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Integrative Life Science Doctoral Program (ILS)

TARGETING KEY SURVIVAL SIGNALING PATHWAYS FOR THE TREATMENT OF LEUKEMIA

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ACADEMIC DISSERTATION

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* The authors contributed equally to this work.

ABBREVIATIONS

AKT	Protein kinase B (PKB)
AML	Acute myeloid leukemia
AUC	Area under the curve
BCL-xL	B-cell lymphoma-extra large
BCL2	B-cell lymphoma 2
BH3	BCL2 homology domain 3
BM	Bone marrow
BP	Base pair
BSA	Bovine serum albumin
c-KIT	SCFR, Mast/stem cell growth factor receptor, CD117
CDK	Cyclin-dependent kinases
CML	Chronic myeloid leukemia
COSMIC	Catalogue of Somatic Mutations in Cancer
CR	Complete response
CTG	CellTiter Glo
DNA	Deoxyribonucleic acid
DSS	Drug sensitivity score
ELISA	Enzyme-linked immunosorbent assay
ELN	European LeukemiaNet
EMA	European Medicines Agency
EPO	Erythropoietin
ET	Essential thrombocytosis
FAB	French-American-British classification
FBS	Fetal bovine serum
FC	Flow cytometry
FDA	Food and Drug Administration
FLT3	Fms Related Tyrosine Kinase 3
GM-SCF	Granulocyte-macrophage colony-stimulating factor
HER2	Human epidermal growth factor receptor 2
HIES	Hyper-IgE syndrome
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
Hsp90	Heat shock protein 90
HC-FC	High-content flow cytometry
IL	Interleukin
JAK	Janus kinase
JMML	Juvenile myelomonocytic leukemia

LGL	Large granular lymphocytes
MAPK	Mitogen activated protein kinase
MNC	Mononuclear cells
MPN	Myeloproliferative neoplasm
mTOR	The mammalian target of rapamycin
NES	Normalized enrichment score
NGS	Next generation sequencing
NHL	Non-Hodgkin's lymphoma
NK cell	Natural killer cell
O/N	Over night
ORF	Open reading frame
PCR	Polymerase chain reaction
PIAS	Protein inhibitors of activated STATs
PTP	Protein tyrosine phosphatase
PV	Polycythemia vera
RT	Room temperature
RTK	Receptor tyrosine kinase
SCID	Severe combined immunodeficiency
SD	Standard deviation
SHP2	Tyrosine-protein phosphatase non-receptor type 11, PTPN11
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
T-ALL	T-cell acute lymphoblastic leukemia
TBS-T	Tris-buffered saline and Tween 20
TCGA	The Cancer Genome Atlas
TK	Tyrosine kinase
TPO	Thrombopoietin
T-PLL	T-cell prolymphocytic leukemia
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World health organization
WT	Wild type

ABSTRACT

Advances in molecular biology and sequencing technologies have revolutionized the understanding of molecular and genetic factors in leukemia pathogenesis. Each leukemia patient harbors a unique set of genetic abnormalities resulting in impaired regulation of cell growth and differentiation. Increased cell proliferation and survival is commonly mediated through overactive signaling cascades, such as JAK/STAT, PI3K/mTOR and MAPK pathways. Understanding the pathobiology of leukemia has led to the development of small molecule inhibitors that can directly bind to target proteins and inhibit aberrant signaling. However, the molecular landscape of rare types of leukemia has not been comprehensively studied and our understanding of the relationship between cancer genotype, phenotype and drug function is limited.

In this study we aimed to better understand the molecular background of large granular lymphocytic (LGL) leukemia and T-cell acute lymphoblastic leukemia (T-ALL) using exome and targeted sequencing. By testing patient derived leukemic cells with high-throughput drug screening platform, we further aimed to assess whether identified genetic abnormalities and/or phenotypes are linked to drug sensitivity. Finally, we developed a flow cytometry-based drug screening platform to assess the sensitivity of acute myeloid leukemia (AML) samples at a more detailed cell population level.

In study I, we aimed to identify driver mutations in *STAT3* mutation negative LGL leukemia patients. Earlier, our research group discovered that 40% of LGL leukemia patients had activating point mutations in the *STAT3* gene. By using exome sequencing we identified novel *STAT5B* mutations in two patients. We then sequenced the *STAT5B* gene in an additional 209 patients using targeted sequencing and identified altogether 4/211 patients to carry a *STAT5B* mutation (N642H and Y640F). Functional studies showed that both mutations resulted in constitutive activation of STAT5B.

In study II, we investigated the molecular drivers of an adult T-ALL patient and identified three mutations in *STAT5B*. To investigate the prevalence of *STAT5A, STAT5B* and *STAT3* mutations in T-ALL, we sequenced these genes from 68 patients with targeted amplicon sequencing. From this larger cohort, six patients carried

STAT5B mutations (8%), whereas no *STAT3 or STAT5A* mutations were discovered. Our index patient, who had three *STAT5B* mutations, showed significantly higher BCL-xL expression and high *ex vivo* sensitivity to a BCL2/BCL-xL inhibitor navitoclax.

In study III, using a functional reporter assay we screened 306 oncology compounds to find potential hits that can decrease the cellular activity of STAT3 and studied whether *STAT3* mutations confer a distinct drug response profile. The most potent targeted compounds inhibiting STAT3 activity were cyclin-dependent kinase (CDK), mammalian target of rapamycin (mTOR), heat shock protein 90 (Hsp90), and Janus kinase (JAK) inhibitors. Amongst these compounds only the Hsp90 inhibitors effectively inhibited both the mutant and interleukin induced wild type STAT3 phosphorylation and activity.

In study IV, we developed high-content flow cytometry (HCFC) based drug screening assay to assess cell population specific drug responses in heterogenous AML samples. Using the assay, we were able to simultaneously measure the population specific drug responses of leukemic blasts, more mature leukemic cells and healthy cells from the same sample. The data showed that targeted therapies have different efficacies towards leukemic AML cells at distinct stages of myeloid differentiation. Particularly, BCL2 inhibitor venetoclax was highly toxic to immature blasts but was not effective against more differentiated monocytic and granulopoietic cells.

In conclusion, we identified novel *STAT5B* mutations in two different lymphoproliferative diseases, T-ALL and LGL leukemia. Furthermore, we developed a modern flow cytometry-based drug screening platform to assess primary AML sample sensitivities at a single cell level.

INTRODUCTION

The rapid development of molecular biology methods and tools from the 1960s to 1980s led to the identification of several oncogenes such as *SRC* [1], *RAS* [2], *HER2* [3] and the *BCR-ABL* fusion gene.[4] The understanding of the molecular pathobiology behind cancer resulted in effective targeted therapies. In leukemia, the progress is best illustrated in chronic myeloid leukemia (CML) in which the specific inhibition of BCR-ABL fusion gene product with imatinib has led to outstanding treatment results.[5] In the beginning of the 21st century, whole genome sequencing methods revolutionized the understanding of cancer-associated genes.[6] Simultaneously, an increasing number of compounds specifically inhibiting aberrant mutated proteins have been developed. Besides a few exceptions, these novel drugs, however, have not led to significant advances in clinical outcomes.

Many driver mutations result in overactive JAK/STAT, MAPK and mTOR/AKT signaling. These pathways regulate cell proliferation, cell survival and anti-apoptotic BCL2 family members. It has become evident that the specific inhibition of a single molecule rarely results in good treatment outcomes. The poor responses illustrate that our functional understanding of the disease mechanism is limited and that specific inhibition of a mutated protein does not directly translate into clinical benefit.[7] Furthermore, significant intra- and inter-tumoral differences and complex signaling networks make it difficult to predict which patients benefit from targeted treatment. The current research interests include finding good biomarkers for targeted therapies and better understanding the functional effects of drugs.

This study focused on the identification of novel driver mutations in rare hematological malignancies, adult T-ALL and LGL leukemia. Furthermore, it aimed to link genetic and phenotypic characteristics to drug responses in *STAT3* mutated leukemia samples and in AML patient samples.

1 REVIEW OF THE LITERATURE

1.1 Formation of blood cells

1.1.1 Hematopoiesis

Blood, the vital body fluid that carries oxygen and nutrients to tissues, is composed of plasma and different blood cells. Blood cells can be divided into three major groups each with their own unique functions: red blood cells (erythrocytes) carry oxygen to tissues, platelets (thrombocytes) form blood clots to stop bleeding and white blood cells (leukocytes) are responsible for defending the body against infections.[8] All blood cells are formed from a small number of hematopoietic stem cells (HSCs) through a process called hematopoiesis [9]. In adults, hematopoiesis takes place in the marrow of specific bones including the cranium, vertebrae, pelvis and sternum. Hematopoiesis starts with an asymmetric cell division of a pluripotent HSC, which leads to the formation of an identical self-renewing daughter cell and a progenitor cell.[9] Progenitor cells are then committed to either myeloid lineage differentiation, which gives rise to granulocytes, macrophages, red blood cells and platelets, or lymphoid lineage differentiation leading to the development of T- and B-cells (**Figure 1**). During the differentiation process progenitor cells lose the capacity of self-renewal distinguishing them from HSCs.

1.1.2 Cytokines

Proliferation, maturation and function of hematopoietic cells are controlled by cytokines, hormones and other factors.[10] For example, during infection or low oxygen levels, specific molecules are produced to stimulate the proliferation of white or red blood cells, respectively. In the bone marrow (BM) cytokines (interferons, chemokines, interleukins, and tumor necrosis factors) are secreted predominantly by stromal cells such as fibroblasts, osteoblasts, adipocytes, endothelial cells and macrophages.[11] Some exceptions involve erythropoietin (EPO) and thrombopoietin hormone (TPO) that are produced in the kidneys and in the liver, respectively.[8] Thus, secreted signaling molecules can be present locally at the site where they are produced (autocrine or paracrine signaling) or they can circulate in the blood and have an effect throughout the body (endocrine signaling). The most important

cytokines for the maintenance and self-renewal of HSCs and early myeloid and lymphoid progenitors include stem cell factor (SCF also named as c-Kit ligand) and fms like tyrosine kinase 3 ligand (FLT3-L).[10] Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 act on a wide range of hematopoietic cells and are important in myeloid lineage differentiation. Common cytokines that activate the immune response and proliferation of lymphocytes include IL-2, IL-7, IL-15 and IL-21 (**Figure 1**).[8]

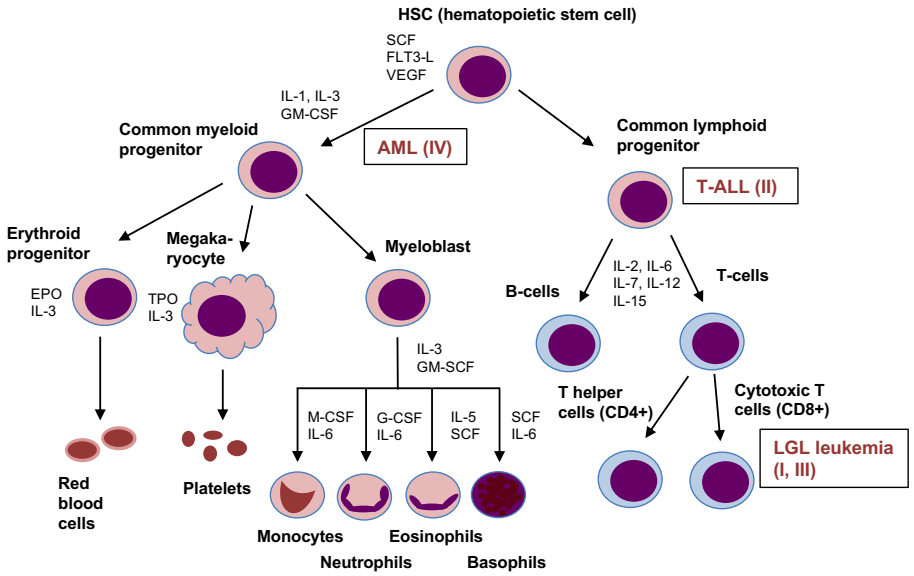

Figure 1. Schematic diagram of hematopoiesis. Hematopoietic stem cells (HSC) give rise to lineage committed progenitor cells (myeloid or lymphoid lineage) that form all mature cells in the blood. The most important cytokines for different cell types are presented in the graph. Different leukemia subtypes studied in this thesis are indicated in red.

The biological effects of cytokines are mediated through the binding of cytokines to cell membraneπ receptors resulting in the activation of intracellular signaling cascades. Altogether, hematopoiesis is a process in which several signaling pathways interact with each other in a balanced way. However, accumulation of mutations and genetic aberrations as we age can shake this equilibrium. Activating mutations in receptor tyrosine kinases (RTKs) can lead to constant proliferative signaling and deletions in tumor suppressor genes can remove the negative feedback loops essential for maintaining homeostasis. Ultimately, aberrant signaling can lead to fatal diseases such as leukemia.

1.2 Survival signaling pathways

Three important signal transduction pathways that are commonly activated by cytokine receptors include Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphatidylinositol 3-kinase/AKT/ mammalian target of rapamycin (PI3K/AKT/mTOR) and mitogen activated protein kinase (MAPK) pathways. They have a central role in cell proliferation and differentiation, and can regulate antiapoptotic BCL2 family members and thus apoptosis.[12–15] Due to their critical function, these pathways are commonly deregulated in leukemia. In this thesis the above-mentioned pathways are referred to as key survival signaling pathways even though many other important pathways are also involved in leukemia pathogenesis.

1.2.1 JAK/STAT pathway

JAKs and STATs were identified between 1989 and 1994 as components of a signal transduction cascade that transferred interferon and cytokine signaling from the cell surface receptors via JAKs and STATs to the nucleus.[16] The human genome encodes four JAK (JAK1, JAK2, JAK3 and TYK2) and seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). Their activation is mediated by more than 30 membrane receptors.[17] While all JAK kinases have shown to be deregulated in some hematological malignancy, amongst the 7 STAT family members, STAT3 and STAT5B are mainly associated with leukemia.[18]

In the absence of stimulation, STATs reside in the cytosol as inactive monomers. Upon cytokine stimulation, JAKs that are bound to the membrane receptors, phosphorylate the receptor chains that then serve as docking site for STAT monomers leading to STAT phosphorylation and dimerization (**Figure 2**).[19] STAT dimers translocate to the nucleus where they bind to specific DNA consensus sequences resulting in modified gene expression.[20] After completing their task, the signaling cascade is inhibited by negative feedback loops. STAT activity is suppressed by three major mechanisms which include (I) protein tyrosine phosphatases (PTPs) that can directly dephosphorylate STATs at their tyrosine residue,[21] (II) suppressors of cytokine signaling 1 to 7 (SOCS1–7) that can bind to JAKs or membrane receptors and block their function,[22] and (III) protein inhibitors

of activated STATs (PIAS) that interfere the interaction of STATs with DNA (**Figure 2**).[23] Mutations in these genes can lead to overactive survival signaling and are commonly observed in leukemias. For example protein tyrosine phosphatase PTPN11, also called SHP2, is mutated in 34% of juvenile myelomonocytic leukemia (JMML)[24] and in 4% of AML patients.[25]

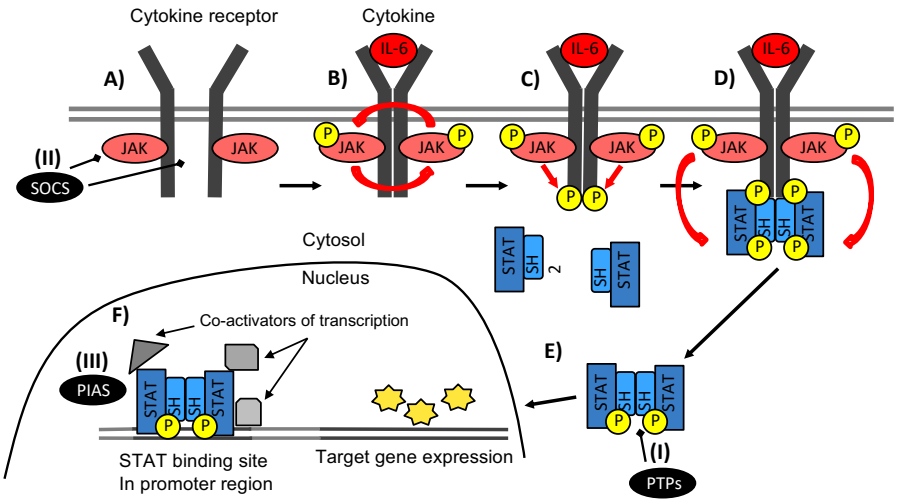

Figure 2. Activation of the JAK-STAT pathway and its inhibition (I-III). A) JAKs are attached to membrane receptors **B)** Cytokines bind to receptors causing conformational changes in the receptor and JAK phosphorylation **C)** Activated JAKs phosphorylate membrane receptors **D)** STAT monomers bind to the activated receptor and JAKs phosphorylate the tyrosine residue of STATs **E)** STATs dissociate from their receptors and form dimers via their SH2 domain **F)** The STAT dimer migrates to the nucleus and binds to specific promoter regions inducing expression of target genes. Adapted from *Molecular Biology of Cell, Alberts et al., 4th ed., figure 15-63*

The importance of the individual members of the JAK/STAT signaling has been extensively studied in knockout mice. *JAK1* null mice die within 24 h following birth.[26] Interestingly, analysis of their blood revealed impaired growth of thymocytes and mature B- and T-cells whereas no alterations were observed in other blood cells. *Ex vivo* experiments also demonstrated that *JAK1* is needed for IL-7 stimulated lymphoid maturation, thus, demonstrating the importance of the IL-7/JAK1/STAT axis in lymphoid development.[26] *JAK2* knockout mice, on the other hand, die prenatally due to the impaired erythropoiesis. This finding is supported by the fact that erythropoietin receptor (EPOR) signals through JAK2.[27,28] Interestingly, *JAK2* deficient progenitor cells can still repopulate the lymphoid compartment of

irradiated mice. This suggests that *JAK2* is essential in red blood cell production and myeloid cell functioning but not in lymphocyte development.[27] JAK3 is activated only by receptors that contain the common gamma chain (γc) subunit such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors.[29] In contrast to other JAK kinases that are expressed in most tissues, *JAK3* expression is mostly restricted to NK- and T-cells.[30,31] Accordingly, loss of *JAK3* causes impaired NK- and T-cell function both in mice and humans.[32] Experiments with *TYK2* deficient mouse model shows that *TYK2* is redundant with other JAKs in the activation of cytokine receptor signaling and is not so crucial in hematopoiesis.[33] However, *TYK2* deficient mice show decreased T-cell and macrophage function.[33]

Amongst the STAT knockdown mouse models, only *STAT3* deficiency is lethal causing mice to die early in embryogenesis prior to gastrulation.[34] However, silencing *STAT3* specifically in T-cells resulted in severely impaired proliferative response to IL-2 and IL-6.[35,36] STAT5A and STAT5B have very high amino acid similarity (>90%) and they are located next to each other in human chromosome 17.[37] By using mice completely deficient of S*TAT5A* and *STAT5B*, John O'Shea and colleagues showed that STAT5 is essential for the normal development of all lymphoid lineage cells.[38] In a different study, *STAT5B* deficiency caused marked decrease in T- and NK-cell proliferation in response to IL-2 and IL-15 whereas *STAT5A* deficiency had similar but a more modest effect.[39] In line with these observations, overexpression of *STAT5B* results in expansion of CD8+ T-cells in mice.[40]

The molecular characterization of primary immunodeficiency diseases has further emphasized the essentiality of the JAK/STAT family members in the immune system. Hyper IgE syndrome (HIES) is a primary immunodeficiency characterized by eczema, infections of the lung and elevated serum IgE levels. In 2007, genetic studies revealed that the disease is caused by heterozygous dominant-negative mutations in *STAT3* resulting in severe reductions of T helper 17 cells (Th17).[41] Moreover, mutations in *JAK3* is known to cause severe combined immunodeficiency (SCID).[42] Taken together, *JAK1*, *JAK3*, *STAT3* and *STAT5B* are essential for proper lymphoid cell function whereas *JAK2* is more essential for erythroid and myeloid lineage cells.

1.2.2 JAK/STAT pathway in leukemia

Many cancers including hematological malignancies have been associated with overactive JAK/STAT signaling. In 2005, several research groups identified a unique somatic mutation in *JAK2* (*JAK2* V617F) in 80% of polycythemia vera (PV) patients[43] and also in 30-50% of essential thrombocytosis (ET) and primary myelofibrosis patients (PMF).[44,45] Prompted by these observations, researchers tried to find other mutated components of the JAK/STAT pathway that would lead to overactive JAK/STAT signaling in hematological malignancies. A few years later *JAK1*, *JAK2* and *JAK3* mutations were identified in a sub-fraction of ALL and AML patients.[46–48] Additionally, novel mutations in cytokine receptor genes (e.g. *IL-7Rα*[49], *CSF3R*[50], *MPL*[51], and *CRLF2*[52]) were identified and shown to result in constitutive JAK/STAT signaling. However, *STAT3* and *STAT5* mutations were not detected in cancers until 2011.[53] This was partly surprising as already in 1998 and 1999, artificially generated point mutations were shown to increase STAT3[54] and STAT5B[55,56] activity and cause cellular transformation in a laboratory setting. *STAT3* mutations were first identified in inflammatory hepatocellular adenomas (IHCA) in 2011[57] and soon after in 40% of LGL-leukemia patients by Koskela et al. .[58] However, *STAT5A or STAT5B* mutations were not described in cancers before the studies presented in this thesis.

1.2.3 Targeting JAK/STAT pathway

Targeting JAK/STAT signaling is a tempting approach to treat leukemia. Depending on the genetic lesions, the best strategy might vary and involves the inhibition of membrane receptors, fusion genes, JAKs or STATs. Inhibition of the *BCR-ABL* fusion gene in CML results in decreased STAT5 phosphorylation, and in parallel inhibits the PI3K/AKT/mTOR and MAPK pathways.[59] Similar outcomes have been obtained by targeting the FLT3 membrane receptor in AML.[60] The discovery of *JAK2* mutations in myeloproliferative neoplasms (MPNs) prompted the development of JAK inhibitors. Type I JAK inhibitors target the ATP-binding site of JAKs in active conformation whereas type II inhibitors target the ATP-binding site in inactive conformation.[61] Currently, all clinically tested drugs belong to type I inhibitors and differences in the specificities to JAKs are the basis for different compounds. Commercially available drugs include ruxolitinib, a JAK1/JAK2 inhibitor, which is used to treat myelofibrosis[62] and PV[63]. Two other JAK inhibitors,

tofacitinib and baricitinib, have been approved to treat methotrexate resistant rheumatoid arthritis (RA).[64,65] As the JAK/STAT pathway has a major role in lymphocyte function, several ongoing clinical studies are investigating the therapeutic potential of JAK inhibitors in autoimmune diseases such as psoriasis, vitiligo, alopecia areata and graft versus host disease.[61] From the novel type II JAK2 inhibitors, NVP-BBT594 and NVP-CHZ868, have demonstrated potent efficacies in preclinical models, but they might also induce profound cytopenia limiting their clinical use.[66,67]

The direct inhibition of STAT3 or STAT5 with SH2 domain antagonists is a rational strategy to silence STAT activity. By high-throughput screening of protein–protein interactions, LLL12, OPB-31121, STA-21 and Stattic, have been identified to directly bind to STAT3 and hamper its dimerization.[68–70] A phase I study of an oral STAT3 inhibitor OPB-51602 was carried out with refractory solid and hematological malignancies.[71,72] However, only modest activity was observed and the long-term administration was limited due to toxic side effects such as neutropenia and lactic acidosis. The Gunning research group from the University of Toronto have generated promising small molecule inhibitors that bind to either STAT3 or STAT5A/B thus inhibiting their dimerization and activation. The group recently identified salicylic acid containing small molecules (BP-1108, BP-1075 and 13a) which effectively inhibit STAT5A/B activity in MV4-11 and K562 leukemia cell lines.[73,74] However, no clinical trials have been conducted so far with STAT5 inhibitors. Altogether, direct STAT inhibitors have not yet led to useful therapies partly because they are pharmacologically challenging targets. Toxicities are also expected since STAT proteins are abundantly expressed throughout the body. Thus, improved drug delivery and target specificity remain a big challenge for direct STAT inhibition.

Another strategy to identify novel JAK/STAT pathway inhibitors is to repurpose commonly used drugs which might indirectly inhibit STAT function. The antipsychotic drug pimozide was shown to inhibit STAT5A in *BCR-ABL*$^{+}$ cell lines[75] and recent high-throughput compound screens identified methotrexate and piperlongumine as potential JAK/STAT3 pathway inhibitors.[76,77] However, these drugs usually have hundreds of targets and STAT3 inhibition might be obtained only with overly high drug concentrations.

1.2.4 MEK/ERK and PI3K/mTOR pathways

In addition to STAT activation, JAKs and cytokine receptors can in parallel activate other signal transduction pathways (**Figure 3**).[18] Cytokine receptors commonly activate the PI3K/AKT/mTOR and MAPK pathways, which converge at the nucleus to regulate gene expression (**Figure 3**). The main proteins in the MAPK signaling chain involve Ras, Raf, MEK and ERK. When one of these proteins is mutated it can result in overactive signaling and cancer formation. As an example, some of the three *RAS* genes (*KRAS*, *NRAS*, *HRAS*) are commonly mutated in solid cancers and hematological malignancies.[78] According to the Catalogue of Somatic Mutations in Cancer (COSMIC), RAS is mutated in 33% of cancers and the mutation results in the activation of downstream proteins Raf-MEK-ERK.[79] Trametinib is a MEK inhibitor that has been approved for the treatment of *BRAF* mutated melanoma.[80] However, resistance often occurs to monotherapy, and the combination therapy with BRAF inhibitor dabrafenib plus MEK inhibitor trametinib has resulted in a better treatment response.[81] This illustrates how the inhibition of the same pathway at two distinct nodes can result in superior treatment outcome. In AML, RAS genes (*NRAS* and *KRAS*) are mutated in 12% of the patients[25], but downstream inhibition of MEK with trametinib has not proven to be a viable strategy in AML.[82]

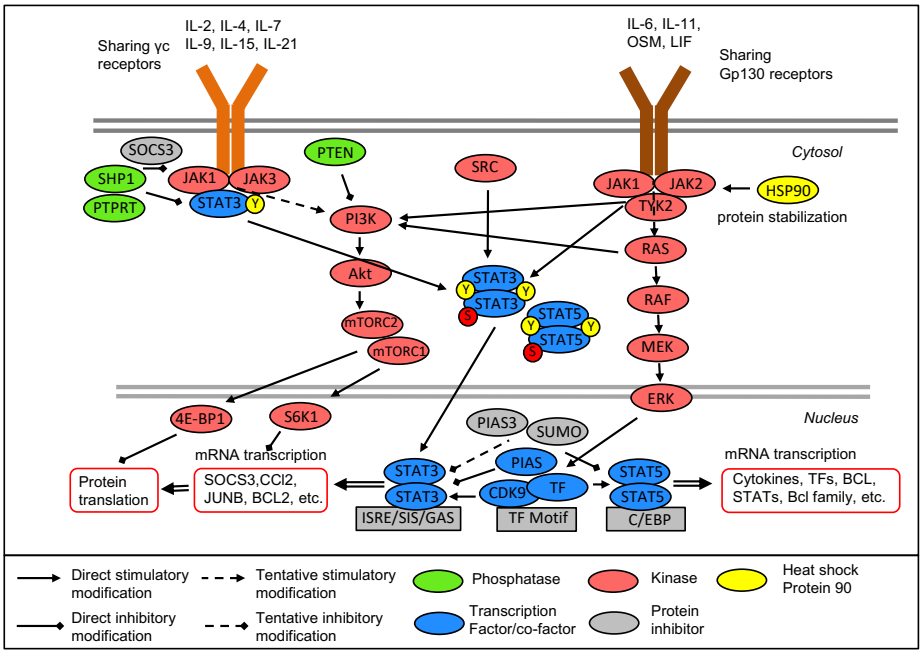

Figure 3. Overview of survival signaling pathways. Membrane receptors are activated by cytokines causing STAT monomers to be phosphorylated by JAKs. Phosphorylated STATs translocate to the nucleus and bind to 9-11 bp long ISRE/GAS/CEPB elements. With the help of transcriptional co-regulators, many oncogenes are overexpressed by STAT3 and STAT5. STAT activation can be inhibited by preventing kinase function via overexpression of proteins such as SOCS and SHP1 or by interfering STAT binding in the nucleus (PIAS). In parallel, cytokine receptors can also activate PI3K/AKT/mTOR and MAPK/ERK pathways. *Adapted from Cell Signaling Technology pathway images. www.cellsignal.com*

The PI3K/AKT/mTOR pathway regulates the cell cycle and proliferation. PI3K phosphorylates AKT, which can in turn activate several downstream targets including mTOR. S6K1 and 4E-BP1 are targets of mTOR and they promote protein synthesis and gene transcription (**Figure 3**). In lay terms, the PI3K/AKT/mTOR pathway can be considered as a volume knob that can decrease or increase all major cell functions. The PI3K/AKT/mTOR pathway is often deregulated in cancer by mutations or deletions in genes like *PTEN* and *PI3K*.[83,84] Moreover, high AKT phosphorylation is associated with poor prognosis in many cancers including AML.[85] Targeting of the PI3K/AKT/mTOR pathway involves, AKT inhibitors, mTORC1 inhibitors (rapalogs), mTOR inhibitors, PI3K inhibitors and dual AKT/mTOR inhibitors .[86] Rapalogs (temsirolimus, everolimus) and PI3K inhibitors (ibrutinib) are in clinical use whereas AKT and dual AKT/mTOR inhibitors are currently tested in clinical trials. Targeting several nodes of different pathways might be crucial to overcome treatment resistance and to obtain synergistic effects. As an example, inhibition of PI3K/mTOR and JAK2/STAT5 pathways in triple-negative breast cancer cell lines leads to synergistic effect and provides rationale to combine these drugs in a clinical setting.[87]

1.2.5 Regulation of apoptosis by BCL2 family members

Programmed cell death, termed apoptosis, is a tightly regulated cellular process. It is characterized by cell shrinkage, chromatin condensation and formation of apoptotic bodies, which are ultimately engulfed by phagocytes.[88] Apoptosis is vital in several normal processes but its impaired function is a hallmark of cancer.[89] Apoptosis is divided into two major pathways, intrinsic and extrinsic, both of which lead to the activation of caspases. Extrinsic or death cell receptor pathway is triggered by external cytokines such as FAS-L and TNF-α that bind to their cell membrane receptors to induce apoptosis. In contrast, cells initiate the intrinsic

pathway in response to stress which can be triggered by radiation, toxins, hypoxia, viral infections[88] and the absence of certain cytokines (e.g. IL-3[90] and IL-6[91]). The intrinsic pathway is regulated through BCL2 family members. This family contains three subfamilies: cell death mediators (BAX and BAK), BH3-only proteins that promote cell death (e.g. PUMA, BIM, BID, BAD, NOXA) and antiapoptotic proteins (e.g. BCL2, BCL-xL and MCL1).[92] The balance between these proteins determines whether a cell undergoes apoptosis.[93] Under normal conditions antiapoptotic BCL2 family proteins (BCL2, BCL-xL and MCL1) maintain cell viability by blocking the cell death mediators (BAX and BAK) and BH3-only proteins, thus preventing them from forming pores in the mitochondrial membrane, and subsequently blocking the release of cytochrome C, activation of caspases and apoptosis (**Figure 4**).

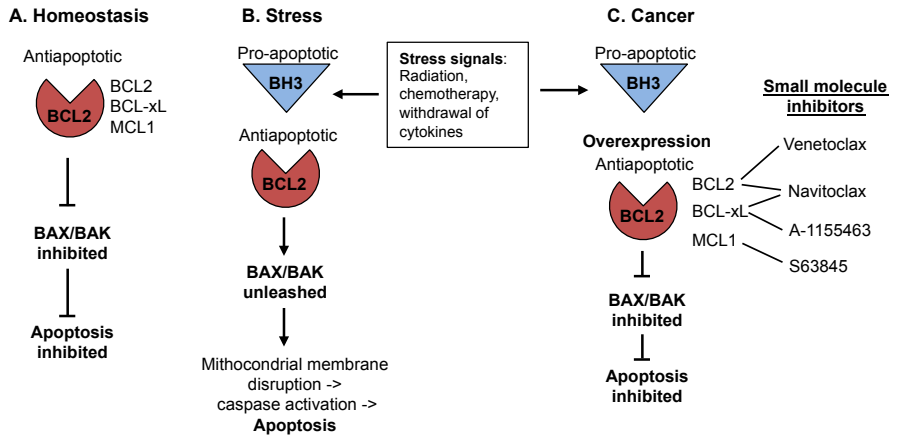

Figure 4. Control of apoptosis by BCL2 family members. A) During homeostasis antiapoptotic BCL2 family members bind to BAX/BAK and BH3-only proteins, thus inhibiting apoptosis. **B)** Upon stressful conditions BH3-only proteins are activated, bound to antiapoptotic BCL2 family members allowing BAX/BAK to initiate apoptosis. **C)** In cancer, antiapoptotic BCL2 family members are overexpressed, thus inhibiting apoptosis also in stressed conditions. Several small molecule inhibitors have been developed, which can specifically inhibit the function of antiapoptotic BCL2 family members. *Adapted from Seminars from Hematology, 2014, Anderson et al.*[94]

Overexpression of antiapoptotic BCL2 family proteins (BCL2, MCL1, BCL-xL) is commonly observed in hematological malignancies and their expression is often regulated by survival signaling pathways.[94,95] High *BCL2* expression is characteristic for many lymphoid malignancies and for AML in which high *BCL2* expression is also associated with poor response to treatment.[96] These observations have led BCL2

to be an attractive target for therapy already for almost three decades after it was described in non-Hodgkin's lymphoma (NHL) in 1985.[97] Obatoclax, a pan-BCL2 inhibitor targeting BCL2, BCL-XL, MCL1 and BCL-W, was one of the first compounds tested in clinical trials in 2007.[98,99] However, the response rates were disappointing partly because of the low affinity of obatoclax to its targets.[100] Later a more potent BCL2/BCL-xL inhibitor navitoclax showed promising results in chronic lymphocytic leukemia (CLL) and NHL, but it resulted in severe grade 3/4 thrombocytopenia stopping its development into clinical use.[101,102] BCL-xL is essential for platelets, and for this reason the BCL2 specific compound venetoclax was developed. Venetoclax showed high response rates in relapsed/refractory (R/R) CLL and received a breakthrough therapy designation from the FDA in 2015.[103] In AML, venetoclax has also shown good results in phase 1/2 clinical trials when combined with hypomethylating agents.[104–106] Moreover, a recent clinical trial with CLL patients showed striking efficacy with venetoclax plus rituximab (anti-CD20 antibody) when compared with the standard of care bendamustine plus rituximab (2 year progression free survival 84.9% vs. 36.3%).[107] This new study demonstrated that the combination strategy results in superior response rates when compared to standard of care or venetoclax monotherapy. Thus, an ongoing interest is to combine venetoclax with other drugs.

In addition to venetoclax, highly specific MCL1 (S63845 and A-1210477) and BCL-xL (A-1155463 and A-1331852) inhibitors have recently been developed (**Figure 4**).[108,109] These new specific BCL2 family inhibitors have the potential to change the current paradigms of leukemia treatment, especially when combined with other targeted drugs or cytotoxic agents.

1.3 LGL leukemia

Large granular lymphocytic (LGL) leukemia is a rare lymphoproliferative disorder characterized by clonal expansion of large granular lymphocytes. Initially, McKennan et al. first described the disorder in 1977 and in 1985 Thomas Loughran named it large granular lymphocyte leukemia.[110,111] The disease can originate from mature CD3+/CD8+ T-cells (85% of cases) or CD3-/CD56+ natural killer (NK)-cells (15%) and thus the disease is subdivided into T-cell or NK-cell types. In some

rare cases T-LGL leukemias can also express both CD3+/CD56+[112] or CD4 with or without CD8 expression.[113,114] In healthy adults, the LGL count in peripheral blood is around 0.2-0.4x10^9/L whereas in LGL leukemia the count is usually between 2 and 20x10^9/L and high cell count persist more than 6 months.[115] Further diagnosis criteria involve evidence of clonality which can be assessed using T-cell receptor (TCR) specific monoclonal antibodies or polymerase chain reaction (PCR) analyses. Since *STAT3* mutations are observed in 30-50% of cases, mutational status can also be used in diagnosis.[116]

It is estimated that LGL leukemia accounts for 2-3% of chronic lymphoproliferative diseases.[117] According to two recent studies, the incidence of LGL leukemia is between 0.2 and 0.72 cases per 1 000 000 individuals and the median age at diagnosis is 66.5 years.[118,119] The majority of the patients have an indolent disease course with a median overall survival of more than 10 years. The most common complications include neutropenia, recurrent infections, splenomegaly and anemia.[116] Moreover, autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus or Sjögren's syndrome are seen in 20-25% of the patients.[117]

1.3.1 Pathogenesis

In normal T-cell homeostasis, activated cytotoxic T-cells become more sensitive to FAS-ligand induced apoptosis the longer they are activated.[120] In contrast to their healthy counterparts, leukemic LGL clone persist in the blood. While the exact mechanism for chronic lymphocytosis is still under investigation some good theories exist. It is hypothesized that clonal LGL expansion arises from chronic antigen stimulation. In aggressive and rare NK-LGL leukemia an Epstein Barr virus infection is commonly detected.[121] In T-LGL leukemia, serologic studies have demonstrated cross-reactivity to human T-cell lymphotropic virus (HTLV) envelope protein BA21 in 30-50% of the patients.[122,123] However, HTLV1 or other virus infections have not been identified in most LGL leukemia patients.

Dysregulation of JAK/STAT3 signaling is proposed to play a fundamental pathogenic role in LGL leukemia. In 2001, constitutive activation of STAT3 was observed in all studied patients[124] and in 2012, activating *STAT3* mutations were detected in 40% of the patients.[58] Since more than half of the patients harbor wild

type (WT) *STAT3*, mutation of *STAT3* alone cannot explain overactive STAT3 or clonal expansion observed in LGL patients. Additionally, high concentrations of IL-6, IL-18, and macrophage inflammatory protein 1 (MIP-1) have been observed in LGL leukemia which can stimulate STAT3 phosphorylation.[125,126] Moreover, the growth of LGL cells has shown to be dependent on IL-15 which is a strong STAT3 activator.[127] Overactive STAT3 signaling may cause the inhibition of homeostatic FAS mediated apoptosis through the upregulation of antiapoptotic BCL2 family proteins.[124] However, PI3K/AKT signaling has also been proposed to mediate resistance through MCL1 independently of STAT3.[128]

1.3.2 Treatment and prognosis

Even though LGL leukemia is a relatively indolent disease, the majority of patients need treatment at some point during disease progression.[116] Disease related deaths are mainly due to infections in patients with severe neutropenia. As supportive care, G-CSF can be used to stimulate neutrophil production.[117,118] Immunosuppressive therapy is the first-line treatment and most clinical experience is obtained with methotrexate and cyclophosphamide. The median overall response rate (ORR) is 50% with these drugs and complete response (CR) is obtained only in 20-30% of the patients of which most relapse.[116] However, reduction of the LGL clone size is often a sufficient treatment goal. Interestingly, one study showed that a patient cohort harboring *STAT3* mutations had an increased response rate to methotrexate but validation is needed in bigger patient cohorts.[129] For refractory patients, there are no treatment recommendations due to limited data. Amongst the individual small clinical trials alemtuzumab (anti-CD52 antibody) and purine analogs (fludarabine, cladribine, bendamustine) have shown the most promising results.[116] Moreover, a recent study with tofacitinib (JAK1/JAK3 inhibitor) showed hematologic response in 6/9 refractory patients with improvement in neutropenia and rheumatoid arthritis symptoms. However, no clear reduction of the leukemic clone was reported.[130]

1.4 T-cell acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is an aggressive disease of immature lymphocytes (lymphoblasts) that can lead to death in a few weeks without treatment. ALL is the most common cancer in children with the majority of cases occurring

during the age of 1-4 years with another peak observed in adults over 60 years.[131,132] According to the Finnish Cancer Registry, 84 individuals were diagnosed with ALL in Finland in 2011 of which 85% were estimated to have the B-cell phenotype and 15% T-cell phenotype.[133] WHO classifies T-ALL and T-cell lymphoblastic lymphoma (T-LL) together, despite their different clinical presentations.[134] T-LL frequently forms bulky mediastinal masses and is distinguished from T-ALL by having less than 20% of blast cells in the BM. T-ALL can be further subdivided into pro-T, pre-T, cortical T, and mature types based on the intrathymic differentiation stage.[134]

1.4.1 Pathogenesis

T-ALL is caused by the accumulation of genetic lesions in lymphoid progenitor cells, leading to their impaired development and increased survival and proliferation. For more than 10 years it has been evident that deregulated NOTCH1 signaling plays a major role in the pathogenesis of T-ALL.[135,136] However, in recent years systematic screening of patients' genome by next generation sequencing has revealed a more precise genomic landscape.[137,138] It is estimated that on average, one T-ALL patient carries approximately 10 biologically relevant genomic lesions.[139] Only 2 of these lesions, *NOTCH1* and *CDKN2A*/*2B*, are detected in more than 50% of the patients, whereas other genes are usually mutated in 10-20% of the patients. Chromosomal rearrangements and activating mutations are commonly found in transcription factors (*TAL1, TLX3, LMO2, BCLB11, LEF1* and *WT1*) and epigenetic regulators (*PHF6, SUZ12, EZH2* and *DNMT3*).[139] Moreover, a recent study estimated that 50% of the patients harbored mutations associated with JAK/STAT, PI3K/AKT or RAS/MAPK pathways.[140] IL-7/JAK/STAT5 signaling is essential in the normal differentiation process of T-cells. Upon IL-7 stimulation, STAT5 translocates to the nucleus where it induces the expression of several target genes including BCL2 family members.[141] Approximately, 12% of adult patients harbor activating mutations in *IL7R* whereas *JAK1* is mutated in 7% of the cases and *JAK3* in 12%.[139] Importantly, IL-7R stimulation results in PI3K/AKT and RAS signaling activation. Moreover, *PTEN* deletions are observed in 10-20% of the patients which results in activated PI3K/AKT signaling, while *KRAS* and *NRAS* are mutated altogether in 10% of T-ALL patients.

1.4.2 Treatment and prognosis

Current treatment of T-ALL relies on high intensity combination chemotherapy (e.g. daunorubicin, dexamethasone, cytarabine, methotrexate, cyclophosphamide and nelarabine) with complex protocols.[134] This has resulted in terrific outcomes in pediatric patients and is one of the success stories in modern medicine. While sixty years ago the median overall survival in pediatric ALL patients was only 2 months,[142] today more than 80% of T-ALL patients are alive after 5 years.[143] However, the remarkable success has not been achieved in adults and only 45% of adult patients are cured.[144] To achieve even these cure rates, hematopoietic stem cell transplantation is used in adult patients with high-risk features, involving patients with relapse and with minimal residual disease (MRD) positivity.[145]

To further improve the treatment outcomes and reduce the toxic side effects, new treatment strategies are warranted. Due to the increased knowledge of molecular level pathophysiology the most attractive approaches are to inhibit overactive NOTCH1 and JAK/STAT signaling. So far, inhibition of NOTCH1 activity with gamma-secretase inhibitors have only shown modest responses in patients and their administration have been limited by gastro-intestinal toxicity.[146,147] However, new therapeutic approaches such as monoclonal antibodies against NOTCH1 receptors or gamma secretase complex may provide better results.[148,149] JAK inhibitors such as ruxolitinib (JAK1/JAK2) and tofacitinib (JAK1/JAK3) are commercially available drugs. Preclinical xenograft mice models have demonstrated a dramatic reduction of the lymphoblast burden when treated with ruxolitinib.[150,151] Interestingly, the reduction was not dependent on the mutational status of the *JAK* or *IL7R* genes raising the question whether all T-ALL lymphoblasts are dependent on the JAK/STAT pathway. Despite the promising results in mice, it is unlikely that ruxolitinib monotherapy alone would be curative.[63] However, combining ruxolitinib with conventional chemotherapeutics is a more promising approach. Ongoing clinical trials with JAK inhibitors in B-ALL (NCT02420717, NCT03117751, NCT02494882) and T-ALL (NCT03613428) will shed light whether combination treatment is a viable treatment strategy.

1.5 Acute myeloid leukemia

In contrast to ALL, AML arises from poorly differentiated myeloid lineage stem/progenitor cells.[152] Due to genetic abnormalities the hematopoietic progenitor cells acquire an increased proliferation rate, resistance to apoptosis and a block in differentiation. The replacement of normal blood cells with leukemic blasts in the bone marrow causes anemia, recurrent infections and bleeding that can lead to death in just a few weeks if not treated. AML is the most common acute leukemia in adults with 20,000 new cases diagnosed in US per year[153] and about 220 in Finland.[133]

AML is diagnosed when at least 20% of the cells in the BM or blood are defined as blasts based on morphological examination.[154] In 1976, a group of French, American and British hematologists divided AML into six different subtypes, M1-M6, based on the differentiation stage of leukemic cells (**Table 1**).[155] Later this classification was updated with the addition of M0 and M7 subtypes.[156,157] In M0 and M1 AML the differentiation block occurs at a very early stage of myeloid progenitor cell development, whereas in M4 and M5 subtypes the differentiation block is "leaky" and leukemic cells show myelomonocytic or monocytic differentiation, respectively. Moreover, in M6 AML the disease starts from the immature erythroid precursors and in M7 AML from platelet precursors called megakaryoblasts. FAB classification was used for decades (1976-2001) but was replaced by the World Health Organization (WHO) classification in 200[154] (updated in 2008[158] and 2016[159]). WHO classification provides a better prognostic value and takes into account significant cytogenetic aberrations and mutations.

Table 1. FAB subtype classification

FAB subtype	Common name	Prevalence	Associated fusion genes
M0	Undifferentiated acute myeloblastic leukemia	5 %	
M1	Acute myeloblastic leukemia with minimal maturation	20 %	
M2	Acute myeloblastic leukemia with maturation	25 %	RUNX1-RUNX1T1
M3	Acute promyelocytic leukemia (APL)	10 %	PML-RARA
M4	Acute myelomonocytic leukemia	20 %	MLL
M4 eos	Acute myelomonocytic leukemia with eosinophilia	5 %	MYH11-CBFB
M5	Acute monocytic leukemia	10 %	MLL
M6	Acute erythroid leukemia	4 %	
M7	Acute megakaryoblastic leukemia	1 %	

*Adapted from publications[152,160]

1.5.1 Pathogenesis

AML is a heterogeneous disease with each patient harboring their own unique set of cytogenetic lesions and mutations. It is estimated that on average AML patients harbor 13 mutations, which is less than most adult cancers.[161] Chromosomal translocations that cause the formation of fusion genes (*RUNX1-RUNX1T1*, *MYH11-CBFB*, *MLL*, *PML-RARA*) have already been recognized for several decades.[162] Fusion genes often associate with FAB subtypes and are powerful determinants of therapy response (**Table 1 and 2**). However, we have only started to understand the mutational heterogeneity of AML within the last 10 years. The first AML genome was sequenced in 2008 by the Washington University group, and it was interestingly, the first whole cancer genome sequenced.[163] In 2013, The Cancer Genome Atlas (TCGA) research network sequenced 200 clinically annotated AML samples along with RNA- and DNA methylation sequencing.[25] Based on these data, the most common genes mutated in AML involve genes that are associated with DNA methylation (*DNMT3A* 26%, *IDH1/2* 20%, *TET2* 9%) and activated signaling (*FLT3*-ITD/TKD 28%, *NRAS*/*KRAS* 14%). Also, nucleophosmin (*NPM1*) was reported to be mutated in 27% of cases. The most recent sequencing study comprised of 1540 AML patients confirmed these findings and additionally demonstrated that the co-occurrence of the mutations can have a prognostic impact in overall survival.[164] Thus, the future interest and challenge will be to define prognostic patient subgroups by taking into account the whole spectrum of genetic lesions of an individual. As a pilot approach, an AML multistage prediction tool was developed (https://cancer.sanger.ac.uk/aml-multistage/) that predicts the disease outcome by taking into account several different parameters.[165]

1.5.2 Treatment and prognosis

Induction therapy for AML has remained unchanged for the last 45 years and the current 5-year OS is 27.5%[166] but is significantly higher in pediatric patients, 76%.[167] The prognosis of adult patients is strongly associated with genetic lesions and the current risk classification of the European LeukemiaNet (ELN), which is based on the WHO classification, divides patients into three risk groups (favorable, intermediate and adverse, **Table 2**).[168] Standard treatment involves 3 days of an anthracycline (idarubicin or daunorubicin) and 7 days of cytarabine aiming for complete remission.[169] If complete remission is reached the patients belonging to

intermediate or adverse risk groups should receive an allogeneic hematopoietic stem cell transplantation (HSCT).[170] This intensive therapy results in 30-40% curative rates in adult patients under 60 years.[171] However, older patients who are usually unfit for intensive chemotherapy are treated with hypomethylating agents such decitabine, azacitidine or low dose cytarabine. These treatments are rarely curative and only 5-15% of patients over 60 years are cured.[171] Moreover, prognosis for relapsed or chemorefractory patients remains dismal.

Table 2. Risk stratification by molecular profiling

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD or with FLT3-ITDlow
	Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITDhigh
	Wild-type NPM1 without FLT3-ITD or with FLT3-ITDlow† (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t(9;22)(q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype,§ monosomal karyotype
	Wild-type NPM1 and FLT3-ITDhigh
	Mutated RUNX1¶
	Mutated ASXL1¶
	Mutated TP53

§ Three or more chromosome abnormalities but not including recurrent translocations

¶ These markers should not be used if they co-occur with favorable risk AML subtypes

The improvement in AML survival can mainly be attributed to supportive care such as blood transfusion and better treatment of infections.[172] Hematologists are well aware that a plateau has been reached with the current treatments and novel therapies are urgently needed. Since the 7+3 combination of cytarabine and daunorubicin in 1973,[173,174] only a few hypomethylating agents have been approved for AML treatment.[175] However, in 2017, the AML community was finally rewarded with the approval of four new drugs. The drugs that were approved in 2017 involve a FLT3 inhibitor (midostaurin[176]), an IDH2 inhibitor (enasidenib[177]), a CD33 antibody toxin conjugate (gemtuzumab ozagamicin[178]) and a nanoscale liposomal particle containing daunorubcin and cytarabine in a synergistic 5:1 molar ratio (CPX-35[179]). While none of these drugs show striking curative effects, the new approvals take AML treatment forward. Moreover, combinations with BCL2 inhibitor venetoclax

plus hypomethylating agents have shown promising results in phase I/II trials with 50% 1-year survival rates in patients over 65 years.[104] Importantly, the current risk classification is based on the anthracycline + cytarabine + allogeneic stem cell transplantation treatment, and it is unclear how classification works with the novel agents. Thus, the future challenge is to find the right patients for the right therapies.

Table 3. Overview of leukemia subtype characteristics studied in this thesis

	Large granular lymphocyte leukemia (LGL leukemia)	T-cell acute lymphoblastic leukemia (T-ALL)	Acute myeloid leukemia (AML)
Incidence	0.2-0.7 cases per 1 million individuals per year, ref. 180	0.13 cases per 100 000 individual per year, ref. 182	4.3 cases per 100 000 individual per year, ref. 166, 211 new cases in Finland 2015, ref. 133
Median age at diagnosis	66.5 years, ref. 180	35 years, ref. 182	68 years, ref. 181
Cell of origin	Mature (post-thymic) lymphocytes T-cell phenotype (87%, CD3+CD8+) NK cell phenotype (13%, CD3-CD56+) Rare phenotypes: CD4+ T-cells, CD3+CD56+ T-cells, ref. 180	Lymphoid lineage progenitor cells early T-lineage progenitors (ETP,CD4-CD8-), early cortical thymocytes (CD1a+CD4+CD8+), late cortical thymocytes (CD4+CD8+CD3+), ref. 183	Myeloid lineage progenitor cells Immunophenotype: CD117 (94% of cases), CD33 (81%), CD34 (62%). ref. 25
Survival time	Median overall survival 9-10 years, ref. 180	5-year survival rate 44.2% ref. 182	5-year survival rate 27.4% ref. 166 In pediatric patients 76%, ref. 167
Clinical manifestations	Cytopenias, neutropenia (50%), splenomegaly (25%) autoimmune disorders (15-40%), rheumatoid arthritis (11-36%), ref. 180	Neutropenia, anemia, thrombocytopenia, mediastinal thymic masses, meningeal infiltration of leukemic cells, ref. 134	Excess bruising and bleeding, recurrent infections, anemia, cytopenias, neutropenia, fewer ref. 181
Major molecular drivers	STAT3 mutation (40-70%) STAT5B mutation (2%), ref. 180	NOTCH1 (60%), CDKN2A/CDKN2B (70%), RUNX1 (15%), TLX1/TLX3 (30%) IL7R/JAK1/JAK3 (30%), PHF6 (30%), ref. 183	Chromosomal translocations: PML-RARA (10%), RUNX1-RUNX1T1 (3.5%), CBP-MYH (6%) and MLL-X (5.5%) Mutations: FLT3 (28%), NPM1 (27%), NRAS/KRAS (12%), RUNX1 (10%), IDH1/IDH2 (20%), TP53 (8%), ref. 25
Treatment	Methotrexate, cyclophosphamide, ciclosporin A, ref. 180	Hyper-CVAD (cyclophosphamide, vincristine, Adriamycin, and dexamethasone), nelarabine, allogeneic hematopoietic stem cell transplantation, ref. 134	"7+3" regimen cytarabine + anthracycline (daunorubicin, idarubicin), allogeneic hematopoietic stem cell transplantation, patients unfit for intensive chemotherapy: azacitidine, low dose cytarabine, decitabine ref. 181
Emerging therapies	Purine analogs (fludarabine, cladribine), JAK inhibitors (tofacitinib), multicytokine inhbitor (BNZ-1: IL-2, IL-9, IL-15), CD52 antibody (alemtuzumab) ref. 180	NOTCH1 inhibitors, BCL2 inhibitor (venetoclax), JAK inhibitors (Tofacitinib), ref. 134	FLT3 inhibitors (midostaurin, quizartinib), IDH1/IDH2 inhibitors (ivosidenib, enasidenib), CD33 antibody-drug conjugate (gemtuzumab ozogamicin), CPX-351 (liposomal cytarabine+daunorubicin), BCL2 inhibitor (venetoclax) ref. 181

*Used references[25,133,134,166,167,180–183]

1.6 Methodologies to identify new treatment strategies

1.6.1 Next generation sequencing

Sanger sequencing, developed in 1977, was used for decades as a gold standard to analyze PCR amplified 100-800 base pair (bp) long DNA fragments.[184] Sequencing of the first human genome was accomplished in 2003[185] with the Sanger method and it was estimated to cost 3 billion dollars.[186] In the last decade, the technological

advances have revolutionized the speed and cost of genetic sequencing. Today, the human genome can be sequenced with less than 1,000 US dollars. Next generation sequencing (NGS) techniques produce thousands or millions of DNA sequence reads and can cover the whole genome. The three main NGS applications in human genomics involve whole-genome sequencing (WGS), whole exome sequencing (WES) and targeted sequencing panels.

Protein coding genes constitute only approximately 1% of the genome but up to 85% of disease causing mutations are estimated to reside within the exome.[187] Therefore, exome sequencing provides relevant information but with a lower price and with decreased computational analysis requirements compared to WGS. Targeted sequencing panels usually contain 10-500 target gene regions, which have been selected for a specific study question. The advantage of this method is the relatively low cost and processing time. Targeted sequencing panels have become commonly used in clinical practice as the majority of cancer driver mutations have been identified. To identify mutations with very low variant allele frequency (<0.05%), targeted amplicon sequencing can be used.[184] The ultra-deep sequencing of PCR products allows the researcher to sequence 16-1,536 targets in the same run producing 150-1500 bp long reads.

Powerful sequencing techniques have produced huge amounts of information from cancer genomes. The TCGA project, which began in 2006, is a big coordinated effort to identify genetic mutations in cancer by using large-scale genome sequencing. Currently, the database covers more than 11,000 tumor samples from 33 cancer types.[188] Taken together, the sequencing era has provided large amounts of information and insight into the heterogeneity of cancer, and accelerated the development of targeted therapies and precision medicine. However, the functional effect and correlation between drugs and mutations cannot be evaluated solely based on sequencing studies.

1.6.2 Ex vivo drug sensitivity and resistance testing

The idea to test a patient's cancer cells *in vitro* to guide treatment decisions is an attractive concept. Similar testing is routinely used to characterize infecting bacteria and response to antibiotics. From the late 1970s, several studies investigated the

correlation between *ex vivo* efficacy of chemotherapeutics and clinical outcome with varying results.[189–191] While some studies showed good predictive accuracy none of the approaches have yet translated into common clinical practice.[192,193]

Due to the development of new targeted compounds and technological advances, the approach has become relevant again in leukemia research. In 2013, Tyner et al. described a high-throughput drug-testing platform in which they tested 151 leukemia patient samples to a panel of 66 small molecule inhibitors.[194] Enriched mononuclear cells of BM samples were seeded on 96-well plates and after 3-day drug treatment cell viability was measured with the MTS reagent. Results demonstrated that individual leukemic samples have specific drug response profiles and up to 70% of the samples exhibited good sensitivity to a specific targeted kinase inhibitor. This approach led to an ongoing clinical trial for relapsed AML patients (NCT01620216). A similar study was published by the personalized medicine cancer group at the Institute for Molecular Medicine Finland and the Hematology department at Helsinki University Hospital in 2013.[195] The drug sensitivity results were used to guide treatment decisions of eight relapsed/refractory patients and included drugs such as trametinib, sunitinib, ruxolitinib, sorafenib, clofarabine and vinblastine. According to ELN criteria, 3 out of 8 patients had a response to *ex vivo* guided therapy. Additionally, the study correlated mutational profiles of 26 AML patients to *ex vivo* drug responses and demonstrated that *FLT3* and *RAS* mutations are associated with increased sensitivity to FLT3 and MEK inhibitors, respectively. The data suggests that *ex vivo* drug-screening platforms can also be useful to identify possible biomarkers when combined with sequencing data and which could be further validated in a clinical setting. Similarly, recent studies have identified significant and novel associations between mutation status and *ex vivo* drug response in other hematological malignancies such as T-cell prolymphocytic leukemia (T-PLL)[196], multiple myeloma[197] and CLL.[198]

Most of the current platforms measure the overall BM sample sensitivity using reagents that measure cell proliferation and/or cell death.[195,198] However, these methods cannot measure cell differentiation and cannot take into account sample heterogeneity. In AML, the BM is often comprised of blast cells and more mature leukemic and healthy cells. An effective drug should target the immature blast cells which presumably have self-renewing capacity. In contrast, targeting only the more

mature leukemic or healthy cells will not result in good treatment outcome. To overcome these limitations immunofluorescence microscopy and flow cytometry-based assays have been implemented in drug sensitivity testing. In 2017, Superti-Furga and colleagues used an automated immunofluorescence microscopy-based platform to test blast specific drug responses.[199] First, they retrospectively evaluated whether pharmascopy is predictive of clinical outcomes. Amongst the 20 tested AML samples, ten had achieved complete remission and ten were non-responders to induction therapy. Interestingly, results showed that blast specific responses (predictive accuracy 78.1%) and relative blast specific responses (88.1%) were good predictors of clinical outcome, whereas overall BM sensitivity was less predictive (68.5%). The study also used the pharmascopy platform to guide treatment decisions of relapsed/refractory patients with varying hematological malignancies. In their interim analysis, 15 of the 17 patients receiving guided treatment had on overall response rate of 88% compared to 24% response rate in patient cohort treated according to physician recommendation. Similarly, a high-throughput flow cytometry-based approach has been used to evaluate blast specific responses and differentiation by commercial companies, such as Notable Labs and ViviaBiotech.[200–202] However, none of the companies have thus far published their results for clinical correlation. While the recent drug testing studies have been promising, the direct translation of *ex vivo* sensitivity to clinical outcome remains controversial.

2 AIMS OF THE STUDY

The overall aim of this study was to identify novel cell signaling associated mutations in LGL-leukemia and T-ALL. By screening patient derived leukemic cells and cell lines with a high-throughput drug screening platform we further aimed to assess whether the identified genetic abnormalities and/or phenotypes are linked to drug sensitivity.

Specifically, the aims were:

1. To identify pathogenic mutations in *STAT3* negative LGL leukemia patients.

2. To determine the prevalence of *STAT5A, STAT5B* and *STAT3* mutations in T-cell acute lymphoblastic leukemia paired with *ex vivo* drug sensitivity testing.

3. To identify compounds that can inhibit the cellular function of mutant STAT3 in lymphoproliferative disorders using high-throughput drug sensitivity testing.

4. To develop a high-content flow-cytometry based drug testing platform to identify drugs specifically targeting leukemic blasts in acute myeloid leukemia.

3 MATERIALS AND METHODS

3.1 Patient material and cell lines

3.1.1 Patient samples (I-IV)

Bone marrow (BM) or peripheral blood (PB) samples were collected from LGL leukemia, T-ALL and AML patients and healthy controls. The studies were approved by the Helsinki University Hospital ethics committee (permit numbers 239/13/03/00/2010 and 303/13/03/01/2011), the National Supervisory Authority for Welfare and Heath, the Cleveland Clinic (Cleveland, Ohio) and the Penn State Hershey Cancer Institute (Hershey, Pennsylvania) in compliance with the Declaration of Helsinki. All patients and healthy control donors gave written informed consent.

Study I patient cohort consisted of 211 patients with LGL leukemia: 173 patients had T-LGL and 38 patients NK-LGL leukemia. Twenty-three samples were from Finland, 87 from the Cleveland Clinic and 101 samples from the Penn State Hershey Cancer Institute. Two Finnish patients were exome sequenced and only *STAT3/STAT5A/STAT5B* genes were sequenced for the other samples. In study III, 8 LGL leukemia samples were obtained from Finland and used for drug sensitivity testing.

In study II, the prevalence of *STAT3/STAT5A/STAT5B* mutations were analyzed in T-ALL by sequencing 64 adult and 4 pediatric patients. Exome sequencing and drug screening was performed to our index patient and *STAT3/STAT5A/STAT5B* targeted sequencing for other patients.

In study IV, drug sensitivity testing and exome sequencing was performed for 32 AML BM samples obtained at diagnosis, relapse or refractory stage. Thirty samples were from Finland and two samples from Haukeland University Hospital, Bergen, Norway.

3.1.2 Sample processing and cell separation (I-IV)

Mononuclear cells (MNCs) were isolated from fresh BM or PB within using Ficoll gradient centrifugation (GE Healthcare). In studies I and III, CD4+ and CD8+ T cells were enriched from MNCs with magnetic beads (Miltenyi Biotech) according to the manufacturer's instructions and enriched with an AutoMACS cell sorter (Miltenyi Biotech). In study II, CD3+ T-cells were enriched using the Easy Sep Human CD3 Positive Selection kit (StemCell Technologies). Enriched CD4+ and CD3+ cells were used as controls in sequencing and gene expression studies and CD8+ LGL cells were used in drug screening assays in study III.

LGL, T-ALL and AML samples were cultured in mononuclear cell media (MCM, Promocell), which is a rich media for short-term maintenance of MNCs. The medium was supplemented with 10 µg/ml gentamicin and 2.5 µg/ml amphotericin B. If indicated, IL-2 and IL-15 were added to the culturing medium of CD8+ LGL cells in study III.

3.1.3 Cell lines (I-III)

In studies I and II, HeLa cells were used to assess the functional effects of *STAT5B* mutations. In study III, HEK293 cells containing a STAT3-specific luciferase reporter (HEK293-SIE; Promega) was used to study the effect of compounds on STAT3 activity. Both cell lines were cultured in high glucose DMEM (Gibco) containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium). In study III, seven NK cell lines were used in the drug screening studies. KHYG-1, YT and NK-92 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). KAI3, NK-YS and SNK6 were provided by Dr. John Chan (City of Hope Medical Center) and NKL from Dr. Thomas P. Loughran (University of Virginia). All NK cell lines were cultured in complete RPMI medium (Gibco) supplemented with 2.5 ng/ml recombinant human IL-2.

3.2 DNA and RNA experiments

3.2.1 RNA and DNA extraction (I, II, IV)

DNA was extracted from the MNC fraction and from the enriched T-cell populations (CD4+ or CD8+ or CD3+) with the Genomic DNA Nucleospin Tissue kit (Macherey-Nagel) or DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's instructions. Total RNA was extracted with Nucleospin RNA II kit (Macherey-Nagel) or miRNAeasy Mini Kit (Qiagen). RNA and DNA concentrations were measured with the Qubit 2.0 fluorometer (Life Technologies) and RNA quality with the Agilent 2100 Bioanalyzer (Agilent Technologies).

3.2.2 Exome sequencing (I, II, IV)

DNA libraries were prepared from 3 µg of DNA as earlier described [58]. Exome capture was performed with the Agilent SureSelect v5 Exome, Agilent SureSelect XT clinical research exome (Agilent) or Nimblegen SeqCap EZ v2 capture kits (Roche NimbleGen) and sequenced using the Genome Analyzer II or HiSeq 2500 instruments (Illumina). Following sequencing, the processed and filtered reads were aligned to the GRCh37 human reference genome. Paired samples were used to detect somatic mutations. In study I, CD4+ cell DNA was used as germline control for LGL leukemia samples. In study II, skin DNA was used as germline control for T-ALL samples. Somatic mutations were called with the VarScan2 algorithm.[203] Public variants were filtered out by using dbSNP database version 130 (National Center for Biotechnology Information). Candidate somatic mutations were visually validated with Integrative Genomic Viewer (IGV, Broad Institute, Cambridge, USA).

3.2.3 Amplicon (I, II) and capillary sequencing (I-III)

The prevalence of *STAT5A, STAT5B* and *STAT3* mutations was evaluated with amplicon sequencing. The exon regions were amplified from purified DNA by PCR reaction with the use of primers carrying Illumina compatible adapter sequences (the primer sequences are presented in the supplementary materials of original studies I and II). The PCR reaction was carried out in a volume of 20 µl containing 10 ng of sample DNA, 10 µl of 2x Phusion High-Fidelity PCR Master Mix (Thermo Scientific), 0.05 µM of exon specific primers and 0.5 µM of an adapter primer

carrying Illumina grafting P7 sequence. PCR amplicons were sequenced using Illumina MiSeq instrument with MiSeq Control Software v2.2.29 (Illumina). Sequencing data was processed with an in-house amplicon pipeline. *STAT5B* mutations of the index T-ALL patient were validated with capillary sequencing.

3.2.4 Gene expression analysis (I, IV)

In study I, the gene expression profiles of three *STAT5B* mutated CD8+ LGL samples were compared to 2 *STAT3* mutated, 2 *STAT3/STAT5B* mutation negative and 4 healthy control samples. Illumina Human HT-12 v4 BeadChip expression array was used to measure expression values. The microarrays were red using iScan instrument (Illumina) and analysis was performed with Genome Studio software (Illumina). Normalization and log2 transformation were done with Chipster software (version 2.10). The microarray data is available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1611.

In study IV, public RNA-sequence data from TCGA (http://cancergenome.nih.gov/) was used to measure the expression levels of BCL2 family members in different AML FAB subtypes. To compare the expression levels of *BCL2, BCL2L1* (*BCL-xL*) and *MCL1* between different FAB subtypes, fold change and two-tailed Wilcoxon test followed by Benjamini Hochberg adjustment of P-values was calculated.

3.2.5 RT-qPCR (II)

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to measure the expression levels of *BCL2*, *BCL-xL*, *BCL-xS* and *MCL1*. Total RNA was extracted from the CD3-enriched cells of healthy donors and from BM MNCs of the index T-ALL patient and two T-ALL controls. 224 ng of total RNA was transcribed to cDNA using SuperScript III reverse transcriptase and random primers (Life Technologies) in a 20 µl reaction. qPCR reactions were performed using iQ SYBR Green Supermix (Bio Rad). Expression levels were quantified based on the Pfaffl method[204] and POLR1B and GUSB were used as reference genes to normalize the data. The primer sequences are listed in the supplementary materials of the original study II.

3.3 Functional studies

3.3.1 STAT5B and STAT3 expression constructs (I-III)

STAT5B expression plasmids were generated to evaluate the functional effect of *STAT5B* mutations detected in LGL leukemia and T-ALL. An expression plasmid (pCMV6-XL6) containing the WT coding sequence of *STAT5B* was obtained from Origene. The point mutations (N642H, T648S, D661V, I704L) were introduced into the coding sequence using the GENEART Site-Directed Mutagenesis System (Life Technologies) according to the manufacturer's protocol. Following the production of the single mutation constructs, the double (N642H+T648S, N642H+I704L, T648S+I704L), and triple (N642H+T648S+I704L) mutation constructs were generated. The mutations were confirmed with capillary sequencing. The primer sequences used for the mutagenesis and capillary sequencing are presented in the supplementary materials of the original study II.

STAT3 expression plasmids were generated to identify compounds with the ability to inhibit STAT3 activity. An ORFeome clone of the human *STAT3* isoform 2 coding sequence (NM_003150) in pENTR201 plasmid was obtained from the Genome Biology Unit at the University of Helsinki. D661V and Y640F mutants were generated with PCR site-directed mutagenesis using 5'-phosphorylated primers (Eurofins MWG|operon). Mutations were validated by capillary sequencing, and the coding sequence of the wild type and two mutant *STAT3* were moved from the pENTR vector to a pDEST-40 expression plasmid using Gateway® LR Clonase II enzyme mix (Life Technologies).

3.3.2 Western blot assay and ELISA (I-III)

Total cell lysates were lysed in RIPA buffer (0.1% SDS, 150mM NaCl, Tris 50mM, 1% Triton-X100, 0.5% sodium deoxycholate) supplemented with 1mM sodium orthovanadate and Roche's Complete Protease Inhibitor Cocktail Tablet. Lysates were sonicated 4x1s, protein concentrations measured using the Qubit™ Fluorometer (Life Technologies) after which 20-50 µg of protein was loaded on 10%, 12% or 4-12% concentration gradient SDS-PAGE gels (Bio-Rad). In study I, cytosolic and nuclear fractions of protein lysates of LGL leukemia samples were separated as earlier described.[205] After electrophoresis (100V) the proteins were

transferred to nitrocellulose membranes (Bio-Rad) and blocked with 5% bovine serum albumin (BSA) for 1 h. Primary antibodies were obtained from Cell Signaling Technology; anti-STAT3 cat. 2972, anti-pSTAT3 Y705 cat. 9131, anti-pSTAT3 S722 cat. 9134, anti-STAT5 cat. 9363, anti-pSTAT5 Y699 cat. 9359, anti-AKT cat. 2920, anti-AKT S473 cat. 4058 and loading control antibodies from Sigma-Aldrich; anti-β-actin cat. 20-33 and anti-α-Tubulin cat. T9026. Primary antibodies were diluted in tris-buffered saline and 0.05% Tween 20 (TBS-T) + 5% BSA in dilution range 1:500-1,000 and incubated with the membranes for 1 h or O/N at room temperature (RT). Secondary infrared antibodies (IRDye 800CW and IRdye680LT from LI-COR Biosciences were diluted 1:15,000 in TBS-T + 5% BSA, and incubated with the membranes for 1 h at room temperature (RT). Membranes were visualized with the Odyssey imaging system (LI-COR Biosciences) and band intensities were measured using its application software version 3.0.

In study I, pSTAT5 protein levels were also measured with enzyme-linked immunosorbent assay (ELISA) analysis using the PhosphoTracer STAT5A/B pTyr693/699 + Total ELISA kit (Abcam) according to manufacturer's instructions. All samples were analyzed as duplicates and the signal intensity was measured using the PHERAstar FS reader (BMG Labtech).

3.3.3 STAT5B luciferase reporter assay (I-II)

In study I and II, HeLa cells were transfected in 6-well plates with STAT5B constructs (6 µg) and luciferase reporter plasmid pGL4.52 (1 µg, Luc2P/STAT5RE/Hygro, Promega) using Fugene HD transfection reagent (Promega) in a 3.5:1 Fugene HD:DNA ratio. After 24 h incubation at 37°C, HeLa cells were detached with trypsin and moved onto 96-well plates at a cell density of 10,000 cells/well. The remaining cells were used for Western blot assays. Luciferase activity was measured using One-Glo luciferase detection reagent (Promega) and PHERAstar FS plate reader. The assay was repeated 3 times and the mean fold change in transcriptional activity induced by the different mutations was compared to WT STAT5B induced activity.

3.3.4 Gene silencing experiments (III)

HEK293-SIE cells stably expressing WT or Y640F STAT3 were plated on 384-well plates in complete DMEM medium with cell density of 2,000 cells/well. The plates

were pre-prepared to contain siRNAs and Lipofectamine RNAiMAX (Life Technologies; Thermo Fisher) resulting in a 10 nM siRNA final concentration. siRNAs were obtained from the Ambion® siRNA library and three different siRNAs was used for each gene (sequences presented in the supplementary materials of the original study III). After 72 h the luciferase signal was measured. WT STAT3 containing cells were induced with IL6 (100 ng/ml) 6 h prior reading. Luciferase activity was normalized against cell viability, which was measured from the same plates with CellTiter-Fluor (CTF, Promega) reagent 3 h before reading the luciferase signal. The inhibitory effect was determined relative to the mock siRNA treated cells and both cell lines were normalized to their own controls.

3.4 Assessment of drug potency

3.4.1 Drug potency assessment (II-IV)

The oncology compound collection consisted of FDA/EMA approved anti-cancer drugs and several investigational or pre-clinical compounds. Drug plates were prepared to contain different sets of compounds (up to 306 compounds) that varied depending on the study. The compounds were dissolved in 100% DMSO and dispensed on 384-well plates (Corning) using an acoustic liquid handling device (Echo 550, Labcyte Inc). All compounds were tested in 5-8 different concentrations covering a 1,000-10,000-fold concentration range. Plates contained several DMSO and 100 μM benzethonium chloride containing wells to which the drug response was normalized. In study II and IV, 10,000 MNCs from T-ALL or AML patients were plated on pre-drugged 384-well plates in MCM media. Following a 3-day incubation, the cell viability was measured using CellTiter-Glo luminescent reagent (CTG) and PHERAstar plate reader. Similarly, in study III the viability of the NK cell lines and enriched CD8+ LGL leukemia cells were measured with CTG after 3 days.

In order to compare the drug potencies of several samples and compounds we developed a drug sensitivity score (DSS) as a single measure.[206] DSS is a quantitative drug response metric, which is based on area under the curve (AUC). Furthermore, it captures both the potency and efficacy of the drug by integrating all four curve-fitting parameters: inhibitory concentration (IC_{50}), slope and minimum and

maximum asymptotes. The DSS score ranges from 0 to 50 with 50 being the maximum inhibitory effect with all tested concentrations.

3.4.2 Flow cytometry-based cell viability assay (IV)

Primary AML MNCs were plated on 96-well polystyrene V-bottom drug plates (Thermo Fisher Scientific) at 100,000 cells/well in MCM medium. The plates contained several DMSO controls, 7 drugs (1,000-10,000-fold dilution series) and 27 drug combinations. Following a 3-day incubation, cells were centrifuged (500g, 6 min) and the supernatant was removed by turning the plates upside down. Cell pellets were suspended in 25 µL of antibody master mix containing the following antibodies: BD Biosciences: CD33 (BV421, clone WM53), CD45 (BV786, clone HI30), CD34 (PE-Cy7, clone 8G12), CD14 (APC, clone M5E2) and Cytognos: CD38 (FITC, clone LD38) and mixed in staining buffer (10% FBS and 0.02% NaN3 in RPMI-1640 medium). After 30 min incubation at RT, the cells were washed by adding 100 µL staining buffer followed by centrifugation and supernatant removal. Dead and apoptotic cells were stained with 7-aminoactinomycin D (7-AAD) and PE-Annexin V (BD Biosciences) in 1:50 dilution each in 25 µL Annexin V binding buffer. The plates were incubated for 20 min at RT before FC analysis. Following the incubation with the cell viability dyes, the FC analysis was performed using the iQue Screener PLUS instrument (IntelliCyt). All media/cells were sipped from each well and population counts and cell gating was performed with ForeCyte software 3.0 (IntelliCyt). Analysis was done from viable CD45 positive singlet cells and the gating strategy is illustrated in the supplementary materials of the original study IV.

3.5 Statistical analysis

Differences between drug responses or gene expressions were analyzed with Mann-Whitney U-test. For multiple t-tests p-values were adjusted using the Benjamin-Hochberg method. Statistical dependencies between two variables were assessed with Spearmen rank correlation. Statistical analyses were performed with Prism software version 6.0 or 7.0 (GraphPad software).

4 RESULTS

4.1 Identification of novel *STAT5B* mutations in LGL leukemia

In our earlier study we discovered that 40% of LGL leukemia patients had a somatic gain-of-function mutation in the *STAT3* gene.[58] However, based on the microarray analysis performed, STAT3 target genes were upregulated in the majority of LGL samples, irrespective of the mutational status. Accordingly, Epling-Burnette et al. showed already in 2001 that in their patient cohort STAT3 was phosphorylated in all of the 19 LGL samples, which presumably involved cases with and without *STAT3* mutations.[124] In **study I** we aimed to identify other candidate genes that could lead to overactive JAK/STAT signaling by using exome sequencing.

4.1.1 STAT5B mutations are rare but recurrent in LGL leukemia

We performed exome sequencing of two STAT3 mutation negative patients (**Table 4**, patient 1 and 2). Both patients had a large clone of CD8+ CD3+ CD56+ T-cells (over 90% of total CD8+ cell fraction) based on T-cell receptor (TCR) Vβ analysis.

Table 4. Characteristics of patients with STAT5B mutations

No.	Phenotype	Mutation	Sex	Age	Lymphocyte count at diagnosis, 10^9/L	CD8+ Vβ*	Other disorders	Therapy¥
1	CD3+CD8+CD56+ T-cell	STAT5B Y665F	F	71	7.0	Vb22, 91%	None	No
2	CD3+CD8+CD56+ T-cell	STAT5B Y665F	F	49	12.9	Vb17, 94%	None	No
3	CD3+CD8+CD56+ T-cell	STAT5B N642H	M	74	85.5	Vb21, 91%	Pancytopenia, neutropenia, splenomegaly	Yes
4	CD3-CD56+ NK-cell	STAT5B N642H	M	75	131.8	Vb7, 27% Vb3, 16%	Hemolytic anemia, neutropenia, splenomegaly, MGUS	Yes

M=Male, F=Female
MGUS=Monoclonal gammapathy of unknown significance
*Proportion of total T-cell fraction or CD8+ cell fraction
¥Pharmacologic treatment at any time point

In patient 1, exome sequencing analysis revealed a somatic mutation in bone morphogenic protein receptor type II (BMPR2), and in patient 2 we detected somatic mutations in early B-cell factor 3 (EBF3) and sphingomyelin synthase 2 (SGMS2) (**Table 5**). Strikingly, both patients harbored an identical missense mutation in *STAT5B* that results in an amino acid change at the tyrosine 665 residue (Y665F)

(**Table 5**). The *STAT5B* mutation was located in the SH2 domain (exon 16), which is involved in the dimerization of STAT5B protein (**Figure 5**). The observed mutations were not present in the CD4+ cells derived from the same patients suggesting that mutations occurred at a late stage of lymphocyte differentiation and are specific to the CD8+ LGL cell clone.

Table 5. Mutations identified with exome sequencing from patients 1 and 2

No.	Gene	Chromosome	Position	Mutation type	Amino acid change	CD8+ VAF	CD4+ VAF	Somatic mutation P-value
1	STAT5B	17	40359659	Missense, A->T	Y665F	41%	0%	9.34x10^-14
	BMPR2	2	203379664	Missense, A->G	E195K	52%	0.9%	7.10x10^-19
2	STAT5B	17	40359659	Missense, A->T	Y665F	52%	0%	5.98x10^-14
	SGMS2	4	108820744	Missense, T->C	R157C	42%	0%	1.20x10^-32
	EBF3	10	131761204	Splice-5	NA	36%	0%	4.42^10-13

C=cysteine, E=glutamic acid, F=phenylalanine, K=lysine, NA=not applicable, R=arginine, Y=tyrosine

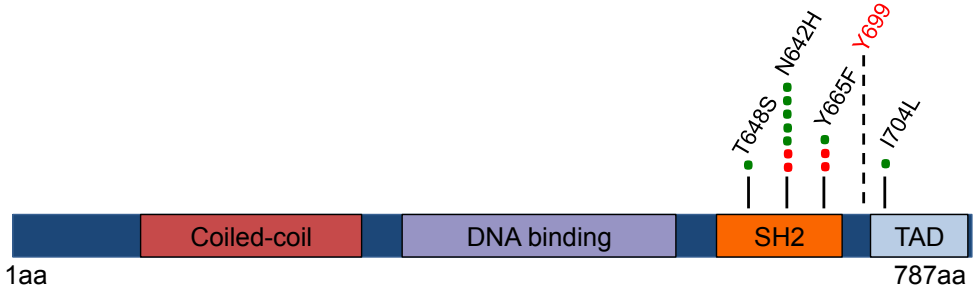

Figure 5. Linear representation of the STAT5B protein structure. Red dots represent mutations observed in LGL leukemia and green dots mutations observed in T-ALL (study II). Tyrosine phosphorylation site (Y699) needed for protein dimerization is marked with red text.

To evaluate the prevalence of STAT5B mutations in a larger patient cohort, we screened an additional 209 LGL leukemia patients, 171 with T-cell phenotype and 38 with NK cell phenotype. We hypothesized that *STAT5B* mutations are enriched in the SH2 domain and its adjacent regions, as shown earlier with *STAT3* mutations [58]. Thus, we performed capillary and amplicon sequencing covering the SH2 domain and the adjacent exons (exons 14-18). As STAT5A and STAT5B have similar protein structures and they can form heterodimers with each other, we also sequenced the SH2 domain of *STAT5A* by capillary sequencing (exons 15-19). The results revealed *STAT5B* mutations (N642H) in two additional patients (**Table 4,** patients 3 and 4). Interestingly, all four patients with *STAT5B* mutations expressed

CD56+ (three cases with T-cell phenotype and one case with NK-cell phenotype) and both patients with the N642H mutation had a rare and aggressive form of the disease manifested with high lymphocyte counts and severe disease related symptoms (**Table 4**). No *STAT5A* mutations were identified in the patient cohort and altogether the prevalence of the *STAT5B* mutations was 2% (4 of 211).

4.1.2 Patients with STAT5B mutations

STAT5B acts in the nucleus, where the phosphorylated and dimerized STAT5B binds to various promoter regions altering gene expression. To study the gene expression profile of the mutated samples we performed microarray analysis of 3 samples with *STAT5B* mutations, 5 samples without *STAT5B* mutations, 2 healthy CD8+ controls and 2 healthy NK cell controls. Interestingly, antiapoptotic *BCL-xL* and *MAPK1*, a major regulator of cell proliferation, were amongst the top 60 overexpressed genes in mutant STAT5B cells when compared to healthy CD8+ cells (**Figure 6A**). Intriguingly, *BCL-xL* is a known STAT5B target gene.[207] However, its expression was also upregulated in LGL samples without STAT5B mutations (**Figure 6A**). These results suggest that high *BCL-xL* expression is more related to LGL leukemia phenotype than to *STAT5B* mutation per se. Western blot analysis showed increased phosphorylation and nuclear localization of STAT5B in the two Y665F mutated patients (**Figure 6B**). This finding was further confirmed with ELISA, which showed high pSTAT5 levels in the same mutated samples. (**Figure 6C-D**). Unfortunately, cell lysates from patients with N642H mutations were not available.

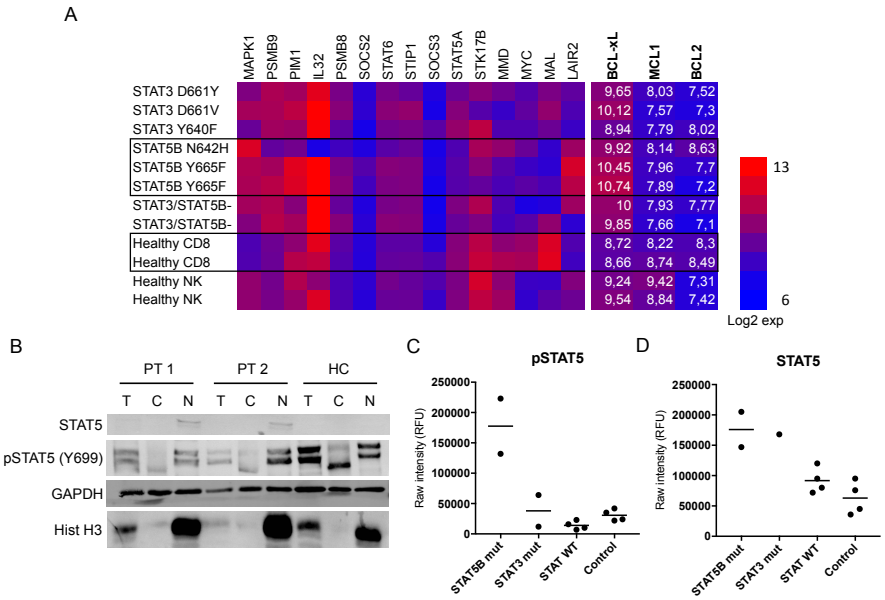

Figure 6. STAT5B is localized in the nucleus of patient samples carrying Y665F mutation A) Gene expression levels of selected genes that were highly up- or downregulated in *STAT5B* mutated cells vs. healthy CD8+ cells. Expression of anti-apoptotic BCL2 family members is also presented. Values are in log2 scale. B) Fresh mononuclear cells of PT1, PT2 and one healthy control were fractionated to nuclear (N), cytosolic (C) and total cell lysates. pSTAT5, STAT5, GAPDH (loading control) and Hist H3 (nuclear localization control) antibodies were used to quantify protein levels. C-D) pSTAT5 and STAT5 levels of 2 patients with *STAT5B* Y665F, 2 patients with STAT3 mutation (Y640F or D661V), 4 patients negative for *STAT3/STAT5* mutations and 4 healthy controls were measured with ELISA. Fresh PB MNCs were used in the analysis.

4.1.3 STAT5B mutations result in increased transcription

Next, we studied whether *STAT5B* mutations lead to increased transcriptional activity in a controlled laboratory setting. STAT5B expression constructs were generated and transfected to HeLa cells together with a STAT5-specific luciferase reporter construct. The N642H mutation increased the luciferase expression over 18-fold and the Y665F mutation over 5-fold when compared to WT STAT5B (**Figure 7A**). Accordingly, N642H and D661V STAT5B showed increased phosphorylation in western blot analysis (**Figure 7B-C**). In conclusion, our results demonstrate that *STAT5B* mutations are rare but recurrent in LGL leukemia and they increase the functional activity of STAT5B.

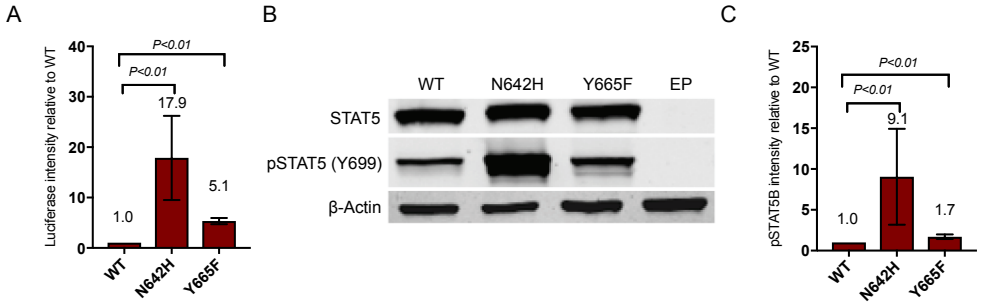

Figure 7. N642H and Y665F increase the transcriptional activity of STAT5B A) HeLa cells were transfected with WT, N642H or Y665F STAT5B expression plasmids together with STAT5B inducible luciferase reporter plasmid. After 24 h the cells were starved for 6 h in 0.2% FBS containing media after which the luciferase activity was measured. B) In parallel, the pSTAT5 and STAT5 levels were measured with western blot analysis from the same bulk transfected cells and C) band intensities were quantified with (Odyssey imaging system; Li-COR Biosciences). All experiments were repeated 3 times and error bars represent the ±SD. P-values were calculated using Mann-Whitney U-test

4.2 *STAT5B* mutations as putative drivers in T-ALL

In **study II**, we aimed to identify novel mutations associated with cell survival pathways in T-ALL. Our index patient was an 18 years old female diagnosed with T-ALL and who relapsed 40 weeks later after allogeneic HSCT. We performed exome- and RNA-sequencing for both diagnosis and relapse samples. To identify somatic mutations, a skin sample was used as a germline control. By exome sequencing mutations were detected in *NOTCH1*, *SUZ12*, *KRAS*, *MED12* and *KDM6A* in both diagnosis and relapse samples. In addition, three somatic missense mutations were identified in *STAT5B* gene (T648S, N642H, I704L) in both samples. The T648S and N642H mutations occurred in the SH2 domain whereas the I704L mutation occurred in the transactivation domain (**Figure 5**). According to RNA-sequencing data, all three mutations were located in the same allele, indicating that the other *STAT5B* allele was wild type. Interestingly, the same N642H mutation was earlier detected in two LGL leukemia patients.

4.2.1 Mutations increase the cellular activity of STAT5B

Approximately 20-30% of T-ALL patients are estimated to harbor mutations in the *IL7R*, *JAK1*, *JAK2* or *JAK3* genes, which are known to activate the IL7R-JAK-STAT5 pathway.[208] However, *STAT5B* mutations were not reported earlier in T-

ALL. Thus, we were interested in focusing on STAT5B mutations. HeLa cells were transfected with different expression plasmids, which contained WT, single, double or triple mutations in *STAT5B* (T648S, N642H, I704L) in all possible combinations. N642H strongly induced the phosphorylation of STAT5B whereas I704L had a smaller effect on it and T648S seemed to have no effect on phosphorylation (**Figure 8A**). Accordingly, the N642H mutation increased the STAT5B-driven luciferase activity 27-fold, I704L 17-fold and T648S 0-fold (**Figure 8B**). HeLa cells with the double mutation N642H+I704L slightly increased STAT5B activity compared to the activity induced by either mutation alone, but no clear additive effect was detected with the other tested combinations (**Figure 8B)**.

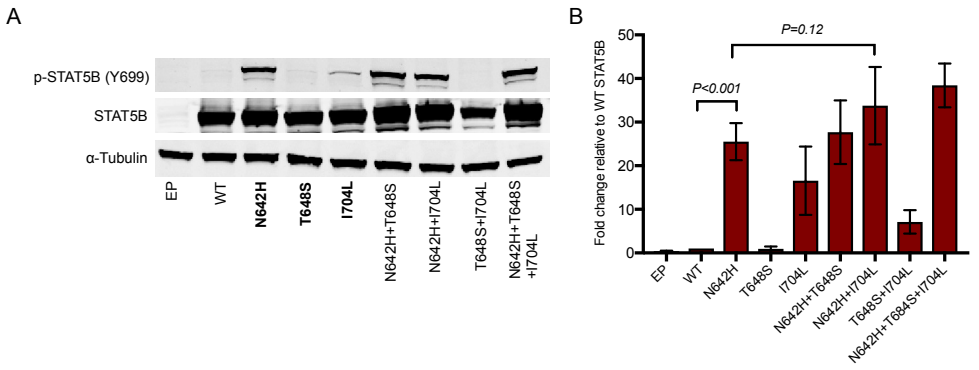

Figure 8. N642H and I704L mutations increase the activity of STAT5B. A**)** HeLa cells were transfected with different STAT5B expression plasmids containing the mutations indicated in the figure. After 24 h incubation cells were lysed and pSTAT5B Y699 levels were quantified with Western blot analysis. B**)** Transcriptional activity of the *STAT5B* mutations. HeLa cells were co-transfected with STAT5B specific reporter construct and STAT5B expression plasmids. After 24 h luciferase activity was measured with OneGlo reagent. Three independent experiments were performed and mean fold change value relative to WT ±SD is presented in the graph. P-values calculated using Student's T-test.

4.2.2 Ex vivo drug sensitivity testing of mutated blasts

We assessed the drug sensitivity of BM blast cells from the index patient sampled at relapse to 264 compounds representing conventional chemotherapeutics and targeted agents. In a 3-day cell viability assay, blast cells were highly sensitive to BCL2/BCL-xL inhibitor navitoclax and glucocorticoids when compared to healthy BM mononuclear cells (**Figure 9A-B**). Blasts also displayed sensitivity to MEK inhibitors, which is likely to be caused by the detected *KRAS* mutation (**Figure 9C**).

Other kinase inhibitors such as mTOR inhibitors, PI3K inhibitors and JAK inhibitors were not effective (**Figure 9D**).

Since the blasts were sensitive to navitoclax we evaluated the gene expression levels of antiapoptotic BCL2 family members (*MCL1*, *BCL2*, *BCL-xL* and *BCL-xS*). Expression levels were compared to CD3+ T-cells enriched from healthy PB and to two T-ALL patients without *STAT5B* mutations. Interestingly, *BCL-xL* expression was 12- and 4-fold higher in the diagnosis and relapsed samples when compared to healthy controls (**Figure 9E**). The results were in line with the increased *BCL-xL* expression levels in LGL leukemia patients with STAT5B mutations (**Figure 6A**). In contrast, *MCL-1*, *BCL-xS* and *BCL2* expression levels were similar across all tested samples. The results suggested that the studied blast cells were particularly dependent on BCL-xL and that the overexpression might be linked to the activating *STAT5B* mutations.

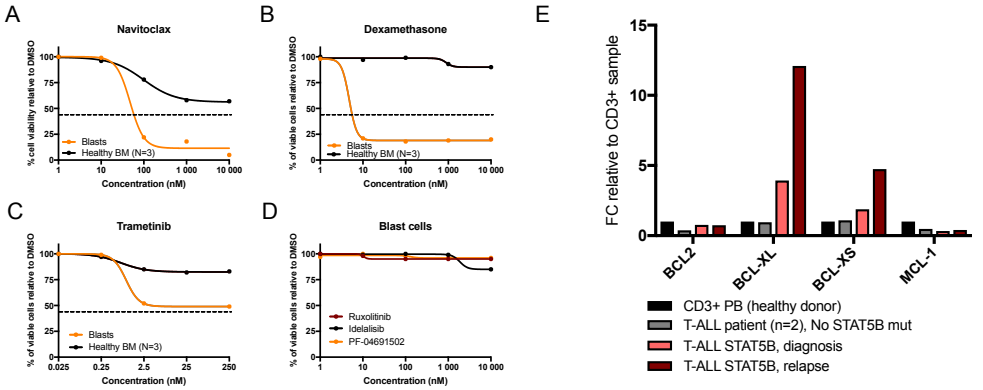

Figure 9. Ex vivo drug sensitivity of blast cells and expression of BCL-family members. A-C) Cell viability of blast cells and healthy BM controls measured with CTG after 3-day incubation with the indicated drugs **D)** Drug sensitivity of blast cells to ruxolitinib-JAK1/JAK2 inhibitor, idelalisib-PI3K inhibitor and PF-04691502-dual mTOR inhibitor. **E)** Measurement of gene expression levels of BCL2 family members in the index patient's blast cells during diagnosis and relapse by quantitative reverse transcription PCR. Healthy CD3+ fraction of PB and two T-ALL patients without *STAT5B* mutation were used as controls.

4.2.3 STAT5B is commonly mutated in T-ALL

To investigate the prevalence of *STAT5A, STAT5B* and *STAT3* mutations in T-ALL we screened 64 adult and 4 pediatric T-ALL patients. The SH2 dimerization and transactivation domains of STAT5B (exons 14-19), SH2 domain of STAT5A (exon

17) and STAT3 (exon 21) were analyzed by targeted amplicon sequencing. The results revealed *STAT5B* mutations in five additional patients but no mutations were detected in *STAT3* or *STAT5A* (**Table 6**). All *STAT5B* mutations were located in the hotspot SH2 domain including four patients with the N642H and one with the Y665F mutations (**Figure 5**). Altogether, 6 out of the 68 patients (8.8%) had a *STAT5B* mutation demonstrating that gain-of-function *STAT5B* mutation is a recurrent event and a putative driver in adult T-ALL.

Table 6. *STAT5B* mutations in the T-ALL patient cohort.

Immunologic subtype	Sex	Age	Mutation	Variant allele frequency (%)
Cortical T	Male	39	N642H	92.3%
Cortical T	Male	44	N642H	39.5%
Mature T	Male	45	Y665F	19.3%
Mature T	Male	59	N642H	47.2%
Pre-T	Male	24	N642H	6.4%
Pre-T	Female	18	N642H I704L T648S	45.9% 48.0% 48.6%

4.3 Drug screening identifies compounds inhibiting STAT3 activity

JAK/STAT3 signaling is commonly deregulated in lymphoproliferative malignancies and is known to contribute to disease progression.[124,209–211] Furthermore, almost half of LGL leukemia patients harbor mutations in the *STAT3* gene, leading to the expression of hyperactive STAT3.[58] Thus, the inhibition of overactive STAT3 is a tempting therapeutic strategy. However, STAT3 mutated primary LGL cells showed no response to JAK inhibitors in our earlier in vitro experiments (data not shown). This led us to hypothesize that mutant STAT3 activity might not be properly inhibited by upstream JAK inhibition, but other compounds could provide alternative ways for inhibition. In **study III** we aimed to identify small molecule inhibitors that could effectively inhibit the cellular activity of mutant STAT3 using a high-throughput drug screening approach.

4.3.1 Mutant STAT3 is active without cytokine stimulation

To identify potential STAT3 inhibitors we used HEK293 cells modified to contain a STAT3 inducible luciferase reporter and that stably expressed either the STAT3 WT or the most common STAT3 mutant, Y640F. In the absence of cytokines, WT STAT3 was not able to produce luciferase signal whereas mutant STAT3 was constitutively active (**Figure 10**). IL-6 stimulation induced WT STAT3 activity and further augmented the activity of mutant STAT3 (**Figure 10**). The results demonstrated that mutant STAT3 is active even without external cytokine stimulation whereas WT STAT3 requires upstream stimulation.

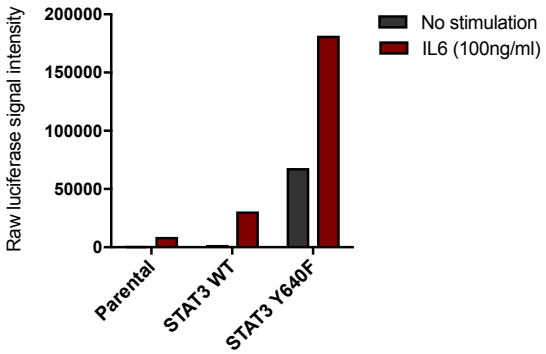

Figure 10. Basal and IL6 induced luciferase signal intensity of different HEK-SIE cell line models. Half of the cells were stimulated with 100ng/ml IL6 for 6 h after the signal intensity was measured. Parental cells do not overexpress STAT3 but contain endogenous STAT3. The means of technical triplicates are presented in the graph.

4.3.2 Drug-screening identifies compounds inhibiting STAT3

Next, we compared the inhibitory effects of 306 approved and investigational oncology compounds between non-stimulated mutant STAT3 and IL6-stimulated WT STAT3 cells. Initial screening with 306 approved and investigational oncology compounds revealed several agents capable of inhibiting mutant STAT3-driven luciferase signal (data not shown). Based on these results we designed a custom panel of 62 drugs and tested it against non-stimulated mutant STAT3 and IL6-stimulated WT STAT3. CDK, mTOR, Hsp90 and JAK inhibitors were the most effective drug classes that inhibited the cellular activity of both WT and mutant STAT3 (**Table 6**). Interestingly, the only drug class that showed clearly reduced efficacy against mutant STAT3 was JAK inhibitors (**Table 6**). Small molecule

STAT3 inhibitors (LLL12, STA-21, STATTIC), that are SH2 domain antagonists, were effective against both mutant and WT STAT3 indicating that the mutation does not alter the binding capabilities of these drugs (**Table 6**). To confirm that the drug targets are involved in mediating STAT3 inhibition, we performed knockdown experiments with siRNAs. In line with the drug screening results, mTOR and HSP90 knockdown caused luciferase signal reduction in WT and mutant STAT3 expressing cells whereas JAK knockdown (JAK1) inhibited only WT STAT3 (**Figure 11**). In conclusion, the data suggest that JAK inhibition has decreased efficacy against non-stimulated mutant STAT3 but the activity can be reduced with other targeted agents.

Table 6. The top 24 compounds inhibiting Y640F STAT3 induced luciferase activity after 6 h drug treatment

#	Drug Name	Drug target	Y640F STAT3* (DSS)	Y640F STAT3 (IC50, nM)	WT STAT3 + IL6^ (DSS)	WT STAT3 + IL6 (IC50, nM)
1	Omacetaxine	Protein synthesis (80 S ribo.)	36,6	14,2	38,4	9,9
2	Alvocidib	Cdk	26,5	33,5	28,7	48,5
3	UCN-01	PKCbeta, PDK1, Chk, Cdk2	20,3	97,6	22,6	126,7
4	SNS-032	Cdk	18,9	116,6	21,8	179,2
5	AZD8055	mTOR	18,6	24,2	20,3	50,2
6	Dactinomycin	RNA synthesis	18,4	6,4	18,9	9,4
7	Ruxolitinib	JAK1/JAK2	13,1	154,9	33,6	19,5
8	Dactolisib	PI3K/mTOR	12,8	13,0	16,6	11,8
9	Momelotinib	JAK1/JAK2	12,4	4036,9	24,5	109,5
10	LLL12	STAT3 SH2 domain	12,4	2334,4	15,8	2297,9
11	INK128	mTOR	12,0	30,1	14,4	31,1
12	Temsirolimus	mTORC1	11,8	1,6	8,2	2,1
13	STA-21	STAT3, SH2 domain	11,5	1971,8	9,3	2169,0
14	Luminespib	HSP90	11,4	5,9	12,0	4,0
15	Lestaurtinib	FLT3, JAK2, TrkA, TrkB, TrkC	10,8	40,9	21,0	19,9
16	BIIB021	HSP90	9,9	52,2	15,3	54,0
17	PF-04691502	PI3K/mTOR	9,1	400,8	15,3	365,0
18	Tofacitinib	JAK1/JAK3	9,1	3200,0	26,0	44,0
19	Everolimus	mTORC1	8,9	3,4	3,2	2,5
20	AZD1480	JAK1/2, FGFR	8,8	794,0	18,6	40,9
21	Bosutinib	Src, Abl inhibitor	8,4	46,8	0,4	10000,0
22	BMS-754807	IGF1R	8,2	75,3	2,6	35,1
23	Omipalisib	PI3K	8,1	0,9	5,9	5,9
24	Stattic	STAT3, SH2 domain	7,4	5774,1	9,5	6227,8

DSS=Drug sensitivity score, based on area under the curve (drugs used in 1,000-100,000 fold concentration ranges)
IC50=concentration of the inhibiton where response is reduced by half
* Luciferase activity of unstimulated Y640F STAT3 cells were measured after 6 h drug treatment
^WT STAT3 cells were stimulated with 100ng/ml IL6 and luciferase activity measured after 6 h drug treatment

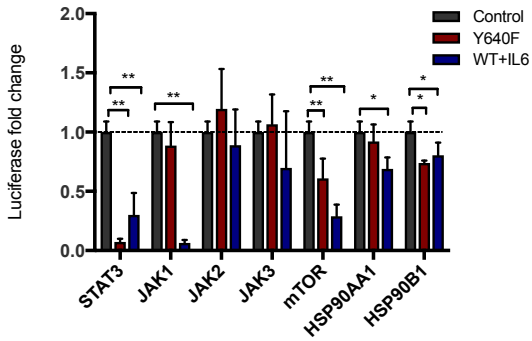

Figure 11. Knockdown of drug targets with siRNAs. Each gene was silenced with 3 different siRNAs in separate wells. Luciferase activity was measured following 3-day incubation. IL6 was added to WT STAT3 cells 6 h prior reading. Fold change is normalized to both cell lines' mock siRNA treated controls separately. The experiment was repeated 2 times and mean ±SD error is represented in the bars. **P*<0.05, ***P*<0.01 (Mann-Whitney U-test)

4.3.3 Hsp90 inhibition decrease STAT3 phosphorylation

Next, we evaluated whether mTOR, Hsp90 and JAK inhibitors decrease the phosphorylation of STAT3. Both cell lines were incubated with the selected drugs for 8 h after which 705-tyrosine and 727-serine phosphorylation levels were measured. While 705-tyrosine phosphorylation is essential for STAT3 dimerization, the 727-serine phosphorylation enhances STAT3 activity.[212] Interestingly, Hsp90 inhibition resulted in decreased 705-tyrosine phosphorylation of both mutant and WT STAT3 whereas JAK inhibitors were only effective against WT STAT3 (**Figure 12**). In contrast, mTOR inhibition did not reduce STAT3 phosphorylation suggesting that its effect is mediated through other mechanism, such as through transcriptional or translational regulation (**Figure 12**).

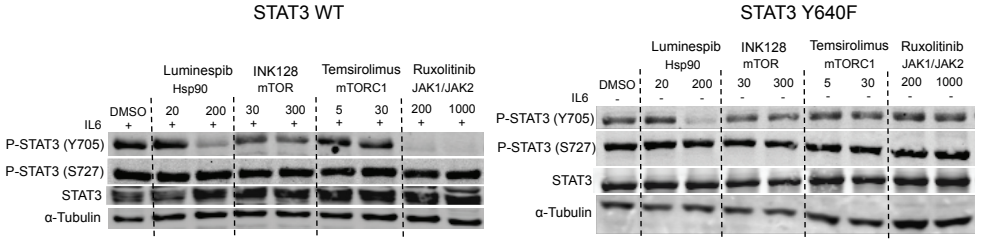

Figure 12. Evaluation of pSTAT3 status after drug treatment. HEK-SIE cell expressing WT or Y640F STAT3 were treated with the indicated drugs and concentrations for 8 h after phosphorylation of Y705 P-STAT3 and S727 P-STAT3 was measured by Western blot. IL-6 (100 ng/ml) was added to WT STAT3 cells 10 minutes prior to the drugs.

4.3.4 Cell viability assessment of STAT3 mutated NK cells

To study whether Hsp90 and JAK inhibitors can decrease the viability of cell lines with naturally occurring STAT3 mutations, we screened seven NK cell lines in the presence of IL-2, which is needed for the survival of the cells in culture. Three cell lines harbored *STAT3* mutations (YT and NK-YS, Y640F mutation; SNK6, D661V mutation) and four WT *STAT3* (KAI3, NK-92, KHYG-1 and NKL). As expected, STAT3 was highly phosphorylated in Y640F mutated cell lines and remained high upon overnight withdrawal of IL-2 in contrast to WT STAT3 containing cell lines (**Figure 13A**). Luminespib and ruxolitinib effectively reduced the viability of all tested cell lines in a 3-day assay in the presence of IL-2 (**Figure 13B**). However, ruxolitinib showed a slightly reduced efficacy to *STAT3* mutated cell lines (**Figure 13B**). In agreement with the results observed with HEK-SIE cells, Hsp90 inhibition dose dependently reduced STAT3 phosphorylation in mutant *STAT3* harboring NK-YS cell line (**Figure 13C**).

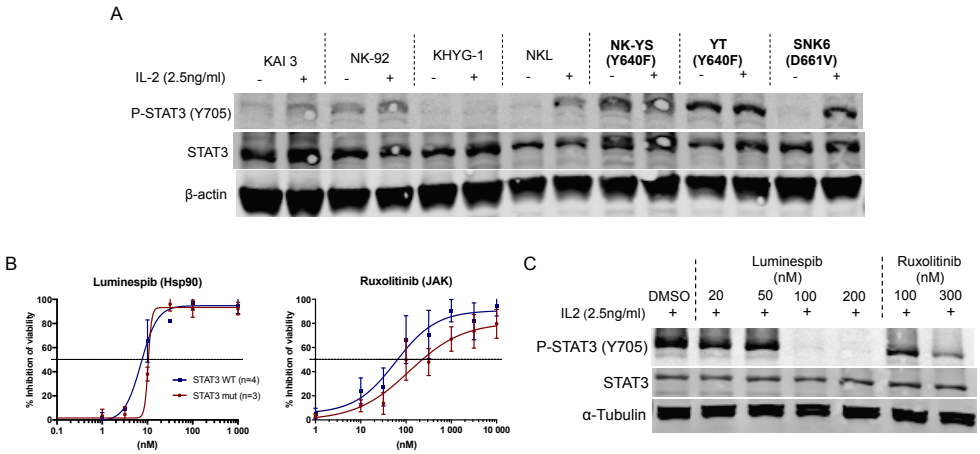

Figure 13. Effect of JAK and Hsp90 inhibition on NK cell lines. A) Basal pSTAT3 status of cell lines with or without *STAT3* mutations in the presence of IL2 (2.5ng/ml) or after 12 h IL2 starvation. B) Cells were incubated with the drugs for 3-days after which cell viability was measured with CTG. The experiment was repeated 3 times and the mean % inhibition of viability is shown for mutant and WT STAT3 cell lines. Error bars represent ±SD of the variation between cell lines in the same group. C) pSTAT3 levels of NK-YS cells treated with indicated drugs for 18 h.

4.3.5 Primary LGL cells are sensitive to Hsp90 inhibitors

To further confirm our findings, we tested whether patient derived LGL leukemia cells are sensitive to Hsp90 and JAK inhibition. In the absence of cytokines CD8+

purified LGL cells with STAT3 mutations were significantly more sensitive to luminespib than WT STAT3 LGL cells or healthy CD8+ cells (P<0.01, **Figure 14A**). Surprisingly, ruxolitinib did not cause decreased cell viability in any samples (**Figure 14A**). Next, we tested the effect of the drugs in the presence of IL-2 and IL-15, two lymphokines associated with the LGL leukemia phenotype,[127] and observed remarkable inhibition of all samples to both luminespib and ruxolitinib (**Figure 14A**). However, the STAT3 mutated samples showed the highest sensitivity to both drugs. Consistent with these findings, IL-2+IL-15 strongly stimulated STAT3 phosphorylation in a STAT3 mutated patient sample and luminespib effectively reduced STAT3 phosphorylation in the presence or absence of cytokines (**Figure 14B-C**). Taken together, our results demonstrate that Hsp90 inhibition is effective at reducing mutant STAT3 activity and phosphorylation across different cell line models and primary patient samples.

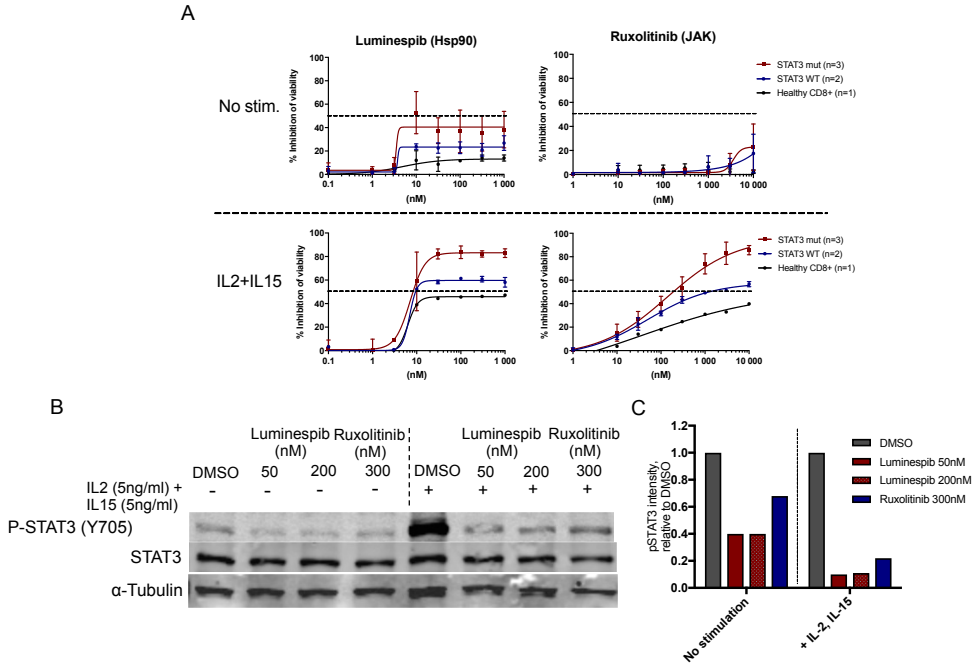

Figure 14. Effect of Hsp90 and JAK inhibition on patient derived LGL leukemia samples. A) CD8+ purified cells of 4 LGL patients with STAT3 mutations (3×Y640F, 1×D661V, VAF>27%), 4 patients without STAT3/STAT5 mutations and 3 healthy CD8+ cells were treated with ruxolitinib or luminespib for 3 days with or without cytokines (IL-2 5ng/ml, IL-15 5ng/ml). B) Patient sample with Y640F mutation was treated with luminespib or ruxolitinib with the indicated concentrations and stimulated with cytokines for 18 h after which pSTAT3 levels were assessed C) pSTAT3 band intensities quantified with Odyssey imaging application software. pSTAT3 intensities were normalized to α-Tubulin and are relative to the DMSO controls.

4.4 Flow cytometry-based drug sensitivity assessment of AML samples

AML BM is comprised of a mixture of cancer cells at distinct stages of myeloid differentiation and of non-malignant cells. Thus, blast-specific drug responses are challenging to measure with conventional cell viability assays, which lyses the cells and measures overall sample sensitivity. In **study IV** we developed a multi-parametric, high-content flow cytometry (HC-FC) based drug-screening platform. By using HC-FC, we simultaneously evaluated the *ex vivo* sensitivity of four different cell populations (blasts, lymphocytes, granulocytes and monocytes) of 32 primary AML samples to seven FDA-approved drugs. The tested drugs were chosen based on their good AML-selective response in our earlier *ex vivo* drug screening studies,[195] and for their ability to inhibit major cell survival pathways: MEK inhibitor (trametinib), JAK1/2 inhibitor (ruxolitinib), mTORC1 inhibitor (everolimus), FLT3/broad range TKI (sunitinib) and BCL2 inhibitor (venetoclax). The standard of care drugs idarubicin and cytarabine were also included in the drug panel as controls. To evaluate the benefit of the new platform, cell viability was measured in parallel with CTG-based assay which measures the overall sensitivity of the sample (**Figure 15.**)

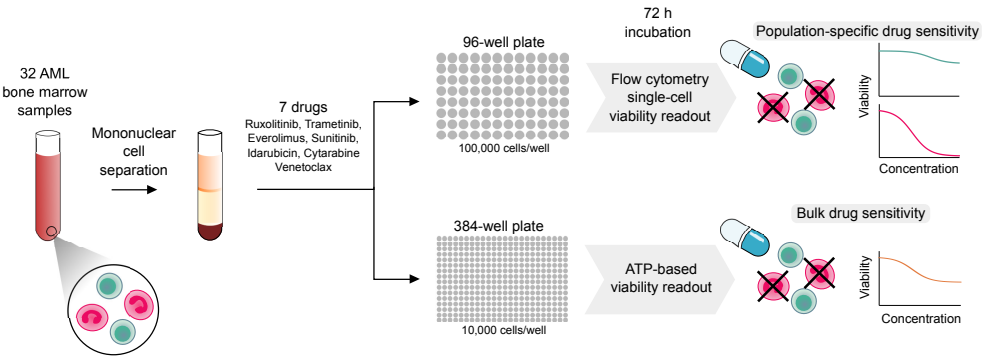

Figure 15. Schematic outline of the experimental setup

4.4.1 Blast-specific drug sensitivity measurement

Immunophenotyping of the AML samples revealed large interpatient variability in the BM cell compositions and as expected, the composition was associated with the FAB subtype (**Figure 16**). Blast cell population size out of the leukocytes (CD45+

population) varied between 17-94% and lymphocytes were present in all samples ranging from 0.5 to 46%. As expected, high numbers of monocytic cells were detected in myelomonocytic/monocytic (M4/5) samples whereas M0/1 samples mainly consisted of blasts and lymphocytes (**Figure 16**).

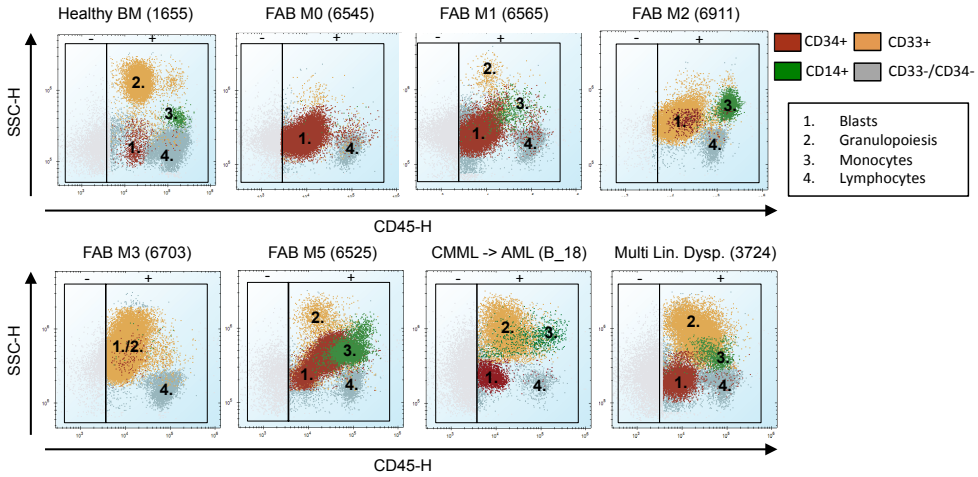

Figure 16. Immunophenotyping of AML samples. To assess the drug sensitivity of the four major leukocyte populations present in the BM, four cell surface markers were used (CD45, CD33, CD34, CD14). SSC vs. CD45 scatter plot visualizes the cell populations present in different AML samples diagnosed at day 0. Blast cells were defined based on CD34 positivity. In the absence of CD34+ cells (35% of cases) blast cells were defined based on CD45dim/SSClow and CD33+ positivity.

To investigate the ability of FC to measure blast-specific drug responses after 3-day drug treatment we compared the results with the overall BM sensitivity measured with CTG based platform. Correlation of the drug sensitivity scores (DSS, based on area under the curve) between CTG and FC was good when the bulk CD45+ leukocyte fraction was used as the FC-readout ($R=0.67$, $P<0.0001$, **Figure 17A**) or when blast-specific drug sensitivities were measured with FC in samples with blast count over 50% ($R=0.71$, $P<0.0001$, **Figure 17B**). However, the correlation was poor when blast-specific drug responses were measured in samples with blast count below 50% ($R=0.17$, $P<0.156$, **Figure 17C**). The poor correlation was largely due to highly different drug sensitivities of the non-blast cell population and AML blasts as shown with an AML patient case (BE_18) with low blast count (**Figure 17D)**. The results demonstrate that CTG based platform cannot accurately measure blast-specific responses in unfractionated AML samples with low blast counts (**Figure 17E**).

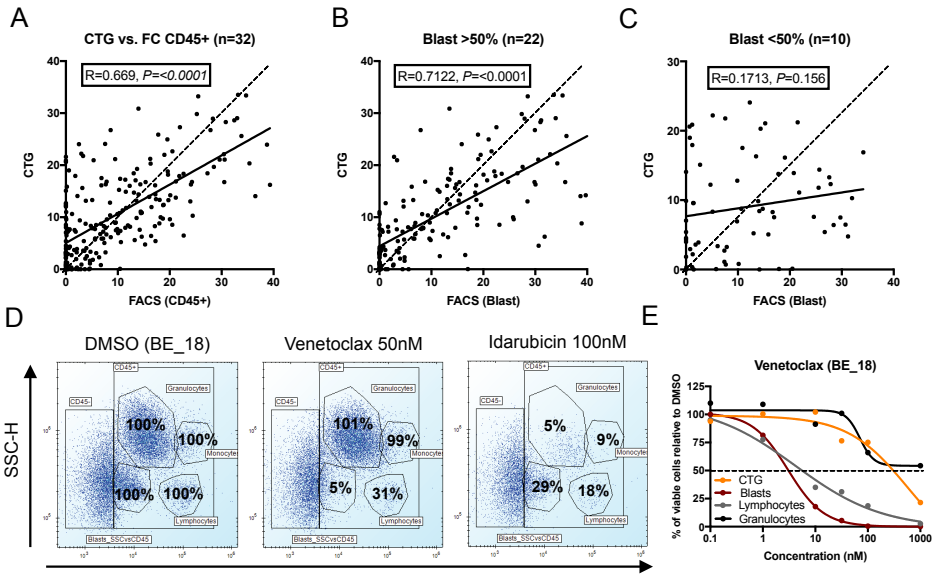

Figure 17. Comparison of the CTG and FC based drug sensitivity platforms. A) Correlation of the CTG *vs.* FC drug sensitivity scores of the tested 32 samples and 7 compounds when using CD45+ leukocytes as FC readout, or B) blasts in samples with clinical blast count >50% or C) blasts in samples with clinical blast count <50%. D**)** Illustration of venetoclax and idarubicin effect on different cell populations in an AML sample with clinical blast count of 20%. The raw number of live cells in each gate was calculated after 3-day drug treatment and normalized to the number of cells present in the DMSO control at day 3. E) Cell viability of different cell populations after 3-day venetoclax treatment measured with FC and CTG assay.

4.4.2 Blasts are sensitive to BCL2 inhibition

To assess whether cell phenotype has an influence on drug responses, we measured the cell population specific drug sensitivities in 32 AML samples. Amongst the tested 7 compounds BCL2 inhibitor venetoclax showed the highest toxicity against blasts with median IC50<5nM whereas monocytes and granulopoietic cells were markedly resistant with IC50>100nM (**Table 7**). In contrast, monocytes were particularly sensitive to ruxolitinib (IC50<100nM) and trametinib (IC50<5nM) whereas lymphocytes and granulocytes lacked kinase inhibitor sensitivity (IC50>1000nM, **Table 7**).

Table 7. Median drug sensitivity scores and IC50 values of each cell population

	Blasts (n=32)		Monocytes (n=16)		Lymphocytes (n=31)		Granulocytes (n=5)	
	DSS	IC50 (nM)	DSS	IC50 (nM)	DSS	IC50 (nM)	DSS	IC50 (nM)
Venetoclax	27.1	3.0	7.1	122.0	18.1	20.3	5.7	113.0
Idarubicin	22.0	28.7	16.1	78.7	16.5	84.0	19.0	41.1
Cytarabine	9.7	894.2	7.5	1071	4.8	2550	9.5	953.7
Ruxolitinib	5.0	302.7	17.2	93.3	0	2511	0	2476
Trametinib	3.0	18.9	25.9	2.4	0	> 250	1.1	165.0
Sunitinib	1.0	321.1	5.7	223.7	0	> 1000	4.4	352.7
Everolimus	0.0	55.6	4.6	7.5	0	> 100	0	> 100

M4/5 AML samples contained high numbers of both leukemic blasts and monocytic cells and these subpopulations showed highly different drug sensitivities particularly to venetoclax and trametinib (**Figure 18A-B**).

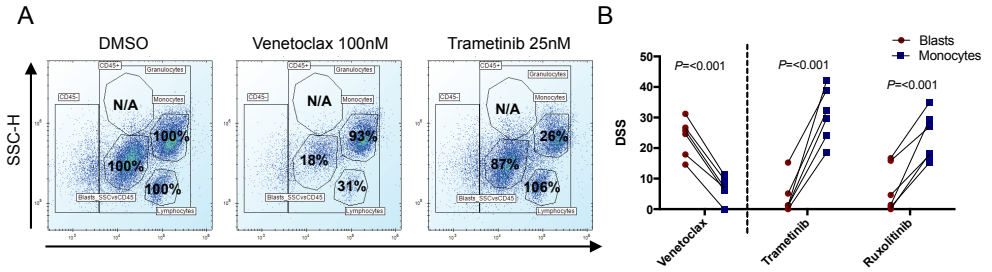

Figure 18. Blast and monocyte specific drug responses in patients with M5 subtype. A) Representative FC scatter plots of venetoclax and trametinib effects on M5 sample after 3-day drug treatment. Percentages indicate the number of gated viable cells normalized to DMSO control treated cells. B) Comparison of the DSS scores of monocytes and blast cells present in the same M5 samples.

4.4.3 Venetoclax response is associated with FAB subtype

While the monocytic cells showed different drug responses we hypothesized that M5 samples should have distinct drug response profiles when overall BM sensitivity is measured with CTG. We re-analyzed our earlier published data of 31 AML samples comprised of M1, M2 and M5 cases that were screened with 302 different compounds. Notably, venetoclax showed the biggest drug sensitivity difference between M1 and M5 amongst the 302 tested compounds (**Table 8, Figure 19A**). Accordingly, the 32 AML samples used in this study showed similar decreasing venetoclax sensitivity from M0 towards M5 subtype when measured with CTG (**Figure 19B**). However, when blast specific sensitivity was measured with FC no clear differences were observed between FAB subtypes (**Figure 19C**). Of note, our

M0/1 sample cohort contained a larger number of chemorefractory samples. When we concentrated only on diagnosis samples a smaller but still significant effect was observed between M1 vs. M5 blasts (**Figure 19D)**. Taken together, monocytic cells blur the blast specific venetoclax sensitivity in unfractionated M4/5 samples. However, M5 blasts still show a decreased sensitivity to venetoclax in an AML sample cohort comprised only of diagnosis samples. As the overall number of the tested AML samples is limited, an expanded number of samples need to be studied to confirm this preliminary association.

Table 8. Drugs with the highest difference in drug sensitivities between M5 vs. M1

#	Drug	Drug target	DSS (M1 vs. M5)	**M1** Mean (n=8)	**M2** Mean (n=15)	**M5** Mean (n=8)	*P*-value	FDR
1	Venetoclax	BCL2	23,0	35,2	21,0	12,2	0,000	0,013
2	Navitoclax	BCl2. BCL-XL	17,0	32,7	22,9	15,7	0,001	0,307
3	Foretinib	C-MET, VEGFR2	9,3	16,9	4,0	7,6	0,159	>0,999
4	Ralimetinib	p38 MAP	8,5	9,8	2,2	1,3	0,018	0,994
286	Ruxolitinib	JAK1/2	-3,7	4,8	2,7	8,5	0,471	>0,999
295	Trametinib	MEK1/2	-5,7	6,5	5,4	12,2	0,199	>0,999
299	PF-00477736	Chk-1	-6,9	5,5	3,9	12,4	0,203	>0,999
300	Pimasertib	MEK1/2	-7,7	7,2	8,6	14,9	0,073	1,000
301	Paclitaxel	Microtubule stabilizer	-8,6	1,3	1,2	9,9	0,006	0,803
302	Docetaxel	Microtubule stabilizer	-10,6	0,5	0,3	11,1	0,006	0,817

Expression of anti-apoptotic BCL2 and MCL1 are considered the most critical feature of AML blast cell survival.[213–215] To investigate whether their expression is correlated with FAB subtypes we analyzed the gene expression data of AML samples using the TCGA dataset (RNA-seq). The TCGA data are derived from unfractionated AML mononuclear cell fractions and thus represent the situation when overall BM sensitivity is measured with CTG. In line with the high overall sensitivity of M0/M1 samples to venetoclax, BCL2 was highly expressed in M0/M1 samples and gradually decreased towards the M5 subtype (**Figure 19E**). Intriguingly, MCL1 showed a contrasting trend from M0 towards M5 subtype (**Figure 19E**). Results indicate that the *BCL2/MCL1* gene expression ratio is associated with FAB subtypes supporting the low *ex vivo* venetoclax sensitivity of M4/5 AML samples. Taken together, AML sensitivity to venetoclax is dependent on the differentiation stage of leukemic cells. Moreover, FC-based phenotypic drug sensitivity testing increases our understanding of *ex vivo* drug effects and may help to identify blast specific treatments.

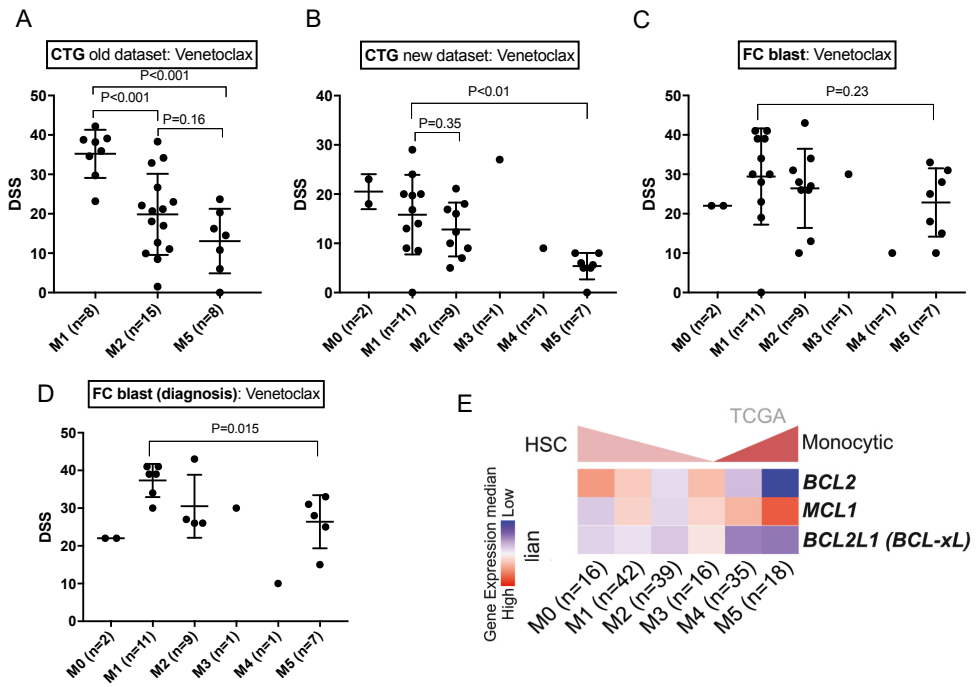

Figure 19. ***Ex vivo*** **venetoclax response is associated with FAB subtype** A) Drug sensitivity of 31 AML samples to venetoclax measured with CTG. Patients are divided into different groups based on FAB subtype. B) Drug sensitivity of AML samples studied here. Overall BM sensitivity measured with CTG or C) blast-specific responses with FC. D) Blast specific venetoclax sensitivity of diagnosis samples E) Log2 gene expression of BCL2 family genes in different FAB subtypes. The data were obtained from TCGA network.

5 DISCUSSION

Advances in sequencing technologies have revolutionized our understanding of leukemia genetics and pathogenesis. However, it has been a challenge to transform this genetic information into clinically actionable strategies. In this thesis we aimed to approach the challenge by evaluating the sensitivity of primary leukemia samples with high-throughput drug sensitivity testing and by combining the information with genetic profiling. In studies I and II, our goal was to identify novel driver mutations in LGL leukemia and T-ALL. Interestingly, we identified novel activating *STAT5B* mutations in both diseases. In study III, we used high-throughput drug sensitivity testing to discover compounds that inhibit mutant STAT3 activity. Amongst the 306 compounds the Hsp90 inhibitors were effective at inhibiting the cellular activity of mutant STAT3. In study IV, we developed an improved *ex vivo* drug sensitivity platform. The flow cytometry-based platform allowed us to measure AML BM drug sensitivity at a detailed cell population level. The study revealed that the effect of BCL2 inhibitor venetoclax is associated with the maturation stage of AML cells.

5.1 *STAT5B* mutations in LGL leukemia and T-ALL

LGL leukemia is an indolent disease of cytotoxic T-cells or NK cells with a poorly characterized molecular pathogenesis. In 2012, our research group discovered that 40% of LGL leukemia patients harbor activating *STAT3* mutations in the SH2 domain that leads to increased STAT3 phosphorylation and transcriptional activity.[58] In study I, we aimed to identify other mutations associated with survival signaling pathways by performing exome sequencing analysis on two *STAT3* negative T-LGL leukemia patients. The approach proved out to be successful as both patients carried a Y665F *STAT5B* mutation. We proceeded with targeted sequencing of the *STAT5B* gene on an additional 209 LGL leukemia patients and discovered two additional patients to harbor *STAT5B* N642H mutations. Both mutations (N642H and Y665F) resulted in an amino acid change in the SH2 domain. Later, Kücük et al. demonstrated that the N642H mutation leads to increased binding affinity between the phosphotyrosine-Y699 and the mutant histidine of the two STAT5B monomers.[216] This causes prolonged persistence of the STAT5B dimer and marked increase in its binding to target genes. Accordingly, in our in vitro assays both

mutations resulted in increased transcriptional activity and phosphorylation. Taken together, *STAT5B* mutations are rare in LGL leukemia (2%), but importantly LGL leukemia was the first cancer in which *STAT5B* mutations were described.

The two patients with the *STAT5B* N642H mutations had a CD56+ phenotype and were the only cases out of the 211 patients with an aggressive and fatal form of the disease. Thus, we hypothesized that *STAT5B* mutations might be associated with an aggressive disease course. Another study conducted later identified *STAT5B* mutations in six out of eleven CD4+ T-LGL leukemia patients demonstrating that *STAT5B* mutations (N642H, Y665F, Q706L and S715F) are enriched in the LGL cells with CD4+ phenotype.[217] Accordingly, STAT5B but not STAT5A is essential for CD4+ T-helper cells due to asymmetric and high expression of STAT5B.[218] Contrary to our findings, CD4+ LGL cells had an indolent disease course. Thus, the association between *STAT5B* mutations and disease aggressiveness remains controversial and may be linked to CD56+ phenotype.

As shown by our study *STAT5B* mutations are rare in LGL leukemia and thus they cannot explain the constitutive JAK/STAT signaling in the majority of STAT3 mutation negative patients.[124,219] This indicates that JAK/STAT signaling can be activated by other means. In 2015, our research group discovered STAT3 mutations outside of the SH2 domain in 4% of the patients.[220] Moreover, a study by Andersson et al. suggested mutations in *PTPRT* and *BCLB11B* as putative driver mutations in LGL leukemia.[219] PTPRT dephosphorylates STAT3 and its malfunction can lead to constitutive STAT3 activity. Furthermore, a recent exome sequencing study consisting of 19 LGL leukemia samples identified 14 genes that were recurrently mutated in LGL leukemia.[221] These genes involved epigenetic regulators, tumor suppressors and cell proliferation related genes. Importantly, many of these genes were linked to JAK/STAT signaling. It is hypothesized, that JAK/STAT3/STAT5 signaling might contribute to FAS-mediated resistance through the upregulation of anti-apoptotic BCL2-family members.[124] However, future studies are warranted to better understand how JAK/STAT3 signaling contributes to disease progression in LGL leukemia.

In our second study, we identified four different *STAT5B* mutations (N642H, T648S, Y665F, I704L) in 6 out of 68 T-ALL patients (8%) included in our cohort. Three of

these mutations were located in the SH2 domain (N642H, T648S, Y665F) whereas the I704L mutation was located in the transactivation domain. Earlier *IL7R*, *JAK1* and *JAK3* mutations have been described in approximately 20-30% of T-ALL patients. These mutations all results in the activation of STAT5B and PI3K/AKT/mTOR pathway.[139] Thus, our discovery of *STAT5B* mutations represents a novel mechanism leading to activation of the IL7R/JAK/STAT5 pathway. After our discovery, Atak et al. identified *STAT5B* mutations in 4 out of 31 T-ALL patients with S434L and N642H mutations.[222] Moreover, a German research group sequenced the *STAT5B* gene of 301 pediatric T-ALL patients and identified the N642H mutation in 19 samples (6.3%).[223] In the same study, *STAT5B* mutations were associated with a higher risk of relapse. Thus, we and others show that *STAT5B* mutations are a recurrent event in adult and pediatric T-ALL and that their acquisition is possibly associated with higher risk of relapse. Additionally studies in mice have shown that overexpression of *STAT5A* and *STAT5B* in the lymphoid compartment results in CD8+ T-cell lymphoblastic lymphomas.[224] This also suggests that *STAT5* overexpression alone can be an oncogenic driver. In a more recent study by Pham et al. N642H *STAT5B* expressing mice rapidly developed transplantable CD8+ lymphomas and leukemias.[225] The disease was characterized by highly invasive and proliferative CD8+ T-cells. Not surprisingly, *STAT5B* mutations have later been detected in other aggressive leukemia and lymphoma subtypes including γδ hepatosplenic T cell lymphomas[226], T cell prolymphocytic leukemia[227] and NK/T cell lymphomas.[216,228] Altogether *STAT5B* mutations seem to be specific to T and NK cell malignancies and might contribute to aggressive disease course as already suggested by our findings in LGL leukemia.

In addition to the relevance of *STAT5B* mutations in disease prognosis, they may also be used to predict drug sensitivity. Our T-ALL index patient with three different *STAT5B* mutations had increased expression of antiapoptotic *Bcl-xL* but no differences in expression were observed in *MCL1* or *BCL2*. Furthermore, the index patient showed good response to Bcl-xL/BCL2 inhibitor navitoclax suggesting that *STAT5B* mutated patients are dependent on BCL-xL. Indeed, *BCL-xL* is a known STAT5B target in mouse Ba/F3 cells,[229] human glioblastoma samples[230] and BCR-ABL transformed K562 erythroleukemia cell line.[231] However, Antohony Letai's group showed that all primary T-ALL patient samples are mainly dependent on BCL-xL except the early T-cell precursor (ETP) ALL subtype, which is more

dependent on BCL2.[232] Their study concludes that the maturation stage of lymphoid blasts determines the dependence between BCL2 and BCL-xL. Additionally, IL-7R activates PI3K/AKT/mTOR signaling, which is proposed to mediate at least partially an increase in *BCL2* expression.[233] Thus, the expression levels of BCL2 family members are probably influenced by several factors involving *STAT5B* mutations. Thus, a single oncogenic driver, such as the *STAT5B* mutations in our index patient, might not be a sufficient biomarker for drug sensitivity but several factors should be simultaneously taken into consideration.

5.2 Targeting the JAK/STAT3 pathway

Constitutive JAK/STAT3 signaling contributes to disease progression in lymphoid malignancies by upregulating the expression of cancer promoting genes.[210,234–236] Mutated or cytokine stimulated JAK kinases are the main activators of STATs, and their direct inhibition has been a successful approach in the clinic. JAK inhibitors are used to treat myelofibrosis and rheumatoid arthritis.[237,238] Moreover, they are actively tested in the treatment of different autoimmune disorders.[61] *STAT3* mutations were recently reported in several T and NK cell lymphomas/leukemias resulting in persistent and increased STAT3 activation.[58,216,228,239–242] In our in vitro assays, mutated STAT3 was active in the absence of cytokines and JAK inhibition did not effectively inhibit mutant STAT3. In study III, we investigated whether some other compounds could reduce the cellular activity of mutant STAT3. Amongst the 306 approved and investigational compounds tested, CDK, mTOR, JAK and Hsp90 inhibitors were the most effective targeted agents inhibiting mutant STAT3 induced luciferase signal. While CDK and mTOR inhibitors decreased STAT3-driven reporter signal they did not reduce STAT3 phosphorylation at Y705 or S727. This suggests that their effect is conveyed through other mechanisms at the transcriptional or translational level.

Ruxolitinib did not inhibit overexpressed mutant STAT3 induced luciferase signal in the absence of cytokine stimulation. Accordingly, ruxolitinib decreased STAT3 phosphorylation to a smaller extent in STAT3 mutation containing NK/lymphoma cell lines compared to the WT STAT3 cells. These results suggest that mutant STAT3 has a basal activity that might be challenging to inhibit solely with JAK

inhibitors. However, in physiological circumstances cytokine activated JAK kinases are presumably responsible for mutant STAT3 hyperactivation. For example, leukemic T-LGLs produce macrophage inflammatory protein-1 (MIP-1) and IL18.[126] Furthermore, peripheral blood cells produce IL-6[125] and all of the above mentioned cytokines induce STAT3 phosphorylation through JAK kinases.[243,244] Accordingly, most lymphocyte cell lines and primary samples are dependent on STAT3 and STAT5B activating cytokines, such as IL-2 or IL-15,[245,246] for *in vitro* growth. We observed that in the presence of cytokines, ruxolitinib effectively decreased the viability of mutated NK-cell lines and primary LGL leukemia samples. In line with these observations, a recent clinical study showed that 50% of LGL leukemia patients with a STAT3 mutation responded to tofacitinib (JAK1/JAK3 inhibitor) treatment.[130,247] The study demonstrates that JAK inhibitors might be a viable treatment strategy even for STAT3 mutated lymphoproliferative malignancies through the inhibition of cytokine induced mutant STAT3 hyperactivation.

In our study, small molecule STAT3 inhibitors (LLL12, STA-21 and Stattic) targeting the SH2 domain inhibited both mutant and WT STAT3 activity. This suggests that mutations in *STAT3* do not alter the binding capabilities of these drugs. However, in the *ex vivo* drug screens conducted at our institute, STAT3 inhibitors, however, showed similar efficacy across a large number of different primary leukemia samples and cell lines (data not shown), which again suggests lack of specificity of the tested STAT3 inhibitors. This presumption is also supported by other studies.[248,249] STAT3 lacks druggable enzyme characteristics and it is located in the cytosol or nucleus making it a difficult drug target. STAT3 is also expressed across a wide variety of tissues, which causes toxicity and drug delivery challenges. So far, OPB-31121 and OPB-51602 are the only STAT3-specific inhibitors that have been tested in early phase clinical trials.[70,71] However, the responses were modest and the toxicity profiles limited their administration. Lately, novel STAT3 inhibitors with higher binding affinities have been developed that might provide better treatment results in the future.[250]

Hsp90 is a chaperon protein that plays an important role in the stabilization of several client proteins of which many are involved in signal transduction.[251] In cancer, Hsp90 has been shown to stabilize abundantly expressed growth factor receptors,

mutated oncogenic proteins[252] and the members of the JAK/STAT and PI3K/AKT/mTOR pathways.[253,254] In our functional assays, Hsp90 inhibitors were able to decrease STAT3 phosphorylation and transcriptional activity of both mutant and WT STAT3 in different cell model systems. Notably, luminespib decreased the basal phosphorylation of mutant STAT3 more effectively than ruxolitinib. Earlier, Hsp90 inhibition has been shown to result in decreased JAK/STAT signaling in JAK2 V617 driven myeloproliferative neoplasms through JAK2 degradation.[255] However, in our cell model systems, the STAT3 was presumably activated by JAK1/JAK3 or by the STAT3 mutation itself. One study suggests, that Hsp90 inhibition causes inhibition of STAT1, -3, -5, -6 tyrosine phosphorylation through decreased expression and phosphorylation of JAK family members in classical Hodgkin lymphoma cells.[256] Thus, it is plausible that Hsp90 inhibition is associated with the inhibition of several JAK kinases and other client proteins that are needed in STAT3 phosphorylation and stabilization. Importantly, luminespib was highly effective at reducing the viability of NK cell lines and primary LGL leukemia samples. Even though, the reduced viability is probably mediated through several different mechanisms, the Hsp90 inhibition might be particularly effective in JAK/STAT3-driven lymphoid malignancies.

In conclusion, we identified Hsp90 inhibitor luminespib to effectively inhibit both the interleukin and STAT3 mutation induced STAT3 activity. However, to date Hsp90 inhibitors have not been approved for cancer treatment despite promising preclinical efficacies. A recent review summarized 15 phase II clinical trials with Hsp90 inhibitors and suggested that the lack of efficacy is linked to dose limiting toxicities resulting in insufficient doses to inhibit malfunctioning oncogenes.[257] In contrast, ruxolitinib has demonstrated efficacy in myeloid neoplasms (MPNs), more specifically in myelofibrosis (PMF) and polycythemia vera (PV), all of which are related to mutations activating JAK2. However, the therapy is not curative but leads to certain clinical benefits but also adverse events such as anemia[237] and decreased NK cell functioning.[258] Therefore, it remains probable that inhibition of JAK/STAT signaling alone does not lead to curative responses but combination strategies remain attractive. For example, by combining JAK/STAT pathway inhibitors with drugs such as methotrexate, glucocorticoids or BCL2 family inhibitors could provide better treatment outcomes in lymphoproliferative disorders with overactive JAK/STAT signaling.

5.3 *Ex vivo* drug sensitivity assessment with flow cytometry

In study IV, we used high-content FC based drug testing to simultaneously measure drug sensitivities of different cell populations present in heterogenous AML BM samples. We observed that monocytic cells abundantly present in acute myelomonocytic (FAB M4) or acute monocytic (FAB M5) leukemia samples were markedly resistant to BCL2 inhibitor venetoclax, whereas immature blasts in the same samples were sensitive. Accordingly, leukemic cells in undifferentiated acute myeloblastic leukemia (FAB M0) and in acute myeloblastic leukemia with minimal maturation (FAB M1) samples were highly sensitive to venetoclax. Our study demonstrates, that FC based drug testing can improve our understanding of drug effects and may help to identify blast specific treatments.

Different cell populations (blasts, lymphocytes, monocytes and granulocytes) present in the studied 32 AML samples had highly different sensitivities to targeted therapies. Amongst the seven drugs, venetoclax had the highest blast specific toxicity whereas monocytes and granulocytes were resistant. In contrast, monocytes showed increased sensitivity to MEK inhibitor trametinib and JAK inhibitor ruxolitinib. Accordingly, Kurtz et al. recently showed that in unfractionated BM samples the efficacies of several kinase inhibitors and venetoclax are associated with blast cell percentages.[259] When extensive molecular profiling was taken into consideration, alongside the FLT3, RAS and NPM1 mutations, the blast cell percentage was the most descriptive factor influencing the *ex vivo* drug sensitivities. Concordantly, Pietarinen et al. showed that in chronic myeloid leukemia CD34+ progenitor cells are sensitive to BCR-ABL-1-inhibitors whereas CD34 depleted cells (mature granulopoietic cells) are resistant.[260] Taken together, we and others show that in AML samples with low blast counts, the blast specific sensitivity can be lost when overall BM sensitivity is measured with conventional cell viability assays.

In line with our results, earlier studies have demonstrated interpatient variability in venetoclax sensitivity in unfractionated AML BM samples.[261–263] Pan et al. demonstrated that *ex vivo* venetoclax sensitivity was largely independent of genetic mutations and cytogenetics with the exception of JAK2 mutations and complex karyotype that were associated with decreased sensitivity.[262] In a study by Kontro et al., high *HOX* gene expression was associated with increased sensitivity.[261]

Interestingly, *HOX* genes are mainly expressed in hematopoietic stem and progenitor cells and their expression decreases during maturation,[264] which suggests that venetoclax is more effective in immature hematopoietic cells. Indeed, in our studies, the undifferentiated acute myeloblastic leukemia (M0) and acute myeloblastic leukemia with minimal maturation (M1) samples were more sensitive to venetoclax compared to the M4/5 samples when overall BM sample sensitivity was measured. Since BCL2 and MCL1 are considered the most important pro-survival factors in AML[213–215] we next evaluated their expression levels in different FAB subtypes. Supporting our drug screening results, M0/1 samples had high *BCL2* gene expression and M4/5 samples high *MCL1* expression. The expression levels are probably influenced by differentiating monocytes, which are more dependent on MCL1 than BCL2.[265] Importantly, our FC drug testing revealed that several M4/5 samples contained venetoclax sensitive blasts as well as venetoclax resistant monocytic cells. Thus, the resistance of monocytes to BCL2 inhibition blurs the blast specific sensitivity of drug screens assessing the viability of unfractionated leukemia samples. While the decreased overall BM sensitivity of M4/5 samples to venetoclax is caused by differentiating monocytes, we still observed a smaller but still significant difference in blast cell venetoclax sensitivity between diagnosis M1 and M5 samples. Thus, the correlation between maturation stage of leukemic blasts and treatment outcomes should be further investigated in a clinical setting. By now *IDH1/2*[266] and *RUNX1* mutations remain the best predictive biomarkers for venetoclax+hypomethylating agent sensitivity in the clinic.[104,105,267] This combination has shown highly promising results in Phase II clinical trials and venetoclax might dramatically change the AML treatment in the near future. To find the responders it might be useful to combine genetic and cell phenotype information.

Predicting treatment outcome based on *ex vivo* assessment has been an attractive concept for decades.[268] However, this approach has so far not been translated into clinical practice. Sample heterogeneity presents some of the challenges. With modern techniques, such as FC used in this study or automated microscopy, sample heterogeneity can be taken into consideration. Superti-Furga and colleagues used an automated immunofluorescence microscopy-based platform to retrospectively predict treatment outcomes of chemorefractory and -sensitive AML samples.[199] The study demonstrated that blast specific drug sensitivity was a better predictor of treatment outcome when compared to overall sample sensitivity. Moreover, marked

improvement in progression free survival was observed when microscopy guided treatment was applied for refractory/relapsed leukemia patients. The data demonstrate, that new techniques can improve the predictive power of *ex vivo* drug screening assays. Another major challenge is to mimic the *in vivo* BM conditions and retain sample viability *in vitro*. Karjalainen et al. showed that conditioned media (CM) derived from HS-5 BM stromal cells, which should more closely resemble the BM microenvironment, had a drastic effect on *ex vivo* drug screening results and cell viability.[269] Increased resistance to specific drugs, particularly to venetoclax, was a result of alternative signaling pathways induced by cytokines present in the media.[269] Similarly, the commercial company Viviabiotech showed that when drugs are tested directly in whole blood samples without mononuclear cell fraction enrichment, the blast cells show lower sensitivities to certain compounds such as topoisomerase II inhibitor idarubicin.[200] It is well acknowledged that patients have a distinct set of genetic alterations, but additionally individuals' BM microenvironment and the expressed cytokines might also vary drastically between patients. Thus, universal-culturing conditions may be difficult to develop. Therefore, screening of leukemic cells directly in the patient's own blood, BM or added serum may be necessary to improve the predictive power. Moreover, many drugs convey their effect through epigenetic modification and differentiation. In the clinical setting these responses are usually observed only after several weeks after treatment initiation. Therefore, the predictive efficacy of such drugs might be challenging to measure in a 3-day drug screening assay. Other future aims include adding antibody-based drugs in the *ex vivo* drug screens which has, however, proven to be a challenge due to their instability.

In summary, phenotype-based screening can increase the functional understanding of drug effects and it can also improve the predictive power of *ex vivo* drug screens. By combining these methodologies with optimal culture conditions and the appropriate drugs, *ex vivo* drug screening might become more common in a clinical setting in the near future.

6 CONCLUSIONS

In this thesis we used exome sequencing to identify novel driver mutations in LGL leukemia and T-ALL. In addition, we assessed which compounds can inhibit the functional activity of mutant STAT3 amongst 306 investigational and pre-clinical compounds. Finally, we developed a flow cytometry-based drug screening platform to assess primary AML sample sensitivity at a detailed cell population level.

1. We identified novel *STAT5B* mutations in 4/211 LGL leukemia patients. Our in vitro assays demonstrated that these somatic mutations result in overactive STAT5B function.

2. We identified similar STAT5B mutations in T-ALL and demonstrated that these events are recurrent in T-ALL. The finding represents a novel mechanism leading to the activation of the IL7R/JAK/STAT5 pathway. We also presented a hypothesis that STAT5B might be involved in the overexpression of anti-apoptotic BCl-xL.

3. Using different cell model systems and a high-throughput drug screening assay, we identified Hsp90 inhibitors to effectively reduce mutant STAT3 activity and phosphorylation. Additionally, Hsp90 inhibitor luminespib was highly effective at reducing the viability of mutant STAT3 NK cell lines and LGL leukemia patient samples.

4. We demonstrate that a flow cytometry-based drug screening platform can accurately measure blast specific drug responses in heterogenous AML patient samples. Furthermore, we show that the resistance of monocytes to BCL2 inhibition by venetoclax may blur the sensitivity of drug screens assessing the viability of unfractionated leukemic samples.

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