence sustaining that MDS and AML derive exclusively from HSPC-intrinsic defects.

Recently, new studies demonstrated that myeloid malignant cells exploit physiological signal and can shape niche cells in the BM microenvironment, in order to create a permissive/self-reinforcing and more favorable niche, supporting malignant cells at the expense of normal HSPCs. In Figure 1, the two last hypotheses are illustrated.

All these concepts have been demonstrated in mice models and in some cases in patients, indicating a fundamental bi-directional interaction among malignant cells and surrounding microenvironment[27].

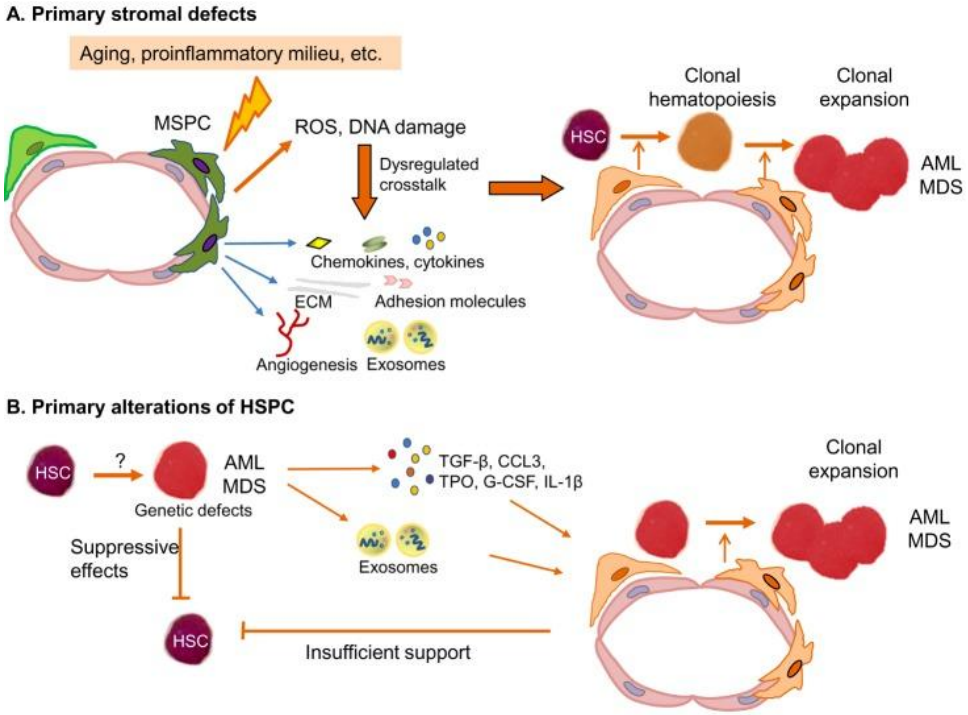


Figure 1. Two models of MDS and AML pathogenesis[29].

2.1 CELL INTRINSIC ALTERATIONS IN MDS AND AML

2.1.1 Cytogenetic alterations in MDS

The identification of karyotype alterations is a key feature of the IPSS and IPSS-R for the prognostication of MDS patients. Chromosomal abnormalities are present in up to 50% of patients and each karyotype has different clinical implications, regulating disease course and treatment[31]. However, cytogenetic heterogeneity prevents the characterization of the impact of rare alterations and combinations of abnormalities.

In contrast to AML, MDS karyotype is characterized by an overweighing of unbalanced alterations, while balanced abnormalities, like inversions and translocations, are rare. Frequently, deletions and monosomies can be detected, leading to the loss of genetic material, whereas its acquisition in the form of total or partial trisomies is less frequent. Unbalanced translocations, leading to gain or loss of genetic material, are usually identified in MDS with multiple abnormalities. These data suggest that in MDS the inactivation or loss of tumor suppressor genes represents the more relevant molecular mechanism, compared to a gain of activity of oncogenes[32].

In MDS patients, cytogenetic abnormalities include:

- del(5q). The most frequent chromosomal abnormalities (up to 15% of diagnosed MDS) is represented by the deletions of the long arm of chromosome 5 (5q)[31]. The deletion affects different genes, including those encoding for ribosomal proteins such as RIBOSOMAL PROTEIN S14 (RPS14), resulting in p53 inactivation. Del(5q) induces also the dysregulation of microRNA (miRNA). MDS with del(5q) arisen following cytotoxic therapy/radiation exposure, show additional chromosomal abnormalities and *TUMOR PROTEIN 53* (*TP53*) mutations with an increased propensity to develop into AML and a shorter overall survival (OS) compared to MDS with *de novo* del(5q)[33];
- chromosome 7 anomalies such as monosomy 7 (-7) and del(7q). These alterations are identified in 10% of *de novo* MDS and 50% of therapy-related MDS[31,34] and correlate with worse prognosis and OS[35]. -7/del(7q) leads to haplo-insufficiency of multiple genes, including the tumor suppressor gene CUT LIKE HOMEOBOX 1 (*CUX1)*[36] and the *MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA PROTEIN 5* (*MLL5*) gene, encoding for a histone methyltransferase;
- trisomy 8 (+8). In the 5% of MDS patients, it is possible to identify +8[37], associated with an intermediate prognostic risk. In MDS pathogenesis, this chromosomal aberration occurs late and

leads to the overexpression of anti-apoptotic proteins. Furthermore, up to 67% of +8 MDS patients show a response to immunosuppressive therapies, suggesting an autoimmune pathophysiology of trisomy 8[38]. The hypothesis is that the overexpression of anti-apoptotic proteins confers an advantage to +8 malignant cells over normal hematopoietic cells. Thus, the MDS clone survive in the autoimmune microenvironment whereas normal cells are eradicated[39];

- Del(20q). The deletion of the long arm of chromosome 20 occurs in less than 5% of MDS patients and is associated with a favorable prognosis[31]. However, the genes lost in the deleted regions of 20q have not been associated with the development of MDS[39];
- sex chromosome abnormalities (-X,-Y). The loss of X/Y chromosomes is an age-related phenomenon and can be associated with hematological diseases[40]. –Y is a frequent chromosomal alteration and it is a potential driver of MDS[41]. However, the prognosis of patients with –Y is very good. –X in female patients is less frequent and the alteration is associated with an intermediate prognosis[35];
- Others: 3q abnormalities which are rare and associated with a short OS, trisomy 13/del(13q), the very rare trisomy and monosomy of 21, isochromosome 17q resulting in the loss of different genes including *TP53,* del(12p), del(11q).

As already described, based on cytogenetic alterations, the IPSS-R established 4 categories of prognostic risk, in the table 1 is reported the MDS cytogenetic scoring system[14].

Table 1. MDS cytogenetic scoring system (IPSS-R)

Prognostic subgroups	**% of patients**	**Cytogenetic abnormalities**	**Median survival (years)**
Very good	4%/3%	−Y, del(11q)	5.4
Good	66%/72%	Normal, del(5q), del(12p), del(20q), double including del(5q)	4.8
Intermediate	13%/19%	del(7q), +8, +19, i(17q), any other single or double independent clones	2.7
Poor	4%/5%	−7, inv(3)/t(3q)/del(3q), double including −7/del(7q), complex: 3 abnormalities	1.5
Very poor	7%	Complex: > 3 abnormalities	0.7

2.1.2 Genetic alterations in MDS

In the last years, modern technology has allowed the identification of genetic rearrangements and driver somatic mutations in more than 50% of cases of MDS, and the majority of these patients show a normal karyotype. In addition, in some cases, mutations are associated with specific morphologic features or clinical outcome[42]. Several studies using high throughput Next Generation Sequencing revealed the landscape of gene mutations which drive the initiation of MDS (driving mutations). Generally, leukemic stem cells of patients who progress to AML, acquire new mutations giving a growth advantage to the malignant clone[43]. However, a careful analysis has also been performed in order to discriminate acquired mutations not involved in the pathogenesis of the disease ('passengers'), which have been identified in HSCs[44]. Genetic alterations usually disrupt different cellular processes and based on their function, the driving mutations are divided into distinct groups:

- RNA-splicing machinery, mutated in 64% of MDS patients with somatic mutations. The mutated genes codify for splicing factors, involved in the splicing process fundamental to remove introns from a pre-mRNA, in order to generate a mature mRNA. The mutated genes include: *SPLICING FACTOR 3B, SUBUNIT 1* (*SF3B1), SERINE/ARGININE-RICH SPLICING FACTOR 2* (*SRSF2), ZINC FINGER CCCH-TYPE, RNA BINDING MOTIF AND SERINE/ARGININE RICH 2 (ZRSR2), U2 SMALL NUCLEAR RNA AUXILIARY FACTOR 1/2 (U2AF1/U2AF2), PRE-MRNA PROCESSING FACTOR 40 HOMOLOG B (PRPF40B), SPLICING FACTOR 1* (*SF1), SPLICING FACTOR 3A SUBUNIT 1 (SF3A1)*. The mutations are mutually exclusive, disrupt the recognition of the 3′-splice site of pre-mRNA, and are most frequently missense and heterozygous[45];
- Epigenetic regulators including genes involved in DNA methylation: *METHYLCYTOSINE DIOXYGENASE 2 (TET2), DNA METHYLTRANSFERASE 3A (DNMT3A), $NADP^+$-DEPENDENT ISOCITRATE DEHYDROGENASES 1* and *2* (*IDH1/2*), and in chromatin modifications: *ENHANCER OF ZESTE 2 POLYCOMB REPRESSIVE COMPLEX 2 SUBUNIT (EZH2), SOMATIC ADDITION OF SEX COMBS LIKE1 (ASXL1*). *TET2* encodes for a protein which regulates DNA demethylation by converting 5-methylcytosine to 5-hydroxymethylcytosine. This process is reduced in *TET2*-mutated myeloid neoplasms[46]. *TET2* mutations occur in 20-30% of MDS patients and the altered methylation pattern silences the expression of genes involved in myeloid differentiation[47]. *IDH1* and *IDH2* genes encode for enzymes catalyzing a reaction in the tricarboxylic acid (TCA) cycle. They protect the cell from oxidative stress. The mutations lead to the accumulation of d-2-hydroxyglutarate (2HG) within the cell, which is thought to have an oncogenic activity. 2HG can

also function as a competitive inhibitor for TET family and for α-KG-dependent dioxygenases, resulting in aberrant DNA methylation[46]. *IDH1/2* mutations are less frequent and occur in 5% of MDS patients[48]. *DNMT3A* and *ASXL1* will be discussed later in this thesis.

- transcription factor and tumor suppressor, mutated in the 15 % of MDS patients with somatic mutations. Mutations of transcriptional factors impair the differentiation and maintenance of HSCs and the genes involved are *TP53, RUNT-RELATED TRANSCRIPTION FACTOR 1 (RUNX1), ETS VARIANT 1 (ETV1), GATA BINDING PROTEIN 2 (GATA2), CCAAT ENHANCER BINDING PROTEIN ALPHA (CEBPA)*. Germ-line mutations of *GATA2*, *CEBPA*, and *RUNX1* are the cause of familial MDS (and AML)[49–51]**.** *TP53* alterations have been identified in 10% of MDS patients and 30–50% of these MDS show a complex karyotype. Indeed, *TP53* mutations have been reported in 'high risk' groups and are associated with an adverse prognosis[52]**;**
- signal transduction/kinases: mutations present in 15% of MDS patients with somatic mutations. These alterations affect genes such as: *FMS RELATED TYROSINE KINASE 3 (FLT3), JANUS KINASE 2 (JAK2), MPL PROTO-ONCOGENE, THROMBOPOIETIN RECEPTOR (MPL), GNAS COMPLEX LOCUS (GNAS), KIT PROTO-ONCOGENE RECEPTOR TYROSINE KINASE* (KIT) in a mutually exclusive manner[52]. *FLT3* and *KIT* encode for receptor tyrosine kinases and mutations are found in <5% of MDS, whereas are commonly seen in *de novo* AML. Mutations in the *JAK2* gene, encoding for a cytoplasmic tyrosine kinase, in particular, the V617F mutation, have been identified in 5% of MDS[53];
- RAS pathway: mutated in 12% of MDS patients with somatic mutations. The genes mutated include *KRAS PROTO-ONCOGENE, GTPase (KRAS), NRAS PROTO-ONCOGENE, GTPase (NRAS), CBL PROTO-ONCOGENE (CBL), NEUROFIBROMIN 1 (NF1), PROTEIN TYROSINE PHOSPHATASE NON-RECEPTOR TYPE 11 (PTPN11).* RAS family members regulate cellular proliferation, and mutations in these signaling regulators contribute to progression to AML[54];
- cohesin complex, mutated in 13% of MDS patients with somatic mutations. The mutually exclusive mutations affect the genes: *CCCTC-BINDING FACTOR (CTCF), STRUCTURAL MAINTENANCE OF CHROMOSOMES 1A (SMC1A), RAD21 COHESIN COMPLEX COMPONENT (RAD21),* which encode for cohesin proteins. The cohesion complex forms a structure that surrounds DNA in order to maintain sister chromatid cohesion and facilitates homologous recombination-mediated DNA repair[55]. The hypothesis underlying MDS pathogenesis, driven by cohesin complex mutations, is that they cause dysregulation of long-range chromatin interactions, which induce gene expression alterations rather than chromosomal aberration[55];

- DNA repair mechanisms: mutated in 10% of MDS patients with somatic mutations. Alterations have been identified in genes such as *ATM SERINE/THREONINE KINASE (ATM), BRCA1/BRCA2-CONTAINING COMPLEX SUBUNIT 3 (BRCC3), DNA CROSS-LINK REPAIR 1C (DCLRE1C), FA COMPLEMENTATION GROUP L (FANCL)*. DNA double-strand breaks are DNA alterations, that if not corrected by DNA repair mechanisms, can influence the formation of chromosomal abnormalities, a feature of MDS[56];
- other mutations: present in 10% of MDS patients with somatic mutations. In the last years, the dysregulation of miRNA biology, affecting post-transcriptional protein expression, the mutations in mithocondrial DNA resulting in the reduced expression of iron transporter, the mutations in the *ATP BINDING CASSETTE SUBFAMILY B MEMBER 7* (*ABCB7*) gene have been identified. In addition, mutations in the *SET-BINDING PROTEIN 1* (SETBP1) gene have been recently reported. SETBP1 regulates the expression of the SET nuclear protein, an oncogene involved in cell proliferation;
- a fraction of MDS (around 10%) does not show mutations.

2.1.3 Cytogenetic alterations in AML

In the 55% of AML patients is possible to detect one or more cytogenetic abnormalities. Thus, they represent a strong prognostic factor considered in the WHO classification[57]. Cytogenetic analysis revealed distinct alterations including translocations, insertions, deletions, inversions, trisomies, monosomies, polyploidy, and other aberrations. According to a cytogenetic analysis performed in a study conducted on 5876 AML patients, the most common karyotype alterations are:

- t(15;17)(q24.1;q21.2) and variants (13%), the balanced translocation characterizes acute promyelocytic leukemia (APL). The breakpoint on chromosome 15 occurs within the *PROMYELOCYTIC LEUKEMIA* (*PML)* gene, whereas on chromosome 17 it occurs in *ALPHA RETINOIC ACID RECEPTOR* gene (*RARA*)[58]. The PML-RARA fusion protein leads to persistent transcriptional repression, thereby blocking the differentiation of promyelocytes[58];
- Trisomy 8 (10%);
- t(8;21)(q22;q22.1) and variants (7%), the balanced translocation (8;21)(q22;q22.1) leads to the formation of a fusion gene composed of *RUNX1*, on chromosome 21, and *RUNX1 TRANSLOCATION PARTNER 1* (*RUNX1T1*), on chromosome 8. WT RUNX1 heterodimerizes with the CORE BINDING FACTOR BETA (CBFB), forming a complex called core binding factor

transcription complex, which regulates gene expression. The chimeric form RUNX1-RUNX1T1 in AML has a dominant activity and represses the expression of target genes of the wild-type complex. The t(8;21)(q22;q22.1) is identified predominantly in AML FAB subtype M2, characterized by hematopoietic cell maturation and is commonly associated with del(9q)[59] or loss of a sex chromosome (-Y or -X);

- 11q23.3 rearrangements (6%). The *LYSINE METHYLTRANSFERASE 2A* (*KMT2A)* gene is altered by the translocations of 11q23.3, which involve another gene from another chromosome. The most common translocation occurs with *MIXED-LINEAGE LEUKEMIA TRANSLOCATED TO CHROMOSOME 3* (*MLLT3)* gene, at 9p21.3. The gene encodes for a DNA-binding protein that methylates histone H3 lysine 4 (H3K4), and the translocation leads to the absence of the H3K4 methyltransferase activity, regulating positively gene expression[60]. Others *KMT2A* partner genes have been identified, including genes encoding for nuclear proteins with DNA-binding and histone acetyltransferase functions, and genes encoding for proteins of the Septin family, which are cytoskeletal proteins fundamental during cytokinesis[61]. 11q rearrangements are associated with acute monoblastic leukemia (AML-M5);
- inv(16)(p13.1q22)/t(16;16)(p13.1q22) (5%). At 16q, the breakpoint occurs in the coding region of *CBFB* gene, which encodes for one protein of the core binding factor transcription complex as already described. At 16 p, the breakpoint interrupts the *MYOSIN HEAVY CHAIN 11* (*MYH11*). The resulting CBFB-MYH11 fusion protein disrupts the function of the RUNX1/CBFB transcription factor and represses the transcription of target genes[62]. The alterations of chromosome 16 are associated with myelomonocytic AML with eosinophilia (FAB subtype M4v).

Based on chromosomal aberrations, it is possible to identify three classes of risk: favorable, intermediate and unfavorable, according to clinical prognosis. The favorable risk class includes several aberrations such as t(8;21)(q22;q22) with RUNX1/RUNX1T1, t(15;17)(q24;q21) with PML/RARA, inv(16)(p13q22) with CBFB/MYH11, the intermediate includes t(9;11)(p22;q23) MLLT3/MLL and normal karyotype, whereas unfavorable risk class includes alterations such as t(6;9)(p23;q34) DEK/NUP214 inv(3)(q21q26) RPN1/EVI1 t(1;22)(p13;q13) RBM15/MKL1 complex karyotype[63].

2.1.4 Genetic alterations in AML

Around 41-45% of patients diagnosed with AML shows a normal karyotype and represents a very heterogeneous group of patients. In the last years, new molecular technologies have allowed the identifications of thousands of mutations in various genes, belonging to genetic and epigenetic pathways. The genes affected encode for myeloid transcription factors (e.g., *RUNX1*, *CEBPA*), tumor suppressor proteins (e.g. *TP53*, *WILMS TUMOR 1 (WT1)* and *PHD FINGER PROTEIN 6* (*PHF6)*), signaling proteins (e.g., *RAS*, *KIT*, *CBL*, and *FLT3*), DNA methylation proteins (e.g. *DNMT3A*, *TET2*, *IDH1/2*, and *SETBP1*) and chromatin modifiers (e.g. *ASXL1*, *EZH2*, *LYSINE DEMETHYLASE 6A (KDM6A)*, *KMT2A/MLL*). The genetic alterations provide fundamental cell-autonomous growth signals to leukemic cells and alter the way of LSCs and their progeny to sense environmental signals. Many of the genes identified have a prognostic value. In addition, mutations in *NUCLEOPHOSMIN 1* **(***NPM1)*, *CEBPA* and *RUNX1* are fundamental also for WHO classification. Mutations with a relevant clinical impact in AML pathogenesis occurs in:

- *NPM1*: encodes for NUCLEOPHOSMIN 1, a protein localized in the nucleolus although it can move from nucleus to cytoplasm. It is a histone chaperone involved in histone assembly and nucleosome assembly. It is also involved in transcription, DNA repair, apoptosis, ribosome biogenesis[57]. Various mutations of *NPM1* have been reported but all of them occur in the C-terminus of the protein, necessary for the nucleolar targeting of *NPM1*, leading to the alterations of the normal nucleus-cytoplasm traffic of *NPM1* and its abnormal accumulation in the cytoplasm. Despite *NPM1*-mediated leukemogenesis is not understood, it is clear that it depends on the perturbation of the nucleophosmin traffic[64]. So far, *NPM1* mutations are the most common single gene alterations identified in the 50-60% of AML with normal karyotype and in the 30% of all AML[65]. In addition, *NPM1* mutations correlate with a favorable outcome also in AML patients aged 60 years[66];
- *FLT3:* it encodes for FMS-LIKE TYROSINE KINASE 3 belonging to the superfamily of tyrosine kinase receptor class 3, which acts as the receptor in the cell membrane for the cytokine FLT3 ligand (FLT3L). The receptor regulates proliferation, differentiation, and apoptosis of hematopoietic cells. *FLT3* mutations are common in AML patients and are associated with poor outcome. Generally, two types of mutations are recognized. 1) Internal tandem duplications of *FLT3 (FLT3-ITD)* which are identified in about the 30% of cytogenetically normal AML and in the 20% of all AML patients. *FLT3-ITD* predict high AML cell number, higher frequency of relapse, and shorter OS[67]. These mutations constitutively activate the tyrosine kinase activity enhancing the

signaling of RAS, MITOGEN ACTIVATED KINASE-LIKE PROTEIN (MAPK) and SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 5 (STAT5). 2) missense mutations in exon 20, the most common is the substitution of an aspartate with a tyrosine at codon 835 (D835Y). These mutations, by changing the conformation of the protein, eliminate its autoinhibitory function, causing a constitutive activation. The influence of missense mutations on prognosis is controversial[68];

- *CEBPA*: it encodes for the CCAAT/ENHANCER BINDING PROTEIN ALPHA, a member of the basic region leucine zipper (bZIP) transcription factor family fundamental for neutrophil development. Two major mutations of *CEBPA* have been identified: the non-sense N-terminal mutation which leads to a truncated isoform with dominant negative properties, and the C-terminal mutation resulting in a decrease of DNA-binding or dimerization activity of CEBPA[69]. When mutations at both C-terminus and N-terminus of *CEBPA* (*CEBPA*^{dm}) are present, the highest effect in accelerating disease development is noticed. *CEBPA*^{dm} AML has been recognized as a distinct entity compared to AML with the single mutation. Indeed only *CEBPA*^{dm} is associated with a favorable prognosis and it is mutually exclusive with *NPM1* mutations[69] and often associated with GATA2 (a gene encoding for a transcription factor regulating hematopoietic stem cell proliferation and megakaryocytopoiesis) mutations[70];

In AML patients, other genes mutated encode for:

- RAS family, including guanine nucleotide binding proteins downstream of tyrosine kinase receptors, which regulates hematopoiesis and became constitutively active in AML[57];
- WT1, a transcription factor which relevance on prognosis remains controversial;
- RUNX1;
- TP53, a cell cycle controller. Despite is the commonest tumor suppressor gene mutated in human cancer, in AML, *TP53* changes are rare and associated with a complex karyotype and a very poor prognosis[71];
- c-KIT, the tyrosine kinase receptor of the stem cells factor. *KIT* mutations are correlated with an increased rate of relapse particularly in patients with t(8;21)[72];
- Epigenetic regulators: the prognostic impact of these mutations is still under investigation. The epigenetic processes involved in AML pathogenesis include DNA methylation, with genes such as *DNMT3A, TET2, IDH1/2* and chromatin modifications with genes, such as *ASXL1*. *TET2* mutations have been identified in the 7.6% of AML and are mutually exclusive with *IDH* mutations [73]**.** *IDH1* mutations are found in the 6-8% of AML and the 10-12% of AML with a normal

karyotype, whereas *IDH2* mutations occur in the 9-11% of unselected AML. The mechanism of *IDH1/2*-induced leukemogenesis is the same already described above for MDS.

2.1.5 Mutation in epigenetic regulators in MDS and AML

As already mentioned, mutations in epigenetic regulatorS are fundamental in the pathogenesis of myeloid malignancies. In particular, genes encoding for the proteins involved in DNA methylation and chromatin modifications have been identified, including *DNMT3A* and *ASXL1*. More recently, *DNMT3A, ASXL1* but also *TET2, TP53, JAK2,* and *SF3B1* have been associated with a phenomenon called clonal hematopoiesis of indeterminate potential (CHIP). Indeed, genome sequencing studies, performed on peripheral blood samples isolated from thousands of individuals without hematological malignancies, identified somatic myeloid malignancy-associated mutations in almost the 10% of people aged over 65 and more than 20% of the person aged over 90 years[74,75]. Despite most of the people with CHIP do not develop any hematologic malignancy, the rate of transformation is 0.5–1% per year, about 13 times higher compared to the incidence of these neoplasms in the rest of population[76]. This suggests that the mutation itself can predispose, but it is not sufficient to induce the development of a hematological disorder[77]. In addition, the preleukemic clones have multilineage differentiation ability, survive chemotherapy and can expand in remission[25].

2.1.5.1 DNMT3A, general features, and structure

DNMT3A encodes for DNA METHYLTRANSFERASE 3A, which transfers a methyl group to specific DNA structures. The gene is located on the chromosome 2 in human and on the chromosome 12 in mouse.

In mammals, DNA methylation occurs at the C5-position of the pyrimidine ring of cytosine residues to form 5-methylcytosine. Usually, repression of gene expression is associated with high DNA methylation[77]. This epigenetic modification regulates different processes like development, stem cell regulation, imprinting, X-chromosome inactivation. Furthermore, aberrant DNA methylation patterns are found in cancer cells.

There are three DNA METHYLTRANSFERASES (MTASES): DNMT1, DNMT3A, and DNMT3B. DNMT1 is the maintenance methyltransferase and regulates the propagation of methylation patterns after DNA replication. On the contrary, DNMT3A, like DNMT3B, is a *de*

novo DNA methyltransferase, meaning that it establishes de novo methylation[78]. DNMT3A has 2 isoforms, DNMT3A1/2, which show different activity on binding and methylating DNA at regulatory sites[79].

The cytosine-C5 MTases activity is located in the C-terminal part of DNMT3A. In the N-terminal part, there is a PWWP domain, which binds DNA, and a Cys-rich domain called ADD (also known as the PHD), which interacts with other proteins and subcellular targeting of the enzyme (Fig.2)[80].

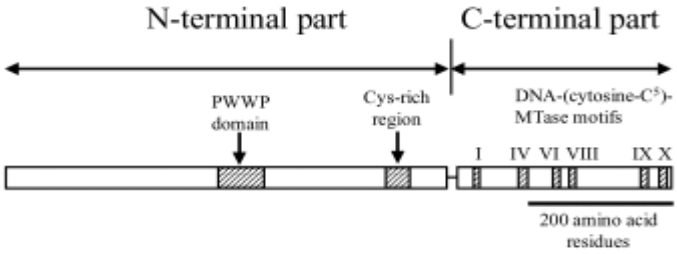


Figure 2. Primary structure of DNMT3A. the MTase activity is in the C-terminal part, while the N-terminal part contains a PWWP and a Cys-rich domain[80].

The mechanisms underlying the enzymatic activity of DNMT3A are not yet understood. The methylation activity is linked to the formation of homo and heterotetramers. One of the most characterized proteins which binds DNMT3A is DNMT3-like (DNMT3L). DNMT3L is an inactive homolog which regulates the oligomeric state of DNMT3A. DNMT3L binds to DNMT3A along a surface with high structural conservation, called tetramer interface. This interface also supports DNMT3A homotetramerization *in vitro*. The disruption of the tetramer interface reduces the methylation activity of the enzyme[81].

DNMT3A plays a crucial role during development inducing the switch from self-renewal towards a differentiation program. It is highly expressed in murine embryonic stem cells (ESCs) and is down-regulated once the cells are more differentiated. The deletion of both DNMT3A/B in ESC leads to inefficient differentiation. In addition, DNMT3A is involved in neural stem cell differentiation in mice[82].

2.1.5.2 DNMT3A in myeloid malignancies

Heterozygous mutations of *DNMT3A* have been identified in patients with myeloid malignancies. In 2010, for the first time, Ley and co-workers identified somatic mutations in *DNMT3A* gene in 22,1% of AML patients with a normal karyotype. In addition, they showed that patients with *DNMT3A* mutations had a shorter OS, compared to patients with other alterations. In AML patients, *DNMT3A* mutations are associated with mutations in other genes such as *FLT3, NMP1,* and *IDH1/2* genes[83]. Meanwhile, *DNMT3A* mutations are present in a mutually exclusive manner with others

alterations, including the chromosomal translocations t(15;17)[83] or mutations of the transcriptional regulator *ASXL1*[84], suggesting a contribution of these alterations on the same epigenetic perturbations.

DNMT3A mutations have been identified also in the 10 % of patients with myeloproliferative neoplasms (MPN) and in 10 % of MDS patients. Also in these patients, *DNMT3A* mutations are often associated with others mutations involving several genes like spliceosome factor *SF3B1*[84] and *IDH1/2*[85]. Walter and collaborators showed that in *de novo* MDS, *DNMT3A* mutations correlate with a worse OS and a more rapid progression to AML[86].

In myeloid neoplasm, the identified *DNMT3A* mutations include missense, frameshift, nonsense, splice-site mutations, and deletions. The most common is the missense mutation R882H, which occurs within the catalytic domain. The altered protein acts like a dominant negative preventing the formation of wild-type active tetramers and resulting in hypomethylation throughout the genome[87].

2.1.5.3 Role of DNMT3A in leukemogenesis

The mechanisms underlying the tumor suppressor activity of DNMT3A are not yet fully elucidated. Recently, it has been demonstrated that the conditional loss of *Dnmt3a* in a mouse model, increases self-renewal and impairs differentiation of HSCs[88]. Furthermore, Mayle and collaborators showed that *Dnmt3a* loss predisposes HSCs to malignant transformation. Indeed, mice transplanted with *Dnmt3a*-deleted HSCs develop different malignancies including MDS, AML, myelofibrosis and lymphocytic leukemia. The common feature was an altered methylation pattern in HSPCs[89]. Moreover, transplanted *Dnmt3a*-delated HSCs acquire other mutations. These acquired mutations can contribute with *Dnmt3a* loss, to drive malignancy. The hypothesis is that *Dnmt3a* mutations arise early and support leukemogenesis, and cells that have acquired additional mutations are selected[89].

Guryanova et al. deeply characterized the phenotype of primary mice in which the conditional loss of *Dnmt3a* in the hematopoietic compartment was induced. The absence of *Dnmt3a* induces myeloid transformation *in vivo*, with aberrant tissue tropism and extramedullary hematopoiesis, especially in the liver. They clearly showed that *Dnmt3a* not only regulates HSC self-renewal but also tissue tropism and restricts myeloid progenitor expansion[90]. The hypothesis is that the self-renewal advantage could potentially serve as a pre-leukemic lesion.

In the leukemogenesis process, the downstream genes involved in DNMT3A activity are still not well characterized. Ferreira and collaborators identified the leukemogenic HOX cofactor, MEIS1. In AML patients with DNMT3A mutations, the promoter of MEIS1 is hypomethylated and the gene

is reactivated. MEIS1 is essential for the development of hematopoietic cells and its transcriptional activity is higher in hematopoietic stem cells and early progenitor cells, and decreases in later stages of hematopoietic development. This pattern appears dysregulated in leukemogenesis, and persistent overexpression of MEIS1 has been associated with poor prognosis in AML patients[91].

The relevance of DNMT3A mutations in the pathogenesis of myeloid malignancies derives also from other evidence observed in humans. Indeed, *DNMT3A* mutations have been identified in the founding hypomethylated clones of patients, suggesting that they could arise first[92]. In addition, *DNMT3A* mutations are also present in phenotypically normal HSCs, indicating that DNMT3A can induce a pre-leukemic state [93] and, as already mentioned, can be found in CHIP. Furthermore, the pre-leukemic clone with *DNMT3A* mutations found at diagnosis in AML patients can be detected also in remission samples, suggesting its resistance to chemotherapy.

2.1.5.4 ASXL1 general features, and structure

ASXL1 encodes for a chromatin-binding protein, a member of the Polycomb group (PcG) proteins. ASXL1 maintains stable repression and activation of homeotic loci and other loci[94]. The gene is on the chromosome 12 in humans and on the chromosome 2 in mice, and it was discovered in mammals after the identification of its homolog gene, the Additional sex combs (*Asx*) gene in *Drosophila melanogaster* flies[95].

In mammals, *ASXL1* is part of a gene family which consists of other 2 genes: *ASXL2* and *ASXL3.* The three proteins show a common protein structure. In the N-terminal region, there is an ASXN domain, in the adjoining region an ASXH domain, while in the middle region, there are ASXM1 and ASXM2 domains, and a PHD domain is present in the C-terminal region. The ASXN and PHD domains are the DNA or histone binding domains of the proteins (Fig.3). The middle domains of the proteins interact with other proteins important for ASXL function[96].

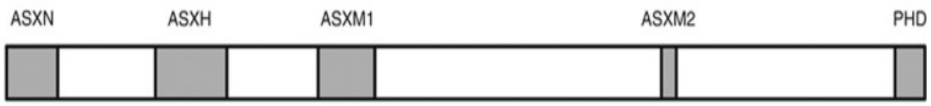


Figure 3. ASXL1 protein structure. The conserved domain architecture is composed of ASXN, ASXH, ASXM1, ASXM2 and PHD domain[96]**.**

ASXL1 regulates transcriptional repression, by interacting with deubiquitinating enzyme BRCA1 associated protein-1 (BAP1), and by recruiting in specific loci, the Polycomb repressor complex 2 (PRC2), which catalyzes the trimethylation of histone H3 (at the lysine 27 H3K27me)[97]. ASXL1 is an epigenetic scaffolding protein, meaning that it is involved in the recruitment of factors to specific loci with histone modification ability. In addition, ASXL1 is involved in transcriptional regulation

also through the interaction with nuclear hormone receptors (NHR), such as retinoic acid receptor[98], inducing or inhibiting their activation.

2.1.5.5 ASXL1 in myeloid malignancies

For the first time in 2009, Gelsi-Boyer and collaborators reported mutations in *ASXL1* gene in the 11% of MDS patients and in the 43% of patients with chronic myelomonocytic leukemia (CMML)[99]. Later, the same research group identified *ASXL1* mutations in the 8% of patients with MPN, especially thrombocythemia and primary myelofibrosis[100] and in the 17% of patients with AML. They also found that *ASXL1* mutations are mutually exclusive with NPM1 mutations, present in the 63% of AML patients of that study[101]. In MDS patients, it has been proved that *ASXL1* mutations correlate with poor clinical outcome[52]. Moreover, the higher incidence of *ASXL1* mutations occurs in MDS that later evolve to AML[102]. In addition, Chou and collaborators showed that AML patients with *ASXL1* mutations are older, have a lower complete remission (CR) rate and that, in some patients, *ASXL1* mutation status can change during disease evolution. Indeed, some of the *ASXL1*-wild patients acquired *ASXL1* mutations during relapse (2 over 109 patients) whereas other patients with *ASXL1* mutations lost them at relapse and/or refractory status (2 over 6 patients)[103].

ASXL1 mutations, identified in myeloid malignancies, are heterozygous and often they include frameshifts, nonsense, deletion/insertion mutations in exon 12, leading to the truncation of the ASXL1 protein to the C-terminal PHD finger, essential for chromatin modifications[99]. The truncated protein should act as a dominant negative, suppressing the wild-type function, or as a gain of function mutant. The ASXL1 mutations result also in a loss of ASXL1 expression[104].

These data reveal that ASXL1 is a tumor suppressor, whose inactivation has a role in myeloid diseases and clinical outcome.

2.1.5.6 Role of ASXL1 in leukemogenesis

Despite evidence proving the role of *ASXL1* in myeloid transformation, the mechanisms of leukemogenesis *ASXL1*-mediated are not fully elucidated. Recent studies showed that ASXL1 regulates hematopoietic cells fate. An *in vitro* study demonstrated that silencing ASXL1 expression, in human $CD34^+$ progenitor cells, perturbs granulo-monocytic differentiation[105]. Furthermore, Abdel-Wahab et al. demonstrated that ASXL1 deficiency in human $CD34^+$ cells leads to the loss of PRC2 mediated histone H3 tri-methylation, inducing the expression of homeobox A

(HOXA) genes, which are known to be involved in myeloid transformation[104]. The same research group studied the role of *Asxl1 in vivo,* by using murine models. They proved that constitutive loss of *Asxl1* induces developmental abnormalities, with 100% of mortality. On the contrary, hematopoietic-specific loss of *Asxl1* results in the development of MDS with cytopenia, dysplasia and an increased total number of stem/progenitor cells, features of human MDS. However, the cells showed impaired self-renewal ability and impaired erythroid and mature myeloid differentiation. In addition, as expected *Asxl1* loss induces a reduction of histone H3 tri-methylation with altered expression of regulators of hematopoiesis, in mice[106]. In another independent study, Wand and collaborators also demonstrated that a full deletion of *Asxl1* in mouse induces developmental abnormalities with a high rate of mortality (80%). Surviving primary *Asxl1* knockout (KO) mice showed features of MDS syndrome with dysplastic neutrophils, multiple lineage cytopenia. Furthermore, $Asxl1^{(-/-)}$ mice showed an increase in the GMP population and an altered HSC pool, characterized by decreased repopulating ability and with a skewed cell differentiation towards granulocytic lineage[107].

All these data suggest that mutations in ASXL1 are loss of function. Nevertheless, a growing body of evidence indicates that *ASXL1* mutations can also result in a gain of function. Indeed, Inoue and collaborators using a mouse model in which *Asxl1* is only mutated demonstrated that C-terminal truncating mutations block myeloid differentiation and promote MDS in mice, with occasional progression to leukemia. In this study, the mutant mice also showed inhibition of histone H3 tri-methylation mediated by PRC2, with the expression of a subset of targets, such as *Homeobox A9* (*Hoxa9*) gene and micro-RNA125a, involved in myeloid transformation[108]. In addition, in a very recent study, the mutually reinforcing interaction of C-terminally truncated form of mutant ASXL1 (ASXL1-MT) with BAP1 has been proved to be fundamental to promote myeloid leukemogenesis. BAP1 expression induces monoubiquitination and stabilization of ASXL1-MT, which in turn activates the catalytic function of BAP1. The formation of this super active ASXL1-MT/BAP1 complex leads to abnormal myeloid differentiation, suggesting that also BAP1 has a tumor-promoting role in myeloid malignancies. Indeed, the same group reported that the depletion of BAP1 inhibits leukemogenicity of ASXL1-MT-expressing myeloid leukemia cells[109]. In another study, it has been proved that the heterozygous overexpression of the truncated ASXL1 protein ($Asxl1^{tm/+}$) confers higher proliferation capacities to hematopoietic cells, *in vitro.* However, during serial transplantation $Asxl1^{tm/+}$ hematopoietic cells decline faster compared to wild-type cells, indicating compromised long-term repopulation capacity, *in vivo*[110]. Interestingly, no blood diseases

in *Asxl1* $^{tm/+}$ mice was observed throughout their life-span, suggesting that *Asxl1* mutation alone was not sufficient for leukemogenesis in that context[110].

Nagare et al. further demonstrated the role of *Asxl1* in hematopoiesis support, proving that *Asxl1* mutations may confer an alteration of function. They demonstrated that the conditional knock-in (KI) of a C-terminal truncated Asxl1 mutant induces myeloid skewing, thrombocytosis, anemia (age-dependent), and dysplasia in mice. In addition, they showed an increased susceptibility to leukemic transformation promoted by co-occurring *RUNX1* mutation or viral insertional mutagenesis, demonstrating that *ASXL1* mutations increase susceptibility for leukemic transformation of HSCs, presenting a novel model for CHIP[111].

Recently, Zhang and collaborators proved the importance of *Asxl1* also in the BM niche compartment. Indeed, they induced the deletion of the gene only in stromal cells. The resulting mice showed an altered ratio between hematopoietic stem and progenitor cells (HSCs/HPCs), with increased myeloid lineage cells. Stromal cells displayed transcriptional alterations of genes involved in HSC/HPC maintenance. In addition, they showed that MSCs isolated from patients affected by chronic myelomonocytic leukemia (CMML) had lower expression of *ASXL1* compared to healthy donors, leading to an impaired hematopoietic support activity with the induction of myeloid differentiation[112].

All this data reveal the essential role of Asxl1 in supporting and maintain normal hematopoiesis. Furthermore, all these distinct studies demonstrated that *Asxl1* alterations affect the myeloid commitment even if in different ways. It is known that a biased myeloid differentiation can promote leukemia formation[113].

2.1.6 Functional alterations of malignant cells in MDS and AML

Malignant cells show several functional abnormalities in both MDS and AML patients, with various consequences. Indeed, the major feature of MDS cells is an increased apoptosis, whereas proliferative/expansion advantage characterizes AML cells.

In the BM of MDS patients, HSPCs express a high level of the TOLL-LIKE RECEPTOR 4 (TLR4), which is central to the activation of inflammatory mediators. TLR4 activation contributes to increased apoptosis with the development of cytopenia in MDS patients[114]. Other two important inflammatory molecules, intermediates in TLR4 signaling, the TLR ADAPTOR E3 UBIQUITIN LIGASE (TRAF6) and the TLR IL-1 RECEPTOR DOMAIN-CONTAINING ADAPTOR

PROTEIN (TIRAP), have been identified as markedly up-regulated in $CD34^+$ cells isolated from MDS patients[115]. TRAF6 is fundamental for the survival and proliferation of MDS HSPCs. Indeed, knockdown of TRAF6 in MDS/AML cell lines or patient samples induces apoptosis and impairment of MDS/AML progenitor function[116]. Moreover, TRAF6 overexpression in mouse HSPCs leads to an MDS-like hematopoietic defect, with associated progression to AML[117]. Furthermore, TRAF6 and TIRAP are constitutively expressed in human MDS del5(q)[118].

In MDS BMs, others apoptosis-inducing ligand/receptor pathways have been found up-regulated, including FAS CELL SURFACE DEATH RECEPTOR (FAS)/FAS ligand (FAS-L), TUMOR NECROSIS FACTOR ALPHA (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL) and its receptors[119,120]. The abnormal susceptibility to apoptosis has been also correlated to the unbalanced expression of the caspase inhibitory protein FLIP (FLICE, FAS-associated death-domain-like IL-1beta-converting enzyme)-inhibitory protein) in $CD34^+$ cells of MDS patients. FLIP suppresses a FAS-L and TRAIL-induced apoptosis[121]. In addition, the NUCLEAR FACTOR KAPPA-LIGHT-CHAIN-ENHANCER OF ACTIVATED B CELLS (NF-κB) activation has been reported in MDS cells, resulting in dysplastic hematopoiesis. On the contrary, in primary human AML stem cells, the activation of the NF-κB pathway regulates positively the survival of leukemic stem cells[122].

It has been also reported that AML cells are able to interact with immune system cells, in order to create an immunosuppressive microenvironment, by deregulating both innate and adaptive immune responses[123]. Indeed, AML cells secrete soluble factors, which reduce the function of lymphocytes T and natural killer cells (NK)[124] and expand regulatory T cells (Treg), an immunosuppressive population of T cells[125], through immunological regulators such as programmed death-ligand 1 (PD-L1). In our laboratory, we previously described the expression of the immune-tolerant enzyme Indoleamine 2,3 dioxygenase 1 (IDO1) in AML cells. Indeed, we demonstrated that AML cells induce T-cell tolerance by inducing the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Treg cells, in an IDO1-dependent manner. This evidence confirms the ability of AML cells to modulate a defective anti-leukemic immune response[126]. In addition, AML cells, contrary to their normal counterpart, produce cytokines such as Interleukin (IL) 10, IL-8, IL-1β, TNF-α, and this production is influenced by chemotherapy[127]. Sanchez-Correa et al. also demonstrated that AML cells produce cytokines such as IL-6 and IL-10. Low levels of IL-6 and high levels of IL-10, detected in the plasma of patients at diagnosis, correlate to a favorable prognosis[128].

In addition, other pathways able to modulate survival of AML cells have been identified, including processes such as cell cycle and oxidative stress. A constitutive activation of PROTEIN KINASE BETA (AKT), as a survival signal (via modulation of the pro-apoptotic factor Bad), has been

observed. Interestingly, the AKT/PHOSPHOINOSITIDE 3-KINASE (PI3K) pathway has also recently been correlated to antioxidant defenses. Indeed, increased activity of PI3K pathway induces the activation of components of the antioxidant defense machinery[129]. The reactivation of β-catenin/Wnt signaling in LSCs has also been identified as fundamental for LSCs self-renewal in AML cells[130].

2.2 CELL EXTRINSIC FACTORS CONTRIBUTING TO MDS/AML PATHOGENESIS

2.2.1 MSC history

In 1991, Caplan et al. coined the definition of Mesenchymal stem cells referring to a population in the BM, that had been identified several years earlier. Indeed, for the first time in 1968, Tavassoli and Crosby demonstrated the osteogenic potential of BM. They transplanted autologous fragments of bone-free BM in heterotopic sites and found that this resulted in the reconstitution of ectopic bone and marrow in the rodent. However, the cell population, progenitor of differentiated bone cells, was not identified[131]. Later, the pivotal work of Friedenstein and coworkers revealed that the osteogenic potential, identified in heterotopic BM transplantation within rodent, was related to a minor population of BM cells. These BM cells were characterized by a rapid adherence to culture vessel and a fibroblast-like shape, which distinguished them from hematopoietic cells. In addition, the authors found that a subpopulation of BM cells was able to form colonies, called colony-forming unit fibroblastic (CFU-Fs)[132]. *In vivo,* transplantation experiments proved the ability of these cells to regenerate bone tissue in serial implants, suggesting also a self-renewal and multilineage potential[133]. Indeed, the progeny of a single BM stromal cell could generate several skeletal tissues including bone, cartilage, adipose, and fibrous tissue. Friedenstein and Owen referred to this cell as an osteogenic stem cell or BM stromal stem cell[134]. In those years, the hypothesis of a stem cell niche, with non-hematopoietic components regulating hematopoiesis, was born[135]. Although this hypothesis was demonstrated by several studies, the concept did not make a large-scale impact until a similar work was published by Pittenger et al. in 1999. In human BMs, they found cells showing features of mesenchymal stem cells, i.e. multilineage potential and clonal ability[136]. Since that moment, the scientific community recognized mesenchymal stem cells as adult

stem cells with differentiation potential[137]. Nowadays, it is preferable to define these cells as mesenchymal stromal cells (MSCs).

2.2.2 MSC identification and general features

MSCs are recognized most of all for their *in vitro* properties. MSCs are characterized by self-renewal and multilineage differentiation ability. Despite in humans, BM remains the main source of MSCs, it is possible to isolate these cells from almost all the post-natal tissues and organs, including the brain, fat, teeth, synovium, blood vessels, and umbilical cord blood[138]. In addition, as adult cells, MSCs are less tumorigenic compared to embryonic stem cells[138]. However, they still have an extended plasticity, demonstrated by MSC ability to differentiate in mesodermal tissues such as tendons, cartilage, bone, and adipose tissue, and in non-mesodermal cell types such as hepatocytes, astrocytes, and neurons[139]. Nevertheless, it is still debated if MSCs isolated from various tissues show the same identical properties. Indeed, different studies pointed out fundamental differences in biological properties of MSCs isolated from different sources. For example, MSCs isolated from umbilical cord blood (UB-MSCs) have a higher rate of proliferation and clonality compared to MSCs isolated from BM (BM-MSCs) and adipose tissue[140].

In 2006, the confusion about the definition of MSCs led the International Society For Cellular Therapy (ISCT) to propose new criteria to define the MSC population. According to these widely accepted proposals, the minimum criteria to identify MSCs are:

- plastic adherence;
- expression of surface markers such as CD105, CD73, CD90 with a frequency higher than 95% and lack of the expression of markers such as CD34, CD14 or CD11b, CD79alpha or CD19 and Human Leukocyte Antigen class II (HLA-DR) (<2%);
- trilineage differentiation towards chondrogenic, adipogenic and osteogenic cells[141].

These criteria, postulated to standardize MSCs isolation *in vitro*, can be used only for human MSCs. Indeed among species, there are differences, and also within the same species, there is heterogeneity. For example, MSCs isolated from different mouse strain show different surface marker expression and different proliferative and differentiation ability[142]. In the last decade, MSCs have been extensively characterized and new surface markers have been identified. MSCs are almost null immunogenic and do not express MAJOR HISTOCOMPATIBILITY COMPLEX II (MHC I) and co-stimulatory molecules, such as CD40, CD40 Ligand (CD40L), CD80, CD86, whereas they express CD29, CD106, and CD44[143]. In particular, BM-MSCs express STRO-1, a

marker of stromal cells, and neural molecules such as CD271 (the receptor of the nerve growth factor) and GD2 (Ganglioside molecule 2). However, *ex vivo* manipulation of MSCs can change their surface marker expression. Indeed, according to Quian et al. CD44 expression is acquired in culture. MSCs freshly isolated from human BMs are $CD44^-$ because only these cells, contrary to $CD44^+$ cells can form colonies (CFU-F)[144]. So far, univocal surface markers, for MSC identification *in vivo*, have not been found yet. In 2007, Sacchetti et al. identified osteoprogenitor cells, $CD146^+/CD45^-$, able to generate CFU-F and to create an ectopic BM niche in mice[145]. Tormin et al. identified CFU-F forming cells enriched in the $CD271^+CD45^-CD146^+$ stem-cell fraction of the BM[146].

As already mentioned, MSCs show several functions *in vitro*, including self-renewal, differentiation, and immune-regulation (that will be largely revised later in this thesis). In particular, self-renewal can be assessed by clonogenic assay and CFU-F colony formation. *In vivo*, it is thought that MSCs can have a role in tissue regeneration, and in the field of regenerative medicine MSCs have drawn attention thanks to their peculiar abilities. Indeed, it has been demonstrated that MSCs, if properly stimulated, can migrate towards damaged tissues (homing), contributing to their repair and modulating the immune response. Furthermore, MSCs are considered hypoimmunogenic or 'immune privileged' cells, due to lack of expression of MHC II and low expression of MHC I, suggesting that MSC transplantation could break down major histocompatibility barriers. However, recent studies showed the presence of antibodies and immune rejection of allogeneic donor MSCs[147]. Beside their physiological role *in vivo*, MSCs show biological properties which can be potentially useful and have clinical applications. Indeed, as reported in Figure 4, MSCs can have a therapeutic effect, mediated by several mechanisms including:

- Homing to the site of inflammation after tissue injury;
- Differentiation;
- Secretion of several molecules, able to recovery injured cells;
- Lack of immunogenicity and immune-regulating ability.

In 2012, 218 trials based on MSCs were ongoing for several therapeutic approaches, such as renal diseases (kidney transplant/injury), cardiovascular disease (myocardial ischemia/infarction), neuromuscular disease (muscular dystrophy, multiple sclerosis, brain injury), gastrointestinal disease (Crohn's disease), liver disease (cirrhosis), autoimmune disease (rheumatoid arthritis, Human immune deficiency virus (HIV)) diabetes, etc. MSCs appeared to be well tolerated and

adverse reactions were not recorded[148]. In 2016, in the database of the US National Institutes of Health, 493 MSC-based clinical trials, either ongoing or complete, were present[149].

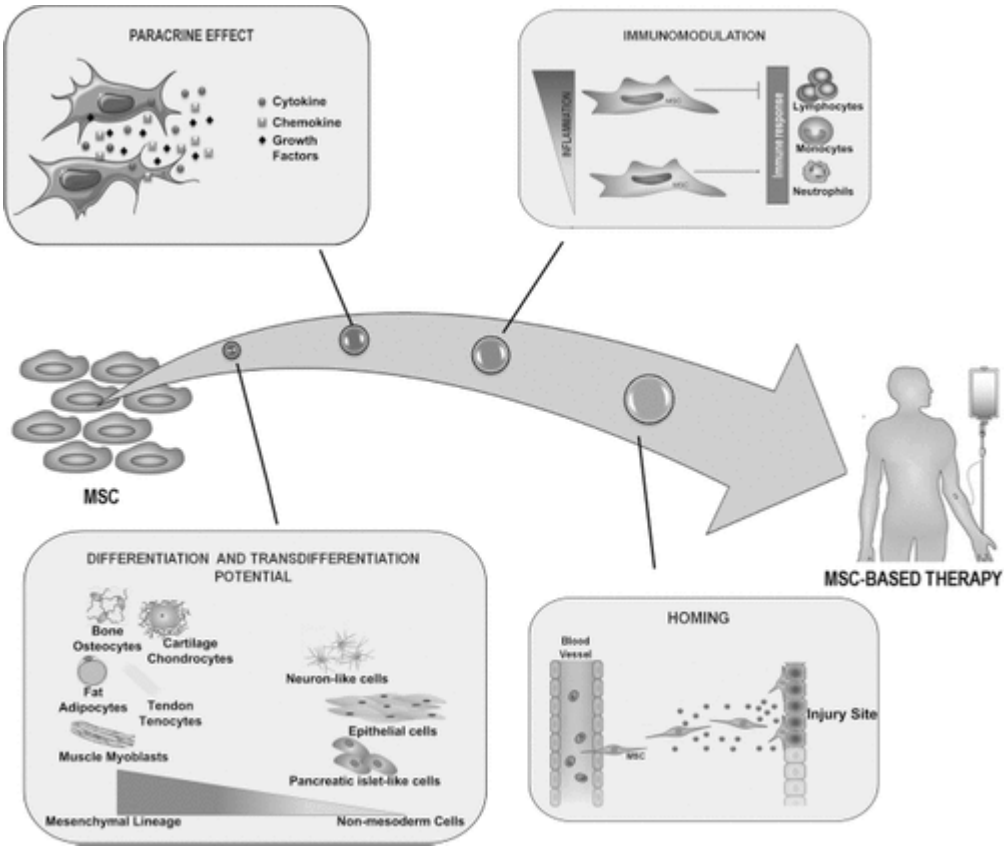


Figure 4. Biological properties justifying MSC clinical use.

2.2.3 MSCs and hematopoiesis

BM is one of the most used sources for MSC isolation. Nevertheless, the procedure to obtain BM blood is invasive and the percentage of MSCs, in healthy BMs, is around 0.001-0.01% and decrease with age[150].

The importance of BM stromal cells in supporting hematopoiesis is known since the pivotal work of Friedestein and coworkers, as already described. Indeed, they identified a nonhematopoietic population which provides a microenvironment, favorable for HSC homing, i.e. the ability of HSCs to migrate in the BM when injected in extra BM sites[133]. Later studies confirmed that BM-MSCs favor the engraftment and homing of HSCs in the BM both in animals and patients when co-transplanted[151,152]. The process of engraftment indicates the ability of the cells to colonize the BM where they can start their activity after transplantation. When human-derived MSCs are

transplanted in mice, they engraft in the BM and differentiate into myofibroblasts, pericytes, BM stromal cells, osteoblasts, osteocytes, and endothelial cells, contributing to the reconstitution of functional components of the BM hematopoietic microenvironment[153].

Despite several studies have demonstrated the role of MSCs in the self-renewal, expansion, proliferation, and differentiation of HSCs *in vitro*, the mechanisms regulating hematopoiesis support, mediated by MSCs, are still under investigation. The hypothesis is that both direct cell-cell contact and release of soluble factors are involved[154].

MSCs produce a wide variety of cytokines. In particular, MSCs secrete cytokines favoring HSC quiescence or inducing their self-renewal rather than differentiation, i.e. LEUKEMIA INHIBITING FACTOR (LIF), STEM CELL FACTOR (SCF), STROMAL CELL-DERIVED FACTOR 1 (SDF-1), BONE MORPHOGENETIC PROTEIN-4 (BMP-4), TRANSFORMING GROWTH FACTOR-*β* (TGF-*β),* FLT-3. Furthermore, MSCs also produce different interleukins, i.e. IL-1, IL6, IL-7, IL-8, 1L-11, IL-12, IL-14, IL-15, and also cytokines, such as GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) and GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF), that influence more mature hematopoietic progenitors[155]. Wagner et al. demonstrated that BM-MSCs have the ability to maintain long-term culture-initiating cell and a $CD34(^{+})CD38(^{-})$ immunophenotype in hematopoietic cells. Furthermore, the authors showed that $CD34^{+}$ cells adhere to the feeder layer formed by BM-MSCs, because of the expression of proteins such as N-CADHERIN, CADHERIN-11, INTEGRINS, VASCULAR CELL ADHESION MOLECULE 1 (VCAM-1), NEURAL CELL ADHESION MOLECULE 1 (NCAM-1), suggesting the importance of cell-cell contact[156].

2.2.4 MSCs *in vivo*: the hematopoietic niche

Most of the MSC characteristics mentioned above refer to cells manipulated *ex vivo*. Despite the ability of BM-MSCs to support and regulate hematopoiesis has been extensively demonstrated *in vitro* and *in vivo*, the characterization of BM-MSCs *in vivo* is still under investigation.

BM-MSCs reside in specialized niches within BM, in which other cell types are present and regulate HSC function. This concept of niche as an "entity in which the stem cell maturation is prevented and the properties of 'stemness' are preserved" was proposed, for the first time in 1978, by Schofield and co-workers[135].

During embryogenesis, the original pool of HSCs is formed and colonizes several anatomical sites (the yolk sac, the aorta-gonadmesonephros region, the placenta, and the fetal liver). At birth, HSCs colonize BM, where HSC pool size is maintained constant, by regulating HSC self-renewal and differentiation[157] within specialized niches. In BM, these niches contain bone cells, neurons, endothelial cells, adipocytes, macrophages, MSCs, extracellular matrix (ECM) components[158]. All these elements cooperate to preserve the multipotency of HSCs, regulating their quiescence, proliferation, differentiation, and localization. To be a component of the HSC niche, a cell has to satisfy the following criteria:

- to be rare;
- to be anatomically close to HSCs;
- to express genes for HSC maintenance;
- to secrete signals able to modify HSC function.

In the last years, new imaging techniques have allowed a better understanding of the HSC niches in mice BM. In human, it is conceivable that the niches are similar to mice niches, as most of HSC biology does not show great differences between humans and mice.

Analysis of BM sections revealed that approximately 85 % of HSCs are localized within 10 μm of sinusoidal blood vessels, suggesting the existence of a perivascular niche[159], whereas a small part of HSCs is located at the endosteum (endosteal niche). In the past, these two niches were considered distinct, nowadays the main idea is that a unique BM niche exists for HSCs. The localization of HSCs is really important because different levels of oxygen are present in BM and hypoxia is thought to be a strong regulator of self-renewal and quiescence. According to hypoxia levels in BM, HSCs close to arterioles, which highly perfuse the endosteum, are associated with quiescence while proliferative HSCs are distant from arterioles[160]. Despite most of the HSCs reside far from the endosteal surface, HSC progenitors have been found also along to endosteal region[161], and HSCs have calcium receptor 'to sense' calcium concentration in the endosteal region[162]. In 1994, Taichma et al. demonstrated that the cells which synthesize bone, called OBs, have a role in HSC maintenance. Indeed, *in vitro* OBs expand hematopoietic progenitors. The manipulation of OBs proved that these cells regulate HSC function, through Notch activation, *in vivo*[163]. Furthermore, osteoclasts, the bone-resorbing cells, have also been shown to correlate with HSC mobilization from BM[164]. However, more recent studies demonstrated that the ablation of OBs *in vivo* did not correlate with a reduced HSCs number[165], indicating that OBs are able to influence HSC function but are not essential for HSC maintenance. The data now suggest that the alterations on HSC

frequency, found following genetic manipulation in osteoblastic cells, reflect more likely indirect effects, rather than the presence of a distinct osteoblastic niche[166].

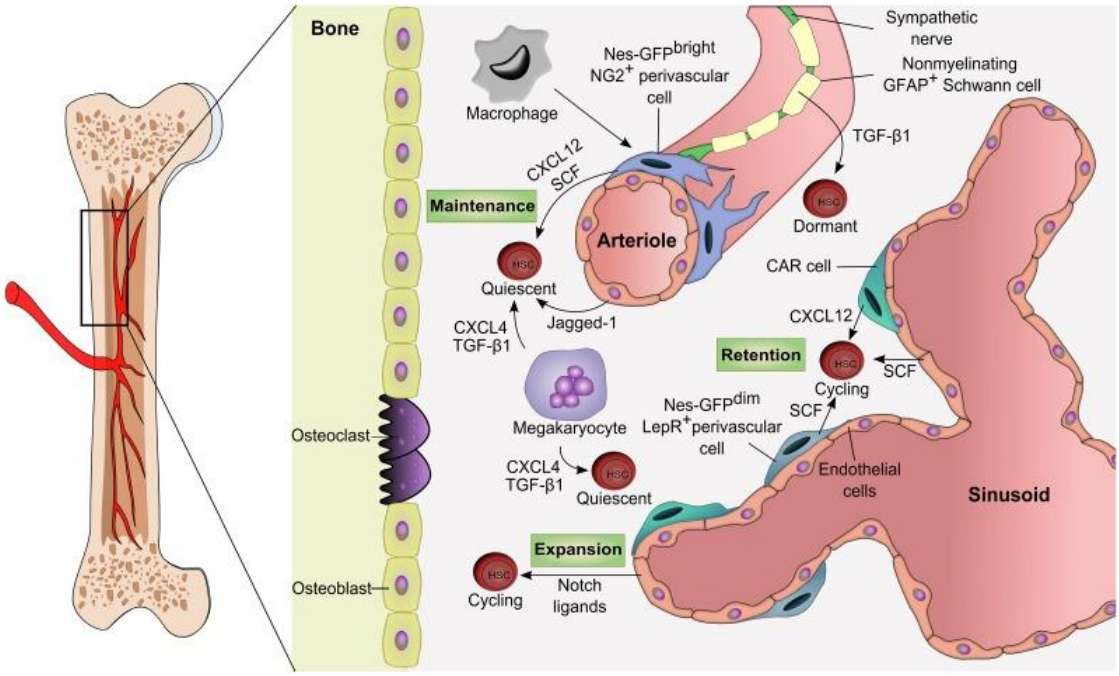


Figure 5. Adult bone marrow niche[160].

In contrast, the preferential localization of HSCs close to vessels *in vivo* reflects the great importance of the perivascular niche. Endothelial cells of sinusoids, a component of the perivascular niche, promote the expansion of long-term reconstituting HSCs[167]. *In vivo*, the depletion of the cytokine receptor subunit, gp130, in hematopoietic and endothelial cells induces bone marrow dysfunction and extramedullary hematopoiesis. The transplantation of gp130-deficient bone marrow cells into wild-type mice results in normal hematopoiesis, whereas the transplantation of wild-type bone marrow cells into irradiated gp130-deficient mice did not revert the hematopoietic alterations, suggesting a peculiar role of gp130 expressing endothelial cells in regulating hematopoiesis[168].

Another component of the perivascular niche is represented by cells resembling MSCs, which have been identified close to vessels. In 2006, Sugiyama et al. defined the mesenchymal progenitors as cells expressing an elevated level of HSC maintenance protein, Cxcl12 (SDF-1), that they called CXCL12-abundant reticular (CAR) cells. CAR cells are quite abundant in BM and are found close to sinusoidal vessels and to endosteum and in close contact to putative HSCs. As expected, these mesenchymal progenitors express other HSC maintenance proteins, such as SCF. In addition, their

depletion results in a partial decrease of HSC cycling, suggesting that CAR cells regulate HSC cycling and self-renewal[169]. In 2007, subendothelial osteoprogenitor cells were identified close to sinusoids. These cells are $CD146^{+,}$ and as already described, show MSCs activity[145]. Furthermore, another putative mesenchymal population was identified by Mendez-Ferrer et al. in mouse BM. Indeed, they found a population of cells, which behave as MSCs, expressing Nestin ($Nestin^{+}$MSCs), a protein typical of nerve cells. $Nestin^{+}$MSCs show CFU-Fs content, multilineage differentiation, and self-renewal ability. $Nestin^{+}$MSCs can be propagated *in vitro* as non-adherent spheres, called mesenpheres. This expansion as spheres is a condition required for neural stem cells, expressing Nestin, to maintain self-renewal ability *in vitro*. Spatially, $Nestin^{+}$MSCs are tightly associated with HSCs and reside close to adrenergic nerve fibers and in perivascular area, so in more central areas of the BM, and with a lower frequency in the immediate vicinity of the endosteum. HSC fate is regulated by $Nestin^{+}$MSCs, which produce HSC maintenance proteins, such as SCF, IL-7, CXCL12, angiopoietin-1 (ANGPT1), VCAM1 and osteopontin (SPP1). The CXCL12/CXCR4 axis regulates HSC mobilization. Treatment with the HSC-mobilizing factor, G-CSF, results in the decreased expression of CXCL12 and other HSC maintenance factors in $Nestin^{+}$ MSCs, while parathormone increases $Nestin^{+}$MSC number and promotes their osteoblastic differentiation. In addition, *in vivo* selective depletion of $Nestin^{+}$MSCs reduce HSC number and BM homing of transplanted HSCs[170]. In mice BM, $Nestin^{+}$MSCs are less abundant than CAR cells and express Cxcl12 as well. According to some authors, $Nestin^{+}$MSCs may be a cell population more primitive than CAR cells[171].

Other regulators of HSC niche are the sympathetic nervous system and adipocytes. In particular, fat cells are negative regulators and their number inversely correlates with hematopoietic activity[172]. On the contrary, the circadian release of noradrenaline, mediated by sympathetic nervous fibers, modulates CXCL12 expression in the bone marrow, regulating HSC mobilization[173]. Furthermore, in the BM, the disruption of adrenergic innervation alters HSC recovery after chemotherapy[174].

The deep comprehension of the network of signaling and cell interactions regulating normal HSC function in the BM niche is compelling and fundamental to understand the nature of the niche in myeloid malignancies. Indeed, for malignant clones and in particular leukemic stem cells (LSCs), the niche represents a sort of sanctuary in which cells find protection during therapy[158].

2.2.5 MSC immunosoppression

Another well-described characteristic of MSCs is their immune regulation ability, which influences both adaptive and innate immunity *in vitro* and *in vivo*[175].

In particular, cells of innate immunity influenced by MSC function, include:

- dendritic cells (DCs). In mammalian immune system, DCs represent the principal antigen presenting cells (APCs). Indeed, their main function is to expose antigens on cell surface to T cells, priming naïve T cells to adaptive immunity. In addition, DCs regulate immune tolerance to self-antigens. During maturations, DCs acquire several surface markers, including MHC I, MHC II and co-stimulatory proteins. *In vitro,* MSCs are able to block DC differentiation from peripheral or cord blood-derived precursors[176]. Likewise, MSCs prevent the typical expression of surface markers such as CD80, CD86, HLA-DR during DC maturation[177]. In addition, MSCs affect also mature DC function, inducing decreased expression of MHC II and others proteins and decreased IL-2 production, which in turn alter APC activity of DCs[178]. In addition, MSCs reduce TNF-α secretion by DCs and hence reduce their pro-inflammatory activity[179]. The negative regulation of DC generation and maturation, mediated by MSCs, is exerted through several mechanisms, including MSCs secretions of IL-6 and PROSTAGLANDIN E2 (PGE-2). Additionally, MSCs impair DC effector properties *in vitro*, by inhibiting the activation of MAPKs, occurring upon TLR4 stimulation[175]. *In vivo,* effects on DC activity have also been reported. Indeed, the injection of DCs and MSCs impairs DC homing to lymph nodes and T cell priming mediated by DCs, in mice[180].
- NKs. The cytotoxic activity and cytokine production of NKs are fundamental for the elimination of virus-infected cells and tumor cells. NKs connect also innate with adaptive immunity, by enhancing T cell mediated immune responses. MSCs block NK cell proliferation and cytotoxicity. Indeed, MSCs inhibit IL-2 induced proliferation of resting NK cells, whereas MSC effects on NK activated cells are less evident[181]. In addition, MSCs inhibit cytolysis mediated by NK cells and their INTERFERON- γ (IFN-γ) production, through HLA-G5 secretion[182]. The inhibition of NK proliferation and immune activity, mediated by MSCs, is also regulated by other mediators including TGF-β1, PGE2, and IDO1[183]. Cell-to-cell contact mechanisms are also involved[175]. It is noteworthy to point out that IL-2 activated NKs, contrary to freshly isolated NKs, are also able to lyse autologous and allogeneic MSCs[181].
- Neutrophils. These phagocyte cells have an high chemotaxis and bactericide activity. When they enter in contact with bacterial components, they start a process called “respiratory burst”, which leads to a rapid release of reactive oxygen species (ROS). *In vitro,* MSCs inhibit apoptosis of

resting and IL8-activated neutrophils, by an IL-6 dependent mechanism, reducing also the "respiratory burst". Cassatella et al. demonstrated that TLR3-activated MSCs have an antiapoptotic effect on neutrophils, *in vitro*. Indeed, TLR3-activated MSCs sustain neutrophil viability and function, through the combined secretion of GM-CSF, IL-6, IFN-β[184].

- Macrophages. These cells, which derive from differentiated monocytes, play a key role in removing dead or dying cells and cellular debris. Macrophages are characterized by a process called polarization, which indicates the ability of the cells to acquire functional programs, based to microenvironment signals. Thus, two phenotypes can be distinguished in macrophages: an M1 phenotype, associated with the protection against bacteria/virus with an anti-tumor activity, and an M2 phenotype, associated with wound healing/tissue repair and exhibiting a pro-tumoral function[185]. *In vitro*, murine MSCs promote macrophage M2 polarization through activation of STAT3 and inhibition of NF- κB[186]. *In vivo*, the interaction of MSCs and macrophages has also been proved. In particular, MSCs recruit and polarize M2 macrophages, through SDF-1 secretion, when murine MSC are transplanted in the pancreas of diabetic mice. The transplantation of MSCs results in a decrease of blood glucose, subsequent to a recovery in the β cell mass. Indeed, M2 macrophages recruited and polarized by MSCs, produce Wnt3a, boosting β cells replication, through Wnt/β-catenin activation[187].

The key components of adaptive immunity, influenced by MSC activity, are:

- T cells. Despite there are different populations, the main common features of T cells are cell proliferation/expansion ability and cytokine production, while cytotoxicity is a particular characteristic of $CD8^+$ cytotoxic T lymphocytes (CTLs). *In vitro,* MSCs are able to inhibit T cell proliferation induced by distinct stimuli, including mitogens, alloantigens[188] and CD3/CD28 mediated activation[189]. This immunosuppressive activity is independent of the use of autologous or allogeneic MSCs. Interestingly, MSCs do not induce T cell apoptosis. On the contrary, MSCs favor their survival in a quiescent state, promoting the arrest of T cells in the G0/G1 phase of the cell cycle[190]. Furthermore, MSCs affect also T cell cytokine production. Indeed, they induce a decrease of IFN-γ production by T helper 1 (Th1) cells *in vitro* e *in vivo*[191] and an increase of IL-4 production by Th2 cells *in vitro*[179], skewing the phenotype from a pro-inflammatory to an anti-inflammatory state[192]. The suppressive activity of MSCs is in part mediated by IDO1 expression and activity, stimulated in turn by IFN-γ/TNF-α producing activated T cells[193,194]. Another component of the T cell family, regulated by MSCs, is represented by CTLs. CTLs are able to kill cancer cells, infected cells or damages cells. MSCs, when added at the beginning of the mixed lymphocyte reaction (MLC), inhibit CTL-mediated lysis by 70%. Interestingly, MSCs are not lysed

by CTLs, suggesting the existence of a mechanism which allows MSCs to escape recognition by CTLs[195]. The last component of the T cell family, influenced by MSCs activity, is the Treg population. Tregs suppress the activation of the immune system, regulating immune homeostasis and tolerance to self-antigens. MSCs are able to induce Tregs[179]**.** In particular, Yal et al. found that MSC-exposed Tregs have an increased immunosuppressive activity, compared to Tregs not pre-cultured with MSC. This effect is potentially due to activation of programmed cell death 1 receptor (PD-1) on Tregs and IL-10 production in MSCs/Treg co-culture system[196]. In addition**,** Selmani et al. demonstrated that HLA-G5 expression in MSCs is also responsible for MSCs-mediated Tregs induction. HLA-G5 regulates also T cell suppression[182]. Interestingly, MSCs can induce Tregs in an indirect way too. In fact, human MSCs induce the production of IL-10 in DCs, which in turn triggers the Treg generation *in vitro*[179].

- B cells. These immune cells are specialized in antigen presentation and antibody production. Despite is widely accepted that MSCs interact with B cells, the results of this interaction are still under debate. It seems that MSCs inhibit B cell proliferation, inducing the arrest of the cell cycle in the G0/G1 phase, *in vitro*[197]. Furthermore, MSCs inhibit B-cell differentiation, associated with impaired immunoglobulin (Ig), i.e. IgM, IgG, IgA, production. In B cells, MSCs also reduce the constitutive expression of the chemokine receptors, such as CXCR4, CXCR5, CXCR7, suggesting that MSCs affect B cell chemotactic ability[197]. Ungerer et al. identified the galectin-9 (gal-9) production, as another mechanism of MSC-mediated immunosuppression. Gal-9, up-regulated in MSCs after IFN-γ stimulation, regulates the anti-proliferative effect of MSCs on T and B cells, suppressing also immunoglobulin release, mediated by B cells, *in vitro.* In contrast, Traggiai et al. found that MSCs promote proliferation and differentiation of B cells upon TLR-9 stimulation, *in vitro*[198]. Regardless of the contrasting results reported in published works, it should be kept in mind that B-cell responses are mainly T-cell dependent. Subsequently, *in vivo,* the outcome of the interaction between MSCs and B cells might be significantly influenced by the alterations of T-cell functions, mediated by MSCs[192].

All these findings highlight the ability of MSCs to interact with several immune cellular components. The MSC-mediated regulation of immune responses could be used as a promising strategy for the treatment of immune-based diseases and to counteract the rejection of tissue transplanted. Pre-clinical studies have already demonstrated the great potential of MSCs. In fact, MSCs enhance the survival of transplants. For example, the prior administration of MSCs increases the survival of a skin graft in baboons[199], and prevent the rejection of allogeneic transplant of the cornea in mice, by reducing the early inflammatory responses[200]. In addition, the administration of

MSCs reduces the clinical manifestations of asthma, by recruiting alveolar macrophages in mice[201], and ameliorates the severity of colitis in mice, by downregulating Th1 response[202]. *In vivo*, the mechanisms regulating MSC immunosuppression are not fully elucidated. According to Zanotti et al., the homing to specific organs or cell-cell contact mechanisms are not essential for MSCs immunosuppression. On the contrary, the release of soluble mediators, acting systematically, is fundamental for the anti-inflammatory and immunosuppressive activity of MSCs[203]. However, it is important to stress that the immunomodulation of MSCs has to be activated by external stimuli, such as pro-inflammatory cytokines, released in the inflammatory sites[204].

The discovery of immunosuppression mediated by MSCs led also the researches to question if MSCs could influence the anti-tumor immune response, within the tumor microenvironment. Interestingly, recent studies demonstrated that MSCs can alter the immune recognition of cancer cells, through the release of immunoregulatory cytokines, such as TGF-β, and others soluble ligands. The soluble mediators bind the activating receptors on CTL, such as decoy molecules, interfering with the correct interaction between tumor cells and lymphocytes. Indeed, MSCs play a key role, both in hematological malignancies and epithelial solid tumors, in regulating anti-tumor immune response and tumor cell growth, as further discussed later in this thesis[205].

2.2.5.1 IDO1 general features

The expression of IDO1 is one of the well-characterized mechanisms of MSC-mediated immunoregulation.

IDO1 is a cytosolic heme-containing enzyme, which catalyzes the first rate-limiting step of tryptophan catabolism, along the kynurenine pathway. It catalyzes the oxidative cleavage of the 2,3 double bond in the indole ring of L-tryptophan, producing N'-formylkynurenine (Fig.6). Another enzyme, with exclusive hepatic localization, called tryptophan 2,3-dioxygenase (TDO), regulates the same reaction[206]. In addition, IDO1 has a homolog, called IDO2.

In 1967, IDO1 was first identified in the rabbit gut, by Yamamoto and Hayaishi. Later studies found that IDO1 is encoded by the gene *INDO*, located on chromosome 8 in human and mouse. IDO1 protein is composed of 407 amino acids, with a molecular weight of 45 kDa. The activity of the protein is linked to the incorporation of the heme prosthetic group into the active site[207].

IDO1 expression and activity have been found in the maternal-fetal interface, mouse epididymis, gut, spleen, lymph nodes, lungs, and thymus[208]. In addition, distinct human cell types express IDO1, including APCc, such as DCs, endothelial cells, fibroblasts, macrophages[209].

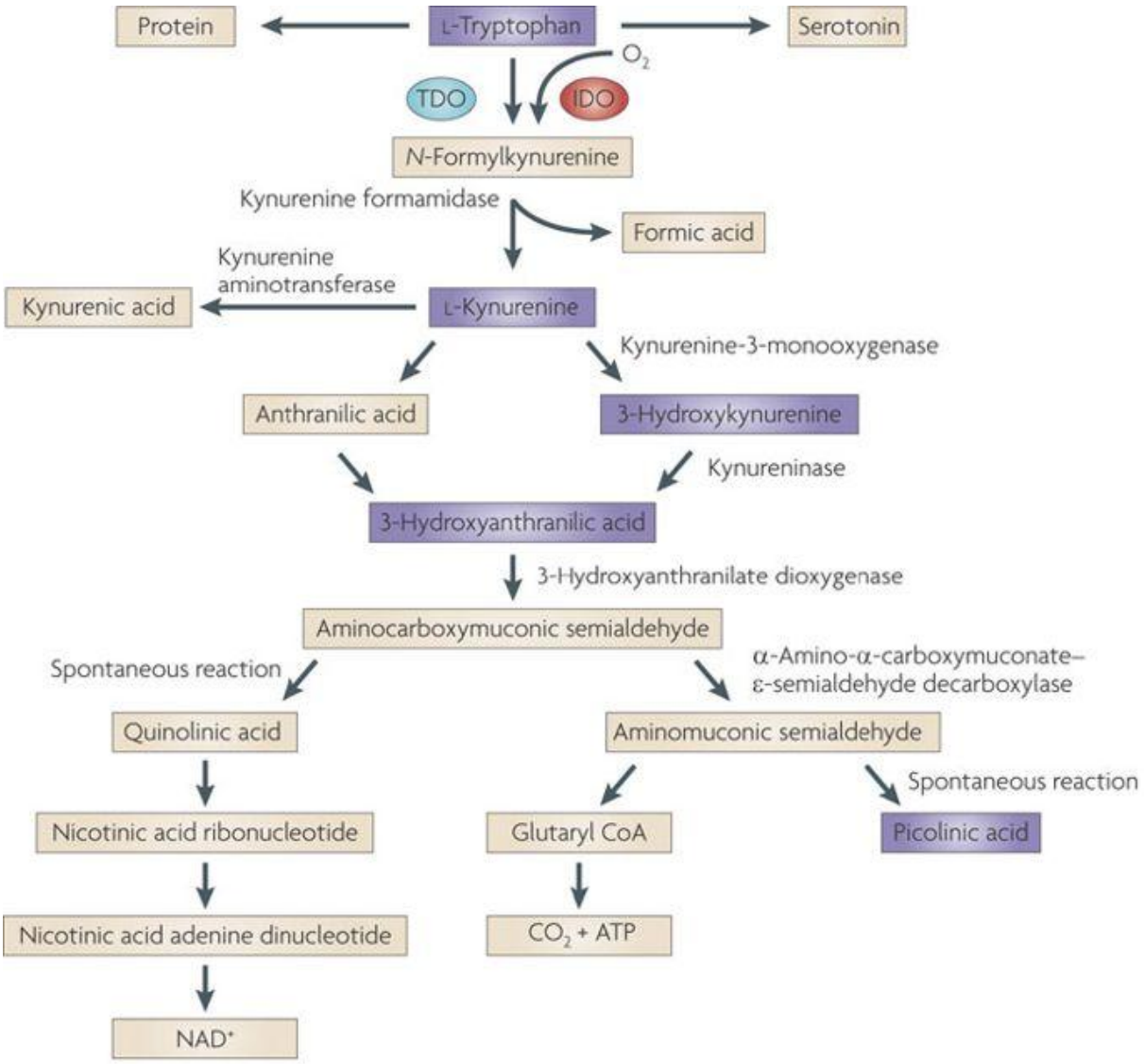


Figure 6. Tyriptophan catabolism along the kyn pathway[391].

2.2.5.2 Tryptophan depletion versus kynurenine production

Tryptophan is an essential amino acid, fundamental for cell survival and in particular for protein synthesis. It is the precursor for serotonin and other molecules in the brain tissue, such as melatonin and niacin. It is also the precursor for the synthesis of the anti-pellagra vitamin nicotinic acid[210]. The kynurenine pathway is the most used way of oxidative degradation of tryptophan and leads to the formation of active metabolites, such as kynurenine, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, picolinic acid, quinolinic acid and nicotinamide adenine dinucleotide (NAD^+)[211]. Tryptophan metabolism plays an essential role in different biological processes, and

disturbed tryptophan metabolism has been correlated to diseases such as arthritis rheumatoid, cardiovascular, neurological and neurodegenerative diseases[212].

The degradation of tryptophan along the kynurenine pathway, mediated by IDO1, has two main consequences: tryptophan deprivation and the accumulation of its active metabolites. Some microorganisms depend on exogenous tryptophan and are sensitive to IDO1 activity. These microorganisms include *Toxoplasma Gondii, Chlamydia pneumoniae,* mycobacteria, group B streptococci, and also viruses such as cytomegalovirus and herpes simplex virus[206]. In addition, in $CD8^+$ T cells, tryptophan starvation is responsible for down-regulation of CD3 zeta-chain, essential for T cell activation. Tryptophan starvation induces also the inhibition of T_H17 cell differentiation[213]. However, tryptophan deprivation mechanism is not enough to explain IDO1 activity. *In vitro,* when the volume of medium is limited, it is conceivable that tryptophan can be degraded, by cells expressing IDO1. Whether this can really occur *in vivo* is not clear. Indeed, plasma tryptophan levels range from 50–100 μM in humans, and diffusion from surrounding tissues can replenish local decreases. Furthermore, the great part of microorganisms can synthesize tryptophan, contrary to immune cells that need it to fight infections. Moreover, during inflammation or tumor growth, dying cells release their content, including tryptophan, providing additional supplies of the amino acid. So it is still unclear to what extent tryptophan starvation accounts as one of the mechanisms which mediates IDO1 induced immunosuppression.

As already mentioned, the degradation of tryptophan along the kynurenine pathway produces also several active metabolites, which have different effects on immune cells. In particular, quinolinic acid and 3-hydroxy-anthranilic acid induce apoptosis of murine $CD4^+$ Th1 cells, whereas CD4+ T Helper 2 (Th2) cells are less sensitive to metabolite-induced apoptosis. The apoptotic effect is Fas mediated[214]. In addition, exogenously added kynurenine and 3-hydroxy-anthranilic acid show anti-proliferative and cytotoxic effects on human T cells[206]. Picolinic acid is also responsible for IDO1-mediated inhibition of proliferation of human $CD4^+$, $CD8^+$ lymphocytes, and NK cells[215]. Ultimately, it is conceivable that both mechanisms, tryptophan starvation and active metabolites production, regulate IDO1-mediated immune regulation.

Recently, the signaling pathways, activated by tryptophan depletion and active metabolites production, have been identified. Indeed, tryptophan depletion, generated by IDO1 activation, acts on two distinct effector pathways: one is inhibited and the other is activated[216]. In particular, the master metabolic regulator mTOR (mTORC1), as well as the T cell receptor regulatory kinase PKC-θ, are inhibited by tryptophan IDO1-mediated catabolism. mTORC1 and PKC-θ are targets of the amino acid-sensing kinase, GLK1[216]. The inhibition of these pathways leads to the induction of

autophagy and impaired activation of T lymphocytes. In contrast, tryptophan degradation by IDO1 leads to the accumulation of uncharged tryptophan-tRNA in cells, which activates stress-response mediated by the general control non-repressed 2 (GCN2) protein. GCN2 is a kinase stimulated by increased levels of uncharged tRNA and, by phosphorylating the eukaryotic initiation factor 2α (eIF2α) kinase, reduces its activity preventing the translation of most RNA transcripts. T cells from GCN2-deficient mice are not susceptible to IDO1-mediated suppression of proliferation *in vitro* and *in vivo*. In addition, IDO1-expressing DCs cannot anergize these T cells[217]. Recently, Barnes et al. found that BLIMP-1 is a mediator of the GCN2 stress response pathway. Indeed, in human monocytes, following pharmacological activation of GCN2 and/or tryptophan-depletion, BLIMP-1 is up-regulated. Furthermore, the authors found that BLIMP-1 inhibits IFN-dependent IDO1 activation, by binding the IFN-responsive sites in the *INDO* promoter. This suggests that BLIMP-1 acts as a negative feedback loop balancing the readout of tolerance and inflammation[218].

The role of kynurenine, resulting from IDO1-mediated tryptophan catabolism, was widely recognized as one of the mechanisms which mediates the effects of IDO1. Nevertheless, it was unclear how kynurenine contributed to inflammatory responses, prior to the identification, by Platten and colleagues in 2011, of the aryl hydrocarbon receptor (AhR), as a physiological receptor for kynurenine[219]. Kynurenine, by binding AHR, triggers its nuclear translocation, leading to the activation of its target genes. The activation of AHR generates T regulatory cells. In fact, CD4 T cells isolated from AhR-null mice fail to generate Tregs when stimulated[220]. AHR function has been implicated in inflammation, immune regulation, and carcinogenesis. In addition, elevated expression of AHR correlates with poor prognosis in cancer patients[219].

2.2.5.3 IDO1 functions

Tryptophan catabolism, regulated by IDO1, has implications in a variety of biological processes and systems, including mammalian reproduction, stem cells, viruses, and the nervous system. Initially, IDO1 was thought to have a role in the defense against infections. However, nowadays IDO1 has been implicated in a more complex regulation of immune responses, contributing to control of excessive immune activation and to establish an acquired immunological tolerance in different settings. The pioneering work by Munn, Mellor and their colleagues demonstrated for the first time the IDO1 involvement in peripheral tolerance, in mammals. Indeed, they found that IDO1-mediated immunosuppression prevents fetal rejection in mice. In particular, the expression of IDO1 in cells of the placenta prevents the destruction of the fetus, mediated by maternal T-cells, during

pregnancy[221]. On the contrary, the inhibition of tryptophan catabolism causes fetal allograft rejection, during pregnancy in mice[222].

Interestingly, IDO1 plays also an essential role in the central nervous system (CNS). The secretion of IFN-γ, by T helper cells, induce IDO1 expression in microglial cells and represents a negative feedback loop to reduce neural inflammation[223]. Recently, astrocytes have also been found to up-regulate IDO1, following stimulation with IFN-γ and TLR3 ligand poly (I:C) (PIC)[224]. In addition, as already mentioned, unbalanced tryptophan metabolism has been found involved in neural degenerative disorders, and tryptophan degradation has been correlated to the onset of diseases, such as Alzheimer's disease, Huntington disease, and even depression. Furthermore, in experimental autoimmune encephalomyelitis (EAE), IDO1 expression in microglia cells promotes a negative feedback loop, which decreases neuroinflammation and subsequent tissue damage. This protective effect is thereby counteracted by the production of some neurotoxic active metabolites of the kynurenine pathway, such as quinolinic acid and 3-hydroxyanthranilic acid. In addition, some of these metabolites, produced during systemic infection, can pass the blood-brain barrier acting as neurotoxins[225].

As already described, the role of IDO1 in the host defense against pathogens has been first characterized. In particular, IDO1 activity is required to reduce pathogen replication *in vitro*. The induction of IDO1 expression in human fibroblast, by IFN-γ treatment, blocks the growth of *Toxoplasma Gondii*, an intracellular parasite[226]. Others pathogens, including fungi, bacteria, and viruses are sensitive to the tryptophan-depleting activity of IDO1, as already mentioned. In particular, Bozza et al. demonstrated the role for tryptophan catabolism during *Candida albicans* infections. They found that mice infected with the fungus express IDO1 in the sites of infection and in DCs and neutrophils. The use of an IDO1 inhibitor increases pro-inflammatory cytokines, Th1 production and decreases Th2 and Treg production in mice[227]. Despite the fundamental role of IDO1 during infections, it is still unclear whether it is always beneficial to the host. HIV and Epstein-Barr virus are able to increase cellular levels of IDO1. It has been suggested that these viruses exploit the immunosuppressive properties of IDO1, to facilitate the immune evasion and virus infection[225].

The immunomodulatory effects of IDO1 are mainly mediated by regulation of adaptive T-cell immunity. Indeed, IDO1 expression in DCs results in multiple effects, including increased T-cell apoptosis and inhibition of T-cell proliferation[228]. IDO1 is also involved in the induction of Tregs, which induces anergy towards specific antigens[229]. In mouse models, the ability of IDO1 to convert T cells $CD25^{-}Foxp3^{-}$ in Tregs $CD25^{+}Foxp3^{+}$ has been demontrated[230].

Despite the essential role of IDO1 in the acquired peripheral tolerance against foreign antigens, in homeostatic conditions, it seems that IDO1 is not responsible for the central tolerance to self-antigens. Indeed, mice *Ido1*$^{-/-}$ or mice treated with IDO1 inhibitors do not develop autoimmune diseases[231].

Another evidence regarding the ability of IDO1 to induce immune tolerance is represented by its ability to prevent the rejection of transplanted tissues/organs in murine models, even with full MHC haplotype mismatches[232]. For example, IDO1 has the potential to promote tolerance in murine models of allogeneic T-cell transfer and pancreatic islet transplantation[233]. In addition, in a rat model, Na et al. demonstrated that the expression of IDO1 in immature DCs, which are pre-injected, is responsible for a prolonged kidney allograft survival[234].

IDO1 is also involved in the regulation of graft versus host disease (GVHD), which is the major cause of mortality after allogeneic transplantation of HSCs, in leukemic patients. Jasperson et al. demonstrated that IDO1 plays a critical role in GVHD, especially in the colon, where IDO1 is highly expressed by epithelial cells in mice. They also showed that mice IDO1 knock out died more quickly from GVHD. T-cell infiltration in the colon showed decreased apoptosis and increased proliferation compared with their controls[235].

IDO1 seems also involved in the regulation of autoimmune responses. In fact, DCs isolated from the synovial joints of patients affected by rheumatoid arthritis, express higher levels of IDO1 than DCs isolated from healthy donors[236]. In addition, in an EAE murine model, IDO1-mediated tryptophan metabolism switches the immune response towards the production of Th2, leading to the repression of inflammation[237].

Finally, IDO1-mediated immunosuppression influences the recognition and elimination of tumor cells, by immune cells. This characteristic will be discussed later in this thesis.

2.2.5.4 Regulation of IDO1 expression

All the mechanisms regulating IDO1 expression are not fully elucidated. However, it is clear that IDO1 is not constitutively expressed, but different stimuli, activating different signaling pathways, induce IDO1 expression in cells[225]. The pro-inflammatory cytokine IFN-γ is one of the most well characterize stimulus, inducing IDO1 expression. The IFN-gamma-inducible expression of the *INDO* gene depends on two elements present in the promoter: 14-base pair interferon sequence response-like elements (ISRE) and 9-base pair palindromic-gamma-activated sequences (GAS). The two sequences are recognized by the transcription factors: INTERFERON REGULATORY FACTOR-1 (IRF-1) and STAT1α (P91)[238]. In addition, *INDO* promoter contains also non-canonical

NF-κB consensus sequences[225]. Interestingly, mutations or deletions, occurring into the ISRE response elements (ISRE1 and ISRE2), induce a decrease in IDO1 expression, when cells are stimulated with cytokines[239]. In particular, the ability of IFN-γ to induce IDO1 is decreased by 50-fold, when the ISRE1 sequence is deleted, while point mutations at two alanine residues of ISRE2 at −111 induce a 4-fold decrease of IFN-gamma-mediated IDO1 expression[240]. The IFN-gamma-inducible expression of IDO1 suggests that this immune regulator counteracts and limits an excessive T cell response, which is activated by IFN-γ itself. Indeed, the pro-inflammatory cytokine induces the activation and proliferation of T cells and, through a negative feedback, it also induces IDO1 expression, which counteracts T cells hyper-activation. In addition, other cytokines, such as IL-6, IL-4, IL-10 stimulate IDO1 expression.

Another receptor/ligand signaling pathways can regulate IDO1 expression. TLRs, the interferon gamma receptor (IFNGR), interferon beta receptor (IFNBR), tumor necrosis factor superfamily members (TNFRs), AHR and transforming growth factor beta receptors (TGFBRs), they can all activate pathways that either induce or maintain IDO1 expression. Stimulation of TLR3 and TLR4 seems specific for inducing IDO1 expression in DCs, whereas TLR7 and TLR8 seem specific for inducing IDO1 in monocytes[241,242]. TNF-α and IFN-β, by binding their receptors (TNFR and IFNBR), activate the non-canonical NF-κB and JAK-STAT signaling, which results in the transcription and translation of IDO1 protein[243]. Finger and coworkers also demonstrated that the interaction of CYTOTOXIC T-LYMPHOCYTE ANTIGEN 4 (CTLA-4)-Immunoglobulin (CTLA-4-Ig) fusion protein with the costimulatory molecule B7 (CD80/86) on DCs, induces IDO1 activity and leads to subsequent tolerogenic rather than immunogenic phenotype of DCs[244]. Furthermore, the CTLA-4 expression on Treg cells can up-regulate IDO1 in DCs[245].

The regulation of IDO1 by TGF-β, unlike the previous mechanisms, induces a long-lasting and stable expression of IDO1 in mouse plasmacytoid dendritic cells (pDCs)[246]. Interestingly, in pDCs TGF-β induced IDO1 activity is independent of its catalytic activity. TGF-β induces IDO1-dependent regulatory functions in pDCs *in vitro,* that are independent of the catalyst function of IDO1[246]. TGF-β signaling activates both the Smad-dependent and Smad-independent pathways, mediated by PI3K, inducing IDO1 biosynthesis [247]. In addition, this signaling generates a positive feedback, sustaining the production of TGF-β and IDO1 through PI3K[246].

The AHR is also involved in IDO1 expression. Indeed, kynurenines produced by IDO1 enzymatic activity bind and activate AHR, as already mentioned[219]. Furthermore, DCs isolated from $AhR^{-/-}$ mouse bone marrows, do not up-regulate IDO1 following LPS treatment[248]. After the interaction with the ligand and translocation to the nucleus, AHR activates several genes, including IDO1, by

its association with dendritic cell responses element (DRE) consensus sequences, present in *INDO* promoter[249].

The IDO1 expression is also negatively regulated. In fact, nitric oxide (NO) reduces IDO1 expression and activity. NO is produced by immune system cells, such as macrophages, to protect the host against viral, bacterial and parasitic infections. As already described for IFN-γ stimulation, the inhibition of IDO1 by NO could counterbalance an excessive response of the immune system. Another negative regulator is represented by IL6, also able to activate IDO1. IL-6 inhibits IFN-gamma-mediated *IDO1 e*xpression in DCs[250], and by inducing the SUPPRESSOR OF CYTOKINE SIGNALING 3 (SOCS3) activity, drives IDO1 proteasomal degradation[251].

2.2.5.5 IDO1 immune escape in tumors

Tumor cells can express several immunogenic antigens on the cell surface. Immune cells recognize these antigens and induce a tumor-specific immune response, in a process called "cancer immunosurveillance". Despite the immunogenicity of some tumor cells, the immune system is not always effective in the eradication of tumors. Indeed, it can also favor a "non protective" immune state to other tumors or it can promote the onset of immunologic anergy and tolerance.

Recently, the new term of "cancer immunoediting" has been proposed to describe the complex cross-talk between tumor cells and immune cells. In this dynamic process, the immune system select for tumor variants that will better survive in an immunologically competent environment. In fact, the immunologic environment imprints tumors, leading to the eliminations of high immunogenicity cells and leaving behind tumor variants of reduced immunogenicity, which are resistant to the tumor-suppressing actions of the immune system. In addition, tumor cells can also actively evade or suppress immune attack. During the immunologic sculpting, alterations in tumor cells, favoring their survival in the immunocompetent host, are probably facilitated by the genetic instability of tumors[252]. The ability of tumor cells to expand in an uncontrolled manner in the immunocompetent host is a process, called "tumor escape".

Recently, IDO1 activity has been proposed as one of the mechanisms favoring the generation of immunological tolerance towards tumor-specific antigens. Many distinct tumors overexpress an active form of IDO1, which induces an immunosuppressive environment, and is associated with poor prognosis[216]. In addition, IDO1 expression into tumor cells avoids their rejection in preimmunized hosts[253]. Muller et al. found that the *INDO* gene is under the negative genetic control of BRIDGING INTEGRATOR 1 (BIN1). BIN1 has been found downregulated in several solid tumors. In different cell types, the absence of Bin1 markedly increases the IFN-γ-induced

expression of IDO1[254], leading to T cell suppression *in vivo*. In tumor cells, IDO1 expression could be constitutive because of mutations i.e. affecting *BIN1*, or it could be induced by pro-inflammatory signals present in the tumor environment.

Interestingly, it has been observed that the inhibition of IDO1 activity has an antitumor effect only in the presence of a fully competent immune system. This suggests that IDO1 acts by inducing an impaired host immune response and by inhibiting a tumor-specific immunity[228]. The mechanisms of IDO1-mediated immune tolerance to tumors seems to act both at the tumor site, where the depletion of tryptophan and the formation of active metabolites reduces clonal expansion and survival of antigen-specific T cells, and at the tumor-draining lymph node, where a tolerogenic effect on T cells is mediated by IDO1-expressing plasmacytoid DCs. In particular, in patients with melanoma IDO1-expressing DCs have been found in sentinel lymph nodes of patients, and are associated with poor clinical outcome[255]. All these findings led to the hypothesis that IDO1 expression could be a prognostic factor. This has been confirmed in several solid tumors including ovarian, endometrial, colorectal cancers[256,257].

Recent findings highlight a more complex role of tryptophan catabolism extending its involvement, beyond adaptive immunoregulation, in metastasis and angiogenesis[216]. Indeed, mice *Ido1*$^{-/-}$ display a reduction of primary or metastatic lung tumor burdens and a better survival. In this mouse model, a reduction of IL-6 levels has been observed, with consequential impairment of myeloid-derived suppressor cells (MDSC)[258], a cell population with protumorigenic functions. In addition, *Ido1*-deficient mice show an angiogenic defect in the lungs, even in the absence of tumors.

2.2.5.6 IDO1 and AML

The evidence that AML cells are susceptible to the action of the immune system is suggested from clinical advantages of allogeneic HSC transplantation (HSCT), in comparison with autologous transplantation. Indeed, donor-derived immune cells are able to destroy host AML cells. Furthermore, in patients affected by chronic myeloid leukemia, the additional infusion of donor-derived lymphocytes increases the rates of complete remission, after post-transplant relapse[259]. In 2017, the results of a phase I/II trial, based on the use of the leukemia-specific vaccine for myeloid malignancies, were published. The infusion of the vaccine induces specific immune responses with clinical manifestations, including molecular remission[260].

Despite all this evidence, highlighting the ability of immune cells to recognize and destroy AML cells, it is also known that AML cells can activate several mechanisms of tumor escape. Indeed, the tumor cell supernatants from primary AML cells, prevent T cell activation and Th1 cytokine

production, through inhibition of NF-κB, MYC Proto-Oncogene, BHLH Transcription Factor (c-Myc), and Retinoblastoma protein (pRb) pathways in T cells[261].

The role of IDO1 in AML-mediated tumor escape has been further emphasized, by Chamuleau et al. They found that high IDO1 mRNA levels in AML cells correlate with shortened OS and relapse-free survival. According to the authors, the inhibition of IDO1 may break immune tolerance, offering new therapeutic options[262]. Recently an immunohistochemical score, based on IDO1 expression, has been proposed to predict early mortality in AML patients[263].

In our laboratory, for the first time Curti and coworkers demonstrated that, contrary to their normal counterpart ($CD34^+$ cells), primary AML cells express an active form of IDO1. IDO1 expressing AML cells are able to produce active metabolites from tryptophan and to inhibit allogeneic T cell proliferation. In addition, Curti et al. also found that in IDO1 not expressing-AML cells, protein expression can be stimulated following IFN-γ treatment. The constitutively IDO1 expression in a significant portion of AML cells indicate IDO1, as a novel pathway of tumor escape in leukemia[264]. The expression of an active form of IDO1 or IDO1 enzymatic activity in AML cells has been confirmed by other groups[265,266]. Interestingly, Noura et al reported that the use of IDO1 inhibitor reduces AML cell proliferation, *in vitro*[266].

In hematological malignancies and in many cancers, including pancreatic ductal adenocarcinoma, ovarian cancer, lung cancer, glioblastoma, melanoma, the accumulation of $Foxp3^+$ Tregs has been found within tumor tissue, and it correlates with worse prognoses[267]. It is still unclear the mechanism regulating this process, but it seems reasonable that IDO1 could represent one of these. Indeed, Curti et al. demonstrated that primary AML cells expressing IDO1 can induce the conversion of $CD4^+CD25^-Foxp3^-$ into $CD4^+CD25^+Foxp3^+$ *in vitro.* In addition, in AML patients at diagnosis IDO1 expression correlates with increased circulating $CD4^+CD25^+FOXP3^+$ T cells[126].

2.2.5.7 IDO1 inhibitors

IDO1 represents a good target for pharmacological drug development for the following reasons:

- IDO1 is a single-chain catalytic enzyme with a well-defined and characterized biochemistry. In addition, other tryptophan catabolizing enzymes, i.e. TDO and IDO2, have a distinct structure and pattern of expression, limiting "off-target" issues;
- mice lacking *Ido1* are viable and healthy, suggesting that IDO1 inhibitors should not be toxic;

- pharmacodynamic evaluation of the drugs can be easily obtained by quantifying blood serum levels of tryptophan and its derivatives in patients[216].

Mechanistically, IDO1 inhibitor function is to spontaneously initiate an immune response against tumor cells, rather than directly kill them[268].

The first molecule, identified as an IDO1 inhibitor, is the 1-methyltryptophan (1-MT). 1-MT is a chiral compound that can exist as both D- and L-stereoisomers (D-1MT, L-1MT), with different properties. In mouse tumor models, preclinical studies have proved that 1-MT is not toxic and shows synergy when combined with chemotherapeutic drugs, in reducing tumor growth[254].

In human, the first Phase I trial was performed treating adult patients affected by refractory solid malignancies with D-1MT[269]. Phase II combination drug trials were also performed in breast or prostate cancer patients. Despite the results are still not accessible, it seems that D-1MT is well tolerated[216].

Nowadays, several IDO1 inhibitors are available. In particular, Epacadostat (INCB024360), produced by Incyte Corporation, is a catalytic inhibitor which competes with tryptophan to bind the enzyme. In different mouse models, Epacadostat reduces tumor growth in wild-type mice but not in *Ido1*$^{-/-}$ mice or nude mice. This suggests that the drug is specific for IDO1 and that a competent immune system is required to mediate IDO1 antitumor effects[270,271]. In 2010, Epacadostat began the first Phase I trials for advanced malignancies.

Since in mouse model of melanoma, the expression of IDO1 and other immune regulatory molecules, i.e. CTLA4 and PD-L1, may be interconnected, the use of combination therapies blocking several of these molecules at the same time, could be attractive[232]. In 2015, Gangadhar et al. published the results of Phase I trial based on the use of Epacadostat with Pembrolizumab, which works as an anti-PD-1 immunotherapy. The combination was used for the treatment of patients affected by non-small-cell lung carcinoma (NSCLC), melanoma, transitional cell carcinoma (TCC), renal cell carcinoma (RCC), endometrial adenocarcinoma (EA), or squamous cell carcinoma of the head/neck (SCCHN). The treatment was safe and reductions in tumor mass, when evaluable, were observed in 15/19 patients[272].

In MDS patients and in AML patients with a myeloblast percentage between 20-30%, a Phase II trial based on the use of Epacadostat is reported. Despite the drug was relatively well tolerated, at the dosages tested no significant clinical activity was observed[273].

In October 2016, 13 trials based on Epacadostat treatment were ongoing. However, the results are not available yet.

2.2.5.8 IDO1 expression in MSCs

MSCs do not constitutively express IDO1, but gain this function following stimulation with the pro-inflammatory cytokine IFN-γ, as already described[193]. IFN-γ alone is sufficient to induce IDO1 expression but the effect is even stronger in combination with TNF-α/IL-1β[274]. The ability of MSCs to respond to pro-inflammatory cytokine by producing IDO1 leads to immunological tolerance, which suppresses the inflammatory responses[275]. In particular, IDO1 expressing MSCs are able to inhibit proliferation, cytotoxicity and cytokine production of NK cells[183] and T cell proliferation[194]. François et al. demonstrated that TNF-α- and IFN-γ-activated MSCs have the ability to inhibit T cell response, *in vitro.* This ability is variable and depends on the different amount of IDO1 levels present in MSCs, isolated from different donors. The authors also proved that IDO1 activity in MSCs favors the conversion of monocytes in IL-10-secreting M2 immunosuppressive macrophages, which amplify MSC-mediated immunosuppression[276].

Based on the role of MSCs and IDO1 activity in tumor progression, it has been hypothesized that IDO1 expressing MSCs could influence tumor growth. Ling et al. induced the expression of human *IDO1*, under the control of an inducible promoter, in murine MSCs. The genetically modified MSCs were then injected in mice together with two distinct tumor cell lines of melanoma and lymphoma. The authors found that IDO1-expressing MSCs promote tumor growth, that is reversed by the administration of IDO1 inhibitor. IDO1-expressing MSCs profoundly reduce both B cells and tumor-infiltrating $CD8^+$ cells. According to the authors, IDO1 inhibition in MSCs could be potentially used to restore immunity against the tumors[277].

In another study, it has been reported that human umbilical cord-derived MSCs (UC-MSCs), manipulated to express IDO1, show a reduced antiapoptotic effect when cultured with two leukemic cell lines. This suggests that IDO1 could reverse the cancer-supportive effect of MSCs[278].

Interestingly, IDO1 expression in MSCs not only influences their immunomodulating ability but also their ability to proliferate and differentiate. Indeed, Croitoru-Lamoury found that IFN-γ, by activating IDO1, inhibits MSCs proliferation and alters their ability to differentiate[279].

2.2.6 Evidence of the role of MSC and other stromal cells in the pathogenesis of AML/MDS in mouse models

Physiologically, BM microenvironment plays a key role in regulating HSC function. In the last years, several studies tested and demonstrated the hypothesis that alterations of niche cells could

promote myeloid transformation, challenging the widely accepted concept that MDS and AML originate exclusively from intrinsic alterations of hematopoietic cells.

In 2007, the pivotal work of Walkley et al. demonstrated that microenvironment does not exert only a bystander effect in myeloid malignancies. Indeed, they showed that the deletion of the retinoic acid receptor γ (RAR-γ) in BM cells induces a myeloproliferative-like syndrome (MPS). In fact, the transplantation of WT BM cells in a RARgamma($^{-/-}$) microenvironment results in MPS, which is partially caused by an increased level of TNF-α. The cells responsible for this phenotype in mice were not identified, however, the significant loss of bone observed, indicates the role of mesenchymal progenitors in this nonhematopoietic cell-intrinsic MPS[280].

An MPS-like disease also develops after constitutive deletion of *Ubiquitin E3 ligase Mind bomb 1* (*Mib1*), which leads to the inhibition of Notch ligand endocytosis. In addition, the transplantation of wild-type bone marrow cells into mice with *Mib1*-null BM microenvironment induces a *de novo* MPS, and the phenotype can be reverted by Notch activation in cells of BM microenvironment[281]. Similarly, mice lacking IκBα (inhibitors of NF-Kb) showed a stroma-mediated myeloid dysregulation, which is not reproducible if the deletion of the gene conditionally occurs only in myeloid precursors[282].

Tyrosine phosphatase SHP-2 (encoded by *PTPN11* gene) mutations are frequently found in Noonan syndrome, associate to an increased risk of leukemia transformation. Dong et al. demonstrated that *Ptpn11* activating mutations in mesenchymal stem/progenitor cells and osteoprogenitors, induce an MPS-like phenotype in mice. The mutated cells produce an excess of the CC chemokine CCL3, which leads to the recruitment of monocytes in BM, where also HSCs reside. Consequently, HSCs are hyperactivated following stimulation with pro-inflammatory cytokines produced by monocytes[283].

Furthermore, also an MDS-like phenotype can be induced in mice by a dysfunction of a well-defined population of mesenchymal progenitors. Indeed, in 2010 Raaijmakers et al. demonstrated in a mouse model that the targeted deletion of *the Dicer 1, Ribonuclease III* (*Dicer I*), a mi-RNA processing endonuclease from osterix-expressing bone progenitor cells, leads to alterations of the hematopoietic system, recapitulating in mice features of human MDS, including the propensity to develop into AML. The loss of Dicer I resulted in a decrease of *SBDS, Ribosome Maturation Factor (Sbds)* expression in mesenchymal/osteoprogenitor cells. *SBDS* encodes for a protein involved in ribosomal maturation and its mutation has been identified in Shwachman-Diamon syndrome (SBDS), a congenital BM failure whit high risk to develop AML. Interestingly, MSCs isolated from MDS patients show lower expression of *DICER I* and *SBDS*, suggesting a possible correlation with

pathophysiological elements found in murine experiments[284]. A recent study further demonstrated the active role of BM microenvironment in myeloid malignancies. Zambetti et al. demonstrated that the targeted deletion of *Sbds* gene in mesenchymal progenitors recapitulates human SBDS in mice, and leads to mitochondria dysfunction and genotoxic stress in HSPCs, mediated by the secretion of damage associated molecular pattern (DAMP) molecules: S100A8 and S100A9. The authors also identified a correlation with the expression of the two DAMP molecules in MSCs, isolated from MDS patients, and the transformation in AML[285].

Recently, genetic alterations, causing a niche-induced AML, have been identified. Indeed, by generating an activating mutation in the *β-actin* gene in osteoprogenitor cells, with subsequent activation of Wnt signaling, Kode and collaborators found that this leads in mice to the development of AML, with impaired myeloid and lymphoid differentiation. The constitutive active β-catenin induces the expression of Jagged 1, the Notch ligand, in osteoblasts. The subsequent activation of Notch signaling in HSPCs promotes the malignant transformation. The authors also speculated about the relevance of the discovery for human disease. Indeed, in the 38% of MDS and AML patients, B-CATENIN signaling is increased in osteoblasts and NOTCH signaling in hematopoietic cells[286]. The following study found that Forkhead Box O1 (FoxO1) expression in osteoblasts mediates the leukemogenic properties of the constitutive activation of β-catenin in mice. At the molecular level, by interacting with β-catenin in osteoblasts, FoxO1 induces the expression of Jagged-1[287]. All these studies support the concept that bone marrow niche is involved in the pathogenesis of myeloid neoplasm in animal models.

2.2.7 Evidence of a niche-induced AML/MDS in humans

As already described, the onset of a niche-induced myeloid malignancy has been demonstrated in mouse models. In human, an evidence supports this concept, indeed in rare cases, donor-derived leukemia completely different from the original leukemic clone can occur in patients, BM transplanted. In the host, preexisting BM niche alterations could initiate leukemogenesis in engrafted cells of donor origin[288]. In addition, as already described, osteoblastic lineage cells from 38% of MDS and AML patients show an increase of β-catenin associated with an higher activation of Notch signaling in hematopoietic cells, which has been correlated to niche-induced leukemogenesis in mice[286].

In addition, *ex vivo* expanded MSCs isolated from MDS and AML patients show several alterations. The first evidence of a putative role of MSCs in myeloid pathogenesis arose from studies that found chromosomal aberrations in 30-70% of MSCs from MDS and AML patients. Surprisingly, in a certain percentage of patients, the cytogenetic abnormalities are different from the hematopoietic alterations, identified in the same patients[289,290]. Chromosomal and genetic alterations of MSCs have been correlated to specific gene-expression programs and disease subtypes, suggesting that the genetic susceptibility of MSCs can play an active role in the progression of MDS and AML[27]. In addition, gene expression is also altered in MSCs isolated from myeloid neoplasm, as described recently in studies based on RNA sequencing[291-292]. However, the functional meanings of these alterations are still under debate and conflicting results have been published. Several studies pointed out normal functionality including differentiation, immunophenotype, expression of adhesion molecules and extracellular matrix proteins, and support of normal hematopoiesis *in vitro*[293,294]. On the contrary, other studies revealed functional alterations of MSCs. Indeed, Geyh et al reported that MSCs isolated from AML patients show growth deficiency and altered osteogenic differentiation ability, with a reduced capacity to support hematopoietic cells. A specific methylation signature was identified in these MSCs, affecting pathways involved in proliferation and cell differentiation[295]. Other studies confirmed the growth deficiency, abnormal osteogenic differentiation and reduced hematopoietic support of MSCs isolated from AML and MDS patients[296–298]. In particular, normal HSCs co-cultured with MSCs isolated from AML patients and then transplanted in mice show a lower repopulating activity compared to HSCs pre-cultured with normal MSCs, suggesting that leukemic MSCs potentially provide a selective support to leukemic cells[299]. In addition, MSCs isolated from patients with myeloid malignancies show increased apoptosis, production of IL-1β and stem cell factor, and reduced expression of adhesion molecules[27]. The immunomodulatory potential is also altered in MSCs isolated form MDS and AML patients. In fact, the inhibitory effect on T cell proliferation is lost when the ratio between lymphocytes and MSCs isolated from AML patients is high[300].

Interestingly, Lopes et al. found that MSCs isolated from *de novo* AML and from AML secondary to MDS, produce several cytokines. The first group was characterized by an increase of VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGFA), CXCL12, RECEPTOR OF PGE2, IDO, IL-1*β*, IL-6, and IL-32, and decreased IL-10 expression, whereas the second group by an increase of IL-6[300]. So far, whether these alterations are predisposing or initiating in human disease is still unclear.

Beside functional alterations of MSCs isolated from MDS and AML patients, in general, MSCs show other mechanisms which can be exploited by leukemic cells to favor their own expansion. In particular:

1. *pro-survival effect.* MSCs are able to promote leukemic cell survival through cytokine production and cell-to-cell interactions[155,301]. In particular, MSCs isolated from AML patients reduce the proliferation of a human AML cell line, K562, modulating cell cycle towards quiescence. This ameliorates K562 cell survival cultured in adverse conditions, such as serum starvation. The PI3K-Akt-Bad signaling pathway has been found to be potentially involved in this antiapoptotic effect[302]. The Notch pathway is also involved in the antiapoptotic effect mediated by MSCs[303]. Similar studies have been performed with primary AML cells. MSCs isolated from healthy donors enhance survival of LSCs *in vitro,* supporting long-term maintenance of LSCs[304]. Ramasamy et al. demonstrated that despite MSCs inhibit K562 proliferation *in vitro*, the transplantation of K562, in conjunction with MSC in mice, results in tumor growth that was much faster compared to that of the group receiving only K562[301].

In a mouse model, Guarnerio et al. demonstrated another mechanism of non-cell autonomous maintenance of AML cells. Indeed, the authors found that the expression of the *Pml* gene in MSCs has a role in sustaining LSCs. In particular, MSCs expressing *Pml* secrete several pro-inflammatory cytokines, such as Cxcl1 and IL-6, inducing a pro-leukemic microenvironment, which favors tumor progression in mice. Interestingly, higher levels of CXCL1 and IL-6 have been found in AML patient serum[305].

Recently, a new mechanism of bone marrow microenvironment support, based on mitochondria transfer, has been identified. Indeed, AML cells contain a higher mitochondrial mass, compared to healthy $CD34^+$ progenitor cells. Furthermore, *in vitro* experiments demonstrated that when co-cultured together, AML patient-derived MSCs transfer their mitochondria to primary AML cells, which increase their mitochondria mass and ATP production, enhancing leukemia cell growth. On the contrary, mitochondira transfer is not observed with healthy $CD34^+$ cells[306].

2. *drug resistance.* BM niche can be a sanctuary helping leukemic cells to evade chemotherapy-induced death. Macanas-Picard et al. demonstrated that MSCs, by secreting soluble factors, protect leukemia cells from cytarabine-induced apoptosis *in vitro* and *in vivo* in a mouse model. The chemoprotective effect was mediated in AML cells by the block of NUCLEOSIDE TRANSPORTER 1 (ENT1) activity, involved in cytarabine cell incorporation[307]. Subsequently, the authors found that the ability of MSCs isolated from AML patients to induce resistance to cytotoxicity induced by Cytarabine (Ara-C) *in vitro,* correlates with the OS. Thus, patients in which

the chemoprotection is active, show worse OS[308]. In another study, it has been found that the adhesion of AML cells to MSCs is required to protect the cells from cytotoxic drug-induced apoptosis, and this drug resistance mechanism is at least in part c-Myc dependent[309]. In addition, Chen et al. demonstrated that the adhesion of the AML cell line, NB4, to MSCs, confers resistance to the anti-proliferative and pro-apoptotic effects of a monoclonal antibody against CD44. In leukemic cells, this mechanism of drug resistance is due to p27(Kip1) down-regulation with the activation of PI3K/Akt signaling, already known to be involved in chemoresistance[310]. Another pathway involved in drug resistance and fundamental in leukemia/bone stromal cell interactions is represented by SDF-1/CXCR4 signaling. The inhibition of CXCR4 reduced the protective effects of stromal cells on apoptosis, induced by chemotherapy in AML cells[311]. All these findings provided a basis for the development of early clinical studies, aimed to overcome the resistance to chemotherapy by interfering with the adhesion of leukemic cells to stromal cells[29]. However, the results are not yet available.

In a mouse model, the expression of *Pml*, in MSCs, has been also correlated with drug resistance of AML cells. In fact, through this non-cell autonomous mechanism, MSC protect LSCs from chemotherapy and the *in vivo* inhibition of Pml was found to be beneficial for the treatment of several AML subgroups, characterized by distinct genetic alterations[305].

In addition, a novel mechanism of drug resistance has been revealed by Moschoi et al. The authors found that the contact-dependent transfer of functional mitochondria from MSCs to AML cells *in vivo*, results in a higher energy production and less depolarization after chemotherapy with a better survival. The mitochondria transfer in normal cord blood $CD34^+$ cells was smaller suggesting a peculiar survival advantage mechanism of AML cells[312].

2.3 MALIGNANT CELLS SHAPE THE NICHE

In mouse models, a considerable amount of evidence has demonstrated that malignant cells can shape the BM microenvironment, to gain some advantage at the expense of normal hematopoiesis. In particular, remodeling of nerve components, endothelial cells, and MSCs have been pointed out in this process. Some of the factors driving remodeling of the BM microenvironment have been revealed, however, most of them are still unknown. Among others, inflammation, a hallmark of cancer, seems to play a role as remodeling factor, observed in different leukemia types. The ability to induce inflammation could potentially reflect specific "normal" immune functions, still present in malignant cells[313].

One of the best examples of bone marrow remodeling in hematological malignancies is represented by multiple myeloma (MM). MM is characterized by the accumulation of malignant plasma cells, which secrete antibody, in the BM. The consequences are elevated serum immunoglobulin and bone tissue loss, which has an impact on prognosis. Bone lesions are the reflection of the infiltration in BM of malignant plasma cells, which remodel bone by producing intrinsic factors or recruiting additional hematopoietic cell. These recruited cells produce factors such as, MIP-1α, IL-3, IL-6, IL-7, SDF-1-α, and VEGF, which alter the balance of bone production/resorption, mediated by osteoblasts and osteoclasts[313].

In a mouse model of chronic myeloid leukemia (CML), it has been demonstrated that leukemic cells produce G-CSF. G-CSF production results in the reduction of Cxcl12 expression in BM stromal cells, with impaired HSC support[314]. Another study reported that CML cells secrete thrombopoietin (TPO) and CCL3. These soluble mediators stimulate MSCs to overproduce osteoblasts, that exhibit impaired HSC maintenance and effectively support LSCs[315].

The niche remodeling process has been identified for myeloid malignancies such as myeloproliferative neoplasms, chromosome Philadelphia negative (Ph-) essential thrombocythemia (ET), polycythemia vera (PV) and myelofibrosis (MF), MDS and AML. Arranz et al. found that murine malignant hematopoietic cells, presenting the mutation V617F in the *JAK2* gene, produce pro-inflammatory cytokines, such as IL-1β, which damage local neurons and microenvironment. The induced neuropathy results in the apoptosis of $Nestin^{+}$MSCs, favoring an increase of myeloproliferation, which could be reverted by the administration of β-adrenergic agonist[316].

Likewise, Medyouf et al. demonstrated *in vitro* that primary human MDS cells can instruct MSCs, isolated from healthy donors, towards an MDS-like behavior. Indeed, MSCs start to produce VEGF, INSULIN-LIKE GROWTH FACTOR (IGF) and EPIDERMAL GROWTH FACTOR (EGF), and

mediators of fibrosis such as LYSYL OXIDASE-LIKE (LOXL), TGF-β, LEUKEMIA INHIBITORY FACTOR (LIF), suggesting a remodeling of microenvironment mediated by MDS cells. In addition, they found that in patient-derived xenograft models, $CD34^+$, isolated from MDS patients, if co-injected with their corresponding MSCs, expanded *in vitro*, show a higher engraftment in mice compared to $CD34^+$ transplanted alone. This suggests that MDS cells need their disease-associated MSCs to propagate the disease *in vivo*[317].

AML cells have also the ability to modify their niche. As described for MDS, healthy MSCs cultured in the conditioned medium of AML cells, show a reduced proliferation and reduced osteogenic differentiation capacity[295]. LSCs are also able to modify the transcriptome of MSCs. Indeed, contrary to their normal counterpart, leukemia stem cells cultured with MSCs isolated from healthy donors, induce an altered expression of cell-cell cross-talk molecules, like CXCL12 and JAG-1. This suggests that LSCs instruct MSCs to provide distinct cross-talk signaling to normal HSCs and to themselves[299]. Frisch et al. found that in an AML mouse model, there is an inhibition of osteoblastic cells associated with reduced plasma level of the bone formation marker, osteocalcin. They identified the chemokine CCL3, produced by malignant cells, as responsible for this bone demineralization. Interestingly, CCL3 mRNA was detected also in malignant cells isolated from AML patients[318].

Kuett et al found that IL-8 is up-regulated in primary AML cells and AML cell lines, within 48 hours of severe hypoxia. IL-8, without acting on AML cells, induces the migration of primary BM-derived MSCs, towards the IL-8 gradient. In addition, AML patients with FLT3-ITD mutations show the highest IL-8 expression, which has been correlated to a worse prognosis[319].

Several studies have highlighted the role of exosomes, cell membrane-derived vesicles containing RNA and protein cargoes, as extrinsic factors able to mediate the cell-cell cross-talk in the BM niche. The role of exosomes in AML niche has also been investigated. It has been found that both primary AML and AML cell lines release exosomes, carrying several coding and noncoding RNAs, relevant to AML pathogenesis. Exosome uptake, by bone marrow stromal cells, alters their function. Indeed, the culture of OP9, a murine stromal cell line, with exosome released by AML cells, alters angiogenic, proliferative, migratory responses and growth factors secretion of OP9[320]. Kumar et al. demonstrated that AML cells can remodel BM niche through exosome secretion *in vivo*, leading to increased number of mesenchymal stromal porgenitors, osteoblast loss and downregulation of hematopoietic stem cell-supporting factors in BM stromal cells. The disruption of exosome secretion reduce leukemia progression in mice[321]. In addition, mitochondria transfer has also been identified as an AML peculiar mechanism of cell-cell interaction. As previously

described, Marlein et al. demonstrated that AML patient-derived MSCs, transfer their mitochondria to AML cells, through AML-derived tunneling nanotubes. The mechanism is triggered by release of superoxide, generated by NADPH OXIDASE-2 (NOX-2) in AML cells. The inhibition of NOX2 prevents the mitochondrial transfer, induces AML apoptosis, and increases the survival of the animals in AML mouse model[306].

2.3.1 HSPCs and MSCs, who does come first in MDS and AML pathogenesis?

For decades, MDS and AML have been considered to originate from mutated HSPCs. In contrast, animal model studies demonstrated that microenvironment alterations can also trigger a myeloid neoplasm. However, translation of this mechanism to human disease represents still a speculation. This 'chicken and egg' mechanism is challenging to prove in human disease and cannot be fully elucidated in animal models[322]. Most likely a single mutation occurring in stromal cells is not sufficient alone to induce a myeloid neoplasm in humans, like in mice. Furthermore, in mouse models, the genetic lesions of stromal cells are global i.e. is present in all stromal cells, whereas in human this scenario is more difficult to take place. It would be unlike that lesions which occur in some stromal cells could induce a myeloid transformation. Reasonably, the BM defect has to be spread, affecting a high number of HSPCs[322]. An exception is represented by secondary AML, in which the exposure to known leukemogenic agents could alter the BM niche function. In addition, also in congenital leukemia predisposition syndromes, like SBDS, a broad disruption of stromal cells can occur in humans. Moreover, aging has been demonstrated to alter cell biology and could modulate stromal cell alterations[323].

According to Schroeder et al. primary stromal cell alteration may potentially arise early and be involved in the initiating phase of myeloid neoplasm. Later malignant cells infiltrate BM, and can actively modify the microenvironment favoring disease progression at the expense of normal hematopoiesis[29].

AIM OF THE STUDY

It is well accepted that MDS and AML originate from intrinsic alterations of hematopoietic stem cells (HSCs) and myeloid progenitors[27,28]. Hematopoietic cells acquire molecular and cytogenetic aberrations due to an unknown trigger and also as consequence of aging, with a subsequent expansion, characterized by the acquisition of additional alterations[25]. However, the mechanisms leading to the replacement of normal hematopoietic cells with malignant cells are still under debate. In the last years, the contribution of BM microenvironment in myeloid malignancies pathogenesis gained more attention. In particular, a better understanding of the myeloid transformation led to the development of two models for MDS and AML pathogenesis: 1) primary alterations of stromal cells induce the disease (niche-induced leukemogenesis), e.g. by promoting an inflammatory or genotoxic microenvironment able to trigger alterations in hematopoietic cells; 2) primary alterations of MDS and AML hematopoietic cells induce changes in the BM stromal cells, turning the microenvironment into a self-reinforcing niche, which supports malignant clones at the expense of healthy hematopoiesis[29]. However, all the mechanisms supporting these two models have just been started to get unraveled. Despite niche-induced leukemogenesis has been extensively demonstrated in mice models[280–283,324], so far, the understanding of MSC role in initiating and supporting myeloid malignancies in humans is still under investigation. Furthermore, all the mechanisms underlying the niche shaping mediated by malignant cells, and the consequences on stromal cells have still to be defined.

The main aim of this thesis is to contribute to a better understanding and further elucidate the interplay involving BM niche and malignant cells. In particular, if intrinsic alterations of the stromal cell, i.e.MSCs, isolated from MDS and AML patients, can promote or support myeloid transformation and if malignant cells, or even pre-malignant cells with the deletion of genes commonly mutated in MDS and AML, can shape their niche and affect stromal cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

The experiments were performed both at the Institute 'L. & A. Seragnoli', Bologna, Italy, and at the Department of Hematology of the Erasmus MC, Rotterdam, The Netherlands. The section of the thesis 'Materials and Methods' is then divided in 2 groups, based on where the experiments were performed. 1) Bologna; 2) Rotterdam.

1.1. Patients and healthy controls

Bone Marrow (BM) samples were obtained at diagnosis from 32 AML patients, (18 males, 14 female, median age 60.5) and 26 MDS patients (17 males, 9 females, median age 78). In addition, BMs from 12 healthy donors (HDs) (9 males, 3 females, median age 42) were used as the controls (see table 4).

1.2. AML cell isolation and culture

AML cells, contained in the mononuclear cell (MNC) fraction, were obtained from BM aspirates (circulating blast>80%) by centrifugation over a Ficoll-Paque gradient (Lympholyte CL5020 1.077 g/ml Cedarlane) and resuspended in culture medium, Roswell Park Memorial Institute medium (RPMI 1640, Lonza), 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-glutamine (Gibco, Thermo Fisher Scientific), and 1% Penicillin/Streptomycin (Pen/Strep) (MP Biomedicals). AML cells were then frozen and used for the experiments

1.3. MSC isolation and Culture

BM-derived MSCs were isolated from BM aspirates of healthy donors (HD-MSCs), patients affected by myelodysplastic syndrome (MDS-MSCs) and acute myeloid leukemia (AML-MSCs) at diagnosis, and were expanded *ex vivo* as previously described[325]. Briefly, the mononuclear cell (MNC) fraction was separated from BM aspirates by centrifugation over a Ficoll-Paque gradient (Lympholyte CL5020 1.077 g/ml Cedarlane), washed twice in PBS and resuspended in culture medium, low-glucose Dulbecco's modified Eagle's medium (DMEM, Lonza), 10% FBS (Thermo Fisher Scientific), 2 mM L-glutamine (Gibco, Thermo Fisher Scientific), and 1% Penicillin/Streptomycin (Pen/Strep) (MP Biomedicals), and plated in flasks at an initial seeding density of 1.6 x 10^5 cells/cm^2. After 1-2 days, the non-adherent cell fraction was removed by washing the flask with a phosphate-buffered saline solution (PBS), and the remaining adherent cells

were cultured until they reached 70%–80% confluence. Then, cells were detached using a trypsin solution, composed of 0.25% trypsin/ 0.1% EDTA in PBS w/o Calcium w/o Magnesium w/ Phenol Red (Aurogene), reseeded at a density of 3.5 x 10^3 cells/cm^2. MSCs were used for experiments within passages 3 to 5. Cell proliferation was assessed by direct cell counts at each passage (P).

1.4. Immunophenotype

To assess HD-, MDS- and AML-MSC immunophenotype, dual-color immunofluorescence was performed with the following anti-human PE- or FITC–conjugated monoclonal antibodies: anti-CD13, anti-CD34, anti-CD19, anti-HLA-DR, anti-CD45, anti-CD44, anti-CD73 (Becton Dickinson), anti-CD29, anti-CD14, anti-CD105 (Biolegend), anti-CD90 (Chemicon). Cell autofluorescence level was used as the negative control. Briefly, 1 x 10^5 cells were washed in FACS buffer (PBS 0.5% FBS) and incubated with the antibodies listed above, at room temperature for 15 min in the dark. Cells were washed in FACS buffer and at least 10,000 events were acquired with a flow cytometer (FACScanto II equipment, Becton Dickinson), equipped with FACSDiva software. The samples were analyzed with FCS Express 4 Flow Research Edition.

To distinguish AML cells from lymphocytes, an immunophenotype was performed with the following anti-human monoclonal antibodies: APC-H7 anti-CD3, APC anti-CD33 (Becton Dickinson). Cell autofluorescence level was used as the negative control and cells were stained as described above.

At least 10,000 events were acquired with a flow cytometer (FACScanto II equipment, Becton Dickinson), equipped with FACSDiva software. The samples were analyzed with FCS Express 4 Flow Research Edition.

1.5. Intracellular stainings

To assess the intracellular expression of proteins, i.e. INDOLEAMINE 2,3-DYOXIGENASE 1 (IDO1), INTERFERON-γ (IFN-γ), α-SMOOTH MUSCLE ACTIN (α-SMA), at least 1 x 10^5 cells were washed in PBS and then fixed with 4% paraformaldehyde (VWR). After another PBS wash to remove paraformaldehyde, cells were washed and permeabilized with 0.1% Saponin (Sigma)[326]. After a final wash with FACS buffer, the following anti-human monoclonal antibodies were used: FITC anti-α-SMA (Abcam), PE anti-IDO1 (eBioscience, Thermo Fisher Scientific), PE anti-IFN-γ (eBioscience, Thermo Fisher Scientific). The incubation with the antibody listed above was performed for 30 minutes at 4°C, in the dark. Cells were washed in FACS buffer and at least 10,000 events were acquired with a flow cytometer (FACScanto II equipment, Becton Dickinson),

equipped with FACSDiva software. The samples were analyzed with FCS Express 4 Flow Research Edition.

1.6. Differentiation potential

For osteogenic differentiation experiments, HD-, MDS- and AML-MSCs were seeded at 3.1 x 10^3 cells/cm^2 and grown on Lab-Tek II coverglass chamber (Nalge-Nunc) for 3 weeks in Osteogenic Differentiation medium (Lonza) containing L-glutamine, dexamethasone, MCGS, *β*-Glycerophosphate, ascorbate, pen/strep, which was replaced every 3-4 days. After 21 days cells were processed for histological staining and total RNA extraction. The histological staining was performed to highlight Calcium deposition, determined by Alizarin red staining as previously described[325]. Briefly, cells were fixed in 10% PFA in PBS at room temperature for 15 min, then rinsed in PBS and distilled water. Fixed cells were incubated with 40 mM Alizarin red solution (Sigma Aldrich) pH 4.2, with gentle shaking at room temperature for 75 min and rinsed with distilled water. Images were collected with an Axiovert 40 CFL microscope (Carl Zeiss Microscopy).

For adipogenic differentiation experiments, HD-, MDS- and AML-MSCs were seeded at 2.1 x 10^4 cells/cm^2 on Lab-Tek II coverglass chamber (Nalge-Nunc) and grown for 3 days in Adipogenic induction medium (Lonza) containing additional h-Insulin, mesenchymal cell growth serum (MCGS), L-glutamine, dexamethasone, 3-isobutyl-I-methylxanthine, indomethacin, pen/strep followed by 3 days in Adipogenic maintenance medium containing L-glutamine, h-Insulin, MCGS, pen/strep. These procedures were repeated for 3 times and after 18 days, cell cultures were processed for histological staining and total RNA extraction. The histological staining identifies fat droplets inside differentiated cells by using the Oil Red O staining method, as previously described[325]. Briefly, cells were fixed in 10% paraformaldehyde (PFA) in PBS at room temperature for 1 h and rinsed in 60% isopropanol. Then isopropanol was removed and the wells were completely dried and stained with a 0.6% (w/v) Oil Red O solution (Sigma Aldrich) with gentle shaking at room temperature for 15 min. Cells were then observed under the microscope (Axiovert 40 CFL, Zeiss) and images were collected to appreciate the staining

1.7. Total RNA Extraction, Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were disrupted in an RLT Buffer solution 1% β-mercaptoethanol (β-ME) and cell lysate was homogenized through a syringe. Total RNA was isolated using an Rneasy Micro kit (Qiagen)

according to the manufacturer's instructions, and quantified by Nanodrop One spectrophotometer (Thermo Fisher Scientific). For cDNA synthesis, 1 µg of denatured total RNA was reverse transcribed using an Improm II kit (Promega) and random hexamers (Promega) in a 20 µl final volume, according to the manufacturer's instruction. qRT-PCR was performed using the ABI-PRISM 7900 Sequence Detection System (Applied Biosystems).

For each PCR run, 3 µl of cDNA product was mixed with 2x Platinum Supermix (Thermo Fisher Scientific) in a total volume of 25 µl. The thermal cycling conditions consisted of an initial stage of 50°C for 2 min, and 95°C for 10 min, 40 cycles of melting at 95°C for 15 s, and annealing and elongation at 60°C for 1 min. Threshold cycle (C_t) values for specific genes (i.e. PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-*γ* (PPAR-*γ)*; RUNT-RELATED TRANSCRIPTION FACTOR 2 (RUNX2), IDO1, IFN-γ) and endogenous reference gene (i.e. GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH)), were determined automatically using 7900 ABI PRISM system (Applied Biosystem). Relative quantification was calculated using *Δ*Ct comparative method[327]. Briefly, the relative level of a specific cDNA was calculated by subtracting the C_t value of the endogenous reference gene from the C_t value of the specific gene. PPAR-γ, RUNX2, IDO1, IFN-γ cDNA levels in control cells were taken as 1. In some experiments, cDNA, synthesized as described before from a Universal RNA (Agilent genomics) was used as a reference value and taken as 1. All reactions were performed in duplicate in a 96-well Optical Reaction Plate. Primer probes, listed in the table 2, were purchased from Applied Biosystems.

Table 2. Primer Probes.

Taqman Gene Expression Assay, Applied Biosystem	
PPAR-γ (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ)	Hs01115513_m1
RUNX2 (RUNT-RELATED TRANSCRIPTION FACTOR 2)	Hs00231692_m1
GAPDH (GLICERALDEIDE-3-FOSFATO DEIDROGENASI)	Hs00266705_g1
IDO1 (INDOLEAMINE 2,3-DIOXYGENASE 1)	Hs00158027_m1
IFN-γ (INTERFERON-γ)	Hs99999041_m1

1.8. Fluorescent in situ hybridization (FISH)

The molecular cytogenetic analysis was performed on BM-derived AML or MDS cells and MDS- and AML-MSCs, isolated as described before. All cells were treated with a hypotonic solution and fixed with a methanol-acetic acid solution (3:1). Fixed cells were dropped on glass slides, washed in saline-sodium citrate (*SSC*) buffer then dehydrated by successive washes with 70, 85 and 100%

ethanol and hybridized over-night with specific probes using HYBrite system (Vysis). After post-hybridization washes, slides were counterstained with DAPI (Vysis) and analyzed using a fluorescence microscope NIKON E1000 equipped with FITC/TRITC/AQUA/DAPI filter sets and the Genikon imaging system software (Nikon Instruments). At least 200 nuclei were analyzed for each sample.

1.9. Cytokine stimulation

HD-, MDS- and AML-MSCs were seeded at 3.5 x 10^3 cells/cm^2. 24 hours later, the following cytokines were added: IFN-γ (50 ng/ml; Thermo Fischer Scientific), TNF-α (10 ng/ml; Endogen), IL6 (10 ng/ml; Endogen), PGE2 (1 ng/ml; Endogen). After 24 hours of stimulation, MSCs were collected and processed for both RNA and protein extraction.

1.10.Western Blotting (WB)

Cells were washed twice with a Washing Buffer, composed of PBS and 1 mM protease inhibitor Phenylmethanesulfonyl fluoride (PMSF; Cell Signaling Technology). Cells were then lysed at 4°C for 30 minutes in Cell Lysis Buffer (Cell Signaling Technology), enriched with PMSF (Cell Signaling Technology) and protease inhibitors (Complete mini Protease Inhibitor cocktail tablet, Roche). The extracted proteins were quantified according to the Bradford method with Coomassie G-250 (Thermo Fisher Scientific) by using Bio-photometer (Eppendorf). 40 μg of proteins were diluted with 2x Laemli sample Buffer 2% β-ME (Bio-Rad) and denatured at 96°C for 5 minutes. Denatured proteins were loaded in a 12% polyacrylamide gel (Mini-PROTEAN® TGX Stain-Free Precast Gels, Bio-Rad) and a marker (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards, Bio-rad) composed of a mixture of ten multicolored recombinant proteins, was used. The protein separation was performed in TGS (10X Tris/Glycine/sodium dodecyl sulfate (SDS)) Running Buffer (Bio-Rad). After the separation, the proteins were transferred from the gel to a nitrocellulose membrane through the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The membrane was then incubated for 1 hour at room temperature with gentle shaking, in a blocking solution of PBS with 3% of milk, 2% of BSA (Sigma) and 0.5% of Tween (Sigma). For the staining, the membrane was incubated with the primary antibody (Ab), diluted in the blocking solution, overnight at 4°C with gentle shaking. After 3 washes in PBS 0.1% Tween, the membrane was then incubated with the secondary antibody, diluted in the blocking solution, for 1 hour at room temperature, with gentle shaking. After 3 washes in PBS 0.1% Tween, protein bands were detected at Chemidoc instrument (Bio-rad) using Amersham ECL Select WB Detection Reagent (GE

Healthcare), according to the manufacturer's protocol. For membrane staining the following antibodies were used: rabbit anti-human IDO1 Ab (1:20000, rabbit; courtesy of Dr. R. Metz, Lankenau Institute for Medical Research, Wynnewood, Pennsylvania) and donkey anti-rabbit secondary Ab (1:20000) (Santa Cruz Biotechnologies); Goat anti-human Actin Ab (1:5,000) and donkey anti-goat secondary Ab (1:20,000) that were used as control staining (Santa Cruz Biotechnologies).

1.11. Co-culture experiments

MSCs were seeded at the density of 2 x 10^4 cells/cm^2 and after 24 hours AML cells were seeded with transwells on MSC layers (1:10) in RPMI (Lonza). After 4 days of co-cultures, AML cells/MSCs were harvested and processed for different experiments. In apoptosis experiments, after 4 days of co-cultures, AML cells were harvested and labeled with Annexin-V/Propidium Iodide (PI) (Annexin-V-FLUOS-kit, Roche). Briefly, cells were washed in PBS and then incubated with Annexin-V-FLUOS/PI incubation buffer with light protection at room temperature for 15 min and analyzed by flow cytometry as described above.

In proliferation experiments, before seeding, AML cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE) (BioLegend). Briefly, cells were washed twice in PBS and incubated with CFSE (5 μM) with light protection at room temperature, for 4 min. Cells were then washed twice in cold medium (RPMI, Lonza) and analyzed by flow cytometry, as described above.

To assess IDO1 expression after 4 days of co-culture, MSCs were harvested and processed to collect RNA or analyzed by flow cytometry after intracellular staining, as described above.

For IFN-γ neutralization experiments, co-cultures were assessed as described and when requested concomitant to AML cell seeding, an anti-human neutralizing antibody direct against IFN-γ (20 μg/ml, BD Pharmingen) or against IFN-γ receptor (CD119, 20 μg/ml, BD Pharmingen) was added on top of MSC layer. After 6 hours, MSCs were collected and processed for total RNA extraction.

1.12. *In vitro* Treg induction

MSCs derived from HDs, AML or MDS patients were seeded and 24 h later were co-cultured for 7 days in RPMI with allogeneic peripheral blood mononuclear cells (PBMCs) (ratio 10:1). After 7 days, PBMCs were harvested and stained using the intracellular staining kit FOXP-3/Transcription Factor Buffer Set (eBioscience, Thermo Fisher Scientific) and with the following monoclonal antibodies (mAbs) PE anti-human FOXP3 (clone PCH101; Thermo Fisher Scientific), APC-H7 CD3 (clone SK7; BD/Pharmingen), FITC anti-CD4 (clone RPA-T4; Thermo Fisher Scientific) and

APC anti-CD25 (clone BC96; eBioscience, Thermo Fisher Scientific). For each sample, isotype-matched irrelevant mAbs staining was used as the control. At least 10,000 events were analyzed by flow cytometry as described above. FoxP3+/CD4+/CD25+ cells were gated on CD4+ cells.

To assess if AML cells influence IDO1-mediated MSC immunosuppression, the same experimental setting was performed with MSCs derived from HDs, AML or MDS patients, pre-cultured for 4 days with AML cells, as described above. When requested, the IDO1 inhibitor Epacadostat, INCB24360 (Incyte Corporation), at the concentration of 50μM was added in the wells with PBMCs, on the top of MSC layers.

1.13. Sample preparation for gene expression profiling (GEP) and GEP analysis

According to manufacturer's protocol, RNA was extracted by column purification (RNA Mini Kit and QIAcube, Qiagen) from BM-derived cells isolated from 61 AML patients (>=80% of circulating AML cells) and from 8 healthy donors. Labeled cDNA was prepared and hybridized to GeneChip Human Transcriptome Array 2.0 (Affymetrix) following the manufacturer's recommendations. Raw data were processed by Expression Console software with Signal Space Transformation Robust Multi-Array average (sst-RMA) normalization.

1.14. Data analysis

Data are presented as mean ± SEM of at least 3 independent determinations. Statistical differences between groups were determined by student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. All analyses were performed using GraphPad Prism software (version 6.0). Differences were considered statistically significant at $p < 0.05$.

Kaplan Mayer model with log-rank two side test for significance was performed to study the impact on survival of different IFN-y levels identified in patients compared to the mean IFN-y expression in HDs. Optimal COX regression model with age (ordinal), AML cytogenetic and molecular risk at diagnosis[328], palliative/non-intensive/intensive induction chemotherapy, de novo/secondary/therapy related AML, and INF-y levels was created with backward conditional method of exclusion of non-significant putative risk factors. Calculations were performed with SPSS v.23.

2.1. Patients and healthy controls

Human BM aspirates were obtained at diagnosis from 17 low-risk MDS patients (median age 70.3), among those 8 showed DNMT3A mutations (median age 67.9) and 10 showed ASXL1 mutations (median age 73.2). HD marrows, obtained from donors for allogeneic transplantation, were used as the controls (median age 46.9) (see table 14). Samples were collected with informed consent, in accordance with the Declaration of Helsinki.

2.2. Mice

All the murine models belong to a C57BL/6 genetic background. Animals were maintained in specific pathogen-free conditions in the Experimental Animal Center of Erasmus MC (EDC).

BM cells were kindly provided by Dr. Siddhartha Mukherjee and collaborators (Columbia University Medical Center, New York, USA). They backcrossed a Dnmt3a$^{f/f}$ conditional knock-out line, carrying a *loxP*-flanked (floxed) copy of the gene Dnmt3a^{329} to C57BL/6 mice and then crossed to Rosa26-CreER^{T2}-deletor line. To induce the excision of the *Dnmt3a* gene in Dnmt3a$^{f/f}$:Rosa26-CreER^{T2+} animals, mice and their controls aged 4-5 months received food with tamoxifen.

BM cells isolated from mice beta-actin-DsRed;Rosa-CreERT2;Dnmt3a^{f}/^{f}, hereafter referred to *Dnmt3a* KO cells were used for transplantation experiments. *Dnmt3a* KO cells were injected competitively with wild-type cells isolated from an SJL mouse (ratio 1:5) into the tail of C57BL/6 mice, lethally irradiated (8.5 cGy). The control of this experiments has been performed injecting fetal liver cells from a C/EBPα-Cre-YFP^{+} CD45.2 mouse into C57BL/6 mice, lethally irradiated (8.5 cGy). After 6 months for the *Dnmt3a* KO recipient and after 8 months for the controls, mice were sacrificed by cervical dislocation

A conditional allele, *loxP*-flanked, targeting the gene *Asxl1* has been also generated. *Asxl1$^{f/f}$* mice were crossed with Mx1-Cre-deletor line. To induce the excision of the gene, Mx1-Cre:Asxl1$^{f/f}$ mice and control mice, Mx1-Cre:Asxl1$^{+/+}$ were injected 5 times every other day with Polyinosinic:polycytidylic acid, poli(I:C), at a dose of 20 mg/kg. BM cells isolated from Mx1-Cre:Asxl1$^{f/f}$ mice, hereafter referred to *Asxl1* KO cells, and control BM cells isolated from Mx1-Cre:Asxl1$^{+/+}$, kindly provided by Dr. Omar Abdel-Wahab and collaborators (Memorial Sloan Kettering Cancer Center, New York, USA) were used for transplantation experiments. 1x10^{6} BM cells were injected into the tail of lethally irradiated (10.5 cGy) CD45.1 recipients (B6/SJL). After 10 weeks the *Asxl1* KO transplanted mice and their controls were sacrificed by cervical dislocation.

Animal studies were approved by the Animal Welfare/Ethics Committee of the EDC in accordance with the legislation in the Netherlands.

2.3. Mice sample collection

Peripheral blood (PB), BM and spleen of the mice were collected.

PB was obtained by submandibular puncture into microhematocrit capillary tubes (Becton Dickinson, BD, New Jersey, USA) with EDTA. Automated PB counts were obtained using a Scil Vet ABP Plus (Horiba, Kyoto, Japan) according to the manufacturer's instruction.

Spine, tibiae, femurs were isolated and excess soft tissue was removed. With the use of pestle and mortar, the bones were crushed and washed few times in PBS with 0.5% FBS (FACS Buffer) and passed through a 40-μm filter into a collection tube. For BM cell collection, cells were spun down and then red blood lysis was performed in a minimal volume of ACK lysing buffer (Cambrex) for 4 minutes on ice and washed once with FACS Buffer. For bone fraction (BF) cells collection, the bones left in the 40-μm filter were incubated with collagenase for 45 minutes at 37°C, while vortexing every 15 minutes. After incubation bones were rinsed in FACS Buffer and passed again through a 40-μm filter. BF cells collected were washed in FACS Buffer and spun down.

The spleen was removed, crushed, passed through a 40-μm filter, washed in FACS Buffer and spun down. Cells were then processed for red blood cell lysis in ACK lysing buffer (Cambrex) for 4 minutes on ice and then washed once with FACS Buffer.

2.4. Flow cytometry

All the stainings were performed in FACS Buffer for 20 minutes on ice, in the dark.

To identify LSK and define chimerism, BM cells were first stained with a cocktail of biotin-labeled antibodies anti-murine lineage (Lin) markers: CD8a, CD3, Gr-1, B220, Mac-1, Ter119, CD4. After incubation cells were washed and then incubated with Pacific Orange-conjugated streptavidin (Life Technologies) and the following antibodies: Pacific Blue (PB) anti-Sca-1, APC anti-c-Kit, PE anti-CD45.1 and APC-Cy7 anti-CD45.2 (all mouse antibodies and from Biolegend). Dead cells were gated out with 7AAD staining. For OP9 sorting, after co-cultures the samples were stained as described above for LSK. OP9 were sorted directly in Trizol (Sigma).

To distinguish hematopoietic stem and progenitor cells (HSPCs), cells were stained with the antibodies described for LSK staining with the addition of mouse PE-Cy7 anti-CD150 and Alexa Fluor (AF) 700 anti-CD48.

To identify differentiated cells, including Granulocytes, Monocytes, and B-cells, peripheral blood (PB), BM cells and cells isolated from the spleen were stained with the following murine antibodies: APC anti-Gr-1, PE-Cy-7 anti-Mac-1, PB anti-B220, PE anti-CD45.1 and APC-Cy7 anti-CD45.2. Dead cells were excluded based on 7AAD staining. All the antibodies were purchased from BD Bioscience. For PB cell analysis, after staining, red blood cells were lysed with an IO-test 3 lysing solution (Beckman Coulter) for 10 minutes at room temperature in the dark.

To analyze stromal cells, BM cells were incubated with the following mouse antibodies: APC-Cy7 anti-CD45.1 and anti CD45.2, PO anti-Ter119, PE Texas Red anti-CD31, PB anti-Sca-1, PE anti-CD51.

For all the experiments, the indicated populations were FACS purified using a FACS ARIA III cell sorter (BD Bioscience).

All FACS samples were analyzed using a BD LSR II Flow cytometer (BD Bioscience) and analyzed with Flow Jo.

2.5. Murine co-cultures

For the co-culture experiments, BM cells isolated from Rosa-CreERT2;Dnmt3a^{f}/^{f} mice and their controls Rosa-CreERT2;Dnmt3a^{+}/$^{+}$, and from Mx1-Cre:Asxl1$^{f/f}$ mice and their controls Mx-Cre:Asxl1$^{+/+}$ (kindly provided by Dr.Mukherjee and collaborators and Dr. Abdel-Wahab and collaborators, respectively) were used.

OP9 cells were seeded at 2 x 10^4 cells/cm^2 in DMEM (Gibco, Thermo Fisher Scientific) with 10% FCS, 1% P/S. The day after LSK cells, isolated as already described from the BM of mice listed above, were seeded on top of OP9 at a ratio of 1:10 respectively, in DMEM, 20 ng/ml SCF (PeproTech) and 100 ng/ml FLT3 (PeproTech). After 6 days of co-cultures, the cells were collected and OP9 were FACS purified and the processed for total RNA extraction.

2.6. Total DNA extraction, polymerase chain reaction (PCR)

To assess the deletion of the genes, DNA was extracted from BM-derived cells and samples were genotyped. Nucleic acid isolation was performed according to the manufacturers' protocol with Trizol and Gentlute LPA (Sigma).

For *Dnmt3a* genotyping, after DNA extraction, a PCR was performed with Q5 polymerase kit and the following thermal cycling conditions: 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 66°C for 20 s, and 72°C for 20 s, and then 72°C for 2 min. The WT allele was detected as a band of 200 bp while the excision of the gene was detected as a band of 420 bp. The floxed allele, i.e. with

2 LoxP site flanking the gene, still present whem the recombination has an efficiency lower than 100%, was detected as a band of 330 bp. For *Asxl1* genotyping, after DNA extraction, a PCR was performed with Taq polymerase kit and the following thermal cycling parameters: 95°C for 4 min, followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and then 72°C for 5 min. The primer pair F3/R3 identified the WT allele as a band at 200 bp, and the genetically modified floxed allele, still present if the recombination has an efficiency lower than 100%, as a band of 380 bp. Excision after recombination was assessed with the primer pair RecF3/RecR3, which detects a band of 700 bp. The PCRs were performed in a thermal cycler machine Veriti (Applied Byosistem), and the primers (Thermo Fisher Scientific) listed above were used (Tab.3).

Table 3. PCR primers.

Dnmt3a 5'-3'		*Asxl1 5'-3'*	
D3a1Lox6	GATTGCTACAGGTGAGTGGCATG	F3	CAGCCGTTTTACCACAGTTT
D3a2Lox5	GGTCTTCAGGTTATTCCGGTG	R3	AGGGAAAGGGACAGAATGAC
D3aCommon2	GAAGGTTACCCCAGAAGTAACG	RecF	ACGCCGGCTTAAGTGTACACG
		RecR	GACTAAGTTGCCGTGGGTGCT

2.7. Total RNA extraction, RNA sequencing (RNA seq) and Analysis

RNA isolation was performed with Trizol and Gentlute LPA (Sigma), following the manufacturer's instructions. Similar amounts of RNA, measured on the Bio-Analyzer using an RNA 6000 Pico Kit (Agilent Technologies), were used for cDNA synthesis which has been performed using a SMART-seq v4 Ultra Low RNA Kit (Clontech Laboratories) for Illumina sequencing. Amplified cDNA was then processed to create a library according to TruSeq Nano DNA Library Prep kit, in which different adapters were ligated to DNA fragments to distinguish the different samples. For sequencing, the library was denaturated using the cBot 2 System (Illumina) and the samples were then sequenced using a HiSeq machine (Illumina).

Quality of RNA-seq data was assessed with different parameters, including the number of aligned bases, base composition, coverage coefficient, and full-length transcript coverage.

Demultiplexing was performed using the CASAVA software (Illumina) and the adaptor sequences were trimmed with Cutadapt (*http://code.google.com/p/cutadapt/*). Human transcripts were aligned to the gencode transcriptome (hg19) using the splice aware aligner STAR. The mouse samples were aligned to the mouse gencode transcriptome (mm10) using the same aligner. Subsequently, abundance estimation was performed using cufflinks. The resulting measurements are expressed in

FPKM (fragments per kilobase of exon per million fragments mapped). The raw unnormalized counts were measured using HTSeq-count using the recommended union mode. Differential expression analysis was performed using the Deseq2 package in the R environment. The R environment was also used to perform principle component analysis and hierarchical clustering. Finally, gene set enrichment analysis (GSEA) was performed based upon the FPKM values of the samples. The databases used for GSEA are the curated gene sets (C2) and the gene ontology gene sets (C5) both from the GSEA database. The Signal2Noise performance metric was used in combination with 10000 gene-set based or if applicable phenotype based permutations. Gene sets were considered differentially expressed between KO transplanted mice and control mice when the associated value of False Discovery rate (FDR) was <0.25.

RESULTS

RESULTS

Although some studies have already reported genetic and functional alterations of MSCs isolated from MDS (MDS-MSCs) and AML patients (AML-MSCs)[289,290,295], the functional meanings of these alterations are still under debate and conflicting results have been published. It is still unclear whether MSC abnormalities, identified by some authors, contribute directly to the onset and development of malignant clone or whether the defects are the consequence of tumor expansion in the bone marrow microenvironment. Furthermore, some authors reported the ability of malignant clone to actively shape their niche, in order to favor their expansion at the expense of normal hematopoiesis.

To further elucidate these mechanisms, we decided to investigate:

1) intrinsic alterations of MSCs which could be directly responsible for malignant cells expansion and progression;
2) alterations of hematopoietic cells that could induce changes in stromal cells, including MSCs, able to create a more favorable microenvironment for tumor progression.

1 INTRINSIC ALTERATIONS OF MSCs

In the last years, the contribution of BM stromal cells to the pathogenesis of hematological malignancies gained more attention. However, contradictory data have been published about the characterization of MSCs isolated from AML and MDS patients. We investigated the biological and functional properties of MSCs isolated from AML and MDS patients, to elucidate the presence of alterations, which could in turn influence tumor cell behavior.

1.1 MDS- and AML-MSCs can be isolated but they show aberrant proliferation ability

After density-gradient centrifugation, we obtained mononuclear cells (MNCs) from bone marrows of MDS (n=25) and AML patients (n=31) at diagnosis and from BM of HDs (n=10) as controls. For MSC isolation, MNCs were seeded at high density and MSCs were obtained because of their ability to adhere to the plastic of the flask (Fig.). However, we identified some differences between HDs

derived MSCs (HD-MSCs), MDS patients derived MSCs (MDS-MSCs) and AML patients derived MSCs (AML-MSCs).

Table 4. The biological, cytogenetic and molecular characteristics of HDs, MDS and AML patients

patient ID	cytogenetics	Molecular	Gender	age (years)	Risk	MSCs/MNCs	cytogenetic alteration in MSCs
AML01	46,XX,t(6;9) (p22;q34)[20][1]	NPMwt FLT3-ITD	F	45	high[2]	0.97	ND
AML02	46,XY[20]	ND	M	80	int	ND	ND
AML03	46,XY[20]	NPMmut FLT3wt	M	63	low	1.5	ND
AML04	46,XY,inv(16) (p13q22)[20]	NPMwt FLT3wt TP53wt	M	55	low	0.1	negative
AML05	46,XX[20]	NPMmut. FLT3wt TP53wt	F	63	low	ND	ND
AML06[3]	*ND*	*NPMmut FLT3-ITD TP53wt*	*M*	*70*	*int*	*ND*	*ND*
AML07	46,XY,t(15;17) (q22;q22)[20]	ND	M	39	na	0.2	negative
AML08	46,XX[20]	NPMmut FLT3-ITD/TKD	F	47	int	1.3	ND
AML09	46,XX[20]	NPMmut FLT3-ITD	F	61	int	0.7	ND
AML10	*47,XY,+8[20]*	*NPMwt FLT3wt*	*M*	*68*	*int*	*ND*	*ND*
AML11	*46,XY[20]*	*NPMmut FLT3-ITD TP53wt*	*M*	*73*	*int*	*ND*	*ND*
AML12	47,XY,+der(3) del(3)(p11), t(10;11;19) (p12;q23;q13) [20]	NPMwt FLT3wt	M	17	high	ND	negative
AML13	44,XX,+der(3) t(3;20)(p12;p11),del(5)(q13q33), -7,-13,t(13;20) (q12;p11), -17,der(21) t(17;21) (q11;q22), +mar[14]/ 45,XX,t(1;16) (q12;q11),del(5) (q13q33),del(6) (q21q25), -7,add(22)(q13)[6]	NPMwt FLT3wt TP53mut	F	60	high	0.3	ND
AML14	46,XX,t(16;16) (p13;q22)[20]	NPMwt. FLT3wt TP53wt	F	46	low	ND	ND
AML15	46,XY,t(6;11) (q27;q23)[20]	NPMwt FLT3wt TP53wt	M	19	high	5.6	ND

AML16	46,XY,inv(16)(p13q22)[18]/47,XY,inv(16)(p13q22),+22[2]	NPMwt FLT3wt TP53wt	M	26	low	ND	ND
AML17	46,XX,t(9;11)(p22;q23)[20]	NPMwt FLT3wt	F	55	int	0.1	ND
AML18	*47,XX,+X, add(7)(q34)[19]/ 47,XX,+X[1]*	*ND*	*F*	*72*	*int*	*ND*	*ND*
AML19	46,XX[20]	FLT3-ITD	F	61	high	6.9	ND
AML20	46,XX[20]	FLT3-ITD	F	73	high	0.5	ND
AML21	*47,XX,del(5)(q22q33),+8[20]*	*ND*	*F*	*62*	*high*	*ND*	*ND*
AML22	46,XY[20]	FLT3-ITD	M	79	high	6.4	ND
AML23	46,XX[20]	NPMmut FLT3-TKD	F	76	low	5.6	ND
AML24	46,XX,der(4)t(1;4)(q32;q31)[20]	NPMwt FLT3wt TP53wt	F	55	int	3.7	ND
AML25	*46,XY[20]*	*NPMwt FLT3-ITD TP53wt*	*M*	*31*	*int*	*ND*	*ND*
AML26	ND	NPMwt FLT3wt TP53wt	M	57	NA	0.5	ND
AML27	*ND*	*NPMmut FLT3wt TP53wt*	*M*	*59*	*low*	*ND*	*ND*
AML28	46,XY,-7,+21[20]	NPMwt FLT3wt TP53wt	M	74	high	ND	ND
AML29	46,XX[20]	NPMwt FLT3wt TP53wt	M	69	int	ND	ND
AML30	47,XY,inv(16)(p13q22),+8[14]/46,XY,inv(16)(p13q2)[6]	NPMwt FLT3wt TP53wt	M	56	low	ND	ND
AML31	46,XY,t(6;9)(p22;q34)[20]	NPMwt FLT3-ITD	M	75	int	3.2	ND
AML32	*ND*	*ND*	*F*	*71*	*ND*	*ND*	*ND*
MDS1	46,XX[20]		F	79	low/int[4]	0.8	ND
MDS3	46,XY[20]		M	62	int2/ high	ND	ND
MDS4	46,XY[20]		M	78	int1/ low	0.5	ND
MDS5	46,XY[20]		M	86	low/ low	3.3	ND
MDS6	46,XX[20]		F	61	high/ high	2.7	ND
MDS9	46,XY[20]		M	79	low/ low	1.2	ND
MDS11	*46,XY,del(20)(q11q13)[20]*		*M*	*53*	*int1/int*	*ND*	*ND*

MDS12	71-74,XXX, +der(2)t(2;17) (p11;p12), del(5)(q13q33), +der(5)del(5) (q13q33), -7[5]/46,XX[5]		F	73	high/ high	6.7	negative
MDS15	46,XX,del(5) (q13q33)[5]/ 46,XX, del(5)(q13q33), del(11)(q21q25) [3]		F	76	int1/ low	5.6	ND
MDS16	46,XY[20]		M	80	low/ low	2.6	ND
MDS17	46,XY[20]		M	68	high/ very high	6	ND
MDS18	46,XY[20]		M	66	low/ low	8.3	ND
MDS21	46,XY[20]		M	78	low/ low	ND	ND
MDS22	46,XY[30][5]		M	61	low/ low	4.4	ND
MDS24	46,XX[20]		F	83	low/ low	2.5	ND
MDS25	*46,XX,del(20) (q11q13)[20]*		*F*	*81*	*low/ low*	*ND*	*ND*
MDS26	46,XY[20]		M	54	low/ low	8.9	ND
MDS32	46,XX[20]		F	67	low/ low	2.5	ND
MDS33	ND		M	76	int1/ high	3.6	ND
MDS34	46,XY[20]		M	90	int1 /low	3.5	ND
MDS35	46,XX[20]		F	83	low/ low	2.5	ND
MDS36	47,XY,+8[20]		M	82	int2/ very high	1.8	ND
MDS38	46,X,idic(X) (q13)[16]/45,X, -X[2]/47,X, del(X)(q13), +idic(X)(q13) [1]/47,del(X) (q13),idic(X) (q13)+idic(X) (q13)[1]		F	86	int1/ low	1.1	ND
MDS39	46,XY[20]		M	87	int1/int	*ND*	*ND*

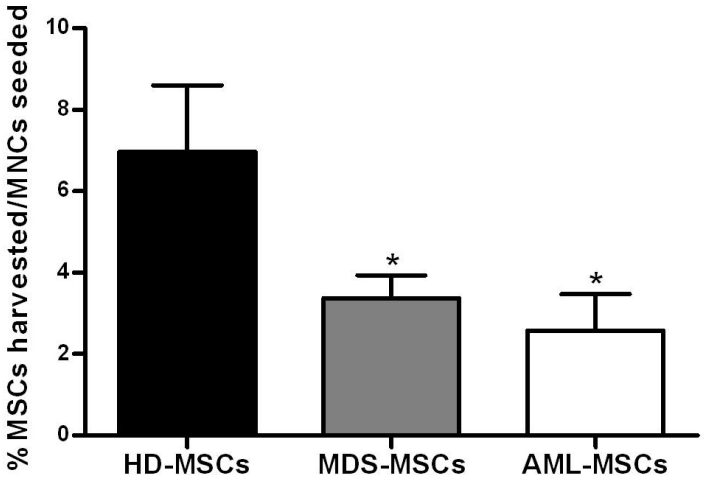
MDS43	49,XY,+1,del(5)(q13q33),+der(5)del(5)(q13q33),+11[18]/47XY,del(5)(q13q33),+der(5)del(5)(q13q33)[1]/46XY,del(5)(q13q33)[1]		M	79	int2/high	ND	negative
MDS45	46,XY,del(13)(q12q14)[3]/46,XY[17]		M	65	high/very high	ND	negative
HD01	46,XY	/	M	22	/	ND	ND
HD02	46,XY	/	M	19	/	ND	ND
HD03	46,XY	/	M	43	/	8.4	ND
HD04	46,XY	/	M	44	/	2.2	ND
HD05	46,XY	/	M	32	/	5.4	ND
HD06	46,XY	/	M	41	/	2	ND
HD07	46,XX	/	F	62	/	0.5	ND
HD08	46,XY	/	M	60	/	14.3	ND
HD09	46,XX	/	F	43	/	9.4	ND
HD10	46,XX	/	F	52	/	7	ND
HD11	46,XY	/	M	22	/	11.2	ND
HD12	46,XY	/	M	38	/	ND	ND

ND, not determined

[1] karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2016)[330,331];
[2] risk evaluation following ELN2017[328];
[3]Patients in which MSCs were not isolated are indicated in grey and italic font;
[4] risk evaluation following IPSS/IPSS-R [13,332];
[5] FISH 8: positive in 4% of interphase nuclei.

First, the isolation of MSCs from patients was found to be more difficult. Indeed, we successfully isolated MSCs from all the HDs, whereas we obtained MSCs from 84% of MDS patients and from only 75% of AML patients (Tab.4). In addition, we isolated fewer MSCs from patients compared to HDs. Indeed, by normalizing the number of MSCs isolated after the first passage (P1) for the number of (BM-isolated) MNC seeded, we showed that the frequency of rescued MSCs, was significantly lower in the AML group than in the HDs (**$p<0.001$), whereas for the MDS group the frequency had an intermediate value (*$p<0.05$ vs HD; not significant vs AML) (Fig.7a). These results suggested a lower percentage of MSC precursors in MDS and AML bone marrows.

A



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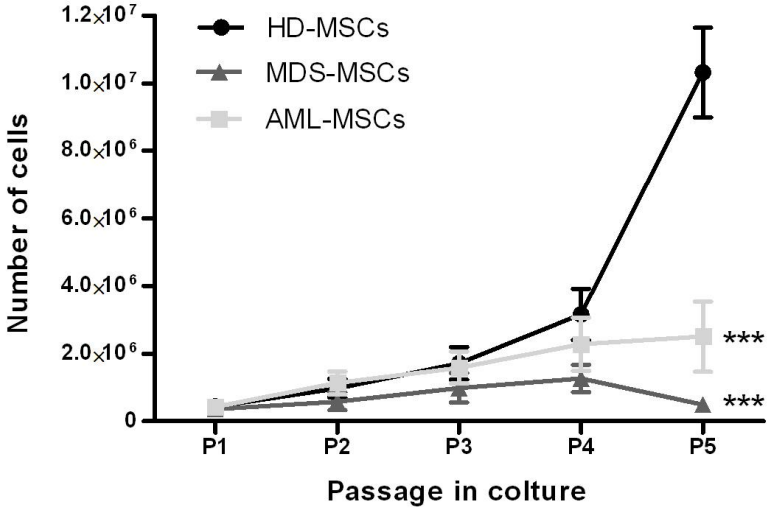


Figure 7. The frequency of rescued MSCs and their growth ability in culture. A) Percentage of MSCs, isolated at passage 1, normalized for the number of BM-derived mononuclear (MNCs) cells seeded. Results are expressed as mean ± SEM of independent samples of HDs (n=7), MDSs (n=19), AMLs (n=16) (*, $p<0.05$;**, $p<0.01$, vs HD-MSCs). **B)** Direct counts of the living cells in BM-derived MSC cultures, obtained from healthy donors (HD-MSCs), MDS patients (MDS-MSCs) and AML patients (AML-MSCs) at each passage (P). Results are expressed as mean ± SEM of at least 7 independent samples (***, $p<0.001$ vs HD-MSCs).

Second, we found that expansion dynamics were very different among the 3 groups. At 80% of confluency, MSCs, plated and cultured with the same conditions, were detached and counted excluding dead cells with erythrosine. As shown in Figure 7b, HD-MSCs had an exponential

growth while AML-MSCs and in particular MDS-MSCs did not show it. Indeed, cell number increased at the first passages and then remained almost constant.

Despite those difficulties, the isolated MSCs showed a typical fibroblastoid elongated shape (Fig.8).

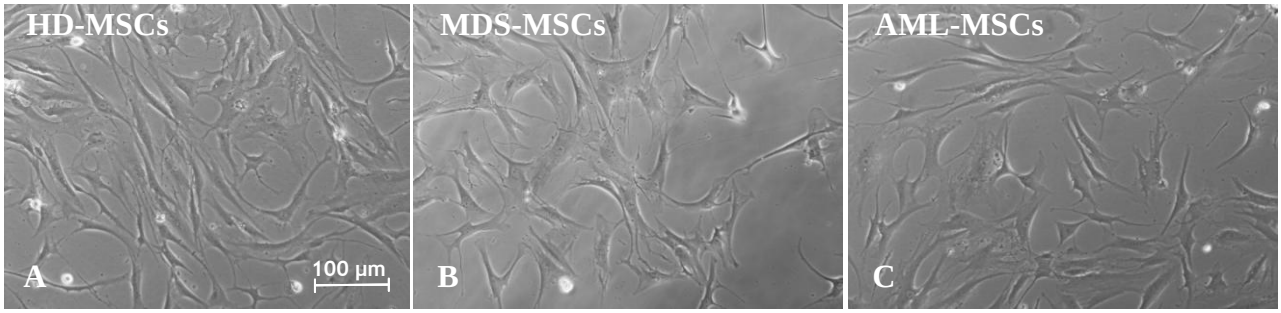


Figure 8. MSCs show normal fibroblastoid elongated shape. Examples of the exponential growth in culture of HD-MSCs (A), MDS-MSCs (B) and AML-MSCs (C). Images were obtained with an inverted microscope. Magnification: 10X; scale bar: 100 µm.

In addition, we found that the time required to reach 80% of confluency was significantly higher for MSCs isolated from patients, in particular from AML patients. Indeed, AML-MSCs needed 4 days more (10,6 ± 4,7) compared to HD-MSCs (6,8 ± 1,3), as shown in the Table 5. This result confirmed the defect of growth of MSCs isolated from patients.

Table 5. MSCs isolated from MDS and AML patients require more days to reach 80% of confluency in culture.

HD-MSCs p1→ HD-MSCs p1+1	
Sample	*Average of days in culture*
HD58	6 ± 0,8
HD65	6 ± 2,5
HD66	7 ± 1,2
HD71	5 ± 1
HD79	3,8 ± 1,4
HD82	6,2 ± 0
HD88	7,5 ±0,6
HD89	7,5 ± 0,7
Average ± SEM	**6,8± 1,3**

MDS-MSCs p1→ MDS-MSCs p1+1	
Sample	*Average of days in culture*
MDS5	9,2 ± 3
MDS6	6,6 ± 1,5
MDS12	10,3 ± 0,6
MDS17	8,5 ± 0,7
MDS18	10,5 ± 3,5
MDS26	8 ± 1,7
Average ± SEM	8,9**** ± 1,8**[1]
AML-MSCs p1→ AML-MSCs p1+1	
Sample	*Average of days in culture*
AML1	6 ± 1
AML2	9,7 ± 6
AML3	8,75 ± 2,2
AML4	4,5± 1,3
AML7	7,75 ± 1,5
AML8	12,6 ± 3,8
AML13	8 ± 6,7
AML19	17±5,6
AML22	19±8,5
AML23	13±7
Media tot ± SEM	**10,6* ± 4,7**

[1] p<0.05;**, p<0.01 vs HD-MSCs

1.2 MDS- and AML-MSCs show a normal immunophenotype

Since the lack of a unique marker for MSC identification, the International Society for Cellular Therapy (ISCT) proposed the expression of specific surface markers and the lack of hematopoietic markers as one of the criteria to identify the MSC population[141]. Indeed, the cells have to be positive or negative for a combination of markers which can be expressed individually also in other cell types.

Therefore, we analyzed by flow cytometry the immunophenotype of MSCs isolated from MDS/AML patients, in order to verify if they still have an immunophenotype comparable to HD-MSCs.

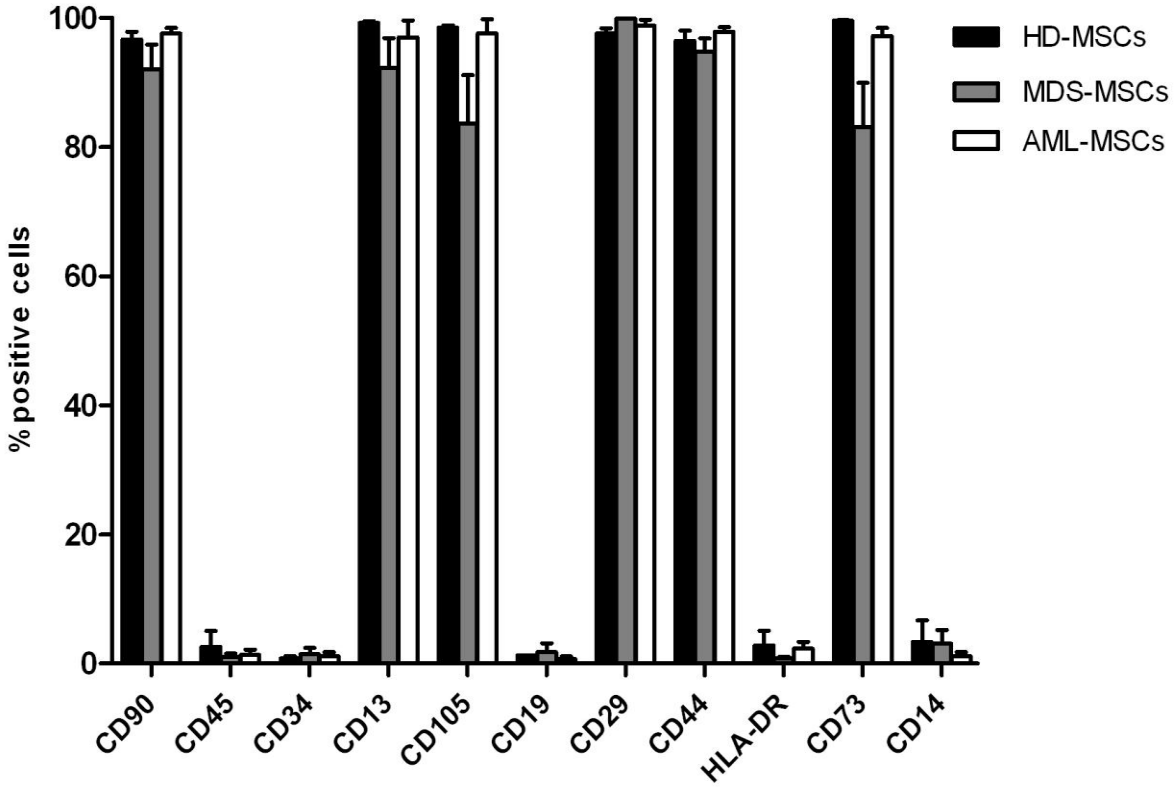


Figure 9. MSCs isolated from MDS and AML patients display typical surface markers. The histograms represent the percentage of MSCs positive for the indicated markers, evaluated by flow cytometry. Mean ± SEM of HD-MSCs (n=9), MDS-MSCs (n=6) and AML-MSCs (n=4).

As shown in Figure 9, MDS and AML-MSCs, in a comparable way to HD-MSCs, expressed typical MSC markers and did not express hematopoietic markers. Indeed, the cells were positive for CD90, CD73, CD105, CD13, CD29, CD44 and negative for CD45, CD34, CD19, HLA-DR, and CD14. However, MDS-MSCs showed variable expression of the markers CD105 and CD73, but the differences were not statistically significant (Fig.9).

1.3 MDS- and AML-MSCs have a normal differentiation ability

Another criteria proposed by ISCT to identify MSCs is represented by their ability to differentiate versus adipogenic, cartilaginous and osteogenic lineage *in vitro*[141].

Controversial results about the differentiation ability of MSCs isolated from patients with hematological disorders have been published[333,334]. In order to assess the differentiation ability of MSCs isolated from MDS and AML patients, we induced the cells to differentiate towards adipogenic and osteogenic lineage. After 3 weeks of culture, the differentiation was assessed by: 1) differentiation-specific histological staining, 2) evaluating the expression levels of differentiation master genes.

The osteogenic-specific differentiation was evaluated by Alizarin Red S histological staining. This organic compound is a red dye, which highlights the presence of extracellular calcific deposition, typical of osteogenic cells. As shown in Figure 10, we observed Alizarin Red positive staining in osteogenic-differentiated HD/MDS/AML- MSC cultures, without differences among the 3 groups. The positive staining was not present in undifferentiated cultures. We then confirmed the results by evaluating the expression of *RUNT-RELATED TRANSCRIPTION FACTOR 2* (*RUNX2*) by quantitative real-time-PCR (qRT-PCR). As expected, we found that differentiated cells express similar levels of *RUNX2* mRNA, without differences among the groups.

The adipogenic differentiation was evaluated by Oil Red O histological staining. The substance is a fat-soluble dye, which stains lipids. In fact, the lipidic vacuoles of adipocytes appear red after the staining with Oil Red O. As shown in Figure 10, adipogenic-differentiated HD/MDS/AML-MSCs displayed a similar Oil Red O staining. On the contrary, undifferentiated cultures were negative for the staining. The evaluation of *PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA* (*PPAR-γ)* expression by qRT-PCR confirmed the results, and no significant differences in induction levels were detected between HD, AML and MDS groups. We concluded that MDS-MSCs and AML-MSCs display a normal and comparable differentiation capacity.

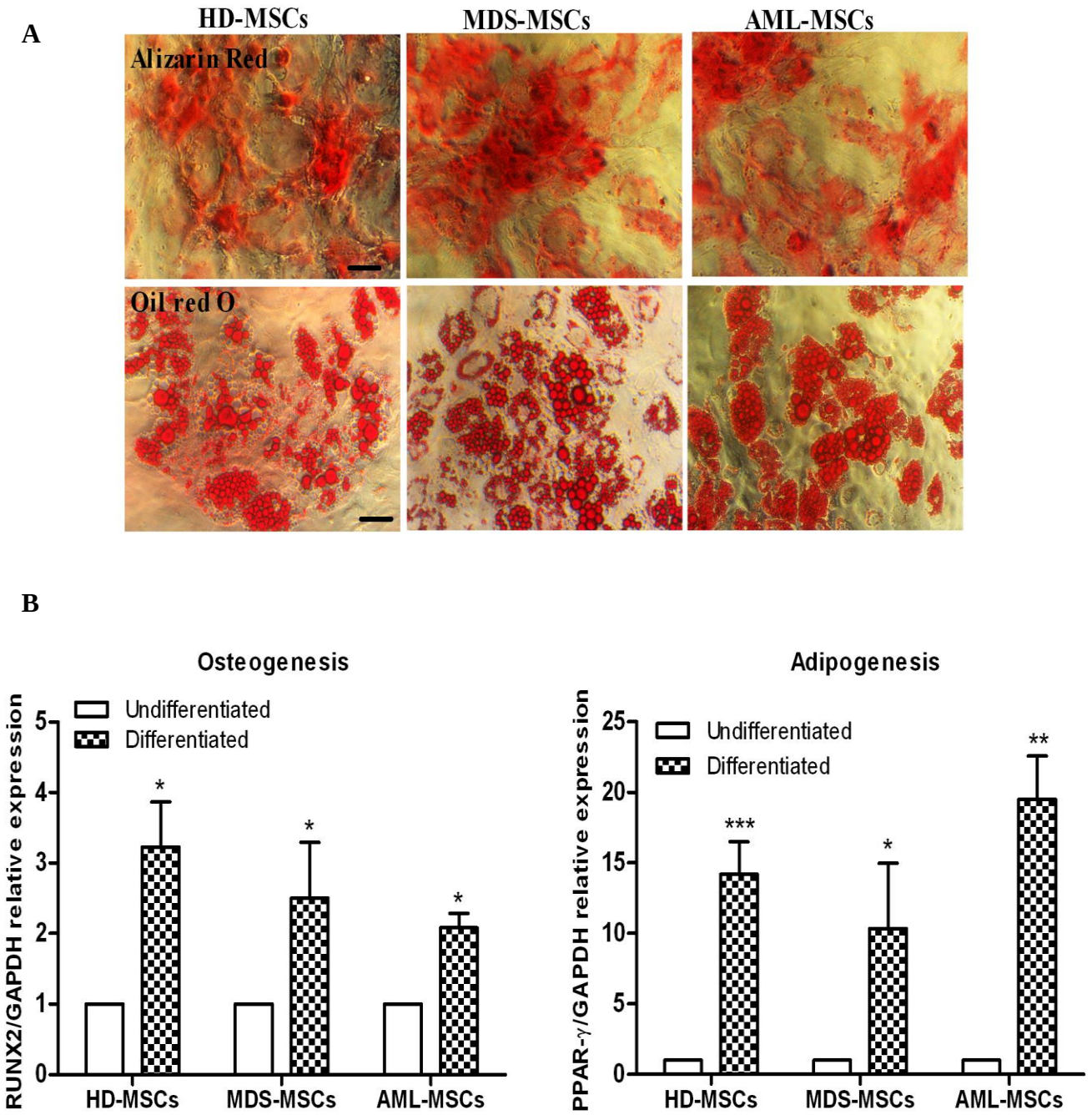


Figure 10. MSCs isolated from MDS and AML patients maintain their ability to differentiate versus adipogenic and osteogenic lineage. A) Microphotos of HD-MSCs, MDS-MSCs, AML-MSCs stained with Alizarin Red S (upper) and Oil Red O (lower) after 3 weeks of culture in osteogenic and adipogenic conditions. Magnification: 10X; scale bar: 100 μm. **B)** After 3 weeks of culture, evaluation by qRT-PCR of *RUNX2* and *PPAR-γ* expression, differentiation master genes for osteogenic and adipogenic differentiation respectively. The expression was calculated based on the ΔC_t method and the expression levels of *RUNX2* and *PPAR-γ* in undifferentiated cells were taken as 1). Results are expressed as mean ± SEM of 7 independent experiments (* $p < 0.05$, ** $p < 0.01$, ***. $p < 0.001$ vs undifferentiated cells).

1.4 MDS- and AML-MSCs do not harbor tumor-specific cytogenetic aberrations

Tumor-specific genetic alterations have been characterized in BM cells derived from hematological patients. On the contrary, the presence of molecular alterations in MSCs isolated from MDS/AML patients has not been yet fully elucidated and conflicting results have been published[293,335]. In MDS/AML-MSCs we decided to detect cytogenetic alterations, by FISH analysis, characterizing hematopoietic cells isolated from the same patients at diagnosis. As shown in table 4, most of our patients (16 out of 23 MDS and 10 out of 24 AML) did not show cytogenetic aberrations in MNCs, so they were not suitable for FISH analysis on MSCs. However, in some of MDS patients and in half of AML patients we could detect chromosomal alterations typical of myeloid malignancies (Tab.4). Interestingly, in the analyzed samples (n=3 MDS, n=3 AML), we found that neither MDS-MSCs or AML-MSCs present the same chromosomal alterations, typical of myeloid malignancies, like those detected in MNCs at diagnosis (Figure 11). Indeed, as shown in Figure 11, two example of FISH analysis of BM-derived hematopoietic cells, highlighted the presence of:

- the deletion of the *RETINOBLASTOMA 1 (RB1*) gene, while MSCs isolated from the same MDS patient were negative for the deletion;
- the inversion of the chromosome 16, inv(16), whereas MSCs isolated from the same AML patient were negative for the inversion.

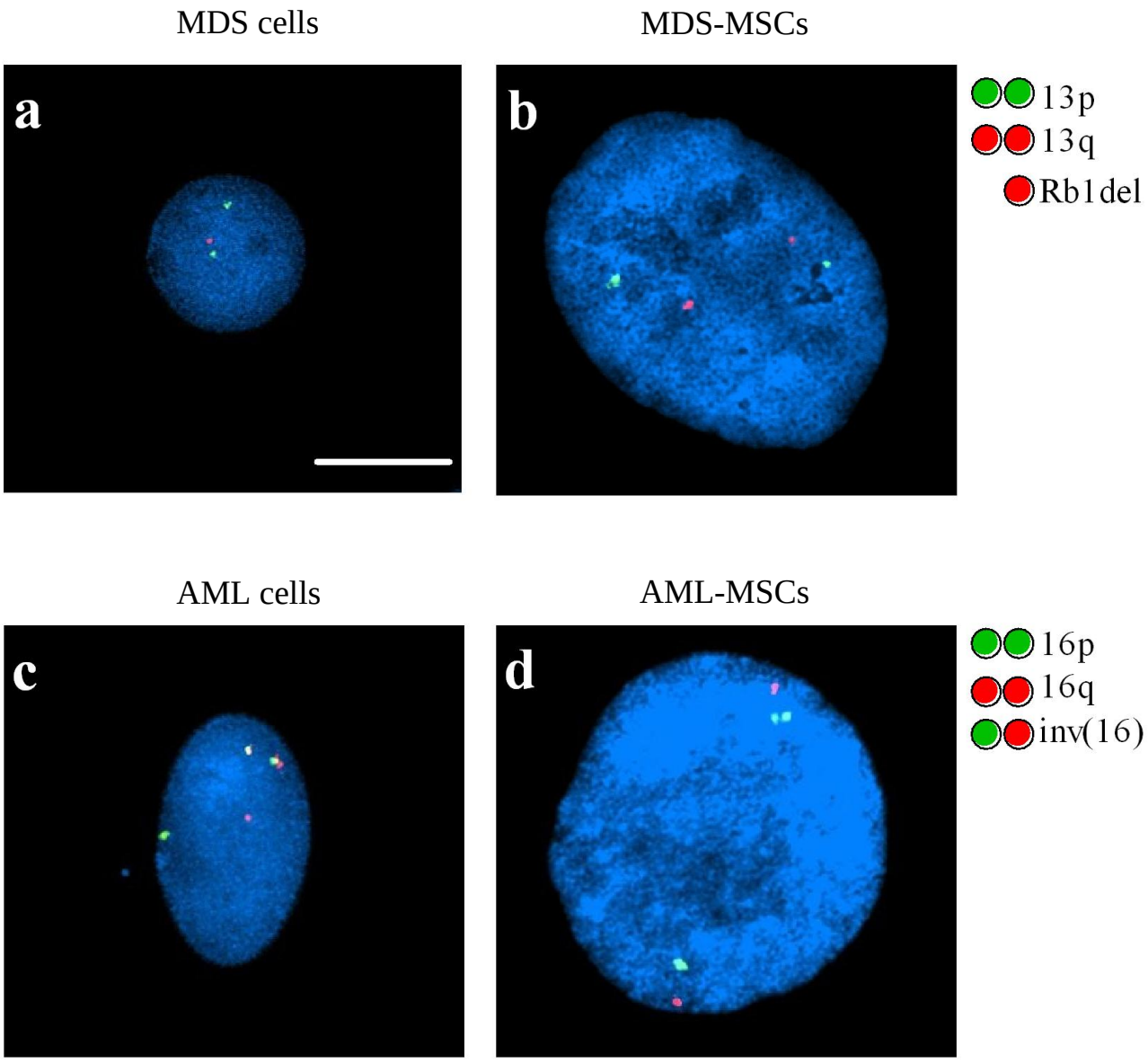


Figure 11. Cytogenetic abnormalities of BM-derived hematopoietic cells were not found in MSCs isolated from the same MDS/AML patients. Representative samples analyzed by FISH: Image **A)** MDS cell positive for the deletion of *RETINOBLASTOMA 1 (RB1*) gene on chromosome 13**; B)** MSC isolated from the same patient do not show *RB1* deletion; **C)** AML cell positive for the inversion of chromosome 16, inv(16); **D)** MSCs isolated from the same patient are negative for inv(16). Magnification (100X). Scale bar 10 μm.

1.5 MDS- and AML-MSCs support leukemic cell viability and proliferation

In the past years, several studies have proved that MSCs are able to support tumor growth. In particular, HD-MSCs promote leukemic cell survival, through cytokine production and cellular interaction[301,336].

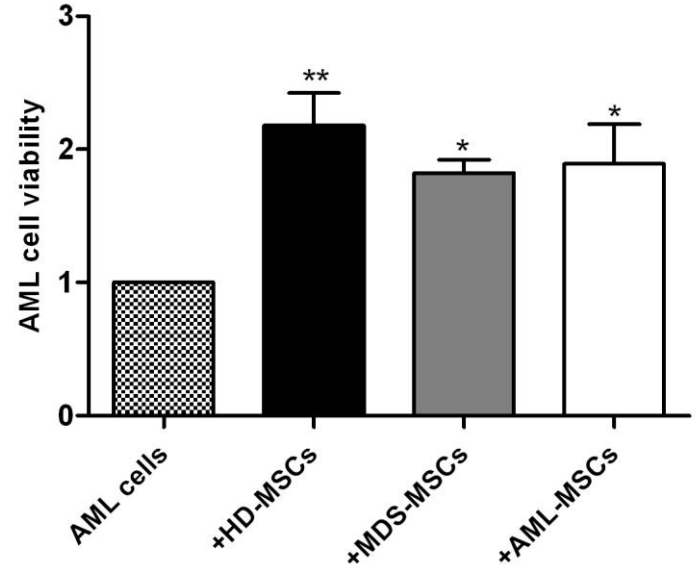
We tested whether MDS and AML-MSCs still maintain this ability by evaluating the effect that MSCs have on apoptosis and cell proliferation of malignant cells. We decided to use only AML cells, to avoid bias due to the heterogeneity of the samples.

For apoptosis assay, AML cells were seeded on the top of the HD/MDS/AML-MSC layers and co-cultured for 4 days. The apoptosis was then evaluated by flow cytometry, after Propidium iodide (PI)/Annexin V staining. The Annexin V detects the externalization of phosphatidylserine on apoptotic cells, these phospholipids are usually in the cytoplasm-facing cell membrane but they are exposed in the early stages of apoptosis. The second component, PI, is a DNA-binding dye able to enter in the cells when their membranes are not intact, characteristic of both necrosis and late apoptosis. After staining with both probes, living cells are double negative, necrotic cells are PI^+, early apoptotic cells are Annexin V^+ and late apoptotic cells are double positive for the staining[337]. In our analysis, we considered double negative living cells.

As shown in Figure 12a, MDS- and AML-MSCs retained their pro-survival effect on leukemic cells and no differences were seen between them and with HD-MSCs. Indeed, as HD-MSCs, MDS- and AML-MSCs protected AML cells from spontaneous apoptosis in culture.

We then evaluated the effect of MSCs on AML cell proliferation, detected by Carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) staining. CFSE is a non-fluorescent pro-dye and a cell permeant. Once internalized in living cells, its acetate group is cleaved resulting in the green fluorescent dye carboxyfluorescein, which is membrane impermeant. The succinimidyl ester group reacts with intracellular amines to generate covalent dye-protein conjugates[338]. When a CFSE-labeled cell divides, daughter cells receive half of the carboxyfluorescein-conjugated molecules and thus each cell division is assessed by measuring the decrease of cell fluorescence by flow cytometry. We stained AML cells with CFSE, we performed co-culture as described above and we assessed AML cell proliferation by flow cytometry. Interestingly, we found that MDS and AML-MSCs slightly but significantly favored leukemic cell proliferation without differences among the groups (Fig.12b).

A



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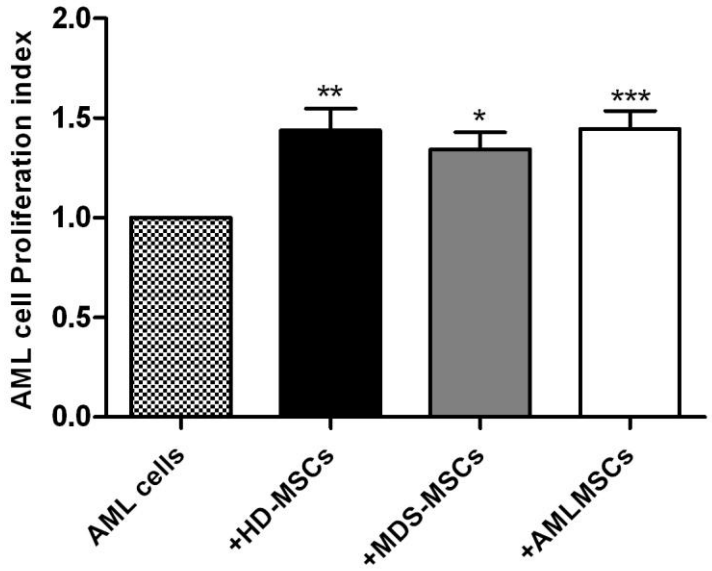


Figure 12. MSCs isolated from MDS and AML patients favor AML cell viability and proliferation. A) The histograms represent the percentage of cells double negative for Annexin/PI staining, evaluated by flow cytometry. Results are presented as Fold Induction, obtained considering the viability of AML cells cultured alone as 1 (mean ± SEM of at least 5 independent experiments). * $p < 0.05$; ** $p < 0.01$ vs AML cells alone. There are no significant differences between MSC groups. **B)** Cell proliferation of AML cells labeled with CFSE was analyzed by flow cytometry. Proliferation index is a parameter provided by the analysis software FCS Express 4 Flow. Results correspond to Fold Induction, evaluated by taking the proliferation of AML cell cultured alone, as 1. * $p < 0.05$; ** $p < 0.01$ vs AML cells alone. The differences are not significant between MSC groups.

It has been demonstrated that human MSCs, exposed to tumor microenvironment, acquire expression of tumor-associated fibroblast (TAFs) antigens, such as alpha SMOOTH MUSCLE ACTIN (α-SMA) both *in vivo* and *in vitro*. Indeed, MSCs acquire TAF phenotype after direct co-culture with tumor cells or with tumor-conditioned media, affecting tumor cell survival[339,340].

Since we found that MDS- and AML-MSCs support cell viability and proliferation, we decided to investigate the expression of α-SMA in MSCs isolated from HDs, MDS and AML patients, by flow cytometry. We found that HD-MSCs show α-SMA expression in about 70% of cells, with some variability between different samples (Min 40%, Max 99%). MDS- and AML-MSCs expressed slightly higher levels of α-SMA with less variability in all sample analyzed, compared to HD-MSCs (Fig.13a). However, the differences were not statistically significant, probably due to control sample variability. Next, we decided to investigate whether α-SMA expression, in MSCs, could be modulated by AML cells, as already demonstrated for other tumor cells[339]. To this aim, we assessed α-SMA expression in HD- and AML-MSCs, after co-culture with AML cells. Initially unanticipated, we did not identify any difference in α-SMA expression in MSCs co-cultured with or without AML cells and between HD- and AML-MSCs (Fig.13b).

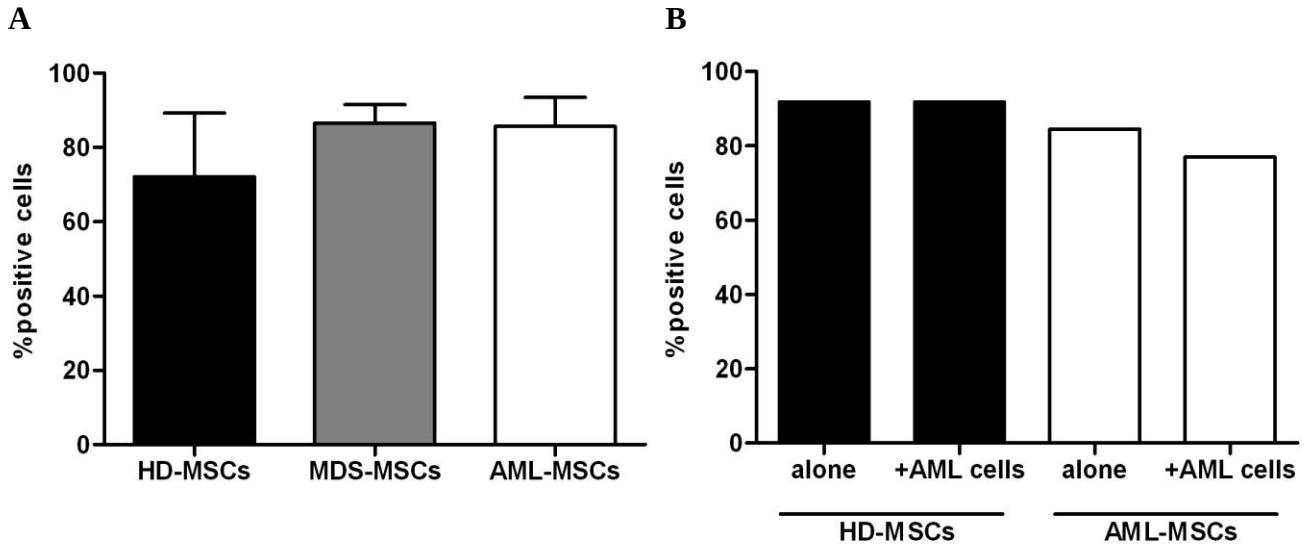


Figure 13. MSCs isolated from HDs, MDS and AML patients express comparable α-SMA levels, not modulated by AML cells. A) Percentage of HD-, MDS- and AML-MSCs positive to α-SMA, evaluated by flow cytometry. Mean ± SEM of HD-MSCs (n=3), MDS-MSCs (n=3) and AML-MSCs (n=4). **B)** Percentage of HD- and AML-MSCs, cultured alone or with AML cells, positive to α-SMA evaluated by flow cytometry (n=1).

1.6 MDS- and AML-MSCs induce Treg

MSCs have extensively been characterized for their immunosuppressive capacity acting on innate and adaptive immunity[341]. In particular, MSCs are able to induce $CD4^+CD25^+HighFOXP3^+$ regulatory T cells (Treg)[342]. This subpopulation of T cells plays a key role in the suppression of the immune response. For a functional characterization, we investigated if MSCs isolated from MDS and AML patients still maintain or have a higher/lower ability to induce Treg cells. For the experiments, we used peripheral blood mononuclear cells (PBMCs) isolated after density-gradient centrifugation from buffy coat. The buffy coat is the portion of an anticoagulated blood sample, containing most of the white blood cells and platelets. We seeded PBMCs on HD-, MDS- or AML-MSC layers and after 7 days of co-culture, the percentage of $CD3^+CD4^+CD25^+FOXP3^+$ was assessed by flow cytometry. As shown in Figure 14, despite MDS-MSCs induced slightly less Treg formation, we demonstrated that MDS and AML-MSCs retain their ability to induce Treg formation with no differences among the groups, suggesting that the cells maintaned their immunosuppressive activity.

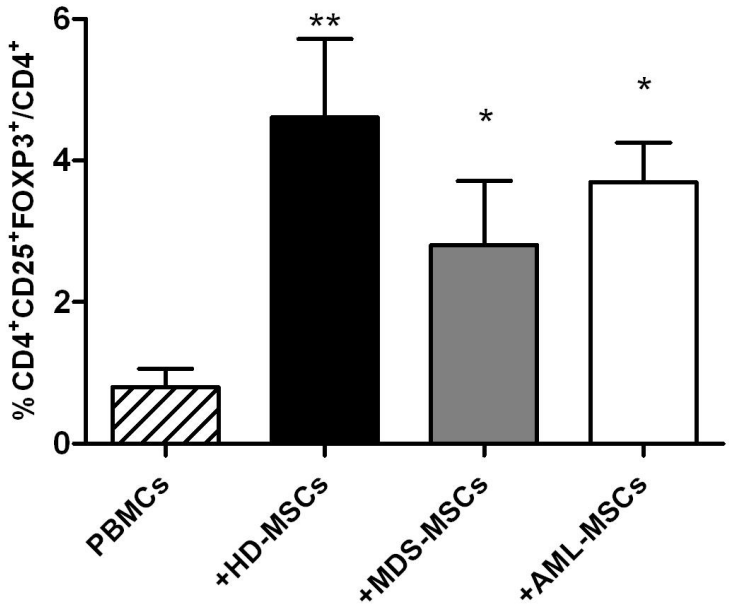
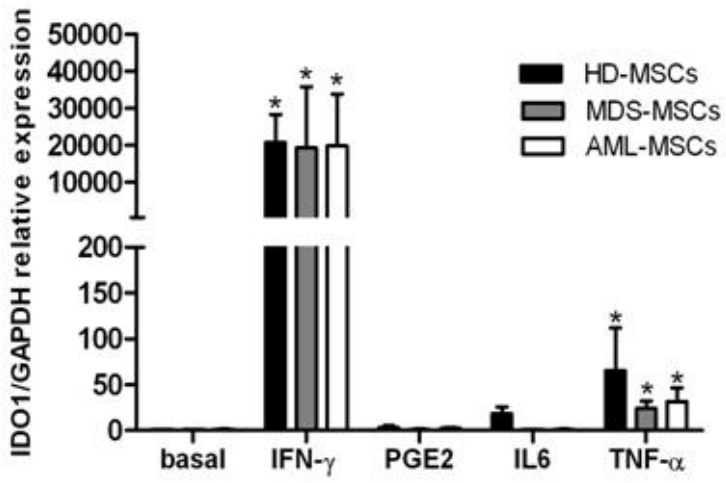


Figure 14. MSCs isolated from MDS and AML patients maintain their ability to induce Tregs. The bars represent the percentage of $CD4^+CD25^+FOXP3^+$ T cells (gated on $CD4^+$) evaluated by flow cytometry (*, $p < 0.05$ vs PBMCs cultured alone). Values are indicated as mean ± SEM of at least 3 independent experiments. The differences are not significant among the 3 groups.

1.7 MDS- and AML-MSCs up-regulate IDO1 after cytokine stimulation

IDO1 is a major player in the immunosuppressive activity of MSCs and IDO1 enzymatic activity in MSCs correlates with higher frequencies of $CD4^+CD25^+FOXP3^+$ Treg in a renal allograft mouse model[343]. IDO1 expression is not constitutively but is induced by several stimuli, including pro-inflammatory cytokines. In particular, it is known that in HD-MSCs, IDO1 is up-regulated after stimulation with IFN-γ and TNF-α[276]. We wondered if also MSCs, isolated from MDS and AML patients were able to up-regulate IDO1 following cytokine stimulation. We also tested other pro-inflammatory cytokines, such as PGE2 and IL-6, which are increased in serum of AML patients and/or are secreted by leukemic cells[128,344]. We treated HD/MDS/AML-MSCs with pro-inflammatory cytokines for 24 hours and we assessed the expression of mRNA and protein of IDO1. As shown in Figure 15a, MDS- and AML-MSCs efficiently up-regulated IDO1 mRNA after treatment with IFN-γ and also TNF-α, and to a lower extent after IL-6 stimulation. We confirmed the results at the protein level by performing WB, indeed IDO1 expression is mostly induced by IFN-γ but also to a lower extent, by TNF-α and IL-6 exposure in HD- and AML-MSCs (Fig.15b).

A



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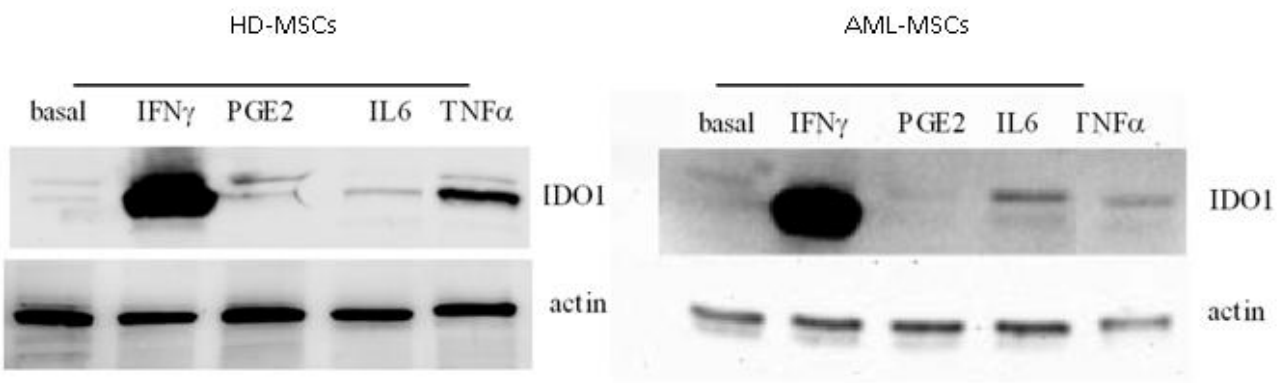


Figure 15. MSCs isolated from MDS and AML patients express IDO1 after stimulation with pro-inflammatory cytokines. A) *IDO1* mRNA expression evaluated by qRT-PCR in HD-MSCs and AML-MSCs after 24 hours of treatment with IFN-γ (50 ng/ml), TNF-α (10 ng/ml), PGE-2 (1 ng/ml) and IL-6 (10 ng/ml). The expression was evaluated based on the ΔC_t method and expression level of *IDO1* in cells untreated was taken as 1. Results are expressed as mean ± SEM of at least 3 independent experiments (* $p < 0.05$, vs basal). **B)** Representative Western Blot (WB) in HD-MSCs and AML-MSCs, after 24 hours of treatment with IFN-γ, TNF-α, PGE-2, and IL-6 as described before. Abbreviation: IFN, interferon, TNF tumor necrosis factor, IL, interleukin, PGE2, prostaglandin E2.

2 MALIGNANT HEMATOPOIETIC CELLS AFFECT THE NICHE

We hypothesized that pre-malignant and malignant hematopoietic cells can modify their own niche, which in turn could promote their selection and expansion. To gain a deeper insight into this cross-talk, we investigated:

1) the role of inflammatory pathways in the interplay between MSCs and AML cells;
2) the effects of hematopoietic-specific molecular alterations on the transcriptome of stromal cells.

2.1 Study of the inflammatory microenvironment in AML

It is known that AML patients are characterized by immune dysregulation including aberrant cytokine production, immune cell dysfunction and altered ratio of immune cell subsets[123]. In addition, AML cells are capable to induce an immunosuppressive/tolerant microenvironment marked by dysregulation of both innate and adaptive immune responses[125,261,345]. It has been recently described that inflammatory pathways can affect not only immune system cells but also stromal cells in the BM. Indeed, leukemic cells are able to modulate BM cells, through pro-inflammatory cytokines, such as CCL-3[315,318] and IL-1β[316].

For these reasons, we decided to investigate the pathways involved in inflammation with a particular focus on IDO1, a well-known regulator of immune tolerance.

2.1.1 AML cells produce IFN-γ

It has been extensively described the ability of AML cells to produce different soluble mediators. Indeed, several studies reported that, unlike normal hematopoietic cells, leukemic cells from AML patients constitutively express cytokines such as GM-CSF, IL-1, IL-6, G-CSF, TNF-α, SCF and IL-8[346–349]. We wondered if AML cells could secrete other pro-inflammatory cytokines. During a gene expression profiling (GEP) screening, performed on mononuclear cells (MNCs) isolated from BM of 61 AML patients at diagnosis (circulating blasts >=80%), we found for the first time that AML cells express IFN-γ. MNCs isolated from BM of healthy donors were used as controls (Fig. 16a). In particular, 38% (23/61) of the analyzed AML samples showed IFN-γ expression levels higher than the mean IFN-γ expression in MNCs isolated from HDs. This group included also 13.1% (8/61) of outliers, with a very high IFN-γ expression (Fig. 16b).

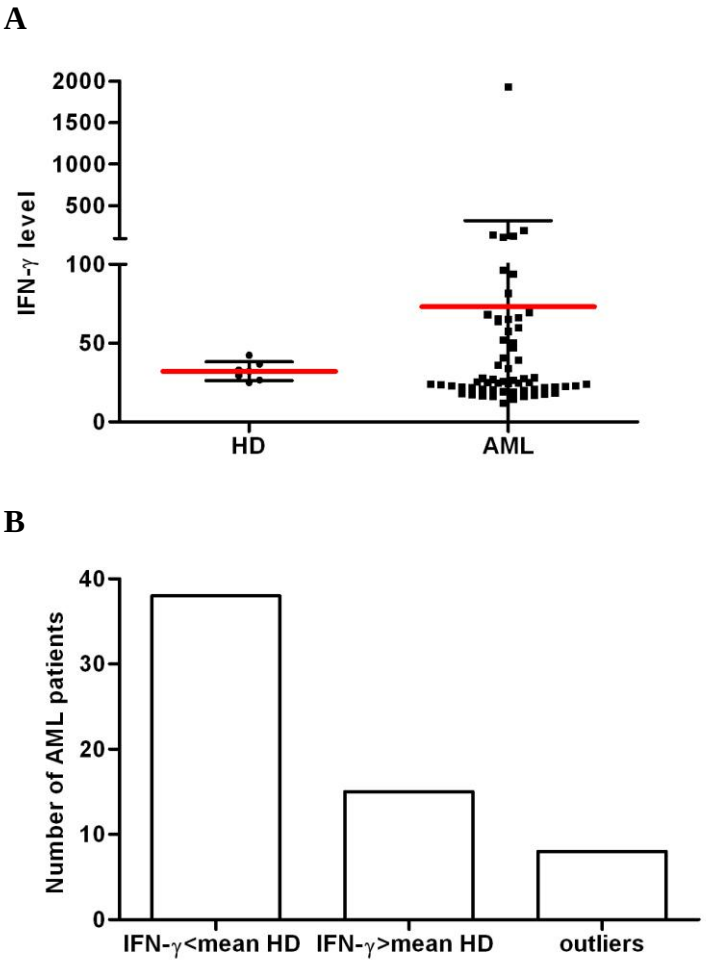


Figure 16. AML cells express IFN-γ. A) Results of gene expression profile (GEP) analysis performed on BM-derived MNCs isolated from HDs and AML patients at diagnosis. Red bars represent the average of IFN-γ expression, HD n=8, AML n=61. **B)** The columns represent the number of patients with an IFN-γ expression lower than the mean IFN-γ expression in HDs (38/61), higher than the mean in HDs (15/61), and outliers (8/61).

We then decided to divide AML samples into 3 groups, based on their IFN-γ expression, relative to the mean IFN-γ expression in MNCs isolated from HDs:

1) AML samples with lower expression than the mean (IFN-γ < mean HDs);
2) AML samples with higher expression than the mean (IFN-γ > mean HDs);
3) outliers.

In order to search for correlation between IFN-γ levels and clinical outcome, we analyzed the available clinical data of 60 patients, relevant for diagnosis, risk stratification, and disease response according to European LeukemiaNet (ELN) 2017 (Tab.6), including age at diagnosis, secondary or *de novo* AML, cytogenetic risk, *FLT3* and *NPM* mutations, and complete remission[328].

Table 6. Clinical data of AML patients analyzed for IFN-γ expression.

	IFN-γ <mean HDs number of patients	**IFN-γ >mean HDs** number of patients	**IFN-γ outliers** number of patients
age >65	18	**5**	**1**
secondary	6	3	1
Hyper[1]	18	6	**5**
low K risk[2]	19	9	5
medium K risk	8	1	1
high K risk	8	4	2
***FLT3* mut**	9	3	2
***NPM* mut**	7	3	0
low total risk[3]	4	0	1
interm total risk	3	5	3
high total Risk	27	**9**	**4**
RC[4]	16	**9**	**5**
RFR[5]	10	3	2
N	37	15	8

[1] hyperleukocytosis;
[2] risk classes according to cytogenetics[328];
[3] total risk;
[4] remission complete (RC);
[5] relapse-free rate (RFR)

Interestingly, we found that AML samples with lower expression of IFN-γ compared to the mean of HDs included a higher percentage of 'high risk' and elderly patients, parameters known to significantly correlate with a worse prognosis. On the contrary, patients with higher expression of IFN-γ compared to the mean of HDs were younger and showed higher rates of complete remission. We then performed an overall survival curve. Patients in which the follow up was not available (n=4) and patients receiving supportive care were excluded (n=1). The characteristics of this subpopulation (n=56) were comparable to that already described in Tab. 6. We found that AML patients with higher IFN-γ expression compared to the mean of HD show a better overall survival compared to patients with lower IFN-γ, and in particular, the outliers have the best overall survival (Fig.17). Multivariable analysis indicated that besides disease cytogenetic and molecular low-risk and full chemotherapy, IFN-γ expression is an independent parameter to determine a better overall survival in our cohort of AML patients. Furthermore, we also assessed the percentage of stem cell transplantation (SCT) among the 3 groups. Despite SCT cannot be considered a prognostic factor at diagnosis, it could still affect the OS of patients considering that most of the outliers are young patients. However, we could not identify a statistically relevant difference in the number of patients receiving SCT in the 3 groups (data not shown).

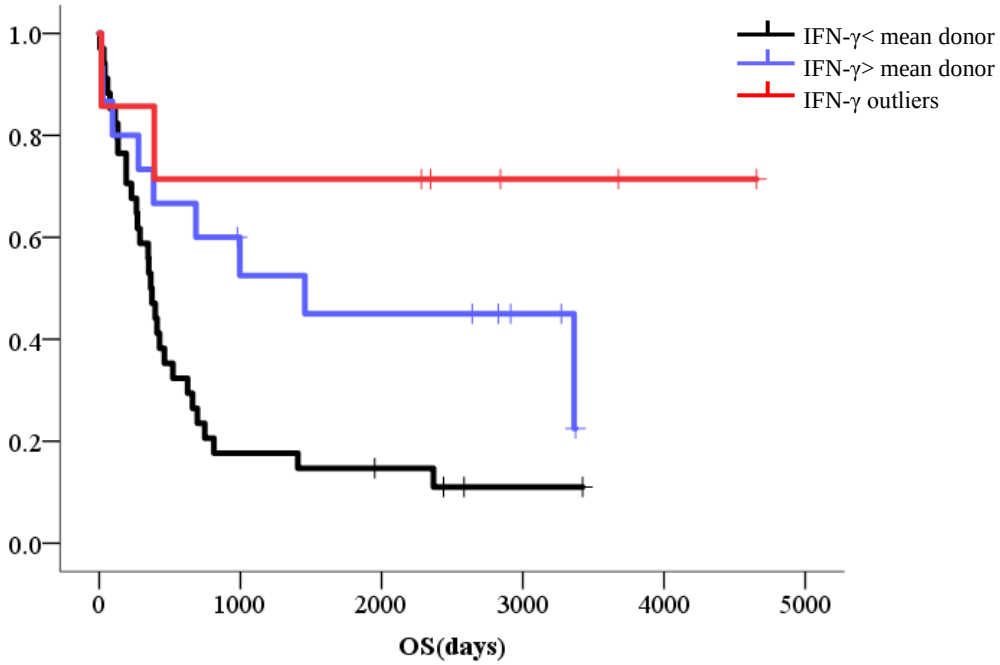


Figure 17. AML patients expressing IFN-γ show a better overall survival. The graph represents the overall survival (days) of AML patients (n=56) expressing different levels of IFN-γ compared to healthy donors.

2.1.2 AML cells induce the expression of IDO1 in MSCs

To gain insight into AML cells/MSCs interaction, after the discovery of AML cells producing IFN-γ, we decided to investigate the impact that this soluble mediator could have on BM microenvironment, by performing co-cultures.

First, we confirmed IFN-γ production in culture in AML cells isolated from BMs of patients (circulating blasts>80%). We performed co-cultures of AML cells and HD- and AML-MSCs, as previously described, and we evaluated IFN-γ production. We detected IFN-γ in 78% (11 samples over 14 tested) of samples analyzed in culture with or without MSCs (Fig.18). As shown in Figure 18, we assessed IFN-γ mRNA levels by qRT-PCR. AML cells cultured alone express IFN-γ, and in co-culture with HD- and AML-MSCs we observed a trend of up-regulation of the cytokine, but it was not statistically significant. Next, we tested IFN-γ protein levels by flow cytometry and we used as negative control MNCs isolated from buffy coat, while as positive controls MNCs treated with phorbol 12-myristate 13-acetate (PMA), Ionomycin (IM) and Brefeldin A (BFA). PMA and IM stimulate cytokine production and BFA disrupts Golgi function, limiting cytokine secretion. We confirmed also at the protein level, that AML cells produce IFN-γ which is not modulated by the co-culture with MSCs contrary to what we observed for IFN-γ mRNA (Fig.18).

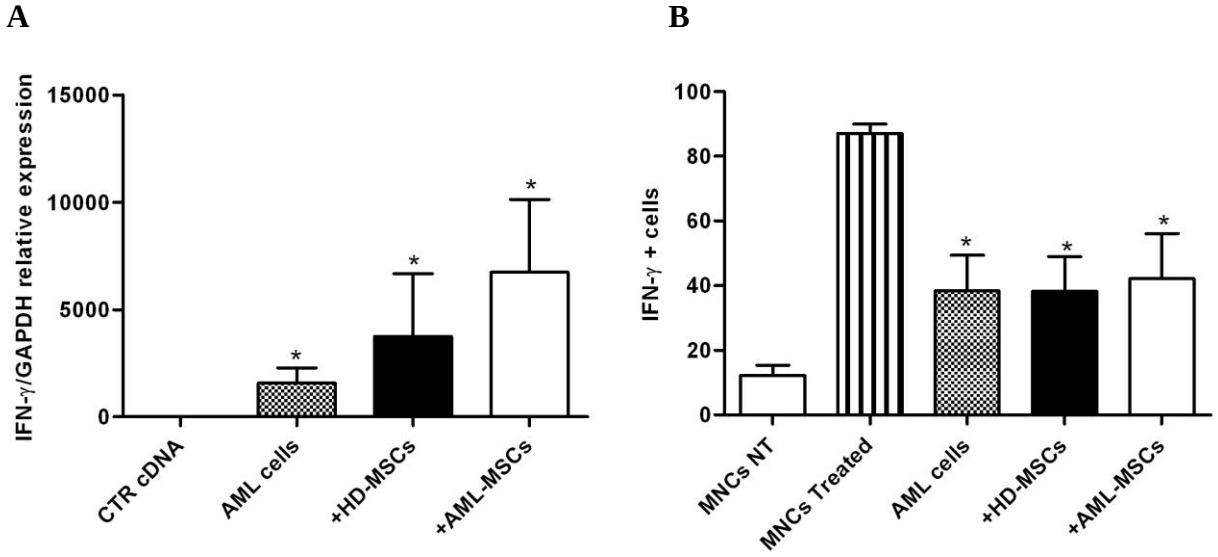


Figure 18. AML cells produce IFN-γ in culture with and without HD/AML-MSCs. AML cells seeded on MSC layers, after 4 days were harvested and analysed. **A)** mRNA expression evaluated by qRT-PCR . The expression was calculated based on the ΔC_t method and expression levels of *IFN-γ* in a control cDNA, composed of a pool of human cDNAs, was taken as 1. Results are expressed as mean ± SEM of at least 4 independent experiments (* $p < 0.05$, vs CTR cDNA). The differences are not significant between the groups. **B)** protein expression assessed by flow cytometry. The bars represent the percentage of IFN-γ positive cells. MNCs isolated from Buffy Coats and not treated (NT) were used as negative controls. MNCs treated with phorbol 12-myristate 13-acetate (PMA) 11 µg/µl, Ionomycin (IM) 0,5 µg/ml for 4 hours and subsequently with Brefeldin A (BFA) 2 µg/ml overnight were used as positive control. Results are expressed as mean ± SEM of at least 4 independent experiments (* $p < 0.05$, vs MNCs NT). The differences are not significant between the groups.

Despite we used AML samples in which leukemic cells replaced the BM, we further characterized the cellular population of BM responsible for IFN-γ production. Based on the immunophenotype of BM-derived AML cells at diagnosis, we distinguished AML cells from the other component able to produce high levels of IFN-γ, i.e. $CD3^+$ lymphocytes. As shown in the example in Figure 19, almost the 90% of the living BM-derived cells was composed of myeloid AML cells $CD3^-CD33^+$, which were responsible for IFN-γ production in AML BM, evaluated by flow cytometry.

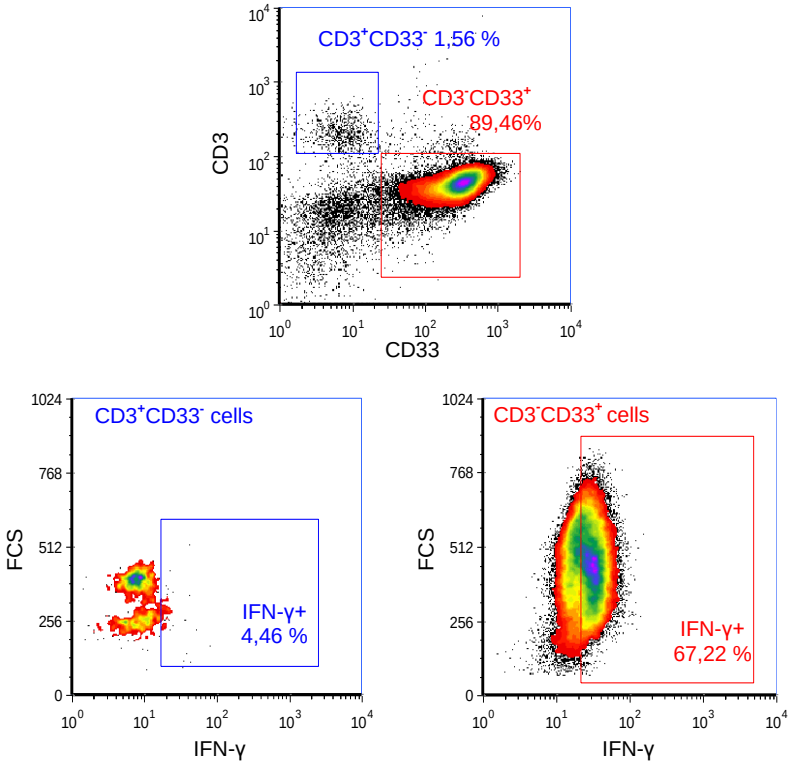
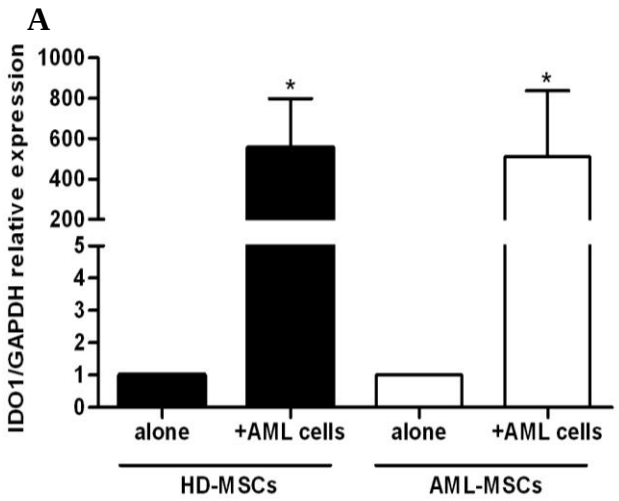


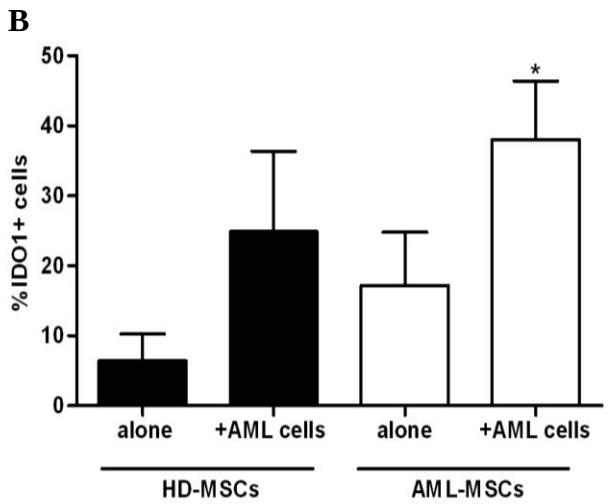
Figure 19. In AML BM, IFN-γ is produced exclusively by AML cells. The figure reports an example of IFN-γ production, assessed by flow cytometry, in two different cell types in an AML BM (circulating blast >90%), characterized by leukemic cells $CD33^+$. In the example, lymphocytes, $CD3^+CD33^-$, do not produce IFN-γ whereas AML cells, $CD3^-CD33^+$, produce IFN-γ.

Next, we also checked IFN-γ production in AML cell lines, including OCI-AML3, HL-60, THP-1, Kasumi-1, KG-1, HT93A, and Nomo1 by qRT-PCR. However, we could not detect IFN-γ mRNA in any of the AML cell lines tested (data not shown), suggesting that IFN-γ expression represents a peculiar mechanism of primary AML samples.

We then wondered if IFN-γ^+ cells could induce IDO1 expression in MSCs. We focused on IDO1 because of its immunotolerant properties. Indeed, it is involved in the tumor escape of several solid tumors[253] and in AML it could promote a favorable environment for leukemic cells. In addition, we have previously described that IDO1 is mainly up-regulated after IFN-γ exposure.

We evaluated IDO1 expression in HD- and AML-MSCs after co-cultures with AML cells, separated by a transwell system. We assessed mRNA expression by qRT-PCR, and interestingly we found that HD- and AML-MSCs efficiently up-regulated IDO1 in the presence of AML cells (Fig.20a). Next, we confirmed the results at the protein level by flow cytometry. We found that both HD-MSCs and AML-MSCs up-regulate IDO1, but only for AML-MSCs in a statistically significant way (Fig.20b). Indeed, HD-MSCs showed more variability suggesting a different responsiveness to AML cells stimulation. WB analysis further confirmed the results. As shown in figure 20c, a clear band corresponding to IDO1 protein, is up-regulated in AML-MSCs and to a lower extent in HD-MSCs, co-cultured with AML cells.





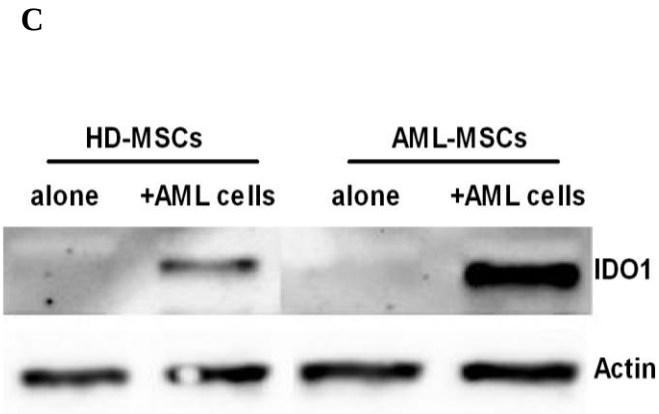


Figure 20. MSCs express IDO1 after co-cultures with AML cells. After 4 days in a transwell system of co-culture, MSCs were collected and RNA and proteins were extracted. **A)** *IDO1* mRNA expression evaluated by qRT-PCR. The expression was calculated based on the ΔC_t method and expression level of *IDO1* in cells cultured alone was taken as 1. Results are expressed as mean ± SEM of at least 6 independent experiments (* $p < 0.05$, vs cells cultured alone).
B) IDO1 protein expression was evaluated by flow cytometry. The bars represent the percentage of IDO1 positive cells. Results are the mean ± SEM of at least 4 independent experiments (* $p < 0.05$, vs cells cultured alone).
C) representative WB of proteins extracted from MSCs after co-cultures with AML cells.

2.1.3 IFN-γ induces IDO1 expression in MSCs in co-culture with AML cells

Since in co-culture experiments we used a transwells system to maintain separate AML cells and MSCs, we could speculate that the induction of IDO1 expression in co-culture was induced by soluble mediators. Thus, our hypothesis was that IFN-γ, produced by AML cells, was the main regulator of IDO1 in our co-culture system.

In order to confirm this hypothesis, we performed co-culture with a neutralizing IFN-γ antibody. Moreover, we decided to assess the effect of IFN-γ antibody on early IDO1 up-regulation, shortening the timing of co-cultures from 4 days to 6 hours, and focusing only on AML-MSCs to better represent the leukemic microenvironment. First, we checked if IDO1 expression was already inducible after only 6 hours of co-cultures. Interestingly, we found that AML-MSCs express IDO1 also after such a short time of co-culture with AML cells (data not shown). We then performed co-cultures adding the IFN-γ neutralizing antibody, direct against the cytokine, and we assessed IDO1 mRNA expression in AML-MSCs by qRT-PCR. As shown in Figure 21, the qRT-PCR analysis demonstrated that the addition of the anti-IFN-γ antibody reduces significantly the induction of IDO1 in AML-MSCs. Therefore, we concluded that AML cells modulate IDO1 expression in MSCs, by producing IFN-γ.

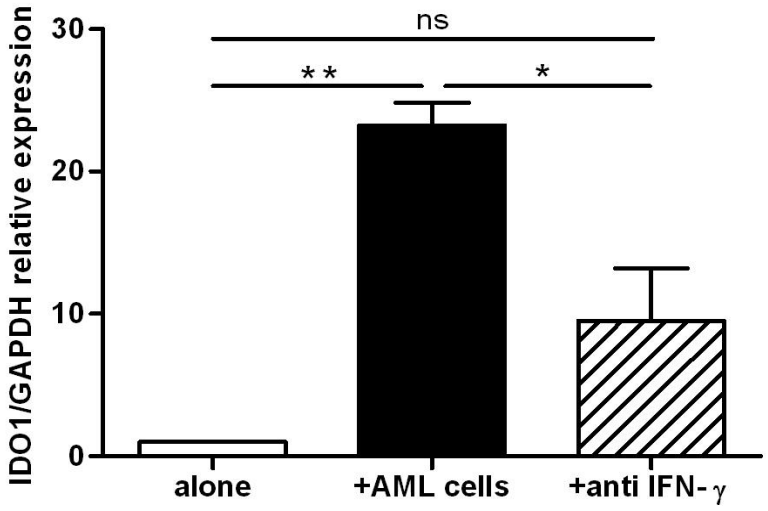


Figure 21. The neutralization of IFN-γ reduces IDO1 expression in AML-MSCs in co-culture with AML cells. *IDO1* mRNA expression, evaluated by qRT-PCR, in AML-MSCs after co-culture with AML cells w/wo anti-IFN-γ antibody (20 μg/ml). The expression was calculated based on the ΔC_t method and expression level of *IDO1* in cells cultured alone was taken as 1. Results are expressed as mean ± SEM of 3 independent experiments (* $p < 0.05$, ** $p < 0.1$ vs cells cultured alone; ns: not significant).

A pilot experiment, performed with an antibody which recognizes an extracellular region of the IFN-γ receptor, confirmed this conclusion (Fig.22).

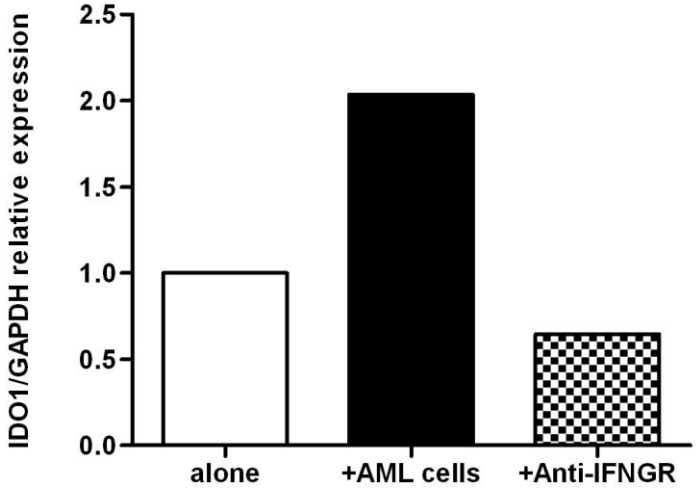


Figure 22. The neutralization of IFN-γ receptor inhibits IDO1 up-regulation in AML-MSC in co-culture with AML cells. *IDO1* mRNA expression, evaluated by qRT-PCR, in AML-MSCs after co-culture with AML cells w/wo the antibody direct against the IFN-γ receptor (20 μg/ml). The *IDO1* expression was calculated based on the ΔC_t method and expression levels of *IDO1* in cells cultured alone was taken as 1, n=1.

2.1.4 MSCs induce Tregs, after AML cells co-culture, in an IDO1 dependent manner

IDO1 activity contributes to immune tolerance through various mechanisms among which the induction of Tregs[216]. In addition, MSCs exert their immunomodulatory properties through IDO1 expression. We hypothesized that IFN-γ producing AML cells favor an immunotolerant milieu, mediated by the induction of IDO1 in MSCs. In particular, we decided to investigate if Tregs are induced by IDO1 expressing AML-MSCs, pre-cultured with IFN-γ^+ AML cells.

We performed co-cultures of AML cells and AML-MSCs in a transwell system, as previously described, to induce IDO1 expression. We then removed AML cells and we added PBMCs isolated from buffy coats. After 7 days, we collected PBMCs and we evaluated by flow cytometry the percentage of $CD3^+CD4^+CD25^+FOXP3^+$ cells (Tregs).

Interestingly, we found that Tregs were induced by MSCs pre-cultured with AML cells. When we added to the culture an IDO1 inhibitor, to definitively prove the role of the enzyme, we found that the induction of Tregs mediated by AML-MSCs, pre-cultured with IFN-γ^+ AML cells, was totally IDO1 dependent. Indeed, the addition of its specific inhibitor almost abrogated Tregs induction. (Fig.23).

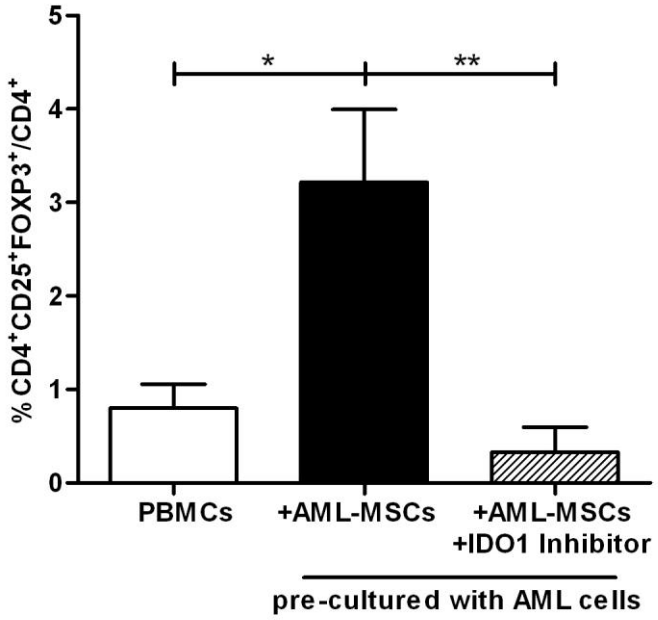


Figure 23. AML-MSCs, pre-cultured with AML cells, induce Tregs in an IDO1 dependent way. Peripheral blood mononuclear cells (PBMCs) seeded on AML-MSCs layer, pre-cultured with AML cells, w/wo IDO1 inhibitor, Incyte (50 μM). The bars represent the percentage of $CD3^+CD4^+CD25^+FOXP3^+$ T cells (gated on $CD4^{+)}$) evaluated by flow cytometry (* $p < 0,05$, ** $p<0,01$). Values are indicated as mean ± SEM of at least 3 independent experiments.

2.2 Study of transcriptional alterations in BM microenvironment

Massive transcriptome sequencing of MSCs isolated from low-risk MDS (LR-MDS) patients showed transcriptional modifications of the cells with signs of cellular stress and up-regulation of genes involved in inflammation[292]. It is unknown whether these transcriptional alterations are intrinsic or if they are caused by mutated hematopoietic cells. Previous studies demonstrated that the deletion of *Dnmt3a* or *Asxl1*, 2 common mutated genes in human MDS, in hematopoietic cells is responsible for an MDS-like phenotype in mice[90,106], suggesting a peculiar role of these genes in MDS pathogenesis. However, it is still unclear the mechanism underlying the malignant transformation, mediated by *Dnmt3a* and *Asxl1*. In addition, in human MDS, mutations in DNMT3A and ASXL1 genes occur frequently, arise early during disease development, and are responsible for the CHIP phenomenon. However, it is still unclear what gives advantages to cells harboring these mutations. The involvement of BM cells seems reasonable.

Therefore, we wondered if hematopoietic cells, harboring *Dnmt3a* or *Asxl1* deletions, could influence the niche in order to favor their own expansion. In particular, based on findings of transcriptome modifications in MSCs isolated from MDS patients, we decided to investigate a possible/putative impact that the hematopoietic deletion of *Dnmt3a* and *Asxl1* have on the transcriptome of stromal cells. To this aim, we used three different approaches. First, we transplanted BM cells characterized by the hematopoietic-specific loss of *Dnmt3a* and *Asxl1*, hereafter referred to *Dnmt3a* and *Asxl1* KO cells, in wild-type recipient mice. We then analyzed the transcriptome modifications in stromal cells, when possible in OBs and MSCs specifically, isolated from transplanted mice. Next, we performed *ex-vivo* co-cultures of OP9, a murine stromal cell line, and *Dnmt3a* KO and *Asxl1* KO hematopoietic stem progenitor cells (HSPCs) to simulate the *in vivo* interaction. Finally, we compared the results of *in vivo* transplantation experiments with the results obtained from massive transcriptome sequencing of highly purified MSCs isolated from low-risk MDS patients, in order to identify similarities relevant to human disease (Fig.24).

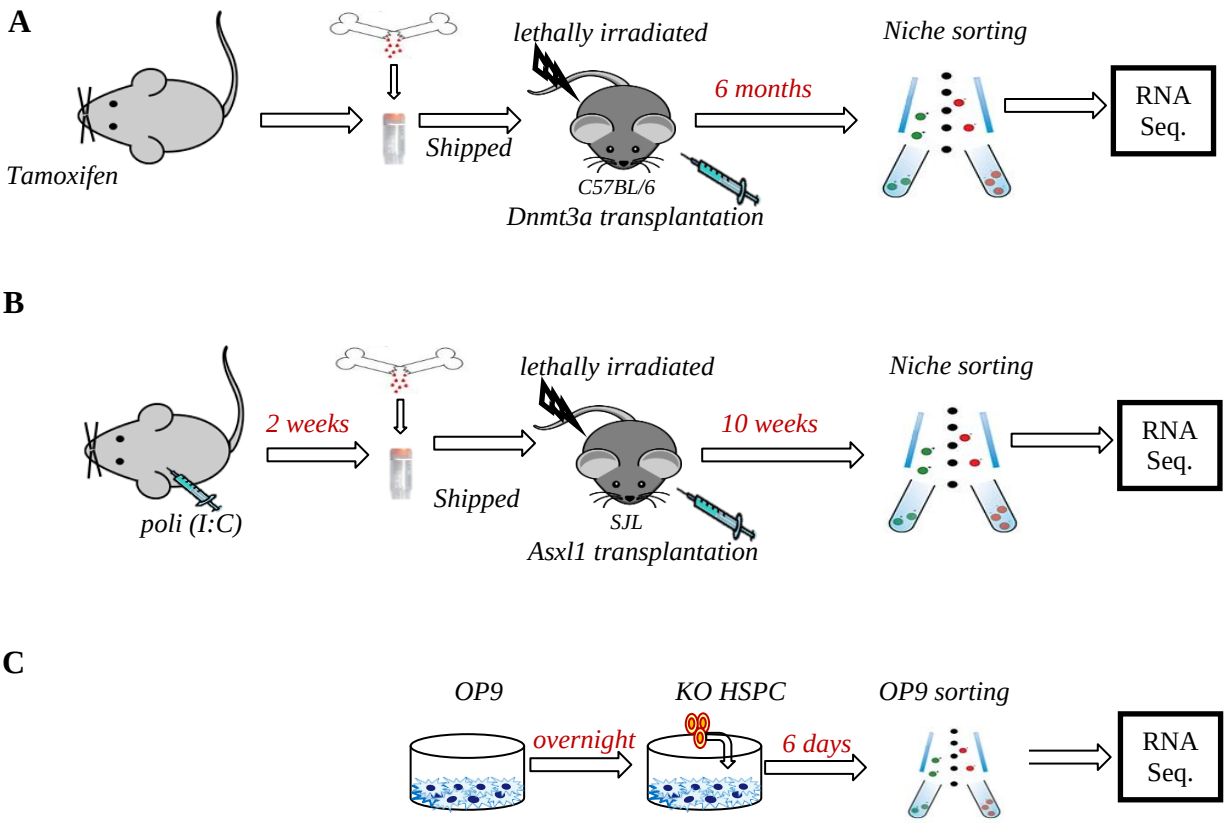


Figure 24. Experimental plans. A) *Dnmt3a* transplantation model. **B)** *Asxl1* transplantation model **C)** co-culture assay model.

2.2.1 *In vivo* experiments

As described in further details in 'materials and methods' section, we transplanted *Dnmt3a* and *Asxl1* KO cells in wild-type (WT) mice. To avoid alterations due to the disease itself, we sacrificed the mice before the onset of the hematological disease, and we collected stromal cells, such as OBs and MSCs when possible. We then performed massive transcriptome sequencing (RNA sequencing) of OBs and MSCs.

2.2.1.1 The loss of *Dnmt3a* in hematopoietic cells affects stromal cells *in vivo*

For these experiments, BM-derived cells isolated from Rosa26-CreERT2;Dnmt3a$^{f/f}$ mice, kindly provided Dr.Mukherjee and collaborators (Columbia University Medical Center, New York, USA) were used. The cells carry the deletion of *Dnmt3a* and hereafter will be referred to *Dnmt3a* KO cells. In the construct, the expression of the recombinase Cre is under the control of the ubiquitous

promoter Rosa26, on the contrary, Cre activity is inducible with tamoxifen. Indeed, Cre is fused to a mutant estrogen ligand-binding domain (ERT2) that requires tamoxifen for recombinase activity. Therefore the ubiquitous excision of *Dnmt3a* was induced by the administration of tamoxifen. Fetal liver cells in which Cre recombinase was expressed under the control of a not-inducible promoter (c/EBPα) in a wild-type *Dnmt3a* context, were used as controls.

We first confirmed the *Dnmt3a* deletion by genomic PCR. The excision of the gene in KO cells was detected as a band of 420 bp whereas the wt allele in control cells was detected as a band of 200 bp (Fig.25). Next, we transplanted *Dnmt3a* KO and control BM cells in wt mice and after 6 months, before the outcome of an hematologic disorder, KO transplanted mice and their controls were sacrificed. BM was collected in order to FACS-purify MSC and OB populations for RNA sequencing. To identify and isolate MSCs and OBs, we used the gating strategy which distinguishes OBs and MSCs as followed: living cells, $CD45^{+}$, $Ter119^{-}$, $CD31^{-}$, $Sca\text{-}1^{+}$, $CD51^{+}$ (MSC)/CD51- (OB)[350]. Unexpectedly due to a technical problem, we could not identify the final Sca-1+ and CD51+/- populations and subsequently, without distinguishing MSC and OB cells, we sorted only the living, $CD45^{-}$, $Ter119^{-}$, CD31- cells, hereafter referred to stromal cells. Therefore, we performed RNA sequencing of the stromal population isolated. Next, we checked the identity of the cells sorted based on gene expression, obtained with RNA seq. Although the expression levels were slightly different, the cells sorted expressed, as expected low level of the endothelial marker CD31 and expressed, at variable levels, transcripts encoding for membrane proteins, such as *Sca-1*, known markers of stromal cells, such as *receptors of Leptin, Platelet derived growth factor (Pdgf)* and *parathyroid hormone 1 (Pth1r),* and niche factors such as *Cxcl12* and *c-kit ligand* (*kitl*) (Fig.25). However, we also found a fraction of CD45 expressing cells, probably due to an hematopoietic contamination. These data suggest that we isolated a highly enriched population of stromal cells.

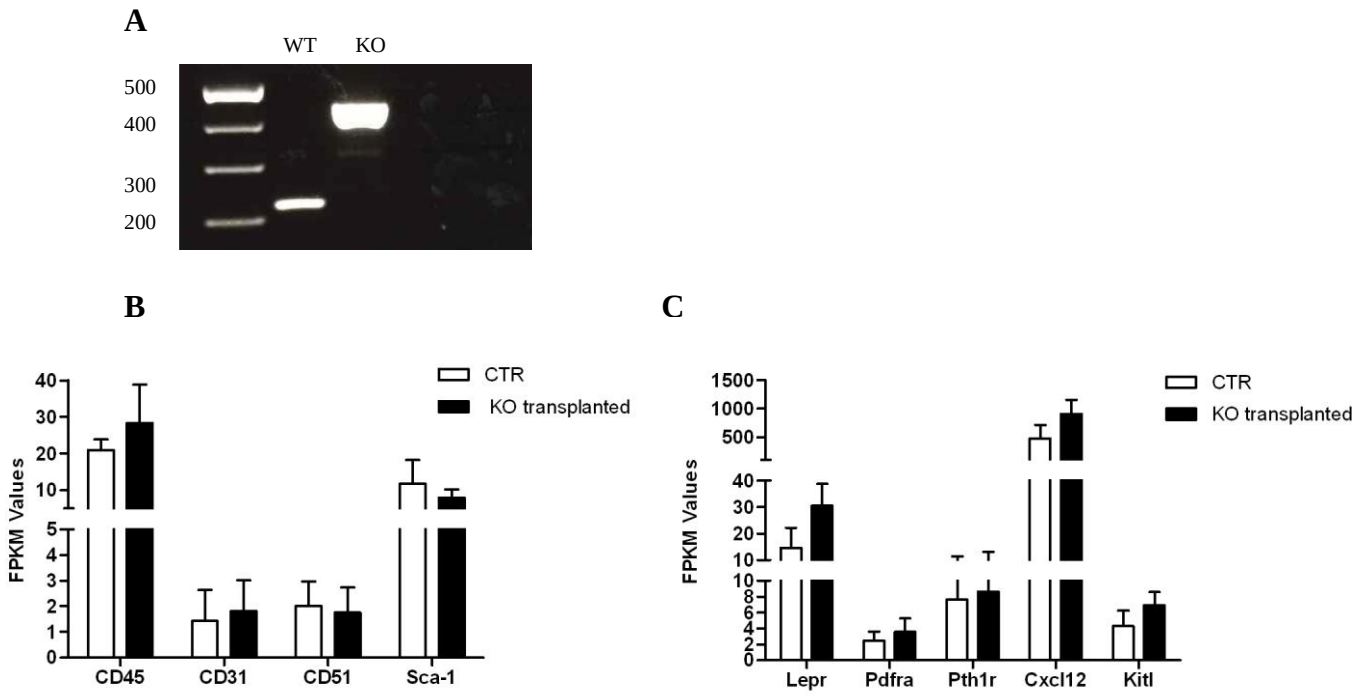


Figure 25. Genotyping of BM cells and molecular characterization of isolated stromal cells. A) PCR of WT and *Dnmt3a* KO BM-derived cells. The WT allele in WT cells (first lane) was detected as a band of 200 bp, whereas the deleted allele in KO cells (right lane) as a band of 420 bp. Gene expression evaluated by RNA sequencing of stromal cells isolated from CTR and *Dnmt3a* KO transplanted mice: **B)** expression of surface markers used for cell identification. CD45 (*PTPRC*: protein tyrosine phosphatase, receptor type), CD31 (*PECAM-1*: platelet/endothelial cell adhesion molecule-1), CD51 (ITGAV: Integrin subunit alpha V), Sca-1 (Ly6a: Lymphocyte antigen complex, locus A); **C)** expression of stromal cell markers *Lepr* (Leptin receptor) *Pdgfra* (Platelet derived growth factor receptor alpha), *Pth1r* (parathyroid hormone 1 receptor), and hematopoiesis supporting cytokines *Cxcl12* (stromal cell-derived factor 1) *Kitl* (c-kit ligand). B)C) Results are expressed as fragments per kilobase of exon per million fragments mapped kilobase (FPKM). The bars represent mean +/- SEM, *Dnmt3a* KO transplanted mice n=4, CTR mice n=4

For RNA seq experiments, we then performed principal component analysis (PCA), a statistic tool which reduces multiple variables of a dataset to fewer dimension helping to interpret the relations among the samples. PCA showed a uniform clustering of stromal cells isolated from control mice, suggesting transcriptional homogeneity (Fig.26). In contrast, a more heterogeneous clustering characterized the transcriptome of stromal cells isolated from *Dnmt3a* KO transplanted mice (Fig.26).

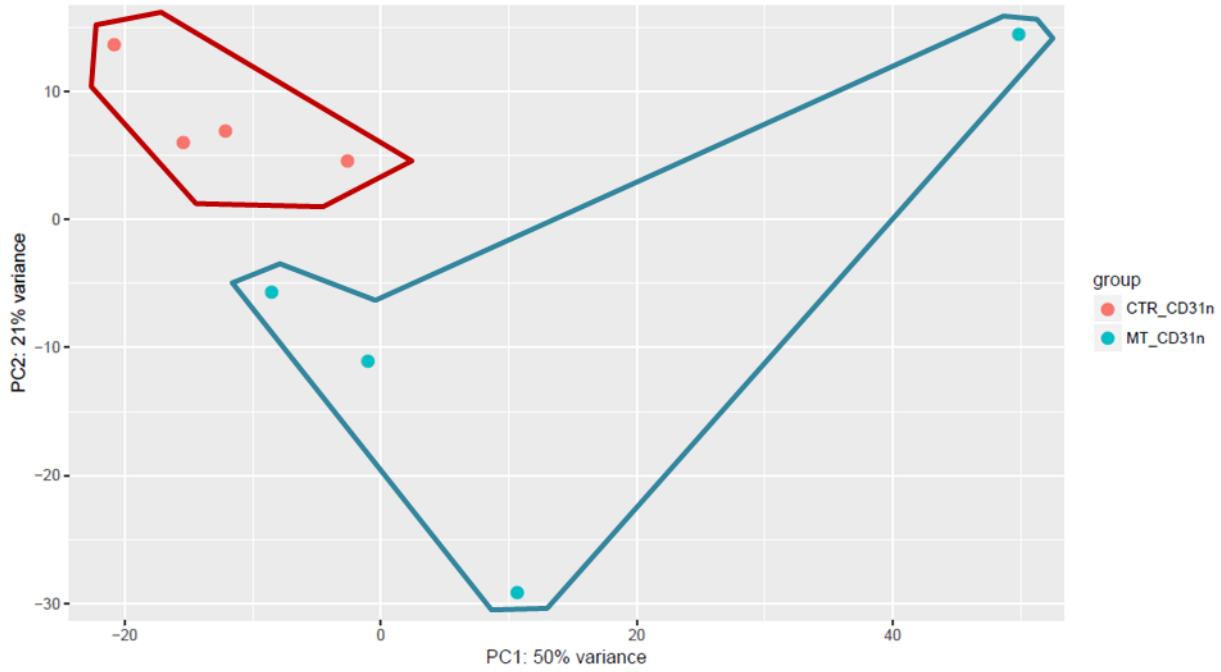


Figure 26. Principal component analysis reveals a heterogeneous clustering of *Dnmt3a* KO transplanted mice transcriptome. Principal component analysis was performed on the fragment counts. *Dnmt3a* KO transplanted mice n=4, CTR mice n=4.

Next, we analyzed the results of transcriptome sequencing by performing two different analyses: 1) differential gene expression analysis, in which the expression of every gene is evaluated. The transcript of stromal cells isolated from *Dnmt3a* KO transplanted mice were compared to transcript of cells isolated from control mice, in order to identify up-regulated or down-regulated genes; 2) gene set enrichment analysis (GSEA), which based on the differential gene expression analysis, defines the pathways (gene sets) underlying the different transcriptional patterns of the cells sequenced, in order to better understand the biological processes involved.

The differential gene expression analysis identified 455 genes differentially expressed. We considered the expression significantly different when the p-value, normalized for the number of reads (p_{adj}), was lower than 0.05 ($p_{adj}<0.05$). In particular, we found 337 up-regulated and 118 down-regulated genes in stromal cells isolated from *Dnmt3a* KO transplanted mice. Based on p_{adj}, the first 10 most up- and down-regulated genes were represented in Table 7. However, it is required

a more detailed analysis, which could highlight the relevance of the genes found differentially expressed.

Table 7. 10 first genes up-regulated and down-regulated in stromal cells isolated from *Dnmt3a* KO transplanted mice.

Gene name	Avg *Dnmt3a* KO transp. mice [1]	Avg CTR	FC[2]	Gene name	Avg *Dnmt3a* KO transp. mice	Avg CTR	FC
Up-regulated				**Down-regulated**			
Dzank1	0,58	0,00	23,56	*Jchain*	0,66	185,47	-7,65
Itgbl1	1,91	0,00	23,50	*Dhx33*	2,31	12,14	-1,96
Fam149a	0,80	0,00	9,70	*Mepe*	0,01	4,66	-8,19
BC018473	16,73	0,10	8,34	*Zfp770*	0,00	1,03	-8,70
Sned1	2,55	0,46	2,96	*BC068281*	0,00	1,63	-4,73
Zswim5	0,45	0,00	5,83	*Ctla4*	0,00	3,63	-9,84
Cpeb3	6,07	0,92	3,19	*Tmtc4*	0,00	1,42	-6,48
Gm684	1,65	0,01	5,46	*Zfp12*	0,03	3,09	-5,85
Fas	10,28	2,61	2,70	*Pard6b*	0,00	1,07	-8,42
Ypel4	77,71	23,10	2,33	*St14*	0,00	0,82	-8,30

The genes are listed based on p_{adj} values.
[1] *Dnmt3a* KO transplanted mice n=4, CTR mice n=4
[2] Fold change (FC)

For the GSEA analysis, we considered differentially expressed those gene sets with a false discovery rate (FDR) lower than 25%. The FDR indicates the estimated probability that a gene set represents a false positive.

The GSEA analysis highlighted differences between the 2 groups with 281 pathways enriched in stromal cells isolated from *Dnmt3a* KO transplanted mice and 276 enriched in stromal cells from control mice (Tab.8).

Table 8. The 10 pathways most enriched in stromal cells isolated from *Dnmt3a* KO transplanted mice and control mice.

Enriched in *Dnmt3a* KO transplanted[1]			Enriched in CTR		
NAME	**NES[2]**	**FDR[3]**	**NAME**	**NES**	**FDR**
WELCH_GATA1_TARGETS	2,3	0,00	REACTOME_TRANSLATION	- 2,03	0,00
BIOCARTA_AHSP_PATHWAY	2,26	0,00	REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_ TARGETING_TO_MEMBRANE	- 2,02	0,00
REACTOME_AMYLOIDS	2,24	0,00	REACTOME_PEPTIDE_CHAIN_ELONGATION	- 2,01	0,00
STEINER_ERYTHROCYTE_MEMBRANE_GENES	2,16	0,02	KEGG_RIBOSOME	- 2,01	0,00
VALK_AML_CLUSTER_7	2,11	0,02	REACTOME_INFLUENZA_LIFE_CYCLE	- 2,0	0,00
STEGER_ADIPOGENESIS_DN	2,08	0,02	REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATI ON	- 2,0	0,00
IVANOVA_HEMATOPOIESIS_MATURE_CELL	2,08	0,02	REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_ REGULATION	-1,99	0,00
HERNANDEZ_MITOTIC_ARREST_BY_DOCETAXEL _1_UP	2,04	0,03	REACTOME_NONSENSE_MEDIATED_DECAY_ENHANCED_BY_ THE_EXON_JUNCTION_COMPLEX	-1,96	0,00
DAZARD_UV_RESPONSE_CLUSTER_G24	2,03	0,03	REACTOME_METABOLISM_OF_RNA	-1,91	0,00

[1] *Dnmt3a* KO transplanted mice n=4, CTR mice n=4.
[2] Normalized enrichment score (NES)
[3] False discovery rate (FDR) values.

In stromal cells isolated from *Dnmt3a* KO transplanted mice, we found enrichment in several pathways, among which we pointed out:

- adipogenesis;
- pathways active in various cancers, such as lung carcinoma, AML, breast, thyroid and liver cancer, retinoblastoma, brain tumor;
- inflammation: pathways mediated by NF-kb and pathways activated in response to inflammatory cytokines, in particular to IFN, IL-6, TNF, IL22, IL17;
- epidermal growth factor (EGF) response;
- cellular stress in response to UV.

We also identified many pathways enriched in stromal cells isolated from control mice, among which:

- RNA processing, involving rRNA, mRNA, and tRNA, and protein translation;
- metabolism, including the Krebs cycle, respiratory electron transport, and the functionality of mitochondria.
- myc activity and mitosis.

2.2.1.2 The hematopoietic-specific loss of *Asxl1* affects stromal cells *in vivo*

In parallel, we performed *in vivo* experiments similar to those described before by using BM-derived cells isolated from Mx1-Cre;Asxl1$^{f/f}$ mice, with hematopoietic-specific deletion of *Asxl1 (*hereafter referred to *Asxl1* KO cells), kindly provided by Dr. Abdel-Wahab and collaborators (Memorial Sloan Kettering Cancer Center, New York, USA). In this construct, the excision of *Asxl1* is mediated by the recombinase *Cre* under the control of a promoter (Mx-1) active in hematopoietic cells following poli I:C stimulation. BM cells isolated from Mx1-Cre;Asxl1$^{+/+}$ mice were used as controls.

Before transplantation, we first checked *Asxl1* deletion by genomic PCR (Fig.27), WT allele in control cells was detected as a band of 200 bp, while the excision of the gene generated a band of 700 bp. In addition, in *Asxl1* KO cells due to an efficiency of recombination lower than 100%, the allele containing *Asxl1* gene still flanked by the two recombinase sites, was still detectable as a band of 380 bp (Fig.27).

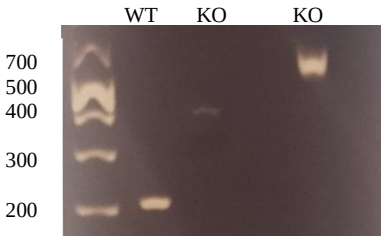
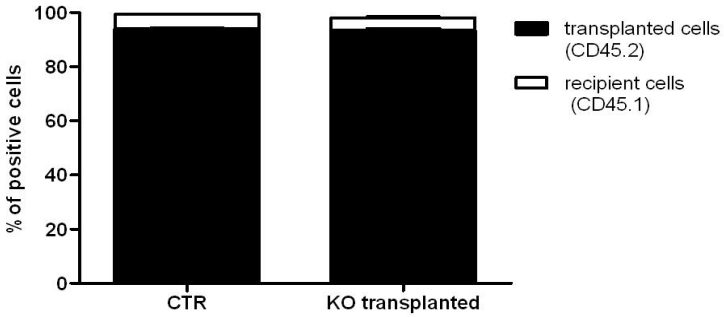


Figure 27. *Asxl1* KO cells show the deletion of the gene. A) PCR of WT and *Asxl1* KO BM-derived cells. The WT allele was detected as a band of 200 bp in WT cells (first lane) the floxed allele in *Asxl1* KO cells (central lane) as a band of 380 bp, whereas the deleted allele in *Asxl1* KO cells (right lane) as a band of 700 bp.

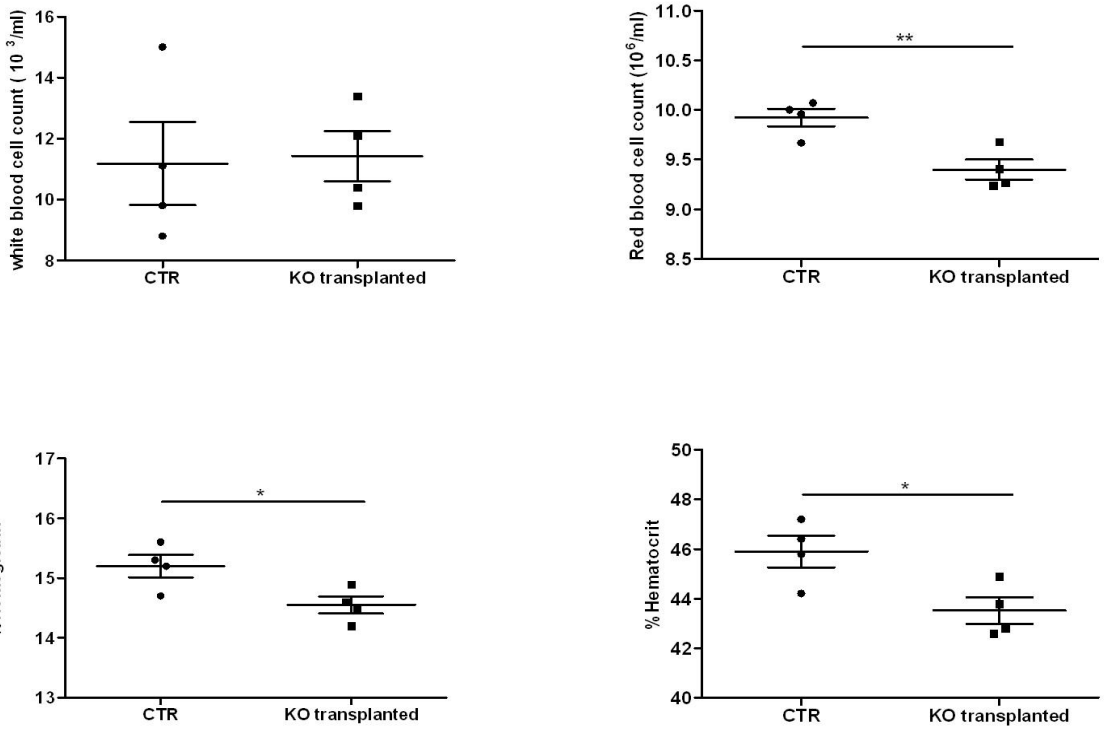
Asxl1 KO cells and their controls were then transplanted into WT mice. It has been already described that the hematopoietic-specific loss of *Asxl1* in mice aged 6-12 months results in anemia, hypocellularity of BM and spleen with extramedullary hematopoiesis (EHM) in liver and leukopenia. The leukopenia is predominantly caused by a decrement in mature B cells, neutrophils, and monocytes[106]. For this reason, *Asxl1* KO transplanted mice and their controls were sacrificed after 10 weeks, before the onset of the hematologic disease. We analyzed blood parameters and cellular composition of several tissues/organs, including spleen, BM and peripheral blood (PB), which was collected just before sacrifice. In particular, we evaluated the chimerism of PB assessing the percentage of circulating hematopoietic donor cells ($CD45.2^{+}$), and the composition of hematopoietic precursors, including HSCs in BM, and myeloid committed cells, including B cells, neutrophils and monocyte in PB, BM and spleen, based on the expression of the surface markers Gr-1, Mac-1 and B220.

Asxl1 KO transplanted mice did not show sign of MDS, with a normal myeloid commitment (data not shown), but they had some alterations. Indeed, despite a comparable white blood cells count, *Asxl1 KO* transplanted mice showed a significant decrease of red blood cells, hemoglobin and hematocrit, compared to control mice, suggesting anemia (Fig.28).

A



B



C

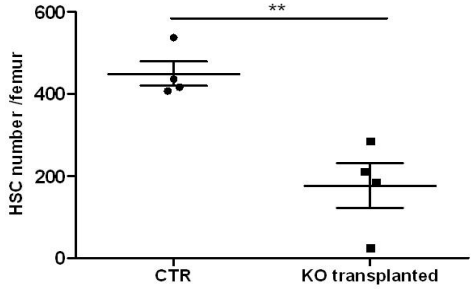


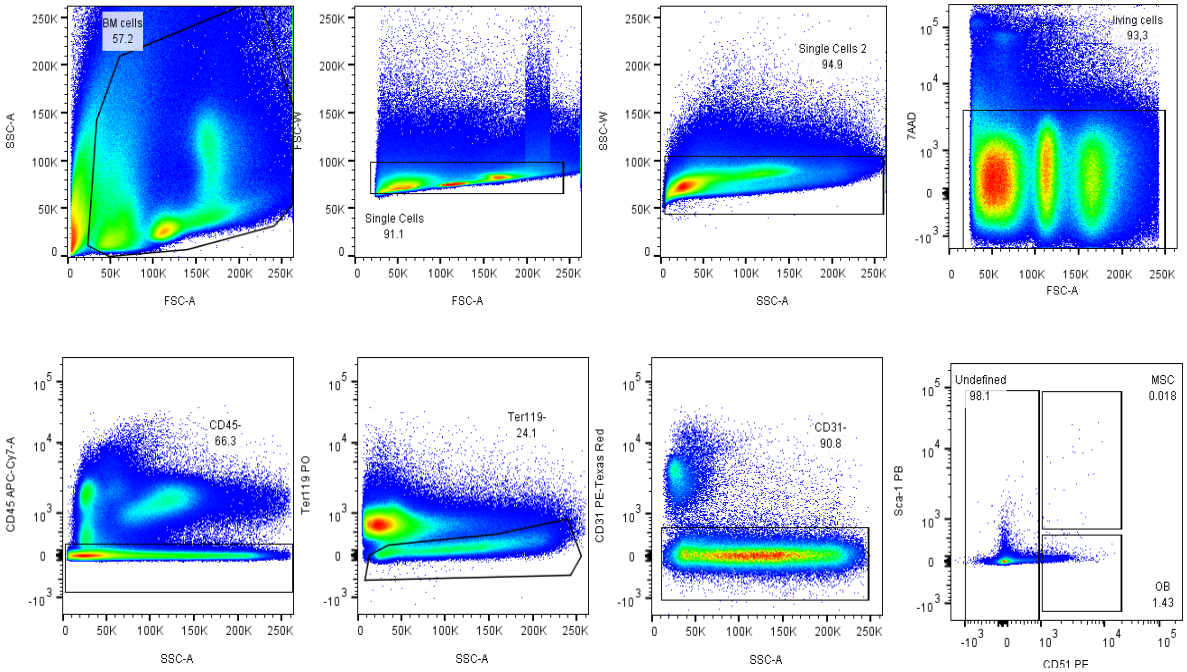
Figure 28. Mice phenotype at sacrifice. A) Chimerism of KO transplanted and control mice in the peripheral blood based on CD45.1/2 expression. **B)** *Asxl1 KO* transplanted mice had a comparable white blood cell count but a decreased red blood cells, hemoglobin, and hematocrit. **C)** *Asxl1 KO* transplanted mice showed lower HSC number per femur. B)C) *Asxl1* KO transplanted mice (n=4) control mice (n=4) (*p<0,05, ** p<0,01). B)C)D) The data are mean +/- SEM, *Asxl1* KO transplanted mice n=4, CTR mice n=4.

Interestingly, we found a decrease in HSC number (per femur), consistent with the study of Wang and collaborators, who reported a decrease of HSCs in germline *Asxl1* KO mice[107].

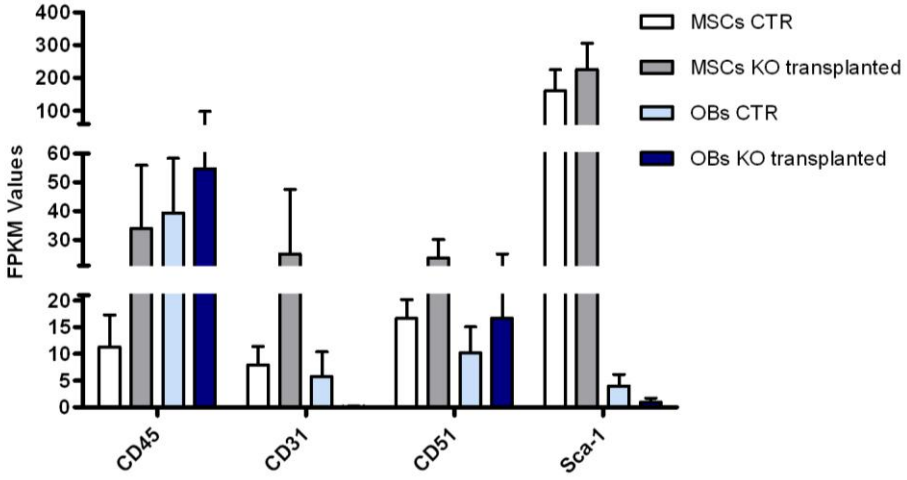
BM and bone fraction cells were collected, as described in the "materials and methods" section, in order to FACS-purify MSC and OB populations for RNA sequencing. As already described, we distinguished the 2 populations based on the following strategy: living cells, $CD45^+$, $Ter119^-$, $CD31^-$, Sca-1^+, $CD51^+$ (MSC)/$^-$ (OB)[350] (Fig.29a) and in this case we successfully obtained both OBs and MSCs and we performed RNA seq.

First of all, we validated the experiment checking gene expression of markers used for sorting, (i.e. CD45, CD31, CD51, Sca-1) MSC and OB established markers (i.e. the *receptors of Leptin, Pdgf, Pth1r, Gremlin-1 like protein, Osterix* and niche factors produced by stromal cells (i.e. *Cxcl12, Kitl)*. As expected, the cells were positive for stromal and niche factors and the expression of Sca-1 and CD51 reflected the sorting strategy. Indeed, MSCs were $CD51^+$ and Sca-1^+ while OBs were $CD51^+$ and Sca-1^-. Thus, despite a slight contamination of $CD45^+$ hematopoietic cells, we successfully sorted two pure populations (Fig.29b).

A


BM cells
57.2
SSC-A
FSC-A
Single Cells
91.1
FSC-W
Single Cells 2
94.9
SSC-W
living cells
93,3
7AAD
CD45-
66.3
CD45 APC-Cy7-A
Ter119-
24.1
Ter119 PO
CD31-
90.8
CD31 PE-Texas Red
Undefined
98.1
MSC
0.018
OB
1.43
Sca-1 PB
CD51 PE

B


FPKM Values
MSCs CTR
MSCs KO transplanted
OBs CTR
OBs KO transplanted
CD45
CD31
CD51
Sca-1

C

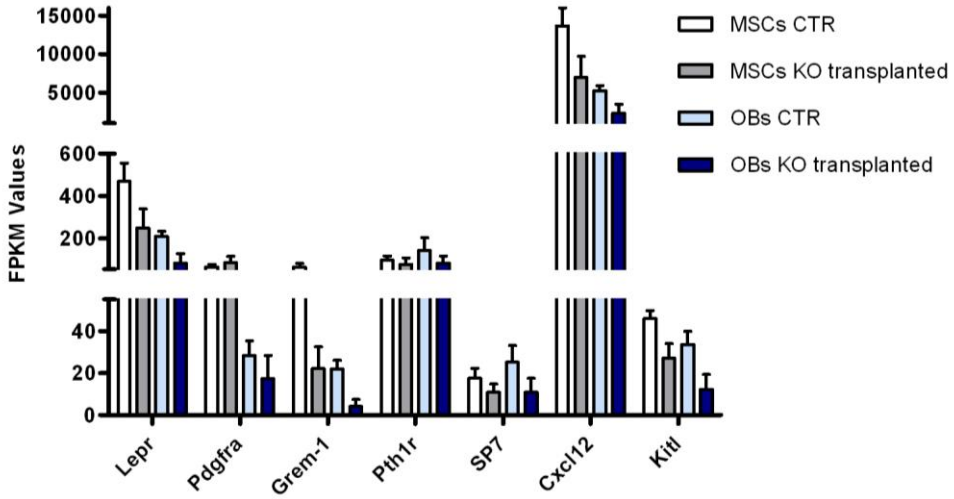

FPKM Values
MSCs CTR
MSCs KO transplanted
OBs CTR
OBs KO transplanted
Lepr
Pdgfra
Grem-1
Pth1r
SP7
Cxcl12
Kitl

Figure 29. Isolation and molecular characterization of MSCs and OBs. A) Representative FACS plots of gating strategy used to isolate OBs and MSCs: 7AAD-, CD45-,Ter119-, CD31-, CD51+, Sca-1-/+. Ter-119 (Lymphocyte antigen 76). Gene expression evaluated by RNA sequencing of OBs and MSCs, isolated from CTR and *Asxl1* KO transplanted mice. Transcriptional validation of the isolated stromal cells based on: **B)** expression of surface markers used for cell identification; **C)** expression of stromal cell markers, *Grem1* (Gremlin-1 like protein), *Sp7* (Osterix), and hematopoiesis supporting cytokines. B)C) Results are expressed as FPKM. The data are mean +/- SEM, *Asxl1* KO transplanted mice n=4, CTR mice n=3.

Principal component analysis of all transcriptomes showed that the different populations did no cluster together, indicating a heterogeneity of the transcriptomes (Fig.30).

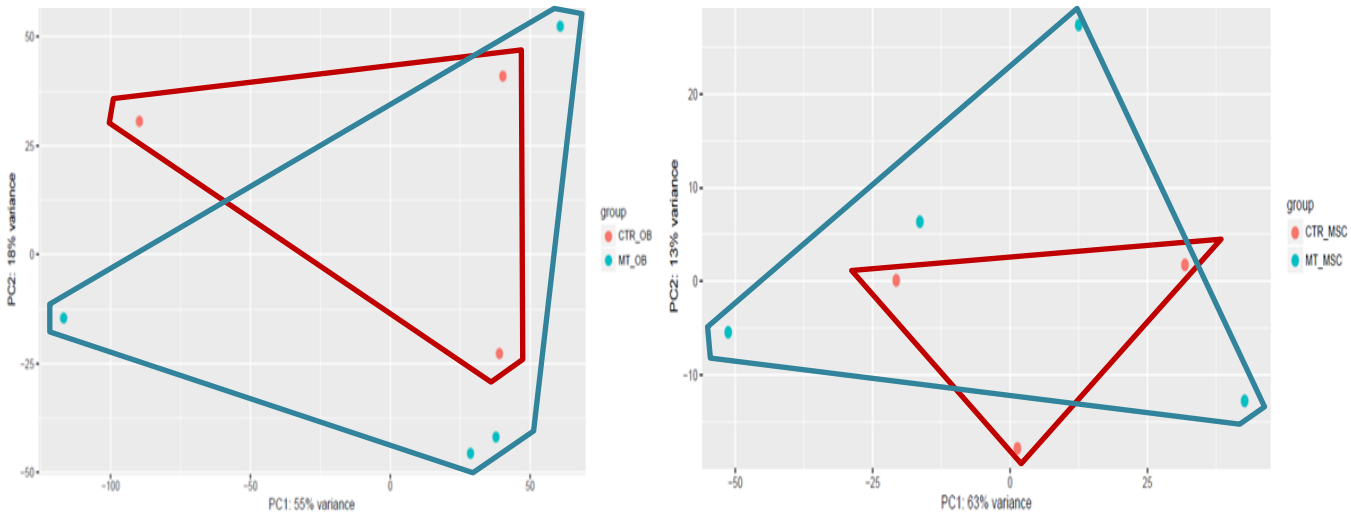


Figure 30. Principal component analysis revealed not clustered patterns of *Asxl1* KO transplanted mice transcriptome. Principal component analysis was performed on the fragment counts. *Asxl1* KO transplanted mice n=4, CTR mice n=3.

We then performed differential gene expression analysis by comparing MSCs and OBs isolated from *Asxl1* KO transplanted mice with cells isolated from their controls. The results showed 15 genes down-regulated and 1 gene up-regulated (p_{adj}<0.05) in OBs isolated from *Asxl1* KO transplanted mice (Tab.9a). The MSC population showed 31 genes differentially expressed (p_{adj}<0.05): 20 were up-regulated and 11 were down-regulated in cells isolated from *Asxl1* KO transplanted mice (Tab.9b). However, further studies are required to elucidate which genes could be potentially relevant for malignant transformation.

Table 9. Genes up-regulated and down-regulated in OBs and MSCs isolated from *Asxl1* KO transplanted mice and control mice.

9.a

OBs[1]			
Gene name	**Avg *Asxl1* KO transp. mice[2]**	**Avg CTR**	**FC**
Up-regulated			
Tuba8	20,59	0,00	11,01
Down-regulated			
Sspo	0,00	2,53	-11,13
Emcn	0,00	10,84	-9,62
Epx	0,00	13,77	-10,52
Fam181b	0,00	14,56	-9,75
Hcn2	0,01	7,85	-9,03
Plcd4	0,00	6,22	-9,84
Ube2ql1	0,00	8,72	-9,83
Cdhr3	0,00	7,21	-10,13
Lrrn1	0,00	12,43	-11,42
Rasl10b	0,00	14,87	-11,22
Scg3	0,00	9,39	-10,11
Gap43	0,00	14,95	-9,62
Ptpro	0,00	5,26	-10,87
Nrxn1	0,00	1,07	-9,29

9.b

MSCs							
Gene names	**Avg *Asxl1* KO transp. mice**	**Avg CTR**	**FC**	**Gene names**	**Avg *Asxl1* KO transp. mice**	**Avg CTR**	**FC**
Up-regulated				**Down-regulated**			
Sox13	5,94	0,03	6,42	*Zfp618*	0,01	4,83	-9,03
9130023H24Rik	22,20	4,66	2,24	*Cry1*	0,00	1,36	-9,09
Tbc1d30	1,15	0,01	6,63	*Ttf1*	1,55	7,71	-2,36
A430035B10Rik	0,81	0,01	7,09	*Phlpp2*	0,00	0,60	-8,61
Etv4	1,55	0,00	7,82	*Zfp217*	0,00	2,26	-8,77
Ncmap	4,36	0,00	8,56	*Col2a1*	0,01	2,34	-8,02
Slc7a4	115,66	3,49	5,20	*Kif5c*	0,60	3,61	-2,60
Mfsd9	2,02	0,00	8,90	*Tjp2*	0,00	4,05	-6,48
Lect1	32,50	0,53	6,05	*1700017G19Rik*	0,01	1,51	-5,91
Ptchd1	4,79	0,01	8,08	*Gm15694*	0,00	4,20	-8,19
Ankrd13d	3,27	0,00	8,85	*Nox4*	1,97	11,14	-2,54
Kcnj2	2,40	0,00	10,14	*Zfp618*	11,14	1,97	-2,54
Zfp770	0,75	0,01	7,07				
Gramd1c	1,26	0,00	8,61				
Prx	4,96	0,00	9,25				
AA415398	11,56	1,71	2,60				
Arhgap10	7,66	1,77	1,91				
Cdkn2b	4,80	0,00	8,23				
Trib3	3,58	0,00	8,57				
Usp31	2,14	0,45	2,20				

The genes are listed based on p_{adj} values.
[1] Osteoblasts (OBs)
[2] *Asxl1* KO transplanted mice n=4, CTR mice n=3
[3] Fold change (FC)
[4] Mesenchymal stromal cells (MSCs)

We next performed the GSEA analysis. In the OB population, we identified 570 gene sets enriched in cells isolated from *Asxl1* KO transplanted mice and 632 enriched in cells isolated from control mice (Tab.10). In contrast, in the MSC population, only 2 pathways were enriched in cells isolated from *Asxl1* KO transplanted mice and 1 was enriched in cells isolated from control mice (Tab.11).

Table 10. The 10 pathways most enriched in OBs isolated from *Asxl1* KO mice and control mice.

OBs					
Enriched in *Asxl1* KO transplanted[1]			**Enriched in CTR**		
NAME	**NES**[2]	**FDR**[3]	**NAME**	**NES**	**FDR**
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	2,40	0,0	KEGG_ASTHMA	-1,96	0,07
KONG_E2F3_TARGETS	2,39	0,0	ZHAN_MULTIPLE_MYELOMA_MS_UP	-1,87	0,17
SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	2,36	0,0	NABA_CORE_MATRISOME	-1,85	0,16
REACTOME_PEPTIDE_CHAIN_ELONGATION	2,36	0,0	PICCALUGA_ANGIOIMMUNOBLASTIC_ LYMPHOMA_UP	-1,85	0,13
KEGG_RIBOSOME	2,35	0,0	LIN_TUMOR_ESCAPE_FROM_IMMUNE_ATTACK	-1,84	0,11
REACTOME_TRANSLATION	2,33	0,0	MEISSNER_NPC_HCP_WITH_H3K27ME3	-1,83	0,12
ISHIDA_E2F_TARGETS	2,31	0,0	GAURNIER_PSMD4_TARGETS	-1,84	0,11
ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	2,3	0,0	NABA_ECM_GLYCOPROTEINS	-1,84	0,10
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_ TARGETING_TO_MEMBRANE	2,29	0,0	KIM_GLIS2_TARGETS_UP	-1,79	0,14
KOBAYASHI_EGFR_SIGNALING_24HR_DN	2,29	0,0	MIKKELSEN_ES_HCP_WITH_H3_UNMETHYLATED	-1,78	0,14

[1] *Asxl1* KO transplanted mice n=4, CTR mice n=3.
[2] Normalized enrichment score (NES)
[3] False discovery rate (FDR) values.

Table. 11 The pathways enriched in MSCs isolated from *Asxl1* KO transplanted mice and control mice.

MSCs					
Enriched in KO transplanted[1]			**Enriched in CTR**		
NAME	**NES**[2]	**FDR**[3]	**NAME**	**NES**	**FDR**
WEBER_METHYLATED_LCP_IN_SPERM_UP	1,92	0,0	CHOI_ATL_ACUTE_STAGE	-1,96	0,7
BROWN_MYELOID_CELL_DEVELOPMENT_UP	1,86	0,1			

[1] *Asxl1* KO transplanted mice n=4, CTR mice n=3.
[2] Normalized enrichment score (NES)
[3] False discovery rate (FDR) values.

In the OB population, isolated from *Asxl1* KO transplanted mice we found enrichment in several pathways, including gene sets involved in:

- Translation and transcription;
- Cell cycle;
- Apoptosis;

- Myc activity;
- Cancers;
- Metabolism, including Krebb cycle, respiratory electron transport
- Cellular stress in response to UV;
- Telomere maintenance, packaging, and shortening.

Pathways enriched in the OB population isolated from control mice resulted mainly involved in:

- Hypoxia;
- Inflammation;
- Adipogenesis;
- Response to EGF.

No common enriched pathways were identified, between MSCs and OBs.

2.2.1.3 RNA sequencing data comparisons of *in vivo* experiments

With the aim to identify common genes or pathways dysregulated in stromal cells by the hematopoietic loss of *Dnmt3a* and *Asxl1*, we compared the results of *in vivo* experiments, performed by transplanting *Dnmt3a/Asxl1* KO cells and their controls in WT recipient.

First, we compared gene expression analysis of stromal cells isolated from *Dnmt3a* KO transplanted mice with that of OBs isolated from *Asxl1* KO transplanted mice and we could not identify any overlap. When we compared the results of stromal cells isolated from *Dnmt3a* with that of MSCs isolated from *Asxl1* KO transplanted mice, we found 2 genes (*Tjp2*, *Zfp770*) in common. However, they were up-regulated in MSCs isolated from *Asxl1* KO transplanted mice, while they were down-regulated in stromal cells isolated from *Dnmt3a* KO transplanted mice.

Second, we compared the GSEA analysis and we found similarities between stromal cells and OBs isolated from *Dnmt3a* and *Asxl1* KO transplanted mice, respectively. Indeed, 28 pathways were enriched (Tab.12), and interestingly, the common pathways were mainly related to telomere maintenance, packaging, and shortening, meiotic recombination and to cellular stress in response to UV, suggesting an ongoing stress in stromal cells, potentially determined by *Dnmt3a*- and *Asxl1*-null cells in BM niche.

Table 12. 28 pathways enriched in stromal cells isolated from *Dnmt3a* KO transplanted mice and OBs from *Asxl1* KO transplanted mice.

WELCH_GATA1_TARGETS
BIOCARTA_AHSP_PATHWAY
REACTOME_AMYLOIDS
STEINER_ERYTHROCYTE_MEMBRANE_GENES
VALK_AML_CLUSTER_7
IVANOVA_HEMATOPOIESIS_MATURE_CELL
DAZARD_UV_RESPONSE_CLUSTER_G24
REACTOME_PACKAGING_OF_TELOMERE_ENDS
LIANG_SILENCED_BY_METHYLATION_UP
REACTOME_RNA_POL_I_PROMOTER_OPENING
SANCHEZ_MDM2_TARGETS
OLSSON_E2F3_TARGETS_DN
GENTILE_UV_HIGH_DOSE_UP
HASLINGER_B_CLL_WITH_6Q21_DELETION
DAZARD_UV_RESPONSE_CLUSTER_G2
LY_AGING_MIDDLE_DN
REACTOME_METABOLISM_OF_PORPHYRINS
XU_HGF_SIGNALING_NOT_VIA_AKT1_48HR_DN
REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_ AT_THE_CENTROMERE
REACTOME_MEIOTIC_SYNAPSIS
REACTOME_MEIOTIC_RECOMBINATION
DACOSTA_LOW_DOSE_UV_RESPONSE_VIA_ERCC3_XPCS_UP
OHASHI_AURKB_TARGETS
CHEMELLO_SOLEUS_VS_EDL_MYOFIBERS_UP
REACTOME_TELOMERE_MAINTENANCE
GRAHAM_CML_QUIESCENT_VS_NORMAL_QUIESCENT_UP
GREENBAUM_E2A_TARGETS_UP
WIEMANN_TELOMERE_SHORTENING_AND_CHRONIC_LIVER_DAMAGE_UP

We also compared the GSEA analysis of stromal cells isolated from *Dnmt3a* KO transplanted mice with that of MSCs isolated from *Asxl1* KO transplanted mice and we found 1 pathway (BROWN_MYELOID_CELL_DEVELOPMENT_UP) that was enriched in both the data set. Furthermore, when we compared the pathways enriched in cells isolated from the control mice, we found only 2 pathways (REACTOME_ASPARAGINE_N_LINKED_GLYCOSYLATION, KEGG_N_GLYCAN_BIOSYNTHESIS) in common between stromal cells and OBs isolated from *Dnmt3a* and *Asxl1* control mice and any in common with MSCs isolated from *Asxl1* control mice. Nevertheless, the comparison of the two set of experiments *in vivo* highlighted the enrichment of many pathways with opposite trends. Indeed, 104 pathways were enriched in cells isolated from

Dnmt3a KO transplanted mice and in OBs isolated from control mice, for example, pathways involved in the response of inflammation such as SCHOEN_NFKB_SIGNALING, SANA_TNF_SIGNALING_UP. Moreover, 206 pathways were enriched in stromal cells from *Dnmt3a* control mice and OBs from *Asxl1* KO transplanted mice, for example, pathways regulated by Myc such as DANG_MYC_TARGETS_UP, DANG_REGULATED_BY_MYC_UP. This finding suggests that most of the effects of the two mutations on stromal cells are very different and opposite.

2.2.2 *In vitro* experiments

We proposed to simulate transcriptome modulation found *in vivo* and to understand if the dysregulations identified in mice were directly induced by *Dnmt3a/Asxl1* KO LSK (Lin-, sca-1+, c-kit+), a population enriched in HSPCs, which are known to be in close contact with stromal cells *in vivo*[170]. To this aim, we performed *in vitro* experiments based on co-culture of OP9, a murine stromal cell line, and LSK isolated from BM cells with the hematopoietic-specific deletion of *Dnmt3a* and *Asxl1*. After co-cultures, OP9 were collected, RNA was extracted and massive transcriptome sequencing was performed.

2.2.2.1 The loss of *Dnmt3a* in hematopoietic cells doesn't affect the transcriptome of OP9

As BM-derived cells with the hematopoietic-specific deletion of *Dnmt3a* (hereafter referred to *Dnmt3a* KO cells), we used cells isolated from Rosa26-CreERT2;Dnmt3a$^{f/f}$ mice, and BM-derived cells isolated from Rosa26-CreERT2;Dnmt3a$^{+/+}$ mice were used as controls. Cells were kindly provided by Dr. Mukherjee and collaborators. In *Dnmt3a*-null cells, we first confirmed *Dnmt3a* excision by genomic PCR, as described before (data not shown). Subsequently, from BM-derived *Dnmt3a* KO cells and their controls, we FACS-isolated LSK cells, based on the lack of expression of lineage commitment markers, i.e. CD8a, CD3, Gr-1, B220, Mac-1, Ter119, CD4, and the expression of Sca-1 and c-kit. We then performed co-cultures with LSK cells and the murine stromal cell line, OP9 at the ratio 1:10 respectively. After 6 days we collected OP9 for RNA extraction and sequencing and we analyzed the LSK phenotype by flow cytometry. In particular, we collected the cells in the supernatant of the co-culture and we stained the cells for Lineage commitment antigens, Sca-1, and c-kit detection. We found that most of the cells were still undifferentiated, negative for lineage markers, but the LSK population was dramatically reduced

(data not shown). In addition, we observed during co-cultures a profound increase of $CD45^+$ hematopoietic cell number. Indeed, at the beginning of co-cultures, LSK number was 10 times less than OP9, 6 days later the number of hematopoietic cells $CD45^+$ was higher, almost 3 times more than OP9 cells (Fig.31) without differences between KO and controls.

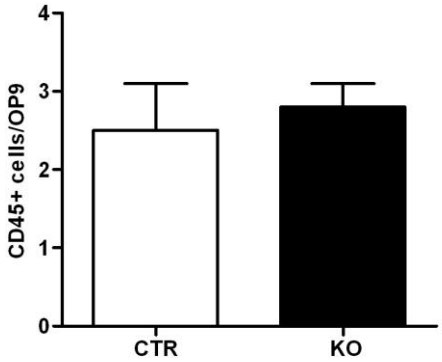


Figure 31. Hematopoietic cells expanded after co-cultures. Ratio between the number of hematopoietic cell ($CD45^+$) and the number of OP9 FACS purified. The data are mean +/- SEM (n=2)

In parallel, after co-cultures, we FACS-purified the OP9 population according to the negative expression for CD45 and LSK markers, such as lineage commitment antigens and c-kit, with the exception of Sca-1, whose expression has been described in OP9 cells (Fig.32). Next, we performed RNA seq of the population isolated. We then first validated the experiment checking for the expression of markers used for sorting. We found that OP9 FACS-isolated cells were negative for CD45, lineage markers, c-kit and positive for Sca-1. In addition, we also checked for the expression of markers which characterize OP9 cells. As expected, the cells did not express CD34, CD31, CD11b, CD31, CD34, CD86, FLK1 whereas expressed CD44, CD29, Sca-1 (Fig.32). In conclusion, after co-cultures, OP9 cells were sorted without an hematopoietic contamination.

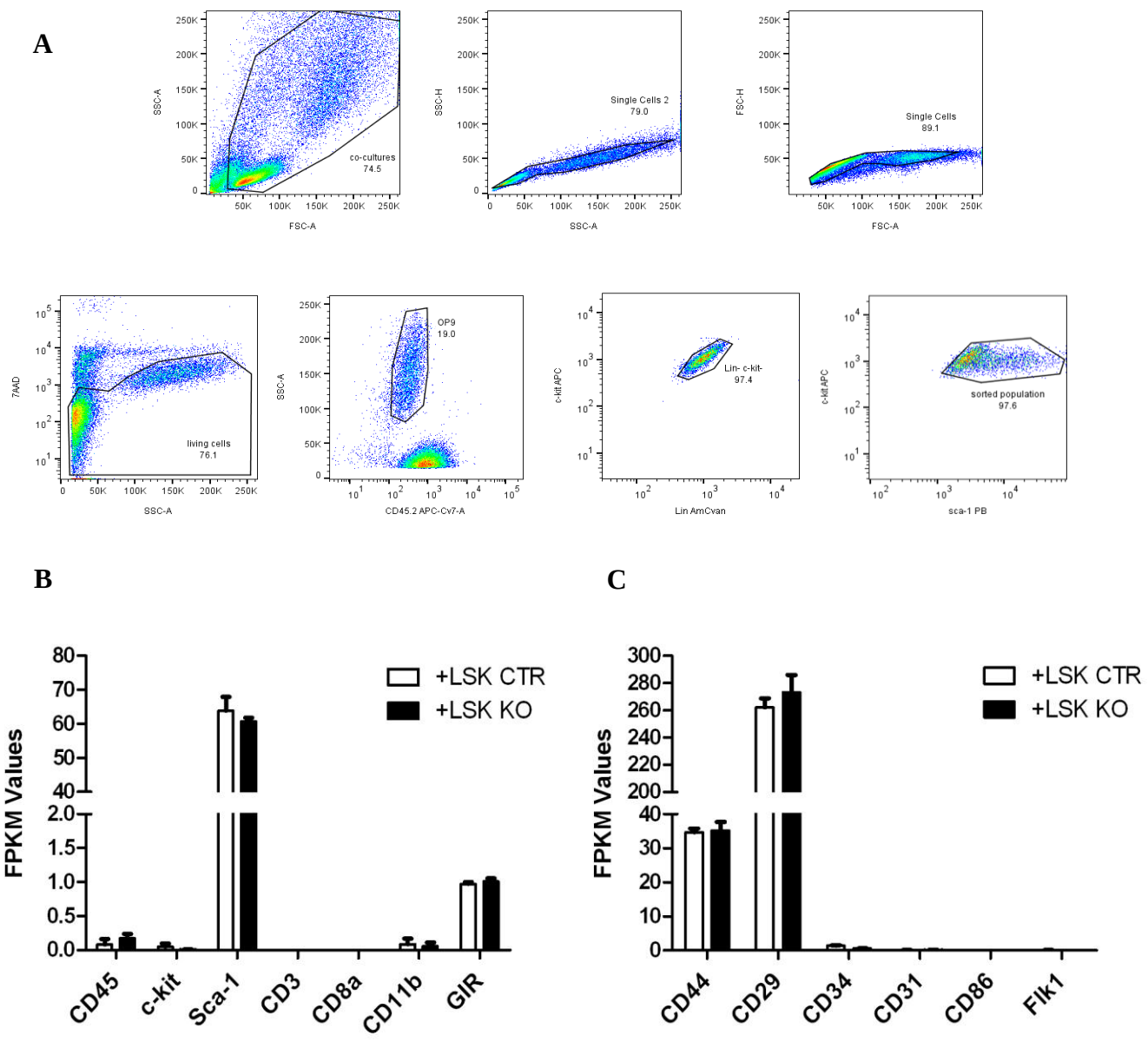


Figure 32. Isolation and molecular characterization of OP9. A) Representative FACS Plot of gating strategy used to isolate OP9: 7AAD-, CD45-, Lineage -,c-kit-, Sca-1+. c-kit (kit: proto-oncogene receptor tyrosine kinase), Lineage cocktail was composed of CD3 (CD3 Antigen) CD8a (CD8a antigen), CD11b (ITGAM: Integrin subunit alpha M), Gir (GPR83: G protein-coupled receptor 83). Gene expression evaluated by RNA sequencing of OP9 after co-cultures: **B)** expression of surface markers used for cell isolation; **C)** expression of OP9 cell markers. CD44 (CD44 Antigen), CD29 (ITGB1 Integrin Subunit Beta 1), CD34 (CD34 Antigen), CD86 (CF86 Antigen), Flk1 (FDR Kinase Insert domain receptor). B)C) Results are expressed as FPKM. The data are mean +/- SEM (n=2).

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Initially unanticipated the differential gene expression analysis did not reveal any gene differentially expressed (p_{adj}<0.05) between OP9 in contact with LSK *Dnmt3a* KO or controls. We then considered to use the p-value without normalization, indeed the small number of samples allowed a less stringent statistical analysis. In this second analysis, we identified 233 genes differentially expressed, 98 up-regulated and 135 down-regulated in OP9 in contact with LSK *Dnmt3a* KO cells

(Tab.13). A further analysis is fundamental to identify genes which could potentially be involved in myeloid transformation.

Table 13. 10 first genes up-regulated and down-regulated in OP9 in contact with *Dnmt3a* KO LSK

Gene name	OP9+KO LSK[1]	OP9+CTR LSK	FC[2]	Gene name	OP9+KO LSK	OP9+CTR LSK	FC
Up-regulated				**Down-regulated**			
Lmntd2	0,42	0,00	7,26	*Mpo*	0,56	2,12	-1,97
Plbd1	0,21	0,00	5,75	*Malat1*	92,76	133,50	-0,52
Hspa4l	1,96	0,79	1,30	*Ankrd6*	0,00	0,38	-7,31
Ahnak	16,19	15,33	0,32	*Rcsd1*	0,00	0,47	-6,69
Tns4	0,12	0,00	6,38	*Dna2*	0,21	1,20	-2,27
Cpa6	0,50	0,00	6,10	*Slc7a3*	0,00	0,25	-6,48
Prpf18	15,10	10,79	0,48	*Zfp12*	4,36	7,38	-0,73
Plxdc1	0,39	0,00	6,40	*Lcp2*	0,01	0,35	-5,68
Evc	8,97	4,87	0,84	*Car2*	0,00	0,66	-6,50
Ctu1	7,79	3,82	1,04	*Col17a1*	0,00	0,21	-6,46

The genes are listed based on p-values.
[1] n=2
[2] Fold change (FC)

The GSEA analysis revealed only 7 pathways significantly enriched in OP9 in contact with CTR LSK (Tab.14) and 0 enriched in OP9 in contact with *Dnmt3a* KO cells.

Table 14. The pathways enriched in OP9 in contact with CTR LSK.

Enriched in OP9+CTR LSK		
NAME	**NES[1]**	**FDR[2]**
ZHAN_MULTIPLE_MYELOMA_PR_UP	-1,84	0,10
KANG_DOXORUBICIN_RESISTANCE_UP	-1,82	0,08
ISHIDA_E2F_TARGETS	-1,82	0,06
KONG_E2F3_TARGETS	-1,81	0,05
ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	-1,75	0,15
INAMURA_LUNG_CANCER_SCC_UP	-1,73	0,19
GAVIN_FOXP3_TARGETS_CLUSTER_P6	-1,72	0,21

[1] n=2
[2] Normalized enrichment score (NES)
[3] False discovery rate (FDR) values

We also compared the results of *in vitro* experiments with the results of *Dnmt3a* KO cell transplantation experiments. In OP9, in contact with LSK *Dnmt3a* KO, and stromal cells isolated from *Dnmt3a* KO transplanted mice, we found 12 genes differentially expressed: 4 (*Rnf11, Frmd4a, Tab3, Dstyk*) were up-regulated and 1 (*Zfp12*) was down-regulated in both groups. The other 7

genes showed opposite expression levels. The comparison of the GSEA analysis did not highlight any similarity between *in vitro* and *in vivo* experiments.

In conclusion, in our experimental settings, we could not identify profound modulations in the OP9 transcriptome, mediated by *Dnmt3a* KO HSPCs *in vitro*.

2.2.2.2 The hematopoietic-specific loss of *Asxl1* doesn't affect the transcriptome of OP9

In parallel, co-culture experiments, similar to those described before were performed with *Asxl1*-null LSK. In particular, we isolated LSK cells from BM cells of Mx1-Cre;Asxl1$^{f/f}$ mice (hereafter referred to *Asxl1* KO cells). LSKs isolated from BM cells of Mx1-Cre;Asxl1$^{+/+}$ mice were used as controls. Cells were kindly provided by Dr. Abdel-Wahab and collaborators.

We first confirmed *Asxl1* excision in *Asxl1*-null cells, by genomic PCR, as described before (data not shown). From BM-derived *Asxl1* KO cells and their controls, we FACS-isolated LSK cells and we performed one co-culture with OP9 cells, at the ratio 1:10 respectively. After 6 days, we collected OP9 cells and cells in the supernatant, and by evaluating the percentage of CD45^{+} cells, we observed a huge increase of the hematopoietic cell number (Fig.33), comparable to the *Dnmt3a* co-cultures. *Asxl1* KO hematopoietic cells expanded less than controls.

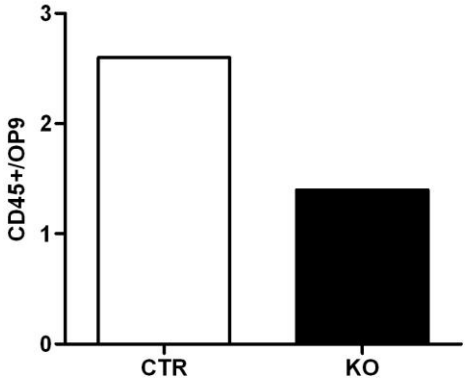

Figure 33. Hematopoietic cells expanded after co-cultures. Ratio between the number of hematopoietic cells (CD45+) and OP9 FACS-purified (n=1).

Next, we FACS-purified OP9 cells, as previously described, and we performed RNA sequencing. We than validated the experiment checking gene expression of markers used for sorting and markers which characterize OP9 cells, as described before. We concluded that the OP9 population we sorted was pure, without hematopoietic contamination.

We then performed the differential gene expression analysis. Interestingly, we could not identify any gene differentially expressed (p_{adj}<0.05) between OP9 in contact with LSK *Asxl1* KO and CTR

LSK. We decided to analyze the data set based on p-value, without any normalization, as well as we did for *Dnmt3a* co-cultures. We found 40 genes differentially expressed, 15 were up-regulated and 25 were down-regulated in OP9 in contact with *Asxl1* KO LSK (Tab.15). Also, in this case, a deep analysis is fundamental to identify genes with a relevance in malignant transformation.

Table 15. Genes up-regulated and down-regulated in OP9 in contact with *Asxl1* KO LSK. The genes are listed based on p-value.

Gene name	OP9+KO LSK[1]	OP9+CTR LSK	FC[2]	Gene name	OP9+KO LSK	OP9+CTR LSK	FC
Up-regulated				**Down-regulated**			
Abcc4	10,77	6,94	0,62	*Prex1*	0,01	0,49	-5,23
Arap2	1,36	0,54	1,32	*Plac8*	1,24	12,05	-2,66
Uggt1	19,14	15,15	0,33	*Fam101a*	0,07	1,82	-5,10
Nxf2	0,62	0,02	5,00	*Dock2*	0,00	0,39	-5,00
Aldh1l2	8,43	5,66	0,57	*Os9*	56,56	69,78	-0,31
Pcnx	1,39	0,68	0,99	*Egln1*	36,51	48,11	-0,39
Dcbld2	11,37	8,29	0,45	*Adm*	82,12	110,22	-0,46
Fam208b	2,93	1,74	0,71	*Nrros*	0,29	1,54	-2,30
Ifit1	6,28	3,27	0,96	*Cd34*	0,94	3,51	-1,84
Trip11	4,18	2,63	0,67	*Ccr2*	0,23	1,01	-2,09
Zcchc24	55,37	46,31	0,27	*Itgb7*	0,68	2,55	-2,08
Nrp2	8,36	5,31	0,63	*Fam162a*	147,87	196,38	-0,49
Irgm2	3,85	1,82	1,09	*Pfkp*	20,91	27,37	-0,40
Nsd1	1,19	0,62	0,87	*Gpnmb*	20,08	26,66	-0,41
Spc25	60,15	45,52	0,41	*Higd1a*	29,97	42,61	-0,48
				Prkcg	0,00	0,67	-4,64
				Rcn3	294,91	344,01	-0,25
				Ptprc	0,12	0,53	-2,32
				Zfp128	1,03	2,42	-1,23
				Plekha2	17,15	21,91	-0,36
				Nln	11,64	16,57	-0,44
				Ctsa	106,53	124,14	-0,23
				Ctsd	855,26	945,37	-0,16
				Kdm4b	17,36	22,65	-0,42
				Cxcl12	2617,84	2881,28	-0,13

[1] n=1
[2] Fold change (FC)

We then performed the GSEA analysis and we found only 3 gene sets differentially expressed between OP9 in contact with LSK *Asxl1* KO and in contact with CTR LSK. 1 pathway was enriched in OP9 in contact with KO LSK (BERGER_MBD2_TARGETS) and 2 were enriched in OP9 in contact with WT cells (WEBER_METHYLATED_ICP_IN_SPERM_DN, RASHI_RESPONSE_TO_IONIZING_RADIATION_6).

Subsequently, we compared the co-culture results with the results of *in vivo* experiments, performed by transplanting *Asxl1* KO cells in WT recipients. We could not identify genes whose expression was modulated in both OBs/MSCs isolated from *Asxl1* KO transplanted mice and OP9 cells. We

also compared the results of *Asxl1* co-cultures with those of *Dnmt3a* co-cultures and we found 2 genes (*cd34*, *Prex-1* fpkm<1) up-regulated in OP9 in contact with both LSK *Dnmt3a* and *Asxl1* KO cells.

When we compared the GSEA analysis we found that in common between OP9 in contact with *Asxl1* KO LSK and OBs isolated from *Asxl1* KO transplanted mice, there was 1 pathway enriched (WEBER_METHYLATED_ICP_IN_SPERM_DN), whereas no overlap with MSCs. Also when we compared the GSEA results of *Asxl1* co-cultures with the GSEA obtained from *Dnmt3a* co-culture experiments we did not find any similarities. In our experimental settings, we did not identify profound modulations of OP9 cell transcriptome induced by *Asxl1* KO HSPC *in vitro*.

2.2.3 LR-MDS patients

We subsequently performed a comparison between transcriptome sequencing results. Indeed, we compared the transcriptome of stromal cells obtained from *in vivo* transplantation experiments with that of MSCs isolated from LR-MDS patients. The aim was to elucidate among the different modifications of transcriptome identified, which were in common and potentially relevant for human MDS.

2.2.3.1 LR-MDS patient characteristics

In his laboratory, Dr. Raaijmakers' group performed massive RNA sequencing of the transcriptome of highly purified MSCs, isolated from low-risk MDS patients (MDS-MSCs), to study alterations in the context of *Dnmt3a* and *Asxl1* mutations in human bone marrows (unpublished data).

MSCs were FACS-purified according to the following surface marker expression: living cells, $CD45^-$, $CD31^-$, $CD235a^-$, $CD271^+$, $CD105^+$, from BM cells isolated from low-risk MDS patients harboring *DNMT3A* (n=8) or *ASXL1* mutations (n=10), at diagnosis. Highly purified MSCs isolated from healthy donors (HD-MSCs, n=10) were used as controls. Patient and healthy donor characteristics were summarized in Table 16.

Table 16. Patient and healthy donor characteristics

16.a

Patient ID	Age	WHO[1]	Cytogenetics	BM Blasts %	IPSS[2]	Genetic aberrations
MDS627	80	RCMD	46,XY[20]	3	0	*ASXL1, TET2*[3]
MDS008	67	RCMD	46,XX[20]	2	0.5	*ASXL1, EZH2*[4]
MDS069	76	CMML-1	46,XY,der(13)t(1;13)(q11;p11)[10]	7	1.0	*ASXL1, IDH2*[5], *SF3B1*[6], *TET2*
MDS079	59	RCMD	47,XY,t(2;14)(q3?7;q2?2),idic(21)(p1?2),+idic(21)(p1?2)[8]	2	1.0	*ASXL1*
MDS135	75	RCMD-RS	46,XX[20]	1	0	*ASXL1, TET2*
MDS183	62	MDS-U	47,XX,+8[12]	2	0.5	*ASXL1, IDH2, SRSF2*[7]
MDS203	81	RCMD-RS	46,XY,del(5)(q13q31)[7]/47,XY,del(5)(q13q31),+8[4]/46,XY[1]	2	0.5	*ASXL1, SF3B1*
MDS646	74	RAEB-1	46,XX[20]	5	1	*ASXL1*
MDS144	79	RCMD	47,XY,+19[6]/48,idem,+21[3]/46,XY[4]	4	1.0	*ASXL1, U2AF1*[8]
MDS101	79	RCMD-RS	46,XY[21]	1	0.0	*ASXL1, DNMT3A,SF3B1*
MDS610	64	RCMD-RS	46,XX[20]	3	0.5	*TET2, DNMT3A, SF3B1, RUNX1*[9]
MDS061	59	RAEB-1	46,XY[20]	8	0.5	*DNMT3A, SF3B1, TET2*
MDS006	62	RAEB-1	46,XX[20]	5	1	*DNMT3A, SRSF2*
MDS118	55	RARS	46,XX[20]	0	0	*TET2, DNMT3A, SF3B1*
MDS247	59	RCMD-RS	46,XY[20]	0	0	*DNMT3A , SF3B1*
MDS158	80	RCMD-RS	46,XX[20]	0	0	*DNMT3A, SF3B1 , TET2*
MDS182	85	RCMD	46,XY,del(5)(q13q33)[10]/46,XY[10]	2	0.0	*DNMT3A, TET2*

16.b

Healthy donors	Age	Cytogenetics	BM blasts	Genetic aberrations
N10410	58	NE[10]	NP[11]	NE
N10513	40	NE	NP	NE
N12723	48	NE	NP	NE
N15863	42	NE	NP	NE
N14207	61	NE	NP	NE
N16237	40	NE	NP	NE
N11703	39	NE	NP	NE
N08276	58	NE	NP	NE
N14167	48	NE	NP	NE
N12066	35	NE	NP	NE

[1] WHO classification 2008[351]
[2] International Prognostic scoring system (IPSS)[13]
[3] *TET* methylcytosine Dioxygenase 2 (TET2)
[4] *EZH2* (Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit)
[5] *IDH2* (Isocitrate Dehydrogenase, NADPH, mitochondrial 2)
[6] *SF3B1* (Splicing Factor 3b Subunit 1)
[7] *SRSF2* (Serine And Arginine Rich Splicing Factor 2)
[8] *U2AF1 (*Small Nuclear RNA Auxiliary Factor 1)
[9] *RUNX1* (Runt Related Transcription Factor 1)
[10] NE, not evaluated
[11] NP, not present

RNA sequencing was performed on MSCs and modulation of distinct genes and pathways were identified in MSCs isolated from MDS patients, strengthening the hypothesis that MSCs are altered in BM of patients affected by myeloid malignancies. In addition, differences were identified between alterations in MSCs isolated from *DNMT3A* and *ASXL1* mutated patients (unpublished data).

2.2.3.2 *Dnmt3a* KO transplanted mice and MDS patients

We compared the results of RNA sequencing of stromal cells isolated from *Dnmt3a* KO transplanted mice with that of MSCs isolated from *DNMT3A* mutated MDS patients, in order to identify common alterations induced by *Dnmt3a* mutated cells, relevant to human MDS.

First, we compared gene expression. We found 31 overlapping genes: 7 genes (*Ankrd16, Ptp4a3, Thsd4, Crot, Ppp1r3b, Tbc1d17, Slfn1*) were up-regulated whereas 4 genes *(Dnajc2, Hbegf, Ppt2, Xrn2)* were down-regulated in both MDS-MSCs and stromal cells isolated from *Dnmt3a* KO transplanted mice. The other 20 genes showed inverse trends: up-regulated in one group and down-regulated in the other (Tab.17). However, a further investigation is required to understand if those genes are really relevant in MDS.

Table 17. Genes differentially expressed in common between murine experiments and human data. Genes are listed based on p_{adj} values of human MSCs.

Gene name	Avg MDS-MSCs[1]	Avg HD-MSCs	FC[2]	Padj[3]	Avg *Dnmt3a* KO transp. mice[4]	Avg CTR mice	FC	padj
Tmtc4	0,05	1,12	3,73	0,00	0,00	1,42	-6,48	0,00
Tnfrsf17	0,00	5,90	5,05	0,00	0,00	1,80	-7,04	0,02
Ankrd16	0,16	1,06	2,45	0,00	5,56	1,61	2,07	0,00
Arrdc3	86,42	29,33	-2,19	0,00	44,67	17,91	1,79	0,02
Dnajc2	12,75	5,22	-2,19	0,00	15,02	42,71	-1,35	0,04
Nlrc4	0,00	0,28	4,62	0,00	0,00	0,34	-6,03	0,05
Map1lc3b	27,16	11,96	-1,87	0,00	261,71	137,03	1,42	0,04
Cx3cr1	0,00	0,73	4,54	0,00	0,00	1,25	-9,05	0,01
Nsg1	13,65	1,02	-3,52	0,00	3,40	1,10	2,10	0,00
Ptp4a3	0,02	1,62	3,60	0,00	117,38	43,68	1,98	0,00

[1] HD-MSCs n=10, MDS-MSCs n=8
[2] Fold change (FC)
[3] $p_{adj}<0,05$
[4] *Dnmt3a* KO transplanted mice n=4, CTR mice n=4.

Second, we compared the GSEA analysis. Interestingly we found 68 pathways enriched both in MDS-MSCs and stromal cells isolated from *Dnmt3a* KO transplanted mice. The common pathways were mainly involved in adipogenesis, cellular stress in response to UV, telomere maintenance/packaging and inflammation mediated by NF-kB and in response to TGF-β1 and IL-22 (Tab.18), suggesting a common stress in those cells and the potential activation of pathways in response to inflammatory stimuli. On the contrary, stromal cells isolated from control mice and HD-MSCs did not show overlapping gene sets. We also identified dissimilarities, indeed 48 pathways showed opposite trends: enriched in MDS-MSCs and stromal cells isolated from control mice.

Table 18. 68 pathways enriched in MSCs isolated from LR-MDS and stromal cells isolated from *Dnmt3a* KO transplanted mice.

BURTON_ADIPOGENESIS_1	**ZHENG_IL22_SIGNALING_UP**
NIELSEN_MALIGNAT_FIBROUS_HISTIOCYTOMA_UP	HAHTOLA_CTCL_CUTANEOUS
GNATENKO_PLATELET_SIGNATURE	MAHADEVAN_IMATINIB_RESISTANCE_UP
HAHTOLA_MYCOSIS_FUNGOIDES_UP	DEMAGALHAES_AGING_UP
KEEN_RESPONSE_TO_ROSIGLITAZONE_DN	**ALTEMEIER_RESPONSE_TO_LPS_WITH_MECHANICAL_ VENTILATION**
LIANG_SILENCED_BY_METHYLATION_UP	JECHLINGER_EPITHELIAL_TO_MESENCHYMAL_ TRANSITION_UP
VERRECCHIA_RESPONSE_TO_TGFB1_C1	HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN
SMIRNOV_CIRCULATING_ENDOTHELIOCYTES_IN_CANCER_UP	ZHENG_RESPONSE_TO_ARSENITE_UP
GRAHAM_CML_QUIESCENT_VS_NORMAL_DIVIDING_UP	CHUNG_BLISTER_CYTOTOXICITY_DN
HAHTOLA_MYCOSIS_FUNGOIDES_CD4_UP	DAVIES_MULTIPLE_MYELOMA_VS_MGUS_DN
DAZARD_UV_RESPONSE_CLUSTER_G2	**BURTON_ADIPOGENESIS_8**
SESTO_RESPONSE_TO_UV_C3	OLSSON_E2F3_TARGETS_DN
BURTON_ADIPOGENESIS_PEAK_AT_2HR	GEORGANTAS_HSC_MARKERS
CHEN_ETV5_TARGETS_SERTOLI	WINZEN_DEGRADED_VIA_KHSRP
RAGHAVACHARI_PLATELET_SPECIFIC_GENES	REACTOME_AMINE_LIGAND_BINDING_RECEPTORS
DAZARD_UV_RESPONSE_CLUSTER_G28	REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_ NUCLEOSOMES_AT_THE_CENTROMERE
LIN_TUMOR_ESCAPE_FROM_IMMUNE_ATTACK	JI_CARCINOGENESIS_BY_KRAS_AND_STK11_DN
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_ GRANULOCYTE_DN	SARRIO_EPITHELIAL_MESENCHYMAL_TRANSITION_DN
VERHAAK_AML_WITH_NPM1_MUTATED_UP	**DAZARD_UV_RESPONSE_CLUSTER_G24**
AMIT_SERUM_RESPONSE_60_MCF10A	RICKMAN_HEAD_AND_NECK_CANCER_C
SCHOEN_NFKB_SIGNALING	PETRETTO_CARDIAC_HYPERTROPHY
PRAMOONJAGO_SOX4_TARGETS_UP	DALESSIO_TSA_RESPONSE
GENTILE_UV_HIGH_DOSE_UP	BROWN_MYELOID_CELL_DEVELOPMENT_UP
HERNANDEZ_MITOTIC_ARREST_BY_DOCETAXEL_2_UP	LIN_SILENCED_BY_TUMOR_MICROENVIRONMENT
REACTOME_RNA_POL_I_PROMOTER_OPENING	DAUER_STAT3_TARGETS_UP
REACTOME_AMYLOIDS	**REACTOME_TELOMERE_MAINTENANCE**
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_SUSTAINDED_ IN_ERYTHROCYTE_UP	MAHADEVAN_GIST_MORPHOLOGICAL_SWITCH
BERENJENO_TRANSFORMED_BY_RHOA_FOREVER_UP	GRAHAM_NORMAL_QUIESCENT_VS_NORMAL_DIVIDING_UP
PODAR_RESPONSE_TO_ADAPHOSTIN_UP	LIANG_SILENCED_BY_METHYLATION_2
HALMOS_CEBPA_TARGETS_UP	ADDYA_ERYTHROID_DIFFERENTIATION_BY_HEMIN

REACTOME_PACKAGING_OF_TELOMERE_ENDS	REACTOME_MEIOTIC_RECOMBINATION
VART_KSHV_INFECTION_ANGIOGENIC_MARKERS_UP	SATO_SILENCED_BY_DEACETYLATION_IN_PANCREATIC_ CANCER
CROONQUIST_STROMAL_STIMULATION_UP	HELLEBREKERS_SILENCED_DURING_TUMOR_ANGIOGENESIS
BROWNE_HCMV_INFECTION_2HR_DN	GROSS_HYPOXIA_VIA_ELK3_ONLY_UP

2.2.3.3 *Asxl1* KO transplanted mice and MDS patients

We compared the results obtained from the RNA seq of OBs and MSCs isolated from *Asxl1* KO transplanted mice with those obtained from the RNA seq of MSCs isolated from MDS patients with *ASXL1* mutations, in order to identify overlapping alterations which could point at mesenchymal alterations induced by *Asxl1* mutated cells, relevant to human MDS.

First, we compared gene expression considering the genes differentially expressed according to p_{adj}. We found 1 gene, *Cdh3*, down-regulated in both OBs isolated from *Asxl1* KO transplanted mice and MDS-MSCs. We also identified 4 genes in common in MSCs isolated from *Asxl1* KO transplanted mice and MDS-MSCs: 2 up-regulated (*Cry1, Usp31*) and 1 down-regulated (*Tbc1d30*), while the other gene showed opposite trend (*Trib3*). Due to the small number of mice (n=3 for control mice) in the murine experiment, we decided to perform a less statistically stringent analysis considering also the genes differentially expressed according to the normal p-value, without any normalization, whereas for human we continued to consider only the p_{adj}. With this second analysis, we then identified other genes in common. Indeed OBs isolated from *Asxl1* KO transplanted mice and MDS-MSCs showed other 21 genes differentially expressed: 12 genes were down-regulated (*Crabp1, Acy3, Pls1, Fam81a, Hs3st1, Actr3b, Atp6v1c2, Dlec1, Rasgrp3, Ablim2, Trpm3, Ryr3*) in both groups, whereas 9 genes showed inverse trend: up-regulated in one group and down-regulated in the other (Tab.19a). When we compared the genes differentially expressed (p-value) in MSCs isolated from *Asxl1* KO transplanted mice with MDS-MSCs (p_{adj}), we found other 40 genes in common: 14 were up-regulated (*Nrip3, Mfsd2a, Dennd5a, Svil, Itga5, Btbd1, Upp1, Cda, Pprc1, Col11a1, Cxxc5, S100a6, Fn1, Gfpt2*) and 6 were down-regulated (*Uvssa, Syt12, Wdr78, Mtmr4, Aifm2, Nynrin)* in both groups. In contrast, the other 20 genes showed an opposite trend of expression (Tab. 19b). However, the meaning of this finding is still unclear and further analyses are necessary to elucidate the role of those genes in MDS.

Table 19. 10 first genes differentially expressed in MSCs isolated from LR-MDS and stromal cells isolated from *Asxl1* KO transplanted mice.

19.a

Gene name	Avg MDS-MSCs[1]	Avg HD-MSCs	FC[2]	Padj[3]	Avg OBs *Asxl1* KO transp. Mice[4]	Avg OBs CTR mice	FC	pval
Crabp1	0,01	13,20	-9,07	0,00	0,00	10,09	-7,90	0,00
Acy3	0,00	3,29	-8,25	0,00	1,19	20,92	-4,49	0,05
Spib	0,02	2,60	-7,32	0,00	0,97	0,00	7,56	0,01
Pls1	0,00	0,22	-6,92	0,00	0,15	12,02	-5,59	0,04
Fam81a	0,01	0,42	-5,14	0,00	0,00	0,37	-6,18	0,04
Csmd2	0,41	0,02	4,81	0,00	0,00	0,50	-8,51	0,00
Hs3st1	0,00	1,09	-7,13	0,00	0,12	10,70	-7,01	0,00
Actr3b	0,07	0,50	-3,86	0,00	0,00	1,70	-7,91	0,00
Atp6v1c2	0,00	0,09	-3,39	0,00	0,00	1,57	-6,82	0,02
Mss51	0,54	0,45	-4,29	0,00	0,64	0,00	5,47	0,02

19.b

Gene name	Avg MDS-MSCs	Avg HD-MSCs	FC	padj	Avg MSCs *Asxl1* KO transp. mice[5]	Avg MSCs CTR mice	FC	pval
Shisa2	40,75	1,41	5,91	0,00	4,52	13,54	-1,61	0,04
Nrip3	4,90	0,65	4,48	0,00	0,73	0,00	7,93	0,03
Dmc1	0,11	0,95	-2,96	0,00	1,53	0,00	8,04	0,00
Uvssa	0,09	0,78	-3,81	0,00	0,46	2,52	-2,40	0,01
Mfsd2a	31,11	4,27	3,46	0,00	1,05	0,00	7,21	0,02
Syt12	0,02	0,68	-4,69	0,00	0,45	3,69	-3,01	0,05
Dennd5a	12,01	4,20	2,54	0,00	16,52	6,75	1,29	0,00
Nr1d1	32,68	5,07	3,55	0,00	23,24	61,20	-1,19	0,05
Svil	6,10	2,58	1,70	0,00	8,00	2,43	1,80	0,03
Pvrl2	27,69	7,30	2,87	0,00	10,16	25,00	-1,21	0,04

Genes are listed based on p_{adj} values of human MSCs.
[1] HD-MSCs n=10, MDS-MSCs n=10
[2] Fold change (FC)
[3] $p_{adj}<0,05$
[4] *Asxl1* KO transplanted mice n=4, CTR mice n=3.

Second, we compared the results of the GSEA analysis of OBs/MSCs isolated from *Asxl1* KO transplanted mice with the results of the GSEA analysis of MDS-MSCs isolated from *ASXL1* mutated patients. Interestingly, we found 30 pathways enriched in MDS-MSCs and OBs. On the contrary, there was no overlap when comparing the OBs isolated from control mice and HD-MSCs. However, we also identified 149 pathways with an opposite trend: enriched in OBs from *Asxl1* KO transplanted mice and enriched in HD-MSCs. When we compared MSCs isolated from *Asxl1* KO transplanted mice and MDS-MSCs we found 1 pathway in common (BROWN_MYELOID_CELL_DEVELOPMENT_UP).

The gene sets enriched in MDS-MSCs and murine OBs were mainly associated with cellular stress in response to UV and to MYC activity, suggesting a general stress of the cells and the potential activation of MYC targets. Furthermore, two pathways related to inflammation were also enriched in both MDS-MSCs and OBs isolated from *Asxl1* KO transplanted mice (Tab.20). Nevertheless, most of the pathways related to inflammation were not enriched in OBs isolated from *Asxl1* KO transplanted mice but were enriched in OBs isolated from control mice. This could suggest that these gene sets have a less fundamental role in the alterations, mediated by the hematopoietic-specific deletion of *Asxl1*.

Table 20. 30 pathways enriched in both MSCs isolated from LR-MDS and OBs isolated from *Asxl1* KO transplanted mice.

DAZARD_UV_RESPONSE_CLUSTER_G2
LIANG_SILENCED_BY_METHYLATION_UP
GENTILE_UV_HIGH_DOSE_UP
SHIN_B_CELL_LYMPHOMA_CLUSTER_8
SESTO_RESPONSE_TO_UV_C0
BIOCARTA_EIF_PATHWAY
TIEN_INTESTINE_PROBIOTICS_6HR_UP
DAZARD_UV_RESPONSE_CLUSTER_G24
SESTO_RESPONSE_TO_UV_C1
SHIPP_DLBCL_VS_FOLLICULAR_LYMPHOMA_UP
HU_GENOTOXIC_DAMAGE_24HR
HUMMERICH_BENIGN_SKIN_TUMOR_UP
MENSSEN_MYC_TARGETS
PID_ARF6_DOWNSTREAM_PATHWAY
FLOTHO_PEDIATRIC_ALL_THERAPY_RESPONSE_UP
COLLER_MYC_TARGETS_UP
YIH_RESPONSE_TO_ARSENITE_C2
DAZARD_UV_RESPONSE_CLUSTER_G4
SANA_RESPONSE_TO_IFNG_DN
SCHLOSSER_MYC_TARGETS_AND_SERUM_RESPONSE_UP
KARLSSON_TGFB1_TARGETS_UP
CHAUHAN_RESPONSE_TO_METHOXYESTRADIOL_UP
PID_MYC_ACTIV_PATHWAY
DACOSTA_LOW_DOSE_UV_RESPONSE_VIA_ERCC3_XPCS_UP
ZAMORA_NOS2_TARGETS_UP
ZHOU_TNF_SIGNALING_30MIN
BILANGES_SERUM_RESPONSE_TRANSLATION
REACTOME_RNA_POL_I_PROMOTER_OPENING
HOLLEMAN_VINCRISTINE_RESISTANCE_ALL_DN
CAIRO_PML_TARGETS_BOUND_BY_MYC_UP

DISCUSSION

DISCUSSION

Myeloid malignancies, including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), are a heterogeneous group of clonal diseases, characterized by hematopoietic insufficiency. It is thought that MDS and AML originate from alterations of hematopoietic stem cells (HSCs) and myeloid progenitors[27,28]. Thus in the past, the main focus of the researchers was hematopoietic cell characterization and the development of novel therapies, specific for malignant cell alterations. However, the contribution of the bone marrow (BM) microenvironment to MDS and AML pathogenesis, has recently gained more attention, challenging the evidence sustaining that MDS and AML derive exclusively from hematopoietic-intrinsic defects. Indeed, like their healthy counterpart, malignant HSC reside in highly regulated, dynamic and complex cellular niches within the BM microenvironment, which provides signals fundamental for the regulation of HSC functions[352]. Stromal cells and in particular mesenchymal stem cells (MSCs) are a key component of the BM microenvironment, with a fundamental role in supporting normal hematopoiesis[353] and a unique immunomodulating ability[354]. The indoleamine 2,3-dioxygenase 1 (IDO1) enzyme is one of the key regulators of immunomodulation mediated by MSCs.

In the last years, a better understanding of the myeloid transformation led to the development of two pathophysiological models for MDS and AML: 1) primary alterations of stromal cells induce the disease (niche-induced leukemogenesis); 2) primary alterations of MDS and AML hematopoietic cells induce changes in the BM stromal cells, turning the microenvironment into a self-reinforcing niche, which supports malignant clones at the expense of healthy hematopoiesis[29]. However, all the mechanisms supporting the 2 pathophysiological models have just been started to get unraveled.

Niche-induced leukemogenesis has been extensively demonstrated in mice models[280–283,324], so far, the understanding of MSC role in supporting myeloid malignancies in humans is still under investigation. Although genetic and functional alterations have been identified in MSCs isolated from MDS (MDS-MSCs) and AML patients (AML-MSCs)[289,290,295], the functional meanings of these alterations are still under debate and conflicting results have been published. Furthermore, it is not completely clear whether MDS-MSCs and AML-MSCs share common features contributing to disease pathogenesis.

In the first part of this thesis, we characterized and compared MSCs isolated from MDS (MDS-MSCs) and AML (AML-MSCs) patients with MSCs isolated from healthy donors (HD-MSCs), as a

reference control. We showed that both MDS- and AML-MSCs can be isolated and expanded *in vitro.* However, we highlighted some functional differences between HD-MSCs and patient-derived MSCs, but also between MDS-MSCs and AML-MSCs. First, the isolation of MSCs from patients resulted more difficult, especially from AML patients. Indeed, we were unable to isolate MSCs from a substantial fraction of AML patients (25%, 8 out of 32), while almost all HD and 84% of MDS samples yielded MSCs. Second, we isolated fewer MSCs from patients than from HDs. Indeed, the frequency of rescued MSCs, i.e. the number of MSCs isolated at P1 normalized to the number of BM-isolated mononuclear cells (MNCs), was significantly lower in the AML group than in the HD group, whereas in MDS group the frequency was intermediate. Of note, we could not identify a correlation between these features and a defined cytogenetic/molecular subgroup or patient age (see table 4). Together these data led us to hypothesize that a lower percentage of MSC precursors characterizes MDS and AML BMs.

We also found that MDS-MSCs and AML-MSCs show a significantly decreased proliferation potential, compared to that of HD-MSCs. In particular, the proliferation rate of MDS-MSCs is also lower than that of AML-MSCs, even if not significantly. Other studies analyzed the proliferation pattern of MSCs and while some authors recorded a reduced expansion potential[295,297,334,355], others did not find any difference in the proliferation rate of MDS- and AML-MSCs, in comparison with that of HD-MSCs[356,357]. In particular, it was suggested that the growing defect in MSCs isolated from MDS patients was associated with cell senescence[358]. However, our unpublished data indicated that fetal bovine serum supplementation partially rescued MDS-MSC proliferation capacity, suggesting that MDS-MSCs displayed an intrinsic proliferation signaling defect, making them more dependent on growth factors.

Despite these differences, MDS and AML-MSCs showed a normal morphology and also the immunophenotyping did not highlight differences with HD-MSCs. Furthermore, when we induced the differentiation of MDS and AML-MSCs towards adipogenic and osteogenic lineage, we found a comparable differentiation ability among MDS-, AML- and HD-MSCs. Controversial results, about the differentiation ability of MSCs isolated from patients with hematological disorders, have been published. Indeed, according to some authors MSCs isolated from patients with hematological diseases, such as acute lymphoblastic leukemia (ALL)[359] and AML[333], show altered differentiation ability, whereas Klaus et al. demonstrated that the differentiation potential toward adipocytes/chondrocytes/osteoblasts of MDS-MSCs do not differ from that of HD-MSCs[355]. The heterogeneity of the hematological diseases and the use of different experimental procedures could explain the differences found among the studies. In our research, for the first time, we compared at

the same time and in the same experimental conditions, the differentiation potential of MSCs derived from MDS and AML patients. However, as expected MDS samples showed a median age higher than AML and HD samples, while this difference was not recorded when we compared AML samples with HD samples. Despite adipogenic differentiation seems increased in aged MSCs[352], we found that HD-, MDS- and AML-MSC differentiation ability was maintained unchanged, regardless of the median age of the subjects. Indeed, when we compared samples of similar age, we obtained similar results (data not shown). In conclusions, we demonstrated that MDS- and AML-derived MSCs both met the MSC criteria proposed by the International Society for Cellular Therapy (ISCT)[141].

We also studied the presence of cytogenetic alterations, found in neoplastic cells, in MDS- and AML-MSCs, isolated from the same patients. The data in the literature about this issue are controversial. Flores-Figueroa et al. reported that MDS-MSCs show the same cytogenetic alterations characterizing the disease[293], whereas Blau et al found that MDS- and AML-MSCs do not show the clonal cytogenetic alterations found in hematopoietic cells isolated from the same individual, but they have distinct chromosomal aberrations. According to the authors, this suggests an enhanced genetic susceptibility of MSCs, which may indicate a potential role of MSCs in the pathophysiology of MDS/AML[289,335]. In addition, MDS and AML patients, with genetic abnormalities in their *in vitro* expanded MSCs, show a worse overall survival and a worse disease free-survival than normal karyotype. However, other groups found that despite MSCs harbor severe chromosomal alterations, they maintain normal functional properties, including their ability to support normal hematopoiesis[294]. The majority of patients analyzed by FISH in this study had a normal karyotype (see table 4). FISH was performed in MDS and AML cases, where an aberration was identified in neoplastic cells, at diagnosis. In these cases, MDS- and AML-MSCs did not show the cytogenetic abnormalities, found in malignant cells isolated from the same patients. Nonetheless, we could not exclude that MSCs presented alterations different than their hematopoietic counterpart, but we could only conclude that both MDS- and AML-MSCs did not share a common precursor with the original malignant clone.

Next, we assessed the ability to support hematopoiesis of patients derived MSCs, in term of viability and proliferation of AML cells in culture with MDS- and AML-MSCs. Gey et al. demonstrated that MDS-MSCs are defective in hematopoiesis supporting functions[298], as well as AML-MSCs, which display insufficient hematopoietic support and diminished ability to support progenitor cells in long-term culture-initiating cells (LTC-ICs). However, conflicting results have also been published reporting a normal hematopoiesis-supporting activity of MDS-MSCs[294] and

AML-MSCs[360]. Furthermore, aged MSCs exhibit reduced hematopoietic support[361]. We found that MDS- and AML-MSCs support AML cell viability and proliferation, as well as HD-MSCs. We could speculate that the MSC capacity to sustain leukemic cell viability and proliferation, demonstrated *in vitro*, could contribute to disease progression *in vivo*.

Next, we decided to test the immunomodulatory properties of MDS- and AML-MSCs, since aberrant immune responses have been associated with the pathophysiology of MDS and AML. In particular, we decided to investigate the ability of MDS and AML patient-derived MSCs to induce Tregs, which have been recognized as essential contributors of cancer progression able to suppress anti-tumor immune responses[267]. Previous studies on this issue have been limited and controversial, with some authors reporting a normal immunomodulatory ability[355,362] and others reporting impaired immunosuppressive properties of MSCs isolated from hematological patients[363,364]. We found that MDS-MSCs and AML-MSCs are able to induce Tregs in a comparable manner to HD-MSCs, suggesting that the cells maintain unaltered their immunosuppressive activity.

(These results, about MDS and AML patients-derived MSCs, were included in a manuscript, recently published in Stem Cell Research & Therapy[365]*).*

IDO1 is a key regulator of MSC-mediated immunoregulation. We wondered if also MDS- and AML-MSCs maintain the ability to up-regulate IDO1, following stimulation. We used pro-inflammatory cytokines, such as IFN-γ, TNF-α, PGE-2, and IL-6. PGE-2 and IL-6 have been found increased in serum of AML patients and/or are secreted by leukemic cells[128,344], whereas François et al. demonstrated that HD-MSCs up-regulate IDO1 after stimulation with IFN-γ and TNF-α[276]. However, there are no studies reporting IDO1 expression in MSCs, derived from MDS and AML patients. We found that IDO1 is up-regulated both at mRNA and protein level in MDS-and AML-MSCs, in a comparable manner to HD-MSCs.

The characterization of MDS- and AML-MSCs highlighted some functional differences but also unchanged functions among the 2 groups, which could influence the support of the BM microenvironment to leukemia cells. Thus, we could speculate that the intrinsic defects, related to the reduced efficiency of isolation/proliferation rates, the support to AML cells and the immunosuppressive function *in vitro,* could all potentially turn the BM microenvironment as supportive for leukemic cells.

However, as already mentioned, the other proposed mechanism of MDS/AML pathogenesis is represented by the ability of malignant cells to shape BM niche, which in turn can promote their selection and expansion, at the expense of normal hematopoiesis. Nevertheless, this theory is still under investigation and new mechanisms involved have to be unrevealed. To contribute to a deeper

investigation and with the aim to identify new pathways involved in this mechanism, in this thesis, we investigated:

- the role of inflammatory pathways in the interplay between MSCs and AML cells, in AML patients;
- the effects of hematopoietic-specific alterations on the transcriptome of stromal cells, in mice.

In MDS BMs, both innate and adaptive immune systems are overly active, and several cytokines are secreted in the BM microenvironment[366]. Furthermore, it is also known that AML cells, unlike normal hematopoietic cells, can secrete several cytokines, which in turn stimulate their growth[127]. The production of soluble mediators not only affects AML cell growth, but also the function of surrounding cells, such as stromal cells in the BM. Indeed, Frisch et al. demonstrated that AML cells, by producing CCL3, induce a demineralization of BM which impairs normal hematopoiesis, in a mouse model of myeloid leukemia[318]. This feature has been demonstrated also in other myeloid malignancies such as MDS, myeloproliferative neoplasms, chronic myeloid leukemia, multiple myeloma[313–317]. Thus, we investigated if AML cells isolated from AML patients at diagnosis could produce pro-inflammatory cytokines. For the first time, by performing a gene expression profiling (GEP) screening in BM-derived MNCs isolated from AML patients at diagnosis, we showed that 38% of AML samples analyzed (23/61) express higher levels of IFN-γ, compared to the mean IFN-γ expression in MNCs isolated from HDs. Interestingly, IFN-γ expression in AML cells is preserved in culture. Indeed, AML cells maintained in culture for 4 days express both mRNA and protein of IFN-γ, regardless of the presence or not of HD- and AML-MSCs. Unlike GEP data, obtained on freshly isolated MNCs, these data, obtained from cultures, should exclude the chance of cytokine production due to an ongoing infection in AML patients.

In literature, data about IFN-γ production by myeloid malignant cells are controversial. Indeed, IFN-γ production has been reported in BM cells of MDS patients[367], while several groups reported low-level or not enhanced expression of IFN-γ in AML cells[127,367]. In particular, according to Sepehrizadeh et al., IFN-γ production increases in AML cells after chemotherapy rather than at the steady state, i.e. pre-chemotherapy[127]. It is noteworthy that the majority of the studies investigated others cytokines. Interestingly, IFNs in the tumor microenvironment can modulate the progression and regression of malignancies. Indeed, the expression of IFN-stimulated genes (ISGs) has been identified in patients with different types of tumors, resistant to chemotherapy and radiation therapy, suggesting a pro-survival effect of IFNs on tumor cells[368]. However, antitumor effects, due to IFN-mediated activation of the immune system, have also been reported. In particular, in mice

during spontaneous or induced tumorigenesis, IFN-γ is responsible for tumor surveillance, and mice deficient for IFN-γ receptor (*Ifngr1*) or *Stat1* develop tumors more rapidly compared to wild-type mice[369]. We further demonstrated that in AML BM-derived cells isolated at diagnosis, IFN-γ mRNA levels higher than the IFN-γ mean expression in MNCs isolated from HDs, correlate with a better overall survival. In contrast, patients with low IFN-γ expression showed a worse overall survival.

Next, we investigated the possibility that IFN-γ producing AML cells could shape the niche. Since IDO1 is up-regulated by IFN-γ in MSCs isolated from healthy donors, and we demonstrated that AML-MSCs maintain this ability, we decided to verify if IFN-γ^+ AML cells could induce IDO1 expression in AML-MSCs. We found that IFN-γ^+ AML cells stimulate both mRNA expression and protein production of IDO1 in AML-MSCs. Interestingly, high expression of IDO1 in the tumor or draining lymph nodes is considered an adverse prognostic factor in several cancers, such as AML, melanoma, colon and ovarian cancer, and brain tumors[268]. In particular, in the tumor microenvironment, the expression of IDO1 by tumor cells themselves or by tumor-associated cells, such as endothelial cells or DCs, may confer some non-immune survival advantage on the tumors but more reasonable may represent a mechanism of tumor-escape mediated by immune tolerance induction[216]. Based on all these findings, we assessed if IDO1 induction in AML-MSCs, mediated by IFN-γ producing AML cells, could induce Tregs, well-known regulators of immune tolerance. Interestingly, we found that the increase in Tregs, mediated by AML-MSCs, is IDO1-dependent. Beyond this indirect effect, mediated by IDO1 expressing AML-MSCs, in our laboratory Curti et al. previously demonstrated that AML cells can also directly induce Treg generation, in an IDO1-dependent manner. In addition, IDO1 expression in AML cells correlates with increased circulating $CD4^+CD25^+Foxp3^+$ T cells in patients at diagnosis[126]. We hypothesized that the increased level of Tregs, induced by IDO1 expressing AML-MSCs pre-cultured with IFN-γ^+ AML cells, could induce in turn a tolerogenic microenvironment, in which tumor growth is promoted. Indeed, Tregs not only mediate IDO1 immunosuppressive effect on adaptive immunity but also represent one of the major suppressive mechanisms in tumors. Tregs lead to the development of an immunosuppressive microenvironment, favoring immune evasion and thus cancer progression[370]. Furthermore, increased level of Tregs within tumor tissue correlates with worse prognosis in many cancers[267]. We identified a novel mechanism of niche shaping mediated by AML cells, which by producing IFN-γ activate MSCs immunosuppression, through IDO1 up-regulation with subsequent Tregs generation. The discrepancy related to the correlation of high IFN-γ with a better OS in AML patients could be due to the concentration of IFN-γ. Our hypothesis is that the concentration of IFN-γ is fundamental

to generate a pro- or anti-tumor response in AML BMs. Indeed, in metastasis mouse model the antitumor effect of IFN-γ shows a bell-shaped dose-response curve[371]. Likewise, the activation of the immune response, i.e. the induction of various markers, mediated by IFN-γ, also exhibits bell-shaped dose-response curves[372]. In particular, a dose-response curve has also been identified in patients, affected by different malignancies, receiving IFN-γ, for the ability of the cytokine to induce lymphocyte and monocyte activation, Fc receptor expression, antibody-dependent monocyte cytotoxicity[373]. To better clarify this hypothesis, further experiments are required and are ongoing at the moment. In particular, the amount of IFN-γ produced by AML cells will be quantified *in vitro* and *in vivo* in AML patients. IFN-γ doses will be correlated with up-regulation of IDO1 and others IFN-γ genes and with Tregs induction.

As a further step to investigate HSC-driven model of BM shaping in the pathogenesis of myeloid malignancies, we wondered if hematopoietic cells, harboring specific alterations, could influence/alter the niche, in order to favor their own expansion. In particular, we decided to study if the deletion of *Dnmt3a* and *Asxl1* in hematopoietic cells could affect the transcriptome of wild-type stromal cells *in vitro* and in mouse models.

As already mentioned, *Dnmt3a* and *Asxl1* mutations are present in 10% of MDS and 20% of AML patients[77,99]. Moreover, mutations related to these epigenetic regulators of transcription are the most frequent in clonal hematopoiesis of indeterminate potential (CHIP)[374]. The hematopoietic-specific deletion of these genes induces an MDS-like phenotype in mice, with a malignant expansion overcoming the normal hematopoiesis. Whether the competitive fitness of pre-leukemic cells, harboring *Dnmt3a* and *Asxl1* mutations, is merely driven by cell-intrinsic mechanisms or whether the microenvironment is also involved, is currently unexplored[313]. However, it is conceivable that the BM microenvironment takes part in the malignant expansion. Furthermore, it is also known that MSCs isolated from MDS patients show profound transcriptome alterations compared to HD-MSCs, with the disruption of pathways involved in several processes, including inflammation[292]. Whether these are primary alterations of stromal cells or whether are induced by malignant clones, is still unclear.

To this aim, we transplanted *Dnmt3a*- and *Asxl1*-null BM cells, hereafter referred to *Dnmt3a* and *Asxl1* KO cells, in wild-type mice recipient. Then, we isolated stromal cells, when possible both MSCs and osteoblasts (OBs), from KO transplanted mice and their controls, and we performed whole-transcriptome sequencing. We subsequently performed the differential gene expression analysis and the gene set enrichment analysis (GSEA), comparing the transcriptome of cells isolated from KO transplanted mice with that of their controls, in order to identify modulations of gene

expression. Nevertheless, transplantations with *Dnmt3a* KO cells were not performed with their proper controls. Indeed, control cells were not BM cells but fetal liver cells in which the recombinase, Cre, was under the control of another promoter, i.e. C/EBPα promoter. In contrast, in *Dnmt3a* KO cells the recombinase was under the control of the ubiquitous Rosa26 promoter. However, in both control cells, *Dnmt3a* was WT and all the mice were irradiated in the same way, leading to the same effect on stromal cells. In addition, due to a technical issue during the procedure of FACS-purification, we could not isolate both MSCs and OBs, but we isolated and sequenced the all population CD31-, containing cells, called generically stromal cells. These issues make the experiment suboptimal. However, we found that the loss of *Dnmt3a* and *Asxl1* in hematopoietic cells induces different modifications in stromal cell transcriptome, with the disruption of pathways involved in distinct biological processes. The comparison of the significantly enriched gene sets (obtained with the GSEA) identified in stromal cells isolated from *Dnmt3a* KO transplanted mice and in OBs isolated from *Asxl1* KO transplanted mice, highlighted some similarities regarding the disruption of pathways mainly involved in telomere maintenance and stress in response to ultraviolet radiation (UV). UV induces many potentially harmful effects on cells. UV interacts with endogenous cellular components, resulting in the generation of ROS and damage to proteins and lipids. In particular, UV type B can interact directly with DNA causing damage[375]. Another feature of UV stimulation is the activation of pathways involved in cell differentiation, growth, and senescence[376]. The activation of UV-related gene programs in BM niche cells recapitulates features of a general condition of stress and alterations which did not depend on the specific mutations occurring in hematopoietic cells. Beside these common pathways, the comparison of the GSEA results obtained from *Dnmt3a/Asxl1* KO transplantation experiments revealed that most of the enriched gene sets were not in common, but specific of stromal cells isolated from *Dnmt3a* KO transplanted mice and OBs isolated from *Asxl1* KO transplanted mice. In addition, most of the gene sets showed inversely trend of enrichment, specifically enriched in cells isolated from the controls of an experiment and from the KO transplanted mice of the other experiment. This suggests a markedly different effect on the transcriptome of stromal cells, mediated by the absence of *Dnmt3a* and *Asxl1* in hematopoietic cells. It is well known that the hematopoietic-specific loss of *Dnmt3a* or *Asxl1* leads to the development of a disease MDS-like in mice[90,106]. Despite a common dysplasia, cytopenia, and impaired erythroid maturation, distinct features characterize conditional *Dnmt3a* and *Asxl1* KO mice. Indeed, conditional *Dnmt3a* KO mice show hypercellular BM, mature myeloid progenitors expansion and hepatomegaly with associated extramedullary hematopoiesis (EMH)[90]. In contrast, conditional *Asxl1* KO mice show hypocellular BM, leukopenia due to impaired myeloid

commitment and EMH in the liver and especially in the spleen[106]. An increased number of hematopoietic stem and progenitor cells (HSPCs) characterize both mouse models. Moreover, in serial competitive transplantation assays, *Dnmt3a* KO cells have an advantage on the wild-type cells, indicating an increased stem cell function[90]. Furthermore, in a recent study, it has been demonstrated that the loss of *Dnmt3a* immortalizes HSCs, which can be transplanted 12 times in serial transplantation experiments. This demonstrates that the loss of *Dnmt3a* confers to the HSCs a self-renewal potential, which exceeds that of WT HSCs[377]. On the contrary, *Asxl1* KO cells have a disadvantage in serial competitive transplantations, suggesting a reduction in self-renewal ability[106]. All these findings point out the presence of critical intrinsic differences between hematopoietic cells harboring the two deletions, which induce phenotypes with some dissimilarities in mice. Thus, not surprisingly the differences we have highlighted in stromal cell transcriptome could be explained by this issue.

In parallel, we performed *in vitro* experiments to simulate the *in vivo* findings and to investigate if the alterations found in stromal cells of transplanted mice were induced by HSPCs, which are known to be in contact with stromal cells *in vivo*[170]. Thus, we assessed co-cultures with OP9 and *Dnmt3a* KO and *Asxl1* KO LSK (Lineage$^-$ Sca-1$^+$ C-kit$^+$), a cell subset enriched in HSPCs, and their controls, as described in the materials and methods section. However, in our experimental settings, LSK KO did not induce profound alterations in the transcriptome of stromal cells. Different reasons may explain this issue. Indeed, we performed the co-cultures for 6 days but this interval of time maybe was not sufficient to perturb the transcriptome of OP9. In addition, we used LSK cells, whereas *in vivo* we transplanted the whole BM in mice. We can not exclude that another type of hematopoietic population could alter gene expression in OP9. Indeed, it is known that stromal cells interact and regulate also the function of progenitor and mature cells, which in turn may influence stromal cells[378]. In addition, a body of evidence also supports the finding that myeloid transformation can derive also from alterations of early progenitors, such as granulocyte macrophage progenitors in AML and lymphoid-primed multipotent progenitors[379,380], and granulocyte macrophage progenitor-like subset, in blast crisis chronic myeloid leukemia (CML)[381]. Furthermore, it has been published that in human MDS not only HSCs play a pivotal role in the pathogenesis of the disease. Indeed, myeloid-derived suppressor cells (MDSCs) are expanded. MDSC are a heterogeneous population with myeloid origin composed of myeloid progenitors and immature macrophages, immature granulocytes and immature dendritic cells. In MDS bone marrows, MDSCs induce ineffective hematopoiesis, mediated by the production of hematopoietic suppressive cytokines[382]. For these reasons, the co-cultures experiments could be performed again

with a different subset of cells. Finally, the *ex-vivo* experiments we performed could represent a too excessive simplistic picture of the BM niche, due to the presence of only two different populations. It is possible that hematopoietic cells may affect indirectly stromal cells, by inducing alterations in another component of the BM niche, which in turn alters the stromal compartment. Indeed, BM microenvironment is a network of signaling and interaction between different cells. Many cell types including osteoblastic, perivascular, endothelial and mesenchymal cells contribute to the HSC niche, regulating HSC survival and function. Recently, also bone marrow-resident macrophages (Mφs) emerged as an important component of the HSC niche[383].

Next, we also compared the results of the GSEA performed on differentially expressed transcripts identified in stromal cells isolated from *Dnmt3a/Asxl1* KO transplanted mice and identified in highly purified MSCs, isolated from low-risk MDS patients, with *DNMT3A* and *ASXL1* mutations. MSCs isolated from HDs were used as the controls for MDS-MSCs. When we compared the results of the GSEA of stromal cells isolated from *Dnmt3a* KO transplanted mice with those of the GSEA of MDS-MSCs, isolated from patients with *DNMT3A* mutations, we found pathways enriched in both groups. The pathways included those related to UV response and to response to inflammatory stimuli, i.e. response to TGF-β and IL22, and NF-kB targets. The modulation of inflammatory pathways was really interesting and could be *Dnmt3a*-specific because OBs isolated from *Asxl1* KO transplanted mice did not show enrichment in pathways involved in inflammatory response. On the contrary, these pathways were mainly enriched in OBs isolated from control mice. It is known that aberrant methylation patterns regulate inflammation. For instance, DNMT3A restrains mast cell in inflammatory response[384]. Furthermore, DNMT3A regulates the expression of several cytokines, and DNMT3A-mediated DNA hypermethylation at the promoter region of IL-1β, IL-6, and IL-8 reduces their expression in lung cancer cells[385]. In turn, methylation activity is regulated by inflammation[386]. We also identified pathways showing opposite trends in stromal cells isolated from *Dnmt3a* KO transplanted mice and in MDS-MSCs, isolated from patients with *DNMT3A* mutations. Nevertheless, this is not surprising because humans had a full-blown disease and most of the patients showed other mutated genes. These issues could explain the differences found in the GSEA analysis.

We then compared the GSEA results obtained from RNA sequencing of OBs isolated from *Asxl1* KO transplanted mice with that of MDS-MSCs, isolated from patients with *ASXL1* mutations. The comparison revealed some similarities, with pathways involved in UV response and Myc activity, enriched in both groups. In contrast, the Myc related gene sets were not enriched in stromal cells isolated from *Dnmt3a* KO transplanted mice but were enriched in stromal cells isolated from

control mice, suggesting a specific mechanism of *Asxl1* loss. Over the past years, the role of MYC in normal and malignant hematopoiesis has been largely investigated. This transcription factor regulates different processes of hematopoiesis, such as differentiation and survival of HSCs. The Myc family members show differential expression in hematopoietic lineages: c-Myc and n-Myc transcripts are found in LT-HSCs and in most progenitor subsets. In contrast, l-Myc expression is found in more mature cells, such as macrophages[387]. Deregulation of *MYC* expression has been described in many types of human lymphoma and leukemia[388]. In mouse models, the ablation of *c-Myc* in hematopoietic cells leads to the accumulation of HSCs. This failure to initiate normal stem cell differentiation is caused by increased HSC adhesion to the niche[389]. In addition, c-Myc plays a role in drug resistance. MSCs protect leukemia cells from the apoptotic effect of mitoxantrone, an antineoplastic agent, through c-Myc dependent mechanisms[309]. c-MYC is also involved in the positive regulation of human MSC proliferation and differentiation[390]. However, the activation of Myc targets in MSC and OB cells has to be clarified yet. We also identified pathways showing opposite trends in OBs isolated from *Asxl1* KO transplanted mice compared to MDS-MSCs, isolated from patients with *ASXL1* mutations. As already described, the differences could be due to the fact that humans had a full-blown disease and most of the patients showed more than one mutated gene.

Overall our findings highlighted the ability of *Dnmt3a* and *Asxl1* hematopoietic-specific loss to induce different and profound modifications in the transcriptome of BM niche cells *in vivo*. Further studies are necessary to confirm and understand the biological and pathological meaning of these alterations.

In conclusion, this thesis provides novel insights into the mechanisms regulating the cross-talk between malignant/pre-malignant cells and BM stromal cells in myeloid malignancies. We demonstrated that MDS- and AML-MSCs have intrinsic, distinct functional abnormalities and they actively support AML cell. Thus, we could speculate that MDS- and AML-MSCs could potentially convert the BM microenvironment from hostile to supportive for tumor growth. On the other hand, we demonstrated that also malignant cells can shape their niche. Indeed, we found that AML cells, producing IFN-γ, induce the expression of IDO1 in AML-MSCs, which in turn induce Tregs production, favoring an immunotolerant milieu. Furthermore, we found that pre-leukemic cells with *Dnmt3a* or *Asxl1* deletion, transplanted in wild-type recipients, are able to induce distinct modification of stromal cell transcriptome, suggesting that *Dnmt3a*- and *Asxl1*-null hematopoietic cells are able to shape their own niche. These experimental findings highlight the relevance of the interplay between malignant cells and BM cells. We pointed out that it is a bi-directional cross-talk,

in which stromal cells can influence malignant cells and malignant/pre-malignant cells can instruct stromal cells. Nevertheless, with the results in our hands, it seems reasonable that AML cells or pre-leukemic cells first shape their niche, which in turn influences normal hematopoiesis and malignant progression. Certainly, further studies are required to further elucidate this process.

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