SUPPLEMENTAL METHODS

CAR T cell production

CD33 and CD123 CAR-encoding lentiviral vectors were produced by transient transfection of the lenti-X 293T packaging cell line. Briefly, lenti-X 293T cells were plated into poly-D lysine coated 15-centimeter plates (BD Biosciences). The following day, lenti-X 293T cells were transfected using lipofectamine 3000 (ThermoFisher Scientific) with plasmids encoding the CAR along with packaging and envelope vectors (pMDLg/pRRE, pMD-2G, and pRSV-Rev). Lentiviral supernatants were harvested at 24 and 48 hours post-transfection, centrifuged at 3000 RPM for 10 minutes to remove cell debris, frozen on dry ice and stored at -80°C. Human PBMCs from normal donors were obtained via an NIH-approved research protocol and activated with a 1:1 ratio of CD3/CD28 microbeads (CD3/CD28 human T-expander Dynabeads, Thermo Fisher Scientific, Cat# 11141D) in AIM-V media containing recombinant human IL-2 40 IU/mL and 5% fetal bovine serum (FBS) for 24 hours. Activated T cells were resuspended at 2 million cells in 2 mL of lentiviral supernatant plus 1 mL of fresh AIM-V media with protamine sulfate 10 mcg/mL and IL-2 100 IU/mL in 6-well plates. Plates were centrifuged at 1000 x g for 2 hours at 32°C and incubated overnight at 37°C. A second transduction was performed on the following day by repeating the same transduction procedure described above. CD3/CD28 beads were removed on the third day following transduction, and cells were cultured at 300,000 cells/mL in AIM-V containing IL-2 100 IU/mL with fresh IL-2-containing media added every 2-3 days until cells were removed for analysis on day 8 or 9. Transduction efficiency for *in vivo* cell line xenograft and patient-derived xenograft (PDX) model studies shown in Figures 4 and 5 are listed in Supplemental Table 2.

Production of clinical-grade CD33CART used in Figure 7 was performed using CD4+/CD8+ T cells selected from a healthy donor apheresis using the Miltenyi Prodigy system. One hundred million selected T cells were activated with TransAct beads, then transduced at moi (multiplicity of infection) of 20 with clinical-grade CD33 CAR construct lentivirus manufactured at

the Children's Hospital of Philadelphia vector core facility. CD33CART cells were expanded for 7 days, formulated in CryoStor10, and cryopreserved using a controlled-rate freezer. CD33CART was tested for sterility and replication-competent lentivirus and confirmed to be free of *Mycoplasma* and other bacteria via Gram staining prior to cryopreservation.

In vitro cytokine production assays of CD33CART

Target tumor cells and transduced CAR+ T cells in a 1:1 ratio were washed 3 times with phosphate-buffered saline (PBS) and resuspend in RPMI culture medium at 1e6 cells/mL. 100ul of tumor cells with 100ul of CAR positive T cells were loaded into each well of a 96-well plate. T cell only and tumor cell only controls were set up. All tests were performed in duplicate or triplicate. Cells were incubated for 18 hours at 37°C, and 120 uL of the culture supernatant was removed for detection of cytokine production. Cytokine levels in supernatants were measured using either ELISA kits (R&D Systems) or a multiplex assay (Meso Scale Discovery) following the manufacturers' instructions.

In vitro cytotoxicity assays of CD33CART

To assess potential *in vitro* killing of AML cells by CD33CARTs, we performed CellTiter-Glo 3D cell viability assays (Promega) per the manufacturer's instructions. Luciferase-expressing human CD33+ AML (MOLM-14, MV4;11, U937) and CD33-negative B-ALL (NALM-6) cell lines were plated in RPMI/10% fetal bovine serum media alone or with irrelevant target GFP-transduced T cells [(-)TC], one of six experimental CD33CARTs, or one of two CD123CARTs (positive AML killing control) at 1:1 ratio in an opaque-walled and cell culture-treated 96-well plate format and incubated at 37°C with 5% CO_2. Each condition was tested with CAR T cells produced from 2 different normal T cell donors in at least 4 technical replicates. Luminescence was measured with a Synergy 2 multi-detection plate reader (BioTek) on individual plates every 24 hours for 96 hours. Data were normalized to baseline (day 0) leukemia cell-only luminescence

readings for each cell line, and the percentage of cell killing at each time point was calculated and displayed graphically in Prism.

In vivo animal studies of CD33CART

All animal studies were conducted on Institutional Animal Care and Use Committee (IACUC)-approved research protocols. GFP/luciferase-expressing (+) AML cell lines and xenografted primary human AML specimens were injected IV into NSG and busulfan-conditioned NSGS mice, respectively. Luciferase+ AML cell line xenograft models in NSG mice were followed by bioluminescent imaging using the Xenogen IVIS Lumina (Caliper Life Sciences or Perkin-Elmer) for quantification of baseline and post-CD33CART treatment leukemia burden. NSG mice were injected intraperitoneally with 3 mg D-luciferin (Caliper Life Sciences) and imaged 4 minutes later with an exposure time of 1 minute. Living Image Version 4.1 software (Caliper Life Sciences) was used to analyze the total bioluminescent signal flux for each mouse as photons/second. For all experiments, mice were randomly assigned to treatment groups prior to administration of CAR T cells. At study termination, murine bone marrow, spleen, and/or liver were harvested for quantification of human AML and CD33CART cells by flow cytometry or immunohistochemical analysis as described.[41,42] With the exception of experiments which were terminated early for tissue analysis as indicated, xenograft mice were generally followed for quantification of leukemia and T cell counts for 40-60 days. Animals were sacrificed at a pre-determined study endpoint or if development of xenogeneic GVHD necessitated euthanasia as per IACUC guidelines. Some MOLM14 xenograft studies were followed for 6 months post-CD33CART treatment. In some studies, CD33CARTs were prestimulated/activated with Dynabeads (Invitrogen/Thermo Fisher) or TransAct beads (Miltenyi) according to manufacturer's instructions.

Pediatric AML PDX models were created as previously described.[41,42] Briefly, 6-8 week-old male or female NSGS mice were conditioned with 30 mg/kg busulfan intraperitoneally at 24 hours prior to IV injection of 2.5 million human AML cells harvested from spleens of secondary

PDX models. Once mice demonstrated at least 1% human CD45+ CD33+ AML cells in peripheral blood by flow cytometry, they were treated IV with saline, 1 x 10e6 (-)TC, or 1 x 10e6 of one of six CD33CARTs (a cell dose previously optimized in previously established preclinical CAR studies) [42] and followed by weekly retro-orbital venous bleeding for flow cytometric quantification of human CD45+ CD33+ AML cells and CD3+ CAR T cells. Studies were terminated and animals sacrificed after 3-8 weeks depending upon the rate of AML progression in saline-treated control animals.

Flow cytometry analyses

Human AML and CAR T cells were quantified by flow cytometry analysis of harvested end-study organs from PDX mice using a BD FACSVerse flow cytometer and FlowJo (TreeStar) or Cytobank analysis with data display and statistical analysis in Prism as described.[41,42] Human CD45-APC, CD33-PE, and CD3-V450 antibodies (all from EBioscience/ThermoFisher) were used for detection of AML and CAR T cells in xenograft mice. AML cell line and PDX cell CD33 and CD123 site density quantification utilized CD33-PE or CD123-PE antibodies (EBioscience) and CountBright beads (Invitrogen). Additional T cell immunophenotyping was performed using CD4, CD8, CD25, CD71, PD-1, CCR7 (CD197), and CD45RA (all from EBioscience), Tim-3 (Biolegend), and LAG-3 (R&D Systems) antibodies.

SUPPLEMENTAL TABLES

Supplemental Table 1. CD33 CAR transduction efficiency for *in vivo* cell line and patient-derived xenograft experiments shown in Figures 4 and 5 and Supplemental Figure 2.

CAR T cell type	Transduction efficiency (%)
GFP negative control [(-)TC]	88.3
gemtuzumab CD28/CD3ζ	31.6
gemtuzumab 4-1BB/CD3ζ	19.9
lintuzumab CD28/CD3ζ	68.3
lintuzumab 4-1BB/CD3ζ	78.4
M195 CD28/CD3ζ	49.4
M195 4-1BB/CD3ζ	72

Supplemental Table 2. CD33 genotypes of AML cell lines. Assessment of CD33 rs12459419 single nucleotide polymorphisms on human AML cell lines used in *in vitro* and *in vivo* studies was performed using polymerase chain reaction as described.[48,49]

AML cell line	CD33 genotype
MOLM14	CC
MV4;11	CT
THP-1	CC
U937	CT

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Induction of activation markers on CD33CARTs varies as a function of CD33 single-chain variable fragments and co-stimulatory domains. (A) Expression of the CD25 (IL-2Rα) subunit was evaluated on CD4+CAR+ and CD8+CAR+ T cells on day 7 of expansion. CD123CARTs were used as positive AML killing controls. **(B)** Expression of the CD71 transferrin receptor was evaluated as in panel A. Representative histograms of T cells expressing the indicated CARs are presented (one of two independent T cell donors).

Supplemental Figure 2. *In vivo* dose titration of CD28/CD3ζ-based CD33CARTs in MOLM14 xenograft model. Luciferase-transduced MOLM14 cells (1x10e6) were injected IV via tail vein into NSG mice on Day 0 as in Figure 3. Once engraftment was documented by bioluminescent imaging (BLI) on Day 7, mice were randomized to IV treatment with saline, GFP-transduced T cells [(-)TC], or one of the CD33CARTs as designated (at 1x10e6, 5x10e6, or 1x10e7 total cells/mouse). Mice were followed by weekly BLI and sacrificed when a pre-determined maximal radiance level of 1x10e10 photons/s/cm^2/sr was detected, indicative of leukemia progression. Subcurative anti-leukemia effects were observed at lowest cell dosing of all three gemtuzumab- (gem), lintuzumab- (lin), and M195-based CD33CARTs. Curative effects of gem- and lin-CD33CARTs were seen with higher cell dosing, while M195-CD33CARTs were not effective even with highest cell dosing tested. 28z = CD28/CD3ζ endodomains.

Supplemental Figure 3. *In vivo* assessment of CD33CARTs in MV4;11 xenograft model. Experiments were conducted as in Supplemental Figure 3 using 1x10e6 luciferase-transduced MV4;11 cells injected into NSG mice (day 0) and injected with 5x10e6 CD33CARTs on day 19 after AML engraftment and followed by bioluminescent imaging. Gemtuzumab (gem)-based CD33CARTs were ineffective at leukemia eradication. The lintuzumab (lin)-CD28/CD3ζ

CD33CART was moderately more effective than the lintuzumab-4-1BB/CD3ζ CD33CART in this model, although neither was curative against this relatively CD33-dim AML cell line. 28z = CD28/CD3ζ endodomains, BBz = 4-1BB/CD3ζ endodomains. X = deceased animal.

Supplemental Figure 4. *In vivo* assessment of CD33CARTs in THP-1 xenograft model. Experiments were conducted as in Supplemental Figures 3 and 4 using 1x10e6 luciferase-transduced THP-1 cells injected into NSG mice (day 0) and injected with 5x10e6 CD33CARTs on day 15 after AML engraftment and followed by bioluminescent imaging. Gemtuzumab (gem)-based CD33CARTs were partially effective at leukemia eradication. Best inhibition of leukemia proliferation was again observed with the lintuzumab (lin)-CD28/CD3ζ CD33CART in the CD33-bright THP-1 AML cell line. 28z = CD28/CD3ζ endodomains, BBz = 4-1BB/CD3ζ endodomains.

Supplemental Figure 5. *In vivo* activity of CD33CARTs against MOLM14. Studies were performed as in Figure 4 using three different normal T cell donors. (**A**) Lintuzumab-CD28/CD3ζ (lin-28z) or lintuzumab-4-1BB/CD3ζ (lin-BBz) CD33CART were cultured *in vitro* in the absence (-) or presence (+) of CD33+ MOLM14 cells for 24 hours. Representative plots showing staining of CD33CARTs with a recombinant CD33 Fc fusion protein antibody are shown with indicated percentages of positively-stained cells in each quadrant (left). The mean fluorescence intensity of CD33CART staining in the absence or presence of antigen is presented for 3 independent donors. Means are indicated by horizontal lines. (**B**) CD33CARTs were co-cultured with GFP/luciferase+ MOLM14 cells at a 1:1 ratio for 24 hour as in panel A. Representative dot plots showing the percentages of CD33CART cells relative to GFP+ AML are shown for one representative donor. (**C**) Induction of CD25 surface expression on CD33CARTs co-incubated without (-) and with (+) MOLM14 cells for 24 hours was evaluated by flow cytometry analysis. Quantification of CD25+/CD4+ and CD25+/CD8+ CD33CART percentages are shown for a representative T cell

donor. (**D**) Similar assessment of CD71+/CD4+ and CD71+/CD8+ cells was also performed. (**E**) Summary data for (C) and (D) are shown in the lower panels (n=3 normal T cell donors) with statistical analysis by Student's t-test for each -/+ MOLM14 pair comparison. *p<0.05; **p<0.01; ***p<0.001.