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Retargeting T cells to GD2 pentasaccharide on human tumors using bispecific humanized antibody

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Abstract

Anti-disialoganglioside GD2 IgG antibodies have shown clinical efficacy in solid tumors that lack human leukocyte antigens (e.g. neuroblastoma) by relying on Fc-dependent cytotoxicity. However, there are pain side effects secondary to complement activation. T-cell retargeting bispecific antibodies (BsAb) also have clinical potential, but it is thus far only effective against liquid tumors. In this study, a fully humanized hu3F8-BsAb was developed, in which the anti-CD3 huOKT3 single chain Fv fragment (ScFv) was linked to the carboxyl end of the anti-GD2 hu3F8 IgG1 light chain, and was aglycosylated at N297 of Fc to prevent complement activation and cytokine storm. *In vitro*, hu3F8-BsAb activated T cells through classic immunological synapses, inducing GD2-specific tumor cytotoxicity at femtomolar EC₅₀ with >10⁵-fold selectivity over normal tissues, releasing Th1 cytokines (TNF α , IFN γ and IL2) when GD2⁽⁺⁾ tumors were present. In separate murine neuroblastoma and melanoma xenograft models, intravenous hu3F8-BsAb activated T cells *in situ* and recruited intravenous T cells for tumor ablation, significantly prolonging survival from local recurrence or from metastatic disease. Hu3F8-BsAb, but not control BsAb, drove T cells and monocytes to infiltrate tumor stroma. These monocytes were necessary for sustained T-cell proliferation and/or survival and contributed significantly to the antitumor effect. The *in vitro* and *in vivo* antitumor properties of hu3F8-BsAb and its safety profile support its further clinical development as a cancer therapeutic, and provide the rationale for exploring aglycosylated IgG-scFv as a structural platform for retargeting human T cells.

Keywords

Disialoganglioside GD2; Bispecificity; Neuroblastoma; Immunotherapy; Aglycosylation

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INTRODUCTION

Neuroblastoma is a solid tumor model in which monoclonal antibody (MAb) 3F8 specific for the pentasaccharide on diasialoganglioside GD2 has been highly successful in controlling chemoresistant microscopic disease (1). These MAbs activate antibody-dependent FcR-mediated cytotoxicity by recruiting natural killer (NK) cells, myeloid cells and monocyte effectors. The National Cancer Institute program for prioritization of cancer antigens has ranked GD2 at the 12th position in the list of top 75 cancer antigens (2). Its rank becomes higher when only directly targetable antigens are selected. Malignant solid tumors such as melanoma, soft tissue sarcomas, osteosarcoma, and small-cell lung cancer (SCLC) express GD2, although with more heterogeneity than neuroblastoma (3). More recently, GD2 was discovered on neural stem cells, mesenchymal stem cells, and breast cancer stem cells (4). GD2 is rarely expressed in normal tissues except neurons, skin cells and pain fibers. Among patients treated with anti-GD2 antibodies that were followed past 2 decades, there were no long-term side effects (5). A recent randomized phase III trial confirmed the efficacy of anti-GD2 MAb ch14.18, when combined with GM-CSF and IL2, in preventing neuroblastoma relapse among patients in first complete remission (6). Mouse 3F8 (m3F8) was successfully humanized (hu3F8) (7) with a near 10-fold slower K_{off} compared to ch14.18. In the phase I trial, hu3F8 showed high tolerability and low immunogenicity (8). Even among patients previously sensitized to m3F8, human anti-human antibody response remained low or undetectable after repeated challenges with hu3F8.

T cells can suppress or eradicate human cancers (9). Although both m3F8 and hu3F8 could recruit NK cells and myeloid cells for tumor cytotoxicity, without FcR, T cells are not involved (1). The absence or downregulation of human leukocyte antigen (HLA) expression in neuroblastoma is well known for escaping classic T-cell immunity. The low clonal frequency of cytolytic T cells and their inability to home to tumor sites have further emboldened neuroblastoma to elude T cell-based tumor surveillance (1). With few exceptions, T cells are inefficient or incapable of targeting carbohydrate epitopes.

Bispecific antibodies (BsAb) recruit T cells for tumor cytotoxicity through HLA-non-restricted CD3-mediated activation. By engaging polyclonal T cells BsAb can overcome the low clonal frequency of classic T cell-mediated antitumor immunity. BsAb specific for CD3 and tumor antigens, such as CD19, HER2, or EGFR have successfully retargeted T cells (10,11). They induce cytotoxic synapse formation in T cells, mobilizing perforin and granzyme to kill tumors (12). CD3 engagement can also induce T-cell proliferation and generation of effector cytokines to potentiate the antitumor effect (13). Picogram quantities of BsAb can exert significant antitumor effects *in vitro* as well as *in vivo* in preclinical animal models and in patients (10). These antitumor mechanisms can even recruit naïve T cells and stimulate the generation of tumor-specific T cells at tumor sites.

Yet, BsAb could overactivate T cells to discharge toxic cytokine storms, analogous to the overwhelming toxicity from anti-CD28 superagonist antibody (14). OKT3 (muromonab-CD3, Orthoclone OKT3) is a mouse anti-CD3 antibody with decades long safety record in humans (15). It is a proven agent for activating human T cells for *in vitro* expansion. It has also been successfully humanized (huOKT3) to reduce immunogenicity (16). OKT3 has

been used to build BsAb for a number of tumor models, many safely tested in the clinic (17). Various forms of BsAb have been explored; among them, monovalent and bivalent forms made either chemically or genetically. Blinatumomab (BiTE AMG103, CD19-CD3 BsAb), an example of a tandem monovalent scFv, is highly effective at extremely low doses (0.06 mg/m²/day) in the treatment of patients with PreB ALL and NHL with mild cytokine storm and no autoimmune phenomenon, except for the expected depletion of B cells (10). However, by bolus injection it engenders substantial CNS toxicities, although the underlying mechanism remains unclear. In animal models, long-term treatment of mice with BiTE antibody did not result in T-cell anergy or sustained cytokine release (18). BiTE technology has since been applied to other tumor targets, including MSCP (CSPG4) for melanoma, EpCAM for pancreatic CA, CEA for epithelial cancers, and EGFR for colorectal cancer (11,19). Thus far, activation of T cells by BiTE is restricted to tumors expressing the proper target antigen, and clinical efficacy limited to tumors of the blood through targeting CD19 (13). Despite these encouraging preclinical and clinical studies, tandem scFvs have unique drawbacks. Their size (~50 KDa) (20) and their inability to bind neonatal FcRn leads to short serum half-lives. Thus, they require continuous infusion over 4 to 8 weeks to be clinically effective. In addition, as monovalent molecules, scFvs directed at tumor antigens need to have substantially higher affinity.

We and others have previously shown that IgG-scFv (**Figure 1A**) as a tetravalent format for bispecific antibodies can penetrate solid tumors (21,22). Here, the bivalent IgG is derived from a tumor-specific antibody, while scFv with a second specificity is attached to the carboxyl end of the light chain. For most tumor-selective antibodies directed at carbohydrates, bivalency is necessary for optimal tumor targeting, since their Fabs are rarely in the nM or sub-nM range. Similar to IgG (160 KDa), the molecular size of IgG-scFv (~210 KDa) is in favorable balance between systemic clearance and vascular extravasation to achieve maximal tumor uptake (20), while being denied entry into the central nervous system (CNS) because of the blood brain barrier (BBB). In addition, the human IgG backbone allows a reproducible and FDA-approved affinity purification method, as well as binding to FcRn to enhance serum half-life (23).

In this report, we describe the first humanized anti-GD2 BsAb using an IgG-scFv format by attaching the huOKT3-scFv to the carboxyl end of the hu3F8 IgG1 light chain. The IgG backbone was aglycosylated to prevent Fc receptor-mediated cytokine storm (14) as well as pain side effects secondary to complement activation (24). We provide evidence that this hu3F8-BsAb has excellent antitumor activity both *in vitro* and *in vivo*. The IgG-scFv format may provide a versatile platform for building cell-engaging BsAb.

MATERIALS AND METHODS

Cloning and Expression of BsAbs

The hu3F8-BsAb format was designed as a huOKT3 scFv fusion to the C-terminus of the light chain of a human IgG1 (21). Nucleotide sequences encoding V_H and V_L domains from our hu3F8, and the OKT3 scFv were synthesized by GenScript with appropriate flanking restriction enzyme sites, and were subcloned into a mammalian expression vector. Two control BsAbs were built on the same platform, Herceptin-huOKT3 and hu3F8-C825 (22).

Linearized plasmid DNA was used to transfect CHO-S cells (Invitrogen) for stable production of BsAb, similar to that described in our previous report (25). Hu3F8-BsAb titer was determined by ELISA using antigen GD2 and CD3⁽⁺⁾ Jurket cell line, and stable clones with highest expression were selected.

The BsAb producer line was cultured in OptiCHO medium and the mature supernatant harvested. A protein A affinity column (GE Healthcare) was used to purify hu3F8-BsAb as previously described (7), and BsAb was dialyzed into 25 mM sodium citrate, 0.15 M NaCl, pH 8.2 and frozen in aliquots at -80°C. The purity of hu3F8-BsAb was evaluated by both SDS-PAGE (7), and size-exclusion high-performance liquid chromatography (SE-HPLC).

In vitro binding kinetics studies by surface plasmon resonance

In vitro binding affinity for GD2 was assayed by Biacore T-100 Biosensor as previously described (7). Separately, for CD3 affinity, CD3 recombinant protein [CD3δε-Fc (26), also produced in CHO-S cells] as the active surface, and blank as the reference, were immobilized using the Amino Coupling kit (GE Healthcare). Purified hu3F8-BsAb and control antibodies, diluted in HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.05% v/v Surfactant P20), were injected over the sensor surface. The data were analyzed using the Biacore T-100 evaluation software, and the apparent association on rate constant (k_{on}), dissociation off rate constant (k_{off}) and equilibrium dissociation constant ($K_D = k_{off}/k_{on}$) were calculated.

Cell lines

The cell lines LAN-1 and M14 were obtained from University of California, Los Angeles; NMB-7 from Dr. SK Liao of McMaster University, Hamilton, Ontario, Canada. SKNJB, SKNJC2, SKEAW were developed at MSK and all others in Table 1 were purchased from ATCC. They were authenticated by short tandem repeat profiling using PowerPlex 1.2 System (Promega), and periodically tested for mycoplasma using a commercial kit (Lonza). The luciferase-labeled tumor cell lines IMR-32-Luc and M14-Luc were generated by retroviral infection with a SFG-GFLuc vector.

Cytokine release assay

Cytokine release was assayed as previously described (25), using human PBMCs isolated from healthy donor whole blood (New York Blood Center).

Cell cytotoxicity (⁵¹chromium release assay)

Cell cytotoxicity was assayed by ⁵¹Cr release as previously described (25), and EC₅₀ was calculated using SigmaPlot software. Effector T cells were purified from human PBMCs using Pan T cell isolation kit (Miltenyi Biotec), and then activated and expanded with CD3/CD28 Dynabeads (Invitrogen) according to manufacturer's protocol.

Xenograft Studies

All animal procedures were performed in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines. The immunodeficient mice colony BALB-

Rag2^{-/-}IL-2R- γ c-KO (DKO) was maintained at MSK under sterile conditions, and provided with Sulfatrim food. *In vivo* experiments were performed with 6-10 week old mice, and each treatment group included 5 mice. Human PBMCs were prepared as previously described (25). All PBMC samples had similar percentages of T-cell subpopulations (30-50% CD3 positive). PBMC depletion of subpopulations was done with either CD56 or CD14 Microbeads (Miltenyi Biotec), and <0.1 % of target populations remaining after depletion.

(A) c tumor plus sc effector cells (1:1 mixing)—Purified PBMCs or T cells (ATC or freshly prepared) were mixed with IMR-32-Luc cells (1:1 ratio, 5 million each) in Matrigel (BD Biosciences) and implanted subcutaneously (sc) into DKO mice in the right flank. Treatment was started according to the schedules indicated in the Results and Figures. ATCs or freshly prepared T cells were also given IL2 (1000 U ip, 2x/wk x 2 wks) to maintain T-cell survival in the mice. Tumor size was measured by calipers twice per week, and tumor volumes were calculated using the approximated formula (length $\times$ width $\times$ width). % growth was then calculated.

(B) c tumor plus iv effector cells—5 million M14-Luc cells in Matrigel were implanted sc into DKO mice, and other procedures similar to that described in (A) above.

(C) iv tumor plus iv effector cells—IMR-32-Luc cells (0.5 million) or M14-Luc cells (1 million) were inoculated into DKO mice intravenously (iv) via the lateral tail vein. For IMR-32 xenografts, both T-cell groups (ATCs) were given I-2 (1000 U ip, 2x/wk x 3 wks). For M14 xenografts, all effector cell groups (ATCs or PBMCs) were given IL2 (1000 U sc, 2x/wk x 2 wks). Tumor growth was assessed by luciferin bioluminescence once a week. Bioluminescence imaging was conducted using the Xenogen In Vivo Imaging System (IVIS) 200 (Caliper LifeSciences). Briefly, mice were injected iv with 0.1 mL solution of D-luciferin (Gold Biotechnology, 30 mg/mL stock in PBS). Images were collected 1-2 min post injection using the following parameters: a 10-60 seconds exposure time, medium binning, and an 8 f/stop. Bioluminescence image analysis was performed using Living Image 2.6 (Caliper LifeSciences).

(D) Immunohistochemistry staining—The immunohistochemical (IHC) detection was performed at the MSK Molecular Cytology Core Facility using Discovery XT processor (Ventana Medical Systems). Paraffin-embedded tumor sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with CC1 buffer (Ventana Medical Systems) and sections were blocked for 30 minutes with Background Buster solution (Innovex). Anti-CD3 (DAKO, cat# A0452, 1.2 ug/ml), anti-CD4 (Ventana, cat#790-4423, 0.25ug/ml), CD8 (Ventana, cat#790-4460, 0.35ug/ml) and anti-CD68 (DAKO, cat#M0814, 0.02ug/ml) antibodies were applied and sections were incubated for 5 h, followed by 60 minutes incubation with biotinylated goat anti-rabbit IgG (Vector labs, cat#PK6101) for CD3, CD4 and CD8 antibodies or biotinylated horse anti-mouse IgG (Vector Labs, cat# MKB-22258) for CD68 antibodies at 1:200 dilution. The detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer's instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific). Images were captured from tumor sections using Nikon ECLIPSE Ni-U

microscope and NIS-Elements 4.0 imaging software, and counts of IHC-positive cells were averaged from two randomly selected fields (200X magnifications).

Statistics

Differences between samples indicated in the figures were tested for statistical significance by Student's *t*-test, and $p < 0.05$ was considered statistically significant.

* Additional experimental details are described in Supplemental Materials and Methods.

RESULTS

Hu3F8-BsAb design

We designed hu3F8-BsAb using the IgG-scFv format (**Figure 1A**). The heavy chain was identical to that of a hu3F8 IgG1 (7) except for a N297A mutation to remove glycosylation. The light chain was constructed by extending a hu3F8 IgG1 light chain with a C-terminal (G₄S)₃ linker followed by huOKT3 scFv. The DNA encoding both heavy chain and light chain was inserted into a mammalian expression vector, transfected into CHO-S cells, and stable clones of high expression selected. Supernatants were collected from shaker flasks and purified by protein A affinity chromatography. Under reducing conditions, hu3F8-BsAb showed two bands around 50 KDa; besides the heavy chain, huOKT3 scFv fusion increased hu3F8 light chain MW to ~50 KDa (**Figures 1B**). By SEC-HPLC a major peak (85%) showed a MW of 210 KDa, the rest being aggregates removable by gel filtration (**Figures 1C**). Hu3F8-BsAb remained stable after multiple freeze and thaw cycles when assayed by SDS-PAGE and SEC-HPLC (data not shown).

Hu3F8-BsAb binding to both tumor cells and T cells

By FACS hu3F8-BsAb was equally efficient as parental hu3F8 IgG1 in binding to GD2⁽⁺⁾ neuroblastoma cell line LAN1 (**Figure 2A**), although 100-fold less in binding to CD3⁽⁺⁾ T cells when compared to parental huOKT3 IgG1(N297A) (**Figure 2B**). This is consistent with our observation that light chain-anchored scFv has lower avidity for T cells than regular huOKT3 IgG1, in order to minimize nonspecific cytokine release (see below).

The binding affinity by Biacore of hu3F8-BsAb to GD2 (7) showed a k_{on} of $1.57 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, a k_{off} of $9.12 \times 10^{-4} \text{ s}^{-1}$, an overall K_D of 5.8 nM, comparable to that of parental hu3F8 ($1.79 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $2.91 \times 10^{-3} \text{ s}^{-1}$, 16.3 nM, respectively). For CD3 (26), hu3F8-BsAb had a k_{on} of $5.43 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, a k_{off} of $1.05 \times 10^{-1} \text{ s}^{-1}$, an overall K_D of 194 nM, less avid than parental huOKT3 IgG1(N297A) ($1.68 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $1.09 \times 10^{-1} \text{ s}^{-1}$, 64.6 nM, respectively). In summary, hu3F8-BsAb gained in GD2 avidity but displayed a decreased k_{on} for CD3 binding compared to huOKT3.

Hu3F8-BsAb redirected T-cell killing of human tumor cell lines

In a standard 4-hour ⁵¹Cr release assay, hu3F8-BsAb demonstrated potent cytotoxicity against *MYCN* amplified neuroblastoma cell line IMR32 ($EC_{50} = 25 \text{ fM}$, **Figure 3A**). Activated T cells (ATC) plus two negative control BsAbs of the IgG-scFv format, hu3F8-C825 (22) (anti-GD2-DOTA(metal)) and HER2-BsAb (Herceptin-huOKT3) showed

negligible cytotoxicity (**Figure 3A**). When an extensive panel of human tumor cell lines (including neuroblastoma, melanoma, small-cell lung cancer, osteosarcoma, breast carcinoma, Ewing's sarcoma, colon carcinoma, ovarian carcinoma, and rhabdomyosarcoma) were tested, hu3F8-BsAb killing potency (nM to fM range) correlated with tumor GD2 expression by FACS (**Table 1**). Based on the $>10^5$ difference in EC₅₀ on tumor cells versus normal human cardiac myocytes, hepatocytes, adrenal cortical cells, renal mesangial cells, or pulmonary alveolar epithelial cells, the safety margin for hu3F8-BsAb redirected T-cell cytotoxicity was quite wide (**Figure 3B**).

GD2-specific activation of T cells by Hu3F8-BsAb

To further investigate the mechanism of Hu3F8-BsAb-mediated cytotoxicity, we imaged primary human T cells together with IMR32 targets in the presence of various BsAb reagents. Hu3F8-BsAb induced stable T cell-target cell conjugate formation, accompanied by a rapid and robust Ca²⁺ flux within the T cell (**Figure 4A and 4B**). This phenomenon, which is characteristic of cytolytic synapse formation, was not observed in the presence of either hu3F8-C825 or HER2-BsAb, indicating that recognition of both CD3 and GD2 by the BsAb was a prerequisite. To assess the structure of Hu3F8-induced contacts in more detail, we imaged T cells on supported lipid bilayers containing purified GD2 and the adhesion molecule ICAM-1, which binds to the $\alpha_L\beta_2$ integrin LFA-1. These surfaces elicited T cell Ca²⁺ flux in a strictly Hu3F8-BsAb-dependent manner, validating their utility as a GD2⁽⁺⁾ target-cell proxy (**Figure 4C**). Total internal reflection fluorescence microscopy revealed that the contacts formed by Hu3F8-BsAb-treated T cells on the GD2 surfaces contained a central accumulation of TCR surrounded by a peripheral ring of filamentous actin (**Figure 4D and 4E**). This radially symmetric architecture was also observed on positive control bilayers containing directly coated huOKT3, and is characteristic of mature immunological synapses. By contrast, T cells treated with hu3F8-C825 or HER2-BsAb exhibited disorganized contacts with GD2 bilayers similar to that of no antibody treatment (**Figure 4D and 4E**). Taken together with the killing assays described above, these data strongly suggest that Hu3F8-BsAb induces the formation of bona fide cytolytic synapses.

PBMCs after 24 hrs of hu3F8-BsAb activation released pro-tumoricidal Th1 cytokines (TNF α , IFN γ and IL2) only in the presence of GD2⁽⁺⁾ tumor cells, while the Th2 cytokine (IL10) release was less intense (**Figure 4F**). Similar results were obtained with freshly isolated peripheral T cells instead of PBMCs (data not shown).

Efficacy of hu3F8-BsAb in humanized mice

For *in vivo* therapy studies, BALB-*Rag2*^{-/-}IL-2R- γ c-KO (DKO) mice were used for human xenografts (27). In three different humanized mouse xenograft models (sc tumor plus sc effector cells, sc tumor plus iv effector cells, iv tumor plus iv effector cells), iv hu3F8-BsAb showed high activity against established tumors, with either early (starting on day 4-5) or late (starting on day 10) treatment. No clinical neuro-toxicities were observed.

(A) sc effector cells plus sc tumors (1:1 mixing) to simulate T cells residing within tumor (Figure 5A)—IMR-32-Luc (luc = luciferase reporter) cells were mixed (1:1) with either T cells (freshly isolated or ATC), or PBMCs (unactivated from the buffy

coat) and planted subcutaneously. On day 4, treatment with BsAb (5 ug iv, 2x/wk x 2 wks) was started and tumor size was measured. While control BsAb (hu3F8-C825) plus PBMCs had minimal effect, hu3F8-BsAb plus PBMCs was curative (see survival curves, **Supplemental Figure S1**). As effectors, PBMCs were superior to purified fresh T cells, and more effective than ATC in this tumor model.

(B) iv effector cells plus sc tumor to simulate homing of T cells to soft tissue tumor (Figure 5B)—M14-Luc melanoma cells (*BRAF* mutated) were planted sc, and hu3F8-BsAb (40 ug iv, 2x/wk x 3 wks) was started on day 5, while fresh PBMCs (1×10^7 cells iv, q wk x 3 wks) started on day 7. In this more stringent model, hu3F8-BsAb without effector cells or control BsAb with PBMCs was ineffective. Hu3F8-BsAb with PBMCs was curative (**Supplemental Figure S1**).

(C) iv effector cells plus iv tumor to simulate circulating T cells against metastatic disease—In **Figure 5C**, IMR-32-Luc cells were inoculated intravenously to mimic metastatic model, hu3F8-BsAb treatment was started on day 4 (40 ug iv, 2x/wk x 3 wks), 1×10^7 ATC cells (half iv and half ip) started on day 6 (q wk x 3 wks) and tumor luciferin bioluminescence signal was recorded and quantified weekly. ATC in combination with hu3F8-BsAb suppressed tumor progression.

In **Figures 5D and 5E**, DKO mice implanted intravenously with M14-Luc melanoma cells were treated with BsAbs started on day 10 (5 ug, 2x/wk x 2 wks), in combination with either PBMCs or ATCs started on day 12 (1×10^7 cells iv, q wk x 2 wks). Mice treated with saline (Control), BsAb only, or effector cells plus control BsAb had equally rapid tumor progression. Hu3F8-BsAb plus effector cells suppressed tumor progression, regardless if effector cells were PBMCs or ATCs; survival was significantly improved (**Supplemental Figure S1**).

In summary, using freshly isolated or activated T cells we demonstrated that hu3F8-BsAb tumor cytotoxicity indeed functioned through T cells *in vivo*. Fresh PBMCs from healthy donors, which mimicked a more realistic clinical situation, showed even better efficacy with hu3F8-BsAb.

The importance of monocytes for antitumor activity

To address the cellular mechanism as to why PBMCs were more effective than T cells alone, we tested whether monocytes could participate in T-cell tumor infiltration using the sc tumor model described in **Figure 5B**. Tumors were collected 5 days after iv PBMCs and immunohistochemistry (IHC) performed (**Figure 6A**). In the PBMCs plus control BsAb tumors, only circulating T cells in the blood vessels and none inside the tumor were detected. In contrast, PBMCs plus hu3F8-BsAb tumor clearly demonstrated T-cell tumor infiltration by CD3⁽⁺⁾ staining, including both CD4⁽⁺⁾ and CD8⁽⁺⁾ populations. Furthermore, CD68⁽⁺⁾ monocytes in the PBMC population were also found infiltrating the tumor stroma, but only when hu3F8-BsAb was given.

The importance of monocytes in the antitumor effect *in vivo* was tested using the tumor model described in **Figure 5A**, by comparing PBMCs and purified T cells to NK-depleted

PMBCs, and monocyte-depleted PBMCs. While depleting NK cells (CD56⁺ population) showed comparable antitumor effect as PBMCs, depleting monocytes (CD14⁺ population) resulted in a substantial loss in tumor suppression (**Figure 6B**). *In vitro*, depleting monocytes markedly decreased Th1 cytokine release (TNF α and IFN γ) (**Figure 6C**) and tumor cytotoxicity (**Figure 6D**). When T cells were identified in the monocyte-depleted PMBC group by IHC, their numbers were reduced substantially in tumors harvested 9 days after starting BsAb treatment (**Figure 6E** and **Supplemental Figure S2**). We interpret these results to suggest a critical role of monocyte tumor infiltration in sustaining T-cell infiltration, survival or proliferation, directly or indirectly through cytokines, and contributed significantly to the exceptional antitumor effect of BsAb (28).

DISCUSSION

In this study, we described the successful engineering of hu3F8-BsAb to engage polyclonal T cells to target the pentasaccharide of GD2 on tumors not recognizable by classic T cells because of low or absent HLA expression. Using an IgG-scFv platform with proven ability to penetrate solid tumors, a fully humanized BsAb was built, riding on decades of safety records of OKT3 and m3F8, carrying the N297A mutation to prevent Fc receptor-mediated cytokine storm and pain side effects from complement activation. The potency of this BsAb, as well as its tumor selectivity and *in vivo* efficacy in preclinical models was exceptional.

In order to exploit the Fc-independent T cell-mediated effectors, we adopted an IgG-scFv BsAb platform to develop the hu3F8-BsAb. We surveyed a number of uniquely different bivalent formats, including chemical conjugation (29), dual-variable-domain (DVD), or attaching huOKT3 scFv to different positions in the hu3F8 IgG (C-terminal of heavy chain or C-terminal of light chain) (30), and found that the last option gave the best functionality. Although this format has been previously described (21), it has never been used for engagement of T cells for immunotherapy. This hu3F8-BsAb represents a log-fold improvement over the monovalent anti-GD2 5HLDS(15)BA(Y) BsAb (BiTE format) (25), primarily due to the far superior innate binding affinity (K_D) to GD2 (5.8 nM vs 250 nM), resulting in substantially higher *in vitro* killing potency (EC_{50} in fM range vs pM range). Since hu3F8-BsAb is four times the size of the monovalent BiTE format and retains the binding to FcRn (see below), its half-life is measured in days instead of minutes (data not shown). This should obviate the need for continuous infusion to be clinically effective. Indeed, hu3F8-BsAb had much better *in vivo* efficacy than 5HLDS(15)BA(Y), despite dosing only 2-3 times vs 6-7 times weekly (**Supplemental Figure S3A** and **Figure S3B**). The bigger size of the hu3F8-BsAb could also reduce the likelihood of leakage into the CNS and neurotoxicity, which is a major adverse side effect of Blinatumomab.

The study of cellular mechanism behind how hu3F8-BsAb worked *in vivo*, especially in the presence of PBMCs, revealed an important role of monocytes during this T cell-mediated antitumor effect. In the presence of hu3F8-BsAb, T cells extravasated to infiltrate solid tumors (**Figure 6A**). Monocytes followed the track of T cells. Once inside the tumor, these monocytes/macrophages sustained T-cell proliferation and/or survival and also contributed to its antitumor effect (**Figure 6B** and **Figure 6E**). The co-migration of T cells and monocytes into the tumor stroma was highly suggestive of a cross talk between the different

infiltrating white-cell populations, possibly through chemokines or cytokines. Since monocytes or macrophages could not by themselves exploit the tumor specificity of the BsAb (since this BsAb was devoid of all Fc receptor binding), this cross talk was most likely orchestrated by tumor-infiltrating T cells. The role of auxiliary leukocytes in supporting T cell-mediated tumor ablation has been observed recently by other groups, where macrophages and IFN γ were shown to be critical for eradicating large solid tumors by destroying stroma cells that support tumor growth (31).

OKT3 can cause cytokine release syndrome, although manageable and rarely reaching the life-threatening “cytokine storms” experienced by superagonist anti-CD28 TGN1412 (14). Cytokine storm is known to be Fc γ R-dependent, with severity mainly correlating with IL2 and/or IL6 release (14,32,33). The IgG-scFv platform was purposely adopted to reduce CD3 binding to T cells (**Figures 2B**), and N297A (aglycosylation) introduced to eliminate all Fc γ R binding to accessory cells, thereby minimizing spontaneous T-cell activation and cytokine release in the absence of tumor targets (**Figures 4F**). In fact, IL2 and IL6 released by hu3F8-BsAb were comparable to the equivalent monovalent BiTE format, and less than the chemical conjugate of hu3F8-IgG x OKT3 (**Supplemental Figure S4**). While the single N297A mutation eliminated Fc γ R binding, it did not affect protein A or FcRn affinities (34,35), or *in vivo* pharmacokinetics (36). Indeed, hu3F8-BsAb had no Fc γ R binding by Biacore, no ADCC and minimal CMC functions *in vitro*, while retaining Biacore binding to FcRn (data not shown). Since aglycosylated OKT3 is known to be much less mitogenic, triggering only partial signaling both *in vitro* and *in vivo* (15), and aglycosylated humanized anti-CD3 antibody (Otelixizumab) (35) was safe in humans (37), we expect hu3F8-BsAb to have an acceptable safety profile. Another critical consideration is the pain side effects observed during anti-GD2 therapy (5,6), generally ascribed to complement activation (24). Removing CMC in hu3F8-BsAb should reduce this side effect. The absence of pain side effect when T cells were driven by GD2-chimeric antigen receptors (CAR) further supported exploration of this T-cell approach (38). Moreover, aglycosylated BsAb can be produced in non-mammalian or cell-free systems providing significant advantages in speed and cost during manufacture, bypassing problems associated with glycan heterogeneity of conventional antibodies (39).

One highlight of hu3F8-BsAb is the exceptional potency against a wide range of different types of GD2⁽⁺⁾ tumor cells (**Table 1**). With *in vitro* EC₅₀ at femto-molar concentrations (<50 molecules of hu3F8-BsAb per T cell), its potency is more than 1000 fold higher than that of the parental hu3F8 IgG in a typical ADCC assay using CD16-transfected NK92 cells (7). This *in vitro* potency translated into a robust and enduring antitumor effect in xenograft models, prolonging tumor-free survival (**Supplemental Figure S1**). It is reassuring that T cells in the presence of hu3F8-BsAb displayed >10⁵ safety margin (based on EC₅₀) against normal tissue cells including cardiac myocytes, hepatocytes, adrenal cortical cells, renal mesangial cells, or pulmonary alveolar epithelial cells (**Figure 3B**). Similar to m3F8 (5) and hu3F8 IgG1 (40), although hu3F8-BsAb plus T cells showed cytotoxicity to GD2⁽⁺⁾ neurons (data not shown), the absence of acute or long-term neuronal toxicity in anti-GD2 clinical trials was likely due to the blood-brain-barrier.

Although many different platforms of T cell engaging BsAb have been described, only one (Catumaxomab) has been approved for malignant ascites by the European Medicines Agency (EMA), and none so far has been proven to be superior when one balances potency and toxicity (19). When compared to the only anti-GD2 BsAb (Ektomab), built using the Catumaxomab platform and now in preclinical testing, hu3F8-BsAb has substantial differences. Ektomab was built from mouse ME261, with low affinity for GD2 compared to 3F8 (41) and cross-reactive with GD3 (42). Its anti-CD3 was derived from a rat IgG2b antibody. These murine and rat components are expected to induce neutralizing antibodies in humans, and unlikely to allow repeated administrations. Ektomab was built using a quadroma-based format known to be relatively inefficient (19). Its *in vitro* potency (43) was substantially (>500 fold based on EC₅₀) lower than that of hu3F8-BsAb. More importantly, it was made with high Fc affinity for human FcR to enhance binding to dendritic cells (44) in order to produce its vaccination effect (45). With this intact Fc function, it runs a higher risk of cytokine storm syndrome. For example, for Catumaxomab, doses above 150 ug over 6 hours have encountered dose-limiting toxicities consistent with cytokine storm (46).

In summary, this report shows the first demonstration of a successful IgG-scFv BsAb platform for engaging T cells to target carbohydrates for cancer immunotherapy. This BsAb for retargeting T cells is built with structural considerations for bivalency towards the target, no Fc function, minimal spontaneous cytokine release, and long serum half life. With the excellent antitumor activity both *in vitro* and *in vivo*, and the large safety margin, hu3F8-BsAb has considerable clinical potential. This platform may be relevant for other tumor or antigen systems, and likely has broad implications for therapeutic lymphocyte-redirecting bispecific antibodies in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. (A) Schematic diagram of hu3F8-BsAb in IgG-scFv format, (B) Reduced SDS-PAGE, (C) SE-HPLC chromatography: major peak (16.310 min) is the fully-paired BsAb (MW 210 KDa), salt buffer peak (25 min).

Figure 2. Hu3F8-BsAb binding to cells. FACS histograms of binding to (A) LAN-1 neuroblastoma, (B) activated T cells (ATC). Upper left histograms recorded the concentrations of antibodies ($\mu\text{g}/10^6$ cells) and Rituxan was used as negative control (MFI set at 5).

Figure 3. Hu3F8-BsAb redirected T-cell killing by ^{51}Cr release assay of (A) IMR-32 neuroblastoma cells, and (B) five normal human primary cells. ATC to target cell ratio was 10:1.

Figure 4.

Hu3F8-BsAb activation of T cells. **(A-B)** Human T cells loaded with Fura2-AM were preincubated with BsAb and added to wells containing GD2⁽⁺⁾ IMR32 target cells. **(A)** Representative time-lapse montages (time (m:ss) below the last montage) showing T cells contacting individual IMR32 cells (IMR) in the presence of BsAb reagents. Ca²⁺ concentration within the T cells is displayed in pseudocolor, blue indicating low and red indicating high concentrations. **(B)** Average single cell Ca²⁺ responses in T cells attached to IMR32 targets plotted against time. Each curve represents the average of 10 aligned responses. **(C)** Human T cells loaded with Fura2-AM were preincubated with BsAb reagents and plated on bilayers containing ICAM-1 and either GD2 or huOKT3. Ca²⁺ responses were quantified by calculating the average Fura ratio over 4 imaging fields (~ 120 cells) during the plateau phase of the response. **(D-E)** Human T cells preincubated with BsAb reagents and plated on bilayers containing ICAM-1 and either GD2 or huOKT3, were fixed and stained for CD3 and F-actin and then imaged using TIRF microscopy. **(D)** Representative images of CD3 accumulation and F-actin ring formation. Scale bars = 10 μ m. **(E)** Quantification of F-actin ring formation by clearance ratio (n = 20 cells per sample). Error bars denote standard error of the mean (SEM). **(F)** Cytokine release from PBMCs activated by hu3F8-BsAb in the absence or presence of IMR-32 neuroblastoma cells. Three whole IgGs (huOKT3, huOKT3-aGlyco and hu3F8) were used as controls. Mean + SD.

Figure 5. Efficacy of hu3F8-BsAb in humanized DKO mice. Treatment schedules, doses of BsAbs (hu3F8-BsAb or control hu3F8-C825) and effector cells (PBMC, ATC or fresh T cells) were detailed in the Methods and Results. Data shown as mean + SEM (n = 5); * $p < 0.01$ determined by Student's t test when treatment groups (ATC/PBMC + hu3F8-BsAb) were compared with the corresponding control groups, respectively. **(A)** *sc tumor plus sc effector cells (1:1 mixing) model*: % tumor growth of IMR-32 neuroblastoma. **(B)** *sc tumor plus iv effector cells model*: % tumor growth of M14 melanoma. **(C-E)** *iv tumor plus iv effector cells model*: **(C)** Bioluminescence changes of IMR-32 neuroblastoma during treatment. **(D)** Bioluminescence changes of M14 melanoma during treatment and representative images at day 31 **(E)**.

Figure 6.

The role of monocytes in antitumor activity. **(A)** M14 sc tumor model as detailed in Figure 5B, with treatments of one dose of PBMCs (2×10^7 cells iv) at day 15, and two doses of BsAbs (40 ug iv) at days 13 and 16. Representative images of IHC staining of tumor sections collected 5 days after iv PBMCs were shown. **(B)** sc tumor model as detailed in Figure 5A, with 5 millions each of IMR-32 mixed with PBMCs, or subpopulations processed from 5 million PBMCs. PBMCs plus hu3F8-C825 as control group, while all other four groups plus hu3F8-BsAb. Treatment schedule of BsAbs (5 ug iv) were indicated in the figure. **(C)** Cytokine release from the same PBMC subpopulations as described in Figure 6B, activated by hu3F8-BsAb in the presence of M14 cells. **(D)** ^{51}Cr release of the same PBMC subpopulations with M14 cells (E:T = 50:1) in the presence of hu3F8-BsAb. PBMC subpopulations were first incubated with BsAb for 18 h before ^{51}Cr -labeled target cells were added for 4 h. **(E)** Tumors (from groups in Figure 6B) were harvested 9 days after starting BsAb treatment, and CD3 $^{+}$ T cells by IHC staining of tumor sections were counted from two randomly selected fields (200X magnifications).

Table 1

Potency of hu3F8-BsAb

Tumor Type	Cell Line Name	GD2 Expression (MFI)*	EC50 (ng/ml)**	EC50
Neuroblastoma	LAN-1	1467	0.0009	4.5 fM
Neuroblastoma	NMB7	1476	0.001	5 fM
Neuroblastoma	IMR32	1229	0.005	25 fM
Melanoma	HT-144	1227	0.016	80 fM
Small Cell lung Cancer	NCI-H524	2437	0.02	100 fM
Melanoma	SKMEL1	576	0.05	250 fM
Melanoma	M14	745	0.08	400 fM
Neuroblastoma	SKNBE(1)N	509	0.08	400 fM
Osteosarcoma	U2OS	283	0.3	1.5 pM
Neuroblastoma	SKNBE(2)C	478	0.5	2.5 pM
Osteosarcoma	CRL1427	371	0.8	4 pM
Small Cell lung Cancer	NCI-H69	232	0.8	4 pM
Neuroblastoma	SKNJB	196	2	10 pM
Melanoma	SKMEL28	24	4	20 pM
Neuroblastoma	SKNJC2	28	20	100 pM
Breast Carcinoma	MCF7	6	21	105 pM
Ewings	SKEAW	102	150	750 pM
Small Cell lung Cancer	NCI-H345	38	320	1.6 nM
Colon Carcinoma	SW620	6	700	3.5 nM
Breast Carcinoma	AU565	8	>1000	>5 nM
Breast Carcinoma	MDA-MB-361	6	>1000	>5 nM
Breast Carcinoma	SKBR3	7	>1000	>5 nM
Breast Carcinoma	MDA-MB-231	5	>1000	>5 nM
Breast Carcinoma	MDA-MB-468	6	>1000	>5 nM
Ewings	SKES-1	24	>1000	>5 nM
Ovarian Carcinoma	OVCAR3	4	>1000	>5 nM
Ovarian Carcinoma	SKOV3	5	>1000	>5 nM
Rhabdomyosarcoma	HTB82	8	>1000	>5 nM

* FACS analysis using hu3F8 IgG1, with Rituxan as negative control (MFI set at 5).

** 4 hour ⁵¹Cr release assay at 10:1 E:T ratio. Maximum antibody concentration at 1 ug/ml. EC50 (concentration of antibody at half maximal killing) was calculated using SigmaPlot.