Precision glycocalyx editing as a strategy for cancer immunotherapy

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Cell surface sialosides constitute a central axis of immune modulation that is exploited by tumors to evade both innate and adaptive immune destruction. Therapeutic strategies that target tumor-associated sialosides may therefore potentiate antitumor immunity. Here, we report the development of antibody–sialidase conjugates that enhance tumor cell susceptibility to antibody-dependent cell-mediated cytotoxicity (ADCC) by selective desialylation of the tumor cell glycocalyx. We chemically fused a recombinant sialidase to the human epidermal growth factor receptor 2 (HER2)-specific antibody trastuzumab through a C-terminal aldehyde tag. The antibody–sialidase conjugate desialylated tumor cells in a HER2-dependent manner, reduced binding by natural killer (NK) cell inhibitory sialic acid-binding Ig-like lectin (Siglec) receptors, and enhanced binding to the NK-activating receptor natural killer group 2D (NKG2D). Sialidase conjugation to trastuzumab enhanced ADCC against tumor cells expressing moderate levels of HER2, suggesting a therapeutic strategy for cancer patients with lower HER2 levels or inherent trastuzumab resistance. Precision glycocalyx editing with antibody–enzyme conjugates is therefore a promising avenue for cancer immune therapy.

cancer immune therapy | trastuzumab | sialic acid | Siglec | ADCC

Therapies that enhance the immune response to cancer are proving revolutionary in the fight against intractable tumors (1–3). Immune cells integrate signals from activating and inhibitory receptors to determine their response to a challenging target—activating signals alert them to the presence of pathology whereas inhibitory signals tell the cell that it has confronted a "healthy self." Successful tumors evolve mechanisms to thwart immune cell recognition, often by overexpressing ligands for inhibitory receptors (4, 5). This discovery has led to new therapeutic strategies aimed at blocking inhibitory immune cell signaling, as embodied in the clinically approved T-cell checkpoint inhibitors nivolumab and ipilimumab, which target PD-1 and CTLA-4, respectively (1, 6). Ongoing preclinical studies have focused on combining therapies targeting multiple immunologic pathways. For example, antibodies against PD-1/PD-L1 in combination with those targeting other T-cell checkpoints demonstrate improved antitumor activity in syngeneic tumor models (7–10). Powerful complements to these interventions are emerging therapies targeting innate immune cells, particularly natural killer (NK) cells (11), macrophages (12), and dendritic cells (13, 14). It is likely that combination therapies that simultaneously stimulate activating pathways and limit inhibitory pathways, across both the adaptive and innate immune compartments, will ultimately transform clinical treatment of cancer.

The "sialome" is a central axis of immune modulation that has not yet been explored in the context of cancer therapy (15–17). When sufficiently abundant, glycans terminating in sialic acid residues create a signature of healthy self that suppresses immune activation via several pathways (18). Most prominently, cell-surface sialosides can recruit sialic acid-binding Ig-like lectins (Siglecs), found on most types of leukocytes, to the immunological synapse (19). Thus, sialylation status plays an important role in a cell's ability to trigger or evade immunological recognition.

Up-regulation of sialosides has long been correlated with poor prognosis and decreased immunogenicity of tumors (16, 18, 20), but the mechanistic significance of this widespread tumor phenotype was unclear. Several recent studies implicate cancer hypersialylation as a mechanism of immune evasion mediated by Siglecs (21–26). For example, we found that dense populations of sialylated glycans can recruit NK cell-associated Siglecs to the immune synapse (Fig. 1*A*) (22). Like PD-1, the Siglecs-7 and -9, expressed on NK cells, possess cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that mediate suppression of signals from activating NK cell receptors (Fig. 1*A*) (27–29). Accordingly, hypersialylation of tumor targets protects them from innate NK cell killing, as well as antibody-dependent cell-mediated cytotoxicity (ADCC) in a Siglec-7–dependent manner (22). Likewise, enzymatic removal of sialic acids by treatment of tumor cells with sialidase potentiates NK cell-mediated killing, as does inhibition of Siglec-7 or -9 with blocking antibodies (22, 23, 25).

Sialylation of cancer cell glycans also disrupts the interaction of the NK-activating receptor natural killer group 2D (NKG2D) with its cognate ligands, thus reducing NK-activating signals derived from tumor cells (Fig. 1*A*) (21). Conversely, removal of cell-surface sialic acids enhances NK cell activation by increasing NKG2D-ligand binding (21). Thus, during the microevolutionary process of tumor progression, hypersialylation provides a selective advantage by reducing NK activating signals while enhancing NK inhibitory signals emanating from the immune synapse.

Given the critical protective role of sialic acid against innate immune surveillance and ADCC, we reasoned that tumor-specific desialylation could be a powerful intervention that potentiates

Significance

Successful tumors are able to evade the immune system, which is otherwise capable of killing transformed cells. Therapies that prevent this evasion have become revolutionary treatments for incurable cancers. One mechanism of evasion is the presentation of sugars, called sialic acids, within the cell surface's sugar coating, or glycocalyx. Here, we designed biotherapeutic molecules, termed "antibody–enzyme conjugates," that selectively remove sialic acids from tumor cells. The antibody directs the enzyme to the cancer cells, the enzyme cleaves the sugars, and then the antibody directs immune cells to kill the desialylated cancer cells. The conjugate increased tumor cell killing compared with the antibody alone. Editing the cancer cell glycocalyx with an antibody–enzyme conjugate represents a promising approach to cancer immune therapy.

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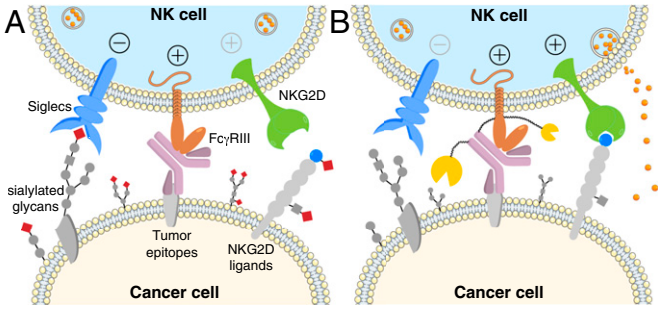

Fig. 1. A glycocalyx approach to cancer immunotherapy targeting the sialic acid axis of immune modulation. (*A*) In sialic acid-overexpressing cancer cells, hypersialylated glycans interact with NK inhibitory receptors, leading to inhibition of NK cell activation. (*B*) Removal of cell-surface sialic acids by an antibody–sialidase conjugate (T-Sia) abolishes the interaction of sialylated glycans and NK-inhibitory Siglec receptors and increases the binding between NK-activating NKG2D receptor and its ligands, thereby enhancing the tumor cell susceptibility to NK cell-mediated ADCC.

cancer cytolysis by NK cells. Here, we report that an antibody–enzyme conjugate (AEC) can selectively edit the tumor cell glycocalyx and potentiate NK cell killing by ADCC, a therapeutically important mechanism harnessed by many antibody cancer drugs (30, 31). We chemically fused a recombinant sialidase to the human epidermal growth factor receptor 2 (HER2)-targeting therapeutic monoclonal antibody trastuzumab (Tras). The antibody–sialidase conjugate desialylated tumor cells in a HER2-dependent manner, destroyed ligands for inhibitory Siglecs while enhancing NKG2D binding, and amplified NK cell killing compared with Tras alone (Fig. 1*B*). Precision glycocalyx editing with AECs may therefore add new breadth to the growing list of cancer immune therapy concepts.

Results

Evaluation of *Vibrio cholerae* Sialidase Activity on ADCC. *V. cholerae* produces a sialidase particularly well suited for our purposes; it is known to desialylate Siglec ligands and is capable of cleaving a variety of sialic acid linkage types (23, 25). *V. cholerae* sialidase was expressed and purified as described previously (32, 33). The purity of the protein was determined by SDS/PAGE and electrospray ionisation mass spectrometry (ESI-MS) analyses (*SI Appendix*, Fig. S1 *A* and *B*, respectively). In accord with previous reports (32), ∼15 mg of enzyme was purified from 1 L of cultured cells, with an in vitro hydrolytic activity of 10.5 ± 0.8 U/mg (a unit is defined as the amount of enzyme required to release 1 μmol of methylumbelliferone per minute) as measured with the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNeuNAc). To confirm that *V. cholerae* sialidase removes sialic acids from cell-surface glycans, we tested its effects on FITC-*Sambucus nigra* agglutinin (SNA) labeling of multiple breast cancer cell lines (Fig. 2*A*). We also evaluated the effects of *V. cholerae* sialidase treatment on cell labeling with receptor-Fc chimeras comprising the ectodomains of Siglec-7, Siglec-9, or NKG2D (29, 34). Desialylation of various breast cancer cell lines by *V. cholerae* sialidase significantly reduced binding of Siglec-7-Fc (Fig. 2*B*) and Siglec-9-Fc (Fig. 2*C*) chimeras while increasing binding to NKG2D-Fc (Fig. 2*D*).

To determine the effects of sialidase treatment on cancer cell susceptibility to ADCC, we assayed three breast cancer cell lines expressing various levels of HER2 (SKBR3, HER2^{+++}; MDA-MB-453, HER2^{++}; and BT-20, HER2^{+}) (*SI Appendix*, Fig. S2) in the presence of 30 nM Tras using purified human peripheral blood NK cells (*SI Appendix*, Fig. S3). All three cell lines showed enhanced killing after sialidase treatment, with the most pronounced effect observed with HER2-low BT-20 cells (*SI Appendix*, Fig. S4). We speculate that, for HER2-high cell lines, such as SKBR3, high levels of opsonization likely lead Fc receptors to dominate the interaction between immune and tumor cells, resulting in lower effects from sialidase treatment. To validate that the enhanced ADCC was due to sialidase enzymatic activity, we performed a similar experiment using heat-inactivated *V. cholerae* sialidase, which showed no activity in the in vitro fluorogenic enzyme assay or on cell-surface sialosides (*SI Appendix*, Fig. S5 *A*–*E*). No enhancement in ADCC was observed after treatment of breast cancer cells with catalytically inactive enzyme (*SI Appendix*, Fig. S5*F*).

Construction of a Trastuzumab–Sialidase Conjugate. Having identified a sialidase capable of cleaving Siglec-7 and -9 ligands while also activating NKG2D ligand activity, we next sought to target the enzyme to HER2-expressing cells via Tras conjugation. In principle, localizing sialidase activity to tumor cell surfaces in this fashion would potentiate NK cell killing while limiting nonspecific desialylation of bystander cells. A key concern in designing the trastuzumab–sialidase conjugate (T-Sia) was to identify a site for enzyme attachment that would not undermine binding to FcγRIII (CD16), the activating receptor on NK cells that initiates ADCC. We took inspiration from the field of antibody-drug conjugates (ADCs) where sites of attachment have been tailored to avoid interference with immune effector functions (35). Accordingly, we chose to link *V. cholerae* sialidase near the C terminus of Tras's heavy chain, far from the C_H2 domain at which FcγRIII binds (36, 37). We used the aldehyde tag method for site-specific conjugation based on precedents of its use in the assembly of protein–protein chemical fusions (38), as well as site-specific ADCs (35). We obtained Tras bearing a C-terminal aldehyde tag as previously described (39). The functionalized antibody was first coupled to aminooxy-tetraethyleneglycol-azide (aminooxy-TEG-N_3) (Fig. 3*A* and *SI Appendix*, S6*D*). Separately, *V. cholerae* sialidase was nonspecifically functionalized on lysine residues with bicyclononyne-*N*-hydroxysuccinimide ester (BCN-NHS). After an overnight reaction, excess linker was removed, and the extent

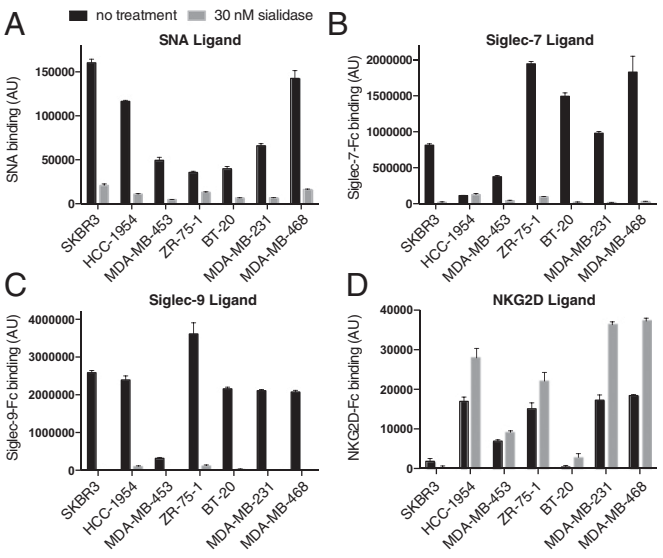

Fig. 2. Levels of SNA, Siglec-7, Siglec-9, and NKG2D ligands on breast cancer cell lines. (*A*) Analysis of cell-surface sialylation levels of different breast cancer cell lines with or without sialidase treatment. (*B*) Ligand levels of Siglec-7 on different breast cancer cell lines with or without sialidase treatment. (*C*) Ligand levels of Siglec-9 on different breast cancer cell lines with or without sialidase treatment. (*D*) Ligand levels of NKG2D on different breast cancer cell lines with or without sialidase treatment.

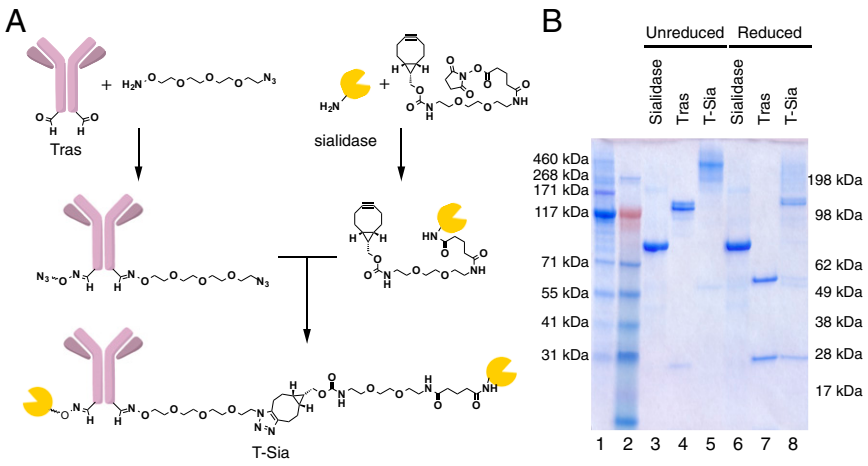

Fig. 3. Preparation and characterization of antibody–sialidase conjugate. (*A*) Humanized trastuzumab (Tras) bearing an aldehyde tag was produced by genetically introducing the consensus sequence SLCTPSRGS at the C terminus of the heavy chain. The cysteine residue within the sequence was converted to formylglycine in situ by action of ER-resident formylglycine generating enzyme (FGE) during expression. The aldehyde-tagged Tras was coupled to the heterobifunctional linker aminooxy-TEG-N_3 by oxime ligation. Separately, recombinant *V. cholerae* sialidase was reacted at lysine side chains with bicyclononyne-*N*-hydroxysuccinimide ester (BCN-NHS). Finally, the two functionalized proteins were conjugated via copper-free click chemistry. (*B*) SDS/PAGE analysis of sialidase, Tras, and trastuzumab–sialidase conjugate (T-Sia) under nonreducing (lanes 3, 4, and 5) and reducing (lanes 6, 7, and 8) conditions visualized by Coomassie staining. Multiple bands observed for Tras and T-sia reflect heterogeneity in antibody glycosylation. Prestained protein ladder: lanes 1 and 2.

of BCN-NHS modification of the sialidase was determined by ESI-MS (*SI Appendix*, Fig. S6*B*). Finally, Tras adorned with the azide-functionalized linker was conjugated to BCN-functionalized *V. cholerae* sialidase via copper-free click chemistry (Fig. 3*A*) (38). The desired conjugate (T-Sia) was purified using a size-exclusion column, and its apparent molecular mass (~312 kDa, two sialidase molecules per IgG) was confirmed by SDS/PAGE (Fig. 3*B*). Both reducing SDS/PAGE and ESI-MS analysis confirmed that the sialidase was covalently linked to the heavy chain of Tras (Fig. 3*B* and *SI Appendix*, Fig. S6*E*). Sialidase activity of T-Sia was evaluated using the fluorogenic in vitro assay. To our delight, more than 85% of the enzymatic activity remained after the chemical conjugation process (*SI Appendix*, Fig. S7).

T-Sia Selectively Desialylates HER2-Positive Cells. To determine whether T-Sia can specifically remove sialic acids from HER2-expressing cells, HER2-positive SKBR3 cells and HER2-negative MDA-MB-468 cells were mixed and treated with either T-Sia or buffer alone. The cells were then labeled with Alexa Fluor 647-labeled anti-HER2 antibody, FITC-SNA, and DAPI. As shown in the microscopy images of Fig. 4 and *SI Appendix*, Fig. S8, treatment with 6 nM T-Sia for 1 h resulted in selective desialylation (as determined by loss of SNA labeling) of SKBR3 cells. We quantified the change in SNA labeling by flow cytometry analysis as well (Fig. 4, *Right*). Sialosides on MDA-MB-468 cells were minimally affected, suggesting that T-Sia is capable of tumor-selective glycocalyx editing (Fig. 4). At a dose 10 times higher, however, 60 nM T-Sia demonstrated more promiscuity, partially desialylating the HER2-negative MDA-MB-468 cells (Fig. 4). This observation underscores the utility of antibody targeting to achieve a high local concentration of cell surface enzyme activity at low conjugate doses and further demonstrates how antibody conjugation might limit off-target effects of a cell surface-editing enzyme in an in vivo setting.

T-Sia Potentiates ADCC Compared with Trastuzumab Alone. To assess the activity of T-Sia in orchestrating ADCC, we performed cytotoxicity assays with BT-20 cells and peripheral blood NK cells, comparing Tras, T-Sia, and free sialidase at comparable concentrations. As shown in Fig. 5*A*, T-Sia significantly potentiated the killing response compared with Tras alone at various effector/target (E/T) ratios. NK cells showed minimal killing of BT-20 targets in the presence of sialidase alone. A similar cytotoxicity trend was observed using whole peripheral blood mononuclear cells (PBMCs) (*SI Appendix*, Fig. S9*A*). Depletion of NK cells from PBMCs eliminated both Tras and T-Sia–mediated cytotoxicity, confirming their central role in the ADCC response (Fig. 5*B*). We performed similar experiments using breast cancer cell lines with various HER2 levels: SKBR3 and HCC-1954, $HER2^{+++}$; MDA-MB-453, $HER2^{++}$; ZR-75–1, BT-20, and MDA-MB-231, $HER2^{+}$; and MDA-MB-468, HER2-negative. For cells with low levels of HER2 ($HER2^{+}$), T-Sia was significantly more potent than Tras alone in mediating cell killing (Fig. 5*C* and *SI Appendix*, Fig. S10). By contrast, cells that express high levels of HER2

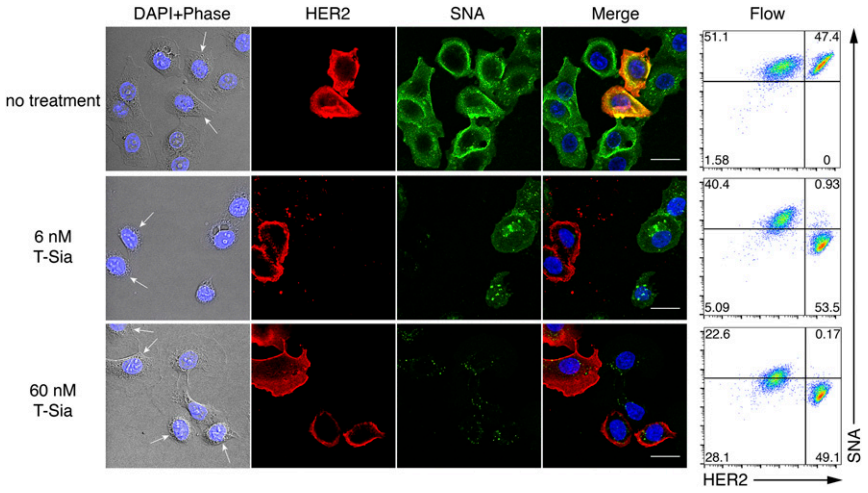

Fig. 4. Visualization and quantitation of T-Sia's tissue-specific selective activity. Cell-surface sialic acid on the HER2-high expressing cell line, SKBR3 (white arrows), can be selectively removed using 6 nM T-Sia in the presence of HER2-negative cell lines MDA-MB-468. SKBR3 and MDA-MB-468 cells were cocultured and then treated or not with T-Sia for 1 h and costained with Alexa Fluor 647-labeled anti-HER2 antibody, FITC-SNA, and DAPI before imaging by confocal microscopy (*Left*). Cell-surface sialylation and HER2 levels were also quantitatively determined by flow cytometry (*Right*). (Scale bars: 25 μm.)

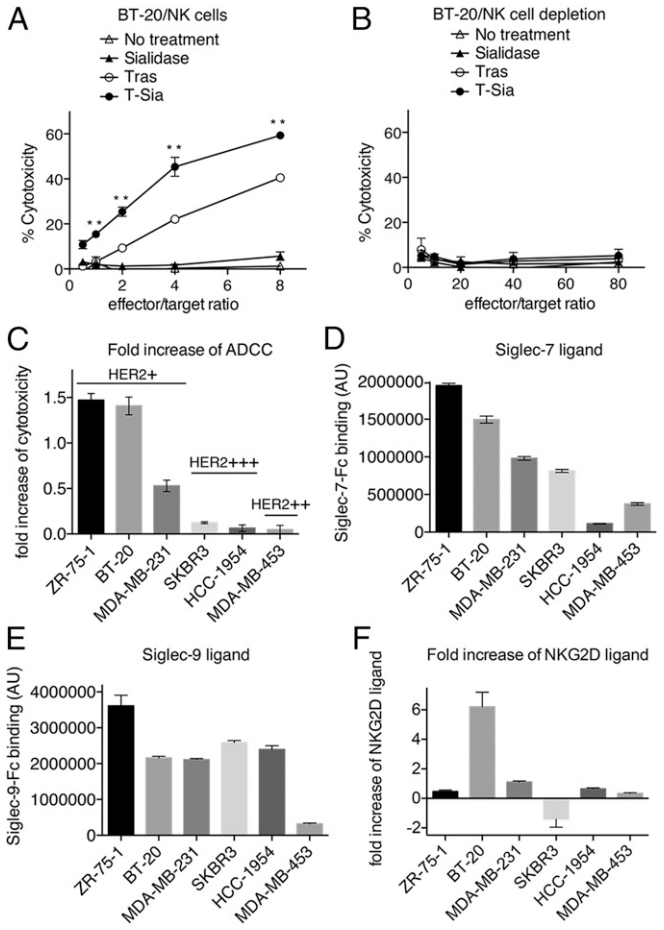

Fig. 5. In vitro activity of trastuzumab and T-Sia against different HER2-expressing cancer cells. (*A*) Cytotoxicity assays performed with BT-20 cells in the absence or presence of sialidase (30 nM), Tras (30 nM), and T-Sia (30 nM) using NK cells at various E/T ratios. (*B*) Cytotoxicity assays performed with BT-20 cells in the absence or presence of sialidase (30 nM), Tras (30 nM), and T-Sia (30 nM) using NK cell-depleted PBMCs. (*C*) The trend seen in the enhancement of ADCC correlated with Siglec-7-Fc (*D*) and Siglec-9-Fc (*E*), but not NKG2D-Fc (*F*) binding levels for various breast cancer cell lines.**$P < 0.005$.

($HER2^{+++}$) showed only modest enhancements in killing (10% to 15%) by T-Sia compared with Tras alone. These data indicate that cancer cells expressing lower levels of HER2, which might not elicit effective ADCC when sialylated, can be rendered vulnerable to NK cell killing once sialosides are removed.

ADCC Potentiation Correlates with Siglec Ligand Abundance. Previous studies have suggested that hypersialylation of cancer cells results in the reduced binding of activating receptor NKG2D, as well as enhanced binding of inhibitory receptors Siglec-7 and Siglec-9, thus reducing NK-mediated cytotoxicity (21–23). We noted that potentiation of killing by T-Sia versus Tras correlated highly with Siglec-7 ligand abundance (Fig. 5*D*) and weakly with Siglec-9 ligand abundance (Fig. 5*E*), as determined by flow cytometry using Siglec-Fc chimeras. Treatment of BT-20 and ZR-75-1 cells—those with the highest expression of Siglec-7 and Siglec-9 surface ligands—with T-Sia enhanced ADCC by more than twofold compared with Tras alone. In contrast, the conjugate offered little enhancement of ADCC against MDA-MB-453 cells, which have the lowest expression of Siglec-7 and Siglec-9 ligands. Earlier, we showed that sialidase treatment of cancer cells increases binding of NKG2D-Fc (Fig. 2*D*), which we considered as another possible contributor to ADCC enhancement. However, there was no correlation between sialidase-dependent enhancement of NKG2D ligand activity and ADCC enhancement by T-Sia versus Tras (Fig. 5*F*). Collectively, these data indicate that loss of Siglec ligands contributes more to T-Sia's ADCC enhancement than does the concomitant gain of NKG2D binding activity.

To further investigate the roles of Siglec-7, Siglec-9, and NKG2D in modulating ADCC, we tested the effects of blocking antibodies in NK cell-killing assays with Tras. Anti–Siglec-7 and anti–Siglec-9 antibodies significantly enhanced NK cell cytotoxicity against BT-20 cells in the presence of Tras, but not with a mixture of Tras and sialidase (*SI Appendix*, Fig. S11). By contrast, blocking the NKG2D receptor had no effect on ADCC in the presence of Tras but diminished ADCC in the presence of Tras and sialidase (*SI Appendix*, Fig. S11). These results indicate that Siglec ligands protect tumor cells from ADCC and reinforce the notion that sialic acids mask NKG2D ligands.

T-Sia Remodels the Tumor–NK Cell Synapse. The NK cell response is the result of a calculation based on engagement of activating and inhibitory receptors colocalized at the immune synapse. In previous work, we demonstrated that hypersialylated cancer cells recruit Siglec-7 to the NK cell synapse, resulting in inhibitory signaling through its ITIM domain (22). To assess the effects of Tras or T-Sia treatment on NK cell receptor distribution, we treated SKBR3 cells with the conjugate and imaged NK cell-target synapses formed during the process of ADCC. Cells were fixed and labeled with fluorescent antibodies against Siglec-7, HER2, and FcγRIII and imaged by fluorescence microscopy. With Tras treatment, Siglec-7 colocalized with FcγRIII at the immune synapse formed with NK cells, consistent with its role as an inhibitory receptor of NK cell activation (Fig. 6). In contrast, SKBR3 cells treated with T-Sia showed little recruitment of Siglec-7 despite an efficient recruitment of FcγRIII. Thus, T-Sia effectively remodels the NK–cancer cell synapse while promoting ADCC.

T-Sia Is More Potent than Tras Alone Against Cells with Low Levels of HER2. To compare the potency of T-Sia to direct ADCC versus Tras alone, we measured the dose response for cytotoxicity using four different breast cancer cell lines: SKBR3 ($HER2^{+++}$), ZR-75-1 ($HER2^{+}$), BT-20 ($HER2^{+}$), and MDA-MB-468 (HER2-negative). Compared with Tras, T-Sia was more cytotoxic against all three HER2-expressing cell lines at an E/T ratio of 4. Interestingly, T-Sia's advantage was most pronounced with cells expressing low levels of HER2 (ZR-75-1 and BT-20) whereas the conjugate was only slightly more potent in eliciting ADCC against cells with high HER2 levels (Fig. 7) (EC_{50} values shown in *SI Appendix*, Table S1). Little lysis of the HER2-negative MDA-MB-468 cells was observed with either Tras or T-Sia (Fig. 7).

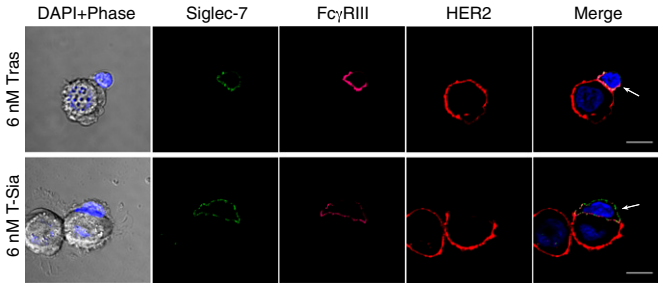

Fig. 6. Fluorescence microscopy analysis of Siglec-7 distribution on NK cells with trastuzumab or T-Sia treatments. Siglec-7 displayed recruitment to the NK cell (white arrows) synapse with Tras treatment. After removing sialic acids on SKBR3 cells using T-Sia, Siglec-7 recruitment to the NK–tumor synapse was lost. (Scale bars: 10 μm.)

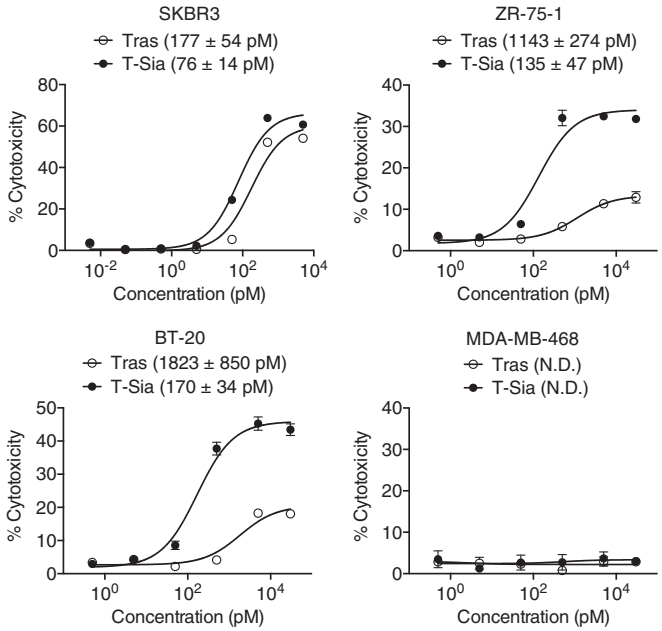

Fig. 7. Cytotoxic activity of NK cells against different HER2-expressing cancer cells with trastuzumab or T-Sia as a function of concentration. Cytotoxicity assays were performed with NK cells and target cells at a 4:1 ratio in the presence of different concentrations of Tras or T-Sia. Cytolytic activity was determined by measuring the amount of lactate dehydrogenase (LDH) released into cultured media. Error bars represent SD of triplicate samples. N.D., not determined.

For all HER2-expressing cell lines, T-Sia was more potent than a mixture of Tras and unconjugated sialidase, underscoring the importance of tumor-targeted cell-surface localization (*SI Appendix*, Table S1).

Discussion

Trastuzumab is heralded as a triumph of precision medicine. However, although 74% of breast cancer patient biopsies show HER2 expression, only those patients with the highest levels of HER2 expression (~20%) have been found to respond and are thus eligible for this treatment (40). And of those considered eligible, only about 18% will respond at all to the single agent alone, and combination therapies reach 50% to 80% response rates at their best (41). Therefore, the vast majority of HER2-positive breast cancer patients (>80%) are either ineligible for, or unresponsive to, trastuzumab therapy. Much effort has been directed toward identifying mechanisms underlying trastuzumab's lack of efficacy in this large patient population, but a possible role for the cancer glycocalyx has been essentially ignored.

Our data suggest that sialylated glycans can protect breast cancer cells from trastuzumab-mediated ADCC by NK cells, raising the intriguing possibility that the sialic acid axis of immune modulation may contribute to clinical drug resistance. We targeted this axis with a type of AEC that functions as a precision cancer immune therapy. Treatment of tumor cells with T-Sia destroyed ligands for NK cell inhibitory Siglecs, enhanced binding of the activating receptor NKG2D, and also recruited FcγRIII to the tumor-immune synapse. Interestingly, T-Sia's benefits compared with trastuzumab alone were most pronounced with tumor targets expressing low levels of HER2 and higher levels of Siglec ligands. This observation supports a model in which NK cells engage in a calculation at the synapse that integrates inputs from both activating and inhibitory signals. Tumor targets with high HER2 levels can deliver a strong activating signal through FcγRIII, with or without Siglec inhibition. For these tumor types, sialidase treatment is not expected to substantially boost the NK cell response. But tumor cells with lower HER2 levels can counter these activating signals with recruitment of Siglecs to the immune synapse. In this situation, blocking Siglec engagement by precision glycocalyx editing can tip the scale toward NK cell activation, which has potentially significant implications regarding the ability to treat patients with HER2 levels deemed too low for a trastuzumab response.

T-Sia can also expose tumor cells to other immune pathways. There are 14 members of the human Siglec family, and 9 of these possess ITIM motifs. They are expressed on almost every type of immune cell, and their engagement has been linked to the suppression of B cells, T cells, neutrophils, macrophages, and dendritic cells (26, 42–44). Further, the ITIM-containing PILRα immune receptor recognizes sialylated mucin-type ligands and is thought to negatively regulate neutrophil infiltration during inflammation (45). Therefore, T-Sia may enable tissue-specific panactivation of the innate immune system and potentially act as a bridge to adaptive immunity as well. And sialylated glycans also modulate humoral immunity. They recruit complement factor H to cell surfaces and thereby block the alternative complement cascade, protecting cells from complement-mediated lysis (46). The multitude of immune functions that are modulated by sialosides provide ample selective pressure to enrich a hypersialylated phenotype during the course of tumor evolution and, conversely, a rich opportunity for cancer immune therapy.

Materials and Methods

Expression and Purification of Sialidase. *Escherichia coli* C600 transformed with plasmid pCVD364 containing the *V. cholerae* sialidase gene was a gift from Eric R. Vimr, University of Illinois, Urbana–Champaign (32, 33). Cells were grown in 2xYT media, supplemented with ampicillin (100 μg/mL) at 37 °C for 12 h. After incubation, the cells were harvested by centrifugation at 4,700 × *g* for 10 min, and the pellet was resuspended in osmotic shock buffer [20% (vol/vol) sucrose, 1 mM EDTA, 30 mM Tris·HCl, pH 8.0] and shaken gently for 10 min at room temperature. The cells were collected by centrifugation (9,000 × *g* for 10 min), and the pellets were resuspended in ice-cold pure water. After a 10-min incubation at 4 °C, the supernatant was obtained by centrifugation at 9,000 × *g* for 10 min. To purify the protein, the sample was further concentrated using an Amicon ultrafiltration device (membrane molecular mass cutoff, 30,000 Da), reconstituted in 0.02 M Tris·HCl buffer (pH 7.6), and loaded onto a HitrapQ-HP anion-exchange column (17-1154-01; GE Healthcare Life Sciences). The protein was eluted with a gradient of NaCl in 0.02 M Tris·HCl buffer (pH 7.6) at a flow rate of 5 mL/min. The protein fractions, with expected molecular mass as determined by SDS/PAGE, stained with Coomassie brilliant blue were collected and pooled. Endotoxins were removed using a high-capacity endotoxin removal spin kit (88275; Thermo Fisher Scientific), and the endotoxin concentration of the sample was determined by an LAL chromogenic endotoxin quantitation kit (88282; Thermo Fisher Scientific).

Activity Assay of Sialidase using MuNeuNAc. Hydrolytic activity of sialidase was identified as previously described (47). Briefly, sialidase [10 ng in Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+}, pH 7.0] was added to a 100-μL solution containing 1 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNeuNAc, Biosynth International Inc.) in DPBS buffer with Ca^{2+} and Mg^{2+} (pH 7.0). After incubation for 10 min at 37 °C, the mixture was diluted with 50 μL of 500 mM Na_2CO_3 buffer, pH 10.5. Fluorescence was read with a fluorescence spectrophotometer (excitation 325 nm; emission 420 nm).

Preparation of T-Sia. Purified *V. cholerae* sialidase (2 mg/mL in DPBS buffer with Ca^{2+} and Mg^{2+}, pH 7.0) was reacted with 12 equivalents of bicyclononyne-*N*-hydroxysuccinimide ester (BCN-NHS) at 4 °C overnight. Excess linker was removed using a PD-10 Desalting Column (17-0851-01GE; Healthcare Life Sciences). The degree of labeling was determined by ESI-MS (*SI Appendix*, Fig. S6*B*). Humanized Tras with C-terminal aldehyde-tag was produced as described previously (39). T-Sia was prepared by first coupling Tras with C-terminal aldehyde-tag (120 μM) to aminooxy-tetraethyleneglycol-azide (aminooxy-TEG-N_3) (10 mM) in 100 mM ammonium acetate buffer, pH 4.5, at 37 °C for 10 d, followed by buffer exchange into DPBS buffer with Ca^{2+} and Mg^{2+} (pH 7.0) using a PD-10 Desalting Column (17-0851-01; GE Healthcare Life Sciences). The resulting conjugate was then coupled to labeled sialidase at a 1:28 molar ratio at 120 mg/mL total protein concentration in DPBS buffer with Ca^{2+} and Mg^{2+}

(pH 7.0). After a 3-d incubation at room temperature, T-Sia was purified by size exclusion chromatography with a Superdex 200. The purified product was analyzed by SDS/PAGE gel and ESI-MS.

Cell Cytotoxicity Assay. Cell cytotoxicity assays were performed essentially as described previously (48). Antibody-dependent cell-mediated cytotoxicity (ADCC) was analyzed by measuring lactate dehydrogenase (LDH) release from breast cancer cells as a result of the ADCC activity of peripheral blood mononuclear cells (PBMCs) or NK cells. Tumor cells (target cells) were coincubated with PBMCs or NK cells (effector cells) at various effector/target (E/T) ratios in the presence or absence of sialidase or mAbs in triplicate. In a typical experiment, 100 μL of PBMCs or NK cells were added to a V-bottom 96-well plate containing 100 μL of target cells at 2×10^5 cells per milliliter. After 4 h, supernatants were collected, and LDH release was measured using an LDH cytotoxicity assay kit (88954; Thermo Fisher Scientific) according to the manufacturer's protocol. The absorbance at 490 nm was measured with a SpectraMax i3x (Molecular Devices). Specific lysis was calculated as 100 × (experimental target cell release − effector cells spontaneous release − target cells spontaneous release)/(target cells maximum release − target cells spontaneous release). For antibody-blocking experiments, purified NK cells were preincubated with 5 μg/mL blocking antibody for 1 h at 37 °C before adding to target cells.

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1. Vanneman M, Dranoff G (2012) Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer* 12(4):237–251.
2. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12(4):252–264.
3. Restifo NP, Dudley ME, Rosenberg SA (2012) Adoptive immunotherapy for cancer: Harnessing the T cell response. *Nat Rev Immunol* 12(4):269–281.
4. Bhatia A, Kumar Y (2014) Cellular and molecular mechanisms in cancer immune escape: A comprehensive review. *Expert Rev Clin Immunol* 10(1):41–62.
5. Raulet DH, Vance RE (2006) Self-tolerance of natural killer cells. *Nat Rev Immunol* 6(7): 520–531.
6. Melero I, et al. (2015) Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat Rev Cancer* 15(8):457–472.
7. Curran MA, Montalvo W, Yagita H, Allison JP (2010) PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci USA* 107(9):4275–4280.
8. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G (2013) Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. *Cancer Res* 73(12):3591–3603.
9. Woo SR, et al. (2012) Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* 72(4):917–927.
10. Sakuishi K, et al. (2010) Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 207(10):2187–2194.
11. Rezvani K, Rouce RH (2015) The application of natural killer cell immunotherapy for the treatment of cancer. *Front Immunol* 6:578.
12. Weiskopf K, Weissman IL (2015) Macrophages are critical effectors of antibody therapies for cancer. *MAbs* 7(2):303–310.
13. Palucka K, Banchereau J (2012) Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* 12(4):265–277.
14. Chester C, Marabelle A, Houot R, Kohrt HE (2015) Dual antibody therapy to harness the innate anti-tumor immune response to enhance antibody targeting of tumors. *Curr Opin Immunol* 33:1–8.
15. Boligan KF, Mesa C, Fernandez LE, von Gunten S (2015) Cancer intelligence acquired (CIA): Tumor glycosylation and sialylation codes dismantling antitumor defense. *Cell Mol Life Sci* 72(7):1231–1248.
16. Büll C, Stoel MA, den Brok MH, Adema GJ (2014) Sialic acids sweeten a tumor's life. *Cancer Res* 74(12):3199–3204.
17. Fuster MM, Esko JD (2005) The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nat Rev Cancer* 5(7):526–542.
18. Varki A, Gagneux P (2012) Multifarious roles of sialic acids in immunity. *Ann N Y Acad Sci* 1253:16–36.
19. Macauley MS, Crocker PR, Paulson JC (2014) Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol* 14(10):653–666.
20. Brossart P, et al. (2001) The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res* 61(18):6846–6850.
21. Cohen M, et al. (2010) Sialylation of 3-methylcholanthrene-induced fibrosarcoma determines antitumor immune responses during immunoediting. *J Immunol* 185(10): 5869–5878.
22. Hudak JE, Canham SM, Bertozzi CR (2014) Glycocalyx engineering reveals a Siglec-based mechanism for NK cell immunoevasion. *Nat Chem Biol* 10(1):69–75.
23. Jandus C, et al. (2014) Interactions between Siglec-7/9 receptors and ligands influence NK cell-dependent tumor immunosurveillance. *J Clin Invest* 124(4):1810–1820.
24. Läubli H, et al. (2014) Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer. *Proc Natl Acad Sci USA* 111(39):14211–14216.
25. Nicoll G, et al. (2003) Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. *Eur J Immunol* 33(6):1642–1648.
26. Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7(4):255–266.
27. Avril T, Floyd H, Lopez F, Vivier E, Crocker PR (2004) The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. *J Immunol* 173(11):6841–6849.
28. Nicoll G, et al. (1999) Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes. *J Biol Chem* 274(48): 34089–34095.
29. Angata T, Varki A (2000) Siglec-7: A sialic acid-binding lectin of the immunoglobulin superfamily. *Glycobiology* 10(4):431–438.
30. Seidel UJ, Schlegel P, Lang P (2013) Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. *Front Immunol* 4:76.
31. Suzuki M, Kato C, Kato A (2015) Therapeutic antibodies: Their mechanisms of action and the pathological findings they induce in toxicity studies. *J Toxicol Pathol* 28(3): 133–139.
32. Vimr ER, Lawrisuk L, Galen J, Kaper JB (1988) Cloning and expression of the Vibrio cholerae neuraminidase gene nanH in Escherichia coli. *J Bacteriol* 170(4):1495–1504.
33. Taylor G, Vimr E, Garman E, Laver G (1992) Purification, crystallization and preliminary crystallographic study of neuraminidase from Vibrio cholerae and Salmonella typhimurium LT2. *J Mol Biol* 226(4):1287–1290.
34. Cosman D, et al. (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14(2):123–133.
35. Drake PM, et al. (2014) Aldehyde tag coupled with HIPS chemistry enables the production of ADCs conjugated site-specifically to different antibody regions with distinct in vivo efficacy and PK outcomes. *Bioconjug Chem* 25(7):1331–1341.
36. Morgan A, et al. (1995) The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, Fc gamma RI and Fc gamma RIII binding. *Immunology* 86(2):319–324.
37. Woof JM, Burton DR (2004) Human antibody-Fc receptor interactions illuminated by crystal structures. *Nat Rev Immunol* 4(2):89–99.
38. Hudak JE, et al. (2012) Synthesis of heterobifunctional protein fusions using copper-free click chemistry and the aldehyde tag. *Angew Chem Int Ed Engl* 51(17):4161–4165.
39. Rabuka D, Rush JS, deHart GW, Wu P, Bertozzi CR (2012) Site-specific chemical protein conjugation using genetically encoded aldehyde tags. *Nat Protoc* 7(6):1052–1067.
40. Lee AH, Key HP, Bell JA, Hodi Z, Ellis IO (2011) Breast carcinomas with borderline (2+) HER2 immunohistochemistry: Percentage of cells with complete membrane staining for HER2 and the frequency of HER2 amplification. *J Clin Pathol* 64(6):490–492.
41. Bartsch R, Wenzel C, Steger GG (2007) Trastuzumab in the management of early and advanced stage breast cancer. *Biologics* 1(1):19–31.
42. Ohta M, et al. (2010) Immunomodulation of monocyte-derived dendritic cells through ligation of tumor-produced mucins to Siglec-9. *Biochem Biophys Res Commun* 402(4): 663–669.
43. Perdicchio M, et al. (2016) Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells. *Proc Natl Acad Sci USA* 113(12):3329–3334.
44. Ikehara Y, Ikehara SK, Paulson JC (2004) Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *J Biol Chem* 279(41):43117–43125.
45. Kuroki K, et al. (2014) Structural basis for simultaneous recognition of an O-glycan and its attached peptide of mucin family by immune receptor PILRα. *Proc Natl Acad Sci USA* 111(24):8877–8882.
46. Blaum BS, et al. (2015) Structural basis for sialic acid-mediated self-recognition by complement factor H. *Nat Chem Biol* 11(1):77–82.
47. Parker RB, McCombs JE, Kohler JJ (2012) Sialidase specificity determined by chemoselective modification of complex sialylated glycans. *ACS Chem Biol* 7(9):1509–1514.
48. Decker T, Lohmann-Matthes ML (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 115(1):61–69.