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Improving the anti-acute myeloid leukemia activity of CD123-specific Engager T cells by MyD88 and CD40 costimulation

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Running Title: Inducible costimulation to enhance Engager T cell function

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CONFLICT OF INTEREST

AV, CB, SG, and MPV have patent applications in the field of T-cell and gene-modified T-cell therapy for cancer. SG is a consultant for Catamaran Bio, Nektar Therapeutics, and TESSA Therapeutics, on the Scientific Advisory Board of Tidal, and a DSMB member of Immatics.

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Data sharing statement
Most data are available within the article or its supplementary materials. Any additional data are available upon reasonable request from the authors.

Key words:
Immunotherapy, Inducible costimulation, CD123, bispecific T cell engager, leukemia, AML, MyD88, CD40
ABSTRACT

The outcome of acute myeloid leukemia (AML) remains poor, and immunotherapy has the potential to improve this. T cells expressing chimeric antigen receptors (CARs) or bispecific T cell engagers targeting CD123 are actively being explored in preclinical and/or early phase clinical studies. We have shown that T cells expressing CD123-specific bispecific T cell engagers (CD123.ENG T cells) have anti-AML activity. However, like CAR T cells, their effector function diminishes rapidly once they are repeatedly exposed to antigen-positive target cells. Here we sought to improve the effector function of CD123.ENG T cells by expressing inducible costimulatory molecules consisting of MyD88 and CD40 (iMC), MyD88 (iM), or CD40 (iC), which are activated by a chemical inducer of dimerization (CID). CD123.ENG T cells expressing iMC, iM, or iC maintained their antigen specificity in the presence of CID as judged by cytokine production (IFNγ, IL-2) and their cytolytic activity. In repeat stimulation assays, activating iMC and iM, in contrast to iC, enabled CD123.ENG T cells to secrete cytokines, expand, and kill CD123-positive target cells repeatedly. Activating iMC in CD123.ENG T cells consistently improved anti-tumor activity in an AML xenograft model. This translated into a significant survival advantage in comparison to mice that received CD123.ENG or CD123.ENG.iC T cells. In contrast, activation of only iM in CD123.ENG T cells resulted in donor-dependent antitumor activity. Our work highlights the need for both toll-like receptor (TLR) pathway activation via MyD88 and provision of costimulation via CD40 to consistently enhance the antitumor activity of CD123.ENG T cells.
INTRODUCTION

Acute myeloid Leukemia (AML) is a disease with poor prognosis due to its high relapse rate and treatment related mortality (1-4). Adoptive immunotherapy has the potential to improve outcomes in AML, but overlapping antigen expression between tumor cells and healthy tissues as well as T cell persistence in a hostile tumor microenvironment are problematic (5, 6). CD123 presents a promising immunotherapy target for AML due to its high expression on leukemia stem cells (LSCs) and lower expression on normal hematopoietic cells (HPCs) (7-9). Several T cell-based immunotherapy approaches are currently being developed to target CD123, including T cells expressing chimeric antigen receptors (CARs) or strategies involving bispecific antibodies (BiTEs, DARTs, ENGs) (8, 10, 11). We and others have previously reported on a T cell platform that secretes bispecific engagers (ENG T cells) against solid tumors and hematological malignancies(10, 12-14). CD123-specific ENG T cells (CD123.ENGs) secrete a bispecific antibody consisting of two single chain variable fragments (scFvs), one able to bind CD123 and the other specific for CD3ε (10). We have shown that CD123.ENG T cells have anti-AML activity in preclinical models (10). However, the effector function of ENG T cells, like that of CAR T cells, decreases rapidly upon repeated tumor exposure (15-17).

Several approaches are being pursued to increase the ability of CAR and ENG T cells to sequentially kill tumor cells (18-21). These include transgenic expression of molecules such as cytokines or co-stimulatory molecules (22, 23), or knocking
out negative regulators (24). We and others have shown that activating an inducible costimulatory molecule, consisting of a myristoylation-targeting sequence, MyD88 lacking its TIR domain, the cytoplasmic domain of CD40, and two tandem FKBP12v36 domains (iMC), significantly improves the effector function of CAR T cells, including their ability to repeatedly kill tumor cells (18, 21, 25).

Here we explore whether an inducible costimulation system can be utilized to enhance the effector function of CD123.ENG T cells and determine the individual contribution of MyD88 and CD40. To achieve this, we generated retroviral vectors encoding CD123.ENG and inducible MyD88 (iM), inducible CD40 (iC), or iMC. We demonstrate that activation of iM and iMC improves the effector function of CD123.ENG T cells in vitro. However, for consistent benefit in vivo, activation of both MyD88 and CD40 was required in CD123.ENG T cells.

METHODS

Cell lines and culture conditions

The MOLM-13 cell line was purchased from Leibniz Institute (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). MV-411, Kg1a, THP-1, K562 and HEK 293T were purchased from ATCC (American
Type Culture Collection, Manassas, VA). MOLM-13, MV-411, Kg1a, THP-1 and K562 cells expressing an enhanced GFP firefly luciferase fusion gene (MOLM-13.GFP.ffluc and K562.GFP.ffluc) were generated by transducing cells with a retroviral vector encoding GFP.ffluc (26-28).

**Generation of retroviral vectors**

The generation of SFG retroviral vectors encoding i) CD20 and CD123.ENG (CD20.T2A.CD123.ENG), ii) CD20 and CD19.ENG (CD20.T2A.CD19ENG), and iii) inducible costimulatory molecules encoding a myristoylation sequence, two FKBP dimerizer domains, and MyD88 (iM), CD40 (iC) or MyD88.CD40 (iMC) with an HA-tag have been previously reported (10, 13, 18, 25). Additional details are described in the Online Supplementary Appendix.

**Generation of ENG T cells**

All methods involving human subjects were carried out in accordance with the Declaration of Helsinki. Human peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained under a St. Jude Children’s Research Hospital IRB approved protocol, after acquiring informed consent. PBMCs were stimulated on CD3 (1µg/mL, Miltenyi Biotec, Bergisch Gladbach, Germany) and αCD28 (1µg/mL, Miltenyi Biotec) antibodies-coated non-tissue culture treated 24-well plates (144530, Thermo Fisher Scientific, Waltham, WA). Human IL-7 and IL-15 (10ng/mL and 5ng/mL respectively, Biological Research Branch, National Cancer Institute, Frederick, MD) were added to cultures on day 2. On day 3, T
cells were transduced with retroviral particles on retronectin (T100A, Takara Clontech, Mountain View, CA) coated plates in the presence of IL-7 and IL-15. On day 5 transduced T cells were harvested and were subsequently expanded with IL-7 and IL-15. Non-transduced (NT) T cells were activated with CD3/CD28 and expanded in parallel with IL-7 and IL-15. Cells were cultured for 7-10 days prior to being used for in vitro or in vivo experiments.

**Xenograft AML model**

All animal experiments were performed on a protocol approved by the St. Jude Children’s Research Hospital's Institutional Animal Care and Use Committee in accordance with the American Association for Laboratory Animal Science. Additional details are described in the Online Supplementary Appendix.

**Statistical analysis**

Data were summarized using descriptive statistics. Measurement data were presented as mean ± standard deviation (SD). To examine overall differences in outcomes between constructs, ANOVA test was used. This overall test was followed by pairwise comparisons using the T-test and when appropriate ANOVA test was performed.

Generalized Estimating Equation (GEE) was used to access the overall difference in outcomes with repeated measurements over time, to account for the intra-subject correlation. A two-sided-significance level of P<0.05 was used for all statistical tests. Adjustment for multiple testing was not performed due to
the small sample size and the exploratory nature of the analysis. For the mouse experiments, survival, determined from the time of tumor cell injection, was analyzed by the Kaplan–Meier method and by the log-rank test. Statistical analyses were conducted with SAS 9.4 and GraphPad Prism 8 (GraphPad software).

Additional experimental procedures are described in the Online Supplementary Appendix.

RESULTS
Generation of CD123.ENG T cells expressing iMC, iM, and iC
CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, CD123.ENG.iC, and CD19.ENG.iMC T cells were generated by transduction with retroviral vectors depicted in Figure 1A. Seven to 10 days post transduction, transduction efficiency was evaluated by determining CD20 expression via flow cytometry analysis. Mean transduction efficiency was 57.4% (range:52-63.4%) with no significant differences between constructs (n=12; Figure 1B, Supplementary Figure 2A). Expression of iM, iC, and iMC was confirmed by western blot for the HA-tag (Figure 1C). Post transduction, all T cell populations expanded and there were no statistically significant differences (n=6; Supplementary Figure 2B). To confirm that CD123.ENG T cells secrete CD123.ENG protein, effector T cells were plated on a recombinant CD123 protein coated plate with +/- 0.5nM CID. The concentration of secreted CD123.ENG protein was determined by ELISA
after 24 hours. Effector T cells encoding CD123.ENG produced significant amounts of CD123.ENG protein in contrast to CD19.ENG.iMC T cells (p<0.01; Figure 1D). In addition, exposure to CID significantly increased CD123.ENG protein secretion of CD123.ENG T cells endowed with an inducible costimulation domain (iMC, iM, or iC) (p<0.05). T-cell subset analysis revealed that while CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, and CD123.ENG.iC T cells had a higher percentage of CD8-positive T cells than NT and CD19.ENG.iMC T cells, this did not reach statistical significance (Figure 1E). CD45RO and CCR7 cell surface markers were used to differentiate between naïve-like (N), central memory (CM), effector memory (EM) and terminally differentiated effector memory T cells (EMRA). All CD123.ENG expressing CD8+ T cells except CD123.ENG.iMC CD8+ T cells had a significantly (p<0.05) lower percentage of naïve-like T cells in comparison to NT T cells. There were no statistically significant differences in the different CD4+ T cell populations. (Figure 1F).

**CD123.ENG T cells expressing iMC, iM, or iC maintain antigen specificity**

To evaluate whether constructs expressing inducible costimulation (CD123.ENG.iMC, CD123.ENG.iM, and CD123.ENG.iC T cells) maintained antigen specificity like CD123.ENG T cells, we performed co-culture assays in the presence or absence of 0.5nM CID with CD123-positive (MOLM-13.ffLUC, MV-4-11.ffLUC, Kg1a.ffLUC) AML cells (Supplementary Figure 2C). NT and CD19.ENG.iMC T cells served as control effector T cells, while media and CD123-negative K562.ffLUC as control for target cells. Only CD123.ENG,
CD123.ENG.iM, CD123.ENG.iC and CD123.ENG.iMC T cells produced significant amounts \((n=3, p<0.05)\) of IFN\(\gamma\) and IL-2 in the presence of MOLM-13, MV-4-11, or Kg1a in comparison to NT and CD19.ENG.iMC T cells, confirming antigen specificity (Figure 2A, B, Supplementary Figure 3A). CD123.ENG.iM, CD123.ENG.iC and CD123.ENG.iMC T cells secreted higher amounts of IFN\(\gamma\) and IL-2 in the presence of CID; however, this did not reach statistical significance (Figure 2A-B, Supplementary Figure 3A). Next, we examined the cytolytic activity of each T cell population using a luciferase-based cytotoxicity assay. Effector T cells were plated in the presence of CD123-positive (MOLM13.ffLUC, MV-4-11.ffLUC and Kg1a.ffLUC) or CD123-negative targets (K562.ffLUC) at a 1:1 E:T ratio. All constructs secreting CD123 ENG (CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM and CD123.ENG.iC) recognized and killed CD123+ targets but did not have antitumor activity against K562.ffluc (CD123 negative). The addition of CID did not confer additional antitumor activity in these conditions. Effector T cell controls (CD19.ENG.iMC and NT T cells) showed no cytotoxicity towards CD123 positive or negative targets (Figure 2C, Supplementary Figure 3B).

To further differentiate functional differences between CD123.ENG T cell constructs, we performed CD107a degranulation assay and analyzed TCF1 expression after exposing effector cells to CD123.ENG protein +/- CID. Our analysis showed increased degranulation in the groups secreting CD123.ENG in the presence of CD123+ targets in comparison to NT cells, without any
statistically significant differences between CD123.ENG constructs (Supplementary Figure 4A). In addition, TCF-1 expression was consistent across constructs and was not influenced by the addition of CID (Supplementary Figure 4B,C).

Finally, we evaluated fold expression of genes associated with T cell function, activation and cell migration using qPCR analysis. In the presence of CID and after overnight incubation, CD123.ENG.iMC T cells maintained a similar gene expression profile to CD123.ENG T cells (n=4 p=ns). However, CD123.ENG.iM and CD123.ENG.iC T cells had significant upregulation of TIGIT, TIM3 and CCR5 in the presence of CID in comparison to iMC T cells (n=4, p< 0.05). In addition, CD123.ENG.iC T cells expressed higher levels of MyD88, Blimp1, TCF7, Traf6 and EOMES consistent with a more differentiated phenotype (Figure 2 D,E).

**iMC and iM enhance the effector function of CD123.ENG T cells in the setting of chronic antigen exposure**

Having observed limited benefit of activating iMC, iM, or iC with CID after a single exposure to tumor cells, we evaluated the effector function of CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, and CD123.ENG.iC in a sequential re-stimulation assay to mimic chronic antigen exposure (Supplementary Figure 4D). MOLM-13.GFP.ffluc cells or Kg1a.GFP.ffluc cells were co-cultured with effector T cells at an E:T ratio of 1:1 +/- CID. Every 3-4 days, the presence of
MOLM13.GFP.ffluc or Kg1a.GFP.ffluc cells was determined by a luciferase assay, and fresh tumor cells +/- CID were added if tumor cells had been killed (n=9 and n=3 respectively). T cells were also enumerated. A subset of donors that were cocultured with MOLM13.GFP.ffluc underwent flow cytometric immunophenotype analysis (n=3) and media was collected for cytokine analysis 24 hours after coculture (n=3). In the absence of CID, all CD123.ENG T cell populations were able to kill target cells for 2-5 stimulations depending on target cells used (Figure 3A,B left panels). T cell expansion followed a similar pattern and cells failed to expand after 2-5 stimulations (Figure 3C,D left panels). The presence of CID did not affect antitumor activity or T cell expansion for the CD123.ENG and CD123.ENG.iC groups. In comparison, CD123.ENG.iM or CD123.ENG.iMC T cells were able to kill target cells for 5 to 11 stimulations depending on whether they were exposed to MOLM13.GFP.ffluc or Kg1a.GFP.ffluc (Figure 3A,B right panel). In the presence of CID, CD123.ENG.iM or CD123.ENG.iMC T cells continued to expand for 6-10 stimulations in comparison to CD123.ENG or CD123.ENG.iC T cells that stopped expanding after 2-4 stimulations (Figure 3C,D). Sequential immunophenotypic analysis in a subset of donors revealed that the expansion in CD123.ENG.iM and iMC groups was driven by an expansion of CD8-positive T cells (Supplementary Figure 5A,B). CD19.ENG.iMC T cells had no cytolytic activity and did not expand in the presence of CID, confirming antigen specificity (Figure 3A,C).

To determine the cytokine expression profile in the setting of serial stimulation, we measured a Th1/Th2 cytokine panel including Th1: IFNγ, TNFα, IL-2, GM-
CSF; Th2: IL-4, IL-5, IL-6, IL-10, IL-13; 24 hours after each stimulation (Figure 4A). CD123.ENG.iMC T cells secreted significantly higher amounts of IFN-γ than CD123. ENG, CD123.ENG.iM and CD123.ENG.iC T cells. In addition, iMC cells secreted significantly higher levels of TNF-α and GM-CSF compared to CD123. ENG and CD123.ENG.iM T cells after the first stimulation. CD123.ENG.iMC T cells consistently maintained this increased secretion through stimulation 5. In addition, CD123.ENG.iMC consistently secreted higher levels of IL13 with every stimulation.

**CD123.ENG.iMC and CD123.ENG.iM T cells become predominantly CD8+ T_{CM} and transiently overexpress TIM3 after multiple stimulations**

We determined the CD4:CD8 ratio as well as T_{N}, T_{CM}, T_{EM}, and T_{EMRA} distribution after stimulation. All populations were predominantly CD8+, with CD123.ENG.iMC and CD123.ENG.iM T cells becoming predominantly T_{CM} subsets, and CD123.ENG and CD123.ENG.iC T cells having a higher percentage of T_{EM} and T_{EMRA} (Figure 5A, Supplementary Figure 5). To further characterize cells and determine exhaustion phenotype of T cells, we examined cell surface expression of PD1, TIM3 and LAG3 on CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, and CD123.ENG.iC T cells before each stimulation. While iMC and IM T cells expressed increased TIM3 between the 2nd
and 4th stimulation (Supplementary Figure 6), increased concomitant expression of 2 exhaustion markers was not observed (Figure 5B).

CD123.ENG.iMC T cells consistently improve the antitumor activity of CD123.ENG T cells in vivo

To evaluate the antitumor activity of CD123.ENG T cells expressing iMC, iM, and iC we used our established MOLM-13 NSG xenograft model that does not require prior sublethal irradiation. Mice were injected with 5x10^4 MOLM-13.GFP.ffluC cells iv, and on day 7 received a single iv dose of 1x10^7 CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, CD123.ENG.iC, or CD19-ENG.iMC T cells (n=10 mice per group; 2 T cell donors except n=5 mice for CD19-ENG.iMC T cells; 1 T cell donor). CID was given i.p. every 3 to 4 days starting on the day of the T-cell injection (Figure 6A). Tumor burden was tracked by serial bioluminescence imaging. CD123.ENG.iMC T cells had significant antitumor activity in both donors, whereas CD123.ENG.iM T cells only controlled the tumor in 1 out of 2 donors (Figures 6-7). This resulted in a significant survival advantage in comparison to mice that had received CD123.ENG T cells for both donors for CD123.ENG.iMC T cells and for one donor for CD123.iM T cells (CD123.ENG vs CD123.ENG.iMC T cells: p=0.0031, donor 1; p=0.0019, donor 2; CD123.ENG vs CD123.ENG.iM T cells; p=0.51, donor 1; p=0.0019, donor 2) (Figure 6B). In contrast, mice treated with CD123.ENG.iC T cells had no survival advantage in comparison to CD123.ENG T cells treated mice.
CD19.ENG.iMC T cells had no antitumor activity, demonstrating that the benefit of CID in vivo is strictly antigen dependent, as observed in vitro. The weight of mice without tumors remained overall stable for long-term survivors (Supplementary Figure 7C). However, 2/10 mice of the CD123.ENG.iM T cell group had to be euthanized due to non-tumor related morbidities on day 46 (paraphimosis) and 68 (weight loss, presumptive graft vs host disease (GvHD)), and 2/10 mice of the CD123.ENG.iMC T cell group on day 68 and 97 (weight loss, presumptive GvHD). We confirmed these findings in a THP-1 model, where the groups receiving either CD123.ENG.iM or CD123.ENG.iMC and CID had disease control, while control groups had progression (CD123.ENG vs CD123.ENG.iMC or CD123.ENG.iM p<0.0001) (Supplementary Figure 8).

To evaluate if the effect of iMC and iM activation in CD123.ENG T cells on in vivo T cell expansion and persistence, we performed the same experiment as described above with T cells genetically modified to express GFP.ffluc instead of the tumor cells. Within the first 10 days post infusion, we observed a significant greater expansion and persistence of CD123.ENG.iMC T cells as judged by area under the curve analysis (AUC; p<0.001) in comparison to CD123.ENG and CD123.ENG.iM T cells (Figure 8 A, B, Supplementary Figure 9).

CD123.ENG.iMC T cells do not induce increased killing of normal CD123-positive hematopoietic progenitors and recognize primary AML blasts.
To determine whether expressing iMC, iM or iC could increase potential on-target/off-tumor toxicity of CD123.ENG T cells against bone marrow CD123+/CD34+ hematopoietic cells (HPCs; **Supplementary Figure 10**) we performed a standard colony forming unit assay (CFU) at E:T ratios of 1:1 and 5:1 in the presence of CID. At both of E:T ratios, CD123.ENG.iMC cells did not induce higher myelotoxicity than NT T cells in CFU-GEMM, CFU-E, CFU GM (**Figure 8C**, n=3 technical replicates, p=ns), except for BFUs at 1:1 ratio (p=0.0038). CD123.ENG.iC T cells had some degree of myelotoxicity at both ratios and in all groups.

Lastly, we set out to determine if we could generate genetically modified T cells from 3 diagnostic bone marrow samples of pediatric patients with AML and to evaluate if they recognized CD123-positive autologous AML blasts. We thawed primary bone marrow samples and evaluated CD123 surface expression on AML blasts via flow cytometry (**Figure 8D**). We isolated CD3-positive T cells using magnetic bead separation. The CD3-negative, AML blast containing cell fraction was frozen. We then generated CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, CD123.ENG.iC, or CD19-ENG.iMC T cells, and cocultured these effector T cells with the primary, thawed AML blasts in the presence of 0.5 nM CID. After 24 hours, media was collected and limited cytokine evaluation by ELISA was performed by determining IFN-γ secretion. Only CD123.ENG and CD123.ENG.iMC T cells consistently produced IFN-γ compared to NT and CD19-ENG.iMC T cells (**Figure 8E**, n=3, p<0.01 for CD123.ENG.iMC).
DISCUSSION

Here we show that inducible activation of MyD88 and CD40 signaling in CD123.ENG T cells enhances their persistence and sequential killing capabilities without impacting antigen specificity. In the presence of CID, CD123.ENG.iMC had superior and sustained effector function in vitro and in vivo compared to CD123.ENG, CD123.ENG.iM and CD123.ENG.iC T cells.

Adoptive immunotherapy strategies to target AML are actively being explored, focusing on antigens such as CD123, CD33, CLL-1, LeY, among others (29). CD123 is an attractive target due to its high expression on LSCs and relatively low expression on normal hematopoietic cells. CD123-ENG T cells have been described as one of the approaches to target AML, but its application has been limited by modest T cell persistence, likely due to the absence of costimulation (10). Optimal T cell activation requires signal 1 delivered by the T cell receptor (TCR) recognition of antigen-specific peptide through major histocompatibility complex, signal 2 provided by costimulatory or inhibitory molecules expressed on the surface of antigen presenting cells (APC) followed by cytokine signal (signal 3) critical for T cell expansion and response (30-33). Augmenting T cell effector
function by transgenic expression of costimulatory molecules has been explored by several groups (18, 22, 25). We have previously shown that constitutively expressing CD80 and 41BBL on the surface of CD19.ENG T cells enhances their effector function, resulting in increased Th1 cytokine secretion, T cell expansion and superior antitumor activity (15). In addition, several groups have explored the use of inducible costimulatory systems, such as the MyD88.CD40 receptor controlled by CID (18, 21, 25). MyD88 is an adaptor for pathways downstream of TLR and IL-1, leading to functional outputs such as the activation of NFκB and MAP (34). CD40 is a costimulatory molecule member of the tumor necrosis factor receptor (TNFR) family that acts through TRAF mediated pathways that can have overlap with MyD88 (35, 36). Together, they have shown to be effective in increasing CAR T cell activity and function. Mata et al and Foster et al showed that, in the presence of CID, two different types of first-generation CAR T cells (HER2ζ or PSCAζ) that also expressed an inducible MyD88 and CD40 receptor, had enhanced antitumor activity in vitro and in vivo (18, 21). Collison-Pautz et al and Prinzing et al further demonstrated that constitutive provision of MyD88/CD40 costimulation in CAR T cells resulted in increased expansion and antitumor activity of CAR T cells (35, 36). Our work explores if provision of inducible MyD88 and/or CD40 costimulation can improve the effector function of CD123.ENG T cells and determines which costimulatory domain combination is the most effective in maintaining T cell persistence and sustained antitumor activity, without additional myelotoxicity as evidenced by CFU assays.
We demonstrate that expressing inducible MyD88 and/or CD40 switches do not change the antigen specificity or the immunophenotype of CD123.ENG T cells. All CD123.ENG T cells acquired a predominantly CD8+ effector memory phenotype, both in healthy donor T cells cocultured with tumor cell lines and using an autologous system of primary T cells and blasts.

To further characterize these cells, we determined LAG3, TIM3 and PD1 expression, 5 days after stimulation with fresh tumor cells. Inhibitory receptors like LAG3, PD1 and TIM3 have been shown to dampen T cell response and have been associated with reduced Th1 cytokine secretion (37). These markers remained largely at baseline for CD123.ENG.iMC until the 5th stimulation, when there was a slight increase in TIM3+LAG3+ as well as PD1+LAG3+ expression on CD8+ T cells that did not reach statistical significance. Consistent with these findings, CD123.iMC T cells secreted increased Th1 cytokines, specifically IFN-γ and TNF-α and GM-CSF. This increased cytokine secretion persisted throughout repeated stimulations and led to increased persistence.

Expression of MyD88 in combination with CD40, was critical for consistent expansion and antitumor activity by CD123.ENG T cells through multiple stimulations in vitro and in two xenograft AML models. This was only evident in the presence of CID, highlighting the importance of TLR pathway activation to achieve an effective T cell response (18, 21). Response to CD123.ENG.iM in the presence of CID, on the other hand, proved to be both donor and tumor model dependent in vivo.
In summary, we demonstrate that CD123.ENG T cells expressing inducible costimulation maintain antigen specificity and that expression of MyD88 or MyD88 and CD40 confer improved expansion capability and potent antitumor activity in vitro and in vivo. In addition, the ability to remotely control T cell engager secretion amount and T cell persistence while maintaining antigen specificity using this inducible system endows CD123.ENG.iMC cells with a desirable safety feature.
REFERENCES


FIGURE LEGENDS

Figure 1. Generation of CD123.ENG T cells expressing iMC, iM, iC. (A) Schema of vectors. Data throughout the manuscript are represented by the color code of the circles to the left of each construct. (B) Transduction efficiency was determined by CD20 expression (mean% ±SD%: 57.4% ±4.1%, n=12, p=ns). (C) Expression of iMC, iM, and iC was determined by western blot for the HA-tag. (D) CD123.ENG protein production by ENG T cells plated in the presence of CD123 protein +/− 0.5 nM CID was determined by ELISA (n=3, * p<0.05, ** p<0.01, T-test). (E-F) T cells were stained 7 days post transduction for CD4, CD8, CD45RO and CCR7 to determine their immunophenotype (n=5). (E) CD4+ and CD8+ T cell populations. (F) T-cell subsets: naïve-like, CCR7+CD45RO−; central memory (CM), CCR7+CD45RO−; terminally differentiated (TemRA), CCR7−CD45RO−; and effector memory (EM), CCR7−CD45RO+).

Figure 2. Functional characterization of CD123.ENG.iM, CD123.ENG.iC and CD123.ENG.iMC T cells. (A,B) Effector cells were cocultured with media, K562 (CD123 -), MOLM-13 (CD123+) or Kg1a (CD123+) cells at a 1:1 E:T ratio in the presence or absence of 0.5nM CID for 24 hours. Supernatants were collected and evaluated for (A) IFNγ and (B) IL-2 by ELISA. All T cells expressing CD123.ENG had a statistically significant increase in IFNγ and IL-2 secretion in comparison to CD19.ENG.iMC, when exposed to CD123-positive target cells (n=3, *p<0.05,**p<0.01 paired t-test). (C) To determine antitumor activity, effector cells were cocultured with target cells expressing firefly luciferase (MOLM-13.
ffluc and Kg1a.ffluc) at an E:T ratio of 1:1. Tumor cell lysis was determined using a luciferase assay (n=3 p=ns for all CD123 ENG expressing constructs). (D) Heatmap depicting fold expression increase in a panel of 14 genes by qPCR in the presence or absence of CID. Gene expression was normalized to 18S RNA, and GAPDH. E) Fold-expression increase for PD1 and CCL4.

**Figure 3. CD123 ENG.iMC and CD123 ENG.iM T cells retain in the presence of CID their effector function in repeat stimulation assays.** Effector T cell populations were co-cultured with either MOLM-13.ffluc or Kg1a.ffluc cells at an E:T ratio of 1:1 +/- 0.5 nM CID. Antitumor activity was assessed using luciferase assay. Fresh tumor cells were added every 5 days resetting the E:T ratio to 1:1 until T cells no longer killed tumor cells. (A,B) Heat maps showing antitumor activity of effector cells in the presence of (A) MOLM13.ffluc (n=9) and (B) Kg1a.ffluc (n=3) (dark blue=100% killing, white= 0% killing). (C,D) Fold expansion of effector T cells after exposure to (C) MOLM13.ffluc (n=6) or (D) Kg1a.ffluc (n=3).

**Figure 4. CD123 ENG.iMC T cells secrete increased Th1 cytokines upon stimulation.** (A) Heatmap showing cytokine secretion by effector T cells after stimulation as measured by multiplex assay (n=3; 2-way ANOVA).
Figure 5. Immunophenotype of CD123.ENG T cells expressing iMC, iM, iC after repeat stimulation. (A) Figure representation of changes in immunophenotype in CD8+ populations of CD123.ENG.iM and CD123.ENG.iMC T cells post stimulations in the presence of CID (n=3). Pie Graphs were plotted using SPICE software (38) (National Institute of Allergy and Infectious Diseases). (B) Summary of percentage of effector cells that stained double positive for an inhibitory receptor (TIM3+LAG3+, PD1+LAG3+ or TIM3+PD1+) after repeated stimulations in the presence of CID.

Figure 6. CD123.ENG.iMC and CD123.ENG.iM T cells have potent antitumor activity in vivo. MOLM-13.GFP.ffluc bearing mice received on day 7 a single iv dose of 1x10^7 n=10 animals per group, 2 T cell donors for CD123.ENG, CD123.ENG iMC, CD123.ENG iM, or CD123.ENG iC T cells, n=5, 1 T cell donor for CD19.ENG.iMC; 4 doses of CID were given i.p. every 3-4 days. (A) Experimental scheme and quantitative bioluminescence data for Donor 1 and Donor 2. (B) Kaplan-Meier survival curve. (** p<0.01, log rank test).

Figure 7. Representative IVIS images of AML xenograft model. (A) Donor 1: Representative IVIS images. Stars represent animals requiring euthanasia due to non-tumor related morbidities (*paraphimosis, **GvHD). (B) Donor 2:
Representative IVIS images. Stars represent animals requiring euthanasia due to non-tumor related morbidities.

**Figure 8. CD123.ENG.iMC T cells expand and persist in an in vivo AML model, do not have increased myelotoxicity, and recognize primary autologous AML blasts.** (A-B) T cell persistence analysis: MOLM-13 bearing mice (n=5 per group) received on day 7 a single iv dose of $1 \times 10^7$ CD123.ENG, CD123.ENG iMC, or CD123.ENG iM T cells expressing GFP.fFluc; 4 doses of CID were given i.p. every 3-4 days. (A) Quantitative bioluminescence data. (B) Area under the curve (AUC) analysis for Day 0 to 10 post T cell infusion (**p<0.001, 2-way ANOVA). (C) CFU Assays: at indicated effector T cells were incubated with HPCs for 4 h at E:T ratios of 5:1 and 1:1, plated on semisolid media, and BFU. CFU-GEMM, CFU-E and CFU-GM were enumerated after 12–14 days. (*p <0.05, ** p<0.01, 2-way ANOVA). (D-E) Primary autologous T cells recognize primary AML blasts: (D) Flow cytometry analysis for CD123, CD3 of bone marrow samples of pediatric patients with AML. (E) Coculture assay in the presence of 0.5nM CID at a 1:1 E:T ratio of 1:1 with indicated effector T cells and autologous AML blasts. After 24 hours media was obtained for IFNγ ELISA (**p<0.05, **p<0.01, Generalized linear model).
Figure 1
Figure 3
Figure 4
SUPPLEMENTARY METHODS

Cell lines and culture conditions

Cell lines were grown in RPMI 1640 (ThermoFisher Scientific, Waltham, MA). Cells were regularly tested for mycoplasma contamination and for authenticity using the ATCC Cell line Authentication service (STR Analysis, ATCC, Manassas). HEK 293T cells were grown in IMDM (Thermo Scientific, Waltham, MA). All media was supplemented with 10-20% Fetal Bovine Serum (ThermoFisher Scientific, Waltham, MA) and 2 mmol/L Glutamax (ThermoFisher Scientific, Waltham, MA).

Generation of retroviral vectors

We generated a panel of retroviral vectors encoding CD20, CD123.ENG, and MyD88 (iM), CD40 (iC) or MyD88.CD40 (iMC), and CD20, CD19.ENG, and iMC. Briefly, cDNAs encoding a E2A self-cleaving peptide and iM, iC, or iMC were subcloned into an SFG retroviral vector encoding CD20.T2A.CD123.ENG (Figure 1A); iMC was also subcloned into an SFG retroviral vector encoding CD20.T2A.CD19.ENG. The sequence of all cloned constructs was confirmed by sequencing performed by Hartwell Center DNA Sequencing Core at St. Jude Children’s Research Hospital with Big Dye® Terminator (v3.1) Chemistry on Applied Biosystems 3730XL DNA Analyzers (Thermo Fisher Scientific, Waltham). RD114-pseudotyped retroviral particles were generated by transient transfection in HEK 293T cells as previously described (29).
**Flow cytometric analysis**

For flow cytometry analysis, we utilized the following monoclonal antibodies: αCD20-APC (BD Biosciences, San Jose, CA, Clone, 2H7), αCD4-Alexa-Flour 700 (BD Biosciences, SK3), αCD8-APC-H7 (BD Biosciences, SK1), αCCR7- Pacific blue (Biolegend, San Diego, CA, G043H7), αCD45RO-PerCP-Cy5.5 (BD Biosciences, UCHL1), αPD1-BV605 (BD Biosciences, EH12.1), αTIM3-PE-Cy7 (Biolegend, F38-2E2), αCD123-APC (BD Biosciences, 7G3), αLAG3-PE (R&D Systems, FAB2319P), αCD34-PE (BD Biosciences, 581). Half a million transduced or NT T cells were washed with 1X PBS and the cells were incubated with 3µL of antibody and 0.5µL of Fixable Live Dead aqua stain (Thermo Fisher Scientific, L34965) on ice and protected from light for 30 mins. Cells were washed with 1X PBS+2% FBS. Flow cytometry was performed on a FACS Canto II or FACS LYRIC Cytometer (BD Biosciences, San Jose, CA) and the Data was analyzed on FlowJo 10.5.3 software (BD Biosciences, San Jose, CA).

**Western blot**

Three million transduced T cells were washed with 1X PBS and lysed in buffer containing 1X RIPA buffer and protease inhibitors (Thermo Fisher Scientific). Protein concentrations were estimated using BCA Protein Assay (Thermo Fisher Scientific) using BSA standards. Samples were denatured in Laemmli buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol at 95°C for 5 minutes. Cell lysates (3µg/lane) were run on a 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) and transferred to nitrocellulose membrane iBlot mini (Thermo Fisher Scientific) using iBlot® 2 Gel Transfer system (23V, 4 minutes). Membranes were blocked on 1% Casein with 1X Tris buffer Saline (Bio-Rad) and then
probed with an αHA-tag-HRP (Cell signaling Technology, Danvers, MA). An anti-beta-actin (Santa Cruz Biotechnology, Dallas, TX, sc-47778) was used to detect beta-actin as a control. Blots were developed using Clarity ECL substrate (Bio-Rad) and imaged on a Li-COR Odyssey FC imaging System (Li-COR Biosciences, Lincoln, NE).

**Cytotoxicity assay**
Transduced or NT T cells were washed with 1X PBS and co-cultured with MOLM-13.GFP.ffluc or MV-4-11.GFP.ffluc or Kg1a.GFP.ffluc (CD123-positive) or K562.GFP.ffluc (CD123-negative) cells in a 24 well tissue culture dish (Corning) at an E:T ratio of 1:1 and incubated overnight. Cells were mixed thoroughly and 100 µl of the cells were incubated with D-Luciferin. Luminescence was measured on a Tecan Infinite® 200 (Tecan, Mannedorf, Switzerland) and analyzed using Magellan Software (Tecan).

**CD107a degranulation assay**
MOLM13 (CD123-positive) cells were labeled with Cell Trace Violet ™ (Invitrogen, C34557) and incubated with NT T cells and CD123.ENG, CD123.ENG.iC, CD123.ENG.iM and CD123.ENG.iMC T cells at an E:T ratio of 1:2 with GolgiStop (BD Biosciences, 554724), αCD107a (LAMP-1, BD biosciences, H4A3) for five hours in the presence or absence of CID (0.5 nmol/L, B/B Homodimerizer, AP20187, Takara Bio). Cells were then washed and analyzed on FACS Lyric flow cytometer.
TCF-1 staining

Effector T cells were plated on CD123 protein coated plate overnight. Eighteen to 24 hours later, T cells were harvested and stained for TCF-1(C63D9, Cell Signaling Technology.) using eBioscience™ FoxP3 transcriptional buffer set (00-5523-00, ThermoFisher Scientific) according to manufacturer's instructions. Samples were subsequently analyzed using flow cytometry.

ELISA to determine CD123.ENG protein concentration

Non-Transduced and Transduced ENG.T cells were plated on a non-tissue culture treated plate coated with recombinant CD123 protein (1µg/mL, ab158757, Abcam, Cambridge, UK), in the presence or absence of CID (0.5 nM, B/B Homodimerizer, AP20187, Takara Bio). Co-culture supernatants were collected after 24 hours. ELISA to detect CD123.ENG protein was performed as previously described. (10).

Cytokine ELISA

5x10^5 CD123.ENG T cells or CD123.ENG.iC, CD123.ENG.iM or CD123.ENG.iMC T cells were plated at an E:T ratio of 1:1 with CD123-positive (MOLM-13) or CD123-negative (K562) target cells in the absence or presence of CID (0.5 nmol/L, B/B Homodimerizer, AP20187). Co-culture supernatants were collected after 24 hours and used for cytokine analysis. IFNγ and IL2 concentrations were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.
Repeat stimulation and cytokine multiplex assay

$5 \times 10^5$ NT, CD19-ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells were plated at an E:T ratio of 1:1 with CD123-positive (MOLM-13.GFP.ffluc) or CD123-negative (K562.GFP.ffluc) target cells in the absence or presence of CID (0.5 nmol/L, B/B Homodimerizer, AP20187). After 3 days, T cell antitumor activity was determined using a luciferase-based assay. Fresh tumor cells were added to adjust the E:T ratio to 1:1, and the stimulation was repeated until T cells no longer killed tumor cells. Culture supernatants were harvested from a coculture done in parallel, and cytokine production was measured by a custom human Cytokine/Chemokine Multiplex assay containing analytes for GM-CSF, IFN$\gamma$, IL-10, IL-13, IL-15, IL17A, IL-2, IL-4, IL-5, IL-6, IL-8, CXCL10, CCL2, CCL3, CCL, CCL5 and TNF$\alpha$ (EMD Millipore, Chicago, IL) as per manufacturer’s instructions.

Quantitative PCR

Non-Transduced and Transduced ENG.T cells were plated on a non-tissue culture treated plate coated with recombinant CD123 protein (1 µg/mL, ab158757, Abcam, Cambridge, UK), in the presence or absence of CID (0.5 nM, B/B Homodimerizer, AP20187, Takara Bio) for 12 hrs. T cells were harvested and total RNA was extracted using an automated RNA purification system (Maxwell ® RSC simplyRNA Cells Kit and RSC instrument; Promega, Madison WI). cDNA was synthesized from total RNA using SuperScript IV VILO KIT (ThermoFisher Scientific). For gene expression analysis, a panel of 14 genes with 18S RNA and GAPDH as housekeeping genes were evaluated by a TaqMan Gene expression array (ThermoFisher Scientific) using TaqMan Universal PCR
Master Mix (ThermoFisher Scientific) and Quantstudio 6 Real-Time PCR system (ThermoFisher Scientific). Gene expression was normalized to 18S RNA, and GAPDH and relative quantification were calculated with DDCT method. Primers used are listed in Supplementary Figure 1.

Colony forming unit (CFU) assay
NT, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells were co-cultured with CD34-positive bone marrow cells (Lonza, Basel, Switzerland) at an E:T ratio of 1:5 and 1:1 for 4 hours and were then plated in the presence of MethoCult (H4434, Stem Cell Technologies, Vancouver, Canada) and CID in a 6-well SmartDish® (Stemcell Technologies) and incubated for 12-14 days at 37°C. Plates were imaged using a Nikon C2 point-scanning confocal Microscope (Nikon, Tokyo, Japan) using a 4X objective. BFU-E (Burst Forming Unit – erythrocyte) and CFU-E (Colony forming unit – erythrocyte), CFU-GEMM (granulocyte, erythrocyte, monocyte and megakaryocyte) and CFU-GM (granulocyte and monocyte) were enumerated using machine learning pixel classifier with Random Forest algorithm on AIVIA 8.0 Image Analysis software (DRvision, Bellevue WA).

Primary cell coculture
Pediatric AML samples were obtained under St. Jude Children’s Research Hospital IRB approved protocol, after informed consent was obtained in accordance to the Declaration of Helsinki. Three bone marrow samples of patients with active AML (M1, M5a, M4E0) were thawed and evaluated for CD123 expression using flow cytometry. AML cells were
separated from T cells by CD3 selection using MACS beads (Miltenyi Biotec, CD3 Microbeads, 130-050-101). The CD3-negative fraction containing AML cells was immediately refrozen, and CD3-positive T cells were expanded in RPMI media supplemented with 10% FBS and human IL-2 (100IU/mL Peprotech, 200-02) for 3 days. Expanded T cells were then activated with plate-bound CD3 (1µg/mL, Miltenyi Biotec, Germany) and CD28 (1µg/mL, Miltenyi Biotec, Germany) antibodies on non-tissue culture treated 24-well plates. CD19-ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells were generated by retroviral transduction as described above. Day 10 post transduction, ENG T cells were cocultured with freshly thawed AML blasts and after 24 hours media was collected to determine IFNγ production by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Xenograft AML models**

To determine the effective dosage and efficacy of the inducible switch, 8 to 10-week old male or female NSG mice (NOD.Cg-Prkdcscid/Il2rgtm1Wjl/SzJ; St. Jude Children’s Research Hospital, Memphis) were injected intravenously (iv) with 5x10⁴ MOLM-13.GFP.ffluc or 3x10⁶ THP-1.ffluc via tail vein injection. On Day 7 mice received a single iv dose of 1x10⁷ CD19-ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells. Each mouse received 100µg of CID intraperitoneally (ip) on the day of T cell injection and then every 3-4 days for a total of 4 doses. Untreated animals served as control. AML progression was tracked by bioluminescence imaging using an IVIS-200 system (IVIS® Systems, Xenogen Corp, Alameda, CA.). For the persistence study, MOLM-13 cells were injected iv, and on day 7 animals received a single iv dose of
$1 \times 10^7$ CD123.ENG, CD123.ENG.iMC, or CD123.ENG.iM T cells modified to express GFP.fflux and received CID ip every 3-4 days for a total of 4 doses. Euthanasia was performed as prior determined time points or when animals met euthanasia criteria in accordance with St. Jude Children’s Research Hospital’s IACUC.
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Supplementary Figure 1. Primers utilized in qPCR analysis. Table including primers utilized in qPCR analysis in Figure 2D.
Supplementary Figure 2: Characterization of CD123.ENG.icostim T cells and CD123+ AML cell lines. (A) Representative FACS histogram showing Non-transduced (NT), CD19.ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM and CD123.ENG.iC T cells stained for CD20. (B) Expansion of T cells transduced with CD123.ENG and CD123.ENG.icostim constructs compared to NT and CD19.ENG.iMC controls. (n=3 p=ns). (C) Representative FACS histogram of CD123 expression (αCD123-PE, BD Biosciences, 7G3) on AML cell line MOLM-13, Kg1a, THP-1 and MV-4-11.
Supplementary Figure 3: CD123.ENG.icostim T cells recognize MV4-11 tumor cells (CD123+). (A) Effector cells were cocultured in the presence of media, K562 or MV-411 cells at a 1:1 E:T ratio (+/- 0.5nM CID) for 24 hours. (n=3, comparison between CD123 ENG expressing constructs: p=ns). (B) Luciferase-based cytotoxicity assay in which effector cells were co-cultured in the presence of luciferase tagged MV4-11 or K562 for 24 hours. (n=3, p=ns).
Supplementary Figure 4. Functional characterization of CD123.ENG.IMC T cells. (A) To elucidate functional differences between CD123.ENG T cell constructs, effector cells were co-cultured with MOLM-13 cells at an E:T ratio of 1:2 for 5 hours in the presence or absence of CID and determined degranulation by CD107a expression (n=3, difference between p=ns) (B, C) TCF-7 expression on effector cells. (B) summary plot (n=3 p=ns), (C) representative dot plots. (D) Experimental schema of serial stimulation assay.
Supplementary Figure 5. Changes in CD4:CD8 Ratio in CD123.ENG.iM and CD123.ENG.iMC after multiple stimulations with CD123+ AML cells (A) Graphic representation of changes in CD4:CD8 ratios after 5 stimulations. Pie charts generated in SPICE software. (n=3) (B) Representative dot plots showing CD4 and CD8 staining.
Supplementary Figure 6: Expression of TIM3, PD1 and LAG3 on CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM and CD123.ENG.iC T cells. (A) Expression of TIM3, PD1 and LAG3 at baseline and subsequent stimulations (B) Representative histograms for TIM3, PD1 and LAG3 at baseline on CD8 T cells (C) Representative histograms for TIM3, PD1 and LAG3 at Stim 1 on CD8 T cells
Supplementary Figure 7: Weights in grams of animals corresponding to experiments outlined in Main Figure 7.
Supplementary Figure 8: CD123.ENG.iMC and CD123.ENG.iM T cells have potent antitumor activity in vivo in a THP-1 AML model. THP-1.GFP.fluc bearing mice received a single iv dose of 1x10^7 effector T cells on day 7 (n=5 animals per group), a total of 4 doses of CID were given i.p. every 3-4 days. (A) Experimental scheme and representative IVIS images. Stars represent animals requiring euthanasia due to non-tumor related morbidities (**= non tumor related mortality/GVHD). (B) Bioluminescence curves. (C) AUC for day 0 to day 53 comparing all groups to the tumor only control (** p<0.01, **** p<0.0001.)
Supplementary Figure 9: *In vivo* expansion of CD123.ENG, CD123.ENG.iMC, and CD123.ENG.IM T cells. This is a Supplementary Figure for the *in vivo* experiment shown in Figure 8A and 8B. Representative IVIS images.
Supplementary Figure 10. Flow cytometry analysis of CD34+ cells. Representative plot for flow Cytometry analysis of hematopoietic cells (HPCs) stained for CD34 and CD123 utilized in CFU assays in Figure 7C.