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Retinoic Acid and proteotoxic stress induce myeloid leukemia progenitors cell death overcoming the protective effects of the bone marrow niche mesenchymal cells.

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1. SUMMARY

The biological issue - Acute myeloid leukemia (AML) is a group of diseases due to chromosomal and genetic mutations that impair myeloid progenitor differentiation. Some leukemic blasts express mutant proteins sensitizing them to pharmacologically induced proteotoxic stresses. Indeed, our lab demonstrated that in the presence of the differentiating agent Retinoic acid, the ER stress inducer Tunicamycin and the oxidative stress inducer Arsenic Trioxide trigger cell death of AML cell lines and primary blasts bearing the FLT3-ITD mutation. Whereas Retinoic acid and Arsenic trioxide are the current therapy for acute promyelocytic leukemia (APL), Tunicamycin has never been used in clinical practice. Therefore, in order to develop a new therapeutic strategy, my Ph.D. project investigated the proteasome inhibitor Bortezomib, which induces ER stress and is already clinically approved for multiple myeloma and mantle cell lymphoma. **Results -** AML cell lines were treated with low doses of Retinoic Acid (R), Bortezomib (B), and Arsenic Trioxide (A). FLT3-ITD$^+$ cell lines and primary blasts are highly sensitive to the triple combination RBA but not to the single agents. The cytotoxic effect is mostly due to the generation of oxidative stress and the suppression of the pro-survival branches of the Unfolded Protein Response (UPR). Additionally, the role of the bone marrow niche was examined. In an *in vitro* co-culture system, bone marrow stromal cells (BMSCs) protect AML from RBA toxicity by attenuating oxidative stress. However, pharmacological doses of ascorbic acid (Vit C) used as an adjuvant pro-oxidant agent, tamper with this protection. Importantly, combined treatment efficacy was confirmed *in vivo*. Furthermore, the combination of RBA with some promising targeted therapies also provided encouraging results. **Conclusion -** FLT3-ITD$^+$ AML cells are sensitive to RBA combination. Notably, the bone marrow niche can protect leukemic blasts from oxidative stress, but additional Vit C hampers this protective effect. These results stress the importance of

studying AML in the context of the bone marrow niche and open to possible applications in AML treatment.

2. INTRODUCTION

2.1 Hematopoiesis

Hematopoiesis is a process starting from multipotent precursor cells named Hematopoietic Stem Cells (HSCs). They have both self-renewal capacity and the ability to produce all types of mature blood cells by activating multi-lineage differentiation programs. It begins during the first weeks of embryogenesis and in these early stages HSCs are located mainly in the yolk sac, then also in fetal liver and spleen. In adults the preferential site for hematopoiesis is the bone marrow and this is a long-life mechanism producing billions of terminally differentiated blood cells every day.
The first studies on murine HSCs functional characterization were made in 1961 by Till and McCulloch.[1] They performed a colony forming units-spleen assay (CFU-S) by injecting bone marrow cells from healthy mice in irradiated recipient mice. Ten days after injection they observed the presence of discrete nodules in the spleen of irradiated mice, consisting in colonies of rapidly proliferating hematopoietic tissue. The identification of both undifferentiated and lineage-specific cells within the nodules demonstrated for the first time that bone marrow cells are able to proliferate and generate all the differentiated blood cells. In the following years, the development of multiparameter fluorescence-activated cell sorting (FACS) technique, allowed the purification of HSCs from mice and their enrichment from humans according to the expression of specific surface markers. Indeed, murine HSCs are identified by a marker combination [$Lin^{neg/low}$, $Thy1.1^{low}$, $c\text{-}Kit^{high}$, $Sca\text{-}1^{+}$] and a similar combination detects human HSCs [Lin^{-}, $Thy1^{+}$, $CD34^{+}$, $CD38^{neg/low}$].[2] Furthermore, HSC can be classified on the basis of their differentiation potential. Long -term HSCs (LT-HSCs) have an indefinite self-renewal capacity and give rise to short-term HSCs (ST-HSCs). The latter maintain their self-renewal property for eight weeks and eventually produce multipotent progenitors (MMPs). MMPs have a more restricted differentiative

potential and generate oligolineage-restricted progenitors (CLPs and CMPs, common lymphoid and myeloid progenitors respectively), which ultimately produce all the differentiated blood cells through irreversible steps of maturation. Thus, according to the classical developmental hierarchy, CMPs give rise to megakaryocytes/platelets and erythrocytes (from megakaryotic/ erythroid progenitors, MEP) and to monocytes/macrophages and granulocytes (from progenitors called GMP). Instead, CLPs generate T and B lymphocytes and natural killer (NK) cells. Interestingly, dendritic cells can originate from both CMPs and CLPs.[3] Despite of this pyramidal differentiation pathways, starting from stem cells, proceeding through multipotent and oligopotent progenitors and resulting in mature cells, more recent studies based on new cell-sorting scheme have defined an alternative progenitor hierarchy across human development. In particular, this work has identified human multi-lymphoid progenitors (MLPs) as early precursors with lymphoid (T, B, NK) and myelomonocytic potential, rather than CLPs. Moreover, the authors have demonstrated that progenitor architecture is not the same across human development. Indeed, oligopotent progenitors with myeloid potential were present in fetal liver, but they were absent in the bone marrow, dominated by unilineage progenitors with myeloid or erythroid potential. Therefore, the bifurcation step between CMPs and CLPs fates is challenged by these findings highlighting highly heterogeneous CMPs, mostly composed of unipotent myeloid/erythroid progenitors and little megakaryotic potential. Instead megakaryotic/erythroid committed progenitors are derived from multipotent cells.[4]

In studying HSCs and adult hematopoiesis, it is crucial to keep in mind that HSCs are rare cells: in murine bone marrow 1:5000 cells are LT-HSCs and 1:1000 are ST-HSCs and multipotent progenitors. These cells are situated in a complex bone marrow microenvironment in which both hematopoietic and non-hematopoietic cells are surrounded by extracellular matrix. Within the bone marrow we can find mesenchymal stem and progenitor

cells, osteoprogenitor cells, perivascular stromal cells, endothelial cells, adipocytes, unmyelinated Schwann cells and cells of immune system. In this intense and heterogeneous microenvironment, a tight regulated spatial organization has a pivotal role in orchestrating HSCs maintenance and fate decisions. Bone marrow is highly vascularized, with arterioles in close proximity to the endosteum and sinusoids immersed in a dense network of reticular stromal cells. Even though the exact location of HSCs within the bone marrow still remains elusive, some recent studies have shown that these cells are preferentially positioned in perisinusoidal areas.[5,6] In these regions, endothelial cells and perivascular stromal cells are mainly required for HSCs maintenance and long-term repopulating activity. Indeed, they produce factors such as angiopoietin, chemokine CXCL12 and stem cell factor (SCF). In particular, CXCL12-expressing mesenchymal progenitors –CXCL12-abundant reticular (CAR) cells, leptin receptor ($Lepr^+$) and $Nestin^+$ stromal cells– are in close proximity with HSCs, thus regulating their functions.[7] Of note, $Nestin^+$ mesenchymal stem cells are physically associated with both HSCs and sympathetic nerve fibers. They not only express essential genes for hematopoietic stem cells maintenance, but also regulate their mobilization from the bone marrow through clock-controlled rhythmic oscillations of *Cxcl12* expression.[8] Moreover, an innovative recent study combining single-cell RNA sequencing and spatial transcriptomics revealed CAR cells specific perivascular localization, thus stressing their role in the formation of micro-niches supportive for hematopoietic activity.[9] Furthermore, HSC niche maintenance also relies on osteolineage, adipocytes and macrophages.[10–12] Thus, healthy HSCs rely on complex bone marrow microenvironment, where multiple cell-cell interactions dictate and fine tune hematopoietic cell fate decisions.

2.2 Leukemia

Leukemia is a hematologic malignancy due to hematopoietic stem/progenitor cells block of differentiation and dysregulated proliferation. This event derives from the occurrence of chromosomal aberrations and gene mutations that impair the maintenance of the wide blood cell repertoire and its normal functionality. Leukemia arises when an HSC accidentally acquires some leukemia-specific mutations, hence becoming a pre-leukemic stem cell (pre-LSC). As shown in a work by Corces-Zimmermann et al.,[13] leukemogenic mutations have been identified in many genes, including DNA methyltransferase 3A (DNMT3A), Ten-eleven translocation 2 (TET2) Isocitrate Dehydrogenase 1 and 2 (IDH1/2) and FMS-like tyrosine kinase-3 (FLT3). Intriguingly, there is evidence of patterns in mutation acquisition, with some alterations (for instance in the TET2 gene) probably occurring as early events, whereas others (such as FLT3-ITD mutation) later on, leading to leukemia progression.[13] The transition from pre-LSC to fully transformed leukemic stem cell (LSC) is a multi-step process in which further aberrations are acquired over time. These alterations affect transcription factors, epigenetic or metabolic factors and proteins involved in signal transduction or cell cycle regulation. Therefore, LSCs have the capability to initiate leukemia in murine transplantation models because they are self-renewing cells with blocked differentiation, reduced programmed cell death and altered signaling and metabolism.[14] While both pre-LSCs and LSCs maintain their self-renewal capacity, they also generate leukemic blasts. Indeed, the leukemic bulk is composed of highly proliferating immature blasts that invade bone marrow and also peripheral circulation. Over the years the interest on origin of LSCs has been constantly increasing and three main models have been proposed.[15] The *stem cell model* assesses that only self-renewing stem cells sustain cancer, thus LSCs are a homogeneous pool and they have to be the target for therapy; Conversely, the *stochastic model* considers the plasticity of stemness. In this model LSCs are a phenotypically

heterogeneous pool of stem, progenitor or precursor cells having the property of self-renewal. Lastly, according to the *dominant sub-clone model*, LSCs are genetically and phenotypically heterogeneous and a specific LCS clone (or more than one) becomes dominant during disease progression.
These three models are not mutually exclusive and they can be preferentially applied to different leukemias. Indeed, leukemias can be distinguished on the basis of both progression speed and cell compartment specifically affected. Therefore, we may classify this disease into four main categories:

- Acute myeloid leukemia (AML)
- Acute lymphoblastic leukemia (ALL)
- Chronic myeloid leukemia (CML)
- Chronic lymphocytic leukemia (CLL)

Acute forms AML and ALL are characterized by fast prognosis due to the abnormal proliferation and differentiation of myeloid and lymphoid blasts respectively. Fast progression depends on the fact that the process of leukemogenesis affects HSCs or very immature progenitors. This provokes the accumulation of malignant cells within the bone marrow, peripheral blood and sometimes in other organs. Consequently, depletion of several terminally differentiated cells from diverse lineages has dramatic effects on patients, who suffer leukocytosis and bone marrow failure. AML is the most common acute leukemia among adults, while ALL has a higher incidence in childhood. The diagnosis is established by the presence of 20% or more blasts in the bone marrow or peripheral blood. If left untreated, the death of the patient may occur in a few months.[16,17]
In Western countries CLL is the most common type of leukemia affecting adults, with a median age at diagnosis of 70 years old. It is a lymphoproliferative disease, almost always characterized by the expansion and accumulation of mature $CD5^+$ $CD23^+$ B lymphocytes within peripheral blood, secondary lymphoid tissues and bone marrow.[18] CML, a myeloproliferative neoplasm affecting elderly

people, is characterized by the uncontrolled growth of myeloid cells at different stages of maturation. The vast majority of patients have a peculiar cytogenetic lesion that is translocation t(9;22), leading to the production of the BCR-ABL1 fusion oncoprotein. There are three pathological phases: chronic phase, accelerated phase, and blast crisis. The latter occurs when blasts detected are more than 20%. Although most of the patients are in the chronic phase, if they remain untreated, they will gradually or directly progress to more severe stages.[19]

2.3 Acute Myeloid Leukemia: classification and molecular landscape

AML is a group of very heterogeneous diseases because it can arise from a plethora of chromosomal and genetic aberrations. The first attempt to encompass this complexity and categorize different subgroups of patients dates back to 1976, when seven French, American and British hematologists distinguished six AML groups.[20] This FAB classification, essentially based on morphological and cytochemical parameters, was then improved in 1986 by adding immunophenotyping criteria.[21] Nowadays, this convention is still used for diagnosis, while cytogenetic markers are the most important features for risk stratification and treatment choice. Clinical guidelines in AML established in 2013 distinguish three groups of cytogenetic risk: favorable, intermediate and high risk.[22] The favorable-risk group includes patients carrying translocations and inversions such as t(8;21), t(15;17), inv(16)/t(16;16) and also patients with normal karyotype and NPM1 mutation in the absence of FLT3-ITD or biallelic CEBPA mutation.[23,24] This patients are usually treated with conventional chemotherapy and 90% of them achieve complete remission. The intermediate-risk group, which is the most numerous category (approximately 45% of AML cases), includes patients with no identifiable cytogenetic abnormalities. In this situation molecular

risk stratification is possible, especially by analyzing genes such as NPM1, FLT3, MLL, CEBPalpha, as well as alterations in the expression levels of BAALC, MN1, ERG and AF1q.[25] The high risk group comprises individuals with karyotype aberrations such as inv(3), t(3;3), t(6;9), TP53 mutation as well as cases with normal cytogenetics and FLT3-ITD mutation. This group shows high therapy resistance and frequent relapses, with an overall survival between 5 and 15%.[26,27] AML molecular biomarkers can be overall divided into four different classes: chromosomal rearrangements, genetic mutations, epigenetic and protein alterations. The next pages will provide some details about the most common, well-known aberrations in AML.

Chromosomal rearrangements

MLL-AML - Mixed Lineage Leukemia (MLL) gene, located at chromosome 11 (11q23), encodes for a histone methyltransferase which has a critical role in embryogenesis and in hematopoiesis. Indeed, MLL mediates the methylation of histone H3 on lysine residue 4 (H3K4) which positively regulates expression of genes undergoing this modification.[28] Moreover, although MLL is endowed with a DNA-binding domain, it has been shown to interact with DNA through Menin, a DNA-binding protein. In leukemia, the MLL gene can be fused to several partner genes, including AF4, AF9, AF10, ENL and ELL.[29,30] The interaction of these MLL chimeras with Menin allows deregulated transcription of genes such as HoxA9 and HoxA10, which are upregulated in MLL-AML. Importantly, MLL translocations are present in both AML and ALL, accounting for about 10% of human acute leukemia cases.[31]

CBF-AML - AML with cytogenetic aberrations in the core binding factor CBF account for 15-20% of adult cases. This subgroup is characterized by either the t(8;21) or the inv(16)/t(16;16), producing RUNX1-RUNXT1 (AML1-ETO) and CBFB-MYH11 fusion genes respectively.[32] In normal conditions RUNX1/CBFbeta is a heterodimeric transcription factor composed of alpha and beta subunits. Chimeric proteins have a dominant negative activity on the

wild type complex because they compete for heterodimerization and for RUNX1 binding sites on DNA.[33]
PML-RARA – The translocation t(15;17) (q24;q21), generating the fusion protein PML-RARA, is the hallmark of Acute Promyelocytic Leukemia (APL), an aggressive AML subtype accounting for 10-15% of AML cases.[34] RARA gene encodes for the receptor of all-trans retinoic acid (ATRA): when RARA is bound to its ligand ATRA, it acts as a transcription factor for key target genes for differentiation. Retinoid X Receptor Protein (RXR) heterodimerizes with RARA and normally, in absence of ligands, shut down target genes by recruiting corepressors with histone deacetylase activity.[35] PML gene encodes for PML protein, which has a critical role in the formation of the PML-nuclear body and acts as a tumor suppressor protein.[36] PML-RARA fusion oncoprotein represses transcription by recruiting N-CoR/SMRT complexes, and physiological concentrations of ATRA (10^{-7} M) are not enough to de-repress differentiation genes. However, pharmacological doses of ATRA (10^{-6} M) can revert the transcriptional repression and promote PML-RARA degradation by proteasome.[37] Remarkably, ATRA administration promotes leukemic promyelocytes differentiation into granulocytes. The combination of ATRA with arsenic trioxide (ATO) is even more effective than ATRA alone, because ATO induces PML-RARA degradation and apoptosis, with an excellent outcome for patients.[35,38]

Genetic mutations
FLT3 – The Fms-like tyrosine kinase 3 gene encodes a type III transmembrane tyrosine kinase receptor which is expressed in hematopoietic early progenitor cells, playing a key role in their survival and differentiation decisions.[39] FLT3 receptor, upon FLT3 ligand (FL) binding, switches from a monomeric to a homo-dimeric state and, as a consequence, some sites of the receptor are phosphorylated by its intracellular tyrosine kinase domain. This event elicits a signal transduction cascade resulting in downstream activation of MAP kinase, STAT, and AKT/PI3 kinase, which

regulate cell fate in terms of proliferation, differentiation, and apoptosis. Mutations in the FLT3 gene are found in 30% of AML patients. The most common FLT3 mutation is the internal tandem duplication of its iuxtamembrane domain, FLT3-ITD.[40] This alteration causes misfunction of the auto-inhibitory domain, thus maintaining the receptor in a constitutively active conformation that culminates in a ligand-independent cellular proliferation. Whereas also wild-type FLT3 has been shown to be upregulated in AML as a favorable prognostic factor, FLT3-ITD is expressed in about 25% of AML patients in correlation with an extremely poor prognosis.[41,42] Additional FLT3 mutations that affect the tyrosine kinase domain (FLT3-TKD) are less common. Therefore, the FLT3 receptor is an interesting factor in the development of molecular targeted therapies. As discussed in the next paragraph (1.4), in the last decade a new promising class of therapeutic strategies based on FLT3 inhibitors has emerged.

NPM1 – Nucleophosmin is a protein with nucleolar localization which acts as a chaperone and shuttles between the nucleus and cytoplasm. It is involved in the regulation of several cellular functions such as genomic stability, ribosome biogenesis, centrosome duplication, and stabilization of tumor suppressor ARF protein.[43] NPM1 mutations affect its C-terminal domain resulting in a misfolded protein delocalized in the cytoplasmic compartment (NPM1c). Moreover, NPM1c has a dominant negative activity on its wild-type counterpart and the total loss of all above mentioned regulatory functions leads to leukemic transformation. Mutations in the NPM1 gene are present in 53% of AML patients and they are associated with favorable prognosis. Instead, the co-occurrence of NPM1c and FLT3-ITD alterations correlates with a worse prognosis.[44,45]

CEBPA – CCAAT-/enhancer binding protein alpha gene is expressed in myelomonocytic cells and it encodes a transcription factor specifically required for granulocytic differentiation. This genetic lesion, carried by 5-14% of AML cases, is mostly detected as a double (biallelic) mutation, more rarely as a single,

heterozygous one. Furthermore, CEBPA double mutation commonly appears in the absence of FLT3-ITD and it has favorable prognosis. It has been demonstrated that patients carrying double mutation without FLT3-ITD have better overall survival compared to those with the single mutation.[46,47]

TP53 – This gene encodes tumor protein p53, an essential DNA-binding protein with tumor suppressor activity. Indeed, upon different cellular stresses, p53 induces cell cycle inhibition, DNA repair, and in some circumstances, apoptotic cell death. TP53 mutations are found in a wide range of solid tumors and they are less frequent in hematological malignancies (approximately 5% of cases).[48] Importantly, TP53 alteration is the most common genetic lesion in AML with a complex karyotype (CK-AML), otherwise quite rare in the case of a single chromosomal abnormality.[49,50] This genetic lesion is usually found in old patients with poor outcomes.[51]

Epigenetic alterations

DNMT3A – The DNA methyltransferase 3A gene encodes a methyltransferase enzyme involved in HSCs self-renewal and myeloid differentiation regulation. It catalyzes the apposition of a methyl group to the cytosine residue of CpG dinucleotides, resulting in gene silencing. Genetic alterations of DNMT3A are found in 18-22% of AML cases and since they are present at the pre-LSC level, it has been concluded that this is an early lesion in AML pathogenesis.[52–54]

IDH1/2 – These two genes encode isocitrate dehydrogenases, which normally catalyze the production of alpha-ketoglutarate from isocitrate in the citric acid cycle. Alpha-ketoglutarate is then used by TET proteins to perform histone demethylation. However, IDH1/2 mutated enzymes generate another reaction product, 2-hydroxyglutarate.[55] The latter acts as an onco-metabolite inhibiting TET2 activity. This results in DNA hypermethylation and blocked histone demethylation, thus differentiation genes repression and clonal expansion of progenitor cells pool.[56,57] IDH1/2 gene mutations are present in 15-20% of AML patients.

TET1/2 – Tet-eleven translocation genes encode for TET1 and TET2 proteins, which convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) during DNA demethylation. Mutations affecting TET2 result in DNA hypermethylation, the expansion of HSCs progenitors, and abnormal differentiation. These alterations are found in 10-20% of AML cases.[58,59]
Besides the genetic aberration of DNA sequences encoding epigenetic factors, also epigenetic markers have been shown to play a prominent role in leukemias onset and progression.[60] Among them, altered DNA methylation is the most frequent. However, alterations in histone modifications and non-coding RNAs are also deeply involved in these processes.[61,62]

2.4 AML treatment: state of the art and future perspectives

Until just a few years ago there has been a huge gap between understanding AML biology and translating this knowledge into good treatment options. Indeed, although several cytogenetic, genetic, and epigenetic hallmarks of the disease have revealed tremendous heterogeneity among patients, intensive chemotherapy followed by allogeneic stem cell transplantation (SCT) has been the only AML standard of care for almost 40 years. The 3+7 regimen (3 days of daunorubicin plus 7 days of cytarabine) provided 5-year survival of 30-35% in younger patients (<60 years) and 10-15% in the older ones (>60 years).[63,64] The first turning point was in the late 1980s, when all trans retinoic acid (ATRA) and arsenic trioxide (ATO) were discovered to have strong anti-APL effects.[65] Moreover, both agents were even more effective when combined with chemotherapy, indeed few years later the combination of idarubicin (IDA) and ATRA became the standard of care for APL.[66] In the early 2000s, MD Anderson showed that the combination of ATRA and ATO was better than ATRA-IDA, resulting in complete remission rate higher than 90%.[67,68]

Except for this exceptional case, the conventional 3+7 regimen remained the only treatment choice until 2017, since when the US Food and Drug Administration (FDA) approved nine new agents for AML.[69] Especially for core binding factor AML (CBF-AML), the approval for a combination of chemotherapy (high dose cytarabine) and CD33-targeted monoclonal antibody (gemtuzumab ozogamicin, GO) improved the estimated 5-year survival from 50% to 75%.[70] Another noteworthy example is the BH3 mimetic Venetoclax, a selective inhibitor of the anti-apoptotic protein BCL-2. High BCL-2 expression in AML has been related to chemotherapy resistance and most frequent relapses.[71] By mediating the dissociation of BCL-2 from pro-apoptotic BH3 proteins BIM and BID, this strong inhibitor promotes the activation of the intrinsic apoptotic pathway. Whereas it has shown moderate efficacy as a monotherapy, Venetoclax is currently used in combination with hypomethylating agents or low-dose cytarabine for patients older than 75 with comorbidities.[72,73] IDH inhibitors and FLT3 inhibitors are also valuable targeted therapies recently approved. Ivosidenib and Enasidenib are small molecules inhibiting mutant IDH1 and IDH2 respectively. Currently used as single agents for both newly diagnosed and relapsed/refractory (R/R) AML patients, they are still under investigation for combination strategies including chemotherapy, which might improve their efficiency.[69] As for FLT3 inhibitors, type I and type II generations have been distinguished. The first category comprises multi-kinase inhibitors such as Midostaurin and Gilteritinib, which are active against both FLT3-ITD and FLT3-TKD mutations. The second group includes Sorafenib and Quizartinib, specific for FLT3-ITD genetic lesion.[74] Nowadays, Midostaurin plus chemotherapy is the frontline treatment for newly diagnosed patients with FLT3 mutations whereas Gilteritinib has been approved for the R/R ones.[75,76] Sorafenib is used as maintenance therapy after allogeneic SCT, instead Quizartinib has been approved for R/R FLT3-mutated AML in Japan.[77,78] However, despite good initial responses to therapy, FLT3 inhibitors used as monotherapy have shown limited effectiveness because of the

development of resistance, leading to leukemia relapse within months or even weeks.[79,80] In a remarkable work by McMahon *et al.* (2019),[81] samples from Gilteritinib clinical trial have been analyzed through next-generation and single-cell DNA sequencing. They have detected not only treatment-driven emergent mutations activating the RAS/MAPK signaling pathway in FLT3-mutated subclones, but the expansion of alternative wild-type FLT3 subclones. The emergence of heterogeneous patterns of secondary mutations is defined clonal selection and is driven by the therapy itself. Both clonal diversity at leukemia initiating cells (LICs) level and clonal evolution during AML progression are the subject of intense study. High throughput single cell genomics has allowed to come to a deeper understanding of AML clonal architecture, a complex hierarchy of different clones which is reshaped over time.[82] This is exactly a Darwinian process, where specific AML clones, not predominant or even not detected at diagnosis, gain a proliferative advantage that is pushed by therapy-mediated selective pressure. Therefore, the emergence of secondary mutations is one of the mechanisms driving drug resistance and patient relapse. In this context, the study of clonal evolution through periodic monitoring - at diagnosis, during treatment, and at remission- definitely would give the opportunity to dynamically modify treatment choices, hopefully avoiding tumor relapse. Since every AML patient has a particular pattern of mutations changing over time, an approach based on personalized medicine would be needed. In the era of new targeted therapies for AML, an increased number of treatment options has just started making it possible. Both clinic and biology research have been focusing on drug resistance mechanisms. It is now clear that monotherapies are not long-term effective, and combined treatment strategy seems to be the only way to prevent relapse of the disease. Indeed, new therapeutic approaches combining FLT3 inhibitors with either chemotherapy or Venetoclax are under investigation.

2.5 ER stress and the UPR in AML

All eukaryotic cells are endowed with the endoplasmic reticulum (ER), a big organelle organized in a complex network of tubules and cisternae surrounding the nucleus and spreading all over the cytoplasm.[83] The ER has three essential functions: it is responsible for Calcium storage and homeostasis, thus mediating a plethora of calcium-dependent signaling pathways;[84] it is the first site for lipid biosynthesis;[83] and lastly, but mostly relevant for the purpose of this thesis, it is specialized in synthesis, folding, maturation, and post-translational modification of secreted and transmembrane proteins. Indeed, the ER lumen is an oxidating compartment containing protein chaperones, oxidoreductases, and glycosylating enzymes. This is the ideal environment where newly translated polypeptides can reach their native structure as mature proteins.[85] Unfolded and misfolded proteins are recognized by chaperones -such as BiP- undergoing multiple folding attempts. Occasionally this process fails, thus defective proteins are degraded by the proteasome through the ERAD system (ER-associated degradation).[86] However, some diverse events, like increased protein folding demands, starvation, oxidative stress, and hypoxia can perturb ER homeostasis, a condition known as ER stress. This is characterized by proteostasis alteration due to the excessive accumulation of misfolded and unfolded proteins. ER stress is detected by IRE1alpha, PERK, and ATF6, three stress sensors located in the ER membrane which activate the Unfolded Protein Response (UPR). This is a compensative network of pathways aimed to restore homeostasis.[87] However, if the stress is too intense to be solved, the response switches from pro-survival to pro-apoptotic.[88] BiP is normally bound to the stress sensors, but in the presence of numerous unfolded/misfolded proteins, it binds them in order to avoid their toxic aggregation. As BiP releases the sensors, they get activated.[89] IRE1alpha (inositol-requiring enzyme 1 alpha) undergo homo-dimerization and trans-auto-phosphorylation, hence becoming an active endonuclease that performs non-canonical

splicing of XBP1 mRNA. Therefore, spliced-XBP1 (sXBP1) enters the nucleus and acts as a transcription factor for the expression of chaperones, the components of the ERAD system, and lipid biosynthesis.[90] This is functional to increase ER protein folding capacity as well as reduce ER overload. However, if this adaptive UPR is not enough to solve ER stress, IRE1alpha operates as an endonuclease for many other RNAs. This massive RNA degradation is called RIDD (Regulated IRE1-Dependent Decay) and it leads to apoptosis.[91] Similar to IRE1alpha, PERK (protein kinase RNA-like endoplasmic reticulum kinase), once released by BiP, is also activated through homo-oligomerization and trans-autophosphorylation. Active PERK phosphorylates eIF2, a factor that is crucial for translation initiation, on its alpha subunit. This results in a general inhibition of protein translation, which is a measure to reduce ER clients load while the responses driven by IRE1alpha, ATF4 and ATF6 increase ER folding capacity. Phosphorylation of eIF2alpha, while reducing general translation, promotes that of specific mRNAs, such as the one encoding ATF4.[92,93] The latter is a transcription factor for protein folding, autophagy, and anti-oxidant response genes. However, upon unsolved ER stress, ATF4 can also induce the pro-apoptotic factor CHOP, which, among other functions, suppresses anti-apoptotic BCL-2 expression, ultimately resulting in cell death.[94] The P-eIF2alpha/ATF4/CHOP axis is regulated by a negative feedback mechanism, in which CHOP promotes transcription of the protein phosphatase subunit GADD34 which de-phosphorylate eIF2 alpha allowing resumption of protein translation. This feedback must be finely coordinated with increased folding capacity of the ER, since if translation restart to early, ER stress will become too strong leading to apoptotic cell death. Indeed, this axis is crucial in the life-death decision mediated by the UPR. The third UPR sensor is ATF6 (activating transcription factor 6), which is translocated from ER to the Golgi apparatus upon the loss of BiP binding. Then, Golgi proteases S1P and S2P cleave ATF6, hence releasing an active form (ATF6f) that can enter the nucleus to induce transcription of several

pro-survival UPR target genes.[95] These pathways are particularly interesting from the perspective of new therapeutic strategies for AML.[96] Indeed, whereas the role of the UPR in solid tumors is well known, its importance in leukemogenesis and in drug resistance is lately being revealed. A study by Schardt *et al*. on a cohort of 122 AML patients has shown UPR activation in 25% of cases.[97] Another intriguing article has demonstrated that JUN transcription factor, which is frequently overexpressed in AML patients, plays a pivotal role in AML pathogenesis as it regulates the UPR. They found that this protein is activated in response to ER stress, thus it induces UPR effectors such as XBP1, ATF4, and CHOP. Moreover, they have confirmed *in vivo* that XBP1 and ATF4 are paramount for AML cell survival.[98] In support of this evidence, it has been observed in AML samples that the XBP1 promoter is hypomethylated, suggesting that this gene is maintained transcriptionally active.[99] These findings have promoted the study of drugs that directly or indirectly alter proteostasis in AML. This is possible by choosing between two modalities: inhibiting the adaptive UPR or inducing the activation of terminal UPR. These studies have stressed how targeting UPR could be a successful strategy to treat AML.[96]

2.6 Oxidative stress in AML and its link with proteotoxic stress

The primordial Earth's atmosphere -dating back to 4.6 million years ago- was mainly composed of hydrogen, helium, and carbon dioxide. Later, the composition changed to the air we breathe today, made up of nitrogen (78,08%), oxygen (20,95%), argon (0,93%), carbon dioxide (0,03%), and other gases (0,01%). A key point for life on our planet was the emergence of oxygen, as it paved the way for the evolution of eukaryotes. However, since it is a highly reactive element, the production of reactive oxygen species (ROS) is a relevant side effect of aerobic metabolism. Superoxide (O_2^-), hydrogen peroxide ($\cdot H_2O_2$), and hydroxyl radical (OH^-) are toxic by-products of cellular respiration able to oxidize and damage proteins,

lipids, and DNA.[100] Mitochondrion is the first site for ROS production. However, they are also produced by specific enzymes such as cyclo-oxygenase (Cox), myeloperoxidase (MPO), lipoxygenase, and NADPH oxidases (Nox). Notably, Nox family is the only professional ROS producer, as it has exclusively evolved to generate them.[101] This finding supports the idea that ROS are not only a leftover from ATP production. Indeed, beyond damage signal, redox signaling has recently emerged as an important ROS activity regulating cell response to growth factors and other stimuli. At physiological concentrations, H_2O_2 is a crucial messenger mediating several cellular events, including shape changes, initiation of proliferation, and recruitment of immune cells.[102] Noteworthy, the enzymatic activity of some tyrosine phosphatases as well as serine/threonine phosphatases is tuned by reversible oxidation of specific cysteine residues.[103–105] Redox state is also important in deciding cell fate. FoxO subfamily of transcription factors maintains low ROS levels in HSC in order to keep them in quiescence.[106] Instead, a progressive ROS increase is required for myeloid progenitor differentiation.[107,108] Since redox homeostasis is crucial for cell functions, its continuous tight regulation ensures the balance between ROS production and elimination. Upon oxidative stress, when the amount of these reactive species consistently increases, the master regulator of the anti-oxidant response NRF2 activates a transcription program to restore redox homeostasis. Indeed, NRF2 protein is oxidized by ROS in the cytoplasm, is released by its inhibitor Keap1, and enters the nucleus where it induces the expression of antioxidant and cytoprotective enzymes. Some of them are glutathione (GSH), glutamate-cysteine ligase catalytic (GCLC), heme-oxygenase 1 (HMOX1), peroxiredoxin, superoxide dismutase 1 (SOD1), and catalase (CAT).[109] Remarkably, pieces of evidence demonstrate that oxidative stress and ER stress are deeply interconnected. Within the ER, the formation of disulfide bonds is driven by protein disulfide isomerase (PDI) and ER oxidase 1 alpha (ERO1) enzymes.[110] Whereas PDI accepts electrons thereby oxidizing a thiol group of a protein

cysteine residue, ERO1 transfers electrons from PDI to molecular oxygen, hence re-oxidizing PDI for another cycle and producing ROS. Moreover, cellular ROS induce calcium release from the ER, thus calcium uptake by mitochondria stimulates again ROS production. Upon ER stress, CHOP can activate ERO1alpha, which in turn favors calcium release.[111,112] Not surprisingly, the UPR effector PERK also activates NRF2, showing how these cellular stresses are related.[113] An elegant work by Kaufman and colleagues has shown that PERK-mediated cell death upon ER stress is due to the activation of P-eIF2 alpha/ATF4/CHOP pathway and an aberrant increase in protein synthesis resulting in ATP exhaustion and strong oxidative stress.[114] Another interesting work has demonstrated that PERK protein is required at the mitochondria-associated ER membranes (MAMs), points of physical and functional connection between the ER and mitochondria.[115] These findings support the idea that proteostasis is regulated by a very complex network in which different pathways are connected and alternatively activated, based on stimulus type and intensity. To explain the reliance of AML on their redox state, a clarification on leukemic energy metabolism is needed. All cancer cells rely on glycolysis, but they tend to not convert pyruvate through the mitochondrial metabolism of the TCA cycle followed by OXPHOS. Rather, cancer cells produce lactate, and this phenomenon is known as the Warburg effect, an important anabolic mechanism of tumors.[116,117] AML cells also display high glucose consumption but depend on oxidative phosphorylation energy supply (OXPHOS).[118,119] However, it has been demonstrated that they have higher mitochondrial mass and a lower spare respiratory capacity.[120] Therefore, AML blasts are particularly vulnerable to oxidative stress as they exhibit elevated ROS levels. On the other hand, oxygen radicals have been demonstrated to be crucial for leukemic transformation in cells carrying FLT3-ITD mutation. Indeed, this oncoprotein activates serine/threonine kinase AKT, leading to the stabilization of $p22^{phox}$, which is a regulatory subunit for NOX1-4 enzymes. Moreover, FLT3-ITD activates STAT5, hence inducing

NOX4 expression. As a consequence, increased NOX-derived ROS provokes DNA damage and mutagenesis.[121] Furthermore, a recent work has shown that chemotherapy-resistant AML cells have increased ROS levels and high OXPHOS status.[122]
Therefore, redox homeostasis is certainly a druggable mechanism in AML. Besides approved conventional chemotherapeutics and oxidative stress inducers -like arsenic trioxide- which elevate ROS provoking cell death, alternative approaches point to OXPHOS pharmacological inhibition. An exquisite work by Pollyea *et al.* has reported the efficacy of a combination of venetoclax and azacitidine for *de novo* elderly AML patients. The results show toxicity on LSCs due to amino acids depletion and consequent OXPHOS inhibition.[123]

2.7 The leukemic bone marrow niche

As discussed above, clonal plasticity and evolution is the main driver of AML progression and therapy resistance. Furthermore, leukemic blasts are able to completely re-shape the bone marrow microenvironment in order to create a self-reinforcing niche, at the expense of normal hematopoietic cells.[124] A key role in this context is played by mesenchymal stem cells (MSCs) and by bone marrow stromal cells. They establish intense crosstalk with leukemic blasts, and their reprogramming renders them able to orchestrate AML cells survival. Nonetheless, all cell populations located within the bone marrow take part in this process. Now we will provide some examples to explain the complex interplay between AML and the bone marrow niche. We have previously stressed the supportive role of the sympathetic nervous system (SNS) toward $Nestin^{+}$ mesenchymal niche cells. In turn, these stromal cells are important for HSCs survival. A work by Hanoun and colleagues has shown that leukemic cells provoke neuropathy of SNS in a model of MLL-AF9 AML. They have found the expansion of stromal cells primed for osteoblastic differentiation and the exhaustion of mesenchymal progenitors supporting HSCs.[125] The transformation of the bone

marrow niche in a leukemia-permissive microenvironment is also due to exosome secretion. Exosomes are vesicles through which cells mutually exchange proteins, lipids, and nucleic acids, hence they have an essential role in cell-cell communication. AML-derived exosomes were shown to block osteolineage and bone formation *in vivo* by inducing DKK1, a suppressor of normal hematopoiesis. Moreover, they also provoked the downregulation of cytokines and growth factors essential for HSC survival. Notably, the inhibitor of Rab27a -involved in exosome release- prolonged life of AML engrafted mice.[126] In addition, another work showed that AML can transfer ER stress to bone marrow MSCs through extracellular vesicles, thus shaping a pro-leukemic microenvironment.[127] Indeed, as for solid tumors, leukemic blasts have high energy metabolism and proliferation rate, thus they continuously experience harsh conditions like hypoxia, ER stress, and starvation. In AML, neo-angiogenesis is sustained by vascular endothelial growth factor (VEGF) and angiopoietin, which are produced by both leukemic and bone marrow stromal cells.[128,129] Importantly, also endothelial cells support AML progression, and increased vascular permeability favors drug resistance.[130] Moreover, AML blasts exploit adipocytes for their own energy supply. Together with OXPHOS, fatty acids oxidation (FAO) is another metabolic pathway AML relies on. Thus, leukemic cells establish crosstalk with adipocytes aimed to induce lipolysis and obtain free fatty acids.[131,132] Additionally, an impressive strategy especially used by AML to survive chemotherapy-related oxidative stress induction is mitochondrial transfer. It has been shown that the unidirectional transfer of mitochondria from mesenchymal stem cells to leukemic blasts occurs through either endocytosis or the formation of actin tunneling nanotubes (TNT), ensuring an increase in AML cells' mitochondrial mass by up to 14%.[133,134] Another valuable work by Forte *et al*. has demonstrated that Nestin^{+} MSCs drive AML chemoresistance in a dual mode: by enhancing AML bioenergetic capacity through OXPHOS and inducing glutathione-dependent antioxidant response.[135] Furthermore, immune escape is

another mechanism leading to AML progression and drug resistance.[136,137] Indeed, whereas NK cells and T lymphocytes antitumor activity is impaired, immunosuppressive T regulatory cells and M2 macrophages are recruited by AML blasts. Again, MSCs are involved in this process. Lastly, bone marrow homing is an important point for AML pathogenesis. Likewise normal HSCs, LSCs express CXCR4 receptors on their surface membrane; thus, their bone marrow homing, mobilization and survival depend on CXCL12 chemokine secreted by MSCs.[138] Moreover, AML cells highly express VLA-4 integrin: through the interaction with VCAM1 adhesion molecule on stromal cells, AML blasts receive pro-survival and proliferative signals responsible for chemoresistance.[139]

3. AIMS

AML is a heterogeneous hematologic malignancy characterized by a wide range of chromosomal abnormalities, genetic and epigenetic mutations. Such a complex molecular landscape reflects enormous diversity among patients in terms of symptoms, prognosis and treatment outcome. Indeed, conventional chemotherapy is often poorly effective and relapse of the disease still remains a major issue. Remarkable improvements in understanding AML biology have provided new insights into essential pathways for leukemic cells, hence druggable vulnerabilities. The presence of mutant and fusion proteins that are prone to misfolding and aggregation can render leukemic cells sensitive to pharmacologically-induced proteostasis alteration. Our group has been working on a combination of ER stress and oxidative stress aimed to perturb proteostasis, thus shifting AML balance toward terminal UPR. In the context of APL, it has been demonstrated that the ER stress inducer Tunicamycin (Tm) acts in synergy with the differentiating agent retinoic acid (RA) and the oxidative stress inducer arsenic trioxide (ATO) in inducing APL cells death.[140] Remarkably, the cytotoxic effect due to the combination of these two stresses is accompanied by oncoprotein PML-RARA aggregates formation. Then, triple treatment RTA efficacy has been confirmed in AML subtypes expressing different oncogenic proteins, such as MLL-AF6 fusion protein and FLT3-ITD. In particular, the combination was effective against both AML cell lines and FLT3-ITD$^+$ AML primary blasts obtained from patients.[141] Importantly, this combined treatment did not alter the clonogenic capacity of normal hematopoietic progenitor cells from the bone marrow of healthy donors (Figure 1). The relevance of this work is the use of low doses of each compound. Indeed, the induction of ER stress alone or oxidative stress alone activate adaptive responses allowing cell survival, whereas the triple treatment RTA strongly increases oxidative stress, leading to AML cell death.

Retinoic acid synergizes with the unfolded protein response and oxidative stress to induce cell death in FLT3-ITD$^+$ AML

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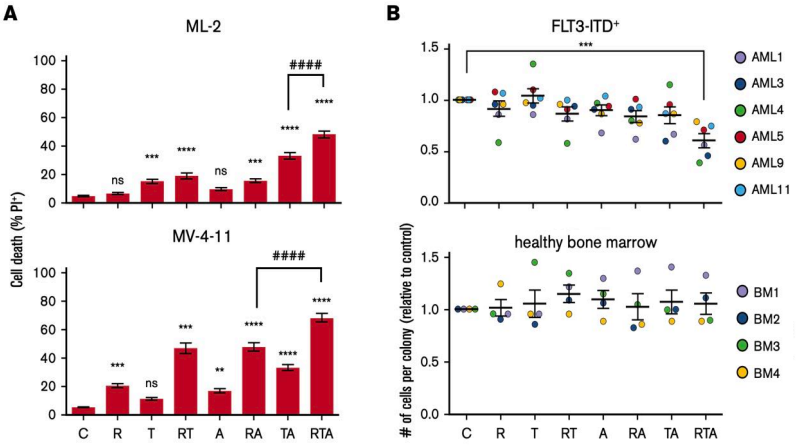


Figure 1. AML cell lines and human primary blasts are sensitive to a combination of retinoic acid and oxidative stress. A) Analysis of cell death by propidium iodide (PI) uptake for ML-2 (upper panel) and MV4-11 (bottom panel) AML cell lines, treated with 10nM RA (R), 50ng/mL Tm (T), and 500nM ATO (A), alone or in combination for 72 hours (n=16 ± SEM). One-way ANOVA vs C (control): **P<0.005, ***P<0.001, ****P<0.0001, ####P<0.0001 Student *t* test. **B)** Colony-forming unit assay for AML blasts from 6 FLT3-ITD$^+$ patients (upper panel) and for mononucleated cells from 4 healthy donors (bottom panel) treated as in A) for 8 days: ***P<0.001 Student *t* test. (Masciarelli, S. *et al*., Blood Advances 2019)[141]

Whereas RA and ATO are used in clinics for the treatment of APL, the N-glycosylation inhibitor Tm has not been approved for clinical studies. Therefore, in order to increase the translational potential of this combined strategy, we have evaluated Bortezomib (Btz) as an alternative ER stress inducer for this combination. Bortezomib is a reversible proteasome inhibitor already approved for the treatment of other hematologic malignancies -multiple myeloma and mantle cell lymphoma. In the last years the interest for proteasome

inhibition in AML has been constantly increasing, and several pre-clinical studies investigating synergism with chemotherapy are actually ongoing.[142] Bortezomib is a reversible inhibitor of the 26S proteasome. The latter is a complex that catalyzes at least 80% of protein degradation in mammalian cells. This machinery has a pivotal role in deciphering the half-life of ubiquitylated proteins, including regulatory and misfolded proteins.[143] Indeed, the proteasome is classified as an ER stress inducer as its activity is downstream of the ERAD pathway, hence it directly regulates ER homeostasis. Therefore, this PhD project investigates the effects of a combination of Retinoic acid, ER and oxidative stress inducers on AML cells, importantly, by using a low concentration of each drug. Furthermore, in order to develop a new combined therapeutic strategy, the possible role of the niche in affecting treatment efficacy is also addressed.

Hence, the present PhD thesis is aimed to:

1) Asses the sensitivity of AML cell lines and human primary blasts to a combination of low doses of Retinoic acid (R), Bortezomib (B), and Arsenic trioxide (A);

2) Investigate cell response to proteostasis alteration, oxidative stress, and molecular pathways regulated by the treatment;

3) Evaluate treatment efficacy in an AML-stromal cells co-culture system and the interplay between leukemic cells and the microenvironment upon treatment;

4) Molecular characterization of cells treated *in vivo*.

4. RESULTS

4.1 The combination of Retinoic acid, ER and oxidative stress inducers (RBA) leads FLT3-ITD$^+$ cell lines and primary blasts to death

The first part of the project was dedicated to the screening of different human AML cell lines, aimed to investigate their sensitivity to Retinoic acid (R), Bortezomib (B), and Arsenic trioxide (A). Table 1 shows a panel of 8 AML cell lines, each one carrying multiple translocations and gene mutations. During this step, a dose-response curve was performed by using R, B and A as single agents. Then, low concentrations having minimal toxicity when used alone were chosen to be tested in combination.

Cell line	Genotype	Sensitive to RBA
MOLM13	**t(9;11)/MLL-AF9; FLT3-ITD**	++++
MV4-11	**t(4;11)/MLL-AF4; FLT3-ITD**	+++
MOLM14	**t(9;11)/MLL-AF9; FLT3-ITD**	++
ML2	**t(6;11)/MLL-AF6; KRAS^{A146T}**	+
OCI-AML2	**t(6;11)/MLL-AF6**	-
OCI-AML3	**DNMT3A^{R882C}; NRAS^{G61}: NPM1^{W288Cfs*12}**	-
HL60	**del TP53; CDKN2A^{R80Ter}; NRAS^{G61L}**	+
NB4	**t(15;17)/PML-RARα; KRAS^{A18D}; TP53^{R248G}**	+

Table 1. Screening of different AML cell lines for their sensitivity to RBA. Cell lines were treated with Retinoic acid (R), Bortezomib (B), and Arsenic trioxide (A) at different concentrations. Symbol + refers to low toxicity of the single drugs as well as high toxicity of the combination RBA; symbol – represents either high toxicity of the single drugs or low sensitivity to the combination.

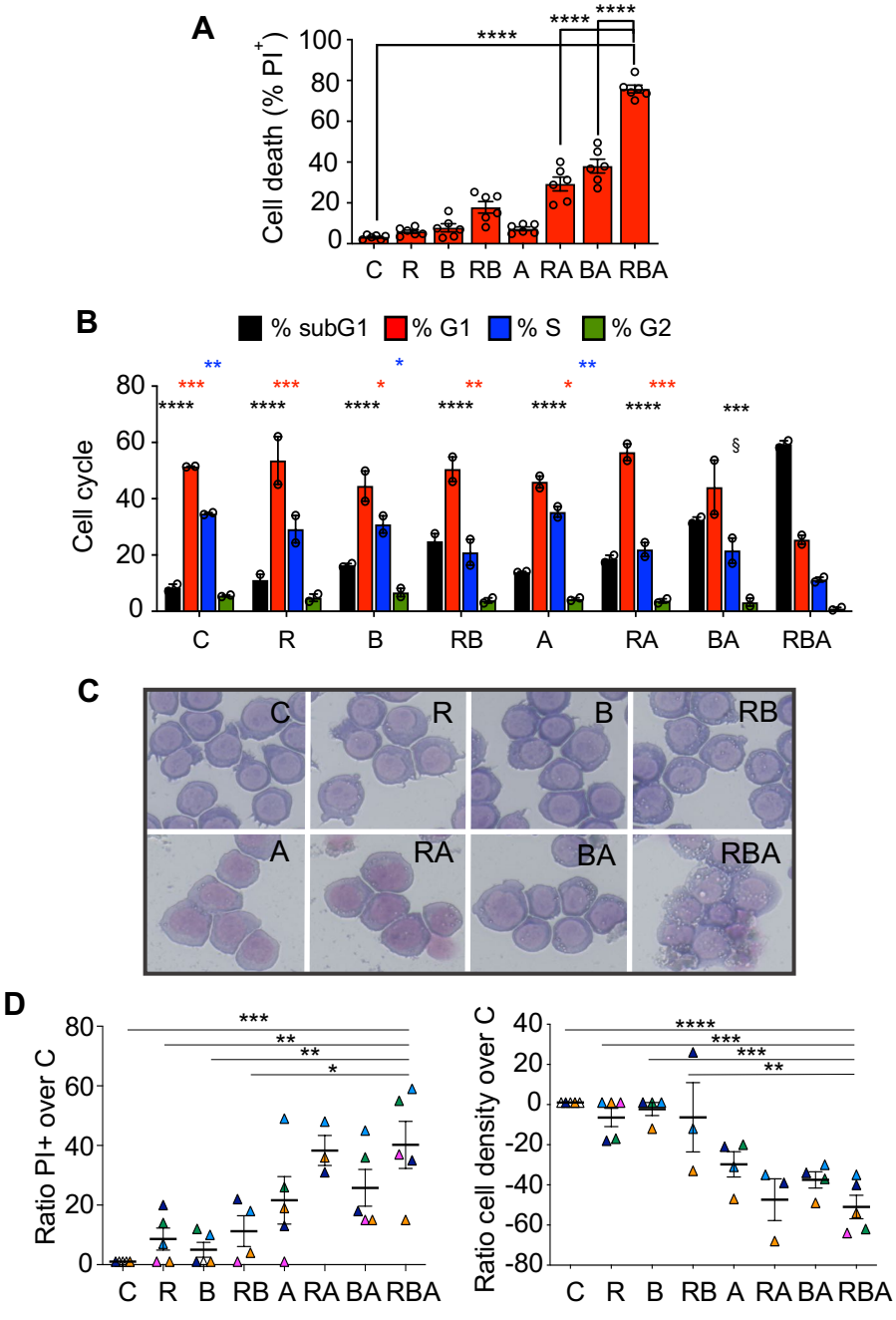

A
Cell death (% PI+)
100
80
60
40
20
0
C R B RB A RA BA RBA
****
B
% subG1
% G1
% S
% G2
Cell cycle
80
60
40
20
0
C R B RB A RA BA RBA
C
C
R
B
RB
A
RA
BA
RBA
D
Ratio PI+ over C
80
60
40
20
0
C R B RB A RA BA RBA
Ratio cell density over C
40
20
0
-20
-40
-60
-80
C R B RB A RA BA RBA

Figure 2 (previous page). FLT3-ITD$^+$ AML cells, both MOLM13 cells line and human primary blasts, are sensitive to the triple treatment RBA. A) MOLM13 AML cells treated for 72 hours with 10nM R, 2.25nM B, and 500nM A, alone or in combination, and propidium iodide exclusion assay measured by FACS (n=6 ± S.E.M., Ordinary one-way ANOVA ****P <0.0001). **B)** Cell cycle analysis of MOLM13 cells 48 hours after treatments. (n=2 ± S.E.M., two-way ANOVA vs RBA: *P <0.05, **P <0.005, ***P <0.003, ****P <0.0001, § subG1 C vs BA: P <0.005). **C)** MOLM13 cell morphology analyzed by Wright-Giemsa staining 72 hours after treatments. **D)** FLT3-ITD$^+$ human primary blasts cell death and cell proliferation upon treatment with 10nM R, 3nM B, and 500nM A, alone or in combination (n=5 ± S.E.M., Ordinary one-way ANOVA *P <0.05, **P <0.005, ***P <0.003, ****P <0.0001).

The results of this screening highlighted that especially MOLM13, MV4-11, and to a lesser extent MOLM14 cell lines, all expressing FLT3-ITD oncoprotein, showed low sensitivity to R, B, and A used as single agents as well as high sensitivity to the triple treatment RBA. On the other hand, the FLT3-ITD$^-$ cell lines resulted either poorly sensitive or totally insensitive to the combination RBA. Specifically, low doses of R, B or A alone were slightly toxic for MOLM13 cell line, whereas the triple treatment provoked a strong cytotoxic effect, suggesting a synergistic interaction among these drugs (**Figure 2A**). Similar though milder responses were found in MV4-11 and MOLM14 cells (data not shown). In MOLM13 cells, in order to understand whether this toxicity was due to cell proliferation inhibition and to which extent, cell cycle analysis was performed 48 hours after treatment. The results showed that the triple treatment RBA induces both significant increase of the subG1 phase (representing dead cells with fragmented DNA) and the decrease of G1 and S phases -comprising resting and dividing cells respectively (**Figure 2B**). These data demonstrate that combined treatment RBA induces both AML proliferation arrest and cell death. Moreover, cell morphology analysis after 72 hours shows the appearance of cell vacuolation strongly supporting RBA effectiveness (**Figure 2C**). Notably, treatment efficacy in terms of cell death and cell proliferation was confirmed in FLT3-ITD$^+$

purified human primary leukemic stem cells ($CD34^+$) obtained from patients (**Figure 2D**).

4.2 Combined treatment RBA generates ER stress and suppresses the adaptive UPR

The study proceeded with the characterization of proteotoxic stress induction in MOLM13 leukemic cell line. In order to investigate the possible proteostasis alteration, leukemic cells were observed 24 hours after treatment by Transmission Electron Microscopy (TEM) analysis. This elegant technique examines cell ultrastructure, thus it is appropriate to detect potential changes in organelles and complex compartments like the ER. From the TEM analysis, ER tubules and cisternae appeared more enlarged, especially upon triple treatment RBA; ER swelling is an adaptive response to ER stress: enlarged ER membranes are aimed to tackle misfolded proteins overload (**Figure 3A**). Furthermore, the analysis of the expression of some ER chaperones was performed. The localization and distribution of calnexin, an abundant ER-resident chaperone which synthesis is upregulated upon ER stress conditions, appeared deeply changed, again confirming ER morphology perturbation (**Figure 3B**). However, BiP protein, a critical chaperone for the pro-survival adaptive UPR pathway, did not seem increased.

A

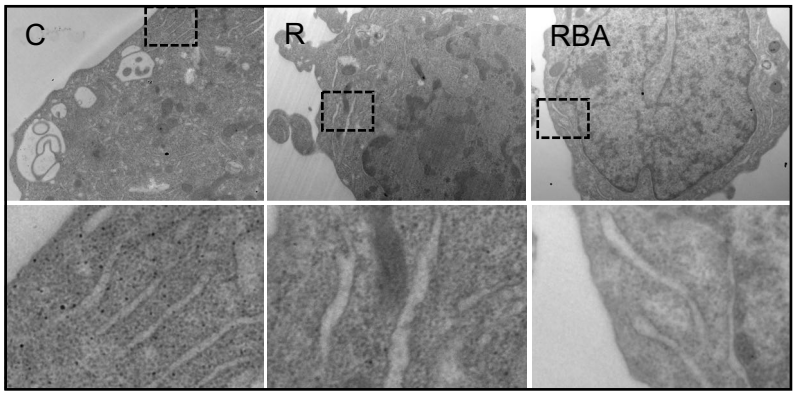


Figure 3 cont.

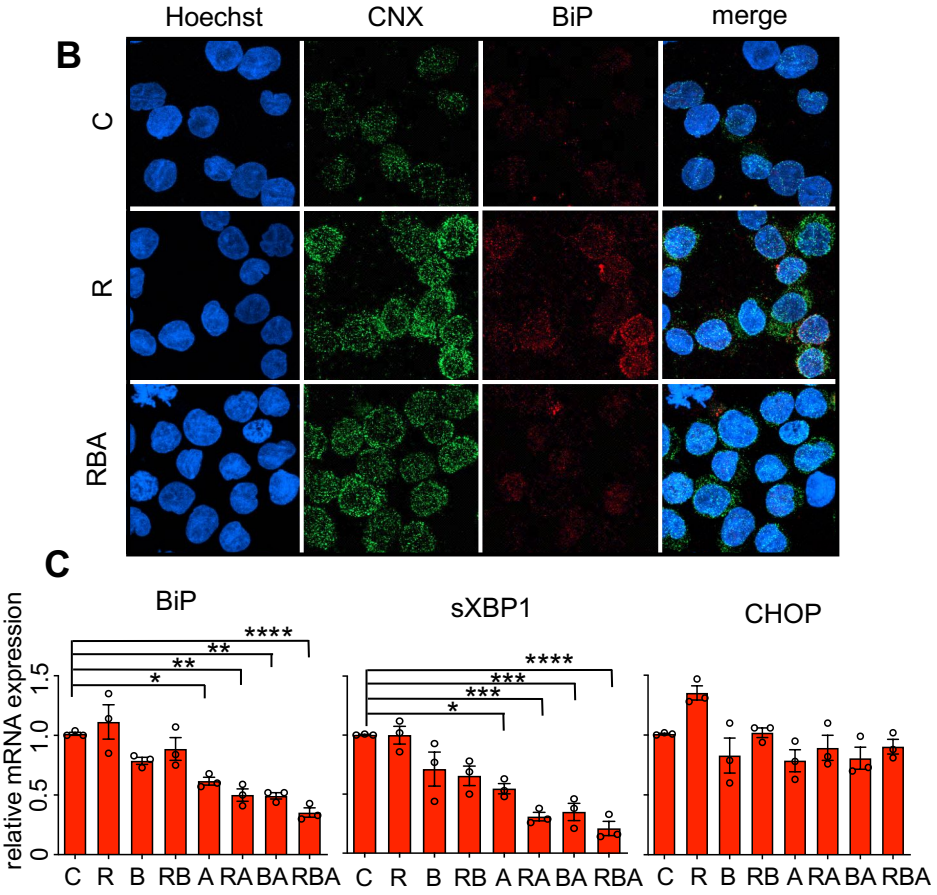
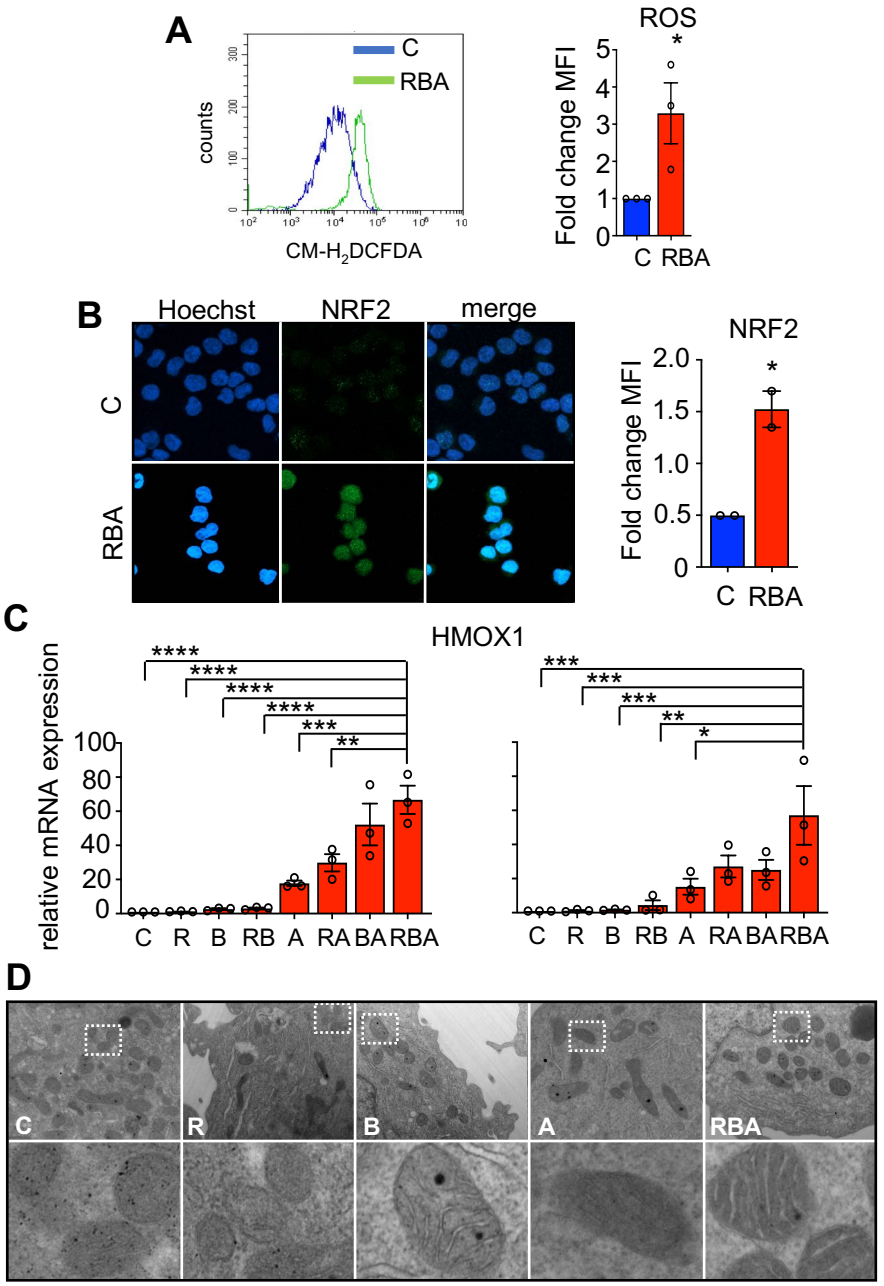


Figure 3. RBA combined treatment generates ER stress without the consequent activation of the pro-survival UPR pathways. A) Previous page. TEM analysis of MOLM13 leukemic cells 24 hours after treatment; Dashed squares indicated in the upper panels are proposed as an enlarged view in the lower panels (magnification 21300x). **B)** Confocal microscopy images of MOLM13 leukemic cells 24 hours after treatment, stained for calnexin (CNX) and BiP chaperones, Obj 40X. **C)** Expression levels of BiP, sXBP1, and CHOP genes 48 hours after treatment measured by real time PCR (n=3 ± S.E.M., Ordinary one-way ANOVA, *P< 0.05, **P< 0.01, ***P< 0.001, ****P<0.0001)

Although leukemic cells showed higher BiP levels upon Retinoic acid administration as single agent, upon triple combination RBA they did not exhibit bigger amount of this chaperone as expected. This finding was corroborated by real time PCR analysis of the expression levels of the three main players of the UPR (**Figure 3C**). BiP and sXBP1 transcripts, that are involved in the pro-survival pathways of the UPR, were downregulated by the combined treatment, whereas CHOP transcript, which is an effector of the pro-apoptotic terminal UPR, remained unchanged. Taken together, these results demonstrate that the combination of low doses of Retinoic acid, Bortezomib and Arsenic trioxide (RBA) generate ER stress; however, the adaptive branch of the UPR which has a pivotal role in cell survival is shut down, hence promoting cell death.

4.3 Combined treatment RBA induces oxidative stress and activates the oxidative stress response

Since ER stress and oxidative stress are tightly linked and they induce each other, and since ATO is an oxidative stress inducer, the induction of oxidative stress was investigated. Thus, cellular reactive oxygen species (ROS) were measured upon treatment. The results showed that leukemic cells treated with RBA have a significant increase in ROS levels compared to untreated cells (**Figure 4A**). Therefore, the potential activation of the NRF2 transcription factor was investigated. As discussed in the Introduction, Nrf2 is the master gene of the oxidative stress response, aimed to restore redox homeostasis upon oxidative stress or toxicants. The activation of the anti-oxidant response upon RBA treatment is demonstrated by the general increase of total NRF2 and its preeminent nuclear localization (**Figure 4B**). After nuclear translocation, NRF2 acts as a transcription factor for several genes by binding antioxidant response elements (ARE) in their promoter region.


A
C
RBA
counts
CM-H2DCFDA
ROS
Fold change MFI
C RBA
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Hoechst
NRF2
merge
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RBA
NRF2
Fold change MFI
C RBA
C
HMOX1
relative mRNA expression
C R B RB A RA BA RBA
D
C
R
B
A
RBA

Figure 4 (previous page). Combined treatment RBA generates oxidative stress and activates NRF2-driven oxidative stress response. A) Peak of fluorescence indicating cellular ROS levels in MOLM13 cell line 48 hours after RBA treatment, measured by flow cytometry (n=3 ± S.E.M., Unpaired t test, *P< 0.05). **B)** NRF2 staining by confocal microscopy, Obj 40X (on the left) and NRF2 fluorescence measurement by flow cytometry (on the right, n= 2 ± S.E.M., Unpaired t test, *P< 0.03). **C)** HMOX1 gene expression levels 24 hours (on the left) and 48 hours (on the right) after treatment (n=3 ± S.E.M., Ordinary one-way ANOVA, *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001). **D)** TEM analysis focused on mitochondria of leukemic cells observed 24 hours after treatment; Dashed squares indicated in the upper panels are proposed as an enlarged view in the lower panels (magnification 21300x).

Among NRF2 numerous target genes, there is heme oxygenase-1 (HMOX1), coding an antioxidant enzyme responsible for heme group catabolism. As expected, all the samples with ATO undergo oxidative stress and upregulate HMOX1 expression. However, triple treatment RBA induces the highest upregulation of the HMOX1 gene (**Figure 4C**). Furthermore, TEM analysis of mitochondria provided additional evidence that the combination RBA is responsible for oxidative damage. Indeed, especially upon triple treatment, mitochondrial cristae appear strongly marked, suggesting that their architecture is changed (**Figure 4D**). Cristae are folds in the inner membrane of mitochondria that are essential for aerobic respiration, as all the reactions of the electron transport chain (ETC), aimed to produce ATP, take place there. This clear rearrangement of cristae may reflect the attempt to recover from the strong oxidative stress induction. Taken together, these results demonstrate that triple treatment RBA induces oxidative stress and the activation of a strong oxidative stress response.

4.4 Cell death induced by combined treatment is rescued by the administration of N-acetyl-cysteine reducing agent

In order to assess the contribution of oxidative stress induction to RBA cytotoxic effect, N-acetyl-cysteine (NAC) reducing agent was used to dampen oxidative stress. NAC, a cysteine precursor, increases glutathione's cellular levels (GSH), which has essential antioxidant activity. Therefore, leukemic cells received the usual treatment with the aforementioned drugs in presence or absence of NAC. The results showed that the reducing activity of NAC almost totally counteracts leukemic cell death (**Figure 5**). Additionally, measurements of ROS levels with or without NAC showed that upon RBA treatment, NAC decreases ROS to a level exactly comparable to ROS of untreated control cells. These results confirm the strong contribution of oxidative stress in cell death induced by RBA treatment.

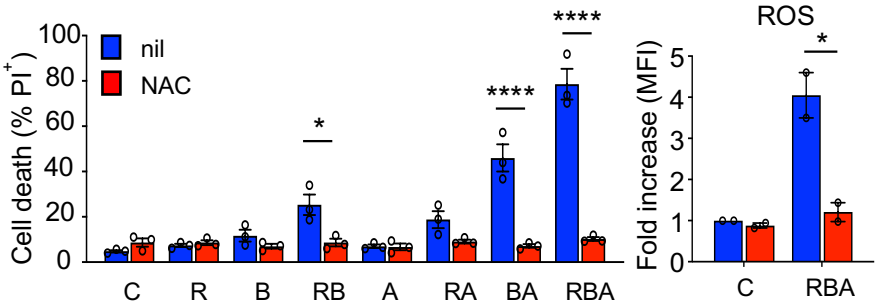

Figure 5. AML cell death mediated by combined treatment RBA is mostly due to oxidative stress. On the left, MOLM13 cell death was measured by propidium iodide exclusion assay 72 hours after treatment with RBA in the absence or presence (blue and red respectively) of N-acetyl-cysteine (n=3 ± S.E.M. two-way ANOVA, *P< 0.03, ****P< 0.0001). On the right, ROS measurement with or without NAC 48 hours after treatment (n=2 ± S.E.M. two-way ANOVA, *P< 0.03).

4.5 Mouse mesenchymal stromal cells protect leukemic cells from RBA toxic effects

As discussed in the Introduction, the bone marrow niche is a rich microenvironment in which normal HSCs reside and communicate with other cell populations through both direct contact and paracrine signals. This crosstalk is essential for determining HSC fate and maintaining a perfect balance between self-renewal and differentiation. Likewise, malignant LSCs deeply interact with the cells populating the niche. However, leukemic cells rapidly alter the composition of this microenvironment in order to survive the immune system attacks, proliferate indefinitely and invade undisturbed bone marrow and other organs. Therefore, these dynamics leading to AML progression strongly challenge treatment outcomes. In particular, several lines of evidence have revealed the crucial role of the bone marrow stromal cells in supporting AML progression and resistance to therapies. Hence, in order to evaluate the efficacy of RBA combination in a condition that approximate pathophysiologic context, an *in vitro* co-culture system was set up. This approach consisted in seeding leukemic cells and bone marrow stromal cells into the same dish and analyzing any eventual change in treatment efficacy. This strategy, aimed to mimic the niche, is a useful method to corroborate *in vitro* characterization by adding a higher level of complexity to our model. Indeed, murine bone marrow stromal cells (BMSC) were isolated from the femur of a healthy mouse. Primary BMSCs were kept in culture with a cocktail of growth factors, including IGF, EGF, FGF, and PDGF. In parallel, the murine stromal cell line MS-5 was also used. Remarkably, murine bone marrow stromal cells, both primary cells and the MS-5 cell line, totally protected MOLM13 leukemic cells from RBA cytotoxicity (**Figure 6**).

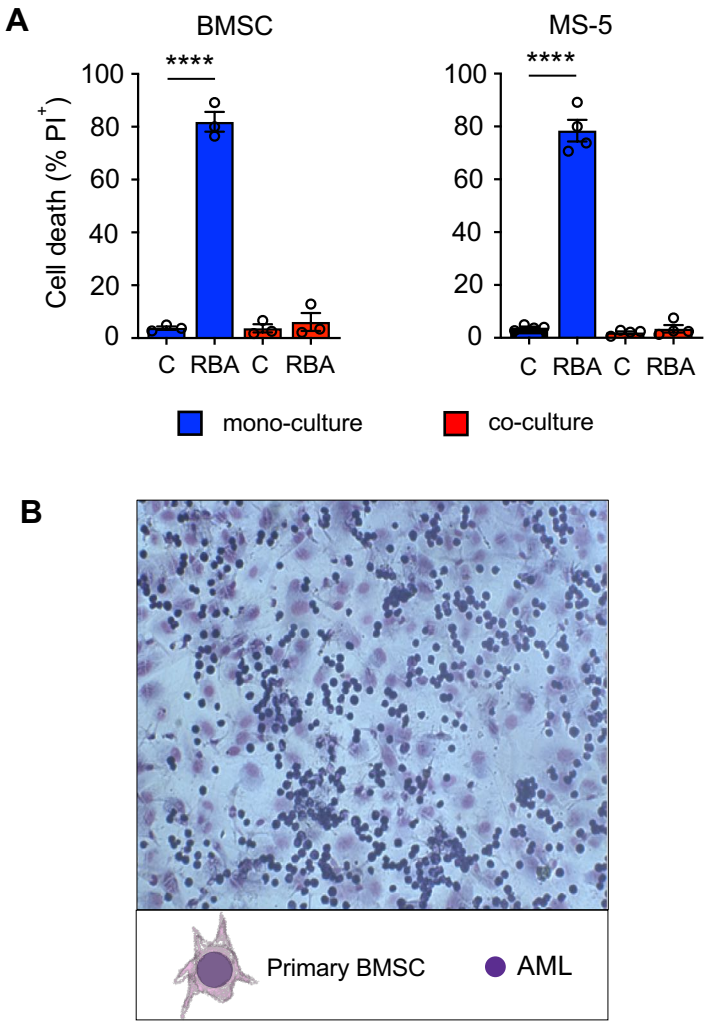

Figure 6. Murine bone marrow stromal cells utterly protect AML leukemic cells from combined treatment RBA cytotoxicity. A) Propidium iodide exclusion assay to measure cell death of MOLM13 leukemic cells when treated as a mono-culture (blue) or a co-culture (red) with primary BMSC and MS-5 stromal cell line (left and right panel respectively, n=3 and n=4 ± S.E.M., Ordinary one-way ANOVA, ****P< 0.0001). **B)** Representative image of MOLM13-BMSCs co-culture system by Wright-Giemsa staining, Obj 20X.

4.6 Pharmacological doses of Ascorbic acid impair protection from stromal cells to AML blasts

The way leukemic cells survive triple treatment RBA in presence of bone marrow stromal cells strongly suggests an intense crosstalk among the two cell populations. These findings are in line with the most recent literature which emphasizes supportive the role of stromal cell during AML progression and chemoresistance. RBA combination showed to induce proteotoxic and oxidative stress. The latter is the ultimate driver of cell death. Nonetheless, in the Introduction are discussed some brilliant papers demonstrating that stromal cells boost AML antioxidant defenses upon chemotherapy through several strategies. Therefore, in order to overcome AML protection by stromal cells, increasing oxidative stress was a valuable option. In literature Ascorbic acid, commonly known as vitamin C (vitC), has emerged for its anti-tumor activity and it has been used in clinical trials with no toxicity.[144,145] Although it is known as an anti-oxidant agent, high doses of vitC have a pro-oxidant role. For this reason, Ascorbic acid seemed an appropriate adjuvant to exacerbate oxidative stress in our conditions. As expected, the combination of RBA and high doses of Ascorbic acid was able to partially overcome the protection given by both primary stromal cells and MS-5 stromal cell line. However, the results obtained from the co-culture with primary BMSCs were characterized by high variability (data not shown). This variable restoration of AML sensitivity was due to the fact that primary BMSCs underwent differentiation within weeks or months. Furthermore, these primary cells were used to the hypoxic bone marrow niche, hence they progressively adapted to the oxygen of the air. This change in oxidative status implied an increased dose of Ascorbic acid to be used over time in our experimental conditions. Instead, MS-5 stromal cell line was obviously stable and did not show variability in the results. Therefore, MS-5 proved to be the ideal cells to study AML-stromal interaction upon treatments. Ascorbic acid was able to partly overcome the protection given by

stromal cells in terms of leukemic cells death and especially in terms of their proliferation (**Figure 7A**). Importantly, MS-5 stromal cells were not affected by this drug combination (**Figure 7B**).

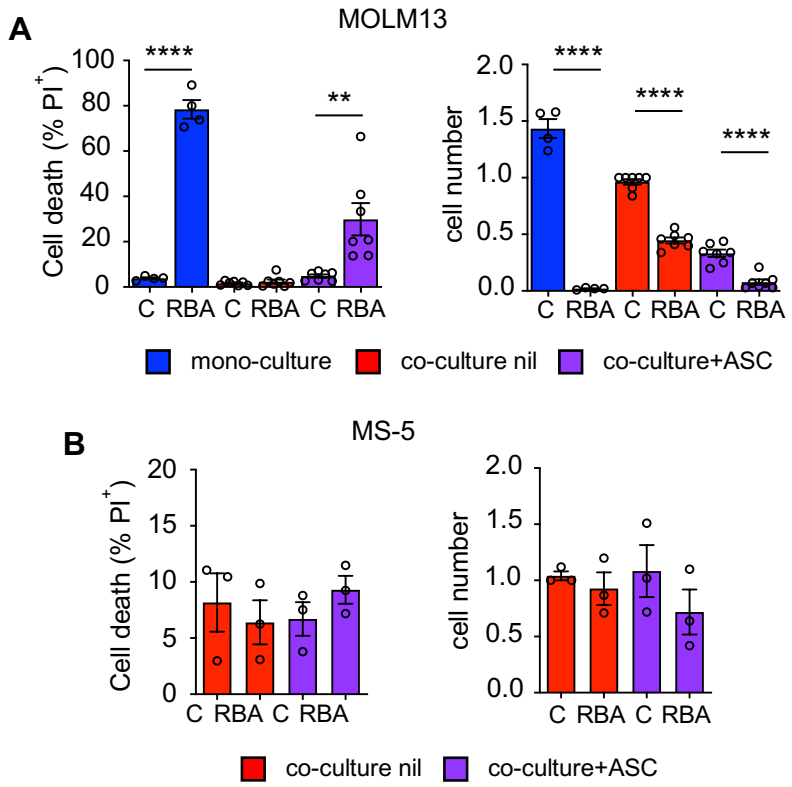

Figure 7. Ascorbic acid partially impairs the protection from stromal cells to leukemic blasts. A) Cell death measured by propidium iodide exclusion assay (on the left) and cell proliferation (on the right) of MOLM13 leukemic cells treated as mono- or co-culture with RBA and 4.5mM Ascorbic acid (ASC), after 72 hours (n=7 ± S.E.M., Ordinary one-way ANOVA **P< 0.01, ****P< 0.005). **B)** Cell death by propidium iodide exclusion assay (on the left) and cell proliferation (on the right) of MS-5 stromal cells treated in co-culture with or without ASC (n=3 ± S.E.M., Ordinary one-way ANOVA).

4.7 Stromal cells trigger the activation of a strong oxidative stress response in leukemic cells

Although the combination of RBA and Ascorbic acid was not toxic for bone marrow stromal cells in terms of cell death and cell proliferation, upon 72 hours of treatment these cells appeared changed in their shape even through the optical microscope. Morphological analysis of stromal cells obtained from the co-culture with leukemic cells gave a preliminary idea of an enlarged and elongated cytoplasm, together with a diverse spatial orientation of these cells upon treatment (**Figure 8**).

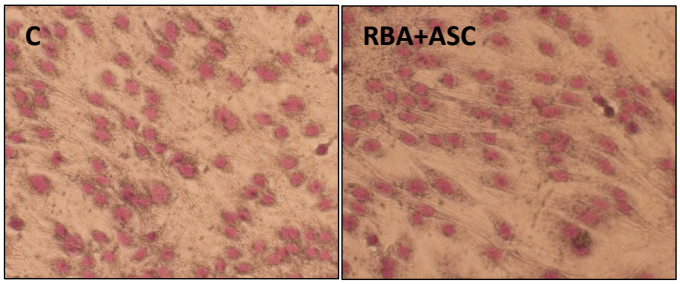

Figure 8. Bone marrow stromal cells change their morphology when they are co-cultured with AML and treated with RBA and Ascorbic acid. MS-5 bone marrow stromal cells obtained from co-culture experiment 72 hours after treatment and stained with Wright-Giemsa reagents. Obj 20X.

Therefore, bone marrow stromal cells morphological changes strongly suggested that these cells undergo potential cytoskeletal rearrangements and this could be a consequence of treatment responsiveness. To further investigate this aspect, as the stromal cells survive the combination of RBA and Ascorbic acid, the possible involvement of the oxidative stress response was evaluated.

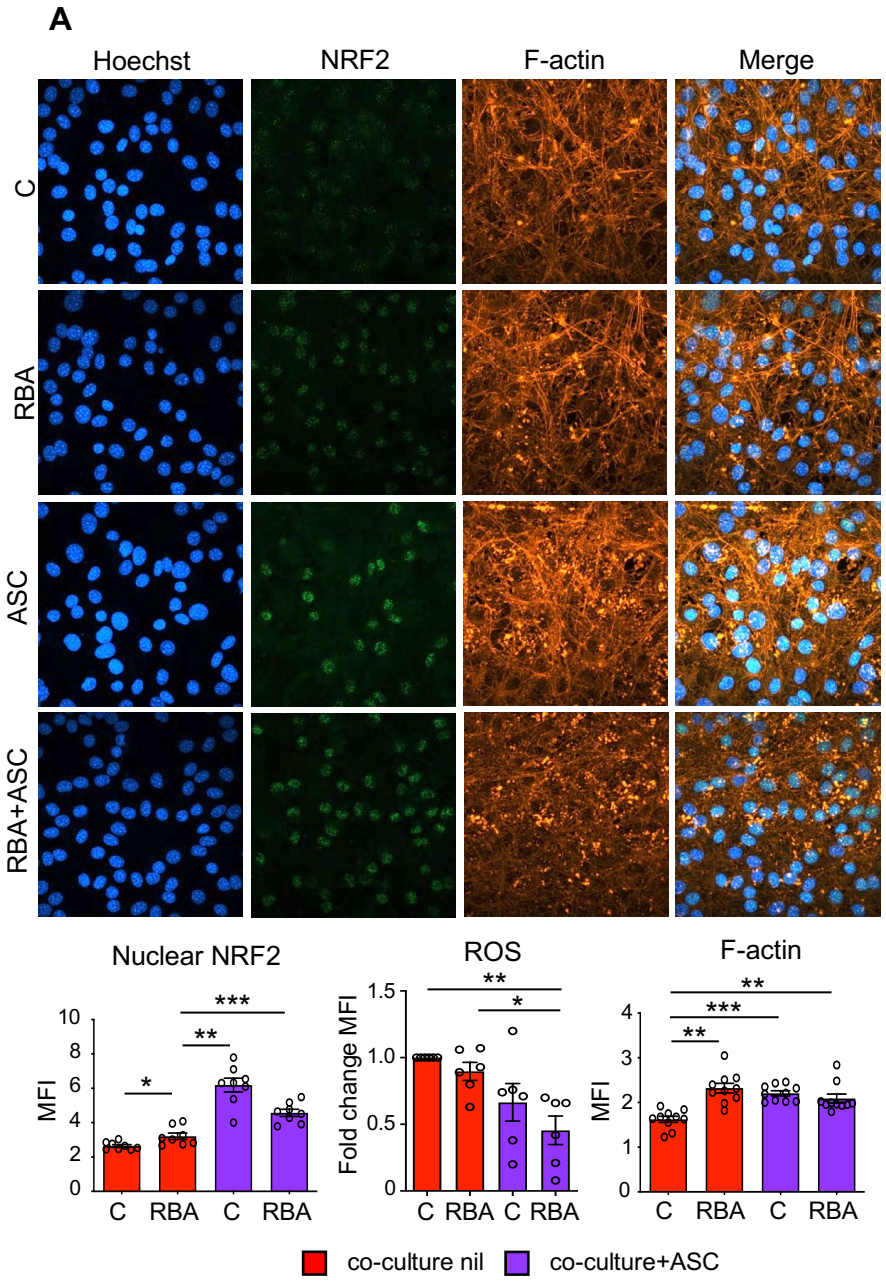

Figure 9 Cont.

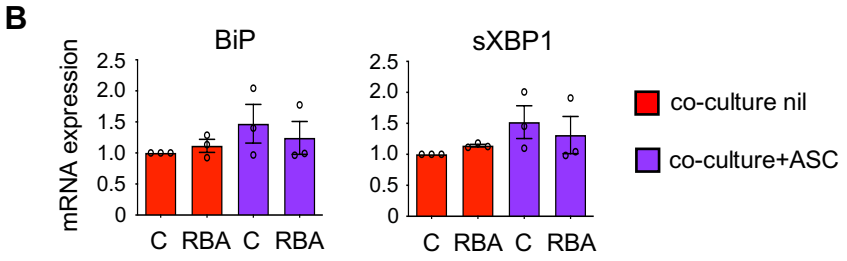

Figure 9 (previous page). Bone marrow stromal cells activate the oxidative stress response and make cytoskeletal rearrangements without UPR activation. A) MS-5 stromal cell line from the co-culture with MOLM13 leukemic cells, treated for 72 hours. Analysis by confocal microscopy of NRF2 and phalloidin staining (Obj 63X), with quantification of mean fluorescence intensity for nuclear NRF2 (left panel), F-actin (right panel), n=8 and n=10 fields ± S.E.M., Ordinary one-way ANOVA, *P< 0.05, **P< 0.01, ***P< 0.0005; Fold change of ROS measurement by flow cytometry (central panel) n=6 ± S.E.M., Ordinary one-way ANOVA, *P< 0.05, **P< 0.01. **B)** Stromal cells BiP and sXBP1 gene expression levels 24 hours after co-culture treatment (n=3 ± S.E.M., Ordinary one-way ANOVA).

Notably, by treating AML-stromal cells co-culture with Ascorbic acid and RBA plus Ascorbic acid, stromal cells show a remarkable increase in nuclear NRF2 levels. This is accompanied by the consequent downregulation of cellular ROS (**Figure 9A**). Therefore, bone marrow stromal cells activate pro-survival anti-oxidant response which is able to tackle treatment toxicity. In parallel, the pattern of the filamentous actin (F-actin) was also analyzed. Indeed, F-actin is responsible for the formation of stress fibers, cytoskeleton networks which regulate cellular shape and the direction of cellular protrusions during critical physiological processes -cell adhesion, migration and division.[146] Our results showed that treating the co-culture with RBA and Ascorbic acid causes stromal cells re-organization of their stress fibers. However, a possible connection between F-actin rearrangement and oxidative stress induction remains to be further elucidated. In order to explore

other pathways eventually involved in stromal cells' treatment response, the study of UPR markers was also addressed. The expression levels of BiP and sXBP1 genes were not significantly changed (**Figure 9B**). These results confirm that the combination of RBA and Ascorbic acid is not toxic for bone marrow stromal cells, as the activation of the NRF2-driven anti-oxidant response allow them to cope with oxidative stress. Nevertheless, their deeply altered morphology could be responsible for the impairment of AML-supportive functions.

In the last decade YAP/TAZ proteins, downstream of the Hippo pathway, have emerged as the primary sensors of cell's structure, shape, and polarity. When the Hippo pathway is inactive, YAP enters the nucleus and promotes cell proliferation or osteoblast differentiation. On the contrary, when YAP is excluded from the nucleus cells experience proliferation arrest and alternative cell fate.[147] Importantly, it has been demonstrated that cell morphology and F-actin regulate YAP sub-cellular localization. Indeed, upon stress fibers disruption YAP is retained within the cytoplasm and it is degraded.[148] This has important consequences on cell fate decisions. Analyzing more carefully stress fibers in our experimental conditions, we observe F-actin de-polymerization upon treatment. Indeed, whereas control cells predominantly have elongated fibers, especially the RBA+ASC combination is associated with the appearance of punctate F-actin and very short fibers (**Figure 10**). Importantly, this is accompanied with reduced nuclear YAP, suggesting YAP signaling inhibition (**Figure 11**). Although the significance of these alterations needs to be further explored and clarified, our preliminary results suggest that stromal cells co-cultured with AML need to preserve their F-actin cytoskeletal network in order to support leukemic cells viability. Moreover, YAP activity could have an indirect role in stromal cell-mediated AML protection.

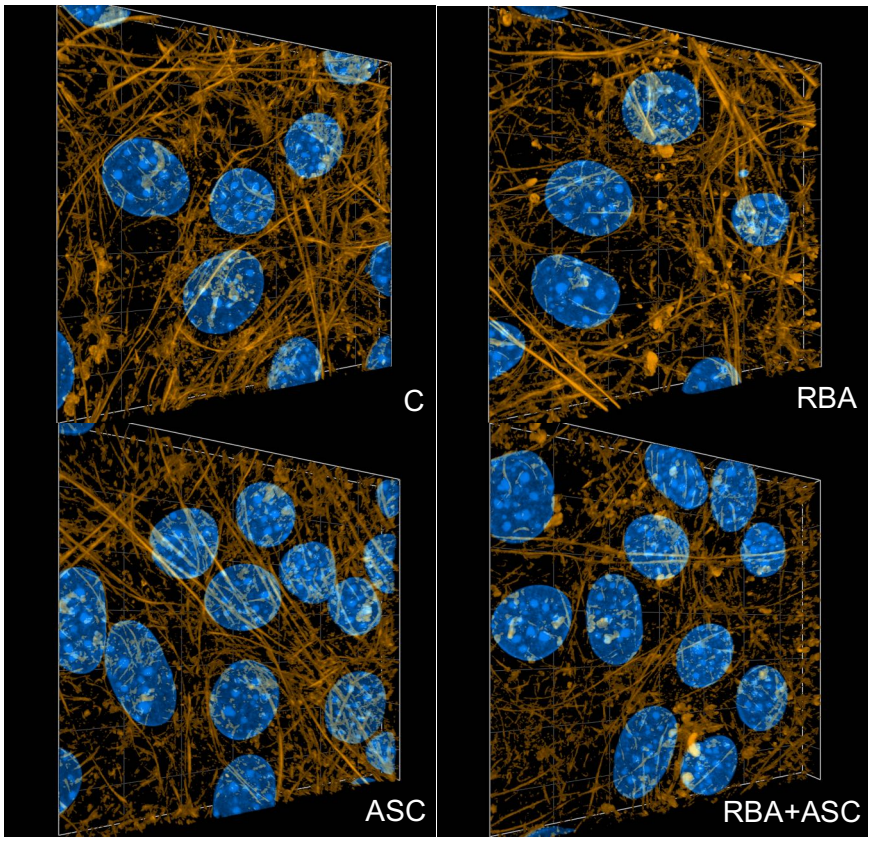

Figure 10. Bone marrow stromal cells co-cultured with AML undergo F-actin de-polymerization upon RBA+ASC treatment. Representative images of stromal cells stained 72h after treatments with phalloidin (orange) and Hoechst (for nuclei, in blue), Obj 63x. The images have been acquired in super resolution mode (SR, AiryScan) and analyzed through 3D processing.

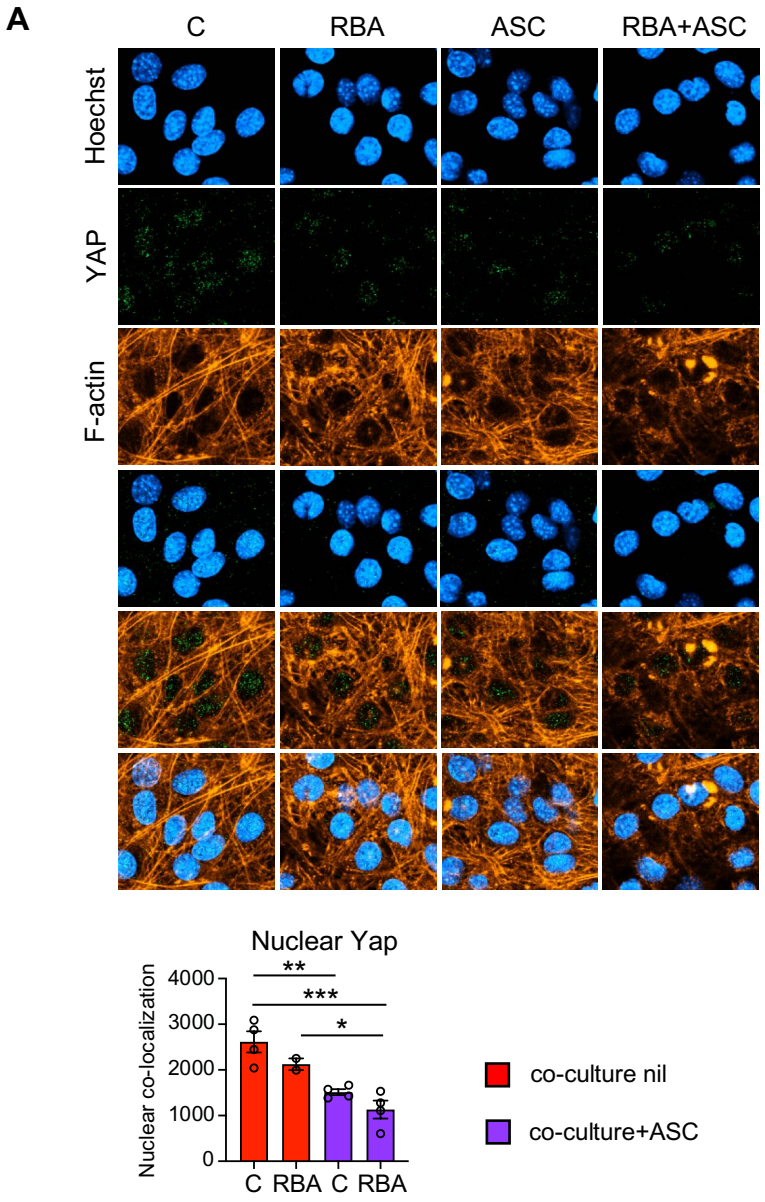

Figure 11 Cont.

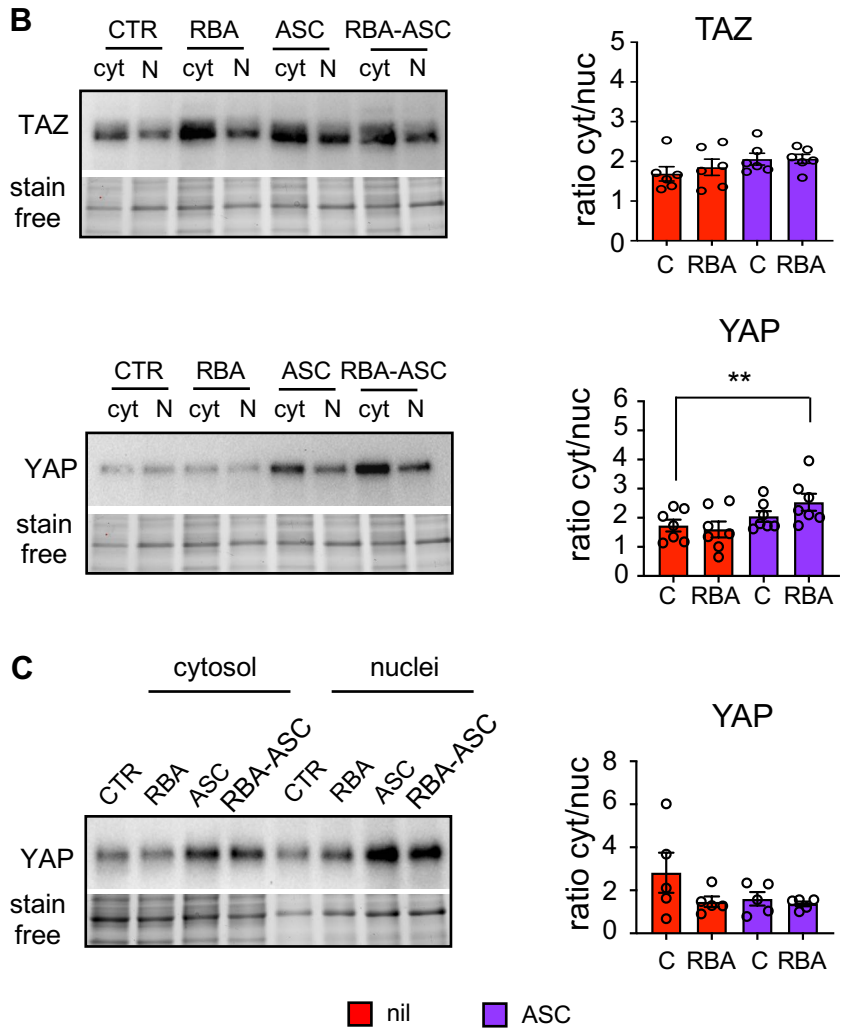

Figure 11 (previous page). Bone marrow stromal cells co-cultured with AML show decreased nuclear YAP upon RBA+ASC treatment. A) Stress fibers degeneration (F-actin, orange) is accompanied by diminished YAP (green) within the nucleus. The graph below shows YAP-nucleus co-localization study obtained from 4 representative fields, for a total of at least 500 cells analyzed (Obj 40x) for each condition (Ordinary one-way ANOVA *P< 0.05, **P< 0.006, ***P<0.0007). **B)** Ratio cytosol/nucleus of TAZ and YAP proteins in

stromal cells treated in co-culture with leukemic cells, obtained by western blot analysis. (Paired T test **P< 0.002). **C)** Ratio cytosol/nucleus of YAP protein in stromal cells treated as a mono-culture, obtained by western blot analysis.

On the other hand, in the co-culture system leukemic cells activate their anti-oxidant defenses as well, probably through stromal cells' support (**Figure 12A**).

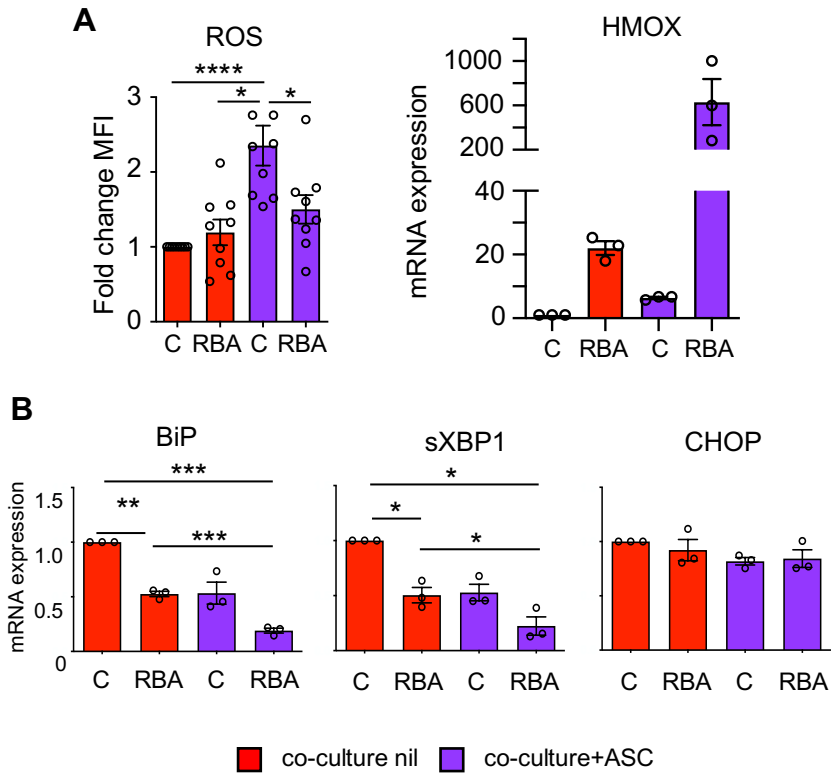

Figure 12. The combination of RBA and Ascorbic acid increases leukemic blasts oxidative stress and impair proteostasis. A) ROS measurement (at 72 hours) and HMOX mRNA expression levels (at 24 hours) of MOLM13 leukemic cells from the co-culture treatment (n=9 and n=3 ± S.E.M., one-way ANOVA,

*P< 0.05, ****P< 0.0001). **B)** Expression levels of the UPR markers BiP, sXBP1, and CHOP in leukemic cells 24 hours after co-culture treatment (n=3 ± S.E.M., one-way ANOVA, *P< 0.05, **P< 0.01, ***P< 0.001).

Only the combination of RBA and Ascorbic acid is able to impact leukemic cells by inducing dramatic oxidative stress and a consequent strong oxidative stress response. Indeed, although Ascorbic acid alone leads to a durable increase in ROS levels which lasts up to 72 hours, only the combination of RBA with ASC activates a vigorous oxidative stress response (as shown by a 600-fold increase of HMOX expression levels compared to control cells). In this context, it is crucial to keep in mind that the highest is oxidative stress, and the strongest will be the anti-oxidant response to cope with it. Notably, the demonstration that stromal cells protect AML is evident by looking at HMOX expression levels in the co-culture. Indeed, in leukemic blasts, HMOX mRNA is upregulated with a 20-fold increase, which is about three times lower than its upregulation in the mono-culture, and ROS levels are lower (compare with Figure 4 panel A and C). This result confirms that AML cells are protected from oxidative stress by stromal cells, and the addition of Ascorbic acid to RBA is necessary to overcome this protection. Indeed, in this case, activation of the anti-oxidant response is not enough, hence leukemic cells cannot cope with such a strong oxidative stress induction. Furthermore, the combination RBA concomitantly alters proteostasis and this, in combination with the oxidative stress provided by Ascorbic acid, renders the protection given by stromal cells not sufficient. Hence AML blasts shut down the pro-survival pathways of the UPR and undergo cell death (**Figure 12B**). These results suggest that AML and bone marrow stromal cells maintain intense crosstalk which is aimed to support leukemic cells upon treatment.

4.8 Stromal cells protective effects toward AML require direct cell-cell contact

In order to investigate the mechanisms underlying AML-stromal cells communication, some experiments with stromal cells conditioned medium were performed. Since several pieces of evidence from literature established that leukemic blasts alter the niche by inducing cell reprogramming in a pro-leukemic manner, we have reason to believe that our co-culture system could recapitulate some of these processes. In other words, the co-culture system is a simplified niche-like space where "messages" mutually sent, in the form of soluble molecules released in the microenvironment, culminate in the activation of mechanisms that protect leukemic cells. Furthermore, it is well known that these processes are enhanced by treatments. For this reason, the medium conditioned by stromal cells treated alone would be different from the one obtained from the co-culture. Thus, in order to assess whether AML-stromal cells crosstalk occurs through direct contact, the medium conditioned for 24 hours in the co-culture experiment with the usual treatments was added to leukemic cells (**Figure 13**).

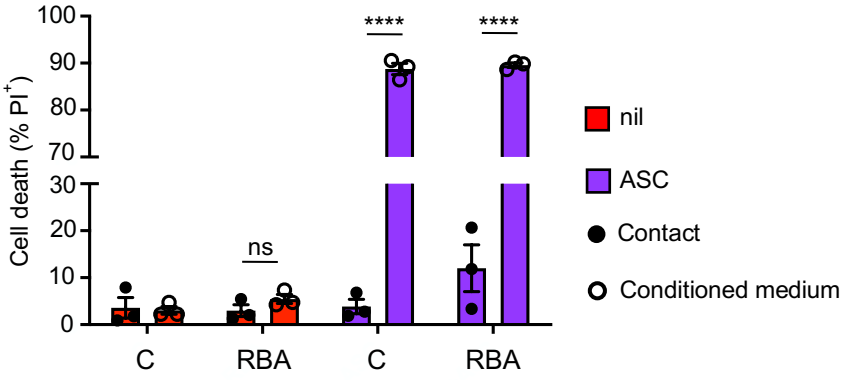

Figure 13. Protective action exerted by stromal cells towards AML requires direct cell-cell contact. Cell death of MOLM13 leukemic cells treated with

conditioned medium (CM) for 48 hours, measured by propidium iodide exclusion assay (n=3 ± S.E.M., one-way ANOVA, ****P< 0.0001)

Interestingly, the results show that the medium conditioned by the co-culture with RBA is not toxic for leukemic mono-culture; instead, the conditioned media from the co-culture with Ascorbic acid alone and the one with Ascorbic acid and RBA were highly toxic for leukemic cells. This suggests that the protection ensured by stromal cells against RBA is a contact-independent mechanism, hence it is probably mediated by factors secreted in the medium. On the contrary, protection from Ascorbic acid plus RBA is ensured by direct cell-cell contact, since the conditioned medium is not enough to promote leukemic cells survival (**Figure 13**). Altogether, these findings confirm that AML blasts rely on bone marrow stromal cells support against proteotoxic and oxidative stress induction, and this protection is partially due to direct cell-cell contact.

4.9 Triple treatment RBA combined with Ascorbic acid has anti-leukemia effects *in vivo*

In parallel with the *in vitro* studies which are the subject of this Ph. D. project, *in vivo* experiments were performed by Dr. Martyna Sniegocka. Indeed, the combination of RBA and Ascorbic acid was tested in a murine model of orthotopic leukemia. In particular, MOLM13 human leukemic cell line was transplanted in immunodeficient NSG (NOD SCID gamma) mice by tail injection. The lack of T cells, B cells and natural killer (NK) cells in these mice allows the engraftment of human AML, hence the modeling of the disease and treatment outcomes. Two days after the injection, mice were divided in control group and treated group, daily treated with vehicle and drugs respectively, and monitored for weeks. This FLT3-ITD^{+} human AML model proved to be extremely efficient - 100% of mice injected were engrafted and developed leukemia. Furthermore, fast progression pointed out the aggressiveness of this

AML subtype, with severe symptoms appearing quickly, about 17 days post injection. The signs of the pathology typically consisted in impaired mobility especially for posterior legs, as AML firstly invaded the bone marrow of femurs, then eventually other bones and organs. Mice were sacrificed as soon as evidence of suffering was noticed. In this context, I also gave my contribution in analyzing human ($CD45^+$) leukemic cells recovered from the mice bone marrow. Importantly, this experiment demonstrated that the combination of RBA and Ascorbic acid is effective in prolonging life of NSG mice engrafted with human FLT3-ITD^+ AML cells. This was accompanied by a significant decrease of leukemic blasts within treated mice bone marrow (**Figure 14**) and no evident toxicity in organs such as kidney, liver and spleen (data not shown). Furthermore, the leukemic cells recovered from the treated mice showed a much higher number of vacuolated cells. Importantly, the murine cells did not seem to be affected. (**Figure 14**)

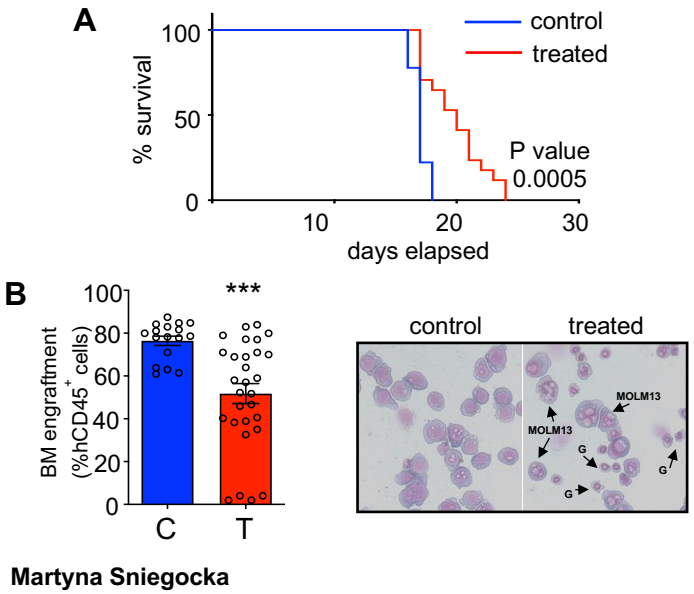

Figure 14. The combination of RBA and Ascorbic acid is effective in prolonging life of NSG mice engrafted with human FLT3-ITD^+ AML cells.

A) Kaplan-Meier curve showing the survival analysis of NSG mice engrafted with MOLM13 leukemic cell line at day 0 and treated (two/three days post-injection) with vehicle (black line, Control) or RBA+ASC (red line, Treated), C=9 and T=17 from two independent experiments performed by Martyna Sniegocka. P-value 0.0005. **B)** On the left, the percentage of human $CD45^+$ leukemic cells obtained from the bone marrow of sacrificed mice, by flow cytometry. T test, ***P< 0.002. On the right, Wright-Giemsa staining of the cells collected from mice bone marrow showing; G indicates healthy murine granulocytes. Obj 20x.

4.10 Other combination strategies: RBA and molecular targeted therapies

The experiments performed *in vivo* reflected the results obtained with the co-culture system. This outcome is extremely remarkable since it emphasizes that the use of drugs directly attacking cancer is not enough to cope with the real complexity characterizing tumor microenvironment. Therefore, the investigation of tumor cells vulnerabilities needs to be combined with the study of the entangled crosstalk with normal cells, constituting a network that underpins drug resistance and relapse. In our context, in order to further explore the communication between AML cells and the niche, additional treatment combinations were tested. As discussed in the Introduction (section **2.4**), several generations of FLT3 inhibitors have been developed, and some of them have been recently approved by FDA for the treatment of FLT3-ITD^+ AML patients. However, their usage as single agents proved to have poor long-term efficacy, hence tumor relapse is still a major issue. It is now clear that combined approaches are the only valuable strategies to tackle the variety of mechanisms leading to AML drug resistance. Therefore, RBA triple treatment was combined with FLT3 inhibitors to evaluate their effect in the co-culture system (**Figure 15**).

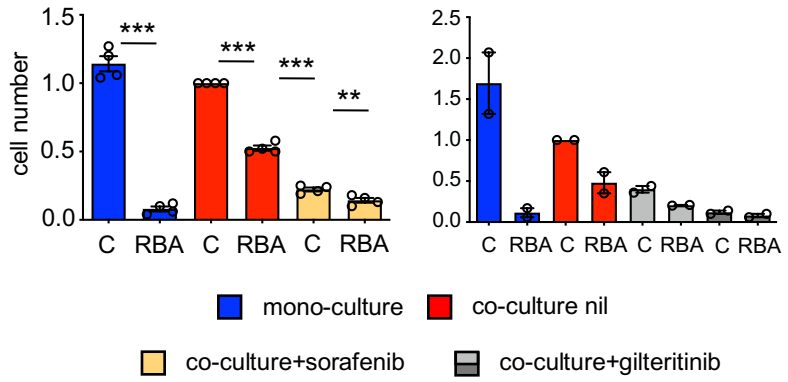

Figure 15. The combination of RBA and FLT3 inhibitors has anti-leukemic effects that bypass the protective action of bone marrow stromal cells. On the left, MOLM13 leukemic cell proliferation after 72 hours of treatment with RBA and Sorafenib 5nM (n=4 ± S.E.M, one-way ANOVA, **P< 0.01, ***P< 0.001, ****P< 0.0001). On the right, MOLM13 leukemic cell proliferation after 72 hours of treatment with Gilteritinib 15nM (light grey bars) and 30nM (dark grey bars); (n=2 ± S.E.M, one-way ANOVA, *P< 0.05, ****P< 0.0001).

Interestingly, leukemic cells are sensitive to the combination of RBA with both Sorafenib and Gilteritinib. Rather than AML cell death, these combined treatments affected leukemic blasts in terms of cell proliferation, with no toxicity for bone marrow stromal cells (data not shown). These results suggest that these combined approaches may slow down disease progression. However, targeting directly leukemic stem cells (LSCs) still remains the most relevant issue as well as an unmet need in AML therapy.

4.11 A combination of RBA and BCL2 inhibitor Venetoclax affects leukemic cells and overcomes stromal cells protection

Since LSCs are more resistant to therapies than leukemic blasts, LSCs are thought to be the responsible for relapses. Thus, identification and targeting of specific pathways that these cells rely

on are valuable strategies to eradicate AML. As discussed in the Introduction (section **2.7**), the supportive role played by bone marrow stromal cells towards leukemic cells consists of multiple alternative mechanisms. Among them, there is the upregulation of the anti-apoptotic pathways including BCL-2 and BCL-XL proteins.[149] Importantly, Jordan and colleagues showed that LSCs obtained from human primary AML samples are mostly dormant cells with low ROS levels and increased BCL-2 expression. Furthermore, LSCs rely on oxidative phosphorylation rather than glycolysis for their energy supply. In particular, these cells uniquely depend on amino acid metabolism to fuel oxidative phosphorylation, thus they are sensitive to BCL-2 inhibitors like Venetoclax.[150,151] The latter has just recently been approved by the FDA, in combination with hypomethylating agents, for the treatment of old/unfit AML patients. In our context, the efficacy of Venetoclax combined with RBA was tested in the co-culture system. Notably, this combination showed good anti-leukemic effects but was not toxic for bone marrow stromal cells (**Figure 16**).

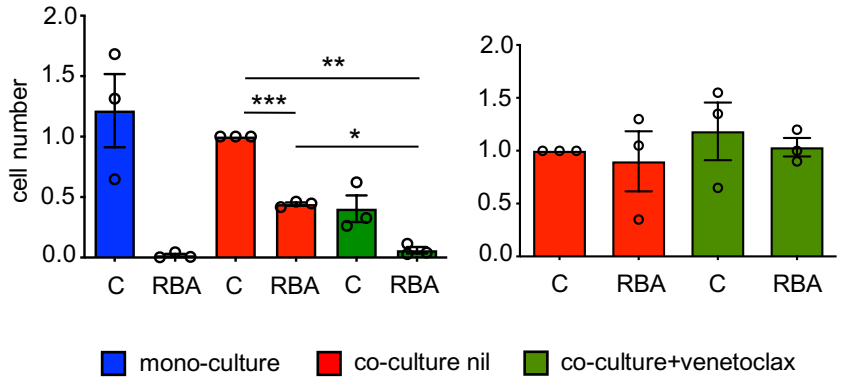

Figure 16. BCL-2 inhibitor Venetoclax combined with RBA has anti-leukemic effects without affecting bone marrow stromal cells. On the left, cell proliferation of MOLM13 leukemic cells treated as mono- or co-culture with RBA and 10nM Venetoclax, after 72 hours (n=3 ± S.E.M., one-way ANOVA *P< 0.05, **P< 0.005, ***P< 0.0005). On the right, cell proliferation of MS-5 stromal cells

treated in co-culture with or without Venetoclax (n=3 ± S.E.M., Ordinary one-way ANOVA).

BCL-2 is a well-known anti-apoptotic protein which prevents mitochondrial outer membrane permeabilization. However, besides its canonical role, BCL-2 is the master regulator of mitochondrial physiology and cellular stress response, especially redox regulation.[152] BCL-2 inhibition has emerged as a promising strategy to eradicate resistant LSCs, hence reducing the risk of relapse. Our first results on anti-leukemic effect of RBA+Venetoclax combination are encouraging and they pave the way for further investigation.

5. DISCUSSION

Acute myeloid leukemia (AML) is a common hematological malignancy due to impaired differentiation and uncontrolled expansion of myeloid progenitor cells. The American association Surveillance, Epidemiology, and End Results (SEER), which in 1973 registered AML incidence of 3.43 per 100.000 people in the US, revealed that this incidence has increased over time – the 2016-update was 4.3 per 100.000 persons-year. European epidemiologic studies reported similar incidence rates. AML is primarily a disease of older adults with a median age at diagnosis of 68 years, and males are more frequently affected than females. Importantly, the 5-year survival rate for AML is 24%, the shortest survival rate among leukemias.[153] Salvage chemotherapy (3+7 regimen) remains the standard of care for *de novo* AML patients. However, elderly people with comorbidities and young unfit patients need other therapeutic approaches. Furthermore, standard chemotherapy does not take into account the extremely heterogeneous AML molecular landscape. For these reasons, chemotherapy effectiveness remains limited. Deep understanding of the complex biology of this disease has allowed the development of molecular targeted therapies. The latter

represent a remarkable improvement compared to chemotherapy, as they target specific AML subtypes on the basis of their particular chromosomic/genetic abnormalities. Nonetheless, besides targeted therapies give good outcomes and patients remission, they are often incapable of avoiding relapses in the long term. Adaptive mechanisms involved in AML multidrug resistance have been explored. Leukemic stem cells (LSCs) are more resistant than leukemic blasts and they are primarily responsible for this adaptation. Notably, the unfolded protein response (UPR) has emerged as a critical regulatory pathway in AML survival and chemoresistance.[154] UPR activation is a strategy used by LSCs to cope with increased metabolic demands due to their high proliferation rate. Moreover, leukemic cells produce high amounts of ROS and some AML subtypes express mutant proteins which are misfolded or misplaced within the cell. These characteristics render AML remarkably vulnerable to pharmacologically induced proteotoxic stress. Therefore, perturbing proteostasis in AML is a promising therapeutic opportunity. In order to lead leukemic cells to death two valuable approaches are possible: inhibiting the adaptive UPR or inducing the terminal UPR.[96] In this context, our group demonstrated that APL cells are sensitive to a combination of the differentiating agent Retinoic acid (RA), the ER stress inducer Tunicamycin (Tm), and the oxidative stress inducer Arsenic trioxide (ATO). Indeed, pharmacological doses of RA can resume granulocytic differentiation in APL patients, and they are successfully curative especially when combined with chemotherapy or ATO. In our lab, it has been demonstrated that RA-mediated differentiation of APL cell lines and human primary blasts sensitizes them to Tunicamycin-induced ER stress. Moreover, low doses of RA, Tm, and ATO used in combination showed a synergistic effect in inducing cell death in both RA-sensitive and RA-resistant APL cell lines. Notably, the pro-survival UPR PERK/P-eIF2alpha/ATF4/CHOP axis is activated as an adaptive response to the combined treatment. Indeed, inhibition of PERK phosphorylation by selective inhibitor GSK2606414 exacerbates

cytotoxic effect.[155] Furthermore, the treatment combining RA, Tm, and ATO has proven to be effective against non-APL AML carrying FLT3-ITD and MLL mutations. In particular, FLT3-ITD$^+$ human primary blasts and AML cell lines with MLL-AF6, MLL-AF9, and FLT3-ITD mutations are highly sensitive to the combination. On the contrary, healthy bone marrow cells are not significantly affected. The presence of these mutant and fusion proteins which are prone to misfolding is a source of intrinsic proteotoxic stress, rendering leukemic blasts particularly vulnerable to these cellular stresses.[141] The use of Retinoic acid in non-APL AML has recently gained revived interest. For decades this field has been focused on terminal granulocytic differentiation as the unique evidence of RA responsiveness. Yet, recent studies have stressed the relevance of other critical points, such as RA-induced loss of clonogenic activity or prolongation of survival *in vivo*. In other words, RA is more likely to initiate AML differentiation rather than leading to terminal granulocytic differentiation like in APL. Nevertheless, although RA alone is not sufficient to achieve AML remission, it is extremely interesting for combined therapeutic strategies. For instance, retinoids and DNA hypomethylating agents have recently shown a synergistic effect in treating AML patients. Other recent studies have tried to identify RA-sensitive AML subtypes on the basis of their driver mutations. One example is given by NPM1-mutated AML cells, where RA may initiate differentiation, and induce TP53 activation with consequent degradation of NPM1-c oncoprotein. Intriguingly, it has been shown that mutated FLT3 protein precludes proper RA signaling, hence the use of FLT3 inhibitors can rescue RA-induced differentiation in AML.[156,157] On the other hand, a work has demonstrated that RA inhibits FLT3-ITD$^+$ AML cells by degrading Chk1 kinase. Chk1 is primarily involved in the DNA damage response (DDR), a cell survival strategy that prevents the progression of cell cycle in case of damage to DNA. Leukemic cells bearing FLT3-ITD mutation have increased ROS levels, hence they are prone to DNA damage. RA has been shown to induce Chk1 downregulation and consequent fatal mitotic catastrophe of these

cells. Moreover, RA and the genotoxic drug SN38 synergize in leading FLT3-ITD$^+$ AML cell death and decreasing tumor burden in a xenograft murine model.[158] Furthermore, the combination of RA and Arsenic Trioxide, which has exquisite curative effects for APL patients, has been extensively explored in the context of AML. For example, NPM1-mutated AML cell lines and human primary blasts undergo cell death upon ATRA-ATO administration, as NPM1-c oncoprotein is degraded in a proteosome-dependent manner. Notably, this combination potentiates the effect of chemotherapy, therefore this approach should be definitely tested in preclinical models.[159] In FLT3-ITD$^+$ AML cells, ATRA and ATO act synergistically in inducing inhibition of FLT3 signaling and leukemic cell death.[160] AML cells have higher basal levels of ROS and their vulnerability to oxidative stress is a matter of fact. Interestingly, together with ROS increase and oxidative damage, ATO can selectively induce FLT3-ITD autophagic degradation and decrease of tumor burden *in vivo*.[161] Interestingly, ATO has been shown to potentiate Gilteritinib cytotoxicity towards FLT3-ITD$^+$ leukemia cells. Monotherapy with Gilteritinib, a new generation FLT3 inhibitor recently approved by FDA, is not able to ensure long-term AML patient remission. *In vitro*, the combination with ATO has a synergistic effect in terms of cell death. Mechanistically, in this case ATO induces ER stress and the activation of the pro-apoptotic branch of the UPR driven by IRE1alpha. Indeed, the alternative addition of the ER stress inducer Tunicamycin to Gilteritinib resulted in similar cytotoxic effect.[162] Altogether, these studies strongly emphasize the therapeutic potential of combining ATRA with ER stress and oxidative stress inducers for AML treatment, with particular focus on FLT3-ITD$^+$ subtypes. The combination of low doses of ATRA, ATO and Tunicamycin we proposed in 2018 perfectly fits in this context. Indeed, in the studies mentioned above the concentrations of ATRA and ATO are higher than those we used with the aim of reducing off target toxic effects. However, while ATRA and ATO are used in clinics for the treatment of APL patients, Tunicamycin has never been used *in vivo*.

Therefore, in order to increase the translational potential of the combination, we have been considering proteasome inhibitor Bortezomib as an alternative ER stress inducer for this combined approach. Human 26S proteasome is a protein complex that is essential for cell proteostasis. Indeed, unfolded and misfolded secretory proteins that fail proper maturation within the ER are retrotranslocated in the cytosol and undergo proteasome-mediated degradation through the ERAD pathway. Importantly, proteasomes also determine the concentration of specific cytosolic proteins with a short half-life, such as cyclines regulating cell cycle progression and the regulator of NF-kB (IkB). In both cases, proteasome activity depends on ubiquitin ligases enzymes which mediate poly-ubiquitin protein tagging required for degradation. Therefore, proteasome inhibition is responsible for the aberrant accumulation of damaged proteins which cause proteotoxic stress. Proteasome inhibition has been demonstrated to be an effective therapeutic strategy in hematologic malignancies. Bortezomib is a transient selective inhibitor of 26S proteasome successfully approved for the treatment of multiple myeloma and mantle cell lymphoma. Hence, its therapeutic role in AML has been recently explored. So far, it has been shown that proteasome inhibition disrupts proliferative cell signaling pathways and synergizes with chemotherapeutics in inducing AML cell lines and primary blasts cytotoxicity.[163] Intriguingly, a strong correlation between sensitivity to Bortezomib and FLT3-ITD allelic burden has been demonstrated both *in vitro* and *in vivo*. Indeed, Bortezomib induces FLT3-ITD autophagy-dependent degradation, hence leading to AML cell death.[164]

The main objective of my Ph.D. thesis was the evaluation of the sensitivity of AML cells to a combination of Retinoic acid, Bortezomib, and Arsenic trioxide. The combined approach enables the use of sub-pharmacological doses of each drug, hence reducing toxicity. These low amounts of drugs have proven to be just slightly detrimental as single agents, but dramatically toxic as a combination against AML cells carrying FLT3-ITD mutation. Increased cytotoxicity upon RBA triple treatment compared to single or

double administrations strongly suggests that these agents have a synergistic effect. Cell cycle analysis clearly illustrates that RBA significantly increases the percentage of dead cells and also determines cell cycle arrest. Of note, $CD34^+$ LSCs isolated from a cohort of 5 FLT3-ITD^+ AML patients and treated *ex vivo* show similar results in terms of cell death and cell proliferation. Although the number of primary samples we used is still little and a larger group of data should be analyzed to strengthen this result, RBA efficacy towards AML stem cells is extremely remarkable. The study of RBA-mediated proteostasis alteration reveals that the triple treatment generates ER stress, as demonstrated by changed distribution of calnexin and BiP ER chaperones and different ultra-structure of ER tubules and sheets. As extensively discussed in the Introduction, in case of ER stress the Unfolded Protein Response (UPR) is usually activated as a pro-survival mechanism to restore ER homeostasis. The protein chaperone BiP and the transcription factor sXBP1 are primarily involved in this adaptive response. However, prolonged or overly intense cellular stresses facilitate the transition to terminal UPR, hence leading to cell death. Our results show that the RBA-mediated ER stress induction ends up in the suppression of the UPR pro-survival pathways. On the other hand, the induction of oxidative stress upon RBA treatment is demonstrated by a significant ROS increase and the consequent activation of the oxidative stress response driven by the master regulator NRF2. The strong upregulation of the HMOX gene, downstream of the NRF2-driven anti-oxidant response, 24 and 48 hours after drug administration, reveals the prolonged attempt to counteract oxidative damage. In line with this, TEM analysis underlines that already at 24 hours mitochondria present marked cristae, suggesting their alteration and potential deterioration, especially in the cells treated with the combination RBA. In literature, the tight connection between ER stress and oxidative stress has been extensively characterized. Proper protein folding and maturation essentially rely on the oxidative state of ER compartment. Conversely, the UPR can regulate redox homeostasis,

either by decreasing ROS to reestablish homeostasis or by increasing them to induce cell death. Our data demonstrate that pharmacologically induced ER and oxidative stress mutually potentiate their effects, hence aggravating leukemic cell death. Moreover, our findings also highlight the important role of Retinoic acid, whose differentiating activity further exacerbate RBA-related cell death. Nevertheless, the experiments we held in presence of the reducing agent N-acetyl-cysteine (NAC) identify ROS induction as the main cause of the RBA cytotoxic effect. This could be explained by considering overwhelming ROS as the key terminal event leading RBA-mediated cell death. Therefore, tampering excessive ROS is sufficient for leukemic blasts to survive the treatment. This result is exactly in line with the results we obtained in the co-culture study, where stromal cells potentiate leukemic anti-oxidant defenses hence allowing AML survival. Indeed, the presence of murine bone marrow stromal cells, both primary and MS-5 cell line, completely protects AML from RBA toxicity. However, further oxidative stress by adding high doses of Ascorbic acid is able to counteract this protection. These findings underline the importance of considering cancer as an integrated system that is surrounded and supported by an adapted tumor microenvironment, rather than just an individual malignant entity. A number of independent lines of evidence have shown that bone marrow stromal cells have a prominent role in AML progression and therapy resistance. This occurs through intense mutual crosstalk which favors niche remodeling in a pro-leukemic manner. For instance, bone marrow stromal cells boost AML oxidative defenses during chemotherapy, hence achieving chemoresistance. Similarly, in our co-culture system, stromal cells activate the anti-oxidant response in the presence of RBA, hence decreasing oxidative stress in AML up to 1/3 of mono-culture levels. Although RBA significantly impacts on leukemic cell proliferation, stromal cell-dependent dampened oxidative stress preserves AML viability. On the contrary, RBA and Ascorbic acid combination alters proteostasis and redox state at such a point to strongly activate NRF2-driven anti-oxidant response in AML, that however is not

enough to avoid leukemic cell proliferation arrest and death. These findings confirm that bone marrow stromal cells ensure blasts survival upon treatment with RBA by boosting their anti-oxidant defenses, but the further addition of Ascorbic acid exacerbates oxidative stress to a level that overcomes this protection. The mechanism through which AML protection is ensured is still not clear in our settings and a deeper analysis of this aspect is needed. Bone marrow stromal cell cytoskeletal rearrangements are an intriguing indication of responsiveness to this combined treatment. Stress fibers re-organization strongly suggests that mechanotransduction could play a prominent role in our context. Indeed, although the mechanism needs to be elucidated, F-actin undergoes a different architecture upon treatments. This could likely represent changes in cell shape, in the pattern of focal adhesions, and eventually in cell proliferation/differentiation. YAP and TAZ proteins form a heterodimer that is a primary sensor of cell's physical nature, and their nuclear translocation regulates a variety of biological processes, including cell growth, survival and mechanotransduction.[147] We show that combined treatment strongly alters stress fibers and decreases YAP expression. The connection between the oxidative stress response and YAP/TAZ pathway is a critical aspect to clarify. However, these indications open up a world of possibilities to be investigated in order to dissect the crosstalk between AML and stromal cells. In literature an increasing number of works have highlighted the role of exosomes in mediating AML progression and therapy resistance.[165] Leukemic cells use exosome-mediated intercellular communication to alter niche composition to their advantage. The transmission of ER stress through exosomes and the consequent commitment of mesenchymal stem cells toward osteoblastic differentiation is an outstanding example of this mechanism.[127,166] Since our combined treatment generates ER stress in AML cells, the investigation of this aspect would be relevant. Furthermore, exosomes can transfer a variety of nucleic acids, including non-coding RNAs. Especially miRNAs have proved to be involved in the crosstalk within the leukemic niche, resulting in

AML maintenance. Intriguingly, an increasing number of circular RNAs has also been identified as a mediator of leukemic progression. Therefore, our system will give us the opportunity to dissect AML-BMSCs crosstalk by investigating these aspects. However, our experiments with conditioned media showed that leukemic cells survival upon the combination of Ascorbic acid and RBA depends on the direct contact with stromal cells. On the contrary, protection from RBA combination alone does not need direct cell-cell contact. These findings demonstrate that upon RBA+Ascorbic acid combined treatment the interaction between AML and stromal cells occurs through multiple modalities. In literature, the mitochondrial direct transfer from BMSCs to leukemic blasts has been well characterized.[133,167] This protective mechanism, that occurs through both endocytosis and dynamic tunneling nanotubes (TNT), requires cell-cell contact. Hence, since our combined treatment generates strong oxidative stress, it is likely that this process is involved in stromal cell-mediated AML protection. Our data suggest a possible formation of dynamic direct connections between AML and stromal cells which need to be further elucidated. Also, the alteration of YAP/TAZ function is an innovative and intriguing aspect to explore in the AML niche. In addition, stromal cells gap junctions have been proven to play a relevant role in intercellular communication within the leukemic bone marrow niche. In particular, in AML-BMSCs co-culture system gap junction disruption by carbenoxolone reduced chemoresistance and increased leukemic cell death.[168,169] Therefore, identifying and targeting the main players in cell-cell communication could provide new therapeutic opportunities to improve AML treatment responsiveness. Moreover, in order to better recapitulate the bone marrow niche *in vitro*, mesenchymal stromal cells can be cultured in new engineered 3D porous scaffolds. These systems efficiently sustain patient-derived $CD34^+$ AML cells for up to three weeks and protect them from chemotherapy, hence they are very suitable for disease modeling. A recent study has compared humanized 3D-niches from AML patients and healthy

donors. The results unraveled the critical role of direct contact among AML and BMSCs (by means of both tunneling nanotubes and gap junctions) in reprogramming stromal cells transcriptome towards pro-oncogenic functions.[170] Importantly, after complete remission the transformed microenvironment reverses to healthy properties.[171] Therefore, different lines of evidence corroborate that targeting niche remodeling is an attractive strategy to potentially reroute the course of AML. The findings we have obtained so far demonstrate that RBA combined with Ascorbic acid show anti-leukemic effect that overcomes bone marrow niche protection. Indeed, this has been confirmed *in vivo* in an orthotopic model of human AML. Therefore, the co-culture system demonstrates to be a reliable way to study the crosstalk between AML and the microenvironment and to test other drug combinations before *in vivo* validation. On the basis of these results, the therapeutic potential of RBA and the BCL-2 inhibitor Venetoclax could be investigated. Although Venetoclax has been approved in combination with hypomethylating agents as the standard of care for older/unfit newly diagnosed AML patients, side effects and relapses still remain a major issue. Our combination strategy allows to reduce drug doses hence minimizing systemic toxicity. Intriguingly, BCL-2 upregulation is one of the mechanisms through which the BM niche support AML cells viability. Furthermore, BCL-2 inhibition has proven to impact amino acid metabolism of LSCs, thus impairing their peculiar way of energy supply. Taken together, these evidences identify Venetoclax as a remarkable candidate to combine with RBA for the study of AML-niche crosstalk. Our first results are encouraging and pave the way to future investigation.

6. MATERIALS AND METHODS

6.1 Cell lines and primary cells cultures and treatments

Human acute myeloid leukemia cell lines MOLM13, MV4-11, MOLM14, ML-2, OCI-AML2, OCI-AML3, HL60 and NB4 were obtained by DSMZ (Leibniz Institute, Germany) and cultured in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 10% heat-inactivated FBS (Gibco, ThermoFisher Scientific, Waltham, MA, USA). AML bone marrow samples were collected at diagnosis in the Department of Biomedicine and Prevention at the University of Rome Tor Vergata after obtaining written informed consent to the study from all patients. Murine mesenchymal stem cell line MS-5 was obtained by DSMZ and cultured in MEM Alpha medium with ribonucleosides and deoxyribonucleosides (Gibco, ThermoFisher Scientific), supplemented with 1% penicillin/streptomycin, 20% heat-inactivated FBS and 2-mercaptoethanol 100uM (Sigma-Aldrich, St. Louis, MO). Primary murine bone marrow stromal cells were obtained from a healthy mouse. After mouse sacrifice, the femurs were flashed with a syringe in order to discard hematopoietic cells. The bones were cleaned, and crushed in a mortar, then the bone matrix was digested by collagenase for 30-40 minutes. Isolated cells were seeded in a medium with 20% FBS enriched with growth factors: 100uM 2-ME, 1X NEAA, N2, B27, 10ng/ml EGF, 40ng/ml IGF, FGF, 40ng/ml PDGF, Oncostatin M (Peprotech, Rocky Hill, NJ, USA). After the first passage, only 2-ME, NEAA, IGF, and EGF were maintained.
All the cultures were maintained at 37°C in a humified atmosphere containing 5% CO_2.
MOLM13 cells were treated with 10nM Retinoic Acid (R), 2.25nM Bortezomib (B) and 500nM Arsenic Trioxide (A) bought from Sigma-Aldrich, alone or in combination, as described in the text. In order to attenuate oxidative stress, cells were pre-incubated 24 hours with N-Acetyl cysteine (NAC) 20mM (Sigma-Aldrich) with pH 7.4 and then re-treated with NAC 20mM together with the above-

mentioned drugs. Primary cells were thawed and the stem cells (CD34+) were isolated by positive selection with CD34 MicroBead Kit UltraPure human (Miltenyi Biotec, Bergisch Gladbach, Germany), by following the manufacturer's instructions. Both CD34+ and CD34- cells obtained from the separation were seeded at a concetration of 10.000 cells/ml in StemSpan Leukemic Cell Culture Kit (STEMCELL Technologies, Vancouver, Canada). After waiting 7 days for cells amplification, they were treated with 10nM R, 3nM B, and 1mM A, then incubated at 37°C with 5% pCO_2. For the co-culture experiments, the MEM Alpha medium in dishes of murine stromal cells almost confluent was changed in RPMI, and 24 hours later this medium was removed in order to add fresh RPMI containing MOLM13 leukemic cells and drugs. For these co-culture experiments Ascorbic acid (by Sigma-Aldrich) was used at 4.5mM, Sorafenib and Gilteritinib (kindly borrowed from Department of Biomedicine and Prevention at the University of Rome Tor Vergata) at 5nM and 15-30nM respectively, and Venetoclax (MedChemExpress, Monmouth Junction, NJ, USA) at 10nM final concentration.

6.2 Cell death, cell cycle, morphologies, and TEM

Leukemic cell lines seeded at 70.000 cells/ml or 100.000 cells/ml depending on their doubling time, were treated with different drugs as described in the Results section. After 72 hours cells were harvested and stained with 10ug/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). In the co-culture experiments, the same procedure was performed for stromal cells upon detachment with 0.05% trypsin/0.53mM EDTA (by Corning, Corning, NY, USA). Both cell death and proliferation were analyzed by flow cytometry (Cytoflex Beckman Coulter, Life Sciences, Brea, CA, USA) and CytExpert v2.2 by Beckman Coulter. 24 hours and 48 hours after treatments, 0.5 $x10^6$ cells were washed with PBS and then fixed o/n with 70% ethanol at +4°C. Then, cells were incubated

at least 3 hours with a solution of propidium iodide with RNases. After this step, cells were analyzed by flow cytometry for their RNA content. In order to analyze cell morphology, 72h hours after treatment about 300.000 leukemic cells were spotted on a slide by using Thermo Shandon Cytospin 3 Centrifuge. Since stromal cells adhere to the dish, they didn't need this step. Cells on the slide/dish were incubated for 2 minutes in solution 1 and for 13 minutes in solution 2 for Giemsa staining (Sigma-Aldrich). Pictures of an appropriate number of representative fields from each sample were acquired by using Zeiss Axiocam 503 color. Zen and Adobe Photoshop (Adobe Systems) were used for the processing of images. In order to analyze the intracellular modifications upon single and combined treatments, about 30-40x10^6 cells for each condition were collected 24 hours after drug administration and centrifuged at 300 rcf for 5 minutes. After two washes, the supernatant was removed while cells were agitated with vortex, resuspended in 250ul glutaraldehyde 2.5%, and stored at 4°C. The TEM analysis was kindly performed by Miglietta and Familiari, Human Anatomy Section of SAIMLAL Department, Sapienza University of Rome.

6.3 RNA extraction and real-time PCR

Total RNA was isolated by TRIzol reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. After quantification and quality check by measuring OD 260-280, 250ng of total RNA was reverse-transcribed with the High-Capacity RNA to cDNA kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) in a final volume of 10ul, then diluted 1:5 to 5ng/ul for all the following PCR. Intercalant dye-based Real-Time PCR was performed by using PowerUp SYBR Green Master Mix (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) in a final volume of 10ul. The reactions, all performed as duplicates, were carried out by using custom oligonucleotides, designed as follows:

H3 (FW: GTGAAGAAACCTCATCGTTACAGGCCTGGT - RW: CTGCAAAGCACCAATAGCTGCACTCTGGAA);
hBiP (FW: TAGCGTATGGTGCTGCTGTC - RW: TTTGTCAGG GGTCTTTCACC);
hCHOP (FW: TGGAAGCCTGGTATGAGGAC - RW: TGTGACCTCTGCTGGTTCTG);
spliced XBP1 (FW: GAGTCCGCAGCAGGTGC - RW: TCCTTCTGGGTAGACCTCTGGGAG). H3 was used as reference gene. On order to detect HMOX1 expression levels, it was used a PrimeTime Std qPCR Assay (Integrated RNA Technologies, Skokie, Illinois, USA) composed of the following primers:
HMOX1 (FW: TCATGAGGAACTTTCAGAAGGG – RW: TGCGCTCAATCTCCTCCT) and the probe (/56-FAM/TGGCCTCCC/ZEN/TGTACCACATCTATGT/3IABkFQ/).
For these reactions, GAPDH was used as reference gene:
GAPDH (FW: ACATCGCTCAGACACCATG – RW: TGTAGTTGAGGTCAATGAAGGG) and probe (/56-FAM/AAGGTCGGA/ZEN/GTCAACGGATTTGGTC/3IABkFQ).
These reactions were performed with the TaqMan Universal PCR Master Mix reagent (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). All PCRs were performed by QuantStudio 7 Flex Real-Time PCR (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) and analyzed with QuantStudio Real-Time PCR Software v1.3 (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA).

6.4 Immunofluorescence analysis

About 250.000 cells were spotted on a slide by using Thermo Shandon Cytospin 3 Centrifuge 24 or 48 hours after treatment. Then cells were fixed in 4% PFA for 10 minutes. Fixed cells were washed and permeabilized with 0.1% TritonX100 in 1%BSA-PBS for 10 minutes. After some washes, cells were stained with primary antibodies in 1%BSA-PBS for 30 minutes at room temperature.

The primary antibodies used are:
Calnexin (C5C9) Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA), 1:100
BiP/GRP78 (610978) Mouse mAb (BD Transduction Laboratories, Franklin Lakes, NJ, USA), 1:100
NRF2 (D1Z9C) XP Rabbit mAb (Cell Signaling), 1:500
YAP (4912S) Rabbit mAb (Cell Signaling) 1:100
Rhodamine Phalloidin R415 (ThermoFisher Scientific) 1:50
Then, cells were washed and incubated with secondary antibodies:
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (ThermoFisher Scientific) 1:500
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (ThermoFisher Scientific) 1:500

After 30 minutes cells were washed and incubated with 1:1000 Hoechst 33342 Solution and 1:3000 TO-PRO-3 Iodide (ThermoFisher Scientific) for 5 minutes for DNA staining. After the washes, the slides were dried and closed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and a cover glass. Confocal images were acquired by Zeiss LSM 900 with Airyscan 2, equipped with ZEN 3.2 Blue Edition. Adobe Photoshop was used for the processing of images. ImageJ software was used in to quantify the mean fluorescence intensity of an appropriate number of fields from each experimental condition.
For the detection of Nrf2 fluorescence by flow cytometry, about 400.000 cells kept in round bottom 2ml tubes were fixed, permeabilized and stained with primary and secondary antibodies as stated above. Then, cells were incubated with Sytox blue fluorescent dye for for dead cells (from ThermoFisher Scientific) and Nrf2 signal was acquired by using Cytoflex Beckman Coulter, from Life Sciences. The results were analyzed with CytExpert v2.2 by Beckman Coulter.
For ROS measurement by flow cytometry around 400.000 cells were harvested at different time points, abundantly washed and incubated with 2uM CM-H_2DCFDA (ThermoFisher Scientific) in

pre-warmed PBS. CM-H_2DCFDA is a general oxidative stress indicator and it derives from H_2DCFDA. This molecule passively diffuses into cells, where it is cleaved by esterases. The thiol-reactive chloromethyl group is oxidized by cellular ROS, thus becoming a fluorescent adduct, and the more ROS are present the more fluorescence detected will be intense. After incubation with CM-H_2DCFDA at 37°C for 30 minutes in the dark, cells were washed and stained with Sytox blue fluorescent dye in order to exclude dead cells. Also in this case, samples were analyzed with Cytoflex Beckman Coulter and the results were analyzed with CytExpert software.

6.5 Western blot

MS5 stromal cells obtained from the co-culture and mono-culture experiments were washed with cold PBS and lysed in a buffer containing 150mM NaCl, 10mM Hepes, 0.25% Sodium Deoxycholate, 1% NP40, and 0.1% SDS. Then the samples were spinned for 5 minutes at 300xg. The supernatant was extracted and the residual nuclear fraction, obtained as a pellet from the centrifugation, was incubated in a buffer containing 25mM Tris pH 7.5, 100mM NaCl, 3mM EDTA, 7% glycerol, and 2% SDS. After the sonication of the nuclear fraction, all the protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated overnight with the following primary antibodies: YAP Rabbit mAb #4912S and TAZ Rabbit mAb #83669S (Cell Signaling Technology, Danvers, MA, USA). Detection of the western blots was performed with ECL western blotting system (GE Healthcare, Sigma-Aldrich St. Louis, MO, USA) and images of the blots were obtained with the ChemiDoc XRS+, using the Image Lab software (Bio-Rad, Hercules, CA, USA).

6.6 Analysis of mice engraftment

In the end of the *in vivo* experiment (discussed in the text) performed by Martyna Sniegocka, bone marrow cells from femurs were analyzed in order to evaluate the engraftment of MOLM13 leukemic cell line in both control and treated NSG mice. Each sample underwent red cells lysis for 5 minutes, then cells were diluted in 2-3ml PBS and passed through 70um nylon cell strainers (by Corning, Corning, NY, USA) to remove small pieces of bones and big debris. After a wash in PBS, cells were counted and used for ROS levels measurement and human CD45 staining. CD45 staining requires a 15 minutes pre-incubation in 1%BSA-PBS. Cell were then incubated with the antibody FITC anti-human CD45 (by BioLegend, San Diego, CA, USA) diluted 1:200 in 1%BSA-PBS. After 30 minutes, the samples were washed and resuspended in PBS. The percentage of human CD45+ cells was measured by flow cytometry (Cytoflex Beckman Coulter) and the results were analyzed with CytExpert software.

6.7 Statistical analysis

In the legend of figures, or under the graphs, "n" indicates biological replicates, intended as the number of repeated independent experiments. All the histograms report the mean of values obtained from each experiment with the error bars representing the standard error of the mean (SEM). Comparisons between two samples were analyzed with a p-value calculated through the student's T-test (unpaired, two-tailed). Instead, ANOVA and multiple comparison tests were used to compare the means between two or more groups of samples. All statistical analyses were performed by using the GraphPad-Prism 6 software (GraphPad Software, La Jolla, CA, USA).

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8. LIST OF PUBLICATIONS

Masciarelli S, Capuano E, Ottone T, Divona M, Lavorgna S, **Liccardo F**, Śniegocka M, Travaglini S, Noguera NI, Picardi A, Petrozza V, Fatica A, Tamagnone L, Voso MT, Lo Coco F, Fazi F. Retinoic acid synergizes with the unfolded protein response and oxidative stress to induce cell death in FLT3-ITD+ AML. Blood Adv. 2019 Dec 23;3(24):4155-4160. doi: 10.1182/bloodadvances.2019000540. PMID: 31834935; PMCID: PMC6929380.

Palombarini F, Masciarelli S, Incocciati A, **Liccardo F**, Di Fabio E, Iazzetti A, Fabrizi G, Fazi F, Macone A, Bonamore A, Boffi A. Self-assembling ferritin-dendrimer nanoparticles for targeted delivery of nucleic acids to myeloid leukemia cells. J Nanobiotechnology. 2021 Jun 9;19(1):172. doi: 10.1186/s12951-021-00921-5. PMID: 34107976; PMCID: PMC8190868.

Liccardo F, Iaiza A, Śniegocka M, Masciarelli S, Fazi F. Circular RNAs Activity in the Leukemic Bone Marrow Microenvironment. Noncoding RNA. 2022 Jul 1;8(4):50. doi: 10.3390/ncrna8040050. PMID: 35893233; PMCID: PMC9326527.

Śniegocka M, **Liccardo F**, Fazi F, Masciarelli S. Understanding ER homeostasis and the UPR to enhance treatment efficacy of acute myeloid leukemia. Drug Resist Updat. 2022 Jul 8;64:100853. doi: 10.1016/j.drup.2022.100853. Epub ahead of print. PMID: 35870226.

Point by point response to the reviewers:

Reviewer Silvia Di Agostino

This thesis is titled “Retinoic Acid and proteotoxic stress induce myeloid leukemia progenitors cell death overcoming the protective effects of the bone marrow niche mesenchymal cells.” by PhD student Francesca Liccardo. The project starts from the solid results developed by the research group where differentiation of human leukemic cell lines induced by retinoic acid (RA) increased their sensitivity to endoplasmic reticulum (ER) stress-inducing drugs (such as chemotherapy or arsenic trioxide) at doses that were not toxic without RA. In this context this project aimed to test the proteasome inhibitor Bortezomib, as inducer of ER stress already clinically approved for multiple myeloma and mantle cell lymphoma, also analyzing the crosstalk between AML cells and bone marrow niche mesenchymal cells in response to the drugs. This study describes a good functional characterization of a triple treatment where each single drug affects a pathway already consolidated in the literature and by the research group. The rationale is solid with a very good prospect of translating this data into an in vivo model. The translational impact is significant. This preclinical finding could be interesting in the context of acquired drug resistance and relapse cases. Overall, this thesis was well written, the “Introduction” is exhaustive and very focused in describing the concepts that are needed in the study. The experiments have good quality with all the appropriate controls, they fully support the aims. Minor observations: - On the title page it would be appropriate to indicate the second tutor as "Dr. Silvia Masciarelli”. -The TEM images are certainly appropriate, very interesting and support the hypotheses- However, for a publication, they should be corroborated with the variation of molecular markers associated with the morphological change, such as by using antibody probing of OXPHOS complexes or by evaluating the

expression of some cristae remodeling factors (referring to the Fig. 4). Any results would strengthen the novelty and robustness of the mechanism. - Both, YAP and TAZ are mechanosensors of cytoskeletal tension and FA formation (such as https://doi.org/10.1038/nrm3416; https://doi.org/10.1083/jcb.201806065). The possible expression and contribution of TAZ to the mechanism should also be evaluated as a future objective.

I thank the reviewer for having accepted my PhD thesis with minor revisions. The modification on the title page has been added as requested. Moreover, the evaluation of a possible alteration in OXPHOS complexes or cristae remodeling factors upon triple treatment RBA is a useful suggestion in view of the publication. To this aim, we will buy the MitoProbe JC-1 Assay Kit to study mitochondrial membrane potential. Indeed, JC-1 dye exhibits potential-dependent accumulation in mitochondria and it is appropriate to detect mitochondrial depolarization. Moreover, the suggestion on the evaluation of TAZ contribution has been addressed. In particular, in order to evaluate YAP/TAZ nucleo-cytoplasmic shuttling, western blot analysis with nucleus/cytoplasm separation has been performed.

Reviewer Gianni Colotti

The thesis "Retinoic Acid and proteotoxic stress induce myeloid leukemia progenitors cell death overcoming the protective effects of the bone marrow niche mesenchymal cells", by Francesca Liccardo, deals with treatment of Acute myeloid leukemia (AML) cell lines with low doses of Retinoic Acid (R)+ Arsenic Trioxide (A), the current therapy for acute promyelocytic leukemia, plus Bortezomib

(B), a proteasome inhibitor. FLT3-ITD+ AML cell lines and primary blasts are highly sensitive to the triple RBA combination, but not to the single molecules, due to oxidative stress and to the suppression of the pro-survival branches of the Unfolded Protein Response (UPR). The bone marrow niche protects AML from the toxic effects of RBA, by attenuating oxidative stress, an effect reverted by the pro-oxidant vitamin C. The data are relevant for the field of oncology, since they open the path towards possible application of Bortezomib in AML treatment. The thesis is logical, clearly organized and well written. The scientific background is more than satisfactory, and the research problem is clearly stated.

I thank the reviewer for having accepted my PhD thesis as it is.