ACUTE MYELOID LEUKEMIA AND THE BONE MARROW MICROENVIRONMENT

FRIENDS OR FOES? ACUTE MYELOID LEUKEMIA AND THE BONE MARROW MICROENVIRONMENT

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TITLE: Friends or foes? Acute myeloid leukemia and the bone marrow microenvironment

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Lay abstract

Acute myeloid leukemia (AML) is an aggressive cancer of the blood and bone marrow, affecting 1,100 Canadians annually. Older patients make up 75% of cases yet have the lowest survival rates due to the lack of tolerable treatments. A novel combination of Venetoclax and Azacitidine (Ven/Aza) has shown great therapeutic promise, however, chemoresistance remains an important concern. Previous studies have implicated fat cells, or adipocytes, in AML chemoresistance, however, their role in Ven/Aza treatment has yet to be studied. Here, we show that adipocytes reduce growth of AML cells, yet enhance their metabolism. In the presence of Ven/Aza, adipocytes induce chemoresistance. We show preliminary data that this chemoprotection may be mediated by the upregulation of mitochondrial MCL-1 protein as inhibition of this protein neutralized the protection. By understanding the relationship between adipocytes and AML chemoresistance, we can target this and re-sensitize AML to Ven/Aza, thereby improving older patient outcomes.

Abstract

Acute myeloid leukemia (AML) is an aggressive cancer of the blood and bone marrow, affecting 1,100 Canadians annually. Older patients make up 75% of cases yet have the lowest survival rates due to the lack of tolerable treatments. Recently, the combination of Venetoclax and Azacitidine (Ven/Aza) has shown great therapeutic promise, however, chemoresistance has become a growing concern. Current evidence points towards a chemoprotective role from the bone marrow (BM) microenvironment, specifically by BM-derived mesenchymal stromal cells (BMSCs) and adipocytes. AML cells can manipulate BMSCs and adipocytes to create a niche that supports its own growth and evades chemotherapy. However, the role of the microenvironment in Ven/Aza chemoresistance has yet to be studied. Our objective was to study the ability of the microenvironment cells to induce AML chemoresistance to Ven/Aza. We employed a 2-dimensional direct contact co-culture system between MOLM-13 AML cells and BMSCs or adipocytes in both the absence and presence of Ven/Aza to determine the effects on the AML cells. In the absence of Ven/Aza, adipocyte co-cultured AML cells showed a 47% reduction in proliferation, 10% reduction in viability, yet a 1.7-fold increase in Maximal respiration when compared to the monocultured cells. In the presence of Ven/Aza, adipocyte co-cultured AML cells showed a significant increase in both proliferation and viability. Preliminary work investigating the mechanism of action of this support points toward an anti-apoptotic mechanism mediated by the upregulation of MCL-1 upon co-culture with adipocytes. Combination of Venetoclax and Tapotoclax, an MCL-1 inhibitor, abrogated the chemoprotection provided by BMSCs and adipocytes. Overall, our data suggests a dual role of adipocytes, where their inhibition or support of AML is context dependent. Therapeutic targeting of mechanisms for adipocyte chemoprotection such as MCL-1 upregulation may re-sensitize AML cells to Ven/Aza, thereby improving patient outcomes.

Acknowledgments

I would like to acknowledge my supervisor, Dr. Tobias Berg, for providing me with the constant resources and support to carry out this project. His guidance was crucial for my success and always provided an open-door policy that I frequently took advantage of. I'd like to thank my committee members Dr. Clinton Campbell and Dr. Gregory Steinberg for their expertise and suggestions over these years to fine-tune this project. A huge thank you to PhD fellow, Dr. Kanwaldeep Singh, for always being there for me both research-wise and emotionally. Kanwal supported my project in every single way, and I could not have it without his knowledge and passion for quality research. Thank you to my best friend and lab colleague Sara Pishyar for her positivity and ability to creatively think about solutions. In addition to my project, she made conducting experiments something to look forward to. In the same notion, thank you to all of those in the Berg lab team, past and present, for being part of this journey with me. Lastly, a huge appreciation for my friends and family who provided love and confidence throughout these last years. Most notably, I truly could not have done this without my life partner Erik Leers for showing unconditional support and being there through good and bad times. He helped me see the light at the end of the tunnel, or in this case, thesis. Thank you to all.

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+	positive
Δ	delta
AML	acute myeloid leukemia
AT	adipose tissue
Aza	azacitidine
BAT	brown adipose tissue
BAX	BCL-2 associated X
BCL-2	B-cell leukemia/lymphoma 2
BM	bone marrow
BMSCs	BM-derived mesenchymal stromal cells
BSA	bovine serum albumin
CD36	fatty acid translocase
cDMEM	complete DMEM
cDNA	complementary DNA
CEBPAα	CCAAT/enhancer-binding protein alpha
cIMDM	complete IMDM
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
cRPMI	complete RPMI
CT	cycle threshold
ctrl	control
DEXA	dexamethasone
DM	differentiating media
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
DNMT3A	DNA methyltransferase 3 alpha

E. coli	Escherichia coli
E86	GP+E86
ETC	electron transport chain
FA	fatty acids
FABP4	fatty acid binding protein 4
FAO	fatty acid oxidation
FBS	fetal bovine serum
FLT3	fms-like tyrosine kinase 3
FLT3-ITD	FLT3 internal tandem duplications
gDNA	genomic DNA
GMP	granulocyte monocyte progenitors
GvHD	graft-versus-host disease
H9M	Hoxa9/Meis1
HSCTs	hematopoietic stem cell transplantations
HSPCs	hematopoietic stem and progenitor cells
IBMX	isobutyl methylxanthine
IC_{50}	half maximal inhibitory concentration
IL	interleukin
INDO	indomethacin
kDa	kilodalton
LDL	low-density lipoprotein
LDs	lipid droplets
LSC	leukemic stem cells
MCL-1	myeloid cell leukemia sequence 1
mono	monoculture
MST	mitochondrial stress test
n	sample size

NFW	nuclease-free water
NGS	next generation sequencing
nM	nanomolar
NPM1	nucleophosmin 1
OCR	oxygen consumption rate
ORO	Oil Red O
OS	overall survival
OXPHOS	oxidative phosphorylation
P/S	penicillin/streptomycin
PA	phoenix ampho
PB	pacific blue
PB	peripheral blood
PFA	paraformaldehyde
PPARγ	peroxisome proliferator-activated receptor gamma
Q	quadrant
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative RT-polymerase chain reaction
RBCs	red blood cells
RPLP0	ribosomal protein lateral stalk subunit P0
rpm	revolutions per minute
RT	room temperature
RvT	reverse transcriptase
SCF	stem cell factor
SEM	standard error of mean
SLM	substrate limited media
TBS-T	tris-buffer saline-tween
TCA	tricarboxylic acid

Tapo	tapotoclax
VCLAD	very long-chain acyl-CoA dehydrogenase
Ven	Venetoclax
V	volts
w.r.t	with respect to
WAT	white adipose tissue
XeF96	extracellular flux analyzer
β-ME	beta-mercaptoethanol
μM	micromolar

Declaration of Academic Achievement

My supervisor, Dr. Tobias Berg, my mentor and PhD fellow Dr. Kanwaldeep Singh, and I designed experiments together. Dr. Singh provided training for Seahorse extracellular flux analyzer and flow cytometry machines. I carried out all experiments, including co-cultures, drug preparation, and end-point assays. Dr. Singh assisted in data analysis. Sara Pishyar and undergraduate lab students assisted in writing out counts of proliferation. Darlene Lane helped with scheduling progress meetings. Dr. Campbell and Dr. Steinberg provided valuable suggestions for future steps at committee meetings.

Chapter 1: Introduction

1.1 Acute Myeloid Leukemia

1.1.1 AML pathophysiology and clinical considerations

Acute myeloid leukemia (AML) is a rapidly progressing cancer in the blood and bone marrow (BM), characterized by the presence of over-proliferative and poorly differentiated leukemic cells (Döhner et al, 2015). Although it is considered a rare disease affecting only 1% of cancer patients in Canada, it is the most common acute leukemia in the adult population, taking the lives of 1,100 Canadians annually (Brian et al., 2018). In normal blood formation, known as hematopoiesis, hematopoietic stem and progenitor cells (HSPCs) undergo differentiation into either common myeloid progenitor (CMP) cells or common lymphoid progenitor (CLP) cells. The process of HSPC differentiation is tightly regulated by growth factors and transcription factors. and requires a precise balance of differentiation or self-renewability of daughter cells to ensure blood formation can continue throughout one's lifetime. CMPs undergo further differentiation into platelets, red blood cells (RBCs), and leukocytes while CLPs differentiate into white blood cells such as T-, B-, and NK-cells (Lowenberg et al., 1999). In AML, mutations arise in the HSCs or CMPs that result in a block in differentiation and undergo uncontrolled proliferation, and these cells are known as myeloblasts (Lowenberg et al., 1999). These non-functional myeloblasts results in a deficiency of the aforementioned differentiated blood cells. The lack of red blood cells, also known as anemia, causes the most commonly presenting symptoms of AML. This includes fatigue, weakness, fever, pallor, and shortness of breath (Marks, 2018). The lack of platelets and leukocytes can

result in bleeding and infections, respectively. As the disease progresses, leukemic cells can infiltrate organs such as the liver, spleen, skin, and lymph nodes, as well as the central nervous system (Marks, 2018).

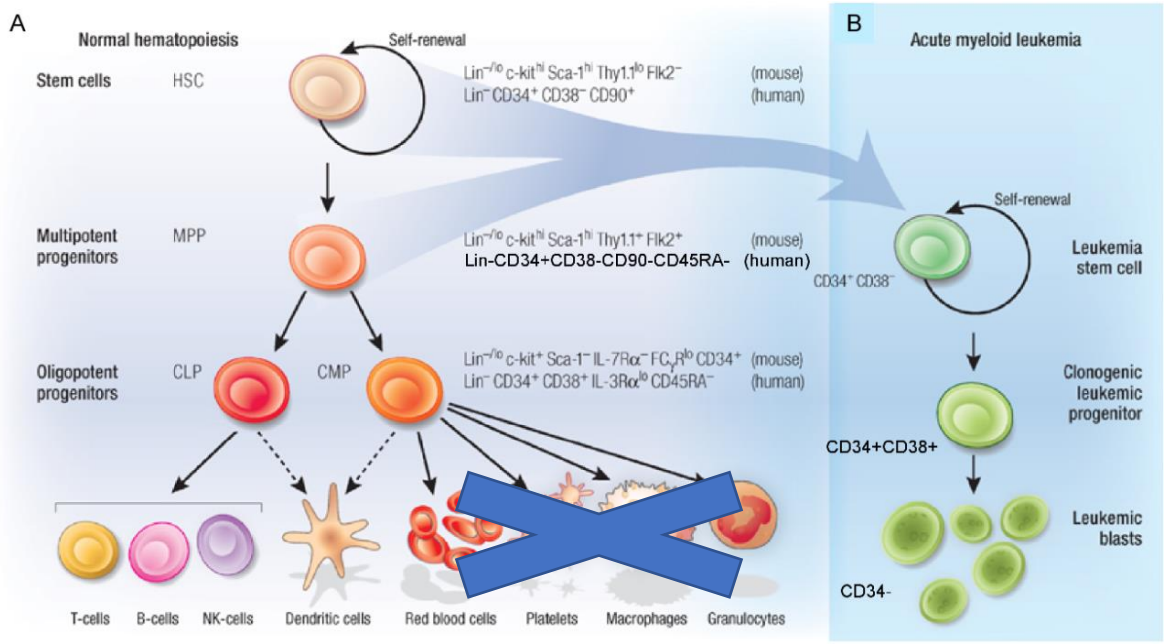

Figure 1. Normal versus AML hematopoiesis. The hierarchy of hematopoetic stem, progenitor, and differentiated cells compared to AML hematopoiesis. The blue X represents the deficiency of differentiated myeloid lineages in patients with AML. Adapted from Thomas & Majeti (2017).

The most common diagnostic test for AML is the identification of leukemic myeloblasts from BM samples and peripheral blood smears (Dohner et al., 2017). AML is diagnosed when ≥20% myeloblasts are present. Based on the WHO classification, AML is classified into one of four subgroups of AML; AML with recurrent genetic abnormalities, AML with myelodysplastic-related changes, AML related to treatment, and AML not otherwise specified (Swerdlow et al., 2016). These classifications are

defined by morphology of blasts, cytogenetic lesions, mutations, and expression of cell surface and cytoplasmic markers using flow cytometry (Swerdlow et al., 2016). Based on the ELN classification, the cytogenetic lesions and mutations AML patients possess are used to stratify patients into 4 risk groups which predict clinical prognosis and recommended treatments: favourable, intermediate I, intermediate II, and adverse (Döhner et al., 2017).

1.1.2 AML heterogeneity and mutations

AML is extremely difficult to treat due to its phenotypic and genetic heterogeneity. This disease can arise from the clonal transformation of BM-resident blood-forming cells called hematopoietic stem and progenitor cells (HSPCs) caused by single or cooperating mutations (Renneville., 2008). Mutations can be classified as class 1 mutations which provide a proliferative, survival advantage to immature myeloblasts or class 2 mutations, which display a block in differentiation and self-renewability (Naoe et al., 2013). One of the most common class 1 mutations is in the fms-like tyrosine kinase 3 (FLT3) receptor, which is overexpressed in approximately one-third of recently diagnosed patients (Kennedy et al. 2020). Specifically, the *FLT3* internal tandem duplications (*FLT3*-ITD) mutation is found in 25% of patients and results in constitutive activation of this receptor, leading to over-proliferation and inhibition of differentiation and apoptosis (DG et al. 2002). Similarly, the class 2 mutation of *nucleophosmin 1* (NPM1) is also one of the most common mutations in AML, accounting for one-third of AML patients (Falini et al. 2007). NPM1 usually resides in the nucleolus and functions as a chaperone for proteins including tumor suppressor proteins and plays multiple roles in

genomic stability, chromatin remodeling, and mRNA processing (Colombo et al. 2011). However, NPM1 mutations lead to this protein being localized to the cytoplasm, disturbing its normal functions. Interestingly, FLT3-ITD is two times more common in NPM1-mutated AML (Verhaak et al. 2005). Unfortunately, having both mutations present results in a worse prognosis for patients. Certain genetic lesions may not fall into either of these categories but are involved in epigenetic modulation, which has gained more attention over the last decade (Kelly et al, 2002). DNA methyltransferase 3 A (DNMT3A) is an enzyme involved in de novo methylation of DNA (Ley et al. 2010). Mutations in this epigenetic protein are found in 20% of patients with AML, leading to the loss of its tumor-suppressive function. Coincidently, DNMT3A mutations are frequently found in patients with both FLT3-ITD and NPM1 mutations and have reduced event-free and overall survival (Bezerra et al. 2020). Although these three mutations are among the most common, they only account for 25% of all cases. Mutations can occur in a plethora of pathways including signaling and kinase pathways, spliceosome complexes, and transcription factors (DiNardo and Cortes, 2016). The vast heterogeneity in AML etiology continues to be a challenge for researchers and explains why it's amongst the most difficult cancers to treat. In addition to mutations, exogenous factors such as radiation, chemotherapy, and cigarette smoking, and endogenous factors such as coexisting hematopoietic disorders, obesity, and congenital conditions, may contribute to the development and influence the prognosis of AML (Aquino, 2002).

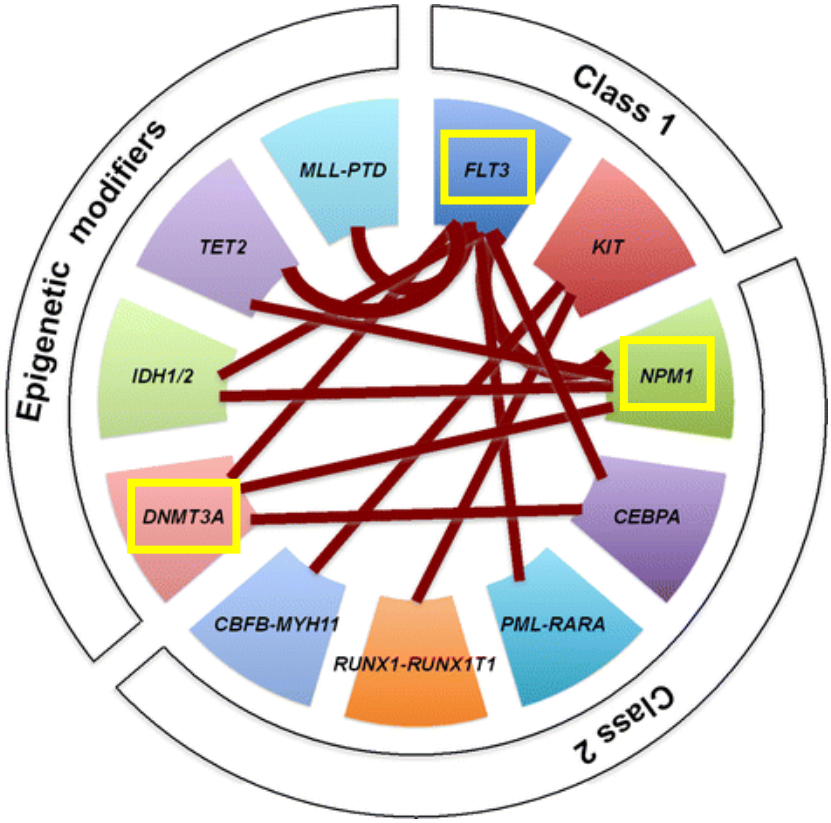

Figure 2. Classes of mutations found in patients with AML. The most common mutations are highlighted in yellow boxes. Adapted from Naoe et al. (2013).

1.2 Treatment of AML

1.2.1 Induction Therapy

Treatment of AML varies patient to patient, depending upon disease status, eligibility for intensive chemotherapy, risk stratification based on genetic lesions, age, and co-existing conditions (Döhner et al., 2017). The goal for AML treatment is to induce remission, defined as <5% myeloblasts, and prevent relapse, the return of leukemic cells. For younger patients and older patients deemed eligible to withstand intensive chemotherapy, standard treatment involves induction therapy, which will empty the BM, allowing for its self-repopulation with healthy blood cells. Induction therapy follows the

"7+3" induction regimen: 7 days of continuous infusion of cytarabine (100-200 mg/m^2) and 3 days of intravenous anthracycline (e.g., Daunorubicin at 60 mg/m^2) (Wiernik et al., 1972). For older eligible patients, considered >60 years, reduced doses of both agents are given. Induction therapy induces complete remission in 60-85% of patients <60 and 40-60% for patients >60 (Stone, 2002).

1.2.2 Consolidation therapy

Remission with induction therapy is the first step of AML treatment. Without additional treatment, leukemic blasts may escape induction therapy and continue to expand, resulting in disease relapse. Consolidation therapy is used to prevent relapse and can be achieved in 2 ways: chemotherapy or hematopoietic stem cell transplantations (HSCTs). Consolidation chemotherapy tends to be administered to low-risk younger patients with favourable genetic profiles given its intensive nature. It involves 2-4 cycles of high-dose cytarabine (1000-1500 mg/m^2). This can cure 60-70% of younger patients (Döhner et al., 2015). Unfortunately, the cure rates in eligible older patients are dishearteningly low, at 10-15% (Döhner et al., 2015). Chemotherapy has been relatively unchanged over the past 3 decades, which fuelled the search for more effective AML treatments. The introduction of HSCTs for treating patients with intermediate or adverse genetic profiles has been pivotal in increasing overall AML patient survival (Cornelissen et al., 2016). Patients in the higher genetic risk profiles are not benefitting to the same extent from intensive consolidation chemotherapy, but are rather considered for stem cell transplantation. In this procedure, the BM is purposely ablated using high-dose cytotoxic therapy to rid it of leukemic blasts. The BM is then repopulated by the transplantation of

healthy HSPCs. There are two types of HSCT: autologous and allogenic. Autologous HSCT involves extracting the patient's own HSPCs and transplanting them back in after chemotherapy, whereas allogenic HSCT involves transplanting HSPCs from a donor with a matching tissue type (Schlenk, 2014). Allogenic HSCT produces an advantage known as graft-versus-leukemia effect where the donor cells identify the patient's leukemic cells as foreign and attack them (Schlenk 2014). However, this can also lead to graft-versus-host disease (GvHD), where the donor cells identify and attack the patient's healthy cells (Schlenk, 2014). Suppression of the patient's immune system using toxic marrow-ablative treatment and the risk of GvHD makes HSCTs restricted to patients younger than 55. Regardless, HSCTs show survival rates of 45-60% for high-risk younger patients, and the risk for relapse in these patients is low at 20% (Schlenk, 2014). Over the past 2 decades, the success and incorporation of HSCTs have risen from 43% of younger patients treated with HSCTs to 71% (Bertoli et al., 2017). Overall, more intensive chemotherapy and increased use of HSCTs have improved the 5-year survival rates of younger patients, sitting at 50% (Oran et al., 2012). Dismally, compare this with older patient's 5-year survival rate of 3-8% (Oran et al., 2012). In addition, though eligible older patients may show remission, they are more likely to relapse than younger patients (Jabbour et al., 2006). What causes this skewed mortality rate towards older patients?

1.2.3 Treatment for older patients with AML

Induction/consolidation chemotherapy and HSCTs have certain criteria that must be met to administer such intensive treatments, which older patients are less likely to reach, deeming them ineligible. There are two categories of factors contributing to

eligibility: patient- and disease-related factors. Patient-related factors include increased age, poor performance status, reduced cognitive function, organ dysfunctions, co-existing comorbid conditions, and elevated white blood cell counts (Malfuson et al., 2008). Disease-related factors include the type of AML, cytogenetic abnormalities, drug resistance gene expression, and increased frequency of epigenetic mutations (Malfuson et al., 2008). Most older patients present with one or more of these conditions, leaving them with extremely limited options for treatment. Prior to 2005, the options were supportive care to treat AML symptoms or low-dose cytarabine, if tolerated. Supportive care includes hydroxyurea treatment to control white blood cell counts, blood product support, and antibiotic treatment (Burnett et al., 2007). These treatments lack anti-leukemic properties, which resulted in overall survival (OS) rates of just 6 weeks. After 2005, hypomethylating agents such as azacitidine (Vidaza^{TM}) (Aza) or decitabine (Dacogen^{TM}) for the treatment of ineligible older patients were introduced (Al-Ali et al., 2014). These agents work by inhibiting methylation of genes involved in differentiation and growth, thereby allowing for their activation in subsequent cell divisions, in hopes of returning leukemic cells to normal function (Silverman, 1994). This form of therapy showed improvements in OS rates (6 weeks vs**.** 8-9 months) (Gardin et al., 2017), but this improvement was not significant enough to be considered the standard of care for these patients, so the search for the best treatment regimen continued (Lancet, 2018).

The lack of treatments available to this population is tragic, given that approximately 75% of patients with AML are >60 (SEER, 2017). As mentioned, there is no standard approach on how to treat ineligible older patients, showcasing the dire need

for more tolerable therapies to improve their survival rate and quality of life. Fortunately, in recent years, a very promising treatment regimen combining Venetoclax and Azacitidine has been reported, showing increased OS rates and remission rates (DiNardo et al., 2020).

1.3 Venetoclax/Azacitidine Co-treatment

Venetoclax is a selective small-molecule B-cell leukemia/lymphoma 2 (BCL-2) inhibitor while Azacitidine is an inhibitor of DNA methylation. BCL-2 is an anti-apoptotic protein as it sequesters and inhibits the pro-apoptotic BCL-2 associated X (BAX) and BIM proteins, preventing mitochondrial apoptosis (Cleary et al., 1986). Studies have shown that a specific population of stem cells, termed leukemic stem cells (LSCs), are responsible for disease onset and long-term self-renewal properties. They are also implicated in disease relapse as they have been shown to evade chemotherapy (Ye et al., 2016). Interestingly, these LSCs show elevated levels of BCL-2, and are dependent on it for survival (Konopleva et al., 2006). Venetoclax works by inhibiting BCL-2, thus disrupting mitochondrial energy production, while Azacitidine works to activate genes involved in differentiation. This co-treatment has been shown to induce remission in 60% of older ineligible patients who were newly diagnosed/had never been treated (DiNardo et al., 2020). They compared Azacitidine alone versus Venetoclax/Azacitidine and found that in older patients with intermediate cytogenetic risk, the co-treatment group had a median OS of 20.8 months while the monotherapy group was at 12.4. Unfortunately, the co-treatment effects are short-lived as the median duration of response is only 11 months (DiNardo et al., 2020). In addition, clinical trials involving older patients who have been

previously treated or are refractory/relapsed samples show a much lower remission rate of 21% (DiNardo et al., 2018).

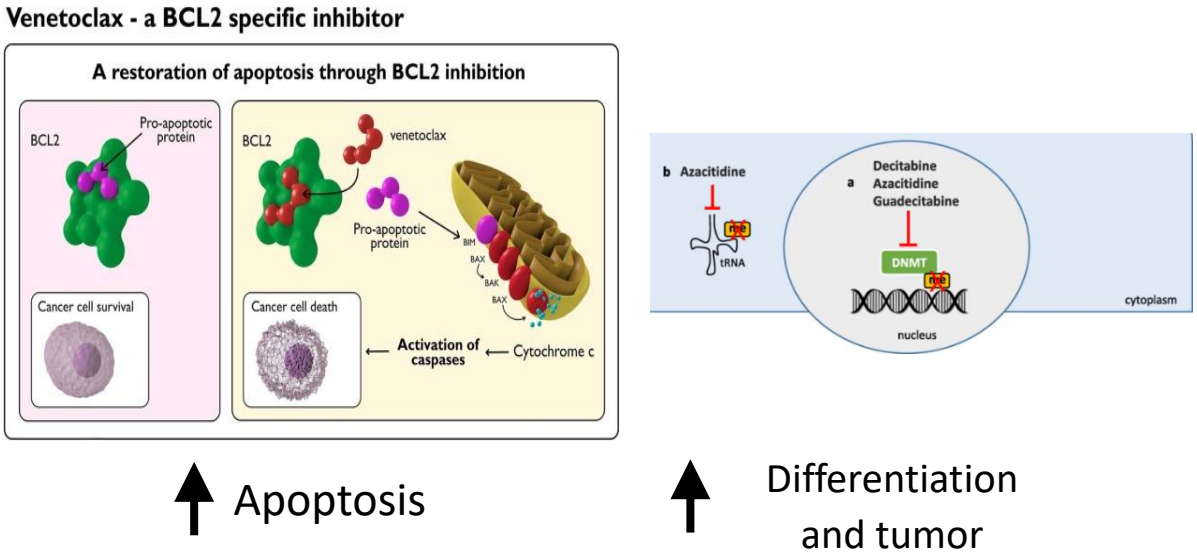

Figure 3. Mechanisms of action of Venetoclax and Azacitidine. Venetoclax is a BCL-2 inhibitor, resulting in mitochondrial and cell apoptosis. Azacitidine is a hypomethylating agent by inhibiting DNA methyltransferase (DNMT) that results in enhanced differentiation patterns and activation of tumor suppressor genes. Adapted from Mihalyoya et al. (2018) on left and Contieti et al. (2020) on right.

1.4 Ven/Aza chemoresistance

Research has attributed this reduced treatment success rate to intrinsic or acquired chemoresistance. There have been many studies investigating the factors associated with resistance such as previous exposure to chemotherapy (DiNardo et al., 2018), certain genetic lesions (Nechiporuk et al., 2019), and differentiation status (Kuusanmäki et al., 2020), but the underlying mechanism of action was unknown until very recently. In 2020, Jordan and colleagues found increased levels of fatty acid (FA) oxidation in LSCs in relapse samples of older patients previously responsive to Venetoclax/Azacitidine. They

suggested that resistance to this co-treatment was due to LSCs' ability to reprogram their metabolism and utilize FAs for energy production instead of glucose or amino acids (Stevens et al., 2020). The reduced efficacy of this co-treatment, one that holds the most therapeutic potential for vulnerable older patients, showcases the need to elucidate the role of metabolism in chemoresistance and to discover pharmaceutical agents to target it.

1.5 Metabolism in AML

AML cells heavily rely on nutrient metabolization to support their rapid, uncontrolled cell division. The ability of AML cells to reprogram their metabolism in response to nutrient availability is a hallmark of chemoresistance (Castro et al., 2019). The four major biological macromolecules include carbohydrates, lipids, proteins, and nucleic acids. These polymers can be broken down into their monomers: monosaccharides, FAs and glycerol, amino acids, and nucleotides. Each of these subunits can be metabolized, yielding various amounts of energy and intermediates (Castro et al., 2019)

Glucose is the most widely available nutrient in cells and thus is a key player in AML metabolism. Glycolysis is the breakdown of glucose into pyruvate, which can be processed in 3 ways: anaerobic glycolysis, mitochondrial respiration, or aerobic glycolysis. Anaerobic glycolysis is the conversion of pyruvate into lactate in the absence of oxygen and produces 2 moles of ATP/glucose. In the presence of oxygen, mitochondrial respiration occurs. Pyruvate can enter the mitochondria and act as a substrate for the tricarboxylic acid (TCA) cycle, producing electron-reducing agents required for oxidative phosphorylation (OXPHOS). OXPHOS transpires at the inner

mitochondrial membrane, where the bulk of ATP production occurs. Although the TCA cycle and OXPHOS pathway is the most efficient form of energy production, AML cells favour aerobic glycolysis, an effect known as the Warburg effect (Warburg, 1956). Regardless of oxygen status, AML cells will preferentially convert pyruvate into lactate, while also maintaining 5% of mitochondrial respiration. Aerobic glycolysis produces the lowest amount of ATP/glucose (Vander Heiden et al., 2009). Although this seems counterintuitive for AML cells to favour the least energy-producing pathway, aerobic glycolysis allows for cancerous cells to perform anabolic processes to synthesize nucleic acids, lipids, and proteins. This process supplies the necessary biomolecules required for highly proliferative cells (Vander Heiden et al., 2009). Unfortunately, LSCs have a distinct metabolic profile that is heavily dependent on OXPHOS compared to bulk AML cells which rely on glycolysis (Lagadinou et al., 2013).

After entering the TCA cycle, carbohydrates, lipids, and proteins are further processed into the electron transport chain (ETC), producing energy through OXPHOS. Studies have shown that LSCs heavily depend on OXPHOS for the bulk of their energy production, as inhibition of this process using BCL-2 inhibitors shows reduced LSC survival (Lagadinou et al., 2013). Coincidently, amino acid uptake and metabolism has been found to be elevated in LSCs, and perturbation of this resulted in oxygen deprivation and selectively killed LSCs (Lagadinou et al., 2013). This suggests that LSCs reliance on OXPHOS may be powered by amino acid metabolism. This correlation led to the investigation of whether Venetoclax/Azacitidine co-treatment targets amino acid metabolism in LSCs. Indeed, Jones et al. (2018) showed that cotreatment reduced the

expression of amino acid transporters, decreased amino acid uptake, and showed reduced LSC counts. This paper was pivotal in elucidating a potential mechanism of action of the cotreatment and showed great therapeutic potential for targeting amino acid metabolism in AML. However, metabolic reprogramming of AML cells resulting in treatment resistance underpins this finding. Stevens et al. (2020) showed that Venetoclax/Azacitidine resistant cells are not affected by amino acid deficiency, but instead display increased levels of FA transport and FA beta-oxidation. This suggests that LSCs can reprogram their metabolism and preferentially utilize fatty acids to amino acids to power OXPHOS. Subsequently, the investigators found that targeting FA metabolism by disrupting FA dehydrogenases or inactivating FA transporters re-sensitized resistant cells to the cotreatment. Metabolism-related chemoresistance is not unique to just this co-treatment. A study by Farge et al. (2017) showed that cytarabine-resistant AML cells possess elevated levels of OXPHOS and that targeting this by inhibiting mitochondrial protein synthesis, fatty acid oxidation, or the ETC reversed this resistance. Overall, these studies provide growing evidence for the therapeutic targeting of energy metabolism unique to LSCs, such as FA metabolism, to better eradicate these chemoresistant cells. The largest source of FAs comes from adipocytes, which are the most abundant cell type in the BM.

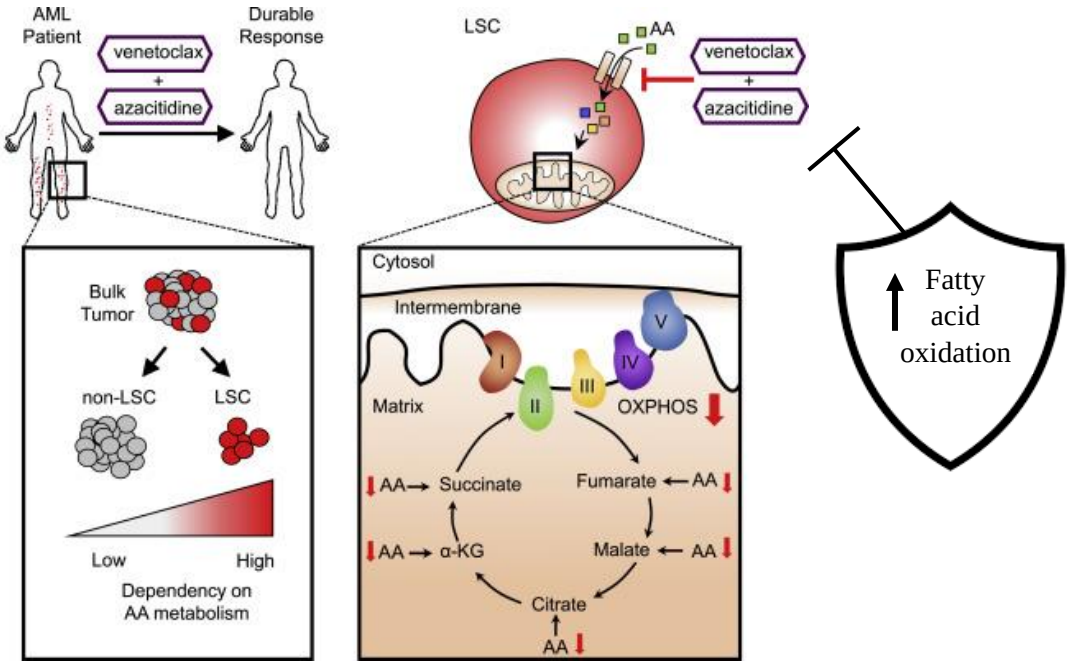

Figure 4. The effects of Ven/Aza on metabolism. Ven/Aza was discovered to reduce amino acid uptake and subsequent oxidative phosphorylation, resulting in clinical responses. However, resistance to Ven/Aza can occur due to the ability of AML cells to enhance fatty acid oxidation. Adapted from Jones et al. (2018)

1.6 Bone marrow microenvironment

The BM microenvironment consists of cell types that do not directly contribute to hematopoiesis, but assist in HSPC self-renewal potential, differentiation, and homing (Ladikou et al., 2020). BM-derived mesenchymal stromal cells (BMSCs) are multipotent stem cells that give rise to these non-hematopoietic cells, such as osteoblasts, osteoclasts, endothelial cells, fibroblasts, chondrocytes, and adipocytes (Ladikou et al., 2020). We are interested in adipocytes, and the BMSCs that give rise to them, as they are responsible for the storage and release of FAs, and are the most prevalent cell types in the BM. There is a direct correlation between adipocyte occupancy in the BM and age. A 20-year-old individual has ~20% of their BM occupied by adipocytes while a 65-year-old individual

shows 65-70% (Justesen et al., 2001). The role of adipocytes in cancer is multi-faceted, but a general finding is the support and nourishment they provide to cancer cells through stimulating FA oxidation and subsequently increasing OXPHOS (Behan et al., 2009). These studies provoke a potential correlation between increased adipocyte presence and the higher incidence of chemoresistance and lower survival rates in older patients.

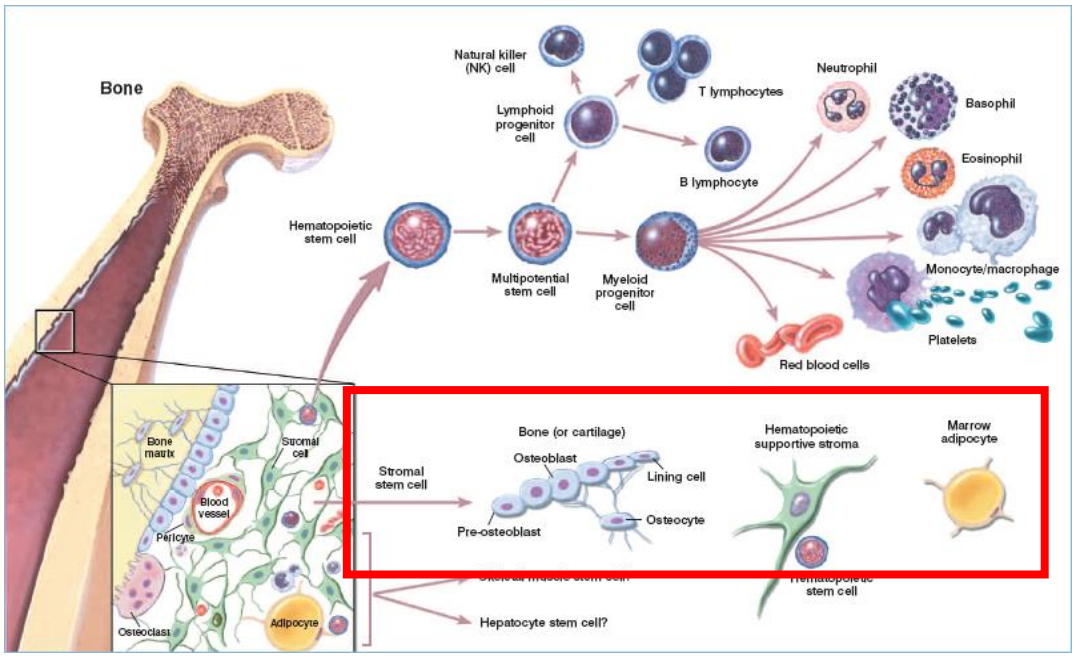

Figure 5. Cell types in the bone marrow. Hematopoietic stem cells and stromal cells reside in the bone marrow and differentiate into many other cell types. The red box highlights BMSCs and the cells it gives rise to, namely adipocytes. Adapted from Domen et al. (2006)

The role of the BM microenvironment in AML has gained traction over the last 5 years, with evidence pointing towards the ability of AML cells to reprogram the BM microenvironment to create a supportive niche to accelerate disease growth. In particular, 4 papers have implicated adipocytes and BMSCs in the AML-induced reprogramming of

the BM microenvironment. The study by Shafat et al. (2017) showed that AML blasts can activate lipolysis of co-cultured adipocytes by inducing phosphorylation of adipocyte lipases. In addition, fatty acid binding protein 4 (FABP4) was found to be elevated in both AML and adipocytes co-cultured together, and inhibiting FABP4 led to reduced AML proliferation. The study by Ye et al. (2016) showed that LSCs expressing the CD36 fatty acid transporter protein are particularly enriched in adipose tissue and are protected from treatment in this microenvironment. They also found that these LSCs released pro-inflammatory cytokines, which induced lipolysis of adipocytes from leukemic mice. Next, a study by Dr. Bhatia's group, a lab from McMaster University, found that BM samples from AML patients show reduced adipocyte occupancy and size in the bone marrow compared to healthy controls of the same age, suggesting that AML disrupts new, or *de novo,* adipogenesis (Boyd et al., 2017). Their findings also showed that inducing adipogenesis actually rescued AML-induced disruption of healthy myelo-erythroid maturation, while inhibiting leukemic progenitors. This highlights the complicated role of adipocytes and shows a dual role of targeting adipocytes in AML; it can restore normal hematopoiesis while disrupting that of leukemic cells. There is less literature regarding the impact of BMSCS on the metabolism of AML cells, but a very recent paper released in November 2020 by Forte et al. (2020) showed that a subpopulation of BMSCs supports LSCs by increasing OXPHOS and the TCA cycle. They also found that AML cells co-cultured with BMSCs show increased resistance to cytarabine treatment.

Taken together, AML cells may be or become resistant to treatment because of their ability to reprogram their surroundings, namely through adipocytes and BMSCs, to create a supportive microenvironment that promotes their own survival and growth.

1.6 MCL-1 in AML hematopoiesis and chemoresistance

One potential mechanism of action for microenvironment-induced chemoresistance to Ven/Aza may be due to the upregulation of the BCL-2 family member myeloid cell leukemia sequence 1 (MCL-1). MCL-1 is an anti-apoptotic protein that works similarly to BCL-2 by sequestering BIM and inhibiting the activation of pro-apoptotic BAX/BAK proteins. It is also essential for proper myeloid differentiation and survival as deletion studies showed loss of HSC, granulocyte monocyte progenitors (GMP) and CMP cells (Zhou et al. 1997). In the context of AML, MCL-1 has been shown to be essential for the survival of both human AML-derived cell lines and primary human AML through gene deletion studies (Glaser et al., 2012). Contrastingly, the same experiments were conducted for BCL-2 or BCL-xL proteins, however, their roles were less impactful than MCL-1 in the survival and expansion of AML cells. Additionally, one study found that the aggressive FLT3-ITD mutation of AML shows upregulation of MCL-1 that results in enhanced survival of LSCs (Yoshimoto et al., 2009). Higher levels of MCL-1 expression have also been shown to predict poor prognosis and relapse for AML patients (Li et al. 2019), which has also been shown for other blood cancers such as multiple myeloma (Wuilleme-Toumi et al., 2005). These studies provide evidence for the essential role of MCL-1 in AML, which set the precedent for researchers to investigate its role in AML chemoresistance.

Most interestingly, Das et al. (2009) created an AML cell line called HL-60/MX2 that overexpresses MCL-1 and found that it was resistant to multiple drugs. In a more recent study, high levels of MCL-1 in *MLL-AF9* mice models of AML overcame sensitivity to daunorubicin, which is most commonly used today for induction chemotherapy for patients with AML (Anstee et al. 2019). In the context of Ven. Figure 6 shows the difference between sensitive and resistant cells. Ven-sensitive lines have lower levels of MCL-1 expression resulting in their inability to sequester BIM, leading to apoptosis. However, in resistant cells, MCL-1 (highlighted in the red box) is elevated, resulting in BIM sequestration, preventing apoptosis, and leading to cell survival. Zhang et al. (2022) showed that Ven-resistant AML cell lines showed higher and more stable levels of MCl-1which also helped maintain their mitochondrial respiration levels. Furthermore, they found that when MCL-1 was genetically silenced and pharmacologically inhibited, Ven sensitivity was restored. Another study showed that combining Ven with arsenic trioxide reduced leukemic proliferation, survival, and apoptosis which was mediated by reduced MCL-1 (Cho et al. 2021). Unfortunately, there is a lack of studies investigating the role of the BM microenvironment-induced resistance and MCL-1. However, one study found that AML cells grown with BMSCs showed resistance to Ven which correlated with enhanced MCL-1 expression, and this too can be reversed using a combination of Ven and MCL-1 inhibitors (O'Reilly et al., 2018). To date, there are no known studies investigating the role of adipocyte-induced chemoresistance to Ven/Aza and whether MCL-1 is involved in this context.

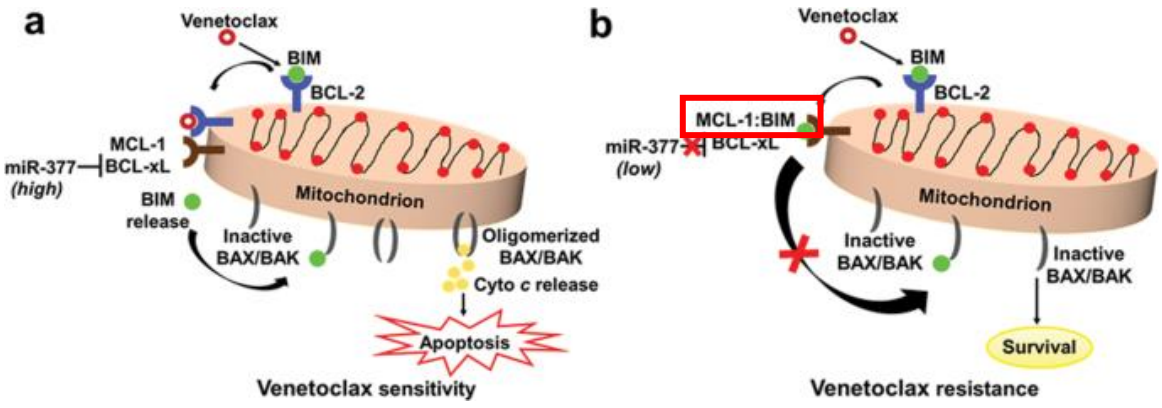

Figure 6. MCL-1-induced Ven/Aza chemoresistance. MCL-1 may confer resistance to Ven/Aza by sequestering pro-apoptotic protein BIM, resulting in cell survival. Adapted from Kapoor et al. (2020)

1.7 Project rationale

This thesis focuses on elucidating the role of the microenvironment on chemoresistance to Venetoclax/Azacitidine co-treatment, one of the only therapies tolerated by older, vulnerable patients with AML. AML cells have been shown to reprogram their own metabolism while also modifying metabolic pathways in cells composing the bone marrow microenvironment, namely adipocytes and BMSCs, to establish a supportive and nourishing niche. If we are able to target and reduce this support, we may be able to increase treatment responses and durations. Our investigation into the role of MCL-1, an anti-apoptotic protein overexpressed in AML patients, in the BM microenvironment may provide insight into how AML cells are able to create a protumoral environment. By understanding the impact of MCL-1 in microenvironment-induced support of AML, we hope to resensitize AML cells to Ven/Aza co-treatment, thereby overcoming treatment resistance and improving patient outcomes. MCL-1

inhibitors have been gaining much interest in AML treatment, specifically in combination with Ven, but have never been studied in the context of Ven/Aza-induced BMSC and adipocyte. In this thesis, we will assess the role of adipocytes and BMSCs in inducing AML chemoresistance to Ven/Aza and to determine the role of MCL-1 in this support.

1.7 Hypothesis

We hypothesize that BMSCs and adipocytes can support the growth and viability of AML cells and that they are capable of inducing resistance to Venetoclax/Azacitidine co-treatment. We further hypothesize that MCL-1 plays a role in the chemoprotective effect induced by these cells. We are therefore pursuing these hypotheses by investigating the following aims:

1.8 Specific aims

1. Investigate the ability of BMSCs/adipocytes to promote the growth of AML cells (Figure 7).

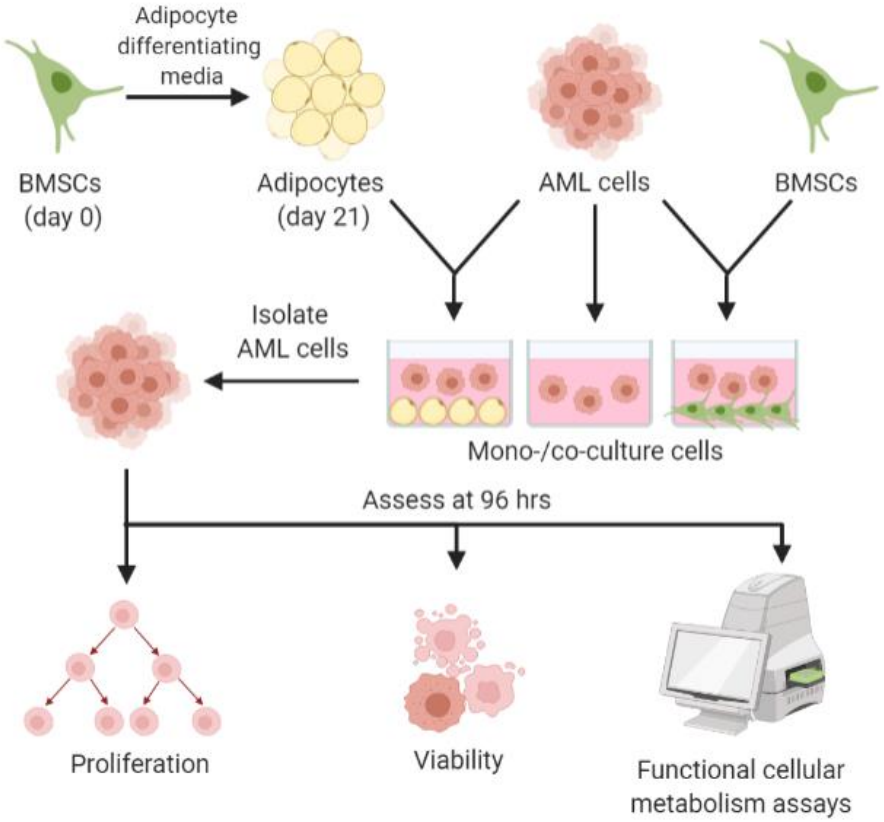

Figure 7. Flowchart for Aim 1. BMSCs will be differentiated into adipocytes using an optimized adipocyte differentiating media. BMSCs and adipocytes will be co-cultured with AML cells, in addition to AML cell monocultures as the control. After 96 hours of co-culture, AML cells will be isolated and measured for proliferation, viability, cell surface markers, and metabolism.

2. Study the ability of BMSCs/adipocytes to induce Ven/Aza chemoresistance in AML cells. If chemoresistance is seen, investigate whether MCL-1 is involved (Figure 8)

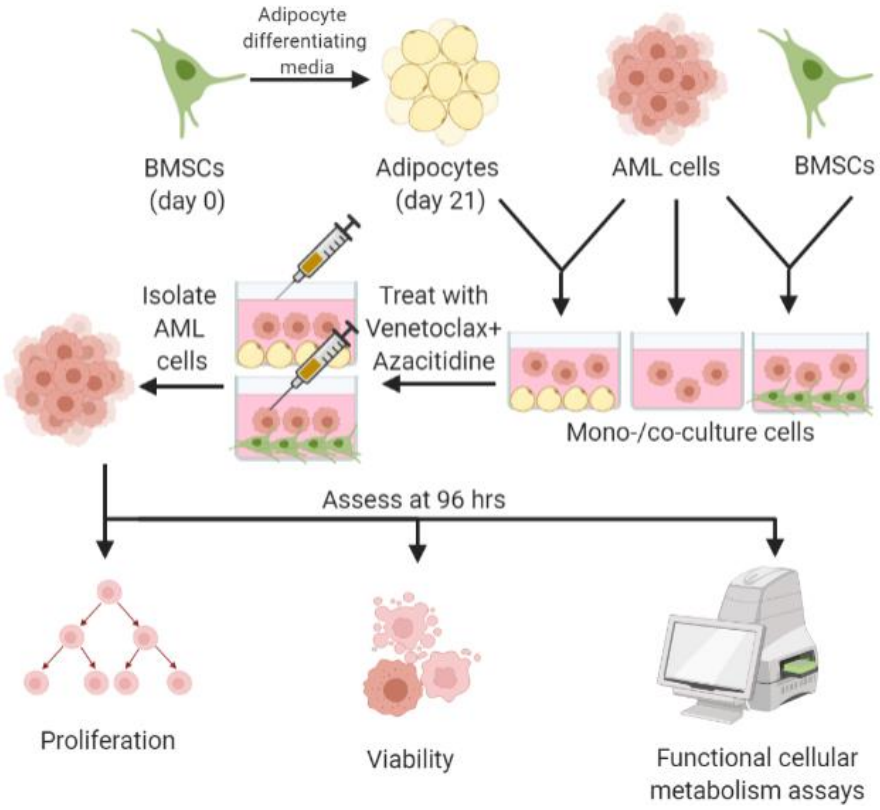

Figure 8. Flowchart for Aim 2. BMSCs will be differentiated into adipocytes using an optimized adipocyte differentiating media. BMSCs and adipocytes will be co-cultured with AML cells, in addition to AML cell monocultures as the control. During the 96 hours, the co-cultures will be treated with Venetoclax/Azacitidine, and then the AML cells will be isolated and measured for proliferation, viability, cell surface markers, and metabolism.

Chapter 2: Materials and Methods

2.1 AML cell culture

2.1.1 AML cell lines

MOLM-13 and HL-60 AML cell lines were used to investigate the specific aims. MOLM-13 carries the *FLT3-ITD* mutation, which occurs in 20% of AML patients while HL-60 harbours a *myc* oncogene. The media used for AML cell lines was RPMI 1640 media+10% fetal bovine serum (FBS)+1% penicillin/streptomycin (P/S)+1%L-glutamine (L-glut) (ThermoFisher). These were combined and filter sterilized. Going forward, this complete RPMI media composition will be termed cRPMI. Frozen vials (-150^{o}C) containing 1-2x10^{6} cells in FBS+20% DMSO (ThermoFisher) freezing solution were thawed and spun down at 1080 revolutions per minute (rpm) for 5 minutes. They were then resuspended in warmed cRPMI and grown in T-75 non-adherent flasks for growth and maintenance and incubated at 37^{o}C. Cells were passaged every 2-3 days once density reached 3-4x10^{6} cells/mL. Cells were used for experiments 2-3 passages after thawing to allow time for viability to recover, which was around 90-95% for MOLM-13 cells and 85-90% for HL-60 cells.

2.1.2 Retrovirally-transduced murine AML cells

We developed two murine models of AML overexpressing Hoxa9/Meis1 (H9M) or MN1, which are oncogenes found in aggressive AML. These H9M- or MN1-transformed murine myeloid progenitor cells can give rise to AML when

transplanted into mice. 4 plasmids containing one of Hoxa9, Meis1-YFP, MN1-GFP, and control-GFP genes will be transformed using heat shock and expanded in Escherichia coli (E. coli) Stbl3 cells. The plasmids will be isolated and will be used to transfect Phoenix Ampho (PA) HEK lentiviral packaging cells. The PA cells will release replication-incompetent lentiviral particles containing the plasmid of interest into the supernatant. GP+E86 (E86) lentiviral packaging cells will be transduced using the PA supernatant. Transduction will be verified using flow cytometry sorting of GFP or YFP for the Meis1-YFP, MN1-GFP, and control-GFP containing E86 cells. Geneticin (ThermoFisher) treatment will be used to verify Hoxa9-containing E86 cells as this plasmid contains a geneticin antibiotic gene. BM progenitor cells isolated from wildtype C57BL/6 donor mice (Jackson Lab) will be transduced by co-culturing them with the transduced E86 cells. There will be 3 co-culture systems with the progenitor cells: Hoxa9 and Meis1 E86 cells cultured together to generate H9M murine AML cells, MN1 alone to generate MN1 murine AML cells, and control-GFP to act as the control. At every step of transduction, we verified the presence of the MN1, Meis1, and GFP-control plasmids using flow cytometry sorting while geneticin treatment was used to select for the Hoxa9 plasmid. After verification and selection of plasmids of interest, cells were frozen in vials at a density of $1x10^6$ cells in DMSO freezing media at -150^{o}C. Going forward, these BM murine myeloid progenitor cells containing the plasmids of interest will be termed murine AML cells.

When needed for experiments, frozen cells were thawed in cRPMI. The generated murine AML cells need to be supplemented with cytokines to promote proliferation and survival. This includes 10 ng/mL human interleukin (IL)-6, 6 ng/mL mouse IL-3, and 100 ng/mL mouse stem cell factor (SCF) (Peprotech). The cytokines were added fresh to pre-warmed media. After thawing cells in cRPMI, cells were spun down at 1080 rpm for 5 minutes then resuspended in cRPMI with cytokines in T-75 non-adherent flasks for growth and maintenance, and incubated at 37^{o}C. Cells were passaged every 2-3 days once density reached 3-4x10^{6} cells/mL. Cells were used for experiments 2-3 passages after thawing to allow time for viability to recover, which was >90% for H9M and MN1 cells.

2.1.3 AML patient samples

BM and peripheral blood (PB) samples were collected from patients and were processed within 1-2 days of receiving the samples. Prior to processing, 2 buffers are require including P.E.F (97% PBS without salts, 1mM EDTA, 3% FBS) (ThermoFisher) and freezing media 90% FBS and 10% dimethyl sulfoxide (DMSO) (ThermoFisher). Measure the volume of blood and dilute the sample with 3 times the volume of PEF (1:4 sample dilution). After diluting, equal parts of Ficoll (Fisher Scientific) to diluted sample needed to be put into a tube, with Ficoll added first, and the diluted sample added very slowly and at an angle to ensure the interface is not disturbed. The sample was then spun down at 1,500 revolutions per minute (rpm) with break and acceleration set to 0, for 20 minutes. After the spin, four visible layers were seen, the red blood cells presented at the

bottom as a pellet, the ficoll as the second layer, the mononuclear layer, followed by the plasma. The mononuclear layer was slowly removed. The removed mononuclear cells were spun down at 1,600 rpm for 5 minutes, with break and acceleration set back to maximum, 9. Once the spin was completed, the supernatant was removed. 10 mL of ammonium chloride was added to lyse the remaining red blood cells, ensuring that everything was homogenized. This was incubated for 10 minutes. Following the incubation period, 20 mL of PEF was added to neutralize the ammonium chloride. This was spun down at 1,600 rpm for 5 minutes. The supernatant was removed, and the sample was resuspended in an appropriate volume of PEF depending on the cell count. If the pellet was large, >5 mL of PEF was used to resuspend. Once the resuspension was complete, the cells were counted using trypan blue exclusion. The total cell number was calculated. 5 million cells/ mL vials, and if possible, 20 million cells/mL vials can also be made, with each vial labeled with the patient sample, type of sample (BM, or PB), date of the sample processing and the concentration of cells. After counting, the cells were spun down at 1,600 rpm for 5 minutes and resuspended in freezing media with the volume equating to the number of vials ready to be frozen. The samples were then placed at -80^{o}C overnight, then transferred to -150 ^{o}C.

The AML patient samples that were utilized are listed in Table 1, with their ID, cytogenetic, and molecular aberration data described. These cells require serum-free IMDM (ThermoFisher) +20% BIT 9500 (StemCell)+10 μg/mL low-density lipoprotein (LDL) (Sigma-Aldrich) +55 μM beta-mercaptoethanol (β-ME)

(Sigma-Aldrich) +1% P/S (ThermoFisher). These were combined and filter sterilized. Going forward, this complete IMDM media composition will be termed cIMDM. These cells also require cytokines to promote proliferation and survival, which includes 10 ng/mL each of human FLT-3, SCF, and IL-3 (Peprotech). The cytokines were added fresh to pre-warmed cIMDM media. When needed for experiments, cells frozen at -150^{o}C were thawed in cIMDM and spun down at 1500 rpm for 5 minutes. They were then resuspended in warmed cIMDM+cytokines in T-75 non-adherent flasks for growth and maintenance and incubated at 37^{o}C. Viability ranges between 50-90% depending on the patient sample. Cells were used for experiments within 1-2 days of thawing.

Table 1. List of patient samples utilized in experiments. The patient sample ID, cytogenetic data, and molecular aberrations through next generation sequencing is listed.

Patient sample ID	Cytogenetics	Molecular aberrations NGS
21001A (Diagnosis)	46,XX[20]	ASXL1, CEBPA, TET2
21003A (Diagnosis)	45,X,-Y,t(8;21)(q22;q22)[2]/45,idem,del(9)(q12q32)[8]	NRAS
21007A (Diagnosis)	45,XY,-7[10]	RUNX1, IDH2, DNMT3A, NRAS, SRSF2
21008A (Diagnosis)	47,XY,+8[9]/46,XY[1]	RUNX1, FLT3, DNMT3A
21029B (Leukapheresis)	46,XY[20]	FLT3, WT1, DNMT3A, (NPM1)
21042B (Leukapheresis)	46,XX[20]	FLT3-ITD, NPM1
21050B (Leukapheresis)	46,XY[20]	FLT3, NPM1, DNMT3A

2.2 Bone marrow-derived mesenchymal stromal cells (BMSCs)

BMSCs require DMEM-F/12 base media+10% FBS+1%P/S+1% L-glut (ThermoFisher). These were combined and filter sterilized. Going forward, this complete DMEM/F12 media composition will be termed cDMEM. When cells were needed for experiments, cells frozen at 150^{o}C were thawed in cDMEM and spun down at 1300 rpm for 5 minutes. They were then resuspended in warmed cDMEM in T-75 adherent flasks for growth and maintenance and incubated at 37^{o}C. Cells were passaged every 2-3 days once confluency was between 70-80%. Cells were used for experiments 2-3 passages after thawing to allow time for viability to recover, which was around >90%.

2.3 Adipogenic differentiation

Adipocytes were differentiated from BMSCs using differentiating media (DM), which needed to be optimized using compositions from well-established studies. All 3 DMs consisted of the same cDMEM media but contained varying concentrations of isobutyl methylxanthine (IBMX), human insulin, indomethacin (INDO), and dexamethasone (DEXA) (Sigma Aldrich). DM1 included 500 μM IBMX, 287 nM insulin, 50 μM INDO, and 1 μM DEXA (Boyd et al., 2017). DM2 included 500 μM IBMX, 100 nM insulin, 0.2 mM INDO, and 1 μM DEXA (Shafat et al., 2017). DM3 included 250 μM IBMX, 574 nM insulin, 100 μM INDO, and 0.5 μM DEXA (Singh, unpublished). The control for this experiment were BMSCs that were left undifferentiated, supplemented with BMSC media alone. Differentiation was induced over 21 days, changing media every 2-3 days. On day 21, adipocytes and undifferentiated BMSCs were stained with

BODIPY (Sigma-Aldrich) or Oil Red O (ORO) (Sigma-Aldrich) to analyze morphology or lysed for RNA for cDNA gene expression analysis using quantitative polymerase chain reaction (qPCR). The optimization data are shown in chapter 3.1. DM3 showed the most adipogenesis, therefore DM3 was used for further differentiation experiments.

2.4 BODIPY and ORO staining for adipogenic efficiency

BODIPY and ORO stain for neutral lipids, which are stored in lipid droplets (LDs), the hallmark of adipocytes. These stains were performed to measure the adipogenesis efficiency of the different DMs.

2.4.1 ORO staining protocol

0.5% ORO solution in 100% isopropanol (Sigma-Aldrich) was prepared and placed on a magnetic stir plate in a dark container overnight. On the day of use, this solution was diluted in distilled water in a 6:4 ratio (0.5% ORO:water) then vacuum filtrated and ready for use. BMSCs that were differentiated into adipocytes for 21 days in 6-well plates were washed on day 21 with DPBS (Corning) twice, then quickly rinsed with 60% isopropanol. 2 ml of ready-to-use ORO solution was placed into the wells and let sit for 45 minutes. The plates were then rinsed indirectly by allowing water to slowly fall into the wells by placing the water stream against the plate not covered by the cells to ensure the adipocyte layer is not removed. The plate was rinsed until the wells become clear and free of ORO to minimize background noise in the imaging. The wells were then visualized using a high-resolution photocopier (Epson Perfection V700 Photo) and analyzed for lipid droplet formation.

2.4.2 BODIPY staining protocol

BMSCs that were differentiated into adipocytes for 21 days in 6-well plates were washed on day 21 with DPBS twice. The cells were fixed with 2 ml of 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) solution per well and left to sit for 20 minutes at room temperature (RT). Wells were then washed twice with DPBS. 2 mL of 1:1000 BODIPY and 1:2000 Hoechst dye (ThermoFisher) in DPBS were added to each well and let sit for 20 minutes at RT. The wells were then rinsed thrice with DPBS and imaged using the Olympus 1X81 microscope to detect lipid droplet formation and size.

2.5 RNA extraction and cDNA synthesis

In order to determine adipogenic efficiency, RNA from adipocyte-specific genes were compared between day 21 adipocytes and undifferentiated BMSCs. 2 wells of day 21 adipocytes and BMSCs that were both confluent in 6-well plates were used for RNA extraction. In order to release these adherent cells from the wells, the media was aspirated from each well, and washed twice with DPBS. 3 mL/well of DPBS was added and a cell scrapper was used to release the adipocytes or BMSCs into the suspension, which were then placed at -80^{o}C until needed for RNA extraction. RNA was extracted from both adipocytes and BMSC lysates using the RNeasy Mini Kit (Qiagen). The *Protocol: Purification of Total RNA from Animal Cells Using Spin Technology* of the RNeasy Mini Kit manual available through https://www.qiagen.com/ca/resources/ was followed. In brief, genomic DNA (gDNA) was removed from thawed adipocyte lysates through the gDNA eliminator. 70% ethanol was added to the flow-through and spun down to dissolve

and wash away the salts. RW1 and RPE solutions were also added to the column to further remove salts and biomolecules. Nuclease-free water (NFW) was used to elute the RNA and was then measured for concentration using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). The BMSC samples had between 100-200 ng/uL of RNA while the adipocytes had <100 ng/uL. These samples were then placed at -80^{o}C until needed for complementary DNA (cDNA) synthesis. Approximately 0.5 μg of total RNA was reverse transcribed into cDNA using the SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher Scientific), following the manufacturer's protocol. In brief, 5x reverse transcriptase (RT) and NFW were added to the RNA samples and were put through the following program: 25^{o}C for 10 mins, 42^{o}C for 60 mins, and 85^{o}C for 5 minutes. The negative controls were RNA samples that had inactive reverse transcriptase (RvT), which was done by denaturing RvT by heating it for 10 mins at 65^{o}C before the RNA and NFW were added. The cDNA was then stored at -20^{o}C until needed for quantitative RT-polymerase chain reaction (qRT-PCR).

2.6 qRT-PCR

1 μL of cDNA was used for each qRT-PCR reaction, using gene-specific TaqMan expression assays (ThermoFisher, PPARγ assay id: Hs01115513_m1; CEBPα assay id: Hs00269972_s1; FABP4 assay id: Hs01086177_m1; CD36 assay id:Hs00354519_m1) and 1x TaqMan™ Fast Advanced Master Mix. These samples were then run on the following program: hold stage consists of 2 mins at 50^{o}C and 10 mins at 95^{o}C, while the PCR stage consists of 40 cycles of 15 seconds at 95^{o}C then 1 min at 60^{o}C. This program was run and measured on the QuantStudio5 Real-Time PCR detection system. The

controls for this include no cDNA and no RT controls. Data was analyzed using the ΔΔ cycle threshold (CT) method described in Livak & Schmittgen (2001). Gene expression was normalized to the internal control ribosomal protein lateral stalk subunit P0 (RPLP0) (ThermoFisher, assay id: Hs00420895_gH). Adipocyte gene expression levels were compared to gene expression levels of BMSCs to determine fold change.

2.7 Co-culture system

3 conditions are needed for co-cultures, which include monocultured AML cells termed monoculture, AML cells cultured on BMSC termed BMSC co-cultures, and AML cells cultured on adipocytes termed adipocyte co-cultures. All co-cultures took place in 6-well plates. Monocultures were set up on the same day of the co-culture (day 0) while BMSCs were prepared 2 days before day 0 of co-culture to allow the cells to adhere and reach >95% confluency. Adipocytes that have been differentiated for 21 days were used for day 0 of co-cultures. On day 0 of the co-culture, $1x10^6$ AML cells were grown alone for the monoculture, on BMSCs, and on adipocytes in media in which the AML cells were grown in. For example, experiments using AML cell lines used cRPMI, murine AML cells used cRPMI with or without cytokines, while AML cells from patient samples used cIMDM with cytokines. Mono/co-cultures were set up for 4 days, passaging the cells and plating back $1x10^6$ AML cells in fresh media on day 2. Proliferation and viability of AML were assessed on day 2 and day 4 using trypan blue exclusion and an automated cell counting machine (Cell Countess™, Thermo Fisher). AML cells were isolated on day 4 and were analyzed for mitochondrial respiration and glycolysis using the Seahorse extracellular flux analyzer (XeF96) (Agilent). Please refer to section 2.4 for

a detailed explanation of how the XeF96 was set up. Data was analyzed using 2-way ANOVA tests (GraphPad Prism 8). Differences were considered significant if *p-value*<0.05.

2.7.1 Venetoclax, Azacitidine, and/or Tapotoclax treatment

Co-cultures were also exposed to treatments such as Ven alone, Aza alone, Tapotoclax (Tapo) (All from Cayman Chemicals) alone or a combination of Ven/Aza or Ven/Tapo. For these experiments, the co-cultures were exposed to treatment for the entirety of the co-culture. Ven, Aza, and Tapo stock solutions were diluted in DMSO and frozen in -20^{o}C. On day 0, when plating the AML cells, Ven, Aza, and/or Tapo were thawed and diluted in the corresponding media that the cells grew in. Ven concentrations used include 100, 500, and 1000 nM, Aza concentrations include 500 and 1000 nM, and the Tapo concentration was 100 nM. The control for the treatment conditions were mono/co-cultures exposed to 0.5% DMSO. Similar to section 2.3, the AML cells were measured for proliferation and viability on day 2 and 4, while metabolism was assessed on day 4.

2.8 Seahorse extracellular flux analyzer (XeF96)

AML cells mono/co-cultured were isolated on day 4 of co-culture experiments and were analyzed using the Seahorse extracellular flux analyzer (XeF96) (Agilent). Specifically, the mitochondrial stress test (MST) was employed to measure mitochondrial respiration and glycolysis. The protocol for setting up and running this assay can be found in the “Seahorse XF Cell Mito Stress Test Kit User Guide” available on

www.agilent.com. In brief, prior to assay day, 96-well seahorse plates were prepared with Cell-Tak™ Cell and Tissue Adhesive (Corning) to make the AML cells adhere to the plates. In addition, the cartridge containing the sensor was also hydrated in seahorse XeF specific RPMI or DMEM media, which needs to be the same base media of the plated AML cells. On the day of the assay, the cells were counted and plated at 60,000 cells/well in 180 μL. This density was determined to be optimal through previous work in the lab. After plating cells, it was spun down at 1080 rpm for 1 minute with full acceleration but no brake to allow for cells to adhere. They were then placed into the Cytation 1/5 Cell Imaging Multi-Mode reader (BioTek Instruments) which uses brightfield microscopy to count the number of cells before the assay. Simultaneously, the cartridge was filled with the reagents needed for the MST assay. The concentrations were different depending on the type of AML cells. The cell lines and murine AML cells required 2.5 uM oligomycin in port A, 0.5 uM FCCP in port B, and 0.5 uM rotenone+0.5 uM antimycin+20 uM of Hoechst dye in port C (All from Cayman Chemicals). The AML cells from patient samples required all the same but 0.25 uM FCCP instead. The cartridge was then calibrated as per the instruction manual, in which the cell plate was added, and the MST assay was initiated. Afterward, the cell plate was placed back in the Cytation 1 instrument to perform a fluorescence-based counting of cells. This allows for the normalization of the values determined by XeF96 based on image-based cell counting to provide a more accurate measure of metabolism. Data was generated using Seahorse Analytics (Agilent) and analyzed using 2-way ANOVA tests (GraphPad Prism 8). Differences were considered significant if $p\text{-}value<0.05$.

2.9 Flow cytometry

After 48 hours of mono/co-culturing, AML cells were isolated and spun down at 1080 rpm for 5 mins. They were then resuspended in 1 mL warm DPBS and 5 µL of 200 µM of JC-1 (ThermoFisher Scientific) was added and let to sit for 30 mins at 37°C. The cells were then washed with DPBS and spun down at 1200 rpm for 5 mins. The supernatant was aspirated, and the cells were resuspended in 100 µL 1x annexin buffer (ThermoFisher Scientific). 5 µL annexin V-pacific blue (PB) antibody (ThermoFisher Scientific) was then added to the cells and incubated at 37°C for 15 mins. Another 400 µL of 1x annexin buffer was added. The last dye added was 5 µL of DRAQ7 (ThermoFisher Scientific). These cells were then analyzed for percent live cells, apoptotic cells, dead cells, and cells expressing mitochondrial apoptosis using the Cytoflex LX flow cytometry machine (Beckman Coulter). PB measures Annexin V, Alexa Fluor700 measures Draq7, and FITC measures JC-1. PE and FITC compensation beads were used to make the flow cytometer gating and voltage constraints accurate. The controls for this experiment include monocultured DMSO control cells that were unstained, stained for only JC-1, stained for only JC-1 and FCCP, and stained for DRAQ7 only. Additional controls include DRAQ7 staining only for cells that show 70-80% viability and annexin V-PB staining only for cells that show <50% viability. Data was analyzed using FlowJo software (BD Sciences) which produced flow plots. For annexinV-PB and DRAQ7 Alexa Fluor700 flow plots, Q4 represents live cells, Q3 represents apoptotic cells, while Q1 and Q2 represent dead cells (Figure 9). Statistics could not be performed given the n=1.

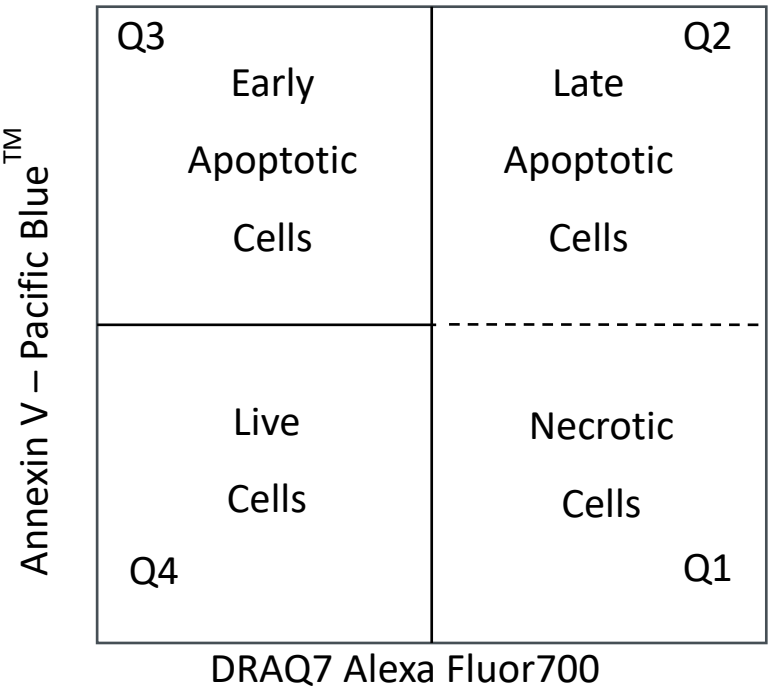

Figure 9. Example flow plot of AnnexinV-PB and DRAQ7 Alexa Fluor700. AML cells were stained for AnnexinV-PB and DRAQ7 Alexa Fluor700 and measured by flow cytometry to determine percentage of live cells (quadrant (Q) 4), early apoptotic (Q3), and dead cells (late apoptotic (Q2) + necrotic cells (Q1)). Flow plots were produced using FlowJo software.

2.10 Protein extraction and quantification

Mono/co-cultured AML cells were isolated and lysed for protein extraction and quantification through western blotting. The number of AML cells ranged given the varying effects of the co-cultures, but a minimum of $1x10^6$ cells was isolated. Throughout isolation, all steps had to be carried out on ice and spun down in a centrifuge set at 4°C. The cells were spun down at 1500 rpm for 10 mins then resuspended in cold DPBS and spun again at 1500 rpm for 5 mins. The supernatant was removed, and the cell pellet was

lysed in lysis buffer (125 mM Tris HCL pH 6.8+4% SDS+20% glycerol dissolved in distilled water). These samples were saved at -80^{o}C until needed for quantification. Prior to quantification, the lysates were thawed and homogenized using insulin needles or pipetting with p200 if needed. Quantification was done using the Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's protocol. In brief, a standard curve was generated using concentrations of bovine serum albumin (BSA) ranging from 25-2000 ug/mL. The protein lysates were prepared in dilutions of 1:5, 1:10, 1:20, and 1:50 and measured on the FLUOstar Omega microplate reader (BMG Labtech) at 562 nM. The concentrations of the protein samples were determined using the equation of the line of best fit of the standard curve.

2.11 Western blotting

All chemicals and reagents used for Western blots were from BioRad, except when specified. Whole protein (30 μg) was prepared in 1x Laemmli protein sample buffer and diluted in lysis buffer, then heated for 10 mins at 95^{o}C. The samples were separated in a 10% acrylamide gel at 60 volts (V) for 30 mins then 120 V for 1-1.5 hrs. The proteins were then wet-transferred to a methanol-activated PVDF membrane (BioCompare) and ran at 90 V for 1.5 hrs in an ice bucket. The membranes were then blocked in 5% skim milk diluted in TBS-Tween (TBS-T) for 1 hour at RT. The membranes were given quick washes with TBS-T three times then incubated with the primary antibodies. MCL-1 (1:500, Cell Signalling Technology) has a molecular weight of 48 kilodaltons (kDa) and is the protein of interest while vinculin (1:1000, Cell Signalling Technology) is 124 kDa and serves as the internal control. Membranes were cut at 75 kDa, thus the segment

containing >75 kDa was incubated with vinculin while the <75 kDa segment was incubated with MCL-1. These were incubated overnight at 4^{o}C. The next day, the membranes were washed three times for 10 mins with TBS-T on the rocker, then incubated with anti-rabbit IgG-HRP linked secondary antibody (1:10,000 Cell Signalling Technology) for 1 hour at RT on the rocker. The membranes were washed again three times for 10 minutes with TBS-T. The enhanced chemiluminescence SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) was placed evenly on the membrane for 2 minutes in the dark and was visualized using the Chemidoc^{TM} MP Imaging System (BioRad). The protein bands were quantified using Image Lab software 6.0 (BioRad). The MCL-1 bands were normalized to that of vinculin loading control to provide semi-quantitative analysis.

Chapter 3: Results

3.1 DM3 shows the greatest adipogenic efficiency

We first optimized the adipocyte differentiating media, using compositions from well-established studies. All 3 DMs consisted of the same base media but contained varying concentrations of IBMX, human insulin, INDO, and DEXA. The control for this experiment were BMSCs that were left undifferentiated, supplemented with BMSC media alone. Differentiation was induced over 21 days, changing media every 2-3 days. On day 21, adipocytes and undifferentiated BMSCs were stained with BODIPY or ORO to analyze morphology or lysed for RNA for cDNA gene expression analysis using qPCR. BODIPY and ORO stain for neutral lipids, which are stored in lipid droplets, the hallmark of adipocytes.

DM3 produced the most adipocytes, represented by the increased ORO and BODIPY staining in cells (Figure 10). DM2 showed the least adipocyte differentiation efficiency, while DM1 showed moderate levels relative to DM3. Additionally, during the differentiation period, DM1 produced a more viscous media, which can increase the risk of adipocytes detaching from the wells. Detachment was seen in both DM1 and DM2, with more seen in DM2. The undifferentiated BMSCs showed little to no ORO or BODIPY staining of LDs, confirming the specificity of DM for adipocyte induction (Figure 10a). The morphological analysis demonstrated that DM3 is superior to DM1 and DM2 for adipocyte induction (Figure 10a).

RNA isolated from adipocytes lysates were reverse transcribed into cDNA then used for gene expression analysis using qPCR. 4 adipocyte-specific genes were assessed:

CCAAT/enhancer-binding protein alpha (CEBPα), peroxisome proliferator-activated receptor gamma (PPARγ), fatty acid translocase (CD36), and fatty acid binding protein 4 (FABP4). Ribosomal protein LO (RPLO) was used as a housekeeping gene. CEBPα and PPARγ are early transcription factors involved in adipogenesis while CD36 and FABP4 are fatty acid transporters found in mature adipocytes. There were insufficient DM2 RNA concentrations to perform qPCR reactions, therefore, only gene expression data for DM1 and DM3 are shown.

DM1 and DM3 showed high expression levels of all 4 adipocyte-specific genes relative to BMSCs (Figure 10a-b). The differences between the two media compositions were not significantly different from each other. As expected, undifferentiated BMSCs did not show expression of the adipocyte-specific genes. In addition, RPLO was consistently expressed between all DMs and the undifferentiated BMSCs, and its expression was used for normalization. This data rationalizes the use of the 4 adipocyte-specific genes and again confirms the specificity of adipocyte induction by the DMs.

Taken together, morphological analysis and gene expression data led to the use of DM3 for subsequent experiments because of its increased efficiency for adipogenic differentiation.

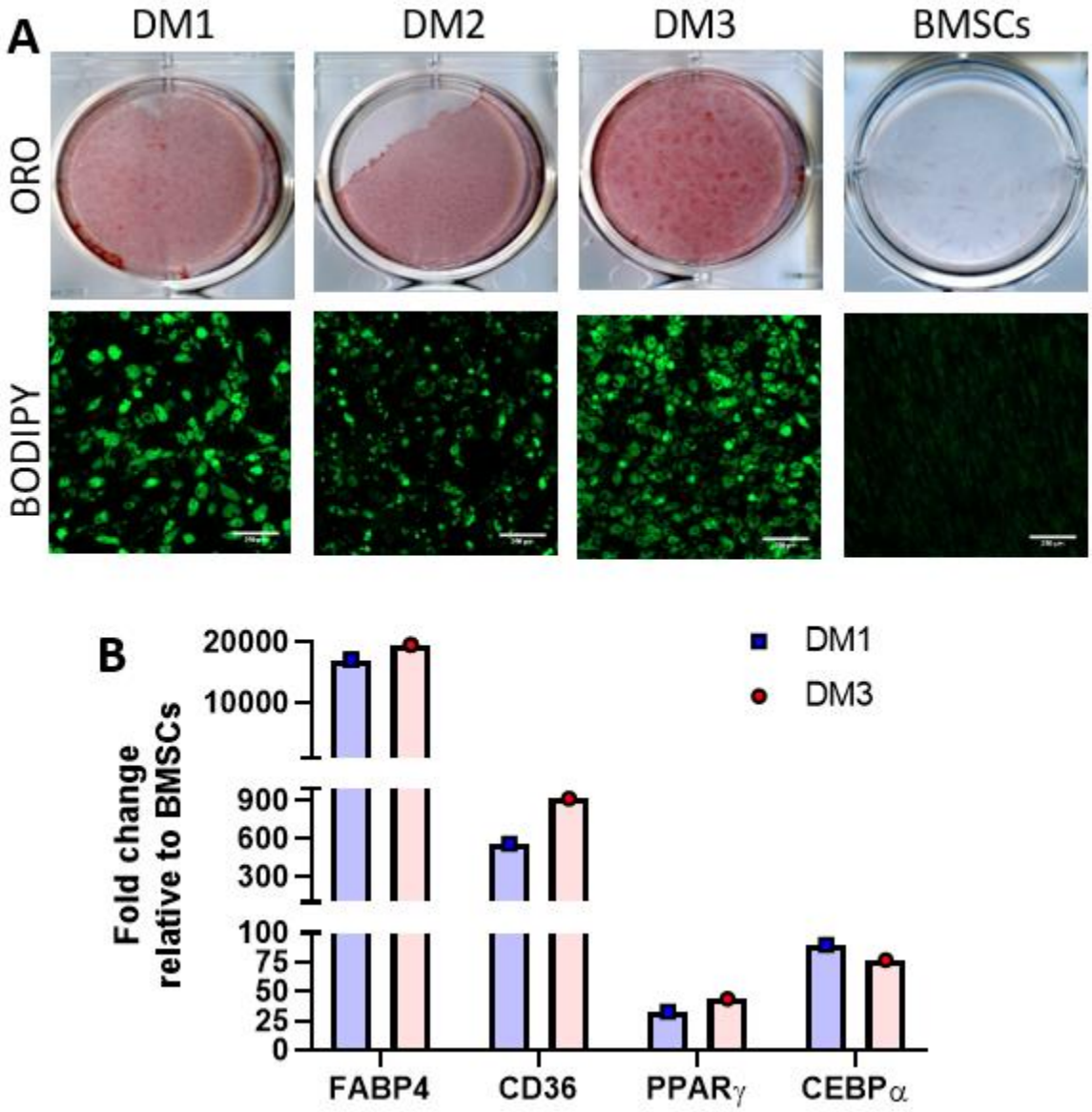

Figure 10. DM3 shows the greatest adipogenic differentiation efficiency.
BMSCs were plated in 6-well plates and were differentiated into adipocytes over 21 days. BMSCs maintained in the media without differentiation inducers acted as the control. Media was changed every 2-3 days. (A) ORO and BODIPY 493/520 stained images of lipid droplets in adipocytes differentiated from 3 varying compositions of differentiating media compared to BMSCs (n=1). (B) Adipocyte-specific gene expression analysis of cDNA from adipocytes differentiated from 2 varying compositions of differentiating media (n=1). PPARγ and CEBPα are early adipogenic transcription factors. FABP4 and CD36 are fatty acid transporters. Gene expression data is represented as fold changes relative to BMSCs. Scale bar represents 250 μm.

3.2 Adipocytes reduce MOLM-13 and HL-60 proliferation and viability, yet enhance MOLM-13 maximal respiration

Once the adipocyte differentiating media was optimized, we next sought to determine the effects of co-culturing AML cells from 2 cell lines, MOLM-13 and HL-60, on BMSCs and adipocytes in terms of proliferation, viability, and metabolism. Upon co-culturing AML cells on BMSCs, only HL-60 cells showed a reduction in proliferation (Figure 11a). However, both cell lines showed >50% and ~15% reduction in proliferation and viability, respectively, when co-cultured on adipocytes (Figure 11a-b). We wanted to determine whether this inhibitory role of adipocytes on AML cell proliferation and viability is similarly reflected in its metabolic activity. Therefore, we employed the Seahorse extracellular flux analyzer (XeF96) to determine the levels of mitochondrial respiration at a cellular level. Interestingly, basal respiration was unaffected by the co-cultures in either cell lines (Figure 11c). The XeF96 metabolic analyzer also allows the measure of maximal respiration, mimicked by the injection of FCCP, which disrupts the proton gradient in the inner mitochondrial membrane. This injection tests a cell's ability to respond metabolically to stress conditions, such as chemotherapy. Surprisingly, MOLM-13 cells showed a substantial 1.5-fold enhancement of maximal respiration when co-cultured on adipocytes (Figure 11d).

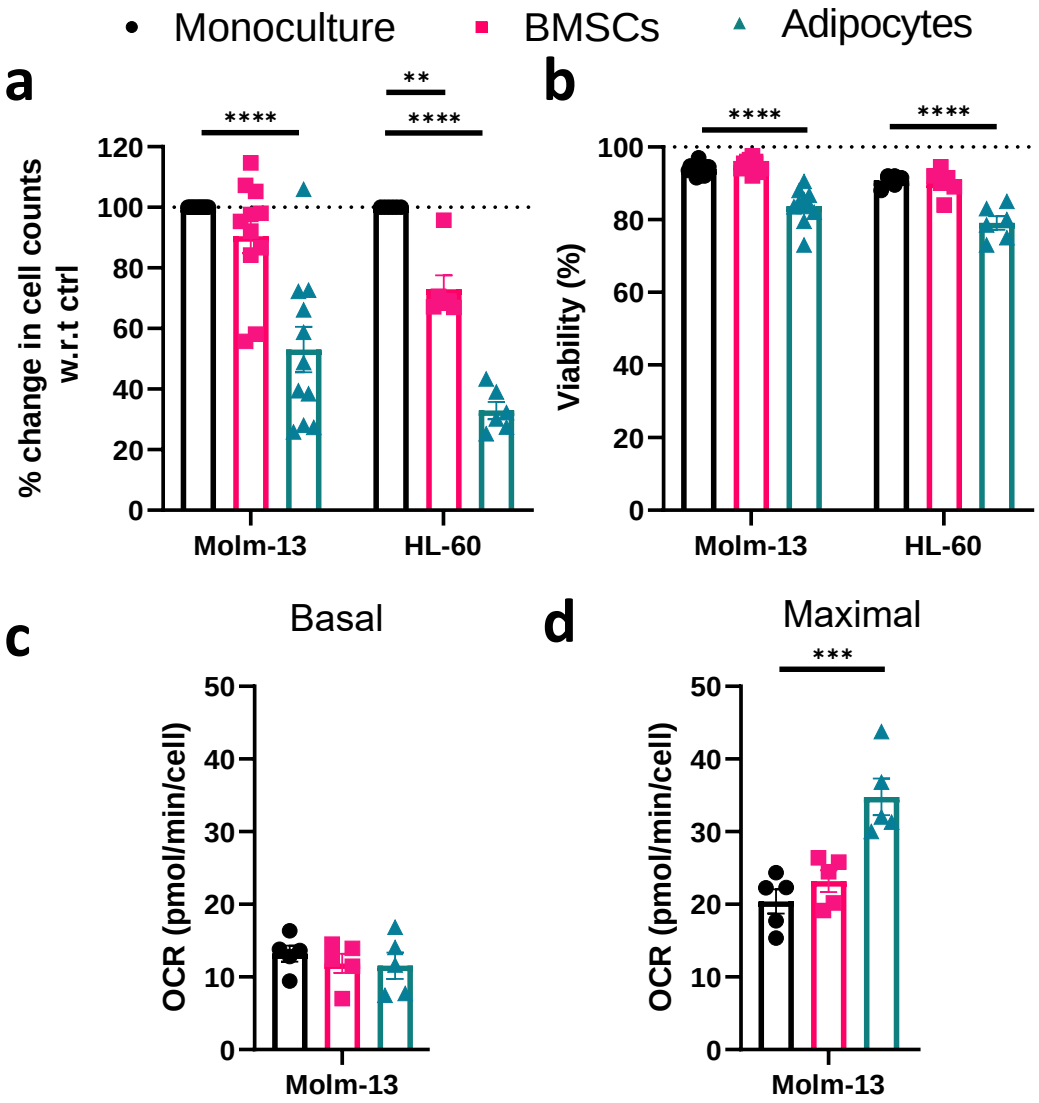

Figure 11. MOLM-13 and HL-60 cells co-cultured on adipocytes show a reduction in proliferation and viability, while only MOLM-13 show enhanced maximal respiration. MOLM-13 and HL-60 AML cells were monocultured or co-cultured on BMSCs and adipocytes for 96 hours, passaging in fresh media at 48 hours. (a) Percentage change in cumulative cell counts of MOLM-13 (n=11) or HL-60 (n=6) cells normalized to DMSO control. (b) Viability of MOLM-13 (n=11) or HL-60 (n=6) cells after 96 hours of mono/co-culture. (c) Basal respiration of MOLM-13 (n=5) or HL-60 (n=1) cells after 96 hours of mono/co-culture. (d) Maximal respiration of MOLM-13 (n=5) cells after 96 hours of mono/co-culture. Data is presented as average±SEM. Significance was measured using 2-way ANOVA tests. BMSCs=bone marrow-derived mesenchymal stromal cells, OCR=oxygen consumption rate.

3.3 Adipocytes reduce H9M and MN1 proliferation, reduce H9M viability, yet enhance MN1 maximal respiration

After discovering the inhibitory role of adipocytes on AML cell proliferation and viability yet enhancement of its maximal respiration, we performed pilot experiments to determine if similar trends are seen in murine models of AML to facilitate future *in vivo* experiments. After successful generation of the H9M and MN1 murine AML cells, we co-cultured these with freshly plated BMSCs and adipocytes. These murine AML cells need to be supplemented with cytokines to promote proliferation and survival. These cytokines include human interleukin (IL) 6, mouse IL-3, and mouse stem cell factor (SCF). Shafat et al. (2017) demonstrated that BMSCs and adipocytes can provide a proliferative advantage for co-cultured AML cells in the absence of cytokines, thereby overcoming the necessity of cytokines for their growth. Therefore, we wanted to reproduce this experiment and setup a co-culture system with and without cytokines between H9M or MN1 cells with either BMSCs or adipocytes.

Unfortunately, we were unable to reproduce their findings as neither BMSCs nor adipocytes were able to support the growth of either H9M or MN1 murine AML cells in the absence of cytokines (data not shown). When culturing the cells in the presence of cytokines, we see a similar trend in both H9M and MN1 conditions as in the human AML cell lines, where BMSCs show slightly reduced proliferation compared to monocultures, but this reduction was much more pronounced in the adipocyte co-culture, by ~70% (Figure 12a). Viability of H9M cells was reduced by ~10% when co-cultured either on BMSCs or adipocytes, while MN1 showed little to no change (Figure 12b). Similar to cell

lines, basal respiration was unaffected by the co-cultures (Figure 12c), however, maximal respiration was enhanced in MN1 cells but slightly reduced in H9M cells (Figure 12d). Although we see similar trends in proliferation, viability, and maximal respiration, more replicates of murine AML cell co-cultures must be performed to gather statistical significance.

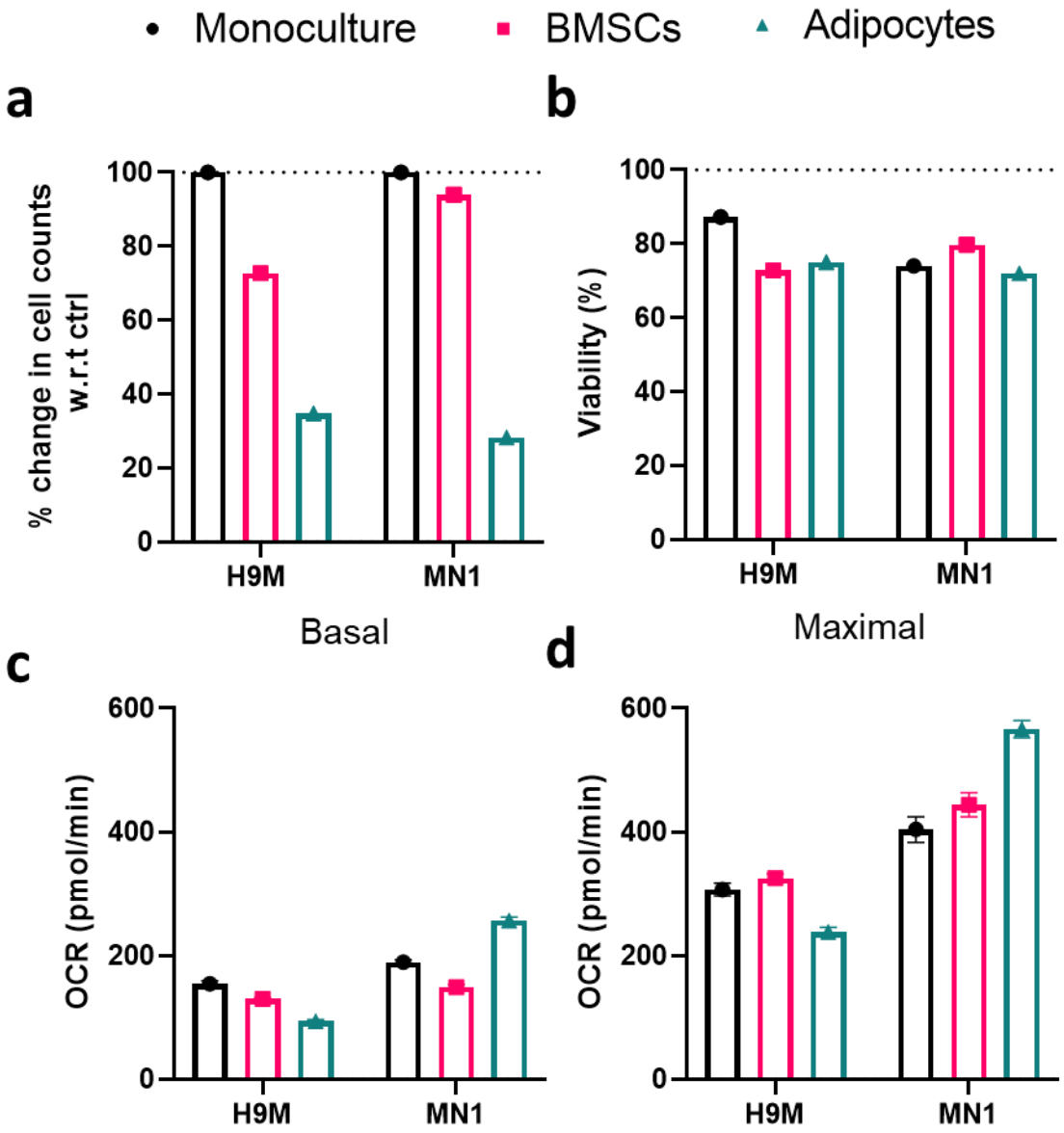

Figure 12. Preliminary data suggests that H9M and MN1 cells co-cultured on adipocytes show reduced proliferation and viability, while only MN1 cells show enhanced maximal respiration. H9M and MN1 AML cells supplemented with IL-6, IL-3, and SCF were monocultured or co-cultured on BMSCs or adipocytes for 96 hours, passaging in fresh media at 48 hours. (a) Percentage change in cumulative cell counts of H9M or MN1 cells normalized to DMSO control. (b) Viability of H9M or MN1 cells after 96 hours of mono/co-culture. (c) Basal respiration of H9M or MN1 cells after 96 hours of mono/co-culture. (d) Maximal respiration of H9M or MN1 cells after 96 hours of mono/co-culture. Data is presented as n=1. Where present, error bars are SEM from 14-16 technical replicates. IL=interleukin, SCF=stem cell factor, BMSCs=bone marrow-derived mesenchymal stromal cells, OCR=oxygen consumption rate.

3.4 Adipocytes reduce primary AML cell proliferation from samples 21029B, 21050B, and 21007A, yet enhance viability of primary AML cells from 21029B

In both AML cell lines and murine AML cells, we show that adipocytes reduce proliferation and viability of co-cultured AML cells, while enhancing their maximal respiration. In order to provide a clinical context, we sought to reproduce our findings in primary AML cells from patient samples. We attempted to perform co-cultures using patient samples 20142B, 21029B, 21050B, and 21007A. However, AML cells from sample 21042B were unable to survive in media thus did not have enough cells to perform a co-culture experiment. Similarly, cells from 21050B also did not survive in media and thus did not have enough cells to have 3 replicates, thus data for this sample is from an n=1. The clinical information of each patient sample can be found in table 1. Similar to murine AML cells, these primary AML cells required 3 cytokines for optimal growth, which included human SCF, FLT3, and IL-3. We created conditions with and without cytokines to determine if the co-culture conditions can overcome the necessity of cytokines for growth compared to the monoculture condition. As with the murine cells,

the co-cultures were unable to overcome the necessity of cytokines for growth as neither the monoculture nor co-culture conditions were able to maintain cell counts in the absence of cytokines (data not shown).

In the presence of cytokines, all 3 patient samples showed a reduction in proliferative ability when co-cultured on adipocytes, however, this was only significant was 21007A. 21007A AML cells grown on BMSCs also showed a significant reduction in proliferation, with a similar trend seen in 21029B (Figure 13a). Contrasting the findings from the previous AML models, patient sample 21029B showed a significant increase in viability when co-cultured on either BMSCs or adipocytes (Figure 13b). 21050B showed an extremely low viability of only 20%, which was greatly enhanced when grown on BMSCs, and to a lesser extent when grown on adipocytes (Figure 13b). Given that 21050B did not grow as well as expected, we did not have cells to perform the XeF96 metabolic assays. Basal respiration rates were largely unaffected by the co-culture in patient samples 21029B and 21007A (Figure 13c). Maximal respiration showed no significant differences in either samples, however, 21007A shows a trend of enhanced maximal respiration when co-cultured on adipocytes (Figure 13d).

Overall, across three different models of AML, including cell lines (Figure 11), murine cells (Figure 12), and primary cells from patient samples (Figure 13), we saw a consistent reduction in AML cell proliferation when co-cultured on adipocytes, which were more pronounced in the murine cells and primary cells. In the cell lines and H9M murine cells, we saw similar reductions in AML cell viability on adipocytes. Lastly,

MOLM-13 cells and MN1 murine cells showed an enhancement of maximal respiration when grown on adipocytes.

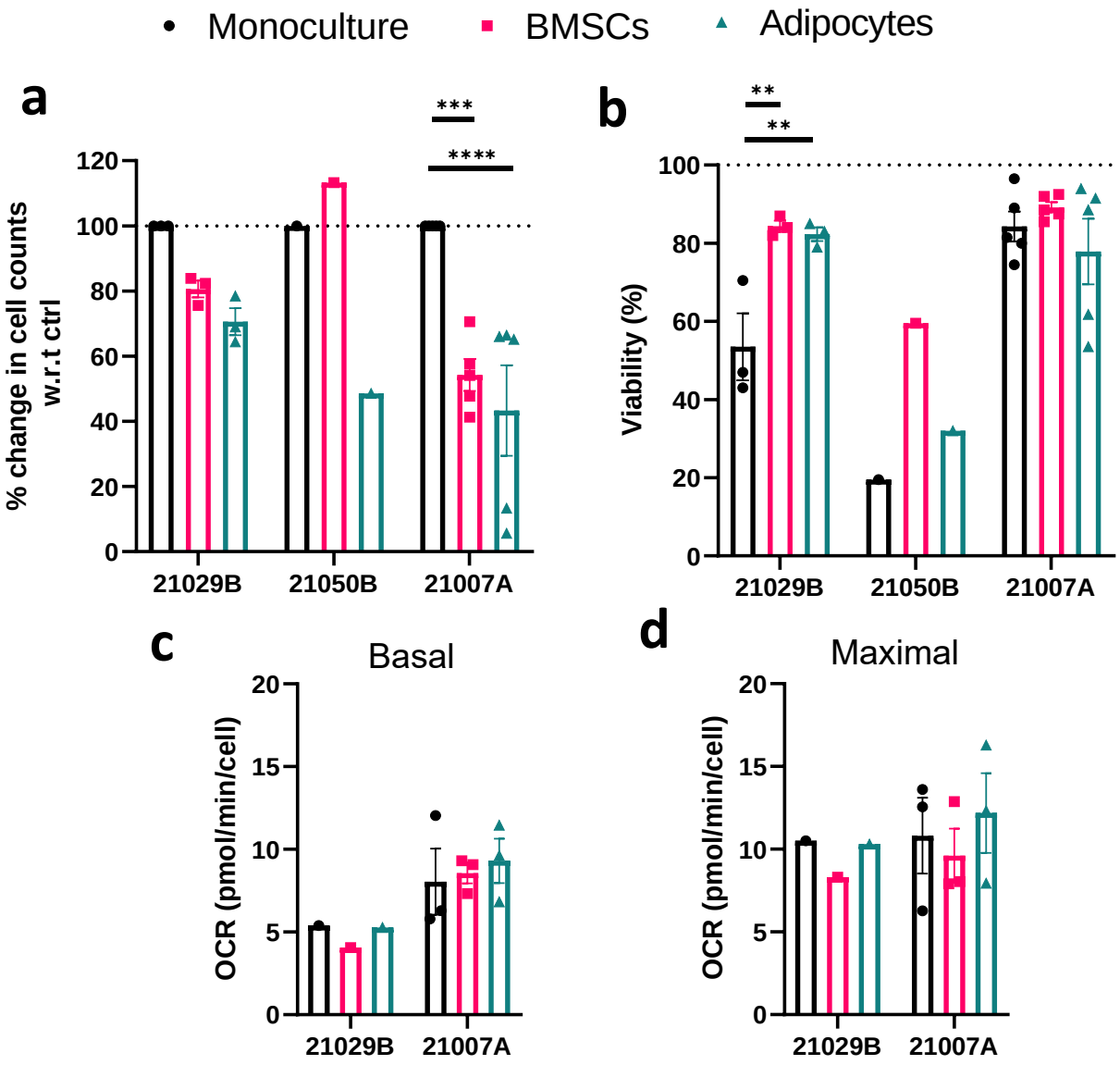

Figure 13. AML cells from patient samples 21029B, 21050B, and 21007A co-cultured on adipocytes show a reduction in proliferation, while 21029B cells show enhanced viability. AML cells from patient samples 21029B, 21050B, and 21007A supplemented with IL-3, FLT3, and SCF were monocultured or co-cultured on BMSCs or adipocytes for 96 hours, passaging in fresh media at 48 hours. (a) Percentage change in cumulative

cell counts of 20129B (n=3), 21050B (n=1), or 21007A (n=5) cells normalized to DMSO control after 96 hours of mono/co-culture. (b) Viability of 20129B (n=3), 21050B (n=1), or 21007A (n=5) cells after 96 hours of mono/co-culture. (c) Basal respiration of 20129B (n=1) or 21007A (n=3) cells after 96 hours of mono/co-culture. (d) Maximal respiration of 20129B (n=1) or 21007A (n=3) cells after 96 hours of mono/co-culture. Data is presented as average±SEM, unless n=1. Significance was measured using 2-way ANOVA tests. IL=interleukin, FLT3=fms-like tyrosine kinase 3, SCF=stem cell factor, BMSCs=bone marrow-derived mesenchymal stromal cells, OCR=oxygen consumption rate.

3.5 BMSCs and adipocytes increase the IC_{50} of Venetoclax in MOLM-13 AML cells

Thus far, we have shown that adipocytes reduce AML cell proliferation and viability, yet enhance maximal respiration. We next sought to determine whether the co-cultures could aid AML cells to be resistant to Ven/Aza treatment. We optimized the experiment first using AML cell lines. Previous work by a post-doctoral fellow in our lab, Kanwaldeep Singh, showed that the MOLM-13 AML cell line was sensitive to Venetoclax treatments at concentrations greater than 100 nM. Therefore, we optimized this experiment first using MOLM-13 cells exposed to only Venetoclax treatment, with the future plan to introduce Azacitidine as a co-treatment. We had 4 conditions: control DMSO (vehicle control) and 100, 500, and 1000 nM of Venetoclax treatment.

As seen in Figure 14, monocultured AML cells showed a dose-dependent reduction in proliferative ability, with an IC_{50} of Venetoclax of <100 nM (Figure 14a). Interestingly, BMSCs also show a dose-dependent reduction in proliferation, however, they increased the IC_{50} to between 100-500 nM. Adipocyte co-cultured AML cells showed the greatest protection from Ven as proliferation was maintained in the presence of 500 nM of Ven. Adipocytes increased the IC_{50} to >1000 nM (Figure 14b). Similarly, BMSCs and adipocytes maintained viability around 90 and 80%, respectively, while

monoculture showed a dose-dependent decrease, with the greatest reduction of 20% occurring at 100 nM of Ven (Figure 14b). There was a trend towards decreased basal and maximal respiration upon treatment with Venetoclax (Figure 14c). This trend was not reverted in the co-cultures and the increased maximum respiration previously seen in adipocyte co-cultures was blunted in the presence of Venetoclax. However, there seemed to be a slight trend towards better maintenance of maximal respiration in the co-cultures (Figure 14d).

Overall, this data suggests that BMSCs and adipocytes can protect and even promote the proliferation, viability, and/or maximal respiration of AML cells when treated with Venetoclax.

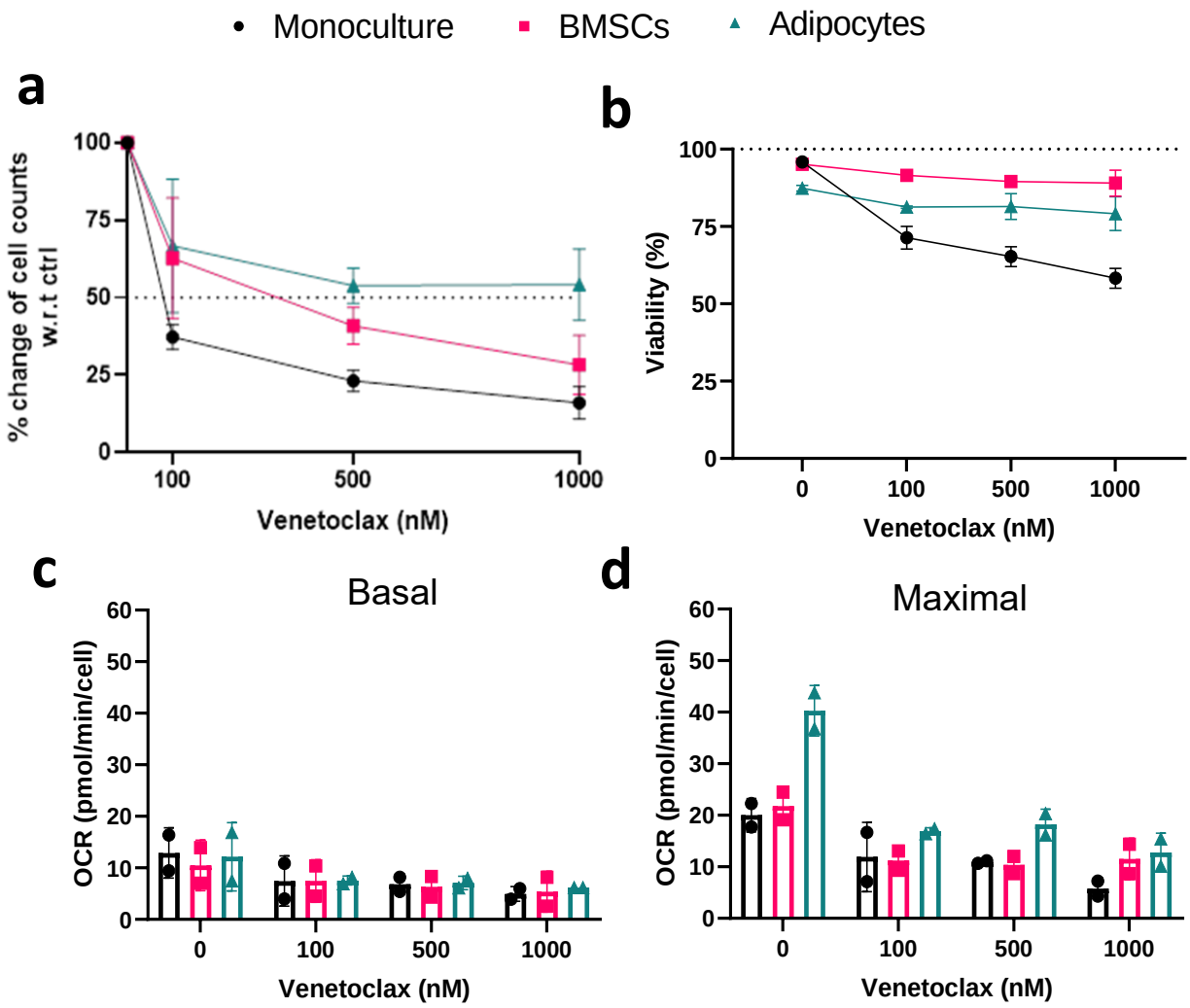

Figure 14. BMSCs and adipocytes increase the IC50 of Venetoclax in MOLM-13 cells. MOLM-13 cells were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0, 100, 500, or 1000 nM of Ven for 96 hours in standard media. The cells were passaged in fresh media with or without treatment at 48 hours. (a) Percentage change in cumulative cell counts of MOLM-13 (n=2) cells normalized to DMSO control after 96 hours of mono/co-culture with or without treatment. The dashed line at 50% represents the IC50. (b) Viability of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. (c) Basal respiration of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. (d) Maximal respiration of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. Data is presented as average±SEM (n=2). BMSCs=bone marrow-derived mesenchymal stromal cells, Ven=Venetoclax, OCR=oxygen consumption rate.

3.6 BMSCs and adipocytes increase the IC_{50} of Venetoclax in MOLM-13 AML cells when grown in substrate limited media

The above chemoresistance experiment was replicated using substrate limited media (SLM) to determine if there are any changes to proliferation, viability, and metabolism of AML cells when exposed to limited nutrients. The only difference between standard media and SLM was that SLM contained human physiological levels of glucose which was 4.5 mmol/litre, while the standard media was 17.5 mmol/litre. All other parameters were kept the exact same.

There were many similarities between SLM and standard media in terms of proliferation. This includes the dose-dependent reduction in proliferation seen in both the mono and co-cultures (Figure 15a). However, the BMSC co-culture did show a greater IC_{50} of between 500 and 1000 nM of Ven in SLM. Adipocytes showed the same enhancement of IC_{50} >1000 nM (Figure 15a). Similar to standard media, BMSCs showed maintenance of viability at all concentrations of Venetoclax however it maintained it at ~76% while standard media showed maintenance at ~90% (Figure 15b). Surprisingly, the adipocyte co-cultures showed reduced viability compared to monocultures when in SLM (Figure 15b), which was opposite of the results in standard media where adipocytes maintained AML cell viability at ~80% across the Venetoclax conditions (Figure 14b). In terms of metabolism, there were almost identical trends observed between standard media and SLM. There were no differences in basal respiration between the mono- and co-culture conditions when treated with Venetoclax (Figure 15c). In the adipocyte co-cultures, maximal respiration showed a similar 2-fold increase in the DMSO control and a

slight increase in the 500 nM and 1 μM Venetoclax treated conditions was observed (Figure 15d). One difference is that in SLM, we also saw a slight increase in maximal respiration when co-cultured with BMSCs for the DMSO control, which was not seen in standard media (Figure 15d). However, the SLM co-culture experiment was only performed once, therefore more biological replicates will be required to provide statistical significance for the above findings.

In summary, BMSCs and adipocytes increase the IC_{50} of Venetoclax in both standard and SLM. In addition, 100 nM Venetoclax was the lowest concentration that showed the protective effects of the co-culture conditions compared to the mono-culture condition. Therefore, going forward, standard media was used for subsequent treatment conditions, and 100 nM was the optimized concentration of Ven to use.

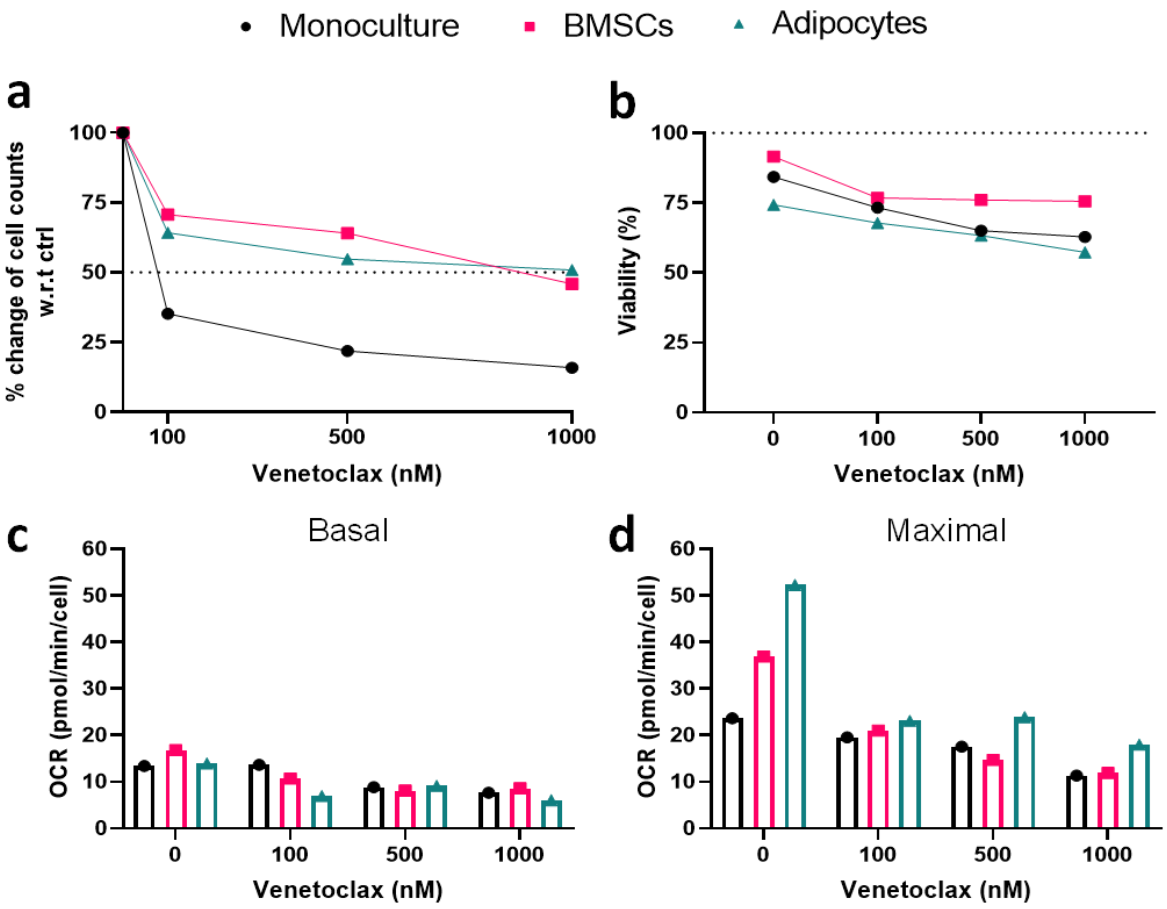

Figure 15. BMSCs and adipocytes increase the IC_{50} of Venetoclax in MOLM-13 cells in substrate limited media. MOLM-13 cells were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0, 100, 500, or 1000 nM of Ven for 96 hours in substrate limited media. The cells were passaged in fresh media with or without treatment at 48 hours. (a) Percentage change in cumulative cell counts of MOLM-13 cells normalized to DMSO control after 96 hours of mono/co-culture with or without treatment. The dashed line at 50% represents the IC50 (b) Viability of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. (c) Basal respiration of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. (d) Maximal respiration of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. Data is presented as n=1. BMSCs=bone marrow-derived mesenchymal stromal cells, Ven=Venetoclax, OCR=oxygen consumption rate.

3.7 BMSCs and/or adipocytes protect MOLM-13 AML cell proliferation, viability, and metabolism from Ven/Aza treatment

After determining the optimal concentration of Venetoclax, we next wanted to determine whether BMSCs and adipocytes can protect AML cells from the co-treatment of Ven/Aza. As previously mentioned, 100 nM of Venetoclax was used. We initially had only tested one concentration of Azacitidine, which was 1 μM as per previous work completed by post-doctoral fellow Kanwaldeep Singh. However, we added in 500 nM of Azacitidine to get closer to the clinical concentration. The controls set up for this experiment included DMSO control, as both Venetoclax and Azacitidine were dissolved in this solution, Venetoclax only, and Azacitidine only conditions. We first performed this experiment in MOLM-13 cells.

In the Venetoclax only condition, adipocytes showed a significant protective effect of proliferation, where the percent response of cell counts relative to DMSO condition was at 70% while the monoculture condition dropped to 30% of the DMSO control (Figure 16a). BMSCs also showed a protective effect, maintaining levels at around 55%. Adipocytes show a similar protective effect in the Azacitidine only condition, however, it was not significant, while BMSCs showed no protective effect as it maintained levels similar to monocultured cells of 20%. In the 1 μM azacitidine co-treatment condition, the percent response of monocultured cell counts relative to the DMSO control was substantially reduced, showing a reduction to 5%. This shows that the co-treatment is more effective compared to the mono-treatments as it reduced proliferative levels lower than that seen for Venetoclax or Azacitidine alone. BMSC

cocultured AML cells also showed a reduction to 5% of DMSO control. However, adipocytes were able to protect AML cells from this reduction as it showed a percent response of 20%, which is a 5-fold protective effect compared to the monocultured cells, however, this difference was not significant (Figure 16a). With the combination of 100 nM Ven with the lower concentration of Aza of 500 nM, we saw the same toxic effect of the co-treatment on mono-cultured and BMSC co-cultured AML cells. Yet, the adipocyte co-cultured cells now showed a 25%, significant protection from Ven/Aza.

As previously mentioned, adipocytes reduce AML cell viability in the DMSO condition to approximately 85% while both monocultured and BMSC-cultured AML cells maintained viability at 95% (Figure 16b). When exposed to Venetoclax, monocultured cells dropped to 70% viability. Both BMSCs and adipocytes protected AML cells from the effects of Venetoclax as it maintained viability at 90% and 80%, respectively. In the Azacitidine only condition, BMSCs protected viability by 10% compared to monoculture's 75% viability, while adipocytes showed no protective effects. When Venetoclax is combined with the higher concentration of Azacitidine, the treatment shows high toxicity against monocultured cells as their viability is around only 10%. Fascinatingly, both BMSCs and adipocytes are able to substantially protect AML cells from this highly toxic co-treatment as they maintain viability around 60-65% (Figure 16b). Despite a lower concentration of Aza, BMSCs and adipocytes maintained viability at 70-75% (Figure 16b)

Similar to the previous paragraph, there was a trend towards lower basal respiration upon treatment with no differences seen between monoculture and the co-

culture conditions. (Figure 16c). Given the low viability of cells upon co-treatment in monocultured cells, there were not enough cells to run metabolism assays using the Seahorse machine, which requires at minimum 300,000 live cells. Therefore, we do not have metabolism data for monocultured cells exposed to the co-treatment. Both BMSCs and adipocytes protected the AML cells from the co-treatment, enough to run the metabolism assay. Still, as there is no monoculture condition, comparisons cannot be made. As shown previously, adipocyte cocultured AML cells show enhanced maximal respiration in the DMSO control group. In the Venetoclax only condition, maximal respiration was almost half of the DMSO levels across the mono- and co-culture conditions, with a slight, but not significant increase in the adipocyte cocultured condition. Azacitidine alone did not affect maximal respiration in monocultured AML cells compared to DMSO control, however, both BMSCs and adipocytes show a slight, but non-significant increase (Figure 16d). As previously mentioned, both BMSCs and adipocytes protect AML cell maximal respiration from the toxicity of the co-treatment, however, it is still overall reduced compared to the DMSO control.

Overall, adipocytes protect AML cell proliferation, viability, and metabolism from the toxic effects of the co-treatment, while BMSCs protect viability and metabolism. This protection is seen in co-treatments of both the higher and lower concentrations of Aza, however, the effects are more pronounced at the lower concentration. Therefore, subsequent treatment co-cultures were prepared using just 100nM of Ven + 500 nM of Aza.

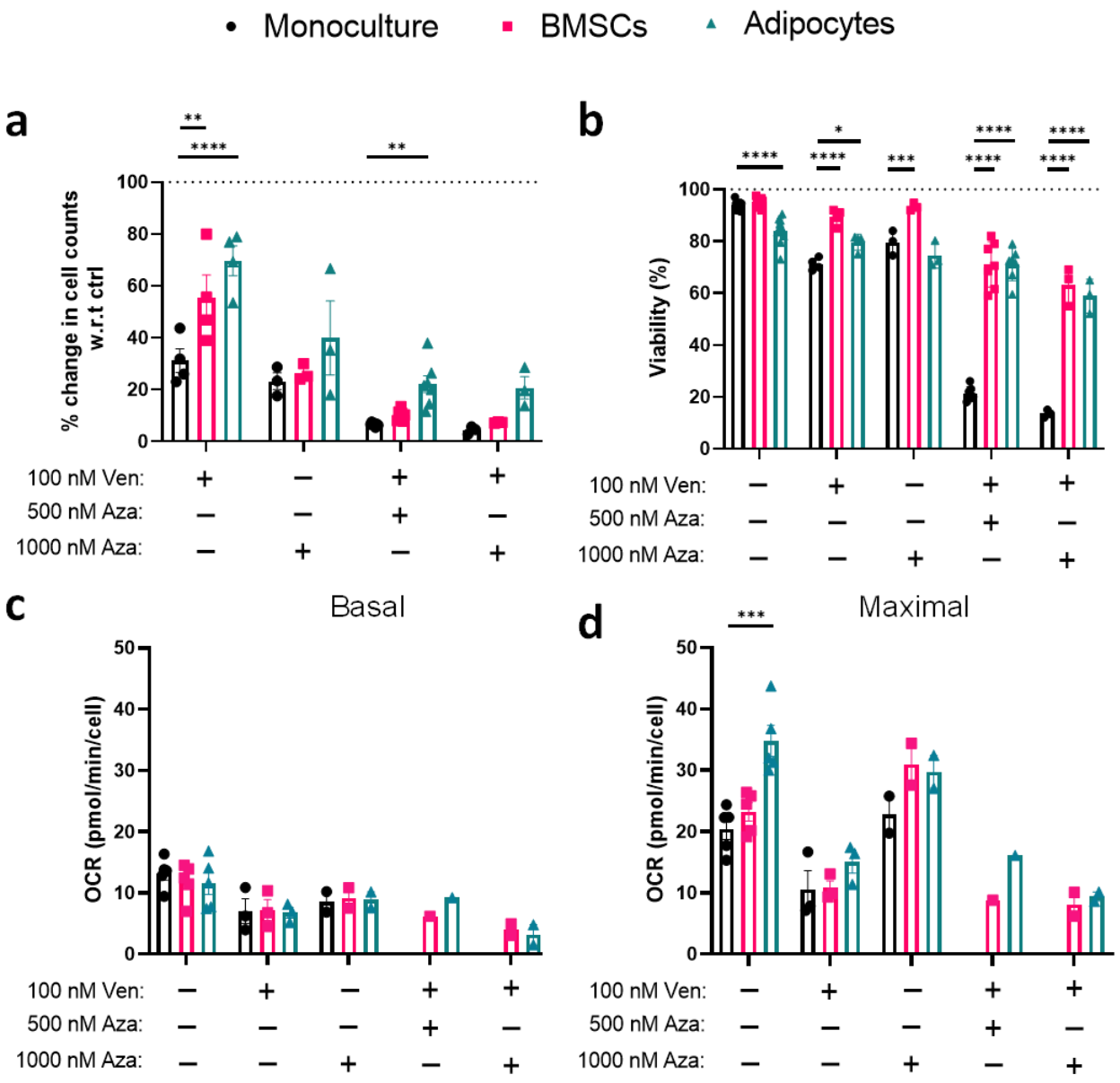

Figure 16. Adipocytes show protection of MOLM-13 proliferation, viability, and metabolism from Ven/Aza, while BMSCs protect viability and metabolism. MOLM-13 cells were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0, 100 nM Ven, 1000 nM Aza, 100 nM Ven+500 nM Aza or 100 nM Ven+1000 nM Aza for 96 hours in standard media. The cells were passaged in fresh media with or without treatment at 48 hours. (a) Percentage change in cumulative cell counts of MOLM-13 cells (DMSO control n=11, Ven only n=4, Aza only n=3, Ven+500 nM Aza

n=6, Ven+1000 nM Aza n=3) normalized to DMSO control after 96 hours of mono/co-culture with or without treatment. (b) Viability of MOLM-13 cells (DMSO control n=11, Ven only n=4, Aza only n=3, Ven+500 nM Aza n=6, Ven+1000 nM Aza n=3) after 96 hours of mono/co-culture with or without treatment. (c) Basal respiration of MOLM-13 cells (DMSO control n=5, Ven only n=3, Aza only n=2, Ven+500 nM Aza n=1, Ven+1000 nM Aza n=2) after 96 hours of mono/co-culture with or without treatment. (d) Maximal respiration of MOLM-13 cells (DMSO control n=5, Ven only n=3, Aza only n=2, Ven+500 nM Aza n=1, Ven+1000 nM Aza n=2) after 96 hours of mono/co-culture with or without treatment. Data is presented as average±SEM, unless n=1. Significance was measured using 2-way ANOVA tests. BMSCs=bone marrow-derived mesenchymal stromal cells, Ven=Venetoclax, Ven=Venetoclax, Aza=Azacitidine, OCR=oxygen consumption rate.

3.8 Adipocytes protect HL-60 AML cell proliferation from Ven/Aza treatment, while BMSCs protect viability

We replicated the co-treatment condition in HL-60 cells as in the DMSO conditions, we saw similar trends of reduced MOLM-13 proliferation and viability upon co-culture with adipocytes, yet saw no enhancement of maximal respiration. We wanted to determine if HL-60's inability to respond to stress conditions would translate to a less protective effect seen with co-cultures. As previously mentioned, we performed only the lower Aza concentration co-treatment of 100 nM Ven+500 nM Aza, but all other parameters were similar to the MOLM-13 experiment.

In Figure 17a, HL-60 cells both mono- or BMSC co-cultured treated with Ven/Aza showed a 20% percent response of cell counts relative to DMSO condition, while adipocyte co-cultured cells showed a 35% response, meaning that it provided a 75% protective effect. Compared to DMSO conditions where adipocytes reduced AML cell viability, there was no difference between the mono and adipocyte co-culture, but

BMSCs provided an almost 20% protection in viability (Figure 17b). Given that HL-60 were not as sensitive to Ven/Aza as MOLM-13 cells were, we had enough cells to run metabolic assays on the mono- and co-cultured conditions. Similar to MOLM-13, basal and maximal respiration was lower overall for the mono/co-culture conditions treated with Ven/Aza compared to the DMSO control group, with no significant differences between mono- and co-culture conditions (Figure 17c-d).

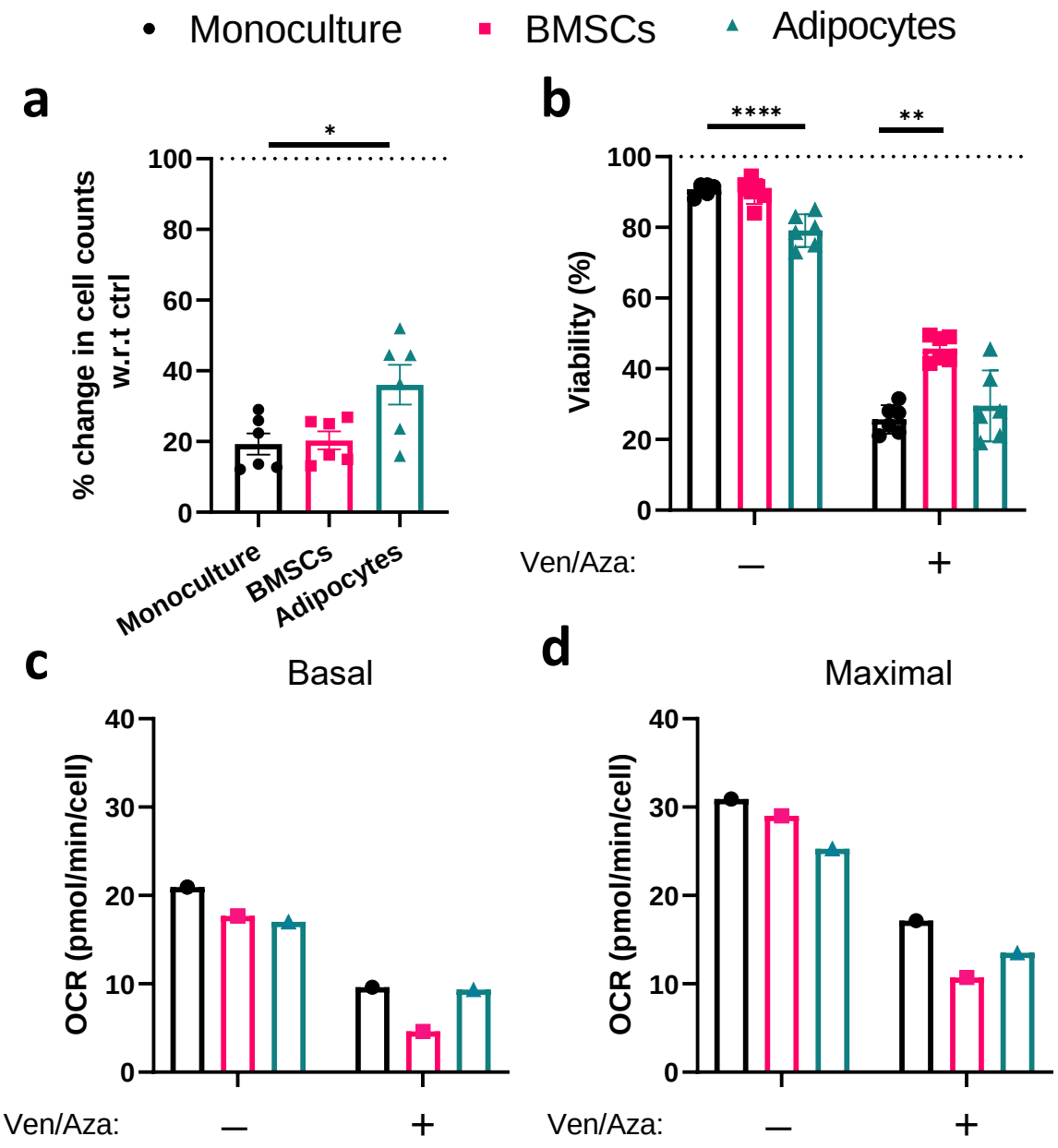

Figure 17. Adipocytes show protection of HL-60 proliferation from Ven/Aza, while BMSCs protect viability. HL-60 were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0 or 100 nM Ven+500 nM Aza for 96 hours in standard media. The cells were passaged in fresh media with or without treatment at 48 hours. (a) Percentage change in cumulative cell counts of HL-60 cells (n=6) normalized to DMSO control after 96 hours of mono/co-culture with or without treatment. (b) Viability of HL-60 cells (n=6) after 96 hours of mono/co-culture with or without treatment. (c) Basal

respiration of HL-60 cells (n=1) after 96 hours of mono/co-culture with or without treatment. (d) Maximal respiration of HL-60 cells (n=1) after 96 hours of mono/co-culture with or without treatment. Data is presented as average±SEM, unless n=1. Significance was measured using 2-way ANOVA tests. BMSCs=bone marrow-derived mesenchymal stromal cells, Ven=Venetoclax, Ven=Venetoclax, Aza=Azacitidine, OCR=oxygen consumption rate.

3.9 Investigation of the effect of co-cultures on the sensitivity of primary AML cells towards Venetoclax/Azacitidine

We next wanted to reproduce these protective effects of adipocytes from the treatment effects of Venetoclax/Azacitidine in primary patient samples. Given the amount of material required for these experiments, these could only be performed in two of the samples collected: 21029B and 21007A. These samples were supplemented with cytokines and exposed to the co-treatment for 96 hours.

Both of these samples only exhibited very modest effects of the co-treatment. While the co-treatment reduced monocultured cell proliferation by more than 90% in MOLM-13 cells (Figure 16a) and 80% in HL60 cells (Figure 17a), the two samples only showed a reduction by 40% in 21029B and 30% in 22007A (Figure 18a). While there was a trend towards a protective effect in the BMSC co-cultures, no protection was seen with adipocytes (Figure 18a). Interpretation was further complicated by a protective effect of both BMSCs as well as adipocytes on the viability of sample 21029B, which was inconsistent with previous observations in other models. These potentially sample-specific differences also made the interpretation of metabolic assays difficult.

Overall, we will need to address these questions again using either optimized concentrations of Venetoclax and/or other more sensitive samples.

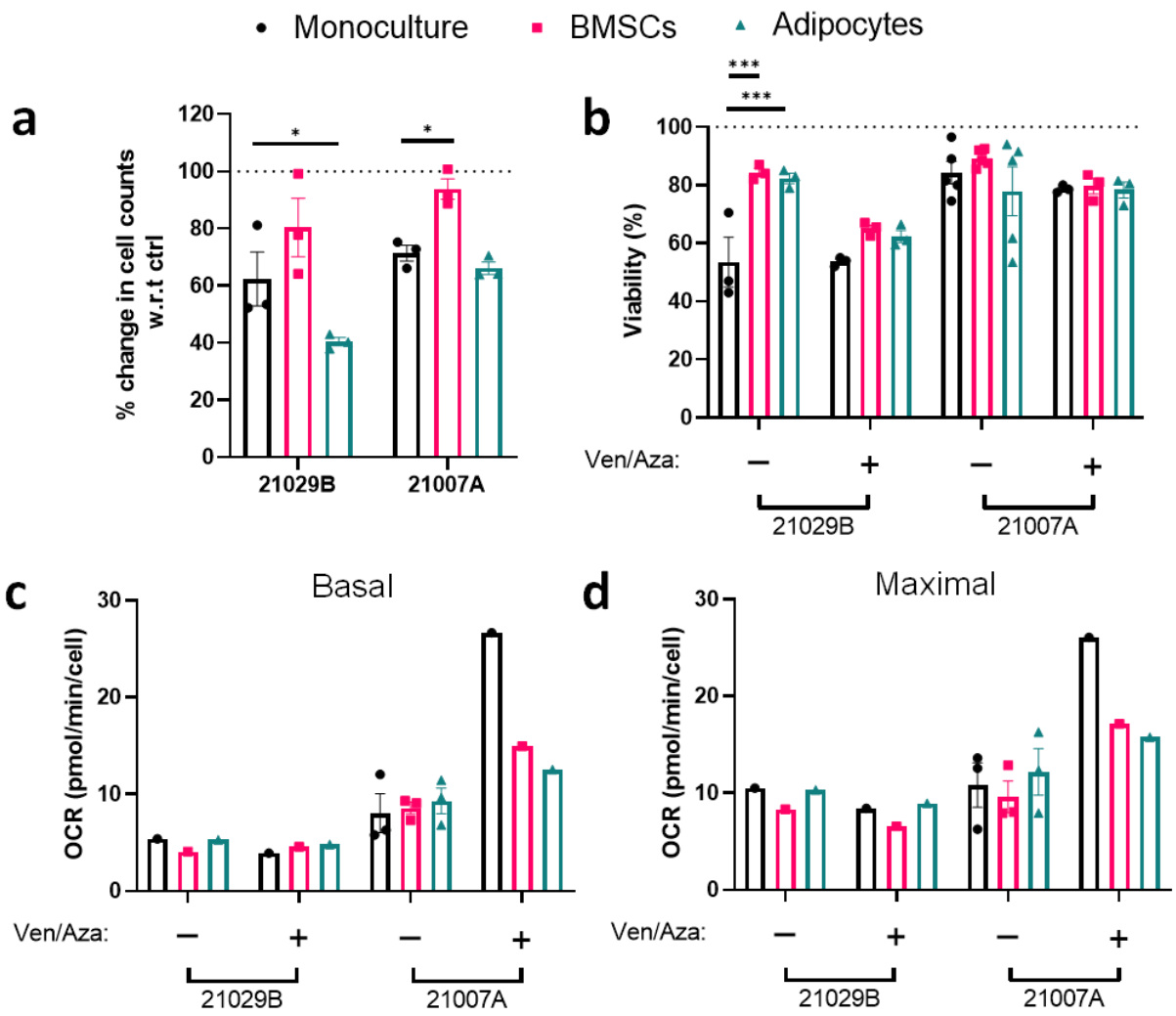

Figure 18. AML cells from patient samples 21029B co-cultured on adipocytes showed a reduction in proliferation while 21007A cells showed enhanced proliferation on BMSCs. AML cells from patient samples 21029B and 21007A supplemented with IL-3, FLT3, and SCF were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0 or 100 nM Ven+500 nM Aza for 96 hours in standard media. The cells were passaged in fresh media with or without treatment at 48 hours. (a) Percentage change in cumulative cell counts of 20129B (n=3) or 21007A (n=3) cells normalized to DMSO control after 96 hours of mono/co-culture. (b) Viability of 20129B (n=3), 21007A untreated (n=5), or 21007A treated with Ven/Aza (n=3) cells after 96 hours of mono/co-culture. (c) Basal respiration of 20129B (n=1), 21007A untreated (n=3), or 21007A treated with Ven/Aza (n=1) cells after 96 hours of mono/co-culture. (d)

Maximal respiration of 20129B (n=1), 21007A untreated (n=3), or 21007A treated with Ven/Aza (n=1) after 96 hours of mono/co-culture. Data is presented as average±SEM, unless n=1. Significance was measured using 2-way ANOVA tests. IL=interleukin, FLT3=fms-like tyrosine kinase 3, SCF=stem cell factor, BMSCs=bone marrow-derived mesenchymal stromal cells, OCR=oxygen consumption rate.

3.10 BMSCs and adipocytes may protect MOLM-13 cells from apoptosis when treated with Ven/Aza

As the findings from the metabolic studies did not reveal mechanistic insight into the protective effect of adipocytes towards a treatment with Ven/Aza, we next wanted to identify if the protective effect is mediated by protection from apoptosis. We therefore performed Annexin-V assays to assess for apoptosis. After the 48 hours of mono/co-culturing, AML cells were isolated and stained for DRAQ7 to assess cell viability and Annexin-V to assess the number of apoptotic cells. While early apoptotic cells only stain for Annexin-V, late apoptotic and necrotic cells additionally stain with DRAQ7. These cells were then analyzed for percent live cells, apoptotic cells, and dead cells using the Cytoflex LX flow cytometry machine

In the DMSO control condition, adipocyte co-cultured AML cells had elevated levels of early and late apoptotic/dead cells of 21%, compared to monocultured cells of 7.5% (Figure 19). In the combination treatment, both BMSCs and adipocytes reduced the percentage of early and late apoptotic/dead cells from 77.5% in the monoculture cells to 59.7% and 61.7%, respectively (Figure 19). This pilot experiment revealed a major reduction in apoptosis both with BMSCs as well as with adipocytes.

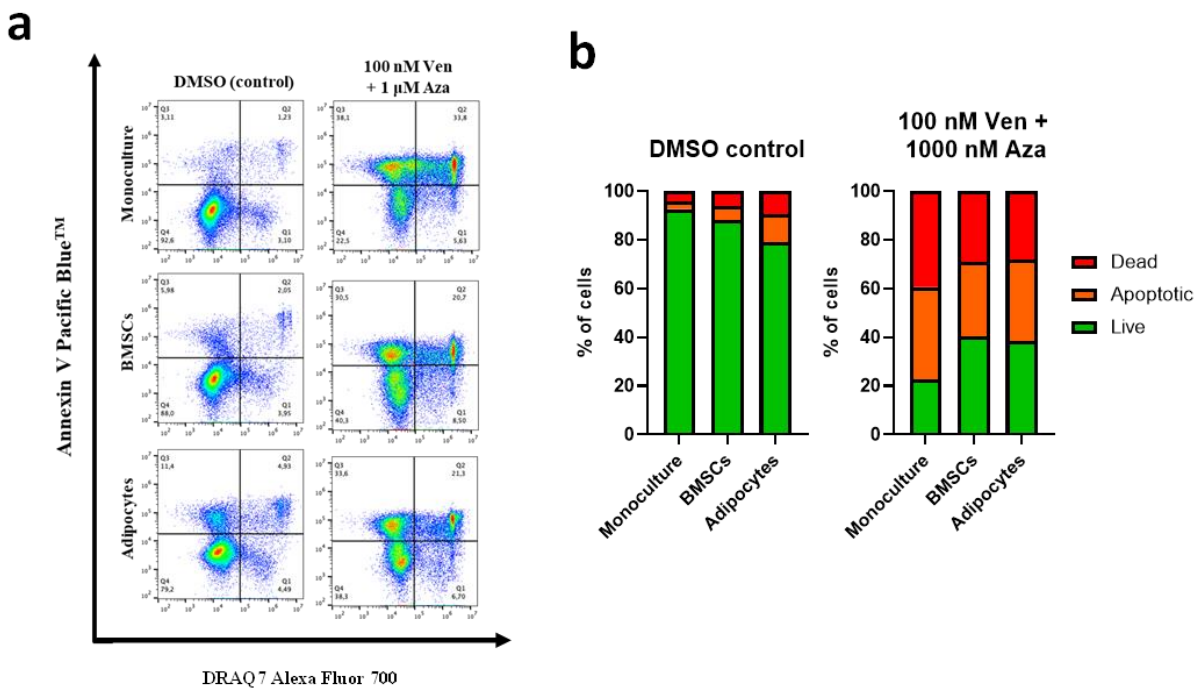

Figure 19. Preliminary data suggests that BMSCs and adipocytes may protect MOLM-13 cells from Ven/Aza-induced apoptosis and necrosis. MOLM-13 cells were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0 or 100 nM Ven+1000 nM Aza for 48 hours in standard media. AML cells were collected and analyzed using the Cytoflex LX flow cytometer. (a) Flow cytometry plots of AML cells stained for DRAQ7 to assess cell viability and Annexin-V to assess the number of apoptotic cells (b) Quantification of flow cytometry gates in Figure 19a to determine the percentage of live cells, apoptotic cells, and dead cells. Data is presented as n=1. Ven=Venetoclax, Aza=Azacitidine.

3.11 Adipocyte co-cultured HL-60 and MOLM-13 AML cells show enhanced MCL-1 expression

MCL-1 is a mitochondrial membrane protein that is in the same family of BCL-2. Studies have shown that Venetoclax resistance may be mediated by MCL-1 overexpression. Therefore, we wanted to determine whether the protection adipocytes confer may be mediated by upregulation of MCL-1. We employed western blot to

determine levels of MCL-1 expression when MOLM-13 cells are mono/co-cultured for 96 hours. OCI-AML3 cells are known to have higher levels of MCL-1 expression (Zhu et al., 2020), therefore were used as the positive control.

As seen in Figures 20a-b, adipocyte co-cultured HL-60 cells show a significant enhancement of MCL-1 protein expression compared to either monocultured or BMSC co-cultured cells, which showed similar levels to each other. MOLM-13 data shows a similar trend of enhanced MCL-1 expression in the adipocyte co-cultured AML cells, albeit to a much lower extent (Figure 20c).

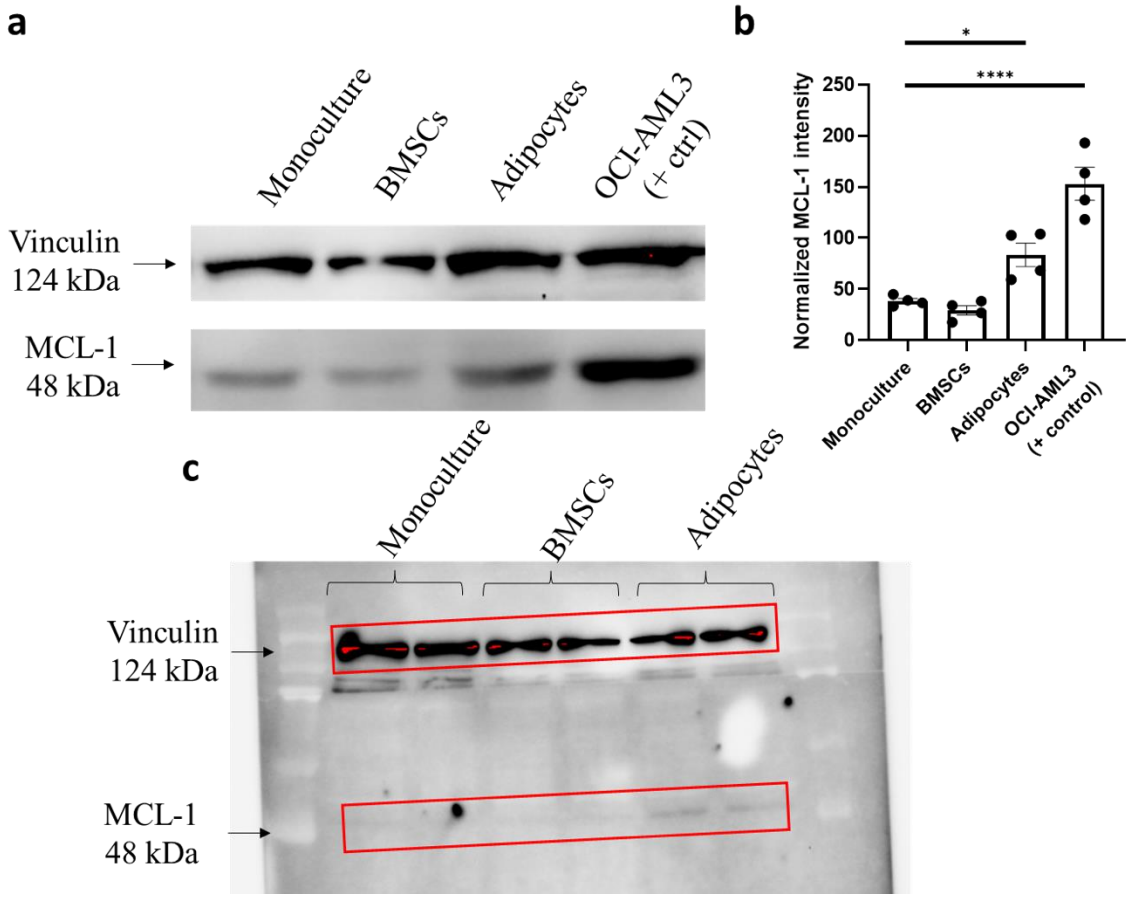

Figure 20. Preliminary data suggests that adipocytes may increase MCL-1 expression in HL-60 and MOLM-13 cells. HL-60 and MOLM-13 AML cells were monocultured or co-cultured on BMSCs and adipocytes for 96 hours. AML cells were isolated and lysed in Tris-HCl+20% glycerol and were analyzed for MCL-1 (48 kDa) protein expression using western blotting. OCI-AML3 cells were used as an MCL-1 positive control and vinculin (124 kDa) was used as the loading control. (a) Representative images of western blots for HL-60 cells mono/co-cultured. (b) Quantification of MCL-1 expression normalized to vinculin from HL-60 cells mono/co-cultured for 96 hours. (c)Western blot of MOLM-13 cells mono/co-cultured for 96 hours. Data is presented as n=1. Ven=Venetoclax, Aza=Azacitidine, BMSCs=bone marrow-derived mesenchymal stromal cells.

3.12 Tapotoclax diminishes the protective effects of BMSC and adipocyte-induced Ven resistance

As findings may be consistent with a mechanism where an upregulation of MCL1 is mediating the protective effect, we next wanted to investigate this functionally by treating the co-culture systems with an MCL1 inhibitor AMG176 (Tapotoclax, Tapo). We thereby wanted to determine whether inhibiting MCL-1 would eliminate the protective effects that adipocytes provide AML. We mono/co-cultured AML cells and treated them with Ven alone, Tapo alone, and Ven/Tapo combination for 96 hours and assessed proliferation and viability.

Consistent with previous data, adipocyte co-cultured cells show a reduction in proliferation by 30%, while BMSC co-cultured cells show a 20% reduction (Figure 21a). When treated with Ven, both BMSCs and adipocytes protect AML cells from its effect by 15% and 40%, respectively. Tapo alone had no significant effects on the proliferative ability of AML cells. With the combination of Ven+Tapo, the protective effects seen from BMSCs and adipocytes could be completely abrogated as proliferation was <5% compared to the DMSO control (Figure 21a). In terms of viability, BMSCs and adipocytes also show protection from Ven treatment by 10%, while Tapo alone has no effect on AML viability (Figure 21b). The protective effects of BMSCs and adipocytes in terms of viability also have been completely eliminated with the combination of Ven with Tapo as viability was reduced to <8%, even in the adipocyte co-cultures (Figure 21b).

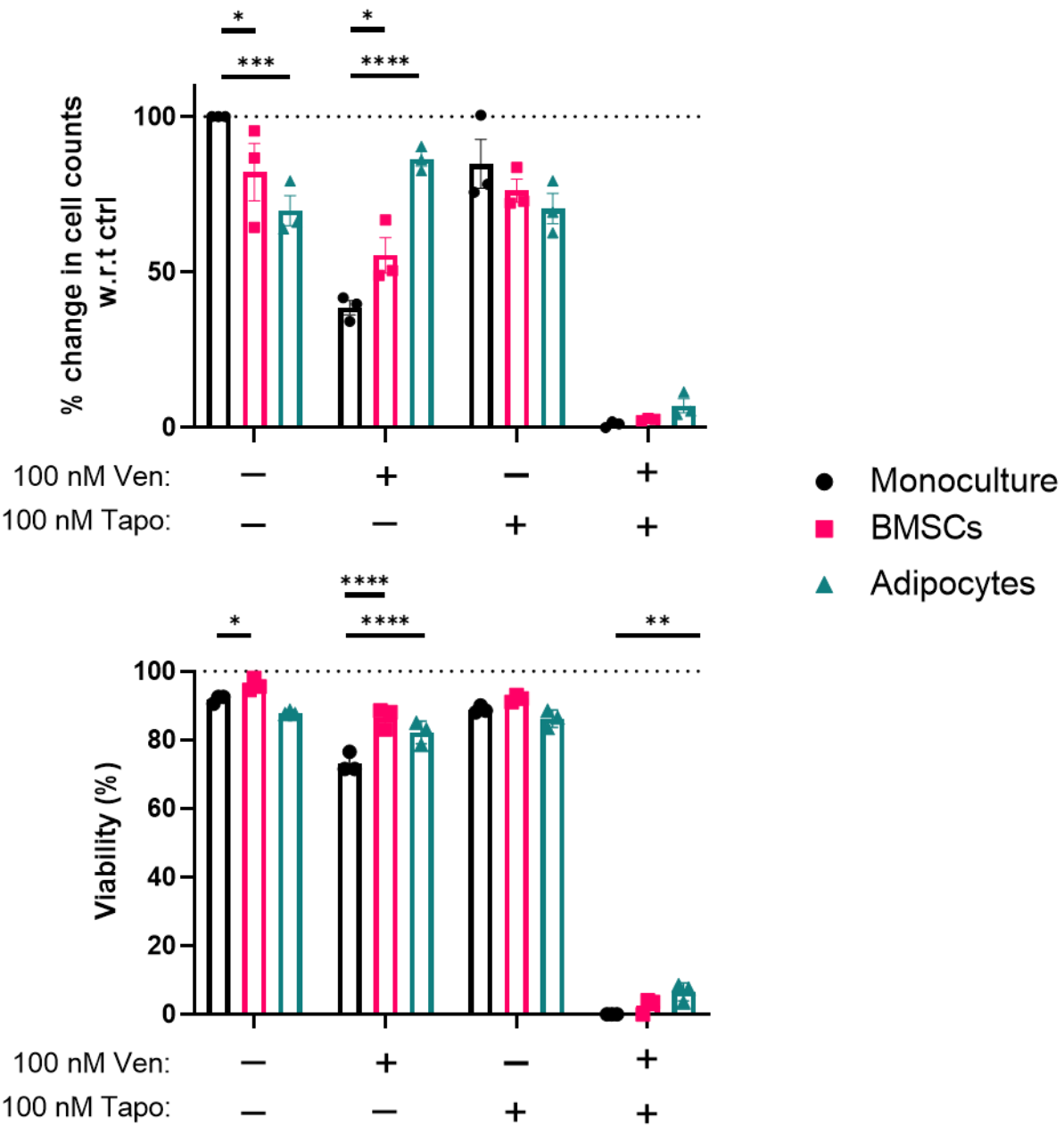

Figure 21. The addition of Tapo to Ven seems to overcome the protective effects of BMSCs and adipocytes on MOLM-13 cell proliferation and viability. MOLM-13 cells were monocultured or co-cultured on BMSCs or adipocytes and were treated with 0, 100 nM Ven, 100 nM Tapo, or 100nM Ven+100 nM Tapo for 96 hours. The cells were passaged in fresh media with or without treatment at 48 hours. (a) Percentage change in cumulative cell counts of MOLM-13 cells normalized to DMSO control after 96 hours of mono/co-culture with or without treatment. (b) Viability of MOLM-13 cells after 96 hours of mono/co-culture with or without treatment. Data is presented as average±SEM (n=3). Significance was measured using 2-way ANOVA tests. BMSCs=bone marrow-

derived mesenchymal stromal cells, Ven=Venetoclax, Ven=Venetoclax, Tapo=Tapotoclax.

To summarize our chemoresistance findings, BMSCs are able to protect AML cell viability and metabolism from the toxicity of Venetoclax/Azacitidine, while adipocytes are able to protect proliferation, viability, and metabolism. Preliminary flow cytometry data showed that both BMSCs and adipocytes may provide this protection through an anti-apoptotic mechanism. Preliminary western data showed that adipocytes enhance MCL-1 expression and when Ven was combined with an MCL-1 inhibitor, the protective effects provided by adipocytes were abrogated.

Chapter 4: Discussion

4.1 Overview

The BM microenvironment is an intricate network of cells and signaling factors that contribute to hematopoiesis and can be disrupted in AML. In this study, we show that in the absence of treatment, adipocytes co-cultured with AML cells reduce AML cell proliferation and viability yet enhance maximal respiration. This was seen in both MOLM-13 and HL-60 AML cell lines and H9M and MN1 murine AML cells. Adipocytes also reduced the proliferative ability of primary AML cells from 3 patient samples, yet did not show a reduction in viability, nor enhancement of maximal respiration. In the context of treatment, both BMSCs and adipocytes provided protection of proliferation and viability from Venetoclax treatment, increasing its IC_{50} value, and this is true in standard and substrate limited media (SLM). In the presence of Venetoclax/Azacitidine (Ven/Aza), adipocytes consistently show protection of MOLM-13 proliferation, viability, and metabolism, while BMSCs protect MOLM-13 viability and metabolism. Adipocytes show protection of HL-60 proliferation, while BMSCs protect viability. Contrastingly, adipocytes enhance the toxic effects of Ven/Aza treatment on 21029B proliferation. Yet, BMSCs protect 21007A proliferation. Through preliminary work, we show that this protection may be through an anti-apoptotic/necrotic mechanism, thus we investigated the BCL-2 family member MCL-1. We provide evidence that adipocytes increase the expression of MCL-1 of co-cultured HL-60 cells, and that when Venetoclax is combined with Tapotoclax, an MCL-1 inhibitor, the chemoprotective effects of both BMSCs and adipocytes is abrogated.

Our findings support the hypothesis that BMSCs and adipocytes can support AML cells to become resistant to Ven/Aza treatment. The finding that adipocytes in the absence of treatment inhibits AML yet allows it to respond to stress conditions better was unexpected and requires further investigation. The preliminary work regarding MCL-1's role in adipocyte-induced chemoresistance was a very interesting aspect of our work and provides a rationale for investigating the correlation between MCL-1 expression in AML and adipocytes contact.

4.2 Adipocytes reduces AML cell line and murine cell proliferation and viability, yet enhance maximal respiration

We first wanted to determine the effects of co-culturing AML cells on BMSCs and adipocytes, and we did so with cell lines due to their ease of use and stability in culture. We showed that both MOLM-13 and HL-60 cells co-cultured on adipocytes were negatively impacted in terms of proliferation and viability, yet these same cells were able to respond to stress conditions better than mono/BMSC co-cultured cells. This contrasts data shown by Yoko et al. (2018) where MOLM-13 cells grown on adipocytes had greater viability than monocultured cells. However, OCI-AML3 cells grown on adipocytes had lower viability when co-cultured on adipocytes. Therefore, there seems to be variability of the effects of adipocytes on AML cells depending on cytogenetic or molecular differences between the AML models. This group also showed that adipocyte-cultured cells have elevated levels of citrate, malate, and fumarate which are products of the Kreb's cycle, which is consistent with the finding of elevated respiration. One possible explanation for the enhancement of maximal respiration is that AML cells have

been shown to induce a lipolytic state in adipocytes, causing them to release their fatty acids (FAs) which AML in turn uses to fuel its own growth (Behan et al, 2020). Thus, with the injection of FCCP to measure maximal respiration, AML cells can continue to use FAs as a source of energy while the BMSC co-culture or monocultures do not have this advantage. However, this theory of utilizing FAs to explain the enhanced maximal respiration does not fit with our finding of the negative impact of adipocytes on AML proliferation and viability as increased respiration usually translates to enhanced growth. Nonetheless, there has been more research discerning the differences between small and large adipocytes, where small adipocytes enter the lipolytic state more easily through the expression of TRPV4, a calcium channel protein in adipocytes (Yang et al., 2020). It would be interesting to determine the role of different adipocyte types on AML cell growth.

We next showed a similar trend of reduced proliferative ability and viability with adipocytes in murine H9M and MN1 cells, both of which require cytokines for optimal growth. Shafat et al (2018) showed that both BMSCs and adipocytes can overcome the necessity of cytokines for the growth of H9M cells. We tried to reproduce these results but were unable to come to the same findings as the cells did not grow in either the mono/co-culture conditions in the absence of cytokines (data not shown).

4.3 Adipocytes reduce the proliferation of primary AML cells from 3 patient samples

Here, we show a trend for reduced proliferative ability across 3 patient samples, however, the viability and metabolism data did not show similar trends to the cell lines or

murine cells. 21050B did not grow as expected and had very low viability by 96 hours. Due to the lack of cells, we were unable to perform metabolism assays on this sample. Despite this, BMSCs and adipocytes did show slight protection, however, this sample had only an n=1. Again, this contradicts Shafat et al. (2018) who did similar work in co-culturing primary AML cells from patient samples with BMSCs and adipocytes. They found in 11 different patient samples, that BMSCs and adipocytes increase AML cell proliferation and viability. They also showed that in 3 different patient samples, adipocyte co-cultured cells show enhanced OCR, however, they did not specify whether this was basal or maximal respiration. Regardless, in our work, the 2 patient samples did not show any differences in respiration, either basal or maximal. The patient samples they used primarily had normal cytogenetics, but information on the molecular aberrations were unavailable. 21007A had a deletion of chromosome 7 while 21029B had a normal karyotype. AML is an extremely heterogenic disease; thus, different patient samples react differently to co-cultures. It would be valuable to conduct co-cultures with AMLs carrying similar cytogenetics or molecular aberrations and determine which ones are responsive to the protective effects of BMSCs/adipocytes.

4.4 BMSCs and adipocytes increase the IC_{50} value of Venetoclax in standard and substrate limited media (SLM)

In this study, we have shown that BMSCs and adipocytes provide MOLM-13 cells protection from Ven treatment in regard to proliferation and viability, with modest support in maximal respiration at higher concentrations. This is true in both standard media and SLM. The rationale for studying the co-culture system at physiological levels

is that in a nutrient abundance such as with high levels of glucose, the AML cells are not starved, and therefore may not need to reprogram their own metabolism, a process known as metabolic plasticity, or reprogram their microenvironment, such as with BMSCs or adipocytes, for access to nutrients. When glucose is abundant, the need for other energy sources such as fatty acids or amino acids diminishes. However, in the human body, glucose is not always readily available, and AML cells have been shown to induce adipocyte lipolysis to use as energy (Shafat et al., 2018). By using SLM, we hoped to mimic nutrient conditions similar to the microenvironment in AML patients which will allow us to extrapolate clinically relevant data. Although we see slightly less support of viability from adipocytes in SLM, there was little to no change seen in terms of support provided by BMSCs and adipocytes.

4.5 BMSCs and/or adipocytes protect MOLM-13 and/or HL-60 AML cell proliferation, viability, and metabolism from Ven/Aza treatment

In the absence of treatment, we showed that adipocytes primarily inhibit AML growth. However, in the presence of treatment, adipocytes show protection of MOLM-13 AML proliferation and viability, while BMSCs protect viability from the toxic effects of Ven/Aza treatment. Due to the high toxicity of the co-treatment, there were not enough cells remaining to perform metabolic assays on. However, both BMSCs and adipocytes did provide enough protection that cells had basal and maximal respiration values, albeit lower than DMSO or monotherapy conditions. The finding that BMSCs protect viability and metabolism is consistent with evidence by Griessinger et al. (2017) and Moschoi et al (2016) who showed that BMSCs can transfer mitochondria to AML cells, in turn

increasing their metabolism and contributing to resistance to cytarabine treatment. Another study also showed that BMSCs in the form of mesenspheres increase the TCA cycle and OXPHOS of AML cells and this had allowed them to evade cytarabine treatment (Forte et al., 2020).

With regard to adipocytes, an early study showed that AML cell lines co-cultured with adipocytes showed protection of viability against 4 chemotherapy agents, including daunorubicin (Behan et al. 2009). One recent study showed that a subpopulation of LSCs from the MLL-AF9 leukemia cell model find shelter in gonadal adipose tissue and release pro-inflammatory cytokines that also have elevated levels of OXPHOS (Ye et al., 2016). This enhancement of OXPHOS, either basal or maximal, was not seen with not seen in adipocytes compared to BMSC co-cultured cells. However, their work showed that these LSCs have high levels of the fatty acid transporter protein CD36 and that this has allowed them to become resistant to 4 different chemotherapeutic drugs including cytarabine and doxorubicin). In the context of Ven/Aza co-treatment, Stevens et al. (2020) showed that Ven/Aza sensitive cells have perturbed amino acid metabolism. However, Ven/Aza resistant cells show an upregulation of FAO. This paper aligns with our work as adipocyte supple FAs to AML cells, allowing them to overcome the toxicity of Ven/Aza.

4.6 Difficulties confirming the enhanced resistance in primary AML samples

With 2 cell lines, we showed that adipocytes protect AML cell proliferation. However, with both patient sample 21029B and 21007A, we did not see protection by adipocytes. In fact, it seemed that adipocytes enhanced the toxic effects of Ven/Aza in terms of 21029B proliferation. BMSCs were able to protect 21007A cells from Ven/Aza

to levels similar to DMSO control, however, overall, both patient samples were more resistant to the co-treatment as it only reduced proliferative ability by 20-30%. In the absence of treatment, 21029B viability was protected by both BMSCs and adipocytes. However, in the presence of treatment, that protection was obviated. It is important to note that the monoculture conditions showed no difference in viability when treated with or without Ven/Aza. The same is seen in 21007A, where there are no changes to viability to either the mono/co-culture conditions in the absence of presence of Ven/Aza. Another unexpected finding was that 21007A monocultured cells show a spike in basal and maximal respiration when treated with Ven/Aza, while this was not seen in the co-cultured cells. These unexpected results can be interpreted that these patient samples are inherently resistant to Ven/Aza, and thus any protective effect provided by BMSCs/adipocytes would not be seen or is masked. Overall, it seems imperative to repeat these experiments with different doses of Venetoclax or other more sensitive samples.

4.7 Preliminary work suggests adipocyte-induced chemoprotection may be through an anti-apoptotic mechanism, which may be mediated by MCL-1

We have provided evidence that BMSCs/adipocytes provide protection of AML cell line from Ven/Aza treatment. Our next steps were to understand the mechanism of action of this support, so we employed flow cytometry to help discern whether this protection was a result of an anti-apoptotic or proliferative mechanism or both. Our preliminary work showed that BMSCs and adipocytes reduce the percentage of MOLM-13 AML cells that are undergoing apoptosis in the presence of Ven/Aza. This is consistent with work done by Konopleva et al. (2002) who showed that HL-60 cells co-cultured with BMSCs

reduced cytarabine-induced apoptosis. Another study found that adipocytes stimulate activation of anti-apoptotic chaperone proteins via MYC activation (Stine et al., 2015) and that the inhibition of FAO reignites apoptosis in AML cells (Jones et al., 2009).

This potential anti-apoptotic mechanism underlying chemoresistance triggered our interest in the anti-apoptotic protein MCL-1. As previously mentioned, MCL-1 is part of the BCL-2 family of anti-apoptotic proteins and has been found to be overexpressed in AML (Dai et al., 2016). We wanted to determine if MCL-1 might be elevated in co-cultures compared to the monocultures. Our western data showed that adipocyte co-cultured AML cells show enhanced levels of MCL-1 expression in MOLM-13 and HL-60 cells, which both showed resistance to Ven/Aza. Anstee et al. (2019) showed that elevated levels of MCL-1 reduced sensitivity to daunorubicin in AML cell lines. They also showed that MCL-1 enhanced resistance to Venetoclax and concluded that the combination of chemotherapeutic drugs combined with inhibitors of MCL-1 may improve patient outcomes. Therefore, we conducted a pilot experiment co-culturing AML cell and exposing them to a combination of Ven with an MCL-1 inhibitor called Tapotoclax. We saw a dramatic drop in both MOLM-13 cell proliferation and viability in the Ven/Tapo combination compared to either monotherapy. Our data is consistent with work done by Ramsey et al. (2018) who showed that in either MCL-1inhibitor-resistant cell lines and Ven-resistant cell lines, the combination of an MCL-1 inhibitor with Ven lowers viability close to 0%, as we have shown. To date, there are no known studies investigating the combination of MCL-1 inhibitors with Ven/Aza in sensitive or resistant lines.

Our preliminary work provides evidence for a role of MCL-1 in adipocyte-induced chemoresistance to Ven/Aza in cell lines. An interesting study looking into the connection between MCL-1 and adipocytes found that leptin, a hormone produced and released by adipocytes, triggers MCL-1 activation and this had inhibited chemotherapy treatment in gallbladder cancer (Wang et al., 2021). The same relationship of leptin triggering MCL-1 activation leading to subsequent cancer growth was seen in ovarian cancer (Chen et al., 2013). With regard to metabolism, MCL-1 has been reported to directly interact with very long-chain acyl-CoA dehydrogenase (VLCAD), which is an enzyme involved in FAO, and found that MCL-1 deletion deregulates FAO of long-chain FAs. To our knowledge, there no known studies investigating the role of MCL-1 in adipocyte-mediated chemoprotection.

4.8 Limitations

In this thesis, we have shown evidence that adipocytes play a dual role in AML inhibition or support depending on the presence or absence of treatment respectively. Our preliminary work suggests that MCL-1 may play a role in this function as its protein levels were enhanced in adipocyte co-cultured AML cells. Furthermore, the addition of an MCL-1 inhibitor abrogated any chemoprotective role BMSCs and adipocytes provide AML cells, providing evidence for further exploration into this potential mechanism of action. Despite these findings, there are some limitations to our work.

Firstly, we did not perform any confirmatory studies into the effectiveness of both Venetoclax and Tapotoclax in terms of their inhibition of BCL-2 and MCL-1,

respectively. To combat this, performing western blots to confirm inhibition to measure target engagement must be completed.

Secondly, the adipocytes we differentiated were from only 1 precursor: BMSCs. Adipocytes can also be differentiated from fibroblasts and other forms of adipocyte precursor cells. Reproducing the experiments performed in this thesis using these alternative adipocyte precursors will allow for more accurate findings.

Thirdly, we did not perform experiments that would provide information on the effects of the co-cultures on normal hematopoiesis. In addition to this, we used healthy BMSCs to differentiate into adipocytes. It would be beneficial to study the differences of the microenvironment cells on normal vs leukemic cells, as well as use BMSCs from AML patients to study how leukemia affects adipogenesis.

Finally, it is important to note that AML is an extremely heterogeneous disease where every single patient is biologically and genetically different from one another, and even within one patient, the leukemic cells can also vary drastically. This makes both research and treatment difficult to perform given the variability in the diseased cells. Although cell lines are easy to use, patient samples may be more encompassing of the complexity of AML, however, each patient sample may respond differently to the same experimental parameters. Overall, disease heterogeneity makes investigations of AML and the role of the microenvironment difficult, but not impossible. Thorough experimental design, large sample sizes, and comparing genetic backgrounds can help overcome this barrier.

4.9 Future directions

Future studies should focus on understanding different factors affecting microenvironment-mediated support of AML cells, the dual role of adipocytes being inhibitory in the absence of treatment yet protective role in the presence of treatment, as well as the role of MCL-1 in adipocyte-mediated chemoprotection. After evidence is seen in *in vitro* models, the next logical step would be to perform these experiments in *in vivo* models.

We first propose investigations into the cause of enhanced maximal respiration rate upon co-culturing with adipocytes. It would be valuable to understand what causes this support, whether it is due to increased oxidation of FAs or another energy source. The use of etomoxir, a fatty acid oxidation (FAO) inhibitor, as an injection in the Mito Stress Test can provide data upon the reliance of AML cells on FAO. If it is determined that FAO is the mechanism of support provided by adipocytes, adding FAO inhibitors during the co-culture period to determine if it would reduce the respiration rate would be interesting.

Although we were unable to reproduce Shafat et al. (2017) work where they showed that BMSCs and adipocytes can overcome the necessity of cytokines, it would be valuable to attempt this again using different concentrations of cytokines. Using a range of cytokine concentrations such as 0, 25, 50, 75, and 100% would help us understand the degree of support BMSCs and adipocytes provide. If cytokine concentrations are at 100%, the protection offered by BMSCs/adipocytes may be masked.

We found that substrate limited media did not largely affect the IC_{50} of Venetoclax when in standard media. Although it was only an n=1, we did see that the support of viability seen with BMSCs and adipocytes in standard media was reduced to levels similar to monoculture in SLM. Therefore, it would be valuable to investigate this change further by investigating media limited in different substrates. For example, limiting amino acids, lipids, glucose, and other important biomolecules, and to compare the effects on the co-culture. This will allow us to determine which biomolecule is necessary for a protective effect to be seen by the co-cultures. Similar to this reasoning, it would be essential to compare the difference between normal and physiological levels of nutrients as the media we used is supplemented with levels of glucose and amino acids that are not seen in the BM. Culturing cells in physiological levels of glucose, amino acids, and lipids and observing the effects on AML cell growth would be beneficial. It would be interesting to see if these physiological levels would alter the inhibition seen by adipocyte son co-cultured AML cells.

As discussed, the lack of proliferative support of our adipocyte co-cultures for the AML cells is inconsistent with some data found in the literature. It remains an open question if these findings may be influenced by the cell type used. Adipocytes differentiated for our experiments were derived from BMSCs from healthy human donors and genetically different from the leukemias used or even cross-species for the murine AML models. It therefore remains to be determined, if BMSCs/adipocytes from individuals with AML would behave differently in these assays and if there may even be a co-dependence of a specific AMLs with cells from their microenvironment. However,

the cell-state of reduced proliferation, but simultaneous increase in maximum respiratory capacity may hint towards interesting alterations in the biology of these AML cells upon co-culture. It remains to be determined which nutrients fuel the increase maximum respiration. In addition, the co-culture may select for or lead to an adaptation of a leukemic stem cell-like state. This could be investigated in the future by subjecting the cells from the co-cultures to functional leukemic stem cell assays such as limiting dilution assays or colony assays.

Studies have shown that certain types of adipocytes, such as small adipocytes, show greater support of AML cells than large adipocytes (Yang et al., 2020). In addition, adipose tissue (AT) can be classified as white AT (WAT) or brown AT (BAT), which have differing functions. WAT has a singular large lipid droplet and functions as energy storage, while BAT has many little lipid droplets and is involved in thermogenesis. Some studies have shown that the BM has a mixed population of WAT and BAT (Krings et al., 2012). It would be important to differentiate which adipocytes are involved in the microenvironment support of AML. If there are differences seen between WAT and BAT or small versus large adipocytes in terms of inducing chemoresistance, it would be interesting to conduct experiments that convert one to another as a way of rescinding this support.

Adipocyte's inhibitory and protective role would be very interesting to see if it is due to a direct cell-to-cell contact mechanism, or a non-contact, communication via soluble factors mechanism. To test this, we suggest performing co-cultures using transwell systems and comparing them to direct contact co-cultures. In addition, with

these co-culture systems, we have focused on the effects of BMSCs and adipocytes on AML cells, however, it would be just as important to investigate the role that AML cells have on these cells. For example, it would be interesting to see the effects of AML on adipocyte size and number.

We were able to see similar trends of reduced AML proliferation and viability with AML cell lines and murine AML cells, however, patient samples did not respond as expected. It would be valuable to have more patient sample data and to group similar samples in terms of cytogenetics and molecular aberrations to determine if certain subgroups of AML depend more on the microenvironment. In addition to this, it would be extremely valuable to compare cell lines that are sensitive versus resistant to Venetoclax and/or Azacitidine. For example, OCI-AML3 is known to be Venetoclax resistant and has higher levels of MCL-1, which is further upregulated with Ven treatment (Pan et al., 2017). It would be interesting to see if using an inhibitor of MCL-1 could make this resistant line sensitive to co-treatments.

These suggested experiments would allow for more insight into the inhibitory and protective role of adipocytes, the effects of changing parameters to determine if chemoresistance would be obviated, as well as provide a potential new combination therapy of MCL-1 inhibitors with Ven/Aza that can benefit older patients with AML.

Chapter 5: Conclusion

AML is an aggressive blood cancer and the major standard of care of chemotherapy faces a huge obstacle of chemoresistance and subsequent relapse. Relapse is even more pronounced in older patients with AML given their comorbidities and ineligibly to more intensive yet curative options, resulting in their low survival rates. The combination of Ven/Aza has shown great promise, however, chemoresistance remains prominent. The BM microenvironment has been gaining lots of interest in its role in supporting AML cells and contributing to such chemoresistance. There are a lack of studies investigating the interplay between BM microenvironment-induced support of AML and Ven/Aza chemoresistance. In this thesis, we hypothesized that BMSCs and adipocytes can support AML cells to become resistant to the co-treatment of Ven/Aza.

Using 2-dimensional co-culture systems with 3 in vitro AML models, I have reported a dual role of adipocytes, where its inhibition or support of AML is dependent on the absence or presence of treatment. In untreated conditions, adipocytes inhibit AML cell proliferation and viability, yet enhance its maximal respiration rates. In the presence of Ven/Aza, BMSCs and/or adipocytes protect AML cell proliferation, viability, and metabolism from the toxic effects of the co-treatment. Through preliminary flow cytometry and western data, we were able to provide evidence of a potential anti-apoptotic mechanism of adipocyte-induced support through the upregulation of MCL-1. With this knowledge, we combined Ven with an MCL-1 inhibitor and were able to abrogate the chemoresistance provided by both BMSCs and adipocytes.

Our work highlights the importance of studying AML in the context of its microenvironment as without studying these crucial cell-to-cell interactions, we would miss a large aspect of chemoresistance. However, there are limitations to our work that must be addressed prior to further investigation into MCL-1. We were unable to show a consistent trend of enhanced maximal respiration by adipocytes in HL-60 and H9M murine cells. However, this can potentially be due to the limited sample size. The same is true for patient samples, however, this too can be due to low sample size and the fact that primary cells have greater difficulty growing in cell cultures. This can be mediated by performing patient-derived xenograft models of AML and treating mice with and without Ven/Aza and observing their differences.

Taken together, our work provides evidence for an inhibitory and supportive role of adipocytes depending on the absence or presence of treatment. This project demonstrates the necessity to understand the BM microenvironment as it is an important niche full of supporting cells and signaling factors that can contribute to AML chemoresistance. By characterizing this support, we can find novel therapeutic targets to overcome treatment resistance, thereby improving patient outcomes for patients with AML.

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