Improving the anti-acute myeloid leukemia activity of CD123-specific engager T cells by MyD88 and CD40 co-stimulation

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Abstract

The outcome of patients with acute myeloid leukemia remains poor, and immunotherapy has the potential to improve this. T cells expressing chimeric antigen receptors 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-acute myeloid leukemia activity. However, like chimeric antigen receptor 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 co-stimulatory molecules consisting of MyD88 and CD40 (iMC), MyD88 (iM), or CD40 (iC), which are activated by a chemical inducer of dimerization. CD123.ENG T cells expressing iMC, iM, or iC maintained their antigen specificity in the presence of a chemical inducer of dimerization, as judged by cytokine production (interferon-γ, interleukin-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 antitumor activity in an acute myeloid leukemia xenograft model. This translated into a significant survival advantage in comparison to that of 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 pathway activation via MyD88 and provision of co-stimulation 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 patients with 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 is a promising immunotherapy target for AML because of its high expression on leukemia stem cells and lower expression on normal hematopoietic cells.[7-9] Several T-cell-based immunotherapy approaches are currently being developed to target CD123, including T cells expressing chimeric antigen receptors (CAR) or strategies involving bispecific anti-

bodies (bispecific T-cell engagers, BiTE®; dual affinity retargeting antibodies, DART; bispecific engagers, ENG).[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 hematologic malignancies.[10,12-14] CD123-specific ENG T cells (CD123.ENG) secrete a bispecific antibody consisting of two single chain variable fragments, 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 co-stimulatory 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 explored whether an inducible co-stimulation system can be utilized to enhance the effector function of CD123.ENG T cells and determined 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 demonstrated 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 the Leibniz Institute (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). MV-411, Kg1a, THP-1, K562 and HEK 293T were purchased from the American Type Culture Collection (Manassas, VA, USA). MOLM-13, MV-411, Kg1a, THP-1 and K562 cells expressing an enhanced green fluorescence protein 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 co-stimulatory molecules encoding a myristoylation sequence, two FKBP dimerizer domains, and iM, iC or iMC with an HA-tag have been previously reported.[10,13,18,25] Additional details are described in the *Online Supplementary Appendix*.

Generation of bispecific engager T cells

All procedures involving human subjects were carried out in accordance with the Declaration of Helsinki. Human peripheral blood mononuclear cells from healthy donors were obtained, after acquiring informed consent, under a St. Jude Children's Research Hospital protocol approved by the hospital's institutional review board. Peripheral blood mononuclear cells were stimulated on CD3 (1 μg/mL, Miltenyi Biotec, Bergisch Gladbach, Germany) and αCD28 (1 μg/mL, Miltenyi Biotec) antibody-coated non-tis-

sue culture treated 24-well plates (144530, Thermo Fisher Scientific, Waltham, WA, USA). Human interleukin (IL)-7 and IL-15 (10 ng/mL and 5 ng/mL, respectively) (Biological Research Branch, National Cancer Institute, Frederick, MD, USA) were added to cultures on day 2. On day 3, T cells were transduced with retroviral particles on plates coated with retronectin (T100A, Takara Clontech, Mountain View, CA, USA) 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 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 acute myeloid leukemia 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 provided in the *Online Supplementary Appendix*.

Statistical analysis

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

A generalized estimating equation was used to determine 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 because of 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 inducible co-stimulatory molecules

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 after transduction, transduction efficiency was evaluated by determining CD20 expression via flow cyto-

metry analysis. Mean transduction efficiency was 57.4% (range, 52-63.4%) with no significant differences between constructs (n=12; Figure 1B, *Online Supplementary Figure S2A*). Expression of iM, iC, and iMC was confirmed by western blot for the HA-tag (Figure 1C). Following transduction, all T-cell populations expanded and there were no statistically significant differences (n=6; *Online Supplementary Figure S2B*). To confirm that CD123.ENG T cells secrete CD123.ENG protein, effector T cells were plated on a plate coated with recombinant CD123 protein with or without 0.5 nM chemical inducer of dimerization (CID). The concentration of secreted CD123.ENG protein was determined by enzyme-linked immunosorbent assay (ELISA) after 24 h. Unlike CD19.ENG.iMC T cells, effector T cells

encoding CD123.ENG produced significant amounts of CD123.ENG protein ($P<0.01$) (Figure 1D). In addition, exposure to CID significantly increased CD123.ENG protein secretion of CD123.ENG T cells endowed with an inducible co-stimulation 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$^+$ T cells than non-transduced and CD19.ENG.iMC T cells, this difference 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 (EMRA) T cells. All CD123.ENG-expressing CD8$^+$ T cells ex-
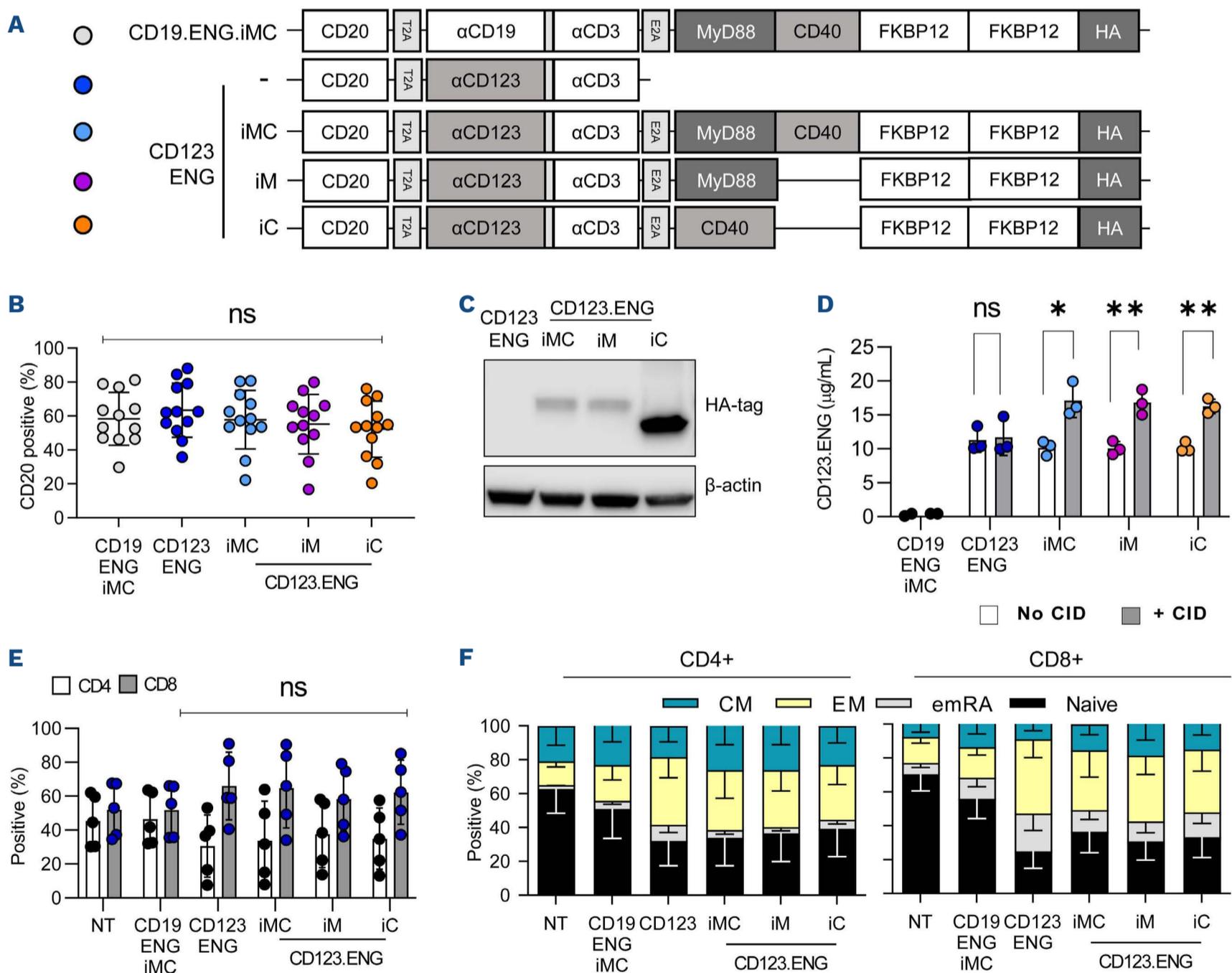

Figure 1. Generation of CD123.ENG T cells expressing iMC, iM, and iC. (A) Schema of vectors. Data throughout the manuscript are represented by the color code of the circles to the left of each construct. (B) Transduction efficiency was determined by CD20 expression (mean% ± standard deviation%: 57.4%±4.1%, 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 engager T cells plated in the presence of CD123 protein with or without 0.5 nM chemical inducer of dimerization was determined by enzyme-linked immunosorbent assay (n=3, *P<0.05, **P<0.01, T-test). (E, F) T cells were stained 7 days after 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 (emRA), CCR7$^-$CD45RO$^-$; and effector memory (EM), CCR7$^-$CD45RO$^+$). CID: chemical inducer of dimerization; iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40; ENG: engager; NT: not transduced.

cept CD123.ENG.iMC CD8+ T cells had a significantly (*P*<0.05) lower percentage of T_N cells in comparison to non-transduced T cells. There were no statistically significant differences in the different CD4$^+$ T-cell populations. (Figure 1F).

CD123.ENG T cells expressing inducible co-stimulatory molecules maintain antigen specificity

To evaluate whether constructs expressing inducible co-stimulation (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 0.5 nM CID with CD123$^+$ (MOLM-13.ffLUC, MV-4-11.ffLUC, Kg1a.ffLUC) AML cells (*Online Supplementary Figure S2C*). Non-transduced and CD19.ENG.iMC T cells served as control T effector cells, while media and CD123$^-$ K562.ffLUC served as controls 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 interferon (IFN)-γ and IL-2 in the presence of MOLM-13,, MV-4-11, or Kg1a in comparison to non-transduced and CD19.ENG.iMC T cells, confirming antigen specificity (Figure 2A, B, *Online Supplementary Figure S3A*). CD123.ENG.iM, CD123.ENG.iC and CD123.ENG.iMC T cells secreted higher amounts of IFN-γ and IL-2 in the presence of CID; however, the difference did not reach statistical significance (Figure 2A, B, *Online Supplementary Figure S3A*). 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$^+$ (MOLM13.ffLUC, MV-4-11.ffLUC and Kg1a.ffLUC) or CD123$^-$ targets (K562.ffLUC) at a 1:1 effector to target (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$^-$). The addition of CID did not confer additional antitumor activity in these conditions. Effector T-cell controls (CD19.ENG.iMC and non-transduced T cells) showed no cytotoxicity towards CD123$^+$ or CD123$^-$ targets (Figure 2C, *Online Supplementary Figure S3B*).

To further differentiate functional differences between CD123.ENG T-cell constructs, we performed a CD107a degranulation assay and analyzed TCF1 expression after exposing effector cells to CD123.ENG protein with or without CID. Our analysis showed increased degranulation in the groups secreting CD123.ENG in the presence of CD123$^+$ targets in comparison to non-transduced cells, without any statistically significant differences between CD123.ENG constructs (*Online Supplementary Figure S4A*). In addition, TCF-1 expression was consistent across constructs and was not influenced by the addition of CID (*Online Supplementary Figure S4B,C*)

Finally, we evaluated fold expression of genes associated with T-cell function, activation and cell migration using quantitative polymerase chain reaction analysis. In the presence of CID and after overnight incubation, CD123.ENG.iMC T cells maintained a similar gene expression profile to that of CD123.ENG T cells (n=4, *P*=ns). However, CD123.ENG.iM and CD123.ENG.iC T cells showed 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 2D, 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 (*Online Supplementary Figure S4D*). 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 with or without 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 with or without CID were added if tumor cells had been killed (n=9 and n=3, respectively). T cells were also enumerated. A subset of donor cells that were co-cultured with MOLM13.GFP.ffluc underwent flow cytometric immunophenotype analysis (n=3) and media were collected for cytokine analysis 24 h after co-culture (n=3). In the absence of CID, all CD123.ENG T-cell populations were able to kill target cells for two to five stimulations, depending on the target cells used (Figure 3A, B left panels). T-cell expansion followed a similar pattern and cells failed to expand after two to five 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 and CD123.ENG.iMC T cells were able to kill target cells for five 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 and CD123.ENG.iMC T cells continued to expand for six to ten stimulations whereas CD123.ENG and CD123.ENG.iC T cells stopped expanding after two to four 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$^+$ T cells (*Online Supplementary Figure S5A, 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).

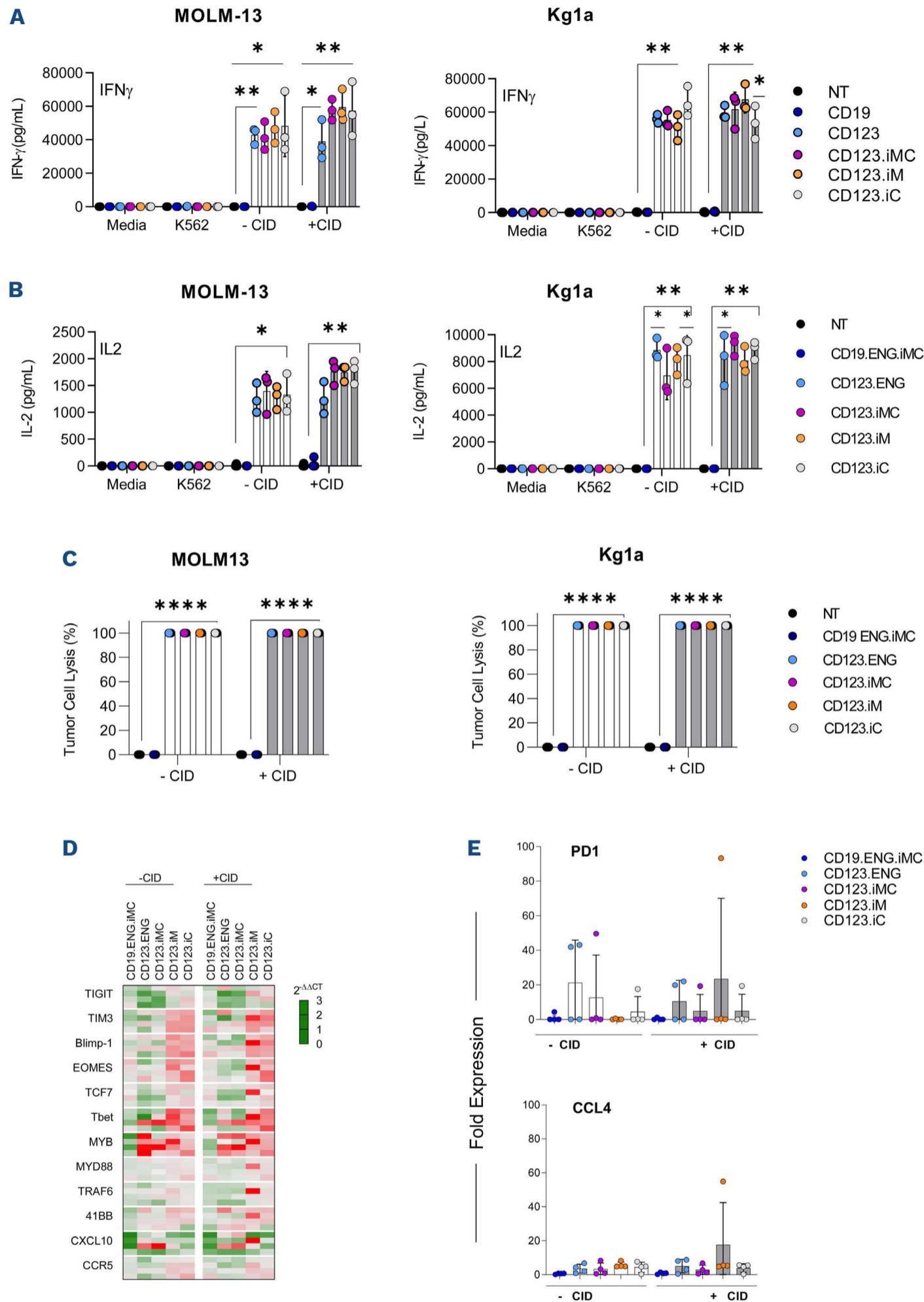To determine the cytokine expression profile in the setting of serial stimulation, we measured a Th1/Th2 panel of

Figure 2. Functional characterization of CD123.ENG.iM, CD123.ENG.iC and CD123.ENG.iMC T cells. (A, B) Effector cells were co-cultured with media, K562 (CD123⁻), MOLM-13 (CD123⁺) or Kg1a (CD123⁺) cells at a 1:1 effector:target (E:T) cell ratio in the presence or absence of 0.5 nM chemical inducer of dimerization (CID) for 24 h. Supernatants were collected and evaluated for (A) interferon-γ (IFN-γ) and (B) interleukin-2 (IL-2) by enzyme-linked immunosorbent assay (ELISA). All T cells expressing CD123.ENG had a statistically significant increase in IFN-γ and IL-2 secretion in comparison to the secretion of T cells expressing CD19.ENG.iMC, when exposed to CD123⁺ target cells (n=3, *P<0.05,**P<0.01 paired *t*-test). (C) To determine antitumor activity, effector cells were co-cultured 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 quantitative polymerase chain reaction in the presence or absence of CID. Gene expression was normalized to 18S RNA, and GAPDH. (E) Fold-expression increase for PD1 and CCL4. iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40; ENG: engager; NT: not transduced.

cytokines including IFN-γ, TNFα, IL-2, and GM-CSF (Th1) and IL-4, IL-5, IL-6, IL-10 and IL-13 (Th2) 24 h after each stimulation (Figure 4A). CD123.ENG.iMC T cells secreted significantly higher amounts of IFN-γ than did 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 IL-13 with every stimulation.

CD123.ENG.iMC and CD123.ENG.iM T cells become predominantly CD8^{+} central memory T cells and transiently overexpress TIM3 after multiple stimulations

We determined the CD4:CD8 ratio as well as T_N, T_{CM}, T_{EM},
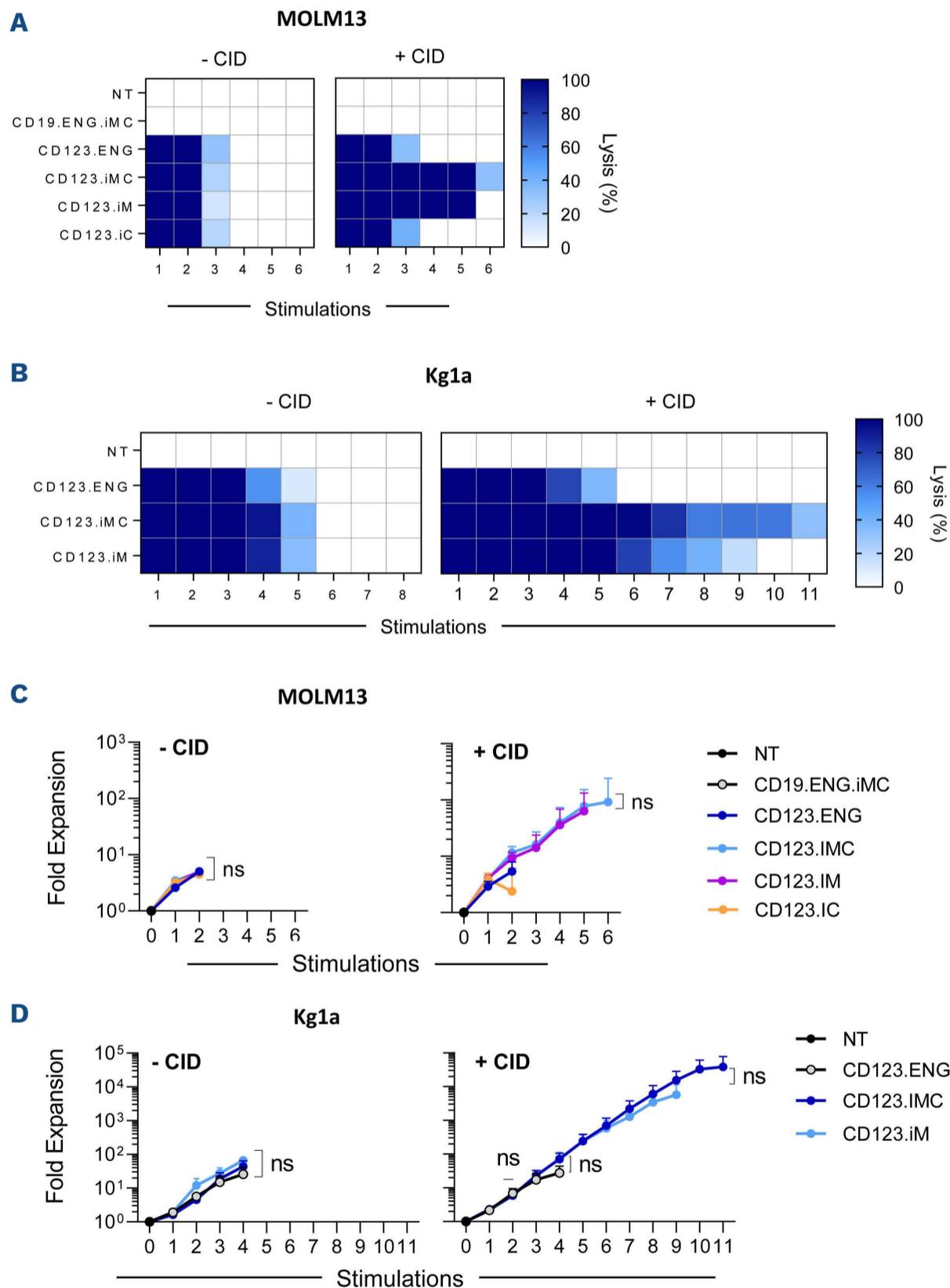
Figure 3. CD123.ENG.iMC and CD123.ENG.iM T cells retain their effector function in the presence of a chemical inducer of dimerization in repeat stimulation assays. Effector T-cell populations were co-cultured with either MOLM-13.ffluc or Kg1a.ffluc cells at an effector:target (E:T) cell ratio of 1:1 with or without 0.5 nM chemical inducer of dimerization. Antitumor activity was assessed using a 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). NT: not transduced; iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40; ENG: engager; ns: not statistically significant.

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, *Online Supplementary Figure S5*). To further characterize cells and determine the 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 second and fourth stimulation (*Online Supplementary Figure S6*), concomitant increased expression of two markers of exhaustion 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 intravenously, and on day 7 received a single intravenous 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 with 2 T-cell donors, except for n=5 mice for CD19-ENG.iMC T cells; 1 T-cell donor). CID was given intraperitoneally 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 one out of two donors (Figures 6 and 7). This resulted in a significant survival advantage over that of 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 that of mice treated with CD123.ENG T cells. CD19.ENG.iMC T cells had no antitumor activity, demonstrating that the benefit
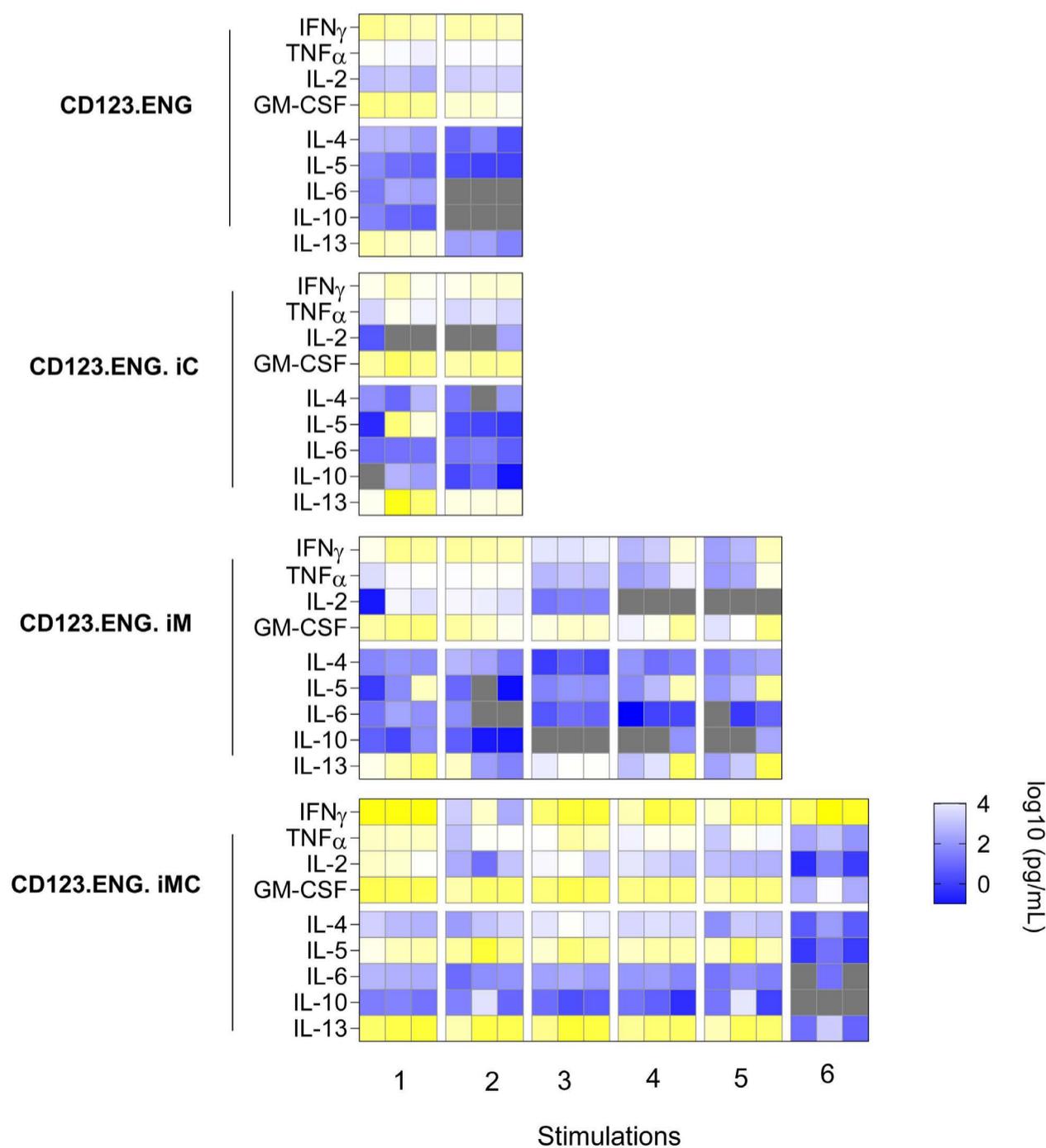

Figure 4. CD123.ENG.iMC T cells secrete increased Th1 cytokines upon stimulation. Heatmap showing cytokine secretion by effector T cells after stimulation as measured by multiplex assay (n=3; 2-way ANOVA). ENG: engager; iC: inducible CD40; iM: inducible MyD88; iMC: inducible MyD88 and CD40; IFN: interferon; TNF: tumor necrosis factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony stimulating factor.
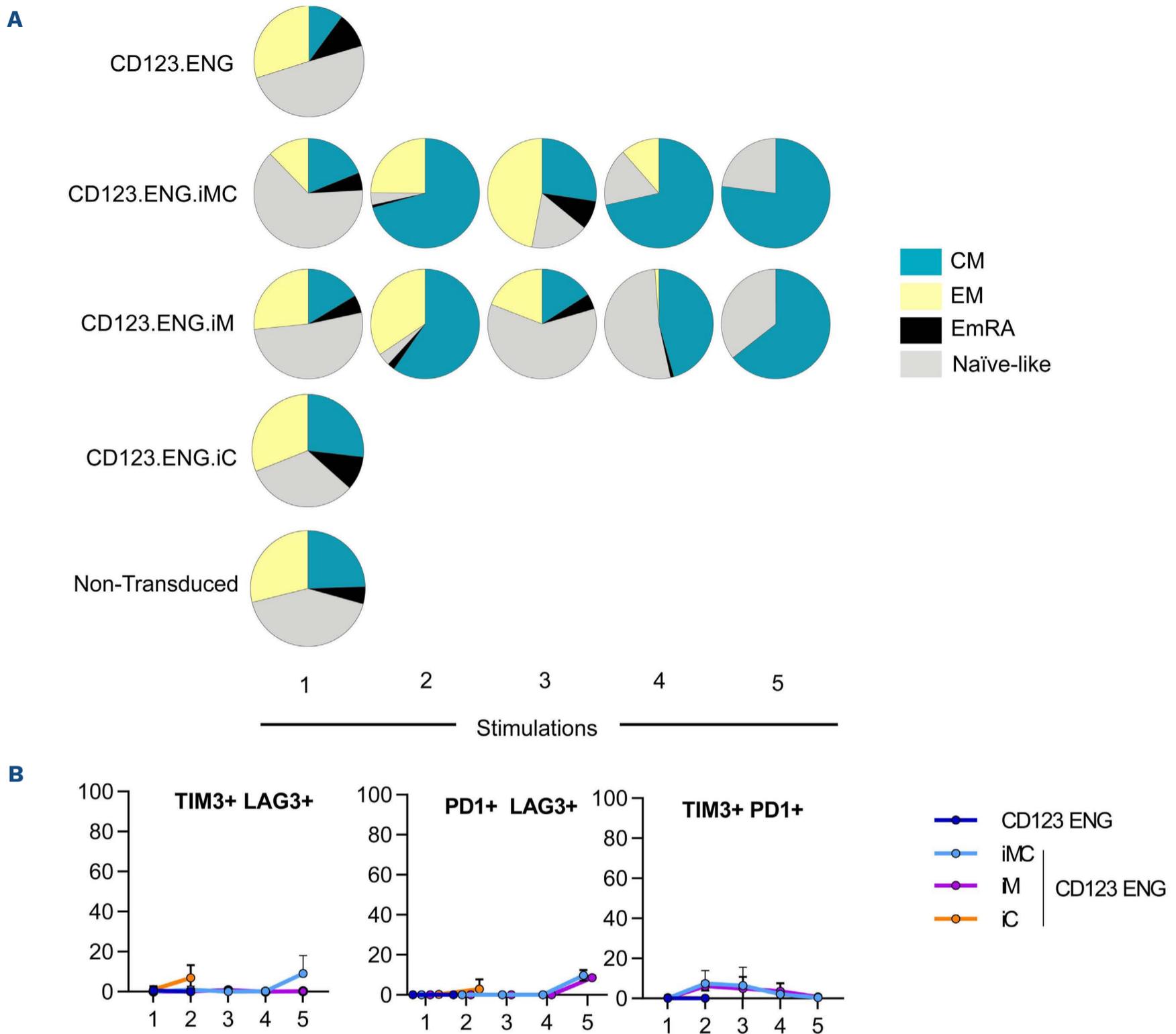
Figure 5. Immunophenotype of CD123.ENG T cells expressing iMC, iM, and iC after repeat stimulation. (A) Pie-chart representation of changes in immunophenotype in CD8+ populations of CD123.ENG.iM and CD123.ENG.iMC T cells after stimulations in the presence of chemical inducer of dimerization (CID) (n=3). The pie-charts 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. iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40; ENG: engager; CM: central memory; EM: effector memory; emRA: terminally differentiated effector memory.

of CID *in vivo* is strictly antigen-dependent, as observed *in vitro*. The weight of mice without tumors remained stable overall for long-term survivors (*Online Supplementary Figure S7C*). However, two of the ten mice in the CD123.ENG.iM T-cell group had to be euthanized due to non-tumor related morbidities on day 46 (paraphimosis) and day 68 (weight loss, presumptive graft-*versus*-host disease), and two of the ten mice in the CD123.ENG.iMC T-cell group on days 68 and 97 (weight loss, presumptive graft-*versus*-host disease). We confirmed these findings in a THP-1 model, in which disease was controlled in the

groups receiving either CD123.ENG.iM or CD123.ENG.iMC and CID, while it progresses in control groups (CD123.ENG *vs.* CD123.ENG.iMC or CD123.ENG.iM; *P*<0.0001) (*Online Supplementary Figure S8*).

To evaluate 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 after infusion, we observed a significantly greater expansion and persistence of CD123.ENG.iMC T cells, as
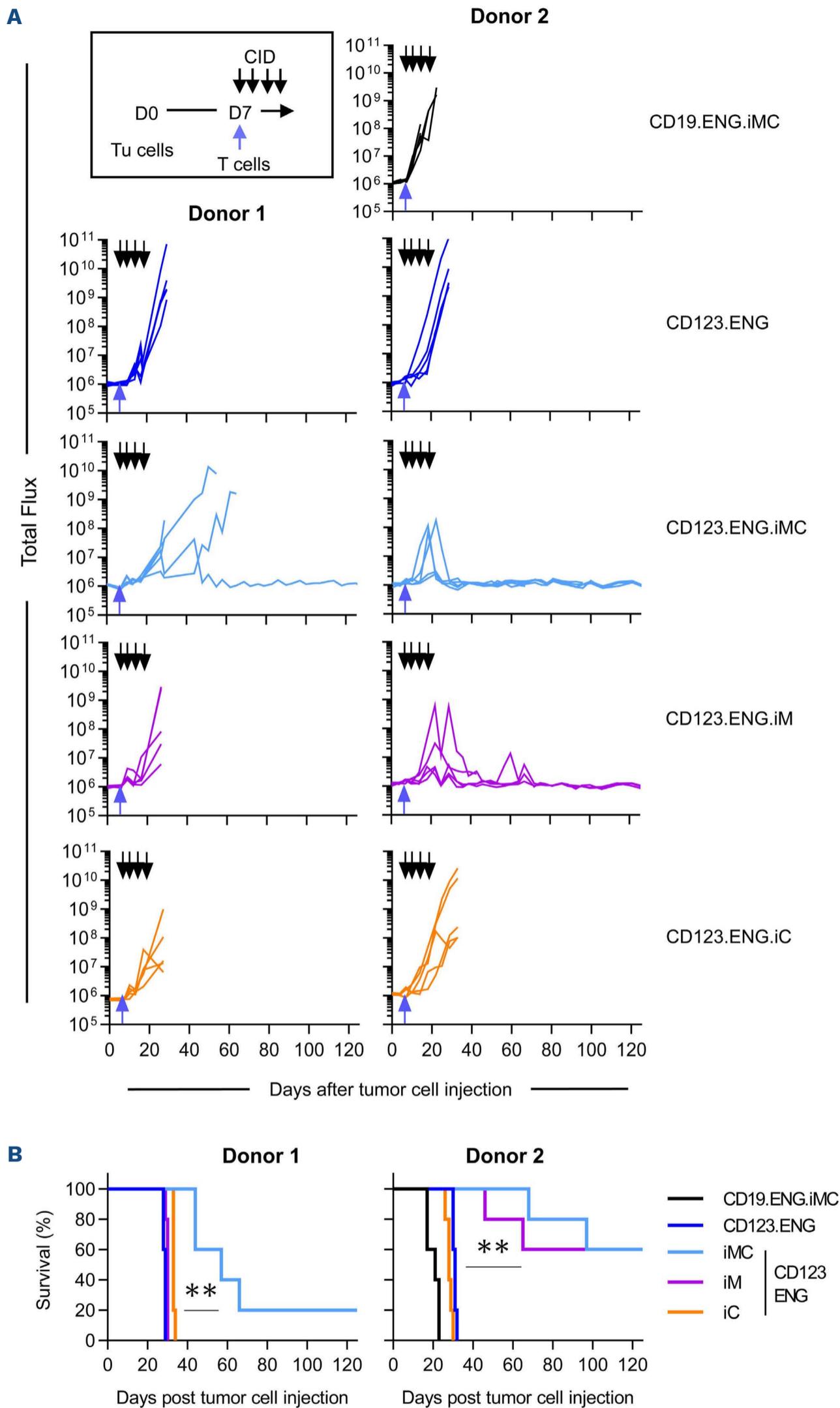
Figure 6. CD123.ENG.iMC and CD123.ENG.iM T cells have potent antitumor activity *in vivo*. MOLM-13.GFP.ffluc-bearing mice received a single intravenous dose of 1×10^7 T cells on day 7 (n=10 animals per group, 2 T-cell donors for CD123.ENG, CD123.ENG iMC, CD123.ENG iM, or CD123.ENG iC T cells; n=5, 1 T-cell donor for CD19.ENG.iMC). Four doses of a chemical inducer of dimerization were given intraperitoneally 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). D0: day 0; D7: day 7; CID: chemical inducer of dimerization; ENG: engager; iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40;

judged by area under the curve analysis (*P*<0.001), in comparison to that of CD123.ENG or CD123.ENG.iM T cells (Figure 8A, B, *Online Supplementary Figure S9*).

CD123.ENG.iMC T cells do not induce increased killing of normal CD123-positive hematopoietic progenitors and recognize primary acute myeloid leukemia 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 (*Online Supplementary Figure S10*) we performed standard colony-forming unit (CFU) assays at E:T ratios of 1:1 and 5:1 in the presence of CID. At both E:T ratios, CD123.ENG.iMC cells did not induce greater myelotoxicity than non-transduced T cells in assays for granulocyte-erythroid-monocyte-megakaryocyte, erythroid, and granulocyte macrophage CFU (n=3 technical replicates, *P*=ns) (Figure 8C), but did show greater toxicity on burst-forming units at a 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 whether we could generate genetically modified T cells from three diagnostic bone marrow samples of pediatric patients with AML and to evaluate whether they recognized CD123⁺ autologous AML blasts. We thawed primary bone marrow samples
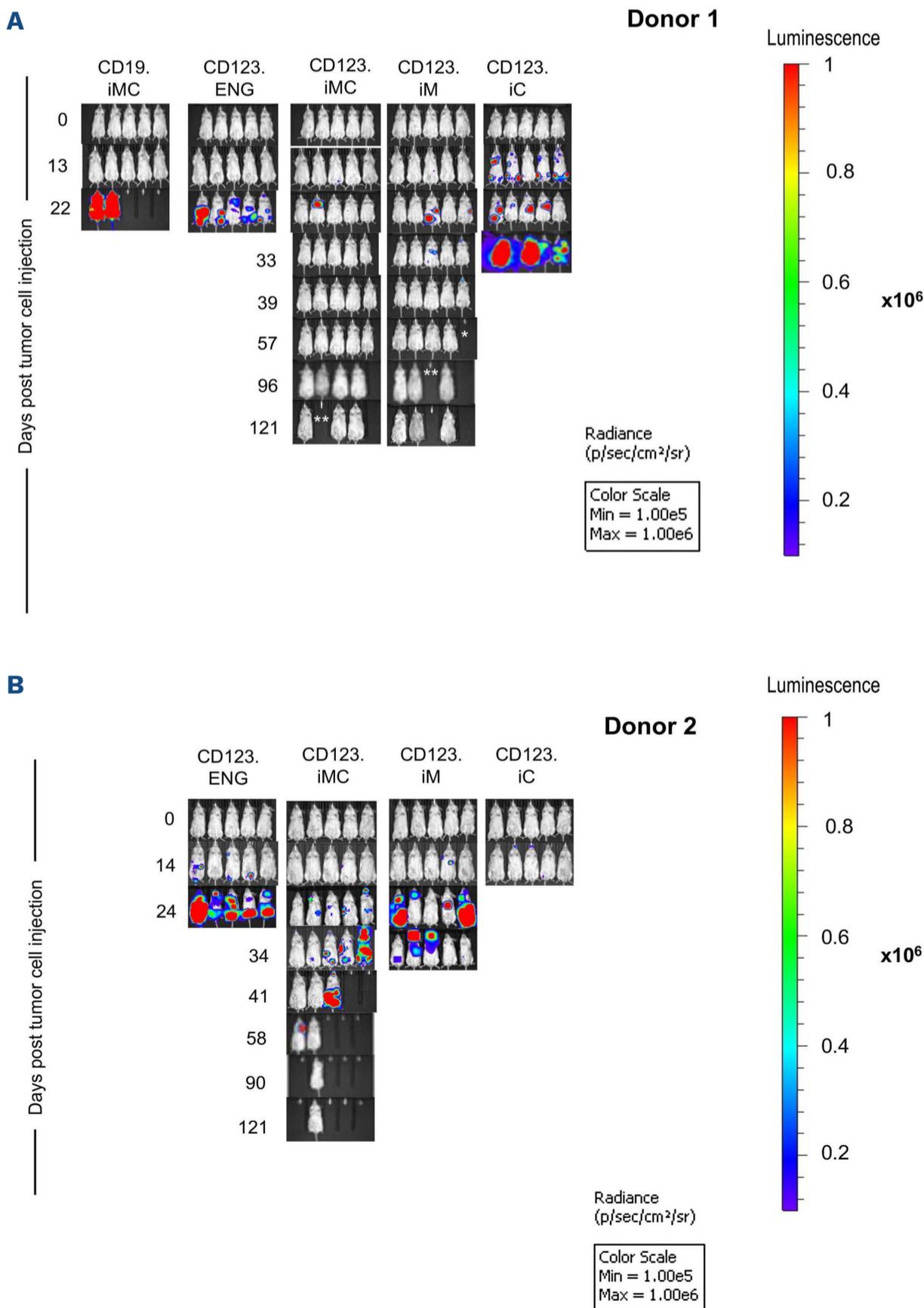

Figure 7. Representative IVIS images of the acute myeloid leukemia xenograft model. (A) Donor 1: representative IVIS images. Asterisks represent animals requiring euthanasia due to non-tumor related morbidities (*paraphimosis, **graft-*versus*-host disease). (B) Donor 2: representative IVIS images. Stars represent animals requiring euthanasia due to non-tumor related morbidities. iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40; ENG: engager.

and evaluated surface expression of CD123 on AML blasts by flow cytometry (Figure 8D). We isolated CD3^{+} T cells using magnetic bead separation. The CD3^{−}, AML blast-containing cell fraction was frozen. We then generated CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM, CD123.ENG.iC, and CD19-ENG.iMC T cells, and co-cultured these effector T cells with the primary, thawed AML blasts in the presence of 0.5 nM CID. After 24 h, media were collected and a limited cytokine evaluation by ELISA was performed by determining IFN-γ secretion. Only CD123.ENG and CD123.ENG.iMC T cells produced consistently more IFN-γ compared to non-transduced and CD19-ENG.iMC T cells (n=3, *P*<0.01 for CD123.ENG.iMC) (Figure 8E).

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 affecting 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 being actively explored, focusing on antigens such as CD123, CD33, CLL-1, LeY, among others.[29] CD123 is an attractive target because of its high expression on leukemia
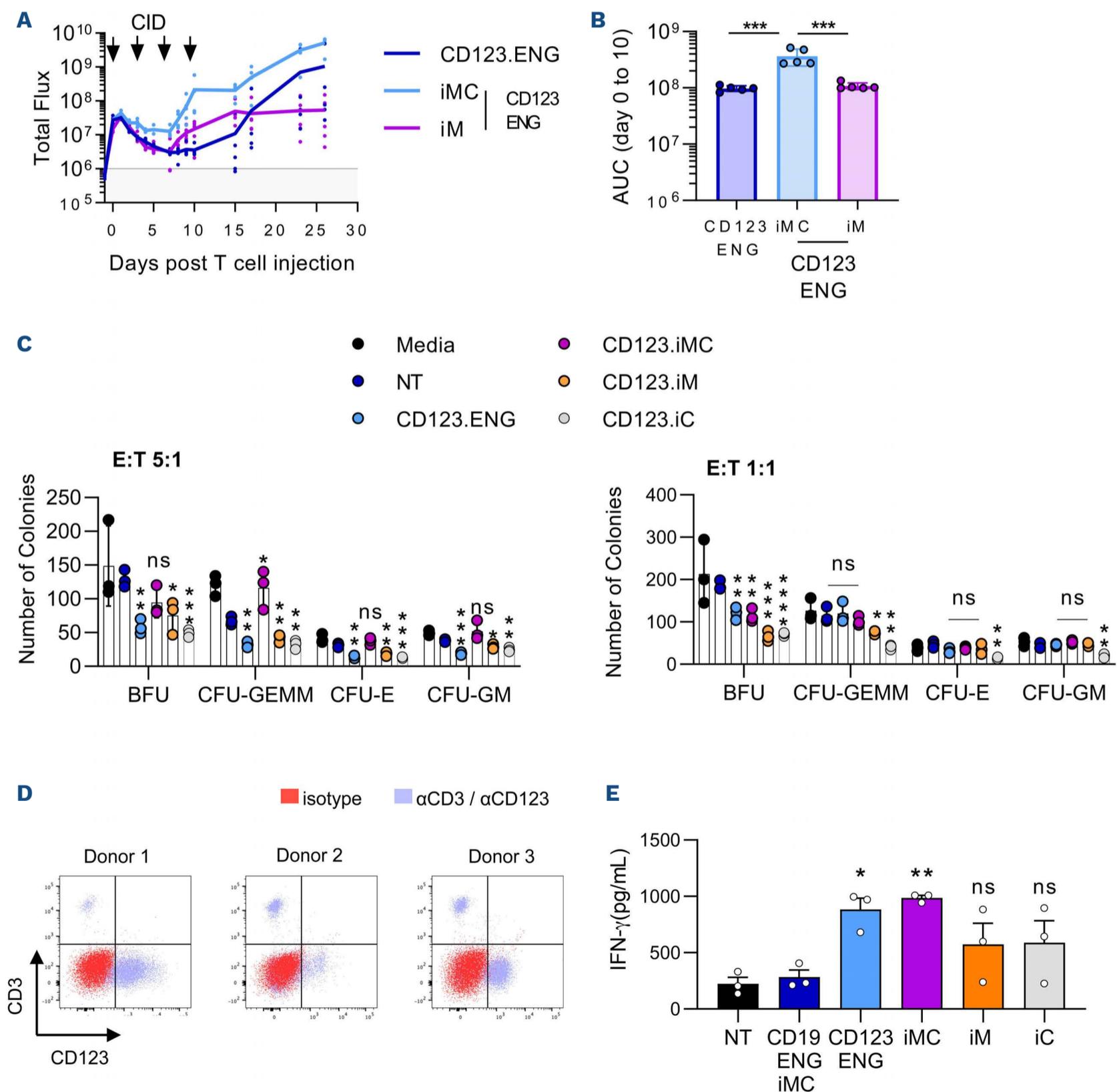
Figure 8. CD123.ENG.iMC T cells expand and persist in an *in vivo* acute myeloid leukemia model, do not have increased myelotoxicity, and recognize primary autologous acute myeloid leukemia blasts. (A,B) Analysis of T-cell persistence: MOLM-13 bearing mice (n=5 per group) received a single intravenous dose of 1x10^{7} CD123.ENG, CD123.ENG iMC, or CD123.ENG iM T cells expressing GFP.ffluc on day 7; four doses of a chemical inducer of dimerization (CID) were given intraperitoneally every 3-4 days. (A) Quantitative bioluminescence data. (B) Area under the curve analysis for day 0 to day 10 after T-cell infusion (***$P<0.001$, 2-way analysis of variance [ANOVA]). (C) Colony-forming unit (CFU) assays: as indicated effector T cells were incubated with hematopoietic cells for 4 h at effector:target (E:T) ratios of 5:1 and 1:1, plated on semisolid media, and burst-forming units, CFU-granulocyte-erythroid-monocyte-megakaryocyte, CFU-erythroid and CFU-granulocyte-macrophage were enumerated after 12–14 days. (*$P<0.05$, **$P<0.01$, 2-way ANOVA). (D,E) Primary autologous T cells recognize primary acute myeloid leukemia (AML) blasts. (D) Flow cytometry analysis for CD123 and CD3 of bone marrow samples of pediatric patients with AML. (E) Co-culture assay in the presence of 0.5 nM CID at an E:T ratio of 1:1 with indicated effector T cells and autologous AML blasts. After 24 h media were obtained for enzyme-linked immunosorbent assay for interferon-γ (*$P<0.05$, **$P<0.01$, generalized linear model). CID: chemical inducer of dimerization; iMC: inducible MyD88 and CD40; iM: inducible MyD88; iC: inducible CD40; ENG: engager; AUC: area under the curve; NT: not transduced; E:T: effector-to-target; ns: not statistically significant; BFU: burst-forming unit; CFU: colony-forming unit; GEMM: granulocyte-erythroid-monocyte-megakaryocyte; E: erythroid; GM: granulocyte-macrophage; IFN: interferon.

stem cells and relatively low expression on normal hematopoietic cells. CD123-ENG T cells have been described as one of the approaches to target AML, but application of this strategy has been limited by modest T-cell persistence, likely due to the absence of co-stimulation.[10] Optimal T-cell activation requires signal 1 delivered by T-cell receptor recognition of antigen-specific peptide through major histocompatibility complex, signal 2 provided by co-stimulatory or inhibitory molecules expressed on the surface of antigen-presenting cells followed by a cytokine signal (signal 3) critical for T-cell expansion and response.[30-33] Augmenting T-cell effector function by transgenic expression of co-stimulatory 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 co-stimulatory 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 co-stimulatory 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 been 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), which 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 co-stimulation in CAR T cells resulted in increased expansion and antitumor activity of the T cells.[35,36] In this study, we explored whether provision of inducible MyD88 and/or CD40 co-stimulation could improve the effector function of CD123.ENG T cells and determined which co-stimulatory domain combination is the most effective in maintaining T-cell persistence and sustained antitumor activity, without additional myelotoxicity as evidenced by CFU assays.

We demonstrated 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 such as LAG3, PD1 and TIM3 have been shown to dampen T-cell responses and have been associated with reduced Th1 cytokine secretion.[37] These markers remained largely at baseline levels for CD123.ENG.iMC until the fifth stimulation, when there was a slight increase in TIM3^{+}LAG3^{+} as well as PD1^{+}LAG3^{+} expression on CD8^{+} T cells, which did not reach statistical significance. Consistent with these findings, CD123.iMC T cells secreted increased amounts of 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 dependent on both the donor and tumor model *in vivo*.

In summary, we demonstrated that CD123.ENG T cells expressing inducible co-stimulation maintain antigen specificity and that expression of MyD88 or MyD88 and CD40 confers improved expansion capability and potent antitumor activity *in vitro* and *in vivo*. In addition, the ability to remotely control the amount of T-cell engager secretion and T-cell persistence while maintaining antigen

specificity using this inducible system endows CD123.ENG.iMC cells with a desirable safety feature.

Disclosures

Contributions

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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.

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