Rational combinatorial targeting by Adapter CAR-T to address inter- and intratumoral heterogeneity in hematologic malignancies

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„Es gibt keine großen Entdeckungen und Fortschritte, solange es noch ein unglückliches Kind auf Erden gibt.“

(Albert Einstein)

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1. Abbreviations

ACT	*adoptive cell transfer*
AdCAR	*adapter chimeric antigen receptor*
ADCC	*antibody-dependent cellular cytotoxicity*
ALL	*acute lymphoblastic leukemia*
AM	*adapter molecule*
AML	*acute myeloid leukemia*
APC	*antigen-presenting cell*
BCMA	*b-cell maturation antigen*
BiTE	*bispecific T-cell engager*
BLI	*bioluminescence imaging*
CAR	*chimeric antigen receptor*
CD	*cluster of differentiation*
CD3z	*t-cell surface glycoprotein CD3 zeta chain*
CDC	*complement-dependent cytotoxicity*
CLL	*chronic lymphocytic leukemia*
CRS	*cytokine-release syndrome*
CTLA-4	*cytotoxic t lymphocyte antigen 4*
DNAM-1	*DNAX accessory molecule-1*
e.g.	*exempli gratia -> for example*
ET	*effector-to-target*
FAB	*fragment antigen binding*
FDA	*food and drug administration*
FITC	*anti-fluorescein isothiocyanate*
FSC	*forward scatter*
GFP	*green fluorescent protein*
hspc	*hematopoietic stem and progenitor cells*
i.v.	*intravenous*
ICANS	*immune effector cell-associated neurotoxicity syndrome*
ICI	*Immune checkpoint inhibitor*
IFN-γ	*interferon-gamma*

IgG	*Immunoglobulin G*
IL-1	*interleukin-1*
IL-12	*interleukin-12*
IL-15	*interleukin-15*
IL-18	*interleukin-18*
IL-2	*interleukin-2*
IL-6	*interleukin-6*
IL-7	*interleukin-7*
LCA	*luciferase-based cytotoxicity assay*
LLE	*linker-label epitope*
LSC	*leukemic stem cell*
MCL	*mantle cell lymphoma*
MFID	*mean fluorescence intensity difference*
MFIR	*mean fluorescence intensity ratio*
MHC	*major histocompatibility complex*
NFAT	*calcineurin-dependent TF Nuclear factor of activated T-cells*
NHL	*non hodgkin lymphom*
NKG2D	*natural killer group 2-member D*
PD1	*programmed cell death protein 1*
PE	*R-phycoerythrin*
r/r	*relapsed/refractory*
s.c.	*subcutaneous*
scFv	*single-chain variable fragment*
SSC	*side scatter*
TAA	*tumor-associated antigen*
TCM	*central memory T cells*
TCR	*t-cell receptor*
TEM	*effector memory T cells*
TEMRA	*effector memory T cells re-expressing CD45RA*
TF	*transcription factor*
TGF-b	*transforming growth factor beta*
TIL	*tumor-infiltrating lymphocyte*
TIM3	*t-cell immunoglobulin and mucin-domain containing 3*

TME	*tumor microenvironment*
TN	*naive t cells*
TNFa	*tumor necrosis factor alpha*
TRUCKs	*t cells redirected for universal cytokine-mediated killing*
TSCM	*stem cell-like memory T cells*
UMAP	*uniform manifold approximation and projection*

2. Summary

CAR-T Cells have completely changed the therapeutic landscape in B-phenotypic malignancies and demonstrated their clinical efficacy in lymphatic leukemia, lymphomas, and myelomas. However, conventional CAR-T cells have their limitations due to a lack of controllability and limited versatility. To achieve temporal and qualitative control of CAR-T function, as well as the ability for multiplex targeting, we have developed the Adapter CAR (AdCAR) system. AdCAR-T cells are redirected to surface antigens via biotinylated adapter molecules (AM) in the context of a specific linker structure, referred to as the Linker-Label Epitope (LLE). AdCAR-T cells are non-functional without the adapter molecules and mediate their anti-cancer function only in the presence of adapter molecules.

In the first project of my thesis the AdCAR System was evaluated in terms of CAR specific activation, cytokine secretion, cytolysis, combinatorial targeting and exhaustion. Alternative target antigens were analyzed by flow cytometry on three NHL cell lines, and AMs were generated by biotinylation of Immunoglobulin G (IgG) 1 against CD19, CD20, CD22, ROR-1, CD276, CD79B, and CD10. AdCAR T cells demonstrated specific activation and cytokine secretion comparable with conventional CD19CAR-T cells. We found that quantitative low molar biotinylation is important to prevent cross-activation. In a CD19 knockout model, we demonstrate the great advantage of combinatorial targeting with the AdCAR in contrast to the mono-targeted CD19CAR to prevent antigen escape.

The second project we focused on combinatorial targeting of Acute myeloid leukemia (AML). AML-associated antigen expression on 30 pediatric AML samples was analyzed by multicolor flow cytometry. CD33, CD38, CD371, IL1RAP and CD123 were identified as the most frequently expressed antigens. High variability among patient samples and also within leukemic cells from the same patient suggests that single antigen targeting is destined to lead to antigen escape and therapy failure. We generated AMs against CD33, CD38, CD123, CD135 and CD371 and validated them *in vitro* and *in vivo* on AML cell lines. In two PDX model preserving the intratumoral heterogeneity of the primary disease, single antigen targeting against either CD33, CD38, or CD371 led to antigen negative relapse of the disease. In contrast, a combinatorial targeting of CD33, CD38, and CD371 resulted in disease clearance

and lasting remission. In conclusion, we were able to shown that the AdCAR technology enables controllable, flexible, combinatorial, and selective targeting.

3. Zusammenfassung

CAR-T-Zellen haben die Therapie von B-phänotypischen-Malignomen vollständig verändert und ihre klinische Wirksamkeit bei lymphatischer Leukämie, Lymphomen und Myelomen gezeigt. Konventionelle CAR-T-Zellen sind allerdings aufgrund mangelnder Kontrollierbarkeit und eingeschränkter Vielseitigkeit nur begrenzt einsetzbar. Um die CAR-T-Funktion besser zu kontrollieren und zeitlich zu begrenzen sowie eine Kombination verschiedener Antigene zu erreichen, wurde das Adapter-CAR (AdCAR)-System entwickelt. AdCAR-T-Zellen funktionieren nur mithilfe biotinylierter Adaptermoleküle (AM) welche eine spezifische Linker-Label-Epitop (LLE)-Struktur aufweisen.

Im ersten Projekt dieser Arbeit wurde das AdCAR-System hinsichtlich CAR-spezifischer Aktivierung, Zytokin Ausschüttung, Zytolyse, Kombinatorik und Erschöpfung untersucht. Oberflächenantigene wurden durchflusszytometrisch an drei NHL-Zelllinien analysiert, und AMs durch Biotinylierung von IgG1 gegen CD19, CD20, CD22, ROR-1, CD276, CD79B und CD10 generiert. AdCAR-T-Zellen zeigten eine spezifische Aktivierung und Zytokin Ausschüttung vergleichbar mit der von konventionellen CD19CAR-T-Zellen. Niedermolare Biotinylierung ist jedoch wichtig, um eine unspezifische Aktivierung zu verhindern. In einem CD19-Knockout-Modell konnte der große Vorteil der Kombinatorik mit dem AdCAR-System gegenüber dem CD19CAR gezeigt werden.

Das zweite Projekt konzentrierte sich auf die Akute Myeloische Leukämie (AML). Die Expression von AML-assoziierten Antigenen auf 30 pädiatrischen AML-Proben wurde mittels Durchflusszytometrie analysiert. CD33, CD38, CD371, IL1RAP und CD123 wurden als die am häufigsten exprimierten Antigene identifiziert. Eine hohe Variabilität zwischen den Patientenproben sowie innerhalb der leukämischen Zellen desselben Patienten legt nahe, dass das Adressieren eines einzelnen Antigens zu einem Therapieversagen führen kann. AMs gegen CD33, CD38, CD123, CD135 und CD371 wurden generiert und mit AdCAR-T Zellen *in vitro* sowie *in vivo* an AML-Zelllinien validiert. In einem PDX-Modell, das die intratumorale Heterogenität der primären Erkrankung bewahrt, führten Monotherapien (CD33, CD38 oder CD371) zu

einem antigen-negativen Rezidiv. Im Gegensatz dazu führte die Kombination von CD33, CD38 und CD371 zu einer anhaltenden Remission. Zusammenfassend wurde gezeigt, dass die AdCAR-Technologie eine kontrollierbare, flexible, kombinatorische und selektive Therapie ermöglicht.

4. Publications

4.1 Accepted publications

1. **Atar, D**., Ruoff, L., Mast, A.-S., Krost, S., Moustafa-Oglou, M., Scheuermann, S., Kristmann, B., Feige, M., Canak, A., Wolsing, K., Schlager, L., Schilbach, K., Zekri, L., Ebinger, M., Nixdorf, D., Subklewe, M., Schulte, J., Lengerke, C., Jeremias, I., Werchau, N., Mittelstaet, J., Lang, P., Handgretinger, R., Schlegel, P., and Seitz, C.M. (2024). Rational combinatorial targeting by adapter CAR-T-cells (AdCAR-T) prevents antigen escape in acute myeloid leukemia. Leukemia *38*, 2183-2195. 10.1038/s41375-024-02351-2.

2. Heinz, A.T., Calkoen, F.G.J., Derbich, A., Miltner, L., Seitz, C., Doering, M., Braun, C., **Atar, D.**, Schumm, M., Heubach, F., Arendt, A.-M., Schulz, A., Schuster, F.R., Meisel, R., Strahm, B., Finke, J., Heineking, B., Stetter, S., Silling, G., Stachel, D., Gruhn, B., Debatin, K.-M., Foell, J., Schulte, J.H., Woessmann, W., Mauz-Körholz, C., Tischer, J., Feuchtinger, T., Handgretinger, R., and Lang, P. (2023). Automated production of specific T cells for treatment of refractory viral infections after allogeneic stem cell transplantation. Haematologica 108, 2080-2090. 10.3324/haematol.2022.281996.

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4. Pecher, A.-C., Hensen, L., Klein, R., Schairer, R., Lutz, K., **Atar, D.**, Seitz, C., Stanger, A., Schneider, J., Braun, C., Schmidt, M., Horger, M., Bornemann, A., Faul, C., Bethge, W., Henes, J., and Lengerke, C. (2023). CD19-Targeting CAR T Cells for Myositis and Interstitial Lung Disease Associated With Antisynthetase Syndrome. JAMA *329*, 2154-2162. 10.1001/jama.2023.8753.

5. Schneidawind, D., Duerr-Stoerzer, S., Liewer, S., Renner, S., Sánchez Navarro, B., **Atar, D.**, Keppeler, H., Beck, R., Hamprecht, K., Kanz, L., Lengerke, C., and Schneidawind, C. (2022). Low Graft Invariant Natural Killer T-Cell Dose Is a Risk Factor for Cytomegalovirus Reactivation After Allogeneic Hematopoietic Cell Transplantation. Transplantation and Cellular Therapy *28*, 513.e511-513.e514. https://doi.org/10.1016/j.jtct.2022.05.011.

6. da Silva, P.B.G., Sieber, L., Mack, N., **Atar, D.**, Kutscher, L.M., Schlegel, P., Ebinger, M., Kawauchi, D., Seitz, C., and Pfister, S.M. (2022). IMMU-11. Evaluation of CAR-T cells targeting CD276 in medulloblastoma. Neuro-Oncology *24*, i83-i83. 10.1093/neuonc/noac079.304.

7. **Atar, D.**, Mast, A.-S., Scheuermann, S., Ruoff, L., Seitz, C.M., and Schlegel, P. (2022). Adapter CAR T Cell Therapy for the Treatment of B-Lineage Lymphomas. Biomedicines *10*, 2420.

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12. Ureña-Bailén, G., Lamsfus-Calle, A., Daniel-Moreno, A., Raju, J., Schlegel, P., Seitz, C., **Atar, D.**, Antony, J.S., Handgretinger, R., and Mezger, M. (2019). CRISPR/Cas9 technology: towards a new generation of improved CAR-T cells for anticancer therapies. Briefings in Functional Genomics *19*, 191-200. 10.1093/bfgp/elz039.

4.2 Manuscripts in preparation

Krost, S.*, **Atar, D**.*, Kristmann, B., Huridou, C., Ruoff, L., Scheuermann, S., Çanak, A., Wolsing, K., Zekri, L., Lang, P., Seitz, C.M. (2024). Pan-B cell targeting by Adapter CAR-T Cells (AdCAR-T) prevents antigen escape in B-phenotypic malignancies.

Mast, A.-S., **Atar, D.**, Ruoff, L., Scheuermann, S., Kristmann, B., Schlegel, P., Seitz, C.M. (2025). AdCAR Tuning to Overcome Immune Escape in AML.

4.3 Poster presentations

Atar, D., Ruoff, L., Mast, A.-S., Krost, S., Moustafa-Oglou, M., et al., Multiplex Targeting by Adapter CAR T Cells (AdCAR-T) to Cope with Inter- and Intratumoral Heterogeneity in Acute Myeloid Leukemia. Poster presented at 1st international iFIT Conference; 21th March 2023; Ferry Porsche Congress Center, Zell am See, Austria.

4.4 Contribution to the publications that comprise this thesis

4.4.1 Adapter CAR-T Cell Therapy for the Treatment of B-Lineage Lymphomas

The original Idea of indirect CAR immunotherapy was developed by Dr. Christian Seitz, Prof. Patrick Schlegel, Prof. Rupert Handgretinger and Prof. Peter Lang. I developed the idea of the manuscript together with Dr. Christian Seitz and Prof. Patrick Schlegel. I performed the experimental design of all experiments in this manuscript by myself. The flowcytometry based antigen screening of the NHL cell lines was done together with Anna-Sophia Mast (Figure 2). Cytotoxicity Assays were performed together with Anna-Sophia Mast, Sophia Scheuermann and Lara Ruoff (Figure 4). I performed all cytokine secretion experiments, flowcytometry based Assays, as well as the CRISPR CAS9 knockout experiments, including the design of the gRNA, shown in this manuscript (Figure 3, Figure 5, Figure 6 and Figure 7). I performed the generation of luciferase-expressing cell lines, including the generation of the respective lentiviral vectors. The original luciferase backbone was kindly provided by Prof. Irmela Jeremias. The interpretation of the data was mainly done by me, Dr. Christian Seitz and Prof. Patrick Schlegel, with the help of Anna-Sophia Mast, Sophia Scheuermann and Lara Ruoff. I did the statistical analysis of the data with the aid of Prof. Patrick Schlegel. All figures and tables were generated by my own. I and Prof. Schlegel wrote the final manuscript which was further improved and revised by all coauthors.

4.4.2 Rational Combinatorial Targeting by Adapter CAR-T-cells (AdCAR-T) Prevents Antigen Escape in Acute Myeloid Leukemia (AML)

The original idea was conceptualized by Dr. Christian Seitz and Prof. Patrick Schlegel. Together with Dr. Christian Seitz, I designed and supervised the whole project. The primary AML samples were kindly provided by Prof. Peter Lang and were categorized by Anna-Sophia Mast. The panel design and multicolor flow cytometry screen of the primary AML samples were performed by Lara Ruoff, Anna-Sophia Mast and myself (Figure 1). I interpreted and generated the inter- and intratumoral heterogeneity with the intellectual input of Dr. Christian Seitz (Figure 4). I designed all *in vitro* cytotoxicity assays and the AM titration. I performed the experiments together with Lara Ruoff, Anna-Sophia Mast, Simon Krost, Moustafa Moustafa-Oglou, Sophia Scheuermann, Beate Kristmann and Maximilian Feige (Figure 2 and Figure 5). I designed and cloned all antibody constructs with the intellectual help of Dr. Latifa Zekri and she kindly provided the original antibody backbone (Figure 2 A). The production of the antibodies and the biotinylation was done together with Lara Ruoff, Simon Krost and Moustafa Moustafa-Oglou. All mice experiments were designed by me and Dr. Christian Seitz. I performed all *in vivo* experiments with the assistance of Aysegül Canak and Kathrin Wolsing (Figure 3, Figure 6 and Figure 7). All figures and tables were generated by my own. Dr. Christian Seitz and I wrote the final manuscript which was further improved and revised by all coauthors.

5. Introduction

5.1 T-Cell immunotherapy of cancer

Modern T cell-based immunotherapy has revolutionized the field of cancer treatment. For decades, the foundations of cancer treatment have been surgery, chemotherapy, and radiation therapy. In contrast, T cell-based immunotherapy has been able to demonstrate its potential, especially in chemo-refractory patients [1].

The knowledge basis for today's cellular immunotherapy emerged at the beginning of the 20th century, when Paul Ehrlich proposed the hypothesis in 1909 that neoplastic cells are constantly generated in the human body, which are eradicated by the immune system itself [2,3]. Later in the 1959 Lewis Thomas and Sir Frank Macfarlane Burnet formulated the hypothesis of "cancer immunosurveillance" [3]. This theory suggested that the immune system identifies and attacks tumor-associated neoantigens, preventing carcinogenesis in a manner similar to transplant rejection [4,5]. Today, we know that T cells play a key role in this type of tumor control. In tumor progression, cancer immunosurveillance is disrupted at various levels. Endogenous T cells have lost their inherent ability to recognize and control tumors [6]. Modern T cell-based immunotherapy targets this malfunctioning T cell compartment [5,7]. This can be achieved through various strategies:

Immune Checkpoint Inhibitors (ICIs): Co-inhibitory receptors, such as Programmed Cell Death 1 (PD-1) and Cytotoxic T Lymphocyte Antigen 4 (CTLA-4), are expressed on the surface of T cells to regulate T cell-mediated immune responses [8]. Nevertheless, tumor cells manipulate these inhibitory molecules to promote tumor tolerance and induce T cell exhaustion [9,10]. Blocking these checkpoint inhibitors can boost the antitumor effect of the T cell compartment [10,11].

Bispecific T Cell Engager (BiTE): BiTEs are bispecific antibody constructs, directed against CD3 on one side and at least one tumor-associated antigen on the other side [12]. This recruits endogenous T cells to the tumor cell, allowing major histocompatibility complex (MHC)-independent formation of a cytolytic synapse, leading to the activation and further recruitment of T cells and ultimately the lysis of the target cell [12,13].

Tumor Vaccination: Through vaccination with mostly personalized tumor antigens, antigen-presenting cells (APCs) present these antigens to T cells via MHC, priming and activating them to build a tumor-specific T cell reservoir and control it [14,15].

Adoptive Cell Transfer (ACT): This includes tumor-infiltrating T cells, extracted from the tumor, expanded *ex vivo*, and reintroduced in a high dose into the patient, exploiting the capability of specific autologous antitumor reactivity [5]. Another strategy of ACT are transgenic T cells with genetically modified T cell receptors to generate a neoantigen-specific TCR. The latest and most innovative achievement in ACT is fully synthetic genetically engineered chimeric antigen receptor (CAR) T cells to target tumor-associated antigens in an MHC-independent way [5,7].

5.2 Evolution of CAR-T cells

The advancement of replication-defective lentiviral or retroviral vectors has opened up new possibilities for adoptive cell therapies [16]. It started with the creation of genetically engineered T-cell receptors (TCRs) to redirect a natural α/β T cell towards a specific tumor-associated antigen (TAA) [17-19]. This is achieved by introducing new α/β genes into T cells, which then re-arrange the α/β TCR of a defined tumor specificity capable of forming a physiological TCR-CD3 immunocomplex [17-19]. A novel approach for MHC independent T cell specificity are chimeric antigen receptor (CAR) T cells [20]. A CAR is a completely artificial construct typically composed of a TAA specific scFv, linked via a hinge (IgG components), to a transmembrane domain (from CD3, CD4, CD8 or CD28 segments), aligned with intracellular TCR signaling domains capable of performing both activating T cells and providing antigen-specific binding [21]. This strategy enables the rapid generation of anti-tumor T cells with a defined phenotype and functional qualities suitable for various cancer types, aiming to enhance therapeutic efficacy and safety [19,22]. The first engineered T-cells with a chimeric molecule occurred between 1989 and 1993, credited to Israeli immunologists Zelig Eshhar and Gideon Gross [23-25]. These **first-generation CARs (1st gen CAR)** contained only a single CD3 ζ-chain or FcεRIγ intracellular domain, lacking any additional costimulatory domains [26]. These complexes were very similar to endogenous TCR. But due to the lack of the costimulatory domain, they lacked the ability to produce sufficient stimulatory cytokines to generate CAR-T cell expansion and sustained antitumor activity *in vivo* [26]. As a result of this insight, **second-**

generation CARs (2nd gen CAR) emerged, in which the co-stimulatory domains CD28 or CD137 (4-1BB) were fused to CD3 ζ [26]. By utilizing two intracellular signaling domains, enhanced antigen-dependent proliferation, interleukin-2 production, and cytotoxic activity could be demonstrated *in vitro* [27,28] as well as a significantly increased expansion and persistence, along with enhanced antitumor activity *in vivo* [29,30]. These successes have led to numerous promising clinical trials with CD19-targeted 2nd gen CAR-T cells in B-cell-mediated malignancies. The major breakthrough came in 2011 when Dr. Carl June's team at the University of Pennsylvania reported that three adult patients with advanced chronic lymphocytic leukemia (CLL) achieved complete or partial remission after CD19-specific CAR-T cell therapy [31-33]. This led to the development of the first Food and Drug administration (FDA)-approved CAR-T cell product, tisagenlecleucel (Kymriah), on August 30th, 2017, for the treatment of pediatric and young adult Acute Lymphoblastic Leukemia (ALL) [33,34]. Followed by three additional approvals of CD19-targeted 2nd gen CAR-T cells for the treatment of different B cell malignancies named axicabtagene ciloleucel (Yescarta), brexucabtagene autoleucel (Tecartus), and lisocabtagene maraleucel (Breyanzi) [35-37]. In 2020 and 2021, two B-cell maturation antigen (BCMA)-specific 2nd gen CAR-T cells for the treatment of multiple myeloma were also approved by the FDA, named idecabtagene vicleucel (Abecma) and ciltacabtagene autoleucel (Carvykti) [38,39]. A detailed overview of all CAR-T cell products approved by the FDA until February 2024 can be found in Table 1.

Brand name	Indications	FDA Approval	Target Antigen Clone	Hinge Transmembrane Costimulatory Domains
Kymriah	r/r B-cell precursor ALL r/r large B-cell lymphoma	August 2017 May 2018	CD19 FMC63	CD8α / CD8α 4-1BB + CD3ζ
Yescarta	r/r large B-cell lymphoma r/r follicular lymphoma	October 2017 March 2021	CD19 FMC63	CD28 / CD28 CD28 + CD3ζ
Tecartus	r/r Mantle Cell Lymphoma r/r B-cell precursor ALL	July 2020 October 2021	CD19 FMC63	CD28 / CD28 CD28 + CD3ζ
Breyanzi	r/r large B-cell lymphoma	February 2021	CD19 FMC63	IgG4 / CD28 4-1BB + CD3ζ
Abecma	r/r Mantle Cell Lymphoma	March 2021	BCMA BB2121	CD8α / CD8α 4-1BB + CD3ζ
Carvykti	r/r Mantle Cell Lymphoma	February 2022	BCMA / dual camel single-domain antibodies	CD8α / CD8α 4-1BB + CD3ζ

Table 1: Overview of FDA-approved CAR-T cell therapies until February 2024 [40]

While all approved CAR-T cells are 2nd gen CARs and demonstrate groundbreaking success in immunotherapy, efforts are being made to improve the outcomes even further especially in non-hematological malignancies. This led to the idea of **third-generation CAR-T (3rd gen CAR)** cells by integrating a second costimulatory signaling domain, such as CD28-41BB or CD28-OX40, to enhance CAR-T cell potency with more substantial cytokine production, anti-tumor ability, and increased T-cell proliferation [26,41]. The idea is that different costimulatory domains possess complementary characteristics that could be utilized synergistically. For instance, CD28 may contribute to a faster expansion of T cells and quicker tumor lysis, while 4-1BB may lead to increased persistence of CAR-T cells in the patient [42,43]. Alternative costimulatory domains such as CD134, CD27 or ICOS also work, but have rarely

been tested in clinical trials [44-46]. Currently, numerous clinical trials with third-generation CAR-T cells are ongoing, primarily for B-phenotypic malignancies targeting CD19 [47-50] or CD22 [51]. Additionally, trials are being conducted for the treatment of myeloid system disorders targeting CD123 [52] and neuroblastomas targeting GD2 [53,54]. However, since the advantage of 3rd generation CARs over 2nd generation CARs is still debated, there is no approved product with a third-generation CAR up to the current standpoint [43]. With increasingly efficient molecular biology methods, there are virtually no limits to the diversity of modern CAR designs. In recent years, various forms of the so-called next-generation CARs have been developed. The most noteworthy are depicted in Figure 1. These include CAR-T cells that simultaneously target two antigens (tandem/dual CARs) [55], immune checkpoint-modulating CAR-T cells (armored CARs) [43], cytokine-expressing CAR-T cells (T cells redirected for universal cytokine-mediated killing - TRUCKs) [56], CAR-T cells with an integrated safety switch to induce apoptosis, complement-dependent cytotoxicity (CDC), or antibody-dependent cellular cytotoxicity (ADCC) upon administration of an exogenous substance (switchable CARs), and "logic CARs" to reduce the on-target off-tumor effect by splitting activation and costimulatory domains (Split-CARs) or by inducing the expression of a second CAR only upon binding of the primary target antigen (inducible CARs) [43,57]. An elegant way to combine some of the mentioned goals with next-generation CARs is to separate antigen recognition from T-cell activation [58]. The idea is to use antigen-specific adapter molecules, such as mAbs, mAb fragments, or antigen-specific ligands, which are either chemically labeled or modified in their amino acid sequence to allow tag- or motif-specific recognition by a CAR-T cell [58,59]. The use of adapter molecules for mediating CAR-specific targeting addresses both safety and efficacy by enabling universal, multiple synchronous, and transient targeting [59-61].

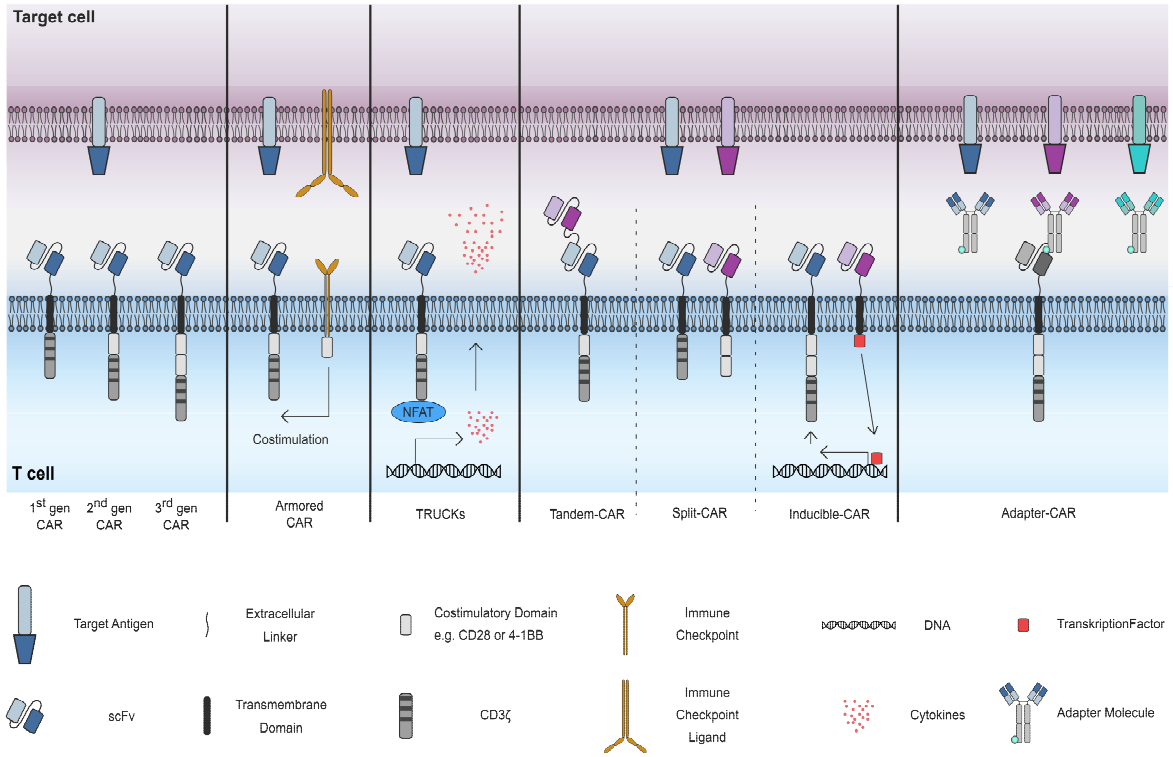

Figure 1: Evolution of CAR-T Cells Design. Adapted from [43,55,57,62-65].
Created with Inkscape 1.2.2 (732a01da63, 2022-12-09).

From left to right: **1st gen CAR**: consisting of an extracellular antigen-recognizing domain single chain variable fragment (scFv) linked to an extracellular Linker, linked to a transmembrane domain, linked to a intracellular CD3z signaling domain [66]; **2nd gen CAR**: equiped with an additional costimulatory domain (e.g., CD28 or 4-1BB) [42]; **3rd gen CAR**: equiped with two additional costimulatory domains (e.g., CD28 or 4-1BB) [63]; **Fourth/Next-generation CAR:** group of CAR-T constructs: **armored CAR**: immune checkpoint-modulating CAR-T cells convert inhibitory signals into activating signals [43]; **TRUCKs**: cytokine-expressing CAR-T cells (T cells redirected for universal cytokine-mediated killing - TRUCKs) [63], **Tandem CAR:** incorporating two different single-chain variable fragments [55]; **Split CAR:** activation through CD3ζ and co-stimulation are split over a 1st gen CAR and a chimeric co-stimulatory CAR, each targeting distinct tumor antigens. Binding of both CARs is required for full T-cell activation [64,65]; **Inducible CAR**: inducing the expression of a second CAR only upon binding of the primary target antigen [57]; **Adapter CAR**: AdCAR-T cells are redirected to surface antigens via biotinylated adapter molecules (AM) in the context of a specific linker structure called a linker-label epitope (LLE) [59,61]. AdCAR-T cells are nonfunctional without the AMs and mediate their anticancer function only in the presence of the AMs, enabling an on/off switch, a universal targeting depending in the chosen AM, multiple synchronic targeting by simultaneous application of multiple AMs, and transient targeting according to the pharmacokinetic and pharmacodynamic characteristics of the different AMs [59-61,67].

5.3 Challenges for T cell-based immunotherapies

Even though CAR-T cell therapies have resulted in dramatic remission rates in several relapsed/refractory hematologic malignancies, these are largely limited to B-phenotypic malignancies [68]. In contrast, no breakthrough has been achieved in the treatment of diseases of the myeloid system or solid tumors [60]. The reasons for this are manifold and vary depending on the tumor entity. However, common problems are often treatment-related toxicities, lack of controllability of the CAR-T activity (safety switch), lack of reliable tumor-associated antigens (antigen heterogeneity), shared antigen expression (on-target/off-tumor effect), antigen escape, lack of efficacy, Immunosuppressive tumor microenvironment (TME) and much more [26,60,68-70]. In the following, some of the mentioned topics will be discussed in more detail, with a special focus on hematologic malignancies especially AML.

5.3.1 Treatment-related toxicities

The most common side effects of CAR-T cell therapy are Cytokine Release Syndrome (CRS) and Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS) [71]. These two side effects are acute toxicities that occur within days to a few weeks after CAR-T cell infusion. CRS arises from the massive interaction of CAR-T cells with target cells and the resulting cytokine storm. Symptomatically, it primarily manifests with fever, and laboratory findings show significantly elevated interleukin-6 (IL-6) and interferon-gamma (IFN-γ) levels in the serum [72-74]. Depending on the severity of CRS, serious complications such as hypotension and hypoxia can occur [75]. Therapeutically, CRS can usually be effectively treated by administering tocilizumab, an IL-6 receptor antagonist and Dexamethasone [76]. Neurotoxicity in the form of ICANS is triggered like CRS by the massive release of CAR-T cell-associated cytokines. ICANS induce symptoms such as confusion, headaches, decreased ability to concentrate, and aphasia [77]. ICANS is preferably treated with the administration of corticosteroids but also with tocilizumab [76]. The main problem with conventional CAR-T cells regarding CRS is the difficulty in controlling the CAR-T cells and their reactions once infused into the patient. Approaches to address this problem include transient expression of CARs through mRNA [78], introduction of suicide genes [79], or controlling CAR expression or function through a pharmacological switch [80,81].

5.3.2 Shared antigens

An important issue is also the On-Target-Off-Tumor-Toxicity (shared antigens). Most tumor-associated antigens (TAAs) targeted by CAR-T cells are expressed not only by the tumor but also by healthy tissues in the body [82,83]. This is one of the reasons why CAR-T cell development against non-B-cell-associated malignancies is so difficult. CD19 appears to be an exclusive antigen of the B-cell lineage and causes few to no off-target effects [84]. There are initial data showing expression of CD19 on human brain mural cells, which could be associated with the possibility of neurotoxicity, but this is still part of current research [85]. Apart from this, the often-long-term B-cell aplasia after CD19 CAR therapy can be compensated for with immunoglobulin substitution [86]. In solid tumors, finding exclusive TAAs is extremely difficult [87]. For example, in an ErbB2-CAR-T cell study, lethal consequences occurred due to the previously unknown low expression in lung tissue [88]. The same issue arises in the development of CAR-T cells against other non-B-cell-associated hematologic diseases such as AML. So far, no clinical breakthrough of CAR-T cell therapy against AML or other myeloid neoplasms has been demonstrated [89,90]. Although some AML-associated markers have been identified so far, such as CD33 (SIGLEC3), CD123 (IL3RA), and CD371 (CLL1 or CLEC12A), CD13, CD7, NKG2D ligand, and CD70 [90]. However, none of these antigens show uniqueness but rather strong expression on healthy cells such as hematopoietic stem and progenitor cells (HSPCs) [60,90-92]. Permanent aplasia of HSPCs would lead to myeloablation and thus to serious risks of infectious complications. This is why all CAR-T cell therapies against AML developed so far are only used as a bridge to transplant [93-95]. To improve this situation, strict control of the function of CAR-T cells would be necessary.

5.3.3 Target heterogeneity and antigen escape

A particularly significant challenge is the inter- and intratumoral heterogeneity of non-B-cell-associated tumors such as AML and solid tumors [87,90]. Especially in AML, addressing a single antigen will not lead to complete remission in most cases due to phenotypic heterogeneity [60]. AML consists of a pool of highly heterogeneous blasts, which, like hematopoiesis itself, are strongly hierarchically organized. Less differentiated stem cell-like cells, known as leukemic stem cells (LSCs), are responsible for disease initiation, relapse, and therapy resistance, while more mature

populations continue to contribute to intratumoral heterogeneity [92,96,97]. Not only the remarkable heterogeneity between different patients but especially the intratumoral heterogeneity of an individual AML is challenging. In most cases targeting only a single antigen leads to relapse, through the outgrowth of pre-existing antigen-negative subclones [98]. Even in CD19-directed CAR-T cell therapy of B-cell-associated malignancies, up to 30% of therapy failures are attributed to antigen escape mechanisms [99]. These antigen escape mechanisms are based on clonal selection of negative clones, trogocytosis, alternative splicing, or epitope masking [100]. This issue can only be addressed by flexible and multiple targeting of several antigens simultaneously or sequentially.

5.4 Ways to overcome main challenges in CAR-T Cell Therapies

Great efforts are being made to improve the current problems and challenges of CAR-T cell therapy [101]. In particular, much work is being done on CAR design, and a variety of so-called Next-Generation CARs have emerged to address therapy-related toxicity, target heterogeneity, the shared antigen problem, and inadequate activity, especially in the area of solid tumors [43,62,78,99]. To increase safety, there are, for example, transient CAR-T cells that show no CAR activity after just a few days due to mRNA electroporation [78]. Inducible CARs, whose expression is under pharmacological control, also enable transient and thus safer use [80,81]. Regarding shared antigen expression, there are now various logic-gated CARs that allow either activation/expression or inhibition due to the recognition of multiple antigens, thereby creating a better therapeutic window [102,103]. Strategies to overcome activity deficiency and increase persistence, especially in solid tumors, include co-expressing proinflammatory cytokines by CAR-T cells (TRUCKs) [43,56,57,62] or integrating receptors that convert inhibitory signals into activating signals (armored CARs) [43]. Ways to overcome antigen escape and disease heterogeneity involve synchronous targeting of multiple targets. This improves long-term remission addressed by several CARs (CD19/CD22) or the integration of multiple binding motifs into one CAR, called Tandem-CAR [104].

5.5 Flexible CAR-T Cells

An elegant approach to address both safety and efficacy is the so-called adapter CAR system (AdCAR) [59]. AdCAR-T cells are redirected to surface antigens via

biotinylated adapter molecules (AM) in the context of a specific linker structure called a linker-label epitope (LLE) [59,61]. AdCAR-T cells are nonfunctional without the AMs and mediate their anticancer function only in the presence of the AMs [60]. The use of AMs to mediate CAR-specific targeting enables AdCAR-T cells to have an on/off switch, a universal targeting depending in the chosen AM, multiple synchronic targeting by simultaneous application of multiple AMs, and transient targeting according to the pharmacokinetic and pharmacodynamic characteristics of the different AMs [59,61 60].

Several groups have developed different kind of adapter CAR-T systems, each with specific advantages and disadvantages [59,61,67,105-107]. For example, anti-fluorescein isothiocyanate (FITC) CAR-T cells have shown efficacy *in vitro* and *in vivo* by using various FITC-tagged Fabs or bifunctional molecules [106]. However, FITC is a strong hapten that does not occur in the human body. High immunogenicity could therefore hinder clinical application [108]. For this reason, our group has developed the AdCAR system, recognizing the endogenous, less-immunogenic vitamin biotin in the context of a special linker moiety called Linker-Label-Epitope (LLE). Adapter molecules are generated by biotinylation of mAbs or mAb fragments (e.g., F(ab)2 or Fab) [59,61]. This allows great flexibility regarding the target antigens, making this technology particularly interesting for highly heterogeneous diseases such as AML [60,109]. Moreover, safety could be significantly increased compared to direct CAR-T cells, as the CAR-T cells are only active in the presence of the adapter molecules, and the duration of activity can be controlled through the pharmacokinetics of the adapter molecules [60,61,110]. It has also been shown that sequential, time-limited activation with treatment-free intervals can also prevent T-cell exhaustion [67,111].

6. Objectives

In this thesis two different projects were pursued.

- The aim of the first project was to evaluate the AdCAR system in B phenotypic malignancies. For this purpose, three different lymphoma cell lines (JeKo-1, Raji, Daudi) were used as a model to demonstrate the flexibility of the system. The cell lines were analyzed by flow cytometry for their surface expression of potential target antigens, followed by testing a total of six different AMs in various *in vitro* cytotoxicity assays. The specific activation of AdCAR-T cells in the presence and absence of AMs was investigated in direct comparison to a 2nd generation CD19CAR. Flow cytometry was used to analyze the early activation markers CD25 and CD69, as well as the immunophenotype via markers CD45RA/CD45RO/CD62L and CD95. In addition, cytokine secretion was examined. Furthermore, the feasibility and advantage of dual targeting using two AMs simultaneously should be demonstrated. This was to be achieved using a CD19-deficient lymphoma cell line generated with CRISPRCas9, to simulate antigen loss of CD19 as one of the most common causes of therapy failure after CD19CAR therapy.

- The aim of the second project was to study inter- and intratumoral heterogeneity on target antigen expression in AML and to proof that combinatorial targeting with the AdCAR can prevent antigen escape. 30 primary pediatric AML bone marrow samples were analyzed by flow cytometry to demonstrate the inter- and intratumoral heterogeneity and the clinical necessity of multiple targeting in AML. Based on our results, 5 different AMs, based on monoclonal antibodies, against the most relevant target antigens were produced in CHO, biotinylated, and chromatographically purified. These were then evaluated in various *in vitro* cytotoxicity assays with 3 different AML cell lines (U937, Molm13, HL60), demonstrating combinatorial targeting with 3 AMs simultaneously. CD33/CD38 double-deficient AML cell lines were generated using CRISPRCas9 for this purpose. Subsequently, all 5 AMs were evaluated *in vivo* with an AML cell line (U937) and finally demonstrated with a

patient-derived xenograft model, which exhibits natural intratumoral heterogeneity, that addressing a single antigen is not sufficient to clear AML *in vivo* and only a combination of multiple antigens can lead to complete remission.

7. Results and discussion

7.1 Adapter CAR-T Cell Therapy for the Treatment of B-Lineage Lymphomas

7.1.1 Target Antigen screening on B-Lineage Non-Hodgkin Lymphoma cell line

The target antigen expression of three B-lineage non-Hodgkin lymphoma cell lines, JeKo-1 (Mantle Cell Lymphoma MCL), Raji, and Daudi (both Burkitt Lymphoma), was analyzed by flow cytometry. To make a semiquantitative statement about the differential expression, the antigens CD19, CD20, CD22, ROR-1, CD276, CD79B, and CD10 were stained with the same fluorophore (R-phycoerythrin (PE)). The selection of antigens was based on literature research and their suitability for CAR-T cell therapy. Not surprisingly, the immunophenotyping of the cell lines showed different antigen profiles for JeKo-1, Raji, and Daudi. The percentage of antigen-positive cells was calculated by Overton subtraction of the normalized histograms and showed significant intra- and intertumoral heterogeneity. For example, CD22, currently a strongly promoted antigen for the therapy of B-cell lymphomas [112], is only expressed to 38% in JeKo-1, while in Raji and Daudi it is expressed at 100%. The mean fluorescence intensity ratio (MFIR) was calculated by dividing the mean fluorescence intensity of the sample by the mean fluorescence intensity of the isotype control. The MFIR can indicate which antigens are particularly suitable as targets for CAR-T cell therapy. It has been shown that a certain density of antigens on the surface is necessary for successful CAR-mediated tumor lysis [113]. While all three cell lines show 99%-100% expression for CD19 and CD20, the MFIR varies widely between the cell lines. For example, for CD19, it ranges from 12-59, and for CD20, it ranges from 84-204. Thus, a CAR-T cell therapy targeted against CD19 in a B-cell lymphoma with low CD19 expression could lead to the selection of antigen-low cells and consequently therapy failure [113]. This intratumoral heterogeneity of antigens and the associated escape mechanisms could be overcome by multi-targeted CAR technologies [59,61,114].

7.1.2 Specific activation of the AdCAR System

The specific activation of AdCAR-T cells in the presence and absence of AMs was investigated in direct comparison to a 2nd generation CD19CAR. Flow cytometry was used to analyze the early activation markers CD25 and CD69, as well as cytokine secretion. Co-incubation experiments were conducted with AdCAR-T cells and JeKo1 in the presence and absence of AMs, and CD19CAR-T cells and JeKo1. CAR-T cells were defined as specifically activated if they were CD25+CD69+ double positive. As expected, CD19CAR-T cells were only activated in the presence of the CD19-positive target cell line JeKo-1. AdCAR-T cells were activated only in the combination of AdCAR-T cells plus the antigen-positive target cell line JeKo-1 plus LLE-CD19-mAb adapter molecule. Specific activation of CAR-T cells after 1 and 5 days was significantly increased compared to unstimulated controls. Unspecific activation via AMs alone could not be induced. We only observed a slight activation of about 10% after 5 days in the presence of LLE-CD19-mAb at 1 ng/mL without the target cells, which could be related to the degree of labeling of the AMs and was later investigated further under 7.1.3. An unspecific upregulation of CD25+CD69+ in AdCAR-T cells after 5 days of incubation with JeKo1 was observed. This could be related to the fact that for expansion, T cells are activated via TransAct, a CD3/CD28 stimulating reagent. It was observed that activated T and CAR-T cells, which were stimulated via the CD3 receptor complex and the costimulatory receptor CD28, induced activating receptors such as DNAM-1 and NKG2D [115]. The expression of DNAM-1 and NKG2D is indirectly supported by common γ-chain cytokines (IL2, IL7, IL15) on activated T cells and CAR-T cells [116]. To support this hypothesis, the expression of DNAM-1 ligands CD112 and CD155 (DNAM-1L) as well as NKG2D ligands, MIC A/B on JeKo-1, was measured, along with the surface expression of activating receptors DNAM-1 (CD226) and NKG2D (CD314) on activated T cells. We found a strong expression of the NKG2D ligands MIC A/B in JeKo-1, but a low expression of the DNAM-1 ligands CD112 and CD155. High expression of CD226 and CD314 was detected on activated T cells. Due to the high expression of CD314 in activated T cells, the NK-like, CAR-independent upregulation of CD25 and CD69 can be explained [117].

To further investigate the specific activation and effector function of AdCAR-T cells, cytokines in the supernatant were measured from the same co-incubation cultures

after 24 hours and after 5 days. For this purpose, a comprehensive 12-cytokine multiplex panel was used, including the following cytokines: GM-CSF, INFα, IFNγ, IL2, IL4, IL5, IL6, IL9, IL10, IL12, IL17a, and TNFα. It was observed that after 24 hours, AdCAR-T cells exhibited significantly lower levels of GM-CSF, IL2, IFNγ, and TNFα compared to CD19CAR T cells. However, after 5 days, no significant differences in the cytokine profile between CD19CAR-T cells and AdCAR-T cells were detectable anymore. This suggests a slightly delayed kinetics of the AdCAR system, which is consistent with our previous *in vitro* and *in vivo* data [59].

7.1.3 Degree of biotinylation matters

Due to the slight unspecific activation of AdCAR-T cells by the adapter molecule, it was suspected that the number of available binding sites for AdCAR-T cells per adapter molecule has an impact on the activation of AdCAR-T cells. It is assumed that a multi-biotinylated adapter molecule could lead to cross-activation of the CARs on the surface of the same T cell or, worse yet, cross-activation and thereby autolysis of the AdCAR-T cells among themselves. To investigate this, IgG1 molecules were hyper-biotinylated as well as biotinylated 1-3-fold through very low molar biotinylation. The hyper and low biotinylated AMs were incubated with the AdCAR-T cells for 1 day and for 5 days. CD25+CD69+ positivity was defined as activation. Overall, the upregulation of CD25 and CD69 by LLE-mAb was slight increased, but was significantly more pronounced with multi-biotinylated LLE-mAb compared to mono-biotinylated LLE-mAb. Indicating a low degree of LLE labeling is important for high antigen specific activation of the AdCAR System.

7.1.4 CAR-T Cell Immune Phenotype and Exhaustion

There is increasing evidence suggests that successful outcomes in patients treated with CAR-T cells depend on the cells' ability to expand and persist after infusion. There are clear associations between sustained clinical remission and patient survival with good *in vivo* expansion and long-term persistence of CAR-T cells [118,119]. In this context, the immune phenotype of the infused CAR-T cells plays a crucial role, as less differentiated T-cell subsets within the product have a significant impact on *in vivo* performance. Therefore, we examined the immune phenotype of CD19CAR-T and AdCAR-T cells without tumor contact, as well as the changes in immune phenotype with tumor contact after one and after five days. Additionally, AdCAR-T

cells were incubated with or without 10 ng/mL LLE-CD19-mAb to assess the influence of the AM itself on the phenotype. Immunophenotypes post-incubation were determined using flow cytometry by staining for cell surface markers CD45RA, CD45RO, CD62L, and CD95. Naive T cells (TN) were defined as (CD45RA+/CD45RO−/CD62L+/CD95−), stem cell-like memory T cells (TSCM) as (CD45RA+/CD45RO−/CD62L+/CD95+), central memory T cells (TCM) as (CD45RA−/CD45RO+/CD62L+/CD95+), effector memory T cells (TEM) as (CD45RA−/CD45RO+/CD62L−/CD95+), and effector memory T cells re-expressing CD45RA (TEMRA) as (CD45RA+/CD45RO−/CD62L−/CD95+).

The baseline phenotype of CD19CAR-T cells and AdCAR-T cells without tumor contact is very similar and distributed as follows: TN 1-2%, TSCM 68-70%, TCM 12-15%, TEM 7-9%, TEMRA 7-8%. With tumor contact, a significant shift towards TEMRA phenotype is already noticeable after 24 hours in CD19CARs and AdCAR-T cells with AM plus tumor, with no discernible difference between CD19CAR-T cells and AdCAR-T cells: TN 0.1%, TSCM 2-5%, TCM 0.9-1%, TEM 18-23%, TEMRA 71-77%. The AM alone seems to have no influence on the phenotype of AdCAR-T cells after 24 hours, but tumor cells without AM show a shift in the AdCAR-T cell phenotype towards TEMRA, from 7.5% to 20.5%. After 5 days of incubation, there is no relevant difference in phenotype between CD19CAR-T cells without tumor and AdCAR-T cells without tumor and AdCAR-T cells plus AM: TN 0.5-1%, TSCM 73-77%, TCM 10-14%, TEM 3-4%, TEMRA 7-11%. The impact of unspecific activation of AdCAR-T cells by the NKG2D ligands MIC A/B in JeKo-1 is evident in the phenotype after 5 days, with a shift towards 57% TEM and 23% TEMRA. Both CD19 CAR-T cells with tumor and AdCAR-T cells with AM and tumor show a shift to 90% (CD19CAR-T) and 99% (AdCAR-T) effector cells after 5 days. In both CD19CAR-T cells and AdCAR-T cells, the proportion of TSCM was significantly reduced. This marked change in immune phenotype suggests a CAR-specific physiological T-cell maturation. Maintaining the TSCM pools would be particularly desirable for the *in vivo* persistence of CAR-T cells. In this regard, the third-generation AdCAR system should be further improved, as it exhibits significantly fewer TSCM after 5 days compared to the second-generation CD19CAR: AdCAR TSCM 0.25%, TCM 0.37% compared to CD19CAR TSCM 2.4%, TCM 8.4%.

To complete the assessment of cell fitness, the exhaustion marker PD-1 was quantified after 24 hours using flow cytometry. It was demonstrated that AdCAR-T cells were activated only in the presence of AM and the corresponding antigen-expressing target cells. There was no difference in PD-1 expression between CD19 CAR-T cells and AdCAR-T cells or in the control groups, where PD-1 expression ranged between 4% and 6%.

7.1.5 Flexible and combinatorial targeting with the AdCAR system

To demonstrate the universal, antigen-specific effector function of AdCAR-T cells *in vitro*, the AdCAR-T cells were titrated on the lymphoma cell lines Raji, Daudi, and JeKo1 at six different effector-to-target (ET) ratios ranging from 5:1 to 0.15:1, with the AM concentration kept constant at 1 ng/mL in all experiments. The cell lines all expressed firefly luciferase and green fluorescent protein (GFP), which were stably integrated into the cells via lentiviral gene transfer. Thus, cytotoxicity could be determined through a luciferase-based cytotoxicity assay (LCA). To demonstrate the specificity of the AdCAR system, non-transduced activated T cells served as negative controls. The CD19 2nd gen CAR was used as a Benchmark. The LLE-CD19-mAb alone did not induce measurable cytotoxic effects at 1 ng/mL.

For all three cell lines, the cytotoxic effect of AdCAR-T cells was significantly higher than the cytotoxic effect of activated T cells. The combination of LLE-CD19 and LLE-CD20 did not show a better cytotoxic effect on the wild-type forms of the cell lines. To demonstrate antigen loss and the superiority of combinatorial targeting with the AdCAR system, a CD19 knockout variant of JeKo1 (JeKo-1CD19KO) was generated using CRISPR-Cas9. In an LCA with the JeKo-CD19KO line, the significant superiority of combinatorial targeting with AdCAR-T cells and LLE-CD19 plus LLE-CD20 over monotargeting with LLE-CD19 or direct CD19CAR could be demonstrated. To demonstrate the flexibility of the AdCAR system, the previously screened alternative target antigens CD19, CD20, ROR-1, CD276, CD79B, and CD10 were targeted at a constant ET ratio of 1.25:1 on the wild-type form of JeKo1 and Raji. It was shown that some of the target antigens are less suitable for CAR-T cell therapy due to their low antigen density. While high cytotoxicity was not achieved for all target antigens with the AdCAR system, it was demonstrated that combinatorial targeting of alternative antigens, such as CD10 in combination with ROR-1 or CD10 in combination with CD20, is feasible and can significantly increase cytotoxic effect

compared to monotargeting. In general, combinatorial targeting was superior in JeKo-1 and Raji.

In summary, it was demonstrated that AdCAR-T cells, utilizing adapter molecules for antigen-specific targeting of the respective antigen-positive lymphoma cell lines, are suitable for *in vitro* use. In terms of their cytolytic properties, cytokine expression profile, and immune phenotype, AdCAR-T cells are comparable to conventional CD19CAR-T cells and thus appear to be a safe and novel therapy. The system is highly flexible and can target almost any antigen using an appropriate adapter molecule. By conducting a histopathological examination of the patient's tumor, an antigen expression profile of the patient could be established, thus allowing for the assembly of a patient-specific panel of adapter molecules for combinatorial targeting. By biotinylating NHL antibody currently used clinically, such as CD19 [120], CD20 [121], CD22 [122], CD37 [123], and CD79B [124], suitable adapter molecules could be generated quickly, potentially preventing antigen escape mechanisms through combination therapy. To conclusively assess this, preclinical *in vivo* experiments should be conducted, and safety and efficacy should be verified in clinical trials.

7.2 Rational Combinatorial Targeting by Adapter CAR-T cells (AdCAR-T) Prevents Antigen Escape in Acute Myeloid Leukemia (AML)

7.2.1 Shared antigen expression in pediatric AML

Targeted immunotherapy of AML is known to be extremely challenging due to shared antigen expression on leukemic cells and healthy HSCs, HSPCs, and on cells of the myeloid lineage, as well as the strong inter- and intra-patient heterogeneity. To investigate antigen expression in pediatric AML and identifying suitable target antigens for CAR-T cell therapy, a total of 30 primary AML samples, including 20 samples at primary diagnosis and 10 at relapse, were screened using multicolor flow cytometry for CD45, CD34, CD3, CD33, CD38, CD123, CD135, CD371, CD276, IL1RAP, Mesothelin, and MICA/B. CD33 and CD38 were identified as the most frequently and highly expressed antigens with the highest mean fluorescence intensity difference (MFID) in 30/30 samples with >50% positive blasts. This was followed by IL1RAP (22/30), CD123 (21/30), CD371 (21/30), CD135 (3/30), and MICA/B (3/30). CD276 and Mesothelin showed no relevant expression. No significant difference was observed between samples at primary diagnosis and relapse samples. IL1RAP has been reported as a new potential antigen for targeted AML therapy in adults and is expressed in both bulk leukemic cells and LSCs in adult AML [125,126]. Therefore, it is already being investigated in a clinical trial (NCT04169022) in adult AML. Our results from pediatric AML samples confirmed high expression in 22 out of 30 samples examined. However, unlike studies in adult AML patients, where leukemic stem cells (LSC) defined as CD34high/CD38low are frequently enriched [92,127], we could identify CD34high/CD38low subpopulations in only 3/30 pediatric patient samples. This is in line with an earlier report [128] and is seen as an important difference between pediatric and adult AML. As mentioned earlier, one of the main problems of targeted immunotherapy in AML is shared antigen expression on healthy cells of the hematopoietic lineage. For this reason, the same antigen panel was also examined in a total of 5 bone marrow samples from healthy donors and compared with the data from AML samples. None of the antigens examined could be found exclusively on AML blasts. All antigens showed expression on healthy cells as well.

CD33, CD38, CD123, CD135, CD371, and IL1RAP are co-expressed in healthy HSPCs (CD45dim/CD34high/CD38high) and myeloid cells, albeit with lower frequency and MFID than on leukemic blasts. In particular, monocytes (CD45high/SSCmedian) express CD33, CD38, CD123, CD135, CD371, and IL1RAP at high frequency and MFID. CD33 and CD38 showed expression in subpopulations of activated T cells. Previous studies in adult AML have identified CD276 [129,130], Mesothelin [131-133], and MICA/B [133] [134]as potential target antigens, which our data in pediatric AML cannot confirm. In summary, our results could not identify an AML-exclusive target antigen in pediatric AML, and stringent safety measures are required to prevent life-threatening, on-target/off-tumor effects such as myeloablation, or at least to be able to respond to them. This could be achieved through innovative CAR design or, as in our case, with the help of the adapter system and fine-tuning of therapy via the pharmacokinetics and dynamics of the adapter molecules.

7.2.2 Pediatric AML is a highly intratumoral heterogeneous disease

To further understand the intratumoral / intra-patient heterogeneity in pediatric AML, the data were analyzed by dimensionality reduction using Uniform Manifold Approximation And Projection (UMAP) based on the expression of CD45, CD3, CD34, CD33, CD38, CD123, CD135, and CD371, as well as forward scatter (FSC) and side scatter (SSC) signals (mean blast percentage 85% (range 76%-92%), average number of AML blasts analyzed per patient: 75,700 (range 28,000-143,000)). With the help of UMAP visualization, the strong intratumoral heterogeneity becomes evident. Using healthy bone marrow samples, the gating strategy was developed, showing how all major hematopoietic cell populations are clustered. In contrast, in the bone marrow samples from AML patients, one leukemic blast cluster dominates, and within this single blast cluster, the antigen expression is highly heterogeneous. Based on these data, a combination of at least two antigens would be required in 6 out of 10 samples to address more than 90% of the blasts. With these data, it has been demonstrated for the first time how strong the intratumoral heterogeneity of antigen expression is in primary pediatric AML, and that targeting a single AML antigen would not be sufficient to reach all leukemic cells for the majority of patients. Considering that antigen-low or antigen-negative cells are one of the main reasons for the failure of targeted immunotherapies, these findings have strong clinical relevance.

7.2.3 Newly generated adapter molecules exhibit antigen-specific activity against AML cell lines

Due to this extensive inter- and intratumoral heterogeneity, personalized targeting is required to cover the disease as a whole. The AdCAR system could be particularly suitable for this purpose, as its flexibility allows for both multiple targeting and minimization of the myeloablative effect due to its natural transient property. To validate the AdCAR system for AML, AMs against 5 clinically relevant AML-associated target antigens, CD33, CD38, CD123, CD135, and CD371, were produced by our self in CHO cells. For this purpose, VH and LH sequences for the mAbs CD33 (hP67.6, Gemtuzumab), CD38 (9A6, Daratumumab), CD123 (7G3, WO2016201065A1), CD135 (4G8 FLT3, EP3623383A1), and CD371 (h6E7.L4H1e. A54, EP3191520A1) were cloned into an Fc-attenuated (L234A/L235A (LALA)) IgG1 backbone to minimize Fc receptor-mediated ADCC and AdCAR-T-independent effects. The ExpiCHO™ expression system (Thermo Fisher) and a pcDNA™3.1 (+) mammalian expression vector were used for production. The antibodies were purified by chromatographic methods and were labeled with biotin-LC-LC-NHS. The final adapter molecules showed high purity on SDS gel and could be flow cytometrically evaluated using a fluorochrome-labeled anti-LLE mAbs. For functional testing of AMs with AdCAR-T cells, three AML cell lines with different antigen expression profiles were used: MOLM13 (CD33high, CD38high, CD123high, CD135high, CD371low), HL60 (CD33high, CD38low, CD123low, CD135low, CD371high), and U937 (CD33high, CD38high, CD123low, CD135low, CD371high). Titration experiments with these cell lines demonstrated highly specific, target antigen-dependent lysis of the cells by AdCAR-T cells even at very low AM concentrations (EC50 range 9.3-647.9 pg/mL for strongly expressed antigens). No nonspecific lysis was observed with AdCAR-T cells alone or when the target antigen was not expressed. Only against U937, a cytolytic effect was observed at high E:T ratios, both with AdCAR-T cells alone and with non- or lightly expressed antigens such as CD123 and CD135. The reason for this was not further analyzed, but it is suspected that there is a similar NKG2D or DNAM1 ligand-dependent cytotoxic effect, similar to what was described in our previous study with JeKo1.

7.2.4 AdCAR-T cells can overcome intratumoral antigen heterogeneity through multiplex targeting in vitro

To demonstrate how AdCAR-T cells can overcome intratumoral antigen heterogeneity of AML through combinatorial targeting with the newly produced AMs, an *in vitro* model was generated by pooling knockout variants for CD33, CD38, or CD33/CD38 combined in AML cell lines using CRISPR/Cas9. AdCAR-T cells were cultured with a 1:1 mixture of wild-type and knockout AML cells (MOLM13WT, MOLM13CD33KO, MOLM13CD38KO, and MOLM13CD33/CD38KO). Initially, it was again shown that in the absence of AMs, lysis does not occur with AdCAR-T cells alone. Addition of individual AMs, either against CD33, CD38, or a combination thereof, specifically led to the elimination of antigen-positive target cells and selection of a pure population of the respective knockout variant, once again demonstrating the high specificity of the AdCAR system. Unexpectedly, even the triple combination of CD33, CD38, and CD123 at an E:T ratio of 1:4 resulted in the selection of a possibly pre-existing CD123 low cell pool. The same experiment was conducted with the U937 cell line and AMs against CD33, CD38, and CD371 individually and in combination. Again, it was observed that a triple-negative pool was selected. This was no longer observed at higher E:T ratios of 1:2 or 1:1, indicating target-independent bystander killing. Nonetheless, this demonstrates the feasibility of simultaneously targeting multiple (n=3) antigens by AdCAR-T cells to address intratumoral heterogeneity in AML.

7.2.5 AdCAR-T cells exhibit specific and flexible in vivo activity

To demonstrate the flexible and specific activity of the AdCAR system with various AMs *in vivo*, a U937 mouse model was established in NSG mice. On day -4, $1x10^6$ U937luc/CD19t (CD33high, CD38high, CD123low, CD135low, CD371high) cells were transplanted via tail vein injection (i.v.). On day 0, a total of $5x10^6$ AdCAR-T cells were injected i.v. subcutaneous (s.c.) injections of 45 micrograms of each respective AM (LLE-aCD33, LLE-aCD38, LLE-aCD123, LLE-aCD135, or LLE-aCD371) were administered twice per week starting from day 0. Untreated mice (tumor only) served as a negative control. Tumor burden was monitored by bioluminescence imaging (BLI). The combination of AdCAR-T cells and one of the AMs targeting CD33, CD38, or CD371 respectively resulted in sustained remission.

Groups receiving a combination of AdCAR-T and AM targeting CD123 or CD135 showed nearly identical BLI signals and thus tumor engraftment as the control group without AdCAR-T cells. These data demonstrate the highly specific *in vivo* activity of AdCAR-T cells for personalized CAR-T therapy. Interestingly, the group treated with the AM targeting CD38 showed no impairment in the anti-leukemic activity of AdCAR-T cells, even though CD38 is known to be expressed on a subset of activated T cells [135]. There are reports describing that anti-CD38 CAR-mediated fratricide negatively influences the expansion and clinical activity of CAR-T cells [136,137]. We did not observe this phenomenon in our AdCAR-T studies. Additionally, during the manufacturing process of AdCAR-T cells, this issue will not arise, as AdCAR-T cells expand in the absence of AMs, which is another advantage of the AdCAR system over conventional CAR-T cells.

7.2.6 Multiple Targeting to avoid antigen negative relapse in AML-PDX model

To simulate native intratumoral heterogeneity and a patient-specific AdCAR-T therapy, we established PDX models of pediatric AML. Tumor cells from patient 21 (CD33high, CD38high, CD123low, CD135low, CD371high) were transduced with a lentiviral vector encoding firefly luciferase and CD19t, and immunomagnetically enriched using CD19 microbeads after two mouse passages. The antigen expression remained identical to the primary leukemia, with a slight decrease in CD123 expression. In the mouse model, 1×10^6 PDX cells were injected via the tail vein on day -3. On day 0, a total of $5x10^6$ CAR-T cells were injected intravenously. Twice a week from day 0 onwards, 45 µg of the respective AMs or their combinations were injected subcutaneously. Tumor growth was monitored by bioluminescence-based imaging (BLI). Interestingly, mono-targeting of CD33, CD38, or CD371 each resulted in rapid disease progression, with median time to reach endpoint criteria compared to untreated PDX mice extended by only 11 days for CD33, 11 days for CD38, and 18 days for CD371. Only the combination of all three AMs, expected to target >95% of leukemic blasts according to our antigen screen, resulted in sustained remission. Flow cytometric analysis of mouse bone marrow showed loss of the respective targeted antigen, indicating antigen escape through selection of antigen-low or antigen-negative subpopulations. Furthermore, significantly higher numbers of AdCAR-T cells were detected in the bone marrow of combination therapy mice

compared to monotherapy groups. These data confirm the prediction from our antigen screen of potential antigen escape or selection of antigen-low populations in AML and clearly demonstrate the potential of multiplex targeting by AdCAR-T cells to overcome antigen escape due to heterogeneous antigen expression. In summary, our findings emphasize that successful clinical translation of CAR-T cells against (pediatric) AML requires stringent safety measures and personalized combinatorial targeting approaches. Additionally, the AdCAR-T platform can be easily adapted to heterogeneous diseases like AML, allowing flexible targeting of different antigens.

8. Concluding remarks

Over the past decade, CAR-T cell therapies have achieved significant success, particularly in treating B-cell malignancies. Nonetheless, their application in other malignant diseases faces challenges, including issues with controlling activity levels and a lack of flexibility in addressing the diversity of diseases. The AdCAR system emerges as a potential solution to these obstacles. Its transient activity, dictated by the pharmacokinetics and dynamics of adapter molecules, ensures enhanced safety compared to traditional CAR-T cells. This system bypasses the need for complex genetic modifications such as suicide switches or inducible constructs, allowing for control through simple adjustments in the concentration and frequency of adapter molecule administration.

In our *in vitro* NHL study, we demonstrated that the AdCAR system can achieve highly specific activity via adapter molecules for targeted antigen recognition when faced with matching antigen-positive cells. The AdCAR-T cells are comparable in cytolytic activity, immunophenotype, exhaustion levels, and cytokine profiles to traditional CD19CARs. Notably, our findings revealed that using two adapters in tandem is not only feasible but also offers superior performance over conventional CD19CAR, particularly in scenarios of heterogenic antigen expression. Moreover, the study underscored the AdCAR-T cells' versatility, which is facilitated by the selection of diverse adapter molecules.

Our subsequent research focused on the inter- and intratumoral heterogeneity in AML. Analyzing 30 pediatric AML samples, we encountered considerable variation both within and between tumors, highlighting the need for highly personalized treatment strategies. We developed and validated adapter molecules targeting five AML-related antigens (CD33, CD38, CD123, CD135, and CD371) in both *in vitro* and *in vivo* settings. In patient-derived xenograft (PDX) models of pediatric AML, which accurately reflects the primary disease's intratumoral heterogeneity, we found that targeting a single antigen with CAR-T cells led to rapid antigen evasion and disease progression. Conversely, a strategy combining attacks against CD33, CD38, and CD371 achieved complete remission.

Furthermore, our research demonstrated the feasibility of rapidly generating adapter molecules (AMs), particularly by leveraging clinically proven antibodies through LLE conjugation.

In conclusion, the AdCAR technology showcases the ability to precisely control the qualitative and quantitative functions of CAR-T cells, offering a path towards multiplex antigen targeting. This advancement marks a significant step forward in the evolution of precision immunotherapy

9. References

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10. Appendix

1st Publication

Adapter CAR T Cell Therapy for the Treatment of B-Lineage Lymphomas

biomedicines

MDPI

Article

Adapter CAR T Cell Therapy for the Treatment of B-Lineage Lymphomas

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Abstract: CD19CAR T cells facilitate a transformational treatment in various relapsed and refractory aggressive B-lineage cancers. In general, encouraging response rates have been observed in B-lineage-derived non-Hodgkin's lymphomas treated with CD19CAR T cells. The major cause of death in heavily pretreated NHL patients is lymphoma progression and lymphoma recurrence. Inefficient CAR T cell therapy is the result of the limited potency of the CAR T cell product or is due to loss of the targeted antigen. Target antigen loss has been identified as the key factor that can be addressed stringently by dual- or multitargeted CAR T cell approaches. We have developed a versatile adapter CAR T cell technology (AdCAR) that allows multitargeting. Screening of three different B-lineage lymphoma cell lines has revealed distinct immune target profiles. Cancer-specific adapter molecule combinations may be utilized to prevent antigen immune escape. In general, CD19CAR T cells become non-functional in CD19 negative lymphoma subsets; however, AdCAR T cells can be redirected to alternative target antigens beyond CD19, such as CD20, CD22, CD79B, and ROR-1. The capability to flexibly shift CAR specificity by exchanging the adapter molecule's specificity broadens the application and significantly increases the anti-leukemic and anti-lymphoma activity. The clinical evaluation of AdCAR T cells in lymphoma as a new concept of CAR T cell immunotherapy may overcome treatment failure due to antigen immune escape in monotargeted conventional CAR T cell therapies.

Keywords: adapter CAR T cell; immune escape; combinatorial immunotargeting; B-cell lymphoma

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1. Introduction

Therapies based on genetically engineered T cells to express a chimeric antigen receptor (CAR) have revolutionized the treatment of relapsed and refractory patients with B-lineage cancers [1]. For the treatment of B-lineage-derived cancers, a total of four CD19- and two BCMA-targeted CAR T cell products with distinct biological properties have been approved by the US FDA over the last 5 years since 2017 and have contributed to improved survival in acute lymphoblastic leukemia, non-Hodgkin's lymphoma, and multiple myeloma [2].

The major acute complications of CD19 CAR T cell therapies are cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), and macrophage activation syndrome (MAS) originating from T cell activation and proliferation with excessive secretion of cytokines and triggering of an immune activation cascade [3]. Despite growing experience in the management of these life-threatening acute toxicities, patients still frequently require intensive care treatment [4]. However, the major causes of death in CAR T cell-treated patients remain leukemia, lymphoma, and multiple myeloma recurrence due to treatment failure [5]. The main drivers of CAR T cell therapy failure are

directly or indirectly linked to the targeted antigen. Target antigen downregulation, point mutations, alternative splicing, and biallelic loss of the antigen have been identified as the drivers of resistance to CD19- and BCMA-CAR T cell therapy in up to 40% of patients [6–10]. Indirect failures are caused by CAR T cell dysfunction while the targeted antigen remains present. In multiple myeloma, antigen shedding and trogocytosis have been shown to induce CAR T cell fratricide. Moreover, the upregulation of immune checkpoint ligands such as (PD-L1) and the hypoxic niche in the bone marrow limit CAR T cell function [11].

We have developed an adapter CAR technology (AdCAR) that allows functional control of the CAR-expressing cells via the introduction of adapter molecules [12,13]. AdCAR T cells are functionally inert without the adapter molecules and mediate anticancer function only in the presence of adapter molecules [13]. Direct effects are defined as downregulation or loss of the targeted epitope of an antigen. Indirect failures are mechanisms in which the CAR T cell function is impaired while the targeted antigen remains present, such as antigen shedding, overstimulation by fratricide, or targeting of healthy tissues. The vast majority of target antigen-dependent CAR T cell treatment failures can be addressed highly efficaciously by multitargeted approaches.

We hypothesized that our AdCAR T cells could target B-lineage lymphomas via adapter molecules targeted to CD19 and alternative antigens present on non-Hodgkin's lymphoma. The advantage over conventional direct CD19 CAR T cells is the possibility to broaden the targets via alternative antigens beyond CD19 and perform combinatorial as well as sequential targeting. We compared the in vitro function of CAR T cells by different measures with a second-generation CD19 CAR and a third-generation AdCAR construct with regards to specific CAR T cell activation, cytokine secretion, cytolysis, and exhaustion. The key feature of AdCAR T cells compared with conventional CAR T cells is the ability for multitargeting, which may overcome antigen immune escape, which is responsible for treatment failure with conventional CD19 CAR T cells. We provide a proof of concept for our AdCAR technology as a platform technology for adoptive T cell therapy to address the major cause of treatment failure with CAR T cells in B-lineage lymphomas.

2. Material and Methods

2.1. Design of CAR Constructs and Lentiviral Vectors

A CD19 single chain variable fragment (scFv) was derived from the murine anti-CD19 mAb clone 4G7. The AdCAR anti-LLE-biotin scFv was derived from the murine mAb clone mBio3. The scFvs were designed in light–heavy (LH) configuration with a standard G4S3 linker. The CD19 was used in a second-generation CAR backbone comprising a IgG4 hinge, a CD8 transmembrane domain, the cytoplasmic costimulatory domain of 4-1BB, and the CD3ζ signaling domain. Both constructs co-expressed truncated epidermal growth factor receptor (tEGFR) downstream from a ribosomal skip P2A site. The custom gene synthesis of the CAR constructs was carried out by GeneArt (ThermoFisher, Waltham, MA, USA). The CAR constructs were subcloned into a second-generation lentiviral transfer vector for generating self-inactivating lentiviral particles (SIN-LV). SIN-LV were produced in Lenti-X 293T cells (Takara) after lipofection (Lipofectamin 3000, ThermoFisher, Waltham, MA, USA) according to the manufacturer's instructions utilizing a second-generation packaging plasmid, the VSV-G envelope plasmid, and the corresponding transfer plasmid for expression of the CD19 or the AdCAR (LLE-CAR). Supernatants containing SIN LV were harvested 24 h after lipofection, concentrated by the Lenti-X concentrator (TaKaRa, Kusatsu, Shiga, Japan) and cryopreserved according to the manufacturer's instructions.

2.2. Isolation of Human Primary T Cells and Transduction

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples acquired from healthy volunteer donors at the University Children's Hospital Tuebingen, by Ficoll centrifugation (Biocoll, Biochrom, Berlin, Germany). T cells were isolated by magnetic separation using anti-CD4 and anti-CD8 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) simultaneously. Isolated T cells were activated with TransAct^{TM}

(Miltenyi Biotec) and cultivated in TexMACS media supplemented with 10 ng/mL IL7 and 5 ng/mL IL15 (Miltenyi Biotec, Bergisch-Gladbach, Germany). TransAct^{TM}-activated T cells were transduced at a multiplicity of infection (MOI) of 3 after 36 h. Transduced T cells were maintained at 0.5–2 $\times$ 10^6 cells/mL in IL7/IL15-supplemented TexMACS® media. On Days 7+, CAR transduction efficiency and the CD4/CD8 ratio were determined by flow cytometry.

2.3. Cell Line Culturing

All lymphoma cell lines (JeKo-1, Raji, Daudi) were purchased from ATCC (Manassas, VA, USA) or DSMZ (Braunschweig, Germany), and Lenti-X 293T cells were purchased from (TaKaRa, Kusatsu, Shiga, Japan). All lymphoma cell lines were maintained in RPMI 1640 media and Lenti-X 293T cells were maintained in DMEM media. Media were supplemented with 10% heat-inactivated fetal bovine serum (ThermoFisher, Waltham, MA, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 units/mL of penicillin and 100 µg/mL of streptomycin (all from Biochrom, Waltham, MA, USA), referred to as complete media.

2.4. Generation of Luciferase-Expressing Cell Lines

Transfer plasmids, based on a third-generation lentiviral transfer vector plasmid encoding firefly luciferase and green fluorescent protein (GFP), were kindly provided by Irmela Jeremias, Helmholtz Center Munich, Germany. LV particles were generated as described above. Cell lines were transduced at a MOI of 3. Transgene expression was confirmed by flow cytometry. Transduced cells were enriched by bulk fluorescence-activated cell sorting.

2.5. Generation of Knockout Cell Lines Using CRISPR/Cas9 Technology

All gRNAs were designed with the online tool "CHOPCHOP" (http://chopchop.cbu.uib.no/, accessed on 16 April 2020) and synthesized by ThermoFisher Scientific. To form a CRISPR/RNP complex that was ready to transfect, the gRNA and the trans-activating CRISPR RNA (tracrRNA) (ThermoFisher, Waltham, MA, USA) were annealed directly before use. The tracrRNA and all gRNAs were dissolved in an appropriate amount of TE buffer (10 mM Tris, 0.1 mM EDTA; pH 7.5) to get working solutions of 100 pmol/µL. These working stocks were aliquoted to avoid freeze–thawing cycles and stored at −20 °C until use. The gRNA and the tracrRNA were mixed in an equimolar concentration in 1× annealing buffer (5× annealing buffer: 30 mM HEPES, 100 mM potassium acetate, and 2 mM magnesium acetate). For each transfection of 2 $\times$ 10^6 cells, 75 picomoles of gRNA and 75 picomoles of tracrRNA were used. The annealing reaction was carried out with a temperature gradient in a thermal cycler (95–25 °C; −0.1°/s). The annealed product (gRNA: tracrRNA) was mixed in an equimolar concentration with TrueCut Cas9 Protein v2 (ThermoFisher, Waltham, MA, USA) and incubated for at least 10 min and for a maximum of 30 min at RT to form the CRISPR/RNP complex. Transfection of the RNP was achieved with the Neon™ Transfection System 100 µL Kit according to the manufacturer's instructions. Knockout cells were enriched by bulk fluorescence-activated cell sorting.

2.6. Luciferase-Based Cytotoxicity Assay (LCA)

Tumor cells were plated in complete RPMI media in white 96-well flat-bottomed plates (Greiner Bio One, Kremsmünster, Austria) with 30,000 cells per well. Synthetic D-luciferin (Sigma Aldrich, St. Louis, MO, USA) was added to each well at 4 µg/mL. Effector cells were plated at the indicated effector to target ratio (E:T). The total volume per well was 200 µL. Therapeutic antibodies or combinations thereof were used at the indicated concentrations. Plates were incubated in a HERAcell incubator (Heraeus, Hanau, Germany) at 37 °C, 95% humidity, and 5% CO_2. Bioluminescence was measured using the Tecan SPARK microplate reader (Perkin Elmer, Waltham, MA, USA) at 37 °C, at the indicated time intervals. Lysis was calculated by the relative luminescence of the testing conditions according to a lysis formula based on a standard dilution series.

2.7. Immunophenotyping of Tumor Cell Lines

Immunophenotyping was performed on a BD™ LSR II flow cytometer. Antibody staining was carried out according to standard operating procedures at 4 °C in a PBS buffer. All commercial antibodies were purchased from (Miltenyi Biotec, Bergisch-Gladbach, Germany). The antibody clone is defined by the clone in brackets. The tumor-associated antigens were CD19 PE (REA675), CD20 PE (REA780), CD22 PE (REA340), ROR-1 PE (REA1051), CD276 PE (REA1094), CD79B PE (REA120), and CD10 PE (97C5). The activating ligands on tumor cells were CD112 APC (REA1195) and CD155 PE (REA1081) (DNAM-1 ligands), and MIC A/B APC (REA10876) (an NKG2D ligand). Indirect staining of therapeutic antibodies was achieved with Anti-Biotin PE (REA746). The isotype controls were IgG1 Isotype PE (REA293) and IgG1 Isotype APC (REA293). Antigen positivity was defined by staining of the tumor cells using primary labelled mAbs compared with the isotype control. Overton positivity was calculated by integral subtraction (specific fluorescence minus fluorescence of the isotype control) using FlowJo 10.4 software.

2.8. Quantification of Cytokine Release

Here, 2.5×10^5 CAR T cells were cultivated with 2.5×10^5 JeKo-1 cells at an ET ratio of 1:1 per well according to the indicated conditions in RPMI 1640-based complete media in a HERAcell incubator (Heraeus) at 37 °C, 95% humidity, and 5% CO_2. Supernatants were collected after 24 and 120 h. Quantification of the cytokines was performed with cytokine capture beads using the MACSPlex® custom cytokine assay with the indicated specificities. Data acquisition was carried out on a MacsQuant® Analyzer 10 SN 2535 flow cytometer and with MACSQuantify® software (2.11.1731.18902) according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany).

2.9. Adapter Molecule Conjugation

Adapter molecule modification was performed at 21 °C for 1 h in 0.1 M $NaHCO_3$ buffer using a 3-fold molar excess of biotin-LC-LC-NHS (ThermoFisher, CAS-No. 89889-52-1, Waltham, MA, USA), followed by separation of the antibody/label mix on a Sephadex G25 column. Protein-containing fractions were pooled, and the concentration was measured as the absorption at 280 nm. Successful conjugation was confirmed by LC-MS and/or by flow cytometry on cell lines expressing the target antigen and secondary staining with a fluorophore-conjugated anti-biotin antibody.

2.10. T Cell Immunophenotyping

The T cells' maturation state was assessed via the expression of CD45RA, CD45RO, CD62L, and CD95. Five different states of maturation were distinguished: $T_N{}^i$, T_{SCM}, T_{CM}, T_{EM}, and T_{EMRA}. T naïve^i ($T_N{}^i$) cells were defined as ($CD45RA^+/CD45RO^-/CD62L^+/CD95^-$). We acknowledge that CD3/CD28 co-activated T cells per se are defined as non-naïve T cells. Thus, we classified T cells with a naïve immunophenotype as $T_N{}^i$. Stem cell-like memory T cells (T_{SCM}) were defined as ($CD45RA^+/CD45RO^-/CD62L^+/CD95^+$), central memory T cells (T_{CM}) were defined as ($CD45RA^-/CD45RO^+/CD62L^+CD95^+$), effector memory T cells (T_{EM}) were defined as ($CD45RA^-/CD45RO^+/CD62L^-/CD95^+$), and effector memory T cells that re-expressed CD45RA (TEMRA) were defined as ($CD45RA^+/CD45RO^-/CD62L^-/CD95^+$). T cells' activation state was assessed using CD25 and CD69. T cells' exhaustion state was assessed by PD-1 expression. All commercial antibodies were purchased from Miltenyi Biotec. The antibody clone is defined by the clone in brackets. The T cell antigens were CD4 VioBlue (M-T466), CD8 APC-Vio770 (REA734), CD45RA VioGreen (REA1047), CD45RO VioGreen (REA611), CD45RO APC-Vio770 (REA611), CD62L PE (REA615), and CD95 APC-Vio770 (REA738). The CAR identification marker-truncated EGFR was measured via EGFR APC (REA688). The activating co-receptors on T cells were CD226 PE (REA1040) (DNAM-1) and CD314 APC (REA797) (NKG2D). The T cell activation markers were CD25 VioBright B515 (REA945) and CD69 VioBlue (REA824). The T cell exhaustion

marker was CD279 PE (REA1165) [PD-1]. The isotype controls were IgG1 Isotype PE (REA293) and IgG1 Isotype APC (REA293).

3. Results

We designed our adapter CAR T cells (AdCAR T), which were targeted to biotin in the context of a specific linker structure, referred to as linker–label–epitope (LLE biotin) in a third-generation CAR T cell format. On the basis of our findings in previous studies, we learned that third-generation signaling was advantageous compared with second-generation signaling for AdCAR T cells [13]. The recognition domain was based on the murine anti-LLE-biotin clone mBio3.

The AdCAR-scFv was used in a light–heavy variable chain (LH) configuration. The CAR backbone consisted of an IgG4 hinge extracellular spacer domain variant, the CD8A transmembrane domain, CD28, and the 4-1BB costimulatory domains, as well as the CD3ζ signaling domain. Our CD19CAR was based on the commonly used murine FMC63 clone in LH-scFv configuration in a second-generation CAR backbone. The CD19CAR comprised an IgG4 hinge extracellular domain variant, the CD8A transmembrane domain, the 4-1BB costimulatory domain, and the CD3ζ signaling domain. A truncated version of the human epidermal growth factor receptor (tEGFR) was integrated downstream from a P2A site suitable for detection and enrichment of the CAR T cells. The CAR design and a schematic illustration of the CD19CAR, the AdCAR, and the tEGFR anchored to the cell membrane are shown in Figure 1a,b. The marker gene tEGFR allowed us to clearly identify the CAR T cell fraction of the whole T cell population by flow cytometry, as depicted in Figure 1c. The transgene expression of the CD19CAR T cell and AdCAR T cell constructs were comparable, indicating similar expression efficiencies.

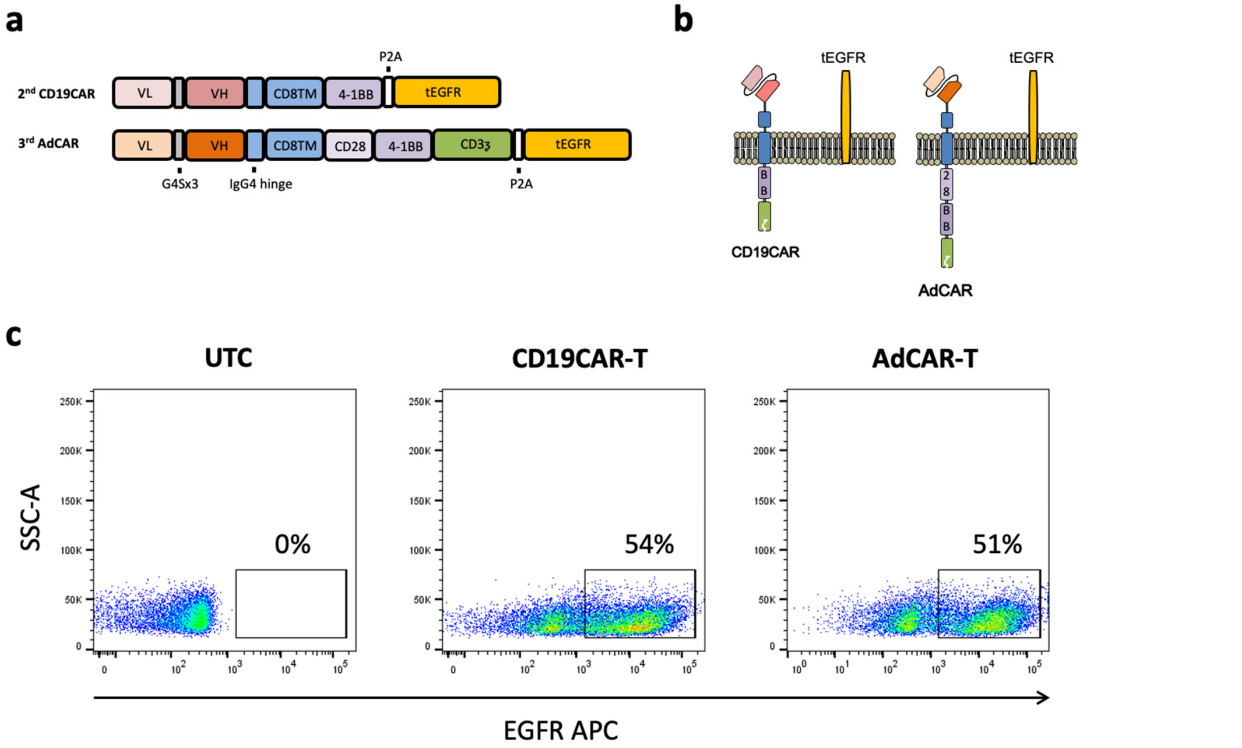
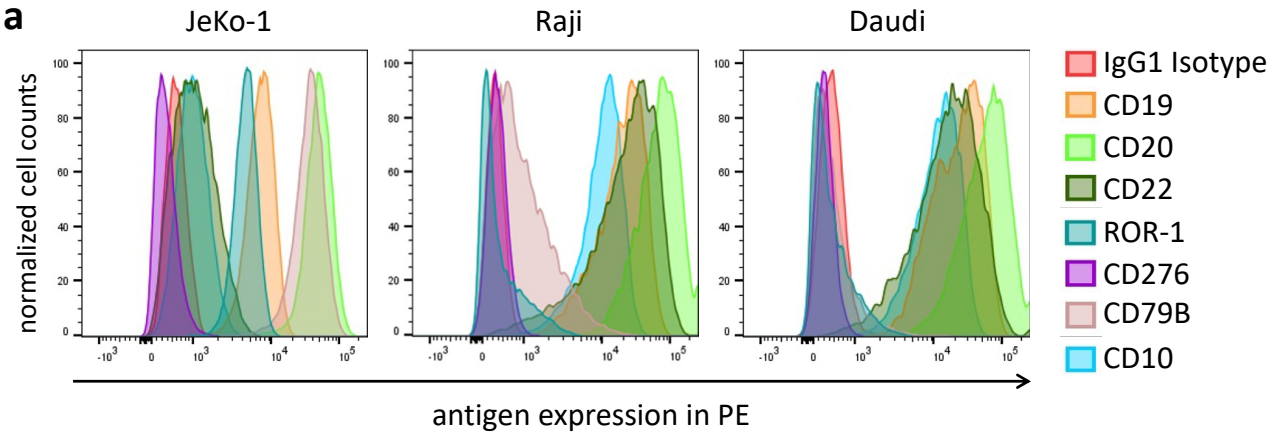


Figure 1. Design and expression profile of second-generation CD19CAR and third-generation AdCAR T cells. (**a**) Illustration of the transgene design of the conventional second-generation CD19CAR and the third-generation AdCAR including the marker gene. (**b**) Schematic illustration of the transgene CAR and marker gene expression in the context of the cell membrane. (**c**) The expression of CAR was determined by flow cytometry utilizing the co-expressed marker gene tEGFR. Representative flow cytometric plots for non-transduced activated T cells (**left**), CD19CAR T cells (**middle**), and AdCAR T cells (**right**) are shown.

3.1. Heterogeneity in the Antigen Expression Profiles of B-Lineage Non-Hodgkin Lymphoma

The essential features of adapter CAR systems include the possibility for transient, universal, simultaneous, and sequential multitargeting. To demonstrate the heterogeneity of B-lineage derived non-Hodgkin's lymphoma, we immunoprofiled three cell lines: JeKo-1 (MCL), Raji, and Daudi (both Burkitt). Immunophenotyping was conducted using primary R-phycoerythrin (PE)-labeled mAbs (Figure 2) of the same fluorophore to estimate differential expression in a semiquantitative manner. The target antigen panel was chosen on the basis of the expression level found in B-lineage lymphomas and their suitability for CAR T cell therapy from our own experience and the literature. We focused on highly expressed antigens found in primary leukemia and lymphomas, such as CD19, CD20, CD22, ROR-1, CD276, CD79B, and CD10. Figure 2a shows the expression of the surface antigens in normalized histograms. The intratumoral heterogeneity is highlighted and visualized in two tables. One table shows a heat map with the fraction of antigen-positive cells as a percentage (Figure 2b), and the second table depicts the suitability of the target antigens for CAR-mediated targeting as a heat map (Figure 2c) by showing the median fluorescence intensity ratio (=MFIR). The intratumoral heterogeneity of antigens in CAR T cell therapy is best addressed by versatile multitargeted CAR technologies.

a



b

antigen positive cells [%]			
Antigen	**Jeko-1**	**Raji**	**Daudi**
CD19	100	100	100
CD20	100	99	100
CD22	38	99	98
ROR1	100	21	8.5
CD276	1.1	4	0.4
CD79b	100	52	8.7
CD10	31	100	100

[%] code
0-10
10-20
20-50
50-90
90-100

c

MFIR			
Antigen	**Jeko-1**	**Raji**	**Daudi**
CD19	12	59	51
CD20	84	204	131
CD22	1.75	72	37
ROR1	7.6	0.84	0.74
CD276	0.6	1.1	0.69
CD79b	62	2.4	0.85
CD10	1.72	30	26

MFIR code
<1.5
1.5-10
10-50
50-100
100-250

Figure 2. Antigen expression profile of the lymphoma cell lines JeKo-1, Raji, and Daudi. (**a**). JeKo (**left**), Raji (**middle**), and Daudi (**right**) were investigated for the surface expression of the target antigens CD19, CD20, CD22, ROR-1, CD276, CD79B, and CD10. (**b**) The fraction of antigen-positive cells (as a percentage) was calculated by Overton subtraction of the histograms. The table shows the percentage of corresponding antigen positive cells. The sample data minus the corresponding isotype control are shown in the heat map. (**c**) The relative expression level of the screened antigens is shown in the heat map as an index calculated from the median fluorescence intensity of the sample divided by the median fluorescence intensity of the isotype control, defined as the MFIR.

3.2. CAR-Specific Activation and Highly Specific Target Cell Lysis

In order to show the activation of AdCAR T cells strictly requires the presence of the AdCAR T cell, a specific adapter molecule, and a corresponding antigen-positive target cell, we performed co-incubation experiments and measured the upregulation of the early

activation marker CD69 and CD25. The specifically activated CAR T cell subset was defined as double $CD25^+CD69^+$ positive. To test the antigen-specific activation of CAR T cells, we tested various combinations of effector cells, adapter molecules, and target cells. Only the combination of CD19CAR T cells in conjunction with the antigen-positive target cell line JeKo-1 activated CD19CAR T cells, and for AdCAR T cells, only the combination of AdCAR T cells plus the antigen-positive target cell line JeKo-1 plus an LLE-CD19 mAb adapter molecule activated the AdCAR T cells. The specific activation ($CD25^+CD69^+$ expression) of CAR T cells measured after 1 and 5 days was significantly increased compared with the unstimulated control. Neither under CD19CAR T cell conditions nor AdCAR T cell conditions was a relevant difference in the expression levels of $CD25^+CD69^+$ found after 1 and 5 days. Non-specific upregulation of $CD25^+CD69^+$ in AdCAR T cells in the presence of JeKo-1 was observed, whereas there was no significant non-specific upregulation of $CD25^+CD69^+$ detected in the presence of LLE-CD19 mAb at 1 ng/mL without the target cells (Figure 3a).

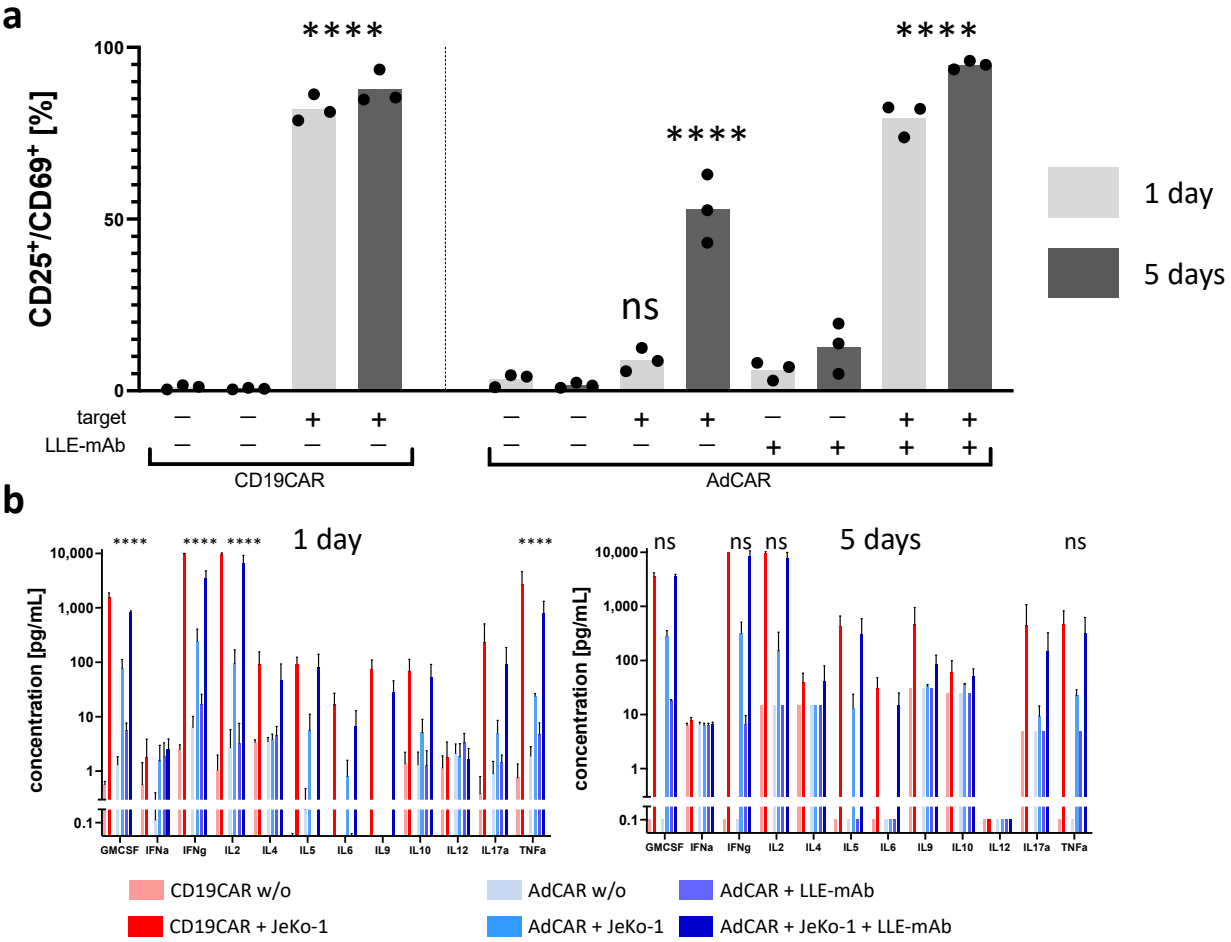


Figure 3. Activation and cytokine secretion profile of AdCAR T cells vs. CD19CAR T cells. (**a**) CD19CAR T cells were incubated for 24 h (light grey) and for 5 days (dark grey) with or without the $CD19^+$ lymphoma cell line JeKo-1. Additionally, AdCAR T cells were incubated for 1 day (light grey) and for 5 days (dark grey) with or without the $CD19^+$ lymphoma cell line JeKo-1, and with or without 10 ng/mL LLE-CD19 mAb. Specific CAR activation after incubation was determined by flow cytometry staining of the cell surface activation markers CD25 and CD69. The double-positive $CD25^+CD69^+$ cells were considered and defined to be the activated cell subset. (**b**) The quantitative levels of the indicated secreted cytokines in the supernatant were measured after 1 day (**left**) and after 5 days (**right**). Data shown in (**a**) represent the mean ± SEM of (n = 1) independent experiments from three different donors. Data shown in (**b**) represent the mean ± SEM of (n = 1) independent experiments in duplicate from three different donors. ns, not significant. ****, $p \leq 0.0001$.

Cytokine secretion was assessed as a secondary indicator of CAR effector function. The cytokine concentrations were measured in the supernatants after 1 day and after 5 days (Figure 3b). The cytokine panel comprised the 12-cytokine multiplex panel: GMCSF, INFα, IFNγ, IL2, IL4, IL5, IL6, IL9, IL10, IL12, IL17a, and TNFα. With regards to the secretion kinetics of the most relevant CAR T cell cytokines GMCSF, IL2, IFNγ, and TNFα, we observed significantly higher cytokine levels secreted by conventional CD19CAR T cells than by AdCAR T cells after 1 day. However, after 5 days, significant differences in the cytokine profile of CD19CAR T cells and AdCAR T cells were no longer detectable. The antigen-specific CAR-mediated cytolysis is illustrated in Figure 4. Antigen-specific CAR activation was further supported by the activation-dependent cytokine secretion, a change in the immunophenotype to an effector cell phenotype T_{EMRA}, and the activation-dependent expression of the exhaustion marker PD-1 (Figure 5).

To demonstrate the universal antigen-specific effector function of AdCAR T cells in vitro, we used adapter molecules in the LLE-mAb format targeted to various tumor-associated antigens expressed by the lymphoma cell lines Raji, Daudi (both Burkitt), and JeKo-1 (MCL). AdCAR T cells were incubated at six different effector-to-target (ET) ratios. The range covered 5:1 to 0.15:1, which corresponded to 1 CAR T cell versus 6.66 cancer cells. The LLE-mAb concentration was used at 1 ng/mL in all experiments per LLE-mAb. Therefore, combinations of LLE-mAb were used at the higher final LLE-mAb concentrations. The target cell lysis was determined by LCA, a luciferase-based cytotoxicity assay. In order to demonstrate the specificity of the AdCAR system, untransduced activated T cells served as negative controls. The LLE-CD19 mAb did not induce any measurable cytotoxic effects at 1 ng/mL. Conventional second-generation CD19CAR T cells served as positive controls and for benchmarking. Both monotargeting with LLE-CD19 only and combinatorial targeting with LLE-CD19 and LLE-CD20 were performed utilizing the wild-type form of the respective cell lines. The cytotoxic effect of the AdCAR T cells was significantly higher than the cytotoxic effect of activated T cells across all three NHL cell lines. However, combinatorial targeting using LLE-CD19 and LLE-CD20 did not outperform monotargeting in the wild-type cell lines (Figure 4a). To demonstrate the superiority of combinatorial targeting, we compared CD19/CD20 dual targeting with AdCAR T cells versus CD19 monotargeting with AdCAR T cells and CD19CAR T cells. Combinatorial CD19/CD20 targeting induced significantly higher cytolysis in the CD19 knockout variant of JeKo-1 (JeKo-1_{CD19KO}) (Figure 4b). Moreover, to minimize non-specific lysis effects in JeKo-1 and in Raji, we demonstrated the capability of targeting alternative target antigens beyond CD19 at a low ET ratio of 1.25:1 (Figure 4c,d). It is noteworthy that some target antigens are more suitable for CAR T cell therapy than others. Targeting of different antigens elucidated different potencies, which were dependent on the antigen expression levels, but also the biological properties of the target antigens' impact on the efficacy to recruit AdCAR T cells to the cancer cells. Thus, despite the capability for universal targeting (one CAR construct for all antigens), not all target antigens managed to recruit AdCAR T cells to cancer cells at the same level and mediate differential potencies. Additionally, we showed that combinatorial targeting of alternative antigens, such as CD10 in combination with ROR-1, or CD10 in combination with CD20, is feasible and can increase the targeting compared with monotargeting. In general, combinatorial targeting was superior compared with monotargeting in JeKo-1 and Raji (Figure 4c,d).

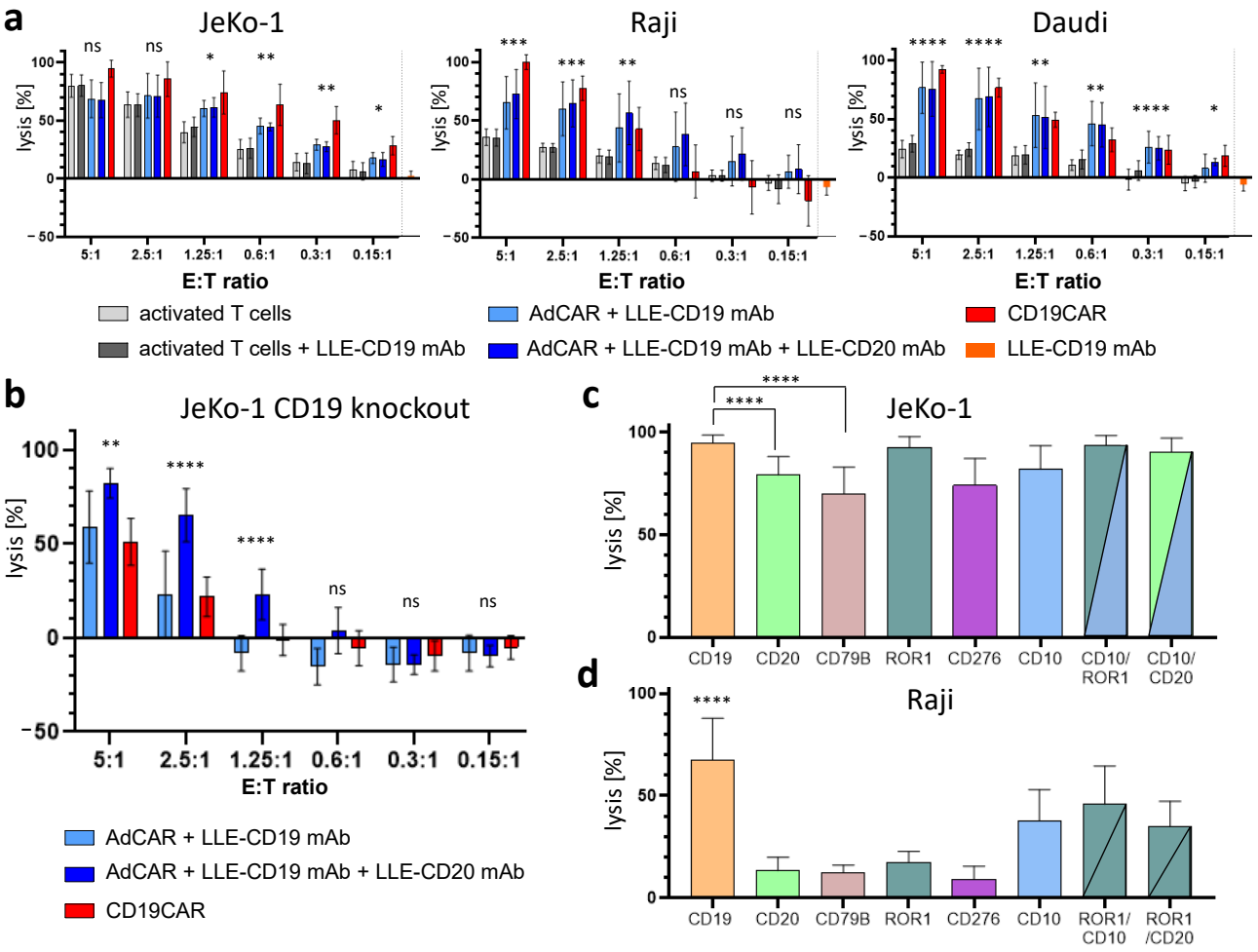


Figure 4. Multitargeting with AdCAR T cells prevents immune evasion in lymphoma. (**a**) Non-transduced activated T cells with or without 1 ng/mL LLE-CD19 mAb, AdCAR T cells with or without 1 ng/mL LLE-CD19 mAb +/− 1 ng/mL LLE-CD20 mAb, or conventional CD19CAR T cells were incubated with JeKo-1 (**left**), Raji (**middle**), and Daudi (**right**) at the indicated E:T ratios. Target cells only were incubated with LLE-CD19 mAb at 1 ng/mL. Target cell lysis was determined by LCA after 48 h. A statistical analysis is presented for the comparison of the AdCAR T cells + LLE-CD19 mAb versus the activated T cells. (**b**) AdCAR T cells with 1 ng/mL LLE-CD19 mAb or 1 ng/mL LLE-CD19 mAb + 1 ng/mL LLE-CD20 mAb, or conventional CD19CAR T cells were incubated for 48 h with a CD19 knockout variant of JeKo-1 (JeKo-1_{CD19KO}) at the indicated E:T ratios. Target cell lysis was determined by LCA after 48 h. A statistical analysis is presented for a comparison of the combinatorial targeting (CD19 + CD20) versus the monotargeting (CD19). (**c**,**d**) To demonstrate the flexibility of the AdCAR system, AdCAR T cells were incubated with either JeKo-1 (**c**) or Raji (**d**) at an E:T ratio of 1.25:1 with 1 ng/mL of the indicated LLE-mAbs. Alternative target antigens can be utilized by AdCAR T cells. Combinatorial targeting was feasible and increased the engagement of the AdCAR T cells with the cancer cells. Target cell lysis was determined by LCA after 48 h. The statistical analysis demonstrates the superiority of CD19 as a target antigen compared with the alternative antigens CD20 and CD79B in JeKo-1. Targeting CD19 mediated the strongest cytolytic effect in Raji. Data shown in (**a**,**b**) represent the mean ± SEM of (n = 3) independent experiments in triplicate from three different donors. Data shown in (**c**,**d**) represent the mean ± SEM of (n = 3) independent experiments from three different donors. Statistical analysis was performed using one-way ANOVA and Tukey's post-hoc test. ns, not significant. *, $p \leq 0.05$. **, $p \leq 0.01$. ***, $p \leq 0.001$. ****, $p \leq 0.0001$.

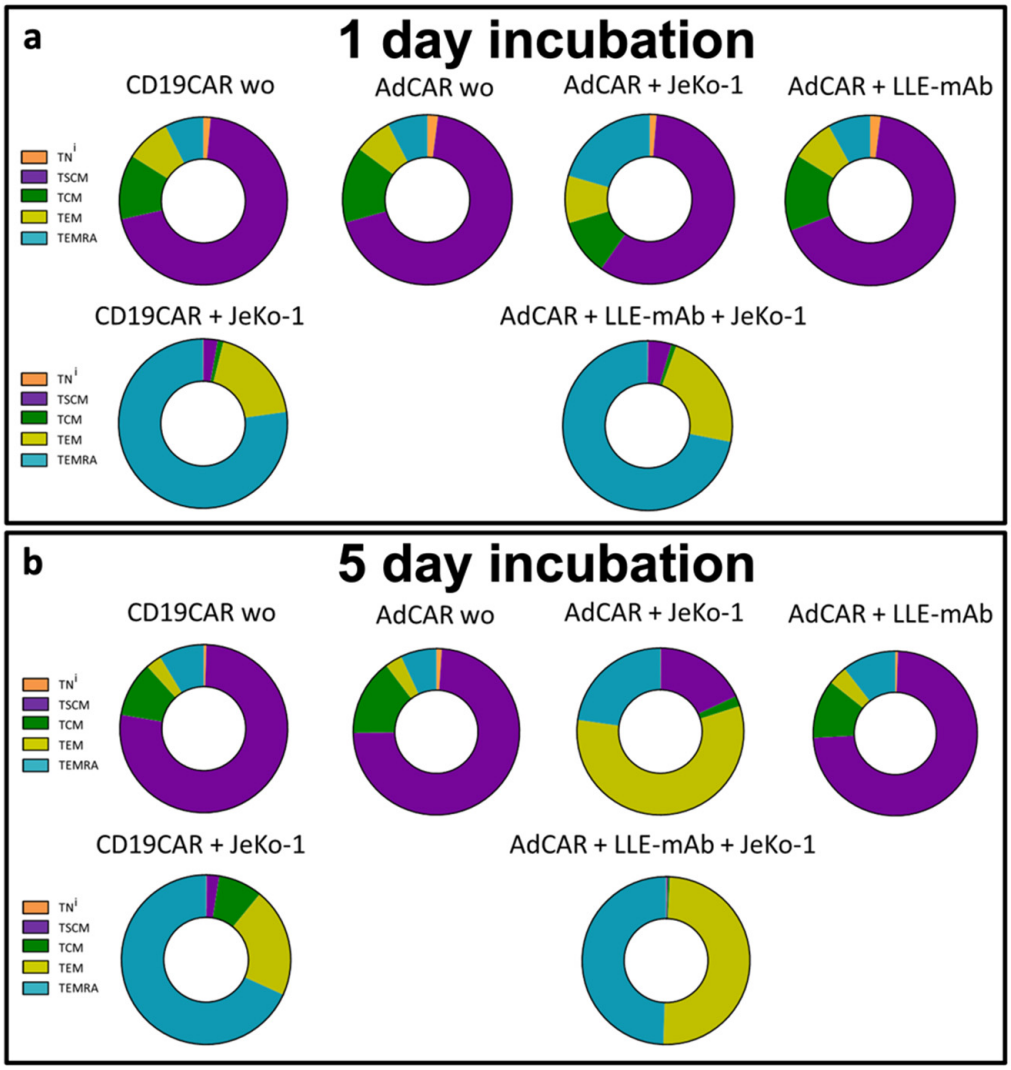


Figure 5. Immunophenotype of AdCAR T cells vs. CD19CAR T cells. CD19CAR T cells were incubated for (**a**) 1 day and (**b**) for 5 days with or without the $CD19^+$ lymphoma cell line JeKo-1. Additionally, AdCAR T cells were incubated for (**a**) 24 h and (**b**) for 5 days with or without the $CD19^+$ lymphoma cell line JeKo-1, and with or without 10 ng/mL LLE-CD19 mAb. Immunophenotypes after incubation were determined by flow cytometry by staining the cell surface markers CD45RA, CD45RO, CD62L, and CD95. Naive T ($T_N{}^i$) cells were defined as ($CD45RA^+/CD45RO^-/CD62L^+/CD95^-$), stem cell-like memory T cells (T_{SCM}) as ($CD45RA^+/CD45RO^-/CD62L^+/CD95^+$), central memory T cells (T_{CM}) as ($CD45RA^-/CD45RO^+/CD62L^+/CD95^+$), effector memory T cells (T_{EM}) as ($CD45RA^-/CD45RO^+/CD62L^-/CD95^+$), and effector memory T cells that re-expressed CD45RA (T_{EMRA}) as ($CD45RA^+/CD45RO^-/CD62L^-/CD95^+$). Data shown in (**a**,**b**) represent the mean ± SEM of (n = 3) independent experiments from three different donors.

3.3. CAR T Cell Immunophenotype, Exhaustion, and CAR-Unrelated Anticancer Effects

At first, CAR T cell activation triggered the primary effector function cytolysis. Secondary effector functions included cytokine secretion support proliferation. Proliferation of CAR T cells induced a significant redistribution of immunophenotypic cell subsets. The composition of and change in the CAR T cell immunophenotype subsets served as indicators of tertiary CAR T cell functions, such as CAR T cell fitness and long-term persistence in patients.

The CAR T cell immunophenotype was assessed on Day 1 and Day 5 after co-incubation with the target cells to understand the physiological shift during the anticancer immune response. A clear shift of the CAR T cell subsets into mature effector T cells

(T_{EMRA}) was specifically induced by contact with target cells. The documented change in the immunophenotype composition to >70% T_{EMRA} plus around 20% T_{EM} cells was very similar for the conventional second-generation CD19CAR T cells and the third-generation AdCAR T cells after 1 day (Figure 5a). The registered T cell subsets' composition after 5 days ws the result of lineage-maturation of cells and the subsequent disproportional proliferation of the CAR T cells maintaining a high proportion of T cell effector populations at 90% and above (Figure 5b). The changes in the immunophenotype indicate physiologic T cell maturation under CAR T cell engagement. In both CD19CAR T cells and AdCAR T cells, the proportion of reconstituting T_{SCM} was significantly reduced. Whereas the immediate maturation into effector cell subsets was required to control the cancer cells, the proliferation and maintenance of T_{SCM} facilitated reconstitution and persistence.

Further, the exhaustion marker PD-1 was assessed by flow cytometry after 24 h (Figure 6a,b). AdCAR T cells were found to be exclusively activated in the presence of (1) antigen specific LLE-conjugated adapter molecules and (2) the corresponding antigen-expressing target cells. There was neither a difference in PD-1 expression between the CD19CAR T cells and the AdCAR T cells in the control groups nor under the testing conditions, in which PD-1 was upregulated from a baseline of around 5% to 30% PD-1 expressing cells after co-incubation with the target cells in the CAR T cell fraction of the cells.

The non-specific activation of the AdCAR T cells and cytolysis of the target cells without the LLE-CD19 adapter molecule is frequently observed in activated T cells and CAR T cells after simultaneous stimulation via the epsilon chain of the CD3 receptor complex and the costimulatory receptor CD28, which induces the expression of activating receptors, such as DNAM-1 and NKG2D [14]. The expression of DNAM-1 and NKG2D is indirectly supported by common γ-chain cytokines (IL2, IL7, IL15) on activated T cells and CAR T cells (Figure 7a) [15].

The abovementioned non-specific lysis of cancer cells by T cells and CAR T cells, which correspond to non-CAR-related anticancer effects in a NK-like mode of action, can be mediated by the activating receptors DNAM-1 and NKG2D. Due to the high expression of the NKG2D ligand MIC A/B in JeKo-1 and the high expression of CD314 in activated T cells (Figure 7b), the NK-like CAR independent recognition of JeKo-1 may be explained. To illustrate the effect of combinatorial targeting effect mediated by the CAR receptor with the AdCAR system, the E:T titration was repeated with a CD19 knockout variant of JeKo-1 (Figure 4b). At the low E:T ratios, the NK-like AdCAR activity is significantly reduced and the specific CAR-mediated effect becomes prominent.

To investigate the reason for the high non-specific activation and cytolysis of the AdCAR T cells targeting JeKo-1, we determined the surface expression of the activating coreceptors DNAM-1 and NKG2D on activated T cells. The cognate ligand expression for DNAM-1, CD112, and CD155 (DNAM-1L), as well as for NKG2D, the MHC Class I chain-related molecules A and B (MIC A/B; NKG2DL) were measured in JeKo-1 (Figure 7a,b). It appears that the high non-specific lysis of JeKo-1 and the long-term non-specific activation of AdCARs by JeKo-1 was due to the high expression of the NKG2D ligands MIC A/B on JeKo-1. Moreover, the degree of LLE labeling (DOL) per adapter molecule seemed to impact the non-specific activation of AdCAR T cells (Figure 7c). We co-incubated the AdCAR T cells without LLE-mAb (red) and with 10 ng/mL of various LLE-mAbs, which were either multibiotinylated (light grey) or monobiotinylated (dark grey), for 1 day (left) and for 5 days (right). Activation of CAR T cells after incubation was determined by flow cytometry by staining for the cell surface markers CD25 and CD69. The increase in the $CD25^{+}CD69^{+}$ double-positive AdCAR T cells was considered to be activated by the LLE-mAb. Overall, the upregulation of CD25 and CD69 was increased by LLE-mAb and was more pronounced with multibiotinylated LLE-mAb compared with monobiotinylated LLE-mAb.

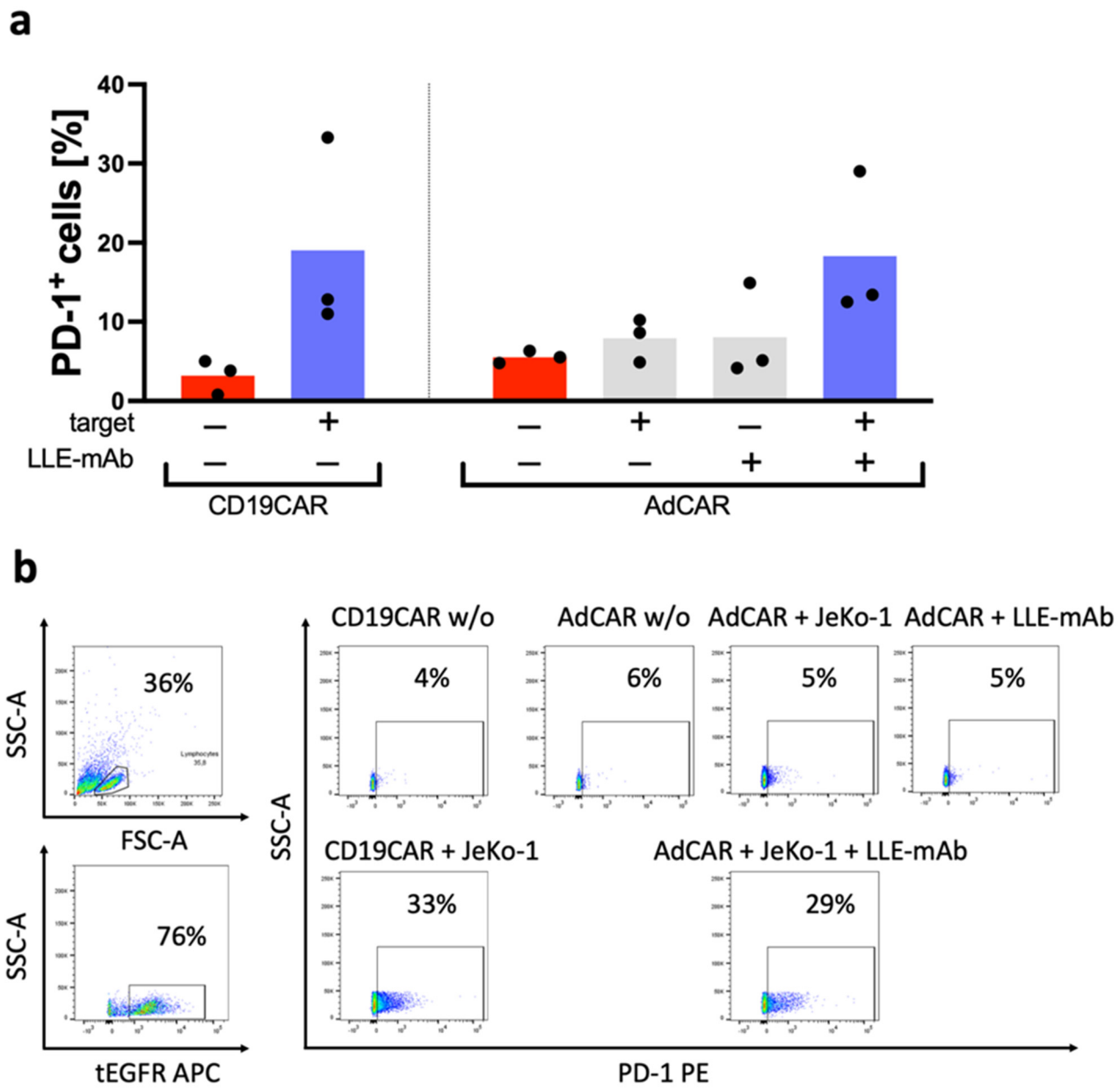


Figure 6. Exhaustion of AdCAR T cells vs. CD19CAR T cells. (**a**) CD19CAR T cells were incubated for 24 h with or without the CD19$^+$ lymphoma cell line JeKo-1. Additionally, AdCAR T cells were incubated for 24 h with or without the CD19$^+$ lymphoma cell line JeKo-1 and with or without 10 ng/mL LLE-CD19 mAb. Exhaustion after incubation was determined by flow cytometry using a PE-labeled PD-1 (CD279) mAb. (**b**) Representative schematic gating strategy of the indicated conditions of CD19CAR and AdCAR expressing cells with and without the target cell line JeKo-1, as well as with or without the LLE-mAb for the AdCAR T cells. PD-1 expression was analyzed and is illustrated for the CAR-positive cells (**left**). Data shown in (**a**) represent the mean ± SEM of (n = 3) independent experiments from three different donors.

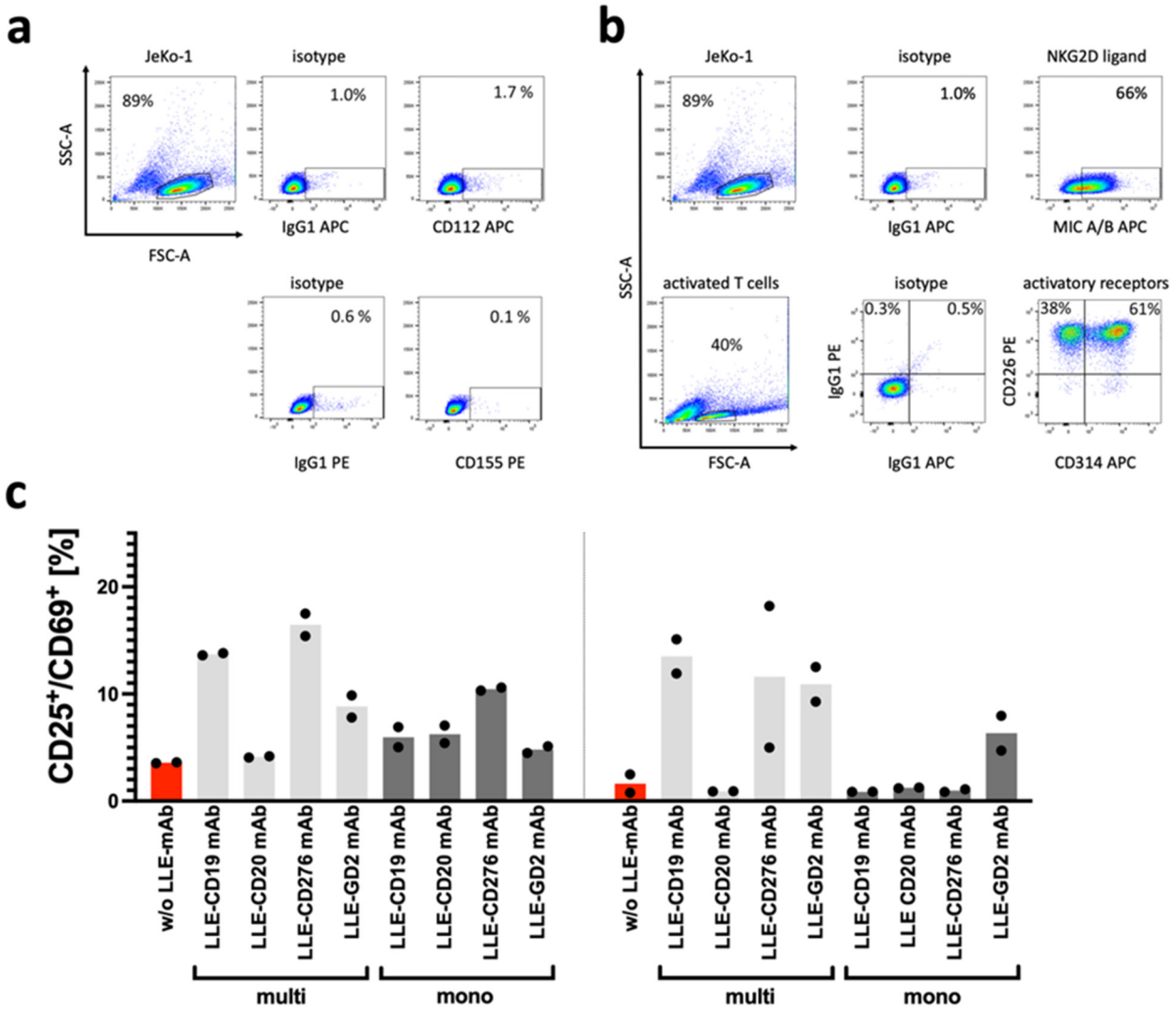


Figure 7. Non-specific activation of AdCAR T cells. (**a**) The surface expression of the DNAM-1 ligands CD112 and CD155 on JeKo-1 was determined by flow cytometry. (**b**) The surface expression of the NKG2D ligands MIC A/B on JeKo-1 and the surface expression of the activating receptors DNAM-1 (CD226) and NKG2D (CD314) on activated T cells was determined by flow cytometry. (**c**) The number of available binding sites for the AdCAR T cells per adapter molecule has an impact on the activation of the AdCAR T cells. To demonstrate that the non-specific activation of AdCAR T cells was enhanced in multi-biotinylated adapter molecules, mono- versus multi-biotinylated adapter molecules were investigated to induce $CD25^+CD69^+$ expression as an indicator of CAR-mediated activation of AdCAR T cells. AdCAR T cells were co-incubated without LLE-mAb (red) and with different LLE-mAbs at 10 ng/mL. The adapter molecules in full-size antibody format were targeted to CD19, CD20, CD276, and GD2. The specific activation status of the CAR T cells was determined by flow cytometry. Data shown in (**b**) represent the mean ± SEM of (n = 2) independent experiments from two different donors.

4. Discussion

Strategies to improve CAR T cell therapy address both safety and efficacy. It must be underscored that to date, the main challenges in CAR T cell therapy, especially in B-lineage cancers, are not around safety [16] but their limited efficacy over a broad range of target antigens [7] and a broad range of tumor entities [13]. While early-onset toxicities can be managed satisfactorily, limited efficacy remains the unmet need [17]. Increasing the efficacy primarily corresponds to solving the antigen question. Subsequently, CAR T cell

characteristics that define the antitumoral performance, such as fitness, exhaustion, and persistence [7,17,18], must be addressed in conjunction to solving tumor entity-specific environmental challenges with regards to the immunosuppressive tumor microenvironment [19,20]. Most tumors will require simultaneous targeting to successfully treat antigen-heterogeneous cancers and prevent pre-existing [21] and evolutionarily driven antigen immune escape [22]. Otherwise, the risk of treatment failure and cancer recurrence is significant due to antigen immune escape mechanisms [23].

In our current in vitro work, we demonstrated that AdCAR T cells can be used for antigen-specific targeting via adapter molecules in the presence of the corresponding antigen-positive target cells in the context of non-Hodgkin's lymphoma cell lines. Obviously, the translational vision is to adapt CAR-mediated targeting to the antigen expression profile of the individual cancer patient by selecting adapter molecules targeted to overexpressed antigens in individual cancers, such as CD19, CD20, CD22, CD37, and CD79B, which are currently used in NHL antibody-based therapies [24–26]. Polyimmunotargeting should contribute to increasing the efficacy of CAR T cell therapy in non-Hodgkin's lymphomas by preventing antigen immune escape, as has been studied with dual-targeted CAR T cells for CD19-CD22 in large B cell lymphoma patients [8] and with trispecific CD19-CD20-CD22-targeted CAR T cells in preclinical models [7]. Moreover, in our previous study, we tested combinatorial immunotargeting utilizing the AdCAR technology in the stably transduced cancerous NK cell line NK-92 with similar functional properties to AdCAR T cells [12]. The advantage of using individual T cell products compared to irradiated cancerous NK cell lines for patient use or primary NK cells are the initial exponential proliferation kinetics of CAR T cells. The fast kinetics can lead to immediate tumor control and the ability to permanently engraft in the patients and become part of the patients' immune system, which may support long-term tumor control. CAR-engineered NK cell lines have been used in clinical trials (NCT00900809, NCT00990717, NCT02944162, and NCT02742727); however, irradiation may limit their anticancer function [27]. NK and T cell CAR effector cells can be regarded as complementary and both provide unique effector functions [28].

Alternative technologies, such as molecular on and off switches [29], or mRNA-based gene delivery for transient targeting will also allow transient polyimmunotargeting [30].

Unsurprisingly, immunophenotyping of the cell lines revealed differential antigen immune profiles for JeKo-1, Raji, and Daudi. In patients, antigen immunoprofiles must be acknowledged and addressed in targeted immunotherapies in the same way. CD19-negative relapses account for around half the number of relapses after CD19CAR T cell therapy, causing treatment failure [21]. By targeting several antigens simultaneously, the chance of a profound response is increased; however, the potency of targeting also impacts the long-term outcomes. Thus, dual- and triple-targeted CAR T cell products have been developed [7]. Adapter CAR T cell technologies allow the targeting of any surface-expressed antigen in a patient-individualized manner. Dual CD19-CD20 targeting via AdCAR T cells outperformed CD19CAR T cells using a JeKo-1^{CD19KO} variant, demonstrating the feasibility of combinatorial targeting and the superiority over single-targeted CD19CAR T cells. Combinatorial CAR targeting strategies may improve the outcome of patients by preventing the development of antigen immune escape variants [31].

The safety profile of AdCAR T cells is high due to its functional inactivity and its self-limiting activity, which is dependent on the pharmacokinetics and dynamics of the adapter molecules. As an example, rituximab, as a full-size antibody, is cleared from the body with a half-life of around 3 weeks [32], whereas blinatumomab (54 kDa) is cleared in (mean ± SD) 1.25 ± 0.63 h t1/2 [33]. The sophisticated elimination of CAR T cells via inducible suicide genes (iCaspase) [34] or the antibody-dependent elimination of CAR T cells by targeting a targetable co-expressed marker gene such as truncated EGFR [35] are less elegant than the functional switch in AdCAR T cells.

Moreover, adapter CAR T cells will allow the targeting of antigens that cannot be targeted by conventional CAR T cells due to dramatic on-target off-tumor toxicities [36,37].

However, most target antigens in B-lineage non-Hodgkin's lymphoma are suitable for long-term CAR targeting [24–26].

In general, there are various indirect CAR approaches with distinct properties, which utilize an extracellular recognition domain that connects the intracellular CAR signaling machinery via linking molecules to cancer-associated target antigens [38]. The underlying principle in most indirect CAR systems is an exclusive artificial antibody-dependent cellular cytotoxicity. The advantage of the AdCAR technology is that it can build on FDA/EMA-approved antibodies that have been widely used in the clinic [13,39].

In our in vitro study, we used a second-generation CD19CAR construct, which is the basic architecture of all four FDA-approved CD19CAR products [2]. It remains uncertain if CD19CAR T cells would benefit from a third-generation architecture [40]. During the development of AdCAR T cells, we confirmed, in previous studies, that indirect CAR T cell constructs including AdCAR T cells and anti-FITC CAR T cells benefit from third-generation costimulatory signaling [13,41].

AdCAR T cells show cancer-dependent activation and cytokine secretion comparable with conventional CD19 CAR T cells. Cancer-dependent engagement is an absolute prerequisite for the application in patients. Logically, the AdCAR T cells showed superior effector function to CD19 CAR T cells in CD19-negative targets, which are a driver of relapse in patients [42]. We showed that AdCAR T cells can target different antigens and increase anticancer activity by using a combination of adapter molecules. Importantly, it was demonstrated that targeting a combination of different antigens does not lead to inhibition of the AdCAR T cells if one targeted antigen is not expressed, which was partially attributed to the favorable binding kinetics of the AdCAR scFv.

The longevity of CAR T cells is dependent on the baseline immunophenotype of a CAR T cell product [43] and the alteration in the immunophenotype and exhaustion upon stimulation of the CAR, which was comparable in the CD19 CAR T cells and the AdCAR T cells. Due to the spontaneous killing capacity of the untransduced T cells and the AdCAR T cells without the addition of adapter molecules, we analyzed the co-expression of important activating receptors in T cells and found the high expression of NKG2D and DNAM-1. In line with our assumptions, we found strong expression of the NKG2D ligands but low expression of the DNAM-1 ligands CD112 and CD155. The expression of the NKG2D ligand MIC A/B sufficiently explained the high background non-specific killing capacity of activated T cells in JeKo-1 and represented a supportive anticancer function in CAR T cells [44].

Additional genetic modifications introducing PD1-CD28 switch receptors [45] or PD1 knockout [46] could increase the potency of AdCAR T cells while still retaining the safety of the CAR T cell product. As we have underscored the importance of multitargeting, there are further implications with AdCAR T cells compared with conventional CAR T cell products. In contrast to conventional CAR T cells, CAR T cell tuning appears to be much safer due to the controllable CAR engagement and may allow us to generate more possible potent CAR T cell products than with conventional CAR T cell technologies. In conclusion, we demonstrated the advantage of multitargeting with AdCAR T cells over conventional CD19CAR T cells. Additional preclinical studies involving patient-derived xenograft models to mimic the heterogeneity of lymphomas in the naturally occurring form will demonstrate its translational potential to overcome immune escape.

Author Contributions: The authors D.A., C.M.S. and P.S. developed the general idea of the indirect CAR immunotherapy project. D.A., A.-S.M., S.S. and L.R. performed the in vitro experiments. D.A. and P.S. designed the experiments. D.A., A.-S.M., S.S., L.R., C.M.S. and P.S. interpreted the data as a whole. D.A. and P.S. drafted and wrote the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data associated with this study are presented in the paper.

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Conflicts of Interest: The employing institution of D.A., A.-S.M., S.S., L.R., C.M.S., and P.S. has received research funding by Miltenyi Biotec GmbH. Furthermore, P.S. and C.M.S. are co-inventors of a patent application focusing on adapter CAR technology.

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2nd Publication

Rational combinatorial targeting by adapter CAR-T-cells (AdCAR-T) prevents antigen escape in acute myeloid leukemia

ARTICLE OPEN

IMMUNOTHERAPY

Rational combinatorial targeting by adapter CAR-T-cells (AdCAR-T) prevents antigen escape in acute myeloid leukemia

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Targeting AML by chimeric antigen receptor T-cells (CAR-T) is challenging due to the promiscuous expression of AML-associated antigens in healthy hematopoiesis and high degree of inter- and intratumoral heterogeneity. Here, we present single-cell expression data of AML-associated antigens in 30 primary pediatric AML samples. We identified CD33, CD38, CD371, IL1RAP and CD123 as the most frequently expressed. Notably, high variability was observed not only across the different patient samples but also among leukemic cells of the same patient suggesting the necessity of multiplexed targeting approaches. To address this need, we utilized our modular Adapter CAR (AdCAR) platform, enabling precise qualitative and quantitative control over CAR-T-cell function. We show highly efficient and target-specific activity for newly generated adapter molecules (AMs) against CD33, CD38, CD123, CD135 and CD371, both in vitro and in vivo. We reveal that inherent intratumoral heterogeneity in antigen expression translates into antigen escape and therapy failure to monotargeted CAR-T therapy. Further, we demonstrate in PDX models that rational combinatorial targeting by AdCAR-T-cells can cure heterogenic disease. In conclusion, we elucidate the clinical relevance of heterogeneity in antigen expression in pediatric AML and present a novel concept for precision immunotherapy by combinatorial targeting utilizing the AdCAR platform.

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INTRODUCTION

Chimeric antigen receptor-expressing T-cells (CAR-T) have transformed the therapeutic landscape in B-phenotypic malignancies, demonstrating clinical efficacy in lymphoblastic leukemia, lymphoma and myeloma [1–4]. In contrast, no clinical breakthrough has been achieved yet in AML and other myeloid neoplasia. Multiple factors contribute to the complexity of targeting AML by CAR-T-cells. AML consists of vastly phenotypically and functionally heterogeneous blasts organized in a hierarchical system. Less differentiated stem-like cells, referred to as leukemic stem cells (LSCs), are responsible for disease initiation, relapse and therapy resistance [5, 6], while more mature bulk populations further contribute to intratumoral heterogeneity and plasticity [7]. CD33 (SIGLEC3), CD123 (IL3RA) and CD371 (CLL1 or CLEC12A) have been identified as potential candidates for CAR-T targeting but unfortunately show highly promiscuous expression profiles with strong overlap in healthy counterpart hematopoietic stem and progenitor cells (HSPCs) [8, 9]. While B-cell aplasia in the context of CD19-targeted CAR-T-cells is clinically manageable, direct targeting of HSPCs leads to profound myeloablation associated with severe risks for infectious complications that cannot be equally resolved by currently available supportive measures. Thus, tight regulation of CAR-T-cell function is needed to allow the regeneration of healthy myelopoiesis after targeting AML with CAR-T-cells. Furthermore, due to phenotypic heterogeneity [10], it appears unlikely that AML can be cured by targeting a single

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antigen. In addition to the already documented escape mechanisms mediated by antigen downregulation, as observed after CD19 targeting in B-lymphoid malignancies, AML relapses are prone to occur from outgrowth of preexisting antigen-negative subclones.

To address these challenges, we recently developed the adapter CAR platform (AdCAR) [11] to provide a highly flexible platform for qualitatively, quantitatively and temporally controlled antigen targeting by T and other effector cells. AdCAR is directed against a biotin tag in the context of a specific linker structure, referred to as a linker-label epitope (LLE), that can be conjugated to any kind of binding molecule (e.g., monoclonal antibodies (mAbs), mAb fragments, natural or synthetic ligands), referred to as an adapter molecule (AM). AdCAR-expressing effector cells are redirected to a surface antigen and activated against the target cell only in the presence of antigen-specific AMs. In addition to tight control of effector cell function, this system allows both simultaneous and sequential targeting of multiple antigens by AdCAR-expressing effector cells, thus paving the road to personalized combinatorial targeting approaches [11–18]. In the present study, we utilized the AdCAR platform to address major limitations of conventional CAR approaches in AML, with a special focus on pediatric AML. We provide a comprehensive overview of both inter- and intratumoral heterogeneity in target antigen expression in pediatric AML and identify preexisting antigen-negative or low subpopulations prone to antigen escape. Importantly, we used a patient-derived xenograft model to show that AdCAR-T-cells targeting multiple antigens ($n = 3$) can induce complete and sustainable remission in heterogeneous AML, thus providing proof-of-concept data demonstrating the vast superiority of the AdCAR-T strategy over single targeted approaches.

METHODS

Generation of novel AMs, mAb production and biotin conjugation

IgG1 against CD33, CD38, CD123, CD135, and CD371 were generated using the ExpiCHO™ expression system (Thermo Fisher). To this end, VH and LH sequences for the respective mAbs were cloned on an Fc-attenuated (L234A/L235A (LALA)) IgG1 backbone in a pcDNA™3.1 $^{(+)}$ mammalian expression vector. Transfection was performed according to the manufacturer's instructions using the high titer protocol. Antibodies in the clarified and filtered supernatant were purified using protein A GraviTrap columns (Cytiva) followed by desalting with PD10 Sephadex G25 columns (Cytiva). Purity was analyzed by SDS gel analysis. Biotin conjugation of purified antibodies was performed using a three-fold molar excess of biotin-LC-LC-NHS (Thermo Fisher), followed by separation of the antibody/label mix on a Sephadex G25 column (Cytiva). Successful conjugation was confirmed by flow cytometry on cell lines expressing the target antigen and secondary staining with a fluorophore-conjugated antibiotin antibody (Miltenyi Biotec) and functional assays.

Isolation of human primary T-cells and CAR-T generation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples acquired from healthy volunteer donors at the University Children's Hospital Tübingen (approved by the Institutional Ethical Review Board 761/2015BO2) by Ficoll-Paque density gradient (Biocoll, Biochrom). T-cells were isolated by magnetic separation using anti-CD4/8 microbeads (Miltenyi Biotec), stimulated with TransAct^{TM} (anti-CD3/28 agonistic signal) (Miltenyi Biotec) and cultivated in TexMACS media supplemented with 10 ng/mL IL7 and 5 ng/mL IL15 (Miltenyi Biotec) for up to 15 days. Transduction with an MOI of 3 was performed 36 h after stimulation. Transduction efficacy was determined on day +7 by flow cytometry with the AdCAR detection reagent PE (Miltenyi Biotec).

Immunophenotyping of bone marrow from pediatric AML patients and healthy donors

Patient characteristics are provided in Supplementary Table 1. This study was approved by the Institutional Ethical Review Board ("Ethikkommission der Medizinischen Fakultät der Eberhard-Karl-Universität und am Universitätsklinikum Tübingen" approval number 819/2017BO1, 674/2017BO2) and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients. Patient samples were treated with DNase 1 (Thermo Fischer) in DPBS prior to staining and incubated with the FcR blocking reagent human (Miltenyi Biotec). A list of antibodies, fluorochromes, dilution factors and live/dead staining used in this study is provided in the supplementary materials. Samples were acquired and unmixed on a Cytek Aurora (CytekBiosciences) 5-laser spectral flow cytometer using the "Cytek Assay Settings" in SpectroFlo® V3.0.3 software and further analyzed using FlowJo 10.8 software. Antigen positivity was defined by fluorescence minus one (FMO) controls. Uniform manifold approximation and projection were calculated using FlowJo 10.8 software and Plugin "UMAP" (arXiv:1802.03426).

Flow cytometry-based cytotoxicity assays

Wild-type, CD33KO, CD38KO, and CD33/CD38KO cells were mixed at a 1:1:1:1 ratio using 1.5×10^5 cells per variant (total cell number 6×10^5). AdCAR-T-cells were added at an E:T ratio of 1:1. AMs only or combinations thereof were added to a final concentration of 10 ng/mL as indicated. Tubes were incubated for the indicated time at 37 °C, 95% humidity, and 5% CO2. At the experimental endpoint, cells were stained for CD33, CD38, CD123, CD135, CD371, CD45 and CD3. 7-AAD was added to exclude dead cells. A list of antibodies, fluorochromes and dilution factors is provided in the supplementary materials. Samples were acquired with a BD FACSCanto™ II flow cytometer (BD Biosciences) using BD FACSDiva™ software and further analyzed using FlowJo 10.8 software. Uniform manifold approximation and projection were calculated using FlowJo 10.8 software and Plugin "UMAP" (arXiv:1802.03426).

Animals, PDX models, and in vivo studies

For all experiments, 6- to 8-week-old female NOD. Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice from Charles River Laboratories were used. The general health status of all animals was monitored daily. All experiments were performed according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) in the animal husbandry facilities of the University Clinic Tübingen. For PDX generation, mono nuclear cells were isolated by Ficoll-Paque density gradient (Biocoll, Biochrom) from fresh bone marrow of pediatric AML patients and transplanted immediately via teil vein injection into NSG mice. After engraftment, AML blasts were isolated from murine bone marrow by magnetic separation using anti-human CD45 microbeads (Miltenyi Biotec), transduced with a lentiviral vector encoding firefly luciferase and CD19t, and retransplanted into a mouse. After the second mouse passage, transduced AML blasts were enriched by magnetic separation using anti-human CD19 microbeads (Miltenyi Biotec), analyzed, and cryopreserved. For in vivo studies, NSG mice were engrafted with either 1×10^6 U937 cells on day −4 or 1×10^6 PDX cells on day −3 via tail vein injection. A total of 5×10^6 CAR-T-cells were injected intravenously on day 0. Twice a week, 45 µg of the indicated AMs or combinations thereof were injected subcutaneously (*s.c.*), beginning on day 0. Tumor growth was monitored by bioluminescence imaging (BLI). A detailed description of the performed in vivo studies is provided in the supplementary materials.

Statistical analysis

Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test. Ns, not significant, $p \le 0.0332$ (*), $p \le 0.0021$ (**), $p \le 0.0002$ (***), $p \le 0.0001$ (****). The full table of the respective statistical analysis is provided in Additional file 1.

RESULTS

Target antigens are heterogeneously expressed in pediatric AML

To study target antigen expression in pediatric AML at single-cell resolution, a representative cohort of primary AML samples ($n = 30$) at diagnosis ($n = 20$) and relapse ($n = 10$), including one matched diagnoses/relapse sample, was analyzed by multicolor flow cytometry for CD45, CD34, and CD3 as well as CD33, CD38, CD123, CD135, CD371, CD276, IL1RAP, mesothelin and MICA/B as potential target antigens. An overview of antigen expression patterns on AML blasts and consents is illustrated in Fig. 1A. Patient characteristics are provided in Supplementary Table 1, the gating strategy in

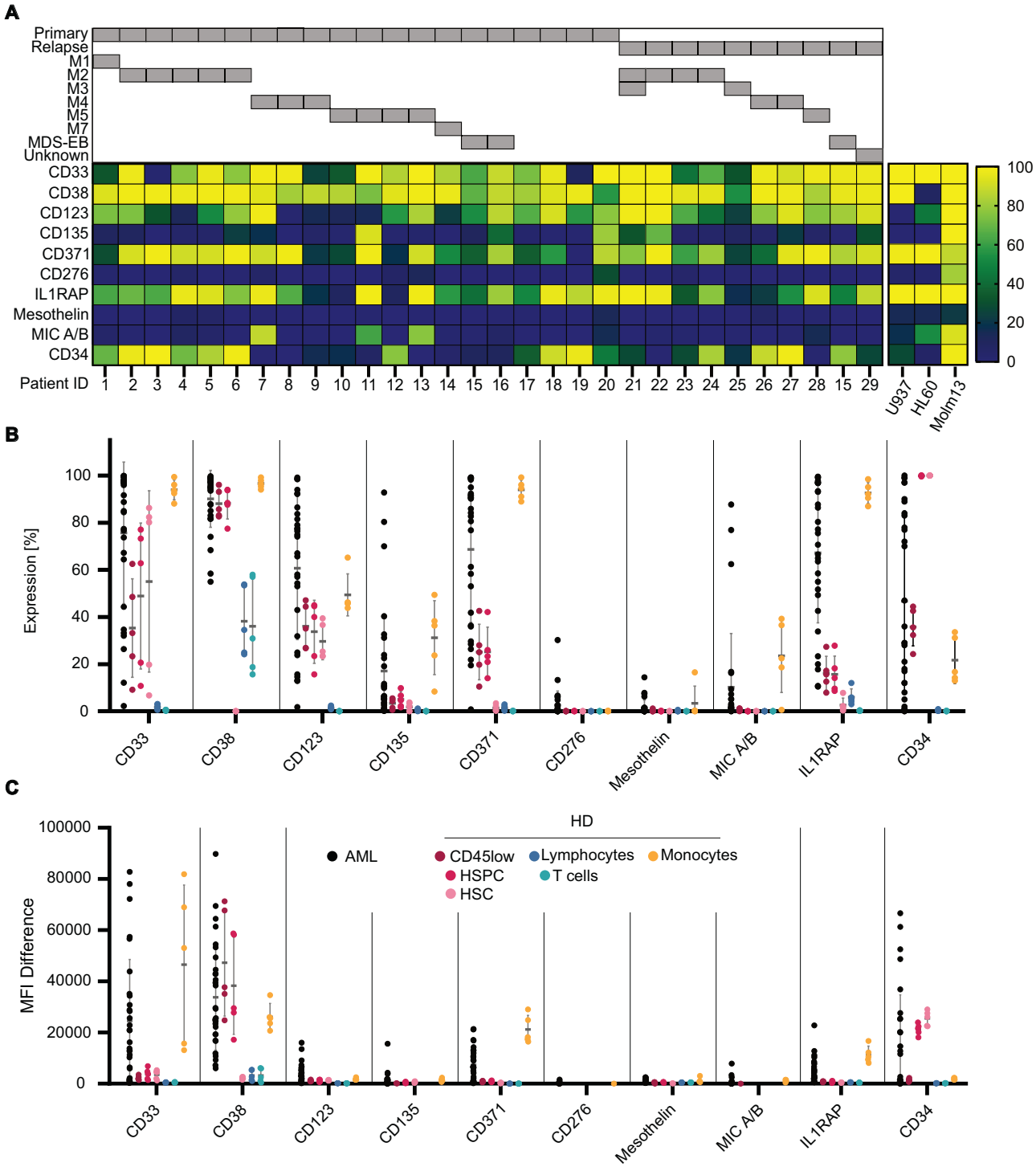


Fig. 1 Expression of target antigens in pediatric AML and healthy bone marrow. A Heatmap visualizing the percentage of leukemic blasts, defined as $CD45^{dim}$ cells within the BM of pediatric AML patients ($n = 30$), expressing the target antigens CD33, CD38, CD123, CD135, CD371, CD276, IL1RAP, Mesothelin, or MIC A/B as well as CD34, analyzed by flow cytometry. Patients are grouped by primary disease vs. relapse as well as FAB classification/MDS-EB. Target expression of the used AML cell lines are visualized as heatmap next to patient data (right). Comparison of target antigen expression between AML blasts and major cell populations (defined as follows: HSC ($CD45^{dim}/CD34^{high}/CD38^{low}$), HSPC ($CD45^{dim}/CD34^{high}/CD38^{high}$), lymphocytes ($CD45^{high}/SSC^{low}$), T cells ($CD45^{high}/SSC^{low}/CD3^{high}$), and monocytes ($CD45^{high}/SSC^{median}$)) in bone marrow from healthy donors ($n = 5$). In **B** the percentage of antigen-positive cells is provided; in **C** the mean fluorescence intensity difference (MFID). Each data point represents an individual patient or heathy donor, and horizontal lines represent the mean value ± standard deviation (SD).

Supplementary Fig. 1A. In line with a previous report [19], only a minority of samples (3/30) showed CD34^{high}/D38^{low} subpopulations reported as LSC enriched in adult AML, suggesting important differences between pediatric and adult patients. In the present cohort, CD33 and CD38 were expressed most frequently in the majority of blasts (23/30 and 30/30 with >50% positive blasts) and at the highest MFI difference (MFID), followed by IL1RAP (22/30), CD123 (21/30), CD371 (21/30), CD135 (3/30) and MICA/B (3/30) (Fig. 1B and C), with no documented difference between samples collected at initial diagnosis versus relapse. In contrast, CD276 and Mesothelin showed no relevant expression. Antigen expression was further compared to expression on healthy blood cell subsets. The gating strategy is provided in Supplementary Fig. 1B. None of the AML-associated antigens were found to be exclusively expressed on AML blasts (Fig. 1B and C). Healthy HSPCs further showed expression of CD33, CD123, CD371 and IL1RAP, although at a lower frequency and MFID than leukemic blasts. In contrast, CD135, CD276, Mesothelin and MICA/B were not expressed on healthy blood cells. CD38 is known to be absent on healthy HSCs but expressed on HSPCs at comparable levels to AML blasts, both in frequency and MFID. Monocytes, one of the most frequent myeloid cell populations, express CD33, CD38, CD123, CD135, CD371 and IL1RAP at high frequency and MFID. CD38 is further expressed at lower levels on lymphocytes and T-cells. Both CD38 and CD33 were found to be expressed on CD3/CD28 activated T-cells (Supplementary Fig. 1D). In conclusion, our analyses reveal no ideal target antigen, which is consistently present on AML blasts but absent on healthy blood cells. High intertumoral heterogeneity in pediatric AML will require multiple target antigens to address the disease as a whole.

Novel adapter molecules demonstrate antigen-specific activity against AML cell lines

To address the intertumoral heterogeneity in AML, a panel of AMs was generated, targeting the AML-associated antigens CD33, CD38, CD123, CD135 and CD371 (Fig. 2A). Targets were prioritized based on our expression data, published pre-clinical and clinical results as well as the availability of validated mAbs. To this end, VH and LH sequences for the mAbs CD33 (hP67.6, gemtuzumab), CD38 (daratumumab), CD123 (7G3, WO2016201065A1), CD135 (4G8 FLT3, EP3623383A1), and CD371 (h6E7.L4H1e. A54, EP3191520A1) were cloned into an Fc-attenuated (L234A/L235A (LALA)) IgG1 backbone [20] to minimize Fc-receptor-mediated ADCC and AdCAR-T-independent effects. After synthesis in CHO cells and purification, mAbs were LLE-conjugated, now referred to as LLE-"target-specificity"mAb (Supplementary Fig. 2). Specific target binding and LLE conjugation were verified by flow cytometry using a fluorochrome-labeled anti-LLE mAb (Fig. 2B). For functional testing of AMs with AdCAR-T-cells, three AML cell lines showing different antigen expression profiles were utilized: MOLM13 (CD33^{high}, CD38^{high}, CD123^{high}, CD135^{high}, CD371^{low}), HL60 (CD33^{high}, CD38^{low}, CD123^{low}, CD135^{low}, CD371^{high}) and U937 (CD33^{high}, CD38^{high}, CD123^{low}, CD135^{low}, CD371^{high}) (Fig. 2B). AM titration experiments demonstrated highly specific, target antigen-dependent lysis of AML cells by AdCAR-T-cells at very low AM concentrations (EC_{50} range 9.3–647.9 pg/mL for highly expressed antigens) (Fig. 2C). Efficient target cell lysis was observed even at a low effector to target (E:T) ratio (Fig. 2D). In contrast, no specific lysis was found for AdCAR-T-cells only or if the target antigen was not expressed (Fig. 2D). Taken together, these data demonstrate that the AdCAR-T platform can easily be adapted to a heterogeneous disease such as AML, allowing flexible targeting of multiple different antigens.

AdCAR-T-cells demonstrate specific in vivo activity, allowing individualized targeting

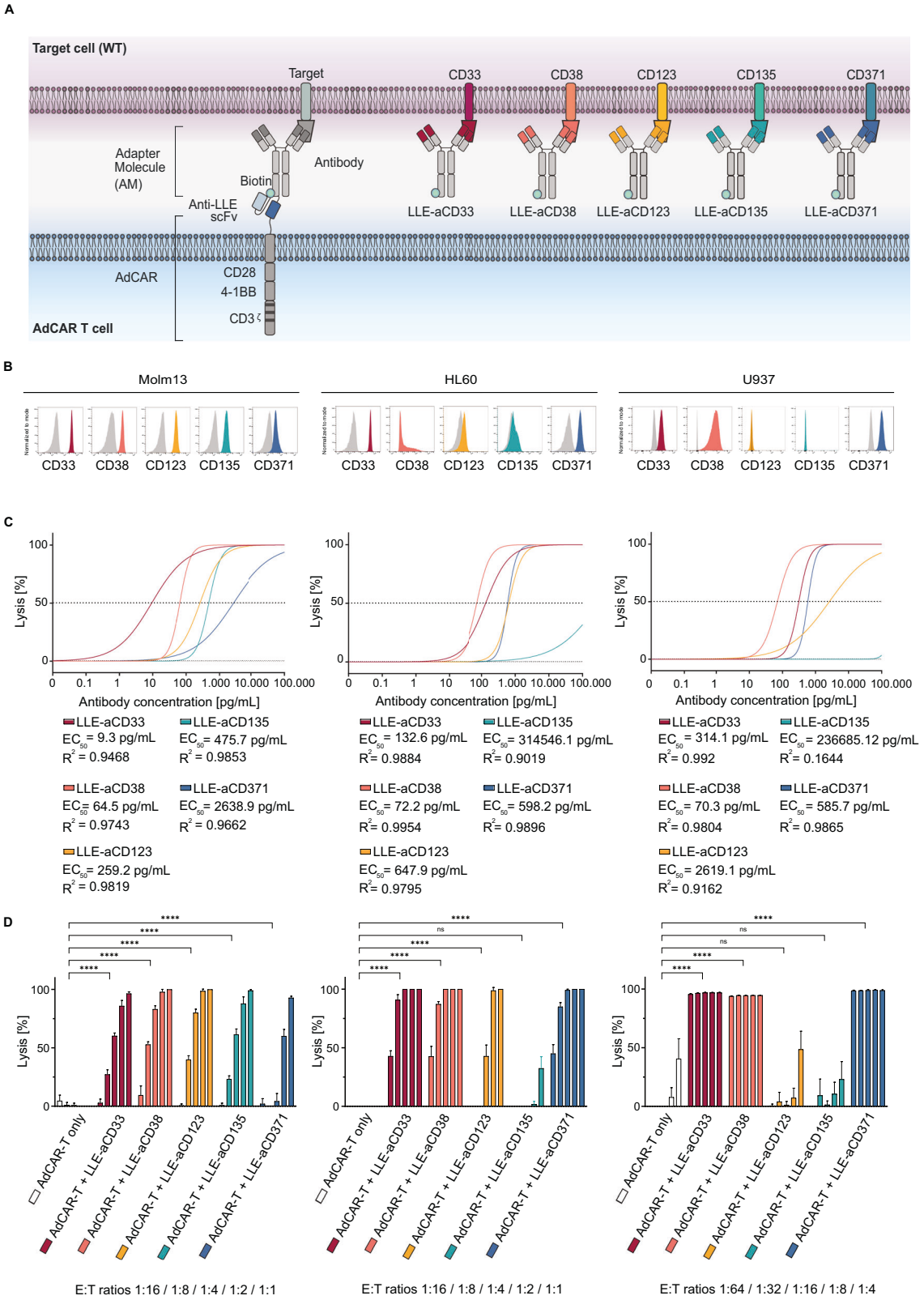
High intertumoral heterogeneity will require personalized targeting approaches based on patient individual antigen expression profiles. After demonstrating specific in vitro activity for different AMs, AdCAR-T-cells were tested in vivo to demonstrate the feasibility of individualized targeting. To this end, NSG mice were engrafted with U973 AML cells (CD33^{high}, CD38^{high}, CD123^{low}, CD135^{low}, CD371^{high}) and treated with AdCAR-T-cells plus AMs targeting CD33, CD38, CD123, CD135 or CD371 (Fig. 3A). Underscoring the potency of AdCAR-T-cells, treatment with AdCAR-T-cells in combination with AMs targeting CD33, CD38 or CD371 induced lasting remission, indicating complete AML cell eradication. In contrast, untreated control groups or treatment with AdCAR-T-cells in combination with AMs in samples with low CD123 or CD135 expression showed no similar effects (Fig. 3A–D). Together, these data demonstrate the specific in vivo activity of AdCAR-T-cells for personalized multiantigen targeting approaches. Of note, although expressed on a subpopulation of activated T-cells, targeting CD38 did not impair the antileukemic activity of AdCAR-T-cells in vivo.

Primary AMLs show a high degree of intratumoral heterogeneity

To further explore antigen heterogeneity in pediatric AML, 10 primary AML samples (mean percentage of blasts 85% (76–92%), mean number of analyzed AML blasts per patient: 75.700 (28.000–143.000)) were analyzed by dimensionality reduction using uniform manifold approximation and projection (UMAP) based on the expression of CD45, CD3, CD34, CD33, CD38, CD123, CD135 and CD371 as well as FSC and SSC signals. As demonstrated for healthy BM (Supplementary Fig. 3), unsupervised clustering accurately differentiates the main cell populations, further validating specific coexpression patterns as described above. In contrast, BMs from AML patients were dominated by leukemic blast clusters (Fig. 4A and Supplementary Fig. 3). Importantly, antigen expression was highly heterogeneous within the analyzed leukemic cell clusters. For example, in the sample shown in Fig. 4A, CD33 was expressed on the majority of blasts, while CD123 expression was restricted to an LSC-like (CD34^{pos}/CD38^{neg}) subpopulation, and CD38 and CD371 expression was documented on subsets of the bulk population (Fig. 4B). Significant intratumoral heterogeneity was confirmed in all analyzed samples (Fig. 4C). In 6 out of 10 samples, combinations of at least two target antigens were identified as needed to allow targeting of >90% blasts (Fig. 4E). A comprehensive overview of coexpression is provided in Additional file 2. Together, these data strongly suggest that mono-antigen targeting is insufficient to treat AML.

Multiplex targeting by AdCAR-T-cells can successfully address intratumoral heterogeneity

To model intratumoral heterogeneity in a controlled fashion, knockout variants for CD33, CD38 or both CD33 and CD38 were generated in AML cell lines using CRISPR/Cas9 (Fig. 5A). Validating the specificity of AM-mediated targeting, knockout of target antigens completely abrogated AdCAR-T lysis, demonstrating the antigen specificity of this targeting approach. To mimic antigen heterogeneity, AdCAR-T-cells were cocultured with a 1:1:1:1 mixture of wild-type and knockout AML cells (MOLM13^{WT}, MOLM13^{CD33KO}, MOLM13^{CD38KO} and MOLM13$^{CD33/CD38KO)}$ (Fig. 5A and B). No specific lysis was found in the absence of AMs (Fig. 5B *left*). The addition of single AMs, either against CD33, CD38 or a combination thereof, specifically led to the elimination of antigen-positive target cells (Fig. 5B). The addition of a third AM targeting CD123 further reduced the CD33- and CD38-negative target population (Fig. 5B *right*). Even triple targeting, CD33, CD38 and CD123, by AdCAR-T-cells at a low E:T ratio of 1:4 resulted in the selection of preexisting or forced CD123^{low} target cells (Fig. 5B). This was not observed at higher E:T ratios, indicating target-independent bystander killing (Fig. 2D). Findings were validated in the U937 cell line, combining AMs against CD33, CD38 and CD371 (Supplementary Fig. 4). Together, this set of data demonstrates the feasibility of simultaneous targeting of multiple ($n = 3$) antigens by AdCAR-T-cells to address intratumoral heterogeneity in AML.



Single antigen targeting of PDX AML results in rapid antigen-negative relapse

To evaluate the relevance of intratumoral heterogeneity in primary disease and simulate patient AdCAR-T therapy, PDX models of pediatric AML were treated with AdCAR-T-cells plus single AMs or a rational combination thereof (Fig. 6A). One PDX was derived from patient No. 21 of our target screening cohort (see Fig. 1A and Fig. 4A). After two mouse passages, including lentiviral transduction for luciferase expression and selection, the antigen expression pattern resembled primary disease, in addition

Fig. 2 Design and in vitro evaluation of novel AML-targeted AMs. A Schematic illustration of the AdCAR-T system. AdCAR-T cells are directed to AML-associated target antigens via LLE-conjugated mAbs (LLEa-CD33, LLEa-CD38, LLEa-CD123, LLEa-CD135, and LLEa-CD371), referred to as AMs. **B** Histograms of target antigen expression (CD33, CD38, CD123, CD135 and CD371) on AML cell lines (Molm13, HL60 and U937) stained with the indicated AM and secondary anti-LLE mAb, analyzed by flow cytometry. **C** Cytotoxicity of AdCAR-T against the indicated luciferase-expressing AML cell lines as determined by LCA after 48 h at an E:T of 1:1 (Molm13, HL60) or 1:4 (U937), mediated by increasing concentrations, logarithmic titration steps from 0.1 pg/mL to 100 ng/mL, of the indicated AMs. EC_{50} values are provided for each AM. **D** Cytotoxicity of AdCAR-T cells against the indicated AML cell lines as determined by LCA after 48 h at fixed AM concentrations (10 ng/mL) and indicated E:T ratios. AdCAR-T cells in the absence of AM served as a negative control. Data shown in C and D represent the mean ± SD of ($n = 6$). Data shown in C were transformed by taking the logarithm of the x values and then fitted by nonlinear regression. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test. ns, not significant. *$p \le 0.0332$ **$p \le 0.021$. ***$p \le 0.002$. ****$p \le 0.0001$. The full table of the statistical analysis is provided in Additional file 1.

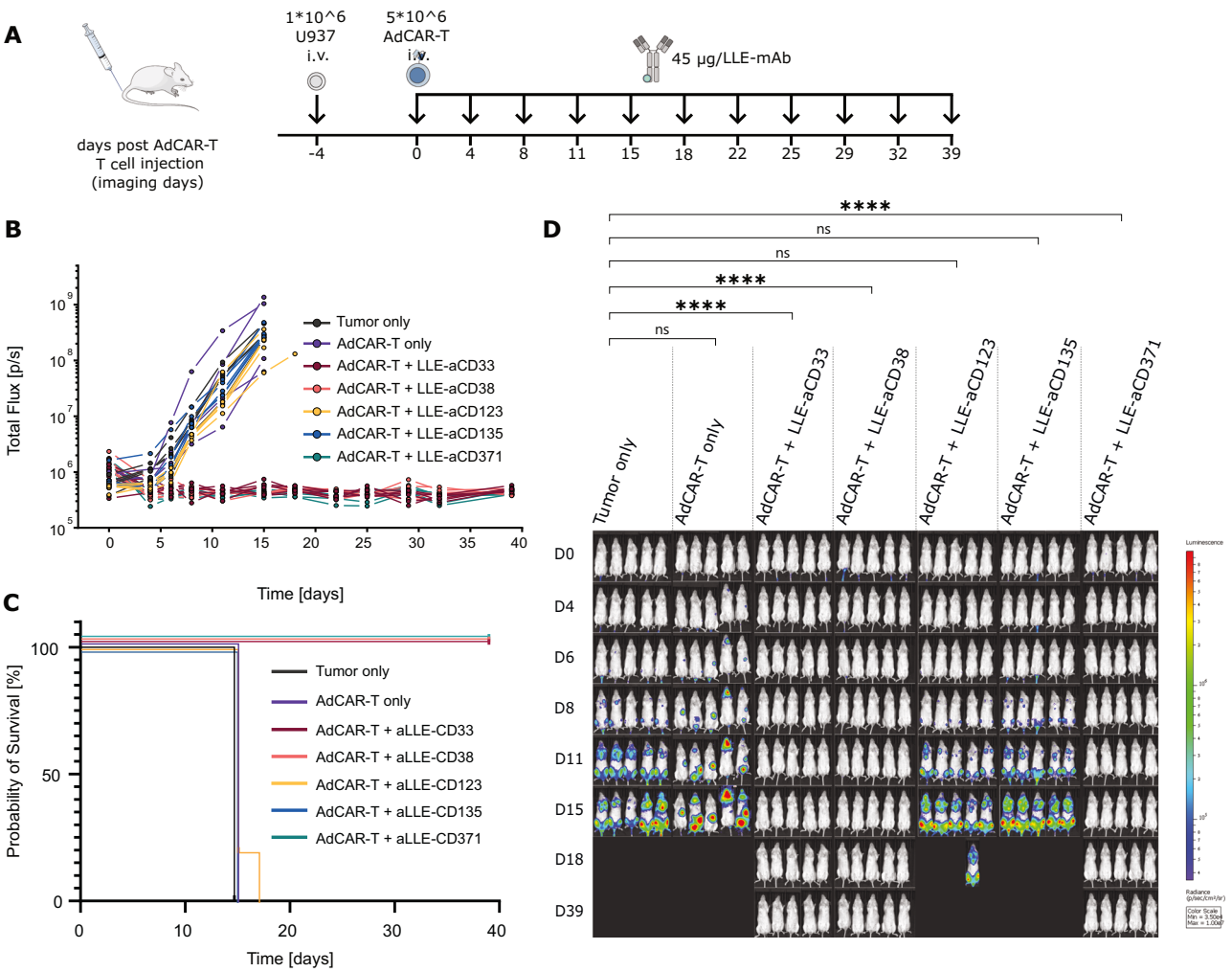


Fig. 3 In vivo validation of target antigen-specific activity of AdCAR-T cells. A Schematic depiction of the in vivo experiment: NSG mice were engrafted with 1×10^6 $U937^{luc/CD19t}$ ($CD33^{high}$, $CD38^{high}$, $CD123^{low}$, $CD135^{low}$, $CD371^{high}$) on day −4 via tail vein injection (i.v.). A total of 5×10^6 AdCAR-T cells were injected i.v. on day 0. Forty-five micrograms of the indicated AM (LLE-aCD33, LLE-aCD38, LLE-aCD123, LLE-aCD135 or LLE-aCD371) was injected subcutaneously (s.c.) twice a week starting on day 0. Untreated mice (tumor only) served as a negative control ($n = 5$ per group). Tumor load was monitored by BLI. Mice were sacrificed when they reached the endpoint criteria. **B** Total flux [photons/second] of in vivo bioluminescence blotted over time for individual animals. **C** Kaplan–Meier curves for reaching endpoint criteria. **D** BLI images at the indicated time points (exposure time 10 sec.). Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test. ns, not significant. *$p \le 0.0332$ **$p \le 0.021$. ***$p \le 0.002$. ****$p \le 0.0001$. The full table of the statistical analysis is provided in Additional file 1.

to a slight decrease in CD123 (Fig. 6B). Underscoring the therapeutic relevance of intratumoral heterogeneity in antigen expression, single targeting against either CD33, CD38 or CD371 led to rapid disease progression, prolonging the median time to reach end-point criteria in comparison to untreated PDX mice by only 11 for CD33, 11 for CD38 and 18 for CD371 days (Fig. 6C–E). In striking contrast, a rational combination of AMs targeting CD33, CD38 and CD371, expected to address >95% of leukemic blasts (Fig. 6B), led to lasting remission in all mice, indicating the potential of this combinatorial treatment to completely eradicate the disease. Combinatorial targeting resulted in significantly increased CAR-T-cell counts in bone marrow in comparison to AdCAR only and single targeting at the endpoint (Supplementary Fig. 5C). Analyses of resistant disease by flow cytometry, gating

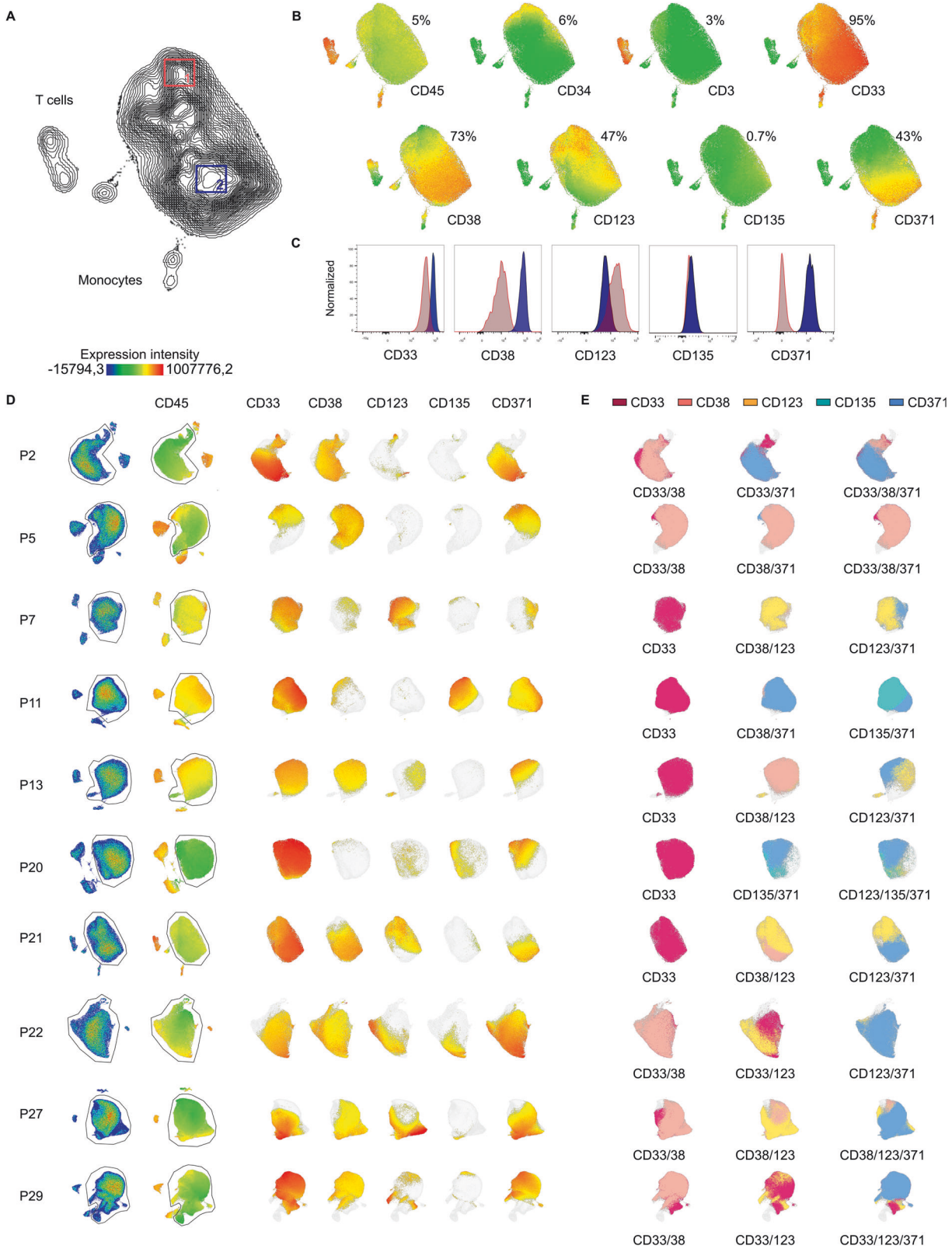


Fig. 4 Intratumoral heterogeneity in target antigen expression in pediatric AML. A UMAP based on the expression of CD45, CD3, CD34, CD33, CD38, CD123, CD135 and CD371 as well as FSC and SSC signals, as determined by flow cytometry, of an exemplary pediatric AML bone marrow sample (P21). **B** Color-coded intensity of CD45, CD3, CD34, CD33, CD38, CD123, CD135 and CD371 expression, with each dot representing one cell. **C** To highlight intratumoral heterogeneity in target antigen expression, expression of CD33, CD38, CD123, CD135 and CD371, plotted as histograms, in two different areas of the AML blast population, gated and labeled as 1 (LSC-like) and 2 (bulk). **D** UMAPs of AML bone marrow samples (P2, P5, P7, P11, P13, P20, P21, P22, P27, and P29); the left row shows bulk bone marrow as a pseudo color dot plot, and the second row shows CD45 expression and AML blast gates, followed by the expression of target antigens CD33, CD38, CD123, CD135 and CD371. **E** Suggestions of possible target combinations to cover most leukemic blasts.

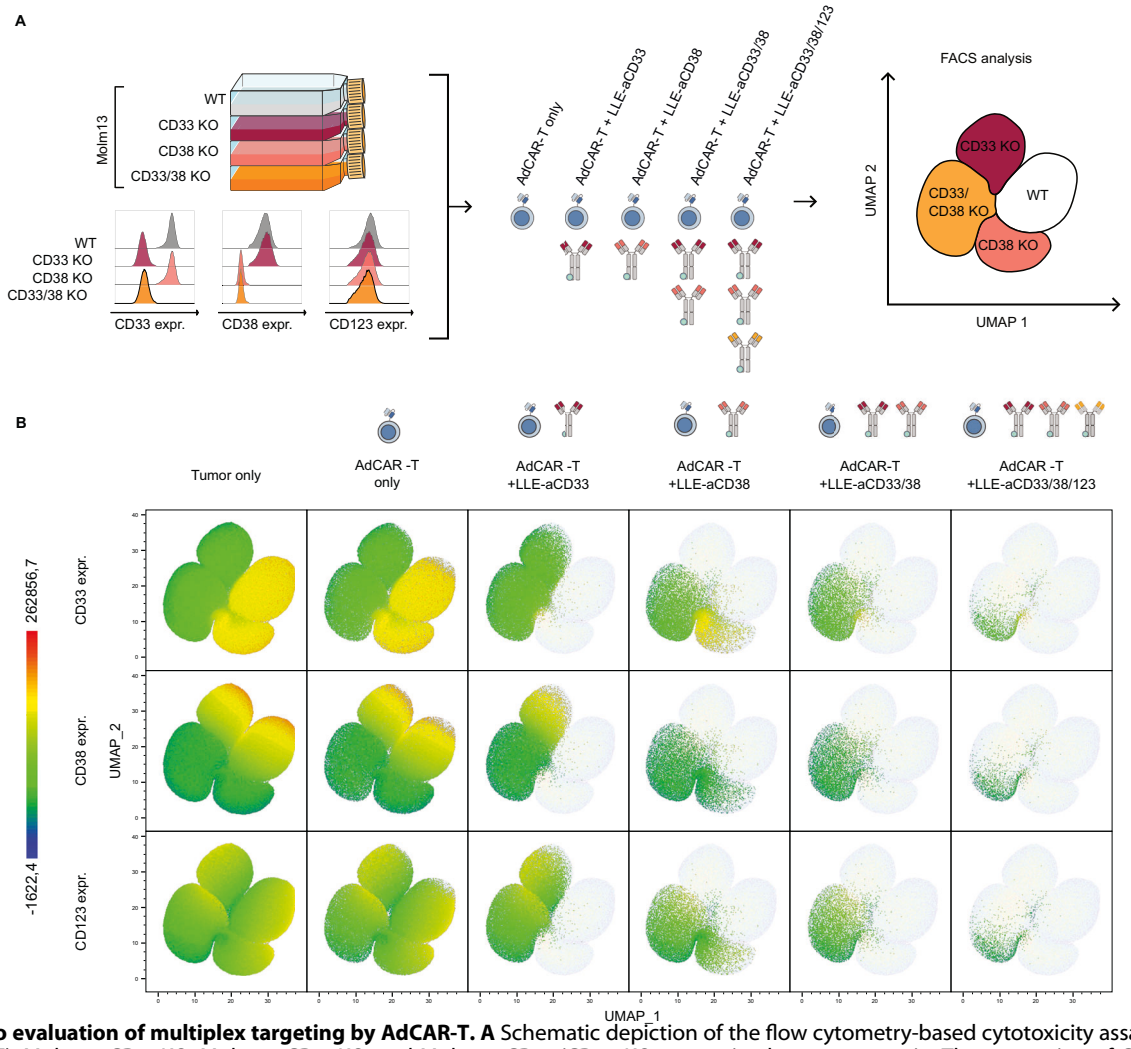


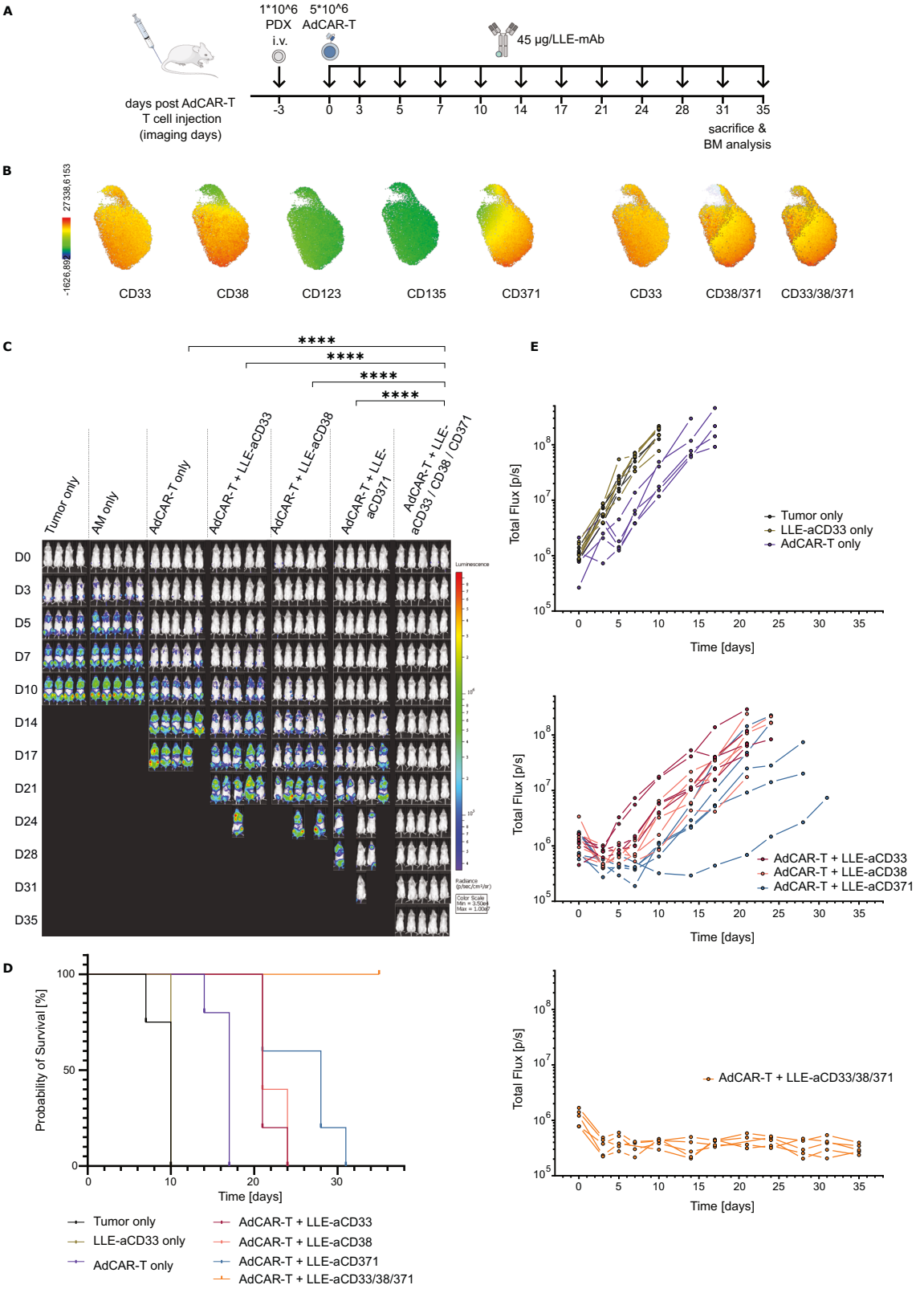
Fig. 5 In vitro evaluation of multiplex targeting by AdCAR-T. A Schematic depiction of the flow cytometry-based cytotoxicity assay: Molm13 wild type (WT), Molm13 CD33KO, Molm13 CD38KO, and Molm13 CD33/CD38 KO were mixed at a 1:1:1:1 ratio. The expression of CD33, CD38, and CD123 in individual cell populations is shown on the lower left. AdCAR-T cells were added at an E:T ratio of 1:1. LLE-aCD33, LLE-aCD38, or LLE-aCD123 as well as combinations thereof were added to reach a final concentration of 10 ng/mL. **B** UMAP representing batched surviving target cells after 48 h of incubation of all conditions ($n = 3$ each condition), based on the expression of CD33, CD38 and CD123, as determined by flow cytometry. From left to right, viable target cells for the indicated conditions plotted in color. From top to bottom, color-coded expression of CD33, CD38, and CD123.

strategy for mouse bone marrow provided in Supplementary Fig. 5 reveals a dramatic loss of detectable target antigen expression according to the specificity of the applied AM, suggesting antigen escape by selection of antigen-low or antigen-negative subpopulations (Fig. 7A). Batched UMAPs of untreated PDX cells, AdCAR-T-cells only and resistant disease after mono-targeting with either anti-CD33, anti-CD38 or anti-CD371 AMs clearly separates different therapeutic conditions, revealing trajectories toward antigen-low or antigen-negative disease enforced by a single targeted therapeutic intervention (Fig. 7B). The therapeutic advantage of combinatorial targeting was verified in a second PDX model (Supplementary Fig. 6A-E). Together, these experiments clearly underscores the potential of multiplex targeting by AdCAR-T-cells to cope with antigen escape due to heterogeneous antigen expression.

DISCUSSION

Targeting AML by CAR-T-cells is notoriously difficult due to shared antigen expression on leukemic cells and healthy HSCs, HSCPs, and cells of the myeloid lineage as well as inter- and intrapatient heterogeneity. In the present study, we evaluated a cohort of primary pediatric AML samples by multicolor flow cytometry to define the landscape of potential target antigens. In line with previous multiomics studies from us and others [8, 9, 19], we demonstrated the expression of the well-described AML-associated antigens CD33 (SIGLEC3), CD38, CD123 (IL3RA) and CD371 (CLL1 or CLEC12A) as well as less frequently CD135 (FLT3) in primary pediatric AML. We further identified IL1RAP as a potential target. IL1RAP expression has been reported in adult-type AML on both bulk leukemic cells and LSCs [21], and IL1RAP-targeted CAR-T-cells are currently being evaluated in a clinical trial (NCT04169022) in adult AML.

However, CD33, CD38, CD123, CD135, CD371 and IL1RAP were coexpressed in healthy HSPCs and myeloid cells, and CD33 and CD38 were coexpressed in subsets of activated T-cells. Previous studies in adult AML have identified CD276, Mesothelin, MICA/B, CD96, LILRB2, CCR1 and CD70 as potential target antigens [8, 22, 23], which could not be confirmed in our pediatric cohort and previously published data. [19] To the best of our knowledge, no AML-exclusive target antigen has been identified in adult or pediatric AML. Targeting promiscuously expressed AML-



associated antigens will result in depletion of antigen-positive cell populations, as demonstrated for B-cell-targeted CAR-Ts. Consequently, stringent safety measurements are needed to prevent or react to life-threatening on-target off-tumor activity, such as myeloablation, as demonstrated in preclinical models [24–26].

Conventional CAR-T design does not allow the control of CAR-T activity. Therefore, current clinical approaches applying CAR-T-cells in AML are limited to a bridge-to-transplant setting, terminating CAR-T-cells by lymphodepletion or activation of kill switches [27]. Transient expression of the CAR, for example, by

Fig. 6 In vivo validation of multiplex targeting by AdCAR-T cells in a PDX model. A Schematic depiction of the in vivo experiment: NSG mice were engrafted with 1×10^6 PDX $^{luc/CD19t}$ (P21) cells on day −3 via tail vein injection (i.v.). A total of 5×10^6 AdCAR-T cells were injected i.v. on day 0. A total of 45 µg of the indicated AM (LLE-aCD33, LLE-aCD38, or LLE-aCD371) or a combination thereof was injected subcutaneously (s.c.) twice a week starting on day 0. Untreated mice (tumor only), mice injected with PBS instead of AM (AdCAR-T only), and mice injected with LLE-aCD33, LLE-aCD38 and LLE-aCD371 but not AdCAR-T (AM only) served as negative controls ($n = 5$ per group, $n = 4$ in tumor only). Tumor load was monitored by BLI. Mice were sacrificed when they reached the endpoint criteria. **B** UMAP based on the expression of CD45, CD3, CD34, CD33, CD38, CD123, CD135 and CD371 as well as FSC and SSC signals, as determined by flow cytometry, of PDX cells prior to injection, expression of CD33, CD38, CD123, CD135 and CD371 on AML blasts (left) and suggestions for combinatorial targeting (right). **C** BLI images at the indicated time points (exposure time 10 s). **D** Total flux [photons/second] of in vivo bioluminescence blotted over time for individual animals. **E** Kaplan–Meier curves for reaching endpoint criteria. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test. ns, not significant. *$p \leq 0.0332$ **$p \leq 0.021$. ***$p \leq 0.002$. ****$p \leq 0.0001$. The full table of the statistical analysis is provided in Additional file 1.

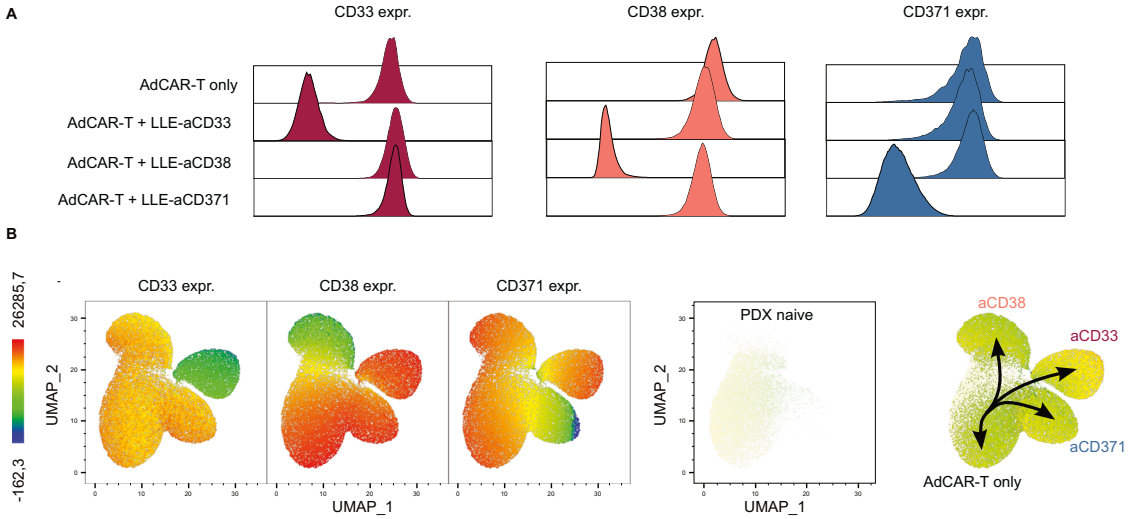


Fig. 7 Analysis of resistance mechanisms to mono-targeting. A Representative flow cytometry analyses of bone marrow from relapsed mice. Expression of CD33, CD38 and CD371 on viable AML blasts for the indicated groups plotted as histograms. **B** UMAP, representing batched viable AML blasts in bone marrow from tumor-only mice, AdCAR-T-only mice and mice treated with AdCAR-T plus LLE-aCD33, LLE-aCD38, or LLE-aCD123 ($n = 3$ each condition), based on the expression of CD33, CD38 and CD123, as determined by flow cytometry. From left to right, expression of CD33, CD38, and CD371 color-coded, spatial localization of treatment naïve PDX cells (= tumor only) and pseudotrajectories enforced by mono-targeting.

mRNA [28], might improve the safety profile but does not convey lasting disease control. More sophisticated approaches address the safety aspect by gene editing, knocking out the target antigen in hematopoietic stem cells to render them resistant to therapy [29], remote controlled inducible CAR expression [30, 31] or synthetic receptors for logic gating [32, 33].

One elegant way to achieve maximal control of CAR-T activity is to split antigen recognition from CAR-T activation. First introduced by the expression of an Fcγ receptor (CD16) [34] or CD16-derived CAR construct [35] in T-cells to enable antibody-dependent cellular cytotoxicity (ADCC), the concept of "adapter"-mediated CAR-T activation was improved via iteratively in subsequent studies [36–40]. We have recently reported on the development of the AdCAR platform, in which the CAR is directed against biotin in the context of a specific linker structure, referred to as a linker-label epitope (LLE). The LLE-tag can be chemically conjugated to any kind of binding molecule (e.g., mAbs, mAb fragments, natural or synthetic ligands), allowing highly flexible and convenient AM generation [11]. Inherent to the design of all "adapter"-CAR systems is the beneficial safety profile, rendering these approaches a perfect fit for AML. Encouragingly, the first clinical data (NCT04230265) targeting CD123 in adult AML demonstrated rapid recovery of white blood cells and neutrophil counts after termination of AM application. Moreover, induction of complete remission was observed in 2 out of 3 patients, underscoring the feasibility of "adapter"-CAR approaches in AML [41].

The second major obstacle to efficient CAR-T therapy in AML is inter- and intratumoral heterogeneity in target antigen expression. In the present study, we provide data on target antigen expression in a cohort of primary pediatric AML patients at single-cell resolution. We detected vast intertumoral heterogeneity, requiring personalized targeting approaches to cover the disease as a whole. Importantly, we demonstrate as a proof-of-concept that the AdCAR platform can be easily adapted to meet these requirements. We manufactured and functionally validated, both in vitro and in vivo, AMs against 5 clinically relevant AML-associated target antigens, CD33, CD38, CD123, CD135 and CD371. The established workflow allows convenient AM generation by LLE conjugation to rapidly broaden the target repertoire, e.g., by building on clinically tested and available binding molecules.

Selected antigens in this study have been intensively investigated in AML in the context of monoclonal antibodies [42, 43], antibody–drug conjugates [44], bispecific antibodies [45–47] and CAR-T-cells [24–26, 41, 48–54]. Particularly targeting CD38 by CAR-T-cells has been challenging due to broad expression not only in early hematopoiesis but also in a subset of activated T-cells, described to be functionally associated with a terminal exhausted phenotype [55]. Previous reports described that anti-CD38 CAR-mediated fratricide negatively impacts CAR-T expansion and clinical activity [56, 57]. Unexpectedly, we found CD33 expression on the majority of activated (CAR-)T-cells, which has previously been described for activated NK cells [58]. We were not able to detect impairment of AdCAR-T-cell function by targeting CD33 or

CD38, either in vitro or in vivo. Since AdCAR-T-cells are expanded in the absence of AMs, AdCAR-T technology allows to exclude CD33 or CD38 dependent CAR signaling and unintended CAR-T activation during manufacturing. This might result in a beneficial cell product.

Beyond intertumoral heterogeneity, we demonstrate dramatic intratumoral heterogeneity of antigen expression in primary pediatric AML. We show that for the majority of patients, targeting a single AML-associated antigen is insufficient to address all leukemic cells. These findings have major clinical implications since antigen-low or antigen-negative subpopulations are a source for antigen escape and subsequent failure to achieve efficient CAR-T therapy, defined by the elimination of all target-positive cells [59]. Using antigen knockout models, we demonstrated that AdCAR-T-cells can be utilized to simultaneously target multiple AML-associated antigens. Validating our hypothesis, we show in PDX models of pediatric AML, resembling intratumoral heterogeneity of the primary disease, that mono-targeting by CAR-T-cells against CD33, CD38 or CD371 results in rapid antigen escape and disease progression. In contrast, we demonstrate for the first time in vivo that rational combinatorial targeting by AMs against CD33, CD38, and CD371 results in the clearance of inherently heterogeneric disease. These experiments clearly underscore the feasibility of utilizing "adapter"-CAR systems, particularly our AdCAR platform, to target multiple antigens in parallel. Antigen evasion by CAR-T-cells or other targeted immunotherapies is an emerging clinical limitation beyond AML [60]. The application of multiple CAR-T products remains challenging, both from a regulatory and a financial point of view. "Adapter"-CAR systems might provide a neat solution, building on a single CAR-T product redirected against a multiplicity of target antigens.

Together, our findings highlight that successful clinical translation of CAR-T-cells against (pediatric) AML will require stringent safety measures as well as personalized combinatorial targeting approaches. We demonstrate that our AdCAR platform meets these requirements, enabling precise qualitative and quantitative control of CAR-T-cell function as well as multiplex antigen targeting, paving the way toward precision immunotherapy. A phase I/II clinical trial in AML is in preparation.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. For original data, please contact christian.seitz@med.uni-heidelberg.de

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AUTHOR CONTRIBUTIONS

DA, PS and CMS designed the study and supervised the project; DA and CMS wrote the manuscript; DA, LR, ASM, SK, MM, SS, BK, MF, and CMS performed the experiments and analyzed and/or interpreted the data; DA, SK, and CMS designed and performed the in vivo experiments with the assistance of AC, KW and LS. KS, LZ, ME, DN, MS, JS, CL, IJ, NW, JM, PL, and RH were involved in designing the experiments and interpreting the data. All authors contributed to the preparation of the manuscript and approved the submitted version.

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ADDITIONAL INFORMATION

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