Targeting CD123 in blastic plasmacytoid dendritic cell neoplasm using allogeneic anti-CD123 CAR T cells

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Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare hematologic malignancy with poor outcomes with conventional therapy. Nearly 100% of BPDCNs overexpress interleukin 3 receptor subunit alpha (CD123). Given that CD123 is differentially expressed on the surface of BPDCN cells, it has emerged as an attractive therapeutic target. UCART123 is an investigational product consisting of allogeneic T cells expressing an anti-CD123 chimeric antigen receptor (CAR), edited with TALEN® nucleases. In this study, we examine the antitumor activity of UCART123 in preclinical models of BPDCN. We report that UCART123 have selective antitumor activity against CD123-positive primary BPDCN samples (while sparing normal hematopoietic progenitor cells) in the in vitro cytotoxicity and T cell degranulation assays; supported by the increased secretion of IFNγ by UCART123 cells when cultured in the presence of BPDCN cells. UCART123 eradicate BPDCN and result in long-term disease-free survival in a subset of primary patient-derived BPDCN xenograft mouse models. One potential challenge of CD123 targeting therapies is the loss of CD123 antigen through diverse genetic mechanisms, an event observed in one of three BPDCN PDX studied. In summary, these results provide a preclinical proof-of-principle that allogeneic UCART123 cells have potent anti-BPDCN activity.

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Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare, aggressive hematologic malignancy that originates from the precursors of plasmacytoid dendritic cells. Presenting signs of BPDCN include skin nodules and tumors, lymph node and splenic enlargement, central nervous system involvement, and circulating leukemia and/or bone marrow infiltration. The biology of BPDCN is not completely understood; patient outcomes have historically been poor and no standard of care has been established. Median survival has been reported to be approximately one to one and a half years, or even shorter in patients who have disseminated disease. Novel therapies are therefore urgently needed for patients with BPDCN.

CD123, the interleukin 3 receptor alpha chain (IL3RA), is the primary low-affinity subunit of the interleukin 3 receptor. CD123 is normally expressed on some endothelial cells, monocytes, plasmacytoid dendritic cells, basophils, and myeloid progenitors. However, one of the major early breakthroughs in BPDCN was the discovery that CD123 is overexpressed in essentially all cases of BPDCN. CD123 has emerged as an attractive therapeutic target given its differential expression on BPDCN cells, as it is expressed at markedly higher levels on BPDCN blasts than on cells in the normal hematopoietic stem cell compartment. The first CD123-targeted therapy tagraxofusp (SL-401, a diphtheria toxin interleukin-3 fusion protein) was recently approved by the FDA specifically for treatment of this rare disease. Last year, an antibody-drug conjugate IMGN632 has received from FDA a fast-track designation for BPDCN therapy, further highlighting the feasibility, importance, acceptable safety, and clinical impact of targeting CD123.

UCART123 is a genetically modified allogeneic T cell product manufactured from healthy donor cells. These modified T cells (CD123CAR+T) express (i) a second-generation chimeric antigen receptor (CAR; CD123 scFv-4-1BB-CD3ε) directed against CD123 and (ii) the “safety switch” RQR8 depletion ligand, combining epitopes from both CD34 and CD20 antigens, which confers susceptibility to rituximab. UCART123 was first analyzed the bone marrow (BM) of 8 patients with newly diagnosed BPDCN to determine CD123 expression in comparison to BM blasts of 28 patients with newly diagnosed acute myeloid leukemia (AML) and 13 AML patients in remission. CD123 expression levels were significantly higher in BPDCN (gated on CD45 + cells, mean fluorescence intensity [MFI] range, 3,484–17,937) than in remission marrows (gated on CD34 + cells, MFI range 232–845; p ≤ 0.01) and in AML blasts (gated on CD45 + cells, MFI range 360–5073; p ≤ 0.01) (Supplementary Fig. 4). Its ubiquitous and high expression in BPDCN samples suggests that CD123 represents a rational target for therapeutic development in BPDCN.

Additional BPDCN samples freshly procured from patients or viably stored at MDACC Leukemia sample bank were obtained for in vitro analysis (Table 1). All samples expressed CD123 although at various levels (MFI range, 1863–136,399) (Fig. 2a). Cytotoxicity assay results showed UCART123 activity against BPDCN samples with high level of specific cytotoxicity against 5 of the 8 samples evaluated (Fig. 2b and Supplementary Fig. 5). In a degranulation assay, CD107α was detected at the surface of UCART123 cells in the presence of BPDCN samples, in the same range as observed with nonspecific stimulation (PMA/ionomycin, Fig. 2c). UCART123 cells, when co-cultured with BPDCN cells, secreted significantly higher levels of IFNγ than when co-cultured with negative control Jurkat cells (Fig. 2d). Furthermore, UCART123 was shown to have minimal toxicity against normal hematopoietic cells in vitro in both a cytotoxicity and a colony formation assay (Supplementary Fig. 6).
Measurement of the percentage of BPDCN cell engraftment by flow cytometry indicated that both doses of UCART123 allowed control of tumor progression, and that the circulating tumor burden was reduced from day 34 after UCART123 injection (Fig. 3c). The presence of circulating tumor cells was evaluated in the long-term surviving mice (i.e., the UCART123 treatment groups) on days 267 and 279 post BPDCN cell injection (Table 2), showing efficient and durable control of tumor progression in surviving mice. In a mouse from the 10×10^6 UCART123 treatment group expiring on Day 150, no BPDCN cells (hCD123/hCD56 double positive cells) were detected in the BM or spleen (Supplementary Fig. 7), indicating that the cause of death was not likely due to BPDCN progression.

The presence of UCART123 cells was assessed in the blood, spleen, and BM of one mouse from the 10×10^6 UCART123 treatment group sacrificed on day 78 (57 days after UCART123 cell injection). UCART123 cells could be detected in the spleen (16.4% CAR^+ cells among viable cells; Supplementary Fig. 8) and in the bone marrow (1.1% CAR^+ cells; Supplementary Fig. 8), indicating long-term persistence.

These results indicate that UCART123 treatment led to long-term survival and elimination of BPDCN in this primary BPDCN xenograft model.

**Elevated cytokine levels correlate with tumor burden.** In a second PDX experiment (PDX-2, using sample BPDCN-3), mice were similarly randomized upon tumor engraftment into four treatment groups: vehicle, 10×10^6 TCRαβ KO T cells, 3×10^6 or 10×10^6 UCART123 cells, each treatment administered as a single tail vein injection (Fig. 4a). All mice treated with UCART123 unexpectedly died 5–7 days after UCART123 injection (day 28 after BPDCN cell injection), while the mice in the vehicle group survived slightly longer (Fig. 4a, b). Circulating hCD123^+ cells indicating high tumor burden were found in all cohorts (Fig. 4c) by day 23. A high level of IFNγ and IL-2 was detected in peripheral blood samples from the mice treated with UCART123 cells 2 days following UCART123 injection (Fig. 4d and Supplementary Fig. 9a). These results suggest that a cytokine surge from activated UCART123 and/or tumor lysis syndrome after UCART123 administration may have contributed to the premature death of mice treated with UCART123.

We therefore repeated the experiment, this time administering the treatment at an earlier time point, day 14 after BPDCN cell injection (Fig. 4e). All mice in the control groups (treated with vehicle or TCRαβ KO T cells) experienced tumor progression by day 34 after primary BPDCN cell injection and were sacrificed. In contrast, the cohorts of mice treated with UCART123 had extended survival, with those treated with the highest dose (10×10^6) of UCART123 cells all alive at the end of the study (day 210), with no detectable circulating tumor cells (Fig. 4f, g). Mice treated with UCART123 experienced cytokine release 2 days after UCART123 injection, but the concentrations of IFNγ and IL2 in the peripheral blood were at least 10-fold lower than that measured in the preceding experiment with the same PDX-2 experiment (Fig. 4h and Supplementary Fig. 9b). This supports the hypothesis that cytokine release syndrome contributed to the premature deaths of the UCART123-treated PDX-2 mice.

To further evaluate if the RQR8 targeting could lead to CAR elimination in vivo and dampen the cytokine release syndrome,
we administered rituximab 10 mg/kg i.p. 2 days after UCART injections (day 19) during 5 days (Supplementary Fig. 10a). Rituximab administration post CAR-T therapy decreased the amount of CAR-T cells in the murine spleens and bone marrow (Supplementary Fig. 10b), supporting the functionality of the safety switch in vivo. IFNγ levels were similarly reduced in peripheral blood from the mice treated with Rituximab post CAR-T therapy (Supplementary Fig. 10c). These results confirm that RQR8 targeting by rituximab leads to CAR-T elimination in vivo associated with abrogation of cytokine production.

Loss of CD123 leading to escape from UCART123 therapy and early relapse in one of the PDX experiments. In the PDX-3 experiment (sample BPDCN-2), which received treatments similar to those administered to PDX-1 and PDX-2 experiments, all vehicle-treated mice succumbed to BPDCN by day 49 after primary BPDCN cell injection, because of high tumor burden. hCD45+/CD123+CD56+ BPDCN cells were detected in the spleen and BM. Mice treated with UCART123 displayed significantly extended survival, as in the PDX-1 and PDX-2 experiments, to days 104–241 (Fig. 5a, b). IFNγ was detected in peripheral blood samples from the mice treated with UCART123 cells 2 days following T cell injection (Supplementary Fig. 11). However, all UCART123-treated mice experienced BPDCN relapse at days 90–155. Flow cytometry analysis showed that the relapses (in all 3 mice tested/cohort) were associated with emergence of CD123-negative circulating BPDCN clones (95–96% CD123 negative) (Fig. 5c). To determine if UCART123 were present at the time of relapse, we performed digital droplet PCR (ddPCR). Spleen cells were collected from vehicle or UCART123-treated mice (1 × 10^6 UCART123 and 10 × 10^6 UCART123) at death. UCART123 cells were readily detectable in several of the mice that died from CD123 negative relapsed tumor (Sample 13, Sample 16 from 1 × 10^6 UCART123 group, and sample 5 from 10 × 10^6 UCART123 group, Supplementary Table 1), consistent with relapse due to the loss of the target and not because of lack of persistence of UCART123 cells. Since relapses at a low UCART123 dose level were associated with CD123+ disease recurrence, we injected secondary recipient mice with 2 × 10^6 relapsed BPDCN tumor cells (CD123+ tumor cells collected from the spleen of a mouse from the group treated with 1 × 10^6 UCART123 in the initial PDX-3 experiment), and randomized upon engraftment into vehicle or 1 × 10^6 UCART123 treatment cohorts (Supplementary Fig. 12a). On day 38 after

Table 2 Circulating tumor (BPDCN) burden in mice after treatment with UCART123.

| Circulating tumor burden at D267 (CD123+ CD56−) (%) | Circulating tumor burden at D279 (CD123+ CD56−) (%) |
|-----------------------------------------------|-----------------------------------------------|
| UCART123 3 × 10^6 group | 0.1 | 0.0 |
| UCART123 10 × 10^6 group | 0.0 | 0.0 |

Circulating tumor burden was measured by flow cytometry in peripheral blood samples from mice collected on the indicated days (post tumor cell injection), using 95% monoclonal CD123-PE antibody (555644, BD Pharmingen) and 95% monoclonal CD56-APC antibody (555518, BD Pharmingen) gating on viable cells (DAPI(−)).

Table 1 Characteristics of primary BPDCN patients’ samples.

| Identifier (sample source) | Clinical Status | Cytogenetic abnormality | Mutations |
|----------------------------|----------------|-------------------------|-----------|
| BPDCN-1 (PB) | Relapse | 46,XY,del(1)(p13),del(2)(p13),add(1)(p13),add(1)(q42),add(2)(p24),-4,add(7)(q11.2),add(17)(p11.2),add(21)(p21.1) | ASXL1, JAK2, TET2, NRAS |
| BPDCN-2 (BM) | Relapse/ refractory | 46,XY,t(1;6)(p21;p36.3),del(5)(q13q33),del(11)(q13q23),del(12)(p11.2p13),add(15)(q15) | None identified |
| BPDCN-3 (BM) | Relapse | 45,XY,-21,add(21)(p13)[9]/46,XY[11] | TET2, IDH2 |
| BPDCN-4 (BM) | Relapse | 46,XY,del(9)(q21)[8]/46,XY[3] | TP53 x2, ZRSR2 |
| BPDCN-5 (PB) | Relapse | 46,XY,add(1)(p11.2),-13,-21,del(1)(p13),add(1)(q42),add(1)(q21q32) | MPL, TET2 |
| BPDCN-6 (BM) | Relapse | 46,XY,del(1)(p13),add(1)(q42),add(2)(p24),-4 (q24=4),add(7)(q11.2),add(17)(p11.2),add(21)(p21.1),-13,-21 | 5,11,13,14,15,16,17,18,19 |

All samples were obtained from patients of male gender. PB peripheral blood, BM bone marrow, FISH fluorescence in situ hybridization.
BPDCN cell injection, all the mice in the vehicle group were sacrificed because of tumor progression. The mice in the UCART123 treatment group died on days 78–99; thus, UCART123 treatment extended survival in mice engrafted with relapsed BPDCN cells (Supplementary Fig. 12b). However, similar to the study with original PDX-3 in cohorts receiving high doses of UCART123, tumor progression and death in all mice of the UCART123 low dose treatment group was now associated with emergence of CD123 negative BPDCN clones (Supplementary Fig. 12c).
**Fig. 4 Evidence of severe cytokine release at high tumor burden in the PDX-2 BPDCN experiment.** 

(a) Experimental design using BPDCN-3 cells. When engraftment was confirmed on day 21 after tumor cell injection (0.1-23.5% circulating BPDCN), mice were randomized into 4 treatment groups (n = 11-12 mice/group) and received treatment as follows: vehicle; 10 × 10⁶ TCRαβ KO T-cells; 3 × 10⁶ UCART123 cells; or 10 × 10⁶ UCART123 cells by single tail vein injection. **(b)** Survival among the treatment groups was estimated by the Kaplan-Meier method. MST = median survival time (in days). The mean ± SD value is presented. **(c)** High baseline and rapidly progressing tumor burden in all cohorts. The mean ± SD value is presented. 

**Molecular analysis of CD123 loss following UCART123 treatment.** To understand the molecular basis for loss of CD123 surface expression in PDX-3 BPDCN cells, we isolated RNA from CD123⁺ blasts from 2 of the vehicle-treated mice and CD123⁻ blasts from 4 of the UCART123 treated mice, and performed RT-PCR and total RNA-sequencing. The cells from all samples were hCD45⁺ and hCD56⁺, indicating they were leukemia cells. These analyses detected the presence of full-length transcripts containing exons 2-12 in both CD123⁺ samples (#1 and #2 in Fig. 6a). In 2 of the 4 CD123⁻ samples, CD123 transcripts were completely absent, as were transcripts of neighboring genes (#3 and #9 in Fig. 6a). RNA-sequencing reads aligned to Genome Browser tracks for CD123 and housekeeping gene GPI showed no reads present for CD123 but reads present for GPI in the 2 samples with CD123 loss (Supplementary Fig. 13a). The Genome Browser tracks for CD123 and housekeeping gene GPI showed no reads present for CD123 but reads present for GPI in the 2 samples with CD123 loss (Supplementary Fig. 13a). The Array-based Comparative Genomic Hybridization (aCGH)
results showed that, compared to sample 1 (CD123\(^+\)), samples 3 and 9 (CD123\(^-\)) had large regional deletions on chromosome X, which includes the CD123 gene (Fig. 6b). In another CD123\(^+\) sample (sample 5), the splicing analysis algorithm MAJIQ\(^{19,20}\) detected CD123 transcripts containing only exons 2–9, indicating premature transcription termination. Interestingly, if translated, this truncated transcript would produce a protein isoform lacking the transmembrane domain in exon 10 (Fig. 6c and Supplementary Fig 13b). Finally, MAJIQ also revealed canonical splicing of exon 2 to exon 3 in all CD123\(^+\) samples but a sharp increase in
Data from aCGH and FISH results further showed that this patient sample harbored TP53 deletion (Supplementary Fig. 14). Gene set enrichment analysis showed that CD123 loss was associated with reduction of expression of genes in SRC/STAT3 pathway, consistent with inability of CD123 receptor to transmit signals from IL-3 ligand (Supplementary Fig. 15). These results indicate that the BPD CN relapses could develop CD123 loss through different genetic mechanisms.

Discussion

Chimeric antigen receptor (CAR) gene therapy from autologous T cells is a breakthrough technology in treating refractory hematologic malignancies. However, there are limitations, including inability to manufacture CAR-T cells from the patient’s T cells, or disease progression prior to completion of cell production and release of the engineered autologous product. T cell dysfunction is known to occur in cancer patients, and several groups have recently described differences in CAR-T cells generated from chronic lymphocytic leukemia (CLL) patients compared with those from a healthy donor. This is thought to contribute to the low response rates observed in this disease group. Allogeneic gene-edited CART products, which do not require human leukocyte antigen (HLA) matching, and minimize the risk of graft versus host disease, offer the potential advantages of healthy donor-derived CAR-T cells as an “off the shelf” product, which overcomes the manufacturing difficulties of producing CAR-T cells for each individual patient.

The cell surface antigen CD123 is highly expressed and is a hallmark of BPD CN, highlighting the role of CD123 as a therapeutic target in BPD CN. In this study, the antitumor activity of allogeneic off-the-shelf CD123 CAR T cells (UCART123) was demonstrated by in vitro and in vivo assays, with both a BPD CN cell line and primary BPD CN samples. Albeit no correlation between CD123 expression in primary BPD CN and in vitro cell lysis could be demonstrated (not shown), the specificity was confirmed by the lack of cytotoxic activity of UCART123 against CD123-negative Daudi cells and a trend towards increasing degranulation, cytotoxic activity and IFNγ release by CART cells against AML cell lines with higher CD123 levels (Supplementary Fig. 3). Treatment with UCART123 cells markedly prolonged survival of the UCART123 treated PDX-mice and eliminated detectable BPD CN cells. Of importance, we have demonstrated long-term persistence of UCART123 in bone marrow and spleen of mice injected with PDX-1 and PDX-3 models (by flow or ddPCR, Supplementary Fig. 8 and Supplementary Table 1). It should be noted that the persistence in humans is expected to be limited by the rejection of the allogenic product upon recovery from lymphodepletion, which cannot be tested in the immunodeficient mouse models. Encouragingly, according to recent reports in relapsed/refractory B-ALL treated with UCART123 and in NHL patients treated with ALLO-501, both products bearing TRAC knock-out generated using TALEN technology, showed high response rates and allogenic CAR-T cell expansion, supporting the notion that even limited CAR-T cell in vivo persistence is sufficient to produce meaningful responses.

Cytokine release syndrome (CRS), resulting from rapid immune activation induced by CAR-T cells, is the most frequent treatment-related toxicity seen in clinical trials. Similar to other immunotherapy products, UCART123 can potentially induce cytokine release syndrome or tumor lysis syndrome. In the PDX-2 experiment, administration of UCART123 to mice with high tumor burden caused a burst of IFNγ secretion and rapid demise of animals, possibly from cytokine release and/or tumor lysis syndromes. This was supported by a second experiment whereby administration of UCART123 cells in mice harboring the same PDX at a lower tumor burden, was associated with drastically lower IFNγ levels and translated into long-term survival of UCART123-treated animals. The lentiviral construct of UCART123 allows expression of the safety switch RQR8, permitting the cells to be eliminated by rituximab (RTX) through Complement-dependent Cytotoxicity (CDC) and Antibody Dependent Cell-mediated Cytotoxicity (ADCC) (Supplementary Fig. 1). Our in vivo data confirm that RQR8 targeting by rituximab leads to CAR-T elimination associated with abrogation of cytokine production (Supplementary Fig. 10). The safety switch should allow elimination of UCART cells in the setting of severe CRS in patients, as confirmed by the efficacy of Rituximab in vitro (Supplementary Fig. 1) and by the efficient depletion in vivo of RQR8-expressing T-cells by rituximab reengineered to mouse IgG2a, reported by Pule et al.

With respect to on-target off-tumor toxicity of CD123-targeting CART, we have shown minimal toxicity of UCART123 against normal hematopoietic cells in vitro (Supplementary Fig. 6). While the toxicity against endothelial and other normal cells was not addressed in this study, preliminary results from autologous CD123 CART clinical trial at the City of Hope indicated lack of bone marrow or other organ toxicity, and no evidence for capillary leak syndrome. Similarly, in an ongoing clinical trial with anti-CD123 antibody-drug conjugate IMGN632, no endothelial or marrow toxicity was seen in AML or BPD CN patients, supporting acceptable safety targeting CD123. Treatment with CAR T-cells targeting the CD19 antigen CART-19) has yielded very high response rates in patients with B-ALL. However, 30–60% of patients relapse after CAR treatment, and among those, 10–20% are CD19-negative relapses. The main underlying mechanism is the selection for preexisting alternatively spliced CD19 isoforms with the compromised CART-19 epitope. In one (PDX-3) out of three PDX studies, mice treated with UCART123 experienced CD123-negative relapse. Unexpectedly, we identified not a single, but three diverse mechanisms of CD123 loss amongst mice that relapsed with CD123- disease: genomic loss of CD123 DNA; a truncated transcript containing only exons 2–9; and predominant alternative splicing (skipping from exon 2 to exon 5) which is reminiscent of CD19 epo gene in B-cell acute lymphoblastic leukemia. The most plausible explanation is the selection of a small pre-existing population of clones lacking CD123, that were enriched upon the selective pressure of UCART123 therapy. It is worth noting that this PDX experiment was derived from a patient whose tumor harbored a TP53 mutation, which could have contributed to genomic instability observed here in different mice engrafted with the same tumor cells. To our knowledge, this is the first reported observation of genomic CD123 loss in an animal study causing early relapses post CD123 targeted CART therapy. While relapses on the tagraxofusp or IMGN632 clinical trials are generally not associated with CD123 loss in BPD CN, this observation could be highly relevant for monitoring strategies targeting CD123, including autologous CART, CD123-ADCs, BiTes, and DARTs.

In summary, allogeneic UCART123 therapy resulted in eradication of BPD CN in vitro and in long-term disease-free survival in a subset of primary BPD CN PDX experiments. Our findings in one of the PDX experiments cautioned that CD123 loss could compromise efficacy of this approach with competitive survival of CD123 negative clone(s). Still, these results provide preclinical
proof-of-principle that allogeneic UCART123 cells have potent anti-BPDCN activity.

Methods

Study approval and ethics statements. Primary PBPCN cells and AML cells were collected from patients who had consented to research protocols approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center (PA14-0157, A Study to Evaluate Patient Samples with Blastic Plasma Cytoid Dendritic Cell Neoplasm). This animal study was performing under Animal Care and Use Protocol (Immunotherapy approaches in leukemia, 00001446-RN00). The production of R&D grade batches of UCART123 was done using commercially available NHPBMC cells ordered from ALLCELLS or HEMACARE. These are normal human peripheral blood mononuclear cells derived from an apheresis collection. Donors have been screened for viral status and the collection protocols and donor-informed consent are approved by an Institutional Review Board (IRB), with strict oversight. HIPAA compliance and approved protocols are also followed. The commercially available NHPBMC cells used to generate the UCART cells described in the manuscript are for Research Purposes Only and were used according to local licenses and regulations (French Ministry of Higher Education, Research and Innovation – French Biotechnology Council).

Cell lines. The PBPCN CAL-1 cells[47] were obtained from Takahiro Maeda (Nagasaki University). MOLM13 acute myeloid leukemia (AML) and Jurkat T cell leukemia cells were obtained from ATCC (Manassas, VA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All cells were validated by short-tandem repeat profiling prior to their use in these experiments, and underwent mycoplasma testing every 6 months. All three cell types were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells were cultured at 37 °C with a 5% CO2-in-air atmosphere.

Patient samples. Primary PBPCN cells and AML cells were collected from patients who had consented to research protocols approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center for analysis of hematologic malignancies (PA14-0157, A Study to Evaluate Patient Samples with Blastic Plasma Cytoid Dendritic Cell Neoplasm). Bone marrow aspirate mononuclear cells from patients with PBPCN were purified by Ficoll density centrifugation. Lymphocytes were isolated by using Lymphocyte Separation Medium. Cells were frozen in FBS and dimethyl sulfoxide and stored in liquid nitrogen until use.

CAR construct. The CAR construct used is described in patent EP3119807B1 (“CD123 specific chimeric antigen receptors for cancer immunotherapy”). We used the scFv derived from the monoclonal antibody Klon4 fused to CD8a hinge and transmembrane domains, the 4-1BB co-stimulatory domain, and the CD3ζ signaling domain.

UCART123 production. R&D grade UCART123 cells were produced by Cellectis using a large-scale manufacturing process. UCART123 cells were derived from a single donor. Two large scale batches (derived from the same donor) were used for in vitro testing, while a single batch was used in all the in vivo experiments. For the data presented in Supplementary Fig 3, three small scale batches were used, from three independently deriving mononuclear cells. Non-transduced T-cells (T-cells) produced by negative selection using a TCRαβ- deficient T-cell population was produced from the same donor and in the same conditions, to be used as control cells. Briefly, PBMCs from healthy donors were thawed and T-cells were activated using CD3/CD28 magnetic beads the day after thawing. Cultures were performed at 37 °C in 5% CO2 in X-vivo15 media, supplemented with 5% Human AB Serum and 20 ng/ml of human IL-2. Cells were transduced with a recombiant lentiviral vector encoding the CD123-targeting CAR, at MOI 5, three days after activation. Two days later the cells were transfected with TALEN™ mRNA (using the Pulse Agile® electroporation system) to knockout the TRAC gene and disrupt TCRαβ expression. Cells are then expanded at 37 °C in 5% CO2 within a WAVE bioreactor CellBag of 2 L or 10 L on the Xcell™ Cell Expansion System W25 for 10 days. At the end of the expansion phase, TCRαβ negative cells are isolated by negative selection using a TCRαβ biotin and anti-biotin magnetic bead system from Miltenyi, using the CliniMACS device. The purified TCRαβ cell population was then resuspended in cryopreservation media, aliquoted in cryovials and stored at below −135 °C.

Cytotoxicity assay. The cytotoxicity activity of UCART123 towards the BPDCN cells (CAL-1 cell line or primary samples) was assessed by measuring the survival of tumor cells when co-cultured with UCART123 cells. The CAL-1[24] MOLM13 cell line was used as a positive control. MOLM13 and CAL-1 cells were stained with carboxyfluorescein succinimidyl ester, co-incubated with UCART123 cells at a 1:1, 3:1, or 10:1 effector-to-target (E:T) ratio for 16 h, and the viability of the target cells was determined by flow cytometry using eFlour 780 viable viability dye. Flow cytometry data were analyzed using by FlowJo V10. The viability of UCART123 treated cells was compared to that of samples co-cultured with non-transduced TCRαβ-deficient (TCRαβ KO) T cells to determine the percentage of specific cell lysis. %Lysis = (1 − viability of UCART123-treated cells / viability of TCRαβ KO T cells-treated cells) × 100%

T cell degranulation assay. UCART123 cells were co-incubated with BPDCN samples, or with CD123+ Jurkat cells as a negative control, at a ratio of 1:1 with an anti-CD107a antibody for 6 h. Co-activator reagents (monensin and anti-CD95 and antiCD28 antibodies) were added to the mixture. UCART123 cells incubated with phosphor myristate acetate (PMA)/ionomycin were used as a positive control of T cell activation. Cells were analyzed for CD107a signal by flow cytometry, gating on viable and CD8+ cells. Flow cytometry data were analyzed using by FlowJo V10.

IFNy release assay. The release of IFNy by activated T cells was analyzed by using a BioLegend LEGENDplex assay. Briefly, TCRαβ KO T cells or UCART123 cells were co-cultured for 25 h at a 1:1 (E:T) ratio with CD123+ (Jurkat), CD123− (MOLM13 or CAL-1), or primary BPDCN cells. The quantity of IFNy released into the culture supernatants was measured. PMA/ionomycin was used as a positive control. The same BioLegend LEGENDplex assay was used to evaluate IFNy and IL-2 release levels in the PDX experiments.

Animals. The animal study was performing under Animal Care and Use Protocol at The University of Texas MD Anderson Cancer Center (Immunotherapy approaches in leukemia, 00001446-RN00). NSGS mice (NOD-SCID-IL2rγnull, 8 weeks old) were used in this study. The mice were female, 8–12 weeks old. Unaltered mice were housed in SPF conditions. Room temperature and humidity were continuously monitored. Mice were monitored at least once daily, and up to twice daily including weekend/holidays to follow eventual morbidity signs: hunched posture, rapid weight loss (more than 20% of body weight from baseline), ruffled fur, impaired mobility (difficulty reaching food and water), limb paresis etc. Animals that exhibited one of these morbid signs were immediately euthanized by compressed CO2.

Patient-derived xenografts. After being quickly thawed in a 37 °C water bath, cryopreserved primary BPDCN cells were resuspended in 10 mL of MEM, Alpha 1X (Corning) supplemented with 20% FBS (Invitrogen), and centrifuged at 300 g for 5 min. Cells were resuspended in mixture of 10 mL of MEM, Alpha 1X containing 20% FBS, 0.1 mg/ml heparin, 10 units/mL DNase (Thermo Scientific), and 10 mM MgSO4 (Sigma). Cells were incubated for 15 min in 37 °C incubator in the above mixture and centrifuged at 300 g for 5 min. After centrifugation, BPDCN cells were resuspended in the injection medium (serum-free RPMI 1640 medium) at a concentration of 2 × 106 BPDCN cells/100 μL. Cells were kept on ice until injection into immunodeficient NSG or NSGS mice via the tail vein (100 μL/mouse). Mice were monitored weekly for evidence of human BPDCN in the peripheral blood. When lymphoid enrichment (i.e., CD56−CD19− MOLM13 or CAL-1, or primary BPDCN cells were detected, mice were randomized into treatment groups ranging from 7 to 12 mice per group. Treatments comprised a single tail vein injection of vehicle; 10 × 106 non-transduced TCRαβ KO T cells; 1 × 106 UCART123 cells; 3 × 106 UCART123 cells; or 10 × 106 UCART123 cells. Rituximab (Genentech, USA) was given i.p. at 10 mg/kg dose for 5 days.

Human tumor cell analysis by flow cytometry. The presence of human tumor cells in viable (DAPI−) cells isolated from mouse blood or spleen was identified by analyzing cell surface expression of ICOS123 and ICOS56. The percentage of engraftment was calculated as indicated below:

Engraftment % = (CD123+ CD56− cells/DAPI− cells) × 100%

For the analysis of CD marker expression, the red blood cells were subjected to lysis in ammonium chloride solution. Samples were incubated with a mixed solution of anti-CD56 and anti-CD56 antibody. After DAPI staining, viable cells (DAPI−) were analyzed by a Gallios flow cytometer (Beckman Coulter). The acquisition was stopped after 5,000 DAPI+ viable cells (if achievable) for each sample. All the events were saved during acquisition. Flow cytometry data were analyzed using by FlowJo V10.

Detection of UCART123 cells by flow cytometry. The presence of UCART123 cells in blood was analyzed using cell surface immunostaining for hCD5 (human T cells, Cat#555352, BD Pharmingen) and for anti-CD123 CAR, using a synthetic protein comprising the extracellular portion of hCD123, fused to a mouse Fc (custom made, LakePharma, Inc). The peripheral blood, spleen, and bone marrow were collected from each mouse and were subjected to lysis in ammonium chloride solution. The red blood cells were subjected to lysis in ammonium chloride solution. Samples were incubated with Fc block master mix at room temperature for 10 min. Then samples were incubated with the CD123-Fc protein (150 ng protein/)
Detection of CD123 expression level in AML, AML remission, and BPDCN patient samples. Multiparameter flow cytometry (MFC) analysis of CD123 expression in neoplastic cells of patients with BPDCN or AML or AML remission was performed on FACS Canto II analyzers (BD Biosciences, Mountain View, CA) using bone marrow aspirate material collected in EDTA. Details of the MFC panel and gating strategies used at MD Anderson Cancer Center were described by our group previously.13 The blast gate was defined on the basis of CD45dim expression (CD34 for patients with AML remission) and side-scatter characteristics and quantified as a percentage of total gated events. CD123 expression was measured using an allophycocyanin-conjugated anti-CD123 (IL-3 receptor α) chain antibody (clone 7G3; BD Pharmingen, BD Biosciences) according to the manufacturer’s recommendation. At least 100,000 events per sample were acquired. Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA). CD123 expression was reported as percentage of positive blasts and as mean fluorescence intensity (MFI) on leukemic blasts.

Digital droplet PCR. UCART123 transcripts in spleen were evaluated with digital droplet PCR (ddPCR) at the timepoint of euthanasia due to sickness or disease progression. RNA was extracted by Qiagen RNeasy Kit and 25–50 ng of RNA was converted into cDNA with SuperScript VILO (Thermo Fisher Scientific). Ten million droplets were generated from cDNA samples (step1) and single molecule in droplets were amplified by PCR reaction (step2) followed by counting the absolute number of fluorescent droplets (step3). Step 1 and step 3 were performed and analyzed with RainDrop and RainDrop Analyst II software (RainDance Technologies/Bio-Rad Technologies). Copy numbers of UCART123 transcript relative to 1.0 × 10⁹ ABL1 transcripts (reference) were calculated.

aGCH assay. The genomic DNA was extracted from CD123- cells from UCART123 treated mice in PDX-3 experiment. The quality of genomic DNA was tested by NanoDrop 2000 (Thermo Scientific). 1.0 μg genomic DNA from CD123 + cells from vehicle-treated mice, which is used as reference, was labeled with Cy3 and Cy5 fluorescent dyes, respectively, by using CytoSure™ HT Genomic DNA Labelling Kit (Cat# 500040, Oxford Gene Technology, UK). Labeled DNA was further purified with Clean-up columns (Cat# 500020, Oxford Gene Technology, UK) to remove unincorporated fluorescent dyes. Labeled DNA was measured the absorbance at 260 nm, 55 nm and 650 nm by NanoDrop 2000. Equal amount of labeled DNA from CD123- cells and CD123+ cells were mixed in hybridization buffer, loaded on 8x80K CytoSure ISCA + SNP array (Cat#200525, Oxford Gene Technology, UK) and hybridized at 65 °C for 22 h in the hybridization oven. After post wash procedures, the array slide was scanned by InnoScan 710 Microarray Scanner (Innopsys, France) and further analyzed using CytoSure Interpret software version 4.8.32 (Oxford Gene Technology, UK).

RT-PCR. RNA was isolated using Qiagen RNeasy Kit and used for RT-PCR or RNA Sequencing. For RT-PCR, cDNAs were prepared with random hexamers using the High Capacity cDNA RT Kit (Life Technologies). CD123 was amplified by semi-quantitative PCR for 30 cycles with primer pairs spanning the coding region. cDNAs were converted into cDNA with SuperScript VILO (Thermo Fisher Scientific). The acquired cDNA was diluted 1:20000 and 10 μL of diluted cDNA was amplified for 30 cycles with primer pairs spanning the coding region. The amplification products were run on 1.5% agarose gel and the band identities were confirmed by gel extraction and Sanger Sequencing.

RNA sequencing analysis. Prior to sequencing, RNA integrity and concentration were determined using Eukaryote Total RNA Nano assay on BioAnalyzer. RNA-Seq was performed on 10 ng to 1 μg of total RNA according to the GeneWiz Illumina Hi-seq protocol for poly(A) selected samples (2 × 150 bp pair-end sequencing, 350 M raw reads per lane). Fastq files of RNA-seq obtained from GeneWiz were mapped using STAR aligner. STAR was run with the option ‘alignSJoverhangMin 8’. We generated STAR genome reference based on the hg19 build. Alignments were then quantified for each mRNA transcript using HTSeq with the Ensembl-based GFF file and with the option ‘-m intersection-strict’. Normalization of the raw reads was performed using the trimmed mean of M-values (TMM). In order to generate splice-graphs, the MAJQ tool (version 1.03) was used. MAJQ was run on the Ensembl-based GFF annotations, disallowing de novo calls. The VIOLA visualization package was used to plot the resulting splice graph, the alternative splicing variants and the violin plots representing the PSI values.

Pathway analysis. Pathway analysis was carried out using the gene set enrichment analysis (GSEA) method12. Gene level RNASeq data were used as input. Samples were divided into CD123+ and CD123- classes. GSEA-R program was used to search against the entire gene set collection of Molecular Signatures Database v6.1.

Statistical analysis. Statistical analyses were performed using Excel 2013, GraphPad Prism V8. Statistical tests were done using the two-tailed unpaired t-test. The statistical test used for each Figure is described in the corresponding Figure legend.

Data availability

The RNA sequencing data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE198471. The remaining data are available within the Article, Supplementary Information or Source Data file. Source data are provided with this paper.

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