Retargeting T Cells to GD2 Pentasaccharide on Human Tumors Using Bispecific Humanized Antibody

Hong Xu1, Ming Cheng1, Hongfen Guo1, Yuedan Chen2, Morgan Huse2, and Nai-Kong V. Cheung1

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-GD3 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 cytotoxic action. In vitro, hu3F8-BsAb activated T cells through classic immunologic synapses, inducing GD2-specific tumor cytotoxicity at femtomolar EC50 with 10-fold selectivity over normal tissues, releasing Th1 cytokines (TNFα, IFNγ, 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.

Introduction

Neuroblastoma is a solid tumor model in which monoclonal antibody (mAb) 3F8 specific for the pentasaccharide on disialoganglioside 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 NCI (Bethesda, MD) 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 sarcoma, 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 for the past two 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; ref. 7) with a near 10-fold slower koff compared with 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 homed 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-nonrestricted 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

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potentiate the antitumor effect (13). Picogram quantities of BsAb can exert significant antitumor effects \textit{in vitro} as well as \textit{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 an 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 \textit{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 are 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/d) in the treatment of patients with pre-B acute lymphoblastic leukemia and non-Hodgkin lymphoma with mild cytokine storm and no autoimmune phenomenon, except for the expected depletion of B cells (10). However, by bolus injection, it engenders substantial central nervous system (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 chondroitin sulfate proteoglycan 4 (CSPG4) for melanoma, EpCAM for pancreatic carcinoma, carcinomaembryonic antigen (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 is 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; ref. 20) and their inability to bind to 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 (Fig. 1A) as a tetravalent format for BsAb can penetrate solid tumors (21, 22). Here, the bivalent IgG is derived from a tumor-specific antibody, whereas 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, because their Fabs are rarely in the nmol/L or sub-nmol/L 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 penetration.
uptake (20), while being denied entry into the 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 Fc receptor to enhance serum half-life (23).

In this article, 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\(_{\text{H}}\) and V\(_{\text{L}}\) 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 transfet CHO-S cells (Invitrogen) for the stable production of BsAb, similar to that described in our previous report (25). Hu3F8-BsAb titer was determined by ELISA using antigen GD2 and the OKT3 scFv, similar to that described in our previous report (25). Hu3F8-BsAb was purified on a Protein A column (GE Healthcare) was used to purify hu3F8-BsAb as previously described (7). The purity of hu3F8-BsAb was evaluated by both SDS-PAGE (7) and size-exclusion high-performance liquid chromatography (SE-HPLC).

In vitro binding kinetic 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 (CD3\(_{\text{c}}\)-Fc; ref. 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 mol/L HEPES, pH 7.4, 0.15 mol/L NaCl, 3 mmol/L 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 (\(k_{\text{on}}\)), dissociation off-rate constant (\(k_{\text{off}}\)), and equilibrium dissociation constant (\(K_D = k_{\text{off}}/k_{\text{on}}\)) were calculated.

Cell lines

The cell lines LAN-1 and M14 were obtained from the University of California, Los Angeles (Los Angeles, CA); NMB-7 from Dr. SK Liao of McMaster University (Hamilton, ON, Canada), SKNB, SKNJC2, and SKEAW were developed at the Memorial Sloan Kettering and all others in Table 1 were purchased from the ATCC. They were authenticated by short-tandem repeat profiling using the PowerPlex 1.2 System (Promega) and were 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 an SFG-GFluc vector.

Cytokine release assay

Cytokine release was assayed as previously described (25), using human peripheral blood mononuclear cells (PBMC) isolated from the whole blood of healthy donors (New York Blood Center).

Cell cytotoxicity (chromium-51 release assay)

Cell cytotoxicity was assayed by \(^{51}\text{Cr}\) release as previously described (25), and EC\(_{50}\) was calculated using SigmaPlot software. Effector T cells were purified from human PBMCs using a Pan T-cell isolation kit (Miltenyi Biotec), and then activated and expanded with CD3/CD28 Dynabeads (Invitrogen) according to the manufacturer’s protocol.

Xenograft studies

All animal procedures were performed in compliance with the Institutional Animal Care and Use Committee guidelines. The immunodeficient mouse colony BALB-Rag2\(^{-/-}\)/IL-2R-γc-KO (DKO) was maintained at MSK under sterile conditions, and provided with Sulfatrim food. In vivo experiments were performed with 6- to 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 deple-tion of subpopulations was done with either CD56 or CD14 Microbeads (Miltenyi Biotec), and <0.1% of target populations remained after depletion.

Subcutaneous tumor plus subcutaneous effector cells (1:1 mixing)

Purified PBMCs, activated T cells (ATCs), or freshly prepared T cells were mixed with IMR-32-Luc cells (1:1 ratio, 5 million each) in Matrigel (BD Biosciences) and implanted s.c. into DKO mice in the right flank. Treatment was started according to the schedule indicated in the Results and figures. ATCs or freshly prepared T cells were also given IL2 (1,000 UI i.p., 2 × wk \(^{-1} \times 2 \text{ weeks}) 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 \(V = 0.5 \times (\text{length} \times \text{width} \times \text{width})\). The percentage of growth was then calculated.

Subcutaneous tumor plus intravenous effector cells

Five million M14-Luc cells in Matrigel were implanted s.c. into DKO mice, and other procedures were similar to those described in the previous paragraph.

Intravenous tumor plus intravenous effector cells

IMR-32-Luc cells (0.5 million) or M14-Luc cells (1 million) were inoculated into DKO mice i.v. via the lateral tail vein. For IMR-32 xenografts, both T-cell groups (ATCs) were given IL2 (1,000 UI i.p., 2 × wk \(^{-1} \times 3 \text{ weeks}) for M14 xenografts, all effector cell groups (ATCs or PBMCs) were given IL2 (1,000 UI s.c., 2 × wk \(^{-1} \times 2 \text{ weeks}) Tumor growth was assessed by luciferin bioluminescense once a week. Bioluminescence imaging was conducted using the Xenogen In Vivo Imaging System (IVIS) 200 (Caliper LifeSciences). Briefly,
mice were injected i.v. with 0.1 ml solution of α-luciferin (Gold Biotechnology; 30 mg/mL stock in PBS). Images were collected 1 to 2 minutes after injection using the following parameters: a 10- to 60-second exposure time, medium binning, and an 8 f/stop. Bioluminescence image analysis was performed using Living Image 2.6 (Caliper LifeSciences).

**Immunohistochemistry staining.** The immunohistochemical (IHC) detection was performed at the MSK Molecular Cytology Core Facility using a 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). Parafﬁn-embedded tumor sections were deparafﬁnized with EZPrep buffer (Ventana Medical Systems), and antigen retrieval with EZPrep buffer (Ventana Medical Systems). Immunohistochemistry staining was performed at the MSK Molecular Cytology Core Facility using a Discovery XT processor (Ventana Medical Systems). The immunohistochemical (IHC) detection was performed at the MSK Molecular Cytology Core Facility using a 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 antigen retrieval with EZPrep buffer (Ventana Medical Systems). Immunohistochemistry staining was performed at the MSK Molecular Cytology Core Facility using a Discovery XT processor (Ventana Medical Systems).

**Results**

**Hu3F8-BsAb design**

We designed hu3F8-BsAb using the IgG-scFv format (Fig. 1A). The heavy chain was identical to that of a hu3F8 IgG1 (7), except for an 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 were selected. Supernatants were 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 the GD2(+) neuroblastoma cell line LAN1.

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**Table 1. Potency of hu3F8-BsAb**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Cell line</th>
<th>GD2 expression (MFI)*</th>
<th>EC50 (ng/mL)b</th>
<th>EC90 (mg/mL)b</th>
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<tr>
<td>Neuroblastoma</td>
<td>LAN-1</td>
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<td>1,229</td>
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<tr>
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<td>0.0200</td>
<td>100 fmol/L</td>
</tr>
<tr>
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<td>0.0500</td>
<td>250 fmol/L</td>
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<td>400 fmol/L</td>
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<tr>
<td>Neuroblastoma</td>
<td>SKMBE(1)N</td>
<td>509</td>
<td>0.0800</td>
<td>400 fmol/L</td>
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<td>Osteosarcoma</td>
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<td>0.3000</td>
<td>1.5 pmo/L</td>
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<tr>
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<td>478</td>
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<tr>
<td>Rhabdomyosarcoma</td>
<td>HTB82</td>
<td>8</td>
<td>&gt;1,000.0000</td>
<td>&gt;5 nmo/L</td>
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</tbody>
</table>

Abbreviation: MFI, mean fluorescence intensity.

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

**Four-hour 51Cr release assay at 10:1 E:T ratio. Maximum antibody concentration at 1 μg/mL. EC50 (concentration of antibody at half maximal killing) was calculated using SigmaPlot.**
(Fig. 2A), although 100-fold less in binding to CD3(+) T cells when compared with parental huOKT3 IgG1(N297A; Fig. 2B). This is consistent with our observation that light chain–anchored scFv has lower avidity for T cells than regular huOKT3 IgG1, to minimize nonspecific cytokine release (see below).

The binding affinity by Biacore of hu3F8-BsAb to GD2 (7) showed a $k_{on}$ of $1.57 \times 10^7/(mol/L)$-s, a $k_{off}$ of $9.12 \times 10^{-4}/s$, an overall $K_D$ of 5.8 nmol/L, comparable with that of parental hu3F8 (1.79 $\times$ 10^7/(mol/L)-s, 2.91 $\times$ 10^{-3}/s, and 16.3 nmol/L, respectively). For CD3 (26), hu3F8-BsAb had a $k_{on}$ of 5.43 $\times$ 10^5/(mol/L)-s, a $k_{off}$ of 1.05 $\times$ 10^{-1}/s, an overall $K_D$ of 194 nmol/L, less avid than parental huOKT3 IgG1(N297A; 1.68 $\times$ 10^6/(mol/L)-s, 1.09 $\times$ 10^{-2}/s, and 64.6 nmol/L, respectively). In summary, hu3F8-BsAb gained in GD2 avidity but displayed a decreased $k_{on}$ for CD3 binding compared with huOKT3.

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

In a standard 4-hour $^{51}$Cr release assay, hu3F8-BsAb demonstrated potent cytotoxicity against the MYCN-amplified neuroblastoma cell line IMR32 (EC$_{50}$, 25 fmol/L; Fig. 3A). ATCs plus two negative control BsAbs of the IgG-scFv format, hu3F8-C825 [ref. 22; anti-GD2-DOTA (metal)] and HER2-BsAb (Herceptin-huOKT3), showed negligible cytotoxicity (Fig. 3A). When an extensive panel of human tumor cell lines (including neuroblastoma, melanoma, SCLC, osteosarcoma, breast carcinoma, Ewing sarcoma, colon carcinoma, ovarian carcinoma, and rhabdomyosarcoma) were tested, hu3F8-BsAb killing potency (nmol/L to fmol/L range) correlated with tumor GD2 expression by FACS (Table 1). On the basis of the $>10^3$ difference in EC$_{50}$ 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 (Fig. 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 Ca$^{2+}$ flux within the T cell (Fig. 4A and B). 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 αβ, integrin LFA-1. These surfaces elicited T-cell Ca2+ influx in a strictly Hu3F8-BsAb-dependent manner, validating their utility as a GD2(+) target-cell proxy (Fig. 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 T-cell receptors surrounded by a peripheral ring of filamentous actin (Fig. 4D and E). This radially symmetric architecture was also observed on positive control bilayers containing directly coated huOKT3, and is characteristic of mature immunologic synapses. In contrast, T cells treated with hu3F8-C825 or HER2-BsAb exhibited disorganized contacts with GD2 bilayers similar to that of no antibody treatment (Fig. 4D and E). 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 hours of hu3F8-BsAb activation, released protumoricidal Th1 cytokines (TNFα, IFNγ, and IL2) only in the presence of GD2(+) tumor cells, whereas the Th2 cytokine (IL10) release was less intense (Fig. 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, DKO mice were used for xenografts (27). In three different humanized mouse xenograft models (s.c. tumor plus s.c. effector cells, s.c. tumor plus i.v. effector cells, and intravenous i.v. tumor plus i.v. effector cells), i.v. 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 neurotoxicities were observed.

Subcutaneous effector cells plus subcutaneous tumors (1:1 mixing) to simulate T cells residing within tumor. IMR-32-Luc (Luc, luciferase reporter) cells were mixed (1:1) with either T cells (freshly isolated or ATC), or PBMCs (unactivated from theuffy coat) and planted s.c. On day 4, treatment with BsAb (5 µg i.v., 2×/wk × 2 weeks) was started and tumor size was measured. Although control BsAb (hu3F8-C825) plus PBMCs had minimal effect, hu3F8-BsAb plus PBMCs was curative (see survival curves; Supplementary Fig. S1). As effectors, PBMCs were superior to purified fresh T cells, and more effective than ATC in this tumor model (Fig. 5A).

Intravenous effector cells plus subcutaneous tumor to simulate homing of T cells to soft-tissue tumor. M14-Luc melanoma cells (BRAF-mutated) were planted s.c., and hu3F8-BsAb (40 µg i.v., 2×/wk × 3 weeks) was started on day 5, while fresh PBMCs (1×107 cells i.v., once weekly × 3 weeks) 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 (Fig. 5B; Supplementary Fig. S1).

Intravenous effector cells plus intravenous tumor to simulate circulating T cells against metastatic disease. In Fig. 5C, IMR-32-Luc cells were inoculated i.v. to mimic metastatic model, hu3F8-BsAb treatment was started on day 4 (40 µg i.v., 2×/wk × 3 weeks), 1×107 ATCs (half i.v. and half i.p.) started on day 6 (once weekly × 3 weeks) and tumor luciferin bioluminescence signal was recorded and quantified weekly. ATC in combination with hu3F8-BsAb suppressed tumor progression.

In Fig. 5D and E, DKO mice implanted i.v. with M14-Luc melanoma cells were treated with BsAbs started on day 10 (5 µg, 2×/wk × 2 weeks), in combination with either PBMCs or ATCs started on day 12 (1×107 cells i.v., once weekly × 2 weeks). 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 (Supplementary Fig. S1).

In summary, using freshly isolated T cells or ATCs, 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 s.c. tumor model described in Fig. 5B. Tumors were collected 5 days after i.v. PBMCs and IHC was performed (Fig. 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 Fig. 5A, by comparing PBMCs and purified T cells to NK-depleted PBMCs, and monocyte-depleted PBMCs. Although depleting NK cells (CD56+ population) showed comparable antitumor effect as PBMCs, depleting monocytes (CD14+ population) resulted in a substantial loss in tumor suppression (Fig. 6B). In vitro, depleting monocytes markedly decreased Th1 cytokine release (TNFα and IFNγ; Fig. 6C) and tumor cytotoxicity (Fig. 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 (Fig. 6E and Supplementary Fig. 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 contributing 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 and lacking the mediators of cytokine storm and pain side effects from complement activation. The potency of this BsAb, as well as its
Figure 4.
Hu3F8-BsAb activation of T cells. A and 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 four imaging fields (≥120 cells) during the plateau phase of the response. D and 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 μm. E, quantification of F-actin ring formation by clearance ratio (n = 20 cells/sample). Error bars denote standard error of the mean (SEM). *** P < 0.001, extremely statistically significant; ns, not statistically significant, between the indicated groups, respectively. F, cytokine release from PBMCs activated by hu3F8-BsAb in the absence or presence of IMR-32 neuroblastoma cells. Three whole IgGs (huOKT3, huOKT3-αGlyco, and hu3F8) were used as controls. Mean ± SD.
tumor selectivity and in vivo efficacy in preclinical models, was exceptional.

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; ref. 30), and found that the last option gave the best functionality. Although this format has been previously described

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) are detailed in Materials and Methods and Results. Data shown as mean ± SEM (n = 5); * P < 0.01 determined by the Student t test when treatment groups (ATC/PBMC + hu3F8-BsAb) were compared with the corresponding control groups, respectively. A, s.c. tumor plus subcutaneous effector cells (1:1 mixing) model: % tumor growth of IMR-32 neuroblastoma. B, s.c. tumor plus i.v. effector cells model: % tumor growth of M14 melanoma. C–E, i.v. tumor plus i.v. effector cells model: C, bioluminescence changes of IMR-32 neuroblastoma during treatment; D, bioluminescence changes of M14 melanoma during treatment; E, representative images at day 31.
(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; ref. 25), primarily due to the far superior innate binding affinity (K_D) to GD2 (5.8 vs. 250 nmol/L), resulting in substantially higher in vitro killing potency (EC50 in fmol/L range vs. pmol/L range). Because 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.

Figure 6.
The role of monocytes in antitumor activity: A, M14 s.c. tumor model as detailed in Fig. 5B, with treatments of one dose of PBMCs (2 × 10^7 cells i.v.) at day 15, and two doses of BsAbs (40 μg i.v.) at days 13 and 16. Representative images of IHC staining of tumor sections collected 5 days after i.v. PBMCs were shown. B, s.c. tumor model as detailed in Fig. 5A, with 5 million 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 μg i.v.) were indicated in the figure. C, cytokine release from the same PBMC subpopulations as described in Fig. 6B, activated by hu3F8-BsAb in the presence of M14 cells. D, %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 hours before %Cr-labeled target cells were added for 4 hours. E, tumors (from groups in Fig 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 (×200 magnifications).
to be clinically effective. Indeed, hu3F8-BsAb had much better \textit{in vivo} efficacy than 5HLD(15)BA(Y), despite dosing only two to three times versus six to seven times weekly (Supplementary Fig. S3A and 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 \textit{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 (Fig. 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 (Fig. 6B and E). 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. Because monocytes or macrophages could not by themselves exploit the tumor specificity of the BsAb (because 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, who showed that macrophages and IFNγ were 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 FcR-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 (Fig. 2B), and N297A (aglycosylation) introduced to eliminate all FcR binding to accessory cells, thereby minimizing spontaneous T-cell activation and cytokine release in the absence of tumor targets (Fig. 4F). In fact, IL2 and IL6 released by hu3F8-BsAb were comparable with the equivalent monovalent BITE format, and less than the chemical conjugate of hu3F8-IgG × OKT3 (Supplementary Fig. S4). Although the single N297A mutation eliminated FcR-binding, it did not affect protein A or FcRn affinities (34, 35), or \textit{in vivo} pharmacokinetics (36). Indeed, hu3F8-BsAb had no FcR binding by Biacore, no antibody-dependent cell-mediated cytotoxicity (ADCC), and minimal complement-mediated cytotoxicity (CMC) functions \textit{in vitro}, while retaining Biacore binding to FcRn (data not shown). Because aglycosylated OKT3 is known to be much less mitogenic, triggering only partial signaling both \textit{in vitro} and \textit{in vivo} (15), and aglycosylated humanized anti-CD3 antibody (otelixizumab; ref. 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 nonmammalian 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 \textit{in vitro} EC\textsubscript{50} at femtomolar concentrations (<50 molecules of hu3F8-BsAb per T cell), its potency is more than 1,000-fold higher than that of the parental hu3F8 IgG in a typical ADCC assay using CD16-transfected NK92 cells (7). This \textit{in vitro} potency translated into a robust antitumor effect in xenograft models, prolonging tumor-free survival (Supplementary Fig. S1). It is reassuring that T cells in the presence of hu3F8-BsAb displayed >10\textsuperscript{5} safety margin (based on EC\textsubscript{90}) against normal tissue cells including cardiac myocytes, hepatocytes, adrenal cortical cells, renal mesangial cells, or pulmonary alveolar epithelial cells (Fig. 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 BBB.

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, and none so far has been proven to be superior when one balances potency and toxicity (19). When compared with 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 ME361, with low affinity for GD2 compared with 3F8 (41) and cross-reactive with GD3 (42). Its anti-C3d 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 \textit{in vitro} potency (43) was substantially (>500-fold based on EC\textsubscript{50}) 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) 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 μg over 6 hours have encountered dose-limiting toxicities consistent with cytokine storm (46).

In summary, this study 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 toward the target, no Fc function, minimal spontaneous cytokine release, and long serum half-life. With the excellent antitumor activity both \textit{in vitro} and \textit{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 BsAb in general.

**Disclosure of Potential Conflicts of Interest**

NK. V. Cheung has ownership interest (including patents) in scFv constructs of anti-GD2 antibodies, therapy enhancing glucan, use of mAb 8H9, methods for preparing and using scFv, GD2 peptide mimics, methods for detecting MRD, anti-GD2 antibodies, generation and use of HLA-A2–restricted peptide-specific mAbs and CARs, high-affinity anti-GD2 antibodies, and multimerization technologies. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Xu, M. Cheng, Y. Chen, M. Huse, NK. V. Cheung

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Retargeting T Cells to GD2 Pentasaccharide on Human Tumors Using Bispecific Humanized Antibody

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