Potent preclinical activity of FLT3-directed chimeric antigen receptor T-cell immunotherapy against *FLT3*-mutant acute myeloid leukemia and *KMT2A*-rearranged acute lymphoblastic leukemia

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Abstract

Chimeric antigen receptor (CAR) T-cell immunotherapies targeting CD19 or CD22 induce remissions in the majority of patients with relapsed/refractory B-cell acute lymphoblastic leukemia (ALL), although relapse due to target antigen loss or downregulation has emerged as a major clinical dilemma. Accordingly, great interest exists in developing CAR T cells directed against alternative leukemia cell surface antigens that may help to overcome immunotherapeutic resistance. The fms-like tyrosine kinase 3 receptor (FLT3) is constitutively activated via *FLT3* mutation in acute myeloid leukemia (AML) or wild-type *FLT3* overexpression in *KMT2A* (*lysine-specific methyltransferase 2A*)-rearranged ALL, which are associated with poor clinical outcomes in children and adults. We developed monovalent FLT3-targeted CAR T cells (FLT3CART) and bispecific CD19xFLT3CART and assessed their anti-leukemia activity in preclinical models of *FLT3*-mutant AML and *KMT2A*-rearranged infant ALL. We report robust *in vitro* FLT3CART-induced cytokine production and cytotoxicity against AML and ALL cell lines with minimal cross-reactivity against normal hematopoietic and non-hematopoietic tissues. We also observed potent *in vivo* inhibition of leukemia proliferation in xenograft models of both *FLT3*-mutant AML and *KMT2A*-rearranged ALL, including a post-tisagenlecleucel ALL-to-AML lineage switch patient-derived xenograft model pairing. We further demonstrate significant *in vitro* and *in vivo* activity of bispecific CD19xFLT3CART against *KMT2A*-rearranged ALL and posit that this additional approach might also diminish potential antigen escape in these high-risk leukemias. Our preclinical data credential FLT3CART as a highly effective immunotherapeutic strategy for both *FLT3*-mutant AML and *KMT2A*-rearranged ALL which is poised for further investigation and clinical translation.

Introduction

Fms-like receptor tyrosine kinase 3 (FLT3, also known as CD135) is a cytokine receptor tyrosine kinase expressed on hematopoietic stem and progenitor cells that regulates proliferation and differentiation.[1] FLT3 is also expressed on the majority of acute myeloid leukemia (AML) and many B-acute lymphoblastic leukemia (B-ALL) cells.[2] Activating mutations in *FLT3* occur in approximately 30% of cases of adult AML and 25-30% of pediatric AML[3,4] and result in ligand-independent constitutive activation of downstream signaling pathways and detectable FLT3 re-

ceptor cell surface protein expression. These mutations most frequently include internal tandem duplications (ITD) within the *FLT3* juxtamembrane domain and, less commonly, missense point mutations in the activation loop/tyrosine kinase domain.[4] *FLT3* mutations are associated with poor disease-free and overall survival in adults and children with AML, although consolidation of chemotherapy-induced initial remissions with allogeneic hematopoietic stem cell transplantation and/or addition of targeted FLT3 inhibitors to chemotherapy (including post-transplant maintenance) have recently improved clinical outcomes.[5,6]

In addition, constitutive wild-type *FLT3* overexpression is common in B-ALL harboring rearrangements in *lysine-specific methyltransferase 2A* (*KMT2A*, formerly *mixed lineage leukemia* [*MLL*]). *KMT2A*-rearranged (*KMT2A*-R) cases make up 10% of ALL across the age spectrum, but account for 70-80% of ALL cases in children <12 months of age who have particularly suboptimal clinical outcomes. Recent data from the Interfant-06 (NCT00550992) and AALL0631 (NCT00557193) clinical trials documented 6-year and 3-year event-free survival rates of 46% and 36%, respectively, in infants with *KMT2A*-R ALL.[7,8] Worse outcomes, with event-free survival <20%, have been reported for patients aged <90 days at diagnosis, those with a diagnostic white blood cell count >300,000 cells/dL, subjects with poor prednisone prophase responses, and cases with positive measurable residual disease at the end of induction which are largely unsalvageable by chemotherapy intensification or hematopoietic stem cell transplantation.[9] Interestingly, highest *FLT3* overexpression has been associated with worst outcomes in infants with *KMT2A*-R ALL,[10] highlighting the potential biological importance of successful therapeutic targeting of FLT3.

Chimeric antigen receptor (CAR) T-cell therapy targeting CD19 (CD19CART) or CD22 (CD22CART) has proven highly successful in patients with relapsed/refractory B-ALL,[11-14] including infants.[15] Despite high rates of initial remission, approximately 50% of patients treated with CD19CART will relapse again within 2 years, often due to target antigen loss and/or lineage switch in *KMT2A*-R ALL.[13,15-17] This phenomenon has led to appreciable interest in alternative strategies that may prevent target antigen escape and increase long-term cure rates, including CAR T cells directed at alternative leukemia cell surface antigens (e.g., TSLPR, BAFFR) and bispecific CAR T cells that recognize and target multiple antigens simultaneously.[18] Initial studies of dual-targeting CD19xCD22 CAR T cells demonstrated robust anti-leukemia activity in preclinical B-ALL models,[19] and current clinical phase I trials of these bispecific immunotherapies (NCT03241940, NCT03289455, NCT03330691, NCT03448393) have reported exciting early results.[20,21] Whether bispecific cellular therapy products will prevent antigen loss relapse and/or induce greater remission duration does, however, remain unknown.

Given the poor clinical outcomes of patients with *FLT3*-mutant AML and *KMT2A*-R ALL and their shared characteristic of *FLT3* overexpression, we hypothesized that immunotherapeutic targeting of the FLT3 cell surface antigen could be highly effective against both high-risk subtypes of leukemia. Herein, we report potent preclinical *in vitro* and *in vivo* activity of new FLT3-targeting CAR T cells (FLT3CART) against *FLT3*-mutant AML and *KMT2A*-R ALL cell lines and patient-derived xenograft (PDX) models. In these preclinical studies, we demonstrate robust activity of new bicistronic dual FLT3- and CD19-targeting CAR T cells (CD19xFLT3CART) in *KMT2A*-R ALL cell lines and PDX models which may provide an alternative therapeutic approach for future clinical investigation.

Methods

FLT3 chimeric antigen receptor construct design and T-cell transduction

FLT3CART, CD19xFLT3CART, and CD19CART were engineered using previously described methodologies[19,22] and as detailed in the *Online Supplementary Methods* with single-chain fragment variable (scFv) derived from an anti-CD135 NC7 antibody.[23] T cells from four healthy donors were utilized for these studies to ensure robustness and reproducibility of results. Transduction efficiency was 52-74% for monovalent FLT3CART and 24-40% for bispecific CD19xFLT3CART across all experimental studies.

Human leukemia cell lines

FLT3-mutant (MOLM-14, MV4;11) and wild-type (THP-1) AML cell lines and *KMT2A*-R (HB11;19, KOPN-8, SEM) and wild-type (NALM-6) ALL cell lines (Figures 1 and 2) were kindly provided by Dr. Martin Carroll at the University of Pennsylvania and Dr. Patrick Brown formerly at Johns Hopkins University or purchased from the DSMZ cell line biorepository (Braunschweig, Germany). Human leukemia cell lines were also lentivirally-transduced with a luciferase/GFP construct and double-sorted for GFP^+ cell selection for use in *in vivo* cell line xenograft model experiments with bioluminescent imaging as described elsewhere.[22,24] All cell lines were assessed regularly for *Mycoplasma* contamination. Cell lines were maintained *in vitro* for no longer than 2 months in RPMI cell culture medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin.

In vitro analyses of FLT3CART and CD19xFLT3CART functionality

Human interleukin-2 (IL-2) and interferon-gamma (IFN-γ)

cytokine production was evaluated via enzyme-linked immunosorbent assays (ELISA; Biolegend) and leukemia cell viability was assessed via a luciferase assay (Promega), conducted as described previously,[22] using luciferase-expressing human ALL or AML cell lines co-incubated *in vitro* with vehicle, mock-transduced T cells, FLT3CART, CD19CART, or CD19xFLT3CART (*Online Supplementary Methods*).

Flow cytometry analyses

Flow cytometry data were collected via FACSVerse and LSRFortessa X-20 flow cytometers (BD Biosciences) and analyzed with FlowJo (BD Biosciences) or Cytobank (Beckman-Coulter). FLT3 cell surface molecules/cell were quantified with CD135-phycoerythrin (PE) antibodies (EBioscience) and QuantiBrite-PE beads (Invitrogen). Other antibodies used for flow cytometry studies are listed in the *Online Supplementary Methods* or below for animal studies.

In vivo analyses of FLT3CART and CD19xFLT3CART functionality

Animal studies were performed at the Children's Hospital of Philadelphia under a protocol approved by the Institutional Animal Care and Use Committee. The ability of FLT3CART to inhibit AML or ALL proliferation *in vivo* was assessed in bioluminescent human leukemia cell line xenograft models or primary PDX models using NOD-scid IL2Rγ^{null} (NSG) or NOD-*scid* IL2Rγ^{null}-3/GM/SF (NSGS) mice as previously described[22,24] and as detailed in the *Online Supplementary Methods*.

Statistical analyses

Statistical analyses and data display were performed using Prism software (GraphPad). Unpaired Student *t*-tests or one-way analysis of variance with Dunnett or Tukey post-tests for multiple comparisons were used to detect differences between or among treatment groups.

Results

Robust *in vitro* and *in vivo* activity of FLT3CART against *FLT3*-ITD acute myeloid leukemia cell lines

In these studies, we first hypothesized that FLT3CART would have potent anti-leukemia effects against *FLT3*-mutant AML. Flow cytometry quantification of surface FLT3 protein expression on human *FLT3*-ITD (MV4;11, MOLM-14) and *FLT3* wild-type (THP-1) AML cell lines demonstrated a range of surface antigen levels (Figure 1A, *Online Supplementary Table S1*). After T-cell transduction with an optimized second-generation FLT3-targeted CAR construct (*Online Supplementary Figure S1A, B*), the *in vitro* activity of FLT3CART against AML cell lines was assessed via cytokine production and viability assays. We observed that FLT3CART induced greater IL-2 and IFN-γ levels when co-incubated with AML cell lines with higher surface expression of FLT3 (Figure 1B), consistent with previous studies of other antigen-targeting CAR T cells.[25,26] FLT3CART also resulted in a significant reduction in the numbers of viable cells of all three AML cell lines tested (Figure 1C).

To evaluate the *in vivo* activity of FLT3CART, NSG mice were engrafted with luciferase-expressing leukemia cell lines, treated with a single dose of FLT3CART ($1x10^7$ total cells, 52-74% transduction efficiency), and followed by bioluminescent imaging to assess effects upon AML proliferation. We observed that FLT3CART potently eradicated leukemia in both MV4;11 and MOLM-14 xenograft models. As with *in vitro* testing, the observed therapeutic effects were greatest against *FLT3*-ITD AML cells. Conversely, FLT3CART had minimal *in vivo* anti-leukemia activity in a *FLT3* wild-type THP-1 xenograft model when compared to saline or mock-transduced T-cell negative controls (Figure 1D).

Robust *in vitro* and *in vivo* activity of FLT3CART against *KMT2A*-rearranged acute lymphoblastic leukemia cell lines

As *KMT2A*-R ALL cells have wild-type *FLT3* overexpression with high levels of FLT3 cell surface protein, we next hypothesized that this B-ALL subtype would also be sensitive to FLT3CART. We first confirmed elevated surface expression of FLT3 on the *KMT2A*-R ALL cell lines HB11;19, KOPN-8, and SEM and low expression on the *KMT2A* wild-type cell line NALM-6 (Figure 2A, *Online Supplementary Table S2*). *In vitro* co-incubation of FLT3CART with *KMT2A*-R, but not control non-*KMT2A*-R, ALL cell lines induced robust cytokine production and decreased viability (Figure 2B, C). As predicted, FLT3CART treatment of luciferase-positive *KMT2A*-rearranged ALL cell line xenograft models demonstrated potent *in vivo* activity with complete leukemia clearance in some models and no anti-leukemia activity detected in the FLT3-low NALM-6 xenograft model (Figure 2D, *Online Supplementary Figure S1C*). These initial data thus accredit FLT3 as an effective immunotherapeutic target not only for AML, but also for *KMT2A*-R ALL.

Potent *in vivo* activity of FLT3CART in patient-derived xenograft models of acute myeloid leukemia and acute lymphoblastic leukemia

Given our goal of potential clinical translation, we further assessed the effects of FLT3CART immunotherapy *in vivo* in PDX models. A single dose of FLT3CART eradicated leukemia in end-study bone marrow and/or spleens of a *FLT3*-mutant AML PDX model with CAR T-cell expansion and persistence detected several weeks later (Figure 3A). Interestingly, FLT3CART was also effective against a *FLT3*

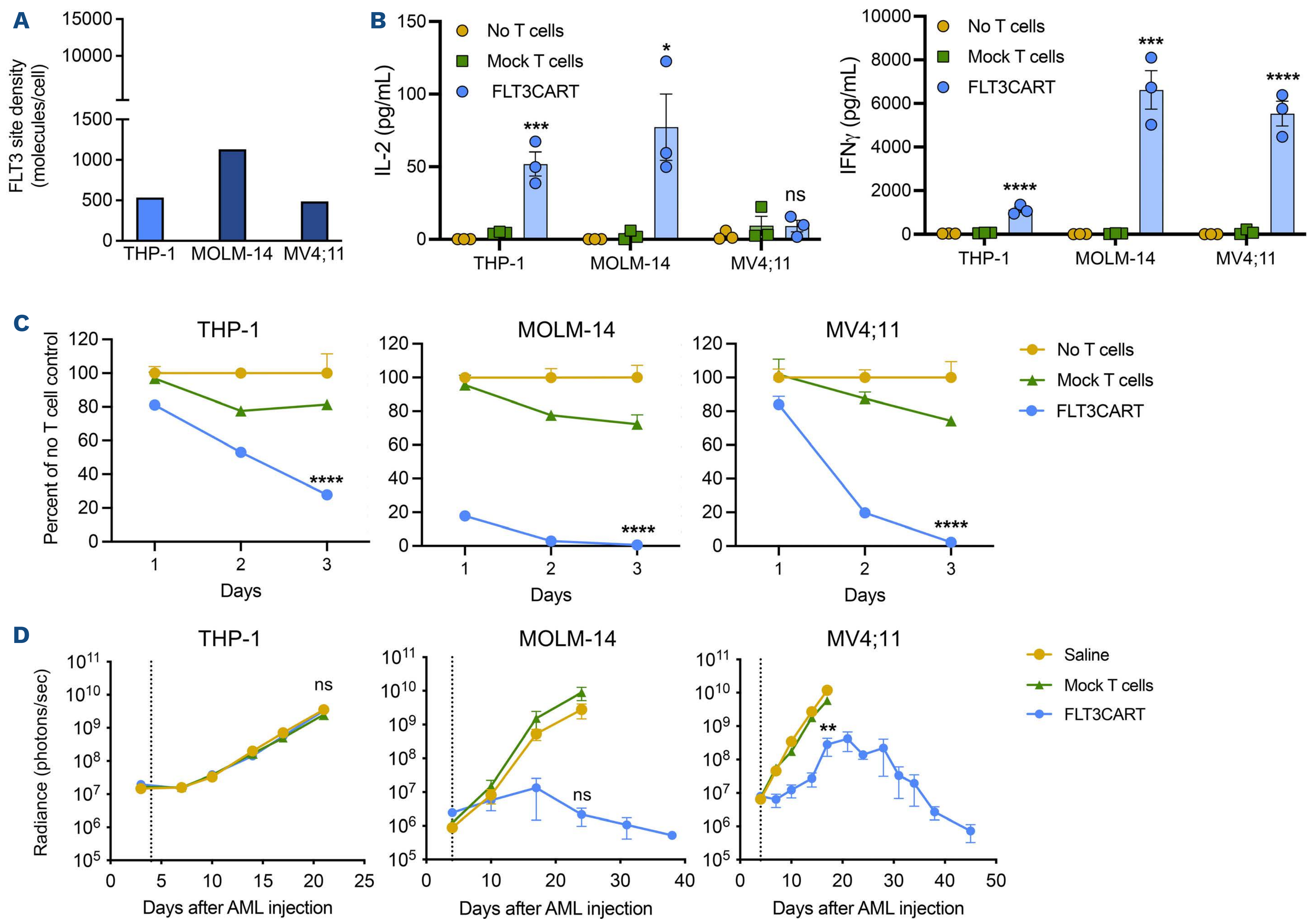

Figure 1. FLT3CART has potent *in vitro* and *in vivo* activity against human *FLT3*-ITD acute myeloid leukemia cell lines. (A) Flow cytometric quantification of FLT3 surface antigen density on acute myeloid leukemia (AML) cell lines. A split Y-axis is utilized to facilitate comparison with acute lymphoblastic leukemia (ALL) cell lines in Figure 2B. (B) AML cell lines were co-incubated *in vitro* with FLT3CART or mock-transduced T cells in a 1:1 ratio (30,000 cells) for 48 hours. Production of human interleukin-2 (IL-2, left panel) and interferon gamma (IFN-γ, right panel) in culture supernatant was measured by enzyme-linked immunosorbent assay. FLT3CART-induced cytokine production was highest with *FLT3*-internal tandem duplication (ITD) AML cell lines MOLM-14 and MV4;11. (C) Viability assays of luciferase-transduced AML cell lines co-incubated with FLT3CART at a 1:1 ratio demonstrate significant inhibition of leukemia cell growth *in vitro* over time with the most complete effects detected against *FLT3*-ITD AML cell lines. Experiments in (B) and (C) were performed with triplicate technical replicates and results are displayed ± standard error of the mean (SEM). (D) Luciferase-transduced AML cell lines ($1x10^6$ cells/mouse) were injected intravenously (IV) into NSG mice. After confirming engraftment via bioluminescent imaging (BLI), cell line xenograft mice were randomized (n=5/group) to receive intravenous saline (yellow), $1x10^7$ mock-transduced T cells (green), or $1x10^7$ FLT3CART (blue) on day 3 or 4 (vertical dashed line). BLI radiance at indicated timepoints is presented graphically ± SEM. FLT3CART displayed *in vivo* anti-leukemia activity against both tested *FLT3*-ITD AML cell line models (MOLM-14 and MV4;11), but not against a *FLT3* wild-type cell line (THP-1) despite some detection of *in vitro* activity as above. Data in (B) and (D) were analyzed by one-way analysis of variance (ANOVA) and in (C) by two-way ANOVA with the Dunnett post-test for multiple comparisons using FLT3CART as the comparator. The statistical significance of the effects of FLT3CART compared to those of the mock T-cell group at the last measured time point is displayed. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns = not significant.

wild-type AML model with somatic monosomy 7 and *PTPN11* mutation (Figure 3B), likely due to its similar level of FLT3 cell surface protein expression. We further observed marked leukemia clearance following FLT3CART treatment of FLT3-expressing infant and adult *KMT2A*-R ALL PDX models with detectable FLT3CART expansion in end-study spleens (Figure 3C, D), but not in a FLT3-low non-*KMT2A*-R ALL PDX model (*Online Supplementary Figure S2A, B*). Of note, FLT3 cell surface antigen expression in most tested *KMT2A*-R ALL models was observed to be greater than in AML models (Figure 3E, *Online Supplementary Figure S2A*). Taken together, these findings underscore the potential broad applicability of FLT3CART immunotherapy for AML potentially regardless of *FLT3* mutation status and further demonstrate potent anti-leukemia activity against *KMT2A*-R ALL.

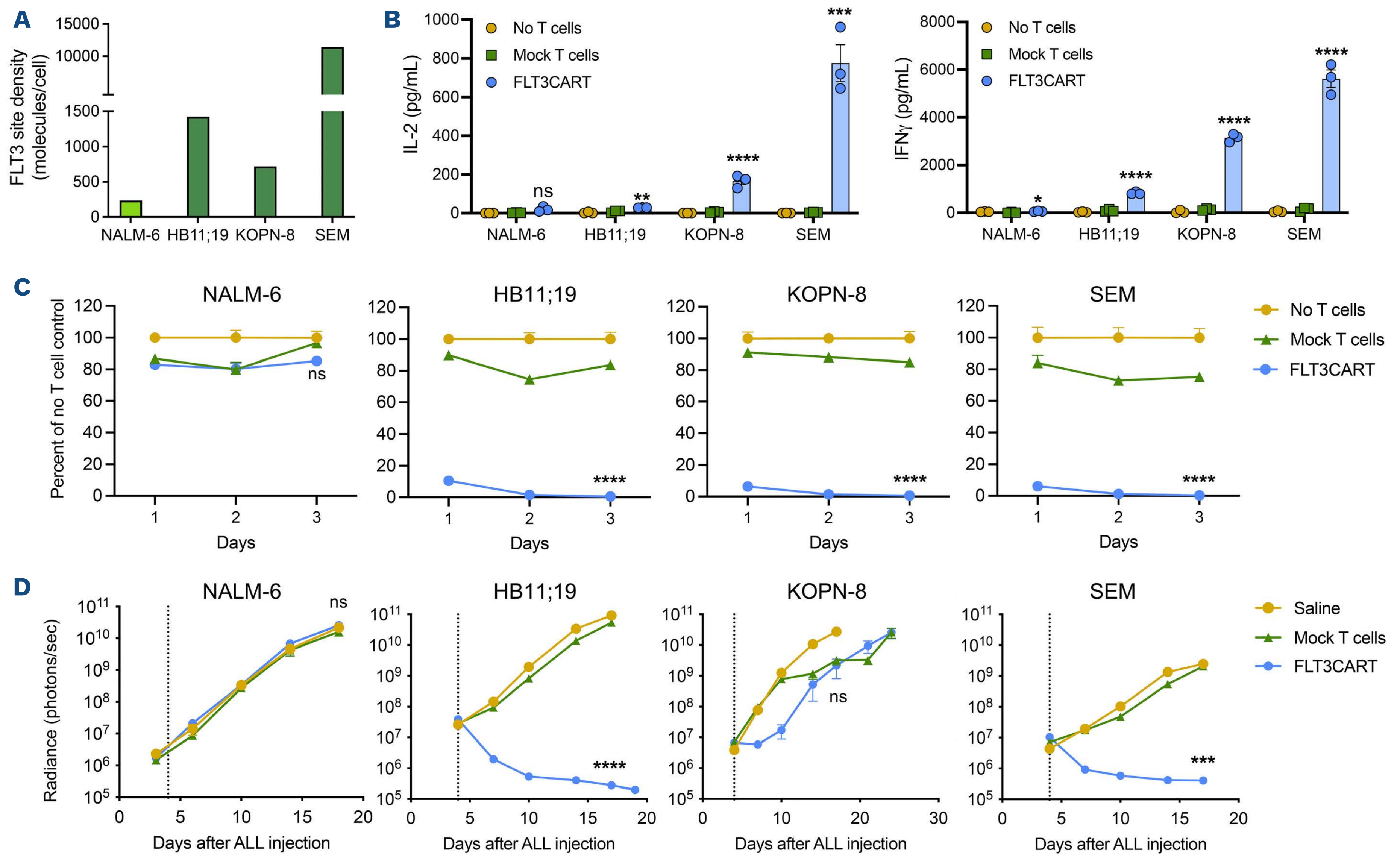

Figure 2. FLT3CART has potent *in vitro* and *in vivo* activity against human *KMT2A*-rearranged acute lymphoblastic leukemia cell lines. (A) Flow cytometric quantification of FLT3 surface antigen density on acute lymphoblastic leukemia (ALL) cell lines showed higher expression on *KMT2A*-rearranged (*KMT2A*-R) ALL cell lines (HB11;19, KOPN-8, SEM) than on *KMT2A* wild-type NALM-6 control cells. *KMT2A*-R ALL cell lines showed equivalent or higher FLT3 surface expression than the *FLT3*-ITD acute myeloid leukemia cell lines assayed (compare to Figure 2A). SEM uniquely has *FLT3* intrachromosomal gene amplification, which explains its almost 10-fold higher number of surface FLT3 molecules.[52] (B) ALL cell lines were co-incubated *in vitro* with FLT3CART or mock-transduced T cells at a 1:1 (30,000 cells) ratio for 48 hours. Production of human interleukin-2 (IL-2, left panel) and interferon gamma (IFN-γ, right panel) in culture supernatant was measured by enzyme-linked immunosorbent assay. FLT3CART-induced cytokine production was highest in co-culture with SEM cells, which had the greatest FLT3 antigen density among ALL cell lines assayed. Minimal IL-2 and IFN-γ production was detected for the negative control NALM-6 cells. (C) *In vitro* live cell imaging of GFP-transduced ALL cell lines demonstrated significant inhibition of cell growth of *KMT2A*-R ALL cell lines HB11;19, KOPN-8, and SEM over time when co-incubated with FLT3CART (blue) at a 1:1 ratio. Experiments in (B) and (C) were performed with triplicate technical replicates and results are displayed ± standard error of the mean. (D) Luciferase-transduced ALL cell lines ($1x10^6$ cells/mouse) were injected intravenously (IV) into NSG mice. After confirming engraftment via bioluminescent imaging (BLI), cell line xenograft mice were randomized (n=5/group) to receive IV saline (yellow), $1x10^7$ mock-transduced T cells (green), or $1x10^7$ FLT3CART (blue) on day 4 (vertical dashed line). BLI radiance at indicated timepoints is displayed graphically ± standard error of the mean. Rapid and sustained FLT3CART-induced inhibition of leukemia proliferation was observed in HB11;19 and SEM cell line models *versus* mock T-cell and saline control treatments, while an initially observed anti-leukemia effect in the KOPN-8 cell line model was not sustained. No effects of FLT3CART were detected in the *KMT2A* wild-type cell line model NALM-6. Data in (B) and (D) were analyzed by one-way analysis of variance (ANOVA) and in (C) by two-way ANOVA with the Dunnett post-test for multiple comparisons using FLT3CART as the comparator. The statistical significance of the effects of FLT3CART compared to those of the mock T-cell group at the last measured time point is displayed. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ns = not significant.

FLT3CART is efficacious *in vivo* in *KMT2A*-rearranged acute lymphoblastic leukemia-to-acute myeloid leukemia lineage-switch in patient-derived xenograft models

Patients with *KMT2A*-R ALL are at increased risk of lineage switch to AML following CD19CART treatment compared to those with non-*KMT2A*-R ALL.[15,17] To assess the activity of FLT3CART in this setting, we created a pair of ALL and AML PDX models from an adolescent with primary chemotherapy-refractory *KMT2A-AFF1* B-ALL who developed lineage-switched CD19-negative *KMT2A-AFF1* AML approximately 3 weeks after tisagenlecleucel administration and was resistant to all further chemoimmunotherapy. In these preclinical studies, FLT3CART equipotently

inhibited leukemia proliferation *in vivo* in both *KMT2A*-R ALL (Figure 4A) and AML (Figure 4B) PDX models, despite a marked reduction in FLT3 surface antigen density after the lineage switch (Figure 4C). These findings suggest that FLT3CART may be a beneficial strategy specifically for patients with *KMT2A*-R ALL who are prone to lineage-switch relapse.

FLT3CART induces minimal detectable on-target/off-tumor toxicity

FLT3 RNA and protein expression has been reported in several tissues including early hematopoietic progenitors, cardiomyocytes, and developing neuronal cells (*https://www.proteinatlas.org/ENSG00000122025-FLT3/tissue*).[27-29] To investigate potential non-hematopoietic on-target/off-tumor toxicities, we first screened for *in vitro* reactivity of FLT3CART co-incubated with normal human tissue induced pluripotent stem cells (including three neural tissue types) via quantification of IFN-γ cytokine production with MOLM-14 as a FLT3-positive control. We detected a small increase in IFN-γ production following co-culture with cardiomyocytes and no evidence of changes with co-culture of other tissue types assessed (*Online Supplementary Figure S3*).

To assess for predicted toxicity due to FLT3 expression in the hematopoietic progenitor compartment,[27,30] human bone marrow CD34$^+$ cells were untreated or exposed to mock T cells or FLT3CART and assayed for colony forma-

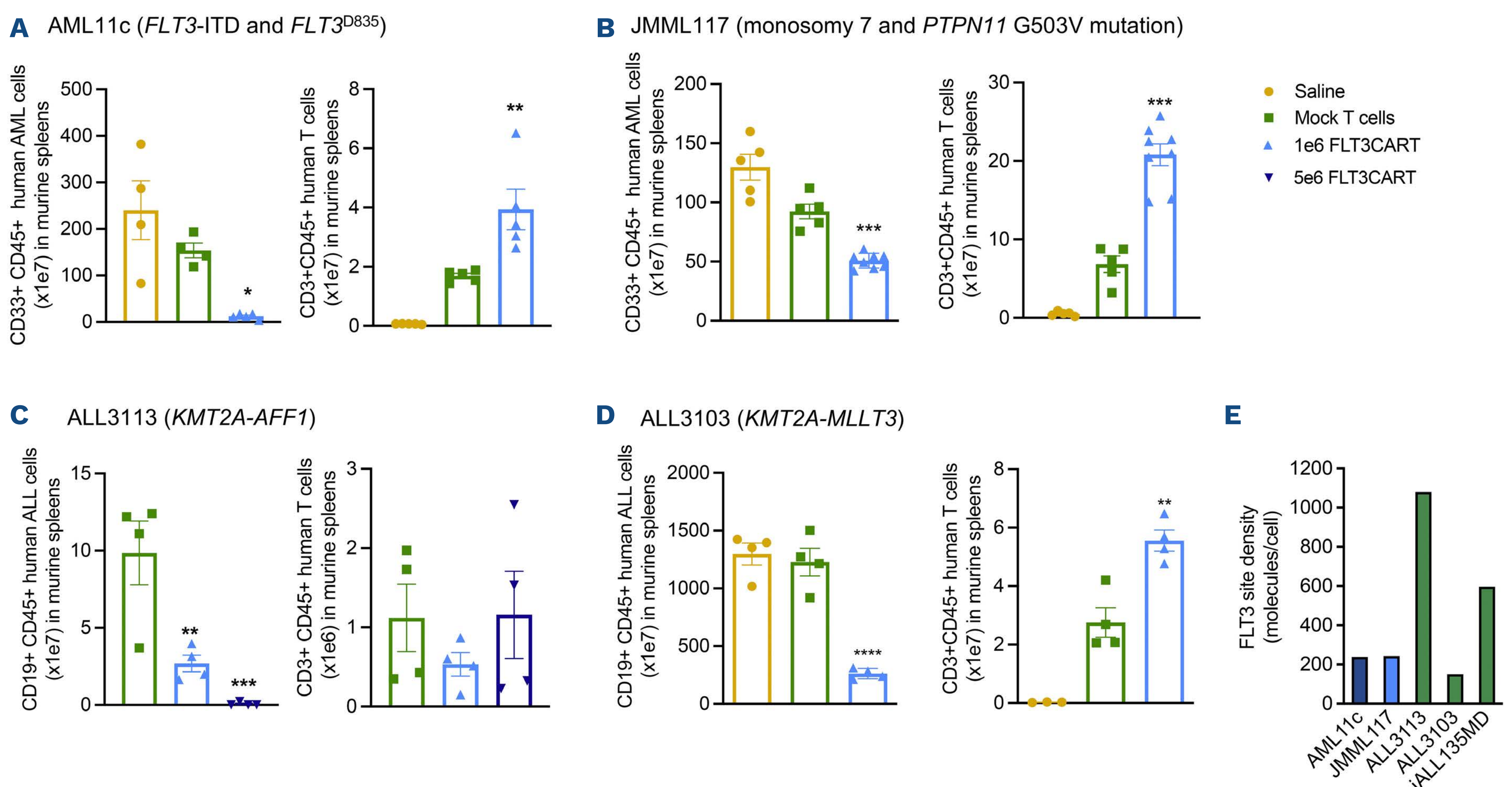

Figure 3. FLT3CART is highly effective against *FLT3*-mutant acute myeloid leukemia and *KMT2A*-rearranged acute lymphoblastic leukemia and patient-derived xenografts *in vivo*. Busulfan-conditioned NSGS mice were engrafted with primary acute myeloid leukemia (AML) cells for the patient-derived xenograft (PDX) models (A) AML11c and (B) JMML117. Once 1-5% CD33$^+$/CD45$^+$ human AML were detectable in peripheral blood, mice were randomized (n=5-8/group) to intravenous (IV) treatment with 1x10^6 FLT3CART (light blue), mock-transduced T cells (green), or saline (yellow). AML (first panel) and CD3$^+$CD45$^+$ T cells (second panel) were quantified in spleens by flow cytometry at the end of the study, which was determined by the rate of AML progression in control mice (6 weeks of treatment for AML11c, 4 weeks of treatment for JMML117). Unconditioned NSG mice were engrafted with *KMT2A*-rearranged (*KMT2A*-R) primary acute lymphoblastic leukemia (ALL) cells for the PDX models (C) ALL3113 and (D) ALL3103, and engraftment was monitored by quantitative flow cytometry analysis of sampled peripheral venous blood. Once human leukemia was detected, mice were randomized (n=4/group) to IV treatment with 1x10^6 (light blue) or 5x10^6 (dark blue) FLT3CART, mock-transduced T cells (green), or saline (yellow). CD19$^+$CD45$^+$ ALL (first panel) and CD3$^+$CD45$^+$ T cells (second panel) were quantified in spleens by flow cytometry at the end of the study, which was determined by rate of ALL progression in control mice (4 weeks and 2 weeks after treatment for ALL3113 and ALL3103, respectively). Please note that a saline control group was not available for model ALL3113 and that a dose of 5x10^6 FLT3CART was also included for this study. FLT3CART was effective in clearing *KMT2A*-R ALL and both *FLT3*-mutant (AML11c) and *FLT3* wild-type (JMML117) AML. (E) Flow cytometric quantification of FLT3 surface antigen density on *KMT2A*-R ALL (green) and *FLT3*-mutant AML (dark blue) and *FLT3* wild-type (light blue) PDX cells. FLT3CART is effective *in vivo* in PDX models with low FLT3 surface expression. Data are displayed ± standard error of the mean and were analyzed by one-way analysis of variance with the Dunnett post-test for multiple comparisons with significance displayed compared to the mock T-cell control group. Absence of a symbol indicates lack of statistical significance. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

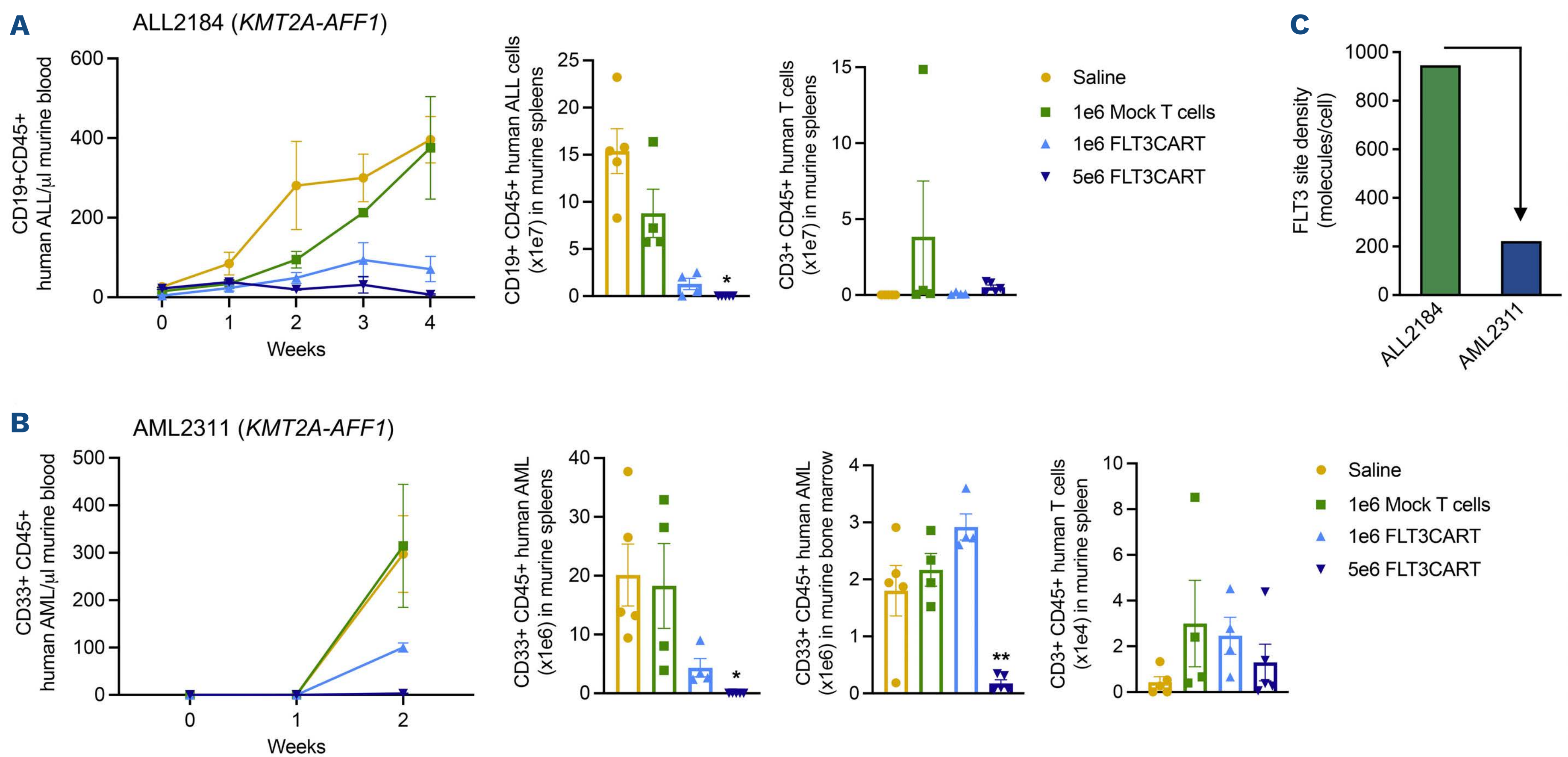

Figure 4. FLT3CART potently inhibits *in vivo* leukemia proliferation in *KMT2A*-rearranged acute lymphoblastic leukemia-to-acute myeloid leukemia lineage switch patient-derived xenograft models. (A) NSG mice were injected intravenously (IV) with *KMT2A*-rearranged (*KMT2A*-R) acute lymphoblastic leukemia (ALL) patient-derived xenograft (PDX) cells and engraftment was monitored by flow cytometry of sampled peripheral blood. Once human leukemia was detected, mice (n=4-5/group) were randomized to IV treatment with 1x10^6 (light blue) or 5x10^6 (dark blue) FLT3CART, 1x10^6 mock-transduced T cells (green), or saline (yellow). CD19$^+$/CD45$^+$ human ALL cells were quantified weekly by flow cytometry of peripheral blood (left panel) and at study endpoint after 4 weeks of treatment in murine spleen (middle panel). Human T cells (CD3$^+$/CD45$^+$) were also enumerated in the spleen (right panel). (B) Busulfan-conditioned NSGS mice were injected with cells from the *KMT2A*-R acute myeloid leukemia (AML) PDX model established from the same patient in (A) following lineage-switch relapse after having been treated with tisagenlecleucel. Once human AML was detected in peripheral blood by flow cytometry, mice (n=4-5/group) were treated with 1x10^6 (light blue) or 5x10^6 (dark blue) FLT3CART, mock-transduced T cells (green), or saline (yellow). Human CD33$^+$/CD45$^+$ AML cells were monitored by flow cytometry in the peripheral blood (left panel) and at study endpoint after 2 weeks of treatment in the spleen (middle-left panel) and bone marrow (middle-right panel). CD3$^+$/CD45$^+$ T cells were also measured in end-study spleens (right panel). FLT3CART cleared leukemia *in vivo* in both *KMT2A*-R ALL and lineage-switched *KMT2A*-R AML relapsed PDX models despite differential FLT3 cell surface expression. (C) Flow cytometric quantification of FLT3 surface antigen density on KMT2A-R ALL (green) and AML (blue) PDX cells demonstrates marked antigen downregulation (arrow) upon lineage-switch relapse. Data in (A) and (B) are displayed ± standard error of the mean and were analyzed by one-way analysis of variance with the Tukey post-test for multiple comparisons with significance displayed compared to the mock T-cell control group. **P*<0.05, ***P*<0.01.

tion. There was no difference in the number of erythroid (CFU-E/BFU-E), myeloid (CFU-GM) or mixed (CFU-GEMM) colonies arising from CD34$^+$ cells exposed to FLT3CART (*Online Supplementary Figure S4A*). Similarly, we detected no increase in IFN-γ or IL-2 production and no alteration in the viability of CD34$^+$/CD38$^+$ or CD34$^+$/CD38$^-$ hematopoietic progenitors with co-culture with FLT3CART (*Online Supplementary Figure S4B-D*). Taken together, we observed minimal off-tumor effects of FLT3CART against hematopoietic or non-hematopoietic human tissues, although caution remains warranted with regard to potential clinical translation.

Bispecific CD19xFLT3CART also has potent *in vitro* and *in vivo* activity against *KMT2A*-rearranged acute lymphoblastic leukemia

Modulation of target antigen surface expression is another well-recognized mechanism of leukemia relapse in clinical experience to date with CD19- and CD22-targeting CAR T cells and antibody-based immunotherapies.[14,31] As a strategy potentially to augment anti-leukemia activity and perhaps also to diminish risk of antigen escape specifically in *KMT2A*-R ALL, we generated bicistronic CD19xFLT3-directed CAR constructs using a single vector. Each dual-targeting construct contained the above-described same FLT3 scFv with 4-1BB/CD3ζ co-stimulatory domains (*Online Supplementary Figure S1A*) and an FMC63-derived CD19 scFv with 4-1BB/CD3ζ (CD19[BBz]xFLT3CART) or CD28/CD3ζ co-stimulatory domains (CD19[28z]xFLT3CART) (Figure 5A, *Online Supplementary Methods*). We confirmed bright flow cytometric CD19 cell surface protein expression on all tested B-ALL cell lines with expectedly negligible expression on AML cell lines (Figure 5B). We observed that short-term co-culture of CD19xFLT3CART with SEM cells

induced similar activation and exhaustion marker expression as was detected with monovalent FLT3CART and CD19CART (*Online Supplementary Figures S6* and *S7*). Co-incubation of CD19xFLT3CART (24-40% transduction efficiency) with CD19-negative AML cell lines induced similar levels of IL-2 and IFN-γ production at 48 hours as monovalent FLT3CART and no appreciable cytokine production with CD19CART (Figure 5C, D). Interestingly, bicistronic CD19(28z)xFLT3CART stimulated greatest cytokine production when co-incubated with *KMT2A*-R ALL cell lines with levels consistently above those in monovalent FLT3CART, CD19CART, or CD19(BBz)xFLT3CART conditions (Figure 5C, D), mimicking the more robust cytokine production that has been observed in preclinical studies of CD19CART designed with 28z *versus* BBz co-stimulatory domains.[32] Similar effects were observed in NALM-6 cells engineered to overexpress FLT3 without or with CD19 deletion (*Online Supplementary Figure S5B*). Furthermore, both CD19xFLT3CART inhibited cell viability of the *FLT3*-mutant AML cell lines MOLM-14 and MV4;11 and of all tested ALL cell lines independently of *KMT2A* mutation status and FLT3 antigen expression (Figure 6). Consistent with cytokine production data, CD19(28z)xFLT3CART also showed faster leukemia cell killing kinetics than those of CD19(BBz)xFLT3CART at early *in vitro* assessment time-points.

In vivo testing of bispecific CD19xFLT3CART against luciferase-expressing *KMT2A*-R ALL cell line SEM induced

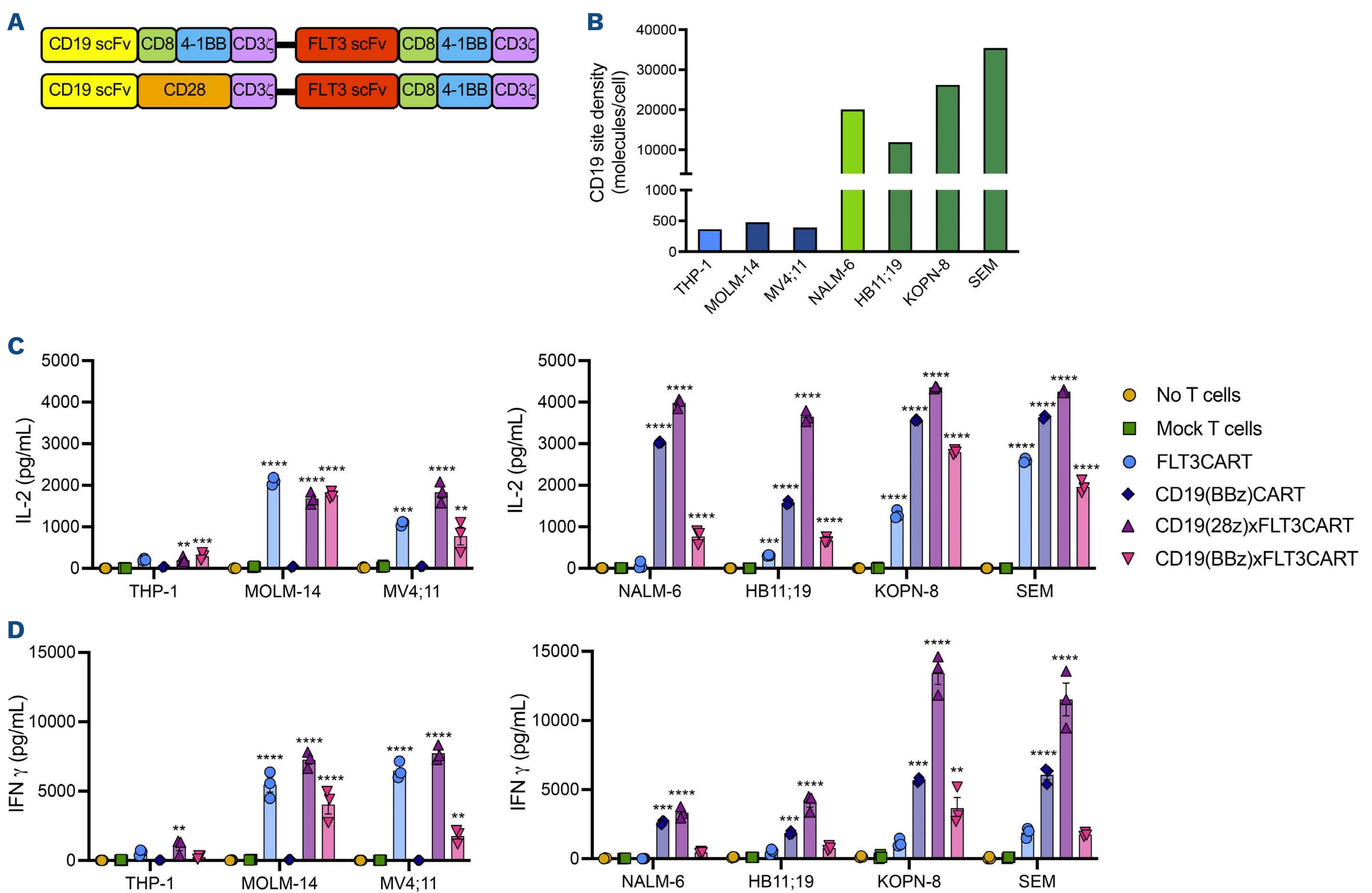

Figure 5. Robust *in vitro* cytokine production of bicistronic CD19xFLT3CART against *KMT2A*-rearranged acute lymphoblastic leukemia. (A) Schematic of bicistronic chimeric antigen receptor constructs containing the same FLT3 scFv (red) with 4-1BB co-stimulatory domain/CD3ζ signaling domain shown in Figure 1A linked with a P2A element (black line) to anti-CD19 scFv FMC63 (yellow) with either 4-1BB/CD3ζ (BBz) or CD28/CD3ζ (28z) co-stimulatory domains. (B) CD19 antigen expression (molecules/cell) is highly enriched on the cell surface of both *KMT2A* wild-type (light green) and *KMT2A*-rearranged (dark green) acute lymphoblastic leukemia (ALL) cell lines, but not expressed on *FLT3* wild-type (light blue) or *FLT3*-mutant (dark blue) acute myeloid leukemia (AML) cell lines, as assessed by quantitative flow cytometry analysis. Luciferase-transduced AML (left panel) and ALL (right panel) cell lines were co-incubated at a 1:1 ratio (30,000 cells) for 48 hours with the indicated CD19CART, FLT3CART, bicistronic CD19xFLT3CART, or mock-transduced T cells. (C) Interleukin-2 (IL-2) and (D) interferon-gamma (IFN-γ) were quantified in culture supernatant by enzyme-linked immunosorbent assay. Cytokine production with dual-targeting CD19xFLT3CART (purple and pink) was either similar or more substantive compared to that with monovalent FLT3CART with the AML and ALL cell lines. Experiments in (C) and (D) were performed in triplicate and results are displayed ± standard error of the mean. Data in (C) and (D) were analyzed by one-way analysis of variance with the Dunnett post-test for multiple comparisons using the mock T-cell group as the comparator. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

rapid eradication of detectable leukemia, facilitating long-term animal survival (Figure 7A, *Online Supplementary Figure S8A*). Notably, sustained anti-leukemia responses with CD19(28z)xFLT3CART mirrored those of monovalent FLT3CART and CD19CART in this model. The long-term response to CD19(BBz)xFLT3CART was slightly more variable, although bioluminescence imaging-detectable leukemia remained below initial engraftment levels. Although development of CD19xFLT3CART was intended primarily for testing in our CD19+ ALL models, we also detected robust inhibition of leukemia proliferation in our luciferase+ CD19− MOLM-14 cell line xenograft model treated with the bicistronic CART (Figure 7B, *Online Supplementary Figure S8B*), suggesting 'OR' logic gating of the bispecific constructs with effective anti-AML activity driven by the FLT3-targeting component.

We then assessed and confirmed the *in vivo* activity of bispecific CD19xFLT3CART in *KMT2A*-R ALL PDX models established from infant (iALL135MD [PAUYJT[33]]) (Figure 8A) and young adult patients (ALL3113) (Figure 8B). In both models, bispecific CD19xFLT3CART quickly cleared human ALL proliferation in murine blood with no detectable leukemia remaining in end-study murine spleens, similar to the curative effects seen with monovalent CD19CART or FLT3CART treatment. Consistent with our recent preclinical observations with CD33CART immunotherapy for AML,[22] peripheral CAR T-cell expansion and plasma IFN-γ levels at early timepoints were appreciably higher in mice treated with CD19(28z)xFLT3CART compared to 4-1BB/CD3ζ-containing monovalent or bicistronic CART. PDX mice treated with CD19(28z)xFLT3CART also showed physical signs of immune activation mimicking cytokine release syndrome with appreciable weight loss compared to that of animals administered negative control or monovalent CART treatment (*data not shown*), coinciding with the observed robust *in vivo* T-cell expansion and IFN-γ peak levels. Affected PDX mice subsequently recovered with supportive care and without pharmacological intervention. Taken together, these *in vitro* and *in vivo* studies corroborate bicistronic CD19xFLT3CART immunotherapy as an alternative approach for *KMT2A*-rearranged ALL.

Discussion

Successful development of immunotherapies for children and adults with relapsed/refractory *FLT3*-mutant AML and *KMT2A*-R ALL is a high priority given these individuals' poor clinical outcomes. Cellular immunotherapy has revolutionized treatment for many patients with CD19-ex-

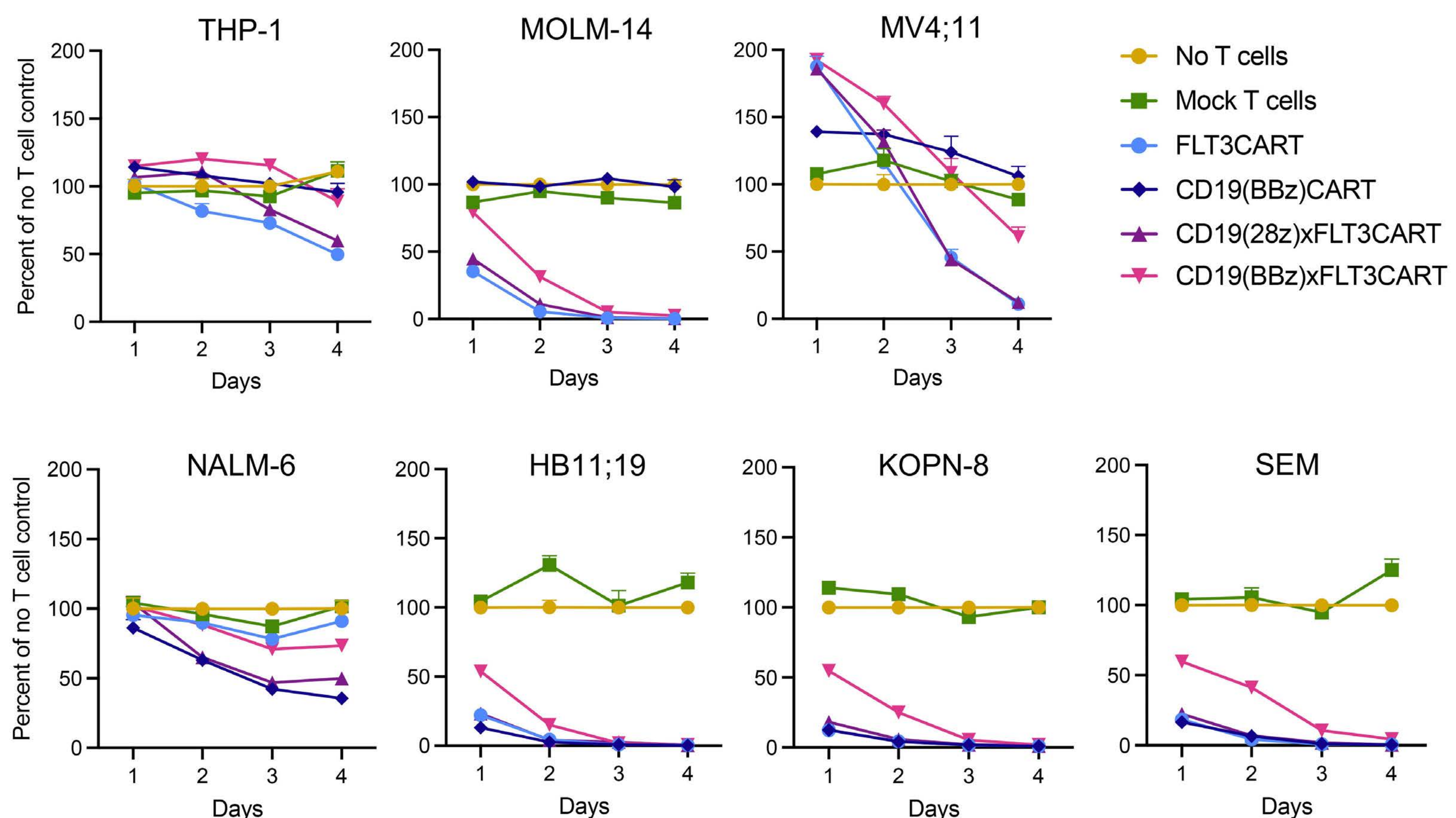

Figure 6. Effective *in vitro* activity of CD19xFLT3CART against *KMT2A*-rearranged acute lymphoblastic leukemia. Inhibition of viability of luciferase-transduced human acute myeloid leukemia (AML, top panel) and acute lymphoblastic leukemia (ALL, bottom panel) cell lines co-incubated with CD19CART, FLT3CART, or one of two CD19xFLT3CART in a 1:1 ratio was assessed via luciferase reporter assay at 24, 48, 72, and 96 hours. FLT3CART and both CD19xFLT3CART significantly inhibited the viability of *FLT3*-ITD AML (CD19-negative) and *KMT2A*-rearranged ALL (CD19-positive) cell lines, suggesting sufficiency of FLT3 targeting in these 'OR'-gated chimeric antigen receptor constructs. Experiments were performed in triplicate and results are displayed ± standard error of the mean. Data were analyzed by two-way analysis of variance with the Dunnett post-test for multiple comparisons using the mock T-cell group as the comparator. Both CD19xFLT3CART induced statistically significant killing at day 4 *versus* mock T-cell controls in all cell lines tested ($P<0.01$).

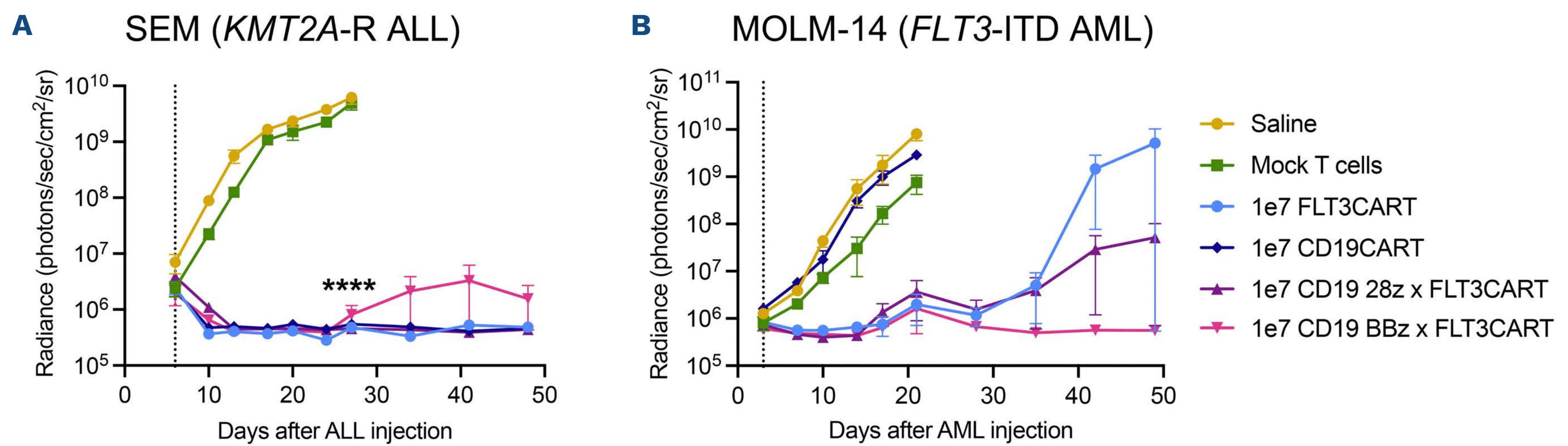

Figure 7. Bispecific CD19xFLT3CART inhibits *in vivo* leukemia proliferation in cell line xenograft models. (A) Luciferase-transduced SEM *KMT2A*-rearranged acute lymphoblastic leukemia (ALL) cells were injected intravenously into NSG mice on day 0. Once engraftment was detected by bioluminescent imaging (BLI), mice were randomized (n=5/group) to receive intravenous treatment (vertical dashed line) with saline, mock-transduced T cells, monovalent CD19CART, FLT3CART, or bicistronic CD19xFLT3CART ($1x10^7$ cell dosing for all groups). Mice were followed by serial BLI twice- or once-weekly with radiance ± standard error of the mean displayed graphically. CD19CART, FLT3CART, and both CD19xFLT3CART resulted in rapid clearance of ALL. (B) In a parallel experiment, luciferase-transduced MOLM-14 *FLT3*-ITD acute myeloid leukemia (AML) cells were injected into NSG mice on day 0. Once engraftment was detected, mice were randomized (n=5/group) to experimental treatment, followed by BLI as described in (A). Monovalent FLT3CART and bicistronic CD19xFLT3CART potently inhibited *in vivo* AML proliferation. As expected for CD19-negative MOLM-14, monovalent CD19CART had no anti-leukemia activity, confirming 'OR' gating logic of the bispecific CD19xFLT3CART seen in Figure 6. Data ± standard error of the mean were analyzed by one-way analysis of variance with significance shown for FLT3CART *versus* the mock T-cell group. Absence of a symbol indicates lack of statistical significance. ****$P<0.0001$.

pressing relapsed/refractory B-cell malignancies.[11-13] However, subsequent relapse with antigen-downregulated or -loss disease following CD19CART or CD22CART treatment has emerged as a substantive barrier to long-term cure.[13,16] It is not yet clear whether new immunotherapies against alternative leukemia target antigens will induce similar mechanisms of resistance. Successful development of CAR T-cell immunotherapies for AML has lagged behind those for B-ALL, in part given concerns about on-target/off-tumor toxicity due to target antigen expression on normal myeloid cells and/or non-hematopoietic tissues.[34]

Interestingly, both *FLT3*-mutant AML and *KMT2A*-R ALL are driven by hyperactive *FLT3* kinase signaling via FLT3 genetic mutation or overexpression and have high cell surface protein expression. Targeting FLT3 activation and downstream signaling with addition of FLT3 inhibitors (e.g., the multi-tyrosine kinase inhibitors, midostaurin and sorafenib, and the more FLT3-selective inhibitors, quizartinib and gilteritinib) to chemotherapy has significantly improved survival in adults and children with *FLT3*-mutant AML.[5,6,35] Conversely, addition of the multi-tyrosine kinase inhibitor lestaurtinib to chemotherapy in the COG AALL0631 phase III trial did not improve outcomes for infants with *KMT2A*-R ALL *versus* chemotherapy, although many patients were shown to have had inadequate exposure to the FLT3 inhibitor because of frequent treatment interruptions. Pharmacodynamic analysis of blood specimens from the subset of infants with robust lestaurtinib-induced FLT3 inhibition demonstrated marked improvement in 3-year event-free survival compared to that of non-inhibited infants,[8] highlighting the therapeutic potential of FLT3 inhibition also in patients with *KMT2A*-R ALL. However, given recent reporting of FLT3-inhibitor resistance mechanisms in patients with *FLT3*-mutant AML, such tyrosine kinase inhibitor-based therapies may not be curative for all patients.[36]

Patients with *KMT2A*-R ALL are also known to be at particularly high risk of lymphoid-to-myeloid lineage switch following CD19CART immunotherapy[15,17] when compared to conventional chemotherapy or immunotherapy with the CD19xCD3 bispecific T-cell engager blinatumomab.[37] This lineage switch predilection presents a unique barrier to cure of these high-risk patients via CD19CART, as well as an opportunity for alternative therapeutic approaches. Successful development of immunotherapies targeting a surface antigen shared by both lymphoid and myeloid leukemias (such as FLT3) would accordingly not only have broader application for a larger subset of patients, but could theoretically also be beneficial in a lineage switch setting.

Here, we report the preclinical development of new FLT3CART immunotherapy with an eye to clinical translation. In these studies we have demonstrated potent *in vitro* and *in vivo* anti-leukemia efficacy against *FLT3*-mutant AML and *KMT2A*-R ALL cell lines, as well as robust FLT3CART-mediated eradication of leukemia *in vivo* in several PDX models of pediatric or young adult *FLT3*-mutant AML, *FLT3* wild-type AML, and *KMT2A*-R ALL. Consistent with other studies of FLT3-directed CAR T-cell immuno-

therapies,[38-42] our FLT3CART showed excellent activity against *FLT3*-ITD AML, as determined via *in vitro* cytokine production and viability metrics, as well as *in vivo* curative effects and long-term survival of treated animals. We were intrigued to discover that flow cytometrically measured FLT3 surface antigen site density was not overtly different in our tested *FLT3*-mutant *versus* non-mutant AML cell lines and PDX models and that FLT3CART also had strong (albeit less complete) activity against *FLT3* wild-type AML. These observations are consistent with data from a recent Children's Oncology Group analysis of primary pediatric AML specimens in which *FLT3*-ITD cases did not have higher FLT3 cell surface expression than non-ITD cases and FLT3 protein levels did not correlate with differential

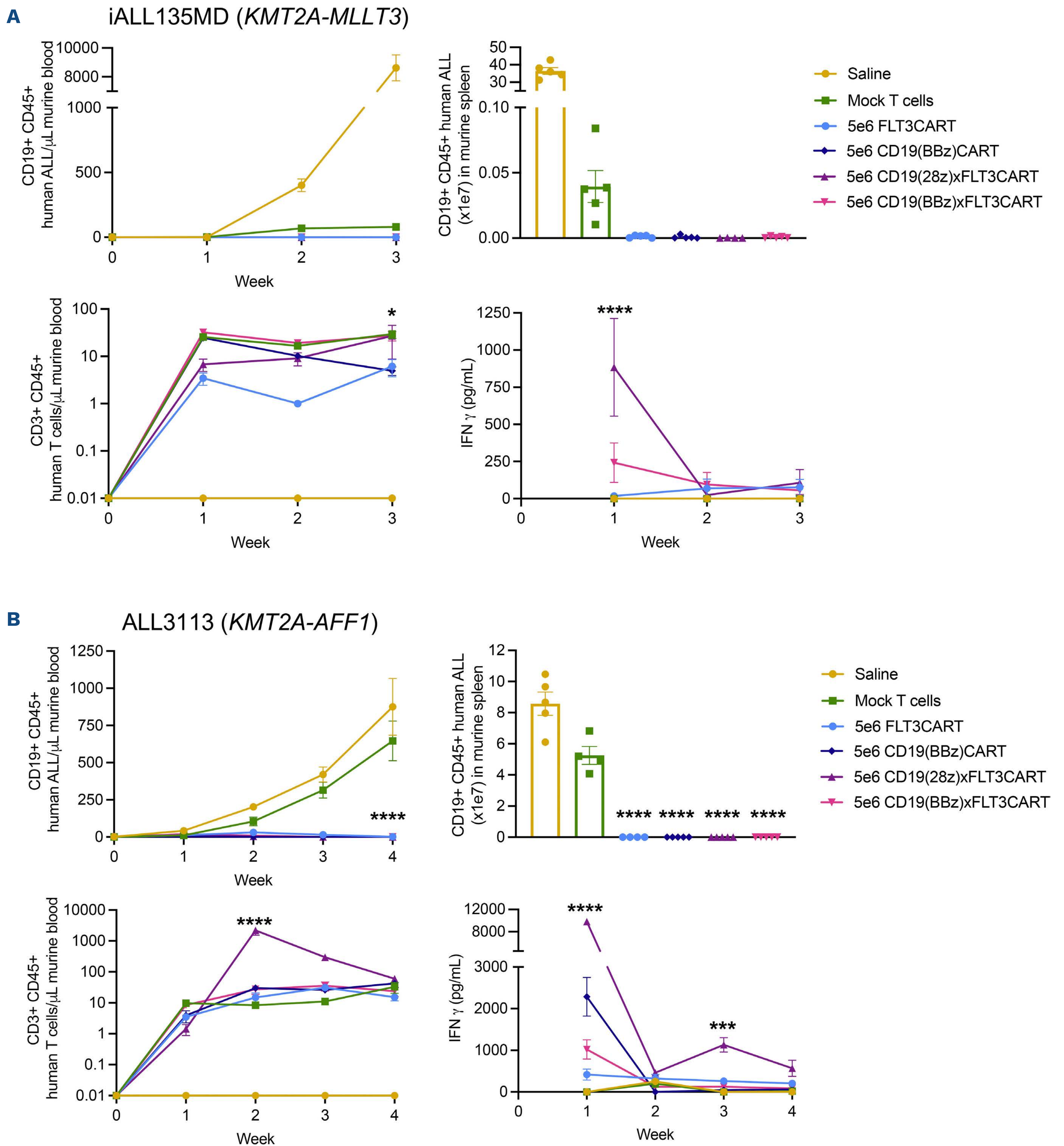

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Figure 8. Bispecific CD19xFLT3CART inhibits *in vivo* leukemia proliferation in patient-derived xenograft models of *KMT2A*-rearranged acute lymphoblastic leukemia. Patient-derived xenograft (PDX) models (A) iALL150MD and (B) ALL3113 were randomized (n=5 mice/group) to intravenous (IV) treatment with saline, mock-transduced T cells, CD19CART, FLT3CART, or one of the bicistronic CD19xFLT3CART ($5x10^6$ cell dosing for all groups) as designated. Weekly quantitative flow cytometric monitoring of $CD19^+/CD45^+$ human acute lymphoblastic leukemia and $CD3^+$ CAR T cells was performed with peripheral venous blood sampling, and interferon gamma (IFN-γ) was measured in prepared plasma by enzyme-linked immunosorbent assay. The study endpoint was determined by the rate of leukemia progression in control mice (3 weeks or 4 weeks of treatment in iALL135MD or ALL3113, respectively). No leukemia was detected in peripheral blood or end-study spleens in either PDX model following treatment with CD19xFLT3CART. An unexpectedly large xenogeneic anti-leukemia effect was observed in the iALL135MD PDX model with mock CAR T-cell *versus* saline treatment, but was not detected in other PDX models. Peripheral blood T-cell expansion and detectable IFN-γ were equal or greater for the bicistronic CD19xFLT3CART than for the monovalent CD19CART or FLT3CART. Data ± standard error of the mean were analyzed by two-way analysis of variance with the Tukey post-test for multiple comparisons with significance shown for FLT3CART *versus* CD19(28z)xFLT3CART for the T-cell and IFN-γ panels and *versus* the mock T-cell group for all other panels. Absence of a symbol indicates lack of statistical significance. *P<0.05, ***P<0.001, ****P<0.0001.

clinical outcomes, as has been reported for CD33 and CD123 antigens.[43-45] Our data highlight potentially wider therapeutic applicability of FLT3CART for patients with AML, which merits further exploration.

To our knowledge, our group is the first to demonstrate efficacy of FLT3-targeting CAR T-cell immunotherapy in FLT3-overexpressing *KMT2A*-R ALL. The importance of target antigen site density for successful treatment of patients with B-ALL with CD19- or CD22-directed immunotherapies is well-established,[25,26] but has not yet been elucidated for alternative leukemia antigens. Our present studies show activity of FLT3CART against leukemias with quantitatively lower FLT3 site density than has been reported for CD19 or CD22, but were not designed to identify a site-density threshold for treatment response or failure. Our observations appear consistent with a recent report of FLT3xCD3 bispecific T-cell-engaging antibodies with preclinical activity against leukemia cell lines with a broad range of FLT3 surface protein levels.[46] It will be critical to elucidate in future studies the degree to which FLT3 site density influences the therapeutic activity of FLT3CART and whether clinical responses differ between patients with ALL or AML or between patients with *FLT3*-mutated or *FLT3* wild-type AML.

Given the propensity for *KMT2A*-R ALL-to-AML lineage switch, we importantly report excellent anti-leukemia activity of FLT3CART in a unique pairing of PDX models established from a patient with chemorefractory *KMT2A*-R ALL who experienced AML lineage switch relapse with retention of the original *KMT2A-AFF1* fusion after receiving tisagenlecleucel. These data further underscore FLT3 as a key immunotherapeutic target in *KMT2A*-R ALL. We hope that ongoing and future studies will shed further light on the potential of FLT3CART to treat or perhaps even prevent these presently universally fatal lineage-switch relapses.[15,17] Expanding upon the clinical potential of FLT3CART for patients with relapsed/refractory *KMT2A*-R ALL, we also report successful development of bispecific CD19xFLT3CART immunotherapy with at least equivalent preclinical activity to that of monovalent CD19CART and FLT3CART. Recent data from clinical phase I studies have raised exciting promise of bispecific CD19xCD22 and CD19xCD20 CAR T-cell immunotherapies for patients with relapsed/refractory B-ALL or lymphoma.[47,48] Further investigation is needed to ascertain whether such approaches have superior long-term clinical efficacy over single antigen-targeting CART and/or prevent antigen escape relapse.

Finally, our preclinical studies suggest a potential therapeutic window for translation of FLT3CART to patients with AML and ALL with largely minimal on-target/off-tumor effects detected against normal non-hematopoietic and hematopoietic tissues. Importantly, although FLT3 expression in neural tissues has been reported, we did not observe any reactivity of our FLT3CART against three different induced pluripotent stem cell-derived neuronal cell lines, nor was neurotoxicity seen *in vivo* in non-human primates by another team studying alternative FLT3 CAR T cells and bispecific antibodies.[40,49] Our results are consistent in terms of both anti-leukemia activity and predicted tolerable hematopoietic toxicity with other studies of FLT3-directed monoclonal antibodies,[50] bispecific antibodies,[46,49,51] and CAR T cells[38,40-42] with *in vitro* and *in vivo* activity against AML cell lines. Caution must nonetheless still be exercised with translation to clinical investigation. Finally, we uniquely report potent activity of our FLT3CART in multiple clinically-relevant leukemia PDX models, including a previously unknown efficacy of FLT3CART and CD19xFLT3CART immunotherapy specifically against *KMT2A*-R ALL and in lineage-switch scenarios. Taken together, our results highlight FLT3 as a critical antigen for cellular immunotherapy in two high-risk leukemia subtypes. Clinical investigation of our optimized monovalent FLT3CART immunotherapy will occur soon via a first-in-human phase I trial.

Disclosures

CDC and TJF have a United States Department of Health and Human Services patent application for FLT3 chimeric antigen receptors (WO2017205747A1). SKT is receiving or has received research funding for unrelated studies from Beam Therapeutics, Gilead Sciences, Incyte Corporation,

and Kura Oncology, has consulted for bluebird bio, and serves on the scientific advisory boards of Aleta Biotherapeutics, Kura Oncology, and Syndax Pharmaceuticals. TJF is a part-time employee of and owns stock options in Sana Biotechnology. The remaining authors have no conflicts of interest to disclose.

Contributions

LMN performed experiments, analyzed and interpreted data, and wrote the manuscript. ZTG and CDC performed experiments, analyzed and interpreted data, and contributed to writing the manuscript. JAC, CAM, LCL, JPL, and MEK performed experiments and analyzed and interpreted data. SKT and TJF conceived and directed the study, analyzed and interpreted data, and wrote and/or edited the manuscript. All authors approved the final version of the manuscript.

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Data-sharing statement

Human leukemia cell lines used in these studies are publicly available through commercial sources and may be made available from the authors upon written request and material transfer agreement approval. The authors are also glad to share guidance regarding protocols and assays used in these studies upon written request.

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