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Bispecific CD33/CD123 targeted chimeric antigen receptor T cells for the treatment of acute myeloid leukemia

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CD33 and CD123 are expressed on the surface of human acute myeloid leukemia blasts and other noncancerous tissues such as hematopoietic stem cells. On-target off-tumor toxicities may limit chimeric antigen receptor T cell therapies that target both CD33 and CD123. To overcome this limitation, we developed bispecific human CD33/CD123 chimeric antigen receptor (CAR) T cells with an "AND" logic gate. We produced novel CD33 and CD123 scFvs from monoclonal antibodies that bound CD33 and CD123 and activated T cells. Screening of CD33 and CD123 CAR T cells for cytotoxicity, cytokine production, and proliferation was performed, and we selected scFvs for CD33/CD123 bispecific CARs. The bispecific CARs split 4-1BB co-stimulation on one scFv and CD3ζ on the other. *In vitro* testing of cytokine secretion and cytotoxicity resulted in selecting bispecific CAR 1 construct for *in vivo* analysis. The CD33/CD123 bispecific CAR T cells were able to control acute myeloid leukemia (AML) in a xenograft AML mouse model similar to monospecific CD33 and CD123 CAR T cells while showing no on-target off-tumor effects. Based on our findings, human CD33/CD123 bispecific CAR T cells are a promising cell-based approach to prevent AML and support clinical investigation.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by excessive clonal proliferation of myeloid cells.[1,2] Treatment options for AML include chemotherapy and allogeneic hematopoietic stem cell transplant (alloHSCT) for patients at high risk of relapse.[3,4] However, the success rate for alloHSCT is limited, with patients suffering from complications such as graft-versus-host disease.[5,6] Therefore, an unmet need remains for the development of new therapies for AML. CD33 and CD123 are highly expressed on AML blasts, with more than 70% expressing both,[7–11] and have been targeted with chimeric antigen receptor (CAR) T cell therapies.[12,13]

CAR T cell therapy has shown significant potential as a cancer therapy.[14,15] CAR T cells have demonstrated remarkable activity for treating relapsed/refractory acute lymphoblastic leukemia, diffuse large B cell lymphoma, and mantle cell lymphoma,[16–19] with the US Food and Drug Administration (FDA) approving CD19 CAR T cells for the treatment of B cell hematologic malignancies. CARs targeted at either CD33 or CD123 have shown potency in preclinical models and clinical trials[20–23] but cannot differentiate between normal and cancerous cells.[24–27] This is primarily due to the heterogeneous nature of AML blasts and the expression of the same targets on hematopoietic stem cells (HSCs).[13,28]

CARs targeting multiple antigens have been reported in preclinical and clinical trials for specifically targeting tumors and controlling relapse.[10,11,29,30] One strategy to achieve this is by using an "AND" gate, which recognizes two different antigens on a single target cell to function. This is done by separating the CD3ζ activation domain and the co-stimulatory domain to separate single-chain variable fragment (scFv) binding domains.[31] The individual constructs can then be co-transduced into a T cell separately or separated by a P2A skip sequence in a single construct and transduced.

In the current study, we report *de novo* production of CD33 and CD123 scFvs with split CAR signals used to create bispecific CARs. These CD33/CD123 bispecific CARs were then screened for optimal proliferation, cytokine production, cytotoxicity, and HSC toxicity *in vitro*. This resulted in a candidate CAR, which we examined *in vivo* using a xenograft AML mouse model to measure efficacy and a

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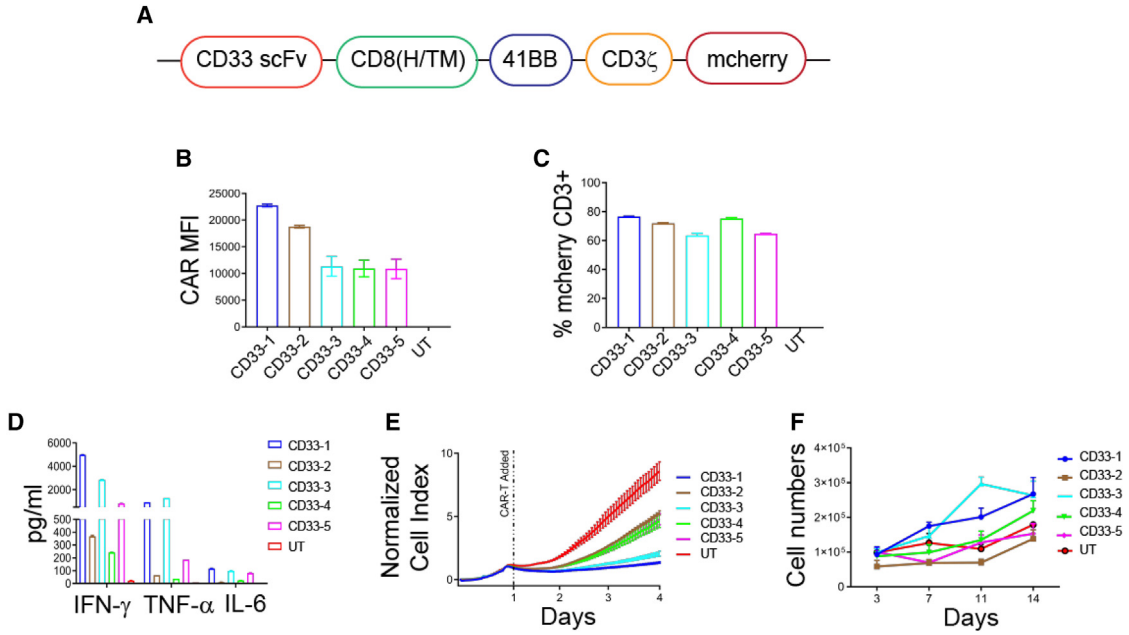


Figure 1. *In vitro* efficacy of anti-CD33 CAR constructs

Healthy donor T cells were transduced with CD33 CAR constructs (CD33-1 through CD33-5) and analyzed using flow cytometry on day 7. (A) Schematic of the CD33 CAR construct. (B) MFI of CAR T cells expressing the mCherry reporter and UT control T cells (mCherry negative). (C) Percentage of CD3 T cells expressing mCherry to indicate transduction levels. (D) IFN-γ, TNF-α, and IL-6 secreted by UT or CAR T cells after 24 h of stimulation with target cells (CHO-CD33). (E) CAR T cells or UT cells co-cultured with target cells, and cytotoxicity was measured using a real-time cell-analysis system. The data are presented as the average normalized cell index over time for triplicate wells. The normalized cell index was calculated as the cell index at a given time point divided by the cell index at the normalized time point, which was day 1 after the addition of T cells. (F) CAR T cells or UT cells were stimulated with target cells and the absolute number of T cells counted over 14 days. Values represent the mean ± SEM. The experimental data shown represent two independent experiments that used T cells from different healthy donors.

$CD34^+$ humanized mouse model to assess safety. We found that the bispecific CAR could control the tumor, similar to monospecific CD33 and CD123 CAR T cells, and had no measurable on-target off-tumor effects.

RESULTS

Selection of novel anti-CD33 and anti-CD123 CAR constructs

We generated novel scFvs for our bispecific CAR using technology as outlined in Figure S1 and materials and methods. These antibodies were tested for antigen specificity by ELISA followed by evaluation of binding specificity using EL4 cells expressing CD33 or CD123 and high-throughput flow cytometry (Figure S2). To confirm the ability of the epitope to induce T cell activation upon binding, we used a Jurkat NFAT-GFP reporter cell line expressing CD16/CD32. The reporter cells were co-cultured with a novel CD33 antibody and Chinese hamster ovary (CHO) cells expressing CD33 with activation measured by GFP expression. We selected two CD33 clones based on either binding affinity or activation efficiency (Figure S3). Evaluation of the immunoglobulin heavy (IgH) and immunoglobulin light (IgL) chain sequences revealed the same IgL chains but different IgH chains for the CD33 clone 6A11. In contrast, CD33 clone 27A3 had the same heavy chain but three different light chains (Figure S4). To identify the most likely correct IgH and IgL pair, we created five anti-CD33 scFvs paired to the CD8 hinge and transmembrane domain, intracellular 41BB co-stimulatory domain, and CD3ζ activation domain. An mCherry tag was used to identify the CAR T cells from non-CAR T cells (Figure 1A). The CD33 targeting CAR T cells were generated by retroviral transduction, as previously reported.[32,33] The five CD33 CAR constructs were given shorter names for easier reference (Figure S4).

The CD33 CAR T cell constructs exhibited a high degree of transduction efficiency with mean fluorescence intensity (MFI) levels >10,000 (Figure 1B) and >65% of the T cells expressing mCherry (Figure 1C). When cultured with CHO-$CD33^+$ target cells (Figure S5), CD33 CAR T cells CD33-1 and CD33-3 demonstrated high expression levels of interferon-γ (IFN-γ) (3,000–4,000 pg/mL), tumor necrosis factor α (TNF-α) (approximately 1,000 pg/mL), and interleukin-6 (IL-6) (approximately 100 pg/mL) compared to those of CAR T cells CD33-2, CD33-4, and CD33-5 (Figure 1D). Furthermore, CD33-1 and CD33-3 CAR T cells demonstrated potent killing (5-fold) (Figure 1E) and proliferation (2-fold) (Figure 1F) against $CD33^+$ target cells compared to that of mock-transduced T cells. There were no clear differences in the proportions of CD4 (60%–90%) or CD8 (10%–25%) T cells (Figure S6A) nor in the proportions of CD8 memory T cells, such as T central memory (TCM; mean 1%), T effector memory (TEM; mean 2%), or TEM CD45RA (TEMRA; mean 70%) (Figure S6B).

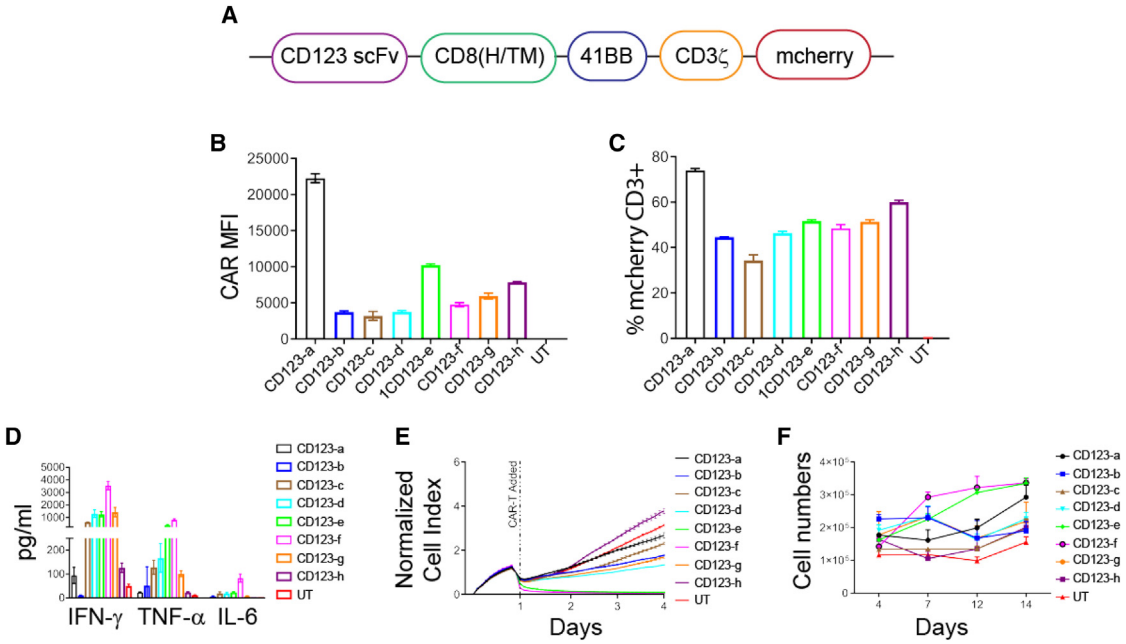


Figure 2. *In vitro* efficacy of anti-CD123 CAR constructs

T cells isolated from peripheral blood mononuclear cells of healthy donors were transduced with CD123 CAR constructs (CD123-a through CD123-h). (A) Schematic of the CD123 CAR construct. (B) MFI of CAR T cells expressing the mCherry reporter and UT control T cells. (C) Percentage of CD3 T cells expressing mCherry to indicate transduction levels. (D) Levels of IFN-γ, TNF-α, and IL-6 secreted by UT or CAR T cells after stimulation with target cells (CHO-CD123). (E) CAR T or UT cells were co-cultured with target cells, and cytotoxicity was measured using a real-time cell-analysis system. The data are presented as the average normalized cell index over time for triplicate wells. (F) CAR T cells or UT cells were stimulated with target cells and the absolute number of T cells determined over 14 days. Values represent the mean ± SEM. The experimental data shown represent two different independent experiments that used T cells from two healthy donors.

For CD123, we selected three hybridoma clones (Figure S7) that showed high levels of binding (99%) and various levels of Jurkat CD16/CD32 T cell activation (7%–39%). IgH and IgL chains were sequenced for clones 3F5, 12H1, and 15A12 and used to generate eight different CD123 CAR T cell constructs (Figure S8). The eight anti-CD123 CAR designs were identical to the CD33 constructs, including the mCherry tag (Figure 2A). Most of the CD123 CAR constructs exhibited similar high degrees of transduction except CD123-a, which showed a much higher MFI (Figure 2B). T cell expression of mCherry was approximately 50% for most of the CD123 CARs (Figure 2C). The CD123 CAR T cells demonstrated variable expression levels of IFN-γ (100–4,000 pg/mL), TNF-α (10–1,500 pg/mL), and IL-6 (10–100 pg/mL) (Figure 2D). CARs CD123-e and CD123-f demonstrated rapid killing at 3-fold higher levels than the UT control and other CARs (Figure 2E). In addition, CD123-f and CD123-g CAR T cells demonstrated enhanced proliferation (2-fold) on days 7 and 14 compared to that of UT (Figure 2F). The prevalence of CD4 (mean 10%) and CD8 T cells (mean 70%) (Figure S9A) and T cell memory markers TCM (mean 0.5%), TEM (mean 1%), and TEMRA (mean 90%) were similar among all the groups (Figure S9B).

CD33 and CD123 CAR pairing optimization

To determine the best combination of CD33 and CD123 scFvs for the bispecific CAR, we selected four anti-CD33 CARs, including CD33-1 and CD33-3, which demonstrated potent effector responses, and CD33-2 and CD33-4, which demonstrated moderate effector responses. Similarly, for CD123 we selected four CARs, including CD123-f and CD123-g, which were highly efficacious at killing, producing cytokines, and proliferation, and CD123-a and CD123-d, which exhibited lower effector responses. We double-transduced T cells with two monospecific CARs, one with 4-1BB and one with CD3ζ, to create an "AND" gate (Figure 3A), resulting in a total of 32 CAR T cell combinations (Figure S10).

We used CHO cells expressing CD33, CD123, or CD33 and CD123 as target cells to evaluate the differences in cytotoxicity and cytokine secretion between the CARs. A normalized cell index on a scale of 0–4, which was the inverse of cytotoxicity with 0–1 representing greater cytotoxicity, was represented via heatmaps. CARs CD33-2 41BB/CD123-f CD3ζ, CD33-1 CD3ζ/CD123-a 41BB, CD33-3 CD3ζ/CD123-a 41BB, and CD33-4 CD3ζ/CD123-f 41BB had an increase in cytotoxicity with the CHO-CD33/CD123 targets compared to either CHO-CD33 or CHO-CD123 alone (Figure 3B). The dotted line in Figure 3B represents the switch in the co-stimulatory domain CD3ξ to 41BB or vice versa for the same set of 16 scFvs. CARs that exhibited no cytotoxicity to target cells only when 41BB co-stimulatory was used to target the antigen were excluded from cytokine analysis.

Cytokine secretion was analyzed and displayed on heatmaps to compare differences across the cell targets. We observed a correlation

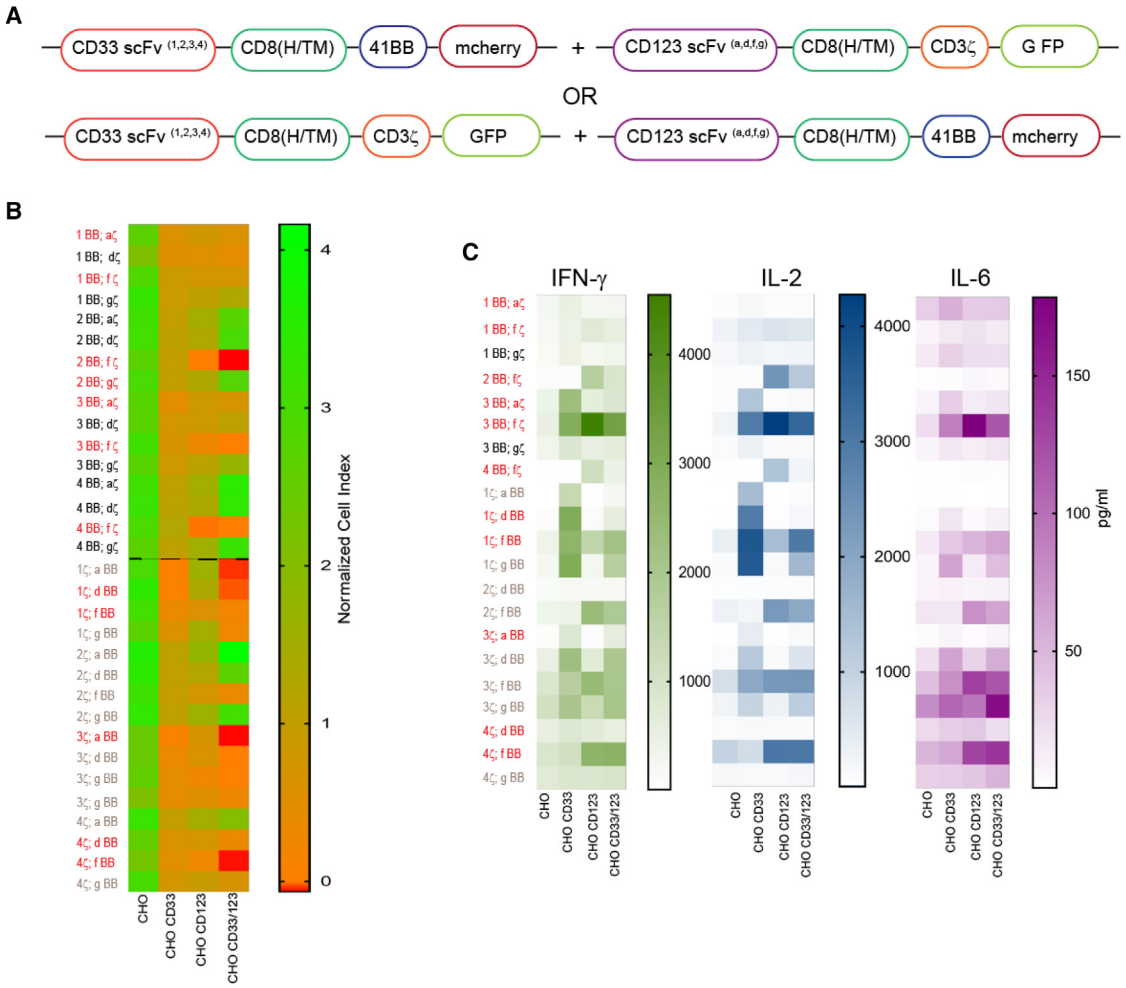


Figure 3. CD33 and CD123 CAR pairing optimization

T cells isolated from peripheral blood mononuclear cells of a healthy donor were transduced twice, first with CD33 CARs with either CD3ζ or 41BB and then again with CD123 CARs with a CD3ζ or 41BB stimulation domain. (A) Schematic of the CD33/CD123 combination CAR constructs. (B) CAR T cells were co-cultured with empty CHO (CHO), CD33-expressing CHO (CHO-CD33), CD123-expressing CHO (CHO-CD123), and CD33/CD123 co-expressing CHO (CHO-CD33/CD123) target cells. Cytotoxicity of the CAR T cells was measured against each cell type indicated using a real-time cell-analysis system. The data are presented as a heatmap of the average normalized cell index at 48 h of co-culture with the target cells. The samples were evaluated in duplicate. (C) Heatmap of the amounts of IFN-γ, IL-2, and IL-6 released by UT or CAR T cells after stimulation with the indicated target cells. Data are representative of two independent experiments with different donors.

between cytotoxicity and cytokine production with IFN-γ levels ranging from 0 to 4,500 pg/mL, IL-2 from 0 to 4,000 pg/mL, and IL-6 from 0 to 350 pg/mL (Figure 3C). CARs listed in red in Figure 3 indicate those selected to generate single-construct bispecific CARs used in the subsequent experiments. These CARs were chosen for their optimal cytotoxicity and cytokine secretion profile when targeting both CD33 and CD123 antigens.

In vitro efficacy of CD33/CD123 bispecific CAR T cells

Using the double transduction data, we selected 12 CAR pairings to generate bispecific CD33/CD123 CARs with "AND" gates (Figure 4A) and evaluated their efficacy and safety *in vitro*. To create the combinatorial bispecific CD33/CD123 CARs, each CAR construct is co-expressed in a single T cell. One CAR has a 41BB co-stimulatory domain while the other has CD3ξ. Recognition of both cognate antigens leads to full activation of the CAR T cell. Full names of the bispecific CAR T cells are available in Figure S11. The CD33/CD123 bispecific CAR constructs exhibited gene transduction ranges of 10%–95% (Figure 4B). To analyze the expression of the individual scFvs (CD33 or CD123), we stained the CAR T cells with CD33 and CD123 antigens conjugated to a fluorophore. The bispecific CAR constructs showed variable CD33 and CD123 scFv expression levels, with MFI ranging from 100 to 150,000 (Figure 4C).

To compare the cytotoxicity of the 12 bispecific CAR T cells, they were incubated with target cells expressing CD33, CD123, or both

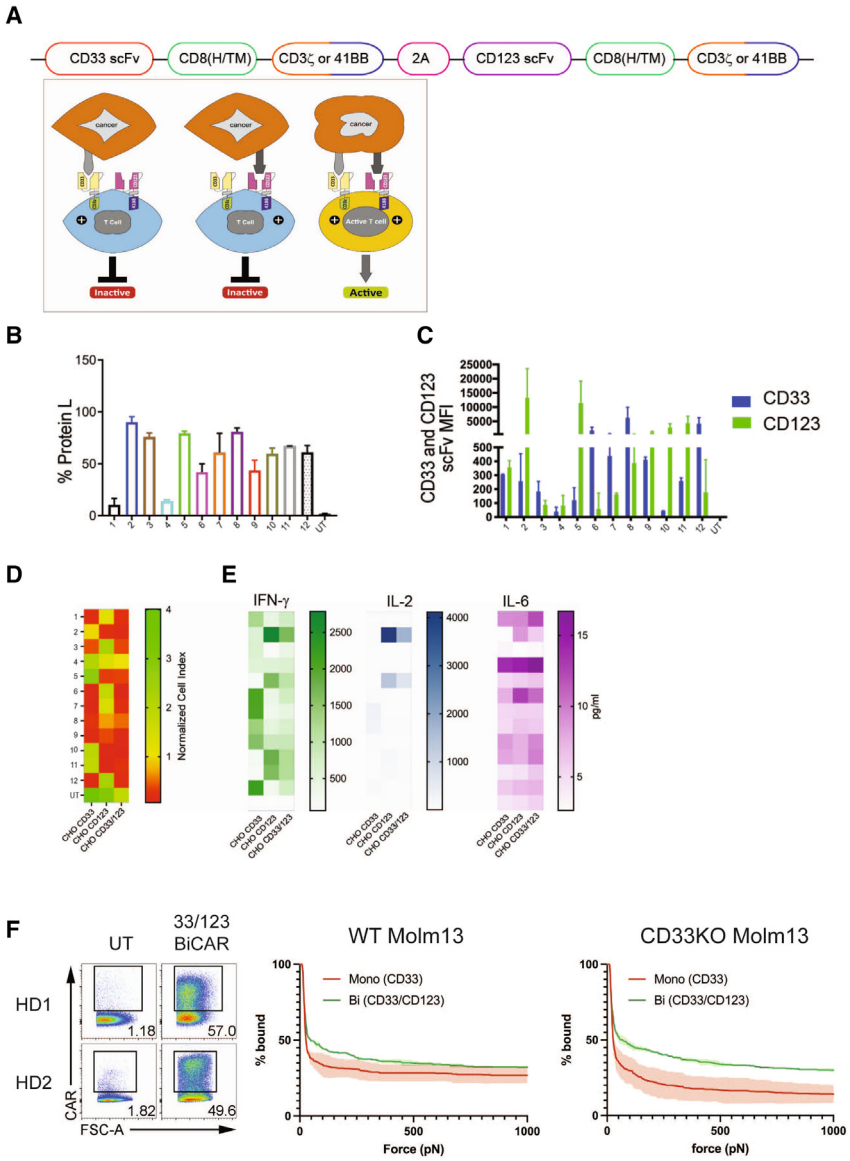
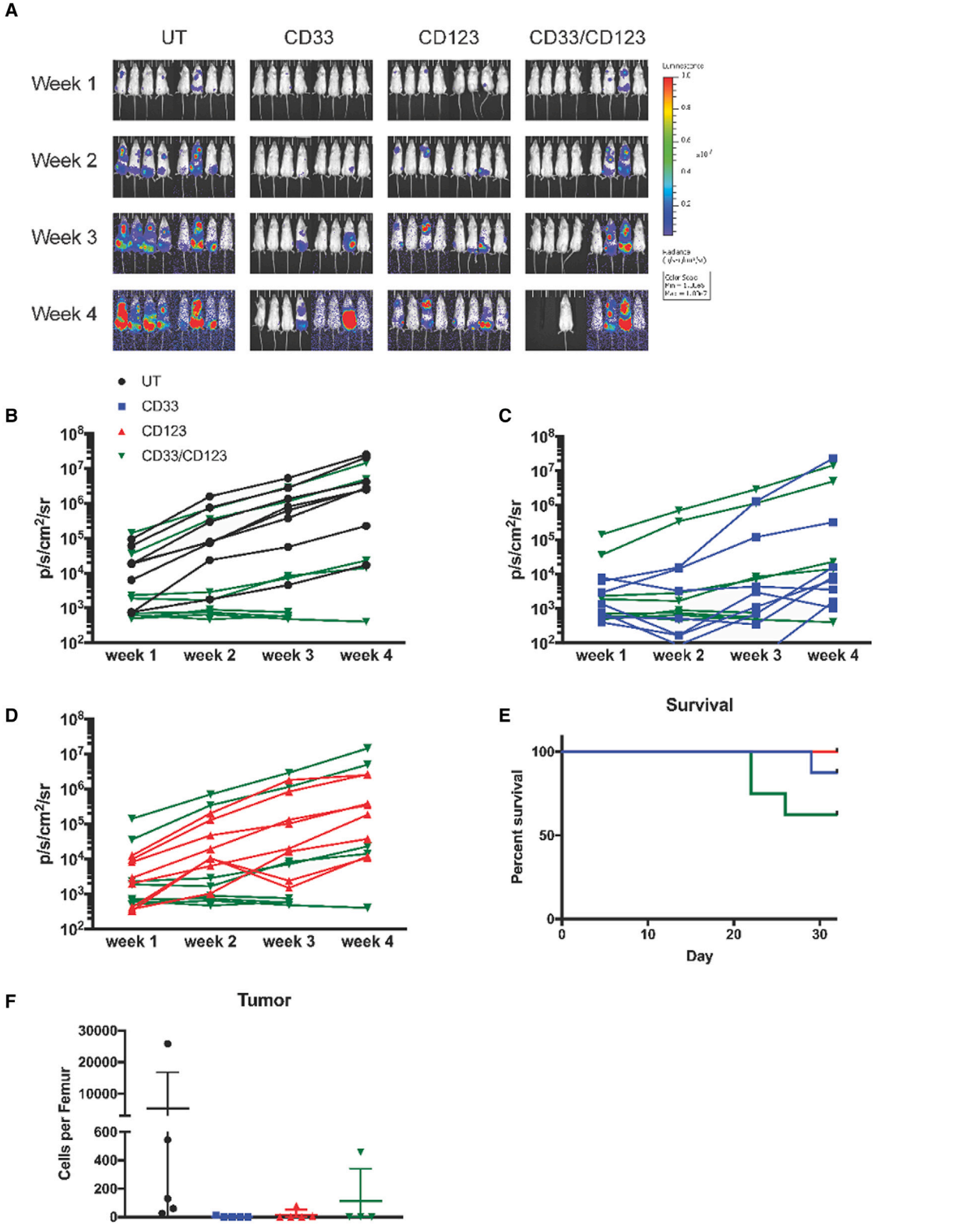


Figure 4. *In vitro* efficacy and binding of CD33/CD123 bispecific CAR constructs

T cells from a healthy donor were transduced with CD33/CD123 bispecific CAR constructs (1–12). (A) Schematic of a bispecific CAR construct and "AND" logic gate. (B) CAR T cells stained with protein L as an indicator of the efficiency of transduction. (C) Bispecific CAR T cells were stained for CD33 or CD123 and by fluorescently labeled antigen using flow cytometry. (D) Bispecific CAR T cells were co-cultured with empty CHO, CD33-expressing CHO (CHO-CD33), CD123-expressing CHO (CHO-CD123), and CD33/CD123 co-expressing CHO (CHO-CD33/CD123) target cells. Cytotoxicity of the CAR T cells was measured against each cell type indicated using a real-time cell-analysis system. Data are presented as a heatmap of the average normalized cell index at 48 h of co-culture with the target cells. The samples were evaluated in duplicate. (E) Heatmap of IFN-γ, IL-2, and IL-6 secreted by UT or bispecific CAR T cells after stimulation with the indicated target cells. (F) CAR transduction as measured by protein L of UT and bispecific CAR 1 in two healthy donors. Bispecific CAR 1 expresses both CD33 and CD123 scFvs as measured by cell-binding avidity assay. Bar graphs represent the mean ± SEM. Experimental data shown represent two independent experiments that used T cells from different healthy donors.

CD33 and CD123. Cytotoxicity ranged from 1 to 4 on a normalized index, with most CAR T cell groups demonstrating higher levels of cytotoxicity with normalized index scores of 0–1 (Figure 4D). Bispecific CAR T cells 1, 3, 6, 7, 8, 9, and 12 targeted CHO cells expressing CD33 better than CHO cells expressing CD123. CARs 7, 8, 9, and 12 cytotoxicity against the CD33 target cells suggests there is some leakiness or recombination with the "AND" gating because these CARs were designed with the co-stimulatory domain on the CD33 scFv. In comparison, bispecific CAR T cells 2, 4, 5, 10, and 11 targeted CHO cells expressing CD123 better than CHO cells expressing CD33. There was also leakiness in the "AND" gating with CARs 2, 4, and 5 because their co-stimulatory domain is on the CD123 scFv. All CAR combinations were effective at killing target cells expressing both CD33 and CD123. Cytokine heatmaps with IFN-γ levels of 0–2,500 pg/mL, IL-2 levels of 0–4,500 pg/mL, and IL-6 levels of 0–20 pg/mL correlated with cytotoxicity (Figure 4E). Bispecific CAR T cell 2 had the highest cytokine IFN-γ and IL-2 production in response to CHO CD123 and CHO CD33/CD123. Bispecific CAR T cell 4 produced higher levels of IL-6 in response to target cells compared to the other CAR combination.

Based on the cytokine and cytotoxicity experiments, we chose to move forward with bispecific CAR 1. While each scFv had a similar MFI expression, we wanted to functionally compare the expression of both CD33 and CD123 scFvs on the cell surface. To this end, we measured CAR/target cell avidity by co-culturing wild-type (WT) Molm13 cells expressing both CD33 and CD123 or CD33KO (knockout) Molm13 cells that only express CD123 with CD33 monospecific or CD33/123 bispecific CAR T cells. We found that the CD33/CD123 bispecific CAR 1 had a binding profile similar to that of the CD33 monospecific CAR with the WT Molm13 target cells (Figure 4F). When co-cultured with the CD33KO Molm13, CAR 1 had significantly higher binding compared to the monospecific CAR. This demonstrates that both CD33 and CD123 CARs are expressed on the cell surface and can bind to their antigen. Therefore, we selected CAR 1 based on its low cytotoxicity against CHO-CD123 target cells, indicating that it would not have off-target effects because the anti-CD123 scFv is together with the co-stimulatory



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domain. The high expression of the construct shown by protein L and expression of both scFvs along with moderate secretion of IFN-γ were also important in its selection.

In vivo tumor control is similar between CD33/CD123 bispecific and monospecific CAR T cells

To evaluate the efficacy of CD33/CD123 bispecific CARs *in vivo*, we employed a xenogeneic human AML mouse model. MV411 cells expressing luciferase were first engrafted into NOD SCID-gamma (NSG) mice. After confirmation of AML burden by bioluminescence imaging (BLI), the mice were treated with UT, CD33 monospecific, CD123 monospecific, or CD33/CD123 bispecific CAR T cells. There was rapid tumor expansion in the UT group by week 2, while the monospecific and bispecific groups were better able to control the tumor (Figure 5A). Four mice in the CD33/CD123 group had lower BLIs than any mouse from the UT group at 3 weeks, while two mice given the bispecific CAR had broken through and had BLI comparable to UT (Figure 5B). Compared to the CD33 monospecific CAR, the bispecific group had similar BLI, with two mice from each group suffering tumor breakthrough (Figure 5C). The CD33/CD123 mice also had BLI similar to that of the group given the CD123 monospecific CAR (Figure 5D). When we examined the overall survival of the mice from all CAR groups, we found no significant difference (Figure 5E). The mice that died in the CD33/CD123 group showed no evidence of tumor by BLI, making it unlikely that they died of disease. After 4 weeks, five mice were sacrificed, and we examined the number of tumor cells in the bone marrow and found no significant differences between the monospecific and bispecific CAR groups (Figure 5F). We also examined CD33/CD123 CAR T cell efficacy against the AML cell line MOLM13. We found a significant increase in survival of mice given the CD33/CD123 bispecific CAR T cells compared to mice given untransduced T cells (Figure S12). These data show that our CD33/CD123 CAR has *in vivo* antitumor efficacy similar to that of either the CD33 or CD123 monospecific CARs, so "AND" gating does not sacrifice potency.

CD33/CD123 bispecific CAR T cells have reduced cytotoxicity against CD34$^+$ cells compared to monospecific CAR T cells

Previous therapies targeting either CD33 or CD123 have been shown to have on-target off-tumor effects.[27,34] To evaluate the risk that mice in the CD33/CD123 bispecific CAR group died of on-target off-tumor toxicities (Figure 5), we intravenously (i.v.) injected 1×10^6 CAR T cells into CD34$^+$ humanized NSG mice and measured their clinical score and body weight. We found the CD123 monospecific CAR group had higher clinical scores compared to the CD33/CD123 bispecific CAR group, suggesting some toxicity of the CD123 monospecific CAR (Figure 6A). There was no significant percentage change in body weight between mice given untransduced, CD33 monospecific, CD123 monospecific, or CD33/CD123 bispecific CAR T cells after 26 days (Figure 6B). Blood was also collected from these mice at weeks 1–4 after CAR injection for complete blood cell count analysis. We found no differences in white blood cell, lymphocyte, monocyte, hematocrit, or platelet counts between any of the treatment groups (Figure 6C).

We also performed a CD34$^+$ colony-forming unit (CFU) assay to evaluate stem cell toxicity of the CD33/CD123 bispecific CAR. We found that the total number of colonies in the bispecific CAR group was comparable to that of CD33 or CD123 monospecific CARs (Figure 7A). However, there were significantly more granulocyte macrophage (GM) colonies after co-culture with CD33/CD123 bispecific CAR T cells compared to either monospecific CAR (Figure 7B). This suggests that the bispecific CAR has a specific benefit for the GM developmental stage. We also evaluated CD34$^+$ cell death after CAR co-culture by flow cytometry. We found a significantly higher percentage of DAPI/caspase double-positive CD34$^+$ cells after co-culture with the CD33 monospecific CAR T cells compared to the CD33/CD123 bispecific CAR T cell group (Figure 7C). The lack of differences between the CD123 monospecific CAR and the CD33/CD123 bispecific CAR groups is probably caused by the short 4-h time point used for this assay because the CD123 kinetics are slower. Together, these data demonstrate that the CD33/CD123 bispecific CAR is less cytotoxic to CD34 stem cells than the CD33 or CD123 monospecific CARs.

DISCUSSION

CD33 and CD123 are abundantly expressed on most AML blasts[11,28,35] but are also present on some normal cells, including myeloid cells, HSCs, and liver Kupffer cells.[35–37] Consequently, CD33-targeted therapies, such as gemtuzumab and ozogamicin, have been associated with myelosuppression and cytopenia.[38,39] Some groups have considered alternative strategies to mitigate the inadvertent effects on HSCs, such as supplanting genetically edited CD34$^+$ cells into patients.[25,40] However, these strategies are cumbersome and expensive. While CD123 expression on HSCs and common myeloid progenitors is low, its expression on blood vessels led to on-target off-tumor toxicity in a CD123-directed CAR T cell therapy clinical trial.[41] Multiple strategies in CAR design have been reported to minimize toxicity and relapse associated with CAR T cell therapy.[42,43] Others have developed CARs expressing both CD33 and CD123 that can signal through either CD33 or CD123 and prevent relapse.[26] Targeting AML using tandem CARs can target HSCs due

Figure 5. CD33/CD123 bispecific CARs have *in vivo* tumor control similar to that of monospecific CARs

NSG mice were injected intravenously with 5×10^5 MV411. Bioluminescence imaging (BLI) was performed to quantify the engraftment and for randomization of the treatment groups. CD33/CD123 bispecific CAR T cells (1×10^6) were injected followed by imaging every week for four additional weeks. Mice were euthanized on week 4, and AML tumor cells from bone marrow were quantified using flow cytometry. (A) BLI of mice over time. (B) Tumor burden, according to BLI analysis, was reported as average radiance (photons/s/cm^2/sr) for UT and bispecific CAR groups. (C) Tumor burden measured by BLI of bispecific CAR compared to CD33 monospecific CAR and (D) bispecific CAR compared to CD123 monospecific CAR. (E) Overall survival of mice given CAR T cells. (F) The number of tumor cells in the bone marrow was analyzed by flow cytometry. For (A)–(E), each point represents one mouse. For (F), n = 5 mice per group.

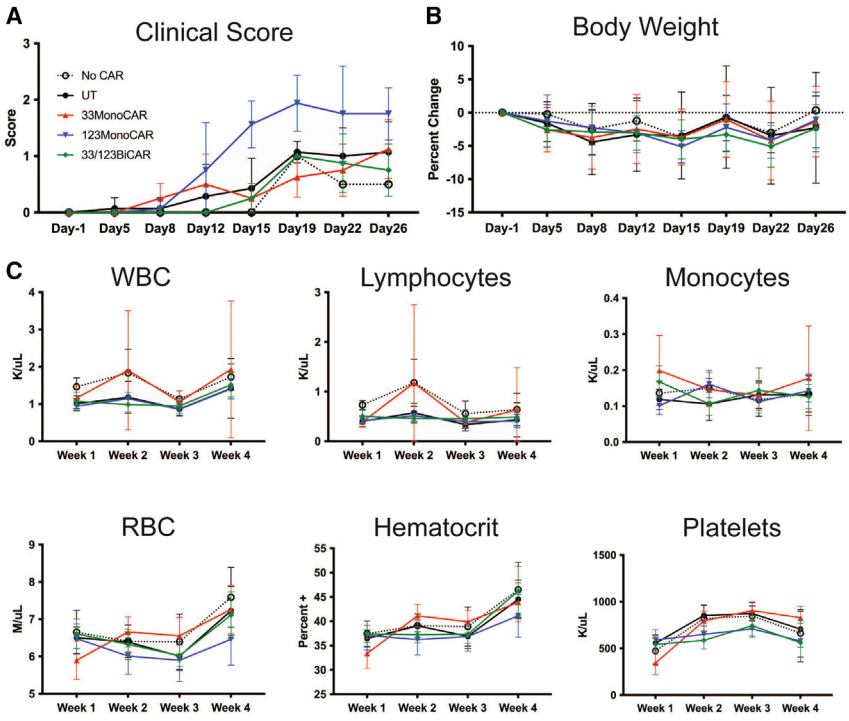

Figure 6. CD33/CD123 bispecific CAR shows no toxicity *in vivo*

(A) hCD34$^+$ humanized NSG mice were i.v. injected with untransduced, monoCAR, or biCAR T cells at day 0. Clinical observations of hCD34$^+$ humanized mice posture and coat were scored. (B) Body weight was then measured at indicated time points, and percentage change was calculated. (C) Complete blood counts were measured at weeks 1–4 after CAR injection. Graphs represent the mean ± SEM. n = 8 mice per group.

to shared antigen expression on AML cells and HSCs.[35] Therefore, engineering T cells with split CARs such that scFv-1 activates CD3ζ and scFv-2 drives only co-stimulatory signals through 41BB offers greater specificity in targeting of AML without HSC toxicity.[10,11,29,30]

Among the limitations of targeting CARs against a single antigen are on-target off-tumor toxicity and loss of antigen or epitope spreading, leading to disease relapse because of antigen escape.[44,45] To overcome these limitations, we presented methods for the preclinical development of bispecific CD33/CD123 CARs to target AML. We first derived multiple *de novo* CD33 and CD123 scFvs using recombinant technology. We then tested the binding affinity of the scFvs to engineered target cells to validate antigen specificity and confirmed their ability to activate the T cells using Jurkat antibody-dependent cytotoxicity reporter cells. Among co-stimulatory molecules, the 41BB co-stimulatory domain has been implicated for its critical role in T cell survival.[46,47] For further screening and to test the *in vitro* efficacies of the bispecific CARs, we individually cloned scFvs into standard monospecific secondary CARs utilizing a second-generation 41BB co-stimulatory domain. Selecting CAR constructs based only on their demonstration of superior *in vitro* efficacy against targets may result in increased toxicity. Therefore, we explored and developed combinations of scFvs with various efficacies to identify the best combination of CARs for targeting AML cells while still refraining from targeting HSCs. While the use of AML cell lines or primary samples with different somatic mutations may have added value to selecting the bispecific CAR, we used CHO cells expressing CD33, CD123, or CD33/CD123 to evaluate the efficiency of the "AND" gating of the CAR T cells.

CAR density is an important factor for inducing ideal CAR signaling.[48] Low levels of CAR expression have been shown to decrease the function of CARs, while overexpression may result in toxic signaling, leading to exhaustion of the CAR T cells.[48,49] While we designed the CARs in the current study to exhibit optimal stimulation through the development of split CARs, we observed variable levels of CD33 and CD123 scFv expression in our bispecific CARs. Consistent with previous studies,[48] the MFI of our CD33 and CD123 scFvs correlated with the *in vitro* killing and cytokine production. We also observed that the CD33 and CD123 scFvs are expressed on the cell surface and can bind to their antigen. This suggests a stoichiometric relationship between CARs and their biological activity of cytotoxicity and cytokine production.

Reduction of myeloid progenitor cells and HSC toxicity has been reported in patients as well as CFU assays of CD33 or CD123 directed therapies.[26,38,40,50] When we examined CD34$^+$ CFUs we found no statistical difference in the number of total colonies, but we did find an increase in the number of GM colonies in the CD33/CD123 bispecific group compared to the monospecific CAR T cells. This suggests that the GM stage of development is less affected by the bispecific CAR compared to the CD33 or CD123 monospecific CARs. We also observed no difference in body weight or complete blood counts after treatment with the CD33/CD123 CAR T cells. This suggests that there are minimal on-target off-tumor effects and that this CAR is safe for further clinical evaluation. Some mice in Figure 5 showed evidence of active disease and expression of CD33 and CD123 after treatment with CD33/CD123 bispecific CAR T cells. This suggests that treatment with bispecific CAR T cells alone would not be curative and that consolidation with HSCT transplantation would be needed. However, we believe this combined treatment would lead to significantly improved patient outcomes.

Affinity and stoichiometry of CARs can influence overall CAR activity.[48,49] We investigated for the first time the switching of CD3ζ and 41BB placement in bispecific CARs. Therefore, optimizing the alignment of co-stimulatory domains to scFvs for split or bispecific CARs is critical for assuring optimal therapeutic response. Future strategies may include modifying the CAR constructs that would allow them to

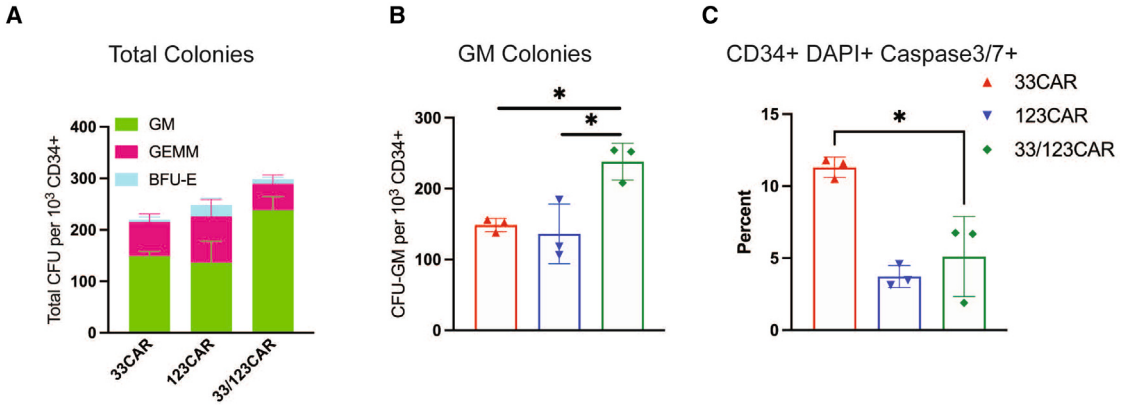

Figure 7. Co-culture of CD33/CD123 bispecific CAR with CD34$^+$ cells shows improved safety compared to the monoCARs

(A and B) CD34$^+$ CFUs were decreased after co-culture with monoCARs versus CD33/123 BiCAR. CD34$^+$ cells were incubated with CAR T cells for 4 h and plated in MethoCult medium. After 14 days, colonies were counted. (C) There is a significant decrease in dead CD34$^+$ cells after co-culture with CD33/CD123 bispecific CAR T cells compared to CD33 MonoCAR T cells. CD34$^+$ cells were incubated with CAR T cells for 4 h and then stained for CD34, caspase-3/7, and DAPI to measure cell death by flow cytometry. Each point represents a unique healthy donor. Bar graphs represent mean ± SEM. * = $p<0.05$

be managed should they lead to overstimulation or lymphopenia. This could include engineering our CD33/CD123 multi-agent CAR constructs with a safety switch, such as a truncated human epidermal growth factor receptor (EGFR). In severe cases, it would be possible to deplete CAR T cells using cetuximab, an FDA-approved monoclonal antibody against EGFR, to correct lymphopenia.

First-generation CAR T cells have been found to support activation. We saw similar activation in our CD3ξ-only CARs as well as in the 41BB CARs without CD3ξ. This may be due to the clustering of the construct on the surface of the T cell allowing for the recruitment of endogenous CD3ξ, resulting in low-level activation. Alternatively, the affinity of this construct to CD123 may allow for some tonic 41BB signaling, which partly activates the T cell. In either case, based on previous work showing that first-generation CARs are not effective in patients, we do not believe that this activation would result in on-target off-tumor cytotoxicity. Target antigen expression levels can also influence CAR T cell functionality, and we plan to examine this in future studies.

Overall, we reported on bispecific CD33/CD123 CAR T cells that showed promise against AML. We demonstrated that optimizing the combination of potent scFvs and the co-stimulatory domain played an essential role in developing a safer bispecific CAR T cell therapy. This meticulously developed CD33/CD123 bispecific CAR has therapeutic potential without on-target off-target toxicity against refractory AML and will be evaluated further in clinical trials.

MATERIALS AND METHODS

Mice

NSG mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the University of South Florida (Tampa, FL). Female mice were used at 8 weeks of age. The mice were intravenously injected via the tail vein with 5×10^5 human AML-derived MV411 cells in a 200-μL volume of phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA). Primary human CAR T cells (1×10^6) in 200 μL of PBS were i.v. injected 2 weeks later. Luciferin (Thermo Fisher Scientific) and *in vivo* imaging using an IVIS imaging system were performed as described.[32,33]

The Jackson Laboratory performed the safety experiments. Female NSG mice were engrafted with human CD34$^+$ cells, and mice which had >25% human CD45$^+$ cells in the peripheral blood 122 weeks post engraftment were used. Mice were i.v. injected by the tail vein with 1×10^6 CAR T cells on day 0. Body weights were collected twice weekly post injection. Whole blood was collected on days 7, 14, 21, and 28 by retro-orbital bleed and analyzed by flow cytometry and complete blood count. On day 28 all mice were sacrificed, and spleen and bone marrow were collected. All animal experiments were performed in accordance with an IACUC-approved protocol.

Cells

The MV411 cell line was maintained in RPMI medium (Thermo Fisher Scientific). The MOLM13 cell line was maintained in RPMI medium (BPS Bioscience, San Diego, CA). CHO cells and mouse EL4 cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific). Jurkat NFAT-GFP reporter cells were purchased from Signosis (Santa Clara, CA) and maintained in RPMI medium. All media were supplemented with glucose, 10% fetal bovine serum, or 10% horse serum for EL4 cells, penicillin, and streptomycin. All media supplements were purchased from Thermo Fisher Scientific. To create target cells, CHO or EL4 cells were transduced with supernatants of SFG gamma retrovirus containing either CD33, CD123, or both, as described.[32,33]

Generation of scFvs

CHO cells expressing human CD33 or CD123 antigens were inoculated into mice. Spleen cells were collected and fused with myeloma

cells to generate hybridomas. To determine antigen binding, monoclonal CD33 and CD123 were co-cultured with CHO cells expressing either CD33 or CD123 for 1 h at 4°C. Cells were washed with PBS, stained with anti-mouse immunoglobulin G (IgG) and analyzed by flow cytometry. These antibodies were further screened for activation by co-culturing EL4-CD33 or EL4-CD123 cells with the antibodies for 1 h at 4°C. Cells were washed with PBS and co-cultured with Jurkat CD16/CD32 NFAT reporter cells overnight at 37°C. Flow cytometry was then performed to measure Jurkat GFP expression. RNA from selected monoclonal hybridomas was isolated and sequenced to determine immunoglobulin sequences and construct scFvs as described.[51]

Genetic CAR constructs and gamma retrovirus

CD33 and CD123 scFvs with either the CD3ζ activation domain or 41BB co-stimulatory domain, and CD33/CD123 bispecific CAR were synthesized and cloned into SFG gamma-retroviral vectors by GENEWIZ (South Plainfield, NJ). Gamma retrovirus was produced as described.[32,33,52] In brief, SFG constructs were transfected into H29 cells using calcium phosphate.[52] Retrovirus was collected by filtering supernatants from the H29 cells through a 0.45-μm filter. RD114 cells were transduced with the retrovirus to produce stable virus-producing cells.[52]

Generation of CAR T cells

CAR T cells were generated by transduction of human T cells as described.[32,33,52] In brief, leukocytes from a healthy human donor were obtained by apheresis (All Cells, Alameda, CA or Stem Cells, Vancouver, Canada) and purified using density gradient centrifugation. T cells were isolated using magnetic beads (Stem Cells) and stimulated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific). Activated T cells were spin-transduced with gamma retrovirus on plates coated with RetroNectin reagent (Takara Bio, Kusatsu, Japan) and cultured with complete RPMI medium supplemented with IL-2. For double transductions, CAR T cells were spinoculated with gamma retrovirus on two different days. CAR T cells were subjected to bead removal after 7–8 days of activation. T cells were expanded using G-REX plates (Wilson Wolf, New Brighton, MN) to increase T cell yield for *in vivo* experiments. Gene transfer or transduction efficiency was estimated based on the number of GFP^+ or $mCherry^+$ cells detected by flow cytometry. To detect transduction and gene transfer for bispecific CAR T cells, the cells were labeled with biotinylated protein L (Thermo Fisher Scientific) followed by streptavidin (Thermo Fisher Scientific), or the CD33 and CD123 antigens were labeled with fluorophore dyes (Creative BioMart, Shirley, NY).

Flow cytometry

For flow cytometry, the following anti-human antibodies were obtained from BD Biosciences (Franklin Lakes, NJ) or Thermo Fisher Scientific: anti-CD3 (clone UCH71), anti-CD4 (clone SK3), anti-CD8 (clone RPA-T8), anti-PD1 (clone J105), anti-CD45RA (clone HI100), anti-CD45RO (clone UCHL1), anti-CCR7 (clone 3D12), anti-CD33 (clone MW53), anti-CD123 (clone 6H6), goat anti-mouse IgG 488, streptavidin 488, and biotinylated protein L. Dead cells were labeled using Fixable Viability Dye eFluor 450 (Thermo Fisher Scientific). Antibody staining was performed at 4°C using human Fc-block in MACS buffer with 0.5% bovine serum albumin (Miltenyi Biotec, Gaithersburg, MD). CountBright absolute counting beads (Thermo Fisher Scientific) were used for cell quantification for some experiments. Live events were acquired on BD FACS Canto II or LSRII flow cytometers (BD Biosciences). Flow-cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytotoxicity, cytokine, and proliferation assays

Cytotoxicity assays were performed using an xCELLigence Real-Time Cell Analysis instrument (ACEA Biosciences, San Diego, CA) according to the manufacturer's instructions. CHO-Empty, CHO-CD33, CHO-CD123, and CHO-CD33/CD123 target cells were seeded at 10,000 cells per well into E-Plate 96 plates (ACEA Biosciences, San Diego, CA) and incubated overnight. CAR T cells (100,000 cells/well) were added to the target cells resulting in an effector/target (E/T) ratio of 10:1. The assays were performed in triplicate. For cytokine analysis, CAR T cells were similarly co-cultured with target cells for 24 h at an E/T ratio of 10:1. Supernatants were harvested and analyzed using a Human Simple Plex Assay Kit (Biotechne, Minneapolis, MN) and an Ella instrument (Biotechne) or a Human Luminex Assay Kit (R&D Systems, Minneapolis, MN) and a Luminex 100 system (Luminex, Austin, TX), according to the manufacturers' instructions. For proliferation assays, CAR T cells were co-cultured in triplicate with target cells at an E/T ratio of 1:1. Total absolute cell numbers in each well were determined on days 1, 7, and 14 using a cell counter (Bio-Rad, Hercules, CA) and trypan blue staining.

Cell-binding avidity assay

WT Molm13 and CD33KO Molm13 cells were attached on poly-L-lysine-coated chips for at least 3 h prior to testing on the z-MoviCell Avidity Analyzer (Lumicks, Amsterdam, the Netherlands). CellTrace far-red-labeled (Thermo Fisher Scientific) CAR T cells from two healthy donors were normalized for transduction efficiency and bound for 5 min prior to ramping up acoustic force. Cell detachment was analyzed using Ocean software. Experiments and analyses were conducted according to manufacturers' recommendations.

Colony-forming unit assay

Granulocyte colony-stimulating factor mobilized human peripheral blood $CD34^+$ cells were purchased from Stem Cells and co-cultured with CAR T cells at an E/T ratio of 10:1. Following incubation, the cells were plated with MethoCult medium (Stem Cells) into dishes according to the manufacturer's instructions and cultured for 14 days. At the end of the culture period, colonies were counted.

Statistics

Data are reported as mean values ± standard error of the mean (SEM). Analysis of variance was used for group comparisons and included Sidak's or Dunn's post test for correction of multiple comparisons. The Mann-Whitney test was used for all other comparisons. Statistical analyses were conducted using Prism software, version 5.04

(GraphPad). Statistical significance was defined by a two-tailed p value of <0.05.

DATA AND CODE AVAILABILITY

The data supporting the findings of this study are available on request from the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.100751.

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AUTHOR CONTRIBUTIONS

M.L.D. conceived the idea and funded the study. J.C.B., B.S., and M.L.D. designed the study and wrote the manuscript. J.C.B., B.S., P.V., M.L., E.V.C., T.G., K.R., K.S., B.Y., and B.C.B. performed experiments and acquired and analyzed data. M.L.D., M.V.M, and J.A.G.-P. provided supervision of the study. All authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

M.L.D. has received research funding from Novartis, Kite/Gilead, and CRISPR. M.L.D. receives fees from Synthekine, Adicet, Bellicum, Capistan, and Syncopation. M.L.D. has stock or stock options with Adaptive Biotechnologies, Adicet, and Bellicum. M.L.D. has licensed inventions to CRISPR and Atara. M.L.D. has patents related to the CAR described.

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