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Single-cell dissection of immune system aging and acute myeloid leukemia

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Single-cell Dissection of Immune System Aging & Acute Myeloid Leukemia

Emin Onur Karakaşlar

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Single-cell Dissection of Immune System Aging and Acute Myeloid Leukemia

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Contents

1	Introduction	1
2	Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging	13
3	Utilizing Epigenetic Regulators to Improve HSC-based Lentiviral Gene Therapy	37
4	A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML	59
5	Mapping AML Heterogeneity: Multi-cohort transcriptomic analysis identifies novel clusters and divergent ex-vivo drug responses	89
6	Resolving inter- and intra-patient heterogeneity in <i>NPM1</i> -mutated AML at single-cell resolution	109
7	Discussion	135
	Summary	149
	Samenvatting	151
	Curriculum Vitae	153
	Acknowledgments	155
	List of Publications	157



Chapter 1

Introduction

What is Hematopoiesis and how it supports the immune system?

Hematopoiesis sustains the continuous generation of blood cells from a rare pool of hematopoietic stem cells (HSCs). With ageing, HSCs gradually acquire somatic mutations that confer a competitive growth advantage, skew differentiation toward the myeloid lineage and predispose carriers to myeloid malignancies. Although individual driver lesions are well catalogued, an integrated, cellular-resolution map that explains how ageing, clonal hematopoiesis and genetic lesions reshape the transcriptional and epigenetic landscape of hematopoietic differentiation and create vulnerabilities that can be therapeutically exploited is still lacking.

In this thesis, we combine single-cell multi-omics, functional perturbations and computational modelling to (i) chart the molecular trajectories of normal and perturbed hematopoiesis across the lifespan, (ii) dissect the mechanisms that expand pre-leukemic clones, and (iii) translate these insights into precision-medicine strategies for myeloid malignancies.

1.1 What is Hematopoiesis and how it supports the immune system?

All cells of a human (or a multi-cellular organism) share almost exactly the same DNA. This is because all cells are offspring of the original zygote, a consequence of mitosis. Although all cells share the same blueprint, each has different responsibilities. To puppet a cell, DNA has specialized regions called genes to encode for proteins. These genes are transcribed into messenger RNAs (mRNA), which are then translated into proteins by ribosomes¹. In each cell, genes are expressed at different scales, thereby controlling protein abundance. The heritable changes in gene expression that occur without changes in the DNA sequence are called epigenetics. This has been described by Waddington in 1942 in his seminal paper as The Epigenotype², using this term without knowing how genes are functioning.

“We need merely to assume that changes in the genotype produce correlated changes in the adult phenotype, but the mechanism of this correlation need not concern us.”

– C. H. Waddington

In his classical view, Waddington’s landscape models cell differentiation as a marble rolling down a hill with branching valleys. The marble starts at the top as a pluripotent cell and, as it rolls down, its path becomes increasingly restricted by the landscape’s contours, representing cell differentiation regulated by epigenetics. Each valley corresponds to a specific cell fate. Early in development, the marble can switch paths, but as it progresses, commitment deepens and plasticity decreases. This explains how cells gradually lose potential and commit to specific lineages. Remarkably, this simple analogy still resonates with our understanding of cell biology today. For instance, blood production or hematopoiesis, one of the most studied systems in animals, begins in the bone marrow with a hematopoietic stem cell (HSC) and closely resembles the valley analogy (Figure 1.1). Over time, HSCs divide and differentiate into various blood cell types, creating the entire blood system. To exemplify, an adult human produces approximately 10^{11} new blood cells each day to maintain steady-state conditions³. This process is heavily regulated to ensure a balanced production of different types of blood cells.

Hematopoiesis bifurcates into two major branches: the myeloid and lymphoid lineages. The myeloid lineage accounts for most cells in the human blood system, especially erythrocytes (or red blood cells) (RBCs) compose 99% of blood cells⁴, and 84% of all human cells⁵. RBCs are responsible for carrying oxygen from the lungs to the entire body and returning carbon-dioxide back from the body to the lungs. During their differentiation, mature RBCs dissolve their nuclei,

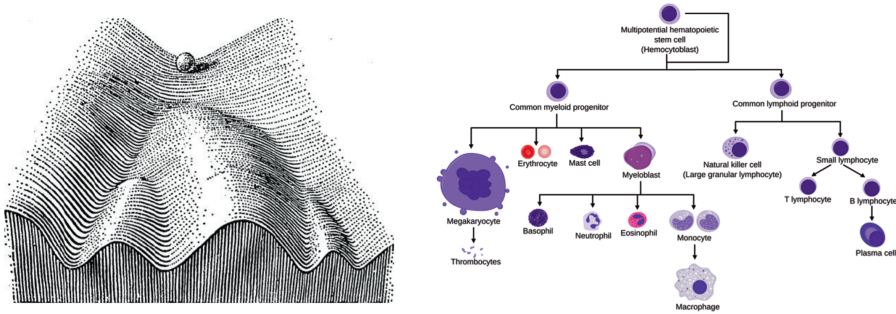


Figure 1.1: Waddington Valley Analogy (left) and hematopoiesis starting from a stem cell (right)

thereby losing their DNA and organelles such as mitochondria to maximize their efficiency for oxygen transport. This process, however, hinders their replication ability and significantly limits their lifespan to around 120 days⁶. Therefore, constant blood production is required in order to maintain the high RBC count.

The remaining myeloid - and lymphoid - cells are white blood cells (WBCs). These cells consist of granulocytes (e.g. neutrophils, basophils, eosinophils), monocytes, and lymphocytes (B cells, T cells, and natural killer cells). In contrast to mature RBCs, white blood cells have nuclei and retain the ability to produce offspring. A recent study by Soerens *et al.* demonstrated that healthy T-cell clones from mice can persist across multiple generations when collected and transferred to successive genetically identical hosts over 10 years, indicating an extraordinary population expansion and longevity well beyond their organismal lifespan⁷. WBCs in the myeloid lineage are responsible for innate immunity, meaning that they are activated within minutes to hours to provide the first line of defense against a threat, such as a pathogen or tissue damage. Neutrophils, for example, can engulf and digest pathogens or cell debris with a process called phagocytosis, or even sacrifice themselves in order to capture and neutralize pathogens with a structure called Neutrophil Extracellular Traps (NET), which consists of their inner parts released when neutrophils undergo cell death⁸. In addition to granulocytes, WBCs of the myeloid lineage consist of monocytes, which can differentiate into macrophages upon leaving the bloodstream and entering peripheral tissues, and dendritic cells (DCs). Monocytes, macrophages and DCs are also able to engulf and digest pathogens and cell debris by phagocytosis, and DCs play a key role in initiating adaptive immune response by presenting antigens to T-lymphocytes.

The second main branch is the lymphoid lineage. In this lineage, T- and B-lymphocytes are responsible for adaptive immunity. Adaptive immunity takes longer to be activated and is highly specific. T- and B-lymphocytes allow the immune system to adapt to specific antigens or pathogens encountered in the environment. Adaptive immunity is also crucial for the clearance of senescent cells, which are old or damaged and no longer function properly. The clearance of senescent cells is of utmost importance as these cells can be pro-inflammatory and disturb other cells or even lead to cancer if not cleared or signaled for apoptosis (self-killing)⁹. To produce B lymphocytes, HSCs in the bone marrow give rise to pro- and pre-B cells, which then differentiate into mature B cells. Mature B cells express B-cell receptors and differentiate into plasma cells upon encountering a specific antigen, thereby releasing their B-cell receptors as soluble antibodies¹⁰. Lineage-committed progenitors derived from HSCs migrate from the bone marrow to the thymus, where they mature into T cells¹¹. B cells produce antibodies, which are soluble proteins that specifically bind and neutralize pathogens, while T cells can recognize infected cells via their membrane-bound T-cell

How hematopoiesis can be measured?

receptor. Upon antigen recognition, T cells start to release cytokines. T cells can also release cytotoxic molecules (cytotoxic T cells) and induce direct cell death. Other T cells only release cytokines (helper T cells) to stimulate other immune cells such as B cells, granulocytes, macrophages and NK cells. NK cells are innate lymphocytes, that target and destroy tissue cells that lack specific cell surface markers.

Overall, hematopoiesis is a complex system which resembles Waddington's landscape, where hematopoietic stem cells roll down through the valleys of different progenitor cells that differentiate into specific blood cell types. Understanding the intricacies of hematopoiesis holds immense potential for treating patients with hematological disorders and enhancing regenerative medicine approaches. The Waddington landscape will be further discussed in the final chapter of this thesis by taking recent emergent evidence into account.

1.2 How hematopoiesis can be measured?

Various technologies can be used to assess hematopoiesis, for instance a Complete Blood Count (CBC) test is a fundamental lab test that offers important insight into different blood elements like RBCs, WBCs, and platelets. This assessment is used in routine diagnostics to screen for abnormalities such as anemia or leukopenia, which may be indications for abnormal blood cell production, or even hint at severe cases of hematological malignancies⁴. More sophisticated techniques like flow cytometry allow for a deeper exploration of hematopoiesis. Using fluorescently labeled antibodies, this technique generates detailed profiles of cell surface markers, providing critical insights into both normal and abnormal distributions within hematopoiesis. For instance, CD34 is a well-known cell surface marker for hematopoietic stem and progenitor cells (HSPC) in healthy hematopoiesis in humans¹². By sorting CD34⁺ cells from bone marrow, researchers can study early hematopoiesis. Other proteins like CD14 and CD11c are markers for more mature cells such as monocytes (CD14⁺CD11c⁺), or dendritic cells (CD14⁺CD11c⁺)¹³. CD3, CD4 and CD8 are canonical markers for T-cell subsets (CD3⁺CD8⁺ and CD3⁺CD4⁺)¹⁴. The presence or absence of specific markers and their combined expression on specific cell types allow researchers to characterize hematopoiesis.

There are also other methods which could be utilized for characterizing the molecular mechanisms underlying hematopoiesis, such as RNA sequencing (RNA-seq) and Assay for Transposase-Accessible Chromatin using Sequencing (ATAC-seq)^{15,16}. For example, RNA-seq allows researchers to analyze gene expression profiles in hematopoietic cells, revealing the transcriptional programs that regulate differentiation and maturation. Meanwhile, ATAC-seq maps regions of accessible chromatin (open or closed), and gives hints about active regulatory elements¹⁷. Together, these techniques provide an opportunity to interrogate the transcriptional and epigenetic landscape that governs hematopoiesis on an unprecedented scale.

Building upon these powerful methodologies, single-cell technologies have revolutionized the study of hematopoiesis. While traditional bulk sequencing only provides averaged data across different cell populations, potentially obscuring rare or transient cell states, single-cell technologies offer researchers to analyze these states and conditions at cellular resolution. For example, single-cell RNA sequencing (scRNA-seq) allows researchers to measure gene expression in individual cells. Initially starting with only a few hundreds of cells, single-cell atlases are nowadays generated for millions of cells¹⁸, and perhaps orders of magnitude more in the future. The growing scale of these datasets allow researchers to uncover previously undetected rare cell types¹⁹, track and trace developmental lineages^{20,21}, and characterize disease progression with higher accuracy.

Single-cell RNA-seq was extensively utilized in this thesis to determine changes or irregularities in the hematopoietic system in healthy individuals or leukemia patients, respectively.

1.3 How hematopoiesis changes with age?

As we grow older, our hematopoietic system undergoes systemic changes. For instance, toddlers usually have more lymphoid cells²², whereas elderly have a myeloid bias²³. This is not surprising as we are exposed to pathogens and our adaptive immunity trained during life. Starting from a naïve state, T- and B-cells are trained by encountering new antigens, which are most often pathogens. Upon activation, naïve T and B cells differentiate into effector and memory cells. This process makes clearance of previously encountered pathogens fast and efficient but comes with a cost. The naïve T-cell pool decreases with age, meaning that there are less cells available that can fight and be trained against new pathogens^{24–26}.

After early adolescence, the thymus, which is the organ responsible for producing naïve T-cells, starts to gradually shrink and it becomes a very small organ in older adults. This raises the question whether shrinkage occurs because the number of HSCs that seed the thymus decreases as we age, or does the thymus accumulate damage over time, making it incapable of producing naïve T-cells, which in turn leads to its shrinkage. The answer to this question remains elusive, but reduced infiltration of HSCs in the thymus could be caused by the myeloid bias in older individuals. The age-associated bias toward myeloid cells may also be explained at least partly by Clonal Hematopoiesis with Indeterminate Potential (CHIP), which is another age-related phenomenon. CHIP is instigated by the acquisition of somatic mutations in genes like *DNMT3A*, *TET2* or *ASXL1*. HSCs with these genes mutations have a competitive growth advantage and produce more myeloid offspring²⁷. A CHIP mutation is found in less than 1% of healthy individuals under the age of 50²⁸, and more than 30% of elderly people above 80^{27,29}. The HSC clones that have these canonical mutations have evolutionary advantage as their expansion rates are higher compared to those HSCs lacking these mutations. For instance, HSCs with *DNMT3A* mutations, especially hotspot mutations, are known to promote heavy myeloid skewing^{30,31}. As a result, clonal diversity, the number of genetically distinct blood-forming stem and myeloid progenitor cell clones, in older adults is significantly reduced compared to younger individuals^{32,33}.

It has been hypothesized that expansion of myeloid cells can lead to chronic inflammation as a result of a more pro-inflammatory environment, which is a hallmark of aging³⁴. This effect does not only occur in humans but also exists in other mammals such as mice. For example, the murine homolog hotspot mutation in *Dnmt3a* is also associated with monocytic expansion and a MDS/AML-related phenotype²². Additionally, in Chapter 2 of this thesis, aging of the immune system has been investigated in two different mouse models (C57BL/6, and New Zealand Obese). Aggregate transcriptional and chromatin signatures were obtained from sorted blood cells, and AP-1, which is a protein complex that regulates inflammation, was shown to be upregulated in monocytes²⁶. Furthermore, CHIP has been linked not only to hematological malignancies but also to age-associated disorders such as cardiovascular disease, and autoimmune diseases such as rheumatoid arthritis and type-II diabetes, all diseases characterized by chronic inflammation²⁷. These associations highlight the clinical relevance of myeloid expansion, positioning it as a potential mechanistic bridge between chronic inflammation and the pathogenesis of diverse age-related diseases.

To summarize, aging affects the fine balance of hematopoiesis and often skews it towards the myeloid lineage due to clonal expansion. A bias toward myeloid cells also leads to a reduced

pool of naive lymphoid cells, making the immune system even more fragile. As a consequence of this decrease, senescent cells and pathogens are cleared less efficiently and may lead to a pro-inflammatory environment and chronic inflammation. These conditions create the perfect environment for malignant cells to arise and proliferate and are probably the main reason why the incidences of myeloid malignancies increase with age.

1.4 Hematopoiesis and Myeloid Malignancies

CHIP significantly increases the chance of developing myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML)^{27,28}. CHIP mutations such as somatic mutations in DNMT3A or TET2, are commonly detected in MDS and AML. Morphology plays a central role in the diagnosis of MDS and AML and is one of the first steps in evaluating bone marrow and peripheral blood. For instance, French American British (FAB) classification was introduced in 1976 as systematic classification system for diagnosing AML based on cell morphology³⁵. Nowadays, with the advent of next generation sequencing (NGS) technologies, diagnosis of AML is performed by morphology combined with cytogenetic tests to measure chromosomal abnormalities such as translocations and extensive molecular screening for somatic mutations in a large panel of genes. The latest World Health Organization (WHO) classification includes 13 subtypes of AML based on recurrent genetic abnormalities³⁶. However, despite detailed classification at diagnosis, the treatment options for the different subtypes are still lagging behind.

The first-line treatment of most AML patients who are fit is induction chemotherapy followed by consolidation chemotherapy or allogeneic stem cell transplantation for patients with high-risk AML^{37,38}. Elderly patients and patients who are unfit are treated with less intensive regimens, which nowadays often include hypomethylating agents combined with Venetoclax as BCL-2 inhibitor. Patients treated with Venetoclax have shown different response profiles to this drug³⁹⁻⁴¹. For instance, in Chapter 4, using bulk-RNA sequencing to analyze AML samples from public cohorts and our internal cohort, we showed higher *ex-vivo* resistance to Venetoclax for AML patients with monocytic phenotype. This shows that phenotypic information, which is also present in the old FAB classification, has still prognostic value for patients. Recent studies have shown that RNA-seq profiles of AML patients not only enable the retrieval of such information⁴², but also provide broader opportunities for patient data analysis. For instance, in Chapter 5 of this thesis, we analyzed RNA-seq data from over 1,200 AML patients to explore transcriptional heterogeneity across genetically defined subtypes. Our analysis was able to reconstruct the major genetic abnormality classes described in the WHO 2022 classification based solely on gene expression profiles⁴³. Importantly, we found that even within well-defined entities such as *NPM1*-mutated AML, patients could be further stratified into transcriptionally distinct clusters. In Chapter 6, we pursued detailed analyses of *NPM1*-mutated AML patients at transcriptomics and cell surface protein levels at single-cell resolution and demonstrated differences in maturation and myeloid skewing between the clusters. These results, consistent with recent studies⁴⁴, suggest that the current classification system may underrepresent the molecular diversity within AML and highlight the potential of RNA-seq to refine disease taxonomy.

In conclusion, genetic abnormalities disturb the landscape of normal hematopoiesis and may eventually lead to hematological malignancies. Especially, myeloid malignancies are associated with aging due to accumulation of somatic mutations in HSCs, which give them a competitive growth advantage and higher chance to acquire additional genetic abnormalities, ultimately leading to malignant transformation and AML.

1.5 Thesis Outline

An overview of the scientific chapters is shown in Figure 1.2.

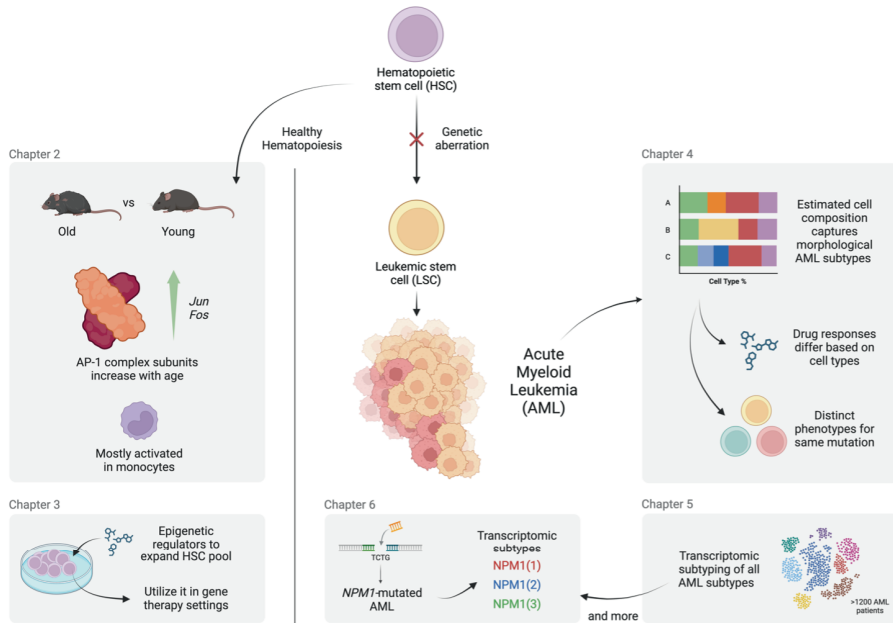


Figure 1.2: Overview of the scientific chapters of the thesis

In **Chapter 2**, we analyzed how the immune system is changing with age in two different mouse strains using peripheral blood mononuclear cells from 3 different time points. One of the mouse strains represents healthy aging (C57BL/6), whereas “unhealthy” aging occurs in New Zealand Obese (NZO) mice, since this strain is prone to get type I diabetes. We performed flow cytometry, ATAC-Seq as well as single and bulk RNA-Seq to uncover the common signatures of immune-aging in these two mouse models. Our computational models indicated that the naive T-cell pool significantly decreases with age in both strains. Notably, we found that the genes encoding the AP-1 transcription factor complex are significantly upregulated and that their promoters became more accessible with age. Our single cell analyses showed that these genes were mostly activated in monocytes with an increasing abundance with age. Functional data showed that upon myeloid cell activation via poly(I:C), JUN protein levels and their promoter-binding activity increased more significantly in spleen cells from old compared to young mice.

Homeostatic balance of immune cells is crucial for many diseases. For instance, in Severe Combined Immunodeficiency (SCID), hematopoietic stem cells (HSCs) transplantation enables the regeneration of both lymphoid and myeloid lineages, effectively reconstituting adaptive and innate immune functions. Without such intervention, patients with SCID lack functional T and B cells, leaving them highly susceptible to infections. However, the low yield of HSCs that can be collected from these patients remains a limitation. Therefore, expansion of HSCs and efficient lentiviral transduction *ex-vivo* to enable gene therapy for patients with inherited immune deficiencies is

References

highly desired. In **Chapter 3**, we utilized two epigenetic regulators (Quisinostat and CPI203) to expand the HSC pool *ex-vivo* and validated these findings using single-cell RNA sequencing analysis and *in vivo* xenotransplantation assays. Importantly, we showed that Quisinostat increased total numbers as well as LV transduction efficiencies of HSCs and early progenitor cells.

The derailing of the hematopoiesis via genetic aberrations might result in heavy myeloid expansion and cause leukemia. In **Chapter 4**, we performed deconvolution on bulk RNA-seq samples of more than 1200 AML cases and showed that the estimated cell proportions are in concordance with morphological diagnosis according to FAB classification. Furthermore, using *ex-vivo* drug responses from one of the studies with matching RNA-seq data, we showed that drugs have different responses for given cell proportions. Notably, *NPM1*-mutated AML cases, a single subtype in the latest WHO classification, had different cell type proportions and *ex-vivo* drug responses to a drug called Venetoclax, which is a *BCL2* inhibitor used in the clinic as first-line treatment option for elderly patients and patients who are frail and cannot be treated with intensive chemotherapy. Our results demonstrate that bulk-RNA seq can potentially guide diagnostics and pave a way for better precision medicine.

In **Chapter 5**, we used the same RNA-seq dataset to create a map of AML based on similarity in their gene expression profiles. Patients belonging to the same genetic WHO classes are closely clustered, indicating that genetic subtyping of recurrent abnormalities can also be performed by transcriptional profiling. Notably, we found additional transcriptional clusters for various single entities in WHO such as *NPM1*-mutated AML, which can be explained at least partly by different co-mutational profiles. We showed that different co-mutations like *TET2* and *IDH1/2* mutations could result in similar gene expression profiles, whereas other mutations such as *FLT3-ITD* and *DNMT3A* mutations lead to different transcriptional signatures.

As we've discovered the molecular heterogeneity of *NPM1*-mutated AML, we wanted to investigate further into characterizing these differences at higher resolution. Therefore, in **Chapter 6**, we performed single-cell RNA-seq and spectral flow cytometry using a custom panel of antibodies on 16 *NPM1*-mutated AML, which were intentionally selected from different clusters based on bulk transcriptomics. Our single-cell RNA-seq data suggest that myeloid differentiation still continues in *NPM1*-mutated AML, but that myelopoiesis is heavily skewed towards progenitor cells with early or more mature phenotypes in the distinct gene expression clusters identified by bulk transcriptomics. These findings were confirmed by flow cytometry, thereby demonstrating that transcriptional signatures of *NPM1*-mutated AML can also be distinguished by cell surface markers. We also identified two small HSC-like cell clusters representing leukemic stem cells (CD34⁺CD38⁺CD45RA⁺) and healthy or pre-leukemic HSCs (CD34⁺CD38⁺CD45RA⁻) and demonstrated that leukemic stem cells are present at variable levels in distinct transcriptomic clusters of *NPM1*-mutated AML.

Finally, in **Chapter 7**, I discuss the overall outlook of the thesis and how results are related to molecular understanding of hematopoiesis and its derailing in a wider scope. The chapter concludes with a reflection on future research directions, emphasizing the importance of personalized medicine in treating hematological disorders.

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Chapter 2

Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging

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2.1 Abstract

Diverse mouse strains have different health and life spans, mimicking the diversity among humans. To capture conserved aging signatures, we studied long-lived C57BL/6J and short-lived NZO/HILtJ mouse strains by profiling transcriptomes and epigenomes of immune cells from peripheral blood and the spleen from young and old mice. Transcriptional activation of the AP-1 transcription factor complex, particularly *Fos*, *Junb*, and *Jun* genes, was the most significant and conserved aging signature across tissues and strains. ATAC-seq data analyses showed that the chromatin around these genes was more accessible with age and there were significantly more binding sites for these TFs with age across all studied tissues, targeting pro-inflammatory molecules including *Il6*. Age-related increases in binding sites of JUN and FOS factors were also conserved in human peripheral blood ATAC-seq data. Single-cell RNA-seq data from the mouse aging cell atlas Tabula Muris Senis showed that the expression of these genes increased with age in B, T, NK cells, and macrophages, with macrophages from old mice expressing these molecules more abundantly than other cells. Functional data showed that upon myeloid cell activation via poly(I:C), the levels of JUN protein and its binding activity increased more significantly in spleen cells from old compared to young mice. In addition, upon activation, old cells produced more IL6 compared to young cells. In sum, we showed that the aging-related transcriptional activation of *Jun* and *Fos* family members in AP-1 complex is conserved across immune tissues and long- and short-living mouse strains, possibly contributing to increased inflammation with age.

2.2 Introduction

Age-related changes in the immune system reduce older individuals' ability to generate protective responses to immunological threats and lead to increases in diseases and infections^{1,2}. Increased inflammation with age (i.e., *inflammaging*) is one of the hallmarks of immune system aging that is conserved across humans and mice as well as across tissues and strains³; however, the drivers of this aging signature are mostly unknown⁴. Human immune aging studies have mostly been limited to blood since it is easy to access and gives an opportunity to study the status of the peripheral immune system with minimal invasiveness. These studies have uncovered significant age-related changes in gene expression levels in whole blood, as well as in blood-derived peripheral mononuclear cells (PBMCs) and sorted immune cells⁵⁻⁷. Through genomic profiling, we and others have uncovered that pro-inflammatory molecules are activated with age, whereas molecules related to T-cell homeostasis and signaling are downregulated^{4,5,7-10}. Although these studies have described significant age-related changes in transcriptional regulatory programs of immune cells, including the activation of pro-inflammatory programs, they have not pinpointed potential upstream regulators of these genomic alterations.

Mouse models are essential in aging research for establishing age-related changes in various tissues, drivers of these changes, and ways to delay or reverse these changes¹¹. Most aging studies utilize the long-living laboratory strain C57BL/6J (B6) with a median life span of 901 days for males and 866 days for females¹². However, there is significant diversity among mouse strains in terms of health and life span that could be exploited to uncover signatures of aging conserved among diverse human populations. An example of such a strain is New Zealand Obese (NZO/HILtj), which resembles human aging in multiple aspects. First, similar to humans, NZO females live longer than males¹³, where the median lifespan is 576 days for females and 423 days for males¹². Second, NZO mice develop obesity and hence are used as models in Type 2 diabetes (T2D), insulin resistance, and obesity¹⁴ research. Obesity is an epidemic in human populations and a factor that accelerates the mechanisms of aging, significantly contributing to unhealthy aging. Thus, NZO and B6 represent "unhealthy aging" and "healthy aging" models, respectively, enabling us to uncover biomarkers of aging that are consistent despite differences in genetic backgrounds and life and health spans. In these two strains, we characterized the effects of age on the immune system by profiling peripheral blood leukocytes (PBL) and the cells from the spleen, the largest lymphoid organ, both of which harbor post-differentiated immune cells affected by aging. We also profiled naive and memory CD8⁺ T cells sorted from the spleen, given the importance of CD8⁺ T cells in human aging^{5,7}.

In these strains and tissues, we studied the effects of age on the transcriptome (via RNA-seq), epigenome (via ATAC-seq), and cell compositions (via flow cytometry) by generating and comparing data from young (3 months) and old (18 months) animals. Together, our data uncovered transcriptional activation of *Jun* and *Fos* members of the AP-1 complex with age as the most conserved biomarker of immune aging in long- and short-living mouse strains and across spleen and blood immune cells. Our results, including functional data on JUN binding and IL6 production, suggest that AP-1 complex activation may be a driver of inflammaging. Data from these two mouse strains and human PBMCs can be queried at: <http://immune-aging.jax.org>.

2.3 Results

Profiling blood and spleen immune cells of young and old long-lived B6 and short-lived NZO mice

To comprehensively study the effects of aging on the mouse immune system, we generated flow cytometry, RNA-seq, and ATAC-seq data from circulating immune cells (PBLs) and from the spleen in B6 and NZO strains. These strains were selected as representatives of each end of the longevity spectrum of laboratory mice to mimic the heterogeneity in individuals' life spans and to uncover conserved immune aging signatures in long and short-living animals. PBL and spleen samples were collected from young (3 months) and old (18 months) mice, which also allows direct age comparison to existing single-cell mouse transcriptomic data from the Tabula Muris Senis atlas¹⁵ (Figure 2.1a). CD8+ T cells are significantly affected with age among the human peripheral immune cells^{4,7}, where aging affects both naive and memory compartments⁷. For this reason, we also sorted and profiled epigenomes and transcriptomes of naive and memory CD8+ T cells from the spleen to study whether age-related changes in the human CD8+ cells⁷ also exist in mice (markers used are summarized in Figure 2.1a right panel). Samples that passed quality control (QC) were used in downstream analyses: 74 flow cytometry samples (39 B6, 35 NZO), 103 RNA-seq samples (49 B6, 54 NZO), and 90 ATAC-seq samples (38 B6, 52 NZO) (summarized in Table S1, Figure S1A). After QC and filtering, 96,623 consensus ATAC-seq peaks from all cell/tissue types and 18,294 expressed genes from RNA-seq samples were used in downstream analyses. Using flow cytometry, we characterized PBL and spleen cell compositions for a total of 36 cell types including total B, CD8+, and CD4+ T cells and their subsets (Table S2).

Principal component analysis (PCA) revealed that RNA-seq, ATAC-seq, and flow cytometry samples were first separated by tissue/cell type and then by strain as expected (Figure S1B–D). We quantified how much variation is attributable to meta-data in each modality using principal variance component analysis (PVCa)¹⁶. Tissue/cell type explained most of the variation in both RNA-seq (~55%) and ATAC-seq (~41%) data, followed by strain differences (16% in RNA-seq, 19% in ATAC-seq) (Figure S1E). Age explained 20% of the variation in the flow cytometry data, due to significant remodeling of PBL and spleen cell compositions with age, whereas it explained around 5% of the variation in RNA-seq and ATAC-seq data. Only ≤2% of the variation in each modality was attributable to biological sex (Figure S1E). These data and analyses suggest that cell type and strain are the main drivers of variation in mouse genomics data, followed by the age of the animals.

Naive T-cell decline is the most significant and conserved age-related cell compositional change

Using flow cytometry, we first quantified the proportion of B, CD4+, and CD8+ T cells within the spleen and PBL (Table S2). The myeloid compartment (granulocytes, monocytes, and dendritic cells-DCs) and NK cells constitute the remainder of the cells (henceforth labeled as “innate” cells). The T-cell subsets were further stratified into effector memory (EM, CD44^{high} CD62L⁻), effector (EMRA, CD44^{low} CD62L⁻), central memory (CM, CD44^{high} CD62L⁺), and naive (CD44^{low} CD62L⁺) cells (Figure S2A). Given their importance in T-cell homeostasis, signaling^{10,17}, and in human aging⁷, we also quantified IL7R+ and PD1+ cells^{4,18}.

The majority of mouse spleen cells (45%) and PBLs (37%) were composed of B cells (Figure 2.1b). Total B and CD8+ T-percentages did not change significantly with age, whereas CD4+ T cells declined significantly in PBL (Figure 2.1c). Regarding T-cell subsets, the most significant change was the decline of naive T cells, observed in both B6 and NZO, in both tissues (PBL, spleen), and in both CD4+ and CD8+ compartments (Figure 2.1d, Figure S3A). We also detected significant

Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging

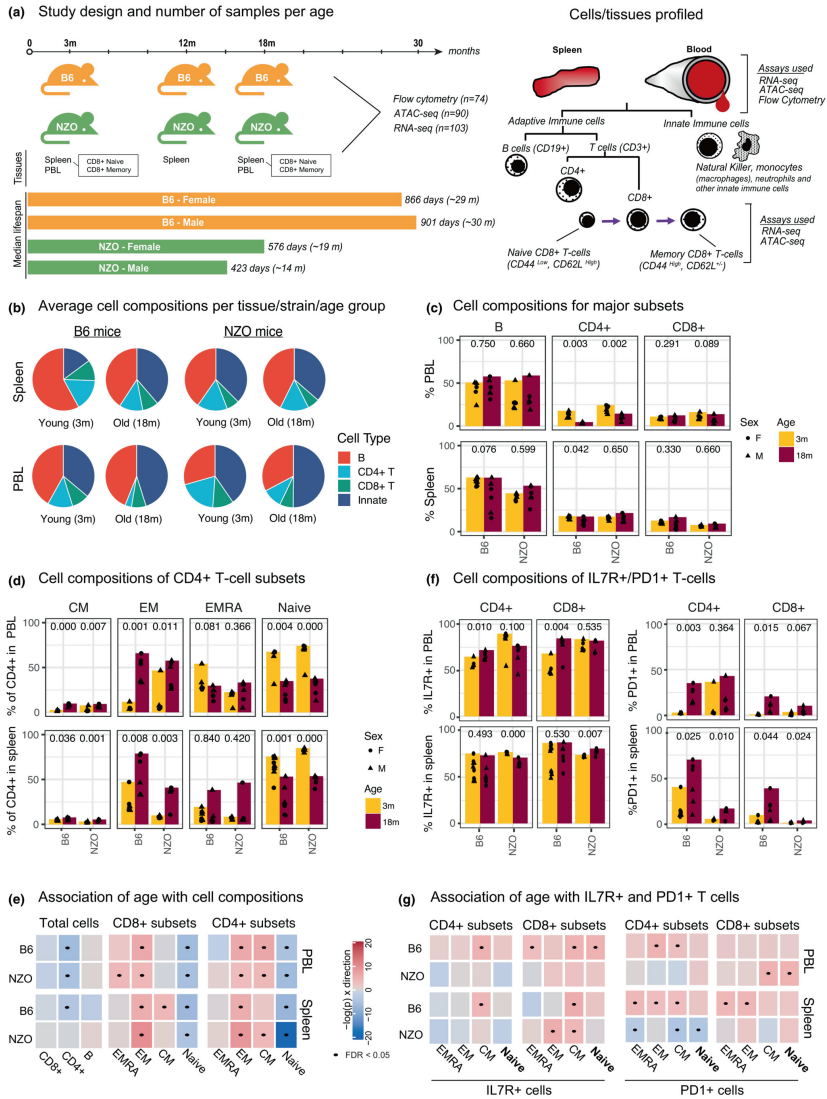


Figure 2.1: (a) Schematic of study design. PBL and spleen were collected from 3- and 18-month-old mice, and CD8+ T cells were sorted from spleen. We also collected and profiled spleen from 12-month-old mice. From these samples, ATAC-seq, RNA-seq, and flow cytometry data were generated. Right panel: markers used for sorting/staining in our study. (b) Overall cell composition pie charts for PBL and spleen from young and old mice for B, CD4+ T, and CD8+ T cells. The remaining cells were designated as “innate.” (c) Changes in the cellular composition of major cell types in mouse spleen and PBL with age (x-axis represents age in months, and each dot represents an animal). The jitter around points is for plotting purposes to avoid overlapping dots. (d) Similar to (c) for CD4+ T-cell subsets (CM: Central Memory, EM: Effector Memory, EMRA: Effector Memory re-expressing CD45RA). (e) Summary for the association of age with a given cell type using linear models. Each cell type was colored according to the slope of change per unit of age (in months) and the direction of the slope—red for positive (increase with age) and blue for negative (decrease with age) slopes, with darker colors indicating steeper slope (i.e., more significant changes). Significant associations (FDR 5%) are marked with a dot. (f) Cell composition changes of IL7R+ and PD1+ T-cell subsets. (g) Linear models that associate IL7R+ and PD1+ T-cell percentages to age similar to (e). p-values in c-d-f are calculated using unpaired t-test.

Results

increases in EM populations in both compartments. Regression models that associate age with the percentage of each cell type confirmed that declines in naive CD4+ and CD8+ T cells and increases in EM CD4+ and CD8+ T cells were the most significant and conserved age-related changes in cell composition (Figure 2.1e). Interestingly, these cell compositional changes were similar between the two tissues (PBL, spleen) and between the two strains despite the significant difference in their life spans. Previous mouse studies similarly reported declines in naive T cells and increases in memory T cells both in spleen¹⁹ and blood²⁰. Naive T-cell decline with age partially stems from insufficient homeostatic proliferation due to thymus shrinkage and continuous activation of naive T cells²¹.

The majority of CD4+ and CD8+ T cells were IL7R+ in both tissues and strains, and there was a slight increase in IL7R+ cells in naive and memory T-cell subsets, especially in the central memory (CM) component (Figure 2.1g, Figure S3B). On the contrary, percentages of PD1+ T cells increased with age in T-cell subsets (Figure 2.1f,g). The increases in PD1+ cell percentages were more significant in long-lived B6 compared to short-lived NZO at 18 months of age (Figure 2.1g, Figure S3B), suggesting that the increases in PD1+ T-cell percentages might not strictly relate to the longevity of the organism. Together, these data and analyses showed that PBL and spleen cell compositions significantly change with age, and most of these changes are conserved between the two mouse strains, where declines in naive T cells and increases in EM cells are the most significant and conserved age-related changes.

Transcriptional activation of Jun/Fos members of the AP-1 complex is a conserved aging signature

We conducted differential gene expression analyses between old and young animals for all studied cells/tissues: spleen, PBL, and naive and memory CD8+ T cells (Table S3, FDR = 0.05 and $|\log\text{FC}| > 1$).

In the spleen tissue, 1960 genes were differentially expressed (DE) in B6 and 1795 in NZO, 675 of which were shared between strains. In PBL, we detected 455 DE genes in B6 and 862 DE genes in NZO, whereas in naive CD8+ T cells, 826 and 1718 genes were DE with age in B6 and NZO, respectively. Functional annotation of DE genes using immune modules²² and cell-specific gene sets from human PBMC scRNA-seq data showed that genes associated with inflammation and the myeloid lineage were activated with age in both strains in PBL and spleen (Table S4, Figure S4A), whereas naive T cell-related genes were inactivated with age—in alignment with cell compositional changes. The most significantly upregulated genes in memory CD8+ T cells included age-associated T-cell (Taa) markers⁴, notably *Gzmk* and *Ifng*, in both strains; and memory CD8+ DE genes were enriched in marker genes associated with this cell type (Figure S4B, C). To uncover the most conserved signatures of immune aging, we first compared age-related gene expression changes between the two strains within each tissue, which revealed a positive and significant correlation suggesting that most age-related changes are conserved (correlation coefficient $R = 0.44$ to 0.61 , $p < 2.2 \times 10^{-16}$ for all tissues) (Figure 2.2a).

To uncover genes that are the most significantly and robustly associated with aging, we calculated a novel similarity score based on the magnitude of association of genes (MAG) to a phenotype, which was originally developed to assess the similarity between diseases²³. First, we calculated MAG scores for each gene based on their association with aging across the 4 studied cells/tissues as well as the two strains (Figure 2.2b, Table S5). Interestingly, the top three most consistently and significantly aging-associated genes (all upregulated) were *Fos*, *Fosb*, and *Jun*, which are members of the Activator Protein-1 (AP-1) complex. Gene Set Enrichment analyses of genes sorted with respect to their MAG score confirmed that AP-1 genes were significantly enriched among

the most conserved aging genes (NES = 2.096, $p = 0.005$) and showed that 5 genes contributed the most to this enrichment: Fos, Fosb, Jun, Junb, and Maff (Figure 2.2c). In alignment, DE genes for each cell/tissue type were also enriched for AP-1 genes (Figure S4C). For example, the expression of Fos gene increased significantly across all 4 cell types/tissues in both strains: ~7-fold and ~5-fold increases in NZO and B6 spleen, respectively (Figure 2.2d, Table S3). To study the timing of age-related changes, we have also collected spleen samples from both strains at 12 months. Interestingly, these data showed that these genes were activated at 12 months, suggesting that some of these changes might start in middle-aged animals (corresponding to 40–50 years in humans) (Figure 2.2d). Overall, activation of Jun/Fos family genes was more significant in shorter-living NZO animals in both tissues. We conducted PCA analyses using only these 5 genes (Figure 2.2e) and observed that the biggest variation in these data (captured in PC1, explains 93% of the variation) is associated with aging, since young and old samples clearly separated in PC1 across all 4 cell/tissue types. PC2 was associated with tissue/cellular origin. These results reinforce the strong association of these genes with aging.

AP-1 is a protein complex that regulates transcriptional responses to diverse stimuli including stress and infections^{24–26}. Structurally AP-1 is a heterodimer composed of proteins that belong to different subfamilies including Fos (Fos, FosB, Fra-1, Fra-2), Jun (Jun, JunB, and JunD), and ATF (ATFa, ATF-2, and ATF-3) families^{27,28}. Closer analyses of distinct protein families in the AP-1 complex showed that genes in the JUN/FOS subfamilies were specifically and most significantly activated with age, whereas members of ATF and MAF subfamilies were mostly not associated with age (Figure 2.2f,g, Figure S4D, Tables S3 and S6). Together, these data show that the activation of Jun/Fos members in the AP-1 complex is a highly conserved aging signature detected in both strains (B6, NZO) and tissues (PBL, spleen), across all four cell/tissue types, including T-cell subsets (naive and memory CD8+).

Although age-related changes are significantly conserved between strains (Figure S4E, F), we detected and studied molecules that are differentially expressed with age only in one strain (i.e., passing the statistical significance threshold only in one strain) and we annotated these genes using WikiPathways and GO terms (Tables S7 and S8). Genes that are upregulated specifically in NZO were enriched for oxidative phosphorylation, mitochondrial translation (genes encoding mitochondrial ribosomal proteins), NFkB (Rela, Relb, Nfkb in spleen), and insulin signaling (Igf1r, Irs2 in PBL), as well as inflammation (e.g., Tnf, Nlrp3, and caspases in spleen). These data suggest that NZO animals experience several hallmarks of aging (inflammaging, mitochondrial dysfunction, deregulated nutrient sensing) more significantly than the B6 strain at the same age, which is in alignment with the shorter lifespan of NZO animals and their increased risk for T2D and obesity.

Chromatin accessibility at the Jun/Fos promoters and at their binding sites increases with age

We uncovered differentially accessible (DA) ATAC-seq peaks with age and mapped these to the closest transcription start site (TSS) (Table S9, FDR = 0.05 and $-\log_{10}(\text{FC}) > 1$, Tables S10 and S11 for strain-specific DA peaks). As expected, genes associated with opening peaks overlapped significantly with genes upregulated with age, whereas closing peak genes overlapped significantly with genes downregulated with age (Figure S5A). Functional enrichment of DA peaks was also in alignment with differential expression results, where the most consistent and strong age-associated signal was the epigenetic activation of pro-inflammatory molecules (Figure S5B). We detected significant increases in chromatin accessibility levels around the promoters of Jun/Fos family genes in older samples; this age-related epigenetic activation of Jun/Fos genes was statistically significant and was conserved across strains, tissues, and cell types (Figure 2.3a, Figure S5C for spleen, Figure S6 for PBL and CD8+ subsets).

Results

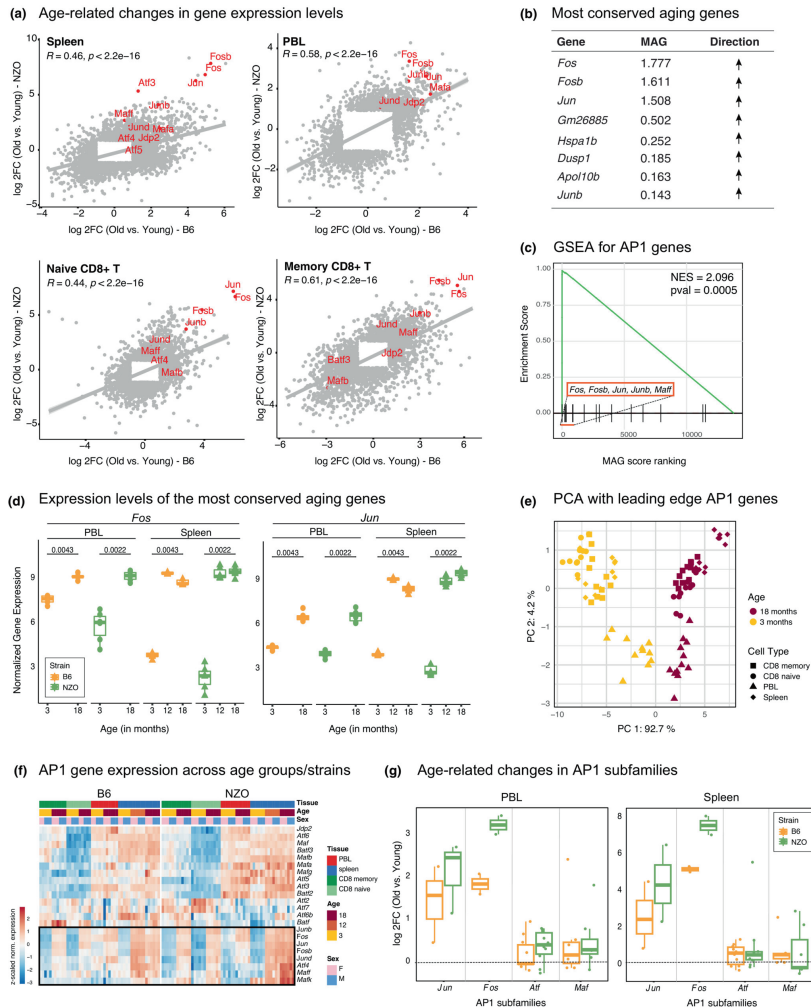


Figure 2.2: (a) Comparison of fold changes for differentially expressed genes (FDR < 0.05) in two strains in each tissue/cell type. Note that transcriptional changes with age are highly conserved across strains. AP-1 complex genes are highlighted in red. (b) Top conserved aging genes (sorted by MAG scores) and the direction of changes with age (upregulated in all cases). (c) Geneset enrichment analysis (GSEA) for MAG-ranked genes using AP-1 complex members as the gene set. Note that top MAG genes are enriched in the AP-1 complex. Leading age genes are denoted. The green line represents the Running Enrichment Score for GSEA, the black lines show where the members of the AP-1 complex appear in the ranked list of genes, and the red box indicates the leading-edge subset. (d) *Fos* and *Jun* expression levels at different ages. Expression values were log(cpm) normalized; p-values are calculated using Wilcoxon rank sum test. (e) PCA plot using the expression of leading-edge genes (*Fos*, *Fosb*, *Jun*, *Junb*, *Maf*), note that these five genes clearly separate old samples (dark red) from young ones (yellow) across tissues and strains. Note that the first PC in these data is associated with age across tissues and strains. (f) Gene expression levels of AP-1 complex genes across all samples. The black rectangle shows the AP-1 member genes that are consistently upregulated with age. Libraries were normalized using cpm function from edgeR package, and the gene expression values were z-transformed. (g) Log2 fold changes (positive values refer to upregulation with age) of AP-1 subunits in PBL (left) and spleen (right). Every dot maps to a gene in the corresponding AP-1 subunit. Note that *Jun*/*Fos* members are the most strongly affected with aging, all of which are significantly upregulated in both B6 and NZO.

Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging

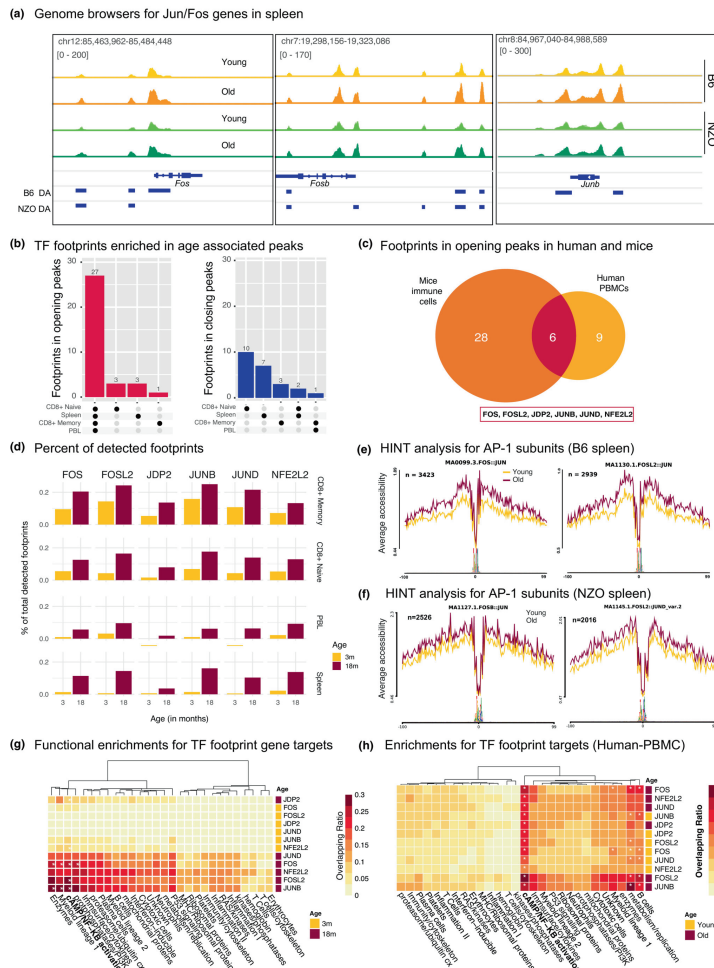


Figure 2.3: (a) Genome browser tracks that show chromatin accessibility data at the *Fos*, *Fosb*, *Junb* genes in spleen cells. Note that in both strains there is increased chromatin accessibility in older spleen cells at these loci. Bars underneath represent differentially accessible (DA) peaks in B6 and NZO strains loci. All genome browser tracks are scaled to the same value which is depicted on the left top corner. (b) Upset plots that summarizes overlaps among enriched transcription factor (TF) footprints across tissue/cell types (adjusted $q < 0.05$ using BiFET). Left panel: 27 out of 34 TF footprints enriched within opening peaks are shared across tissue/cell types. Right panel: TF footprints enriched within closing peaks are mostly cell type specific. (c) Overlap of footprints enriched in opening peaks in human and mice. 6 proteins were common including five AP-1 members (*FOS*, *FOSL2*, *JDP2*, *JUNB*, *JUND*), and a co-factor of the complex *NFE2L2*. (d) Percent of footprints detected for the shared 6 TFs in each age group and cell type. Counts are normalized with respect to the total number of footprints detected. Note that there are more binding events for these TFs with age. (e, f) Chromatin accessibility levels at the *JUN*/*FOS* footprints for (e) B6 spleen and (f) NZO spleen cells. Note that in older spleen cells, these sites are more accessible. The number of footprints used in each analysis is listed at the top left corner. Functional enrichment of gene targets for conserved age-associated TFs using immune modules in B6 PBL (g) and human PBMCs (h). Values show the percent of overlap between gene targets and module genes; significant enrichments are indicated with an asterisk ($FDR < 0.05$). Note the increase in NFkB pathway in older samples.

Results

To uncover whether binding events of Jun/Fos TFs are also affected, we conducted footprinting analyses in ATAC-seq data, which in silico infers TF binding events by integrating chromatin accessibility patterns with the underlying TF sequence motifs²⁹. First, we called TF footprints³⁰ in mouse samples from pooled young and old animals in four different tissue/cell types using known TF motifs³¹, as well as in human PBMCs from young and old subjects^{7,9}. Samples were pooled to increase the depth of sequencing, increasing the number and quality of detected footprints³². Next, we identified TF footprints enriched in peaks that were activated (i.e., opening) or inactivated (i.e., closing) with age in each tissue/cell type³². Interestingly, TF footprints enriched in opening peaks were largely shared across the 4 cell types in mice (27 out of 34 were detected in all studied cells/tissues), in contrast TF footprints enriched in closing peaks were cell/tissue type-specific (Figure 2.3b). Among the footprints that were enriched in opening peaks with age, six were also enriched in opening peaks from human PBMCs (Figure 2.3c, Table S12). Remarkably, this included five AP-1 complex members (FOS, FOSL2, JDP2, JUNB, JUND) as well as NFE2L2 (i.e., NRF2), a protein that interacts with -JUN and contributes to the regulation of the NLRP3 inflammasome³³. The baseline activated JUN/FOS status in older mice can be an important modulating element of the biological responses of the aging immune cells. Interestingly, the chromatin accessibility levels around the promoters of these TFs increased in men with aging—inferred from PBMC RNA-seq data from our previous study⁷; notably older men experience accelerated myeloid activation and “inflammaging” compared to older women (Figure S7A). In alignment with the footprinting results, motif enrichment analyses confirmed that opening peaks with age were enriched in motifs of these TFs, particularly JUN/FOS families both in human and mouse immune cells (Figure S7B, Table S13).

In addition to the enrichment of these six TFs among opening peaks, footprints for these TFs made up a larger proportion of all detected footprints in older samples across the four tissues (Figure 2.3d, Table S14). We also compared the cleavage profiles from the ATAC-seq libraries in young and old mice, to get insights into chromatin accessibility profiles at the binding sites of JUN/FOS TFs^{29,34}. Chromatin around their binding sites was more accessible in cells from old mice compared to cells from young mice, which was observed in tissues (PBL, spleen) (Figure 2.3e,f) and in CD8+ subsets in both strains (Figure S7C, D). To understand which cellular functions are modulated by the increased “binding” of these TFs, we identified their gene targets based on the distance to TSS. These gene targets included members of the Nf-KB pathway (Rel, Rela, Nfkbiz), pro-inflammatory cytokines and chemokines (Il1b, Il6, Il15, Cxcl10), genes expressed by activated myeloid (Cd86, Cd44, Il7r, S100a11) and lymphoid cells (Cd44, Cd28)³⁵, cytotoxic molecules (Gzmk, Gzmb, Klrg1), and plasma cell marker Cd38 (Table S15). Our results suggest that these TFs modulate important immune responses in both innate and adaptive immune cells. These gene targets were functionally enriched in pro-inflammatory immune modules and pathways (e.g., Nf-KB activation) across mouse tissues and strains (Figure 2.3g, Figure S7E). Similarly, in human PBMCs, TF gene targets included pro-inflammatory (FOSL2, LMNA, CASP8, NFKBIZ), cytotoxic (GNLY, PRF1, GZMB), and activated cell markers (CD44, IL7R)³⁵, and were enriched in pro-inflammatory pathways/modules (Figure 2.3h, Table S16). These results support the previously reported cross-regulation of AP-1 complex and NF-kB pathways in myeloid cells^{36,37} and provide further insight into other pathways and functions potentially regulated by these TFs in both myeloid and lymphoid cells. Together, our findings nominate increased JUN/FOS TF activity as a conserved biomarker of immune system aging involved in regulating pro-inflammatory and effector molecule functions, thereby potentially contributing to inflammaging.

Expression of Jun/Fos increases with age across all immune cell types from the spleen

To uncover whether age-related transcriptional activation of Jun/Fos genes stems from specific cell types, we reanalyzed single-cell RNA-seq data from the Tabula Muris Senis consortium³⁸ us-

Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging

ing spleen cells from young (3 months) and old (18 months) B6 mice. We annotated spleen cells as B, T, NK cells, or macrophages using known marker genes (Figure 2.4a). In alignment with our flow cytometry data (Figure 2.1b), the majority of spleen cells were composed of B cells, followed by T cells and innate immune cells (Figure S8A, B). Next, we studied the activation of the most conserved aging genes (Jun/Fos/Fosb) (Figure 2.2b) in these single cells. The expression of these molecules increased with age across all immune cell types (Figure 2.4b,c, Figure S8C). Their activation with age was observed at both the level of expression and the frequency of expression (Figure 2.4c, Figure S8C). For example, older B, T, and macrophage cells expressed Fos and Jun at significantly higher levels than their younger counterparts. In addition, the percentage of cells that expressed these molecules also increased. For example, ~78% of older macrophages expressed Fos, compared to 43% of young macrophages (Figure S8C). We also conducted differential expression analyses at the single-cell level between young and old mice for T, B, macrophage, and NK cells using MAST. These results also confirmed that these genes are significantly upregulated with age across the four cell types studied here (Figure 2.4d, Table S17). Together, these data showed that age-related activation of Jun/Fos family genes are a conserved signature of cellular aging across different immune cell types, where macrophages express these molecules most significantly, potentially to regulate increased inflammatory responses with age.

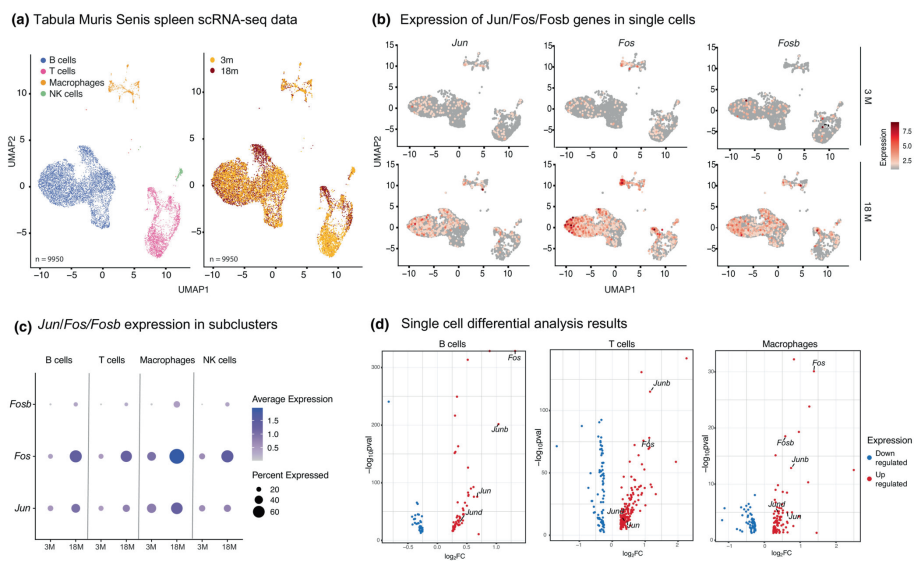


Figure 2.4: (a) Left: UMAP for the Tabula Muris Senis spleen data using young (3-month, 3 m) and old (18-month, 18 m) B6 mice spleen ($n = 2$, per age group). Cell types that have <100 cells were removed from the downstream analyses, resulting in four major cell types; B cells, macrophages, natural killer (NK) cells, and T cells ($n = 9950$ cells). Right: Cells are annotated with respect to the age of the animals. (b) The expression levels of Jun, Fos, Fosb genes in log-normalized scale in young (3 m, top) and old cells (18 m, bottom). Note the increase in expression of these molecules across all cell types. (c) Dotplots summarizing the expression of Jun, Fos, and Fosb genes in immune cell subsets. The size of the dot represents the proportion of cells expressing that specific gene, and the color of the dot represents the level of expression. Note the increase in expression/abundance of these molecules with age across all subsets. (d) Volcano plots that summarize differential expression result from scRNA-seq data. Red dots represent significantly upregulated genes; blue dots represent significantly downregulated genes ($p = 0.05$). Note that Jun and Fos family members are significantly upregulated across cell types.

JUN protein production and binding increase with age upon stimulation via Toll-like receptors

An increase in the transcription of genes that form complexes may not necessarily indicate in-

Results

creased function. This is particularly true for the AP-1 protein complex, being a heterodimer composed of FOS, JUN, and ATF protein families and whose activity depends on the formation of the complex and post-translational modifications such as phosphorylation³⁹. To understand whether age-related transcriptional/epigenetic activation of Jun/Fos affects protein levels and AP-1 function, we studied JUN protein expression via Western blotting and protein binding via a JUN transcription factor functional assay ELISA kit (Table S18). We used splenocytes and not sorted immune cell subsets for these assays since a robust immune response requires crosstalk between different immune cells^{40,41}. First, we quantified JUN binding activity in nuclear extracts from the B6 spleen cells upon stimulation using (1) anti-CD3/anti-CD28 to stimulate T cells; (2) LPS to stimulate B cells and monocytes via TLR4; and (3) poly(I:C) to stimulate monocytes via TLR3. The JUN binding activity level did not significantly change between age groups upon T-cell stimulation; however, it significantly increased with age upon TLR-mediated stimulation (Figure 2.5a), particularly upon poly(I:C) stimulation, which activates monocytes ($p = 0.0063$). TLR3 activation by poly(I:C) has been shown to regulate inflammatory responses in tissues⁴². To complement the binding assay, we also quantified the level of JUN protein in nuclear and cytosolic fractions of the spleen before and after stimulation by Western blotting for JUN and loading markers LamininB1 for nuclear extracts and GAPDH for cytosolic extracts. In the nuclear extract, even before the activation of cells, splenocytes from older animals had more JUN protein compared to those from younger animals (Figure 2.5b). Upon poly(I:C) stimulation, nuclear and cytosolic JUN protein levels increased in both young and old splenocytes as expected; however, the increases were more significant in older cells (Figure 2.5b), in alignment with their transcriptional and epigenetic activation (Figures 2 and 3). Together, our data demonstrate that when activated via TLR3 spleen cells from older mice have increased JUN protein and JUN binding compared to cells from younger mice.

In pooled splenocytes, poly(I:C) stimulation led to the greatest increase in JUN binding, which stimulates monocytes via TLR3. Monocytes govern the innate immune responses by initiating inflammation, through the production of pro-inflammatory cytokines including IL6. AP-1 complex components are important in regulating inflammation⁴³; however, their role in inflammaging is unknown. To further explore this connection, we focused on the IL6 cytokine—a canonical marker of inflammaging⁴⁴. Footprinting analyses established that footprints for JUN/FOS TFs bound around the IL6 promoter, and there were more footprints for these TFs around the IL6 locus with age (Figure 2.5c). To further characterize the downstream effects of increased JUN activity with age, we studied whether there is increased IL6 protein production with age in TLR3-activated splenocytes. For this, splenocytes were stimulated *in vitro* with poly(I:C) for 1 day and the supernatant was assayed for IL6. As expected, poly(I:C) stimulation led to increased IL6 levels both in young and in old splenocytes; however, this inflammatory response was significantly higher in older cells with age ($p = 0.00654$) (Figure 2.5d). Interestingly, spleen cells from older females produced significantly more IL6 than spleen cells from older males ($p = 0.01$, Table S19). scRNA-seq data from spleen³⁸ showed that the expression levels of Toll-like receptor genes did not change significantly with age (Figure S8D), suggesting that the increased inflammatory responses upon stimulation are modulated by downstream regulators of TLR signaling, not via the changes in the receptor expression levels. These data confirm that aged spleen cells are more pro-inflammatory in nature and that increased production and binding of JUN/FOS TFs with age is a potential regulator of these increased inflammatory responses.

Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging

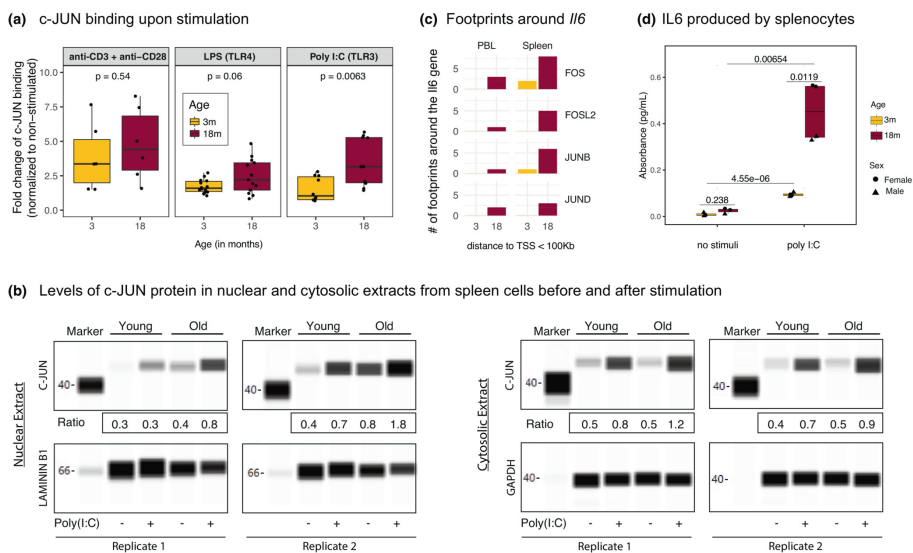


Figure 2.5: (a) JUN binding levels upon different stimuli (anti-CD3+ anti-CD28, LPS, and poly(I:C)) for young and old B6 mice spleen. JUN binding levels were normalized to the non-stimulated samples. Note the more pronounced increase in JUN binding in old spleen cells. p-values are calculated using the Wilcoxon rank sum test. (b) Western blot of c-JUN proteins from the nuclear and cytosolic extracts of male B6 mice with and without poly(I:C) stimulation. Note that c-JUN levels increase with age in nuclear extracts with age both at baseline and upon poly(I:C) stimulation. The ratio of JUN protein to the housekeeping protein (LAMININ B1 for nuclear extract and GAPDH for cytosol) was quantified and included under the gels (labelled Ratio). (c) Number of footprints detected around the *IL6* gene (100 kb upstream and 100 kb downstream of the TSS from each side) in B6 PBL and spleen for JUN/FOS TFs. Note the increased binding events around this molecule with age. (d) IL6 cytokine levels secreted by splenocytes of young (3 months) and old (18 months) B6 mice spleen. Note the increased production of IL6 with age upon poly(I:C) stimulation. p-values are calculated using unpaired t-test.

2.4 Discussion

To uncover conserved biomarkers and regulators of immune aging, we comprehensively profiled diverse immune cells and tissues in long-living B6 and short-living NZO mouse strains. NZO mice develop T2D and obesity and can thus be considered a model for “unhealthy aging.” Despite differences in their health and life spans, our results show significant agreement in age-related changes between the two strains both in flow cytometry (cell compositional) and genomic (cell-intrinsic) data. Using a novel scoring metric, we uncovered that transcriptional activation of JUN/FOS genes of the Activating Protein-1 (AP-1) complex—particularly the upregulation of *Jun*, *Fos*, and *Fosb* genes—is the most conserved aging signature across the two mouse strains, diverse immune tissues, and cell types. AP-1 is a transcription factor (TF) complex that regulates gene expression programs in response to diverse stimuli, including stress, viral infections²⁷, and pro-inflammatory signals in concert with the NF-KB pathway^{36,37,45}. ATAC-seq data from the same tissues revealed that there is also epigenetic activation of *Jun/Fos* genes with age. Chromatin accessibility at their promoters increases with age in addition to the chromatin accessibility increases at their binding sites. Western blot data showed that there is more nuclear JUN protein in older splenocytes compared to younger ones even at baseline. Furthermore, when myeloid cells within splenocytes are stimulated via TLR3 (poly(I:C)), older cells produce more JUN and there is more JUN binding compared to younger cells. These data suggest that transcriptional activation of *Jun/Fos* genes, whose protein products form the AP-1 complex, is a conserved signature of immune aging that increases the level of JUN protein production and binding with age.

Increased inflammation with age (i.e., inflammaging) is one of the hallmarks of aging observed in multiple mouse tissues³ and also in human cells^{9,46}, including the increased levels of IL6 in human serum⁴⁷. Several lines of evidence indicate that the activation of *Jun/Fos* genes with age manifests most prominently in the myeloid compartment, specifically monocytes that govern the inflammatory responses. First, poly(I:C) primarily activates monocytes via TLR3. TLR3 is a highly conserved molecule that recognizes double-stranded (ds) RNA associated with a viral infection and induces the activity of the interferon response and pro-inflammatory molecules in myeloid cells⁴⁸. Second, single-cell RNA-seq data from Tabula Muris Senis³⁸ showed that, although the gene expression levels of *Jun/Fos* members increase significantly across all immune cell types with age, older macrophages express these molecules at higher percentages compared to other immune cell types. Footprinting analyses nevertheless suggest that these TFs can target and potentially activate important molecules across distinct immune cell types: pro-inflammatory, cytotoxic, and effector molecules in both myeloid and lymphoid cells. Among the pro-inflammatory molecules, we further studied IL6 as a biomarker of inflammaging. Footprinting analyses demonstrated that JUN/FOS binding events (i.e., footprints) in the vicinity of *Il6* locus increase with age. Furthermore, older spleen cells produce more IL6 upon TLR3-stimulation compared to younger ones. Together with the increased JUN protein production and binding in these cells, these results suggest that age-related activation of JUN/FOS TFs with age is a potential upstream regulator of inflammaging.

Blood samples are the most frequently used tissue in human aging studies; we compared age-related changes detected in mice blood samples with the changes detected from human PBMCs. At the cell compositional level, human PBMCs were composed of more T cells and less B and innate cells compared to mice PBLs. In both humans and mice, there is a significant remodeling with age within T cells including decreases in naive and increases in memory subsets. However, in human PBMCs this decline is more striking for naive CD8+ T cells²¹ whereas in mice both CD4+ and CD8+ T-cell compartments were similarly affected with age. A prominent age-related change we previously detected in human PBMCs was the chromatin closing and reduced

expression of IL7R and its downstream molecules in the IL7 signaling pathway that stem from the decline of IL7R+ CD8+ cells⁷. In mouse tissues and strains studied here, we detected neither the downregulation of the IL7r gene nor a decline in the percentages of IL7R+ T cells. This discordance between human and mouse CD8+ T-cell aging patterns might be attributable to differences in their antigenic challenges—unlike humans, laboratory mice live in a highly controlled environment (strict diet, unchallenged immune system). In terms of the activation of AP-1 complex members, Jun/Fos genes were not significantly upregulated with age in bulk PBMCs; however, we detected more TF footprints for these molecules with age. Furthermore, similar to mice, these TF footprints were in the vicinity of pro-inflammatory and effector molecules in human PBMCs, suggesting that increased regulatory activity of these TFs might be a conserved signature of aging. Recent single-cell studies from human and mouse (B6) immune cells have uncovered conserved expansion of GZMK+ CD8+ T-cell populations with age⁴. In the present study, we detected the upregulation of marker genes for this T-cell population in both strains with age, suggesting that this aging signature is conserved across different strains of mice regardless of their life span.

B6 and NZO strains exhibit differences in terms of the life span and health span of female and male animals, where NZO males live shorter and develop T2D (Figure 2.1a). However, we did not observe significant sex differences in the aging signatures described here, likely because the cohort was not powered to detect sex differences as this was not our main objective. Bigger cohorts will be needed to delve into differences between female and male immune system aging in different mouse strains and their potential implications for human immune aging and vaccine responses. However, we did observe an interesting sex dimorphism in the production of pro-inflammatory IL6 upon stimulation, where female splenocytes from old B6 mice produce significantly more IL6 compared to splenocytes from old males (Figure 2.5d). In alignment with these results, previous studies suggested that testosterone has a suppressing effect on Jun/Fos genes and this plays a role in sex differences observed in human vaccine responses^{49,50}. Another human study showed that chromatin accessibility around AP-1 members decreases upon influenza vaccination in blood-derived immune cells—both with trivalent influenza vaccine (TIV) and AS03-adjuvanted H5N1 vaccine in young adults⁵¹. Interestingly, this epigenetic remodeling around the AP-1 complex members boosted responsiveness to vaccines for other viruses—Zika and Dengue⁵¹. Here, we show chronic activation of AP-1 complex members with age. In human PBMCs, AP-1 member gene expression does not significantly increase with age, though increases were more pronounced in men. However, we detected more binding events for JUN/FOS TFs in PBMCs from older adults compared to PBMCs from younger adults, suggesting that the binding activity of these molecules might also be affected with age in human immune cells. Future studies in older adults will be important to uncover the extent of JUN/FOS remodeling with age and the contribution of AP-1 members to reduced vaccine responsiveness in older adults.

Interestingly for spleen samples, cell compositional changes at the major population level were more modest in shorter-living NZO compared to longer-living B6 (total B, total T). However, within subsets of T cells, there was significant remodeling with age and naive CD4+ decline with age was the most significant for NZO spleen samples. On the contrary, Jun and Fos genes are significantly upregulated in the NZO spleen samples; hence, these gene expression patterns are more likely to be driven from cell-intrinsic changes rather than cell compositional changes. This is also in alignment with the fact that we detected the upregulation of these genes in sorted naive and memory CD8+ T cells as well as in single-cell RNA-seq data from the spleen. Together, these data suggest that the activation of Jun/Fos genes is a cell-intrinsic aging signature and does not always align with cell compositional changes. Although in this study, we focused on most conserved signatures of immune aging, we uncovered strain-specific aging patterns that could serve as a resource for future studies. In sum, NZO mice experience several hallmarks of aging (inflam-

Methods

maging, mitochondrial dysfunction, deregulated nutrient sensing) more significantly than the B6 strain at the same age, which is in alignment with the shorter lifespan of NZO animals and their increased risk for T2D and obesity. Several genetic variants have been linked to these syndromes in the NZO mice (*Pcp1*⁵², *Lepr*⁵³, *Ifi202b*⁵⁴, *Abcg1*⁵⁵), which might also contribute to the accelerated aging phenotype observed in the NZO mice compared to B6.

2.5 Methods

Animals and housing

C57BL/6J (stock 000664) and NZO/HILtJ (stock 002105) animals were obtained from The Jackson Laboratory and kept in individually ventilated cages with free access to food (5KOG, LabDiet) and water. The pathogen-free room (health status report attached) was kept between 20°C and 22°C, with a 12-h light:dark cycle. Spleen and blood samples were obtained immediately after euthanasia through cervical dislocation. The mouse study was approved by The Jackson Laboratory's Institutional Animal Care and Use Committee.

Flow cytometry data generation and analyses

Data are obtained from spleen and peripheral blood lymphocytes (PBLs) of C57BL/6J and NZO/HILtJ mouse strains, hereafter B6 and NZO, respectively, at ages 3, 12, and 18 months. Cells were stained with (i) CD8 FITC, Clone 53-6.7 BD Bioscience Cat 553031, used at 1:240 final concentration; (ii) CD3e PE, Clone 145-2C11 eBioscience (Now ThermoFisher) cat 12-0031-85, used at 1:240 final concentration; (iii) CD62L PE-Cy7, Clone MEL-14 Tonbo Biosciences Cat 60-0621-U100, used at 1:480 final concentration; (iv) CD44 APC-Cy7, Clone IM7 Tonbo Biosciences Cat 25-0441-U100, used at 1:240 final concentration and for 30 min at 4°C. Propidium Iodide used for cell viability at 0.5 µg/ml.

Then, cells were sorted on a FACSaria II (BD Biosciences). Briefly, doublets were gated out, Viable PI- cells were gated, CD3e+, CD8+ cells were gated and subdivided into CD44Low, CD62LHigh Naive cells and CD44High, CD62L+/- Memory cells. Up to 50,000 cells were sorted for ATAC-seq, remaining cells collected for other RNA preparations. Cells were collected in tubes coated with fetal bovine serum (FBS). The percentages of B, CD4, CD8, Naive CD4, Central memory CD4, Effector memory CD4, Effector memory RA CD4, Naive CD8, Central memory CD8, Effector memory CD8, Effector memory RA CD8 were measured. In addition, within each of these cell types, the percentages of IL7R+ and PD1+ cells were measured. After summing up B, CD4+, and CD8+ T cells, we labeled the rest of the percentages as monocytes since NK cells, neutrophils, and other cell types compose only ~5% of spleen and PBL. Naive and memory CD8 T cells were sorted from spleens as follows: Spleens were removed from mice and teased apart in nylon mesh bags in PBS with 2% FBS, 5 mM EDTA, and 0.02% Sodium Azide (FACS Buffer). The cells were lysed with "Gey's Buffer" (a modification of GBSS as described in the attached paper where the sodium chloride was exchanged with ammonium chloride at the same molar concentration) for 5 minutes and then washed with FACSBuffer and counted to determine concentration. Cells were stained at approximately 108/mL with CD8 FITC, CD3e PE, CD62L PE-Cy7, and CD44 APC for 30 min at 4 degrees. Cells were washed and resuspended for sorting in FACS Buffer. I sorted Naive (CD62L+, CD44Low) and Memory (CD62L+/-, CD44High) CD8+, CD3e+ cells. To quantify cell compositional changes with age, we built linear models where age is the independent variable and cell type percentage is the dependent variable. For each model, we computed the slope of change per increase in age by 1 unit and a corresponding p-value, which is later corrected using

Benjamini-Hochberg procedure.

RNA isolation and QC

Total RNA was isolated from 1 million cells using the RNeasy Mini kit (Qiagen), according to the manufacturers' protocols, including the optional DNase digest step. For samples with fewer than 1 million cells, RNA was isolated using the RNeasy Micro kit (Qiagen). Sample concentration and quality were assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific) and the RNA 6000 Nano and Pico LabChip assays (Agilent Technologies).

RNA library generation

30 ng of total RNA, with the addition of 6 μ l ERCC Spike-In Control Mix 1 (Ambion Thermo Fisher) diluted 1:10,000, was used for library construction. Libraries were prepared by the Genome Technologies core facility at The Jackson Laboratory using the KAPA mRNA HyperPrep Kit (KAPA Biosystems), according to the manufacturer's instructions. Briefly, the protocol entails isolation of polyA containing mRNA using oligo-dT magnetic beads, RNA fragmentation, first and second strand cDNA synthesis, ligation of Illumina-specific adapters containing a unique barcode sequence for each library, and PCR amplification. Libraries were checked for quality and concentration using the D5000 assay on the TapeStation (Agilent Technologies) and quantitative PCR (KAPA Biosystems), according to the manufacturer's instructions.

RNA sequencing

Libraries were pooled and sequenced 75 bp single-end on the HiSeq 4000 (Illumina) using HiSeq 3000/4000 SBS Kit reagents (Illumina), targeting 40 million reads per sample. We obtained RNA-seq data from spleen, PBL and sorted T cells (derived from spleen) of B6 and NZO mouse strains at age 3, 12, and 18 months. Single-end RNA-seq reads were aligned to the mouse genome (mm10) with Bowtie 2⁵⁶ and counts were generated with RSEM⁵⁷. To normalize the raw counts count-per-million (cpm) function from edgeR package is used and the genes that are $\log(\text{cpm}) < 1$ and expressed less than 2 samples were excluded from rest of the analyses. For differential analysis pipeline, however, raw counts are used with the default options of edgeR package⁵⁸, and via TMM normalization.

ATAC-seq library generation

ATAC-seq libraries were prepared using 50,000 cells, as previously described⁵⁹, with the following modifications: Digitonin was added to the transposition reaction at a final concentration of 0.01%; the transposition reaction was purified using the Genomic DNA Clean & Concentrator-10 kit (Zymo Research Corporation); PCR amplification was carried out using the Nextera DNA Library Prep (Illumina) Index Adapters, Nextera PCR Master Mix, and PCR Primer Cocktail for 10 cycles of PCR; PCR reaction was purified using 1.7 $\times$ SPRI beads (Agencourt AMPure XP, Beckman Coulter). Libraries were checked for quality and concentration using the DNA High-Sensitivity LabChip assay (Agilent Technologies) and quantitative PCR (KAPA Biosystems), according to the manufacturer's instructions. Libraries were pooled and sequenced 75 bp paired-end on the NextSeq 500 (Illumina) using NextSeq High Output Kit v2 reagents (Illumina).

ATAC-seq data analyses

We obtained ATAC-seq data from spleen, PBL and sorted T cells from spleen of B6 and NZO mouse strains at age 3, 12, and 18 months. Paired-end ATAC-seq reads were quality trimmed using Trimmomatic⁶⁰ and trimmed reads were aligned to mouse genome (mm10) using BWA⁶¹. After preprocessing and quality filtering, peaks were called on alignments with MACS2 using the BAMPE option⁶². The consensus peakset for PCA and PVCA plots were generated gathering

Methods

all peaks from all tissue/cell types, whereas for differential accessibility analyses the samples of the same tissue were merged to generate one consensus peak set by using R package DiffBind⁶³. Peaks only present in at least two samples were included in the analysis. Raw read counts were normalized using the cpm function via the log option turned on from edgeR package⁵⁸.

Statistical methods

Principal variance component analysis (PVCA) was used in order to determine the sources of variability in flow cytometry data¹⁶, which combines the strengths of principal component analysis (PCA) and variance component analyses (VCA). So, using PVCA the proportions variances were attributed to each factor. To compare the normalized gene expressions and peak counts across different tissues and cell types, Wilcoxon rank sum test was used.

Differential analyses

To identify differentially expressed genes and differentially accessible peaks between age groups, we used the R package edgeR⁵⁸ was used. It fits a generalized linear model (GLM) that includes age as a continuous independent variable and read counts from either ATAC-seq or RNA-seq as dependent variables to test for the effect of age on read counts. We stratified data by tissue and strain and fit GLM within strata. In addition to the age, we included sex as a covariate, which did not yield any statistically significant results. p-Values for the age effect were adjusted using the Benjamini–Hochberg procedure, and genes or peaks with FDR-adjusted p-Value < 0.05 were considered differential.

To uncover genes that are the most significantly and robustly associated with aging, we calculated a similarity score based on the magnitude of association of genes (MAG²³) to a phenotype. The MAG score was calculated by computing the geometric mean of the inverse of ranks of a gene for the two strains. The genes were then ranked based on the summation of the MAG score for each gene across the four studied cells/tissues. This ranked gene list was provided as input to the gene set enrichment analysis program using AP-1 genes and age-associated T-cell markers as gene sets.

Enrichment analyses

Immune modules were obtained from human PBMCs²². Human and mouse orthologs were identified using R package biomaRt⁶⁴. Consensus peaks were annotated using HOMER⁶⁵ and gene-based analyses were restricted to promoter peaks annotated to the nearest transcription start sites (TSS) of expressed genes. Hypergeometric p-value is calculated for each module of the inflammation genesets. Then, p-values are adjusted for multiple hypothesis testing using Benjamini–Hochberg correction. For all analyses, modules that have FDR-adjusted p-value < 0.05 considered as enriched.

Single-cell data analyses

We have downloaded 10× single-cell RNAseq spleen data of Tabula Muris Senis³⁸ from USCS browser (<https://cells.ucsc.edu/?ds=tabula-muris-senis+droplet+spleen>) and transferred it into R environment (v4.0.5). Next, we selected 3- and 18-months samples (2 samples per age point) and ran the standard Seurat (v4.0.2) pipeline with the default parameters (log normalization, 10 PCs, and UMAP for dimensionality reduction). Then, we removed the cell types which had less than 100 cells in total along with the doublet cluster, remaining cells from four major cell types (NK, Macrophage, B, and T cells) were used in all single-cell related analyses. We detected the cluster cell types based on their respective marker genes here: B cell (*Blnk*, *Cd79a*, *Cd79b*), T cell (*Cd3d*, *Cd3e*, *Cd3g*), NK (*Ncr1*, *Gzma*), and Macrophage (*Itgax*). We used MAST to compare young (3 mo) and old (18 mo) mice spleen samples at single-cell level.

Footprinting analyses

ATAC-seq data from spleen and PBL were scanned for TF footprints using the PIQ algorithm³⁰. This method integrates genome-wide TF motifs (i.e., position weight matrices) with chromatin accessibility profiles to generate a list of potential TF binding sites that are bound by a TF. The method also produces a quality score for each footprint (positive predictive value). Only the TF footprints with positive predictive values >0.9 are used in downstream enrichment analyses.

Before footprint calling, we merged samples of the same sex, strain, tissue and age group to increase read depth, which improved the quality and detection power of PIQ. In addition to that we used SAMtools⁶⁶ to randomly downsample aligned reads from each merged data set to 50 M reads to minimize the impact of the high correlation between library depth and footprint positive predictive values. We used a set of motifs available in the JASPAR 2016 database ($n = 454$ for human and $n = 189$ for mouse)⁶⁷. Finally, footprint calls were further filtered to include in analyses only those associated with TFs that are expressed in the PBL or spleen. For each cell type, we applied BiFET³² to identify TFs whose footprints are significantly more detected in opening/closing peaks compared to background peaks (peaks whose chromatin accessibility do not change with age). In each tissue, we selected TFs whose BiFET q -values are <0.05 for at least two samples. We followed the same protocols for our previously published human chromatin accessibility data⁹ and merged PIQ calls of young (<40 years) and older individuals (>65 years). For AP-1 complex related TF analyses, we selected each subunit from JASPAR annotated files. Then, the locations of these TFs were annotated to their closest TSS using ChIPseeker package⁶⁸ (v1.27.3) to uncover the most effected sites. Finally, we calculated enrichment scores of these sites using hypergeometric p -value test and our immune signature gene sets. We used HINT-ATAC⁶⁹ which is part of the Regulatory Genomics Toolbox (RGT). The consensus peak files for young (3 months) and old mice (18 months) along with respective merged bam files were provided as input to the HINT program. To find TFs associated with a particular cellular condition, we checked for motifs overlapping with predicted footprints to find motif predicted binding sites (MPBS) with JASPAR motifs. Finally, HINT was used to generate average ATAC-seq profiles around binding sites of a TF.

Cell stimulation and immunoblotting experiments

Mice spleens were removed aseptically and transferred to a Petri dish where they were minced and filtered through a 40- μ m nylon cell strainer containing DMEM medium. The cellular suspension was centrifuged to yield a pellet and then depleted of erythrocytes by resuspending the cells in pre-chilled red blood cell lysis solution (Sigma). The spleen cells were washed twice in DMEM medium containing 25 mM HEPES, 1 mM l-glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated FCS. Viability of the spleen cells was +90%. Five million spleen cells per condition were suspended at 1×10^6 /mL in 10-mL Petri dishes pre-coated with 100 μ g anti-CD3/CD28 or in culture medium supplemented or not with either LPS (1 μ g/mL) or Poly(I:C) (both high and low molecular weight, 10 μ g/mL each) (Invivogen). After 2 h, cells were lysed, and the nuclear extracts were isolated using Nuclear Extraction Kit (Abcam) according to the manufacturer's instructions. Protein content of the nuclear extracts was quantified by PierceTM Coomassie (Bradford) Protein Assay Kit (Thermo-Fisher) and the activation of the JUN transcription factor was assayed by c-Jun Transcription Factor Assay Kit (Abcam) according to the manufacturer's instruction. Secretion of IL-6 was quantified from the supernatant of 10 million spleen cells stimulated as above, after 24 h using Mouse IL-6 ELISA kit (Abcam).

The capillary immunoblotting analysis was performed, using Wes (ProteinSimple, Santa Clara, CA, USA), according to the ProteinSimple user manual. The lysates of the primary male mice splenocytes were mixed with a master mix (ProteinSimple) to a final concentration of 1 $\times$ sample

Acknowledgments

buffer, 1 × fluorescent molecular weight marker, and 40 mM dithiothreitol and then heated at 95°C for 5 min. The samples, blocking reagents, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate (ProteinSimple), and separation and stacking matrices were also dispensed to the designated wells in a 25-well plate. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated. A capillary immunoblotting analysis was carried out at room temperature, and the instrument's default settings were used. Capillaries were first filled with a separation matrix followed by a stacking matrix, with about 40 nL of the sample used for loading. During electrophoresis, the proteins were separated by molecular weight through the stacking and separation matrices at 250 volts for 40–50 min and then immobilized on the capillary wall, using proprietary photo-activated capture chemistry. The matrices were then washed out. The capillaries were next incubated with a blocking reagent for 15 min, and the target proteins were immunoprobed with primary antibodies followed by HRP-conjugated secondary antibodies. The antibodies of GAPDH (sc-25778, 1:200, Santa Cruz Biotechnology), Lamin B1 (12586S, 1:100; Cell Signaling Technology), and c-Jun (9165S, 1:50; Cell Signaling Technology) were diluted in an antibody diluent (ProteinSimple).

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Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging

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2.8 Appendix

The appendix was shortened due to environmental considerations. To minimize unnecessary printing and reduce material use, extensive data including 8 supplementary figures and 19 supplementary tables are provided online at: <https://doi.org/10.1111/ace1.13792>.



Chapter 3

Utilizing Epigenetic Regulators to Improve HSC-based Lentiviral Gene Therapy

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3.1 Abstract

The curative benefits of autologous and allogeneic transplantation of hematopoietic stem cells (HSCs) have been proven in various diseases. However, the low number of true HSCs that can be collected from patients and the subsequent in vitro maintenance and expansion of true HSCs for genetic correction remain challenging. Addressing this issue, we here focused on optimizing culture conditions to improve ex vivo expansion of true HSCs for gene therapy purposes. In particular, we explored the use of epigenetic regulators to enhance the effectiveness of HSC-based lentiviral (LV) gene therapy. The histone deacetylase inhibitor quisinostat and bromodomain inhibitor CPI203 each promoted ex vivo expansion of functional HSCs, as validated by xenotransplantation assays and single-cell RNA sequencing analysis. We confirmed the stealth effect of LV transduction on the loss of HSC numbers in commonly used culture protocols, whereas the addition of quisinostat or CPI203 improved the expansion of HSCs in transduction protocols. Notably, we demonstrated that the addition of quisinostat improved the LV transduction efficiency of HSCs and early progenitors. Our suggested culture conditions highlight the potential therapeutic effects of epigenetic regulators in HSC biology and their clinical applications to advance HSC-based gene correction.

3.2 Introduction

Hematopoietic stem cells (HSCs) have been used successfully in allogeneic transplantation settings to treat hematological and immunological disorders, as they maintain homeostasis through their unique capacity of self-renewal and multipotency¹⁻³. Over the past two decades autologous HSC-based gene therapy has emerged as a compelling alternative to allogeneic transplantation as it is not dependent on finding a matched donor and avoids any risk of graft-versus-host disease. This has proven successful for several inherited diseases such as immune deficiencies, hematological disorders, but also metabolic diseases through *ex-vivo* manipulation of the patient's own HSPCs⁴⁻⁷. Lentiviral vectors (LVs) have emerged as vectors of choice in clinical settings for safer and more efficient transgene delivery to HSCs compared to γ -retroviral vectors that were used in initial gene therapy trials. Recently, gene editing has emerged as another powerful tool providing a platform for therapeutic correction of monogenic diseases. Proof of concept studies using CRISPR-Cas9 gene editing in hematopoietic stem and progenitor cells (HSPCs) have shown promise to treat hematological disorders⁸⁻¹⁰. Nevertheless, critical challenges such as the efficiency of targeting long-term HSCs, on- and off-target side effects and safety still need to be addressed.

For successful treatment outcome of hematological and immunological disorders, correction of long-term HSCs and progenitors is required to ensure long term correction of the disease¹¹. Both viral gene addition therapy as well as homology-directed recombination (HDR)-based gene editing approaches have shown preferential genetic correction of more proliferating progenitors, rather than quiescent HSCs *in vitro*, possibly due to better chromatin accessibility of relevant loci^{12,13}.

Combinations of hematopoietic cytokines are commonly used in human HSC cultures to promote HSPC expansion for gene therapy purposes¹⁴. Although these cytokines support short-term maintenance of HSCs, they fail to expand functional HSCs, particularly in genetic manipulation settings.

Great effort has been put to develop culture conditions for HSC expansion *in vitro* by incorporation of small molecules such as UM171¹⁵, StemRegening-1¹⁶ or using chemically defined cytokine free medium¹⁷. Recent approaches of using various epigenetic regulators for HSC expansion *in vitro* have been reported. Epigenetic regulation is an essential intrinsic cue for normal haematopoiesis and cell fate decisions under physiological conditions¹⁸. Small molecules targeting chromatin regulating proteins, such as histone deacetylase inhibitors (HDACi), DNA methylating agents, and Bromodomain and Extra-Terminal motif domain inhibitors (BETi) have been successfully used in culturing protocols of human HSCs¹⁹⁻²³. These findings have highlighted the importance of epigenetic regulators as a therapeutic target for HSC expansion and gene therapy purposes.

3.3 Results

CPI203 and Quisinostat support expansion of phenotypic HSCs *in vitro*

In a clinical gene therapy settings, HSPCs often are cultured in serum free medium supplemented with the essential cytokines stem cell factor (SCF), thrombopoietin (TPO), Fms-like tyrosine kinase 3 (FLT3) and Interleukin-3 (IL3)⁵. We have previously showed the adverse effect of IL3 on *ex vivo* HSC expansion and subsequently low engraftment in mice, suggesting exclusion of IL3 from culture protocols¹⁴.

CD34⁺ cells from cord blood were expanded for 4 days in a cytokine cocktail of SCF, TPO and FLT3

Results

(called STF), with or without addition of Quisinostat (STF+Q) or CPI203 (STF+CPI203) (Figure 1A). Flow cytometry analysis showed that addition of Quisinostat increased the expression of the HSC markers CD34, CD90, and CD201, while low expression of CD38 and CD45RA were maintained in presence of both CPI203 and Quisinostat (Figure 3.1B). This resulted in substantial increase of the frequency and count of $CD34^+CD38^{-/lo}CD90^+CD45RA^{-}CD201^+$ phenotypic HSCs (Figure 3.1C-D); in contrast, STF cytokine cocktail supported expansion of multipotent progenitors (MPPs) and different lineage progenitors after four days (Figure 3.1D-E).

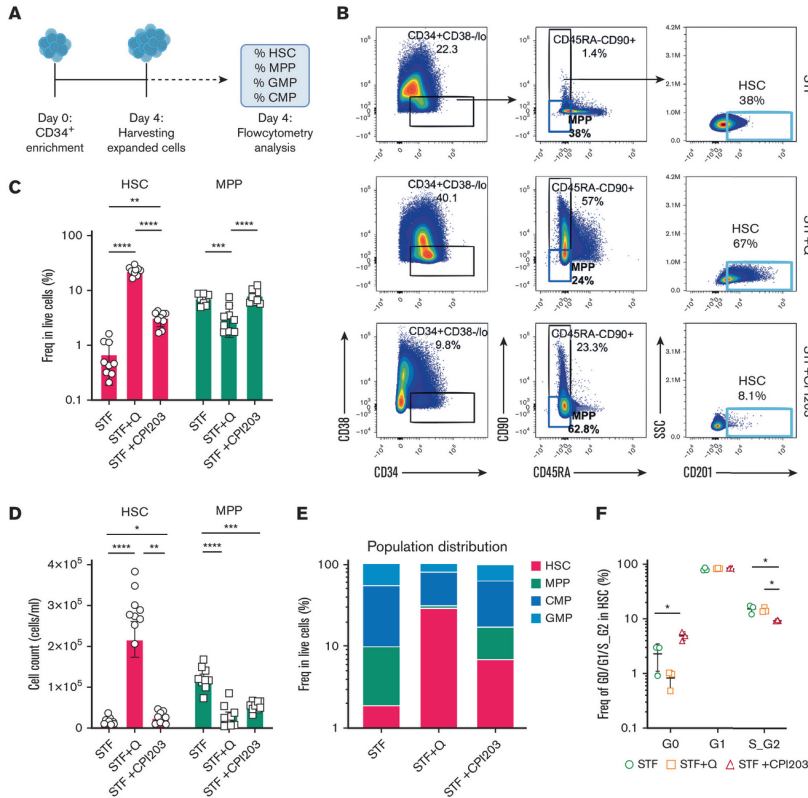


Figure 3.1: CPI203 and quisinostat support ex vivo expansion of HSCs. (A) Schematic experimental design. CD34⁺ cells were enriched from cord blood and cultured in a cytokine-supplemented medium with quisinostat (0.5 nM) or CPI203 (150 nM) for 4 days. (B) Representative gating strategy of expanded HSCs (CD34⁺CD38^{-/lo}CD90⁺CD45RA⁻CD201⁺) and MPPs (CD34⁺CD38⁻CD90⁺CD45RA⁻) in the indicated culture conditions. (C) The graph indicates the frequency of HSC and MPP after 4 days of culture in the indicated culture conditions (n = 9; mean ± standard deviation [SD]). (D) HSC and MPP count after 4 days of culture in the indicated culture conditions (n = 9; mean ± SD). (E) Stacked bar plot showing the composition of HSPCs expanded with only cytokines and in the presence of quisinostat and CPI203. (F) Frequency of cell cycle stages in the expanded HSCs (n = 3; mean ± SD). Statistical significance was determined by a 2-way analysis of variance (ANOVA) test for multiple comparisons: *P < .05; **P < .01; ***P < .001; ****P < .0001. CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor.

Both HDAC and BET inhibitors have been clinically evaluated as antitumor agents, mainly due to their antiproliferative effect on cancer cells by inducing G0/G1 cycle arrest through P13K-AKT pathway³⁰ and targeting MYC, respectively³¹. In this study, increasing concentrations of Quisinostat reduced the total nucleated count (TNC) but increased the frequency of phenotypic HSCs

(Supplementary Figure 3.1B-C). Addition of CPI203 reduced total cell counts and CD34⁺ numbers after four days of culture (Supplementary Figure 3.1D). This is consistent with the observed significant increase of G0 frequency and a decrease of proliferative states of S/G2 in HSCs, whereas no changes in cell cycle states were observed in the Quisinostat-treated HSCs (Figure 3.1F).

Quisinostat increases LV transduction efficiency *in vitro*

LV-based gene therapy in HSCs has proven to be safe and effective for correction of different blood disorders. Nevertheless, often clinical applications are limited by suboptimal transduction efficiency especially in true HSCs, due to their dormant state.¹¹ Various transduction protocols containing small molecules such as Prostaglandin E₂³², Cyclosporin H¹² and poloxamer F108 (LentiBOOST)³³ have been reported to improve transduction efficiency *in vitro* and *in vivo*.

To assess the efficiency of our suggested HSC expansion culture conditions for LV based gene therapy settings, CD34⁺ were transduced with LV carrying green fluorescent protein (GFP), in presence of either CPI203 or Quisinostat (Figure 3.2A). Both flow cytometry and vector copy number analyses showed that addition of Quisinostat in culture increased the LV transduction efficiency significantly in total cells (Figure 3.2B-C, Supplementary Figure 3.2A). Notably, Quisinostat has more than doubled the transduction efficiency in HSCs (Figure 3.2D-E) and other progenitors in comparison with other culture conditions (Supplementary Figure 3.2B).

HDACs impact gene expression status, by increasing acetylation level, leading to more transcriptionally active and accessible chromatin. Upon the introduction of Quisinostat, we have confirmed that the acetylation level of lysine 27 on Histone 3 (H3K27) in CD34⁺, HSCs, and MPPs has significantly increased (Figure 3.2F). This finding provides a potential explanation for the observed enhanced transduction efficiency.

In vivo repopulating potential of transduced HSPCs from an optimized cell culture

To evaluate the repopulation potential and *in vivo* engraftment capabilities of cultured HSPCs, we preformed xenotransplantation assays, which are the accepted gold standard for assessing multilineage potential and self-renewal of human HSCs, as reliable *in vitro* assays to address stem cell functionality are currently absent³⁴.

We transplanted 5x10⁴ and 2.5x10⁴ expanded cells from the conditions 'STF', 'STF+CPI203' and 'STF+Q' into recipient NSG mice (Figure 3.3A). All mice (5 out of 5) injected with Quisinostat- and CPI203-expanded cells showed signs of human engraftment ($\geq 0.1\%$ of huCD45⁺) at both cell doses, whereas mice that received cells expanded in cytokine cocktail only showed consistent engraftment solely at higher cell doses. Significantly higher human chimerism (huCD45⁺) in BM was detected in Quisinostat and CPI203 culture groups when mice received lower cell dose (Figure 3.3B-C). Given this difference, the subsequent sections will primarily focus on the results from mice receiving the lower cell dose of 25,000 cells. Peripheral blood analysis over time showed higher human chimerism in mice receiving CPI203-cultured cells (Figure 3.3D). Interestingly, transplantation of CPI203-expanded cells also resulted in substantial increased engraftment in the thymus (Thy), whereas no differences were observed in the spleen (Sp) (Figure 3.3E). The frequency of human CD34⁺ was significantly higher in BM in the CPI203-expanded culture group (Figure 3.3F). Distinct multilineage output in different organs demonstrated that both Quisinostat and CPI203 cultured cells yielded improved lymphoid development in BM, with significantly higher frequency of CD19⁺, common lymphoid progenitors (CLPs) and Pro-B cells (Figure 3.3F). Strikingly, cells expanded with CPI203 exhibited enhanced engraftment in thymus comparing to other organs (Supplementary Figure 3.3 A), accompanied by an increased frequency of CD3⁺, CD4⁺CD8⁺, and TCRab⁺ cells (Figure 3.3G).

Results

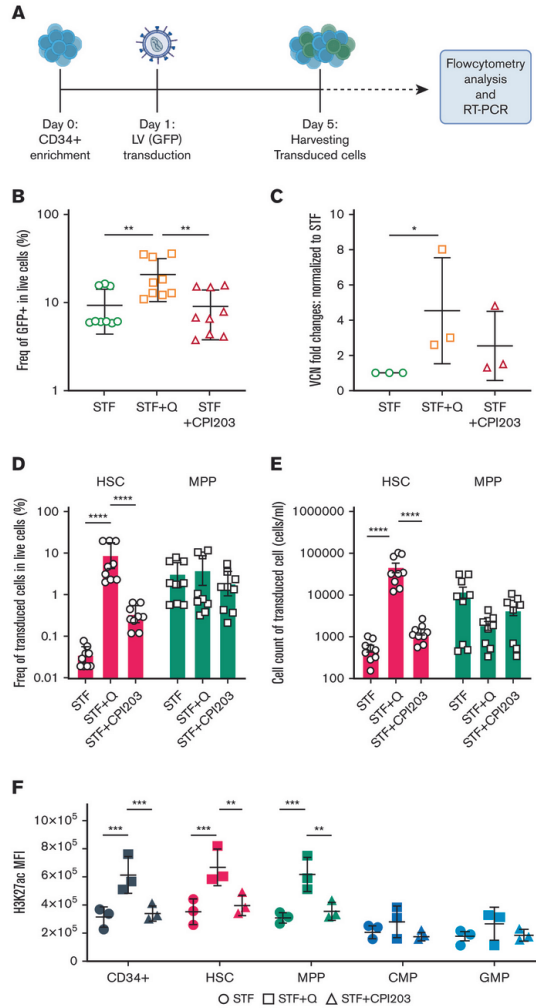


Figure 3.2: **Quisinostat increases LV transduction efficiency.** (A) Schematic experimental design. CD34+ cells were transduced with LV-GFP 24 hours after stimulation in cytokine-supplemented medium alone or the presence of quisinostat (0.5 nM) or CPI203 (150 nM). Cells were harvested for flow cytometry analysis, DNA isolation, and reverse transcription polymerase chain reaction (RT-PCR), 4 days after transduction. (B) Frequency of GFP+ 4 days after transduction in live cells ($n = 9$; mean $\pm$ SD). (C) VCN was determined by RT-PCR ($n = 3$; mean $\pm$ SD; * $P < .05$; unpaired t test). (D) Frequency of transduced HSCs and MPPs in the indicated culture conditions are shown ($n = 9$; mean $\pm$ SD). (E) Cell counts of transduced HSCs and MPPs ($n = 9$; mean $\pm$ SD). (F) The plot shows the mean fluorescence intensity of H3K27ac in different populations of expanded cells in the indicated culture conditions ($n = 3$; mean $\pm$ SD). Statistical significance was determined by 2-way ANOVA test with multiple comparisons: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$. H3K27ac, acetylation level of lysine 27 on histone 3.

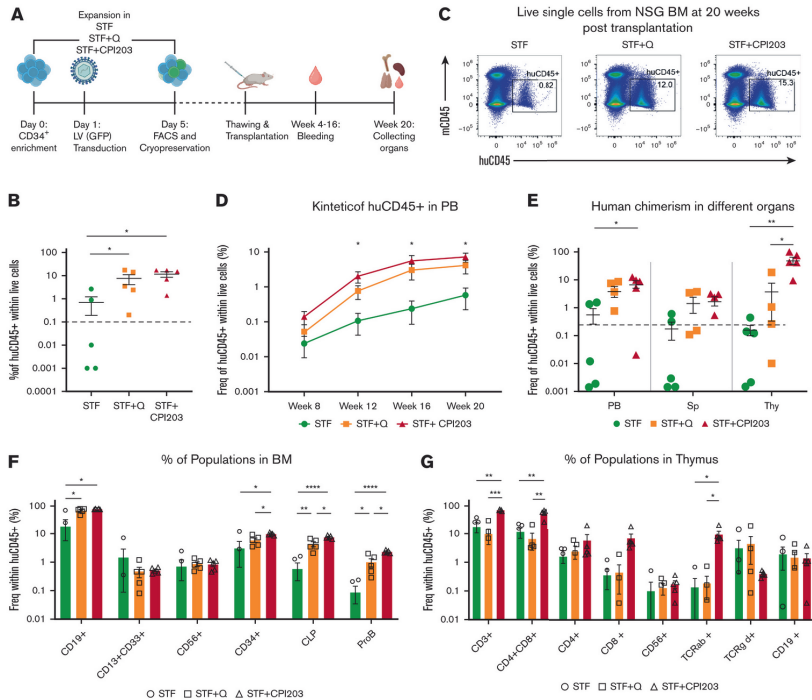


Figure 3.3: CPI203 and quisinostat support expansion of functional HSCs. (A) Schematic overview of primary transplantation. A total of 25 000 and 50 000 nucleated expanded cells in the indicated culture conditions were transplanted per mouse. (B) Human engraftment in the BM of NSG mice at week 20 after transplantation (n = 5 mice per group; mean $\pm$ standard error of the mean [SEM]; * P < .05). Statistical significance was calculated by 1-way ANOVA, with multiple comparisons. (C) Representative fluorescence-activated cell sorter (FACS) plots of huCD45 in NSG BM 20 weeks after transplantation. (D) Kinetics of chimerism in PB over time (n = 5 mice per group; significance of STF vs STF + CPI203 is shown in the plot). (E) Human engraftment in different organs 20 weeks after transplantation (n = 5 mice per group). (F) Frequency of different populations in BM (n = 5 mice per group). (G) Frequency of different populations in the Thy (n = 5 mice per group; mean $\pm$ SEM). Statistical significance was calculated by 2-way ANOVA with multiple comparisons: * P < .05; ** P < .01; *** P < .001; **** P < .0001.

Results

Subsequently, we examined the frequency of transduced cells (% GFP⁺) in engrafted cells in the BM (Figure 3.4A). In alignment with our *in vitro* results, mice that received Quisinostat-expanded cells, exhibited a greater frequency of transduced huCD45⁺ in BM, PB and spleen, with a marginal increase observed in thymus when compared to other culture conditions (Figure 3.4B). Further analysis has also revealed higher transduction efficiency across various lineages in BM (Figure 3.4C). Secondary transplantation was performed to confirm long-term repopulation capacity of expanded cells in BM (Figure 3.4D-E), and engraftment of transduced cells in BM (Figure 3.4F). The data confirm that addition of CPI203 or Quisinostat maintain HSC self-renewal.

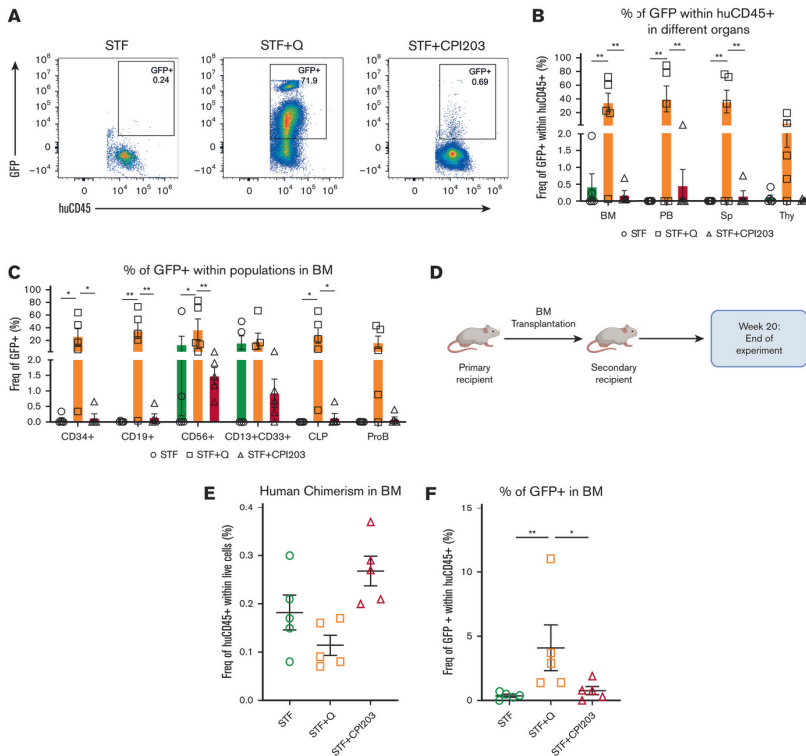


Figure 3.4: Quisinostat supports better engraftment of transduced cells in vivo. (A) Representative FACS plots of BM at 20 weeks after transplantation in primary mice. (B) Frequency of GFP⁺ cells in different organs (n = 5 mice per group; mean ± SEM; **P < .01). Statistical significance was calculated using 2-way ANOVA and multiple comparisons. (C) Frequency of transduced populations within huCD45⁺ in the BM at 20 weeks after transplantation in primary NSG mice (n = 5 mice per group; mean ± SEM; *P < .05, **P < .01). Statistical significance was calculated by 2-way ANOVA and multiple comparisons. (D) Schematic design of the secondary transplantation into NSG mice. (E) Human chimerism in the BM at 20 weeks after transplantation (n = 5 mice per group; mean ± SEM). Statistical significance was calculated using the Mann-Whitney test. (F) Frequency of transduced cells (GFP⁺) in the BM (n = 5 mice per group; mean ± SEM; *P < .05, **P < .01). Statistical significance was calculated using the Mann-Whitney test. CLP, common lymphoid progenitor.

scRNA-seq reveals epigenetic regulators support expansion of HSCs upon LV transduction

To reveal cellular heterogeneity within CD34⁺ cells among different culture conditions, we performed single cell RNA-sequencing (scRNA-seq). Specifically, we set out to compare our culture conditions as well as the effect of LV transduction on the cellular composition of the total CD34⁺

cells.

Enriched CD34⁺ cells (hereafter referred to as unstimulated CD34⁺) were used as a reference control, along with six samples that were cultured and transduced in medium supplemented with cytokines alone, Quisinostat or CPI203 (Figure 3.5A). After manual annotation of unstimulated CD34⁺ cells into multiple different progenitor cell types based on gene expression profiles, all samples were integrated and analyzed using Seurat. We identified 13 major clusters (Figure 3.5B-C) corresponding to several well recognized early differentiation stages of CD34⁺ cells. Based on the marker genes adopted from Dong *et al*²⁷, and top differential expressed genes (DEGs) (Supplementary Table 2), we identified the *bona fide* HSC population as having high expression of *AVP* and *HLF* genes, while lacking lineage specific genes such as *MPO*, *DUTT*, *GATA2*. The populations with lower expression of *HLF* and *AVP* were identified as MPP, as they have been proposed to be early progenitors¹⁷. Furthermore, progenitors of distinct lineages of myeloid, lymphoid, erythroid (Eryth) and megakaryocytic cells (Mks) were identified using their unique gene signatures (Figure 3.5C and Supplementary Figure 3.4A).

Comparing HSPC composition among different culture conditions, a higher frequency of HSCs was observed in culture conditions with CPI203 and Quisinostat compared to the STF condition (Figure 3.5 D-E, and Supplementary Figure 3.4D). In cultures supplemented with Quisinostat and CPI203, expression of HSC signature genes of *HLF*, *RORA* and *HOXA9* was higher compared to cytokine-only (STF) expanded cells, while *AVP* gene expression decreased in CPI203 expanded cells (Supplementary Figure 3.4C). Moreover, consistent with our flow cytometry analysis (Figure 3.1B), Single cell transcriptomics data confirmed higher expression of *CD34*, *THY1* and *PROCR* genes in Quisinostat-expanded cells, while expression of *CD38* gene was decreased (Figure 3.5F).

In accordance with our *in vivo* results, the scRNA-seq data also demonstrated the increase in frequencies of lymphoid multipotent progenitors (LMPPs), B cells, common myeloid progenitors (CMPs) and Mks in CPI203 expanded cells (Figure 3.5E, and Supplementary Figure 3.4D). Collectively, our *in vivo* and *in vitro* results suggest that addition of CPI203 in HSC expansion protocol not only has improved *ex vivo* expansion of HSCs, but has also supported the priming of cells towards the lymphoid lineage. On the other hand, the addition of Quisinostat reduced the frequency of granulocyte monocyte progenitors (GMPs), while increasing erythroid progenitors (MEPs) and myeloid progenitors (CMPs) (Figure 3.5E, and Supplementary Figure 3.4D).

Results

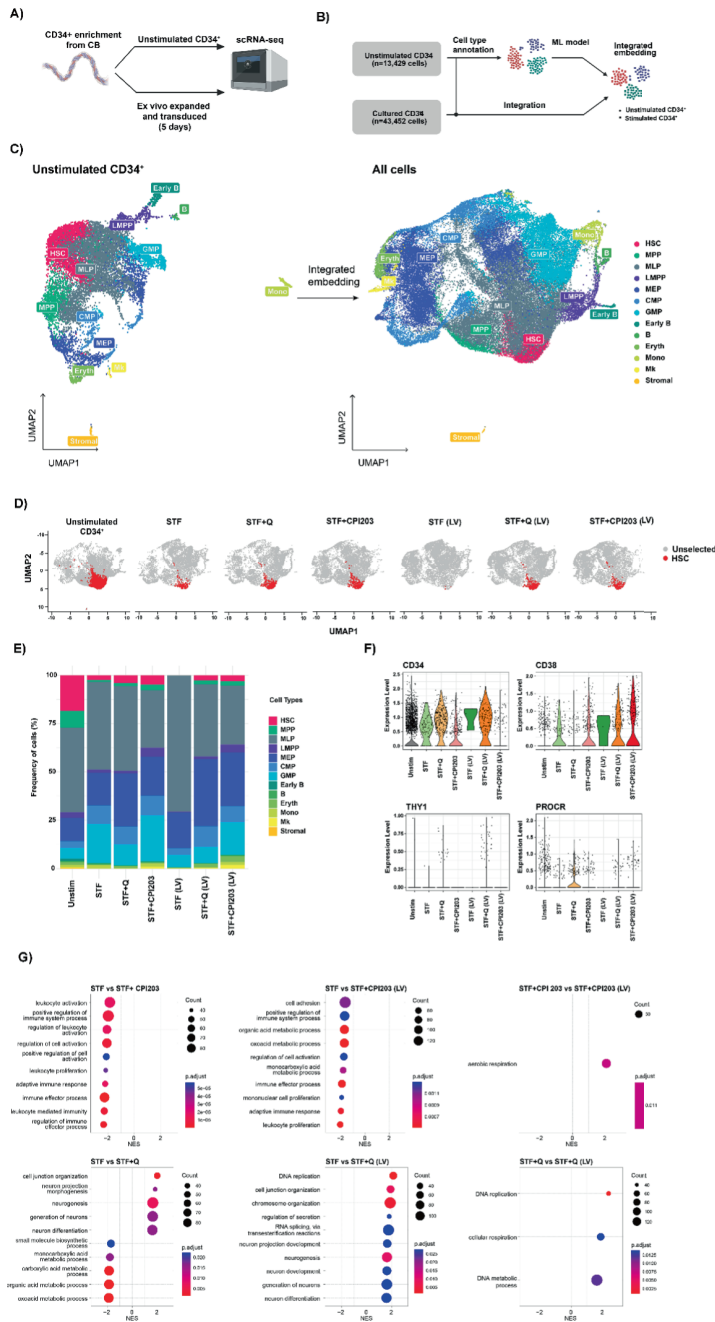


Figure 3.5: **Single-cell transcriptome analysis reveals quisinostat and CPI203 maintain HSCs upon LV transduction in CD34+.** (A) Schematic experimental design. (B) Mock-up schematic of cell-type projection. (C) Uniform manifold approximation and projection for dimension reduction (UMAP) plots of unstimulated CD34+ cells and integrated UMAP for all conditions. (D) UMAP displaying HSCs for each condition. (E) The bar plot represents population distribution. (F) Violin plots displaying the expression levels of selected HSC surface marker genes in the indicated conditions. (G) Gene set enrichment analysis and molecular processes of various conditions interrogated by scRNA-seq. LMPP, lymphoid multipotent progenitor; MEP, megakaryocyte-Eryth progenitor; ML, myeloid-lymphoid progenitor; Mono, monocyte.

Of note, our data showed higher frequency of MEPs, Eryth and Mks upon LV transduction, suggesting LVs could skew cells towards the erythroid lineage in culture conditions containing Quisinostat or CPI203. In contrast, higher frequency of myeloid-lymphoid progenitors (MLPs) have been detected in cytokine-expanded cells (Supplementary Figure 3.4D). This possible lineage skewing upon LV transduction warrants further investigation.

Finally, single cell transcriptomics results showed LV transduction in itself induces cell proliferation (Supplementary Figure 3.4B) but reduces the frequency of the HSC population compared to non-transduced conditions. This decrease is more prominent in STF condition, while inclusion of Quisinostat and CPI203 in the LV transduction protocol has helped to maintain HSCs (Figure 3.5D, and Supplementary Figure 3.4D). LV transduction induces innate immune response via indirect activation of interferon (IFN) responses through different pathways such as Toll-like receptors (TLRs)³⁵, DNA damage response³⁶ or DNA sensor responses³⁷. We have detected elevated gene expression related to the interferon response and DNA damage response, such as *TP53*, *IFITM2*, *IFITM3*, *IFI16*, *TMEM173*, *DDB2* in expanded cells. However, addition of CPI203 results in a reduction of their expression. Additionally, no changes in expression of TLRs was observed (Supplementary Figure 3.4E). Using Gene set enrichment analysis and checking the cellular processes associated with the differentially expressed genes (Figure 3.5G), we noted that the addition of Quisinostat to the LV transduction condition, resulted in differential expression of genes associated with DNA replication and chromosome organization, in line with the functional effects observed.

Collectively, these findings indicate that addition of Quisinostat and CPI203 enhances the expansion of hematopoietic stem cells (HSCs) while simultaneously priming cells for erythroid and lymphoid lineages, respectively. Furthermore, our study highlights the impact of lentiviral (LV) transduction on cellular heterogeneity, revealing a significant loss of HSCs with the commonly employed protocol in clinical settings.

3.4 Discussion

Working towards improving HSC expansion protocols for gene therapy purposes, in this study we have evaluated existing and newly tested culture conditions for HSC expansion and gene modification. We incorporated the small molecules CPI203—a BETi that has been previously reported by Hua *et al*¹⁹—and the HDACi Quisinostat for testing *ex vivo* expansion of HSCs and their genetic modification. BET proteins interact with acetylated lysins at transcriptional start sites that regulate transcription through RNA polymerase II complex formation. BETi regulates transcription by blocking BET protein binding to histones. Therapeutic benefits of BETis have been investigated in several cancer trials, however, their clinical progression has been challenging and none have received regulatory approval³⁸. On the other hand, Quisinostat, an HDACi targeting HDAC I and II, has been evaluated in several clinical trials for different malignancies³⁹⁻⁴¹. Recent research

Methods

suggests Quisinostat inhibits the cancer cell self-renewal by re-establishing expression of histone linker H1.0⁴². H1 is highly conserved and involved in embryonic stem cell differentiation and mammalian development, although the role of H1 linker in HSCs development and fate decisions needs further investigation.

We have reported improved culture conditions for *ex vivo* expansion of true HSCs, which could be implemented in lentiviral transduction protocols for gene therapy purposes. We showed that the inclusion of epigenetic regulators of Quisinostat and CPI203 increased expansion of HSCs. In addition, the positive effect of Quisinostat on transduction efficiency provides a major benefit for the gene therapy field. This not only ensures long-term correction of true HSCs, which is the ultimate goal of gene therapy, but also reduces the costs associated with viral vectors, since less lentiviral virus would be required to achieve a desired vector copy number.

Importantly, our scRNA-seq data reveals the stealth effect of LV transduction on loss of HSCs, particularly in the conventional clinical protocol of STF-cytokine-expanded cells. LV transduction induces an innate immune response via indirect activation of interferon (IFN) responses through different pathways such as Toll-like receptors (TLRs)³⁵, DNA damage response³⁶ or DNA sensor responses³⁷. Even though LVs can escape the innate immune responses⁴³, they can be detected through TLRs³⁵ in HSPCs or trigger p53 signaling followed by robust induction of IFN responses⁴³. Human CD34⁺ cells express *TLR3*, *TLR4*, *TLR7*, *TLR8* and *TLR9* to detect infection and induce innate immune response immediately³⁵. Upon viral infection under physiological conditions, IFN- α induction leads to proliferation as a response to clear infections⁴⁴. In addition, results from mouse studies showed induction of IFN- γ impairs HSC self-renewal and restore of HSCs number upon viral infection⁴⁵. However, in our study no changes in *TLRs*' gene expressions were found. Strikingly, the benefit of CPI203 in gene editing can be investigated as it reduces interferon responses through p53 and reduced DNA damage responses.

Furthermore, our *in vivo* study suggests that CPI203 improved lymphoid development in BM and significantly in thymus without compromising other lineages, a benefit that would be valuable for patients with an impaired immune system. Moreover, based on our single cell transcriptomics data, Quisinostat primed HSCs toward the erythroid lineage, which also can hold promises for gene therapy targeting blood disorders.

In the realm of clinical trials focused on HSC-based gene therapy, substantial financial investments are dedicated to producing Good Manufacturing Practice (GMP) grade LV batches. Frequently, the expenses associated with generating these batches reach into the millions of dollars, even for a small number of patients. Compliance with regulatory standards mandates full-scale test runs in meticulously controlled clean room environments. Consequently, the limited availability of GMP-grade virus for patient treatment significantly amplifies the overall costs associated with gene therapy medicines, emerging as a critical concern within the field⁴⁶. This underscores the pressing need for advancements in HSC transduction protocols to alleviate the financial burden and streamline the production of gene therapies. Here we have reported on enhanced culture conditions that not only improve transduction efficiency but also preserve the integrity of HSCs. Such optimization is imperative for achieving a more favorable clinical outcome and emphasizes the importance of refining current protocols for expanded hematopoietic cell therapies.

3.5 Methods

Human cells and CD34⁺ enrichment

Human cord blood was obtained after informed consent from Leiden University Medical Centre. Mononuclear Cells (MNCs) were obtained from cord blood by density centrifugation using Ficoll-Amidotrizoat. CD34⁺ cells were positively selected using the human CD34 UltraPure MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol.

CD34⁺ cell culture

100,000-250,000 enriched CD34⁺ cells/ml (>90% purity) were cultured in X-vivo15 medium (Lonza) supplemented with recombinant huSCF (300ng/ml), huTPO (100ng/ml), huFLT3 (100ng/ml) (Miltenyi Biotec), and small molecules of CPI203 (150nM) (Selleckchem) and Quisinostat (0.1, 0.5 and 1nM) (MedChemExpress) After 4-5 days of culture, expanded cells were harvested and counted using a nucleocounter 3000 (Chemometec) for subsequent immunophenotyping, transplantation in mice, and scRNA-seq.

Lentivirus production

HEK 293T cells were seeded at density of 4×10^6 cells in 10cm² plate. After 24 hours, cells were transfected using X-tremeGENE HP DNA transfection reagent (Sigma-Aldrich) at a mass ratio of 1:3 (DNA:X-tremeGene HP), with the plasmids. The amount of each viral component per million of cells was: 2.5 μ g of pSIN.SF.EGFP.WPRE; 1.25 μ g of pMDLg/pRRE; 0.6 μ g of pRSV-Rev; 0.75 μ g of pMD2.VSVG. Supernatant was harvested, clarified with 0.45 μ m filter (Whatman) at 24h and 48h post transfection. Supernatant from different time points were pooled together. Virus concentration was performed using Vivaspin20 (Sartorius) according to the manufacturer's instruction.

Lentivirus transduction

One day prior to transduction, CD34⁺ cells were cultured in cytokine supplemented X-vivo15 medium in combination with CPI203 (150nM) or Quisinostat (0.5nM). After overnight stimulation, lentiviral particles carrying GFP transgene were added to the cells at MOI of 1.5 as well as Lentiboost (Sirion) final concentration of 1ug/ml followed by spinoculation at 32 °C for 1 hour at 800 g. Four days post-transduction, transduced cells were used for *in vitro* and *in vivo* approaches.

Determination of vector copy number (VCN) by RT-qPCR

Quantitive (q)PCR was used for the quantitative analysis of proviral DNA copies in transduced cells using Hiv-psi and Albumine as housekeeping gene. Genomic DNA of transduced cells was extracted using GeneElute Mammalian Genomic DNA kit (Sigma-Aldrich). qPCR was performed using TaqMan Universal Master Mix II (Thermofisher) in combination of specific probes from universal library (Roche) on the Quantstudio system (Thermo Fisher Scientific).

Mice

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were purchased from Charles River (France). All animal experiments were approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie Dierproeven, CCD).

Primary and secondary transplantations into NSG mice

For the primary transplantations, *ex vivo* expanded CD34⁺ cells (25,000 or 50,000 total nucleated cells per mouse) in Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Gibco) were transplanted by tail vein injection into pre-conditioned recipient NSG mice (n=5). For the secondary transplantations, bone marrow (BM) cells from primary recipient mice from each group were pooled and 1/7th of the pooled cells were injected into pre-conditioned secondary recipient NSG mice (n=5). 6-8-week-old recipient mice were conditioned with two consecutive doses of 25mg/kg Busulfan (Sigma-Aldrich) (48h and 24h prior transplantation). Mice used for transplan-

Methods

tation were kept under specific pathogen-free conditions. The first four weeks after transplantation mice were fed with additional DietGel recovery food (Clear H₂O) and antibiotic water containing 0.07 mg/mL Polymixin B (Bupha), 0.0875 mg/mL Ciprofloxacin (Bayer b.v.). Peripheral blood (PB) from the mice was drawn by tail vein puncture every 4 weeks until the end of the experiment. At the end of the experiment, PB, thymus, spleen and BM were harvested. Mice were euthanized via CO₂-asphyxiation.

Flow cytometry

Ex vivo cultured cells were stained with the live/dead marker Zombie (Biolegend) according to the manufacturer's instruction. Subsequently, cells were stained with antibodies listed in Supplementary Table 1, and incubated for 30 min at 4°C in the dark in FACS Buffer (PBS pH 7.4, 0.1% azide, 0.2% BSA). Single cell suspensions from murine BM, thymus and spleen were prepared by squeezing the organs through a 70 μ M cell strainer (BD Falcon). Erythrocytes from PB and spleen were lysed in NH₄Cl (8.4 g/L)/KHCO₃ (1 g/L). Single cell suspensions were counted and stained as described above. When 7AAD and KI67 staining was needed, intracellular staining was performed using the Transcription Factor Staining Buffer Set (eBioscience), according to manufacturer's instruction. All cells were measured on an Aurora spectral flow cytometer (Cytex). The data was analysed using FlowJo software (Tree Star).

Single cell analysis quality control (QC) pipeline

QC and downstream analyses of scRNA-seq data were performed using Seurat version 4²⁴. The quality control pipeline before integration consists of three steps per sample: 1) soft filtering, 2) removal of low-quality cluster, and 3) doublet detection. In soft filtering, Seurat objects were created with cells expressing at least 200 genes and with the genes expressed at least in 3 cells. Then, standard Seurat command list was run to detect low quality clusters. We removed a cluster only if it had higher than 15% mitochondrial and less than 1000 mRNA in median. Next, we ran DoubletFinder v3 per sample with 7.5% detection rate and filtered for the singlets²⁵. To integrate the samples, CCA mode of Seurat pipeline was used with most variable 2000 features. Finally, after integration we filtered cells with higher than 15% mitochondrial mRNA.

Cell type predictions for stimulated cells

After QC, we subsetted the unstimulated CD34⁺ cells (n=13,429). Then, these cells were clustered again with a higher resolution parameter (resolution=1.2) to ensure precise annotation in downstream projections of stimulated samples. Furthermore, we checked cluster stability with *clustree* R package²⁶. To annotate each cell cluster, we used a combination of cord blood markers from Dong *et al*²⁷ and differentially expressed genes (DEGs). Top 20 DEGs per cell cluster from unstimulated cells are provided in Supplementary Table 2. To project these annotations onto stimulated samples, we trained a random forest model with 300 trees using unstimulated CD34⁺ cells via 2000 integrated features. Cross-validation on out-of-bag data showed that HSCs could be predicted with an accuracy of 81.2%.

Differential expression and gene set enrichment analysis

Differential expression analysis was conducted with MAST²⁸ with a cut-off of at least 25% difference in cells expressing a given gene. Then, clusterProfiler²⁹ was used along with biological processes subset of GO terms for gene set enrichment analysis. Pathway p-values are adjusted with Benjamini-Hochberg procedure. Pathways enriched with adjusted p<0.05 are selected. ggplot2 library was used for all visualizations unless indicated otherwise.

Statistics

Statistics were calculated and graphs were generated using GraphPad Prism9 (GraphPad Software). Statistical significance was determined by standard two-tailed Mann-Whitney U tests, unpaired t-tests and ANOVA tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

3.6 Acknowledgments

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3.7 Author Contributions

P.T designed, performed, analyzed experiments, and wrote the manuscript; E.O.K performed bioinformatic analysis; K.C.B supported in vivo experiments. B.A.E.N, S.A.V and M.C.J.A.s.E assisted with mouse experiments. M.L.H provided cord blood for this study. E.V.D.A supported with scRNA seq experimental design; K.P.O and F.J.T.S provided supervision and revision of the manuscript. All authors have read and agreed to the final version of manuscript.

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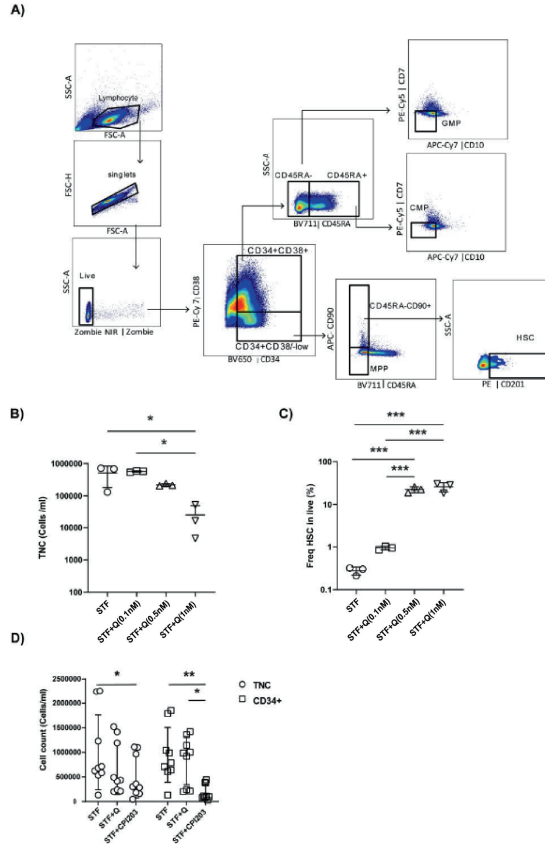
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3.9 Appendix

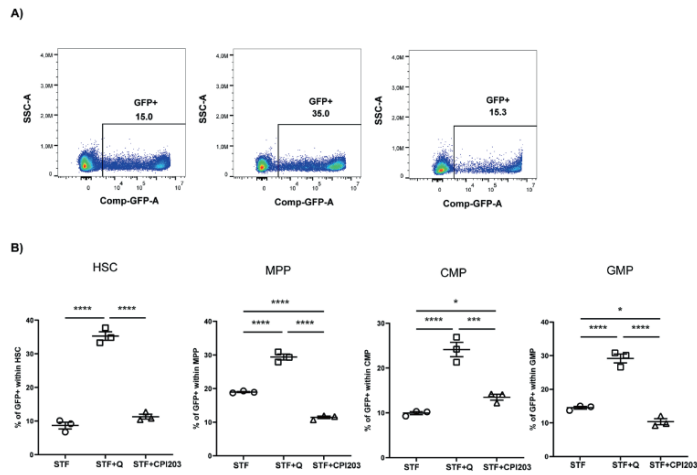
3.9.1 Supplementary Tables

The appendix was shortened due to environmental considerations. To minimize unnecessary printing and reduce material use, extensive data including 11 supplementary tables are provided online at: <https://doi.org/10.1182/bloodadvances.2024013047>.

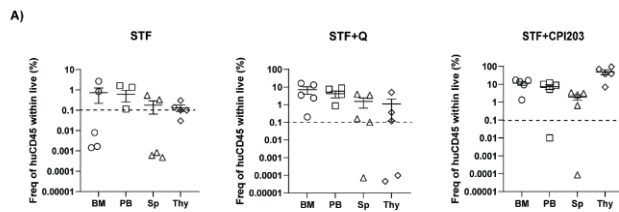
3.9.2 Supplementary Figures



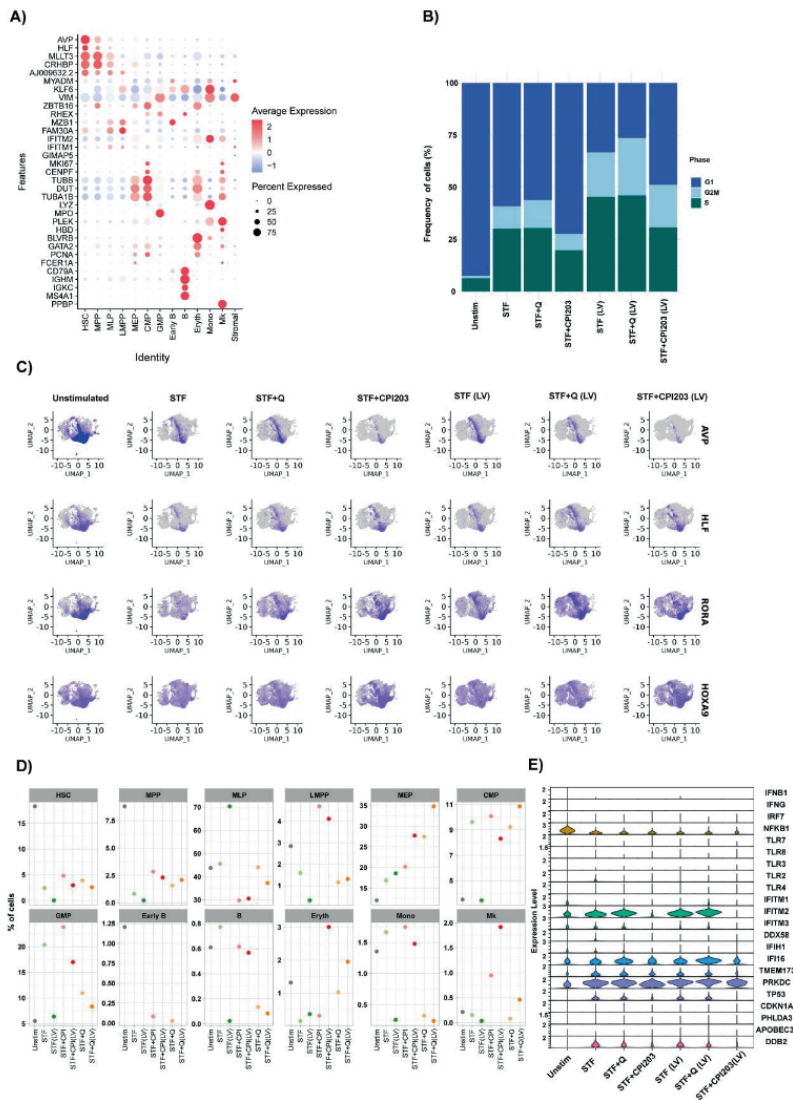
Supplementary Figure 1. **Ex vivo expansion of HSPCs.** A) Representative gating strategy for phenotypic analysis of HSPCs. B) Total nucleated cell count (TNC) after 4-day culture in presence of different concentration of Quisinostat (n=3, Mean±SD). C) Frequency of HSC after 4-day culture in different concentration of Quisinostat (n=3, Mean±SD). D) TNC and CD34+ cell count after 4 days of expansion in indicated culture condition (n=9, Mean±SD). Significant statistical was determined by two-way anova with multiple comparison *p<0.05, **p<0.01, ***p<0.001)



Supplementary Figure 2. LV transduction efficiency in vitro. A) Representative FACS plots displaying GFP expression in live cells, 4 days post transduction. B) Frequency of GFP+ in different population of HSC, MPP, CMP and GMP (n=3, Mean±SD, *p<0,05, ***p<0,001, ****p<0,0001. Statical significance was calculated by two-way anova with multiple comparison)



Supplementary Figure 3. Human engraftment in organs from primary transplanted NSG mice. A) Frequency of huCD45+ in different organs at week 20 post transplantation.



Supplementary Figure 4. Single cell transcriptome of human HSPCs. A) Expression of key marker genes in each cell population (Marker gene set was adopted from Dong et al). B) Stacked bar plot showing cell cycle analysis C) Feature plots showing HSC signature genes expression per condition. D) Plots displaying frequency of different population in indicated conditions. E) Expression of genes inducing interferon response



Chapter 4

A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML

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4.1 Abstract

The diagnostic spectrum for AML patients is increasingly based on genetic abnormalities due to their prognostic and predictive value. However, information on the AML blast phenotype regarding their maturational arrest has started to regain importance due to its predictive power for drug responses. Here, we deconvolute 1350 bulk RNA-seq samples from five independent AML cohorts on a single-cell healthy BM reference and demonstrate that the morphological differentiation stages (FAB) could be faithfully reconstituted using estimated cell compositions (ECCs). Moreover, we show that the ECCs reliably predict *ex-vivo* drug resistances as demonstrated for Venetoclax, a *BCL-2* inhibitor, resistance specifically in AML with CD14+ monocyte phenotype. We validate these predictions using LUMC proteomics data by showing that *BCL-2* protein abundance is split into two distinct clusters for NPM1-mutated AML at the extremes of CD14+ monocyte percentages, which could be crucial for the Venetoclax dosing patients. Our results suggest that Venetoclax resistance predictions can also be extended to AML without recurrent genetic abnormalities and possibly to MDS-related and secondary AML. Lastly, we show that CD14+ monocytic dominated Ven/Aza treated patients have significantly lower overall survival. Collectively, we propose a framework for allowing a joint mutation and maturation stage modeling that could be used as a blueprint for testing sensitivity for new agents across the various subtypes of AML.

4.2 Introduction

Acute myeloid leukemia (AML) is an aggressive hematological cancer of the myeloid lineage. AML is caused by a combination of relatively few genetic alterations that are predominantly somatically acquired and cooperatively induce a maturation arrest in combination with rapid uncontrolled proliferation of immature myeloid precursor cells. The prognosis of AML is highly dependent on the presence of such recurrent genetic alterations and varies from > 90% cure rates to less than 10%¹. Therefore, the current WHO classification primarily defines AML subtypes according to the presence of eleven recurrent genetic aberrations (RGA) changes and an added heterogeneous umbrella subtype composed of a highly diverse set of relatively rare RGAs³⁻⁶. Only AML cases that lack any detected RGA are characterized according to their maturation stage according to the French American-British (FAB) cytomorphological/cytochemical classification².

Recently, the maturation and differentiation stage of AML blasts has gained importance due to a striking association with sensitivity or resistance to new drugs⁷⁻⁹. AML differentiation stage can be assessed by diagnostic flow cytometry more accurately and objectively than by cytomorphological examination alone. Current standardized flow cytometry for AML diagnosis, analyzes few carefully selected differentiation markers, sufficient for accurate immunological AML classification¹⁹. However, gene expression profiling by bulk RNA-sequencing (RNA-seq) analyzes many more markers (several thousands) and is an attractive alternative technique as it allows both calling of genetic aberrations and estimation of cell subsets, i.e. estimated cell composition (ECC) from the same sample¹⁰⁻¹². Reported attempts to estimate ECCs by deconvoluting bulk AML samples utilizing single-cell RNA-seq as an *in-silico* reference, were mostly focused on detection of survival differences without performing thorough validation of the ECCs and/or used leukemic samples as reference¹³⁻¹⁵, which prevents assessing whether ECCs are tissue- or sample-specific.

Here, we perform deconvolution, a technique in which you try to estimate each cell type within the total gene expression profile of a bulk mixture, of 1350 AML transcriptomic samples via a healthy single-cell reference while validating our findings with our in-house (LUMC) flow cytometry data. Of note, we demonstrate that the ECCs recapitulate the entire FAB landscape (M0-M7). Then, using these ECCs we predict *ex-vivo* drug resistance data from literature and show the agreement of these results at protein level with the help of LUMC proteomics data in AML patients, for whom we also had acquired gene expression data. To conclude, we hereby propose a transcription-based single-cell guided deconvolution framework to assess the drug effectiveness to different maturational stages of AML. We also provide our framework as a CRAN R package available at <https://github.com/eonurk/seAMLess>.

4.3 Results

Deconvolution pipeline recapitulates healthy and malignant hematopoiesis

We initially created a healthy bone marrow (BM) reference atlas from single-cell transcriptomics data (Figure 4.1A). For this purpose, we integrated data from 69 101 cells covering 439 genes from three publicly available datasets (two full-transcriptome and one targeted) from two studies^{16,17} (Figure 4.1B, Methods). T-cells and B-cells formed separate clusters in the UMAP plot, whereas the myeloid lineage cells clustered together. To differentiate early myelopoiesis, we distinguished more than 3 349 hematopoietic stem cells (HSC) and 1 432 erythro-myeloid progenitors (EMP), 2 939 lymphoid multipotent progenitors (LMPP), as well as 3 508 granulocytes-monocytes progeni-

Results

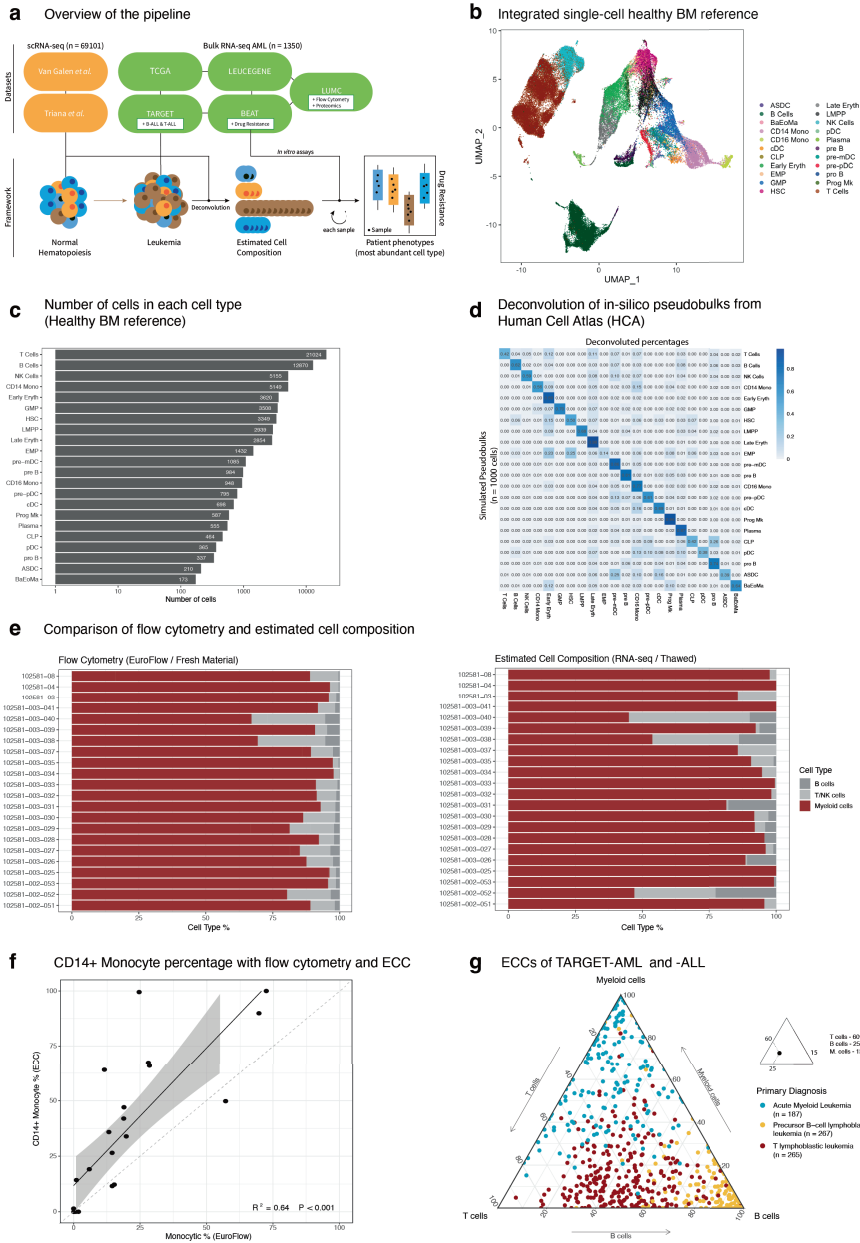
tors (GMP) (**Figure 4.1C**). Inter-individual variability, possibly due to age differences or technical differences, mostly affected T- and B- cells and did not influence overall clustering (**Figure S1A**). Homogeneous distribution of the studies on the UMAP plot showed successful integration of the different datasets (**Figure S1B**).

We next performed *in silico* experiments to validate our deconvolution set-up. We first used the healthy BM reference to create *in silico* mixed bulk samples with known cell compositions. Pseudobulk profiles were simulated with one abundant cell type (80% of the cells) with the remaining cells being a mix created by random selection (Methods). Then, MuSiC¹² with the default settings was used to deconvolute the simulated pseudobulk profiles into their respective cell types (**Figure S1C**). To validate on an independent dataset, we also simulated pseudobulks from 40 000 healthy bone marrow cells across 8 donors of the human cell atlas (HCA)¹⁸ (**Figure S1D**) and performed deconvolution via MuSiC. All simulated pseudobulk profiles were successfully deconvoluted into their respective cell types (matching to the most abundant cell type) apart from EMP (**Figure 4.1D**). For this profile, the annotations were shared mostly among EMP (14%) as well as HSC (25%) and Early Erythrocytes (23%). This discrepancy might be explained by the transcriptional similarity of these cell types (**Figure 4.1B**).

Next, we analyzed our LUMC diagnostic flow cytometry data (EuroFlow¹⁹ panels; see Methods) for 22 AML samples with matched bulk RNA-seq data. An overview of mean fluorescence intensity (MFI) values for these samples' abnormal cells after staining with antibodies for 33 markers distributed over 7 tubes (1 Orientation + 6 AML assignment tubes) is shown in **Figure S1E** (Supplementary Table S1, Figure S7). In line with our expectations, the most abundant cell types for all samples were in the myeloid lineage for both flow cytometry analyses and estimated cell compositions (ECCs) (**Figure 4.1E**, Supplementary Table S4). To quantify whether monocytic AML can be accurately distinguished from AML with more stem cell-like phenotypes, we plotted CD14+ monocyte percentages as determined by deconvolution against MFI values of antibodies of monocytic markers (CD11b, CD64, IREM2, and CD14) on all BM cells without gating, and observed statistically significant correlations for 3 markers (CD11b, CD64, IREM2), with CD64 being most significant ($R^2 = 0.43$, $P < 0.001$) (**Figure S1F**). Also, percentages of AML cells assigned to the monocytic subset by EuroFlow panels and ECCs showed statistically significant correlations ($R^2 = 0.64$, $P < 0.001$) (**Figure 4.1F**).

A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML

Figure 1



Results

Figure 4.1: **a** The overview of the study. Integrated cells for healthy bone marrow (BM) dataset were collected from two studies^{17,18}. Five independent bulk transcriptomic AML cohort (TCGA-LAML, BEAT-AML, TARGET-AML, LEUCEGENE and LUMC) samples (n = 1,350) were deconvoluted. Drug resistance data (n = 122) from BEAT-AML were predicted using ECCs. Additional ALL samples (n = 532) from TARGET study were used for validating deconvolution framework. Proteomics (n = 39) and flow cytometry samples (n = 22) from LUMC cohort were used for further validation of the framework. **b** UMAP plot of integrated healthy BM (n = 69,101) cells. Annotations are lifted via Azimuth framework. T and B cell subset were merged, resulting into 22 cell types (abbreviations; GMP: Granulocyte Monocyte Progenitors, LMPP: Lymphoid Primed Multipotent Progenitors, NK: Natural Killer, EMP: Erythroid Megakaryocyte Progenitor, pDC: Plasmacytoid Dendritic Cell, CLP: Common Lymphoid Progenitor, HSC: Hematopoietic Stem Cell, BaEoMA: Basophil Eosinophil Mast Progenitor, Prog Mk: Progenitor Megakaryocyte, pre-mDC: Precursor Myeloid Dendritic Cell, ASDC: AXL+ Dendritic Cell). **c** Barplot for cells per cell type of the healthy BM. X-axis is in log10 scale. **d** Heatmap showing the fraction of deconvoluted cell types for simulated pseudobulks from HCA subset. Each row indicates a simulated pseudobulk with an overabundant cell type (80%) and adds up to 1, and each column is a cell type from the healthy BM reference. **e** Cellular composition of the same samples via flow gating of orientation tube (ALOT) and ECCs (RNA-seq). **f** Percentage of monocytic subsets measured by flow cytometry (fresh material) and ECCs (thawed for RNA-seq). **g** Ternary plot showing the ECCs of TARGET AML and ALL cohorts (n=719), each dot represents a sample and corners indicate a major cell type and colors indicate the primary diagnosis of each leukemic sample (Supplementary Table S1).

Lastly, we downloaded publicly available TARGET AML and ALL (B-ALL and T-ALL) data and deconvoluted these samples (n=719) with the healthy BM reference (Figure 4.1G, Supplementary Table S1) to show that different leukemic phenotypes could be captured by deconvolution. The ECC of the matching cell type of origin was significantly higher for the different acute leukemias (Figure S1G), most prominent for B-ALL, confirming the ECCs' ability to capture the patients' immune phenotypes at major cell type levels. Together, these benchmarking and validation analyses demonstrated that given a healthy single cell BM transcriptomic atlas, the cell type proportions can be recapitulated faithfully via deconvolution from bulk transcriptomic leukemia cases.

Deconvolution of bulk AML transcriptomics reveals the dominating immune fraction

To investigate heterogeneity in cell composition of AML, we next applied our framework to deconvolute five independent bulk transcriptomic AML studies, i.e. TCGA-LAML²⁰ (n=151), BEAT-AML²¹ (n=460), TARGET-AML²² (n=187), LEUCEGENE²³ (n=452) and our cohort LUMC¹⁰ (n=100) totaling in 1350 samples from 1267 patients (Figure S2A). The results are shown in Figure 4.2A as a heatmap where each sample was decomposed into the 22 cell types from the healthy BM reference. We also added information on patients' clinical blast counts, FAB², WHO 2016⁴, and ELN 2017⁶ classes for an overarching picture of AML landscape, and we also calculated the stemness score²⁴, which is a gene expression signature for patient prognosis trained on engraftment capacity of AML in immunodeficient mice (Supplementary Table S2). Each sample was assigned to the most abundant cell type as determined by deconvolution (AML phenotype) and arranged according to their fractions within each phenotype. The data showed a clear dominance of one immune cell type for most of the cases, indicating that maturational arrests and lineage skewing are leukemic properties that can be readily assessed using transcriptome sequencing. The majority of AML cases was estimated to be dominated by myeloid cells (Figure 4.2A, S2A, S2B, S3A, S3D). As notable exceptions, a few pediatric AML samples from the TARGET cohort showed ECC profiles dominated by T-cells or B-cells. As previously reported these cases can nevertheless be considered as a special subgroup of pediatric AML, and specifically T-cell dominated patients were recognized for poor survival²⁵. Furthermore, in line with previous reports¹⁶, patients with acute promyelocytic leukemia (APL), classified as AML-M3 by FAB, were correctly assigned to have a cell composition dominated by granulocyte-monocyte progenitors (GMPs). Besides APL, there are also AML cases dominated by GMP and large groups of AML assigned as monocytic AML or AML with an earlier HSC or EMP phenotype, again demonstrating that bulk transcriptomics can be used to capture the stage of arrest of AML during hematopoiesis by deconvolution.

Deconvolution of bulk AML transcriptomics agrees upon stemness score

To comprehensively visualize changes in ECCs in relation to metadata, ECCs of AML samples

were visualized using a UMAP plot. As expected, samples with similar ECCs clustered, as shown after annotating the samples for their most abundant cell type (**Figure 4.2B** – left panel). When superimposing the deconvoluted percentages of 6 major cell types on the same UMAP plot (**Figure 4.2B** – right panels), gradual shifts in cell composition became apparent, in particular, APL samples populated the extreme extension of the GMP cluster. Moreover, a gradient towards monocytic outgrowth and conversely to a high stemness was also observed. Comparisons with the previously published stemness score showed an overall agreement with ECCs, with patients dominated by HSCs and EMPs having a statistically significantly higher stemness score than patients dominated by CD14+ monocyte and GMPs (**Figure S3B**). Late Erythrocyte also had high stemness score, albeit with large variation. By dividing AML into cases with high and low stemness scores, we observed similar compositional changes with abundance of HSCs and EMPs in AML with high stemness scores in all cohorts. Late erythrocytes, however, were not consistently enriched in all cohorts. Furthermore, we noticed that in TARGET, T cells are abundant in AML with high stemness scores (**Figure S3C**). This raises the question whether the stemness score, which has been trained on adult AML, is useful to predict prognosis of pediatric patients particularly considering that large subtypes of adult AML such as APL and AML with mutated NPM1 are rare in pediatric AML. The consistent enrichment of HSCs and EMPs in AML with high stemness scores in all cohorts, however, further supports correct determination of the cell composition of AML by deconvoluting bulk transcriptomics.

Results

Figure 2

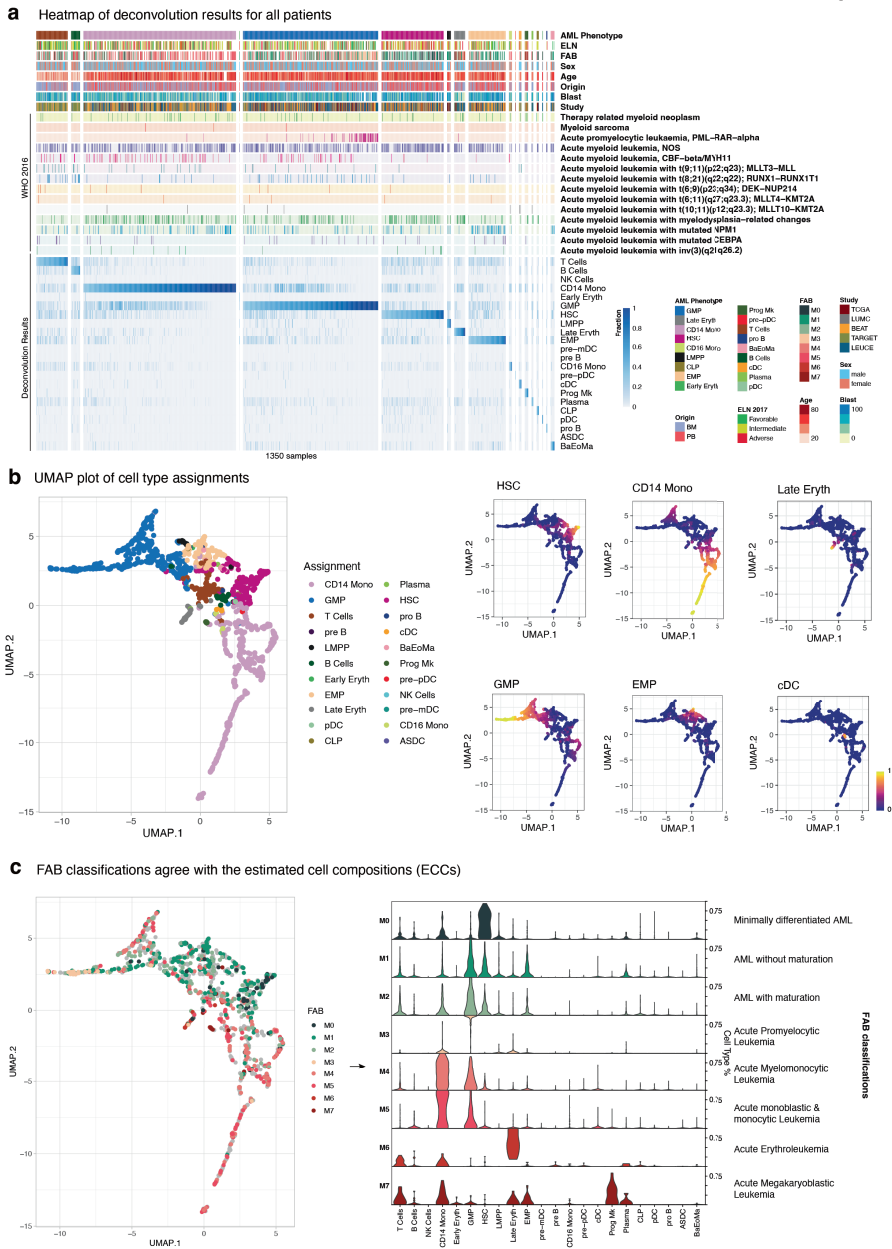


Figure 4.2: **a** Heatmap showing the deconvoluted fractions for of five AML cohorts (TCGA-LAML, BEAT-AML, TARGET-AML, LEUCEGENE and LUMC). Each column represents a sample, which is deconvoluted into 22 cell types (bottom part with blue) via healthy single cell BM reference. Top part of the annotations shows the provided meta data (FAB, WHO and ELN classifications, origin, sex and reported blast percentages) for these cohorts. Each patient was assigned to its most abundant deconvoluted cell type (AML phenotype) and samples within each assignment were sorted according to the assigned phenotype. **b** UMAP plot of deconvolution levels annotated via most abundant cell type as the left panel; and on the right, ECCs of 6 out of 22 cell types were shown in continuous scale (HSC, CD14+ monocyte, Late Erythrocytes, GMP, EMP and cDC). **c** Similar to UMAP plot in (b) but colored with FAB classifications (left panel), and the fractions of cell types for each of these FAB classifications (right panel).

Estimated cell composition recapitulates FAB classes

Clear associations were also observed between cell type assignments by ECC and FAB classification status (left panel of **Figure 4.2C**). Furthermore, distinct distributions of cell type-defining gene expression profiles became evident for each FAB AML type by plotting continuous values of deconvolutions rather than categorical assignments: M0 (minimally differentiated AML) cases had high levels of the HSC-defining signature, whereas these levels decreased and EMP- and GMP signatures appeared in M1 (AML without maturation) and M2 (AML with maturation). As expected, M3 (APL) cases were almost completely dominated by the GMP signature, while M4 (Acute myelomonocytic Leukemia) and M5 (Acute monoblastic/monocytic leukemia) samples resembled CD14+ monocyte cells and GMP. Noticeably, a few samples that were dominated by the GMP signature were assigned to AML M4 or M5 by morphological assessment (top left corner). Further inquiry in LEUCEGENE, which used more detailed FAB annotations, revealed that these AML samples were enriched for M5a (Fisher exact $p = 3.08 \times 10^{-8}$) and M4Eo (Fisher exact $p = 2.06 \times 10^{-6}$) (**Figure S3E**). AML M5a are dominated by monoblastic cells² and AML M4Eo are characterized by myelomonocytic marrow infiltration with eosinophils containing abnormal immature granules³⁴. Both AML subtypes are more differentiated than GMP, but less differentiated and clearly distinct from AML M4 and M5b, which contain more mature promonocytic or monocytic cells. The small groups of M6 (Acute Erythroleukemia) and M7 (Acute Megakaryoblastic Leukemia) AML were dominated by the signatures of Late Erythrocytes and Megakaryocyte Progenitors, respectively. These data demonstrated and confirmed that our single cell guided deconvolution strategy successfully captures the maturational arrest of AML cells at different differentiation stages of hematopoiesis.

Estimated cell composition captures genetic subtype-specific resistances to various drugs

To explore whether ECCs convey information on drug resistances, *ex vivo* drug response data of 122 small molecule inhibitors provided as area under the curves (AUC) for 363 AML samples from BEAT were downloaded. A higher AUC indicates that cancer cells are relatively resistant since higher drug concentrations are needed to induce cell death. To understand whether drug resistance of AML samples can be predicted by ECCs, we trained random forest (RF) models per drug via a leave-one-out cross validation (LOOCV) setting. For each RF model Spearman ρ values were calculated (Supplementary Table S5-S7). The strongest correlation was observed for the BCL-2 inhibitor Venetoclax²⁷ (ABT-199) drug (Spearman $\rho = 0.509$). Drugs like EGF-R inhibitor Erlotinib (Spearman $\rho = 0.376$) and the mTOR pathway inhibitor Rapamycin (Spearman $\rho = 0.368$) are amongst the top 10 drugs for which resistance could be best predicted (**Figure S4A**).

Next, we asked how changes in each cell type affect drug responses and therefore univariately associated ECC to drug resistance (**Figure 4.3A**, Supplementary Table S8). This analysis revealed that most change in resistance occurs across maturational states. For instance, CD14+ Monocytic AML are more resistant to Venetoclax, whereas more immature cell subsets are more sensitive. This trend was also clear after overlaying the AUC values of Venetoclax onto the UMAP plot (**Figure 4.3B**) or when comparing AUC values across states (**Figure S5A**) (one-way ANOVA $p =$

Results

6.31×10^{-13} ; Supplementary Table S9).

We also stratified RF-based predictions according to WHO classifications for all drugs (**Figure S4B, S5B**, Supplementary Table S10). Notably, drug responses to the Erlotinib and Rapamycin showed statistically significant correlations with AML with mutated NPM1 ($n = 77$, $R^2 = 0.29$, $P = 3.7 \times 10^{-7}$) and inv(3) ($n = 6$, $R^2 = 0.91$, $P = 3.2 \times 10^{-3}$), respectively. Also, responses to Flavopiridol (Spearman $\rho = 0.325$), a CDK kinase inhibitor, showed significant correlations with ECC within the group of AML-NOS ($n = 77$, $R^2 = 0.31$, $P = 3.7 \times 10^{-7}$) while showing higher resistance towards AML with more stem cell like cell phenotypes (**Figure S5C, S5D**). These annotations also revealed that PML-RARA-carrying APL samples ($n = 8$) are dominated by GMPs are sensitive for Venetoclax, whereas AML with CBFB-MYH11 ($n = 7$) had the best fit ($R^2 = 0.78$, $P = 0.009$) (**Figure 4.3C**), although the small sample size of CBFB-MYH11 cases prohibits drawing robust conclusions. Larger groups of AML, however, such as NPM1 mutated cases ($n = 55$, $R^2 = 0.33$, $P < 0.001$), and AML-NOS ($n = 42$, $R^2 = 0.17$, $P = 0.007$), together accounting for approximately 58% of AML, also showed a clear trend for Venetoclax resistance (**Figure 4.3C**). Also, within the group of NPM1 mutated samples, which is the largest class of AML, CD14+ monocyte dominated cases were most resistant to Venetoclax (**Figure S5E, S5F**; one-way ANOVA $p = 7.6 \times 10^{-4}$; Supplementary Table S11). In summary, these findings suggest that information on the ECCs of AML will yield therapeutic implications even within one genetic subtype.

a Drug resistance associations with ECCs



Results

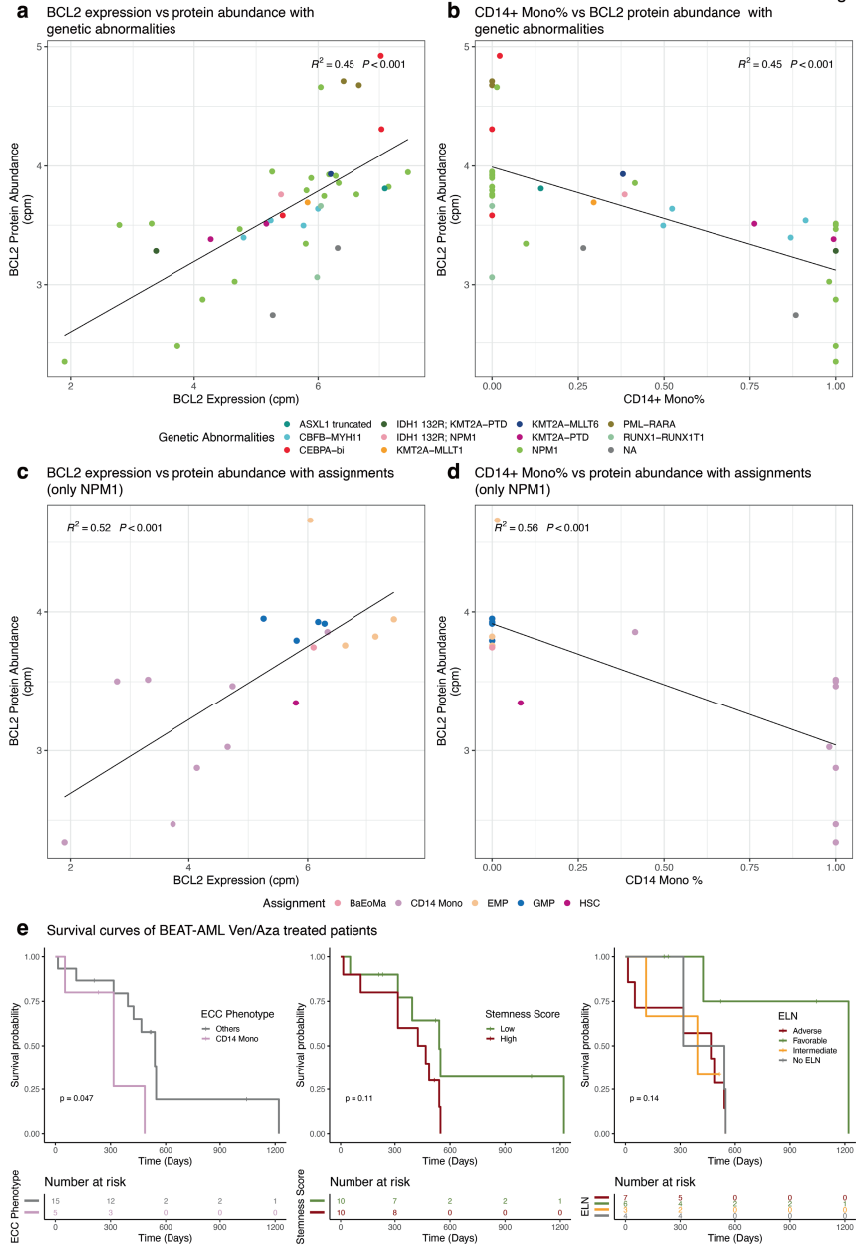
Figure 4.3: **a** Dot plot for association of normalized AUC and ECCs (top 30 drugs). Bigger bubble corresponds to higher correlation, and colors indicate coefficient of the associations. Asterix denotes statistical significance **b** Samples with normalized Venetoclax resistance data are shown on the UMAP plot, along with phenotype map from (Fig. 2b). Patients without available data were shown as gray dots for the sake of completeness. **c** Random Forest predictions of Venetoclax resistance per WHO class. **d** Venetoclax resistance vs *BCL-2* expression; also, CD14+ monocyte percentages for these patients were annotated. Expression data was count-per-million (cpm) normalized. **e** Univariate analyses of different attributes (i.e., CD14+ monocyte%, reported blast percentages etc.) vs normalized Venetoclax resistance. P-values were corrected using Benjamini-Hochberg procedure and the names of the attributes are shown for FDR>0.01. **f** Similar to (e); A multivariate model is associated with different attributes, and the names of the attributes with P>0.01 is shown. Full list of attributes' p-values and estimates are provided in Supplementary Table for both (e) and (f).

Estimated CD14+ monocyte percentages predict Venetoclax resistance better than *BCL-2* mRNA expression

Since Venetoclax is targeting the anti-apoptotic *BCL-2* protein, we next checked *BCL-2* mRNA expression levels in AML and overlaid the CD14+ monocyte percentages for these cases (Figure 4.3D). The data showed that low *BCL-2* expression indeed correlated with strong resistance to Venetoclax. However, a few samples were resistant despite relatively high *BCL-2* expression (samples at upper right corner in Figure 4.3D). As the majority of these cases had a CD14+ monocyte phenotype, we univariately associated *BCL-2* expression and CD14+ monocyte percentage to investigate which factor best explains the Venetoclax response (Figure 4.3E). We also associated metadata such as reported blast percentages, ELN status and primary diagnosis to determine their effect sizes and significance (Supplementary Table S12). This analysis demonstrated that both CD14+ monocyte percentages (adjusted $P = 1.7 \times 10^{-18}$) and *BCL-2* mRNA expression (adjusted $P = 4.8 \times 10^{-14}$) were associated significantly with the Venetoclax response. Furthermore, we investigated whether previously reported MAC Score³⁵ (albeit in gene expression) was more associated to Venetoclax resistance compared to *BCL-2* gene expression alone ($R = -0.54$, and $R = -0.53$), but the results were similar, and the estimated CD14+ monocyte percentages had a stronger correlation ($R = 0.60$) (Figure S6E). Next, we created a multivariate model to investigate whether the significance of CD14+ monocyte percentages diminishes along with the presence of *BCL-2* expression in the same model (Figure 4.3F, Multivariate Venetoclax Tab in Supplementary Table 13). In this model, CD14+ monocyte percentages remained significantly associated with Venetoclax resistance ($P = 1.92 \times 10^{-5}$), while *BCL-2* expression was above the significance threshold of 0.01 ($P = 0.015$). In conclusion, these results indicate that cellular composition is a more robust marker than *BCL-2* mRNA expression to predict Venetoclax resistance, specifically for AML from NOS and NPM1 mutated patients.

A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML

Figure 4



Discussion

Figure 4.4: **a** *BCL-2* expression vs protein abundance ($n = 39$) annotated with genetic abnormalities ($R^2 = 0.45$, $P < 0.001$). Each dot represents a sample. **b** CD14+ monocytes percentage vs *BCL-2* protein abundance annotated with genetic abnormalities ($R^2 = 0.45$, $P < 0.001$). **c** Similar to **a**; but only limited to NPM1 mutated samples ($n = 18$) and annotated with patients' AML phenotypes (most abundant cell type) ($R^2 = 0.52$, $P < 0.001$) **d** Similar to **b**; but only limited to NPM1 mutated samples ($n = 18$) and annotated with patients' AML phenotypes (most abundant cell type) ($R^2 = 0.56$, $P < 0.001$). **e** Kaplan-Meier curves of Ven/Aza treated BEAT-AML patients. ECC based annotation (left), stemness score (middle) and ELN classification (right). Log-rank test was used to calculate p-values.

Estimated CD14+ monocyte percentages associates with *BCL-2* protein abundance

Next, we compared the effects of *BCL-2* gene expression and CD14+ monocyte percentages with *BCL-2* protein abundance within each sample. For this purpose, we used our LUMC produced proteomics data, and after batch correction (Figure S6A, S6B, see Methods) for matched AML cases ($n = 39$; LUMC) to correlate the abundance of Apoptosis regulator *BCL-2* protein vs gene expression (Figure S6C) and CD14+ monocyte percentages (Figure S6D). The data showed that *BCL-2* gene expression and CD14+ monocyte percentages both correlated with *BCL-2* protein abundance to a similar extent ($R^2 = 0.45$, $P < 0.001$). We also overlaid plots for *BCL-2* expression and CD14+ monocyte percentages with information on patients' genetic abnormalities (Figure 4.4A). Based on their low AUC for Venetoclax response (Figure 4.3B), two AML cases with PML-RARA had high *BCL-2* protein expression ($n=2$, dark green). We also confirmed the strong variability in AUC for Venetoclax response within the group of AML with mutated NPM1 ($n=18$, green) by showing two distinct groups at the extremes of CD14+ monocyte percentages which correlated with *BCL-2* protein expression (Figure 4.4B). Within the group of AML patients with mutated NPM1, CD14+ monocyte percentage associated stronger with both *BCL-2* protein abundance ($R^2 = 0.56$, $P < 0.001$) than *BCL-2* gene expression ($R^2 = 0.52$) (Figure 4.4C, 4D). In conclusion, the data demonstrate the relevance of our deconvolution approach on bulk RNA-Seq to separate AML, especially those with mutated NPM1, with high and low monocyte percentages to predict the patient's response to Venetoclax.

Estimated CD14+ monocyte phenotype captures Venetoclax treated patient prognosis

To test whether deconvolution approach associates with clinical outcome, we utilized BEAT-AML study and selected Venetoclax/Azacitidine (Ven/Aza) treated patients with overall survival and RNA-seq data ($n=20$). Deconvolution revealed that 5 of these patients had a CD14+ monocytic phenotype and these patients had a significantly worse overall survival ($p=0.047$) compared to others (Figure 4.4E; Supplementary Table S14). On the contrary, stemness score²⁴ ($p=0.11$) and ELN classification ($p=0.14$) were not able to fully split these patients with the notable exception of ELN favorable patients. In summary, this analysis highlights the clinical significance of employing ECC-based annotation for Ven/Aza treated patients.

4.4 Discussion

In this work, we utilized single cell guided deconvolution to decompose bulk transcriptomics data from five independent AML cohorts and show that the obtained estimated cell compositions (ECCs) faithfully reconstitute the FAB landscape (M0-M7). Moreover, using same-sample flow cytometry, we were able to validate our deconvolution framework. Hence, following our previous work using deep transcriptomics to call various types of leukemia-defining genetic aberrations^{10,26}, our current findings further underpin the power of transcriptomic-based approaches as a comprehensive and versatile platform for AML diagnosis. We next illustrated the potential use of our deconvolution framework for precision medicine applications, by correlating

the estimated ECC to the results of an *ex vivo* drug resistance screening of 122 small-molecule inhibitors in the BEAT-AML study. For the *BCL-2* inhibitor Venetoclax we show that higher levels of the estimated ECC subset 'CD14+ monocyte' correspond to a higher resistance, and intriguingly, that estimated CD14+ monocyte levels is a better explanatory variable of resistance to Venetoclax than *BCL-2* expression alone. Nevertheless, using same-sample LUMC proteomics data in 39 patients, we show that the estimated CD14+ monocyte levels accurately mark *BCL-2* protein expression, and that for *NPM1*-mutated patients the presence or absence of a CD14 monocytic outgrowth corresponds with a distinct *NPM1* protein abundance. Lastly, we show that CD14 monocyte phenotype correlates with poor survival outcome. Our findings may potentially have important implications on drug use especially for genetically uncharacterized patients (AML, NOS) currently accounting for ~40% of all AML as well as other well-characterized patients such as *NPM1* mutated samples.

Kuusanmaki *et al.* reported that monocytic differentiation of AML reduced sensitivity to Venetoclax *ex vivo*⁸, and also with a recent paper they show *ex-vivo* drug responses correlate with AML response in clinic²⁹. Similarly, Pei *et al.* reported that the different monocytic subclones in vivo created resistance to Venetoclax treatment⁹, and also recently, White *et al.* showed that *BCL-2* inhibitor resistance could be predicted via the genes associated with monocytes. Recently, using the flow cytometry data from untreated MDS patients Ganan-Gomez *et al.* showed that after relapse and becoming secondary AML (sAML) patients, those patients with less matured cell types (EMP) before treatment had a faster complete remission (CR) and longer relapse free status compared to more matured cell type (GMP) with Venetoclax treatment, supporting our hypothesis that ECCs could also predict Venetoclax resistance for MDS and sAML patients²⁸. Collectively, these studies suggest that Venetoclax has different resistances at different maturational stages, and especially higher resistance for patients with CD14+ monocyte dominated phenotype as we have shown in this manuscript, and we provide an open-source framework, *seAMLess*, for replicating our results or applying it to other clinically relevant datasets.

A limitation of our deconvolution strategy is that it cannot distinguish the cancerous cell types as it uses a healthy bone marrow as a reference. Although it is conceptually appealing, we have three rationales behind not using a cancerous reference. First, without mutation calling for all cells, one cannot be sure whether a cell is cancerous or not. Strategies like predicting cancer cells based on their transcriptional similarity of cells with mutation calling, proposed by Van Galen *et al.*¹⁶, adds another level of ambiguity to already not perfect deconvolution pipelines. Secondly and more importantly, heterogeneity of AML causes further sub-clustering within individual AML cases (e.g. UMAP plots of Triana *et al.*, 2021), therefore creating a not well-characterized cell type signature but rather patient specific clusters^{17,19}. Lastly, using healthy subsets as reference allows our framework to provide more interpretable and intuitive results for clinicians and doctors, as it reports immune phenotypes and percentages on contrary to score-based prognostic values^{15,24,26}. To summarize, we believe our proposed pipeline could be a blueprint for assessing new drugs' resistances on different cell types of AML and along with our framework, they may provide better insights for clinicians and help paving the way into precision medicine in AML.

4.5 Methods

Creating the healthy BM single-cell reference

We downloaded three different healthy BM datasets from two different studies, namely Van Galen *et al.*¹⁶ (full-transcriptome, n=6,915), and Triana *et al.*¹⁷ (full-transcriptome, n = 13,165; 462

Methods

targeted mRNA, $n=49,057$). Then, all cell labels were uplifted up via *Seurat* package³⁰ (v4.0.3) default query annotation pipeline to match with the Triana's full-transcriptome cell labels as it had the most recent and detailed labels. Next, *Seurat*'s integration pipeline with CCA was run and the cells that were labeled as doublets/multiplets were removed from the down-stream analyses, and this yielded a healthy BM atlas of 69,130 cells in total, covering 439 genes. Also, we used the 40,000 cell subset of HCA provided by *SeuratObject* package (v4.0.2) for the validation analyses (Figure 4.1C, 1D).

Different schemes of creating pseudobulks

To create a cancerous-like pseudobulk profile from healthy BM reference and HCA subset, we selected a total of 1,000 cells for each profile, majority (80%) of them coming from a cell-type (over-abundant) and the rest of the cells were distributed according to inverse proportion of the numbers of cells for the remaining cell types. To achieve this, first integrated counts were exponentiated to make them non-log scale and *slice_sample* function from *dplyr* package (v1.0.7) was used with a replacement option. Then, these non-log scale cell counts were summed up to create the pseudobulk profiles. For individual-based pseudobulk, *AggregateExpression* from *Seurat* package was used.

Flow Cytometry data

AML cases were stained with fluorescent antibodies and analyzed by flow cytometry for diagnosis, prognosis, and disease monitoring of AML in the diagnostic laboratory of the department of Hematology in the Leiden University Medical Center. The flow cytometric test has been developed and performed according to EuroFlow standard operating procedures (<http://www.euroflow.org>)¹⁹. EuroFlow antibody combinations have been tested against references databases of normal cells from healthy individuals and allow multidimensional identification and distinction of aberrant cells from normal cell populations. The flow cytometric test includes 8 tubes with different antibody combinations, i.e. one ALOT tube (acute leukemia orientation tube) and 7 AML tubes (AML1-7). The ALOT tube contains antibodies against CD3, CD45, MPO, CD79, CD19 and CD7. The AML tubes contain antibodies against CD16, CD13, CD34, CD117, CD11b, CD10, HLA-DR and CD45 (AML-1), CD35, CD64, CD34, CD117, CD300e/IREM2, CD14, HLA-DR and CD45 (AML-2), CD36, CD105, CD34, CD117, CD33, CD71, HLA-DR and CD45 (AML-3), NuTdT, CD56, CD34, CD117, CD7, CD19, HLA-DR and CD45 (AML-4), CD15, NG2, CD34, CD117, CD22, CD38, HLA-DR and CD45 (AML-5), CD42a and CD61, CD203c, CD34, CD117, CD123, CD4, HLA-DR and CD45 (AML-6). AML tube 7 contains antibodies against CD41, CD25, CD34, CD117, CD42b, CD9, HLA-DR and CD45, but this tube has not been used to stain AML cases analyzed in this study.

Obtaining bulk transcriptomic data and their preprocessing

We have downloaded the non-normalized count matrices (htseq-counts) and the meta files of the four discovery cohorts (TCGA-LAML, BEAT-AML, TARGET-AML and TARGET-ALL) from <https://portal.gdc.cancer.gov>. For LEUCEGENE, count data was downloaded from their dedicated site (<https://data.leucegene.iric.ca/>) along with their provided meta data. All meta/count data were pre-processed using *R* (v4.1.0). For the meta data, genomic aberration labels were relabeled to the main AML WHO 2016 classes, non-AML samples were removed from the down-stream analyses, ELN-classes were relabeled according to ELN 2017 recommendations. For the count data, ERCC spike-ins and mitochondrial genes were removed, and the count matrix was then sorted according genes standard deviation in order to remove the duplicated genes that had less variation thus providing less information, and lastly the gene ensembl ids were converted to gene symbols. Before converting ensembl ids into gene symbols, the stemness score for each

patient was calculated via count-per-million (cpm) normalized libraries, and these libraries were normalized using *cpm* function from *edgeR* package³¹ (v.3.34.1).

Our 100 AML samples (LUMC) deposited to EGA with accession number EGAS00001003096 and they are accessible upon request. QC benchmark analyses for these samples were done in our previous paper¹⁰. Therefore, we ran default *HT-SEQ* pipeline (v0.11.2) with paired-end option aligning fastq files to *hg38* to obtain the count matrix. All above mentioned preprocessing steps (filtering, gene name conversion) were also conducted for these samples as well before deconvolution.

Deconvolution pipeline

To deconvolute the simulated pseudobulks and bulk RNA-seq AML patients, we used *MuSiC*¹² package (v0.1.1) as it benchmarked highly and consistently across different cell types at various settings¹¹ and had an easy-to-use open-source (GPL-3) implementation (<https://github.com/xuranw/MuSiC>). We used non-log scaled count values as inputs and set the normalization option to false. Patients were assigned to the groups (e.g., GMP, CD14+ monocyte etc.) according to their most abundant deconvoluted ECCs. In the heatmap (Figure 4.2A), patients were re-ordered according to their ECCs within each assignment.

UMAP of estimated cell compositions

First, to obtain reproducible results with umap plots, we set a seed to 2 as UMAP procedure involves random initialization. Then, we ran *umap* function with default parameters from *umap* package (v0.2.7) and used the first two reduced dimensions to create the plots. All related figures were plotted using *ggplot2* package (v3.3.5).

Drug resistance predictions via Random Forest

BEAT-AML has drug resistance data for 122 small-molecule inhibitors, we downloaded these from their manuscript (Supplementary Table S5). Then, each drug response was min-max normalized, then matched with their available RNA-seq samples. Next, drug resistances were predicted with the deconvoluted ECCs. Random forest algorithm from *randomForest* package (v4.6-14) with default parameters was used for the predictions. Each sample within each drug was predicted at leave-one-out cross-validation settings. Then, for each drug, Spearman ρ values were calculated between predicted and actual drug resistance values. To stratify the drugs according to their primary diagnosis of WHO classification, samples within each diagnosis are selected and then each drugs predicted, and normalized drug resistance were associated, then the correlation and p-values were calculated using *summary* function in base R.

Venetoclax Association Analysis

First, each attribute was associated to standardized (min-max normalized) Venetoclax resistance from BEAT-AML study at univariate settings using *lm* function in R environment (v4.1.0) (Supplementary Table S12). Then, p-values and coefficients were calculated using *summary* function and then p-values were multiple hypothesis corrected using Benjamini-Hochberg procedure. For multivariate models, FAB classification, and ECC levels (except CD14+ monocyte) were excluded as only 76 out of 460 samples of BEAT-AML had FAB classifications and as other ECCs are not independent of CD14+ monocyte percentages (as the question is whether CD14+ monocyte levels are independently predictive of Venetoclax resistance given *BCL-2* expression in the same model). Again, p-values and coefficients were calculated with *summary* function and plotted in a volcano plot (Figure 4.3F, Supplementary Table S13).

Survival analysis

Methods

ggsurvival and *survminer* R packages was used for producing Kaplan Meier curves. P-values were calculated with log-rank test. For ECC graph, patients were annotated with their most abundant cell type. Stemness score is split into low and high categories using the median value.

Proteomics Sample preparation

Cell lysis, digestion and TMT labeling was performed as described in Paula and et al³²¹¹. Cell lysis was performed using 5% SDS lysis buffer (100 mM Tris-HCl pH7.6) and 5 U benzonase nuclease (Thermo Scientific) with incubation at 95 °C for 4 minutes. Protein concentration was determined using Pierce BCA Gold protein assay (Thermo Fisher Scientific). 100 µg protein of each sample was then reduced with 5 mM TCEP. Reduced disulfide bonds were alkylated using 15 mM iodoacetamide. Excess iodoacetamide was quenched using 10 mM DTT. Protein lysates were precipitated using chloroform/methanol; resulting pellets were re-solubilized in 40 mM HEPES pH 8.4 and digested using TPCK treated trypsin (1:12.5 enzyme/protein ratio) overnight at 37 °C. Peptide concentration was then determined using Pierce BCA Gold protein assay.

The different samples, and reference samples, were arranged into five TMTpro 16plex sets. The peptides were labeled with TMTpro Label Reagents (Thermo Fisher Scientific) in a 1:4 ratio by mass (peptides/TMT reagents), total volume was 35 µL, for 1 h at RT. Excess TMT reagent was quenched with 5 µL 6% hydroxylamine for 15 min at RT. Samples corresponding to a TMT set were pooled and lyophilized.

Each TMT sample (80 ug) was fractionated by high pH reverse phase chromatography on a Zorbax RRHD Eclipse Plus C18 2.1x150 mm 1.8-micron column, at 800 ul/min using an Agilent1200 binary HPLC system, equipped with a UV detector. The mobile phases were 10 mM Ambic pH 8.4 (A) and 10 mM Ambic/Acetonitrile 20/80 pH 8.4 (B). The gradient was from 2% to 90%B in 35 minutes. 20 fractions were collected in a circular fashion, i.e., collection per vial for 20 sec before moving to the next collection vial. After collection in the last vial collection is continued in the first vial. Fractions were subsequently freeze dried.

Mass spectrometry

TMT-labeled peptide fractions were dissolved in water/formic acid (100/0.1 v/v) and analyzed by on-line C18 nanoHPLC MS/MS with a system consisting of an Ultimate3000nano gradient HPLC system (Thermo, Bremen, Germany), and an Exploris480 mass spectrometer (Thermo) as in Rossi et al³³. Fractions were injected onto a cartridge precolumn (300 µm × 5 mm, C18 PepMap, 5 µm, 100 Å, and eluted via a homemade analytical nano-HPLC column (50 cm × 75 µm; Reprosil-Pur C18-AQ 1.9 µm, 120 Å (Dr. Maisch, Ammerbuch, Germany)). Solvent A was water/formic acid 100/0.1 (v/v). The gradient was run from 2% to 40% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 120 min. The nano-HPLC column was drawn to a tip of 10 µm and acted as the electrospray needle of the MS source. The mass spectrometer was operated in data-dependent MS/MS mode with a cycle time of 3 seconds, with the HCD collision energy at 36 V and recording of the MS2 spectrum in the orbitrap, with a quadrupole isolation width of 1.2 Da. In the master scan (MS1) the resolution was 120,000, the scan range 350-1200, at standard AGC target @maximum fill time of 50 ms. A lock mass correction on the background ion $m/z=445.12003$ was used. Precursors were dynamically excluded after $n=1$ with an exclusion duration of 45 s, and with a precursor range of 20 ppm. Charge states 2-5 were included. For MS2 the first mass was set to 110 Da, and the MS2 scan resolution was 45,000 at an AGC target of 200% @maximum fill time of 60 ms.

Proteomics data processing and down-stream analysis

In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer

version 2.4 (Thermo Electron), and submitted to the Uniprot database (Homo sapiens, 20596 entries), using Mascot v. 2.2.07 (www.matrixscience.com) for protein identification. Mascot searches were performed with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and the enzyme trypsin was specified. Up to two missed cleavages were allowed. Methionine oxidation and acetyl on protein N-terminus were set as variable modifications. Carbamidomethyl on Cys and TMTpro on Lys and N-terminus were set as fixed modifications. Protein and peptide FDR were set to 1%. Normalization was on the total peptide amount. The 5 TMT-16plex analyses were normalized to each other by the bridge samples.

First, the abundance data is log-cpm transformed to stabilize variance among samples and then to ensure dealing with the technical batch effects, we ran *removeBatchEffect* function from *limma* package (v3.48.3) providing batch information. Then, we overlaid the transformed protein abundances onto a PCA plot to ensure that there is no batch related clustering, as can be observed from the positioning of the TMT control samples before (**Figure S6A**) and after correction (**Figure S6B**). The down-stream analyses were then done using only the primary AML (n=39). Next, the transformed BCL-2 protein abundance was associated with the BCL-2 expression from LUMC cohort (**Figure 4.4A**) and CD14+ monocyte percentage within each sample (**Figure 4.4B**) (Supplementary Table S14). R^2 and p-values were calculated using *stat_poly_eq* function from *ggpmisc* (v0.4.5) package and lines were drawn with *geom_smooth* function from *ggplot2* package (v3.3.5) via method option set to linear model.

4.6 Acknowledgements

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4.7 Author Contributions

M.J.R., E.v.d.A., and M.G. conceptualized the study. Resources were provided by E.v.d.A. Methodology was developed by E.O.K., E.v.d.A., P.v.A., and A.M.O. The investigation was performed by E.O.K., J.S., M.Z., M.G., E.v.d.A., and P.v.A. Visualization was carried out by E.O.K. Funding acquisition, project administration, and supervision were led by E.v.d.A., with additional supervision from M.J.R. and M.G. The original draft was written by E.O.K., M.G., and E.v.d.A. All authors contributed to the review and editing of the manuscript.

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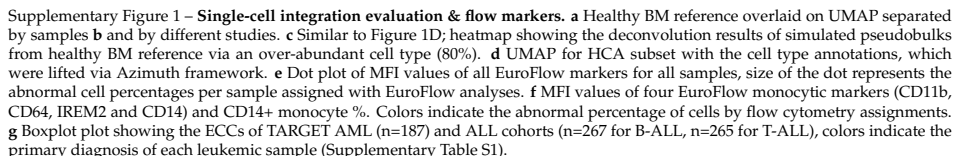
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4.9 Appendix

4.9.1 Supplementary Tables

The appendix was shortened due to environmental considerations. To minimize unnecessary printing and reduce material use, extensive data including 14 supplementary tables are provided online at: <https://doi.org/10.1038/s41698-024-00596-9>.

4.9.2 Supplementary Figures



A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML

Figure S2

a Patient meta summary from all studies

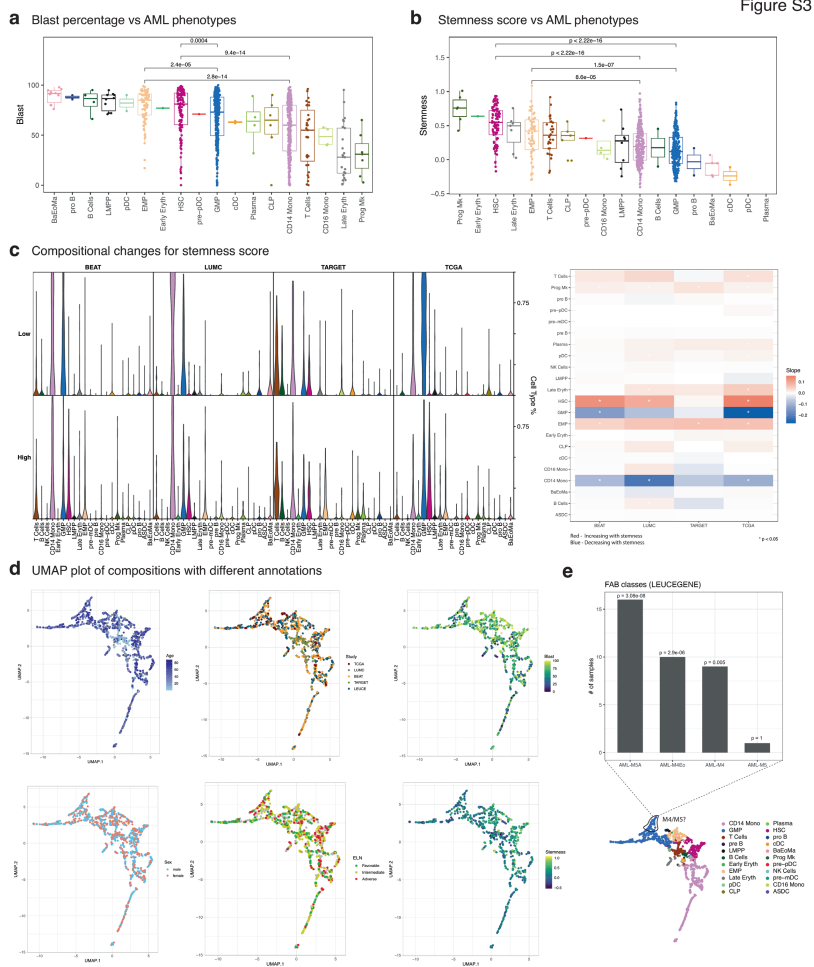
Characteristic	Overall, N = 1,267 ^a	BEAT, N = 412 ^a	LEUCE, N = 452 ^a	LUMC, N = 96 ^a	TARGET, N = 156 ^a	TCGA, N = 151 ^a
Age	55 (0, 89)	62 (2, 87)	57 (17, 87)	NA (Inf, -Inf)	9 (0, 23)	56 (22, 89)
Unknown	97	1	0	96	0	0
Sex						
female	567/1,267 (45%)	183/412 (44%)	195/452 (43%)	44/96 (46%)	77/156 (49%)	68/151 (45%)
male	700/1,267 (55%)	228/412 (56%)	257/452 (57%)	52/96 (54%)	79/156 (51%)	83/151 (55%)
FAB						
M0	51/761 (6.7%)	5/90 (5.6%)	27/381 (7.1%)	0/0 (NA%)	4/140 (2.9%)	15/150 (10%)
M1	189/761 (25%)	7/90 (7.8%)	129/381 (34%)	0/0 (NA%)	17/140 (12%)	36/150 (24%)
M2	138/761 (18%)	10/90 (11%)	54/381 (14%)	0/0 (NA%)	37/140 (26%)	37/150 (25%)
M3	56/761 (7.4%)	11/90 (12%)	30/381 (7.9%)	0/0 (NA%)	0/140 (0%)	15/150 (10%)
M4	155/761 (20%)	24/90 (27%)	59/381 (15%)	0/0 (NA%)	43/140 (31%)	29/150 (19%)
M5	145/761 (19%)	31/90 (34%)	69/381 (18%)	0/0 (NA%)	30/140 (21%)	15/150 (10%)
M6	14/761 (1.8%)	0/90 (0%)	10/381 (2.6%)	0/0 (NA%)	2/140 (1.4%)	2/150 (1.3%)
M7	13/761 (1.7%)	2/90 (2.2%)	3/381 (0.8%)	0/0 (NA%)	7/140 (5.0%)	1/150 (0.7%)
Unknown	506	322	71	96	16	1
ELN						
Adverse	197/686 (29%)	148/390 (38%)	0/0 (NA%)	0/0 (NA%)	9/148 (6.1%)	40/148 (27%)
Favorable	203/686 (30%)	107/390 (27%)	0/0 (NA%)	0/0 (NA%)	64/148 (43%)	32/148 (22%)
Intermediate	286/686 (42%)	135/390 (35%)	0/0 (NA%)	0/0 (NA%)	75/148 (51%)	76/148 (51%)
Unknown	581	22	452	96	8	3
Blast	72 (0, 100)	60 (0, 98)	77 (1, 99)	75 (15, 99)	74 (14, 100)	71 (0, 100)
Unknown	144	109	31	0	4	0
Primary Diagnosis						
Acute myeloid leukemia with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); RPN1-EV1	17/1,267 (1.3%)	8/412 (1.9%)	7/452 (1.5%)	2/96 (2.1%)	0/156 (0%)	0/151 (0%)
Acute myeloid leukemia with mutated CEBPA	37/1,267 (2.9%)	28/412 (6.8%)	0/452 (0%)	0/96 (0%)	9/156 (5.8%)	0/151 (0%)
Acute myeloid leukemia with mutated NPM1	163/1,267 (13%)	96/412 (23%)	0/452 (0%)	27/96 (28%)	7/156 (4.5%)	33/151 (22%)
Acute myeloid leukemia with myelodysplasia-related changes	213/1,267 (17%)	88/412 (21%)	114/452 (25%)	11/96 (11%)	0/156 (0%)	0/151 (0%)
Acute myeloid leukemia with t(10;11)(p12;q23.3); MLLT10-KMT2A	9/1,267 (0.7%)	0/412 (0%)	0/452 (0%)	0/96 (0%)	9/156 (5.8%)	0/151 (0%)
Acute myeloid leukemia with t(8;11)(q27;q23.3); MLLT4-KMT2A	5/1,267 (0.4%)	0/412 (0%)	0/452 (0%)	0/96 (0%)	5/156 (3.2%)	0/151 (0%)
Acute myeloid leukemia with t(8;9)(p23;q34); DEK-NUP214	8/1,267 (0.6%)	3/412 (0.7%)	3/452 (0.7%)	1/96 (1.0%)	1/156 (0.6%)	0/151 (0%)
Acute myeloid leukemia with t(8;21)(q22;q22); RUNX1-RUNX1T1	60/1,267 (4.7%)	9/412 (2.2%)	20/452 (4.4%)	3/96 (3.1%)	21/156 (13%)	7/151 (4.6%)
Acute myeloid leukemia with t(8;11)(p22;q22); MLLT3-MLL	35/1,267 (2.8%)	9/412 (2.2%)	8/452 (1.8%)	4/96 (4.2%)	12/156 (7.7%)	2/151 (1.3%)
Acute myeloid leukemia, CBF-beta/MYH11	101/1,267 (8.0%)	26/412 (6.3%)	29/452 (6.4%)	10/96 (10%)	28/156 (18%)	8/151 (5.3%)
Acute myeloid leukemia, NOS	492/1,267 (39%)	91/412 (22%)	215/452 (48%)	35/96 (36%)	64/156 (41%)	87/151 (58%)
Acute promyelocytic leukaemia, PML-RAR-alpha	62/1,267 (4.9%)	17/412 (4.1%)	29/452 (6.4%)	2/96 (2.1%)	0/156 (0%)	14/151 (9.3%)
Myeloid sarcoma	3/1,267 (0.2%)	3/412 (0.7%)	0/452 (0%)	0/96 (0%)	0/156 (0%)	0/151 (0%)
Therapy related myeloid neoplasm	62/1,267 (4.9%)	34/412 (8.3%)	27/452 (6.0%)	1/96 (1.0%)	0/156 (0%)	0/151 (0%)

^a Median (Range) or Frequency (%)

b AML studies on ternary plot



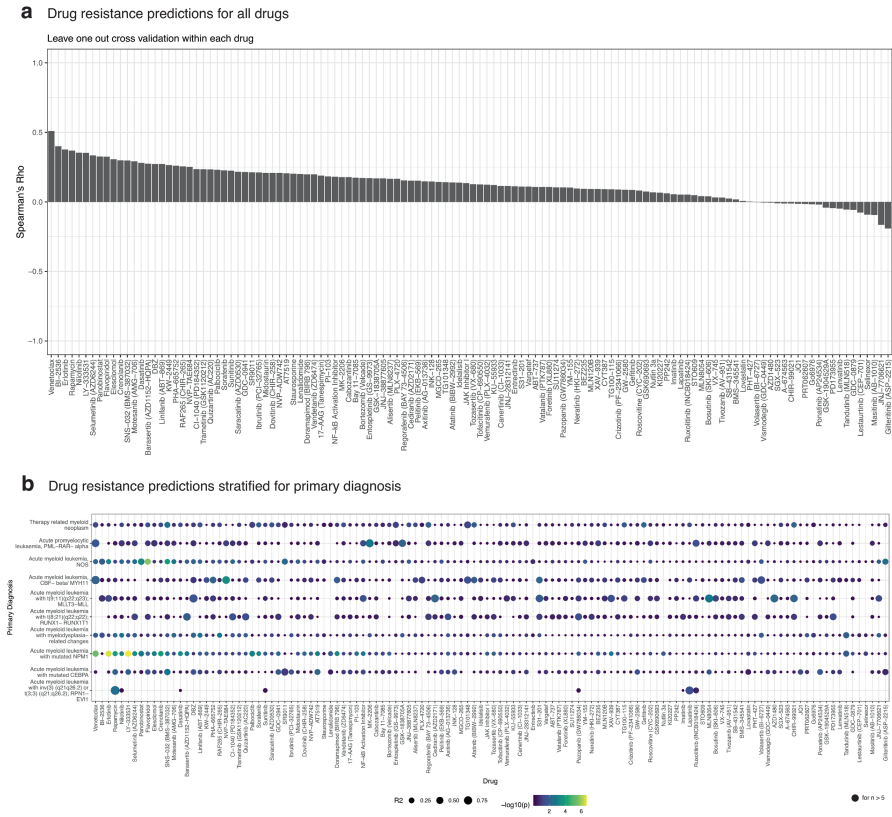
Supplementary Figure 2 – Overview of AML cohort. a Detailed table for sample characteristics for age, sex, FAB, ELN, and primary diagnosis (WHO) for each cohort. Missing data is labeled as 'Unknown' for each attribute. *AML, NOS patients represent %39 of WHO classes. b Ternary plots like Figure 1G for all five AML cohorts.



Supplementary Figure 3 - **Stemness score and meta data on UMAP.** **a** Box plots of blast percentage vs AML phenotype and **b** Stemness score vs AML phenotype. Boxplots were sorted according to median values. **c** Compositional changes of each cohort (except LEUCEGENE as it lacks entire list of genes to calculate stemness score) for low and high stemness score (split by median). Violin plots show the deconvoluted cellular percentages for 22 cell type and heatmap on the right panel summarizes the changes. A linear model is fit to calculate the slope and significance per cell type vs. stemness, and a red slope indicates more of that cell type with higher stemness score, and blue is the vice versa. Asterisk indicates a statistically significant trend ($P \leq 0.05$). **d** UMAP plots of deconvoluted percentages (ECCs) annotated via age, study, reported blast level, sex, ELN and stemness score. **e** Bar plot of extended FAB classifications of LEUCEGENE patients with GMP assignment and M4/5 morphology

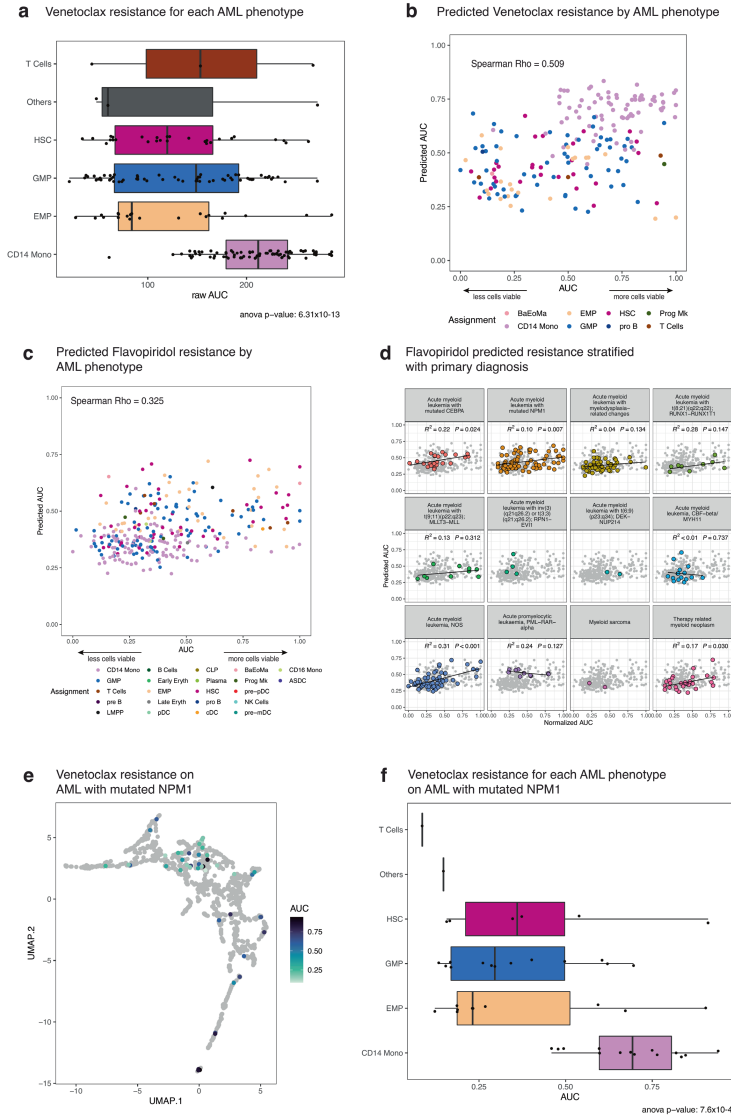
A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML

Figure S4



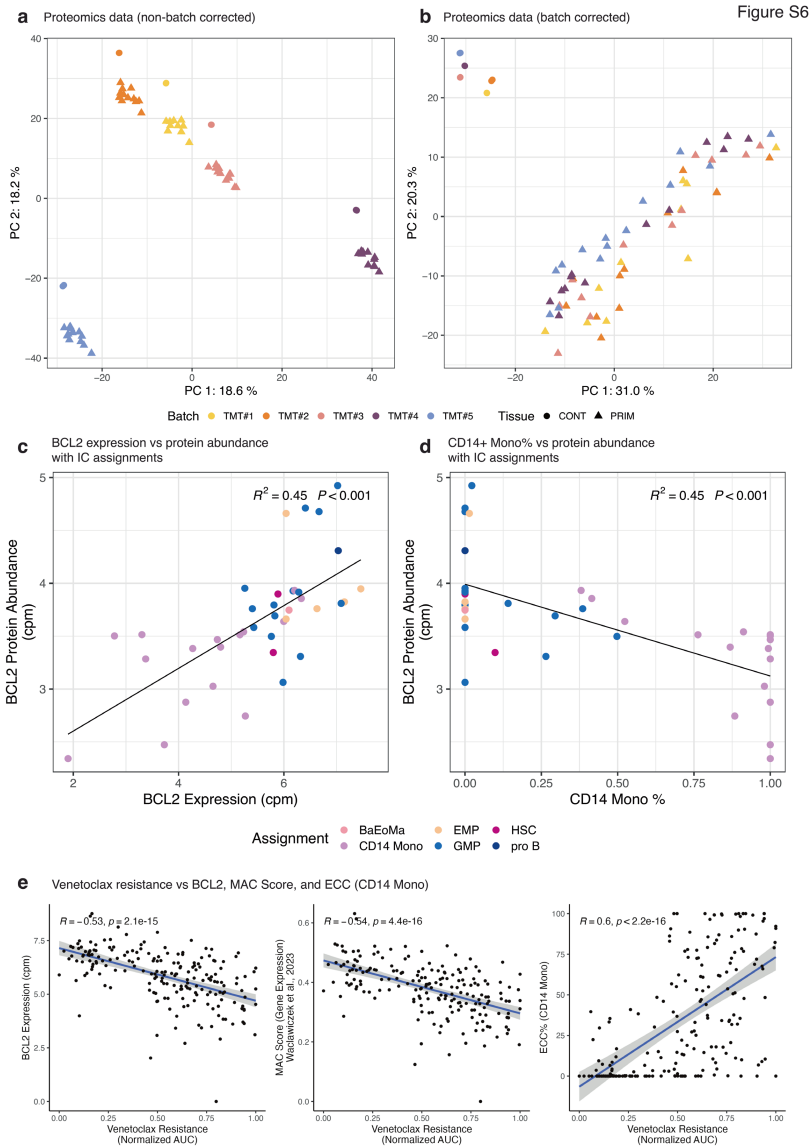
Supplementary Figure 4 - Summary plots for ex-vivo drug response predictions. **a** Spearman ρ values for the predictions of the drug resistance of 122 small molecule inhibitors from BEAT-AML (see Supplementary Table S7). For each drug, the resistance per sample is predicted via random forest algorithm using ECCs at leave-one-out cross validation setting. **b** Dot plot showing the stratified associations with predicted resistance values. Colors of the dot show the significance and sizes indicate the correlation strength. Groups with more than 5 samples were included in the plot.

Figure S5



Supplementary Figure 5 - Venetoclax response on different WHO classes. **a** Venetoclax resistance split by AML phenotypes. All drugs' one-way anova p values are provided in Supplementary Table S10. **b** Venetoclax resistance predictions with random forest annotated with patients' AML phenotype. **c** Flavopiridol resistance predictions with random forest annotated with patients' AML phenotype. **d** Flavopiridol resistance predictions are faceted and highlighted by WHO classes, classes with >5 samples were fit linear models thus R2 and p values are shown. **e** UMAP plot showing Venetoclax resistance only for NPM1 mutated samples **f** Venetoclax resistance split by AML phenotype only for NPM1 mutated samples.

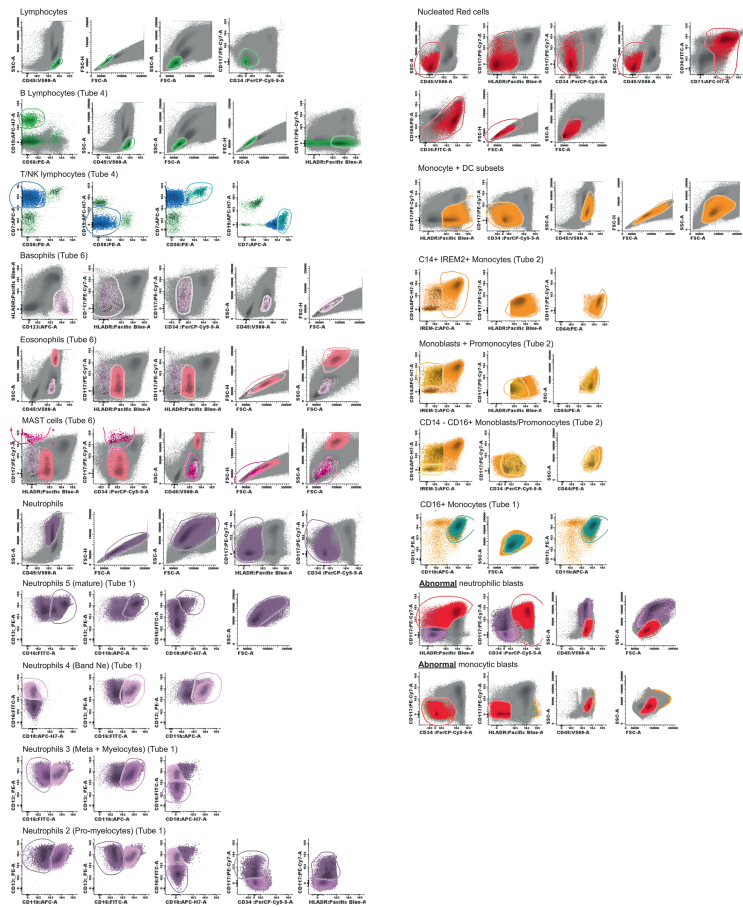
A Transcriptomic-based Deconvolution Framework for Assessing Differentiation Stages and Drug Responses of AML



Supplementary Figure 6 - QC plots & BCL-2 expression for proteomics. **a** PCA plot of proteomics data (primary AML $n = 39$) without batch correction with TMT controls and **b** same samples with batch correction. All samples are log-cpm normalized. **c** BCL-2 expression vs protein abundance annotated with patients' AML phenotypes ($R^2 = 0.45$, $P < 0.001$). **d** CD14+ monocyte percentage vs BCL-2 gene expression annotated with patients' AML phenotypes ($R^2 = 0.45$, $P < 0.001$). **e** Venetoclax resistance in ex-vivo compared to: BCL-2 gene, MAC Score, BCL-2/(BCL-xL+MCL1) calculated with corresponding gene signatures, and the estimated CD14 Mono percentages.

a Flow gating strategy on Euroflow Panel

Figure S7



Supplementary Figure 7 - **Flow gating strategy for EuroFlow panels.** a An example of EuroFlow gating strategy on one of our flow samples. Seven tubes (ALOT orientation tube + 6 AML assignment tubes) were used per patient to sort normal-like cell (lymphocytes, eosinophils, neutrophils etc.) and abnormal cell subsets (abnormal neutrophilic blast, abnormal monocytic blast). Different colors indicate related cell (sub)types.



Chapter 5

Mapping AML Heterogeneity: Multi-cohort transcriptomic analysis identifies novel clusters and divergent ex-vivo drug responses

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5.1 Abstract

Subtyping of acute myeloid leukaemia (AML) is predominantly based on recurrent genetic abnormalities, but recent literature indicates that transcriptomic phenotyping holds immense potential to further refine AML classification. Here we integrated five AML transcriptomic datasets with corresponding genetic information to provide an overview (n=1224) of the transcriptomic AML landscape. Consensus clustering identified 17 robust patient clusters which improved identification of *CEBPA*-mutated patients with favourable outcomes, and uncovered transcriptomic subtypes for *KMT2A* rearrangements (2), *NPM1* mutations (5), and AML with myelodysplasia-related changes (AML-MRC) (5). Transcriptomic subtypes of *KMT2A*, *NPM1* and AML-MRC showed distinct mutational profiles, cell type differentiation arrests and immune properties, suggesting differences in underlying disease biology. Moreover, our transcriptomic clusters show differences in ex-vivo drug responses, even when corrected for differentiation arrest and superiorly capture differences in drug response compared to genetic classification. In conclusion, our findings underscore the importance of transcriptomics in AML subtyping and offer a basis for future research and personalised treatment strategies. Our transcriptomic compendium is publicly available and we supply an R package to project clusters to new transcriptomic studies.

5.2 Introduction

In acute myeloid leukaemia (AML), recurrent genetic abnormalities (RGA) have been identified through systematic genomic studies^{1–5}. Based on these RGAs, the World Health Organization (WHO 2022) and International Consensus Classification (ICC 2022) define several AML subtypes, as well as a heterogeneous subtype of AML with myelodysplasia-related changes (AML-MRC)^{6,7}. RGAs are essential for risk-stratification and are increasingly targeted with drugs^{8,9}.

AML subclassification is genetics-based, but transcriptomics holds immense potential to refine AML classification further^{1–3,10–12}. Transcriptomic studies have led to the discovery of *CEBPA*-mutated AML^{13,14}, and *NPM1*-mutated AML subtypes with different cell differentiation arrests and ex-vivo drug responses^{15,16}. Similar stratification would be beneficial for AML-MRC, given its heterogeneity^{17,18}. Still, a comprehensive examination of AML subtypes defined by gene expression has yet to be performed. Furthermore, the differentiation arrest state is known to modify drug response in AML¹⁹, and failing to account for this effect when comparing drug responses could skew conclusions.

Therefore, we integrated five mRNAseq datasets with corresponding genetic aberration data and annotated cases according to WHO and ICC 2022 standards. We outline AML's transcriptomic landscape and define transcriptional subtypes with distinct gene expressions, genetic aberrations, and cell type arrests. We relate the clusters to ex-vivo drug responses independently of differentiation arrest and show how they superiorly capture differences in response compared to genetic classification. We provide all harmonised data and a transcriptional cluster predictor for future research. Our study underscores the importance of incorporating transcriptomic data in AML classification.

5.3 Results

Multi-cohort AML gene expression compendium

We collected 1224 RNAseq samples from adult (BEAT, TARGET, TCGA, Leucegene, LUMC) and paediatric (TARGET) cohorts with corresponding genetic and clinical data (**Figure 5.1A**). We quantified gene expression with the same pipeline and corrected counts for cohort, sex and source tissue (**Supplemental Figure 1**). Sample classification by their genetic data according to the WHO (**Figure 5.1C**) and ICC was successful for 97% of the samples. In line with previous reports, frequencies of the AML subtypes were similar for the adult cohorts but different between paediatric and adult cohorts (**Supplemental Table 1**), confirming that our dataset is representative of the AML landscape^{40,5}.

Transcriptomics define 17 AML clusters

Next, we assigned AML cases to 17 transcriptional clusters using consensus clustering (**Figure Supplemental Figures 2 & 3**). We named the clusters based on genetic diagnoses (**Figure 5.1B**, **Supplemental Figure 4 & 5**). As expected, the distribution over the clusters was different for paediatric and adult cohorts, exemplified by the large percentage of paediatric samples in the *KMT2A*^T clusters (26%), and adult samples in the *NPM1*^T (93%) and AML-MRC^T (94%) clusters (**Supplemental Table 1**, **Supplemental Figure 6**). However, samples of identical AML genetic subtypes from adult and paediatric cohorts clustered together, indicating that the 17 clusters capture differences in AML biology.

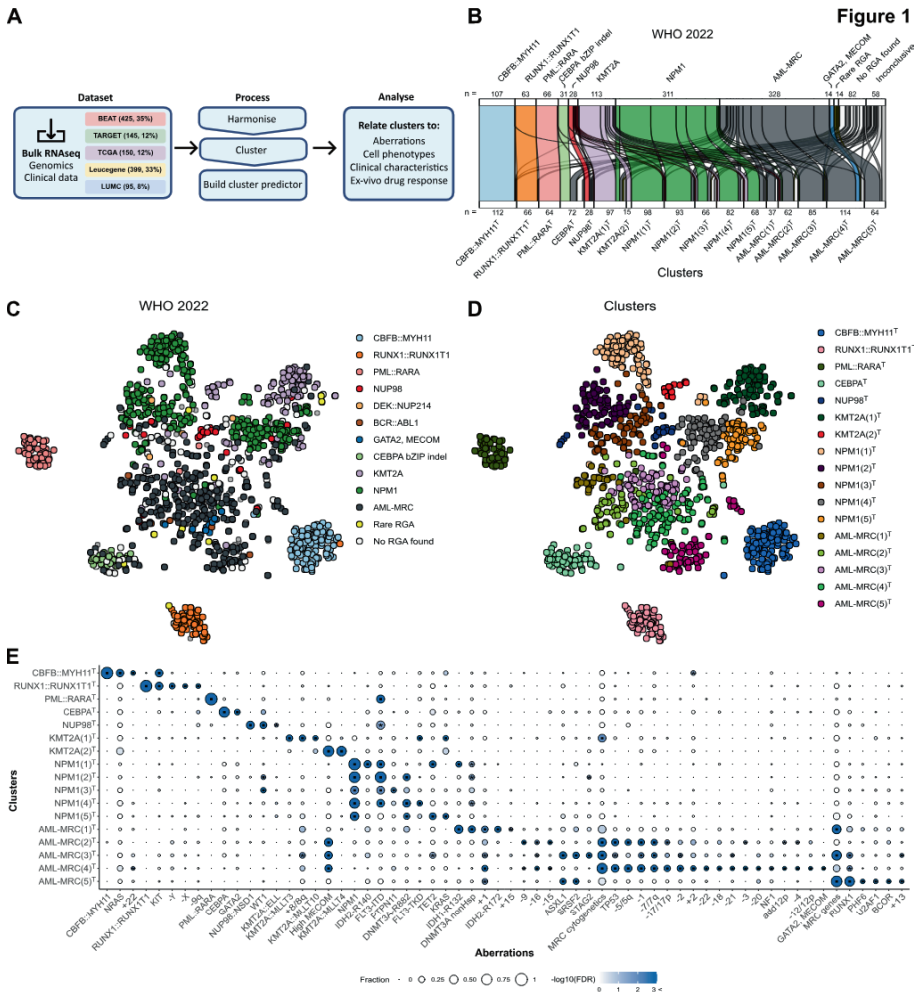


Figure 5.1: Transcriptomic analysis further stratifies AML. **A** Flowchart of the used data and methods. **B** Sankey plot showing the assignment of WHO 2022 diagnoses over the identified clusters. **C** tSNE visualisation of the gene expression of patient samples. Each dot represents a patient. The samples are coloured according to the WHO 2022 subtyping of AML. **D** The same tSNE visualisation as in **C**, but samples are coloured according to the 17 clusters. **E** Dot plots that show enriched aberrations in the 17 clusters. The dots are coloured according to the adjusted p-value. The dots are sized according to the sample fraction with the aberration in the cluster. The x-axis shows the aberrations, and the y-axis shows the clusters. We only visualised enriched aberrations that occurred in at least 10% of the patients in a cluster.

We examined clustering robustness using the stability score (**Supplemental Figure 7**). Median clustering stability was high (0.97-1.00), with AML-MRC(3)^T showing the lowest stability. A correlation test revealed a significant but weak correlation ($\rho = 0.18$, $p\text{-value} < .001$) between blast percentage and clustering robustness, but blast percentage varied greatly in clusters. tSNEs generated using different MVGs (**Supplemental Figure 8**) were stable from 500 to 2500 features. These results show that clustering was robust and only weakly influenced by blast percentage.

We developed a transcriptional cluster predictor using uncorrected counts as input (accuracy = 0.90), demonstrating the persistence of expression patterns. The quality of the predictor was further improved (accuracy = 0.95) by including a reject option (10% rejected) (**Supplemental Figure 9**).

Next, we tested for enrichment of mutations, fusions and cytogenetic aberrations ($n=102$) (**Figure 5.1E**, p -values and frequencies in **Supplemental Table 2**). Four transcriptomic clusters corresponded to singular genetic AML subtypes: RUNX1::RUNX1T1^T (RUNX1::RUNX1T1: 94%, FDR<.001), CBFB::MYH11^T (CBFB::MYH11: 95%, FDR<.001), PML::RARA^T (PML::RARA: 100%, FDR<.001), and NUP98^T (NUP98::NSD1: 45%, FDR<.001). Risk-stratification for survival based on transcriptional subtypes performed similarly to genetics (**Supplemental Figure 10 & 11**). We identified no enrichment for BCR::ABL1 and DEK::NUP214, possibly due to their limited occurrence. For KMT2A rearrangements, CEBPA mutations, NPM1 mutations, and AML-MRC, we found evidence that transcriptomics can refine subtyping, as described below.

Transcriptome analysis identifies two KMT2A-related clusters

The WHO classification defines a single KMT2A-rearranged subtype (KMT2A-r), while the ICC recognises KMT2A::MLLT3 and other KMT2A fusions as distinct^{6,7}. We identified two KMT2A fusion clusters. KMT2A(1)^T was significantly enriched for KMT2A::MLLT3 (31%, FDR<.001), KMT2A::MLLT10 (19%, FDR<.001) and any KMT2A fusion (67%, FDR<.001), while KMT2A(2)^T was enriched for KMT2A::MLLT4 (67%, FDR<.001) and high MECOM expression (80%, FDR<.001) (**Figure 5.2A & B**). Interestingly, we found cases with NPM1 mutations and trisomy 8/8q localised in KMT2A(1)^T, indicating that these lead to KMT2A fusion-like gene expression.

The genes *LAMP5*, and *ADCY9* showed high expression in KMT2A(1)^T and low expression in KMT2A(2)^T (**Figure 5.2B**), and all have been shown to contribute to AML pathogenesis^{41,42}. Additionally, the transcription factor (TF) *ETV2* was highly expressed in KMT2A(1)^T, while the TF *ERG* displayed high expression in KMT2A(2)^T. The cell type scores revealed KMT2A(1)^T to have a significantly higher promonocyte-like score (FDR<.001), while KMT2A(2)^T was more hematopoietic stem cell (HSC)-like (FDR<.001) (**Figure 5.2B, Supplemental Figure 12**). FAB annotations showed similar results for KMT2A(1)^T, which had a high M5 (monocytic leukaemia) fraction (90%), while KMT2A(2)^T was more mixed (**Figure 5.2C**). Overall, we found that gene expression-based separation of KMT2A-r did not align with the ICC 2022 classification.

The CEBPA^T cluster indicates a favourable prognosis

As acknowledged in the ELN2022, patients with a CEBPA bZIP inframe mutation have a favourable prognosis^{8,43}. We identified a transcriptional CEBPA^T cluster significantly enriched for mutated CEBPA cases (72%, FDR<.001), with 42% of the samples having a CEBPA bZIP indel, either as single mutation or combined with an N-terminal frameshift mutation (**Figure 5.3A & B**). The remaining samples contained other mutations in the bZIP area or N-terminal region or had no detectable CEBPA mutation. Of note, a single CEBPA bZIP indel case resided outside the CEBPA^T cluster. This patient had an IDH-R132 mutation with a VAF=0.47, while the CEBPA bZIP

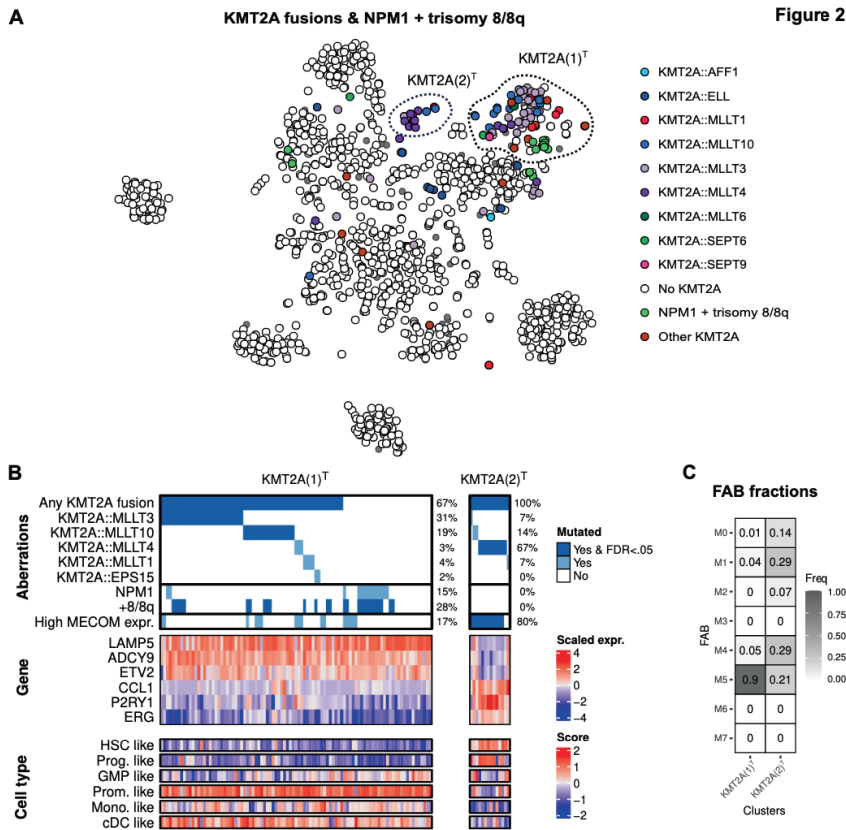


Figure 5.2: Transcriptome analysis identifies two *KMT2A*-related clusters. **A** tSNE visualisation of patient samples, coloured according to *KMT2A*-fusion or *NPM1* mutation and trisomy 8. **B** Waterfall plot of aberrations in the *KMT2A* clusters, including the percentage of samples with the aberration. The plot is combined with heatmaps showing the expression of marker genes and cell type scores. The columns are samples, which are split according to transcriptional clusters. **C** Fraction of FAB annotations per cluster. HSC = hematopoietic stem cells, Prog. = progenitor, GMP = granulocyte-monocyte progenitor, Prom. = promonocytes, Mono. = monocytes, cDC = conventional dendritic cells

in-frame mutation had a VAF=0.21. This finding suggests that the *IDH*-R132 mutation dominates the expression pattern, placing this case in cluster AML-MRC(1)^T. Conversely, all *CEBPA*^T cluster patients showed similar favourable outcomes (log-rank test: p-value=.80), irrespective of whether the *CEBPA* bZIP inframe mutation was detected (Figure 5.3C). The *CEBPA*^T cluster thus marks patients with a favourable outcome regardless of *CEBPA* mutation detection, which the *CEBPA*^T expression profile can detect.

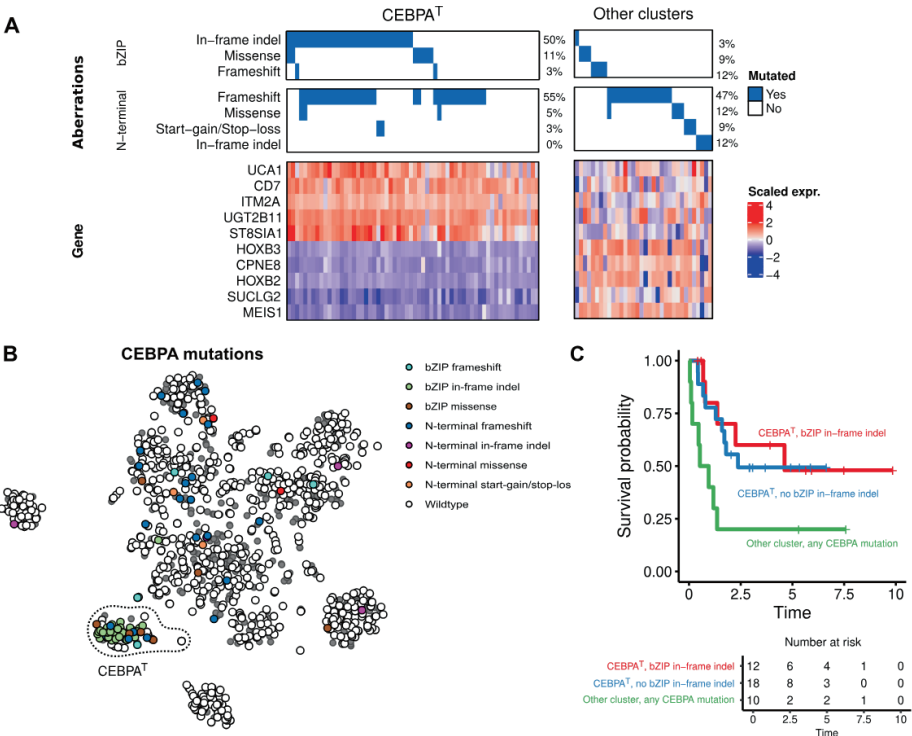


Figure 5.3: The *CEBPA*^T cluster indicates a favourable prognosis. **A** Waterfall plot and gene expression heatmap of all samples in the *CEBPA*^T cluster and samples with a *CEBPA* mutation located outside the *CEBPA*^T cluster. **B** tSNE visualisation of patient samples, coloured according to the type of *CEBPA* mutation. For samples with multiple *CEBPA* mutations, we used the ordering as in **A** to decide which mutation to display. **C** Kaplan-Meier curve of the survival of BEAT and TCGA patients in and outside the *CEBPA*^T cluster.

Gene expression profiling identifies five transcriptional *NPM1*-related clusters

The 2022 WHO and ICC classifications include one subtype of *NPM1*-mutated AML^{6,7}. However, we identified five clusters enriched for mutated *NPM1* (Figure 5.4). We observed elevated expression of *HOXA3*, *HOXB5*, and *MEIS1* (Figure 5.4A), which has been earlier associated with *NPM1* mutations⁴⁴. Interestingly, *NPM1* mutation-lacking samples generally also exhibited high expression of these genes, suggesting that there are alternative mishaps that disrupt these genes leading to *NPM1* mutated-like AML.

NPM1(1)^T exhibited the highest percentage (95%, FDR<.001) of *NPM1* mutated samples and was significantly enriched for *IDH1*-R132 (25%, FDR<.001), *IDH2*-R140 (37%, FDR<.001), and *TET2* (33%, FDR<.001) co-mutations (Figure 5.4A). *NPM1*(2)^T samples were enriched for *FLT3*-ITD mu-

Results

tations (84%, FDR<.001), but *FLT3*-ITD was also enriched in NPM1(1)^T, NPM1(3)^T and NPM1(4)^T (42-43%, all FDR<.001). Additionally, NPM1(4)^T and NPM1(5)^T had a significantly lower variant allele frequency for mutated *NPM1* (**Figure 5.4B**). We found two *NPM1::MLF1* cases in our compendium, which both clustered in NPM1(3)^T. *NPM1::MLF1* has been shown to localise in the cytoplasm⁴⁵, like mutated *NPM1*, possibly leading to a similar expression profile as *NPM1*-mutated cases.

Each of the *NPM1*-related clusters exhibited unique marker genes (**Figure 5.4A**). For instance, *FTO* expression was high in NPM1(1)^T. Additionally, *LYRM1*, *ADAM8*, and *DNAJC13* were elevated in NPM1(2)^T, NPM1(4)^T, and NPM1(5)^T, respectively. NPM1(3)^T had a less distinct expression pattern, suggesting a more heterogeneous cluster. Also, we observed differential expression of TFs (*RUNX1*, *PRDM16*, *SPI1*)^{46,47} – even in samples lacking the *NPM1* mutation – and TF expression aligned with cell differentiation stages.

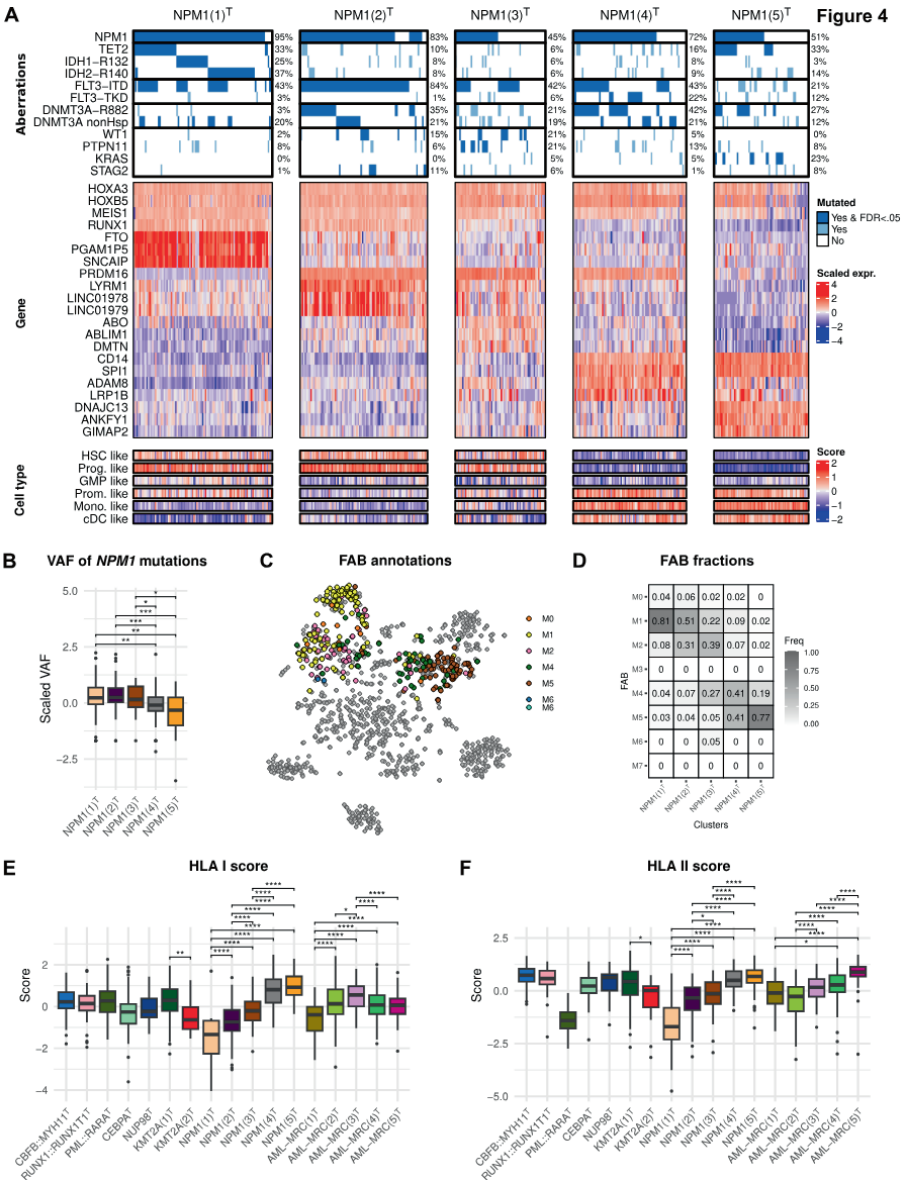
NPM1(1)^T and NPM1(2)^T displayed a HSC-like expression pattern, NPM1(3)^T was mixed, whereas NPM1(4)^T and NPM1(5)^T were more differentiated (**Figure 5.4A**, **Supplemental Figure 12**). FAB annotations showed additional differences, with NPM1(5)^T containing fewer M4 (myelomonocytic leukaemia) but more M5 (monocytic leukaemia) cases than NPM1(4)^T (**Figure 5.4C & D**). Using scores for HLA I and HLA II antigen-presenting cells³⁹ we found NPM1(1)^T to have significantly lower HLA I (FDR<.001) and HLA II (FDR<.001) scores than the other clusters (**Figure 5.4E & F**). NPM1(1)^T and NPM1(5)^T patients were significantly older (FDR<.05), while NPM1(3)^T patients were younger (FDR<.01) (**Supplemental Figure 6F**). Our findings emphasise the existence of distinct *NPM1*-related subsets, highlighting the limitations of relying solely on genetic classification.

Gene expression profiling identifies five transcriptional AML-MRC-related clusters

The ICC 2022 divides AML-MRC into three groups based on *TP53* mutations, myelodysplasia-related gene mutations, and cytogenetic abnormalities⁷. Our study identified five gene expression-based AML-MRC related clusters (**Figure 5.5**), with varying fractions of *TP53* mutations, MRC gene mutations, and cytogenetic abnormalities.

Despite sharing these mishaps, each cluster had unique characteristics (**Figure 5.5A**). AML-MRC(1)^T was characterised by *IDH1*-R132 (49%, FDR<.001) and *IDH2*-R170 (27%, FDR<.001) mutations. A *DNMT3A* and *IDH1/2* mutated subtype has been reported⁵, but 41% of the AML-MRC(1)^T cases lacked *DNMT3A* mutations. AML-MRC(2)^T, AML-MRC(3)^T, and AML-MRC(4)^T were all enriched (FDR<.001) for *TP53* mutations, cytogenetic abnormalities and high *MECOM* expression, and AML-MRC(3)^T also contained a large fraction of mutated MRC genes (65%, FDR<0.001). AML-MRC(5)^T stood out with the highest fraction of mutated MRC genes cases (81%, FDR<.001) and the lowest fraction of *TP53* mutations (6%) and cytogenetic abnormalities (34%).

Mapping AML Heterogeneity: Multi-cohort transcriptomic analysis identifies novel clusters and divergent ex-vivo drug responses



Results

Figure 5.4: Gene expression profiling identifies five transcriptional *NPM1*-related clusters. **A** Waterfall plot of aberrations in the *NPM1*-related clusters, including the percentage of samples with the aberration. The plot is combined with heatmaps showing the expression of marker genes and cell type scores. The columns are samples, which are split according to transcriptional clusters. **B** Boxplot showing the scaled variant allele frequency (VAF) of mutated *NPM1* from the BEAT, LeuceGene, and LUMC cohorts. The VAF was scaled per gene and study to allow for a combined analysis. We used a two-sided Wilcoxon test to test for statistical differences and Benjamini-Hochberg to adjust p-values for multiple testing. **C** tSNE visualisation of patient samples, coloured according to the FAB annotation. Only *NPM1*-related clusters are coloured; the rest are in grey. **D** Fraction of FAB annotations per cluster. **E** and **F** show boxplots of HLA I and HLA II antigen presenting cell scores, respectively. Tests were performed as in **B**, but were only done between the two KMT2A^T, the five *NPM1^T and the five AML-MRC^T clusters. FDR values: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001. HSC = hematopoietic stem cells, Prog. = progenitor, GMP = granulocyte-monocyte progenitor, Prom. = promonocytes, Mono. = monocytes, cDC = conventional dendritic cells, VAF = variant allele frequency*

We found marker genes for all clusters (**Figure 5.5A**). For instance, high *SRSF12* marked AML-MRC(1)^T, and *LINC00865* marked AML-MRC(5)^T. AML-MRC(2)^T presented high glycophorin genes and *UROD* expression, suggesting an association with acute erythroid leukaemia^{48–50}. Distinct cell differentiation scores further highlighted differences (**Figure 5.5A–C**, **Supplemental Figure 12**). For example, AML-MRC(1)^T showed high progenitor-like scores, with 65% M1 (minimal maturation) cases, and AML-MRC(3)^T showed a more differentiated pattern, with 54% M2 (significant maturation) cases. AML-MRC(2)^T was the only cluster with M6 (erythroid leukaemia) – in line with high expression of erythrocyte cell markers – and M7 (megakaryocytic leukaemia) cases. Additionally, high cytolytic cell infiltration has been reported for AML-MRC cases³⁹. Using the same score (**Figure 5.5D**) we found that the cytolytic infiltration was significantly (FDR<.05) lower for AML-MRC(1)^T and higher for AML-MRC(2)^T compared to the other AML-MRC clusters. Our results demonstrate that different AML-MRC transcriptomic clusters can be identified, showing genetic enrichments that do not necessarily align with the ICC 2022 classification.

AML clusters exhibit cell type-independent differences in ex-vivo drug responses

Finally, we assessed the drug sensitivity of the transcriptional AML subtypes. Using ex-vivo drug response data, we discovered 101 drug-cluster combinations with significantly lower resistance (FDR<.05), of which 21 combinations remained statistically significant (FDR<.05) when adjusting for cell differentiation status (**Figure 5.6**, **Supplemental Figure 13**, **Supplemental Table 3**).

The ex-vivo drug responses between *NPM1*-related clusters were often divergent, exemplified by venetoclax and selumetinib (**Figure 5.6A,B**). *NPM1*(1)^T, *NPM1*(2)^T and *NPM1*(3)^T mostly responded positively to tyrosine kinase inhibitors and CDK kinase inhibitors. *NPM1*(4)^T and *NPM1*(5)^T samples were more sensitive to PI3K and MAPK kinase inhibitors. We also found drugs where only one cluster was responsive, exemplified by axitinib for *NPM1*(4)^T where this effect remained significant (FDR<.05) when controlled for cell type scores (**Figure 6C**).

Several drugs demonstrated favourable ex-vivo responses in KMT2A(1)^T compared to the other clusters, exemplified by idelalisib. For KMT2A(2)^T we found no significant responsive drugs, but testing was limited due to small cluster size. For the AML-MRC clusters, most drugs showed strong resistance. Still, specific drugs were more effective for AML-MRC(1)^T, AML-MRC(4)^T, and AML-MRC(5)^T (**Figure 5.6A, B**), suggesting potential for targeted treatments in this diverse, high-risk patient group.

Next, we examined if transcriptional clusters provide insights beyond genetic classifications. Comparing the AUCs of each drug between groups, we found 71 drugs with significantly different (FDR<.05) median AUCs between the clusters, while only 21 drugs were significantly different between ICC 2022 classes (**Figure 5.6D**). Additionally, 57 of the 101 cluster-drug combinations remained significant (FDR<.05) when cluster membership and ICC 2022 diagnosis were included in a LM, suggesting that the transcriptional clusters offer information beyond genetic classification.

Mapping AML Heterogeneity: Multi-cohort transcriptomic analysis identifies novel clusters and divergent ex-vivo drug responses

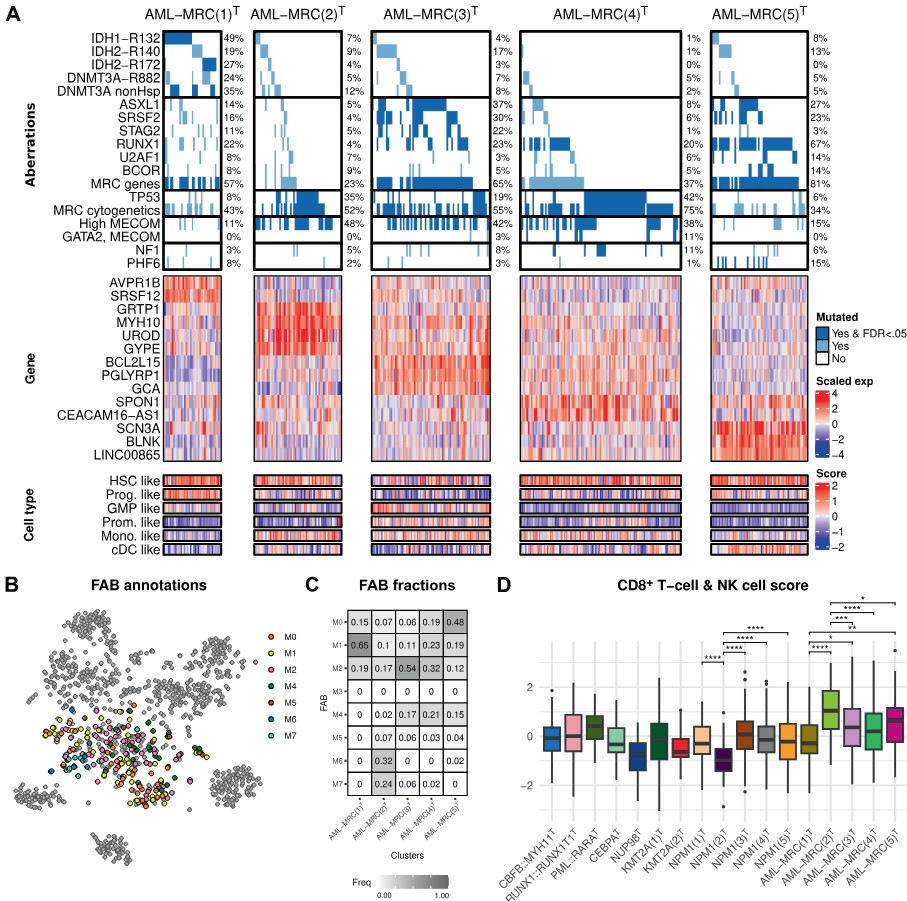


Figure 5.5: Gene expression profiling identifies five transcriptional AML-MRC-related clusters **A** Waterfall plot of aberrations in the AML-MRC-related clusters, including the percentage of samples with the aberration. We did not plot enriched individual large chromosomal mishaps. The plot is combined with heatmaps showing the expression of marker genes and cell type scores. The columns are samples, which are split according to transcriptional clusters. MRC genes are *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, or *ZRSR2*. Cytogenetic abnormalities are the ICC 2022 aberrations that define AML-MRC with cytogenetic abnormalities. **B** tSNE visualisation of patient samples, coloured according to the FAB annotation. Only AML-MRC clusters samples are coloured; the rest are in grey. **C** Fraction of FAB annotations per cluster. **D** Boxplots of cytolytic cell score per cluster. We used a two-sided Wilcoxon test to test for statistical differences and Benjamini-Hochberg to adjust p-values for multiple testing. Tests were performed only between the two *KMT2A*^T and the five *NPM1*^T and the five AML-MRC^T clusters. FDR values: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001. HSC = hematopoietic stem cells, Prog. = progenitor, GMP = granulocyte-monocyte progenitor, Prom. = promonocytes, Mono. = monocytes, cDC = conventional dendritic cells

Results

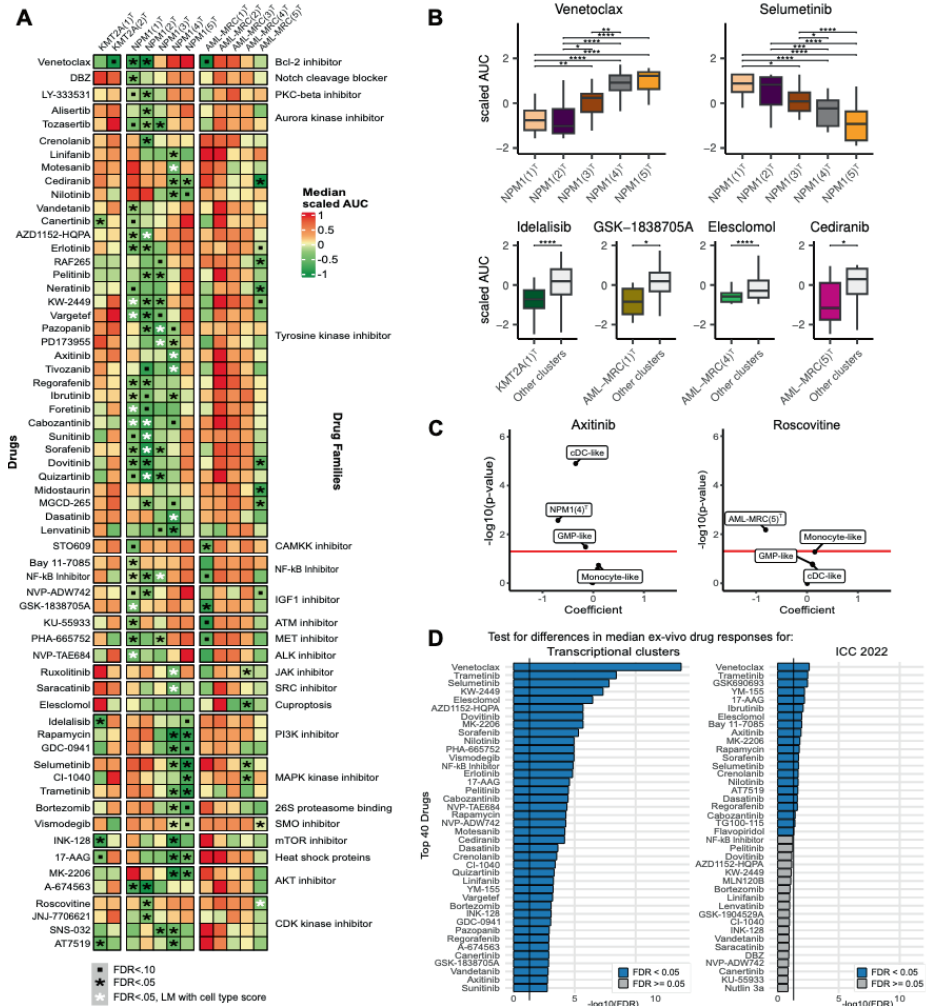


Figure 5.6: AML clusters exhibit cell type-independent differences in ex-vivo drug responses **A** Heatmap coloured according to the median scaled area under the curve (AUC) of the ex-vivo drug response per drug and cluster. On the left is the drug name, and on the right is the drug family. A green colour indicates a lower median AUC for the drug for the samples in the cluster compared to the other clusters, indicating a strong drug response. Red indicates a higher median AUC, meaning a weak drug response. **B** Boxplots showing ex-vivo drug responses for a selection of drugs. We performed significance testing using a two-sided Wilcoxon test. FDR values: * < 0.05, ** < 0.01, *** < 0.05, **** < 0.001. **C** Plots of the multivariate linear models with cluster membership and the six cell type scores as independent variables and AUC as the dependent variable. On the x-axis, the coefficient of the variables in the models is shown, and the y-axis shows the $-\log_{10}$ of the p-value for each variable. The shown p-values are not corrected and are for visualisation to indicate variable importance in the multivariate model. The corrected p-values of the cluster membership variable are shown in **Supplemental Table 3**. The red line indicates a p-value of 0.05. **D** Barplots of the top 40 drugs with highest corrected p-values for Kruskal-Wallis tests between ex-vivo drug response and clusters or ICC 2022 diagnosis to test if there were significant differences in the median AUCs. All p-values were corrected with Benjamini-Hochberg.

Overall, our findings offer novel opportunities for targeted therapy in AML. We observed effective drug responses even after adjusting for differentiation status, possibly allowing gene expression-based subtypes to guide treatment strategies.

5.4 Discussion

This study presents an overview of transcriptomics in AML and provides a framework for transcriptional subtyping. We integrate multiple cohorts to identify 17 robust transcriptional subtypes that subclassify ~75% of our datasets' patients. We make the harmonised data and a cluster predictor publicly available, facilitating future research.

For the CEBPA^T cluster, we show that patients without a detected *CEBPA* bZIP indel mutation still have similar favourable survival. Patients without the canonical *CEBPA* mutation in the CEBPA^T cluster could be explained by undetected mutations, given the complexities of *CEBPA* sequencing. Also, *CEBPA* hypermethylation has been described to lead to a similar expression profile⁵¹. The use of the CEBPA^T gene signature for risk stratification could be a relevant alternative to detect these favourable-outcome patients.

KMT2A(1)^T mainly featured *KMT2A* with the fusion partners *MLLT3*, *MLLT10* and *MLLT1* – all TFs in the super elongation complex whose perturbation leads to disrupted hematopoietic lineage commitment⁵². In contrast, KMT2A(2)^T featured *KMT2A::MLLT4*, which is thought to cause leukaemia by promoting self-association⁵³. Interestingly, *MLLT3*, *MLLT10* and *MLLT1* all fuse a specific region of *KMT2A*, but *MLLT4* shows less specificity⁵⁴. Collectively, these results suggest that two types of oncogenic mechanisms involving *KMT2A* fusions exist that may be marked with unique gene expression patterns.

We identified five *NPM1*-related clusters, further underpinning findings of transcriptional heterogeneity among *NPM1*-mutated patients^{15,16,55}, but also providing additional insight into co-mutations and detailed subtypes. We observed several samples from *NPM1*-related clusters that lacked the *NPM1* mutation. Several rare *NPM1* fusions, like *NPM1::MLF1*, have been described to lead to cytoplasmic localisation of *NPM1*, comparable to the canonical *NPM1* frameshift^{45,56}. These non-canonical mishaps could lead to a *NPM1*-mutated-like presentation and similarities in survival and drug response should be explored. Additionally, NPM1(1)^T was mutually exclusive enriched for *IDH1/2* and *TET2* co-mutations. *IDH1/2* mutations lead to an aberrant alpha-ketoglutarate metabolism and are functionally complementary to *TET2* loss-of-function mutations⁵⁷. While NPM1(1)^T and NPM1(5)^T show significant enrichments for *TET2*, only NPM1(1)^T shows this mutual exclusivity with *IDH1/2*. This suggests that only NPM1(1)^T is driven by aberrant alpha-ketoglutarate metabolism, which should be further studied using metabolomics.

Similarly, cytogenetic abnormalities, AML-MRC mutations and high MECOM expression were found in all AML-MRC clusters, but lead to different gene expression. A possible explanation could be clonal architecture and the differentiation state of the cell acquiring the leukemic aberration, both known to influence the biology of the resultant leukaemia^{58,59}. To our knowledge, we are the first to show different gene expression-based subgroups in AML-MRC, with divergent drug responses. Accurate identification of these clusters requires gene expression analysis, showing the relevance of our work.

We found no additional survival differences between other clusters. However, data availability limited the survival analysis, and different treatment protocols across studies could have led to

Methods

confoundment. Survival differences between transcriptional subtypes should thus be further explored in one large cohort. However, we did find marked differences in drug responses between the clusters. Ideally, new studies should test in-patient efficacy of drugs with good ex-vivo responses in transcriptional subtypes. Furthermore, transcriptional AML subtyping could aid specialists in the complex field of clinical care and lead to multidisciplinary tailored-based treatment advice⁶⁰.

In conclusion, the transcriptional subtypes reveal heterogeneity in AML not captured by genetic classification. Integration of transcriptomics into AML research and diagnostics could improve disease understanding and lead to more treatment options.

5.5 Methods

Transcriptomic data

We acquired transcriptomics data of primary AML patients from blood or bone marrow from BEAT^{3,20} (n = 425), TARGET² (n = 145), TCGA¹ (n = 150), and Leucegene^{11,21–23} (n = 399), and our in-house LUMC²⁴ dataset (n = 95). Data statements and methods for transcriptome sequencing are available in the referenced studies.

We acquired quantified gene expression for BEAT, TARGET and TCGA from www.portal.gdc.cancer.gov (release 36) and implemented the same pipeline for Leucegene and LUMC to harmonise quantification. In short, FASTQ files were aligned and quantified with STAR²⁵ to the GRCh38 reference genome²⁶, using Gencode v36²⁷ as the gene annotation index which included 60 600 genes.

Gene expression count data were corrected with Combat-Seq²⁸ for the variables “cohort”, “sex”, and “tissue”. We split Leucegene for the batch correction into Leucegene.stranded and Leucegene.unstranded, since different sequencing libraries were used. We removed 8057 genes that were not detected in all cohorts, leaving 52 603 genes. Finally, we removed genes detected in less than 200 samples or with less than 300 counts leaving 41 862 genes for our final dataset. We normalised the corrected count data using the geometric mean and variance stabilising transformation (VST)²⁹ and quantified the remaining cohort-specific variation using kBET³⁰.

Genetic and patient data

We acquired genetic data for the samples from the referenced studies in the form of mutation and fusion calling, and cytogenetics data and clinical data on sex, age, blast percentage, and survival. Blacklisted fusions as reported by Arriba³¹ were removed from the fusion calling data.

We harmonised the data by standardising the annotation of gene names, fusion genes, and karyotyping. Using genetic data, we subclassified samples according to the WHO 2022⁶ and ICC 2022⁷. Samples for which we found no RGA and all genetic data available were annotated as “No RGA found”. We classified samples with missing data and “No RGA found” as “Inconclusive”.

Clustering

We employed consensus clustering^{32,33} on the batch-adjusted gene expression. First, we created a weighted nearest neighbour graph³⁴ using the 2000 most variable genes (MVGs). MVGs were selected via the median absolute deviation from samples with a blast percentage over 70% to minimise tumour microenvironment effects. Using the Leiden algorithm³⁵ – with seed and resolution varied per iteration – we generated 300 cluster assignments from the graph for each n.clusters

ranging from 10 to 20, totaling 3300 assignments.

From these 3300 assignments we created a consensus matrix with values ranging between 0 and 1 based on pairwise co-clustering. We then converted this matrix into a distance matrix (1 - consensus matrix) and conducted Ward.D2 hierarchical clustering. The final cluster count was determined based on the individual separation of WHO classes and clusters displaying differential traits.

Cluster stability

To evaluate per sample clustering stability, we devised a stability score. We constructed a consensus matrix for each n_clusters (300 assignments) and subtracted each co-clustering value from 1 if it was below 0.5. Then, we took the mean of all values per sample as the stability score, which ranged from 0.5 to 1, with higher scores indicating less clustering ambiguity. To investigate correlation between cluster stability and blast percentage we performed a Spearman correlation test. Additionally, we generated tSNEs using 100 to 2500 MVGs to visually assess cluster stability.

Cluster prediction

To predict cluster assignments we trained a one-vs-rest SVM per cluster. As input we used the uncorrected gene expression of the 2000 MVGs used for clustering. To select hyperparameters and evaluate performance we utilised 5x5 nested cross-fold validation.

To improve predictions we included a reject option using a minimum distance to the decision boundary. We determined this distance by looping over possible minimum values for the predictions of the inner fold. We selected the minimum value with the highest Kappa for the accepted inner fold samples and an accuracy < 0.5 for the rejected inner folds samples.

The final model was trained on the whole dataset, using 5-fold cross-validation to select hyperparameters and the minimum decision boundary distance.

Differential gene expression analysis

Differential gene expression analysis between the clusters was performed using DESeq2²⁹ using the corrected gene counts. We performed one-versus-rest analyses to identify differentially expressed genes in one cluster compared to all others. We annotated genes as transcription factors or coding for cell surface proteins using public databases^{36,37}.

Aberration enrichment analysis

To test if aberrations occurred more in a cluster than in others we first removed aberrations found in only one cohort or which occurred in less than 1% of the samples. We also included high *MECOM* expression in the analysis (VST expression > 6 , based on the tail of a *MECOM* expression density plot). We tested for enrichment per aberration by performing a one-sided Fisher exact test for one cluster versus all others and adjusted p-values using the Benjamini-Hochberg (BH) procedure. We considered aberrations with a false discovery ratio (FDR) < 0.05 enriched.

Survival analysis

We performed survival analysis using right censored overall survival data by generating Kaplan Meier (KM) curves on BEAT and TCGA survival data, comparing different groups of patients with the log rank test. We also performed Cox-regression using BEAT, TARGET and TCGA survival data for different patient groups and included cohort, sex and age as co-variables to analyse hazard ratios.

Acknowledgements

Expression based score

We created cell type scores to assess the differentiation arrest of AML samples, using the mean expression of 30 marker genes for six haematological cells³⁸. Additionally, we created immune phenotype scores for cytolytic infiltration and HLA I and HLA II antigen presenting cells using the mean expression of marker genes³⁹.

Drug response analysis

To analyse drug response differences, we used ex-vivo drug response data of 331 BEAT³ samples, quantified as area under the curve (AUC). We excluded drugs with less than 200 samples or missing data for any cluster, leaving 103 of the 123 drugs. We used a Kruskal-Wallis test for each drug with the AUC as response and clusters as groups to compare the average drug response per cluster. Drugs with a significant difference ($FDR < .05$) were analysed with one-sided Wilcoxon tests to identify clusters with low AUCs. Additionally, we performed a Kruskal-Wallis test for each drug with the ICC 2022 diagnosis as groups, to compare with clusters as groups.

Multivariate linear models (LM) were evaluated per drug to test if clusters were sensitive to a drug when adjusted for cell type, with AUC as response and cluster membership (one-versus-rest) and the six cell type scores as explaining variables. Similarly, we fitted LMs but with cluster membership and ICC 2022 diagnoses as variables. We considered clusters sensitive to a drug if the cluster membership's FDR was below 0.05 and the LM coefficient was negative. All p-values were corrected using BH.

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5.7 Author Contributions

M.J.R., M.G., and E.B.A. conceived and designed the project; E.B.A. acquired funding; E.B.A. performed project administration; M.G., E.B.A., H.V., R.R.B., C.J.M.H., P.B. performed oversight and management of resources (data generation, collection, transfer, infrastructure, data processing); J.F.S. performed computational and statistical analyses; J.F.S., E.B.A., M.G., E.O.K., E.S.-L. performed analyses and interpretation; J.F.S. performed and structured data visualisation; M.J.R., M.G. and E.B.A. provided supervision and scientific direction; J.F.S. wrote the manuscript; and all authors critically reviewed the manuscript and figures.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available at <http://www.osf.io/wq7gx>.

CODE AVAILABILITY

All code used to generate results is available on reasonable request. The predictor is available at www.github.com/jeppe-severens/AMLmapR as an R package.

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Mapping AML Heterogeneity: Multi-cohort transcriptomic analysis identifies novel clusters and divergent ex-vivo drug responses

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5.9 Appendix

The appendix was shortened due to environmental considerations. To minimize unnecessary printing and reduce material use, extensive data including 13 supplementary figures and 4 supplementary tables are provided online at: <https://doi.org/10.1038/s41375-024-02137-6>.



Chapter 6

Resolving inter- and intra-patient heterogeneity in *NPM1*-mutated AML at single-cell resolution

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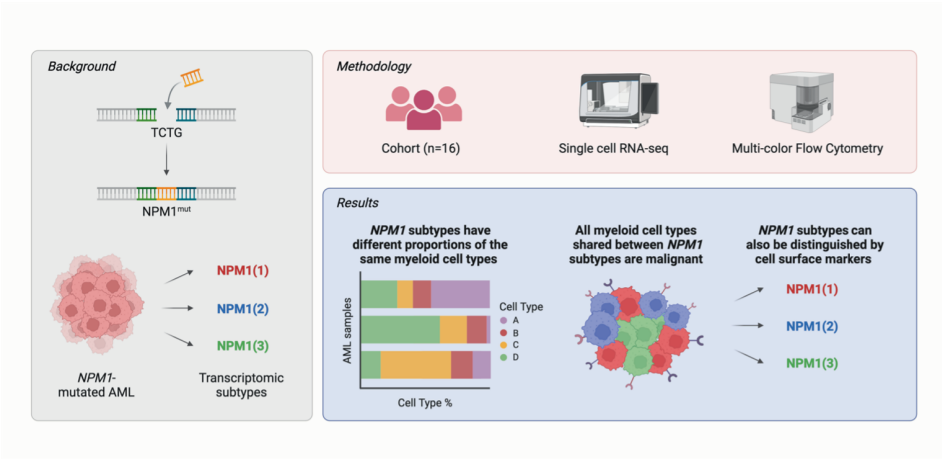
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6.1 Abstract

NPM1-mutated AML is one of the largest entities in international classification systems of myeloid neoplasms, which are based on integrating morphologic and clinical data with genomic data. Previous research, however, indicates that bulk transcriptomics-based subtyping may improve prognostication and therapy guidance. Here, we characterized the heterogeneity in *NPM1*-mutated AML by performing single-cell RNA-sequencing and spectral flow cytometry on 16 AML belonging to three distinct subtypes previously identified by bulk transcriptomics. Using single-cell expression profiling we generated a comprehensive atlas of *NPM1*-mutated AML, collectively re-constituting complete myelopoiesis. The three *NPM1*-mutated transcriptional subtypes showed consistent differences in the proportions of myeloid cell clusters with distinct patterns in lineage commitment and maturational arrest. In all samples, leukemic cells were detected across different myeloid cell clusters, indicating that *NPM1*-mutated AML are heavily skewed but not fully arrested in myelopoiesis. Same-sample multi-color spectral flow cytometry recapitulated these skewing patterns, indicating that the three *NPM1*-mutated subtypes can be consistently identified across platforms. Moreover, our analyses highlighted differences in the abundance of rare hematopoietic stem cells suggesting that skewing occurs early in myelopoiesis. To conclude, by harnessing single-cell RNA-sequencing and spectral flow cytometry, we provide a detailed description of three distinct and reproducible patterns in lineage skewing in *NPM1*-mutated AML that may have potential relevance for prognosis and treatment of patients with *NPM1*-mutated AML.

GRAPHICAL ABSTRACT



6.2 Introduction

Acute Myeloid Leukemia (AML) is a heterogeneous malignant disease characterized by uncontrolled proliferation of immature myeloid precursor cells in the bone marrow. The latest WHO and ICC classifications stratify adult AML into various categories that are mainly based on distinct recurrent genetic aberrations, with one of the largest entities being *NPM1*-mutated AML^{1,2}. A 4 base pair frameshift mutation in *NPM1* is sufficient to drive malignant transformation. Although *NPM1*-mutated AML generally have a relatively favorable prognosis³, other co-mutations are also important and may affect the prognosis and treatment response of AML patients. For example, patients who acquire *FLT3-ITD* and *DNMT3A*^{R882} co-mutations in addition to the *NPM1* mutation have a significantly worse prognosis⁴.

In line with others who also reported on different gene signatures, we previously identified three distinct subtypes of *NPM1*-mutated AML by bulk-transcriptomics, each showing distinct ex-vivo drug sensitivity profiles, and enrichments for different co-mutations⁵⁻⁸. Patients with the transcriptional *NPM1*(1) subtype had mutually exclusive co-mutations with either *IDH1/2* or *TET2*, whereas AML samples with *NPM1*(2) or *NPM1*(3) subtypes showed strong enrichments for *FLT3-ITD* or *DNMT3A*^{R882} co-mutations, respectively. These observations raise the question whether the three distinct transcriptional subtypes in *NPM1*-mutated AML are the result of different co-mutational patterns or whether the three subtypes reflect progenitor cells arrested at different stages in myelopoiesis with variable susceptibility to acquire certain type of co-mutations.

Most large-scale gene expression studies, including ours, leveraged RNA sequencing data in bulk of AML samples in which healthy and malignant hematopoiesis may co-exist^{9,10}. Recent single-cell RNA sequencing revealed that leukemic cells with different maturational arrests carry different gene mutations^{10,11}, and that these leukemic cell subsets in AML typically exhibit phenotypes reminiscent of healthy hematopoietic cell types, albeit often accompanied with aberrant characteristics^{10,12-14}. Consequently, to advance our understanding of *NPM1*-mutated AML, single-cell analyses are essential for investigating and comparing intra-patient heterogeneity at a resolution that can highlight transcriptional subtypes identified in large-scale bulk sequencing studies.

In this study, we investigated whether the distinct *NPM1*-mutated AML subtypes as revealed by bulk transcriptomics are characterized by subtype-specific cell clusters or whether the same cell clusters are present in different proportions. To investigate and compare cellular heterogeneity, we employed single-cell RNA sequencing and spectral flow cytometry on 16 *NPM1*-mutated AML selected from the three distinct subtypes. Using both single-cell modalities, we identified various leukemic cell clusters resembling different maturation stages during normal hematopoiesis. These leukemic cell clusters were present in different proportions in all three transcriptional subtypes. Leukemic cells resembling early hematopoietic stem and multipotent progenitor cells dominated in two *NPM1* subtypes, whereas leukemic cell subsets with more mature myeloid phenotypes were enriched in the third subtype. Our data support a model in which the three *NPM1* transcriptional subtypes originate from different progenitor cells with variable capacity to produce more differentiated myeloid offspring. Since AML cells with different maturation phenotypes have shown variable *ex-vivo* drug responses, our data may be relevant for the prognosis and treatment of patients and provide preliminary insight into the etiology of *NPM1*-mutated AML.

6.3 Results

Single-cell atlas of *NPM1*-mutated AML reconstitutes myelopoiesis

To investigate the identity and cellular composition of the three *NPM1* transcriptional subtypes, we performed single-cell RNA sequencing on samples from 16 *NPM1*-mutated AML patients. All AML samples contained high blast levels (77-99%) and were specifically selected from each of the three major gene expression-based clusters as previously identified for *NPM1*-mutated AML by bulk RNA sequencing. In total, we selected 7, 5 (including relapse sample AML08), and 4 AML from the transcriptional *NPM1*(1), *NPM1*(2), and *NPM1*(3) subtypes, respectively (Figure 6.1a). All patient-derived samples featured the hotspot frameshift mutation in *NPM1*, and co-mutation patterns varied across the three *NPM1* subtypes as previously described⁵ (Figure 6.1b, Supplementary Table 1, see Methods).

After stringent quality control and doublet removal, single-cell RNA sequencing (scRNA-seq) data from 83,162 total cells were integrated and clustered into 15 cell clusters (Figure 6.1c, S1, S2a-d, see Methods). Subsequent annotation of these clusters was performed using various strategies. First, we used Azimuth¹⁵ to project a healthy bone marrow atlas onto our data (Figure S2e). This allowed us to identify the most resembling cell subset in healthy hematopoiesis for each of our identified clusters. Given that particularly the myeloid lineage is affected by disease, we refer to ‘-like’ subsets for clusters mostly resembling granulocyte-monocyte progenitors (GMPs), monocytes, dendritic cells (DCs), and erythroid cells. While clusters with mature myeloid cell populations, e.g. monocytes and erythrocytes, strongly resembled their healthy counterparts, most clusters with characteristics of progenitor cell populations could not be faithfully projected onto the healthy bone marrow atlas (Figure S2f). We next compared the obtained cell clusters to a previously published dataset by van Galen *et al.*¹² containing 16 AML with diverse genetic lesions, including 5 *NPM1*-mutated AML (Figure S2g). Many of our cell clusters resembled one of the 6 cell types previously described in this dataset, including hematopoietic stem cell (HSC)-like, progenitor-like, GMP-like, promonocyte-like, monocyte-like, and conventional dendritic cell (cDC)-like leukemic cells.

The combined analysis resulted in 11 myeloid cell clusters including one long-term HSC-like (LT-HSC-like) cluster expressing *CD34*, one lymphoid-primed multipotent progenitor-like (LMPP-like) cluster, one progenitor-like cluster, three GMP-like clusters in different cell cycle states (non-cycling, S, and G2), two monocyte-like clusters (*CD14*⁺ and *CD16*⁺ monocytes), one DC-like cluster and two erythrocyte-like clusters (early and late erythrocytes) (Figure S2g, h). In addition, two lymphoid cell subsets clustered separately from the myeloid cells (B-cells and NK/T cells), which is in line with previously published AML studies^{5,6}.

Next, we performed additional analyses to explore the identity of two remaining cell clusters with a relatively high mitochondrial RNA content that passed our stringent quality control (Figure S2c). A similar cell cluster was observed in a recent single-cell transcriptomics study including 10 *NPM1*-mutated AML¹³. Since a relatively high content of mitochondrial RNAs may indicate poor data quality, we evaluated to what extent our clustering was affected by variability in mitochondrial content. After adjusting for mitochondrial content, the two high-mito clusters persisted as independent clusters (Figure S2i, j). Moreover, projecting the cells of each individual patient into a separate map revealed that high-mito cells were present within each AML sample (Figure S3). Collectively, these data suggest that the two cell clusters with a high content of mitochondrial RNAs are not the result of poor-quality data of one or a few samples but represent a consistent signal across all AML samples.

Finally, for each cell cluster, differentially expressed genes (DEGs) were identified by comparing its gene expression profile with the other cell clusters (Figure 6.1g, Supplementary Table 2). We plotted the DEGs identified for high-mito cells using an independent healthy bone marrow atlas¹⁸ and demonstrated that these cells exhibited signatures reminiscent of early progenitors (Figure S2k). Overall, expression of the DEGs was stronger in the more mature myeloid cell clusters than in the hematopoietic stem cell or progenitor cell clusters. Of note, the DEGs identified for the progenitor-like cell cluster were also expressed by early erythrocyte-like cells, suggesting a common lineage origin and early commitment of progenitor-like cells towards the erythrocyte lineage.

To conclude, our single-cell transcriptomics atlas revealed 13 myeloid cell clusters in *NPM1*-mutated AML representing different maturation signatures and states varying from early to late myeloid differentiation.

Transcriptional subtypes of *NPM1*-mutated AML exhibit distinct cell type compositions

To investigate whether the cell clusters were exclusively present in one of the three transcriptional *NPM1*-mutated AML subtypes, or whether these cells were present in all three subtypes in different proportions, we checked the distribution of the three *NPM1*-mutated AML subtypes over the UMAP plot (Figure 6.1d). This analysis showed a clear difference in distribution between the three subtypes due to differences in abundance of specific cell clusters. This observation was further corroborated when the composition of each AML sample was quantified, with samples from the same subtype showing relatively similar cell cluster compositions, and samples from different subtypes exhibiting notable differences in cell cluster composition (Figure 6.1e, S2l). Moreover, in alignment with our previous observations, *NPM1*(1) and *NPM1*(2) samples had more abundant cell clusters with early progenitor phenotypes, whereas cell clusters resembling more mature monocytes and DCs were dominating in *NPM1*(3) samples (Figure 6.1f). Of note, although both *NPM1*(1) and *NPM1*(2) subtypes were more abundant in cell clusters with early progenitor phenotypes, there were also differences between the two subtypes. For instance, the two high-mito clusters were more abundant in *NPM1*(1) samples (8-33%, Wilcoxon rank-sum test $P = 0.005$ and $P = 0.05$ for high-mito and -G2, respectively), whereas *NPM1*(2) samples had higher fractions of LT-HSC-like and LMPP-like cell clusters albeit not significant. Overall, the single-cell transcriptomics data revealed that all 13 cell clusters, representing different myeloid differentiation stages, were present in different proportions in the three *NPM1*-mutated AML subtypes.

NPM1(2) samples resemble multipotent hematopoietic stem cells

To further explore the difference in composition between *NPM1*(1) and *NPM1*(2) subtypes, we compared the expression of *CD34*, which has been used as marker to annotate LT-HSC-like cells. The data showed that *CD34* expression was mostly restricted to *NPM1*(2) samples (Figure 6.2a). To validate this finding, *CD34* expression was analyzed in primary *NPM1*-mutated AML samples from BEAT-AML, an independent bulk-transcriptomics cohort¹⁹. This analysis confirmed that *NPM1*(2) samples had higher expression of *CD34* (Wilcoxon rank-sum $P = 0.0009$, Figure 6.2b, Figure S4a). Since previous studies associated *FLT3-ITD* status with high *CD34* expression^{12,20}, we also separately analyzed *NPM1*-mutated AML samples of BEAT-AML based on their *FLT3-ITD* status (Figure S4b). The data showed that *CD34* expression was higher in *NPM1*(2) samples compared to other subtypes, irrespective of *FLT3-ITD* status ($P = 0.09$).

To further characterize the differences between the *NPM1* clusters, we calculated the stemness score²¹ for our AML samples per cell cluster by creating pseudo-bulks from single-cell data. While the stemness score was shown to decline along the myeloid maturation trajectory, *NPM1*(2) samples had consistently higher stemness scores across all cell clusters compared to *NPM1*(1) and

Results

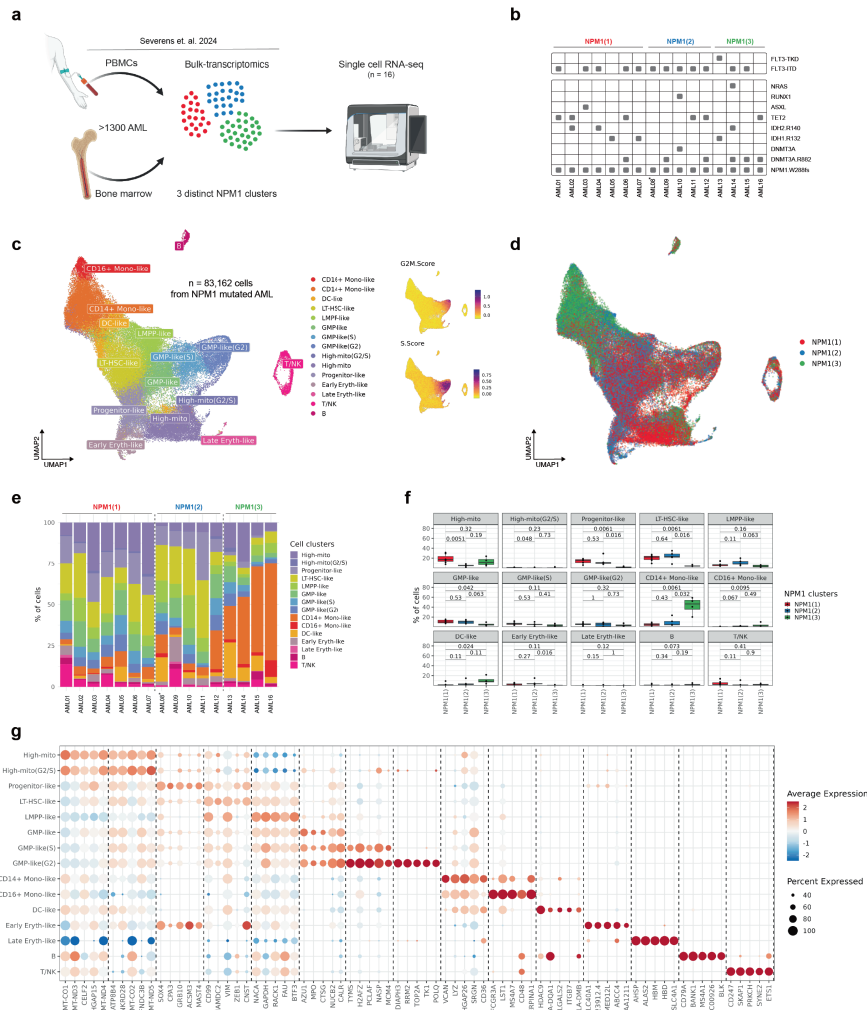


Figure 6.1: AML heterogeneity between NPM1 subtypes by single-cell RNA-sequencing **a** Experimental design – 16 AML cases representing the three distinct transcriptional NPM1 subtypes identified by bulk transcriptomics were selected for single cell RNA-sequencing. **b** Gene mutations detected in our cohort of 16 AML cases collected at primary diagnosis (n=15) or relapse (n=1; AML08*). **c** UMAP plot showing cells from 16 AML cases (n=83,162) colored by annotated cell types accompanied by cell cycle information **d** UMAP plot colored by NPM1 subtype **e** Barplot showing cell cluster distribution per patient. Patients were grouped based on their NPM1 subtypes. Relapse sample AML08* belongs to the NPM1(2) subtype. **f** Boxplots showing percentages of cell clusters per sample grouped by NPM1 subtypes. Boxplots colored by NPM1 subtype. **g** Heatmap showing the top 5 up-regulated DEGs per cell cluster. Dot color indicates average expression per cluster and size shows the percentage of cells expressing the respective gene for that cluster.

NPM1(3) subtypes (Figure 6.2c). Cross-checking the individual genes contributing to the stemness score revealed that *CD34* expression strongly correlated with stemness score (Figure S4c-d, Pearson $r = 0.62$). Altogether, our data indicate that *NPM1*(2) samples have the highest stemness scores and that they seem to retain transcriptional characteristics of multipotent HSCs along differentiation towards more committed progenitor and mature myeloid cells.

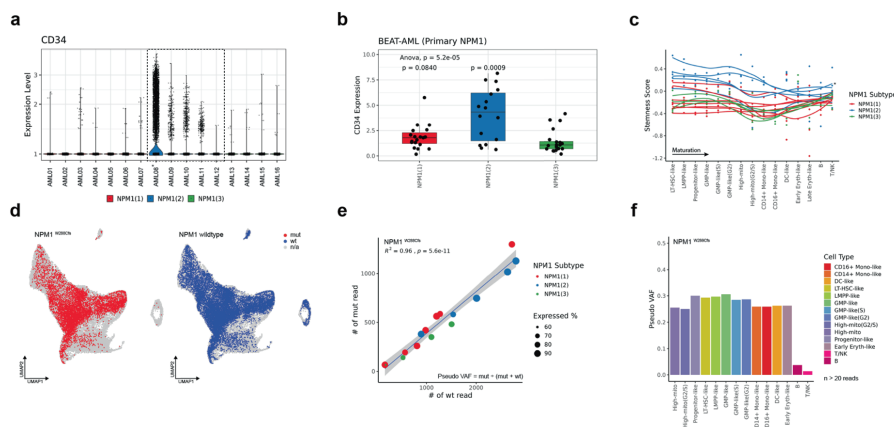


Figure 6.2: AML in *NPM1*(2) subtype resemble hematopoietic stem cells or early progenitor cells **a** Violin plot for *CD34* gene expression per sample. *NPM1*(2) samples, including one relapse sample (AML08*) are highlighted. **b** *CD34* gene expression of *NPM1*-mutated AML at diagnosis of the BEAT-AML cohort. p values are calculated using the Wilcoxon rank-sum test with *NPM1*(3) samples as reference. **c** Stemness score of each cell cluster calculated per sample. To visualize per sample trends smoothed lines were fitted across cell types. Cell clusters are ordered based on their maturation stage. **d** UMAPs plots annotated with cells expressing mutated *NPM1*^{W288Cfs} (left) or wild type *NPM1* allele (right). **e** Scatter plot of mutant (mut) and wildtype (wt) *NPM1* allele counts per sample. Samples are colored by their respective *NPM1* subtype. **f** Barplot of *NPM1*^{W288Cfs} pVAF for all cell types, except for late erythrocytes in which less than 20 reads were detected. All myeloid cell clusters are malignant, whereas lymphoid cell clusters mostly lack the mutation.

Myeloid cell clusters in *NPM1*-mutated AML samples are leukemic

To investigate whether the identified cell clusters represent leukemic cells, we performed variant calling in single-cell RNA-seq data for the known hotspot mutations *NPM1*^{W288Cfs} and *DNMT3A*^{R882H}. Mutated and wildtype *NPM1* reads were detected in all AML samples (Figure 6.2d, S4e). In the two lymphoid cell clusters (B and T/NK cell clusters), most single-cells had only wildtype *NPM1* transcripts, whereas mutant *NPM1* transcripts were detected in many single-cells in all myeloid cell clusters (Figure S4f). Since all samples were selected for high blast percentages (77-99%) by routine morphology and high VAFs (27-38%) for mutant *NPM1* reads by bulk RNA-Seq, we expect that all cells that share gene expression profiles with cells in which mutant *NPM1* reads have been detected are leukemic cells. However, it cannot be excluded that within the various cell clusters, healthy or preleukemic cells co-localize with leukemic cells. Furthermore, the call rates of transcripts across cell clusters were concordant with the expression of the *NPM1* gene (Figure S4g).

Read distributions showed that most cells had only one read covering either the mutant or wild-type *NPM1* allele (Figure S4h). We therefore determined pseudo variant allele frequencies (pVAF) by calculating the ratio of mutant reads to the total number reads for a given number of cells.

Results

All 16 *NPM1*-mutated AML samples (Figure 6.2e, $R^2 = 0.96$, $P = 5.6 \times 10^{-11}$) and all myeloid cell clusters (Figure 6.2f) had similar pVAF. We also re-analyzed *NPM1*-mutated AML samples from Sergi-Beneyto *et al.*¹⁰, who used targeted genotyping to increase the coverage of *NPM1*. pVAF distributions after targeted genotyping closely resembled those in our data, supporting the robustness of pVAF values (Figure S4m-p). Cell clusters with more mature myeloid phenotypes had slightly lower pVAF compared to early progenitor cell clusters, but these differences were not significant (Figure S4i, $P = 0.75$). This slight decrease in pVAF was also observed when samples were compared between the three *NPM1* subtypes, with *NPM1*(3) samples having overall lower pVAF (Figure S4j). Also, *NPM1*(3) samples in BEAT-AML had lower VAF_{DNA} than *NPM1*(1) and *NPM1*(2) subtypes, but the difference was not statistically significant (Figure S4k). Despite the low number of single cells with *DNMT3A*^{R882H}, mutant reads for this hotspot mutation were detected in all cell clusters present in AML samples with this mutation (Figure 6.1b, Figure S4l). Overall, for each sample, we detected mutant *NPM1* transcripts in all myeloid cell clusters, thereby confirming the leukemic origin of the single cells in these clusters.

Heterogeneity of *NPM1*-mutated AML captured by spectral flow cytometry

To investigate whether *NPM1*-mutated AML from the three transcriptional subtypes also displayed different cell surface markers, we performed spectral flow cytometry using an antibody panel against 19 cell surface markers (Figure 6.3a, S5a, see Methods). For this purpose, antibodies against well-established hematopoietic stem or progenitor cell and myeloid differentiation markers were used (CD33, CD45, CD34, CD38, CD117, CD45RA, CLL-1, GPR56, CD14, CD16, CD61, CD11c, CD163, CD68, CD85k-LILRB4) as well as antibodies against HLA class I and II molecules (HLA-DR, -DQ, -DP), for which low gene expression was observed particularly in the *NPM1*(1) subtype⁵ (Figure S5b).

We used this spectral flow cytometry antibody panel to stain the same set of 16 *NPM1*-mutated AML cases as analyzed by single-cell transcriptomics. After removal of doublets, dead cells and lymphocytes, CD33⁺ and/or CD34⁺ cells were gated, and data were analyzed for a total of 433,646 cells. On average, lower cell numbers were acquired for *NPM1*(1) samples than *NPM1*(2) and *NPM1*(3) samples (Figure S6a), and *NPM1*(3) samples had higher number of cells showing strong expression of multiple markers (Figure S6b).

Similar to single-cell RNA-seq data, we projected all 433,646 single-cells from the 16 *NPM1*-mutated AML samples acquired by spectral flow cytometry onto a UMAP plot and annotated the cells based on the *NPM1* subtype as measured by bulk transcriptomics. Like single-cell RNA-seq results, the data showed that cells from the three *NPM1*-mutated AML subtypes were differently distributed over the UMAP plot (Figure 6.3b), and that single-cells from the same *NPM1* subtype co-localized (Figure S6c). To further characterize the phenotype of the AML cells, we performed clustering with PhenoGraph,²² revealing 33 different cell clusters (Figure 6.3c, see Methods). Cell cluster abundances differed between the three *NPM1* subtypes (Figure S6c-d). Various cell clusters were significantly more abundant in *NPM1*(1) samples (clusters 4, 7, 11, 14), whereas other cell clusters were mainly present in *NPM1*(3) samples (clusters 2, 10, 12, 17, 21, 22, 23) (Figure S6e), illustrating that the AML sample composition differed between the three *NPM1* subtypes.

NPM1 subtypes display different cell surface markers

Based on what is known about marker expression during normal hematopoiesis from hematopoietic stem cells to more mature myeloid cell populations^{23–25}, we grouped the 33 flow cytometry clusters into 5 major AML phenotypes, i.e. Hematopoietic Stem Cell-like (HSC-like) (CD34⁺CD38⁺Lin⁻), Early Progenitor-like (CD34⁺CD117⁺CD45RA⁺Lin⁻, CD34⁺CD117⁺CD45RA⁻Lin⁻, CD34⁺CD117⁻CD45RA⁺Lin⁻), Late Progenitor-like

Resolving inter- and intra-patient heterogeneity in *NPM1*-mutated AML at single-cell resolution

(CD34⁺CD117⁺CD45RA⁺Lin⁻), Mono-like (CD14⁺CD11c⁺, CD16⁺CD11c⁺, CD68⁺CD11c⁺), and DC-like (HLA-DQ⁺CD11c⁺) AML cells. The Mono- and DC-like clusters were defined by expression of at least one lineage (Lin) marker, including CD14, CD16, CD68 and/or CD11c, whereas all progenitor cell clusters were negative for these lineage markers (Figure 6.3d).

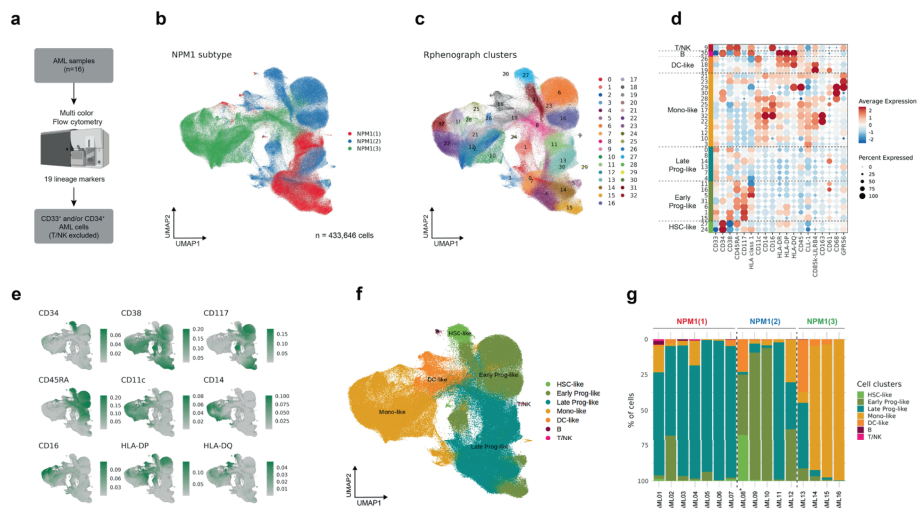


Figure 6.3: AML heterogeneity between *NPM1* subtypes by spectral flow cytometry a Chart illustrating the strategy for spectral flow cytometry. The same 16 AML samples analyzed by single-cell RNA-sequencing were also measured by spectral flow cytometry. b UMAP plot of AML cells (n=433,646) measured by spectral flow cytometry data colored by *NPM1* subtypes. c UMAP plot for 33 *PhenoGraph* clusters (k = 60). d Dot plot showing all 19 markers. Each marker is scaled across clusters. Colored bars on the left indicate cell type annotations. e Expression of 9 cell surface markers (CD34, CD38, CD117, CD45RA, CD11c, CD14, CD16, HLA-DP and HLA-DQ). f UMAP plot colored with cell type annotations. g Barplot showing cell type distribution per patient. Patients are grouped based on their *NPM1* subtypes. The relapse sample belongs to the *NPM1*(2) subtype (AML08*).

To corroborate these cluster annotations and as a data quality check, we selected CD33⁺ or CD34⁺ cells from our single-cell transcriptomics data and associated the flow cytometry markers with their corresponding gene expression (Figure S6f, see Methods). All markers used to annotate the 5 major AML phenotypes showed significant correlations between gene and protein expression (Figure 6.3e, S6f). Having ensured that marker expression correlated with their corresponding genes, we next correlated the markers of the 33 flow cytometry clusters with the clusters found in scRNA-seq data (Figure S6g). This analysis yielded similar patterns with the same 5 major AML cell subsets in Figure 6.3d. Of note, high-mito clusters from single-cell RNA-seq data correlated with late progenitor-like cells (CD33⁺CD34⁺CD117⁺CD45RA⁺Lin⁻). Next, we projected the 5 major cell subsets onto the UMAP plot of the flow cytometry data (Figure 6.3f) and demonstrated their distribution within each of the 16 AML samples (Figure 6.3g).

The flow cytometry data supported single-cell RNA-seq results, demonstrating that AML cells with early progenitor-like phenotype dominated in *NPM1*(2) samples, whereas *NPM1*(1) samples mainly contained AML cells with late progenitor-like phenotypes, and AML cells with more mature monocyte- or DC-like phenotypes were abundantly present in *NPM1*(3) samples. The only discrepant sample was AML11, which has been identified as *NPM1*(2) sample by bulk transcriptomics. Further inspection of this sample confirmed lower *KIT* (CD117) gene expression in this sample as compared to other *NPM1*(2) samples (Figure S6h), which may explain its resemblance

Discussion

to NPM1(1) by spectral flow cytometry. Overall, the spectral flow cytometry data confirmed that there is intra-patient heterogeneity in *NPM1*-mutated AML samples and that inter-patient heterogeneity largely overlapped with our identified transcriptional *NPM1* subtypes.

HSC in *NPM1*-mutated AML can be leukemic, but also healthy or preleukemic

Two cell clusters in the flow cytometry data resemble HSCs characterized by CD34⁺CD38⁺CD45RA⁻ (cluster 24) or CD34⁺CD38⁻CD45RA⁺ (cluster 27) expression (Figure 6.4a). Cluster 24 was mainly present in NPM1(1) samples (Wilcoxon rank-sum $P = 0.048$ and $P = 0.024$), whereas cluster 27 was more dominant in NPM1(2) samples, reaching the significance threshold when compared to NPM1(1) (Figure 6.4b, Wilcoxon rank-sum $P = 0.05$). Since we observed a relatively high contribution of the relapse sample to cluster 27, we repeated the analyses after excluding AML08, which confirmed that these leukemic cells can be readily identified in AML samples at diagnosis (Figure S6i-l).

To explore whether these cell clusters represent leukemic or non-leukemic cells, we made use of Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) data from Sergi-Beneyto *et al.*¹⁰. We first selected the 7 *NPM1*-mutated AML samples from their cohort and re-integrated these samples. Next, we projected the cells on a UMAP plot colored by author's original cell type annotations, which resulted in various clusters including lymphoid (B-, T- and NK-cells) and AML cells, which clearly clustered separately (Figure 6.4c). Besides lymphoid cell clusters, there was another healthy or preleukemic cell cluster identified (cluster 16), which consists of cells mostly lacking the *NPM1* mutation (Figure 6.4d, S7a). This cluster had high CD34 protein expression (Figure 6.4e, S7b), and was present in all 7 *NPM1*-mutated AML samples (Figure S7c). Since this cell cluster had the same phenotype as cell cluster 24 in our flow cytometry data (CD34⁺CD38⁺CD45RA⁻), our analyses suggest that cluster 24 represents healthy or preleukemic HSCs that are mainly present in NPM1(1) samples.

To show that early progenies indeed carry the *NPM1* mutation in NPM1(2) samples, we selected CD34⁺ cells within this subtype ($n=3,668$) and demonstrated that the majority of these cells exhibit LT-HSC-like, LMPP-like or Progenitor-like phenotypes (Figure 6.4f, S7d), and that the mutant *NPM1* allele was detected across all different clusters and patients, thereby confirming that cell cluster 27 in the flow cytometry data (CD34⁺CD38⁻CD45RA⁺) represents leukemic cells (Figure 6.4g, S7e). Lastly, we additionally stained two AML samples from each of the three transcriptional *NPM1* subtypes as well as relapse sample with a polyclonal antibody against human mutant NPM1 (S7f-g, Supplementary Table 3). The data confirmed that cells with a CD34⁺CD38⁻CD45RA⁺ phenotype are leukemic, as they stained positive for the intracellular mutant NPM1 protein, whereas cells with a CD34⁺CD38⁺CD45RA⁻ phenotype were negative for intracellular mutant NPM1, supporting that these cells are healthy or preleukemic HSCs (Figure 6.4h, i). The data also showed that healthy or preleukemic HSC populations were detectable at low frequencies in all AML samples, whereas leukemic HSC-like AML cells were present in NPM1(2) and NPM1(3) samples, but absent or barely detectable in analyzed NPM1(1) samples.

6.4 Discussion

NPM1-mutated AML accounts for ~30% of adult AML patients and it is a single entity in recent WHO and ICC 2022 classification^{1,2}. Nevertheless, previous studies demonstrated that this genetic subclass harbors significant phenotypic heterogeneity with potential prognostic and therapeutic significance^{5,6,9}. Here, we followed up our recent work in which analyzed >1200 bulk transcrip-

Resolving inter- and intra-patient heterogeneity in *NPM1*-mutated AML at single-cell resolution

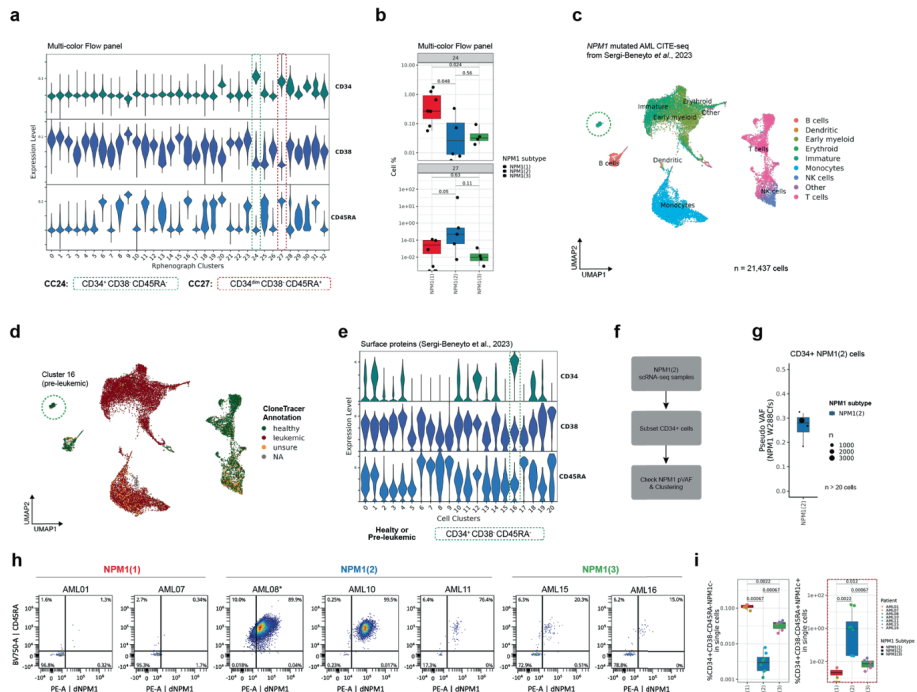


Figure 6.4: AML in *NPM1*(1) subtype contain healthy or preleukemic HSCs **a** Cell surface expression of CD34, CD38 and CD45RA on all cell clusters as identified by spectral flow cytometry. Two CD34⁺CD38⁻ cell clusters resembling HSC or early progenitor cells (CC24 and CC27) are highlighted. **b** Percentages of cells within each sample for CC24 and CC27 grouped by *NPM1* subtypes. p-values were calculated using the Wilcoxon rank-sum test. **c** UMAP plot of integrated cells from 7 *NPM1*-mutated AML at diagnosis of Sergi-Beneyto *et al.*¹ analyzed by CITE-seq. Cells colored by reported cell type annotations. **d** Same UMAP colored with CloneTracer annotations indicating malignant cells. Cluster 16 is highlighted as healthy or preleukemic cell subset. **e** Surface expression of CD34, CD38 and CD45RA as measured by CITE-Seq for all cell clusters. **f** Strategy for single-cell RNA-seq samples to show pVAF in CD34⁺ cells of *NPM1*(2) subtype. **g** *NPM1*^{W288Cfs} pVAF for CD34⁺ *NPM1*(2) cells. **h** Intracellular mutant *NPM1* (x-axis) and surface CD45RA (y-axis) expression for CD34⁺CD38⁻ gated cells in 7 AML including relapse sample AML08 by flow cytometry. *NPM1*(1) samples have been selected specifically for clear presence of cluster 24. **i** Percentage of leukemic (CD34⁺CD38⁻CD45RA⁻*NPM1*c⁺) and healthy or preleukemic (CD34⁺CD38⁻CD45RA⁺*NPM1*c⁻) cells in total viable cells. Note that healthy or preleukemic HSCs are present at small frequencies in all AML samples, whereas leukemic HSC-like AML cells are detectable in *NPM1*(2) and *NPM1*(3) samples, but not or barely in analyzed *NPM1*(1) samples.

Discussion

tomics data of AML cases and identified three major transcriptional subtypes of *NPM1*-mutated AML. In the current manuscript, we leveraged same-sample AML single-cell RNA sequencing and spectral flow cytometry data to show that these transcriptional subtypes can be traced back to differences in proportions of cell subsets, ranging from early hematopoietic stem or progenitor cells to more committed progenitors and mature myeloid cell types. Overall, the results suggest that in *NPM1*-mutated AML, myelopoiesis continues with leukemic cells skewed towards different myeloid cell types.

Based on our single-cell analyses, we hypothesize that AML belonging to the NPM1(2) subtype originate from early progenitor cells producing myeloid offspring. Flow cytometry data showed that CD45RA and CD117, which are early progenitor markers in normal hematopoiesis²³, are often present in leukemic cell populations in NPM1(2) subtype. scRNA-seq data also demonstrated that early progenitor-like AML cells are most frequent in NPM1(2) subtype, and that all leukemic cell populations in this subtype have higher stem cell scores than leukemic cell populations in AML of the NPM1(1) and NPM1(3) subtypes. Since monocyte- and DC-like AML cells have also been detected in the NPM1(2) subtype, our data suggest that at least a proportion of the early progenitor-like AML cells retain the capacity to differentiate into more mature myeloid cells.

In contrast to AML belonging to NPM1(2), CD34, CD45RA and CD117 were absent or low on all or the majority of leukemic cell populations in NPM1(1) and NPM1(3) samples. Flow cytometry and scRNA-seq data showed that AML cells with more mature phenotypes, expressing high levels of maturation markers (CD11c, CD14, CD16, HLA-DR/DQ/DP, LILRB4, CD163, CD68), are most abundant in samples of the NPM1(3) subtype. This suggests that these AML may have developed from progenitor cells that are more primed to the monocytic lineage, such as GMP cells, monoblasts or promonocytes²³, and that these progenitor cells, upon acquiring the *NPM1* mutation, retain the ability to differentiate into mature myeloid cells. An alternative possibility is that NPM1(3) samples originate from the same early progenitor-like cells as NPM1(2) samples, but that the progenitor cells in NPM1(3) samples are less arrested and therefore produce more mature myeloid offspring. This possibility may be supported by our scRNA-seq data showing that besides mature monocyte-, macrophage- and DC-like AML cells, leukemic cells with earlier progenitor-like phenotypes are also present in samples of the NPM1(3) subtype, albeit in smaller proportions than in NPM1(2) samples. Due to the abundant presence of mature AML cells, NPM1(3) cases could be more resistant to treatment with the BCL2-inhibitor Venetoclax, as a monocytic phenotype in AML has been associated with higher resistance to Venetoclax and lower overall survival rates^{9,26–29}.

Flow cytometry data also shows absence of CD34, CD45RA and CD117 on all or the majority of cell populations in AML of the NPM1(1) subtype, suggesting that these AML may have originated from cells that are more committed than the early progenitor-like AML cells in the NPM1(2) subtype. Our scRNA-seq data shows that in particular cells with a high mitochondrial RNA content are abundantly present in the NPM1(1) subtype. A similar subset was recently reported by Naldini *et al.* for *NPM1*-mutated AML samples¹³. Our data shows evidence that AML cells with a high content of mitochondrial RNAs resemble progenitor cells that are more committed, such as CMP, GMP or myeloblasts, yet lack the characteristic markers to define the cell type of origin. Interestingly, the functionally complementary IDH1/2 and TET2 co-mutations, that are enriched in NPM1(1) subtype, have previously been linked to a disturbed alpha-ketoglutarate metabolism which leads to genome-wide hypermethylation³⁰. This might explain upregulation of Fat mass and obesity-associated protein (FTO) gene expression as previously reported for the NPM1(1) subtype⁵, encoding a mRNA demethylase potentially counteracting this global hypermethylation. This suggests that cells with a high content of mitochondrial RNAs, which are notably abundant in NPM1(1) samples, may exhibit reduced expression of various cell surface markers as a result of

extensive hypermethylation.

As described above, AML cells with a high content of mitochondrial RNA may represent late progenitor cells such as CMP- or GMP-like cells or myeloblast-like cells that are more committed to the granulocytic lineage²³. The hypothesis that AML belonging to the *NPM1*(1) subtype may have originated from more differentiated progenitor cells than AML of the *NPM1*(2) subtype is supported by flow cytometry data showing a small healthy or preleukemic cell cluster similar in phenotype to healthy HSC (CD34⁺CD38⁺CD45RA⁻) in AML of all three *NPM1* subtypes, whereas another small cell cluster resembling leukemic stem cells (CD34⁺CD38⁺CD45RA⁺) is present in AML of the *NPM1*(2) and *NPM1*(3) subtypes, but barely detectable in *NPM1*(1) samples. Kersten *et al.*³¹ demonstrated that CD34⁺CD38⁺CD45RA⁺ cells are leukemic stem cells (LSCs) that are present in CD34-positive AML, but absent in CD34-negative AML. We demonstrated that healthy (or preleukemic) HSCs as well as LSCs are often present in *NPM1*-mutated AML, which are typically negative for CD34, but that frequencies are extremely low and that LSCs can be absent or barely detectable in *NPM1*(1) samples. However, our cohort is small, and our observations need to be confirmed by deep analysis of a larger panel of *NPM1*-mutated AML.

The current study also has some limitations. First, our panel of AML includes both bone marrow and peripheral blood samples. Although bone marrow and peripheral blood specimens are largely similar in cellular compositions³², it cannot be excluded that small differences between the three *NPM1* subtypes are attributed to sample source. Secondly, in our previous analysis with bulk-transcriptomics, the size of the compendium allowed us to subdivide *NPM1*(2) and *NPM1*(3) each in two further subtypes⁵. For instance, *NPM1*(3) could be distinguished into two clusters dominated by CD14⁺ or CD16⁺ monocyte-like AML cells, indicating that there is still underlying heterogeneity, and that future single-cell studies are warranted to deduce the mechanisms underlying these additional *NPM1* subtypes. Thirdly, for variant calling, we recommend interpreting results from read-level pseudo VAF (pVAF) values on cell clusters, since variant calling on single-cells relies on only a few reads and may therefore be heavily biased and lead to wrong interpretations. Yet, pVAF values in our experiments were not affected by variable *NPM1* gene expression between different cell types. Furthermore, although we convincingly demonstrated that AML cells with a high content of mitochondrial RNAs have similar *NPM1* pVAF levels as other leukemic cell clusters, thereby confirming their leukemic origin, our antibody panel used for spectral flow cytometry lacked any strong positive lineage markers for these cells. Our single-cell transcriptomics data provide an opportunity to refine our flow cytometry panel, and since the high mito clusters lack most cell surface markers, other flow cytometry protocols may be considered requiring cell fixation and permeabilization to allow inclusion of antibodies against RUNX1, FOXPI, PBX3, and MEIS1, which have been identified as transcription factors that are highly expressed. (see Supplementary Table 2).

In conclusion, our data showed that AML in the three transcriptional *NPM1* subtypes previously identified by bulk transcriptomics contained different proportions of the same leukemic cell types resembling early progenitor or more mature myeloid cells. Our data favor a model in which AML of the three *NPM1* subtypes originate from different progenitor cells with variable capacity to produce more differentiated offspring. This model suggests that *NPM1*-mutated AML belonging to different transcriptional subtypes may differ in susceptibility to standard or new anti-cancer therapies, and our model may thus have potential relevance for prognosis and treatment of patients with *NPM1*-mutated AML.

6.5 Methods

AML samples

All sixteen peripheral blood and bone marrow samples were obtained from patients with AML with high blast levels (77-99%) at diagnosis (n=15) or relapse after chemotherapy (AML08, n=1) with written informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation and cryopreserved in the Leiden University Medical Center (LUMC) Biobank for Hematological Diseases, and approval to use *NPM1*-mutated AML for this research has been obtained by the LUMC Institutional Review Board (protocol no. RP24.056).

Sample preparation for single-cell RNA-seq

Single-cell RNA-seq was performed using the 10X Genomics Chromium Next GEM Single-cell 3' Kit v3.1 (PN-1000268) and 10X Genomics Chromium Next GEM Chip G Single-cell Kit (PN-1000120) according to the manufacturer's instructions (User Guide CG000315 Revision D). The libraries were pooled and sequenced on a NovaSeq 6000 platform (Illumina) using a 300-cycle kit S4 flow cell with v1.5 chemistry.

Downstream analysis of single-cell RNA-seq data

CellRanger v7.0.0 was run on all samples with the human reference genome hg38. For all QC *Seurat* v4 was used¹⁵. Our QC pipeline had three steps per sample: 1) soft filtering, 2) low quality cluster removal, and 3) doublet detection. In soft filtering, *Seurat* objects were created with cells expressing at least 200 genes and with the genes expressed at least in 3 cells. Then, standard *Seurat* command list with default parameters was run to detect low quality clusters. Clusters with >15% mitochondrial and <1000 mRNA in median were removed. Next, *DoubletFinder* v3¹⁶ was run for each sample with 7.5% detection rate and then singlets were selected. To integrate the samples, CCA mode of *Seurat* pipeline was used with most variable 2000 features. Finally, after integration we filtered cells with >15% mitochondrial mRNA.

We used standard *Seurat* commands to scale and normalize the data on integrated features. The first 30 principal components were used to create UMAP plots. We used *clustree*¹⁷ to determine the optimal cluster number, based on *FindClusters* with resolutions sweeping from 0 to 1.2. We chose res=0.5, as clusters became stable (Figure S1a). Next, we merged two clusters (CC5 and CC12) into one GMP-like cluster as one of these clusters (CC12) had high expression of HSP-genes yet still retained its cell-type specific properties (Figure S1c, d).

Spectral flow cytometry

AML patient derived bone marrow or peripheral blood mononuclear cells were thawed, purified by ficoll-paque and stained with a 23-color antibody panel on the same day as data acquisition. Cells were stained with Zombie Red viability dye (Biolegend) for 15 minutes at room temperature (RT), washed, incubated for 15 minutes in PBS supplemented with 2.5% human serum (Sanquin) and subsequently stained with fluorochrome-conjugated antibodies in Brilliant stain buffer plus (BD) for 30 minutes at 4°C. The antibodies used in the spectral flow cytometry marker panel were included in Supplementary Table 1. The staining protocol to measure intracellular mutant NPM1 by flow cytometry has been described in the extended methods.

6.6 Acknowledgments

Authors would like to thank Peter van Balen for his help on data acquisition and transfer, and Leon Mei and Davy Cats for their help on data upload. This study received financial support from a strategic investment from the Leiden University Medical Center, integrated within the Leiden Oncology Center and conducted within the Leiden Center for Computational Oncology, and partly by the Dutch Cancer Society (project number 15152). EBA was supported by a personal grant from the Dutch Research Council (NWO; VENI: 09150161810095). The funding entities played no part in determining the study's design, data collection, analysis, interpretation, manuscript composition, or the decision to submit it for publication. *BioRender* icons were used in graphical abstract, Figure 6.1a, 3a.

6.7 Author Contributions

EBA, MG, MJTR designed the project; EBA acquired funding; EMA, NES, MG, SK and HV helped with data generation and transfer; EOK, EMA performed computational and statistical analyses; EOK created all figures; EOK, RSHZ implemented variant calling pipeline; EOK, EMA, RSHZ, JFS, EBA, MG, and MJTR supported data exploration and interpretation; MJTR, MG and EBA provided supervision and scientific direction; EOK, EBA and MG wrote the manuscript; and all authors critically reviewed the manuscript and figures.

DATA AVAILABILITY

All single-cell RNA-sequencing data was submitted to EGA with the accession number EGAS50000000332, and they are accessible upon request (due to privacy rules). Processed version of the scRNA-seq data for all 16 *NPM1*-mutated AML samples were uploaded to Figshare under the DOI: <https://doi.org/10.6084/m9.figshare.26189771>.

CODE AVAILABILITY

All source codes are available at <https://github.com/eonurk/scNPM1>. Our AML variant calling pipeline for single-cell RNA-seq data is also open-sourced and available under the same repository.

6.8 References

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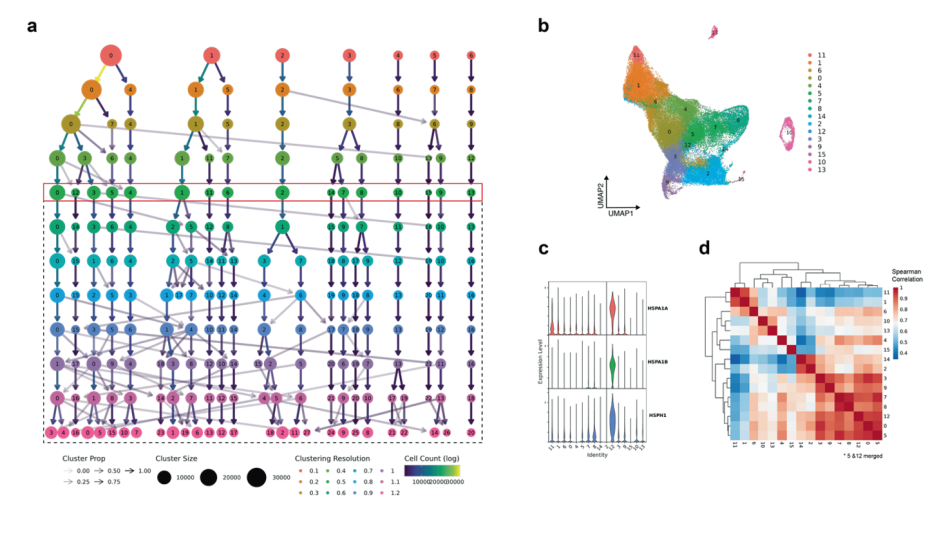
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6.9 Appendix

6.9.1 Supplementary Tables

To minimize unnecessary printing and reduce material use, extensive data including 14 supplementary tables are provided online at: <https://doi.org/10.1038/s41698-024-00596-9>.

6.9.2 Supplementary Figures

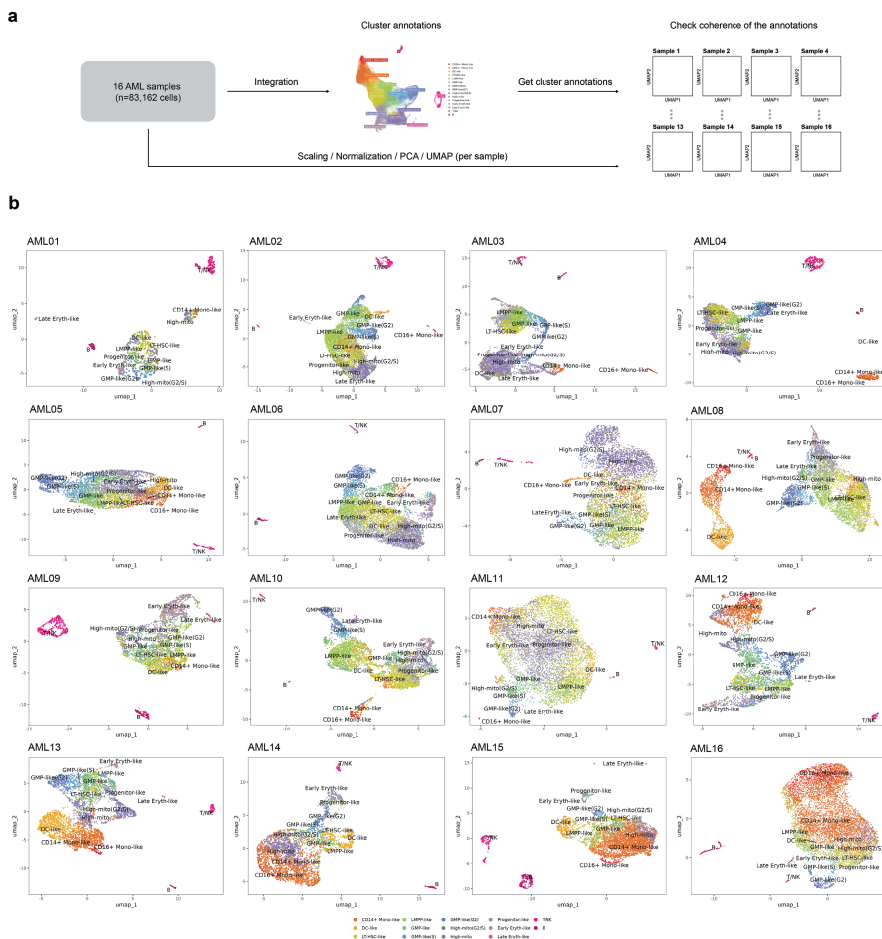


Supplementary Figure 1 - Distinct cell clusters in *NPM1*-mutated AML by single-cell RNA-sequencing. **a** Clustering tree for determining optimum clustering resolution. Selected resolution ($r=0.5$) is highlighted via red lines. **b** UMAP plot of *NPM1*-mutated AML atlas colored by clusters at selected resolution. **c** Heat shock protein (HSP) genes across cell clusters, indicating presence of a stress response probably due to technical artifacts in CC12. **d** Spearman correlations among cell clusters. Note that CC12 and CC5 were merged in further downstream analysis to recapitulate biology rather than technical artifacts, resulting in a total of 15 cell clusters.

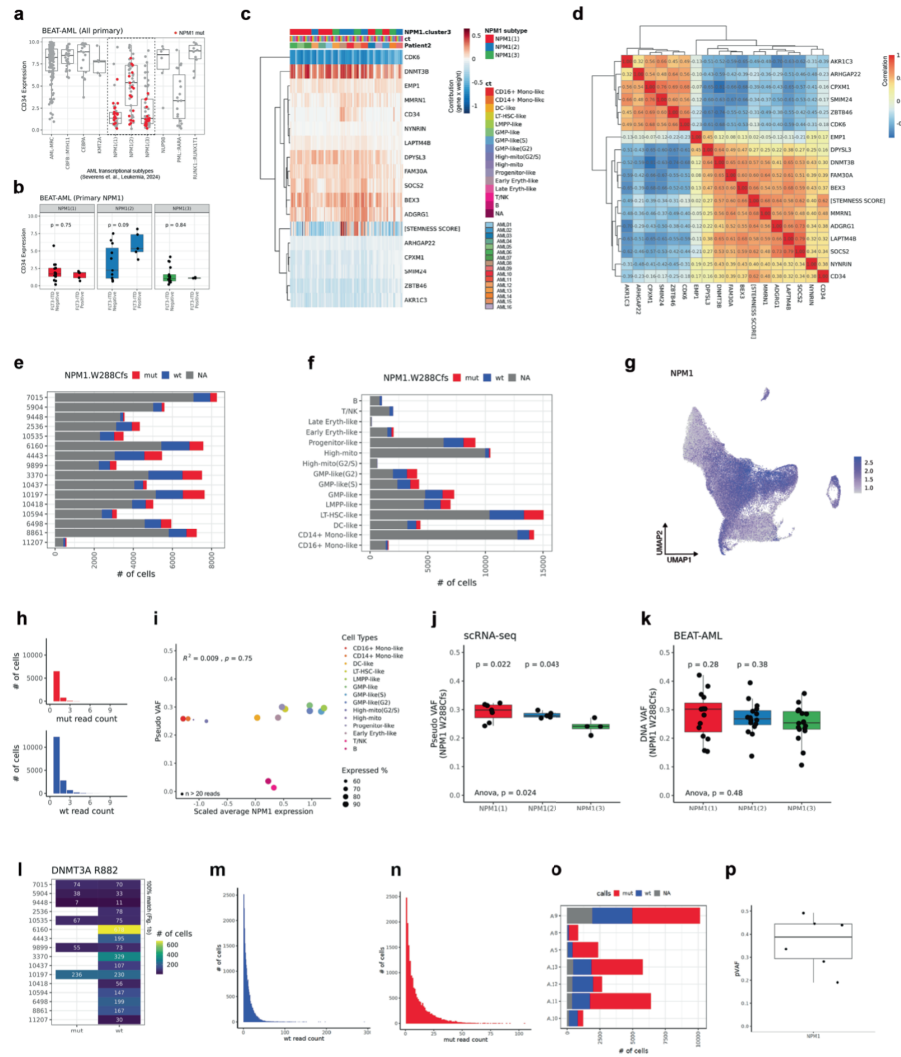
Supplementary Figure 2 - Quality control and annotation of cell clusters in *NPM1*-mutated AML by single-cell RNA-sequencing. **a**, **c** Standard quality control (QC) metrics for all AML samples and cell cluster. **b**, **d** Barplot for the number of cells per patient and cell cluster. **e** UMAP plot of *NPM1*-mutated AML atlas annotated by Azimuth at $> 90\%$ prediction score **f** Barplot showing the distribution of Azimuth annotations per cluster **g** Heatmap of AML cell type-specific genes as identified by van Galen *et al.*² per cell cluster **h** *CD34* expression across cell clusters **i** UMAP plot of the *NPM1*-mutated AML cell atlas after regressing out high mitochondrial RNA content **j** Barplot showing the distribution of cell type annotations after correction for high mitochondrial RNA content. Cell clusters characterized by a high content of mitochondrial RNAs do not merge with other cell clusters after regressing out the high content of mitochondrial RNAs. **k** Expression of DEGs specific for cell clusters with a high content of mitochondrial RNAs in an independent cohort of healthy bone marrow samples of Suo *et al.*³ **l** Distribution of all cells for each separate AML sample on the *NPM1*-mutated AML cell atlas.



Resolving inter- and intra-patient heterogeneity in *NPM1*-mutated AML at single-cell resolution

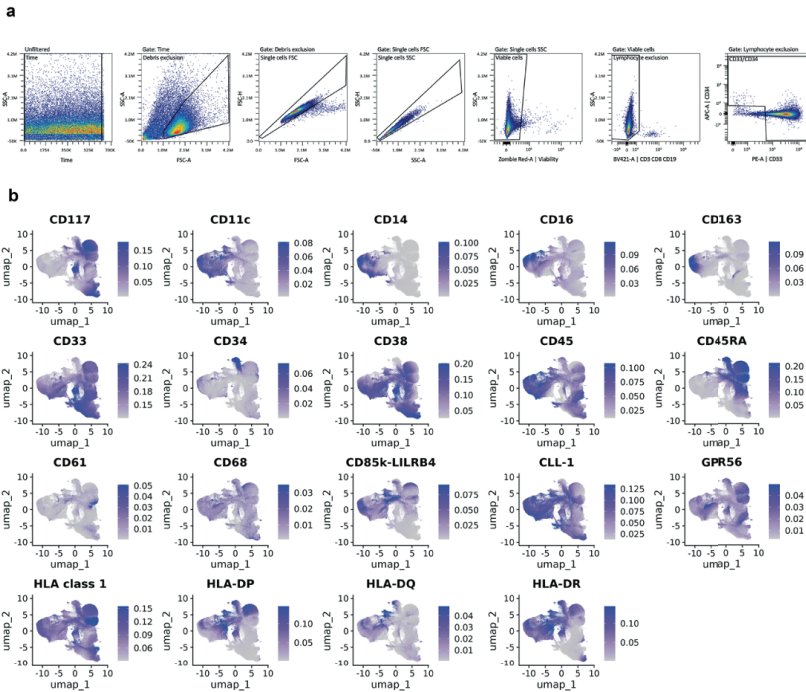


Supplementary Figure 3 - Quality control for integration and high-mito subsets. **a** Sketch showing the performed procedure for analyzing quality of integration. Seurat pipeline was run 16 AML samples independently. Then each sample's cells were colored by the integrated cell type annotations to check for inconsistent cell clusters that merged randomly **b** UMAP plots showing all *NPM1*-mutated AML samples. All AML samples are obtained at primary diagnosis except for AML08, which is a relapse sample. Cells are colored by cell annotations from integrated cell atlas.



Supplementary Figure 4 - **Detection of stemness and mutant alleles in *NPM1* subtypes by single-cell RNA-sequencing.** **a** *CD34* gene expression in AML samples at diagnosis of the BEAT-AML cohort. *NPM1* subtypes are highlighted via a dashed line. Red dots indicate presence of the *NPM1* mutation. **b** *CD34* gene expression across *NPM1* subtypes in the BEAT-AML cohort divided based on FLT3-ITD status. **c** Contribution (gene expression x coefficient of that gene from Ng. *et al*⁴) of individual genes to the stemness score for each pseudo-bulk. 15 pseudo-bulks profiles were created for each sample (one per cell type) from single cell RNA-seq data. [Stemness Score] in the heatmap indicates the final stemness score for each pseudo-bulk. **d** Correlation of each gene with stemness score. Note that *CD34* expression is strongly correlated with stemness score ($R = 0.62$). *MMRN1* and *ADGRG1* also showed strong correlations with stemness score with $R = 0.68$ and $R = 0.64$, respectively. **e** Barplot showing the percentage of cells with mutated (red) or wild-type (blue) *NPM1* alleles for each patient **f** and cell cluster. Gray indicates that no reads were found for *NPM1*. **g** *NPM1* expression on the UMAP plot. Note that *NPM1* expression is low for monocyte-like AML cells and AML cells with a high content of mitochondrial RNAs, and that this affects the sensitivity to call mutant *NPM1* alleles in these cell clusters. **h** Distribution of mutant (mut) and wildtype (wt) reads in individual cells. Most cells have been annotated as mutant or wildtype based on one single read. **i** *NPM1*^{W288Cfs} pVAF with *NPM1* expression per cell cluster. **j** *NPM1*^{W288Cfs} pVAF across *NPM1* subtypes **k** VAF_{DNA} for *NPM1*-mutated AML at diagnosis of the BEAT-AML cohort split by *NPM1* subtype. p-values were calculated using the Wilcoxon rank-sum test. **l** Heatmap showing the number of cells with detectable reads for mutated or wild-type DNMT3A allele. Note that mutant DNMT3A alleles have only been detected in AML samples that are positive for the respective mutation as shown in Figure 1b. **m-p** Similar analysis for *NPM1*-mutated AML samples of Sergi Beneyto *et al*.

Appendix

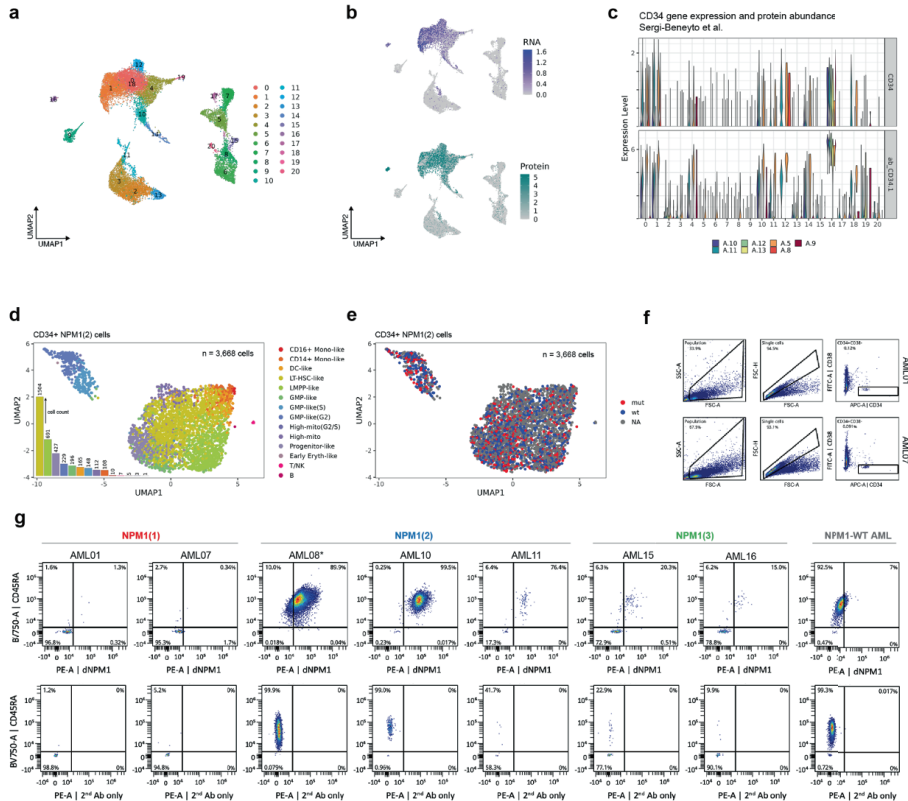


Supplementary Figure 5 - Marker expression in cell clusters by spectral flow cytometry. **a** Gating strategy for a representative AML in flow cytometry analysis. **b** UMAP plots showing expression of the 19 markers in cell clusters as measured by spectral flow cytometry. For each marker, maximum values were capped at 95% quantile.

Supplementary Figure 6 - AML heterogeneity between NPM1 subtypes by spectral flow cytometry and single-cell RNA-sequencing. **a** Number of cells measured by spectral flow cytometry per AML sample. **b** UMAP plot showing total expression of all 19 markers as measured by spectral flow cytometry. **c** Distribution of cells on the UMAP plot for each AML sample using the *ggsdensity* package. **d** Heatmap showing clustered percentages for each cell cluster per AML sample. Cell percentages were transformed with *log1p* function for better representation. **e** Proportion of individual cell clusters in samples across NPM1 subtypes. Pairwise p-values were calculated using the Wilcoxon rank-sum test. **f** Scatter plots showing normalized cell surface expression by spectral flow cytometry (y-axis) versus corresponding gene expression by single-cell RNA-sequencing (x-axis) for each sample. **g** Similar to (f) showing the proportion of individual cell clusters in samples by spectral flow cytometry versus single-cell RNA-sequencing on a heatmap plot. **h** *KIT* gene expression for each sample by scRNA-seq. AML11 has lower *KIT* gene expression than other NPM1(2) samples. **i** UMAP plot of spectral flow cytometry data without the relapse sample (same $k=60$). **j** Cell cluster distributions from original versions of flow data to new clusters without the relapse sample. Alluvial plot showing the cells going from original clusters (left) to the new cell clusters (right). Note that most cell clusters are unchanged but renumbered. **k** Heatmap showing the same distribution but normalized across new clusters for better visibility of the renumbering of the cell clusters. Please note that this is especially useful for clusters with low number of cells. **l** UMAP plot of flow cytometry data of only primary AML samples. Two previously detected HSC-like clusters were indicated with orange and red. Dashed line indicates the Rphenograph cluster.

6





Supplementary Figure 7 - Analysis of an independent cohort of *NPM1* mutated AML. **a** UMAP plot of integrated cells from 7 *NPM1*-mutated AML at diagnosis of Sergi-Beneyto *et al.* analyzed by CITE-seq. Cell clusters are represented by different colors. **b** mRNA expression and surface expression of *CD34* by CITE-seq. **c** Similar to (b) with a violin plot and split per sample. Note that *CD34* surface expression is consistently high for CC16 across samples. **d** *CD34*⁺ cells from *NPM1*(2) samples in our own AML cohort analyzed by scRNA-seq colored by each cell type. For each cell type, numbers of *CD34*⁺ cells are shown. *CD34*⁺ cells are most abundant in LT-HSC-like and LMPP-like AML cells. **e** The mutant *NPM1*^{W288Cfs} allele is present in all cell types identified in *CD34*⁺ *NPM1*(2) cells. **f** Gating strategy for single *CD34*⁺*CD38*⁻ cells. **g** Intracellular mutant *NPM1* was detected using a rabbit polyclonal anti-human mutant *NPM1* IgG as first antibody and a goat anti-rabbit IgG as secondary antibody. The top panel shows intracellular mutant *NPM1* (x-axis) and surface *CD45RA* (y-axis) expression on *CD34*⁺*CD38*⁻ gated AML cells for 7 AML. The lower panel shows results after staining with the secondary antibody only. The 7 AML include relapse sample AML08 and a *NPM1* wild-type AML case as control.



Chapter 7

Discussion

This dissertation set out to study immune aging and hematopoiesis, with the overarching aim of understanding how these processes converge upon leukemogenesis in acute myeloid leukemia (AML). In conducting studies, we have explored how molecular pathways drive immune senescence and inflammation, how epigenetic manipulations can preserve or enhance hematopoietic stem cell (HSC) function, and how dissecting molecular data from AML at bulk or single-cell resolution can characterize the disease heterogeneity.

7.1 Immune Aging and Hematopoietic Decline: Mechanisms and Therapeutic Opportunities

In Chapter 2, we systematically studied immune aging in mice. Using transcriptional profiling and chromatin accessibility data, we demonstrated that older immune cells exhibit increased AP-1 activity, correlating with enhanced accessibility of the AP-1 binding site in genes encoding pro-inflammatory cytokines such as IL-6. AP-1 activity and accessibility of AP-1 binding sites in pro-inflammatory cytokine genes were also increased with age in short-lived (NZO) and long-lived (B6) mouse strains. Human data also showed that the accessibility profiles of *JUN* and *FOS*, together encoding the dimeric AP-1 transcription factor, are similar to those of mice. This suggests that AP-1 activation might be an evolutionarily conserved immune signature of aging. Our data refine the term inflammation by placing AP-1 transcription factors as one of the central nodes orchestrating pro-inflammatory gene programs. Yet, further studies are warranted to grasp whether these changes are predominantly driven by metabolic states (e.g., obesity, T2D) or by intrinsic epigenetic drift in immune cells.

Many studies show that inflammation is related to specific immune subsets like monocytes or macrophages¹⁻³. In the context of aging, this myeloid bias is associated with age-related clonal hematopoiesis, which is often driven by recognizable somatic mutations but can also occur in the absence of currently identifiable canonical driver variants⁴⁻⁷. Extensive studies on aging of the immune system demonstrated that a clonal mutation is found in less than 1% of individuals below 50 years of age⁵, but in more than 30% of those above 80 years^{6,7}. For instance, recent single-cell multi-omics data from bone marrow samples of multiple myeloma patients with a *DNMT3A* hotspot mutation revealed that these patients have a clonal expansion in the myeloid lineage⁸, suggesting that increased presence of myeloid cells may affect the entire bone marrow microenvironment, including cells interacting with lymphoid-derived plasma cells. As we age, numbers of myeloid-biased HSCs may increase, and this might be the fundamental culprit that eventually leads to systemic deterioration of the immune system. This leads to the fundamental question of whether myeloid skewing at the HSC level might induce aging of the immune system, and how this can be prevented.

If myeloid skewing in HSCs is indeed causal for immune aging, two complementary strategies may hold therapeutic potential: depleting myeloid-biased HSCs or expanding HSCs with more balanced output of lymphoid- and myeloid-biased HSCs. For instance, Ross *et al.*⁴ recently demonstrated that antibody-mediated depletion of myeloid-biased HSCs in aged mice reduced their dominance in the bone marrow and restored a more balanced pool of HSCs. This shift promoted the expansion of common lymphoid progenitors and increased the production of naive CD4⁺ and CD8⁺ T cells as well as naive B cells. Functionally, these changes translated into stronger vaccine-induced T-cell and antibody responses, improved control of viral infections, and a reduction in exhausted or age-associated immune subsets. Plasma levels of pro-inflammatory cytokines and other proteins elevated with age also decreased toward more youthful levels. Together, these

findings show that restoring the HSC balance not only corrects lineage skewing but also rejuvenates adaptive immunity at both the cellular and functional level, suggesting a promising strategy to delay immune aging and its associated morbidities.

Another way in which output and skewing of HSCs can be influenced is through epigenetic modulation. In Chapter 3, we investigated epigenetic manipulation of HSCs *ex vivo*. Using a BET inhibitor (CPI203) and HDAC inhibitor (Quisinostat), we demonstrated improved HSC expansion and viral transduction efficiency, which is critical for the success of gene therapy protocols. scRNA-seq data along with machine learning models showed that HSC pools were enriched after both treatment conditions. Additionally, Quisinostat unexpectedly skewed HSC differentiation toward the erythroid lineage, suggesting a potential advantage of using this drug for gene therapies to restore red blood cell disorders. Taken together, these findings indicate that epigenetic regulators originally developed for cancer treatment can, in lower dosages, be used to modulate stemness and lineage potential. The next step then would be to apply these gene therapy protocols in preclinical models to evaluate the safety and long-term engraftment potential of epigenetically modified HSCs.

7.2 Recurring computational ambiguities in analysis of cancer data: Context and Method Layers

A myeloid bias in hematopoiesis can also be the result of oncogenesis, like in the case of acute myeloid leukemia (AML). AML is characterized by abnormal expansion of malignant myeloid cells in the bone marrow, which blocks normal hematopoiesis. In Chapter 4, we tried to characterize this extreme derailment of hematopoiesis by applying deconvolution of bulk RNA-seq from over a thousand AML patients using a healthy bone marrow single-cell atlas as reference. This approach allowed us to calculate estimated cell compositions (ECCs) that recapitulated classical AML differentiation stages and revealed lineage arrests consistent with FAB subtypes. Importantly, these ECCs were not only descriptive of disease heterogeneity but also predictive of treatment outcomes. For example, samples enriched for CD14⁺ monocytes displayed resistance to the BCL-2 inhibitor Venetoclax, a pattern that outperformed BCL-2 expression alone. At the same time, the study highlighted critical challenges: leukemic blasts often deviate transcriptionally from their healthy counterparts, and results can shift depending on the reference atlas or resolution of the deconvolution method. So, our analyses revealed two recurring layers of ambiguity in analyzing cancer datasets: **context** (disease-specific phenotypes diverging from canonical healthy states) and **methods** (algorithm choice, modality, resolution).

As a concrete example of methods-related ambiguities, there are many algorithms to perform deconvolution on bulk RNA-seq data. In our study, we employed an algorithm called MuSiC⁹, based on its benchmark performance¹⁰ and that it is open-sourced, which allowed us to build the algorithm into our already existing HAMLET pipeline¹¹. Nevertheless, algorithm choice can strongly affect estimated fractions. Similar to our study, Zeng *et al.*¹² also deconvoluted bulk RNA-seq AML samples and systematically compared different algorithms by assessing how well estimated cell compositions correlated with matched single-cell RNA-seq data. In their evaluation, CIBERSORTx¹³ achieved the highest concordance, with MuSiC ranking second. Together, these findings underscore that while deconvolution is a powerful tool to dissect cell type composition in bulk data, interpretation must be tempered by awareness of algorithm-specific biases and limitations.

As an example of context-related ambiguities, cancer samples add further complexity because

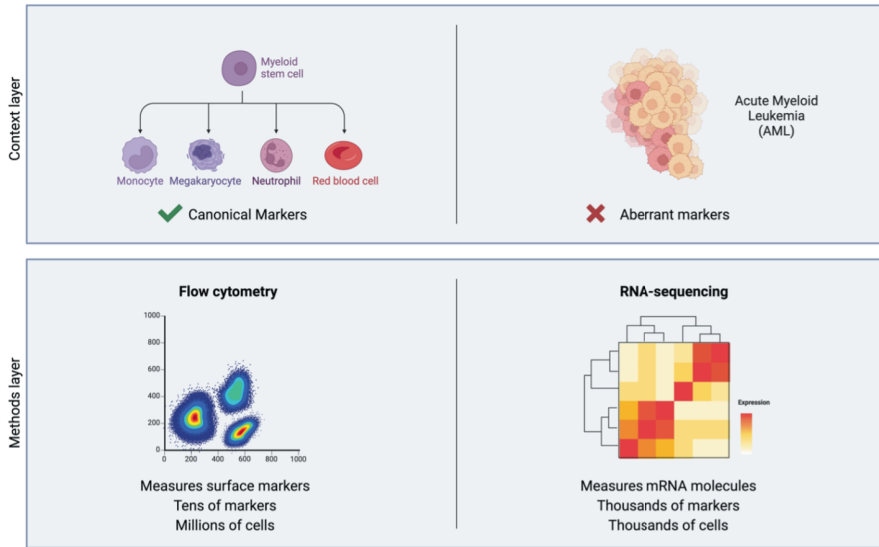


Figure 7.1: Examples for the two layers of ambiguity in analyzing cancer data.

aberrant phenotypes diverge from canonical healthy states¹⁰. Due to this complexity, AML samples are hard to phenotype by deconvolution. For instance, we used a detailed healthy bone marrow sample to deconvolute our in-house RNA-seq samples and correlated them with more coarse spectral flow cytometry phenotypes to understand the cell type compositions. However, although at a high level (e.g. monocytes) the methods agreed with each other, they do not perfectly match for detailed subsets due to technical differences, i.e. flow panels measure the presence of, in the order of ten, highly discriminative cell surface markers for millions of single cells, whereas bulk RNA-seq measures thousands of mRNA molecules for thousands of cells per sample (Figure 7.1).

These two layers also recurred in other chapters of the thesis while analyzing the different types of cancer datasets. For instance, in **Chapter 5**, we used bulk RNA-Seq from 1224 AML patients and demonstrated that the major AML subtypes in the World Health Organization (WHO) 2022 classification, which are defined by genetic aberrations, can also be successfully identified by gene expression profiling. However, we did experience ambiguities caused by the choice of methods when patients had to be assigned to robust clusters based on their transcriptional profiles. First, we identified either three major or five more refined transcriptional clusters of *NPM1*-mutated AML, which could be partly explained by co-mutational profiles. Secondly, the choice of clustering resolution was adhered to a balance between capturing the granularity of transcriptional heterogeneity and maintaining biological interpretability. A resolution that is too fine has the risk of overfitting to transcriptomic noise, while a resolution that is too coarse could obscure meaningful subgroups, particularly those driven by co-mutational patterns. Therefore, we evaluated clustering stability using consensus clustering and silhouette scores, ensuring that the resulting AML clusters were both robust and biologically relevant.

In **Chapter 6**, we used single cell RNA-seq and spectral flow cytometry to characterize the cellular

signatures of *NPM1*-mutated AML. Using 19 markers in flow cytometry, we were able to replicate the distinct transcriptional subtypes of *NPM1*-mutated AML, even though they were based on the expression of more than 20,000 genes measured by single cell RNA-Seq. However, methodological ambiguities also emerged. While the majority of markers showed good concordance between mRNA expression and protein abundance, some did not. For example, CD61 displayed robust protein staining despite weak or inconsistent transcript detection in the single-cell RNA-seq data. Such transcript-protein mismatches are well documented. Correlations between mRNA and protein are often modest, reflecting both technical limitations (e.g. mRNA drop-out, or degradation) and biological regulation (e.g. post-transcriptional control)^{14,15}. Another example of context layer in this chapter was a cluster of “High-mito” cells, enriched within one of the *NPM1*-mutated AML subtypes, which showed elevated mitochondrial gene expression yet remained below the conventional 15% threshold used to flag dying or low-quality cells. Their atypical profile, which was neither fully consistent with technical artifacts nor with canonical early progenitor phenotypes, illustrates how disease-specific metabolic states can complicate interpretation of cellular identities in AML.

Taken together, these findings indicate that analyses of cancer datasets are consistently influenced by two recurring and interacting sources of ambiguity: methodological and contextual. Methodological ambiguities arise from differences in algorithmic design, parameter choices, and modality-specific biases, while contextual ambiguities stem from malignant phenotypes that deviate from canonical healthy states. Careful recognition and transparent reporting of these limitations are essential to ensure that computational analyses yield results that are both robust and biologically meaningful.

7.3 Redefining Leukemia-Initiating Cells: Lessons from *NPM1* Mutations

A lot of the research in the field of AML is focused on the *leukemic stem cell* (LSC). A LSC is a malignant cell with self-renewal capacity that initiates the leukemia upon acquiring a genetic aberration. However, the true definition of a LSC is rather ambiguous. It was first shown by Dick *et al.* in mice with severe combined immunodeficiency (SCID) that the engraftment potential of leukemic human cells is much higher for CD34⁺CD38⁻ leukemic cells than CD34⁻ or CD34⁺CD38⁺ leukemic subsets¹⁶. Yet, as shown in **Chapter 4**, by performing single cell RNA-seq on human cord blood, CD34⁺CD38⁻ cells are still heterogeneous in HSC subsets, and this also holds for leukemic cells¹⁷. For instance, latest mice experiments have shown that knock-in of the *NPM1* hotspot mutation in more differentiated progenitor cell types (e.g. MPP, GMP) can also lead to engraftment and full-blown leukemia, albeit at a lower efficiency compared to earlier stem cell subsets¹⁸. These findings support that LSCs do not necessarily need to have a CD34⁺CD38⁻ phenotype, and that LSCs can also reside in AML cells with other phenotypes. Later, Dick *et al.* defined two gene signatures for LSCs commonly referred to as *stemness score*¹⁹. However, these signatures include *CD34* as one of the most important genes. In **Chapter 6**, we showed that *CD34* gene expression levels strongly correlate with stemness scores across the transcriptional clusters of *NPM1*-mutated AML²⁰, and that *NPM1* mutations are not confined to classical LSCs but detectable across all myeloid cell types. While this distribution does not imply uniform leukemia-initiating capacity, it suggests that leukemic potential is not exclusively restricted to immunophenotypically defined LSCs, but may also emerge from progenitors capable of retaining or re-acquiring stem-like features within a largely preserved differentiation hierarchy.

This principle of stemness plasticity is further exemplified by *NPM1*-mutated AML, where a single molecular lesion is sufficient to reprogram progenitors toward self-renewal. NPM1 mutations, most commonly a 4-bp TCTG insertion (type A) in exon 12, occur in ~30% of adult AML. The frameshift mutation disrupts a nuclear retention signal and creates a nuclear export signal, leading to mis-localization of the nucleolar chaperone to the cytoplasm. The mis-localization of NPM1 (NPM1c) represents both a defining biomarker and an unresolved mechanistic driver. Upon gaining the *NPM1* mutation, myeloid progenitor cells acquire long-term self-renewal. *NPM1*-mutated AML is associated with a distinctive transcriptional program characterized by high expression of *HOXA*, *HOXB*, and cofactors such as *MEIS1*²¹. Shuttling NPM1c back to the nucleus with XPO1 inhibitors extinguishes the *HOXA/MEIS1* program, drives maturation and extends survival in mouse models²².

Recent studies have further refined this mechanism by showing that NPM1c does not merely activate *HOX* genes indirectly, but can also directly regulate oncogenic transcription. In particular, a residual nuclear fraction of NPM1c has been shown to bind a restricted set of active chromatin regions, including the *HOXA* and *HOXB* clusters and *MEIS1*, where it cooperates with XPO1/CRM1, RNA polymerase II, and the MLL1–Menin transcriptional machinery to sustain the leukemic self-renewal program^{23,24}. These observations provide a more direct explanation for the characteristic *HOXA/HOXB*-high state of *NPM1*-mutated AML and also help explain the marked sensitivity of this leukemia subtype to Menin inhibition^{23,24}.

At the same time, indirect mechanisms likely contribute in parallel. Earlier studies showed that NPM1c-driven cytoplasmic mislocalization of key nuclear regulators such as PU.1 and CTCF perturbs differentiation-associated transcriptional control and chromatin architecture, thereby further reinforcing leukemic identity^{25,26}. Together, these findings indicate that NPM1c-mediated stemness is mechanistically multifactorial, reflecting both direct chromatin-level activation of leukemic transcriptional hubs and indirect rewiring of transcription factor and chromatin regulator localization.

Wild-type NPM1 transiently coats unduplicated centrosomes and is released after CDK2–cyclin E phosphorylates Thr199^{27,28}. In *NPM1*-mutated leukemic cells, the cytoplasmic mutant form exhibits elevated Thr199 phosphorylation and a heightened dependence on this modification for G₂/M progression²⁹.

While wild-type NPM1 participates in centrosome licensing, whether mislocalized NPM1c accumulates within centrosome-proximal compartments in primary blasts remains unclear; thus, any proposed centrosome-mediated link to *HOX* control should be viewed as a hypothesis that warrants testing. It would be interesting to address this hypothesis using advanced spatial proteomics (e.g. imaging mass cytometry) integrated with single-cell or spatial transcriptomics, to map centrosome-associated interactors and downstream kinases that could connect NPM1c export to leukemic identity. Taken together, these observations reinforce a central theme: leukemic potential is not limited to LSCs as initially thought but can also be conferred to myeloid progenitor cells other than HSCs through acquisition of mutations such as NPM1c that hijack stemness programs.

7.4 General Discussion and Future Perspective

7.4.1 Understanding the identity of a cell

A human cell is a product of 3.8 billion years of evolution. Arguably, the most complex machine in existence. Millions of proteins work seamlessly to establish intrinsic functions and accommodate to external conditions. A cell consists of different organic molecules, and particularly mRNAs and proteins play an essential role. As outlined in the **Introduction**, lineage identity is imposed not by changes in the DNA sequence but by reversible epigenetic programs such as DNA methylation, histone modifications and higher-order chromatin remodeling that modulate gene accessibility. This epigenetic regulatory layer has first been conceptualized by Waddington in 1942³⁰. Today, thousands of scientists around the world work on these epigenetic changes and try to further map the effects of these changes on cells. Perhaps the most striking example of these endeavors is reversing cell identity to stem cells by only 4 transcription factors³¹. This discovery propelled the epigenetics field, underscoring that cell identity can be reprogrammed by external cues, much like changing the software on fixed hardware. In other words, what cells do is “programmable” without altering the underlying genome. For instance, in **Chapter 3**, we used two different epigenetic modifiers to enhance the yield of HSCs when co-cultured with stem cell factor. Particularly Quisinostat significantly increased the HSC pool without interfering with the ability to transduce these cells with a lentivirus.

Building on this concept of programmability, totipotency represents the ultimate plasticity. It is a uniquely defining feature of cells in an early developing multi-cellular organism, which allows a single cell, the zygote, to create all the types of cells making a fully grown organism. In his classical view, Waddington used a landscape analogy to describe cell fates during development. He used the example of a marble rolling down from a hill. As it rolls down, the marble encounters valleys, which represent different developmental pathways. Once the marble enters a valley, it becomes more committed to a certain cell fate and the way back to a less differentiated state is much harder. This analogy captures the broad intuition, but modern single-cell data suggest refinements are needed. For instance, transcriptional entropy of a cell is correlated with its maturation stage³². High entropy, characterized by many genes that are expressed at similar levels, is associated with more stem cell phenotypes. In contrast, low entropy, characterized by a few genes that dominate expression, is associated with more differentiated cell phenotypes. This is similar to each fork taken by the rolling-marble in a valley which reduces the uncertainty of the outcome. However, rather than a simple continuum of stable fates, differentiation is better described as an attractor landscape, in which gene-regulatory dynamics stabilize discrete cell identities and intermediate states are usually transient. Along trajectories, cells can pass through intermediate configurations, but these are typically transient and low probability. Consistent with this, many surface markers, and their mRNA proxies, exhibit near on/off distributions created by feedback, cooperativity, and thresholds; stable intermediate expression is rare, usually confined to transient states at bifurcations.

Single-cell studies further show that differentiation often proceeds through groups of closely related cell states that form recognizable clusters. Within each cluster, cells have similar phenotypes, illustrating the top-down dynamics that happen in cellular differentiation. Beyond transcriptional and epigenetic states, physical and bioelectric properties also constrain differentiation trajectories. For instance, the cell surface is a thin layer with almost constant capacitance, but varying resistance. Interestingly, resting membrane potential (V_M) also correlates with differentiation³³, with depolarized cells (i.e. chondrocytes) being more prone to cellular division and hyperpolarized cells being less likely to undergo cell division, e.g. neurons, or skeletal muscle cells³⁴. A strik-

ing example of these bioelectric signals affecting cellular differentiation was shown by Levin et al. in planaria. These worms can be cut many times and still regenerate their entire body. Yet remarkably, altering their endogenous bioelectric signals can change the pattern of regeneration. Levin and colleagues demonstrated that by artificially modulating transmembrane voltage gradients, they could control the head size of planaria and could induce regeneration of multiple heads or, conversely, fail to regenerate a head at all^{35,36}. These findings support the view that bioelectric signals serve as a type of pattern memory, guiding cells toward specific morphological outcomes. In other words, the top-down influence of bioelectric states can override default molecular cues, reshaping an organism's anatomy despite a constant genomic blueprint. In the context of hematopoiesis and leukemogenesis, these perspectives suggest that stemness, lineage bias, and even clonal architecture may be governed not only by genetic and epigenetic alterations but also by emergent top-down constraints, from attractor dynamics to bioelectric patterning.

7.4.2 Towards a system-level understanding of biology

Hematopoiesis is a complex system that requires strict regulation. It is a dynamic process that changes throughout the lifespan of an animal. The disruption of this top-down process during hematopoiesis is most prominent in cancer settings. For instance, in **Chapter 4**, we show that AML with distinct morphology by microscopy are dominated by distinct cell types identified from molecular data (RNA-seq), indicating that internal changes in the leukemic blasts are reflected at system scale which can be seen with a microscope. Throughout the thesis, we showed that hematopoiesis can be deregulated and skewed towards the myeloid lineage as a consequence of aging or genetic abnormalities. This bias can be simply explained by competition between different myeloid- and lymphoid-skewed HSC clones. Clones with an evolutionary advantage (e.g. faster division rate) repopulate the hematopoietic system with more exponentially growing cells. This is supported by recent studies in humans with decades of genomics data^{37,38}. Clones with a growth advantage dominate the hematopoietic system and as a consequence, clonal diversity decreases significantly³⁹. These findings promote important questions in the field of systems immunology. As we age, the thymus involutes and naive T-cell output declines, while clonal hematopoiesis of indeterminate potential (CHIP) mutations, most commonly in *TET2* or *DNMT3A*, become more prevalent. *TET2*-mutant clones consistently show myeloid-biased differentiation in both humans and mice, whereas *DNMT3A* mutations primarily expand multipotent HSCs, with myeloid skewing occurring more specifically with hotspot mutations at sites such as R882. This shift toward myeloid output reduces the pool of common lymphoid progenitors⁸ (CLPs) and which in return may limit the number of HSCs available to seed the thymus. An open question is whether such CHIP-associated changes directly contribute to progressive thymic shrinkage during adulthood.

Another important question is how hematopoietic alterations contribute to other cancer types. For example, a recent paper revealed that in blastic plasmacytoid dendritic cell neoplasm (BPDCN), a rare but aggressive malignancy that often presents as skin lesions, the initial cells may originate from the bone marrow harboring only a CHIP mutation. Subsequently, these cells could acquire additional genetic mutations upon exposure to ultraviolet light in the skin⁴⁰. Another recent study revealed that interleukin-4 (IL-4) signaling within the bone marrow promotes the development of immunosuppressive myeloid cells, which facilitate tumor progression in non-small cell lung cancer (NSCLC)⁴¹. Both studies demonstrate that only a systems-level perspective can capture how hematopoietic alterations drive disease processes beyond the primary tissue, and how therapeutic strategies must therefore target the entire immune network.

This systemic understanding is important not only for cancers but also for other lethal diseases. For instance, the “Berlin Patient,” was the first individual who was accidentally cured of HIV after allogeneic hematopoietic stem cell transplantation (HSCT) for his AML. As of late 2024, seven patients have been cured from HIV by allogeneic HSCT⁴². These cases highlight the potential of bone marrow-derived interventions in treating even viral infections. Altogether, these examples highlight that hematopoiesis functions as a central hub influencing diverse systemic diseases. Understanding these interactions requires a systems-immunology perspective that integrates clonal dynamics, immune regulation, and organism-wide consequences.

7.4.3 What can we do for a true system understanding?

“Math is to physics what AI will be to biology.”

– Demis Hassabis

Although criticized by many experts, reductionist ideas can converge to reality. A great example to this is neural networks. Inspired by the neurons in the brain, a layer of simple perceptron can approximate any function⁴³. Cascades of these simple models are capable of understanding language, visual and audio clues and even creating new simulated worlds^{44,45}. Similarly, a cell can be seen as a “bag of information”, where the information can represent any molecule of the hardware such as DNA, RNA, or protein. With the digitization of the cells’ hardware, we can create computational models that are capable of understanding the statistical relations between these molecules and render the actual *algorithm*. Simulating molecular interactions with AI and creating a virtual cell to understand cell-cell interactions and eventually understanding the system seems to be the direction we are heading. One early example of these models is called Evo⁴⁶, where the aim is to predict the next base in a given bacterial genome via a hybrid transformer architecture. For instance, this foundation model is capable of predicting essentiality of the genes in knock-out experiments. Still, Evo is trained only on DNA sequence, which captures part of the biological “vocabulary” but not the full regulatory language of the cell.

Understanding how one cell reacts to certain stimuli will not be sufficient for system level understanding of multi-cellular life. To solve the language of cells, multi-omics single-cell data will be truly essential. We know that the molecular pathways are simply the means (or ‘sentences’) of the language, yet they are mostly undiscovered. These pathways are not yet truly understood at single cell level, mostly due to technical difficulties such as drop-out, sequencing biases or not having truly enormous single-cell multi-omics experiments. In the coming decade, a major goal will be to use artificial intelligence to understand cellular regulation from a systems-level perspective. Along the same trajectory, the same authors introduced Evo2⁴⁷, which was trained on a vastly larger corpus of genomes, spanning bacteria to apes and humans, and extended to RNA data. Evo2 can operate at the scale of entire chromosomes and has already been used not only for variant effect prediction but also for *de novo* generative design. Strikingly, a recent study demonstrated that Evo2-generated bacteriophage genomes, despite harboring hundreds of novel mutations, could assemble into fully functional infectious phages, some even outperforming wild-type strains in competition assays⁴⁸. This proof-of-concept shows that genome language models are not restricted to passive prediction but can actively design novel biological entities, pushing reductionist “bags of information” toward system-level engineering.

An analogy of infamous System I and II thinking⁴⁹ from Daniel Kahneman can also be applied to cells. A single cell is mostly reactive and does not have the capability of long-term thinking and planning and therefore remains at the System I level. However, multi-cellular or complex

Concluding Remarks

organisms can plan and act ahead, which suggests that multi-cellularity and top-down regulation is essential for System II level. Therefore, integration of single-cell insights with top-down, system-level approaches is critical for deciphering how multi-cellular organisms acquire System II-like abilities. We are at a point in time where we can control System I level of cells through molecular or cellular manipulations (Figure 7.2). For instance, we can edit the genome via gene therapy or CRISPR, or we can change the epigenetic landscape of cells and change their identity back to their stem cell state. Still, we are not fully at the level of system manipulation, although the first glimpses are already visible, e.g. designing organoids or rewiring electrical signals of multi-cellular organisms. By combining comprehensive multi-omics data with artificial intelligence and large-scale modeling, we can begin to bridge the gap between the reactive nature of individual cells and the emergent, forward-planning capabilities of entire tissues and organisms. This will be the challenge of this century.

Lastly, System III level is a new phenomenon that represents the next step in the evolutionary timeline, with the potential to create entirely new biological entities. While System II encompasses the ability to rewire existing multicellular organisms at the tissue or organ level and balancing back the homeostasis, System III goes one step further by integrating breakthroughs in synthetic biology, regenerative medicine, computational modeling, perhaps robotics, and AI to sculpt novel or profoundly re-engineered forms of life. Recent work with Evo2 demonstrates that genome models can already generate functional bacteriophages from scratch, hinting that System III is no longer theoretical but also an emerging reality. This entails orchestrating development, physiology, and even behavior in ways that fundamentally transcend current natural boundaries. For example, combining whole-organ engineering, dynamic bioelectrical patterning, and self-organizing organoids with advanced computational designs could allow us not only to repair complex tissues, but also to produce emergent biological systems.

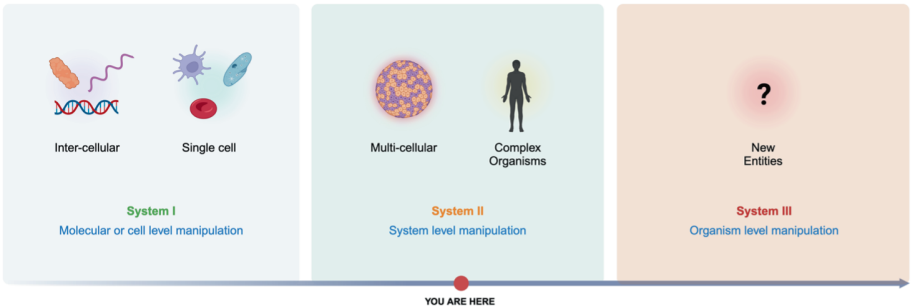


Figure 7.2: A simple schematic of manipulation levels.

7.5 Concluding Remarks

In conclusion, this dissertation has provided a comprehensive exploration of immune aging and hematopoiesis, revealing critical intersections that predispose to leukemogenesis in acute myeloid leukemia (AML). By integrating transcriptional profiling, chromatin accessibility analyses, and advanced computational techniques across murine and human models, this work helps our understanding of inflammaging through the potential of epigenetic interventions to rejuvenate

hematopoietic stem cell function. Moreover, the detailed investigation into the novel subtypes of AML using bulk and single-cell methodologies underscores the intrinsic challenges posed by methodological and contextual ambiguities, while offering new insights into the dynamic interplay between genetic aberrations and cellular identity.

In the last century, we began to understand how the hematopoietic system functions and how blood cells arise, operate, and sometimes fail. We are now recognizing how age-related changes in the hematopoietic system can drive skewing toward the myeloid lineage, thereby increasing the risk of cancer. Today, we are on the verge of understanding blood cells and the hematopoietic system from a more holistic perspective. These new insights, together with the ingenious tools that have been developed, may one day enable the full eradication of lethal blood cancers and perhaps eventually other cancer types as well. Once among the deadliest subtypes of AML, acute promyelocytic leukemia can now be effectively treated with differentiation-inducing drugs, and immunotherapy has revolutionized the treatment of B-cell lymphomas, with newer forms such as TCR-T cell therapy beginning to find their way into AML treatment.

Looking ahead, the future of hematology and cancer research is bright. Global collaborations, relentless scientific rigor, and continuous innovation will reshape our ability to understand the mechanisms underlying blood cancers. Curative treatments are no longer just a dream but becoming a real possibility. We are quickly entering an era where what once seemed impossible is turning into reality.

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Summary

The continuous production of blood cells, known as hematopoiesis, sustains our immune system throughout life. This process relies on rare hematopoietic stem cells (HSCs), which generate all mature blood lineages. However, as individuals age, these stem cells accumulate somatic mutations that confer selective growth advantages, skew differentiation toward the myeloid lineage, and increase the risk of developing myeloid malignancies such as acute myeloid leukemia (AML). Although many of the individual genetic drivers involved in this process are known, how they interact with aging to create therapeutic vulnerabilities remains poorly understood.

This thesis investigates how aging and genetic lesions reshape the transcriptional and epigenetic landscape of blood cell development, with the goal of translating these insights into precision medicine strategies for myeloid cancers. By integrating bulk and single-cell omics technologies with functional assays and computational modeling, this work characterizes key molecular changes in hematopoiesis during aging and malignant transformation. The findings show that aging consistently alters immune cell composition, diminishes the naïve T-cell pool, and activates pro-inflammatory transcriptional programs, particularly through upregulation of the AP-1 complex in monocytes. At the same time, the research demonstrates that epigenetic interventions can expand the HSC pool, providing potential avenues to improve stem cell transplantation and gene therapy.

In the context of AML, the thesis reveals that transcriptional profiling recapitulates known dynamics such as FAB and offers even a more granular view of disease heterogeneity than genetic classifications alone. Patients with identical genetic lesions exhibited distinct transcriptional states that correlated with treatment responses and differentiation trajectories, uncovering substructures within established diagnostic categories. These results suggest that current classification systems may underrepresent the molecular diversity of AML.

Overall, this work bridges the gap between normal aging processes and cancer development, highlighting how age-associated changes in hematopoiesis generate vulnerabilities that could be therapeutically targeted. The research proposes a framework for refining leukemia classification through integrative molecular profiling and lays the foundation for interventions that may prevent or delay the onset of age-related hematological malignancies, moving the field closer to truly personalized treatment strategies.

Samenvatting

De continue productie van bloedcellen, ook wel hematopoëse genoemd, ondersteunt ons immuunsysteem gedurende het hele leven. Dit proces is afhankelijk van zeldzame hematopoëtische stamcellen (HSC's), die alle rijpe bloedceltypen kunnen genereren. Naarmate mensen ouder worden, hopen deze stamcellen echter somatische mutaties op die groeivoordelen geven, de differentiatie verschuiven naar de myeloïde lijn en het risico verhogen op het ontwikkelen van myeloïde maligniteiten zoals acute myeloïde leukemie (AML). Hoewel veel van de individuele genetische drijfveren bekend zijn, is het onduidelijk hoe deze factoren in wisselwerking met veroudering therapeutische kwetsbaarheden creëren.

Dit proefschrift onderzoekt hoe veroudering en genetische afwijkingen het transcriptomische en epigenetische landschap van bloedcelontwikkeling veranderen, met als doel deze inzichten te vertalen naar precisiegeneeskunde voor myeloïde kankers. Door bulk- en single-cell omics technologieën te combineren met functionele assays en computationele modellen, karakteriseert dit onderzoek de belangrijkste moleculaire veranderingen in de hematopoëse tijdens veroudering en kwaadaardige transformatie. De bevindingen laten zien dat veroudering consequent de samenstelling van immuuncellen verandert, de naïeve T-celpopulatie vermindert en pro-inflammatoire transcriptieprogramma's activeert, met name via opregulatie van het AP-1 complex in monocyten. Tegelijkertijd toont het onderzoek aan dat epigenetische interventies de HSC-populatie kunnen uitbreiden, wat mogelijke aanknopingspunten biedt voor verbetering van stamceltransplantatie en genterapie.

In de context van AML laat dit proefschrift zien dat transcriptomische profilering bekende klinische indelingen zoals FAB kan reconstrueren, maar ook een fijnmaziger beeld geeft van de ziekteheterogeniteit dan genetische classificaties alleen. Patiënten met identieke genetische afwijkingen vertoonden verschillende transcriptionele toestanden die samenhangen met behandelingsresponsen en differentiatiepaden. Dit onthult substructuren binnen bestaande diagnostische categorieën en suggereert dat de huidige classificatiesystemen de moleculaire diversiteit van AML mogelijk onderschatten.

Samenvattend overbrugt dit werk de kloof tussen normale verouderingsprocessen en kankeronwikkeling, en benadrukt hoe leeftijdsgebonden veranderingen in de hematopoëse therapeutische kwetsbaarheden genereren. Het onderzoek stelt een raamwerk voor om leukemieclassificatie te verfijnen via integratieve moleculaire profilering en legt de basis voor interventies die mogelijk het ontstaan van leeftijdsgerelateerde hematologische maligniteiten kunnen voorkomen of vertragen. Hiermee komt precisiegeneeskunde voor deze ziekten een stap dichterbij.

Curriculum Vitae

Onur was born in Soma, Turkey, on July 8th, 1994. He attended primary school in Kastamonu and later ranked 266th among more than 900,000 students in the nationwide high school entrance examination in 2008, earning admission to A.U. Fen Lisesi, a private science high school in Ankara.

In 2012, he ranked 1,044th nationwide in the YGS, taken by more than 1.8 million candidates, and was admitted to the Electrical and Electronics Engineering program at Bilkent University in Ankara. He later pursued a Master's degree in Computer Science at Bilkent University, where his research focused on predicting cancer biomarkers from H-NMR spectroscopy data acquired during brain surgery. This project marked his first introduction to bioinformatics.

Following his Master's studies, he moved to the United States to join The Jackson Laboratory, where he worked on deciphering the molecular mechanisms of immune system aging. During this time, he contributed to multiple publications and gained extensive experience in single-cell transcriptomics and epigenomics.

Driven by a desire to deepen his expertise in computational biology, Onur then relocated to the Netherlands to pursue a PhD at Leiden University Medical Center. His doctoral research focused on characterizing hematopoietic aging and acute myeloid leukemia using single-cell multi-omics technologies, integrating multiple omics modalities with functional data. His work aimed to uncover the transcriptional and epigenetic mechanisms governing immune system aging and malignant transformation, with the broader goal of improving precision medicine strategies for acute myeloid leukemia.

Currently, Onur works as a postdoctoral researcher in the group of Dr. Erik van den Akker at Leiden University Medical Center. He leverages his expertise in myeloid malignancies and machine learning to investigate the cellular and molecular mechanisms of lymphoid cancers, with the aim of identifying novel biomarkers and therapeutic targets.

Acknowledgments

A PhD is a war with many mental battles. You win some and lose some. In the end, what matters is winning the war, and that is impossible without the help of many people.

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Veni, vidi, vici.

List of Publications

1. **Karakaslar, E. O.**, Argiro, E. M., Struckman, N. E., Shirali, R. H. Z., Severens, J. F. *et al.* Resolving inter- and intra-patient heterogeneity in *NPM1*-mutated AML at single-cell resolution. *Leukemia* (2025).
2. Tajer, P., **Karakaslar, E. O.**, Canté-Barrett, K., Naber, B. A. E., Vloemans, S. A. *et al.* Utilizing epigenetic regulators to improve HSC-based lentiviral gene therapy. *Blood Adv.* **8**, 4936–4947 (2024).
3. Severens, J. F., **Karakaslar, E. O.**, van der Reijden, B. A., Sánchez-López, E. *et al.* Mapping AML heterogeneity–multi-cohort transcriptomic analysis identifies novel clusters and divergent ex-vivo drug responses. *Leukemia* **38**, 751–761 (2024).
4. Ravichandran, S.*, Erra-Diaz, F*, **Karakaslar, E. O.***, Marches, R. *et al.* Distinct baseline immune characteristics associated with responses to conjugated and unconjugated pneumococcal polysaccharide vaccines in older adults. *Nat. Immunol.* **25**, 316–329 (2024).
5. **Karakaslar, E. O.**, Severens, J. F., Sánchez-López, E., van Veelen, P. A., Zlei, M. *et al.* A transcriptomic based deconvolution framework for assessing differentiation stages and drug responses of AML. *NPJ Precis. Oncol.* **8**, 105 (2024).
6. **Karakaslar, E. O.***, Katiyar, N.*, Hasham, M., Youn, A., Sharma, S., Chung, C. *et al.* Transcriptional activation of Jun and Fos members of the AP-1 complex is a conserved signature of immune aging that contributes to inflammaging. *Aging Cell* **22**, e13792 (2023).
7. Cheong, J. G., Ravishankar, A., Sharma, S., Parkhurst, C. N., Grassmann, S. A., Wingert, C. K., Laurent, P., Ma, S., Paddock, L., Miranda, I. C., **Karakaslar, E. O.** *et al.* Epigenetic memory of coronavirus infection in innate immune cells and their progenitors. *Cell* **186**, 3882–3902.e24 (2023).
8. **Karakaslar, E. O.** & Ucar, D. cinaR: A comprehensive R package for the differential analyses and functional interpretation of ATAC-seq data. *bioRxiv* 2021.03.05.434143 (2021).
9. Cakmakci, D., **Karakaslar, E. O.**, Ruhland, E., Chenard, M. P., Proust, F., Piotto, M. *et al.* Machine learning assisted intraoperative assessment of brain tumor margins using HRMAS NMR spectroscopy. *PLoS Comput. Biol.* **16**, e1008184 (2020).
10. **Karakaslar, E. O.**, Coskun, B., Outilaft, H., Namer, I. J. & Cicek, A. E. Predicting carbon spectrum in heteronuclear single quantum coherence spectroscopy for online feedback during surgery. *IEEE/ACM Trans. Comput. Biol. Bioinform.* **17**, 533–544 (2019).

