Enhancing the anti-tumor efficacy of Bispecific T cell engagers via cell surface glycocalyx editing

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Abstract: Bispecific T-cell engager (BiTE)-based cancer therapies that activate the cytotoxic T cells of a patient's own immune system have gained momentum with the recent FDA approval of Blinatumomab for treating B cell malignancies. However, this approach has had limited success in targeting solid tumors. Here, we report the development of BiTE-sialidase fusion proteins that enhance tumor cell susceptibility to BiTE-mediated cytolysis by T cells via selective desialylation at the T cell-tumor cell interface that results in better immunological synapse formation. We show that a BiTE-sialidase fusion protein targeting human epidermal growth factor receptor 2 (HER2) exhibits remarkably increased efficacy in terms of killing HER2 positive tumor cells when compared to the BiTE alone. This enhanced function is seen both *in vitro* and in an *in vivo* xenograft solid tumor model. We feel that BiTE-sialidase fusion proteins have great potential as candidates for the development of next generation bispecific T-cell engaging molecules for cancer immunotherapy.

Introduction

A central theme in cancer immunotherapy is the activation of a patient's own immune system for tumor control. Bispecific T cell engagers (BiTEs) are off-the-shelf agents that recruit endogenous CD8+ and CD4+ T cells capable of eradicating tumor cells in a manner that is independent of the major histocompatibility complex (MHC)(1). A BiTE molecule consists of two single-chain variable fragments (scFvs), one targeting a tumor-associated antigen, while the other binds to CD3 on T cells. These two scFvs are covalently connected by a small linker peptide. Blinatumomab, which targets the CD19 antigen present on B cells, is the first BiTE approved by the US Food and Drug Administration (FDA), and is used to treat B-cell precursor acute lymphoblastic leukemia (ALL) in patients with residual cancer following chemotherapy(2).

However, as with most T cell-based therapies, the promise of BiTEs in the treatment of solid tumors has yet to be realized(3). In addition to the problem of limited penetration into the tumor tissue, T cell-based therapies must overcome the immunosuppressive tumor microenvironment, where T-cell suppression is orchestrated by tumor cells and the neighboring stromal myeloid and lymphoid cells(4). In this unique microenvironment, limited availability of nutrients and accumulated metabolic waste products lead to alterations in cell-surface epitopes of both tumor and immune cells, which subsequently alters their interactions and ultimately leads to T cell exhaustion and poor tumor control(5). Therefore, enabling approaches that target the molecular and cellular components of the immunosuppressive tumor microenvironment may transform T cell-based cancer treatments, including those based on T cell engaging technologies.

Aberrant glycosylation is a hallmark of cancer(6-9). Tumor cells often upregulate characteristic glycoforms with terminal sialic acid, which can then act as glyco-immune checkpoints to suppress immune activation(10, 11). Sialosides attenuate immune cell activation and effector function by recruiting sialic acid-binding Ig-like lectins (Siglecs) that are found on most leukocytes to the immunological synapse, where they can trigger inhibitory signaling(12, 13). In addition, sialo-glycans expressed on T cells and antigen-presenting cells (APCs) may interact with CD28 on T cells to compete with its binding to CD80 on APCs, resulting in suppression of the co-stimulation required for T cell activation and survival(14).

Previous studies by Adema and coworkers demonstrated that selective inhibition of cell-surface sialylation in the tumor microenvironment via intra-tumoral administration of an unnatural sialic acid mimic is a powerful intervention that potentiates cancer killing by T cells, while reducing the infiltrating regulatory T cells and myeloid suppressor cells(15). Sialoside blockade enhanced antigen-specific CD8+ T cell-mediated cytolysis of tumor cells, in part by facilitating clustering of tumor cells with T cells. Inspired by this and other work indicating the importance of hyper-sialylation in T cell-induced killing(16), herein, we report the development of BiTE-sialidase fusion proteins that specifically remove sialo-glycans at the T cell-tumor cell interface, leading to increased T cell-dependent tumor cell cytolysis. We also demonstrate that the enhanced tumor cell cytolysis is independent of the inhibitory sialo-glycan-Siglec

signaling, but leads to stronger immunological synapse formation induced by BiTEs. Furthermore, using a solid tumor xenograft mouse model, we show that a HER2-targeting BiTE-sialidase fusion protein exhibits superior efficacy in the *in vivo* control of tumor proliferation when compared to the parent HER2-targeting BiTE.

Results

Removal of sialic acids on the surface of tumor cells enhances BiTE-mediated tumor cell killing by T cells

To evaluate whether desialylation may enhance the susceptibility of tumor cells to BiTE-mediated cytotoxicity by T cells, we first constructed a BiTE molecule from a HER2-targeting ScFv 4D5 and a human CD3-targeting ScFv (4D5 BiTE). We then treated HER2 positive SK-BR-3 human breast cancer cells with a sialidase derived from *Bifidobacterium longum subspecies infantis (B. infantis)* to remove cell-surface sialic acids. Staining with FITC-*Sambucus nigra agglutinin* (SNA), that binds preferentially to sialic acid attached to terminal galactose in an α-2,6 linkage, confirmed the success of cell-surface desialylation (Fig. 1A). Next, we incubated SK-BR-3 cells with 4D5 BiTE and PBMCs from healthy human donors in the presence or absence of *B. infantis* sialidase. At different effector (E) to target (T) ratios the addition of sialidase markedly potentiated the 4D5 BiTE-induced tumor cell killing by T cells compared with 4D5 BiTEs alone (Fig. 1B and Figure S1A). The improvement of the BiTE-induced killing was sialidase dose-dependent (Figure S1B). A similar trend was observed when another HER2 positive breast cancer cell line, MCF-7, was used as the target cell and when 4D5 BiTE treatment was combined with a sialylation inhibitor, P-3Fax-Neu5Ac (Figure 1B and 1C). To verify the enhancement of BiTE-induced killing following sialidase treatment, another BiTE molecule, PSMA BiTE, that targets Prostate-Specific Membrane Antigen (PSMA) was constructed. Again, strong cytotoxicity enhancement was observed when sialidase and BiTEs were added simutaneously (Figure 1D). To confirm that the effect of BiTEs on tumor cell killing is mediated through their interaction with T cells, we repeated the killing assay with MCF-7 cells using purified T cells. To our satisfaction, the sialidase treatment indeed led to better BiTE-mediated target cell killing (Figure S1C). Moreover, the addition of the sialidase also significantly enhanced IFN-γ secretion and T cell activation (Figure 1E and F).

Tumor cell desialylation promotes stronger BiTE-mediated immune synapse (IS) formation between T cells and tumor cells

To elucidate the mechanism underlying the potentiation of BiTE-induced cytotoxicity by desialylation, we first investigated if the sialoglycan-Siglec (Sialic acid-binding immunoglobulin-type lectin) inhibitory pathway is involved. Through their interaction with sialylated glycans aberrantly expressed on tumor cells, immune cell-associated Siglecs trigger signaling cascades to suppress immune cell activation and effector functions(17, 18). Stanczak *et al.* observed increased T cell-dependent

cytotoxicity of blinatumomab, a CD19-targeting BiTE, and catumaxomab, an Epithelial Cell Adhesion Molecule (EpCAM) targeting trifunctional antibody, against target tumor cells deficient in sialic acid(16). They argued that the increased potency is due to the alleviation of the inhibitory signals mediated by the sialoglycan-Siglec-9 interaction. However, consistent with previous reports, we found that T cells from PMBCs of multiple healthy donors expressed negligible levels of Siglec-7 and Siglec-9 as compared to their CD3 negative counterparts that mainly consist of B cells, NK cells, monocytes and dendritic cells (Figure 2B). Nevertheless, we did observe a slight up-regulation of both Siglec-9 and Siglec-7 following T cell activation. When compared to the expression of these Siglecs on freshly isolated CD3 negative cells, the expression of Siglec-7 and -9 on activated T cells was still minimal (Figure 2A). To further test if the Siglec-9 inhibitory pathway plays a role in BiTE-induced T cell killing, a Siglec-9 blocking antibody was added with 4D5 BiTEs and the level of target cell killing was analyzed. In contrast to sialidase addition, blocking of the Siglec-9 signals did not increase cytotoxicity, indicating a negligible role of Siglec-9 in the BiTE-induced T cell killing (Figure 2C). Although the killing process mediated by BiTEs is often considered independent of the CD28 costimulatory signal, several studies have reported therapeutic advantages when T cell engager molecules are coupled with an additional CD28 recruiting moiety(19-21). In addition, as demonstrated by Paulson and coworkers, sialoglycans can block the interaction of CD28 with CD80, resulting in dampened costimulatory signaling(14). To investigate whether the enhanced BiTE-induced killing seen with desialylation is affected by CD28 co-stimulation, the CD28-CD80 interaction was blocked by adding a high affinity ligand of CD80, recombinant human CTLA-4. However, even with the addition of high concentrations of CTLA-4 no change in the enhanced cytolysis was detected (Figure 2D).

Formation of the BiTE-induced immunological synapse (IS) between target cells and T cells is the essential mode of action of BiTEs. We hypothesized that the removal of cell-surface sialosides may lead to stronger BiTE-induced IS formation between target tumor cells and T cells, and thus, promote better tumor cell killing. Accumulation of the TCR-CD3 complex and F-actin at the synapse is a hallmark of a stable and functional cytolytic IS in T cells. To test whether desialylation can enhance IS formation, we imaged the IS formed between T cells with sialidase-treated and non-treated SK-BR-3 cells by staining F-actin and CD3ζ. The resulting immunofluorescence was imaged by confocal microscopy. As shown in Figure 2F, we observed visually larger BiTE-induced IS formation between T cells and desialylated SK-BR-3 cells. To assess the stability of the IS formed, we calculated the relative CD3 fluorescence intensity at the IS and the relative area of the IS. The IS formed by sialidase-treated tumor cells and T cells showed significantly stronger CD3 accumulation and larger IS contact area compared to IS formed by untreated tumor cells and T cells (Figure 2G and H). The same trend was observed for BiTE-induced IS formation between SKOV-3 cells and T cells, with stronger IS formed following sialidase treatment. (Figure S2).

The interaction between CD2 and CD58 is known to play a critical role in the formation of a productive immunological synapse(22-24). We found that the inhibition of this interaction with an anti-CD2 blocking antibody partially reversed the

cytotoxicity enhancement from the sialidase addition, further suggesting that the desialylation triggers stronger target cell killing by facilitating a tighter interaction between target tumor cells and T cells (Figure 2E).

HER2-targeting BiTE-sialidase fusion protein selectively desialylates HER2-positive cells

Having confirmed that sialidase treatment potentiates T cell-dependent tumor cell cytolysis induced by BiTE, we next sought to specifically target sialidase to the tumor cell-T cell interface via BiTE conjugation. Confining sialidase activity to the IS would potentiate tumor cell killing while limiting nonspecific desialylation of bystander cells in the tumor microenvironment. Importantly, sialyl-Lewis X, a sialylated tetrasaccharide, is essential for leukocyte tethering and rolling en route to sites of inflammation and tumor tissues. Nonspecific desialylation would destroy this glycan epitope on leukocytes, thereby hindering their tumor homing, and accordingly effective tumor control(25-27). Toward this end, we constructed 4D5 BiTE–*B. infantis* sialidase fusion proteins in which sialidase was introduced onto either the N terminus (Sialidase-4D5 BiTE) or the C terminus (4D5 BiTE-sialidase) of 4D5 BiTE, respectively (Figure 3A). To test whether the fusion protein can successfully remove sialic acids from the surface of tumor cells, SK-BR-3 human breast cancer cells (HER2+++) and SKOV-3 human ovarian cancer cells (HER2+++) (Figure S3A) were treated with Sialidase-4D5 BiTE or 4D5 BiTE-sialidase, respectively, followed by staining with the α-2,6-sialic acid-binding lectin SNA. Desialylation was evidenced by decreased SNA binding compared to the untreated controls, and was seen in both SK-BR-3 and SKOV-3 cells when treated with either fusion protein (Figure 3B). To determine whether the sialidase fusion proteins can selectively desialylate HER2 positive cells in the presence of HER2 negative cells, we mixed SKOV-3 (HER2+++) and MDA-MB-468 (HER2-) cells, followed by the addition of 4D5 BiTE-sialidase. We observed that 4D5 BiTE-sialidase, at both 5 nM and 50 nM concentrations, selectively desialylates HER2 positive SKOV-3 cells while sparing the HER2 negative MDA-MB-468 cells, thus, confirming selectivity for HER2-specific targeting (Figure 3C).

HER2-targeting BiTE and sialidase fusion protein shows better *in vitro* cytotoxicity than HER2 BiTE alone

We then compared the cytotoxicity mediated by both fusion proteins to that of the original 4D5 BiTE. At the same concentration of 4 nM, 4D5 BiTE-sialidase induced a higher level of T cell-dependent cytolysis of SK-BR-3 and SKOV-3 cells than the other two BiTE constructs (Figure 4A and B). Specifically, in a dose-response assay, 4D5 BiTE-sialidase induced significantly stronger T cell-mediated killing of SK-BR-3 and SKOV-3 cells with a 10fold and 3fold lower EC50, respectively, than that of 4D5 BiTE (4D5 BiTE EC50 = ~200 pM) (Figure 4C and D). Consistent with these findings, when SK-BR-3 cells were used as target cells, 4D5 BiTE-sialidase induced the highest T cell activation as measured by the expression of T cell activation markers CD25 and CD69

and the degranulation marker CD107a. (Figure 4E to g and K). Also, the strongest cytokine release was observed for 4D5 BiTE-sialidase-treated T cells compared to T cells treated with 4D5 BiTE or Sialidase-4D5 BiTE (Figure 4I to J). A similar trend was seen for SKOV-3 cells, with 4D5 BiTE-sialidase inducing the strongest T cell activation (Figure S4).

We further tested the BiTE-sialidase-mediated killing of cell lines with different cell-surface HER2 expression levels: MDA-MB-231 (+), MDA-MB-435 (+) and MDA-MB-468 (-) (Figure S3A). At 4 nM concentration, which is beyond saturation level, compared to 4D5 BiTE alone, 4D5 BiTE-sialidase strongly augmented the killing of cells with low levels of HER2 (HER2+), e.g., MDA-MB-231 and MDA-MB-435. Under this condition, stronger enhancements in killing were achieved than those measured for HER high (HER2+++) cells (SK-BR-3 and SKOV-3 cells) (94-203% *vs.* 22-24%) (Figure 4L and Figure S3D and E). These observations suggest that desialylation can increase the susceptibility of cells that would normally be relatively resistant to BiTE-mediated T cell killing. Significantly, 4D5 BiTE-sialidase did not trigger the killing of HER2 negative MDA-MB-468 cells or murine melanoma B16-F10 cells that expresses abundant sialoglycans, indicating exclusive specificity towards HER2 positive cells (Figure 4M and Figure S3B)

A previous study by Bertozzi and coworkers demonstrated that a trastuzumab-sialidase conjugate enhanced the killing of HER2 positive tumor cells by NK cells, potentially through blocking the inhibitory Siglec-7 signaling(28). Inspired by this pioneering study, we tested whether 4D5 BiTE-sialidase could also induce stronger NK cell-directed tumor cell killing. We incubated NK-92MI cells with SK-BR-3 cells in the presence of 4D5 BiTE or 4D5 BiTE-sialidase. Whereas 4D5 BiTE had no effect on NK-92MI-induced killing of tumor cells, 4D5 BiTE-sialidase significantly enhanced NK-mediated SK-BR-3 cell cytolysis (Figure S5).

4D5 BiTE-sialidase enables better tumor control than 4D5 BiTE in a xenograft immune deficient mouse model

Having demonstrated the superiority of 4D5 BiTE-sialidase compared to 4D5 BiTE in terms of the induction of T cell-mediated cytolysis of tumor cells *in vitro*, we then sought to determine if this enhanced efficacy is also seen *in vivo*. We chose a human tumor murine xenograft model using the NOD-*Prkdc*$^{\text{em26Cd52}}$*IL2rg*^{em26Cd22}/NjuCrl coisogenic (NCG) immunodeficient mouse to compare the antitumor immunity induced by these two BiTE constructs(29). On day 0, NCG mice were injected subcutaneously (s.c.) with 2.5 million SK-BR-3-luc cells followed by intraperitoneal (i.p.) administration of 5 million hPBMCs. On day 7, these NCG mice were divided into three groups and then received an intravenous (i.v.) infusion of PBS, 4D5 BiTE, or 4D5 BiTE-sialidase, respectively (Figure 5A). Blood was collected from each mouse 5 hrs after BiTE administration and the serum IFN-γ level was measured. We found that the serum IFN-γ level in the 4D5 BiTE-sialidase group was significantly higher than the level in the other two groups, whereas the IFN-γ level in the 4D5 BiTE treated group barely increased over the level in the PBS control group (Figure 5C). The BiTE

administration was continued twice per week until day 41. A second dose of 2 million hPBMCs per mouse was given on day 16. During this treatment course tumor growth was monitored by longitudinal, noninvasive bioluminescence imaging. As shown in Figures 5B and 5D, the administration of 4D5 BiTE-sialidase significantly delayed tumor cell proliferation *in vivo* compared to the 4D5 BiTE treatment and PBS control. Remarkably, by the end of the treatment regimen, tumors in two mice receiving the 4D5 BiTE-sialidase treatment were completely eradicated (Figure 5E).

BiTE-sialidase fusion proteins specific for CD19 and PSMA trigger enhanced *in vitro* cytotoxicity and T cell activation

Finally, to evaluate whether the BiTE-sialidase fusion format can be applied to improve the efficacy of BiTEs targeting other tumor-associated antigens, we designed and constructed two additional BiTE-sialidase molecules. The first was based on the FDA-approved drug Blinatumomab that targets CD19, a cell surface marker on B cells and B cell malignancies. The second was based on BiTEs against prostate-specific membrane antigen (PSMA), a target for prostate cancer treatment. As shown in Figure 6A, compared to Blinatumomab (CD19 BiTE), the Sialidase fusion counterpart exhibited much stronger cytotoxicity toward CD19 positive Raji cells with a five-fold lower EC50 (0.80 pM *vs.* 4.26 pM). Using the same concentration of 5 pM, CD19 BiTE-sialidase induced much higher T cell activation and degranulation than Blinatumomab (Figure 6B to E). Consistent with better T cell activation, CD19 BiTE-sialidase also triggered stronger cytokine release (Figure 6F to H). For another CD19 positive cell line, NALM-6, much stronger killing was also observed for CD19 BiTE-sialidase compared to the Blinatumomab treated group (Figure S6). Likewise, PSMA BiTE-sialidase also induced better killing of PSMA positive PC3 cells and stronger T cell activation compared to PSMA BiTE (Figure S7A and B)

Discussion

The concept of cancer progression facilitated by hypersialylation was introduced more than five decades ago, but the idea of pursuing desialylation as a therapeutic strategy for cancer treatment has met with only mixed results since the earliest attempts in the 1970s(30-32). Recently, the enthusiasm for this idea has been reinvigorated by the innovative work of Bertozzi and coworkers(28, 33). As demonstrated by Bertozzi, Laubli, *et al.*, selective removal of sialylated Siglec-ligands in the tumor microenvironment using an antibody-sialidase conjugate enhanced anti-tumor immunity and suppressed tumor progression *in vivo* in several mouse tumor models(33, 34). Mechanistically, desialylation facilitated the conversion of tumor-associated macrophages with immunosuppressive phenotypes to their antitumoral counterparts(34).

Here, we demonstrate that targeted desialylation is also beneficial to BiTE-based therapy, which so far has only shown limited success in the treatment of solid tumors. Not only does a BiTE-sialidase fusion protein with a sialidase conjugated to a HER-2

targeting BiTE possess enhanced T cell engaging capabilities, but also triggers superior T-cell dependent cytotoxicity against target cancer cells both *in vitro* and *in vivo* in a xenograft solid tumor model. Worthy of pointing out, the observed cytotoxicity enhancement of BiTEs resulting from desialylation seems to be independent of the inhibitory Siglec signaling pathways. Instead, desialylation elicits stronger immunological synapse formation between the engaged T cells and target cells. Indeed, several studies published recently revealed that bulky glycans on the surface of tumor cells result in suboptimal synapse formation induced by both CAR-T cells and BiTEs(35).

Although we have evidence that the desialylation-induced cytotoxicity enhancement *in vitro* within a short window of 24 hours is independent of the Siglec signaling, recent studies showed that tumor-infiltrating T cells may express high levels of Siglec-9(16). In addition, 48 hours following T-cell receptor stimulation, T cells upregulate Siglec-5 to counteract activation signals(36). Therefore, desialytion by BiTE-sialidase fusion proteins may further facilitate long-term tumor cell killing by interrupting the inhibitory sialoglycan-Siglec interaction on T cells and other immune cells. Finally, the overexpression of sialosides on tumor cells is known to contribute to tumor metastasis(37-39). There is a possibility that BiTEs-Sialidase could suppress metastasis by causing targeted desialylation(40). These hypotheses are currently under investigation in our lab.

To date, many efforts have been devoted to improving the efficacy of T cell-engaging therapies. One strategy is to include the CD28 costimulatory signal within the T cell-engaging process by either adding a CD28-engaging molecule or constructing trispecific molecules with both the CD3 and CD28 binding moieties(19-21). Others have tried to incorporate immune checkpoint inhibitors (ICIs)(41). BiTE-secreting CAR-T cells and BiTE-encoding oncolytic virus have also been developed in an effort to achieve better efficacy(42, 43). Distinct from these strategies, through the development of BiTE-sialidase fusion proteins we show that the efficacy of BiTEs can be significantly enhanced through targeted cell-surface glycocalyx editing. As illustrated in this work, the BiTE-sialidase fusion principle is universally applicable to BiTEs targeting various tumor-associated targets and therefore opens a new door for improving multiple types of T cell-engaging therapies. We anticipate that BiTE-sialidase fusion proteins will prove promising agents that can be combined with other anti-cancer modalities such as ICIs and adoptive cell transfer to achieve better tumor control.

Methods:

Cell lines and cell culturing

SK-BR-3 cells, MCF7 cells, PC3 cells, Raji cells, SKOV-3 cells, MDA-MB-435 cells, MDA-MB-231 cells, MDA-MB-468 cells, NALM-6, NK92MI were obtained from ATCC and they were cultured as suggested. Expi293f cells were purchased from Thermo Fisher Scientific and cultured according to the protocol. For culturing of the

isolated human PBMCs, AIM V™ Medium (Gibco™ 12055091) supplemented with 10% FBS was used. All cells were cultured in the incubator at 37°C supplemented with 5% CO2.

General gene cloning procedures

The protein sequences of ScFv targeting human CD3, CD19, HER2 and PSMA were obtained from publicly available patents and the protein sequences were reverse-translated and codon-optimized to DNA sequences. All ScFv sequences were synthesized from IDT. The sequence of *B. infantis* sialidase was kindly gifted from George Peng Wang's lab. For the molecular cloning process, the difference sequences were assembled together using NEBuilder HiFi DNA Assembly (New England BioLabs, E2621). For BiTE molecules, two separate ScFv sequences were connected with a GGGGS linker. For the BiTE and sialidase fusion proteins, the sialidase sequence was conjugated to the BiTE sequence through a 2x GGGGS linker.

Expression of BiTEs, *B. infantis* sialidase and BiTE-sialidase fusion proteins

All BiTEs, sialidase and BiTE-sialidase fusion proteins were fused with a 6x his tag at the C terminus for purification. For all BiTE and BiTE-sialidase fusion proteins, the expression was done in Expi293f cell system (Thermo Fisher Scientific). The transfection and handling of the cells were done according to the manufacturer's protocol. *B. infantis* sialidase was expressed in BL21 E. coli. For purification, all proteins were purified using Ni-NTA (nickel-nitrilotriacetic acid) resin from QIAGEN. After the incubation of Expi293 media supernatant with the Ni-NTA resin, the Nickle charged resin was washed with PBS and 20 mM imidazole. Proteins were eluted with 250 mM imidazole and were concentrated and buffer-exchanged to PBS before use. The concentration of all proteins was determined by Qubit Protein Quantification Assay (Thermo Fisher Scientific, Q33211)

Desialylation by *B. infantis* sialidase, 4D5 BiTE-sialidase fusion proteins and P-3Fax-Neu5Ac

For the removal of sialic acids by B. infantis sialidase or 4D5 BiTE-sialidase fusion proteins, 0.5 million cells were suspended in 100 mL DMEM without the serum. 1.5 mg sialidase or the fusion proteins were added in each sample and each sample was incubated at 37 oC for an hour. After the incubation, cells were washed twice by DPBS before they were used for killing experiments or staining. The Endo F3 treatment process is the same as the sialidase treatment. For the desialylation by inhibitor P-3Fax-Neu5Ac (R&D Systems, 117405-58-0), SK-BR-3 cells were cultured in T25 flask with the addition of 100 mM P-3Fax-Neu5Ac for three days.

Human PBMC and T cell isolation

Human PBMCs were collected from blood samples of multiple healthy donors. Briefly, equal amount of DPBS with 2 mM EDTA was used to dilute the blood samples. Then, the mixture was carefully added to Ficoll (Ficoll® Paque Plus, GE Healthcare, 17-1440-02) for gradient separation. After centrifuge at 650g for 30 min with minimal acceleration and deceleration setting, the middle layer was collected and washed twice with DPBS supplemented with 2 mM EDTA. Further T cells isolation from human PBMCs was done with EasySep™ Human T Cell Isolation Kit (STEMCELL Technologies, 100-0695) according to the manufacturer's protocol.

Cell cytotoxicity measurement by lactate dehydrogenase (LDH) release

T cell cytotoxicity induced by BiTEs and BiTE-sialidase fusion proteins was measured by lactate dehydrogenase (LDH) release using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1780). 10000 tumor cells and 50000 hPBMCs per well in 100 μL media were exposed to different treatments and incubated in 96 well plates at 37 ºC. After 24 hours of coincubation, 50 μL of media supernatant from each well was transferred to a new flat bottom 96 well plate and LDH release was measured using the supplier's protocol. Specific killing was calculated as suggested in the supplier's protocol with background subtraction and total lysis comparison. For the NK-92MI cytotoxicity measurement, different ratios of NK-92MI cells and SK-BR-3 cells were incubated with or without 4D5 BiTE (5 μg/ml) or 4D5 BiTE-sialidase (10 μg/ml) for 18 hours at 37 ºC. Then, the cytotoxicity was measured and calculated by the same LDH release kit according to the protocol.

Cytokine release and T cell surface activation marker measurement

For T cell cytokine release measurement, as with the cytotoxicity experiment, 10000 tumor cells and 50000 hPBMCs were co-incubated per well in 96 well plates with different treatments in 100 mL media at 37 ºC for 24 hours. Then, 20 mL of supernatant from each well was diluted in 100 mL DPBS and used for IFN-γ, IL-2 and TNFα measurement. The ELISA measurement was done by ELISA MAX™ Sets. IFN-γ, IL-2 and TNFα kits (BioLegend) and the experiments were done according to the manufacturer's protocol. The exact concentration was calculated from a standard curve. For the cell surface activation marker measurement, 80000 tumor cells and 400000 hPBMCs were co-incubated per well in 12 well plates with different treatments in 1 mL media at 37 ºC for 24 hours. Following incubation, cells from each well were resuspended and stained with anti-CD3-PE, anti-CD69-FITC, anti-CD25-APC or anti-CD107a-pacific blue (All from biolegend and were added at 1:200) for 30 min at 4 ºC. Cells were then washed twice with FACS buffer (PBS with 2.5% BSA) before being analyzed using flow cytometry. Data analysis and mean fluorescence intensity calculation were done by Flowjo.

Flow cytometric analysis of Siglec-7 and Siglec-9 expression

Human PBMCs were collected from four healthy human donors. 0.5 million freshly isolated human PBMCs were suspended in 100 μL FACS buffer (PBS with 2.5% BSA) and each sample was stained with anti-CD3-PE. Each sample was also stained with either anti-Siglec-7-APC or anti-Siglec-9-APC (All from biolegend and were added at 1:200). After incubation for 30 min at 4 ºC, cells were washed twice with FACS buffer before being analyzed using flow cytometry. Positive population percentage of both Siglec-7 and Siglec-9-stained samples was analyzed by Flowjo. For T cells activated by BiTEs, 80000 tumor cells and 400000 hPBMCs were coincubated per well in a 12 well plates with or without the BiTEs and sialidase treatment in 1 mL media at 37 ºC for 24 hours. Following incubation, cells were resuspended and stained as described earlier for Siglec-7 and Siglec-9 expression analysis.

Staining of human CD3ζ and actin for confocal imaging

Briefly, 0.4 million tumors cells were treated with 4 nM 4D5 BiTE or 4 nM 4D5 BiTE and 15 μg/ML sialidase in 100 μL DMEM without the serum for 1 hr at 37 ºC. After the incubation, all the samples were washed twice using PBS before incubating with 0.4 million hPBMCs in 500 μl PBS for 30 min at 37 ºC. Then all of the cells were transferred in 1 ml PBS to the coverslips in 12 well plates and incubated at 37 ºC for 30 min to let cells attach to the coverslip. 1 ml 4% PFA was added to each well and incubated with shaking for 20 min at room temperature (RT) for cell fixing, and then each well was washed twice with ice cold PBS. Washing took place at RT for 10 min with shaking. After fixation, 1 mL 0.1% PBS-Triton100 was added to each well for 10 min with shaking at RT to permeabilize the sample. PBST was used for washing for three times, each time with shaking at RT for 5 min. Next, 1 mL 2.5% FBS-PBST was used to block each sample for 50 min with shaking at RT. Then, anti-CD247(CD3ζ) antibody (Sigma-Aldrich, 12-35-22-00) was diluted in FACs buffer at 1:200 and anti-actin antibody (Novus Biologicals, NBP267113) was diluted in 1:500. 500 mL of each diluted antibody was added to samples and incubated for an hour at RT with shaking. PBST was used for washing for three times before anti-rabbit 488 (Invitrogen, 35553) and anti-mouse 594 ((Invitrogen, A-11005) secondary antibody was diluted and used for staining at RT for 30 min with shaking. Finally, samples were washed three times and each coverslip was transferred to a glass slide with mounting oil. Finger nail oil was used to seal the coverslip. Samples were analyzed on a Zeiss LSM880 with a 63x oil lens (NA 1.4). The relative mean fluorescent intensity (MFI) of CD3ζ accumulation and relative contact area of IS was calculated by imageJ.

Desialylation detection from SNA staining

For SNA staining, 0.5 million cells with or without desialylation were suspended in 100 μL HBSS buffer (Sigma-Aldrich, H6648) supplemented with 5 μM CaCl2 and MgCl2. SNA-FITC was added at 1:200 and DAPI was added at 1:2000 to each sample and the mixture was incubated on ice for 30 min before washing twice with HBSS buffer. Samples were then analyzed by FACS. Desialylation was analyzed in DAPI negative

live cell populations using Flowjo.

SK-BR-3-Luc cell xenograft tumor model

All animal experiments were approved by the TSRI Animal Care and Use Committee. 15 NCG (6 weeks old male) mice (Charles Rivers Laboratories) were injected with 5×10^6 human PBMCs (intraperitoneally) and 2.5×10^6 SK-BR-3 cells (subcutaneously) on Day 0. On Day 6, mice were imaged by BLI and divided into groups based on similar tumor burden within each group. One Day 7, Three groups were intravenously (i.v) treated with PBS, 6 μg 4D5 BiTE, and 10 μg 4D5 BiTE-sialidase, respectively. Blood was collected from each mouse 5 hrs following BiTE administration and the serum IFN-γ level was measured using ELISA MAX™ (Biolengend). Drug treatment was continued twice a week, mouse received a second dose of 2×10^6 human PBMCs (intraperitoneally) and each on day 16. Tumor burden was imaged multiple times throughout the whole study process. For the BLI imaging, 200 μL 15 g/L D-Luciferin, Potassium Salt (GoldBio) was injected intraperitoneally in each mouse and mice were imaged by IVIS imaging system (PerkinElmer) after 10 mins.

Statistical analysis

Unless specified elsewhere, results are shown using GraphPad Prism version 8.0.0 with standard error of the mean (SEM) as error bars, each dot represents a biological replicate. P values were calculated using the built-in data analysis function of Microsoft excel.

Author contributions

Z.Y., P.W. and R.L., conceived and designed research studies. Z.Y. and P.W. wrote the paper and analyzed the data. Z.Y., G.G., Y.H., C.W. and Y.S. performed most of the experiments mentioned in the paper.

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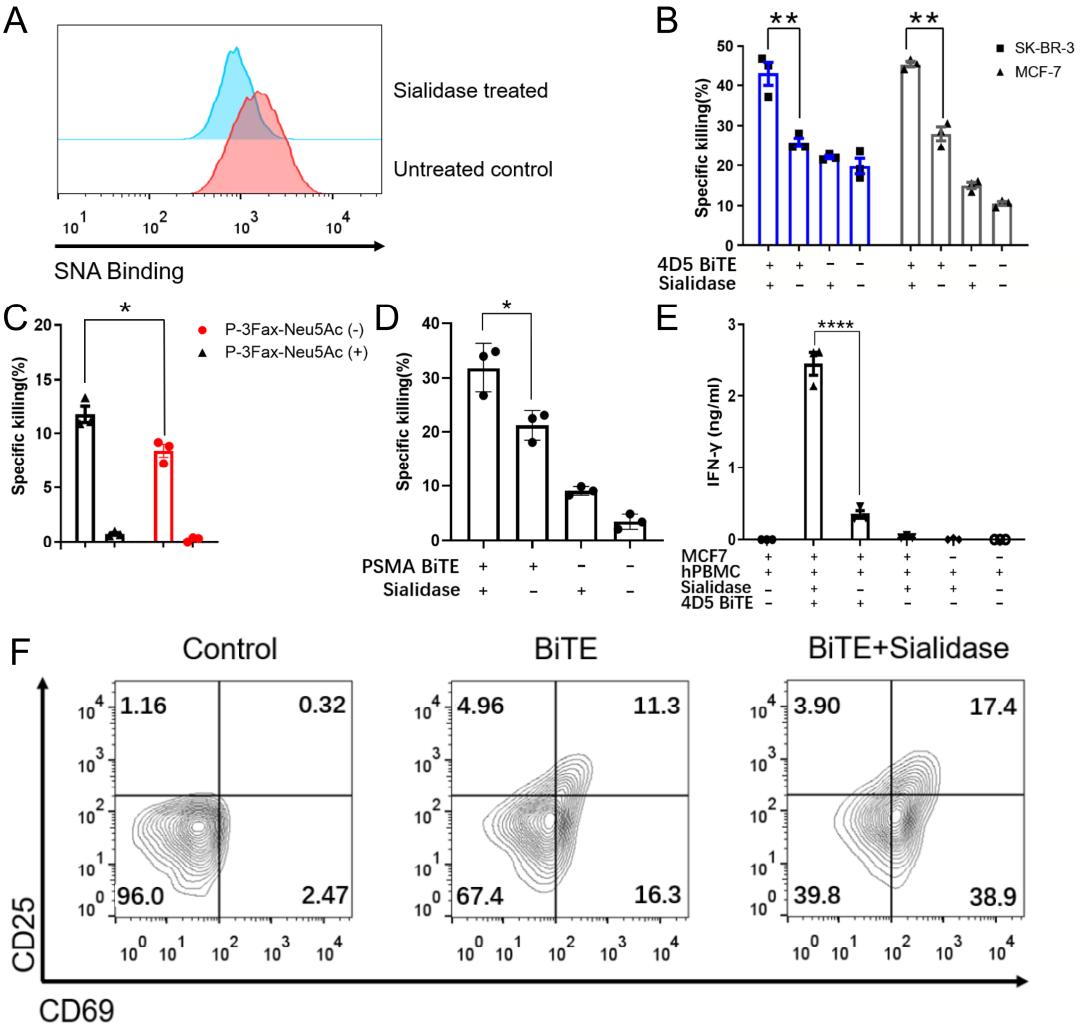

Figure 1: Sialic acid removal enhanced BiTE-induced T cell cytotoxicity and activation (A) Measuring sialic acid levels on the surface of SK-BR-3 cells after the treatment of *B. infantis* sialidase with sialic acid binding lectin SNA. (B) Killing of SK-BR-3 cells and MCF7 cells induced by 4D5 BiTEs with or without sialidase treatment. (C) Killing of SK-BR-3 cells induced by 4D5 BiTEs + hPBMCs with or without prior treatment with 100 μM sialylation inhibitor P-3FAX-Neu5Ac. (D) Killing of PSMA positive PC3 cells induced by PSMA targeting BiTEs with or without the sialidase treatment. (E) IFN-γ release was measured as an indicator of BiTE-induced T cell activation by incubating MCF7 cells with 4D5 BiTEs + hPBMCs with or without the addition of sialidase. (F) CD25 and CD69 expression level was measured in T cells with or without the presence of 4D5 BiTEs and sialidase. The E to T ratio used for all experiments in Figure 1 is 5 to 1. Mean values show three independent experiments with standard error of the mean (SEM) as error bars. For statistical analysis, unpaired Student t test with Welch correction was applied (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$).

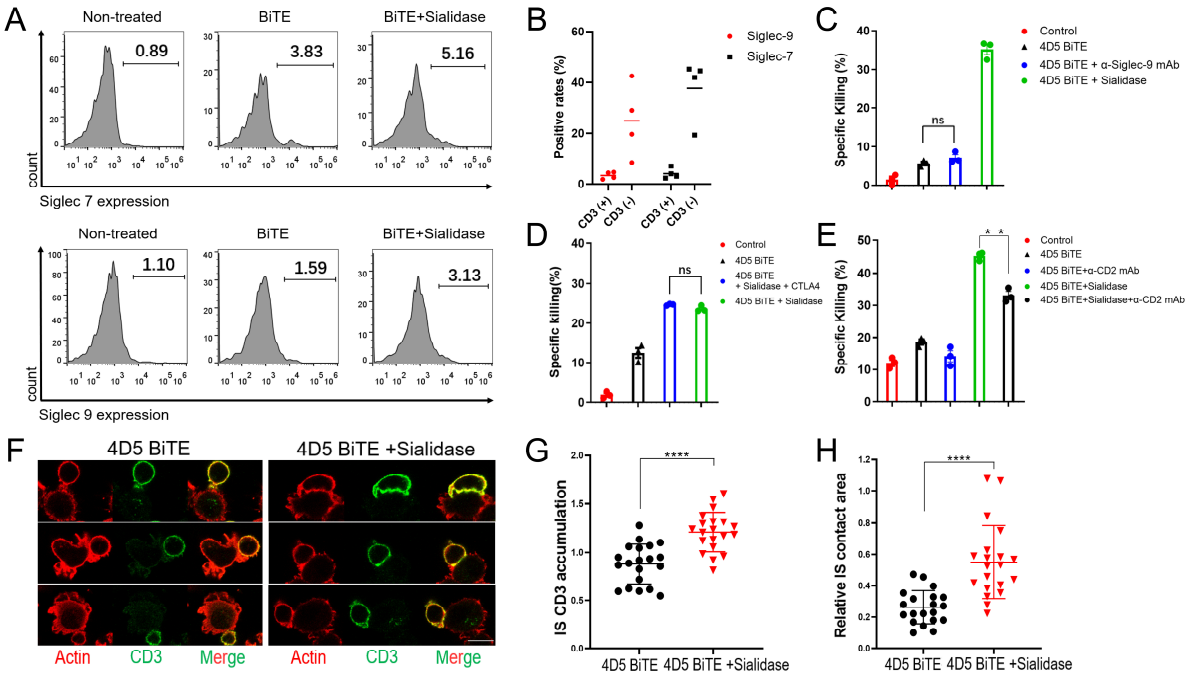

Figure 2: Desialylation promotes stronger BiTE-mediated immune synapse (IS) formation rather than suppressing the inhibitory Siglec signaling. (A) Siglec-7 and -9 expression levels were measured on human T cells with or without BiTE-induced activation, and with or without sialidase treatment. (B) Siglec-7 and -9 expression levels were measured on T cells and CD3 negative cells in PBMCs from healthy human donors. (C) SK-BR-3 cell killing induced by 4D5 BiTEs was measured with or without the addition of sialidase or anti-siglec-9 antibody. (D and E) SK-BR-3 cell killing induced by 4D5 BiTEs was measured in the presence or absence of sialidase with added recombinant CTLA-4 (D) and (E) Anti-CD2 blocking antibody. (F) Staining of CD3ζ and actin to visualize the immune synapses formed by T cells and tumor cells by confocal microscopy. Two groups, with and without sialidase treatment of the tumor cells, were imaged. Scale bar =10 μm. (G) CD3 accumulation at the IS was calculated by dividing the mean fluorescence intensity (MFI) at the IS by the MFI of the rest of the membrane. (H) Relative IS contact area was calculated by dividing the area of the IS by the area of the rest of the T cell membrane. All analysis was done using ImageJ. Mean values show three independent experiments with standard error of the mean (SEM) as error bars. For statistical analysis, unpaired Student t test with Welch correction was applied (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$).

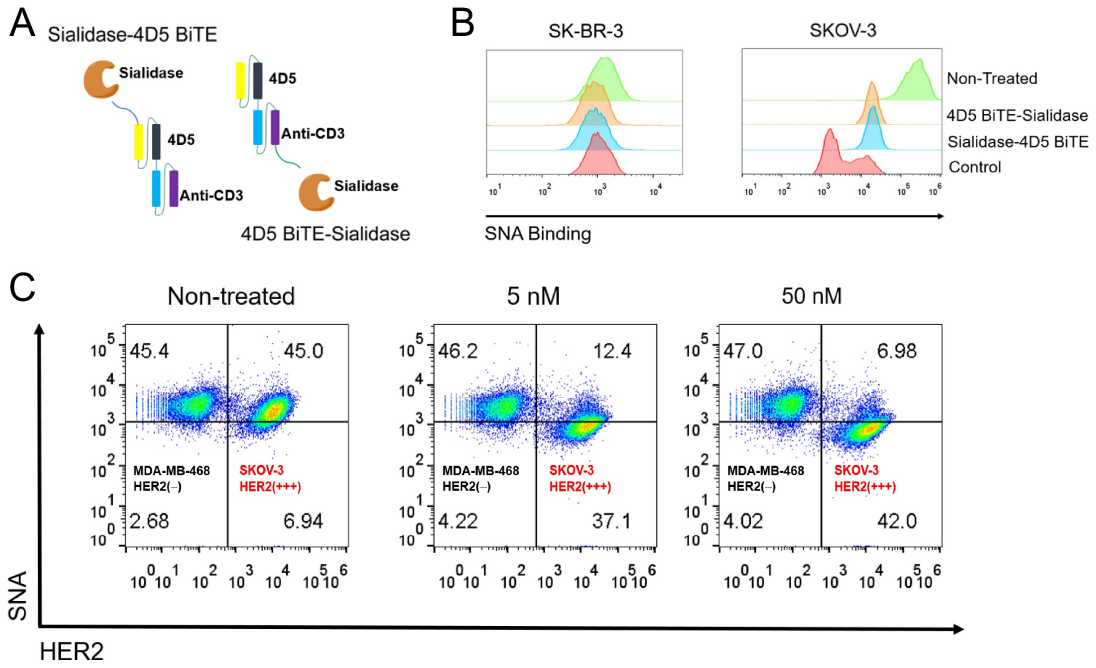

Figure 3: Construction of 4D5 BiTE-sialidase fusion proteins for selective desialylation of HER2 positive cells. (A) Two fusion proteins are constructed by conjugating sialidase to either the N or C terminus of the 4D5 BiTE. (B) Measuring sialic acid levels on the surface of SK-BR-3 and SKOV-3 cells after the treatment of the fusion proteins and staining with FITC-SNA. Control represents cells not stained with SNA. (C) HER2 positive SKOV-3 cells and HER2 negative MDA-MB-468 cells were mixed together and treated with 5 nM or 50 nM 4D5 BiTE-sialidase. The cell-surface sialylation level was measured by FITC-SNA staining and flow cytometry analysis.

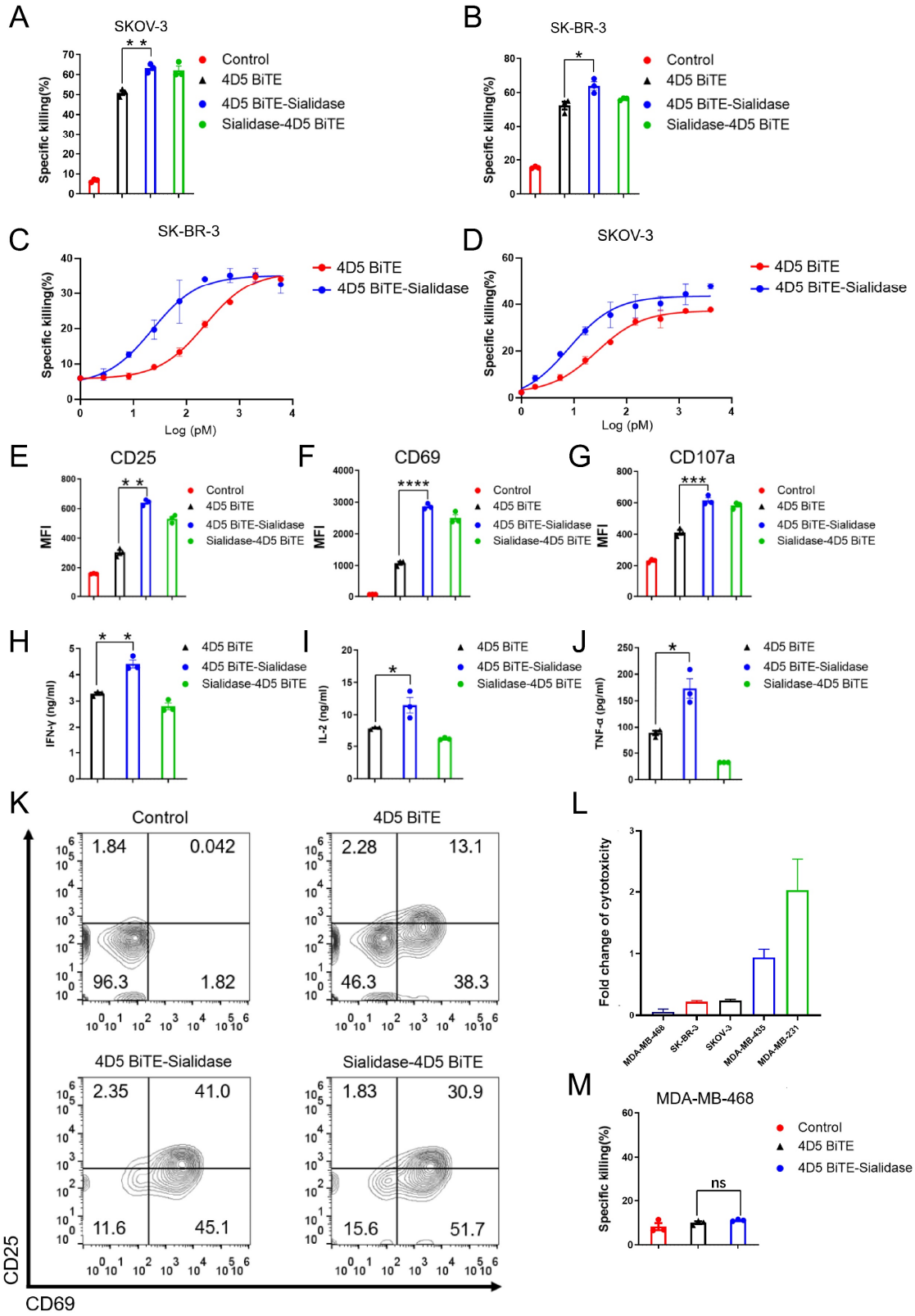

Figure 4: 4D5 BiTE-sialidase fusion proteins exhibit better activities than 4D5 BiTE alone for HER2 positive target cell killing and T cell activation. (A) and (B) The specific lysis of HER2 positive SK-BR-3 cells and SKOV-3 cells with 4 nM 4D5 BiTE or sialidase fusion proteins at the effector: target ratio of 5:1. (C) & (D) Dose dependent

targeted killing using 4D5 BiTE or 4D5 BiTE-sialidase against SK-BR-3 and SKOV-3 cells. (E), (F), (G) & (K) CD25, CD69 and CD107a expression level was measured in T cell populations in the presence of SK-BR-3 cells and 4 nM 4D5 BiTE or the fusion protein. (H), (I) & (G) IFN-γ, IL-2 and TNF-α release were measured for 4D5 BiTE- or fusion protein-induced T cell activation in the presence of SK-BR-3 cells. (L) The cytotoxicity enhancements induced by 4D5 BiTE-sialidase as compared to 4D5 BiTE for cell lines with different HER2 expression levels. (M) The specific lysis of MDA-MB-468 cells under 4 nM 4D5 BiTE or 4D5 BiTE-sialidase at the effector: target ratio of 5:1. For statistical analysis, unpaired Student t test with Welch correction was applied (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$).

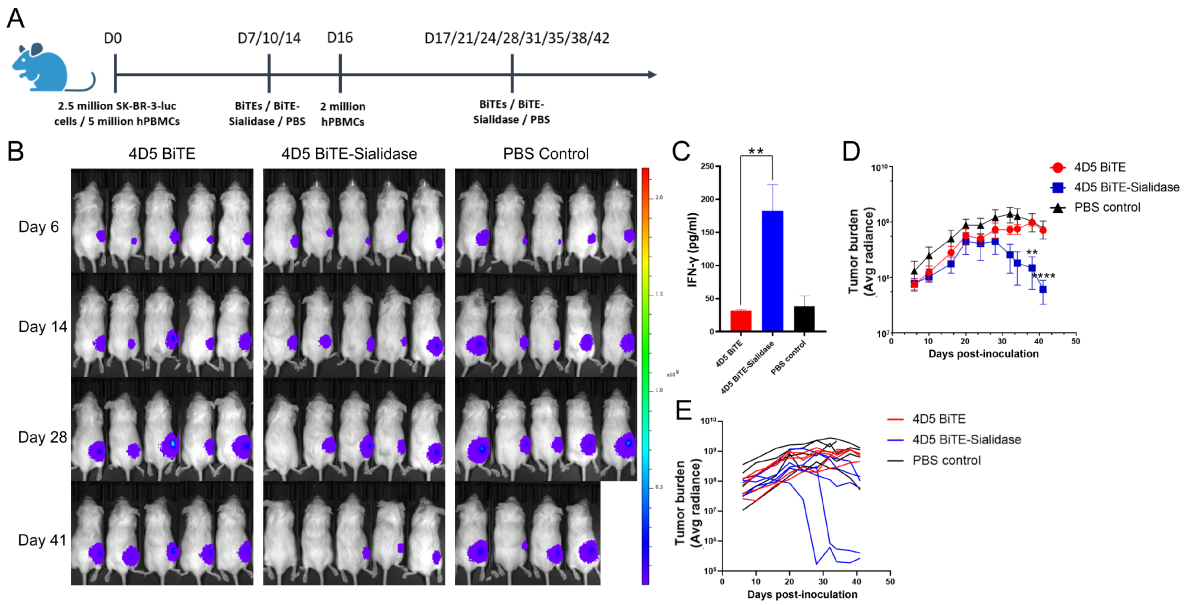

Figure 5: 4D5 BiTE-sialidase exhibits better tumor control *in vivo* than 4D5 BiTE. (A) Scheme for treatment protocol. (B) Bioluminescence was measured twice a week to visualize changes in tumor volume. On day 41, one mouse in the PBS control group died. (C) Serum IFN-γ release was measured 5 hours after the first drug treatment. (D) & (E) Bioluminescence was measured and calculated for each mouse as an indication of tumor burden. Tumor progression was followed by plotting change in the group average (D) and the individual (E) values over time. One-way ANOVA was used to analyze the differences among groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$).

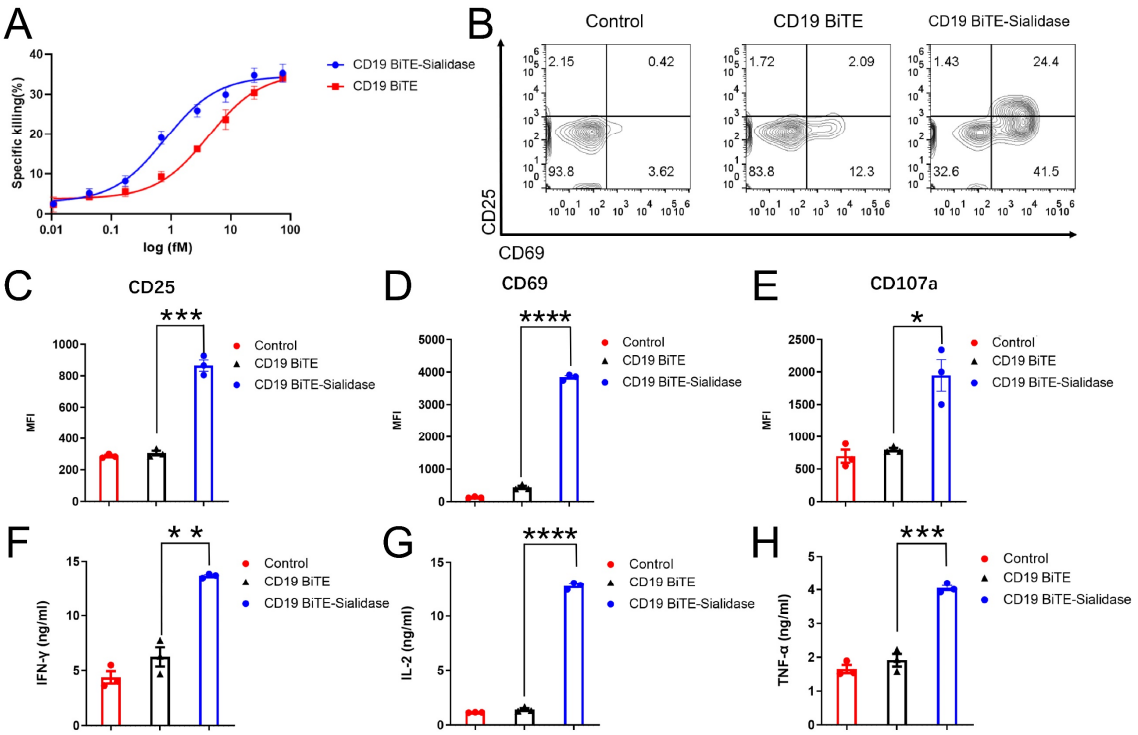

Figure 6: CD19 BiTE-sialidase exhibits superior activities than CD19 BiTE to induce *in vitro* tumor cell killing and T cell activation. (A) Dose-dependent killing of Raji cells induced by CD19 BiTE or CD19 BiTE-sialidase at the effector: target ratio of 10:1. (B), (C), (D) & (E) CD25, CD69, and CD107a expression levels were measured in T cell populations by flow cytometry analysis. (F), (G) & (H) IFN-γ, IL-2 and TNF-α release was measured for CD19 BiTE- or fusion protein-induced T cell activation in the presence of Raji cells. For statistical analysis, unpaired Student t test with Welch correction was applied (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001).