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 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 AML³,⁴ 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.\textsuperscript{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.\textsuperscript{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- and 3-year event-free survival rates of 46% and 36%, respectively, in infants with KMT2A-R ALL.\textsuperscript{7,8} Worse outcomes, with event-free survival <20%, have been reported for patients aged <90 days at diagnosis, those with a diagnostically 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.\textsuperscript{9} Interestingly, highest FLT3 overexpression has been associated with worst outcomes in infants with KMT2A-R ALL,\textsuperscript{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,\textsuperscript{11-14} including infants.\textsuperscript{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.\textsuperscript{16} 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.\textsuperscript{18} Initial studies of dual-targeting CD19xCD22 CAR T cells demonstrated robust anti-leukemia activity in preclinical B-ALL models,\textsuperscript{19} and current clinical phase I trials of these bispecific immunotherapies (NCT03241940, NCT03289455, NCT03330691, NCT03448393) have reported exciting early results.\textsuperscript{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 \textit{in vitro} and \textit{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 (CD19xCD23CART) 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, CD19xCD23CART, and CD19CART were engineered using previously described methodologies\textsuperscript{19,22} and as detailed in the \textit{Online Supplementary Methods} with single-chain fragment variable (scFv) derived from an anti-CD135 NC7 antibody.\textsuperscript{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 CD19xCD23CART 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\textsuperscript{+} cell selection for use in \textit{in vivo} cell line xenograft model experiments with bioluminescent imaging as described elsewhere.\textsuperscript{22,24} All cell lines were assessed regularly for Mycoplasma contamination. Cell lines were maintained \textit{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 CD19xCD23CART functionality

Human interleukin-2 (IL-2) and interferon-gamma (IFN-\(\gamma\))
cytokine production was evaluated via enzyme-linked immunosorbent assays (ELISA; Biologend) and leukemia cell viability was assessed via a luciferase assay (Promega), conducted as described previously,22 using luciferase-expressing human AML 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 IL2Rγnull (NSG) or NOD-scid IL2Rγnull-3/GM/SF (NSGS) mice as previously described22,24 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 (1x10⁶ 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...
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 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 (1x10⁶ 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), 1x10⁷ mock-transduced T cells (green), or 1x10⁷ 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.
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. 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

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. (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 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 (1x10⁶ 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), 1x10⁷ mock-transduced T cells (green), or 1x10⁷ 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.
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-
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 bispecific CAR (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 [PAUYJT33]) (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 pre-clinical 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 7. Bispecific CD19xFLT3CART inhibits in vivo leukemia proliferation in cell line xenograft models. (A) Luciferase-transduced SEM KMT2A-R 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. However, subsequent relapse with antigen-downregulated or -loss disease following CD19CART or CD22CART treatment has emerged as a substantive barrier to long-term cure. 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. 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. 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, 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. Patients with KMT2A-R ALL are also known to be at particularly high risk of lymphoid-to-myeloid lineage switch following CD19CART immunotherapy when compared to conventional chemotherapy or immunotherapy with the CD19xCD3 bispecific T-cell engager blinatumomab. 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, 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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clinical outcomes, as has been reported for CD33 and CD123 antigens.43-45 Our data highlight potentially wider therapeutic applicability of FLT3CAR 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 FLT3CAR 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 FLT3CAR 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 FLT3CAR 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 FLT3CAR to treat or perhaps even prevent these presently universally fatal lineage-switch relapses.55,57 Expanding upon the clinical potential of FLT3CAR for patients with relapsed/refractory KMT2A-R ALL, we also report successful development of bispecific CD19xFLT3CAR immunotherapy with at least equivalent preclinical activity to that of monovalent CD19CAR and FLT3CAR. Recent data from clinical phase I studies have raised exciting promise of bispecific CD19xCD22 and CD19xCD2O 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 FLT3CAR 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 FLT3CAR 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 cells38,40-42 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 FLT3CAR in multiple clinically-relevant leukemia PDX models, including a previously unknown efficacy of FLT3CAR and CD19xFLT3CAR 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 FLT3CAR immunotherapy will occur soon via a first-in-human phase I trial.

Disclosures
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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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