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

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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,

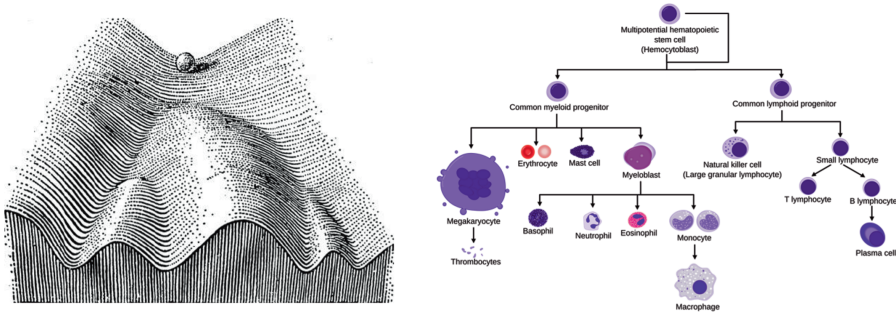


Figure 1.1: Waddington Valley Analogy (left) and hematopoiesis starting from a stem cell (right)

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.

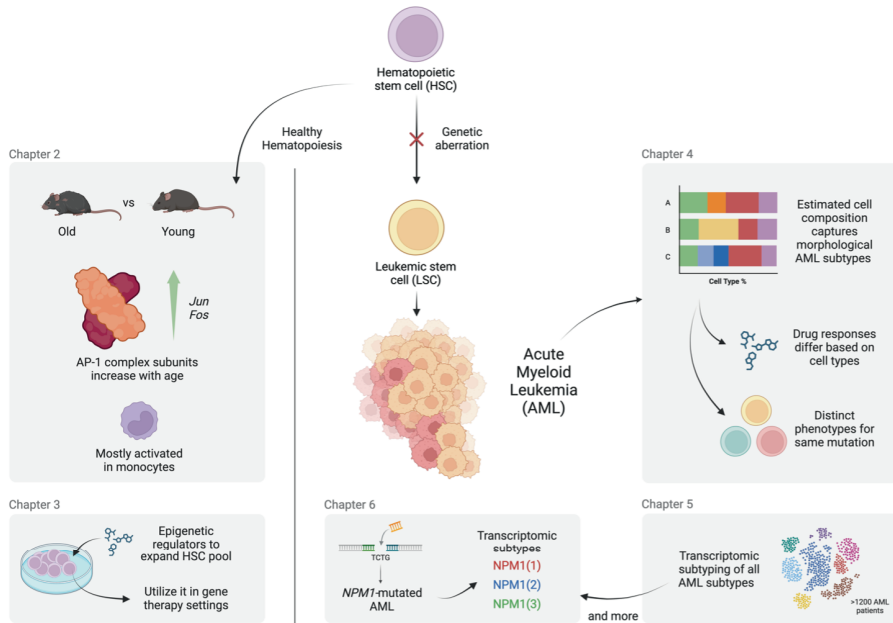


Figure 1.2: Overview of the scientific chapters of the thesis

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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