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Next-generation immunotherapy to counteract T-cell exhaustion

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I hereby declare, that the submitted thesis entitled:

Next-generation immunotherapy to counteract T-cell exhaustion

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I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

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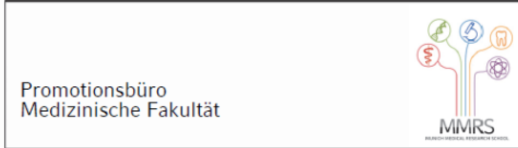

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List of abbreviations

AdCAR	adapter chimeric antigen receptor
ALL	acute lymphoblastic leukemia
AM	adapter molecule
AML	acute myeloid leukemia
AP-1	activator protein 1
BCMA	B-cell maturation antigen
BCP	B-cell precursor
BiTE	bispecific T-cell engager
BsAb	T cell-recruiting bispecific antibody
CAR	chimeric antigen receptor
CD3ζ	T-cell surface glycoprotein CD3 zeta chain
CPI	checkpoint inhibitor
CRS	cytokine-release syndrome
DART	dual affinity retargeting antibody
DLBCL	diffuse large B-cell lymphoma
EMA	European Medicines Agency
EOMES	Eomesodermin
FDA	Food and Drug administration
HSCT	hematopoietic stem cell transplantation
ICAHT	immune effector cell-associated hematotoxicity
ICANS	immune effector cell-associated neurotoxicity syndrome
ICP	inhibitory checkpoint molecule
IFNγ	Interferon gamma
IL-1	interleukin-1
IL-12	interleukin-12
IL-15	interleukin-15
IL-18	interleukin-18
IL-2	interleukin-2
IL-6	interleukin-6
ITAM	immunoreceptor tyrosine-based activation motif
KLRG1	killer cell lectin-like receptor subfamily G member 1
LAG3	lymphocyte activation gene 3
LSC	leukemic stem cell
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MRD	minimal residual disease
NFAT	calcineurin-dependent TF Nuclear factor of activated T-cells
NR4A	nuclear receptor subfamily 4A
PD1	programmed cell death protein 1

Ph-	Philadelphia chromosome-negative
r/r	relapsed/refractory
scFv	single-chain variable fragment
T-bet	T-box transcription factor
TAA	tumor-associated antigen
TAM	tumor-associated macrophages
TCF-1	T-cell factor-1
TCR	T-cell receptor
T_{EX}	terminally exhausted T cells
TF	transcription factor
TFI	treatment-free interval
TGF-β	transforming growth factor beta
TIL	tumor-infiltrating lymphocyte
TIM3	T-cell immunoglobulin and mucin-domain containing 3
TNFα	tumor necrosis factor alpha
TOX	thymocyte selection-associated high mobility group box protein
T_{PEX}	progenitor exhausted T cells
Treg	regulatory T cells
WT1	Wilms-Tumor-Protein

List of publications

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

Publication I:
"Adapter CAR T cells to counteract T-cell exhaustion and enable flexible targeting in AML"
Daniel Nixdorf, Monika Sponheimer, Dominik Berghammer, Fabian Engert, Ulrika Bader, Nora Philipp, Maryam Kazerani, Tobias Straub, Lisa Rohrbacher, Lucas E. Wange, Sandra Dapa, Daniel Atar, Christian Seitz, Katharina Brandstetter, Andreas Linder, Michael von Bergwelt-Baildon, Heinrich Leonhardt, Jörg Mittelstaet, Andrew Kaiser, Veit Bücklein and Marion Subklewe
Leukemia, April 2023, doi: 10.1038/s41375-023-01905-0

Publication II:
"T-cell exhaustion induced by continuous bispecific molecule exposure is ameliorated by treatment-free intervals"
Nora Philipp, Maryam Kazerani, Alyssa Nicholls, Binje Vick, Jan Wulf, Tobias Straub, Michaela Scheurer, Amelie Muth, Gerulf Hänel, **Daniel Nixdorf**, Monika Sponheimer, Malte Ohlmeyer, Sonja M. Lacher, Bettina Brauchle, Anetta Marcinek, Lisa Rohrbacher, Alexandra Leutbecher, Kai Rejeski, Oliver Weigert, Michael von Bergwelt-Baildon, Sebastian Theurich, Roman Kischel, Irmela Jeremias, Veit Bücklein and Marion Subklewe
Blood, September 2022, doi: 10.1182/blood.2022015956

During my thesis work I also contributed to the following publications which have been accepted in peer-reviewed journals, however, are not part of this thesis:

"STING agonism turns human T cell into interferon-producing cells but impedes their functionality"
Niklas Kuhl, Andreas Linder, Nora Philipp, **Daniel Nixdorf**, Hannah Fischer, Simon Veth, Gunnar Kuut, TengTeng Xu, Sebastian Theurich, Thomas Carell, Marion Subklewe, Veit Hornung
EMBO Reports, January 2023, doi: 10.15252/embr.202255536

"Protective immune trajectories in early viral containment of non-pneumonic SARS-CoV-2 infection"
Kami Pekayvaz, Alexander Leuning, Rainer Kaiser, Markus Joppich, Sophia Brambs, Aleksander Janjic, Oliver Popp, **Daniel Nixdorf**, Valeria Fumagalli, Nora Schmidt, Vivien Polewka, Afra Anjum, Viktoria Knottenberg, Luke Eivers, Lucas E. Wange, Christoph Gold, Marieluise Kirchner, Maximilian Muenchhoff, Johannes C. Hellmuth, Clemens Scherer, Raquel Rubio-Acero, Tabea Eser, Flora Deak, Kerstin Puchinger, Niklas Kuhl, Andreas Linder, Kathrin Saar, Lukas Tomas, Christian Schulz, Andreas Wieser, Wolfgang Enard, Inge Kroidl, Christof Geldmacher, Michael von Bergwelt-Baidon, Oliver T. Keppler, Mathias Munschauer, Matteo Iannacone, Ralf Zimmer, Philipp Mertins, Norbert Hubner, Michael Hoelscher, Steffen Massberg, Konstantin Stark and Leo Nicolai
Nature Communications, February 2022, doi: 10.1038/s41467-022-28508-0

In addition, I worked extensively on a project combining the innate immune pathway STING and T-cell recruiting bispecific antibodies to target AML. At time of writing, a manuscript entitled: "**STING activation improves T-cell engaging immunotherapy of acute myeloid leukemia**" is in preparation. This manuscript is not part of the presented doctoral thesis.
Andreas Linder*, **Daniel Nixdorf***, Niklas Kuhl, Teng Teng Xu, Ignazio Piseddu, Thomas Carell, Roland Kischel, Marion Subklewe*, Veit Hornung*
Manuscript in preparation
*contributed equally

Contribution to the publications

1.1 Contribution to paper I

I am the first author of this manuscript and therefore significantly contributed to the whole project and manuscript. I was involved in study design, planned the experiments, was involved in conducting all the experiments, interpreted the data, designed all the figures, wrote the entire manuscript, and conducted the review process.

1.2 Contribution to paper II

I contributed to the design of the studies which led to the establishment of a long-term culture system to mimic T-cell dysfunction due to chronic antigen stimulation in the context of T-cell recruiting bispecific antibodies. In addition, I assisted in data interpretation throughout the whole project.

2. Introductory summary

2.1 T cell-based immunotherapy of cancer

The era of T cell-based immunotherapy has led to a paradigm shift in how to treat patients with cancer. Conventional therapies like chemotherapy, are capable of curing a substantial percentage of patients relying on classical dose-response relationships. T cell-based immunotherapy on the other hand has shown breakthrough character results in chemo-refractory patients. The key principle of modern T cell-based immunotherapy is to specifically re-program a misfunctioning T-cell compartment which lost its inherent ability to detect and control tumors, towards regaining its potential to recognize malignant cells and coordinate a concerted anti-tumor response [1].

Allogeneic hematopoietic stem cell transplantation (HSCT) was the first immunotherapy to demonstrate an effective T cell graft versus tumor response in 1979 [2]. Different strategies harnessing the intrinsic properties of T cells in a multitude of ways have evolved in the last decades (Figure 1). The major platforms of current T-cell immunotherapy include: 1) checkpoint inhibitors (CPI) aiming to re-activate pre-existing T-cell responses for example by blocking programmed cell death protein 1 (anti-PD1 antibodies); 2) dendritic cell vaccination to induce anti-tumor-specific T-cell responses; 3) T cell-recruiting bispecific antibodies (BsAbs) specifically designed to direct endogenous T cells to the tumor sites; 4) adoptive cell transfer including tumor-infiltrating lymphocytes (TILs), T-cell receptor (TCR)-transgenic T cells and genetically engineered chimeric antigen receptor (CAR) T cells to target and execute anti-tumor functions [1, 3-5].

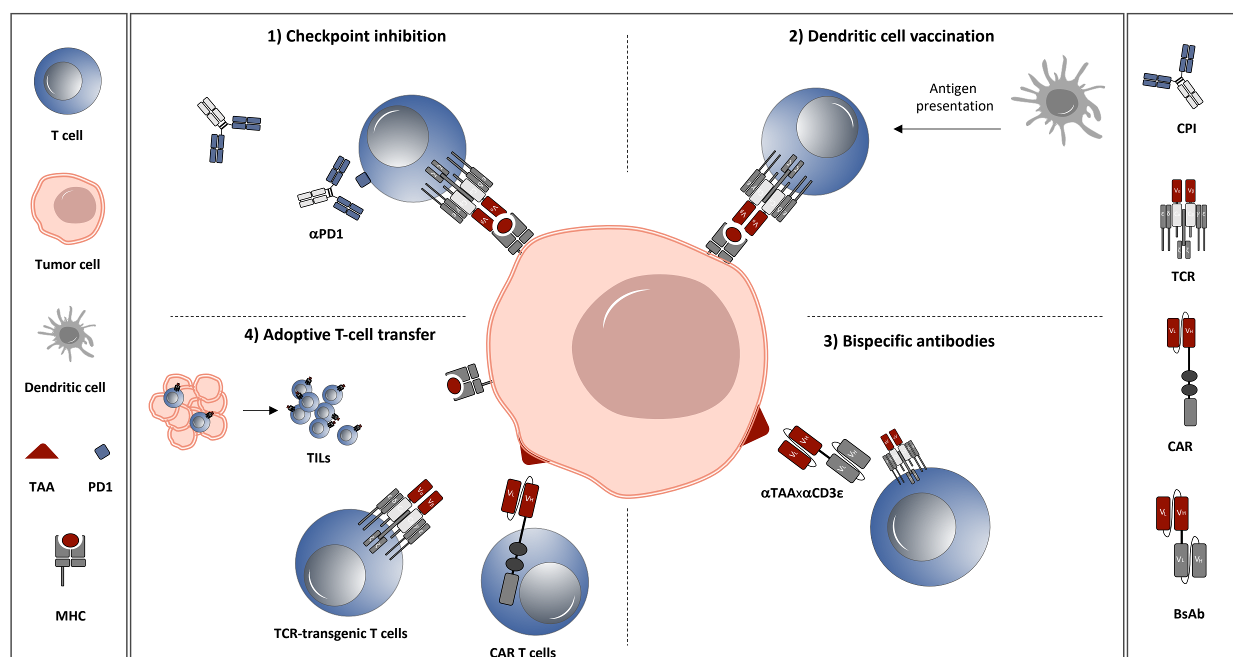

Figure 1: Concepts of T cell-based immunotherapy. TAA = tumor-associated antigen, PD1 = programmed cell death protein 1, MHC = major histocompatibility complex, CPI = checkpoint inhibitor, TCR = T-cell receptor, CAR = chimeric antigen receptor, BsAb = bispecific antibody. Adapted from [1, 5].

2.1.1 T cell-recruiting bispecific antibodies in hematologic malignancies

One way to redirect T cells and bring them into close proximity to tumor cells is BsAbs [6]. In essence, these molecules are designed as a bridging module between tumor cells and endogenous T cells, activating the latter, and leading to their proliferation and execution of effector function. The formation of an immunological synapse accompanied by the secretion of cytolytic molecules results in tumor cell lysis [7]. BsAbs usually consist of two single-chain variable fragments (scFv) fused by a flexible linker, however a variety of different formats of varying molecular size have been described, including serum half-life extended versions to prolong application intervals or multi-specific versions simultaneously targeting more than one target antigen [8].

From the first report of a BsAb in 1985 [9] it took 30 years until the Food and Drug Administration (FDA)/European Medicines Agency (EMA) approved the first CD19xCD3 BsAb blinatumomab for patients with Philadelphia chromosome-negative (Ph-) relapsed/refractory (r/r) B-cell precursor (BCP)-acute lymphoblastic leukemia (ALL), which was later on amended to minimal residual disease $(MRD)^+$ BCP-ALL [10]. Blinatumomab is directed against the tumor-associated antigen (TAA) CD19 and the CD3 part of the human TCR [10]. The use of blinatumomab significantly improved the median overall survival of Ph^- BCP-ALL patients compared to standard-of-care chemotherapy [11] and resulted in response rates of 43% in r/r patients and >80% in MRD^+ patients [12, 13]. Since then, the field of BsAbs has gathered momentum leading to an ever-increasing number of clinical trials [14, 15], and to date two additional constructs have been approved for hematologic malignancies (mosunetuzumab: CD20xCD3 BsAB for r/r follicular lymphoma [16] and teclistamab: B-cell maturation antigen (BCMA)xCD3 for r/r multiple myeloma [17]).

Apart from CD19, CD20, and BCMA, a number of different BsAbs against targets across the board of hematologic malignancies are being explored [5]. These include for example the CD33 targeting bi-specific T-cell engager (BiTE) AMG 330 [18-20], the anti CD123 directed dual affinity retargeting antibody (DART) flotetuzumab [21, 22], an FLT3 directed BiTE [23], or even molecules against major histocompatibility complex (MHC) presented intracellular targets like Wilms-Tumor-Protein (WT1) in acute myeloid leukemia (AML) [24].

2.1.2 CAR T cells in hematologic malignancies

With the development of gene transfer techniques like viral vectors, the concept of genetically modified T cells emerged. Following the successful generation of TCR-transduced T cells [25, 26], a novel concept of genetically modified T cells, known as chimeric antigen receptor (CAR) T cells, was introduced. First described in 1993 and formerly termed “T bodies” [27] or “chimaeric TCRs” [28] and later “first-generation CARs” [29], these modified T cells represented a simplistic way to generate a TCR-surrogate by combining an extracellular ligand-binding domain with a T-cell surface glycoprotein CD3 zeta chain (CD3ζ). While first-generation CARs lacked efficacy due to missing co-stimulation [30-32], second-generation CARs benefitted from the incorporation of co-stimulatory domains to provide both activation as well as co-stimulation signals [33-35].

Since then, a plethora of different CAR designs emerged, improving on many different aspects such as efficacy, persistence, or toxicity. Classically, the backbone of a CAR construct consists of an extracellular antigen recognition domain comprised of antibody-derived single chain variable fragments (scFv), however, any ligand binding domain may be used. The antigen binding domain is incorporated in the membrane via a combination of a linker of variable length (hinge domain), followed by a transmembrane domain connecting the extracellular part to the intracellular signaling domains. These signaling domains usually consist of a co-stimulatory domain and a CD3ζ activation domain to promote CAR T-cell activation, proliferation, and persistence [36]. Similar to BsAbs, CAR T cells are artificially directed against their respective target cells and induce lysis via the secretion of cytolytic molecules [37].
More advanced CAR designs as highlighted in Figure 2 include for example: additional co-stimulatory domains (third-generation CARs); cytokine-secreting CARs ("armored CARs") to improve CAR T-cell efficacy or persistence; dual-targeting concepts to target more than one target antigen simultaneously ("tandem-CARs"); or "logic-gated" CARs to reduce off-tumor targeting by splitting activation and co-stimulatory domains, or by inducing the expression of a second CAR only upon primary target antigen binding. In addition, Adapter CAR (AdCAR) T cells have been developed, which are only activated and targeted to tumor cells upon bridging with a small adapter molecule (AM) such as full-length antibodies or Fab-fragments [36, 38] (**Publication I: Nixdorf et al., 2023**).

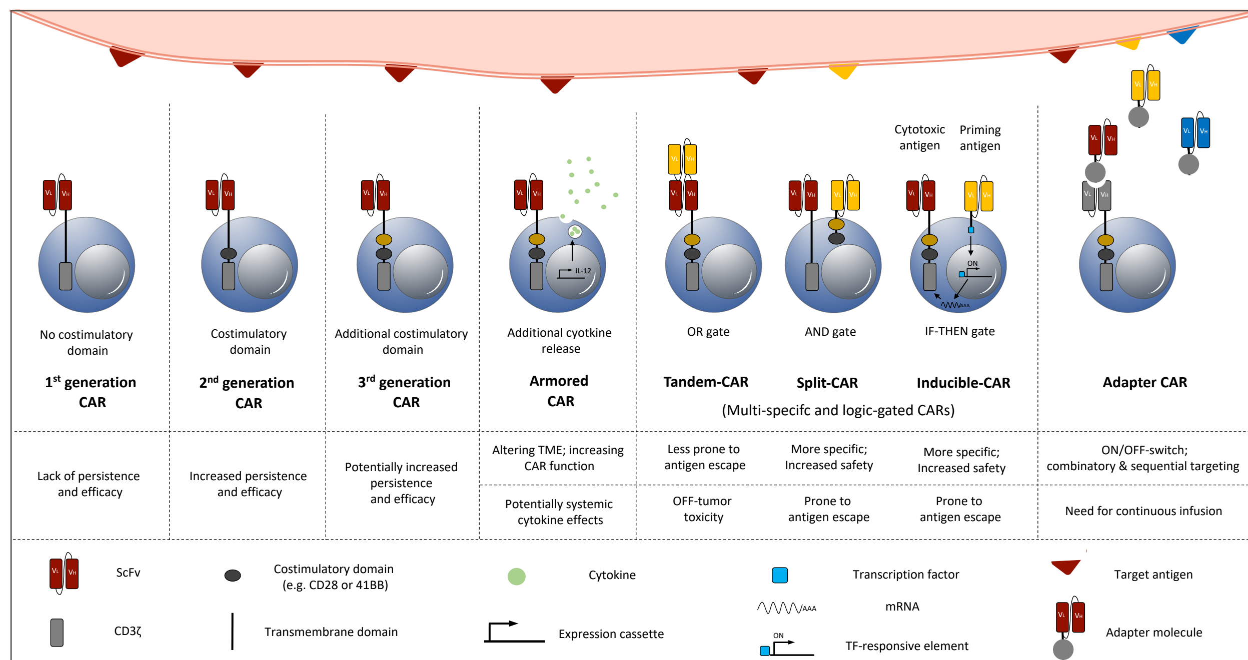

Figure 2: The evolution of CAR T cell designs. ScFv = single-chain variable fragment, TF = transcription factor. Adapted from [36, 38].

While many of the novel CAR designs are still in pre-clinical stages or on the verge of early clinical trials, their development was sparked by the tremendous success of second-generation CAR constructs in hematologic malignancies [39-41]. First and foremost, the first two FDA-approved CAR T-cell constructs, tisagenlecleucel [42] and axicabtagene ciloleucel, [43] both targeting CD19, showed remarkable response rates for patients with r/r ALL and diffuse large B-cell lymphoma (DLBCL) of > 80%. As of writing, 6

different CAR constructs have been approved by the FDA for various indications [36] including follicular lymphoma [44], mantle cell lymphoma [45], and multiple myeloma [46, 47]. Importantly, decade-long remissions have been reported [48] and CAR T-cell therapy has already advanced to second-line therapy in DLBCL [49, 50] and was even shown to be highly effective in first-line therapy [51], clearly demonstrating its potential for early disease stages.

2.2 Challenges for T cell-based immunotherapies and modes of resistance in hematologic malignancies

Despite the overwhelming initial response rates of BsAbs and CAR T cells, mainly in the setting of B-cell malignancies, accumulating data showed that fewer than 50% of patients experience durable remission [13, 43, 52-57]. Reasons are multi-faceted, differing between tumor entities, choice of target antigens, or the applied T-cell immunotherapy. In the following, an overview of the major challenges in developing more advanced, safe, and efficacious T-cell recruiting immunotherapies for the vast spectrum of hematological malignancies with a special focus on CAR T cells and T-cell dysfunction will be given.

2.2.1 Target-heterogeneity

An important aspect slowing the development of T-cell therapies for non-B-cell hematologic malignancies like AML is the huge inter- and intra-patient heterogeneity. Extensive data on the expression patterns of potential AML target antigens revealed that the identification is challenging due to the heterogeneity between patients and the co-expression of potential target antigens on healthy tissue. Only a subset of patients will therefore benefit from single-targeting strategies, requiring the manufacture of multiple different approaches to address all different AML subtypes [58, 59] (**Publication I: Nixdorf et al., 2023 [60]**).
In addition, co-expression of TAAs on healthy tissue potentially directly influences the efficacies of T-cell therapy, as it was reported that surprisingly high doses of the CD33-targeting BsAb AMG 330 were used in a recent phase I trial compared to blinatumomab [5, 19]. These observations are in line with our data (**Publication I: Nixdorf et al., 2023**), showing that receptor-mediated endocytosis of internalizing antigens like CD33 acts as a form of antigen sink, depleting AMs of small molecular weight, thereby influencing the efficacy of AdCAR T cells.

2.2.2 Target-antigen escape

Approximately 30-50% of relapses after CD19 CAR T therapy in ALL and DLBCL are associated with CD19 loss or downmodulation [53, 61-64]. The emergence of antigen-low or -absent tumor cells can happen in various ways, including mutation and clonal selection [65-67], alternative splicing [66], post-transcriptional mechanisms [68], trogocytosis [69], antigen shedding [70] or cell lineage switching [71].
Due to the differences in CAR vs TCR signaling, CAR T cells require higher antigen densities for full-scale T-cell activation, therefore being especially prone to antigen escape variants. Since BsAbs are reported to require similar if not higher antigen densities than CAR T cells to function [72], it is somewhat surprising that CD19 loss after unsuccessful blinatumomab treatment was reported to be less frequent

compared to post-CAR T-cell therapy [72, 73]. Data on potential antigen-escape post-T-cell immunotherapy for non-B-cell malignancies is therefore highly anticipated.

2.2.3 Therapy-related toxicities

Since patients treated with modern T-cell therapy are often in bad overall shape due to comorbidities, age, or previous treatment regimes, any further therapy-related toxicities are of concern. The major therapy-related toxicities observed to date in T cell-based therapy of B-cell malignancies are cytokine-release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS) [74, 75], and the ill-defined immune effector cell-associated hematotoxicity (ICAHT) which can lead to profound cytopenias [76, 77]. CRS is defined by high serum levels of inflammatory cytokines (e.g. interleukin-6 (IL-6) and interleukin 1 (IL-1)) resulting in sepsis-like symptoms. ICANS, however, is not well described but seems to occur as a result of endothelial dysfunction in the central nervous system leading to neurotoxicities [78, 79]. CRS and ICANS are usually treated with steroids or with IL-6 antagonists like tocilizumab, however, other means to temporarily intervene or prevent these toxicities, while preserving CAR activity, are highly anticipated [80].

Long-lasting B-cell aplasia caused by CD19 CAR persistence has also been observed but can be compensated with immunoglobulin replacement therapy [81]. Noteworthy, CD19 is exclusively expressed throughout the whole B-cell lineage and is absent on other healthy tissues [82, 83]. For other hematologic malignancies, like AML, potential target-antigens are often also expressed in healthy cells such as hematopoietic stem- and progenitor-cells, monocytes, or macrophages, potentially leading to profound on-target/off-tumor toxicities through engagement with CAR- or BiTE-redirected T cells [58, 59]. In fact, the reasons why CAR T cell therapy in AML is so far mainly used as a bridge to transplant, are the accompanying profound cytopenia and myeloablation, putting patients at high risk for fatal infectious complications [84-86].

2.2.4 Hostile tumor-microenvironment

In addition to the aforementioned aspects, the efficacy of T cell-based immunotherapy depends on a variety of other parameters. Tumor cells have evolved mechanisms to adapt to and escape from immunotherapy, by providing an immunosuppressive tumor-microenvironment. This protective habitat comprises a combination of various immunosuppressive cell types including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), or regulatory T cells (Tregs) [87], providing tumor-promoting cytokines, chemokines, and growth factors [88]. In addition, the upregulation of inhibitory receptors like PD1 [89], the generation of a pro-inflammatory cytokine milieu [90], or impaired T-cell trafficking [91, 92], renders T cells dysfunctional.

2.2.5 T-cell dysfunction

A functional pool of T cells is a prerequisite for the efficacy of T cell-based immunotherapy. As such, it is crucial to unravel the mechanism involved in T-cell dysfunction and decipher ways to prevent or revert T cells from progressively and hierarchically losing their function ("T-cell exhaustion").

The concept of T-cell exhaustion was originally described in the context of murine and human chronic viral infections [93, 94]. Continuous antigen exposure renders T cells dysfunctional, losing their effector functions starting from reduced secretion of interleukin-2 (IL-2), accompanied by decreased cytotoxic and proliferative potential and decreased secretion of interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) [95]. At the same time, sustained up-regulation of inhibitory checkpoint molecules (ICPs) including T-cell immunoglobulin and mucin domain 3 (TIM3), lymphocyte activation gene 3 (LAG3), and PD1, as well as defective metabolic programs can be observed [95-97].

T-cell dysfunction is a broadly used term for likely interconnected T-cell states with distinct functional, transcriptional, and epigenetic features [98]. While different forms of T-cell dysfunction with varying definitions and transitory states, including senescence, exhaustion, or anergy have been described, the term will be used in the following synonymously to T-cell exhaustion as a consequence of continuous overstimulation of T-cells.

A complex and highly debated transcriptional and epigenetic network underlies the observed dysfunctional phenotypes in T cells. These networks differ substantially from effector or memory T cells, and it is still unclear whether exhausted T cells are derived from effector cells or reflect a separate branch of T cells potentially originating and imprinting an "exhaustion program" on T cells of any differentiation state [98-101]. One possibility for T-cell differentiation trajectories is outlined in Figure 3: Upon antigen encounter and activation, naïve T cells undergo differentiation into effector cells that either further differentiate into killer cell lectin-like receptor subfamily G member 1 (KLRG1) positive terminally differentiated effector cells or long-lived memory progenitor cells [102-104]. A fraction of the effector cells retains their capacity for longevity and in case of antigen persistence gives rise to progenitor-exhausted T cells (T_{PEX}) with expression of T-cell factor-1 (TCF-1). T_{PEX} ($PD1^{hi}TIM3^{low}TCF\text{-}1^{+}$) are capable of effector function, and self-renewal, and have been identified to be the origin of effector cells after PD1 checkpoint blockade [105-108]. Whether these progenitor cells are equally important in the context of BsAb- or CAR- therapy remains to be elucidated. Over time, T_{PEX} will undergo a number of not yet defined transitory states to finally develop into terminally exhausted (T_{EX}) T cells ($PD1^{hi}TIM3^{hi}TCF\text{-}1^{-}$), progressively losing effector function and experiencing cell growth arrest and potentially a more fixed and epigenetically imprinted state [109-114].

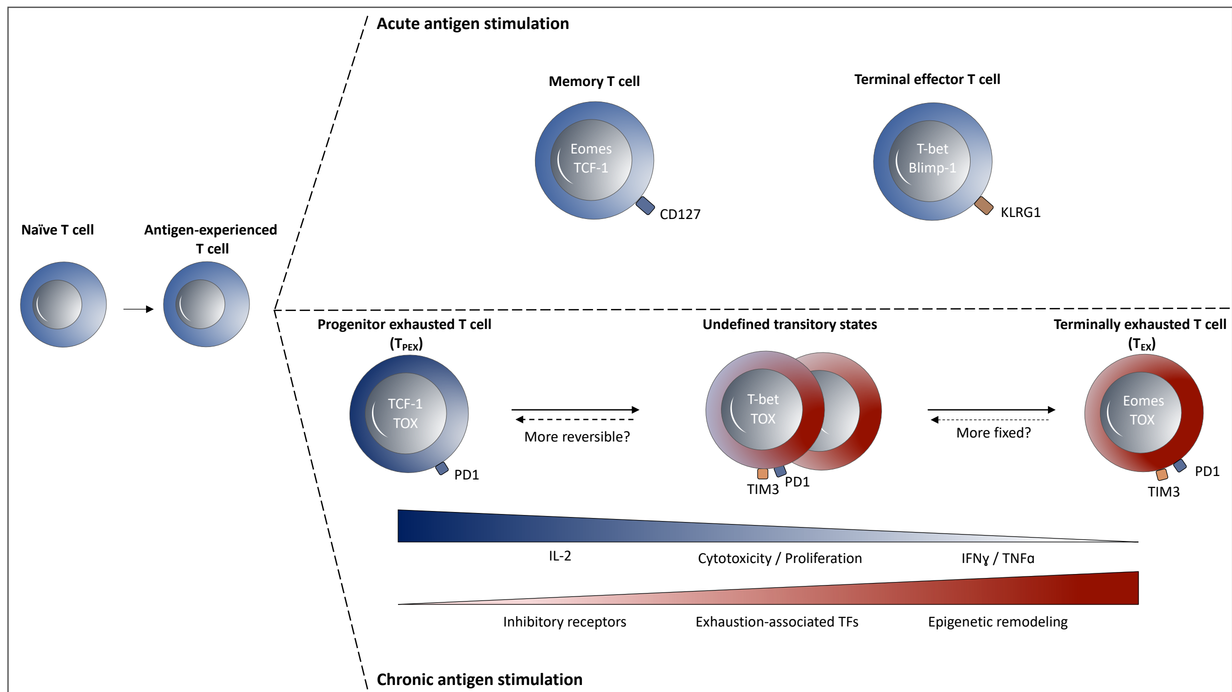

Figure 3: The concept of T-cell exhaustion. IL-2 = Interleukin-2, TCF-1 = T-cell factor-1, TOX = Thymocyte selection-associated high mobility group box protein, T-bet = T-box transcription factor, EOMES = Eomesodermin, KLRG1 = killer cell lectin-like receptor subfamily G member 1, IFNγ = interferon gamma, TNFα = tumor necrosis factor alpha, PD1 = programmed cell death protein 1, TIM3 = T-cell immunoglobulin and mucin-domain containing-3. Adapted from [95, 98, 111, 115, 116].

The different T-cell states are controlled by a delicate interplay of transcription factors (TFs) that are shared with other effector and memory T-cell types [100, 117]. The major TFs associated with T-cell exhaustion are T-box transcription factor (T-bet), Eomesodermin (Eomes), and the calcineurin-dependent TF Nuclear factor of activated T-cells (NFAT)/activator protein 1 (AP-1) axis. In exhausted T cells, the balance of NFAT and members of the AP-1 family (Fos-Jun) is skewed towards NFAT, leading to an increased amount of partnerless NFAT inducing TF thymocyte selection-associated high mobility group box protein (TOX) and nuclear receptor subfamily 4A (NR4A). This shifted core transcriptional network is one of the drivers of ICP induction such as PD1 and installs an exhaustion-associated program on the T cells [95, 118-123].
Similar to viral infections, features of T-cell dysfunction have been discovered in cancer immunology [89, 124-126]. Importantly, preclinical CAR T cell studies [127-129] [130] and our own studies on BsAbs (**Publication II: Philipp et al., 2022),** as well as AdCAR T cells (**Publication I: Nixdorf et al., 2023**) revealed similar mechanisms upon continuous antigen exposure. In addition, T-cell exhaustion has been observed in many patients receiving BsAbs [131] or CARs [132, 133]. Gaining data on the plasticity and revertability of dysfunctional T-cell states will likely advance the development of T cell-based immunotherapy [115, 116].

2.3 Ways to improve T cell-based immunotherapy

To overcome the current roadblocks of T cell-based immunotherapy, tremendous efforts are being made. Strategies are multifold and examples will be outlined in the following, especially highlighting the potential of AdCAR T cells to handle target heterogeneity, therapy-related toxicities, and T-cell dysfunction.

In principle, the modular nature of CAR-redirected T cells allows for a variety of genetic engineering approaches. For instance, the CAR T-cell design itself can be adjusted to fine-tune target affinities or cytokine secretion, thereby influencing toxicities and persistence [134-136]. Decreasing the signal strength of CAR T cells by truncating the CD3ζ domain to contain only one of three immunoreceptor tyrosine-based activation motif (ITAM) domains can result in enhanced persistence and efficacy in animal models [137].

Ways to circumvent the hostile tumor microenvironment encompass strategies like: checkpoint blockage [138-140], engineered switch receptors to turn unfavorable PD1 interactions into CD28 co-stimulatory responses [141, 142], genetic ablation of checkpoint molecules like PD1 [143], dominant negative transforming growth factor beta (TGF-β) receptors to modulate the immunosuppressive milieu [144] or specifically targeting the tumor-associated macrophages to disturb their negative impact on T cells and counteract T-cell dysfunction [145]. Increased T-cell trafficking into the tumor site can be achieved by equipping T-cells with chemokine receptors [146-150] and efficacy might be enhanced by so-called "armored" CAR T cells constitutively secreting cytokines such as IL-12, IL-15, IL-18 [151-155] or even BsAbs [156, 157].

Insufficient efficacy of T cell-based immunotherapy, target-antigen escape, and disease heterogeneity is being tackled by the development of multi-specific constructs such as bispecific [158] or tandem-CAR T cells [159], which simultaneously target more than one antigen. Rationally designed "logic-gated" approaches as outlined in Figure 2 allow for better therapeutic windows. Only upon simultaneous targeting of two different antigens, a full-scale T-cell activation is achieved, thereby reducing off-tumor targeting, however, at the potential cost of antigen escape [156, 160, 161].

While many of these approaches show promising results, their application requires time- and cost-intensive genetic engineering. This has sparked the development of Adapter CAR T cells combining the flexibility of BsAbs with CAR T cells. Intriguingly, we (**Publication I: Nixdorf et al., 2023**) and others [162-167] have shown that AdCAR T cells are only activated upon bridging the CAR T cells to the tumor cells via the addition of adapter molecules. While efforts are being made to design "kill switches" to permanently ablate the CAR T cells in the event of life-threatening toxicities, the AdCAR platform has the advantage of being effectively turned on and off, by controlled addition of the AMs. In fact, prevention and termination of CRS by the use of AdCAR T cells were recently shown in animal models [165] and data from the first phase I study targeting AML with an anti-CD123 adapter CAR platform demonstrated recovery of neutrophil counts upon AM withdrawal [168].

Moreover, these platforms were shown to be highly versatile in the use of AMs of different specificity, in parallel [167], or sequentially (**Publication I: Nixdorf et al., 2023**), inferring a window of opportunity to deal with target antigen escape and antigen heterogeneity.

Importantly, we and others have shown that precisely timed activation and termination of T cells can be used to prevent T-cell exhaustion. Intermittent stimulation of T cells by treatment-free intervals (TFIs) prolonged overall effector function and led to the transcriptional rejuvenation of BsAb-treated cells *in vitro* and *in vivo* [169-172] (**Publication II: Philipp et al., 2022).** Interestingly, our group observed comparable effects for AdCAR T cells after disruption of continuous antigen exposure (**Publication I: Nixdorf et al., 2023**). This is in line with data from another CAR study showing that an inducible CAR, which is prone to T-cell dysfunction due to tonic signaling, can be transcriptionally, epigenetically, and functionally reinvigorated by resting periods [127], implying that T-cell dysfunction is a process of plasticity and not necessarily a fixed cell state. Of note, we did observe transcriptional differences between BsAb- and AdCAR-redirected T cells, potentially caused by the CAR manufacturing process shifting the T cells further down the differentiation path compared to naïve BsAb-treated T cells. Whether this has consequences on the potential of reinvigoration requires further investigation.

These data show the overarching advantage of optimized treatment schedules for dealing with dysfunction in T cell-based immunotherapy and are in line with the observation that the preservation of a more naïve-like T-cell phenotype is beneficial for T-cell immunotherapy [173-177]. We, therefore, envision the next generation of T cell-based immunotherapy to take flexible treatment schedules into consideration and widen its success in and beyond hematologic malignancies.

2.4 Summary of publications

2.4.1 Publication I: Adapter CAR T cells to counteract T-cell exhaustion and enable flexible targeting in AML

The development of CAR T-cell therapies in AML is facing challenges due to target antigen heterogeneity, therapy-related toxicities, and T-cell dysfunction. In the main publication of this doctoral thesis, we, therefore, developed and evaluated an adapter CAR T-cell platform for targeting AML. We generated Fab-based adapter molecules (AMs) which act as a bridging module between the tumor cells and the AdCAR T cells. Only upon simultaneous engagement of target (AML) and effector (AdCAR) cells by the AMs, do the AdCAR T cells get activated, leading to T-cell proliferation and target elimination.

Utilizing AML cell lines, we demonstrated the *in vitro* efficacy of the platform in eliminating AML cells using αCD33, αCD123, and αCLL-1 AMs. We confirmed the results with primary AML samples and were able to demonstrate similar efficacy of AdCAR T cells *in vivo* compared to a conventional CD33-directed CAR T-cell construct.

The small-molecular weight AMs used in this study have a short half-life allowing for specific activation and termination of CAR T-cell functions, thus potentially providing a wider therapeutic window. We, therefore, characterized *in vitro* surface retention kinetics of AMs on AML cell lines and primary samples, demonstrating pronounced receptor-mediated internalization of all three AMs. Our data hint towards a dual role when targeting internalizing antigens with AMs. On the one hand, we identified AM internalization as a form of antigen sink, contributing to the elimination of AM, thereby limiting AdCAR T-cell

function, especially for lowly expressed target antigens. On the other hand, the short half-life is likely to beneficially influence AdCAR on/off-switch dynamics.

Along these lines, we demonstrated for the first time in an *ex vivo* long-term co-culture system of primary AML cells and healthy donor-derived AdCAR T cells that sequential application of AMs of different specificity is feasible and efficacious. A universal approach like this has the potential to cover the heterogeneity observed in AML patients as well as deal with potential antigen escape variants.

We next set out to establish an *in vitro* model to mimic continuous antigen stimulation to monitor long-term AdCAR T-cell functionality in AML. Importantly, while we demonstrated that chronic AdCAR T-cell stimulation led to T-cell dysfunction, similar to BsAbs or conventional CAR T cells, we demonstrated that the introduction of treatment-free intervals counteracted AdCAR T-cell dysfunction resulting in prolonged effector function as well as transcriptional reprogramming associated with a more functional T-cell phenotype.

As T-cell exhaustion, safety profile, and target antigen heterogeneity are well-known restrictions for T cell-based immunotherapy in AML, the AdCAR platform might offer effective strategies to ameliorate these limitations.

2.4.2 Publication II: T-cell exhaustion induced by continuous bispecific molecule exposure is ameliorated by treatment-free intervals

The concept of T-cell exhaustion is well appreciated in the context of chronic viral infections or CAR T-cell therapy, however, little is known in the context of BsAb therapy. We therefore explored the effects of chronic antigen stimulation on T cells, by continuously exposing them to the CD19xCD3 T cell-recruiting antibody blinatumomab and $CD19^+$ tumor cells.

To this end we performed a longitudinal analysis of T cells from patients with r/r B-cell precursor ALL, treated for up to 28 days with continuous infusion of blinatumomab. We observed a decrease in cytotoxicity and IFNγ secretion when analyzing these T cells *in vitro*, well in line with evolving T-cell exhaustion. To mechanistically study the effects of continuous BsAb treatment on T-cell function, we next developed an *in vitro* model system to co-culture human T cells in the presence of BsAbs and the DLBCL cell line OCI-Ly-1 for 28 days. In concordance with the patient data, we observed evolving features of T-cell exhaustion, including pronounced upregulation of inhibitory checkpoint molecules (PD1, TIM3, LAG3), decreased effector cytokine secretion, decreased T-cell proliferation and cytotoxicity, impaired metabolic functions as well as the establishment of a distinct transcriptional program characterized by down-regulation of memory- and stemness-related genes and a shift towards exhaustion-related genes.

In line with the treatment-free interval data from Publication I, we then hypothesized that intermittent BsAb stimulation of T cells has the potential to delay or even revert the evolving exhaustion-associated phenotype induced by chronic antigen exposure. Intriguingly, functional data revealed a rejuvenation of T cells as evidenced by down-regulation of inhibitory checkpoint molecules, increased cytokine secretion, increased T-cell proliferation and cytotoxicity as well as increased metabolic capacity. In addition, the

transcriptional program observed during T-cell exhaustion was partly reverted leading to a shift in expression of memory-associated genes, indicating a more functional state. Lastly, we demonstrated that only intermittently stimulated T cells were capable of eliminating leukemic cells in an *in vivo* patient-derived xenograft ALL mouse model.

Together, we highlight the importance of counteracting T-cell dysfunction in BsAb therapy and propose treatment-free intervals to develop more efficacious treatment schedules.

3. Paper I

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Adapter CAR T cells to counteract T-cell exhaustion and enable flexible targeting in AML

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Although the landscape for treating acute myeloid leukemia (AML) patients has changed substantially in recent years, the majority of patients will eventually relapse and succumb to their disease. Allogeneic stem cell transplantation provides the best anti-AML treatment strategy, but is only suitable in a minority of patients. In contrast to B-cell neoplasias, chimeric antigen receptor (CAR) T-cell therapy in AML has encountered challenges in target antigen heterogeneity, safety, and T-cell dysfunction. We established a Fab-based adapter CAR (AdCAR) T-cell platform with flexibility of targeting and control of AdCAR T-cell activation. Utilizing AML cell lines and a long-term culture assay for primary AML cells, we were able to demonstrate AML-specific cytotoxicity using anti-CD33, anti-CD123, and anti-CLL1 adapter molecules in vitro and in vivo. Notably, we show for the first time the feasibility of sequential application of adapter molecules of different specificity in primary AML co-cultures. Importantly, using the AML platform, we were able to demonstrate that chronic T-cell stimulation and exhaustion can be counteracted through introduction of treatment-free intervals. As T-cell exhaustion and target antigen heterogeneity are well-known causes of resistance, the AdCAR platform might offer effective strategies to ameliorate these limitations.

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INTRODUCTION

CAR T-cell therapies have revolutionized the treatment of various B-cell malignancies [1–3]. Although allogeneic stem cell transplantation first demonstrated the power of adoptive T-cell transfer for combating leukemia, in AML, CAR T-cell therapy is only slowly progressing. The main challenges to the successful use of CAR T cells in the AML setting are inter- and intra-patient heterogeneity in the target antigen expression profile and a lack of leukemia-restricted target antigens. The latter translates into increased toxicity and safety issues [4, 5]. Due to the lack of fast and efficient safety switches to circumvent on-target-off leukemia toxicity, current CAR T constructs are mainly used as a bridge to transplant strategy [6, 7].

Another cause of CAR T-cell failure is T-cell dysfunction, the reasons for which are multifaceted, ranging from the quality of the T cells at the time of apheresis [8, 9], remodeling by the tumor microenvironment [10–14], chronic antigen exposure [15, 16], also caused by the intrinsic resistance of AML cells due to impaired death receptor signaling [17], and tonic CAR T-cell signaling [18, 19].

Building on the previously described Adapter CAR™ T-cell platform [20] we therefore developed a fragment antigen-binding region (Fab)-based approach that enables flexibility of targeting and control of AdCAR T-cell activation. Using an AdCAR directed against a biotinylated adapter molecule (AM) in the context of a specific linker, we demonstrate that AdCAR T cells are highly functional against multiple AML cell lines in vitro and in vivo, and against primary AML (pAML) cells, by utilizing a long-term culture system in combination with αCD33, αCD123, and αCLL-1 AMs.

The majority of AML-associated target antigens get internalized, hence we also studied receptor-mediated endocytosis of various AM formats and its impact on half-life and cytotoxicity. In addition, we provide the first evidence that serial use of AMs against different target antigens is feasible and highly potent in eliminating pAML cells in ex vivo long-term co-cultures. To

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counteract CAR T-cell exhaustion as a result of continuous (CONT) stimulation, we sought to advantageously utilize the AM as an on–off switch for implementing treatment free intervals (TFIs). Importantly, we could show that the AdCAR T-cell platform allows the use of TFIs that abrogate target-induced T-cell dysfunction.

METHODS

In vitro cytotoxicity assays and pAML culture

AdCAR T cells or untransduced (mock) T cells were co-cultured with MV4-11, HL-60, or OCI-AML-3 cells at varying E:T ratios in the presence of either αCD33, αCD123, αCLL-1, or αCD19 AMs. Specific lysis was assessed by flow-cytometry on Cytoflex S/LX instruments (Beckman Coulter, Brea, CA, US) at the iFlow Core facility, Munich, and calculated relative to conditions without AMs or mock T cells. pAML long-term co-culture assays were performed as described [21]. For serial targeting experiments, AMs were either replenished or exchanged for an AM with different target specificity every 3 days by exchanging 50% of the cell culture medium with 2x complete blast medium containing the AM.

Internalization assays

αCD33-AM_{Fab} was labeled with pHrodo Red Avidin (ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions. MV4-11 cells were labeled with 500 ng/ml αCD33-AM_{Fab}-pHrodo and 1:1000 Hoechst 33342 (ThermoFisher Scientific) for 15 min at 4 °C. Unbound AM was removed and cells were incubated for 6 h at 4 °C or 37 °C on poly-D-lysine-coated glass-bottomed two-well ibidi slides (ibidi GmbH, Gräfelfing, Germany). Images were acquired on a Nikon TiE microscope. Instrument settings are outlined in detail in the supplements.

Indirect internalization assay. AML cells were treated for 15 min at 4 °C with an AM, after which time unbound AM was removed. Cells were incubated for 0–6 h at 4 °C or 37 °C in R10. At each time point, cells were washed and stained with anti-biotin-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and AquaLive/Dead (Invitrogen) for 15 min at 4 °C. T cells were analyzed by flow-cytometry. The percentage of surface-bound AMs was calculated based on the MFI at 37 °C compared to controls.

In vitro long-term AdCAR T-cell stimulation and treatment-free intervals

Healthy donor (HD)-derived AdCAR T cells were co-cultured with irradiated (2.5 Gy) OCI-AML-3 cells in R10 (E:T = 1:4; 1×10^6 cells/ml) in the presence of 10 ng/ml αCD33-AM_{Fab}. After 3 days, half of the medium was exchanged with fresh medium containing αCD33-AM_{Fab} and irradiated target cells (E:T = 1:2). On day 7, human T cells were isolated using the EasySep Human CD3 Positive Selection Kit II (Stemcell, Vancouver, Canada) according to manufacturer's instructions. A fraction of isolated T cells was used for functional cytotoxicity and proliferation assays and immunophenotyping [22]. The remaining T cells were re-cultured with irradiated target cells (E:T = 1:4). To implement TFIs, the cultures were split and treated with or without αCD33-AM_{Fab} for a further 7 days. A third CONT round of αCD33-AM_{Fab} stimulation was performed until day 21. Co-culture supernatants were harvested to quantify human cytokine secretion.

In vivo studies

All experiments were performed according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) in the animal husbandry facilities of Miltenyi Biotec. General health status was monitored daily. NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ mice were engrafted with OCI-AML-2 cells via tail vein injection on day −5. AdCAR T or conventional CD33CAR T cells were injected intravenously on day 0. αCD33-AM_{Fab} was intraperitoneally administered daily. Leukemia growth was monitored twice per week by bioluminescence imaging (BLI). A detailed description of the in vivo studies is provided in the supplements.

RESULTS

AdCAR T cells mediate specific lysis of AML cell lines

One major obstacle to CAR T-cell-based immunotherapy in AML is the heterogenous expression profile of target antigens. We analyzed 32 pAML samples at initial-diagnosis for their expression of the target antigens CD33, CD123, and CLL-1. The majority expressed these antigens at high levels, however, we did observe samples in which a minority of cells expressed one of the three antigens (Fig. 1A, Supplementary Fig. S1A and Table 1). Hence, to make CAR T-cell therapy applicable in all AML subtypes and at the same time counteract antigen escape variants, a CAR T platform allowing to address several target antigens either in parallel or sequentially is desirable. We therefore established an AdCAR T-cell platform allowing flexible targeting of various antigens by uncoupling antigen recognition and T-cell activation (Fig. 1B).

Lentiviral transduction of human T cells with the AdCAR construct was consistent and highly efficient (% transduction efficiency: 55 ± SEM), whereas $CD4^+$ T cells were significantly more susceptible to lentiviral transduction than $CD8^+$ T cells (Fig. 1C and Supplementary Fig. S1B).

HD-AdCAR T cells were co-cultured with different AML cell lines (MV4-11, HL-60, OCI-AML- 3) expressing CD33, CD123, and CLL-1 at various levels (Fig. 1D) in the presence of target-antigen-specific Fab- or Ab-based AMs. Specific cytotoxicity was observed against all three AML cell lines and was dependent on target-antigen specificity, antigen density, AM concentration, and E:T ratio (Fig. 1E and Supplementary Fig. S1C–F).

Addition of the control αCD19-AM_{Fab} did not result in nonspecific lysis (Fig. 1E), and we found no target-antigen-independent cytotoxicity for the AMs (Supplementary Fig. S1E). AdCAR T-cell activation was measured by the secretion of the effector cytokines IFN-γ, TNF, and IL-2, and was observed only in the presence of target-antigen-specific AMs and corresponding target cells (Fig. 1F).

AdCAR-mediated cytotoxicity against pAML cells: impact of receptor-mediated internalization on AdCAR T-cell efficacy

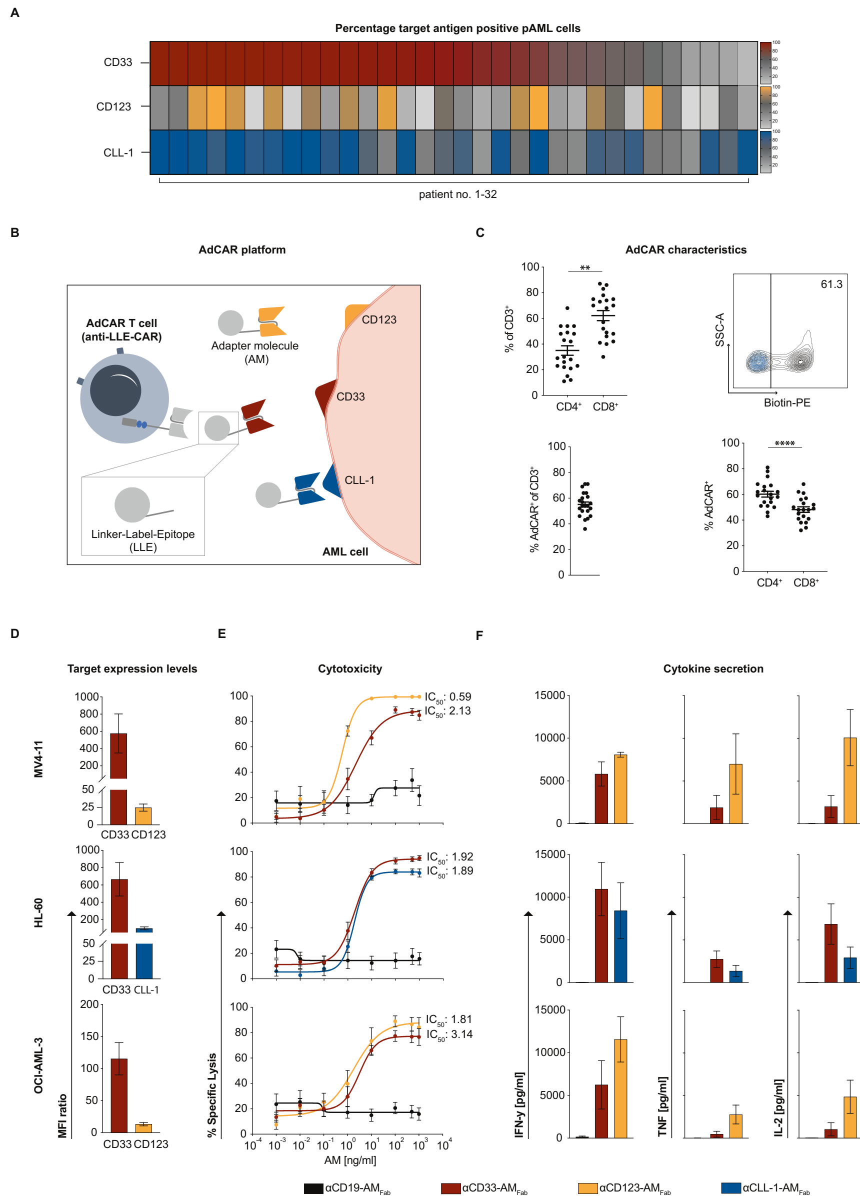
Next, we assessed whether AdCAR T cells can effectively target and lyse pAML cells. We focused on CD33 as the target antigen and observed high and specific lysis of pAML cells (% specific lysis: 75 ± SEM at 100 ng/ml αCD33-AM_{Fab}) after 3 days of co-culture with AdCAR T cells using our previously described pAML culture system [21]. Target elimination was AM dose and E:T ratio dependent (Fig. 2A). Notably, AdCAR T cells generated from HD or pAML T cells were equally effective in vitro (Supplementary Fig. S2C), suggesting the vector system is suitable for clinical applications.

However, over an extended period of 12 days, we observed an outgrowth of pAML cells with faster kinetics at lower AM concentrations (Fig. 2B). In line with this, T-cell proliferation peaked at around day 6 (Supplementary Fig. S2A) and the percentage of PD1/TIM3/LAG-3^+ T cells declined over time, indicating a loss of T-cell activation (Supplementary Fig. S2B).

Because the AMs were added only at the start of the co-cultures, we speculated that the available AMs were consumed by target antigen receptor-mediated endocytosis. We assessed the surface retention of αCD33-AM_{Fab} bound to the CD33 receptors of MV4-11 AML cells, by labeling the cells with AMs in the absence of AdCAR T cells. We observed almost complete loss of receptor-bound AMs within 72 h, with even faster kinetics after prior removal of unbound AM, suggesting that free AM was binding to recycled or newly synthesized antigen receptors (Fig. 2C).

To further validate our findings, αCD33-AM_{Fab} was coupled to a pH-sensitive dye. Confocal microscopy confirmed that internalization of AMs occurred rapidly at 37 °C (Fig. 2D). Furthermore, a deficiency of CD33 receptors, as well as blocking vesicle transport with monensin, completely disrupted internalization by MV4-11 cells, hinting convincingly at receptor-mediated endocytosis of the AMs (Supplementary Fig. S2D).

To quantitatively describe this phenomenon, we characterized the internalization kinetics of different AM formats (Fab vs Ab



based). We observed a rapid decline of all analyzed AMs within the first 2 h (Fig. 2E, F and Table 2), with kinetics dependent on the AML cell line, AM format, and the target antigen. Interestingly, Fab-based AMs had shorter half-lives than the Ab-based formats, and, after 6 h, only 19%, 8%, and 8% of the initial surface-bound concentrations of αC33-AM_{Fab}, αCD123 AM_{Fab} (both on MV4-11), and αCLL-1-AM_{Fab} (on HL-60), respectively, were detected. To validate that AM internalization also occurs in a clinically relevant setup, we confirmed the results with pAML cells (Supplementary Fig. S2E). Of note, based

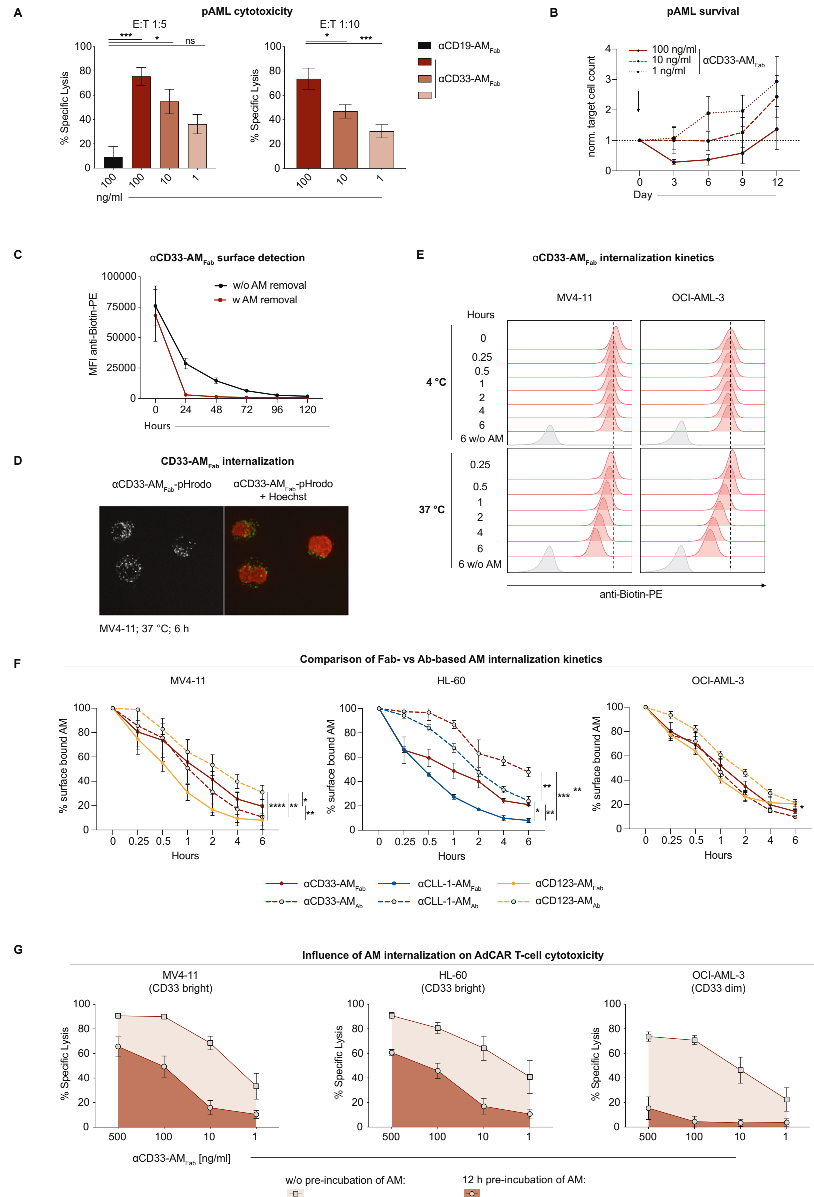
Fig. 1 AdCAR T cells mediate specific lysis of AML cell lines. **A** Percentage of CD33-, CD123- and CLL-1-positive pAML cells assessed by surface marker staining with biotinylated AMs and subsequent secondary staining ($n = 32$). **B** Schematic illustration of the AdCAR T-cell platform recognizing an AML cell via AMs directed against the target antigens CD33, CD123, or CLL-1. **C** AdCAR characteristics after transduction and 14 days of expansion in IL-7/IL-15. The CD4/CD8 ratio of the AdCAR T-cell product was determined by flow-cytometry ($n = 20$). The transduction efficiency of AdCAR T cells was measured by biotin-PE staining. A representative contour plot depicts the percentage of AdCAR$^+$ fraction in black (untransduced cells in blue). **D** Target antigen expression on AML cell lines determined by surface marker staining with biotinylated AMs and subsequent secondary staining ($n = 3$). MFI ratios were calculated based on corresponding controls without AMs. **E** AdCAR T-cell-mediated cytotoxicity after 48 h ($n = 4$–12) against the AML cell lines MV4-11, HL-60, and OCI-AML-3 (E:T = 1:1) in co-cultures containing αCD33-AM$_{Fab}$, αCD123-AM$_{Fab}$, or αCLL-1-AM$_{Fab}$ at concentrations ranging from 1 pg/ml to 1000 ng/ml. Target-irrelevant αCD19-AM$_{Fab}$ was used as a control AM. Specific lysis was calculated relative to the mock T-cell condition. **F** Secretion of IFN-γ, TNF, and IL-2, determined by cytometric bead array (CBA) analysis, from corresponding cytotoxicity assays at an AM concentration of 10 ng/ml ($n = 3$). All graphs present the mean ± SEM. Statistical analysis: paired *t*-test; ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Table 1. Patient characteristics.

Patient no.	Age	ELN	FAB	% positive			MFI ratio		
-	-	-	-	CD33	CD123	CLL1	CD33	CD123	CLL1
1	68	adverse	N/A	99.9	43.8	99.8	104.1	2.6	55.9
2	69	adverse	N/A	99.6	51.5	99.5	204.7	4.6	144.9
3	N/A	N/A	N/A	99.5	90	96.3	105.3	19.4	78.5
4	28	adverse	N/A	99.4	99.4	82.8	418.4	68.9	43.2
5	N/A	N/A	N/A	99.2	85.4	99	84.9	8.3	79.8
6	39	N/A	N/A	99	3.99	87.7	41.7	4.3	25.7
7	N/A	N/A	N/A	98.9	82.6	90.8	20.1	3.2	14.3
8	71	adverse	M5	98.7	5.43	98.2	237.7	1.9	198.4
9	79	adverse	M1	98.1	73.2	99	116.7	26.6	377.7
10	N/A	N/A	N/A	98.1	33.1	88.3	41.7	4.3	25.7
11	N/A	N/A	N/A	98.1	78.1	97	141.4	28.9	94.2
12	N/A	N/A	N/A	97.5	34.8	68.3	39.8	6.1	12.6
13	77	adverse	M1	95.7	91.7	47.1	35.9	26.6	6.2
14	N/A	N/A	N/A	95.7	21.7	95.4	187.3	9.4	152.8
15	N/A	N/A	N/A	94.8	3.81	48.8	18.8	1.3	3.9
16	70	adverse	M1	92.5	63.4	66.5	74.7	25.7	34.1
17	N/A	N/A	N/A	91.6	43.9	76.6	11.7	4.1	8.5
18	81	adverse	M2	87.6	25.6	37.6	14.0	3.5	4.6
19	N/A	N/A	N/A	86.6	34.1	94.5	73.9	6.2	125.3
20	49	adverse	M4	83.5	81	55.5	37.5	29.4	17.9
21	N/A	N/A	N/A	79.2	99.1	100	50.4	172.0	1363.9
22	57	adverse	N/A	69	33	48.4	73.7	22.5	41.6
23	34	adverse	M0	67.9	24.7	44.4	65.1	6.1	14.2
24	N/A	adverse	N/A	65.8	75.1	78.2	25.1	26.8	81.2
25	74	intermediate	N/A	63.5	61.6	72.5	22.5	26.4	45.2
26	83	adverse	N/A	62.9	11.6	81.6	22.3	2.0	157.7
27	63	adverse	M0	58.4	97.1	52.6	16.2	56.4	17.1
28	N/A	adverse	N/A	50.5	51.1	48.8	22.0	27.6	24.1
29	66	adverse	M5a	31.7	1.93	40	21.7	4.0	40.7
30	N/A	N/A	N/A	30.5	7.67	89.6	6.0	2.4	43.9
31	N/A	N/A	N/A	28.6	53.9	67.2	4.4	5.6	13.7
32	N/A	N/A	N/A	18.5	29	95.9	62.1	1.5	100.7

on the detection of surface-bound AM we cannot exclude AM-dissociation and potentially other elimination pathways, having contributed to the decrease of AM.

To gain a deeper understanding into whether and how AM internalization influences AdCAR T-cell cytotoxicity, we performed co-culture cytotoxicity assays with three AML cell lines expressing high or low target antigen levels. AML cells were labeled with αCD33-AM$_{Fab}$ and AdCAR T cells were added 12 h later. Consistent with the internalization study, cytotoxicity was also dependent on AM level, as lysis of target cells declined upon prolonged pre-incubation of AML cells with AMs (Fig. 2G). Importantly, this effect was most pronounced if target antigen densities were low (OCI-AML-3 cell line), leading to almost complete loss of AdCAR T-cell-mediated cytotoxicity, even at initial αCD33-AM$_{Fab}$ concentrations



of 500 ng/ml (decrease of specific lysis: 73% to 15% ± SEM). We did not observe long-term downmodulation of CD33 expression levels by AM internalization (Supplementary Fig. S2F).

In summary, we showed that AdCAR T cells efficiently lyse pAML cells. However, we describe AM internalization as a common phenomenon for a variety of Fab- and Ab-based AM formats that target known internalizing antigens. AM internalization reduced their respective serum levels, thereby contributing to a form of "antigen sink" that impairs AdCAR T-cell cytotoxicity, especially at low levels of target antigen.

Fig. 2 AdCAR-mediated cytotoxicity against pAML cells: impact of receptor-mediated internalization on AdCAR T-cell efficacy. A AdCAR T-cell-mediated cytotoxicity after 72 h ($n = 3–13$) against pAML cells co-cultured on irradiated MS5 feeder cells (E:T = 1:5 and 1:10) in the presence of 100, 10 or 1 ng/ml αCD33-AM$_{Fab}$. αCD19-AM$_{Fab}$ at 100 ng/ml served as a negative control. Specific lysis was calculated relative to the AdCAR T-cell condition without AM. **B** Growth of pAML samples in long-term co-cultures with AdCAR T cells (E:T = 1:10) for 12 days with initial (one-time) addition of 100, 10 or 1 ng/ml αCD33-AM$_{Fab}$. pAML cell counts over time are plotted as normalized target cell count relative to starting conditions on day 0 ($n = 4–9$). **C** Levels of receptor-bound αCD33-AM$_{Fab}$ were monitored daily for 5 days on MV4-11 cells stained with 500 ng/ml AM. AM was added on day 0 for 15 min at 4 °C, unbound AM was either removed from the supernatant or not ($n = 3–4$). **D** Internalization assay. Left: Representative confocal image of MV4-11 cells stained for 6 h at 37 °C with 500 ng/ml αCD33-AM$_{Fab}$ coupled to pHrodo Red Avidin (gray). Right: Nuclei were stained with Hoechst 33342 (red) and merged with the pHrodo Red Avidin (green) channel. AM internalization can be seen as puncta located in the cytoplasm. Control conditions at 4 °C did not yield a measurable pHrodo Red Avidin signal (data not shown). Three independent experiments were performed. **E** Representative example of flow-cytometry-based indirect internalization assay of αCD33-AM$_{Fab}$ at 4 °C and 37 °C for 6 h on MV4-11 and OCI-AML-3 cells. AML cells were labeled for 15 min at 4 °C with 500 ng/ml AM. The unbound AM was removed and the percentage of surface-bound AM (red histograms) was assessed at each indicated time point by secondary staining with anti-Biotin-PE antibody. **F** Quantitative representation of the internalization assay described in **E** ($n = 6$). The kinetics of internalization of Fab- and Ab-based AMs were compared on AML cell lines. **G** The influence of AM internalization on AdCAR T-cell-mediated cytotoxicity ($n = 6$). AML cell lines were pre-incubated for either 0 or 12 h with 500–1 ng/ml αCD33-AM$_{Fab}$ before addition of AdCAR T cells (E:T = 1:1). Cytotoxicity was assessed by flow-cytometry after an additional 48 h. Data are plotted as mean ± SEM. Statistical analysis: **A** Ordinary one-way ANOVA with Dunnett's comparison; **F** Mixed-effects analysis with Geisser–Greenhouse correction. ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Table 2. Adapter molecule internalization kinetics.

half-lives [min]	MV4-11	HL-60	OCI-AML-3
aCD33-AM$_{Fab}$	86	127	73
aCD33-AM$_{Ab}$	64	88	65
aCD123-AM$_{Fab}$	36	N/A	43
aCD123-AM$_{Ab}$	56	N/A	71
aCLL-1-AM$_{Fab}$	N/A	18	N/A
aCLL-1-AM$_{Ab}$	N/A	86	N/A

AdCAR T cells allow for sequential targeting of pAML cells

Based on the short half-lives of the AMs, we examined whether repetitive AM dosing prolongs AdCAR T-cell functionality and leads to better elimination of pAML cells compared to single administration. Owing to the heterogeneity of target antigen expression in AML, we took advantage of the versatility of the AdCAR technology, which enables a sequential application of AMs against different target antigens. AMs against CD33, CD123, or CLL-1 were used once at a concentration of 10 ng/ml or replenished every third day until day 12 of co-cultures of pAML cells and AdCAR T cells (E:T = 1:10). Additional experiments included a dose increase to 100 ng/ml and/or a switch to an AM of different target specificity on day 6 of co-culture (10 or 100 ng/ml), followed by AM replenishment on day 9.

All AMs reduced leukemia growth compared to αCD19-AM$_{Fab}$ controls, underlining the specificity and potency of the AdCAR platform. However, a single addition of 10 ng/ml of αCD33-AM$_{Fab}$ or αCLL-1-AM$_{Fab}$ was insufficient to stop leukemia growth (Fig. 3A, B and Supplementary Fig. S3C, D).

Repetitive administration of AMs at 10 ng/ml further delayed (αCD33-AM$_{Fab}$) or halted (αCD123-AM$_{Fab}$ or αCLL-1-AM$_{Fab}$) leukemia outgrowth compared to a single addition of the AM.

Convincingly, an increase in dose of αCD33-AM$_{Fab}$ or αCLL-1-AM$_{Fab}$ from 10 to 100 ng/ml on day 6 of co-culture resulted in almost complete elimination of AML blasts (Fig. 3A, B), highlighting the potential for individually adjusting treatment conditions based on response to therapy or on target antigen levels.

Interestingly, switching from αCD33-AM$_{Fab}$ on day 6 to 10 ng/ml of either αCD123-AM$_{Fab}$ or αCLL-1-AM$_{Fab}$ was effective, demonstrating comparable efficacy in eliminating pAML cells to 100 ng/ml αCD33-AM$_{Fab}$. AdCAR T-cell-mediated cytotoxicity was further enhanced not only by changing the target specificity but also by increasing the respective AM doses to 100 ng/ml (Fig. 3A–D). Notably, targeting the same pAML samples first with αCLL-1-AM$_{Fab}$ followed by αCD33-AM$_{Fab}$ or αCD123-AM$_{Fab}$ did not result in higher lysis compared to continuous αCLL-1-AM$_{Fab}$ targeting, as opposed to starting the treatment with αCD33-AM$_{Fab}$ (Supplementary Fig. S3C–E).

The effects on AdCAR T-cell cytotoxicity were accompanied by a trend for increased T-cell proliferation after AM switching or dose increases (day 9), as well as increased expression of activation markers, indicative of pronounced and sustained AdCAR T-cell activation (Supplementary Fig. S3A, B, F, G).

These results collectively show that the use of highly modular AdCAR technology potently and specifically eradicated pAML cells in a dose- and time-dependent manner.

Fab molecules efficiently activate AdCAR T cells in vivo

Next, we aimed to translate our findings to a clinically relevant AML in vivo model by testing whether Fab-based AMs were able to efficiently direct AdCAR T cells against a highly aggressive OCI-AML-2 model. Therefore, HD-AdCAR T cells were expanded in vitro for 8 days and transferred to NSG mice bearing OCI-AML-2 leukemias (Fig. 4A). The mice were injected daily with αCD33-AM$_{Fab}$. AdCAR T cells were readily activated in vivo using Fab-based AMs. Convincingly, AdCAR T cells showed equipotency in controlling leukemia growth compared to conventional CD33CAR T cells, as quantified by BLI (Fig. 4B, C).

Treatment-free intervals prolonged AdCAR T-cell function

T-cell exhaustion is an emerging cause of CAR T-cell failure. We previously developed an in vitro system to monitor T-cell dysfunction induced by continuous bispecific antibody (BsAb) exposure in a clinically relevant B-cell lymphoma model [22]. It is unknown if AdCAR T cells react to CONT stimulation in the same way as conventional CAR T cells. Here, we adapted the long-term stimulation system to further evaluate AdCAR T-cell exhaustion in the context of AML. AdCAR T cells were co-cultured for 21 days with OCI-AML-3 in the presence of αCD33-AM$_{Fab}$. AdCAR T cells were isolated on days 0, 7, 10, 14, 17, and 21, and their cytotoxicity against OCI-AML-3 cells was assayed in co-cultures. We observed progressive AdCAR T-cell dysfunction over time, starting between day 10/14 of co-culture (Fig. 5A; 82% specific lysis on day 0 vs 13% on day 21), supporting our hypothesis that prolonged periods of AdCAR T-cell activation lead to loss of effector function.

As previously shown by us and others, intermittent T-cell stimulation using TFIs or molecular and chemical switches can prolong T-/CAR T-cell functionality, mainly through transcriptional and epigenetic remodeling [19, 22, 23].

Hence, we compared continuously to intermittently stimulated AdCAR T cells in our AML-optimized in vitro dysfunction

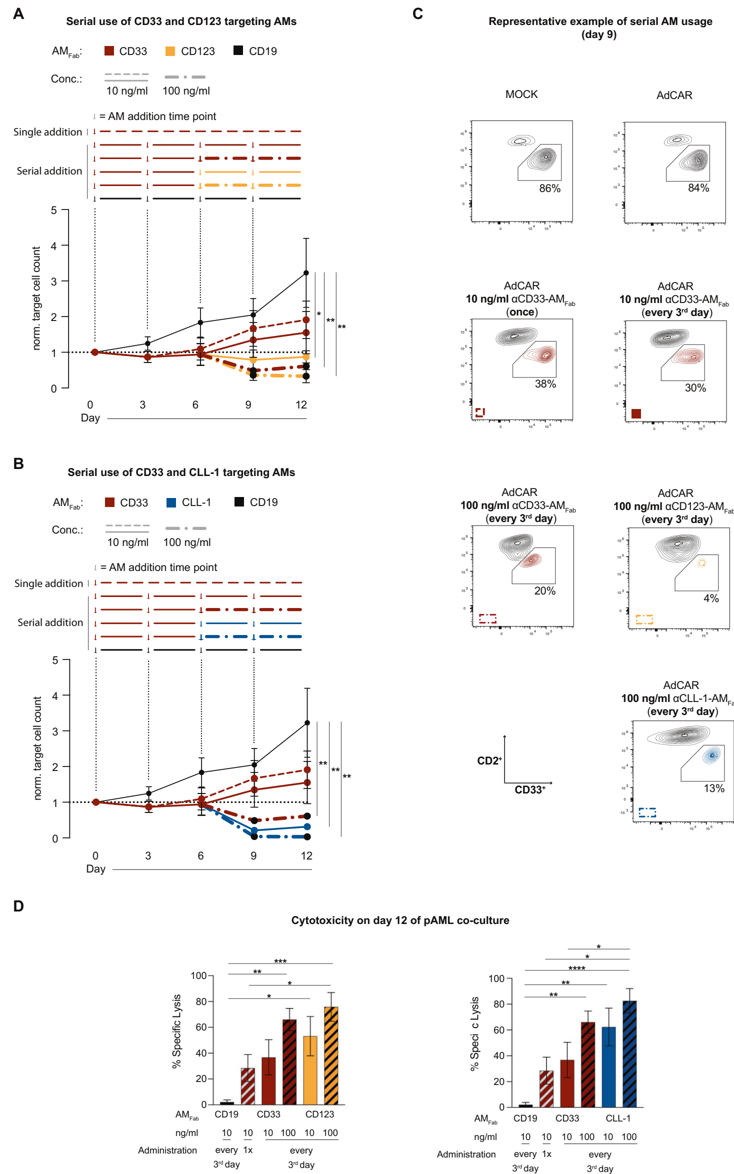


Fig. 3 AdCAR T cells allow for sequential targeting of pAML cells. A, B Long-term (12 days) co-cultures of AdCAR T cells and pAML cells (E:T = 1:10). pAML cell counts over time are plotted as normalized target cell count relative to starting conditions on day 0 ($n = 7$). αCD19-AM_{Fab} was replenished every third day at 10 ng/ml and served as a control. αCD33-AM_{Fab} was either applied once (10 ng/ml; dotted red line) or every third day until day 6 (solid red line). On days 6 and 9, the AM dose was either maintained at 10 ng/ml or increased to 100 ng/ml (bold dotted line). Alternatively, AMs were switched to AMs of different target specificity (αCD123-AM_{Fab} or αCLL-1-AM_{Fab}) on days 6 and 9 (10 or 100 ng/ml). **C** Representative flow-cytometry data from day 9 of co-culture. T cells and pAML cells were distinguished by CD2 and CD33 staining, respectively. Doublets, as well as dead cells were excluded, as described. **D** Corresponding AdCAR T-cell-mediated cytotoxicity on day 12 of co-culture. Specific lysis was calculated relative to the AdCAR T-cell condition without AM. Data are presented as mean ± SEM. Statistical analysis: Ordinary one-way ANOVA with Dunnett's comparison; ns $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

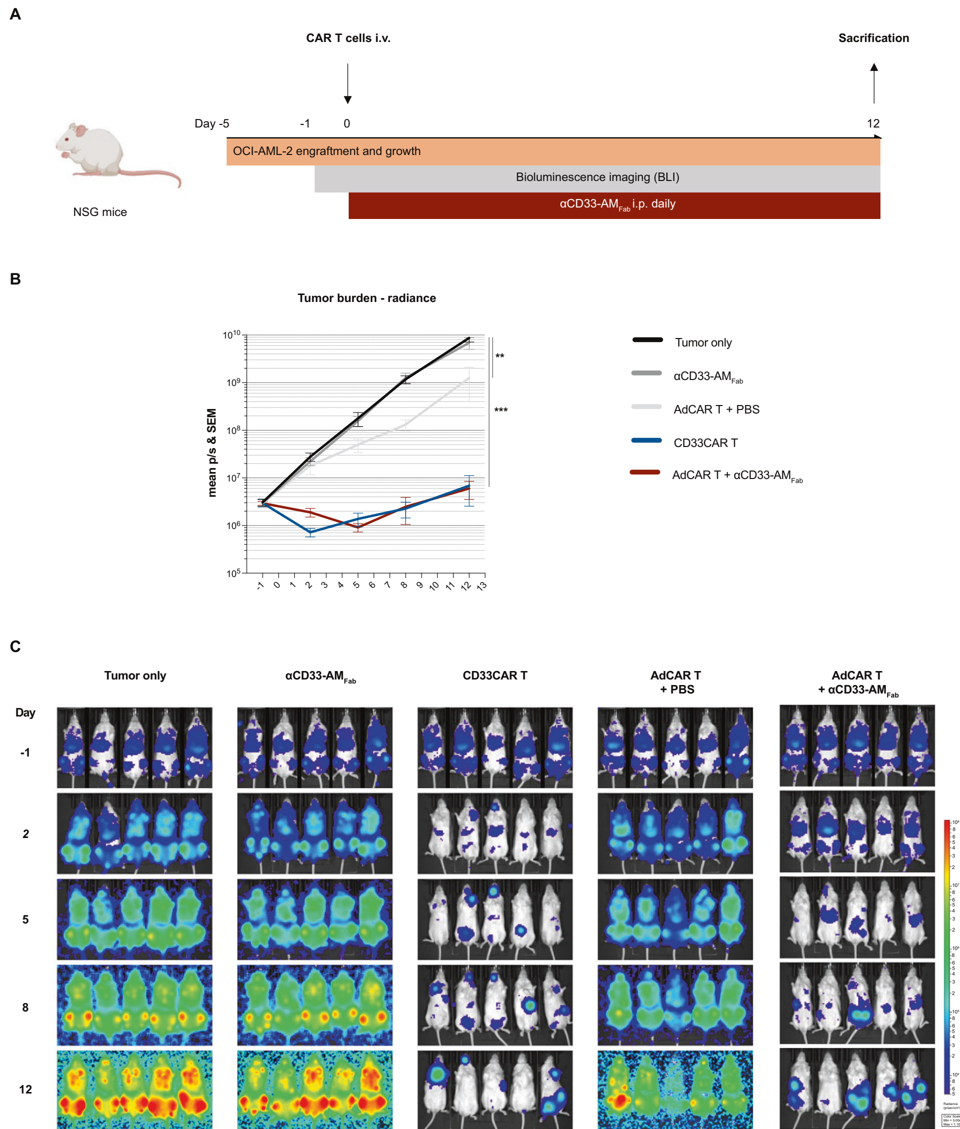
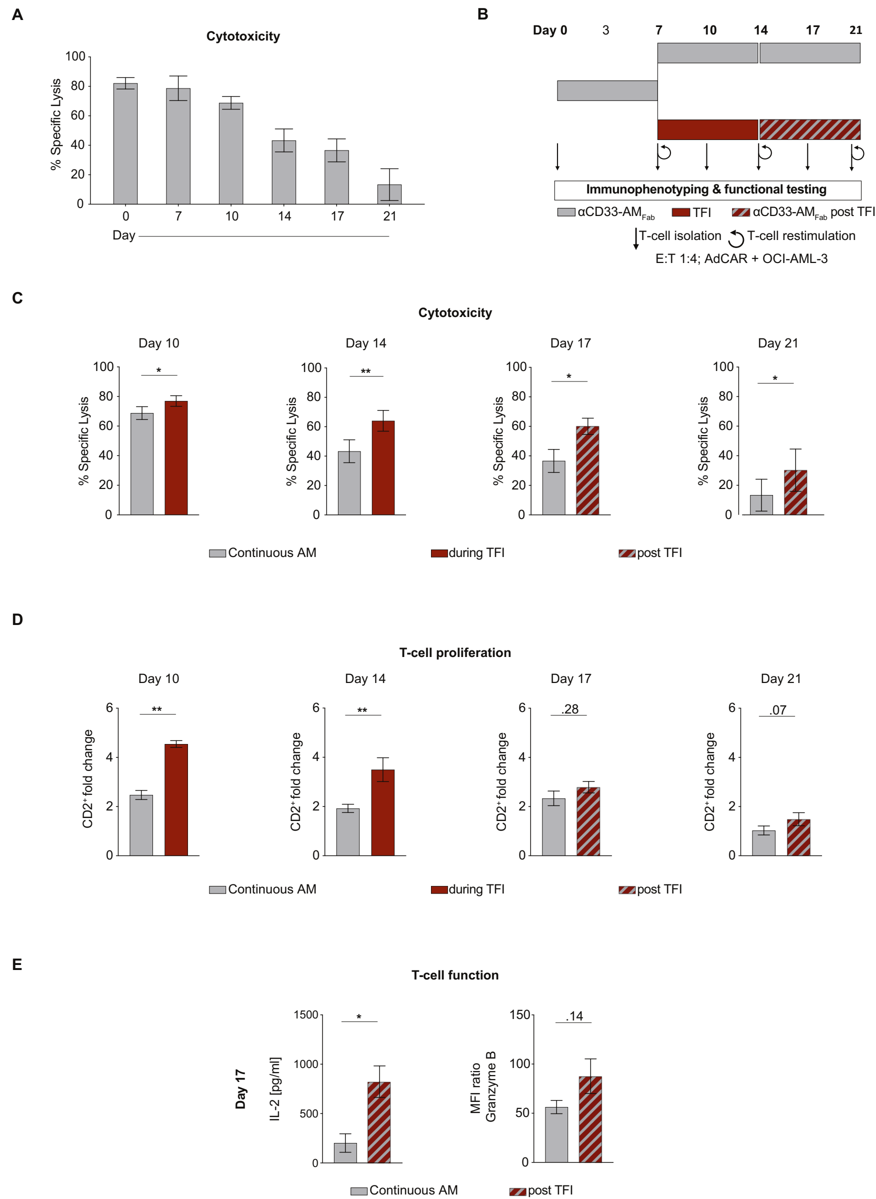


Fig. 4 Fab molecules efficiently activate AdCAR T cells in vivo. A Schematic representation of the in vivo experimental timeline: NSG mice were inoculated on day −5 with luciferase-expressing OCI-AML-2 tumor cells followed by injection of AdCAR/CAR T cells on day 0. A second-generation conventional CD33CAR T-cell construct served as control. AdCAR/CAR T-cell functionality was assessed regularly by BLI of OCI-AML-2 cells. **B** In vivo BLI of OCI-AML-2 cells. **C** Bioluminescence images ($n = 5$ mice per group). Data are plotted as mean ± SEM. Statistical analysis: Ordinary one-way ANOVA with Tukey's comparison; ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

model for 21 days. In both conditions, AdCAR T cells were co-cultured with OCI-AML-3 target cells in the presence of αCD33-AM_{Fab} for 7 days. Then, AdCAR T cells were either exposed for another 7 days to AMs and OCI-AML-3 cells or cultured in the absence of AMs. All cultures were then treated for a further 7 days with αCD33-AM_{Fab} (Fig. 5B). AdCAR T cells were again isolated on days 0, 7, 10, 14, 17, and 21, and their cytotoxicity was assayed in co-cultures.



We observed significantly improved AdCAR T-cell-mediated cytotoxicity against OCI-AML-3 cells during and after the TFI (specific lysis ± SEM: day 14, CONT vs TFI = 43% vs 64%; day 17, CONT vs post TFI = 36% vs 60%; Fig. 5C). In addition, AdCAR T cells intermittently exposed to AMs demonstrated greater proliferation, IL-2 secretion, granzyme B production, and increased expression of PD1 and LAG-3 (Fig. 5D, E and Supplementary Fig. S4A). The AdCAR receptor was not differentially expressed between the two treatment modes (Supplementary Fig. S4B). Whereas T-cell subset analysis revealed a shift towards an effector memory subtype

Fig. 5 Treatment-free intervals prolong AdCAR T-cell function in vitro. **A** AdCAR T cells were continuously stimulated for 21 days with 10 ng/ml αCD33-AM_{Fab} in the presence of irradiated OCI-AML-3 cells (E:T = 1:4; $n = 3$–12). OCI-AML-3 cells and AMs were replenished every third day. AdCAR T cells were isolated at the indicated days and cytotoxicity against OCI-AML-3 cells (E:T = 1:1) after 72 h was assessed by flow-cytometry. **B** Timeline and overview of the continuous and intermittent stimulation of AdCAR T cells co-cultured with OCI-AML-3 cells over 21 days. **C** Cytotoxicity of AdCAR T cells isolated from co-cultures against OCI-AML-3 cells at the indicated days ($n = 3$–12; E:T = 1:1; 10 ng/ml αCD33-AM_{Fab}). **D** T-cell proliferation expressed as fold change in $CD2^+$ cells compared to conditions without AM ($n = 5$–12). **E** IL-2 secretion determined by CBA analysis of co-culture supernatants on day 17 ($n = 6$) and granzyme B expression of $CD8^+$ AdCAR T cells isolated on day 17 and transferred to 72 h cytotoxicity assays ($n = 5$). Data are presented as mean ± SEM. Statistical analysis: paired t-test; ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

during CONT AM stimulation (Supplementary Fig. S4C), the resting period had no effect on this, indicating that the functional improvement is not driven by only a subpopulation of AdCAR T cells.

In summary, we showed that continuous AdCAR T-cell stimulation leads to a decrease in effector functions, which can be abrogated by the use of TFIs.

Treatment-free intervals lead to transcriptional reprograming of AdCAR T cells

To better understand how functional superiority is established, we performed bulk RNA sequencing on isolated AdCAR T cells from CONT- and TFI-treated cells at days 0, 14, and 21 from co-cultures from three individual donors. An analysis of differentially expressed genes (DEGs) of day 14 AdCAR T cells identified 115 significantly upregulated and 219 downregulated genes under TFI conditions versus CONT ($p < 0.01$; Fig. 6A). Unsupervised clustering showed markedly different gene expression patterns under the two treatment modes, indicating transcriptional reprograming. Most importantly, genes related to activation (IL2RA, CD70, LAG3) and cell cycle (CDK1, GMMN, E2F1, CDC45) were downregulated in day 14 TFI-treated AdCAR T cells compared to CONT-stimulated cells, consistent with functional rest (Fig. 6A, B). Interestingly, these genes remained downregulated in day 21 CONT-stimulated AdCAR T cells, indicating a progressive loss of cellular activity due to sustained antigen stimulation. In contrast, other genes related to T-cell activation (CD69, CD44, CD45, Jak1) were upregulated on day 14 TFI-treated relative to CONT-treated AdCAR T cells, pointing towards a better effector function. Pathway comparison of day 14 TFI- and CONT-treated AdCAR T cells was consistent with downregulation of cell cycle (E2F targets, normalized enrichment score, NES = − 3.35; G2M checkpoint, NES = − 3.21; MYC targets V1, NES = − 2.74; mitotic spindle, NES = − 2.25; $p < 0.05$) and metabolism-associated genes (OXPHOS, NES = − 2.08; glycolysis, NES = − 2.04; $p < 0.05$), highlighting AdCAR T-cell quiescence during TFIs (Fig. 6C). Compared to a model of chronic LCM virus infection [15], gene set enrichment analysis (GSEA) revealed a shift towards memory-related from effector-related genes in day 14 TFI-treated AdCAR T cells (Fig. 6D; GSE9650, NES = − 2.41, false-discovery rate $q = 0.0$).

These data imply that day 14 TFI-treated AdCAR T cells undergo rejuvenation through transcriptional reprograming. Convincingly, although we observed progressive downregulation of cell cycle (CDK1) or activation markers (IL2RA) in CONT-stimulated AdCAR T cells, resting periods led to a re-expression of these markers on day 21 TFI-treated cells (Fig. 6E). Overall, genes and pathways downregulated during day 14 TFI were upregulated again at day 21 (and vice versa; Supplementary Fig. S5A–C). GSEA showed that in contrast to day 14, effector-related versus memory-related genes were enriched at day 21 (Supplementary Fig. S5D). Collectively, these data suggest that day 21 TFI-treated AdCAR T cells are more functional than CONT-treated cells and have a greater potential for being re-activated upon αCD33-AM_{Fab} re-exposure. Notably, the re-expression of cell-cycle-related genes in day 21 TFI-treated AdCAR T cells did not reach the level on day 0, indicating that T-cell dysfunction cannot be completely reversed.

DISCUSSION

Translating the success of CD19-directed CAR T cells in B-cell neoplasms to myeloid malignancies, in particular AML, remains challenging. The lack of leukemia-restricted antigens in the myeloid compartment has hampered the advancement of T-cell-recruiting strategies, including BsAbs and CAR T cells, in AML. In addition, inter- and intra-patient heterogeneity at the genetic and protein levels renders this disease more challenging to treat using novel immunotherapeutic strategies [24]. To overcome these barriers, more individualized and safer treatment regimens are necessary.

We therefore optimized our recently developed AdCAR T-cell technology [20] for the treatment of AML. Using Fab- and Ab-based AMs against three different AML target antigens (CD33, CD123, and CLL-1), we demonstrate the specificity and efficacy of AdCAR T cells against AML cell lines and pAML samples in vitro and in vivo.

Although efficacy was already achieved in clinical trials using CAR T cells directed against CD123 or combinations of CD33/CLL-1, these approaches will only benefit a minority of patients owing to their application as a bridge to transplant for avoiding profound on-target/off-leukemia activities [6, 7]. To capitalize on the flexibility of our AdCAR T-cell platform, we demonstrated for the first time here, in an ex vivo long-term pAML model, that sequential use of AMs with different target specificity is feasible. AdCAR T cells could be readily re-targeted against CD33, CD123 or CLL-1 and activated in an AM dose-dependent manner. Notably, we cannot rule out that the observed functional benefits of eliminating pAML cells by AdCAR T cells after AM switching were impacted by the intrinsic differences of the AMs, rather than resulting from targeting antigen-escaped tumor cells.

In the context of B-cell malignancies, the mechanisms of CD19 escape variants have been well described [25]. Therefore, it appears likely that antigen escape variants will also occur in AML after single-antigen targeting, either due to pre-existing target antigen dim/neg clones or adaptive escape mechanisms. Although the sequential administration of CD19-, CD20-, or CD22-directed CAR T cells has proven efficacy [26–28], these approaches require manufacturing of multiple CAR T-cell products, emphasizing the need for flexible targeting of multiple antigens to overcome the evolution of escape variants that besets the use of a single CAR T-cell platform.

Furthermore, the majority of current mono- or dual-targeting CAR T constructs designed for AML do not integrate safety switches. Therefore, these constructs come with the inherent risk of potentially life-threatening cytokine release syndrome (CRS), ICANS and severe hematotoxicity. In fact, high-grade CRS and myeloablation have been observed in a number of pilot CAR T-cell studies targeting AML and accordingly, are commonly used as a bridge to transplant strategy [7, 29]. Hematotoxicity has also been reported utilizing CD33 or CD123 BsAb [30, 31], albeit our knowledge on long-term impact is still limited. Hence, we expect our AdCAR T cells to also mediate target-antigen dependent hematotoxicity, however, in contrast to conventional CAR T constructs, the AdCAR platform allows limited exposure and thereby a possibly beneficial safety profile.

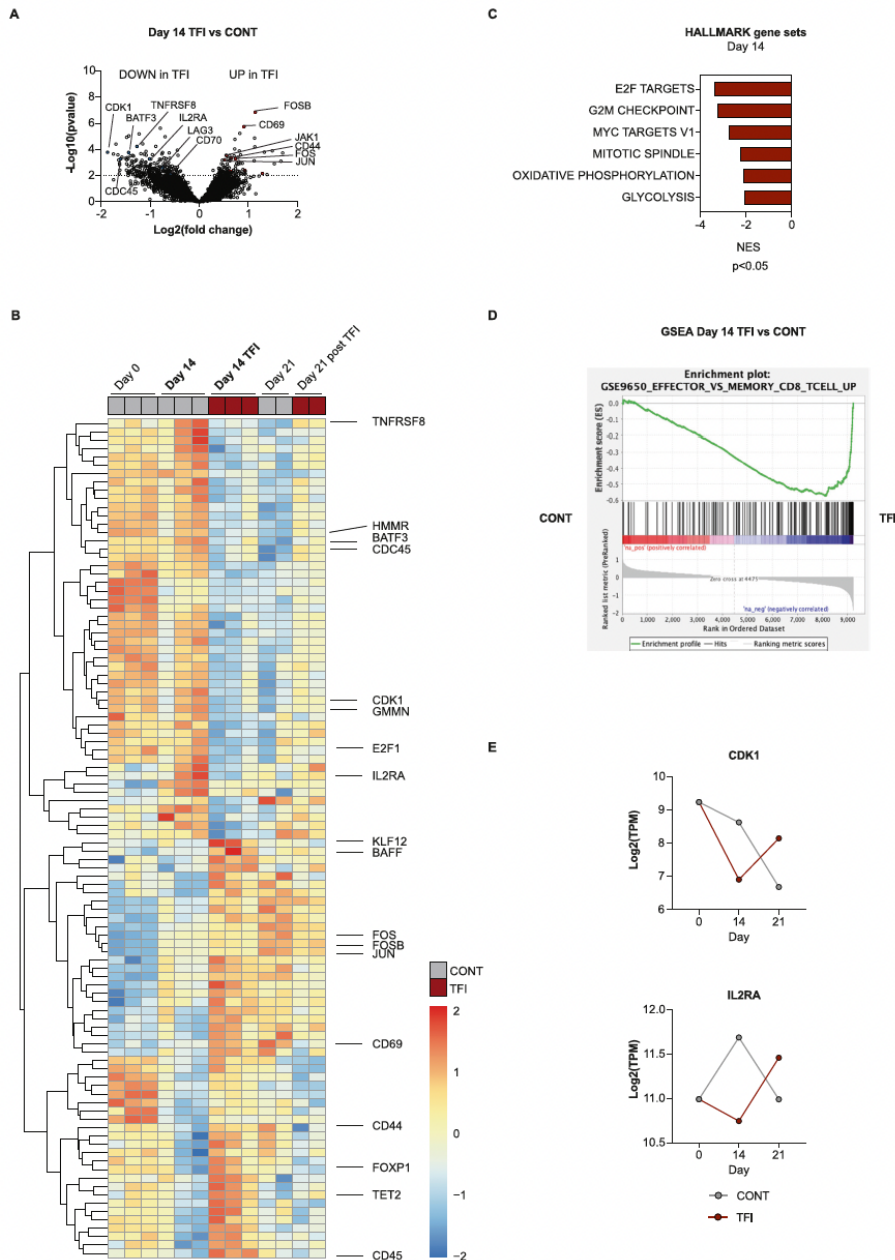


Fig. 6 Treatment-free intervals lead to transcriptional reprograming of AdCAR T cells. **A** Volcano plot of DEGs in day 14 TFI-treated versus CONT-treated AdCAR T cells; $p < 0.01$. Selected genes are highlighted in blue (downregulated) or red (upregulated). **B** Heatmap with hierarchical clustering of the top 100 DEGs in day 14 TFI-treated versus CONT-treated AdCAR T cells; $p < 0.01$. Selected genes are indicated. **C** Hallmark gene set analysis of day 14 TFI-treated versus CONT-treated AdCAR T cells; $p < 0.05$. **D** GSEA of day 14 TFI-treated versus CONT-treated AdCAR T cells using MSigDB and the gene set GSE9650_EFFECTOR_VS_MEMORY_CD8_TCELL_UP [15]. **E** Log_2(TPM) values of CDK1 and IL2RA over time for TFI-treated and CONT-treated AdCAR T cells. DEG = differentially expressed gene; NES = normalized enrichment score; GSEA = gene set enrichment analysis.

The AdCAR T-cell technology relies on AMs of low molecular weight (Fab molecules), which, in contrast to Ab-based AM formats, have substantially shorter half-lives (1–2 h vs days to weeks) [32, 33]. Interestingly, a recent report highlighted the possibility for preventing or arresting CRS in an adenocarcinoma mouse model using low-molecular-weight AMs [34]. Consistent with that, we readily observed cessation of AdCAR T-cell activation in pAML co-culture assays when αCD33-AM_{Fab} addition was stopped, suggesting similar efficacy of our platform in controlling therapy-related toxicity. Future studies will need to address, if intermittent application of AdCAR T cells will allow restoration of healthy hematopoiesis within the treatment free intervals.

In addition to stability and renal clearance of the AM [35], we identified receptor-mediated endocytosis as another variable that determines bioactivity and serum half-lives. As the majority of AML-associated target antigens are myeloid lineage antigens, the binding of which leads to internalization, we studied this mechanism as a potential antigen sink that affects AM pharmacokinetics. As hypothesized, receptor-mediated endocytosis led to AM depletion, thereby influencing AdCAR T-cell potency. While on a systemic level the elimination is likely governed by renal clearence of these small molecular weight AMs, we think that AM internalization on a cellular level contributes to the controlled activation/termination of AdCAR T cells. We believe this to be a broadly applicable mechanism in T-cell-based immunotherapy of myeloid malignancies, that potentially applies to BsAbs as well as AdCAR T cells. In fact, antigen sink effects might have contributed to the need for much higher doses of the CD33-targeting BsAb AMG330 compared to blinatumomab in a recent phase I trial [24]. Future AM dosing regimens should therefore take this effect into account.

Another emerging factor for the failure of T-cell-based immunotherapies is T-cell dysfunction due to chronic antigen exposure [15, 16] or tonic CAR T-cell signaling [18, 19]. In a study by Weber et al., [19], transient rest was shown to reinvigorate exhausted, tonically signaling GD2-CAR T cells through epigenetic remodeling. Importantly, we recently identified similar features for BsAbs [22], indicating that T-cell dysfunction can be induced through different signaling pathways. Based on these observations, we hypothesized that AdCAR T cells follow the same principles. However, the short half-lives of the AMs provide the AdCAR T cells an on–off switch, an inherent advantage over conventional CAR T cells that makes implementation of resting periods through TFIs possible (comparable to intermittent BsAb treatment).

Indeed, in an AML-optimized long-term culture system recapitulating 21 days of continuous AdCAR T-cell stimulation, we observed progressive loss of effector functions. Interruption of AM exposure for 7 days resulted in rejuvenation of AdCAR T cells compared to CONT-stimulated cells, which was reflected by restored effector function and transcriptional remodeling.

When we compared AdCAR T cells after intermittent AM exposure to BsAb-activated T cells, also after a TFI [22], we observed differences in the transcriptome. Although puzzling at first, we believe that the two scenarios cannot be compared as AdCAR T cells are stimulated during the initial production protocol. Interestingly, more recent developments in CAR T-cell production have focused on maintaining a more naïve-like phenotype, thereby conserving the original T-cell subset composition at the time of leukapheresis. In that sense, our AdCAR T cells rather resemble conventional CAR T cells, suggesting that optimized manufacturing protocols will likely influence the efficacy of AdCAR T-cell rejuvenation by TFIs [8, 36, 37].

In summary, we established a highly potent and flexible AdCAR T-cell platform for T-cell-based immunotherapy in AML. Owing to its modular design and the use of low-molecular-weight adapters, the platform allows targeting of different AML-associated target antigens and controlled T-cell activation. The possibility of intermittent activation counteracts AdCAR T-cell exhaustion. Our data support the use of AdCAR T cells in an early clinical trial for patients with relapsed/refractory AML, and addresses questions that are most likely pertinent to other disease entities.

DATA AVAILABILITY

The RNA-seq data discussed in this publication have been deposited in the GEO database under the accession code GSE221070. The datasets generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

MS, VB, AK, JM, and DN designed the study and supervised the project; DN and MS wrote the manuscript; DN, MSponheimer, FE, UB, DB, SD, and KB, performed the experiments and analyzed and/or interpreted the data; DN, MS, VB, AK, JM, FE, UB, DB, MK, NP, LR, DA, CMS, AL, MvB, and HL were involved in designing the experiments and interpreting the data. LW performed the library preparation for bulk RNA-Seq and, in collaboration with TS, conducted the bioinformatic analysis; FE and UB designed and performed the in vivo experiments with the assistance of DN. All authors contributed to the preparation of the manuscript and approved the submitted version.

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MS receives industry research support from Amgen, Bristol-Myers Squibb/Celgene, Gilead, Janssen, Miltenyi Biotec, Morphosys, Novartis, Roche, Seattle Genetics, and Takeda, and serves as a consultant/advisor to AvenCell, CDR-Life, Ichnos Sciences, Incyte Biosciences, Janssen, Molecular Partners, and Takeda. She serves on the speakers' bureau at Amgen, AstraZeneca, BMS/Celgene, Gilead, GSK, Janssen, Novartis, Pfizer, Roche, and Takeda. VB has received research funding from Miltenyi Biotec, Novartis, and Pfizer, and has served as a consultant/advisor to Novartis, Amgen, and Gilead. He serves on the speakers' bureau at Novartis and Pfizer. AK (now employed by BioNTech SE) and JM (employed by Miltenyi Biotec) hold a patent on the AdCAR platform. CMS holds a patent on the AdCAR platform and has received research funding from Miltenyi Biotec. FE, UB, and SD are employed by Miltenyi Biotec. MvB has received research support from and serves on the speakers' bureau at Gilead, Miltenyi Biotec, MSD Sharpe & Dohme, Roche, Mologen, Novartis, Astellas, and BMS. HL is co-founder and shareholder of Tubulis GmbH. DN, MSponheimer, DB, MK, TS, NP, LR, DA, KB (now employed by Bristol-Myers Squibb), and AL declare no relevant conflicts of interest.

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4. Paper II

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

T-cell exhaustion induced by continuous bispecific molecule exposure is ameliorated by treatment-free intervals

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KEY POINTS

- **Continuous exposure to a CD19xCD3 bispecific molecule induces T-cell exhaustion.**
- **Treatment-free intervals transcriptionally reprogram and functionally reinvigorate T cells.**

T-cell–recruiting bispecific molecule therapy has yielded promising results in patients with hematologic malignancies; however, resistance and subsequent relapse remains a major challenge. T-cell exhaustion induced by persistent antigen stimulation or tonic receptor signaling has been reported to compromise outcomes of T-cell–based immunotherapies. The impact of continuous exposure to bispecifics on T-cell function, however, remains poorly understood. In relapsed/refractory B-cell precursor acute lymphoblastic leukemia patients, 28-day continuous infusion with the CD19xCD3 bispecific molecule blinatumomab led to declining T-cell function. In an in vitro model system, mimicking 28-day continuous infusion with the half-life–extended CD19xCD3 bispecific AMG 562, we identified hallmark features of exhaustion arising over time. Continuous AMG 562 exposure induced progressive loss of T-cell function (day 7 vs day 28 mean specific lysis: 88.4% vs 8.6%; n = 6; *P* = .0003). Treatment-free intervals (TFIs), achieved by AMG 562 withdrawal, were identified as a powerful strategy for counteracting exhaustion. TFIs induced strong functional reinvigoration of T cells (continuous vs TFI-specific lysis on day 14: 34.9% vs 93.4%; n = 6; *P* < .0001) and transcriptional reprogramming. Furthermore, use of a TFI led to improved T-cell expansion and tumor control in vivo. Our data demonstrate the relevance of T-cell exhaustion in bispecific antibody therapy and highlight that T cells can be functionally and transcriptionally rejuvenated with TFIs. In view of the growing number of bispecific molecules being evaluated in clinical trials, our findings emphasize the need to consider and evaluate TFIs in application schedules to improve clinical outcomes.

Introduction

T-cell–recruiting bispecific antibodies comprise a novel immunotherapeutic platform for the treatment of hematological malignancies and are currently being investigated in clinical trials.[1] The CD20xCD3 bispecifics glofitamab[2,3] and mosunetuzumab[4] have shown encouraging results in phase-I trials in patients with indolent or aggressive lymphomas. In patients with acute myeloid leukemia (AML), the CD123xCD3 molecule flotetuzumab demonstrated promising response rates.[5] To date, the only FDA-approved bispecific molecule is the CD19xCD3 molecule blinatumomab for treatment of relapsed/refractory (r/r) and minimal residual disease-positive (MRD$^+$) B-cell precursor acute lymphoblastic leukemia (BCP-ALL).[6-9]

Bispecific antibodies are administered using varying application schedules (continuous to weekly infusions) to achieve serum levels that support efficient T-cell recruitment. Owing to their short circulation half-lives, both flotetuzumab and blinatumomab are administered to eligible patients by 28-day continuous intravenous infusion (c.i.v.) during the first cycle.[5,9] Rates of response to blinatumomab in clinical trials were significantly higher compared with standard-of-care chemotherapy, with 43% in r/r and 81% in MRD$^+$ patients.[9,10] These results are encouraging; however, they also highlight that a considerable portion of patients remain refractory to therapy. To improve the response to bispecific antibodies, a better understanding of resistance mechanisms is urgently needed.

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First clinical evidence that highly functional T cells are required for successful bispecific molecule therapy was provided by a phase-II follow-up analysis of r/r BCP-ALL patients treated with blinatumomab. Response to blinatumomab positively correlated with $CD3^+$ T-cell expansion,[11] whereas the frequency of regulatory T cells prior to therapy negatively correlated to response.[12] Furthermore, enrichment of exhausted T cells was reported in r/r BCP-ALL patients unresponsive to blinatumomab.[13]

T-cell exhaustion has been described in chronic viral infection in mice,[14,15] humans,[16,17] and, more recently, in cancer.[18-20] Upon continuous antigen exposure, T cells coexpress inhibitory checkpoint molecules including programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin domain 3 (Tim-3), and lymphocyte activation gene 3 (LAG-3). Concomitantly, T cells enter a state of hypo-responsiveness, characterized by gradual loss of functions such as cytokine secretion, proliferation, and cytotoxicity.[16,21-23] Translating this concept into the clinical setting of bispecific therapies, we hypothesized that T-cell exhaustion occurs during continuous exposure to bispecific antibodies and contributes to resistance to therapy.

We therefore investigated the relevance of continuous long-term bispecific molecule stimulation on T-cell function and exhaustion in an in vitro model system using a CD19xCD3 half-life–extended bispecific molecule (AMG 562) for proof of concept. We demonstrate that continuous stimulation with bispecifics over 28 days induces T-cell exhaustion and we provide evidence that disruption of this stimulation using treatment-free intervals (TFIs) maintains high T-cell functionality and induces transcriptional reprogramming. Finally, we report improved antileukemic efficacy of TFI-stimulated vs continuously stimulated T cells in a patient-derived xenograft (PDX) ALL mouse model.

Methods

Assessment of ex vivo T-cell function of r/r BCP-ALL patients

T cells were isolated from peripheral blood mononuclear cells using the Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and cocultured with the $CD19^+$ ALL cell line REH in culture medium (supplemental Table 1 and supplemental Materials and Methods, available on the *Blood* Web site) at an effector/target ratio (E:T) of 1:3 and 0.5 ng/mL blinatumomab or control construct. After 72 hours, cells were stained with AquaLiveDead and with antibodies against CD2, CD4, CD8, and CD19. Blinatumomab-mediated lysis of REH cells was determined using flow cytometry as described in Equation 1. On day 6, IFN-γ secretion was analyzed in supernatants via cytometric bead array (CBA):

$$\% \text{ specific lysis} = \left(1 - \frac{CD19^+ \text{ target cell count blinatumomab}}{CD19^+ \text{ target cell count control construct}}\right) \times 100. \tag{1}$$

28-day stimulation of healthy donor T cells with AMG 562

Healthy donor (HD) T cells were isolated from peripheral blood mononuclear cells using the Human T Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada) and cocultured with irradiated OCI-Ly1 cells in culture medium (E:T = 1:4) containing 5 ng/mL AMG 562. On day 3, culture medium, target cells, and AMG 562 were replenished. On day 7, T cells were isolated from cultures (stimulation cycle 1) and functionally tested, as detailed below. Remaining T cells were recultured with OCI-Ly1 cells and AMG 562, as described above (stimulation cycle 2). The T cells underwent 4 stimulation cycles. For experiments implementing TFIs, T cells were cocultured with OCI-Ly1 cells in absence of AMG 562 during stimulation cycles 2 (days 7-14) and 4 (days 21-28).

Immunophenotyping of T cells

On days 3 and 7 of each stimulation cycle, cultures were stained with AquaLiveDead and antibodies against CD2, CD4, CD8, T-cell receptor αβ (TCRαβ), PD-1, Tim-3, and LAG-3. Additionally, cells were intranuclearly stained for the transcription factor TOX. Corresponding isotype controls were used. Median fluorescence intensity (MFI) ratios were calculated with Equation 2:

$$\text{MFI ratio} = \frac{\text{MFI stained sample}}{\text{MFI isotype control}}. \tag{2}$$

Quantification of cytokine secretion

Cytokine levels in supernatants were measured using CBA. In some experiments, isolated T cells were restimulated with phorbol myristate acetate (PMA; 20 ng/mL) and ionomycin (750 ng/mL) in the presence of GolgiStop/GolgiPlug solution containing monensin (25 nM) and brefeldin A (10 ng/mL; all Sigma-Aldrich, St. Louis, MO) for 4 hours at 37°C with 5% CO_2. Subsequently, cells were stained with AquaLiveDead and antibodies against CD2, CD4, and CD8, then permeabilized and stained intracellularly with antibodies against IFN-γ and TNF-α or corresponding isotype controls.

Proliferation and cytotoxicity assays

T cells were cocultured with hCD19-Ba/F3 or OCI-Ly1 cells (E:T = 1:1) and 5 ng/mL AMG 562 or control construct. After 72 hours, cells were stained with AquaLiveDead and antibodies against CD2, CD4, CD8, and CD19. AMG 562-mediated lysis of $CD19^+$ target cells and T-cell proliferation was calculated as described in Equations 1 and 3:

$$\text{Fold change} = \frac{CD2^+ \text{ cell count day3}}{CD2^+ \text{ cell count day0}}. \tag{3}$$

Cytotoxicity assays against OCI-Ly1 cells were incubated for 4 hours with GolgiStop/GolgiPlug solution followed by staining with AquaLiveDead and antibodies against CD2, CD4, CD8, and CD19. Then, cells were permeabilized and stained intracellularly for granzyme B or isotype control.

Metabolic stress tests

T cells were stimulated for 48 hours with CD3/CD28 Dynabeads (Thermo Fisher Scientific, Waltham, MA). After bead depletion, 2.5×10^5 T cells/well were plated on a poly-D-lysine-coated 96-well plate. Mitochondrial and glycolysis stress tests were performed on a Seahorse XFe96 Analyzer using corresponding kits (Agilent, Santa Clara, CA). Metabolic rate was normalized to cell count using a Cytation 1 reader (BioTek Instruments, Inc., Winooski, VT).

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Bulk RNA sequencing

5×10^5 viable $CD3^+$ T cells were sorted and lysed in Trizol (Sigma-Aldrich). RNA was isolated using the RNA Clean and Concentrator-25 Kit (Zymo Research, Freiburg, Germany). Libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs, Frankfurt, Germany).

In vivo studies

NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice were engrafted with PDX-ALL-265 cells expressing enhanced firefly luciferase and GFP. On day 28, mice received human T cells stimulated in vitro with AMG 562 for 14 days either continuously or with a TFI from day 7 to 14. Subsequently, mice were treated weekly with AMG 562 or control construct (cBiTE). Leukemia burden was monitored twice weekly by bioluminescence imaging.[24,25] The study protocol was reviewed and approved by local government authorities.

Statistical analysis

Prism 8 v.8.4.3. (471) (GraphPad Software, LLC) was used for data visualization and statistical analysis. The Mann-Whitney test, the Kruskal-Wallis and Dunn's multiple comparisons test, or two-way ANOVA and Sidak's multiple comparisons test was used for statistical testing, as specified in the respective figure legends. Not significant (ns) $P > .05$; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P \leq 0.0001$.

Results

T cells of r/r ALL patients receiving blinatumomab show signs of dysfunction

We hypothesized that continuous exposure to bispecific molecules leads to T-cell exhaustion in patients. Hence, we tested ex vivo T-cell function of r/r BCP-ALL patients that received blinatumomab c.i.v. over 28 days (Table 1). Patient T cells showed high cytotoxicity and IFN-γ secretion prior to blinatumomab treatment. However, both significantly decreased ex vivo during blinatumomab c.i.v. (Figure 1A, mean specific lysis: prior = 73.1% vs during = 31.5%; $P = .0052$). Longitudinal analysis of T-cell function during the first blinatumomab cycle revealed a significant decline in ex vivo cytotoxicity and IFN-γ secretion within the first 2 weeks of blinatumomab c.i.v. Interestingly, T-cell function increased upon cessation of blinatumomab therapy (Figure 1B, mean specific lysis: day 0 = 73.1% vs day 14 = 17.4%; $P = .0176$; day 0 vs post = 48.5%; ns). These observations support the hypothesis that continuous exposure with bispecifics may lead to impaired T-cell function in patients.

Establishment of a continuous T-cell stimulation system in vitro with the CD19xCD3 bispecific AMG 562

Next, we aimed to translate the clinical setting into a stable, reproducible in vitro model system. We therefore established long-term T-cell stimulation with the CD19xCD3 bispecific molecule AMG 562 over 28 days. The diffuse large B-cell lymphoma cell line OCI-Ly1 was identified as suitable $CD19^+$ target cell line with slow AMG 562-mediated lysis kinetics to ensure the continued presence of the target antigen for at least 3 days (Figure 1C; mean specific lysis: SEM cells = 91.0% vs OCI-Ly1 cells = 20.8%; $P = .0073$). OCI-Ly1 cells express high levels of CD19, and HD T cells in coculture with OCI-Ly1 showed robust AMG 562-mediated activation and proliferation after 72 hours (Figure 1D-F; supplemental Figure 1B-C). Furthermore, costimulatory and coinhibitory molecule expression in OCI-Ly1 was comparable to primary ALL samples (n = 20, Figure 1G). The following data are based on 28-day cocultures with HD T cells and OCI-Ly1 cells in the presence of AMG 562, unless otherwise indicated (Figure 2A).

Continuous T-cell stimulation with AMG 562 over 28 days induces T-cell exhaustion in vitro

Immunophenotyping during AMG 562 stimulation showed that only a minor fraction of T cells coexpressed the inhibitory receptors (IRs) PD-1, Tim-3, and LAG-3 at baseline. However, after 7 days of AMG 562 stimulation, more than 60% of $CD4^+$ and $CD8^+$ T cells coexpressed 3 IRs. Coexpression was maintained until day 28 of continuous stimulation (Figure 2B; supplemental Figure 2A-B; mean % $CD8^+$ T cells: day 0 = 0.05% vs day 7 = 64.0%; $P = .004$; day 0 vs day 28 = 42.7%). Coexpression of IRs was accompanied by gradual upregulation of the transcription factor TOX (Figure 2B; supplemental Figure 2A-B; mean MFI ratio of $CD8^+$ T cells: day 0 = 4.2 vs day 28 = 12; $P = .0001$). Importantly, TOX upregulation was accompanied by progressive T-cell dysfunction. Comprehensive functional analysis revealed T-cell dysfunction starting approximately 2 weeks after AMG 562 stimulation commenced. Cytokine secretion into culture supernatants decreased significantly over the course of AMG 562 stimulation (Figure 2C; supplemental Figure 2E, mean IL-2 secretion: day 3 = 16 000 pg/mL vs day 24 = 75 pg/mL; $P < .0001$). We also observed that coexpression of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells upon restimulation with PMA/ionomycin declined over time (Figure 2D, IFN-γ^+TNF-α^+ mean of $CD8^+$: day 0 = 19.6%, day 7 = 23.1%, day 21 = 9.9%). Furthermore, AMG 562-mediated T-cell proliferation and cytotoxicity against hCD19-Ba/F3 cells were highest with T cells extracted from the coculture on day 7, but significantly decreased with further AMG 562 stimulation (Figure 2E; $CD2^+$ fold change after 3 days of assay: day 7 = 2.1 vs day 28 = 0.05; $P < .0001$; mean specific lysis: day 7 = 88.4% vs day 28 = 8.6%; $P = .0003$). Similar results were observed in cytotoxicity assays against OCI-Ly1 cells (Figure 2F; supplemental Figure S2C). Importantly, T-cell exhaustion occurred upon continuous BsAb stimulation using different target cell lines and BsAbs (supplemental Figure 2F-G). Together, we found that continuous stimulation with AMG 562 for up to 28 days induced T-cell exhaustion.

TFIs reinvigorate T-cell function in vitro

We then hypothesized that disruption of continuous antigen exposure might allow the functional recovery of T cells and to ameliorate exhaustion. We therefore implemented resting periods (the aforementioned TFIs) between days 7-14 and days 21-28 by removing AMG 562 from cocultures and compared the function of T cells that had undergone TFIs with cells continuously (CONT) stimulated with AMG 562 (Figure 3A). During TFIs, a significantly smaller fraction of $CD4^+$ and $CD8^+$ T cells coexpressed PD-1, Tim-3, and LAG-3 (Figure 3B; supplemental Figure 3A, mean % of $CD8^+$ T cells at day 14: CONT = 57% vs TFI = 21.8%; $P = .013$). This effect was transient and coexpression was reestablished upon restimulation with AMG 562 (Figure 3B; supplemental Figure 3A, mean % of $CD8^+$ T

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Table 1. Characteristics of r/r BCP-ALL patients prior to blinatumomab therapy

Patient	Age	Sex	Ph	Interval ID - blinatumomab (mo)	Prior therapies	Cytoreductive therapy prior to blinatumomab	Start of cytoreductive therapy prior to blinatumomab	Prior allo-SCT	Blast % (BM) prior to blinatumomab	Days sampled under blinatumomab
R1	37	m	–	21	GMALL induction I, II and consolidation I conditioning therapy for allo-SCT: TBI (3×6 Gy), cyclophosphamide	Vin/Dex	day −12	+	54	48
R2	35	m	–	9	GMALL induction I, II; HAM, FLAG	Vin	day −17	–	30 (PB)	14/21
R3	51	m	–	32	GMALL protocol (<55a, complete)	Cy	day −4	–	40	0/7/28
R4	32	m	–	5	GMALL induction I, II	–	–	–	52	1/8/28
R5	30	f	–	32	GMALL protocol (<55a, complete); repeated GMALL induction I	–	–	–	5	1/7/22
R6	70	f	+	18	EWALL elderly (complete, including dasatinib)	Cy/Dex	day −9	–	88	−2/12/28
R7	68	f	–	4	GMALL elderly (induction I, II)	–	–	–	24	0/7/42
R8	32	f	–	11	GMALL induction I, II and consolidation I conditioning therapy for allo-SCT: TBI (3×4 Gy), etoposide	–	–	+	70	0/21
R9	58	m	–	14	GMALL protocol (<55a, until consolidation IV)	FLAG-Ida	day −10	–	60	−1/7/44
R10	34	m	–	22	GMALL induction I, II and consolidation I conditioning therapy for allo-SCT: FLAMSA - TBI (2×4 Gy)	–	–	+	8	−1/7
R11	22	m	–	7	GMALL induction I, II and consolidation I FLAG-Ida	Cy/Dox/Vin	day −8	–	2	8/28/44
R12	20	m	–	46	CoALL protocol (complete); GMALL induction I, II and consolidation I, imatinib	–	–	–	70	0/16/22
R13	72	m	–	5	GMALL elderly (induction I, II, and consolidation I, II)	Cy	day −4	–	39	0/8/15

allo-SCT, allogeneic stem cell transplantation; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; BM, bone marrow; Cy, cyclophosphamide; Dex, dexamethasone; Dox, doxorubicin; EWALL, European Working Group on Adult ALL; f, female; FLAG-Ida, fludarabine, Ara-C, G-CSF–idarubicin; FLAMSA, Fludarabin, Ara-C, Amsacrine; GMALL, German Multicenter Study Group For Adult ALL; ID, initial diagnosis; m, male; mo, month; PB, peripheral blood; Ph, Philadelphia chromosome; r/r, relapsed/refractory; TBI, total body irradiation; Vin, vincristine.

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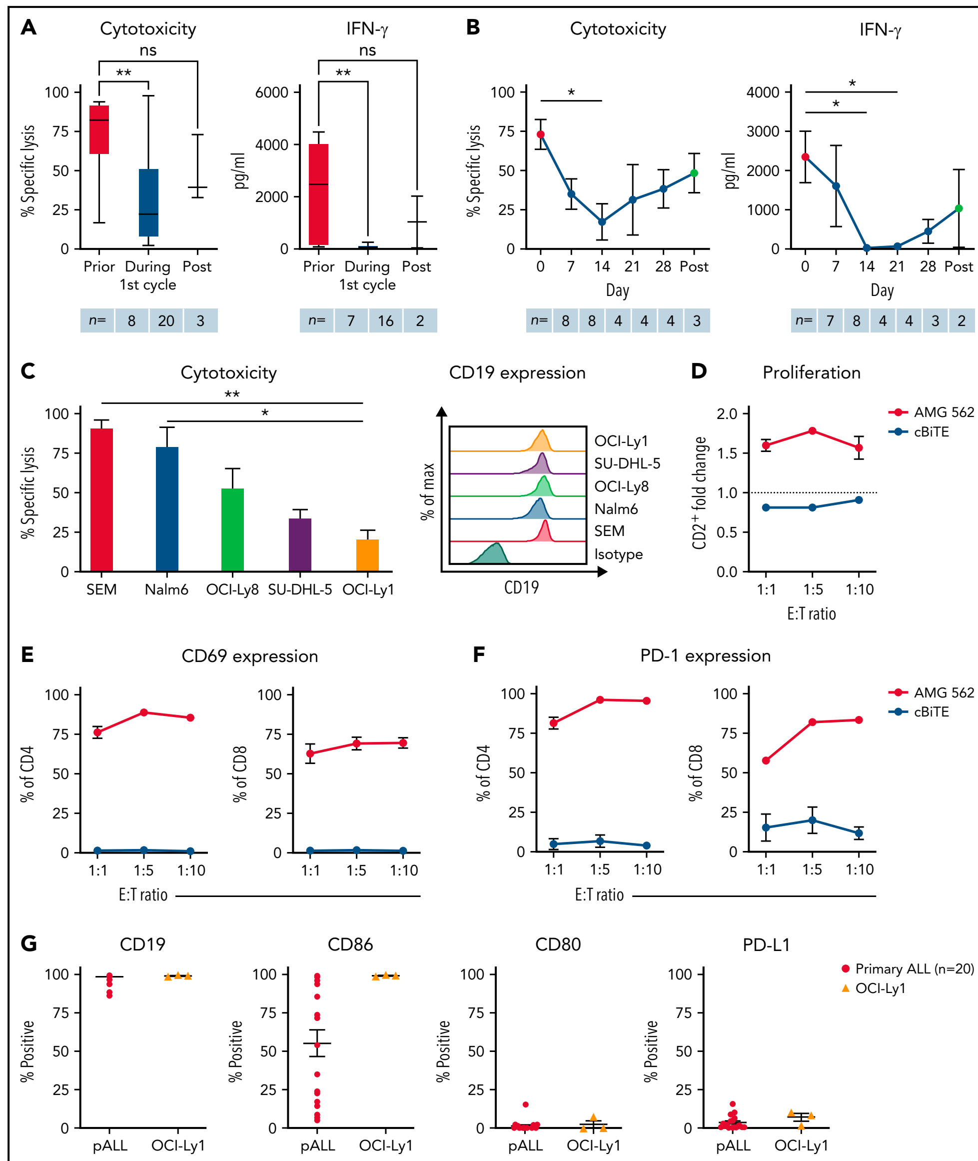

Figure 1. T cells of blinatumomab-treated ALL patients show signs of exhaustion ex vivo. (A) Blinatumomab-mediated cytotoxicity (n = 8–20) on day 3 and IFN-γ secretion (n = 7–16) on day 6 of T cells against REH cells (blinatumomab/control BiTE = 0.5 ng/mL, E:T = 1:3). The T cells were isolated from ALL patients prior to ("pre"), during and after the first cycle of blinatumomab therapy. (B) Blinatumomab-mediated cytotoxicity (n = 3–8) on day 3 and IFN-γ secretion (n = 2–8) on day 6 of T cells against REH cells (blinatumomab/control BiTE = 0.5 ng/mL, E:T = 1:3). The T cells were isolated from ALL patients at different timepoints during the first cycle of blinatumomab therapy. (C) AMG 562-mediated cytotoxicity (n = 3–6) and of HD T cells against ALL (SEM, Nalm6) and diffuse large B cell lymphoma (OCI-Ly8, SU-DHL-5, OCI-Ly1) cell lines (supplemental Table 1) after 4 days (AMG 562/control BiTE = 5 ng/mL, E:T = 1:3). Representative histograms of CD19 expression on ALL and diffuse large B cell lymphoma cell lines are shown. (D) $CD2^+$ fold change (n = 3) of HD T cells and (E-F) percentage of $CD69^+$ and PD-1^+ among $CD4^+$ and $CD8^+$ T cells after 3 days of cytotoxicity assay (AMG 562/control BiTE = 5 ng/mL) against OCI-Ly1 cells; n = 3. (G) Percentage of $CD19^+$, $CD86^+$, $CD80^+$, and PD-$L1^+$ primary ALL (n = 20) and OCI-Ly1 cells (n = 3). Box-plot whiskers indicate minima and maxima, and boxes represent the lower quartile, the median, and the upper quartile. All other graphs present mean ± SEM values. Statistical analysis: Kruskal–Wallis and Dunn's multiple comparison test (A–C); $^{ns}P > .05$; $*P < .05$; $**P < .01$. ALL, acute lymphoblastic leukemia; cBiTE, control BiTE, bispecific control construct; E:T, effector/target ratio; HD, healthy donor; ns, not significant; pALL, primary ALL; ± SEM, standard error of the mean.

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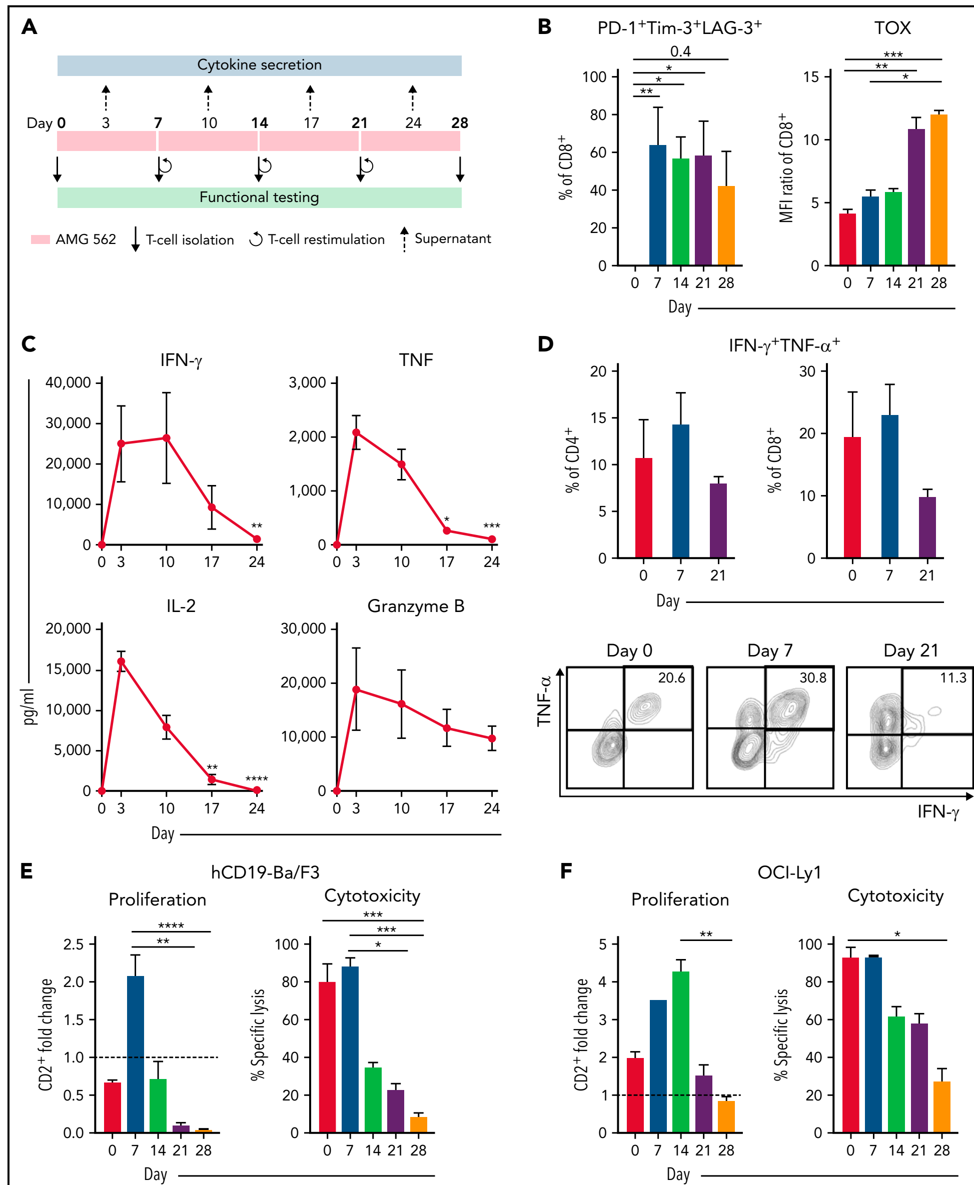

Figure 2. Continuous stimulation with AMG 562 induces T-cell exhaustion. (A) Timeline of continuous T-cell stimulation with AMG 562 and functional testing over 28 days. (B) Percentage of $CD8^+$ T cells coexpressing PD-1, Tim-3, and LAG-3 and the MFI ratio of TOX during continuous AMG 562 stimulation; n = 6. (C) Cytokine and granzyme B levels in coculture supernatants determined by CBA; n = 3–9. Significant differences compared with day 3 are indicated. (D) Percentage of IFN-γ and TNF-α double-positive $CD4^+$ and $CD8^+$ T cells after PMA/ionomycin restimulation. Representative examples of $CD8^+$ T cells from 1 donor are shown; n = 3. (E-F) AMG 562-mediated $CD2^+$ fold change (n = 3) and cytotoxicity against hCD19-Ba/F3 cells (E) or OCI-Ly1 cells (F) after 3 days (AMG 562/cBiTE = 5 ng/mL, E:T = 1:1); n = 6. Data are mean ± SEM values. Statistical analysis: Kruskal–Wallis and Dunn's multiple comparison test (B,C,E,F); $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$. CBA, cytometric bead array; E:T, effector/target ratio; LAG-3, lymphocyte activation gene 3; MFI, median fluorescence intensity; PD-1, programmed cell death protein 1; PMA, phorbol myristate acetate;± SEM, standard error of the mean.

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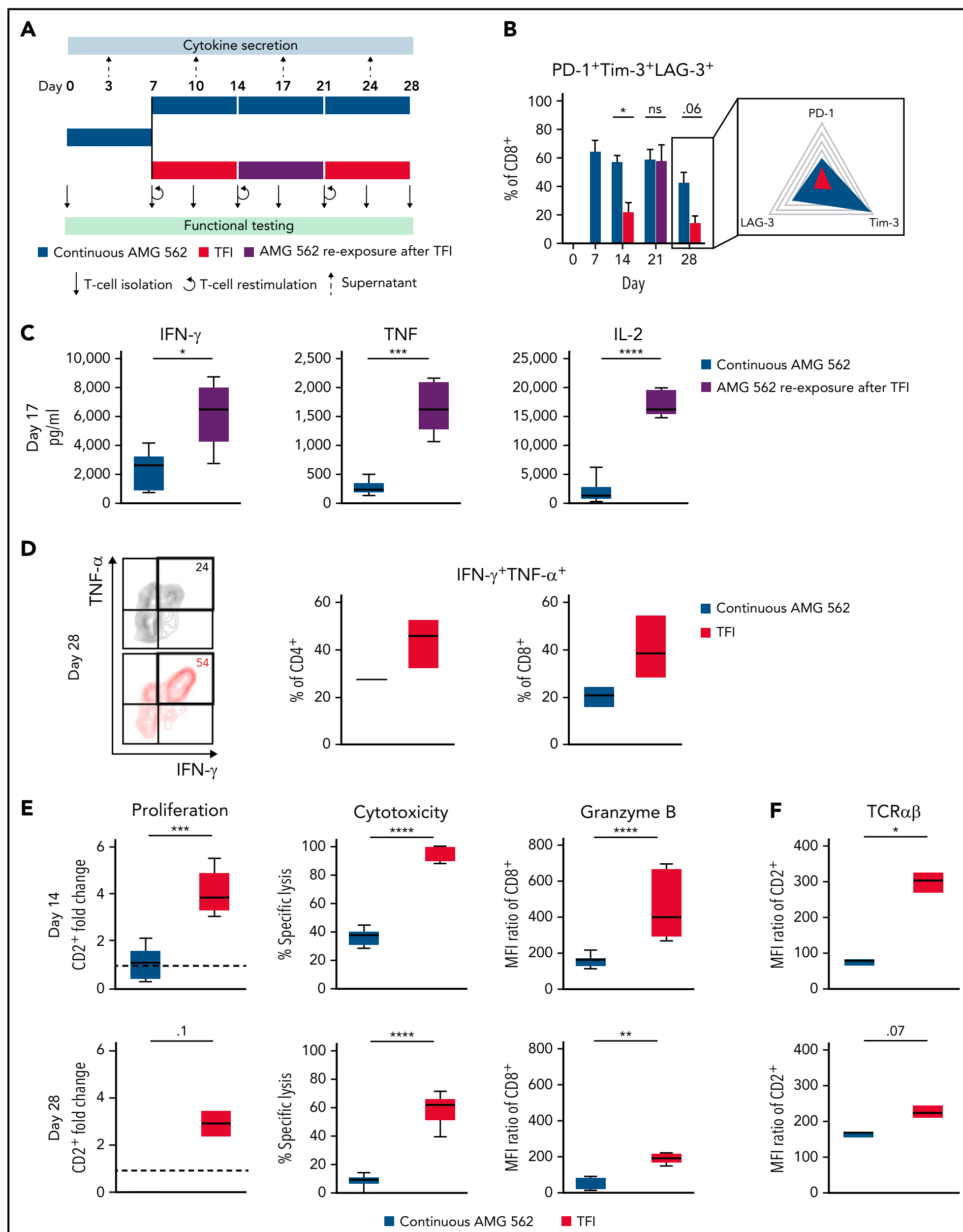

Figure 3. TFIs reinvigorate T-cell function. (A) Timeline of continuous vs TFI T-cell stimulation with AMG 562 over 28 days. (B) Percentage of CD8$^+$ T cells coexpressing PD-1, Tim-3, and LAG-3; n = 6. The spider plot (right) indicates coexpression on day 28 in continuously stimulated vs rested T cells from 1 representative donor. (C) Cytokine levels determined by CBA in coculture supernatants on day 17; n = 6. (D) Percentage of IFN-γ and TNF-α double-positive CD4$^+$ and CD8$^+$ T cells after PMA/ionomycin restimulation on day 28 of coculture; n = 3. Representative plots of CD8$^+$ T cells from 1 donor are shown. (E) AMG 562-mediated CD2$^+$ fold change (n = 3), cytotoxic capacity against hCD19-Ba/F3 cells (n = 6) and granzyme B expression (n = 6) of isolated T cells after 14 or 28 days of coculture. (F) TCR

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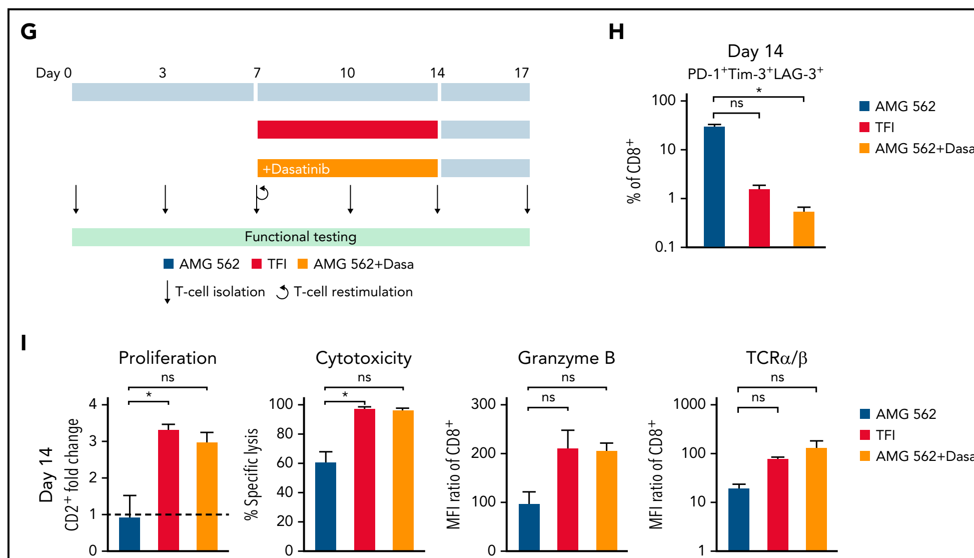

Figure 3 (continued) expression of T cells (n = 3) quantified by immunophenotyping during coculture. (G) Timeline of AMG 562+dasatinib-mediated T-cell stimulation in comparison with continuous or TFI stimulation over 17 days. Dasatinib = 100 nM. (H) Percentage of CD8$^+$ T cells coexpressing PD-1, Tim-3, and LAG-3; n = 3. (I) T-cell proliferation, cytotoxicity, Granzyme B, and TCR expression; n = 3. Boxplot whiskers indicate minima and maxima, and boxes represent the lower quartile, the median, and the upper quartile. Bar graphs present mean ± SEM values. Statistical analysis: 2-way ANOVA and Sidak's multiple comparison test (B,C,E-F,H–I); ^{ns}P > .05; $*P$ < .05; $**P$ < .01; $***P$ < .001; $****P$ < .0001. CBA, cytometric bead array; LAG-3, lymphocyte activation gene 3; ns, not significant; PD-1, programmed cell death protein 1; PMA, phorbol myristate acetate; ± SEM, standard error of the mean; TCR, T-cell receptor; TFI(s), treatment-free interval(s).

cells at day 21: CONT = 58.7% vs TFI = 57.8%; P > .99). Despite the reexpression of IRs, secretion of cytokines on day 17 of the long-term culture was significantly higher in TFI-stimulated T cells (Figure 3C, mean IL-2 secretion: CONT = 1889 pg/mL vs TFI = 17 724 pg/mL; P < .0001). In concordance, TFIs increased the percentage of IFN-γ^+TNF-α^+ T cells upon PMA/ionomycin restimulation as compared with continuously stimulated T cells on day 28 of culture (Figure 3D, mean % of CD8$^+$ T cells: CONT = 20.6% vs TFI = 38.2%). Also, TFIs markedly reinvigorated AMG 562-mediated T-cell proliferation on days 14 and 28 of long-term culture (Figure 3E, mean CD2$^+$ fold change after 3 days of assay; day 14: CONT = 1.1 vs TFI = 4.1; P = .002; day 28: CONT = 0.06 vs TFI = 2.8; P = .09). This was accompanied by significantly increased cytotoxicity (mean specific lysis; day 14: CONT = 34.9% vs TFI = 93.4%; P < .0001; day 28: CONT = 8.6% vs TFI = 58.7%; P < .0001) and granzyme B production (mean MFI ratio of CD8$^+$ T cells; day 14: CONT = 144.5 vs TFI = 451.8; P < .0001; day 28: CONT = 45.5 vs TFI = 196.1; P = .0038). Importantly, T-cell exhaustion and reinvigoration by a TFI could be reproduced by using the AML cell line Molm-13 and a CD33xCD3 half-life extended bispecific molecule for continuous or TFI-stimulation (supplemental Figure 3G). We also observed that T cells upregulated TCRαβ during TFIs (Figure 3F, mean MFI ratio of CD2$^+$; day 14: CONT = 74.3 vs TFI = 299.6; P = .016), pointing toward a possible mechanism behind the enhanced function in TFI-stimulated T cells. Notably, inhibition of TCR signaling by intermittent addition of the Src kinase inhibitor dasatinib[26,27] during continuous AMG 562 stimulation led to similar results (Figure 3G-I; supplemental Figure 4).

TFIs maintain high metabolic fitness of T cells

Highly functional T cells have significant energy demands, and T-cell exhaustion confers changes in the T cell's metabolic program.[28] We therefore hypothesized that exhausted and TFI-stimulated T cells possess distinct metabolic phenotypes and compared the metabolic profiles of continuously AMG 562-stimulated with TFI-stimulated T cells. Indeed, loss of effector function in continuously stimulated T cells (Figure 2) was accompanied by mitochondrial impairment (Figure 4A, oxygen consumption rate [OCR], in pmol/min/1000 cells; mean maximal OCR: day 0 = 3.3 vs day 14 = 1.0; P = .0169). Strikingly, on day 14, TFI-stimulated T cells showed significantly higher basal and maximal mitochondrial respiration and spare respiratory capacity (SRC; Figure 4B, OCR in pmol/min/1000 cells; mean maximal OCR: CONT = 3.9 vs TFI = 13.5; P = .0079; SRC: CONT = 2.2 vs TFI = 8.5; P = .0317). Furthermore, TFI-stimulated T cells maintained higher glycolytic capacity and glycolytic reserve (Figure 4C, mean glycolytic reserve in mpH/min/1000 cells: CONT = 0.2 vs TFI = 0.6; P = .0397). Similar effects were observed even after 28 days of continuous vs TFI AMG 562 stimulation (supplemental Figure 3E-F). Overall, these data indicate that continuously AMG 562-stimulated T cells are metabolically impaired, whereas a TFI maintains high metabolic fitness.

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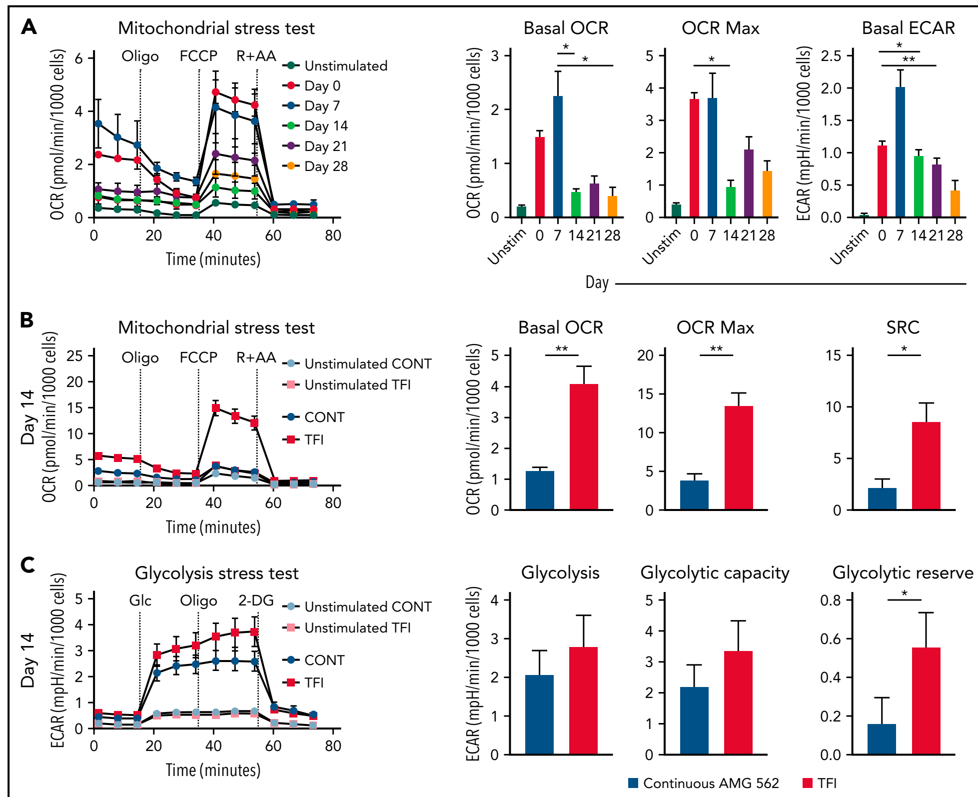

Figure 4. TFIs maintain high T-cell metabolic fitness. (A) Kinetic plot and corresponding bar graphs of normalized OCR obtained during mitochondrial stress test of T cells continuously stimulated with AMG 562; n = 3. (B) Kinetic plot and corresponding bar graphs of normalized OCR obtained during mitochondrial stress test of T cells after 14 days of continuous vs TFI AMG 562 stimulation; n = 5. (C) Kinetic plot and corresponding bar graphs of normalized ECAR obtained during glycolysis stress test of T cells after 14 days of continuous vs TFI AMG 562 stimulation; n = 5. All graphs present mean ± SEM values. Statistical analysis: Kruskal–Wallis and Dunn's multiple comparison test (A); 2-way ANOVA and Sidak's multiple comparison test (B-C); $*P < .05$; $**P < .01$. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; ±SEM, standard error of the mean; TFI(s), treatment-free interval(s).

TFIs induce transcriptional reprogramming of T cells

Next, we performed bulk RNA sequencing of T cells after continuous AMG 562 stimulation or with TFIs to identify transcriptional profiles that drive the profound functional and metabolic differences observed. Samples were processed in 3 batches as shown in supplemental Figure 6I. Unbiased principal component analysis revealed separate clustering of samples according to timepoint and/or treatment (Figure 5A). Differentially expressed gene analysis of day-14 TFI vs CONT T cells identified 1902 significantly upregulated and 2603 downregulated genes ($P_{adj} < .05$). Unsupervised clustering of the top 100 differentially expressed genes showed striking similarity in gene expression patterns in unstimulated (day 0) and day-14 TFI T cells (Figure 5C), suggesting transcriptional reprogramming. Intriguingly, memory-related genes were highly enriched on day 14 of the TFI stimulation (*TCF7*, *IL7R*, and *SELL*; Figure 5B-D). Genes related to cell cycle (*CCNB1* and *CDK1*) and activation (*IL2RA*) were downregulated in day-14 TFI vs CONT T cells (Figure 5B-C). In line with functional reinvigoration (Figure 3), genes involved in T-cell exhaustion (*NR4A3*, *IRF4*, *PDCD1*, *LAG3*) were downregulated in day-14 TFI compared with continuously stimulated T cells (Figure 5B). Pathway analysis of day-14 TFI vs CONT T cells confirmed downregulation of the cell cycle (G2M checkpoint, normalized enrichment score [NES] = −2.47, P_{adj} = 6.3E^{-10}) and metabolism (MTORC1 signaling, NES = −2.27, OXPHOS, NES = −2.03; P_{adj} = 6.3E^{-10}) in line with T-cell quiescence during a TFI (Figure 5E). Gene set enrichment analysis also showed enrichment of memory- compared with effector-related genes identified in a chronic lymphocytic choriomeningitis virus infection model[29] in day-14 TFI-stimulated T cells (Figure 5F; GSE9650, NES = −1.95, false-discovery rate q = 0.0). Together, these data suggest that day-14 TFI-stimulated T cells were functionally and transcriptionally rejuvenated during the TFI.

Interestingly, after 7 days of restimulation with AMG 562 (day-21 TFI) T cells reexpressed genes involved in activation, growth

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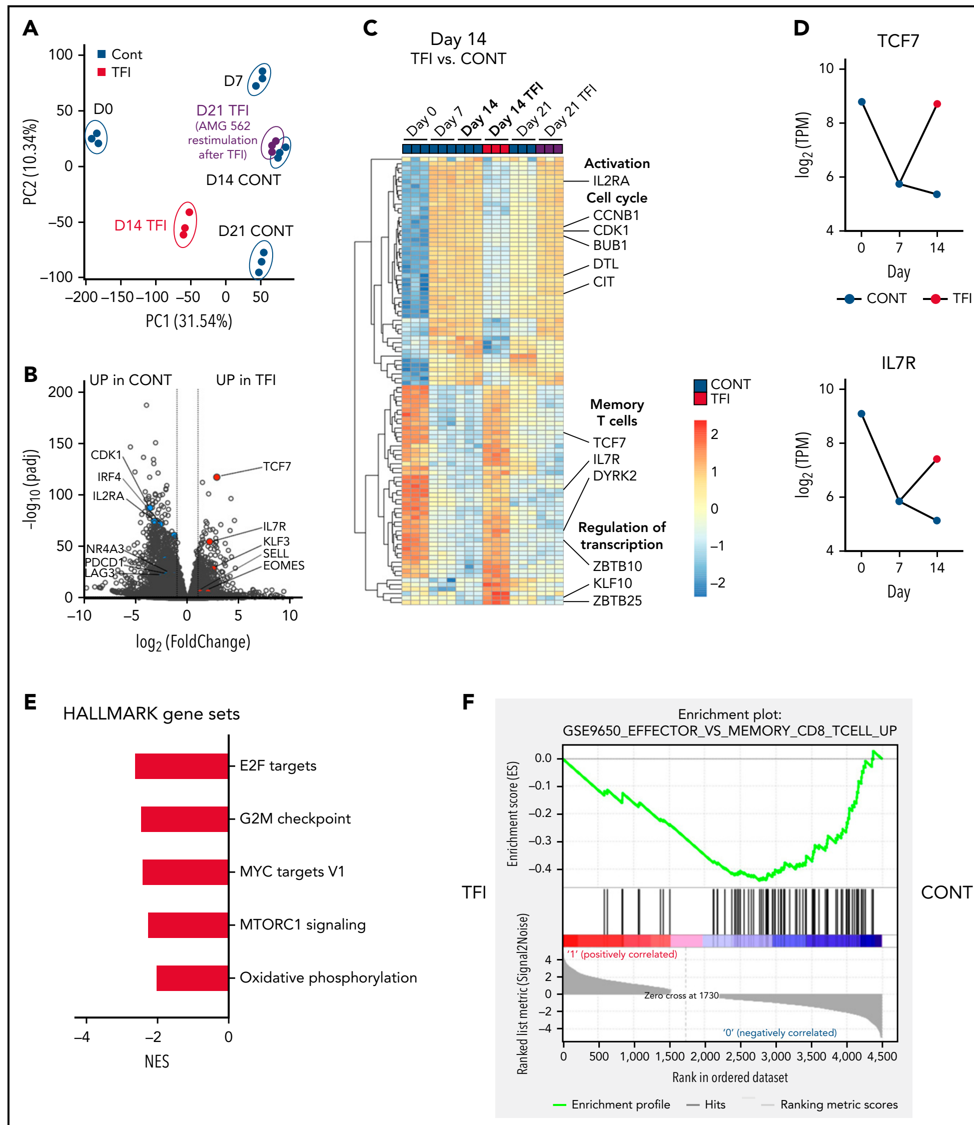

Figure 5. T cells are transcriptionally reprogrammed during TFIs. (A) Principal component analysis. (B) Volcano plot of day 14 TFI vs CONT T cells; $P_{adj} < .05$. Selected genes are highlighted as significantly downregulated (blue) or significantly upregulated (red) in TFI vs CONT cells. (C) Heatmap with hierarchical clustering of the top 100 differentially expressed genes in day-14 TFI vs CONT T cells; $P_{adj} < .05$. Selected genes are highlighted. (D) Log_2(TPM) expression level of *TCF7* and *IL7R* across timepoints 0, 7, and 14 days in TFI vs CONT T cells. (E) Pathways enriched in day-14 TFI vs CONT T cells; $P_{adj} < .05$. (F) Gene set enrichment analysis of day-14 TFI vs CONT T cells using MSigDB and the gene set GSE9650_EFFECTOR_VS_MEMORY_CD8_TCELL_UP.[29] Line plots present mean ± SEM values. CONT, continuously; NES, normalized enrichment score; ±SEM, standard error of the mean; TFI(s), treatment-free interval(s).

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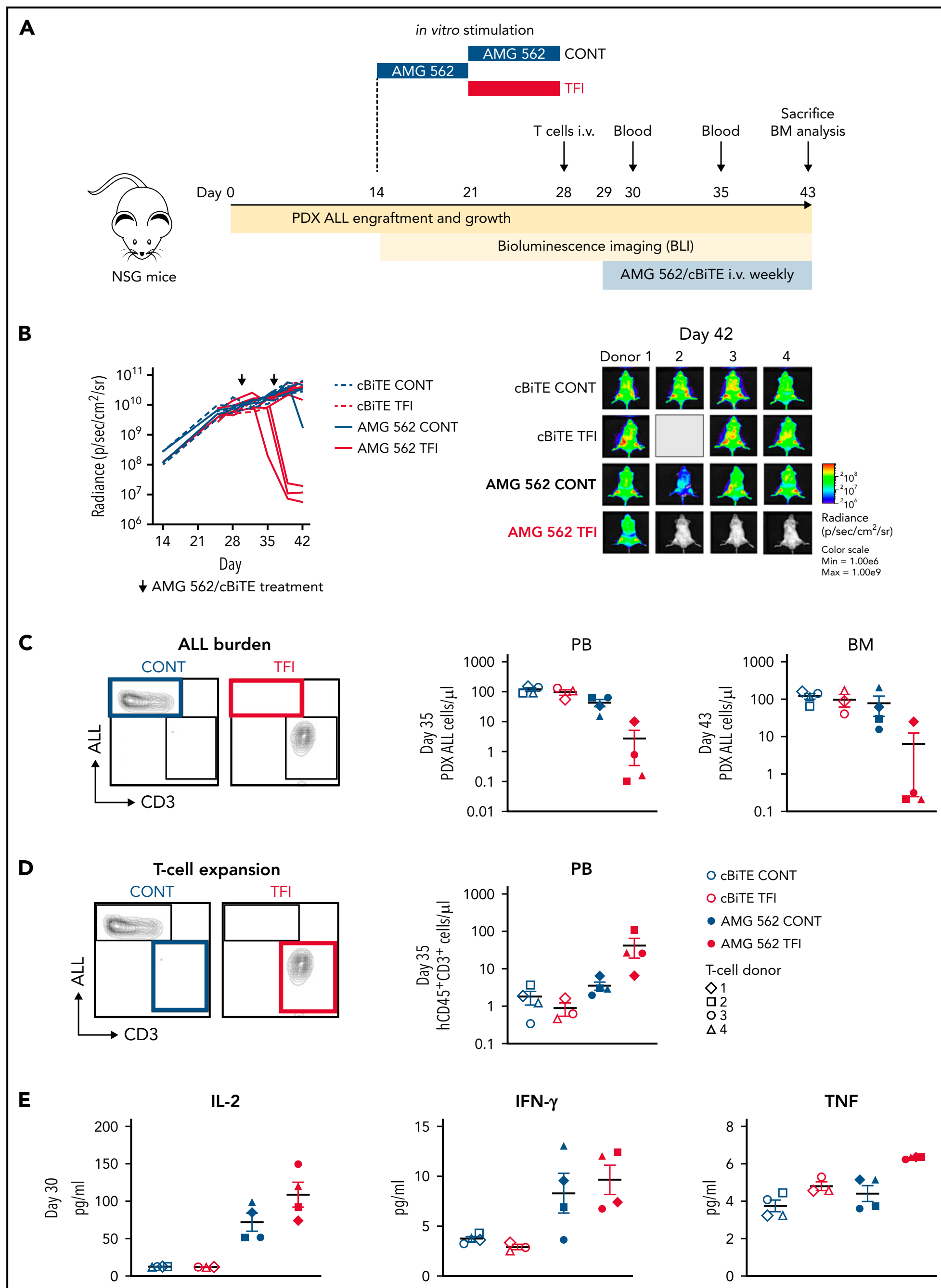

Figure 6.

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(*DPP4* and *SLC3A2*) and cell cycle (*CDK1* and *PLK1*) whereas exhaustion-related genes (*LAG-3*, *BTLA*, and *NFATC1*) were downregulated compared with continuously stimulated cells (supplemental Figure 6A-C). This confirmed the enhanced effector function in day-21 TFI vs continuously stimulated T cells, although the effect was less pronounced compared with day 14 (supplemental Figure 3B-D). Furthermore, we found upregulation of natural killer receptor genes in day-21 CONT vs TFI T cells, which has recently been linked to dysfunctional chimeric antigen receptor (CAR) T-cell states[30] (supplemental Figure 6H). Pathway analysis revealed that identical pathways downregulated on day-14 TFI-receiving T cells were enriched by day 21 and effector- rather than memory-related genes were enriched in a gene set enrichment analysis (supplemental Figure 6D-E). These data suggest that day-21 TFI T cells are reactivated upon AMG 562 reexposure and less exhausted than continuously stimulated T cells. However, we observed downregulation of effector-associated genes (*GZMK* and *IL-2*) and enrichment of exhaustion-associated genes (*TOX* and *CD244*) in day-21 TFI vs day-7 T cells (supplemental Figure 6F-G). These observations underline that upon restimulation with AMG 562 after a TFI T-cell function tends to decrease again (supplemental Figure 3B-F) and support the notion that TFIs have the potential to delay but not to fully prevent exhaustion.

TFIs improve AMG 562-mediated leukemia control in vivo

Lastly, we aimed to confirm the in vivo relevance of a TFI for enhanced T-cell function. Thus, HD T cells were stimulated in vitro for 14 days continuously with AMG 562 or with a TFI, and subsequently transplanted into NSG mice bearing PDX-ALL cells (Figure 6A). Strikingly, T cells receiving a TFI, but not those continuously stimulated, were able to clear the leukemia in mice receiving AMG 562 treatment, as quantified by bioluminescence imaging and peripheral blood) and bone marrow analysis (Figure 6B-C; supplemental Figure 7; mean PDX-ALL cells/μL in bone marrow on day 43 post-ALL engraftment: CONT = 75.0 vs TFI = 6.2). We also observed greater expansion of TFI T cells in peripheral blood 6 days after initial AMG 562 injection in vivo (Figure 6D; mean $CD3^+$ cells/μL: CONT = 3.5 vs TFI = 41.5) and higher levels of human cytokines in murine plasma 1 day after first AMG 562 injection in vivo (Figure 6E; mean concentration of IL-2: CONT = 71.9 pg/mL vs TFI = 108.6 pg/mL). Together, these data confirm that a TFI preserved the high antileukemic activity of T cells in vivo, whereas continuously stimulated T cells failed to control the tumors.

Discussion

T-cell-recruiting bispecific antibodies have shown promising response rates in patients. However, a considerable portion of patients remain refractory to therapy.[9] One contributor to resistance to therapy is preexisting T-cell dysfunction in patients.[11,13] Independent of initial T-cell compartment composition, however, continuous antigen stimulation via the TCR,[29,31] or tonic CAR signaling,[26,32] have been postulated as major causes of T-cell exhaustion. Therefore, to improve patient outcomes, a deeper understanding of the development of T-cell function during therapy with bispecifics is needed. We demonstrate here that ex vivo T-cell function of r/r BCP-ALL patients deteriorated during blinatumomab c.i.v., particularly within the first 14 days of therapy. In a study in pediatric ALL patients, those that showed no response to blinatumomab on day 15 also failed to reach MRD negativity on day 29.[33] This hints that response occurs early during treatment and leads to the hypothesis that continuous exposure to bispecifics induces T-cell exhaustion. We are the first to comprehensively analyze T-cell exhaustion in the context of bispecifics using a stable 28-day in vitro model system. We could thereby mimic chronic exposure to a bispecific antibody over a clinically relevant time period and monitor T-cell function in a standardized manner. Using this system, we observed gradual upregulation of TOX and multiple IRs over the course of AMG 562 stimulation, accompanied by progressive decline in cytokine secretion, T-cell proliferation, and cytotoxicity. These findings recapitulate well-known hallmark features of exhaustion identified in chronic viral infection and cancer,[16,23,34] underlining the suitability of our model system to study T-cell exhaustion.

Strategies to preserve T-cell function and achieve durable antitumor responses are urgently needed. Using our model system, we show that a powerful strategy to maintain T-cell function is disruption of continuous bispecific stimulation with TFIs. Using this strategy, we report a sustained high level of secretion of effector cytokines, T-cell proliferation, and cytotoxicity over 28 days, with metabolic reinvigoration and transcriptional reprogramming of T cells. In concordance, recent studies demonstrated the functional reinvigoration of a tonically signaling GD2-CAR by invoking resting periods.[26] Furthermore, a phase I/II study reported an improved safety profile for r/r AML patients receiving intermittent flotetuzumab treatment in combination with stepwise dosing during week 1, dexamethasone pretreatment, and tocilizumab.[5] Another phase I/II study, treating Ph^+ ALL patients with dasatinib[26,27] in combination with blinatumomab c.i.v., reported an overall survival rate of 95%[35] and an increase in peripheral lymphocytes,[36] consistent with our observation that T-cell function can be reinvigorated to a similar extent using TFIs or pharmacological inhibition of TCR signaling with dasatinib (Figure 3; supplemental Figure 4). Together, these studies underline the potential of TFIs for reinvigorating T cells and for fine-tuning T-cell responses in patients.

We show improved expansion and tumor control of in vitro TFI-stimulated T cells using an ALL-PDX mouse model. While this model system underlines the relevance of the rested T-cell phenotype in vivo, suitable mouse models need to be developed in

Figure 6. TFIs improve AMG 562-mediated control of ALL in vivo. (A) Timeline of in vivo experiment: PDX-ALL cells were transplanted into NSG mice. T cells (4 donors) were stimulated in vitro for 14 days continuously or with TFI cells (days 7-14) and subsequently injected into NSG mice 28 days post engraftment. Mice were treated with AMG 562/control BiTE = 5 ng/mL on days 1 and 8 post T-cell injection. T-cell function and ALL burden was analyzed via bioluminescence imaging and flow cytometry. (B) Quantification of bioluminescence imaging signals (left panel) and images of mice on day 42 after engraftment (right panel). See supplemental Figure 7B for images of all timepoints. (C) Flow cytometry analysis of PDX-ALL cells detected in PB on day 35 and in BM on day 43. Representative plots from 1 T-cell donor are shown. (D) $CD3^+$ T-cell expansion in PB on day 35. Representative plots from 1 T-cell donor are shown. (E) Human cytokine levels detected in murine plasma on day 30. All graphs present mean ± SEM values. BM, bone marrow; cBiTE, control BiTE, bispecific control construct; CONT, continuously; PB, peripheral blood; PDX-ALL, patient-derived xenograft acute lymphoblastic leukemia; NSG, NOD.Cg-$Prkdc^{scid}$ $IL2rg^{tm1Wjl}$/SzJ; ±SEM, standard error of the mean; TFI(s), treatment-free interval(s).

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future studies to mimic continuous vs intermittent BsAb administration in vivo.

Targeting PD-1/PD-L1 to overcome T-cell exhaustion has shown efficacy in cancer. The combination of a CD33xCD3 BiTE® construct with disruption of PD-1/PD-L1 signaling increased antitumor responses in preclinical AML models.[37] Similar findings in CAR T cells[38-40] led to the initiation of clinical trials.[41] Intriguingly, T-cell reinvigoration through TFIs was much more efficient than continuous AMG 562 + nivolumab (a PD-1 inhibitor) treatment, the latter showing no to modest T-cell reinvigoration (supplemental Figure 5). In concordance, PD-1 blockade alone failed to reverse phenotypic and transcriptomic hallmarks of exhaustion in CAR T cells.[26] This highlights the need to deepen our understanding of the molecular networks involved in T-cell exhaustion.

T-cell metabolism is closely associated with effector function.[28,42,43]. In metabolic stress tests TFIs significantly enhanced T-cell metabolic fitness, along with increased effector function. In line with these observations, enhanced expansion, persistence, and survival of BBζ vs 28ζ CAR T cells have been linked to higher mitochondrial capacity.[44] In chronic lymphocytic leukemia patients, high metabolic fitness of CAR T cells prior to infusion correlated with response to therapy,[45] underlining the importance of metabolic fitness for successful immunotherapy.

High SRC, observed in TFI-stimulated T cells, is a hallmark feature of memory T cells,[28] and is consistent with upregulation of memory-associated genes in day-14 TFI-stimulated T cells. Transcriptional reprogramming of T cells during a TFI recapitulates findings made in transiently rested CAR T cells[26] and preclinical models of resolved chronic infection,[46] and confirmed the superior function of TFI- compared with continuously AMG 562-stimulated T cells. Intriguingly, we found that reinvigorated function in day-14 TFI-stimulated T cells coincided with sustained expression of *TCF7* (encoding TCF-1). TCF-1 is expressed in precursor-exhausted T cells (T_{PEX}) with the potential to self-renew and to give rise to terminally exhausted TCF-1-negative T cells (T_{EX}). Furthermore, T_{PEX} cells retain the ability to be functionally reinvigorated by checkpoint blockade.[47-52] Our findings support the notion that *TCF7* expression correlates with T-cell function and distinguishes T_{EX} from functional T_{PEX} cells.

In summary, we identified T-cell exhaustion as a potential mechanism contributing to resistance to bispecifics therapy in a preclinical model. The reinvigorating effect of TFIs on T cells suggests the relevance of resting periods between bispecific antibody treatment cycles. Hence, the implementation of TFIs should be considered for the design of administration schedules in the future. In light of the similarities between the exhausted T-cell phenotypes in chronic viral infections and CAR T-cell and bispecific antibody therapy, TFIs might be applicable to other T-cell–based immunotherapies.

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Authorship

Contribution: M. Subklewe, V.B., R.K., and N.P. designed the study and supervised the project; N.P., M.K., V.B., B.V., T.S., and M. Subklewe wrote the manuscript; N.P., A.N., J.W., M. Scheurer, A. Muth, G.H., and M.O. performed experiments and analyzed and/or interpreted the data; M. Subklewe, V.B., R.K., N.P., M.K., D.N., S.M.L., B.B., S.T., I.J., G.H., M. Sponheimer, A. Marcinek, L.R., A.L., and K.R. were involved in research design and data interpretation; O.W. and M.v.B.-B. critically reviewed and discussed the data. M.K. performed the library preparation for bulk RNA-seq and, in collaboration with T.S., conducted the bioinformatic analysis; B.V. and I.J. designed and performed the in vivo experiments.

Conflict-of-interest disclosure: M. Subklewe has received industry research support from Amgen, Gilead, Miltenyi Biotec, Morphosys, Roche, and Seattle Genetics, and has served as a consultant/advisor to Amgen, BMS, Celgene, Gilead, Pfizer, Novartis, and Roche. She sits on the advisory boards of Amgen, Celgene, Gilead, Janssen, Novartis, Pfizer, and Seattle Genetics, and serves on the speakers' bureau at Amgen, Celgene, Gilead, Janssen, and Pfizer. V.B. has received research funding from Miltenyi Biotec, Novartis, and Pfizer, and has served as a consultant/advisor to Novartis, Amgen, and Gilead. He serves on the speakers' bureau at Novartis and Pfizer. R.K. is employed at Amgen Research Munich, Germany. S.M.L. receives research funding from Roche. M.v.B.-B. has received research support from and serves on the speakers' bureau at Gilead, Miltenyi Biotec, MSD Sharpe & Dohme, Roche, Mologen, Novartis, Astellas, and BMS. K.R. received research funding from Gilead and honoraria from Gilead and Novartis. O.W. has received research funding from Roche. He serves on the speakers' bureau at Janssen and sits on the advisory board of Epizyme. S.T. has served as a consultant/advisor to Amgen, BMS, GSK, Janssen, Pfizer, Sanofi, and Takeda. The remaining authors declare no competing financial interests.

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Footnotes

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The online version of this article contains a data supplement.

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