CD28 and 41BB costimulation enhance the effector function of CD19-specific engager T cells

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ABSTRACT

T cells expressing CD19-specific chimeric antigen receptors (CARs) with endodomains that encode a signaling domain derived from CD3ζ and CD28 or 41BB have potent antitumor activity in early phase clinical studies for B-cell malignancies. Besides CD19-specific CARs, other approaches are actively being pursued to redirect T cells to CD19, including recombinant bispecific T-cell engager (BiTE) proteins or T cells genetically modified to express BiTEs (engager [ENG] T cells). Since BiTEs provide no costimulation, we investigated here if provision of costimulation through CD28 and 41BB enhances the effector function of CD19-ENG T cells. CD19-ENG T cells expressing CD80 and 41BBL on their cell surface (CD19-ENG.41BBL/CD80 T cells) were generated by retroviral transduction. CD19-ENG.41BBL/CD80 T cells retained their antigen specificity and had superior effector function compared to both unmodified T cells and CD19-ENG T cells expressing either CD80, 41BBL or no costimulatory molecule, as judged by cytokine (IFNγ and IL2) production, T-cell proliferation, and their ability to sequentially kill target cells. *In vivo*, CD19-ENG.41BBL/CD80 T cells had superior antileukemia activity in the BV173 xenograft model resulting in a survival advantage in comparison to CD19-ENG T cells. Thus, provision of costimulation is critical for the effector function of ENG T cells.

INTRODUCTION

Hematological malignancies of B-cell origin are an important cause of cancer-related mortality since the prognosis of relapsed or refractory disease remains poor (1-4). In recent years, immunotherapeutic approaches have shown promise in the treatment of $CD19^+$ hematological malignancies, including the adoptive transfer of T cells expressing CD19-specific chimeric antigen receptors (CARs) or the infusion of bispecific antibodies (BiTEs) to redirect T cells to $CD19^+$ tumor cells (5-16).

Although CD19-targeting CAR T cells and BiTEs have been successful in clinical studies, however, both have been associated with toxicities including cytokine release syndrome (CRS) and neurotoxicity (7,10,11,14,17,18). Thus, exploration of alternative strategies to redirect the immune system towards $CD19^+$ malignancies are needed. For example, T cells, genetically modified to secrete CD19-specific BiTEs (CD19-ENG T cells), kill not only $CD19^+$ cells, but also recruit bystander T cells to tumor cells in an antigen specific manner (19,20). Although CD19-ENG T cells had antitumor activity in preclinical animal models, consistent IL2 production and T-cell expansion *in vivo* was dependent on the presence of costimulatory molecules on the cell surface of tumor cells (19).

Because most $CD19^+$ malignancies do not express costimulatory molecules on their cell surface (19), we explored here if expressing the costimulatory

molecules CD80 and/or 41BBL on the cell surface of CD19-ENG T cells enhanced their effector function. Our results indicate that costimulation with CD80 and 41BBL is required for optimal antigen-dependent CD19-ENG T-cell activation.

MATERIALS AND METHODS

Cell lines and culture conditions

The Ph-positive chronic B lymphoblastic leukemia (ALL) cell line BV173 (German Collection of Microoganisms and Cell Cultures (DSMZ), Braunschweig, Germany), and the ALL cell line Nalm 6 (DSMZ) were used as $CD19^+$ targets. The generation of firefly luciferase (ffLuc)-expressing BV173 (BV173.ffLuc) were described previously (21,22). K562 (chronic myelogenous leukemia; ATCC, Manassas, VA), and KG1a (acute myelogenous leukemia; ATCC) were used as negative controls. All cell lines were grown in RPMI 1640 (Thermo Scientific, Waltham, MA) except for KG1a (IMDM; Thermo Scientific). 293T cells (ATCC) were used for packaging retroviral vectors and grown in DMEM. All media was supplemented with 10-20% FBS (Thermo Scientific) and GlutaMAX-I (2 mmol/L; Invitrogen, Carlsbad, CA), and all cell lines were grown in standard (37°C, 5% CO2) tissue culture incubators.

Cell lines were purchased between 2012 and 2016. The Characterized Cell Line Core Facility at MD Anderson Cancer Center, Houston, Texas, performed cell line validation. Once thawed, cell lines were kept in culture for a maximum of three months before a new reference vial was thawed. All cell lines were tested on a regular basis for mycoplasma and were negative.

Generation of retroviral vectors

The generation of SFG retroviral vectors encoding the CD19- or EphA2-ENG molecule and mOrange were previously described (19,23). MSCV retroviral vectors encoding CD80, 41BBL, or 41BBL and CD80 were generated by subcloning CD80 from pORF.CD80 (Invivogen, San Diego, CA, USA), and/or 41BBL from pORF.41BBL (Invivogen) into MSCV-I-GFP(M) (provided by the late Elio Vanin, Northwestern University Feinberg School of Medicine, Chicago, IL). RD114-pseudotyped retroviral particles were generated as previously described (24).

Generation of engager T cells

All methods involving human subjects were carried out in accordance to the Declaration of Helsinki. Human peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained under a Baylor College of Medicine IRB approved protocol, after acquiring informed consent. Retroviral transduction was done as previously described (25,26). PBMCs were stimulated on OKT3 (1µg/mL; CRL-8001, ATCC) and CD28 (1µg/mL; BD Bioscience) antibody-coated, non-tissue culture treated 24-well plates. Human interleukin 7 (IL7; 10ng/mL; Peprotech, Rocky Hill, NJ) and human interleukin 15 (IL15; 5ng/mL; Peprotech, Rocky Hill, NJ) were added to cultures on day 2. On day 3, T cells were transduced with retroviral particles on RetroNectin-coated plates (Takara Bio USA, Mountainview CA) in the presence IL7 (10ng/mL) and IL15 (5 ng/mL). T cells were subsequently expanded with IL7 and IL15. Non-transduced (NT) T cells were activated with OKT3/CD28 and expanded in parallel with IL7 and IL15.

Cells were cultured for 7-10 days prior to being used for *in vitro* or *in vivo* experiments.

Flow cytometric analysis

Monoclonal antibodies (mAb) for the following markers were used for fluorescence activated cell sorting (FACS) analysis as described elsewhere (26): 41BBL (Clone C65-485; BD Biosciences, San Jose, CA) conjugated with GAM-APC antibody (BD Biosciences; cat. 550826), CD80-PerCP (eBioscience, San Diego, CA; cat. 46080942); CD3-APC (clone HIT3a; cat. 555342), CD4-PECy7 (clone SK3; cat. 560909), CD8-APCH7 (clone SK1; cat. 560179), CCR7-FITC (clone 150503; cat. 561271), CD62L-APC (clone DREG-56; cat. 559772), CD95-Pacific Blue (clone DX2; cat. 562616), and CD45RO-PercP (clone UCHL1; cat. 560607) (all BD Biosciences, Mountain View, CA). Isotype controls used were IgG1-FITC, IgG1 APC, IgG1Pe.Cy7, IgG1APC H7, IgG1 Pac Blue and IgG1 PercP.Cy7 (all from BD Biosciences). 10,000 cells (non-transduced [NT] or genetically modified) per sample were analyzed by a FACSCalibur instrument (BD Biosciences) using Cell Quest Software (BD Biosciences) and a BD Canto II instrument (BD Biosciences) using FACSDiva software (BD Biosciences) and analyzed using Kaluza Analysis 1.3 (Beckman Coulter) and FlowJo v10 (FlowJo LLC).

Coculture assays and ELISA

NT or genetically modified effector T cells were plated at a 2:1 effector to target (E:T) ratio with $CD19^+$ (BV173) or $CD19^-$ (K562) target cells. Coculture supernatants were collected after 48 hours, snap frozen, and stored for cytokine analysis at a later time. IFNγ and IL2 concentrations were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxic activity of ENG T cells against targets was determined by standard ^{51}Chromium (Cr) release assay. $1x10^6$ target cells were labeled with 50μCi ^{51}Cr and incubated for 1 hour. Targets were then washed and $5x10^3$ cells were cocultured with effector T cells at different effector to target (E:T) ratios. Supernatants were analyzed for radioactivity using a Wizard gamma counter Model 2470 (Perkin Elmer, Shelton CT) reader after 4 hour incubation. Percent lysis was calculated as previously described (21).

Sequential killing assay

To determine the cytolytic activity of T cells after repeat stimulations, we performed a sequential killing assay with GFP-positive tumor cell lines (BV173 and Nalm 6) as outlined in **Supplementary Fig. S4**. $1.5x10^5$ GFP-positive tumor cells were plated at different E:T ratios (1:4 and 1:8) with T cells in a 48-well plate. Every 3 to 4 days, cells were mixed in the well, and a small aliquot of cells was removed for analysis. The remaining cells were washed once, resuspended

in fresh, cytokine-free RPMI and $1.5x10^5$ fresh tumor cells were added. Cells were stained with 7-AAD (BD Biosciences; cat. 559925) and anti-CD3-APC (BD Biosciences) before enumerating viable GFP^+ (tumor) and $CD3^+$ T cells by FACS analysis using Countbright Absolute Counting Beads (Life technologies, Eugene, OR). Coculture assays were also performed in a 24-well plate format in which tumor cells and T cells were plated at a 1:1 E:T ratio ($5x10^5$ cells each per well). After 5 days, cells were mixed in the well, and a small aliquot of cells was removed for analysis. The remaining cells were washed once, resuspended in fresh, cytokine-free RPMI and $5x10^5$ fresh tumor cells were added.

***In vivo* experiments**

Animal experiments were performed on a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee in accordance to the American Association for Laboratory Animal Science. Eight to 10-week old NSG mice (NOD.Cg-Prkdcscid/Il2rgtm1Wjl/SzJ; JAX Mice, Bar Harbor, ME) were sublethally irradiated with 120 cGy 24 hours before the intravenous (i.v.) injection of $3x10^6$ BV173.ffLuc cells. Mice were treated with $1x10^6$ CD19-ENG, CD19-ENG.CD80.41BBL or EphA2-ENG.CD80/41BBL T cells given i.v. on day 7 after tumor cell injection. The same model was used to track T cells *in vivo*, but in this case, unmodified BV173 cells and T cells genetically modified to express an enhanced green fluorescent protein ffLuc fusion gene (eGFP.ffLuc) were injected. Mice were imaged using the IVIS® system (IVIS, Xenogen Corp.,

Alameda, CA) as previously described (21), and euthanized at predefined endpoints (antitumor activity: day 80; T-cell persistence: day 100) or when they met euthanasia criteria in accordance with the Center for Comparative Medicine at Baylor College of Medicine.

Statistical analysis

Data were summarized using descriptive statistics. Comparisons of continuous variables among three or more groups were made by one-way ANOVA, while comparisons between two groups were made by t-test or Wilcoxon rank-sum test when appropriate. Multiple comparisons were adjusted by the Holm's method. Survival times from tumor cell injection in the mouse experiments were analyzed by the Kaplan–Meier method and the Gehan-Wilcoxon test. GraphPad Prism 5 software (GraphPad software, Inc., La Jolla, CA), SAS 9.4, and R 3.3.2 were used for statistical analysis. P values < 0.05 were considered statistically significant.

RESULTS

Generation and characterization of CD19-ENG T cells

We generated T cells expressing CD19-ENG, CD19-ENG and CD80 (CD19-ENG.CD80), CD19-ENG and 41BBL (CD19-ENG.41BBL), and CD19-ENG with both 41BBL and CD80 (CD19-ENG.41BBL/CD80) by transducing CD3/CD28-activated T cells with one or two retroviral vectors encoding the respective transgenes (**Fig. 1A**). Five to seven days post transduction, genetically modified T cells were enumerated by FACS analysis for mOrange (CD19-ENG), CD80, and 41BBL expression (**Fig. 1B,C**). Mean mOrange expression ranged from 53.9% (±6.2%) to 79% (±3%) with no significant differences between transduced T-cell populations. CD19-ENG.CD80 (mean 55.9% ±4.4%) and CD19-ENG.41BBL/CD80 (mean 53.6% ±13%) T cells showed significantly higher expression of CD80 molecules when compared to CD19-ENG ($p=0.0002$ and $p=0.004$) and CD19-ENG.41BBL T cells ($p=0.0002$ and $p=0.003$). CD19-ENG.41BBL (mean 62.8% ±14%) and CD19-ENG.41BBL/ CD80 T cells (mean 86.7% ±8%) expressed significantly more 41BBL than CD19-ENG and CD19-ENG.CD80 T cells ($p=0.004$ and $p=0.0002$, respectively). These results indicate the successful generation of CD19-ENG T cells coexpressing CD80 and/or 41BBL.

Ten to 14 days post transduction, CD19-ENG T-cell populations were stained for CD3, CD4, CD8, CD45RO, CCR7, TIM3, LAG3, and PD-1 to determine if expression of costimulatory molecules changes their phenotype. The ratio of $CD8^+$ to $CD4^+$ T cells was 3 to 1 for T cells expressing CD19-ENG, and T cells

had predominantly an effector memory RA (EMRA; $CD45RO^{-}$ $CCR7^{-}$) phenotype (**Fig. 2A,B; gating strategy in Supplementary Fig. S1B**). Expressing CD80 and/or 41BBL on CD19-ENG T cells changed neither CD8:CD4 T-cell ratio nor phenotype. Non-transduced (NT) T cells had a similar CD8:CD4 ratio, but had a predominately naïve (N; $CD45RO^{-}$ $CCR7^{+}$) and central memory (CM; $CD45RO^{+}$ $CCR7^{+}$) phenotype (**Fig. 2A,B)**. PD-1 and LAG3 expression in T cells transduced with CD19-ENG, CD19-ENG.CD80, CD19-ENG.41BBL, or CD19-ENG.41BBL.CD80 was not significantly different from NT T cells, and no T-cell population expressed TIM3 (**Fig. 2C,D; gating strategy in Supplementary Fig. S1C**). Thus, expression of CD80 and/or 41BBL on the cell surface of CD19-ENG T cells does not change their phenotype. To further investigate the naïve cell population of NT T cells, we performed staining for CD62L and CD95, demonstrating a high percentage of T cells with a stem cell memory-like phenotype ($CD45RO^{-}$ $CCR7^{+}$ $CD62L^{+}$ $CD95^{+}$ cells; **Supplementary Fig. S2**), which is consistent with previous findings by others (27-29).

CD80 and 41BBL enhance antigen-dependent IFNγ and IL2 production

To evaluate if expressing CD80 and/or 41BBL on the cell surface of CD19-ENG T cells enhanced their effector function, we performed cytotoxicity assays and determined the production of IFNγ and IL2 after exposure to $CD19^{+}$ target cells. CD19-ENG T cells killed $CD19^{+}$ (BV173) but not $CD19^{-}$ (KG1a) tumor cells, and expression of CD80 and/or 41BBL did not change their specificity or cytolytic

activity (**Fig. 3A**). This was confirmed for a second $CD19^+$ target cell (Nalm 6; **Supplementary Fig. S3**). To confirm that expression of CD80 and 41BBL did not result in nonspecific target cell killing, we expressed both molecules in T cells transduced with an ENG molecule specific for an irrelevant antigen (EphA2-ENG). EphA2-ENG.41BBL/CD80 T cells did not kill BV173 or KG1a (**Fig. 3A**).

To determine IFNγ and IL2 production after antigen exposure, CD19-ENG, CD19-ENG.CD80, CD19-ENG.41BBL, CD19-ENG.41BBL/CD80, or EphA2-ENG.41BBL/CD80 T cells were cultured with $CD19^+$ (BV173) or $CD19^-$ (K562) cell lines, two cell lines that do not express the costimulatory molecules CD80, CD86, or 41BBL (19). After 24 hours, the concentration of IFNγ and IL2 in culture media was determined by ELISA. CD19-ENG.41BBL/CD80 T cells secreted significantly more IFNγ ($p=0.037$) and IL2 ($p=0.011$) in comparison to CD19-ENG T cells (**Fig. 3B**). Although CD19-ENG.CD80 and CD19-ENG.41BBL T cells also secreted more IFNγ and IL2 than CD19-ENG T cells, this difference did not reach significance. EphA2-ENG.41BBL/CD80 T cells did not produce significant levels of IFNγ and IL2 in the presence of $CD19^+$ or $CD19^-$ targets, confirming antigen specificity (**Fig. 3B**). Thus, expression of CD80 and 41BBL on the cell surface of CD19-ENG T cells is required to significantly enhance their ability to secrete IFNγ and IL2 after antigen-specific T-cell activation.

CD80 and 41BBL enhance sequential antitumor activity

To evaluate if provision of costimulation enhanced the sequential killing capability of CD19-ENG T cells, we performed a sequential killing assay (outlined in **Supplementary Fig. S4**). BV173 or Nalm 6 cells were cocultured with different CD19-ENG T cell populations at an initial E:T ratio of 1:4 or 1:8. Every 3 to 4 days, T cells and tumor cells were enumerated before washing cells, resuspending in cytokine-free media, and adding fresh tumor cells. For BV173 at both E:T ratios and for Nalm 6 at an E:T ratio of 1:4, CD19-ENG.41BBL/CD80 T cells killed target cells up to the 7th time fresh tumor cells were added. CD19-ENG.CD80 T cells killed target cells up to the 5th, CD19-ENG.41BBL T cells up to the 4th, and CD19-ENG T cells up to the 2nd time, respectively (**Fig. 4A-C; Supplementary Fig. S5A**). For Nalm 6, at an initial E:T ratio of 1:8, CD19-ENG.41BBL.CD80 T cells could kill targets for up to the 4th time fresh tumor cells were added, with other CD19-ENG T cell populations being able to kill up to the 3rd time (**Supplementary Fig. S5B**). CD19-ENG.41BBL/CD80 T cells had the most consistent antitumor activity against both targets at both E:T ratios in comparison to CD19-ENG T cells (**Fig. 5B**). CD19-ENG.41BBL/CD80 T cells were also the only population that expanded significantly more than CD19-ENG in the presence of both targets and E:T ratios (**Fig. 5A,B**). Thus, our analysis demonstrated the following T-cell effector rank order: CD19-ENG.41BBL/CD80 > CD19-ENG.CD80 = CD19-ENG.41BBL > CD19-ENG T cells (**Fig. 5**). Due to the superior effector function of CD19-ENG.41BBL.CD80 T cells in all performed *in vitro* assays, we selected these cells for our *in vivo* experiments. Improved

cytolytic activity of CD19-ENG.41BBL/CD80 and CD19-ENG.CD80 T cells was also confirmed in a coculture assay in which T cells were re-exposed to tumor cells every 5 days (**Supplementary Fig. S6**). Although CD19-ENG.41BBL/CD80 T cells were effective against both targets, T-cell expansion was limited in the presence of Nalm 6 cells. We, therefore, investigated whether a difference in PD-L1 expression existed between BV173 and Nalm 6 cells. At baseline, neither cell line expressed PD-L1, whereas IFNγ exposure induced PD-L1 expression in Nalm 6 cells but not in BV173 cells (**Supplementary Fig. S7**).

CD19-ENG.41BBL/CD80 T cells have superior antitumor activity *in vivo*

To evaluate the antitumor activity of CD19-ENG.41BBL/CD80 T cells, NSG mice were injected with $3x10^6$ BV173.ffLuc cells, and on day 7 received a single i.v. dose of $1x10^6$ CD19-ENG, CD19-ENG.41BBL/CD80, or EphA2-ENG.41BBL/CD80 T cells. Whereas CD19-ENG T cells had no antitumor activity at a cell dose of $1x10^6$ as previously reported (19), $1x10^6$ CD19-ENG.41BBL/CD80 T cells had potent antitumor activity in 9/10 mice as judged by bioluminescence imaging (**Fig. 6A,B**). Long-term follow up to 80 days revealed no weight loss or leukemia recurrence, resulting in a significant survival advantage of CD19-ENG.41BBL/CD80 T cell treated mice ($p<0.0001$; **Fig. 6C,D**). EphA2-ENG.41BBL/CD80 T cells had no antitumor activity.

CD19-ENG T cells do not expand in the BV173 leukemia model *in vivo*.(19) To evaluate if CD19-ENG.41BBL/CD80 T cells expand *in vivo* in an antigen-

dependent manner, NSG mice were injected with $1x10^6$ BV173 cells, and on day 7 received a single i.v. dose of $1x10^6$ CD19-ENG.41BBL/CD80 or EphA2-ENG.41BBL/CD80 T cells, that were also genetically modified to express eGFP.ffLuc. Control mice that did not receive any tumor were injected with eGFP.ffLuc-expressing CD19-ENG.41BBL/CD80 T cells. Within the first 5 days post T-cell injection, CD19-ENG.41BBL/CD80 T cells expanded significantly in the femurs of tumor-bearing mice, as judged by bioluminescence imaging, in contrast to mice without tumors (**Fig. 7A, B**). EphA2-ENG.41BBL/CD80 T cells expanded much more slowly than CD19-ENG.41BBL/CD80 in tumor-bearing mice, demonstrating that the early expansion of CD19-ENG.41BBL/CD80 T cells post injection is antigen-specific. Starting 7 days post injection, no statistical significant difference in the bioluminescence signal among all three groups was seen. Long-term follow-up revealed that all three T-cell populations expanded until day 13. Afterwards, T cells in the CD19-ENG.41BBL/CD80 T cells +/- tumor groups contracted and persisted in low numbers until the end of the end of the experiment (day 93 post T-cell injection) with no evident weight loss (**Supplementary Fig. S8A-C**). Area under the curve analysis revealed a significantly greater ($p<0.01$) expansion and persistence of CD19-ENG.41BBL/CD80 T cells in the presence of tumor cells (**Supplementary Fig. S8B**). Tumor-bearing mice that had received EphA2-ENG.41BBL/CD80 T cells needed to be euthanized on day 13, which is consistent with tumor progression (**Fig. 6**).

DISCUSSION

In this manuscript, we demonstrate that the expression of CD80 and 41BBL on the cell surface of CD19-ENG T cells enhances their effector function. Expression of CD80 and 41BBL had no impact on antigen specificity, but improved antigen-dependent cytokine secretion (IFNγ and IL2), T-cell expansion, and antitumor activity of CD19-ENG T cells.

Optimal T-cell activation requires antigen-specific CD3ζ stimulation (signal 1) and costimulation (signal 2). Upon proper costimulation, T cells produce cytokines or induce cytokine production by neighboring cells (signal 3), which is critical for their expansion (30-33). Comparisons of the effector function of T cells that express 1st generation CARs, containing only a CD3ζ endodomain, to 2nd generation CARs, whose endodomains contain the CD3ζ signaling domain plus those from costimulatory molecules such as CD28, 41BB, OX40, or CD27, has highlighted the need of costimulation for proper T cell activation (34-36). Whether T cells expressing a CAR that encodes two costimulatory endodomains (3rd generation CAR) have superior effector function than 2nd generation CAR T cells remains controversial and depends on the used tumor model (37-39).

Besides incorporating costimulatory signaling domains into CARs, investigators have also explored the expression of costimulatory molecules on the cell surface of CAR T cells (40). A study by Zhao and colleagues suggests that T cells expressing CAR.CD28.ζ and 41BBL have superior effector function in comparison to T cells expressing CAR.ζ, CD80 and 41BBL, or CAR.41BB.CD28.ζ on their cell surface (41). Here, we explored if provision of costimulatory molecules improved the effector function of CD19-ENG T cells, which secrete BiTEs that only activate CD3ζ. We successfully generated CD19-ENG T cells expressing CD80 and/or 41BBL. Expression of both molecules did not change the phenotype of CD19-ENG T cells and did not result in upregulation of exhaustion markers (PD-1, LAG3, TIM3), which has been observed in T cells that express CARs that are constitutively active (tonic signaling) (42). However, expressing CD19-ENG in T cells led to a decrease of naïve ($CD45RO^{-}$ $CCR7^{+}$) T cells in comparison to NT T cells, which might reflect the presence of residual B-cells in our culture system at the time of transduction, resulting in T-cell activation through CD19-ENGs. Although CD19-ENG T cells did not produce significant amounts of IFNγ in the absence of $CD19^{+}$ target cells, baseline tonic signaling by CD19-ENG bound to the T-cell surface cannot be excluded as another mechanism for a decrease of naïve T cells. Further studies are needed to investigate the mechanism of these phenotypic changes including molecular and epigenetic studies (43,44).

Expression of both CD80 and 41BBL was necessary for a significant increase in antigen-dependent IL2 production in comparison to CD19-ENG T cells. This differs from CARs in which a single costimulatory endodomain is sufficient (45,46). However, it is consistent with findings by others that expression of CD80 and 41BBL is required on the cell surface of CAR.ζ T cells for significant antigen-dependent IL2 production (40,41). Expression of both CD80 and 41BBL was also required for optimal cytolytic activity and expansion of CD19-ENG T cells in the sequential killing assays we performed. In these assays, CD19-ENG T cells expressing either CD80, 41BBL or no costimulatory molecule were re-exposed to tumor cells every 3 to 4 days to mimic the *in vivo* situation in which tumor cells are exposed to tumor cells constantly when they first arrive at tumor sites. All CD19-ENG T-cell populations expanded better in the presence of BV173 than Nalm 6, most likely due to the upregulation of PD-L1 on Nalm 6 cells in the presence of IFNγ. Here we explored only one combination of costimulatory molecules. In the setting of CAR.ζ T cells, investigators have compared the benefits of expressing CD80 with several members of the tumor necrosis factor ligand family, including 41BBL, CD70, OX40L, and CD30L (40). Their results indicate that combining CD80 and 41BBL costimulation is most effective in enhancing the effector function of CAR.ζ T cells (40).

In vivo, expression of CD80 and 41BBL on the cell surface of CD19-ENG T cells resulted in a significant increase in their antitumor activity, confirming the *in vitro* sequential killing assay result. This result was not due to nonspecific tumor killing

because control EphA2-ENG.41BBL/CD80 T cells had no antitumor activity. CD19-ENG.41BBL/CD80 T cells did not induce significant xenogenic graft versus host disease (GvHD), as judged by weight and/or fur loss, during the 80-day observation period post T-cell injection nor was it observed in the T-cell persistence experiment with a follow-up of 93 days post T-cell injection. Future studies are planned to confirm our findings by performing a detailed histological analysis of organs of euthanized mice.

Besides expressing costimulatory molecules on the cell surface of ENG T cells, other strategies could be explored to provide costimulation. For example, others have generated fusion proteins that consist of a tumor-associated antigen (TAA)-specific scFv and the ectodomain (ECD) of CD80 or 41BBL (32,33). T cells incubated with BiTEs, TAA.scFv-CD80.ECD, and TAA.scFv-41BBL.ECD recombinant proteins result in improved IFNγ production and T-cell proliferation in comparison to T cells that were incubated with BiTEs and TAA.scFv-CD80.ECD (33,47). These results mirror our findings that provision of two costimulatory signals results in enhanced effector function after BiTE-mediated T-cell activation.

In summary, our study demonstrates that provision of CD80 and 41BBL enhances the effector function of CD19-ENG T cells. Our results are informative not only for the future clinical development of ENG T cells for hematological and

448 solid malignancies, but also for immunotherapeutic approaches that rely on the
449 infusion of recombinant BiTE proteins or oncolytic viruses that are genetically
450 engineered to produce BiTEs.
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Figure Legends

Figure 1: Generation of CD19-ENG T cells expressing CD80 and/or 41BB. **(A)** Scheme of retroviral constructs. **(B)** Representative dot plots for CD19-ENG (mOrange), CD80 (PercP) and 41BBL (APC) 5 to 7 days post transduction of CD3/CD28-activated T cells with retroviral vectors. **(C)** Mean and SD of mOrange, CD80, and 41BBL expression (n=3; * $p<0.05$ compared to CD19-ENG T cells, two-tailed t-test).

Figure 2: Immunophenotype and exhaustion marker expression in CD19 ENG T cells. 10 to 14 days post transduction, flow cytometric analysis was used to determine (**A**) Frequency of $CD8^+$ and $CD4^+$ T cells subsets (n=3, non-significant (NS), one-way ANOVA); (**B**) Frequency of naïve ($CD45RO^-$ $CCR7^+$), effector memory ($CD45RO^+$ $CCR7^-$), central memory ($CD45RO^+$ $CCR7^+$), and EMRA ($CD45RO^-$ $CCR7^-$) T cells (n=6; naïve and EMRA T cells: NT vs CD19-ENG +/- costimulatory molecules: $p\leq0.001$, one-way ANOVA); Frequency of Tim3, PD-1, and LAG3 expression on (**C**) $CD4^+$ cells and (**D**) $CD8^+$ cells (n=3, NS, one-way ANOVA).

Figure 3: Antigen specificity and cytokine secretion by CD19-ENG T cells. (**A**) Cytotoxicity assays were performed using CD19-ENG, CD19-ENG.CD80, CD19-ENG.41BBL, CD19-ENG.41BBL/CD80 and EphA2-ENG, 41BBL/CD80 T cells as effectors and $CD19^+$ (BV173) and $CD19^-$ (KG1a) tumor cells as targets

at a E:T ratio of 10:1. Percent lysis is plotted using Tukey box and whisker plots. (n=4; assay performed in triplicates). (**B**) Effector T cells were cocultured with $CD19^+$ (BV173), $CD19^-$ (KG1a) tumor cells, or media only at a ratio of 2:1. After 48 hours, IFNγ and IL2 production was determined by ELISA and plotted using Tukey box and whisker plots (n=5; assay performed in duplicates, significance at $p<0.05$; one-way ANOVA).

Figure 4: CD19-ENG.41BBL/CD80 T cells have improved ability to sequentially kill $CD19^+$ target cells. Sequential killing assays were performed as outlined in the material and methods section and **Supplementary Fig S2**. Absolute cell count of live tumor cells was obtained by flow cytometry using Countbright counting beads. (**A,B**) Live tumor cell number was plotted relative to tumor cell number at the start of each stimulation; (**A**) BV173 (n=3), (**B**) Nalm6 (n=4). (**C**) Summary data presented as a heatmap denoting no antitumor activity for all donors (yellow), donor-dependent antitumor activity (blue/yellow), or antitumor activity in all donors (blue). Numbers 1-7 under cell line names denote stimulation number. White boxes represent values not determined. The Wilcoxon rank sum test was used to determine significance ($p<0.05$).

Figure 5: CD19-ENG.41BBL/CD80 T cells have improved antigen-dependent proliferative capacity. (**A**) Sequential killing assays were performed as outlined in the material and methods section and **Supplementary Fig S2**. Absolute cell count of live T cells was obtained by flow cytometry using Countbright counting

668 beads. Fold T-cell expansion at each sequential stimulation is plotted. (**B**)
669 Proliferation comparison table. Statistical significance was determined using the
670 Wilcoxon rank sum test. Orange boxes denote comparison of T-cell numbers
671 (dark orange: p <0.05, light orange p=NS). Blue boxes denote comparison of
672 tumor cell numbers between different conditions (dark blue: p<0.05, light blue
673 p=NS).
674
675 **Figure 6: CD19-ENG.41BBL/CD80 T cells have improved antitumor activity**
676 **when compared to CD19 ENG T cells *in vivo***. NSG mice were sublethally
677 irradiated and injected i.v. with BV173.ffLuc. On day 7, mice received one i.v.
678 dose of $1x10^6$ CD19-ENG (n=5), CD19-ENG.41BBL/CD80 (n=9), or EphA2-
679 ENG.41BBL/CD80 (n=5) T cells. Tumor growth was monitored by
680 bioluminescence imaging. (**A**) Images of representative animals. (**B**)
681 Bioluminescence signal (radiance = photons/sec/cm^2/ sr) over time is plotted. (**C**)
682 Shown are weights of animals over the course of the experiment. (**D**) Kaplan-
683 Meier survival curves for injected mice. Statistical significance was determined
684 using the Wilcoxon rank sum test (p<0.0001).
685
686 **Figure 7: CD19 ENG.41BBL/CD80 T cells expand *in vivo*.** NSG mice were
687 sublethally irradiated and injected i.v. with BV173. On day 7, mice received one
688 i.v. dose of $1x10^6$ CD19-ENG.41BBL/CD80 (n=5) or EphA2-ENG.41BBL/CD80
689 (n=4) T cells that were genetically modified with eGFP.ffLuc. Mice without tumors
690 received one i.v. dose of $1x10^6$ CD19-ENG.41BBL/CD80 (n=5). (**A**) Images of

representative animals over time are shown. (**B**) Plots of bioluminescence signal (radiance = photons/sec/cm^2/sr) of femurs (*p<0.05 for day 1, ***p<0.001 for days 3 and 5, p=ns for day 7, by Gehan-Wilcoxon test).

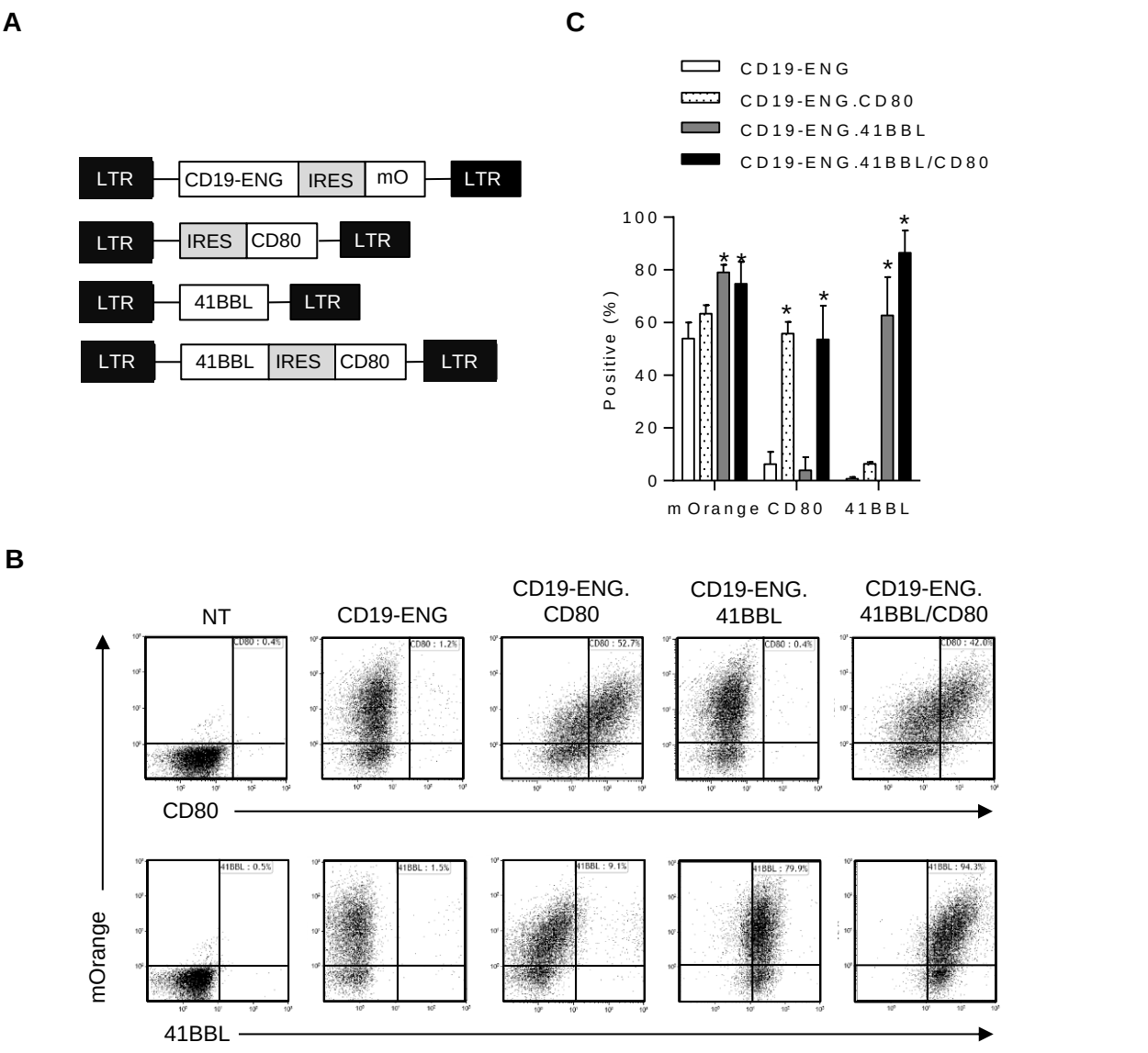


Figure 1

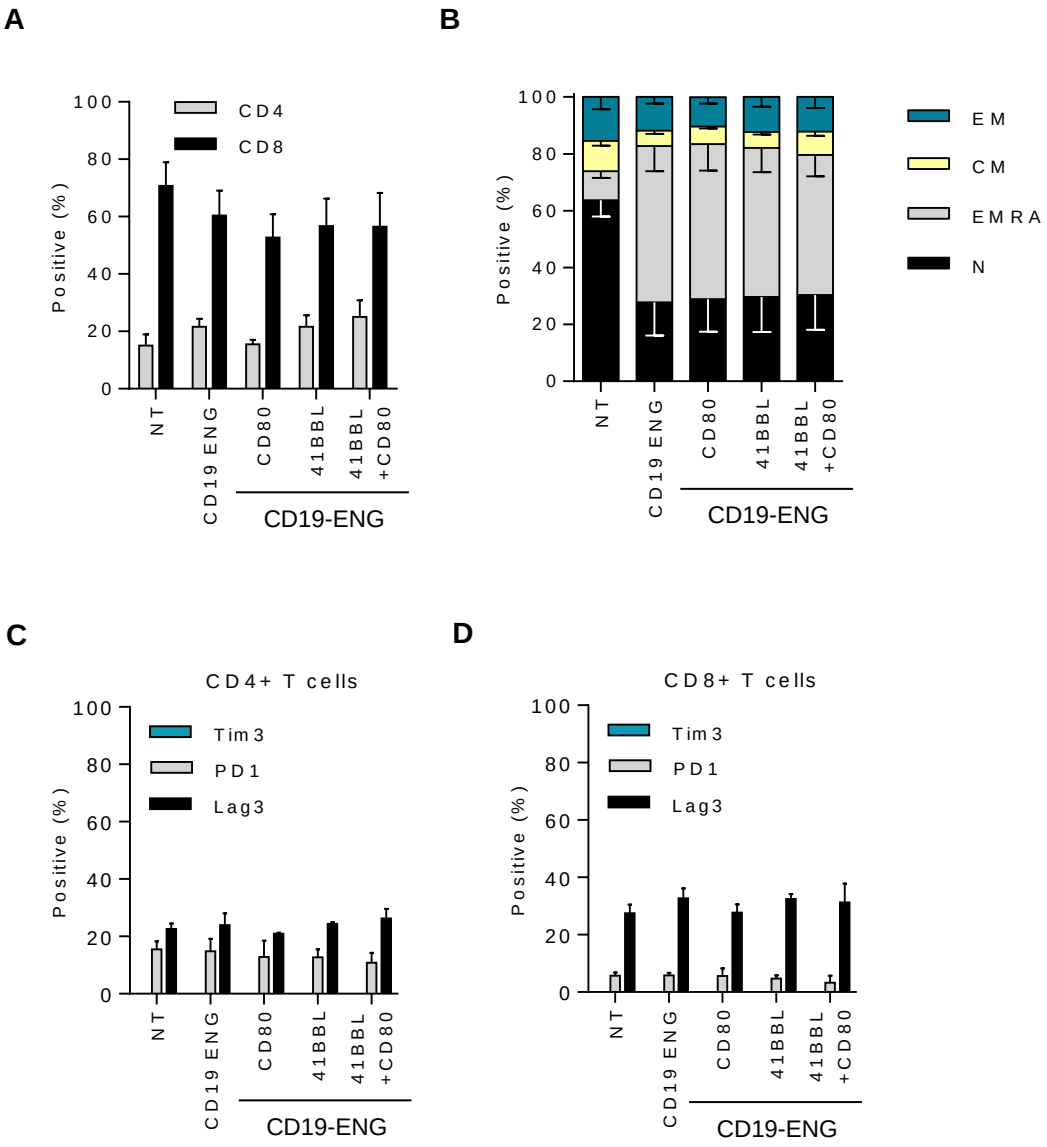


Figure 2

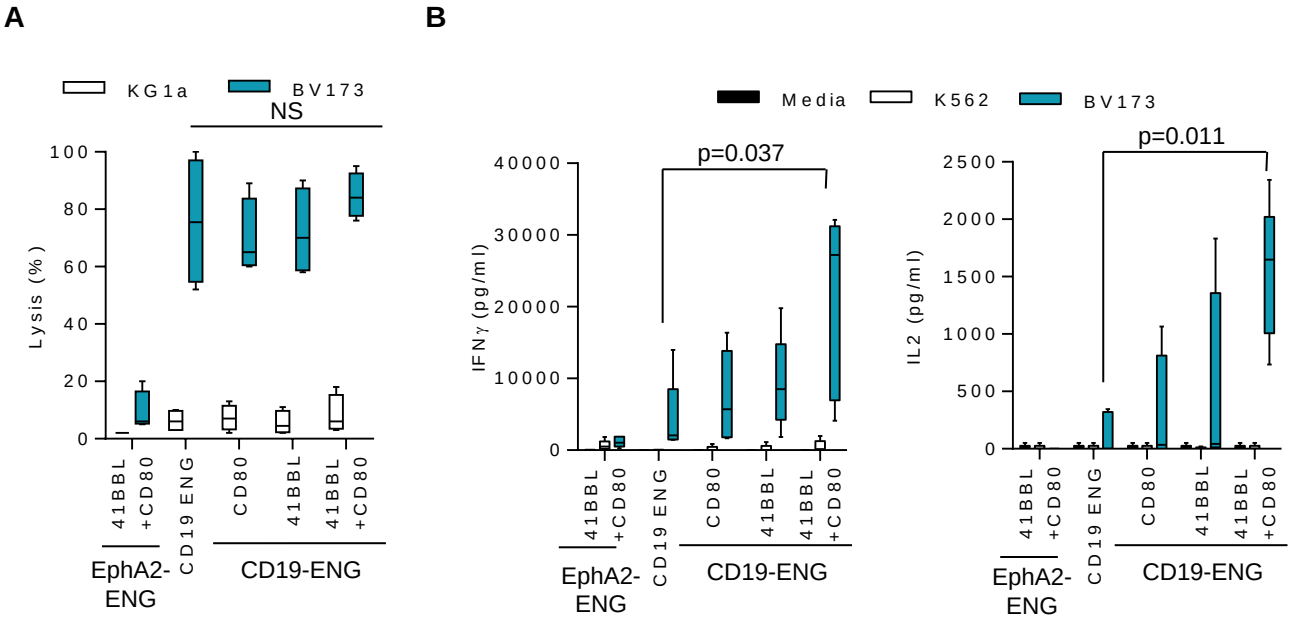
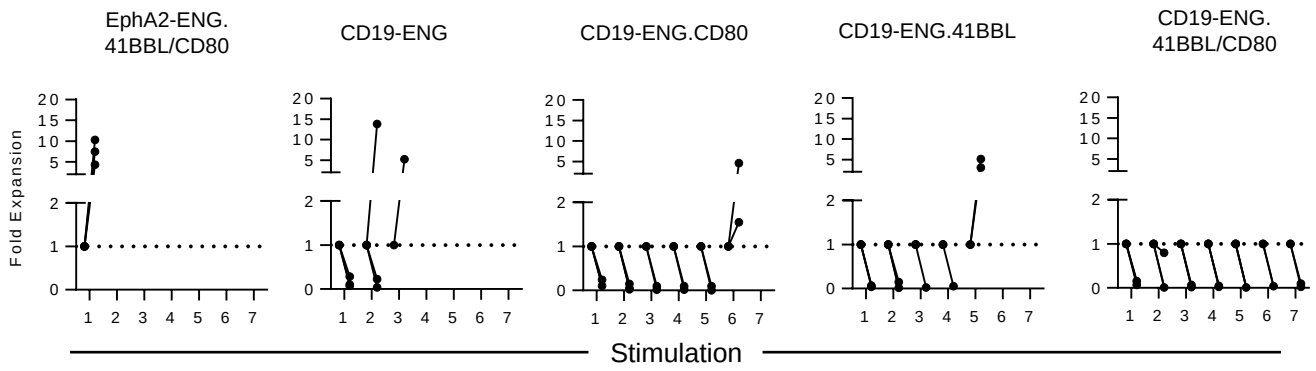


Figure 3

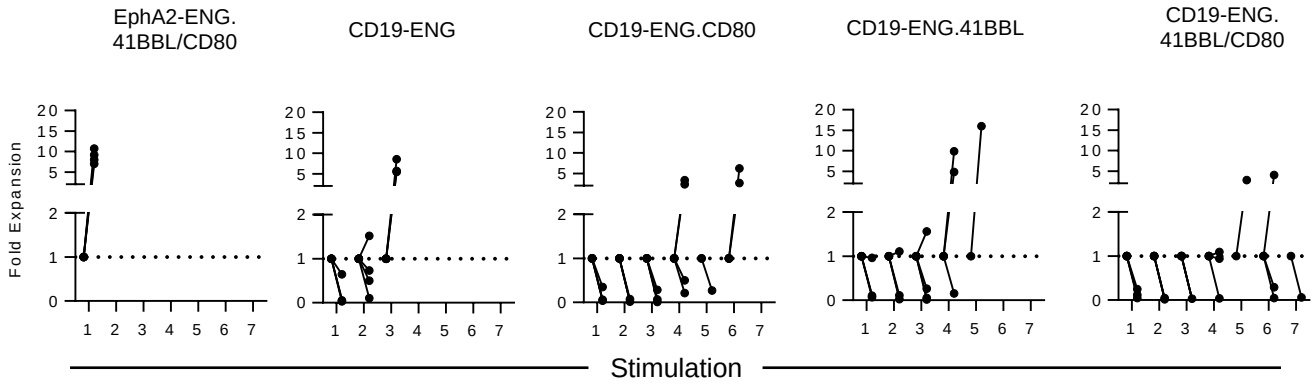
A

BV173; E:T ratio 1:4



B

Nalm6; E:T ratio 1:4



C

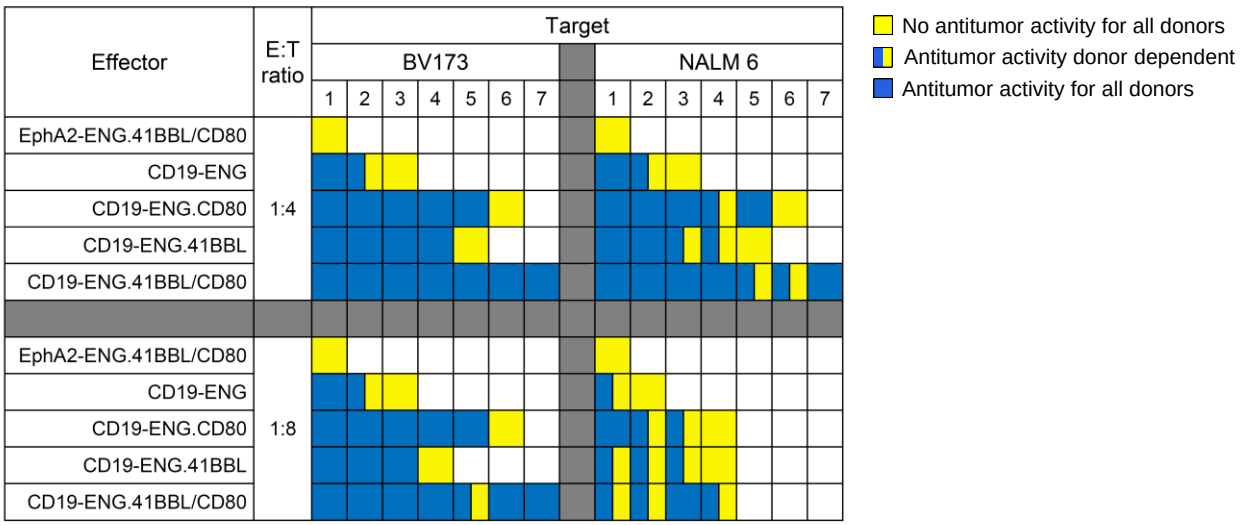
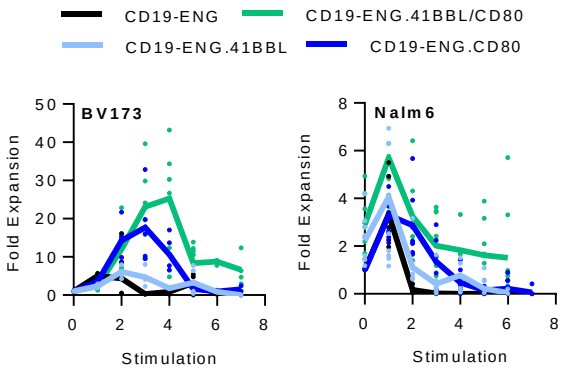


Figure 4

A



B

		CD19-ENG							
		-	CD80	41BBL	41BBL CD80	-	CD80	41BBL	41BBL CD80
E:T ratio		1:4				1:8			
Target		BV173							
	-		0.007	0.031	0.001		<0.001	0.054	<0.001
	CD80	0.360		0.299	0.899	<0.001		0.004	0.669
	41BBL	0.571	0.157		0.140	0.014	0.058		0.002
	41BBL.CD80	0.021	0.056	0.002		<0.001	0.209	0.002	
Target		Nalm6							
	-		0.027	0.137	<0.001		0.094	0.554	0.003
	CD80	<0.001		0.528	0.029	0.015		0.341	0.173
	41BBL	0.001	0.547		0.004	0.001	0.564		0.024
	41BBL.CD80	<0.001	0.005	0.001		<0.001	0.001	0.002	

T -cell comparison: p<0.05; NS

Tumor cell comparison: p<0.05; NS

Figure 5

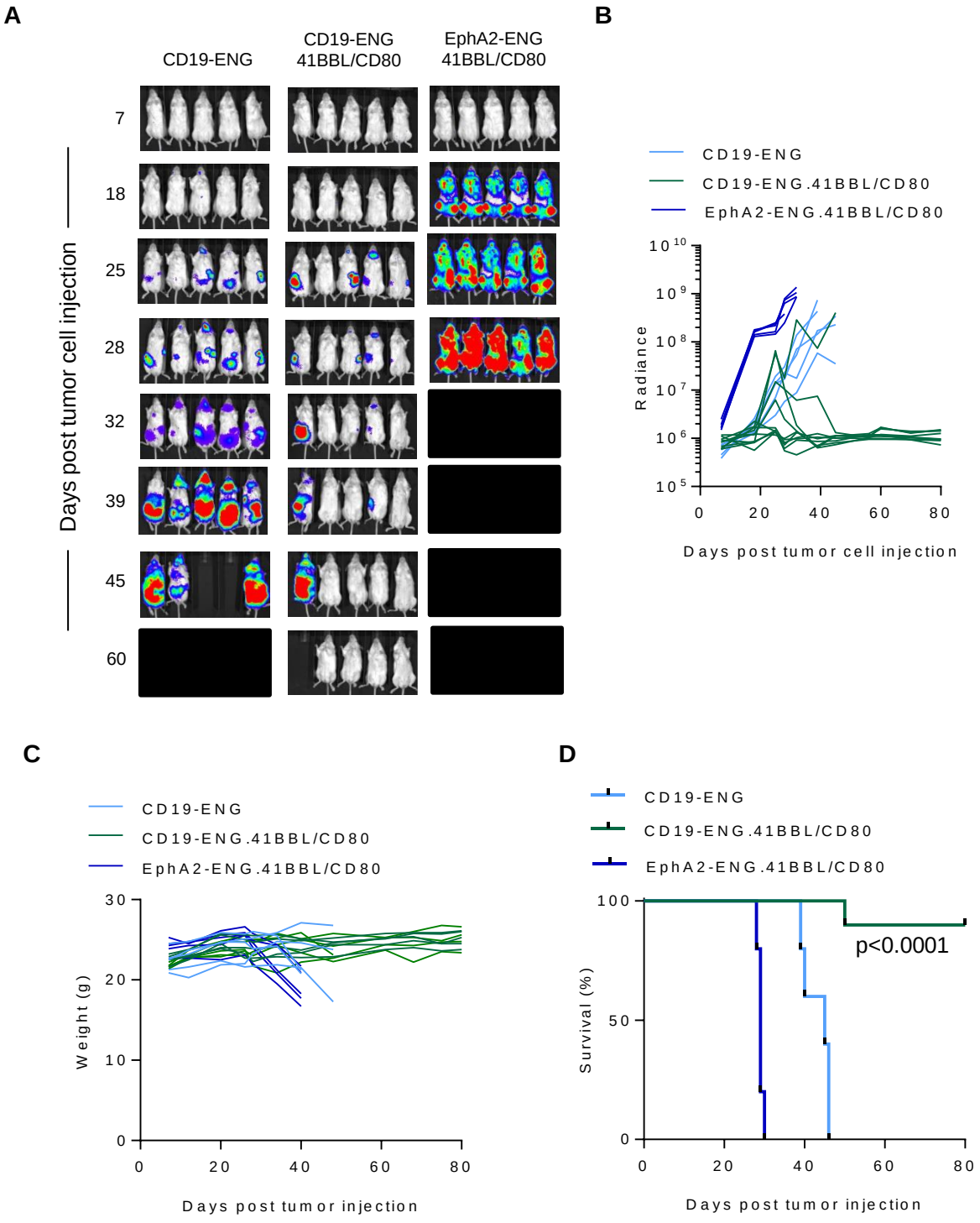
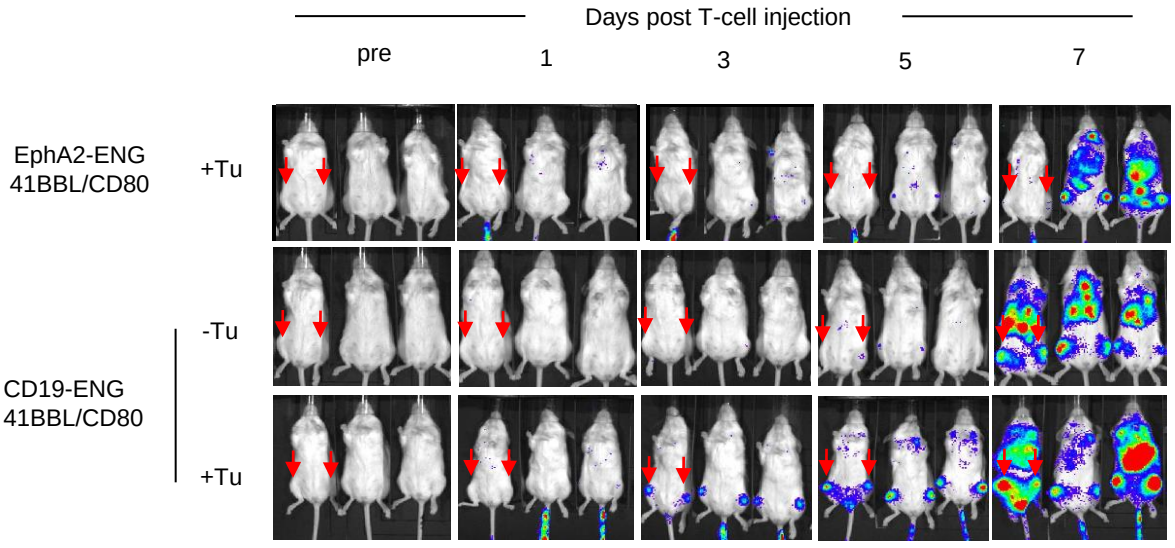


Figure 6

A



B

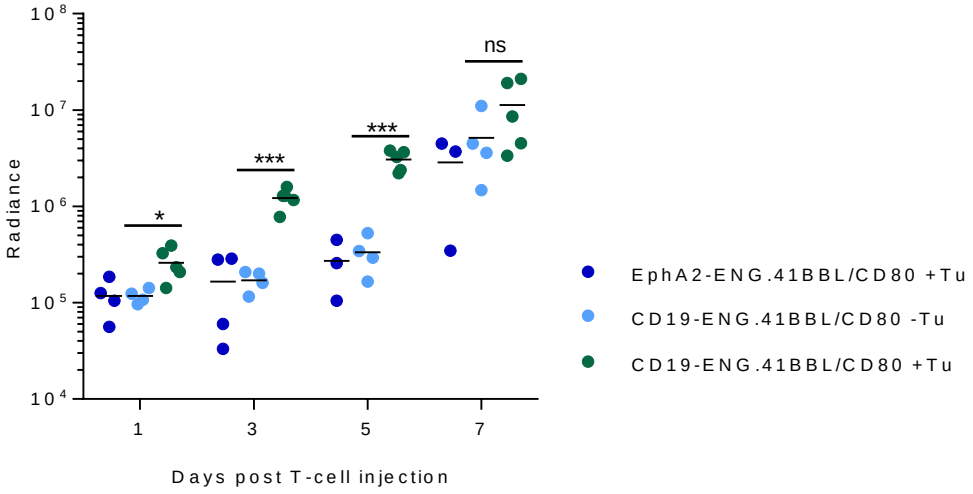


Figure 7

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CD28 and 41BB costimulation enhances the effector function of CD19-specific engager T cells

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