Studies of Ligands and Receptors Regulating Acute Myeloid Leukemia

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Abstract:

Acute myeloid leukemia (AML) is the most common leukemia in adults and is coupled to a poor prognosis, with a five-year overall survival of around 20% and a high risk of relapse. In AML, immature myeloid cells accumulate rapidly in the bone marrow impairing normal blood cell development. Current treatments fail to eradicate the leukemia stem cells, which is a self-renewing population of leukemia cells responsible for disease progression and relapse. Hence, improved treatments are needed in AML that more efficiently target the leukemia stem cells. In this thesis, I have studied ligand and receptor interactions to find novel dependencies of AML cells that can be therapeutically targeted.

To find selective regulators of leukemia cells, in article I, we performed an *in vitro* cytokine screen using AML and normal bone marrow cells. We identified interleukin 4 (IL4) as a selective negative regulator of AML cells, and showed that IL4 induces apoptosis of AML cells in a Stat6-dependent manner. By using two *in vivo* approaches, injections of IL4 into leukemic mice and enforced expression of IL4 by leukemia cells, we showed that IL4 reduced the leukemia burden and increased survival, suggesting that IL4 has therapeutic potential in AML.

In article II, we found that Toll-like receptor 1 (TLR1) is upregulated on the surface of AML cells compared to normal bone marrow cells. Agonistic stimulation of the TLR1/TLR2 complex with the synthetic lipopeptide PAM3CSK4 triggered NFκB-dependent differentiation and p38 MAPK-dependent apoptosis of AML cells. Upon transplantation of human and murine PAM3CSK4-treated AML cells, mice survived longer and had decreased leukemia cell numbers in the bone marrow compared to controls. Hence, agonistic stimulation of TLR1/TLR2 has therapeutic potential in AML.

In article III, we performed a cytokine screen using murine AML cells with an *in vivo* readout of leukemia-initiating activity using arrayed molecular barcoding. To this end, we developed lentiviral barcodes that allowed for *in vivo* competition of treated leukemia cells that could be traced to separate cytokine stimulations. We identified TNFSF13 as a positive regulator of AML-initiation, and showed that TNFSF13 suppresses apoptosis and promotes NFkB-dependent cell cycling. TNFSF13 is secreted by mature myeloid cells, suggesting a supporting role of these cells in AML initiation.

In article IV, we further investigated the anti-leukemic effects of IL4 *in vivo* and found that IL4 expands F4/80^{+} macrophages that kill AML cells. These macrophages showed a gene expression signature enriched for phagocytosis. Accordingly, in macrophage differentiation cultures with IL4, increased phagocytosis of AML cells was observed. Moreover, combined blockade of the cell surface molecule CD47 and IL4 stimulation resulted in enhanced macrophage-mediated phagocytosis of AML cells, revealing a new immunotherapeutic opportunity in AML.

In conclusion, this thesis has identified critical interactions between receptors on AML cells and ligands in the microenvironment, findings that might translate into new therapies in AML.

Key words: acute myeloid leukemia, cytokines, interleukin 4, TLR1, TNFSF13, macrophages

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Original articles

This thesis is based on the following articles:

Article I

Peña-Martínez P, Eriksson M, Ramakrishnan R, Chapellier M, Högberg C, Orsmark-Pietras C, Richter J, Andersson A, Fioretos T, Järås M. **Interleukin 4 induces apoptosis of acute myeloid leukemia cells in a Stat6-dependent manner.** *Leukemia* 2017; 32:588-596.

Article II

Eriksson M, Peña-Martínez P, Ramakrishnan R, Chapellier M, Högberg C, Glowacki G, Orsmark-Pietras C, Velasco-Hernández T, Lazarević V, Juliusson G, Cammenga J, Mulloy JC, Richter J, Fioretos T, Ebert BL, Järås M. **Agonistic targeting of TLR1/TLR2 induces p38 MAPK-dependent apoptosis and NFkB-dependent differentiation of AML cells.** *Blood advances* 2017; 1(23):2046-2057.

Article III

Chapellier M, Peña-Martínez P, Ramakrishnan R, Eriksson M, Talkhoncheh MS, Orsmark-Pietras C, Lilljebjörn H, Högberg C, Andersson-Hagström A, Fioretos T, Larsson J, Järås M. **Arrayed molecular barcoding identifies TNFSF13 as a positive regulator of acute myeloid leukemia-initiating cells.** In revision, *Haematologica*.

Article IV

Peña-Martínez P, Eriksson M, Ramakrishnan R, Chapellier M, Högberg C, Jansson C, Gisselson Nord D, Järås M. **Interleukin 4 induces macrophage-mediated phagocytosis of leukemia cells by macrophages.** Manuscript.

Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BCP-ALL	B cell precursor ALL
CAR	Chimeric antigen receptor
CD	Cluster of denomination
CHIP	Clonal hematopoiesis of indeterminate potential
CLL	Chronic lymphoid leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
DNMT	DNA methyltransferase
EMP	Erythroid-myeloid progenitor
FAB	French, American and British
FACS	Fluorescence-activated and cell sorting
GMP	Granulocyte-macrophage progenitor
H3K4	Histone 3, lysine 4
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cell
Ig	Immunoglobulin
IL	Interleukin
IL13Ra1	IL13 receptor alpha 1
IL1RAP	IL1 receptor accesory protein
IL2Rg	IL2 receptor gamma
IL4Ra	IL4 receptor alpha
Lin	Lineage
LMPP	Lymphoid-primed multipotent progenitor
LSC	Leukemia stem cell
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythroid progenitor
MHC	Major histocompatibility complex
MLL-R	MLL-rearranged
MPP	Multi-potent progenitors
MRD	Measurable residual disease
NBM	Normal bone marrow
NGS	Next generation sequencing
NK	Natural killer
NOD	Nonobese diabetic
NSG	NOD-SCID/g_c^{null}
SCID	Severe combined immune deficiency
TCR	T cell receptor
T_H2	T helper cell type 2
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
WHO	World health organization

Populärvetenskaplig sammanfattning

Akut myeloisk leukemi (AML) är en typ av blodcancer som uppkommer i omogna blodceller som ger upphov till vårt medfödda immunförsvar. AML är en heterogen och komplex sjukdom som orsakas av specifika genetiska förändringar i vårt DNA. Dessa förändringar gör att omogna blodceller, som skapar mogna blodceller, delar sig okontrollerat och förlorar förmågan att mogna. Dessa omogna celler ackumulerar i benmärgen och förhindrar blodets normala funktion, vilket bland annat leder till blodbrist och infektioner hos patienterna.

De genetiska förändringarna i AML-cellerna används till att klassificera patienterna och styra behandling, eftersom de påverkar sjukdomens utveckling och prognos. De senaste åren har sekvensering av DNA från patienter lett till en större kunskap om de genetiska förändringarna och hur de påverkar sjukdomen. AML-patienter behandlas med intensiv kemoterapi bestående av cytostatika-läkemedel som inte har förändrats nämnvärt de senaste årtiondena, och överlevnaden har främst ökats med hjälp av bättre patientvård. Då cytostatikan inte dödar alla leukemicellerna finns det en stor risk för patienterna att återfalla i leukemi. Benmärgstransplantation användes till de patienter som har den sämsta prognosen i AML, men denna behandling är associerad med stora risker. Därför finns det ett stort behov av att hitta sårbarheter i AML som kan utnyttjas för att ta fram nya behandlingar som botar patienter.

Leukemicellerna i AML expanderar främst i benmärgen, där de omvandlar miljön till fördel för sin egen överlevnad. I denna miljö finns proteiner som tillväxtfaktorer och cytokiner, ansvariga för cell-cell kommunikation. Dessa proteiner påverkar cellernas beteende genom att binda till receptorer på cellytan, som aktiverar signalering, vilket förändrar cellens genetiska uttryck. Interaktioner mellan leukemiceller och externa molekyler öppnar möjligheter att rikta behandlingar mot signaler som är viktiga för deras överlevnad.

Målet med denna avhandling var att hitta och karakterisera nya sårbarheter i AML. I **artiklarna I** och **III** utvecklade vi ny metodik för att identifiera cytokiner som påverkar AML-celler. I **artikeln I** undersökte vi effekten av 114 cytokiner på normala omogna blodceller och leukemiceller samtidigt. Interleukin 4 (IL4) hade den största negativa effekten på AML-cellerna. IL4 inducerade celldöd i AML men påverkade inte normala benmärgsceller. När vi behandlade leukemiska möss med IL4, minskade leukemicellerna i benmärgen, vilket ledde till ökad överlevnad. Transplantation av leukemiceller som utsöndrar höga nivåer av IL4 resulterade i kraftig minskning av AML-celler i benmärgen och längre överlevnad.

I **artikeln IV** studerade vi effekt av IL4 i djurmodeller av AML i mer detalj. I möss med bristfälligt immunförsvar saknades den antileukemiska effekten av IL4, vilket indikerar att IL4 stimulerar immunceller som attackerar AML-celler. Vi upptäckte en ökning av makrofager i benmärg och mjälte när IL4 överuttrycktes. Makrofager är en immuncell som normalt äter upp bakterier och skadade celler genom en process som heter fagocytos. Kombinerad blockering av CD47, en molekyl på cellytan, och IL4-stimulering, gav kraftigt ökad fagocytos av AML-celler.

I **artikeln III** sökte vi efter cytokiner som påverkar AML-cellernas leukemi-initierande förmåga. Vi utvecklade en teknik för att markera cellerna med en genetisk streckkod som tillät oss att efter transplantation av leukemiceller till möss spåra effekter till specifika cytokiner. Vi hittade att TNFSF13 hade en positiv effekt på AML-initiering, eftersom den gav ökad celldelning och minskade celldöd.

I **artikeln II** fokuserade vi på ytreceptorn TLR1, och identifierade att uttrycket är högre på AML-celler än i normala blodstamceller. TLR1 binder normalt molekyler som kommer från bakterier, och aktiverar immunsignalering. Vi visade att aktivering av TLR1 i AML resulterade i utmognad av cellerna och celldöd.

Sammanfattningsvis, i denna avhandling har jag studerat och karakteriserat tidigare okända mekanismer som AML-celler är beroende av och identifierat sårbarheter som i framtiden förhoppningsvis kan utnyttjas för att utveckla nya botemedel för AML.

Resumen divulgativo

La leucemia mieloide aguda (LMA) es un tipo de cáncer que afecta a las células sanguíneas encargadas de nuestras respuestas de defensa innata. La LMA es una enfermedad clínica y biológicamente heterogénea, cuyo origen se da en las mutaciones que ocurren en el ADN de estas células. Estas mutaciones provocan que las células encargadas de formar la sangre, o células madre hematopoiéticas, se dividan de forma incontrolada y se detengan en fases precoces de maduración, dando lugar a una acumulación de células inmaduras en la médula ósea llamadas blastos. Esta acumulación compromete el normal funcionamiento de la médula ósea y la producción de sangre, ocasionando que los pacientes sientan fatiga derivada de un estado anémico y tengan un elevado riesgo de infecciones y hemorragias.

Las mutaciones que ocurren en las células hematopoiéticas albergan información que se usa para clasificar y tratar a los pacientes, ya que afectan al pronóstico y desarrollo de la enfermedad. En los últimos años, las técnicas de secuenciación de ADN han experimentado un gran desarrollo que nos permite conocer con más detalles los cambios mutacionales que ocurren en las células leucémicas y las implicaciones que estos tienen en la enfermedad. Sin embargo, también han puesto de manifiesto la complejidad de la LMA, así como la necesidad de mejorar su tratamiento. Los pacientes de LMA reciben una quimioterapia intensiva que apenas ha cambiado en las últimas décadas, y el incremento en supervivencia se ha debido principalmente a la mejora de las condiciones de hospitalización. Además, esta quimioterapia no erradica por completo las células leucémicas, por lo que el riesgo de recaída en pacientes de LMA es alto. En casos con pronóstico poco favorable, tras los ciclos de quimioterapia se realiza un transplante de médula ósea, pero este tratamiento conlleva altos riesgos asociados. Por ello, hay una necesidad de descubrir nuevas vulnerabilidades que se puedan traducir en tratamientos más efectivos que puedan ayudar a curar por completo la LMA.

Las células leucémicas residen en la médula ósea, donde transforman el microambiente que las rodea para su propia supervivencia. En este espacio también se encuentran moléculas como los factores de crecimiento o las citocinas, encargadas de la comunicación entre células. Estas moléculas afectan al comportamiento de las células a través de receptores de superficie específicos, que traducen el mensaje en señales intracelulares que afectan a la expresión genética. Por tanto, estas interacciones abren la posibilidad de dirigir los tratamientos contra las señales que permiten la supervivencia de las células de LMA.

En esta tesis, el principal objetivo ha sido encontrar y caracterizar nuevas interacciones de moléculas y sus receptores en las células de LMA. Con este fin, en los **artículos I** y **III** se ha llevado a cabo un cribado de citocinas para describir nuevas dependencias de las células leucémicas. En el caso del **artículo I**, se evaluó el efecto de 114 citocinas en la proliferación de células hematopoiéticas normales y células de LMA. La interleucina 4 (IL4) mostró el mayor efecto negativo en células leucémicas, mientras que no tuvo efecto en las células hematopoiéticas. La estimulación de células de LMA con IL4 activa señales que inducen a la muerte celular programada, denominada apoptosis. El tratamiento de ratones con LMA con IL4 resultó en una reducción de células leucémicas en la médula ósea, que se tradujo en un incremento en su supervivencia. Además, cuando se forzó a las células de LMA a expresar IL4 en los ratones, el efecto fue más marcado, con un aumento drástico del tiempo de supervivencia y la desaparición casi completa de la leucemia.

Por ello, en el **artículo IV** estudiamos más en profundidad este efecto de IL4 *in vivo*. Usando modelos de ratones con un sistema inmune deficiente, el efecto de IL4 se vio reducido, indicando que la IL4 estimula al sistema inmune para atacar a las células leucémicas. Analizando la composición de la médula ósea y el bazo de los ratones que expresan la IL4, detectamos un incremento en el porcentaje de macrófagos, un tipo celular encargado de combatir infecciones y limpiar los tejidos de células dañadas mediante un proceso de ingestión y digestión denominado fagocitosis. Además, al bloquear la molécula de superficie CD47 en las células leucémicas, los macrófagos estimulados con IL4 fagocitaron más eficientemente a las células de LMA.

En el **artículo III**, realizamos otro cribado de citocinas, pero en este caso se evaluó su efecto *in vivo*. Para ello, desarrollamos una técnica para marcar las células leucémicas mediante códigos de barras genéticos, que nos permiten identificar las células *a posteriori* según el efecto del tratamiento recibido y su desarrollo en ratones. De este modo, identificamos la citocina TNFSF13 como una molécula que afecta positivamente a la iniciación de la LMA, promoviendo la división celular y suprimiendo el mecanismo de apoptosis en las células leucémicas.

En el **artículo II**, estudiamos el receptor de superficie TLR1, ya que su expresión es mayor en células leucémicas que en células hematopoiéticas normales. TLR1 es un receptor encargado de detectar la presencia de moléculas bacterianas, activando señales inmunológicas. Al estimular TLR1 en las células de LMA con una de estas moléculas, las células activan mecanismos de apoptosis y de diferenciación celular.

En conjunto, esta tesis ha estudiado y caracterizado nuevas dependencias y vulnerabilidades en LMA que resultan de la estimulación de receptores en las células leucémicas. Con un mayor conocimiento de la biología de la LMA, se abre la posibilidad de desarrollar nuevos tratamientos que ayuden a mejorar el pronóstico de los pacientes.

Hematopoiesis

> "The modern haematologist, instead of describing in English what he can see, prefers to describe in Greek what he can't." – Richard Asher (1959).[*]

To carry out the diversity of functions that our blood is responsible for, different populations of cells are needed to ensure optimal performance under both normal physiological conditions and during stress. The cells in our blood carry oxygen, defend us from pathogens and help us to heal our wounds, to name a few of their tasks. Therefore, a high cell turnover is needed to maintain and produce the different cell populations. This process is termed hematopoiesis, from the old Greek αἷμα, blood, and ποιεῖν, to make.

The hematopoietic hierarchy and blood lineages

Blood cell populations have been classified differently depending on diverse criteria. One historical classification was based on the colour of the blood cells after sedimentation by centrifugation: white blood cells, also referred to as leukocytes, and red blood cells, also named erythrocytes. The former includes nucleated blood cells that carry out defence functions against infections, while the latter comprises enucleated cells that carry oxygen. This division was based on the mere observation of blood cells, and provides a broad separation based on their function.[1] This classification was also the basis for what later was called "leukemia", combining the Greek words λευκός, white, and αἷμα, blood.

A more precise and scientific classification of blood cells is based on their origin and maturation. Again, two broad populations are defined: lymphoid cells, which include T cells, B cells and natural killer (NK) cells; and myeloid cells, which include monocytes, granulocytes, thrombocytes/platelets and erythrocytes. These two branches of blood cells arise from hematopoietic stem cells (HSCs), but were initially thought to split early in the hematopoietic process and remain separated along their differentiation into mature cells. However, this hypothesis has recently been challenged.

[*]Richard Asher (1912-1969), British endocrinologist and haematologist.

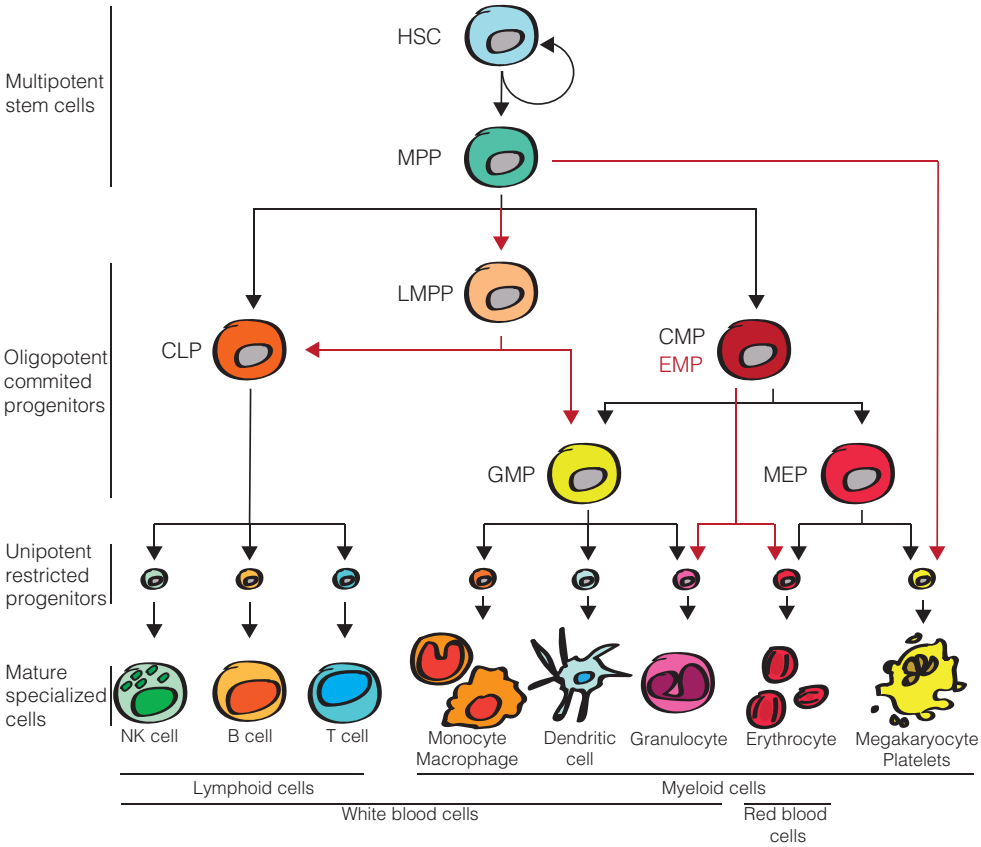

Figure 1. The hematopoietic tree. Hematopoiesis is a hierarchical process with HSCs located at the top. HSCs differentiate into the progenitors of each blood lineage (black lines), which recently has become more complicated as new differentiation pathways have been revealed (red lines). Full names for abbreviations can be found on page 10.

Hematopoiesis has classically been depicted as a hierarchical tree in which HSCs are at the top, and as the cells go down and branch apart, they differentiate into more restricted lineage progenitors (figure 1). With more advanced assays that have allowed us to study the process at higher resolution, it has become evident that cellular fate decisions are more intricate than first anticipated. The HSCs are characterized by their self-renewal capacity, which ensures that at least one daughter cell remains a HSC after cell division. More specifically, self-renewal of HSCs can occur by symmetrical cell divisions, generating two HSCs, or asymmetrically, with one of the daughter cells differentiating into the next stage of development.[2] Downstream of HSCs are multi-potent progenitors (MPPs),[3] which have a limited life-span, as evaluated by transplanting the cells into sublethally irradiated immunodeficient mice. Previously, MPPs were thought to split into two major progenitors types: the

common lymphoid progenitor (CLP)[4] and the common myeloid progenitor (CMP).[5] However, recent studies have described a progenitor cell type that is primed towards the lymphoid potential, but it is also able to produce cells of the myeloid lineage.[6-8] This progenitor population has been named as lymphoid-primed multipotent progenitors (LMPPs)[6], and have redefined the classical view of hematopoiesis. Hence, the dichotomy between lymphoid and myeloid cells now seems to take place at a more committed stage than previously thought.

The myeloid cell development, or myelopoiesis, has also been revisited recently. CMPs have been shown to not be the only cell type capable of generating megakaryocyte precursors, which can arise also directly from HSC populations.[9-11] Therefore, CMPs have been redefined as a more restricted erythroid-myeloid progenitor (EMP) that generates erythrocytes and certain granulocytes. Megakaryocyte-erythroid progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) were thought to arise from CMPs, but now these progenitor populations are hypothesized to come from different populations. GMPs can arise from LMPPs, and are the progenitors for neutrophils and monocytes.[12]

Within the lymphoid branch, CLPs arise from LMPPs, and give rise to more mature lineage restricted progenitors generating mature lymphoid cells.[13] It is worth noting that the final steps of maturation of lymphoid cells, with the exception of NK cells, take place out of the bone marrow: B cells migrate to the spleen and lymph nodes, whereas T cells migrate to the thymus.

Studying hematopoiesis

The hierarchical organization of hematopoiesis has been defined by transplanting various cell populations into irradiated mice and studying their repopulating capacity, and also by studying their differentiation fate *in vitro*. To identify and characterize various hematopoietic cell populations, it has been instrumental to identify cell surface markers that distinguish the various cell populations.

Cell surface markers

The plasma membrane contains a variety of molecules responsible for functions such as cell communication, attachment to the extracellular matrix, or recognition by other cells in the organism. Some of these molecules are displayed in a cell type-specific manner, allowing for the identification of cell populations by detecting their immunophenotype using antigen specific antibodies. This is the basis for a method termed fluorescence-activated and cell sorting (FACS), in which antibodies specific for cell surface molecules are conjugated to fluorescent dyes that allow for staining

and separation of cells. When combining several antigen-specific antibodies, FACS allows for the identification of very specific and rare cell populations.

Since HSCs are undifferentiated cells, they are enriched within lineage negative (Lin^-) cells as they lack expression of markers that define mature cell populations. HSCs express CD34,[14] but this marker is also expressed on a variety of progenitor cell populations. Therefore, there have been many efforts aimed at elucidating a combination of markers that best isolates HSCs, and currently human adult HSCs are defined as $Lin^-CD34^+CD38^-CD45RA^-Thy1^+Rho^{lo}CD49f^+$, as these cells are able of long-term engraftment and multi-lineage repopulation of immunodeficient recipient mice.[15] More mature populations lose or gain markers, allowing for the detection of cell populations in different maturation steps.

A major challenge this phenotypic classification encounters is to define whether the cell population is truly pure, consisting of only HSCs, for example. Therefore, FACS should ideally be combined with prospective isolation of cell populations that can be functionally characterized. Single cell studies coupled to cell surface markers have advanced our knowledge of hematopoiesis, in combination with transplantation experiments that assess their clonal capacity, and with genome sequencing analysis, which helps elucidate their gene expression programs critical for their functions.

Mice and transplantation assays

Mice are an indispensable tool to study hematopoiesis, and as many other techniques, we have improved and tailored them to our needs. Mice can be used as recipients of human cells, establishing what is termed a xenograft (from the Greek ξένος, alien): a tissue from one species transplanted into another.

To allow for the repopulation of human cells in recipient mice, the host immune system from the mouse needs to be defective, as otherwise the human grafted cells would be attacked and destroyed, in a similar process as human immune cells attack transplanted organs in graft-versus-host disease.[16] The severe combined immune deficiency (SCID) strain of mice has been key to much of the success of xenotransplantation models. The lymphocytes in these mice are arrested at an early step of development due to a mutation in the *Prkdc* gene affecting the recombination of the T cell receptor (TCR) and immunoglobulins (Ig), making them susceptible to infections but also to transplantation of foreign tissues across species barriers.[17] Further crossing of these mice with nonobese diabetic (NOD) mice, which have a partial deficiency in NK cells[18] and decreased macrophage activity,[19] generated the NOD-SCID strain with a more severely compromised immune system.

The NOD-SCID mouse model allows for engraftment of human hematopoietic cells,[20] but residual immune activity still dampers the reconstitution. By crossing of

NOD-SCID mice with mice mutant for the interleukin (IL) 2 receptor gamma-chain (IL2Rg), the NOD-SCID-γ_c^{null} (NSG) mouse strain was generated.[21] The mutation in the IL2Rg gene disrupts the signalling of multiple interleukins that are important for immune cell development, allowing for higher engraftment of human cells. A challenge with xenograft models that limits the repopulation of human cells in mice is a partial lack of cross-reactive murine growth factors needed to fully support human cells. Although some cytokines that regulate cell communication are cross-reactive between species, many are species specific, which means that critical signals supporting human cells are lacking. To this end, transgenic NSG mice expressing human cytokines have been developed to enhance human cell engraftment and in particular myeloid cell reconstitution.[22-24]

Table 1. Mouse strains and immune cell functions.

Mouse strain	Immune cell function			
	B cell	T cell	NK cell	Macrophages
C57BL/6	✓	✓	✓	✓
SCID	✗	✗	✓	✓
NOD	✓	🡻	🡻	🡻
NOD-SCID	✗	✗	🡻	🡻
NSG	✗	✗	✗	🡻

✓: functional; ✗: impaired; 🡻: decreased function.

Regulation of hematopoiesis

Hematopoiesis is a tightly controlled process, regulated both by external (extrinsic) and intracellular (intrinsic) factors. To ensure optimal function under various conditions, hematopoietic cells respond to a variety of regulators that define their final output. In steady (or normal) state, HSCs are mostly quiescent and rarely divide. Instead, the generation of mature blood cells is maintained by the progenitors that divide rapidly and ensure a continuous output of cells.[25,26] This organization is important because HSCs are a rare long-lived population that is in charge of maintaining the whole hematopoietic system throughout the life of an individual, and by delegating this task to more mature and transient progenitors, they ensure that errors that originate from DNA replication and stress are kept at a minimum at the HSC level.[27,28]

Extrinsic regulators of HSCs

Adult HSCs are physically located in the bone marrow surrounded by multiple other cells and structures, which provides a microenvironment often referred to as the bone marrow stem cell niche.[29] Within the bone, HSCs are found in the trabecular zone in close proximity to the endothelium of small capillaries and mesenchymal stromal

cells,[30,31] anchored to their niche by cell surface molecules. In addition, a number of other components also regulate HSCs, such as nerve fibres,[32] osteoclasts,[33] and osteoblasts.[34] The composition of the HSC niche is still not completely resolved, and many aspects remain elusive because of the challenges associated with studying the niche without disruption of its structure and the difficulties associated with localizing the rare HSCs.[31]

A variety of molecules from different cell types of the niche play a crucial role in signalling to HSCs, either by keeping them dormant or activating their cell cycling. A major type of communication between cells are cytokines, molecules that are secreted and bind to cell surface receptors on target cells. The pairing of the cytokine with its receptor activates a signal transduction in the cells that affects their behaviour, mainly by modifying gene expression. One example of an important cytokine for HSC maintenance in the bone marrow is CXCL12 and its receptor CXCR4, which are essential for hematopoiesis. CXCL12 is secreted by stromal cells in the niche, and binds to CXCR4 on the surface of HSCs, resulting in retention of HSCs in the bone marrow.[35,36]

Under stress conditions that require increased blood cell output, such as infections or bleedings, HSCs are stimulated by danger signals that along with other mediators ensure an increased production of certain cell types that provides protection.[37,38] These signals can directly act on HSCs, such as stimulation of Toll-like receptors (TLRs) by microbial particles,[39] or indirectly, by pro-inflammatory cytokines such as interferons and IL1.[40] However, if these signals are sustained for a long time, e.g. during chronic inflammation, HSCs are altered and can lose their self-renewal capacity and produce more myeloid cells, which might set a founding ground for hematopoietic malignancies.[41]

Intrinsic regulators of HSCs

Gene expression is a tightly regulated process that defines not only the cellular response to environmental cues but also its fate and development. One of the main regulators of gene expression are transcription factors, proteins with the ability to bind to specific DNA sequences and control gene expression.

In the context of HSCs, transcription factors are key regulators of quiescence and differentiation. Transcription factors often act in complexes comprising several regulatory networks, including multiple transcription factors that together instruct the fate of the cells.[42] For example, during hematopoietic development the transcription factors GATA-1 and GATA-2 antagonize the myeloid transcription factor PU.1 to favour megakaryocyte/erythrocyte lineage development.[43] In addition, the order in which different transcription factors are expressed is critical for cell differentiation.[44] However, recent findings suggest that the HSC behaviour is more

complex, highlighting HSCs as a more heterogeneous cell population than previously anticipated. How HSC fate decisions are made is partially an elusive process, as recent studies show that HSCs are biased towards certain differentiation pathways.[45,46] It is likely that these fate decisions are influenced *in vivo* by a balance between the temporal and dosage conditions of transcription factors and external signals.

Another level of HSC gene regulation comes from epigenetics, inheritable changes in gene expression that are not a result of variations in the DNA sequence.[47] Epigenetic changes are caused, for example, by methylation of cytosine residues that can change transcriptional activity, histone acetylation that opens the chromatin to facilitate transcription, or RNA-mediated gene regulation.[48] The rapid advancements of sequencing techniques in recent years, such as chromatin immunoprecipitation sequencing and bisulfite sequencing, allows for studying these processes in more detail, and thereby increasing our understanding of how epigenetics shape cellular fates. For example, DNA methylathion is maintained by a group of enzymes named DNA methyltransferases (Dnmt), and defects in the function of these enzymes alter the behaviour of HSCs.[49] Loss of *Dnmt1* abolishes the lymphoid potential of HSCs because expression of myeloerythroid genes cannot be suppressed,[50] and mutations in *Dnmt3a* impair differentiation and causes expansion of HSCs in bone marrow as a result of genomic hypomethylation.[51]

Malignant hematopoiesis

Despite regulation of hematopoietic stem and progenitor cell (HSPC) activity and repair mechanisms during DNA replication, genetic alterations in the DNA strand of HSPCs occur. Whereas most genetic alterations do not give a proliferative advantage to the cells, others might eventually lead to transformation of a normal cell into a malignant clone. The cell of origin in these diseases can be a HSC, as in the case of most myeloid malignancies; or at more mature stages, as in most B cell malignancies.[52] This thesis focuses on malignancies arising from HSCs.

Clonal hematopoiesis

As we age, DNA alterations are acquired in cells during replication and accumulate over time. If a genetic alteration causes a certain benefit to the cell, then the progeny will have a selective advantage, for example by providing protection against apoptosis or increased proliferation. As these cells expand, they can acquire new mutations that might provide an even stronger selective advantage, e.g. by acquisition of self-renewal or immune evasion; and thus causing diseases such as cancer. A group of cells that originate from a single HSPC is termed a clone, as they share the same cellular origin.

If a mutation happened at that HSPC, then the descendant cells will inherit that mutation in their DNA, allowing for tracing of the cells that originated from the same precursor.

Clonality and barcode tracking

To study the dynamics of blood cell production over time from individual HSCs, either single cell transplantations of HSCs or genetic labelling can be applied in a transplantation setting. For genetic labelling, molecular barcodes can be introduced into HSCs that are unique DNA sequences that will allow for tracing of daughter cells from single HSCs by sequencing.[53] Such clonal tracking experiments have allowed for a more detailed understanding of hematopoiesis, and have highlighted the heterogeneity that exists at the HSC level; such as lineage biases from individual HSCs.[54] By analysing the clonal output over time, different HSC dynamics have been described. After transplantation of barcoded HSCs, a first wave of lineage-biased clones deriving from short-lived HSCs arises. At later times, balanced multilineage populations arise from single long-term HSCs.[55,56]

Clonal hematopoiesis of indeterminate potential

In recent years, several studies have performed sequencing-based detection of somatically acquired mutations to study clonal hematopoiesis in humans.[57] In older individuals, mutations characteristic of hematological diseases can be detected in up to 10% of healthy individuals over 60 years old.[58,59] These mutations, such as *DNMT3A* and *TET2*, confer a proliferative advantage to the HSCs resulting in increased clonal output. This is what has been termed clonal hematopoiesis of indeterminate potential (CHIP).[60] Therefore, CHIP is correlated with an increased risk of hematological cancer, but surprisingly, these individuals have also been found to have a higher risk of ischemic stroke, heart disease and overall mortality.[58] This has been associated with deficiencies in the mature cells descending from the mutated HSCs, as for example *TET2* mutated macrophages secrete higher levels of IL1β, which is a key cytokine in the development of artherosclerotic plaques.[61] These findings pose then the question whether these individuals should be regarded as healthy, and whether screening programmes to identify them should be initiated.[62]

CHIP is strongly correlated with the age of the individual.[63] The most commonly mutated genes in individuals with CHIP is *DNMT3A*, followed by *TET2, ASXL1, JAK2, TP53* and *SF3B1*.[58] These genes are as well among the most commonly mutated genes in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), suggesting that these mutations provide a founding ground for the development of hematological diseases. Moreover, the majority of these genes are associated with the epigenetic state of the cells, highlighting alterations in epigenetics as a regulatory and permissive mechanism of malignant transformation. As discussed before, *DNMT3A* mutations lead to hypomethylation of the genome, whereas *TET2*

leads to hypermethylation. Interestingly, recent studies have shown that mutated *TET2* activity can be repaired by pharmacological treatment with vitamin C, restoring the normal epigenetic state, as it acts as a co-factor for *TET2* enzymatic activity.[64] This finding suggests that pharmacological protection against malignant transformation might be possible, hampering potential leukemia development. The evolution from CHIP to an overt hematological malignancy will probably become a topic of intense research in the coming years, as questions remain such as how CHIP-associated HSCs acquire secondary cooperating lesions leading to disease or other mechanisms contributing to malignant transformation.

Myelodysplastic syndromes

MDS are a group of diseases in which malignant clonal hematopoiesis with abnormal morphology, or dysplasia, results in low blood cell counts, also referred to as cytopenias.[65] About one third of patients with MDS progress into acute myeloid leukemia,[66] showing the close relationship between the two diseases. Indeed, many of the mutations found in MDS are also characteristic of AML, such as *TET2*, *DNMT3A*, *ASXL1* and *IDH1*.[67] However, certain mutations such as splicing factor mutations are more prevalent in MDS than in AML.

Most of MDS patients present with 1–4 oncogenic driver mutations.[67] Many of these mutations occur in genes that are part of the spliceosome or related to epigenetic regulation.[66] Interestingly, by studying how mutations occur in the cells, certain patterns emerge. Mutations that affect the same pathway tend to be mutually exclusive, either because they are redundant or lethal to the cells. But mutations that happen in different pathways can also be mutually exclusive if they affect the same oncogenic processes. For example, *TET2* and *IDH2* mutations are exclusive of each other, as both affect DNA methylation.[68] Patterns of mutational co-occurrence also emerge, and can be associated with disease outcome. As an example, mutations in *SF3B1* and *SRSF2* affect RNA splicing, but they differ in their set of co-mutated genes and result in different disease phenotypes.[67] This suggests that mutations in the same pathway can have different functional outcomes that are further defined by other mutations. Understanding the mechanistic basis for such interactions might reveal new treatment opportunities allowing for targeting of cancer cell dependencies.

Leukemia

The term leukemia refers to a group of cancers that are manifested in various mature and immature blood cell populations. When the transformed cells belong to the lymphoid lineage, it is referred to as a lymphocytic/lymphoblastic leukemia; whereas if the cells belong to the myeloid lineage, then it is a myeloid leukemia. Acute

leukemia develops fast, with a rapid increase in immature blood cells; whereas chronic leukemia progresses over months or years, with a slight accumulation of somewhat mature cells over time.

Lymphocytic leukemia

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children, representing about one third of all childhood cancers. Today the survival for children with ALL is around 90%, but infants (children below 1 year of age) and adults do worse.[69] ALL affects B cells or T cells, with B cell precursor ALL (BCP-ALL) being the most common type in children, accounting for about 85% of the cases.

BCP-ALL arises early in life, with the genetic lesion often occurring already *in utero*. After birth, additional genetic changes transform the cells into BCP-ALL.[70] The mechanistic basis for the mutational changes still remain unclear, but might be related to a defective RAG-mediated recombination. However, a recent model for BCP-ALL suggests that infections might play a dual role in its development: early-life infections would have a protective role, as the immune system is trained to trigger normal immune responses; while exposure to infections later in life, without early pre-exposure, create a defective immune response that correlates to BCP-ALL development.[70] Epidemiological studies support these findings, as children who attend day-care early in life and are exposed to common infections have a decreased risk of ALL.[70]

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults and affects mature B cells. Most CLL patients have a favourable outcome and can live many years even without treatment, but the disease is heterogeneous and some develop a rapid fatal disease.[71] CLL cells are dependent on signals from the environment, such as B cell receptor stimulation, to escape apoptosis and survive.[72] Hence, treatments targeting B cell receptor signalling are effective and improve patient outcome.[71,72]

Myeloid leukemia

Chronic myeloid leukemia (CML) arises when a HSC harbours a chromosomal translocation between chromosomes 9 and 22, giving rise to the *BCR/ABL1* fusion gene. This alteration disrupts the maturation process of the HSCs and leads to a clonal expansion and accumulation of immature myeloid cells in the bone marrow. The introduction of tyrosine kinase inhibitors (TKIs) blocking BCR/ABL1 has dramatically increased the survival of the patients. Without treatment, CML progresses from a chronic phase into blast crisis, which is an acute leukemia with rapid proliferation of immature cells. With TKIs, CML patients have a normal lifespan but are generally dependent on lifelong treatment.[73]

The *BCR/ABL1* fusion protein has constitutive kinase activity and activates multiple downstream signalling pathways, which results in reduced apoptosis and increased growth-factor independent proliferation.[74] As the CML cells are dependent on the kinase activity of *BCR/ABL1*, blocking this activity by TKIs inhibits the effects of the fusion protein, leading to disease remission. However, resistance to treatment can emerge as certain mutations that affect the kinase domain of *BCR/ABL1* render the TKIs ineffective. To also target resistant clones, second and third generation TKIs have been developed. In patients failing multiple TKIs, allogeneic HSCT is considered.[75]

Blast crisis is manifested in the myeloid lineage in two thirds of cases, but it can also be manifested in lymphoid cells, as well as cells with phenotypes of both lineages.[75] This strongly suggests that malignant transformation occurs in a HSC with potential for both lineages. How CML develops into blast crisis is still poorly understood, but it has been associated with additional genetic alterations and *BCR/ABL1*-induced genomic instability.[74] Despite the efficacy of TKIs, CML stem cells are resistant to the treatment by mechanisms still unclear. Therefore, although many patients achieve disease remission, treatment cannot be discontinued as this would lead to disease relapse.[76]

Acute myeloid leukemia

"Science and everyday life cannot and should not be separated." – Rosalind Franklin.*

AML is characterized by increased proliferation of immature myeloid blast cells, and is the most common form of acute leukemia in adults. Each year, around 350 new cases of AML are diagnosed in Sweden, with an incidence of 3.7 new cases per 100 000 inhabitants per year in Europe.[77] The five year overall survival is around 30% and treatment has remained similar for decades. Hence, there is a strong medical need to develop new treatments. To this end, we need to better understand the mechanisms of leukemogenesis and the vulnerabilities of leukemia cells.

Clinical features

In AML, myeloid-committed hematopoietic cells are arrested in different stages of differentiation, with immature blasts accumulating in the bone marrow and blood, hampering normal blood production.[78] When the myeloid blast count reaches 20% of the cells in bone marrow or peripheral blood, it is diagnosed as AML, otherwise, the disease might be diagnosed as MDS depending as well on other features such as dysplasia.[79] The risk of AML increases with age, accounting for about 80% of the acute leukemias that occur in adults, and it has a 5-year survival rate of approximately 50% for patients below 60 years of age, and around 25% for those over 60 years.[80]

Similar to other types of leukemia, AML is associated with several vague symptoms in patients caused by reduced numbers of normal blood cells. These range from vulnerability to infections and sensitivity for bruising in the early stages, to fatigue and anemia at later stages. Ultimately, the diagnosis of AML requires a bone marrow biopsy to determine not only the percentage of blasts but also the mutations they carry. The major risk factor for AML is age, but previous exposure to chemotherapy related to previous diseases such as MDS also increases the risk of developing what is termed as secondary AML.[81]

* Rosalind Franklin (1920-1958), British chemist and crystallographer.

Classification of AML

AML is a heterogeneous disease and diverse classification efforts have been made to stratify patients according to clinical features.[82] In the early 1980s, a group of French, British and American (FAB) hematologists proposed a classification based on the blast morphology, differentiation stage and myeloid cell type affected, termed FAB groups M0–M7 (table 2).[83] However, although this classification also incorporates certain cytogenetic features, it does not take into account the diversity of genetic alterations present in AML.

The world health organization (WHO) classification system for AML is more complex but carries more prognostic information than the FAB system. The WHO system includes multiple recurrent genetic abnormalities found in AML that have prognostic value, as well as other factors related to AML development such as prior hematological malignancies or therapy (table 2).[84] The improved WHO classification has been possible due to advancements in sequencing techniques that have allowed researchers and clinicians to better understand the impact these genetic alterations have on patient outcome. Nevertheless, due to the high mutational diversity of AML, some patients do not fall into any of the WHO categories, and therefore the FAB classification is still used for these cases, although it provides less prognostic information.[85]

Genetic alterations in AML

Due to advances in next generation sequencing (NGS) techniques, and efforts such as the Cancer Genome Atlas, a heterogeneous mutational landscape of AML has been revealed. In around 50 to 60% of AML patients, structural chromosomal changes are detected, whereas the remaining patients have a normal karyotype.

Chromosomal alterations

Cytogenetics, and more recently RNA sequencing, are useful tools to identify chromosomal abnormalities in AML. Chromosomal changes in AML include insertions and deletions such as del(5q),[86] trisomy in chromosomes 8 and 21,[87] or inversion of chromosome fragments. In AML, around 10% of the cases present with a complex karyotype, harbouring 3 or more chromosomal aberrations, which is coupled with poor prognosis.[88]

Table 2. Different classification systems of AML.

FAB classification		
M0	Undifferentiated acute myeloblastic leukemia	
M1	Acute myeloblastic leukemia with minimal maturation	
M2	Acute myeloblastic leukemia with maturation	
M3	Acute promyelocytic leukemia	
M4	Acute myelomonocytic leukemia	
M5	Acute monocytic leukemia	
M6	Acute erythroid leukemia	
M7	Acute megakaryoblastic leukemia	
WHO classification		
AML with recurrent genetic abnormalities		Fusion gene name
	AML with t(8;21)(q22;q22)	*RUNX1/RUNX1T1*
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;p22)	*CBFB/MYH11*
	Acute promyelocytic leukaemia with t(15;17)(q22;q12)	*PML/RARA*
	AML with t(9;11)(p22;q23)	*MLLT3/MLL*
	AML with t(6:9)(p23;q34)	*DEK/NUP214*
	AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2)	*RPN1/EVI1*
	AML (megakaryoblastic) with t(1:22)(p13;q13)	*RBM15/MKL1*
	AML with mutated NPM1	
	AML with mutated CEBPA	
AML with myelodysplasia-related changes		
	Therapy-related myeloid neoplasms	
	Acute myeloid leukaemia, not otherwise specified	
Proposed genomic classification by Papaemmanuil *et al*., 2016		
AML with *NPM1* mutation		
AML with mutated chromatin, RNA-splicing genes, or both		
AML with *TP53* mutations, chromosomal aneuplody, or both		
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;p22)		*CBFB/MYH11*
AML with biallelic *CEBPA* mutations		
AML with t(15;17)(q22;q12)		*PML/RARA*
AML with t(8;21)(q22;q22)		*RUNX1/RUNX1T1*
AML with t(x;11)(x;q23)		*MLL* fusion genes
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)		*GATA2*, *MECOM (EVI1)*
AML with IDH2R172 mutations and no other class-defining lesions		
AML with t(6;9)(p23;q34)		*DEK/NUP214*
AML with driver mutations but no detected class-defining lessions		
AML with no detected driver mutations		
AML meeting criteria for ≥2 genomic subgroups		

The most common chromosomal abnormalities found in AML are translocations, leading to rearrangement of the genome and the creation of fusion genes. Many of these rearrangements are recurrent and used for classification of AML, and carry as well prognostic information (table 2). Of these chromosomal abnormalities, the translocation t(15;17)(q24;q21), which gives rise to the *PML/RARA* fusion gene that encodes a protein that can be therapeutically targeted, is a paradigm for the efficacy of targeted therapies.

The presence of the *PML/RARA* fusion gene classifies the subgroup acute promyelocytic leukemia, or M3 according to the FAB classification. This subtype of AML used to have the worst survival, mainly because patients suffered of severe bleedings.[89] Mechanistically, *PML/RARA* decreases the levels of the myeloid transcription factor PU.1 and the tumour suppressor PTEN, leading to a differentiation block and leukemia initiation, respectively.[90] Today, these patients have an overall survival of nearly 80% due to the introduction of a targeted therapy against *PML/RARA*.[91] By adding all-trans retinoic acid into the chemotherapy treatment, the differentiation block of the leukemic blasts is rescued, facilitating the maturation process and rendering the blasts sensitive to chemotherapy.[92] Retinoic acid restores the levels of PU.1, which is crucial for myeloid differentiation, and this consequently restores the levels of PTEN, thereby neutralizing the oncogenic activity of *PML/RARA*.[90] Thus, this treatment is an example of the success of targeted therapies for a specific subtype of AML. However, targeted therapies directed to mutated proteins are not expected to be effective in other subtypes of leukemia, in which leukemogenesis is driven by loss of function mutations.

Gene mutations

In normal karyotype AML, leukemogenesis is driven predominantly by point mutations in genes. Although AML has a heterogeneous mutational landscape, it is one of the cancers with the lowest mutational burden per patient; on average 13 gene mutations.[93] Despite the diversity in genes that are found mutated in AML, only 23 are so far identified as recurrently mutated, including genes such as *DNMT3A*, *FLT3*, *NPM1*, *IDH2*, and *TET2*.

A recent study by Papaemmanuil *et al.*[94] that analysed the mutational landscape in a total of 1540 patients, the largest patient cohort to date, identified new associations between molecular alterations and prognosis. The study proposed 11 AML subgroups with distinct phenotypic and clinical features, of which 3 are groups with a heterogeneous set of mutations (table 2). Moreover, this study highlighted the importance of co-mutations that occur in AML and the profound effect they have on patient survival. One such example is the interaction between *NPM1*, *FLT3* and *DNMT3A* mutations. In the presence of coexisting *NPM1* and *DNMT3A* mutations,

the acquisition of internal tandem duplications in *FLT3* conferred a much worse prognosis to patients compared to cases with only one of the genes mutated. These findings suggest that patients should be exhaustively evaluated regarding the mutations present in their AML cells. This knowledge can then be used in translational efforts to improve patient outcome, tailoring the treatments available to the prognostic information conferred by the mutations.

Epigenetic regulators

The focus on epigenetics is increasing with advances in sequencing technologies, and it is now accepted that epigenetic deregulation is a hallmark of AML.[95] Interestingly, this deregulation in AML cells is found even in cells that lack a mutation in known epigenetic regulators,[93] highlighting the importance of these processes in AML biology.

DNA methylation represses gene expression by the addition of a methyl group to cytosine residues of the DNA. This can either prevent the binding of transcription factors or the recruitment of proteins that silence gene expression. Hypermethylation in AML is associated with silencing of tumour-suppressor genes,[48] and mutations that result in loss of *DNMT3A* cause hypomethylation resulting in expression of genes that facilitate AML development.[96, 97] DNA methylation patterns differ among AML patients, and similar to genetic alterations they can be used for classification of specific subtypes.[98]

Epigenetic changes can also occur by modifications of histone proteins that regulate chromatin accessibility. Histone acetylation opens the chromatin to facilitate transcription in a process regulated by acetyltransferases and deacetylases that associate with transcription factors.[48] Histone methylation on the other hand does not activate or repress transcription *per se*, but act as a mark for the recruitment of methyl-binding proteins. Different patterns of methylation are associated with different stages of transcription.[99] For example, methylation of histone 3 on lysine 4 (H3K4) is a marker of transcriptional activation regulated by the *KMT2A* gene, also known as *MLL*.[*]

MLL-rearranged leukemia

Chromosomal rearrangements affecting *MLL* are present in about 10% of all acute leukemias, and more than 130 fusion partners to *MLL* have been identified as of today, of which 9 represent more than 90% of the *MLL*-rearrangements (*MLL-R*).[100]

[*] The name *MLL* will be used throughout this thesis and in the articles included. *MLL* is the traditional name, standing for *Mixed-Lineage Leukemia* due to the presence of translocations affecting this gene in leukemias with mixed phenotypes. *KMT2A* is now the official gene name, standing for *Lysine-specific Methyltransferase 2A*, referring to its protein function.

MLL fusion genes act as oncogenes that give rise to an aggressive disease associated with poor prognosis. *MLL-R* are found in both ALL and AML, and certain fusion partners are more prevalent in one of the diseases, as well as in certain age groups. For example, *MLL-R* account for up to 80% of infant ALL cases, where *MLL-AF4* is the most common fusion gene.[101] In adult AML, *MLL-R* are found in 10% of the cases, with *MLL-AF9* being the most common translocation.[102]

The fusion gene encodes a fusion protein in which MLL has lost the domain responsible for H3K4 methylation and is frequently fused to proteins of the elongation complex.[48] The MLL fusion protein is recruited into a complex that contains the methylase DOT1L, which methylates histone 3 on position 79, a mark for transcriptional activation.[103] This leads to a deregulated expression of *MLL* target genes, including certain *HOX* genes, critical for HSC biology.[104]

Treatment of AML

Chemotherapy is the first treatment given to AML patients, termed induction therapy, and is used to achieve complete remission, defined as less than 5% blasts in the bone marrow.[80] The chemotherapy consists of a continuous infusion of cytarabine for 7 days, in combination with an anthracycline, usually danorubicin, for the 3 first days.[105] These drugs disrupt DNA replication and therefore selectively target rapidly dividing cells but have substantial toxic side-effects also to normal cells.

Complete remission is achieved in about 80% of the patients below 60 years of age, but in patients over 60 years of age, only around 60% will achieve complete remission.[80] In addition, due to the toxic side effects of chemotherapy, not all patients are eligible for standard treatment, especially among the elderly. In these cases, low doses of cytarabine are given, and hypomethylating agents such as azacitidine have also been approved recently for this patient group.[106]

Measurable residual disease (MRD) is another parameter that is used to evaluate and stratify patients post-therapy as it allows for a more robust assessment of treatment outcome. MRD measures the presence of leukemia cells in a range of 1 to 10^4–10^6 bone marrow cells.[107] MRD in AML is measured by either flow cytometry or by PCR in cases with known genetic abnormalities. Moreover, NGS approaches are being explored as they allow for detection of the genetic lesions with high sensitivity.[108]

Despite achieving complete remission, many patients will relapse without further consolidation treatment. Therefore, post-induction therapy is given, either as additional chemotherapy courses for favourable risk patients, or as hematopoietic stem cell transplantation (HSCT) for the high risk groups.[109] In patients with complete remission, post-induction therapy reduces the relapse rate to about 50% of

the patients, translating to a cure rate of approximately 45% for patients diagnosed with AML.[80]

HSCT is only considered for patients with a poor prognosis because it is associated with a high mortality risk. The transplantation might trigger a graft-versus-host effect, a life-threatening condition in which the donor's immune system reacts against the host.[16] Moreover, to improve engraftment and suppress the disease, patients undergo an immunosuppressive treatment prior to HSCT. The aim of the treatment is to achieve a graft-versus-leukemia effect, in which the donor immune cells will attack and destroy the malignant cells.[110] The graft effects are mediated mainly by T cells,[111] which by peptides displayed on major histocompatibility complexes (MHC) recognize self from foreign. Each person generally has a different set of MHC molecules inherited from their parents, and these need to be properly matched between donor and host to reduce the potential side-effects. This setting of transplantation is termed allogeneic HSCT.

In patients that do not receive HSCT, IL2 and histamine can be used to activate the immune system and deplete residual AML cells in post-induction therapy.[112] This treatment activates NK and T cells that are suppressed by the leukemia cells,[113] and clinical trials show special efficacy in monocytic forms of AML, as these blasts express histamine receptors that impact the effectiveness of treatment.[114]

Resistance and relapse in AML

Relapse rates remain high in AML patients, with 50% relapsing after treatment.[80] Not all AML cells present at diagnosis are equally sensitive to chemotherapy,[115] and in some cases the cells that have relapse potential can be found already at diagnosis.[116] Different hypotheses have been postulated to explain the therapy resistance of relapsing cells in AML. A recent study showed that one of the main characteristics of relapsing cells is that they retain stem cell-like transcriptional programs,[117] highlighting this mechanism as a potential therapeutic target to reduce relapse. In addition, evaluation of these stemness markers could allow for patient stratification in different risk groups.[118] However, other mechanisms have also been proposed for the arise of resistance, such as microenvironment protection[119] or drug efflux from the cells by membrane pumps.[120] In particular, p53 aberrant function, either by mutations in its gene *TP53* or by deregulation through other pathway members,[121] protects AML cells from chemotherapy-induced apoptosis. *TP53* mutations are present in around 8% of AML cases, and are coupled to complex karyotypes, and associated with the worst survival outcomes.[93,94]

Malignant transformation

Cell of origin and leukemia stem cells

Leukemia stem cells (LSCs) are defined as cells that can give rise to leukemia when transplanted into an irradiated murine host. LSCs are responsible for disease maintenance, and are often a more immunophenotypic immature subpopulation of cells within the cancer that has the ability to self-renew.[122,123] This ability suggests that leukemia might arise from HSCs, which also have self-renewal capacity and accumulate mutations prior to malignant transformation.[123-125] The stemness gene expression signature observed in AML cells with relapsing capacity also supports this theory, as they can give rise to leukemia again after treatment demonstrating self-renewal capacity.[117] Hence, treatments that would efficiently target LSCs could cure AML patients by eradicating the source of relapse (figure 2).

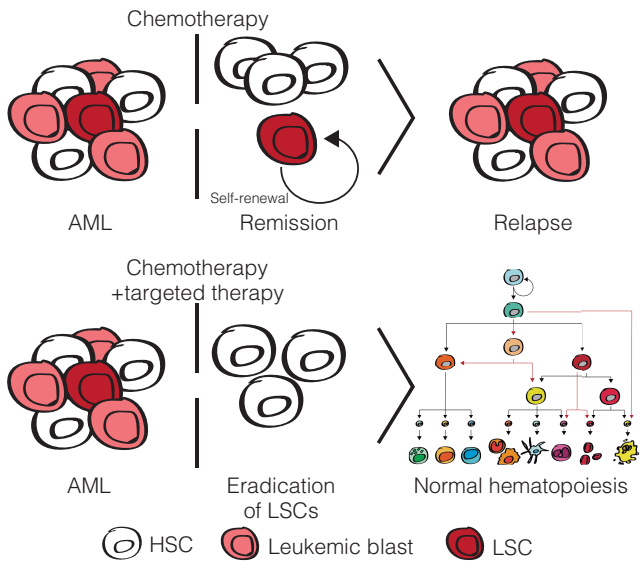

Figure 2. Targeted therapies against LSCs. Conventional chemotherapy is uneffective in eradicating LSCs, which are the source for relapse. Novel therapeutic agents that can target and eradicate LSCs would be able to cure patients and restore normal bone marrow function.

Experimental studies have demonstrated that also progenitors can be transformed into a LSC by acquisition of self-renewal properties.[126] For example, the fusion gene *MLL-AF9* has been shown to transform both HSCs and GMPs into leukemic cells with full leukemogenic capacity, but the AML arising from each population had different

phenotypic properties, as the HSC-derived leukemias were coupled with worse prognosis and a different gene expression.[127]

The LSCs and their progeny are as well organized in a hierarchy, similar to normal hematopoiesis,[128,129] and therefore eradicating LSCs harbours potential for AML cure. As for HSCs, the vast majority of LSCs are $CD34^{+}CD38^{-}$, but they have more plasticity as in some patients leukemia-initiating cells can also be found in the $CD34^{+}CD38^{+}$ and $CD34^{-}$ compartments, showing a less defined hierarchy.[130] For example, $CD34^{-}$ LSCs are enriched in patients with *NPM1* mutations.[131]

Temporal order of mutations

Mutations arise mostly randomly in the genome, and accumulate over time creating a unique set of mutations for a given cell and its progeny. When a mutation confers a selective growth advantage to the cell, it is termed an oncogenic driver mutation, and this constitutes the first step of transformation.[132]

Mutations in epigenetic regulators associated with AML can sometime be found in older individuals with CHIP, and most of these will not develop AML.[59,133] These mutations are rarely found alone, suggesting that they arise early in time in AML patients but are not enough to completely transform an hematopoietic cell into a leukemic state.[94] With time, cooperating driver mutations will appear in the same cell, progressing into a leukemic clone.[134] In AML, the most common initiating driver mutations are *DNMT3A*, *TET2* and *ASXL1*; whereas mutations in genes like *IDH1*, *NPM1* and *FLT3* are cooperating driver mutations that arise as secondary events (figure 3).[125,135] In the case of chromosomal translocations that encode fusion genes, these aberrations are often so aggressive that fewer extra cooperating mutations are needed for the development of AML.[93]

Besides the driver mutations, most mutations occur outside coding regions and will not confer any advantage or disadvantage to the cell. These mutations are termed passenger mutations, and are captured in the genome when a leukemic clone arises and develops.[125,134] Because mutations are random events, a complex evolution of subclones can emerge, creating heterogeneity in the tumour, recognized as an important prognostic factor for patients.[136]

Clonality and tumour heterogeneity

The acquisition of additional mutations in cancer cells creates subclones that can evolve independently. Over time, such subclones can develop into a dominating clone, while others will remain as a small population of cells. The progression of these clones depends on how the mutations affect survival and clonal fitness.[123]

Tumors evolve in an analogous manner to organism populations, being affected by the local environment, creating a selection process. The best adapted subclone will outcompete other subclones based on the 'survival of the fitness' principle.[137] In solid tumours, this is reflected by a genetic heterogeneity within the mass of the tumour, with up to 70% of the detected mutations not present in the dominating major clone.[138] Tumour heterogeneity has a negative impact on patient survival,[139] not only because subclones might support disease progression,[140] but also because it can affect treatment selection. When sampling a tumour, the detection of certain biomarkers can provide relevant prognostic information. However, if such a biomarker is representative only of a subclonal population, then the treatment selected might not be effective against the majority of cancer cells.

In leukemia, there is not as much spatial limitation for tumour growth as in solid tumours. Therefore, it was thought that subclones would arise and dominate through selective sweeps, a model of cancer known as linear evolution.[141] However, with advances in NGS techniques allowing for detection of small subclones, leukemia has emerged as a complex cancer with a mixture of subclones, resembling the model of branching evolution observed in solid tumours.[142,143] These subclones can also influence the dominant clone resulting in a more aggressive disease, for example by secreting pro-leukemic factors that nurture the dominant clone.[144]

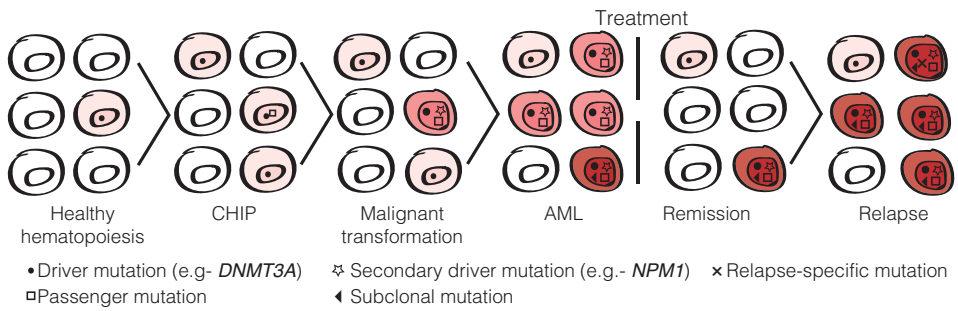

Figure 3. Evolution of AML over time. A HSC acquires a driver mutation (•) that causes a slight proliferative advantage giving rise to CHIP. The progeny of this cell can acquire passenger mutations (□), that will be captured in the genome when a secondary driver mutation (✡) transforms the cell into a LSC. Subclones with additional mutations (◂) will arise during AML development, which might survive treatment and cause relapse, as depicted here.

Chemotherapy treatment puts a selective pressure on the subclonal populations that will eradicate only those cells that are sensitive to the treatment, but resistant subclones will remain and contribute to relapse. The clone contributing to relapse can either be a dominant clone of the primary AML that has acquired relapse-related mutations, or it can be a subclone already present in the primary cancer that survived treatment and then expanded (figure 3).[116] This once more highlights the importance of monitoring subclonal populations for determining patient treatment and thereby

improve outcome, by targeting common vulnerabilities.[145] Moreover, recent studies have also shown that the pre-leukemic CHIP clones persist after treatment and that their presence is correlated with a higher incidence of relapse in patients.[146,147]

Microenvironment

Upon AML development the bone marrow microenvironment is altered, promoting leukemia cell growth over normal HSCs.[148] The leukemia cells are in part responsible for this alteration, by remodelling the microenvironment to their own benefit.[149,150] Both autocrine[151] and paracrine[152] aberrant cytokine secretion supports leukemia cell survival, creating a selective expansion of malignant cells over normal HSCs. Deregulated expression of cytokine receptors on the cell surface of AML cells also contributes increased sensitivity to cytokines promoting their survival, such as IL1 receptor accessory protein (IL1RAP) and IL1 signalling.[153,154] However, this also opens up possibilities for targeting the AML cells by attacking their dependencies, and several approaches have been developed, as discussed in the next chapter.

Non-hematopoietic cells in the bone marrow microenvironment also show alterations that might be permissive for leukemia progression, and even create favourable conditions for leukemia initiation.[155,156] How the microenvironment can promote AML is not well understood, but might be related factors such as hematopoietic stress and chronic inflammation.[157] Moreover, the microenvironment plays an important role on LSC survival during chemotherapy, adding more complexity to the resistance mechanisms that favour AML persistence and relapse.[119]

Functional studies in AML

Patient-derived xenotransplantations

Transplantation of AML samples from patients into mice has allowed for the study of many aspects of leukemia biology that cannot be directly assessed in humans. For example, this has been key for the definition of LSCs as cells capable of initiating leukemia upon serial transplantations[122]. However, only about two thirds of AML samples engraft in NSG mice. Notably, samples from high-risk AML patients generally show higher engraftment capacity in xenotransplantations, in particular cases with mutated *FLT3*. In contrast, low-risk AML samples show generally lower engraftment in these assays and are associated with long disease latency.[158] By transplantation into NSG mice expressing human cytokines supporting myeloid cell development, improved engraftment can be achieved.[159] Hence, the ability and dynamics of AML in the transplanted mice seem to reflect patient outcome, with

patients in which AML samples engrafted in NOD-SCID mice showing poorer survival than those that did not engraft.[160-162]

An interesting application of xenografts is the possibility of drug testing on tumour samples in mice prior to treatment of patients (figure 4).[163] Some studies have transplanted large numbers of AML samples into mice to test drug efficacy across a panel of leukemias with different genetic backgrounds, mimicking a clinical trial.[164-166] This approach allows for prospective investigation of AML sample characteristics that can predict drug response and disease development across a heterogeneous set of samples, in order to tailor clinical trials to improve patient outcome.[167] In other approaches, tumour samples from a single patient have been transplanted into different mice in order to test multiple drugs separately, allowing for subsequent personalized treatment for the patient.[168,169]

Nevertheless, patient-derived xenografts have several limitations. As mentioned before, only the most aggressive forms of AML show robust engraftment.[170] Moreover, as it often takes several months to achieve high engraftment and test drugs in mice, this is still too long to translate findings into a personalized treatment for the patients. Furthermore, the inter-species barrier might bias tumour development. This has been partially solved with the generation of humanized mice that express human cytokines,[23] whereas other approaches have tried to create scaffolds seeded with human stromal cells in order to mimic the human bone marrow niche.[171] In addition, the immunodeficient nature of the recipient mice makes them less suitable for testing of immunotherapies depending on immune cell recruitment.

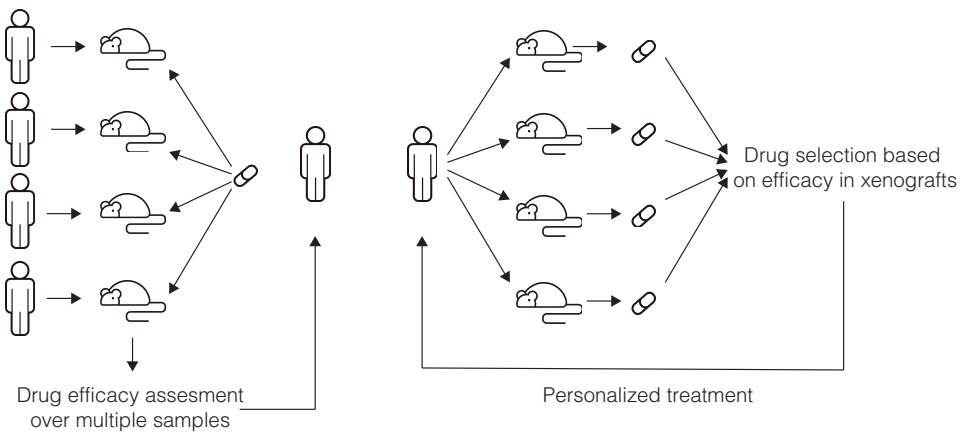

Figure 4. Use of patient-derived xenografts for drug testing. Big scale transplantation of multiple patient sampes allows for assesment of drug efficacy, whereas multiple xenografts from a single patient allows for drug testing in a strategy to find a personalized treatment.

Mouse models of AML

Although xenograft models allow for work with primary samples from human AML, they are limited by low access to patient samples and species incompatibilities. Instead, syngeneic mouse models of AML can be used to study other aspects of AML biology. Several methods have been used to develop models of AML, in which different genetic lesions giving rise to the disease are introduced in hematopoietic mouse cells.

Transgenic mice harbour the genetic alterations in their genome and produce offspring that carries the same genetic alteration.[172] In such models, the mice have often been engineered to express the gene in a tissue specific and/or inducible manner. For example, an inducible model of *MLL-AF9* AML was used to study the cell of origin for AML.[173] By sorting different hematopoietic cell populations and transplanting them into wild type mice, the *MLL-AF9* transgene could be expressed at different stages of development. As previous work had suggested,[127] the *MLL-AF9*-induced AML arising from HSCs was more aggressive than those arising from GMPs.

Another approach to introduce genetic lesions into hematopoietic cells are retroviral transductions. In this strategy, a replication-deficient retrovirus is used to deliver and express a copy of the gene of interest following viral integration in the genome of the transduced cells. The cell population to be studied needs to be purified and cultured *ex vivo* during the time of transduction, and then transplanted in recipient mice. Although this method poses some issues, such as cell manipulation, non-physiological gene dosage, and a risk of insertional mutagenesis; it is widely used as it is fast and convenient, and can be applied to both human[174] and mouse[126] hematopoietic cells.

MLL-AF9 driven AML model

Much of the work in this thesis has been done using a mouse model of AML driven by the *MLL-AF9* fusion gene. This model of AML has been widely used and characterized.[126,175] Mouse GMPs are transformed by retroviral delivery of *MLL-AF9*, retaining a gene expression pattern similar to their normal counterparts, but with the addition of HSC-related genes that conferred self-renewal capacity to the cells. In addition to the bone marrow, AML cells with LSC activity can also be found in the spleen of transplanted mice.[176] Upon serial transplantations, this *MLL-AF9* AML model gives rise to AML with shorter latency, possibly because the cells with stem cell activity are enriched.[177,178]

Novel therapeutic approaches in AML

> "If you know the enemy and know yourself, you need not fear the result of a hundred battles. If you know yourself but not the enemy, for every victory gained you will also suffer a defeat. If you know neither the enemy nor yourself, you will succumb in every battle." – Sun Tzu.*

The modest improvement in AML treatment warrants the development of new therapies that improves the survival of patients. New treatments strategies exploit different approaches, ranging from targeting the molecular alterations to directing the immune system towards the AML cells.

Molecularly targeted therapies

With increased understanding of AML pathology, and with the success of retinoic acid treatment in AML cases harbouring *PML/RARA* fusion genes, major efforts in developing new treatments that target the molecular aberration driving the disease have been explored. However, this approach is challenging in AML as it is a heterogeneous disease both genetically and immunophenotypically.[179] Despite these difficulties, a few new drugs have recently been approved for AML patients.

For *de novo* AML cases harbouring *FLT3* mutations, midostaurin has recently been approved for treatment. Midostaurin is a multi-target protein kinase inhibitor that decreases the constitutional kinase activity caused by the mutations.[180] The combination of midostaurin and chemotherapy resulted in an increased 4-year overall survival in patients with *FLT3* mutations,[181] present in about 28% of AML patients.[93]

In cases of relapsed AML with *IDH2* mutations, enasidenib has recently been approved. Mutations in *IDH2* and *IDH1* are found in around 20% of AML patients,[93] and affect cell metabolism creating an oncometabolite, 2-hydroxyglutarate, that inhibits epigenetic enzymes like TET2, altering the epigenetic landscape of the cell.[182] Enasidenib is administered as a monotherapy in patients with relapsed AML and inhibits the mutated *IDH2*, restoring its normal function and resulting in differentiation of the AML blasts.[183] Because of the promising results observed in

* Sun Tzu (6th century BC), Chinese military strategist and philosopher.

patients with *IDH2* inhibitors, where good responses were observed and survival was prolonged with enasidenib treatment, *IDH1* inhibitors are also being developed.[184]

Despite the success of midostaurin and enasidenib, other targeted approaches have not shown similar efficacy when translated to patients from pre-clinical models. Such an example are DOT1L inhibitors in *MLL-R* AML cases, where treatment of patients has shown only modest effects.[185] To improve these type of targeted therapies, it is hypothesized that targeting multiple AML pathways with different drugs that act synergistically could improve treatment,[179,186] and recent studies suggest that this strategy has great potential.[145,164,187] Thus, combination therapies might improve AML outcome, possibly by minimizing the risk of drug resistance.

Immunotherapy

Besides suppressing normal hematopoiesis, AML cells, as well as other types of cancer cells, evade immune destruction. The remaining healthy bone marrow cells are a putative source for therapeutic intervention if they can be directed against the AML blasts. Strategies aimed at overcoming immune evasion by reactivating the immune system against cancer cells comprise the field of immunotherapy.

Immunotherapies show high clinical potential as demonstrated by HSCT in which donors cells of the immune system elicit a graft-versus-leukemia effect, but the development of new immunotherapies in AML has been challenging. The low mutational burden in AML results in low expression of neoantigens not found in healthy tissues that can be targeted, and the heterogeneity of AML gives rise to a repertoire of antigens that are different across patients, hindering the development of therapies that can be broadly applied.[188,189]

Immune evasion in cancer

The mechanisms by which cancer cells evade the immune system are diverse. Immunosuppressive cytokines can be secreted by both AML cells[190] or other bone marrow cells that have been affected by the leukemogenic environment.[191] T and NK cell mediated cytotoxicity is especially affected by this immunosuppression,[192,193] which AML cells exploit to avoid immune-mediated killing. The IL2 and histamine treatment for relapsed patients of AML discussed in the previous chapter is an example of immunotherapy that targets this immunosuppression.

In addition to the secretion of immunosuppressive factors, AML cells also escape immune recognition by cell-cell interactions and cell surface molecules. This mechanism combines defective antigen presentation by MHC molecules and

stimulation of inhibitory receptors on immune cells.[194,195] Therefore, other approaches in immunotherapies exploit the interactions between malignant and immune cells, mostly by blocking antibodies, to counteract immune cell inhibition and rescue their function.[196,197]

Antibody-based treatments

Antibodies directed to cell surface molecules can recognize targets with high specificity. Different therapeutic approaches have been developed that involve antibodies, ranging from blocking cell-to-cell interactions to antibody-drug conjugates. An ideal antibody target should be a cell surface molecules with higher expression on AML cells than on normal HSCs, in order to avoid targeting healthy cells and cause toxicity.

Gemtuzumab ozogamicin is an immunoconjugate that combines an anti-CD33 antibody with a chemotherapeutic drug. Upon binding of the antibody part to CD33, the whole molecule is internalized and the drug is released internally causing cell death.[198] It was initially approved for older patients that experienced relapse, but subsequent studies led to its withdrawal due to toxicity and lack of improvement on patient survival.[198] However, it was re-approved in 2017, based on studies supporting efficacy in newly diagnosed AML with favourable and intermediate risk.[199]

Upregulation of surface molecules by AML cells renders them vulnerable for antibody-based targeting, as in the case of IL1RAP. This cell surface molecule is upregulated on CML[200] and AML[153] stem cells, and participates in IL1 signalling, a cytokine important for AML cell growth.[201] Engineered antibodies targeting IL1RAP not only block IL1 signalling, but also recruit immune cells through the fragment crystallizable region, triggering antibody-dependent cellular cytotoxicity by NK cells.[202] Thus, upregulation of this surface molecule can be targeted by a dual mechanism of action leading to AML stem cell clearance.

Immune checkpoints regulate immune cell activation, preventing deregulated responses. Cancer cells often exploit these checkpoints to avoid immune detection, for example by upregulation of the cell surface molecule CD47.[203] CD47 is expressed on the surface of every cell as a marker of self and binds to SIRPα on macrophages, inhibiting phagocytosis by providing a so-called “don’t eat me” signal to macrophages.[204] Antibodies targeting CD47 are already in clinical trials for AML patients,[205] and has potential to be effective also in solid tumours.[196]

Immune cell engineering

Other immunotherapeutic strategies are focused on engineering the immune cells as living therapeutic agents against cancer cells. The most successful example are chimeric antigen receptor- (CAR-) T cells, which have shown remarkable efficacy in ALL. In this approach, healthy T cells from the patient are purified and modified *ex vivo* by genetic engineering to express an artificial receptor targeting specific cell surface molecules, and then the CAR-T cells are transplanted back into the patient.[206] In ALL, CAR-T cells have predominantly been directed against CD19, a marker exclusively expressed by B cells, leading to complete eradication of both healthy and malignant B cells.[207] Patients receiving such CAR-T cells will experience B cell aplasia for an extended period of time, as the CAR-T cells are long lived and will continuously deplete $CD19^+$ cells. This is manageable by transfusion of IgG containing antibodies effective against a variety of pathogens.[208]

In AML, CAR-T cell therapies have been more challenging to develop, because cell surface molecules on AML cells are also found on healthy HSCs and normal myeloid cells.[209] However, CAR-T cells against CLL-1 have shown therapeutic potential in pre-clinical models,[210] and the first successful CAR-T cell treatment in AML has been reported recently this year, targeting the surface molecule NKG2D on AML cells.[211] In order to increase specificity and efficacy, CAR-T cells targeting two surface molecules, CD33 and CD123, have been tested, demonstrating proof of concept for this approach in AML.[212]

Solid tumours are more challenging for CAR-T cells due to poor infiltration and inhibitory signals from the tumour microenvironment.[213] To overcome this challenge, CAR-macrophages have been developed, as these cells better infiltrate solid tumours, and have been shown to elicit anti-cancer immune responses in xenograft models of ovarian cancer.[214] Whether this approach could be applied as an AML therapy remains to be evaluated, but it shows the potential of directing the immune system towards malignant cells.

Interleukin 4

> "I didn't want to just know names of things. I remember really wanting to know how it all worked." – Elizabeth Blackburn.*

Interleukins are a group of cytokines first described to be secreted by leukocytes for supporting hematopoietic cell development and differentiation. Defects in interleukins are coupled to a number of immune deficiencies, as they regulate key processes ranging from lymphocyte development to immune responses. Although the source and function of many cytokines still remain partially unclear, there are more than 40 interleukins described.[215]

Interleukin 4 was first described in the context of its proliferative effect on B cells.[216] Since then, many more functions and cell types have been recognized to be influenced by IL4, ranging from memory and learning[217] to metabolism and thermogenesis[218]. Here, the effects of IL4 on the immune system and cancer will be discussed.

IL4 pathway

Upon binding IL4, the IL4 receptor alpha (IL4Ra) chain dimerizes either with the IL2Rg, creating the IL4R complex type I, or with the IL13 receptor alpha chain I (IL13Ra1), creating the IL4R complex type II (figure 5).[219] The IL4R complex type I is only found on hematopoietic cells since the IL2Rg is expressed exclusively on hematopoietic cells, whereas the IL4R complex type II complex also can be found on non-hematopoietic cells.

Although the two IL4R complexes signal by partially different kinases, their activation results in STAT6 phosphorylation and activation.[220] STAT6 then forms a homodimer that is translocated to the nucleus and regulates gene expression.[221] In addition to STAT6, the IL4R complex type I also activates the IRS1/2 pathway due to the spatial conformation of the intracellular part.[222] IL4 is closely related to IL13, as they share around 25% sequence homology and signal through a common receptor chain. However, only IL4 can activate the IL4R complex type I.[219]

* Elizabeth Blackburn (1948), Australian biochemist.

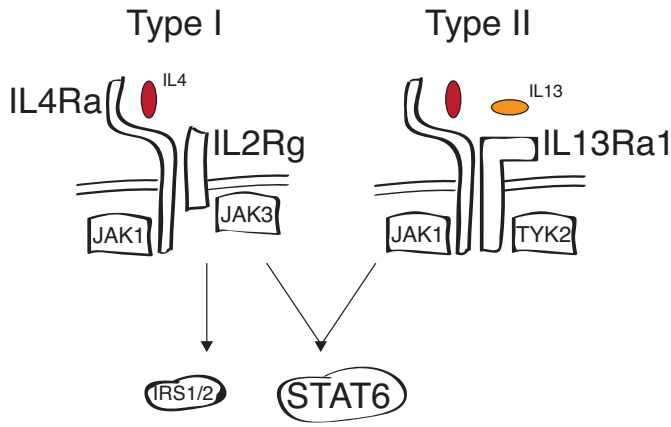

Figure 5. IL4 receptors and pathway. Upon binding of IL4 to the IL4Ra chain, the complex dimerizes either with the IL2Rg or the IL13Ra1, generating two distinct receptor complexes. The spacial conformation of the IL4R complex type I allows for intracellular binding of IRS1/2, but this is not possible in the IL4Ra complex type II as the IL13Ra1 chain blocks the binding sites for IRS1/2. IL13 binds to the IL13Ra1 chain, which only dimerizes with the IL4Ra.

Functions of IL4

The first described function of IL4 was its proliferative effect on B cells, but since then IL4 has been described to have effects on almost every immune cell.[223] The most prominent effect of IL4 is on T cells, where it promotes differentiation of $CD4^+$ T cells into a T helper 2 (T_H2) phenotype.[222] T_H2 cells secrete more IL4, creating a positive feedback loop and activating as well other cells that generate an immune response against parasites. Among the cells implicated in this response, IL4 causes IgE switching in B cells and granule release in mast cells. Recently, IL4 has also been shown to activate NK cells and enhance their cytotoxic capacity,[224] but it remains to be determined if all NK cells can respond to IL4 or only a subset.

IL4 has an important role in macrophage activation through a process termed alternative activation of macrophages.[225] Macrophages exist in a continuum between M1 and M2 macrophages, where M1 macrophages are proinflammatory cells responsible for fighting bacteria and M2 macrophages are anti-inflammatory cells responsible for tissue repair.[226] IL4 causes activation and differentiation of macrophages into M2 phenotypes.

IL4 and cancer

The first studies of IL4 in cancer found a striking anti-tumour effect of this cytokine on cancer cell lines transplanted into xenograft models.[227] However, further studies also described a pro-tumorigenic effect of IL4, as higher expression of T_H2 cell-derived cytokines correlated with tumour development.[227] This contradictory role of IL4 might arise from the different approaches used: when IL4 was ectopically expressed in the cancer cells, it showed anti-tumour effects; while when its secretion was deregulated by the mutations present in cancer cells, it showed pro-tumour effects. It is hypothesized that this is due to the stronger stimulatory effect of IL4 on the immune cells when its secretion is enforced in the cells, but the exact mechanism and cell responsible for this has not been clearly elucidated.[227] On the other hand, deregulated expression of IL4 by the mutational changes in cancer cells favours the maintenance of a T_H2 polarization state, which promotes tumour growth.[228]

In leukemia, early studies showed negative effects of IL4 *in vitro* on the growth of several subtypes of acute leukemia,[229-232] but so far IL4 has not been evaluated in clinical trials involving patients with acute leukemia. However, IL4 has been assessed in clinical trials for non-Hodgkin lymphoma, where some patients exhibited partial responses upon treatment.[233] So far, no biomarkers that could stratify the patients have been reported.[234] Thus, the full potential of IL4 remains poorly studied, in particular in acute leukemia.

Present investigation

> "We're like children who always want to take apart watches to see how they work." – Ernest Rutherford.[*]

The overall aim of my PhD studies has been to identify novel dependencies of AML cells by studying of ligands and receptors, and evaluate their therapeutic potential in mouse models and primary patient samples of AML.

Aims of the study

The main objectives of this thesis were:

- To use *in vitro* cytokine screening to find selective negative regulators of AML cells (**article I**).

- To elucidate whether upregulation of TLR1 on AML cells can be exploited as a therapeutic target in AML (**article II**).

- To use an arrayed molecularly barcoded approach to identify cytokines regulating leukemia-initiating cells (**article III**).

- To investigate the anti-leukemic role of IL4 *in vivo* (**article IV**).

[*] Ernest Rutherford (1871-1937), New Zealander physicist.

Summary of articles

Article I

Interleukin 4 induces apoptosis of acute myeloid leukemia cells in a Stat6-dependent manner

Deregulated cytokine expression in the leukemic niche promotes leukemia progression while suppressing normal hematopoiesis. With an improved understanding of how cytokines regulate AML cell biology, new therapeutic opportunities might emerge. To identify new cytokines that suppress AML cells, we performed a competitive screen where we evaluated the effect of 114 cytokines on the proliferation of primitive AML and normal bone marrow (NBM) cells. We identified IL4 as a selective negative regulator of leukemia cells, inhibiting AML cell survival. Gene expression analysis revealed upregulation of Stat6 target genes as well as apoptosis gene signatures. Disrupting *Stat6* with CRISPR/Cas9 genetic engineering rendered the cells partially resistant to apoptosis. We also evaluated IL4 *in vivo* by intraperitoneal injections of IL4 and transplantation of AML cells expressing IL4 ectopically, with both approaches resulting in prolonged survival and reduced leukemia burden in mice. Human AML samples treated with IL4 also exhibited reduced cell growth and increased apoptosis, whereas NBM cells were less affected. These results show that IL4 harbours therapeutic potential in AML by inducing apoptosis and inhibiting leukemia cell growth.

Article II

Agonistic targeting of TLR1/TLR2 induces p38 MAPK-dependent apoptosis and NF B-dependent differentiation of AML cells

Upregulation of cell surface molecules on AML cells can be exploited as selective targets on leukemia cells over normal HSCs. We studied the expression of TLR1 and TLR2 on AML patient samples and found upregulation of these molecules in comparison to corresponding normal bone marrow cells. Stimulating the TLR1/TLR2 complex *in vitro* with synthetic agonists induced apoptosis and differentiation of both murine *MLL-AF9* leukemia cells and human primary AML samples. We studied the mechanism behind these effects and found differential signalling pathways being involved. Apoptosis of AML cells was dependent on p38 MAP kinase signalling whereas differentiation was dependent on NFkB signalling. Agonistic stimulation of TLR1/TLR2 *in vivo* resulted in increased apoptosis and reduced leukemia burden. Altogether, we identified TLR1/TLR2 stimulation as a potential therapeutic target on AML cells.

Article III

Arrayed molecular barcoding identifies TNFSF13 as a positive regulator of acute myeloid leukemia-initiating cells

To further characterize the role of cytokines on leukemia initiation, we evaluated the effect of the cytokine library used in **article I** on AML cells coupled to an *in vivo* read-out of leukemia-initiating activity. However, this approach would require a large number of mice and would not be feasible with 114 independent cytokines tested in triplicates. To reduce the number of mice, we developed arrayed molecular barcodes that allowed us to study the effect of multiple *ex vivo* cytokine treatments combined with an *in vivo* readout of leukemia development in a single mouse by linking each barcode to one treatment. We sequenced the cells to determine the contribution of individual barcodes in the leukemia cells harvested from mice, and found that Tnfsf13 had a positive effect on AML cells by suppressing apoptosis and promoting cell cycle progression in an NFkB-dependent manner. By using *Tnfsf13*$^{-/-}$ mice, we found that Tnfsf13 is important for AML-initiation as well as normal myelopoiesis. Tnfsf13 was not secreted by AML cells but by mature myeloid cells, suggesting a role for the microenvironment in AML initiation. Furthermore, TNFSF13 supported the survival of human AML cell lines. In conclusion, we demonstrate the potential of arrayed molecular barcoding as a tool for evaluating stem cell functionality, and identified novel roles of Tnfsf13 in AML biology.

Article IV

Interleukin 4 induces phagocytosis of leukemia cells by macrophages

Normal immune cells present in the leukemic bone marrow harbour therapeutic potential that can be directed against AML cells. In this study, we evaluated the effects of IL4 *in vivo* as a potential immunotherapy against AML. We transplanted IL4-secreting murine AML cells into *IL2Rg*$^{-/-}$ deficient mice, which cannot respond to IL4, and found that the *in vivo* anti-leukemia effect of IL4 that we described in **article I** was predominantly microenvironment dependent. Upon analysing bone marrow and spleens of mice exposed to ectopic expression of IL4, we found an increase in macrophages. Depletion of macrophages in mice transplanted with IL4-secreting leukemia cells rescued the leukemic phenotype, demonstrating that macrophages are mediators of the *in vivo* anti-leukemic effect of IL4. *In vitro* differentiation assays with both murine and human monocytes in the presence of IL4 resulted in increased phagocytosis of AML cells by macrophages. RNA sequencing of macrophages exposed to IL4 *in vivo* revealed an M2 phenotype and an enrichment of phagocytosis signatures. We also found an IL4-induced, Stat6-dependent upregulation of CD47 on AML cells. By combining CD47 blocking treatment on AML cells and IL4 stimulation of macrophages, we could increase leukemia cell

phagocytosis by macrophages, showing combinatory treatment potential. This study shows that IL4 has *in vivo* anti-leukemia potential by activating macrophages that kill AML cells by phagocytosis.

General conclusions and future directions

Although our knowledge of AML biology has increased substantially the last decade, this has not translated into new therapies effective in the majority of patients. In this thesis, we have searched for AML cell vulnerabilities that can be exploited to eradicate leukemia. In particular, we have focused on interactions between ligands and receptors and their effects on leukemia cells. We have successfully applied two cytokine screening approaches that have identified IL4 and TNFSF13 as new regulators of AML cells, and we have described TLR1 as an upregulated cell surface molecule that can be therapeutically targeted. In addition, we have also evaluated IL4 as an immunotherapy that activates macrophages, and the crosstalk between AML and macrophages through CD47.

As we found that stimulation of TLR1 and IL4Ra on AML cells results in apoptosis, the question is why leukemia cells express these molecules at all. One hypothesis is that they are present on the AML cells as they mirror the normal cells that are transformed into malignant cells, thus reflecting then the phenotype of the cell of origin. Alternatively, these molecules might be upregulated by the genetic events that give rise to leukemia, as multiple pathways are deregulated. Either way, the ligands for TLR1 and IL4Ra are not present in high concentrations in the microenvironment unless an external stimulus is present. Therefore, AML cells do not experience any selective pressure that would select for downregulation of these receptors, which opens the possibility to exploit these vulnerabilities.

For IL4, direct administration of this cytokine is a strategy that could be exploited for therapeutic purposes, similar to IL2 and histamine. This approach has previously been explored for other diseases,[233] but can be challenging. Cytokines have a short lifespan in circulating blood, making it difficult to achieve an optimal concentration, and they can lead to toxic effects such as cytokine storms. To solve this issue, we speculate that conjugating IL4 to an antibody directed against AML cells would potentially increase efficacy and reduce toxicity due to a targeted delivery. We will explore this strategy in future studies, findings that might translate into new therapeutic opportunities.

IL4 concentration increases upon certain conditions such as allergy, asthma and parasitic infections. Interestingly, there are reports that all these conditions are associated with reduced risk of acute leukemia,[235-237] but further studies with larger patient cohorts are needed to evaluate if the reduced risk is linked to higher IL4 levels.

As allergy, asthma and parasitic infections trigger complex immune responses, other mechanisms than IL4 might also boost cancer surveillance. As we found that IL4 causes macrophage stimulation that results in increased phagocytosis of AML cells, this opens an interesting possibility to enhance macrophage-based immunotherapies that are currently being developed, such as CD47 blockers.

Agonistic targeting of TLR1/TLR2 on AML cells causes differentiation and apoptosis of leukemia cells, but it also showed negative side effects to normal myeloid progenitor cells. Interestingly, there have been reports of patients that experience spontaneous remission of AML after sepsis,[238,239] a condition in which infections cause a dangerous systemic immune response. Targeting TLR1/TLR2 with an agonistic antibody would mimic the stimulation with bacterial molecules, which could be further engineered as a bi-specific antibody targeting at the same time an AML-specific cell surface molecule. This strategy would reduce the risks associated with TLR1/TLR2 stimulation of normal cells.

The arrayed barcode screen used in article III in the context of cytokine screening can also be used to evaluate the effect of other molecules in AML initiation or other aspects of AML biology, and has potential to support large-scale molecule libraries. Moreover, it could be applied in a variety of fields, in which a competitive *in vivo* readout of *ex vivo* manipulated cells is needed.

In conclusion, this thesis has identified critical interactions between receptors on AML cells and ligands in the microenvironment, findings that have revealed previously unknown vulnerabilities in AML. Moreover, this thesis highlights the importance of extrinsic factors for leukemia cells, and how they can be explored for therapeutic purposes, findings that might be translated into new treatments in the future.

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References

1. Hajdu SI. A note from history: The discovery of blood cells. *Annals of clinical and laboratory science* 2003; **33**(2)**:** 237-238.
2. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood* 2015; **125**(17)**:** 2605.
3. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell* 2007; **1**(6)**:** 635-645.
4. Hoebeke I, De Smedt M, Stolz F, Pike-Overzet K, Staal FJ, Plum J*, et al.* T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34(+)CD38(-)CD7(+) common lymphoid progenitors expressing lymphoid-specific genes. *Leukemia* 2007; **21**(2)**:** 311-319.
5. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000; **404:** 193.
6. Adolfsson J, Månsson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT*, et al.* Identification of Flt3+ Lympho-Myeloid Stem Cells Lacking Erythro-Megakaryocytic Potential: A Revised Road Map for Adult Blood Lineage Commitment. *Cell* 2005; **121**(2)**:** 295-306.
7. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nature Immunology* 2010; **11**(7)**:** 585.
8. Helft J, Anjos-Afonso F, van der Veen AG, Chakravarty P, Bonnet D, Reis e Sousa C. Dendritic Cell Lineage Potential in Human Early Hematopoietic Progenitors. *Cell Reports* 2017; **20**(3)**:** 529-537.
9. Woolthuis CM, Park CY. Hematopoietic stem/progenitor cell commitment to the megakaryocyte lineage. *Blood* 2016; **127**(10)**:** 1242.
10. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G*, et al.* Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* 2016; **351**(6269)**:** aab2116.
11. Carrelha J, Meng Y, Kettyle LM, Luis TC, Norfo R, Alcolea V*, et al.* Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature* 2018; **554**(7690)**:** 106-111.
12. Psaila B, Barkas N, Iskander D, Roy A, Anderson S, Ashley N*, et al.* Single-cell profiling of human megakaryocyte-erythroid progenitors identifies distinct megakaryocyte and erythroid differentiation pathways. *Genome Biology* 2016; **17:** 83.
13. Jensen CT, Strid T, Sigvardsson M. Exploring the multifaceted nature of the common lymphoid progenitor compartment. *Current opinion in immunology* 2016; **39:** 121-126.
14. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. *Proceedings of the National Academy of Sciences of the United States of America* 1992; **89**(7)**:** 2804-2808.

15. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 2011; **333**(6039)**:** 218-221.
16. Ferrara JLM, Levine JE, Reddy P, Holler E. Graft-versus-Host Disease. *Lancet* 2009; **373**(9674)**:** 1550-1561.
17. Bosma MJ, Carroll AM. The SCID mouse mutant: definition, characterization, and potential uses. *Annu Rev Immunol* 1991; **9:** 323-350.
18. Kataoka S, Satoh J, Fujiya H, Toyota T, Suzuki R, Itoh K*, et al.* Immunologic Aspects of the Nonobese Diabetic (NOD) Mouse: Abnormalities of Cellular Immunity. *Diabetes* 1983; **32**(3)**:** 247.
19. Piganelli JD, Martin T, Haskins K. Splenic macrophages from the NOD mouse are defective in the ability to present antigen. *Diabetes* 1998; **47**(8)**:** 1212-1218.
20. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* 1997; **94**(10)**:** 5320-5325.
21. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K*, et al.* NOD/SCID/γcnull mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 2002; **100**(9)**:** 3175.
22. Chen Q, Khoury M, Chen J. Expression of human cytokines dramatically improves reconstitution of specific human-blood lineage cells in humanized mice. *Proc Natl Acad Sci U S A* 2009; **106**(51)**:** 21783-21788.
23. Wunderlich M, Chou FS, Link KA, Mizukawa B, Perry RL, Carroll M*, et al.* AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia* 2010; **24**(10)**:** 1785-1788.
24. Rongvaux A, Willinger T, Martinek J, Strowig T, Gearty SV, Teichmann LL*, et al.* Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol* 2014; **32**(4)**:** 364-372.
25. Fleming WH, Alpern EJ, Uchida N, Ikuta K, Spangrude GJ, Weissman IL. Functional heterogeneity is associated with the cell cycle status of murine hematopoietic stem cells. *The Journal of cell biology* 1993; **122**(4)**:** 897-902.
26. Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M*, et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 2008; **135**(6)**:** 1118-1129.
27. Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M*, et al.* Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 2000; **287**(5459)**:** 1804-1808.
28. Boulais PE, Frenette PS. Making sense of hematopoietic stem cell niches. *Blood* 2015; **125**(17)**:** 2621.
29. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells* 1978; **4**(1-2)**:** 7-25.
30. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* 2006; **6**(2)**:** 93-106.
31. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature* 2014; **505:** 327.

32. Mendez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* 2008; **452**(7186)**:** 442-447.
33. Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, Blin-Wakkach C. Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med* 2012; **209**(3)**:** 537-549.
34. Shiozawa Y, Taichman RS. Getting blood from bone: An emerging understanding of the role that osteoblasts play in regulating hematopoietic stem cells within their niche. *Experimental hematology* 2012; **40**(9)**:** 685-694.
35. Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood* 1998; **91**(12)**:** 4523-4530.
36. Greenbaum A, Hsu Y-MS, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN*, et al.* CXCL12 Production by Early Mesenchymal Progenitors is Required for Hematopoietic Stem Cell Maintenance. *Nature* 2013; **495**(7440)**:** 227-230.
37. Mirantes C, Passegue E, Pietras EM. Pro-inflammatory cytokines: emerging players regulating HSC function in normal and diseased hematopoiesis. *Experimental cell research* 2014; **329**(2)**:** 248-254.
38. Zhao Jimmy L, Ma C, O'Connell Ryan M, Mehta A, DiLoreto R, Heath James R*, et al.* Conversion of Danger Signals into Cytokine Signals by Hematopoietic Stem and Progenitor Cells for Regulation of Stress-Induced Hematopoiesis. *Cell Stem Cell* 2014; **14**(4)**:** 445-459.
39. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S*, et al.* Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity* 2006; **24**(6)**:** 801-812.
40. Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk LV, Zhang S*, et al.* Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. *Nature cell biology* 2016; **18**(6)**:** 607-618.
41. Pietras EM. Inflammation: a key regulator of hematopoietic stem cell fate in health and disease. *Blood* 2017; **130**(15)**:** 1693-1698.
42. Sieweke MH, Graf T. A transcription factor party during blood cell differentiation. *Current Opinion in Genetics & Development* 1998; **8**(5)**:** 545-551.
43. Zhang P, Behre G, Pan J, Iwama A, Wara-aswapati N, Radomska HS*, et al.* Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proceedings of the National Academy of Sciences* 1999; **96**(15)**:** 8705-8710.
44. Iwasaki H, Mizuno S, Arinobu Y, Ozawa H, Mori Y, Shigematsu H*, et al.* The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev* 2006; **20**(21)**:** 3010-3021.
45. Copley Michael R, Beer Philip A, Eaves Connie J. Hematopoietic Stem Cell Heterogeneity Takes Center Stage. *Cell Stem Cell* 2012; **10**(6)**:** 690-697.
46. Hoppe PS, Schwarzfischer M, Loeffler D, Kokkaliaris KD, Hilsenbeck O, Moritz N*, et al.* Early myeloid lineage choice is not initiated by random PU.1 to GATA1 protein ratios. *Nature* 2016; **535:** 299.

47. Deans C, Maggert KA. What Do You Mean, "Epigenetic"? *Genetics* 2015; **199**(4)**:** 887-896.
48. Wouters BJ, Delwel R. Epigenetics and approaches to targeted epigenetic therapy in acute myeloid leukemia. *Blood* 2016; **127**(1)**:** 42.
49. Cedar H, Bergman Y. Epigenetics of haematopoietic cell development. *Nature Reviews Immunology* 2011; **11:** 478.
50. Broske AM, Vockentanz L, Kharazi S, Huska MR, Mancini E, Scheller M*, et al.* DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nat Genet* 2009; **41**(11)**:** 1207-1215.
51. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS*, et al.* Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* 2012; **44**(1)**:** 23-31.
52. Johnsen HE, Bergkvist KS, Schmitz A, Kjeldsen MK, Hansen SM, Gaihede M*, et al.* Cell of origin associated classification of B-cell malignancies by gene signatures of the normal B-cell hierarchy. *Leukemia & Lymphoma* 2014; **55**(6)**:** 1251-1260.
53. Glimm H, Ball CR, von Kalle C. You can count on this: barcoded hematopoietic stem cells. *Cell Stem Cell* 2011; **9**(5)**:** 390-392.
54. Lu R, Neff NF, Quake SR, Weissman IL. Tracking single hematopoietic stem cells in vivo using high-throughput sequencing in conjunction with viral genetic barcoding. *Nat Biotech* 2011; **29**(10)**:** 928-933.
55. Kim S, Kim N, Presson Angela P, Metzger Mark E, Bonifacino Aylin C, Sehl M*, et al.* Dynamics of HSPC Repopulation in Nonhuman Primates Revealed by a Decade-Long Clonal-Tracking Study. *Cell Stem Cell* 2014; **14**(4)**:** 473-485.
56. Cheung AM, Nguyen LV, Carles A, Beer P, Miller PH, Knapp DJ*, et al.* Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. *Blood* 2013; **122**(18)**:** 3129-3137.
57. Holstege H, Pfeiffer W, Sie D, Hulsman M, Nicholas TJ, Lee CC*, et al.* Somatic mutations found in the healthy blood compartment of a 115-yr-old woman demonstrate oligoclonal hematopoiesis. *Genome Research* 2014; **24**(5)**:** 733-742.
58. Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG*, et al.* Age-Related Clonal Hematopoiesis Associated with Adverse Outcomes. *N Engl J Med* 2014.
59. Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF*, et al.* Clonal Hematopoiesis and Blood-Cancer Risk Inferred from Blood DNA Sequence. *N Engl J Med* 2014.
60. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP*, et al.* Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 2015.
61. Fuster JJ, MacLauchlan S, Zuriaga MA, Polackal MN, Ostriker AC, Chakraborty R*, et al.* Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science* 2017; **355**(6327)**:** 842-847.
62. Abelson S, Collord G, Ng SWK, Weissbrod O, Mendelson Cohen N, Niemeyer E*, et al.* Prediction of acute myeloid leukaemia risk in healthy individuals. *Nature* 2018; **559**(7714)**:** 400-404.
63. Bowman RL, Busque L, Levine RL. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. *Cell Stem Cell* 2018; **22**(2)**:** 157-170.

64. Cimmino L, Dolgalev I, Wang Y, Yoshimi A, Martin GH, Wang J*, et al.* Restoration of TET2 Function Blocks Aberrant Self-Renewal and Leukemia Progression. *Cell* 2017; **170**(6)**:** 1079-1095.e1020.
65. Adès L, Itzykson R, Fenaux P. Myelodysplastic syndromes. *The Lancet* 2014; **383**(9936)**:** 2239-2252.
66. Bejar R, Steensma DP. Recent developments in myelodysplastic syndromes. *Blood* 2014; **124**(18)**:** 2793.
67. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P*, et al.* Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood* 2013; **122**(22)**:** 3616-3627; quiz 3699.
68. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A*, et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 2010; **18**(6)**:** 553-567.
69. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. *The Lancet* 2013; **381**(9881)**:** 1943-1955.
70. Greaves M. A causal mechanism for childhood acute lymphoblastic leukaemia. *Nature Reviews Cancer* 2018.
71. Chiorazzi N, Rai KR, Ferrarini M. Chronic Lymphocytic Leukemia. *New England Journal of Medicine* 2005; **352**(8)**:** 804-815.
72. Kipps TJ, Stevenson FK, Wu CJ, Croce CM, Packham G, Wierda WG*, et al.* Chronic lymphocytic leukaemia. *Nature reviews Disease primers* 2017; **3:** 16096.
73. Jabbour E, Kantarjian H. Chronic myeloid leukemia: 2018 update on diagnosis, therapy and monitoring. *American journal of hematology* 2018; **93**(3)**:** 442-459.
74. Quintás-Cardama A, Cortes J. Molecular biology of bcr-abl1–positive chronic myeloid leukemia. *Blood* 2009; **113**(8)**:** 1619-1630.
75. Apperley JF. Chronic myeloid leukaemia. *The Lancet* 2015; **385**(9976)**:** 1447-1459.
76. Zhou H, Xu R. Leukemia stem cells: the root of chronic myeloid leukemia. *Protein & Cell* 2015; **6**(6)**:** 403-412.
77. Visser O, Trama A, Maynadie M, Stiller C, Marcos-Gragera R, De Angelis R*, et al.* Incidence, survival and prevalence of myeloid malignancies in Europe. *European journal of cancer (Oxford, England : 1990)* 2012; **48**(17)**:** 3257-3266.
78. Ferrara F, Schiffer CA. Acute myeloid leukaemia in adults. *Lancet* 2013; **381**(9865)**:** 484-495.
79. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK*, et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* 2010; **115**(3)**:** 453-474.
80. Burnett A, Wetzler M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol* 2011; **29**(5)**:** 487-494.
81. De Kouchkovsky I, Abdul-Hay M. 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer Journal* 2016; **6:** e441.
82. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T*, et al.* Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 2017; **129**(4)**:** 424-447.

83. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia : A report of the french-american-british cooperative group. *Annals of Internal Medicine* 1985; **103**(4)**:** 620-625.
84. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM*, et al.* The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 2016; **127**(20)**:** 2391-2405.
85. Walter RB, Othus M, Burnett AK, Löwenberg B, Kantarjian HM, Ossenkoppele GJ*, et al.* Significance of FAB subclassification of "acute myeloid leukemia, NOS" in the 2008 WHO classification: analysis of 5848 newly diagnosed patients. *Blood* 2013; **121**(13)**:** 2424-2431.
86. Van den Berghe H, Cassiman JJ, David G, Fryns JP, Michaux JL, Sokal G. Distinct haematological disorder with deletion of long arm of no. 5 chromosome. *Nature* 1974; **251**(5474)**:** 437-438.
87. Fröhling S, Döhner H. Chromosomal Abnormalities in Cancer. *New England Journal of Medicine* 2008; **359**(7)**:** 722-734.
88. Stölzel F, Mohr B, Kramer M, Oelschlägel U, Bochtler T, Berdel WE*, et al.* Karyotype complexity and prognosis in acute myeloid leukemia. *Blood Cancer Journal* 2016; **6:** e386.
89. Coombs CC, Tavakkoli M, Tallman MS. Acute promyelocytic leukemia: where did we start, where are we now, and the future. *Blood Cancer Journal* 2015; **5:** e304.
90. Noguera NI, Piredda ML, Taulli R, Catalano G, Angelini G, Gaur G*, et al.* PML/RARa inhibits PTEN expression in hematopoietic cells by competing with PU.1 transcriptional activity. *Oncotarget* 2016; **7**(41)**:** 66386-66397.
91. Ades L, Guerci A, Raffoux E, Sanz M, Chevallier P, Lapusan S*, et al.* Very long-term outcome of acute promyelocytic leukemia after treatment with all-trans retinoic acid and chemotherapy: the European APL Group experience. *Blood* 2010; **115**(9)**:** 1690-1696.
92. Raelson JV, Nervi C, Rosenauer A, Benedetti L, Monczak Y, Pearson M*, et al.* The PML/RAR alpha oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. *Blood* 1996; **88**(8)**:** 2826-2832.
93. Network TCGAR. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. *New England Journal of Medicine* 2013; **368**(22)**:** 2059-2074.
94. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND*, et al.* Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med* 2016; **374**(23)**:** 2209-2221.
95. Li S, Garrett-Bakelman FE, Chung SS, Sanders MA, Hricik T, Rapaport F*, et al.* Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. *Nature Medicine* 2016; **22:** 792.
96. Russler-Germain David A, Spencer David H, Young Margaret A, Lamprecht Tamara L, Miller Christopher A, Fulton R*, et al.* The R882H DNMT3A Mutation Associated with AML Dominantly Inhibits Wild-Type DNMT3A by Blocking Its Ability to Form Active Tetramers. *Cancer Cell* 2014; **25**(4)**:** 442-454.
97. Yang L, Rodriguez B, Mayle A, Park HJ, Lin X, Luo M*, et al.* DNMT3A Loss Drives Enhancer Hypomethylation in FLT3-ITD-Associated Leukemias. *Cancer Cell* 2016; **29**(6)**:** 922-934.

98. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ*, et al.* DNA Methylation Signatures Identify Biologically Distinct Subtypes in Acute Myeloid Leukemia. *Cancer Cell* 2010; **17**(1)**:** 13-27.
99. Greer EL, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nature Reviews Genetics* 2012; **13:** 343.
100. Meyer C, Burmeister T, Gröger D, Tsaur G, Fechina L, Renneville A*, et al.* The MLL recombinome of acute leukemias in 2017. *Leukemia* 2017; **32:** 273.
101. Winters AC, Bernt KM. MLL-Rearranged Leukemias—An Update on Science and Clinical Approaches. *Frontiers in Pediatrics* 2017; **5:** 4.
102. Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. *Nat Rev Cancer* 2007; **7**(11)**:** 823-833.
103. de Boer J, Walf-Vorderwülbecke V, Williams O. In focus: MLL-rearranged leukemia. *Leukemia* 2013; **27:** 1224.
104. Lawrence HJ, Christensen J, Fong S, Hu YL, Weissman I, Sauvageau G*, et al.* Loss of expression of the Hoxa-9 homeobox gene impairs the proliferation and repopulating ability of hematopoietic stem cells. *Blood* 2005; **106**(12)**:** 3988-3994.
105. Dombret H, Gardin C. An update of current treatments for adult acute myeloid leukemia. *Blood* 2016; **127**(1)**:** 53.
106. Schuh AC, Dohner H, Pleyer L, Seymour JF, Fenaux P, Dombret H. Azacitidine in adult patients with acute myeloid leukemia. *Crit Rev Oncol Hematol* 2017; **116:** 159-177.
107. Schuurhuis GJ, Heuser M, Freeman S, Béné M-C, Buccisano F, Cloos J*, et al.* Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood* 2018; **131**(12)**:** 1275.
108. Roloff GW, Lai C, Hourigan CS, Dillon LW. Technical Advances in the Measurement of Residual Disease in Acute Myeloid Leukemia. *Journal of Clinical Medicine* 2017; **6**(9)**:** 87.
109. Schlenk RF. Post-remission therapy for acute myeloid leukemia. *Haematologica* 2014; **99**(11)**:** 1663-1670.
110. Cornelissen JJ, Blaise D. Hematopoietic stem cell transplantation for patients with AML in first complete remission. *Blood* 2016; **127**(1)**:** 62.
111. Szyska M, Na I-K. Bone Marrow GvHD after Allogeneic Hematopoietic Stem Cell Transplantation. *Frontiers in Immunology* 2016; **7:** 118.
112. Brune M, Castaigne S, Catalano J, Gehlsen K, Ho AD, Hofmann WK*, et al.* Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial. *Blood* 2006; **108**(1)**:** 88-96.
113. Cuapio A, Post M, Cerny-Reiterer S, Gleixner KV, Stefanzl G, Basilio J*, et al.* Maintenance therapy with histamine plus IL-2 induces a striking expansion of two CD56(bright) NK cell subpopulations in patients with acute myeloid leukemia and supports their activation. *Oncotarget* 2016; **7**(29)**:** 46466-46481.
114. Aurelius J, Martner A, Brune M, Palmqvist L, Hansson M, Hellstrand K*, et al.* Remission maintenance in acute myeloid leukemia: impact of functional histamine H2 receptors expressed by leukemic cells. *Haematologica* 2012; **97**(12)**:** 1904-1908.

115. Ojamies PN, Kontro M, Edgren H, Ellonen P, Lagstrom S, Almusa H, *et al.* Monitoring therapy responses at the leukemic subclone level by ultra-deep amplicon resequencing in acute myeloid leukemia. *Leukemia* 2017; **31**(5)**:** 1048-1058.
116. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, *et al.* Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* 2012; **481:** 506.
117. Shlush LI, Mitchell A, Heisler L, Abelson S, Ng SWK, Trotman-Grant A, *et al.* Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature* 2017; **547:** 104.
118. Ng SW, Mitchell A, Kennedy JA, Chen WC, McLeod J, Ibrahimova N, *et al.* A 17-gene stemness score for rapid determination of risk in acute leukaemia. *Nature* 2016; **540**(7633)**:** 433-437.
119. Tabe Y, Konopleva M. Role of microenvironment in resistance to therapy in AML. *Current hematologic malignancy reports* 2015; **10**(2)**:** 96-103.
120. Chapuy B, Koch R, Radunski U, Corsham S, Cheong N, Inagaki N, *et al.* Intracellular ABC transporter A3 confers multidrug resistance in leukemia cells by lysosomal drug sequestration. *Leukemia* 2008; **22**(8)**:** 1576-1586.
121. Quintás-Cardama A, Hu C, Qutub A, Qiu YH, Zhang X, Post SM, *et al.* p53 pathway dysfunction is highly prevalent in acute myeloid leukemia independent of TP53 mutational status. *Leukemia* 2016; **31:** 1296.
122. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**(6464)**:** 645-648.
123. Grove CS, Vassiliou GS. Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer? *Disease models & mechanisms* 2014; **7**(8)**:** 941-951.
124. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 1997; **3:** 730.
125. Jan M, Snyder TM, Corces-Zimmerman MR, Vyas P, Weissman IL, Quake SR, *et al.* Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Science translational medicine* 2012; **4**(149)**:** 149ra118.
126. Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, *et al.* Transformation from committed progenitor to leukaemia stem cell initiated by MLL–AF9. *Nature* 2006; **442**(7104)**:** 818-822.
127. Krivtsov AV, Figueroa ME, Sinha AU, Stubbs MC, Feng Z, Valk PJ, *et al.* Cell of origin determines clinically relevant subtypes of MLL-rearranged AML. *Leukemia* 2013; **27**(4)**:** 852-860.
128. Hope KJ, Jin L, Dick JE. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol* 2004; **5**(7)**:** 738-743.
129. Goardon N, Marchi E, Atzberger A, Quek L, Schuh A, Soneji S, *et al.* Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. *Cancer Cell* 2011; **19**(1)**:** 138-152.
130. Quek L, Otto GW, Garnett C, Lhermitte L, Karamitros D, Stoilova B, *et al.* Genetically distinct leukemic stem cells in human CD34– acute myeloid leukemia are arrested at a

hemopoietic precursor-like stage. *The Journal of Experimental Medicine* 2016; **213**(8)**:** 1513-1535.

131. Taussig DC, Vargaftig J, Miraki-Moud F, Griessinger E, Sharrock K, Luke T*, et al.* Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood* 2010; **115**(10)**:** 1976-1984.
132. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer Genome Landscapes. *Science* 2013; **339**(6127)**:** 1546-1558.
133. Young AL, Challen GA, Birmann BM, Druley TE. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nature Communications* 2016; **7:** 12484.
134. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC*, et al.* The origin and evolution of mutations in acute myeloid leukemia. *Cell* 2012; **150**(2)**:** 264-278.
135. Xie M, Lu C, Wang J, McLellan MD, Johnson KJ, Wendl MC*, et al.* Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 2014; **20**(12)**:** 1472-1478.
136. Mengelbier LH, Karlsson J, Lindgren D, Valind A, Lilljebjörn H, Jansson C*, et al.* Intratumoral genome diversity parallels progression and predicts outcome in pediatric cancer. *Nature Communications* 2015; **6:** 6125.
137. Gillies RJ, Verduzco D, Gatenby RA. Evolutionary dynamics of carcinogenesis and why targeted therapy does not work. *Nat Rev Cancer* 2012; **12**(7)**:** 487-493.
138. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E*, et al.* Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *New England Journal of Medicine* 2012; **366**(10)**:** 883-892.
139. Morris LG, Riaz N, Desrichard A, Senbabaoglu Y, Hakimi AA, Makarov V*, et al.* Pan-cancer analysis of intratumor heterogeneity as a prognostic determinant of survival. *Oncotarget* 2016; **7**(9)**:** 10051-10063.
140. Cleary AS, Leonard TL, Gestl SA, Gunther EJ. Tumour cell heterogeneity maintained by cooperating subclones in Wnt-driven mammary cancers. *Nature* 2014; **508:** 113.
141. Davis A, Gao R, Navin N. Tumor evolution: Linear, branching, neutral or punctuated?(). *Biochimica et biophysica acta* 2017; **1867**(2)**:** 151-161.
142. Campbell PJ, Pleasance ED, Stephens PJ, Dicks E, Rance R, Goodhead I*, et al.* Subclonal phylogenetic structures in cancer revealed by ultra-deep sequencing. *Proceedings of the National Academy of Sciences* 2008; **105**(35)**:** 13081-13086.
143. Gawad C, Koh W, Quake SR. Dissecting the clonal origins of childhood acute lymphoblastic leukemia by single-cell genomics. *Proc Natl Acad Sci U S A* 2014; **111**(50)**:** 17947-17952.
144. Hyrenius-Wittsten A, Pilheden M, Sturesson H, Hansson J, Walsh MP, Song G*, et al.* De novo activating mutations drive clonal evolution and enhance clonal fitness in KMT2A-rearranged leukemia. *Nature Communications* 2018; **9**(1)**:** 1770.
145. Saito Y, Mochizuki Y, Ogahara I, Watanabe T, Hogdal L, Takagi S*, et al.* Overcoming mutational complexity in acute myeloid leukemia by inhibition of critical pathways. *Science translational medicine* 2017; **9**(413).

146. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc Natl Acad Sci U S A* 2014; **111**(7)**:** 2548-2553.
147. Rothenberg-Thurley M, Amler S, Goerlich D, Köhnke T, Konstandin NP, Schneider S*, et al.* Persistence of pre-leukemic clones during first remission and risk of relapse in acute myeloid leukemia. *Leukemia* 2018; **32**(7)**:** 1598-1608.
148. von der Heide EK, Neumann M, Vosberg S, James AR, Schroeder MP, Ortiz-Tanchez J*, et al.* Molecular alterations in bone marrow mesenchymal stromal cells derived from acute myeloid leukemia patients. *Leukemia* 2017; **31**(5)**:** 1069-1078.
149. Lane SW, Scadden DT, Gilliland DG. The leukemic stem cell niche: current concepts and therapeutic opportunities. *Blood* 2009; **114**(6)**:** 1150.
150. Kumar B, Garcia M, Weng L, Jung X, Murakami JL, Hu X*, et al.* Acute myeloid leukemia transforms the bone marrow niche into a leukemia-permissive microenvironment through exosome secretion. *Leukemia* 2017; **32:** 575.
151. Dokter WH, Tuyt L, Sierdsema SJ, Esselink MT, Vellenga E. The spontaneous expression of interleukin-1 beta and interleukin-6 is associated with spontaneous expression of AP-1 and NF-kappa B transcription factor in acute myeloblastic leukemia cells. *Leukemia* 1995; **9**(3)**:** 425-432.
152. Kim JA, Shim JS, Lee GY, Yim HW, Kim TM, Kim M*, et al.* Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. *Cancer Res* 2015; **75**(11)**:** 2222-2231.
153. Askmyr M, Ågerstam H, Hansen N, Gordon S, Arvanitakis A, Rissler M*, et al.* Selective killing of candidate AML stem cells by antibody targeting of IL1RAP. *Blood* 2013; **121**(18)**:** 3709-3713.
154. Mitchell K, Barreyro L, Todorova TI, Taylor SJ, Antony-Debre I, Narayanagari SR*, et al.* IL1RAP potentiates multiple oncogenic signaling pathways in AML. *J Exp Med* 2018; **215**(6)**:** 1709-1727.
155. Raaijmakers MHGP, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA*, et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukemia. *Nature* 2010; **464**(7290)**:** 852-857.
156. Dong L, Yu W-M, Zheng H, Loh ML, Bunting ST, Pauly M*, et al.* Leukaemogenic effects of Ptpn11 activating mutations in the stem cell microenvironment. *Nature* 2016; **539**: 304-308.
157. Zambetti NA, Ping Z, Chen S, Kenswil KJ, Mylona MA, Sanders MA*, et al.* Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. *Cell Stem Cell* 2016; **19**(5)**:** 613-627.
158. Paczulla AM, Dirnhofer S, Konantz M, Medinger M, Salih HR, Rothfelder K*, et al.* Long-term observation reveals high-frequency engraftment of human acute myeloid leukemia in immunodeficient mice. *Haematologica* 2017; **102**(5)**:** 854-864.
159. Ellegast JM, Rauch PJ, Kovtonyuk LV, Müller R, Wagner U, Saito Y*, et al.* inv(16) and NPM1mut AMLs engraft human cytokine knock-in mice. *Blood* 2016; **128**(17)**:** 2130-2134.

160. Rombouts WJ, Martens AC, Ploemacher RE. Identification of variables determining the engraftment potential of human acute myeloid leukemia in the immunodeficient NOD/SCID human chimera model. *Leukemia* 2000; **14**(5)**:** 889-897.
161. Pearce DJ, Taussig D, Zibara K, Smith LL, Ridler CM, Preudhomme C*, et al.* AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML. *Blood* 2006; **107**(3)**:** 1166-1173.
162. Griessinger E, Vargaftig J, Horswell S, Taussig DC, Gribben J, Bonnet D. Acute myeloid leukemia xenograft success prediction: Saving time. *Experimental hematology* 2018; **59:** 66-71.e64.
163. Gao H, Korn JM, Ferretti S, Monahan JE, Wang Y, Singh M*, et al.* High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nature Medicine* 2015; **21:** 1318.
164. Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A*, et al.* Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nature Biotechnology* 2010; **28:** 275.
165. Wunderlich M, Mizukawa B, Chou F-S, Sexton C, Shrestha M, Saunthararajah Y*, et al.* AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood* 2013; **121**(12)**:** e90.
166. Chen WC, Yuan JS, Xing Y, Mitchell A, Mbong N, Popescu AC*, et al.* An Integrated Analysis of Heterogeneous Drug Responses in Acute Myeloid Leukemia That Enables the Discovery of Predictive Biomarkers. *Cancer Research* 2016; **76**(5)**:** 1214.
167. Townsend EC, Murakami MA, Christodoulou A, Christie AL, Köster J, DeSouza TA*, et al.* The Public Repository of Xenografts Enables Discovery and Randomized Phase II-like Trials in Mice. *Cancer Cell* 2016; **29**(4)**:** 574-586.
168. Chen Z, Cheng K, Walton Z, Wang Y, Ebi H, Shimamura T*, et al.* A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature* 2012; **483:** 613.
169. Malaney P, Nicosia SV, Davé V. One mouse, one patient paradigm: New avatars of personalized cancer therapy. *Cancer Letters* 2014; **344**(1)**:** 1-12.
170. Sanchez PV, Perry RL, Sarry JE, Perl AE, Murphy K, Swider CR*, et al.* A robust xenotransplantation model for acute myeloid leukemia. *Leukemia* 2009; **23:** 2109.
171. Antonelli A, Noort WA, Jaques J, de Boer B, de Jong-Korlaar R, Brouwers-Vos AZ*, et al.* Establishing human leukemia xenograft mouse models by implanting human bone marrow-like scaffold-based niches. *Blood* 2016; **128**(25)**:** 2949-2959.
172. Kohnken R, Porcu P, Mishra A. Overview of the Use of Murine Models in Leukemia and Lymphoma Research. *Frontiers in oncology* 2017; **7:** 22.
173. Stavropoulou V, Kaspar S, Brault L, Sanders MA, Juge S, Morettini S*, et al.* MLL-AF9 Expression in Hematopoietic Stem Cells Drives a Highly Invasive AML Expressing EMT-Related Genes Linked to Poor Outcome. *Cancer Cell* 2016; **30**(1)**:** 43-58.
174. Barabé F, Kennedy JA, Hope KJ, Dick JE. Modeling the Initiation and Progression of Human Acute Leukemia in Mice. *Science* 2007; **316**(5824)**:** 600-604.
175. Cozzio A, Passegue E, Ayton PM, Karsunky H, Cleary ML, Weissman IL. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev* 2003; **17**(24)**:** 3029-3035.

176. Somervaille TCP, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell* 2006; **10**(4)**:** 257-268.
177. Miller PG, Al-Shahrour F, Hartwell KA, Chu LP, Järås M, Puram RV*, et al.* In Vivo RNAi screening identifies a leukemia-specific dependence on integrin beta 3 signaling. *Cancer Cell* 2013; **24**(1)**:** 45-58.
178. Puram RV, Kowalczyk MS, de Boer CG, Schneider RK, Miller PG, McConkey M*, et al.* Core Circadian Clock Genes Regulate Leukemia Stem Cells in AML. *Cell* 2016; **165**(2)**:** 303-316.
179. Estey E, Levine RL, Löwenberg B. Current challenges in clinical development of "targeted therapies": the case of acute myeloid leukemia. *Blood* 2015; **125**(16)**:** 2461.
180. Stone RM, Manley PW, Larson RA, Capdeville R. Midostaurin: its odyssey from discovery to approval for treating acute myeloid leukemia and advanced systemic mastocytosis. *Blood advances* 2018; **2**(4)**:** 444-453.
181. Levis M. Midostaurin approved for FLT3-mutated AML. *Blood* 2017; **129**(26)**:** 3403.
182. Medeiros BC, Fathi AT, DiNardo CD, Pollyea DA, Chan SM, Swords R. Isocitrate dehydrogenase mutations in myeloid malignancies. *Leukemia* 2016; **31:** 272.
183. Stein EM, DiNardo CD, Pollyea DA, Fathi AT, Roboz GJ, Altman JK*, et al.* Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood* 2017; **130**(6)**:** 722-731.
184. Okoye-Okafor UC, Bartholdy B, Cartier J, Gao EN, Pietrak B, Rendina AR*, et al.* New IDH1 mutant inhibitors for treatment of acute myeloid leukemia. *Nat Chem Biol* 2015.
185. Stein EM. Molecularly targeted therapies for acute myeloid leukemia. *Hematology American Society of Hematology Education Program* 2015; **2015:** 579-583.
186. Shafer D, Grant S. Update on rational targeted therapy in AML. *Blood Reviews* 2016; **30**(4)**:** 275-283.
187. Kurtz SE, Eide CA, Kaempf A, Mori M, Tognon CE, Borate U*, et al.* Dual inhibition of JAK1/2 kinases and BCL2: a promising therapeutic strategy for acute myeloid leukemia. *Leukemia* 2018.
188. Anguille S, Van Tendeloo VF, Berneman ZN. Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia. *Leukemia* 2012; **26:** 2186.
189. Goswami M, Hensel N, Smith BD, Prince GT, Qin L, Levitsky HI*, et al.* Expression of putative targets of immunotherapy in acute myeloid leukemia and healthy tissues. *Leukemia* 2014; **28**(5)**:** 1167-1170.
190. Hong C-S, Sharma P, Yerneni SS, Simms P, Jackson EK, Whiteside TL*, et al.* Circulating exosomes carrying an immunosuppressive cargo interfere with cellular immunotherapy in acute myeloid leukemia. *Scientific Reports* 2017; **7**(1)**:** 14684.
191. Qianshan T, Ying P, Yiping W, Huiping W, Shudao X, Qing L*, et al.* Regulatory T cells-derived IL-35 promotes the growth of adult acute myeloid leukemia blasts. *International Journal of Cancer* 2015; **137**(10)**:** 2384-2393.
192. Buggins AGS, Milojkovic D, Arno MJ, Lea NC, Mufti GJ, Thomas NSB*, et al.* Microenvironment Produced by Acute Myeloid Leukemia Cells Prevents T Cell Activation and Proliferation by Inhibition of NF-κB, c-Myc, and pRb Pathways. *The Journal of Immunology* 2001; **167**(10)**:** 6021-6030.

193. Lion E, Willemen Y, Berneman ZN, Van Tendeloo VF, Smits EL. Natural killer cell immune escape in acute myeloid leukemia. *Leukemia* 2012; **26**(9)**:** 2019-2026.
194. Chen Daniel S, Mellman I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* 2013; **39**(1)**:** 1-10.
195. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E*, et al.* Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in Cancer Biology* 2015; **35:** S185-S198.
196. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS*, et al.* The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A* 2012; **109**(17)**:** 6662-6667.
197. Veuillen C, Aurran-Schleinitz T, Castellano R, Rey J, Mallet F, Orlanducci F*, et al.* Primary B-CLL resistance to NK cell cytotoxicity can be overcome in vitro and in vivo by priming NK cells and monoclonal antibody therapy. *Journal of clinical immunology* 2012; **32**(3)**:** 632-646.
198. Godwin CD, Gale RP, Walter RB. Gemtuzumab ozogamicin in acute myeloid leukemia. *Leukemia* 2017; **31:** 1855.
199. Rowe JM, Löwenberg B. Gemtuzumab ozogamicin in acute myeloid leukemia: a remarkable saga about an active drug. *Blood* 2013; **121**(24)**:** 4838.
200. Järås M, Johnels P, Hansen N, Ågerstam H, Tsapogas P, Rissler M*, et al.* Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. *Proc Natl Acad Sci U S A* 2010; **107**(37)**:** 16280-16285.
201. Carey A, Edwards DKt, Eide CA, Newell L, Traer E, Medeiros BC*, et al.* Identification of Interleukin-1 by Functional Screening as a Key Mediator of Cellular Expansion and Disease Progression in Acute Myeloid Leukemia. *Cell Rep* 2017; **18**(13)**:** 3204-3218.
202. Ågerstam H, Karlsson C, Hansen N, Sanden C, Askmyr M, von Palffy S*, et al.* Antibodies targeting human IL1RAP (IL1R3) show therapeutic effects in xenograft models of acute myeloid leukemia. *Proc Natl Acad Sci U S A* 2015; **112**(34)**:** 10786-10791.
203. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, Jr.*, et al.* CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* 2009; **138**(2)**:** 286-299.
204. Okazawa H, Motegi S, Ohyama N, Ohnishi H, Tomizawa T, Kaneko Y*, et al.* Negative regulation of phagocytosis in macrophages by the CD47-SHPS-1 system. *Journal of immunology (Baltimore, Md : 1950)* 2005; **174**(4)**:** 2004-2011.
205. Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L*, et al.* Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential. *PLoS One* 2015; **10**(9)**:** e0137345.
206. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science* 2018; **359**(6382)**:** 1361-1365.
207. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood* 2015; **125**(26)**:** 4017-4023.
208. Andrew D, A. PM. Hypogammaglobulinemia due to CAR T-cell therapy. *Pediatric Blood & Cancer* 2018; **65**(4)**:** e26914.

209. Taussig DC, Pearce DJ, Simpson C, Rohatiner AZ, Lister TA, Kelly G*, et al.* Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood* 2005; **106**(13)**:** 4086-4092.
210. Wang J, Chen S, Xiao W, Li W, Wang L, Yang S*, et al.* CAR-T cells targeting CLL-1 as an approach to treat acute myeloid leukemia. *Journal of Hematology & Oncology* 2018; **11:** 7.
211. Sallman DA, Brayer J, Sagatys EM, Lonez C, Breman E, Agaugué S*, et al.* NKG2D-based chimeric antigen receptor therapy induced remission in a relapsed/refractory acute myeloid leukemia patient. *Haematologica* 2018.
212. Petrov JC, Wada M, Pinz KG, Yan LE, Chen KH, Shuai X*, et al.* Compound CAR T-cells as a double-pronged approach for treating acute myeloid leukemia. *Leukemia* 2018; **32**(6)**:** 1317-1326.
213. Newick K, O'Brien S, Moon E, Albelda SM. CAR T Cell Therapy for Solid Tumors. *Annual Review of Medicine* 2017; **68**(1)**:** 139-152.
214. Klichinsky M, Ruella M, Shestova O, Kenderian SS, Kim MY, O'Connor R*, et al.* Abstract 4575: Chimeric antigen receptor macrophages (CARMA) for adoptive cellular immunotherapy of solid tumors. *Cancer Research* 2017; **77**(13 Supplement)**:** 4575-4575.
215. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E*, et al.* Interleukins, from 1 to 37, and interferon-g: Receptors, functions, and roles in diseases. *Journal of Allergy and Clinical Immunology* 2011; **127**(3)**:** 701-721.e770.
216. Howard M, Farrar J, Hilfiker M, Johnson B, Takatsu K, Hamaoka T*, et al.* Identification of a T cell-derived b cell growth factor distinct from interleukin 2. *J Exp Med* 1982; **155**(3)**:** 914-923.
217. Gadani SP, Cronk JC, Norris GT, Kipnis J. Interleukin-4: A Cytokine to Remember. *Journal of immunology (Baltimore, Md : 1950)* 2012; **189**(9)**:** 4213-4219.
218. Berndt JD. The IL-4 Brown Out. *Science signaling* 2014; **7**(331)**:** ec170-ec170.
219. Junttila IS. Tuning the Cytokine Responses: An Update on Interleukin (IL)-4 and IL-13 Receptor Complexes. *Frontiers in Immunology* 2018; **9**(888).
220. Pesu M, Takaluoma K, Aittomaki S, Lagerstedt A, Saksela K, Kovanen PE*, et al.* Interleukin-4-induced transcriptional activation by stat6 involves multiple serine/threonine kinase pathways and serine phosphorylation of stat6. *Blood* 2000; **95**(2)**:** 494-502.
221. Wick KR, Berton MT. IL-4 induces serine phosphorylation of the STAT6 transactivation domain in B lymphocytes. *Molecular immunology* 2000; **37**(11)**:** 641-652.
222. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 Receptor: Signaling Mechanisms and Biologic Functions. *Annual Review of Immunology* 1999; **17**(1)**:** 701.
223. Paul WE. History of interleukin-4. *Cytokine* 2015; **75**(1)**:** 3-7.
224. Kiniwa T, Enomoto Y, Terazawa N, Omi A, Miyata N, Ishiwata K*, et al.* NK cells activated by Interleukin-4 in cooperation with Interleukin-15 exhibit distinctive characteristics. *Proceedings of the National Academy of Sciences* 2016; **113**(36)**:** 10139-10144.
225. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; **3**(1)**:** 23-35.

226. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation* 2012; **122**(3)**:** 787-795.
227. Li Z, Chen L, Qin Z. Paradoxical roles of IL-4 in tumor immunity. *Cell Mol Immunol* 2009; **6**(6)**:** 415-422.
228. Ellyard JI, Simson L, Parish CR. Th2-mediated anti-tumour immunity: friend or foe? *Tissue Antigens* 2007; **70**(1)**:** 1-11.
229. Manabe A, Coustan-Smith E, Kumagai M, Behm FG, Raimondi SC, Pui CH*, et al.* Interleukin-4 induces programmed cell death (apoptosis) in cases of high-risk acute lymphoblastic leukemia. *Blood* 1994; **83**(7)**:** 1731-1737.
230. Yamashita U, Tanaka Y, Shirakawa F. Suppressive effect of interleukin-4 on the differentiation of M1 and HL60 myeloid leukemic cells. *Journal of leukocyte biology* 1993; **54**(2)**:** 133-137.
231. Harada M, Akashi K, Shibuya T, Takamatsu Y, Niho Y. Role of interleukin-4 in the negative regulation of proliferation of chronic myelomonocytic leukemia cells. *Annals Of The New York Academy Of Sciences* 1991; **628:** 156-164.
232. Akashi K, Shibuya T, Harada M, Takamatsu Y, Uike N, Eto T*, et al.* Interleukin 4 suppresses the spontaneous growth of chronic myelomonocytic leukemia cells. *The Journal of clinical investigation* 1991; **88**(1)**:** 223-230.
233. Wiernik PH, Dutcher JP, Yao X, Venkatraj U, Falkson CI, Rowe JM*, et al.* Phase II study of interleukin-4 in indolent B-cell non-Hodgkin lymphoma and B-cell chronic lymphocytic leukemia: a study of the Eastern Cooperative Oncology Group (E5Y92). *Journal of immunotherapy (Hagerstown, Md : 1997)* 2010; **33**(9)**:** 1006-1009.
234. Martin P, Leonard JP. IL-4 as therapy for non-Hodgkin's lymphoma: possibilities but major challenges. *Leuk Lymphoma* 2007; **48**(7)**:** 1259-1260.
235. Rivera-Luna R, Cardenas-Cardos R, Martinez-Guerra G, Ayon A, Leal C, Rivera-Ortegon F. Childhood acute leukemia and intestinal parasitosis. *Leukemia* 1989; **3**(11)**:** 825-826.
236. Linabery AM, Jurek AM, Duval S, Ross JA. The Association Between Atopy and Childhood/Adolescent Leukemia: A Meta-Analysis. *American Journal of Epidemiology* 2010; **171**(7)**:** 749-764.
237. Zhou M-H, Yang Q-M. Association of asthma with the risk of acute leukemia and non-Hodgkin lymphoma. *Molecular and Clinical Oncology* 2015; **3**(4)**:** 859-864.
238. Müller CI, Trepel M, Kunzmann R, Lais A, Engelhardt R, Lübbert M. Hematologic and molecular spontaneous remission following sepsis in acute monoblastic leukemia with translocation (9;11): a case report and review of the literature. *European Journal of Haematology* 2004; **73**(1)**:** 62-66.
239. Trof RJ, Beishuizen A, Wondergem MJ, Strack van Schijndel RJ. Spontaneous remission of acute myeloid leukaemia after recovery from sepsis. *The Netherlands journal of medicine* 2007; **65**(7)**:** 259-262.

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