Aus der Medizinischen Klinik und Poliklinik III des Klinikums
Der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. rer. nat. Dr. med. Michael von Bergwelt-Baildon

Dissertation
zum Erwerb des Doctor of Philosophy (Ph.D.)
an der
Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

From small molecule to cellular immunotherapy: Therapeutic approaches for Leukemia

vorgelegt von:

Lisa Rohrbacher

aus:

Lam, Deutschland

Jahr:

2022

Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

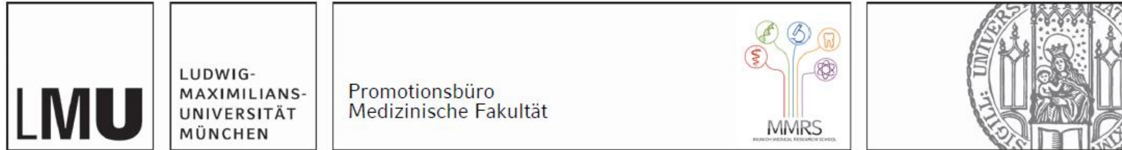
First supervisor: *Prof. Dr. Marion Subklewe*
Second supervisor: *Prof. Dr. Sebastian Kobold*
Third supervisor: *Prof. Dr. Vigo Heissmeyer*

Dean: **Prof. Dr. med. Thomas Gudermann**

Datum der Verteidigung:

17.10.2022

Affidavit



Affidavit

Rohrbacher, Lisa

Surname, first name

I hereby declare, that the submitted thesis entitled:

From small molecule to cellular immunotherapy: Therapeutic approaches for Leukemia

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

München, 12.1.2022

Place, date

Lisa Rohrbacher

Signature doctoral candidate

Confirmation of congruency

Confirmation of congruency between printed and electronic version of the doctoral thesis

Doctoral Candidate: Rohrbacher, Lisa

I hereby declare, that the submitted thesis entitled:

From small molecule to cellular immunotherapy: Therapeutic approaches for Leukemia

is congruent with the printed version both in content and format.

München, 12.1.2022
Place, date

Lisa Rohrbacher
Signature doctoral candidate

Table of contents

Affidavit III
Confirmation of congruency IV
Table of contents V
List of publications VIII
1 Introductory Summary 1
1.1 Hematopoiesis 1
1.2 Leukemia 2
1.3 Targeted Immunotherapy 4
1.3.1 Immunomodulation (Kinase inhibition) 4
1.3.2 T-cell based immunotherapy 6
1.4 Aim 9
1.5 Summary of publications 11
1.5.1 Publication I: The PI3K∂-Selective Inhibitor Idelalisib Induces T- and NK-Cell Dysfunction Independently of B-Cell Malignancy-Associated Immunosuppression[139] 11
1.5.2 Publication II: "A modular and controllable T cell therapy platform for acute myeloid leukemia"[140] 12
2 Publications 15
2.1 Publication I 15
2.2 Publication II 27
3 References 42
4 Acknowledgements 50

List of abbreviations

ACT	Adoptive cell therapy
ALL	Acute lymphocytic leukemia
Allo-SCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
BCR	B-cell receptor
BM	Bone marrow
CAR	Chimeric antigen receptor
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CR	Complete remission
CTLA-4	Cytotoxic T lymphocyte antigen 4
DNA	Deoxyribonucleic acid
ELN	European LeukemiaNet
FDA	U.S. Food and Drug Administration
FLT3	FMS-like tyrosine kinase
GvHD	Graft-versus host disease
GvL	Graft-versus leukemia
HD	Healthy donor
HSC	Hematopoietic stem cell
IDH	Isocitrate dehydrogenase
IFNγ	Interferon gamma
IGHV	Immunoglobulin heavy-chain variable region gene
IL	Interleukin

ITD	Internal tandem duplication
LAG-3	Lymphocyte activation gene 3
LUC	Luciferase
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
NK cell	Natural killer cell
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PI3K	Phosphoinositide-3 kinase
r/r	Relapsed/refractory
SAR	Synthetic antigen receptor
scFv	Single chain variable fragment
SCT	Stem cell transplantation
TAA	Tumor-associated antigen
taFv	Tandem scFv construct
TCR	T-cell receptor
TIL	Tumor-infiltrating lymphocyte
TK	Tyrosine kinase
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TNF	Tumor necrosis factor
TP53	Tumor protein 53
Treg	Regulatory T cells
TRUCK	T cells redirected for universal cytokine-mediated killing
WHO	World Health Organization

List of publications

This thesis includes two publications which have been accepted for publication in peer-reviewed journals:

Publication I:

“The PI3K∂-Selective Inhibitor Idelalisib Induces T- and NK-Cell Dysfunction Independently of B-Cell Malignancy-Associated Immunosuppression”

Lisa Rohrbacher, Bettina Brauchle, Ana Orginc Wagner, Michael von Bergwelt-Baildon, Veit L. Bücklein and Marion Subklewe

Frontiers in Immunology, March 2021, doi: 10.3389/fimmu.2021.608625

Publication II:

“A modular and controllable T cell therapy platform for acute myeloid leukemia”

Mohamed-Reda Benmebarek, Bruno L. Cadilha, Monika Herrmann, Stefanie Lesch, Saskia Schmitt, Stefan Stoiber, Abbass Darwich, Christian Augsberger, Bettina Brauchle, Lisa Rohrbacher, Arman Oner, Matthias Seifert, Melanie Schwerdtfeger, Adrian Gottschlich, Felicitas Rataj, Nadja C. Fenn, Chrisitan Klein, Marion Subklewe, Stefan Endres, Karl-Peter Hopfner & Sebastian Kobold

Leukemia, January 2021, doi: 10.1038/s41375-020-01109-w

1 Introductory Summary

1.1 Hematopoiesis

The cells of the immune system, as well as all other cellular components of the human blood, are derived from hematopoietic stem cells (HSCs) (Fig.1)[1-4]. HSCs are capable of asymmetric cell division, which means they are able to self-renew and thereby maintain their number while also differentiating into a cascade of progenitor cell stages to replenish the entire blood system[2, 5-7]. With every differentiation step, progenitor cells continuously lose their multilineage potential and become lineage-restricted progenitors with limited capacity to divide[8]. HSCs produce to two distinct types of progenitor cells. On the one side, there is the common lymphoid progenitor, from which lymphoid cell lines like B cells, T cells, and Natural Killer (NK) cells evolve. On the other side, there is the common myeloid progenitor cell, from which myeloid cell lines like monocytes, erythrocytes, and mast cells evolve[5, 9, 10]. Differentiation arrest and uncontrolled proliferation lead to subsequent failure of the healthy blood-building system and to various blood cell diseases like leukemia.

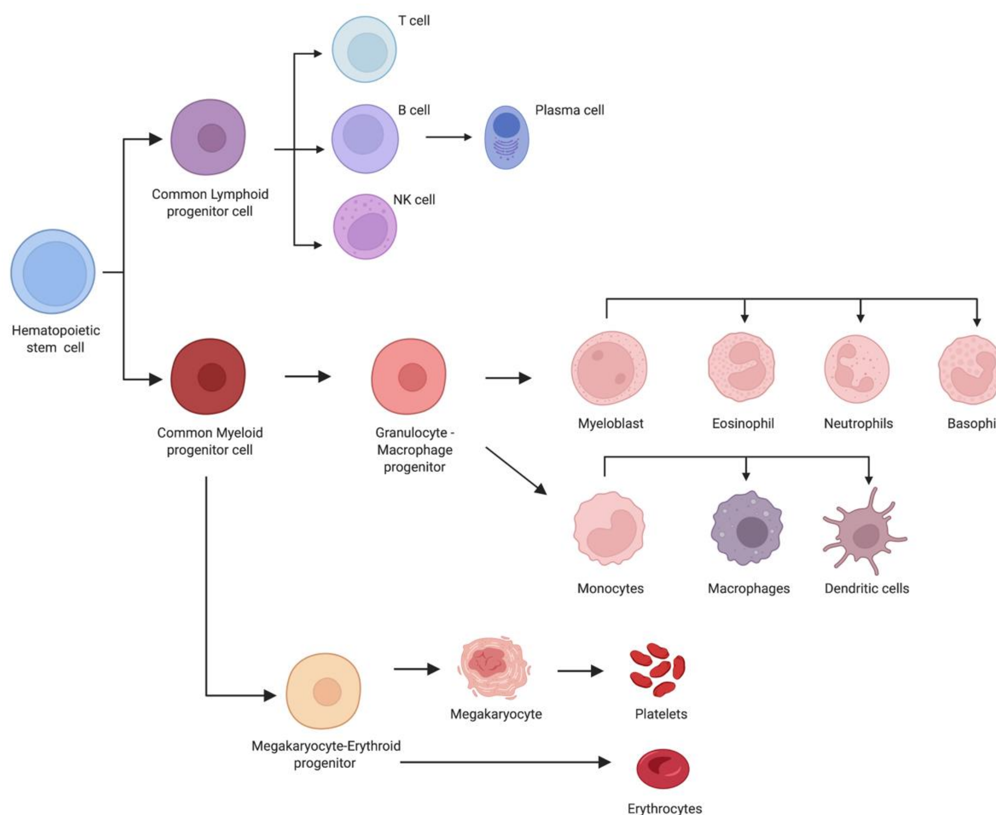


Figure 1: Schematic presentation of the hematopoietic system (Adapted Zhang et al., 2019[2], created with BioRender.com)

1.2 Leukemia

Leukemia is the common name for a variety of malignant hematologic disorders, which are defined by disrupted differentiation and uncontrolled proliferation of primitive or atypical cells in the blood and bone marrow[11, 12]. While the exact cause of leukemia, as in many other cancers, is unknown, there is evidence that the accumulation of multiple driver mutations disrupts the regulation of cell differentiation and death[12-15]. The genetic lesions lead to a maturation arrest that enables leukemic cells to continuously proliferate, and prevent the apoptosis seen in normal blood cells[16]. Risk factors underlying these alterations can be both acquired and inherited. These include other hematologic disorders, genetic disorders like Down syndrome, as well as prior DNA-damaging therapies with topoisomerase II-inhibitors, alkylating agents, or radiation therapy for a previous malignant disorder[12, 17].

Depending on the rate of disease progression and the predominant type of blood cell affected, leukemias are classified into four major subtypes: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL)[11, 18].

Acute leukemias are characterized by a rapid clonal expansion and accumulation of abnormal, immature, and non-functional blood cells which prevent the maturation of healthy blood cells. The fast progression of this disease requires immediate treatment. AML is caused by uncontrolled proliferation and impaired differentiation of myeloid progenitor cells[19-21]. With a prevalence of 4.3 newly diagnosed cases per 100.000 people per year, AML is the most common form of acute leukemias in adults[22]. The incidence of AML increases with age leading to a median age of 68 years at diagnosis[22]. Clinical manifestations of AML include accumulation of malignant, partially differentiated myeloid cells within the bone marrow (BM), peripheral blood (PB), and infrequently in other organs[17]. Therefore, morphologic assessment of bone marrow aspirates and blood smears are used as diagnostic procedures to classify AML. The diagnosis of AML is made by the presence of ≥ 20% leukemic blasts in the PB or BM, or regardless of the blast count, in the presence of unique genetic abnormalities like chromosomal translocations in the bone marrow cells[12, 17, 21, 23]. AML can be classified according to different systems. The World Health Organization (WHO) classification categorizes AML according to morphologic, cytogenetic, and genetic properties. The European LeukemiaNet (ELN) risk stratification system incorporates cytogenetic abnormalities and genetic mutations to provide prognostic information for patients,

which are divided into three risk groups to predict relapse-free and overall survival[24-26].

The standard treatment paradigm for patients with AML consists of intensive induction chemotherapy, also known as the “7+3” regimen[17, 21, 27, 28]. Induction chemotherapy leads to complete remission (CR) in 40-50% of patients ≥ 60 years of age and 60-80% of younger patients[17, 29-32]. However, the risk of relapse caused by the persistence of chemo resistant-leukemic cells remains high. The aim of post-remission consolidation therapies is to eliminate these cells[33]. Post-remission treatment consists of additional chemotherapy for patients with a favorable genetic risk profile or allogeneic stem cell transplantation for patients with a non-favorable genetic risk profile[17, 28, 34, 35]. The current five-year relative survival rate of patients with AML is 29.5%[22]. However, progress in the understanding of the pathophysiology of this disease has occurred rapidly over the past years, leading to the approval of various new agents for different indications in AML. These agents include several targeted therapies like venetoclax, FLT3 or IDH inhibitors, TP53 modulators, and others revolutionized the treatment of patients with AML[23, 31, 36-39]. Combinatorial treatments of previously mentioned therapeutic strategies are currently under investigation in several clinical trials and may improve treatment options even further[40].

Chronic leukemias are characterized by a slow but excessive accumulation of partially mature, abnormal blood cells crowding out healthy cells in the BM[41]. CLL is caused by clonal proliferation of typically CD5+ B cells and their accumulation in PB, BM, lymph nodes, and spleen[42-45]. With a prevalence of 4.9 newly diagnosed cases per 100.000 people a year, CLL is the most frequent chronic leukemia among adults. The incidence of CLL increases with age leading to a median age of 65 to 70 years at diagnosis[46, 47]. As symptoms due to marrow infiltration are rare in this slowly developing hematologic disorder, most CLLs are detected during routine blood tests[48, 49]. The diagnosis of CLL is made by the presence of ≥ 5000 B cells per µL of PB and the immunophenotypic assessment of the blood cells for co-expression of CD5, CD19, CD20, and CD23[42, 48, 50]. Patients with CLL undergo clinical risk stratification according to the staging systems developed by Rai and Binet[49, 51-55], the former being more frequently used in the US and the latter mainly used in Europe. Furthermore, the mutational status of TP53 and IGHV provide prognostic information to predict the aggressiveness of disease and survival[54, 56-60]. When it comes to the treatment of CLL, newly diagnosed patients that present with asymptomatic and early-stage disease should be monitored until disease

progression. Patients with symptoms or advanced disease require treatment[61, 62]. Monotherapy with alkylating agents like Chlorambucil was the therapeutic "gold standard" for several decades[42, 61, 63]. Nowadays it is mainly used as an affordable option to achieve palliation in unfit or elderly patients[61]. Current first-line treatment regimens consist of the monoclonal therapeutic antibodies rituximab or obinutuzumab as part of either chemotherapy or targeted therapy with ibrutinib or venetoclax[61, 64-67]. The current five-year relative survival rate of patients diagnosed with CLL is 87.2%[46].

1.3 Targeted Immunotherapy

Treatment of leukemia depends on several factors, like the patient's age and fitness, leukemia subtype including genetics, clinical presentation, and progression of the disease. Unfortunately, with current treatment regimens, many patients still relapse due to the persistence of chemo-refractory leukemia-initiating cells. Therefore, the need for novel therapeutic approaches remains high. In 1891, William B. Coley first came up with the idea to deploy the patient's immune system to target tumor cells dates. He observed that the injection of a combination of inactivated *Streptococcus pyogenes* and *Serratia marcescens,* also referred to as Coley's toxin, can stimulate an immune response against tumors[68, 69]. However, due to the tumor cells' capability to escape recognition by the immune system, immunotherapies achieved only limited clinical efficacy, wherefore surgery, chemo- and radiotherapy were adopted as standard treatment regimens over decades[70, 71]. As the understanding of the key mediators of the immune system evolved also novel therapeutic strategies for the treatment of leukemia evolved. Immunotherapeutic strategies to treat leukemia include various approaches, ranging from strategies to stimulate effector immune cells to boost the patient's own immune system to strategies counteracting immunosuppressive mechanisms. These strategies encompass immunomodulators and adoptive cell therapy (ACT)[72].

1.3.1 Immunomodulation (Kinase inhibition)

Tyrosine kinases (TKs) are a family of molecules transferring phosphate groups from ATP to tyrosine residues in downstream proteins leading to the activation of intracellular signaling cascades[73, 74]. TKs regulate a variety of intracellular functions like cell growth, proliferation, differentiation, and apoptosis[73-76]. Mutation, deregulation,

or overexpression of these TKs plays a major role in oncogenesis and has been reported in several hematologic malignancies[73, 74, 77-79].

Therefore, targeting of TKs involved in these signaling cascades has emerged as a promising treatment strategy. Tyrosine kinase inhibitors (TKI) are targeted oral medications used to disrupt signal transduction pathways of kinase proteins contributing to oncogenesis and tumor growth[78-80].

The first FDA-approved TKI for oncology, imatinib, is used to treat Philadelphia-chromosome-positive CML[80, 81]. Since the approval, the interest in TKIs as a cancer treatment has grown. As of now, over 50 TKIs have been FDA-approved, several of them as a medication for hematologic diseases[82, 83]. All TKIs basically share the same mode of action. They specifically inhibit tyrosine phosphorylation of various oncogenic proteins by competitive blockade of the kinases' ATP-binding site, thereby regulating aberrant downstream signaling cascades involved in cancer proliferation, invasion, and angiogenesis[84].

Overexpression or constitutive activation of multiple TKs and downstream effectors are nowadays known to be key players in the pathogenesis of AML[38, 85, 86]. A promising target for TK inhibition in AML is the FMS-like tyrosine kinase 3 (FLT3) gene[87]. About 30% of all AML patients present with mutations in this gene. They can be distinguished into FLT3- tyrosine kinase point mutations (FLT3-TKD) or FLT3 internal tandem duplication mutations (FLT3-ITD)[88-90]. Both mutations cause uncontrolled signaling through different pathways and thereby render the survival of the malignant cells dependent on FLT3-signaling pathways[91-95]. Hence, blocking FLT3 kinase activity is a promising treatment strategy for AML.

Overexpression or constitutive activation of B-cell receptors (BCRs), several TKs, and downstream mediators also play a major role in the pathogenesis of B-cell malignancies like CLL (Fig. 2)[64, 96-99]. Deregulation of these pathways leads to apoptosis resistance and thereby to prolonged survival of malignant cells. Idelalisib is an immensely specific small-molecule inhibitor of the phosphoinositide-3 kinase catalytic subunit delta (PI3K∂). PI3K∂ activation leads to phosphorylation of the serine/threonine kinases AKT and its downstream effector mTOR and thereby is an important regulator of malignant B-cell proliferation and enhanced survival[100-103]. Idelalisib competitively blocks the ATP-binding site of the catalytic domain of PI3K. As a result, downstream signaling cascades including BCR and C-X-C chemokine receptors type 4 and 5 signaling are abrogated. This in turn leads to reduced trafficking,

homing capacity, and apoptosis of malignant cells[104, 105]. In 2014, Idelalisib, in combination with Rituximab, received approval for the first-line treatment of CLL patients with the 17p deletion or TP53 mutation and in the relapsed/refractory (r/r) CLL setting[105-107]. Furthermore, Idelalisib was approved for patients with r/r follicular B-cell non-Hodgkin lymphoma or r/r small lymphocytic lymphoma with at least two preceding lines of therapy[107, 108].

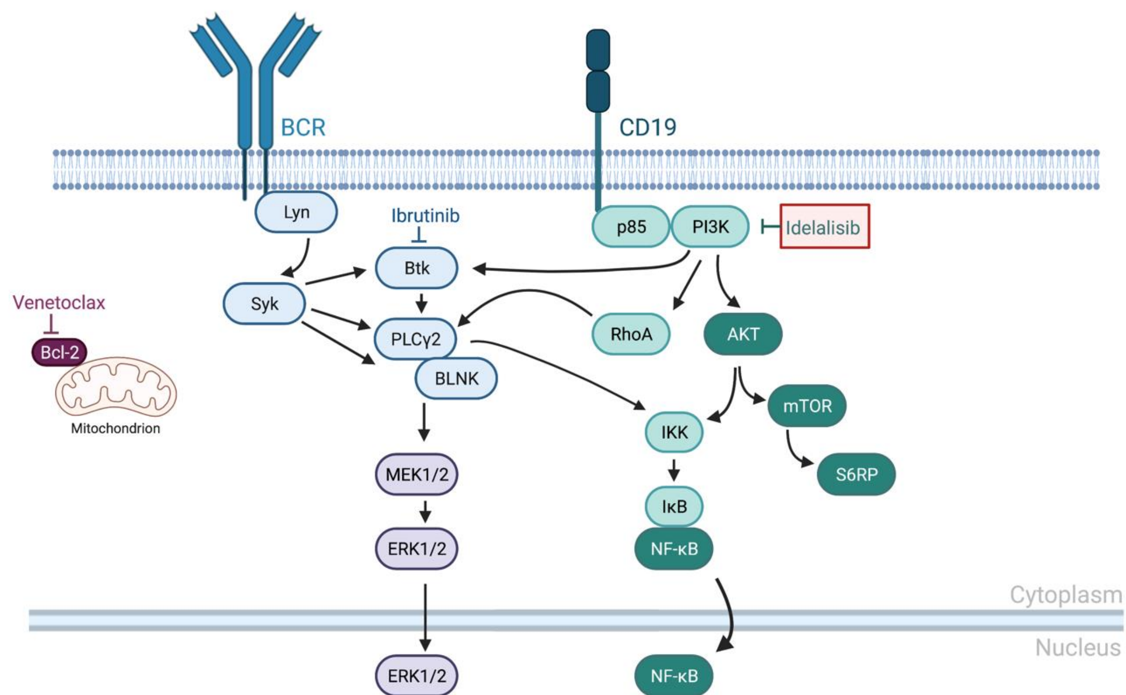


Figure 2: Simplified illustration of signaling networks downstream of CD19 and BCR. (Adapted from Skanland et al., 2020[109], created with BioRender.com)

1.3.2 T-cell based immunotherapy

1.3.2.1 Allogeneic stem cell transplantation

The most potent T-cell based treatment strategy for malignant diseases like chronic and acute leukemias is allogeneic stem cell transplantation (allo-SCT). To date, it is the best anti-leukemic option for patients with AML with intermediate or high-risk genetic markers and the only curative treatment option for (r/r) AML patients[33, 110, 111]. This treatment regimen consists of intensive conditioning chemotherapy in combination with or without whole-body irradiation[112, 113]. The goal of this preconditioning therapy is to reduce the leukemic burden and to weaken the patient's own immune system to allow engraftment of donor hematopoietic cells. The

transplanted hematopoietic stem cells replace the stem cells in the bone marrow and reconstitute normal hematopoiesis[112]. Furthermore, donor alloreactive T cells eliminate residual leukemic cells in a process called graft-versus-leukemia (GvL) effect[34, 114-116]. However, donor alloimmune responses can also affect healthy tissue. This is known as graft-versus-host-disease (GvHD). Balancing the GvL against the GvHD risk, without reducing the efficiency of the GvL effect and possible treatment failures has been a major hurdle of this strategy[117, 118]. Furthermore, age, comorbidities, toxicity, and availability of a suitable donor translate into a minority of AML patients that undergo transplantation[26, 119].

Another strategy, which reduces the risks of alloreactive side effects is to use the patients' own T cells to fight cancer cells (Fig. 3).

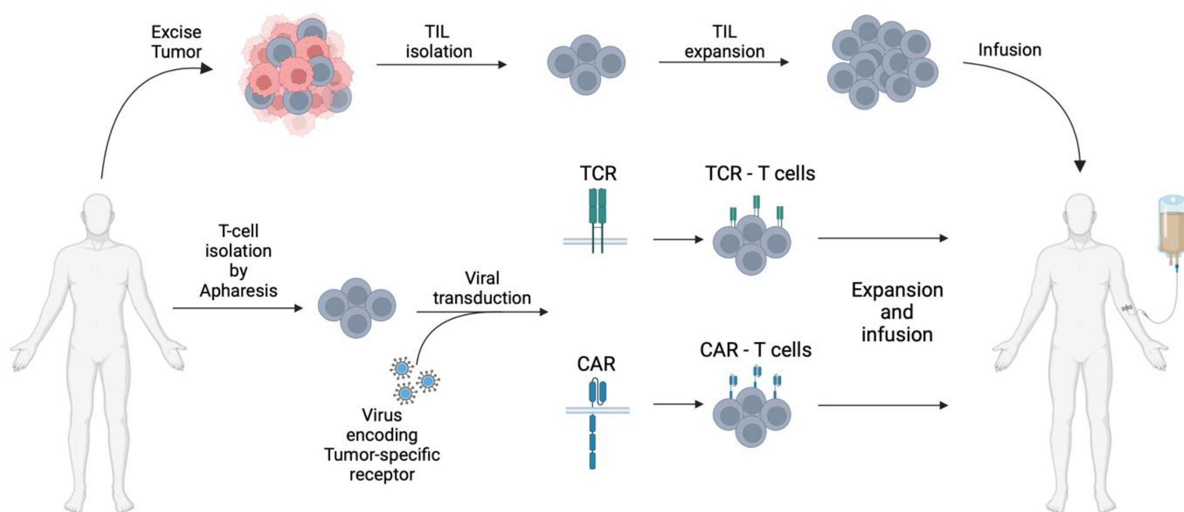


Figure 3: Illustration of different ACT approaches for cancer treatment, including tumor-infiltrating lymphocytes or genetically modified TCR- or CAR-T cells. (Adapted from Zah et al, 2017[120], created with BioRender.com)

1.3.2.2 Adoptive T-cell transfer

Adoptive T-cell transfer involves the isolation, expansion, and re-infusion of autologous or allogeneic T cells[121]. Therefore, sufficient proliferation, persistence, and survival must be achieved in order to ensure efficient anti-tumoral effector functions[122, 123]. Three different modalities of ACT have developed from the idea to utilize the patient's own immune system to fight cancer cells. These modalities differ in the source of the T cells, as well as the need to genetically engineer the harvested T cells[122]. For the

first approach, T cells are directly isolated out of the tumor tissue. This approach is also known as tumor-infiltrating lymphocyte (TIL) therapy[124]. For TIL therapy, the harvested T cells need to be selected for specificity, expanded, activated, and ultimately reinfused into the patient[123]. The advantage of this approach is that it is independent of genetic engineering. However, there are several prerequisites for successful TIL therapy, like the existence of a tumor-reactive T-cell population in the tumor as well as accessibility for biopsy to be able to isolate a sufficient amount of these T cells[125]. To overcome these limitations, T-cell receptor (TCR) engineered T cells and chimeric antigen receptor (CAR) T cells were developed[126]. Both of these approaches rely on T-cell isolation from the PB by apheresis, followed by genetic engineering to render T cells specific for a suitable tumor-associated antigen (TAA)[124]. The major difference between these approaches is restriction by TCR-major histocompatibility complex (MHC) interaction[127]. TCR therapy utilizes the natural mechanism of T-cell activation and therefore relies on the recognition of tumor-specific epitopes presented by the MHC complexes on the tumor cell surface[124]. CAR T cells in contrast can target virtually any surface molecule presented by a tumor cell and are MHC-independent[127]. This is achieved by the architecture of the CAR. The composition of the CAR has evolved over the past couple of years (Fig. 4)[128-130]. First-generation CARs consisted of a target binding single-chain variable fragment (scFv) linked to a cytoplasmic signaling domain containing the CD3□ chain of the TCR[131, 132]. These two segments are chained together by a spacer and the transmembrane domain. However, these CAR T cells were not capable of mounting persistent anti-tumor responses due to their limited signaling capability[133, 134]. To improve activation, persistence, and successful expansion of the genetically modified cells, a costimulatory signaling domain, mostly consisting of CD28 or 4-1BB, was added to the original structure[135, 136]. These second-generation constructs represent the foundation for the currently clinically approved CAR T-cell therapies. However, there are various combinations in pre-clinical clinical settings, for example, third-generation CAR T cells contain two costimulatory domains, and fourth-generation CAR T cells, also referred to as TRUCKs, even include transgenes for cytokine secretion or additional costimulatory ligands[137, 138].

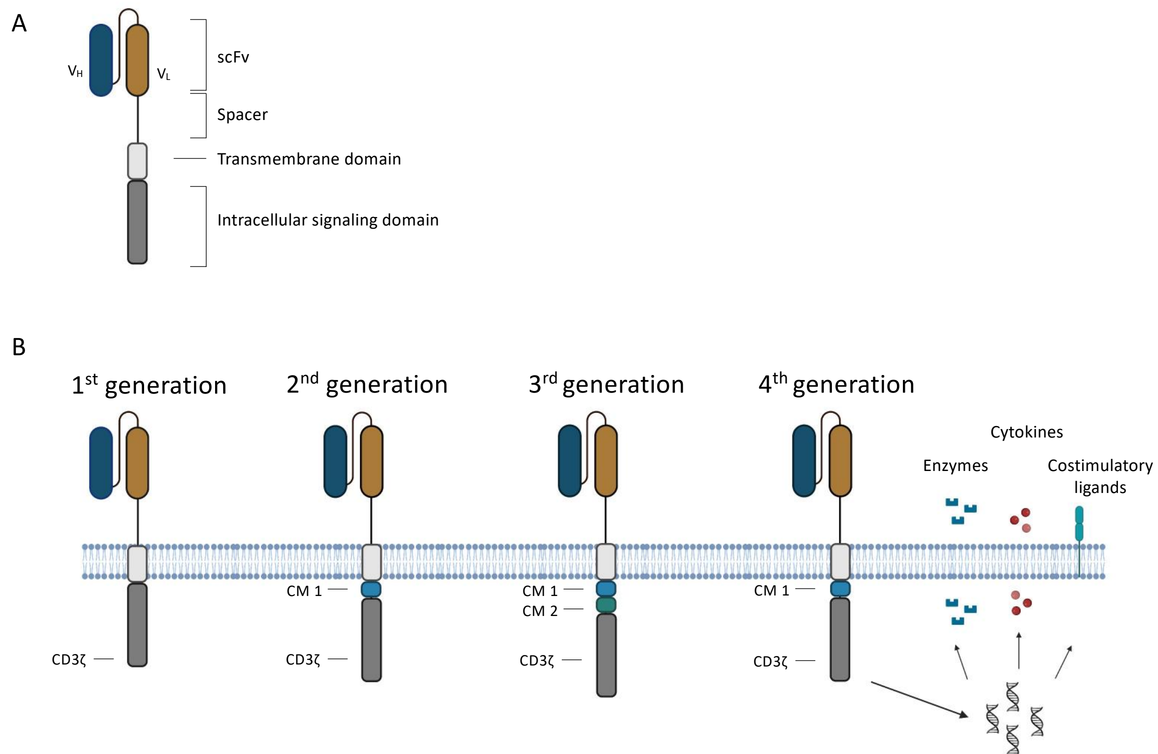


Figure 4: A) The general structure, depicting the major components of CARs. B) Overview of different CAR generations. CM = Costimulatory Molecule (Adapted from Stock et al, 2019[129], created with BioRender.com)

1.4 Aim

It is well known that T cells play a key role in the immune system and the fight against cancer. Therefore, T-cell-based immunotherapies became a major focus of cancer research over the past years and have revolutionized the field of oncology.

T-cell function impacts the efficacy of these promising treatment approaches. Within these projects, we aimed to develop strategies to improve T-cell-based immunotherapy in leukemia. We therefore investigated cellular immune modulation through small inhibitors and studied a modular CAR T platform in the context of leukemia. In the first part of this thesis, we wanted to understand how interfering with one of the most commonly activated signaling cascades in CLL will modulate the phenotype and function of healthy immune cells, especially T and NK cells. In the second part, we tried to improve CAR T cell therapy for patients with AML. Therefore, an adjustable and controllable adoptive T-cell therapy platform, which provides the benefit of a higher safety profile and eventually allows a personalized choice of the target antigen, was developed and extensively tested.

Perspectively, we believe that immunotherapy will only be successful by combinatorial approaches and that the combination of T-cell modulation with synthetic immune modulation will be needed to successfully improve the clinical outcome of patients with leukemia. The gained insights might serve as a rationale for further combinatorial strategies for patients with leukemia.

1.5 Summary of publications

1.5.1 Publication I: The PI3K∂-Selective Inhibitor Idelalisib Induces T- and NK-Cell Dysfunction Independently of B-Cell Malignancy-Associated Immunosuppression[139]

In this publication, we examined how idelalisib treatment might contribute to the elevated rate of opportunistic infections seen in CLL patients. We therefore looked into the impact of idelalisib on different cell types in both healthy donors (HD) and patients with CLL. Analysis of CFSE dilution showed no impact of idelalisib on the proliferative capacity of T cells. Subset composition of T cells was also not affected. Expression analysis revealed that coculture with idelalisib led to a significant downregulation of various inhibitory checkpoint molecules on the surface of T cells and Tregs. To assess whether these downregulations are indicative of impaired function, T-cell mediated tumor cell lysis and cytokine secretion were evaluated in cocultures with idelalisib. Cultivation of T cells with idelalisib led to a significantly reduced specific lysis of tumor targets. This was accompanied by decreased secretion levels of perforin and granzyme B, as well as IL-10, TNF, and IFNγ.

To see if other types of immune cells are impacted as well, we analyzed the effect of PI3K∂ blockade on NK cells. Coculture with idelalisib reduced the proliferative capacity of NK cells, especially affecting the cytotoxic NK-cell compartment. Moreover, blockade of PI3K∂ impaired two different apoptotic pathways used by NK cells. Perforin/granzyme B-mediated, as well as Fas-FasL-mediated tumor cell lysis were significantly reduced upon coculture with idelalisib.

Further studies on the effect of idelalisib on other types of innate immune cells showed that PI3K∂ blockade does not alter the phagocytic capacity of monocytes. Also, Seahorse analysis of the oxidative burst upon activation of neutrophils revealed no effect of PI3K∂ blockade.

After *in vitro* evaluation of idelalisib, *ex vivo* studies were performed. T cells harvested from 15 primary CLL samples were analyzed in proliferation and cytotoxicity experiments. Coculture with idelalisib had no effect on the proliferation of these T cells. However, idelalisib significantly impaired the cytotoxic capacity of these T cells towards tumor cells. Furthermore, the degranulation of granzyme B, as well as the expression level of PD-1, were significantly reduced upon culture with idelalisib. This was accompanied by a significant decrease in the secretion of IL-10, IL-4, IL-6, and IFNγ.

I contributed to this manuscript by collecting data for patient characterization and performing the experiments which led to the key findings of this publication. More precisely, I cultivated all cell lines that were used and collected the healthy donor PBMCs used for the experiments. Furthermore, I conducted the *in vitro* proliferation assays, the cytometric bead arrays, T-cell cytotoxicity assays, NK-cell cytotoxicity assays, phagocytosis assays, as well as the neutrophil activation assays. Also, I performed the experiments on T-cell function of cells derived from CLL patients. In addition, I interpreted the data, designed the figures, and wrote the manuscript. (Figures 1,2,3,4,5 and 6; Supplementary Figures S1 and S2).

1.5.2 Publication II: "A modular and controllable T cell therapy platform for acute myeloid leukemia"[140]

In this study, published by Benmebarek et al in Leukemia 2021, a flexible and controllable platform for adoptive T-cell therapy of AML evolved. This approach combines T cells, transduced to express synthetic agonistic receptors (SAR), with tandem scFv (taFv) adapter molecules. The SAR contains an inert extracellular domain (EGFRvIII) as an antigen-binding domain linked to the signaling domains CD28 and CD3ζ inside the cell. The SAR ectodomain is absent on naturally existing T cells and therefore requires a specifically engineered adapter molecule for activation. Furthermore, the lack of natural ligands confers reduced toxicity and, if needed, SAR T cell function can be abrogated by the administration of FDA-approved drugs like cetuximab. To further improve safety, previously used bispecific antibodies were replaced with taFv constructs with a shorter half-life. These confer two specificities, one targeting AML and the other one targeting EGFRvIII on the SAR-transduced T cell. Two tumor target antigens were tested in this study, namely CD33 and CD123.
In a first step, binding studies to assess the dissociation dynamics of both constructs were performed. Analysis was performed via flow cytometry. Both constructs were designed with the same backbone with a low affinity for T cells and a high affinity for the tumor target antigen. The *in vitro* experiments showed that only SAR T cells that were in contact with both, the tumor target and the adapter taFv construct were activated and produced IFNγ. Furthermore, an increase in T-cell proliferation, as well as upregulation of PD-1 was measured in these SAR T-cell cultures. In contrast,

control T cells cultured with target cells and taFv constructs did not show any signs of activation.

Next, we tested SAR T- cell function in *in vitro* cytotoxicity assays. SAR T cells were incubated with different leukemic cell lines and 1µg/mL of adapter taFv molecule. Only SAR T cells, but not control T cells mediated efficient tumor-cell lysis. To gain more insight into the way SAR T cells function, we evaluated and characterized synapse formation via fluorescence microscopy. Significantly more conjugates were formed in the SAR T-cell cultures compared to control T-cell cultures. Strong F-actin, CD11a-LF1, and Granzyme B accumulation, indicated functionality of the immune synapse. We could also show the efficiency and functionality of the safety switch used in this platform. In absence of taFv redosing, the molecule was cleared from the circulation. In contrast, repeated redosing maintained SAR activity. An advantage of the short half-life of the adapter molecules is the modularity and flexibility of this platform. We again demonstrated the modularity of this system as the same T cells could be redirected towards tumor cells by sequentially targeting multiple AML-associated antigens.

In the next step, we wanted to validate SAR T-cell function in *ex vivo* experiments. We performed long-term coculture assays with HD T cells transduced to express the SAR and primary AML blasts. SAR T cells in combination with the CD33-taFv molecule mediated specific AML cell lysis over time. We could further validate our findings in an autologous setting. Patient-derived SAR T cells cocultured with both taFv adapter molecules specifically targeted their own AML blasts. This was accompanied by increased expression levels of PD-1, TIM-3, and CD69 on the SAR T cells. In addition, the SAR-taFv combination also demonstrated cytolytic activity towards $CD34^{+}CD38^{-}$ leukemic stem cells.

As a last step, the *in vitro* and *ex vivo* findings were validated in *in vivo* mouse studies. Two different NSG mouse models were established. The models mainly differed in the AML cell line that was engrafted into the mice. The mice in both models were treated with the genetically engineered T cells in combination with the CD33-taFv molecule. This treatment led to major responses. Complete remission was achieved in two out of seven mice in the MV4-11 model and one out of five mice in the THP-1 model. Overall survival significantly improved in both models when compared to the group that received SAR T cells plus a CD19-taFv control construct. The modularity of this platform was also tested and validated *in vivo*.

I contributed to this manuscript by collecting information for patient characterization, including cytogenetic and molecular data. Furthermore, I was involved in the performance of long-term culture assays with patient-derived AML blasts. I set up allogeneic cocultures, performed flow cytometric analysis, and helped with the interpretation of the data generated from these experiments. Moreover, I was involved in the autologous cocultures by isolation the T cells for the generation of SAR-T cells and setting up cocultures of transduced cells and patient-derived AML blasts. I also performed the flow cytometric analysis of these experiments, harvested supernatant for ELISA quantification, and helped with the interpretation of the data generated from these experiments. (Figure 4 A, B, C, and D; Supplementary Fig. 3E).

2 Publications

2.1 Publication I

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 March 2021
doi: 10.3389/fimmu.2021.608625

The PI3K∂-Selective Inhibitor Idelalisib Induces T- and NK-Cell Dysfunction Independently of B-Cell Malignancy-Associated Immunosuppression

*Lisa Rohrbacher[1,2], Bettina Brauchle[1,2], Ana Ogrinc Wagner[1,2], Michael von Bergwelt-Baildon[1,2,3], Veit L. Bücklein[1,2] and Marion Subklewe[1,2,3]**

[1] *Laboratory for Translational Cancer Immunology, Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany,* [2] *Department of Internal Medicine III, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany,* [3] *German Cancer Research Center (DKFZ), German Cancer Consortium (DKTK), Heidelberg, Germany*

OPEN ACCESS

Edited by:
Roberto Benelli,
San Martino Hospital (IRCCS), Italy

Reviewed by:
Aaron James Marshall,
University of Manitoba, Canada
Vassilios Lougaris,
University of Brescia, Italy

Correspondence:
Marion Subklewe
marion.subklewe@med.uni-muenchen.de

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *21 September 2020*
Accepted: *11 February 2021*
Published: *15 March 2021*

Citation:
Rohrbacher L, Brauchle B, Ogrinc Wagner A, von Bergwelt-Baildon M, Bücklein VL and Subklewe M (2021) The PI3K∂-Selective Inhibitor Idelalisib Induces T- and NK-Cell Dysfunction Independently of B-Cell Malignancy-Associated Immunosuppression. Front. Immunol. 12:608625. doi: 10.3389/fimmu.2021.608625

B-cell receptors, multiple receptor tyrosine kinases, and downstream effectors are constitutively active in chronic lymphocytic leukemia (CLL) B cells. Activation of these pathways results in resistance to apoptosis and enhanced survival of the leukemic cells. Idelalisib is a highly selective inhibitor of the PI3K p110∂ isoform and is approved for the treatment of CLL in patients with relapsed/refractory disease or in those harboring 17p deletions or tp53 mutations. Despite the initial excitement centered around high response rates in clinical trials of idelalisib, its therapeutic success has been hindered by the incidence of severe opportunistic infections. To examine the potential contribution of idelalisib to the increased risk of infection, we investigated the effects of idelalisib on the immune cell compartments of healthy donors (HDs) and CLL patients. PI3K∂ blockade by idelalisib reduced the expression levels of inhibitory checkpoint molecules in T cells isolated from both HDs and CLL patients. In addition, the presence of idelalisib in cultures significantly decreased T-cell-mediated cytotoxicity and granzyme B secretion, as well as cytokine secretion levels in both cohorts. Furthermore, idelalisib reduced the proliferation and cytotoxicity of HD NK cells. Collectively, our data demonstrate that both human T and NK cells are highly sensitive to PI3K∂ inhibition. Idelalisib interfered with the functions of T and NK cell cells from both HDs and CLL patients. Therefore, idelalisib might contribute to an increased risk of infections regardless of the underlying B-cell malignancy.

Keywords: cancer immunotherapy, chronic lymphocytic leukemia, idelalisib, immune effector cells, PI3K inhibition

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by impairment of the immune system and is therefore associated with an increased susceptibility to opportunistic infections (1–5). Several factors contribute to this increased risk profile: CLL cells compromise the development of healthy B cells, cause immunosuppression due to their close proximity to effector cells,

modulate T-cell function, and cause immunoglobulin deficiency (6–9). Therefore, immunotherapeutic approaches are indicated and first-line treatments consist of rituximab or obinutuzumab as part of either chemoimmunotherapy or targeted therapy with ibrutinib and venetoclax (10). The latter target B-cell receptor (BCR) signaling and downstream receptor tyrosine kinases, which play a key role in the pathogenesis of CLL (11–17).

Idelalisib is a potent small-molecule inhibitor of phosphoinositide 3-kinases (18, 19). PI3K is one of the most commonly activated kinases in the BCR signaling cascade (20–23). Class I PI3Ks are comprised of a regulatory subunit and one of four catalytic subunits (p110 α, β, γ, and ∂)(24–27). These isoforms differ in tissue expression: PI3Kα and PI3Kβ are ubiquitously expressed, whereas PI3Kγ and PI3K∂ are highly enriched in the hematopoietic compartment (19, 21, 25, 28–30). Mechanistically, PI3K∂ activates the serine/threonine kinases AKT and mammalian target of rapamycin (mTOR), which leads to proliferation, differentiation, and enhanced survival of the cancer cells (19, 24, 25, 31–33). Idelalisib binds to the ATP-binding pocket of the catalytic subunit of PI3K, thereby specifically abrogating downstream PI3K∂/AKT signaling and inducing apoptosis of malignant cells (34, 35). Idelalisib, has been evaluated in conjunction with rituximab in a randomized, double-blind, phase III study in patients with CLL (ClinicalTrials.gov Identifier: NCT01539512). Due to high response rates of 81%, idelalisib was approved by the US Food and Drug Administration for the first-line treatment of CLL patients with the 17p deletion or TP53 mutation, and also in the relapsed or refractory (r/r) setting (35, 36).

However, after the initial excitement, three clinical trials involving idelalisib reported a high rate of adverse events, including severe diarrhea, liver toxicity, pneumonitis, severe colitis, and serious infections (37–42) (ClinicalTrials.gov Identifiers: NCT01539512, NCT01732913, NCT01569295). Although a high rate of opportunistic infections in CLL patients is well-documented (43–45), idelalisib treatment not only increased the incidence but also added other immune-related adverse events (46). In a randomized phase III trial in r/r CLL, patients were treated with Rituximab plus Idelalisib (IDELA/R-to-IDELA) vs. Rituximab monotherapy (placebo/R). The group with IDELA/R-to-IDELA had a higher incidence of infection or infestation: 53.6 vs. 23.1%, with lower respiratory tract infection in 23.6 vs. 11.1% (47). Opportunistic infections were a common cause with 5 vs. 1 patient presenting with pneumocystis jirovecii pneumonia, 2 vs. 0 patient presenting with cytomegaly virus infection and 22 patients with fungal infection in the IDELA/R-to-IDELA group. Confirmatory data were reported from another phase III trial comparing ofatumumab with and without Idelalisib in pretreated CLL patients with serious infections being more common in the ofatumumab plus idelalisib group: pneumonia was reported in 23 vs. 8 patients, sepsis in 11 vs. 1 patient, and pneumocystis jirovecii pneumonia in 8 vs. 1 patient (48). This resulted in 22-treatment-related deaths in the ofatumumab plus Idelalisib vs. only 6-treatment-related deaths in the ofatumumab group.

These observations suggest for an additive negative impact of idelalisib on immune effector cells. Although many studies have focused on the influence of idelalisib on CLL cells, less is known about the impact of PI3K∂ blockade on healthy immune cells.

In this study, we investigated the immunomodulatory influence of idelalisib on the adaptive cellular immune compartment. To start dissecting the effect of idelalisib on cellular immune responses independently of its impact on CLL cells, we isolated T and natural killer (NK) cells from healthy donor (HD) peripheral blood (PB). We analyzed changes in the proliferative behavior, cytokine secretion, and cytotoxic capacity of cocultures in the presence of idelalisib. In a second step, we sought to replicate these findings with samples from CLL patients. Our data suggest that idelalisib interferes with T- and NK-cell function, thereby adding to the already increased rate of opportunistic infections in CLL patients.

MATERIALS AND METHODS

Idelalisib and Key Reagents

Idelalisib was provided by Gilead Sciences. A 10 mM stock solution of idelalisib was prepared in dimethyl sulfoxide (DMSO; Serva, 20385.01) and stored at −20°C. The used concentrations of 0.05, 0.5, and 1 µM were chosen to mimic the peak plasma concentrations observed in patients after 150 mg twice daily administration of idelalisib (49). Sources of all key reagents are listed in **Supplementary Table 1**.

Patients

PB samples from HDs and patients with CLL were collected with written consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilian University of Munich. Patient characteristics are summarized in **Table 1**. The patients had a median age of 64.5 years with a percentage of female patients of 46.7%.

Cell Lines

Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were authenticated by their short tandem repeat profile. All cell lines were tested monthly for *Mycoplasma* contamination with the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-705) according to the manufacturer's instructions. Cells were passaged twice a week and cultured in RPMI 1640 medium (PAN Biotech, P04-16500) media supplemented with 10% fetal calf serum (Thermo Fisher Scientific, 10270106), 1% HEPES (Carl Roth, HN78.1) and 1% penicillin-streptomycin-glutamine (PSG) (Thermo Fisher Scientific, 10378016) at 37°C in a 5% CO_2 atmosphere. Cells were used within 2 months of thawing.

Abbreviations: PI3K∂, Phosphoinositide 3-kinase delta; PB, Peripheral blood; PBMC, Peripheral blood mononuclear cell; CLL, Chronic lymphocytic leukemia; HD, Healthy donor; NK cell, Natural killer cells; AKT, Protein kinase B, or PKB; mTOR, mammalian target of rapamycin; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; PD-1, Programmed cell death protein 1; LAG-3, Lymphocyte activation gene 3; IL-2, Interleukin 2; IL-10, Interleukin 10; TNF, Tumor necrosis factor; IFN-γ, Interferon-γ; FasL, Fas ligand; MFI, Median fluorescence intensity; SEM, Standard error of mean.

TABLE 1 | Patient characteristics.

PT	Gender	Age	RAI stage	Binet stage	TP53 mutational status	Del(17p13)	IgVH mutational status	Del(13q14)
1	M	64	III	B	−	−		
2	M	74	III	C	−	−		+
3	M	55	III	C	−	−		
4	F	66	0	A			+	+
5	M	48	I	A			+	+
6	F	81	I	A				−
7	F	59	I	A			+	+
8	M	60	II	B	−	−	+	+
9	F	59	III	C	−	−		+
10	M	82	II	B	−	−		−
11	F	69	I	B	−	−		−
12	F	67	III	C	−	−		−
13	M	73	IV	C	−	−		−
14	-	80						
15	M	75	III	C	−	−		−

+, positive; −, negative; blank, not determined.

Source of Primary Cells

HDs had a median age of 28.4 years with a percentage of females of 48.3%. To overcome the age discrepancy, we extended the trial on an elderly HD cohort ($n = 4$, **Supplementary Figure 2**). The median age of this cohort was 62 years with a percentage of 50% females. PB mononuclear cells (PBMCs) from HD were isolated by density gradient centrifugation (using Biochrom separating solution, L6115) from PB and either cryopreserved at <-80°C in cell culture medium (described above) containing 10% DMSO, or directly used for experiments. T cells were negatively isolated from frozen HD PBMCs with the human Pan T Cell Isolation Kit (Miltenyi Biotec, 130-096-535) and cultured in cell culture medium. NK cells were either isolated negatively with the human NK Cell Isolation Kit (Miltenyi Biotec, 130-092-657) or with the EasySep Human CD56 Positive Selection Kit II (Stemcell Technologies, 17815) from fresh HD PBMCs and cultured in NK MACS Medium (Miltenyi Biotec, 130-114-429) supplemented with 5% human serum (Sigma–Aldrich, H6914). Monocytes were positively isolated from fresh HD PBMCs with human CD14 Microbeads (Miltenyi Biotec, 130-050-201). Neutrophils were negatively isolated from fresh HD PB with the EasySep Direct Human Neutrophil Isolation Kit (Stemcell Technologies, 19666).

HD-derived immune cells were cultured with 0.05, 0.5, or 1 μM idelalisib or with the DMSO concentration that matches the DMSO concentration of the drug-treated cultures as vehicle controls.

Flow Cytometry

All measurements were conducted on a CytoFLEX flow cytometer (Beckman Coulter) and analyzed using FlowJo software (BD Biosciences, version 10; RRID: SCR_008520). All antibodies used in the following experiments are listed in **Supplementary Table 1**. Median fluorescence intensity (MFI) was determined, and the MFI ratio (MFI sample/MFI isotype control) was calculated (**Supplementary Figure 1**).

In vitro Cell Proliferation Assay

HD T cells were stained using the CellTrace CFSE Proliferation Kit (Thermo Fisher Scientific, C34554) according to the manufacturer's instructions. For stimulation, CD3/CD28 Dynabeads (Thermo Fisher Scientific, 11131D) at a bead-to-cell ratio of 1:2 and 30 U/mL interleukin-2 (IL-2; R&D Systems, 202-IL-010/CF) were added to the culture for 5 days. T cell subsets were discriminated by the expression of the chemokine receptor CCR7 in combination with the naïve cell marker CD45Ra (**Supplementary Figure 2**). NK cells were stimulated by addition of 500 U/mL IL-2 for 10 days. Fold change was calculated as: "Viable NK cell count day 10"/"Viable NK cell count day 0."

Cytometric Bead Array

HD T cells were stimulated as described above. After 3 days, the secretion of interferon-γ (IFN-γ), tumor necrosis factor (TNF), IL-2 and interleukin 10 (IL-10) was measured by analyzing the cell culture supernatant in a Th1/Th2 cytometric bead array (BD Biosciences, 551809) (50, 51). The assay was performed according to the manufacturer's instructions using flow cytometry.

T-Cell Cytotoxicity Assay

The cytotoxic capacity of T cells was assessed in two different assays. In the first assay, HD T cells were activated as described above. After 3 days the secretion of the cytolytic molecules perforin and granzyme B was analyzed by multiparameter flow cytometry. In the second assay, the T-cell recruiting antibody-mediated lysis of the target cell line HL-60 at an effector– to–target ratio (E:T) of 1:3 was measured. Either an anti-CD33 bispecific T-cell-recruiting antibody (5 ng/mL) (52), recognizing CD3 on T cells and CD33 on target cells, or no antibody was added to the coculture. After 72 h, target and T cell counts were analyzed by flow cytometry. Lysis was calculated according to the

formula %lysis = 100 − [#target cells (with antibody)]/[#target cells (without antibody)]*100.

NK-Cell Cytotoxicity Assay

The cytotoxic capacity of NK cells was assessed in two different assays. In the first assay, the cell line K562 was stained with calcein AM (Thermo Fisher Scientific, C3100MP) according to the manufacturer's instructions. Freshly isolated HD NK cells were cocultured with the stained K562 cells at an E:T ratio of 10:1 in presence of idelalisib or DMSO. After 4 h, the fluorescence intensity of the co-culture supernatant was measured on a microplate reader (excitation: 485 nm; emission: 535 nm). Lysis was calculated according to the formula $[(F_{test}) - (F_{spontaneous})/(F_{maximum}) - (F_{spontaneous})] \times 100$, where $F_{spontaneous}$ represents the fluorescence intensity of calcein released from target cells in medium alone, and $F_{maximum}$ is the fluorescence intensity of calcein released from target cells lysed in medium containing 2% Triton X-100 (Sigma–Aldrich, X100-5ML), each measured in at least three replicate wells. In the second assay, freshly isolated HD NK cells were cocultured with K562 or Jurkat cells at an E:T ratio of 5:1 for 20 h in presence of idelalisib or DMSO. After 20 h, specific lysis was analyzed by multiparameter flow cytometry (Beckman Coulter CytoFLEX S flow cytometer). The percentage of lysis was determined as the target cell count of idelalisib-treated relative to the control cultures.

Phagocytosis Assay

Monocyte function was assessed by measuring phagocytosis of pHrodo Green *E.coli* BioParticles (Thermo Fisher Scientific, P35366). Freshly isolated monocytes were incubated with the pHrodo particles for 2 h, then phagocytosis was analyzed by flow cytometry.

Neutrophil Activation Assay

Neutrophil function was analyzed over 4 h in a standard Seahorse XF neutrophil activation assay according to the manufacturer's instructions.

Statistics

Statistical analyses were performed using GraphPad Prism Software Version 8.4.2. As statistical test to compare the two treatment groups Wilcoxon matched signed-rank test was used. *P*-values and the number of replicates performed to derive the data are indicated in the figure legends.

RESULTS

Inhibition of the PI3K∂ by Idelalisib Reduces the Expression Levels of Inhibitory Checkpoint Molecules in CD3$^+$ T Cells and Treg Cells

As PI3K∂ was previously shown to be important for T-cell signaling, we evaluated the effect of PI3K∂ blockade on T-cell proliferation. We found that proliferation of CD4$^+$ and CD8$^+$ T cells (**Figure 1A**) and the respective subsets (**Figures 1B,C**) was not affected by idelalisib. Next, we analyzed the expression levels of several inhibitory checkpoint molecules in T cells and Tregs after stimulation for 72 or 120 h in the presence of idelalisib. The expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and programmed cell death protein 1 (PD-1) was significantly downregulated in idelalisib-treated T cells and Tregs in comparison to the DMSO vehicle control cells (**Figures 1D–G**). Our results demonstrate that proliferation of T cells is not solely dependent on signaling through PI3K∂ and thus is not susceptible to inhibition of PI3K∂ by idelalisib. Furthermore, our data demonstrate that blockade of PI3K∂ signaling reduced expression of inhibitory checkpoint molecules in T cells and Tregs.

Inhibition of PI3K∂ by Idelalisib Leads to Decreased T-Cell-Mediated Cytotoxicity Against Target Tumor Cells and Reduced Secretion of IL-10, TNF, and IFN-γ

To test whether the downregulation of inhibitory checkpoint molecules in T cells also mirrors reduced T-cell effector function, we analyzed the effect of PI3K∂ blockade on T-cell cytotoxicity and cytokine secretion. The cytotoxicity of T cells against target HL−60 cells was significantly reduced in a coculture in the presence of idelalisib (**Figure 2A**). To further investigate if this reduction in the cytolytic capacity was due to a decrease in secretion of cytolytic molecules, T cells were analyzed for perforin and granzyme B secretion after 72 h stimulation in the presence of idelalisib. There was a significantly lower degranulation of perforin and granzyme B in the cultures containing idelalisib (**Figure 2B**). Next, we evaluated the effect of PI3K∂ blockade on the secretion of different cytokines after stimulation for 72 h. Secretion levels of IL-10, TNF, and IFN-γ were significantly reduced in the presence of idelalisib. In contrast, secretion of IL-2 was slightly increased (**Figure 2C**). Taken together, these results demonstrate that blockade of PI3K∂ signaling with idelalisib has a negative impact on the effector functions of T cells, such as cytotoxicity and cytokine secretion.

Inhibition of PI3K∂ by Idelalisib Reduces NK-Cell Proliferation and the Percentage of Cytotoxic NK Cells

As we found that inhibition of PI3K∂ significantly impairs important functions of the T-cell compartment, we sought to evaluate if similar effects can be seen in NK cells, the cytotoxic lymphocytes of the innate immune system. The addition of idelalisib to IL-2 stimulated NK cells led to a decreased NK-cell expansion in comparison to the DMSO-treated control (**Figure 3A**). Furthermore, idelalisib affected the proliferation of the cytotoxic NK-cell population over the 10-day period. As the percentage of dead cells in both conditions is not significantly different, this observation is most likely due to a reduced proliferation instead of increased apoptosis in the idelalisib treated condition (media %dead cells 1 μM idelalisib vs. corresponding DMSO control: 24.3 vs. 25.5, $n = 12$, data not shown). The cytokine producing CD56^{bright}CD16^{neg} compartment showed no differences between

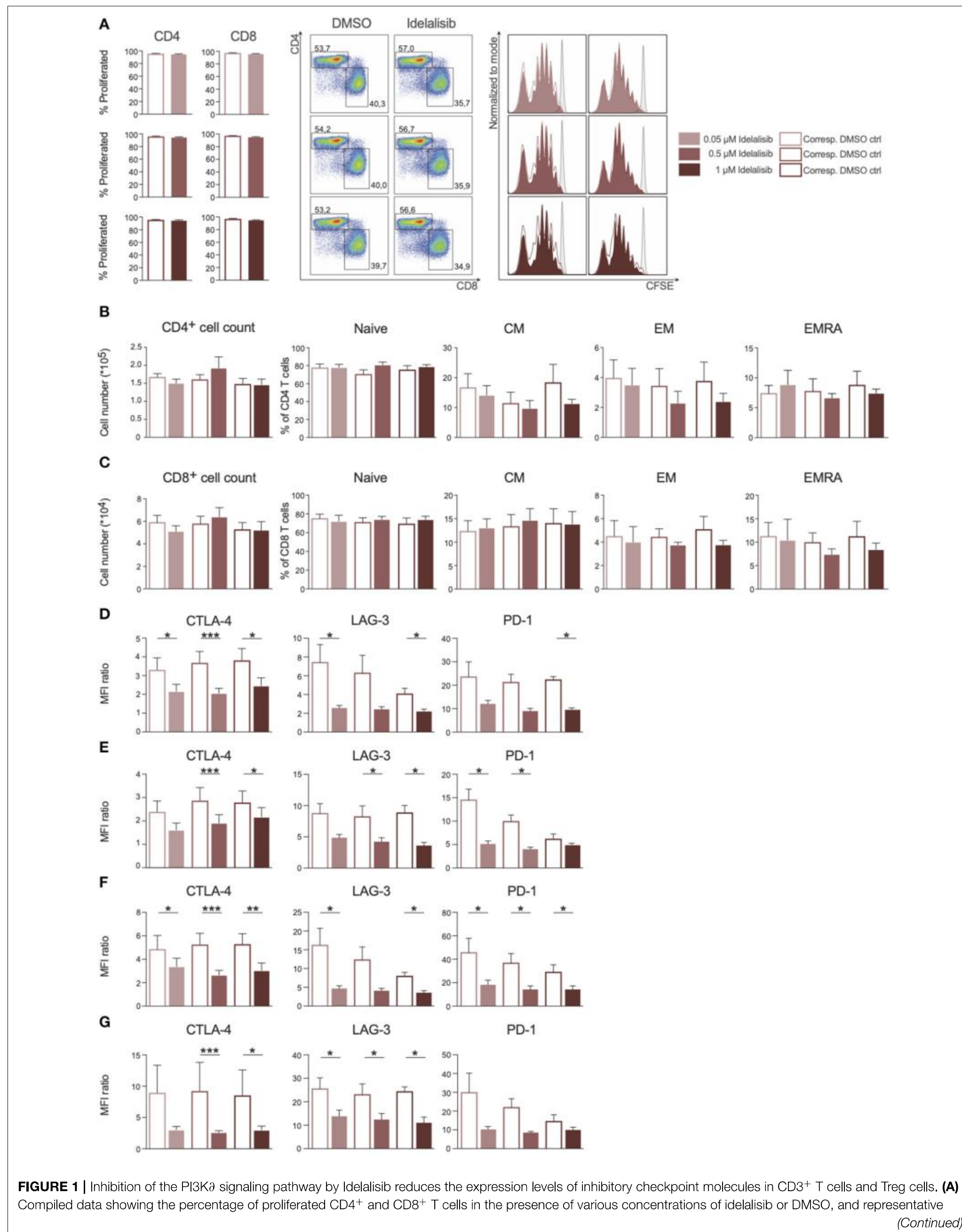


FIGURE 1 | Inhibition of the PI3Kδ signaling pathway by Idelalisib reduces the expression levels of inhibitory checkpoint molecules in CD3+ T cells and Treg cells. **(A)** Compiled data showing the percentage of proliferated CD4+ and CD8+ T cells in the presence of various concentrations of idelalisib or DMSO, and representative *(Continued)*

FIGURE 1 | flow cytometry dot plots and histograms. **(B,C)** Bar charts depicting the development of CD4$^+$ and CD8$^+$ T-cell subsets: naïve, central memory (CM), effector memory (EM), and terminally differentiated effector memory cells re-expressing CD45RA (ERMA) after 6 days of coculture, with different concentrations of idelalisib or DMSO as vehicle control. **(D–G)** Bar graphs of MFI ratios showing the expression levels of checkpoint molecules CTLA-4, LAG-3, and PD-1 in CD4$^+$ T cells, CD8$^+$ T cells, CD4$^+$ Tregs and CD8$^+$ Tregs after stimulation for 3 or 5 days with IL-2 and CD3/CD28 activation beads. Error bars represent mean ± SEM; $^*p \le 0.05$, $^{**}p \le 0.005$, $^{***}p \le 0.0005$; n = 6–12 HDs.

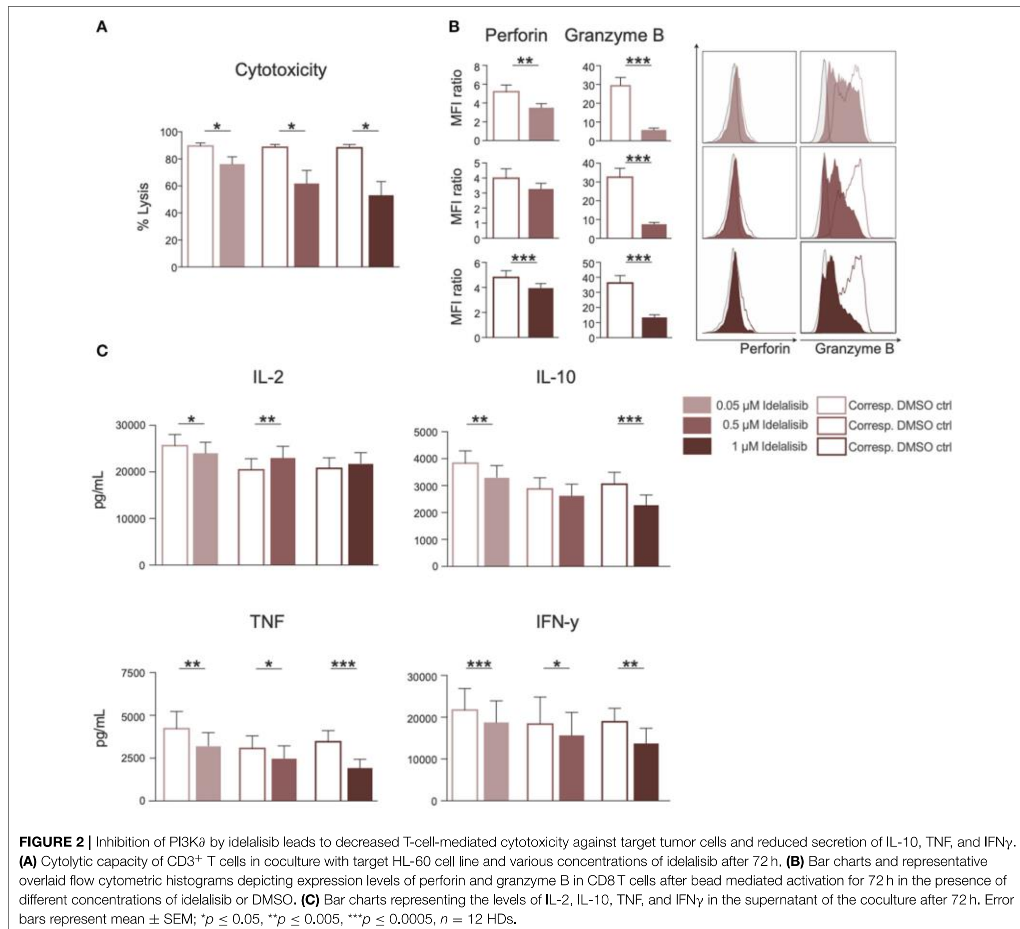


FIGURE 2 | Inhibition of PI3K∂ by idelalisib leads to decreased T-cell-mediated cytotoxicity against target tumor cells and reduced secretion of IL-10, TNF, and IFNγ. **(A)** Cytolytic capacity of CD3$^+$ T cells in coculture with target HL-60 cell line and various concentrations of idelalisib after 72 h. **(B)** Bar charts and representative overlaid flow cytometric histograms depicting expression levels of perforin and granzyme B in CD8 T cells after bead mediated activation for 72 h in the presence of different concentrations of idelalisib or DMSO. **(C)** Bar charts representing the levels of IL-2, IL-10, TNF, and IFNγ in the supernatant of the coculture after 72 h. Error bars represent mean ± SEM; $^*p \le 0.05$, $^{**}p \le 0.005$, $^{***}p \le 0.0005$, n = 12 HDs.

cultures (**Figure 3B**). However, the proliferative capacity of the cytotoxic CD56^{dim}CD16^{bright} subset was reduced upon PI3K∂ blockade (**Figure 3C**).

Inhibition of PI3K∂ by Idelalisib Decreases NK-Cell Cytotoxicity

To evaluate if this reduction translates into impaired cytotoxicity, we analyzed the cytolytic capacity of NK cells toward target cells in presence of idelalisib. NK cells are capable of killing target cells via two different apoptotic pathways, either through perforin- and granzyme- mediated lysis or through death receptor ligation with, for example, the Fas ligand (FasL). To investigate if idelalisib interferes with both apoptotic pathways, we separately cocultured two target cell lines with HD NK cells. The cytotoxicity of NK cells toward target K562 cells, which are lacking the MHC class I antigen and are thus killed via granzyme B-mediated lysis, was

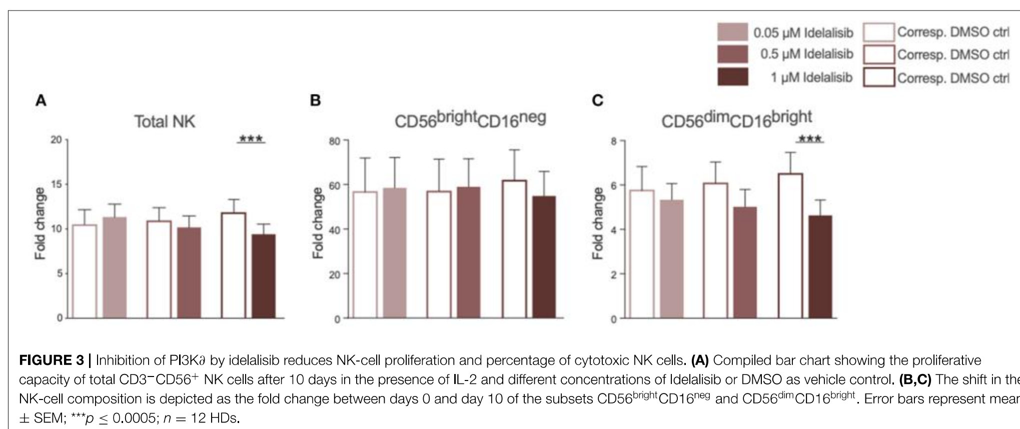


FIGURE 3 | Inhibition of PI3K∂ by idelalisib reduces NK-cell proliferation and percentage of cytotoxic NK cells. **(A)** Compiled bar chart showing the proliferative capacity of total CD3−CD56+ NK cells after 10 days in the presence of IL-2 and different concentrations of Idelalisib or DMSO as vehicle control. **(B,C)** The shift in the NK-cell composition is depicted as the fold change between days 0 and day 10 of the subsets $CD56^{bright}CD16^{neg}$ and $CD56^{dim}CD16^{bright}$. Error bars represent mean ± SEM; ***$p \leq 0.0005$; $n = 12$ HDs.

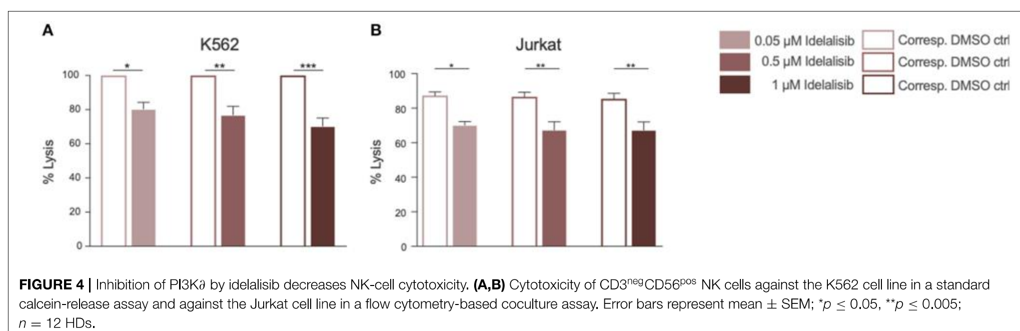


FIGURE 4 | Inhibition of PI3K∂ by idelalisib decreases NK-cell cytotoxicity. **(A,B)** Cytotoxicity of $CD3^{neg}CD56^{pos}$ NK cells against the K562 cell line in a standard calcein-release assay and against the Jurkat cell line in a flow cytometry-based coculture assay. Error bars represent mean ± SEM; *$p \leq 0.05$, **$p \leq 0.005$; $n = 12$ HDs.

significantly reduced (**Figure 4A**). Lysis of target Jurkat cells, which are killed in a Fas–FasL-dependent manner, was also significantly decreased in the presence of idelalisib (**Figure 4B**). Together our data show that PI3K∂ signaling is important for NK cell-mediated lysis of cancer cells *in vitro* and that blockade by idelalisib significantly reduces the cytolytic capability of NK cells.

Inhibition of PI3K∂ by Idelalisib Alters Neither the Phagocytic Capacity of Monocytes Nor the Activation of Neutrophils

To further investigate the impact of idelalisib on other innate immune cells, we studied the effect of PI3K∂ blockade on the phagocytic capacity of monocytes and neutrophil activation in two independent short-term assays. No differences in the phagocytosis rates of monocytes were detected (**Figure 5A**). Neutrophil activation was measured via the generation of reactive oxygen species, termed an "oxidative burst," in a Seahorse XF neutrophil activation assay. Neutrophils in the idelalisib-treated group did not show a significant difference in oxygen consumption rates in comparison to the vehicle-treated group (**Figure 5B**). These data indicate that PI3K∂ blockade does not have a direct impact on monocyte phagocytic capacity and neutrophil activation in these short-term assessments.

Inhibition of PI3K∂ by Idelalisib Has a Negative Impact on Effector Functions of T Cells Derived From CLL Patients at the Time of Initial Diagnosis

Next, we analyzed if the previously described effects of PI3K∂ inhibition could also be seen if CLL patient cells were treated in culture with idelalisib. To this end, we isolated T cells from cryopreserved PBMCs of CLL patients at time of initial diagnosis. Again, patient samples treated with the vehicle DMSO served as controls. PI3K∂ blockade by idelalisib did not alter the proliferative capacity of T cells isolated from CLL PBMCs (**Figure 6A**). The cytotoxicity of CLL T cells against

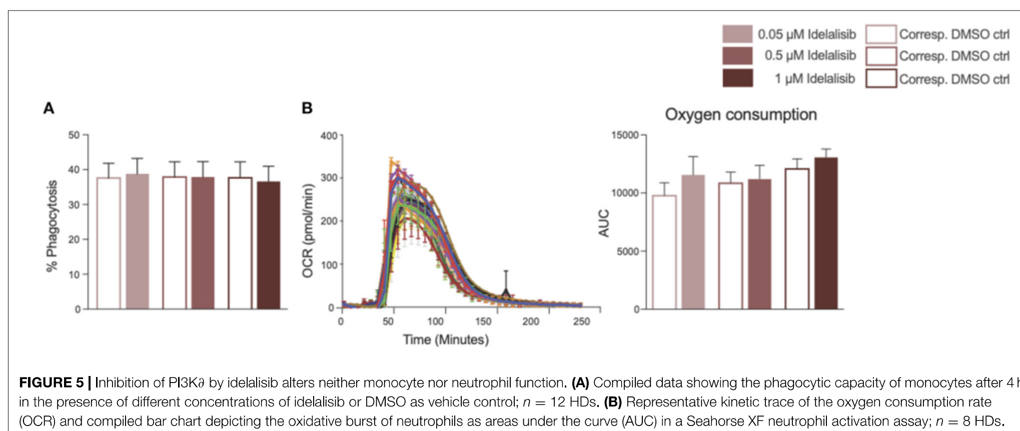


FIGURE 5 | Inhibition of PI3K∂ by idelalisib alters neither monocyte nor neutrophil function. **(A)** Compiled data showing the phagocytic capacity of monocytes after 4 h in the presence of different concentrations of idelalisib or DMSO as vehicle control; $n = 12$ HDs. **(B)** Representative kinetic trace of the oxygen consumption rate (OCR) and compiled bar chart depicting the oxidative burst of neutrophils as areas under the curve (AUC) in a Seahorse XF neutrophil activation assay; $n = 8$ HDs.

target HL-60 cells was significantly reduced in a coculture treated with idelalisib (**Figure 6B**). This could be further verified by a significant decrease in granzyme B secretion in a culture containing the inhibitor (**Figure 6C**). Furthermore, PD-1 expression was significantly decreased in the idelalisib-treated T-cell group (**Figure 6D**). Next, we evaluated the effect of PI3K∂ blockade on the secretion of different cytokines after coculture for 72 h with the target HL-60 cell line. Cytometric bead array analysis of CLL T cells revealed significantly reduced secretion levels of IL-10, IL-4, IL-6, and IFN-γ (**Figure 6E**). Taken together, these results demonstrate that blockade of PI3K∂ signaling with idelalisib has a negative impact on the effector functions of CLL T cells, such as cytotoxicity and cytokine secretion. However, differences in age are possible cofounding variables.

DISCUSSION

PI3K∂ inhibition by idelalisib has proven to be highly effective in the treatment of r/r CLL patients. However, this clinical success is somewhat diminished by the increased rate of opportunistic infections in these patients (37–42). The factors contributing to this observation are incompletely understood.

In the present study, we evaluated the effect of PI3K∂ blockade by idelalisib on the non-malignant human immune cell compartment of healthy individuals. First, we analyzed the effects of idelalisib on T cells and Tregs. PI3K∂ blockade by idelalisib did not have an impact on the proliferative capacity of HD T cells. However, we observed a significant decrease in the expression levels of the inhibitory checkpoint molecules PD-1, CTLA-4, and LAG-3 in both T cells and Tregs in cultures containing idelalisib. Our findings are supported by previous studies, which described PI3K∂ as the main transducer of PI3K signaling in human T cells (25, 53) and Tregs (54). Next, we evaluated if these findings correlated to impaired T-cell function. We demonstrated that secretion of IL-10, TNF, and IFNγ by idelalisib-treated HD T cells was significantly reduced. This is consistent with previous studies that looked at the impact of other PI3K∂-blocking agents on cytokine secretion of T cells in mice (25, 53, 55). Furthermore, we observed that PI3K∂ blockade significantly decreased the cytolytic capacity of HD T cells. This manifested through a significantly reduced secretion of the cytolytic molecules perforin and granzyme B, as well as a significant decrease in antibody-mediated target cell killing. Together, our data show that idelalisib severely impairs the functions of T cells and Tregs isolated from HDs. Combined with the findings of a previous study by Chellappa and colleagues (56), our data support the hypothesis that idelalisib leads to T and NK cell dysfunction. Therefore, our *in vitro* observations might reflect the increased rate of opportunistic infections in treated CLL patients. Idelalisib exposure led to a significant decrease in the expression of PD-1, which is often described as a marker for T-cell activation. This might be an indicator for reduced T-cell activity and in turn a dampened immune response, which might be a factor behind the increased rates of infections measured in idelalisib-treated CLL patients.

Emerging data from clinical trials suggests that the improved T-cell-mediated antitumor response and the impressive clinical outcome in CLL might be due to reduced Treg numbers and their reduced suppressive function in idelalisib-treated patients (57, 58). Conversely, inhibiting the suppressive activity of Tregs can also lead to severe adverse autoimmune effects (56, 59).

As a next step, we wanted to evaluate the effect of idelalisib on NK cells, the cytotoxic lymphocytes of the innate immune system. Previous studies in mice with defective PI3K∂ have suggested that PI3K plays a critical role in NK-cell effector function (60, 61). Furthermore, Zebedin and colleagues demonstrated that selective inhibition of PI3K∂ in mice leads to impaired degranulation and target cell killing by NK cells (62). Therefore, we wanted to evaluate whether PI3K∂ blockade also has a negative impact on the human NK-cell compartment. In our study, we observed that idelalisib reduced NK-cell proliferation. Moreover, PI3K∂ inhibition led to a decrease in

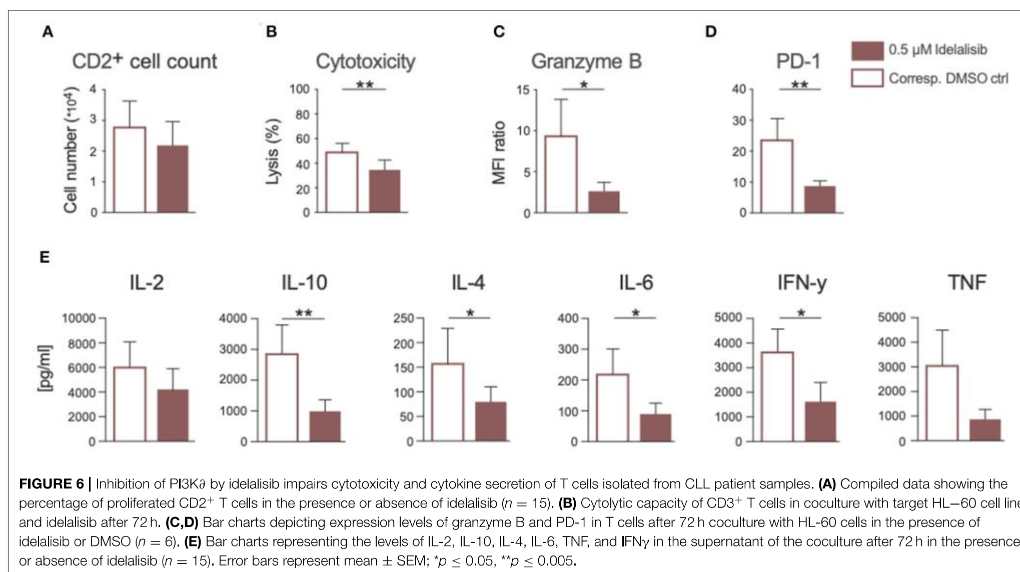


FIGURE 6 | Inhibition of PI3K∂ by idelalisib impairs cytotoxicity and cytokine secretion of T cells isolated from CLL patient samples. **(A)** Compiled data showing the percentage of proliferated $CD2^+$ T cells in the presence or absence of idelalisib ($n = 15$). **(B)** Cytolytic capacity of $CD3^+$ T cells in coculture with target HL−60 cell line and idelalisib after 72 h. **(C,D)** Bar charts depicting expression levels of granzyme B and PD-1 in T cells after 72 h coculture with HL-60 cells in the presence of idelalisib or DMSO ($n = 6$). **(E)** Bar charts representing the levels of IL-2, IL-10, IL-4, IL-6, TNF, and IFNγ in the supernatant of the coculture after 72 h in the presence or absence of idelalisib ($n = 15$). Error bars represent mean ± SEM; $^*p \leq 0.05$, $^{**}p \leq 0.005$.

the percentage of cytotoxic NK cells, which also translated into reduced target cell killing by NK cells. We show that two different apoptosis pathways are affected. Idelalisib impaired cell death through secretion of cytolytic molecules, as well as cell death via the Fas–FasL pathway. NK cells are an important part of the innate immune system and play a key role in the defense against infections. Taken together, our data demonstrate a decrease NK-cell proliferation and cytolytic activity by idelalisib and that this might contribute to the increased frequency of infectious events observed in clinical trials.

CLL has been associated with profound defects in T-cell function and synapse formation (63–66). These T-cell defects are thought to result in failure of antitumor immunity and increased susceptibility to infections (67, 68). In view of our findings on healthy T cells, PI3K∂ inhibition might have an even more pronounced effect on the immune response of CLL patients, contributing to an elevated risk of severe side effects such as opportunistic infections. To confirm the clinical relevance of our findings and that these effects might indeed contribute to the increased rate of infections during idelalisib therapy, we isolated T cells from cryopreserved PBMCs collected from CLL patients at the time of initial diagnosis. As expected, the proliferative capacity of these T cells was lower compared to healthy T cells. Idelalisib had only a minor impact on the proliferation on T cells from CLL patient. However, we observed a significant decrease in the cytolytic capacity of CLL T cells treated with idelalisib. This was supported by our finding of a significantly reduced level of secreted granzyme B. Consistent with our data from HDs, PD-1 expression in T cells was significantly decreased in the presence of idelalisib, most likely as a result of reduced T-cell activation. In line with these findings, we also observed a significantly reduced secretion of IL-10, IL-4, IL-6, and IFN-γ; secretion of IL-2 and TNF also appeared to be affected, albeit to a lesser extent. As proinflammatory cytokines are mainly secreted by Tregs, the decrease in IL-10 secretion might indicate impaired Treg suppressive function, which could also contribute to a higher risk of autoimmune diseases. Furthermore, reduced TNF and IFNγ secretion serves as an indicator of less activated, less functional, or even exhausted T cells.

Our data demonstrates that both human T and NK cells are highly sensitive to PI3K∂ inhibition. Idelalisib interfered with the functions of T and NK cells from both HDs and CLL patients. In summary our *in vitro* data suggest that idelalisib-induced impairment of T and NK-cell function contributes to an increased rate of infections regardless of the underlying B-cell malignancy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by PB samples from HDs and patients with CLL were collected with written consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilian University of Munich. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS, VB, and MB-B: conceptualization and funding acquisition. LR, BB, and AO: methodology. LR, BB, and AO: investigation. LR: writing—original draft. AO, BB, VB, and MS: writing, review, and editing. LR, BB, and MS: review. MS: supervision. All authors read and approved the final manuscript.

FUNDING

This study was supported by funds from the international doctoral program iTarget: Immunotargeting of Cancer funded by the Elite Network of Bavaria (to LR), the German Research Council (DFG) within the Collaborative Research Center (SFB) 1243 Cancer Evolution (project A10: to BB), the Else Kröner-Fresenius-Stiftung (Cancer Immunotherapy, Clinician Scientist Program Munich, to VB), and Gilead Sciences Inc.

ACKNOWLEDGMENTS

We acknowledge the iFlow Core Facility of the university hospital Munich (INST 409/225-1 FUGG) for assistance with the generation of flow cytometry data. We also thank Prof. Dr. Sebastian Theurich for his support with the Seahorse Analyzer.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.608625/full#supplementary-material

Supplementary Figure 1 | Descriptive Plots. **(A)** Flow cytometry histogram: calculation of the MFI ratio (Median fluorescence intensity ratio). **(B)** Gating strategy T-cell subset discrimination. The four subsets were discriminated as follows: naïve T cells: $CD45Ra^+/CCR7^+$, memory T cells: $CD45Ra^-/CCR7^+$, effector T cells: $CD45Ra^-/CCR7^-$, and terminally differentiated effector memory T cells: $CD45Ra^+/CCR7^-$. **(C)** Representative flow cytometry histograms, MFI ratio: CTLA-4 (idelalisib = 1.9; DMSO = 4.7), LAG-3 (MFI ratios: idelalisib =2.3; DMSO = 19.5), and PD-1 (MFI-ratios: idelalisib = 13.7; DMSO = 39.3).

Supplementary Figure 2 | Inhibition of PI3Kδ by idelalisib in an older reference cohort. **(A)** Compiled data showing the percentage of proliferated $CD4^+$ and $CD8^+$ T cells in the presence of various concentrations of idelalisib or DMSO. **(B–E)** Bar graphs of median fluorescence intensity (MFI) ratios showing the expression levels of checkpoint molecules CTLA-4, LAG-3, and PD-1 in $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ Tregs, and $CD8^+$ Tregs after stimulation for 3 days with IL-2 and CD3/CD28 activation beads. **(F)** Cytolytic capacity of $CD3^+$ T cells in coculture with HL-60 cell line and various concentrations of idelalisib after 72 h. **(G)** Bar charts depicting expression levels of perforin and granzyme B in $CD8^+$ T cells after stimulation with CD3/CD28 beads for 72 h in the presence of various concentrations of idelalisib or DMSO. **(H)** Cytotoxicity of $CD3^-CD56^+$ NK cells against the K562 cell line in a flow cytometry-based coculture assay. Error bars represent ± SEM, $n = 4$ HDs in all depicted assays.

Supplementary Table 1 | Key Resources.

REFERENCES

1. Johnston PB, Kay NE. Pathogenesis of Impaired Cellular Immune Function in CLL. In: Faguet GB, editor. *Chronic Lymphocytic Leukemia: Molecular Genetics, Biology, Diagnosis, and Management*. Totowa, NJ: Humana Press (2004) p. 109–21. doi: 10.1007/978-1-59259-412-2_5
2. Goldman D. Chronic lymphocytic leukemia and its impact on the immune system. *Clin J Oncol Nurs*. (2000) 4:233–4.
3. Morrison VA. Infectious complications in patients with chronic lymphocytic leukemia: pathogenesis, spectrum of infection, and approaches to prophylaxis. *Clin Lymphoma Myeloma*. (2009) 9:365–70. doi: 10.3816/CLM.2009.n.071
4. Molica S. Infections in chronic lymphocytic leukemia: risk factors, and impact on survival, and treatment. *Leukemia Lymphoma*. (1994) 13:203–14. doi: 10.3109/10428199409056283
5. Itala M, Helenius H, Nikoskelainen J, Remes K. Infections and serum IgG levels in patients with chronic lymphocytic leukemia. *Eur J Haematol*. (1992) 48:266–70. doi: 10.1111/j.1600-0609.1992.tb01805.x
6. Fraietta JA, Beckwith KA, Patel PR, Ruella M, Zheng Z, Barrett DM, et al. Ibrutinib enhances chimeric antigen receptor T-cell engraftment and efficacy in leukemia. *Blood*. (2016) 127:1117–27. doi: 10.1182/blood-2015-11-679134
7. Dhalla F, Lucas M, Schuh A, Bhole M, Jain R, Patel SY, et al. Antibody deficiency secondary to chronic lymphocytic leukemia: should patients be treated with prophylactic replacement immunoglobulin? *J Clin Immunol*. (2014) 34:277–82. doi: 10.1007/s10875-014-9995-5
8. Freeman CL, Gribben JG. Immunotherapy in chronic lymphocytic leukaemia (CLL). *Curr Hematol Malig Rep*. (2016) 11:29–36. doi: 10.1007/s11899-015-0295-9
9. Wadhwa PD, Morrison VA. Infectious complications of chronic lymphocytic leukemia. *Sem Oncol*. (2006) 33:240–9. doi: 10.1053/j.seminoncol.2005.12.013
10. Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol*. (2019) 94:1266–87. doi: 10.1002/ajh.25595
11. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med*. (2005) 352:804–15. doi: 10.1056/NEJMra041720
12. Bernal A, Pastore RD, Asgary Z, Keller SA, Cesarman E, Liou HC, et al. Survival of leukemic B cells promoted by engagement of the antigen receptor. *Blood*. (2001) 98:3050–7. doi: 10.1182/blood.V98.10.3050
13. Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. (2002) 100:4609–14. doi: 10.1182/blood-2002-06-1683
14. Herishanu Y, Perez-Galan P, Liu D, Biancotto A, Pittaluga S, Vire B, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*. (2011) 117:563–74. doi: 10.1182/blood-2010-05-284984
15. Ferrer G, Montserrat E. Critical molecular pathways in CLL therapy. *Mol Med*. (2018) 24:9. doi: 10.1186/s10020-018-0001-1
16. Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: are we making headway? *Nat Rev Clin Oncol*. (2018) 15:273–91. doi: 10.1038/nrclinonc.2018.28
17. Randis TM, Puri KD, Zhou H, Diacovo TG. Role of PI3Kdelta and PI3Kgamma in inflammatory arthritis and tissue localization of neutrophils. *Eur J Immunol*. (2008) 38:1215–24. doi: 10.1002/eji.200838266
18. Zirlik K, Veelken H. Idelalisib. Recent results. *Cancer Res*. (2018) 212:243–64. doi: 10.1007/978-3-319-91439-8_12
19. Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ, et al. CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. *Blood*. (2011) 117:591–4. doi: 10.1182/blood-2010-03-275305

20. Fruman DA. Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. *Curr Opin Immunol*. (2004) 16:314–20. doi: 10.1016/j.coi.2004.03.014
21. Herman SE, Gordon AL, Wagner AJ, Heerema NA, Zhao W, Flynn JM, et al. Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood*. (2010) 116:2078–88. doi: 10.1182/blood-2010-02-271171
22. Ringshausen I, Schneller F, Bogner C, Hipp S, Duyster J, Peschel C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cδ. *Blood*. (2002) 100:3741–8. doi: 10.1182/blood-2002-02-0539
23. Pauls S, Lafarge S, Landego I, Zhang T-t, Marshall A. The phosphoinositide 3-kinase signaling pathway in normal and malignant B cells: activation mechanisms, regulation and impact on cellular functions. *Front Immunol*. (2012) 3:224. doi: 10.3389/fimmu.2012.00224
24. Hawkins PT, Stephens LR. PI3K signalling in inflammation. *Biochim Biophys Acta*. (2015) 1851:882–97. doi: 10.1016/j.bbalip.2014.12.006
25. Koyasu S. The role of PI3K in immune cells. *Nat Immunol*. (2003) 4:313–9. doi: 10.1038/ni0403-313
26. Okkenhaug K. Signaling by the phosphoinositide 3-kinase family in immune cells. *Annu Rev Immunol*. (2013) 31:675–704. doi: 10.1146/annurev-immunol-032712-095946
27. Fruman DA, Cantley LC. Phosphoinositide 3-kinase in immunological systems. *Semin Immunol*. (2002) 14:7–18. doi: 10.1006/smim.2001.0337
28. Beer-Hammer S, Zebedin E, von Holleben M, Alferink J, Reis B, Dresing P, et al. The catalytic PI3K isoforms p110gamma and p110delta contribute to B cell development and maintenance, transformation, and proliferation. *J Leukoc Biol*. (2010) 87:1083–95. doi: 10.1189/jlb.0809585
29. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol*. (2003) 3:317–30. doi: 10.1038/nri1056
30. Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, et al. P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci USA*. (1997) 94:4330–5. doi: 10.1073/pnas.94.9.4330
31. Hoellenriegel J, Meadows SA, Sivina M, Wierda WG, Kantarjian H, Keating MJ, et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood*. (2011) 118:3603–12. doi: 10.1182/blood-2011-05-352492
32. Berning P, Lenz G. The role of PI3K inhibitors in the treatment of malignant lymphomas. *Leukemia Lymphoma*. (2020) 2:1–11. doi: 10.1080/10428194.2020.1839654
33. Hemmings BA, Restuccia DF. PI3K-PKB/Akt pathway. *Cold Spring Harb Perspect Biol*. (2012) 4:a011189. doi: 10.1101/cshperspect.a011189
34. Somoza JR, Koditek D, Villaseñor AG, Novikov N, Wong MH, Liclican A, et al. Structural, biochemical, and biophysical characterization of idelalisib binding to phosphoinositide 3-kinase δ. *J Biol Chem*. (2015) 290:8439–46. doi: 10.1074/jbc.M114.634683
35. Yang Q, Modi P, Newcomb T, Quéva C, Gandhi V. Idelalisib: first-in-class PI3K delta inhibitor for the treatment of chronic lymphocytic leukemia, small lymphocytic leukemia, and follicular lymphoma. *Clin Cancer Res*. (2015) 21:1537–42. doi: 10.1158/1078-0432.CCR-14-2034
36. Markham A. Idelalisib: first global approval. *Drugs*. (2014) 74:1701–7. doi: 10.1007/s40265-014-0285-6
37. Okkenhaug K, Graupera M, Vanhaesebroeck B. Targeting PI3K in cancer: impact on tumor cells, their protective stroma, angiogenesis, and immunotherapy. *Cancer Discov*. (2016) 6:1090–105. doi: 10.1158/2159-8290.CD-16-0716
38. Louie CY, DiMaio MA, Matsukuma KE, Coutre SE, Berry GJ, Longacre TA. Idelalisib-associated enterocolitis: clinicopathologic features and distinction from other enterocolitides. *Am J Surg Pathol*. (2015) 39:1653–60. doi: 10.1097/PAS.0000000000000525
39. Weidner AS, Panarelli NC, Geyer JT, Bhavsar EB, Furman RR, Leonard JP, et al. Idelalisib-associated colitis: histologic findings in 14 patients. *Am J Surg Pathol*. (2015) 39:1661–7. doi: 10.1097/PAS.0000000000000522
40. Coutre SE, Barrientos JC, Brown JR, de Vos S, Furman RR, Keating MJ, et al. Management of adverse events associated with idelalisib treatment: expert panel opinion. *Leuk Lymphoma*. (2015) 56:2779–86. doi: 10.3109/10428194.2015.1022770
41. Cheah CY, Fowler NH. Idelalisib in the management of lymphoma. *Blood*. (2016) 128:331–6. doi: 10.1182/blood-2016-02-702761
42. Barr PM, Saylors GB, Spurgeon SE, Cheson BD, Greenwald DR, O'Brien SM, et al. Phase 2 study of idelalisib and entospletinib: pneumonitis limits combination therapy in relapsed refractory CLL and NHL. *Blood*. (2016) 127:2411–5. doi: 10.1182/blood-2015-12-683516
43. Teh BW, Tam CS, Handunnetti S, Worth LJ, Slavin MA. Infections in patients with chronic lymphocytic leukaemia: mitigating risk in the era of targeted therapies. *Blood Rev*. (2018) 32:499–507. doi: 10.1016/j.blre.2018.04.007
44. Nosari A. Infectious complications in chronic lymphocytic leukemia. *Mediterr J Hematol Infect Dis*. (2012) 4:e2012070. doi: 10.4084/mjhid.2012.070
45. Williams AM, Baran AM, Meacham PJ, Herr M, Valencia HE, Newsom C, et al. High risk of infections in chronic lymphocytic leukemia patients treated with B-cell receptor inhibitors. *Blood*. (2016) 128:3203. doi: 10.1182/blood.V128.22.3203.3203
46. Hanlon A, Brander DM. Managing toxicities of phosphatidylinositol-3-kinase (PI3K) inhibitors. *Hematology*. (2020) 2020:346–56. doi: 10.1182/hematology.2020000119
47. Sharman JP, Coutre SE, Furman RR, Cheson BD, Pagel JM, Hillmen P, et al. Final results of a randomized, phase iii study of rituximab with or without idelalisib followed by open-label idelalisib in patients with relapsed chronic lymphocytic leukemia. *J Clin Oncol*. (2019) 37:1391–402. doi: 10.1200/JCO.18.01460
48. Jones JA, Robak T, Brown JR, Awan FT, Badoux X, Coutre S, et al. Efficacy and safety of idelalisib in combination with ofatumumab for previously treated chronic lymphocytic leukaemia: an open-label, randomised phase 3 trial. *Lancet Haematol*. (2017) 4:e114–e26. doi: 10.1016/S2352-3026(17)30019-4
49. *CHMP Assessment Report of Zydelig*. London: European Medicines Agency (2014) 44:1–132.
50. Schmidt RLJ, Jutz S, Goldhahn K, Witzeneder N, Gerner MC, Trapin D, et al. Chloroquine inhibits human CD4+ T-cell activation by AP-1 signaling modulation. *Sci Rep*. (2017) 7:42191. doi: 10.1038/srep42191
51. Arnold KB, Szeto GL, Alter G, Irvine DJ, Lauffenburger DA. CD4+ T cell-dependent and CD4+ T cell-independent cytokine-chemokine network changes in the immune responses of HIV-infected individuals. *Sci Signal*. (2015) 8:ra104. doi: 10.1126/scisignal.aab0808
52. Herrmann M, Krupka C, Deiser K, Brauchle B, Marcinek A, Ogrinc Wagner A, et al. Bifunctional PD-1 × αCD3 × αCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia. *Blood*. (2018) 132:2484–94. doi: 10.1182/blood-2018-05-849802
53. Soond DR, Bjorgo E, Moltu K, Dale VQ, Patton DT, Torgersen KM, et al. PI3K p110delta regulates T-cell cytokine production during primary and secondary immune responses in mice and humans. *Blood*. (2010) 115:2203–13. doi: 10.1182/blood-2009-07-232330
54. Patton DT, Garden OA, Pearce WP, Clough LE, Monk CR, Leung E, et al. Cutting edge: the phosphoinositide 3-kinase p110 delta is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol*. (2006) 177:6598–602. doi: 10.4049/jimmunol.177.10.6598
55. Okkenhaug K, Patton DT, Bilancio A, Garcon F, Rowan WC, Vanhaesebroeck B. The p110delta isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells. *J Immunol*. (2006) 177:5122–8. doi: 10.4049/jimmunol.177.8.5122
56. Chellappa S, Kushekhar K, Munthe LA, Tjonnfjord GE, Aandahl EM, Okkenhaug K, et al. The PI3K p110delta Isoform Inhibitor Idelalisib Preferentially Inhibits Human Regulatory T Cell Function. *J Immunol*. (2019) 202:1397–405. doi: 10.4049/jimmunol.1701703
57. D'Arena G, Laurenti L, Minervini MM, Deaglio S, Bonello L, De Martino L, et al. Regulatory T-cell number is increased in chronic lymphocytic leukemia patients and correlates with progressive disease. *Leuk Res*. (2011) 35:363–8. doi: 10.1016/j.leukres.2010.08.010
58. Hanna BS, Roessner PM, Scheffold A, Jebaraj BMC, Demerdash Y, Ozturk S, et al. PI3Kdelta inhibition modulates regulatory and effector T-cell differentiation and function in chronic lymphocytic leukemia. *Leukemia*. (2019) 33:1427–38. doi: 10.1038/s41375-018-0318-3
59. Lampson BL, Kasar SN, Matos TR, Morgan EA, Rassenti L, Davids MS, et al. Idelalisib given front-line for treatment of chronic lymphocytic leukemia

causes frequent immune-mediated hepatotoxicity. *Blood*. (2016) 128:195–203. doi: 10.1182/blood-2016-03-707133
60. Guo H, Samarakoon A, Vanhaesebroeck B, Malarkannan S. The p110 delta of PI3K plays a critical role in NK cell terminal maturation and cytokine/chemokine generation. *J Exp Med*. (2008) 205:2419–35. doi: 10.1084/jem.20072327
61. Mace EM. Phosphoinositide-3-kinase signaling in human natural killer cells: new insights from primary immunodeficiency. *Front Immunol*. (2018) 9:445. doi: 10.3389/fimmu.2018.00445
62. Zebedin E, Simma O, Schuster C, Putz EM, Fajmann S, Warsch W, et al. Leukemic challenge unmasks a requirement for PI3Kdelta in NK cell-mediated tumor surveillance. *Blood*. (2008) 112:4655–64. doi: 10.1182/blood-2008-02-139105
63. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. *Haematologica*. (2017) 102:562–72. doi: 10.3324/haematol.2016.151100
64. Görgün G, Holderried TAW, Zahrieh D, Neuberg D, Gribben JG. Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. *J Clin Invest*. (2005) 115:1797–805. doi: 10.1172/JCI24176
65. Vardi A, Stamatopoulos K, Hadzidimitriou A. T cells in chronic lymphocytic leukemia: can they fight? *Oncotarget*. (2017) 8:99209–10. doi: 10.18632/oncotarget.22277
66. Roessner PM, Seiffert M. T-cells in chronic lymphocytic leukemia: guardians or drivers of disease? *Leukemia*. (2020) 34:2012–24. doi: 10.1038/s41375-020-0873-2
67. Riches JC, Davies JK, McClanahan F, Fatah R, Iqbal S, Agrawal S, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood*. (2013) 121:1612–21. doi: 10.1182/blood-2012-09-457531
68. Bonyhadi M, Frohlich M, Rasmussen A, Ferrand C, Grosmaire L, Robinet E, et al. *In Vitro* engagement of CD3 and CD28 corrects t cell defects in chronic lymphocytic leukemia. *J Immunol*. (2005) 174:2366–75. doi: 10.4049/jimmunol.174.4.2366

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Rohrbacher, Brauchle, Ogrinc Wagner, von Bergwelt-Baildon, Bücklein and Subklewe. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

2.2 Publication II

Leukemia (2021) 35:2243–2257
https://doi.org/10.1038/s41375-020-01109-w

ARTICLE

Acute myeloid leukemia

A modular and controllable T cell therapy platform for acute myeloid leukemia

Mohamed-Reda Benmebarek[1] · Bruno L. Cadilha[1] · Monika Herrmann[2] · Stefanie Lesch[1] · Saskia Schmitt[2] · Stefan Stoiber[1] · Abbass Darwich[3] · Christian Augsberger[2] · Bettina Brauchle[2,4] · Lisa Rohrbacher[2,4] · Arman Oner[1] · Matthias Seifert[1] · Melanie Schwerdtfeger[1] · Adrian Gottschlich[1] · Felicitas Rataj[1] · Nadja C. Fenn[2] · Christian Klein[5] · Marion Subklewe[2,4,6] · Stefan Endres[1,6,7] · Karl-Peter Hopfner[8] · Sebastian Kobold[1,6,7]

Received: 17 June 2020 / Revised: 9 November 2020 / Accepted: 1 December 2020 / Published online: 7 January 2021
© The Author(s) 2021. This article is published with open access

Abstract

Targeted T cell therapy is highly effective in disease settings where tumor antigens are uniformly expressed on malignant cells and where off-tumor on-target-associated toxicity is manageable. Although acute myeloid leukemia (AML) has in principle been shown to be a T cell-sensitive disease by the graft-versus-leukemia activity of allogeneic stem cell transplantation, T cell therapy has so far failed in this setting. This is largely due to the lack of target structures both sufficiently selective and uniformly expressed on AML, causing unacceptable myeloid cell toxicity. To address this, we developed a modular and controllable MHC-unrestricted adoptive T cell therapy platform tailored to AML. This platform combines synthetic agonistic receptor (SAR) -transduced T cells with AML-targeting tandem single chain variable fragment (scFv) constructs. Construct exchange allows SAR T cells to be redirected toward alternative targets, a process enabled by the short half-life and controllability of these antibody fragments. Combining SAR-transduced T cells with the scFv constructs resulted in selective killing of $CD33^+$ and $CD123^+$ AML cell lines, as well as of patient-derived AML blasts. Durable responses and persistence of SAR-transduced T cells could also be demonstrated in AML xenograft models. Together these results warrant further translation of this novel platform for AML treatment.

Keypoints

- Modular platform enabling controlled targeting of AML by SAR-transduced T cells in combination with tandem scFv constructs.
- Efficient lysis of primary AML blasts in vitro and strong antitumoral effects and T cell persistence in xenograft models.

Introduction

With high relapse rates and few targeted therapeutic options, there is a need develop novel solutions for the treatment of acute myeloid leukemia (AML). While standard therapy (induction chemo- and consolidation therapy)

These authors contributed equally: Mohamed-Reda Benmebarek, Bruno L. Cadilha

Supplementary information The online version of this article (https://doi.org/10.1038/s41375-020-01109-w) contains supplementary material, which is available to authorized users.

✉ Sebastian Kobold
Sebastian.kobold@med.uni-muenchen.de

[1] Center of Integrated Protein Science Munich (CIPS-M) and Division of Clinical Pharmacology, Department of Medicine IV, Klinikum der Universität München, LMU, Munich, Germany

[2] Department of Medicine III, Klinikum der Universität München, LMU, Munich, Germany

[3] Mucosal Immunology and Microbiota Lab, Humanitas Clinical and Research Center, Milan, Italy

[4] Laboratory for Translational Cancer Immunology, Gene Center, LMU Munich, Munich, Germany

[5] Roche Innovation Center Zurich, Schlieren, Switzerland

[6] German Center for Translational Cancer Research (DKTK), Partner Site Munich, Munich, Germany

[7] Einheit für Klinische Pharmakologie (EKLiP), Helmholtz Zentrum München, German Research Center for Environmental Health (HMGU), Neuherberg, Germany

[8] Gene Center, LMU, Munich, Germany

SPRINGER NATURE

does offer a curative first-line therapy to those eligible [1], leukemic stem cells (LSCs) drive disease relapse in the majority of responders [2]. In spite of significant advances, including allogeneic stem cell transplantation and a growing molecular tailoring of treatment toward driver pathways such as FLT3 [3], the prognosis of relapsed or refractory AML remains poor.

Immunotherapy that promotes the killing of tumor cells by cytotoxic T lymphocytes has entered clinical routine for hematological malignancies in recent years [4–6]. In acute lymphocytic leukemia (ALL), bispecific antibodies utilized for the recruitment of cytotoxic T cells to $CD19^+$ leukemic cells have been shown to be an effective approach, and are now part of the standard-of-care [7]. Similarly, anti-CD19 chimeric antigen receptor (CAR) T cell therapy has been approved in ALL and diffuse large B cell lymphoma based on unprecedented response rates [8–10]. The cornerstone of these treatments is a broadly expressed target antigen on tumor cells that is harnessed to redirect T cells toward the cancer or leukemic cell [11]. In the case of B cell neoplasia, the target antigens, CD19 or CD20, are restricted to the B cell lineage, and the potentially adverse side effect of B cell depletion has been manageable [11, 12]. In contrast, myeloid lineage antigens are much less suited as target structures, as the absence of myeloid cells or of major myeloid lineages is associated with a high morbidity and mortality rate [13]. Thus, there is a need to render AML targeting by T cells either modular or conditional to prevent excessive and life-threatening toxicities whilst enabling clinical activity.

CD33 is an antigen expressed in more than 99% of AML cases, therefore offering a targeted therapeutic modality with the potential to induce remission [14, 15]. CD33 is expressed by all early myeloid progenitors ($CD34^+$ $CD33^+$), thus LSCs that acquired one or more of their transforming events following commitment to the myeloid compartment are targetable [16]. Moreover, CD33 has been shown to be expressed on the majority of $CD34^+$ $CD38^-$ LSCs of AML patient blasts [17, 18]. Along these lines, CD33-targeting should also be able to eradicate chemo-resistant LSCs. However, antigen negative escape variants cause disease relapse in many targeted therapies, emphasizing the need for sequential or even multiple targeting [19].

The pan-T cell-CD33-targeting bispecific T cell engager (BiTE) AMG330 recently showed encouraging results in a phase I trial [14]. In addition, several AML-specific CAR T cells are currently in clinical trials (NCT03971799; NCT04010877; NCT04156256). However, none of these strategies targeted truly AML-specific antigens but rather antigens either only expressed on subsets of AML cells or co-expressed by normal myeloid progenitors [20, 21]. Although potentially effective, all aforementioned T cell strategies are, once deployed, non-reversible and would therefore benefit from a capability to control T cell activity.

An alternative approach is to transduce T cells with a synthetic agonistic receptor (SAR) composed of an inert extracellular domain (EGFRvIII -referred to as E3) acting as a unique antigen receptor fused to intracellular T cell-activating domains that can be specifically activated by an engineered BiAb [22]. Because SARs have no known natural ligands, this reduces the likelihood of unforeseeable toxicity. Triggering of SAR by the BiAb is dependent on it binding its second specificity, i.e., a selected tumor-associated antigen on the tumor cell. This binding allows for BiAb molecules to aggregate, enabling crosslinking of the SAR. This activates the T cells and directs T cell-mediated lysis [22]. This tumor-killing activity is limited by the supply and half-life of the BiAb. Notably, SAR T cells, unlike CAR T cells, can be removed from the circulation if needed, by using FDA-approved monoclonal antibodies, without having to rely on the addition of a suicide gene [22]. With these favorable properties, SAR T cells developed to target AML could overcome the hurdles of toxicities and escape variants.

To enable better control over T cell activity for AML and ALL indications, we reasoned that we could replace the BiAb (IgG) with tandem scFv (taFv) constructs as these would be more controllable and safer due to their shorter half-life [23, 24]. Here, to test this, we developed novel taFv constructs made up of two scFvs linked by a $(G_4S)_4$ linker. These constructs have dual specificities: one to target AML (via binding CD33 or CD123), and the other to target the SAR-expressing T cell (via binding E3 – which is the inert extracellular domain of the T cell-activating SAR).

We could show that T cells expressing the SAR construct can, in a reversible manner, be selectively activated in the presence of AML cells ($CD33^+$ or $CD123^+$) and the taFv molecule. We demonstrate that, unlike a conventional BiTE (anti-CD33–anti-CD3), which activates pan-T cells, our E3-specific constructs activate only SAR-transduced T cells – giving additional control over effector cell modifications, phenotype and dosage. Importantly, we highlight substantial activity of the platform in primary AML-blast cultures and in different AML-xenograft models, underpinning the translational potential of the approach.

Methods

Animal experimentation

4-week-old female NSG mice (NOD.Cg-Prkdcscid Il2rgtm1WjI/SzJ) were purchased from Charles River (Sulzfeld, Germany). MV4-11-LUC-GFP and THP-1-LUC-GFP xenograft models were established by intravenously injecting 2×10^6 or 10^6 cells, respectively into the tail vein. taFv molecules were delivered intraperitoneally as indicated. 10^7 T cells were given intravenously as indicated. All

animal experiments were approved by the local regulatory agency (Regierung von Oberbayern). Prior to treatment mice were randomized according to tumor burden. Endpoints were registered by an observer blinded to the treatment groups as previously defined [25]. More than 15% weight loss after experiment start or a decrease in general health condition (decreased mobility, general weakness, hunched posture or ungroomed hair) are defined as humane surrogate endpoints for survival and are later referred to as survival of mice. In vivo imaging approach outlined in supplementary methods.

Binding studies

Apparent dissociation constants (K_D) were measured by calibrated flow cytometry on a Guava easyCyte 6HT instrument (Merck Millipore, Burlington, MA, USA) with 3.0 to 3.4 µm Rainbow Calibration particles (BioLegend, San Diego, CA, USA) as calibration control [26]. After normalization, data points were fitted to a one-site specific binding model. Expression and purification of molecules is outlined in supplementary methods.

Cell lines

PL-21, THP-1, MOLM-13, MV4-11, E.G7-OVA, and SEM cell lines were purchased from ATCC (USA). The E.G7-OVA cell line was modified to express full-length human EGFRvIII (Uniprot Entry P00533 AA 1-29, 298–646), resulting in E.G7-EGFRvIII cells. Luciferase-eGFP (LUC-GFP) overexpressing cell lines PL-21-LUC-GFP, THP-1-LUC-GFP and MV4-11-LUC-GFP were generated according to previously described protocols [22]. Antigen quantification of cell lines are summarized in Supplementary Table 1A. 293Vec-Galv and 293Vec-RD114 were a kind gift of Manuel Caruso, Québec, Canada and have been previously described [27]. All human cell lines were short tandem repeat profiled in house to verify their origin. Cells were used for a time period no longer than two months.

Cytotoxicity assays

T cells were incubated with tumor cell lines and taFvs at indicated effector-to-target ratios and concentrations. Following a 24 h coculture, the BioGlo Assay (Promega, USA) system was used according to the manufacturer's protocol.

Confocal microscopy

Blinded confocal imaging and conjugate quantification were carried out following the selection of 10 representative areas of each slide. Cells in or out of conjugate within each area were quantified and a ratio thereof subsequently determined. For each conjugate, the position of the microtubule organizing center (MTOC) was observed, and its polarization to the immune synapse, or lack thereof, was noted. The ratio of polarized to nonpolarized MTOCs was used to determine the ratio of functional synapses out of all conjugates formed.

Flow cytometry

Flow cytometry was carried out according to previously published protocols [28]. For cell number quantification CountBright® absolute counting beads (Life Technologies) were added. Samples were analyzed with flow cytometers from BD, Canto II and Fortessa (BD Bioscience, Germany) and a Beckman Coulter CytoFLEX for the long-term cultures. Surface antigen density of cell lines and constructs was evaluated with QIFIKIT (Agilent Dako, Santa Clara, CA, USA). Flow cytometry data were analyzed with FlowJo V10.3 software or GuavaSoft, version 3.1.1 (Merck Millipore). Staining approach outlined in supplementary methods.

Generation of T cell activating fusion constructs and T cell transduction

SAR construct generation was previously described [22]. SAR-transduced T cells will be referred to as SAR T cells. An anti-CD33–CD28–CD3ζ (anti-CD33 CAR) was generated with the same humanized scFv against CD33 used for the taFv construct [29]. Transduction and expansion of primary human T cells was carried out following a previously described protocol [25]. Virus production methods outlined in supplementary methods.

Interferon-γ release assays

Human T cell stimulation assays were set up at indicated concentrations and effector-to-target ratios. IFN-γ was quantified by ELISA (BD Bioscience).

Long-term coculture assays

AML blasts were cultivated for 3 days before coculture. Allogeneic healthy donor T cells were incubated with patient-derived AML blasts at indicated effector-to-target ratios and concentrations. Untransduced T cells were used to control for allogeneic effect. Patient blasts were otherwise cultured according to the previously described protocol [30].

Patient and healthy donor material

After written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilians-Universität (Munich, Germany), peripheral blood (PB) or bone marrow

(BM) samples were collected from healthy donors and AML patients. At initial diagnosis or relapse, samples were analyzed at the Laboratory for Leukemia Diagnostics of the Klinikum der Universität München as previously described [31, 32]. Patient characteristics are summarized in Supplementary Table 2A, B.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism software V8.3.1 (San Diego, CA, USA). Differences between experimental conditions were analysed as described in figure P values < 0.05 were considered to be significant. Data are shown as mean values SEM of a minimum of three biological replicates or independent experiments, as indicated. For in vitro experimentation with healthy donor or patient samples, no statistical methods were used to predetermine sample size. These were chosen based on prior experience with this experimental design and patient sample availability. For in vivo experimentation sample sizes were used in accordance with prior experience with the models used.

Results

Tandem scFv-mediated effects on SAR T cell activation, proliferation and differentiation

Based on our previous results, we hypothesized that the SAR platform could be developed specifically for AML targeting and treatment [22]. We began by recombinantly generating bispecific anti-E3–anti-CD33 and anti-E3–anti-CD123 taFv molecules. We envisioned that these E3-targeting molecules could efficiently and selectively redirect SAR-expressing T cells to AML blasts (Fig. 1A).

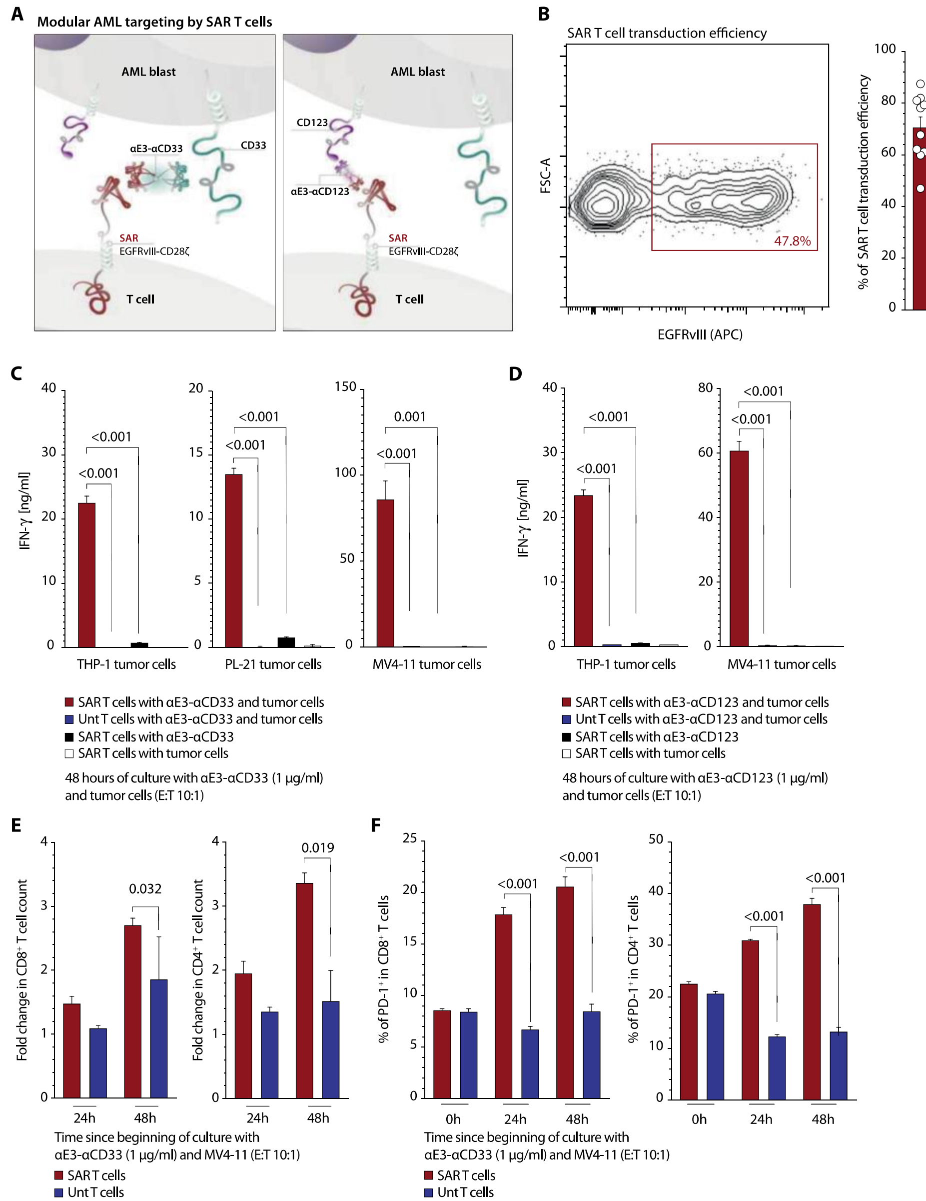
The E3 SAR could be retrovirally transduced into human T cells from healthy donors with high efficiencies (Fig. 1B and Supplementary Table 1B). The novel anti-E3–anti-CD33 molecule was designed to have a high affinity for the target cells (CD33 $K_D = 19.5$ nM), and a lower affinity for the T cells (E3 $K_D = 235.8$ nM) so that aggregates could form more easily on the target cells. The binding properties and apparent dissociation constants of the anti-E3–anti-CD33 molecule to both its targets were analyzed by flow cytometry (Supplementary Fig. 1A and 2A). Similarly, the anti-E3–anti-CD123 molecule was designed using the same backbone as the CD33-targeting one and served as an additional AML-specific targeting taFv molecule to demonstrate the modularity of the platform (CD123 $K_D = 32$ nM) (Supplementary Fig. 1A and 2A). We additionally generated an anti-E3–anti-CD19 molecule to serve as a non-AML-targeting control construct (CD19 $K_D = 4.9$ nM) (Supplementary Fig. 1A and 2A). The anti-CD3–anti-CD33 control has been previously characterized [33]. Purified proteins were analyzed by SDS-PAGE and analytical size exclusion chromatography and protein stability was assessed by fluorescence-based thermal shift assay (Supplementary Fig. 1B to E).

In vitro, taFv-mediated T cell activation is strictly dependent on antibody aggregation on the target cell and their presentation to the T cell in a polyvalent form [34]. To assess this conditional T cell activation upon targeting of the SAR molecule, we incubated SAR T cells with the anti-E3–anti-CD33 construct in the absence or presence of three CD33-expressing AML cell-lines, PL-21, THP-1, and MV4-11, with untransduced (unt) T cells serving as a control. Only SAR T cells in the presence of the taFv construct as well as the target antigen were shown to produce IFN-γ, whereas unt T cells were not stimulated, even in the presence of both taFv and target molecules (Fig. 1C). The anti-E3–anti-CD123 taFv was similarly evaluated, demonstrating both comparable and conditional T cell activation (Fig. 1C, D).

Congruently, SAR T cell activation following coculture with target AML cells resulted in enhanced proliferation of both $CD4^+$ and $CD8^+$ SAR T cells when compared to other T cell and taFv controls (Fig. 1E). We further observed upregulation of the T cell activation marker PD-1 specifically for SAR T cells compared to the control T cells following coculture with target AML cells and taFv (Fig. 1F). Following activation in culture, SAR T cells were also observed to have a mixture of effector and effector memory phenotypes, similar to the control T cells (Supplementary Fig. 2B)

SAR T cells form functional immunological synapses to mediate efficient tumor-cell lysis

CD33-expressing tumor cells were effectively targeted and lysed by anti-E3–anti-CD33 and anti-E3–anti-CD123-activated SAR T cells, but not unt T cells (Fig. 2A and Supplementary Fig. 2C). To dissect the mode of action of SAR T cells in these settings, we analyzed the interface between both cell types. Cell conjugates and synapses formed between the T cells and tumor cells were labeled and quantified. SAR T cell conjugates occurred significantly more frequently than unt T cell-target cell conjugates (Fig. 2B). To probe the nature of the immunological synapse (IS), we assessed F-actin and CD11a-LFA-1 accumulation. Strong accumulation of F-actin is indicative of a functional immune synapse, which was observed to span the entire area of the synapse (Fig. 2C). A moderate accumulation



of the LFA-1 signal was also seen at the IS, although the signal was also observed across the T cell surface. IS functionality was judged by the polarization of the MTOC, or lack-thereof, as well as the organization pattern of the T cell-associated tyrosine kinase, Lck. Significantly more SAR T cell-target cell conjugates had a polarized MTOC compared to unt T cell control conjugates (Fig. 2B, C). Moderate Lck accumulation

◀ **Fig. 1 SAR T cells can be bound and triggered by tandem scFvs to induce T cell activation and proliferation. A** Schematic overview of the SAR construct as well as the modular composition of anti-E3–anti-CD33 and anti-E3–anti-CD123 molecules and CD33 and CD123 target structures. **B** Transduction efficiency flow cytometry plot and SAR expression data in T cells from healthy donors. **C** SAR and unt T cells were cocultured with THP-1, PL-21, or MV4-11 tumor cells and anti-E3–anti-CD33 molecule, with hIFN-γ readout 48 h after coculture. **D** SAR and unt T cells were cocultured with THP-1 or MV4-11 tumor cells and anti-E3–anti-CD123 molecule, with hIFN-γ readout 48 h after coculture. **E** The proliferation rate of the T cells was determined by flow cytometry analysis with surface staining for CD3, CD4, CD8, and EGFR after coculture. **F** SAR and UT T cells were cocultured with MV4-11 tumor cells at a 10:1 E:T ratio. Anti-E3–anti-CD33 taFv was added at a concentration of 1 μg/ml. Readouts were carried out at 0, 24, and 48 h time-points. PD-1 expression of SAR and UT $CD4^+$ and $CD8^+$ T cells over time (0, 24, and 48 h) is shown. Statistical analysis was performed with unpaired two-tailed Student's *t* test. Experiments in subfigures (**B–F**) show mean values ± SEM and are representative of three independent experiments.

was observed at the IS, however a dispersed signal could also be seen (Fig. 2C). SAR T cells also showed granzyme B accumulation and degranulation at the IS, demonstrating formation of a mature and functional IS (Fig. 2C).

Modular, selective and reversible activation of SAR T cells and their applied safety switches

Due to the antigen heterogeneity in AML, and because of toxicities associated with the targeting of myeloid lineage antigens, cell therapy approaches need to be modular and controllable [35, 36]. To show selectivity advantages of the SAR platform over BiTE, SAR T cells were serially titrated in a peripheral blood mononuclear cell (PBMC) mix, then cocultured with target cells and either a pan-T cell-targeting molecule (anti-CD3–anti-CD33) or a SAR-specific one (anti-E3–anti-CD33). The selective activation of SAR T cells was evident when the SAR–PBMC mix was cocultured with an anti-E3–anti-CD33 molecule, as IFN-γ levels decreased with lower concentrations of SAR T cells in the mix (Fig. 3A). This titrated T cell activation effect was lost when the anti-CD3–anti-CD33 molecule was employed at equivalent total cell numbers. Furthermore, the anti-E3–anti-CD33 construct did not mediate any T cell activation when incubated with a pure PBMC mix devoid of SAR T cells, whereas the anti-CD3–anti-CD33 molecule was non-selective in activating $CD3^+$ T cells in the PBMC mix, as expected (Fig. 3A).

An intrinsic safety switch of the SAR platform is that the activity of SAR T cells is strictly dependent on the presence of the taFv construct. Contrary to CAR T cells, the activity of which is irreversible in the presence of the target antigen, SAR T cell activity should quickly dissipate with clearance of the taFv. Indeed, we found that, following cocultures with MV4-11 tumor cells, SAR T cell activity was reversible over time in the absence of taFv redosing, unlike human anti-CD33 CAR T cells. Importantly, repeated dosing of the taFv molecule could maintain SAR activity at comparable levels to that of the CAR (Fig. 3B). This data indicates that engineering the half-life of the taFv molecule would enable control over SAR activity.

The relatively short half-life of the taFv molecule should also enable modularity of the platform, i.e., the sequential targeting of multiple antigen types that would allow for more refined patient-specific tailoring of the treatment. Modularity was demonstrated when the same SAR T cells were redirected toward AML cells expressing multiple targets. SAR T cells were cocultured with $CD33^+$ $CD123^+$ THP-1 cells. Through the addition, exchange or depletion of anti-E3–anti-CD33 or anti-E3–anti-CD123-targeting molecules we could show modularity by sequentially redirecting SAR T cells toward different AML targets (Fig. 3C and Supplementary Fig. 3A).

Overall, this approach has the potential to target a multitude of AML-associated antigens with a level of flexibility and controllability that is superior to that of CAR T cells. These advantages together with the aforementioned safety facets make this platform a promising modality for the targeting of myeloid lineage antigens.

SAR–taFv combination can mediate specific cytotoxicity against patient-derived AML blasts and leukemic stem cells

To further translate the potential of our approach, we assessed SAR T cell activity against patient-derived AML blasts. A long-term coculture assay system was utilized to evaluate SAR T cell-mediated cytotoxicity over time. AML blasts were specifically targeted by SAR T cells in the presence of the anti-E3–anti-CD33 molecule, whereas control T cell and taFv combinations were not (Fig. 4A, B and Supplementary Fig. 3B, C). We applied a similar setup to test the efficacy of the approach in an autologous AML patient setting. We could successfully isolate, culture and transduce patient-derived T cells with the SAR (Supplementary Fig. 3D). Their capacity to target their own blasts in the presence of either anti-E3-anti-CD33 or anti-E3-anti-CD123 taFvs was demonstrated, with similar effects to what was already shown in the allogeneic setting (Fig. 4C). SAR T cell activity was also assessed by expression of the markers PD-1, TIM-3, and CD69 after 3 days of coculture. In the presence of the taFv and AML blasts, SAR T cells upregulated PD-1, TIM-3, and CD69 (Fig. 4D and Supplementary Fig. 3E). In addition, we could show that the SAR–taFv combination could also effectively target $CD34^+$ $CD38^-$ LSC (Fig. 4E, F). The data obtained supports the clinical application of the platform as it shows the efficacy of the approach in targeting patient blasts and LSCs.

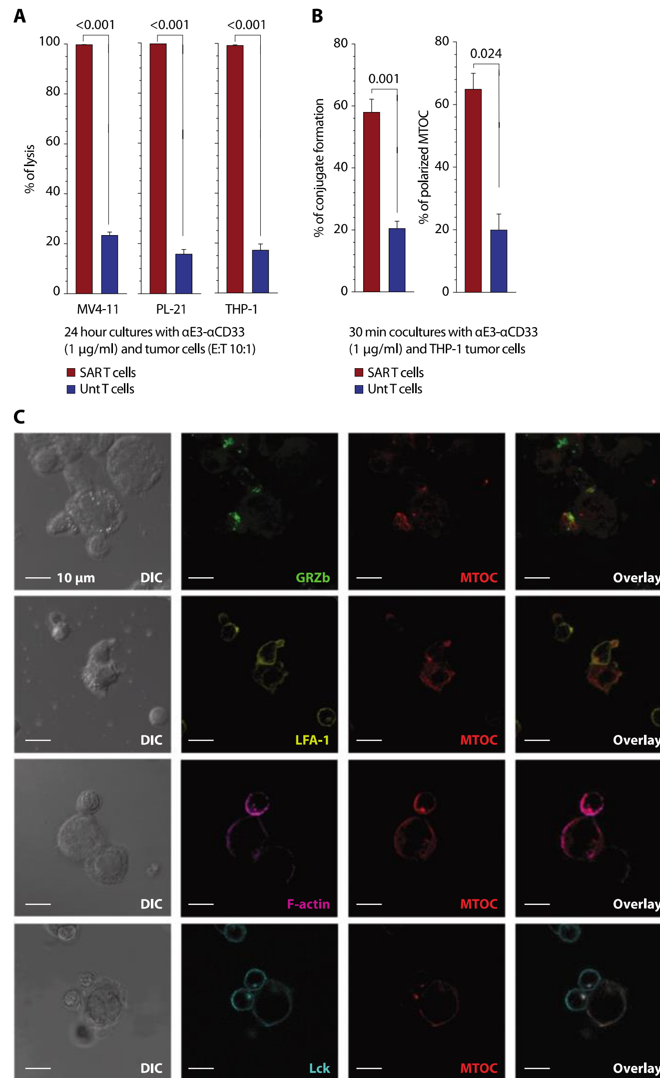


Fig. 2 SAR T cells selectively form functional immunological synapses to mediate efficient tumor cell lysis. A SAR and unt T cells were cocultured with THP-1, PL-21, or MV4-11 tumor cells with anti-E3–anti-CD33. Following coculture, the BioGlo Luciferase assay was used to calculate the percentage of cells lysed—values shown were normalized to the AML only control condition which was taken as 0 % lysis. **B** SAR or unt T cells were cocultured with THP-1 tumor cells in a V-well plate before transfer to a poly-L-lysine-coated slide. Cells were allowed to adhere for 30 min before fixation and permeabilization. The percentage of T cells conjugated with tumor cells was quantified, as well as the percentage of those conjugates with a polarized MTOC. **C** Double Immunofluorescence labeling was carried out to characterize the polarization of the MTOC, Granzyme B, LFA-1 and F-actin at the SAR T cell IS. For statistical analysis the unpaired two-tailed Student's *t* test was used. Experiments in subfigures (**A** and **B**) show mean values ± SEM and are representative of at least three independent experiments. Subfigure (**D**) is representative of three independent experiments. Leica TCS SP5 confocal system with a HCX PL APO CS 63x/1.4 oil objective was used for image acquisition on Leica application suite v2.7.3.9723. Tumor cells were GFP positive. Fluorochromes used: MTOC (AF594) Granzyme B (AF647); F-actin (AF647); LFA-1 (AF647); Lck (AF647). For z-axis image reconstruction (stacking) confocal sections were taken 0.2 μm apart.

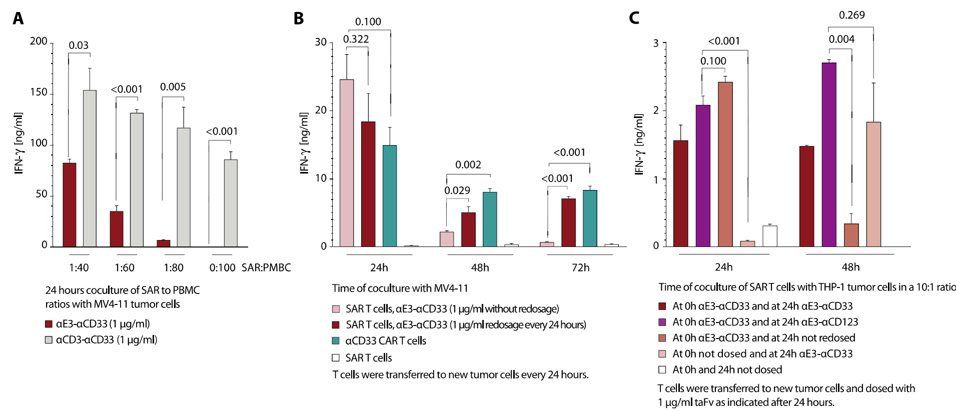


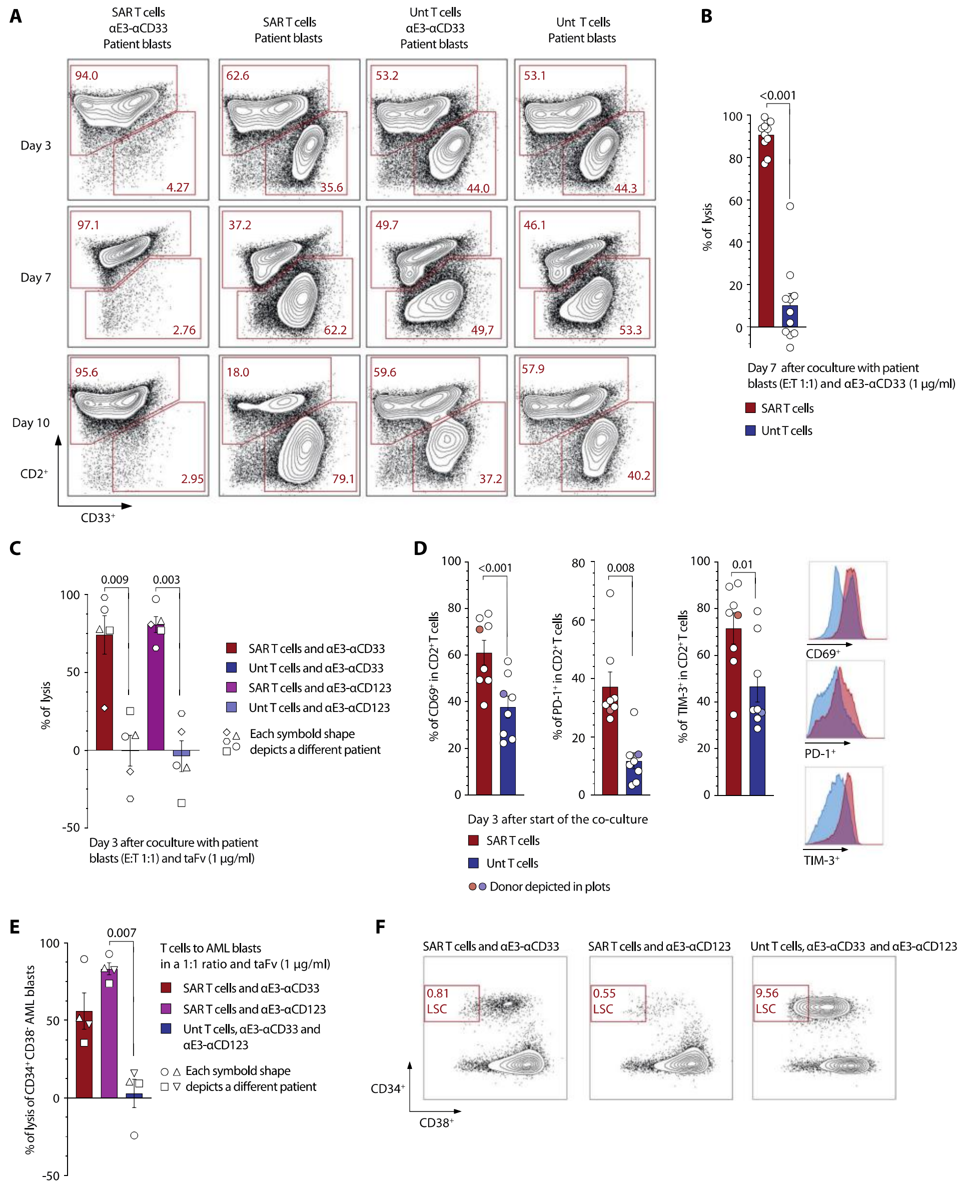
Fig. 3 Modular, selective and reversible activation of SAR T cells and their applied safety switches. A SAR T cells were serially titrated (1:40, 1:60, 1:80, 0:100) in a PBMC mix. Cells were then cocultured with MV4-11 tumor cells (E:T 10:1), with either a pan-T cell (anti-CD3–anti-CD33, 1 μg/ml) or a SAR-specific molecule (anti-E3–anti-CD33, 1 μg/ml). **B** MV4-11 tumor cells were repeatedly cocultured with SAR T cells with or without redosage of the constructs (1 μg/ml). Anti-CD33 CAR T cells were used as a control and cocultured with tumor cells following the same procedure (no taFv was added) (E:T 10:1). **C** A modularity stress test was carried out using anti-E3–anti-CD33 and anti-E3–anti-CD123 molecules (1 μg/ml). SAR or unt T cells were cocultured with THP-1 tumor cells (E:T 10:1). Readouts were carried out at 24 or 48 h. At assay start, cocultures received either anti-E3–anti-CD33 molecules, anti-E3–anti-CD123 molecules, or no molecules. At 24 h, cocultures were either redosed with the same taFv, redosed with the other taFv against a different target, dosed for the first time with either molecule, or not redosed after initial dosing. At each time point, supernatants were collected and subjected to a hIFN-γ ELISA readout. For statistical analysis, the unpaired two-tailed Student's *t* test was used. Experiments show mean values ± SEM and are representative of three independent experiments.

Treatment with the SAR-taFv combination can efficiently eradicate leukemia and enhance survival in vivo

To probe the in vivo function of the SAR–taFv combination, we took advantage of xenograft models of leukemia by engrafting two different AML cell-lines, THP-1-LUC-GFP and MV4-11-LUC-GFP, into NSG mice (Fig. 5A, D). In the MV4-11 model, mice treated with the SAR T cell and anti-E3–anti-CD33 taFv combination experienced major responses to the therapy, with improved tumor control in all treated mice, and a complete response observed in two out of seven mice, which was not seen under any of the negative control conditions. A direct comparison against aCD33-CAR-treated mice was also carried out in this model. While a strong antitumoral response could also be observed in the CAR-treated group, the mice developed severe toxicity (appeared to be non-disease related, likely graft-versus-host disease) and were subsequently taken out of the experiment (Fig. 5B, C). In the THP-1 model, a strong antitumoral response was also observed with the SAR T cell and taFv combination, with one out of five mice clearing the disease (Fig. 5E, F).

Moreover, overall survival was significantly improved in the SAR with anti-E3–anti-CD33 treatment group compared to SAR with anti-E3–anti-CD19 (i.e., non-AML targeting) treatment group in both MV4-11 ($p = 0.009$) and THP-1 ($p = 0.010$) models (Fig. 5B, F). Ex vivo phenotyping of SAR T cells at the experimental endpoint (70 days post transfer) revealed prolonged persistence in the treated mice of the THP-1 model. These SAR^+ T cells predominantly possessed an effector memory phenotype. CD25 and CD69 staining revealed a higher expression in $CD4^+$ and $CD8^+$ subsets in the BM compared to the spleen, which correlated with observed tumor burden. PD-1 staining revealed very high expression levels in both the BM and spleen (Supplementary Fig. 4A–D). Together these data indicate that the SAR platform can mediate substantial therapeutic activity in relevant AML xenograft models.

To demonstrate the modularity of the SAR-taFv combination in vivo, we treated THP-1-bearing mice with SAR T cells plus an anti-E3-anti-CD33 or anti-E3-anti-CD123 taFv. We found that mice continuously treated with either taFv showed comparable anti-tumoral efficacy to mice where taFv treatment was switched after four doses, indicative that the targeting moiety can indeed be changed without T cell reinfusion over the course of treatment. In contrast, in mice where taFv treatment was ceased after four doses, the disease quickly progressed, reaching comparable levels to that of mice that received no taFv treatment (Supplementary Fig. 5A, B). This demonstrates the reversibility of T cell activation induced by the taFv modules, which ceases with module decay.



Discussion

Donor T cell alloreactivity driving the graft-versus-leukemia effect is a major mechanism behind the curative effect of allo-SCT, and supports the notion that T cells are crucial effector cells in the context of AML therapy [37, 38]. Importantly, there is strong preclinical and clinical evidence showing that T cell-based treatment is an

◀ **Fig. 4 SAR–taFv combination can activate SAR T cells to mediate specific cytotoxicity against patient AML blasts and LSCs. A** Patient-derived AML blasts targeted by SAR T cells (E:T 1:1) and an anti-E3–anti-CD33 taFv (1 µg/ml), or with controls (SAR T cells and patient blasts, unt T cells with anti-E3–anti-CD33 and patient blasts, unt T cells and patient blasts). In a long-term coculture assay set-up, flow cytometry-based readouts were taken after 3, 7, and 10 days. Cells were stained for CD2 and CD33, to differentiate the T cells and AML blasts respectively. **B** The percentage lysis of patient-derived AML blasts ($n = 11$) by SAR T cells and taFv was calculated as a ratio and compared to unt cells and AML blasts. **C** Patient-derived AML blasts targeted by autologous SAR T cells (E:T 1:1) and either an anti-E3–anti-CD33 taFv (1 µg/ml) or an anti-E3–anti-CD123 taFv (1 µg/ml), or with controls (SAR T cells and patient blasts, unt T cells with either taFv and patient blasts, unt T cells and patient blasts). In a coculture assay set-up, flow cytometry-based readouts were taken after 3 days. Cells were stained for CD2 and CD33, to differentiate the T cells and AML blasts respectively. **D** Following coculture (at day 3), T cells were also stained for CD69, PD-1 and TIM-3. **E** Short-term coculture (18 h) assays were set-up between 5×10^5 patient blasts and SAR T cells (E:T 1:1) and an anti-E3–anti-CD33 (1 µg/ml) or an anti-E3–anti-CD123 (1 µg/ml) molecule, or with controls (SAR T cells only, anti-E3–anti-CD33 and anti-E3–anti-CD123 molecules only, patient blasts only, unt T cells with anti-E3–anti-CD33 and anti-E3–anti-CD123 molecules, unt T cells with AML blasts). To show efficiency of LSC killing, blasts were stained for CD45, CD34, and CD38, and lysis of the $CD34^+CD38^-$ LSC population was quantified as a ratio over unt T cells with patient blasts as a control condition. **F** Representative flow cytometry plots from coculture experiment described in subfigure (**E**). For statistical analysis, the paired two-tailed Student's *t* test was used. Experiments show mean values ± SEM. Experiments in subfigures (**A**, **B**, and **D**) are representative of six independent long term coculture (LTC) experiments, with multiple patients used per LTC. Experiments in subfigure (**C**) are representative of two independent coculture experiments, with two to three patients used per coculture. Experiments in subfigure E are representative of four independent short term coculture experiments. Patient information for each experiment is listed in supplementary Table 2. CD33 and CD123 patient expression data is depicted in supplementary Fig. 4.

effective means of targeting and eliminating AML, including LSCs [39].

Our studies demonstrate that SAR T cells can be redirected by a SAR-specific taFv construct toward the aberrantly expressed AML antigens CD33 and CD123. We could show that SAR T cells are able to specifically recognize multiple targets on several AML cell lines, demonstrating in vitro and in vivo efficacy. We also showed induction of a functional synapse (MTOC polarization, F-actin area), whereas Lck and LFA-1 organization patterns were comparable to those reported for the IS of CAR T cells [40]. This targeted specificity and cytolytic capacity was furthermore demonstrated by the successful targeting of patient-derived AML blasts and LSCs. The potent anti-leukemic activity observed with the SAR platform, in in vivo models and against patient-derived AML blasts, is comparable to those observed in the preclinical testing of AML-targeting BiTEs and CARs [30, 41, 42].

Despite its similarly broad expression on myeloid progenitors and some normal B cell and activated T cell populations, CD33 remains a valuable antigen for the targeting of AML due to its overexpression on blasts in all AML [43, 44]. Low CD33 antigen density in subsets of patient blasts remains a caveat of targeting this antigen [17]. To tackle this, we designed the taFv molecule with a comparably higher CD33 binding affinity, resulting in better targeting of blasts with low CD33 surface expression. A higher affinity for the tumor antigen also means a taFv matrix can be formed on the surface of the AML cells, upon which SAR T cells, with their lower affinity to the taFv, can mediate serial tumor cell killing more efficiently [33, 45]. This design also minimizes antibody trapping in T cell-containing tissues, such as the spleen or lymph nodes, reducing the potential for off-target toxicity [46, 47].

To date, anti-AML CAR T cells have shown limited efficacy in the clinic [48, 49], with on-target off-tumor toxicity being especially problematic in the context of targeting CD33 [50, 51]. To overcome these challenges, highly modular and controllable approaches, as well as those that can make normal hematopoiesis resistant to targeted therapy are needed. One such approach could generate hematopoietic systems unaffected by CD33-targeted therapy [52, 53]. Our SAR platform repurposed for AML, remains, as previously described, highly modular and controllable [22]. Through the direct comparison of a pan-T cell targeting molecule with a SAR-specific one, we could substantiate the claim that nonengineered T cells are not affected by the platform. This level of controllability means the SAR platform distinguishes between two T cell populations in the patient (engineered and nonengineered), which can be carefully selected and tailored. Once the T cell arm of the therapy is adoptively transferred, the redirection and subsequent activation of SAR T cells is completely dependent on the taFv. Clearance of the taFv, in the event of toxicity or on-target-off-tumor activity, would reverse SAR T cell activity. Further or sequential targeting of the AML through the redirection of pre-existing SAR T cells could then be achieved through the introduction of a new taFv with a different AML specificity. In the event of target downregulation as an escape mechanism following treatment (the most prevalent resistance mechanism observed following blinatumomab treatment in ALL patients), platform modularity would again be advantageous. Furthermore, an additional safety layer is ensured by the unique expression of EGFRvIII on SAR T cells (otherwise only expressed on pathologic tissues, such as gliomas), thus depletion with cetuximab as another safety switch is possible if required [22]. Taken together, the SAR platform aligns the advantages of antibody therapy (controllable dosing and reversibility) with that of adoptive T cell therapy (potent anti-tumoral effectors).

Many approaches have emerged attempting to make CAR T cells more modular and controllable. These

include but are not restricted to the SUPRA CAR (with tunable availability and affinity) [54], CAR T cells using the synNotch receptor (that can induce transcription of CAR expression to target a second antigen) [55], bispecific CARs (can target two tumor antigens interchangeably) [56] and suicide CAR T cells (such as those using the inducible caspase 9 system, whereby small molecules can activate apoptosis independent of CAR activation) [57]. The SUPRA CAR has the potential to improve the broader applicability and modularity of CAR T cells. The published data however does not show durable in vivo efficacy, while the controllability data (in sparing cells with lower antigen expression) is suboptimal.

◀ **Fig. 5 Treatment with the SAR–taFv combination can efficiently eradicate leukemia and enhance survival in vivo. A** Schematic overview of the experimental setup for (**B** and **C**). NSG mice were inoculated i. v. with 2×10^6 MV4-11-LUC-GFP tumor cells. Mice were treated with a single i. v. injection of T cells. Antibody treatment was given by several i. p. injections of the anti-E3–anti-CD33 molecule (2.8 µg/injection) or a control anti-E3–anti-CD19 molecule (2.8 µg/injection), as indicated by the arrows in the figure. Treatment groups were as follows: SAR T cells and anti-E3–anti-CD33 ($n = 7$), SAR T cells and anti-E3–anti-CD19 ($n = 6$), SAR T cells only ($n = 5$), anti-E3–anti-CD33 only ($n = 6$), PBS ($n = 6$), and anti-CD33 CAR T cells ($n = 5$). **B** Percentage survival readout. † indicates sacrifice of mice suffering from CAR-related toxicity. **C** In vivo imaging data displaying luminescent signal in counts for all experimental groups from treatment day onwards (Days 0, 7, 14, 17, 21, 28, and 42). **D** Schematic overview of the experimental setup for (**E** and **F**). NSG mice were inoculated i. v. with 10^6 THP-1-LUC-GFP tumor cells. Mice were treated with a single i. v. injection of T cells with or without the anti-E3–anti-CD33 molecule (2.8 µg /injection) or a control anti-E3–anti-CD19 molecule (2.8 µg /injection). Treatment groups were as follows: SAR T cells and anti-E3–anti-CD33 ($n = 5$), SAR T cells and anti-E3–anti-CD19 ($n = 5$), SAR T cells only ($n = 5$), anti-E3–anti-CD33 only ($n = 5$), and PBS ($n = 5$). **F** Percentage survival readout. **G** In vivo imaging data displaying luminescent signal in all experimental groups from treatment day onwards (Days 0, 24, 28, 38, 45, 52). For statistical analysis of survival data, the log-rank test was applied. All in vivo experiments were carried out twice. One representative experiment is shown per xenograft model.

Furthermore, its applicability and tailoring toward specific disease settings, such as AML, is yet to be shown [54]. Bispecific CAR T cells are already in clinical testing (anti-CLL-1–anti-CD33; NCT03795779), though given the interpatient LSC diversity, it is unlikely that any two-antigen combination would suffice in eradicating disease across patient cohorts. By comparison, our platform gives more freedom in tailoring a patient-specific combination therapy. As mentioned, the AML setting stands to benefit from improved target selectivity. Application of the synNotch CAR system could improve safety and reduce myeloid toxicity [55]. Despite this, the system still lacks modularity, an important feature for improved targeting of heterogeneous leukemic stem cell populations in AML. A big challenge in the CAR system is autonomous signaling [58]. This is in contrast to the SAR-taFv platform which provides a functionally inert molecule only triggered by the addition of a specific taFv but not by any other known molecule in the body.

Furthermore, many of the modular CAR approaches (such as switchable CAR T cells) rely on the introduction of a neoepitope for selective targeting [59], which comes with immunogenicity issues driving either anti-drug immune responses and dampening activity or potentially triggering toxicities or all of it. SAR T cells and their triggering taFv are fully human or humanizable proteins which markedly reduces immunogenicity risks. Despite the appeal, suicide systems in CARs is a rather brute approach that eliminates all effector cells and requires additional genetic modifications. Importantly, the ability to deplete CAR T cells in the event of toxicities remains to be demonstrated clinically. These approaches have been comprehensively reviewed by Darowski et al. [60].

Perhaps the greatest challenge hindering the success of adoptive T cell therapy in AML is target specificity, which results from disease heterogeneity and diverse antigen expression on LSCs. A recent AML proteomic and transcriptomic study revealed a series of differentially expressed AML-specific antigens, out of which came the rationale that systematic therapeutic combinations would be ideal in the context of AML therapy [18]. This approach was given clinical relevance after a coexpression profile of LSC markers was described for AML patients [17]. The authors found CD33, CD123, CLL1, TIM3, and CD244 to be ubiquitously expressed on AML cells both at diagnosis and relapse stages, and further stressed the benefits of a dual targeting approach for AML. Thus, despite CD33 being expressed on the vast majority of LSCs, the importance of a modular approach with the capacity to simultaneously, or, in the event of antigen escape (or clonal heterogeneity), sequentially target other AML-specific antigens is clear, and is further evidenced by previous work [2, 61].

A strength of our platform—its modularity, stands to benefit from the significant research that has already been carried out on many AML targets as stand-alone targeted therapies [16, 46, 62]. Thus, the repurposing of this knowledge might be a fast and effective method to accelerate the pre-clinical development of the approach. As specific taFv molecules can be tailored individually, the potential for combinatorial approaches will only be limited by the testing and approval of the separate molecules. The SAR platform still stands to benefit from certain optimizations. Amongst these is the modulation of SAR surface expression, an approach that has been successfully applied to the CAR T cell setting [63]. In addition, while advantageous, the short half-life of the taFv will likely require regular infusions, which could present hurdles in the form of practicality and cost.

Collectively, we could comprehensively demonstrate that AML-specific taFvs can be effectively used to target AML in a controllable manner. While further development and more extensive testing are required before its application in a clinical setting, the SAR platform undoubtedly offers new solutions to the ever-challenging AML disease setting.

Acknowledgements Binje Vick and Irmela Jeremias provided patient-derived xenografted cells for LSC experiments. We acknowledge Life Science Editors for their editorial assistance. This study was supported by the Marie-Sklodowska-Curie Program Training Network for the Immunotherapy of Cancer funded by the H2020 Program of the European Union (Grant 641549, to SE and SK), the Marie-Sklodowska-Curie Program Training Network for Optimizing

Adoptive T Cell Therapy of Cancer funded by the H2020 Program of the European Union (Grant 955575, to SK), the Hector foundation, the International Doctoral Program i-Target: Immunotargeting of Cancer funded by the Elite Network of Bavaria (SK and SE); Melanoma Research Alliance Grants 409510 (to SK); the Else Kröner-Fresenius-Stiftung (SK); the German Cancer Aid (SK); the Ernst-Jung-Stiftung (SK); LMU Munich's Institutional Strategy LMUexcellent within the framework of the German Excellence Initiative (SE and SK); the Bundesministerium für Bildung und Forschung Project Oncoattract (SE and SK); by the European Research Council Grant 756017, ARMOR-T (to SK), by the German Research Foundation (DFG to SK), the Fritz-Bender Foundation (to SK) and the José-Carreras Foundation (to SK).

Author contributions MRB, KPH, and SK designed the experiments and interpreted the data. MH, SS, NF, and MRB generated and characterized the molecules. MRB, BLC, MS, and SS performed cell line-based assays. MRB and AD performed synapse imaging and conjugation assays. FR contributed to cell line generation. CK and SE provided critical feedback and support. MRB, BLC, SL, AO, MS, and AG performed the in vivo studies. MRB and BLC evaluated the data. CA, BB, LR, and MS contributed to patient characterization including cytogenetic and molecular data, and interpreted the data generated from primary AML samples. SK supervised the project. BLC designed the figures. MRB, BLC, and SK wrote the manuscript with input from all authors.

Compliance with ethical standards

Conflict of interest SK, CK, and SE are inventors of several patents in the field of immuno-oncology including one patent application on the SAR platform. SK and SE received research support from TCR2 Inc and Arcus Bioscience for work unrelated to this manuscript. Parts of this work have been performed for the doctoral thesis of MRB at the Ludwig-Maximilians-Universität München. The authors declare no other conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Estey EH. Acute myeloid leukemia: 2014 update on risk-stratification and management. Am J Hematol. 2014;89:1063–81.
2. Yilmaz M, Wang F, Loghavi S, Bueso-Ramos C, Gumbs C, Little L, et al. Late relapse in acute myeloid leukemia (AML): clonal evolution or therapy-related leukemia? Blood Cancer J. 2019;9:7.
3. Yeung YA, Krishnamoorthy V, Dettling D, Sommer C, Poulsen K, Ni I, et al. An optimized full-length FLT3/CD3 bispecific antibody demonstrates potent anti-leukemia activity and reversible hematological toxicity. Mol Ther 2020;28:P889–900.
4. Lim WA, June CH. The principles of engineering immune cells to treat cancer. Cell. 2017;168:724–40.
5. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science. 2015;348:62–8.
6. Benmebarek M-R, Karches HC, Cadilha LB, Lesch S, Endres S, Kobold S. Killing mechanisms of chimeric antigen receptor (CAR) T Cells. Int J Mol Sci. 2019;20:1–21.
7. Kantarjian H, Stein A, Gökbuget N, Fielding AK, Schuh AC, Ribera J-M, et al. Blinatumomab versus chemotherapy for advanced acute lymphoblastic leukemia. N Engl J Med. 2017;376:836–47.
8. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med. 2018;378:439–48.
9. June CH, Sadelain M. Chimeric antigen receptor therapy. N Engl J Med. 2018;379:64–73.
10. Stoiber SC, Bruno L, Benmebarek M, Lesch S, Endres S, Kobold S. Limitations in the design of chimeric antigen receptors for cancer therapy. Cells. 2019;8:472.
11. Brudno JN, Kochenderfer JN. Toxicities of chimeric antigen receptor T cells: recognition and management. Blood. 2016;127:3321.
12. Lesch S, Benmebarek M-R, Cadilha BL, Stoiber S, Subklewe M, Endres S, et al. Determinants of response and resistance to CAR T cell therapy. Semin Cancer Biol. 2019;65:80–89.
13. Lichtenegger FS, Krupka C, Köhnke T, Subklewe M. Immunotherapy for Acute Myeloid Leukemia. Semin Hematol. 2015;52:207–14.
14. Ravandi F, Walter RB, Freeman SD. Evaluating measurable residual disease in acute myeloid leukemia. Blood Adv. 2018; 2:1356–66.
15. Ehninger A, Kramer M, Röllig C, Thiede C, Bornhäuser M, von Bonin M, et al. Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia. Blood Cancer J. 2014;4:e218–e.
16. Walter RB, Appelbaum FR, Estey EH, Bernstein ID. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. Blood. 2012;119:6198–208.
17. Haubner S, Perna F, Köhnke T, Schmidt C, Berman S, Augsberger C, et al. Coexpression profile of leukemic stem cell markers for combinatorial targeted therapy in AML. Leukemia. 2019;33:64–74.
18. Perna F, Berman SH, Soni RK, Mansilla-Soto J, Eyquem J, Hamieh M, et al. Integrating proteomics and transcriptomics for systematic combinatorial chimeric antigen receptor therapy of AML. Cancer Cell. 2017;32:506–19.e5.
19. Jackson HJ, Brentjens RJ. Overcoming antigen escape with CAR T-cell therapy. Cancer Discov. 2015;5:1238–40.
20. Levine JH, Simonds EF, Bendall SC, Davis KL, Amir E-AD, Tadmor MD, et al. Data-driven phenotypic dissection of AML reveals progenitor-like cells that correlate with prognosis. Cell. 2015;162:184–97.
21. Taussig DC, Pearce DJ, Simpson C, Rohatiner AZ, Lister TA, Kelly G, et al. Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. Blood. 2005;106:4086–92.
22. Karches CH, Benmebarek M-R, Schmidbauer ML, Kurzay M, Klaus R, Geiger M, et al. Bispecific antibodies enable synthetic agonistic receptor-transduced T cells for tumor immunotherapy. Clin Cancer Res. 2019;25:5890–900.
23. Portell CA, Wenzell CM, Advani AS. Clinical and pharmacologic aspects of blinatumomab in the treatment of B-cell acute lymphoblastic leukemia. Clin Pharmacol 2013;5 Suppl 1:5–11.
24. Labrijn AF, Janmaat ML, Reichert JM, Parren PWHI. Bispecific antibodies: a mechanistic review of the pipeline. Nat Rev Drug Discov. 2019;18:585–608.

25. Kobold S, Steffen J, Chaloupka M, Grassmann S, Henkel J, Castoldi R, et al. Selective bispecific T cell recruiting antibody and antitumor activity of adoptive T cell transfer. J Natl Cancer Inst. 2014;107.
26. Benedict CA, MacKrell AJ, Anderson WF. Determination of the binding affinity of an anti-CD34 single-chain antibody using a novel, flow cytometry based assay. J Immunol Methods. 1997;201:223–31.
27. Ghani K, Wang X, de Campos-Lima PO, Olszewska M, Kamen A, Rivière I, et al. Efficient human hematopoietic cell transduction using RD114- and GALV-pseudotyped retroviral vectors produced in suspension and serum-free media. Hum Gene Ther. 2009;20:966–74.
28. Voigt C, May P, Gottschlich A, Markota A, Wenk D, Gerlach I, et al. Cancer cells induce interleukin-22 production from memory CD4+ T cells via interleukin-1 to promote tumor growth. Proc Natl Acad Sci. 2017;114:12994–9.
29. Kuan C-T, Wikstrand CJ, Archer G, Beers R, Pastan I, Zalutsky MR, et al. Increased binding affinity enhances targeting of glioma xenografts by EGFRvIII-specific scFv. Int J Cancer. 2000;88:962–9.
30. Krupka C, Kufer P, Kischel R, Zugmaier G, Bögeholz J, Köhnke T, et al. CD33 target validation and sustained depletion of AML blasts in long-term cultures by the bispecific T-cell–engaging antibody AMG 330. Blood. 2014;123:356–65.
31. Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S, Zellmeier E, et al. Acute myeloid leukemia with biallelic CEBPA gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. J Clin Oncol. 2010;28:570–7.
32. Büchner T, Hiddemann W, Wörmann B, Löffler H, Gassmann W, Haferlach T, et al. Double induction strategy for acute myeloid leukemia: the effect of high-dose cytarabine with mitoxantrone instead of standard-dose cytarabine with daunorubicin and 6-thioguanine: a randomized trial by the German AML Cooperative Group. Blood. 1999;93:4116–24.
33. Herrmann M, Krupka C, Deiser K, Brauchle B, Marcinek A, Ogrinc Wagner A, et al. Bifunctional PD-1 × αCD3 × αCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia. Blood. 2018;132:2484–94.
34. Brischwein K, Parr L, Pflanz S, Volkland J, Lumsden J, Klinger M, et al. Strictly target cell-dependent activation of T cells by bispecific single-chain antibody constructs of the BiTE class. J Immunother. 2007;30:798–807.
35. Martelli MP, Sportoletti P, Tiacci E, Martelli MF, Falini B. Mutational landscape of AML with normal cytogenetics: Biological and clinical implications. Blood Rev. 2013;27:13–22.
36. Anguille S, Van Tendeloo VF, Berneman ZN. Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia. Leukemia. 2012;26:2186–96.
37. Zilberberg J, Feinman R, Korngold R. Strategies for the identification of T cell-recognized tumor antigens in hematological malignancies for improved graft-versus-tumor responses after allogeneic blood and marrow transplantation. Biol Blood Marrow Transpl. 2015;21:1000–7.
38. Sweeney C, Vyas P The graft-versus-leukemia effect in AML. Front Oncol. 2019;9.
39. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, Lassailly F, Tettamanti S, Spinelli O, et al. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. Leukemia. 2014;28:1596–605.
40. Davenport AJ, Cross RS, Watson KA, Liao Y, Shi W, Prince HM, et al. Chimeric antigen receptor T cells form nonclassical and potent immune synapses driving rapid cytotoxicity. Proc Natl Acad Sci USA. 2018;115:E2068.
41. Kenderian SS, Ruella M, Shestova O, Klichinsky M, Aikawa V, Morrissette JJD, et al. CD33-specific chimeric antigen receptor T cells exhibit potent preclinical activity against human acute myeloid leukemia. Leukemia. 2015;29:1637–47.
42. Friedrich M, Henn A, Raum T, Bajtus M, Matthes K, Hendrich L, et al. Preclinical characterization of AMG 330, a CD3/CD33-bispecific t-cell–engaging antibody with potential for treatment of acute myelogenous leukemia. Mol Cancer Ther. 2014;13: 1549–57.
43. Walter RB. Investigational CD33-targeted therapeutics for acute myeloid leukemia. Expert Opin Investig Drugs. 2018;27:339–48.
44. Garnache-Ottou F, Chaperot L, Biichle S, Ferrand C, Remy-Martin J-P, Deconinck E, et al. Expression of the myeloid-associated marker CD33 is not an exclusive factor for leukemic plasmacytoid dendritic cells. Blood. 2005;105:1256–64.
45. Hoffmann P, Hofmeister R, Brischwein K, Brandl C, Crommer S, Bargou R, et al. Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct. Int J Cancer. 2005;115:98–104.
46. Leong SR, Sukumaran S, Hristopoulos M, Totpal K, Stainton S, Lu E, et al. An anti-CD3/anti–CLL-1 bispecific antibody for the treatment of acute myeloid leukemia. Blood. 2017;129: 609–18.
47. Bortoletto N, Scotet E, Myamoto Y, D'Oro U, Lanzavecchia A. Optimizing anti-CD3 affinity for effective T cell targeting against tumor cells. Eur J Immunol. 2002;32:3102–7.
48. Cummins KD, Frey N, Nelson AM, Schmidt A, Luger S, Isaacs RE, et al. Treating relapsed / refractory (RR) AML with biodegradable Anti-CD123 CAR modified T cells. Blood. 2017;130 (Suppl 1):1359.
49. Luo Y, Chang L-J, Hu Y, Dong L, Wei G, Huang H. First-in-man CD123-specific chimeric antigen receptor-modified T cells for the treatment of refractory acute myeloid leukemia. Blood. 2015; 126:3778-.
50. Cummins KD, Gill S. Chimeric antigen receptor T-cell therapy for acute myeloid leukemia: how close to reality? Haematologica. 2019;104:1302–8.
51. Wang Q-S, Wang Y, Lv H-Y, Han Q-W, Fan H, Guo B, et al. Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia. Mol Ther. 2015;23:184–91.
52. Kim MY, Yu K-R, Kenderian SS, Ruella M, Chen S, Shin T-H, et al. Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid leukemia. Cell. 2018;173:1439–53.e19.
53. Borot F, Wang H, Ma Y, Jafarov T, Raza A, Ali AM, et al. Gene-edited stem cells enable CD33-directed immune therapy for myeloid malignancies. Proc Natl Acad Sci. 2019;116: 11978.
54. Cho JH, Collins JJ, Wong WW. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. Cell. 2018;173:1426–38.e11.
55. Roybal Kole T, Rupp Levi J, Morsut L, Walker Whitney J, McNally Krista A, Park Jason S, et al. Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. Cell. 2016;164:770–9.
56. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat Med. 2018;24:20–8.
57. Straathof KC, Pulè MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. Blood. 2005;105:4247–54.
58. Gomes-Silva D, Mukherjee M, Srinivasan M, Krenciute G, Dakhova O, Zheng Y, et al. Tonic 4-1BB costimulation in

chimeric antigen receptors impedes T cell survival and is vector-dependent. Cell Rep. 2017;21:17–26.
59. Rodgers DT, Mazagova M, Hampton EN, Cao Y, Ramadoss NS, Hardy IR, et al. Switch-mediated activation and retargeting of CAR-T cells for B-cell malignancies. Proc Natl Acad Sci. 2016;113:E459.
60. Darowski D, Kobold S, Jost C, Klein C. Combining the best of two worlds: highly flexible chimeric antigen receptor adaptor molecules (CAR-adaptors) for the recruitment of chimeric antigen receptor T cells. mAbs. 2019;11:621–31.
61. Thol F, Schlenk RF, Heuser M, Ganser A. How I treat refractory and early relapsed acute myeloid leukemia. Blood. 2015;126: 319–27.
62. Gill S, Tasian SK, Ruella M, Shestova O, Li Y, Porter DL, et al. Preclinical targeting of human acute myeloid leukemia and myeloablation using chimeric antigen receptor–modified T cells. Blood. 2014;123:2343–54.
63. Frigault MJ, Lee J, Basil MC, Carpenito C, Motohashi S, Scholler J, et al. Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. Cancer Immunol Res. 2015;3:356–67.

3 References

1. McComb, S.; Thiriot, A.; Akache, B.; Krishnan, L.; Stark, F., Introduction to the Immune System. *Methods Mol Biol* **2019,** *2024*, 1-24.
2. Zhang, P.; Zhang, C.; Li, J.; Han, J.; Liu, X.; Yang, H., The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications. *Stem Cell Research & Therapy* **2019,** *10* (1), 327.
3. Jiang, N.; Chen, M.; Yang, G.; Xiang, L.; He, L.; Hei, T. K.; Chotkowski, G.; Tarnow, D. P.; Finkel, M.; Ding, L.; Zhou, Y.; Mao, J. J., Hematopoietic Stem Cells in Neural-crest Derived Bone Marrow. *Sci Rep* **2016,** *6*, 36411.
4. Walasek, M. A.; van Os, R.; de Haan, G., Hematopoietic stem cell expansion: challenges and opportunities. *Ann N Y Acad Sci* **2012,** *1266*, 138-50.
5. Seita, J.; Weissman, I. L., Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* **2010,** *2* (6), 640-53.
6. Giebel, B., Cell polarity and asymmetric cell division within human hematopoietic stem and progenitor cells. *Cells Tissues Organs* **2008,** *188* (1-2), 116-26.
7. Benveniste, P.; Frelin, C.; Janmohamed, S.; Barbara, M.; Herrington, R.; Hyam, D.; Iscove, N. N., Intermediate-Term Hematopoietic Stem Cells with Extended but Time-Limited Reconstitution Potential. *Cell Stem Cell* **2010,** *6* (1), 48-58.
8. Rieger, M. A.; Schroeder, T., Hematopoiesis. *Cold Spring Harb Perspect Biol* **2012,** *4* (12).
9. Bryder, D.; Rossi, D. J.; Weissman, I. L., Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol* **2006,** *169* (2), 338-46.
10. Iwasaki, H.; Akashi, K., Hematopoietic developmental pathways: on cellular basis. *Oncogene* **2007,** *26* (47), 6687-6696.
11. Leukemia - American Cancer Society.
12. Juliusson, G.; Hough, R., Leukemia. *Prog Tumor Res* **2016,** *43*, 87-100.
13. Ramdas, B.; Mali, R. S.; Palam, L. R.; Pandey, R.; Cai, Z.; Pasupuleti, S. K.; Burns, S. S.; Kapur, R., Driver Mutations in Leukemia Promote Disease Pathogenesis through a Combination of Cell-Autonomous and Niche Modulation. *Stem Cell Reports* **2020,** *15* (1), 95-109.
14. Stratton, M. R.; Campbell, P. J.; Futreal, P. A., The cancer genome. *Nature* **2009,** *458* (7239), 719-24.
15. Hanahan, D.; Weinberg, R. A., The Hallmarks of Cancer. *Cell* **2000,** *100* (1), 57-70.
16. Sell, S., Leukemia: stem cells, maturation arrest, and differentiation therapy. *Stem Cell Rev* **2005,** *1* (3), 197-205.
17. De Kouchkovsky, I.; Abdul-Hay, M., 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J* **2016,** *6* (7), e441.
18. Davis, A. S.; Viera, A. J.; Mead, M. D., Leukemia: an overview for primary care. *Am Fam Physician* **2014,** *89* (9), 731-8.
19. Döhner, H.; Weisdorf, D. J.; Bloomfield, C. D., Acute Myeloid Leukemia. *N Engl J Med* **2015,** *373* (12), 1136-52.
20. Kavanagh, S.; Murphy, T.; Law, A.; Yehudai, D.; Ho, J. M.; Chan, S.; Schimmer, A. D., Emerging therapies for acute myeloid leukemia: translating biology into the clinic. *JCI Insight* **2017,** *2* (18).
21. Pelcovits, A.; Niroula, R., Acute Myeloid Leukemia: A Review. *R I Med J (2013)* **2020,** *103* (3), 38-40.

22. Acute Myeloid Leukemia - Cancer Stat Facts. https://seer.cancer.gov/statfacts/html/amyl.html.
23. Estey, E. H., Acute myeloid leukemia: 2019 update on risk-stratification and management. *Am J Hematol* **2018,** *93* (10), 1267-1291.
24. Jentzsch, M.; Bill, M.; Grimm, J.; Brauer, D.; Backhaus, D.; Goldmann, K.; Schulz, J.; Niederwieser, D.; Platzbecker, U.; Schwind, S., Allogeneic stem cell transplantation mitigates the adverse prognostic impact of high diagnostic BAALC and MN1 expression in AML. *Ann Hematol* **2020,** *99* (10), 2417-2427.
25. Jentzsch, M.; Grimm, J.; Bill, M.; Brauer, D.; Backhaus, D.; Goldmann, K.; Schulz, J.; Niederwieser, D.; Platzbecker, U.; Schwind, S., ELN risk stratification and outcomes in secondary and therapy-related AML patients consolidated with allogeneic stem cell transplantation. *Bone Marrow Transplant* **2021,** *56* (4), 936-945.
26. Döhner, H.; Estey, E.; Grimwade, D.; Amadori, S.; Appelbaum, F. R.; Büchner, T.; Dombret, H.; Ebert, B. L.; Fenaux, P.; Larson, R. A.; Levine, R. L.; Lo-Coco, F.; Naoe, T.; Niederwieser, D.; Ossenkoppele, G. J.; Sanz, M.; Sierra, J.; Tallman, M. S.; Tien, H. F.; Wei, A. H.; Löwenberg, B.; Bloomfield, C. D., Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **2017,** *129* (4), 424-447.
27. Braess, J., [Acute myeloid Leukemia]. *Dtsch Med Wochenschr* **2016,** *141* (24), 1748-1751.
28. Döhner, H.; Estey, E. H.; Amadori, S.; Appelbaum, F. R.; Büchner, T.; Burnett, A. K.; Dombret, H.; Fenaux, P.; Grimwade, D.; Larson, R. A.; Lo-Coco, F.; Naoe, T.; Niederwieser, D.; Ossenkoppele, G. J.; Sanz, M. A.; Sierra, J.; Tallman, M. S.; Löwenberg, B.; Bloomfield, C. D., Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **2010,** *115* (3), 453-74.
29. Yates, J. W.; Wallace, H. J., Jr.; Ellison, R. R.; Holland, J. F., Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. *Cancer Chemother Rep* **1973,** *57* (4), 485-8.
30. Burnett, A.; Wetzler, M.; Löwenberg, B., Therapeutic advances in acute myeloid leukemia. *J Clin Oncol* **2011,** *29* (5), 487-94.
31. Kantarjian, H., Acute myeloid leukemia--major progress over four decades and glimpses into the future. *Am J Hematol* **2016,** *91* (1), 131-45.
32. Short, N. J.; Rytting, M. E.; Cortes, J. E., Acute myeloid leukaemia. *Lancet* **2018,** *392* (10147), 593-606.
33. Sweeney, C.; Vyas, P., The Graft-Versus-Leukemia Effect in AML. *Frontiers in Oncology* **2019,** *9*.
34. Loke, J.; Malladi, R.; Moss, P.; Craddock, C., The role of allogeneic stem cell transplantation in the management of acute myeloid leukaemia: a triumph of hope and experience. *Br J Haematol* **2020,** *188* (1), 129-146.
35. Richard, F. S., Post-remission therapy for acute myeloid leukemia. *Haematologica* **2014,** *99* (11), 1663-1670.
36. Kadia, T. M.; Ravandi, F.; Cortes, J.; Kantarjian, H., Toward Individualized Therapy in Acute Myeloid Leukemia: A Contemporary Review. *JAMA Oncol* **2015,** *1* (6), 820-8.
37. Kadia, T. M.; Ravandi, F.; O'Brien, S.; Cortes, J.; Kantarjian, H. M., Progress in acute myeloid leukemia. *Clin Lymphoma Myeloma Leuk* **2015,** *15* (3), 139-51.
38. Daver, N.; Venugopal, S.; Ravandi, F., FLT3 mutated acute myeloid leukemia: 2021 treatment algorithm. *Blood Cancer Journal* **2021,** *11* (5), 104.

39. Lai, C.; Doucette, K.; Norsworthy, K., Recent drug approvals for acute myeloid leukemia. *Journal of Hematology & Oncology* **2019,** *12* (1), 100.
40. Erba, H. P., Finding the optimal combination therapy for the treatment of newly diagnosed AML in older patients unfit for intensive therapy. *Leuk Res* **2015,** *39* (2), 183-91.
41. Society, A. C., Chronic Lymphocytic Leukemia.
42. Hallek, M.; Shanafelt, T. D.; Eichhorst, B., Chronic lymphocytic leukaemia. *Lancet* **2018,** *391* (10129), 1524-1537.
43. Kay, N. E.; Hampel, P. J.; Van Dyke, D. L.; Parikh, S. A., CLL update 2022: A continuing evolution in care. *Blood Rev* **2022**, 100930.
44. Chiorazzi, N.; Chen, S. S.; Rai, K. R., Chronic Lymphocytic Leukemia. *Cold Spring Harb Perspect Med* **2021,** *11* (2).
45. Scarfò, L.; Ferreri, A. J.; Ghia, P., Chronic lymphocytic leukaemia. *Crit Rev Oncol Hematol* **2016,** *104*, 169-82.
46. Chronic Lymphocytic Leukemia - Cancer Stat Facts.
47. Shanafelt, T. D.; Rabe, K. G.; Kay, N. E.; Zent, C. S.; Jelinek, D. F.; Reinalda, M. S.; Schwager, S. M.; Bowen, D. A.; Slager, S. L.; Hanson, C. A.; Call, T. G., Age at diagnosis and the utility of prognostic testing in patients with chronic lymphocytic leukemia. *Cancer* **2010,** *116* (20), 4777-87.
48. Strati, P.; Jain, N.; O'Brien, S., Chronic Lymphocytic Leukemia: Diagnosis and Treatment. *Mayo Clin Proc* **2018,** *93* (5), 651-664.
49. Ghia, P.; Ferreri, A. M.; Caligaris-Cappio, F., Chronic lymphocytic leukemia. *Crit Rev Oncol Hematol* **2007,** *64* (3), 234-46.
50. Parikh, S. A., Chronic lymphocytic leukemia treatment algorithm 2018. *Blood Cancer Journal* **2018,** *8* (10), 93.
51. Rai, K. R.; Jain, P., Chronic lymphocytic leukemia (CLL)-Then and now. *Am J Hematol* **2016,** *91* (3), 330-40.
52. Rai, K. R.; Jain, P., Advances in the clinical staging of chronic lymphocytic leukemia. *Clin Chem* **2011,** *57* (12), 1771-2.
53. Binet, J. L.; Leporrier, M.; Dighiero, G.; Charron, D.; D'Athis, P.; Vaugier, G.; Beral, H. M.; Natali, J. C.; Raphael, M.; Nizet, B.; Follezou, J. Y., A clinical staging system for chronic lymphocytic leukemia: prognostic significance. *Cancer* **1977,** *40* (2), 855-64.
54. Hallek, M.; Al-Sawaf, O., Chronic lymphocytic leukemia: 2022 update on diagnostic and therapeutic procedures. *Am J Hematol* **2021,** *96* (12), 1679-1705.
55. Rai, K. R.; Sawitsky, A.; Cronkite, E. P.; Chanana, A. D.; Levy, R. N.; Pasternack, B. S., Clinical staging of chronic lymphocytic leukemia. *Blood* **1975,** *46* (2), 219-34.
56. Hamblin, T. J.; Davis, Z.; Gardiner, A.; Oscier, D. G.; Stevenson, F. K., Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **1999,** *94* (6), 1848-54.
57. Damle, R. N.; Wasil, T.; Fais, F.; Ghiotto, F.; Valetto, A.; Allen, S. L.; Buchbinder, A.; Budman, D.; Dittmar, K.; Kolitz, J.; Lichtman, S. M.; Schulman, P.; Vinciguerra, V. P.; Rai, K. R.; Ferrarini, M.; Chiorazzi, N., Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* **1999,** *94* (6), 1840-7.
58. Zenz, T.; Eichhorst, B.; Busch, R.; Denzel, T.; Häbe, S.; Winkler, D.; Bühler, A.; Edelmann, J.; Bergmann, M.; Hopfinger, G.; Hensel, M.; Hallek, M.; Döhner, H.; Stilgenbauer, S., TP53 mutation and survival in chronic lymphocytic leukemia. *J Clin Oncol* **2010,** *28* (29), 4473-9.
59. Rossi, D.; Khiabanian, H.; Spina, V.; Ciardullo, C.; Bruscaggin, A.; Famà, R.; Rasi, S.; Monti, S.; Deambrogi, C.; De Paoli, L.; Wang, J.; Gattei, V.; Guarini, A.; Foà, R.; Rabadan,

R.; Gaidano, G., Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. *Blood* **2014,** *123* (14), 2139-47.
60. Stilgenbauer, S.; Schnaiter, A.; Paschka, P.; Zenz, T.; Rossi, M.; Döhner, K.; Bühler, A.; Böttcher, S.; Ritgen, M.; Kneba, M.; Winkler, D.; Tausch, E.; Hoth, P.; Edelmann, J.; Mertens, D.; Bullinger, L.; Bergmann, M.; Kless, S.; Mack, S.; Jäger, U.; Patten, N.; Wu, L.; Wenger, M. K.; Fingerle-Rowson, G.; Lichter, P.; Cazzola, M.; Wendtner, C. M.; Fink, A. M.; Fischer, K.; Busch, R.; Hallek, M.; Döhner, H., Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood* **2014,** *123* (21), 3247-54.
61. Hallek, M., Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol* **2019,** *94* (11), 1266-1287.
62. Hallek, M.; Cheson, B. D.; Catovsky, D.; Caligaris-Cappio, F.; Dighiero, G.; Döhner, H.; Hillmen, P.; Keating, M. J.; Montserrat, E.; Rai, K. R.; Kipps, T. J., Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* **2008,** *111* (12), 5446-56.
63. Chemotherapeutic options in chronic lymphocytic leukemia: a meta-analysis of the randomized trials. CLL Trialists' Collaborative Group. *J Natl Cancer Inst* **1999,** *91* (10), 861-8.
64. Sharma, S.; Rai, K. R., Chronic lymphocytic leukemia (CLL) treatment: So many choices, such great options. *Cancer* **2019,** *125* (9), 1432-1440.
65. Patel, K.; Pagel, J. M., Current and future treatment strategies in chronic lymphocytic leukemia. *J Hematol Oncol* **2021,** *14* (1), 69.
66. Scheffold, A.; Stilgenbauer, S., Revolution of Chronic Lymphocytic Leukemia Therapy: the Chemo-Free Treatment Paradigm. *Curr Oncol Rep* **2020,** *22* (2), 16.
67. FDA approves Imbruvica (ibrutinib) for the first-line treatment of chronic lymphocytic leukemia.
68. Coley, W. B., The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin Orthop Relat Res* **1991,** (262), 3-11.
69. Strohl, W. R.; Naso, M., Bispecific T-Cell Redirection versus Chimeric Antigen Receptor (CAR)-T Cells as Approaches to Kill Cancer Cells. *Antibodies (Basel)* **2019,** *8* (3).
70. Drake, C. G.; Jaffee, E.; Pardoll, D. M., Mechanisms of immune evasion by tumors. *Adv Immunol* **2006,** *90*, 51-81.
71. Yang, Y., Cancer immunotherapy: harnessing the immune system to battle cancer. *J Clin Invest* **2015,** *125* (9), 3335-7.
72. Garber, H. R.; Mirza, A.; Mittendorf, E. A.; Alatrash, G., Adoptive T-cell therapy for Leukemia. *Mol Cell Ther* **2014,** *2*, 25.
73. Du, Z.; Lovly, C. M., Mechanisms of receptor tyrosine kinase activation in cancer. *Mol Cancer* **2018,** *17* (1), 58.
74. Lemmon, M. A.; Schlessinger, J., Cell signaling by receptor tyrosine kinases. *Cell* **2010,** *141* (7), 1117-34.
75. Cadena, D. L.; Gill, G. N., Receptor tyrosine kinases. *Faseb j* **1992,** *6* (6), 2332-7.
76. Paul, M. K.; Mukhopadhyay, A. K., Tyrosine kinase - Role and significance in Cancer. *Int J Med Sci* **2004,** *1* (2), 101-115.
77. Ghosh, S.; Marrocco, I.; Yarden, Y., Roles for receptor tyrosine kinases in tumor progression and implications for cancer treatment. *Adv Cancer Res* **2020,** *147*, 1-57.
78. Krause, D. S.; Van Etten, R. A., Tyrosine Kinases as Targets for Cancer Therapy. *New England Journal of Medicine* **2005,** *353* (2), 172-187.

79. Esteban-Villarrubia, J.; Soto-Castillo, J. J.; Pozas, J.; San Román-Gil, M.; Orejana-Martín, I.; Torres-Jiménez, J.; Carrato, A.; Alonso-Gordoa, T.; Molina-Cerrillo, J., Tyrosine Kinase Receptors in Oncology. *Int J Mol Sci* **2020,** *21* (22).
80. Shimada, A., Hematological malignancies and molecular targeting therapy. *Eur J Pharmacol* **2019,** *862*, 172641.
81. de Kogel, C. E.; Schellens, J. H., Imatinib. *Oncologist* **2007,** *12* (12), 1390-4.
82. Roskoski, R., Jr., Properties of FDA-approved small molecule protein kinase inhibitors: A 2021 update. *Pharmacol Res* **2021,** *165*, 105463.
83. Roskoski, R., Jr., Properties of FDA-approved small molecule protein kinase inhibitors: A 2022 update. *Pharmacol Res* **2022,** *175*, 106037.
84. Jiao, Q.; Bi, L.; Ren, Y.; Song, S.; Wang, Q.; Wang, Y. S., Advances in studies of tyrosine kinase inhibitors and their acquired resistance. *Mol Cancer* **2018,** *17* (1), 36.
85. Burchert, A., Maintenance therapy for FLT3-ITD-mutated acute myeloid leukemia. *Haematologica* **2021,** *106* (3), 664-670.
86. Kiyoi, H.; Naoe, T., Biology, clinical relevance, and molecularly targeted therapy in acute leukemia with FLT3 mutation. *Int J Hematol* **2006,** *83* (4), 301-8.
87. Kindler, T.; Lipka, D. B.; Fischer, T., FLT3 as a therapeutic target in AML: still challenging after all these years. *Blood* **2010,** *116* (24), 5089-102.
88. Kiyoi, H.; Kawashima, N.; Ishikawa, Y., FLT3 mutations in acute myeloid leukemia: Therapeutic paradigm beyond inhibitor development. *Cancer Sci* **2020,** *111* (2), 312-322.
89. Levis, M., FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? *Hematology Am Soc Hematol Educ Program* **2013,** *2013*, 220-6.
90. Kottaridis, P. D.; Gale, R. E.; Linch, D. C., Flt3 mutations and leukaemia. *Br J Haematol* **2003,** *122* (4), 523-38.
91. Moore, M. A.; Dorn, D. C.; Schuringa, J. J.; Chung, K. Y.; Morrone, G., Constitutive activation of Flt3 and STAT5A enhances self-renewal and alters differentiation of hematopoietic stem cells. *Exp Hematol* **2007,** *35* (4 Suppl 1), 105-16.
92. Daver, N.; Schlenk, R. F.; Russell, N. H.; Levis, M. J., Targeting FLT3 mutations in AML: review of current knowledge and evidence. *Leukemia* **2019,** *33* (2), 299-312.
93. Megías-Vericat, J. E.; Ballesta-López, O.; Barragán, E.; Martínez-Cuadrón, D.; Montesinos, P., Tyrosine kinase inhibitors for acute myeloid leukemia: A step toward disease control? *Blood Rev* **2020,** *44*, 100675.
94. Brandts, C. H.; Sargin, B.; Rode, M.; Biermann, C.; Lindtner, B.; Schwäble, J.; Buerger, H.; Müller-Tidow, C.; Choudhary, C.; McMahon, M.; Berdel, W. E.; Serve, H., Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer Res* **2005,** *65* (21), 9643-50.
95. Lee, B. H.; Tothova, Z.; Levine, R. L.; Anderson, K.; Buza-Vidas, N.; Cullen, D. E.; McDowell, E. P.; Adelsperger, J.; Fröhling, S.; Huntly, B. J.; Beran, M.; Jacobsen, S. E.; Gilliland, D. G., FLT3 mutations confer enhanced proliferation and survival properties to multipotent progenitors in a murine model of chronic myelomonocytic leukemia. *Cancer Cell* **2007,** *12* (4), 367-80.
96. Al-Sawaf, O.; Fischer, K.; Eichhorst, B.; Hallek, M., Targeted Therapy of CLL. *Oncol Res Treat* **2016,** *39* (12), 768-778.
97. Ferrer, G.; Montserrat, E., Critical molecular pathways in CLL therapy. *Mol Med* **2018,** *24* (1), 9.
98. Chiorazzi, N.; Rai, K. R.; Ferrarini, M., Chronic lymphocytic leukemia. *N Engl J Med* **2005,** *352* (8), 804-15.

99. Herman, S. E.; Gordon, A. L.; Wagner, A. J.; Heerema, N. A.; Zhao, W.; Flynn, J. M.; Jones, J.; Andritsos, L.; Puri, K. D.; Lannutti, B. J.; Giese, N. A.; Zhang, X.; Wei, L.; Byrd, J. C.; Johnson, A. J., Phosphatidylinositol 3-kinase-δ inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood* **2010,** *116* (12), 2078-88.
100. Lannutti, B. J.; Meadows, S. A.; Herman, S. E.; Kashishian, A.; Steiner, B.; Johnson, A. J.; Byrd, J. C.; Tyner, J. W.; Loriaux, M. M.; Deininger, M.; Druker, B. J.; Puri, K. D.; Ulrich, R. G.; Giese, N. A., CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. *Blood* **2011,** *117* (2), 591-4.
101. Hoellenriegel, J.; Meadows, S. A.; Sivina, M.; Wierda, W. G.; Kantarjian, H.; Keating, M. J.; Giese, N.; O'Brien, S.; Yu, A.; Miller, L. L.; Lannutti, B. J.; Burger, J. A., The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* **2011,** *118* (13), 3603-12.
102. Koyasu, S., The role of PI3K in immune cells. *Nat Immunol* **2003,** *4* (4), 313-9.
103. Berning, P.; Lenz, G., The role of PI3K inhibitors in the treatment of malignant lymphomas. *Leuk Lymphoma* **2021,** *62* (3), 517-527.
104. Somoza, J. R.; Koditek, D.; Villaseñor, A. G.; Novikov, N.; Wong, M. H.; Liclican, A.; Xing, W.; Lagpacan, L.; Wang, R.; Schultz, B. E.; Papalia, G. A.; Samuel, D.; Lad, L.; McGrath, M. E., Structural, biochemical, and biophysical characterization of idelalisib binding to phosphoinositide 3-kinase δ. *J Biol Chem* **2015,** *290* (13), 8439-46.
105. Yang, Q.; Modi, P.; Newcomb, T.; Quéva, C.; Gandhi, V., Idelalisib: First-in-Class PI3K Delta Inhibitor for the Treatment of Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia, and Follicular Lymphoma. *Clin Cancer Res* **2015,** *21* (7), 1537-42.
106. Trials.gov, C., A Randomized, Double-Blind, Placebo-Controlled Study of Idelalisib in Combination With Rituximab for Previously Treated Chronic Lymphocytic Leukemia (CLL).
107. Markham, A., Idelalisib: First Global Approval. *Drugs* **2014,** *74* (14), 1701-1707.
108. Miller, B. W.; Przepiorka, D.; de Claro, R. A.; Lee, K.; Nie, L.; Simpson, N.; Gudi, R.; Saber, H.; Shord, S.; Bullock, J.; Marathe, D.; Mehrotra, N.; Hsieh, L. S.; Ghosh, D.; Brown, J.; Kane, R. C.; Justice, R.; Kaminskas, E.; Farrell, A. T.; Pazdur, R., FDA approval: idelalisib monotherapy for the treatment of patients with follicular lymphoma and small lymphocytic lymphoma. *Clin Cancer Res* **2015,** *21* (7), 1525-9.
109. Skånland, S. S.; Cremaschi, A.; Bendiksen, H.; Hermansen, J. U.; Thimiri Govinda Raj, D. B.; Munthe, L. A.; Tjønnfjord, G. E.; Taskén, K., An in vitro assay for biomarker discovery and dose prediction applied to ibrutinib plus venetoclax treatment of CLL. *Leukemia* **2020,** *34* (2), 478-487.
110. Pohlen, M.; Groth, C.; Sauer, T.; Görlich, D.; Mesters, R.; Schliemann, C.; Lenz, G.; Müller-Tidow, C.; Büchner, T.; Berdel, W. E.; Stelljes, M., Outcome of allogeneic stem cell transplantation for AML and myelodysplastic syndrome in elderly patients (⩾60 years). *Bone Marrow Transplant* **2016,** *51* (11), 1441-1448.
111. Hamilton, B. K.; Copelan, E. A., Concise review: the role of hematopoietic stem cell transplantation in the treatment of acute myeloid leukemia. *Stem Cells* **2012,** *30* (8), 1581-6.
112. Thomas, E. D.; Buckner, C. D.; Banaji, M.; Clift, R. A.; Fefer, A.; Flournoy, N.; Goodell, B. W.; Hickman, R. O.; Lerner, K. G.; Neiman, P. E.; Sale, G. E.; Sanders, J. E.; Singer, J.; Stevens, M.; Storb, R.; Weiden, P. L., One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. *Blood* **1977,** *49* (4), 511-33.

113. Bacigalupo, A.; Vitale, V.; Corvò, R.; Barra, S.; Lamparelli, T.; Gualandi, F.; Mordini, N.; Berisso, G.; Bregante, S.; Raiola, A. M.; Van Lint, M. T.; Frassoni, F., The combined effect of total body irradiation (TBI) and cyclosporin A (CyA) on the risk of relapse in patients with acute myeloid leukaemia undergoing allogeneic bone marrow transplantation. *Br J Haematol* **2000,** *108* (1), 99-104.
114. Zilberberg, J.; Feinman, R.; Korngold, R., Strategies for the identification of T cell-recognized tumor antigens in hematological malignancies for improved graft-versus-tumor responses after allogeneic blood and marrow transplantation. *Biol Blood Marrow Transplant* **2015,** *21* (6), 1000-7.
115. Weiden, P. L.; Flournoy, N.; Thomas, E. D.; Prentice, R.; Fefer, A.; Buckner, C. D.; Storb, R., Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med* **1979,** *300* (19), 1068-73.
116. Giralt, S.; Estey, E.; Albitar, M.; van Besien, K.; Rondón, G.; Anderlini, P.; O'Brien, S.; Khouri, I.; Gajewski, J.; Mehra, R.; Claxton, D.; Andersson, B.; Beran, M.; Przepiorka, D.; Koller, C.; Kornblau, S.; Kørbling, M.; Keating, M.; Kantarjian, H.; Champlin, R., Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood* **1997,** *89* (12), 4531-6.
117. Ferrara, J. L.; Levine, J. E.; Reddy, P.; Holler, E., Graft-versus-host disease. *Lancet* **2009,** *373* (9674), 1550-61.
118. Rowe, J. M., Graft-versus-disease effect following allogeneic transplantation for acute leukaemia. *Best Pract Res Clin Haematol* **2008,** *21* (3), 485-502.
119. Juliusson, G.; Karlsson, K.; Lazarevic, V.; Wahlin, A.; Brune, M.; Antunovic, P.; Derolf, A.; Hägglund, H.; Karbach, H.; Lehmann, S.; Möllgård, L.; Stockelberg, D.; Hallböök, H.; Höglund, M., Hematopoietic stem cell transplantation rates and long-term survival in acute myeloid and lymphoblastic leukemia: real-world population-based data from the Swedish Acute Leukemia Registry 1997-2006. *Cancer* **2011,** *117* (18), 4238-46.
120. EUGENIA ZAH, Y. Y. C., Engineering Cancer-Fighting T Cells. **2017**.
121. Melief, C. J., Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. *Adv Cancer Res* **1992,** *58*, 143-75.
122. Rosenberg, S. A.; Restifo, N. P., Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **2015,** *348* (6230), 62-8.
123. Dudley, M. E.; Wunderlich, J. R.; Shelton, T. E.; Even, J.; Rosenberg, S. A., Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. *J Immunother* **2003,** *26* (4), 332-42.
124. Met, Ö.; Jensen, K. M.; Chamberlain, C. A.; Donia, M.; Svane, I. M., Principles of adoptive T cell therapy in cancer. *Semin Immunopathol* **2019,** *41* (1), 49-58.
125. Rosenberg, S. A.; Packard, B. S.; Aebersold, P. M.; Solomon, D.; Topalian, S. L.; Toy, S. T.; Simon, P.; Lotze, M. T.; Yang, J. C.; Seipp, C. A.; et al., Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med* **1988,** *319* (25), 1676-80.
126. Benmebarek, M. R.; Karches, C. H.; Cadilha, B. L.; Lesch, S.; Endres, S.; Kobold, S., Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells. *Int J Mol Sci* **2019,** *20* (6).
127. Harris, D. T.; Kranz, D. M., Adoptive T Cell Therapies: A Comparison of T Cell Receptors and Chimeric Antigen Receptors. *Trends Pharmacol Sci* **2016,** *37* (3), 220-230.
128. Subklewe, M.; von Bergwelt-Baildon, M.; Humpe, A., Chimeric Antigen Receptor T Cells: A Race to Revolutionize Cancer Therapy. *Transfus Med Hemother* **2019,** *46* (1), 15-24.

129. Stock, S.; Schmitt, M.; Sellner, L., Optimizing Manufacturing Protocols of Chimeric Antigen Receptor T Cells for Improved Anticancer Immunotherapy. *International Journal of Molecular Sciences* **2019,** *20* (24), 6223.
130. Tokarew, N.; Ogonek, J.; Endres, S.; von Bergwelt-Baildon, M.; Kobold, S., Teaching an old dog new tricks: next-generation CAR T cells. *British Journal of Cancer* **2019,** *120* (1), 26-37.
131. Jensen, M. C.; Riddell, S. R., Designing chimeric antigen receptors to effectively and safely target tumors. *Curr Opin Immunol* **2015,** *33*, 9-15.
132. Ajina, A.; Maher, J., Strategies to Address Chimeric Antigen Receptor Tonic Signaling. *Mol Cancer Ther* **2018,** *17* (9), 1795-1815.
133. Brocker, T.; Karjalainen, K., Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. *J Exp Med* **1995,** *181* (5), 1653-9.
134. Gong, M. C.; Latouche, J. B.; Krause, A.; Heston, W. D.; Bander, N. H.; Sadelain, M., Cancer patient T cells genetically targeted to prostate-specific membrane antigen specifically lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. *Neoplasia* **1999,** *1* (2), 123-7.
135. Krause, A.; Guo, H. F.; Latouche, J. B.; Tan, C.; Cheung, N. K.; Sadelain, M., Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. *J Exp Med* **1998,** *188* (4), 619-26.
136. Porter, D. L.; Levine, B. L.; Kalos, M.; Bagg, A.; June, C. H., Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* **2011,** *365* (8), 725-33.
137. Brentjens, R. J.; Curran, K. J., Novel cellular therapies for leukemia: CAR-modified T cells targeted to the CD19 antigen. *Hematology Am Soc Hematol Educ Program* **2012,** *2012*, 143-51.
138. Scarfò, I.; Maus, M. V., Current approaches to increase CAR T cell potency in solid tumors: targeting the tumor microenvironment. *J Immunother Cancer* **2017,** *5*, 28.
139. Rohrbacher, L.; Brauchle, B.; Ogrinc Wagner, A.; von Bergwelt-Baildon, M.; Bücklein, V. L.; Subklewe, M., The PI3K∂-Selective Inhibitor Idelalisib Induces T- and NK-Cell Dysfunction Independently of B-Cell Malignancy-Associated Immunosuppression. *Frontiers in Immunology* **2021,** *12*.
140. Benmebarek, M. R.; Cadilha, B. L.; Herrmann, M.; Lesch, S.; Schmitt, S.; Stoiber, S.; Darwich, A.; Augsberger, C.; Brauchle, B.; Rohrbacher, L.; Oner, A.; Seifert, M.; Schwerdtfeger, M.; Gottschlich, A.; Rataj, F.; Fenn, N. C.; Klein, C.; Subklewe, M.; Endres, S.; Hopfner, K. P.; Kobold, S., A modular and controllable T cell therapy platform for acute myeloid leukemia. *Leukemia* **2021,** *35* (8), 2243-2257.

4 Acknowledgements

First of all, I would like to thank Marion Subklewe for her support and supervision during the time I spent in her group. Thank you for giving me the opportunity to work on really exciting projects and perform my doctoral thesis in your lab.

A big thanks also goes to iTarget "Immunotargeting of Cancer" International Doctoral Program, not only for the financial support but also for the great seminars, workshops, and get-togethers.

Thank you to Veit Bücklein for your help with abstract writing and poster design, for looking up patient data and especially for your moral support.

Another thanks goes to the technicians Sabine Sandner, Simone Pentz, and Marina Leeping for taking care of general lab work so I could fully focus on my projects. I also want to thank the physicians associated to our group and the technicians in the diagnostic lab.

A huge thanks goes to the whole AG SUBKLEWE group! Thank you for the great working environment, fruitful discussions, and endless willingness to help whenever I needed it!
A special THANK YOU goes to Bettina, Anetta, Nora, Daniel, and Gerulf: the best office mates I could have asked for! It has always been fun to work with you! Thank you for your never-ending support and, more than anything, for your friendship!

To my parents and my twin sister. Without your financial and especially moral support, I would not be where I am today. You are the best family anyone could ever ask for! THANK YOU FOR EVERYTHING!!!

My biggest thanks goes to Martin (aka Merlin). Thank you for never stopping to believe in me and for enduring all my moods and quirks!

To all my friends I did not mention - MERCI FÜR OIS!