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CD123 CAR T cells for the treatment of myelodysplastic syndrome

Brett M. Stevens[a,1], Wei Zhang[a,1], Daniel A. Pollyea[a], Amanda Winters[b], Jonathan Gutman[a], Clayton Smith[a], Elizabeth Budde[c], Stephen J. Forman[c], Craig T. Jordan[a], and Enkhtsetseg Purev[1,a]

[a]*Division of Hematology, Department of Medicine, University of Colorado, Aurora, CO;* [b]*Department of Pediatrics, University of Colorado, Aurora, CO;* [c]*Hematology and Hematopoietic Cell Transplantation, T Cell Immunotherapy Research Laboratory, Beckman Research Institute at City of Hope National Medical Center, Duarte, CA*

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Myelodysplastic syndrome (MDS) is a group of heterogeneous disorders caused by ineffective hematopoiesis and characterized by bone marrow dysplasia and cytopenia. Current treatment options for MDS are limited to supportive care, hypomethylating agents, and stem cell transplant. Most patients eventually succumb to the disease or progress to leukemia. Previously, we found that CD123 can be used to delineate MDS stem cells in patients at high risk for MDS and that the CD123-positive population is biologically distinct from normal hematopoietic stem cells. Furthermore, selective targeting of MDS stem cells can dramatically reduce tumor burden in preclinical models. On the basis of these findings, we propose CD123 as a candidate target for chimeric antigen receptor (CAR) T-cell therapy in high-risk MDS patients. To test this concept, we employed a CAR lentiviral vector containing a CD123-specific single-chain variable fragment in combination with the CD28 costimulatory domain, CD3ζ signaling domain, and truncated epidermal growth factor. Utilizing this system, we illustrate that CD123 CAR can be expressed on both healthy donor and MDS patient-derived T lymphocytes with high efficiency, leading to the successful elimination of MDS stem cells both in vitro and in patient-derived xenografts. These results provide the concept for the use of CD123-targeted CAR T cells as a therapeutic option for patients with MDS. © 2019 ISEH – Society for Hematology and Stem Cells. This is an open access article under the CC BY-NC-ND license. (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Myelodysplastic syndrome (MDS) is a hematological disorder resulting from stem cell–driven clonal growth of pathological hematopoietic progenitors and ineffective hematopoiesis [1]. The annual incidence of MDS is in excess of 20 per 100,000 people [2]. The pathological progression of MDS is described with each distinct stage of disease evolution and characterized by increasingly aberrant biological features. The International Prognostic Scoring System (IPSS-R) is utilized to define life expectancy and leukemic progression [3,4]. It incorporates degree of pancytopenia, cytogenetic abnormalities, and number of blasts. Early stage, also known as very low-risk and low-risk MDS, is characterized by low IPSS-R scores. Intermediate-, high-, and very high-risk MDS patients have high IPSS-R scores with profound pancytopenia, unfavorable cytogenetic abnormalities, and increased blast count. Approximately 25% of high- and very high-risk patients will progress to acute myeloid leukemia (AML) within a year [5]. Low-risk MDS patients are typically managed with supportive care. Upon acquisition of high-risk features, patients are typically treated with hypomethylating agents (azacitidine or decitabine), but response rates are only about 30% and resistance develops within 2 years. Thus, development of new and improved therapies is critical.

Like most myeloid malignancies, MDS is thought to arise from mutations in early hematopoietic stem or

Offprint requests to: Enkhtsetseg Purev, Division of Hematology, Blood Cancer and Bone Marrow Transplant Program, Department of Medicine, University of Colorado Anschutz Medical Campus, 1665 Aurora Court, Aurora, CO 80045; E-mail: enkhtsetseg.purev@ucdenver.edu

[1]BMS and WZ contributed equally.

W.Z., B.S., C.J. and E.P. planned the research and wrote the manuscript; W.Z. performed the experiments; W.Z and B.S. analyzed data; E.B., S.J.F and A.W. contributed with vital reagents and materials. D.A.P., J.G., C.S., and E.P. participated in subject screening, enrollment, and patient care.

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progenitor cells. Thus, to investigate more effective MDS therapies, we have focused on properties inherent to malignant hematopoietic stem cells (HSCs). Notably, although malignant stem cells in more advanced diseases such as AML have received significant attention, MDS stem cells remain much less characterized [6–8]. This is likely due to the lack of good experimental models that adequately mimic human disease, as well as the relatively low abundance of tumor cells that can be isolated from patients. Nonetheless, defining therapies that effectively target MDS stem cells is of undeniable importance, and has been the focus of our efforts. We recently reported a detailed study of high-risk MDS patients in which specific molecular and cellular characteristics were described [6]. Of particular importance to the present study, we found that the cell surface antigen CD123 is upregulated on MDS stem cells. This is a notable observation because, although CD123 is expressed at low levels on normal HSCs, its expression was also found to be upregulated in AML, implying that the antigen is activated during pathogenesis [9]. Indeed, previous studies have described a gradual increase of CD123 in low-, intermediate-, and high-risk MDS [10]. Our studies illustrate that expression of CD123 coincides with major changes in the molecular biology of MDS stem cells, which include a strong increase in protein synthesis machinery and changes in energy metabolism [6]. These findings indicate a clear progression toward the emergence of AML. Thus, not only does expression of CD123 provide a means to identify and target MDS stem cells; it also represents a strategy for eradicating the most clinically problematic stage of the disease.

On the basis of the findings outlined above, we sought to develop therapies that could directly target MDS cells expressing CD123. Namely, we chose to pursue the cell therapy-based approach of chimeric antigen receptor (CAR) T cells. CARs are synthetic constructs that, when introduced into T cells, enable recognition and killing of target cells in a highly specific and major histocompatibility complex (MHC)–independent manner. This is accomplished through an antigen recognition motif that is typically derived from a target-specific antibody, which, in turn, is fused to an intracellular CD3 signaling domain (CD3ζ). Incorporation of a T-cell costimulatory (e.g., CD28, 4-1BB) domain dramatically increases the potency of CAR T cells [11–14].

For the present study, we generated a CD123 CAR consisting of a single-chain variable fragment derived from a monoclonal antibody specific for CD123 [9]. This fragment is fused in-frame to the human immunoglobulin G4 Fc region followed by the CD28 co-stimulatory domain, CD3ζ signaling domain, and truncated epidermal growth factor (EGFRt) [9,15]. CD123 CAR T cells have exhibited efficacy in eradicating AML in preclinical studies [9], and are currently being evaluated in a clinical trial to treat patients with relapsed/refractory AML and blastic plasmacytoid dendritic cell neoplasm (BPDCN) (NCT:02159495) [16]. Given the expression of CD123 in MDS stem cells, we investigated targeting CD123 as a potential strategy for treating patients with high-risk MDS.

Methods

Generation of CAR lentiviral vector

CD123 CAR and control CD19 CAR constructs were described previously [9]. CD123 and CD19 lentiviral vectors were produced in 293T cells. In short, 293T cells were plated 1 day prior to transfection in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS) to achieve 80% confluence on the day of transfection. On the day of transfection, the appropriate amounts of lentiviral DNA and CAR vector in 2 × Hepes-buffered saline 2 × HBS $CaCl_2$ were added to the 293T culture. Three days after transfection, viral supernatant was harvested, centrifuged at 2,000 rpm for 10 min at 4°C, filtered through a 0.45-μm vacuum filter, mixed with a 25% volume of PEG-it Virus Precipitation Solution 5X (System Biosciences, Palo Alto, CA) (4°C), and stored at 4°C. Next day, the solution was centrifuged at 2,540 rpm at 4°C for 30 min, aspirated, and centrifuged again at 2,540 rpm at 4°C for 20 min. All trace fluid was carefully removed, and the pellet was resuspended in 1/300th of the original volume with cold sterile 1 × phosphate-buffered saline (PBS) and stored at −80°C until ready for use.

Healthy donor and patient-derived T-cell transduction

The University of Colorado Institutional Review Board approved the tissue bank protocol (IRB Protocol 06-0720), and all subjects gave informed consent in accordance with the Declaration of Helsinki. Healthy donor-derived T cells were obtained from fresh peripheral blood mononuclear cells (PBMCs) using Ficoll–Paque PREMIUM (GE Healthcare, Chicago, IL). Patient-derived T cells were obtained from cryopreserved peripheral blood from our MDS patient tissue bank. T cells were selected from the tissue samples using human CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified CD3+ cells were cultured in X-VIVO15 medium (Lonza Group, Basel, Switzerland) with 10% heat-inactivated fetal bovine serum (Corning Inc., Corning, NY) and 50 units/mL interleukin (IL)-2 (PeproTech, Rocky Hill, NJ) and 0.5 ng/mL IL-15 (PeproTech). Cultured T cells were stimulated with CD3/CD28 Dynabeads (Gibco Biosciences) and then transduced with CD123 CAR or left untransduced (mock). After 6–8 days, CD3/CD28 Dynabeads were removed from T cells, and CD123 CAR expression in transduced T cells was determined by flow with anti-CD3-FITC (BioLegend, San Diego, CA), anti-human EGFRt (BioLegend), biotin–Protein L (GenScript, China), streptavidin-PE (BD Biosciences, San Diego, CA), and Live/Dead-APC/Cy7 viability dye (Invitrogen Thermo Fisher Scientific, Waltham, MA).

CAR T-cell cytotoxicity and cytokine assays

To assess CAR T cell-mediated cytotoxicity, 5×10^4 EGFR+ CD123 CAR T cells were co-cultured with equal number of MDS-L cells or bone marrow cells derived from MDS patients in X-VIVO 15 medium with 10% FBS for 48 hours at 37°C with 5% CO_2. For MDS-L cell line killing assays in vitro, expression of CD123, CD3, and EGFRt was evaluated by flow cytometry to determine the number of MDS-L cells and CAR T cells in co-culture using the following antibodies: anti-CD123-APC (BD Biosciences), anti-CD3-FITC (BioLegend), and anti-EGFRt-BV421 (Biolegend). For primary MDS cell killing assays, numbers of MDS stem cells and CAR T cells were assessed by flow cytometry with the following antibodies: anti-CD34-PE/Cy7 (BD Biosciences), anti-CD38-PE-CF594 (BD Horizon), anti-CD123-APC (BD Biosciences), anti-CD3-FITC (BioLegend), and anti-EGFRt-BV421 (BioLegend). Viability of each cell population was determined by Live/Dead-APC/Cy7 (Invitrogen Thermo Fisher Scientific, MA). For the pro-inflammatory cytokine release assay, co-culture supernatants were collected at 48 hours, and analyzed with the V-PLEX Plus Proinflammatory Panel1 (human) Kit (Meso Scale Diagnostics LLC, MA). To assess CAR T-cell cytokine response to target cells, intracellular tumor necrosis factor (TNF)-α expression and degranulation were measured in CAR T cells that were co-cultured with target cells. In brief, equal numbers of CD123 CAR T cells and target tumor cells (effector-to-target [E:T] ratio of 1:1), either MDS-L cells or MDS patient bone marrow samples, were co-cultured in X-VIVO 15 medium with 10% FBS, Golgistop Protein Transport Inhibitor (BD Biosciences), and anti-human CD107A-BV510 (BD Horizon) for 5 hours at 37°C with 5% CO_2. The cells were then harvested and labeled with anti-CD123-BV711 (BD Biosciences), anti-CD3-FITC (BioLegend), anti-CD4-PE/Cy7 (BioLegend), and anti-CD8-APC (BioLegend), as well as subjected to viability staining with Live/Dead Far Red-APC/Cy7 (Life Technologies, Carlsbad, CA). After the cells were fixed with Cytofix/Cytoperm (BD Biosciences), intracellular staining for cytokines was performed using anti-TNF-α-V450 (BD Biosciences).

Colony formation assay

CD123 CAR T cells, CD19 CAR T cells, and untransduced mock T cells were generated from healthy donor peripheral blood mononuclear cells (PBMCs) and co-cultured with autologous CD34+ cells in X-VIVO 15 medium with 10% FBS for 48 hours at an E:T ratio of 1:1. Cells were washed once, resuspended in cell resuspension solution (R&D Systems), added to human methylcellulose complete medium (R&D Systems), and cultured with 2,000 CD34+ cells/mL per plate. Eight days later, colonies of BFU-E, CFU-GEMM, and CFU-GM were counted manually. Images with all plates were captured with the BIO-RAD ChemiDoc MP Imaging System with an 0.8-sec exposure.

Generation of MDS xenograft model

Animal experiments were performed under protocols approved by the University of Colorado Institutional Animal Care and Use Committee (Protocol B-103416(09)1E). To generate patient-derived xenograft (PDX) mice, each NSG-S mouse was conditioned with 1.5 μg busulfan in 100 μL of sterilized FACS buffer (10% heat-inactivated fetal bovine serum [Corning] in PBS) via intraperitoneal injection 24 hours prior to xenograft transplant. Xenograft cells were resuspended in sterile PBS with OKT3 (BioXCell, NH, 1 μg/10^6 cells) prior to intravenous tail vein injection. High-risk MDS patient bone marrow cells (1.5×10^6 cells from MDS patient 1 or 0.5×10^6 cells from MDS patient 6, per mouse) were used as sources of xenograft. Engraftment was confirmed in a randomly chosen mouse that was adoptively transferred with high-risk MDS patient bone marrow cells starting 8 weeks after MDS cell injection. Mouse femurs were flushed. Collected cells were assessed by flow cytometry using anti-human CD45-Alexa Fluor 700 (BD Biosciences), anti-CD34-PE/Cy7, anti-CD38-PE-CF594, and anti-CD123-APC. Xenograft engraftment was determined as greater than 50% hCD45+ in total flushed PDX mouse bone marrow cells and the presence of hCD45+/CD34+/CD38+/CD123+.

Flow cytometry

All flow cytometry analyses were performed with the BD FACSCelesta flow cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo LLC, Ashland, OR).

CD123 epitope density quantification

Quantum MESF beads were purchased from Bangs Laboratories Inc. (Fishers, IN). Staining and quantification of CD123 expression were performed according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5.04 (GraphPad, San Diego, CA). Unpaired Student *t* tests were used to identify significant differences between treatment groups.

Results

Generation of CD123 CAR T cells

For these studies we utilized a previously characterized CD123 CAR construct that has been reported to be effective in eliminating AML cells both in vitro and in vivo [9]. CD123 CAR contains an scFv that recognizes CD123, followed by a CD28 co-stimulatory domain, CD3ζ signaling domain, and EGFRt domain that serves as an epitope tag to identify successfully transduced T cells. As a control, we used an scFv that recognizes CD19 [9,15], cloned into the same vector backbone. To generate CAR T cells, we utilized the method outlined in Supplementary Figure E1A (online only, available at: www.exphem.org). Briefly, CD3-positive cells were isolated from healthy donor PBMCs, followed by T-cell stimulation with IL-2, IL-15, and CD3/CD28 dynabeads. T cells were then transduced in the presence of protamine sulfate and the lentiviral vector overnight before 7 to 10 days culture in IL-2 and IL-15. We evaluated transduction efficiency via flow

cytometry through measurement of EGFRt and protein-L. Transduction efficiencies up to 74% were obtained (multiplicity of infection [MOI] = 1.0) (Supplementary Figure E1B). Both CD4+ and CD8+ subpopulations were effectively transduced with CD123 and CD19 CAR and confirmed in two different healthy donors (Supplementary Figure E1A, B).

CD123 CAR T cells eradicate CD123+ MDS-L cells in vitro

To examine the antitumor function of CD123 CAR T cells, we first examined the tumoricidal function of healthy donor-derived CD123 CAR T cells against the MDS-L cell line [17]. MDS-L was derived from an MDS patient with a large proportion of cells exhibiting the CD34+/CD123+ immunophenotype. CD123+ MDS-L cells were effectively eliminated after co-culture with CD123 CAR T cells for 48 hours at an E:T ratio of 1:1. In contrast, no killing was observed when MDS-L cells were co-cultured with CD19 CAR T cells, with mock T cells, or without any T cells (Figure 1A, B). These results indicate efficient elimination of CD123+ MDS-L cells by CD123 CAR T cells. To further examine the antitumor function of CD123 CAR T cells, intracellular production of the inflammatory cytokine TNF-α, was examined in CD123 CAR T cells, CD19 CAR T cells and mock T cells after co-culture with MDS-L at an E:T ratio of 1:1 or without tumor cell for 5 hours. A significant and robust increase in TNF-α+ cells was detected in response to the CD123 CAR T-cell product after co-culture with MDS-L cells. This was not observed in CD19 CAR T cells or mock T cells (Figure 1C; Supplementary Figure E1C). In addition, an increased number of CD123 CAR T cells exhibited surface expression of CD107A+, a surrogate marker for degranulation, suggesting activation of

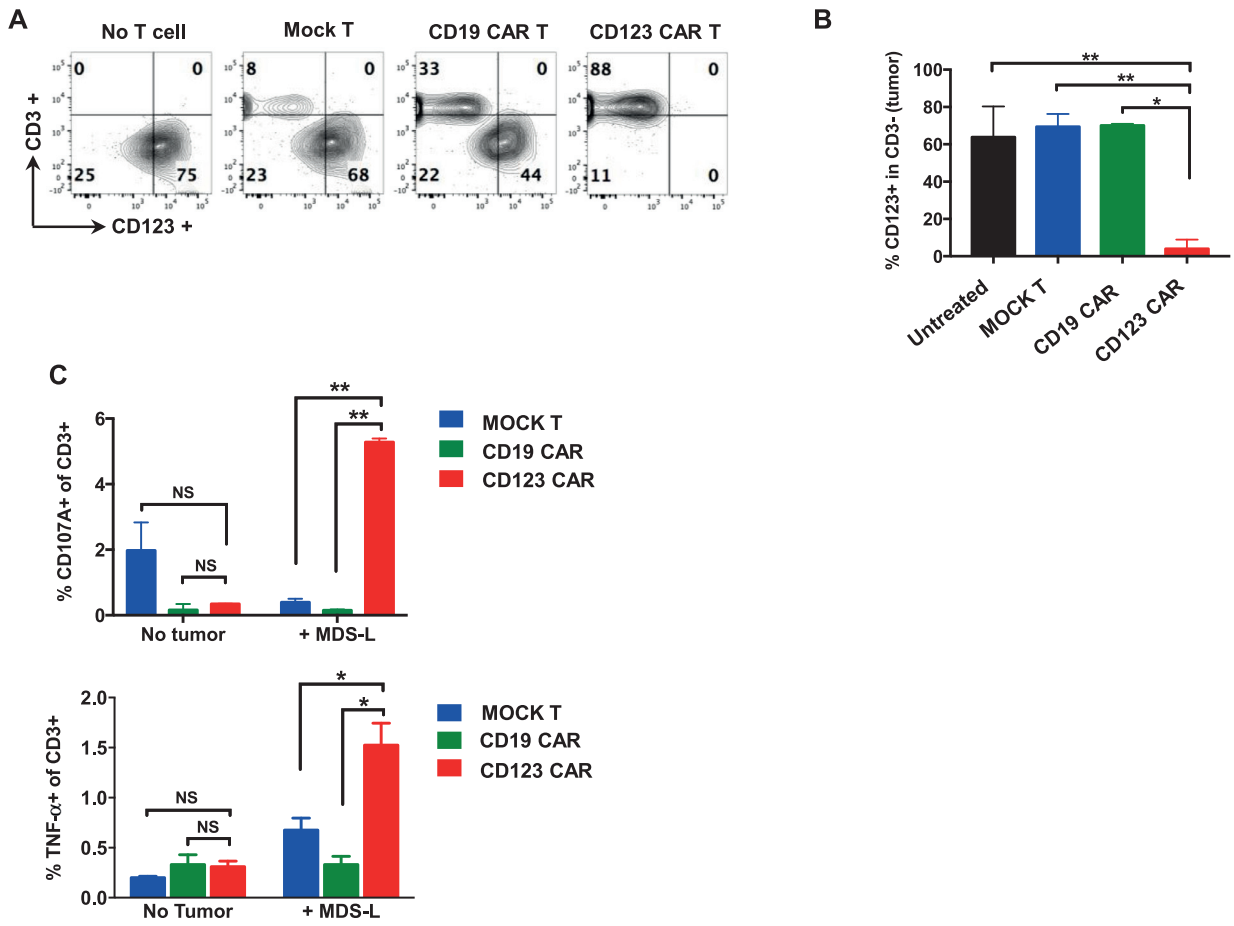

Figure 1. Healthy donor-derived CD123 CAR T cells target and eliminate CD123+ MDS-L cells. **(A)** MDS-L cells were co-cultured for 48 hours at an E:T ratio of 1:1 with mock T cells, CD19 CAR T cells, or CD123 CAR T cells; stained for CD3 and CD123; and analyzed by flow cytometry (mean ± SD of duplicates, $n = 2$ healthy donors with three to five technical duplicates). **(B)** Statistical analyses of flow cytometry revealing elimination of CD123+ MDS-L cells by CD123 CAR T cells (*red*), but not mock T cells (*blue*) or CD19 CAR T cells (*green*). **(C)** Mock T cells (*blue*), CD19 CAR T cells (*green*), and CD123 CAR T cells (*red*) were cultured with and without MDS-L cells for 5 hours at an E:T ratio of 1:1. The presence of CD123 antigen on MDS-L cells leads to an increase in CD107A+ and TNF-α+ in CD123 CAR T cells, but not in mock T cells and CD19 CAR T cells (mean ± SD of duplicates, unpaired parametric *t* test. NS: $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$).

Table 1. Characterization of samples from patients with primary high-risk myelodysplastic syndrome

Patient	Age/sex	Mutation	Karyotype	Blasts (%)
1	77/M	IDH2, ASXL1, U2AF, NRAS	Gain of 1q21 sequences	10
2	82/M	NRAS	Gain of 8	5
3	92/M	DNMT3A, BCOR, TET2	Normal karyotype	15
4	55/F	SF3B1	Normal karyotype	8
5	62/F	ASXL1, KDM6A, STAG2, TET2	Normal karyotype	6
6	65/M	ASXL1, PTPN11, TET2	Monosomy 7	5
7	79/M	RUNX1, TET2	Normal karyotype	10
8	71/M	NA	Deletion of 7q31,deletion of 3q21, deletion of 20q12, gain of 11q23	5

CD123 CAR T cells in the presence of CD123-expressing cells (Figure 1C; Supplementary Figure E1C).

Targeting of CD123 MDS stem cells in patient specimens

Given the efficacy of CD123 CAR T cells in targeting CD123+ cell lines, we next investigated the activity of CD123 CAR T cells against primary MDS specimens (Table 1). We recently reported that CD123 is upregulated during MDS pathogenesis and indicates a stage of disease evolution in which malignant stem cells acquire multiple molecular properties associated with AML [6]. Primary MDS stem cells are characterized as CD34+/CD38−/CD123+. Initial studies were performed using specimens from four different patients (patients 1−4) with high-risk MDS (Figure 2A). CD123 CAR T cells generated from healthy donors were co-cultured with each specimen (patients 1−4)

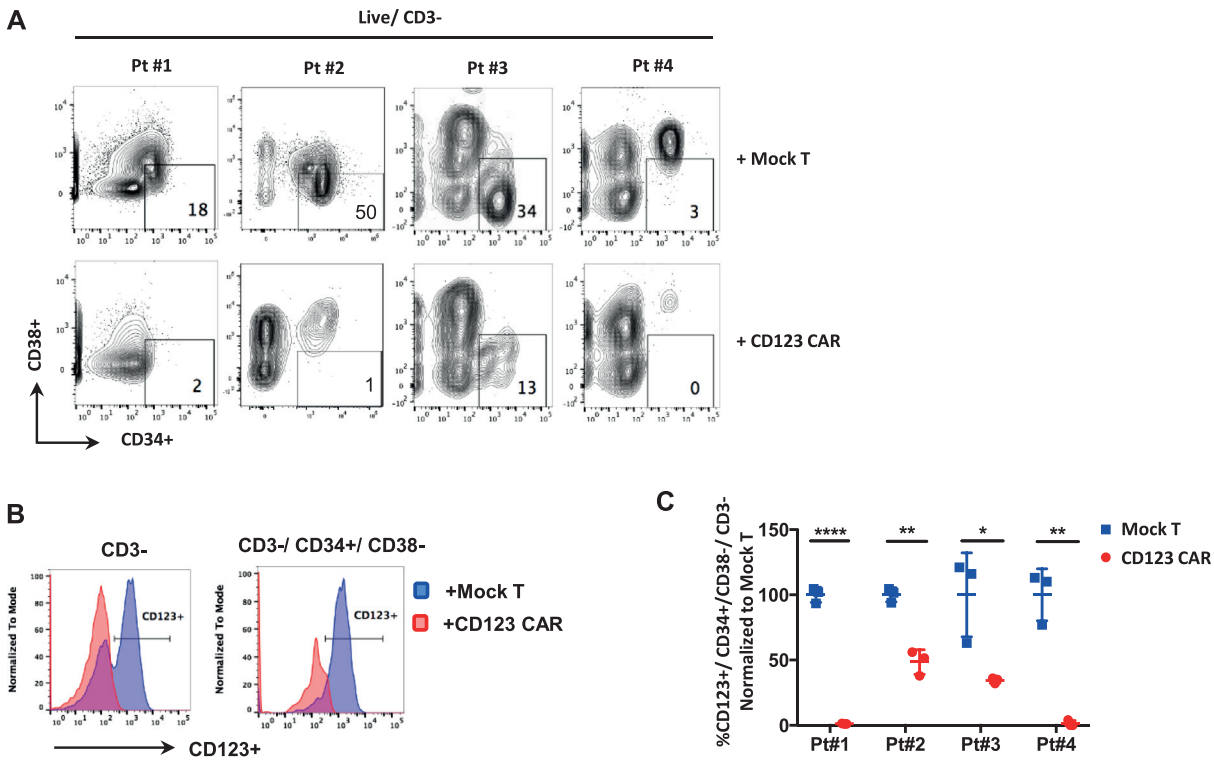

Figure 2. Healthy donor-derived CD123 CAR T cells target and eliminate bone marrow−derived primary CD123+ MDS stem cells in vitro. **(A)** Primary MDS cells from four high-risk MDS patients were co-cultured with healthy donor-derived mock T cells or CD123 CAR T cells for 48 hours at an E:T ratio of 1:1. Primitive cells (CD34+CD38−) in MDS patient bone marrow samples were then analyzed by flow cytometry. **(B)** Representative flow analyses revealing elimination of MDS stem cells (CD34+/CD38−/CD123+) and MDS bone marrow cells (CD3−/CD123+) by CD123 CAR T cells (*red*) and not by mock T cells (*blue*), indicated by disappearance of the CD123+ subpopulation. **(C)** Statistical analyses of flow cytometry revealing elimination of MDS stem cells in bone marrow samples from four different patients by healthy donor-derived CD123 CAR T cells (*red*), compared with mock T cells (*blue*), expressed as the percentage of CD123+CD34+CD38 cells normalized to mock T cells ($n = 3$, mean ± SD of duplicates, unpaired parametric t test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

for 48 hours. We observed 50%–100% elimination of primary MDS stem cells by calculating the proportion of MDS stem cells (CD34+/CD38–/CD123+) after normalizing to mock treatment (Figure 2B, C). These data suggest that CD123 CAR T cells exhibit efficient and specific antitumor activity against CD123-expressing cell lines and primary MDS cells.

Autologous CD123 CAR T cells successfully eliminate high-risk MDS disease in vitro

Although previous experiments suggest the specificity of CD123 CAR T cells [9], the possibility of allogeneic, nonspecific targeting of CD123-expressing cells cannot be excluded from the experiments illustrated in Figures 1 and 2. In addition, use of allogeneic T cells

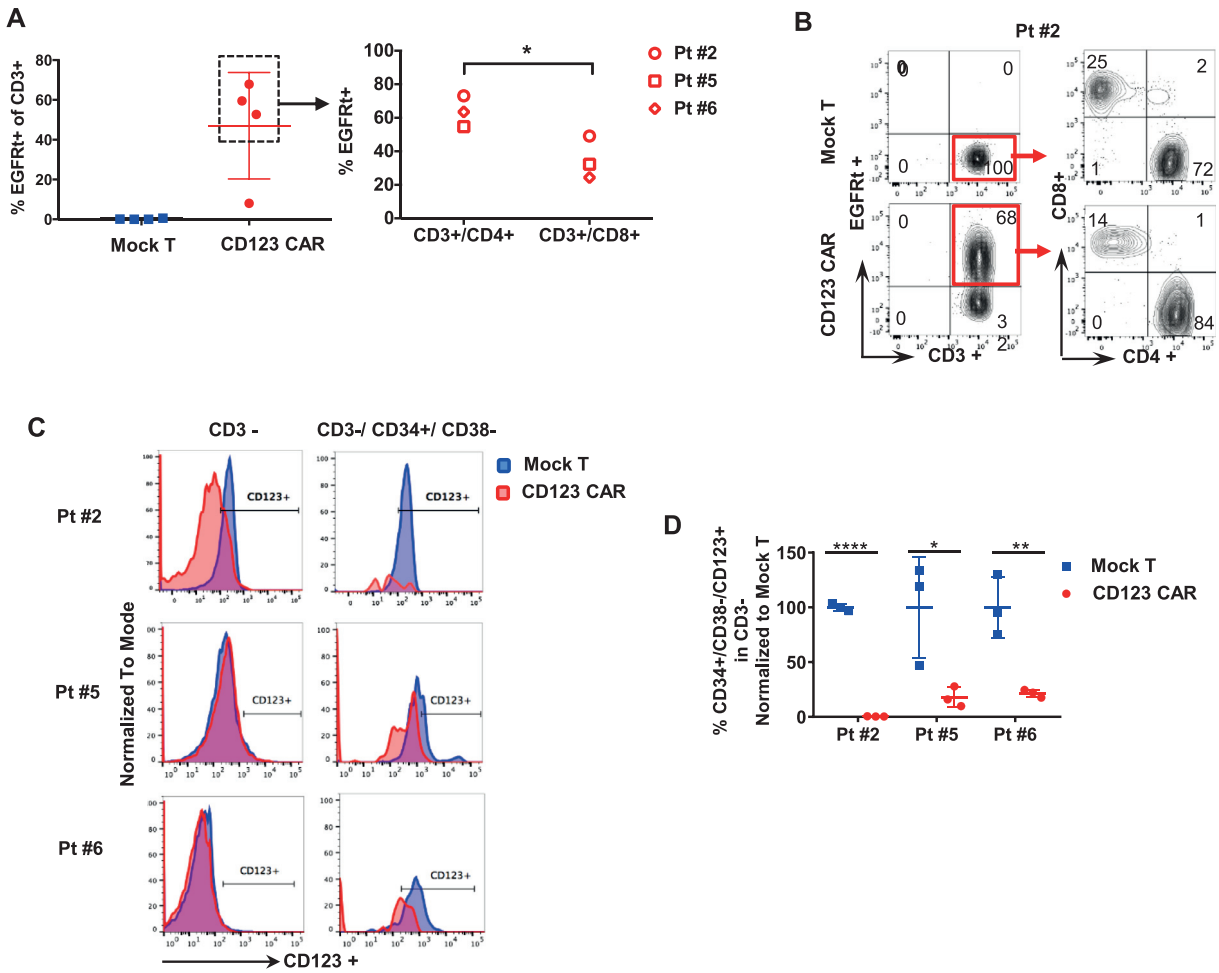

Figure 3. High-risk MDS patient T cells successfully transduced to express CD123 CAR and target-matched bone marrow–derived MDS stem cells in vitro. **(A)** Peripheral blood T cells from four high-risk MDS patients (patients 1, 2, 5, and 6) were transduced with CD123 CAR. CD123 CAR T cells (*red*) or mock T cells (*blue*) were analyzed by flow cytometry. Successful >50% transduction (%EGFRt+ cells) was achieved in three patients' (patients 2, 5, and 6) derived T cells. One patient (patient 1) had 9% transduction efficiency. CD123 CAR T cells from patients 2, 5, and 6 were further analyzed for CD4 and CD8. Both CD4 and CD8 populations expressed CD123 CAR. **(B)** Representative flow cytometry revealing transduction efficiency of CD4+ and CD8+ subpopulations in CAR T cells (EGFRt+) and nontransduced mock T cells for patient 2. The *red box* denotes populations further gated for CD4 and CD8 expression. **(C)** Histograms of CD123+ cells in MDS bone marrow (CD3–) and MDS stem cells (CD3–/CD34+/CD38–) of patients 2, 5, and 6 after co-culture with autologous CD123 CAR T cells (*red*) or mock T cells (*blue*) for 48 hours. **(D)** Primary MDS cells from patients 2, 5, and 6 were co-cultured with autologous mock T cells (*blue*) or CD123 CAR T cells (*red*) for 48 hours at an E:T ratio of 1:1. MDS stem cells (CD34+/CD38–/CD123+) in bone marrow (CD3–) are significantly decreased after co-culture with autologous CD123 CAR T cells, but not mock T cells. Percentage normalized to mock T-cell treatment ($n = 3$, mean ± SD). **(E)** Degranulation as detected by cell surface CD107A is significantly higher in CD123 CAR T cells (*red*) compared with mock T cells (*blue*) after co-culture with an autologous bone marrow sample at an E:T ratio of 1:1 for 5 hours ($n = 3$, mean ± SD) in patient 2. **(F, G)** TNF-α production as detected by intracellular flow cytometry is significantly higher in CD123 CAR T cells (*red*) than in mock T cells (*blue*) after co-culture with an autologous bone marrow sample in the total CD3+ T-cell population **(F)** and both CD4+ and CD8+ subpopulations **(G)** at an E:T ratio 1:1 for 5 hours ($n = 3$, mean ± SD) in patients 2 and 8. (H) Secretion of TNF-α, IFN-γ, and IL-2 in culture supernatant of MDS bone marrow sample with autologous CD123 CAR T cells (*red*) is significantly higher compared with that of mock T cells (*blue*) when co-cultured for 48 hours at an E:T ratio of 1:1 for patients 2 and 6 and for 72 hours at an E:T ratio of 2:1 for patient 5 ($n = 3$, mean ± SD, unpaired parametric *t* test, $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$).

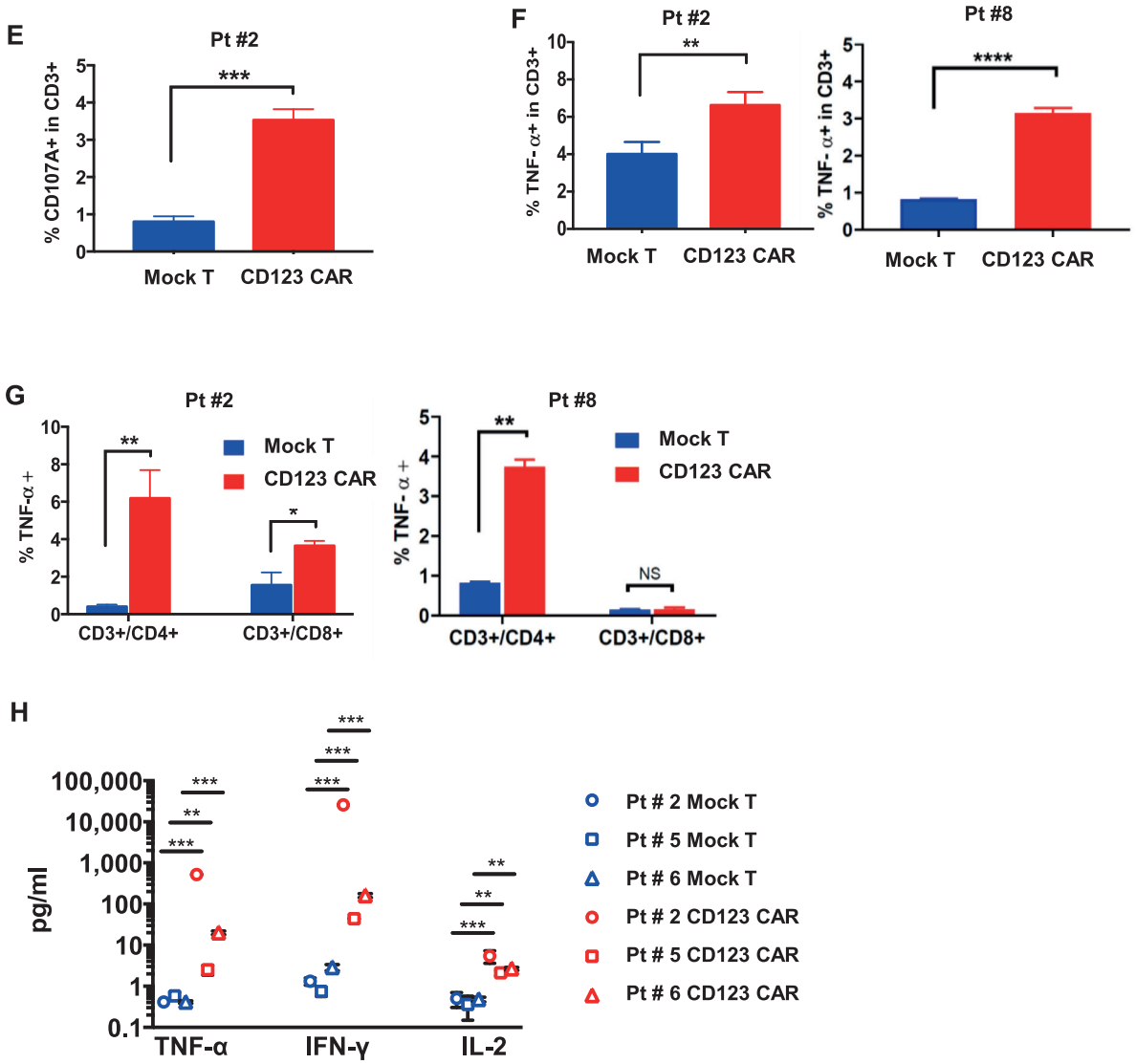

Figure 3 Continued.

significantly complicates any potential clinical application. Therefore, we sought to determine if autologous T cells derived from high-risk MDS patient T cells can also be used to generate effective CD123 CAR T cells to eliminate CD123+ MDS cells. To our knowledge, CAR T cells have never been created from MDS patients; thus we sought to test the suitability of autologous lymphocytes.

Peripheral blood T cells were derived from four high-risk MDS patients to generate CD123 CAR T cells as described above. Greater than 50% transduction efficiency was achieved with three patients (patients 2, 5, and 6) after 7 days of transduction, and 9% transduction efficiency was observed with T cells from the fourth patient (patient 1) (Figure 3A). Both CD4+ and CD8+ T-cell subpopulations were transduced (Figure 3A, B). These efficiencies are similar to those achieved in T cells from AML patients transduced with CD123 vectors [9].

Next, to test the functional activity of autologous CAR T cells, we co-cultured CD123 CAR and mock T cells with autologous MDS bone marrow samples for 48 hours at an E:T ratio of either 1:1 or 2:1. We observed elimination of both bulk tumor (labeled CD3−/CD123+) and MDS stem cells (labeled CD34+/CD38−/CD123+)(Figure 3C, D). Notably, as illustrated in Figure 3C and D, overall cytotoxicity for MDS stem cells ranged between 80% and 100% with residual cells remaining at 0.37 ± 1.97% in patient 2, 17.83 ± 27.34% in patient 5, and 21.33 ± 16.2% in patient 6 when normalized to mock T-cell treatment. Additionally, mock T cells did not present significant killing efficacy against autologous target cells when compared with the bone marrow–only group in either of the two patient samples tested (Figure 3D). Epitope densities of CD123 in

remaining CD34+/CD38−/CD123+ cells after co-culture were analyzed for patient 6. A significant decrease in the CD123 epitope density was observed after treatment of CD123 CAR T cells in comparison to mock T cells or no T cells. The CD123 CAR T-cell treatment-resistant cells exhibited average CD123 for specimens expressing 10,000-15,000 CD123 molecules/cell, indicating a threshold of CD123 CAR T-cell activity at about 10,000 CD123 molecules/cell (Supplementary Figure E2, online only, available at www.exphem.org).

To further evaluate the functional activity of autologous generated CAR T cells, we next examined degranulation and secretion of cytokines. Degranulation, as detected by cell surface CD107A expression, was elevated in CD123 CAR T cells, but not in mock T cell after 5 hours of co-culture in a representative patient sample after incubation with autologous MDS bone marrow samples (Figure 3E). Similarly, intracellular levels and secretion of TNF-α were also increased in both patient samples (Figures 3F, G). The increase in TNF-α production was found in both CD4+ and CD8+ subpopulations (Figure 3G). In addition, a panel of human pro-inflammatory cytokines was analyzed from the supernatant of the cytotoxicity assay, where autologous CD123 CAR T cells were co-cultured with MDS bone marrow samples for 5 hours. Significant increases in TNF-α, interferon (IFN)-γ, and IL-2 were observed in all three patient-derived CD123 CAR T-cell products compared with mock T cells (Figure 3H). However, no significant difference in intracellular IFN-γ was observed by flow cytometry (Supplementary Figure E3, online only, available at: www.exphem.org). Taken together, these results indicate that autologous CD123 CAR T cells are effective at eliminating MDS stem cells in patient-derived specimens in vitro.

Autologous CD123 CAR T cells successfully eliminate high-risk MDS disease in a patient-derived xenograft model in vivo

To evaluate the activity of CD123 CAR T cells in the context of a preclinical in vivo model, we next established a patient-derived xenograft (PDX) to assess the eradication of MDS stem cells. Recently, we reported that high-risk MDS specimens can successfully engraft immune-deficient NSG-S mice when the animals are conditioned via pretreatment with busulfan and T cells depleted from the specimen prior to engraftment [6]. Using this approach, we established cohorts of mice engrafted with primary MDS marrow derived from patients 1 and 6 (Figure 4A, B). Notably, both specimens were able to engraft into mouse marrow with high efficiency as assessed by the expression of human CD45 in comparison to the absence of hCD45 in the pretransplant mice. The specimen from MDS patient 1 represented 69% of marrow cells at 8 weeks posttransplant with 7% composed of CD34+ cells. Additionally, 12% of hCD45+ MDS cells and 15% of hCD45+/CD34+ progenitor cells were CD123+ (Figure 4A). The specimen from MDS patient 6 achieved a level of 68% engraftment at 21 weeks post-transplant, with 25% of cells expressing CD34. CD123 expression was higher in this specimen, with 40% and 52% positive cells in the CD45+ and CD45+/CD34+ populations, respectively (Figure 4B).

For animals transplanted with the MDS patient 1 specimen, autologous CAR T cells were administered 10 weeks postengraftment. Mice received either autologous CD123 CAR T cells or mock T cells ($n = 10$ of each group, 7×10^4 CD123 CAR T cells or mock T cells for each animal). At 4 weeks after T-cell infusion, 26.75 ± 6.52% of human CD45+ cells in the mouse peripheral blood and 28.38 ± 4.61% of human CD45+ cells in bone marrow were CD123 CAR T cells as assessed by the expression of the EGFRt marker (Figure 4C). Analysis of bulk MDS cells (hCD45+/CD123+), MDS progenitors (hCD45+/CD34+/CD123+), and MDS stem cells (hCD45+/CD34+/CD38−/CD123)+ revealed a significantly decreased MDS tumor burden after CD123 CAR T-cell treatment (Figure 4D). The decreased MDS tumor burden was further corroborated by performing histological analyses of bone marrow from MDS PDX mice 4 weeks after CD123 CAR T-cell infusion (Figure 4G). The images reveal a reduction in tumor burden when mice were treated with CD123 CAR T cells compared with mock T cells.

The cohort of animals engrafted with the second high-risk MDS patient specimen (patient 6) exhibited an immunophenotype similar to that of specimen 1, as illustrated in Figure 4A and B. At 2 weeks post transplant, all animals were sacrificed to assess MDS tumor cell eradication, and CAR T-cell persistence in xenograft mouse bone marrows ($n = 3$ of mock group, $n = 6$ of CAR T group). CAR T cells represented 24.6 ± 4.44% and 17 ± 4.69% in bone marrow and peripheral blood respectively (Figure 4E), indicating CD123 CAR T-cell persistence 2 weeks post infusion. CD123 CAR T-cell persistence was accompanied by the eradication of MDS cells, as reflected by significant decreases in the percentage of bulk tumor cells (hCD45+/CD123+), MDS progenitors (hCD45+/CD34+/CD123+), and MDS stem cells (hCD45+/CD34+/CD38−/CD123+) (Figure 4F). Histological examination confirmed the elimination of MDS cells in mice treated with CD123 CAR T cells, but not mock T cells (Figure 4H).

Normal CD34+ cells retain colony-forming capacity after treatment with CD123 CAR T cells

To examine the potential toxicity of CD123 CAR T-cell treatment of normal hematopoietic stem cells, healthy donor-derived CD123 CAR T cells, CD19 CAR T cells and untransduced mock T cells were

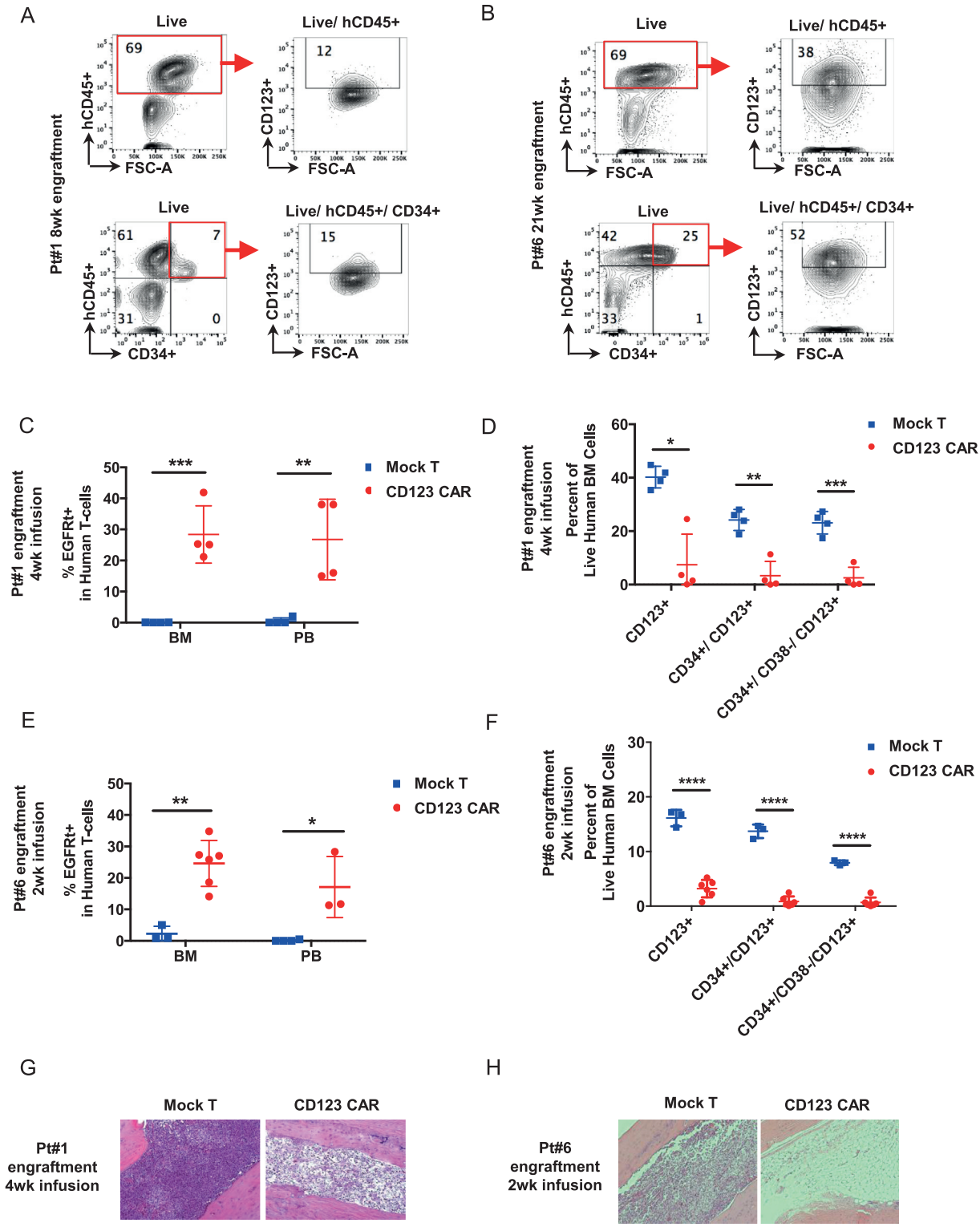

Figure 4. Eradication of MDS tumor cells in patient-derived xenograft by autologous CD123 CAR T cells. **(A, B)** Primary MDS cells from two high-risk MDS patients (patients 1 and 6) were used to generate PDX mice. Representative flow cytometry analysis of each patient sample for CD123+ population in both hCD45+ and hCD45+/CD34+ populations after 8 or 22 weeks of engraftment is shown for patient 1 **(A)** and patient 6 **(B)**. *Red boxes* denote engrafted human cells (hCD45+). **(C, D)** PDXs from patient 1 were treated with autologous CD123 CAR T cells (*red*) or mock T cells (*blue*). Four weeks later, mice were analyzed for persistence of CD123 CAR T cells in bone marrow and peripheral blood **(C)** and elimination of MDS stem cells **(D)**. **(E, F)** PDXs from patient 6 were treated with autologous CD123 CAR T cells (*red*) or mock T cells (*blue*). Two weeks later mice were analyzed for persistence of CD123 CAR T cells in bone marrow and peripheral blood **(E)** and elimination of MDS stem cells **(F)**. (Mean ± SD, unpaired parametric *t* test, $n = 4$ for each group of mock or CD123 CAR T cells for patient 1 xenografts; $n = 3$ for group of mock T cells and $n = 5$ for group of CD123 CAR T cells for patient 6 xenografts.) **(G, H)** Representative morphology of MDS xenograft bone marrow revealing MDS tumor eradication at 2 or 4 weeks post-CD123 CAR T-cell infusion in MDS PDX mice patient 1 **(G)** and patient 6 **(H)**. (Mean ± SD, unpaired parametric *t* test. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.)

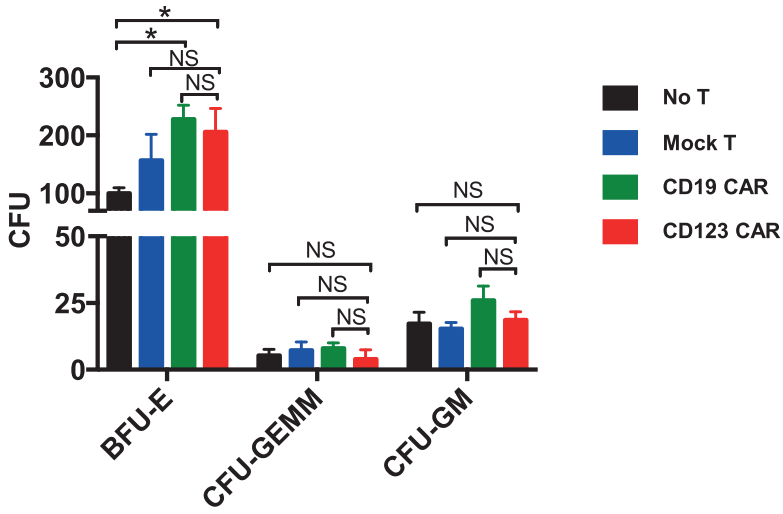

Figure 5. Normal CD34+ cells retain colony-forming capacity after treatment with CD123 CAR T cells. Healthy donor CD34+ cells were cultured with autologous mock T cells (*blue*), CD19 CAR T cells (*green*), or CD123 CAR T cells (*red*) for 48 hours at an E:T ratio of 1:1. CD34+ cells were then analyzed for colony formation. No significant adverse effect was observed in BFU-E, CFU-GEMM, or CFU-GM colony formation after co-culture with CD123 CAR T cells, CD19 CAR T cells, or mock T cells after 8 days. ($n = 3$, mean ± SD of triplicates, unpaired parametric t test, NS: $p > 0.05$, $*p \leq 0.05$.)

co-cultured with autologous matching CD34+ cells for 48 hours at an E:T ratio of 1:1. Cells were then plated in human methylcellulose complete medium to examine colony formation with 2000 CD34+ cells per plate. Eight days later, colony units of BFU-E, CFU-GEMM, and CFU-GM were manually counted, and statistical analyses were performed. Exposure to CD123 CAR T cells did not have any adverse effect on CD34+ cell stemness (Figure 5A). This result suggests normal stem/progenitor cells are not significantly affected by treatment with autologous CD123 CAR T cells.

Discussion

Developing strategies to treat high-risk MDS and prevent progression to acute leukemia remains a major challenge. To date, MDS therapies have largely been limited to the use of hypomethylating agents, which are not curative and do not prevent progression to AML. MDS is a disease of stem cell origin, and the relative paucity of good models to study MDS pathogenesis and malignant stem cell biology has also been a significant challenge. However, relatively recent studies have begun to define the properties of MDS stem cells derived from primary patient specimens [7,8,18–20]. Studies have reported that MDS stem cells can be identified immunophenotypically including the expression of CD99 [7] and IL1RAP [8] on the surface and that these epitopes can be therapeutically targeted. Furthermore, MDS stem cells have been found to exhibit unique and targetable molecular properties including increased PAK1 and IL-8 signaling [21,22]. Our work has described specific molecular changes that accompany late stages of MDS stem cell pathogenesis and identified upregulation of CD123 as a coincident event in the evolution of MDS stem cells [6]. CD123 has previously been the subject of numerous studies in the AML field because of its selective expression on AML stem cells. Thus, multiple approaches based on targeting CD123 are being investigated [9,23–25].

In the present study, we utilized a CAR T-cell strategy to target CD123. CAR T-cell therapy has previously resulted in a high remission rate with CD19-directed CAR T cells, namely, with pediatric and adult acute lymphoblastic leukemia (ALL), with complete remission (CR) of near 90% [26] and near 60% with non-Hodgkin's lymphoma [27]. CAR T-cell therapy may offer a better prognosis for relapsed patients with hematologic malignancies than chemotherapy. In addition to CD19, there are growing numbers of targets that have been validated with Ab-based therapy and are being translated as targets for CAR-based therapy for patients with various malignancies.

The findings in this study illustrate several important scientific principles. First, we found for the first time that autologous T cells derived from MDS patients can be successfully transduced and are highly active in multiple functional assays. Importantly, we obtained effective transduction of both CD4- and CD8-positive T cells from MDS patient specimens. Second, we developed and characterized a xenograft model in which high-risk MDS specimens engraft to high levels and thus provide an in vivo model for characterization of MDS stem cell behavior. Third, we found that autologous T cells are highly active in our xenograft model

and selectively target the CD123-positive population. Equally important, CD123 CAR T cells did not exhibit appreciable targeting of normal hematopoietic stem and progenitor cells. Although at least one previous study reported harm to normal primitive cells [28], it appears that the specific design and affinity of a given CAR construct can be tuned for a level of reactivity that maintains sufficient selectivity to be functionally useful. Indeed, a threshold of antigen density for CAR T cells has been reported previously [29]; hence, it is perhaps not surprising that malignant cells can be differentiated from normal progenitors given that CD123 expression is known to be substantially higher on malignant populations [6,23,25,30]. In our studies, we observed some surviving CD123+ cells. Using epitope density analysis, we found that CD123 CAR T cells are able to eliminate autologous target cells with CD123 epitope density at about 10×10^3 or higher. This threshold is consistent with reports from other groups, and likely explains the survival of $CD123^{dim}$ nonprimitive healthy progenitors [31].

It is important to note that although eradicating CD123-positive MDS stem cells may substantially reduce disease burden and pathogenesis, one would expect that residual CD123 negative MDS stem cells might remain. Indeed, Li et al. reported a progressive increase in CD123 expression as diseases evolves from low- to intermediate- to high-risk stages [10], indicating that MDS stem cells in low-risk disease will not be targeted by CD123 CAR T cells. However, given the fact that most MDS patients are generally advanced in age, reducing the risk of progression to AML and decreasing symptoms associated with high-risk MDS may still have highly significant clinical impact.

Conclusions

The preclinical studies herein provide a strong rationale for testing CD123 CAR T cells for the treatment of high-risk MDS patients. Expression of CD123 provides a validated target antigen for cell-based therapy and also serves as an excellent biomarker to assess the efficacy of CAR T therapy. Furthermore, as a somewhat less acute disease than AML, the setting of MDS provides a clinical context in which various strategies for optimizing CAR T use may be evaluated.

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Conflict-of-interest disclosure

The authors declare no competing final interest.

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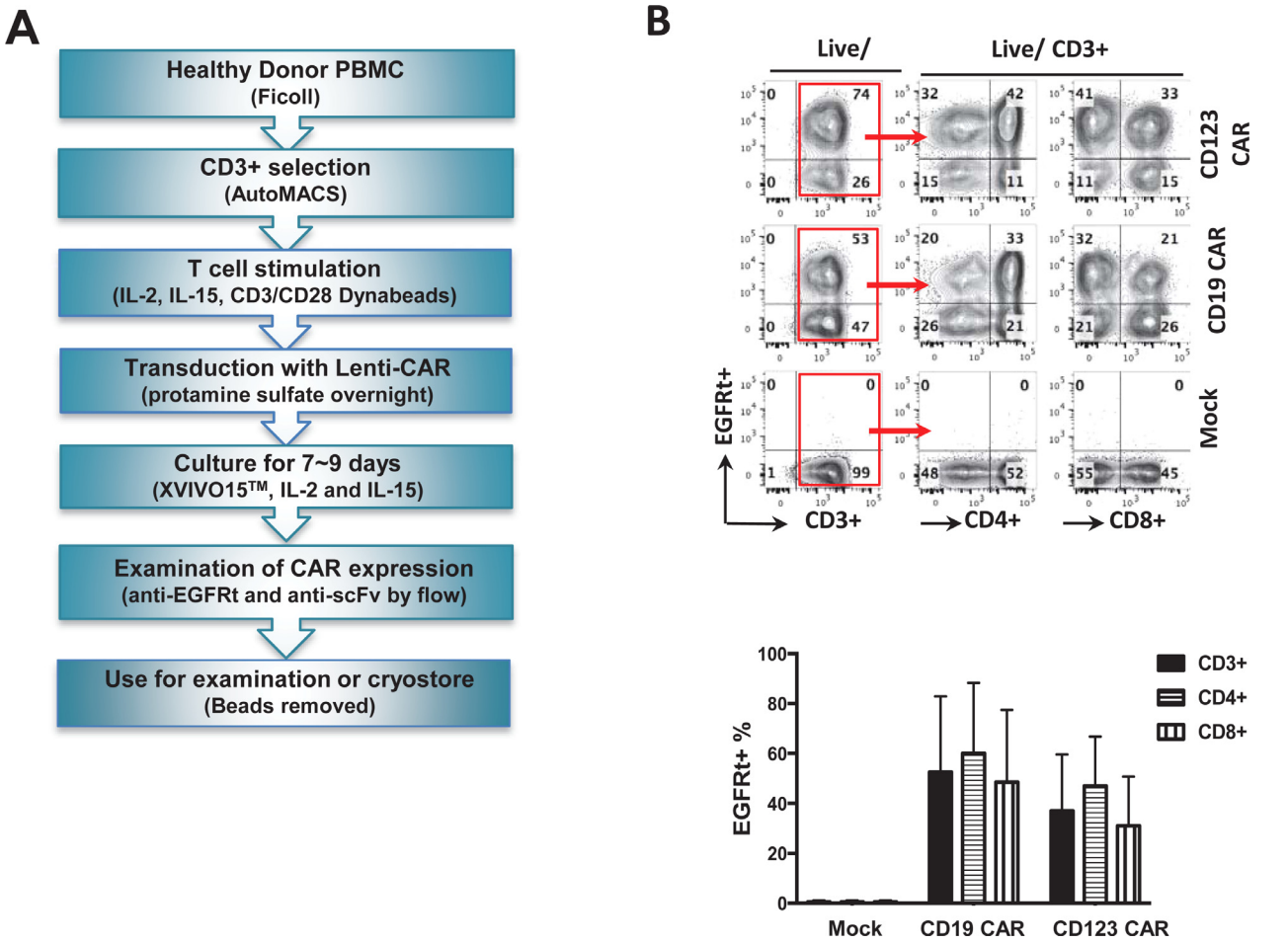
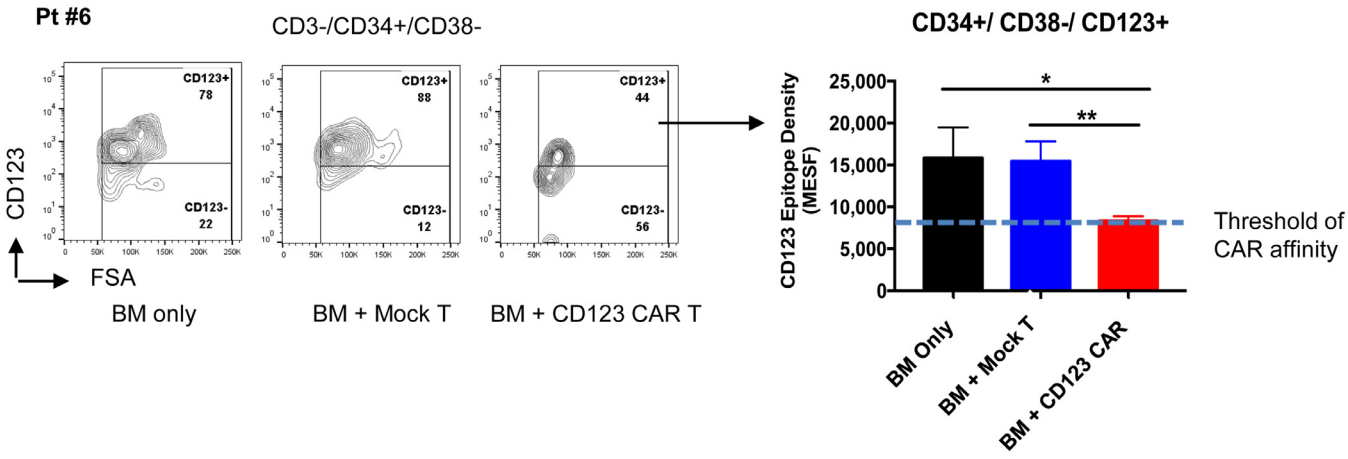
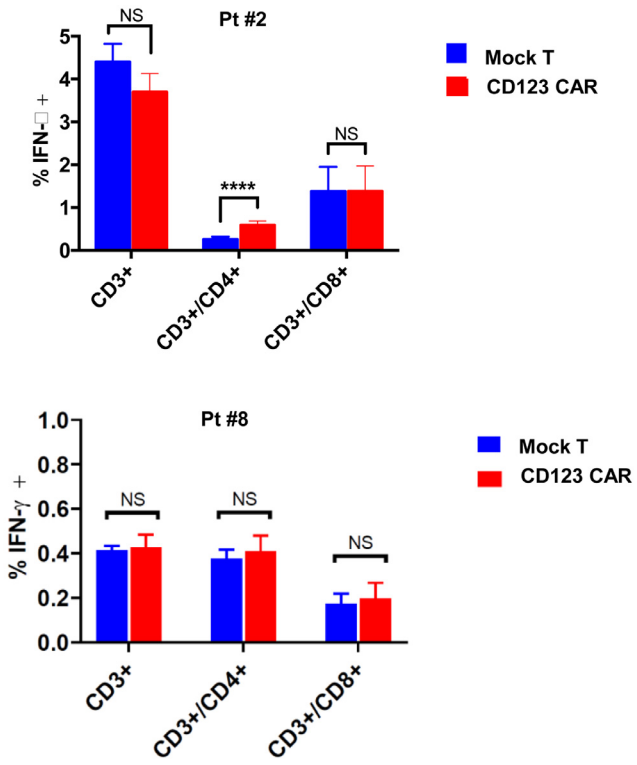


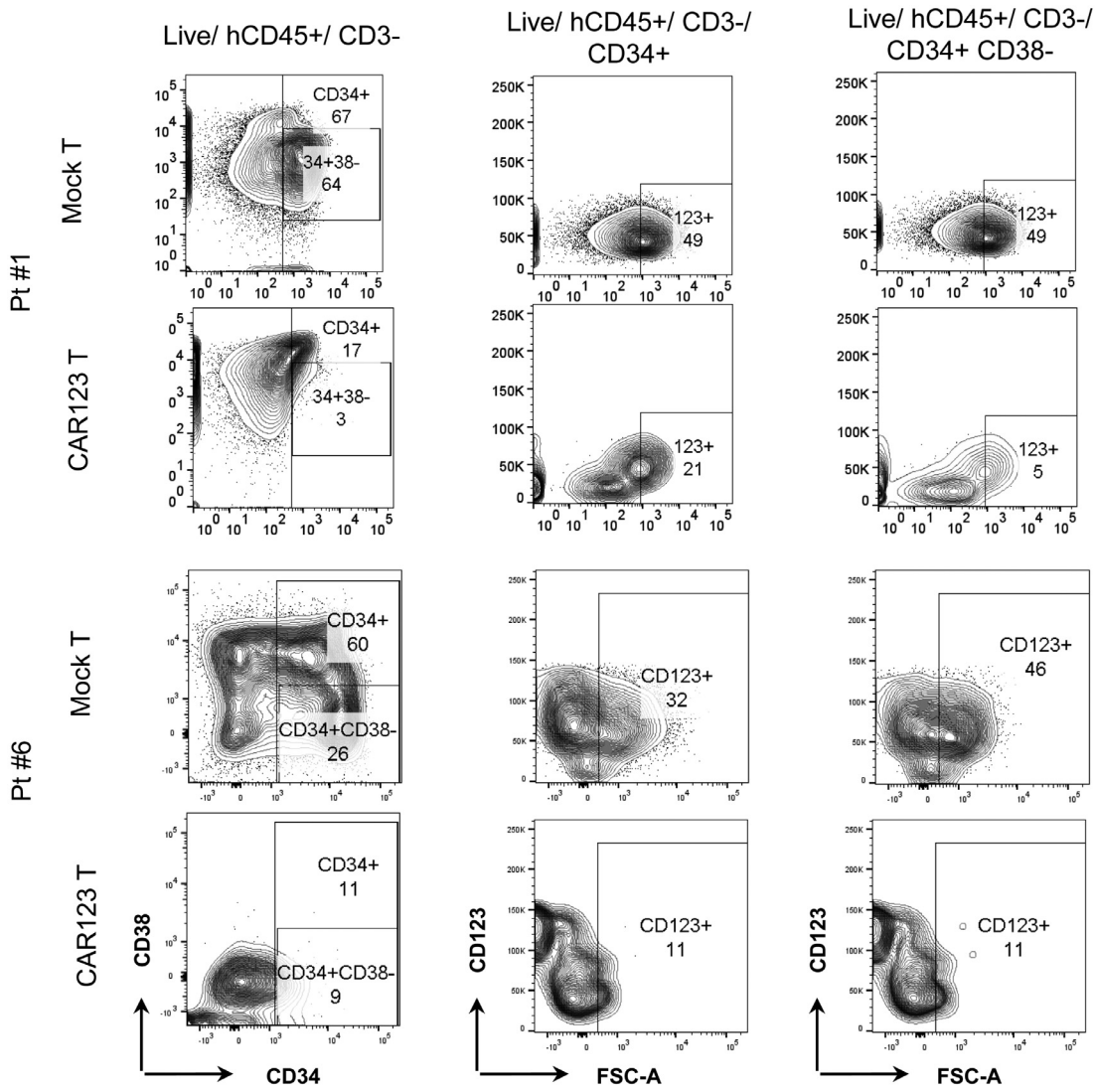
Supplementary Figure E1. Production of CD123 and CD19 CAR T cells. **(A)** Schema of CD123 and CD19 CAR T-cell production. **(B)** Representative flow analysis and statistical analyses of CD123 CAR- and CD19 CAR-transduced healthy donor T cells or mock T cells from healthy donor and average of transduction efficiencies in two different healthy donors (mean ± SD, unpaired parametric *t* test). Gating strategies are indicated by the *red boxes* and *arrows*.



Supplementary Figure E2. CD123 epitope density required for CD123 CAR T-cell efficacy. Primary MDS cells from patient 6 were co-cultured with autologous mock T cells or CD123 CAR T cells for 48 hours at an E:T ratio of 1:1. MDS stem cells (CD34+/CD38−/CD123+) were analyzed by flow cytometry. Residual CD34+/CD38−/CD123+ cells detected in bone marrow cells after co-culture with autologous CD123 CAR T cells, mock T cells, or no T cells were analyzed for epitope density of CD123. Epitope density of CD123 in CD34+/CD38−/CD123+ cells after co-culture with autologous CD123 CAR T cells is significantly decreased compared with that after co-culture with mock T cells or no T cells, indicating a threshold of CD123 CAR T-cell affinity at about 10,000 CD123 molecules/cell. ($n = 3$, mean ± SD, unpaired parametric *t* test, $*p \leq 0.05$, $**p \leq 0.01$.)



Supplementary Figure E3. Intracellular IFN-γ production in CD123 CAR T cells and mock T cells after co-culture with autologous MDS bone marrow sample. CD123 CAR or mock T cells derived from patients 2 and 8 were co-cultured with autologous MDS samples at an E:T ratio 1:1 for 5 hours, stained for INF-γ, and analyzed by flow cytometry. Intracellular IFN-γ was increased in CD3+/CD4+ subpopulation, but not in the CD3+/CD8+ subpopulation or the total CD3+ cells in CD123 CAR T cells compared with mock T cells derived from patient 2. No significant differences were found between T-cell compartment when comparing CD123 CAR T cells and mock T cells derived from patient 8. (n = 3, mean ± SD, unpaired parametric *t* test. NS: $p > 0.05$, ****$p \leq 0.0001$.)



Supplementary Figure E4. Representative histograms of flow cytometry analyses of persisting CD123 CAR T cells in peripheral blood (PB) and bone marrow (BM) of xenografted mice.