Bispecific antibody does not induce T-cell death mediated by chimeric antigen receptor against disialoganglioside GD2

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ABSTRACT

Chimeric antigen receptors (CAR) and bispecific antibodies (BsAb) are two powerful immunotherapy approaches for retargeting lymphocytes toward cancer cells. Despite their success in lymphoblastic leukemia, solid tumors have been more recalcitrant. Identifying therapeutic barriers facing CAR-mediated (CART) or BsAb Redirected T (BsAb-T) cells should facilitate their clinical translation to solid tumors. Novel lentiviral vectors containing low-affinity or high-affinity 4-1BB second-generation anti-GD2 (disialoganglioside) CARs were built to achieve efficient T cell transduction. The humanized anti-GD2 × CD3 BsAb using the IgG-scFv platform was described previously. CART and BsAb-engaged T cells were tested for viability, proliferation, and activation/exhaustion marker expression, and in vitro cytotoxicity against GD2(+) tumor cells. The antitumor effect of CAR-grafted and BsAb-T cells was compared in a human melanoma xenograft model. The majority of high CAR density T cells were depleted upon exposure to GD2(+)-target cells while the BsAb-T cells survived. The in vitro cytotoxicity of the surviving CART cells was inferior to that of the BsAb-T cells. Using low-affinity CARs, inclusion of the 4-1BB co-stimulatory domain or exclusion of a co-stimulatory domain, or blocking PD1 did not prevent CART cell depletion. Both CART cells and BsAb-T cells penetrated established subcutaneous human melanoma xenografts; while both induced tumor regression, BsAb was more efficient. The fate of T cells activated by BsAb differs substantially from that by CAR, translating into a more robust antitumor effect both in vitro and in vivo.

Introduction

It is well known that T cells are effective for treating human cancers. Learning how to exploit these cells is essential. To mediate antitumor function, T cells need to infiltrate tumors, survive the tumor microenvironment, and perform their tumoricidal activity. Results of the previous studies have suggested that a combination of CD4(+) and CD8(+) T cells together should provide optimal antitumor effect, but both populations need to survive the microenvironment. Furthermore, inducing tumor cytotoxicity without excessive activation is critical to prevent activation-induced cell death (AICD). Currently, two approaches have been pursued to redirect polyclonal T cells: chimeric antigen receptors (CAR) and bispecific antibodies (BsAb). Their relative merit for each tumor target system is yet unclear.

Disialoganglioside (GD2) is an established target that is constitutively expressed in neuroblastoma and many other tumors. Antibody based immunotherapies directed at this target have proven efficacy. Cytotoxic effect of neuroblastoma immunotherapy is mostly mediated by NK cells and myeloid cells. T cells engineered against neuroblastoma have also shown promise. Since GD2 is present on multiple human cancers, novel GD2-directed immunotherapies can have broad clinical implications.

Similar to other solid tumor systems, CAR-retargeted T cells toward GD2 have met initial success, but T cell exhaustion and depletion upon antigen exposure soon became apparent. Various strategies have been developed to bypass AICD, including modifying the structure of CAR to avoid Fc receptor-mediated fratricide, incorporating 4-1BB instead of CD28 in the cytoplasmic domain of CAR, constitutive activation of akt/PI3K pathway, and antibody-mediated checkpoint blockade. Measuring both immune (i.e. T cell tumor infiltration and survival/persistence) and therapeutic (tumor regression) endpoints have been used for the assessment of antitumor effect. In our study, we directly compare functional efficacy of CAR versus BsAb in directing T cells to mediate antitumor effect and to study the role of CAR density versus CAR affinity in overcoming hurdles in implementing these T-cell based therapies.

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Supplemental data for this article can be accessed on the publisher’s website.

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Results

Anti-GD2 CART cells are depleted upon stimulation with GD2(+) target cells in vitro

To generate an anti-GD2 CART cell product, T cells generated from frozen PBMCs were transduced with the hu3F8CAR lentiviral vector. Human T cell transduction efficiency with hu3F8CAR was generally ≥80%.

To assess the GD2 specificity of the CART cells, T cell activation was studied using irradiated GD2(+) IMR32luc and GD2(−) THP1 targets. CART cells produced both T helper-1 (TH1) cytokines (interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and interleukin-2 (IL2)) and TH2 cytokines (IL4 and IL6) in an antigen-specific manner (Fig. S1). Unexpectedly, analysis of T cell viability within 24 h showed that the majority of cells expressing high levels of hu3F8CAR (CARhi) were depleted when exposed to GD2(+) IMR32luc target cells but remained intact when GD2(−) THP1 targets were used (Fig. 1A and B). In contrast, in the presence of BC119 BsAb, exposure of untransduced T cells to GD2(+) or GD2(−) target cells did not compromise survival or cell proliferation (Fig. 1C). Antigen-specific loss of CART cells followed the same dynamics when exposed to solid-phase bound purified GD2 or anti-3F8-idiotype antibody A1G4 whereas those exposed to solid-phase-bound control ganglioside GD3 or control anti-5F11-anti-idiotype antibody 1G8 survived (Fig. S2). In addition, analysis of the surviving cells revealed that while PD1 and LAG3 were significantly upregulated on CARhi cells when stimulated with IMR32luc, their expression following stimulation with GD2(−) targets were modest (Fig. 1D and F). Similarly, BsAb-stimulated T cells upregulated their PD1 and LAG3 expression in an antigen-specific manner (Fig. 1E and G). However, this PD1 overexpression on BC119-stimulated T cells did not compromise cell survival suggesting that these markers might be activation rather than exhaustion markers in these experiments. Analysis of PD1 and LAG3 expression over time showed that these markers were upregulated as early as 5 h after stimulation with target cells (Fig. S3). Furthermore, incubation of CART cells with a PD1-blocking antibody pembrolizumab did not prevent cell loss upon exposure to IMR32luc targets (data not shown).

Figure 1. Anti-GD2 CART cells are depleted upon stimulation with GD2(+) target cells in vitro. (A, B) T cells that were transduced with the hu3F8CAR (anti-GD2) and expanded for 8 d thereafter were stimulated with irradiated GD2(+) IMR32luc or THP1 (GD2−) cells. After 1, 2, and 4 d, cells were counted and CAR expression was assessed by anti-idiotype antibody (A1G4). Dead cells were excluded by Annexin-V staining. Absolute cell number was calculated based on cell count and flow cytometry. (C) T cells that were cultured similar to CART cells but were not transduced (UntT) were stimulated with irradiated IMR32luc (GD2+) or THP1 (GD2−) cells in the presence of BC119 (0.01 μg/mL). After 1, 2, and 4 d, cells were counted and CAR expression was assessed by anti-idiotype antibody (A1G4). Dead cells were excluded by Annexin-V staining. Absolute cell number was calculated based on cell count and flow cytometry. Absolute cell number was calculated as explained for CART cells. (D–G) One day after stimulation, cells were assessed by flow cytometry. The anti-idiotype A1G4 antibody was used to stain CART cells. CARlo, CARneg, and CARneg cells were then gated for the expression of PD1 and LAG3. FACS plots are gated on human CD3(+) T cells. Data were generated from triplicates. Student t test was used for statistical analysis.
Lower-affinity anti-GD2 CARs cannot prevent CART cell depletion upon antigen exposure

Humanized 3F8 (hu3F8) and 5F11(F104) were both anti-GD2 antibodies that were affinity-matured to generate hu3F8 (D32H-E1K) and 5F11(Y104) species, respectively. The affinity ($K_D$) of the scFv format of hu3F8, hu3F8(D32H-E1K), 5F11(F104), and 5F11(Y104) is 153.4 nM, 3.6 nM, 19.8 nM, and 6.02 nM, respectively.

To investigate whether CARs with lower affinities can prevent CART cell overstimulation and subsequent death, the scFv of hu3F8 and hu3F8(D32H-E1K) or 5F11(F104) and 5F11(Y104) were used for generation of CARs with different affinities.

Following exposure to GD2(+) targets, a near complete CARhi cell depletion for all four CART cell types were observed (Fig. 2A and B). Since CART cells with higher CAR expression were more susceptible to depletion, we quantified the average minimal number of CAR molecules on a given T cell population that predisposed them to depletion upon antigen encounter. The minimum detection threshold by Quantum bead quantification was around 4,000 molecules per cell. The maximum CAR expression exceeded 2,000,000 molecules per cell. A depletion threshold was defined as the CAR density that separated top 5% from the bottom 95% in the surviving population.

GD2-specific CART cell depletion severely compromises CART cell-mediated cytotoxicity in short-term killing assays

Following antigen exposure for 4 d with either irradiated IMR32luc or THP1 cells, hu3F8CART cells or BC119-redirected T cells were tested against IMR32luc targets in a 4-h
51Cr-release assay. Whereas THP1-exposed CART cells lysed target cells efficiently, IMR32luc-exposed CART cells lost their cytotoxic function almost completely (Fig. 4A). To confirm the compromised cytotoxicity of the surviving CARlo cells, CART cells were sorted for CARhi, CARlo, and untransduced (CARneg) cells and their cytotoxicity against IMR32luc was determined in a 4-h chromium release assay. While CARhi cells demonstrated cytotoxicity, CARlo cells had compromised killing of target cells (Fig. 4B). In contrast to CART cells, exposure of T cells to BC119 in the presence of either irradiated GD2(CD2)IMR32luc or GD2(CD2) THP1 cells did not affect their cytotoxicity (Fig. 4C).

Surviving CART cells retain significant antitumor cytotoxicity in long-term killing assays

Depletion of CARhi T cells severely affected antitumor cytotoxicity in a short-term 51Cr-release assay. The antitumor potential of the remaining CAR with low density (CARlo) cells was tested in a long-term killing assay. Hu3F8CART cells or untransduced T cells (+/− of BC119) were exposed to GD2(+) IMR32luc cells for 3 d at a low effector:target ratio (E:T = 1). On day 3, cells were harvested, counted, and mixed with fresh IMR32luc targets at the same E:T ratio. After 4 days, cells were counted and assessed by flow cytometry. Annexin V was used to exclude dead cells and staining for CD3 was used to differentiate between CD3(+) T cells and CD3(−) targets. Target cell killing over the first 3 d reflected the combined antitumor cytotoxicity by CARhi cells and the slow antitumor cytotoxicity by the surviving CARlo cells. After antigen induced depletion of CARhi cells on day 1, the surviving CART cells harvested after 3 d were CARlo. The long-term killing effect (from day 3 to 7) of CARlo cells was then quantified. As shown in Fig. 5, even though CARhi T cells died after antigen exposure, the tumor cells in contact with CARhi T cells were also eliminated, a fate similarly shared by target cells exposed to BC119-redirected T cells. From day 3 through day 7, the surviving CARlo T cells were able to kill target cells although at significantly lower efficiency than BC119-activated T cells. In the control groups, target cells exposed to untransduced T cells in the absence of BC119 grew rapidly and overtook the T cells. These data confirmed the residual functionality of CARlo cells that could be underestimated in a 4 h 51Cr-release assay.

Anti-GD2 bispecific antibody redirected T cells outperform CART cells against tumor cells in vivo

To compare the functionality of hu3F8CART cells versus T cells redirected by BC119 against tumor cells in vivo, a GD2(+) melanoma cell line M14luc was xenografted subcutaneously in immunodeficient DKO mice. Hu3F8CART cells or untransduced T cells that were cultured for 7 d in vitro were used for in vivo experiments. Transduction efficiency of T cells for in vivo experiments determined on day 7 post-transduction was confirmed to be more than 80%. Subpopulation analysis showed that the percentage of CD4+ T cells was slightly higher than CD8+ T cells. Most of the cells expressed surface markers of central memory cells (80% by FACS) (Fig. 6A and B). T cells were injected intravenously on day 7, 14, and 21 after tumor inoculation. BC119 was injected one day before and one day...
after each T cell injection. To support T cell survival in vivo, low dose IL-2 was injected to all mice including the controls. Mice receiving either BC119 alone or untransduced T cells alone were used as controls. Neither BC119 nor untransduced T cells by itself conferred any antitumor effect, whereas the combination of the two provided rapid shrinkage of the

Figure 4. GD2-specific CART cell depletion severely compromises their function in short-term killing assays. (A) T cells that were transduced with the hu3F8CAR and expanded thereafter for 8 d were stimulated with irradiated IMR32luc (GD2+) or THP1 (GD2−) cells. Four days later, hu3F8CART cells were tested against IMR32luc targets in a 4-h $^{51}$Cr-release assay. (B) T cells that were transduced with the hu3F8CAR and expanded thereafter for 8 d were sorted for the CARhi, CARlo, and CARneg populations and were tested in a 4-h $^{51}$Cr-release assay. (C) Untransduced T cells were similarly stimulated with irradiated target cells but in the presence of BC119 (0.01 μg/mL). Four days later, BC119-activated T cells were tested against IMR32luc targets in a 4-h $^{51}$Cr-release assay in the presence of BC119 (0.01 μg/mL).

Figure 5. Surviving CART cells retain a significant antitumor cytotoxicity in long-term killing assays. (A) Hu3F8CART cells or untransduced T cells (+− 0.01 μg/mL of BC119) were exposed to GD2(+) IMR32luc cells in triplicates for 3 d at an E:T = 1. On day 3, cells were harvested, counted, and mixed with fresh IMR32luc targets (E:T = 1). After 4 more days, cells were counted and assessed by flow cytometry. Dead cells were excluded by Annexin V and CD3 staining was used to differentiate between CD3(+) T cells and CD3(−) targets. Photo micrographs of cultures at day 7 are shown. (B, C) Number of live target cells was calculated based on cell count and flow cytometry.
established tumors. In contrast, tumor mass in the CART cell group, despite initial fast growth when compared with the BC119-redirected T cell group, also showed regression with longer follow-up (Fig. 6C and D). These results are consistent with the in vitro observations of the two phases of cytotoxicity, short-term and long-term, confirming a small advantage of BC119-redirected T cells over hu3F8CART cells in this particular melanoma model.

The fate of CART cells and BsAb-engaged T cells differs at the tumor site in vivo

Infiltration of T cells into solid tumor masses is a prerequisite for a robust therapeutic response. To study if hu3F8CART cells and BC119-redirected T cells could penetrate tumors, mice were killed on day 22 and 25 of the experiment. Splenocytes and tumor infiltrating lymphocytes (TILs) were assessed by flow cytometry. The percentage of TILs in the BC119-engaged T cell group was significantly higher than that in the CART cell group (Fig. 7A and B). The ratio of CD4⁺ and CD8⁺ T cells in the spleen of mice in BsAb-engaged versus CART cell groups was comparable. Surprisingly, whereas the percentage of CD4⁺ and CD8⁺ TILs was nearly equal in the BC119-T cell group, almost all of the hu3F8CART cells were expressing CD8⁺ (Fig. 7A and C). Numerical advantage of TILs having a balanced CD4⁺ and CD8⁺ T cell phenotype in the BC119-redirected versus CART cell group could partly explain the superior antitumor effect mediated by BsAb versus CAR in these in vivo experiments.

Discussion

By directly comparing CAR versus BsAb in redirecting T cells toward GD2, we showed that CAR was associated with substantial T cell death, resulting in lower antitumor potency. This depletion was antigen-specific, induced within 24 h after exposure to solid phase antigen, cell bound antigen, or anti-idiotype antibodies, not preventable by 4-1BB-signaling, or by anti-PD1 checkpoint blockade. Furthermore, exhaustion and depletion was preferential for T cells with high CAR density and was unaffected by lowering scFv affinity. In vivo, the input CD4⁺ versus CD8⁺ ratio was maintained when TILs were driven by BsAb, and substantially reduced when driven by CAR. BsAb driven T cells demonstrated a more robust antitumor effect in vivo without evidence of increased toxicity.

The phenomenon of AICD for T cells is well known. With GD2 CART cells the evidence is unequivocal. The immunology behind AICD is vital to the central property of the immune system to put brakes on run-away immune cells to prevent autoimmunity. The signaling pathways for AICD of T cells have been well defined. For CART cells they include phosphorylation of ERK, AKT, and Stat6. Various strategies have been developed to bypass CART cell AICD, such as modifying CAR structure, constitutive activation of survival pathways,
and using immune checkpoint inhibitors.\textsuperscript{15} In our CAR design, we avoided the CH2-CH3 FcR binding domain, incorporated 4-1BB instead of CD28, and applied anti-PD1 antibodies. Yet, none of these methods was able to alleviate AICD of CART cells. Instead, we directed our efforts to determine the role of CAR density and affinity to study the following endpoints: T cell tumor infiltration, T cell phenotype inside the tumor, and antitumor effect \textit{in vitro} and against tumor xenografts in mice. Both density and affinity could enhance T cell activation and hence AICD. Our findings were unexpected. While high density CART cells died, low density CART cells persisted in the presence of GD2(+) tumors and were able to mediate an effective although delayed antitumor effect. One implication of these findings is the identification of a CAR density threshold which could serve as the receptor ceiling for CART cell therapy in the clinic. The other surprising result was the inability to avoid T cell exhaustion and T cell death despite endpoints following depletion of T cells at the tumor site compared with cells homing to the spleen.\textsuperscript{22}

To maintain comparability, we kept near identical T cell subset ratios and central memory characteristics for CART versus BsAb-driven T cells at the time of adoptive transfer. We also used a relatively short duration for \textit{in vitro} culture before \textit{in vivo} administration to ensure that the T cells were not terminally differentiated. In our experiments, we observed a balanced CD4\textsuperscript{+} and CD8\textsuperscript{+} TIL phenotype in the BsAb-treated mice; however, CD4\textsuperscript{+} TILs were almost absent in the CART cell-treated mice. Since CD4\textsuperscript{+} T cells play important roles in antitumor response (both direct cytotoxicity and providing help for CD8\textsuperscript{+} T cells\textsuperscript{23,24}), their absence among CAR TILs could compromise antitumor response that is CD4\textsuperscript{+} T cell dependent.

Our data support an advantage of BsAb over CAR in redirecting T cells for tumor therapy. Although there was no obvious difference in their toxicity profiles in these preclinical models, a direct comparison in patients will be necessary before the final verdict on their clinical utility can be decided. While both modalities use polyclonal T cells, CART cell technology is patient-specific where each injection requires custom synthesis, distinct from BsAb which is manufactured and distributed like any other pharmaceutical drug. Unlike liquid tumors, TILs have to survive in the tumor microenvironment and if they cannot persist, single CART injections will unlikely have long-term benefit. Multiple CART cell injections would substantially increase the cost of treatment, defeating its original premise.

Figure 7. The fate of CART cells and BsAb-engaged T cells differs at the tumor site \textit{in vivo}. (A–C) Mice treated similarly as in Fig. 5 but in the absence of \textit{in vivo} IL2 injection were killed one day before and 2 d after the third T cell injection. Splenocytes and tumor infiltrating lymphocytes (TILs) were assessed by flow cytometry the same day. Data were pooled (n = 7 and n = 6 for the CART cell and untransduced T cells plus BC119 (UntT cell + BC119) groups, respectively). Human CD45(+) cells were gated for analysis.
that genetically modified killer cells, unlike BsAb, should persist for years. BsAbs have been through or undergoing phase I clinical trials. The anti-CD19 BsAb format called BiTE® (Blinatumomab) was recently FDA approved. Main toxicities have been cytokine release syndrome, as was observed for CART cells.\(^2\) The GD2-BsAb used in these studies was built with a silent Fc to reduce or to prevent AICD and cytokine storm. If clinically proven to be safe, it could be an alternative or replacement of CART cells.

Materials and methods

**CAR design and vectors**

Humanized 3F8 (hu3F8) and 5F11(F104) are two anti-GD2 antibodies that were affinity-matured to generate hu3F8 (D32H-E1K) and 5F11(Y104) species, respectively.\(^1\)–\(^3\) The anti-GD2 scFv encoding sequences were cloned into an anti-CD19 scFv encoding sequences were cloned into an anti-CD19 CAR construct in place of the anti-CD19 scFv. The original CD19 CAR contained the human CD8\(^+\) leader, CD8\(^+\) hinge-transmembrane domain, the human 4-1BB molecule, and the CD3 \(\zeta\) endodomain, as described previously.\(^4\) The hu3F8, hu3F8(D32H-E1K), 5F11(F104), or 5F11(Y104) CAR cassettes were inserted into BE plasmid\(^2\) using In-Fusion cloning method (Clontech, Mountain View, CA). To generate a 1st-generation hu3F8 CAR, the 4-1BB domain was removed using PCR. The rest of the CARs are 2nd-generation. All experiments were performed with 2nd-generation CARs unless otherwise specified.

**Lentivirus production**

Lentiviruses were produced by transfection of 2 \times 10^7 293T cells using 20 \(\mu\)g lentiviral vector, 10 \(\mu\)g pVSV-G, 20 \(\mu\)g pCMV-dR8.91,\(^2\) and 100 \(\mu\)L PolyJet (SignaGen, Rockville, MD) in 175 cm\(^2\) tissue culture flasks. Briefly, plasmid DNA and PolyJet reagent were mixed with 1.5 mL DMEM medium in two separate 50 mL conical tubes. After 5 min, PolyJet solution was added to the DNA mixture and left at room temperature for 20 min. DNA-PolyJet complex was then admixed with 2 \times 10^7 293T cells resuspended in 1.5 mL DMEM supplemented with 10% fetal bovine serum (FBS, Life Technologies), penicillin/streptomycin, and glutamate. The resulting mixture was incubated at 37°C in a rotator for 30 min before transferred to flasks with 15 mL DMEM culture medium. Medium was exchanged in 24 h. Virus harvested 48 and 72 h after transfection was combined, centrifuged at 25,000 rpm (100,000 G) for 2 h, and resuspended in 1 mL RPMI culture medium.

**T cell transduction**

T cells were purified from human PBMCs using a Pan T-cell isolation kit (Miltenyi Biotec, Auburn, CA) and expanded with CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. RPMI supplemented with 10% heat-inactivated FBS and 30 IU/mL interleukin-2 (IL-2) was used for T cell culture. Three days after activation, 1–4 \times 10^6 of human T cells were transduced with 1 mL of concentrated virus supplemented with protamine sulfate (4\(\mu\)g/mL) and IL-2 (30 IU/mL) followed by rotator shaking for 1 h at 37°C. The concentrated virus was generated by centrifugation of 35 mL of viral supernatant at 100,000 G speed for 2 h. Cells were then seeded into retronectin (Takara Bio USA)-coated (50 \(\mu\)g/mL, 250 \(\mu\)L/well) 24-well plates (1 million/well in 2 mL total volume), centrifuged at 800 g/32°C/90 min, and transferred to incubator at 37°C with 5% CO\(_2\). After 14–16 h, fresh media and IL-2 were added to the cells. For experiments with BsAb, T cells were similarly treated but not transduced with the virus. T cells were split every 2 d for 4–5 d in IL-2 containing media. Then, T cells were harvested, washed, and resuspended in PBS for injection.

**Bispecific antibodies (BsAb)**

The hu3F8 BsAb was designed using VH/VL domains from hu3F8 antibody and huOKT3 scFv fused to the C-terminus of the light chain of a human IgG1 as described previously.\(^17\)–\(^19\) The hu3F8 \(\times\) huOKT3 BsAb was named biclone-119 (BC119).

**Cytotoxicity assay (chromium-51 release assay)**

GD2-expressing (IMR32luc, obtained from American Type Culture Collection (ATCC), Manassas, VA) and GD2-negative leukemia cells (THP1) were cultured in RPMI1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS, Life Technologies) at 37°C in a 5% CO\(_2\) humidified incubator. Adherent cells were harvested with EDTA/trypsin. T cells were purified from human PBMC using Pan T cell isolation kit (Miltenyi Biotec). Target tumor cells were labeled with sodium 51Cr chromate (Amersham, Arlington Height, IL) at 100 \(\mu\)Ci/10^6 cells at 37°C for 1 h. After the cells were washed twice, 5,000 target cells/well were mixed with 50,000 effector cells (hu3F8 CAR or non-modified T) cells at E:T = 10:1 in the presence of serial dilutions of BsAb antibodies, and incubated in 96-well polystyrene round-bottom plates (BD Biosciences) in a final volume of 25 \(\mu\)L/well, at 37°C for 4 h. After centrifugation at 400 g for 10 min, the released 51Cr in the supernatant was counted in a \(\gamma\)-counter (Packed Instrument, Downers Grove, IL). Percentage of specific lysis was calculated using the formula 100% (experimental cpm – background cpm)/total cpm – background cpm), where cpm represented counts per minute of ^51Cr released. Total release was assessed by lysis with 10% SDS (Sigma, St Louis, Mo), and background release was measured in the absence of effector cells.

**Cytokine release assay**

The hu3F8 CART cells or unmodified T cells in the presence of hu3F8-BsAb (80,000/per well) were co-cultured with irradiated IMR32luc or THP1 cell lines (80,000/ per well) in 37°C in 24 well plate. Supernatants were harvested after 24 h of culture. Concentration of cytokines were assessed using an ELISA based cytokine assay kit (OptEIA™ human cytokine set, BD Biosciences, San Jose, CA) according to the manufacturer’s instructions.
**FACS analysis**

Hu3F8/hu3F8(D32H-E1K) and 5F11(F104)/5F11(Y104) CAR expression was detected with rat anti-idiotypic antibodies A1G4 and 1G8 (conjugated to Alexa Fluor 647 (Thermo Fisher Scientific)), respectively. Antihuman antibodies against CD3, CD4+, CD8β, CD45RO, CCR7, LG3, PD1, and CD127 (BD Biosciences) were used to define T cell subpopulations. Stained cells were processed with a FACScalibur instrument (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR). The number of CAR molecule expression on T cells was determined using Quantum™ Simply Cellular® anti-Rat IgG (Bangs Laboratories, Inc.) microbeads according to the manufacturer’s instructions.

**In vivo studies**

All mouse experiments were performed in compliance with the Institutional Animal Care and Use Committee guidelines. The immunodeficient mice BALB-Rag2−/− IL-2R−γc-KO (DKO) were either purchased from Taconic Inc. (Hudson, NY) or maintained at MSK and provided with Amoxicillin food. For tumor challenge experiments, 6- to 8-week-old male mice were inoculated subcutaneously with 4 × 106 GD2+ M14 luc melanoma cell line in Matrigel (BD Biosciences). Activated and transduced hu3F8CAR or untransduced T cells that were in culture for 7 d were intravenously injected weekly for 3 weeks starting 7 d after tumor inoculation. To support T cell survival in vivo, 1000 IU of interleukin-2 (IL2) was subcutaneously injected 3 times per week unless otherwise specified. Tumor size was measured weekly using Peira TM900 imaging device (Peira, Belgium).

**Disclosure of potential conflicts of interest**

For conflict of interest disclosure, anti-GD2 antibodies have been licensed by Memorial Sloan Kettering Cancer Center (MSK) to Ymabs Therapeutics Inc.; both MSK and NK Cheung have financial interest in this company.

**Acknowledgments**

We thank Hoa Tran and Zhihao Wu for their technical support.

**Funding**

This work was supported in part by Funds from Enid A. Haupt Endowed Chair, Kids Walk for Kids with Cancer NYC, Isabella Santos Foundation, Katie Find a Cure Foundation, the Robert Steel Foundation, NIH/NCI Cancer Center Support under Grant P30 CA008748, and funds from Ymabs Therapeutics Inc.

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