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# Roles Of Oxidative Stress And Dna Methylation In Cigarette Smoking-Induced Accelerated Acute Myeloid Leukemia Progression

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# Roles of oxidative stress and DNA methylation in cigarette smoking-induced accelerated acute myeloid leukemia progression

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## DEDICATION

To all the English as a second language students and students trying to find a way to obtain a higher education. It is possible, do not lose hope, do not give up.

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**Abstract**

ROLES OF OXIDATIVE STRESS AND DNA METHYLATION IN CIGARETTE SMOKING-INDUCED ACCELERATED ACUTE MYELOID LEUKEMIA PROGRESSION

Mary Carmen Figueroa, B.S.

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Acute myeloid leukemia (AML) is a commonly diagnosed cancer in smokers. When current or former smokers have AML, they have worse survival compared to never smoking patients. This has been observed clinically for decades, but then it is unknown how smoking leads to worsened AML survival. Smoking causes oxidative stress and altered DNA methylation that persists for decades in peripheral blood mononuclear cells, but these changes from smoking have not been evaluated in the context of AML. We hypothesize that smoking-induced molecular changes, including altered DNA methylation associated with poor AML prognosis, promote AML. We developed a novel model to study cigarette smoke exposure (SE) in AML-bearing mice. Using our model, we investigated the impact of SE on AML progression and how smoking cessation compared to continued SE. Cigarette smoke condensate (CSC) treatment of AML cells in vitro was used to study how chemicals from cigarette smoke in the absence of high heat combustion directly impact AML cells. Our SE model and CSC treated AML cells resulted in accelerated leukemic progression in mice. SE and CSC exposed AML cells were examined for altered DNA methylation and for oxidative stress. Evaluation of non-smoking and SE samples revealed changes in protein expression involved in AML progression

and treatment resistance. This work shows that cigarette smoking enhances leukemic progression, induces oxidative stress in AML, alters DNA methylation that is associated with poor prognosis in AML, alters the bone marrow microenvironment to promote the growth of myeloid progenitor cells, and activates oncogenic signaling in AML cells. My work highlights the potential for using smoking as a behavioral biomarker to help tailor treatment in AML patients.

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## Chapter I: Introduction

### *Acute myeloid leukemia*

Acute myeloid leukemia (AML) is a common form of cancer that develops from hematopoietic cells in the bone marrow. Hematopoiesis is constantly ongoing, and mutations in hematopoietic stem cells can lead to improper production of mature blood cells. AML is characterized by clonal expansion of undifferentiated, blast cells and decreased differentiated blood cells. Myelodysplastic syndrome (MDS) is a condition where there are low number of mature differentiated cells, and this may develop into AML. Over 20,000 patients are diagnosed with AML in the United states annually (1). AML is the most lethal major subtype of leukemia, with a five-year survival rate of 30%, which highlights the need to discover more effective treatment options for these patients (1).

The standard of care for AML has been used for decades (2), but there have been advances in treatment in recent years. AML patients generally receive cycles of the 7+3 chemotherapy regimen, with 7 days of cytarabine with 3 days including daunorubicin in the infusion (3). Cytarabine is a nucleoside analog that hinders DNA replication and since cancer replicate at a faster rate than normal cells, there is increased cytotoxicity in cancer cells. Daunorubicin is an anthracycline that intercalates into double-stranded DNA, which impedes synthesis of DNA and RNA (4). The 7+3 regimen is physically hard for patients to tolerate, and some patients are not suitable to receive this treatment; in these patients, hypomethylating agents can also be given (5). Following chemotherapy, stem cell transplants are also an option when there are compatible bone marrow donors are

available. To avoid potential risk of graft versus host disease (GVHD) occurring, AML patients that receive stem cell transplants must continue immune suppressive drugs that leaves patient vulnerable to potential infections; additionally, acute and chronic GVHD can be lethal for AML patients. While these treatments have been used for decades in patients, AML relapse rates are still around 50% and mortality rates are high (6, 7). Given the high relapse rates, there is a need for better understanding of AML drivers.

Attempts to gain understanding of AML have uncovered that there are genetic mutations that can lead to different survival rates in patients. The most common oncogenic mutation is the fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) mutation that confers the worst survival in AML patients (8-10). The FLT3-ITD mutation occurs in 27% of AML patients with an additional 5% of AML patients carrying mutations in the tyrosine kinase domain of FLT3 (FLT3-TKD). The FLT3-ITD mutation occurs in the juxtamembrane domain and leads to constitutive activation of the FLT3 receptor. Interestingly mutation of Nucleophosmin 1 (NPM1) confers a better survival only if it is not accompanied by the FLT3-ITD mutation (9). Other common mutations include: isocitrate dehydrogenase (IDH) 1/2, DNA methyltransferase 3A (DNMT3A), p53, runt-related transcription factor 1 (RUNX1), and GATA-binding factor 2 (GATA2) (9). Aside from specific mutations, there are other factors that are used try to define AML (11).

Leukemias are not classified in stages when diagnoses, like solid tumors, but there are other methods that have been created to define AML subtypes. The French-American-British (FAB) classification is based on what cell and how mature the cells from which the

AML arose. The World Health Organization has made their own classification system that is based on the presence of specific mutations and chromosomal rearrangements, but also includes other categories similar to FAB. AML patients can receive different care dependent on their subtype. Greater understanding of AML biology has expanded the treatment options for patients.

To meet the need for more effective treatment options in AML, there have been recent FDA approvals which include targeted therapies for select patient populations. Midostaurin, a small molecule FLT3 inhibitor, gained FDA approval for treatment of FLT3-mutant AML patients in 2017 (12, 13). Another promising drug, Venetoclax, is a BCL-2 inhibitor that was recently approved for AML treatment (14, 15). While treatment advances against molecular targets have improved patient outcomes, there are additional factors beyond specific gene mutations, such as environmental factors, that contribute to worse outcomes in patients and need to be addressed in parallel to novel therapeutic strategies.

***Cigarette smoking and AML***

Cigarette smoking is the most preventable cause of cancer, but cigarette smoking is still associated with a third of global cancer deaths (16, 17). The majority of smoking research focuses on lung cancer, which leaves many other conditions and patients overlooked. Cigarette smoking is associated with the development of various cancers, and one of the most commonly diagnosed form of cancer in smokers is AML. Any history of smoking

increases the lifetime risk of developing AML, and this risk is positively correlated with the rate and duration of the smoking habit (18).

Smokers not only have increased incidence of AML, but current and former smoking AML patients have worse survival compared to patients that have never smoked (19-21). In fact, this phenomenon of worse survival has been observed in several retrospective studies of AML patients; however, these retrospective studies lack detailed information on the precise smoking habits of the patients. Since the previous studies were retrospective in design, there is still a need to understand the mechanism driving the poor survival outcomes among AML patients with a history of smoking.

***DNA methylation and smoking***

Methylation of DNA is an important epigenetic process that helps regulate gene expression in cells (22). The methylation of cytosine resulting in 5-methylcytosine (5mC) is the predominant DNA methylation mark. The only other DNA methylation event in eukaryotic cells is that of adenosine, which has only been identified during embryonic development (23). In general, increased DNA methylation within promoters of genes is considered to decrease gene transcription, but as more is learned about these methyl marks, the complex nuances of DNA methylation are discovered. Since DNA methylation marks are found throughout the genome, their function must extend past transcriptional regulation of promoters. DNA methylation outside of genes have been associated with helping to maintain genome stability (24). DNA methylation within genes but outside of

promoter regions have been suggested to play a role in gene splicing (24). There has been a growing appreciation for DNA methylation of enhance regions within and outside of genes, that may play major roles in controlling cellular differentiation (25, 26). Additionally, the presence of 5mC can impair the binding of transcription factors and recruit the binding of certain proteins (22, 24). Given the broad functionality of DNA methylation, it is unsurprising that there are several mechanisms that help regulate DNA methylation.

Methylation marks can be added or removed by various proteins which act to regulate the expression of genes. DNMT1 is the most widely expressed DNMT family member and is involved in maintaining genomic DNA methylation (22). DNMT3A and DNMT3B are isoforms of DNMT3 that both function to add new methyl groups to cytosines and have been found to help maintain DNA methylation with DNMT1 (24, 27). Methyl marks are added by DNMT proteins and removed by ten-eleven translocation (TET) enzymes through a series of reactions (28). TETs work through alpha ketoglutarate (α-KG) dependent reactions to remove methyl marks from cytosine. In general, TET erasure of DNA methylation is thought that first 5mC is oxidized to 5-hydroxymethylcytosine, then converted to 5-formylcytosine, 5-carboxylcytosine, until the cytosine is finally back to its unmarked state. Although, the full molecular process of TET-mediated DNA methylation is not yet fully decoded. Lastly, IDH enzymes convert isocitrate into α-KG, which is required for TET-mediated removal of DNA methylation marks. There are other proteins that are important to consider for DNA methylation.

Methyl-CpG-binding domain proteins (MBDs) can bind to 5mC and can recruit other proteins to the DNA region where they are bound. When MBD proteins are bound to methylated DNA, they can interfere with binding of proteins involved in gene transcription (29). MBD binding can have other effects including chromatin remodeling, histone methylation, and recruitment of DNA methyltransferases (DNMTs). These mechanisms provide insight into methods that DNA methylation is involved in regulating gene expression.

DNA methylation is an impermanent epigenetic modification that is necessary for proper cellular functions and its dysregulation can have serious consequences. For example, absence of functioning DNMT1, 3A, and 3B were found to be lethal in neonatal and embryonic stages in genetic mouse models (24). Different tissues have distinct DNA methylation patterns, and continued maintenance of the DNA methylation patterns are needed (30). Global levels of DNA methylation can change over time with aging, but do not cause major changes in cellular differentiation since few tissues are differentiating in adults, besides hematopoietic differentiation. While DNA methylation is a regulated process in cells, changes to DNA methylation can occur through unintentional consequences of cellular damage.

Damage to DNA is another mechanism known to cause changes in DNA methylation. When DNA damage occurs at methylated CpG sites, an unmethylated cytosine can replace a previously 5mC and would result in loss of methylation. Another common DNA lesion is the oxidation of guanine, which is often caused by increased levels of reactive oxygen species.

When the 8-oxoguanine is replaced, nearby methylated cystines may be replaced, during the DNA repair process, causing decreased DNA methylation. An additional DNA oxidation event that occurs is the oxidation of 5mC into 5-hydroxymethylation (5hmC), which is not equivalent to 5mC and will not recruit proteins that would normally bind to 5mC (31, 32). Although 5hmC still contains a methyl mark, several proteins that normally interact with 5mC will not bind to 5hmC (33). Sources of DNA damage can come from environmental exposures, such as smoking and cigarette smoke exposure (SE).

Cigarette smoking causes global changes in DNA methylation. Altered DNA methylation from smoking can be tracked in peripheral blood cells of smokers. Smoking-induced altered DNA methylation can persist decades after quitting smoking. The alterations in DNA methylation are random and specific genes are not consistently found to have altered DNA methylation, with the exception of aryl hydrocarbon receptor repressor (AHRR) (34). In this instance, the hypomethylation of AHRR is used as a biomarker for smoking and second-hand cigarette smoke exposure as it is routinely has the same results from smoke exposure. Interestingly, no other genes have been identified as consistent targets for having altered DNA methylation from smoking (35). The lack of consistency suggests that broad, varied epigenetic changes are occurring, rather than consistent alteration of a specific molecular pathway(36, 37).

***DNA methylation and cancer***

Specific DNA methylation patterns are necessary for proper differentiation of cell types, and dysregulated DNA methylation is found in many cancers (30, 38). Hematopoietic stem cells are constantly producing new blast cells that go on to differentiate into mature leukocytes. AML is characterized by increased immature, undifferentiated blast cells, so it is unsurprising that several common mutations found in AML patients are involved in DNA methylation regulation, such as TET2, DNMTs, and IDH1/2 (39, 40).

While TET2 mutations are commonly found in multiple cancer types, they have not been determined to be solely able to induce carcinogenesis (41, 42). Loss of function mutation in TET2, in a mouse model, was sufficient to alter hematopoietic stem and progenitor cell differentiation (42). This study also found progressive accumulation of DNA damage in TET mutant cells (42). Thus, wildtype TET2 acts as a tumor suppressor that preserves DNA methylation and the integrity of the genome required for cellular differentiation.

The carefully balanced DNA methylation needed for cellular differentiation, it is unsurprising that there are mutations and altered function of DNMTs found in cancer. The most notable DNMT mutation in AML is that of DNMT3A that is found in about 22% of AML patients and confers a worse survival rate (40). DNMT3A mutations that result in gains or losses of function have been observed in AML patients, and the DNMT3A mutations have been implicated in leukemogenesis (43). DNMT3A mutations are found in myelodysplastic syndrome and clonal hematopoiesis of indeterminate potential, which emphasizes the importance of DNMT3A function in normal hematopoietic cell differentiation (43, 44).

Mutations in DNMT3B can inhibit its activity and increase genomic instability, which is a hallmark of cancer (27). While DNMT1 is not frequently mutated in AML, there is increased expression of DNTM1 during malignant transformation and inhibition of DNMT1 can decrease frequency of cancer stem cells (45). Mutations of DNMTs can inhibit their ability to use S-adenosylmethionine, which is the methyl donor used for DNA methylation (24).

Mutations in either IDH 1 or IDH2 are found in approximately 20% of AML patients (46). The IDH1/2 mutations commonly found in AML are single nucleotide, missense mutations in the active site (47). The mutations result in decreased binding to isocitrate, which results in decreased α-KG. TET enzyme reactions are dependent on alpha ketoglutarate, so IDH1/2 mutations also inhibit TET activity. Mutant IDH1/2 convert α-KG to D-2-hydroxygluterate (D2HG) (47). Not only are α-KG levels decreased due to their conversion to D2HG, but D2HG also inhibits α-KG-dependent enzymes, including TET enzymes. Increased D2HG levels have been found to inhibit cell differentiation (47). The consequent inhibition of TET functions from IDH1/2 mutations may explain why TET2 and IDH1/2 mutations are not both found together within the same AML patient. The recognized importance of IDH dysregulation in AML is highlighted by the approval of two IDH inhibitors for use in AML patients (46).

DNA methylation is important for maintaining proper gene expression and is altered in cancer, including leukemias (38, 48). There have been AML associated changes in DNA methylation that have been linked to patient survival, unrelated to mutations (49, 50). Chromatin structure and other epigenetic marks, including histone methylation, can also impact DNA methylation patterns in the absence of mutant DNMTs (51). It is not well

understood how the altered DNA methylation patterns arise in AML patients, but lifestyle factors, such as cigarette smoking or CSE, may be a contributor.

***Oxidative stress and smoking***

The levels of reactive oxygen species (ROS) and antioxidants are carefully balanced within a cell. When a cell produces ATP through the electron transport chain, ROS is released as a byproduct. Therefore, in healthy, functional cells, there is a maintained level of antioxidants constantly present in cells to quench ROS. Another source of ROS is from NADPH oxidase (NOX) complexes. NOX complexes were first discovered in immune cells as a defense mechanism, where the cell releases a cytotoxic "burst" of ROS to kill another cell. Elevated levels of ROS will lead to oxidative stress, and eventually oxidative damage. With enough oxidative damage occurs this can also lead to cell death. Reversely, elevated levels of antioxidants may keep cells alive under conditions where apoptosis would be appropriate.

Oxidative stress, and oxidative damage can be initiated through cigarette smoking, where the damage is evident in tissues throughout the body, not just lungs. Cigarette smoke increases levels of ROS through harmful chemicals coupled with the high heat combustion (52-54). A study with over 250 participants found that general peroxides, a species of ROS, within serum positively correlated with the number of cigarettes smoked per day in individuals who smoked (55). The damaging effects of oxidative stress are well

documented; however, the long-term impact of elevated oxidative stress specifically from smoking is unknown.

***Oxidative stress and Leukemia***

A homeostatic balance of reactive oxygen species (ROS) and antioxidants are normally maintained, but when there is an imbalance this results in oxidative stress. Alterations in redox levels can lead to enhanced leukemic progression (56). When elevated ROS are elevated from internal or external factors, there are cellular antioxidant defenses to protect the cell from oxidative damage. NRF2 is a key regulator of redox balance; when ROS levels are increased, NRF2 will be released by KEAP1, translocate to the nucleus, and induce the transcription of antioxidant response elements (ARE). One important ARE is heme oxygenase-1 (HO-1), which is an antioxidant involved in metabolizing heme. Increased levels of antioxidants can also result in oxidative stress that promotes aggressive leukemia.

Oxidative stress is a hallmark of cancer and can activate oncogenic signaling within cells. In fact, oxidative stress is implicated in the promotion of leukemia where it can contribute to genomic instability due to oxidative damage of DNA. Mutations such as FLT3-ITD, the most common oncogenic mutation in AML, can also lead to increased intracellular ROS from the activation of NOX complexes (57). Increased antioxidants in response to elevated ROS have also been identified as a mechanism of resistance to treatment in leukemia (56, 58). In summary, oxidative stress can increase leukemia progression and treatment resistance through different mechanisms.

***Hypothesis and research goals***

Several clinical studies have shown that any history of cigarette smoking is linked with worsened survival in AML patients, but no steps have been taken to help these patients. Although there are known oxidative damage from smoking or chemical components of cigarette smoke, it is unknown how they may be contributing to AML. Thorough examination of smoking in this disease will uncover a mechanism of how smoking is promoting AML and aid a population of vulnerable patients that would benefit from additional targeted interventions.

I hypothesize that cigarette smoking induces molecular changes, including altered DNA methylation associated with poor AML prognosis, that promote AML. We addressed this hypothesis by:

- Understanding the impact of cigarette smoking on progression and treatment response of AML
    - Within AML patient treatment response
    - AML progression with smoke exposure (SE)
    - AML progression with cigarette smoke condensate (CSC)
    - Treatment response with SE
- Defining DNA methylation alterations from cigarette smoking in AML
    - Altered DNA methylation from SE
    - Altered DNA methylation from CSC

- Describing how cigarette smoking alters hematopoietic cells populations and antioxidants in the microenvironment
    - Hematopoietic cell populations in immune competent model
    - Hematopoietic cell populations in immune deficient model
    - Changes in redox with SE in the microenvironment
- Elucidating changes in protein expression and antioxidants in AML because of SE
    - Changes in ROS with SE
    - Changes in redox with CSC
    - Changes in pro-leukemic protein expression with SE

Together this work provides rationale for using smoking as a behavioral biomarker of poor prognosis in AML that should be used to tailor treatment in patients.

## Methods and Materials

*Patient Cohort*

Cohort of 99 newly diagnosed, treatment naïve AML patients bearing the FLT3-ITD mutation and with smoking status available were studied in this single center retrospective review. This cohort of patients were treated at UT MD Anderson Cancer Center between 2012 and 2017. Data were collected under protocols DR09-0223 and PA12-0395 for retrospective data collection in patients with FLT3 mutated AML. Patient record review was approved by MD Anderson Cancer Center Institutional Review Board and consent from patients in compliance with the Declaration of Helsinki. Patients with available cytogenetics, smoking status, and progression free survival were used in this study. Patients were defined as never smokers or ever smokers, which included current and former smokers, based on if smoking history was listed in the medical record. Univariate analysis was used to determine differences in patient AML attributes. Chi-square test was used to determine differences in treatment response. Patient and AML characteristics described in Table 1.

*Cell lines and Reagents*

Luciferase-tagged FLT3-ITD–mutant AML cell lines MOLM-13 and MOLM-14 cell lines were provided by Hayashibara Biochemical Laboratories (Okayama, Japan) and MOLM14 cells were provided by Dr. Mark Levis, Johns Hopkins University School of Medicine, Baltimore, MD, respectively. All cells were cultured in 5% CO2 at 37°C. in RPMI 1640 medium (25-506N, Gen Clone) with 10% FBS (SH-30256.01, Hyclone), 1% L-glutamine (25030-081m Gibco), and 1% penicillin/streptomycin (400-109, Gemini). ML-1 and OCI-AML3 cell lines were purchased

from American Type Culture Collection and their media contained 1% sodium pyruvate and 1% non-essential amino acids. All AML cell lines were maintained at cell densities of 0.2-2.0x$10^6$ cells/ml and in non-adherent flasks (Olympus, 25-213).

Cells incubated at 37 C with 5% $CO_2$, were mycoplasma free, and were tested with MycoAlert PLUS (Lonza) at least every 6 months. Cell lines were validated annually by STR DNA fingerprinting using Promega 16 High Sensitivity STR Kit (Catalog #DC2100). Cell counts and viability were determined by trypan blue exclusion assay on Vi-CELL XR Cell Viability Analyzer (Beckman Coulter).

Alamar blue assay was seeded with 50,000 cells/well on day 14 of DMSO or CSC treatment and treated with drug. Stock 100x Alamar blue solution was made from resazurin salt (R7017, Sigma) in sterile PBS and stored at 4C. 72 hours post treatment, 1X Alamar blue and 18 hours after the addition of Alamar Blue the plate was read 18 hours after Alamar blue addition for fluorescence on CLARIOstar Plus plate reader (BMG Labtech). Fluorescence was normalized to DMSO control. Inhibitory concentrations 50% (IC50) were calculated using GraphPad Prism Sigmoidal, 4PL with no special handling of outliers.

Cigarette smoke condensate (CSC) was purchased from Murty Pharmaceuticals, Inc. CSC was aliquoted upon receival and stored at -80. CSC stock was 40mg/ml and cells were dosed with 10ug/ml or vehicle DMSO every passage.

*Mouse studies*

All mouse studies and techniques performed were conducted in accordance with Institutional Animal Care and Uses Committee (IACUC) at MD Anderson Cancer Center (protocol 00000638-RN02). All experiments began when mice were between at 4-8 weeks of age. NOD-SCID mice were purchased from the MD Anderson Department of Experimental Radiation Oncology. Male and female mice were used in AML-bearing experiments. Mice were housed in stable temperature with 12hr light dark cycles. Mice were fed facility chow and water ad libitum. Mice had 100,000 MOLM13 or 200,000 MOLM14 cells resuspended in sterile 100ul PBS (SH-30256.01, Hyclone) introduced via tail-vein injection. Daunorubicin (D-6660, LC Labs) was administered twice weekly at 2 mg/kg via tail-vein. Bioluminescent images were taken using IVIS Spectrum ten minutes after D-luciferin potassium salt (LUCK, GoldBio) 150mg/kg subcutaneous injections. Living Image software (Caliper Life Sciences) used to quantify bioluminescent images.

Mice were euthanized at set end points or when they began to show moribund symptoms. Mice underwent carbon dioxide (3 Liter/minute flow rate for at least 5 minutes), along with either cervical dislocation or exsanguination via cardiac puncture as a secondary method. Mouse livers were flash frozen in liquid nitrogen immediately after collection. Blood was collected via cardiac puncture and collected in heparin coated tubes (41.1393.105, Sarstedt AG & co). Spleens and bone marrow were run through 40um cell strainers (352340, Falcon). Blood, bone marrow, and spleen had red blood cells were diluted 1:10 in ACK lysis buffer (118-156-101, Quality Biological) twice before cells were used for any assays.

*Cigarette smoke exposure*

Mice were exposed to cigarette smoke 5 days/week, 24 cigarettes daily, 11 puff/cigarette, 3 puffs/minute using the whole-body smoke exposure chamber. 30 ml puffs were given for 2 seconds following the exposure protocols from the International Organization of Standardization (59). Bias flow rate of 2 ml/minute was constant in between puffs air was given to the mice. 3R4F and 1R1F research cigarettes were purchased from the University of Kentucky, Tobacco Research and Development Center. Control mice were removed from normal housing with water and food restricted during SE of smoking mice. Flexiware Software V7 (SCIREQ) or newer was used to control and monitor the CSR and InExpose unites. Particulate density of air pumped into whole body chamber was monitored throughout exposures via Flexiware software. SCIREQ InExpose and SCIREQ Cigarette smoking robot (CSR) were housed in Radioisotope Fume Hood (6082200, Labconco) that was certified per the AIHA/ANSI lab ventilation standard with no air recirculation.

Calbiotech Mouse/Rat Cotinine ELISA kit (CO096D-100) was used to measure cotinine levels. Mouse urine samples were collected throughout the smoke exposure; matched non-smoking and SE samples were collected immediately following the daily SE. Samples were stored at -80C until thawed for assay. Plate absorbance was read on CLARIOstar Plus plate reader (BMG Labtech) at 450nm wavelength. Levels of cotinine were determined based on the logarithmic curve created from the standard values.

*Reduced Representative Bisulfide sequencing*

Genome wide methylation analysis: advances in methods for mapping of DNA methylation genome wide have recently became available and been proved highly efficient. Thus, we applied reduce representation methods to evaluate DNA methylation genome wide in genomic DNA of AML-bearing mice spleens. RRBS (Reduced Representation Bisulfite Sequencing) (60, 61) was utilized to resolve methylated cytosines in four AML-bearing mouse spleens or two MOLM13 samples and these data used to compare global changes in DNA methylation from SE or CSC treatment. Genomic DNA was obtained with PureLink Genomic DNA Mini Kit (Invitrogen, K1802001). Advantages of RRBS in comparison to other methods are that it allows for deep coverage of a subset of single CpG sites in CpG-rich regions and thus sensitive quantitation of methylation states, DNA amounts required for the method are as low as 1ng, and batch effects are easier to eliminate due to internal normalization. All steps in library preparation were performed using Ovation RRBS Methyl-Seq kit (Tecan Group Ltd., Zurich, Switzerland). In brief, up to 100ng of genomic DNA was digested with MspI, and Illumina-compatible cytosine-methylated adaptor were ligated to the enzyme-digested DNA. Size-selected fragments were bisulfite-converted, and library preparation done by PCR amplification. RRBS libraries were then visualized in Bioanalyzer High Sensitivity DNA chips (Agilent, Santa Clara, CA), and those passing quality control were subsequently sequenced as 75bp single-ended reads in an Illumina MiSeq200 instrument and 50 bases single-read protocol on Illumina HiSeq 3000 instrument for the CSC or SE samples respectively. 49-85 million reads were generated per sample.

Mapping: The adapters were removed from 3' ends of the reads by Trim Galore! (version 0.4.1) (62) and cutadapt (version 1.9.1) (63). Then the reads were mapped to mouse genome mm10 by the bisulfite converted read mapper Bismark (version v0.16.1) (64) and Bowtie (version 1.1.2) (65). 92-94% reads were mapped to the mouse genome, with 66-68% uniquely mapped. 33-56 million uniquely mapped reads were used in the final analysis. Methylation Calling: The methylation percentages for CpG sites were calculated by the bismark_methylation_extractor script from Bismark and an in-house Perl script. Differential Methylation: the differential methylation on CpG sites was statistically assessed by R/Bioconductor package methylKit (version 0.9.5) (66). The CpG sites with read coverage ≥ 20 in all the samples were qualified for the test. The significance of differential methylation on gene level was calculated using Stouffer's z-score method by combining all the qualified CpG sites inside each gene's promoter region (defined as -1000bp to +500 of TSS) and was corrected to FDR by Benjamini & Hochberg (BH) method.

*Visualization of RRBS data*

AHRR DNA methylation was prepared based on bedgraph and loaded into the UCSC Genome Browser (67). PDF image was downloaded, and Adobe Photoshop was used to add text labels for clarity. Bubble plots were used by using the GO terms for the genes from DAVID bioinformatics Resources 6.8. Then GO terms and associated FDR corrected p-values were analyzed using REVIGO R package (68). SimRel was used as the semantic similarity measure.

*TCGA analysis*

Raw bisulfite sequencing and patient survival were first described in Ley et al, 2013 NEJM. Only patients with DNA methylation sequencing data were included. High and low DNA methylation of gata2 was defined by being in the highest or lowest quartile of gata2 DNA methylation levels of the patients in the cohort. Log-rank test was performed on GraphPad Prism V8 or newer.

*Protein Assessment*

Samples were lysed using radioimmunoprecipitation assay (RIPA) buffer with added protease inhibitor (11836153001, Roche) and samples were run on polyacrylamide gels. Protein concentrations were determined using Bradford Assay (5000113, 5000114, 5000115, Bio-Rad) and bovine serum albumin (A7906, Sigma-Aldrich) was used to create the standard curve. Bottom well absorbance values were measured on CLARIOStar Plus at 590nm wavelength for Bradford assay quantification. Gels were then transferred onto polyvinylidene difluoride (PVDF, 620177, Bio-Rad) membranes. Anti-mouse (7076S, Cell Signaling) and anti-rabbit (7074S, Cell Signaling) horseradish peroxidase-conjugated secondary antibodies were used and detected on a ChemiDoc Touch imaging system (1708370, Bio-Rad). Antibodies used: HO-1 (Enzo, ADI-SPA-895), actin (Sigma A2066), GATA2 (Cell Signaling, 4595), BCL-2 (Cell Signaling, 15071T), Histone H3 (Millipore Sigma, 06-755), alpha-tubulin (Cell Signaling, 2125S), AHRR (Abcam, ab108518), DNMT1 (Active Motif, 61467), and gamma H2AX (Cell Signaling, 9718S).

*Flow cytometry*

All samples were run on the BD LSRFortessa flow cytometer with BD FACSDiva software. Cell viability was determined by negative staining with Ghost Dye Violet (13-0870, Tonbo Biosciences) for 10 minutes at room temperature. Cells were then stained with 10uM H2DCFDA (c6827, Life Tech), 10mM dihydroethidium (D11347, Life Tech), 500nM CellROX Deep Red, 5uM MitoSOX (Invitrogen, C10491) Red Mitochondrial Superoxide Indicator (M36008, Thermo) and 20uM monochlorobrimane (69899, VWR) protected from light for 30 minutes at 37°C. Fc receptor binding inhibitor polyclonal antibody (14-9161-73, Thermo Scientific) was used for 10 minutes before antibody staining of PBMC, bone marrow, and spleen samples. PE/Cyanine7 anti-mouse CD4 (100421,biolegend), PE/Cyanine7 anti-mouse CD8a Antibody (100721, Biolegend), PE/Cyanine5 anti-mouse Ly-6G/Ly-6C (108409, biolegend), PE/Cyanine5 anti-mouse/human CD11b Antibody (101209, Biolegend), PE/Cyanine5 anti-mouse/human CD45R/B220 Antibody (103209, Biolegend), Pacific Blue™ anti-mouse Ly-6A/E (Sca-1) Antibody (108119, Biolegend), PE/Cyanine7 anti-mouse CD150 (SLAM) Antibody (115913, Biolegend), APC/Cyanine7 anti-mouse CD117 (c-kit) Antibody (105825, Biolegend), APC anti-mouse CD48 Antibody (103411, Biolegend), PE/Cyanine7 anti-mouse TER-119/Erythroid Cells Antibody (116221, Biolegend), Flt-3/Flk-2/CD135 Antibody (A2F10) [DyLight 680] (NBP1-43352FR, Novus Biologicals), PE/Cyanine5 anti-mouse CD8a Antibody (100709, Biolegend), PE/Cyanine5 anti-mouse CD4 Antibody (100409, Biolegend), PE/Cyanine5 anti-mouse TER-119/Erythroid Cells Antibody (116209, Biolegend). Data were analyzed using FlowJo version 8 or newer.

Single cell splenocyte suspension from mice at day 17 post AML engraftment were used for cell mass cytometry. 1x10^6 cells/sample were washed with PBS, then surface antibodies (listed below) were added for 1 hour. Then samples were stained with 5uM Cisplatin (201192A, Fluidigm) for 5 minutes. A 1.6% Paraformaldehyde (50-980-487, Fisher Scientific) solution was used for 10 minutes at room temperature and supernatant was removed. Cold 100% methanol (A411, Fisher) was added to the samples and samples were stored at –80°C until 3 days prior to running. Samples were washed thrice in PBS with 0.5% bovine serum albumin (A7906, Sigma-Aldrich) with 0.02% sodium azide (2628-22-8, Sigma). The intracellular antibody mix (listed below) was added with the total volume being 50ul. Samples were incubated for one hour at room temperature. Samples were incubated 125uM Ir-intercalator (201198, Fluidigm) in 1.6% Paraformaldehyde overnight at 4°C. Samples were filtered through 35um capped cell strainers (352235, Corning) and stored at 4°C until run on Fluidigm Helios.

FlowJo was used to define viable cells, remove debris, and doublets. Markers with positive staining on positive control, untreated MOLM13 cells, were used in analysis. FlowJo was also used to export CD45 positive and negative populations within samples. Live singlets were exported as .fcs files onto R Studio 3.3.2. CyTOF Kit R package was used for Rphenograph analysis (69).CyTOF Kit R package was used for Rphenograph analysis (69).

Table 1 Mass Cytometry Antibodies

| TaggedAbs.description | cat | Source |
|---|---|---|
| Ly-6C 89Y | 128002 | BioLegend |
| CD45 112Cd | 3112001B | DVS-Fluidigm |
| ERK1 139La | MAB1940 | R&D |
| iNOS 141Pr (CXNFT) | 14-5920-82 | Invitrogen |
| B220 116Cd | 70-0452-U500 | Tonbo |
| DNMT3B 142Nd | 695202 | BioLegend |
| PARP (cleaved) 143Nd | 3143011A | DVS-Fluidigm |
| c-Myc 145Nd | 5605BF | CST |
| HIF-1a 147Sm | A700-001CF | BETHYL |
| Survivin 151Eu | AF6471 | R&D |
| HO-1 152Sm | ADI-OSA-110-F | Enzo |
| Caspase3(Cleaved) 153Eu | 9579BF | CST |
| p21 154Sm | P1484 | Sigma |
| RUNX1 155Gd | 659302 | BioLegend |
| IL-6 156Gd | 3156011B | DVS-Fluidigm |
| Bcl-2 159Tb | 658702 | BioLegend |
| Mcl-1 160Gd | 5453BF | CST |
| LSD1 161Dy | PA5-11308 | Invitrogen |
| TIM-3(Ms)162DY (MDA) | 70-5870-U500 | Tonbo |

| | | |
|---|---|---|
| IL-10 163Dy | 554704 | BD |
| CD34 164Dy | 343502 | BioLegend |
| IFNg 165Ho | 3165002B | DVS-Fluidigm |
| p-ERK 167Er (MDA) | 4370BF | CST |
| CXCR4 169Tm | 306502 | BioLegend |
| Bax(2D2) 171Yb | 633602 | BioLegend |
| Ki67 172Yb | 3172024B | DVS-Fluidigm |
| CD11b 173Yb | 301302 | BioLegend |
| p-FLT3 174Yb | 3464BF | CST |
| TNFa 175Lu | 3175023B | DVS-Fluidigm |
| CD11c(Ms) 209Bi | 3209005B | DVS-Fluidigm |

*Gene expression assessment*

mRNA was isolated using kit (Zymo Research, R1054) following manufacturer protocol and stored at -20 C until use. RNA and DNA concentrations were determined using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). 1000ug of RNA was used to generate complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-rad, 1708890). cDNA was diluted 1:1 with template buffer and 1ul per well was loaded per well with triplicates per gene. Forget-Me-Not EvaGreen qPCR Master Mix (Biotium, 31041) was used with primers listed in Table 2 below were used at 10uM concentration

for forward and reverse primers. Fluorescence of wells was read on LightCylcer 96 (Roche) instrument with 40 heat cycles with single melting peaks. Cycle threshold values were analyzed using LC96 software (Roche) and $2^{-\Delta\Delta CT}$ method compared to matched controls.

Table 2 Primer sequences

| **Primer** | **Sequence** |
|---|---|
| DNMT1 Forward | GGCGGCTCAAAGATTTGGAA |
| DNMT1 Reverse | CAGGTAGCCCTCCTCGGATA |
| DNMT3A Forward | CACCGGCCATACGGTGG |
| DNMT3A Reverse | GGTCTTTGGAGGCGAGAGTT |
| DNMT3B Forward | TGGAGCCACGACGTAACAAA |
| DNMT3B Reverse | GGTAAACTCTAGGCATCCGTCA |
| TET2 Forward | CTCAGCAGCAGCCAATAGGA |
| TET2 Reverse | CTGTCTGGCAAATGGGAGGT |
| HO-1 Forward | AAGACTGCGTTCCTGCTCAAC |
| HO-1 Reverse | AAAGCCCTACAGCAACTGTCG |
| GAPDH Forward | TGCACCACCAACTGCTTAGC |
| GAPDH Reverse | GGCATGGACTGTGGTCATGAG |
| Actin Forward | CTGTGGCATCCACGAAACTA |
| Actin Reverse | CGCTCAGGAGGAGCAATG |
| AHRR Forward | CTCCTTAAGCACAGACGGCA |

| | |
|---|---|
| AHRR Reverse | ACCACTCAATCTGGCACCTG |
| GATA2 Forward | GTCGTCCGAACCATCCCAAC |
| GATA2 Reverse | GGCTTACAGGGTAGGAGCTG |
| EXO5 Forward | ATCAGAGGTTAGGGGAAGGC |
| EXO5 Reverse | TGAAATGACAAGGCTCGGGAA |
| SGK3 Forward | GCAGTGCGTTCGGCTC |
| SGK3 Reverse | TCGGAGCTGGGAATGCTTAC |
| TICAM2 Forward | ACATTAACCCCTGACTCACAGC |
| TICAM2 Reverse | CTCACCCTTGGAGGTCGC |
| PANO1 Forward | TCCACAGATGTCCGGTGAAC |
| PANO1 Reverse | CAGCGGATGCTCCGATCC |
| NRF2 Forward | TTCCCGGTCACATCGAGAG |
| NRF2 Reverse | TCCTGTTGCATACCGTCTAAATC |

*Seahorse Metabolic Assays*

At least one day before assay was run, Seahorse plate was coated with 22.4 ug/mL CellTak (Corning, 354240) for 20 minutes, washed twice gently with sterile water, and then stored at 4C until day of use. Seahorse Cartridge (Agilent, 102416-100) was hydrated with sterile water overnight and then switched to Seahorse calibrant (Agilent, 100840-000) one hour before assay was run. Day of assay Seahorse media (Agilent, 103680-100) was

prepared with 10uM Glucose and 2mM L-Glutamine with pH 7.4. On day 7 or 14 of DMSO or CSC treatment, AML cells were seeded on Seahorse plate (room temperature). AML cells were seeded at 80,000 cells/well. ALL cells were seeded at 160,000 cells/well. For mitochondrial stress assay: Oligomycin was used at 1.5uM, Carbonyl cyanide-4 phenylhydrazone (FFCP) was used at 1uM, and Rotenone and Antimycin were used at 0.5uM in wells. For glycolytic stress assay: glucose free media used with added 2mM L-Glutamine with final pH of 7.4, Glucose was used at 10mM concentration, then Oligomycin was used at 1uM concentration, and 2-deoxy-glucose (2-DG) was used at 50nM. Plates were read on Seahorse XF Analyzer (Agilent) with Wave software.

*Statistics*

GraphPad Prisms version 7 was used for all other statistical analyses unless otherwise indicated.

## Chapter II: Cigarette Smoke and Condensate Exposure promotes AML progression

### Background

The relationship between cigarette smoking and cancer has mostly focused on lung cancer, which has left many other cancer types overlooked, including acute myeloid leukemia (AML). AML is one of the most commonly diagnosed forms of cancer in smokers. Cigarette smoking increases risk of developing AML by 1.4-fold for current and 1.2-fold for former cigarette smokers (18, 70, 71). Clinical studies have observed that a history of smoking also results in decreased survival compared to AML patients that have never smoked (19, 21, 72). While it has been clinically observed for decades that current and former smoking AML patients have worse survival, it is unknown how smoking impacts the progression of AML.

Studies that have observed worse survival in AML patients with any history of smoking have been retrospective studies and therefore have not monitored how the leukemia progressed in the different patients. This leaves the possibility that worsened survival in smoking AML patients is due to comorbidities associated with smoking rather than the aggressiveness of the disease. Furthermore, if smoking impacts the survival of patients that bear mutations that confer worse prognosis, such as the fms-like receptor internal tandem duplication (FLT3-ITD) mutation. It is unknown if difference in AML survival is due to changes in the rate of growth of the leukemia cells. Another possibility is that AML in smokers is more resistant to chemotherapy, rendering chemotherapy treatment less effective. This chapter details investigation of the effects of cigarette smoking on the progression and treatment response of FLT3-ITD AML.

**Results**

To understand if smoking worsened the survival of FLT3-ITD AML patients, we collaborated with physicians from the Department of Leukemia at UT MD Anderson Cancer Center to investigate the survival of FLT3-ITD AML patients with histories of smoking. A population of treatment naïve FLT3-ITD AML patients treated at UT MD Anderson Cancer Center whose smoking status was available was used. These patients were then classified as having never smoked (N=59) or had ever smoked (N=40) based on what was documented in their medical records. There were no significant differences in age of diagnosis or sex in never and ever smoking AML patients; nor were there major differences in their blast, blood, or platelet counts (Table 3). There was a trend of the ever smoker AML patients as being older than never smoked patients (p-value 0.07). By univariate analysis, ever smoked patients had no differences in cytogenetics or ELN 2017 risk classification (Table 3). There were no statistical differences between the type of treatment patients received (Table 3).

We assessed treatment response in FLT3-ITD AML patients differed with a smoking history. The overall complete response rates of ever smoked patients were decreased compared to patients that had never smoked (p-values: 0.005, 0.001 respectively; Table 4). Ever smokers had worse survival rates, with the median survival of ever smokers being 18 months versus 58 months for never smokers (P-value: 0.0092, *Figure 1*a). When patients who received FLT3 inhibitor-based treatment, there was a trend of possible decreased survival (p-value: 0.1366, *Figure 1*b). The median survival of ever smoker patients was 18 months compared to 26 for never smoker patients. While overall response to FLT3 inhibitor-

based treatment was not significantly impacted, the complete remission and complete remission with incomplete count recovery were significantly impacted (p-values: 0.03, 0.04 respectively; Table 4). The decreased response rates of ever smoking patients suggests that their AML is resistant to chemotherapy, but it is unknown if their disease progression was also impacted by smoking.

| Characteristic | Treatment Naïve FLT3-ITD$^{mut}$ AML | | |
|---|---|---|---|
| | *Never smoked* | *Ever Smoked* | *P* |
| **N** | **59** | **40** | |
| Age, years | 59 [29-83] | 68 [33-87] | **0.07** |
| Sex (Male) | 22 (37) | 17 (43) | 0.6 |
| De novo AML | 49 (83) | 29 (73) | 0.21 |
| sAML/t-AML | 10 (17) | 11 (28) | . |
| AML with dysplasia | 25 (43) | 18 (45) | 0.80 |
| BM blasts (%) | 70 [21-96] | 60 [15-93] | 0.16 |
| WBC (x10$^{9}$/L) | 7.3 [0.3-122] | 5.4 [0.6 -56] | 0.23 |
| Platelets | 27 [3-324] | 34 [2-205] | 0.12 |
| Cytogenetics | | | |
| Complex | 3 (5) | 5 (13) | 0.18 |
| Diploid | 35 (59) | 26 (65) | 0.57 |
| -7/-5 | 1 (2) | 3 (8) | 0.15 |
| 11q | 1 (2) | 1 (3) | 0.80 |
| Others | 16 (27) | 9 (23) | 0.60 |
| Insufficient | 6 (10) | 1 (3) | 0.14 |
| ELN 2017 | | | |
| Favorable | 21 (36) | 14 (35) | 0.95 |
| Intermediate | 16 (27) | 11 (28) | 0.97 |
| Adverse | 22 (37) | 15 (38) | 0.98 |
| Treatment | | | |
| High intensity | 37 (63) | 19 (48) | 0.13 |
| Low intensity | 22 (37) | 21 (53) | . |
| FLT3i-based | 37 (63) | 26 (65) | 0.82 |

Table 3 Demographic Characteristics of Treatment naive FLT3-ITD AML patients.

Values are n (%) or median [range]. High intensity treatment includes treatment with the combination of cytarabine and idarubicin or the addition of a nucleoside analogue to the combination. Low intensity treatment includes treatment with hypomethylating agents, low-dose cytarabine, or targeted therapies. Abbreviations: R/R AML, relapsed/refractory AML; t-AML: therapy-related AML; ELN, European LeukemiaNet; BM, bone marrow. T-test was used for continuous variable and chi-square for categorical variables.

| Best Response | Treatment Naïve FLT3-ITD$^{mut}$ AML | | |
|---|---|---|---|
| | *Never smoked* | *Ever Smoked* | *P* |
| ORR | 55 (93) | 29 (73) | **0.005** |
| • CR | 44 (75) | 17 (43) | **0.001** |
| • CRi/CRp | 9 (15) | 12 (30) | 0.08 |
| • PR/HI | 2 (3) | 0 | 0.24 |
| No response | 4 (7) | 11 (28) | . |
| FLT3i-based treatment (N) | 37 | 26 | |
| ORR | 35 (95) | 22 (85) | 0.18 |
| • CR | 26 (70) | 11 (42) | **0.03** |
| • CRi/CRp | 7 (19) | 11(42) | **0.04** |
| • PR/HI | 2 (5) | 0 | 0.23 |
| No response | 2 (5) | 4 (15) | **.** |

Table 4 FLT3-ITD AML patient response rates

Values are n (%). Abbreviations: N, number; ORR. Overall response rate; CR, complete remission; CRi, complete remission with incomplete count recovery; PR, partial response; HI, hematological improvement; FLT3i, FLT3 inhibitor. Chi-square analysis was used for categorical variables.

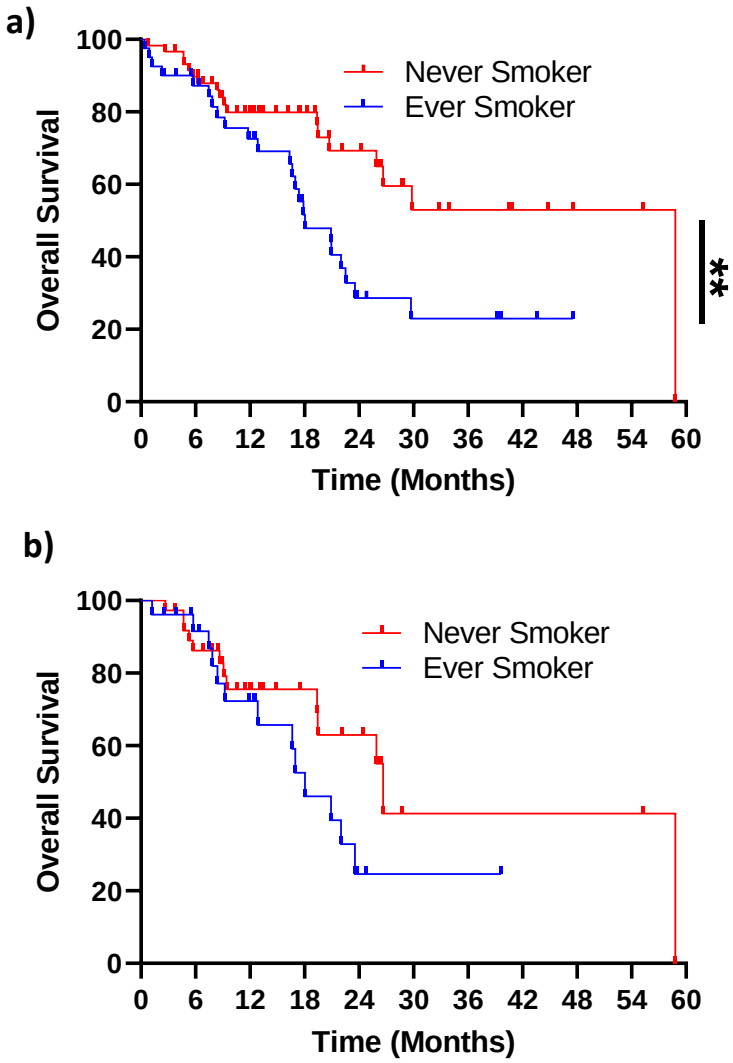



Figure 1 Ever and Never smoking FLT3-ITD AML patient survival

Ever and Never smoking FLT3-ITD AML patient survival. a) Treatment naïve FLT3-ITD AML patient survival with either general chemotherapy or FLT3-inhibitor based treatment for ever or never smoking patients (N=40,59 respectively; p-value: 0.0092). b) Treatment naïve FLT3-ITD AML patient survival with FLT3-inhibitor based treatment (N=37, 26 respectively; p-value: 0.137).

To study how cigarette smoking contributes to worsened AML survival, we developed models to study the impact of smoke exposure on AML bearing mice (Figure *2*a). NOD-SCID mice received cigarette smoke exposure (SE) starting two weeks before being challenged with luciferase-tagged human FLT3-ITD AML cell lines, while non-smoking control mice were exposed only to air and leukemic burden was monitored via bioluminescent imaging (Figure *2*a). SE mice experienced enhanced leukemic burden three days post injection, when evidence of engraftment can be detected in two FLT3-ITD cell lines, MOLM13 and MOLM14 ($p$-value<0.0001, <0.001 respectively; Figure *2*b,c). Increased leukemic burden was observed in MOLM14 24 days after AML introduction ($p$-value<0.05; Figure *2*d). Our SE model assumed continued smoking after AML is established, but we also wanted to understand how AML progression was impacted if smoking cessation was introduced (Figure *2*e). Smoking cessation mice had reduced leukemic burden one week after SE was halted compared to continued SE mice (Figure *2*f, $p$-value: 0.025). This result is promising rationale for AML patients to quit smoking even after being diagnosed with AML, but we needed to understand how closely our SE model reflected human smoking.

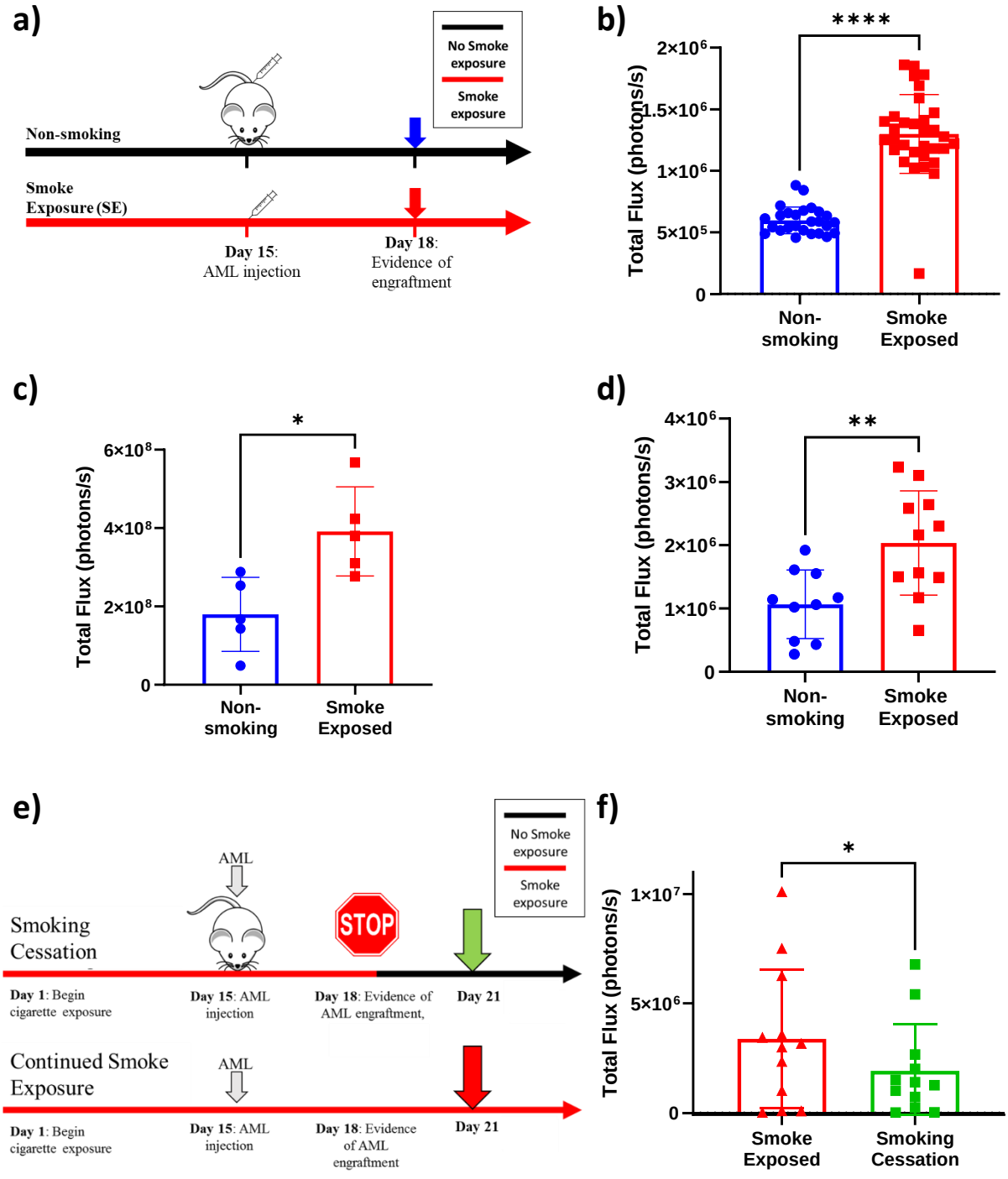



Figure 2 SE enhances leukemic burden

Figure 2 SE enhances leukemic burden

a) Schematic of SE and AML introduction timeline for mouse modeling of smoking. b) Bioluminescent flux shown of MOLM13 bearing mice (N=15,20). c) Leukemic burden of MOLM14-bearing mice 3 days post injection (N=7). d) Leukemic burden of MOLM14-bearing mice 24 days post AML introduction (N=5). e) Schematic of smoking cessation versus smoke exposed regimen. f) Leukemic burden of MOLM13-bearing mice 7 days after AML introduction (N=10).

We wanted to understand how the chemicals from cigarettes could impact AML progression in the absence of the high heat combustion during normal smoking. Luciferase-tagged MOLM13 cells were treated in vitro with 10ug/ml cigarette smoke condensate (CSC) or vehicle DMSO in vitro for two weeks, to mimic the two-week whole body SE that the mice received before AML was introduced into the mice, before being orthotopically introduced into mice (Figure *2*a). The CSC dosing was chosen based on a study that used the condensate in non-transformed human bronchial cells, this dose was not transformative, and after months of treatment induced changes in gene expression found in human lung cancer (73).

For our in vitro treated AML, leukemic burden was significantly enhanced throughout the experiment until mice became moribund (Figure 2b). This demonstrates that chemicals from cigarette smoke are impacting the AML to be more aggressive. Although, the in vitro cultured CSC treated cells did not show changes in proliferation or viability before or after the cells were introduced into mice (Figure 2c-d); this result was also seen in MOLM14 cells (Figure 2e-f). The CSC- induced enhanced leukemic burden is evidence that components of cigarettes promote AML to grow aggressively when introduced into the microenvironment.

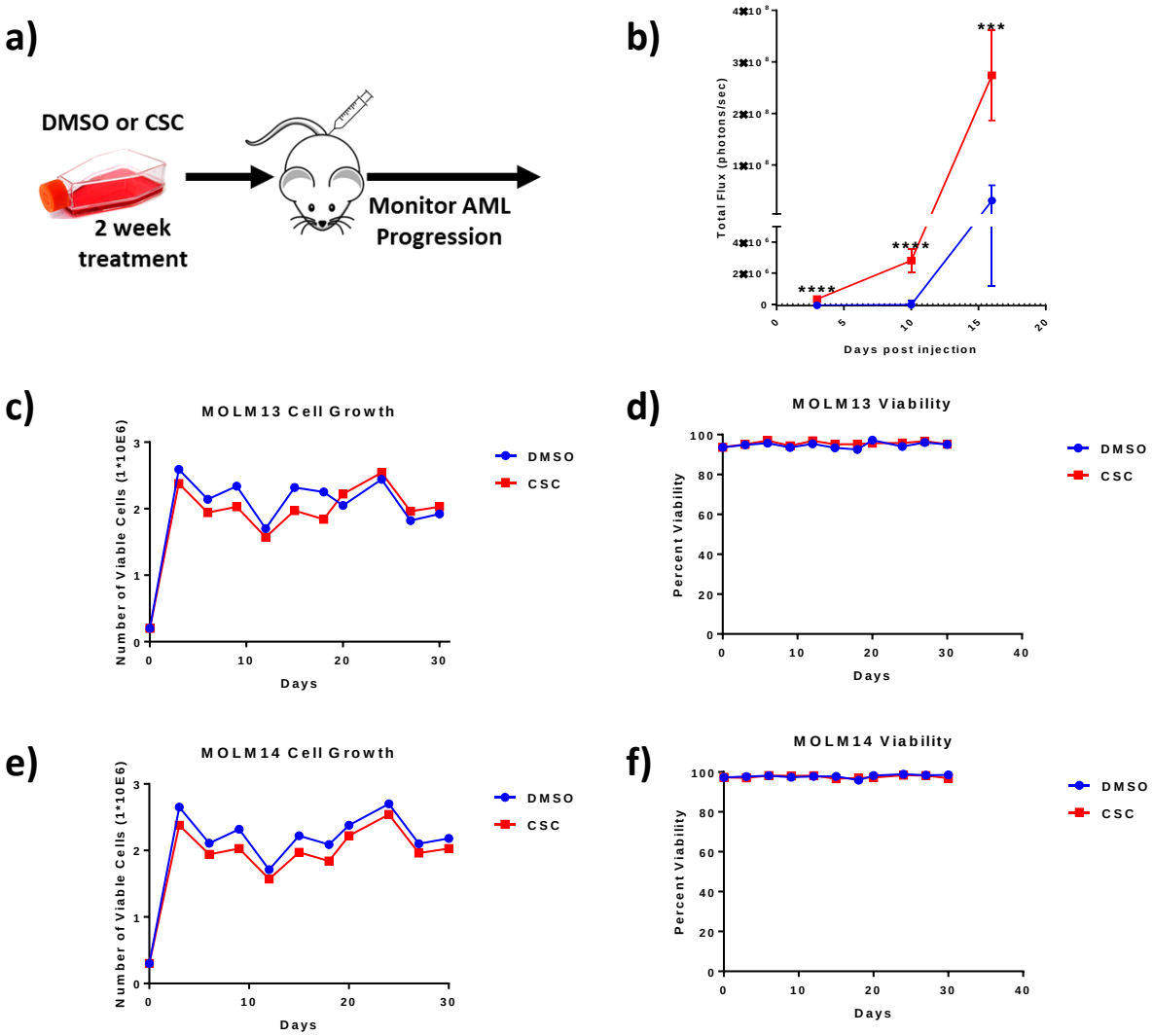



Figure 3 CSC-induced enhanced AML progression

a) Schematic of in vitro treatment before introduction into mouse. b) Bioluminescence of mice bearing MOLM13-luciferase tagged cells that were pre-treated with either DMSO or CSC. c) MOLM13 viable cell growth in vitro with DMSO or CSC treatment. d) MOLM13 cell viability in vitro with DMSO or CSC treatment. e) MOLM14 viable cell growth in vitro with DMSO or CSC treatment. f) MOLM14 cell viability in vitro with DMSO or CSC treatment.

Since FLT3-ITD AML patients had worse response to chemotherapy, we wanted to understand if chemotherapy treatment efficacy would be impacted by smoke exposure. We began investigating the impact that CSC would have on treatment response of daunorubicin, using the two-week DMSO or CSC we treated performed a 72- hour dose response experiment. We observed no major differences in the cell growth of MOLM13 and MOML14 cells treated with doses of daunorubicin ranging from 500-3.9 nM (

Figure 4Figure 4a-b). We also assessed treatment response to Quizartinib, a second-generation tyrosine kinase inhibitor, that is being used in clinical trials for AML patients with FLT3 mutations (74, 75). Quizartinib (5nM-39pM) treated DMSO and CSC cell did not show major differences in the rate of growth, viability, or sub G1 population (Figure 4c-h). Given that the CSC treated cells did not show differences in growth until introduced into mice, the tumor microenvironment is likely needed to closely mimic smoking in AML patients.

We next wanted to understand how treatment efficacy would be impacted with SE. A daunorubicin treatment, of 2mg/kg daunorubicin treatment twice weekly via tail vein, was added to our SE model to include a chemotherapy treatment used in AML patients. In the first biological replicate, there was increased leukemic burden in the SE mice treated with daunorubicin compared to the non-smoking mice (Figure 5a-b). Unfortunately, some of the non-smoking mice were lost due to non-leukemia related incidents that underpowered the results of the experiment. In the subsequent replicate of the daunorubicin treated AML-bearing mice, we did not observe a difference in leukemic burden (Figure 5c) that was seen in the first replicate. When data from previous smoke exposure experiments were pooled for comparison, the data indicates that the daunorubicin treatment regimen, frequency and or dose, is not enough to reduce leukemic burden compared to untreated mice (Figure 5d).

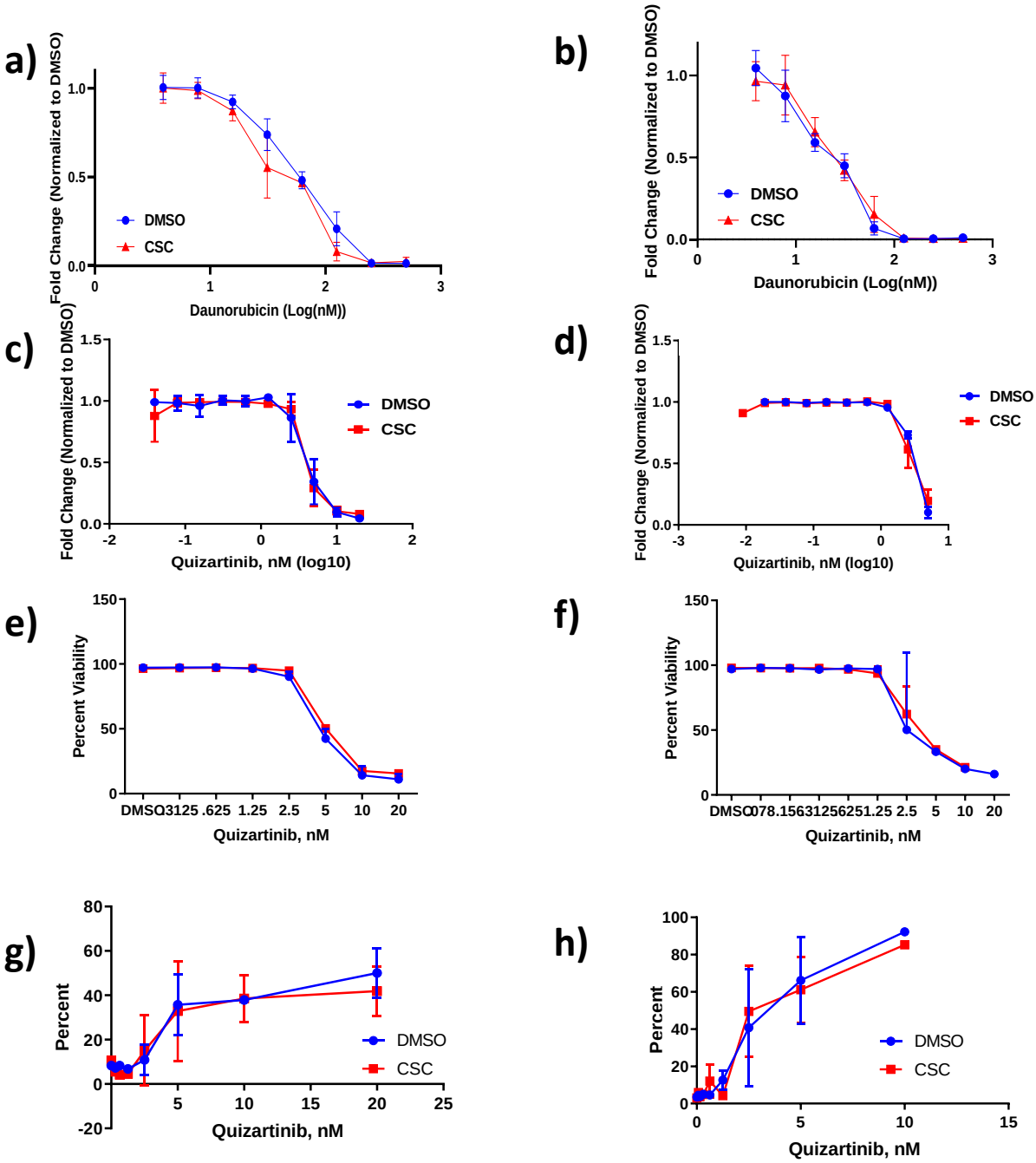



Figure 4 In vitro AML treatment response with smoke exposure

Figure 4 In vitro AML treatment response with smoke exposure.

a) 72- hour daunorubicin cell growth, measured by Alamar blue assay, of MOLM13 DMSO and CSC treated cells. b) 72- hour daunorubicin cell growth, measured by Alamar blue assay, of MOLM14 DMSO and CSC treated cells. c) 72- hour Quizartinib cell growth, measured by Alamar blue assay, of MOLM13 DMSO and CSC treated cells. d) 72- hour Quizartinib cell viability, measured by Alamar blue assay, of MOLM14 DMSO and CSC treated cells. e) 72- hour Quizartinib cell death, measured by ghost dye, of MOLM13 DMSO and CSC treated cells. f) 72- hour Quizartinib cell death, measured by ghost dye, of MOLM14 DMSO and CSC treated cells. g) 72- hour Quizartinib cell death, measured by sub G1 DNA via propidium iodine fluorescence, of MOLM13 DMSO and CSC treated cells. g) 72- hour Quizartinib cell death, measured by sub G1 DNA via propidium iodine fluorescence, of MOLM14 DMSO and CSC treated cells.

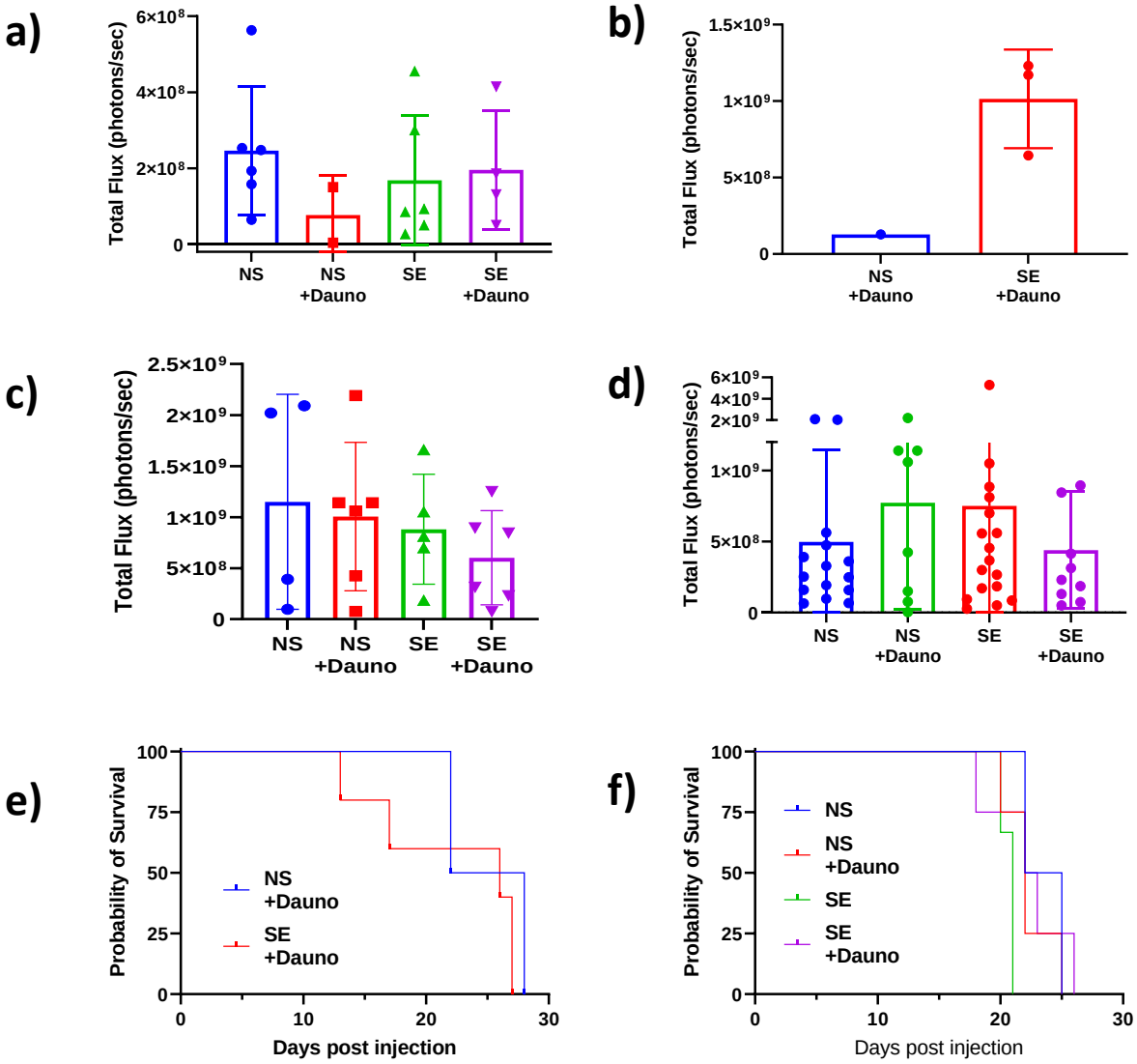



Figure 5 In vivo AML Daunorubicin treatment response with smoke exposure

Figure 5 In vivo AML Daunorubicin treatment response with smoke exposure.

a) Quantified bioluminescence imaging 17 days post AML introduction into mice, in untreated or daunorubicin treated NS or SE mice. b) Quantified bioluminescence imaging 24 days post AML introduction into mice, in daunorubicin treated NS or SE mice. c) Replicate 2: quantified bioluminescence imaging 17 days post AML introduction into mice, in untreated or daunorubicin treated NS or SE mice. d) Quantified bioluminescence imaging 17 days post AML introduction into mice, in untreated or daunorubicin treated NS or SE mice, including data from untreated NS and SE mice experiments. e) Survival curve of experiment from a-b. e) Survival curve of experiment from c.

Although we did not observe a difference in leukemic burden with daunorubicin treatment, we were interested to see if there were would an impact on survival. From the first experiment survival was used as an endpoint only in the daunorubicin treated mice. We did not observe a worsened survival in SE mice, but the experiment was underpowered as mentioned above (Figure 5e). When the experiment was repeated, the survival of daunorubicin treated mice was not extended compared to untreated mice (Figure 5f). The untreated SE mice deceased before all the other groups but was not statistically significant. Further studies will be needed in order to determine if AML treatment efficacy is impacted by smoking.

**Discussion**

This work is the first to describe that AML progression is enhanced by smoking. Several previous studies had only conducted retrospectively described worsened survival of smoking AML patients, but none have looked at the leukemic progression of smoking and non-smoking patients. Additionally, this work is the first to describe that a history of smoking worsens survival of FLT3-ITD AML patients; thus, describing a novel subpopulation of AML patients who have a worse prognosis than the already poor survival associated with FLT3-ITD AML.

Our data also bypasses possible differences in socioeconomic or racial disparities between ever and never smoker patients that may have affected the care in the AML patient cohorts from other studies. There are significant differences in smoking habits among

races, where Caucasian and Asian males smoke at higher rates than any other racial subgroup (76). Furthermore, almost 75% of smokers in the U.S.A. are at or below the federal poverty level (77, 78). This is important to consider since cancer patients from low socioeconomic status are diagnosed with more advanced cancer across different cancer types(79). All of these factors could contribute to worsened outcomes in AML smoking patients but were not a part of our animal experiments.

The enhanced leukemic burden from CSC treated AML when introduced into mice also highlights that the chemicals from cigarette smoking promote AML, which is relevant for other tobacco products like e-cigarettes that are deemed safer due to the lower burning temperature even though they produce many of the same chemicals as cigarettes (52). While other tobacco products were not utilized in this study, their use may be able to promote worse AML outcomes.

Since the FLT3-ITD AML patients had overall worse treatment response, this indicates that the leukemia is not responding to the treatment; thus, enhanced progression of AML is leading to worse survival in ever smoking AML patients, rather than being attributed to health complications related from ever smoking. The patient cohort was treated with chemotherapy and some also received TKI-based treatment as well. Overall, the decreased complete response rate to TKI-based therapy in addition to those treated with only chemotherapy suggests that smoking induces treatment resistance against general chemotherapy and targeted therapy.

Our in vivo and in vitro smoke exposure studies did not produce differences in treatment efficacy, but this is likely due to weaknesses within our models. Since we are modelling aggressive FLT3-ITD AML, our cell lines harbor this mutation and result in a short life expectancy for our mouse models. Our AML-bearing model is so aggressive that we may be missing differences in leukemic burden due to the rapid rate of leukemic growth within the mice. At later time points within our model, the levels of leukemic burden may be too high for differences between groups to be evident without being deadly to the mouse host.

The two daunorubicin treatment experiments in SE mice had contradictory results, which makes it hard to interpret the true impact of SE on daunorubicin efficacy. Our first replicate of the experiment suggested that SE mice did not respond to daunorubicin as well as non-smoking, but it lacked statistical power due to the low number of mice. It is important to note that we have consistently observed large variation in leukemic burden at later time points in the AML-bearing mice, so we may have lost mice that would have higher leukemic burden and mimicked the results in the subsequent experiment. In the second replicate there were two mice in the non-smoking daunorubicin treated group that had high leukemic burden, and they were difficult to inject; thus, they may not have consistently received the full dose of daunorubicin. Additionally, since the daunorubicin treatment failed to reproducibly reduce leukemic burden compared to untreated mice, a new treatment regimen is necessary before we can study the impact of SE on treatment efficacy.

Our in vitro AML experiments did not show differences in treatment efficacy with CSC treatment, but in vitro CSC treatment only demonstrated promotion of AML when

introduced into mice; thus, the tumor microenvironment is necessary to more accurately capture the effects that smoking causes in AML.

The FLT3-ITD AML patient data provides rational for a history of cigarette smoking to be used as a behavioral biomarker for AML patients who have poor prognosis, even within this subpopulation known to have poor survival outcomes. The establishment of a behavioral biomarker would be a novel, cost effective way to predict outcome, compared to sequencing and tailoring treatments based on mutations. When physicians are aware that an AML patient has any history of smoking, they can plan to give the patient more aggressive treatment compared to the standard treatment regimen.

Knowing that a history of smoking promotes the acceleration of AML has broad implications; this could lead to more careful observation of ever smoking AML patients by physicians, and potentially tailored treatment options to help improve the survival of ever smoking AML patients.

## Chapter III: Cigarette Smoke Exposure Alters DNA Methylation in AML

### Background

DNA methylation is a dynamic process and methylation of CpG sites on DNA is an important epigenetic modification used by cells to regulate chromatin accessibility and gene expression. Generally, it is thought that hypomethylation of a gene will increase expression of a gene and hypermethylation will decrease the expression. However, gene methylation does not strictly correlate inversely with expression as previously expected. To facilitate DNA methylation, DNA methyltransferases (DNMT) are a family of enzymes responsible for the addition of methyl marks onto CpG sites. Reversely, Ten-eleven translocation (TET) enzymes remove methyl marks from DNA through a series of reactions that convert 5-methylcytosine (5mC) and ultimately leave the cytosine unmodified. The location of DNA methylation also plays a role with the effect on gene expression. Several studies suggest that the methylation of transcription start sites is more predictive of the expression of the gene, rather than the overall gene DNA methylation levels. Furthermore, there are many different additional factors that can impact the expression of genes, such as enhancers, histone modifications, and levels of proteins that regulate transcription. Overall, transcriptional regulation is necessary for the proper maintenance of cells and its dysfunction can lead to abnormal growth advantages. DNA methylation patterns are one factor which are known to change through aging and can be impacted by various environmental factors (80). It is important to understand the significance of altered DNA methylation beyond healthy, homeostatic functions within healthy cells and apply our understanding to how transcription can be dysregulated in cancer.

Cancer cells are known to have dysregulated epigenetic marks, and non-mutational epigenetic reprogramming is being recognized as an emerging hallmark of cancer (81). Altered DNA methylation can be found in numerous types of cancer, including AML (38). In AML, a common mutation in AML is that of DNMT3A, which is an important protein in establishing DNA methylation. DNMT3A has important implications during development and cell differentiation (61).

AML is characterized by undifferentiated cells of myeloid lineage, and studies have found that inducing differentiation of leukemic cells can decrease leukemic burden. DNA methylation plays a critical role in the proper differentiation, and impairments in the DNA methylation can inhibit differentiation of hematopoietic cells. Given the importance of DNA methylation in differentiation, it is unsurprising that it is often dysregulated in a range of cancer types, including leukemia.

Global changes in DNA methylation have been observed from environmental exposures including cigarette smoke exposure. Cigarette smoking causes significant changes in DNA methylation in tissues beyond the lungs, including peripheral blood cells. This phenomenon of altered DNA methylation has been observed to persist decades after people have quit smoking (82). One of the best recognized hallmarks of smoking or smoke exposure is the hypomethylation of aryl hydrocarbon receptor repressor (AHRR) (83). AHRR has been found to be consistently hypomethylated due to smoking or cigarette smoke exposure in infants, children, and adults (83-85). While these global changes in DNA methylation have been observed, their role in cancer remains unknown.

Within AML patients it has been discovered that DNA methylation patterns have prognostic value, independent of mutations. Currently DNA methylation sequencing is not routinely conducted in cancer patients, but as bisulfite sequencing become more common it will be able to provide further insight into AML prognosis. DNA methylation patterns is an overlooked opportunity to gain insight into their disease progression of cancer patients. It is unknown what is driving the AML patients to have DNA methylation patterns associated with worse prognosis. Even though smoking is known to alter DNA methylation, there are currently no DNA methylation data sets of AML patients with smoking status available. In this chapter, we will investigate how cigarette smoking impacts the DNA methylation of FLT3-ITD AML using a mouse model.

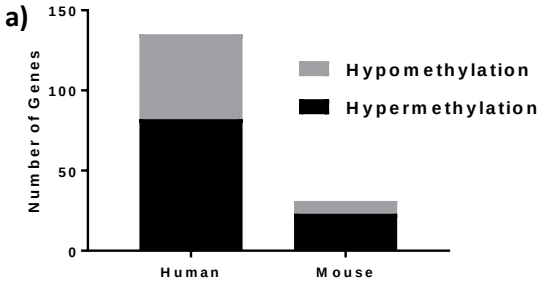
**Results**

To understand how smoking impacts AML DNA methylation, we started by performing reduced representative bisulfite sequencing (RRBS) on genomic DNA from MOLM13-bearing mice spleens 17 days post AML introduction. Over 200 genes had significantly altered DNA methylation from SE, and the DNA was matched to either the human AML or mouse genome (Figure 6a). There were many genes gains and losses of methylation, the top genes with altered DNA methylation are shown in Figure 6b. Notably, SE did produce a hypomethylation of AHRR which indicates that our SE model is able to mimic human smoking (Figure 6 c, d).

Subsequently, we wanted to comprehend what the cellular functions were associated with genes with SE-induced altered DNA methylation. Principle component analysis of the biological processes associated with the gene ontology terms of the genes with altered DNA methylation revealed that there were several pathways that had similar functions are to each other (Figure 6 e, f). For the bubble plots, the size of the bubble is related to the number of genes that are related to that biological pathway, while the color is related to the FDR corrected p-value of altered DNA methylation from SE. The distance is determined from semantic similarity scores of all the biological processes relative to all others on the graph. Many genes involved in transcription, differentiation, nucleic acid binding, differentiation, and response to reactive oxygen species had altered DNA methylation. These pathways whose genes had altered DNA methylation may provide insight into how smoking enhances AML.

Next we wanted to understand if CSC treatment was inducing changes in DNA methylation and how it compared to SE. Since the cells that produced enhanced leukemic burden when introduced into mice were only exposed to CSC for two weeks in vitro, we chose to investigate if this treatment was able cause changes in DNA methylation. RRBS uncovered that there were only twenty genes that had significantly altered DNA methylation from CSC treatment, but ten of those genes overlapped with genes that had SE-altered DNA methylation. Of those ten overlapping genes, eight genes were hypermethylated (Figure 6i); the other two genes had altered DNA methylation, but CSC and SE induced opposite directional changes in methylation.

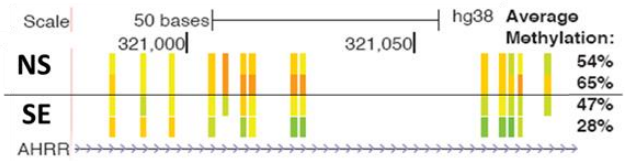
a)



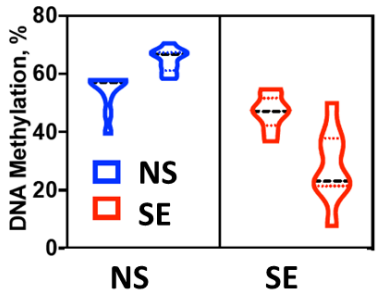

b)

| Hypomethylation | | Hypermethylation | |
|---|---|---|---|
| MBOAT2 | 4.37E-08 | SLC25A22 | 1.42E-14 |
| EXO5 | 1.46E-07 | COL4A1 | 1.42E-14 |
| GATA2 | 5.88E-07 | SGK3 | 4.10E-14 |
| CFAP70 | 1.14E-06 | 1-Mar | 5.69E-14 |
| ACOT4 | 1.33E-06 | PENK | 3.23E-13 |
| ADPRH | 1.20E-05 | MAP1LC3A | 4.24E-13 |
| AHRR | 1.49E-05 | BTBD6 | 1.51E-11 |
| TMEM176A | 3.29E-05 | STARD13 | 1.55E-11 |
| PDE4D | 3.38E-05 | TICAM2 | 2.18E-10 |
| MGMT | 1.20E-04 | PANO1 | 2.18E-10 |

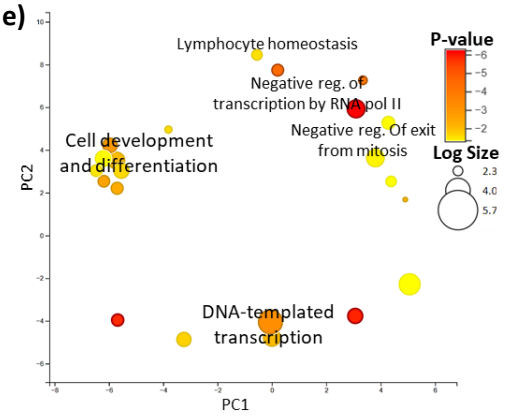
c)



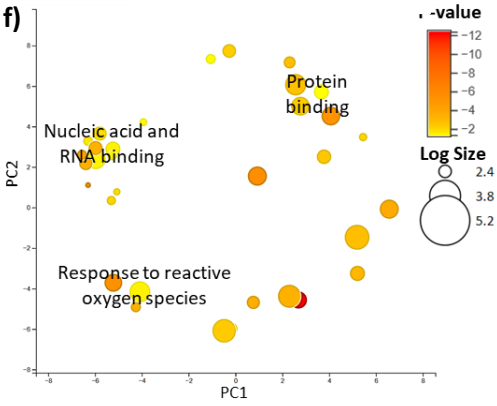

d)



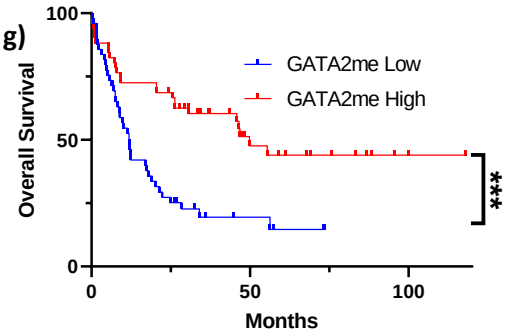

e)




f)




g)




h)

| | # of cases | # of cases, deceased | Median Months Overall |
|---|---|---|---|
| High GATA2 Methylation | 50 | 25 | 49.8 |
| Low GATA2 Methylation | 50 | 40 | 11.8 |
| High GATA2 Expression | 42 | 27 | 16.3 |
| Low GATA2 Expression | 43 | 30 | 15.8 |

i)

| | SE | CSC |
|---|---|---|
| Gene | p-value | p-value |
| MAP1LC3A | 2.0E-06 | 4.2E-13 |
| MGA | 2.0E-06 | 3.5E-06 |
| SFRP1 | 6.7E-05 | 1.2E-02 |
| METTL24 | 4.3E-03 | 3.4E-06 |
| PCP4L1 | 5.3E-03 | 2.0E-02 |
| LINC01122 | 8.0E-03 | 2.2E-04 |
| AGBL2 | 1.8E-02 | 5.2E-06 |
| IL15 | 2.8E-02 | 5.3E-06 |

Figure 6: CSE alters AML DNA methylation of genes that could be prognostic for AML patients. A) Histogram with total number of genes with altered DNA methylation across promoter regions in either the human, AML or mouse, host cells. B) Table with top hyper- and hypomethylated genes from CSE in AML cells. C) Graphic showing the methylation levels of a portion of *Ahrr* from 4 mouse samples. D) Violin plot showing overall DNA methylation levels for *Ahrr* from RRBS. E and F) Principal component analysis of hypomethylated or hypermethylated genes, respectively, based on GO terms and similarity scores. G) TCGA data from 10.1056/NEJMoa1301689 NEJM 2013 study. Patients with quartile of high or low *Gata2* methylation survival depicted (N=50/group; p-value: 0.0002). H) Table with genes with median survival of patients based on GATA2 gene methylation (shown in E) or GATA2 expression (data not shown). NS: Non-smoking; SE: Smoke Exposed. ***=p-value<0.001.

We looked for the gene expression of the top genes with altered DNA methylation in mouse spleens of MOML13-bearing mice from another biological replicate. We observed no statistically significant difference in the expression of the genes (data not shown). The RNA quality was also dubious as the quantification cycle (Cq) values for the loading controls were all >25. Since we had seen some concordance between SE and CSC altered genes we were interested in understanding if there were any changes in the expression of genes that had altered DNA methylation from SE. The expression of top genes with SE-altered DNA methylation was also investigated in CSC treated cells to see if chemicals from smoking may impact these genes. Quantitative PCR revealed that there were not changes in the gene expression in the CSC treated cells, in either MOLM13 or MOLM14 CSC treated cells (Figure 7a-b). This is not unexpected given that changes in DNA methylation do not strictly correlate to gene expression. No correlation was found when we probed the TCGA AML cohort from Figure 6g to see how the expression of GATA2 compared to the methylation levels (Figure 7c, d). Overall SE and CSC induced changes in DNA methylation, some of which were shared, and that several of the biological processes of those genes are relevant for AML.

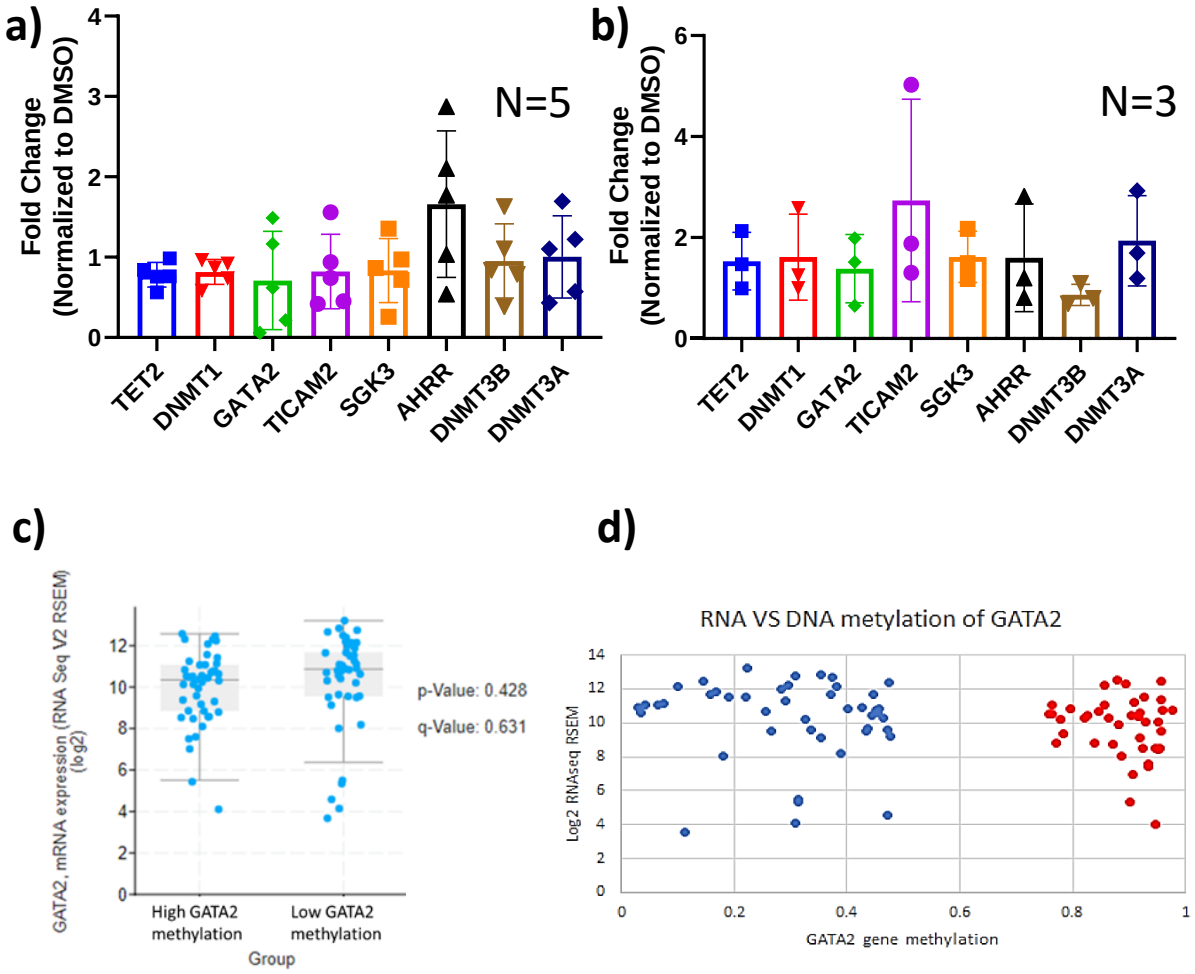



Figure 7 SE-altered DNA methylation did not change gene expression

a) Expression of select SE-altered genes in CSC treated MOLM13. b) Expression of select SE-altered genes in CSC treated MOLM14. c) Overall expression of GATA2 in high or low methylation from patients shown in Figure 6g. d) Expression versus methylation of GATA2 in patients shown in Figure 6g. Gene expression from C and D are based on RNA sequencing data from TCGA data from 10.1056/NEJMoa1301689 NEJM 2013 study and each group contains 50 patient values.

**Discussion**

From this chapter we have described that cigarette smoking can cause global changes in AML that are relevant to AML progression and survival. Currently there are no DNA methylation data sets of AML patients with smoking status available. Thus, it is unknown what driving the changes in DNA methylation associated with worse survival of AML patients. Therefore, understanding the causes of DNA methylation patterns that predict poor survival in AML patients may be an overlooked opportunity to gain prognostic insight into patients.

In Figueroa et al. 2013 they categorized AML patients into clusters based solely on their DNA methylation patterns and were able to find that these subgroups had different survival and that this was independent of mutational status of the patients (49). When comparing the altered DNA methylation from their clusters with worse survival, there were a couple of genes that also had SE-altered DNA methylation that overlapped with three clusters that had the worst prognosis, clusters 2, 8, and 15 (Table *5*). There were several genes that were similar or closely related between our SE and their clusters. It is unknown what is causing the changes in DNA methylation that predict worse outcomes in the AML patients from that study, but our model was able to induce a few of them even with our short-term SE exposure.

Similarly, our observation of GATA2 hypomethylation, independent of increased gene expression, in AML patients may be an indicator of poor outcomes. A study observed GATA2 hypomethylation in hematopoietic stem cells in aged mice compared to young mice,

but this study also found an increase in GATA2 expression (86). Another study found that upon fifteen months of  in vitro CSC exposure to bronchial epithelial cells, the CSC epigenetically primed the cells to become more tumorigenic when an oncogene was introduced in the cells; furthermore, GATA4 was among the genes that had significantly altered DNA methylation from CSC treatment (73). Both GATA2 and GATA4 have important roles in differentiation of tissue and are not normally expressed in the same tissue.  Their role in differentiation rather than their direct functions may make them good targets for dysregulation to then prime tissue to become cancerous.

| DNA methylation alteration | Gene | Cluster | Gene from Figueroa 2010 |
|---|---|---|---|
| Hyper | DNM3 | 2 | DNM3 |
| | GALP | 2 | GALP |
| | BTBD6 | 15 | BTBD3 |
| | N4BP3 | 15 | N4BP1 |
| | SMAD1 | 15 | SMAD3 |
| | TUBB8 | 15 | TUBB6 |
| | ZFHX4 | 15 | ZFHX2 |
| | PHF21B | 15 | PHF2 |
| | RBPMS | 15 | RBPMS2 |
| | ZNF432 | 15 | ZNF434 |
| | CYP26A1 | 8 | CYP26C1 |
| | THBS1 | 8 | THBS4 |
| Hypo | CACNG8 | 15 | CACNG5 |

Table 5 SE-altered DNA methylation compared to DNA methylation alterations associated with poor AML prognosis.

The left most column defines the directional change in DNA methylation that was found in both for the genes listed. Genes with altered DNA methylation from MOLM13-bearing mice in the left column and genes whose altered DNA methylation was found to be associated with poor outcomes in the right column (49). Three clusters with the highest hazard rations from the 2010 paper are listed in the middle column.

A notable SE and CSC hypermethylated gene is METTL24 (methyltransferase like 24). Methyltransferase-like (METTL) proteins have been found to be dysregulated in different cancer types, and recently a METTL3 inhibitor demonstrated preclinical efficacy in AML (87, 88). Thus, demonstrating a potential for alterations in epigenetic marks from health behaviors, including smoking, as prognostic indicators of treatment response is significant and warrants further investigation.

The concordance from CSC and SE induced altered DNA methylation is important and suggests that chemicals from cigarette smoke in the absence of high heat combustion, such as e-cigarettes, can mimic changes from cigarette smoking. E-cigarette use has not yet been studied in the context of leukemia, but our findings suggest that chemicals from cigarette smoking cause similar changes to cigarette smoking. There has been some interest in understanding how tobacco use, besides just cigarette smoking, impact DNA methylation. One study evaluated global changes in DNA methylation in buccal cells in the mouth of non-smokers, cigarette smokers, and participants that used moist snuff on a BeadChip array (89). This study found only 41 sites that had altered DNA methylation only in moist snuff users and had about 400 genes with altered DNA methylation shared with cigarette smokers. When the pathways of genes with altered DNA methylation from smoking and moist snuff use. Notably, they found that genes with tobacco use altered DNA methylation were involved with development, inflammation, cell growth, and mitotic regulation which overlap with many of the pathways we found to have altered DNA methylation in AML from SE (89). Another study has tried to assess changes in DNA methylation shared between

cigarette smoking, non-combustible tobacco use, and e-cigarette use in serum of healthy human participants (90). This study used methylation PCR to measure DNA methylation levels of several specific sites known to be altered in cigarette smoking, and they did not find similarities in methylation know to be altered from smoking, including AHRR. Hence, our data is relevant to e-cigarette use and its potential to induce alterations in DNA methylation. Discoveries from this work will provide insight into the impact of tobacco products in cancer.

There was no single biological process that was significantly altered with SE, likely due to the broad impact of smoking on global DNA methylation levels. The biologic processes associated with genes that had SE-altered DNA methylation, revealed many interesting pathways. Among the biologic processes with altered DNA methylation, there were many involved in nucleic acid binding, transcription factors, chromatin remodeling, response to reactive oxygen species, and differentiation of different cell types. Although these genes had altered DNA methylation we do not know if they impacted the expression.

Canonically, increased DNA methylation should decrease expression of a gene and vice versa, but this is not always consistent (30, 91). Traditional analysis of DNA methylation does not account for different populations within samples that have different genome methylation, which can inaccurately measure DNA methylation of genes. Advances in methylation sequencing analysis are attempting to understand how to overcome these challenges and improve the predicted gene expression based on DNA methylation (92). These techniques may eventually be applicable for understanding changes within cancer

clones that drive tumor progression. RRBS is an important tool for evaluating DNA methylation, but there are SE-induced epigenetic changes that RRBS is unable to detect. RRBS is focused on promoter regions of genes and would not detect DNA methylation changes in enhancer regions found outside of gene promoters. Several studies have noted the importance of enhancer DNA methylation in altering gene expression (25, 26, 93, 94). Specialized software to help interpret DNA methylation and gene expression has also demonstrated the importance of gene enhancer DNA methylation in regulating gene expression (95).

Beyond changing the expression of specific genes, SE-altered DNA methylation can cause changes to chromatin structure that impact the accessibility of DNA regions and thus the ability for them to be transcribed. Chromatin interactions can be evaluated through other methods, such as Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) (96). Another possible change from smoking is altering hydroxymethylcytosine (5hmC) levels. Changes in 5hmC would be undetected through RRBS, because oxidative bisulfite sequencing is needed to distinguish between these DNA modifications (97). More investigation, and perhaps multiple concurrent techniques, are needed to understand the full impact of smoking-induced altered DNA methylation.

In this chapter we have focused on the DNA methylation changes of AML cells specifically, because these have been described in other work and the AML cells experienced most of the changes in DNA methylation. Although there were changes in DNA methylation in the mouse tumor microenvironment, changes in normal spleen tissue of

AML patients is not documented and makes it difficult to draw any conclusions from any comparisons. Additional examination of altered DNA methylation in cells from the tumor microenvironment should be explored in AML patients.

## Chapter IV: SE alters hematopoietic cells populations and antioxidants in the microenvironment

### Background

While it is known that cigarette smoking increases incidence of AML, the impact of cigarette smoking on the tumor microenvironment is not well understood (18, 70). Leukemia develops in the bone marrow microenvironment. Within bone marrow, hematopoietic stem cells (HSCs) are continuously producing cells for the immune system, when a mutation occurs in an HSC there is clonal expansion of cells that carry these mutations. This makes HSCs susceptible to creating malfunctioning progenitor cells that can disrupt the production of normal hematologic cell populations. A pre-neoplastic example of this, is clonal hematopoiesis of indeterminate potential (CHIP). Clinical observations have found that smoking increased occurrence of CHIP (98). CHIP is not considered a pre-malignant state, but it can develop into MDS and or AML.

The impact of smoke exposure on bone marrow is not fully understood, yet smoking is associated with increased white blood cell counts. (99). One study found that in human smokers there were decreased progenitor cells in the bone marrow, but this human study had few participants, the participants were had a range of age, and the duration of smoking was not controlled for in their smoking population (100). Similarly, another study conducted in mice with 9 months of SE observed decreased hematopoietic stem, progenitor, and mesenchymal stromal cell populations compared to control mice (101); this study also observed that SE mesenchymal stromal cells were able to expand HSCs when co-cultured in vitro. These studies broadly looked for changes in hematopoietic stem cells and did not

examine hematopoietic multipotent progenitor cells that would give insight into the types of mature cells that would be produced in the bone marrow.

To understand how smoking may alter the bone marrow microenvironment to promote AML in humans, C57BL/6J mice were used to evaluate the impact of cigarette smoking in an immunocompetent mouse model. There have been studies that have used these mice for smoking studies, but they have focused on the impact on the lungs, asthma, and airway epithelial cells (52, 102, 103). Importantly, our study differs in that we chose to assess how myeloid progenitor and myeloid blast cells, the cells in which AML arises, are affected by smoke exposure.

Our AML-bearing SE model utilizes non-obese diabetic severe combined immune deficiency (NOD-SCID) mice, which has not been used in studies related to cigarette smoking. NOD-SCID mice bear the *Prkdc*$^{scid}$ mutation that leads to immunodeficiency that is characterized by absence of mature B and T cells (104). We chose to utilize immunodeficient mice because they can be used for xenograft models and allowed for the use of human, luciferase-tagged AML cells. Leukemic burden can be easily measured through bioluminescent imaging. This is in contrast to identifying CD45 positive cells via flow cytometry from bleeding the mice; which can place undue stress on the animals, especially as they are handled daily. The impact of smoking has not been previously evaluated in NOD-SCID mice.

We also wanted to understand if our SE model was capable of recapitulating changes that occur in human smokers. In humans and rodents, smoking increases levels of

cotinine, the major metabolite of nicotine, within serum and urine of smokers and those exposed to cigarette smoke. To confirm a significant level of smoke inhalation in the mice, we utilized this clinical parameter to equate our SE to human smoking. Another interesting finding from human studies is that cigarette smoking and tobacco products cause oxidative stress through increased levels of ROS and by altering antioxidant responses (53, 105-107). Heme-oxygenase-1 (HO-1) is a small heat shock protein and antioxidant transcribed by NRF-2 in response to increased cellular ROS and known to be elevated in FLT3-ITD+ AML. Also, within bladder cancer patients, patients who smoked had increased levels of HO-1 that positively correlated with the intensity of their smoking habits (108); in the same study, patients with high HO-1 expression had more aggressive cancer progression. High expression of HO-1 has also been associated with worse survival in AML patients (109). Thus, SE-mediated HO-1 expression may play a role in aggressive AML. Given our knowledge of the molecular changes that occur in humans from smoke exposure, we began by looking at the levels of cotinine, ROS, and HO-1, to examine how smoke exposure is

In summary, this chapter will examine how cigarette smoking alters the hematopoietic cell populations and molecular changes in the microenvironment.

**Results**

We began by using immune competent C57BL/6J mice to evaluate the impact of cigarette smoking on hematopoietic populations within the bone marrow. After four and a half weeks of smoke exposure, the time point when SE AML-bearing mice would typically begin becoming moribund, the non-leukemia bearing immunocompetent mice were sacrificed. When cell lineage was evaluated, only myeloid cells were significantly increased in response to smoking (Figure 8a). There was increased lineage negative, sca positive, c-kit positive (LSK) cells, but not in the long-term hematopoietic stem cell (HSC) or multipotent

progenitor 1 (MPP1) cells (Figure 8b). There was also increased MPP3 and MPP4 cells, which are likely to differentiate into mature myeloid cells (Figure 8c) (110). Subsequently we evaluated the impact of SE in the immunodeficient NOD-SCID mice that we had used for our AML-bearing models. When the bone marrow of these mice was evaluated in a pilot experiment, it appeared that there may be an increase in LSK, MPP2, and MPP3 cells (Figure 9

Figure 9a). When the experiment was repeated with more mice, the differences were not observed in different cell populations (Figure 9b). The samples were stained by two different people and on two different flow cytometers. Additionally, only the second replicate used an Fc-block to reduce non-specific antibody binding. Even with these

differences between the assay performances, both replicates resulted in similar insignificant differences in hematopoietic stem cell populations (Figure 9c).

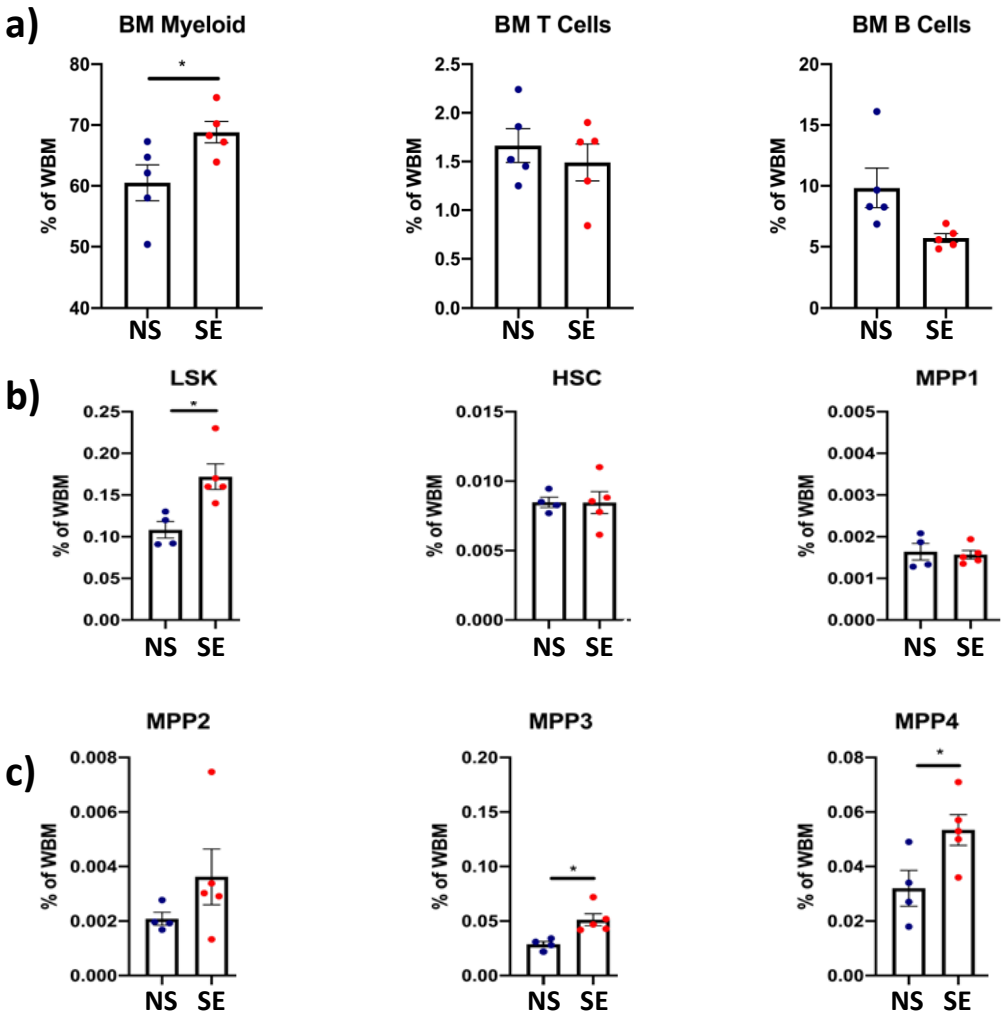



Figure 8 Hematopoietic cell populations in the bone marrow of immune competent mice.

a) Lineages of cells within the bone marrow. b) Percentages of whole bone marrow with stem cell populations. c) Multipotent progenitor populations in bone marrow of non-smoking or smoke exposed mice.

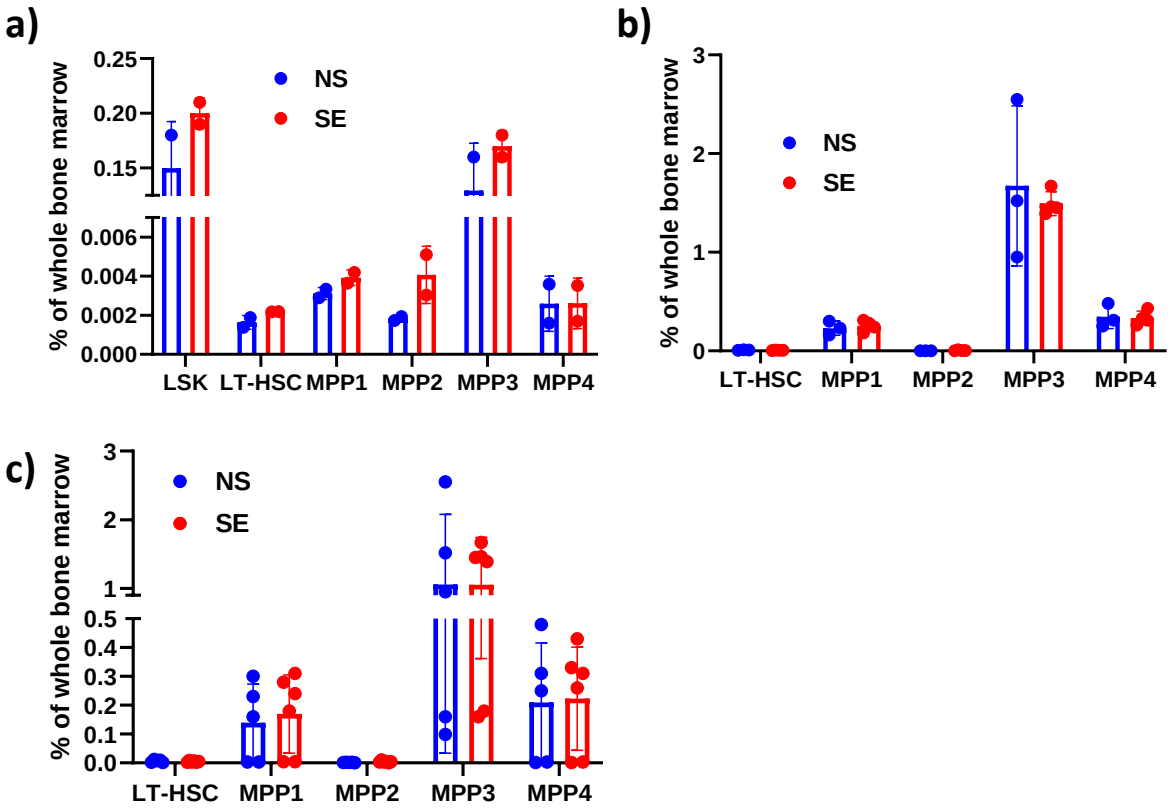



Figure 9 Hematopoietic cell populations in the bone marrow of immune deficient mice

a) Hematopoietic stem cell populations from first replicate (N=2). b) Hematopoietic stem cell populations from second replicate (NS=3, SE=4). c) Combined hematopoietic stem cell populations from a) and b).

Throughout these experiments, urine samples were collected immediately following daily SE to measure cotinine levels in mice. Cotinine levels were significantly elevated throughout the experiment in SE mice (Figure 12a). Comparing the SE level of urine cotinine in humans would be found in moderate smokers (111, 112).

Next we evaluated differences in reactive oxygens species of SE mice. Using immunocompetent mice with one week of smoke exposure, blood was collected immediately following daily SE. Peripheral blood mononuclear cells (PBMCs) levels of general peroxides were not altered, but there was a trend of increased superoxide levels (p-value: 0.087; a, b). Four and a half weeks after SE had begun, the mice were sacrificed and their PBMCs and spleen ROS levels were examined. In these sample there were no significant differences in general peroxide or superoxides with SE (Figure 10c-d).

When immune deficient mice were examined for ROS levels, this was conducted when the mice began to display moribund symptoms approximately five weeks after SE began. When PBMCs and spleen samples were evaluated for ROS levels, there were no significant changes in general peroxide or superoxide levels (Figure 11a-d).

Subsequently we evaluated oxidative stress in tissue samples from AML-bearing SE mice. At the time of euthanasia, spleens and PBMCs from SE mice did not show significantly increased ROS levels via flow cytometry. We evaluated mouse tissue for changes in proteins involved in oxidative stress response. HO-1 expression as measured by immunoblotting in spleens from NS versus SE mice was elevated (Figure 12b-d). Increased presence of antioxidant response element, HO-1, indicated that there was dysregulated redox with SE.

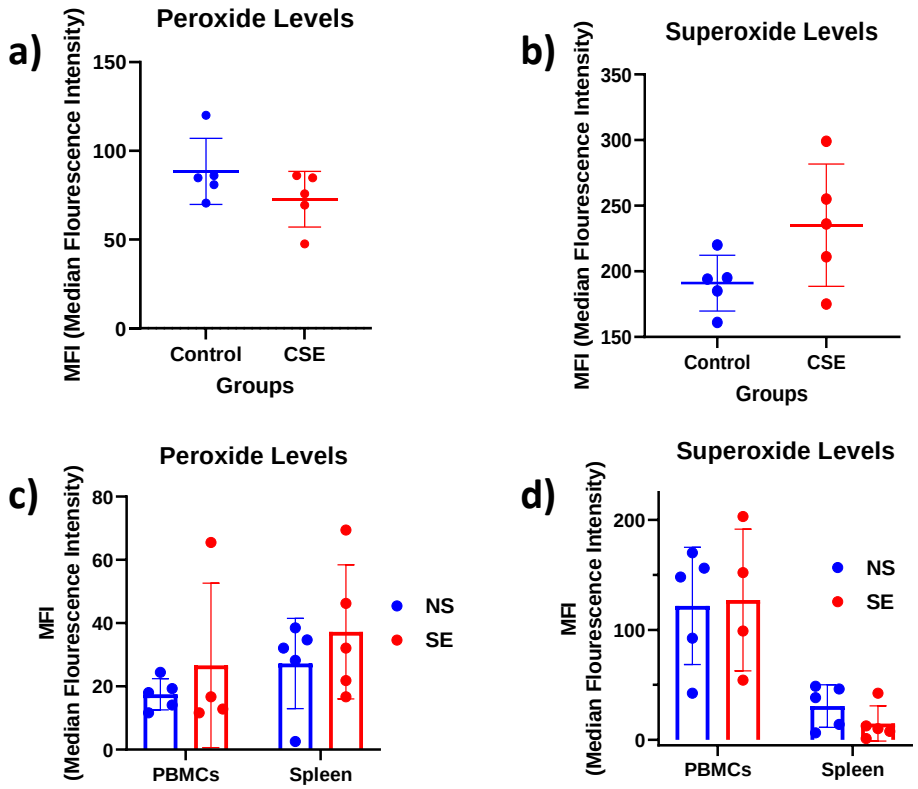



Figure 10 Levels of reactive oxygen species with SE in immunocompetent mice

a) Levels of intracellular general peroxides in PBMCs. b) Levels of intracellular superoxides in PBMCs. c) Levels of intracellular peroxides in PBMCs or spleen samples after 30 days of SE began. d) Levels of intracellular superoxides in PBMCs or spleen samples after 30 days of SE began.

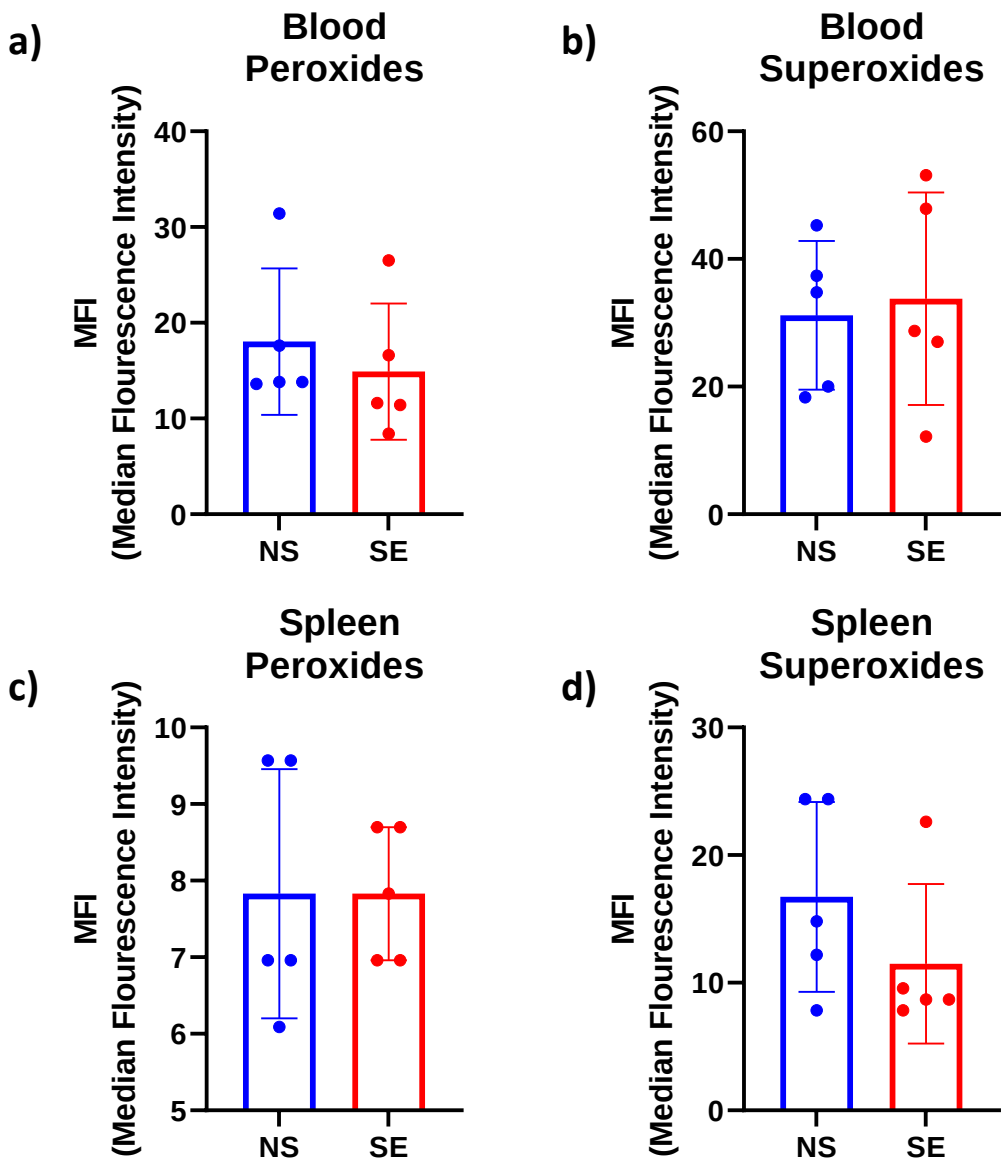



Figure 11 Levels of reactive oxygen species with SE in immunodeficient mice

a) Levels of intracellular general peroxides in PBMCs 16 days after leukemic introduction. b) Levels of intracellular superoxides in PBMCs 16 days after leukemic introduction. c) Levels of intracellular peroxides in spleen samples 16 days after leukemic introduction. d) Levels of intracellular superoxides in spleen samples 16 days after leukemic introduction.

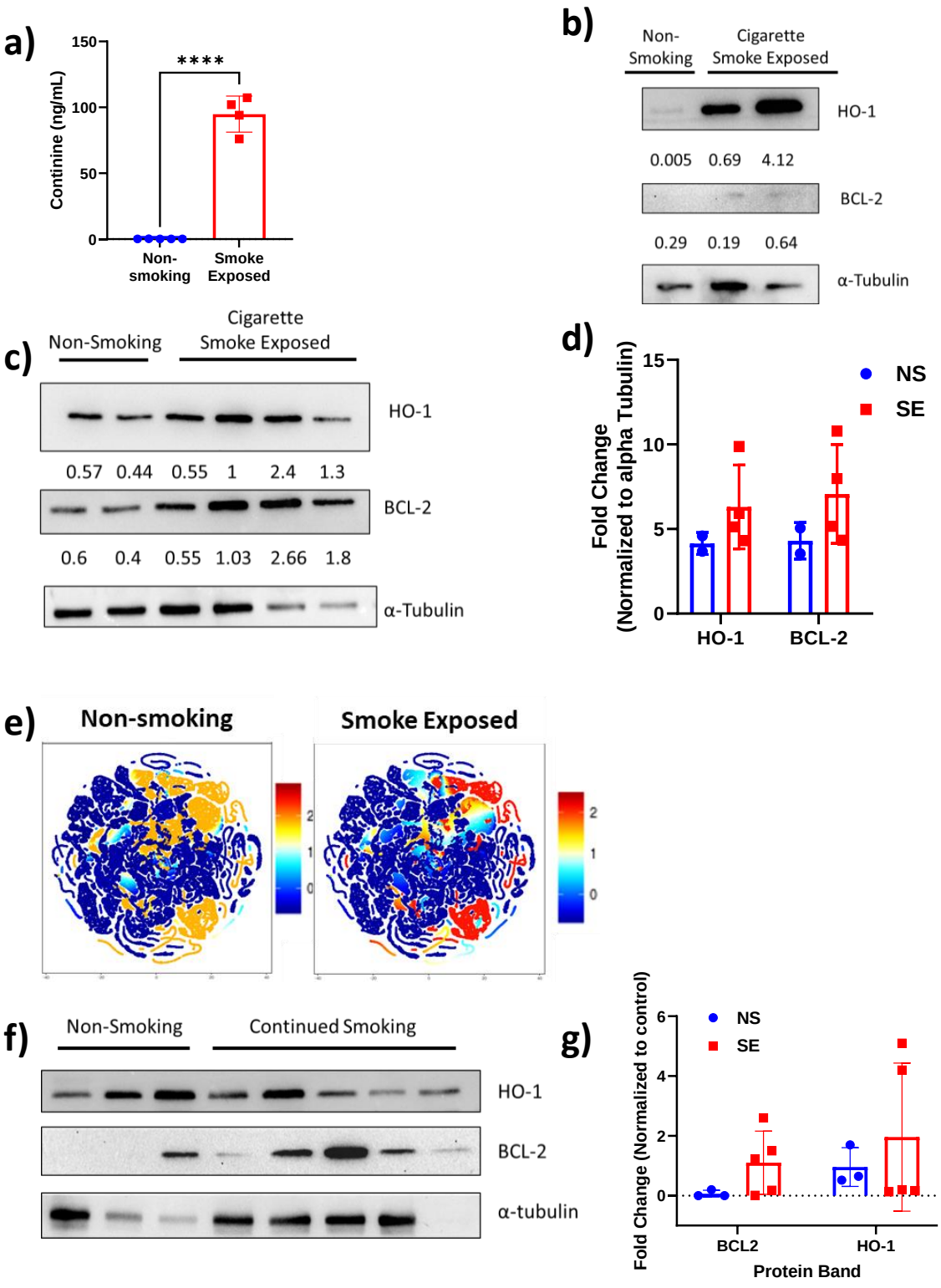



Figure 12 Cotinine and protein changes in AML-bearing mouse spleens with SE

Figure 12 Cotinine and protein changes in AML-bearing mouse spleens with SE

a) Urinary cotinine levels as measured by rodent ELISA. b) Western blot of MOLM13-bearing mouse spleen. c) Different western blot of MOLM13-bearing mouse spleen. d) Quantification of protein changes from b) and c). e) Tsne plot of HO-1 expression of MOLM13-bearing mouse spleen from mass cytometry. f) Western blot of MOLM14-bearing mouse spleen. g) Quantification of protein changes from immunoblot show in f).

Furthermore, mass cytometry revealed increased HO-1 in SE MOLM13-bearing mice (Figure 12e, N=6/group). Elevated HO-1 has been attributed to aggressive AML progression, so we evaluated the expression of other proteins associated with poor survival outcomes in AML patients. SE MOLM13 samples had increased expression of BCL-2, which has been linked to poor survival in AML patients (Figure 12b-d). This trend of increased expression of HO-1 and BCL-2 was also seen in samples from SE MOLM14 mice (Figure 12f, g). Increased expression of HO-1 and BCL-2 are both associated with worse outcomes and treatment resistance in AML (108, 109, 113, 114).

**Discussion**

In AML, the tumor microenvironment can contribute to the development and progression of cancer; therefore, it is critical to understand how smoking impacts the cellular components of the tumor microenvironment, the myeloid progenitor cell population, as well as changes in redox that can promote AML.

While increased leukocytes are commonly observed in smokers, these studies have not extensively investigated changes within the bone marrow of smokers. Another factor to consider when investigating the bone marrow microenvironment is age, since aging reduces the amount of hematopoietic cells in the bone marrow (115). Controlled animal studies can better account for these factors in comparison to human studies.

In our studies, we found that SE alters the bone marrow microenvironment to promote the growth of myeloid cells in immunocompetent mice. Similarly, we have previously shown using a different smoking inhalation model that exposed C57BL/6 mice to 4 cigarettes per day for 4 months, resulted in increased myeloid cells in the bronchoalveolar lavage fluid (116). The lack of changes in the immune deficient mice suggests that a fully functioning immune model is critical to capture changes in hematopoiesis that may be relevant to humans.

The increased urinary cotinine levels in our SE mice demonstrate that our smoking model is eliciting changes in mice that are expected. Based on the cotinine levels, the mice have levels of cotinine similar to those that have moderate human smokers (111, 117, 118); although this should not be used as a basis for a strict comparison, since there are differences in human and rodent metabolism. The full effects of SE on the mice need to be evaluated with other markers.

When oxidative stress was assessed, changes in ROS were not observed in samples from immune competent or immune deficient mouse samples. The trend of increased superoxides was only seen in blood samples from mice immediately following their smoke exposure. At time of sacrifice, the mice are at least 24 hours after their last smoke exposure, which may be too long after the exposure to detect increased ROS from SE. ROS are highly unstable and transient, making it difficult to capture.

Effects from elevated ROS, such as antioxidant responses may be a better indicator of oxidative stress. HO-1 is an antioxidant response element that is increased in response to

elevated ROS, so it is a more stable indicator of oxidative stress (119, 120). Elevated HO-1 may be occurring as an adaptive response to the continued SE. These changes were observed along with increased expression of BCL-2, an antiapoptotic protein. Since SE increased BCL-2 levels, smoking AML patients may be good candidates to receive BCL-2 inhibitor treatment, such as Venetoclax. The increased expression of HO-1 and BCL-2 proteins suggests that SE is inducing expression of proteins that increase leukemia progression and are involved in treatment resistance in AML. This emphasizes the potential utility of understanding the mechanism of smoking-enhanced AML progression.

## Chapter V: Changes in protein expression and antioxidants in AML from SE

### Background

While the link between smoking and AML has been observed clinically, no studies have defined the changes induced in AML from smoking. Retrospective studies have linked complex karyotypes and RUNX1 mutations to smoking in AML patients, but there has been no mechanism linking smoking-induced changes to worse survival (19, 121). Herein I will describe changes in AML from smoking that promote leukemic progression.

External sources of ROS, such as from dysfunctional adipocytes or smoking, can increase intracellular cellular ROS within AML. As described in the previous chapter, we saw increased levels of HO-1 and BCL-2 induced by SE, and expression of these proteins are both linked to worse survival in AML patients. These increases were seen in samples that contained a mixture of mouse and human AML cells; so, it is unknown if this increased expression is happening within the host mouse cells, AML cells, or both.

There are proteins that promote AML progression and treatment resistance. In addition to redox response and epigenetic signaling, we were curious to know how the expression of proteins involved in activation of proliferative signaling and anti-apoptotic proteins were altered with SE. For example, the activate phosphorylated FLT3 (p-FLT3) receptor promotes proliferation in AML. By understanding how cellular pathways are being impacted in AML from smoking, we will gain a sense of ways that smoking is aiding AML.

It is important to understand if smoking is working to promote AML cells broadly, or if there are subpopulations of AML that are responding more readily to smoking. So far, our

data has shown increased leukemic burden with SE and CSC treated AML cells, so both cigarette smoke exposure methods enhance AML progression. CSC enhanced leukemic progression demonstrates that AML cells are promoted by the chemicals in cigarette smoke and that it is not solely from smoking priming the microenvironment for AML to develop. We also showed that continued smoke exposure increased leukemic burden compared to mice with smoking cessation, which demonstrates a positive correlation in leukemic progression with SE. The specific molecular changes from smoking in AML are unknown, and in this chapter will explore oxidative stress and the expression of proteins that promote AML progression from SE and CSC. Understanding these changes will provide novel insight into how smoking that is promoting AML.

**Results**

In vitro CSC treated cells resulted in enhanced leukemic burden when introduced into mice, without any smoke exposure to the tumor microenvironment (Figure 3b). We wanted to understand what changes were occurring with the two-week CSC treatment that allowed the AML to rapidly expand once in the mice. We began by comparing the protein expression of CSC treated cells. We were curious to understand if there were changes in expression of genes with SE-altered DNA methylation or genes involved in redox in AML cells treated with CSC. No consistent changes in expression of HO-1, NRF-2, genes with altered DNA methylation, or genes involved in regulation of DNA methylation (Figure 13a, b). When protein expression of HO-1 was probed through immunoblotting, there were inconsistent

changes found at the 14 day CSC time point in MOLM13 and MOLM14 cell (Figure 13c, d). Although, there were significantly increased levels of general peroxides (DCF), mitochondrial superoxides (MitoSox), and glutathione (mBCL) as detected by flow cytometry after two weeks of exposure to CSC in MOLM13 and MOLM14 cells (Figure 13e, f). Being cultured continuously in the CSC may allow for elevated ROS to be captured, which we were unable to observe with SE.

Given the increased mitochondrial superoxide levels, we investigated the mitochondrial respiration rates. No significant changes were observed in the oxygen consumption rate in MOLM13 or MOLM14 CSC treated cells when mitochondrial respiration was tested (Figure 14a, b). We did discover consistent significant increased extracellular acidification rates (ECAR) during the mitochondrial stress assay in MOLM13 and MOLM14 cells treated with CSC treatment (Figure 14c, d). This led us to investigate the glycolytic rates with CSC treatment. ECAR of CSC treated cells was elevated in MOLM13 and MOLM14 cells after 7 and 14 days of treatment (Figure 15a-d). The fold change in ECAR showed the overall increased changes in glycolytic rates of in vitro CSC treated cells (Figure 15e, f). These findings gave insight into what changes chemicals from cigarette smoke were able to induce in AML, but these may not be the same changes that occur with in vivo smoke exposure.

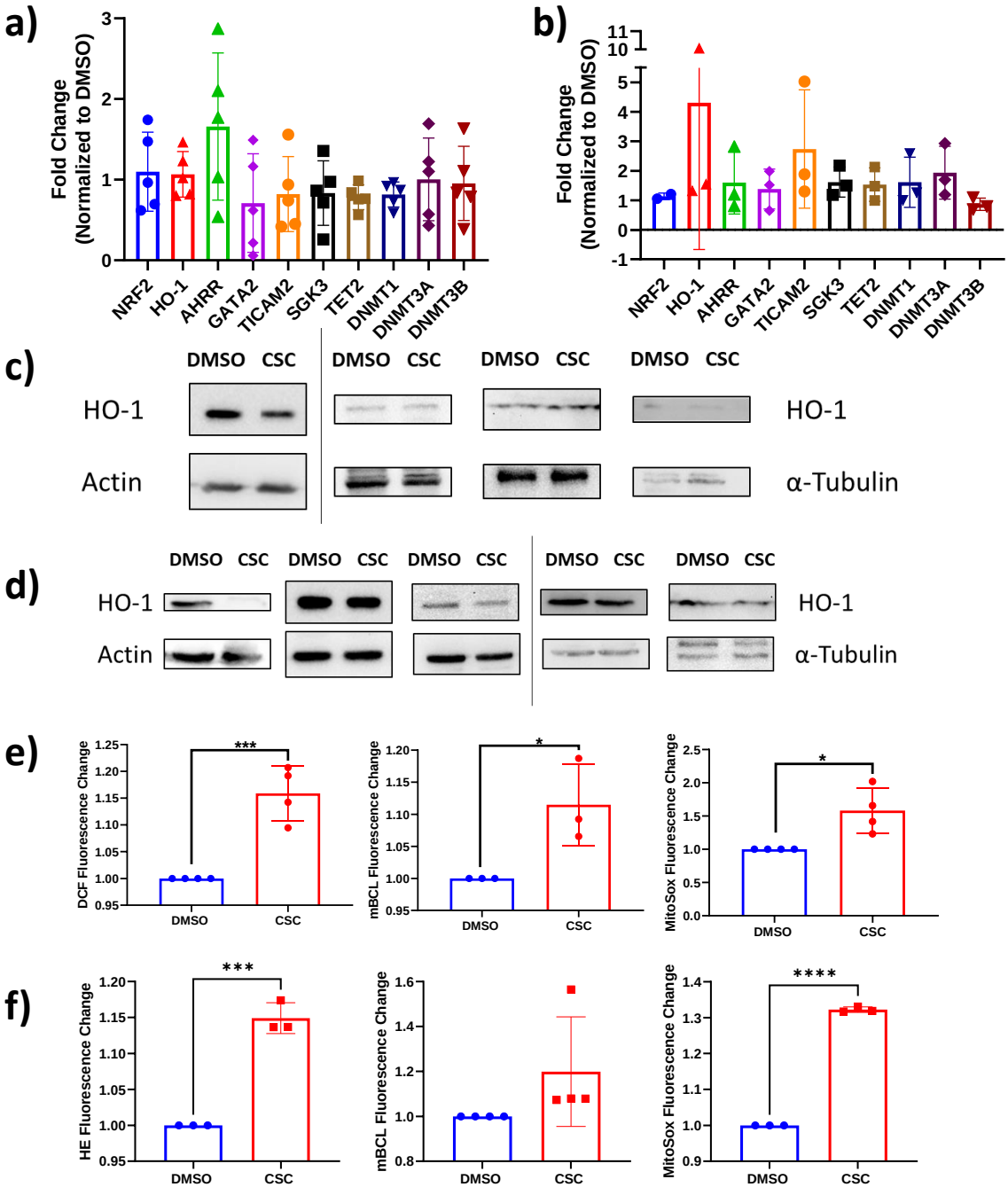



Figure 13 Oxidative stress in AML cells after two-week CSC treatment

Figure 13 Oxidative stress in AML cells after two-week CSC treatment

Gene expression of redox genes, genes with SE-altered DNA methylation in AML, and genes involved in regulation of DNA methylation in MOLM13 a) and MOLM14 b) cells (N=3, 5 respectively). c) Normalized median fluorescence intensity to matching DMSO control of general peroxides (DCF), glutathione (mBCL), and mitochondrial superoxides (MitoSox) in MOLM13 cells after 14 days of CSC or DMSO treatment. d) Normalized median fluorescence intensity to matching control of general superoxides (HE), glutathione (mBCL), and mitochondrial superoxides (MitoSox) in MOLM14 cells with two-week CSC or DMSO treatment. e) Western blot of MOLM13 cells with two-week CSC or DMSO treatment with Actin or alpha-tubulin as loading control. e) Western blot of MOLM14 cells with two-week CSC or DMSO treatment with Actin or alpha-tubulin as loading control.

*=p-value<0.05; **=p-value<0.01; ***=p-value<0.001; ****=p-value<0.0001.

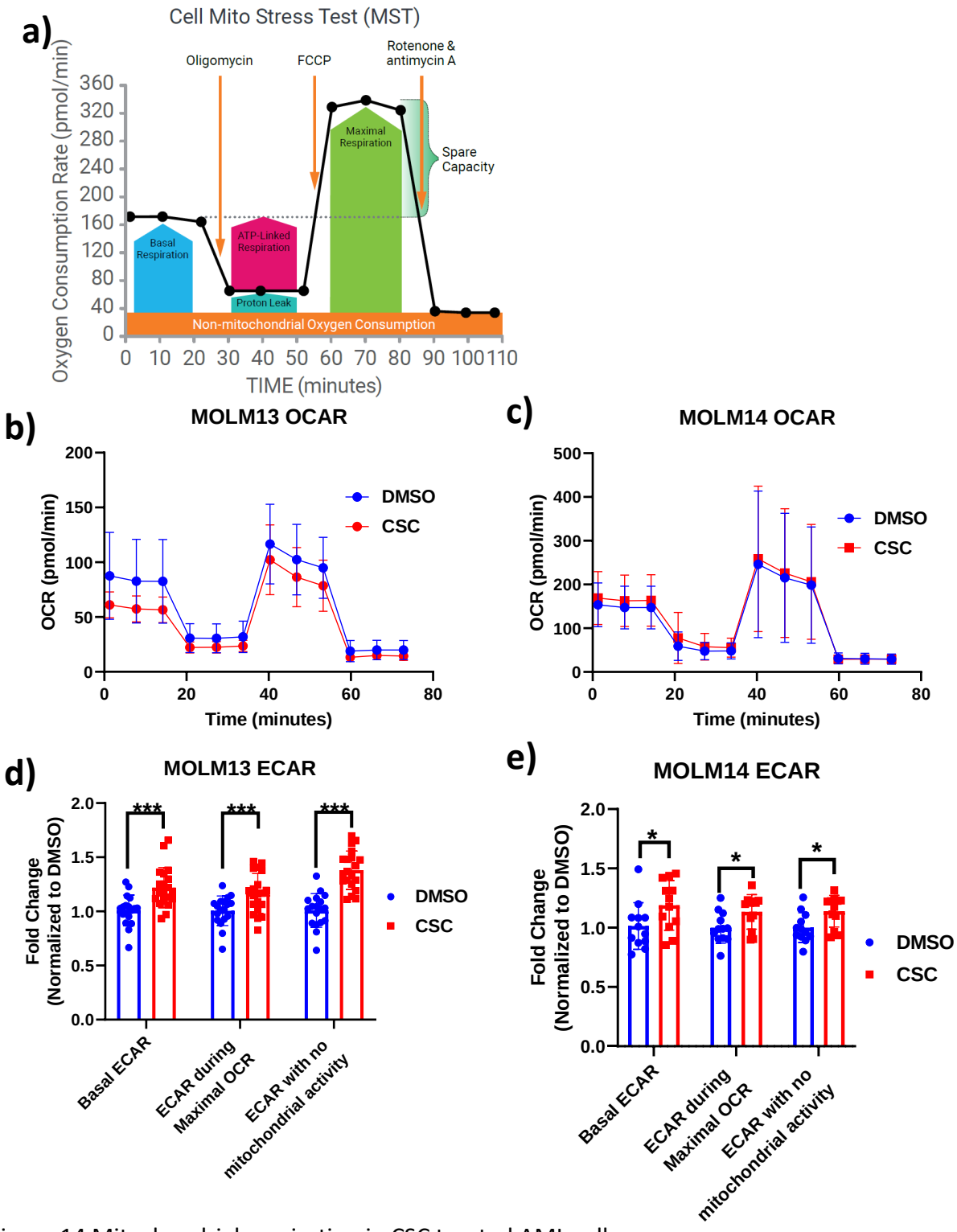



Figure 14 Mitochondrial respiration in CSC treated AML cells

Figure 14 Mitochondrial respiration in CSC treated AML cells

a) Schematic of mitochondrial stress assay from (122). b) Oxygen consumption rates (OCR) of MOLM13 cells with 14-day in vitro treatment of CSC or DMSO, N=3. c) OCR of MOLM14 cells with 14-day in vitro treatment of CSC or DMSO, N=3. d) Extracellular acidification rate (ECAR) of MOLM13 cells with 14-day in vitro treatment of CSC or DMSO, N=3. e) ECAR of MOLM14 cells with 14-day in vitro treatment of CSC or DMSO, N=3. ECAR data is normalized to DMSO for either 0, 40, or 70-minute timepoint of mitochondrial stress assay. *=p-value<0.05; ***=p-value<0.001.

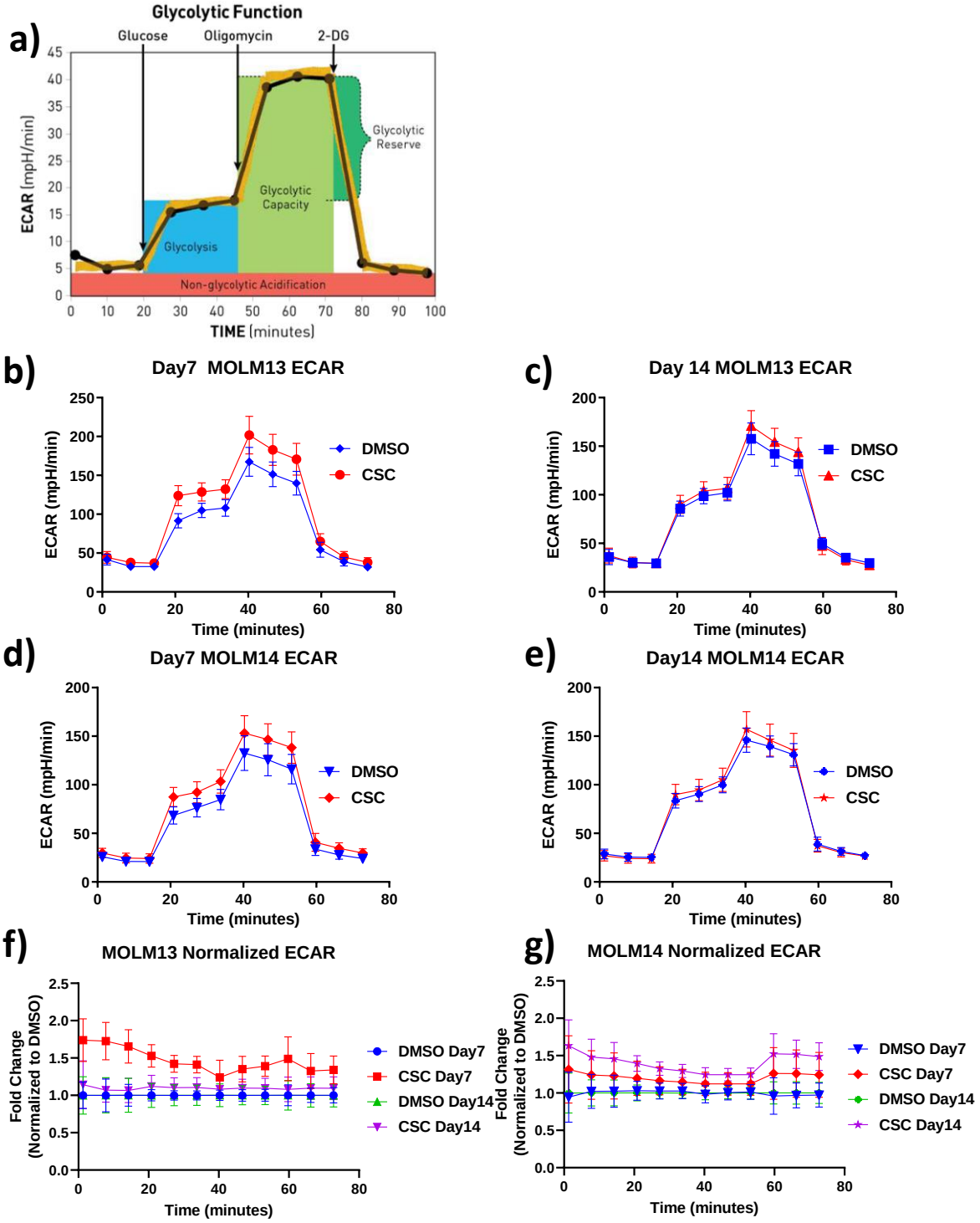



Figure 15 Glycolytic rate of CSC treated AML cells

Figure 15 Glycolytic rate of CSC treated AML cells

a) Schematic of glycolytic stress assay from (123). Extracellular acidification rate (ECAR) of MOLM13 cells with 7 b) or 14-day c) in vitro treatment of CSC or DMSO; representative replicate shown. ECAR of MOLM14 cells with 7 d) or 14-day e) in vitro treatment of CSC or DMSO; representative replicate shown. ECAR data normalized to DMSO of glycolytic stress assay in MOLM13 f) and MOLM14 g) cells with 7 or 14 days of CSC treatment (N=2).

We wanted to understand how SE was impacting ROS levels in AML in leukemia-bearing mice. We looked at intracellular levels of ROS within CD45+ AML cell from spleen and PBMCs of SE mice. There were no significant differences found in general peroxide or superoxide levels in spleen or PBMC from SE and non-smoking mice samples (Figure 16). While changes in intracellular ROS were not detected, other molecular changes that promote AML could be induced by SE.

Using mass cytometry, we were able to determine if changes in expression were found in CD45 positive or negative cells. Using mass cytometry analyses of MOLM13 bearing mice exposed to SE or NS, we focused on human CD45 positive AML cells from spleen samples. RphenoGraph analysis revealed that non-smoking and SE had differences in the percent of cells found within clusters based on expression of proteins: CD34, p-ERK1/2, RUNX1, MCL-1, DNMT3B, HIF-1α, p21, and p-FLT3 (Figure 17a). When the differences in cell populations found within the clusters were examined, SE increased the number of cells in clusters that expressed proliferative signaling and decreased clusters with low signaling (Figure 17a). Differences in cluster population were predominantly observed in the human CD45 positive cells, and similar to the findings in Figure 17A the CD45 negative cell populations did not show major differences (Figure 17b, c).

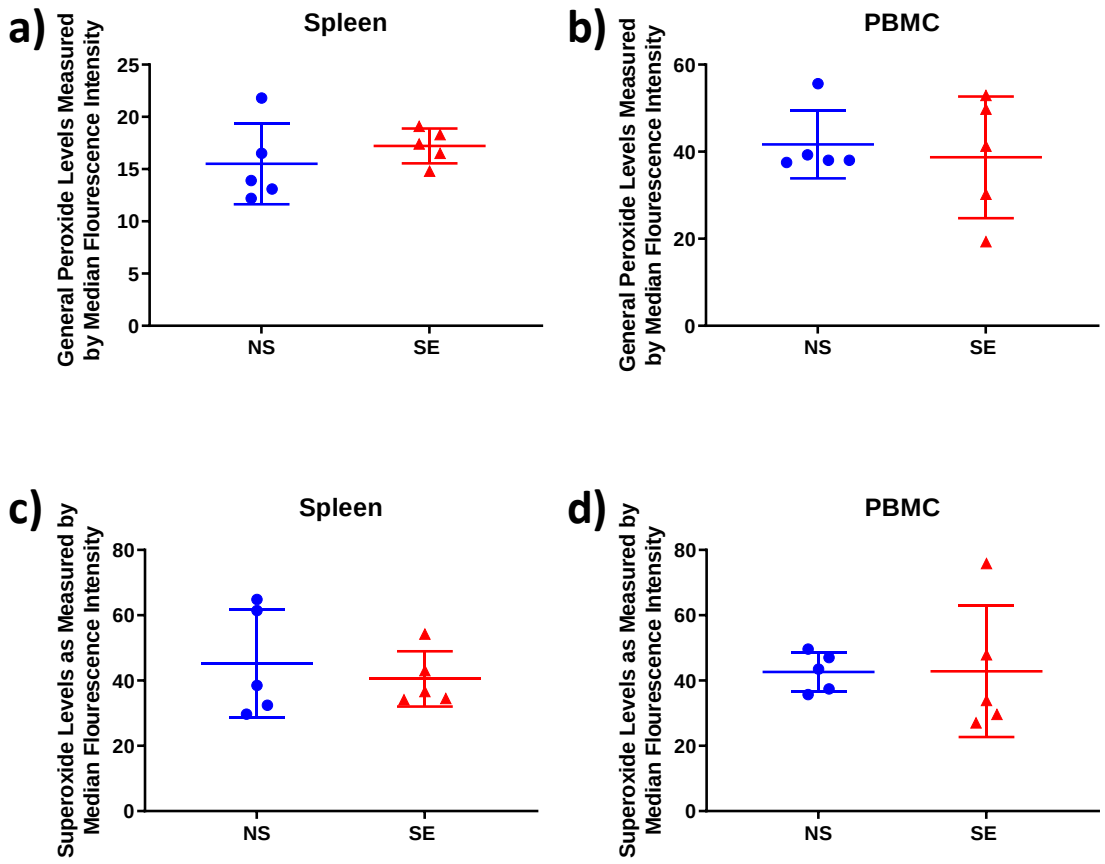



Figure 16 MOLM13 ROS levels in non-smoking and SE AML-bearing mice

General peroxide levels as measured by CellROX DeepRed intracellular staining of AML cells from MOLM13-bearing mouse spleen a) and peripheral blood mononuclear cells (PBMC) b). Superoxide levels as measured by dihydroethidium intracellular staining of AML cells from MOLM13-bearing mouse spleen c) and PBMCs d). NS= non-smoking; SE= smoke exposed.

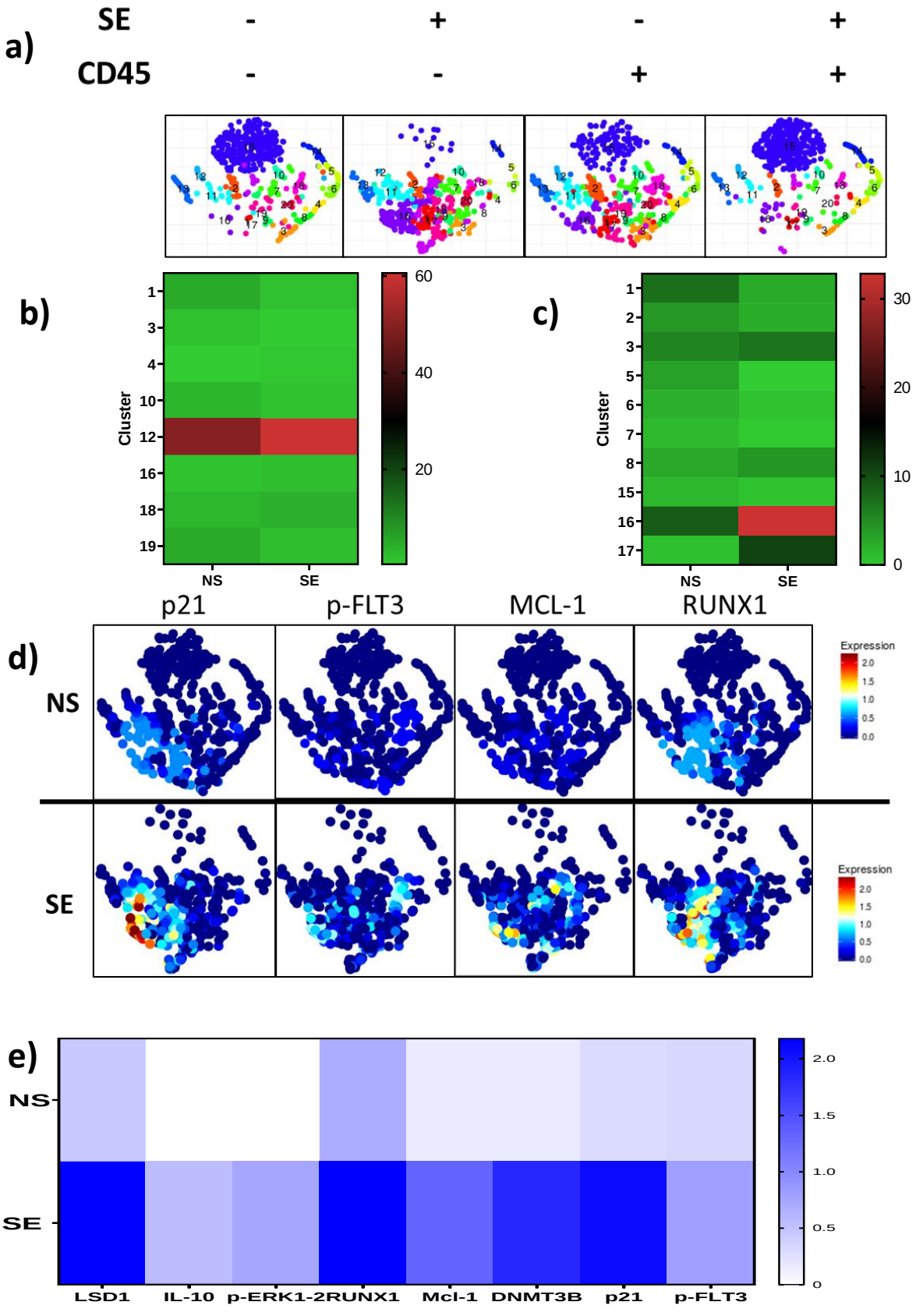

a)
SE - + - +
CD45 - - + +
b)
Cluster
1 3 4 10 12 16 18 19
NS SE
60 40 20
c)
Cluster
1 2 3 5 6 7 8 15 16 17
NS SE
30 20 10 0
p21 p-FLT3 MCL-1 RUNX1
d)
NS
SE
Expression
2.0 1.5 1.0 0.5 0.0
e)
NS
SE
LSD1 IL-10 p-ERK1-2 RUNX1 Mcl-1 DNMT3B p21 p-FLT3
2.0 1.5 1.0 0.5 0

Figure 17 Smoking increases AML populations expressing pro-leukemic signaling

a) Tsne plots of CD45 positive or negative cells from spleens of non-smoking or smoke exposed (SE) MOLM13-bearing mice. Two mouse samples combined each group in the analysis. Heat maps of percentages of CD45 negative b) or CD45 positive c) cells from the total number of cells present in all of the clusters depicted in a. d) Tsne plots with expression of p21, p-FLT3, MCL-1, and RUNX1 in CD45 positive samples from non-smoking (NS) or SE depicted in A. e) Overall median expression of RUNX1, MCL1, DNMT3B, P21, P-FLT3 in CD45 positive cells from NS or SE clusters depicted in a.

The clusters that were more populated in the SE samples expressed higher levels of P-FLT3, MCL-1, RUNX1, and p21 (Figure 17d). Overall expression of LSD1, IL-10, p-ERK1/2, RUNX1, MCL-1, DNMT3B, p21, and p-FLT3 were elevated in AML cells from SE samples (Figure 17e). Increased expression of these proteins implicates altered epigenetic regulation, activated proliferative signaling, and anti-apoptotic pathways in contributing to SE enhanced AML.

**Discussion**

Cigarette smoking is known to induce oxidative stress, but we are the first to describe the effects of smoking within AML cells. No significant changes in ROS were observed in CD45+ cells (Figure 16); this is likely due to the timing of when the samples are collected as discussed in Chapter III. Oxidative stress was instigated by CSC treatment, emulating the effects seen in SE samples. Increased levels of general peroxides, mitochondrial superoxides, and or superoxides were observed after 2 weeks of CSC treatment (Figure 13e, f). There was also an increase in glutathione, the most abundant intracellular antioxidant (Figure 13e, f). Although we did not observe a consistent increase in HO-1 expression with CSC treatment, others have found increased HO-1 gene expression in human bronchial epithelial cells treated with cigarette smoke extract (124). Moreover, the increased levels of glutathione are likely an adaptive response to elevated ROS from the CSC. Inconsistency in protein and gene expression from CSC treatment are due to volatility of the CSC. Since many of the chemicals are unstable and highly reactive, it is hard to create specific reproducible effects that are captured at the same timepoint.

While mitochondrial respiration was not significantly impacted, the glycolytic activity of the AML cells was increased with CSC. Increased glycolytic activity is notable, because it is an anerobic process of producing energy in cells which is relevant given the hypoxic conditions of bone marrow. Additionally, stemness of hematopoietic stem cells have been found to have increased glycolytic activity compared to more mature hematopoietic cells (125, 126); this could be linked to smoking increasing rates of CHIP. Since the CSC is inducing these changes in AML in vitro,

Mass cytometry of smoke exposed AML-bearing mice revealed that many of the changes from smoking are occurring within AML cells and not the microenvironment. Moreover, it was revealed that SE increased the overall expression of MCL-1, DNMT3B, and RUNX1, which are both therapeutically relevant for AML. MCL-1, an antiapoptotic protein, is important for AML survival. MCL-1 inhibitors are currently being investigated for AML treatment, especially in the setting of resistance to BCL-2 inhibitor Venetoclax resistance (113). RUNX1 mutations occur in 10% of AML patients and are associated with inferior prognosis (127). Increased occurrence of RUNX1 translocations have also been found in blood cells of smokers, which suggests there may be a novel role for RUNX1 in smoking-associated AML (121). Together these data reveal that cigarette smoking can induce changes within AML that contribute to aggressive disease progression in a FLT3-ITD AML-bearing mouse model.

## Chapter V: Discussion

*Overall Summary*

Acute myeloid leukemia (AML) is one of the most common cancers in cigarette smokers. Any history of cigarette smoking in AML patients confers a worse survival compared to patients that have never smoked. Smoking associated worsened survival has also been seen in patients that harbor the oncogenic FLT3 internal tandem duplication (FLT3-ID) mutation that has the worst survival. Smoking induces oxidative stress in circulating blood and altered DNA methylation in peripheral blood mononuclear cells can be followed even after decades of quitting. We hypothesize that cigarette smoking induces molecular changes, including altered DNA methylation associated with poor AML prognosis, that promote AML.

Whole body cigarette smoke exposure (SE) and chemicals from cigarette smoke, via cigarette smoke condensate (CSC) treatment, were able to accelerate the progression of AML in mice. Within FLT3-ITD AML patients we also discovered that there is decreased response to chemotherapy, so the worsened survival in ever smoking patients was due to their disease progression instead of smoking related comorbidities.

When DNA methylation was evaluated in SE AML-bearing mice samples, over two hundred genes with altered DNA methylation were found in AML cells. These genes were involved in numerous pathways, many are involved in chromatin rearrangement, nucleoside binding, and response to oxidative stress. Hypomethylation of GATA2 was induced by SE, and AML patients with low GATA2 methylation showed decrease survival. Although it is unknown how different DNA methylation patterns arise in AML, our SE-altered DNA

methylation overlapped with some genes that are associated with poor prognosis in AML patients.

The role of the tumor microenvironment in smoking-accelerated AML is not well understood. In immune competent mice we found that SE increased number of myeloid stem and progenitor cells. In immune deficient AML-bearing mice tissue samples, we observed increased protein expression of heme oxygenase 1 (HO-1) and BCL-2. These changes in the microenvironment can promote AML, but we did not observe them directly in AML.

To evaluate the direct impact of smoking on AML, we used in vitro CSC treated cells that mimicked the in vivo SE. CSC treatment resulted in increased reactive oxygen species (ROS) and glutathione levels. Within SE AML-bearing mouse samples, mass cytometry allowed us to evaluate changes in AML cells. There was increased expression of proteins involved in activating proliferation, treatment resistance, and epigenetic proteins.

Herein we described how cigarette smoking and cigarette smoke condensate enhance AML progression, altered AML DNA methylation, induced oxidative stress, and increased pro-leukemic expression in AML cells.

**Future Studies**

This work has built the groundwork for the investigation of tailored treatment for ever smoking AML patients, further work must be conducted to rescue smoking enhanced AML progression.

We produced evidence of oxidative stress from cigarette smoke exposure, in SE AML-bearing mice and in AML cells directly treated with CSC. Oxidative stress in leukemia cells can drive progression and treatment resistance; hence modulating redox in AML is a therapeutic intervention may be useful for treating smoking AML patients. BCL-2 and heme oxygenase-1 (HO-1) protein levels were elevated in tissue samples from SE AML-bearing mice and levels of reactive oxygen species and glutathione levels were increased with cigarette CSC treatment. Since increased BCL-2 levels was found with SE, smoking patients may benefit from BCL-2 inhibitor treatment. NRF2 inhibition decreased expression HO-1 and increased treatment efficacy of chemotherapy in FLT3-ITD AML cells (58). Consequent change from smoking induced oxidative stress may be better targets for former smoking patients, since the oxidative damage would have occurred throughout the duration of the smoking.

Oxidative stress from SE should be evaluated further in AML, since oxidative damage can impact DNA methylation as well (128, 129). We have started to examining tissues for 8-oxoguanine, the most common oxidative DNA lesion, from non-smoking and SE mice, but needs further investigation. Activity and expression of DNA methyltransferases (DNMTs) should be examined. Increase ROS can enhance the activity of DNMTs, which could be

another way that smoking leads to altered DNA methylation. Furthermore, altered histone methylation has been associated with poor prognosis of AML was found in smoking AML patients (130). This suggests that smoking AML patients may be uniquely suited for epigenetic modification, including through treatment of hypomethylating agents, to help interfere with epigenetic patterns that predict poor prognosis in AML patients.

SE increased myeloid stem and progenitor cells in the bone marrow of immune competent mice, which suggests that SE produces a microenvironment that promotes the growth of these cells. The evaluation of hematopoietic cell populations should be repeated with a longer SE time frame and with a model of smoking cessation to understand how this relates to ever smokers having increased AML incidence; this experiment may also shed light on why smokers have increased rates of clonal hematopoiesis of indeterminate potential (CHIP). Additionally, since differences in hematopoietic cell populations in immune competent and immune deficient mice were observed, a syngeneic model of AML may necessary to fully capture SE induced changes of the tumor microenvironment and how they impact AML progression.

E-cigarettes have recently become popular and may exhibit similar impacts on AML as traditional cigarette smoking, but these patterns may not be observed for many years. The concordance between CSC and SE on AML progression, oxidative stress, and altered DNA methylation suggest that chemicals from smoking can induce similar molecular changes; thus, these findings are not only relevant to cigarette smoking, but also for tobacco products broadly. With the alarming rise in e-cigarette use which have unknown

consequences, it is of upmost importance to gain understanding of the effects of smoking products.

This work serves as evidence of the potential for using behavioral biomarkers for tailoring cancer treatment. Currently treatments are largely based on cancer type, with some targeted therapies for patient that harbor mutations. Additionally, this work supports the consideration of lifestyle interventions for cancer patients.

## Addendum I: Cigarette smoking and Philadelphia positive acute lymphoblastic leukemia progression and tyrosine kinase inhibitor treatment response

I began working on this project during the spring of my second year and continued until my fourth year as a branch of my main thesis project. Acute Lymphoblastic leukemia (ALL) is an adult and pediatric cancer. When either parent smokes, their children develop ALL at higher rates and they have worse survival compared to patients with parents that never smoked (131, 132). The BCR-ABL mutation, also known as Philadelphia positive (Ph+), is an oncogenic mutation that drives aggressive leukemic growth and is found in many ALL patients. A recent retrospective study conducted in adult Ph+ ALL patients found that if patients had significantly worse survival if they had smoked at least one pack year of smoking in their lifetime (133); worsened survival was seen in patients that received targeted tyrosine kinase inhibitor (TKI) treatments for Ph+ ALL. Additionally, this study found an inverse correlation between smoking habits and response to treatment. The BCR-ABL fusion mutation is also able to increase levels of reactive oxygens species in cells. Smoking increases levels of reactive oxygen species (ROS) in peripheral blood cells of smokers. Oxidative stress, such as from increased levels of ROS can promote leukemia progression and treatment response. It is unknown how smoking is contributing to Ph+ ALL progression or TKI treatment response. We hypothesized that smoking elevated ROS in Ph+ ALL that resulted in enhanced leukemic progression and TKI resistance.

**Methods**

*Cell lines*

Z119 and Z33 obtained from Dr. Estrov's lab at MD Anderson. Grown in RPMI 1640 with 10% FBS, 1% L-Glutamine, 1% Penicillin-Streptomycin, 1% Sodium Pyruvate, and 1% Non-essential Amino Acids. All other conditions followed those listed in Methods and Materials chapter.

Viability was determined by fluorescent ghost dye exclusion measured on BD LSRFortessa. Viability was also studied using Alamar blue using the methods mentioned in AML cells outlined in Materials and Methods chapter, but 100,000 cells/well were seeded for ALL cells.

*Patient Derived Xenograft (PDX) bearing SE mouse model*

NSG-IL2 mice used to for PDX engraftment. Mice were challenged with $0.75x10^6$ viable cells/mouse in 200ul of sterile PBS via tail-vein injection. The same daily smoking regimen were used as described in Materials and Methods chapter. Mice were exposed to SE starting two-weeks prior to ALL introduction and only continued for three days post leukemic introduction. Leukemic burden was tracked via blood sample evaluation of CD45 positivity. Blood was collected via tail-vein or facial vein bleeding.

**Results and Discussion**

Similar to the smoke exposure (SE) of mice previously described, we exposed mice to SE for two weeks before leukemic introduction (Figure *18*). A patient derived xenograft (PDX) from a Ph+ ALL patient was used to challenge mice with leukemia. There was increased leukemic progression in SE mice as the experiment progressed (Figure *18*). At the time of sacrifice of the ALL-bearing mice, there was a significant increase in the ALL cells found in peripheral blood samples from SE mice.

Next we evaluated the impact of in vitro CSC treatment on Imatinib, a TKI approved for use in ALL patients, efficacy in ALL. Using a viability assay that relied on metabolic activity, we did not observe differences in treatment response with CSC (Figure 19). When an assay that measures viability via exclusion of a fluorescent dye instead, there was only increased ALL viability at the 40uM dose (Figure 19). Unfortunately, we were unable to finish these experiments in multiple cell lines. Since the BCR-ABL mutation and smoking are associated with increased ROS, we measured the intracellular peroxide and superoxides of cells (134). There was increased peroxides and superoxide levels in an ALL cell line. Since there was increased ROS, we wanted to understand if DNA was being damaged. We observed increased expression of γH2AX, a marker of DNA damage (Figure 19).

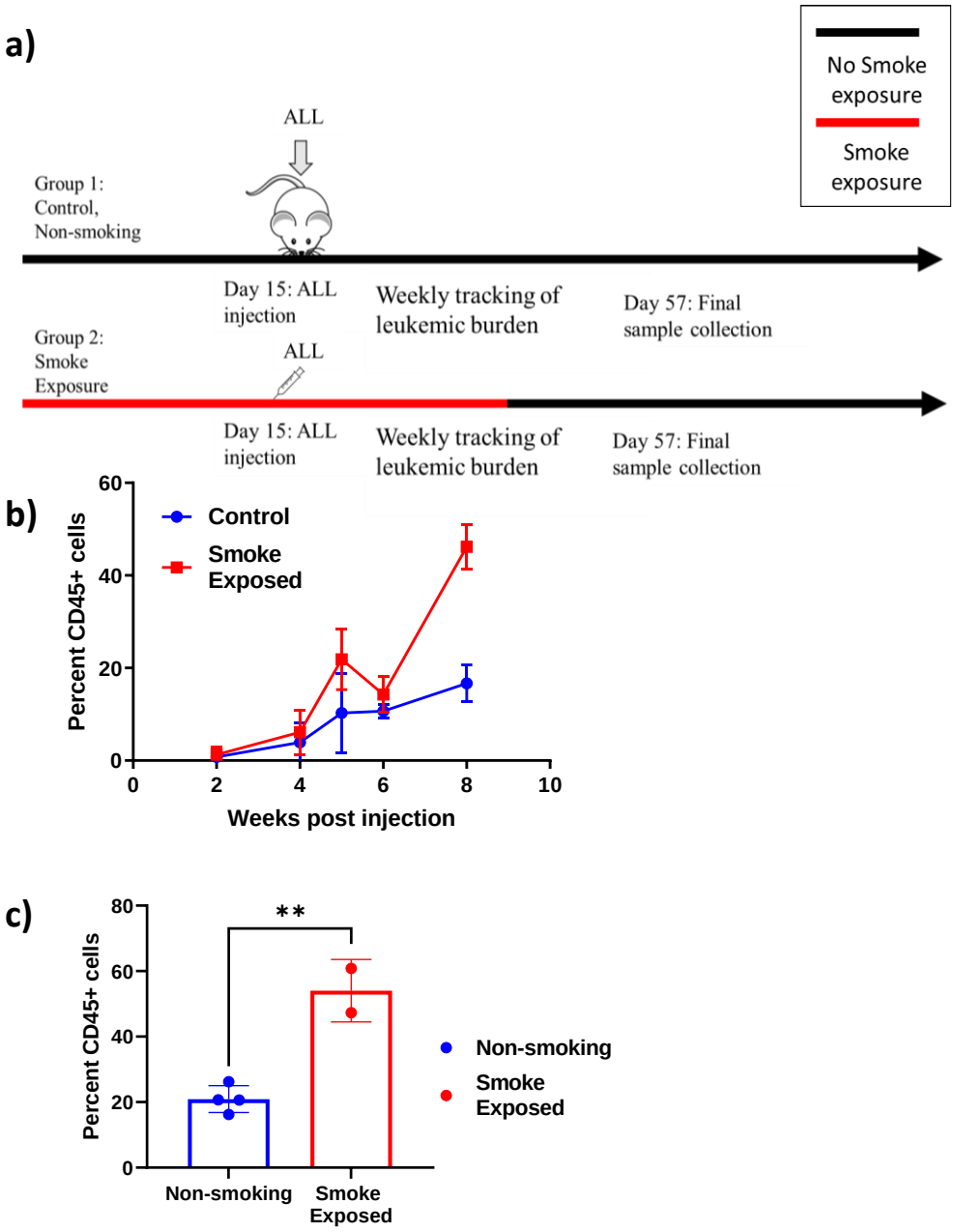



Figure 18 Smoking increases leukemic progression of Ph+ ALL

Figure 18 Smoking increases leukemic progression of Ph+ ALL

a) Schematic of smoke exposure of patient derived xenograft (PDX) bearing mice. b) CD45 positivity of peripheral blood samples from PDX throughout the experiment. c) Percent CD45 positive peripheral blood samples taken at time of sacrifice. **=p-value<0.01

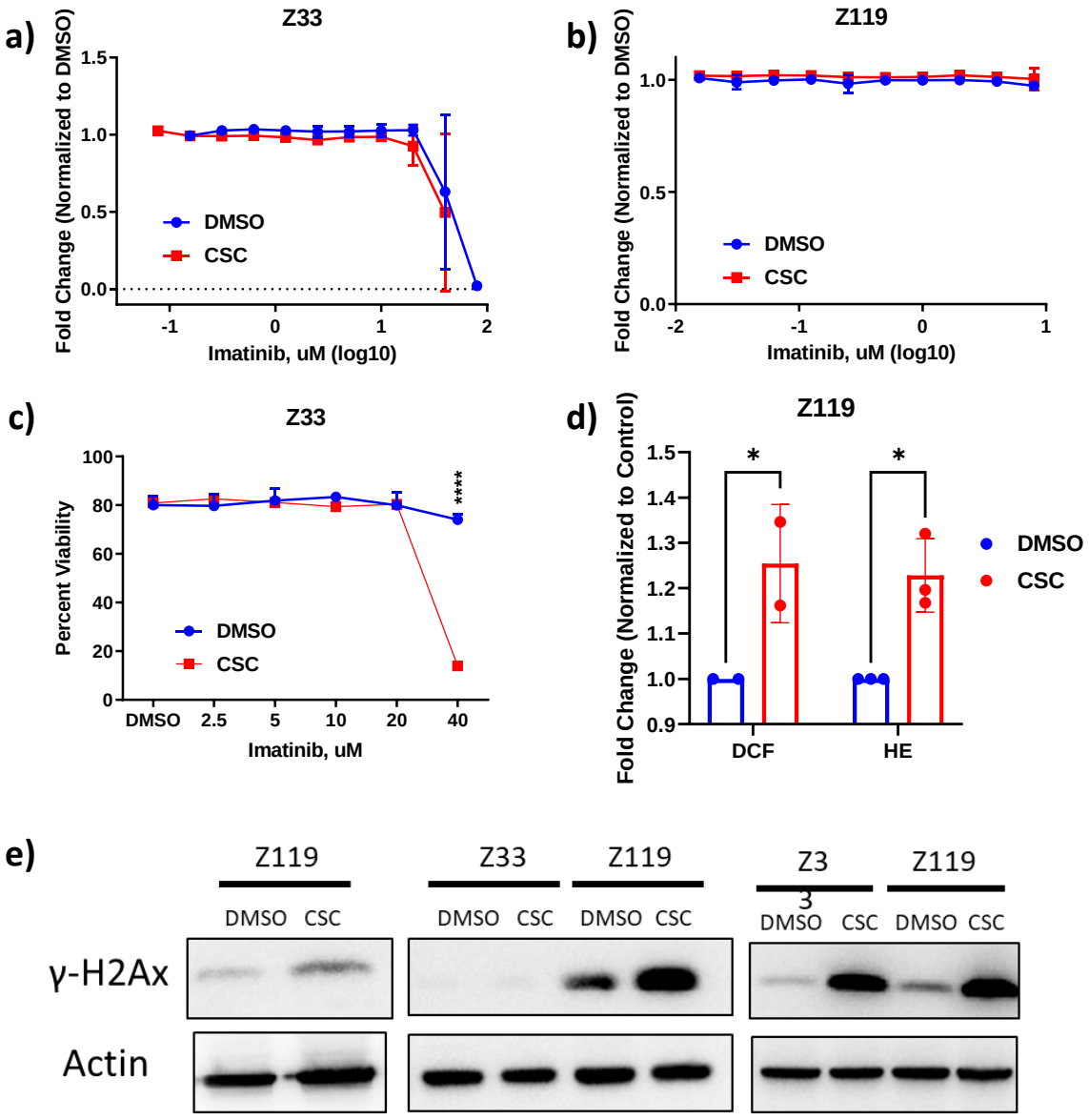



Figure 19 Imatinib treatment response, elevated ROS, and DNA damage in CSC treated Ph+ ALL

Figure 19 Imatinib treatment response, elevated ROS, and DNA damage in CSC treated Ph+ ALL

Imatinib treatment response as measured by Alamar blue assay in Z33 a) and Z119 b) cells. Imatinib treatment response as measured by ghost dye exclusion via flow cytometry in Z33 c) cells. d) Intracellular general peroxides (DCF) and superoxides (HE) levels of Z119 cells treated with CSC for 14 days. Values normalized to DMSO control sample of each replicate. e) Western blots of Z119 or Z33 cells after one week (left), two weeks (middle), and 2 months of 10ng/ml CSC or DMSO treatment. *=p-value<0.05; ***=p-value<0.001.

Overall, we found that cigarette smoke exposure promoted ALL, CSC increased ROS levels, CSC caused DNA damage, and CSC treated cells showed resistance to cytotoxicity from high dose Imatinib treatment.

**Addendum II: Adipocyte promoted acute myeloid leukemia**

During my rotation until the end of the summer semester of my second year, I investigated the role of adipocytes in promoting AML. There are modifiable health behaviors that are associated with development of AML. There is increased risk of developing AML in obese patients. Obesity rates have been increasing drastically over the past decades and have been linked to unhealthy dietary habits and sedentary lifestyles. Moreover, in pediatric AML patients they have found that obese patients do worse than normal weight patients (135). This shows that there is a role for modifiable health behaviors having an impact in AML progression and is a potential area for intervention in patients.

This had garnered interest in understanding the role of adiposity and AML progression. Adipocytes are present and develop within the yellow marrow, which increases with adiposity. Adipocyte and AML interactions have been investigated to understand their role in aiding leukemia. There have been studies that showed protective roles of adipocytes against chemotherapy for leukemia (136). It has also been found that elevated superoxide levels from NOX2 in AML can drive the transfer of mitochondria from adipocytes (137). A weakness among many of the adipocyte and AML studies is that they conducted using rodent adipocytes and human AML cells. We chose to use two different human adipocyte models along with human AML cells.

**Methods and Materials**

*Adipocyte differentiation and culturing:*

SGBS cells obtained from Dr. M. Wabitsch from the University of Ulm, Germany. SW872, liposarcoma, cells were purchased from AATC. Adipocyte base media (0F) for adipocytes is DMEM/F-12 with 2.5mM L-Glutamine, 15mM HEPES, 16.5uM Biotin, 85uM Pantothenate, and 1mM penicillin/streptomycin. For the growth of undifferentiated cells, the cells were kept in base media with 10% heat inactivated FBS. For the first 3 days of adipocyte differentiation, the cells were cultured in the base media with added 2uM Rosiglitazone, 25nM Dexamethasone, 250uM IBMX, 0.001mg/mL Transferrin, 20nM insulin, 0.2nM triiodothyronine, and 100nM Cortisol. For the rest of the differentiation and subsequent experiments the cells were kept in the 3F media comprised of 0F base media with 0.001mg/mL Transferrin, 20nM insulin, 0.2nM triiodothyronine, and 100nM Cortisol. All media and added components were sterile filtered through 0.2uM filter.

*Transwell plating and time course*

On day 14 of adipocyte differentiation, adipocytes were gently removed from adherent flask using TryPLE (Gibco, 12604013) and viable cell counts were determined via Vi-cell. Then 0.1x10^6 cells/ml were seeded into a 6 well plate at 3mL in differentiation media. The cells were allowed to adhere to the plate overnight. Viable cell concentrations were determined using trypan blue exclusion assay on Vi-cell XR. MOLM13 and ML-2 cells

were seeded at 0.2x10^6 cells/ml and OCI-AML3 cells were seed at 0.1x10^6 cells/ml at 1ml per transwell insert. The AML cells were resuspended in media that was 1:1 3FC adipocyte differentiation media and corresponding AML media. Control cells were seeded in transwells without any feeder cells. The AML cells were carefully resuspended for viability and cell counting daily at 1:10 dilution on Vi-cell XR.

**Results and Discussion**

Since adipocytes and AML cells are not always in direct contact within the body, we wanted to understand if adipocytes could promote AML without cell-to-cell contact. Using transwell co-culturing of differentiated adipocytes we were able to study if adipocytes were able to promote AML without direct contact. Co-culturing MOLM13, ML-2, and OCI-AML3 cells resulted in enhanced leukemic growth (Figure 20). Viability of ML-2 and OCI-AML3 cells was also increased at later timepoints (Figure 20). This suggest that AML cells are supported by secreted factors released by adipocytes when cultured together. It is notable that the increased progression and viability were more pronounced when the control cells

There were plans to evaluate changes in reactive oxygen species (ROS) since obesity increases ROS levels, but the transwell culturing resulted in too few cells for these assays. Cytokine and lipids in the media from co-culture experiments were also intended to be examined for factors that could promote AML.

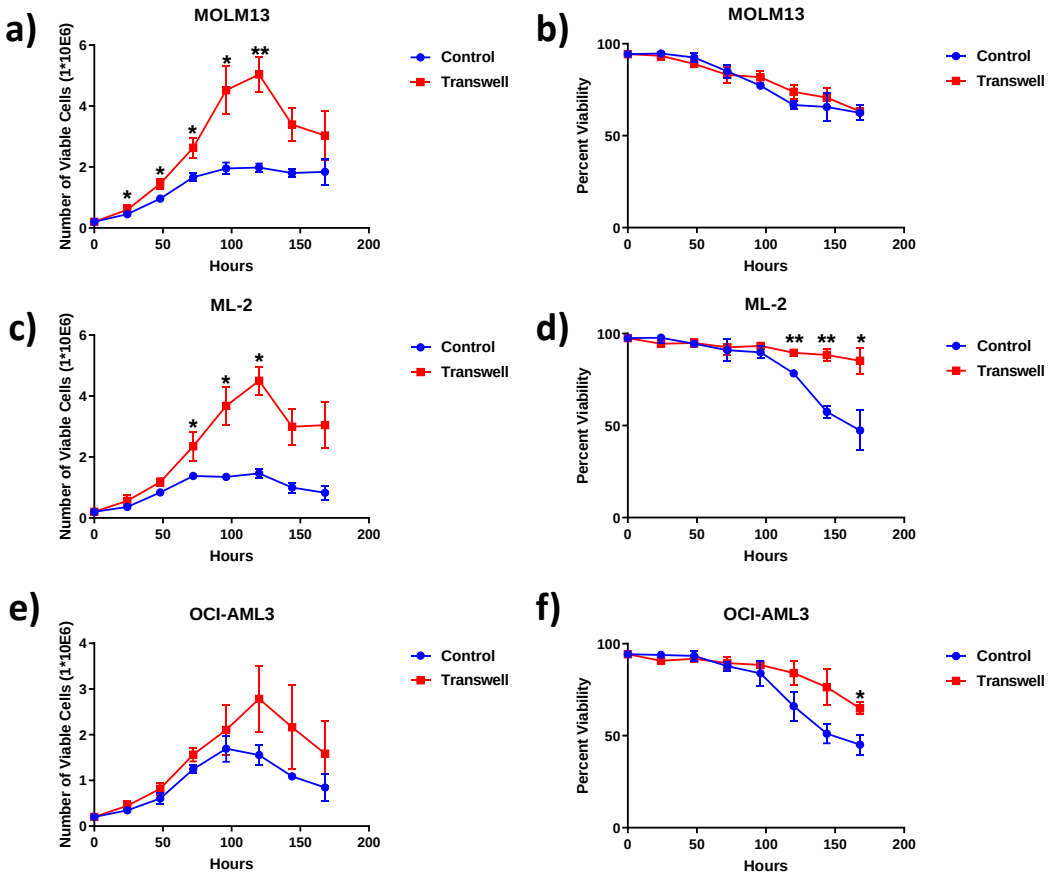



Figure 20 Transwell co-culturing of AML with adipocytes

Viable cell growth a) and total viability b) of MOL13 cells. Viable cell growth c) and total viability d) of ML-2 cells. Viable cell growth e) and total viability f) of OCI-AML3 cells.

*=p-value<0.05; **=p-value<0.01; ***=p-value<0.001; ****=p-value<0.0001.

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## VITA

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