EFFECTS OF LSD1 INHIBITION ON AML IMMUNOGENICITY

INVESTIGATION OF THE EFFECTS OF LSD1 INHIBITION ON AML IMMUNOGENICITY AND T CELL-MEDIATED IMMUNE RESPONSES

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Lay Abstract

Acute myeloid leukemia (AML) is an aggressive blood cancer characterized by the rapid growth of abnormal blood cells in the bone marrow. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an important treatment for AML that involves taking blood stem cells from a healthy donor and transplanting them into an AML patient. Meanwhile, immune cells such as T cells from the donor can help destroy leukemia cells. However, AML frequently reappears after allo-HSCT and new therapies are needed to prevent the disease from coming back. The present study investigates whether blocking a protein called lysine-specific demethylase 1 (LSD1) can increase T cells' ability to identify and kill cancer cells. The results demonstrate that treatment with an LSD1 blocker called bomedemstat can enhance the recognition of AML cells by T cells, thereby enhancing their immune response. These findings suggest that blocking LSD1 is a promising approach to enhance the effectiveness of allo-HSCT.

Abstract

Acute myeloid leukemia (AML) is an aggressive hematological malignancy. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) represents a curative treatment option for AML. Alloreactive donor T cells can produce the graft-versus-leukemia (GVL) effect which represents a major therapeutic benefit of allo-HSCT. However, evading from allogeneic immune surveillance, potentially through the downregulation of human leukocyte antigen class II (HLA-II) antigen presentation machinery, can contribute to AML relapse. Lysine-specific demethylase 1 (LSD1) is an emerging epigenetic therapeutic target in AML. The present study aims to explore whether LSD1 inhibition can enhance AML immunogenicity to promote T-cell mediated immune response.

The immunological effects of a clinical-stage LSD1 inhibitor bomedemstat (IMG-7289) were examined in both human and murine AML cell models *in vitro*. The results demonstrated that bomedemstat treatment significantly enhanced the expression of class II transactivator (*CIITA*). It subsequently led to the upregulation of HLA-DR in certain human AML cell lines when stimulated by IFN-γ. Bomedemstat also markedly upregulated the expression of CD86 in all human AML cell lines tested. The study also demonstrated that bomedemstat treatment significantly increased the production of pro-inflammatory cytokines IL-12 and CXCL-10.

In murine AML models, bomedemstat concurrently upregulated the expression of major histocompatibility complex class II (MHC-II) and CD86 in H9M-transformed cells without IFN-γ stimulation. This effect was not observed in MN1-transformed cells. Bomedemstat-treated H9M cells were subsequently shown to induce antigen-dependent T cell activation. Functional assays revealed that bomedemstat treatment sensitized H9M cells to antigen-dependent immune killing effect mediated by $CD4^+$ T cells.

In conclusion, the current study demonstrates both phenotypically and functionally that LSD1 inhibition by bomedemstat treatment can enhance AML immunogenicity. This is evident by the increased antigen presentation, co-stimulation and production of inflammatory cytokines. These findings suggest that LSD1 inhibition may be a relevant strategy to pursue as a maintenance therapy after allo-HSCT.

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I would like to thank my committee members, Dr. Jonathan Bramson and Dr. Mark Larché, for their continued support and invaluable contributions to this project. Their profound expertise in immunology is crucial in shaping this project. Their thoughtful and constructive feedback not only helped me develop my scientific communication skills but also allowed me to think more critically about the results I obtained.

I would also like to thank my lab mentor, Dr. Kanwaldeep Singh, for providing all the necessary training on laboratory techniques and for helping me design the experiments. His technical expertise has greatly shaped my research skills. I also thank Dr. Joanne Hammill and Rebecca Burchett for providing technical assistance for T cell experiments. I would like to thank Darlene for offering administrative support, which has made the day-to-day life in the lab so much easier. To all other members of the Berg Lab, past and present, I am truly grateful for being able to share this journey with you. I wish each and every one of you the best of luck in all your future endeavours.

Starting the project amidst a global pandemic is certainly challenging. Therefore, I would like to take this opportunity to thank my family and all my friends for their companionship as I navigated through and overcame academic and personal obstacles. I will forever be grateful for the intellectual and personal growth I have experienced during this challenging yet fruitful period of time.

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List of all Abbreviations and Symbols

7AAD	7-aminoactinomycin D
Allo-HSCT	allogeneic hematopoietic stem cell transplantation
AML	acute myeloid leukemia
ANOVA	analysis of variance
AOL domain	amine oxidase-like domain
APC	antigen-presenting cell
ATRA	all-trans retinoic acid
CCL-1	C-C motif ligand 1
cDNA	Complementary DNA
CIBMTR	Center for International Blood and Marrow Transplant Research
CIITA	class II transactivator
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL-9/10	CXC motif chemokine ligands 9 and 10
CXCR3	CXC chemokine receptor 3
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNMT3A	DNA methyltransferase 3A
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ELN	European LeukemiaNet
ERV	endogenous retrovirus
E:T	effector-to-target
FAB system	French-American-British system
FACS	fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FLT3	fms-like tyrosine kinase 3
FLT3-ITD	FLT3 internal tandem duplications
gDNA	genomic DNA
GFI-1	growth factor independence 1
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HDAC	histone deacetylase
H3K4	histone 3 lysine residue 4
HLA	human leukocyte antigen
H9M	Hoxa9 and Meis1
HMA	hypomethylating agent
HSC	hematopoietic stem cell
ICAM-1	intercellular adhesion molecule-1
IDH	isocitrate dehydrogenase
IFN-γ	interferon-γ
IL	interleukin
IL-2RA	IL-2 receptor α
IRF	interferon regulatory factor

L-glut	L-glutamine
LPS	lipopolysaccharide
LSD1	lysine-specific demethylase 1
MACS	magnetic-activated cell sorting
MCP-1	monocyte chemoattractant protein 1
MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MiHA	minor histocompatibility antigen
MIP-1α	macrophage inflammatory protein 1α
MN1	Meningioma-1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFW	nuclease-free water
NPM1	nucleophosmin
NuRD complex	nucleosome remodelling and deacetylase complex
OS	overall survival
OVA	ovalbumin
qPCR	quantitative polymerase chain reaction
RCF	relative centrifugal force
RFS	relapse-free survival
PBS	phosphate-buffered saline
PD-1	programmed death-1
PD-L1	programmed death-ligand 1
P/S	penicillin/streptomycin
RIC	reduced-intensity conditioning
RPMI-1640	Roswell Park Memorial Institute-1640
SCF	stem cell factor
SD	standard deviation
STAT1/5	signal transducer and activator of transcription 1 and 5
TBI	total body irradiation
T-bet	T-box transcription factor
TCF1	T cell factor 1
TCP	tranylcypromine
TET2	tet methylcytosine dioxygenase 2
Th1 cells	type 1 T helper cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin domain and mucin domain 3
TNF-α	tumor necrosis factor-α
TLR3	toll-like receptor 3
YFP	yellow fluorescent protein
M	molar
mM	millimolar
μM	micromolar
nM	nanomolar
mL	milliliter

Declaration of Academic Achievement

I designed all the experiments presented in this thesis with Dr. Tobias Berg and Dr. Kanwaldeep Singh. I carried out the experiments by myself. H9M, MN1 and H9M-*Irf8*-KO cell lines were generated by Dr. Singh while H9M-OVA and MN1-OVA cells were generated by me. Undergraduate students Brendan Lee contributed to some qPCR experiments. I optimized the T cell isolation procedures based on protocols provided by Dr. Joanne Hammill and Rebecca Burchett.

Chapter 1: Introduction

1.1 Acute Myeloid Leukemia

1.1.1 Pathophysiology

Acute myeloid leukemia (AML) is an aggressive hematological malignancy with a 5-year survival rate of around 30% (Sasaki et al., 2021). It is characterized by the rapid clonal expansion of immature myeloid blast cells in the bone marrow and blood (Khwaja et al., 2016). The accumulation of leukemic cells can have detrimental effects on the normal functions of the hematopoietic system, which include the production of red blood cells, platelets and various types of immune cells. This would result in cytopenia, which often leads to clinical symptoms such as fatigue, uncontrolled bleeding, and frequent infections (Ferrara & Schiffer, 2013).

AML cells possess distinct phenotypes that differentiate them from healthy blood cells. During normal hematopoiesis, hematopoietic stem cells (HSCs) can undergo self-renewal or differentiate into fully functional blood cells. This process is tightly regulated by lineage-specific transcriptional factors, cytokines, growth factors and the bone marrow microenvironment (Orkin & Zon, 2008). However, HSCs and/or hematopoietic progenitor cells can acquire genetic mutations, chromosomal rearrangement and epigenetic changes that ultimately lead to malignant transformation (Khwaja et al., 2016). An older classification system, namely the French-American-British (FAB) system classifies AML into 8 subtypes, from M0 to M7, based on leukemia cell morphology (Schiffer & Stone, 2003). Current classification systems such as the International Consensus Classification of Myeloid Neoplasms and Acute Leukemias consider the underlying genetic abnormalities,

which can better facilitate the diagnosis, treatment and research of AML (Arber et al., 2022). Among the recurrent genetic abnormalities, somatic mutations are identified in over 96% of AML patients (Papaemmanuil et al., 2016), suggesting the critical roles of driver mutations in the initiation and progression of the disease.

1.1.2 Recurrent mutations in AML

Based on their roles in leukemogenesis, recurrent genetic mutations can be generally sorted into 3 main classes. Class I mutations include genes that participate in cell signalling and kinase pathways and contribute to unrestricted cell proliferation and survival (Di Nardo & Cortes, 2016). Fms-like tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase (RTK) that promotes the survival and proliferation of hematopoietic progenitor cells (Stirewalt & Radich, 2003). *FLT3* internal tandem duplication (*FLT3*-ITD) is one of the most commonly found recurrent mutations, occurring in approximately 25% of AML patients. *FLT3*-ITD causes constitutive activation of the receptor that leads to ligand-independent proliferation of leukemic blasts (Gary Gilliland & Griffin, 2002). Class II mutations involve genes responsible for the regulation of cell differentiation and self-renewal. An example of this type of mutation would be Nucleophosmin (*NPM1*) mutations, which occur in about 30% of AML patients and are often found in conjunction with other driver mutations like *FLT3*-ITD and DNA methyltransferase 3A (*DNMT3A*) (Falini et al., 2020). Mutant NPM1 has been shown to reinforce the blocked differentiation state of leukemic cells through the overexpression of homeobox (HOX) genes (Brunetti et al., 2018). A third class of mutations includes epigenetic regulators like *DNMT3A* and isocitrate dehydrogenase (*IDH*). In addition to shaping the epigenetic profiles of AML, these mutations also contribute to the immunogenicity of AML (Dufva et al., 2020).

The genomic profiles of AML patients are associated with varied responses to treatment and prognostic outcomes. Therefore, the European LeukemiaNet (ELN) AML risk stratification system has classified these recurrent genetic mutations, along with other cytogenetic abnormalities and clinical factors, into favourable, intermediate and adverse-risk categories (Döhner et al., 2022). The system provides valuable information to help clinicians determine treatment options and intensity and predict patient prognosis.

1.1.3 AML Treatment

The main clinical objects in the treatment of AML are inducing complete remission and preventing relapse. AML treatment is usually carried out in 3 different phases: induction and consolidation, after which a maintenance phase can follow. During the induction phase, patients are given the "7+3" chemotherapy regimen, which consists of 7 days of continuous infusion of cytarabine (100–200 mg/m^2) and 3 days of daunorubicin (60 mg/m^2). This is generally followed by higher doses of cytarabine (1000-1500 mg/m^2) for consolidation treatment (Döhner et al., 2022). This therapeutic approach, developed in 1973, remains the standard of care for AML today (Short et al., 2018). It has a cure rate of 30 to 40% in patients under the age of 60. For older patients, however, the outcomes are markedly inferior due to differences in disease biology and comorbidities leading to reduced treatment intensity (Kantarjian et al., 2021). This highlights the urgent need for novel and effective AML treatments that are less toxic particularly for older patients.

One recent advancement in AML treatment is the approval of FLT3 inhibitors as both induction and maintenance therapies for patients with FLT3 mutations. The addition of the FLT3 inhibitor midostaurin to the standard "7+3" regimen significantly improved the 5-year overall survival (OS) from 43.1% to 50.8% in a global randomized phase III

clinical trial (Stone et al., 2015). Currently, there are a number of FLT3 inhibitors either approved for clinical use or undergoing clinical trials (C. C. Smith, 2019). The success of FLT3 inhibitors encourages further development of targeted therapies for characterized driver mutations in AML.

Once complete remission is achieved, maintenance therapies are administered to prevent relapse and improve overall survival. Different therapeutic strategies have been proposed and examined in the clinic with varying degrees of success. These therapies include low-dose chemotherapy, targeted therapy, hypomethylating agents and immunotherapy (Reville & Kadia, 2021).

Allogeneic hematopoietic stem cell transplantation (allo-HSCT), which is the transplantation of HSCs from a healthy donor, represents another standard post-remission therapy. Despite being a potentially curative treatment option, relapse after allo-HSCT is of major concern and strategies to prevent post-transplant relapse are needed to further improve patient outcomes.

1.2 Allogeneic stem cell transplantation

1.2.1 Clinical development of allo-HSCT

Pioneered by Dr. E. Donnall Thomas in 1957, early attempts at allo-HSCT were met with significant clinical challenges, including graft rejections, post-transplant relapses and the development of graft-versus-host disease (GVHD) (Granot & Storb, 2020). In the 1970s, laboratory studies and clinical observations led to a better understanding of the roles of human leukocyte antigen (HLA) molecules in stem cell transplantation (Thomas et al., 1975). HLAs are human designations of the major histocompatibility complex (MHC)

molecules that present antigens to T cells to initiate diverse immune responses. As HLAs are highly polymorphic proteins, they also help the immune system distinguish foreign cells from self cells. A mismatch between the HLA types of the donor and the recipient can trigger host immune responses against donor stem cells and cause graft rejection. It can also prompt alloreactive donor-derived T cells to attack the otherwise healthy non-hematopoietic tissues of the recipient, leading to the development of GVHD (Fürst et al., 2019). Advances in HLA-typing technology, in particular moving from serological typing to molecular typing as well as typing additional loci, allowed for more precise matching of HLA groups between the donor and the recipient. This led to the successful transplantation of stem cells from unrelated donors, which have become the most common source of stem cells nowadays (Granot & Storb, 2020). In addition, the development of better conditioning regimens and immunosuppressants further improved transplant success and patient outcomes (Granot & Storb, 2020). Today, allo-HSCT has become standard of care and a cornerstone of AML treatment for many patients. A systematic review and meta-analysis of 24 clinical trials showed that allo-HSCT significantly reduced patient mortality by 27% and 17% in patients with adverse and intermediate risk profiles, respectively. For patients with a favourable risk profile, however, allo-HSCT in first complete remission has no clear clinical benefits (Koreth et al., 2009). Even in patients over the age of 60, allo-HSCT provides better long-term survival benefits compared to chemotherapy consolidation treatment alone, increasing the 5-year OS from 13.8% to 29%. Nevertheless, this is accompanied by a significant increase in treatment-related mortality during the first 9 months after transplant (Ustun et al., 2019). Therefore, the benefits of allo-HSCT in older patients are more nuanced and have to be determined on a case-by-case basis.

1.2.2 The Graft-versus-leukemia effect

For younger patients with adverse and intermediate genetic risk profiles, myeloablative conditioning is used to allow for more thorough eradication of leukemia cells prior to allo-HSCT. In older patients and patients with other comorbidities, reduced-intensity conditioning (RIC) regimens are preferred in order to lower toxicity and complications (Luo et al., 2022). While the use of RIC has been shown to decrease treatment-related mortalities, it has also been associated with a significantly higher risk of relapse (Scott et al., 2021).

After myeloablative conditioning, patients are then engrafted with stem cells from healthy donors to reconstitute the hematopoietic system. Allogeneic T cells transferred along with the graft can eliminate residual malignant cells and produce the so-called graft-versus-leukemia (GVL) effect (Sweeney & Vyas, 2019). Mechanistically, donor-derived T cells primarily carry out their anti-leukemic effect in fully matched recipients by recognizing minor histocompatibility antigens (MiHAs) presented in the context of identical HLA molecules. MiHAs are self-derived polymorphic peptides that differ in amino acid sequence between the donor and recipient. They help donor T cells recognize non-self leukemia cells in the recipient and induce antigen-specific alloreactive immune response. GVHD occurs when donor T cells recognize MiHAs presented by non-hematopoietic tissues (Jenq & Van Den Brink, 2010). During haploidentical (half-matched) allo-HSCT, donor-derived T cells can additionally recognize both self and non-self peptides presented by non-self HLA molecules (Falkenburg & Jedema, 2017). While this greatly expands the repertoire of alloreactive T cells capable of mediating the GVL effect, it also significantly increases the risk of developing GVHD. Therefore, T cell depletion

with post-transplant cyclophosphamide and immunosuppressants are required to allow for haploidentical transplant (Luznik et al., 2008). Overall, the GVL effect represents a major therapeutic benefit of allo-HSCT, particularly for patients receiving RIC regimens. To prevent post-transplant relapse, maintenance therapies currently in development generally aim to enhance the post-transplant GVL effect without exacerbating GVHD.

1.2.3 Patient outcomes after allo-HSCT

Over the past few decades, increased scientific knowledge and improved clinical supportive care have led to better outcomes after allo-HSCT. The Center for International Blood and Marrow Transplant Research (CIBMTR) collects and analyzes data from transplant centres around the world to better inform and improve the outcomes of transplant patients. Most recent CIBMTR data showed that the 3-year survival rate for AML patients who have received allo-HSCT is around 50%, compared to 35% observed twenty years ago. The outcomes are comparable in both pediatric and adult AML patients (D'Souza et al., 2020). AML patients who have survived at least 2 years after the initial transplant are expected to remain in remission for an extended period of time, with a 10-year survival rate of 84% (Wingard et al., 2011). Therefore, the first 2 years after allo-HSCT are critical in determining the long-term outcomes of transplant recipients. Interestingly, the 2-year period correlates with the amount of time required for complete immune reconstitution in transplant recipients. Following myeloablation-induced immunological injury, donor-derived innate immune cells are expected to recover within the first 100 days. However, the functional recovery of T cells and B cells may take up to 2 years (Figure 1) and this recovery process has been directly linked to patient outcomes (Velardi et al., 2020).

Therefore, promoting post-transplant immune reconstitution is another strategy to improve the overall success of allo-HSCT.

The leading causes of death for allo-HSCT recipients can vary depending on the stages after transplantation. Among the patients who succumbed within 100 days (early stage), transplantation-related mortalities, including organ failure, infection and acute GVHD collectively contribute to over 60% of patient deaths. For patients who passed away after the 100-day mark (intermediate to late stage), recurrence of the primary disease becomes the primary cause of death, accounting for over 50% of deaths alone (D'Souza et al., 2020). To ensure the long-term effectiveness of allo-HSCT, preventing relapse is of utmost priority and serves as the main objective for post-transplant maintenance therapies.

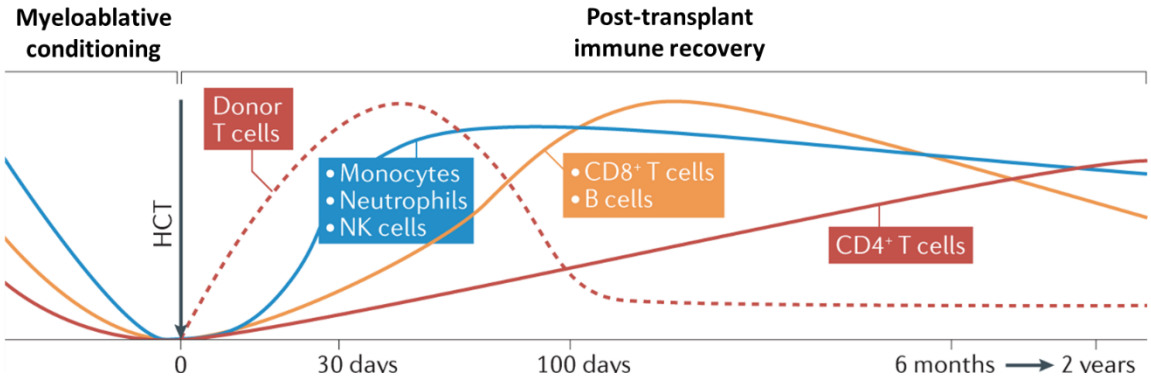


Figure 1. Timing of immune recovery after allo-HSCT. The reconstitution of immune system by donor hematopoietic stem cells occurs in a stepwise manner with innate immune cells being the first ones to recover. Donor-derived T cells initially transferred with the graft are mainly responsible for mediating alloreactive anti-leukemic response. Adapted from Velardi *et al.* (2020).

1.3 Immunological considerations for post-transplant maintenance

1.3.1 Post-transplant immune evasion mechanisms

Despite the powerful GVL effect provided by donor T cells, residual leukemia cells can still evade allogeneic immune surveillance and contribute to AML relapse. Recent studies have analyzed clinical samples from relapsed patients and several post-transplant immune evasion mechanisms have been proposed. The most prevalent phenotype involves a significant downregulation of HLA-II molecules, which occurs in about 50% of relapsed AML patients receiving HLA-matched allo-HSCT (Figure 2). This is accompanied by a decreased expression of class II transactivator (*CIITA*), a master regulator of HLA-II expression. There were no significant changes in HLA-I expression after relapse (Christopher et al., 2018; Toffalori et al., 2019). In patients with haploidentical allo-HSCT, the irreversible genomic loss of HLA alleles is a frequent occurrence observed in 20% to 50% of cases (Wu et al., 2022).

The downregulation of HLA-II in relapsed AML cells indicates the presence of immune selection pressure that leads to the emergence of a leukemia cell population more resistant to the GVL effect. More specifically, it may impair T cell recognition of leukemia cells by alloreactive $CD4^+$ T cells and thus leads to immune evasion. $CD4^+$ T cells are known to promote anti-tumor response by activating and sustaining $CD8^+$ cytotoxic T cell response. Polarized $CD4^+$ type 1 T helper (Th1) cells can also directly mediate anti-tumor immunity by secreting effector cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Tay et al., 2020). Therefore, evading from $CD4^+$ T cells through the downregulation of HLA-II may compromise the GVL effect and contribute to post-transplant relapse.

On the other hand, $CD8^+$ T cells isolated from relapsed patients showed significant exhaustion phenotypes. This is characterized by an increased expression of inhibitory immune checkpoint receptors including programmed death-1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and T cell immunoglobulin domain and mucin domain 3 (TIM-3) (Hutten et al., 2018). In particular, $PD\text{-}1^{hi}$ $TIM\text{-}3^+$ T cells isolated from relapsed transplant recipients showed functional impairment demonstrated by a reduced expression of effector cytokines including interleukin-2 (IL-2), IFN-α and TNF-α. They are found to be strongly associated with post-transplant relapse (Kong et al., 2015). $PD\text{-}1^{hi}$ $TIM\text{-}3^+$ T cells have been characterized as terminally exhausted T cells. They represent a distinct T cell population that cannot be reinvigorated by existing immune checkpoint blockade therapies (Zhang et al., 2014). The presence of terminally exhausted alloreactive T cells may therefore limit the efficacy of using immune checkpoint inhibitors as post-transplant maintenance therapy (Köhler et al., 2021).

In conclusion, HLA-II downregulation and T cell exhaustion are two major contributors to post-transplant relapse. Maintenance therapies in development should seek to target these mechanisms to enhance and prolong the GVL effect.

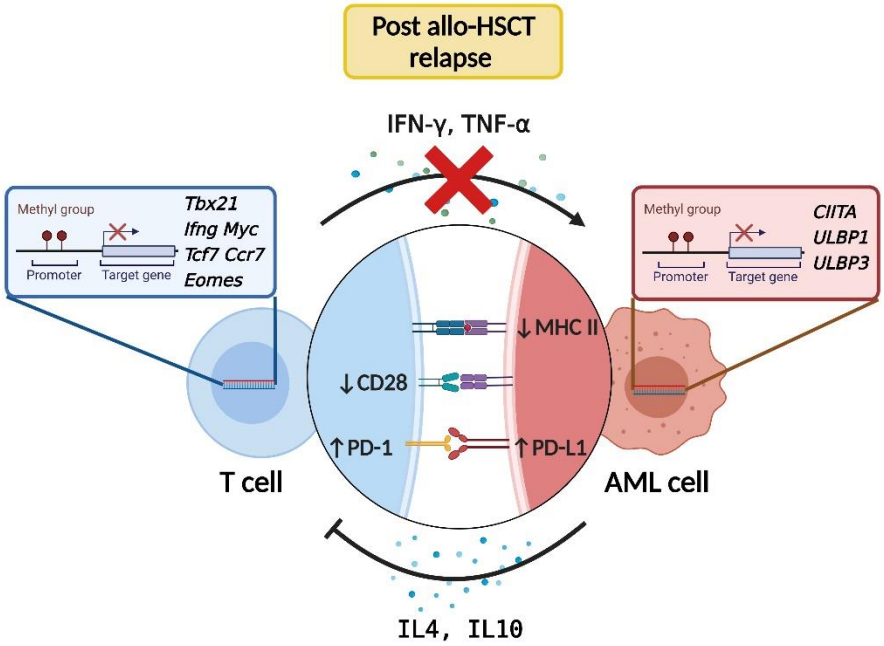


Figure 2. Post-transplant AML immune evasion mechanisms. The downregulation of HLA-II molecules represents a distinct mechanism by which leukemia cells can evade alloreactive $CD4^+$ T cells immune surveillance and potentially contribute to AML relapse. AML cells may also upregulate inhibitory programmed death-ligand 1 (PD-L1) and anti-inflammatory cytokines like IL-4 and IL-10 to further suppress T cell activity (Norde et al., 2011; Park et al., 2006). The exhaustion of donor-derived T cells can also impair the post-transplant GVL effect. Both HLA-II downregulation and T cell exhaustion can be influenced by epigenetic reprogramming (Yan et al., 2022). *Imagine created from BioRender.com*

1.3.2 Epigenetic regulation of immune evasion

Epigenetics is the study of gene expression changes caused by structural alterations in chromatin. Common epigenetic modifications include DNA methylation, histone methylation and acetylation (Bird, 2007). Recent studies suggest that the aforementioned immune evasion mechanisms could be driven by epigenetic changes.

Comprehensive immunogenomic analysis showed that the hypermethylation of *CIITA* at its promotor region strongly associates with low HLA-II expression (Figure 2). It was further revealed that DNA methylation levels are highest in AML cells harboring *NPM1*, *IDH1/2* and tet methylcytosine dioxygenase 2 (*TET2*) mutations (Dufva et al., 2020). *IDH1/2* and *TET2* mutations are mutually exclusive in AML and can lead to similar global DNA hypermethylation events (Figueroa et al., 2010). Treatment with a hypomethylating agent decitabine can reverse the epigenetic silencing of *CIITA* and re-upregulate HLA-II in AML cell line MOLM-13 (Dufva et al., 2020). Histone deacetylation is also involved in the epigenetic silencing of *CIITA* and often directly correlates with DNA hypermethylation (Morimoto et al., 2004). Treatment by the histone deacetylase (HDAC) inhibitor sodium butyrate moderately enhanced the expression of HLA-II in KG-1 AML cells (Santana Carrero et al., 2019).

At the same time, epigenetic reprogramming can drive and reinforce T cell exhaustion (Figure 2). In terminally exhausted murine T cells, de novo DNA methylation was shown to suppress the expression of genes responsible for T cell effector functions, including *Ifng*, *Tbx21* (encodes for T-box transcription factor; T-bet), and *Tcf7* (encodes for T cell factor 1; TCF1). This subsequently prevented reinvigoration by immune checkpoint inhibitors (Ghoneim et al., 2017). Reduced histone acetylation levels at the *Ifng* and *Il2* loci were also associated with diminished effector function in exhausted T cells (Zhang et al., 2014). Treatment with hypomethylation agents and HDAC inhibitors can reverse the silencing of effector genes and restore T cell effector functions (Ghoneim et al., 2017; Zhang et al., 2014). Therefore, targeting T cell exhaustion through epigenetic mechanisms represents a promising strategy to rescue the GVL effect.

1.3.3 Current post-transplant maintenance therapies

Given the important roles of epigenetic regulators in post-transplant relapse, there has been growing interest in using epigenetic inhibitors as post-transplant maintenance therapy.

Hypomethylating agents (HMAs) like azacitidine and decitabine produce active metabolites that can work to reduce DNA methylation by acting as cytosine analogues during DNA replication. Incorporated azanucleotides can covalently bind DNMT1 and reduce DNA methylation (Stomper et al., 2021). Despite their ability to enhance HLA-II expression and reinvigorate exhausted T cells, a phase III randomized clinical trial with azacitidine as maintenance therapy did not improve relapse-free survival (RFS) or overall survival (OS) in high-risk AML patients (Oran et al., 2020). Nevertheless, the design of this clinical trial exhibited multiple limitations and further investigation to determine the true therapeutic benefits of HMAs maintenance is ongoing.

HDAC inhibitors are another class of epigenetic therapy currently being explored as maintenance strategies. In addition to enhancing T cell effector functions, HDAC inhibitors were shown to upregulate the co-stimulatory molecule CD86 and the intercellular adhesion molecule-1 (ICAM-1) in AML cells. Both molecules are essential for the activation and recruitment of T cell response (Maeda et al., 2000). A completed phase I/II clinical trial with the HDAC inhibitor panobinostat demonstrated favourable outcomes in allo-HSCT recipients (2-year RFS = 75%; 2-year OS = 81%). A randomized phase III clinical trial is underway (Bug et al., 2017).

Given the heterogeneous nature of AML, additional epigenetic targets are desired to increase the number of available treatment options. Meanwhile, it is equally important

to characterize the differential responses of AML subtypes to various therapeutic candidates during preclinical studies. This can aid in designing targeted clinical trials to develop personalized maintenance approaches.

1.4 Lysine-specific demethylase 1 (LSD1)

1.4.1 Biochemical basis of LSD1

Originally identified in 2004 as an essential regulator for histone methylation (Shi et al., 2004), LSD1 has emerged as a promising therapeutic target in AML. As a transcriptional repressor, LSD1 catalyzes the demethylation of mono and di-methylated histone 3 lysine residue 4 (H3K4me1/2) to decrease chromatin accessibility and thereby suppress gene expression. This process is carried out through its association with the repressive CoREST or the nucleosome remodelling and deacetylase (NuRD) complex. On the other hand, LSD1 can also activate gene transcription by associating with androgen receptor or estrogen receptor to demethylate H3K9me1/2 residues (Fang et al., 2019). Structurally, LSD1 consists of 3 major domains: the SWIRM domain, the Tower domain and the amine oxidase-like (AOL) domain (Figure 3A). The long, protruding Tower domain is indispensable for the interaction between LSD1 and the CoREST complex. The AOL domain contains a deep catalytic centre that confers spatial specificity for the insertion of mono and di-methylated H3K4 residues (Figure 3B). The demethylation activity of LSD1 is dependent on its co-factor flavin adenine dinucleotide (FAD), which is a strong oxidizing agent situated inside the catalytic centre. K661 residue is necessary for

the binding of FAD, with which it forms hydrogen bonds via a conserved water molecule (Figure 3C) (Chen et al., 2006).

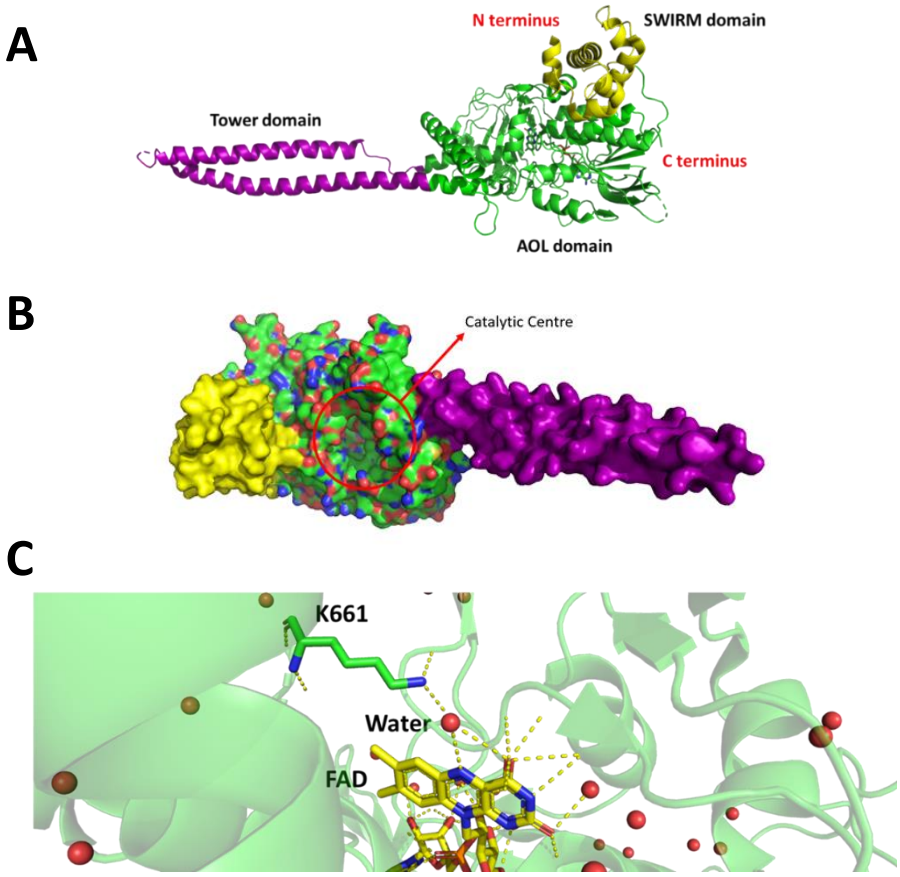


Figure 3. Crystal structure of lysine-specific demethylase (LSD1: PDB 2HKO). (A) The three major domains of LSD1 are shown in different colours: the SWIRM domain (yellow), the AOL domain (green) and the Tower domain (purple). (B) The catalytic centre (highlighted in red) is a deep and narrow pocket that can provide specificity for mono or di-methylated lysine residues. (C) K661 is hydrogen-bonded to a water molecule (red), which is hydrogen-bonded to the N5 atom of the FAD molecule. *Imagines created with PyMol.*

1.4.2 Roles of LSD1 inhibition in AML pathogenesis

Although not a common driver mutation itself, LSD1 is highly expressed in the majority of AML cases (Niebel et al., 2014). It was first identified as a targetable epigenetic regulator in AML due to its role in inhibiting myeloid differentiation and maintaining the clonogenic potential of leukemia stem cells (Harris et al., 2012). LSD1 inhibition increased H3K4me2 levels and selectively enhanced the expression of genes involved in myeloid differentiation. Subsequently, pharmacological inhibition of LSD1 by tranylcypromine (TCP) synergized with all-trans retinoic acid (ATRA) to induce the differentiation of both murine and human AML cells (Schenk et al., 2012). It was further demonstrated in murine AML models that LSD1 is recruited by the transcriptional repressor growth factor independence 1 (GFI1) to suppress the expression of genes regulated by PU.1, an essential transcriptional factor in myeloid differentiation. LSD1 inhibition led to significant upregulation of PU.1 target genes, including *Gfi1b* and interferon regulatory factor 8 (*Irf8*) (Barth et al., 2019).

The LSD1 inhibitor TCP is a monoamine oxidase inhibitor initially approved for treating mood disorders. It was later discovered to irreversibly inhibit LSD1 activity by forming a covalent bond with its co-factor FAD (Yang et al., 2007). Currently, a number of TCP-based LSD1 inhibitors are undergoing clinical trials for treating AML, often in combination with other therapeutic agents. Most of these inhibitors demonstrated good safety profiles and modest response rates during phase I studies, providing support for further clinical investigation (Fang et al., 2019).

1.4.3 Immunomodulatory effects of LSD1 inhibition

More recently, LSD1 inhibition has been found to exhibit diverse immunomodulatory properties that could be exploited to enhance the GVL effect. In studies conducted on both murine and primary human AML cells, treatment by LSD1 inhibitors led to a marked upregulation of the co-stimulatory molecule CD86 (Barth et al., 2019). In the context of solid tumors, LSD1 inhibition was shown to increase the expression of endogenous retroviruses (ERVs), which are non-infectious retroviral gene fragments making up around 8% of the human genome (Johnson, 2019). The upregulation of ERVs activated intracellular toll-like receptor 3 (TLR3) signalling and induced a type 1 interferon response in melanoma cells. As a result, treatment with LSD1 inhibitors enhanced the immunogenicity of tumor cells and sensitized them to T cell-mediated anti-tumor activity when combined with immune checkpoint inhibitors (Sheng et al., 2018). Similarly, in breast and small cell lung cancers, LSD1 inhibition enhanced tumor immunogenicity by increasing the production of T cell-attracting (CXCL-9/10), which subsequently promoted T cell infiltration into the tumor microenvironment (Hiatt et al., 2022; Qin et al., 2019). It was further demonstrated that LSD1 inhibition can reprogram tumor-associated macrophages to exhibit pro-inflammatory M1 phenotypes (Tan et al., 2019). Altogether, the combination of LSD1 inhibitors and immune checkpoint inhibitors significantly reduced tumor growth by stimulating anti-tumor T cell immunity. Given the high efficacy demonstrated in preclinical studies, the combination therapy has received tremendous translational interest and is undergoing clinical trials for treating numerous types of cancers (Noce et al., 2023).

In addition, LSD1 may directly influence T cell activity. In $CD4^+$ T cells, LSD1 was shown to be involved in GFI1-mediated suppression of Th1 cell programming. LSD1 inhibition directly drove Th1 polarization, which was demonstrated by the upregulation of Th1-associated transcriptional factors and the increased production of type 1 cytokines IFN-γ, TNF-α and IL-2 (Suzuki et al., 2016). Independent of its role in epigenetic regulation, LSD1 was also found to directly interact with and antagonize TCF1, which is essential for the persistence of T cell effector functions. LSD1 inhibition was able to promote and sustain the cytotoxic activity of tumor-infiltrating $CD8^+$ T cells and thereby enhancing anti-tumor immunity (Liu et al., 2021). TCF1 expression is responsible for maintaining a population of stem-like progenitor exhausted $CD8^+$ T cells more responsive to immune checkpoint blockade (Escobar et al., 2020). Therefore, LSD1 inhibition could be a promising approach to prevent T cell exhaustion, which may be relevant in post-transplant maintenance.

1.5 Project rationale

Relapse of the primary disease remains the leading cause of mortality after allo-HSCT, indicating the urgent need for safer and more effective post-transplant maintenance therapies. HLA-II downregulation and T cell exhaustion are two major mechanisms by which residual leukemia cells can evade immune surveillance from alloreactive donor-derived T cells and lead to AML relapse. As both immune evasion mechanisms are influenced by epigenetic modifications, pharmacological inhibitors targeting epigenetic regulators may serve as ideal candidates for post-transplant maintenance. More specifically, the expression of HLA-II is generally restricted to professional antigen-presenting cells (APCs) of myeloid lineages under non-inflammatory conditions (Roche & Furuta, 2015).

Therefore, restoring HLA-II expression in AML may be a promising strategy to selectively enhance the GVL effect.

LSD1 is a promising therapeutic target in AML due to its role in regulating myeloid differentiation. In murine AML cells, LSD1 is involved in GFI1-mediated repression of PU.1-target genes, which include *Irf8* (Barth et al., 2019). Together, PU.1 and IRF8 positively regulate *CIITA* expression to promote dendritic cell maturation, suggesting that LSD1 inhibition may enhance HLA-II expression in AML cells and restore their antigen-presenting capability (M. A. Smith et al., 2011). In addition, LSD1 inhibition may have a role in reinvigorating terminally exhausted T cells. Emerging evidence shows that the T cells dysfunctions are reinforced by epigenetic remodelling. Decreased chromatic accessibility at T cell effector function genes, including *Tbx21*, *Ifng*, *Tcf7*, strongly associates with terminal exhaustion phenotypes and prevents reinvigoration by immune checkpoint blockade (Franco et al., 2020). In particular, *Tbx21* has been established as a master regulator of T cell effector function (Intlekofer et al., 2005). LSD1 is recruited by GFI1 to decrease H3K4 methylation levels at *Tbx21* loci, potentially contributing to the transcriptional silencing of T-bet (Suzuki et al., 2016). Therefore, LSD1 inhibition may unleash the epigenetic block on terminally exhausted T cells to rescue their effector functions.

In conclusion, because of its effects in immune activation, LSD1 inhibition may represent an interesting strategy in post-transplant maintenance. With a number of LSD1 inhibitors currently undergoing clinical trials for AML treatment and demonstrating favourable safety profiles in phase I studies, exploration of their immunomodulatory effects may provide additional therapeutic benefits for allo-HSCT recipients. The

availability of clinical-stage LSD1 inhibitors would also provide opportunities for speedy clinical translation to meet the urgent need for effective post-transplant maintenance therapies. The current project has specifically investigated the ability of LSD1 inhibitors to enhance antigen presentation in AML cells and augment T cell-mediated anti-tumor immunity.

1.6 Hypothesis and specific aims

The hypothesis underlying the project is that LSD1 inhibition may enhance T cell-mediated anti-leukemia immunity after allo-HSCT. More specifically, it is hypothesized that LSD1 inhibition can enhance class II antigen presentation and co-stimulation in AML cells, which would in turn promote antigen-specific $CD4^+$ T cell activation and immune killing. To investigate these hypotheses, the present project has studied the following aims:

1. **Characterize the effects of LSD1 inhibition on relevant immunological factors in human and murine AML cells *in vitro* (Figure 4).**

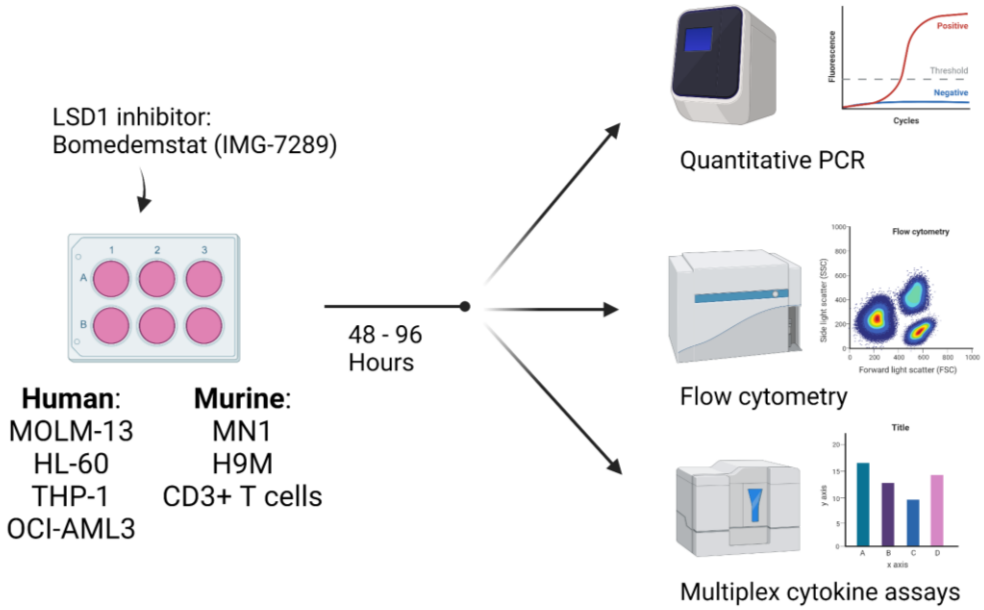


Figure 4. Assessing the effects of bomedemstat on relevant immunological factors *in vitro*. The immunomodulatory effects of a clinical-stage LSD1 inhibitor bomedemstat on both human and murine AML cells were assessed using quantitative polymerase chain reaction (qPCR), flow cytometry and multiplex cytokines assays.

2. **Conduct *in vitro* functional assays with murine AML and T cells to examine the effects of LSD1 inhibition on T cell-mediated anti-leukemia immune response (Figure 5).**

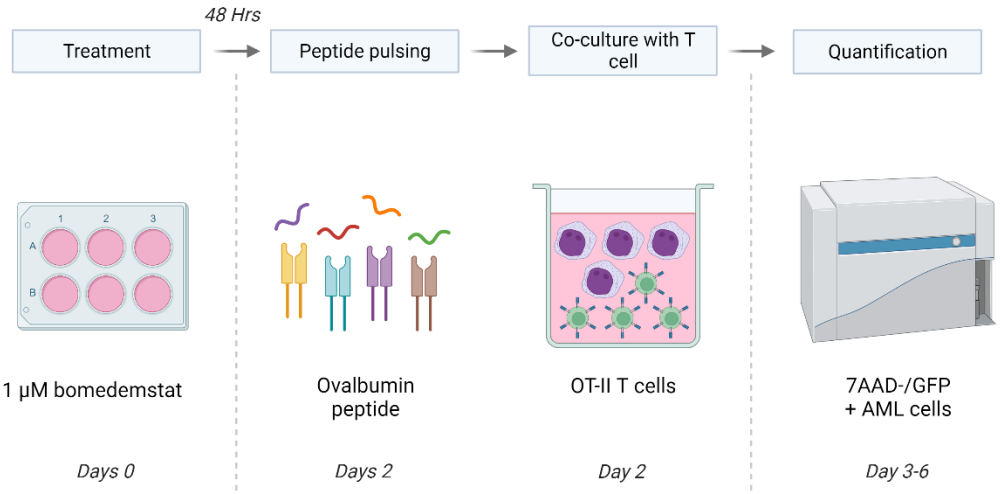


Figure 5. *In vitro* AML and T cell co-culture assays for functional assessment. Murine AML cells were pre-treated with bomedemstat and co-cultured with antigen-specific T cells. Flow cytometry were used to assess T cell killing, activation and proliferative capability.

Chapter 2. Methods and Materials

2.1 AML cell culture

2.1.1 Human AML cell lines

Three human AML cell lines representing various molecular subtypes of AML were used to assess the effects of LSD1 inhibition on HLA-II and CD86 expression. MOLM-13 cells (FAB M5a) harbor an *FLT3-ITD* mutation in addition to a *KMT2A-MLLT3* fusion gene. HL-60 cells (FAB M2) carry the *c-myc* oncogene. OCI-AML3 (FAB M4) cells carry *NPM1* and *DNMT3A* mutations. All three cell lines are HLA-DR negative at baseline. An additional AML cell line THP-1 carrying the *KMT2A-MLLT3* fusion gene was assessed for cytokine production upon LSD1 inhibition.

All human AML cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 media +10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (P/S) + 1% L-glutamine (L-glut). This media recipe is hereinafter referred to as the human AML media. The human cell AML lines were cryopreserved in 90% FBS + 10% dimethyl sulfoxide (DMSO) and stored at -150 ℃. Prior to experiments, the frozen cells were thawed and washed with cRPMI media. They were then seeded in T-75 non-adherent flasks at a density of 0.5-1x10^5 cells/mL. Cells were incubated at 37 ℃ with 5% CO_2 and were passaged every 2-3 days once the density reached 2-3x10^6 cells/mL.

2.1.2 Generation of murine AML cell lines

Two murine AML cell lines were generated by overexpressing oncogenes *Meningioma-1* (MN1) or *Hoxa9* and *Meis1* (H9M) in lineage-depleted murine bone marrow cells through retroviral transduction. More specifically, bone marrow cells were

first harvested from C57BL/6J mice (Jackson Laboratory). They were lineage-depleted via magnetic-activated cell sorting (MACS) using the mouse Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's protocol. The lineage-depleted bone marrow cells were co-cultured with GP+E86 viral packaging cells transduced with pSF91-*MN1*-IRES-eGFP (for MN1 cells), or MSCV-*Hoxa9*-PGK-neo and MSCV-*Meis1*-IRES-YFP (for H9M cells). Transformed MN1 and H9M cells were selected through fluorescence-activated cell sorting (FACS) based on enhanced green fluorescent protein (eGFP) and yellow fluorescent protein (YFP) expression, respectively. As the *Hoxa9* plasmid contains a geneticin-resistant gene, the H9M cells were further selected with 600 μg/mL of geneticin for a week. Selected MN1 and H9M cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% FBS, 1% P/S and 1% L-glut. As the murine AML cells are cytokine-dependent, the media was additionally supplemented with 6 ng/mL murine IL-3, 10 ng/mL human IL-6, and 100 ng/mL murine stem cell factor (SCF) (Peprotech). The cytokine composition is together referred to as murine AML cytokines and the media is referred to as the BM-AML media. To study the impact of IRF8 depletion, another H9M cell line were generated from *Irf8*-knockout (*Irf8*-KO) mice (B6(Cg)-*Irf8*$^{tm1.2Hm}$/J, Jackson Laboratory). This cell line is termed the H9M-*Irf8*-KO cells. The generation of stable H9M-*Irf8*-KO cells required a slight modification to previous protocols. Lineage-depleted bone marrow cells were first transduced with *Hoxa9* and were selected with 600 μg/mL of geneticin for a week until their viability recovered. They were then transduced with *Meis1* and were FACS-sorted based on YFP expression. To study T cell-mediated anti-leukemia immunity in an antigen-specific context, additional MN1 and H9M cell lines were generated from mice transgenic for

ovalbumin (OVA)(C57BL/6-Tg(CAG-OVAL)916Jen/J, Jackson Laboratory). The two cell lines are termed MN1-OVA and H9M-OVA cells to indicate the transgenes they carry. H9M-OVA cells were generated following the same protocol as H9M-*Irf8*-KO cells as attempts to deliver both oncogenes at the same time failed to give rise to stable OVA-expressing H9M murine AML cells. All animal experiments were approved by the Animal Research Ethics Board under the Animal Utilization Protocol 20-10-35.

2.2 Pharmacological inhibition of LSD1

The effects of LSD1 inhibition were assessed using the clinical-stage, irreversible small molecule inhibitor bomedemstat (also referred to as IMG-7289 or simply IMG) provided by the industry collaborator Imago Biosciences. Bomedemstat was dissolved in DMSO at a stock concentration of 20 mM and stored at -30 ℃. For the treatment of human and murine AML cells, the stock solutions were diluted with their corresponding media and thoroughly mixed with the cell culture. Concentrations of bomedemstat used in this project included 1 nM, 10 nM, 100 nM and 1 μM. The maximum clinically relevant concentration of bomedemstat was previously determined by Imago Biosciences.

2.3 Quantitative PCR (qPCR)

2.3.1 RNA extraction and complementary DNA (cDNA) synthesis

qPCR was used to determine the effects of bomedemstat treatment on the expression of various markers in human AML cell lines. The cell lines were seeded at a density of $2.5x10^5$ cells/mL in 4mL cRPMI media and treated with 1 nM, 10 nM, 100 nM, and 1 μM of bomedemstat or vehicle (DMSO) for 48 hours. Cell cultures were collected and centrifuged at 250 relative centrifugal force (rcf) for 5 minutes and washed with 4mL

phosphate-buffered saline (PBS). The cell pellets can be stored at -80 ℃ prior to any experimental analysis. Total RNA was extracted from the cell pellets using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. Briefly, the cell pellets were lysed and homogenized with the Buffer RLT Plus containing β-mercaptoethanol. The cell lysates were transferred to the genomic DNA (gDNA) Eliminator spin column and centrifuged for 30 seconds at 8,000 rcf. 1 volume of 70% ethanol was added to each tube containing the flow-through. The samples were transferred to RNeasy spin column and washed with Buffer RW1, followed by Buffer RPE. The flow-through was discarded after each wash. Finally, RNA was eluted from the spin column with 30 µL of nuclease-free water (NFW). The concentrations and purities of the RNA samples were determined using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). The RNA samples can be stored at -80 ℃ until further analysis. To synthesize cDNA, 500 ng of RNA from each sample was reverse-transcribed into cDNA using the SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher Scientific). More specifically, 2 µL of 10X SuperScript III Enzyme Blend and 4 µL of 5X VILO Master Mix solution were added to 500 ng of RNA. Each sample was topped up with NFW for a total reaction volume of 20 µL. Negative controls include samples without the addition of reverse transcriptase. Then, the samples were amplified with the Mastercycler nexus PCR Thermal Cycler (Eppendorf) using the following program: 25 ℃ for 10 mins, 42 ℃ for 60 mins, and 85 ℃ for 5 minutes. The cDNA sample can be stored at -30 ℃.

2.3.2 qPCR

The expression of genes of interest was measured using the pre-designed TaqMan™ Real-Time PCR Assays (ThermoFisher Scientific). The following markers were

studied in this project: *CIITA* (Assay ID: Hs00172106_m1), *CXCL10* (Assay ID: Hs00171042_m1), *IL12B* (Assay ID: Hs01011518_m1). *ABL1* (Assay ID: Hs01104728_m1) was used as the housekeeping gene for normalization. The reaction mix contains 5 µl of 2X TaqMan™ Fast Advanced Master Mix, 4 µL of NFW and 1 µL of cDNA from each sample, for a total volume of 10 µL. Negative controls include samples without reverse transcriptase or template cDNA. Real-time PCR amplification was performed using the QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific). The following program was used: 20 seconds of holding at 95 ℃ for polymerase activation, followed by 15 seconds at 95 ℃ for denaturation and 60 seconds at 60 ℃ for annealing/extension, for a total of 40 cycles. Data were analyzed using the comparative Ct (ddCt) method (Livak & Schmittgen, 2001). Briefly, the relative expression levels of the target genes were first normalized to the internal housekeeping control *ABL1*. The resulting dCt values were compared to the DMSO control group and gene expression level changes were expressed as log2 fold change relative to the control group.

2.4 Flow cytometry

To prepare for flow cytometric analysis, the cell cultures were collected and washed with staining buffer which contains PBS, 10% FBS and 1 nM EDTA (PEF). For staining with fluorescent conjugated antibodies, cells were resuspended in 200 µL of PEF. The following antibodies were used in the present study:

Table 1. List of antibodies utilized in this study.

Target	Fluorophore	Clone	Dilution	Source
Human HLA-DR	Pacific Blue	Immu-357	1:40	Beckman Coulter

Human CD86	PerCP-eFluor 710	IT2.2	1:200	ThermoFisher Scientific
Mouse MHC-II I-A/I-E	AF700	M5/114.15.2	1:200	BioLegend
Mouse CD86	BV421	GL1	1:200	BD Biosciences
Mouse CD3	APC	17A2	1:200	BioLegend
Mouse CD25	PE	3C7	1:200	BioLegend
Mouse CD69	PE-cy7	H1.2F3	1:200	BioLegend
Mouse CD107a	BV421	1D4B	1:100	BioLegend
Mouse CD3	PE-cy7	145-2C11	1:200	BioLegend

To assess the effects of bomedemstat treatment on the surface expression of HLA-DR and CD86, Human AML cell lines were seeded at a density of $2.5x10^5$ cells/mL in 4 mL cRPMI media and treated with 100 nM and 1 μM of bomedemstat or vehicle (DMSO) for 48 hours. The cell cultures were equally divided and 10 ng/mL of human IFN-γ (Peprotech) was added to 2 mL of cell culture for 24 hours. Murine AML cells were seeded at a density of $1.25x10^5$ cells/mL in 4 mL cDMEM media. They were treated with 100 nM and 1 μM of bomedemstat or vehicle (DMSO) for 48 hours. For compensation analysis, single-stained controls were set up using the UltraComp eBeads™ Compensation Beads (ThermoFisher Scientific). After staining with fluorescent conjugated antibodies, the samples were incubated in the dark for 30 minutes at room temperature. The cells were

then washed and resuspended in 200 µL of PEF. Dead cells were excluded using 7-aminoactinomycin D (7AAD; BD Biosciences). Flow cytometry was performed using a CytoFLEX LX Flow Cytometer (Beckman Coulter) and the data were analyzed using FlowJo software (version 10; Tree Star).

2.5 Multiplex cytokines analysis

The effects of bomedemstat-treatment on human AML cytokine production were measured using the Human Cytokine 71-Plex Discovery Assay (Eve Technologies). Human AML cell lines were seeded at a density of $2.5x10^5$ cells/mL in 4mL cRPMI media and treated with 100 nM bomedemstat or vehicle (DMSO) for 48 hours. 1 mL of cell culture was taken from each condition and centrifuged at 3,000 rcf at 4 ℃ for 5 minutes. 500 µL of supernatant were taken from each sample and centrifuged again with the same parameters. Finally, 200 µL of supernatant were taken from the middle of the sample tube and stored at -80 ℃ before being shipped to the company.

2.6 T cell isolation

To isolate T cells for functional analysis, spleens from OT-II transgenic mice (B6.Cg-Tg(TcraTcrb)425Cbn/J, Jackson Laboratory) were harvested and transferred to a 50 mL conical tube containing 10 mL of cold T cell media. The recipe for the T cell media is as follows: 500 mL RPMI + 50 mL FBS + 5 mL HEPES + 5 mL L-glut + 5 mL non-essential amino acids + 5 mL sodium pyruvate + 1 mL P/S. The complete T cell media is further supplemented with 5 ng/mL human IL-2 (hIL-2), 5 ng/ml human IL-7 (hIL-7), and 50 µM 2-mercaptoethanol before being used for experiments. The splenocytes were isolated by smashing the prepared spleen with a sterile plunger of a 3 mL syringe in a 70

μm cell strainer placed on top of a 50 mL conical tube. The strainer was flushed with 10 mL cold PBS and the conical tube was centrifuged at 400 rcf for 10 minutes. After discarding the supernatant, the cell pellet was resuspended in 3 mL sterile ACK lysis buffer for red blood cell lysis. The ACK buffer contains 0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA. The cell suspension was incubated for 5 minutes at room temperature and 10 mL cold PBS were added to stop the lysis. After centrifuging at 400 rcf for 5 minutes, the supernatant was removed and the cell pellet was resuspended in 20 – 30 mL of cold PBS to determine the total cell number. Pan T cells or $CD4^+$ T cells were purified via MACS using the mouse Pan T Cell Selection Kit or the $CD4^+$ T Cell Selection Kit (Miltenyi Biotec) respectively according to the manufacturer's protocols. The purified T cells were cryopreserved using the same protocol or cultured in complete T cell media until further analysis.

2.7 T cell activation assays

H9M-OVA and MN1-OVA cells were pre-treated with 1 μM of bomedemstat for 48 hours. The cells were resuspended in complete T cell media supplemented with 25% murine AML cytokines at a density of $1x10^6$ cells/mL. $CD4^+$ OT-II cells were resuspended in complete T cell media at densities of $1x10^6$ cells/mL. Murine AML cells and T cells were co-cultured at 1:1 E:T ratio in 200 μL final volume for 72 hours. The co-cultures were then washed with PBS and stained with anti-CD3 (APC), CD25 (PE), CD69 (PE-cy7) and CD107a (BV421) antibodies. Single-stained controls were set up using the UltraComp eBeads™ Compensation Beads (ThermoFisher Scientific). The samples were incubated in the dark for 30 minutes at room temperature. The cells were then washed and resuspended in 200 μL of PEF. Dead cells were excluded using 7AAD (BD Biosciences). Flow

cytometry was performed using a CytoFLEX LX Flow Cytometer (Beckman Coulter) and data were analyzed using FlowJo software (version 10; Tree Star).

2.8 T cell proliferation assay

MN1 and H9M AML cells were pre-treated with 1 µM of bomedemstat for 48 hours. Around 2 million cells were pelleted and resuspended in 2 mL pre-warmed PBS. They were pulsed with 1µg/mL of $OVA_{323-339}$ peptides for 50 minutes and washed twice with PBS. Then, the cells were resuspended in complete T cell media supplemented with 25% murine AML cytokines at a density of $1x10^6$ cells/mL. Naïve $CD4^+$ OT-II T cells were resuspended in 1 mL PBS at a density of $1x10^6$ cells/mL. 1 µL of CellTrace™ stock solution was added to the cell suspension. The cells were incubated for 20 minutes at 37°C in the dark. 5 mL of pre-warmed T cell media were added to the staining solution and incubated for 5 minutes. The fluorescent-stained T cells were pelleted and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. Murine AML cells and naïve T cells were co-culture at 1:1 E:T ratio in 200 µL final volume for 96 hours. The co-cultures were then washed with PEF and stained with anti-CD3 (PE-cy7) antibody. Single-stained controls were set up using the UltraComp eBeads™ Compensation Beads (ThermoFisher Scientific). The samples were incubated in the dark for 30 minutes at room temperature. The cells were then washed and resuspended in 200 µL of PEF. Dead cells were excluded using SYTOX™ Red Dead Cell Stain (ThermoFisher). Flow cytometry was performed using a CytoFLEX LX Flow Cytometer (Beckman Coulter) and data were analyzed using FlowJo software (version 9.7.6; Tree Star).

2.9 *In vitro* T cell killing assay

H9M-OVA and MN1-OVA cells were pre-treated with 1 µM of bomedemstat for 48 hours. For antigen-pulsing, MN1 and H9M AML cells were pre-treated with 1 µM of bomedemstat for 48 hours. Around 2 million cells were pelleted and resuspended in 2 mL pre-warmed PBS. They were pulsed with 1µg/mL of $OVA_{323-339}$ peptides for 50 minutes and washed twice with PBS. Then, the cells were resuspended in T cell media supplemented with 50% murine AML cytokines at a density of $1x10^6$ cells/mL. Naïve OT-II T cells were resuspended in complete T cell media at densities of $2x10^6$ cells/mL, $1x10^6$ cells/mL, $0.5x10^6$ cells/mL and $0.2x10^6$ cells/mL. Finally, 100 µL of AML cell culture were combined with 100 µL of T cell culture, giving effector-to-target (E:T) ratios of 2:1, 1:1, 1:2 and 1:5. Controls for normalization included treated and non-treated murine AML cells in 200 µL mono-culture. Each condition was conducted in duplicate. The cells were cultured for 24 hours and then transferred to 5 mL round bottom tubes. 5 µL of 7AAD (BD Biosciences) and 40 µL of CountBright™ Absolute Counting Beads (ThermoFisher Scientific) were added to each tube. Flow cytometry was performed using a CytoFLEX LX Flow Cytometer (Beckman Coulter) where 2,500 bead events were collected. Data were analyzed using FlowJo software (version 10; Tree Star) The absolute numbers of 7AAD- and GFP+/YFP+ positive leukemia cells were determined using the cell-to-bead ratios. The percentages of killing were calculated as follows:

$$\%Killing = \left(1 - \frac{Absolute\#\ of\ AML\ cells\ in\ co\_culture}{Absolute\#\ of\ AML\ cells\ in\ monoculture}\right) \times 100\%$$

2.10 Statistical analysis

Statistical significance was determined using GraphPad Prism software (v. 9.5.1). Differences were considered statistically significant if the p values were equal or smaller than 0.05. Where applicable, Student's t-test was used to compare the means between two groups. One-way analysis of variance (ANOVA) was used to compare the means between three or more groups while adjusting for multiple comparisons using Dunnett's method.

Chapter 3. Results

3.1 Bomedemstat treatment enhances *CIITA* expression in human AML cell lines.

Class II transactivator (CIITA) is a master regulator of HLA class II expression which is downregulated in relapsed allo-HSCT recipients (Christopher et al., 2018). CIITA expression levels strongly associate with HLA-II expression. Epigenetic silencing of CIITA through DNA methylation has been shown to inhibit HLA-II expression in some AML cells (Dufva et al., 2020). In this project, it is hypothesized that LSD1 may impact CIITA epigenetically by reducing the expression of IRF8, which has previously been found to be regulated by LSD1 inhibition (Barth et al., 2019). Therefore, HLA-DR negative human AML cell lines were treated with the clinical-stage LSD1 inhibitor bomedemstat for 48 hours and the expression changes of *CIITA* were assessed through qPCR. Across all three human AML cell lines tested, bomedemstat treatment significantly upregulated *CIITA* expression in a dose-dependent manner. In HL-60 and OCI-AML3, bomedemstat led to a maximum of 2 $\log_2$ increase of *CIITA* at 100 nM while the upregulation of *CIITA* is relatively less pronounced in MOLM-13 (Figure 6). Given the essential role of CIITA in HLA-II expression, bomedemstat could therefore enhance HLA-II expression through the upregulation of CIITA in human AML cells.

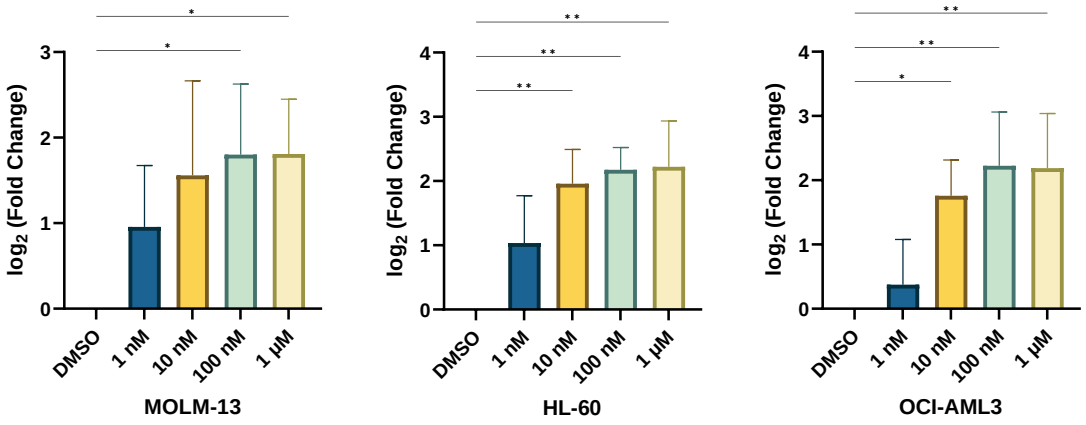
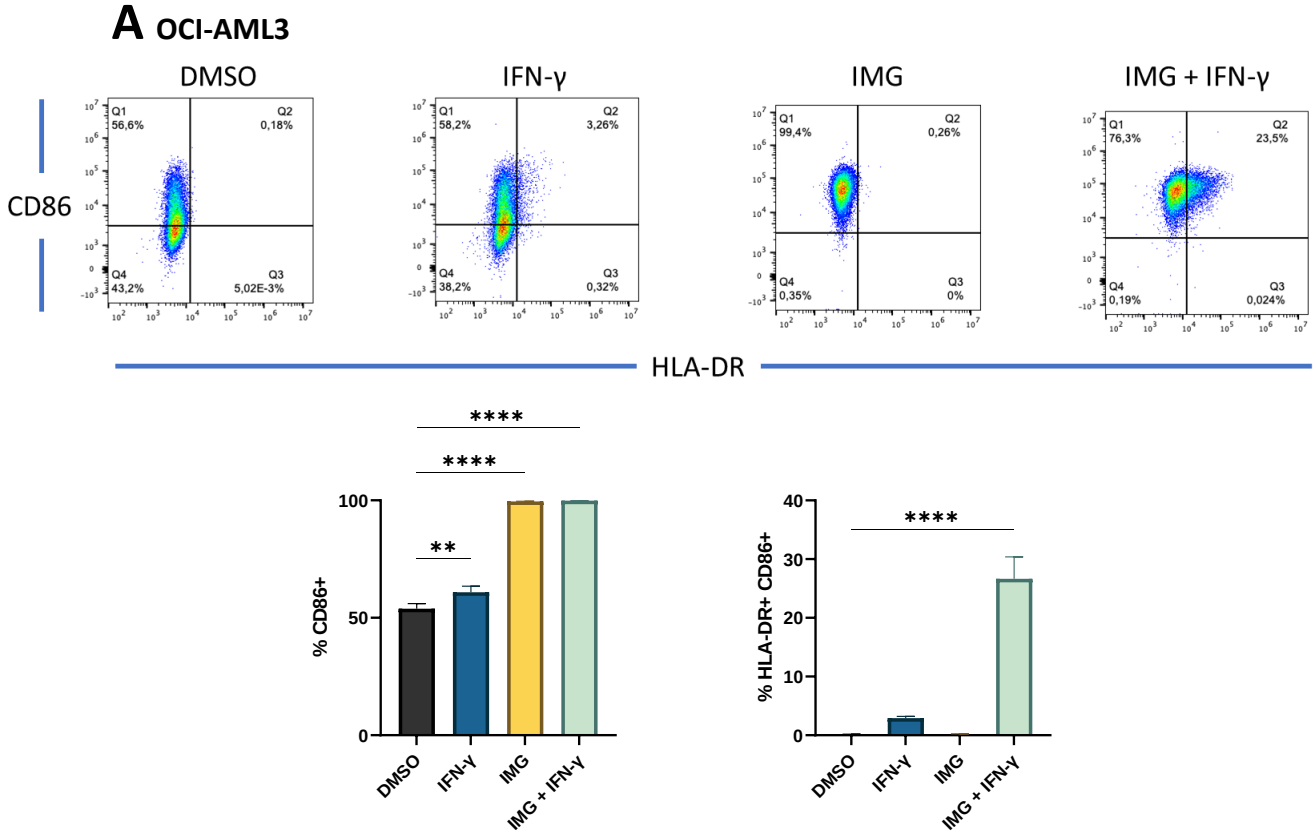


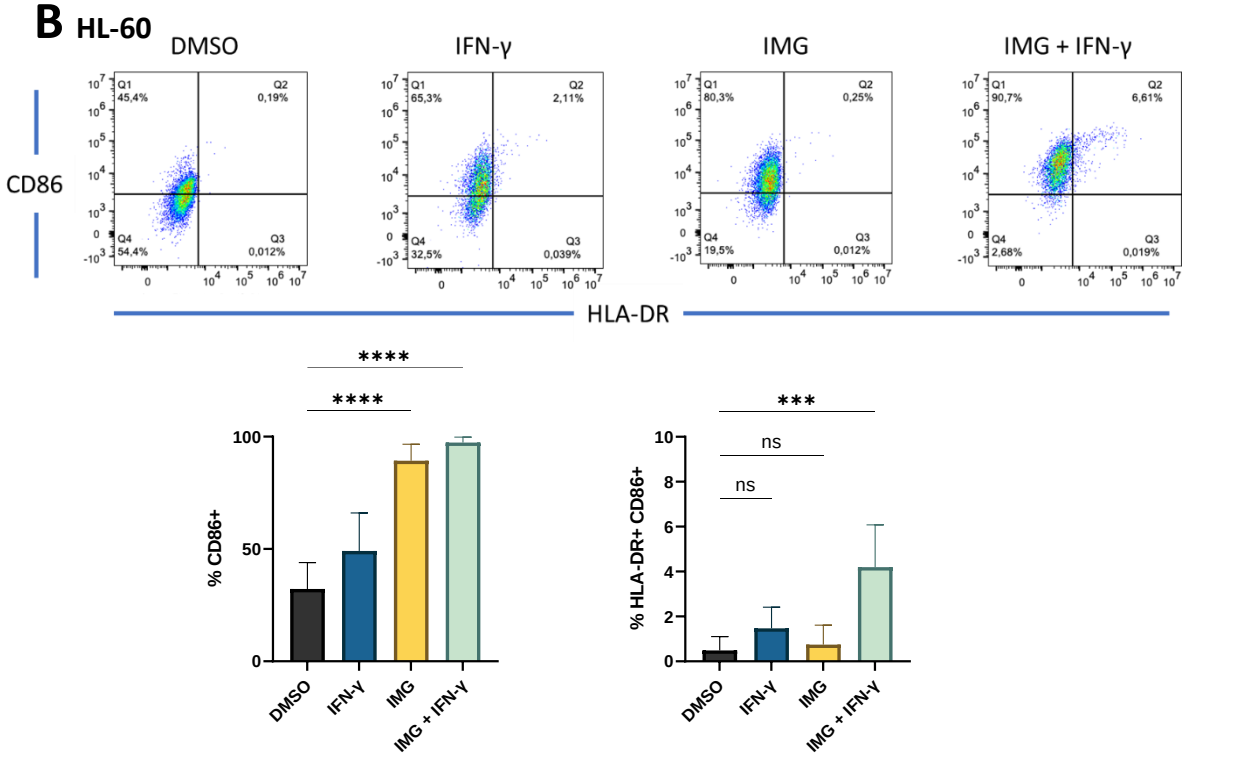
Figure 6. Upregulation of *CIITA* in human AML cell lines in response to bomedemstat. Relative mRNA expression of *CIITA* compared to the DMSO control group. Data are normalized to *Abl1* expression. Columns represent the mean ± SD of at least three independent experiments. *p ⩽ 0.05, **p ⩽ 0.01. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test.

3.2 HLA-DR and CD86 are increased in human AML cells upon treatment with bomedemstat in combination with IFN-γ stimulation

Next, the effects of LSD1 inhibition on the surface expression of HLA-DR were assessed using flow cytometry. Human AML cell lines were treated with 100 nM of bomedemstat for 48 hours. They were then stimulated with 10 ng/mL IFN-γ for another 24 hours. Despite the increased *CIITA* expression, bomedemstat treatment by itself did not upregulate HLA-DR in OCI-AML3 and HL-60 cells (Figure 7A-B). However, when additionally stimulated with 10 ng/mL of IFN-γ, bomedemstat led to significant surface expression of HLA-DR in OCI-AML3, increasing from 0% at baseline to nearly 25% (Figure 7A). The upregulation of HLA-DR was less pronounced in HL-60 cells (Figure 7B). For MOLM-13 cells, the treatment led to a smaller but also significant 5% increase in

HLA-DR expression, without IFN-γ stimulation (Figure 7C). The addition of IFN-γ did not synergistically enhance HLA-DR expression. In all three AML cell lines, bomedemstat treatment led to marked upregulation of CD86, reaching almost 100% at the highest concentration. Together, the results show that LSD1 inhibition can concurrently upregulate HLA-DR and CD86 expression in AML cells and thereby potentially promoting immune recognition by $CD4^+$ T cells.




B HL-60
DMSO
IFN-γ
IMG
IMG + IFN-γ
CD86
HLA-DR
Q1 45,4%
Q2 0,19%
Q4 54,4%
Q3 0,012%
Q1 65,3%
Q2 2,11%
Q4 32,5%
Q3 0,039%
Q1 80,3%
Q2 0,25%
Q4 19,5%
Q3 0,012%
Q1 90,7%
Q2 6,61%
Q4 2,68%
Q3 0,019%
****
****
% CD86+
100
50
0
DMSO
IFN-γ
IMG
IMG + IFN-γ
***
ns
ns
% HLA-DR+ CD86+
10
8
6
4
2
0
DMSO
IFN-γ
IMG
IMG + IFN-γ

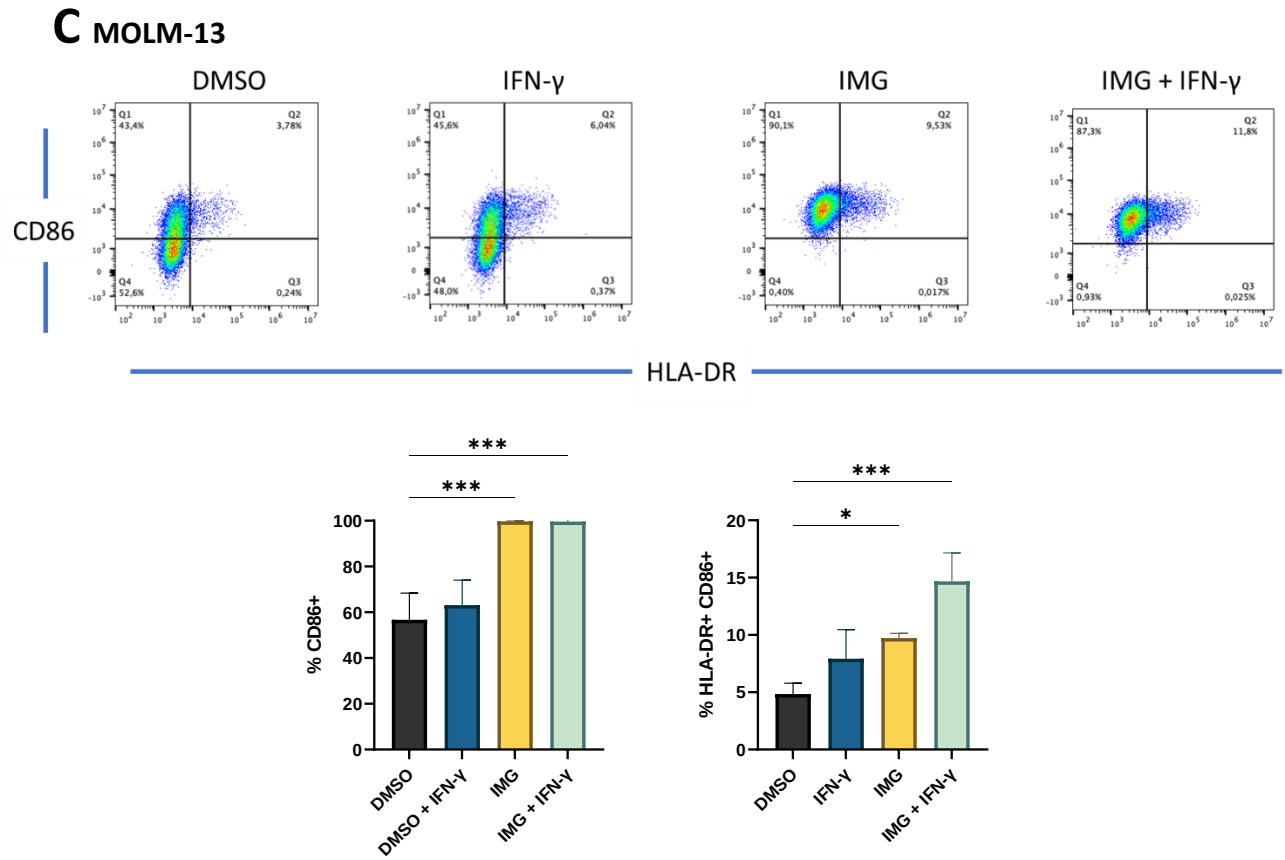

C MOLM-13
DMSO
IFN-γ
IMG
IMG + IFN-γ
CD86
HLA-DR
Q1 43,4%
Q2 3,78%
Q4 52,6%
Q3 0,24%
Q1 45,6%
Q2 6,04%
Q4 48,0%
Q3 0,37%
Q1 90,1%
Q2 9,53%
Q4 0,40%
Q3 0,017%
Q1 87,3%
Q2 11,8%
Q4 0,93%
Q3 0,025%
***
***
% CD86+
100
80
60
40
20
0
DMSO
DMSO + IFN-γ
IMG
IMG + IFN-γ
***
*
% HLA-DR+ CD86+
20
15
10
5
0
DMSO
IFN-γ
IMG
IMG + IFN-γ

Figure 7. Bomedemstat treatment enhances class-II antigen presentation and co-stimulation in human AML cell lines. Representative flow plots for CD86 and HLA-DR expression in (A) OCI-AML3, (B) HL-60 and (C) MOLM-13. The cells were treated with 100 nM bomedemstat (IMG) for 48 hours, followed by stimulation with 10 ng/mL of IFN-γ for 24 hours. Columns represent the mean ± SD of at least three independent experiments; *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001, ****p ⩽ 0.0001. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test.

3.3 Upregulation of Th1-promoting cytokines by bomedemstat.

Regulated by a variety of cytokine signals, $CD4^+$ T helper cells can specialize into different subsets and perform diverse functions in the immune system (Liew, 2002). Among them, Th1 cells can exert direct anti-tumor immunity through the secretion of Th1-type cytokines, including IFN-γ and TNF-α (Tay et al., 2020). Promoting Th1 response should further enhance $CD4^+$ T cell-mediated immune response against leukemia cells. Therefore, qPCR was performed to assess the effects of bomedemstat treatment on the production of cytokines that can help drive Th1 response. IL-12 is the primary cytokine responsible for the polarization towards Th1 immunity. IL-12 is a heterodimer consisting of the p35 (encoded by *IL12A*) and the p40 (encoded by *IL12B*) subunits. It is mainly produced by macrophages and dendritic cells in response to bacterial and viral infections. As the expression of *IL12B* is restricted to activated immune cells, it is generally used as a marker for the production of biologically active IL-12 (Trinchieri, 2003). When treated with bomedemstat, *IL12B* was significantly upregulated in MOLM-13 and HL-60 cells in a dose-dependent manner. No significant changes were observed for OCI-AML3 (Figure 8A). On the other hand, CXC chemokine ligands 10 (CXCL-10), also known as IFN-γ-

induced protein 10 (IP-10), is a T cell-attracting chemokine produced by many cell types when stimulated by IFN-γ (Tokunaga et al., 2018). CXCL-10 is responsible for the chemotaxis and tissue infiltration of $CD8^+$ cytotoxic T cells and Th1 cells through its interaction with the CXC chemokine receptor 3 (CXCR3). CXCL-10/CXCR3 signalling can further contribute to Th1 polarization via signal transducer and activator of transcription 1 and 5 (STAT1/5) (Karin et al., 2016). In the context of allo-HSCT, however, CXCL-10 has also been implicated in the development of GVHD, potentially by increasing tissue infiltration of alloreactive donor T cells (Choi et al., 2012; Piper et al., 2007). In MOLM-13 and HL-60 cells, *CXCL10* was strongly upregulated in response to bomedemstat treatment, demonstrating over 3 $\log_2$ fold-changes at the highest concentrations in both cell lines (Figure 8B). The differences in *CXCL10* expression for OCI-AML3 cells were not statistically significant.

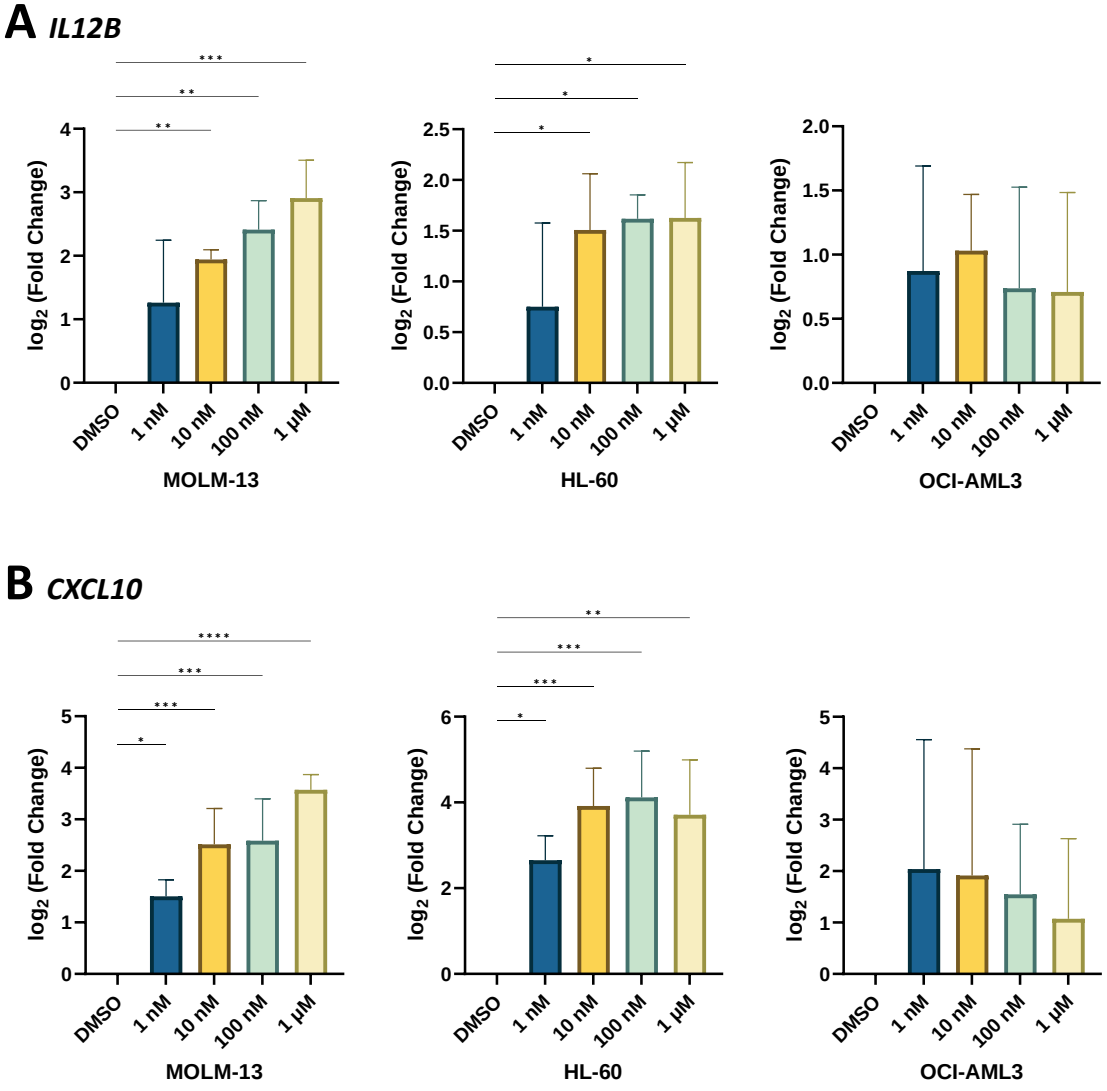


Figure 8. Expression of *IL12B* and *CXCL10* in response to bomedemstat. Relative mRNA expression of (A) *IL12B* and (B) *CXCL10* compared to the DMSO control group in MOLM-13, HL-60 and OCI-AML3. Data are normalized to *Abl1* expression. Columns represent the mean ± SD of three independent experiments. *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001, **** ⩽ 0.0001. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test.

3.4 Increased production of inflammatory cytokines and chemokines in response to bomedemstat.

Given the increased expression of *IL12B* and *CXCL10*, the effects of bomedemstat treatment on cytokine production in human AML cells were measured more broadly using a multiplex cytokine assay. Human AML cell lines MOLM-13, HL-60, OCI-AML3 and THP-1 were treated with 100 M of bomedemstat for 48 hours and the supernatants from the cell culture were collected for analysis by an external laboratory service provider (Eve Technologies). While there was some heterogeneity in the regulation of the cytokines between the different cell lines, some consistent trends were observed. Again, there was a major increase in CXCL-10 production in 3 of the 4 analyzed cell lines. (Figure 9A-D). These differences were statistically significant for MOLM-13 and THP1. In HL-60 cells, CXCL-10 increased from 221.6 ± 108.9 ng/mL at baseline to 13036.0 ± 8042.5 ng/mL in the treatment group (Figure 9B). While this was the strongest overall increase observed for any cytokines in this assay, statistical testing yielded a p value of 0.0873 due to the large SD between biological replicates. On the other hand, the production of IL-12B was significantly increased in HL-60 (Figure 9B).

In addition, bomedemstat significantly increased the production of members of the CC chemokine family, including chemokines CCL-1 (I-309), CCL-2 (monocyte chemoattractant protein 1: MCP-1), CCL-3 (macrophage inflammatory protein 1α: MIP-1α) and CCL-22 (macrophage-derived chemokine: MDC) (Figure 9A-D). These chemokines are secreted by activated monocytes or macrophages upon stimulation with inflammatory signals like IFN-γ and lipopolysaccharide (LPS). They are generally

responsible for the recruitment of lymphocytes and other monocytes to the site of inflammation and help sustain inflammatory response (Rollins, 1997).

Overall, these results confirm that bomedemstat treatment enhanced the production of Th1-promoting cytokines in AML cells (Tay et al., 2020). It also increased a number of inflammatory cytokines, suggesting that LSD1 has a generally pro-inflammatory effect on these leukemic cells.

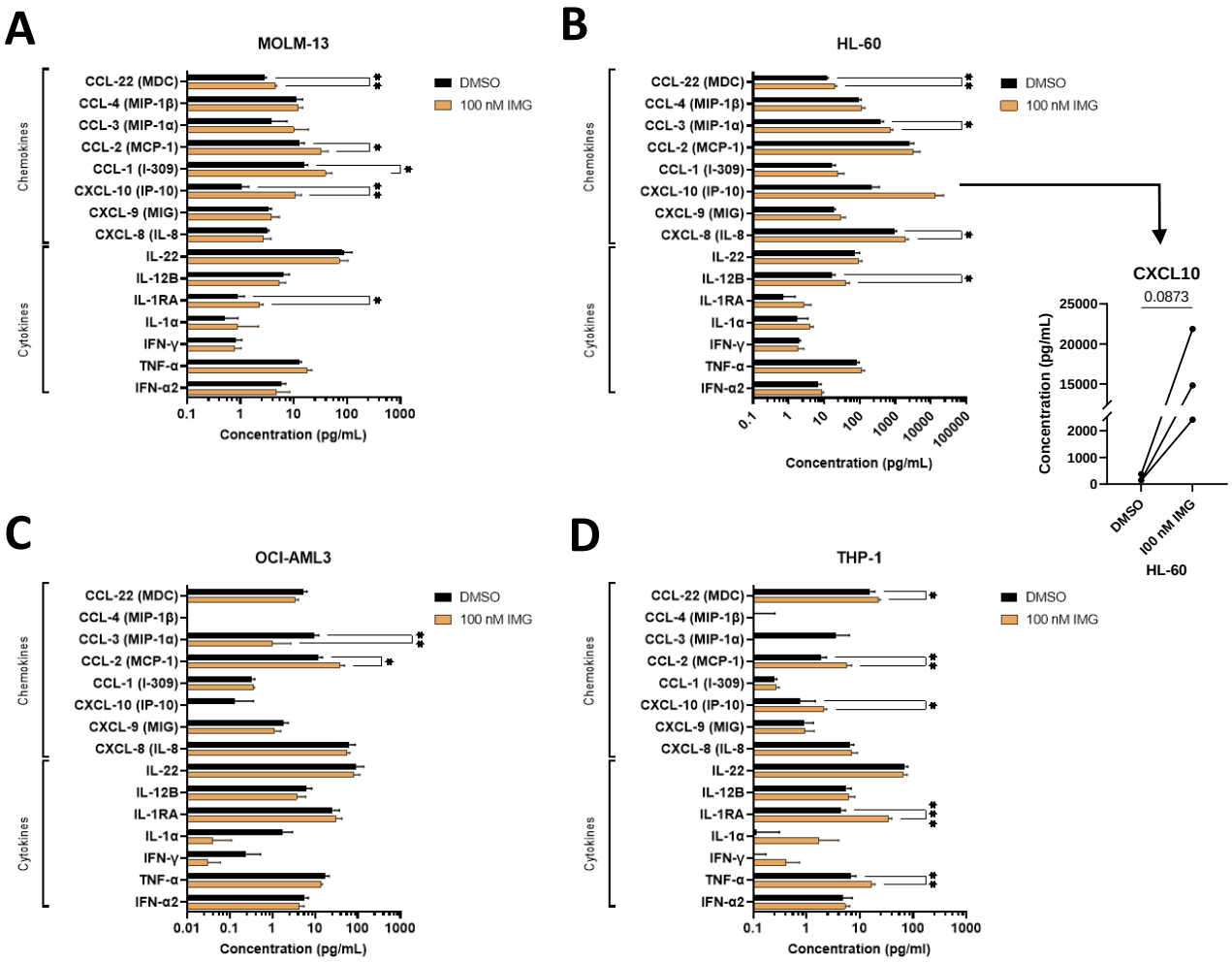


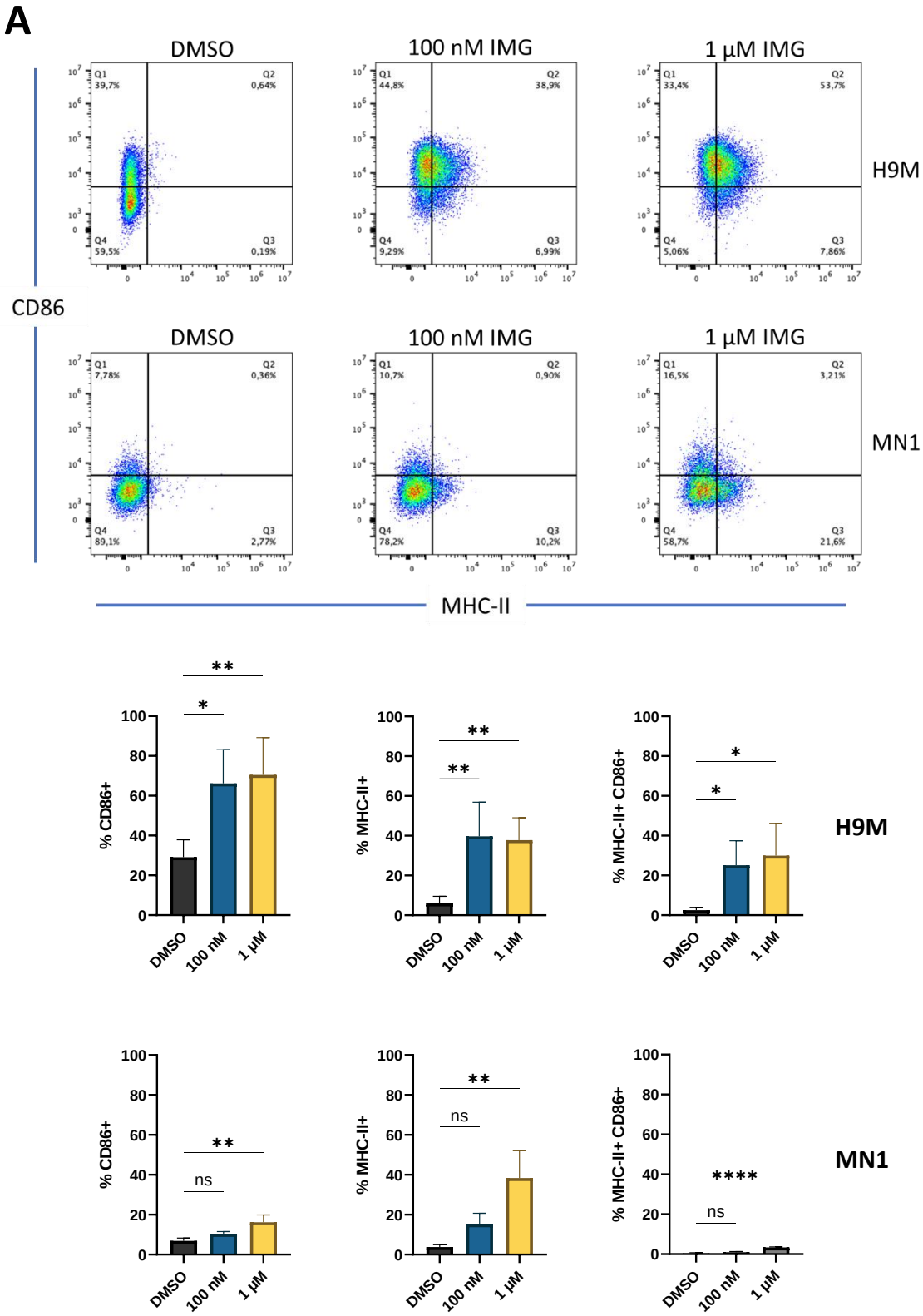
Figure 9. Cytokine production in human AML cell lines upon bomedemstat treatment. Multiplex cytokine analysis of cytokine and chemokine production in (A) MOLM-13, (B) HL-60, (C) OCI-AML3, (D) THP-1 in response to DMSO or 100 nM bomedemstat

treatments. Columns represent the mean ± SD of three independent experiments, presented on a log10 scale. (B) Concentrations of CXCL-10 are additional presented on a linear scale. *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001. Statistical significance was determined using Student's t test.

3.5 Bomedemstat upregulates MHC-II and CD86 expression in murine AML cells.

To facilitate functional experiments *in vitro* and potentially *in vivo* studies, the project made use of syngeneic murine AML models that are versatile platforms to functionally investigate the role of molecular factors in AML. The present study examined the effects of LSD1 inhibition by bomedemstat in myeloid progenitor cells transformed by retroviral transduction with the combination of *Hoxa9* and *Meis1* (H9M) or with *MN1*. These transformed myeloid progenitors can be expanded in cytokine-dependent cultures and have been previously shown to give rise to AML in irradiated syngeneic murine hosts. This allows them to serve as physiologically relevant models for AML (Barth et al., 2019). In H9M cells, bomedemstat significantly upregulated the expression of MHC-II (I-A/I-E) from 7.6% at baseline to 35.1% at 1 μM concentration. It also increased the expression of CD86 from 25.4% to 64.9%. Together the percentage of MHC-II$^+$/CD86$^+$ double-positive cells increased from 3.1% at baseline to 22.0% at 1 μM. In MN1 cells, bomedemstat also significantly increased the expression of MHC-II from 3.8% to 38.4% at 1 μM. There was also a moderate upregulation of CD86 from 6.9% to 16.2% at the same concentration. However, there was only a minimal increase in MHC-II$^+$/CD86$^+$ double-positive cells, going up from 0% to only 3% at 1 μM (Figure 10A).

In order to study T cell anti-tumor activity in an antigen-specific context, additional transformed myeloid progenitors were generated from mice that constitutively express the well-established model antigen ovalbumin (C57BL/6-Tg(CAG-OVAL)916Jen/J, Jackson Laboratory). The two cell lines are termed H9M-OVA and MN1-OVA to indicate the transgenes they carry. Consistent with previous results, bomedemstat treatment significantly increased the co-expression of MHC-II and CD86 in H9M-OVA cells from 2% to 38% at 1 μM. In MN1-OVA cells, however, bomedemstat did not lead to any major increase in MHC-II expression, while only minimally upregulating CD86 by 4%. Consequently, there was again a negligible number of MHC-II$^+$/CD86$^+$ double-positive cells in the MN1 model after treatment with Bomedemstat (Figure 10B).


A
DMSO
100 nM IMG
1 µM IMG
H9M
MN1
CD86
MHC-II
% CD86+
% MHC-II+
% MHC-II+ CD86+
DMSO
100 nM
1 µM
ns

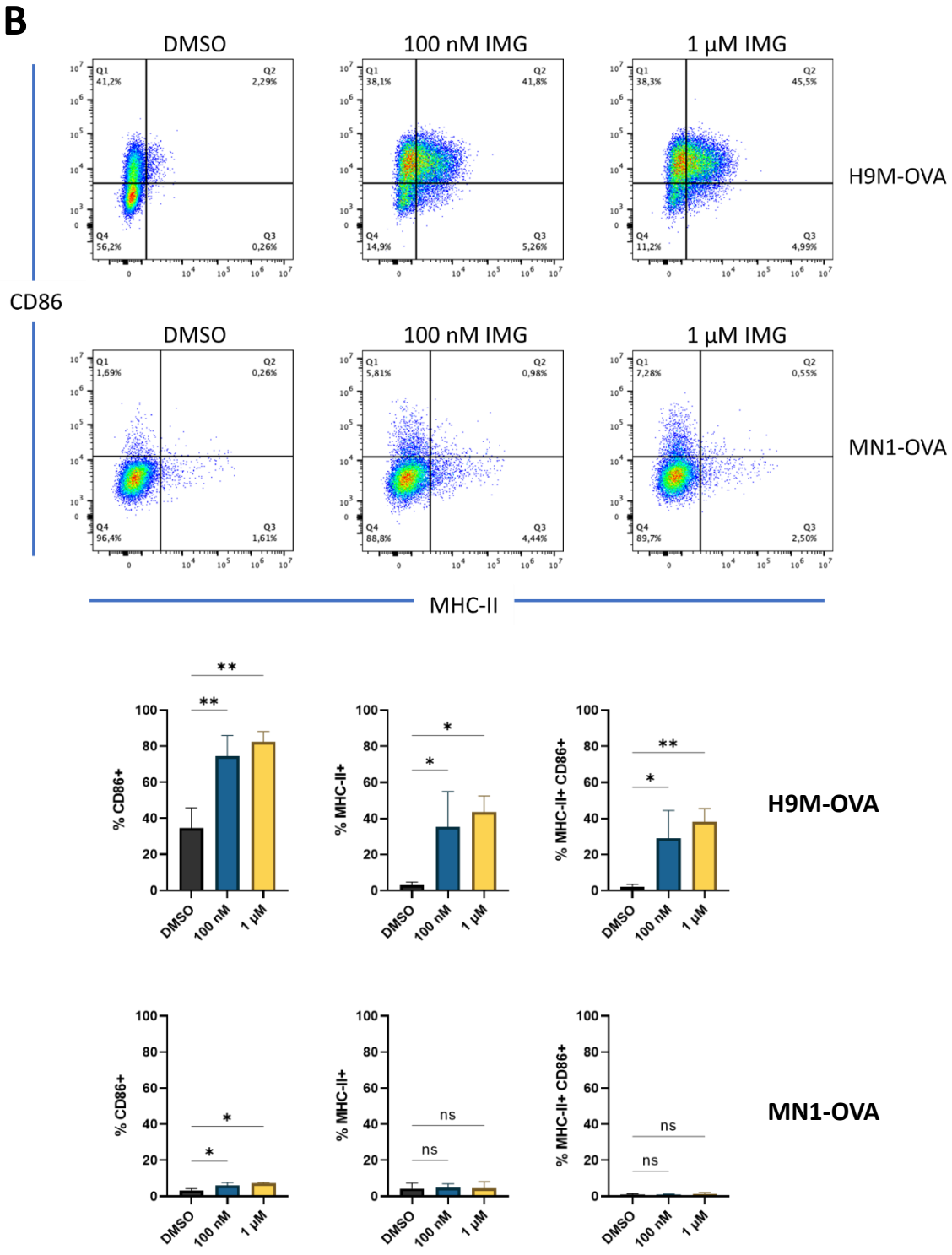


Figure 10. Bomedemstat treatment enhances class-II antigen presentation and co-stimulation in murine AML cell lines. (A) Representative flow plots of CD86 and MHC-II expression in H9M (top row) and MN1 (bottom row) cells. Columns represent the

mean ± SD of at least three independent experiments. (B) Representative flow plots of CD86 and MHC-II expression in H9M-OVA (top row) and MN1-OVA (bottom row) cells. Columns represent the mean ± SD of at least three independent experiments; *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001, ****p ⩽ 0.0001. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test.

3.6 *Irf8* expression is essential for bomedemstat-induced MHC-II upregulation in H9M

IRF8 has previously been shown to be repressed in MN1 cells, which is not overcome by LSD1 inhibition (Sharma et al., 2014). However, in H9M transformed cells a major increase in IRF8 transcription factor activity is observed upon inhibition of LSD1 (Barth et al., 2019). IRF8 is also established as an essential positive regulator of MHC-II along with its transcriptional partner PU.1 (Smith et al., 2011). It was therefore hypothesized that the differences in the effect of LSD1 inhibition between myeloid progenitors transformed by MN1 and the ones by H9M may partially be explained by differences in the upregulation of *Irf8*. To examine the role of *Irf8* expression in bomedemstat-induced MHC-II and CD86 upregulation, H9M-transformed progenitor cells from *Irf8* knockout mice (B6(Cg)-*Irf8*$^{tm1.2Hm}$/J, Jackson Laboratory) were generated (H9M-*Irf8*-KO). Bomedemstat treatment led to a similar upregulation of CD86 in H9M-*Irf8*-KO cells, increasing from 15% to 94.7% at 1μM concentration. However, the upregulation of MHC-II was completely abrogated, showing no significant increase even at the maximum concentration (Figure11A-B). This result therefore demonstrates that the upregulation of MHC-II, but not CD86, by bomedemstat treatment is dependent on *Irf8* expression. Inhibiting LSD1 may regulate MHC-II and CD86 expression through different

mechanisms. This however also suggests that the differences observed between H9M- and MN1-transformed cells described in the previous section cannot be fully explained by the differential activity of IRF8 between the two models.

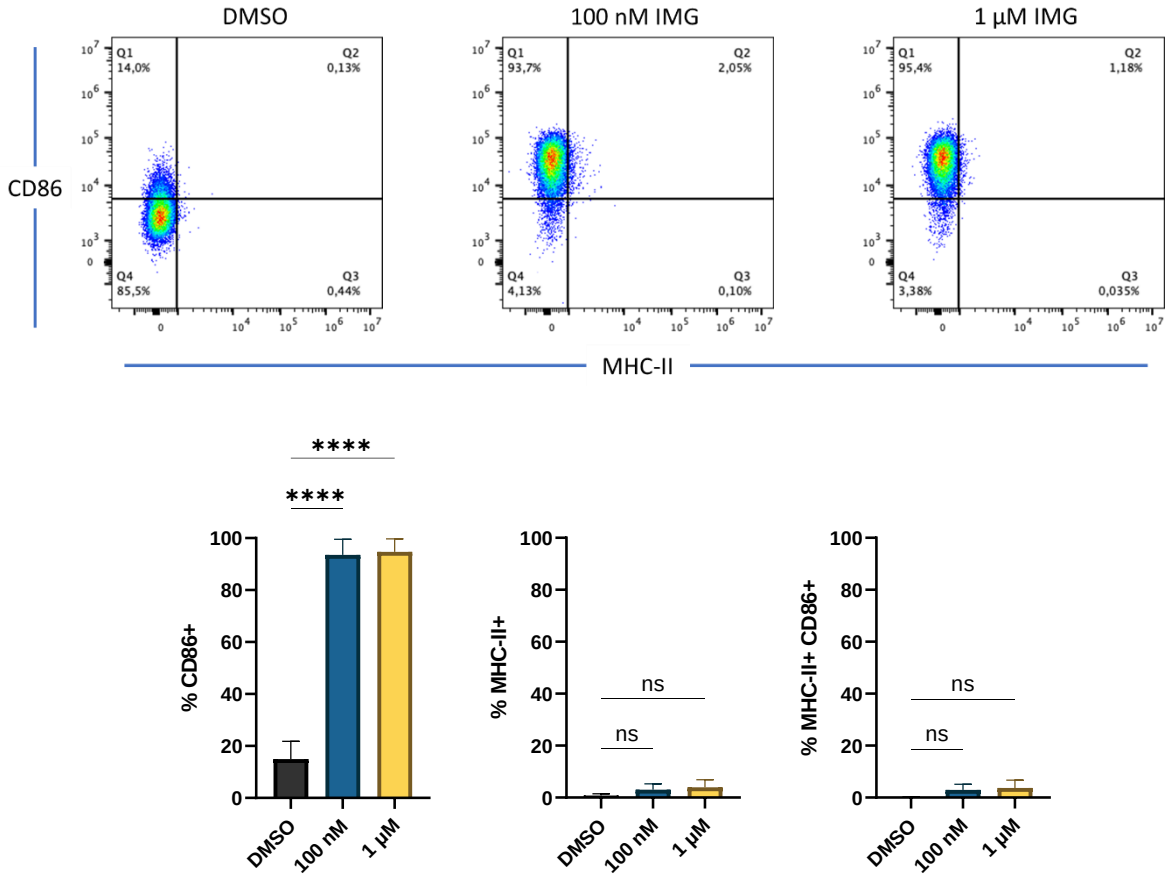


Figure 11. MHC-II and CD86 expression in H9M-*Irf8*-KO cells. Representative flow plots of CD86 and MHC-II expression in H9M-*Irf8*-KO cells. Columns represent the mean ± SD of at least three independent experiments; ****p ⩽ 0.0001. Statistical significance determined by one-way ANOVA followed by Dunnett's multiple comparison test.

3.7 Bomedemstat-treated H9M-OVA cells induce the activation of antigen-specific $CD4^+$ T cells.

To further investigate whether the concurrent upregulation of MHC-II and CD86 activate naïve $CD4^+$ T cells, H9M and H9M-OVA cells were co-cultured with MACS-purified $CD4^+$ OT-II T cells for 72 hours. T cell activation can be demonstrated by the expression of the canonical activation markers CD69 and CD25, which are measured through flow cytometry. CD69 is a type II C-type lectin rapidly expressed on T cell membrane upon TCR stimulation. It is one of the earliest T cell activation markers, the expression of which can be detected as early as 2-3 hours post-stimulation (Cibrián & Sánchez-Madrid, 2017). CD25, also known as IL-2 receptor α (IL-2RA), is one of three subunits that make up the high-affinity IL-2 receptor. CD25 expression is moderately induced upon TCR engagement, and is subsequently enhanced by autocrine IL-2 signaling (Malek, 2008). Bomedemstat-treated H9M-OVA cells significantly induced the expression of both CD69 and CD25 on $CD4^+$ OT-II T cells after 72 hours of co-culture compared to the un-treated H9M-OVA cells (Figure 12A-B). No significant changes were observed for T cells co-cultured with control H9M cells lacking OVA antigens. The exclusion of exogenous IL-2 for the duration of the co-culture had no significant effect on the expression of CD25 and CD69 (Figure 12A-B). This suggests that the upregulation of CD25 was not driven by exogenous IL-2.

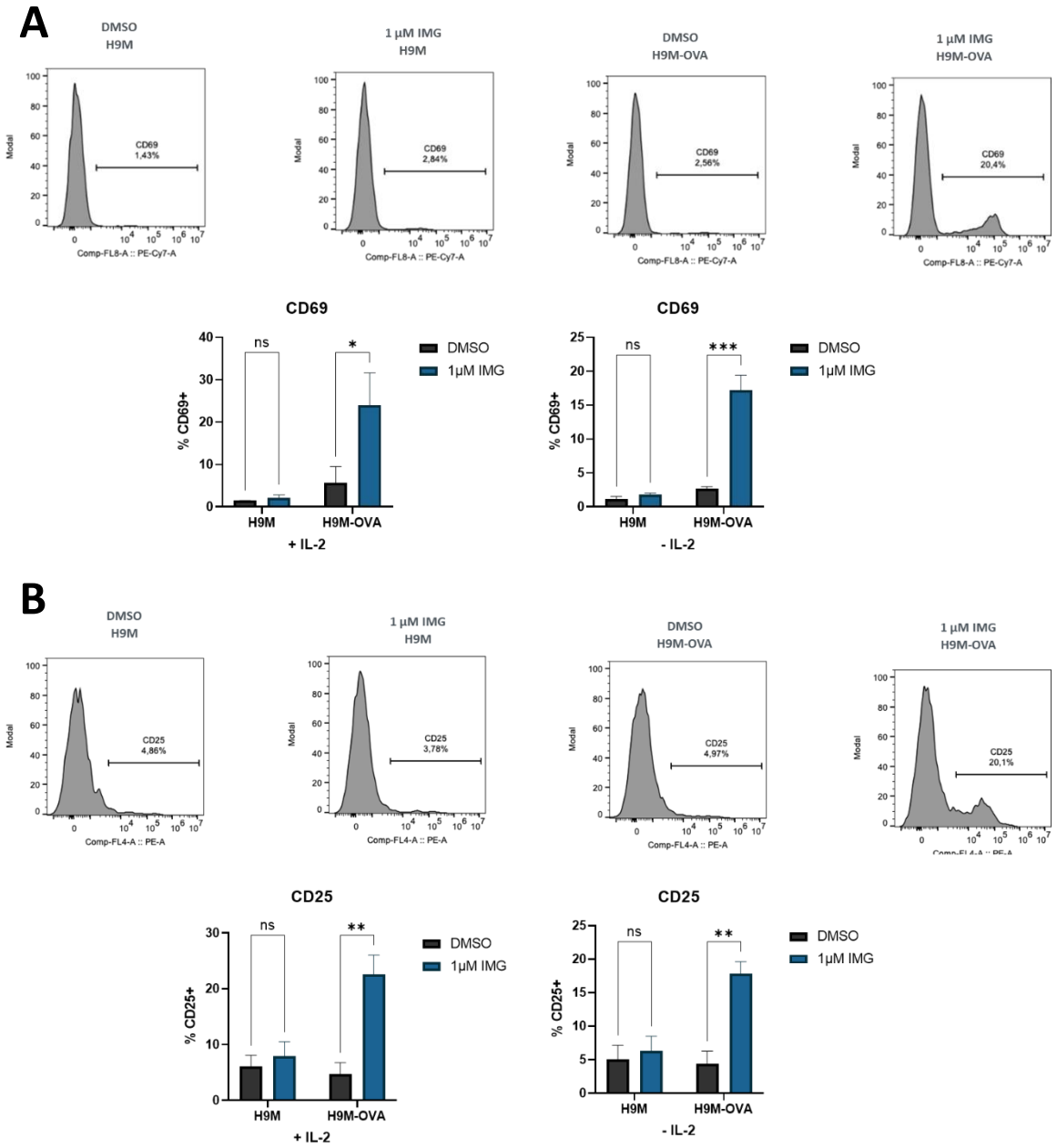


Figure 12. Bomedemstat-treated H9M-OVA cells induce antigen-dependent T cell activation. Representative flow plots and expression levels of (A) CD69, (B) CD25 in $CD4^+$ OT-II T cells after 72 hours of co-culture, with or without IL-2. Columns represent the mean ± SD of at least three independent experiments; *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001. Statistical significance was determined using Student's t test.

3.8 Enhanced T Cell Proliferation Induced by Bomedemstat-Treated H9M cells.

T cell activation subsequently leads to T cell proliferation, a process mostly driven by autocrine IL-2 signalling (Pennock et al., 2013). Therefore, *in vitro* functional assays were conducted to determine whether LSD1 inhibition can increase the ability of H9M cells to induce T cell proliferation. CellTrace Yellow is a cell-permeable dye that allows for the tracking of cell proliferation through fluorescent dilution (Tempany et al., 2018). To examine the effects of bomedemstat treatment on antigen-dependent T cell proliferation, $CD4^+$ OT-II T cells were stained with CellTrace Yellow and co-cultured with bomedemstat-treated, antigen-pulsed H9M and MN1 cells. The results showed that bomedemstat-treated H9M cells significantly induced T cell proliferation after 96 hours of co-culture. No proliferative effect was observed with bomedemstat-treated MN1 cells (Figure 13A-B). However, bomedemstat-treated H9M cells also induced T cell proliferation in the absence of OVA antigens (Figure 13B). It was hypothesized that exogenous IL-2 included in the co-culture media could drive T cell proliferation and therefore, the co-culture was also conducted in the absence of IL-2. The removal of exogenous IL-2 from the co-culture did not diminish the observed proliferative effect as bomedemstat-treated H9M cells led to similar levels of $CD4^+$ T cell proliferation, regardless of antigen-pulsing (Figure 13C). Therefore, in this experimental setup, bomedemstat treatment in murine H9M AML cells could have promoted T cell

proliferation by a mechanism that is not solely dependent on the OVA antigens.

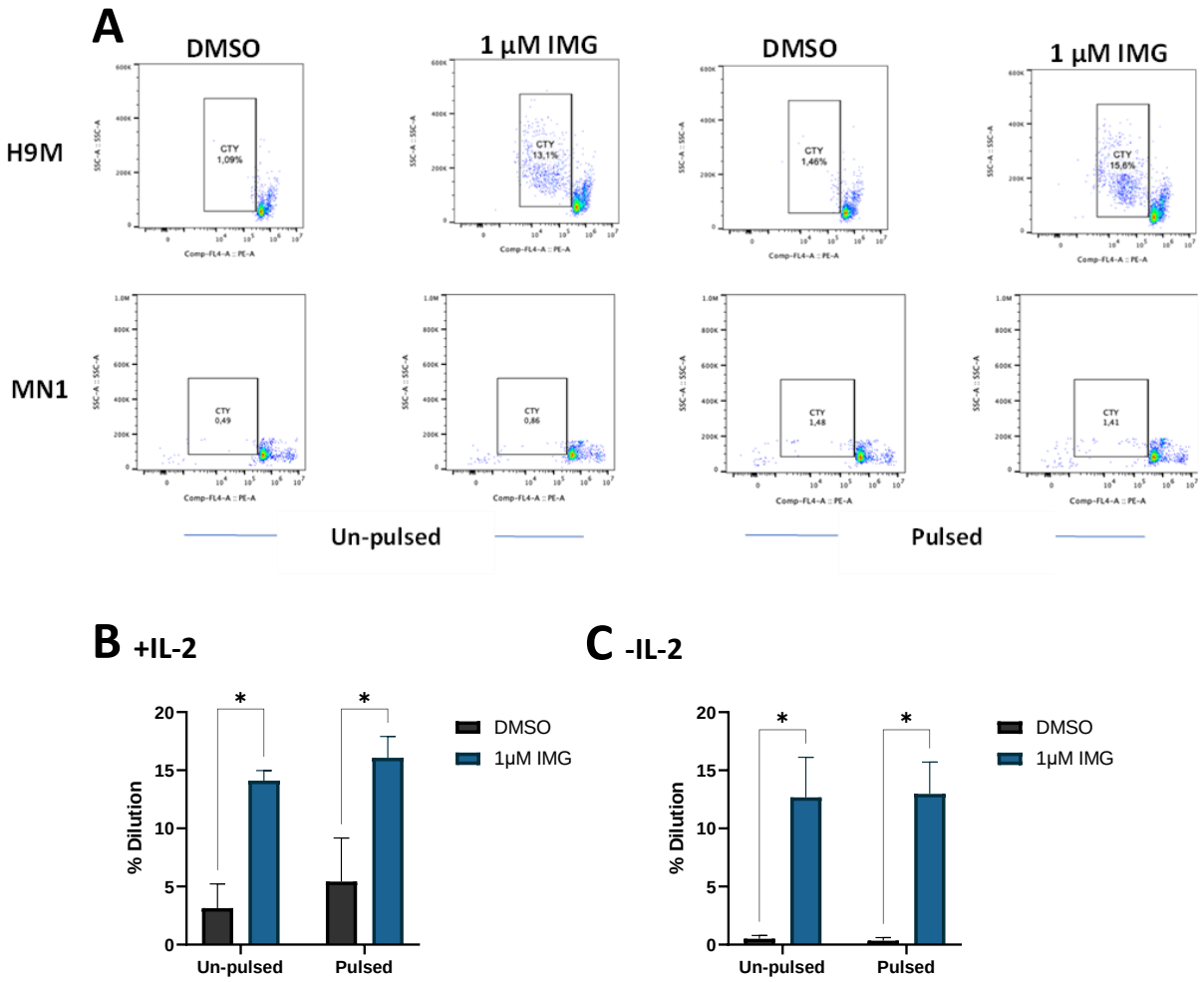


Figure 13. CD4$^+$ T cell proliferation induced by bomedemstat-treated H9M cells. (A) Representative flow plots of the proliferation of CD4$^+$ OT-II T cells co-cultured with bomedemstat-treated H9M (top row) and MN1 (bottom row). T cell proliferation is demonstrated by the dilution of CellTrace Yellow fluorescent dye. (B) Proliferation of T cells co-cultured with H9M with or without antigen-pulsing. (C) Proliferation of T cells in the absence of IL-2. Columns represent the mean ± SD of at least three independent experiments; *p ⩽ 0.05. Statistical significance was determined using Student's t test.

3.9 Bomedemstat treatment sensitizes antigen-pulsed H9M cells to T cell-mediated immune killing effect.

The activation of $CD4^+$ T cells may subsequently lead to the initiation of anti-leukemia immune response. To examine immune killing in an antigen-specific context, bomedemstat-treated murine AML cells were pulsed with $OVA_{323-339}$ peptides. They were then co-cultured with OT-II T cells at various E:T ratios overnight. OT-II T cells contain T cell receptors (TCRs) specific for $OVA_{323-339}$ peptide presented in the context of MHC-II. The degree of killing effect was measured by comparing the absolute numbers of live AML cells in co-culture to those in mono-culture without T cells. The results showed that pre-treatment with bomedemstat significantly enhanced the killing of H9M cells at the 2:1, 1:1 and 1:2 ratio. More specifically, the killing of bomedemstat-treated H9M cells directly correlated with E:T ratios, with a maximum of 20% killing observed at the 1:1 E:T ratio (Figure 14). Meanwhile, there was minimal killing effect for antigen-pulsed H9M cells without bomedemstat treatment. Additionally, no significant killing was observed for MN1 cells, regardless of bomedemstat pre-treatment (Figure 14).

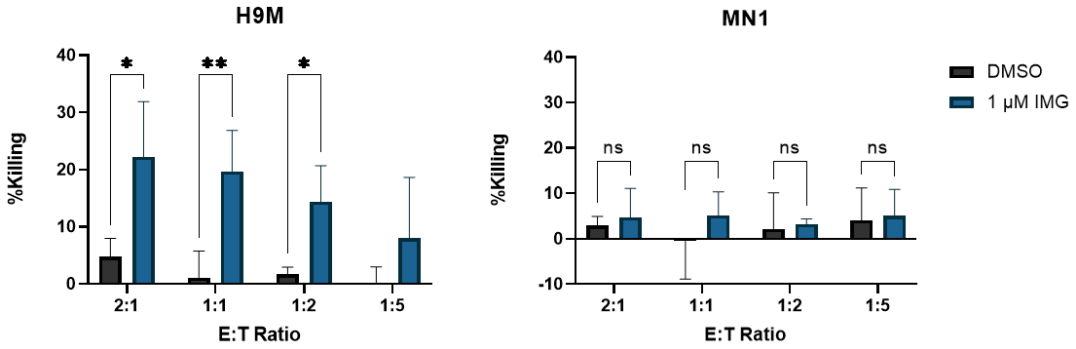


Figure 14. Bomedemstat treatment sensitizes antigen-pulsed H9M cells to T cell-mediated killing effect. Percentages of killing of $OVA_{323-339}$ -pulsed H9M and MN1 cells by OT-II T cells, with or without bomedemstat pre-treatment. Columns represent the mean ± SD of at least three independent experiments; *p ⩽ 0.05, **p ⩽ 0.01. Statistical significance was determined using Student's t test.

3.10 H9M-OVA cells are sensitized to antigen-dependent, $CD4^+$ T cell-mediated immune killing by bomedemstat treatment.

As there were concerns (in particular raised by the previously described proliferation experiment) that the peptide pulsing technique only incompletely loads MHC-II molecules in the presence of other high-affinity peptides such as peptides derived from retrovirally overexpressed human MN1. The constitutive expression of OVA in this setting may help displace MN1-derived peptides to ensure proper loading of OVA antigens. Therefore, the killing assay was repeated again with H9M-OVA and MN1-OVA cells. Similar to previous observations, there were around 20% immune killing effects on bomedemstat-treated H9M-OVA cells at the 2:1 and 1:1 ratio (Figure 15A). Notably, there was increased background killing of H9M-OVA cells without bomedemstat treatment at the 2:1 ratio, which may be due to the higher level of antigen

presentation in this model. Consistent with previous results, there were no significant differences in the killing of MN1-OVA cells.

Meanwhile, in the absence of OVA antigens, no significant killing was observed for H9M cells, regardless of bomedemstat treatment (Figure 15B). To further confirm that the killing effect was mediated by $CD4^+$ T cells, the killing assay was set up using MACS-purified $CD4^+$ OT-II T cells. A comparable 20% killing was achieved at the 1:1 E:T ratio (Figure 15C), indicating that $CD4^+$ T cells were indeed responsible for generating the killing effect against bomedemstat-treated H9M-OVA cells. Together, these results suggest that bomedemstat treatment can sensitize murine H9M cells to $CD4^+$ T cell-dependent immune killing effect.

The study further investigated potential killing mechanisms mediated by $CD4^+$ T cells. The release of cytotoxic granules from secretory lysosomes represents a major killing mechanism by cytotoxic T cells. Degranulation was assessed by measuring the surface expression of CD107a, which is a lysosome-associated membrane glycoprotein (Betts & Koup, 2004). Although a small upregulation of CD107a was observed on OT-II T cells co-cultured with bomedemstat-treated H9M and H9M-OVA, the expression was not significant compared to naïve $CD4^+$ T cells in mono-culture (Appendix 1). Indeed, CD107a expression is a feature of a subset of $CD4^+$ T cells with degranulation capability. These cells are termed cytotoxic $CD4^+$ T cells and mainly differentiate from the Th1 lineage (Takeuchi & Saito, 2017). The relatively short period of the co-culture may not be sufficient for the differentiation of cytotoxic $CD4^+$ T cells. Therefore, the early killing effect observed in this experimental setup may be mediated by factors other

than the release of cytotoxic granules. Nevertheless, it was also suggested that the timing of the readout might be too long to detect CD107a expression since the marker is quickly internalized after its initial expression (Vahlne et al., 2008). Further optimization of the experimental protocol is needed to properly detect the expression of CD107a. This may involve the inclusion of anti-CD107a antibodies for the duration of the co-culture and the addition of monensin to prevent CD107a degradation (Bryceson et al., 2010).

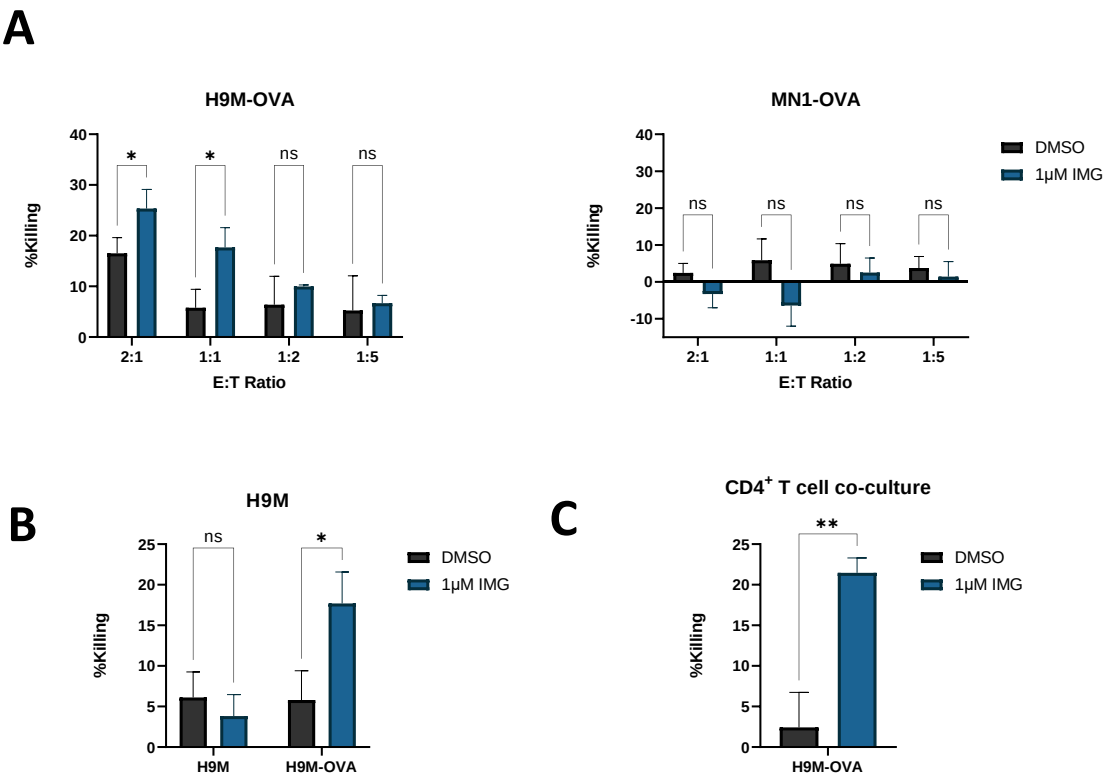


Figure 15. H9M-OVA cells sensitized to T cell-mediated killing effect by bomedemstat. (A) Percentages of killing of H9M-OVA and MN1-OVA cells by OT-II T cells, with or without bomedemstat pre-treatment. (B) Percentages of killing of H9M in the absence of OVA antigens at 1:1 E:T ratio. (C) Percentages of killing of H9M-OVA cells by $CD4^+$ OT-II T cells at 1:1 E:T ratio. Columns represent the mean ± SD of at least three independent experiments; *$p \leqslant 0.05$, **$p \leqslant 0.01$. Statistical significance was determined using Student's t test.

Chapter 4. Discussion

4.1 Roles of LSD1 in regulating MHC-II and CD86 expression.

The downregulation of HLA-II is a major mechanism by which AML cells can potentially evade immune surveillance from donor T cells and contribute to post-transplant relapse. The current project seeks to propose a novel therapeutic strategy that can restore HLA-II expression in human AML cells to promote $CD4^+$ T cell-mediated anti-leukemia immune response. The inhibition of LSD1, an epigenetic regulator of histone methylation, was previously shown to induce epigenetic reprogramming and increase the expression of IRF8, which is an upstream regulator of CIITA. Therefore, it was hypothesized that LSD1 may upregulate *CIITA* and enhance HLA-II expression in human AML cells. To date, the results have demonstrated that LSD1 inhibition by a clinical-stage small molecule inhibitor bomedemstat can increase the expression of *CIITA* in human AML cell lines MOLM-13, HL-60 and OCI-AML3. Subsequently, bomedemstat treatment also led to a significant upregulation of HLA-DR in OCI-AML3 and HL-60 cells when stimulated by IFN-γ, while there was only a moderate upregulation of HLA-DR in MOLM-13 in the absence of IFN-γ. The variations observed between different AML cell lines may be dependent on the epigenetic landscape and transcriptional control of CIITA.

There are three functional promotors that can independently regulate CIITA expression and are highly conserved in both human and murine cells. Promoter I specifically regulates CIITA expression in myeloid cells and is under the synergistic transcriptional control of IRF8 and PU.1 (Smith et al., 2011). Promoter III activity is

restricted to cells of the lymphoid lineage while promotor IV is dependent on IFN-γ signalling (Figure 16) (Morris et al., 2000). Although LSD1 inhibition might be sufficient to upregulate CIITA through IRF8-controlled promoter I, the results show that IFN-γ stimulation is still required to induce the surface expression of HLA-II in human AML cells. As all three cell lines studied in this project are resistant to IFN-γ-induced HLA-II upregulation in the absence of bomedemstat, epigenetic silencing of promoter IV may inhibit HLA-II expression in human AML cells. Indeed, DNA methylation of CIITA promotor IV cells has been shown to prevent IFN-γ-induced HLA-II expression in MOLM-13 cells, a process which can be reversed by HMA treatment (Dufva et al., 2020). Therefore, it may be reasonable to infer that, in HL-60 and OCI-AML3 cells, epigenetic modification at promoter IV through histone methylation may also inhibit the transcription of CIITA, and subsequently HLA-II expression. Chromatin immunoprecipitation ChIP analysis may be employed to examine histone methylation levels at CIITA promoters before and after bomedemstat treatment to provide mechanistic insights.

In murine AML cells, bomedemstat significantly induced the expression of MHC-II in H9M even without IFN-γ stimulation. It was then demonstrated that the upregulation is dependent on IRF8 as bomedemstat failed to induce MHC-II expression in H9M-*Irf8*-KO cells. However, preliminary results showed that MHC-II expression can still be induced in H9M-*Irf8*-KO by IFN-γ stimulation (Appendix 2). This suggests that LSD1 and IFN-γ may influence MHC-II expression through different mechanisms. It might be interesting to knock down IRF8 in human AML cells to determine whether it is required for bomedemstat-induced HLA-DR upregulation in human cells as well.

This would also help determine which promoter(s) of CIITA may be essential for bomedemstat-induced HLA-II expression in human AML cells.

The co-stimulatory molecule CD86 was potently upregulated in response to bomedemstat treatment in all three human cell lines, reaching nearly 100% at 100 nM concentration. It was also significantly upregulated in H9M-*Irf8*-KO cells, suggesting that CD86 expression is not dependent on IRF8 expression in this context, but may rather be a direct target of LSD1 inhibition. Indeed, PU.1 has been established as an essential activator of CD86 expression that can act independently from other known CD86 regulators including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IRF4 (Kanada et al., 2011). There is a subset of PU.1 sites colocalized with GFI1 sites which are repressed through LSD1 recruitment, which may contribute to the suppression of CD86 in this setting (Barth et al., 2019).

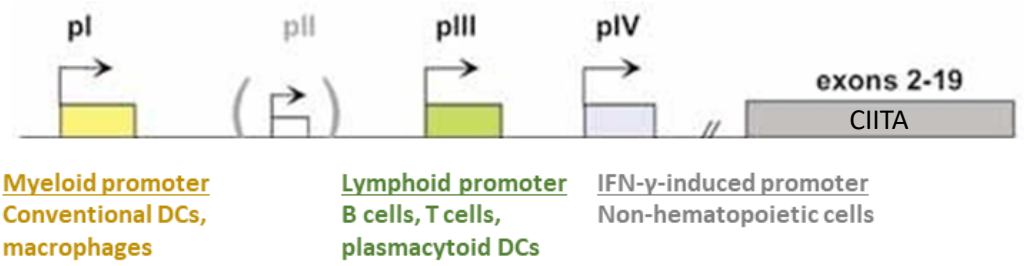


Figure 16. Three independent promoters regulating CIITA expression. Promoter I is only active in myeloid cells and is under the transcriptional control of IRF8 and PU.1. Promoter III activity is restricted to cells of the lymphoid lineage. Promotor IV is dependent on IFN-γ signalling and is indispensable for IFN-γ-induced MHC-II expression in non-hematopoietic cells (LeibundGut-Landmann et al., 2004). Adapted from LeibundGut-Landmann *et al.*

4.2 LSD1 inhibition shows a pro-inflammatory effect in AML cells.

The current project examined the effects of LSD1 inhibition by bomedemstat on AML cytokine production through qPCR and multiplex cytokine assays. In MOLM-13 and HL-60, bomedemstat treatment significantly increased the production of Th1-type cytokine IL-12 and chemokine CXCL-10. Both inflammatory markers may help enhance $CD4^+$ T cell-mediated anti-leukemia effect by driving the polarization of Th1 cells, which are highly desired in this context due to their direct anti-tumor immunity. In addition, bomedemstat also led to the upregulation of several pro-inflammatory chemokines, including MCP-1, MIP-1α and MDC. These chemokines are responsible for the recruitment and tissue infiltration of monocytes and T cells, thereby contributing to the maintenance of inflammatory response (Rollins, 1997). While these chemokines are typically induced by IFN-γ, there was no significant IFN-γ production in any of the four cell lines tested. Therefore, the trigger for bomedemstat-induced inflammatory response may be endogenous to the cells themselves. LSD1 inhibition has been previously shown to upregulate ERVs and activate TLR3 signalling (Sheng et al., 2018). However, in this study, there was also no significant production of type 1 interferons, which are typically associated with TLR3 activation. Ongoing studies in the lab are looking at the expression of ERVs and genes involved in intracellular anti-viral pathways to propose potential mechanistic explanations for the inflammatory response induced by bomedemstat.

In the setting of allo-HSCT, the activation of alloreactive donor T cells relies on effective antigen presentation and co-stimulation by patient-derived APCs, which are

best triggered by inflammatory signals. As a result, the post-transplant GVL effect is optimally induced in an inflammatory milieu (Falkenburg & Jedema, 2017). The generally pro-inflammatory effects of LSD1 inhibition may therefore be desired for post-transplant maintenance in this context. Nevertheless, the increased production of inflammatory cytokines may also increase the risk of developing GVHD. In murine acute GVHD models, IFN-γ-induced chemokines like MIP-1α, MCP-1 and CXCL-10 are highly upregulated in GVHD-target organs such as the skin, liver, spleen and gastrointestinal tract. This is accompanied by a significant infiltration of CCR5 and CXCR3-expressing $CD8^+$ and $CD4^+$ T cells into the target organs (Ichiba et al., 2003; New et al., 2002). Blocking the interactions between chemokines and their receptors has been shown to reduce tissue damage and ameliorate GVHD (He et al., 2008; Murai et al., 1999). Therefore, chemokine-induced tissue infiltration of T cells plays a central role in the development of GVHD. In humans, CXCL-10 has been established as a clinical diagnostic marker for both acute and chronic GVHD (Ahmed et al., 2015; Kariminia et al., 2016). *In situ* expression of CXCL-10 drives the infiltration of $CXCR3^+$ $CD8^+$ cytotoxic T cells and $CD4^+$ Th1 cells into target tissues of GVHD, which strongly associates with inflammation-associated tissues damages (Croudace et al., 2012; Piper et al., 2007). Therefore, the massive upregulation of CXCL-10 in response to bomedemstat treatment may be of particular concern but it is not yet clear whether the effect is restricted to AML cells. Further investigation should look at the effects of bomedemstat on cytokine production in non-hematopoietic cells. One recent study has shown that LSD1 inhibition by tranylcypromine significantly increased the production of CXCL-10 in human umbilical cord-derived mesenchymal stromal cells, suggesting a

generalized pro-inflammatory effect of LSD1 inhibition (Mardani et al., 2022).

4.3 LSD1 inhibition enhances T cell activation by leukemic cells.

In the setting of fully matched allo-HSCT, the activation of alloreactive T cells requires the presentation of non-self MiHAs in identical HLA molecules by patient-derived APCs. Therefore, most alloreactive T cells would be naïve at the time of transplant, encountering non-self antigens for the first time (Falkenburg & Jedema, 2017). Compared to memory T cells, naïve T cells have a significantly higher threshold for activation. Therefore, this project made use of naïve T cells in functional assays to better emulate the initial antigen encounter in an HLA-matched setting.

In the current study, naïve OT-II T cells were co-cultured with murine AML cells to examine the effects of bomedemstat on antigen-specific T cell response *in vitro*. The results showed that bomedemstat-treated H9M-OVA induced the activation of $CD4^+$ OT-II T cells, as demonstrated by the expression of activation markers CD25 and CD69. This suggests that the upregulation of MHC-II and CD86 in murine H9M cells by bomedemstat are able to induce antigen-specific T cell activation. Subsequently, bomedemstat treatment significantly sensitized antigen-pulsed and OVA-expressing H9M cells to T cell-mediated immune killing effect.

Nevertheless, the underlying mechanisms for the early immune killing effect observed in this study remain to be elucidated. The release of cytotoxic granules is a major killing mechanism by $CD4^+$ cytotoxic T cells. However, the expression of the degranulation marker CD107a was not detected after 72 hours of co-culture. Although further optimization of the experimental protocol is required to more accurately detect

CD107a expression, it is possible that the early killing effect is mediated by mechanisms other than degranulation. In fact, naïve $CD4^+$ T cells rapidly produce a large amount of TNF-α within a few hours of TCR-dependent activation, while other effector molecules like IFN-γ and granzyme B are only found in activated effector and memory $CD4^+$ T cells (Ohshima et al., 1999; Wolint et al., 2004). The production of TNF-α can be readily detected through enzyme-linked immunosorbent assay (ELISA) and intracellular cytokine staining. In addition, $CD4^+$ T cells can also induce the apoptosis of target cells through the Fas/Fas Ligand signaling pathway, which represents another potential contact-dependent immune killing mechanism in this context (Malyshkina et al., 2017).

4.4 Antigen-independent mechanisms driving T cell proliferation

The capacity to proliferate is another hallmark of T cell activation which can be tracked through the dilution of permanent fluorescent dye. In this study, bomedemstat-treated H9M cells significantly induced the proliferation of $CD4^+$ OT-II T cells after 96 hours of co-culture. However, proliferation was also observed in the absence of OVA antigens, suggesting that the effect was not specific to this antigen. Removing exogenous IL-2, which can drive T cell proliferation *in vitro*, did not diminish the proliferative effect.

Non-antigen-dependent proliferative processes may play a role in this model. For example, $CD4^+$ T cells are known to undergo slow homeostatic cell divisions in both lymphopenic and lymphocyte-sufficient environment *in vivo*. The process occurs approximately every 3-4 days and is dependent on adequate IL-7 signalling (Min, 2018). Homeostatically proliferating $CD4^+$ T cells also do not acquire the early activation marker CD69 (Min et al., 2005). The timing of the proliferative effect observed in this model

resembles that of $CD4^+$ T cells undergoing homeostatic proliferation. Therefore, it is plausible that IL-7 may have driven the proliferation of OT-II T cells in conjunction with other activating signals. That being said, IL-7 is required for culturing naïve T cells *in vitro* since they quickly undergo apoptosis in the absence of IL-7 signalling. On the other hand, IL-15 is also known to drive T cell proliferation. One previous study has shown that treatment with the FLT3 inhibitor sorafenib can increase IL-15 production in human AML cells and thus enhance the activation of $CD8^+$ T cells to promote the GVL effect (Mathew et al., 2018). Therefore, it is important to further examine the cytokine profiles of long-term co-culture to identify potential drivers for antigen-independent T cell proliferation.

In addition, the proliferation may be dependent on other antigens present in the retrovirally transformed progenitor model. Importantly, no proliferation was observed when MN1-transformed cells were used for stimulation, which suggests that the observed proliferation is still dependent on the presence of treated leukemia cells, potentially through their expression of MHCII and CD86, or soluble factor secreted by the leukemia cells. Some $CD4^+$ OT-II T cells employed in this study have rearranged endogenous TCR receptors which could be activated by other antigens presented by MHC-II. Using *Rag2*-deficient OT-II cells in this model may help restrict the activation of TCR to OVA-specific interactions.

4.5 Limitations

There are several limitations in the current study. The current study only employed a pharmacological approach to inhibit LSD1. Additional testing using genetic

inhibition techniques may be desired to confirm the observed immunomodulatory effects associated with LSD1 inhibition.

The activation of allogeneic immune response is a complicated process which may not be accurately represented by the model. Naive T cells with potential alloreactivity become activated upon encountering a wide variety of MiHAs presented by both hematopoietic and non-hematopoietic cells. During mismatched allo-HSCT, donor T cells can additionally recognize both self and pathogen-derived foreign antigens presented by non-self HLAs from the recipient. This greatly expands the repertoire of alloreactive T cells to include previously activated, pathogen-specific memory T cells, which require a much lower activation threshold compared to naive T cells (Amir et al., 2010). This process is further complicated by the gradual replacement of recipient APCs with donor-derived APCs during the immune recovery process, which alters the conditions required for the activation of alloreactive T cells (Falkenburg & Jedema, 2017). Therefore, the induction of allogeneic immunity is a complex and dynamic process involving a diverse range of antigens and effector cells in various functional states. In this project, the hypothesis is that LSD1 inhibition enhances class II antigen presentation to augment $CD4^+$ T cell-mediated anti-leukemia immunity. To examine this hypothesis, the study made use of a well-established model involving $OVA_{323-339}$-specific OT-II T cells. While this model provides a simple and standardized approach for studying antigen-specific immune response, it may not be sufficient to capture the intricacies of alloreactivity and fully represent the immunological landscape in transplant recipient. That being said, previous functional experiments involving MHC-mismatched murine

AML and T cells were met with technical challenges, most likely due to the lack of defined antigens and relatively low frequencies of alloreactive T cells in the naïve setting. Therefore, it is important to develop novel functional models that can better represent the complexity of allogeneic immunity. One option may be to immunize murine hosts with MHC-disparate AML cells to enrich for alloreactive T cells prior to *in vitro* analysis. Meanwhile, it is also essential to perform additional functional assays with human AML cells and allogeneic T cells to determine the therapeutic potential of bomedemstat treatment in the context of post-transplant maintenance.

Secondly, the study only examined the effects of bomedemstat treatment on HLA-II and CD86 expression in human and retrovirally transformed murine models. Given the vast heterogeneity of AML, it is imperative to investigate the effects of bomedemstat also in primary AML patient samples. In particular, relapsed AML cells after allo-HSCT represent a distinct population of cells that emerges under the immune selection pressure from alloreactive donor T cells. Therefore, there are phenotypic differences between AML at initial diagnosis and those found during post-transplant relapse. Ideally, future study should assess the effects of bomedemstat on paired samples obtained before and after allo-HSCT to better determine its clinical relevance in the setting of post-transplant maintenance.

4.6 Future directions

The current project has demonstrated the positive effects of bomedemstat treatment on antigen-dependent immune killing and T cell activation. However, the model utilized in the study also showed the ability of bomedemstat to induce T cell

proliferation in a manner that may not be strictly antigen-specific. Therefore, future experiments should aim to investigate the underlying mechanisms by which bomedemstat treatment in murine AML cells may lead to T cell proliferation. Multiplex cytokine assays can be used to identify potential drivers of TCR-independent T cell proliferation. To rule out the effect of endogenous TCR-signaling, T cells will be harvested from *Rag2*-deficient OT-II mice and subjected to functional analysis. As bomedemstat treatment also led to significant CD86 upregulation, it would be interesting to explore whether the proliferative effect can be attenuated by blocking CD86 using cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) Fc chimeric protein. Meanwhile, as naïve T cells require significantly higher activation thresholds, certain hallmarks for T cell activation, such as proliferation and cytokine production, may not be readily observed in the current model. Generating effector and memory T cells from OT-II splenocytes through antigen pulsing may serve as a more convenient alternative for functional assessment.

Given the encouraging effects demonstrated in human and murine AML cell lines, future studies should focus on determining the therapeutic potential and clinical relevance of bomedemstat treatment using primary AML patient samples. Currently, experiments are underway to assess the effects of bomedemstat on HLA-II and CD86 expression in patient samples. Particular focus will be given to post-transplant relapsed AML samples with HLA-II downregulation. Overall, *in vitro* characterization of primary AML samples will help identify patterns in treatment response and potentially relate them to the genetic profiles and specific subtypes of AML. This will help determine the cohorts of patients that are most likely to benefit from bomedemstat maintenance.

Meanwhile, some of the patient samples in the Cancer Stem Cell Bank, including those obtained from allo-HSCT recipients, have the ability to engraft irradiated immunodeficient murine hosts. These samples will be transplanted with allogeneic T cells with or without bomedemstat to emulate the post-transplant GVL effect and enable *in vivo* testing of treatment response in a physiologically relevant model. Special attention will also be given to monitoring the development of GVHD, particularly caused by increased chemokine production, to ensure the safety of bomedemstat treatment in a xenogeneic context. Previously, murine CXCL-10 has been shown to induce tissue infiltration of human T cells in a xenogeneic GVHD model (Kawasaki et al., 2018).

Last but not least, T cell exhaustion continues to play a central role in the immune evasion of cancer cells, not only in the context of allo-HSCT. As the development of exhausted T cells requires repeated antigen stimulation, it may be reasonable to deduce that a substantial portion of exhausted T cells in the tumor microenvironment are specific to tumor antigens. Therefore, reinvigorating exhausted T cells is a promising strategy to enhance anti-tumor immunity. Epigenetic reprogramming has been shown to reinforce exhaustion phenotypes, which presents epigenetic inhibitors like bomedemstat as promising candidates for rescuing exhausted T cells. Nevertheless, there is currently a lack of convenient and representative models for assessing bona fide T cell exhaustion *in vitro*. Future studies investigating pharmacological approaches for T cell reinvigoration may require the development of novel and accurate models for T cell exhaustion.

Chapter 5. Conclusion

Allo-HSCT provides a curative treatment option for AML patients, particularly those with adverse and intermediate risk profiles. However, relapse of the primary disease remains the leading cause of mortality after transplant and novel therapeutic strategies are urgently needed to improve patient outcomes. Immune evasion from the alloreactive donor T cell-mediated GVL effect significantly contributes to post-transplant relapse, which presents immunomodulatory strategies as potential maintenance therapies. This work sets out to examine the immunomodulatory effects of LSD1 inhibition by bomedemstat, in the hope of leveraging them to enhance the GVL effect.

To date, the results have shown that bomedemstat treatment significantly increases the expression of CIITA, the master regulator of HLA-II expression. It subsequently led to the expression of HLA-DR in specific human AML cell lines with additional IFN-γ stimulation. Bomedemstat also markedly upregulates the expression of CD86 across all the human AML cell lines tested. In addition, the study also demonstrates that bomedemstat treatment significantly increases the production of IL-12 and CXCL-10, which can help promote Th1 immunity and facilitate T cell trafficking, respectively. It additionally increases the production of chemokines that may help sustain the inflammatory response. Overall, the results demonstrate that LSD1 inhibition by bomedemstat has the potential to enhance all three signals required for T cell activation, including antigen presentation, co-stimulation and cytokine production.

In murine AML models, bomedemstat treatment can significantly upregulate the

expression of MHC-II and CD86 in H9M cells, even without IFN-γ stimulation. It has been further demonstrated that the upregulation of MHC-II, but not CD86, is dependent on IRF-8 expression. Bomedemstat-treated H9M-OVA cells can subsequently induce activation of naïve T cells in an antigen-dependent manner, as evidenced by the upregulation of CD25 and CD69. When subjected to *in vitro* functional assay, bomedemstat treatment can sensitize H9M-OVA and OT-II peptide-pulsed H9M cells to antigen-dependent T cell-mediated immune killing effect. Nevertheless, in the current model, bomedemstat treatment has led to the non-specific proliferation of T cells. Efforts are currently underway to address potential confounding factors in the model.

In conclusion, the pharmacological inhibition of LSD1 in AML by bomedemstat has demonstrated diverse immunomodulatory effects which may be relevant in post-transplant maintenance. Particularly, enhancing class II antigen presentation in AML represents a targeted approach to augment the GVL effect. However, heightened inflammation is often associated with an increased risk of GVHD. Rigorous pre-clinical studies are needed to assess the efficacy and safety of bomedemstat treatment to ensure a seamless clinical translation. Balancing the GVL effect with GVHD continues to be a significant clinical challenge. Depending on each specific case, preventing AML relapse may have priority over mitigating GVHD since the former is associated with more serious outcomes. Clinical decisions can be informed by laboratory findings to determine the risk situation of individual patients and provide truly personalized post-transplant maintenance approaches.

References

Ahmed, S. S., Wang, X. N., Norden, J., Pearce, K., El-Gezawy, E., Atarod, S., Hromadnikova, I., Collin, M., Holler, E., & Dickinson, A. M. (2015). Identification and validation of biomarkers associated with acute and chronic graft versus host disease. *Bone Marrow Transplantation 2015 50:12*, *50*(12), 1563–1571. https://doi.org/10.1038/bmt.2015.191

Amir, A. L., D'Orsogna, L. J. A., Roelen, D. L., Van Loenen, M. M., Hagedoorn, R. S., De Boer, R., Van Der Hoorn, M. A. W. G., Kester, M. G. D., Doxiadis, I. I. N., Falkenburg, J. H. F., Claas, F. H. J., & Heemskerk, M. H. M. (2010). Allo-HLA reactivity of virus-specific memory T cells is common. *Blood*, *115*(15), 3146–3157. https://doi.org/10.1182/BLOOD-2009-07-234906

Arber, D. A., Orazi, A., Hasserjian, R. P., Borowitz, M. J., Calvo, K. R., Kvasnicka, H. M., Wang, S. A., Bagg, A., Barbui, T., Branford, S., Bueso-Ramos, C. E., Cortes, J. E., Dal Cin, P., DiNardo, C. D., Dombret, H., Duncavage, E. J., Ebert, B. L., Estey, E. H., Facchetti, F., … Tefferi, A. (2022). International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood*, *140*(11), 1200–1228. https://doi.org/10.1182/BLOOD.2022015850

Barth, J., Abou-El-Ardat, K., Dalic, D., Kurrle, N., Maier, A. M., Mohr, S., Schütte, J., Vassen, L., Greve, G., Schulz-Fincke, J., Schmitt, M., Tosic, M., Metzger, E., Bug, G., Khandanpour, C., Wagner, S. A., Lübbert, M., Jung, M., Serve, H., … Berg, T. (2019). LSD1 inhibition by tranylcypromine derivatives interferes with GFI1-mediated repression of PU.1 target genes and induces differentiation in AML. *Leukemia*, *33*(6), 1411–1426. https://doi.org/10.1038/s41375-018-0375-7

Betts, M. R., & Koup, R. A. (2004). Detection of T-Cell Degranulation: CD107a and b. *Methods in Cell Biology*, *75*(75), 497–512. https://doi.org/10.1016/S0091-679X(04)75020-7

Bird, A. (2007). Perceptions of epigenetics. *Nature 2007 447:7143*, *447*(7143), 396–398. https://doi.org/10.1038/nature05913

Brunetti, L., Gundry, M. C., Sorcini, D., Guzman, A. G., Huang, Y. H., Ramabadran, R., Gionfriddo, I., Mezzasoma, F., Milano, F., Nabet, B., Buckley, D. L., Kornblau, S. M., Lin, C. Y., Sportoletti, P., Martelli, M. P., Falini, B., & Goodell, M. A. (2018). Mutant NPM1 Maintains the Leukemic State through HOX Expression. *Cancer Cell*, *34*(3), 499-512.e9. https://doi.org/10.1016/j.ccell.2018.08.005

Bryceson, Y. T., Fauriat, C., Nunes, J. M., Wood, S. M., Björkström, N. K., Long, E. O., & Ljunggren, H. G. (2010). Functional analysis of human NK cells by flow cytometry. *Methods in Molecular Biology (Clifton, N.J.)*, *612*, 335–352. https://doi.org/10.1007/978-1-60761-362-6_23/FIGURES/23_4_189279_2_EN

Bug, G., Burchert, A., Wagner, E. M., Kröger, N., Berg, T., Güller, S., Metzelder, S. K., Wolf, A., Hünecke, S., Bader, P., Schetelig, J., Serve, H., & Ottmann, O. G. (2017). Phase I/II study of the deacetylase inhibitor panobinostat after allogeneic stem cell

transplantation in patients with high-risk MDS or AML (PANOBEST trial). *Leukemia 2017 31:11*, *31*(11), 2523–2525. https://doi.org/10.1038/leu.2017.242

Chen, Y., Yang, Y., Wang, F., Wan, K., Yamane, K., Zhang, Y., & Lei, M. (2006). Crystal structure of human histone lysine-specific demethlase 1 (LSD1). *Proceedings of the National Academy of Sciences of the United States of America*, *103*(38), 13956–13961. https://doi.org/10.1073/pnas.0606381103

Choi, J., Ziga, E. D., Ritchey, J., Collins, L., Prior, J. L., Cooper, M. L., Piwnica-Worms, D., & DiPersio, J. F. (2012). IFNγR signaling mediates alloreactive T-cell trafficking and GVHD. *Blood*, *120*(19), 4093–4103. https://doi.org/10.1182/BLOOD-2012-01-403196

Christopher, M. J., Petti, A. A., Rettig, M. P., Miller, C. A., Chendamarai, E., Duncavage, E. J., Klco, J. M., Helton, N. M., O'Laughlin, M., Fronick, C. C., Fulton, R. S., Wilson, R. K., Wartman, L. D., Welch, J. S., Heath, S. E., Baty, J. D., Payton, J. E., Graubert, T. A., Link, D. C., … DiPersio, J. F. (2018). Immune Escape of Relapsed AML Cells after Allogeneic Transplantation. *New England Journal of Medicine*, *379*(24), 2330–2341. https://doi.org/10.1056/nejmoa1808777

Cibrián, D., & Sánchez-Madrid, F. (2017). CD69: from activation marker to metabolic gatekeeper. *European Journal of Immunology*, *47*(6), 946. https://doi.org/10.1002/EJI.201646837

Croudace, J. E., Inman, C. F., Abbotts, B. E., Nagra, S., Nunnick, J., Mahendra, P., Craddock, C., Malladi, R., & Moss, P. A. H. (2012). Chemokine-mediated tissue recruitment of CXCR3+ CD4+ T cells plays a major role in the pathogenesis of chronic GVHD. *Blood*, *120*(20), 4246–4255. https://doi.org/10.1182/BLOOD-2012-02-413260

D'Souza, A., Fretham, C., Lee, S. J., Arora, M., Brunner, J., Chhabra, S., Devine, S., Eapen, M., Hamadani, M., Hari, P., Pasquini, M. C., Perez, W., Phelan, R. A., Riches, M. L., Rizzo, J. D., Saber, W., Shaw, B. E., Spellman, S. R., Steinert, P., … Horowitz, M. M. (2020). Current Use of and Trends in Hematopoietic Cell Transplantation in the United States. In *Biology of Blood and Marrow Transplantation* (Vol. 26, Issue 8, pp. e177–e182). Elsevier Inc. https://doi.org/10.1016/j.bbmt.2020.04.013

Di Nardo, C. D., & Cortes, J. E. (2016). Mutations in AML: prognostic and therapeutic implications. *Hematology*, *2016*(1), 348–355. https://doi.org/10.1182/ASHEDUCATION-2016.1.348

Döhner, H., Wei, A. H., Appelbaum, F. R., Craddock, C., DiNardo, C. D., Dombret, H., Ebert, B. L., Fenaux, P., Godley, L. A., Hasserjian, R. P., Larson, R. A., Levine, R. L., Miyazaki, Y., Niederwieser, D., Ossenkoppele, G., Röllig, C., Sierra, J., Stein, E. M., Tallman, M. S., … Löwenberg, B. (2022). Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood*, *140*(12), 1345–1377. https://doi.org/10.1182/BLOOD.2022016867

Dufva, O., Pölönen, P., Brück, O., Keränen, M. A. I., Klievink, J., Mehtonen, J., Huuhtanen, J., Kumar, A., Malani, D., Siitonen, S., Kankainen, M., Ghimire, B.,

Lahtela, J., Mattila, P., Vähä-Koskela, M., Wennerberg, K., Granberg, K., Leivonen, S. K., Meriranta, L., … Mustjoki, S. (2020). Immunogenomic Landscape of Hematological Malignancies. *Cancer Cell*, *38*(3), 380-399.e13. https://doi.org/10.1016/j.ccell.2020.06.002

Escobar, G., Mangani, D., & Anderson, A. C. (2020). T cell factor 1 (Tcf1): a master regulator of the T cell response in disease. *Science Immunology*, *5*(53). https://doi.org/10.1126/SCIIMMUNOL.ABB9726

Falini, B., Brunetti, L., Sportoletti, P., & Paola Martelli, M. (2020). NPM1-mutated acute myeloid leukemia: from bench to bedside. *Blood*, *136*(15), 1707–1721. https://doi.org/10.1182/BLOOD.2019004226

Falkenburg, J. H. F., & Jedema, I. (2017). Graft versus tumor effects and why people relapse. *Hematology*, *2017*(1), 693–698. https://doi.org/10.1182/ASHEDUCATION-2017.1.693

Fang, Y., Liao, G., & Yu, B. (2019). LSD1/KDM1A inhibitors in clinical trials: Advances and prospects. In *Journal of Hematology and Oncology* (Vol. 12, Issue 1, pp. 1–14). BioMed Central Ltd. https://doi.org/10.1186/s13045-019-0811-9

Ferrara, F., & Schiffer, C. A. (2013). Acute myeloid leukaemia in adults. *The Lancet*, *381*(9865), 484–495. https://doi.org/10.1016/S0140-6736(12)61727-9

Figueroa, M. E., Abdel-Wahab, O., Lu, C., Ward, P. S., Patel, J., Shih, A., Li, Y., Bhagwat, N., Vasanthakumar, A., Fernandez, H. F., Tallman, M. S., Sun, Z., Wolniak, K., Peeters, J. K., Liu, W., Choe, S. E., Fantin, V. R., Paietta, E., Löwenberg, B., … Melnick, A. (2010). Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation. *Cancer Cell*, *18*(6), 553–567. https://doi.org/10.1016/j.ccr.2010.11.015

Franco, F., Jaccard, A., Romero, P., Yu, Y. R., & Ho, P. C. (2020). Metabolic and epigenetic regulation of T-cell exhaustion. *Nature Metabolism 2020 2:10*, *2*(10), 1001–1012. https://doi.org/10.1038/s42255-020-00280-9

Fürst, D., Neuchel, C., Tsamadou, C., Schrezenmeier, H., & Mytilineos, J. (2019). HLA Matching in Unrelated Stem Cell Transplantation up to Date. *Transfusion Medicine and Hemotherapy*, *46*(5), 326–336. https://doi.org/10.1159/000502263

Gary Gilliland, D., & Griffin, J. D. (2002). The roles of FLT3 in hematopoiesis and leukemia. *Blood*, *100*(5), 1532–1542. https://doi.org/10.1182/BLOOD-2002-02-0492

Ghoneim, H. E., Fan, Y., Moustaki, A., Abdelsamed, H. A., Dash, P., Dogra, P., Carter, R., Awad, W., Neale, G., Thomas, P. G., & Youngblood, B. (2017). De Novo Epigenetic Programs Inhibit PD-1 Blockade-Mediated T Cell Rejuvenation. *Cell*, *170*(1), 142-157.e19. https://doi.org/10.1016/J.CELL.2017.06.007/ATTACHMENT/5EE2F831-EF7A-4780-A03D-63F1F586A157/MMC1.DOCX

Granot, N., & Storb, R. (2020). History of hematopoietic cell transplantation: challenges

and progress. *Haematologica*, *105*(12), 2716–2729. https://doi.org/10.3324/HAEMATOL.2019.245688

Harris, W. J., Huang, X., Lynch, J. T., Spencer, G. J., Hitchin, J. R., Li, Y., Ciceri, F., Blaser, J. G., Greystoke, B. F., Jordan, A. M., Miller, C. J., Ogilvie, D. J., & Somervaille, T. C. P. (2012). The Histone Demethylase KDM1A Sustains the Oncogenic Potential of MLL-AF9 Leukemia Stem Cells. *Cancer Cell*, *21*(4), 473–487. https://doi.org/10.1016/j.ccr.2012.03.014

He, S., Cao, Q., Qiu, Y., Mi, J., Zhang, J. Z., Jin, M., Ge, H., Emerson, S. G., Zhang, Y., & Zhang, Y. (2008). A New Approach to the Blocking of Alloreactive T Cell-Mediated Graft-versus-Host Disease by In Vivo Administration of Anti-CXCR3 Neutralizing Antibody. *The Journal of Immunology*, *181*(11), 7581–7592. https://doi.org/10.4049/JIMMUNOL.181.11.7581

Hiatt, J. B., Sandborg, H., Garrison, S. M., Arnold, H. U., Liao, S. Y., Norton, J. P., Friesen, T. J., Wu, F., Sutherland, K. D., Rienhoff, H. Y., Martins, R., Houghton, A. M. G., Srivastava, S., & MacPherson, D. (2022). Inhibition of LSD1 with Bomedemstat Sensitizes Small Cell Lung Cancer to Immune Checkpoint Blockade and T-Cell Killing. *Clinical Cancer Research*, *28*(20), 4551–4564. https://doi.org/10.1158/1078-0432.CCR-22-1128/707373/AM/INHIBITION-OF-LSD1-WITH-BOMEDEMSTAT-SENSITIZES

Hutten, T. J. A., Norde, W. J., Woestenenk, R., Wang, R. C., Maas, F., Kester, M., Falkenburg, J. H. F., Berglund, S., Luznik, L., Jansen, J. H., Schaap, N., Dolstra, H., & Hobo, W. (2018). Increased Coexpression of PD-1, TIGIT, and KLRG-1 on Tumor-Reactive CD8+ T Cells During Relapse after Allogeneic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation*, *24*(4), 666–677. https://doi.org/10.1016/J.BBMT.2017.11.027

Ichiba, T., Teshima, T., Kuick, R., Misek, D. E., Liu, C., Takada, Y., Maeda, Y., Reddy, P., Williams, D. L., Hanash, S. M., & Ferrara, J. L. M. (2003). Early changes in gene expression profiles of hepatic GVHD uncovered by oligonucleotide microarrays. *Blood*, *102*(2), 763–771. https://doi.org/10.1182/BLOOD-2002-09-2748

Intlekofer, A. M., Takemoto, N., Wherry, E. J., Longworth, S. A., Northrup, J. T., Palanivel, V. R., Mullen, A. C., Gasink, C. R., Kaech, S. M., Miller, J. D., Gapin, L., Ryan, K., Russ, A. P., Lindsten, T., Orange, J. S., Goldrath, A. W., Ahmed, R., & Reiner, S. L. (2005). Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nature Immunology 2005 6:12*, *6*(12), 1236–1244. https://doi.org/10.1038/ni1268

Jenq, R. R., & Van Den Brink, M. R. M. (2010). Allogeneic haematopoietic stem cell transplantation: individualized stem cell and immune therapy of cancer. *Nature Reviews Cancer 2010 10:3*, *10*(3), 213–221. https://doi.org/10.1038/nrc2804

Johnson, W. E. (2019). Origins and evolutionary consequences of ancient endogenous retroviruses. *Nature Reviews Microbiology 2019 17:6*, *17*(6), 355–370. https://doi.org/10.1038/s41579-019-0189-2

Kanada, S., Nishiyama, C., Nakano, N., Suzuki, R., Maeda, K., Hara, M., Kitamura, N., Ogawa, H., & Okumura, K. (2011). Critical role of transcription factor PU.1 in the expression of CD80 and CD86 on dendritic cells. *Blood*, *117*(7), 2211–2222. https://doi.org/10.1182/BLOOD-2010-06-291898

Kantarjian, H., Kadia, T., DiNardo, C., Daver, N., Borthakur, G., Jabbour, E., Garcia-Manero, G., Konopleva, M., & Ravandi, F. (2021). Acute myeloid leukemia: current progress and future directions. *Blood Cancer Journal 2021 11:2*, *11*(2), 1–25. https://doi.org/10.1038/s41408-021-00425-3

Kariminia, A., Holtan, S. G., Ivison, S., Rozmus, J., Hebert, M. J., Martin, P. J., Lee, S. J., Wolff, D., Subrt, P., Abdossamadi, S., Sung, S., Storek, J., Levings, M., Aljurf, M., Arora, M., Cutler, C., Gallagher, G., Kuruvilla, J., Lipton, J., … Schultz, K. R. (2016). Heterogeneity of chronic graft-versus-host disease biomarkers: association with CXCL10 and CXCR3+ NK cells. *Blood*, *127*(24), 3082–3091. https://doi.org/10.1182/BLOOD-2015-09-668251

Karin, N., Wildbaum, G., & Thelen, M. (2016). Biased signaling pathways via CXCR3 control the development and function of CD4+ T cell subsets. *Journal of Leukocyte Biology*, *99*(6), 857–862. https://doi.org/10.1189/JLB.2MR0915-441R

Kawasaki, Y., Sato, K., Nakano, H., Mashima, K., Minakata, D., Yamasaki, R., Morita, K., Ashizawa, M., Yamamoto, C., Hatano, K., Fujiwara, S., Oh, I., Ohmine, K., Muroi, K., & Kanda, Y. (2018). Comprehensive Analysis of Chemokines in Host Organs and Their Corresponding Receptors on Donor T-Cells in Xenogeneic Gvhd Model. *Blood*, *132*(Supplement 1), 5676–5676. https://doi.org/10.1182/BLOOD-2018-99-111773

Khwaja, A., Bjorkholm, M., Gale, R. E., Levine, R. L., Jordan, C. T., Ehninger, G., Bloomfield, C. D., Estey, E., Burnett, A., Cornelissen, J. J., Scheinberg, D. A., Bouscary, D., & Linch, D. C. (2016). Acute myeloid leukaemia. *Nature Reviews Disease Primers 2016 2:1*, *2*(1), 1–22. https://doi.org/10.1038/nrdp.2016.10

Köhler, N., Ruess, D. A., Kesselring, R., & Zeiser, R. (2021). The Role of Immune Checkpoint Molecules for Relapse After Allogeneic Hematopoietic Cell Transplantation. *Frontiers in Immunology*, *12*, 634435. https://doi.org/10.3389/FIMMU.2021.634435

Kong, Y., Zhang, J., Claxton, D. F., Ehmann, W. C., Rybka, W. B., Zhu, L., Zeng, H., Schell, T. D., & Zheng, H. (2015). PD-1hiTIM-3+ T cells associate with and predict leukemia relapse in AML patients post allogeneic stem cell transplantation. *Blood Cancer Journal 2015 5:7*, *5*(7), e330–e330. https://doi.org/10.1038/bcj.2015.58

Koreth, J., Schlenk, R., Kopecky, K. J., Honda, S., Sierra, J., Djulbegovic, B. J., Wadleigh, M., DeAngelo, D. J., Stone, R. M., Sakamaki, H., Appelbaum, F. R., Döhner, H., Antin, J. H., Soiffer, R. J., & Cutler, C. (2009). Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia in First Complete Remission: Systematic Review and Meta-analysis of Prospective Clinical Trials. *JAMA*, *301*(22), 2349–2361. https://doi.org/10.1001/JAMA.2009.813

LeibundGut-Landmann, S., Waldburger, J. M., Krawczyk, M., Otten, L. A., Suter, T.,

Fontana, A., Acha-Orbea, H., & Reith, W. (2004). Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *European Journal of Immunology*, *34*(6), 1513–1525. https://doi.org/10.1002/EJI.200424964

Liew, F. Y. (2002). TH1 and TH2 cells: a historical perspective. *Nature Reviews Immunology 2002 2:1*, *2*(1), 55–60. https://doi.org/10.1038/nri705

Liu, Y., Debo, B., Li, M., Shi, Z., Sheng, W., & Shi, Y. (2021). LSD1 inhibition sustains T cell invigoration with a durable response to PD-1 blockade. *Nature Communications 2021 12:1*, *12*(1), 1–16. https://doi.org/10.1038/s41467-021-27179-7

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, *25*(4), 402–408. https://doi.org/10.1006/METH.2001.1262

Luo, C., Wu, G., Huang, X., Ding, Y., Huang, Y., Song, Q., Hou, Y., Chen, J., Li, X., & Xu, S. (2022). Myeloablative conditioning regimens in adult patients with acute myeloid leukemia undergoing allogeneic hematopoietic stem cell transplantation in complete remission: a systematic review and network meta-analysis. *Bone Marrow Transplantation 2022 58:2*, *58*(2), 175–185. https://doi.org/10.1038/s41409-022-01865-6

Luznik, L., O'Donnell, P. V., Symons, H. J., Chen, A. R., Leffell, M. S., Zahurak, M., Gooley, T. A., Piantadosi, S., Kaup, M., Ambinder, R. F., Huff, C. A., Matsui, W., Bolaños-Meade, J., Borrello, I., Powell, J. D., Harrington, E., Warnock, S., Flowers, M., Brodsky, R. A., … Fuchs, E. J. (2008). HLA-Haploidentical Bone Marrow Transplantation for Hematologic Malignancies Using Nonmyeloablative Conditioning and High-Dose, Posttransplantation Cyclophosphamide. *Biology of Blood and Marrow Transplantation*, *14*(6), 641–650. https://doi.org/10.1016/j.bbmt.2008.03.005

Maeda, T., Towatari, M., Kosugi, H., & Saito, H. (2000). Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. *Blood*, *96*(12), 3847–3856. https://doi.org/10.1182/BLOOD.V96.12.3847

Malek, T. R. (2008). The Biology of Interleukin-2. *Https://Doi.Org/10.1146/Annurev.Immunol.26.021607.090357*, *26*, 453–479. https://doi.org/10.1146/ANNUREV.IMMUNOL.26.021607.090357

Malyshkina, A., Littwitz-Salomon, E., Sutter, K., Zelinskyy, G., Windmann, S., Schimmer, S., Paschen, A., Streeck, H., Hasenkrug, K. J., & Dittmer, U. (2017). Fas Ligand-mediated cytotoxicity of CD4+ T cells during chronic retrovirus infection. *Scientific Reports 2017 7:1*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-08578-7

Mardani, F., Saad, W., El-Hachem, N., Bikorimana, J. P., Kurdi, M., Shammaa, R., Talbot, S., & Rafei, M. (2022). LSD1 Inhibition Enhances the Immunogenicity of Mesenchymal Stromal Cells by Eliciting a dsRNA Stress Response. *Cells*, *11*(11). https://doi.org/10.3390/CELLS11111816

Mathew, N. R., Baumgartner, F., Braun, L., O'Sullivan, D., Thomas, S., Waterhouse, M., Müller, T. A., Hanke, K., Taromi, S., Apostolova, P., Illert, A. L., Melchinger, W., Duquesne, S., Schmitt-Graeff, A., Osswald, L., Yan, K. L., Weber, A., Tugues, S., Spath, S., … Zeiser, R. (2018). Sorafenib promotes graft-versus-leukemia activity in mice and humans through IL-15 production in FLT3-ITD-mutant leukemia cells. *Nature Medicine*, *24*(3), 282–291. https://doi.org/10.1038/nm.4484

Min, B. (2018). Spontaneous T Cell Proliferation: A Physiologic Process to Create and Maintain Homeostatic Balance and Diversity of the Immune System. *Frontiers in Immunology*, *9*(MAR), 1. https://doi.org/10.3389/FIMMU.2018.00547

Min, B., Yamane, H., Hu-Li, J., & Paul, W. E. (2005). Spontaneous and Homeostatic Proliferation of CD4 T Cells Are Regulated by Different Mechanisms. *The Journal of Immunology*, *174*(10), 6039–6044. https://doi.org/10.4049/JIMMUNOL.174.10.6039

Morimoto, Y., Toyota, M., Satoh, A., Murai, M., Mita, H., Suzuki, H., Takamura, Y., Ikeda, H., Ishida, T., Sato, N., Tokino, T., & Imai, K. (2004). Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-γ in haematopoietic tumour cells. *British Journal of Cancer 2004 90:4*, *90*(4), 844–852. https://doi.org/10.1038/sj.bjc.6601602

Morris, A. C., Spangler, W. E., & Boss, J. M. (2000). Methylation of Class II trans-Activator Promoter IV: A Novel Mechanism of MHC Class II Gene Control. *The Journal of Immunology*, *164*(8), 4143–4149. https://doi.org/10.4049/JIMMUNOL.164.8.4143

Murai, M., Yoneyama, H., Harada, A., Yi, Z., Vestergaard, C., Guo, B., Suzuki, K., Asakura, H., & Matsushima, K. (1999). Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. *The Journal of Clinical Investigation*, *104*(1), 49–57. https://doi.org/10.1172/JCI6642

New, J. Y., Li, B., Koh, W. P., Ng, H. K., Tan, S. Y., Yap, E. H., Chan, S. H., & Hu, H. Z. (2002). T cell infiltration and chemokine expression: relevance to the disease localization in murine graft-versus-host disease. *Bone Marrow Transplantation 2002 29:12*, *29*(12), 979–986. https://doi.org/10.1038/sj.bmt.1703563

Niebel, D., Kirfel, J., Janzen, V., Höller, T., Majores, M., & Gütgemann, I. (2014). Lysine-specific demethylase 1 (LSD1) in hematopoietic and lymphoid neoplasms. *Blood*, *124*(1), 151–152. https://doi.org/10.1182/BLOOD-2014-04-569525

Noce, B., Di Bello, E., Fioravanti, R., & Mai, A. (2023). LSD1 inhibitors for cancer treatment: Focus on multi-target agents and compounds in clinical trials. *Frontiers in Pharmacology*, *14*. https://doi.org/10.3389/FPHAR.2023.1120911

Norde, W. J., Maas, F., Hobo, W., Korman, A., Quigley, M., Kester, M. G. D., Hebeda, K., Falkenburg, J. H. F., Schaap, N., De Witte, T. M., Van Der Voort, R., & Dolstra, H. (2011). PD-1/PD-L1 interactions contribute to functional T-cell impairment in patients who relapse with cancer after allogeneic stem cell transplantation. *Cancer Research*, *71*(15), 5111–5122. https://doi.org/10.1158/0008-5472.CAN-11-

0108/649713/AM/PD-1-PD-L1-INTERACTIONS-CONTRIBUTE-TO-FUNCTIONAL-T

Ohshima, Y., Yang, L.-P., Avice, M.-N., Kurimoto, M., Nakajima, T., Sergerie, M., Demeure, C. E., Sarfati, M., & Delespesse, G. (1999). Naive Human CD4+ T Cells Are a Major Source of Lymphotoxin α. *The Journal of Immunology*, *162*(7), 3790–3794. https://doi.org/10.4049/JIMMUNOL.162.7.3790

Oran, B., de Lima, M., Garcia-Manero, G., Thall, P. F., Lin, R., Popat, U., Alousi, A. M., Hosing, C., Giralt, S., Rondon, G., Woodworth, G., & Champlin, R. E. (2020). A phase 3 randomized study of 5-azacitidine maintenance vs observation after transplant in high-risk AML and MDS patients. *Blood Advances*, *4*(21), 5580–5588. https://doi.org/10.1182/BLOODADVANCES.2020002544

Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell*, *132*(4), 631–644. https://doi.org/10.1016/J.CELL.2008.01.025

Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V. I., Paschka, P., Roberts, N. D., Potter, N. E., Heuser, M., Thol, F., Bolli, N., Gundem, G., Van Loo, P., Martincorena, I., Ganly, P., Mudie, L., McLaren, S., O'Meara, S., Raine, K., Jones, D. R., … Campbell, P. J. (2016). Genomic Classification and Prognosis in Acute Myeloid Leukemia. *New England Journal of Medicine*, *374*(23), 2209–2221. https://doi.org/10.1056/NEJMOA1516192/SUPPL_FILE/NEJMOA1516192_DISCLOSURES.PDF

Park, H. H., Kim, M., Lee, B.-H., Lim, J., Kim, Y., Lee, E. J., Min, W. S., Kang, C. S., Kim, W. Il, Shim, S. I., & Han, K. (2006). *Intracellular IL-4, IL-10, and IFN-g Levels of Leukemic Cells and Bone Marrow T Cells in Acute Leukemia*. www.annclinlabsci.org

Pennock, N. D., White, J. T., Cross, E. W., Cheney, E. E., Tamburini, B. A., & Kedl, R. M. (2013). T cell responses: naïve to memory and everything in between. *Advances in Physiology Education*, *37*(4), 273. https://doi.org/10.1152/ADVAN.00066.2013

Piper, K. P., Horlock, C., Curnow, S. J., Arrazi, J., Nicholls, S., Mahendra, P., Craddock, C., & Moss, P. A. H. (2007). CXCL10-CXCR3 interactions play an important role in the pathogenesis of acute graft-versus-host disease in the skin following allogeneic stem-cell transplantation. *Blood*, *110*(12), 3827–3832. https://doi.org/10.1182/BLOOD-2006-12-061408

Qin, Y., Vasilatos, S. N., Chen, L., Wu, H., Cao, Z., Fu, Y., Huang, M., Vlad, A. M., Lu, B., Oesterreich, S., Davidson, N. E., & Huang, Y. (2019). Inhibition of histone lysine-specific demethylase 1 elicits breast tumor immunity and enhances antitumor efficacy of immune checkpoint blockade. *Oncogene*, *38*(3), 390–405. https://doi.org/10.1038/s41388-018-0451-5

Reville, P. K., & Kadia, T. M. (2021). Maintenance Therapy in AML. *Frontiers in Oncology*, *10*, 3255. https://doi.org/10.3389/FONC.2020.619085/BIBTEX

Roche, P. A., & Furuta, K. (2015). The ins and outs of MHC class II-mediated antigen processing and presentation. *Nature Reviews Immunology 2015 15:4*, *15*(4), 203–

216. https://doi.org/10.1038/nri3818

Rollins, B. J. (1997). Chemokines. *Blood*, *90*(3), 909–928. https://doi.org/10.1182/BLOOD.V90.3.909

Santana Carrero, R. M., Beceren-Braun, F., Rivas, S. C., Hegde, S. M., Gangadharan, A., Plote, D., Pham, G., Anthony, S. M., & Schluns, K. S. (2019). IL-15 is a component of the inflammatory milieu in the tumor microenvironment promoting antitumor responses. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(2), 599–608. https://doi.org/10.1073/pnas.1814642116

Sasaki, K., Ravandi, F., Kadia, T. M., DiNardo, C. D., Short, N. J., Borthakur, G., Jabbour, E., & Kantarjian, H. M. (2021). De novo acute myeloid leukemia: A population-based study of outcome in the United States based on the Surveillance, Epidemiology, and End Results (SEER) database, 1980 to 2017. *Cancer*, *127*(12), 2049–2061. https://doi.org/10.1002/CNCR.33458

Schenk, T., Chen, W. C., Göllner, S., Howell, L., Jin, L., Hebestreit, K., Klein, H. U., Popescu, A. C., Burnett, A., Mills, K., Casero, R. A., Marton, L., Woster, P., Minden, M. D., Dugas, M., Wang, J. C. Y., Dick, J. E., Müller-Tidow, C., Petrie, K., & Zelent, A. (2012). Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. *Nature Medicine*, *18*(4), 605–611. https://doi.org/10.1038/nm.2661

Schiffer, C. A., & Stone, R. M. (2003). *Morphologic Classification and Clinical and Laboratory Correlates*. https://www.ncbi.nlm.nih.gov/books/NBK13452/

Scott, B. L., Pasquini, M. C., Fei, M., Fraser, R., Wu, J., Devine, S. M., Porter, D. L., Maziarz, R. T., Warlick, E., Fernandez, H. F., Soiffer, R. J., Alyea, E., Hamadani, M., Bashey, A., Giralt, S., Geller, N. L., Leifer, E., Hourigan, C. S., Gui, G., … Horwitz, M. E. (2021). Myeloablative versus Reduced-Intensity Conditioning for Hematopoietic Cell Transplantation in Acute Myelogenous Leukemia and Myelodysplastic Syndromes—Long-Term Follow-Up of the BMT CTN 0901 Clinical Trial. *Transplantation and Cellular Therapy*, *27*(6), 483.e1-483.e6. https://doi.org/10.1016/J.JTCT.2021.02.031

Sharma, A., Yun, H., Jyotsana, N., Chaturvedi, A., Schwarzer, A., Yung, E., Lai, C. K., Kuchenbauer, F., Argiropoulos, B., Görlich, K., Ganser, A., Humphries, R. K., & Heuser, M. (2014). Constitutive IRF8 expression inhibits AML by activation of repressed immune response signaling. *Leukemia 2015 29:1*, *29*(1), 157–168. https://doi.org/10.1038/leu.2014.162

Sheng, W., LaFleur, M. W., Nguyen, T. H., Chen, S., Chakravarthy, A., Conway, J. R., Li, Y., Chen, H., Yang, H., Hsu, P. H., Van Allen, E. M., Freeman, G. J., De Carvalho, D. D., He, H. H., Sharpe, A. H., & Shi, Y. (2018). LSD1 Ablation Stimulates Anti-tumor Immunity and Enables Checkpoint Blockade. *Cell*, *174*(3), 549-563.e19. https://doi.org/10.1016/j.cell.2018.05.052

Shi, Y. Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., & Shi, Y. Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell*, *119*(7), 941–953. https://doi.org/10.1016/j.cell.2004.12.012

Short, N. J., Rytting, M. E., & Cortes, J. E. (2018). Acute myeloid leukaemia. *The Lancet*, *392*(10147), 593–606. https://doi.org/10.1016/S0140-6736(18)31041-9

Smith, C. C. (2019). The growing landscape of FLT3 inhibition in AML. *Hematology*, *2019*(1), 539–547. https://doi.org/10.1182/HEMATOLOGY.2019000058

Smith, M. A., Wright, G., Wu, J., Tailor, P., Ozato, K., Chen, X., Wei, S., Piskurich, J. F., Ting, J. P. Y., & Wright, K. L. (2011). Positive regulatory domain I (PRDM1) and IRF8/PU.1 counter-regulate MHC class II transactivator (CIITA) expression during dendritic cell maturation. *Journal of Biological Chemistry*, *286*(10), 7893–7904. https://doi.org/10.1074/jbc.M110.165431

Stirewalt, D. L., & Radich, J. P. (2003). The role of FLT3 in haematopoietic malignancies. *Nature Reviews Cancer 2003 3:9*, *3*(9), 650–665. https://doi.org/10.1038/nrc1169

Stomper, J., Rotondo, J. C., Greve, G., & Lübbert, M. (2021). Hypomethylating agents (HMA) for the treatment of acute myeloid leukemia and myelodysplastic syndromes: mechanisms of resistance and novel HMA-based therapies. *Leukemia 2021 35:7*, *35*(7), 1873–1889. https://doi.org/10.1038/s41375-021-01218-0

Stone, R. M., Mandrekar, S., Sanford, B. L., Geyer, S., Bloomfield, C. D., Dohner, K., Thiede, C., Marcucci, G., Lo-Coco, F., Klisovic, R. B., Wei, A., Sierra, J., Sanz, M. A., Brandwein, J. M., de Witte, T., Niederwieser, D., Appelbaum, F. R., Medeiros, B. C., Tallman, M. S., … Dohner, H. (2015). The Multi-Kinase Inhibitor Midostaurin (M) Prolongs Survival Compared with Placebo (P) in Combination with Daunorubicin (D)/Cytarabine (C) Induction (ind), High-Dose C Consolidation (consol), and As Maintenance (maint) Therapy in Newly Diagnosed Acute Myeloid Leukemia (AML) Patients (pts) Age 18-60 with FLT3 Mutations (muts): An International Prospective Randomized (rand) P-Controlled Double-Blind Trial (CALGB 10603/RATIFY [Alliance]). *Blood*, *126*(23), 6–6. https://doi.org/10.1182/BLOOD.V126.23.6.6

Suzuki, J., Maruyama, S., Tamauchi, H., Kuwahara, M., Horiuchi, M., Mizuki, M., Ochi, M., Sawasaki, T., Zhu, J., Yasukawa, M., & Yamashita, M. (2016). Gfi1, a transcriptional repressor, inhibits the induction of the T helper type 1 programme in activated CD4 T cells. *Immunology*, *147*(4), 476–487. https://doi.org/10.1111/IMM.12580

Sweeney, C., & Vyas, P. (2019). The Graft-Versus-Leukemia Effect in AML. *Frontiers in Oncology*, *9*(November), 1–19. https://doi.org/10.3389/fonc.2019.01217

Takeuchi, A., & Saito, T. (2017). CD4 CTL, a Cytotoxic Subset of CD4+ T Cells, Their Differentiation and Function. *Frontiers in Immunology*, *8*(FEB), 194. https://doi.org/10.3389/FIMMU.2017.00194

Tan, A. H. Y., Tu, W. J., McCuaig, R., Hardy, K., Donovan, T., Tsimbalyuk, S., Forwood, J. K., & Rao, S. (2019). Lysine-specific histone demethylase 1A regulates macrophage polarization and checkpoint molecules in the tumor microenvironment of triple-negative breast cancer. *Frontiers in Immunology*, *10*(JUN), 1–17. https://doi.org/10.3389/fimmu.2019.01351

Tay, R. E., Richardson, E. K., & Toh, H. C. (2020). Revisiting the role of CD4+ T cells in cancer immunotherapy—new insights into old paradigms. *Cancer Gene Therapy*. https://doi.org/10.1038/s41417-020-0183-x

Tempany, J. C., Zhou, J. H. S., Hodgkin, P. D., & Bryant, V. L. (2018). Superior properties of CellTrace Yellow™ as a division tracking dye for human and murine lymphocytes. *Immunology and Cell Biology*, *96*(2), 149–159. https://doi.org/10.1111/IMCB.1020

Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, F. L., Neiman, P. E., Lerner, K. G., Glucksberg, H., & Buckner, C. D. (1975). Bone-Marrow Transplantation. *Https://Doi.Org/10.1056/NEJM197504172921605*, *292*(16), 843. https://doi.org/10.1056/NEJM197504172921605

Toffalori, C., Zito, L., Gambacorta, V., Riba, M., Oliveira, G., Bucci, G., Barcella, M., Spinelli, O., Greco, R., Crucitti, L., Cieri, N., Noviello, M., Manfredi, F., Montaldo, E., Ostuni, R., Naldini, M. M., Gentner, B., Waterhouse, M., Zeiser, R., … Vago, L. (2019). Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nature Medicine*, *25*(4), 603–611. https://doi.org/10.1038/s41591-019-0400-z

Tokunaga, R., Zhang, W., Naseem, M., Puccini, A., Berger, M. D., Soni, S., McSkane, M., Baba, H., & Lenz, H. J. (2018). CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation – A target for novel cancer therapy. In *Cancer Treatment Reviews* (Vol. 63, pp. 40–47). W.B. Saunders Ltd. https://doi.org/10.1016/j.ctrv.2017.11.007

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology 2003 3:2*, *3*(2), 133–146. https://doi.org/10.1038/nri1001

Ustun, C., Le-Rademacher, J., Wang, H. L., Othus, M., Sun, Z., Major, B., Zhang, M. J., Storrick, E., Lafky, J. M., Chow, S., Mrózek, K., Attar, E. C., Nand, S., Bloomfield, C. D., Cripe, L. D., Tallman, M. S., Appelbaum, F., Larson, R. A., Marcucci, G., … Artz, A. S. (2019). Allogeneic hematopoietic cell transplantation compared to chemotherapy consolidation in older acute myeloid leukemia (AML) patients 60–75 years in first complete remission (CR1): an alliance (A151509), SWOG, ECOG-ACRIN, and CIBMTR study. *Leukemia 2019 33:11*, *33*(11), 2599–2609. https://doi.org/10.1038/s41375-019-0477-x

Vahlne, G., Becker, S., Brodin, P., & Johansson, M. H. (2008). IFN-γ Production and Degranulation are Differentially Regulated in Response to Stimulation in Murine Natural Killer Cells. *Scandinavian Journal of Immunology*, *67*(1), 1–11. https://doi.org/10.1111/J.1365-3083.2007.02026.X

Velardi, E., Tsai, J. J., & van den Brink, M. R. M. (2020). T cell regeneration after immunological injury. *Nature Reviews Immunology 2020 21:5*, *21*(5), 277–291. https://doi.org/10.1038/s41577-020-00457-z

Wingard, J. R., Majhail, N. S., Brazauskas, R., Wang, Z., Sobocinski, K. A., Jacobsohn, D., Sorror, M. L., Horowitz, M. M., Bolwell, B., Rizzo, J. D., & Socié, G. (2011).

Long-term survival and late deaths after allogeneic hematopoietic cell transplantation. *Journal of Clinical Oncology*, *29*(16), 2230–2239. https://doi.org/10.1200/JCO.2010.33.7212

Wolint, P., Betts, M. R., Koup, R. A., & Oxenius, A. (2004). Immediate Cytotoxicity But Not Degranulation Distinguishes Effector and Memory Subsets of CD8+ T Cells. *The Journal of Experimental Medicine*, *199*(7), 925. https://doi.org/10.1084/JEM.20031799

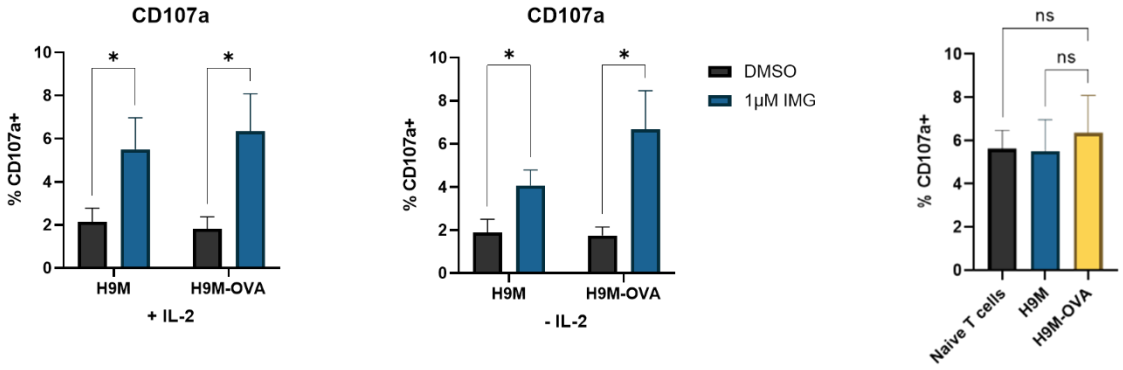
Wu, H., Shi, J., Luo, Y., Yu, J., Lai, X., Liu, L., Fu, H., Ouyang, G., Xu, X., Xiao, H., Huang, H., & Zhao, Y. (2022). Assessment of Patient-Specific Human Leukocyte Antigen Genomic Loss at Relapse After Antithymocyte Globulin–Based T-Cell–Replete Haploidentical Hematopoietic Stem Cell Transplant. *JAMA Network Open*, *5*(4), e226114–e226114. https://doi.org/10.1001/JAMANETWORKOPEN.2022.6114

Yan, Y., Upadhyaya, R., Zhang, V. W., & Berg, T. (2022). Epigenetic maintenance strategies after allogeneic stem cell transplantation in acute myeloid leukemia. *Experimental Hematology*, *109*, 1-10.e1. https://doi.org/10.1016/J.EXPHEM.2022.03.003

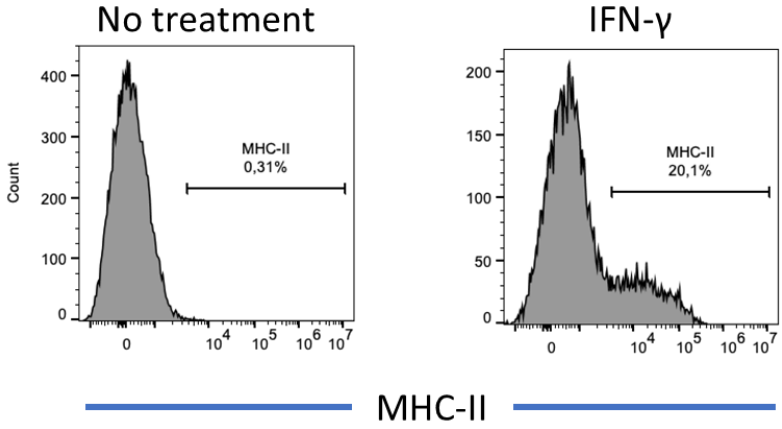
Yang, M., Culhane, J. C., Szewczuk, L. M., Jalili, P., Ball, H. L., Machius, M., Cole, P. A., & Yu, H. (2007). Structural basis for the inhibition of the LSD1 histone demethylase by the antidepressant trans-2-phenylcyclopropylamine. *Biochemistry*, *46*(27), 8058–8065. https://doi.org/10.1021/BI700664Y/ASSET/IMAGES/MEDIUM/BI700664YN00001.GIF

Zhang, F., Zhou, X., Dispirito, J. R., Wang, C., Wang, Y., & Shen, H. (2014). Epigenetic Manipulation Restores Functions of Defective CD8+ T Cells From Chronic Viral Infection. *Molecular Therapy*, *22*(9), 1698–1706. https://doi.org/10.1038/MT.2014.91

Appendices



Appendix 1. Expression of CD107a on CD4$^+$ OT-II T cells in co-culture. Expression of CD107a in CD4$^+$ OT-II T cells after 72 hours of co-culture, with or without IL-2. Columns represent the mean ± SD of at least three independent experiments; *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001. Statistical significance was determined using Student's t test.



Appendix 2. MHC-II expression in H9M-*Irf8*-KO cells stimulated by IFN-γ. Representative flow plots of MHC-II expression in H9M-*Irf8*-KO cells after 48 hours of stimulation with 10 ng/mL of murine IFN-γ.