REGULAR ARTICLE

blood advances

FLT3-directed BiTE molecules vs CAR T cells in AML: costimulatory signals mitigate T-cell exhaustion

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Key Points

- The costimulatory profile of AML cells determines the quality and persistence of T-cell responses to BiTE molecules.
- CAR T cells demonstrate improved long-term fitness via intrinsic costimulation, reducing dependence on AML-derived signals.

T-cell–based immunotherapies have revolutionized treatment paradigms in B-cell malignancies, yet their translation to acute myeloid leukemia (AML) has been hindered by a scarcity of tumor-restricted antigens and the risk of on-target off-leukemia toxicity. FLT3 has emerged as a promising therapeutic target with limited expression in healthy hematopoietic tissues. Here, we performed a head-to-head preclinical comparison of an FMS-like tyrosine kinase 3 (FLT3)-directed bispecific T-cell engager (BiTE) molecule and second-generation FLT3-specific chimeric antigen receptor (CAR) T cells. Both approaches induced potent cytotoxicity against AML cell lines and primary patient-derived cells but spared healthy hematopoietic stem and progenitor cells in vitro. Despite similar short-term efficacy, prolonged antigen exposure demonstrated progressive functional decline and metabolic exhaustion; however, CAR T cells maintained cytotoxic capacity and proliferative potential over time. In AML xenograft models, CAR T cells achieved superior tumor control, prolonged survival, and greater T-cell infiltration than BiTE molecule–treated counterparts. Transcriptomic profiling of T cells recovered from the bone marrow further revealed a distinct exhaustion-associated gene signature in samples from mice that had been treated with the FLT3 BiTE molecule. Importantly, provision of CD86-mediated costimulation enhanced antitumor activity of BiTE-redirected T cells in vitro and in vivo. These findings establish FLT3 as a viable and selective immunotherapeutic target in AML and underscore the functional and transcriptional differences between BiTE molecule–redirected T cells and CAR T cells. Moreover, they reveal a critical role for costimulatory signaling in sustaining the efficacy of T-cell–based therapies in vivo, offering a rationale for improving T cell–redirection strategies in myeloid malignancies.

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The RNA sequencing data generated and analyzed during this study have been deposited in the Gene Expression Omnibus database (accession number GSE298033).

Original data are available from the corresponding author, Marion Subklewe (marion.subklewe@med.uni-muenchen.de), on request.

The full-text version of this article contains a data supplement.

Introduction

Despite the recent approval of several novel targeted small-molecule agents for the treatment of acute myeloid leukemia (AML), overall survival remains poor, and relapse rates continue to be high. Allogeneic hematopoietic stem cell (HSC) transplantation remains the most effective curative strategy for AML,[1] primarily through the graft-versus-leukemia effect mediated by donor T cells. However, only a subset of patients are eligible for transplant, and relapse even after allogeneic HSC transplantation, continues to pose a major therapeutic challenge. T-cell–based immunotherapy has demonstrated unprecedented efficacy in $CD19^+$ B-cell malignancies, leading to the approval of the bispecific T-cell engager (BiTE) blinatumomab in 2014,[2] followed by 2 CD19-directed chimeric antigen receptor (CAR) T-cell therapies in 2017.[3,4] These successes have established T cells as powerful therapeutic tools and have spurred intensive efforts to translate similar strategies to other hematologic malignancies, including AML. The latter has shown to be a challenging entity for synthetic, T-cell–based immunotherapy, primarily due to the lack of leukemia-specific target antigens and high risk of on-target, off-leukemia toxicity arising from common antigen expression on healthy hematopoietic cells.[5,6]

In a previous study, our group demonstrated that FMS-like tyrosine kinase 3 (FLT3) presents a favorable expression profile due to its broad expression in AML bulk cells and limited expression within the hematopoietic compartment.[7,8] Importantly, expression on AML bulk and leukemic stem cells is observed independent of FLT3 mutational status.[9-11] Several ongoing phase 1/2 clinical trials (eg, ClinicalTrials.gov identifier: NCT05143996, NCT05023707, and NCT06786533) are currently investigating FLT3-directed T-cell–based therapies, including T-cell engagers (TCEs) and CAR T cells, for the treatment of AML. TCE molecules offer key advantages such as off-the-shelf availability and dosing flexibility and allow for treatment-free intervals that might help mitigate T-cell exhaustion.[12] Another advantage of TCE molecules is the possibility to combine them with other agents, as shown recently by Hänel et al.[13] By contrast, CAR T-cell therapy requires individualized manufacturing, beginning with patient-specific leukapheresis, followed by ex vivo genetic modification, expansion, and quality control procedures, all of which introduce logistical complexity and substantial time delays before treatment can start. However, CAR T cells incorporate built-in costimulatory domains that can enhance T-cell activation and cytotoxicity, a particularly relevant feature given the low expression of costimulatory ligands on AML cells[14] and the diminished T-cell function observed in patients with AML.[15,16] Despite these conceptual differences, no preclinical study has directly compared the functional properties of TCE molecule– and CAR-redirected T cells that target the same antigen with an identical single-chain variable fragment. Here, we systematically evaluate both approaches in parallel using a FLT3-directed BiTE molecule (FLT3×CD3) and a second-generation FLT3-specific CAR T-cell construct (FLT3-CD28-CD3ζ) with the same single-chain variable fragment, and therefore identical binding affinity for FLT3. This study aims to delineate mechanistic differences between these 2 platforms, with the goal of informing rational development and clinical prioritization of T-cell–based immunotherapies against AML.

Methods

Patient and healthy donor (HD) samples

Patient characteristics are summarized in Table 1.

Cell lines

Cell lines were initially obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) or the American-Type Culture Collection (Manassas, VA). Cells were cultured using standard techniques and reagents. Cell line authentication and mycoplasma tests were performed regularly. Cells were used within 2 months of thawing.

Table 1. Patient characteristics

Patient no.	Sex	Age, y	ELN (2017)	FLT3 mutation	MFI ratio CD135
1	M	76	Adverse	ITD	4.4
2	F	45	Adverse	ITD	1.5
3	M	57	Adverse	–	5.9
4	M	83	Adverse	ITD	3.2
5	F	50	Adverse	–	4.8
6	F	82	Adverse	–	5.9
7	F	77	Adverse	–	5.7
8	M	66	Adverse	–	2.5
9	M	69	Adverse	ITD	5.4
10	F	55	Adverse	ITD	8.0
11	M	28	Adverse	–	18.7
12	M	68	Adverse	TKD	4.1
13	M	73	Adverse	–	4.8
14	M	86	Adverse	–	6.8
15	M	73	Adverse	–	10.6

F, female; ITD, internal tandem duplication; M, male; MFI, median fluorescence intensity; TKD, tyrosine kinase domain.

Experimental FLT3 BiTE

The anti-FLT3 BiTE molecule (FLT3×CD3) was kindly provided by Amgen Inc. A range of BiTE molecule concentrations was evaluated by titration. A concentration of 5 ng/mL of BiTE molecule was used for all experiments to achieve saturation. A control BiTE construct was used as a negative control for all experiments. Peripheral blood mononuclear cells from HDs or patients with primary AML (pAML) were isolated by density gradient centrifugation. HD T cells were negatively isolated using the EasySep human T-cell isolation kit (Stemcell Technologies, Vancouver, Canada). T cells from patients with AML were isolated using the EasySep Human CD3 Positive Selection kit II (Stemcell Technologies). T cells were cryopreserved for all experiments.

Generation of CAR T cells

The anti-FLT3 CAR vector (FLT3-CD28-CD3ζ) was kindly provided by Amgen Inc. T cells were isolated as described earlier. Isolated T cells were cultured in TexMACS medium (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 10 ng/mL interleukin-17 (IL-7)/IL-15 (PeproTech, Hamburg, Germany). T cells were activated for 24 hours with TransAct (Miltenyi Biotec). Lentiviral transduction was performed at a multiplicity of infection of 10, and cells were cultured 12 to 14 days before cryopreservation. Transduction efficiency was assessed by staining with an anti-ID antibody followed by a fluorescent secondary anti–immunoglobulin G2b.b antibody and subsequent flow cytometric analysis. Comparable transduction efficiencies were observed throughout all experiments. Untransduced (UT) T cells that underwent the same procedure but did not have contact with the virus were used as a negative control for all CAR T-cell experiments. The same T-cell donors were used for BiTE molecule and CAR assays (supplemental Figure 1A).

T-cell cytotoxicity assays

The cytotoxicity of BiTE molecule– or CAR-redirected T cells against various AML cell lines was assayed in cocultures with different effector-to-target (E:T) ratios. Ex vivo cytotoxicity assays against pAML samples were performed as previously described.[17] Cells were counted by flow cytometry, and specific lysis was calculated using the following equation:

$$\%\ \text{specific lysis} = \left(1 - \frac{\text{CD33}^{+}\text{target cell count BiTE or CAR}}{\text{CD33}^{+}\text{target cell count cBiTE or UT}}\right) \times 100.$$

T-cell proliferation

The proliferation of CellTrace Far Red–labeled BiTE molecule–treated T cells or CAR T cells was assayed in cocultures with various AML cell lines at an E:T ratio of 1:5 or upon stimulation with CD3/CD28 Dynabeads (Thermo Fisher Scientific, Waltham, MA) at a bead-to-cell ratio of 1:2. Flow cytometric analysis was performed after 72 hours.

Colony-forming unit (CFU) assay with healthy bone marrow cells

HSCs were isolated from the bone marrow of HDs using the EasySep Human CD34 Positive Selection kit II (Stemcell Technologies). Isolated $CD34^{+}$ cells were cocultured with T cells plus BiTE molecule, CAR T cells, or medium only at an E:T ratio of 10:1 for 6 hours, in duplicates. After incubation, 1 portion of the cocultures was used for flow cytometric readout, the remaining cell suspensions were plated in duplicate in MethoCult H4434 medium containing several recombinant cytokines (Stemcell Technologies). After incubation for 14 days, the number of colonies was assessed by counting using a THUNDER imaging system (Leica, Wetzlar, Germany).

In vitro long-term culture (28 days)

HD-derived T cells or CAR T cells were cocultured with irradiated (2.5 Gy) Monocytic Osteogenic Leukemia model 13 (MOLM13) cells in R10 (E:T of 1:5, 1×10^6/mL). For conditions involving a BiTE molecule, 5 ng/mL FLT3-BiTE molecule was added to the coculture. On day 3, culture medium, irradiated target cells, and BiTE molecule (if appropriate) were replenished. On day 7, T cells were isolated using the EasySep Human CD3 Positive Selection kit II (Stemcell Technologies). A fraction of the isolated T cells was used for functional testing and phenotypic characterization. The remaining T cells were recultured as described earlier. This stimulation process was repeated 4 times.

Coculture supernatants were collected to quantify cytokine secretion.

Metabolic stress tests

T cells were stimulated with TransAct (Miltenyi Biotec) for 48 hours. T cells (2×10^5 per well) were plated per well onto a poly-D-lysine–coated 96-well plate. The metabolic stress test was performed on a Seahorse XFe96 Analyzer (Agilent, Santa Clara, CA) using consecutive injections of oligomycin, BAM15, and 2-deoxy-D-glucose plus rotenone/antimycin A plus Hoechst 3342 (Sigma-Aldrich, St Louis, MO). Metabolic rate was normalized to cell count using a Cytation 1 reader (BioTek Instruments, Inc, Winooski, VT).

In vivo studies

Four-week-old NXG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/Rj) were purchased from Janvier (St Berthevin, France). OCI-AML3-LUC-GFP xenograft models were established by IV injecting 1×10^6 cells into the tail vein. BiTE or half-life extended BiTE molecules were delivered intraperitoneally (IP) daily. HD T cells (5×10^6) or HD CAR T cells (1×10^7, with a transduction efficiency of 50%) were administered IV, 4 days after AML engraftment. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern). Before treatment, mice were randomized according to tumor burden. More than 15% weight loss after the start of the experiment or a decrease in general health condition (decreased mobility, general weakness, hunched posture, or ungroomed hair) are defined as surrogate mouse end points for human survival.

RNA sequencing

A second in vivo study was performed as described earlier. Eighteen days after T-cell transfer, all mice were euthanized. Human T cells were isolated from the murine bone marrow by fluorescence-activated cell sorting (MoFlo Astrios, Beckman Coulter, Brea, CA). A total of 5000 T cells or CAR T cells were transferred into 50 μL of buffer RLT Plus (Qiagen, Venlo, The Netherlands) containing 1% β-mercaptoethanol and stored

at −80°C. RNA isolation and library preparation were performed according to the Prime-seq protocol.[18]

AML and healthy donor (HD) samples were collected with written informed consent in accordance with the Declaration of Helsinki and approval by the institutional review board of the Ludwig Maximilian University of Munich.

Results

BiTE molecule vs CAR T-cell construct: similar cytotoxicity against various FLT3$^+$ AML cell lines, divergent T-cell activation profiles

FLT3 expression and mutational status vary greatly among patients with AML. To compensate for these variations while testing the in vitro functionality of the BiTE molecule and CAR, a panel of cell lines with different genetic backgrounds (Figure 1A) and FLT3 expression levels (Figure 1B) was used. Of note, FLT3 expression level was not associated with mutational status, as has been shown previously.[19] Both BiTE molecule and CAR induced similar specific lysis in the FLT3$^+$ cell lines MOLM-13, OCI-AML3, and MV4-11 when cocultured with HD T cells (Figure 1C, left). Lysis was FLT3 specific, with minimal cytotoxicity observed against the FLT3$^-$ leukemic cell line, HEL.92.1.7 (Figure 1D). T-cell activation was measured by the secretion of the effector cytokines interferon gamma (IFN-γ), tumor necrosis factor (TNF), IL-2, and granzyme B in cocultures with the FLT3$^+$ cell line MOLM-13 (Figure 1E). Although cytokine secretion levels varied slightly between the 2 platforms, the differences were not statistically significant. T-cell proliferation in cocultures with OCI-AML3 (Figure 1F) was greater for CAR T cells than for BiTE molecule–redirected T cells. To determine whether this proliferative advantage was caused by previous activation of the CAR T cells during their manufacture, we tested UT cells in combination with the BiTE molecule and showed that T-cell proliferation was greater when using preactivated UT T cells for recruitment with the BiTE molecule (Figure 1F). A metabolic stress test to evaluate metabolic fitness of the T cells at baseline, before being subjected to any experimental setup, revealed an increased basal and maximal respiration, as well as the higher glycolytic capacity of CAR T cells compared with the unmanipulated T cells used for BiTE molecule recruitment by the BiTE molecule (Figure 1G-H). Taken together, these data indicate that the required preactivation of CAR T cells during the manufacturing process gives them a "head start" over unmanipulated T cells that are recruited by the BiTE molecule.

BiTE molecule and CAR T cells efficiently eliminate pAML cells with minimal off-leukemia toxicity against healthy HSCs

Next, we assessed whether CAR T cells and BiTE molecule–redirected T cells can effectively target and lyse pAML cells with different levels of FLT3 expression and mutational status in vitro (Figure 2A; Table 1).

In an allogeneic assay setup with HD T cells, the use of BiTE molecule and CAR led to comparable specific lysis of pAML cells after 6 days of coculture (Figure 2B). Similar results were achieved when testing an autologous assay setup in which patient-derived T cells were used to target autologous AML blasts (Figure 2C). Regarding T-cell activation via cytokine secretion in allogeneic cocultures, CAR T cells secreted higher levels of IFN-γ (Figure 2D). By contrast, secretion levels of TNF, IL-2, and granzyme B were comparable between BiTE molecule– and CAR-redirected T cells. To test whether the specificity shown with AML cell lines could also be observed using primary patient samples, BiTE molecule– and CAR-redirected T cells were cocultured with HD bone marrow mononuclear cells. Both constructs led to low lysis (Figure 2E). To further test for on-target off-leukemia toxicity, we repeated the previous assay with isolated healthy CD34$^+$ HSCs and found again low levels of cytotoxicity. After 14 days of coculturing BiTE molecule– and CAR-redirected T cells with healthy CD34$^+$ HSCs, no changes in CFU assays were observed (Figure 2G; supplemental Figure 1B). Furthermore, experiments with mixtures of pAML cells and HD bone marrow mononuclear cells revealed efficient pAML cell lysis with minimal impact on healthy bone marrow (Figure 2H). Overall, both BiTE molecule–recruited T cells and CAR T cells efficiently kill pAML cells with limited on-target off-leukemia toxicity.

Functional decline of BiTE molecule– and CAR-redirected T cells through continuous antigen exposure

T-cell exhaustion is an emerging as a cause of immunotherapy failure. To test whether BiTE molecule– and CAR-redirected T cells are equally affected by continuous antigen stimulation, we used our previously developed in vitro system to monitor T-cell dysfunction induced by chronic antigen stimulation.[12,20] BiTE molecule– and CAR-redirected HD T cells were cocultured with FLT3$^+$ MOLM-13 cells for 28 days (Figure 3A). CD2$^+$ T cells were isolated on days 0, 7, 14, 21, and 28, and used for functional testing. Immunophenotyping (supplemental Figure 1C) showed that only a minor fraction of BiTE molecule– and CAR-redirected T cells coexpressed the inhibitory receptors programmed cell death protein 1, lymphocyte activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin domain containing 3 at baseline. On day 7 of the long-term culture, an increase in the coexpression of the immune-inhibitory checkpoints was observed (Figure 3B). The supernatant of the coculture was measured for various cytokines on days 3, 10, 17, and 21. A constant decrease in IFN-γ, TNFα, IL-2, granzyme B, perforin, granzyme A, granulysin, and Fas-ligand secretion was measured for CAR T cells throughout the coculture. By contrast, the highest BiTE molecule–mediated cytokine secretion was measured on day 10, and then similarly decreased toward day 28 (Figure 3C; supplemental Figure 1D). CAR-mediated cytotoxicity against MOLM-13 cells was stable throughout the coculture experiment, whereas BiTE molecule–mediated cytotoxicity decreased after 21 days (Figure 3D). An analysis of T-cell proliferation revealed a peak expansion of CAR T cells after 7 days of coculture followed by a continuing decline toward day 28. BiTE molecule–mediated T-cell proliferation was slightly delayed in comparison and peaked after 14 days of coculture before slowing sharply by day 28 (Figure 3E). To test whether continuous antigen stimulation had a similar effect on the metabolic fitness of BiTE molecule– and CAR-redirected T cells, a metabolic stress test was performed. Indeed, the mitochondrial function was impaired with both treatment strategies. Glycolytic activity also decreased toward the end of the coculture; however, CAR T cells maintained higher levels of glycolysis than T cells

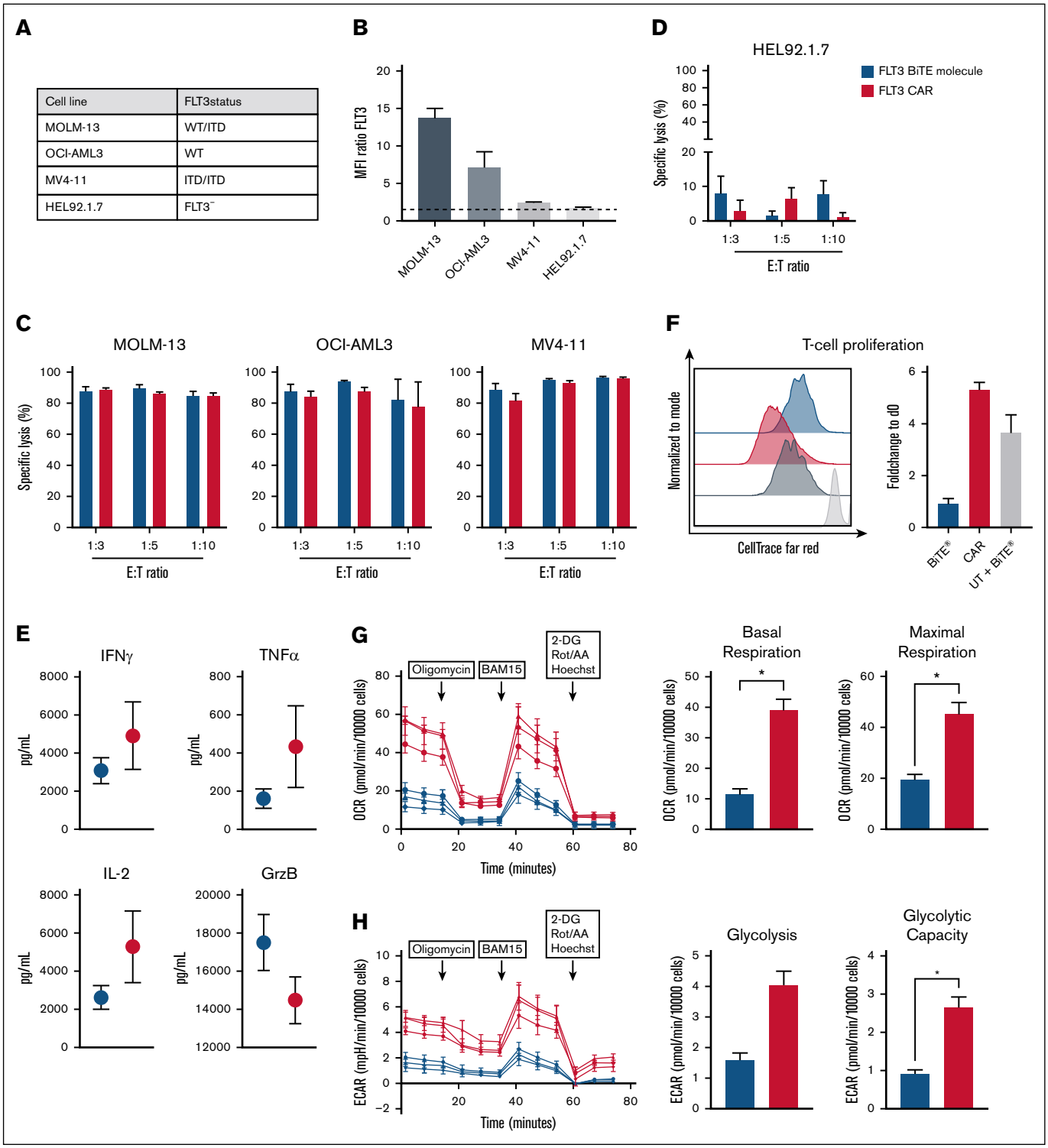
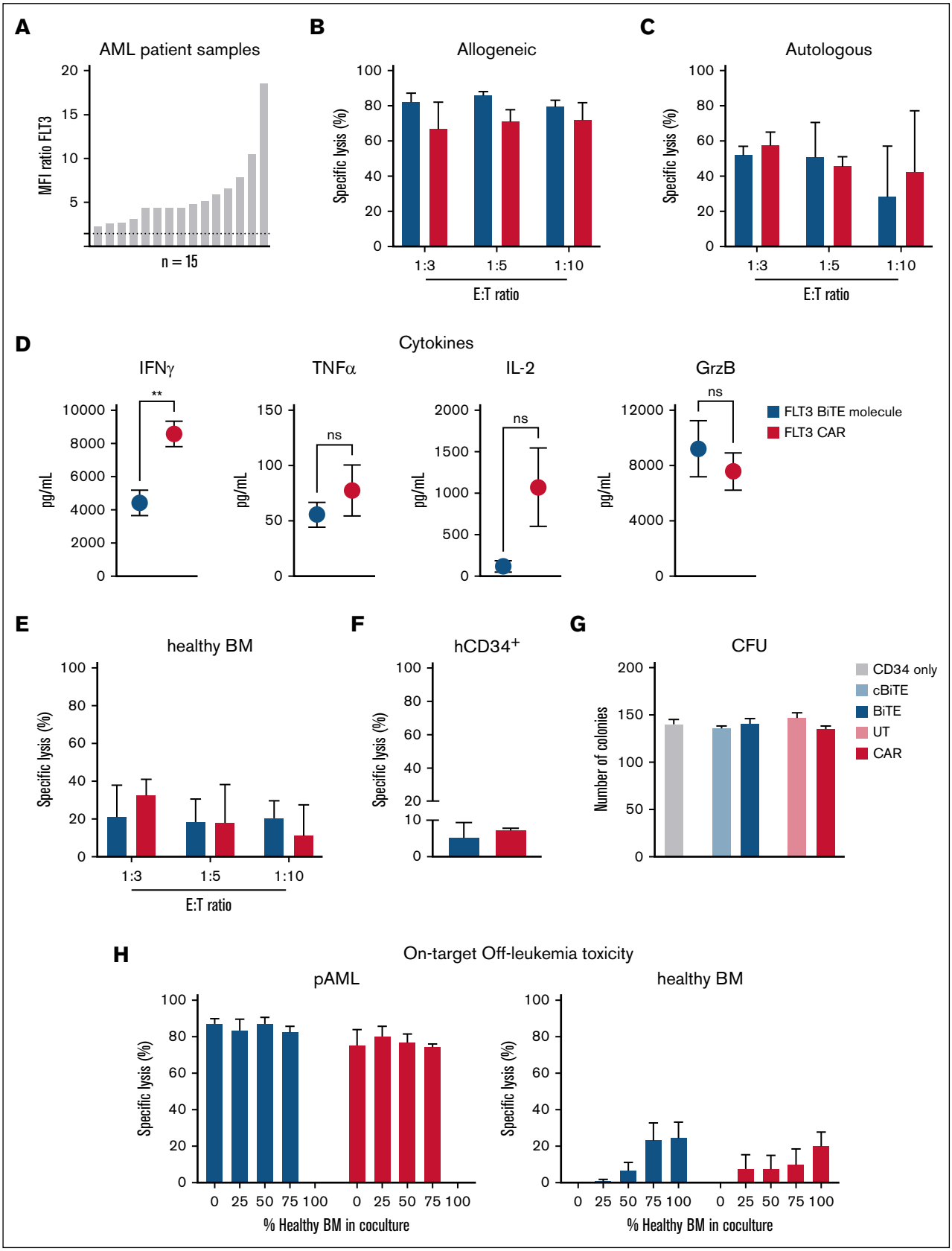


Figure 1. BiTE molecule and CAR induce similar cytotoxicity against various AML cell lines and show divergent T-cell activation profiles. (A) Genetic backgrounds of AML cell lines used for the following experiments. (B) FLT3 expression levels of different leukemic cell lines assessed by flow cytometry. (C) BiTE molecule– and CAR-mediated cytotoxicity of HD T cells against the FLT3^{+} AML cell lines MOLM-13, OCI-AML3, MV4-11, and (D) the FLT3^{-} leukemic cell line HEL.92.1.7 after 72 hours (n = 6) at different E:T ratios. (E) Secretion of IFN-γ, TNF, IL-2, and granzyme B (GrzB) determined by cytometric bead array (CBA) analysis (n = 6), from corresponding cytotoxicity assays at an E:T ratio of 1:3. (F) Representative histogram of Far Red dilution and reciprocal depiction of HD T-cell proliferation (n = 3) after coculture with OCI-AML3 cells for 5 days with comparison with UT cells plus BiTE molecule (dark gray) and UT cells alone as control (light gray). (G) Seahorse assay. Mitochondrial respiration reflected by OCR of unmanipulated HD T cells used for evaluating of BiTE molecule and HD CAR T cells at baseline. (H) Seahorse assay. Glycolysis evaluated as the extracellular acidification rates (ECAR) of unmanipulated HD T cells used for testing of BiTE molecule and HD CAR T cells at baseline. All graphs represent the mean ± standard error of the mean (SEM) values. Statistical analysis: paired *t* test (panels G-H); nonsignificant *P* value (ns_*P*) > .05; *P < .05. 2-DG, 2-Desoxy-D-glucose; ITD, internal tandem duplications; MFI, median fluorescence intensity; MFI ratio, MFI of sample to MFI of isotype control; OCR, oxygen consumption rates; WT, wild type.


A
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n = 15
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GrzB
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FLT3 BiTE molecule
FLT3 CAR
E
healthy BM
F
hCD34+
G
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Number of colonies
CD34 only
cBiTE
BiTE
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On-target Off-leukemia toxicity
pAML
healthy BM
% Healthy BM in coculture

Figure 2.

redirected by the BiTE molecule (Figure 3F). Overall, these data indicate that continuous antigen stimulation reduces T-cell function in both BiTE molecule– and CAR-redirected T cells. Notably, CAR T cells were still capable of lysing tumor cells after 28 days of continuous stimulation.

In comparison with BiTE molecule–activated T cells, CAR T cells show superior antileukemia activity in an AML xenograft model

Next, we were curious to see whether we could reproduce our aforementioned results in vivo. To this end, we used xenograft models of leukemia by engrafting the AML cell line OCI-AML3-LUC-GFP into NXG mice (Figure 4A). Upon engraftment, equivalent numbers of HD T cells or CAR T cells were administered via the tail vein, followed by IP BiTE or control BiTE injection in the appropriate groups.

In this model, mice treated with CAR T cells experienced major responses to the therapy, with improved tumor control in all treated mice (Figure 4B). However, 2 mice developed severe toxicity, which appeared to be unrelated to disease, likely graft-versus-host disease, and were subsequently excluded from the experiment. Although a strong antitumoral response could also be observed in the BiTE molecule–treated group (Figure 4B), overall survival was lower than in the CAR T-cell treatment group (Figure 4C). Ex vivo analysis at the individual experimental end points revealed an increase of human $CD45^+CD3^+$ ($hCD45^+CD3^+$) T cells in the blood of the CAR-treated mice. In comparison, the blood and bone marrow of the BiTE molecule–treated mice contained higher frequencies of $hCD45^+CD33^+$ leukemic cells (Figure 4D-E). Furthermore, analysis of the spleens revealed higher numbers of $hCD45^+CD3^+$ in the CAR-treated group, demonstrating stronger T-cell proliferation (Figure 4F). Taken together, these data indicate a benefit of CAR T-cell treatment over BiTE molecule treatment in our in vivo model.

BiTE molecule–redirected T cells exhibit a more exhausted transcriptional profile than CAR T cells in an NGS mouse model of AML

To identify transcriptional profiles that drive the functional and metabolic differences between CAR T cells and BiTE molecule–redirected T cells, we repeated the previously described in vivo AML xenograft model to harvest the T cells for bulk RNA sequencing. Based on our previous experiments, T cells were isolated from the bone marrow of mice 18 days after treatment was initiated (Figure 5A). Unbiased principal component analysis revealed separate clustering of the samples according to the treatment platform. Differential gene expression analysis identified 387 genes upregulated and 86 genes downregulated in CAR T cells compared with T cells from mice treated with the BiTE molecule (adjusted P value <.01, fold change of >1 or less than −1). Among the markedly upregulated genes in the BiTE molecule–treated samples, we found several were related to T-cell exhaustion (*PDCD1*, *CTLA4*, *LAG-3*, *TIGIT*, *TOX*, and *TOX2*; Figure 5B-C). Among the markedly upregulated genes in CAR T cells were genes related to enhanced T-cell signaling (*CD9*, *CMIP*, *TNFSF10*, *FOS*, *RAB27B*, and *NCAM1*; Figure 5B-C). Reactome analysis further revealed an upregulation of processes involved in synapse formation and cytokine signaling in CAR T cells compared with T cells from mice treated with the BiTE molecule (Figure 5D). Gene set enrichment analysis also showed enrichment of effector- vs exhaustion-related genes in CAR T cells (Figure 5E; GSE9650, normalized enrichment score [NES], 1.2; false discovery rate, $q = 0.005$).[21] Furthermore, pathway analysis revealed upregulation of several Hallmark gene sets related to the cell cycle (G2M checkpoint: NES, 1.43; $P = .002$) and metabolism (glycolysis: NES, 1.28; $P = .005$; fatty acid metabolism: NES, 1.29; $P = .008$) in CAR T cells (Figure 5F). Together, these data suggest that CAR T cells were more functional and less exhausted than BiTE molecule–redirected T cells in our in vivo AML xenograft model.

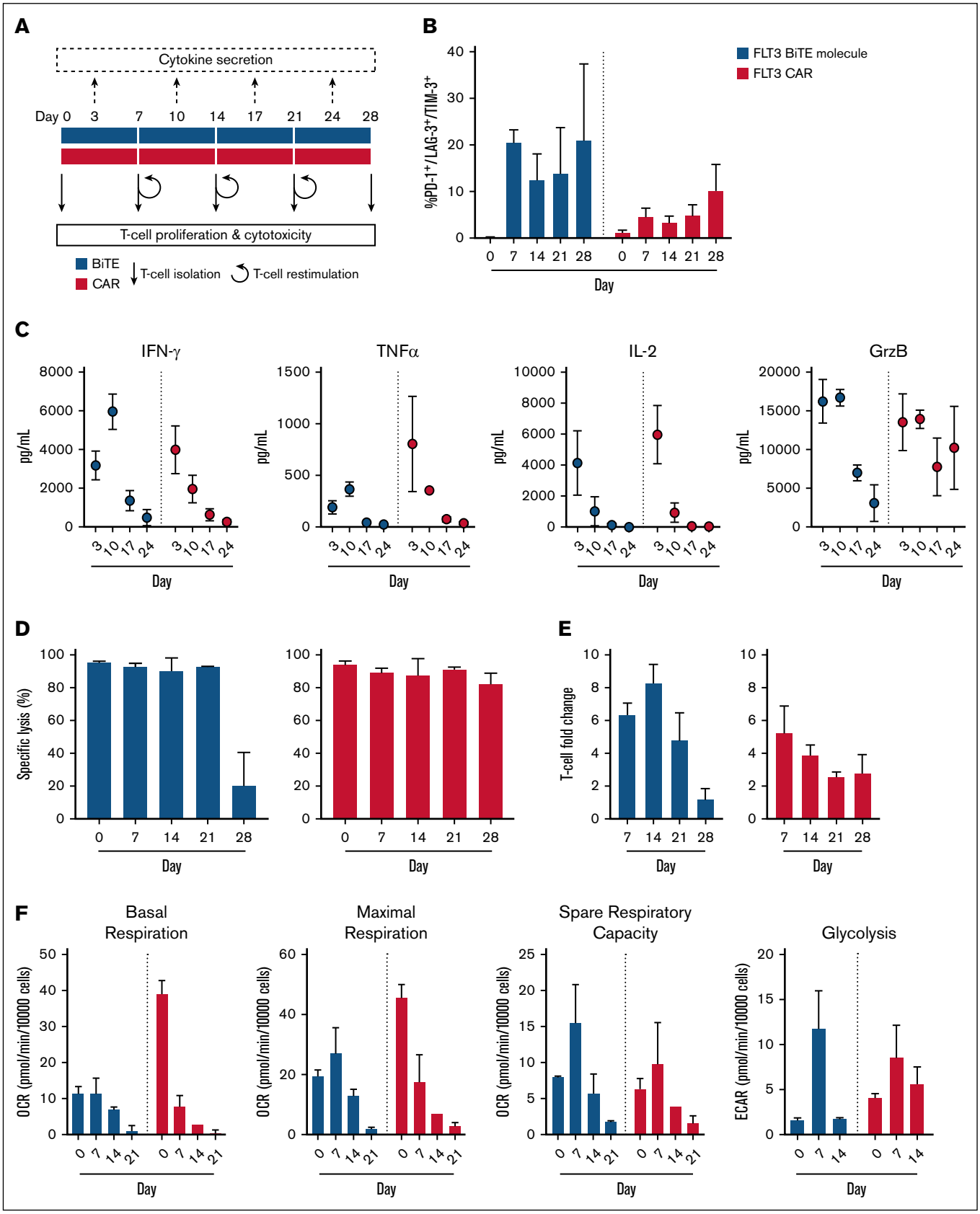
Positive costimulatory signals enhance BiTE molecule–mediated T-cell efficacy in vivo

We hypothesized that BiTE molecules are less capable of counteracting T-cell exhaustion and preventing AML outgrowth compared with CAR T cells, due to the absence of a costimulatory domain. Conceptually, BiTE molecules resemble first-generation CAR T cells, relying on the costimulatory profile of target cells. By contrast, second-generation CAR constructs include a built-in CD28 costimulatory domain, enabling T-cell activation independently of the target cell's costimulatory status.

To test this, we used our established Ba/F3 model,[14] in which murine Ba/F3 cells overexpress human CD86. Expression of CD86 enhanced BiTE molecule–mediated cytotoxicity, whereas CAR-mediated killing remained unaffected (supplemental Figure 1E). We then translated this finding to CD86-overexpressing OCI-AML3 cells for further in vitro testing. Again, positive costimulation increased BiTE molecule– but not CAR-mediated lysis of AML cells (Figure 6A).

Next, we compared BiTE molecule and CAR constructs in vivo using OCI-AML3-LUC-GFP cells expressing CD86 as target cells (OCI-AML3-LUC-GFP-$CD86^{high}$; Figure 6B). Both treatment groups showed comparable responses up to day 40 (Figure 6C-D). As expected, some mice developed severe, non–disease-related

Figure 2. BiTE molecule and CAR T cells efficiently eliminate pAML cells with minimal off-leukemia toxicity. (A) FLT3 expression levels of pAML samples used for the following experiments: BiTE molecule– and CAR-mediated cytotoxicity against pAML cells in an (B) allogeneic and (C) autologous setting after 6 days of coculture (allogeneic, n = 12; autologous, n = 2-3). (D) Secretion of IFN-γ, TNF, IL-2, and GrzB determined by CBA analysis (n = 6), from corresponding allogeneic cytotoxicity assays at an E:T ratio of 1:5. (E) BiTE molecule– and CAR-mediated cytotoxicity against healthy bone marrow mononuclear cells (BMMCs) after 72 hours (n = 6) at different E:T ratios. (F) Cytotoxicity of BiTE molecule– and CAR-redirected T cells against HD $hCD34^+$ BMMCs after 6 hours at an E:T ratio of 10:1. (G) CFU after coculture of BiTE molecule– and CAR-redirected T cells with $CD34^+$ cells isolated from healthy BMMCs for 6 hours at an E:T ratio of 10:1. The total number of colonies was determined 2 weeks after plating. (H) On-target off-leukemia cytotoxicity of BiTE molecule– and CAR-redirected T cells against a mixture of pAML cells and healthy BMMCs. Bar and dot graphs represent the mean ± SEM values. Statistical analysis: 2-way analysis of variance and the Šidák multiple comparison test (panel D); ns_$P > .05$; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. cBiTE, control BiTE; MFI, median fluorescence intensity; MFI ratio, MFI of sample to MFI of isotype control.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TNFα
IL-2
GrzB
pg/mL
Day
D
Specific lysis (%)
Day
E
T-cell fold change
Day
F
Basal Respiration
Maximal Respiration
Spare Respiratory Capacity
Glycolysis
OCR (pmol/min/10000 cells)
ECAR (pmol/min/10000 cells)
Day

Figure 3.

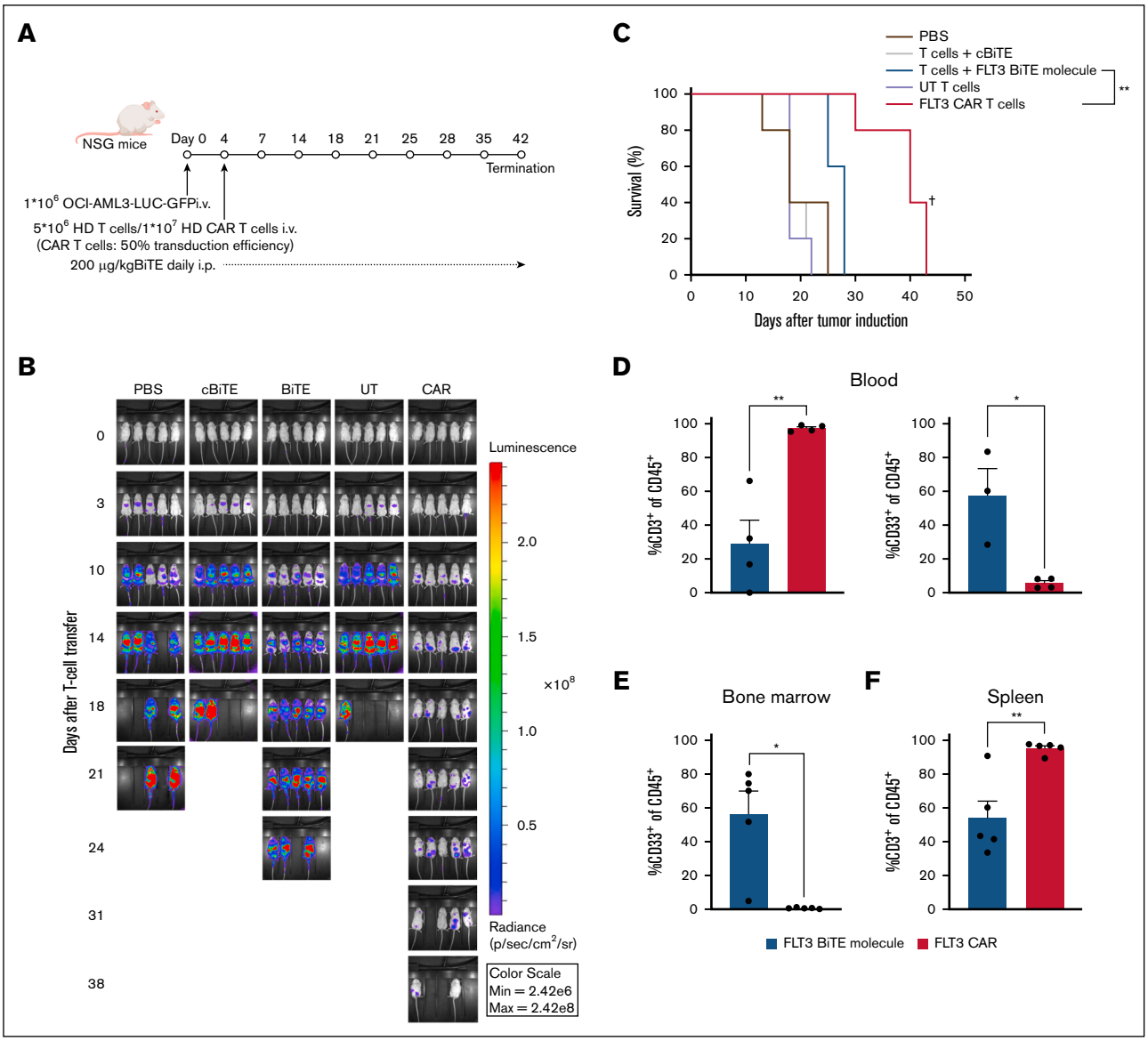

Figure 4. Superior antileukemia activity of CAR T cells in an AML xenograft model. (A) Schematic overview of the experimental setup. NXG mice were inoculated IV with 1×10^6 OCI-AML3-LUC-GFP tumor cells. Mice were treated with a single IV injection of T cells. BiTE molecule was given by daily IP injections (200 µg/kg per injection) as indicated by the arrows in the figure. A total of 5×10^6 HD T cells or 1×10^7 HD CAR T cells (50% transduction efficiency) were given IV as indicated. Treatment groups were as follows: PBS (n = 5), cBiTE (n = 5), BiTE (n = 5), UT (n = 5), and CAR (n = 5). (B) In vivo images with luminescence intensity counts for all experimental groups from treatment day onward (days 0, 3, 10, 14, 18, 21, 24, 31, and 38). (C) Kaplan-Meier plot of survival probability. (D) Analysis of blood samples on day 14 after the start of treatment. Frequencies of $hCD3^+$ and $hCD33^+$ cells were determined by flow cytometry. (E) Analysis of tumor burden in the bone marrow. Frequencies of $hCD33^+$ cells were determined by flow cytometry. (F) Analysis of T-cell homing to the spleens of mice. Frequencies of $hCD3^+$ cells were determined by flow cytometry. Bar and dot graphs represent the mean ± SEM values. Statistical analysis: log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon testing (panel C); Kruskal-Wallis and Dunn multiple comparison testing (panels D-E); ns_$P > .05$; *$P < .05$; **$P < .01$. †Mice were euthanized because of non–disease-related toxicity. cBiTE, control BiTE; PBS, phosphate-buffered saline.

Figure 3. Functional decline of BiTE molecule– and CAR-redirected T cells through continuous antigen exposure. (A) Timeline of continuous T-cell stimulation with BiTE molecule or CAR and functional testing over 28 days. (B) Percentage of $CD3^+$ T cells coexpressing PD-1, TIM-3, and LAG-3. (C) Secretion levels of IFN-γ, TNF, IL-2, and GrzB measured in coculture supernatants determined by CBA. (D) BiTE molecule– and CAR-mediated cytotoxicity against MOLM-13 cells. (E) BiTE molecule– and CAR-mediated T-cell proliferation expressed as $CD2^+$ fold change (n = 3) upon coculture with MOLM-13 cells. (F) Bar graphs of normalized OCR and ECAR obtained during the metabolic stress testing, showing basal respiration, maximal respiration, spare respiratory capacity, and glycolysis. Bar graphs represent the mean ± SEM values. OCR, oxygen consumption rates; PD-1, programmed cell death protein 1; TIM-3, T-cell immunoglobulin and mucin domain containing 3.

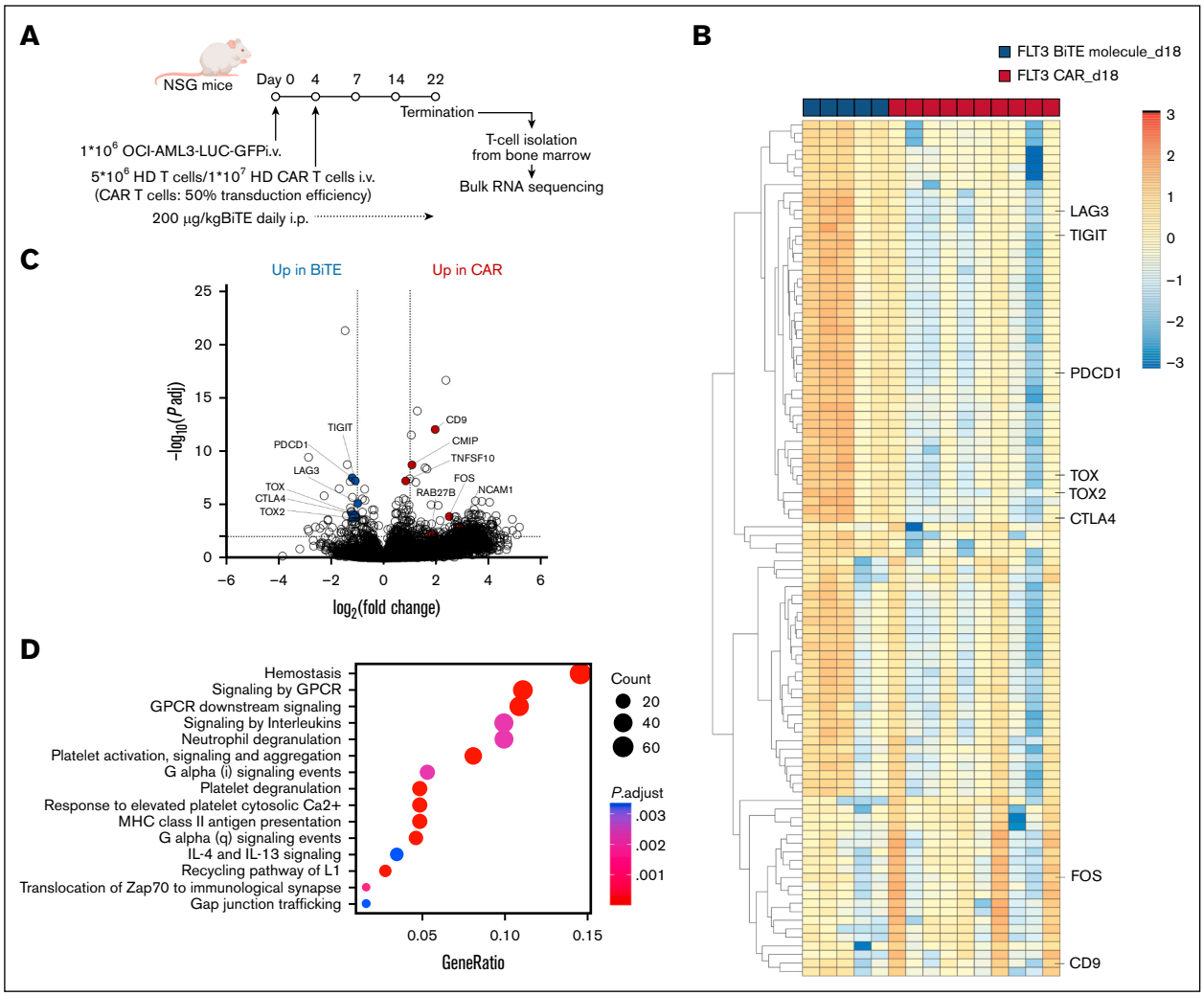

Figure 5. BiTE molecule–redirected T cells exhibit a more exhausted transcriptional profile. (A) Schematic overview of the experimental setup. NXG mice were inoculated IV with 1 × 10^6 OCI-AML3-LUC-GFP tumor cells. Mice were treated with a single IV injection of T cells. BiTE molecule was given by daily IP injections (200 µg/kg per injection) as indicated by the arrows in the figure. A total of 5 × 10^6 HD T cells or 1 × 10^7 HD CAR T cells (50% transduction efficiency) were given IV as indicated. Treatment groups were as follows: PBS (n = 5), cBiTE (n = 5), BiTE (n = 5), UT (n = 5), and CAR (n = 5). The experiment was terminated 18 days after the start of the treatment. T cells were isolated from the bone marrow of mice and sorted for bulk RNA sequencing. (B) Heat map depicting the top 100 differentially expressed genes in day-18 CAR vs BiTE molecule–redirected T cells; adjusted *P* value (P_{adj}) < .05. Selected genes are highlighted. (C) Volcano plot of day-18 CAR vs BiTE molecule–redirected T cells; P_{adj} < .05. Selected genes are highlighted as significantly downregulated (blue) and upregulated (red) in day 18 CAR vs BiTE-redirected T cells. (D) Pathways enriched in day 18 CAR vs BiTE molecule–redirected T cells; P_{adj} < .05. (E) Gene set enrichment analysis of day 18 CAR vs BiTE-redirected T cells using the Molecular Signatures Database (MSigDB) and the gene sets GSE9650_EFFECTOR_VS_MEMORY_CD8_TCELL_UP.[21] (F) Hallmark gene sets upregulated in CAR T cells compared with BiTE molecule–redirected T cells. cBiTE, control BiTE; CTLA4, Cytotoxic T-Lymphocyte-Associated Protein 4; FOS, FBJ Murine Osteosarcoma Viral Oncogene Homolog; GPCR, G-protein coupled receptor; PDCD1, Programmed Cell Death 1; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TOX, Thymocyte selection-associated high mobility group box.

toxicity, likely graft-versus-host disease, and were excluded from further analysis.

Ex vivo end point analysis revealed similar infiltration of $hCD45^+CD3^+$ T cells into the bone marrow (Figure 6E) and spleen (Figure 6F) of both treatment groups. Notably, the addition of CD86-mediated costimulation improved survival in BiTE molecule–treated mice compared with our initial in vivo experiment without CD86 (Figure 6G). These results suggest that costimulation is a critical factor for enhancing T-cell proliferation, persistence, and antitumor efficacy in BiTE molecule–based therapies in vivo.

Discussion

T-cell–based immunotherapies have transformed treatment paradigms in hematologic malignancies, particularly B-cell malignancies, against which CD19-targeted CAR T-cell therapies have achieved remarkable clinical success.[22,23] However, the translation of this success to AML has been limited, primarily due to the scarcity of leukemia-specific antigens and the potential for on-target off-leukemia toxicity. Albeit still early, innovative engineering approaches such as IL-18–secreting CAR T cells have recently been explored in AML, showing feasibility and first signs of activity in a pilot study.[24] FLT3 has emerged as an attractive target in AML

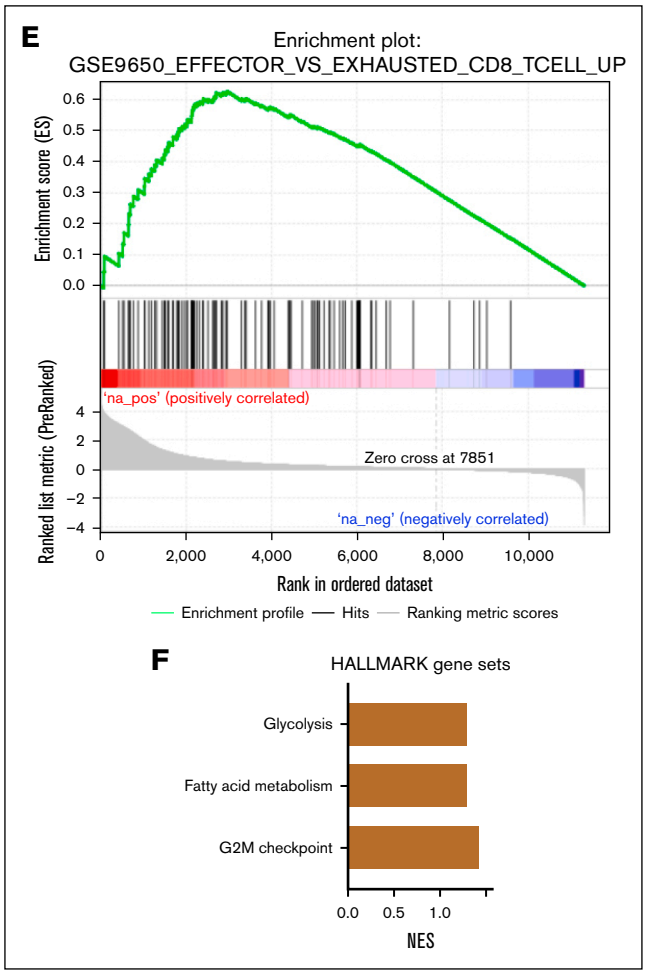

Figure 5 (continued)

due to several key features. First, FLT3 is overexpressed in most AML cases, regardless of its FLT3 mutational status, with both mutant FLT3 and wild-type FLT3 leukemic cells often expressing the antigen on the cell surface.[7,10,11,25] Second, and particularly relevant for therapeutic applications, FLT3 expression can be upregulated further through FLT3 tyrosine kinase inhibitors such as quizartinib or gilteritinib, offering a unique opportunity for synergistic combinations.[26-28] Third, FLT3 expression is largely restricted to early hematopoietic progenitors and is absent in long-term repopulating HSCs, reducing the risk of long-lasting hematopoietic toxicity.[25,29,30]

To validate FLT3 as a safe target antigen in AML, we tested our FLT3 CAR T cells and BiTE molecule against primary hematopoietic precursor cells. Cytotoxicity and progenitor function were assessed using bone marrow–derived $CD34^+$ hematopoietic stem and progenitor cells. Neither FLT3 CAR T cells nor BiTE molecule–redirected T cells impaired colony formation in CFU assays, indicating a preserved clonogenic capacity. These data, together with minimal cytolysis in healthy bone marrow cocultures, suggest a lack of significant off-leukemia toxicity and are in line with previous observations that long-term HSCs lack FLT3 surface expression. To further rule out bystander effects, we coincubated $FLT3^+$ AML blasts with healthy bone marrow cells. Unlike $CD33^+$ or $CD123^+$ targeted approaches,[31,32] FLT3 targeting did not trigger collateral damage to surrounding healthy cells, even in the presence of excess antigen load. This is consistent with early data from a trial using a FLT3 monoclonal antibody in patients with AML with measurable residual disease.[33] Nonetheless, neither our in vitro assays nor our in vivo model using immunodeficient mice can comprehensively evaluate the risk of on-target off-leukemia toxicity or AML-related toxicities caused by inflammatory cytokines. This question will need to be investigated further in future studies using humanized mouse models.

Having established the therapeutic potential and safety profile of FLT3 as a target in AML, we compared the efficacy of 2 different immunotherapy platforms, CAR T cells and BiTE molecules, for efficacy in short- and long-term cultures against various AML cell lines and also pAML samples. Our experimental setting was unique for having a CAR construct (FLT3-CD28-CD3.z) and a BiTE molecule (FLT3×CD3) with an identical single-chain variable region for comparison.

Both constructs induced FLT3-specific cytotoxicity against AML cell lines in short-term cultures, consistent with previous reports of antigen-restricted killing by FLT3-directed TCEs and CAR T cells.[7,34] CAR T cells showed stronger cytokine release, proliferation, and metabolic activity in vitro compared with BiTE molecule–activated T cells, likely due to their preactivated state after manufacturing, a finding consistent with other studies comparing unmanipulated and ex vivo expanded T-cell products. This difference was abolished when preactivated T cells were used in assays involving BiTE molecules, resulting in equivalent cytotoxicity against AML cell lines and underlining the relevance of the culture conditions before coculture.

To assess functional persistence under sustained antigen exposure, we next undertook long-term coculture assays designed to model T-cell exhaustion. Notably, under chronic stimulation, a known driver of T-cell dysfunction,[12,20,35] CAR T cells retained cytotoxicity longer than BiTE molecule–redirected T cells, which exhibited earlier and more pronounced loss of effector function alongside upregulation of exhaustion markers such as *PDCD1*, *TOX*, and *LAG-3*.[35-38] Transcriptomic profiling confirmed this phenotype; BiTE molecule–redirected T cells displayed a transcriptional program consistent with exhaustion, whereas CAR T cells maintained signatures linked to activation, metabolism, and cell cycle progression. These differences likely reflect the integrated costimulatory signaling domains (CD28 or 4-1BB) in second-generation CAR constructs, which enhance T-cell persistence and resistance to exhaustion.[39-42]

We found that providing CD86-mediated costimulation at AML targets enhanced BiTE molecule–induced T-cell responses both in vitro and in vivo. This further supports emerging strategies combining TCEs with targeted costimulatory agonists, such as CD19-CD28 fusion proteins paired with glofitamab, which have shown enhanced antitumor activity in preclinical lymphoma models.[43] Early-phase clinical trials are already evaluating such combinations, which are demonstrating safety, feasibility, and promising response rates.

Taken together, our data highlight the intrinsic advantages of CAR T cells in AML, particularly their prolonged effector function and superior in vivo persistence. At the same time, they point to a rational path forward for BiTE molecule–based strategies: combining TCEs with targeted costimulatory signals. Approaches

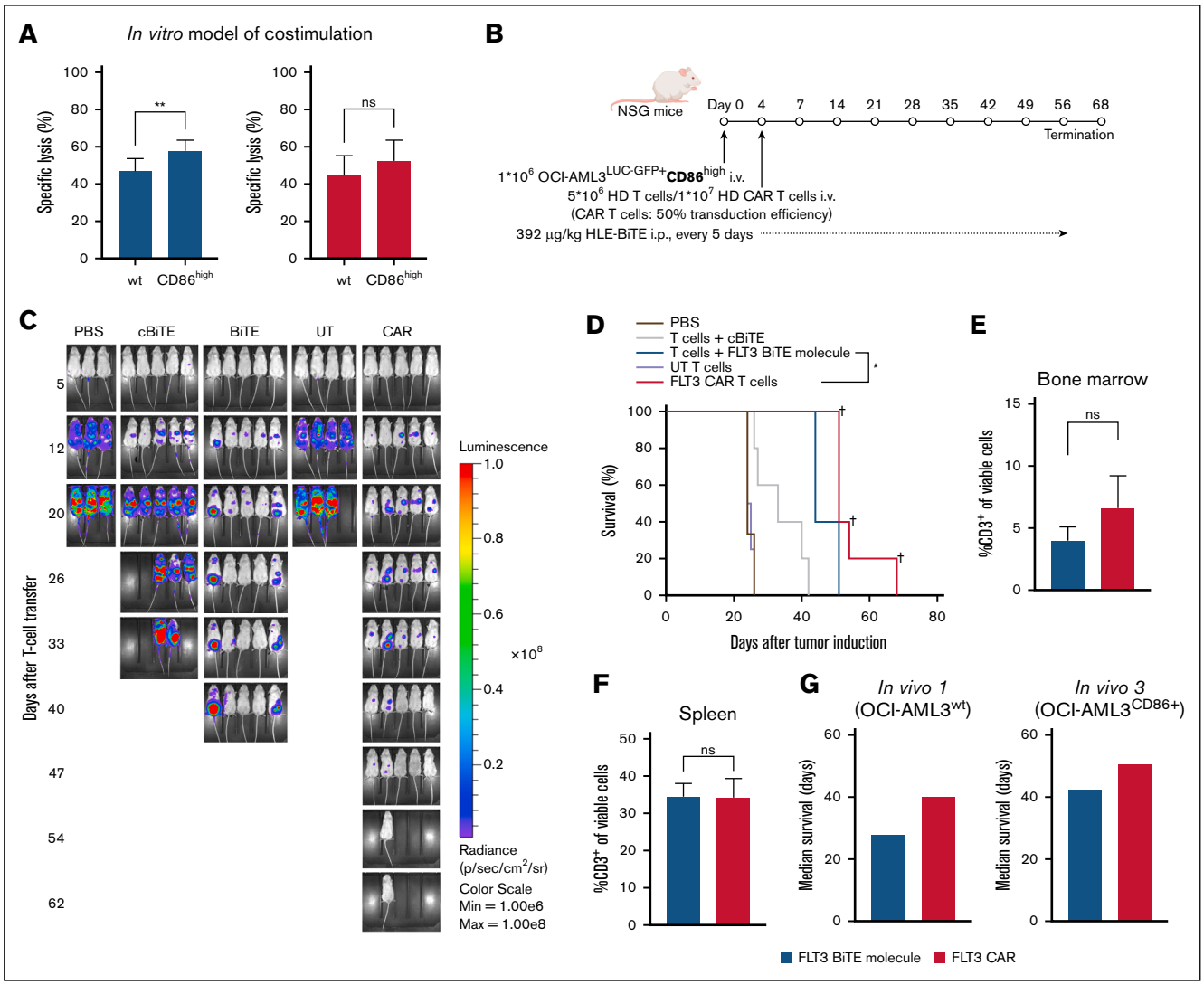

Figure 6. Positive costimulatory signals enhance BiTE molecule–mediated T-cell efficacy in vivo. (A) In vitro cell line model demonstrating the effect of positive costimulation by CD86 on BiTE molecule– and CAR-mediated cytotoxicity. (B) Schematic overview of the experimental setup. NXG mice were inoculated IV with 1 × 10^6 OCI-AML3-LUC-GFP-CD86^{high} tumor cells. Mice were treated with a single IV injection of T cells. The half-life extended (HLE) BiTE molecule was given by IP injections (392 µg/kg per injection) every 5 days. 5 × 10^6 HD T cells or 1 × 10^7 HD CAR T cells (50% transduction efficiency) were given IV as indicated. Treatment groups were as follows: PBS (n = 5), cBiTE (n = 5), BiTE (n = 5), UT (n = 5), and CAR (n = 5). (C) In vivo images with luminescence intensity counts for all experimental groups from treatment day onward (days 5, 12, 20, 26, 33, 40, 47, 54, 62). (D) Kaplan-Meier plot of survival probability. (E-F) Analysis of murine bone marrow and spleen. Frequencies of hCD3$^+$ cells were determined by flow cytometry. (G) Comparison of median survival of HLE BiTE molecule–treated and CAR-treated mice between in vivo 1 (Figure 4; without additional costimulation) and in vivo 3 (Figure 6; with additional costimulation). Statistical analysis: log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon testing (panel D); Kruskal-Wallis and Dunn's multiple comparison testing (panels A,E-F); ns_$P > .05$; *$P < .05$; **$P < .01$. †Mice were euthanized because of non–disease-related toxicity. max, maximum; min, minimum; ns, not significant; PBS, phosphate-buffered saline; wt, wild type.

akin to CD19–4-1BB or CD19-CD28 constructs plus glofitamab[43] might improve functional durability and broaden the clinical reach of BiTE molecule immunotherapy in myeloid malignancies.

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Authorship

Contribution: M.S., V.B., and L.R. designed the study and supervised the project; L.R., D.N., V.B., and M.S. wrote the manuscript; L.R., D.N., H.S., and M.E.K. performed experiments and analyzed and/or interpreted the data; M.S., V.B., L.R., D.N., B.B., N.P., G.H., A.M., G.M., R.L.G., and T.A. were involved in research design and data interpretation; S.T. and S.K. critically reviewed and discussed the data; D.R. performed library preparation for bulk RNA sequencing; M.K. conducted the bioinformatic analysis in collaboration with T.S.; and F.M., A.G., and G.V.H. performed the in vivo experiments.

Conflict-of-interest disclosure: M.S. has received industry support from Amgen, Bristol Myers Squibb (BMS)/Celgene, Gilead/Kite, Johnson & Johnson, Miltenyi Biotec, Novartis, Roche, Seattle Genetics, and Takeda; serves as a consultant/advisor for AbbVie, Crossbow, Debiopharm, Gilead/Kite, Interius, Johnson & Johnson, Molecular Partners, Novartis, and Otsuka; and serves on the speakers bureau at Amgen, BMS/Celgene, Gilead/Kite, Miltenyi Biotec, Novartis, Roche, and Takeda. S.T. received honoraria from Amgen but not related to this project. S.K. has received honoraria from Plectonic, TCR2 Inc, Miltenyi Biotec, Galapagos, Cymab, Novartis, BMS, and GSK; is an inventor of several patents in the field of immuno-oncology; and has received research support from TCR2 Inc, Tabby Therapeutics, Catalym GmbH, Plectonic GmbH, and Arcus Biosciences for work unrelated to the manuscript. S.K. received license fees from TCR2 Inc and Carina Biotech. T.A. and R.L.G. were employed by Amgen. V.B. has received research funding from Gilead/Kite and Miltenyi Biotec; has received educational grants from BMS, Novartis, Takeda, and Roche; has served as a consultant/advisor for Amgen, Gilead/Kite, Novartis, Pfizer, and Priothera; and serves on the speakers bureau for Novartis and Pfizer. The remaining authors declare no competing financial interests.

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