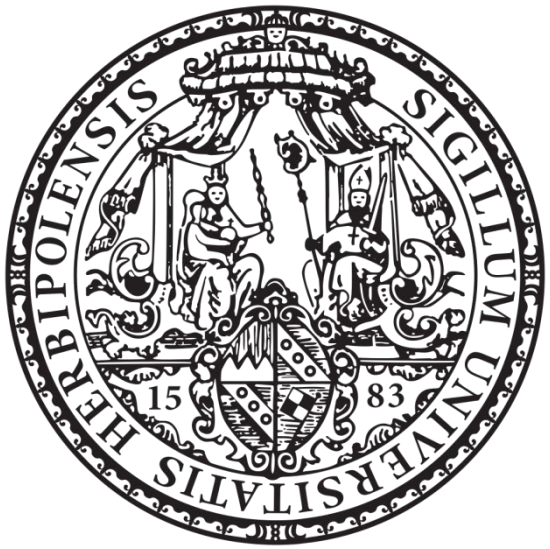

Chimeric antigen receptor (CAR)-modified T cells targeting FLT3 in acute myeloid leukemia (AML)

Chimäre Antigen Rezeptor (CAR)-modifizierte T-Zellen gegen FLT3 bei Akuter Myeloischer Leukämie (AML)

Thesis for a doctoral degree
at the Graduate School of Life Science,
Julius-Maximilians-Universität Würzburg,
Section- Infection and Immunity

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Affidavit

I hereby confirm that my thesis entitled ‘Chimeric antigen receptor (CAR)-modified T cells targeting FLT3 in acute myeloid leukemia (AML)’ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Hiermit erkläre ich an Eides statt, die Dissertation ‘Chimäre Antigen Rezeptor (CAR)-modifizierte T-Zellen gegen FLT3 bei Akuter Myeloischer Leukämie (AML)‘ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Acknowledgements

I would like to convey my warmest gratitude to my supervisor, Dr. Michael Hudecek for his guidance, generous contribution of knowledge and experience, valuable comments and encouragement from the start until the end of my doctoral project and thesis work. I would like to sincerely thank to my co-supervisors, Prof. Hermann Einsele, Prof. Thomas Herrmann and Prof. Christoph Rader for their constructive suggestions throughout my doctoral research and for being part of my thesis committee.

I would like to sincerely thank to the Graduate School of Life Science (GSLS), University of Wuerzburg for providing me PhD fellowship, financing conferences, retreats, spring schools and transferrable skills programs which I attended throughout my doctoral research. I am thankful to Dr. Gabriele Blum Oehler and entire GSLS team for all the support. I am greatly thankful to Universitätsklinikum Würzburg for providing research facility and financial support.

I would like to thank all past and present members of AG Hudecek for providing lively work environment. During this project work, I have been tirelessly supported and encouraged by AG Hudecek members. I am thankful to all the collaborators, who were involved in the project and helped in development of my research project. A special thanks to Irene Garcia-Cadenas for a knowledge transfer which helped me to establish fruitful progression in this project. Words cannot suffice my gratitude towards my dear friends, Maddy, Ravi, Benjamin, Mohindar, Manu, Ankit and Srikkanth who are always being with me to support and advise me in every possible ways.

Lastly, I am extremely thankful to my family for their endless love, moral support and encouragement, without which I would have never been able to complete this important step of my life.

Table of contents

Summary

Adoptive immunotherapy using chimeric antigen receptor (CAR)-modified T cells targeting CD19 has shown remarkable therapeutic efficacy against B cell leukemia and lymphoma, and provided proof of concept for therapeutic potential in other hematologic malignancies. Acute myeloid leukemia (AML) is an entity with an unmet medical need for effective and curative treatments. Therefore, there is a strong desire for development of potentially curative CAR-T cell immunotherapy for AML treatment.

FMS-like tyrosine kinase 3 (FLT3) is a homodimeric transmembrane protein expressed uniformly by AML blasts. FLT3 plays a vital role in the survival of AML blasts and is a key driver of leukemia-genesis in AML cases with internal tandem duplication (FLT3-ITD) and tyrosine kinase domain (TKD) mutations. These attributes suggest that FLT3 could be an excellent target for CAR-T cell immunotherapy. Here, we engineered human CD4+ and CD8+ T cells to express FLT3-specific CARs and demonstrate that they confer potent reactivity against AML cell lines and primary AML blasts that express either wild-type FLT3 or FLT3-ITD. Further, we show that FLT3 CAR-T cells exert potent anti-leukemia activity in xenograft models of AML and induce complete remissions.

We also demonstrate that FLT3-expression on FLT3-ITD+ AML cells can be augmented by FLT3 inhibitors, which lead to increased recognition by CARs and improved efficacy of FLT3 CAR-T cells. We confirmed this principle with three different FLT3 inhibitors which are at distinct stages of clinical development i.e. Phase II/III clinical trial (crenolanib, quizartinib) and clinically approved (midostaurin). Further, we observed the strongest anti-leukemia activity of FLT3 CAR-T cells in combination with crenolanib *in vivo*.

FLT3 is known to be expressed by normal hematopoietic stem and progenitor cells. We evaluated FLT3-expression on normal hematopoietic stem cells (HSCs) using flow cytometry and confirmed lower level of FLT3-expression on HSCs and progenitors compared to AML cells. As anticipated, we found that FLT3 CAR-T cells recognize normal HSCs *in vitro* and *in vivo*, and compromise normal hematopoiesis, suggesting that adoptive therapy with FLT3 CAR-T cells will require successive CAR-T cell

depletion and allogeneic HSC transplantation (HSCT) to reconstitute the hematopoietic system. Moreover, an FLT3 inhibitor treatment does not increase FLT3-expression on HSCs. Accordingly, we demonstrate that the depletion of FLT3 CAR-T cells is possible with inducible Caspase 9 (iCasp9) safety switch.

Collectively, our data establish FLT3 as a novel CAR target in AML with particular relevance in high-risk FLT3-ITD+ AML. Our data demonstrate that FLT3 CAR-T cells act synergistically with FLT3 inhibitors in FLT3-ITD+ AML. i.e. FLT3 inhibitors-induced upregulation of FLT3 in FLT3-ITD+ AML cells enhances their recognition and elimination by FLT3 CAR-T cells. Due to recognition of normal HSCs, the clinical use of FLT3 CAR-T cells is likely restricted to a defined therapeutic window and must be followed by CAR-T cell depletion and allogeneic HSCT for hematopoietic reconstitution. The data provide rational to use FLT3 CAR-T cells in combination with FLT3 inhibitors to augment the anti-leukemia efficacy of FLT3 CAR-T cells in high-risk FLT3-ITD+ AML patients, and to mitigate the risk of relapse with FLT3-negative AML variants, which could otherwise develop under therapeutic pressure. The data provide proof of concept for synergistic use of CAR-T cell immunotherapy and small molecule targeted therapy and encourage the clinical evaluation of this combination treatment in high-risk patients with FLT3-ITD+ AML.

Zusammenfassung (summary in German)

Adoptive Immuntherapie, die Chimäre- Antigenrezeptor (CAR) –modifizierte, gegen CD19 gerichtet T-Zellen verwendet, hat eine bemerkenswerte therapeutische Wirksamkeit gegen B-Zell-Leukämien und -Lymphome und großes therapeutisches Potenzial für die Behandlung anderer hämatologischer Erkrankungen gezeigt. Die Akute Myeloische Leukämie (AML) ist hierbei eine Entität, für die es bisher an wirksamen und kurativen Therapien fehlt und für die die Entwicklung einer potentiell kurativen CAR-T-Zellimmuntherapie von großer Bedeutung ist.

FMS-like tyrosine kinase 3 (FLT3) ist ein homodimeres Transmembranprotein, das von AML-Blasten uniform exprimiert wird. FLT3 spielt eine wichtige Rolle beim Überleben von AML-Blasten und ist ein Schlüsselfaktor in der Leukämie-Genese bei AML-Fällen mit interner Tandem-Duplikation (FLT3-ITD) und Tyrosinkinase-Domänen (TKD)-Mutationen. Diese Eigenschaften legen die Vermutung nahe, dass FLT3 ein ausgezeichnetes Target für die CAR-T-Zell-Immuntherapie darstellen könnte. Daher setzten wir dort an und modifizierten humane $CD4^+$ und $CD8^+$ T-Zellen, um FLT3-spezifische CARs zu exprimieren, und konnten nachweisen, dass diese eine starke Reaktivität gegen AML-Zelllinien und primäre AML-Blasten besitzen, die entweder den FLT3-Wildtyp oder FLT3-ITD exprimieren. Weiterhin konnten wir zeigen, dass FLT3 CAR-T-Zellen in AML-Xenograft-Modellen eine starke anti-Leukämie-Aktivität besitzen und vollständige Remissionen hervorrufen können.

Zudem gelang der Nachweis, dass die FLT3-Expression auf $FLT3\text{-}ITD^+$ AML-Zellen durch FLT3-Inhibitoren verstärkt werden kann, was zu einer erhöhten Erkennung durch die CARs und einer verbesserten Wirksamkeit von FLT3-CAR-T-Zellen führt. Wir konnten dieses Prinzip mit drei verschiedenen FLT3-Inhibitoren belegen, die sich in unterschiedlichen Stadien der klinischen Entwicklung befinden, d. h. aus einer Klinischen Phase II / III-Studie (Crenolanib, Quizartinib) und einem klinisch zugelassenen Inhibitor (Midostaurin). Darüber hinaus beobachteten wir die stärkste anti-Leukämie-Aktivität von FLT3 CAR-T-Zellen in einer Kombination mit Crenolanib in vivo.

Es ist bekannt, dass FLT3 von normalen hämatopoetischen Stamm- und Vorläuferzellen exprimiert wird. Wir untersuchten die FLT3-Expression in normalen hämatopoetischen Stammzellen (HSCs) mittels Durchflusszytometrie und bestätigten im Vergleich zu AML-Zellen eine niedrigere FLT3-Expression auf HSCs und Vorläuferzellen. Wie erwartet, zeigte sich, dass FLT3 CAR-T-Zellen normale HSCs *in vitro* und *in vivo* erkennen und die normale Hämatopoese beeinträchtigen, was darauf hindeutet, dass eine adoptive Therapie mit FLT3 CAR-T-Zellen eine sukzessive CAR-T-Zell-Depletion und allogene HSC-Transplantation erfordert, um das hämatopoetische System wiederaufzubauen. Darüber hinaus erhöht die Behandlung mit einem FLT3-Inhibitor nicht die FLT3-Expression auf den HSCs. Dementsprechend konnten wir aufzeigen, dass die Depletion von FLT3 CAR-T Zellen mit einer induzierbaren Caspase 9 (iCasp9) als „Sicherheitsschalter“ möglich ist.

Zusammenfassend etablieren unsere Daten FLT3 als ein neuartiges CAR-Target in der Behandlung von AML mit besonderer Relevanz für die Hochrisiko-FLT3-ITD$^+$ AML. Unsere Daten zeigen, dass FLT3 CAR-T-Zellen synergistisch mit FLT3-Inhibitoren in FLT3-ITD$^+$ AML wirken, d.h. eine FLT3-Inhibitoren-induzierte Hochregulation von FLT3 in FLT3-ITD$^+$ AML-Zellen bewirkt und dies die Erkennung und Eliminierung durch FLT3-CAR-T-Zellen verstärkt. Durch ihre Eigenschaft der Erkennung von normalen HSCs ist die klinische Verwendung von FLT3 CAR-T-Zellen wahrscheinlich auf ein definiertes therapeutisches Fenster beschränkt und muss durch eine anschließende CAR-T-Zell-Depletion und eine allogene HSCT zur Rekonstitution des hämatopoetischen Systems ergänzt werden. In Anbetracht der Daten scheint es sinnvoll, FLT3-CAR-T-Zellen in Kombination mit FLT3-Inhibitoren zu verwenden, um die anti-leukämische Wirksamkeit von FLT3-CAR-T-Zellen bei Hochrisiko-FLT3-ITD$^+$ AML-Patienten zu erhöhen und das Risiko eines Rückfalls mit FLT3-negativen AML-Varianten zu verringern, die sich sonst therapiebedingt entwickeln könnten. Die Daten stellen ein Proof-of-Concept für den synergistischen Einsatz von CAR-T-Zell-Immuntherapie und niedermolekularen Inhibitoren dar, der eine klinische Evaluation dieser Kombinationsbehandlung bei Hochrisikopatienten mit FLT3-ITD$^+$ AML erstrebenswert macht.

1. Introduction

1.1 Adoptive immunotherapy of acute leukemia

Leukemia is one of the major cancers of the blood and bone marrow and affects a significant number of adults and children worldwide. It is classified based on the type of blood cells affected: In acute lymphoblastic leukemia (ALL), lymphoid cells are affected and in acute myeloid leukemia (AML), myeloid cells are affected. ALL is the most common leukemia in children, accounting for about 75% of pediatric leukemia cases. Among adults, AML (32%) is more common type of leukemia. The 5-year survival rate of acute leukemia patients is very poor, particularly in patients diagnosed with AML (26.6%)[1,2].

Novel immunotherapeutic approaches have revolutionized the field of cancer therapy[3,4]. In hematology, advances in immunotherapy have been made mainly in the treatment of B-cell malignancies i.e. acute lymphoblastic leukemia (ALL) and lymphoma. In B-cell malignancies, monoclonal antibodies (mAb), antibody drug conjugates (ADC) (anti-CD20 mAb Rituximab[5] and anti-CD22 mAb Inotuzumab ozogamicin[6]), bi-specific T-cell engagers (BiTE, Blinatomumab[7]) and chimeric antigen receptor (CAR)-T cells (CD19 CAR-T[8,9]) have been approved in United States (US) and Europe to treat patients[3]. Among these immunotherapeutic approaches, treatment with CD19 CAR-T cells is the most effective in relapsed/refractory (r/r) leukemia and lymphoma patients, and adoptive transfer (Figure 1.1) of these CAR-T cells has led to durable remissions[10-13].

Although considerable advancements have been made in AML treatment, hematopoietic stem cell transplantation (HSCT) remains the only potentially curative therapeutic approach[3,4]. However, frequent leukemia relapse after HSCT and treatment-related morbidity and mortality remain major concerns. Therefore, there is a medical need to develop novel and potentially curative therapies for AML patients[1].

Novel immunotherapeutic agents to treat AML are subject of intense translational research. The most recent treatment approaches which are currently in clinical trials include dendritic cell vaccination, ADCs (gemtuzumab ozogamicin[14]), T cell recruiting

antibodies (AMG330, CD3xCD33 BiTE[15]), checkpoint inhibitors (CTLA4, PD-1/PDL-1 inhibitors[15]) and engineered T cells, including transgenic TCR engineered T cells and CAR-T cells[3,4,13,16]. However, immunotherapy in AML is complex due to disease-related inherent challenges such as clonal heterogeneity, absence of a well-established AML-specific target antigen, low mutational burden, and intrinsic resistance mechanisms of AML blasts against immune responses[4]. Personalized, targeted immunotherapy may help to improve responses and achieve desired clinical outcomes[3,4,17].

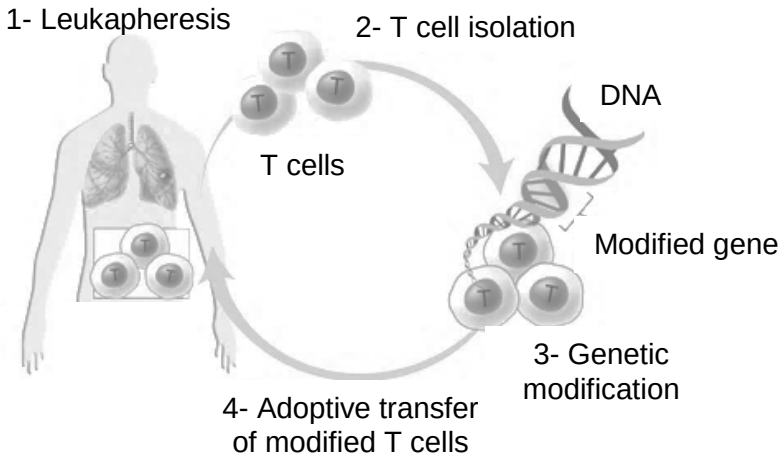


Figure 1.1: Principle of adoptive immunotherapy using CAR-T cells. A patient undergoes leukapheresis to obtain peripheral blood mononuclear cells (PBMCs). T-cell subsets required for genetic modification are isolated from a patient's own PBMC, activated and transduced using a viral vector encoding an antigen specific receptor (i.e CAR). Further, T cells are expanded *ex vivo* and re-infused into the patient. (Image is modified from https://lungevity.org/for-patients-caregivers/lung-cancer-101/treatment-options/immunotherapy, retrieved on 26.07.2018).

Clinical trials with CAR-T cells targeting CD19 in ALL and lymphoma, and BCMA in multiple myeloma, have shown potent anti-tumor activity and sustained remissions. More recently, the approvals of CD19 CAR-T cells for patients with ALL (Kymriah®, Novartis), and large B-cell lymphoma (Yescarta®, Kite pharma) by the USFDA (US Food and Drug Administration) and the EMA (European Medicines Agency) have spurred excitement in the field[8,9]. However, the targeted treatment of AML with CARs at efficacies comparable to those in ALL and large B-cell lymphoma remains to be achieved. In this study, we investigated FMS-like tyrosine kinase 3 (FLT3) as a CAR

target antigen, targeted using CAR-modified T cells and carried out pre-clinical evaluations of FLT3 CAR-T cells.

1.2 CAR design and mechanism of action

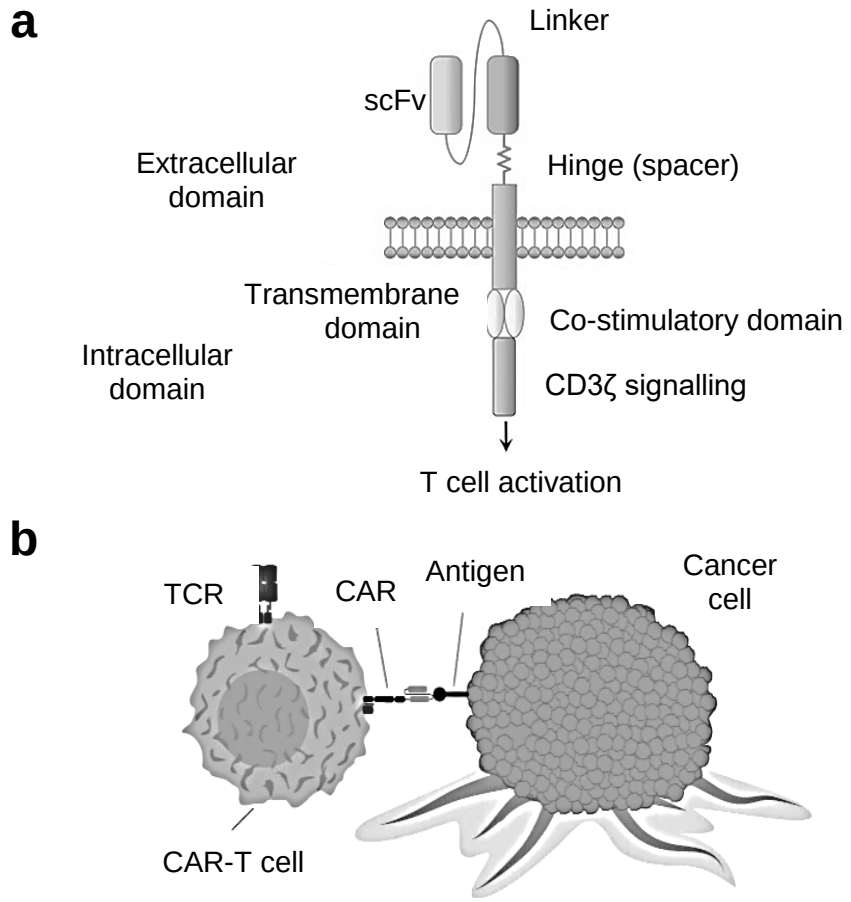


Figure 1.2: CAR design and mode of action of CAR-T cells. (a) General structure of the second generation CAR. The targeting element of a CAR is most commonly derived from a monoclonal antibody (scFv) and fused to an intracellular co-stimulatory domain (CD28 or 4-1BB) and a CD3ζ signaling domain via the hinge region of a human Immunoglobulin G (IgG). The transmembrane domain is most commonly derived from either CD8 or CD28. Image is modified from S. Han et al. (2017)[18] (b) Mode of action of CAR-modified T cells. A CAR binds to specific tumor antigens on the cell surface independent of a major histocompatibility complex (MHC) context. (Image is modified from www.hudeceklab.org, retrieved on 25.04.2018).

CARs are synthetic receptors that combine the antigen specificity of a monoclonal antibody (mAb) with the potent effector mechanisms of T cells[19]. CAR comprise of an

extracellular antigen binding domain derived from the variable heavy and light chains of a mAb. A transmembrane (TM) domain (usually derived from CD8a or CD28) links the extracellular antigen recognition domain to an intracellular signaling domain, most commonly composed of CD3ζ chain and one or more co-stimulatory domains such as CD28, 4-1BB, ICOS or OX40[19] (Figure 1.2a). In some designs, the extracellular spacer domain comprises the hinge and/or part of the Fc domain of a human IgG and provides flexibility to the receptor[20]. CARs can recognize epitopes of cell surface proteins in an MHC-independent manner. Therefore, CAR-based approaches are unaffected by tumor escape from MHC loss variants[21]. However, in comparison to endogenous TCRs, CARs are limited to target antigens that are expressed on the tumor cell surface (Figure 1.2b).

Stable expression of CARs can be achieved in bulk CD3+ T cells, or separately in CD4+ and CD8+ T cells, using genetic engineering tools such as retroviral transduction or transfection[19]. Upon engagement of CAR-modified T cells with target cells, the intracellular signaling domain of a CAR initiates T cell activation through downstream proteins, activating subsequent effector functions such as release of cytotoxic perforin/granzymes, production of cytokines and T cell proliferation[19].

In this study, we designed second generation CARs targeting FLT3 and integrated them into a lentiviral vector. We utilized an EF1/HTLV hybrid promotor to drive CAR expression[22]. Additionally, the CAR transgene was fused to a truncated human epidermal growth factor-receptor (EGFRt) by a T2A ribosomal skip sequence which serves as a transduction marker and a tool for enriching CAR-positive T cells[23].

1.3 Current treatment paradigm and challenges in AML

AML is a clonally heterogeneous hematologic malignancy. Although the understanding of AML pathogenesis, genomic landscape, and prognostic factors has improved, the treatment options have barely changed in the last 20 years. The standard care of treatment for adult AML patients is the front line induction chemotherapy with cytarabine and an anthracycline agent, which is effective and can induce remission[24,25]. Induction is usually followed by consolidation therapy with HiDAC (high dose cytarabine) as a post-

remission therapy in patients with types of AML that have a good prognosis[25-28]. However, HSCT is considered the preferred post-remission therapy for AML types with poor and very poor prognosis, particularly in younger patients who are medically fit to tolerate such an intensive treatment[25,29,30]. The most effective treatment for elderly AML patients who are unfit for intensive chemotherapy and HSCT remains controversial[31,32]. Consequently, treatments with low-dose chemotherapy aimed at prolonging survival are often preferred in these patients[33,34].

Although some of these therapeutic approaches are effective, they are associated with severe side effects. The conventional chemotherapy is severely toxic to normal tissues and associated with acute and long-term side effects. Additionally, the response to these treatments is highly variable and about 50-60% of patients relapse after an initial remission[17]. Similarly, HSCT is associated with transplant related morbidity and mortality due to Graft versus Host Disease (GvHD) or severe infections[25,29]. Therefore, novel immunotherapeutic approaches aim to redirect the immune system against leukemia, while avoiding the systemic toxicity associated with chemotherapy and HSCT are required.

AML is known as a clinically and clonally heterogeneous disease[35], and clonal heterogeneity is one of the major challenges in treating AML. Additionally, acquired drug resistance, acquisition of novel mutations and high relapse rates remain major concerns for AML patients[36]. There is increasing evidence that selective pressure induced on leukemic stem cells (LSC) by chemotherapeutic agents results in selection of chemo-resistant leukemic clones and progression of disease after an initial remission[37]. A recent study of the genomic and epigenomic landscape of AML has shown that, some of the mutations responsible for poor prognosis are present from the beginning of malignancy. An average of 13 genetic mutations were found in *de novo* AML patients, including mutations in genes for signaling pathways, transcription factors, DNA methylation and chromatin modification[38]. In addition to these mutations, AML blasts have shown to acquire novel mutations at the time of relapse[39]. AML sub-clones typically contain diverse sets of cytogenetic aberrations and somatic mutations, resulting in considerable genetic complexity[40]. Mutations in genes like *FLT3, CEBPA* and *NPM-1*

are frequent and have been included in AML classification guidelines to improve prognosis[38]. Thus, the identification of these mutations has led to improved AML classification, more precise decision on the treatment regime and the development of novel targeted drugs, including next generation synthetic inhibitors like FLT3 inhibitors and isocitrate dehydrogenase (IDH) inhibitors[41].

Immunotherapeutic approaches to treat AML are an area of intensive research. This includes the use of vaccines[42], mAbs and ADCs[43], BiTEs[44], dual affinity retargeting (DART) proteins[45,46], donor lymphocyte infusions (DLI)[47,48] and others. More recently, check point inhibitors such as anti-PD1 (nivolumab and pembrolizumab) and anti-CTLA-4 mAbs (ipilimumab) are being evaluated in AML patients with minimal residual disease (MRD) positive or MRD-negative remission with high risk of relapse[49]. Although there is a significant development in immunotherapeutic approaches to treat AML, each of these approaches has inherent limitations and are not able to consistently induce durable responses in AML patients[3].

1.4 Preliminary experience with CAR-T cell therapy in AML

CAR-modified T cells redirect antigen specificity to malignant cells and are an attractive novel approach for the treatment of AML. Pre-clinical activity of CAR-T cells against AML-associated antigens including CD123, CD33, CD44v6, CLL-1, folate receptor β, and Lewis Y has been shown (Table-1).

All of these anti-AML CAR-T cells have shown potent cytotoxicity and cytokine production against AML cells *in vitro* and demonstrated anti-leukemic activity in murine xenografts *in vivo* (Table-1). This has encouraged the clinical evaluation of some of these CAR-T cells and a small number of adults have been treated in early phase clinical trials (Table-2).

Table-1: Pre-clinical activity of CAR-T cells against AML

Target antigen	CAR design	Anti-leukemia activity	High level expression on HSC?	Expression on normal tissue
CD123	32716 or 26292 mAb scFv_4-1BB_ CD3ζ[50]	*In vitro* and *in vivo* in NSG/MOLM-14 and PDX model	yes	Myeloid progenitors, monocytes, dendritic and endothelial cells, platelets, basophils
	32716 or 26292 mAb scFv_CD28_CD3ζ[51]			
CD33	MY96 mAb scFv_4-1BB_CD3ζ[52]	*In vitro* and *in vivo* in NSG/MOLM-14 and PDX model	yes	Kupffer cells, myeloid progenitors
	M-195 mAb scFv_CD28_CD3ζ + IL-12 gene[53]			
CD44v6	BIWA-8 mAb scFv_CD28_CD3ζ[54]	*In vitro* and *in vivo* in NSG/THP-1 murine model	no	Monocytes, keratinocytes, activated T cells
CLL-1	scFv from hybridoma mAb_4-1BB and CD28_CD3ζ[55]	*In vitro* and *in vivo* in NSG/HL-60 and NSG/U-937 murine model	no	Myeloid cells, lung, GI epithelial cells
	scFv from unspecified source_4-1BB_CD3ζ[56]			
FRβ	m909 mAb scFv_CD28_CD3ζ[57]	*In vitro* and *in vivo* in NSG/THP-1 murine model	no	Myeloid cells
Lewis Y	Hu3S193 mAb scFv_ CD28_CD3ζ[58]	*In vitro* and *in vivo* in NSG/OVCAR-3 murine model	yes	Neutrophils, intestinal epithelial cells

scFv: Single chain variable fragment, HSC: Hematopoietic stem cell, CLL-1: C-type Lectin-like Molecule, FRβ: Folate receptor beta, PDX: Patient derived xenograft, GI: Gastro intestinal.

Although promising responses have been observed in early phase clinical trials, there are unresolved challenges such as choosing a suitable target antigen, preventing

resistance to CAR-T cell therapy and defining the optimal clinical parameters for anti-AML CAR-T cell therapy. To address some of these challenges, in this study, we developed CAR-T cells targeting FLT3 with intent of clinical translation of FLT3 CAR-T cells to treat high and intermediate risk AML patients.

Table-2: Pilot clinical trials of CAR-T cells in AML

Target antigen	CAR-T cell dose	CAR construct	Response (No. of Patients)	Center and publication
CD33	$1.12x10^9$ autologous CAR-T cells	Anti-CD33 (scFv)- 4-1BB-CD3ζ CAR	Partial response (1)	Chinese PLA General Hospital, China[59].
CD123	$50\text{-}200x10^6$ autologous CAR-T cells	Anti-CD123 (26293 scFv)-CD28- CD3ζ CAR	Ongoing trial SD (2), PD (1), CR (1), CRm (1), CRi (1), CR- MRD+ (1)	City of Hope, CA,USA[60].
	$6.25x10^5$ per kg body weight allogeneic CAR-T cells	Universal CD123 CAR-T cells (construct unspecified)	Ongoing trial first treated patient died due to capillary leakage syndrome	Weill Cornell Medical College, New York, USA MD Anderson Cancer Center, Texas, USA. (clinicaltrials.gov)
Lewis Y	$0.5\text{-}1.3x\ 10^9$ autologous CAR-T cells	anti-Lew Y (Hu3S193 scFv)-CD28-CD3ζ CAR	SD (2) PD (2), death (1)	Peter MacCallum Cancer Centre Melbourne, Australia[61].

SD: stable disease, PD: progressive disease, CR: complete remission, CRi: complete remission with incomplete blood count recovery, CRm: morphological leukemia free state.

1.5 FLT3 is a candidate CAR target for immunotherapy of AML

FLT3 (CD135) is a type I transmembrane protein expressed uniformly on primary AML blasts independent of cytogenetic and FAB (French-American-British) subtype[62-64]. FLT3 is physiologically expressed on normal hematopoietic stem cells (HSCs), as well as

lymphoid, myeloid and granulocyte/macrophage progenitor cells in humans and plays a vital role in normal hematopoiesis[64,65]. FLT3-expression has also been reported in mature hematopoietic cells such as subsets of dendritic cells and natural killer cells[66,67].

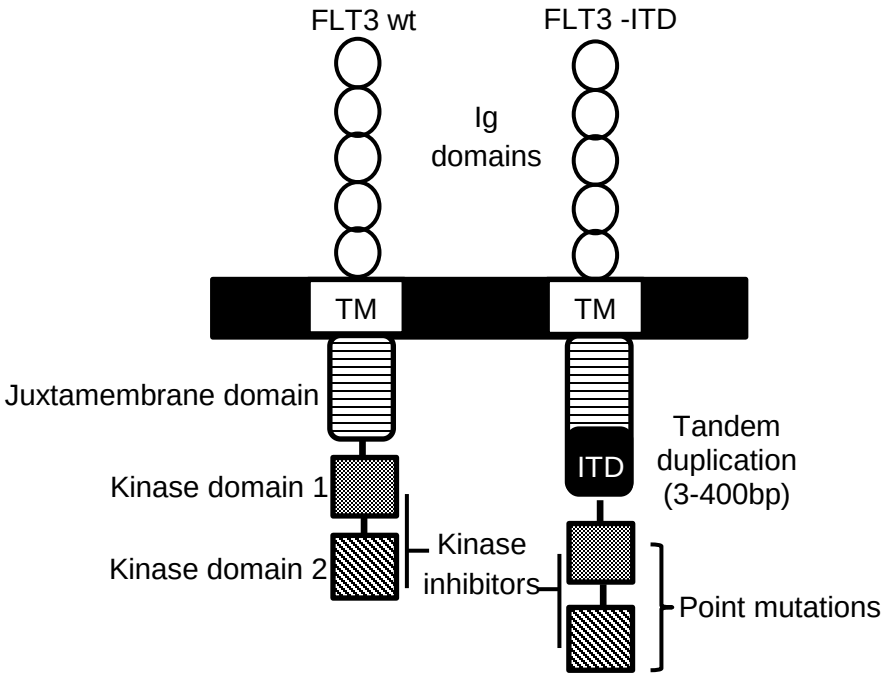


Figure 1.3: Structure of wild type and mutated FLT3 protein. FLT3 is a class III receptor tyrosine kinase. It consists of five extracellular immunoglobulin (Ig)-like domains. The transmembrane domain links the extracellular Ig domains to an intracellular juxta membrane (JM) domain (auto-inhibitory domain). FLT3 has two intracellular tyrosine kinase domains (TKD) which provide tyrosine residues for phosphorylation and receptor down-stream signaling. Internal tandem duplication (ITD) mutations are commonly located in JM domain but can also extend to the TKD. The most frequent point mutations are located in the kinase domains e.g. at position D835, N676 and V592.

More importantly, FLT3 is uniformly present on malignant blasts in AML, providing a target for antibody and cellular immunotherapy[62-64,68-70]. The antigen density of FLT3 protein on the cell surface of AML blasts is in the range of several hundred to several thousand molecules per cell, which is believed to be optimal for recognition by CAR-T cells[71,72]. FLT3 transcripts are universally detectable in AML blasts with different expression levels in distinct AML subtypes[37,73]. Higher FLT3 transcript levels are associated with higher leukocyte counts and higher infiltration of leukemic blasts in the bone marrow[69].

FLT3 is vital for survival and proliferation of AML blasts. Particularly, FLT3 has a pathophysiologic relevance in AML patients that carry activating mutations in the FLT3 intracellular domain of AML blasts[64,69] (Figure 1.3). FLT3 mutations are the most common molecular abnormality in AML and have an unfavorable prognostic impact on the survival of AML patients[64,69,73]. Of these mutations, internal tandem duplication (ITD) in the juxta-membrane domain and point mutations in the tyrosine kinase domain (TKD) occurs in ~30% AML patients[64,69,73,74]. Both of these aberrations lead to constitutive FLT3 activation in a ligand-independent manner and act as gain-of-function 'driver mutations' which contribute to sustaining the disease[75,76]. This suggests that FLT3-ITD^{+} AML is particularly vulnerable and an ideal AML subset for anti-FLT3 immunotherapy since the risk of FLT3$^{negative/low}$ antigen-loss is anticipated to be low. Further, the presence of FLT3-ITD has been linked to poor clinical outcome after induction/consolidation chemotherapy and allogeneic HSCT, therefore outlining a subset of high-risk AML patients that require novel, innovative treatment strategies[30,77].

FLT3 has been pursued as an antibody immunotherapy target, although the FLT3 antigen density on AML blasts is much lower compared to other well-characterized mAb target antigens such as CD20 on lymphoma cells. Thus, an anti-FLT3 mAb approach is not presumed to be optimal for inducing potent antibody-mediated effector functions. Interestingly, the mouse anti-human FLT3 mAbs 4G8 and BV10 have been shown to bind to AML blasts specifically and to a lesser extent to normal HSCs. After Fc-optimization, these mAbs confer specific reactivity against high FLT3 antigen expressing AML blasts in pre-clinical models[71]. Here, we engineered T cells to express FLT3-specific CARs with a targeting domain derived from either 4G8 or BV10 mAb and analyzed the anti-leukemia reactivity of FLT3 CAR-T cells.

1.6 FLT3 inhibitors for AML treatment

FLT3 is a class III receptor tyrosine kinase, which has been pursued as a target for tyrosine kinase inhibitors in AML. There are several FLT3 inhibitors at advanced stages of clinical development. However, the clinical outcomes of single-agent therapy with 'first

generation' FLT3 inhibitors in AML were poor. One of the reasons, at least in part, is the development of resistance against these inhibitors by AML blasts through acquisition of novel secondary mutations in the FLT3 intracellular domain, or FLT3 overexpression to compensate for the inhibition[78-81].

Among the panel of FLT3 inhibitors that are under clinical investigation, crenolanib is a novel FLT3 inhibitor which was initially developed as a selective inhibitor of platelet-derived growth factor receptor (PDGFR)[82]. However, crenolanib also has a high affinity for type III receptor tyrosine kinases. Therefore, crenolanib acts as a specific inhibitor (type-I-inhibitor) of FLT3. Crenolanib targets the active FLT3 kinase conformation and is effective against FLT3 with ITD and TKD mutations[83] which confer resistance to other inhibitors (type-II-inhibitors), that target the inactive kinase conformation. Crenolanib has shown efficacy in heavily pretreated relapsed/refractory AML with FLT3-ITD and TKD mutations, with overall response rate up to ~50% in recently reported phase II clinical trials[84,85]. Crenolanib and other tyrosine kinase inhibitors (TKIs) are also therefore being investigated in combination regimens to exploit potentially synergistic effects.

Quizartinib and midostaurin are 'first-generation' multi-kinase inhibitors[86,87]. Quizartinib treatment improved overall survival of FLT3-ITD$^+$ AML patients that were relapsed after HSCT or after failure of salvage chemotherapy[88]. Interestingly, midostaurin has shown activity against AML blasts expressing mutant FLT3 and is the only USFDA approved FLT3 inhibitor for AML treatment[89].

Monotherapy using FLT3 inhibitors may result in measurable clinical responses, including significant reductions of peripheral blood and bone marrow blasts[90]. However, in most cases, patients become resistant after transient responses[69]. The emergence of novel mutations in tyrosine kinase and/or juxta-membrane domains have been observed frequently after treatment with FLT3 inhibitors which limit the clinical activity of FLT3 inhibitors as a monotherapy, particularly in relapsed/refractory AML[79,80].

One of the most important mechanisms by which AML blasts gain resistance to FLT3 inhibitors is overexpression/upregulation of the FLT3 receptor. This has been observed in AML patients after treatment with the FLT3 inhibitors lestaurtinib or sorafenib[81].

Treatment with the FLT3 inhibitor midostaurin has also resulted in FLT3 upregulation on MOLM-13 AML cells and development of resistance *in vitro*[78]. Interestingly, it has been shown that target antigen density positively correlated to CAR-T cell activation, and higher surface target antigen density translated into enhanced CAR-T cells function[91]. Therefore, we hypothesized that up-regulation of surface FLT3 on AML blasts in response to FLT3 inhibitors can be exploited to enhance anti-leukemic activity of FLT3 CAR-T cells. A combination of FLT3 CAR-T cells with an FLT3 inhibitor could therefore act synergistically. In this study, we investigated this potential synergistic effect using different FLT3 inhibitors.

1.7 Safety mechanisms for depletion of CAR-T cells

T cells equipped with CARs targeting CD19 and BCMA have shown potent activity in hematologic malignancies[8,9,92]. However, the development of highly potent CAR-T cells also raises safety concerns, particularly when the target antigen is also expressed by healthy tissues. In case of CD19 CAR-T cell therapy for ALL and lymphoma patients, such on-target off-tumor toxicity is well-tolerated because B cell aplasia which is side effect of the therapy is clinically manageable[11]. However, FLT3 is expressed by normal HSCs and their progenitors[65] and elimination of these cells by FLT3 CAR-T cells may lead to myeloablation and is anticipated to induce impaired hematopoiesis.

Notably, adoptively transferred CAR-T cells can persist long term to sustain remission, potentially causing ongoing unwanted effects. Therefore an effective and timely restricted pharmacological intervention is desirable to control these side effects[9,11]. Available techniques that can selectively deplete T cells include a herpes simplex virus thymidine kinase (HSV-TK) switch[93], *E.coli*-derived cytosine deaminase (CD) gene[94] and antibody mediated depletion using anti-CD20 mAb (Rituximab)[95], anti-EGFRt mAb (cetuximab)[96] or anti-CD52 mAb (alemtuzumab)[97]. Each of the mentioned techniques has its merits and demerits (Table-3). Here, we sought to utilize a validated cellular safety switch, an inducible caspase 9 (iCasp9)[98].

Table-3: Comparison of suicide genes for adoptive immunotherapy[99]

Suicide gene	Source	Activating agent	Clinically validated	Pros	Cons
HSV-TK	Virus	GCV	yes	Removes alloreactive cells	Gradual onset, Immunogenic
CD	Bacteria	5-FC	yes		
Therapeutic mAbs	Human or mouse-human chimeric	Anti-CD20 mAb, anti-EGFRt mAb, anti-CD52 mAb	no	Rapid onset, Non-immunogenic	On-target toxicity from mAb
iCasp9	Human	Small molecule dimerizer i.e. AP20187	yes	Rapid onset, Non-immunogenic, Use of non-therapeutic agent	Incomplete elimination, although eliminates more than 90% of cells

HSV-TK: herpes simplex virus thymidine kinase, GCV: ganciclovir, CD: cytosine deaminase, 5-FC: 5-fluorocytosin

Caspase 9 is the key initiator of intrinsic apoptosis pathway[100]. The iCasp9 safety switch exploits the central role of caspase 9 in the apoptosis cascade. This suicide gene utilizes a genetically engineered human caspase 9, called iCasp9 that can form homodimers and thereby induce apoptosis, independently of physiological activation. In iCasp9, the physiological caspase activation and recruitment domain (CARD) has been replaced with the FK binding protein (FKBP) with an F36V mutation. Cell-permeable small molecules (i.e. AP20187 and AP1903) can bind to $FKBP_{F36V}$ and induce dimerization of iCasp9, thereby activating apoptosis in human cells[98,101].

The iCasp9 suicide gene has demonstrated depletion of alloreactive T cells after haploidentical stem cell transplantation in patients[98] and shown to improve the safety of CAR-T cells targeting CD19 or CD20 in pre-clinical mouse models[102-105]. Importantly, iCasp9 activation achieved elimination of more than 90% of the T cells within 30 minutes after administration in four ALL patients and ended the GvHD without recurrence[98]. In

this study, we equipped FLT3 CAR-T cells with iCasp9 safety switch and investigated if it could deplete FLT3 CAR-T cells.

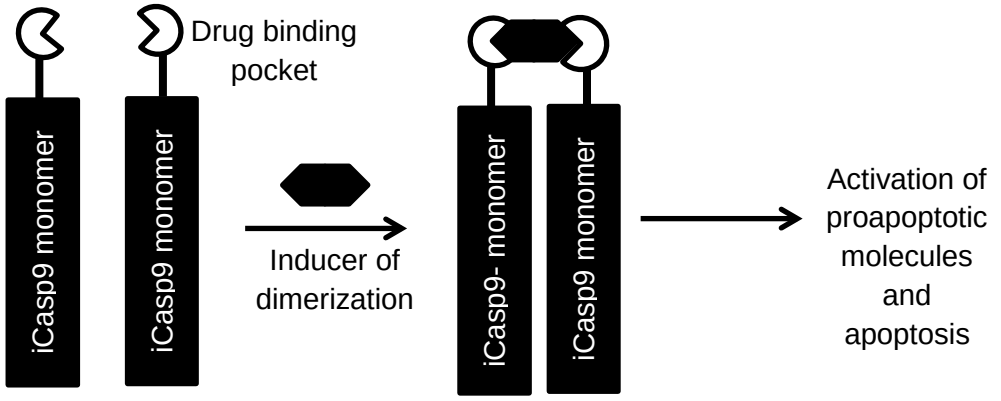


Figure 1.4: Mode of action of iCasp9 safety switch. iCasp9 is a hybrid molecule formed by linking a drug-binding domain with caspase9 (that lack a caspase activation and recruitment (CARD) domain) via a linker. Binding of small molecules (AP1903 or AP20187) leads to dimerization of iCas9, thereby relaying activation signals and initiating apoptosis pathway.

1.8 Study objectives and specific aims

Immunotherapy using CD19 CAR-T cells has shown potent efficacy and durable remissions in B-ALL and lymphoma and provided proof of concept for therapeutic potential in other hematologic malignancies. AML is an entity with unmet medical need of effective curative treatment. Therefore, the main objective of the study is as following:

- Preclinical evaluation of anti-AML CAR-T cells

For an effective CAR-T cell immunotherapy of AML, selection of a target antigen is a critical aspect. The ideal candidate would be one i) that is uniformly expressed by AML blasts ii) absent (or minimally expressed) on healthy cells and tissues and iii) indispensable for AML pathophysiology and therefore less likely to be subject of antigen loss under therapeutic pressure. Based on our preliminary evaluation and literature assessment, FLT3 could be a candidate fulfilling these criteria.

FLT3 is a transmembrane protein and expressed uniformly by AML blasts. Moreover, it is a pathologically more relevant target compared to other available target antigens, particularly in a subgroup of patients with FLT3-ITD driver mutations. Therefore, we hypothesized that

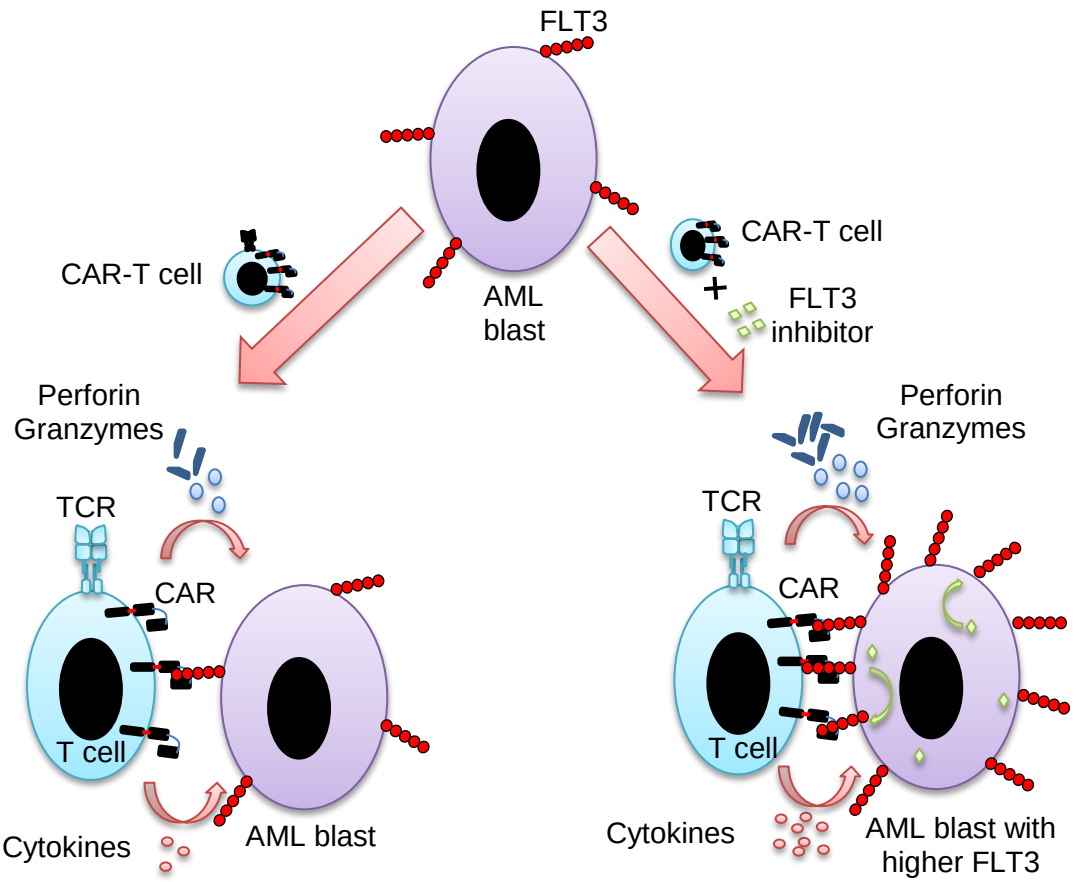
i) FLT3 can be targeted effectively using CAR-modified T cells, including in a subgroup of high-risk FLT3-ITD+ AML patients

FLT3 is pursued as a target of small molecule inhibitors for treatment of AML. However, monotherapy with FLT3 inhibitors did not consistently achieve durable responses. In addition, continuous exposure to an FLT3 inhibitor has been reported to lead to the development of resistance by AML blasts by overexpression of cell surface FLT3 protein. Therefore, we postulated that higher target antigen density on AML blasts due to FLT3 inhibitor treatment can increase anti-leukemia activity of FLT3 CAR-T cells and that combining FLT3 inhibitors with FLT3 CAR-T cells can be a potent anti-AML therapeutic strategy. Therefore, we hypothesized that

ii) FLT3 inhibitors can enhance cell surface FLT3-expression and can be utilized as a synergistic combination partner for FLT3 CAR-T cells

CAR-modified T cells are potent living drugs that can recognize and eliminate healthy cells if they also express the targeted antigen. Therefore, we aimed to evaluate the recognition of healthy hematopoietic cells by FLT3 CAR-T cells. Additionally, we aimed to equip FLT3 CAR-T cells with a potent suicide mechanism to deplete FLT3 CAR-T cells if needed. Based on these hypotheses, the specific aims of the study are:

1) To assess anti-leukemic activity of FLT3 CAR-T cells *in vitro* and *in vivo*
2) To determine if the anti-leukemic efficacy of FLT3 CAR-T cells can be enhanced by combination treatment with FLT3 inhibitors
3) To assess recognition of healthy human hematopoietic cells by FLT3 CAR-T cells
4) To demonstrate ability of a safety switch to deplete FLT3 CAR-T cells



Hypothesis 1: Targeting FLT3 using CAR-modified T cells

Hypothesis 2: FLT3 inhibitors as a synergistic partner for FLT3 CAR-T cells

Figure 1.5: Targeting FLT3 in AML using CAR-T cells. Hypothesis 1: We hypothesized that AML blasts expressing FLT3 (wild type or mutant) can be recognized by FLT3 CAR-T cells leading to T cell activation, cytokine production, perforin and granzymes release and AML cell death. Hypothesis 2: We hypothesized that FLT3 inhibitor treatment can enhance the surface expression of FLT3 protein on AML blasts and thus be better recognized by FLT3 CAR-T cells. Therefore, FLT3 inhibitors can act as a synergistic partner for FLT3 CAR-T cells.

Approach:

To assess the anti-leukemic activity of FLT3 CAR-T cells, we designed second generation CAR-T cells using targeting domains that are derived from anti-FLT3 mAbs (BV10 or 4G8) which have shown strong binding to FLT3 in a pre-clinical evaluation and has been evaluated clinically to treat AML (4G8). We selected target cell lines that represent AML genetic heterogeneity by either expressing wild type FLT3 (THP-1),

heterozygous FLT3-ITD (MOLM-13) or loss of heterozygosity (LOH)-ITD (MV4;11) for the pre-clinical assessment of FLT3 CAR-T cells activity. To corroborate our observation of FLT3 CAR-T cell activity against distinct AML cell lines, we obtained primary AML blasts, evaluated FLT3-expression on them and assessed their recognition by FLT3 CAR-T cells *in vitro*. AML blasts readily engraft and develop systemic leukemia in mice and therefore we exploited murine models to assess *in vivo* anti-leukemic activity of FLT3 CAR-T cells, which is the standard pre-clinical assessment approach in the field. Further, to determine whether FLT3 inhibitors can act as combination partners of FLT3 CAR-T cells, we selected potential candidates from FLT3 inhibitors that are FDA approved (midostaurin) or in advance phase of clinical trials (crenolanib and quizartinib). To dissect subtle differences in FLT3 levels on AML cells after treatment with FLT3 inhibitors, we utilized flow cytometry and super resolution microscopy, and further compared FLT3 CAR-T cell activity against untreated and FLT3 inhibitor treated AML cells *in vitro* and *in vivo*. To assess HSCs toxicity, methods that partly mimic human hematopoiesis (colony formation assays and a murine model of human hematopoiesis) were utilized and further evaluation of icasp9 mediated FLT3 CAR-T cell depletion was carried out.

2. Methods and materials

2.1 Biological material

2.1.1 Healthy human samples

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient centrifugation using Ficoll–Paque solution (Amersham Biosciences, Uppsala, Sweden). Peripheral blood samples were obtained after written informed consent to participate in research protocols approved by the Institutional Review Boards of the University of Würzburg (Germany).

Peripheral blood was mixed with room temperature (RT) DPBS (Dulbecco's phosphate buffer saline) at final volume of 35 mL and carefully added to a 50 mL Leucosep tube that was previously equilibrated with 15 mL of RT Biocoll separating solution. Density centrifugation was performed for 15 min at 310 g, with slow acceleration and deceleration. PBMCs that accumulate above of the leucosep filter were washed twice with cold PBS/EDTA buffer, and centrifuged for 10 min at 220 g at 4°C. The cells were then directly used for isolation of T cells or myeloid cells.

2.1.2 Primary AML samples

PBMCs from AML patients were obtained after written informed consent to participate in research protocols approved by the Institutional Review Boards of the University of Regensburg. Primary AML cells were maintained in RPMI-1640 supplemented with 10% human serum, 2 mM glutamine, 100U/mL penicillin/streptomycin, and a cytokine cocktail including IL-4 (1000 IU/mL), granulocyte macrophage colony-stimulating factor (GM-CSF) (10 ng/mL), stem cell factor (5 ng/mL) and tumor necrosis factor (TNF)-α (10 ng/mL).

2.1.3 Human hematopoietic stem cells

Healthy donor peripheral blood mobilized stem cells were obtained from the Blutspendtdienst, Frankfurt, Germany. Normal HSCs were isolated by staining with an anti-CD34 antibody and magnetic cell separation. HSCs were maintained in RPMI-1640 supplemented with 10% human serum, 2 mM glutamine, 100 U/mL penicillin/streptomycin, and a cytokine cocktail including granulocyte macrophage colony-stimulating factor (GM-CSF) (10 ng/mL) and stem cell factor (5 ng/mL).

2.1.4 Cell lines

The human leukemia cell lines MOLM-13 (ACC 554), THP-1 (ACC 16), MV4;11 (ACC 102), and K562 (ACC 10), JeKo-1 (ACC 553), NALM-16 (ACC 680), KOPN-8 (ACC 552), SEM (ACC 546) and Jurkats (ACC 282) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and U937 cells were obtained from ATCC (the American Type Culture Collection). Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 U/mL penicillin/streptomycin. All cell lines were transduced with a lentiviral vector encoding a firefly luciferase (ffluc)_green fluorescent protein (GFP) transgene to enable detection by flow cytometry (GFP) and bioluminescence imaging (ffLuc). K562/FLT3 cell line was generated by retroviral transduction with the full-length human FLT3 gene. All cell lines were tested regularly for mycoplasma and shown to be negative.

2.2 Vector construction and preparation of viral vectors

We designed second generation CARs that contains an scFv fragment derived from BV10 or 4G8 mAb[22,71] (FLT3 CAR), 32716 mAb[50] (CD123 CAR), FMC63 mAb[11] (CD19 CAR). CAR spacer domains comprised either an IgG4 hinge with short spacer (12 amino acids) or long spacer (229 amino acids of hinge-CH2-CH3). A spacer domain was linked to a human CD28 transmembrane domain, a signaling module derived from either CD28 or 4-1BB and the cytoplasmic domain of CD3ζ. The gene cassette was equipped

with a T2A sequence and a truncated epidermal growth factor receptor (EGFRt) sequence downstream of the CAR gene.

Codon-optimized nucleotide sequences encoding each transgene were synthesized (Geneart, Thermoscientific) and cloned into the epHIV7 lentiviral vector backbone. LentiX cells were co-transfected with each of the lentiviral vector plasmids along with the packaging vectors pCHGP-2, pCMV-Rev2 and pCMV-G using a calcium phosphate based transfection reagent. Culture medium was changed 12-hours after transfection, and lentiviruses (from supernatant) were collected after 72-hour.

In order to determine the viral titer, Jurkat T cells were plated in a 48-well plate at a cell density of $1x10^6$ cells/mL in 250 µL medium. Serial dilutions of lentivirus (0, 0.25, 1, 2.5, 5 or 10µL) were added to different wells and incubated for 4-hours at 37°C. Following incubation, the medium was topped up to 1mL, and the cells cultured for another 48-hours. CAR expression was analyzed by flow cytometry using the EGFRt transduction marker encoded within the lentiviral vector. The lentivirus titer in transforming units (TU)/mL was calculated, and a multiplicity of infection (MOI) of 3-5 was used for the transduction of T cells.

2.3 Preparation of CAR-T cells

2.3.1 T cells transduction with lentiviral vectors and enrichment of CAR+ T cells

Blood samples obtained from healthy donors or AML patients and PBMCs were prepared by Ficoll-Hypaque gradient centrifugation. CD4+ and CD8+ T cells were isolated from PBMCs using CD4+ or CD8+ T cell isolation kits respectively. T cells were activated with anti-CD3/CD28 beads according to the manufacturer's instructions. On day 1, activated T cells were transduced by exposure to lentiviral supernatant (MOI 3-5), supplemented with 0.8 µg/ml polybrene, and centrifuged at 800 g for 45 min at 32°C. T cells were expanded in RPMI, 10% human serum, 2 mM l-glutamine, 1% penicillin-streptomycin and 50 µM β-mercaptoethanol. The culture medium was supplemented with recombinant human IL-2 to a final concentration of 50 U/ml. CAR-transduced T cells

were stained with a biotin-conjugated anti-EGFR antibody followed by anti-biotin beads, and EGFRt-positive T cells were enriched using magnetic separation. After enrichment, CAR-T cells were expanded using a rapid expansion protocol (REP) for some experiments. Briefly, CAR-T cells were stimulated with irradiated (80 Gy) CD19+ B-lymphoblastoid cells (LCL) and irradiated PBMCs (30 Gy) in culture medium supplemented with 50 U/ml IL-2 for 12-14 days before using the CAR-T cells for *in vitro* or *in vivo* experiments.

2.4 Flow cytometric analysis

2.4.1 Phenotyping of T cells, leukemia cells and HSC

Conjugated antibodies specific for CD3, CD4, CD8, CD14, CD19, CD33, CD34, CD38, CD45, CD62L, CD123, CD135 and matched isotype controls were used for staining and analysis by flow cytometry. CAR-positive T cells were detected using mAb against EGFR (Cetuximab, ImClone Systems) that had been biotinylated in-house (EZ-Link™Sulfo-NHS-SS-Biotin, ThermoFisher Scientific, IL; according to the manufacturer's instructions) and streptavidin-PE. Staining with 7-AAD (7-amino actinomycine-D) was used for live/dead cell discrimination as directed by the manufacturer. Flow cytometry analyses were performed on a FACS Canto II and data were analyzed using FlowJo software v9.0.2.

2.4.2 Flow cytometric analysis of FLT3-expression

Cell surface expression of FLT3 (CD135) was analyzed using a conjugated mouse-anti-human-FLT3 mAb and a mouse IgG1 isotype control. In brief, 1×10^6 cells were washed, resuspended in 100 µL PBS/0.5% fetal calf serum and stained with 2 µL of anti-FLT3 mAb or isotype for 30 minutes at 4 °C.

2.5 Analysis of CAR-T cell functions *in vitro*

2.5.1 Cytotoxicity, cytokine secretion and proliferation assays

Cytolytic activity: Target leukemia cells expressing firefly luciferase (ffluc) were washed and incubated in triplicates at 5×10^4 cells/well with effector T cells at various effector/target (E:T) ratios in 96 well flat bottom plates. Luminescence intensities were recorded at specific time points (2, 4, 6, 24 hours) using a luminometer. Specific lysis was determined using the standard formula.

Cytokine secretion: Target and effector cells were plated in triplicates at an effector: target E:T ratio of 2:1 or 4:1, and cytokine production was measured in cell culture supernatants after 24-hours incubation by enzyme-linked immunosorbent assay (ELISA).

Proliferation: T cells were labeled with 0.2 μM carboxy fluorescein succinimidyl ester (CFSE), washed and plated in triplicates with stimulator cells at a ratio of 2:1 or 4:1 in culture medium without exogenous cytokines. After 3 days of incubation, the cells were labeled with an anti-CD4 or CD8 mAb and 7-AAD to exclude dead cells from analysis. Samples were analyzed by flow cytometry for cell division of live T cells as determined by dilution of CFSE. Untransduced T cells were used as a control for calculation of percentage proliferation.

The cytolytic activity of CAR-modified and control T cells against primary AML cells was analyzed in a flow-based cytotoxicity assay. T cells and AML cells were seeded into 96-well plates at E:T ratios ranging from 20:1 to 1:1, with $10x10^3$ target cells per well. After 4-24 hours, the cultures were aspirated, stained with 7-AAD to discriminate live and dead cells and anti-CD3/anti-CD33/anti-CD45 mAbs to distinguish T cells and AML cells. In some experiments, AML blasts were pretreated with 20 nM crenolanib for 5 days prior to the assay. To quantitate the number of residual live AML cells, counting beads (123count eBeads) were used according to the manufacturer's instructions. Flow analyses were performed on a FACS Canto II and data analyzed using FlowJo software.

2.6 *In vivo* anti-leukemia efficacy experiments

2.6.1 Adoptive transfer of T cells in NSG mice engrafted with AML cells

The University of Würzburg Institutional Animal Care and Use Committee approved all mouse experiments. 6 to 8 week-old female NOD/SCID/$\gamma c^{-/-}$ (NSG) mice were obtained from Charles River Laboratories (Sulzfeld, Germany) or bred in-house. Mice were injected intravenously (i.v.) with 1×10^6 ffluc$^+$ MOLM-13 cells through the tail vein, and received injections of $5x10^6$ CAR-modified or control T cells i.v. 7 days after tumor inoculation. Mice were imaged weekly by bioluminescence imaging and blood samples were obtained regularly to assess leukemia progression or regression. Mice were sacrificed if they lost >20% weight or if hind limb paralysis was observed. Bone marrow, spleen and peripheral blood were analyzed by flow cytometry at the end of the experiment. All the animal experiments were repeated at least once. In most experiments, five mice were used in each experimental group to provide 81% power to detect an effect size of 1.75, based on a t-test with a one-sided 0.05 level of significance.

2.6.2 *In vivo* experiments with primary AML blasts and normal HSCs

Six to eight week old female NSG and NSG-3GS mice were obtained from Charles River Laboratories. NSG mice were inoculated with $2.5x10^6$ primary FLT3-ITD$^+$ AML blasts by tail vein injection on day 0. On day 21, engraftment of AML blasts was confirmed in a subgroup of mice by flow cytometric analysis of bone marrow. On day 28, mice received $5x10^6$ CAR-T cells or untransduced T cells (CD4$^+$:CD8$^+$ ratio = 1:1). Peripheral blood was obtained at regular intervals to analyze the frequency of transferred T cells and AML cells. 21 days after T cell injection, peripheral blood, bone marrow and spleen were analyzed by flow cytometry to detect AML cells (CD45$^+$CD33$^+$) and T cells (CD45$^+$CD3$^+$).

NSG-3GS mice were inoculated with $1x10^6$ human GM-CSF-mobilized peripheral blood CD34$^+$ HSCs (i.v. tail vein injection). Peripheral blood was obtained at regular intervals to analyze the frequency of mature human hematopoietic cells (e.g. CD19$^+$ B cells).

Successful engraftment and development of human hematopoiesis was confirmed 8 weeks after HSC injection, and the mice then received $5x10^6$ T cells ($CD4^+$:$CD8^+$ ratio = 1:1) (by i.v. tail vein injection). Peripheral blood was obtained at regular intervals to analyze the frequency of human hematopoietic cells and transferred T cells, and 4 weeks after adoptive T cell transfer, bone marrow from mice were analyzed by flow cytometry to detect HSC and progenitor cells ($CD34^+CD38^-$/$CD34^+CD38^+$), B-cells ($CD45^+CD19^+$) and T cells ($CD45^+CD3^+$).

2.7 Colony formation assay

GM-CSF-mobilized human CD34+CD38- peripheral blood HSCs (>95 % purity) were seeded at $5x10^3$ cells/well in triplicate wells and incubated with CAR-transduced or untransduced T cells (E:T ratio = 5:1) in T-cell medium. After 24 hours, the cell suspension was plated onto methyl cellulose-based medium (Methocult opti H4034) in 6-well plates (SmartdishTM plate). Colonies were evaluated using established criteria according to the manufacturer's instructions and counted under an optical microscope 14 days later.

2.8 FLT3 inhibitor preparation

Crenolanib, Quizartinib and midostaurin were reconstituted in dimethylsulfoxide (DMSO) prior to dilution in cell culture medium or 30% glycerol formal (Sigma Aldrich, Steinheim, Germany) for use in *in vitro* and *in vivo* experiments.

2.9 FLT3 inhibitor treatment

2.9.1 Treatment of leukemia cells with FLT3 inhibitors

Leukemia cells were maintained in RPMI-1640 medium, supplemented with 10 % fetal calf serum, 2 mM glutamine, 100 U/mL penicillin/streptomycin. $1x10^6$/mL leukemia cells were plated in 24-well plates in 2 mL culture media and treated with one of the following FLT3 inhibitors: 10 nM crenolanib, 1 nM quizartinib or 10 nM Midostaurin. Every 7 days,

leukemia cells were adjusted to $1x10^6$/mL in fresh medium and 2 mL of this cell suspension plated per well in 24-well plates. Midostaurin concentration was increased serially (increased by 10 nM weekly) to 50 nM. In some experiments, leukemia cells were labelled with efluoro670 according to the manufacturer's instructions to assess proliferation by flow cytometry.

2.9.2 Treatment of HSCs and primary AML cells with FLT3 inhibitors

$1x10^6$/mL HSCs or primary AML cells were plated in 24-well plates in 2 mL culture media and treated with one of the following FLT3 inhibitors: 10 nM crenolanib, 1 nM quizartinib or 50 nM midostaurin. Flow cytometry analysis was performed on day 3-7 to determine FLT3-expression and percentage of live cells (7-AAD negative cells).

2.10 High resolution microscopy analysis

2.10.1 LabTek slides coating

Poly-D-lysin (PDL) coated slides were used for plating and staining cells. In brief, LabTek slides were treated with 0.5 ml 1 M sodium hydroxide (NaOH) for 15 min at RT. Slides were washed 3 times with sterile water. Slides were then treated with 0.5 ml 100 % ethanol for 15 min and washed 3 times with sterile water. Coating was performed using 33 µg/ml PDL for 1 hour at 37°C, followed by 3 times washing with sterile water (500 µl each time) and air-drying.

2.10.2 Staining AML cells on LabTek slides

$1x10^4$ AML cells were plated in 200 µl media in PDL-coated slides and incubated for 90 min at 37°C. Cells were washed with 500 µl cold PBS and stained with alexafluor 647 labelled anti-FLT3 mAb for 30 min on ice. Cells were then washed twice with 500 µl cold PBS, fixed with 200 µl 4% paraformaldehyde (PFA) and stored at 4°C until direct stochastic optical reconstruction microscopy (dSTORM) analysis[106].

2.11 Generation of iCasp 9 transduced cells

The vector encoding for iCasp9 and GFP was purchased from addgene (vector #15567)[107]. Jurkat T cells were transduced using a retrovirus, and GFP expression was confirmed by microscopy. Next, to facilitate enrichment of iCasp9 positive cells using magnet based selection, GFP was replaced with HER2t and cloned to lentiviral backbone. Lentiviruses were produced, and primary $CD4^+$ and $CD8^+$ T cells were transduced, and subsequently enriched using biotinylated anti-HER2 mAb trastuzumab and anti-biotin beads. Functional analyses were carried out using apoptosis assays.

To generate iCasp9+ FLT3 CAR-T cells, T cells were co-transduced with iCasp9 and FLT3 (4G8) CAR. Primary T cells were stimulated on day 0 and transduced on day 1 by iCasp9-encoding and on day 2 by FLT3 CAR-encoding lentiviruses. On day 9-10, HER2t positive cells were enriched using magnetic cell sorting and functional assays were carried out.

2.11.1 Apoptosis assays

Jurkat or primary T cells (50000 cells/well in 200 µl media) were plated in 96-well plates. Cells were either treated with increasing concentrations of the dimerizer drugs AP1903, AP20187 or rapamycin or left untreated. After 4-hour and 24-hour, the cells were washed and stained with biotinylated anti-HER2 (trastuzumab) followed by streptavidin-PE staining and flow cytometry analysis. 7-AAD was used to distinguish live/dead cells. Counting beads (20 or 30 µl per tube) were added before flow cytometry analysis and 5000 bead events were recorded on flow cytometer. The percentage of live cells was calculated using the following equation:

$$\text{Absolute count} = \frac{\text{cell count} \times \text{Bead concentration}}{\text{Bead count}}$$

2.12 Statistical analyses

Statistical analyses were conducted using Prism Software (GraphPad). Student's t-test was used as a two-sided paired or unpaired test with a confidence interval of 95% and results with a *P* value less than 0.05 were considered significant. Log-rank (Mantel-Cox) testing was performed to analyze differences in survival observed in *in vivo* experiments.

2.13 Non-biological material

2.13.1 Equipment and consumables

Equipment, consumables specification	Supplier
25, 75 cm^2 surface area cell culture flasks	Corning, Kaiserslautern, Germany
96-well half-area plates, Corning® Costar®	Corning, Kaiserslautern, Germany
96-well plate ,white, flat bottom, Corning® Costar®	Corning, Kaiserslautern, Germany
96, 48, 12, 24-well plates, Corning® Costar® U bottom	Corning, Kaiserslautern, Germany
SmartdishTM plate	Stem Cell Technologies, Cambridge, MA
Lab-Tek® Chamber Slides	Sigma Aldrich, Germany.
Centrifuge tubes- 1,2, 15, 50 mL	Geriener Bio-One, Frickenhausen, Germany
Centrifuge, Heraeus Megafuge 40R	Thermo Fisher, Darmstadt, Germany
Biological safety cabinets, Herasafe™ KS	Thermo Fisher, Waltham, MA, USA
CO_2 Incubators, Heracell™ 150i and 240i	Thermo Fisher, Darmstadt, Germany
Gel electrophoresis system, Owl™ Minigel	Thermo Fisher, Darmstadt, Germany
Electrophoresis power supply, Consort E802	Consort, Turnhout, Belgium
Gel imaging system, ChemiDoc™ MP	Bio-Rad, Munich, Germany
Flow cytometer, BD FACSCanto™ II	BD Biosciences, Heidelberg, Germany

Flow cytometry tubes, Röhre 5 mL	Sarstedt, Nümbrecht, Germany
Facs Aria Fusion Cell Sorter	BD Biosciences, Heidelberg, Germany
AutoMACS ProSeparator	Miltenyi, Bergisch Gladbach, Germany
MACS separation columns, 25 LS	Miltenyi, Bergisch Gladbach, Germany
Ice maker	Scotsman, Vernon Hills, IL, USA
Heat block, neoBlock1	neoLab, Heidelberg, Germany
Leucosep tubes	Geriener Bio-One, Frickenhausen, Germany
Multimode multiplate reader ,Infinite 200 PRO	TECAN- Männedorf, Switzerland
Microcentrifuge, Fresco 17	Thermo Fisher, Darmstadt, Germany
Microscope, Primo Vert	ZEISS; Jena, Germany
Shaker Incubator	INFORS DANN, Basel, Switzerland
Ultracentrifuge, Sorvall WX80	Thermo Fisher, Darmstadt, Germany
NanoDrop 2000	Thermo Fisher, Darmstadt, Germany
Refrigerator, -4 and -20 °C	Liebherr, Bulle, Switzerland
Trans-Blot Turbo Transfer Pack	Bio-Rad, Munich, Germany
Ultra-low temperature freezer, -80 °C FORMA 900	Thermo Fisher, Darmstadt, Germany
UV transilluminator	neoLab, Heidelberg, Germany
Vivaspin 6 MWCO 50.000	GE Healhcare, Uppsala, Sweden
Water bath	Memmert, Schwabach, Germany

2.13.2 Software

Software	Application	Company
GraphPad Prism 6	statistical analysis	La Jolla, CA, USA
Living Image 4.4.1	Mouse tumor burden analysis	Caliper Lifesciences, MA, USA
Snapgene 2.8.2	Molecular biology	GSL Biotech LLC, IL, USA
FlowJo X 10.0.7	FACS analysis	Tree Star Inc. Ashland, OR, USA
Microsoft office	Documents preparation	Microsoft, USA
Endnote X5	Bibilography	Thomson Reuters, UK

2.13.3 Chemicals and reagents

2.13.3.1 Molecular biology

Name	Manufacturer
1 Kb DANN Ladder	NEB, Frankfurt am Main, Germany
100 bp DANN Ladder	NEB, Frankfurt am Main, Germany
Ethanol absolute for molecular biology	AppliChem, Darmstadt, Germany
GelRed™ Nucleic Acid Gel Stain	Biotium, Fermont, CA, USA
LB Agar plates with 100 µg/mL Carbenicillin	TEKnova, Hollister, CA, USA
LB broth 1x	Thermo Fisher, Darmstadt, Germany
Restriction enzymes (i.e NheI, EcoRI etc.)	NEB, Frankfurt am Main, Germany
Water, molecular biology grade	AppliChem, Darmstadt, Germany
Crenolanib, quizartinib and midostaurin	SelleckChemicals, Houston, TX
Glycerol formal	Sigma-Aldrich, Steinheim, Germany
AP1903 and AP20187	Clontech (Takara), France

Rapamycine	Thermo Fisher (Invitrogen), Darmstadt, Germany
Sodium Hydroxide	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Sigma-Aldrich, Steinheim, Germany
Isopropyl alcohol	Sigma-Aldrich, Steinheim, Germany
Methanol	Sigma-Aldrich, Steinheim, Germany

2.13.3.2 Cell culture and immunology

Name	Manufacturer
Anti-biotin MicroBeads	Miltenyi, Bergisch Gladbach, Germany
Anti-PE MicroBeads	Miltenyi, Bergisch Gladbach, Germany
D-Luciferin firefly, Potassium Salt	Biosynth, Staad, Switzerland
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
Human Serum	Bayerisches Rotes Kreuz
Fetal calf serum (FCS)	Life Technologies, Darmstadt, Germany
Dulbecco's Phosphate-Buffered Saline (DPBS)	Life Technologies, Darmstadt, Germany
Dynabeads® Human T-Activator CD3/CD28	Life Technologies, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA) 0.5 M	Life Technologies, Darmstadt, Germany
GlutaMax-I 100X	Life Technologies, Darmstadt, Germany
2-Mercaptoethanol	Life Technologies, Darmstadt, Germany
HEPES 1M	Life Technologies, Darmstadt, Germany
Cell trace CFSE	Life Technologies, Darmstadt, Germany
efluoro670 dye	e-bioscience, San Diego, CA

Ionomycine	Sigma-Aldrich, Steinheim, Germany
PBS, pH 7.4, contains TWEEN® 20 (dry powder)	Sigma-Aldrich, Steinheim, Germany
PE Streptavidin 0.2 mg/mL	BioLegend, Fell, Germany
Penicillin/Streptomycinn 10,000 U/mL	Life Technologies, Darmstadt, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany
Polybrene (Millipore, 10 mg/mL)	Merck, Darmstadt, Germany
Recombinant human IL-2 (PROLEUKIN ® S)	Novartis, Basel, Switzerland
RPMI 1640 Medium, GlutaMAX™ Supplement	Life Technologies, Darmstadt, Germany
Trypan blue	Life Technologies, Darmstadt, Germany
123count beads	e-bioscience, San Diego, CA
Methocult opti H4034	Stem Cell Technologies, Cambridge, MA

2.13.4 Media and buffers

T cell medium (CTL)	
1640 RPMI, with 25 mM HEPES and Glutamax	500 mL
Human Serum (heat inactivated at 56 °C for 30 min)	10%
Penicillin/Streptomycinn 10,000 U/mL	100 U/mL
GlutaMax-I 100X	1%
2-Mercaptoethanol 50 mM	0.1%
Mix all components and filter sterilize (0.22 µm)	
Tumor cells medium (LCL)	
1640 RPMI, with 25 mM HEPES and Glutamax	500 mL

FCS	10%
Penicillin/Streptomycinn 10,000 U/mL	100 U/mL
GlutaMax-I 100X	1%

Mix all components and filter sterilize (0.22 µm)

Flow cytometry (FACS) buffer	
DPBS	500 mL
EDTA 0.5 M	0.4%
FSC	0.5%
Sodium azide ($NaN_{3)}$	0.1%
MACS buffer	
DPBS	500 mL
EDTA 0.5 M	0.4%
FSC	0.5%

Mix all components and filter sterilize (0.22 µm)

PBS/EDTA	
DPBS	500 mL
EDTA 0.5 M	0.4%
FSC	0.5%

2.13.5 Commercial kits

Name	Manufacturer
CalPhos Mammalian Transfection Kit	Clontech, Taraka

Pan T Cell Isolation Kit, human	Miltenyi Biotec
CD4+ T Cell Isolation Kit, human	Miltenyi Biotec
CD8+ T Cell Isolation Kit, human	Miltenyi Biotec
ELISA Max™ Set Deluxe (IL-2 and IFN-γ kits)	BioLegend
Endofree Plasmid MAXI and Mini Kit	QIAGEN, Macherey Nagel
PE Annexin V Apoptosis Detection Kit with 7-AAD	BD Biosciences

2.13.6 Flow cytometry antibodies

Ligand	Clone/Poly	Conjugation	Isotype	Manufacturer
FLT3 (CD135)	4G8	AlexaFluor 647	Mouse IgG1a, κ	BD Biosciences
CD123	REA918	vioblue	Mouse IgG2a, κ	Biolegend
CD69	FN50	FITC	Mouse IgG1, k	Biolegend
CD45	DL-101	APC/Cy7	Mouse IgG1, κ	Biolegend
CD34	561	vioblue	Mouse IgG2a, κ	Biolegend
CD38	HIT2	Brilliant Violet 421	Mouse IgG1, κ	Biolegend
CD33	HIM3-4	FITC	Mouse IgG1a; k	Biolegend
CD33	WM53	PE	Mouse IgG1, k	Biolegend
CD19	HIB19	PE	Mouse IgG1a; k	Biolegend
CD19	HIB19	PE/Cy7	Mouse IgG1a; k	Biolegend
CD14	M5E2	PE	Mouse IgG2a, k	Biolegend
CD8	BW135/80	VioBlue	Mouse IgG2a, k	Miltenyi

CD8	BW135/80	FITC	Mouse IgG2a, k	Miltenyi
CD4	M-T466	VioBlue	Mouse IgG1, k	Miltenyi
CD4	M-T466	APC	Mouse IgG1, k	Miltenyi
CD3	BW264/56	vioblue	Mouse IgG2a, k	Miltenyi
CD3	BW264/56	APC	Mouse IgG2a, k	Miltenyi
EGFR (Cetuximab)	C225	Biotinylated	Human IgG1, k	ImClone LLC
EGFR (Cetuximab)	C225	AlexaFluor 647	Human IgG1, k	ImClone LLC
Her2/new	4D5-8	Biotinylated	humanized	Roche

3. Results

3.1 Aim-1: Functional analysis of FLT3 CAR-T cells

3.1.1 FLT3 CAR design and expression in T lymphocytes

To investigate the recognition of AML cells by CAR-modified T cells, we designed CARs that target different extracellular epitopes of FLT3 protein (Figure 3.1a). Healthy donor-derived T cells were genetically modified using lentiviral transduction (MOI = 3-5, n>5 healthy donors) to express FLT3 CAR with targeting domains derived from BV10 or 4G8 mAb.

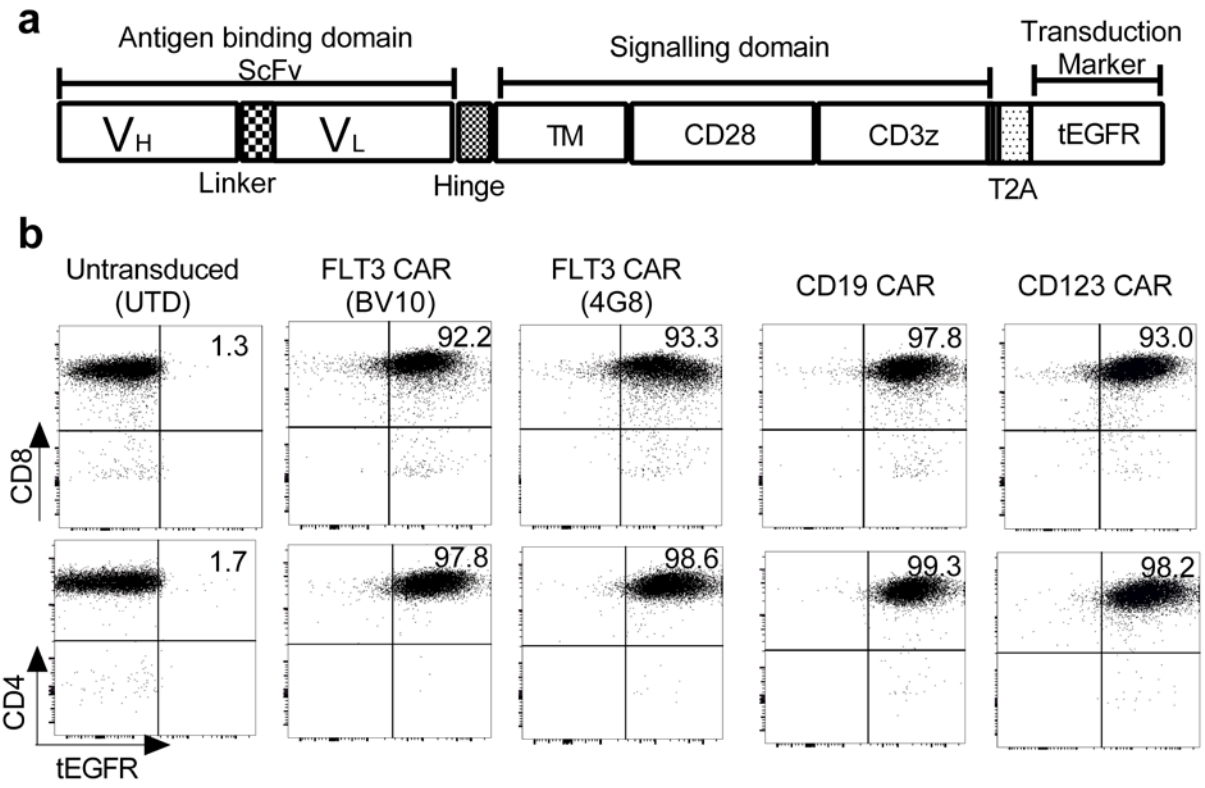


Figure 3.1: CAR constructs and phenotype of CAR-T cells. (a) Design of CARs used in the study. Single chain variable fragments (scFv) were derived from mAbs BV10 or 4G8 (FLT3-CAR), FMC63 (CD19-CAR), and 32716 (CD123-CAR). (b) Dot plots show the expression of the EGFRt transduction marker after enrichment and expansion of $EGFRt^+$ $CD8^+$ and $CD4^+$ T cells, prior to functional testing. Untransduced T cells (UTD) are included as a negative control.

CD19 and CD123 CARs were used as negative and positive controls in the study, respectively. FLT3 CAR, CD19 CAR and CD123 CAR-T cells were generated from the same respective healthy donor T cells for the *in vitro* and *in vivo* studies.

CAR-positive T cells were enriched by magnetic bead selection using an anti-EGFR mAB (cetuximab) at day 9-10 after transduction. More than 90 % CD4+ and CD8+ T cells were CAR+ after the enrichment step (Figure 3.1b). Initially, both FLT3 CARs were designed with either a short (IgG4 hinge, 12 amino acid) or a long (IgG4 CH2-CH3-hinge, 229 amino acids) spacer domain. CAR-T cell functional analysis revealed that the CAR constructs with short spacer showed significantly higher cytolytic activity and cytokine production (data not shown) and therefore we used BV10 and 4G8 FLT3 CAR-T cells with short spacer domains for all further studies.

3.1.2 FLT3 CAR-T cells specifically recognize cell surface FLT3 protein

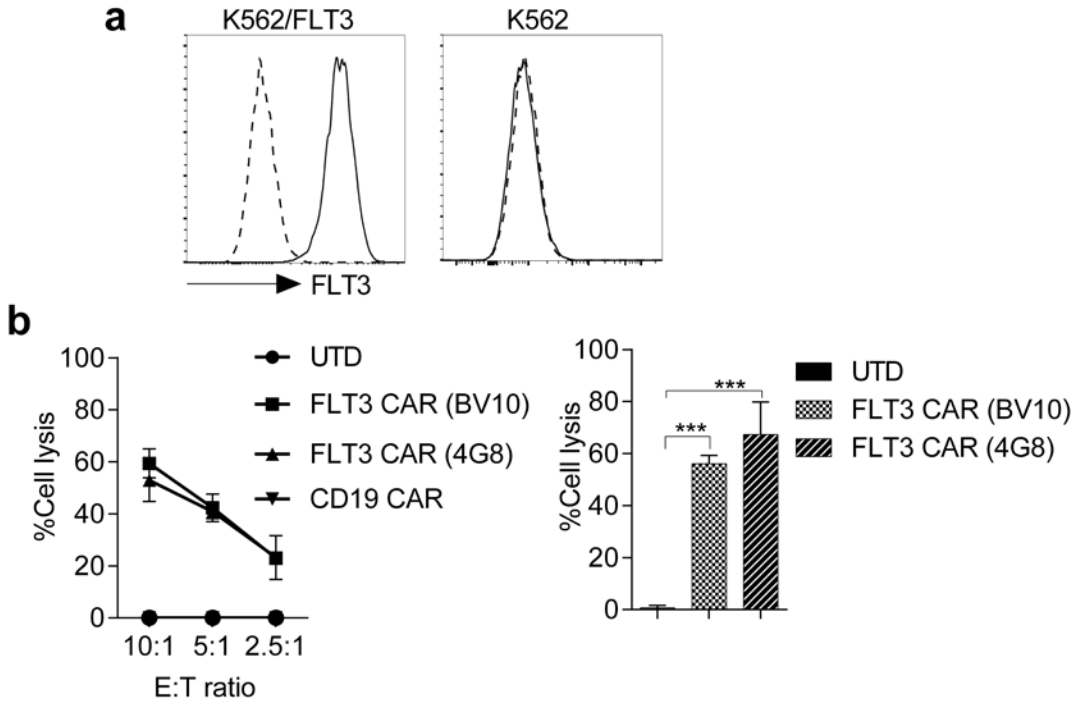


Figure 3.2: Specific-recognition of FLT3 on K562/FLT3 cells by FLT3 CAR-T cells. (a) Flow cytometric analysis of FLT3-expression by FLT3 transduced (and enriched) K562/FLT3 cells and native K562 cells. Staining with anti-FLT3 mAb is represented by solid line and isotype staining by dotted line. (b) Diagram on the left represents the antigen-specific cytolytic activity of CD8+

FLT3 CAR-T cells, analyzed after 4-hour in a bioluminescence-based cytotoxicity assay. The experiment was performed in triplicate wells and values are presented as mean + standard deviation (s.d.). Diagram on the right shows summary data on cytolytic activity of FLT3 CAR-T cells (10:1 E:T ratio) prepared from n=3 different donors. ***p<0.0005 (student's t test).

To assess the specificity of FLT3 CAR-T cells against FLT3 expressing target cells, we genetically engineered K562 (chronic myelogenous leukemia) cells to overexpress wild type (wt) FLT3 protein. A retroviral vector encoding the wt FLT3 sequence was generated by TOPO TA cloning and retroviruses were produced by transfection of platinum A cells with retroviral vector. K562 cells were transduced and FLT3-expression was confirmed using flow cytometry. FLT3-positive cells were further enriched using flow cytometry based cell sorting (Figure 3.2a).

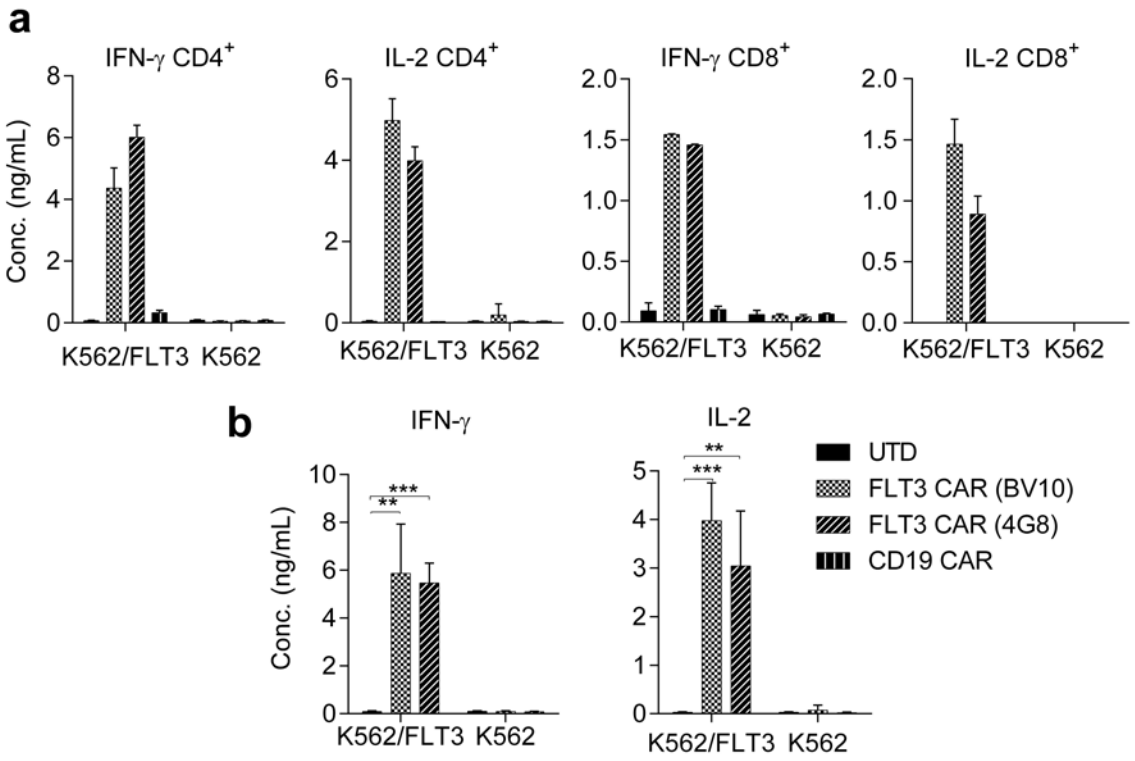


Figure 3.3: IFN-γ and IL-2 production by FLT3 CAR-T cells against K562/FLT3 cells. (a) Cytokine production (IFN-γ and IL-2) by CD4+ and CD8+ FLT3 CAR-T cells. Supernatant obtained after 24-hour co-cultures (2:1 E:T ratio) was analyzed by ELISA. T cells (50,000/well) and target cells (25,000/well) were seeded in triplicate wells. Values are presented as mean ± s.d. (b) Diagrams show IFN-γ and IL-2 production by CD4+ FLT3 CAR-T cells obtained from n=3 different donors. **p<0.005, ***p<0.0005 (student's t test).

Next, we performed detailed *in vitro* T cell functional analysis to assess the specificity of FLT3 CAR-T cells. We observed potent cytolytic activity of both BV10 and 4G8 FLT3 CAR-T cells after 4 hour co-incubation with K562/FLT3 cells (Figure 3.2b). We further confirmed antigen-specific recognition by FLT3 CAR-T cells that were derived from n=3 different healthy donors against K562/FLT3 cells (Figure 3.2b, right graph).

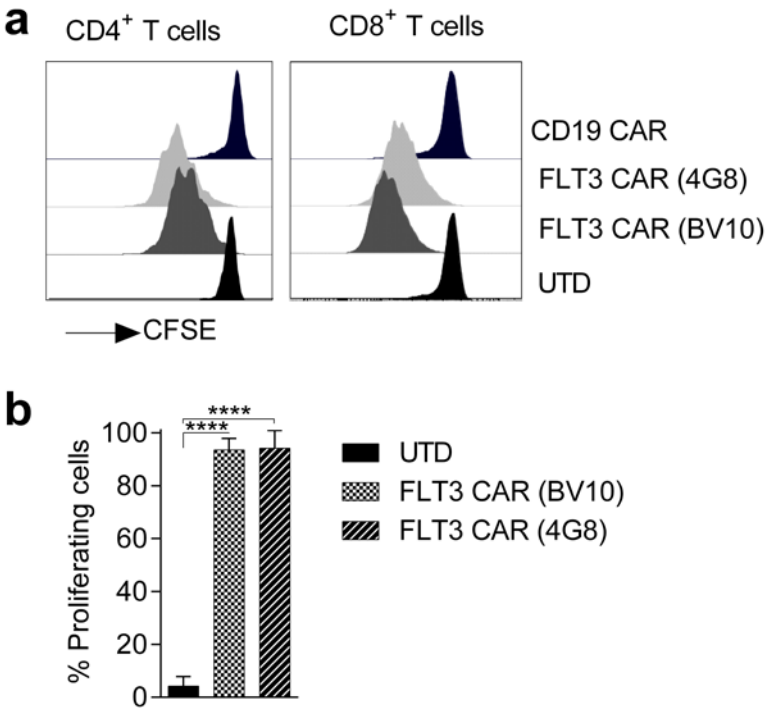


Figure 3.4: Antigen specific proliferation by FLT3 CAR-T cells against K562/FLT3 cells. (a) FLT3 CAR-T cells proliferation was examined by CFSE dye dilution after 72 hour of co-culture with K562/FLT3 target cells. Assay was performed in triplicate wells with 2:1 E:T ratio. Histograms show proliferation of live (7-AAD-) CD4+ or CD8+ T cells. No exogenous cytokines were added to the assay medium. (b) Graph shows the percentage of proliferating cells (normalized to untransduced T cells) in response to stimulation with K562/FLT3. T cells were prepared from n=3 different donors. **** p<.0001 (Student's t test).

Both CD4+ and CD8+ FLT3 CAR-T cells produced high levels of interferon-gamma (IFN-γ) and interleukin-2 (IL-2) after co-culture with FLT3 positive K562 cells (Figure 3.3a, b). Further, FLT3 CAR-T cells showed antigen specific productive proliferation against K562/FLT3 cells (Figure 3.4a, b). We did not observe significant differences in CAR-T cell mediated functions in these experiments although the different FLT3 CARs targeted

different epitopes of FLT3 protein. Of note, we observed slightly higher IL-2 production by BV10 FLT3 CAR-T cells compare to 4G8 FLT3 CAR-T cells. We also did not observe any T cell effector functions by CD19 CAR-T cells against K562/FLT3 (FLT3+CD19-) cells.

In summary, the data shows that CAR-T cells targeting FLT3 show specific recognition of FLT3 protein and exert antigen-dependent effector functions. Furthermore, recognition of FLT3 protein was similar by both the FLT3 CAR-T cell products, independent of targeting domains (BV10 or 4G8) used in a CAR.

3.1.3 FLT3 CAR-T cells exert potent anti-leukemia activity against AML

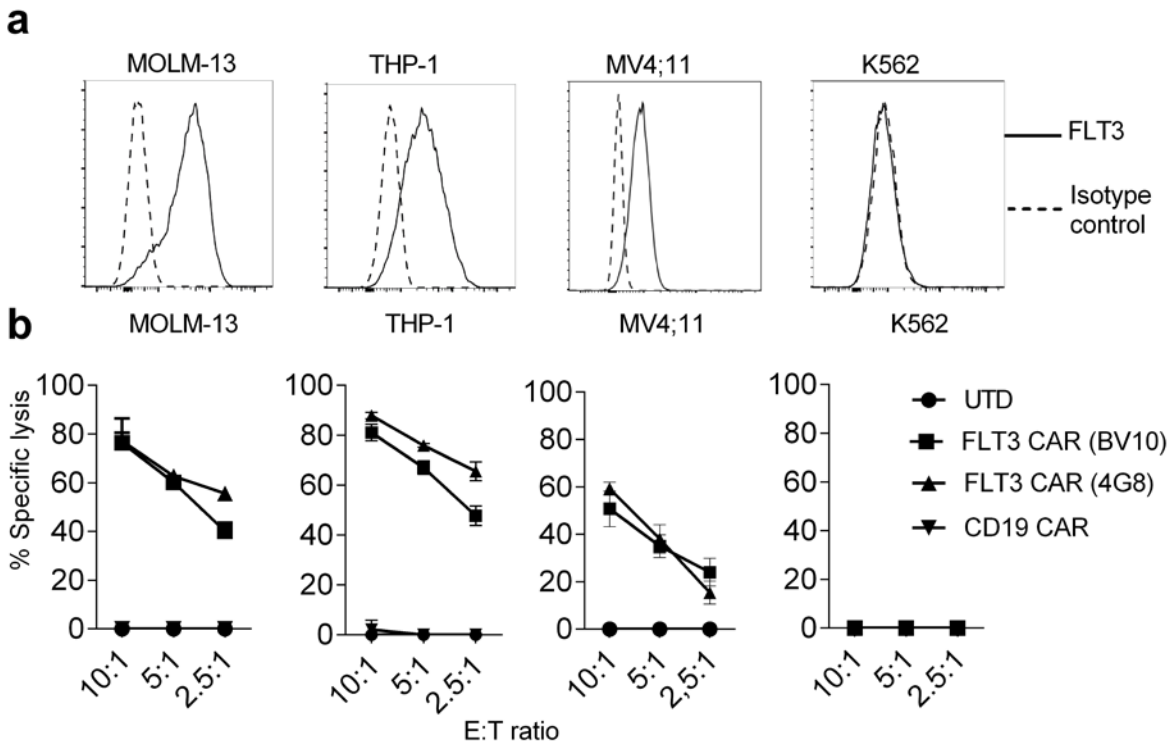


Figure 3.5: Recognition and specific reactivity of FLT3 CAR-T cells against AML cell lines. (a) Flow cytometric analysis of FLT3-expression by AML cell lines (MOLM-13, THP-1, MV4;11). Solid and dotted line represents staining with anti-FLT3 mAb and isotype control, respectively. (b) Specific cytolytic activity in 4 h co-culture assay with AML cell lines as target cells, evaluated by bioluminescence measurement. Values represent mean + s.d. Representative data of T cells prepared from n=3 different donors.

To evaluate the reactivity of FLT3 CAR-T cells against AML cells, we included the AML cell lines THP-1 (wt FLT3), MOLM-13 (heterozygeously FLT3-ITD+) and MV4;11 (loss of heterozygosity FLT3-ITD+) into our analyses[108]. First, we confirmed FLT3-expression by all three AML cell lines using flow cytometry (Figure 3.5a). Next, we carried out functional analyses and observed specific high-level cytolytic activity of CD8+ FLT3 CAR-T cells at multiple effector to target cell ratios (range: 10:1 – 2.5:1) against all three AML cell lines (Figure 3.5b). We observed higher cytolytic activity against THP-1 and MOLM-13 cells compared to MV4;11 due to comparatively low FLT3 protein expression by MV4;11 cells while FLT3 negative K562 cells were not lysed (Figure 3.5b).

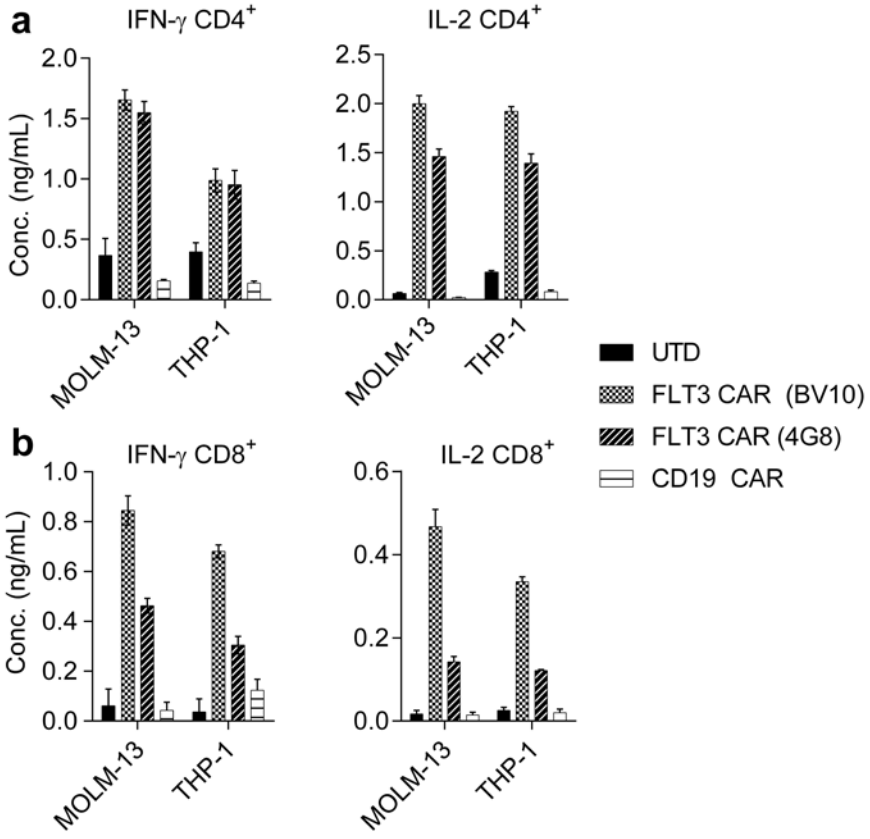


Figure 3.6: Cytokine production by FLT3 CAR-T cells against AML cell lines. (a) Cytokine (IFN-γ and IL-2) production by CD4+ and CD8+ T cells after 24 hour co-incubation with MOLM-13 and THP-1 target cells. ELISA of supernatant was performed in triplicates and values represents mean ± s.d. Representative data of T cells prepared from n=3 different donors.

Further, we continued *in vitro* functional analyses of FLT3 CAR-T cells against MOLM-13 (FLT3-ITD+) and THP-1 (wt FLT3) target cells. We observed high levels of IFN-γ and IL-2 production by CD4+ and CD8+ FLT3 CAR-T cells against both target cell lines (Figure 3.6a, b). FLT3 CAR-T cells underwent productive proliferation after stimulation with both AML cell lines (Figure 3.7a, b), whereas untransduced T cells or CD19 CAR-T cells derived from the same donor only showed background reactivity in both assays (Figure 3.6, 3.7).

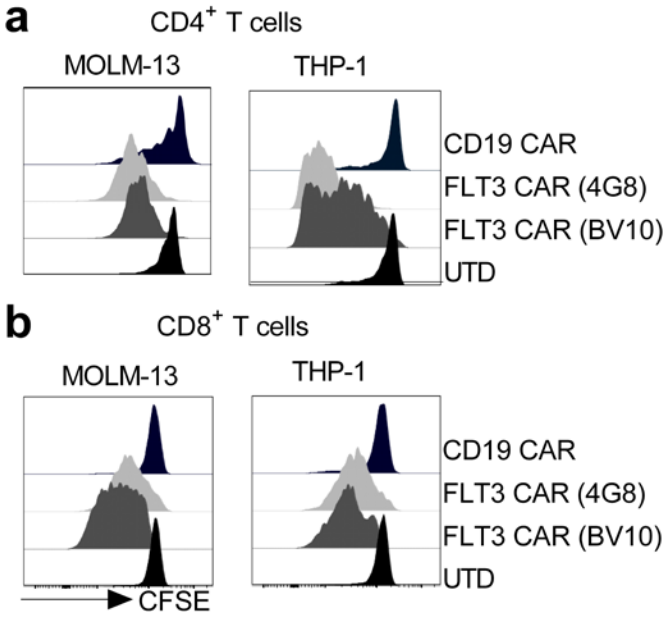


Figure 3.7: Proliferation of CD4+ and CD8+ FLT3 CAR-T cells against AML cell lines. Proliferation of (a) CD4+ and (b) CD8+ T cells examined by CFSE dye dilution within 72 hour after stimulation with MOLM-13 and THP-1 target cells. For analysis, triplicate wells were pooled and live (7-AAD) T cells proliferation was analyzed. No exogenous cytokines were added to the assay medium. Representative data of T cells prepared from n=3 different donors.

In summary, the data shows that FLT3 CAR-T cells mediate potent anti-leukemia activity against AML cells *in vitro*. The recognition of AML cell lines by FLT3 CAR-T cells is independent from the mutation status of the intracellular tyrosine kinase domain, but depends on the antigen density of FLT3 surface protein on AML cells indicated by the mean fluorescence intensity (MFI) in flow cytometry analysis (MOLM-13 = THP-1 > MV4;11) (Figure 3.5a, b).

3.1.4 FLT3 CAR-T cells eradicate primary AML *in vitro*

Next, we sought to assess the recognition of primary AML blasts by FLT3 CAR-T cells. We confirmed expression of FLT3 on patient-derived AML blasts (Pt#1: FLT3-ITD+ and Pt#2: unknown, CD19+) using flow cytometric analysis (Figure 3.8a). In subsequent assays, we observed potent anti-leukemic activity of FLT3 CAR-T cells against patient-derived AML blasts, with strong cytolytic activity leading to eradication of >75% of AML blasts within 4 hours at an effector to target cell ratio of 10:1 (Figure 3.8b).

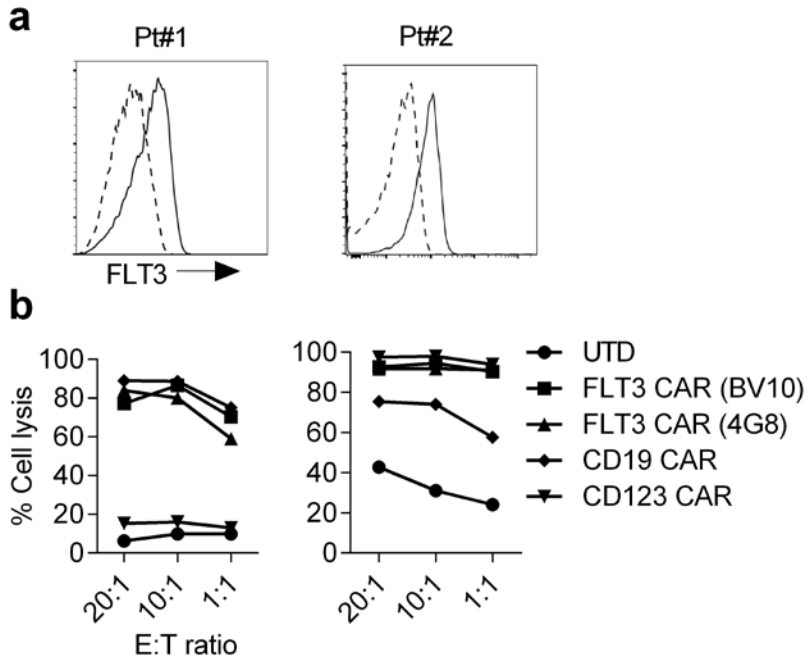


Figure 3.8: Elimination of primary AML blasts by healthy donor derived FLT3 CAR-T cells. (a) Flow cytometric analysis of FLT3-expression by AML blasts (Mutation status- Pt#1: FLT3-ITD+, Pt#2: unknown, but CD19+). Staining with anti-FLT3 mAb is represented by solid line and isotype staining by dotted line. (b) Cytolytic activity in 4 hour co-culture assay with primary AML blasts, evaluated by flow cytometry based assay. Counting beads were used to quantify residual live cells.

Further, healthy donor derived FLT3 CAR-T cells showed a significant amount of IFN-γ production and proliferation after stimulation with allogenic AML blasts (Figure 3.9a, b). We observed cytolytic activity, cytokine production and proliferation by CD19 CAR-T cells against patient #2 blasts due to presence of CD19 protein on the blasts (Figure

3.8b, 3.9a, b). We also included T cells expressing a CAR targeting CD123[50] (an alternative CAR target in AML) in the assays and observed that FLT3 CAR-T cells were as effective as CD123 CAR-T cells.

In previous experiments, we used healthy donor-derived T cells and prepared FLT3 CAR-T cells. However, for clinical application in an autologous setting, FLT3 CAR-T cells should be prepared from patient-derived T cells. Thus, to mimic an AML treatment setup using FLT3 CAR-T cells, we generated autologous FLT3 CAR-T cells (Pt#3). Here, we lentivirally transduced CD3+ bulk T cells (due to lack of enough starting material for CD4+ and CD8+ cells isolation), enriched for EGFRt+ T cells and separated CD4+ and CD8+ T cells for functional analysis. We used AML blasts from the same patient (Pt#3, Figure 3.10a) for evaluating patient-derived FLT3 CAR-T cell functions.

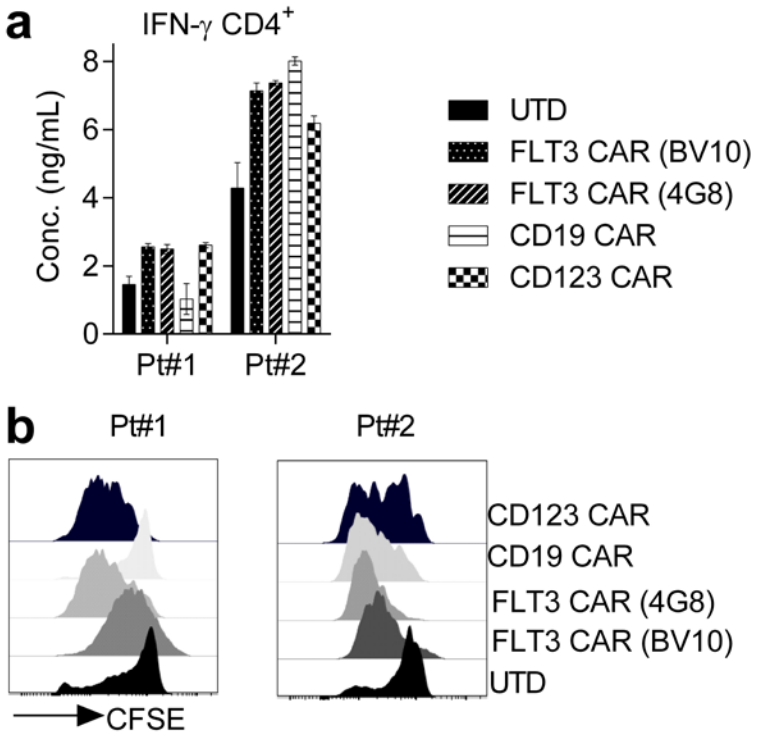


Figure 3.9: IFN-γ production and proliferation mediated by healthy donor-derived FLT3 CAR-T cells against primary AML. (a) IFN-γ production by CD4+ T cells after 24 hour co-incubation with primary AML blasts. ELISA of supernatants after co-incubation was performed in triplicate wells and values represents mean ± s.d. (b) Proliferation of CD4+ T cells examined by CFSE dye dilution within 72 hour after stimulation with primary AML blasts.

Similar to allogeneic FLT3 CAR-T cells, autologous FLT3 CAR-T cells showed potent cytolytic activity, eliminating >70% AML blasts within 4-hour co-incubation at an E:T ratio (1:1- 20:1) (Figure 3.10b). Further, autologous FLT3 CAR-T cells produced IFN-γ and proliferated after co-incubation with AML blasts but showed lower background activity in comparison to allogeneic FLT3 CAR-T cells (Figure 3.10c, d).

In summary, FLT3 CAR-T cells derived from healthy donors and AML patients were similarly effective in eliminating AML cell lines and primary AML cells. Furthermore, the anti-leukemia activity of FLT3 CAR-T cells was equivalent to T cells expressing an analogously designed CAR specific for the alternative AML target antigen CD123.

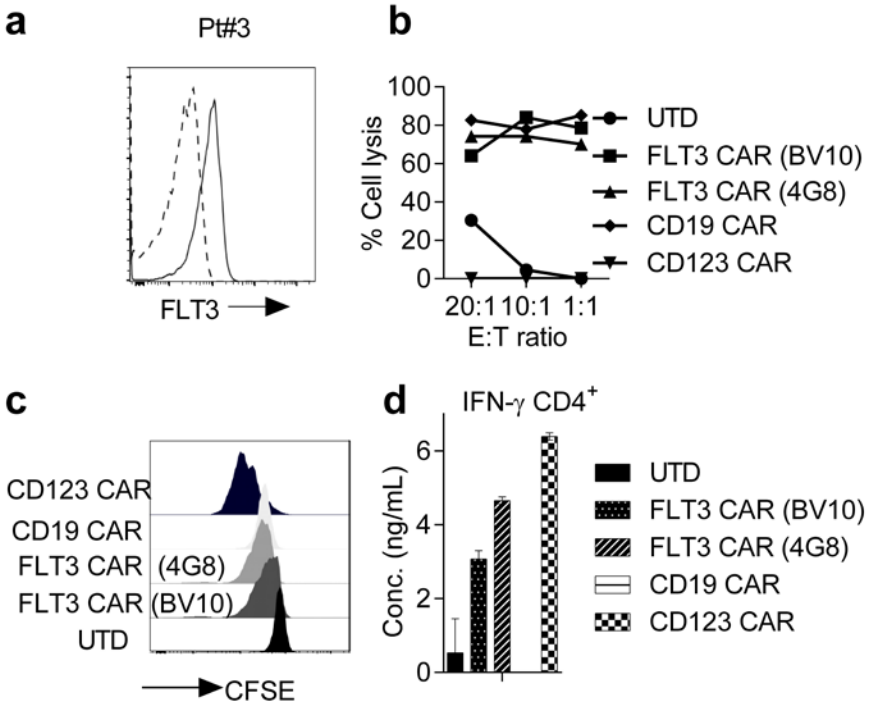


Figure 3.10: Elimination of primary AML blasts by autologous FLT3 CAR-T cells *in vitro*. (a) Flow cytometric analysis of FLT3-expression by AML blasts (Mutation status- Pt#3: FLT3-ITD+). Staining with anti-FLT3 mAb is represented by a solid line and isotype staining by a dotted line. (b) Cytolytic activity of FLT3 CAR-T cells in 4 hour co-culture assay with primary AML blasts (pt#3), evaluated by a flow cytometry-based assay. (c) Proliferation of CD4+ T cells within 72 hour after stimulation with primary AML blasts. (c) IFN-γ production by CD4+ T cells after 24 hour co-incubation with autologous primary AML blasts. T cells and target cells were seeded in triplicates and an ELISA of supernatants was performed after 24-hour co-incubation. Values represent mean ± s.d.

3.1.5 FLT3 CAR-T cells eradicate advance leukemia in murine models *in vivo*

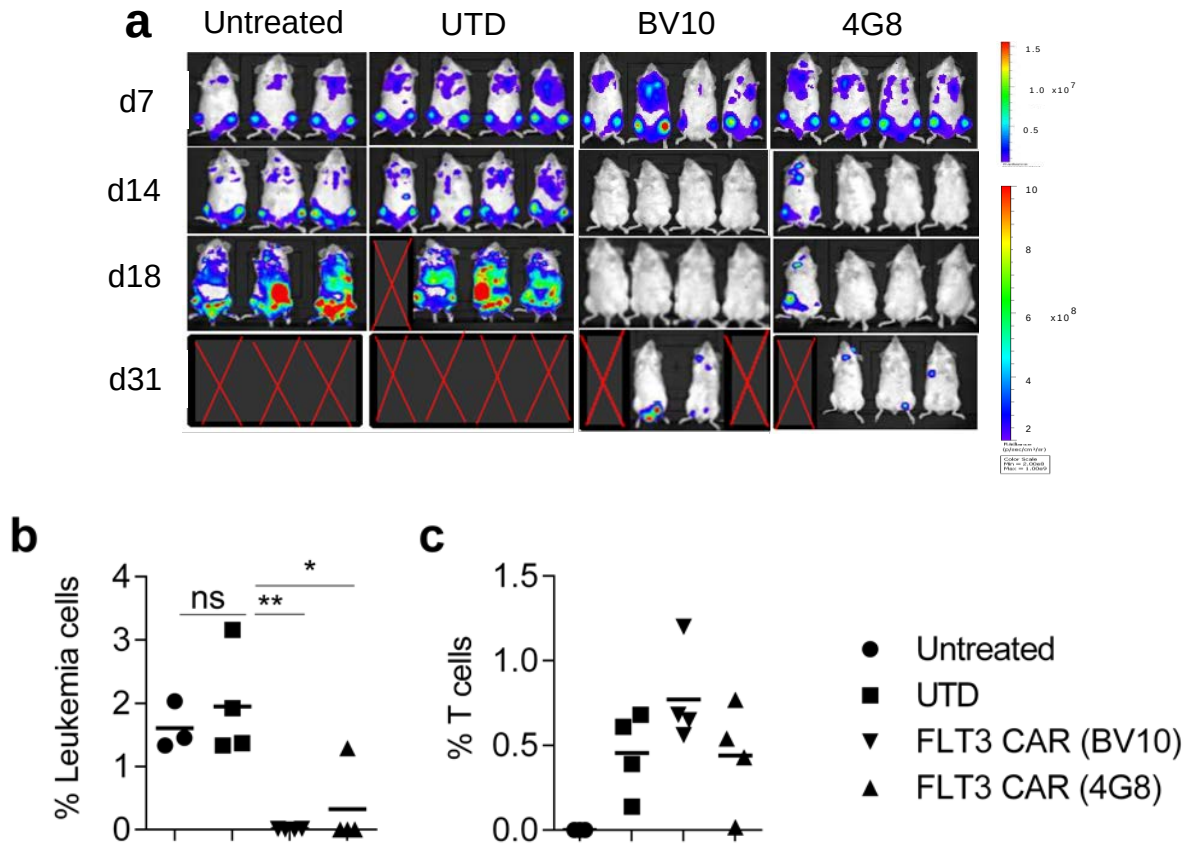


Figure 3.11: Anti-leukemia activity of FLT3 CAR-T cells and CAR-T cell expansion in NSG mice. 1x10^6 ffluc$^+$GFP$^+$ MOLM-13 AML cells were injected to immunodeficient NSG mice on day 0, followed by treatment with a single dose of 5x10^6 CAR-modified or untransduced (UTD) T cells (CD4$^+$:CD8$^+$ ratio = 1:1) on day 7 or were left untreated. (a) Leukemia progression or regression was assessed by serial bioluminesence (BL) imaging. Note the scale indicating upper and lower BL thresholds at each analysis time point (right). (b-c) Flow cytometric anaysis of peripheral blood on day 10 after leukemia inoculation. Shown is the frequency of leukemia cells (GFP$^+$/FLT3$^+$) and transferred T cells (CD45$^+$/CD3$^+$) as percentage of live (7-AAD$^-$) cells. *p<0.05, **p<0.005 (student's t test).

To analyze the anti-leukemia efficacy of FLT3 CAR-T cells *in vivo,* we used immunodeficient NSG mice. We injected the mice with MOLM-13 AML cells (ffLuc+_GFP+) that aggressively expanded to systemic leukemia in the peripheral blood, and showed heavy infiltration of bone marrow and spleen (Figure 3.11a, b). Mice bearing leukemia remained untreated or were treated with a single dose of 5x10^6 FLT3

CAR-modified or untransduced T cells on day 7 (at an equal proportion of $CD4^+$ T cells and $CD8^+$ T cells) (Figure 3.12).

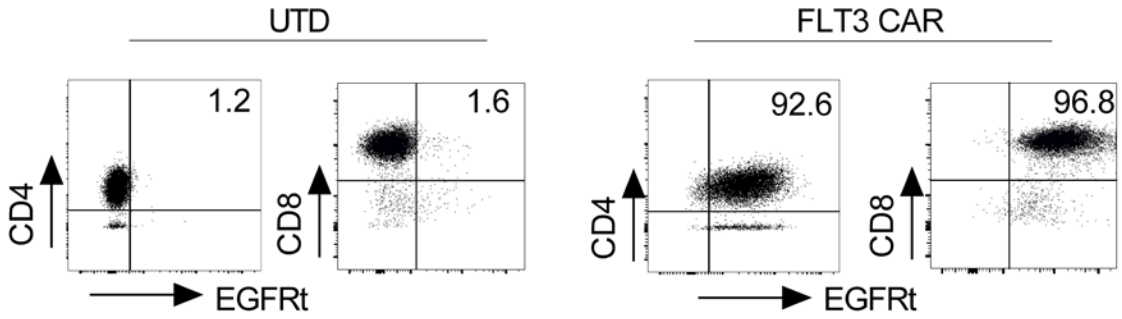


Figure 3.12: Phenotype of T cells injected to leukemia bearing mice. Flow cytometric analysis of CD4, CD8 and EGFRt (CAR) expression by T cells before injection.

We observed a strong anti-leukemia effect by BV10 and 4G8 FLT3 CAR-T cells in all mice where FLT3 CAR-T cells engrafted (Figure 3.11a). In these mice, we observed that FLT3 CAR-T cells could be readily detected in peripheral blood, increased in number during the anti-leukemia response, and were present in bone marrow and spleen at the end of the experiment, confirming persistence for more than 3 weeks after adoptive transfer (Figure 3.13).

We further observed complete elimination of leukemia cells from peripheral blood within 7 days after FLT3 CAR-T cell treatment (Figure 3.11b), and bioluminescence imaging confirmed reduction of leukemia burden and strong systemic anti-leukemia activity (Figure 3.11a, 3.14a). Flow cytometric analyses revealed sustained complete remission of AML from bone marrow, spleen and peripheral blood after treatment with FLT3 CAR-T cells, which translated into superior overall survival compared with mice that received untransduced T cells or no treatment ($p<.05$) (Figure 3.13b, 3.14, 3.15). We observed T cell engraftment failure in one mouse treated with 4G8 FLT3 CAR-T cells in this particular experiment, which in turn resulted in leukemia progression in that mouse (Figure 11a, b, 13b, 14a, 15b).

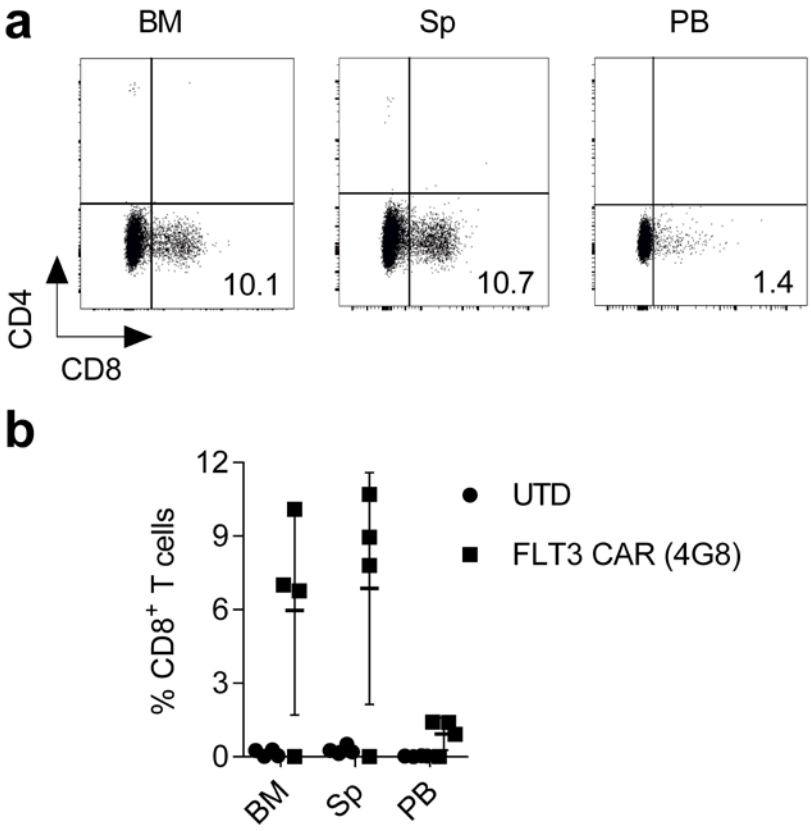


Figure 3.13: Analysis of FLT3 CAR-T cells expansion *in vivo*. (a) Representative dot plots from one mouse treated with FLT3 CAR-T cells illustrating the frequency of T cells in bone marrow (BM), spleen (Sp) and peripheral blood (PB) at the end of experiment. (b) Frequency of $CD8^+$ T cells in mice that were treated with FLT3 CAR-T cells or untransduced T cells at termination of the experiment. Values are presented as mean ± s.d. Data shown are representative for independent experiments with FLT3 CAR-T cell from n=3 donors.

This resulted into higher overall response rate by BV10 CAR-T cells compared to 4G8 FLT3 CAR-T cells [Response rate: BV10 FLT3 CAR-T cells: 100% (n=4/4), vs. 4G8 FLT3 CAR-T cells: 75% (n=3/4), vs. untransduced T cells: 0% (n=0/4), vs. no treatment: 0% (n=0/3)] (Figure 3.14a). However, there was no significant difference in overall survival after BV10 or 4G8 FLT3 CAR-T cell treatment (Figure 3.14b).

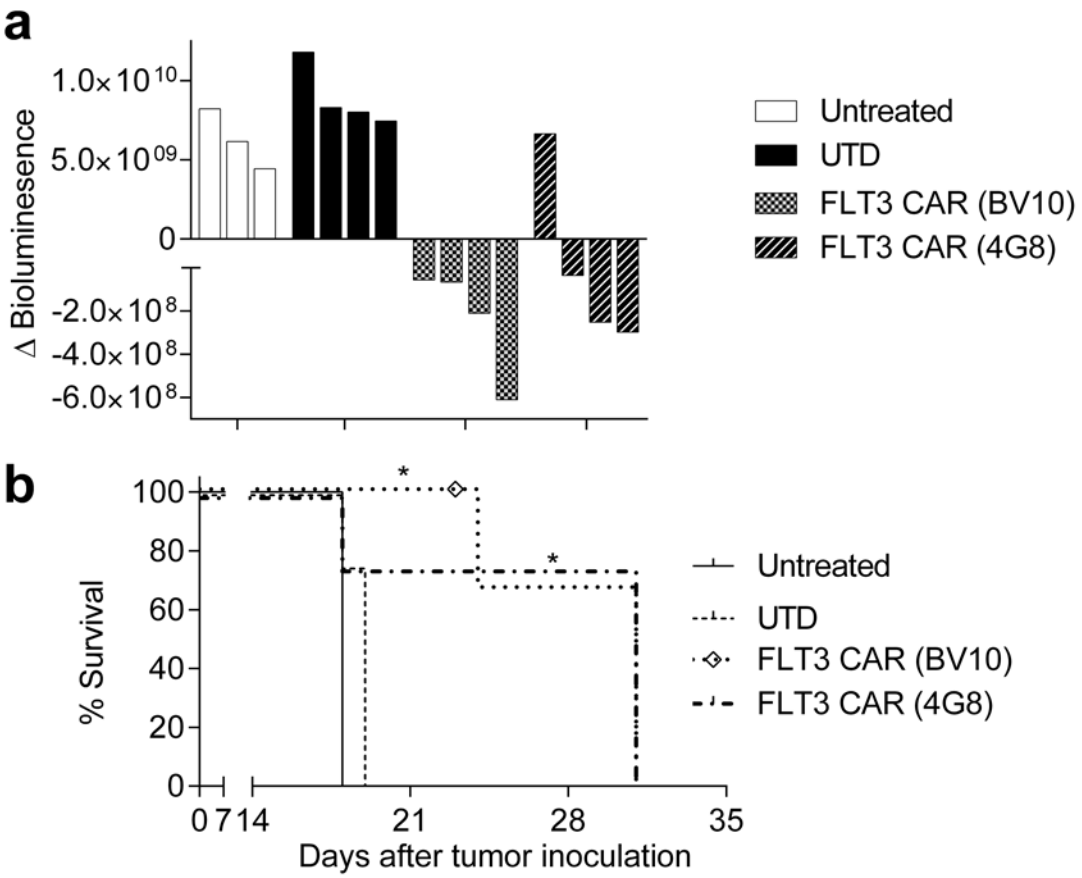


Figure 3.14: Analysis of anti-leukemia efficacy and survival of mice after FLT3 CAR-T cell treatment. (a) Waterfall plot showing the difference (increase/decrease) in absolute bioluminesence values obtained from individual mice between day 7 and day 14 after tumor inoculation. Values were calculated by substracting day 7 BL from day 14 BL. BL values were obtained as photon/sec/cm^2/sr in regions of interest encompassing the entire body of each mouse. (b) Kaplan-Meier analysis of survival. According to protocol, experiment endpoints were defined by relative (%) loss of body weight and total BL values. Diamond denotes experiment independent (not due to tumor or T cells) death of mice. *p<.05 (Log-rank test).

In all mice that responded to FLT3 CAR-T cell therapy, we observed leukemia recurrence in anatomical sanctuary sites (e.g. peritoneum, subcutaneous tissue etc.) at later time points (Figure 3.11a). Similar observations have been reported by others in similar murine models previously[50,109]. Importantly, FLT3 was expressed at similar levels on native and recurring MOLM-13 cells suggesting antigen loss had not occurred (Figure 3.16a, b).

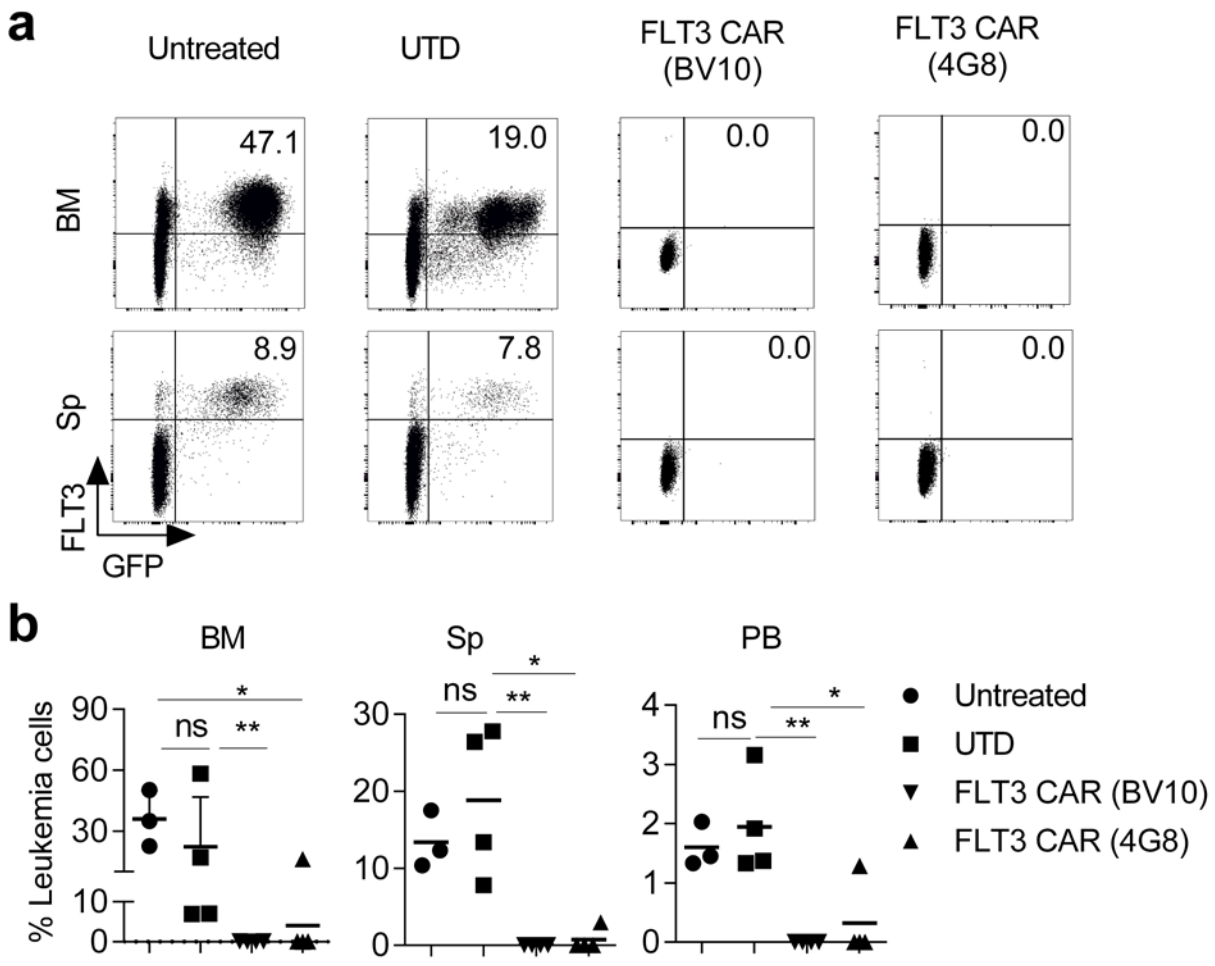


Figure 3.15: Flow cytometric analysis of leukemia elimination from NSG mice. (a) Flow cytometry dot plots show the frequency of MOLM-13 leukemia cells ($GFP^+/FLT3^+$) as percentage of live ($7\text{-}AAD^-$) cells in bone marrow (BM) and spleen (Sp) of one representative mouse per treatment group. (b) Flow cytometric anaysis of BM, Sp and peripheral blood (PB) at the experiment endpoint in each mouse. Diagrams show the frequency of leukemia cells ($GFP^+/FLT3^+$) as percentage of live ($7\text{-}AAD^-$) cells. *p<.05 **p<.005* (Student's t-test). Data shown are representative for results obtained in independent experiments with FLT3 CAR-T cell from n=3 donors.

We analyzed tissue samples obtained from these sanctuary sites for the presence of FLT3 CAR-T cells and could not detect infiltrating FLT3 CAR-T cells, even though they were present in peripheral blood and bone marrow (Figure 3.13a-b, 3.16). However, taken together, a single dose of FLT3 CAR-T cells lead to durable remission of AML in MOLM-13/NSG xenograft model.

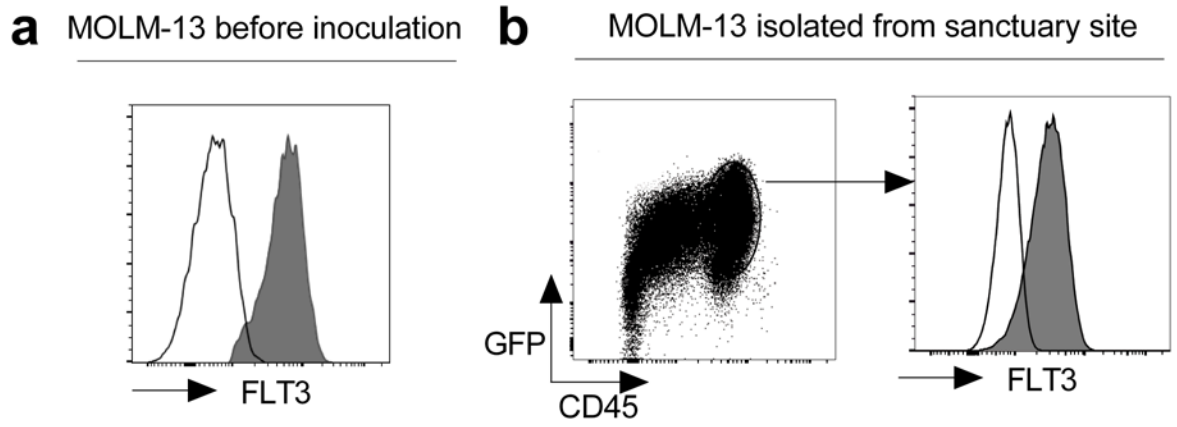


Figure 3.16: Flow cytometric analysis of FLT3-expression on recurring MOLM-13 cells in the NSG mice. FLT3-expression analysis on MOLM-13 cells using flow cytometry (a) before inoculation and (b) MOLM-13 cells isolated from a sanctuary site. Histograms show staining with anti-FLT3 mAb (4G8) (filled) and isotype control antibody (open). Tumor tissue samples from at least one mouse from each treatment group were analyzed. Flow cytometry plots shown here are representative for the FLT3 CAR-T cell treatment group.

Next, we sought to examine the anti-leukemia efficacy of FLT3 CAR-T cells against primary AML blasts *in vivo*. Therefore, we inoculated immunodeficient NSG mice with FLT3-ITD+ primary AML blasts.

We analyzed leukemia engraftment in a subgroup of mice on day 21 (after inoculation) and confirmed the development of leukemia within a 3-week engraftment period (Figure 3.17a) which is consistent with prior work[110]. Mice then received a single dose of $5x10^6$ FLT3 CAR-T cells (equal proportion of CD4+ and CD8+ T cells) on day 28. We observed that FLT3 CAR-T cells engrafted in all mice, could be detected at multiple time points after adoptive transfer and increased in number during the anti-leukemia response (Figure 3.17b). Further, FLT3 CAR-T cells induced complete remission of AML in bone marrow and spleen in all treated mice (Figure 3.18a). We included a group of mice that received CD123 CAR-T cells treatment and observed that FLT3 CAR-T cells showed similar efficacy to CD123 CAR-T cells (Figure 3.18a).

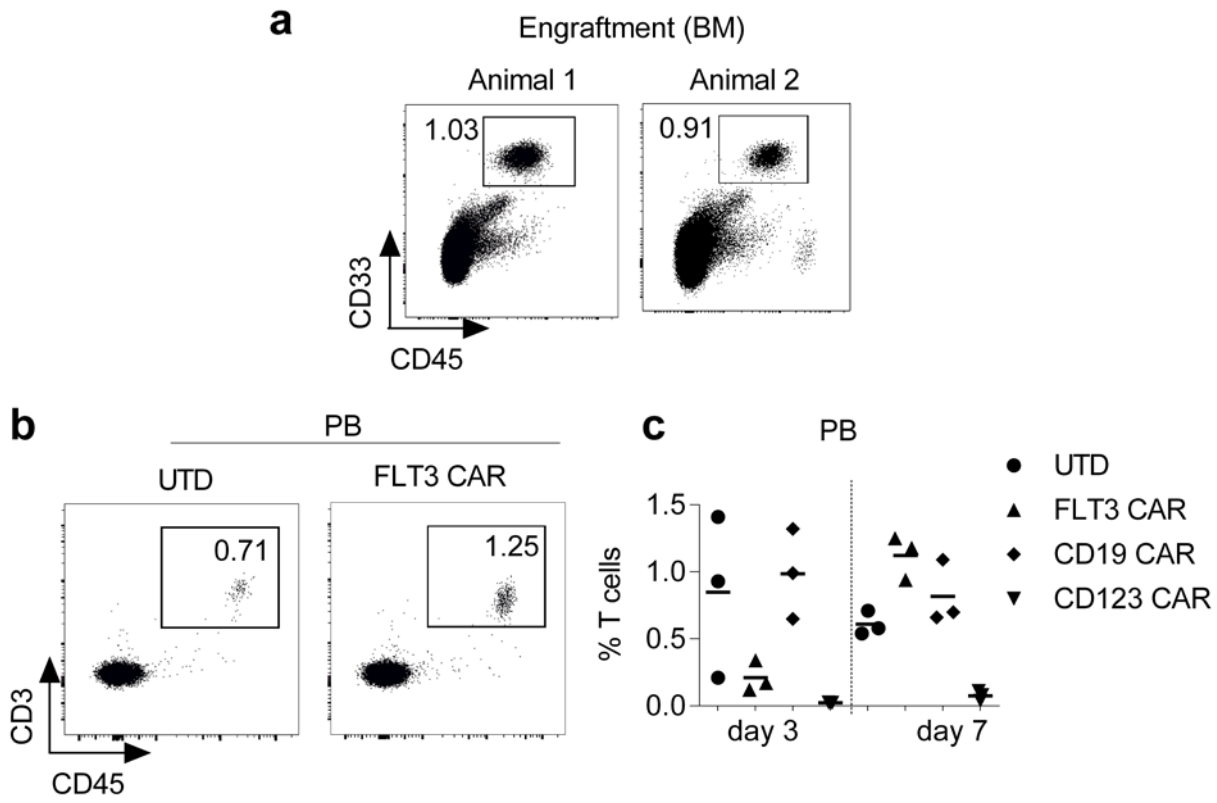


Figure 3.17: FLT3-ITD+ AML blasts engraftment in NSG mice and CAR-T cell expansion. (a) 6-8 week old female NSG mice were injected with FLT3-ITD$^+$ primary AML blasts (2.5x10^6 blasts per mice). Three weeks later, a subgroup of mice (n=2) was analyzed for AML engraftment in bone marrow by flow cytometry. Dot plots are gated on live (7AAD$^-$) cells. (b) At 4 weeks following inoculation with AML blasts, mice received 5x10^6 FLT3 CAR-modified or untransduced T cells (CD4$^+$:CD8$^+$ ratio = 1:1). (c) Frequency of T cells (CD45$^+$/CD3$^+$) as percentage of live (7-AAD$^-$) cells, 3 and 7 days after T cell transfer.

However, FLT3 CAR-T cells showed higher persistence in bone marrow and spleen compared to CD123 CAR-T cells (Figure 3.18b). We did not observe recurrence of AML blasts after FLT3 or CD123 CAR-T cell treatment in this model.

In summary, the data show that FLT3 CAR-T cells cleared leukemia from bone marrow, spleen and peripheral blood and induced durable remission of leukemia in MOLM-13 xenografts and patient-derived xenograft model of adult AML.

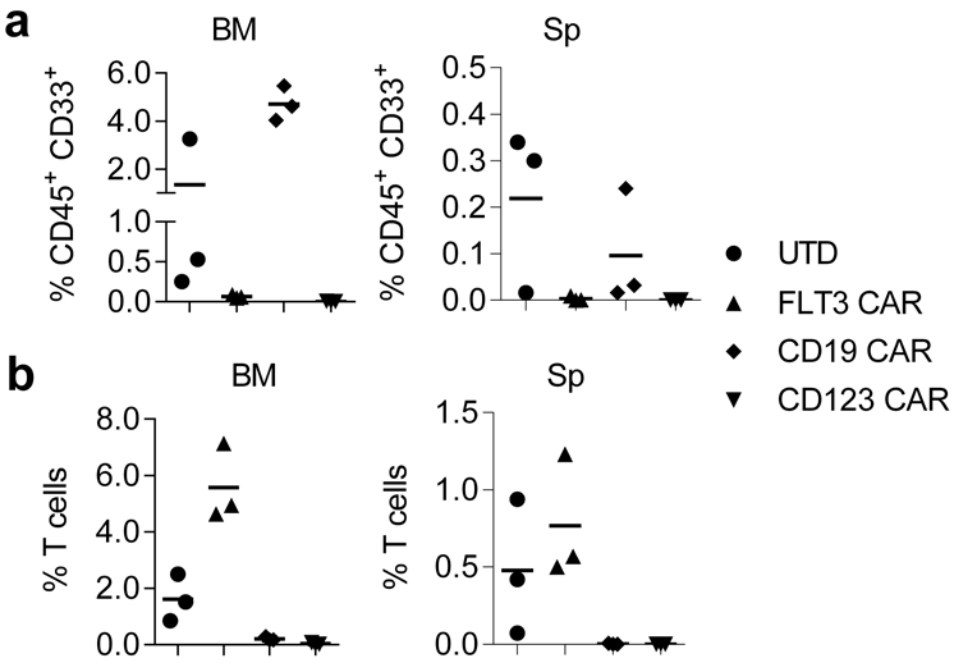


Figure 3.18: Flow cytometric analysis of BM and Sp after FLT3 CAR-T cell treatment. (a) At 3 weeks following T cell transfer (i.e. 7 weeks after AML blast inoculation) mice were sacrificed and the frequency of AML blasts ($CD45^+CD33^+$) and T cells ($CD45^+CD3^+$) in bone marrow (BM) and spleen (Sp) were analyzed by flow cytometry. Diagrams show frequencies as percentage of live ($7AAD^-$) cells. Data shown are representative for results obtained in independent experiments with primary FLT3-ITD^+ AML blasts obtained from n=2 patients.

Collectively, CAR-T cells targeting FLT3 on AML exert potent anti-leukemia activity against AML cell lines independent of FLT3 mutation status. FLT3 CAR-T cells targeting different epitopes on FLT3 extracellular domain were equally effective *in vitro*. FLT3 CAR-T cells recognized and eliminated FLT3-ITD+ primary AML blasts *in vitro* and were equally effective as CD123 CAR-T cells. Furthermore, FLT3 CAR-T cells induce durable remission in xenograft models of AML and induce remission in bone marrow, spleen and peripheral blood.

3.2 Aim-2: To determine synergy between FLT3 CAR-T cells and FLT3 inhibitors

3.2.1 Crenolanib increases cell surface FLT3 protein expression on AML cells

The clinical use of the FLT3 inhibitors lestaurtinib[81] and sorafenib[111] and *in vitro* studies with midostaurin[78] have shown evidence of FLT3 protein up regulation on AML cells after treatment with FLT3 inhibitors. Therefore, we hypothesized that surface FLT3-expression can be enhanced by the FLT3 inhibitor crenolanib and exploited to augment the efficacy of the FLT3 CAR-T cells.

We cultured native MOLM-13 cells (MOLM-13^{Native}) with continuous exposure to 10 nM crenolanib (which is clinically achievable serum level) for a week. We observed significantly higher levels of FLT3 surface protein (analyzed by MFI) compared to MOLM-13^{Native} cells (n=5 experiments, $p<.05$) within a week after starting the *in vitro* drug treatment (Figure 3.19a). Interestingly, the effect is reversible and crenolanib cessation leads to reduction of surface FLT3-expression to base level and FLT3 levels rise again after re-application of the drug (Figure 3.19b). After primary exposure to crenolanib, we observed a moderate cytotoxic effect and slower expansion of efluor670 labelled MOLM-13^{Creno} cells compared to MOLM-13^{Native} cells for approx. 7 days (Figure 3.20a, b). However, despite continuous supplementation to the culture medium, the cytotoxic effect of crenolanib subsequently ceased and the expansion of MOLM-13^{Creno} cells accelerated, suggesting they had acquired resistance to the drug. Thus, we established a cell line resistant to 10 nM crenolanib and further we will call the cell line as crenolanib resistant MOLM-13 (MOLM-13^{Creno}) cells. We cultured MOLM-13^{Creno} cells in the presence of crenolanib for several months and observed stable FLT3-expression over the time.

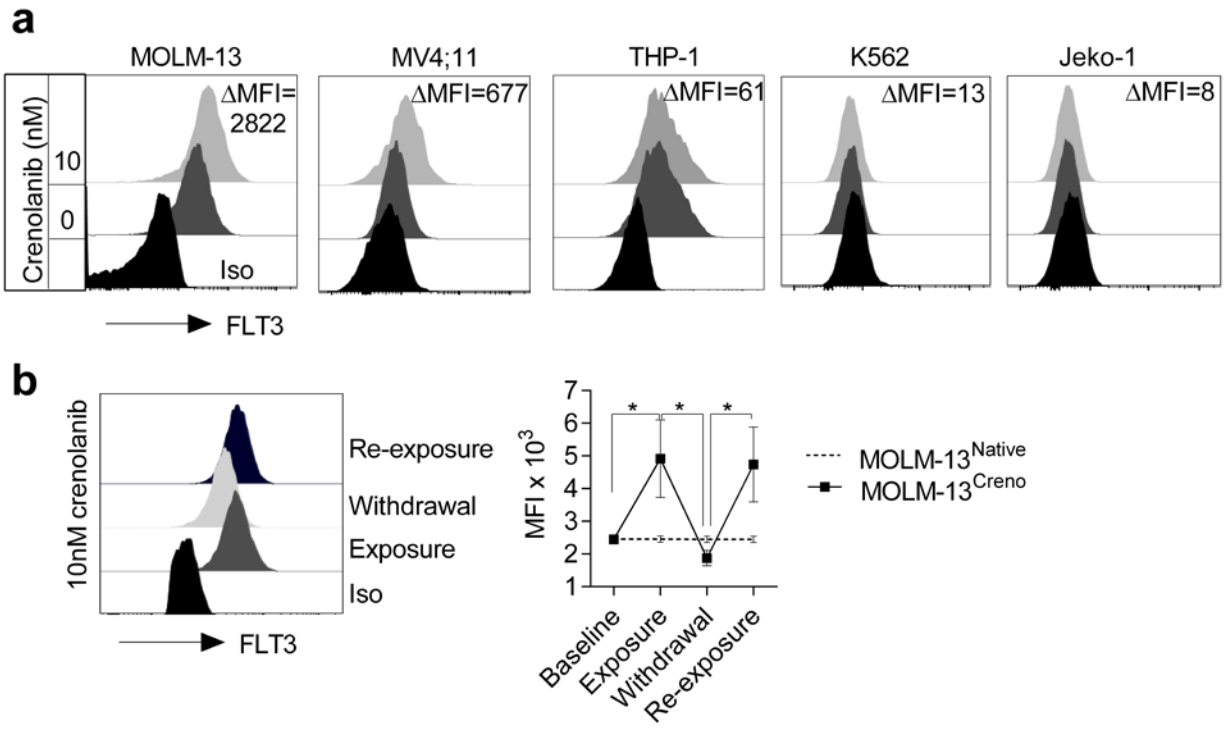


Figure 3.19: FLT3-expression on FLT3-ITD+ AML cell lines after treatment with FLT3 inhibitor crenolanib. (a) Flow cytometric analysis of FLT3-expression on AML and CML cell lines. The cells were cultured in absence or presence of 10 nM crenolanib for 7 days. Histograms show staining with anti-FLT3 mAb (gray) compared to isotype (black). Inset numbers state the absolute difference in MFI of treated/non-treated cells. (b) Histograms show FLT3-expression on MOLM-13 cells after 7 days culture in presence of 10 nM crenolanib (exposure), 2 days after subsequently withdrawing the drug (withdrawal), and 7 days after re-exposure to 10 nM crenolanib (re-exposure). The diagram on the right shows summary data from n=3 experiments. Values are presented as mean ± s.d. **p*<.05 (Student's t-test).

MV4;11 AML cells (FLT3-ITD+, loss of heterozygosity) also showed increased FLT3-expression upon exposure to crenolanib. However, several other cell lines expressing wt FLT3, i.e. THP-1 AML cells, or FLT3 negative cells like JeKo-1 mantle cell lymphoma and K562 erythro-myeloid leukemia did not show increase in FLT3-expression (Figure 3.19a). This observation suggests that the upregulation of FLT3-expression in response to crenolanib specifically occurred in FLT3-ITD+ AML cells (Figure 3.19a).

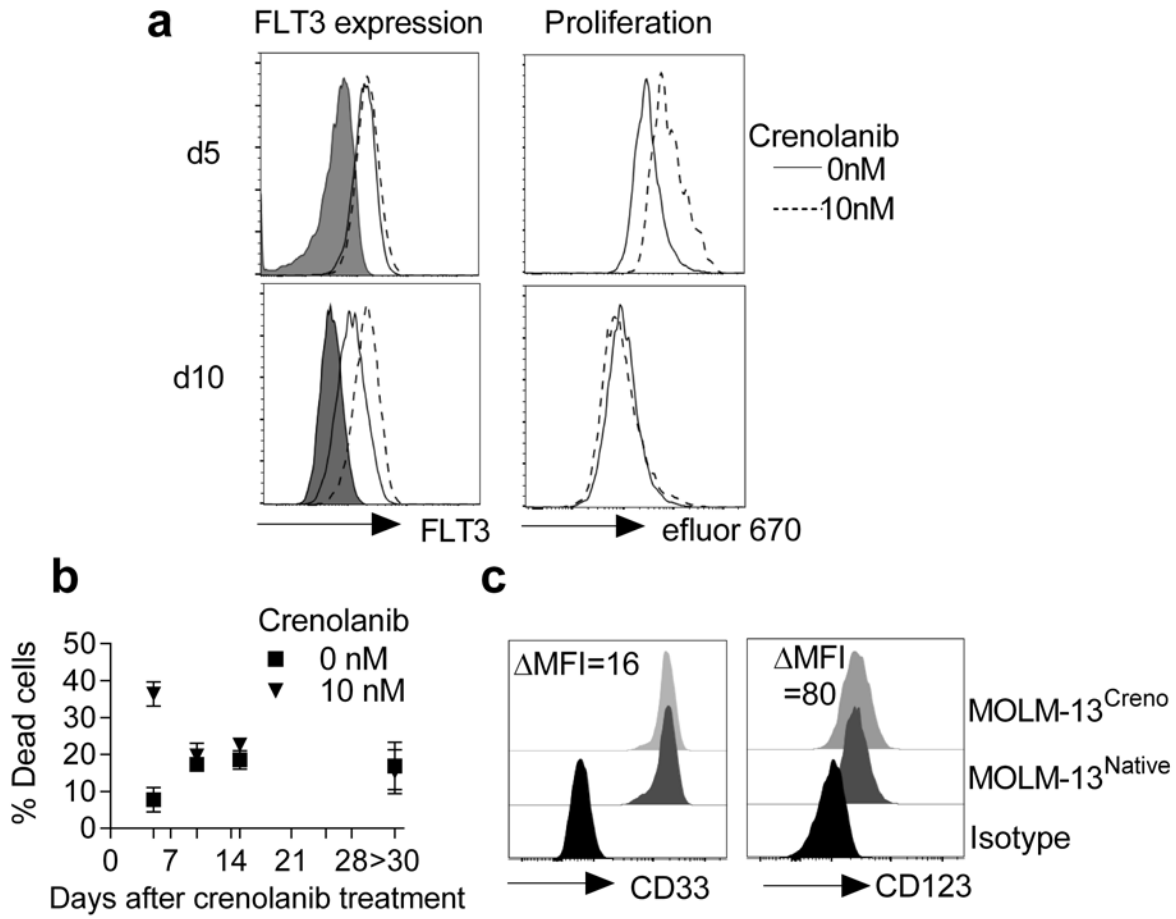


Figure 3.20: Development of resistance to crenolanib by MOLM-13 AML cells *in vitro*. (a-b) $1x10^6$ efluor-670 labelled MOLM-13 cells were plated in 48-well plates (in triplicate wells) on day 0 in 1 mL culture medium with or without 10 nM crenolanib. After 5 and 10 days, the cells were washed and stained for FLT3-expression using the anti-FLT3 mAb 4G8. (a) Analysis of FLT3-expression and proliferation using flow cytometry. The solid line denotes untreated (0 nM) and dashed line represents crenolanib-treated MOLM-13 cells. Data shown are representative for results obtained in n=2 independent experiments. (b) Cell viability after treatment with 10 nM crenolanib (triangle) compared to untreated (square) MOLM-13 cell. The diagram shows the percentage of 7-AAD^+ (dead) cells in the cell population. Cells were washed and fresh culture medium supplemented with crenolanib was added after 7 days. Data shown are mean ± s.d. from n=2 independent experiments. (c) Flow cytometric analysis of CD33 and CD123-expression on $\text{MOLM-13}^{\text{Native}}$ (dark grey) and $\text{MOLM-13}^{\text{Creno}}$ (light grey) cells. Representative data from n=2 independent experiments.

In comparison to FLT3, CD33 and CD123-expression on both MOLM-13 and MV4;11 was not affected by crenolanib and we did not observe any increase in protein expression (Figure 3.20c).

To examine the effect of crenolanib on normal HSCs, we analyzed FLT3-expression on HSC after 7 days of exposure to 10 nM crenolanib. Again consistent with their wt FLT3 genotype, FLT3-expression did not increase (Figure 3.21a). We also assessed HSC viability, which in contrast to MOLM-13 AML cells, did not decrease within 7 days of exposure to 10 nM of the drug (Figure 3.21b).

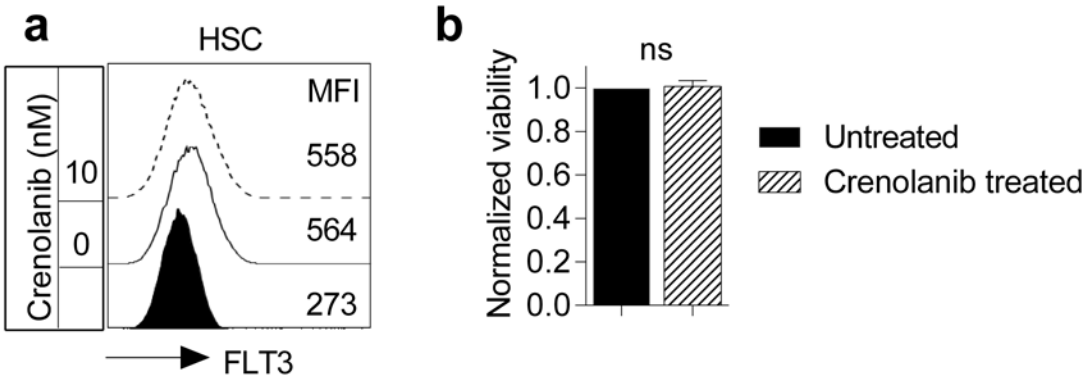
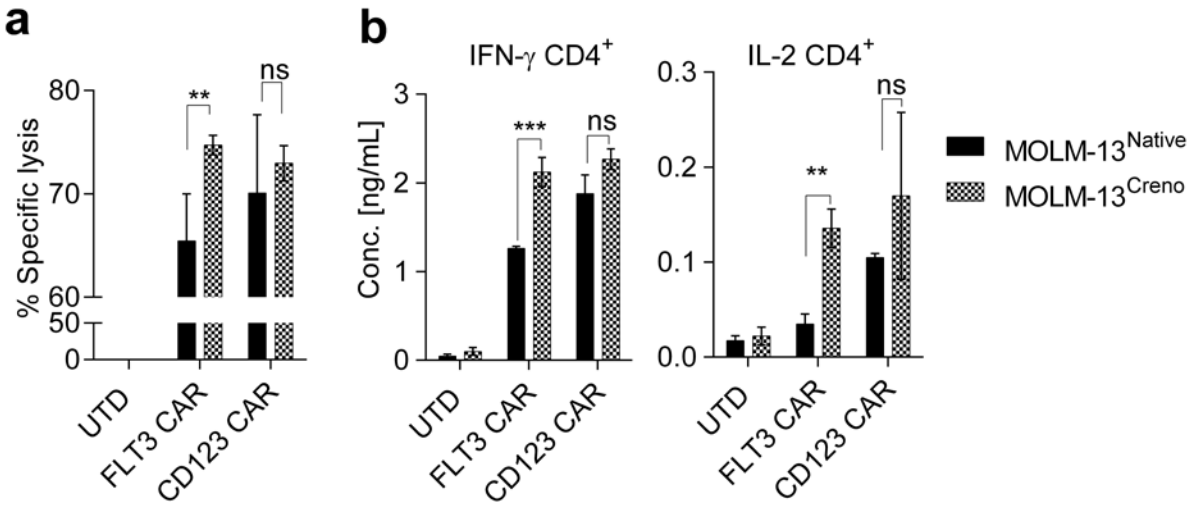
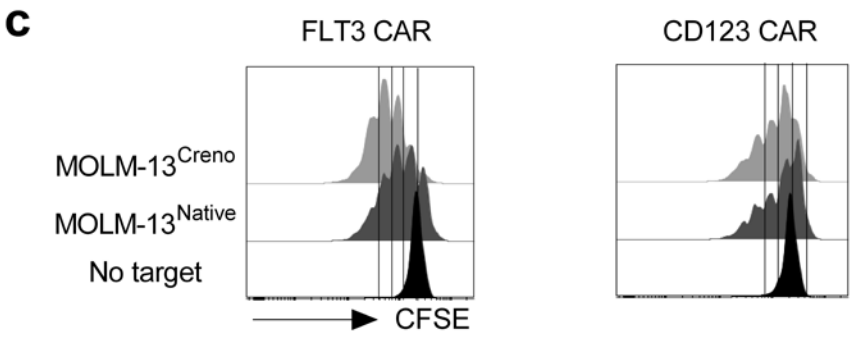


Figure 3.21: FLT3-expression on HSCs after the treatment with crenolanib. (a) Flow cytometric analysis of FLT3-expression on HSC after 7 days of culture in presence of 10 nM crenolanib. Representative data from n=2 independent experiments. (b) Bar diagram shows the frequency of live HSC cells (7-AAD$^-$) after 7 days of crenolanib treatment compared to untreated HSC. Data were normalized to untreated HSCs. Values are presented as mean ± s.d. (Student's t-test, *p<.05).

In summary, the data shows that crenolanib treatment increases surface FLT3 protein exclusively on FLT3-ITD+ AML cells. Crenolanib treatment does not increase FLT3-expression on normal HSC and does not affect CD33 and CD123 protein expression on AML cells.

3.2.2 Higher FLT3-expression on crenolanib-treated MOLM-13 cells leads to enhanced anti-leukemia activity of FLT3 CAR-T cells





	FLT3 CAR					CD123 CAR			
Cell divisions	4	3	2	1		4	3	2	1
MOLM-13^{Native}	10.5	19.3	24.9	27.8		6.5	12.8	15.6	27.9
MOLM-13^{Creno}	23.9	31.4	24.4	12.4		8.6	15.2	19.0	31.9

Figure 3.22: Recognition of MOLM-13Creno and MOLM-13Native AML cells by FLT3 CAR-T cells. (a) Cytolytic activity of FLT3 and CD123 CAR-T cells after 4 hour co-incubation (10:1 E:T ratio). (b) IFN-γ and IL-2 production by FLT3 and CD123 CAR-T cells measured by ELISA after 24 hour incubation at a 4:1 E:T ratio. (c) Proliferation of CD4$^+$ FLT3 and CD123 CAR-T cells assessed by CFSE dye dillution (4:1 E:T ratio). Numbers below histograms indicate the number of cell divisions the proliferating subset underwent, and the fraction (%) of T cells in each gate that underwent ≥4/3/2/1 cell divisions. Data shown are representative for results obtained in independent experiments with FLT3 CAR-T cells prepared from n=2 donors. Assays with MOLM-13^{Creno} were performed in medium containing 10 nM crenolanib. ***p*<0.005, ****p*<0.0005 (Student's t-test).

We examined whether the higher antigen density of FLT3 protein on MOLM-13^{Creno} cells would enhance their recognition by FLT3 CAR-T cells. Our earlier data showed rapid modulation of FLT3-expression upon exposure to and withdrawal of crenolanib (Figure 3.19a, b), suggesting maximum reactivity of FLT3 CAR-T cells against MOLM-13^{Creno} would be accomplished in presence of the drug.

In subsequent functional analyses, we observed superior cytolytic activity of CD8+ FLT3 CAR-T cells against MOLM-13^{Creno} compared to MOLM-13^{Native} cells (p<0.05) (Figure 3.22a). We observed enhanced production of IFN-γ and IL-2 by CD4+ FLT3 CAR-T cells against MOLM-13^{Creno} compared to MOLM-13^{Native} cells (p<0.05), as well as enhanced proliferation of CD4+ FLT3 CAR-T cells after stimulation with MOLM-13^{Creno} compared to MOLM-13^{Native} (Figure 3.22b, c). In particular, FLT3 CAR-T cells that underwent more than two cell divisions were higher after stimulation with MOLM-13^{Creno} compared to MOLM-13^{Native} cells (Figure 3.22c). In contrast to FLT3 CAR-T cells, the anti-leukemia reactivity of CD123 CAR-T cells against MOLM-13^{Creno} and MOLM-13^{Native} was not significantly different (Figure 3.22a, b, c).

We confirmed that upregulation of FLT3 after treatment with crenolanib also occurred on primary FLT3-ITD^{+} AML blasts after 3 days culture with 10 nM and 20 nM crenolanib (Figure 3.23a). Next, we carried out functional analyses and observed increased cytolytic activity of FLT3 CAR-T cells against crenolanib pre-treated FLT3-ITD+ primary AML blasts in a 24 hour co-culture assay (Figure 3.23b).

It has been reported that the use of tyrosine kinase inhibitors may interfere with T cell activation and function[112,113] and therefore we analyzed FLT3 CAR-T cells functions in the presence of crenolanib. We utilized MOLM-13^{Native} and K562/FLT3 cells as target cells in functional analyses and confirmed that crenolanib *per se* did not affect cytolytic activity, IFN-γ and IL-2 production, and proliferation of FLT3 CAR-T cells (Figure 3.24a-c).

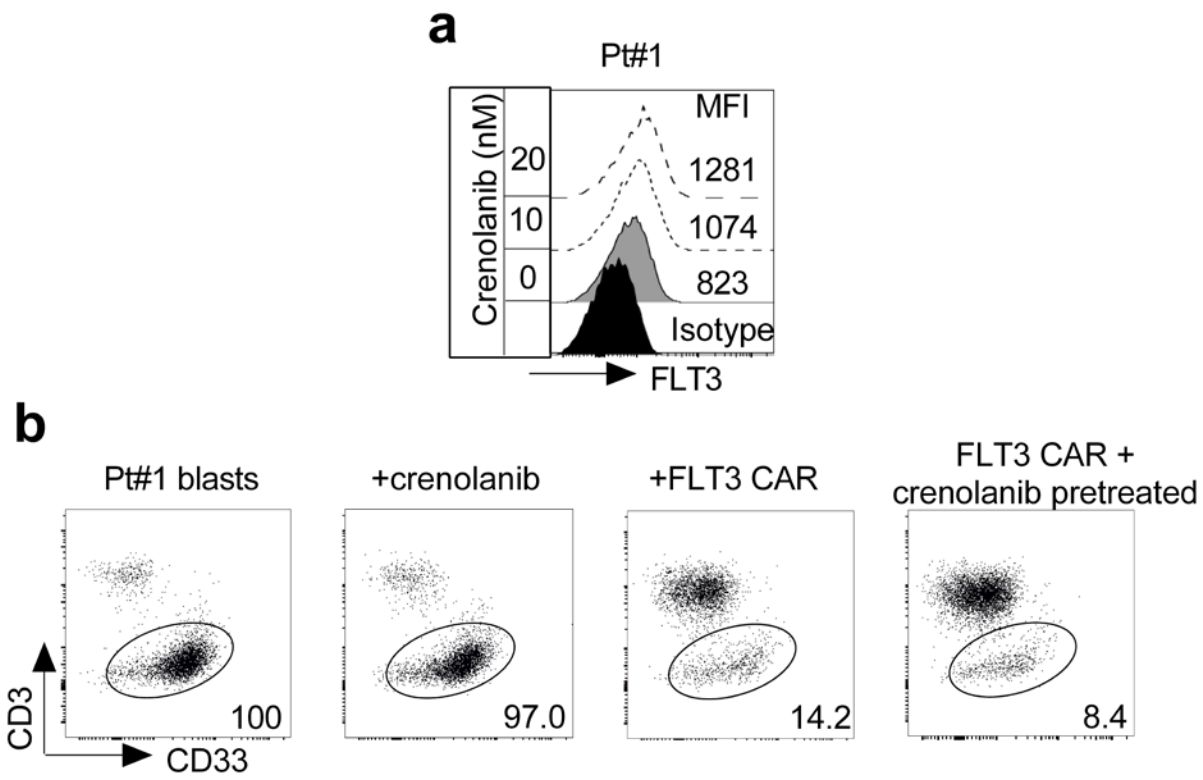


Figure 3.23: FLT3-expression on FLT3-ITD+ primary AML after treatment with crenolanib and cytolytic activity of FLT3 CAR-T cells. (a) Flow cytometric analysis of FLT3-expression on primary FLT3-ITD$^+$ AML (patient #1) after 3 days culture in the presence of 10 nM or 20 nM crenolanib. (b) Dot plots show residual live (7-AAD$^-$) AML blasts that had been either pre-treated with 20 nM crenolanib or left untreated, and subsequently co-cultured with FLT3 CAR-T cells (24-hour incubation at 1:1 E:T ratio with 5,000 target cells/well). Flow cytometric analysis was done on pooled cultures from triplicate wells. Data shown are representative of independent experiments with primary AML blasts from n=2 patients.

Collectively, these data show that the treatment with crenolanib leads to increased expression of surface FLT3 protein, specifically on FLT3-ITD$^+$ AML cells. Consecutively, enhanced recognition of crenolanib treated AML cells results in superior anti-leukemia activity by FLT3 CAR-T cells compared to non-treated AML cells.

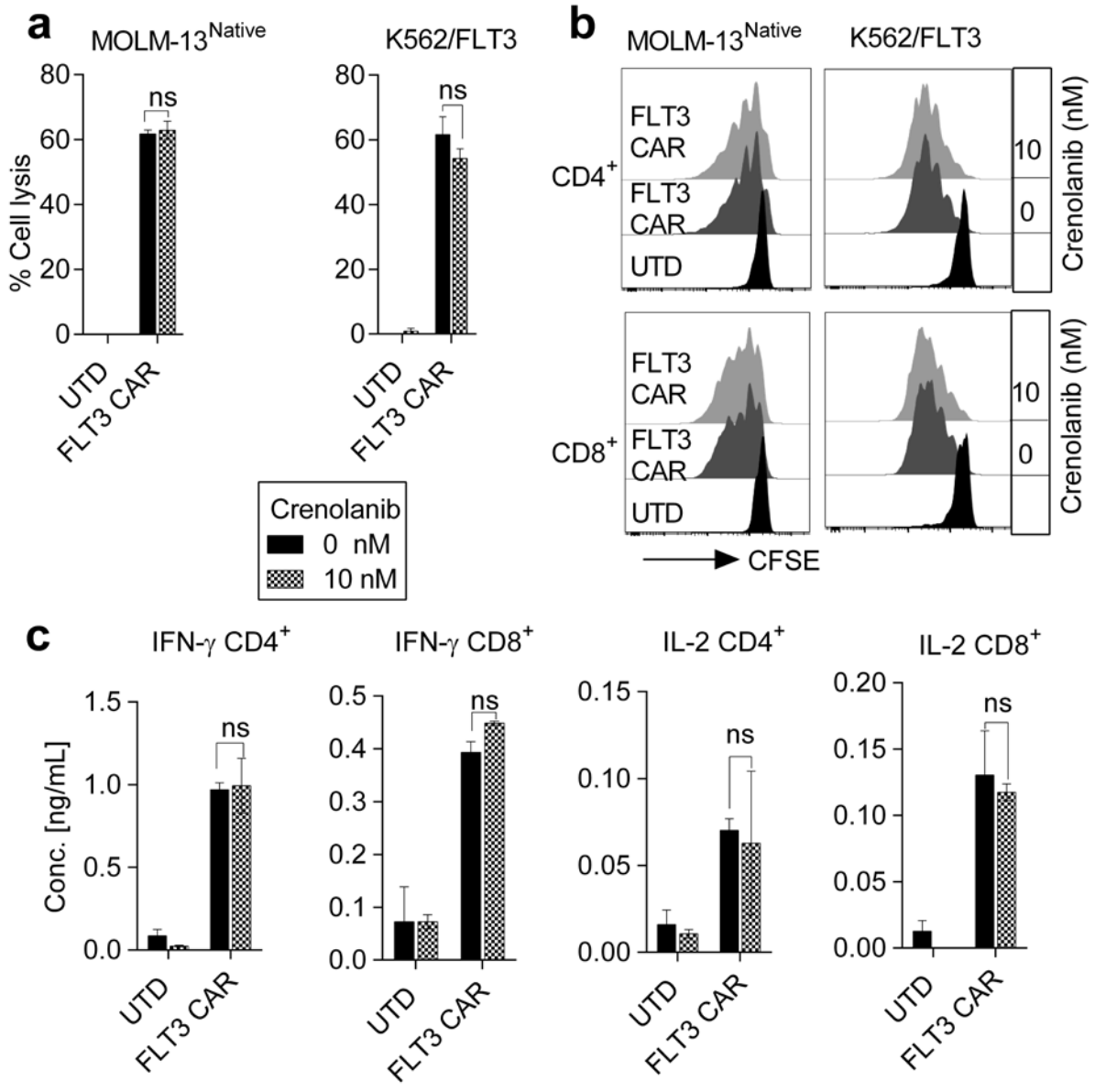


Figure 3.24: Effect of crenolanib on CAR-T cell effector functions. Functional analysis of FLT3 CAR-T cell in the presence or absence of 10 nM crenolanib (a) Cytolytic activity of $CD8^+$ FLT3 CAR-T cells analyzed after 4 hour co-incubation. (b) Proliferation of $CD4^+$ and $CD8^+$ FLT3 CAR-T cells examined by CFSE dye dilution after 72 hour of co-culture. (c) Cytokine production (IFN-γ and IL-2) by $CD4^+$ and $CD8^+$ FLT3 CAR-T cells, supernatants were obtained from 24 hour co-cultures with MOLM-13^{Native} and analyzed by ELISA. Values are presented as mean ± s.d. **p*<0.05 (Student's t-test). Data shown are representative for results obtained in independent experiments with FLT3 CAR-T cells lines prepared from n=2 donors.

3.2.3 FLT3 CAR-T cells and crenolanib act synergistically *in vivo*

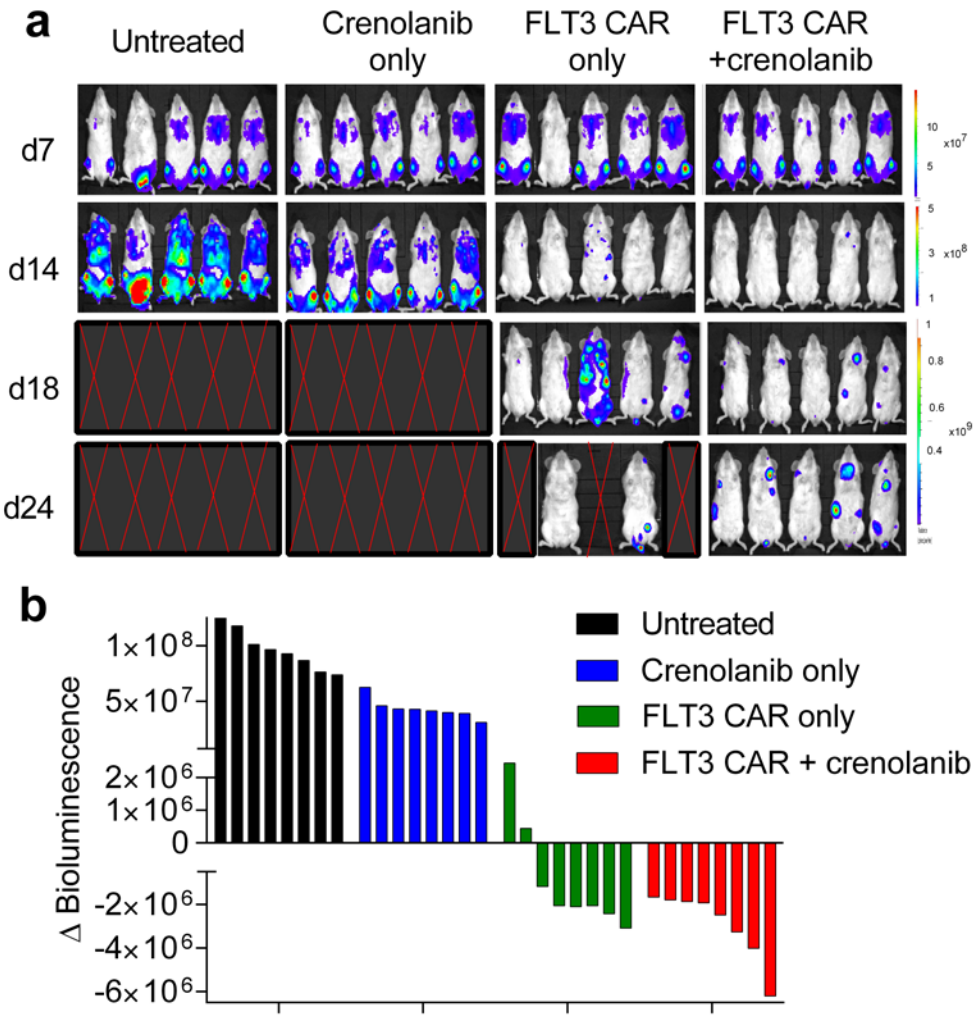


Figure 3.25: Anti-leukemia activity of FLT3 CAR-T cells in combination with crenolanib *in vivo*. NSG mice were injected with 1x10^6 ffluc$^+$GFP$^+$ MOLM-13 cells. After 7 days, mice were treated with FLT3 CAR-T cells alone (5x10^6 cells, CD4$^+$:CD8$^+$ ratio = 1:1), crenolanib alone (15 mg/kg body weight as i.p. injection)[83], or both (combination), or were left untreated. The first administration of crenolanib was given on day 7 and mice received total of 15 doses in 3 consecutive weeks (Monday-Friday). (a) Serial bioluminesence (BL) imaging to assess leukemia progression/regression in each treatment group. Note the scale (right) indicating upper and lower BL thresholds at each analysis time point. (b) Water fall plot showing the difference in absolute BL values obtained from individual mice between day 7 and day 14 after tumor inoculation. BL values were obtained as photon/sec/cm^2/sr. Data shown are combined from two independent experiments with T cells prepared from different donors.

Next, we sought to examine the anti-leukemia effect of FLT3 CAR-T cells in combination with crenolanib in the MOLM-13/NSG xenograft model. To investigate *in vivo* synergy, we injected mice with MOLM-13^{Native} AML cells and treated these mice with either FLT3 CAR-T cells alone, crenolanib alone, the combination of FLT3 CAR-T cells and crenolanib, or left them untreated. We observed potent anti-leukemia efficacy in mice receiving the combination treatment with FLT3 CAR-T cells and crenolanib (Figure 3.25a, b). We observed superior engraftment and *in vivo* expansion of FLT3 CAR-T cells (analyzed by flow cytometry) in mice treated with the combination therapy (Figure 3.26a). We observed a higher overall response rate (combination: n=8/8, 100% vs. FLT3 CAR-T cells alone n=6/8, 75% vs. crenolanib alone n=0/8, 0% vs. no treatment n=0/8, 0%); and faster and deeper remissions as assessed by bioluminescence imaging (Figure 3.25a, b). This translated into improved overall survival of mice receiving the FLT3 CAR-T cells and crenolanib combination, compared to monotherapy with FLT3 CAR-T cells and crenolanib, or no treatment (p<0.05) (Figure 3.27a).

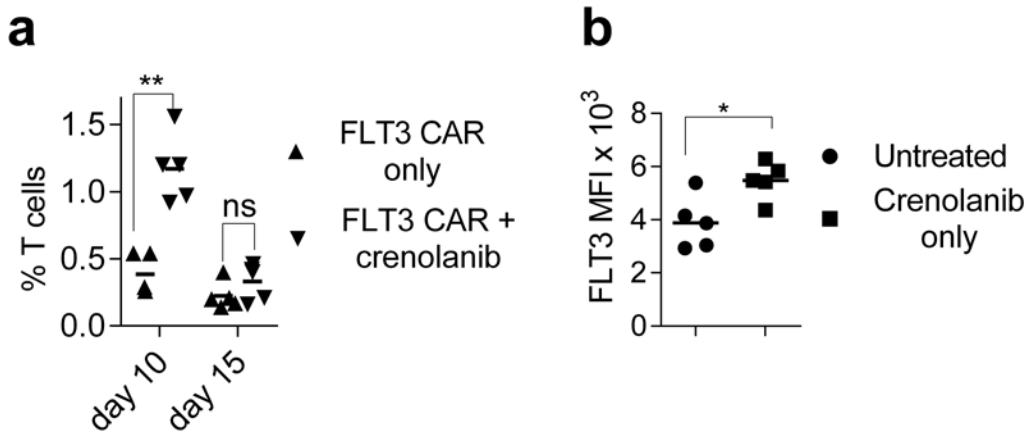


Figure 3.26: FLT3 CAR-T cell persistence and FLT3-expression analysis on MOLM-13 cells. (a) Peripheral blood analysis (on day 11 and 15 after tumor inoculation) of mice treated with FLT3 CAR-T cells alone or in combination with crenolanib. Diagram shows percentage of live (7-AAD$^-$) T cells (CD45$^+$CD3$^+$) in peripheral blood. **p<.005 (Student's t-test). (b) Flow cytometric analysis of FLT3-expression of MOLM-13 cells was performed on the cells obtained from bone marrow of untreated and crenolanib only mice (after 5 doses of crenolanib). Diagram shows MFI of FLT3. Data were analyzed using Student's t-test (*p<.05).

Crenolanib alone only had a minute anti-leukemia effect and leukemia progressed in these mice. FLT3-expression analysis on MOLM-13 cells revealed that cells recovered from peripheral blood and bone marrow at the end of the experiment had uniformly and strongly upregulated FLT3, consistent with our earlier observations *in vitro* (Figure 3.26b).

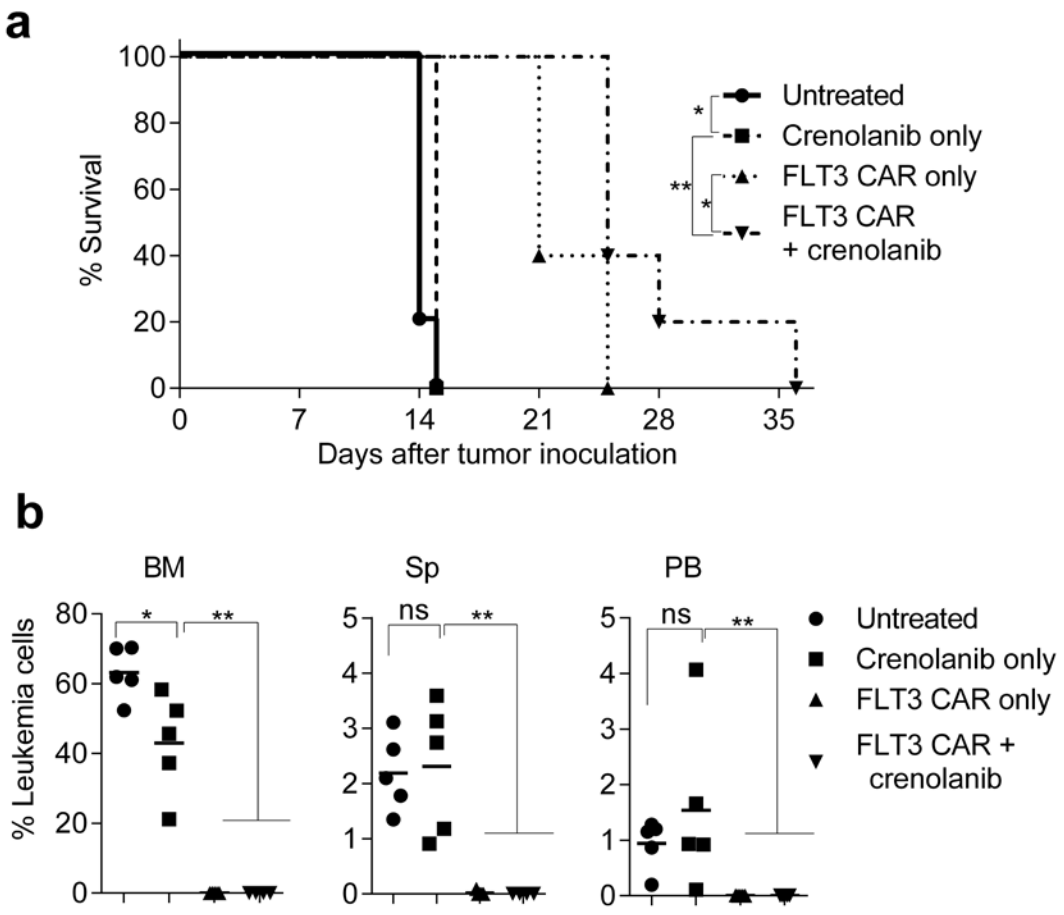


Figure 3.27: Flow cytometric analysis of BM, Sp and PB and Kaplan-Meier survival analysis. (a) Kaplan-Meier analysis of survival. Experiment endpoints were defined by relative (%) loss of body weight and total BL values. **p*<0.05, ***p*<0.005 (Log-rank test). (b) Diagrams show the percentage of live (7-AAD$^-$) leukemia cells (GFP$^+$/CD45$^+$) in BM, Sp, and PB. **p*<0.05, ***p*<0.005 (Student's t-test).

At the end of the experiment, peripheral blood, spleen and bone marrow of mice treated with the FLT3 CAR-T cell/crenolanib combination or FLT3 CAR-T cell monotherapy were completely free from MOLM-13 AML cells, whereas mice treated with crenolanib

monotherapy and untreated mice showed a high degree of leukemia infiltration (Figure 3.27b). We also observed that after combination treatment, mice experienced recurrence of MOLM-13 cells in anatomical sanctuary sites (Figure 3.25a). However, recurrent MOLM-13 cells had not lost FLT3-expression and did not show any downregulation compared to MOLM-13 cells before inoculation (Figure 3.28a, b).

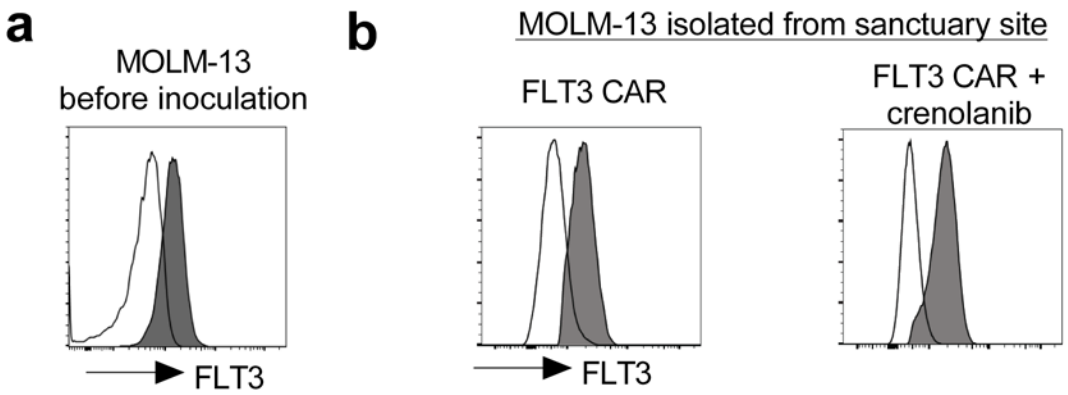


Figure 3.28: FLT3-expression on recurring MOLM-13 cells in the NSG xenograft model. Flow cytometric analysis of FLT3-expression on MOLM-13 cells (a) before inoculation and (b) MOLM-13 cells isolated from a sanctuary site. Histograms show staining with anti-FLT3 mAb (4G8) (filled) and isotype control antibody (open).

In summary, the FLT3 inhibitor crenolanib enhanced FLT3-expression exclusively on FLT3-ITD+ cells. Increased FLT3-expression was stable in the presence of crenolanib. FLT3 CAR-T cells showed a gain of function in combination with crenolanib and crenolanib did not affect the CAR-T cell effector functions.

Collectively, the data shows that FLT3 CAR-T cells and the FLT3 inhibitor crenolanib can be used synergistically as a combination therapy to confer a potent anti-leukemia effect against FLT3-ITD$^+$ AML cells *in vitro* and *in vivo*.

Interestingly, crenolanib is currently in the clinical development phase and has not yet been approved by the FDA for AML treatment. Therefore, we examined if FLT3-expression could be enhanced by other FLT3 inhibitors, preferably ones that are already approved for clinical use in AML (i.e. midostaurin), to assess if these inhibitors could also serve as a synergistic partners for FLT3 CAR-T cell therapy.

3.2.4 Cell surface FLT3-expression on AML cells can be enhanced by midostaurin

We exposed AML cells to FLT3 inhibitor midostaurin (10 nM) for 7 days, and analyzed FLT3-expression by flow cytometry. We observed increased FLT3-expression by MOLM-13 and MV4;11 cells after 7 days of exposure to midostaurin (Figure 3.29a).

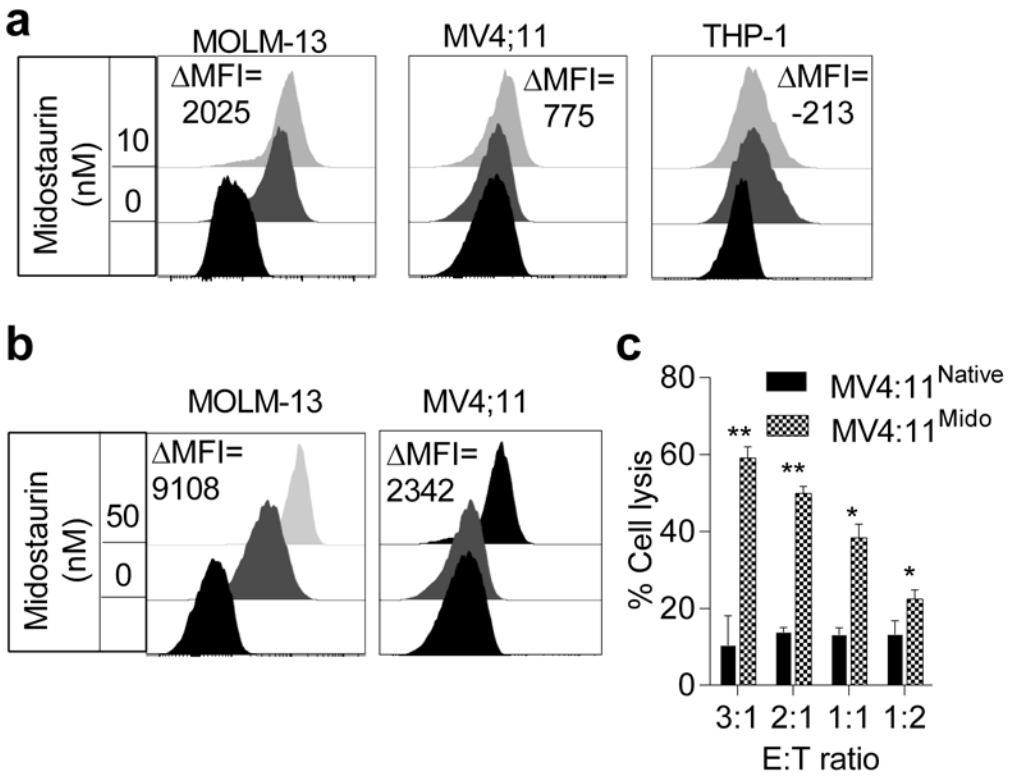


Figure 3.29: FLT3-expression on AML cell lines after midostaurin treatment. (a) Flow cytometric analysis of FLT3-expression on AML cell lines. Cells were cultured in the absence or presence of 10 nM midostaurin for 7 days. Histograms show staining with an anti-FLT3 mAb (gray) compared to isotype (black). Inset numbers state the absolute difference in MFI between treated and untreated cells. (b) The concentration of midostaurin was serially increased from 10 nM to 50 nM (10 nM increase per week). Histograms show FLT3-expression on MOLM-13 and MV4;11 cells after about 1 week culture in presence of 50 nM midostaurin. (c) Cytolytic activity of FLT3 CAR-T cells against native MV4;11 cells and 50 nM midostaurin treated MV4;11 cells ($MV4;11^{Mido}$) after 4 hour co-culture. Data shown are representative of n=3 experiments. Values are presented as mean ± s.d. *p<0.05 **p<0.005 (Student's t-test).

We increased the midostaurin concentration gradually from 10 nM to 50 nM and observed significantly higher FLT3 levels on MOLM-13 and MV4;11 cells compared to untreated cells (ΔMFI= 9108 and 2342 respectively, n=3 experiments) (Figure 3.29b). We cultured MOLM-13 and MV4;11 cells for few months with continuous exposure to 50 nM midostaurin and observed stable higher FLT3 surface protein by flow cytometry. However, removal of the drug led to a decrease in FLT3-expression to baseline level, and re-exposure increased FLT3 protein expression again (data not shown). We also exposed wt FLT3 expressing THP-1 cells to midostaurin and did not observe higher FLT3 protein expression compared to untreated cells (Figure 3.29a).

Next, we carried out *in vitro* functional analyses and observed significantly higher cytolytic activity by CD8+ FLT3 CAR-T cells against MV4;11^{Mido} compared to MV4;11^{Native} cells (Figure 3.29c).

In summary, midostaurin enhances surface FLT3 protein expression in FLT3-ITD+ cells and acts synergistically with FLT3 CAR-T cells *in vitro*.

3.2.5 Midostaurin acts synergistically with FLT3 CAR-T cells *in vivo*

Next, we sought to examine the synergistic anti-leukemia effect of FLT3 CAR-T cells in combination with midostaurin *in vivo*. Therefore, we inoculated mice with MOLM-13^{Native} AML cells and treated them with either FLT3 CAR-T cells alone, midostaurin alone, the combination treatment of FLT3 CAR-T cells and midostaurin, or left them untreated. The combination treatment was administered with 2 different schedules: One group of mice received midostaurin from day 3 after leukemia inoculation (FLT3 CAR + early midostaurin, midostaurin administration commenced prior to FLT3 CAR-T cell transfer) and the other group of mice received midostaurin from day 7 after leukemia inoculation (FLT3 CAR + midostaurin, midostaurin administration commenced at the day of FLT3 CAR-T cell transfer). Both the treatment group received a total of 15 doses of midostaurin.

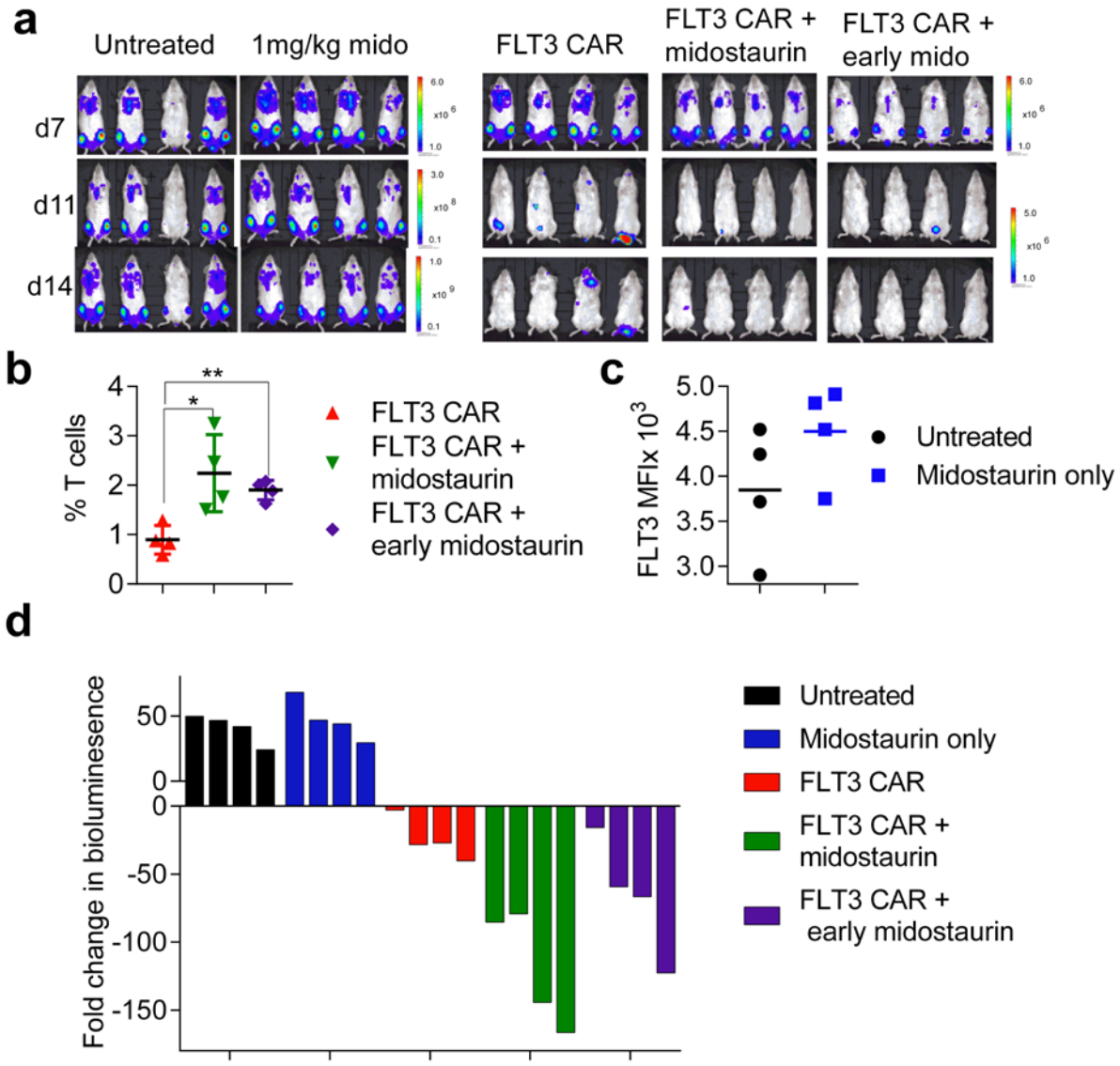


Figure 3.30: Anti-leukemia activity of FLT3 CAR-T cells in combination with midostaurin *in vivo*. 6-8 week old female NSG immunodeficient mice were injected with 1x10^6 ffluc$^+$GFP$^+$ MOLM-13 cells. After 7 days, the mice were treated with a single dose of FLT3 CAR-T cells alone (5x10^6 cells, CD4$^+$:CD8$^+$ ratio = 1:1), midostaurin alone (1 mg/kg body weight as i.p. injection), or both (combination), or were left untreated. Mice from the FLT3 CAR + early mido group received midostaurin even prior FLT3 CAR-T cells. Mice from the FLT3 CAR+ midostaurin group received the first midostaurin dose at the day of FLT3 CAR-T cell transfer. (a) Serial BL imaging to assess leukemia progression/regression in each treatment group. (b) Peripheral blood analysis (on day 11 after tumor inoculation) of mice treated with FLT3 CAR-T cells alone or in combination with midostaurin. The diagram shows percentages of live (7-AAD$^-$) T cells (CD45$^+$CD3$^+$) in peripheral blood. *p<0.05, **p<0.005 (Student's t-test). (c) Flow cytometric analysis of FLT3-expression on MOLM-13 cells was performed on cells obtained from the bone marrow of untreated and midostaurin only treated mice (after 5 doses of midostaurin). The

diagram shows MFI of FLT3. (d) Water fall plot showing the difference in absolute BL values obtained from individual mice between day 7 and day 14 after tumor inoculation.

We observed potent anti-leukemia efficacy in mice that received the combination treatment with FLT3 CAR-T cells and midostaurin (Figure 3.30a). In comparison to mice treated with only CAR-T cells, we observed superior engraftment and *in vivo* expansion of FLT3 CAR-T cells in mice treated with the combination therapy (Figure 3.30b). The mean frequency of FLT3 CAR-T cells in the peripheral blood of mice that received FLT3 CAR-T cells + midostaurin was more than twice as high compared to mice that had received FLT3 CAR-T cells alone (Figure 3.30b) ($p<0.05$). Furthermore, we observed faster and deeper remissions in mice treated with the combination therapy as assessed by bioluminescence imaging (Figure 3.30d). FLT3-expression analysis on MOLM-13 cells revealed that cells recovered from bone marrow had strongly upregulated FLT3 at the end of the experiment (Figure 3.30c). This is consistent with our earlier *in vitro* observation.

We again observed that after combination treatment with midostaurin, mice experienced recurrence of MOLM-13 cells in anatomical sanctuary sites. Although, we did not observe a significant difference in T cell expansion in between the midostaurin early and late treatment groups, we observed a delayed recurrence of MOLM-13 cells in anatomical sanctuary sites in the midostaurin early group (FLT3 CAR+ early mido treatment group).

In summary, midostaurin exerts synergistic anti-leukemia activity in combination with FLT3 CAR-T cells *in vitro* and *in vivo*. Our data suggests that midostaurin can be applied prior or concomitant to FLT3 CAR-T cells.

3.2.6 Quizartinib induces the highest increase in FLT3-expression on AML cells

Next, we sought to exploit the FLT3 inhibitor quizartinib to enhance FLT3-expression on AML cells and compare the effect of quizartinib to that of the other FLT3 inhibitors that we used in this study. Therefore, we exposed AML cells to 1 nM quizartinib for 7 days and analyzed FLT3-expression by flow cytometry. We observed increased FLT3-

expression by MOLM-13 and MV4;11 cells (but not by wt FLT3+ THP-1 cells) after 7 days of exposure to quizartinib (Figure 3.31a). We also exposed AML cells to increasing concentrations of quizartinib and observed that a 1-2 nM concentration is optimal for inducing resistance (data not shown). In subsequent functional analysis, we observed significantly higher cytolytic activity by CD8+ FLT3 CAR-T cells against MV4;11^{Quiza} compared to MV4;11^{Native} cells (Figure 3.31b).

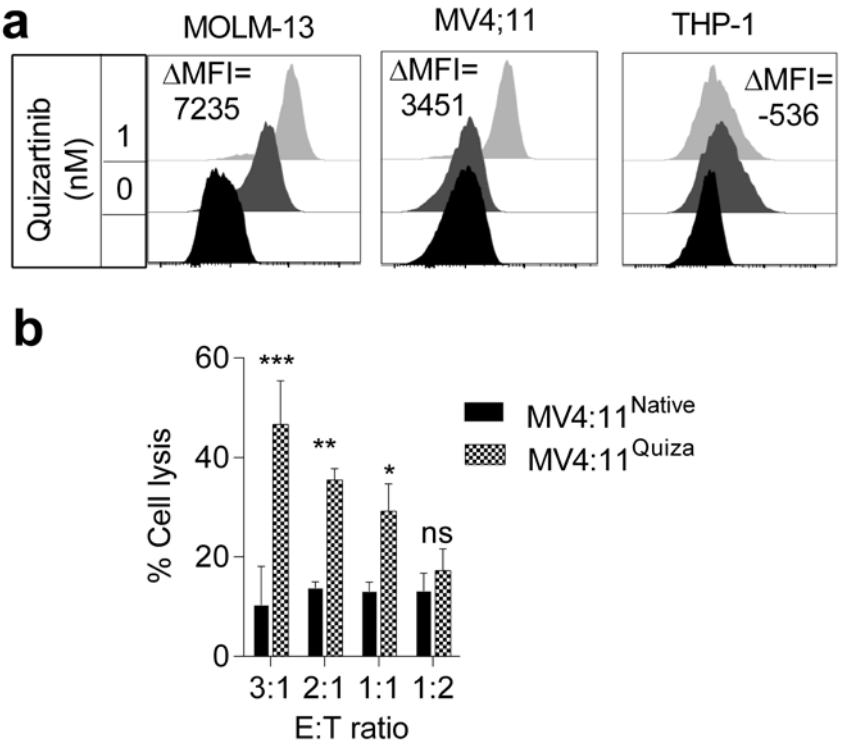


Figure 3.31: FLT3-expression on AML cell lines after quizartinib treatment. (a) Flow cytometric analysis of FLT3-expression on AML cell lines. Cells were cultured in the absence or presence of 1 nM quizartinib for 7 days. Histograms show staining with an anti-FLT3 mAb (gray) compared to isotype (black). Inset numbers state the absolute difference in MFI between treated and untreated cells. (b) Cytolytic activity of FLT3 CAR-T cells against native MV4;11 cells and 1 nM quizartinib treated MV4;11 cells (MV4;11^{Quiza}) after 4 hour co-culture. Data shown are representative of n=3 experiments. Values are presented as mean ± s.d. *p<0.05 **p<0.005 ***p<0.0005 (Student's t-test).

Next, we compared all the three FLT3 inhibitors crenolanib, midostaurin and quizartinib to determine the best synergy partner of FLT3 CAR-T cells that confers the highest

synergistic effect. Therefore, we exposed AML cell lines to 50 nM midostaurin, 10 nM crenolanib or 1 nM quizartinib. We observed significantly higher surface FLT3 protein levels on FLT3-ITD+ cells with any of the three FLT3 inhibitors compared to inhibitor-untreated controls. The highest difference in FLT3 MFI between untreated and treated AML cells was observed with quizartinib treatment followed by crenolanib and midostaurin (ΔMFI: MOLM-13^{Quiza} > MOLM-13^{Creno} > MOLM-13^{Mido}; 11600> 8072> 7836) (Figure 3.32).

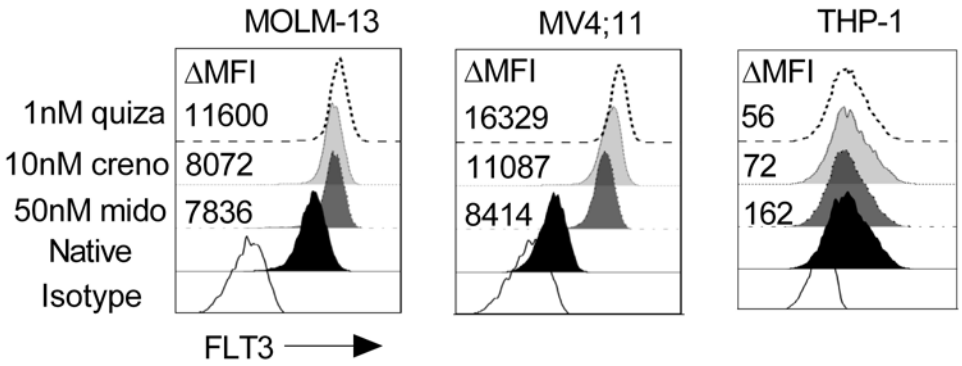


Figure 3.32: FLT3-expression on AML cells after treatment with FLT3 inhibitors. (a) Flow cytometric anaylsis of FLT3-expression on AML cell lines that were cultured in the absence or presence of 50 nM midostaurin, 10 nM crenolanib or 1 nM quizartinib for 8-12 weeks. Inset numbers state the absolute difference in MFI between treated and untreated cells. Representative data of n=2 independent experiments.

In subsequent studies, we aimed to dissect subtle differences at the single AML cell level after treatment with the different FLT3 inhibitors. Therefore, we used high resolution dSTORM microscopy[106] and quantified the number of FLT3 protein molecules on the surface of AML cells. A similar pattern of enhanced surface FLT3 protein after treatment with FLT3 inhibitors was observed with the highest FLT3 protein molecules/μm^2 after quizartinib treatment (Figure 3.33a, b). Surprisingly, we observed a clustering of FLT3 protein on the cell surface after treatment with midostaurin but not with any of the other FLT3 inhibitors (Figure 3.33a). Additionally, the amplification of FLT3 was uniform in the entire MV4;11 cell population after treatment with crenolanib and midostaurin, while quizartinib treatment lead to heterogeneous FLT3 amplification (Figure 3.33b). Of note, the fold change in FLT3 protein levels after FLT3 inhibitor

treatment was higher in MV4;11 cells compared to MOLM-13 cells and was highest after quizartinib treatment (Figure 3.33b).

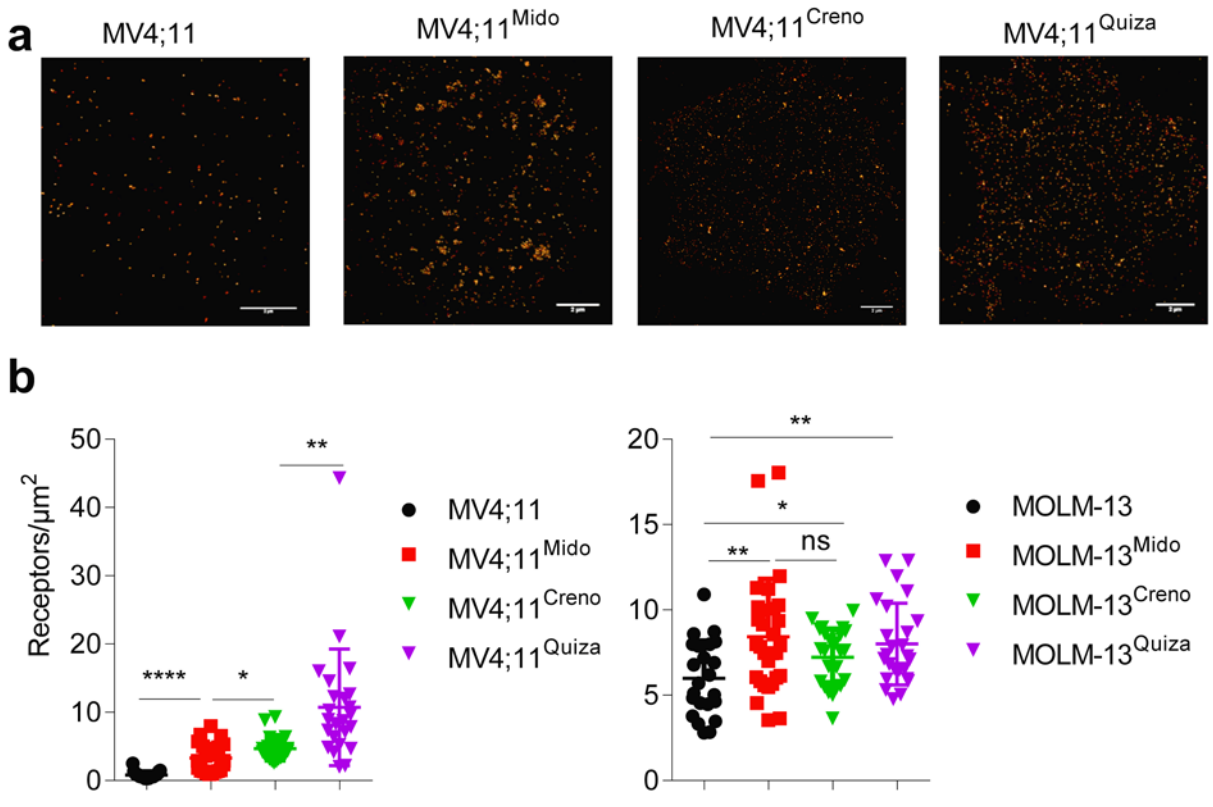


Figure 3.33: dSTORM analysis of FLT3-expression on AML cells after treatment with FLT3 inhibitor. (a) High resolution microscopy (dSTORM) analysis of a single MV4;11 cell after culture in the presence or absence of FLT3 inhibitors. (b) The quantification of FLT3 receptors on native and FLT3 inhibitors treated AML cells. Data shown are representative of n=2 independent experiments. Values are presented as mean ± s.d. *p<.05 **p<.005 ****p<0.0001 (Student's t-test).

3.2.7 Crenolanib is the optimal combination partner for FLT3 CAR-T cells

Next, we carried out functional analyses of FLT3 CAR-T cells against AML cells, either native or treated with different FLT3 inhibitors, for a direct functional comparison.

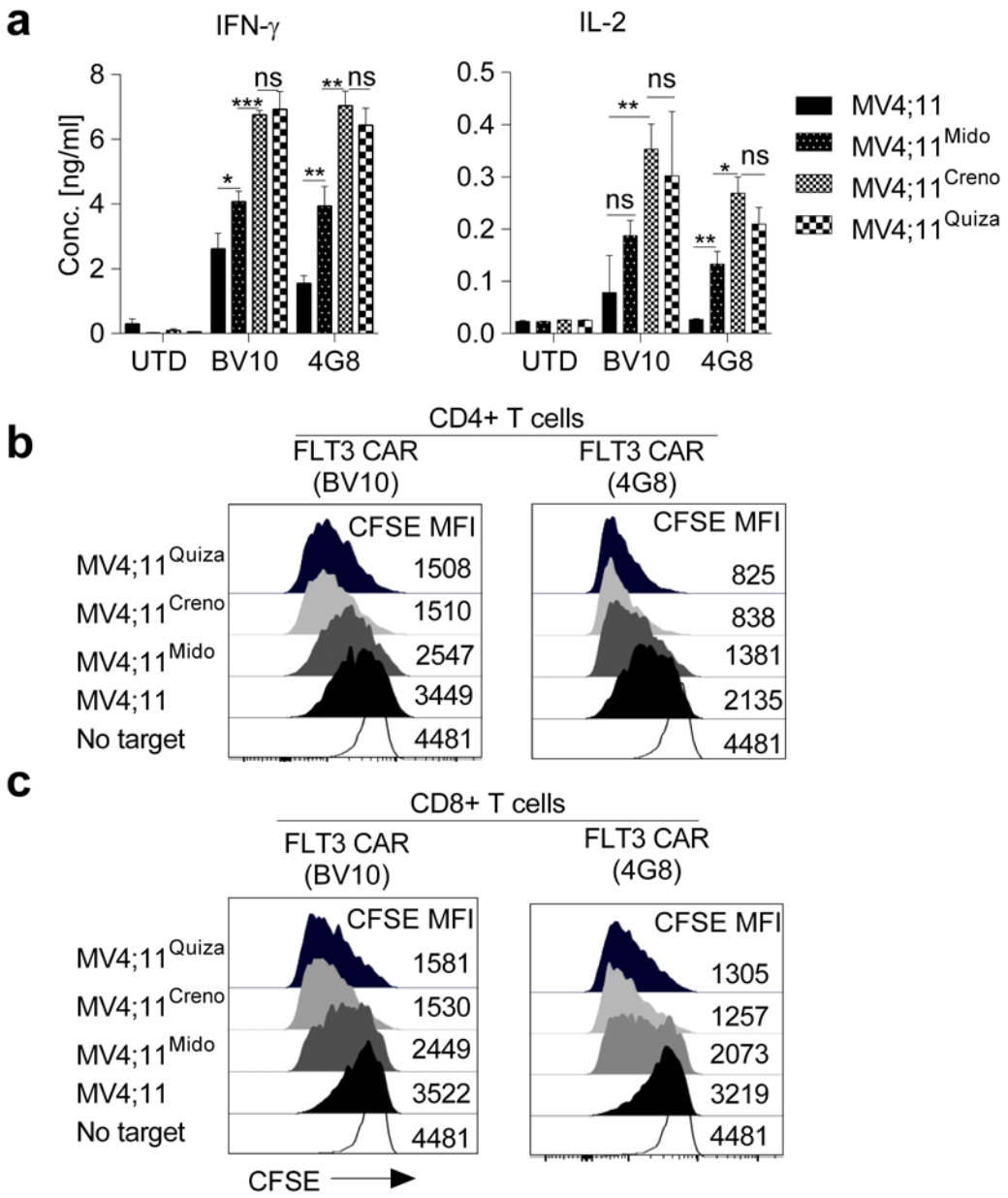


Figure 3.34: FLT3 CAR-T cells activity against native and resistant MV4;11 cells. (a) Cytokine production (IFN-γ and IL-2) by $CD8^+$ FLT3 CAR-T cells (BV10 and 4G8) after co-culture with target cells at an E:T ratio of 1:2. Supernatants were obtained from 24 hour co-cultures and analyzed by ELISA. Values are presented as mean ± s.d. (b) Proliferation of $CD4^+$ FLT3 CAR-T cells and (c) CD8+ FLT3 CAR-T cells (BV10 and 4G8) examined by CFSE dye dilution after 72 hour of co-culture at an E:T ratio of 1:2. Inset numbers show MFI for CFSE. Data shown are representative for results obtained in independent experiments with FLT3 CAR-T cells lines prepared from n=2 donors. **p*<0.05, ***p*<0.005, ****p*<0.0005, *****p*<0.0001 (Student's t-test).

We utilized MV4;11 cells for this comparison to mimic the (comparatively low) FLT3-expression on primary AML blasts. We also carried out the assays at lower effector to target ratio (E:T = 1:2) to partially imitate clinical scenario of T cells to AML blasts proportion in bone marrow of AML patients. Additionally, we included both BV10 and 4G8 FLT3 CAR-T cells for this analysis.

In subsequent functional analysis, we observed significantly higher IFN-γ and IL-2 production by CD8+ FLT3 CAR-T cells against FLT3 inhibitors treated MV4;11 cells compared to native MV4;11 cells (Figure 3.34a). Cytokine production by both FLT3 CAR-T cell variants was significantly higher against MV4;11^{Creno} compared to MV4;11^{Mido} and there was no significant difference in cytokine production between MV4;11^{Creno} and MV4;11^{Quiza} targets (Figure 3.34a). Similarly, CD4+ and CD8+ FLT3 CAR-T cell mediated proliferation was higher (analyzed by MFI of CFSE) against MV4;11^{Creno} compared to MV4;11^{Mido} target cells, while there was no significant difference in proliferation of FLT3 CAR-T cells against MV4;11^{Creno} or MV4;11^{Quiza} targets (Figure 3.34b, c). These observations correlate with FLT3-expression on native MV4;11 cells and FLT3 inhibitor treated MV4;11 cells.

Next, we utilized NSG/MOLM-13 xenografts (described previously) to directly compare FLT3 inhibitor-mediated synergy with FLT3 CAR-T cells *in vivo*. Mice received FLT3 CAR-T cells alone, an FLT3 inhibitor alone, a combination of both or were left untreated. We observed potent anti-leukemia efficacy in mice that received the combination of FLT3 CAR-T cells and an FLT3 inhibitor (Figure 3.35a). In comparison to mice that were treated with FLT3 CAR-T cells only, we observed superior engraftment and *in vivo* expansion of FLT3 CAR-T cells in mice that received the combination therapy (Figure 3.35b). The mean frequency of FLT3 CAR-T cells in the peripheral blood of mice that received FLT3 CAR-T cells and an FLT3 inhibitor was about 2-4 fold higher to that of mice that had received FLT3 CAR-T cells alone ($p<0.005$) (Figure 3.35b).

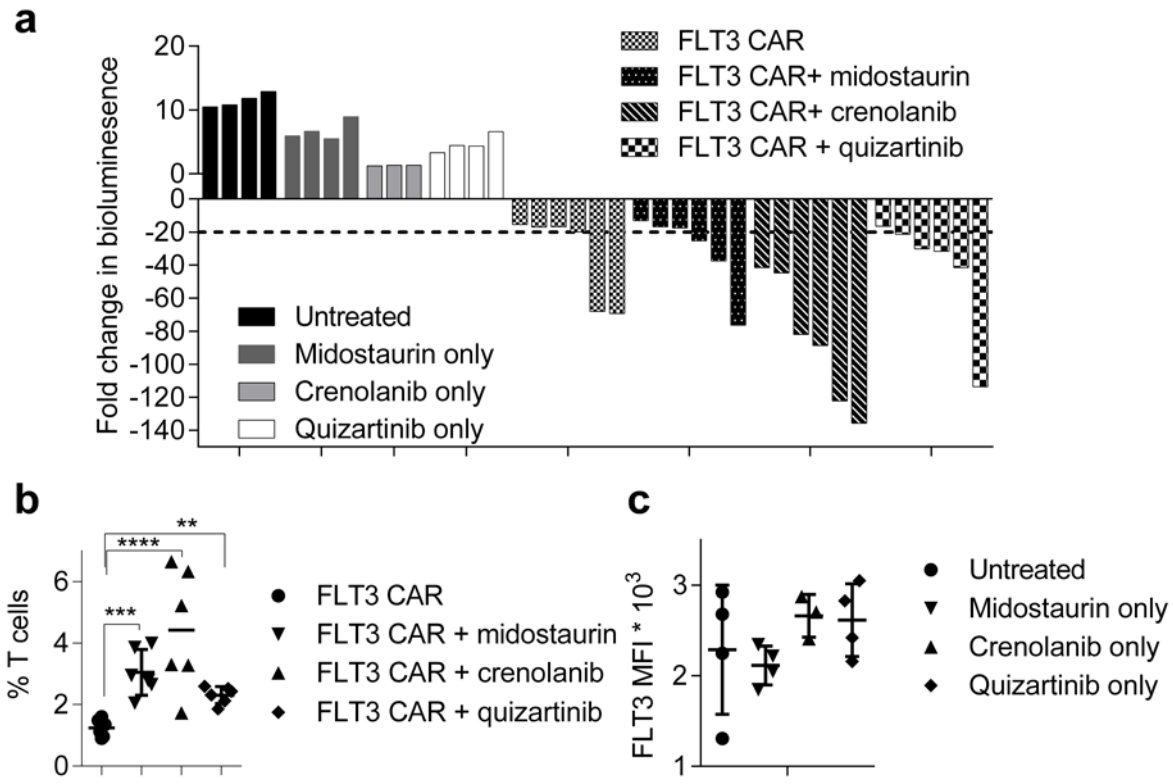


Figure 3.35: Anti-leukemia activity of FLT3 CAR-T cells alone and in combination with FLT3 inhibitors *in vivo*. NSG immunodeficient mice were injected with $1x10^6$ ffluc$^+$GFP$^+$ MOLM-13 cells on day 0. On day 7, the mice received a single dose of FLT3 CAR-T cells ($5x10^6$ cells, CD4$^+$:CD8$^+$ ratio = 1:1), an FLT3 inhibitor, both (combination), or were left untreated. In the combination tretament groups, mice received a total of 15 doses of midostaurin (1 mg/kg) or quizartinib (1 mg/kg) or crenolanib (15 mg/kg). (a) Waterfall plot showing the fold change in absolute bioluminesence values obtained from individual mice between day 7 and day 10 after tumor inoculation. BL values were measured as photons/sec/cm^2/sr. (b) Peripheral blood analysis (on day 10 after tumor inoculation) of mice treated with FLT3 CAR-T cells alone or in combination with an FLT3 inhibitor. The diagram shows the percentage of live (7-AAD$^-$) T cells (CD45$^+$CD3$^+$) in peripheral blood. **p<0.005, ***p<0.0005, ****p<0.0001 (Student's t-test). (c) FLT3-expression analysis on MOLM-13 cells obtained from bone marrow of untreated and FLT3 inhibitor treated mice (after 5 doses of each FLT3 inhibitor). Diagram shows mean fluoresence intensity of FLT3.

The combination of FLT3 CAR-T cells with crenolanib resulted in the highest CAR-T cell expansion with a mean CAR-T cell frequency of 4.4 % in peripheral blood. We observed increased FLT3 levels on MOLM-13 cells isolated from bone marrow of mice that received an FLT3 inhibitor treatment compared to cells isolated from untreated mice. Of

note, crenolanib treated MOLM-13 cells (isolated from bone marrow) showed the highest FLT3-expression (Figure 3.35c).

Further, we observed faster and deeper remissions in mice treated with the combination therapy as assessed by bioluminescence imaging (Figure 3.35a). In the group of mice, that were left untreated or had received an FLT3 inhibitor only, we did not observe a reduction in leukemia burden in any of the mice (response rate: 0/4 = 0%). In the group of mice, that had received FLT3 CAR-T cells only, we observed leukemia reduction in all of the mice (6/6 = 100%); however, in only 2 of 6 mice was the reduction in BL signal (as a marker for leukemia regression) greater than 20 fold (2/6 mice = 33%). In the group of mice that had received FLT3 CAR-T cells + midostaurin, we observed leukemia reduction in all of the mice (6/6 = 100%) and in 3 out of 6 = 50% of mice was the leukemia reduction greater than 20 fold. In the group of mice that had received FLT3 CAR-T cells + quizartinib, we observed leukemia reduction in all of the mice (6/6 = 100%) and in 5 out of 6 = 83.3% of mice was the leukemia reduction greater than 20 fold. The strongest anti-leukemia response was observed in the group of mice that had received FLT3 CAR-T cells + crenolanib with a leukemia reduction in all of the mice (6/6 = 100%) and in 6 out of 6 mice was leukemia reduction greater than 20 fold (Figure 3.35a).

Collectively, the data shows that FLT3 inhibitors act synergistically with FLT3 CAR-T cells and mediate potent synergistic anti-leukemia activity *in vitro* and *in vivo*. Crenolanib and quizartinib exert potent synergy with FLT3 CAR-T cells *in vitro* and we observed higher effector functions of FLT3 CAR-T cells in combination with these drugs compared to midostaurin. Further, crenolanib in combination with FLT3 CAR-T cells induced deepest remission and higher CAR-T cell expansion *in vivo* compare to other FLT3 inhibitors and therefore is an optimal combination partner for FLT3 CAR-T cells.

3.3 Aim-3: Recognition of healthy hematopoietic cells by FLT3 CAR-T cells

3.3.1 FLT3 CAR-T cells do not eliminate normal monocytes

Application of CD19 CAR-T cells in patients with acute lymphoblastic leukemia and lymphoma leads to depletion of normal mature CD19+ B cells and long term B cell aplasia[8,9,11]. FLT3 CAR-T cells have shown potent activity against FLT3+ AML and could eliminate normal tissue cells if they express FLT3. Therefore, we sought to analyze FLT3-expression on mature blood cells of healthy donors to predict possible toxicities after administration of FLT3 CAR-T cells in AML patients. We isolated peripheral blood mononuclear cells from healthy donor blood (n=3) and carried out flow cytometry analyses. We did not detect FLT3 on any of the mature blood cell types including CD3+ T cells, CD19+ B cells, CD33+ myeloid cells, CD56+ NK cells and (remaining) CD45+ granulocytes (Figure 3.36).

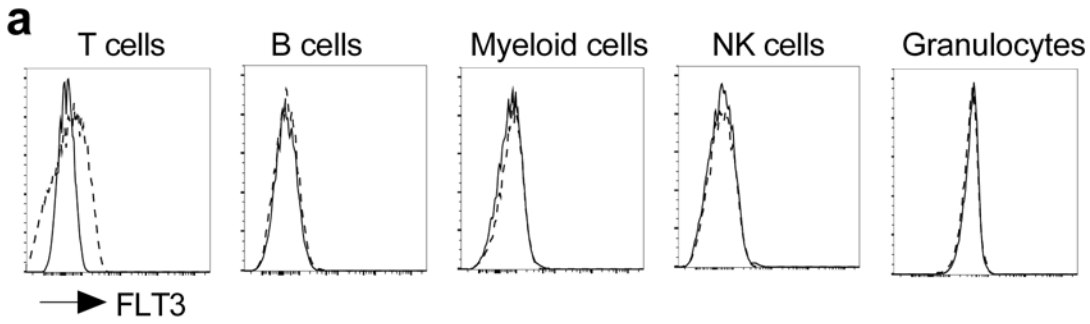


Figure 3.36: FLT3-expression on healthy donor derived PBMCs. Flow cytometric analysis of FLT3-expression on human peripheral blood cells. Staining was performed with a 4G8 mAb (solid line) or an isotype control (zebra line). Data shown are representative for n=3 independent experiments with healthy donor cells.

Next, we thought to analyze the effect of FLT3 CAR-T cells on myeloid cells *in vitro*. We isolated CD14+ myeloid cells from n=3 different healthy donors and analyzed FLT3-expression. We did not observe FLT3-expression on CD14+ myeloid cells (Figure 3.37a). Next, we carried out an *in vitro* cytotoxicity assay and observed that FLT3 CAR-T cells did not lyse myeloid cells, whereas CD123 CAR-T cells eliminated 20-40% myeloid cells after 4 hour co-culture (Figure 3.37b).

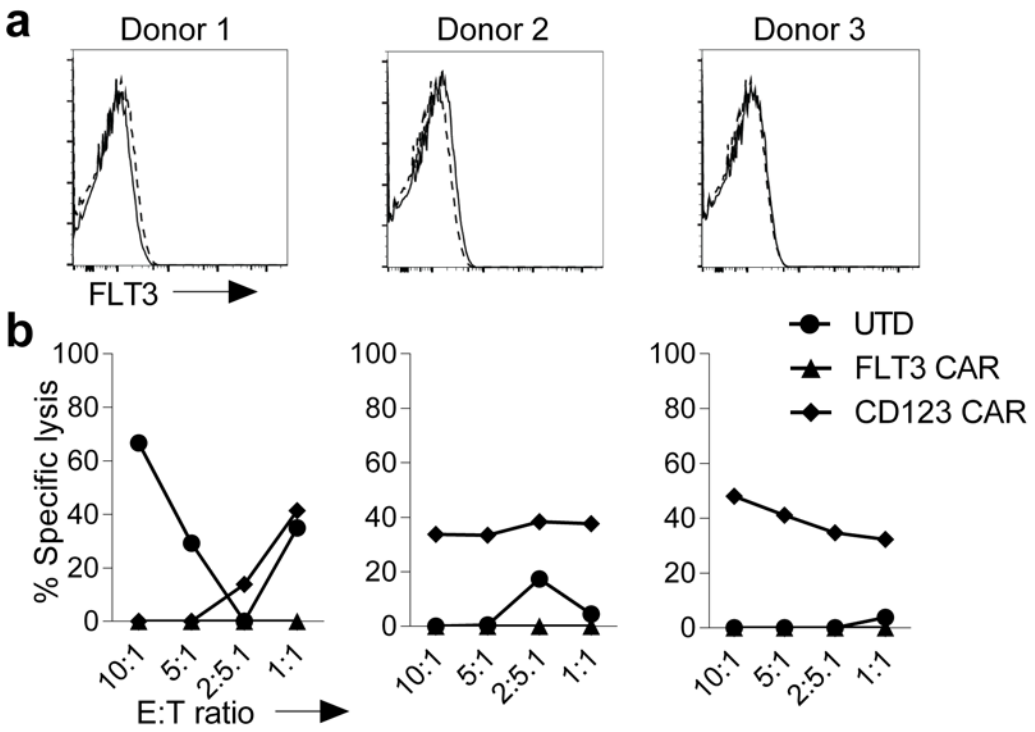


Figure 3.37: FLT3-expression on CD14+ myeloid cells and cytotoxicity mediated by FLT3 CAR-T and CD123 CAR-T cells. Human mature CD14+ myeloid cells were isolated using CD14 microbeads (Miltenyi, Bergisch-Gladbach, Germany) from PBMCs of healthy donors. (a) Flow cytometric analysis of FLT3-expression. Staining was performed with a 4G8 mAb (solid line) or an isotype control (zebra line). (b) Cytolytic activity of FLT3 CAR-T cells and CD123 CAR-T cells against CD14+ myeloid cells analyzed in a flow cytometry based assay after 4-hour of co-culture. The assay was carried out in triplicates and cells were pooled for flow cytometric analysis.

We did not observe proliferation or cytokine production by FLT3 CAR-T cells after co-culture with allogenic CD14+ myeloid cells. In contrast, CD123 CAR-T cells proliferated and produced cytokines after co-incubation with myeloid cells (Figure 3.38a, b).

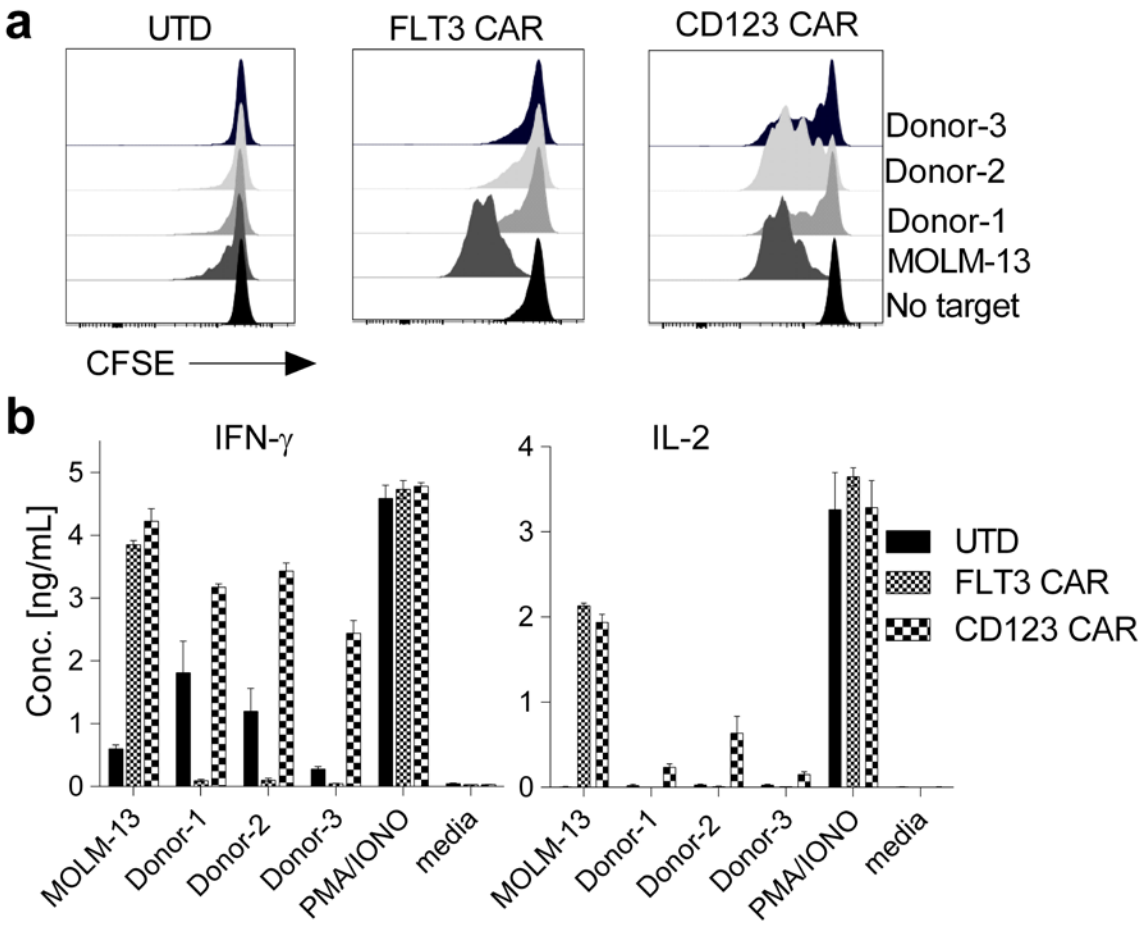


Figure 3.38: Functional analysis of FLT3 CAR-T cells against CD14+ myeloid cells. (a) Proliferation of CD4+ FLT3 and CD123 CAR-T cells (after 72 hour of co-culture), analyzed in CFSE dye dilution assay. (b) IFN-γ and IL-2 production by CD4+ FLT3 CAR-T and CD123 CAR-T cells measured by ELISA in cell culture supernatant obtained after 24-hour co-cultures with myeloid cells. Values are presented as mean ± s.d.

Collectively, the data shows that FLT3 CAR-T cells do not recognize normal myeloid cells *in vitro*, suggesting that FLT3 CAR-T cells will not eliminate mature myeloid cells in AML patients. FLT3-expression on other mature, differentiated, myeloid cell types such as dendritic cells should be performed in future studies to allow for a more accurate prediction of the potential myeloid toxicity of FLT3 CAR-T cells.

3.3.2 FLT3 CAR-T cells compromise normal hematopoiesis *in vitro* and *in vivo*

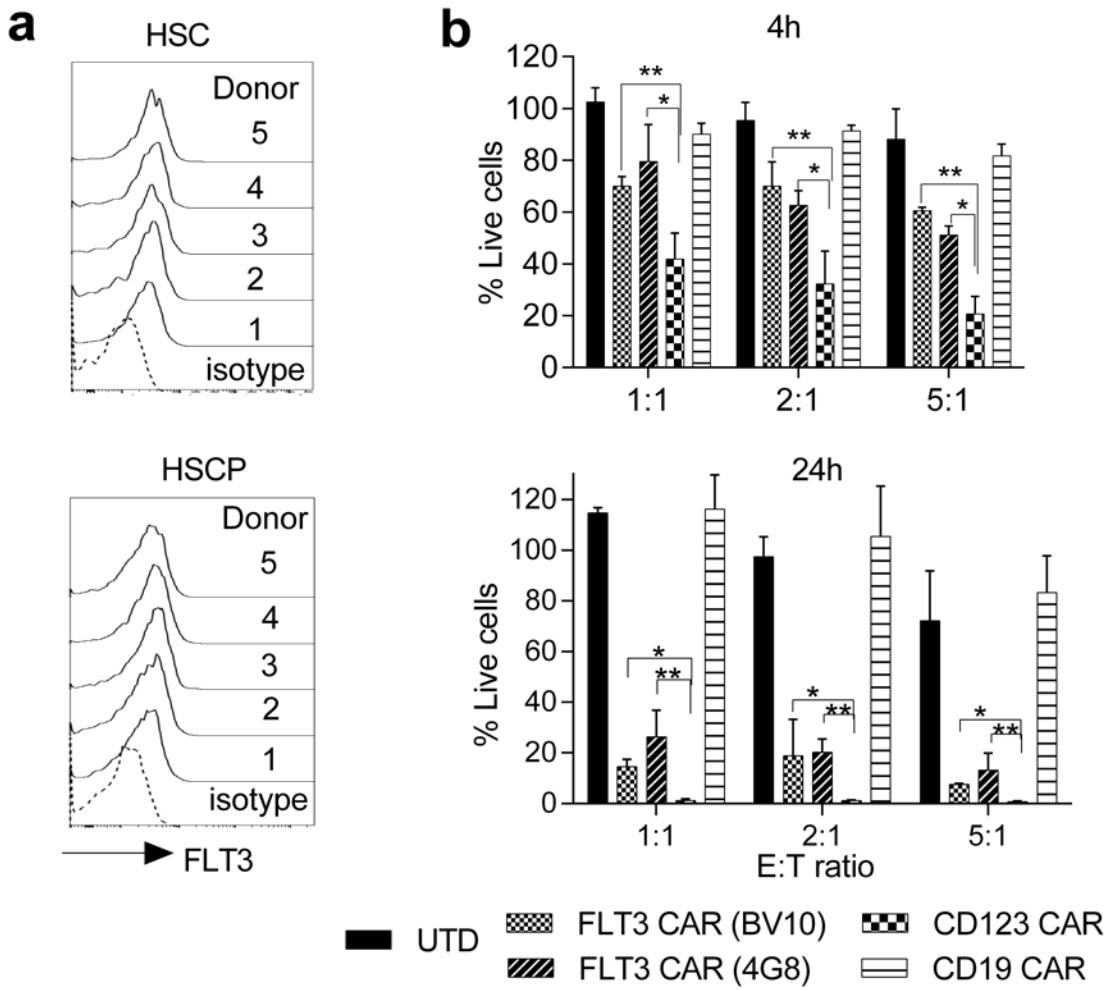


Figure 3.39: FLT3-expression on normal HSC and recognition by FLT3 CAR-T cells. (a) Flow cytometric analysis of FLT3-expression on GM-CSF mobilized peripheral blood HSC (CD34+CD38-) and progenitors (CD34+CD38+) from five different healthy donors. (b) Residual live CD34+ HSC after 4-hour (upper diagram) and 24-hour (lower diagram) co-incubation with FLT3 CAR-T and CD123 CAR-T cells at different effector to target ratio. Assay was performed in triplicate wells with 5,000 target cells/well. Counting beads were used to quantitate the number of residual live HSCs at the end of co-culture. Data shown are mean ± s.d. from n=3 independent experiments with T cells and HSC obtained from different donors. *p<0.05, **p<0.005 (Student's t-test).

FLT3 is known to be expressed by normal HSC and progenitors[65], and therefore, we sought to determine potential collateral damage caused by FLT3 CAR-T cells on normal hematopoietic stem and progenitor cells.

First, we confirmed FLT3-expression on (healthy donor derived) GM-CSF-mobilized peripheral blood $CD34^{+}CD38^{-}$ HSCs and $CD34^{+}CD38^{+}$ progenitor (HSCP) cells. We observed lower FLT3 density on the cell surface compared to AML cell lines and primary AML (Figure 3.39a, 3.5a, 3.8a, and 3.10a). Next, we carried out a flow cytometry-based *in vitro* cytotoxicity assay and observed that at higher E:T ratio, both the FLT3 CAR-T cells lysed approx. 50% and 80% of normal HSCs within 4 and 24 hours, respectively (E:T ratio = 5:1) (Figure 3.39b). We also included T cells expressing a CD123-specific CAR as a reference for the assay.

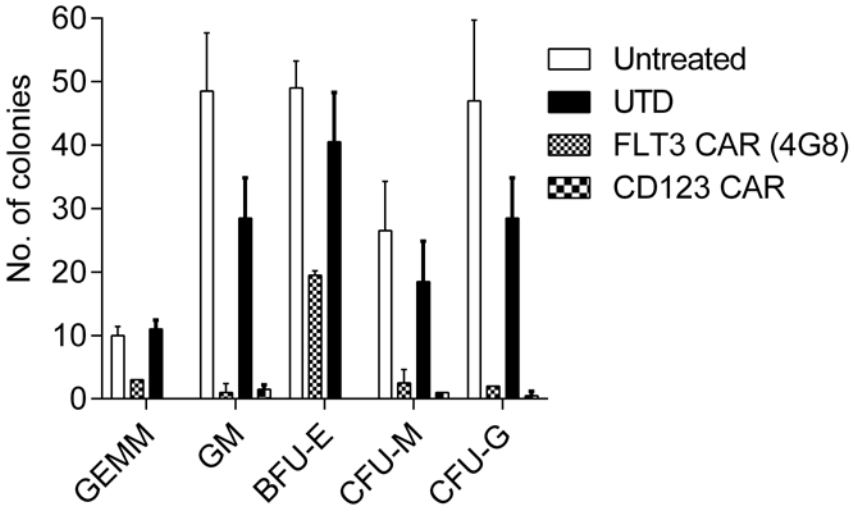


Figure 3.40: Colony formation by normal HSC after treatment with FLT3 CAR-T cells. (a) Colony formation assay performed with residual, live HSCs after 24 hours of co-incubation with CD8+ FLT3 CAR-T, CD123 CAR-T or untransduced T cells. Diagram shows the absolute number of colonies (mean ± s.d.) per 55 mm plate as determined by microscopy on day 14. Data shown are representative of results obtained in independent experiments with T cells from n=3 donors. GEMM (Granulocyte/erythroid/macrophage/megakaryocyte); GM (Granulocyte/macrophage); CFU-E (Colony forming unit-erythroid); CFU-M (Colony forming unit-Macrophage); CFU-G (Colony forming unit-Granulocyte).

CD123 CAR-T cells have been reported to completely eliminate normal HSCs and induce myeloablation[50]. CD123 CAR-T cells exerted a faster and stronger cytolytic effect on normal HSCs compared to FLT3 CAR-T cells and lysed >95% of HSCs within 24-hour (Figure 3.39b).

Next, we performed *in vitro* colony formation assays from residual HSCs at the end of the 24-hour co-culture with FLT3 or CD123 CAR-T cells. We only detected a small number of erythroid colonies after 14 days, while formation of myeloid colonies was completely abrogated after FLT3 CAR-T cell treatment (Figure 3.40).

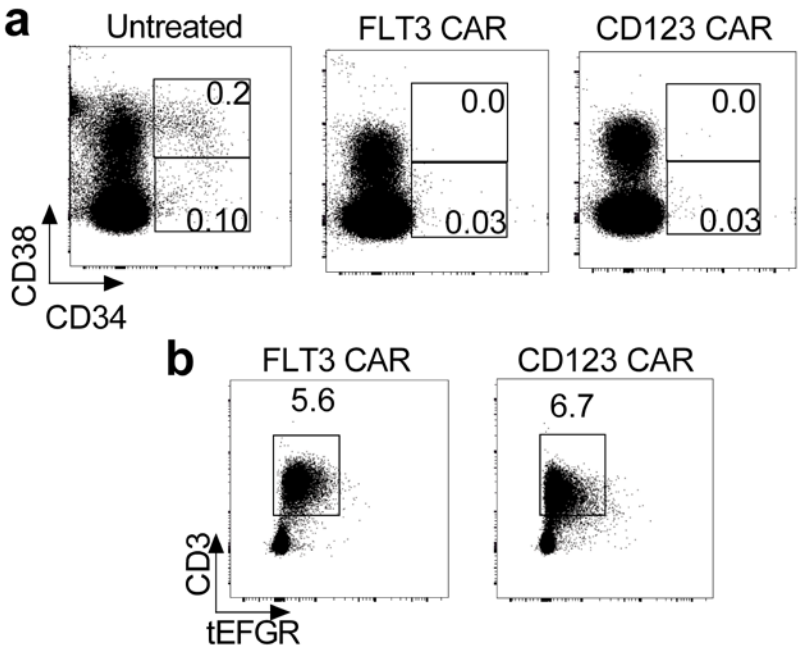


Figure 3.41: Recognition of HSCs by FLT3 CAR-T cells in NSG-3GS mice *in vivo*. (a) Immunodeficient NSG-3GS mice were engrafted with normal HSCs and treated with FLT3 CAR-T or CD123 CAR-T cells after 8 weeks. Dot plots show the frequency of HSCs ($CD34^+CD38^-$) and progenitors ($CD34^+CD38^+$) in bone marrow of mice. (b) Dot plots show CAR+ T cells (CD3+ EGFRt+) in bone marrow of mice. Data shown are representative for n=3 mice in each treatment group.

To support our observations with *in vivo* data, we utilized humanized mouse model to mimic human hematopoiesis in mice. We injected $1x10^6$ CD34+ normal HSCs to NSG-

3GS mice and analyzed peripheral blood to confirm HSC engraftment. After an 8-week engraftment period, mice were treated with a single dose of FLT3 or CD123 CAR-T cells. We observed that normal HSCs and progenitor cells were depleted from bone marrow of mice after treatment with FLT3 CAR-T cells, and similarly after treatment with CD123 CAR-T cells (Figure 3.41, 3.42a). We observed expansion of FLT3 and CD123 CAR-T cells in bone marrow, and depletion of progenitors and mature B cells (due to lack of HSCs) in CAR-T cell treated mice (Figure 3.41, 3.42).

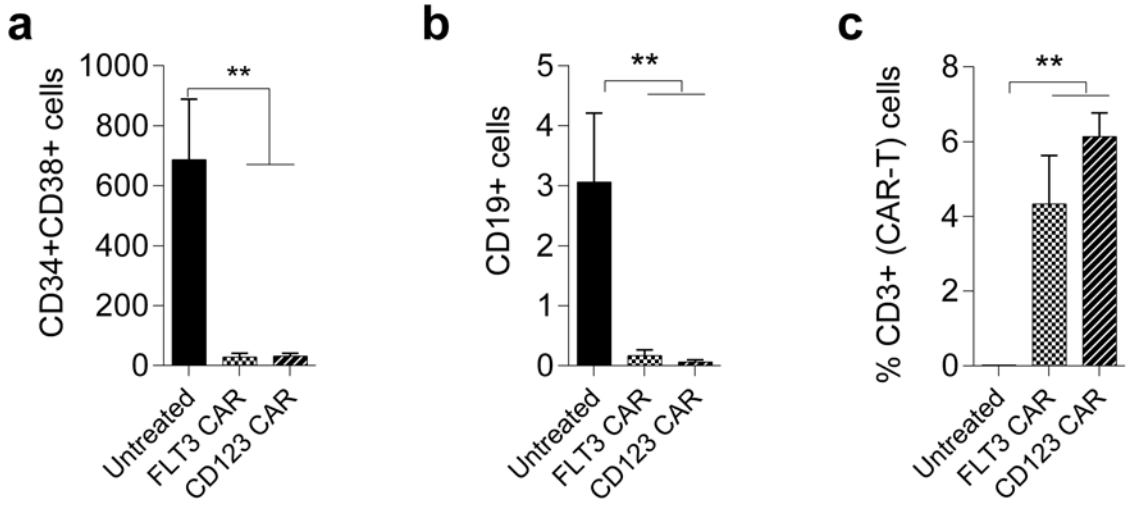


Figure 3.42: The residual human cells after FLT3 CAR-T cells treatment in BM of NSG-3GS mice. Diagrams show (a) absolute number of progenitor cells (b) the percentage of B-cells ($CD19^+$) and (c) CAR-T cells ($CD3^+EGFRt^+$) in the bone marrow of NSG-3GS mice that had been engrafted with human CD34+ HSC. ***p*<0.005 (Student's t-test).

In summary, these data show that FLT3 CAR-T cells recognized and eliminated normal HSCs *in vitro* and *in vivo*. These data suggest that the clinical use of FLT3 CAR-T cells against AML would be restricted to a defined window of time prior to allogeneic HSCT that allows successive CAR-T cell depletion and reconstitution of the hematopoietic system.

3.4 Aim-4: A safety mechanism to deplete FLT3 CAR-T cells

3.4.1 Depletion of Jurkat T cells by iCasp9

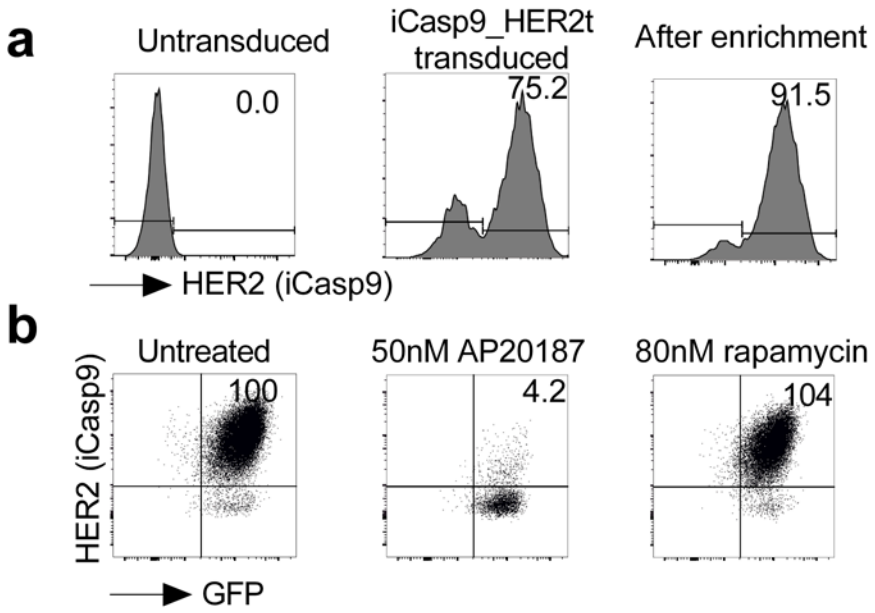


Figure 3.43: iCasp9_HER2t transduction in Jurkat T cells and analysis of apoptosis. (a) Flow cytometric analysis of HER2t (truncated HER2) expression on Jurkat (GFP+) cells after lentiviral transduction and enrichment. (b) iCasp9_HER2t+ Jurkat T cells (also GFP+) were cultured in absence or presence of the dimerizer molecules AP20187 (specific for iCasp9 activation) or rapamycin (negative control). After 24-hour, the cells were washed and flow cytometric analysis was carried out. HER2t-positive cells in flow plots represent residual iCasp9+ Jurkat cells. 7-AAD was used to discriminate live and dead cells. The assay was performed in triplicates and cells were pooled for flow cytometric analysis. Counting beads were used for quantification of residual live cells. Representative plot from n=3 independent experiment.

To achieve the clinical setting described previously, a safety mechanism which enables rapid and complete elimination of FLT3 CAR-T cells is required. Inducible caspase 9 is the strongest suicide gene and therefore we thought to utilize it to achieve CAR-T cell depletion. We modified the iCasp9_GFP addgene plasmid vector (#15567)[107] and replaced GFP with HER2t (iCasp9_HER2t on the same gene cassette under the same

promoter, separated by T2A sequence) to facilitate enrichment of iCasp9-positive T cells using magnetic selection.

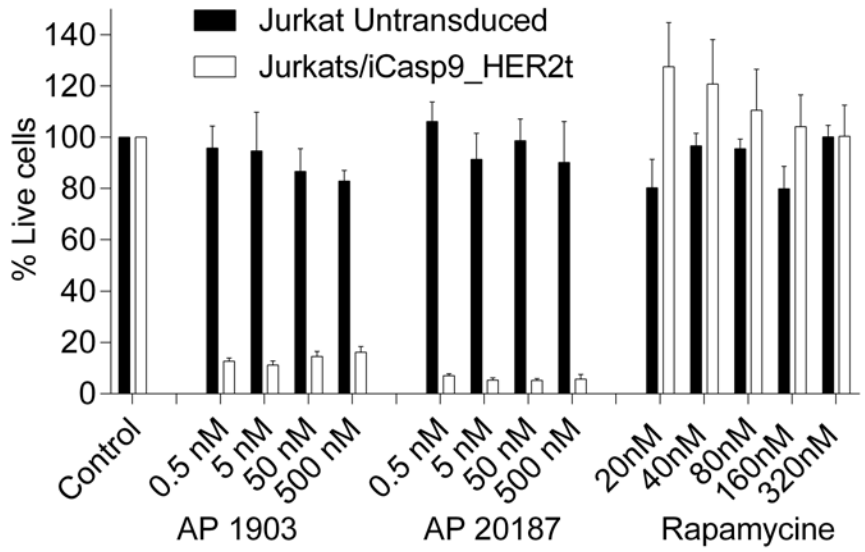


Figure 3.44: Analysis of apoptosis induction in iCasp9_HER2t-positive Jurkat T cells. The graph represents residual live cells after treatment with AP1903 (0.5-500 nM), AP20187 (0.5-500 nM) or rapamycin (20-320 nM) for 24-hour. The data represents mean + SEM from n=3 independent experiments. AP1903 and AP20187: specific dimerizer, Rapamycin: negative control.

Next, we transduced Jurkat T cells (GFP+) with a lentiviral vector encoding iCasp9_HER2t and confirmed HER2t-expression using flow cytometric analysis (Figure 3.43a). We enriched HER2t-positive (and therefore iCasp9+) cells by flow assisted cell sorting. After enrichment, >90% of Jurkat cells were iCasp9_HER2t+ and therefore, we had an internal control of iCasp9_HER2t negative cells (~10% cells) for the apoptosis assay (Figure 3.43a). We cultured iCasp9+ Jurkat cells in the presence of the dimerizer drug AP20187 and observed that >90% cells underwent apoptosis within 24-hour, while untreated and rapamycin treated cells were unaffected (Figure 3.43b). Jurkat cells that highly expressed iCasp9 underwent apoptosis rapidly while low expressing and iCasp9 negative cells were resistant to dimerizer induced apoptosis. We further confirmed a specific induction of apoptosis in Jurkat cells with an alternative dimerizer drug AP1903 (Figure 3.44). Of note, AP20187 induced apoptosis was stronger compared to AP1903 (Figure 3.44).

3.4.2 iCasp9 safety switch induces apoptosis in CAR-modified T cells

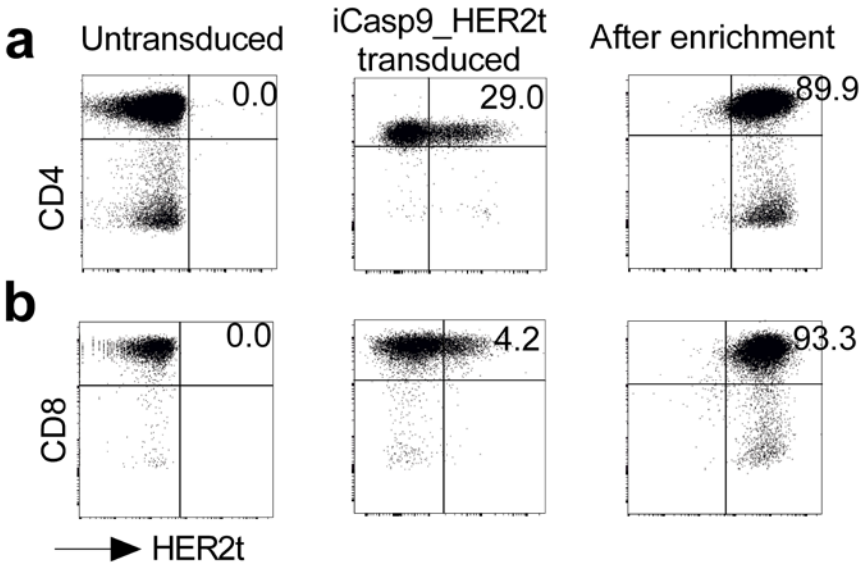


Figure 3.45: Phenotype of iCasp9_HER2t transduced CD4+ and CD8+ T cells. CD4+ and CD8+ T cells were transduced with a lentivirus encoding iCasp9_HER2t. Dot plots from (a) untransduced T cells, CD4+/iCasp9_ HER2t T cells and (b) CD8+/iCasp9_HER2t T cells before and after enrichment. Representative data of T cells prepared from n=2 different donors.

In the subsequent set of experiments, we transduced healthy donor derived CD4+ and CD8+ T cells with a lentivirus encoding for iCasp9_HER2t and enriched HER2t positive cells (Figure 3.45). We cultured CD4+/iCasp9_HER2t T cells and CD8+/iCasp9_HER2t T cells in presence of different concentrations of AP20187 or rapamycin. We observed that T cells that expressed high levels of iCasp9 underwent apoptosis rapidly, specifically with a 10-50 nM dose of AP20187, but remained unaffected by the non-specific dimerizer rapamycin (Figure 3.46). We observed elimination of ~80 % T cells after a 24-hour treatment with 10-50 nM AP20187 (Figure 3.46). However, low iCasp9-expressing T cells were resistant to the dimerizer and did not undergo apoptosis after 72 hour (data not shown). Of note, CD4+ T cells were more susceptible to dimerizer-induced apoptosis, compared to CD8+ T cells (Figure 3.46).

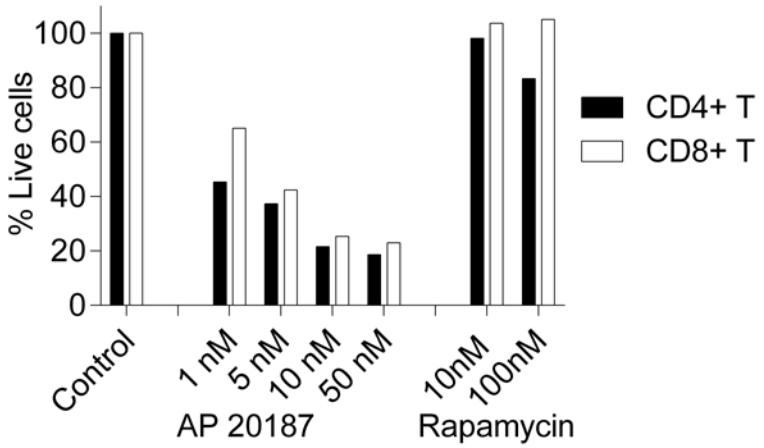


Figure 3.46: Induction of apoptosis in CD4+ and CD8+ T cells. CD4+ and CD8+ T cells were cultured in presence of dimerizers for 24-hour, and flow cytometry analyses were carried out. (a) Residual live cells after treatment with AP20187 (1-50 nM) and rapamycin (10-100 nM). The assay was carried out in triplicates and cells were pooled for flow cytometric analysis. Counting beads were used for quantification of residual live cells. Representative data of T cells prepared from n=2 different donors.

Then, we co-transduced healthy donor-derived CD4+ and CD8+ T cells with iCasp9_HER2t and FLT3 CAR encoding lentiviruses. We enriched iCasp9+ cells and carried out an *in vitro* cytotoxicity assay. We observed significant reduction of cytotoxicity mediated by FLT3 CAR/iCasp9 T cells against MOLM-13 cells after 4-hours and 24-hours in presence of AP20187 (Figure 3.47). However, there was no complete inhibition of cytolytic activity of FLT3 CAR/iCasp9 T cells in presence of AP20187. This is indicative of anti-leukemia activity mediated by low iCasp9 expressing FLT3 CAR-T cells that were resistant to AP20187 induced apoptosis.

In summary, the data shows that a single treatment with AP20187 leads to an elimination of ~80 % CD4+ and CD8+ T cells. High iCasp9-expressing T cells are preferentially undergoing apoptosis while low iCasp9-expressing T cells are resistant to dimerizer-induced apoptosis.

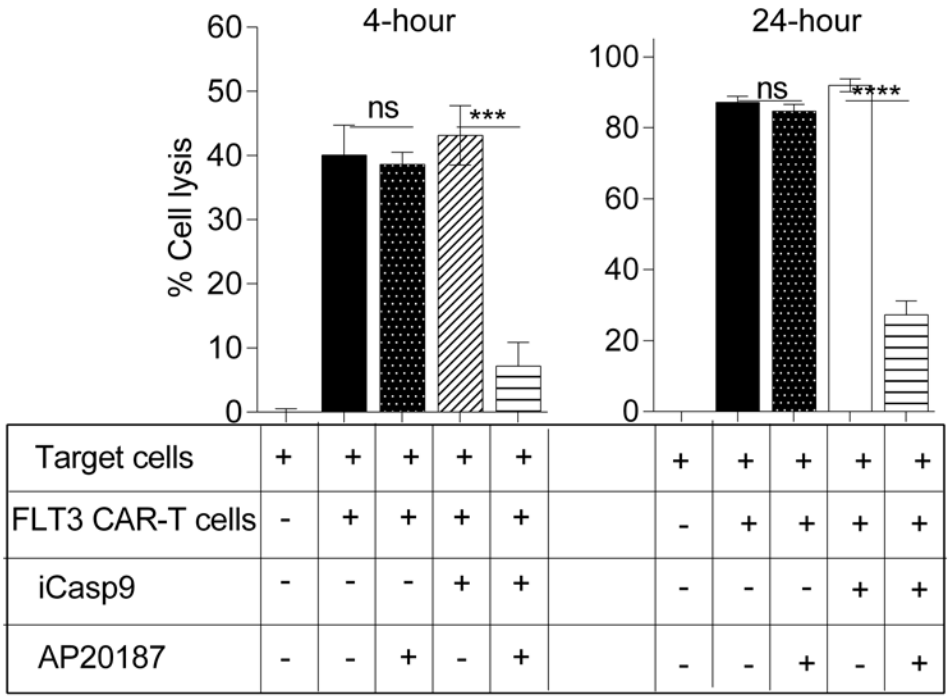


Figure 3.47: FLT3 CAR-T cells mediated cytotoxicity in the presence of AP20187. Cytolytic activity mediated by CD8+ FLT3 CAR-T cells in a 4-hour and 24-hour co-culture assay with MOLM-13 cells at an 10:1 E:T ratio, evaluated by bioluminescence measurement. Values represent mean ± s.d. Representative data of T cells prepared from n=2 different donors. ***p<0.0005, ****p<0.0001 (Student's t-test).

Collectively, the iCasp9 safety switch rapidly eliminates FLT3 CAR-T cells, particularly high iCasp9-expressing cells and significantly reduces anti-leukemia activity of FLT3 CAR-T cells. However, low iCasp9-expressing cells did not reach the threshold for apoptosis induction and were resistant to dimerizer induced activation of apoptosis. Thus, elimination of FLT3 CAR-T cells is incomplete and could cause difficulties in a subsequent engraftment of allogeneic stem cells (as graft) as the remaining CAR-T cells might attack HSC cells of the graft. To overcome this challenge, the gene transfer could be improved to enhance iCasp9 expression, or low iCasp9–expressing CAR-T cells could be sorted out before CAR-T cell transfer. Thus, it will be importnat to formulate FLT3 CAR-T cells products in a way that ensure a reliable and complete elimination of FLT3 CAR-T cells equipped with the iCasp9 safety switch.

3.5 FLT3 CAR-T cells in ALL and MLL

FLT3-expression has been reported in patients with acute lymphoblastic leukemia (ALL) and mixed lineage leukemia (MLL)[62,64,70,114]. To explore additional applications, we wanted to assess if FLT3 CAR-T cells can recognize ALL and MLL.

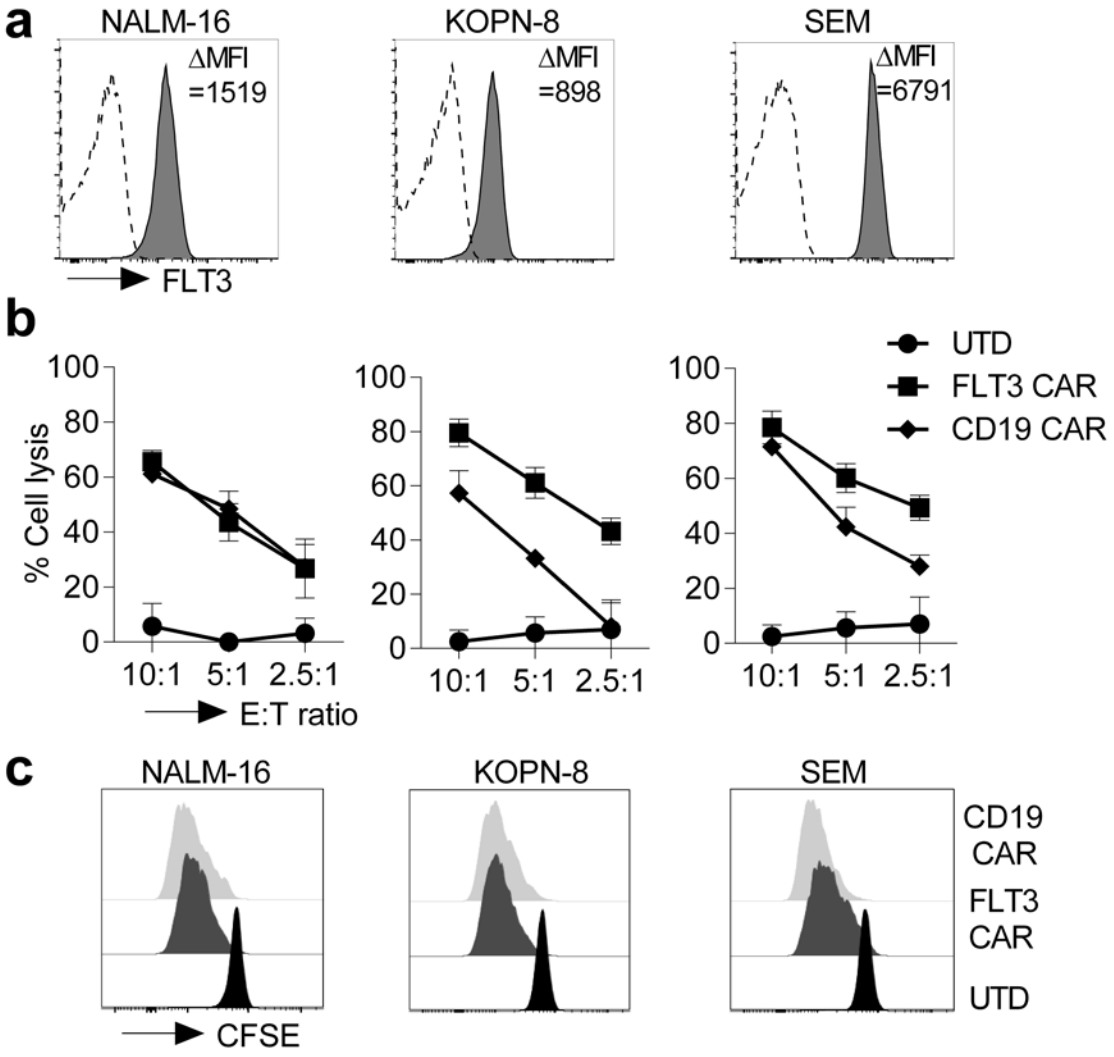


Figure 3.48: Recognition and specific reactivity of FLT3 CAR-T cells against ALL and MLL cell lines *in vitro*. (a) Flow cytometric analysis of FLT3-expression by ALL (NALM-16) cells and MLL (KOPN-8 and SEM) cells. Inset number represent the absolute difference between the MFI of anti-FLT3 and isotype staining. (b) Specific cytolytic activity in a 4-hour co-culture assay with ALL and MLL cell lines as target cells, evaluated by bioluminescence measurement. Values represent mean ± s.d. (c) Proliferation of $CD4^+$ FLT3 CAR-T and CD19 CAR-T cells was examined by CFSE dye dilution after 72 hour of co-culture. Representative data of T cells prepared from n=2 different donors.

To evaluate the activity of FLT3 CAR-T cells against ALL and MLL, we included NALM-16 (wt FLT3+, CD19+ pediatric ALL), KOPN-8 (wt FLT3+, CD19+ infant MLL with KMT2A-MLLT1 fusion gene) and SEM (wt FLT3+, CD19+ pediatric MLL with KMT2A-AFF1 fusion gene) into our analyses. First, we confirmed FLT3-expression by all three cell lines using flow cytometry (Figure 3.48a). Then we carried out functional analyses and observed specific high-level cytolytic activity of CD8+ FLT3 CAR-T cells at multiple effector to target cell ratios (range: 10:1 – 2.5:1) against all three cell lines (Figure 3.48b). Further, FLT3 CAR and CD19 CAR-T cells underwent antigen specific proliferation after stimulation with all three target cell lines, whereas control T cells did not show any proliferation (Figure 3.48c).

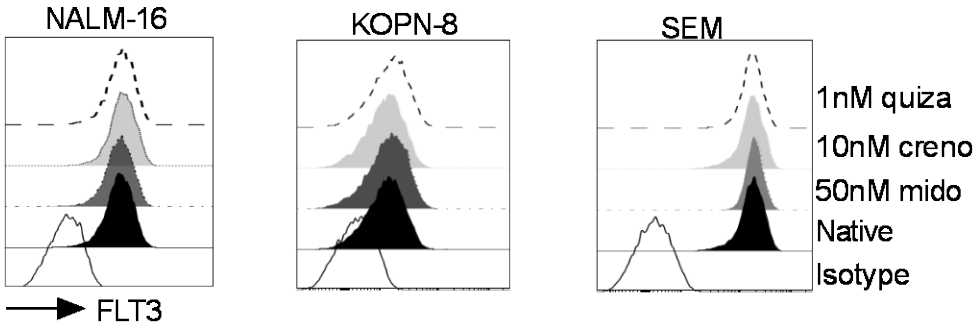


Figure 3.49: FLT3-expression on ALL and MLL cell lines after FLT3 inhibitors treatment. Flow cytometric anaylsis of FLT3-expression on ALL and MLL cell lines that were cultured in absence or presence of 50 nM midostaurin, 10 nM crenolanib or 1 nM quizartinib for 1 week.

Next, we wanted to study if FLT3-expression on ALL and MLL cells could be enhanced by FLT3 inhibitors. Therefore, we exposed wild type FLT3 expressing ALL and MLL cells to FLT3 inhibitors for 7 days. However, we did not observe enhanced FLT3-expression on these cells within the assay period (Figure 3.49).

In summary, the data shows that FLT3 protein is expressed by ALL and MLL cell lines, and FLT3 CAR-T cells exert potent effector functions against these cells. The tested cell lines expressed wild type FLT3, and FLT3-expression on the tested ALL and MLL cells was not increased by FLT3 inhibitors. Collectively, FLT3 CAR-T cells exert specific anti-leukemia activity against FLT3 positive ALL and MLL and can be utilized to treat these malignancies.

3.6 A strategy for clinical application of FLT3 CAR-T cells in AML

Therefore, we propose a clinical application of FLT3 CAR-T cells as illustrated in figure 3.50. We suggest an application of autologous FLT3 CAR-T cells (after lymphodepleting chemotherapy) for a defined period within the therapeutic window to induce complete remission or minimal residual disease (MRD) negativity in AML patients. After that, since we anticipate recognition and depletion of normal HSC by FLT3 CAR-T cells, CAR+ T cells should be removed using a safety switch. This could be followed by allogeneic HSCT to consolidate a Graft versus Leukemia (GvL) effect and reconstitute normal hematopoiesis. In case HSC toxicity is not observed in humans, allogeneic HSCT may not be required. Additionally, this would further extend the application of FLT3 CAR-T cells to induce or sustain complete remission in AML patients after allogeneic HSCT.

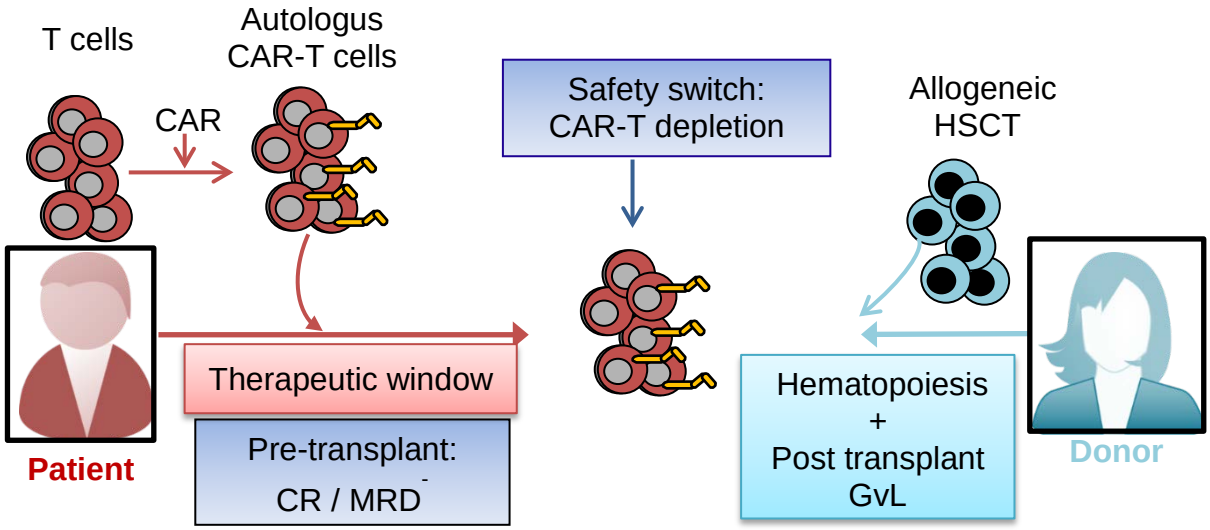


Figure 3.50: Proposed clinical setting of FLT3 CAR-T cells for treatment of AML. Autologous FLT3 CAR-T cells could be used as pre-transplant regime to induce complete remission and/or MRD negativity in AML patients. FLT3 CAR-T cells could be used for a defined period within the therapeutic window and would be depleted afterwards by activating the safety switch. Subsequent, allogeneic HSCT could help to reconstitute normal hematopoiesis. Additionally, the allogeneic graft could also provide post-transplant GvL effect.

In summary, we demonstrate that FLT3 CAR-T cells do not recognize normal peripheral blood cells, including monocytes, and therefore, may not eliminate normal blood cells. However, FLT3 CAR-T cells can be activated by low FLT3-expression on normal HSC

and can eliminate normal HSC along with AML blasts. Thus, this may lead to myeloablation and disruption in normal hematopoiesis even after induction of MRD negativity or complete remission from AML. Therefore, FLT3 CAR-T cells need to be depleted from patient completely before reconstituting normal hematopoiesis by allogeneic HSCT.

4. Discussion

4.1 FLT3 is a novel CAR target in AML

The potent clinical responses achieved by CD19 CAR-T cells in ALL and lymphoma patients with multiple lines of pre-treatments have had transformative scientific and clinical impact in hematology. These results have encouraged an extension of application of CAR-T cells into other hematologic and solid tumors. Interestingly, in spite of the recent outburst of preclinical studies showing potent anti-tumor activity of several AML targeting CAR-T cells, the clinical translation has been much slower and only a small number of AML patients have been treated with CAR-T cells in phase I/II trials[4]. This lack of clinical application highlights inherent challenges and risks in developing CAR-T cell therapy for AML patients.

The important factors that are crucial for successful CAR-T cell immunotherapy of AML include: i) selection of a target antigen that is uniformly expressed on AML blasts and has pathophysiologic relevance in AML, ii) consideration of strategies to alleviate potential on-target off-tumor toxicities, iii) development of approaches to circumvent potential CAR-T cell resistance. We deemed FLT3 amenable to meet the above mentioned criteria and therefore, we pursued FLT3 as a novel CAR target for anti-AML immunotherapy in this study.

Surprisingly, although FLT3 is transmembrane protein, it has attracted little attention as a surface target antigen and efforts have been focused as an intracellular target of tyrosine kinase inhibitors. However, FLT3 is uniformly expressed by AML blasts and present on the cell surface, which makes targeting with CAR-modified T cells feasible. Therefore, we designed CARs from different targeting domains (derived from BV10 and 4G8 mAb) and generated CAR-T cells that recognize FLT3 protein. We reported potent anti-leukemia activity of FLT3 CAR-T cells against AML cell lines expressing wild type and mutant (homozygous/heterozygous ITD^+) FLT3 protein and FLT3-ITD^+ primary AML *in vitro*. We observed that a single dose of FLT3 CAR-T cells resulted in durable remission in AML xenografts (NSG/MOLM-13 and PDX model) after FLT3 CAR-T cell

treatment. Notably, FLT3 CAR-T cell reactivity against primary AML was similar to that of CD123-specific CAR-T cells. CD123 is an alternative AML CAR target and currently being evaluated in pilot clinical trials. Thus, our data suggests that targeting FLT3 could be equally effective as targeting other AML antigens.

Our pre-clinical data suggests that FLT3 CAR-T cell therapy is effective against AML, and may be suited to all AML patients expressing FLT3, independent of mutation status or FAB subtype. In particular, AML patients with FLT3-ITD$^+$ mutations are high risk subgroup and have a high likelihood to benefit from FLT3 CAR-T cell therapy. ITD mutations are crucial for disease progression and therefore, FLT3 in FLT3-ITD$^+$ AML cases is a preferred CAR target and has advantage over the other alternative antigens such as CD33 (siglec-3) and CD123 (interleukin-3 receptor) which are of less pathophysiologic relevance in AML compared to FLT3. Additionally, clinical trials using mAbs against CD33 and CD123 have demonstrated rapid antigen downregulation of these antigens by AML blasts after treatment[115,116]. Therefore, there is concern of antigen downregulation or loss under stronger therapeutic pressure exerted by CD33 or CD123 CAR-T cells. In contrast, antigen downregulation is not observed in a small number of AML patients treated with Fc optimized 4G8 mAb and therefore deemed unlikely to occur after FLT3 CAR-T cells treatment.

Prior studies have demonstrated FLT3-expression in other types of leukemia including, mixed-lineage leukemia (MLL), pre-B-ALL and T-cell acute lymphoblastic leukemia and myelodysplastic syndrome[62-64]. In this study, we demonstrated recognition of MLL and ALL cells by FLT3 CAR-T cells, further increasing the spectrum of hematologic malignancies amenable to adoptive immunotherapy with FLT3 CAR-T cells.

4.2 FLT3 inhibitors as combination partners for FLT3 CAR-T cells

FLT3 is a target for small molecule inhibitors and there is an increasing number of clinical trials that use FLT3 inhibitors in AML[79-81]. Furthermore, the recent approval of the tyrosine kinase inhibitor midostaurin in combination with chemotherapy for newly

diagnosed AML patients has encouraged the development of more specific FLT3 inhibitors[89].

Although the use of FLT3 inhibitors as a monotherapy to treat AML showed promising responses in patients, these were transient responses in majority of patients. This was caused partly by an acquired resistance to FLT3 inhibitor treatment. The development of resistance by AML blasts to FLT3 inhibitors is a major challenge and known to occur through various mechanisms. One mechanism is the development of point mutations i.e. in the activation loop at D835[117,118], the gatekeeper site F691[119], a secondary mutation at position N676[120,121] and others[122]. These mutations disrupt the binding of FLT3 inhibitors to the FLT3 kinase domain, resulting in an acquired resistance, particularly to type II FLT3 inhibitors such as quizartinib, midostaurin, sorafenib and others. This observation further indicates the need for combinatorial therapies in order to prevent resistance toward FLT3 inhibitors and to achieve prolonged remissions.

Interestingly, one of the resistance mechanisms that have been observed after repeated application of the FLT3 inhibitors sorafenib and lestaurtinib in AML patients is the upregulation/overexpression of cell surface FLT3 protein by AML blasts[81,111] and non-responsiveness to the FLT3 inhibitor treatment. Intriguingly, it has been shown that higher surface target antigen density positively correlates with CAR-T cell functions[91]. Hence, there is a conceptual appeal of combinatorial use of FLT3 inhibitors and FLT3 CAR-T cells: when AML blasts acquire resistance to FLT3 inhibitors and upregulate FLT3, they expose themselves to CAR mediated recognition and elimination. Indeed, our data show strong upregulation of FLT3 in FLT3-ITD$^+$ AML cells after treatment with the FLT3 inhibitors crenolanib, quizartinib and midostaurin, and enhanced anti-leukemia reactivity of FLT3 CAR-T cells against FLT3 inhibitor pre-treated FLT3-ITD$^+$ AML cells *in vitro* and *in vivo*. Furthermore, our observation after FLT3 inhibitors treatment is that FLT3 upregulation occurs specifically in AML cells with FLT3-ITD mutation, supporting the critical role of FLT3-ITD in the pathogenesis of FLT3-ITD+ AML. This observation also suggests that a combinatorial use of FLT3 inhibitors with FLT3 CAR-T cell may enhance FLT3 CAR-T cells activity exclusively against mutant FLT3 expressing malignant cells and not against wt FLT3 expressing normal cells. This target selectivity

could reduce the risk of on-target off-tumor reactivity and of targeting healthy tissues. Hence, our data demonstrate for the first time that CAR-T cell immunotherapy and kinase inhibitors can be used synergistically in a hematologic malignancy, and provide proof-of-concept with the combination of FLT3 CAR-T cells and FLT3 inhibitors. Additionally, the combinatorial therapy approach may not only overcome the limitation of FLT3 antigen availability in FLT3-ITD+ AML cells but could also prevent adaptive resistance to an FLT3 inhibitor. Beyond CAR-T cell immunotherapy, our results suggest that a combination of FLT3 inhibitors with FLT3 specific mAb or bispecific T cell engagers (BiTEs, FLT3XCD3) can also provide an alternative for potent immunotherapy of AML. This alternative would be applicable particularly in those patients where the generation of autologous CAR-T cells product fails (reported to be 4-14% patients with CD19 CAR-T therapy clinical trials[123]).

In a recent study, investigators observed FLT3 upregulation after quizartinib treatment in AML cells with activating point mutations in FLT3 TKD, FLT3-ITD mutations or both[111]. Using western blot analysis, the investigators demonstrated that quizartinib enhances the post-translational modification (glycosylation) of immature FLT3-ITD protein in the endoplasmic reticulum, and this mature protein traffics to the cell surface and ultimately resulting in enhanced surface FLT3-expression. The authors also suggest that ligand independent activation and auto-phosphorylation of the FLT3-ITD receptor may prevent the physiological processing that is required for maturation and surface expression of FLT3 protein. They also demonstrated that quizartinib treatment leads to an increase of the FLT3 mRNA level in mutated FLT3+ AML cells. This also suggests a possible feedback response mechanism to compensate for the lack of mature FLT3 receptor in FLT3 mutated cells. It is unknown whether crenolanib and midostaurin enhance surface FLT3 protein by similar or closely related mechanisms and needs to be uncovered in future studies.

In our study, FLT3-expression analysis (after treatment with different FLT3 inhibitors) by flow cytometry and high resolution microscopy revealed that crenolanib and quizartinib led to higher FLT3-expression on AML cells compared to midostaurin. This reflected in significantly higher FLT3 CAR-T cells functionality when used in combination with

crenolanib and quizartinib instead of midostaurin. These data suggest that crenolanib and quizartinib are more effective than midostaurin in enhancing the efficacy of FLT3 CAR-T cell therapy.

Interestingly, crenolanib has the ability to inhibit the active and inactive form of the FLT3 kinase and is therefore a more potent inhibitor of AML blasts with FLT3 TKD mutations than quizartinib. This is owing to the fact that point mutations in FLT3 (i.e. at D835) favor the active kinase conformation which hampers the binding of type II FLT3 inhibitors i.e. quizartinib[117]. Additionally, quizartinib has been shown to inhibit c-Kit and suppress the formation of erythroid colonies more aggressively than crenolanib *in vitro*[124]. Furthermore, our *in vivo* experiments in this study with NSG/MOLM-13 xenografts have shown faster and deeper response for FLT3 CAR-T cell in combination with crenolanib as compared to quizartinib. These observations suggest that crenolanib is the best choice for combination therapy with FLT3 CAR-T cells in patients with ITD and TKD mutations. Our data encourages evaluation of FLT3 CAR-T cells/FLT3 inhibitor combination treatment in a carefully designed clinical trial.

4.3 Myeloablation by FLT3 CAR-T cells and consequences for clinical implementation

Clinical trials targeting CD19, CD22 and BCMA have provided proof for the curative potential of CAR-T cells in hematologic malignancies. However, these trials also uncovered several challenges that affect the safety and limit the efficacy of CAR-T cell therapy. One of these challenges originates from on-target off-tumor recognition by CAR-T cells that causes long-term elimination of healthy cells that express the target antigen[12,125].

Due to a lack of AML restricted surface proteins, leukemia-associated antigens have been the main target of anti-AML CAR-T cells. These antigens are also expressed on normal cells and therefore, recognition of these cells can lead to severe toxicity. In particular, the recognition and elimination of normal HSCs is a legitimate concern with potential anti-AML CAR-T cell candidates and would require to rescue patients with an

allogeneic HSCT to re-establish normal hematopoiesis after anti-AML CAR therapy (Table-4)[4].

Table-4: Expression of AML associated antigen on the healthy cells and potential CAR-T cell toxicities in AML[4]

Antigen	Expression on healthy cells	Potential toxicity
CD33 (siglec-3)	Hematopoietic stem and progenitor cells, mature myeloid cells, macrophages especially kupffer cells	Hematopoietic depletion, myeloablation, sinusoidal obstruction syndrome
CD123 (IL3Rα)	Hematopoietic stem and progenitor cells, endothelial cells, mature myeloid cells, basophils, platelets and low levels on B-lymphocytes	Hematopoietic depletion, myeloablation, cardiac endothelial damage, capillary leak syndrome[126]
CD44v6	Keratinocytes, monocytes, activated T cells	Monocytopenia, skin toxicity
CLL-1	Myeloid cells, lung, GI epithelial cells	Lung, GI and myeloid toxicity
FLT3	Hematopoietic stem and progenitor cells, Cardiomyocytes?* Brain?*	Myeloablation, cardiac and neurotoxicity

*denotes expression as cell surface protein is unknown

In our study, we detected FLT3 protein on HSCs and progenitor cells and confirmed the notion of prior studies, which demonstrated uniform expression of FLT3 on human HSC and progenitor cells obtained from bone marrow and cord blood of healthy donors[62,64,65]. Our flow cytometry data further confirm that FLT3 protein density on normal HSCs is lower compared to AML blasts[22,71]. Although the minimal target antigen density required to induce CAR-T cell activation is unknown, it is presumed to be in the order of (few) hundred molecules per cell[72], which is in the range that has been estimated for FLT3 on HSCs[71]. Indeed, our data show that FLT3 CAR-T cells eradicate the majority of HSCs within a 24-hour co-culture assay, leading to qualitatively and quantitatively impaired hematopoiesis in colony formation assays *in vitro* and in NSG-3GS mice *in vivo*.

Overall, the recognition of normal HSCs by FLT3 CAR-T cells is an anticipated outcome, in line with the previous finding that mAbs, from which we derived the targeting domain for our FLT3 CARs – show uniform binding to normal HSCs[71]. Our data suggest that the clinical use of FLT3 CAR-T cells may be limited to a defined therapeutic window and restricted to a clinical context that allows subsequent reconstitution of the hematopoietic system. Such a window of opportunity is provided in the context of allogeneic HSCT with adoptive transfer of autologous FLT3 CAR-T cells prior to HSCT to reduce leukemia burden and/or induce minimal residual disease (MRD)-negativity, followed by FLT3 CAR-T cell depletion and engraftment of allogeneic donor HSCs. This clinical setting would further enhance the therapeutic efficacy as shown by prior studies in ALL with CD19 CAR-T therapy followed by HSCT[127-129] (without CAR-T cell depletion as it is not required due to lack of CD19 expression on normal HSC).

This approach requires the ability to rapidly and completely remove CAR-T cells to protect incoming normal HSCs. The lymphodepleting chemotherapy prior to HSCT can potentially be used to eliminate CAR-T cells from the patients. Additionally, we have equipped our FLT3 CAR-T cells with an EGFRt depletion marker to facilitate the elimination of CAR-T cells if required. Others have shown that administration of an anti-EGFR mAb (Cetuximab, Erbitux®) can mediate CAR-T cell depletion and reverse CAR-T cell-induced systemic toxicity in immunocompetent mice[96]. Furthermore, we and others have shown that inclusion of an iCasp9 suicide gene accomplished (near)-complete removal of CAR-T cells within few hours after addition of an inducer drug[98,105]. Although iCasp9 mediated depletion of CAR-T cells is rapid, it is not complete and low iCasp9 expressing cells may not undergo apoptosis after dimerizer treatment[98,105]. Therefore, efficient gene transfer methods are required to attain a consistently high gene-transfer rate and ultimately higher intracellular iCasp9 protein levels, equal or higher than the threshold for apoptosis induction. Alternatively, a selection step for enriching high iCaps9 expressing CAR-T cells could be added. Further, use of bi-directional promoters to simultaneously drive the expression of CAR and iCasp9 may be advantageous. This is due to the fact that antigen-specific activation of CAR-T cells induces enhanced expression of the CAR and will therefore also enhance the iCasp9 expression (because

both genes would be on the same expression cassette, driven by the same promoter), which may help to overcome the threshold needed to induce apoptosis. Furthermore, iCasp9 can be combined with lymphodepleting chemotherapy (which is a common practice in HSCT to facilitate HSC engraftment) to induce removal of residual CAR+ T cells prior to HSCT. Additionally, operational models of myeloablative CAR-T cell therapy have been established pre-clinically, shown by alemtuzumab-mediated (anti-CD52 mAb) ablation of CD123 CAR-T cells[97]. These approaches could further help in defining the optimal timing for FLT3 CAR-T cell administration and elimination, and subsequent reconstitution of normal hematopoiesis.

It is uncertain whether a small proportion of $FLT3^{low}$ HSCs may escape elimination by FLT3 CAR-T cells, and whether this diminished pool of HSCs would be capable of replenishing a quantitatively and qualitatively normal hematopoietic system. A recent study suggested that FLT3 CAR-T cells would not deplete HSCs and preserve HSC differentiation *in vivo*[130]. However, the only experiment to validate this statement had been performed in immunodeficient NSG-3GS mice that received simultaneous injections of human HSCs and FLT3 CAR-T cells. It is unclear whether this mode of application actually leads to any interaction of HSC and FLT3 CAR-T cells, especially in the bone marrow. Rather, FLT3 CAR-T cells should have been administered after HSC engraftment and hematopoietic differentiation is established, which takes several weeks in this model[130]. Indeed, our data from the NSG-3GS/HSC model show that normal HSCs are eliminated after adoptive transfer of FLT3 CAR-T cells. Several alternative CAR target antigens including CD33 and CD123 have advanced to the stage of clinical evaluation, but share with FLT3 the challenge of being expressed on normal HSCs[50,131]. CD123 CAR-T cells have been shown to induce myeloablation *in vivo* due to recognition of normal HSCs, consistent with our data that shows rapid and complete elimination of normal HSCs by CD123 CAR-T cells in our co-culture assay *in vitro* and in HSC-engrafted NSG-3GS mice *in vivo*[50]. Recently, a study in a primate model has shown that knocking out CD33 from donor HSC can allow allogeneic HSCT without depleting CD33 CAR-T cells for AML treatment[132]. The exact functional role of CD33 in HSC is not known and these data suggest that it may dispensable for HSC differentiation into early

progenitors. FLT3 is known to have important role in early hematopoiesis and FLT3 knock out mice showed quantitatively impaired hematopoiesis in a murine model[133]. However, mature peripheral blood cells did not differ phenotypically in these knock out mice. These results suggest that FLT3 knock out in HSC may be tolerated, and HSCT using donor derived FLT3 knock out HSC may extend the therapeutic window for FLT3 CAR-T cells, allowing them to induce and sustain durable remissions in AML patients.

It has recently been shown that FLT3 acts as a cytoprotective kinase in cardiomyocytes[134]. It is unknown whether FLT3 surface levels on cardiomyocytes are sufficient for CAR recognition and hence, particular caution must be taken when clinically translating FLT3 CAR-T cell therapy, especially in combination with FLT3 inhibitors. We did not detect FLT3 upregulation after FLT3 inhibitor treatment in AML and non-AML cells that express wt FLT3 (including HSCs), suggesting that a concomitant use of FLT3 inhibitors enhances the selectivity of FLT3 CAR-T cells for FLT3-ITD+ AML blasts compared to HSC and non-AML cells. Whether the human cerebellum expresses FLT3 protein is a subject of discussion and we are currently performing studies using high-resolution microscopy to evaluate FLT3 protein expression in the human brain.

An alternative approach would be to target an antigen that is not expressed by normal HSC and progenitors and thus prevent hematopoietic cell ablation mediated by CAR-T cells. In collaboration with Dr. Christoph Rader (The Scripps, Florida, USA), we have designed CARs targeting an undisclosed antigen (expressed by AML cells). We evaluated the anti-leukemic efficacy of CAR-T cells targeting this undisclosed antigen *in vitro*. We observed recognition and elimination of target antigen-positive AML cell lines *in vitro*. In preliminary experiments, we observed that target antigen is not expressed by normal HSC and CAR-T cells do not recognize normal HSC in cytotoxicity and colony formation assays *in vitro* (data not shown). Further, pre-clinical studies in relevant murine models need to be done to confirm our *in vitro* observations.

4.4 Is antigen loss a threat to FLT3 CAR-T cell therapy? Can FLT3 inhibitors prevent antigen loss in FLT3-ITD+ AML?

An observation from clinical trials with CAR-T cells targeting CD19 and CD22 in ALL is leukemia relapse in a proportion of patients. One of the major reasons for relapse is target antigen loss under therapeutic pressure. Leukemia relapse due to target antigen loss has been observed in 30-60% of all relapses in ALL patients treated with CD19 or CD22 CAR-T cells[9,11,91,135]. The underlying mechanisms of CD19 antigen loss in these patients have been investigated and include: i) acquired mutations (frameshift or missense) in CD19 exon 2-5 leading to truncated CD19 protein which lacks membrane anchorage and therefore lack of cell surface CD19 protein[136] ii) alternative splicing that results in a loss of the epitope targeted by the CAR[137]; iii) lymphoid to myeloid *trans* differentiation of leukemic cells[138]; iv) selective survival of pre-existing antigen-negative leukemia cell clones, as the CAR target antigen is not uniformly expressed on ALL blasts[135]. Additionally, antigen downregulation has also been observed in a myeloma patient treated with anti-BCMA CAR-T cells[139]. This suggests that antigen loss is not exclusive for a particular protein or a disease but can also occur in other malignancies under therapeutic pressure by CAR-T cells. These data highlight the need to select CAR target antigens that are uniformly expressed by malignant cells and ideally of pathophysiologic relevance.

Encouragingly, FLT3 is uniformly expressed in AML blasts, including leukemia stem/initiating cells, suggesting a curative potential of FLT3 CAR-T cell therapy[73,75]. So far, there is no profound clinical experience with FLT3 as an immune target that would allow estimating the risk for mutations in the extracellular FLT3 domain, which may arise during therapy and conceal or remove the epitope recognized by our FLT3 CARs. We observed disease recurrence in anatomical sanctuary sites in NSG/MOLM-13 AML xenograft model. We confirmed that FLT3-expression and the epitope recognized by the FLT3 CAR (extracellular Ig domain 4 of FLT3 which is recognized by 4G8 mAb) had been retained on recurring MOLM-13 cells, but could not detect FLT3 CAR-T cells in sanctuary lesions. Importantly, the lack of T cell persistence is one of the reasons for failure of CD19 CAR-T cell therapy in ALL and lymphoma patients. However, FLT3

CAR-T cells were present in bone marrow, spleen and peripheral blood of the mice at the end of the experiment in our study, confirming persistence of FLT3 CAR-T cells. A reasonable explanation for this observation is that human T cells are unable to migrate through murine endothelial and epithelial barriers, which is in line with previous findings[140]. The recurrence of tumors of hematologic origin in NSG mice has been observed in similar xenograft models (i.e. Raji/NSG lymphoma model[109], MOLM-14/NSG AML model[50]) and therefore does not necessarily reflect leukemia relapse observed in patients.

FLT3-ITD and TKD mutations are known driver mutations in AML and also play an important role in sustaining malignant blasts. Our data and data from clinical trials suggest that continuous application of FLT3 inhibitors can enhance surface FLT3 protein. Thus, combination therapy of FLT3 CAR-T cells with FLT3 inhibitors will not only overcome the limitation of FLT3 antigen availability in FLT3-ITD+ AML but may also prevent opportunities for antigen loss and resulting relapse. However, FLT3 inhibitors do not upregulate FLT3 on wt FLT3-expressing AML cells and may not be able to prevent downregulation or loss of FLT3 in FLT3-wt clones.

Although there is uniform FLT3-expression on AML blasts, due to clonal heterogeneity, there is a potential risk of $FLT3^{-/low}$ AML relapse which may develop from FLT3 wt subclones even in patients that are classified as FLT3-ITD+. In our study, we did not observe $FLT3^{-/low}$ AML escape variants in a primary AML xenograft model. However, it has been shown that FLT3-ITD+ AML subclones show preferential engraftment in NSG mice compared to FLT3 wt subclones and therefore, the NSG mouse model may underestimate this risk[110]. Further preclinical models mimicking antigen loss escape could help to investigate whether FLT3 inhibitors can prevent antigen loss.

Alternatively, the use of tandem or compound CARs targeting two different antigens may prevent antigen loss escape. In our laboratory, we have CARs specific to FLT3 and undisclosed antigen and T cells expressing compound CAR could help to overcome the challenge of antigen loss. Encouragingly, a recent case report has shown remission from AML in a patient treated with a CD33/CLL-1 compound CAR-T cells[141]. This further

demonstrates the feasibility of dual targeting in AML, in our case with CAR-T cells against FLT3/undisclosed antigen.

4.5 Conclusion and perspective: roadmap to clinical translation

In this study, we demonstrated that targeting FLT3 using CAR-T cells eradicate AML blasts *in vitro* and *in vivo*, and that FLT3 CAR-T cells act synergistically with FLT3 inhibitors. Our data suggests that FLT3 CAR-T cells, in combination with FLT3 inhibitors, can provide a curative treatment option for high-risk FLT3-ITD+ AML patients and encourages the clinical evaluation of this combination treatment, preferably FLT3 CAR-T cells with crenolanib in high-risk AML patients.

We observed depletion of normal HSC in cytotoxicity assay *in vitro* and in NSG-3GS mice, and therefore precaution must be taken before the clinical application of FLT3 CAR-T cells in humans. Furthermore, pre-clinical studies to evaluate potential toxicities other than HSC depletion (i.e. cardiac toxicity, neurotoxicity) should also be carried out before an evaluation of the combinatorial therapy approach in higher primates and in humans.

FLT3 CARs used in the study are endowed with the EGFRt transduction marker, and as shown previously, use of cetuximab provides a means for CAR-T cell depletion[96]. Additionally, use of the iCasp9 safety switch in combination with lymphodepleting chemotherapy can deplete FLT3 CAR-T cells. Furthermore, patients will likely undergo HSCT after FLT3 CAR-T cell therapy if depletion of HSC is observed and therefore should have a matched stem cell donor at the time of enrollment in a clinical trial (which is an inclusion criteria in clinical trials that currently use CD123 CAR-T cells to treat AML patients in USA). Moreover, AML patients who are refractory to currently available treatments and/or relapsed after HSCT, and other high risk sub-group of AML patients (FLT3-ITD+ AML) should preferably be enrolled in early phase clinical trials. Lastly, a treatment of other aggressive form of leukemia (i.e. MLL, T-ALL and myelodysplastic syndrome) is a challenge to hematologists and FLT3 CAR-T cell therapy could be extended to these malignancies in the future.

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List of abbreviations

µ	micro (10^{-6})
× *g*	times gravity
µg	microgram
µL	microliter
µM	micromolar
µm	micrometer
nM	nanomolar
˚C	centigrade
7-AAD	7-aminoactinomycin D
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CEBPA	CCAAT/enhancer-binding protein alpha
CFSE	Carboxyfluorescein succinimidyl ester
CO_2	Carbon dioxide
CR	Complete remission
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dSTORM	Direct Stochastic optical reconstruction microscopy
EDTA	Ethylenediaminetetraacetic acid
EF1	Elongation factor 1
eGFP	Enhanced green fluorescent protein
EGFRt	Human truncated epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
E:T	Effector : Target
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
ffluc	Firefly luciferase
FLT3	FMS like tyrosine kinase 3

GMCSF	Granulocyte macrophage colony-stimulating factor
GvHD	Graft versus host disease
GvL	Graft versus leukemia
HS	Human serum
HSC	Hematopoietic stem cell
HSCP	Hematopoietic stem cell progenitors
HSCT	Hematopoietic stem cell transplantation
HTLV-1	Human T-lymphotropic virus 1
iCasp9	Inducible caspase 9
IDH	Isocitrate dehydrogenase 1
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
ITD	Internal tandem duplication
JM	Juxtamembrane
LSC	Leukemic stem cells
LV	Lentiviral
mAb(s)	Monoclonal antibody(ies)
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
min	Minute
mL	milliliter
MLL	Mixed lineage leukemia
mM	millimolar
MOI	Multiplicity of infection
NPM-1	Nucleophosmin-1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD	Progressive disease
PD-1	Programmed cell death protein-1
PDL	Poly D-lysine
PDGFR	Platelet-derived growth factor receptor

PE	Phycoerythrin
PFA	Paraformaldehyde
PMA	Phorbol-12-myristat-13-acetate
PR	Partial response
scFv	Single-chain variable fragment
SD	Stable disease
s.d.	Standard deviation
TCR	T cell receptor
TKI	Tyrosine kinase inhibitor
TKD	Tyrosine kinase domain
TM	Transmembrane
TM-LCL	Lymphoblastoid cell lines, donor initials: TM
USFDA	United states food and drug administration
Wt	Wild type

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1. **Jetani H**, Garcia-Cadenas I, Nerreter T, Thomas S, Rydzek J, Briones J, Bönig H, Herr W, Sierra J, Einsele H, Hudecek M. CAR-T cells targeting FLT3 have potent activity against FLT3-ITD+ AML and act synergistically with the FLT3-inhibitor crenolanib. ***Leukemia*** (2018); 32(5): 1168-1179.

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