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High affinity FRβ-specific CAR T cells eradicate AML and normal yeloid lineage without HSC toxicity

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Abstract

Acute myeloid leukemia (AML) is an aggressive malignancy, and development of new treatments to prolong remissions is warranted. Chimeric antigen receptor (CAR) T-cell therapies appear promising but on-target, off-tumor recognition of antigen in healthy tissues remains a concern. Here, we isolated a high affinity (HA) folate receptor beta (FRβ)-specific scFv (2.48nM K_D) for optimization of FRβ-redirected CAR T-cell therapy for AML. T-cells stably expressing the HA-FRβ CAR exhibited greatly enhanced antitumor activity against $FR\beta^+$ AML *in vitro* and *in vivo* compared to a low affinity (LA) FRβ CAR (54.3nM K_D). Using the HA-FRβ IgG, FRβ expression was detectable in myeloid-lineage hematopoietic cells; however, expression in $CD34^+$ hematopoietic stem cells (HSCs) was nearly undetectable. Accordingly, HA-FRβ CAR T-cells lysed mature $CD14^+$ monocytes, while HSC colony formation was unaffected. Because of the potential for elimination of mature myeloid lineage, mRNA CAR electroporation for transient CAR expression was evaluated. mRNA-electroporated HA-FRβ CAR T-cells retained effective anti-tumor activity *in vitro* and *in vivo*. Together, our results highlight the importance of antibody affinity in target protein detection and CAR development and suggest that transient delivery of potent HA-FRβ CAR T-cells is highly effective against AML and reduces the risk for long-term myeloid toxicity.

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Conflicts of Interest

The authors have no conflicts of interest to disclose related to the work described.

Authorship

RCL and DJP designed experiments and wrote the paper. RCL conducted experiments, analyzed data and prepared the figures. YF and DSD isolated the scFvs and prepared and conducted experiments with soluble FRβ scFvs and IgGs. KS prepared mRNA CAR reagents. MP conducted *in vivo* imaging. AK scored the CFU. All authors have read and approved the submitted manuscript.

Introduction

Acute myeloid leukemia (AML) remains a disease with poor prognosis[1, 2]. Currently, the most effective therapy, allogeneic bone marrow transplant, is not feasible in all patients, may not fully eliminate tumor cells, and carries a substantial risk of GVHD-associated toxicity[3–5]. Therefore, newer safe and effective therapy is needed for AML. Chimeric antigen receptor (CAR) T-cell therapy has recently produced dramatic clinical success in patients with $CD19^+$ acute and chronic lymphocytic leukemia with up to 90% of patients responding[6–8]. CAR T-cells are genetically modified to eliminate tumor cells by linking extracellular tumor antigen-recognition domains, most commonly antibody-derived single-chain variable fragments (scFvs), to intracellular T-cell receptor signaling moieties[9]. Expanding the clinical efficacy of CD19-directed CAR therapy to additional types of cancer will depend foremost on the identification of appropriate cell-surface tumor antigens. In the application of cytolytic CAR T-cells, the largest concern remains toxicity resulting from off-tumor CAR recognition of target protein in healthy tissues.

The high expression of several AML surface antigens, such as CD33 and CD123, on healthy bone marrow (BM) hematopoietic stem cells (HSCs) remains a prominent challenge for developing safe and effective CAR therapy for AML[10–14]. We have previously established that folate receptor beta (FRβ)-specific CAR T-cells can target AML without HSC toxicity[15]. However, these CAR T-cells were functionally limited, effectively controlling AML tumor growth only in the minimal disease setting. Modifying CAR platforms with high affinity scFvs can result in improved anti-tumor activity[16, 17]. However, higher affinity CARs may possess heightened sensitivity to antigen on healthy tissues and present a greater risk for off-tumor toxicity in patients. Indeed, toxicity related to off-tumor recognition has led to patient morbidity in clinical trials using CAR T-cells redirected against Her2[18] and CAIX[19].

Awareness of target tumor antigen expression in normal tissues is exceedingly important for the prediction of possible off-tumor toxicity. Because the antigens targeted in CAR T-cell therapy are commonly self-antigens, with overexpression on tumor cells, it is not uncommon for low levels of antigen to be expressed on normal tissues[20]. Antibodies are commonly used to assess protein expression by tumor and healthy cells using flow cytometry or IHC, however antibody affinity should be carefully considered as lower affinity reagents may be unable to bind low levels of antigen and therefore produce false-negative results.

We hypothesized that our previously described FRβ-specific scFv, which bears low-intermediate affinity (LA) for FRβ, may not promote optimal interaction with FRβ, either in a CAR platform or as a labeling reagent. Therefore, we sought to develop high affinity (HA) reagents for FRβ CAR platform optimization as well as sensitive protein detection in normal tissues to better assess potential risk for toxicity related to targeting FRβ in AML.

Materials and Methods

Surface plasmon resonance analysis

Binding of LA-FRβ or HA-FRβ Fab to recombinant human FRβ (rFRβ) was assessed using Biacore×100 instrument as described[21]. Briefly, purified rFRβ was immobilized on a CM5 sensor chip. LA-FRβ or HA-FRβ scFv, diluted to 0.04, 0.4, 4, 40, or 400 nM, was applied through the cells. The chip was regenerated with 10 mM glycine pH 2.5, 1 M NaCl. The sensorgram was analyzed with BiaEvaluation software, and data were fitted to a 1:1 binding model.

rFRβ binding ELISA

100 ng/well rFRβ was coated on 96-well plates overnight at 4°C. Wells were blocked and 50μL of diluted antibodies were incubated 2h at 37°C in duplicate. 1:1000 goat anti-human IgG-HRP (Jackson ImmunoResearch) for 1h at 37°C and 50μL/well ABTS substrate were used for detection of LA-FRβ and HA-FRβ IgG binding. Absorbance was read at 405 nm.

Lentiviral CAR Production

The HA-FRβ scFv was cloned into previously validated pELNS lentiviral vectors containing CD3ζ or CD28-CD3ζ to create HA-Z and HA-28Z CAR constructs (details and sequences in supplement). Third generation lentiviral vector was produced in 293T (ATCC) as previously described[15, 22]. Briefly, 293Ts were transfected with pRSV-Rev, pMDLg/pRRE, pMD2.G and pELNS CAR plasmids. 24h and 48h supernatants were combined and concentrated using high-speed ultracentrifugation. Lentiviral vectors were titered in 293Ts and stored at −80°C until use.

Cells

All cells were cultured in complete media (CM, RPMI-1640-GlutaMAX, 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin) at 37°C. C30[23] was provided by George Coukos. C30-FRβ was created as described[15]. Human AML cell lines THP1, MV411, and HL60 were provided by Gwenn Danet-Desnoyers. Cell lines used in animal models were confirmed mycoplasma negative before use. De-identified healthy adult BM, $CD34^+$ HSCs and primary human AML were purchased from the University of Pennsylvania Stem Cell and Xenograft Core (SCXC). Peripheral blood (PB) was collected by apheresis from volunteer donors by the University of Pennsylvania Human Immunology Core (HIC) with informed consent according to the Declaration of Helsinki. Whole blood, monocytes, and T-cells were purchased from HIC. T-cells were activated, transduced, and expanded as previously described[15, 22]. Briefly, T-cells were activated with αCD3/αCD28 beads, lentivirally transduced at MOI=10 20h later, and expanded in CM with 50IU/mL IL2 for 10–14 days. Cell size was monitored using a Multisizer-3 Coulter Counter (Beckman Coulter). Rested T-cells (<300fL volume) were used for functional *in vitro* and *in vivo* assays. Transduction frequencies were normalized using untransduced T-cells before each experiment.

Flow Cytometry

Up to 1×10^6 cells were labeled in 100μL staining buffer (2% FBS in PBS) containing relevant antibodies for 30min at 4°C. LA-FRβ and HA-FRβ scFv and IgG were prepared as described and biotinylated *in vitro*. 0.5μg IgG or 1μg scFv was used for primary labeling, and 1:200 Streptavidin (SA)-APC for secondary detection. Washed samples were assessed by flow cytometry using a FACSCantoII flow cytometer (BD) in the presence of 7AAD. Surface CAR was labeled using 0.3μg rabbit-anti-human-IgG(H+L)-biotin (LA-FRβ, HA-FRβ, or CL10 CARs) or 0.3μg goat-anti-mouse-IgG(H+L)-biotin (CD19 CAR) and secondary SA-APC or PE. Marker antibody details in supplement.

CAR binding to rFRβ

GFP-2A-CAR constructs were used to evaluate binding to rFRβ by flow cytometry. rFRβ was biotinylated *in vitro*. 2×10^5 T-cells were incubated with 200ng, 500ng, or 1μg rFRβ-biotin and secondary SA-APC. For CAR T-cell reactivity against rFRβ, 250 or 500ng rFRβ was coated on 96-well plates overnight at 4°C. Wells were washed and 1×10^5 CAR^+ T-cells/well were cultured overnight in 200μL CM. IFNγ secretion was assessed by ELISA.

Cytokine Release and CD69

1×10^5 targets and 1×10^5 CAR^+ T-cells were plated in 200μL CM. After overnight co-culture, supernatants were analyzed by IFNγ ELISA. IFNγ, IL2, MIP1α, TNFα, IL4, and IL10 were assessed using cytokine bead array (BD). In some cases, cell pellets were washed and labeled for CD3, CAR, and CD69 by flow cytometry following co-culture.

Cell Lysis

Cell lines C30, C30-FRβ, THP1, MV411, and HL60 were stably transduced with GFP-2A-firefly luciferase (fLuc) lentiviral vector. 2×10^4 fLuc-targets were cultured in white 96-well plates with CAR^+ T-cells at 5:1, 1:1, and 1:5 E:T ratios in triplicate. After overnight co-culture, residual luciferase activity was measured using the Extended-Glow Luciferase Reporter Gene Assay System (Life Technologies). Percent lysis was calculated as follows [100− ((Average Signal T-cell Treated Wells)/(Average Signal Untreated Target Wells) ×100)].

Degranulation

1×10^5 target cells and 1×10^5 CAR^+ T-cells were plated in 200μL CM with monensin and 5μL/well CD107a-APC and CD107b-APC in triplicate. After 6h co-culture, total cells were labeled for CD3, CAR and 7AAD by flow cytometry.

CFU Assay

2000 healthy adult BM $CD34^+$ HSCs were cultured with 2000 CAR^+ T-cells in V-bottom plates. After 4h, wells were diluted in methylcellulose and plated in duplicate. After 14 days colonies were counted and scored as CFU-GEMM, GM, G, M, or BFU-E.

BM Lysis Assay

1×10^5 $CD34^{(-)}$ BM cells and 1×10^5 CAR^+ T-cells were plated in 200μL CM. After 5h, cells were washed and labeled for CD3, CD33, CD19, FRβ and 7AAD. The phenotype of cells surviving co-culture is indicated in represented flow cytometry plots.

Monocyte Lysis

2×10^4 healthy donor monocytes were labeled with Cr^{51}, washed, and plated with CAR^+ T-cells at the indicated E:T ratios in 6 replicates. Monocyte lysis was assessed after 4h by Cr^{51} release. Spontaneous release was measured in untreated target wells. Maximum lysis was induced with 10% SDS. Percent Lysis = [(Treated–Spontaneous)/(Maximum–Spontaneous) ×100].

Mice

6–8 week old male or female Nod/SCID/γchain$^{-/-}$ (NSG) and Nod/SCID/γchain$^{-/-}$ with transgenic human SF, GM-CSF, and IL3 (NSGs) mice were purchased from, treated and maintained under pathogen-free conditions by the SCXC under protocols approved by the University of Pennsylvania IACUC. We injected 5×10^6 fLuc-THP1 tumor cells subcutaneously (sc) or intravenously (IV) and T-cells intraperitoneally (IP) or IV as indicated in the figure legends. Tumor growth was monitored by caliper measurement (sc models) and bioluminescent imaging as described[15]. Mice were randomized to ensure equal mean tumor burden between groups before treatment. 5 mice per group were treated based on previous tumor models[15]. Researchers were blinded to treatment groups during data acquisition. Peak luminescence is displayed as p/s (IV tumor) or p/s/cm^2 (sc tumor). Peripheral blood sampling was conducted via retro-orbital blood collection under isoflurane anesthesia. 50μL blood was labeled for indicated cell markers and quantified using TRUCount beads (BD).

mRNA CAR

pELNS lentiviral CAR constructs were digested and ligated into PDA mRNA expression plasmids. mRNA was produced *in vitro* as described[24]. T-cells were electroporated with 10μg mRNA/10×10^6 cells using an ECM830 BTX electroporator (Harvard Apparatus) at the following settings: unipulse, 500V, 700μs. “No-RNA” T-cells were electroporated without mRNA. mRNA-CAR expression and functional activity were assessed at the indicated time points following electroporation.

Statistical Analysis

Data was analyzed for significance using an unpaired two-tailed student’s t-test using the Holm-Sidak method without assuming a consistent SD (GraphPad Prism 6). $P < .05$ was considered significant. All error bars represent mean and standard error (SEM) unless otherwise noted in figure legends. Specific sample sizes and experimental replicates are reported in the figure legends. Unless otherwise noted, in vitro assays were repeated at least 3 times to ensure adequate power.

Results

Isolation of a higher affinity FRβ scFv

To identify high affinity FRβ-specific antibodies, we isolated a new scFv through light chain shuffling as previously described[25]. We utilized Biacore ×100 (Figure 1a) to define monovalent affinities of 54.3nM for LA-FRβ[15] and 2.48nM for the new high affinity (HA) FRβ scFv. HA-FRβ IgG displayed increased binding capability to rFRβ protein by ELISA (Figure 1b) and cell-surface FRβ by flow cytometry (Figure 1c). $FR\beta^{+}$ cell lines C30-FRβ, THP1 and MV411 all displayed greater binding to HA-FRβ IgG as visualized by increased MFI compared to LA-FRβ IgG. The HA-FRβ scFv was also able to bind THP1 and MV411, albeit at lower levels compared to the full bivalent IgG, whereas the LA-FRβ scFv could not be visualized by flow cytometry (Figure S2). Although the epitopes recognized by LA-FRβ and HA-FRβ scFvs have not been defined, competition ELISAs demonstrate their ability to inhibit association of the other to rFRβ (Figure S3), suggesting binding at nearby locations.

High affinity FRβ CAR T-cells demonstrate increased binding to rFRβ

The HA-FRβ scFv was cloned into previously validated lentiviral CAR vectors containing either CD3ζ alone or CD28-CD3ζ intracellular signaling domains to create HA-Z and HA-28Z CAR constructs, respectively (Figure 1d and S4a). Primary human T-cells were transduced with lentiviral CAR constructs, and transduction efficiency was determined by labeling for surface CAR expression. Control T-cells were transduced with GFP, CD19-28Z CAR, or CL10-28Z CAR (specific for mouse FRβ). High transduction efficiencies were reproducibly achieved (Figure S4b). GFP-2A-LA-FRβ and HA-FRβ CAR T-cells were labeled with rFRβ, and binding of cell-surface CAR to recombinant antigen was determined by flow cytometry. HA-FRβ CAR T-cells demonstrated high association with rFRβ, whereas this interaction could barely be visualized with LA-FRβ CAR even at high protein concentration (Figure 1e). Accordingly, HA-FRβ CAR T-cells also exhibited increased IFNγ production in response to immobilized rFRβ *in vitro* (Figure 1f).

High affinity FRβ CAR T-cells display increased reactivity against cell surface FRβ

Next we assessed the relative functional reactivity of low and high affinity CAR T-cells against cell surface FRβ by measuring T-cell cytokine secretion, CD69 expression, and lytic activity against $FR\beta^{+}$ cell lines C30-FRβ, THP1 and MV411 and $FR\beta^{(-)}$ cell lines C30 and HL60. Compared to LA-FRβ, HA-FRβ CAR T-cells secreted dramatically increased levels of IFNγ in the presence of $FR\beta^{+}$ C30-FRβ, THP1 and MV411 without activity against negative lines (Figure 2a). HA-FRβ CAR T-cells also produced high levels of IL2 and MIP1α, and moderate to low levels of TNFα, IL4, and IL10 (Figure S5). As depicted in Figure 2b, >90% of LA-FRβ and HA-FRβ CAR^{+} T-cells upregulated CD69 in the presence of high density FRβ (C30-FRβ). However, when encountering antigen at endogenous levels on the AML cell lines, LA-FRβ CAR T-cells displayed lower levels of CD69, whereas nearly all HA-FRβ CAR T-cells were positive. Likewise, both LA-FRβ and HA-FRβ CARs displayed high lytic activity against C30-FRβ (Figure 2c); however, only HA-FRβ T-cells efficiently lysed THP1 and MV411 AML with endogenous FRβ expression.

To determine whether soluble FRβ IgGs could block CAR T-cell activity, we measured IFNγ secretion in response to C30-FRβ in the presence of LA-FRβ or HA-FRβ blocking IgGs. High concentrations (>40ng/1×10^5 target cells) of HA-FRβ IgG completely blocked LA-FRβ CAR T-cell IFNγ secretion (Figure S6). Interestingly, neither low nor high affinity IgG was able to block activity of HA-FRβ CAR T-cells.

High affinity FRβ CAR T cells display exceptional anti-leukemic activity *in vivo*

To determine whether the potent activity of HA-FRβ CAR T-cells against AML could be recapitulated in a mouse model of human disease, we inoculated immunocompromised NSG mice with $FR\beta^+$ fLuc-THP1 human AML. Previously, in mice treated with T-cells at days 8 and 10 of tumor growth, both LA-FRβ and HA-FRβ CAR T-cells led to long-term tumor control[15] (and data not shown). Here, we evaluated FRβ CAR T-cells in mice with large, palpable subcutaneous AML tumors. Surprisingly, both first and second generation HA-FRβ CAR T-cells reproducibly mediated rapid and enduring complete tumor destruction (Figure 3a). LA-FRβ CAR T-cells were ineffective. Consistent with robust *in vivo* activation, peripheral HA-FRβ CAR T-cell numbers were significantly elevated 2 weeks post treatment whereas LA-28Z CAR T-cell counts were not different from control T-cell treated mice (day 29; Figure 3b). However, at day 42, LA-28Z CAR T-cells were present at very high frequency (Figure 3b) while HA-FRβ CAR T-cell numbers from mice that had cleared tumor were low but detectable. Thus, chronic activation of LA-FRβ CAR T-cells in the continued presence of $FR\beta^+$ tumor *in vivo* led to robust expansion, however, likely due to their low functional avidity, did not result in efficient activity against large tumor burden. Alternatively, HA-FRβ CAR T-cells were efficiently activated by $FR\beta^+$ AML, transiently expanded *in vivo*, rapidly cleared tumor and numerically contracted, illustrating an antigen-dependent mechanism of CAR T-cell persistence *in vivo*.

Despite their numerical contraction following clearance of tumor, HA-Z and HA-28Z CAR T-cells were still detectable at on day 92 (Figure S7), suggesting the potential for long-lasting tumor protection. To directly assess this hypothesis, we re-challenged mice that had previously eradicated their primary tumor (HA-28Z) or that were previously untreated (naïve) with 5×10^6 THP1 cells on the opposite flank. Previous treatment led to complete protection against tumor re-challenge (Figure 3c).

High affinity FRβ CAR T-cells are reactive against primary human AML

Importantly, HA-FRβ CAR T-cells also recognized primary human AML. Previous reports found that 70% of AML patients express FRβ[26, 27]. Using HA-FRβ IgG, we confirmed expression of FRβ in 15/16 (93.8%) of AML specimens by flow cytometry, with a mean of 49.8% $FR\beta^+$ blasts (Figure 4a). Four samples with varying FRβ expression were used to assess CAR function. Both LA-FRβ and HA-FRβ CAR T-cells secreted significantly more IFNγ than control T-cells in response to all four patient samples (Figure 4b). For 3/4 primary AML samples, IFNγ secretion by HA-FRβ CAR T-cells was comparable to that achieved using THP1cells, suggesting robust activity against patient tumor. The viability of cryopreserved patient samples was low, and standard lysis assays were not possible. We therefore evaluated CD107 expression on CAR^+ T-cells as a well-accepted surrogate for lytic function[28]. HA-FRβ CAR T-cells exhibited significantly higher CD107 expression

following 6h culture with primary AML (Figure 4c) than control T-cells, confirming the potential for clinical responses in AML patients using potent HA-FRβ CAR T-cells.

High affinity FRβ IgG reveals increasing FRβ expression along myeloid differentiation in healthy hematopoietic cells

We next evaluated the potential for off-tumor recognition of FRβ in healthy tissues. Although previously reported[29], we did not detect FRβ in HSCs using LA-FRβ IgG[15]. We hypothesized that its affinity may not permit sensitive detection of low levels of FRβ described elsewhere. Therefore, we used HA-FRβ IgG to investigate FRβ expression in $CD34^+$ BM HSCs from 5 healthy adults. In contrast to LA-FRβ IgG, we were able to detect very low surface FRβ in HSCs from most donors (Figure 5a). FRβ expression has been previously described in mature myeloid cells,[30–32] however, expression in BM progenitors has not been well characterized. Using myeloid markers (CD123, CD33, CD14), we found increasing FRβ expression during myeloid differentiation, with highest levels found in mature $CD14^+$ monocytes (Figure 5a). Other BM lineages were FRβ-negative (data not shown). In addition, we assayed peripheral blood (PB) for FRβ expression using HA-FRβ IgG. Consistent with previous results, we confirmed lack of expression in PB T-cells, B-cells, NK cells, and granulocytes (Figure 5b). PB FRβ expression was limited to $CD14^+$ monocytes, however, HA-FRβ IgG revealed ~70% (Mean 66.6%, ± 2.44% SEM) of PB monocytes express FRβ, whereas LA-FRβ IgG detection indicated <20% (Mean 15.2%, ± 3.24% SEM) (Figure 5c).

High affinity FRβ CAR T-cells specifically eliminate $FR\beta^+$ myeloid-lineage cells without toxicity against HSCs

To assess HSC toxicity, we conducted colony-forming (CFU) assays following co-culture with CAR T-cells. We did not observe inhibition of total or lineage-specific colony formation by HSCs under any condition (Figure 6a). These results suggest that very low FRβ expression observed in some $CD34^+$ donors was insufficient to activate FRβ-directed CAR T-cells. However, surface FRβ expression increases along BM myeloid differentiation and could activate HA-FRβ CAR T-cells. We co-cultured total $CD34^{(-)}$ BM cells with CAR T-cells and assessed the phenotype of surviving cells after 5h (Figure 6b). Extensive loss of viable myeloid-lineage ($CD33^+$) target cells was not observed, however, HA-FRβ CAR T-cells clearly eliminated $FR\beta^+$ $CD33^+$ myeloid cells (Figure 6b), suggesting that toxicity may be limited to the subset of $FR\beta^+$ BM resident myeloid cells with neighboring $FR\beta^{(-)}$ BM cells selectively spared. In accordance with their high FRβ expression, HA-FRβ CAR T-cells exhibited specific lysis of PB monocytes *in vitro* (Figure 6c).

To assess the impact of targeting FRβ in the native BM microenvironment, NSGs mice reconstituted with human adult BM $CD34^+$ HSCs were treated with HA-FRβ or CD19 control CAR T cells derived from autologous BM T-cells (Figure S8a–b). Similar to *in vitro* experiments, BM HSCs or myeloid progenitor cells were not decreased after treatment with HA-FRβ CAR, however, CD19 control CAR T-cells decreased $CD19^+$ lymphoid-lineage BM cells (Figure S8d–g). Surprisingly, total $CD14^+$ monocytes were not significantly depleted by HA-FRβ CAR T-cells in this model (Figure S8c and h). However, we noted that FRβ expression in $CD14^+$ monocytes from these mice (13.9% $FR\beta^+$) was substantially lower

than that observed in fresh donor PB monocytes (70% FRβ^{+}) (Figure S8i, Figure 5c). Despite this lower overall expression, HA-FRβ CAR T-cells still efficiently depleted FRβ^{hi} monocytes, resulting in decreased expression in monocytes from HA-FRβ CAR T-cell treated mice (mean 5.4% FRβ^{+}) compared to CD19 control treated mice (mean 13.9% FRβ^{+}) (Figure S7i). These data suggest that monocytes with the highest expression of FRβ expressed were depleted by HA-FRβ CAR T-cells *in vivo*.

Transient high affinity FRβ mRNA CAR T-cells retain effective anti-tumor activity

Given that HA-FRβ CAR T-cells can eliminate both FRβ^{+} tumor and healthy myeloid cells but not HSCs, we reasoned that short-term CAR expression during T-cell therapy might allow for upfront tumor cell destruction, with eventual healthy myeloid cell repopulation from normal HSCs. Thus, we developed a transient HA-FRβ CAR model using mRNA electroporation of T-cells. One day post-electroporation, both HA-Z and HA-28Z CARs were highly expressed in T-cells with gradual decrease over time (Figure 7a), coinciding with a similar reduction in THP1 lysis (Figure 7b). mRNA and lentiviral HA-FRβ CAR T-cells displayed similar *in vitro* functional reactivity (Figure S9). We selected first generation HA-FRβ mRNA for *in vivo* T-cell evaluation as we reproducibility observed greater stability of HA-Z mRNA expression and never observed appreciable advantages using CD28 in lentiviral CARs. The activity of HA-Z lentiviral and mRNA CAR T-cells was assessed against disseminated THP1 delivered IV. Although not as robust as lentiviral CAR, HA-Z mRNA CAR T-cells significantly delayed disseminated THP1 tumor growth compared to CD19-Z mRNA T-cells (Figure 7c). These results suggest that mRNA delivery of the HA-FRβ CAR platform could be used transiently to eliminate FRβ^{+} tumor cells.

Discussion

Utilizing a higher affinity scFv, we have greatly optimized our platform for robust targeting FRβ^{+} AML. HA-FRβ CAR T-cells reproducibly out-performed LA-FRβ CAR T-cells as demonstrated by significantly enhanced *in vitro* cytokine secretion, activation marker expression, cell lysis, and *in vivo* anti-leukemic activity.

HA-FRβ IgG allowed sensitive detection of very low levels of FRβ in hematopoietic cells not detectable by LA-FRβ IgG. Our results highlight the importance of antibody affinity when evaluating normal tissue expression for preclinical evaluation of new CAR targets. In addition, the field may find it useful to employ the scFv of the CAR for antibody-based analysis of tissue expression as this may best correlate with CAR recognition. Supporting this notion, we found that target cells not bound by LA-IgG (e.g. MV411 or BM progenitors) were not substantially targeted by LA-FRβ CAR T-cells.

The scFv affinity is considered critically important to CAR T-cell function, with a general consensus that higher affinity reduces the activation threshold, increasing T-cell activity at lower levels of antigen present. However, this aspect of CAR T-cell design is historically under-studied. Chmielewski *et al* developed CARs targeting hErbb2 with scFv affinities ranging from 10^{-7} to 10^{-11} nM, and established a threshold of 10^{-8} below which CAR T-cells responded similarly to all levels of antigen, and above which CAR T-cells only responded to high levels of antigen[16]. Hudecek and colleagues observed increased anti-

tumor efficacy from ROR1-directed CAR T-cells with 5.6×10^{-10}nM K_D compared to 6.5×10^{-8}nM K_D scFvs[17]. We recently reported that FRα CAR T-cells bearing a high affinity scFv were more sensitive to low levels of FRα expression on healthy cells, increasing the risk for toxicity compared to intermediate affinity FRα CAR T-cells[33]. Our experience with FRβ CAR T-cells is in agreement with these findings. LA-FRβ CAR T-cells showed strong *in vitro* function only in response to high levels of antigen in C30-FRβ with reduced reactivity against lower-expressing AML lines THP1 and MV411. HA-FRβ CAR T-cells displayed strong reactivity against C30-FRβ, THP1, and MV411 without large differences in cytokine secretion in response to different levels of FRβ.

We hypothesize that the remarkable difference in LA-FRβ and HA-FRβ CAR T-cell function is related to affinity, however, it is formally possible that they recognize different epitopes of FRβ. For other CAR platforms, epitope proximity to the cell surface can drastically alter CAR performance[34, 35]. Our blocking studies showed that LA-FRβ and HA-FRβ IgGs inhibit the binding capability and/or CAR function of the other platform suggesting a nearby region is recognized. In addition, the two scFvs share identical heavy chain sequences with only 13 amino acid changes in the light chain, an overall 95% sequence homology. The highly similar sequences further suggest these two scFvs do not recognize dissimilar epitopes.

Generally, costimulatory domains provide functional improvement beyond first generation CAR platforms[22, 36–39]. Notably, both HA-Z and HA-28Z FRβ CAR T-cells were highly functional *in vitro* and resulted in complete tumor regression *in vivo*. To our knowledge, this is the first report of second generation CAR activity showing no improved *in vivo* benefit over first generation. We have observed varying levels of costimulatory ligands in AML cell lines[15]. Therefore, costimulation through natural receptors may compensate for the lack of costimulation in HA-Z CARs in the context of targeting AML. Costimulation through TNFR family members (4-1BB, CD27, or OX40) could further increase the persistence of HA-FRβ CAR T-cells beyond Z or 28Z, as reported elsewhere[38, 40, 41]. However, as discussed below, long-term persistence is likely not desirable for HA-FRβ CAR T-cells.

Encouragingly, even with highly potent HA-FRβ CARs, we did not see evidence of HSC destruction using *in vitro* CFU assays or *in vivo* humanized mouse models. This could be important as reports of gross BM toxicity have been described using CAR T-cells directed against AML antigens CD123 and CD33[12, 14]. Therefore, HA-FRβ CAR T-cells could potentially be applied with a reduced risk for BM HSC destruction compared to CD123 or CD33 CAR T-cells. However, FRβ expression does increase along myeloid-lineage differentiation with a distinctly later phase of expression in hematopoietic differentiation compared to CD123 and CD33. Accordingly, HA-FRβ CAR T-cells can lyse more mature myeloid lineage target cells. Therefore, FRβ CAR T-cell therapy may be well suited as an adjunct to be applied following myeloablation in advance of stem cell transplant, a common treatment modality for AML.

While ongoing depletion of healthy B-cells in patients treated with lentiviral CD19 CAR T-cells is managed through IgG replacement therapy, an analogous regimen to cope with lifelong myeloid depletion is not available. As such, long-term persistence of HA-FRβ CAR

T-cells is highly undesirable. Transient CAR expression in T-cells could allow for short-term tumor destruction while avoiding long-term myeloid loss. Multiple doses of HA-FRβ mRNA CAR T-cells significantly delayed THP1 growth *in vivo*. The HA-FRβ CAR sequence is fully human derived, decreasing the propensity for transgene immunogenicity which produced anaphylaxis after repeated administration of murine-derived CAR T-cells[42]. Alternatively, a suicide gene or inducible CAR approach could be used in combination with lentiviral HA-FRβ CAR T-cells to obtain complete tumor destruction before CAR depletion. In any case, transient treatment with HA-FRβ CAR T-cells appears to be a promising therapy for FRβ$^+$ AML while decreasing the risk for long-term myeloid toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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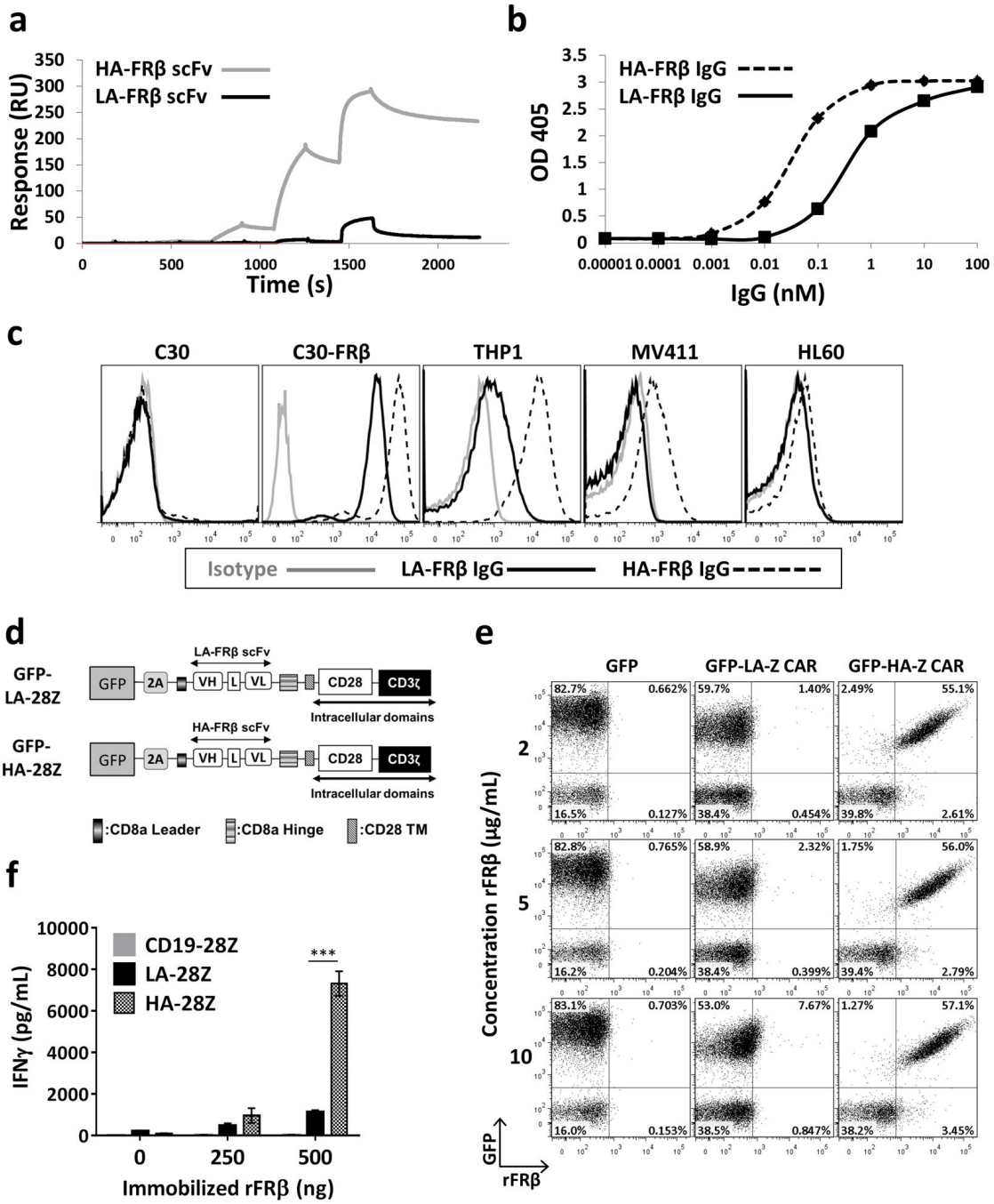

Figure 1. Isolation and characterization of a higher affinity FRβ scFv

(a) Increasing concentrations (0.04, 0.4, 4, 40, and 400 nM) of soluble scFvs were applied to human FRβ-coated chips and affinity was measured by plasmon resonance with Biacore×100. Binding of LA-FRβ and HA-FRβ IgG to **(b)** immobilized rFRβ measured by ELISA or **(c)** cell-surface FRβ measured by flow cytometry in the indicated cell lines. **(d)** Representative schematics of lentiviral CAR constructs (full list in Figure S4a). **(e)** Binding of LA-FRβ and HA-FRβ CAR$^+$ (GFP$^+$) T cells to soluble rFRβ. **(f)** IFNγ secretion following 24h culture of LA-FRβ and HA-FRβ CAR T cells in rFRβ-coated plates. CD19-28Z CAR T

cells were used as a negative control. Error bars represent mean ± SD of triplicate wells. scFv – single chain variable fragment, VH – variable heavy chain, L – linker, VL – variable light chain, TM – transmembrane domain, LA – low affinity FRβ, and HA – high affinity FRβ. (* P < .05, ** P < .01, *** P < .001)

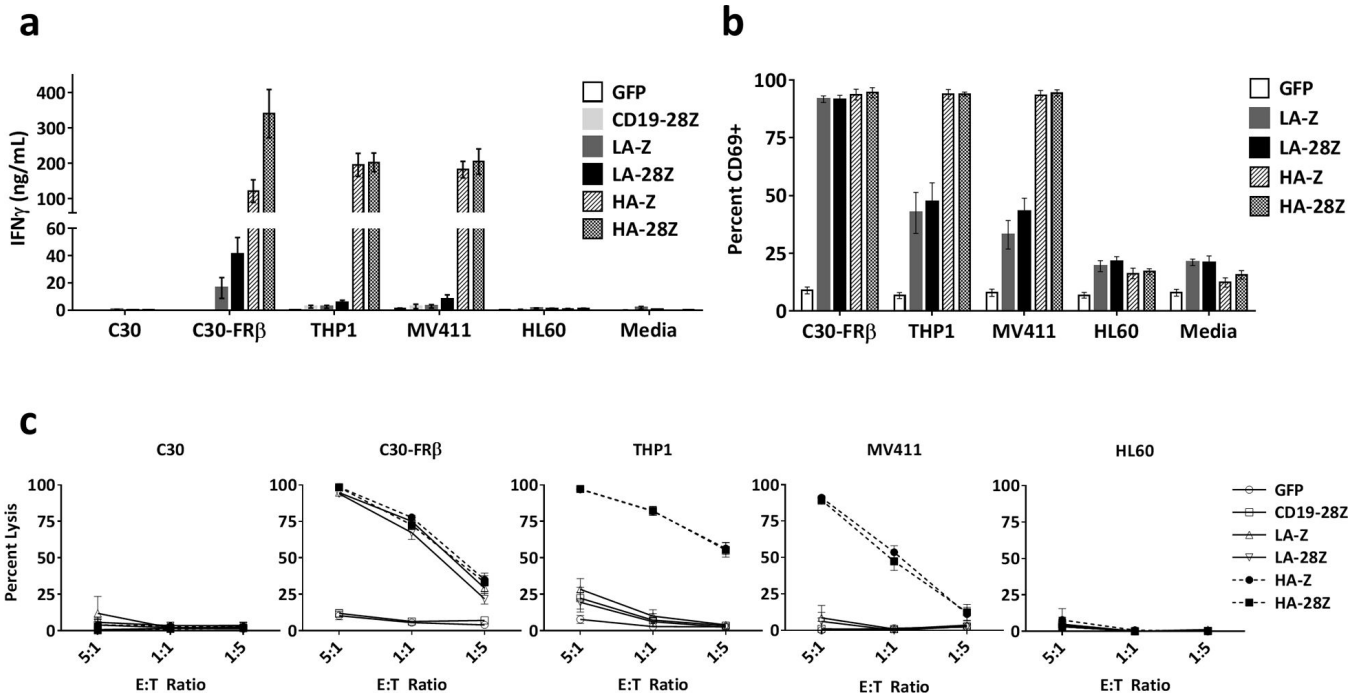

Figure 2. High affinity FRβ CAR T cells demonstrate greater *in vitro* reactivity against FRβ$^+$ cell lines compared to low affinity FRβ CAR T cells

(a) IFNγ secretion following overnight co-culture of LA-FRβ, HA-FRβ, or control CAR T cells with the indicated cell lines. Error bars represent mean ± SEM of n ≥5 independent experiments each performed in triplicate. **(b)** CD69 expression on CAR T cells following overnight co-culture with the indicated target cells. Live, CD3$^+$ CAR$^+$ flow cytometry gates were used to assess percent CD69$^+$ CAR T cells. Error bars represent mean ± SEM of n=4 independent experiments each performed in triplicate. (Note: unstimulated CAR$^+$ T-cells have ~20% CD69$^+$ cells at baseline as revealed in the Media control). **(c)** High lytic activity from HA-FRβ CAR T cells against FRβ$^+$ cell lines compared to LA-FRβ. T cells and fLuc$^+$ target cells were co-cultured at the indicated E:T ratios. Percent lysis was assessed by residual target cell luminescence following overnight culture. Error bars represent mean ± SEM of n ≥5 independent experiments each performed in triplicate. Media indicates T cells cultured without target cells. GFP and/or CD19-28Z CAR T cells were used as controls. SEM – standard error, fLuc – firefly luciferase, LA – low affinity FRβ, HA – high affinity FRβ.

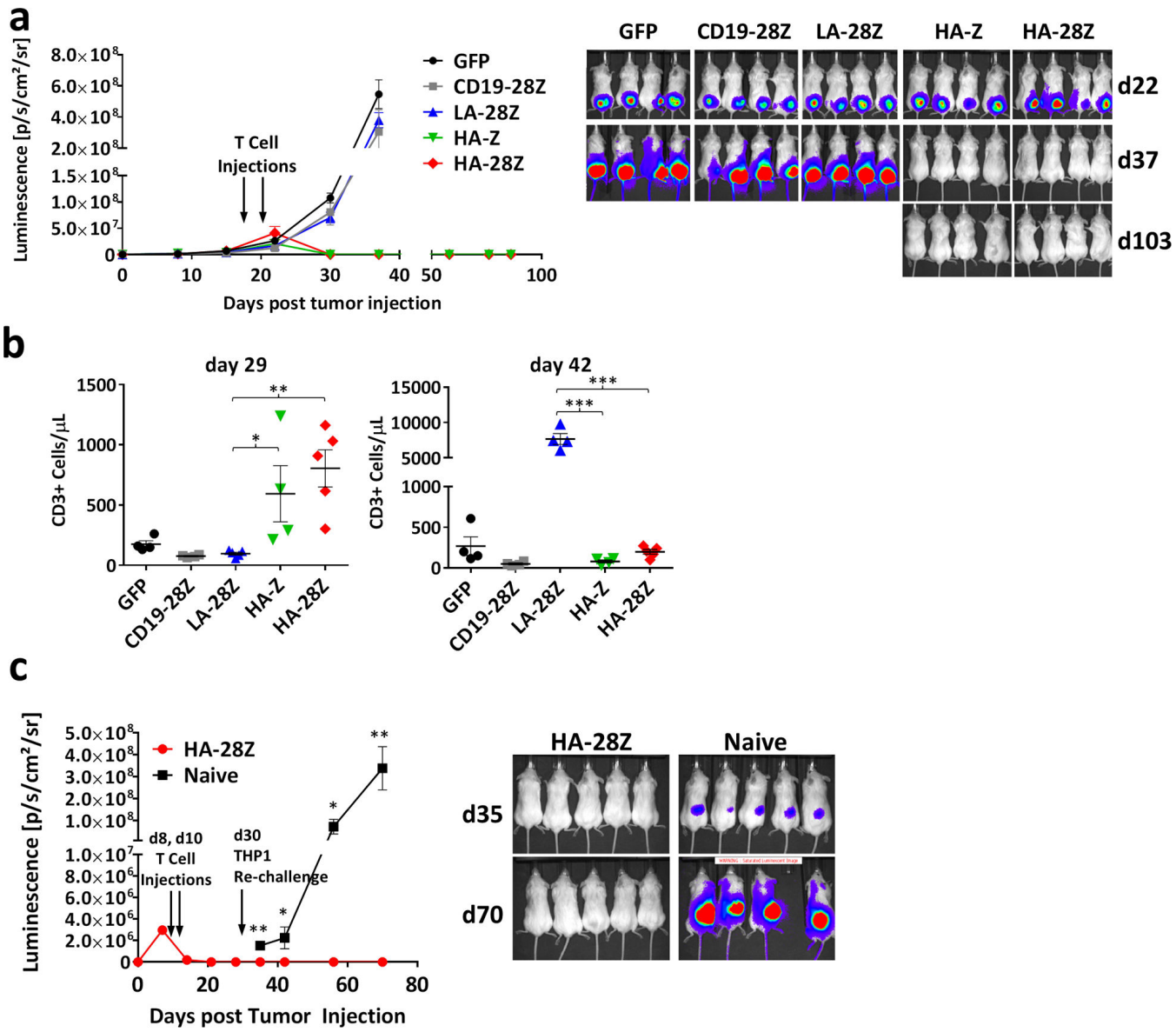

Figure 3. High affinity FRβ CAR T cells display exceptional anti-leukemic activity *in vivo*

In panels a–b, 5×10^6 fLuc-THP1 AML cells were injected sc into flanks of NSG mice. 5×10^6 CAR$^+$ (or GFP$^+$) T cells were injected IP on days 19 and 22 post tumor inoculation. **(a)** Tumor growth was monitored by tumor bioluminescence. **(b)** Peripheral blood T cell quantification on days 29 and 42 post tumor inoculation. Error bars represent mean ± SEM of n=4–5 mice per group. In panel c, mice were injected with 5×10^6 HA-28Z CAR T cells on days 8 and 10 following tumor inoculation. Following tumor clearance on day 30, HA-28Z treated mice were re-challenged and previously untreated (naïve) mice were challenged with 5×10^6 fLuc-THP1. Tumor growth was monitored by bioluminescence **(c)**. Error bars represent mean ± SEM of n=5 mice per group. fLuc – firefly luciferase, NSG – Nod/SCID/γchain$^{-/-}$, sc– subcutaneous, IP – intraperitoneal, SEM – standard error, LA – low affinity FRβ, HA – high affinity FRβ. (* P < .05, ** P < .01, *** P < .001)

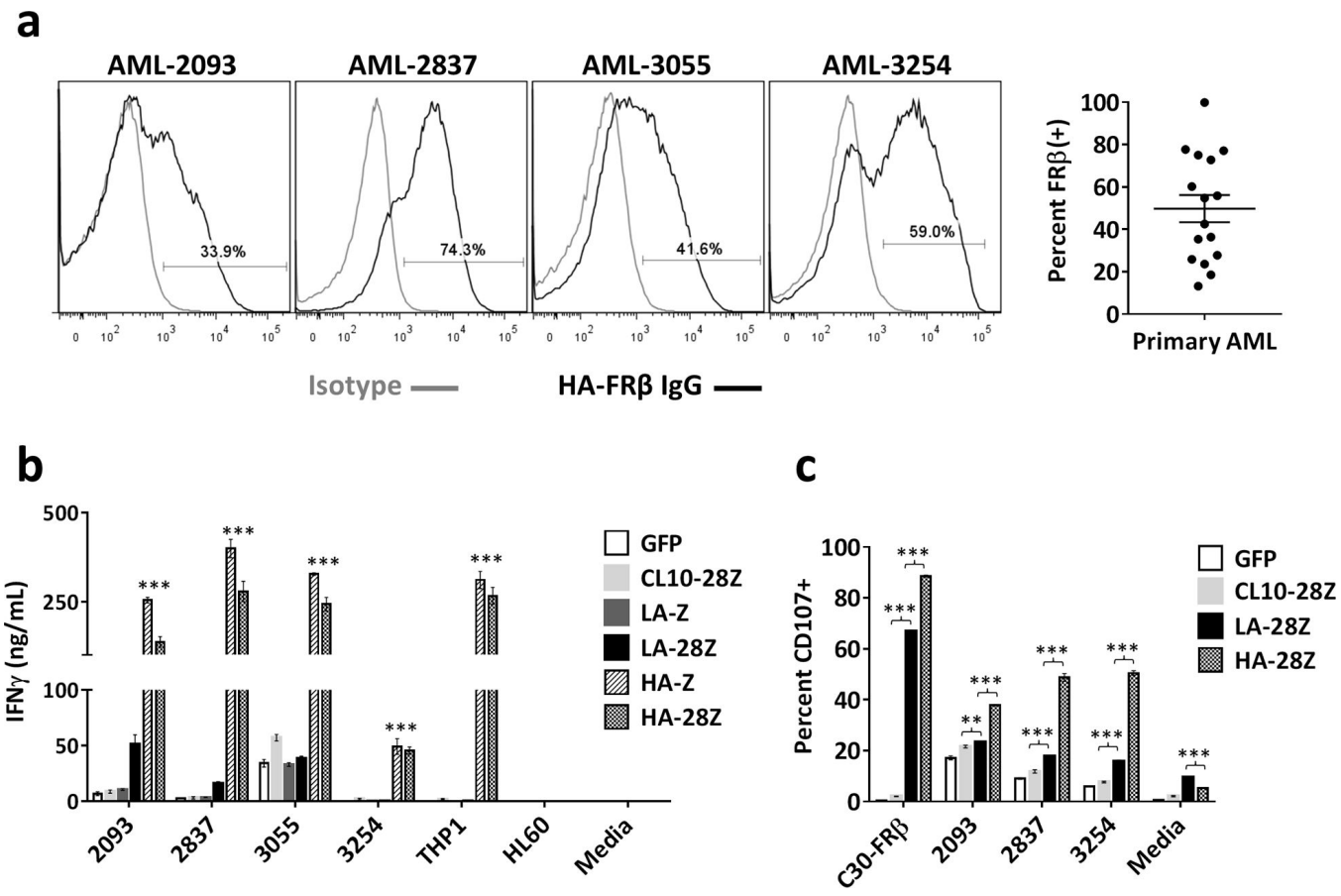

Figure 4. High affinity FRβ CAR T cells are reactive against primary human AML

(a) FRβ expression in primary human peripheral blood cells isolated from AML patients. Live, $CD33^+$ gates were used to assess FRβ expression by flow cytometry. (Gray histogram–isotype, black histogram – HA-FRβ IgG). 4 representative histograms shown. Percent $FR\beta^+$ blasts for n=16 patients is summarized to the right (Mean=49.8%). **(b)** IFNγ secretion after overnight co-culture of indicated primary AML samples with CAR T cells. The CD19 CAR control was replaced with a murine FRβ-specific CL10-28Z CAR T-cell control because of the presence of $CD19^+$ B cells in the patient blood samples. Error bars represent mean ± SD of triplicate wells. One representative of 3 independent experiments shown. (*** P < .001 for both HA-Z and HA-28Z compared to CL10-28Z control CAR T cells) **(c)** CD107 upregulation on CAR^+ T cells following 6h co-culture with primary AML patient cells. Live, $CD3^+$, CAR^+ gates were used to assess percent $CD107^+$. Error bars represent mean ± SD of triplicate wells. One representative of 2 independent experiments shown. SD – standard deviation, LA – low affinity FRβ, HA – high affinity FRβ. (** P < .01, *** P < .001)

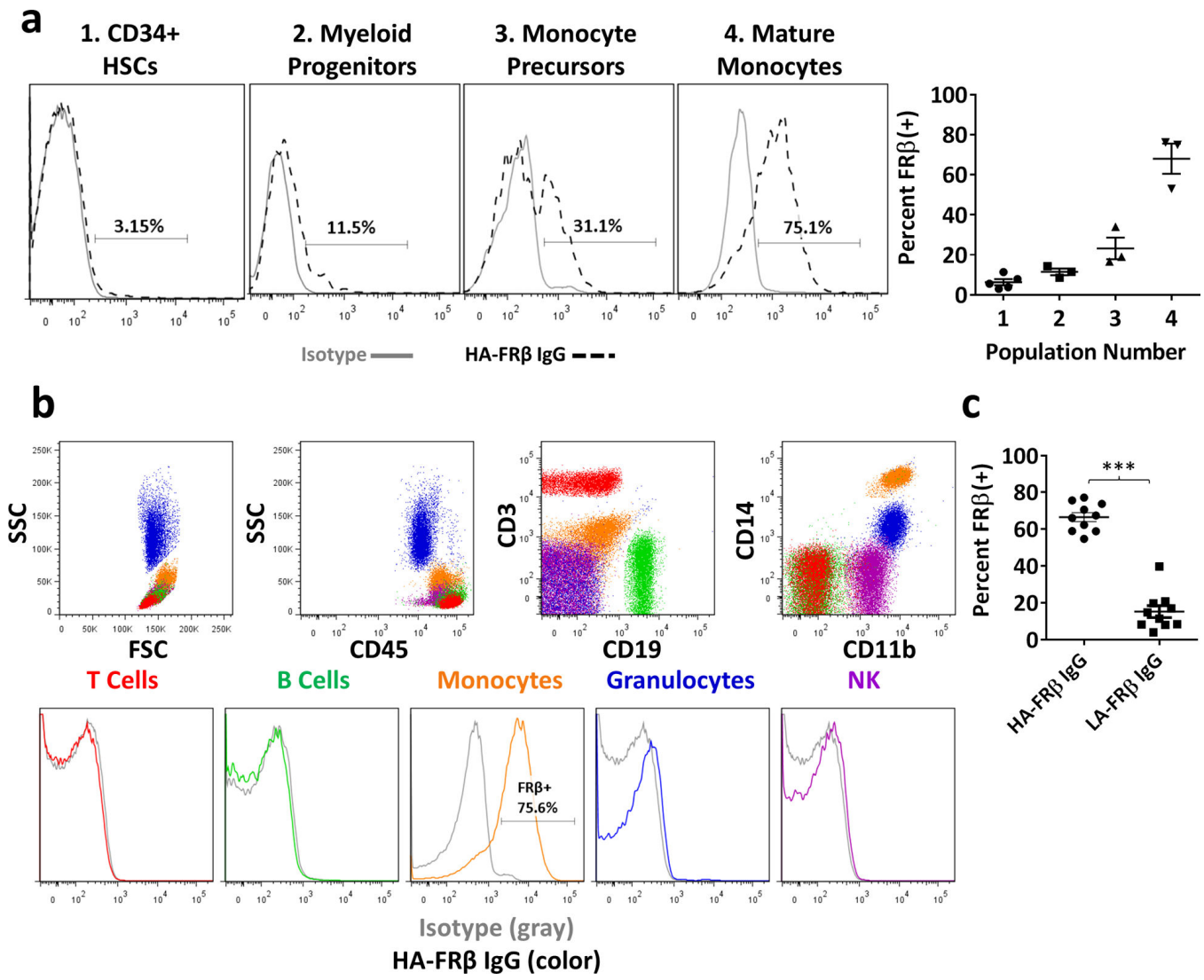

Figure 5. High affinity FRβ IgG reveals increasing expression of FRβ along myeloid differentiation in healthy hematopoietic cells

(a) FRβ expression in healthy adult BM. Representative histograms from one donor shown. Data from 3–5 independent donors is displayed to the right for the following populations: (1) $CD34^{+}$ HSCs (Mean 6.3% $FR\beta^{+}$, n=5). (2) Myeloid progenitors–$CD123^{HI}CD33^{(-)}CD14^{(-)}$ (Mean 11.4% $FR\beta^{+}$, n=3), (3) Monocyte precursors–$CD123^{low}$, $CD33^{+}$, $CD14^{low}$ (Mean 23.2% $FR\beta^{+}$, n=3), and (4) Mature monocytes–$CD123^{(-)}$, $CD33^{+}$, $CD14^{HI}$ (Mean 68.0% $FR\beta^{+}$, n=3). (Gray histogram– isotype, black dashed histogram – HA-FRβ IgG). **(b)** FRβ expression in peripheral blood cells. Upper panels – gating strategy to identify subsets. Lower panels – FRβ expression in indicated subsets. (Gray histogram– isotype, color histograms – HA-FRβ IgG) Red – T cells, Green – B cells, Orange – monocytes, Blue – granulocytes, Purple – NK cells. One representative of 3 independent donors shown. **(c)** Percent FRβ expression detected in peripheral blood monocytes (n=10) using HA-FRβ IgG (mean=66.6%) or LA-FRβ IgG (mean=15.2%) for flow cytometry. Error bars represent

mean ± SEM. BM – bone marrow, HSCs – hematopoietic stem cells, SSC – side scatter, FSC – forward scatter, LA – low affinity FRβ, HA – high affinity FRβ. (*** P < .001)

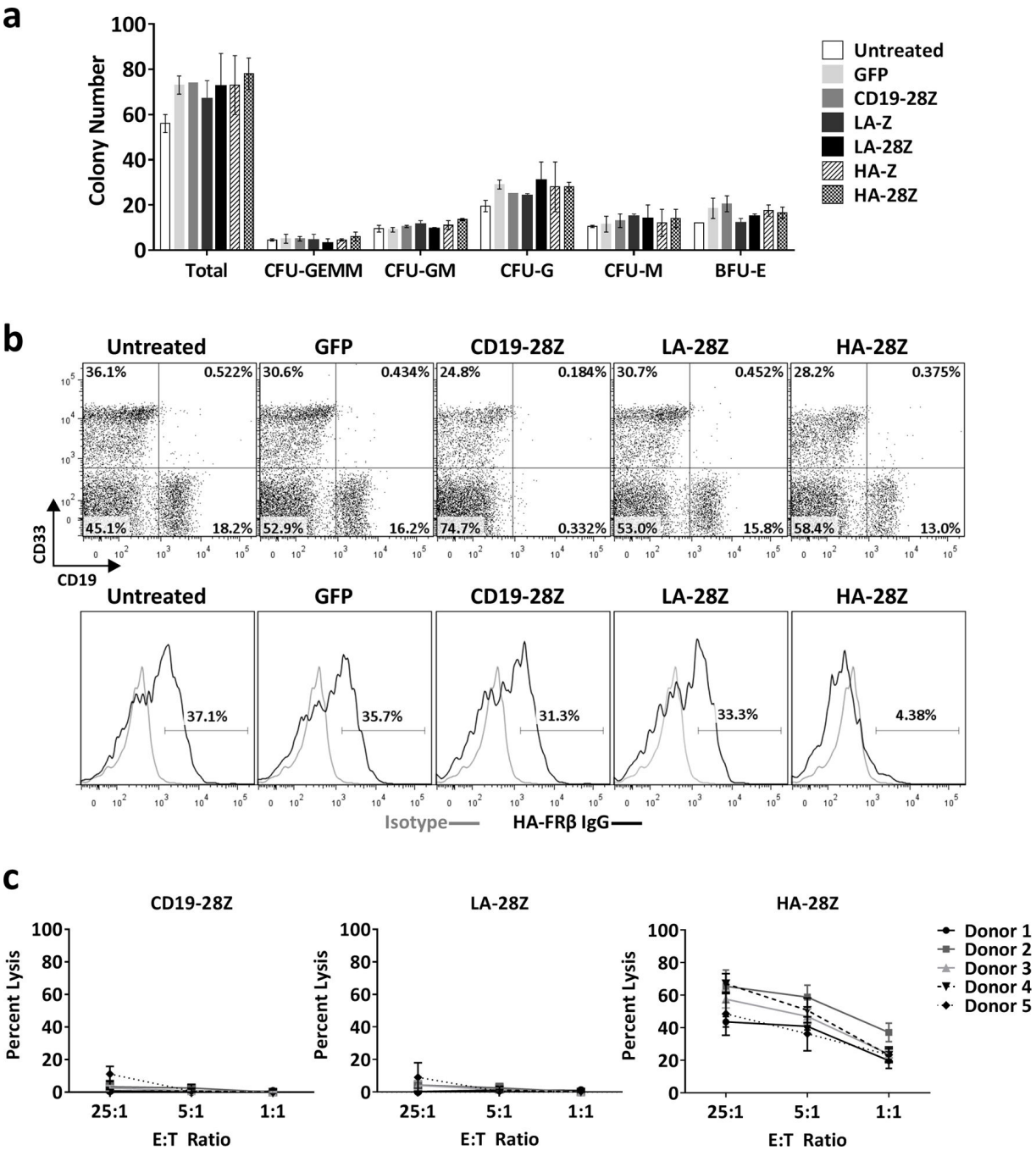

Figure 6. High Affinity FRβ CAR T-cells specifically eliminate FRβ^{+} myeloid lineage cells without toxicity against HSCs

(a) Number of total and lineage specific colonies from CFU assay following 4h co-culture of 2000 CD34^{+} and 2000 CAR^{+} T cells. Error bars represent mean ± SD of duplicate wells. One representative of 4 independent experiments is shown. **(b)** Phenotype of CD34$^{(-)}$ adult BM following 5h co-culture with CAR T cells. Untreated samples were cultured in the absence of T cells. Upper panels - frequency of CD33 and CD19 expression in total live, CD3$^{(-)}$ target cells surviving co-culture with the indicated T cells. Lower panels - FRβ

expression in total live, $CD3^{(-)}CD33^{+}$ myeloid lineage BM target cells surviving co-culture with the indicated T cells. (Gray histogram– isotype, black histogram – HA-FRβ IgG). One representative of 3 independent experiments is shown. **(c)** Percent lysis of isolated $CD14^{+}$ peripheral blood monocytes following 4h co-culture with CAR T cells at 25:1, 5:1, or 1:1 E:T ratios. Error bars represent mean ± SD of 6 replicate wells. 5 independent healthy monocyte donors were assessed. CFU– colony forming unit, GEMM–granulocyte/erythrocyte/monocyte/megakaryocyte, GM–granulocyte/monocyte, G–granulocyte, M–monocyte, BFU-E– erythroid blast forming unit, BM– bone marrow, SD– standard deviation, SEM– standard error, LA – low affinity FRβ, HA – high affinity FRβ.

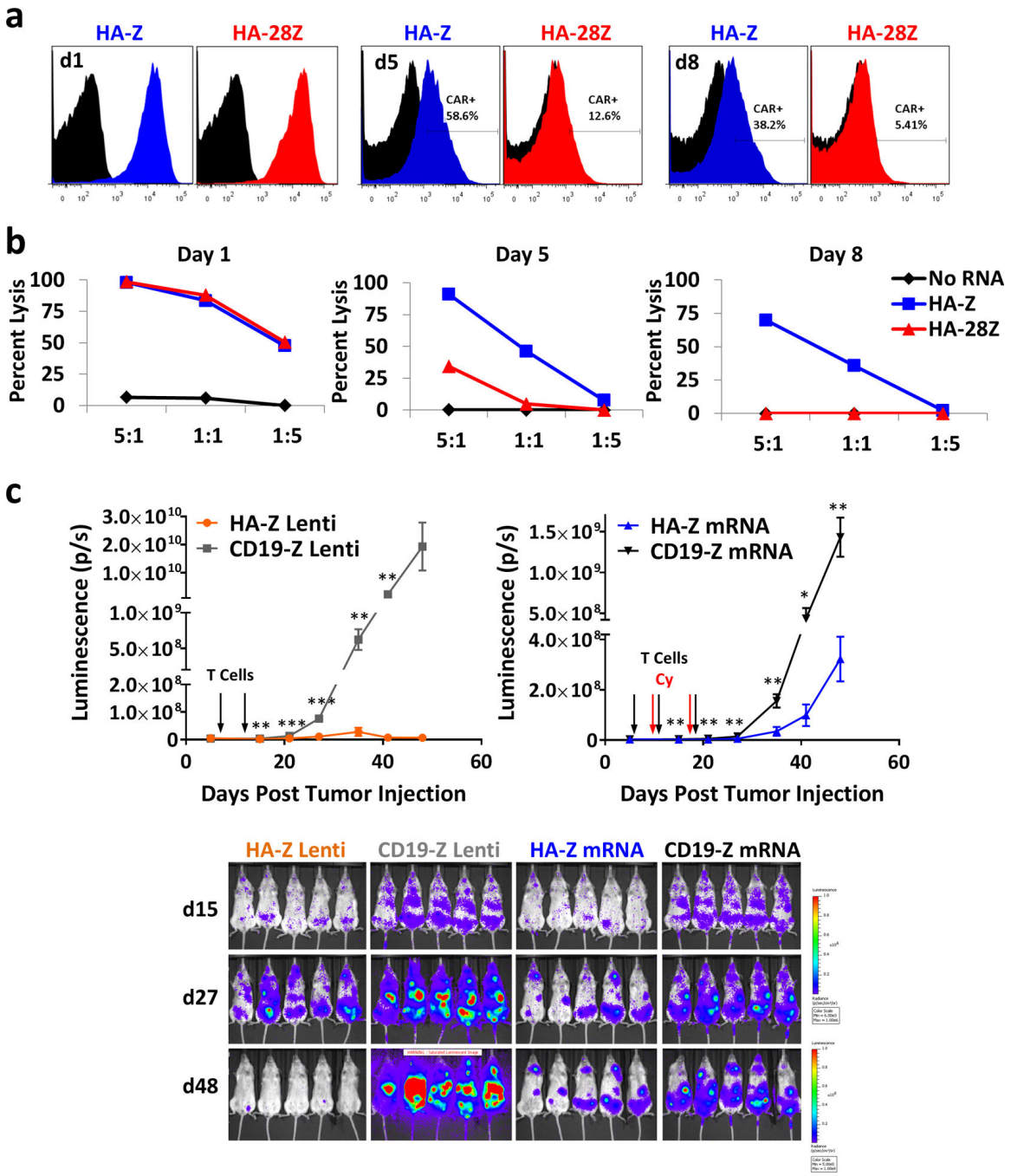

Figure 7. Transient High Affinity FRβ mRNA CAR T cells retain anti-tumor activity against disseminated AML

HA-Z and HA-28Z CAR mRNA was introduced into resting T cells by electroporation. CAR expression **(a)** and THP1 lysis **(b)** was measured on day 1 (left panels), day 5 (middle panels), and day 8 (right panels) following electroporation. "No RNA" (black) represents T cells electroporated in the absence of mRNA. **(c)** Bioluminescence of disseminated THP1 tumor growth in mice treated with mRNA or lentiviral (Lenti) CAR T cells. Mice were inoculated with 5×10^6 fLuc-THP1AML cells via IV injection. Mice received 5×10^6 HA-Z or

CD19-Z Lenti CAR T cells on days 6 and 11, or $10\times10e^6$ HA-Z or CD19-Z mRNA CAR T cells on days 6, 11, and 18 post tumor injection via IV delivery. mRNA CAR T cells were injected 18h post-electroporation. Mice receiving mRNA CAR T cells also received 60mg/kg Cyclophosphamide (Cy) IP between T cell doses (days 10 and 17) to eliminate CAR-negative T cells between doses. Error bars represent mean ± SEM of n=5 mice per group. IV– intravenous, IP– intraperitoneal, SEM– standard error, HA – high affinity FRβ. (* P < .05, ** P < .01, *** P < .001)