GLYCANS: MASTERS OF IMMUNITY, FROM CANCERS TO INFLAMMATORY DISEASE

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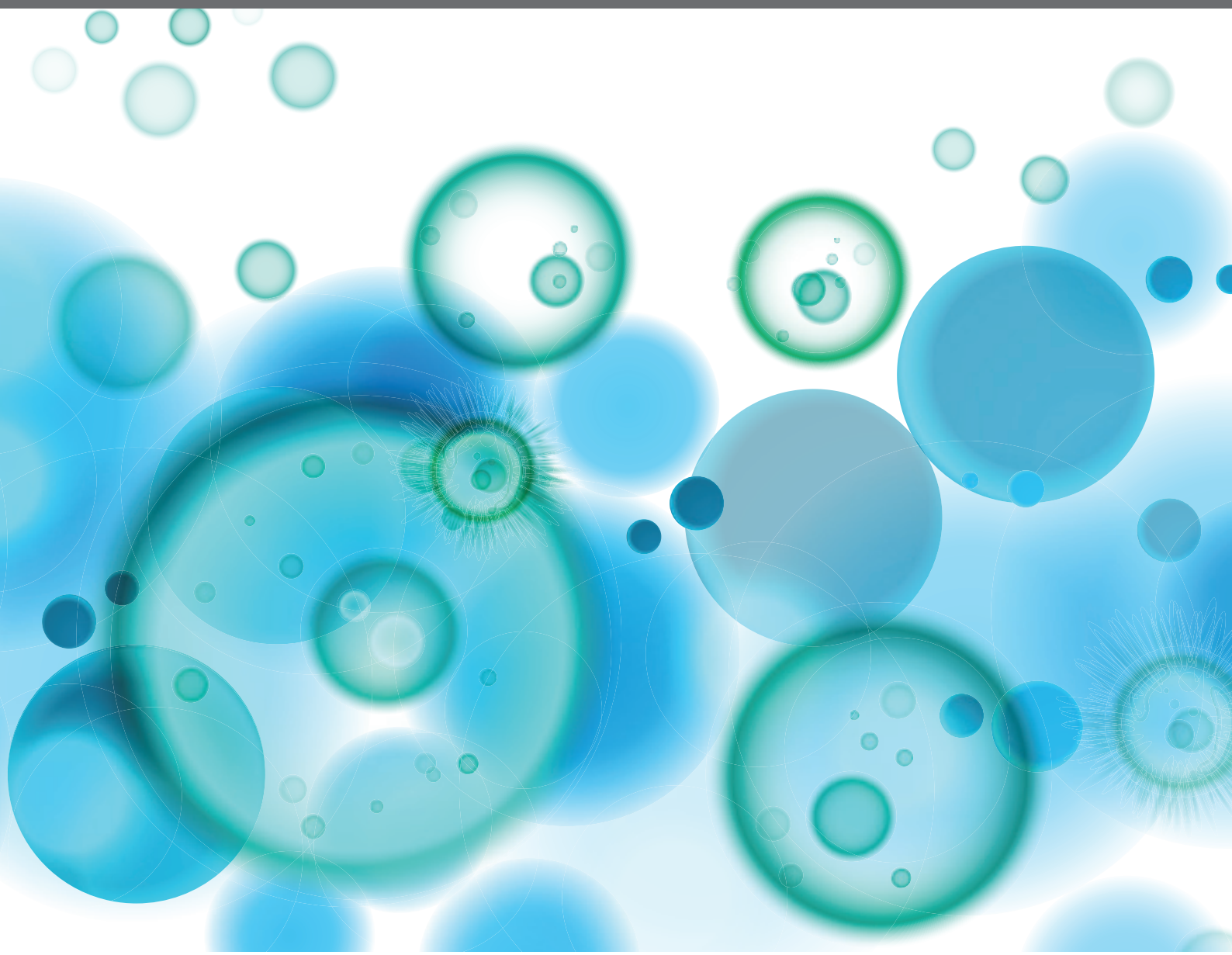

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GLYCANS: MASTERS OF IMMUNITY, FROM CANCERS TO INFLAMMATORY DISEASE

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Table of Contents

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Editorial: Glycans: Masters of immunity, from cancers to inflammatory disease

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Editorial on the research topic
Glycans: Masters of immunity, from cancers to inflammatory disease

As editors of this Research Topic, it was our pleasure to review a wide range of fascinating articles and reviews within the field. In this editorial we summarize the main findings and perspectives detailed within each of the accepted articles.

Hugonnet et al. give an overview on the different functions sialyltransferases have in cancer progression and inhibition of anti-cancer immunity. Hypersialylation – a term used for a cancer-associated increase in intratumoral sialic acid content – has been described many years ago and can be significantly supported by sialyltransferases. However, only recent elucidation of various mechanisms promoting cancer progression including engagement of Siglec receptors, stabilization of receptors and influencing of antigen presentation have led to further investigations to target hypersialylation and sialyltransferases for cancer therapy.

Mucins are well studied carriers of these hypersialylated glycans, and as such, in a hypothesis article, Hitchcock et al. propose to use an antibody against the cancer-associated TAG-72 mucin protein to determine the extent of surgery for patients with colorectal cancer (CRC). Mucins play an important role in cancer progression and metastasis formation. The use of cancer-associated changes in mucins could therefore be a valuable diagnostic and therapeutic target.

Hypersialylated glycans are well-known to engage Siglecs, however there is much complexity beyond this statement. Van Houtum et al. summarize the current role of Siglec receptors in the tumor microenvironment. Interactions of Siglec receptors with sialic acid-containing ligands have recently moved into the focus of a broad interest as the

first Siglec-targeting antibodies as well as sialic acid-reducing compounds have reached early clinical stages of development.

An improved understanding of the Siglec-Ligand interactions described above is likely to prove useful in the application of this axis in regulating immune responsiveness. The current tools being used to explore the Siglec-ligand interaction, and in particular the physiological ligands, is discussed within the mini review by Jiang et al. In this review the advantages and disadvantages of the current methodologies to identify relevant Siglec ligands is summarised, including affinity purification, proximity labelling, and genetic modifications of cells and genome-wide screening, with consideration given to the *cis* or *trans* orientation of the ligand-receptor interaction.

Some of these methodologies are then put into practice by Chang et al. who explore the molecular basis for a Siglec-7 checkpoint axis in chronic lymphocytic leukemia. In this research article the authors identify high levels of Siglec-7 ligands expressed on malignant B-cells predominately on CD43, CD45, and PSGL-1 counter receptors. The interaction of these counter receptors with Siglec-7 is facilitated through a display of disialyl-T O-glycans. This overexpression of the disialyl-T antigen likely results from overexpression of the ST6GalNAc-IV enzyme, a sialyltransferase which further sialylates the sialyl-T antigen (at the core GalNAc residue) to form the disialylated antigen. These decorations of disialyl-T antigen on malignant B-cells may inhibit anti-tumour immunity, in particular NK cell cytotoxicity, providing another example of the sialoglycan-siglec axis in tumour immunity.

Remaining of the subject of sialic acids, Villanueva-Cabelloet al. discuss and analyse the current knowledge on polysialic acid (polySia) and the immune system. Although much has been elucidated about polySia in mammals, such as its role in the central nervous system, the role of polySia in other tissues are not fully understood, including in cells of the immune system. The authors describe the dynamic changes that PolySia presents during differentiation, maturation, and activation of different types of immune cells of the innate and adaptive response. They also discuss PolySia involvement in cellular regulatory mechanisms. The paper addresses various aspects about polySia, including its biosynthesis as well as the tools for the identification and structural characterization of this glycan. Furthermore, the paper discusses various functional aspects in the immune system and its potential therapeutic implications.

Specific glycans, often on specific proteins, have been associated with cell death for decades. Parshenkov and Hennet sought to drill down into the specific pathways associated with lectin-induced cell death *via* these specific glycans. Using three lectins (Wheat Germ Agglutinin, Maackia Amurensis lectin I, Aleuria aurantia lectin) on the same cell line model, the authors demonstrated that caspase-independent but autophagy-dependent death pathways were activated. The authors conclude by arguing that the activation of these pathways may be a useful tool in sensitising tumours to other cytotoxic agents, especially those tumours that become resistant to more classically activated pathways.

Remaining of the subject of lectins, Griffiths et al. show their versatility in using them to develop a diagnostic tool for Invasive Aspergillosis (IA), a disease which is notoriously difficult to identify at an early stage. The authors examined the sequence and expression of four C-type lectin and lectin-like receptors (Dectin-1, Dectin-2, Mincle and Mcl) alongside matched responses to Aspergillus (IL6, TNF) in 42 patients. Correlation analysis revealed novel IA disease risk factors which they used to develop a pre-emptive patient stratification protocol to identify haematopoietic stem cell transplant patients at high and low risk of developing IA.

In an analysis of patients with chronic obstructive pulmonary disease (COPD), Krick et al. identify an inverse correlation and a role of the α2,6-sialyltransferase ST6GAL1 in the production and secretion of IL-6, an important mediator of exacerbations in patients with COPD. The authors use primary patient samples and an *in vitro* system to demonstrate that low levels of ST6GAL1 increase IL-6 levels. Interestingly, they are also able to show that cigarette smoke can decrease ST6GAL1 and increase thereby IL-6.

In another disease of chronic inflammation, Wang et al. explore desialylation on synovial fibroblasts in rheumatoid arthritis (RA). Previous work had shown that RA synovial fibroblasts display lower levels of sialylation and sialyltransferases than healthy controls. Using both *in vivo* and *in vitro* models the authors explore the impact of desialylation on fibroblasts using sialidases on phenotype and function. RNA-seq analysis and protein validation showed fibroblasts became hyper-inflammatory after the removal of cell-surface sialic acids, and displayed impaired migration. The authors therefore argue that hypo-sialylation itself may be a disease driver in RA.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ST6GAL1 and α2-6 Sialylation Regulates IL-6 Expression and Secretion in Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is a systemic disease strongly associated with cigarette smoking, airway inflammation, and acute disease exacerbations. Changes in terminal sialylation and fucosylation of asparagine (N)-linked glycans have been documented in COPD, but the role that glycosyltransferases may play in the regulation of N-linked glycans in COPD has not been fully elucidated. Recent studies suggest that modulation of ST6GAL1 (ST6 beta-galactoside alpha-2,6-sialyltransferase-1), which catalyzes terminal α2-6 sialylation of cellular proteins, may regulate inflammation and contribute to COPD phenotype(s). Interestingly, it has been previously demonstrated that ST6GAL1, a Golgi resident protein, can be proteolytically processed by BACE1 (beta-site amyloid precursor protein cleaving enzyme-1) to a circulating form that retains activity. In this study, we showed that loss of ST6GAL1 expression increased interleukin (IL)-6 expression and secretion in human bronchial epithelial cells (HBECs). Furthermore, exposure to cigarette smoke medium/extract (CSE) or BACE1 inhibition resulted in decreased ST6GAL1 secretion, reduced α2-6 sialylation, and increased IL-6 production in HBECs. Analysis of plasma ST6GAL1 levels in a small COPD patient cohort demonstrated an inverse association with prospective acute exacerbations of COPD (AECOPD), while IL-6 was positively associated. Altogether, these results suggest that reduced ST6GAL1 and α2-6 sialylation augments IL-6 expression/secretion in HBECs and is associated with poor clinical outcomes in COPD.

Keywords: ST6GAL1, COPD, cigarette smoke, bronchial epithelium, inflammation

INTRODUCTION

The glycosylation of proteins and lipids has been shown to be critically involved in the regulation of a variety of physiological and pathological processes in eukaryotic cells (1–3). ST β-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) is a type II membrane protein that is commonly localized in the Golgi apparatus catalyzing the transfer of a sialic acid from Cytidine 5′-monophosphate (CMP)-sialic acid to galactose-containing glycans (4). ST6GAL1 plays an important role in cancer progression and metastasis (5–8). The expression of ST6GAL1 has been determined to be downregulated in some cancers including bladder cancer and upregulated in others such as prostate, lung, and breast cancer (9–11). Interestingly, ST6GAL1 has been shown to regulate Notch1, Hes1, matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) in lung cancer and altered α2-6 sialylation has been linked to lung cancer progression (10). ST6GAL1 has also been documented for its role in other cancer cellular processes including angiogenesis (12, 13), inflammation (14, 15), and apoptotic resistance (16–18).

In the lung, it has been shown that ST6GAL1 mRNA levels were significantly increased in non-small cell lung cancer, whereas other sialyltransferases were downregulated, such as ST3GAL1, ST6GALNAC3, and ST8SIA6 (10). In addition, α2,6-sialylation by ST6GAL1 has been linked to lung cancer progression by mediating tumor invasiveness and protecting cancer cells through hypoxia inducible factor (HIF)-1α signaling (19). Recently, ST6GAL1 was linked to modulating airway mucins and sialylation levels in asthma, which further altered cell proliferation and inflammation in this disease (20). The literature on ST6GAL1 in other chronic lung diseases; however, is limited and its role in underlying lung disease processes has not been fully elucidated.

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death globally and is strongly associated with cigarette smoke and airway inflammation with disease exacerbations being a prognostic factor increasing the mortality of the disease (21–23). Inflammation has been shown to lead to alterations in protein glycosylation (24–27) and assessment of plasma from individuals with COPD demonstrated significant changes in compound glycan structures such as tetra-sialylated and complex-type fucosylated glycoforms (28). In addition, alterations in asparagine (N)-linked glycans have been documented for their role in COPD (29) and the function of α1-antitrypsin (30, 31). Still, studies investigating the role of terminal glycosylation and the potential role of glycosyltransferases in the regulation and function of the N-linked glycans in COPD have not been fully elucidated.

In this study, our goal was to determine the effects of gain- and loss-of-function of ST6GAL1 on the response of human bronchial epithelial cells (HBECs) to inflammatory stimuli, and identify the clinical relevance for changes in circulating ST6GAL1 in smoking, COPD, and acute exacerbations of COPD (AECOPD). Our findings show that loss of ST6GAL1 and α2-6 sialylation increases interleukin (IL)-6 expression/secretion in HBECs similar to cigarette smoke and ST6GAL1 cleavage/secretion blockade. In addition, reduced circulating ST6GAL1, while increased IL-6 levels in the same COPD patient cohort, was shown to associate with prospective AECOPD.

MATERIALS AND METHODS

Study Approval

All protocols were approved by the Institutional Review Board of the University of Alabama at Birmingham and written consent was obtained from each patient enrolled in the study. The research herein was performed in accordance with the Helsinki Declaration.

Study Population

Individuals with COPD as defined previously (32) were recruited by the University of Alabama at Birmingham (UAB) Lung Health Center from May 2017 through December 2018. These patients were classified based on severity of the COPD using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (33), which are as follows: GOLD 1: Mild (FEV1 ≥ 80% predicted); GOLD 2: Moderate (FEV1 between 50 and 79% predicted); GOLD 3: Severe (FEV1 between 30 and 49% predicted); and GOLD 4: Very severe (FEV1 <30% predicted). Subjects were recruited during their stable state and followed prospectively for one year. Data collection included demographic data, smoking history, pre- and post-bronchodilator spirometry using American Thoracic Society (ATS) standards (34), dyspnea assessment using the modified medical research questionnaire (MMRC), respiratory symptom assessment using the Breathlessness, Cough, and Sputum Scale (BCSS), and queried for AECOPD within the previous 12-months prior to the study visit. AECOPD was defined as a persistent worsening of the subject's condition from a stable state that was acute in onset, lasted more than 48 hours, and required additional treatment (35, 36). Inclusion criteria to participate was to have a diagnosis of COPD (35) and willing to sign the inform consent to participate.

Blood Sampling and ST6GAL1 Measurements

Venous blood was sampled during the study visit and processed immediately by centrifugation and collection of the plasma fraction with subsequent storage at −80°C. Specimens were thawed within 6 months and ST6GAL1 levels were measured by enzyme-linked immunosorbent assay (ELISA) utilizing the Human ST6GAL1 ELISA (RAB1722; Sigma, St. Louis, MO; USA) as per provided protocol.

Bronchial Epithelial Cell Cultures

16HBE cells (HBECs), an immortalized human bronchial epithelial cell line, were plated and grown as recently described (37). All treatments were carried out in antibiotic-free Eagle's Minimum Essential Medium (EMEM; ATCC, Manassas, VA; USA) with 1x GlutaMAX (Gibco; Gaithersburg, MD; USA) and 10% fetal bovine serum (Atlas Biologicals; Fort Collins, CO;

USA) on cell culture plates coated with collagen IV. Cigarette-smoked medium/extract (CSE) was prepared by bubbling cigarette smoke through 1.0 ml serum-free EMEM per cigarette, followed by sterile-filtering through a 0.45 μm filter, and subsequent spectrophotometric analysis to define concentration. For experimental purposes, 100% CSE was set at an OD = 1.0 when absorbance was measured at 320 nm. Short-term CSE exposure was performed on HBECs plated at 6.0 x 10^4 cells per well in 12-well plates. Following overnight incubation to allow for cell attachment, the cells were treated with varied concentrations of CSE for 24 hours. Long-term CSE exposure was performed on HBECs following a continuous culture method utilizing 3-day intervals for media changes between passages. Briefly, cells were collected by trypsinization, plated at 1.5 x 10^5 cells per well on a 6-well plate, and treated with 2.5 ml medium containing CSE every 3 days. The remaining cells from each well were washed in cold phosphate buffered saline (PBS) and pelleted at 300 x g. Cell pellets and conditioned media for each time point were stored at -80°C until time of analysis.

For β-site amyloid protein cleaving enzyme 1 (BACE1) inhibitor studies, 16HBE cells were plated as described above followed by a 24-hr recovery period. Then, cells were incubated for 2 hours with either DMSO vehicle or 20 μM LY2886721 (Selleck Chem; UK), which is a small molecule inhibitor of BACE1/2; hereafter referred to as iBACE. After pre-incubation with iBACE, CSE was added and cells were incubated for an additional 72 hours.

Knockdown and Overexpression of ST6Gal in Bronchial Epithelial Cells

HBECs were generated utilizing lentiviral transduction particles containing a non-mammalian shRNA control sequence (pLKO.1-puro SHC002V; Sigma, USA); MISSION shRNA targeted against ST6GAL1 (SHCLNV-NM_003032; Sigma, USA); or an expression cassette for overexpression of ST6GAL1 (M0351; GeneCopoeia; Rockville, MD; USA). Cells were plated at a density of 4.0 x 10^4 cells per well in 24-well plates and infected overnight with 10 TU per cell in Opti-MEM (Gibco, USA) containing 8 μg/ml Polybrene (Sigma, USA). Medium was changed to EMEM with GlutaMAX and 10% fetal bovine serum and cells were allowed to recover for 24 hours. Following the recovery period, cells were selected with puromycin (10 μg/ml) for 3 days, and then maintained in 0.5 μg/ml puromycin. Knockdown and overexpression were confirmed by Western blot and quantitative RT-PCR.

RNA Purification and Quantitative RT-PCR

RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific, Grand Island, NY, USA). For gene expression analysis, qRT-PCR was performed using the following Taqman probes (Life technologies/Applied Biosystems; Carlsbad, CA; USA): Hs00949382 for ST6GAL1; Hs01555410_m1 for IL1β; Hs00174131 for IL6; and Hs00174103_m1 for IL8. Hs02758991 for GAPDH was used as our internal control and the transcript expression data for each gene was normalized to GAPDH.

Flow Cytometry

Sialylation was assessed utilizing FITC-conjugated *Sambucus nigra* agglutinin (SNA)-FITC; Vector Labs; Burlingame, CA; USA), which is a lectin that binds preferentially to α2-6 linked sialic acid as previously described (25). Briefly, HBECs were gently dissociated using Accutase (Gibco, USA), washed with cold Dulbecco's phosphate buffered saline containing 0.1mM calcium (DPBS; Gibco, USA), and stained for 1 hour in the dark at 4°C with 10 μg SNA-FITC per ml DPBS. Cells were washed twice in DPBS and fixed in 1% paraformaldehyde. Stained cells were analyzed on a LSR II Flow cytometer and the data were analyzed utilizing FlowJo software (BD Life Sciences; Franklin Lakes, New Jersey; USA).

IL-6 and IL-8 ELISA

IL-6 and IL-8 cytokine levels in cell culture media were measured utilizing a human IL-6 and a human IL-8 enzyme-linked immunosorbent assays (ELISA) from Invitrogen (Thermo Scientific). Briefly, media from 16HBE cell cultures were collected after treatment at indicated time points, clarified by centrifugation at 500 x g for 10 min at 4°C, loaded onto an assay plate coated with anti-IL-6 or anti-IL-8 capture antibody, and incubated for 2 hours at room temperature. After completing the manufacturer's suggested protocol, absorbance was measured at 450 nm.

ST6GAL1 Slot Blot

Samples were prepared in sample loading buffer containing SDS and DTT. Equal volumes of samples were transferred onto a 0.45μm nitrocellulose membrane under gentle vacuum using Bio-Rad Bio-Dot SF microfiltration apparatus (Bio-Rad, Life Sciences, USA). To ensure equal loading, the supernatants were normalized to total cellular protein following a Bradford assay and loaded equivalently. Slot blots were probed with a monoclonal ST6GAL1 antibody (MA5-11900; Thermo Scientific; Grand Island, NY; USA). The secondary antibody used was a goat anti-mouse IgM antibody conjugated to HRP. Blots were developed using enhanced chemiluminescence SuperSignal West Dura Substrate (Thermo Scientific) and imaged using the GE Imaging System (GE Healthcare). ImageJ software (38, 39) was used to perform densitometry measurements.

Statistics

Data were expressed as mean ± SEM, median [interquartile range or IQR], and counts (percentages). Student's *t* tests were used to analyze group differences for continuous variables; Mann Whitney U tests were used to measure between group differences for ST6GAL1 given its non-Gaussian distribution in the human cohort; and 1-way ANOVA or Kruskal Wallis tests with appropriate post-hoc tests were used to measure between-group differences for analyses that involved three or more groups. Pearson's correlation coefficients were used to measure correlation between ST6GALl and outcomes for COPD in the clinical cohort. Logistic regression models adjusting for post-BD FEV1 percent predicted smoking status (current *vs* not-current) were used to measure the association between ST6GAL1 and

AECOPD at 1-year of follow-up. SPSS (version 26.0, Chicago, IL, USA) and PRISM (Version 9, GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Sialylation Is Reduced by Cigarette Smoke Extract in HBECs

Previous reports have shown that glycosylation is altered in COPD, and reduced following, cigarette smoke exposure and may result in inflammation (24–28, 40). To determine whether cigarette smoke results in changes in ST6GAL1 expression and/or α2-6 sialylation, HBECs were subjected to mRNA transcript analysis and SNA-FITC staining (which preferentially binds to α2-6 sialic acid on terminal galactose/N-acetylglucosamine over the α-2,3 linkage) and flow cytometric analysis following exposure to CSE for 3, 6, and 15 days. Interestingly, mRNA expression was reduced at 3, 6, and 15 day of CSE, reaching statistical significance at 3 and 15 days (**Figure 1A**). Similarly, the levels of α2-6 sialic acid were reduced in the presence of CSE at all three time points analyzed when compared to vehicle-treated cultures (**Figures 1B–D**). These data suggest that ST6GAL1 expression and α2-6 sialylation are reduced following exposure to cigarette smoke extract in HBECs.

ST6GAL1 Overexpression and Knockdown Leads to Alterations in Sialylation in HBECs

To determine the consequences of the gain- and loss-of-function of ST6GAL1 in bronchial epithelium, we stably overexpressed or knocked down ST6GAL1 in bronchial epithelial cell cultures. Overexpression showed a marked increase in relative ST6GAL1 mRNA expression, and significant downregulation following siRNA gene knockdown and clonal selection (**Figure 2A**). CSE reduced the levels of ST6GAL1 expression in the control group; however, it did not statistically change the expression levels in the knockdown or overexpression groups (**Figure 2A**). Assessment of α2-6 sialylation in these stably transfected cultures with and without CSE showed a further reduction following ST6GAL1 knockdown (**Figures 2B, C**). As expected,

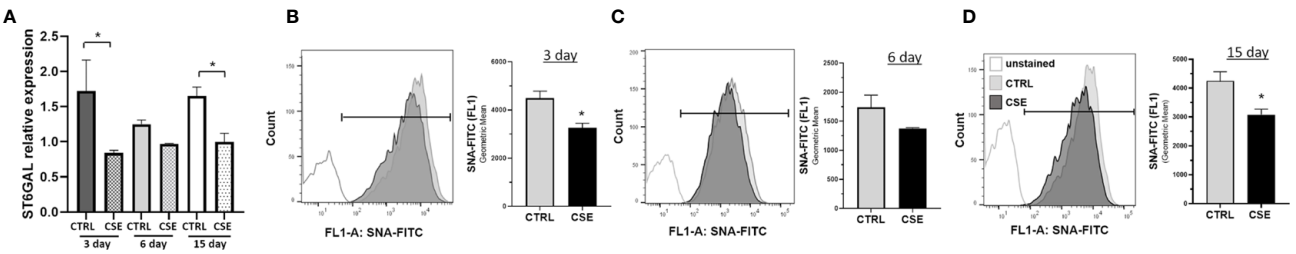

FIGURE 1 | Reduced extracellular sialylation in HBEs following 3, 6 and 15-day CSE exposure. **(A)** Relative mRNA expression of ST6GAL1 at times indicated without (CTRL) and with cigarette smoke extract (CSE). **(B–D)** Flow cytometric histogram showing levels of α2-6 sialylation for HBECs cultured for 3, 6, and 15 days without (CTRL) and with (CSE). Ten thousand events were collected for each group, analyzed by SNA-FITC staining, and shown as the geometric mean of the values. Experiments were performed in triplicate and three separate experiments. SNA, Sambucus Nigra Lectin; and CSE, cigarette smoke extract. All bar graphs are means ± SEM with *p < 0.05.

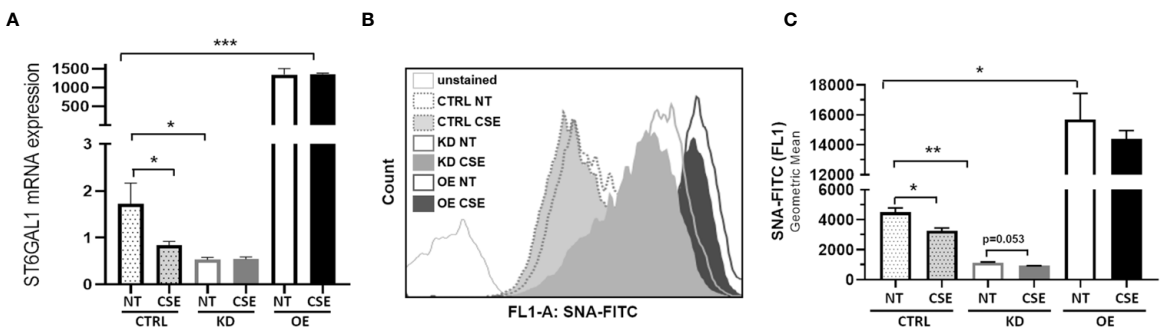

FIGURE 2 | ST6GAL1 overexpression and knockdown leads to alterations in sialylation in HBECs. **(A)** Bar graphs showing ST6GAL1 mRNA levels in stably transfected ST6GAL1 KD and OE cultures compared to control (CRTL) infected cells in the presence or absence of CSE. **(B)** Flow cytometric analysis of α2-6 sialylation using SNA-FITC labeling of HBECs (CTRL, ST6GAL1 KD, and ST6GAL1 OE) following treatment with and without CSE for 3 days. **(C)** Graphical data of the 10,000 events that were collected from **(B)** for each group and analyzed. Abbrv. SNA, Sambucus Nigra Lectin; CTRL, pLKO vector control; KD, ST6GAL1 knockdown; and OE, ST6GAL1 overexpression; NT, no treatment; CSE, cigarette smoke extract; and HBECs, human bronchial epithelial cells. Experiments were performed in triplicate using three separate experiments. All bar graphs are means ± SEM with *p < 0.05, **p < 0.01 and ***p < 0.001.

stable overexpression of ST6GAL1 led to an increase in α2-6 sialylation (**Figures 2B, C**). Following 3 days of CSE exposure, the levels of α2-6 sialylation were shown to significantly decrease further when compared to vehicle treatment in the control (**Figures 2B, C**). The mean α2-6 sialylation was slightly reduced in the ST6GAL1 knockdown (p=0.053) and sialylation changes in the overexpressing cell cultures but did not reach statistical significance. These data suggest that overexpression of ST6GAL1 increases α2-6 sialylation and ST6GAL1 knockdown results in similar ST6GAL1 expression and α2-6 sialylation levels as CSE exposure in the HBEC cultures (**Figure 1**).

ST6GAL1 Knockdown Increases IL-6 Expression and Secretion

Next, we wanted to determine the functional outcomes of altering ST6GAL1 expression on the production of the pro-inflammatory cytokines IL-1β, IL-6, and IL-8. ST6GAL1 knockdown alone led to a significant increase in mRNA levels of IL-1β, IL-6, and IL-8 (**Figure 3A**). Overexpression demonstrated upregulation in IL-8 but changes were not significant for IL-1β or IL-6 (**Figure 3A**). Interestingly, IL-6 protein secretion was higher in ST6GAL1 knockdown cells (**Figure 3B**) compared to the control and ST6GAL1 overexpressing cells, corroborating the changes in mRNA expression. In contrast, IL-8 secretion was not statistically different in the supernatants of the groups (**Figure 3C**), while IL-1β was below the limit of detectability (data not shown). These data suggest that loss of ST6GAL1 leads to increased IL-6 protein levels in HBECs that is not observed with IL-8.

IL-6 Expression and Secretion Is Increased by Cigarette Smoke Exposure in HBECs and Partially Rescued by ST6GAL1 Overexpression

To determine whether CSE affects IL-6 expression and secretion similarly to ST6GAL1 knockdown, we subjected HBECs to CSE for 24 hours. mRNA upregulation (**Figure 4A**) and protein secretion of IL-6 (**Figure 4B**) were observed following CSE exposure in HBECs compared to the controls and is consistent with our findings observed in the ST6GAL1 knockdown cultures (**Figures 3A, B**). When stable ST6GAL1 OE HBECs were exposed to CSE, there was no statistical change in IL-6

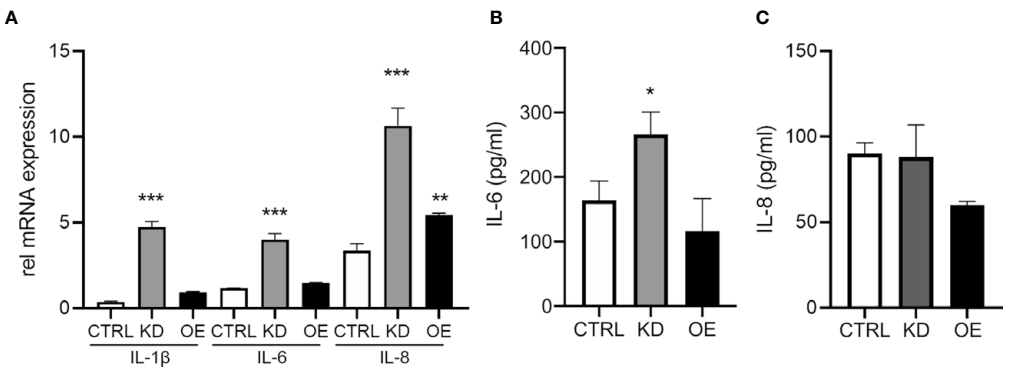

FIGURE 3 | ST6GAL1 knockdown increases IL-6 expression and secretion. **(A)** Relative mRNA transcript levels of IL-1β, IL-6, and IL-8 in CTRL, ST6GAL1 KD, and ST6GAL1 OE HBECs. Analysis of IL-6 **(B)** and IL-8 **(C)** protein levels from supernatants of CTRL, ST6GAL1 KD, and ST6GAL1 OE HBECs using ELISA. CTRL, pLKO vector control; KD, ST6GAL1 knockdown; OE, ST6GAL1overexpression; rel, relative; IL-6, interleukin 6; and IL-8, interleukin 8. All experiments were reproduced 3 times and done in triplicates with bar graphs indicating means ± SEM with *p < 0.05, **p < 0.01 and ***p < 0.001.

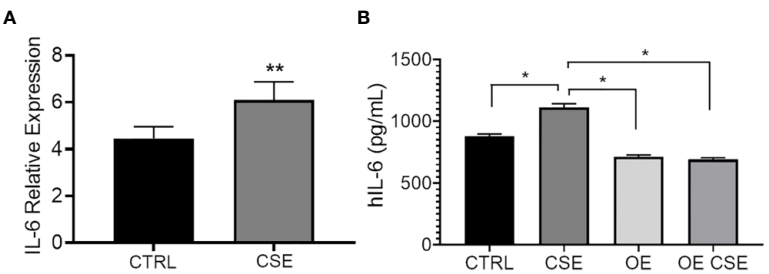

FIGURE 4 | IL-6 expression and secretion is increased following cigarette smoke exposure and attenuated by ST6GAL1 overexpression in HBECs. **(A)** Relative mRNA transcript levels of IL-6 in control (CTRL) and CSE treated HBECs. **(B)** IL-6 protein levels from supernatants of control, and ST6GAL1 OE HBECs exposed to CSE for 24 hours. CTRL, control; CSE, cigarette smoke extract/medium; and OE, ST6GAL1 overexpression. All experiments were reproduced 3 times and done in triplicates with bar graphs indicating means ± SEM with *p < 0.05 and **p < 0.01.

secretion levels when compared to the control group; however, the OE cultures showed a statistically significant reduction in IL-6 secretion compared to CSE alone (in the control group) (**Figure 4B**). Altogether, these findings suggest a potential link between the loss of ST6GAL1 and CSE induced IL-6 production in HBECs that is partially blocked by ST6GAL1 overexpression.

Inhibition of ST6GAL1 Secretion Reduces Sialylation in HBECs Similar to CSE

Previous reports have shown that BACE1 (beta-site amyloid precursor protein cleaving enzyme 1) cleaves and releases ST6GAL1 from the trans-Golgi into the secretory pathway (41, 42). Therefore, we wanted to determine whether BACE1-dependent ST6GAL1 cleavage and secretion was necessary for any of the α2-6 sialylation in HBECs. Both the expression of BACE1 and ST6GAL1 has been shown previously in the lung epithelium (20, 43, 44). BACE1 inhibition in the HBECs resulted in a significant decrease in α2-6 sialylation levels when compared to controls and similar to the level of reduction when compared to CSE (**Figures 5A, B**). Combined, BACE1 inhibition and CSE resulted in more of a reduction in α2-6 sialylation (**Figures 5A, B**). As expected, ST6GAL1 secretion into the HBEC culture medium was reduced by BACE1 inhibition (**Figure 5C**), which was determined by slot blot and densitometry analysis. Interestingly, ST6GAL1 secretion was reduced following CSE and with both iBACE and CSE (**Figure 5C**). These data suggest that cigarette smoke extract exposure or blocking ST6GAL1 cleavage by BACE1 inhibition partially reduces secretion of ST6GAL1 and levels of α2-6 sialylation in HBEC cultures.

BACE1 Inhibition Increases IL-6 Secretion

To determine the effect of BACE1 inhibition on IL-6 secretion, we subjected cells to the same treatments as shown in **Figure 5C** and measured IL-6 levels in culture medium by ELISA. IL-6 secretion from HBECs was increased following BACE inhibition, CSE administration, or both (**Figure 5D**). These findings suggest that blocking ST6GAL1 cleavage and secretion by BACE1 inhibition leads to an increase in IL-6 secretion from HBECs similar to *in vitro* cigarette smoke extract exposure (**Figure 4**).

Circulating ST6GAL1 Levels Are Lower in COPD Patients and Associate With Worse Clinical Outcomes

To determine a potential clinical impact for ST6GAL1 and IL-6, we analyzed plasma levels in 70 COPD subjects, with characteristics displayed in **Table 1**. Median [IQR] circulating ST6GAL1 levels were 1.93 [1.44-2.48] ng/ml, while the mean ± SEM was 2.26 ± 1.33 ng/ml for the cohort. Circulating ST6GAL1 levels positively and significantly correlated with post-BD FEV1 percent predicted (Pearson's correlation coefficient r=0.36,

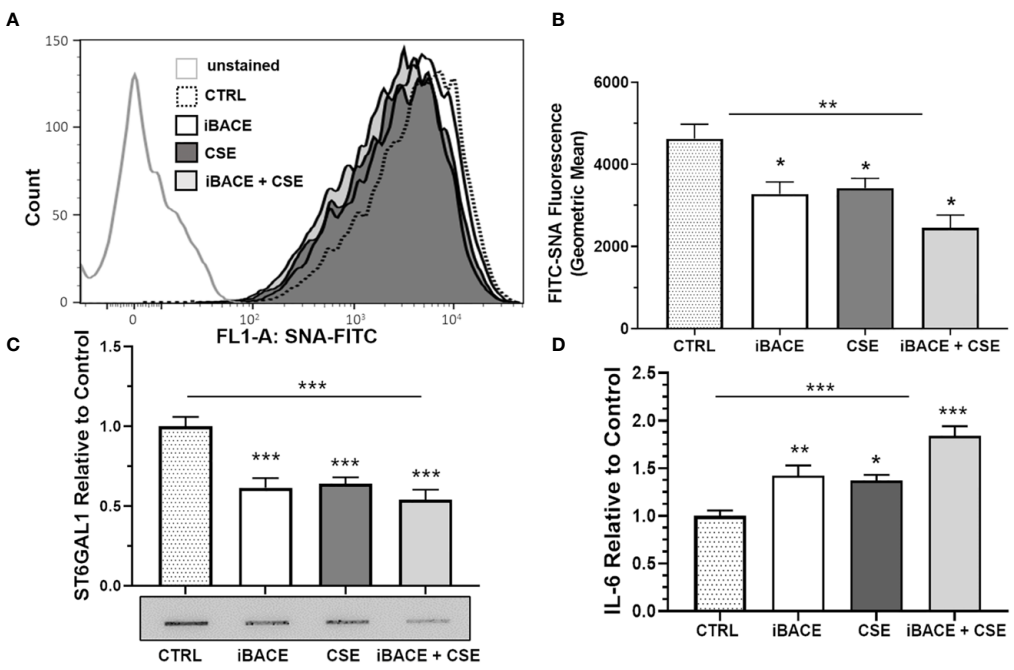

FIGURE 5 | Inhibition of ST6GAL1 secretion reduces α2-6 sialylation and increases IL-6 secretion similar to CSE. **(A)** Flow cytometric analysis of α2-6 sialylation using SNA-FITC labeling of HBECs (CTRL, ST6GAL1 KD, and ST6GAL1 OE) following treatment with and without iBACE and CSE for 72 hours. **(B)** 10,000 events were collected for each group, analyzed, and shown as the geometric mean. **(C)** Densitometry and a representative slot blot of secreted ST6GAL1 collected from conditioned medium following incubation with iBACE, CSE, or both. As a loading control, the supernatants were normalized to total cellular protein. **(D)** IL-6 secretion was determined from conditioned medium using an ELISA kit to the IL-6 ligand following same treatments as **(C)**. Experiments were performed in triplicate using three separate experiments. SNA, Sambucus Nigra Lectin; CTRL, control; iBACE, beta-site amyloid precursor protein cleaving enzyme 1 inhibitor; and CSE, cigarette smoke extract. All bar graphs are means ± SEM with *p < 0.05, **p < 0.01 and ***p < 0.001.

TABLE 1 | Baseline characteristics.

	COPD (n=70)
Age, years	59 ± 9
Male sex	36 (51%)
White race	34 (47%)
Smoking Status	34 (49%)
Current	36 (50%)
Former	2 (3%)
Never	
Pack-year history of smoking	36 ± 25
Post-BD FEV1, pct predicted	65 ± 22
Post-BD FVC, pct predicted	84 ± 16
FEV1/FVC	0.59 ± 0.16
MMRC score	1.8 ± 1.2
BCSS score	4.3 ± 2.7
Median [IQR] plasma ST6GAL1	1.93 [1.44-2.48] ng/ml

Data expressed as mean ± S.E.M. or n (%). BD, bronchodilator; FEV1, forced expiratory volume in 1-second; FVC, forced vital capacity; MMRC, modified medical research council; BCSS, breathlessness, cough, and sputum scale.

p=0.003), post-BD FVC percent predicted (r=0.30, p=0.011), and were inversely associated with GOLD stages (**Figure 6A**). However, ST6GAL1 was not correlated with dyspnea as measured by MMRC (r=0.19, p=0.10) or other respiratory symptoms measured by BCSS (r=0.15, p=0.22). Seven individuals (9.7%) experienced an AECOPD during the 1-year of follow-up. Circulating ST6GAL1 levels were lower among these individuals that experienced an AECOPD compared to the group that was AECOPD-free (**Figure 6B**; Median [IQR]: 1.44 [1.29-1.91] ng/ml *vs* 2.02 [1.48-2.58] ng/ml, p<0.001). In a logistic regression model adjusting for post-BD FEV1 percent predicted and smoking status, circulating ST6GAL1 levels were associated with decreased odds for AECOPD, though this failed to meet statistical significance (OR 0.14, 95%CI 0.02-1.19, p=0.072). Not unexpectedly, plasma IL-6 levels were higher among the AECOPD group compared to the non-AECOPD group (**Figure 6C**; Median [IQR]: 13.1 [8.30-15.1] ng/ml *vs* 7.44 [5.70-11.0] ng/ml, p=0.035). Circulating levels of ST6GAL1 and IL-6 were not associated (r=0.08, p=0.50). These translational findings support our *in vitro* data demonstrating loss of ST6GAL1 and increased secretion of IL-6, which may be linked to prognostic clinical outcomes in COPD patients.

DISCUSSION

Our study is the first to link reduced circulating ST6GAL1 levels and increased IL-6 levels with acute exacerbations in COPD patients. These findings are complemented by our *in vitro* findings indicating that loss of ST6GAL1 results in decreased α2-6 sialylation and increased secretion of IL-6 in HBEC cultures under basal conditions. Our results also showed that exposure to cigarette smoke or BACE1 inhibition resulted in decreased ST6GAL1 secretion and loss of α2-6 sialylation. Conversely, CSE or BACE1 inhibition increased IL-6 expression/secretion consistent with the loss of ST6GAL1. Altogether, these results suggest that loss of ST6GAL1 function, through knockdown or blocking its proteolysis, augments IL-6 secretion in HBECs and is associated with poor clinical outcomes in COPD.

Recently, ST6GAL1 was recognized for its role in asthma (20). In this report, Zhou and colleagues showed that ST6GAL1 regulated airway epithelial cell differentiation and type-2 inflammation in asthma through altered mucin glycosylation and cell proliferation. The role of ST6GAL1 has also been shown in other diseases and their associated complications. For example, increased cigarette smoke exposure was shown to alter sialylation of the fallopian tubes potentially through ST6GAL1 in ectopic pregnancy (40). In addition, changes in protein sialylation were shown to contribute to fatty liver deposition and various inflammatory responses (45). Our findings demonstrate that loss of ST6GAL1 or exposure to cigarette smoke leads to decreased sialylation (**Figures 1**, **2**) and increased IL-6 expression/secretion in bronchial epithelial cells (**Figures 3**, **4**). These findings together highlight the potential importance of ST6GAL1 and α2-6 sialylation in inflammation and other cellular processes in the lung as well as other tissues that may be impacted by inflammatory mediators such as cigarette smoke.

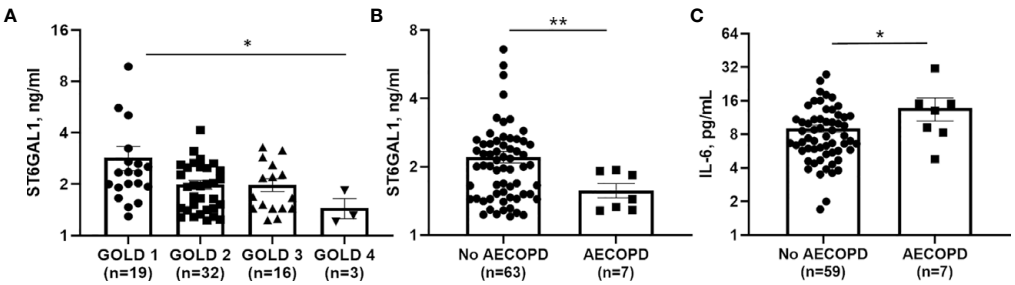

FIGURE 6 | Circulating ST6GAL1 levels are lower in COPD patients and correlate with poor outcomes. **(A)** GOLD group status 1-4 and associated levels of ST6GAL1 in a COPD patient cohort. Circulating levels of ST6GAL1 **(B)** and IL-6 **(C)** and their respective association with acute exacerbations in COPD. Patients were classified based on severity of the COPD using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (30846476), which are as follows: GOLD 1: Mild (FEV1 >= 80% predicted); GOLD 2: Mod (FEV1 between 50 and 79% predicted); GOLD 3: Severe (FEV1 between 30 and 49% predicted); and GOLD 4: Very severe (FEV1 <30% predicted). Shown are the mean ± SEM, where n = subjects per group. *p < 0.05 and **p < 0.01.

Several reports have shown that ST6GAL1, a normally trans-Golgi network resident protein, can be cleaved from its membrane bound form to a soluble/secreted protein by BACE1 (41, 42, 46). BACE1 is the same enzyme identified for cleavage of the amyloid precursor protein involved in the pathogenesis of Alzheimer's disease (47–50). Interestingly, our knowledge of the BACE1/ST6GAL1 interaction is still limited; however, BACE1 has been shown to be responsible for release of ST6GAL1 into the blood (51). BACE1 expression has been shown to affect the sialylation of soluble/cell surface glycoproteins through cleavage of ST6GAL1 (52) suggesting that the soluble form of the ST6GAL1 still has activity when released [others have shown that ST6GAL1 has activity in a secreted/soluble form (53)]. Additionally, secreted/circulating ST6GAL1 has been associated with inflammation (45, 54, 55). Here, we show that inhibition of BACE1 reduced ST6GAL1 secretion and α2-6 sialylation and resulted in augmented IL-6 secretion in HBECs similar to that found with CSE treatment (**Figure 5**).

In a previous report, Nasirikenari and colleagues demonstrated that administration of ST6GAL1 reduced infection in a mouse model of acute lung inflammation, while transient depression of circulating ST6GAL1 accompanied acute airway inflammation (56). In parallel experiments, they showed that inflammatory cytokine release was suppressed by recombinant ST6GAL1 infusion in these mice and suggested a potential role for ST6GAL1 in diseases like COPD. In a small COPD patient cohort, we observed an inverse association between circulating ST6GAL1 levels and lung function and GOLD stages (**Figure 6A**), suggesting that ST6GAL1 may have a protective role in maintaining lung function, potentially through anti-inflammatory mechanisms. We also found that ST6GAL1 levels were lower among the group that experienced an acute exacerbation compared to those that did not (**Figure 6B**) further supporting the hypothesis that ST6GAL1 may have a protective effect in COPD. Since our cohort was small, more studies are needed in a larger patient population to validate of these findings and to determine the use of ST6GAL1 as a potential prognostic marker of disease symptom severity.

In this same patient cohort, increased plasma IL-6 levels were shown to positively associate with acute COPD exacerbations (**Figure 6C**). IL-6 has been shown to be upregulated in COPD exacerbations (24) and was also suppressed/enhanced by the increase/reduction of ST6GAL1 in acute lung injury mouse models studied by Nasirikenari et al. (56). These findings are consistent with our *in vitro* data showing loss of ST6GAL1 led to increased IL-6 expression/secretion (**Figure 3**). Moreover, BACE1 inhibition (reduction in ST6GAL1 cleavage/secretion and α2-6 sialylation) or cigarette smoke exposure resulted in similar increases in IL-6 (**Figure 5**).

Our study is not without limitations. Our translational results were generated from a single center study and contained a relatively small sample size. However, this small sample population was offset by a well-characterized dataset and complete follow-up. In addition, ST6GAL1 was measured from plasma of these patients and not from lung tissue or sputum, which was not practical for this study. Analysis in these samples would have been a more ideal and direct measure. Still, these findings in plasma were robust and improved the overall generalizability to other human cohorts. Finally, studies here were performed with HBECs and we cannot rule out the fact that circulating ST6GAL1 may be coming from other tissues (e.g., liver). In future studies, we plan to utilize primary cells and tissue obtained from COPD subject studies to obtain a more direct measure.

Altogether, our results along with the previous literature suggest that ST6GAL1 potentially has an important role regulating the inflammatory cytokine response in patients diagnosed with COPD. Further, although future studies are needed, our work suggests that circulating ST6GAL1 levels might serve as a potential therapeutic marker of acute COPD exacerbation and of inflammatory lung disease progression.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

All protocols were approved by the Institutional Review Board of the University of Alabama at Birmingham. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK, JB, and JW contributed to the concept and/or design of the study. SH, ME, RD, RZ, SB, PC, SV, EH, JW, JB, and SK contributed to the acquisition of the data and SH, JW, SK, and JB contributed to the analysis and interpretation. SK, SH, JW, and JB drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Survival Advantage Following TAG-72 Antigen-Directed Cancer Surgery in Patients With Colorectal Carcinoma: Proposed Mechanisms of Action

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Patients with colorectal carcinoma (CRC) continue to have variable clinical outcomes despite undergoing the same surgical procedure with curative intent and having the same pathologic and clinical stage. This problem suggests the need for better techniques to assess the extent of disease during surgery. We began to address this problem 35 years ago by injecting patients with either primary or recurrent CRC with ^{125}I-labeled murine monoclonal antibodies against the tumor-associated glycoprotein-72 (TAG-72) and using a handheld gamma-detecting probe (HGDP) for intraoperative detection and removal of radioactive, i.e., TAG-72-positive, tissue. Data from these studies demonstrated a significant difference in overall survival data ($p < 0.005$ or better) when no TAG-72-positive tissue remained compared to when TAG-72-positive tissue remained at the completion of surgery. Recent publications indicate that aberrant glycosylation of mucins and their critical role in suppressing tumor-associated immune response help to explain the cellular mechanisms underlying our results. We propose that monoclonal antibodies to TAG-72 recognize and bind to antigenic epitopes on mucins that suppress the tumor-associated immune response in both the tumor and tumor-draining lymph nodes. Complete surgical removal of all TAG-72-positive tissue serves to reverse the escape phase of immunoediting, allowing a resetting of this response that leads to improved overall survival of the patients with either primary or recurrent CRC. Thus, the status of TAG-72 positivity after resection has a significant impact on patient survival.

Keywords: colorectal carcinoma, TAG-72, surgery, survival, glycosylation, immunosuppression

INTRODUCTION

On a global basis, colorectal carcinoma (CRC) is ranked third in incidence and second in cancer-related mortality (1). Of the non-keratinocytic tumors, in the United States, CRC ranks fourth in overall incidence and second in mortality (2). Approximately 80% of newly diagnosed patients undergo surgical resection with "curative" intent. However, the patient outcome varies despite

having the same surgical procedure performed by the same surgeon and having a tumor with the same morphologic features and pathologic stage (3). The incidence of recurrent CRC varies from 20% to 50% of cases following surgery with curative intent, the majority of which occur within the first 3 years following surgery (4–6). Curative resection of cancer requires the removal of "all" tumor-involved tissues. Differences in patient outcomes can arise from a lack of accurate assessment of the extent of disease by preoperative imaging (7), surgical exploration (8), pathologic staging (9), and tumor biology (10). Together, these variabilities result in an inaccurate evaluation of the individual patient's prognosis. This inaccuracy is not a new problem, as evidenced by over 60 years of findings from second-look surgeries for recurrent disease in otherwise asymptomatic patients (11, 12).

ANTIGEN-DIRECTED CANCER SURGERY

In the late 1980s and 1990s, a series of clinical trials was undertaken with the goal to improve the surgeon's ability to intraoperatively detect and remove tumor-involved tissues from patients with either recurrent or primary CRC (13, 14). The studies combined the use of ^{125}I-labeled murine monoclonal antibodies (mMoAbs) against the tumor-associated glycoprotein-72 (TAG-72) antigen and a handheld gamma-detecting probe (HGDP) for intraoperative detection of TAG-72-positive tissue. We refer to these studies as TAG-72 Antigen-Directed Cancer Surgery (ADCS) rather than the previously described Radioimmunoguided Surgery (RIGS) (15, 16).

In brief, the protocols included blocking the thyroid gland uptake of ^{125}I preoperatively. Early studies called for patients to be injected intravenously with ^{125}I-anti-TAG-72 mMoAb B72.3, whereas patients in later studies received ^{125}I-anti-TAG-72 mMoAbs CC49 or CC83 (15, 16). Serial precordial counts using the HGDP ensured that an optimal tumor-to-background ratio occurred before taking the patient to surgery within 28 days after injection. The surgeon first used traditional exploration techniques (i.e., inspection and palpation) to explore the abdomen and pelvis and then declare the findings and surgical plan. The surgeon then used the HGDP to resurvey the pelvis and abdomen, which often led to the detection of occult (residual) tumors and a change in the surgical plan.

TAG-72 PROTEIN

TAG-72 was originally isolated from xenografts of the human colon carcinoma cell line LS-174T through binding of a mMoAb called B72.3 (17–19). The B72.3-binding isolate was characterized as a high-molecular weight (>1,000,000 kD) protein with extensive glycosylation consistent with a mucin. Further work identified the TAG-72 epitopes recognized by several of these mMoAbs as different O-linked glycan antigenic structures (17, 18). B72.3 binds ovine submaxillary mucin, a glycoprotein rich in the sialyl-Tn (NeuAcα2-6GalNAcα1-Ser/Thr, STn) tumor-associated carbohydrate antigen (TACA). This binding was eliminated by sialidase treatment and inhibited by both STn and the GalNAcα1-Ser/Thr (Tn) precursor structure. In contrast, mMoAbs to the Thomsen-Friedenreich (TF) antigen (Galβ1-3GalNAcα-O-Serine/Threonine) did not inhibit B72.3 binding (20). Additional work by Reddish et al. (21) showed that STn-O-serine dimeric and trimeric clusters were recognized by mMoAb B72.3 better than the monomeric structure. The finding of cross reactivity with dimeric Tn-O-serine is consistent with earlier findings that B72.3 agglutinated red blood cells (22).

The discovery of second-generation mMoAbs to B72.3-purified TAG-72 demonstrated a series of overlapping but unique glycan antigenic epitopes (23–25). We subsequently used this second-generation mMoAb, called CC49, in our ADCS studies. The glycan antigenic epitope recognized by CC49 overlaps with that recognized by the B72.3, as it had a strong reactivity with dimeric STn-O-serine/threonine and less reactivity with Tn-O-serine dimers; it also lacked reactivity to monomeric forms of STn-O-serine or Tn-O-serine (21). In addition, several studies demonstrated that CC49 has a higher binding affinity for the core-1 sialylated glycan NeuAcα2-3Galb1-3GalNAc (sTF) than STn (26–29). The fact that both mMoAbs bind equally to periodate-treated mucins indicates that the overlapping epitopes do not include the sialic acid glycerol side chain in its free or O8′ or 09′ acetylated forms (29).

Immunohistochemical (IHC) staining with either mMoAb B72.3 or CC49 demonstrated TAG-72 expression in various carcinomas and only in normal secretory endometrium (30–32). IHC staining and autoradiography (**Figure 1**) show TAG-72 in the cytoplasm of CRC tumor cells, in their luminal secretions, and in the tumor microenvironment (TME). IHC staining demonstrates TAG-72 dispersed in the plasma membranes of apical, lateral, and basal surfaces of tumor cell luminal debris (**Figures 1A, B**). In addition, TAG-72 occurs in the luminal debris of malignant glands (**Figure 1A**). TAG-72-positive secretions are evidenced by positive IHC staining TAG-72 of mucin lakes in the TME of non-mucinous and mucinous carcinomas, as well as in cytoplasmic vacuoles, and luminal CC49 confirms the IHC staining results with the respective mMoAb (**Figure 1C**) (33). **Figure 1C** depicts the black silver autoradiography grains (ARGs) representing the

Abbreviations: ADCS, antigen-directed cancer surgery; ARGs, autoradiography grains; CRC, colorectal carcinoma; DAMPs, damage-associated molecular patterns; DC, dendritic cell; cDC, conventional dendritic cell; iDC, immature dendritic cell; mDC, mature dendritic cell; pDC, plasmacytoid dendritic cell; DC-SIGN, dendritic cell-specific ICAM-3 grabbing non-integrin; H&E, hematoxylin and eosin; HGDP, handheld gamma detecting probe; IHC, immunohistochemical; IL, interleukin; MGL, macrophage galactose-type lectin; mMoAb, murine monoclonal antibody; MHC, major histocompatibility complex; MUC, mucin; NK, natural killer; OS, overall survival; PAMPs, pathogen-associated molecular patterns; pStage, pathologic stage; RIGS, radioimmunoguided surgery; SAMPs, self-associated molecular patterns; TACA, tumor-associated carbohydrate antigen; TAG-72, tumor-associated glycoprotein-72; TAIR, tumor-associated immune response; Th, T-helper cell; TME, tumor microenvironment; Tn, GalNAcα1-O-Ser/Thr or Thomsen-nouveau; STn, Sialylated Tn; Treg, regulatory T cell; Siglecs, sialic acid recognizing Ig-like lectins.

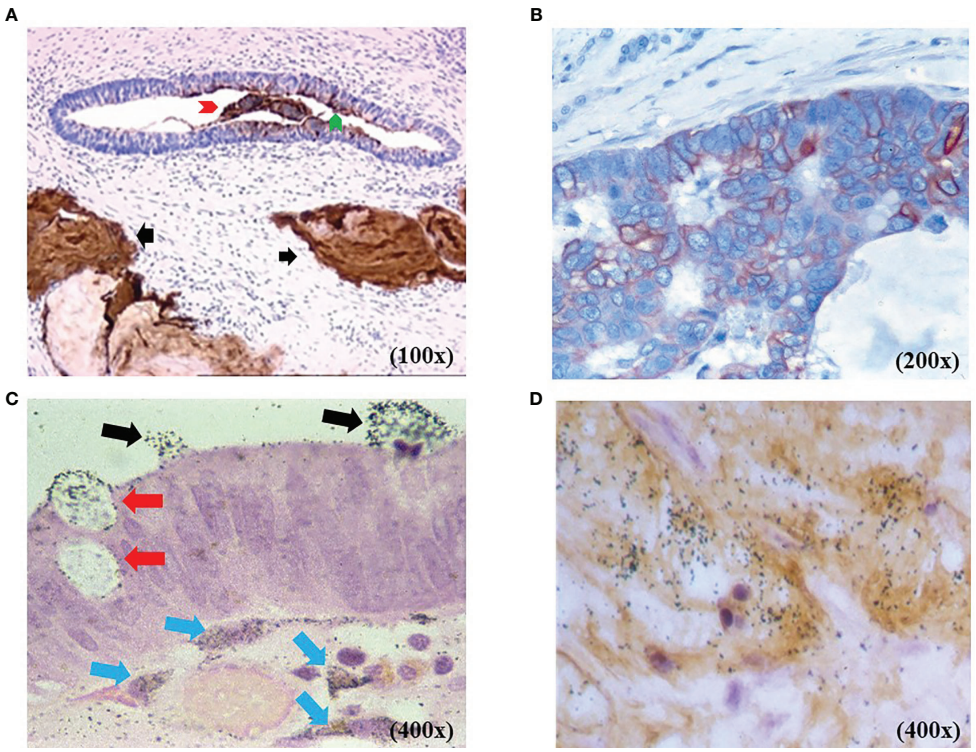


FIGURE 1 | TAG-72 expression in colorectal adenocarcinoma [colorectal carcinoma (CRC)]. **(A)** Non-mucinous CRC with murine monoclonal antibody (mMoAb) CC49 immunohistochemical (IHC) staining (brown) demonstrates TAG-72 in malignant glands and in mucin lakes in the tumor microenvironment (TME) (black arrows). The malignant gland exhibits TAG-72 expression in the luminal contents (red arrow head), cytoplasmic vacuoles, and the tumor cells' luminal surface (green arrow head). **(B)** Non-mucinous CRC with mMoAb CC49 IHC staining (brown) demonstrates TAG-72 outlining the plasma membrane of the malignant cells. **(C)** Black autoradiography silver grains (ARGs) of the injected mMoAb ^{125}I-CC49 demonstrates ^{125}I-CC49-bound TAG-72 in cytoplasmic secretory vesicles (red arrows) in CRC cells as they move from the cytoplasm to the lumen of a malignant gland (black arrows). Below the malignant cells are ARGs in the cytoplasm of dendritic-shaped cells (blue arrows) in TME. **(D)** Co-localization of the injected ^{125}I-mMoAb CC49 (black ARGs) overlapping with the IHC staining (brown) for TAG-72 demonstrates the TAG-72 antigenic epitope in the TME of a CRC.

injected ^{125}I-labeled mMoAb CC49 binding to TAG-72 in secretory vesicles that are migrating to the apical surface and released into the lumen of a malignant gland. In addition, ARGs occur in cells and the extracellular matrix of the TME along the basal surface of the tumor cells. **Figure 1D** demonstrates the co-localization of ARGs and IHC staining of TAG-72 in the TME and cells.

TAG-72 expression in tumor-draining lymph nodes (TDLNs) is similar to that of the tumor itself (**Figure 2**). Reactive germinal centers (**Figures 2A–C**), sinus histiocytosis, and paracortical hyperplasia (not shown) are prominent histologic features in regional and extraregional TDLNs in CRC. In the case of CRC, TAG-72 localizes to the germinal centers of the TDLNs. IHC staining and autoradiography commonly demonstrate TAG-72 in a dendritic or eccentric distribution within the germinal centers (**Figures 2A–C**). IHC staining with mMoAbs B72.3 or CC49 demonstrates that TAG-72 expression is similar to that of other pan-carcinoma antigens in adenocarcinomas (34). In addition, it is important to note that not all CRC tumors take up anti-TAG-72 mMoAbs. The mMoAb B72.3 and mMoAb CC49 localized in 75% and 86% of patients with primary CRC, respectively. In contrast, mMoAb B72.3 and MoAb CC49 localized in 63% and 97% of patients with recurrent CRC (14).

PATIENT SURVIVAL

The overall survival (OS) data provide direct evidence that using radiolabeled anti-TAG-72 mMoAbs to intraoperatively determine the extent of disease had a significant clinical impact with the removal of all the TAG-72-positive tissue (35–40). **Figure 3** depicts the 5-year OS of 212 patients with primary or recurrent CRC intravenously injected with mMoAbs ^{125}I-B72.3 or ^{125}I-CC49 for TAG-72 ADCS (37). The patients were divided into three groups based on the HGDP evidence of tumor at the end of surgery. The largest group of 95 (44.8%) patients had grossly evident tumor and TAG-72-positive tissue remaining upon completing the surgical procedure (black line). Carcinomatosis and unresectable liver or lung metastases were common among the patients with primary CRC in this group. Patients with recurrent CRC in this group who had unresectable pelvic, abdominal, and thoracic metastases did not tend to survive beyond 3 years. In addition, this study demonstrated that mMoAb ^{125}I-B72.3 detected the unresectable disease in patients with recurrent CRC that would have undergone unwarranted surgery based solely on traditional detection techniques (36).

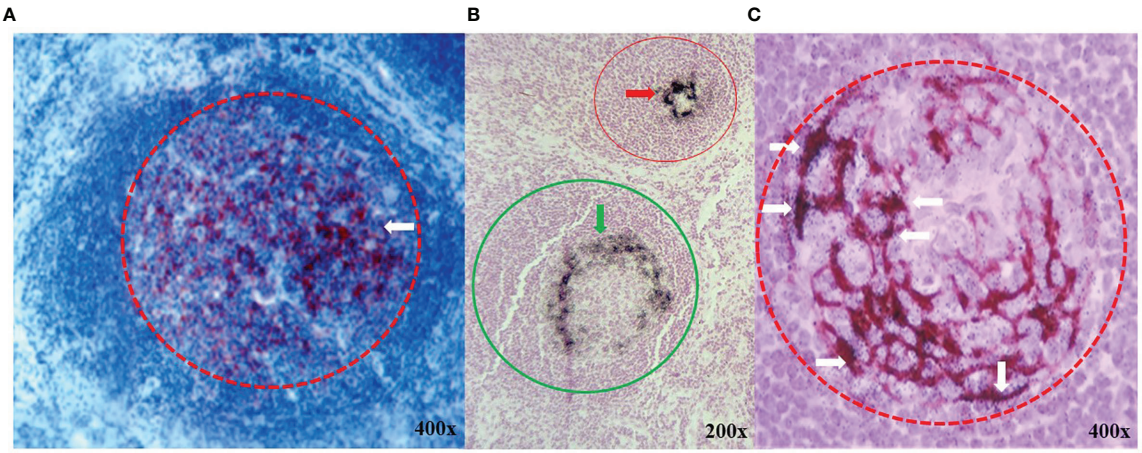


FIGURE 2 | TAG-72 expression in germinal centers of lymphoid follicles in extraregional tumor draining lymph nodes. **(A)** Immunohistochemical (IHC) staining with murine monoclonal antibody (mMoAb) CC49 (dark red) demonstrates early polarization (white arrow) of TAG-72 toward the periphery of a germinal center (red circle) in a reactive lymphoid follicle. **(B)** Black autoradiography silver grains (ARGs) located in demonstrate the injected ^{125}I- mMoAb CC49 in two germinal centers (red and green circles). TAG-72 distribution varies from an acentric pattern (green arrow) in the germinal center of a reactive lymphoid follicle (green circle) as compared to a dense circular peripheral pattern in the germinal center (red arrow) of a smaller lymphoid follicle (red circle). **(C)** mMoAb CC49 IHC staining (brown) and black ARGs demonstrate co-localization of TAG-72 (white arrows) in an acentric dendritic pattern in a reactive germinal center (red dashed circle).

In stark contrast are the 74 (34.9%) patients with primary or recurrent CRC who lacked evidence of gross tumor and TAG-72-positive tissue at the end of surgery (red dotted line). For those patients with primary CRC, the 5-year OS was independent of the pathologic stage (38). For patients with recurrent disease, the 5-year survival identified a population of patients who benefitted from TAG-72 ADCS (13, 14).

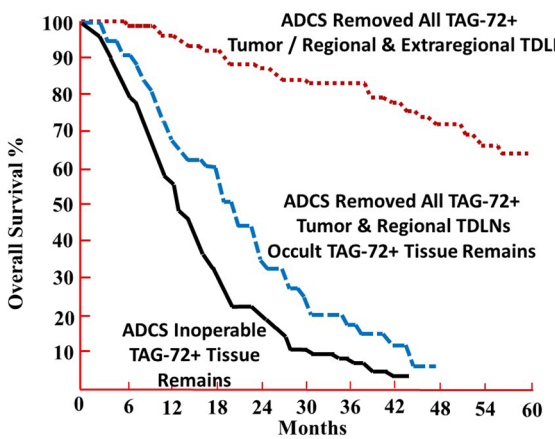


FIGURE 3 | The 5-year overall survival (OS) percentage of patients undergoing TAG-72 antigen-directed cancer surgery. The 5-year OS of 212 patients with recurrent or primary colorectal carcinoma was injected with murine monoclonal antibody (mMoAb) ^{125}I-B72.3 or ^{125}I-CC49 (37). Complete removal of all TAG-72-positive tissue provides a highly significant 5-year OS advantage when compared to those patients with retention of grossly evident tumor and TAG-72-positive tissue ($p < 0.0001$) (black line) or occult TAG-72-positive tissue (no residual grossly evident tumor) ($p < 0.0025$) (blue dashed line) upon completion of surgery.

Figure 3 also identifies a group of 43 (20.3%) patients lacking grossly evident tumor while retaining TAG-72-positive tissue, consistent with unresected lymph nodes, at the end of surgery (blue dashed line). A follow-up study identified the recurrent disease in previously unresected, TAG-72-positive, extraregional TDLNs (41). These results demonstrate the clinical implications of the relationship between TAG-72-positive extraregional TDLNs and the extent of disease in CRC patients. Overall, these results indicate that TAG-72 ADCS detects and localizes occult diseased tissue left behind when only using more traditional surgical techniques (e.g., inspection and palpation).

Analysis of 92 patients with primary CRC injected with mMoAb ^{125}I-CC49 demonstrated a significant difference in the % OS relative to the presence or absence of residual TAG-72-positive tissue beyond 5 years, at 10 years ($p = 0.002$) and 15 years ($p = 0.003$) (**Figure 4**) (16). Analysis of OS *vs.* pathologic stage (pStage) required grouping patients relative to the absence of metastatic disease (pStages 0, I, and II) or presence of metastatic disease (pStages III and IV). As expected, patients with visceral metastases (pStage IV) were significantly ($p = 0.03$) more prevalent among those patients with residual TAG-72-positive tissue at the end of surgery (16).

ANTIGEN-DIRECTED CANCER SURGERY AND EXTENT OF DISEASE

Does TAG-72-positive tissue equate with the presence of shed antigen? The answer to this is yes, and we consider it a significant component of the extent of disease. TAG-72 circulating in patients with adenocarcinomas, including CRC, is demonstrated by the elevated levels of TAG-72 glycoprotein in the serum and effusions

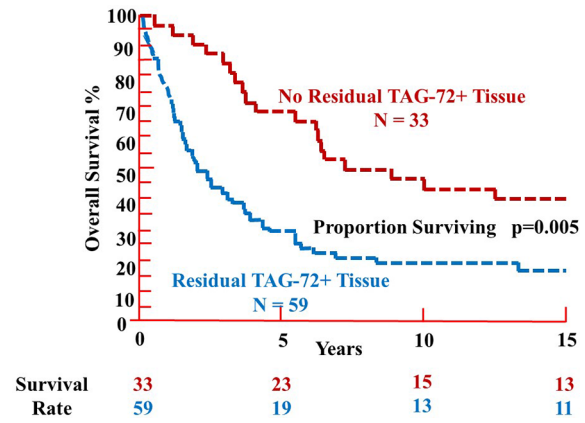


FIGURE 4 | Overall survival of patients with colorectal carcinoma following TAG-72 antigen-directed cancer surgery. Ninety-two primary colorectal carcinoma patients were injected with either murine monoclonal antibody (mMoAb) ^{125}I-CC49 or mMoAb ^{125}I-CC83 (16). There was a significant difference ($p = 0.005$) in the proportion surviving between those patients with no residual TAG-72-positive tissue at the end of surgery (red dashed line) as compared to those patients where residual TAG-72-positive tissue (blue dashed line) remained at the end of surgery. The survival rate at 5 years was 70% for those patients without residual tumor (upper red values) as compared to 32% for patients with residual TAG-positive tissue (lower blue values). However, there is no significant difference in the survival rate after 10 and 15 years of follow-up.

(42, 43) and by IHC staining demonstrating TAG-72 in tumor lymphatic vessels (44). TAG-72 in TDLNs co-localizes mMoAb ^{125}I-CC49 in germinal centers with a crescentic dendritic pattern, suggestive of a T-helper 2 (Th2) immune response or a central distribution (**Figure 2**) rather than the Th1 tumor-associated immune response (TAIR) (11). Together, the TAG-72 ADCS results (reviewed in 13, 14) and the morphologic data support our hypothesis that TAG-72 positivity in the tissue equates with the extent of disease of CRC.

Is TAG-72 ADCS a better predictor of extent of disease than routine pathologic staging based on conventional surgery? Data from our ADCS cases point out that the extent of disease extends beyond what surgeons consider as the normal area of resection. Tissue involved with tumor often goes undetected by preoperative imaging, by the surgeon's visual inspection or manual palpation, and/or by a lack of exploration. In 41 primary CRC cases, the surgeon alone identified 45 sites as compared to 153 sites detected using the HGDP to identify mMoAb ^{125}I-CC49 in the tissue (45). In a similar comparison in 45 cases of recurrent CRC, conventional methods identified 116 sites as compared to 184 sites using the HGDP to identify mMoAb ^{125}I-CC49 in the tissue. The percentage of TAG-72-positive lymph nodes from the area of the gastrohepatic ligament (66%) and celiac axis (~50%) was similar for both primary and recurrent cases. The ratio of tumor-involved liver to these three lymph node-bearing locations was 1:3 and 1:1 in primary and recurrent cases, respectively. Several non-ADCS studies (reviewed by 46) identified metastatic CRC in similar lymph node groups, as noted above, and that removal of these nodes improved patient outcomes. However, recurrent disease occurred in these areas when TAG-72-positive tissue was purported to be negative on frozen sections that remained behind at the end of surgery (41). Similarly, non-ADCS data demonstrated that lymph node recurrences outnumber those in the liver and lungs and occur alone or in combination with distant metastases in over 90% of the 835 cases that developed recurrent CRC out of the 4,023 patients who underwent curative surgery (5).

The ADCS results beg the question: Does TAG-72-positive tissue equate with the presence of metastatic tumor cells? The answer is controversial. Many TAG-72-positive TDLNs lacked tumor cells based on routine pathologic studies, indicating that the answer to this question is no (46, 47). Our results from an unpublished survey of 599 consecutive TAG-72-positive specimens from 92 patients with either primary or recurrent CRC suggest that the answer is no. In non-lymphoid tumor deposits, tumor cells were present in 92.5% (136/147) of hematoxylin and eosin (H&E)-stained sections submitted for routine pathologic examination. In contrast, only 15.7% (71/452) of TAG-72-positive lymph nodes contained tumor cells. This difference was highly significant ($p < 0.00001$). These results appear to support the impression that the lack of tumor cells in TAG-72-positive lymph nodes submitted for routine histopathology was indicative of false positivity (44, 46–48). However, numerous variables account for "false-negative" results, including lymph node size, size and location of the metastasis in the node, and the number of sections taken (9, 49–52).

Secondly, routine tissue processing, sectioning, and H&E staining induce errors in sampling and sensitivity. Thirdly, H&E-negative TAG-72-positive lymph nodes may contain unrecognized tumor cells because a routine single 4–5-mm section evaluated by the pathologist represents less than 0.1% of a 1-cm-diameter node (49). Cytokeratin IHC staining studies, with or without step sectioning, detected tumor cells in otherwise H&E-negative lymph nodes (49, 51, 52). However, even these studies suffer from errors in sampling and sensitivity (49). More sensitive flow cytometry, cell sorting, and RT-PCR studies have identified tumor cells' presence in otherwise "H&E-negative" TAG-72-positive lymph nodes (37, 53–55).

MECHANISMS UNDERLYING SURVIVAL RESULTS FROM TAG-72 ANTIGEN-DIRECTED CANCER SURGERY FOR COLORECTAL CARCINOMA

Aberrant O-Linked Glycosylation of Mucins in Colorectal Carcinoma

Glycosylation is the predominant posttranslation modification of cellular proteins and lipids. The complex process of O-linked-glycosylation of mucins begins with N-acetylgalactosamine (GalNAc) that is O-linked to either serine (S) or threonine (T) amino acids on the peptide that serves as the starting point for addition of sugars to form the precursors of the first four of the eight core structures (**Figure 5**) out of the possible. Aberrant O-glycosylation is a hallmark of carcinomas (56). This process gives

rise to immunogenic glycans that are collectively refereed as tumor-associated carbohydrate antigens (TACAs) (57, 58). Mucins and other glycoproteins concurrently express TACAs, such as the truncated core Tn, STn, T, and ST, as well as Lewis (Le) blood group antigens $Le^{a/x}$ and $Le^{b/y}$ and their sialylated glycoforms (59). A given glycoprotein can express multiple different TACAs (60, 61). The accumulation of Tn, STn, T, and ST (**Figure 5**) is a characteristic feature of CRC and other carcinomas (62). The Tn, ST, and STn containing antigenic epitopes recognized by mMoAbs to TAG-72 occur early in the adenoma–carcinoma sequence and tumor progression (63–66). The accumulation of Tn and STn is associated with a mutation and with hypermethlyation of the *Cosmc* gene whose product is the chaperone protein Core 1 β3GalT-specific molecular chaperone (Cosmc). Cosmc is required for the proper function of the Core 1Gal-Transferase enzyme (C1GalT) to synthesize the T glycan (**Figure 5**) (67). In addition, Tn and STn accumulate with the mutation of the gene for N-acetylglucosamine transferase (C3GnT), which is the only enzyme generating the core 3 precursor glycan (**Figure 5**), and its loss is common in CRC (68). Radhakrishnan et al. (69) observed that hypermethlyation of the promoter of *Cosmc* is the "most prevalent cause" of Tn and STn formation in pancreatic cancer samples. They used an immortalized keratinocytic cell model to examine the phenotype roles of these truncated glycans. Their results demonstrated that truncated O-linked glycans alter cell adhesion and the RAS signaling pathways leading to cell proliferation, tissue invasiveness, and decreased apoptosis. Differences in the expression pattern may well be due to the presence or absence of such mutations.

IHC staining demonstrates that Tn and STn accumulate in over 85% of colorectal, pancreatic, and ovarian carcinomas and over 50% of the carcinomas of the lungs, cervix, esophagus, stomach, and breasts (23, 57, 70–72). Although aberrant glycosylation varies with the tumor type, the expression of the truncated glycans, Tn and T and their sialylated counterparts, is associated with a poor prognosis for patients with a carcinoma including CRC (57, 64–66, 72). This correlates with the observation that aberrant glycosylation, including Tn and STn, suppresses the TAIR (63, 64).

Tumor-Associated Immune Response

The microenvironments of both the tumor and the TDLNs are a complex and intricate network of neoplastic, stromal, and infiltrating immune cells and their products. Through the interaction of these components, tumor cells progress to

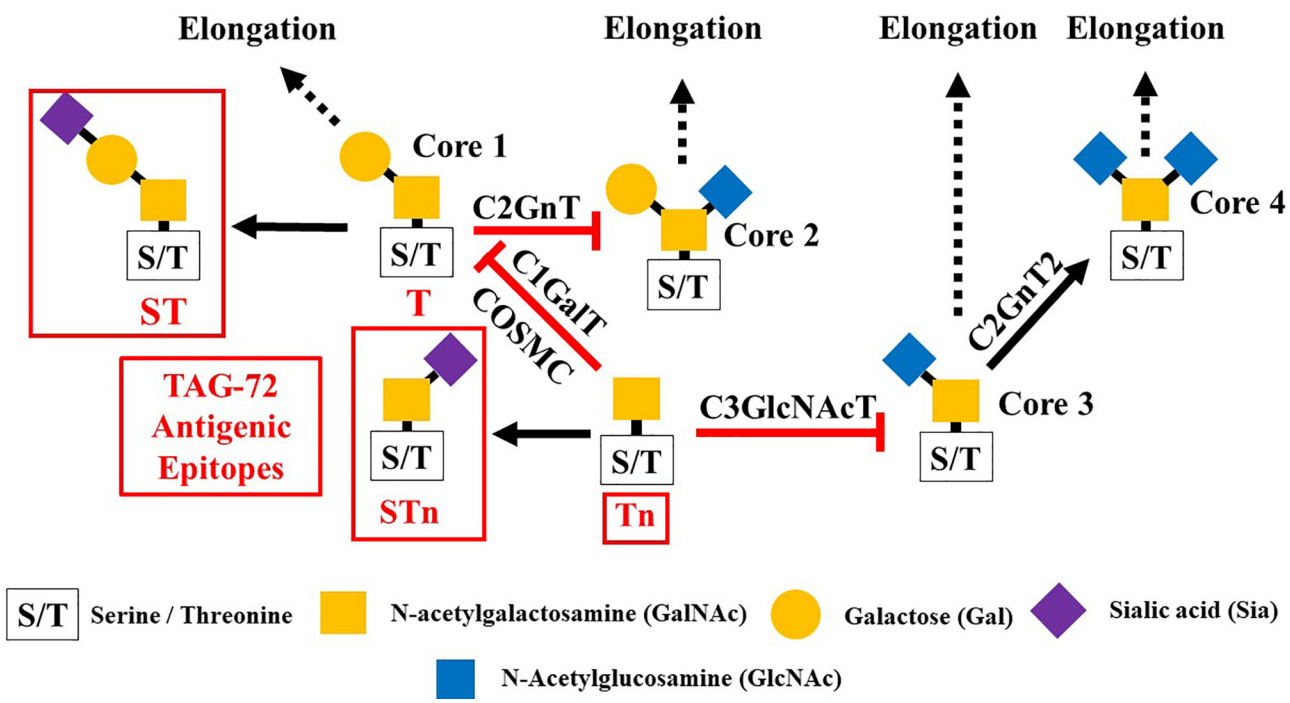


FIGURE 5 | Formation of Core structures in O-linked glycosylation of mucins. Normal O-linked glycosylation begins with the addition N-acetylgalactosamine (GalNAc) to a serine or threonine (S/T) amino acid on core protein. Core 1 Gal-transferase (C1GalT), with the help of the COSMC chaperone protein, adds Gal to the GalNAc-S/T to form the Core 1, which in turn gives rise to Core 2 with the addition of GlcNAc to GalNAc that is catalyzed by Core 2 N-acetylglucosamine transferase (C2GnT). Core 3 N-acetylglucosamine transferase (C3GnT) is the only enzyme that catalyzes the addition of GlcNAc to GalNAc to form Core 3. Core 4 is formed by the addition of another GlcNAc to the GalNAc of Core 3. The subsequent addition of sugars to these Cores [i.e., elongation (dash black arrows)] gives rise to the variable O-glycosylation pattern of mucins. The expression of Tn, STn, T, and ST glycans (red boxes) and loss of Core 3 are often the result of mutations of the transferase genes (⊣) associated with the formation of Cores 1, 2, and 3, and the loss of C2GnT activity is also associated with hypermethylation of the gene for the chaperone protein SMC. The loss of function of one or more of these enzymes accounts for the presence of TAG-72 antigenic epitopes (red boxes) present in various carcinomas.

acquire the potency to evade ongoing immune responses by reducing immune recognition, increasing their resistance against immune attack, and creating an immunosuppressive tumor microenvironment.

The concept of tumor immunoediting helps explain the role of immune cells, especially dendritic cells (DCs), in tumor progression (73, 74). Elimination, or immunosurveillance, the first of the three "E" processes, equates with an effective TAIR that eliminates neoplastic cells. Here, the mechanism of the TAIR "outweighs" those of immunosuppression. The Equilibrium phase represents a balance between the mechanisms of elimination and immunosuppression that can last your years. Escape is the third phase of the immunoediting process and equates with clinically evident disease and suppression of the TAIR.

The TAIR is dependent on the ability of immature dendritic cells (iDCs) to become mature dendritic cells (mDCs) that migrate to TDLNs (**Figure 6**). In the TDLN, DC maturation gives rise to the three signals needed to activate naive CD4+ and CD8+ T cells to become effector T cells needed to combat tumorigenesis. We propose that binding of TACA to iDCs in the TME and TDLN leads to CRC progression and that the ability of TAG-72 ADCS to reverse this suppression of the TAIR accounts for the mechanisms that underlie our results.

Dendritic Cells and the Tumor-Associated Immune Response

DCs aided by natural killer (NK) cells and macrophages (75) play a central role in bridging the innate and adaptive immune responses. Bone marrow precursors give rise to a heterogeneous group of DC subsets that vary in their developmental pathway, location, and phenotype (76). The various subpopulations of DCs are grouped as conventional (cDC1 and cDC2) and plasmacytoid (pDC). These three

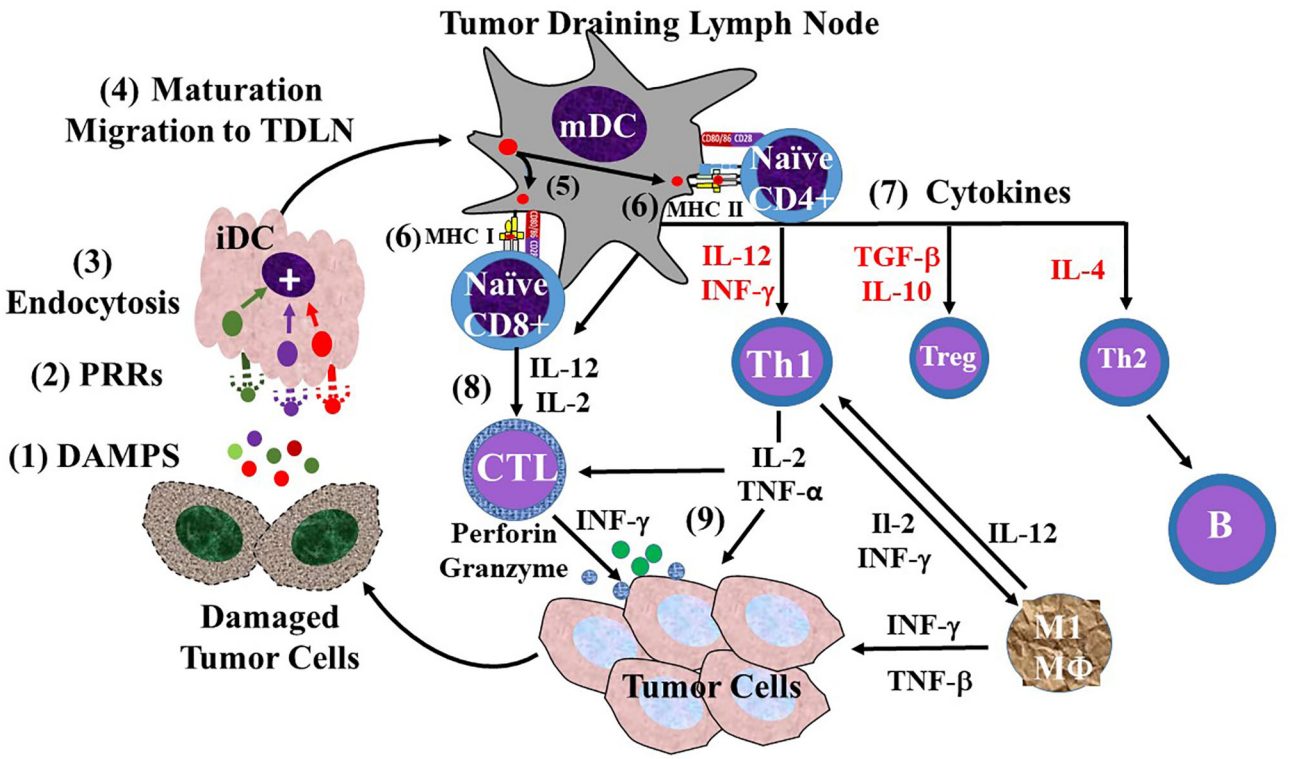


FIGURE 6 | Overview of the sequence of events involving dendritic cells in the tumor-associated immune response (TAIR). The TAIR begins with natural killer (NK) cell and macrophage (not shown) inducing damage of tumor cells that (1) releases damage-associated molecular patterns (DAMPs) into the tumor microenvironment (2). The DAMPs bind to various types of pattern recognition receptors (PRRs) on immature dendritic cells (iDCs) (3), where their endocytosis leads to activation of iDC maturation (4). iDC maturation leads to morphologic changes, decreased endocytic activity, migration lymphatics to a tumor draining lymph node (TDLN). Once in the TDLN, the now mature dendritic cell (mDC) provides the three signals needed for activation of naive T cells. mDCs are capable of cross-presenting antigens (5) to both major histocompatibility complex (MHC I and MHC II) molecules of the surface. Signal 1 (6) is generated by the MHC molecule presentation of the antigen to the T-cell receptor (TCR) on naive CD 4 or CD8 T cells. Signal 2 is generated by the binding of co-stimulatory molecules CD80/86 on the mDC to CD28 on the naive T cells (7). Signal 3 is provided o the release of pro-inflammatory cytokines (e.g., IL-2, IL-12, IFN-γ) that activate naive CD4+ T-helper (Th) cells to differentiate into different populations based on the cytokine milieu (red text). These include IL-2 and TGF-β stimulated CD4 regulatory T cells (Treg), and IL-4 stimulated Th2 cells. However, IL-12 induced differentiation of naive CD4 cells into Th1 cells in the major antitumor effector T cells (8). Activation of naive CD8 by IL-12 and IL-2 leads to their differentiation into CD8+ cytotoxic T lymphocytes (CTLs). The Th1s and CTLs migrate back to the tumor cells *via* blood vessels (9). The tumor-associated immune response (TAIR) involves the Th1 cells activating M1 macrophages (MΦ), CTL release of perforin and granzyme, and release of IL-2 and TNF-α that work together to damage tumor beyond repair.

subsets differ in their expression of pattern recognition receptors (PRRs), surface markers, cytokine expression, and promotion of Th1 *vs*. Th2 *vs*. Th17 immune responses (77). These differentiated DC subsets exist functionally as either immature or mature. Subsets of iDCs are present in all tissues where they sample their environment for non-self molecules, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) released by damaged or dead cells (78), as well as self-associated molecular patterns (SAMPs) (79) that bind to PRRs on iDCs and other antigen-presenting cells. iDCs primarily take up the bound molecular patterns by receptor-mediated endocytosis. This binding activates iDC maturation that leads to morphologic and phenotype changes and migration to lymph nodes.

Normal Tumor-Associated Immune Response

In brief (**Figure 6**), the TAIR, as the first phase of immunoediting, eliminates neoplastic cells. It begins with tumor-associated antigens binding to PRRs on resident NK cell and M1-type macrophages (74). This binding induces tumor cell apoptosis and lysis that releases DAMPs into the TME. DAMPs bind to various PRRs that include carbohydrate-binding lectins and Toll-like receptors on iDCs and macrophages. The resulting activation of iDCs, primarily cDC1s (80), leads to a cascade of events leading to a pro-inflammatory TAIR (81). Activation of iDCs begins with phagocytosis or receptor-mediated endocytosis resulting in DC maturation and migration to TDLNs (82). Balan and Bhardwaj (83) postulated that, once in the TDLN, the now mDCs may transfer antigen-containing vesicles to resident cDC1 and cDC2 cells. The migrated mDCs and the resident DC subpopulations provide naive T cells with the three signals needed for their activation. Signal 1 is the cross-presentation of antigens bound to both major histocompatibility complex (MHC) I and MHC II molecules. MHC I-bound antigens are presented to naive CD8+ T cells by resident DCs and cDC1s (80, 84), while resident counterparts of cDC2s and cDC1s present antigen *via* MHC II molecules to naive CD4+ T cells (75, 85, 86). Signal 2 is generated by the binding of the CD80/86 co-stimulator molecules on mDCs to CD28 co-stimulator molecules of naive T cells. Signal 3 is provided by the release of various pro-inflammatory cytokines, the type of which is dependent on the antigen presented by the mDC. These cytokines include interleukin 12 (IL-12), primarily by cDC1s, as well as interferon gamma (IFN-γ), transforming growth factor beta (TGF-β), IL-4, and IL-10 that activate naive CD4+ Th cells to differentiate into different populations of effector CD4 Th cells based on the cytokine milieu (73, 86). IL-2 and IL-12 provide Signal 3 for activation of CD8+ cells. The effector T cells—CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T cells—in a Th1 response migrate back to the nascent tumor TME. In the TME, the CD8+ cytotoxic T cells release IFN-γ as well as perforin and granzyme that lyse the tumor cells. Th1 cells release pro-inflammatory cytokines support CTL and M1 macrophages provide cytokines for CTLs and M1 macrophage functions. Together, these three cells, among other cells in the TME, damage tumor cells, releasing DAMPs that start the process over again (86, 87).

Tumor-Associated Immune Response Immunosuppression in Colorectal Carcinoma

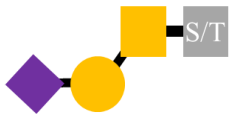
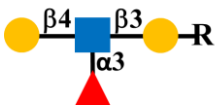
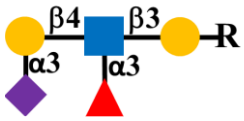
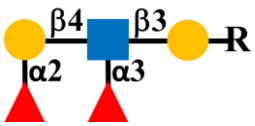
We propose that the interaction of TACAs with innate immune cells plays a central role in the escape phase of the immunoediting characterized by the suppression of the TAIR. The tumor-associated glyco-code correlates with the heterogeneous expression patterns of Tn, STn, and ST and other TACA ligands on glycoproteins and glycolipids of cancer cells and their association with patient prognosis and binding to carbohydrate-binding receptors (i.e., lectins) on the cells of the innate and adaptive immune responses (**Table 1**) (60, 88). Our hypothesis centers on the impact this binding has on the CRC-related TAIR.

Tumor-Associated Carbohydrate Antigen-Associated Lectins

Lectins are carbohydrate-binding proteins that occur on the surface of every cell. Binding of PAMPs by the various types of lectins on leukocytes is critical to the mounting immune response to pathogens (89, 90). Similarly, lectins binding to DAMPs in the form TACAs are critical to mounting a TAIR. The critical lectins on leukocytes that bind TAG-72-associated TACAs in CRC include Sialic Acid Recognizing Ig-like Lectins (Siglecs), the C-type lectin Macrophage Galactose-type Lectin (MGL), and Dendritic Cell-Specific ICAM-3 Grabbing Non-Integrin (DC-SIGN). This binding on iDCs, NK cells, and macrophages leads to the suppression of the TAIR in CRC. The terminal sialic acids on various TACAs, including STn, ST, and mono- or di-sialylated $Le^{x/a}$ and $Le^{y/b}$ glycans, bind to the single carbohydrate recognition domain of Siglecs on DCs, macrophages, NK cells, and monocytes (91, 92). MGL, expressed on both DCs and macrophages, is specific for GalNAc on both Tn and STn (93, 94) (**Figure 7**). DC-SIGN lectins on DCs and macrophages bind high mannose and fucose carbohydrates of non-sialylated Lewis antigens $Le^{x/a}$ and $Le^{y/b}$ on carcinoembryonic antigen (CEA) and other glycoproteins (95–97).

The amount of sialylated glycans on the surface of normal cells far exceeds that of pathogens, and because of this, sialylated glycans are self rather than non-self glycans (SAMPs) that bind Siglecs (79). Leukocytes are the principal carriers of one or more of the 14 functional human Siglecs. Siglecs recognize terminal sialic acids on glycoproteins and glycolipids in a linkage-specific manner (88, 98–100). The single carbohydrate-receptor domain of Siglecs may bind a sialic acid on another glycan on the same cell (cis-binding) or to another cell or extracellular glycan (trans-binding) (92). Siglecs divide into two groups based on their amino acid sequence and chemical phylogeny—CD33-related or non-CD33-related (91). The CD33-related Siglecs predominate on cells of both the innate and adaptive immune responses, where they either activate or suppress the immune response to pathogens and self-antigens (99, 101). Inhibitory Siglecs-2, -3, and Siglecs-5 through 10 have a cytoplasmic tail that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM).

TABLE 1 | Colorectal carcinoma (CRC) tumor-associated carbohydrate antigens (ligands) and the lectin-binding cells Tumor-Associated Carbohydrate Antigens (TACA), Serine/threonine (S/T), N-acetyl-galactosamine (), Sialic Acid (), Galactose (), Fucose (), N acetyl-glucosamine (), Radicals – (i.e., carbohydrates) closer to the serine/threonine (R), Macrophage Galactose-Type Lectin (MGL), Sialic Acid Recognizing Ig-like Lectins (Siglecs), Dendritic Cell-Specific ICAM-3 Grabbing Non-Integrin (DC-SIGN), Macrophage (MΦ), Immature Dendritic Cell (iDC), Monocyte (Mono), Natural Killer (NK) Cell.

TACA	Structure	Lectins	Lectin-Carrying Cells
Tn		MGL	iDC, MΦ
STn		Siglecs MGL	iDC, MΦ, Mono, NK
ST		Siglecs	iDC, MΦ, Mono, NK
Le^X		DC-SIGN	iDC, MΦ
SLe^X		DC-SIGN Siglecs	iDC, MΦ
Le^Y		DC-SIGN	iDC, MΦ

The cytoplasmic tail of Siglecs-14, -15, and -16 contains an immunoreceptor tyrosine-based activation motif (ITAM), while Siglecs-1 and -4 lack both (91).

The relative expression of Siglecs on circulating conventional dendritic cells (cDCs) (Siglecs-2, -3, -7, -9, and -15), plasmacytoid dendritic cells (pDCs) (Siglec-1 and Siglec-5), and macrophages (Siglecs-1, -3, -8, -9, -11, -15, and -16) in a steady state will change based on environmental triggers (92). NK cell subpopulations express Siglecs-7 and -9, and monocytes and their derived DCs and macrophages express Siglecs-1, -3, -7, -9, and -10 (60, 100). ITIM-carrying Siglecs suppress the TAIR, thus serving as a critical immune checkpoint (60, 92, 102) . Also, subsets of T cells express the inhibitory Siglecs-9 and -10 (103). In CRC, the terminal sialic acid residues on STn, ST, $SLe^{x/a}$, and $SLe^{y/b}$ TACA primarily bind to the single carbohydrate recognition domain of Siglecs-3, -7, and -9 on iDCs, macrophages, monocytes, and NK cells and by Siglec-15 on tumor-associated macrophages (TAMs).

MGL (CD301) is a C-type lectin that binds explicitly terminal α- and β-linked galactose and N-acetyl-galactosamine carbohydrates on both Tn and STn TACAs in CRC (104–106). MGL binding of a TACA leads to trimerization of the carbohydrate-binding domains followed by endocytosis of the bound ligand. Conformational changes arising after different binding ligands may activate different signal pathways and account for the ability of MGL to distinguish normal tissue from TACAs (105, 107–109). The expression of MGL is limited to iDCs and macrophages, where it modulates both the innate and adaptive immune responses, including the TAIR (110–112).

Like MGL, DC-SIGN (CD209) is a C-type lectin on subsets of DCs and macrophages (112, 113). DC-SIGN binds fucose (e.g., Lewis antigens) and mannose containing glycolipids and glycoproteins, as well as galactose and glucose to a much weaker extent. This "ligand-binding promiscuity" may arise from DC-SIGN's tetrameric configuration with four independent acting carbohydrate-recognition domains (95). The presence of terminal sialic acid blocks this binding. DC-SIGN plays a critical role in viral infections, especially HIV and bacterial and fungal infections. DC-SIGN's cell adhesion role arises from its ability to bind ICAM-2 on endothelial cells and ICAM-3 on naive T cells (112, 113).

Escaping the Tumor-Associated Immune Response in Colorectal Carcinoma—A Cascade of Effects

CRC cells' ability to escape the TAIR occurs in the microenvironments of both the tumor and the TDLNs (114, 115). The heterogeneous microenvironments in these locations contain many factors released by the tumor cells, stromal cells, and immune cells that suppress the TAIR and promote tumor cell proliferation and metastasis. CRC mucins and their associated TACAs play a critical role in this process by binding to and inhibiting the activity's various innate immune cells that suppress the TAIR. Subsets of iDCs, along with subsets of NK cells, macrophages, and regulatory T cells (Tregs), play a central role in CRC's elimination phase. We hypothesize that the various inhibitory signals arising from the binding of non-sialylated sugars on TACAs to MGL, DC-SIGN, and sialylated TACAs to Siglecs on subsets of these innate immune cells have an additive

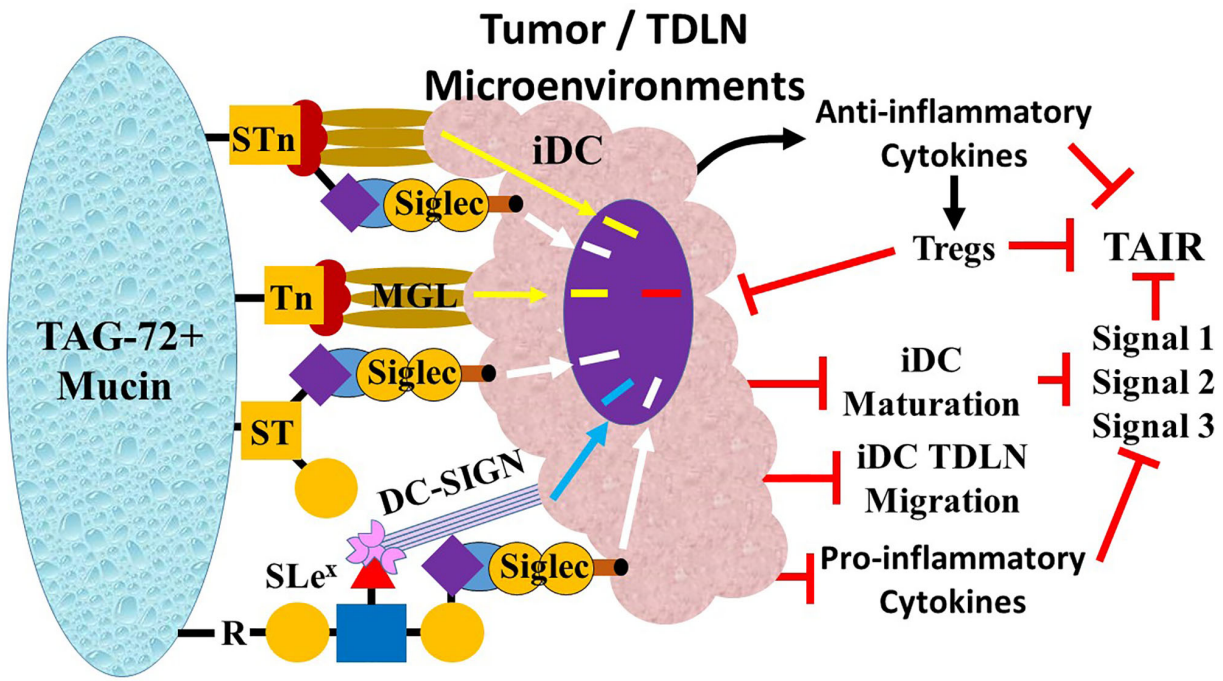


FIGURE 7 | Overview of tumor-associated immune response (TAIR) suppression by Tn, STn, and ST. TAG-72-related suppression of the TAIR begins with the ligands Tn, STn, ST, and SLe^X on the TAG-72-positive mucin molecules binding to lectins on immature dendritic cells (iDCs). The sialic acid carbohydrate on ST, STn, and SLe^x bind to Sialic Acid Recognizing Ig-like Lectins (Siglec), while the galactose on Tn and STN binds to Macrophage Galactose-Type Lectin (MGL), and the fucose of the Lewis antigens (Lex, SLe^x, and Le^y) bind to Dendritic Cell-Specific ICAM-3 Grabbing Non-Integrin (DC-SIGN). The binding generates variable inhibitory signal pathways (MGL, yellow arrow; Siglecs, white arrow; DC-SIGN, blue arrow) that suppresses (⊣) iDC maturation. Inhibition of iDC maturation inhibits iDC migration to tumor draining lymph nodes (TDLNs). Function changes including inhibition of the major histocompatibility complex (MHC) molecule expression (Signal 1), expression of co-stimulatory molecules (Signal 2), and secretion of pro-inflammatory cytokines (e.g., IL-6 and IL-12) (Signal 3) suppress the TAIR. Concurrently, there is increased release of anti-inflammatory cytokines (e.g., IL-10 and TGF-β) (black curved arrow) that further suppresses the T-helper 1 (Th1) TAIR and while activating (short black arrow) Th2, Th17, and regulatory T (Treg) cells. Treg activation suppresses the TAIR by further inhibiting iDC maturation (long black arrow) *via* cytotoxic T lymphocyte-associated protein 4 (CTLA-4) binding to CD80/86 co-stimulatory molecules and induces Th1 anergy by removing IL-2 from the environment. The result is suppression of the TAIR.

effect. In CRCs expressing TAG-72, this effect overcomes the opposing activating signals associated with other DAMPs and SAMPs binding to their respective lectins. TAG-72 in the blood and lymph nodes suggests that immunosuppression is concurrent in the microenvironments of the tumor and TDLNs (116).

Inhibiting Immature Dendritic Cell Maturation

The binding of TACAs to their respective lectins on iDCs multiplies the impact of other tumor-derived cytokines and factors while inhibiting their maturation. In addition, the resulting inhibitory signal modifies activation signals resulting from binding DAMPs and SAMPs to other PRRs (95). iDC subsets express Siglecs-3, -7, -9, and -15, MGL, and DC-SIGN that bind the TACAs carried by the TAG-72 (92, 117). van Vliet et al. (111) demonstrated that the binding of GalNAc on Tn and STn by MGL on iDCs generated a signal that inhibits iDC maturation (111). In addition, MGL binding to Tn carried by CD45 on effector T cells suppresses their activities, further promotes the escape phase of the TAIR, and is associated with reduced OS in Stage III CRC (118, 119). DC-SIGN is primarily expressed on DCs in TDLNs (95, 120, 121) where it binds fucose on non-sialylated Lewis antigens carried by TAG-72 and other ligands such as carcinoembryonic antigen (CEA) (122). These inhibitory signals combine with Siglecs-3, -7, -9, and -15 binding to sialylated TACAs, and these binding events have a wide-ranging impact on the TAIR.

Inhibited iDC maturation leads to limited MHC I and MHC II expression, leading to the defective presentation of tumor antigens to naive CD8+ and CD4+ T cells. Concurrently, there is downregulation of CD80/86 costimulatory molecule expression and release of IL-12 and other cytokines needed for T-cell activation. The result is Th1 anergy for tumor antigens. An increase in the secretion of anti-inflammatory cytokines, including IL-10, TGF-β, and IL-4 promotes the differentiation of Tregs and the Th2 immune response that further suppresses the TAIR (123–125).

Regulatory T Cells

Tregs are CD4+ CD25+ T cells that play a central role in suppressing the TAIR, and their accumulation in the

microenvironments of the tumor and TDLNs portends a poor prognosis in CRC (126). Early studies of TAG-72-positive TDLNs of CRC demonstrated an increase in the CD4+:CD8+ ratio that may well be attributable to an increase in Tregs in these lymph nodes (53). Treg subsets exhibit overlapping mechanisms for suppressing the TAIR (127). Tregs express cytotoxic T lymphocyte-associated protein 4 (CTLA-4) that binds to CD80/86 on iDCs that further inhibits iDC maturation. Tregs' high expression of the interleukin-2 receptor (IL-2R-CD25) consumes IL-2 in the TME and TDLN microenvironment needed for T-cell proliferation. The lack of IL-2 and suppression of the co-stimulation signal 2 further lead to Th1 anergy and an increase in the Th2 response. Tregs suppress the TAIR by release of perforin and granzyme that induces apoptosis of CD4 and CD8 effector T cells and by the release of adenosine triphosphate (ATP) (128).

Tumor-Associated Carbohydrate Antigen Binding to Natural Killer Cells and Macrophages

In the case of NK cells, their concentration in the TME of CRCs appears to be minimal despite the presence of tumor-infiltrating T cells (129). DC-derived IL-12 secretion promotes NK cell cytotoxicity and the production of IFN-γ. Suppression of iDC maturation associated with TACA binding to cDC1 inhibits the production of IL-12 (130, 131). Subsets of NK cells express varying amounts of Siglecs-7 and -9 that bind mono- or di-sialylated Lewis antigens and STn and ST on mucins (132). TACA binding inhibits NK cell cytotoxicity and downregulates the release of the cytokines tumor necrosis factor-alpha (TNF-α), IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (100, 132, 133).

TAMs are a prominent cellular component of the TME of CRCs. They predominantly arise from resident macrophages in the lamina propria of and from circulating monocytes (134). In general, TAMS are either pro-inflammatory (M1) or anti-inflammatory (M2). M1 and M2 TAMS differ in their phenotype and functions; however, individual cell analysis indicates the TAM subsets go beyond the dichotomous M1/M2 in the extent of phenotypic and functional subsets (135). Monocytes and macrophages express Siglecs-3, -7, -9, and -15, MGL, and DC-SIGN binding to TACAs on the TAG-72 positive glycoprotein (136, 137). The binding of TACAs to their lectins on M2 TAMs (73) leads to increased secretion of anti-inflammatory cytokines and chemokines that suppress the TAIR while supporting tumor angiogenesis, tumor cell proliferation, and metastasis (138). However, the clinical implications of the M1:M2 ratio in CRC appear unsettled, especially when looking at differences in location between the invasive front and sites deeper in the tumor (139).

Resetting the Tumor-Associated Immune Response

Immunotherapy joins targeted therapy, surgery, chemotherapy, and radiation therapy as the main strategies for treating CRC. The goal of immunotherapy is resetting the TAIR, i.e., reversing immunoediting (140). Wculek et al. (77) proposed that modulating DC function be added to the list of immunotherapeutic strategies that include activating inert T cells with anti-PD-1, adoptive cellular therapy, CAR-T cell therapy, and vaccines. Each approach depends on functional DCs to present tumor antigens to naive T cells. However, clinical studies directed at TACAs in CRC indicate minimal response (141, 142). In addition, current targeted therapy and immunotherapy have the potential of severe side effects and the development of resistance that are not associated with curative intent surgical resection. In contrast, TAG-72 ADCS with curative intent removes the suppressed TAIR-involved tumor and TDLNs in primary and recurrent CRC. In our opinion, the ^{125}I-labeled mMoAbs to TAG-72 do not themselves reset the TAIR in CRC patients. These mMoAbs serve as a preferential locator of exposed Tn, STn, and ST epitopes on the TAG-72 molecules that are bound to receptors on the cells of the innate and adaptive immune response.

In summary, an accurate assessment of the extent of CRC in a patient needs to be improved. Intravenous injection of a ^{125}I-labeled mMoAb to TAG-72 binds to the antigenic epitope(s) on both tumor cell and extracellular mucin(s) in the TME. The intraoperative use of an HGDP provides the surgeon with a tool to detect tissue inside and outside the normal surgical field that is involved in the disease process. TAG-72-induced suppression of the tumor immune response rather than just the presence of tumor cells is the actual disease process needing correction. This correction is brought about by removal of all radioactive tissue to the point there is no detectable radioactivity at the end of surgery. This allows for the immunoediting tumor immune response to reset itself to the point of increasing patient survival beyond that associated with current staging protocols (**Figures 3**, **4**). In contrast, the inability of removing the radioactive tissue precludes resetting of the tumor immune response and leads to recurrent disease and/or decreased patient survival.

DISCUSSION

The last decade saw researchers define the mechanisms underlying the role of aberrant glycosylation in tumor invasion, metastasis, and evasion of the immune response. Publications describing the pattern of aberrant glycans—the tumor-associated glyco-code—in suppressing the antitumor immune response caused us to reexamine its possible role in explaining the results of our long-term survival data using TAG-72 ADCS for the treatment of patients with primary and recurrent CRC. Our survival results provide prospective clinical data supporting the relationship between the pattern of aberrant glycan expression and patient outcome. Also, our results highlight the importance of the sialoglycan–Siglec relationship in TAIR (143) and the concept of a "sialoglycan-Siglec glyco-immune checkpoint" (144) that TAG-72 ADCS inhibits. In addition, concurrent MGL bindings to GalNAc on Tn and STn leading to a Th2 TAIR further supports our hypothesis.

As with all hypotheses, there are caveats to consider. The first is that of the sample size of our clinical studies. Even though we have safely injected well over 500 patients with mMoAbs to TAG-72, we have limited follow-up data. The endpoints for our Phase I and Phase II clinical studies focused on the impact of changing surgeon behavior in real-time while increasing the accuracy in defining the extent of disease rather than OS altering the adjuvant therapy available before 2000. Also, there was a lack of knowledge to ask the right basic science questions at the time. Secondly, the data presented here are from only one institute. However, results from multiple institutions duplicated the stated endpoints of our study; however, there was no emphasis on obtaining patient survival data. Thirdly, there is no direct evidence, using human tissue samples, that our hypothesis is valid. In addition, our theory is based on results obtained from CRC and may not apply to other carcinomas. However, TAG-72 ADCS supports and extends the conclusion by Perdicchio et al. (145) "that reducing sialylation may provide a therapeutic option to render tumors permissive to immune attack." This statement is true not only for CRC but also for similar carcinomas of gastrointestinal and genitourinary tracts, lungs, and breasts.

The most important caveat is that TAG-72 ADCS is not a "silver bullet." However, our results clearly distinguish subpopulations of patients who vary in their prognosis. Reviewing the numbers, only 90% of primary colorectal adenocarcinomas express TAG-72, only 80% of these patients undergo surgery with curative intent, and mMoAb CC49 localizes in only 86% of these cases. The result is that approximately 62% of patients with primary CRC have a significant chance of improved OS. However, on an annual basis, that equates to over 1 million patients per year worldwide and over 95,000 patients in the US who could benefit from TAG-72 ADCS.

Testing our hypothesis could involve repeating our clinical studies using one of the humanized fragments of the mMoAb CC49 developed in the last decade (14, 146–148). There are many radionuclides that can be used instead of ^{125}I, but it is critical to match the antibody's clearance to that of the radionuclide. Preoperative imaging techniques provide only information in the X-Y plane and often miss small lesion. The use of a ^{125}I-labeled mMoAb provides in-depth information that allowed us to detect and then locate tumor-involved tissue within the liver and in extraregional lymph nodes draining the tumor. Intraoperative imaging combined with the HGDP further improves real-time tumor detection and assessment of surgical resection to ensure the best outcome for the individual patient (149). In addition to using an HGDP, intraoperative imaging confirms adequate removal of the TAG-72 tissue and for accurate sampling of the tissue in the Pathology laboratory (149). This type of study precludes current robotic and laparoscopic approaches and requires a thorough assessment using a gamma detection probe (150). It does not preclude the use of fluorescence-labeled intact or fragments of monoclonal antibodies nor does it preclude the use of bilabeled antibodies (151). Fluorescence-guided surgery is limited by the ability of the fluorophore's light to penetrate tissue, which is not a problem for a radionuclide. However, fluorophores allow for more sensitive assessment of surfaces (e.g., serosal, tissue margins). These combined techniques can be used to provide the researcher with the "correct" tissue for subsequent study possibly by multicolor fluorescent molecular digital imaging of frozen sections. The resulting tissue can also be used to establish patient-derived xenografts (PDXs) in an immunodeficient mouse model (reviewed in (152) that is best suited to assess impact role of TAG-72 in suppressing the TAIR.

Our overall goal is to provide clinicians with tumor-specific information beyond the current staging system to more accurately stratify CRC patients at the molecular level and specifically allow for a more case-by-case determination of the most appropriate cancer-targeted therapies. Such a further refinement in this concept of oncologic theranostics, as it relates to TAG-72, other aberrant glycans, and the resultant TAIR, may well include new innovative and personalized immunotherapies directed at specific glycan targets expressed within individual tumors. This innovative oncologic theranostics would hold significant promise for improving CRC patients' care and long-term outcomes (17). Our results suggest that TAG-72 ADCS will provide the opportunity to obtain appropriate tissues needed for an accurate definition of the roles of aberrant glycans, their lectins, and the specific innate immune cells that express them on the biology of tumor cells. The goal is to develop new molecules and methods to inhibit the immunosuppression associated with aberrant glycosylation.

Our results and those of the authors who wrote the papers referenced here provide proof of the sageness of Dr. Robert M. Zollinger, a giant of American Surgery, who stimulated his younger colleagues with, to paraphrase, "The answer is right in front of you, but you don't know enough to see it." We are still learning.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CH, SP, CM, and EM conceptualized the article. CH wrote the article, and each author edited the article. CM and EM conducted the clinical studies. All authors contributed to the article and approved the submitted version.

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Recent Progress in the Methodologies to Identify Physiological Ligands of Siglecs

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Siglecs, a family of receptor-like lectins, recognize glycoproteins and/or glycolipids containing sialic acid in the extracellular space and transduce intracellular signaling. Recently, researchers uncovered significant contributions of Siglecs in cancer immunity, renewing interest in this family of proteins. Previous extensive studies have defined how Siglecs recognize glycan epitopes (glycotopes). Nevertheless, the biological role of these glycotopes has not been fully evaluated. Recent studies using live cells have begun unraveling the constituents of Siglec ligands. These studies demonstrated that glycoprotein scaffolds (counter-receptors) displaying glycotopes are sometimes just as important as the glycotope itself. These new insights may guide future efforts to develop therapeutic agents to target the Siglec – ligand axis.

Keywords: Siglec, ligand, glycotope, counter-receptor, proximity labeling, cell array, genome-wide knockout/knockdown screening

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INTRODUCTION

Siglecs (sialic acid-binding immunoglobulin superfamily lectins) are a family of type-I transmembrane proteins belonging to the immunoglobulin superfamily (1–5). Most of them are expressed on one or more subsets of leukocytes, and participate in signal transduction by regulating the tyrosine phosphorylation/de-phosphorylation cycle of signal transduction molecules. This regulation is achieved by recruiting tyrosine phosphatase SHP-1 or tyrosine kinase Syk in the cytoplasm. Recent studies have shown that some Siglecs expressed on killer leukocytes (such as Siglec-7 on natural killer cells and Siglec-9 on cytotoxic T cells of tumor patients) work similarly to classical immune checkpoint receptors (e.g., programmed cell death protein-1) (6, 7), and some others expressed on phagocytes (e.g., Siglec-10 on macrophages) work similarly as canonical "do not eat me" receptors (e.g., signal-regulatory protein alpha) (8). The functional parallels between Siglecs and immunomodulatory receptors, particularly regarding cancer immunity, have led to a recent surge in the interest in Siglecs and their ligands.

As the name implies, Siglecs recognize glycans containing sialic acid. Past extensive investigations have contributed to the establishment of Siglec glycan recognition specificities (1, 2, 4, 5). Some questions remain, however, such as whether and how these glycan epitopes

(glycotopes) are displayed on natural glycoconjugates (glycoproteins and/or glycolipids) and which ligand is most significant in a given biological context. Affinity purification is often used to identify the ligand for a lectin. However, the inherently weak interaction between Siglec and the glycotope (dissociation constant usually in the order of 10^{-3} mol/L) renders affinity purification ineffective. Recent *in vitro* studies using innovative methodologies in chemical biology and/or genetics are beginning to reveal the Siglec ligand constituents in the cellular context.

Siglec ligands can be classified into two categories (**Figure 1**): Siglec ligands expressed on the same cells that express the Siglec of interest (cis-ligands), and those on juxtaposing cells interacting with the cells on which Siglec of interest is expressed (trans-ligands). If a Siglec ligand is a glycoprotein, it comprises a glycan epitope being recognized by the Siglec of interest (glycotope), and the protein backbone that displays glycotope (counter-receptor).

In this Mini Review, we summarize recent methodological progress in the identification of physiologically relevant Siglec ligands in cellular contexts. Additionally, we discuss the advantages and disadvantages of these new approaches. For a comprehensive review of Siglec ligands, readers are encouraged to refer to a recent review (9).

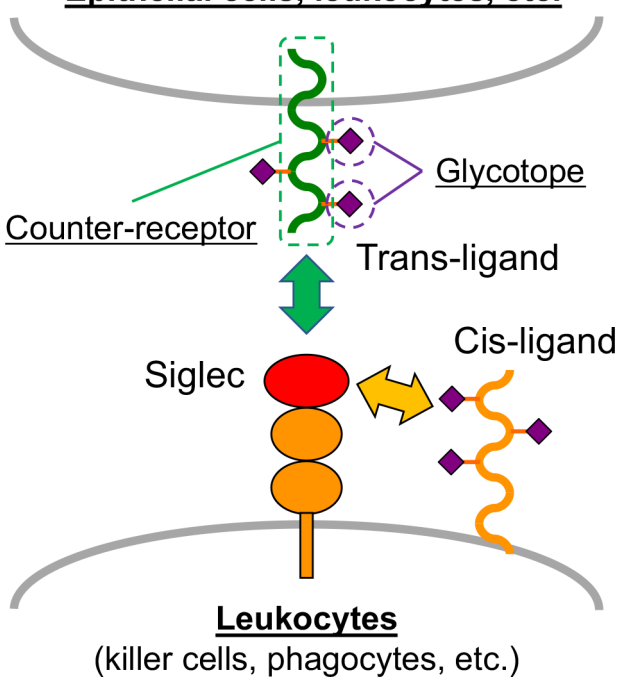

FIGURE 1 | Siglec and Siglec ligand. Most Siglecs are expressed on leukocytes. The ligands on the same cell that express Siglec are called cis-ligands, and those on the juxtaposing cells (e.g., epithelial cells and other leukocytes) are called trans-ligands. Glycoprotein ligands of Siglecs comprise two constituents: glycotope, which directly interacts with Siglec, and counter-receptor, which is the protein backbone that displays the glycotope.

METHODOLOGIES TO IDENTIFY SIGLEC LIGANDS

Affinity Purification With a New Design of Recombinant Siglec Protein

For affinity purification, one has to prepare a solid phase matrix on which recombinant Siglec is immobilized, and use it to enrich Siglec ligand from cell lysate or biological fluid. Traditionally, the protein fusion "tag" of choice in recombinant Siglecs has been the fragment crystallizable (Fc) region of human immunoglobulin G (IgG). This choice is because it facilitates the folding of recombinant protein (thus increasing the yield). Additionally, the recombinant protein containing IgG-Fc can be easily purified with protein A resin (10). Nevertheless, IgG-Fc fusion protein is a homo-dimer (bivalent), which may not be sufficient to compensate for the low binding affinity between Siglec and its ligand by multivalency. An alternative protein fusion tag that allows the formation of a higher oligomer, thus increasing the "avidity," may be useful for affinity purification. A novel protein tag [homo-pentamerization domain of cartilage oligomeric matrix protein (COMP)] has been adopted for the production of recombinant Siglec-8 protein, facilitating the identification of Siglec-8 ligand in the human airway (11). Whether the pentamer (as formed by COMP oligomerization domain) is optimal or other oligomer(s) may perform better is unknown as of yet. Artificially designed helical bundle oligomer tags, forming homo-tetramer, homo-pentamer, or homo-hexamer (12), may be useful to researchers endeavoring to answer this question.

A caveat of the affinity purification approach is that it requires a large amount of recombinant Siglec protein (usually in multimilligrams) for the preparation of the affinity matrix. Also, the affinity purification of integral membrane proteins serving as Siglec ligand requires disruption of the cell membrane by detergent or chaotropic ion, which inevitably dissociates cell surface protein complexes. Many Siglec ligands recently identified *via* affinity purification are soluble proteins (11, 13–15), likely because membrane solubilization leads to loss of the cell surface protein complex, which may be a prerequisite for Siglec –ligand interaction.

Proximity Labeling

To overcome some of the limitations of the traditional affinity purification method, several groups have developed methods to identify Siglec ligands in a cellular context. One approach was to install a photoreactive sialic acid analog on cell surface glycoconjugates, followed by cross-linking and immuno-precipitation of the Siglec of interest. This process is followed by mass spectrometry-based proteomics to identify proteins that are cross-linked with the Siglec (16, 17). This approach revealed biologically relevant ligands for CD22/Siglec-2: CD22 itself as a major cis-ligand, and surface IgM as a major trans-ligand, of CD22. However, to apply this method to other Siglecs, one would have to evaluate whether the reactive group installed on sialic acid is tolerated by the Siglec of interest. Prior knowledge of the sialyltransferase responsible for the biosynthesis of the glycotope recognized by the Siglec may also be required. Hence, a more facile and versatile method may be needed.

Recently, some groups (including ours) developed methods to identify Siglec ligands on the basis of the same chemical principle: proximity labeling of proteins with short-lived tyramide radicals generated by peroxidase (18–20). This chemical principle has been known for decades and adapted for the enhancement of antibody-binding signals in immunohistochemical staining [known as catalyzed reporter deposition or tyramide signal amplification (21)]. However, its application for the identification of protein interacting partners (ligands and cluster) was only recently realized (22).

Here, cells expressing the Siglec ligand are incubated with peroxidase-coupled recombinant Siglec, followed by the addition of tyramide-based labeling compound (often biotin tyramide) and hydrogen peroxide. The addition of hydrogen peroxide generates short-lived tyramide radicals in the vicinity of the Siglec–peroxidase probe (thus in the vicinity of Siglec ligands) (**Figure 2A**). Coupling of peroxidase [horseradish peroxidase (HRP)] with Siglec can be achieved in one of two ways: by preparing Siglec–peroxidase fusion protein (19) or by combining Siglec–Fc with peroxidase-conjugated secondary antibody (18). Biotin-labeled proteins are purified by affinity purification from the cell lysate and identified by mass spectrometry-based proteomics. Studies utilizing this methodology demonstrated glycophorin A acts as a Sialoadhesin/Siglec-1 counter-receptor on human erythrocytes (19), and CD44 acts as a counter-receptor for Siglec-15 on RAW264.7 mouse macrophage cell line (18).

A variation of this protocol, applicable to the identification of cis-ligands (**Figure 2B**), is to use HRP-conjugated (or coupled) antibody against the Siglec of interest (20). This study yielded insight on the mechanism wherein CD22/Siglec-2 regulates B cell signaling. These results complemented results from past studies (23–26) and the chemical biology-based approach mentioned above (16). This method may be considered an implementation of the "selective proteomic proximity labeling using tyramide" method (27) targeting Siglecs.

Yet another variation of this method is to use another peroxidase (APEX) fused with the lectin of interest (28, 29). APEX is an engineered peroxidase developed from cytosolic ascorbate peroxidase of leguminous plants. It folds well in the cytosol of mammalian cells (whereas HRP fails to do so) (30, 31). APEX–galectin-3 fusion protein was used for the identification of both extracellular and intracellular interaction partners of galectin-3 (28). This study confirmed known interaction partners as well as revealed new partners. In principle, Siglec–APEX fusion protein would also be useful for the identification of Siglec ligands.

An advantage of the proximity labeling-based ligand identification approach is that it requires a relatively small amount of recombinant Siglec (on the order of micrograms). Additionally, the number of cells required is small (on the order of 10^6 cells), making it possible to attempt the identification of Siglec ligands not only on cell lines but also on primary cells.

Some caveats of this approach may be as follows: [1] glycolipids are not labeled by tyramide radicals and thus cannot be identified; [2] some proteins poor in tyrosine, which is the primary amino acid labeled by tyramide radical (22), may not be labeled efficiently and thus may not be identified; [3] bystander

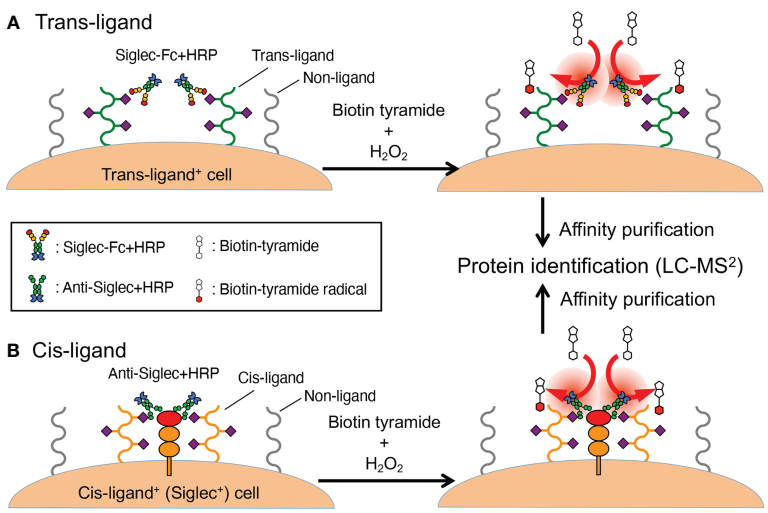

FIGURE 2 | Proximity labeling method. **(A)** A workflow to identify trans-ligands. Cells that express Siglec ligand (as revealed by flow cytometry, microscopy, etc.) are labeled with a recombinant Siglec of interest that is coupled to peroxidase (either as a fusion protein or by way of complexing with a secondary reagent). The cells are washed and then exposed to biotin tyramide and hydrogen peroxide, which generates short-lived tyramide radicals that diffuse a limited distance from the origin before reacting with tyrosine residues in the vicinity (or diminish). This limited diffusion distance ensures selective labeling of the proteins in the proximity of the Siglec ligand, to which the Siglec–peroxidase complex is attached. **(B)** A workflow to identify cis-ligands. The probe used in this workflow is not recombinant Siglec but a peroxidase-coupled antibody that recognizes the Siglec of interest. Otherwise, the overall workflow is similar to **(A)**. In fact, the workflow described in **(A)** can also be applied for the identification of cis-ligand.

proteins in the vicinity of true Siglec ligand are also labeled and identified; [4] some glycoproteins inherently resistant to proteolysis (such as mucins and mucin-like glycoproteins) may not be identified easily *via* mass spectrometry.

Genetically Modified Cell Array

Recent advances in genetic tools, particularly CRISPR/Cas9-based genetic manipulation tools for gene editing and silencing, have been applied to modify glycosylation-related genes. Dr. Henrik Clausen's group has developed extensive libraries of cell lines with modified glycosyltransferase genes. These lines were initially modified with zinc finger nucleases (32), and more recently with CRISPR/Cas9 (33). A recent publication from this group revealed the details of glycotopes recognized by Siglecs and glycosyltransferases involved in their synthesis. They also described the importance of galactose sulfation for the generation of glycotope recognized by several Siglecs (34), which was independently confirmed by another group (35). For several Siglecs, mucin-like glycoproteins appear to be effective counter-receptors.

Although this approach is no doubt powerful, it is not without caveats, as follows: [1] it is labor-intensive to develop and maintain a comprehensive library of cells comprising several sublines in which a single gene (or combination of genes) is disrupted and/or overexpressed; [2] the cell line used as the platform for the library may not be the best model of the cell type of interest.

Genome-Wide Knockout/Knockdown Screening

An extension of the "cell library" approach is to utilize Cas9 and a single guide RNA (sgRNA) library to prepare an *ad hoc* library of gene-disrupted cells in mixture. After library creation, cells showing reduced (or enhanced) Siglec binding are enriched by cell sorting. Finally, researchers seek to identify the genes targeted in the cells (i.e., sgRNA enriched in the cells) that lost (or gained) Siglec binding. A recent study demonstrated this approach is feasible for the identification of Siglec ligands (36). This research revealed that a primary Siglec-7 counter-receptor on the K562 human erythroleukemia cell line is CD43. It also revealed that the cluster of O-glycans on the N-terminus of CD43 is important for recognition by Siglec-7. CD43 was independently confirmed by another group using proximity labeling as the Siglec-7 counter-receptor (37).

An advantage of this approach is that one can reveal unsuspected pathway(s) that regulate the expression of Siglec ligands, providing novel insights into the mechanism regulating Siglec – ligand interactions as well as possibly revealing a novel point of intervention for therapeutic applications. A genome-wide knockout/knockdown screening can, in theory, identify all the factors that contribute to the expression of Siglec ligands. Genome-wide screening using primary cells or live animals [using transgenic mice expressing Cas9 protein (38)] is possible, although a large amount of sgRNA-coding lentivirus may be required (39). A weakness of this approach is that it may not reveal genes essential for Siglec ligand expression in the presence of redundancy (e.g., multiple counter-receptors, alternative biosynthetic pathways, etc.).

DISCUSSION

Although significant methodological progress toward the identification of Siglec ligands in a cellular context has been made in recent years, there is no single method that applies to all biological contexts in which Siglecs are involved. A combination of new and traditional methods (such as glycosylation inhibitors and glycosidases), along with supportive bioinformatics, may prove most efficient in identifying biologically relevant ligands for Siglecs.

Some studies utilizing genome-wide knockout screenings to identify the genes influencing cancer cell sensitivity to NK cells (40), cytotoxic T cells (41), and antibody-dependent cellular phagocytosis by macrophages (42) have revealed some of the genes involved in the sialic acid biosynthetic pathway. Whether Siglecs are involved in the observed phenomenon is not clear. Nevertheless, in-depth analysis of gene lists obtained in these studies may reveal some interesting pathways influencing cancer immunoevasion *via* engaging Siglecs on killer leukocytes and phagocytes.

Identification of Siglec ligands, particularly counter-receptors, could lead to novel therapy options. For example, an antibody that recognizes a counter-receptor carrying a specific glycotope expressed on cancer cells (an equivalent of checkpoint ligand) may complement an immunotherapy that targets the cognate Siglec. One major obstacle in this direction is that, there is no established method to generate an antibody that recognizes a glycotope displayed on a specific protein scaffold. Technological breakthroughs and a platform enabling the development of such antibodies are highly anticipated (43).

AUTHOR CONTRIBUTIONS

TA conceptualized the content of the review and wrote the first draft. H-SJ, S-CZ, CHL, and L-YC performed literature search and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Siglec Signaling in the Tumor Microenvironment

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Sialic acid-binding immunoglobulin-like lectins (Siglecs) are a family of receptors that recognize sialoglycans – sialic acid containing glycans that are abundantly present on cell membranes. Siglecs are expressed on most immune cells and can modulate their activity and function. The majority of Siglecs contains immune inhibitory motifs comparable to the immune checkpoint receptor PD-1. In the tumor microenvironment (TME), signaling through the Siglec-sialoglycan axis appears to be enhanced through multiple mechanisms favoring tumor immune evasion similar to the PD-1/PD-L1 signaling pathway. Siglec expression on tumor-infiltrating immune cells appears increased in the immune suppressive microenvironment. At the same time, enhanced Siglec ligand expression has been reported for several tumor types as a result of aberrant glycosylation, glycan modifications, and the increased expression of sialoglycans on proteins and lipids. Siglec signaling has been identified as important regulator of anti-tumor immunity in the TME, but the key factors contributing to Siglec activation by tumor-associated sialoglycans are diverse and poorly defined. Among others, Siglec activation and signaling are co-determined by their expression levels, cell surface distribution, and their binding preferences for *cis*- and *trans*-ligands in the TME. Siglec binding preference are co-determined by the nature of the proteins/lipids to which the sialoglycans are attached and the multivalency of the interaction. Here, we review the current understanding and emerging conditions and factors involved in Siglec signaling in the TME and identify current knowledge gaps that exist in the field.

Keywords: Siglecs, sialic acids, sialoglycans, tumor microenvironment, immune checkpoint, cancer, immunotherapy

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INTRODUCTION

Humans express 14 members of the Sialic acid-binding immunoglobulin-like lectins (Siglecs) that are divided in two subfamilies based on their sequence similarity and evolutionary conservation. Siglec-1 (also known as sialoadhesin and CD169), Siglec-2 (also known as CD22) Siglec-4 and Siglec-15 have clear orthologous in mammalian species (1–4). The CD33-related Siglecs (Siglec-3 also known as CD33, Siglec-5 to -11, -14 and -16) evolved more rapidly among species. These transmembrane

receptors are mainly present on immune cells, but also on other cell types such as trophoblasts, myelin-forming cells, and stromal cells (1, 3, 4). Siglecs bind their ligands *via* an extracellular N-terminal V-set domain. Intracellularly, most Siglecs (Siglec-3, -5 to -9, and -11) harbor a combination of a membrane proximal ITIM (immunoreceptor tyrosine-based inhibition motif) domain and a membrane distal ITIM-like domain (1–3). Upon Siglec activation, this ITIM domain is phosphorylated by Src family kinases. Phosphorylation subsequently leads to recruitment of SH2-domain containing phosphatases SHP-1 and/or SHP-2, which dephosphorylate downstream components of immune stimulatory pathways, thus inhibiting cellular activation. Interestingly, three Siglecs (Siglec-14, -15 and -16) do not contain ITIM domains, but have a positively charged residue in their transmembrane domain that enables them to complex with ITAM (immunoreceptor tyrosine-based activation motif) containing adaptor proteins, such as DAP10 or DAP12 (2, 3, 5). This leads to recruitment of protein kinases that can phosphorylate downstream targets, eventually triggering downstream signaling pathways (1–3, 5). Besides ITIM- and ITAM-containing Siglecs, Siglec-1 does not contain known intracellular signaling motifs.

The ligands for the Siglecs are constituted by glycans, highly diverse biomolecules composed of various monosaccharides that are linked to membrane bound and secreted glycoproteins, glycolipids, and as recently suggested also small noncoding glycoRNAs (3, 4, 6–9). Siglecs selectively recognize glycans that contain one or more negatively charged sialic acid residues, so called sialoglycans (1, 6). Sialoglycans are present on virtually every human cell, but are aberrantly expressed on tumor cells where they contribute to tumor growth and progression, including metastasis (10). Interestingly, Siglec expression has been reported to be enhanced and/or induced on other cells within the TME, including immune cells (11–16). Concomitantly, the combination of altered Siglec expression on immune cells and aberrant sialoglycan expression on tumor cells, could possibly lead to strong Siglec activation and resulting in immune cell inhibition within the TME (**Figure 1**). The enhanced sialoglycans expression within the TME is clearly described in literature, however, the evidence that this directly translates into more Siglec ligand expression and Siglec signaling remains scarce.

The structure and signaling motifs of Siglec receptors show great resemblance to the well-known inhibitory receptor PD-1 (3, 17) that is used as target in immunotherapy of cancer. Moreover, Siglecs are often co-expressed on T cells with other inhibitory receptors, such as PD-1, TIM-3 and LAG-3 (11). Therefore, the immune inhibitory Siglecs are currently envisioned as potential immune checkpoint receptors that can be targeted in cancer (17–20). Despite Siglecs emerging as an attractive target for cancer immunotherapy, the exact cell and glycobiological conditions that trigger, regulate and control Siglec signaling in the TME remain largely elusive. Here, we will review the current knowledge on factors (co-)determining Siglec signaling and discuss knowledge gaps regarding this Siglec-sialoglycan signaling axis focusing on the TME.

SIGLEC EXPRESSION WITHIN THE TME

Siglec family members are expressed by most cell subsets of the human immune system and by several tissues and cell types

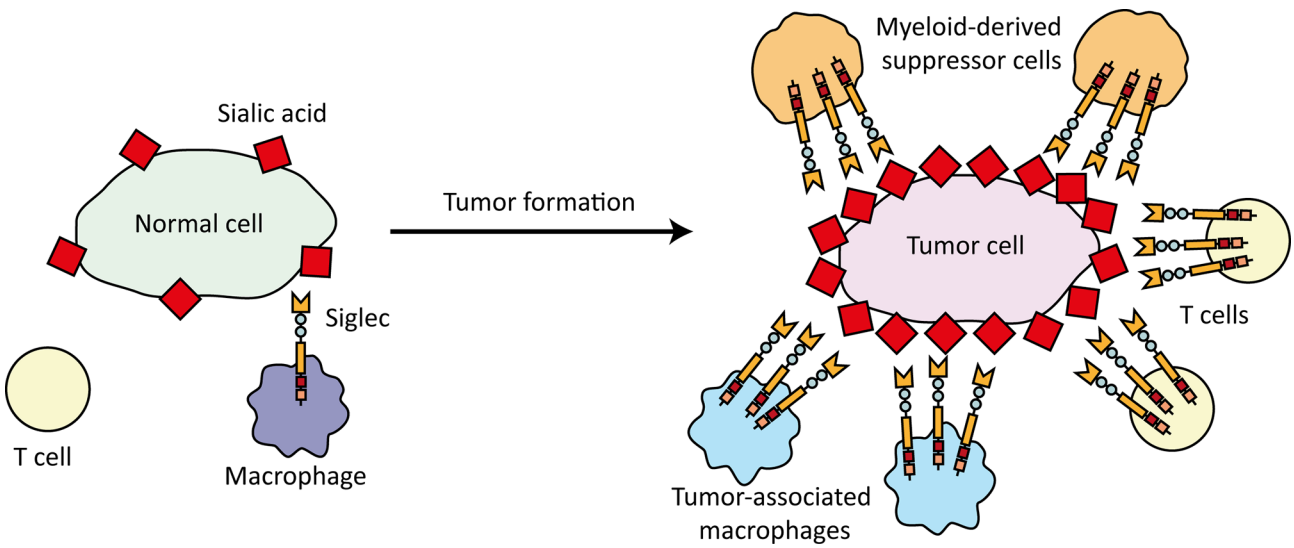

FIGURE 1 | Schematic overview of changes in the Siglec-sialoglycan axis upon tumor formation. Immune cells circulating in healthy tissue express Siglecs and cells in this environment express 'normal' sialic acid (red diamonds) types and levels. Upon tumor development, immune cells infiltrate the TME, such as tumor-associated macrophages, T cells, and myeloid-derived suppressor cells (and many other types). These immune cells display changed and partially enhanced Siglec expression levels and can interact with tumor-associated sialoglycans that are formed and often increased as result of altered glycosylation in cancer cells. Siglec-sialoglycan interactions in the TME can lead to functional modulation of infiltrating immune cells and the anti-tumor immune response.

outside of the immune system (1–4). Some Siglecs have immune cell type specific expression and can therefore be used as differentiation marker such as Siglec-1 that is found mainly on macrophages, Siglec-2 that is specifically expressed by B cells, Siglec-3 that marks cells from the myeloid lineage including microglia cells, or Siglec-8 that is found predominantly on eosinophils (3, 8). In the TME, focus has been mainly on the inhibitory Siglecs-7, -9, and -10 as well as the activating Siglec-15. Siglec-7 and -9 are abundantly present on natural killer (NK) cells with Siglec-7 being a pan-marker for human NK cells and Siglec-9 being present on a population of $CD56^{dim}$ $CD16^{+}$ NK cells (21). Strong interactions of these Siglecs with synthetic and natural multivalent sialoglycan ligands appears to be sufficient to inhibit NK cell activation (22). Accordingly, blocking Siglec-7 and -9 interactions with sialoglycans using monoclonal antibodies increased killing of several tumor cell lines (K562 and HeLa) by peripheral blood NK cells (21). Another example of a Siglec-mediated immune modulatory effect was observed in T cells. Only a small percentage (1-3%) of peripheral blood $CD8^{+}$ T cells express Siglec-7 and/or Siglec-9 (11, 23). Remarkably, Siglec-9 expression on CD8+ T cells present in peripheral blood and tumor tissue of non-small cell lung cancer (NSCLC) patients is upregulated up to 25% and 40% respectively (11). Similarly, tumor-infiltrating $CD8^{+}$ T cells in melanoma patients have also been found to display enhanced levels of Siglec-9 (13). Functionally, Fab fragments targeting Siglec-9 could modulate activation and IL-2 production of staphylococcal enterotoxin B stimulated tumor-infiltrating lymphocytes of primary NSCLC patients (11). Siglec-9 antibodies could increase cytotoxicity of healthy donor $CD8^{+}$ T cells against anti-CD3-loaded P815 tumor cells and enhance IFNγ and TNFα production (13). Besides NK and T cells, immune modulation of Siglec-9 expressing myeloid cells has also been observed. For instance, Rodriguez et al. showed that pancreatic ductal adenocarcinoma-derived sialic acid can cause monocytes to differentiate to macrophages, which was prevented using anti-Siglec-7 and anti-Siglec-9 blocking antibodies (24).

Siglec-15 has emerged as an interesting target for cancer immunotherapy (25). Siglec-15 contains a positively charged residue in its transmembrane domain and is therefore regarded as an activating Siglec (26). Siglec-15 expression was observed on tumor-associated macrophages (TAMs) in the TME of human lung and rectal adenocarcinoma and human hepatocellular carcinoma (27). The interaction between Siglec-15 expressing monocytic THP-1 cells and sialyl-Tn expressing H157 human lung carcinoma cells enhanced TGF-β production. Next to its expression on myeloid cells, Siglec-15 was observed on stromal cells and even on tumor cells (28). Siglec-15 expression on tumor cells was verified by others on several types of cancer, such as lymphoma, gastric cancer and acute myeloid leukemia (29–31). Similarly, Siglec-8 has been reported to be expressed by breast cancer cells (32). Next to that, several Siglecs were found to be expressed on hematological cancers (33–35). Siglec-6 expression was observed on acute myeloid leukemia cell lines, primary acute myeloid leukemia blasts, transformed B-cells in chronic lymphocytic leukemia (33). Siglec-2 expression on acute lymphoid leukemia has also been documented and Siglec-3 is found on blasts in nearly all acute myeloid leukemia patients (34, 35). Besides Siglec expression on immune cells and on tumor cells, Siglecs might also be expressed within the stromal compartment, as Siglec-11 expression was found on human and chimpanzee ovarian stromal cells (36). However, the influence of the stromal compartment on the Siglec-sialoglycan signaling axis remains largely unexplored.

Regulation of Siglec Expression

How Siglec expression is being triggered and regulated is still largely unknown, including the factors contributing to the enhanced expression in the TME (12). Siglec-10 was found to be upregulated on $CD52^{+}$ $CD4^{+}$ T cells upon CD3/CD28 activation (37, 38). Therefore, one factor contributing to enhanced Siglec expression is cellular activation, but this was not investigated within the TME context. Another way tumor cells influence Siglec-10 expression on immune cells has been studied by Li et al. (39). They showed that extracellular vesicles (EVs) isolated from ascites from epithelial ovarian cancer patients could induce Siglec-10 expression on Jurkat T cells. Functionally, treatment of Jurkat T cells with tumor cell derived EVs inhibited PMA/ionomycin-induced protein kinase C activity and impaired phosphorylation of tyrosine kinase zeta-chain-associated protein kinase 70 after activation with anti-CD3, but direct evidence for Siglec signaling is lacking.

Siglec-15 expression was found to be downregulated by IFN-γ on monocyte-derived macrophages and RAW264.7 cells and to be dependent on macrophage-colony stimulating factor (28). Interestingly, also Siglec-1 expression was shown to be influenced by cytokines, as it could be induced by IFN-α and IFN-γ (40). Moreover, a few studies have observed that immune suppressive cytokines can influence Siglec expression, which might explain how the immune suppressive TME affects Siglec expression on immune cells. For instance, De Saint Jean et al. (2017) reported that TGF-β1 can enhance Siglec-1, but not Siglec-5, -7, -9, -10 and -14, expression on monocyte-derived dendritic cells (41). Nagase et al. did not observe increased Siglec-1 expression on the monocytic THP-1 cell line by treatment with TGF-β, but rather after stimulation with IL-1β and TNF-α (42). Another study showed that high-expressing Siglec-1 peripheral blood mononuclear cells (PBMC) derived macrophages ($CD14^{+}$ monocytes differentiated with 10% human AB serum) downregulate Siglec-1 expression upon treatment with tumor culture supernatant from HepG2 cells and upon treatment with recombinant human TGF-β, but not with recombinant human TNF-α or IL-10 (43). Lastly, Calzada-Wack et al. showed that IL-10 treatment of *in vitro* cultured blood monocytes resulted in decreased Siglec-3 expression (44). Furthermore, it has been shown that Siglec-expressing cells can also be recruited to the TME, for example the TME of gliomas can contain many myeloid-derived suppressor cells (MDSCs) that express Siglecs (45). These data show that both monocytic MDSCs and polymorphonuclear MDSCs from the glioma TME express Siglec-3, -5, 7 and -9. Potentially, TME characteristics such as metabolic shift, hypoxic areas and changes in stromal

compartment could all affect Siglec expression and signaling in the TME.

One component that might influence Siglec expression in general and in the TME in particular is the dynamics of their recruitment to the membrane, internalization, recycling, and degradation. Siglec-2 has been shown to be constitutively endocytosed, and several Siglec receptors (Siglec-1, Siglec-2, Siglec-3) have been demonstrated to be internalized after ligand binding or antibody crosslinking, and it was shown that Siglec-2 internalization was controlled by tyrosine residues Tyr (843) or Tyr (863) in its intracellular ITIM motif (46–52). Phosphorylation of the ITIM motifs can induce an intracellular pathway that eventually leads to Siglec degradation. Suppressor of cytokine signaling 3 and Cbl, a RING finger-containing E3 ligase, can bind the phosphorylated ITIM of Siglec-3 (51, 53). This leads to ubiquitination and proteasomal degradation of Siglec-3.

Regarding Siglec recruitment to the membrane, a recent study by Chen et al. showed that N-glycosylation of Siglec-15 affects its localization (54). Treatment with a variety of lysosome inhibitors showed that Siglec-15 was degraded in a lysosome-dependent manner in Siglec-15 overexpressing HEK293T cells. Moreover, inhibition of glycosylation using tunicamycin diminished transportation of Siglec-15 to the cell membrane and promoted lysosomal degradation of the receptor. Interestingly, this process was regulated by glucose uptake. As we will touch upon later, differences in glucose uptake are observed within the hypoxic TME, so how this affects Siglec expression is an interesting topic for future studies (55).

A few studies have observed soluble Siglec receptors, indicating that receptor shedding could contribute to Siglec expression as well. Ito et al. for instance showed presence of the ectodomain of Siglec-9 in the secretome of serum-free conditioned medium from stem cells derived from human exfoliated deciduous teeth (56). Moreover, soluble Siglec-8 has been observed in serum from patients with different forms of eosinophilia (57). However, the contribution and effect of Siglec receptor shedding in the TME remains to our knowledge largely unstudied.

In summary, recent studies support the notion that Siglec expression on immune cells in the TME is enhanced compared to normal physiology. These changes in Siglec expression may be the result of altered cellular signaling and activation, secreted factors such as EVs and cytokines in the TME, as well as altered expression, glycosylation, internalization, and degradation dynamics of Siglecs themselves. In addition, to upregulation of expression, the enhanced presence of Siglec expressing cells could also be due to preferential recruitment to the TME. More detailed investigation is required to understand how Siglec expression and function is controlled under steady state conditions and within the TME, including the transcription factors and epigenetic mechanisms involved.

SIALOGLYCAN LIGANDS FOR SIGLECS IN THE TME

Both healthy cells and cancer cells generate structurally highly diverse sialoglycans that are displayed on the cell surface and on secreted glycoproteins and glycolipids. Early studies already reported changes in sialoglycan expression during cancer development and found sialoglycans on multiple distinct cell types within the TME, including tumor cells and immune cells (6, 10, 15, 16, 58–60). Sialic acids were described to play a role in tumor progression, for instance by masking antigens on tumor cells to prevent immune cell recognition, by avoiding complement activation, hindering physical interactions with immune cells, and by functioning as ligand for immunosuppressive Siglecs (10). As reviewed extensively elsewhere, there are many different ways employed by tumors to change their glycosylation patterns including sialic acid levels (15, 61–65). In addition to tumor cells, Siglec ligands have been observed in the stromal compartment of the TME. For instance, Siglec-7 and -9 ligands were identified on human bone marrow-derived mesenchymal stromal cells (66). Interestingly, Siglec-9 ligands were higher expressed on cancer-associated fibroblasts than on normal fibroblasts. In mice, Siglec-E ligands were found on mesenchymal stromal cells in the presence of tumor conditioned medium and on aortic endothelial cells after lipopolysaccharide (LPS) treatment (67). Siglec-9 ligands were also expressed on human aorta and HUV-EC-C endothelial cells at high glucose levels and Siglec-10 was shown to bind the endothelial protein VAP-1 (67, 68). However, expression of Siglec ligands on stromal cells needs further confirmation *in vivo*.

Printed arrays of synthetic glycans and cell-based arrays have revealed that the individual Siglec family members have unique and partially overlapping binding specificities for distinct 'healthy' or cancer-associated sialoglycan structures (3, 8, 9, 69–71). However, the binding epitome for all the human Siglecs and ligand dynamics in the TME is of tremendous structural diversity and has not been fully mapped (**Figure 2**). Arguably, the glycosylation changes in the TME that result in specific Siglec binding or potentially abrogate binding events largely determine signaling of inhibitory and activating Siglecs.

It is important to note that the increased sialylation within the TME is mostly demonstrated with plant lectin staining and gene expression data of the enzymes involved in the sialylation pathway. Recombinant Siglec proteins are used to show whether these changes in sialoglycans also lead to more Siglec ligand binding, however, data demonstrating how this translates into more Siglec signaling is lacking or mostly indirect.

Many structural aspects of sialoglycans can contribute to Siglec binding. Here, we will summarize the current knowledge on factors implicated in Siglec preferences for specific sialoglycans and identify factors regulating expression of these sialoglycans within the TME (**Figure 2**).

Siglec Binding Preferences for Sialic Acids

All functionally expressed human Siglecs contain a conserved arginine residue in the V-set domain that forms a salt bridge with the carboxylate group of sialic acids and is essential for sialic acid recognition (1, 4). Sequence diversity between the Siglecs further co-determines their glycan fine binding specificities. The term sialic acids refers to a large family of related sugar derivatives that share the same 9-carbon backbone which can undergo extensive natural modification (6, 72). The four core sialic acids are *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid

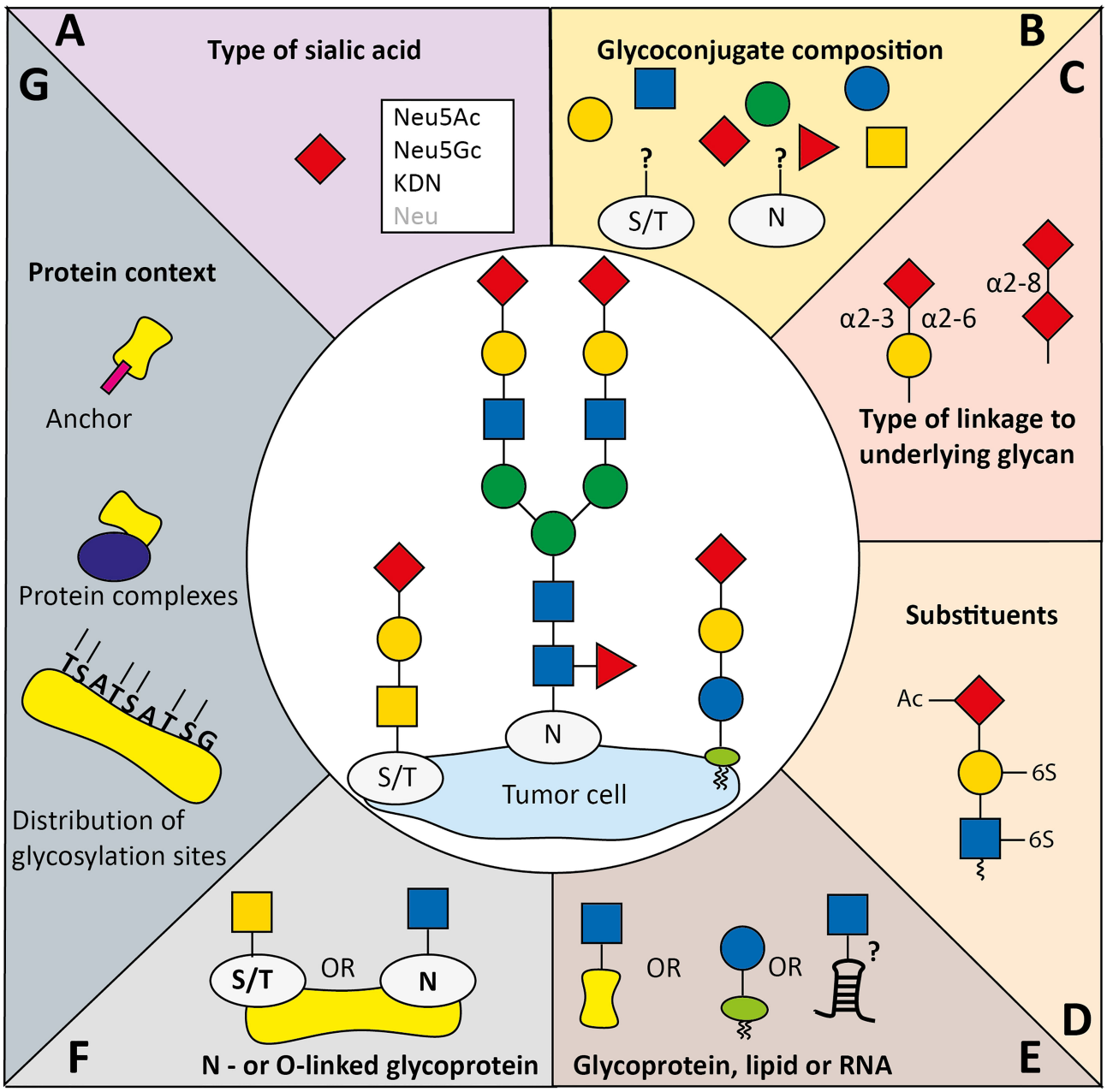

FIGURE 2 | Variables implicated in co-determining Siglec specificity for sialoglycans. Siglecs display specific binding preferences for sialoglycans which is among others determined by **(A)** the type of sialic acid, **(B)** the glycan composition, and **(C)** the type of glycosidic linkage of sialic acid to the penultimate glycan. Siglec binding preferences to sialoglycans can be (co-)determined by **(D)** glycan modifications to the sialic acids or other glycans (Ac: acetylation, 6-S: 6-O-sulfation), **(E)** the glycan carrier backbone which can be lipids, proteins, and potentially RNA, **(F)** the type of protein glycosylation (O-linked to the oxygen atom in serine or threonine, or N-linked to the nitrogen atom in asparagine). Besides, **(G)** the protein backbone, including distribution of glycosylation sites, complexing with other proteins and specific (glycosylated) anchors can provide specific binding context for Siglecs.

(Neu5Gc), 3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN), and neuraminic acid (Neu) which can be further altered by additional modifications (**Figures 2A–D**) (6). Generally human Siglecs prefer Neu5Ac, the most abundant sialic acid in human, but some including human Siglec-2 recognize both Neu5Ac and Neu5Gc with similar affinity (73–76).

Next to the sialic acid core structure, Siglecs feature specific preference for how the sialic acid is linked (α2-3, α2-6, or α2-8 linkages) to underlying glycans. For instance, Siglec-2 has clear preference for α2-6-linkages, Siglec-9 prefers α2-3-linked sialic acid, and Siglec-7 seems to have preference for both α2-3 and α2-8 linkages (3, 9, 69, 71, 77) (**Figure 2C**). Altered expression of

sialyltransferase enzymes during cancer development could change the sialic acid linkage types towards one or the other. For instance, ST6GalNAc-I has been associated with cancer, which leads to α2-6 linked sialyl Tn antigen that has been shown to be recognized by Siglec-7 (9, 78, 79). Siglec-15 has also been suggested to bind Sialyl Tn, although a recent study could not confirm this interaction (9, 26, 80).

Modification of Monosaccharides

Besides the type of the sialic acid core, Siglec binding to its ligands can be influenced by modification of monosaccharides within the sialoglycan (**Figure 2D**) (3, 70). For instance, galactose 6-*O*-sulfation has been identified as critical component for Siglec-3, -5, -8 and -14 binding and 6-O-acetylation was shown to be important for Siglec-9 binding (9, 81–83). Accordingly, expression of the sulfotransferase CHST1 in HEK293 cells that installs a 6-*O*-sulfo group to galactose induced binding of recombinant Siglec-3, -8, and -15 and largely enhanced Siglec-7 binding (9, 82). In line with this, several studies showed that Siglec-7 binds the carbohydrate sialyl 6-sulfo Lewis^x that is expressed by non-transformed colonic cells, rather than sialyl Lewis^x, which is expressed by tumor cells (23, 84, 85). This could result in decreased Siglec activation and concomitant enhanced inflammation (23). It was demonstrated that Siglec-7 on macrophages exerts an immunosuppressive effect upon ligand binding. Besides sulfation, other modifications have also been described to affect Siglec binding. For instance, a study that investigated binding of recombinant Siglec Fc chimeras to biotinylated polyacrylamide probes that were conjugated to sialylated glycans found that fucosylation of sialoglycans can reduce Siglec binding (86). Furthermore, sialic acid O-acetylation has been shown to generally abrogate Siglec binding to sialoglycans (86, 87). Using CRISPR-Cas9-mediated knockouts of Sialic acid acetylesterase and Sialic acid acetyltransferase, a recent study by Grabenstein et al. showed that Siglec-7, -9, -10 and -11 binding to HCT 116 colon cancer cells and A549 lung cancer cells was decreased upon knock out of the Sialic acid acetylesterase (88). Furthermore, knock out of Sialic acid acetylesterase resulted in enhanced NK cell-mediated cytotoxicity in colon and lung cancer cells.

These studies suggest that glycan modifications such as sulfation and acetylation and the regulation of expression and activity of glycan modifying enzymes in the TME can have a strong impact on Siglec interactions with sialoglycans. Further studies into the presence of these modification in the TME compared to healthy tissue and effects on Siglec signaling could reveal a major role for glycan modifications in the tumor immune response.

Influence of Protein/Lipid Context on Siglec Binding

Sialoglycans are attached to different backbones, such as proteins/peptides (either N-linked to asparagine or O-linked to serine or threonine) or lipids (89, 90). Recently Flynn et al. provided evidence that sialoglycans can be attached to conserved small noncoding RNAs (7) (**Figures 2E, F**). Cell surface glycoRNAs were proposed to specifically interact with Siglec-11 and -14, but not with Siglec-5 which shares the same V-set domain sequence with Siglec-14 (7, 91). Siglecs can harbor a very distinct binding preferences for N- and O-linked sialoglycans which is presumably mediated by the variable Siglec C-C' loop (3, 4, 9, 59, 70, 71). Ligand interactions with Siglec-7, and probably the other Siglecs, induced conformational changes in the sialic acid binding site and in the C-C' loop resulting in further interactions with the glycosphingolipid core region itself, indicating that the lipid context is of importance for Siglec-7 binding (92, 93). Furthermore, Siglec-7 binds the two linked sialic acid residues (Neu5Acα2,8Neu5Acα) and most of the other sugar moieties of a GT1b analog, demonstrating that not only the sialic acid monomer is important for binding, but the underlying glycan as well (93). More Siglec preferences have extensively been reviewed elsewhere (3, 8). Thus, Siglec binding is determined by both the context of the protein or lipid backbone and the structure of the accompanied glycans.

The hypothesis that the protein context is important for Siglec binding can be demonstrated by two specific ligands, CD24 and CD52, that were identified for Siglec-10 and which are highly similar in structure (94–96). Both are relatively small glycoproteins, consisting of 31-35 (CD24) or 12 amino acids (CD52) (97–99). They both harbor a glycosylphosphatidylinositol anchor (GPI anchor) and can be released from the cell membrane by the action of phospholipases. Interestingly, the GPI anchor, consisting of a phospholipid tail, a glycan core and a phosphoetanolamine linker to which the protein is attached, contains a glycolipid that can be sialylated as well (100–102). Therefore, CD24 and CD52 are glycoproteins attached to a glycolipid, which makes their context highly interesting for future studies. CD24 and CD52 have been shown to complex with HMGB1, adding even an additional layer of complexity, as binding of Siglec-10 to CD52 has even been shown to be enhanced by HGMB1 (95, 103, 104). Therefore, the context of the glycoprotein can cause binding by third proteins that influence Siglec binding. Both CD24 and CD52 have been shown to inhibit macrophage and T cell immune cell function through Siglec-10 binding (94, 96, 103). Studies showed that the interaction affects a diverse set of processes, such as phagocytosis by TAMs, Lck and ZAP-70 phosphorylation in $CD4^+$ T cells and cytokine secretion by activated T cells.

In summary, the protein/lipid context of a Siglec ligand can be of importance for Siglec binding. At least, we hypothesize that the protein backbone can provide specific binding context for Siglecs (**Figure 2G**) and multivalency for high avidity binding can be created by dense sialoglycan presentation on a protein backbone or *via* dense glycan clusters at the cell membrane, which we will touch upon later in more detail.

Regulation of Siglec Ligands

A remaining knowledge gap is how expression of specific Siglec ligands is regulated within the TME. Multiple factors might play a role here. For instance, the hypoxic environment could influence glycan composition. Human cancer-associated gangliosides have been observed to incorporate more Neu5Gc into their glycans than non-transformed cells (105). Gangliosides are a special class of glycosphingolipids – glycans attached to a

ceramide anchor - that contain at least one sialic acid residue (106). Due to a deletion in the *Cmah* gene, humans are incapable of synthesizing Neu5Gc and are dependent on dietary Neu5Gc (107–109). The increase in ganglioside associated Neu5Gc was suggested to be associated with the hypoxic environment of tumors. Potentially, Siglec binding or loss of binding due to Neu5Gc incorporation can alter signaling in the TME.

Interestingly, hypoxia was shown to induce transcription of the sialic acid transporter Sialin (SLC17A5), which mediates transport of external sialic acid into the cell (110). Moreover, hypoxia was reported to influence expression of sialyltransferases (111, 112). Therefore, Siglec binding could be influenced by the hypoxic environment by adjustment of the main type of sialic acid that is incorporated in sialoglycans, but future studies should elucidate how this exactly affects Siglec activation. Besides gene expression, also enzyme activity of for example specific sialyltransferases could influence incorporation of the type of sialic acid, although additional research is required to further study enzyme activity within the TME and its influence on Siglec activation.

The shift in metabolism that is accompanied with malignant transformation adds an additional layer to regulation of glycosylation in the TME (113). HIF-1α is a key player in regulating cellular energetics within the hypoxic TME as it causes enhanced glucose uptake, which is not only required to generate ATP *via* various biosynthetic pathways, but it is also an important glycosylation precursor (55, 114, 115). HIF-1α suppresses the tricarboxylic acid cycle, ultimately preventing generation of UDP-N-Acetylglucosamine (UDP-GlcNAc), which is required for N-glycan branching (116, 117). Besides, hypoxia also influences sialylation, as it activates biosynthesis of CMP-NeuAc nucleotides through conversion of UDP-GlcNAc to ManNAc by GNE (118). This eventually results in enhanced overall cell surface sialylation. Overall, the changed metabolism that is observed in the TME is intertwined with aberrant glycosylation patterns, and future research should elucidate how this specifically affects Siglec signaling within this hypoxic TME.

Tumor cells have been demonstrated to release EVs able to modulate immune cell activation in the TME (119). Recently, the presence of Siglec ligands on EVs has been shown, which could be an additional manner in which tumor cells influence Siglec ligand expression within the TME (**Figure 3A**) (120). For example, Dusoswa et al. showed that isolated EVs (including exosomes and small membrane budded vesicles) of glioblastoma cell lines specifically express ligands for Siglec-9 (120). Moreover, exosomes derived from ovarian ascites fluid from cancer patients express GD3 on their surface (121). Shenoy et al. reported that expression of GD3 on liposomes inhibits T cell activation in a sialic acid-dependent way. GD3 has been reported to be a ligand of Siglec-7, which is expressed on subsets of T cells, and might be activated by GD3 positive exosomes (122–125). These data imply that T cell inhibition by GD3 can at least in part be Siglec-mediated, but this finding needs further experimental confirmation.

Another interaction with exosomes has been reported for Siglec-1 that is expressed on macrophages and dendritic cells. Siglec-1 is an atypical Siglec, as it has no intracellular signaling motif and has 16 C2 Ig domains that extend the V-set domain far away from the cell membrane (1). Although Siglec-1 does not have intracellular motifs and is mostly described in uptake of α2-3-sialylated proteins or particles, multiple studies have shown that Siglec-1^+ macrophages exert immunosuppressive effects (43, 46, 126, 127). Functionally, Siglec-1 expression on macrophages has been described to be important for antigen transfer to dendritic cells or T cells (43, 46, 127). An *in vivo* mouse study demonstrated that Siglec-1^+ macrophages in tumor-draining lymph nodes bind tumor-derived EVs to physically block further dissemination and subsequent lymphocyte activation (128). Black et al. reported that Siglec-1 also binds a special type of EVs, apoptotic bodies, derived from the EL4 lymphoma cell line (129). Importantly, they showed *in vivo* that *siglec*-$1^{-/-}$ mice had an increased cytotoxic T cell response to apoptotic vesicles displaying ovalbumin protein than wild type C57BL/6. Thus, the data show that Siglec-1 activation could be enhanced by tumor cells secreting EVs displaying Siglec-1 ligands. However, it remains unknown whether Siglec-1 ligands are actively incorporated into membranes of EVs, or whether this is a passive process. Moreover, many factors regarding Siglec signaling induced by tumor-derived EVs remain unknown, such as the strength and kinetics of the activation and how these factors compare to Siglec activation induced by ligands on tumor cells. Furthermore, systemic transport of tumor-derived EVs and the resulting effect of these on Siglec activation outside the TME also requires further research, as well as the comparison between EVs derived from tumors and from healthy tissue.

In summary, Siglec ligand expression within the TME can be affected by various factors, such as hypoxia, its associated change in metabolism and the secretion of Siglec ligand-containing EVs. However, additional research is required to elucidate the contribution of these factors and possible others that have yet to be determined.

SIGLEC CLUSTERING AND MULTIVALENT LIGANDS

Immune inhibitory Siglec signaling requires a few steps that are similar to induction of signaling by PD-1. First of all, PD-1 receptors have been described to require clustering to initiate signaling and literature now supports the idea that Siglecs require clustering as well (**Figure 3D**) (3, 130). Inhibitory Siglecs harbor ITIM domains like the PD-1 receptors to which kinases get recruited upon clustering and activation to phosphorylate the ITIM domains (1, 131). Research has previously shown that intracellular kinases can locally accumulate at receptor clusters, which might also be the case for Siglec receptors (132). Lastly, SHP-1/2 phosphatases are recruited intracellularly to the clusters and these will cause dephosphorylation of downstream molecules to inhibit immune cell activation (133). Localization of the clusters of PD-1 receptors to T cell receptors (TCRs) enhances efficiency of PD-1 mediated suppression, and this might also be true for Siglecs and their downstream targets (130). Here, we will discuss the current evidence for Siglec

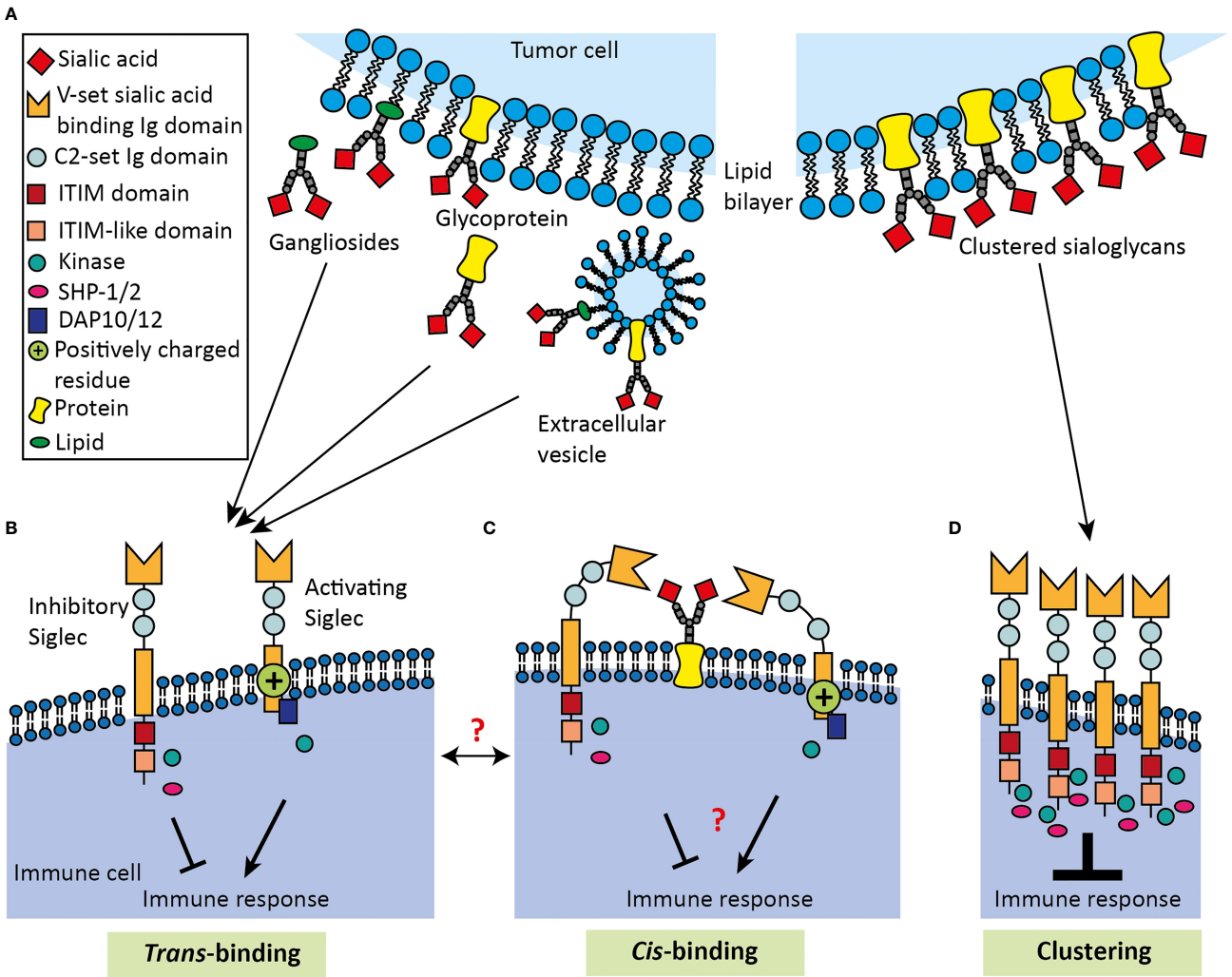

FIGURE 3 | Schematic overview of Siglec binding to *cis*- and *trans*-ligands. **(A)** Tumor derived Siglec *trans*-ligands comprise glycolipids and glycoproteins, which can either be membrane-bound, secreted or present on EVs. Inhibitory and activating Siglecs can bind ligands either in **(B)** *trans*-fashion or in **(C)** *cis*-fashion. Siglecs bind their ligand using the V-set sialic acid-binding Ig domain, which is positioned away from the membrane by a variable number of C2-set Ig domains. Siglecs can bear ITIM and ITIM-like domains, which lead to inhibition of the immune response. In contrast, activating Siglecs can carry a positively charged residue in the transmembrane domain, which can associate with DAP10/12 activating transmembrane adaptor proteins to activate the immune response. *Cis*-ligand interactions probably contribute to immune homeostasis, but remain largely unknown and the dynamics of Siglecs binding in *trans* or *cis* mode requires more investigation. **(D)** Multivalent ligands are capable of clustering Siglecs to induce signaling, causing an intracellular accumulation of signaling molecules that eventually results in enhanced immune modulation.

clustering and that multivalent ligands can be bound by clusters of Siglec receptors.

Siglec Clustering

It has been observed that Siglecs can form foci (such as Siglec-E on neutrophils) or accumulate at cell-cell contact sites, like the Siglec-9 accumulation on neutrophils that was observed at contact sites with LS180 and A549 carcinoma cells (60, 134). Furthermore, several studies used antibodies to cross-link Siglecs on the cell membrane to induce a functional effect (135–138). A typical example that demonstrates the relevance of Siglec clustering is given by Siglec-2. Siglec-2 binds *cis*-ligands resulting in cluster formation that inhibits B cell receptor signaling, which we will discuss in more detail in the section Siglec binding to *cis*-ligands (139–142). Another example was given by Ikehara et al., who transfected Jurkat T cells with Siglec-7 or -9 and observed clustering and partial co-localization of these receptors with CD3 of the TCR (125). Subsequently, they found that Siglec expression leads to reduced phosphorylation of molecules downstream of the TCR following TCR engagement.

Multivalent Siglec Ligands

The monovalent binding affinity of a Siglec for a sialoglycan ligand is relatively low (K_d of 100-300 μM) (4, 8, 59). Multivalent

interactions, the simultaneous binding between clustered receptors and multimeric ligands, increases the binding affinity between Siglecs and sialoglycans as has been shown for Siglec-2 clusters (143–149). For instance, lipid rafts that contain many gangliosides can be seen as a multivalent Siglec ligand. Accordingly, Nicoll et al. reported clustering of unmasked Siglec-7 on NK cells at the contact site with ganglioside GD3-synthase transfected P815 cells (150).

Multivalent Siglec ligands can be formed at the cell surface through clustering of individual sialoglycoproteins or lipids or are formed by densely glycosylated protein such as the mucins (151–155). Mucins are a family of large secreted and membrane-bound glycoproteins consisting for up to 60% of tandem repeat domains formed by repeating serine, threonine and proline sequences (156, 157). Both serine and threonine are acceptors of O-GalNAc-type glycosylation resulting in dense glycosylation of mucins that can account for up to 80% of their total mass. Gel-forming mucins such as MUC2 and MUC5AC form a barrier at the epithelial surfaces and house the microbiome, and membrane-bound mucins have many functions in cell signaling and cellular interactions (157).

CD43 is another example of multivalent Siglec ligand (158, 159). Wisnovsky et al. discovered CD43 as a multivalent ligand for Siglec-7 on the K562 chronic myeloid leukemia cell line using a genome-wide CRISPR screen (158). CD43, or sialophorin, is a mucin-type protein harboring a heavily O-glycosylated extracellular domain (160, 161). This study showed that CD43 can relocalize Siglec-7 on NK cells to the immunological synapse with the K562 cells. In parallel, Yoshimura et al. identified CD43 on K562 cells as Siglec-7 binding partner using biochemical techniques (159).

In summary, binding of Siglecs to their ligands can be strengthened by multivalent interactions and in line with this, several multivalent ligands for Siglecs have been identified.

Multivalent Siglec Ligands Present in the TME

Mucins are overexpressed and aberrantly glycosylated in many types of cancer (162, 163). Several studies have reported Siglec binding to (cancer-associated) mucins suggesting that they can mediate communication between the epithelial surface and the immune system and that they could alter the anti-tumor immune response (151–154). For instance, MUC1 that is overexpressed in many cancer types has been reported to interact with Siglec-1 and Siglec-4, mediating adhesion between Siglec-4 expressing Schwann cells and MUC1 expressing pancreatic cells (152, 154). Beatson et al. reported that the cancer-associated sialyl T glycoform of MUC1 binds with Siglec-9 on myeloid cells and that this interaction can induce a tumor-associated macrophage phenotype (151, 164). Sialyl T-MUC1 instructed macrophages secrete soluble factors (IL-6, M-CSF, PAI-1) associated with tumor progression and display enhanced levels of the inhibitory receptor PD-L1 (151, 164). Moreover, these mucins enabled tumor-associated macrophages to inhibit T cell proliferation. Recently, the interaction of the human Siglecs with a large panel of recombinant mucins and mucin-like proteins decorated with defined O-GalNAc-type glycans was dissected (9). Although Siglec-9 interactions with sialyl T-MUC1 were not confirmed, this study revealed Siglec-4, -7, and -15 interactions with mucins that were largely determined by the mucin O-glycan pattern and type.

Another multivalent Siglec ligand that has been found on tumor cells or secreted in the TME is LGALS3BP (Mac-2 binding protein) (165, 166). LGALS3BP is a heavily N-glycosylated protein and Siglec-5, -9, and -10 have been shown to bind it (166). Importantly, recombinant LGALS3BP was demonstrated to inhibit neutrophil activation and siRNA-mediated reduction of LGALS3BP expression in HT-29 colon cancer cells increased neutrophil-induced apoptosis of the HT-29 cells (166). Interestingly, Koths et al. have shown before that LGALS3BP can form multimeric complexes (167). These data suggest a role for complex formation of Siglec ligands to offer a multivalent Siglec ligand to activate Siglec receptors.

Altogether, Siglecs can accumulate at the cell surface to induce a functional effect. Multivalent ligands increase binding affinity between Siglecs and sialoglycans. Classic example of tumor associated multivalent Siglec ligands are mucins. Smaller Siglec ligands, such as CD24 for Siglec-10, however, have also been identified in the TME that are less likely to act as multivalent ligand (106–108). Potentially, clustering of such ligands within the cell membrane could result in local presentation of Siglec ligands in a multivalent manner. In the same way, glycolipids could possibly organize in lipid rafts to activate Siglecs on the same cell (*cis*) as well as on opposing cells (*trans*) (59).

It would be interesting to study whether there is a critical amount of specific interactions and/or Siglec clustering that is required to trigger Siglec signaling. Also, comparison of the strength and kinetics of Siglec activation by ligands that either offer a multivalent or monovalent ligand would contribute to the current knowledge on Siglec activation by multivalent ligands and would aid in the rational development of Siglec targeting therapeutics. We hypothesize that the multivalency of Siglec ligands can have multiple functions, for example to induce Siglec clustering and thereby have a functional effect or to outcompete *cis*-ligands with a lower affinity. Nevertheless, more research is required to study these hypotheses and elucidate whether and how monovalent ligands can activate Siglecs.

THE FUNCTION OF SIGLEC BINDING TO *CIS*-LIGANDS

In order for Siglecs to interact with *trans*-ligands (**Figure 3B**), for instance on tumor cells, Siglecs have to be available for binding. Cells expressing Siglecs also express themselves sialoglycan ligands on their membrane that can interact in *cis* thereby 'masking' the V-set binding site (59) (**Figure 3C**). This has been shown for most Siglecs and potentially lowers the threshold for Siglec signaling by *trans*-interactions (45, 150, 168–172). Naturally, masking interactions are overcome by the dynamic on/off binding of Siglecs with their ligands, in particular for lower (monovalent) affinity ligands, that enables binding to higher affinity ligands (1, 4). Noteworthy, masking can also be

abolished by sialidases or experimentally by treatment with sialylation inhibitors (17, 173). Furthermore, the C2-set domain repeats allows orientation of the V-set domain away from *cis*-ligands which likely contributes to recognition of *trans*-ligands. The 16 C2 Ig domains that orient the V-set domain of Siglec-1 into the extracellular space were suggested to enable Siglec-1 to mediate intercellular interactions. Still, interactions of Siglec-1 with cis-ligands has been observed (168, 174). However, it remains unknown whether *cis*-ligands only mask Siglec-1 or also activate the receptor. Variation in the C2-set repeat numbers between the Siglecs possibly determines the sensitivity of individual family members for the *cis*- and *trans*-ligands and also endogenous membrane and secreted sialidases (e.g. NEU1, NEU3).

Glycosylation changes in the cell have also been suggested to regulate Siglec binding dynamics for *cis*- and *trans*-ligands (169, 175). Masking/unmasking dynamics under physiological conditions and particularly in the TME and consequences for signaling and immune regulation is likely of key importance in Siglec biology.

Effect of Siglecs Binding to *Cis*-Ligands

One of the main knowledge gaps regarding *cis*-ligands is whether Siglecs can only be activated by *trans*- ligands or also by *cis*-ligands. The best studied Siglec receptor in terms of *cis*-interactions is Siglec-2, which is a receptor intracellularly harboring multiple ITIMs, an ITIM-like domain and a Grb2 binding motif (1, 176). Siglec-2 and B cell receptor (BCR) are present on the membrane as clusters that partially overlap (139). Co-clustering was found to be highly important for Siglec-2-mediated inhibition of BCR signaling as increased clustering of Siglec-2 with the BCR inhibits BCR signaling (139, 177). Siglec-2 is scavenged away from the BCR by *cis*-ligands on neighboring sialylated Siglec-2 receptors present on the same cell membrane leading to enhanced BCR activity (139–142). Collins et al. demonstrated that Siglec-2 on B cells can redistribute to the sites of cell contact with other lymphocytes, despite the presence of *cis*-ligands (178). This indicates that clustered patterns of Siglecs have to be tightly regulated and are affected by both *cis*- and *trans*-ligands (140, 141).

Human Siglec-2 has a high affinity for Neu5Ac as sialic acid and a 6-O-sulfate on the GlcNAc in the underlying glycan (175, 179). When human B cells are activated, the 6-O-sulfotransferases needed for the Siglec-2 ligands are downregulated. This results in a decrease in interactions between Siglec-2 and *cis*-ligands and therefore Siglec-2 becomes available to inhibit the BCR. Accordingly, *in vivo* mouse studies established that mice that lack the α2-6 sialyltransferase *St6gal1* and mice with a mutation of the critical Arginine in the V-set domain of Siglec-2 both showed enhanced BCR inhibition. Murine Siglec-2 on B cells has a similar mechanism to inhibit BCR signaling. Murine Siglec-2 has a preference for Neu5Gc, rather than Neu5Ac (180). It is unmasked by downregulation of the hydroxylase that is responsible for the conversion of Neu5Ac to Neu5Gc, eventually resulting in a reduction of Siglec-2-*cis*-interactions and enhanced BCR inhibition (175, 181). Nevertheless, despite the ability of *cis*-ligands to prevent Siglec-2 to inhibit BCR signaling, Siglec-2 is always able to bind *trans*-ligands on an opposing cell (178, 182, 183). This interaction causes Siglec-2 to translocate to the immunological synapse, where it can inhibit BCR signaling (184, 185).

A recent study by Ballet et al. (2021) has further established Siglec-2 signaling on B cells induced by *cis*-ligands (186). Here, Siglec-2 was shown to associate with β_7 integrin in a sialic acid-dependent manner. Next, recruitment of SHP-1 to Siglec-2 was demonstrated to inhibit β_7 integrin endocytosis and restrain β_7 integrin phosphorylation at the cell surface. The β_7 integrin complexes with the α_4 integrin, and this complex is involved in homing B lymphocytes to gut-associated lymphoid tissue, where B cells are being activated. Indeed, the Siglec-2-SHP-1 axis was shown to enhance B cell homing to the gut-associated lymphoid tissue.

Another study on Siglec signaling induced by *cis*-ligands has recently been performed by Delaveris et al. (2021) (187). Applying synthetic lipid-tethered glycopolypeptides that inserted into cell membranes in combination with Förster resonance energy transfer analysis revealed binding between Siglec-9 and glycopolypeptides in *cis*. Strikingly, Siglec-9 binding to ligands in *cis* reduced LPS-mediated MAPK signaling, cytokine secretion and phagocytosis in macrophages, while the same ligand used in *trans* did not. Of note, these studies on Siglec interactions with *cis*-ligands have not been performed within the context of the TME. Activation of Siglecs by *cis*-ligands in the TME therefore remains to be elucidated.

Relevance of *Cis*-Ligands in the TME

Although it has been established that Siglecs are masked by *cis*-interactions, for many Siglecs the biological role of these interactions is poorly understood. Varki and Angata (2006) proposed that *cis*-interactions set a threshold for recognition of *trans*-ligands with higher affinity or to monitor sialylation on the own cell surface (188). C*is*-interactions could have a gatekeeper function blocking insubstantial interactions and signaling enabling sensing of high affinity or avidity Siglec ligands that lead to a biologically relevant signaling outcome. C*is*-interactions, on the other hand, may exert baseline signaling which in case of the inhibitory Siglec members may support a resting state or return to a resting state after activation. Understanding the physiological relevance of Siglec *cis*-interactions and possible signaling requires further investigation. Likewise, the relevance of Siglec *cis*-interactions in the TME is largely unknown.

It is conceivable that tumor-associated *trans*-ligands outcompete *cis*-ligands either due to higher affinity or avidity for instance. The malignant transformation could potentially guide expression of specific glycan/glycoprotein or higher expression levels and clustering of *trans*-ligands. Altered biosynthesis, or degradation of *cis*-ligands on Siglec-expressing immune cells upon infiltration into the TME could further increase Siglec activation on immune cells upon encounter with tumor cells. For instance, it has been reported that T cell activation can result in a loss of sialylation on core 1-O glycans (189–191). Similarly, IL-2 stimulation of NK the cell line NK-92 reduced gene expression of the sialyltransferases ST8SIA1, ST6GAL1, and ST3GAL1, but increased overall α2-6-linked sialic acids and

poly sialic acid likely due to an increase in the expression of poly sialic acid carrier molecule NCAM/CD56 (192). Occurrence of such alterations in glycosyltransferase and sialyltransferase expression in the TME, but also sialidase expression or changes in nucleotide sugar metabolism could thus alter *cis*-ligand expression. Together with the observed changes in immune cell phenotype like the enhanced Siglec expression on T cells and macrophages in response to factors in the TME, this would enable strong *trans*-interactions with sialoglycans on tumor cells and modulate the immune cell function (11, 28, 94). Clearly, multiple aspects need further investigation to understand how Siglec *cis*-interactions in the TME are altered and the biological effects thereof.

Challenges for the Study of *Trans*- and *Cis*- Siglec Interaction Dynamics

The dynamics of Siglec binding to *trans*- and *cis*-ligands is emerging as a key event in Siglec biology determining their clustering, signaling and biological effects. Studying these dynamics, however, is challenging. To study *cis*-binding-mediated Siglec activation, studies have installed lipid-conjugated glycopolypeptides into cell membranes (187, 193, 194). Similarly, to study *trans*-binding-mediated Siglec activation, Siglec-ligand containing liposomes have been used (195–197). However, addition of Siglec ligands in such manners requires careful interpretation, as this can lead to overexpression of unnatural sialic acids that can be recognized by Siglecs either in *cis* or *trans* and does not represent a natural situation. Besides, novel tools have been developed to remove sialic acids from the cell membrane to assess Siglec binding in either *cis* or *trans* manner. Sialic acid removal from the cell membrane can be established using sialidase or sialic acid mimetics that can enter the cell to inhibit sialyltransferases and thereby prevent transfer of sialic acid onto glycan chains (4, 173).

Nevertheless, altering cellular sialylation by different means can co-affect other factors. For instance, a recent study by Edgar et al. has provided an example that abrogation of sialic acid expression can result in Siglec-independent effects (198). They have shown that the costimulatory molecule CD28 on T cells can bind to sialoglycans in *cis* and in *trans*. The interactions of CD28 with *cis*-ligands limited binding of CD28 to its *trans* protein ligands CD80 and CD86 on antigen-presenting cells thereby negatively affecting co-stimulation. This example illustrates that also the function of non-Siglec receptors can be affected by abrogation of sialic acid expression. Moreover, removal of sialic acids will expose galactose, which can be recognized by galectins, which can modulate the immune response and affect tumor development (199). Similar mechanisms may be in place for other sialoglycan binding receptors, such as factor H and selectins (6). Furthermore, removal of the negatively charged sialic acid molecules could affect total charge of the cell and might therefore affect cellular interactions. Indeed, it was demonstrated that diminished sialic acid expression caused enhanced tumor cell killing by cytotoxic $CD8^+$ T cells, which was explained at least in part by increased clustering of the T cells with tumor cells (200).

Additionally, studies have genetically manipulated the glycosylation machinery to modify sialylation, for example *via* genetic glycoengineering with inducible sialyltransferase expression to tune Siglec ligand expression (11, 201–203). However, this might also affect other processes, as Kohnz et al. have found that knockdown of cytidine monophosphate N-acetylneuraminic acid synthase, an enzyme required for sialic acid activation to be incorporated in glycans, affected levels of more than 200 other gene transcripts including oncogenes (204).

Overall, results have to be carefully interpreted when interrupting Siglec ligands, as these type of experiments are likely to have various side effects.

CONCLUDING REMARKS

The Siglec-sialoglycan axis emerges as important regulator of immune cell-tumor cell interactions in the TME that co-determines the outcome of tumor immunity (17). Qualitative and quantitative changes in Siglec ligand expression as well as Siglec receptors on immune cells in the TME have been linked to immune evasion. However, unraveling the biological effects and consequences of Siglec signaling in the TME remains challenging for several reasons. First, healthy cells and cancer cells can produce a highly diverse Siglec interactome that is formed by the sialoglycan structures, their modifications, their display on particular glycoproteins and glycolipids as well as multivalent higher order binding patterns created by protein glycosylation density and ligand clustering. Deducing the specific and biological meaningful *cis*- and *trans*-interactions of each of the individual Siglec family member is currently ongoing aided by recent technological advances with recombinant (multimeric) Siglec probes, proximity labeling approaches, cell-based glycan arrays, and genome-wide CRISPR screens (9, 22, 158, 173, 205).

Second, the expression patterns and membrane organization of most Siglecs on immune cells in the periphery and the TME (and other tissues) are not well understood. Siglec-2 is the best studied Siglec in that context and shows the importance of *cis*-interactions in tuning B cell receptor activation. Siglec-2 interactions with high avidity ligands in *trans* and downregulation of *cis*-ligands result in clustering around the B cell receptor and potent inhibitory signaling sufficient to block B cell activation (139–142). The membrane organization and interaction partners, recruitment to the membrane, internalization and recycling kinetics of the other Siglecs are less well understood, but should move more into focus as they likely determine their individual signaling modes.

Third, still relatively little is known regarding the signaling of Siglecs and the downstream molecular targets and effects. The immune inhibitory Siglecs that recruit SHP-1 or SHP-2 after ITIM phosphorylation are best described, but the molecular and cellular consequences of their signaling are not fully understood. Likewise, signaling of the activating Siglecs-14, -15, and -16 *via* adaptor proteins needs further dissection as well as other potential signaling residues (Grb2, Fyn kinase sites). Next to understanding the consequences of Siglec signaling and their

integration with other immune signaling pathways, systems to determine whether ligand binding equals signaling are required. Studies with Siglec-Fc chimeras inform on binding, but this does not necessarily correlate with signaling (22). Suematsu et al. developed a reporter system for direct measurement of Siglec activation using a receptor consisting of the extracellular Siglec domains and the transmembrane and intracellular domains of the CD3ζ chain (206). Such cell-based assays could allow direct measurement of Siglec activation in response to a ligand, measure effects of signaling kinetics with different (multivalent) ligands, and shed light into signaling through *trans*- and *cis*-ligand binding dynamics.

Finally, further insight into general aspects of Siglec biology is needed to advance our currently limited understanding of inhibiting and activating Siglec signaling in the TME and the role that hypoxia, altered metabolism and cytokines play in regulating *cis*/*trans*-Siglec ligand and Siglec expression that guide cell-cell communication in the TME. The identification of Siglecs as important immune checkpoints in the TME implies that blocking monoclonal antibodies or other strategies to abolish Siglec-sialic acid interactions and signaling can boost anti-tumor immunity (16, 17, 28, 94). Further research into the diverse Siglec signaling modes in the TME may turn out to be highly awarding to uncover the impact of therapeutic Siglec targeting on the efficacy of cancer immunotherapy.

AUTHOR CONTRIBUTIONS

EH designed and wrote the review, CB edited the review and LC and GA designed, supervised and edited the review. All authors contributed to and approved the submitted version.

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A Novel Strategy to Identify Haematology Patients at High Risk of Developing Aspergillosis

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Invasive Aspergillosis (IA), typically caused by the fungus *Aspergillus fumigatus*, is a leading cause of morbidity and mortality in immunocompromised patients. IA remains a significant burden in haematology patients, despite improvements in the diagnosis and treatment of *Aspergillus* infection. Diagnosing IA is challenging, requiring multiple factors to classify patients into possible, probable and proven IA cohorts. Given the low incidence of IA, using negative results as exclusion criteria is optimal. However, frequent false positives and severe IA mortality rates in haematology patients have led to the empirical use of toxic, drug-interactive and often ineffective anti-fungal therapeutics. Improvements in IA diagnosis are needed to reduce unnecessary anti-fungal therapy. Early IA diagnosis is vital for positive patient outcomes; therefore, a pre-emptive approach is required. In this study, we examined the sequence and expression of four C-type Lectin-like receptors (Dectin-1, Dectin-2, Mincle, Mcl) from 42 haematology patients and investigated each patient's anti-*Aspergillus* immune response (IL-6, TNF). Correlation analysis revealed novel IA disease risk factors which we used to develop a pre-emptive patient stratification protocol to identify haematopoietic stem cell transplant patients at high and low risk of developing IA. This stratification protocol has the potential to enhance the identification of high-risk patients whilst reducing unnecessary treatment, minimizing the development of anti-fungal resistance, and prioritising primary disease treatment for low-risk patients.

Keywords: CLR, *Aspergillus*, aspergillosis, fungal immunology, host-pathogen interactions

INTRODUCTION

Invasive aspergillosis (IA) has become a leading cause of death among immunocompromised patients (1–3). The disease, mainly caused by *Aspergillus fumigatus*, affects ~ 10% of allogeneic stem cell transplant (SCT) patients and ~6% of acute myeloid leukaemia (AML) patients. IA is associated with unacceptably high mortality rates ranging from 30-40% in AML patients and 50-90% in SCT patients

(4–9). IA is particularly prevalent in patients with haematologic malignancies. This is attributed to the profound immune suppression and neutropenia brought about by the extensive therapeutic use of cytotoxic chemotherapies, radiation therapy, requirement for SCT and the use of corticosteroids and immunomodulatory therapies (10). Whilst the diagnosis and treatment of A*spergillus* infections is improving, severe IA-associated morbidity and mortality in haematology patients has led to the widespread, empirical use of anti-fungal prophylaxis in this patient group (11, 12). Current anti-fungal therapeutics can be ineffective, encounter resistance, are poorly tolerated and highly drug interactive, often impacting patient's primary cancer therapies (13).

Improvements in IA diagnostics and the identification of novel risk factors are required to stratify patients prior to infection and enhance the early diagnosis of IA, thus providing a personalised medicine approach that better targets anti-fungal therapy. Assessing a patient's IA risk and then accurately and rapidly diagnosing IA remains challenging. Initially, a patient's underlying clinical conditions will govern IA risk, with patients being grouped into low, medium and high risk cohorts (14). Patients are classified with possible IA, probable IA and proven IA through the presence of host factors, and clinical and microbiological evidence. However, proven IA is often only confirmed post-mortem. A range of routine mycological investigations are available, both novel (*Aspergillus* PCR, galactomannan enzyme immunoassay, 1-3-β-D-glucan detection) and conventional (culture and microscopy), and are usually combined with radiology typical of IA. However, the availability of these tests varies considerably, they can produce false positive results, some lack sensitivity, are not always *Aspergillus* specific, are impacted by anti-fungal therapies, and can require invasive sampling (15, 16). Therefore, an improved strategy to promote the rapid and accurate diagnosis of IA is required.

The accurate, early diagnosis of *Aspergillus* infection is vital for positive patient prognosis (17). Whilst the development of more sensitive assays has improved IA diagnosis, the identification of novel risk factors that increase IA susceptibility is central to promoting a personalised medicine approach to anti-fungal investigations and treatment. Multiple risk-factors for IA have been identified, many of which are associated with the haematology patient population. Neutropenia was the first described IA risk factor and is frequently encountered in haematology patients following primary disease treatment (18). Since then, allogeneic stem cell transplantation, graft versus host disease and respiratory infection have been associated with increased IA susceptibility (19–21). Whilst the identification of these risk factors is associated with a higher prevalence of IA in haematology patients, they do not permit a pre-emptive personalised medicine approach as these risk factors are common and often unavoidable.

The identification of genetic risk factors that increase fungal susceptibility has promoted a pre-emptive approach to determining haematology patient IA risk. These genetic risk factors can be routinely screened for and used to inform a patient's anti-fungal investigations and therapies (22). Genetic mutations that increase fungal susceptibility have been found in innate and adaptive anti-fungal immune components. One of the most important facets of anti-fungal immunity is the C-type Lectin-like Receptor (CLR) family. CLRs are pattern recognition receptors that recognise pathogen associated molecular patterns, specifically carbohydrate structures present in the fungal cell wall (23). CLRs such as Dectin-1 and Dectin-2 are essential for anti-fungal immunity and recognise most, if not all, fungal species that cause human disease (24, 25). Deficiencies in these CLRs have been associated with increased susceptibility to invasive fungal infection (26–28). Upon fungal recognition CLRs induce intracellular signalling and drive the production of cytokines and chemokines, phagocytosis, and respiratory burst (26, 29). Additionally, CLRs have been shown to mediate protective Th1 and Th17 immunity during systemic and mucosal fungal infection (30, 31). Directly determining an individual's functional response to fungi through peripheral blood mononuclear cell (PBMC) functional assays has the capacity to identify immune deficiencies associated with a wide array of genetic mutations (including novel mutations) that predispose the patient to IA.

Stratifying patients according to their IA risk prior to their primary treatment and immune suppression would permit a personalised medicine approach and reduce the empirical prophylactic use of anti-fungal therapies. In this study we investigated CLR status and anti-*Aspergillus* immune response for a small cohort of haematology patients. Samples were collected from 42 AML and SCT patients. Each patient was screened for exonic CLR (Dectin-1, Dectin-2, Mcl, Mincle) mutations and their mRNA expression level was quantified. PBMCs were isolated and functional assays were performed to determine each patient's LPS- and *Aspergillus*-induced cytokine (IL-6/TNF) response. Each patient's CLR status and functional response results were then associated with the incidence of IA and these results were used to identify new IA risk factors. Our research describes a novel strategy that permits the pre-emptive stratification of haematology patients according to their IA susceptibility and drives a personalised medicine approach to their anti-fungal therapy.

MATERIALS AND METHODS

Patient Study Information

The clinical research project was undertaken with sponsorship from Cardiff University, support from the University of Wales teaching hospital haematology and Public Health Wales Microbiology departments, and ethical approval from Health and Care Research Wales (NISCHRC CRC 1351-14). REC reference 14/WA/1119 and IRAS project ID 151136. Written informed consent was obtained from all patients in the study. Whole blood samples were collected from 42 acute-myeloid leukaemia and stem cell transplant patients upon admission to hospital for their primary disease treatment. Fungal disease investigations were undertaken according to local health board guidelines. Of the 42 patients enrolled in the study, 9 developed probable IA according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections

Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) consensus definitions (32). Patient's primary disease, anti-fungal treatment and survival are included in **Table 1**. This study tracked 42 patient's fungal disease status and survival from 7th September 2015 until 9th April 2018. Each patient was anonymised and assigned a number which was consistent throughout the study. The results in this study show patients 1 to 43, as the samples from patient 18 were not processed. Not all assays were undertaken for each patient and statistical analysis was only completed on patient results that met all investigated parameters. Therefore, patient numbers throughout results and analysis vary.

Samples Collected

17.5ml Whole blood was collected from each patient into EDTA tubes (BD) and 2ml whole blood from was collected from each patient into PAXgene blood RNA tubes (Preanalytix) following initial admission to hospital. The 17.5ml whole blood sample was immediately processed for use in the functional assays. The 2ml whole blood in PAXgene blood RNA tubes was stored according to manufacturer's instructions for up to 6 months before being processed in batches.

RNA Isolation

Patient RNA samples were processed in batches of 8 and were not stored for longer than 6 months at -80°C. PAXgene blood RNA tubes were equilibrated at room temperature for 2 h following storage at -80°C before RNA was extracted from the whole blood within the PAXgene blood RNA tubes according to the manufacturer's instructions. The optional DNA digestion step was completed for every patient sample. Typically, ≥3μg RNA was obtained from each patient sample and quantified by nanodrop (Thermo Scientific). The integrity of extracted RNA was confirmed by running patient RNA samples on a 1% agarose gel and visualising the 18S and 28S rRNA bands.

cDNA Generation

Patient RNA was used to generate cDNA using a Quantitect Reverse Transcription Kit (Qiagen). Typically, 2000ng of cDNA was generated for each patient at a final concentration of 100ng/μl for immediate use in Real-Time qPCR. Successful cDNA generation was confirmed by running patient RNA and cDNA samples on a 1% agarose gel. The absence of the 18S and 28S rRNA bands in the cDNA sample indicated successful cDNA generation.

CLR Gene Expression and Sequencing

Gene expression levels of the CLRs Dectin-1 (*CLEC7A*), Dectin-2 (*CLEC6A*), Mcl (*CLEC4D*) and Mincle (*CLEC4E*) was determined by Real-Time qPCR using the Taqman qPCR Mastermix (Thermo Scientific) and CLR gene-specific primer and probe sets (Thermo Scientific) detailed in **Supplementary Table 1**. 100ng of patient cDNA was used in each CLR qPCR reaction. Gene expression normalization was performed against *HPRT1*. Patient CLR exon sequences were determined by PCR amplification of each CLR gene from patient cDNA using the primers detailed in **Supplementary Table 1**. CLR DNA was purified from PCR reaction mixtures using a PCR purification kit (Qiagen) and sent for sequencing at GATC Biotech.

PBMC Culture

17.5ml blood collected into EDTA tubes was added to 50ml conical tubes on top of an equal volume of Ficoll Plus (Sigma) and centrifuged at 400 x g for 30 min with the centrifuge brake reduced to its lowest setting. After centrifugation the layer of PBMCs was removed, washed once with PBS (Life Technologies) and then three times with RPMI 1640 (Life Technologies) before being counted and resuspended in RPMI 1640 supplemented with 10% FBS (Life Technologies), 2% Human Serum (Sigma), 10mM L-glutamine (Life Technologies), 10mM Sodium Pyruvate (Life Technologies) and 100μg/ml Gentamycin (Life Technologies). 100μl of $5x10^6$/ml PBMCs were added to each required well on a 96-well plate (Thermofisher) and rested at 37°C for 4 h prior to the functional assay.

Aspergillus Culture

Aspergillus fumigatus (isolate 13073) was cultured on potato dextrose agar (Sigma) for 7 days before resting conidia (RC) were harvested by vigorous washing with PBS 0.05% Tween 20

TABLE 1 | Patient information including primary demographics, clinical parameters, mortality, and anti-fungal prophylaxis for no evidence of fungal disease (NEF) and invasive aspergillosis (IA) patients.

Parameter		NEF (n=33)	IA (n=9)
Patient Demographics	Female	13	5
	Male	20	4
	Age Median (Range)	59 (21 to 76)	52 (23 to 72)
Clinical parameters	AML	15	4
	SCT	18	5
	Neutropenia* (% of total)	27 (82%)	7 (78%)
Mortality	Total	11	4
	AML	5	1
	SCT	6	3
Anti-fungal prophylaxis	Fluconazole	20	7
	Posaconazole	1	1
	Voriconazole	0	2

**Defined as 10 consecutive days of <$0.5x10^9$/L neutrophils in whole blood within one month prior to a diagnosis of IA or across the duration of the study.*

(Sigma). Harvested RC were counted and stored at 4°C in PBS 0.05% Tween 20 for a maximum of 6 weeks before a new culture was started. For functional assays, RC were grown in RMPI 1640 supplemented with 0.2mg/ml Polymixin B (Sigma) at 37°C 5% CO_2 for 6 hours to generate swollen conidia (SC). *Aspergillus fumigatus* SC were counted and resuspended at $5x10^6$ SC/ml for use in functional assays.

Cytokine Assay

100µl of $5x10^6$/ml PBMCs were stimulated with 100µl of 1µg/ml LPS (Sigma) or 100µl of $5x10^6$ *Aspergillus fumigatus* swollen conidia/ml for 24 h at 37°C 5% CO_2. An unstimulated media-only control was included. After 24 h, supernatant was removed from each well and TNF and IL-6 levels in the supernatants were quantified by ELISA (eBioscience).

Statistical Analysis

Significance was determined using contingency multivariate statistical analysis with Fisher's exact test. If two variables were analysed $*p=0.05$, $**p=0.005$. Where more than two variables were analysed Bonferroni's correction was applied and adjusted p values are described in the Figure legend.

RESULTS

Haematology Patients Display Varied CLR Expression Levels and Mutations

Previous studies have associated SNPs in Dectin-1, Dectin-2 and CARD9 with IA (33–36); therefore, we first aimed to determine the sequence and expression levels of four fungal binding CLRs in our cohort of SCT and AML patients. We screened 42 patients for mutations in the exon coding regions of four CLRs (Dectin-1, Dectin-2, Mincle and Mcl) and identified a Dectin-2 mutation (N170I) (rs1334241354) (34) present in 1 patient and an Mcl mutation (S32G) (rs4304840) present in 17 patients. The Dectin-2 mutation resulted in an early stop codon and loss of the carbohydrate binding region whereas the Mcl mutation only resulted in a single amino acid substitution. We next quantified gene expression of *CLEC7A* (Dectin-1), *CLEC6A* (Dectin-2), *CLEC4D* (Mcl) and *CLEC4E* (Mincle) by Real-Time qPCR for each patient (**Figure 1**). Expression levels of the four CLRs varied considerably from patient to patient and no clear association between CLR expression level and the incidence of IA was observed.

Patient CLR Expression Levels Are Not Associated With IA

In order to investigate whether CLR expression was associated with the incidence of IA, we grouped each patient's CLR results into high (above the median) or low (below the median) and analysed whether high or low CLR expression was associated with IA incidence (**Figure 1**). Here we found that AML patients who developed IA were 10.2 times more likely to have low Dectin-1 expression. In contrast, SCT patients who developed IA were 7.1 times less likely to have low Mcl expression, and 10 times less likely to have low Dectin-1 expression. However, the overall results associating CLR expression with IA incidence across total, AML and SCT patient groups did not provide significant associations.

Patient CLR Mutations Are Not Associated With IA

We next determined whether CLR mutations were associated with IA incidence (**Table 2**). We did not find any association between the incidence of the Mcl S32G mutation and the incidence of IA. Patients with the mutation were equally distributed between the IA group and the no evidence of fungal infection (NEF) group. In addition, as low Mcl expression in SCT patients reduced the likelihood of developing IA within the study, we also examined whether the Mcl S32G affected Mcl gene expression and found no association. The single patient with the Dectin-2 N170I mutation did develop IA (34); however, as this mutation was only present in one patient statistical correlation analysis was not appropriate. Overall, we did not observe any significant association between the CLR mutations and expression identified in this study and the incidence of IA.

Most IA Patients Lack an IL-6/TNF Response to *A. fumigatus*

As the cytokines IL-6 and TNF are vital for a protective anti-*Aspergillus* immune response (37, 38), we next decided to investigate each patient's functional anti-*Aspergillus* immune response. To this end, we isolated patient PBMCs and challenged them with LPS or *Aspergillus fumigatus* SC for 24 h before quantifying the IL-6 and TNF cytokine response (**Figures 2A–C**). Here, our results suggest the majority of patients were able to generate LPS-induced TNF and IL-6; however, fewer patients were able to generate *Aspergillus*-induced TNF and IL-6. Interestingly, whilst all 9 of the IA positive patients produced LPS-induced cytokines, only three IA positive patients (all in the AML cohort) generated *Aspergillus*-induced TNF or IL-6, and only one of these patients produced both cytokines (**Figures 2A, C**). Additionally, only 11 out of 17 AML patients were able to produce any TNF or IL-6 response (**Figure 2C**). This was likely due to AML patient's highly disrupted haematopoietic compartment and lack of mature myeloid cells. However, for SCT patients our data suggests that IA patients may lack a specific anti-*Aspergillus* response. All 5 IA positive SCT patients produced LPS-induced TNF and 4 of the 5 IA positive SCT patients produced LPS-induced IL-6, whilst none of these IA positive patients produced either cytokine following *Aspergillus* challenge (**Figure 2B**). Similarly, the majority (all but one) of the NEF patients produced LPS-induced TNF and/or IL-6, but in contrast to the IA patients, 13 out of 18 NEF patients generated *Aspergillus*-induced TNF and/or IL-6 (**Figure 2B**). The samples used to generate these results were isolated from patients upon admission to hospital for their primary disease treatment. At the time the assays were performed all IA patients in the study had monocyte counts within the normal range. Therefore, this assay may stratify SCT patients according to their risk of developing IA prior to the patients becoming highly susceptible to invasive fungal disease.

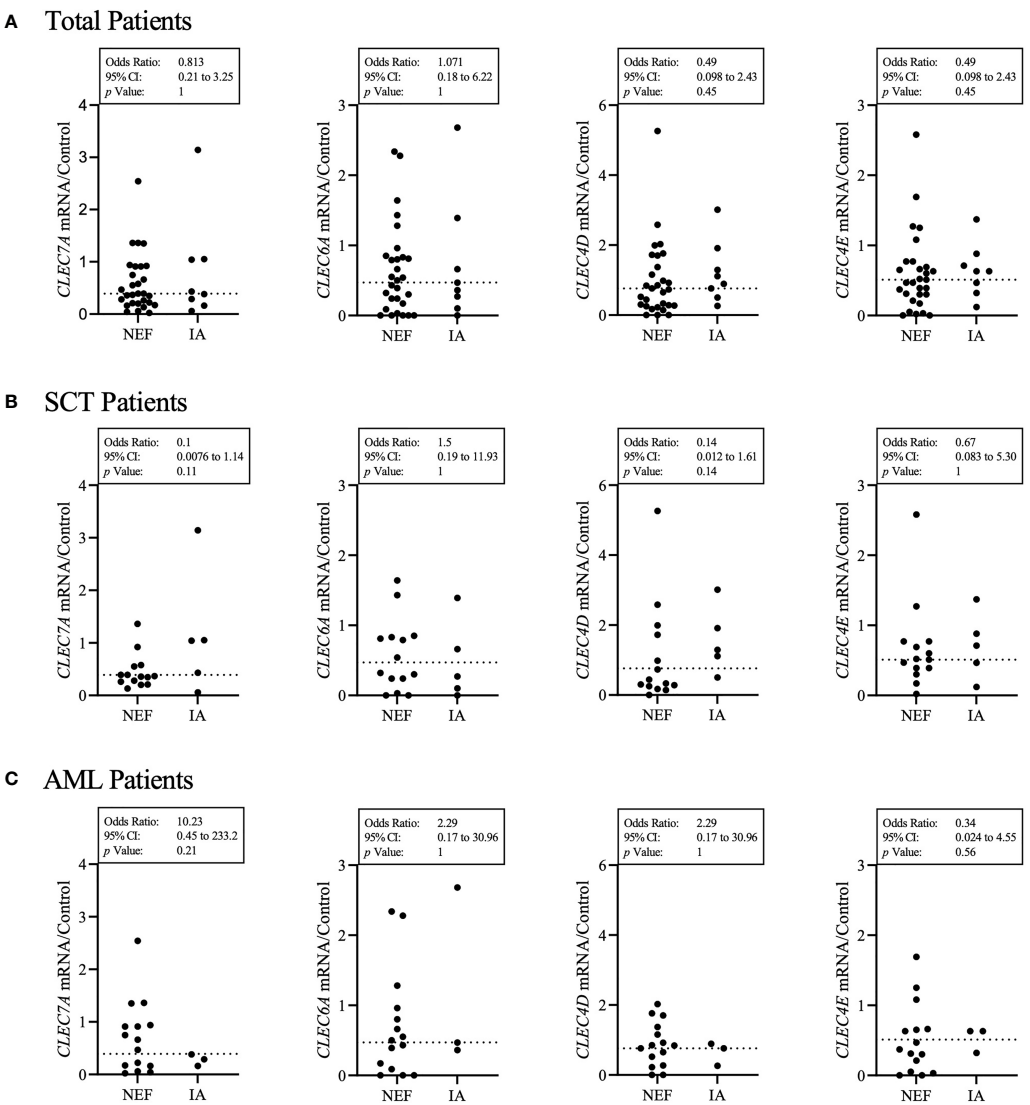

FIGURE 1 | Patient's CLR status does not clearly identify those susceptible to IA. **(A)** Each patient's *HPRT1* and CLR gene expression (*CLEC7A* – Dectin-1, *CLEC6A* – Dectin-2, *CLEC4D* – Mcl, *CLEC4E* – Mincle) was quantified by qPCR. Results displayed were calculated using ΔΔCt comparison against HPRT and CLR results from a healthy control sample. The healthy control CLR results were set at a value of 1 for each CLR. The dotted line represents the median for each CLR gene expression from 37 patients. IA represents probable IA. NEF represents no evidence of fungal disease. **(A)** Displays total patient results, **(B)** displays SCT patient results and **(C)** displays AML patient results. Statistical analysis was produced from a contingency multivariate statistical analysis of low CLR expression associated with the incidence of IA. Results described as low were below the median calculated for each CLR from all patient results. Fisher's exact test was used to identify statistical significance. As two variables were examined, statistical significance was set at $p < 0.05$.

TABLE 2 | Mcl S32G does not affect Mcl gene expression or the incidence of IA.

Patient Group	Parameter	IA	NEF	Odds Ratio	95% CI	*p* Value
Total (42)	Mcl S32G	5/9	12/33	2.19	0.49 to 9.74	0.446
AML (19)	Mcl S32G	2/4	5/15	2	0.21 to 18.7	0.603
SCT (23)	Mcl S32G	3/5	7/18	2.36	0.31 to 17.85	0.618
Patient Group	**Parameter**	**Low Mcl Expression**	**High Mcl Expression**	**Odds Ratio**	**95% CI**	***p* Value**
Total (37)	Mcl S32G	11/19	6/18	2.75	0.72 to 10.48	0.192

*This data was produced from a contingency multivariate statistical analysis of incidence of Mcl mutant against incidence of IA, and incidence of Mcl mutant against Mcl expression. CLR expression as determined in **Figure 1** was used in this analysis. IA represents probable IA. NEF represents no evidence of fungal disease. Fisher's exact test was used to identify statistical significance. As two variables were examined, statistical significance was set at $p < 0.05$.*

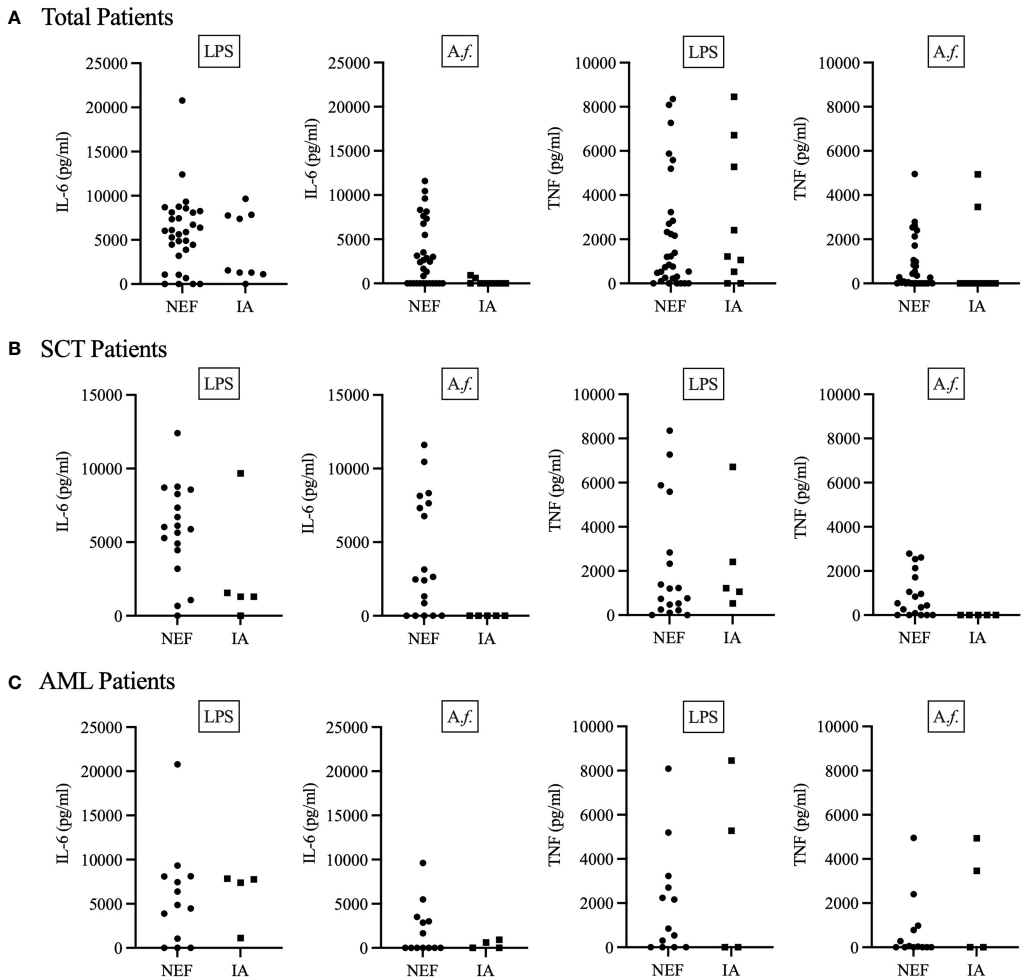

FIGURE 2 | IA patients may lack a specific anti-*Aspergillus* TNF and IL-6 cytokine response. Patient PBMCs were isolated from whole blood and stimulated with 1μg/ml LPS or $5x10^6$ *Aspergillus fumigatus* RC/ml for 24 h. **(A)** Displays total patient results, **(B)** displays SCT patient results and **(C)** displays AML patient results. 24 h after stimulation, supernatant was collected, and the concentration of TNF and IL-6 determined by ELISA. IA represents probable IA. NEF represents no evidence of fungal disease. Statistical analysis for these graphs is presented in **Table 3**. Data from these graphs are the same as in **(A)** from a previous publication from our group (34).

Lack of IL-6/TNF Production as a Risk Factor for IA in SCT Patients

In order to determine whether a patient's functional immune response could be used as a risk factor for IA, we associated each patient's functional response results with the incidence of IA (**Table 3**). Each patient's functional response results were included as the single parameter no *Aspergillus*-induced TNF or IL-6 response. Here, we identified a significant association between patient's functional immune response and the incidence of IA. The strongest association was identified in SCT patients who lacked an *Aspergillus*-induced IL-6 response. This factor defined 5 out of 5 SCT patients who developed IA and only 5 out of 18 SCT NEF patients. Patients who fulfilled these criteria were 27 times more likely to develop IA when compared to SCT patients who generated *Aspergillus*-induced IL-6. SCT patients who did not produce an *Aspergillus*-induced TNF response were also significantly more likely to develop IA, with over 21 times higher IA risk within our study. Here, we identify novel IA functional risk factors that could be used to stratify patients according to their IA susceptibility.

Lack of IL-6/TNF Production Is Not Associated With Mortality

Whilst we have described the significant association between *Aspergillus*-induced TNF and IL-6 and the incidence of IA, it was important to determine whether these results were specific for predicting IA or a more general indicator of poor patient prognosis. Therefore, we associated the incidence of IA and patient's functional immune response results with mortality (**Supplementary Table 2**). Here, we found no association between the incidence of IA or patient's functional immune response results with the incidence of mortality. These results

TABLE 3 | Patients who lack a functional response against *Aspergillus* are more susceptible to IA.

Patient Group	Parameter	IA	NEF	Odds Ratio	95% CI	*p* Value
Total (40)	No *Aspergillus* TNF response	7/9	12/31	5.542	0.98 to 31.25	0.0601
Total (40)	No *Aspergillus* IL-6 response	7/9	12/31	5.542	0.98 to 31.25	0.0601
AML (17)	No *Aspergillus* TNF response	2/4	6/13	1.167	0.12 to 11	1
AML (17)	No *Aspergillus* IL-6 response	2/4	7/13	0.857	0.09 to 8.07	1
SCT (23)	No *Aspergillus* TNF response	5/5	6/18	21.15	1.06 to 445	***0.0137**
SCT (23)	No *Aspergillus* IL-6 response	5/5	5/18	27	1.27 to 575	***0.0075**

*This data was produced from a contingency multivariate statistical analysis of patient PBMC functional assay results as displayed in **Figure 2**. IA represents probable IA. NEF represents no evidence of fungal disease. Fisher's exact test was used to identify statistical significance. Where two variables were examined, statistical significance was set at *p < 0.05; significant values are highlighted bold.*

suggest the TNF and IL-6 functional immune response results are specific risk factors for the incidence of IA within our cohort and not simply identifying patients with a high incidence of mortality.

Combination Risk Factors and IA

As we found a significant association for patient's functional immune response and IA, we next analysed multiple risk factors in combination. Patient's CLR status and functional immune responses were combined and associated with the incidence of IA (**Table 4**). Here, our data shows SCT patients that produced LPS-induced TNF and/or IL-6, lacked *Aspergillus*-induced TNF and/or IL-6 and had high Mcl expression possessed a high risk of IA. Of the 6 patients that fulfilled these parameters, 4 (66%) went on to develop IA. Our data suggests the highest IA risk is present in patients that produced LPS-induced TNF and/or IL-6, lacked *Aspergillus*-induced TNF and/or IL-6 and had high Dectin-1 expression or both high Dectin-1 and high Mcl expression. These parameters stratified 5 patients from the total SCT cohort and 4 developed IA. Interestingly, patient 39 was positive for *Aspergillus* mycology but their radiological investigation did not show evidence of IA; therefore, the patient was not classified as probable IA in accordance with EORTC/MSG guidelines (32). In our study patient 39 had high Mcl and Dectin-1 expression, produced LPS-induced IL-6 and/or TNF and lacked *Aspergillus*-induced IL-6 and/or TNF, suggesting this patient had a high risk of IA. Patient 39 was the only patient stratified into the highest risk cohort that did not have proven IA.

In **Table 4** we also display the incidence of IA within the SCT cohort according to the risk factor parameters used. Here, 5 of 11 (45%) SCT patients with no *Aspergillus*-induced TNF response developed IA, 5 of 10 (50%) SCT patients with no *Aspergillus*-induced IL-6 response developed IA, and 5 of 8 (62.5%) SCT patients with an LPS-induced TNF and/or IL-6 response but lacking an *Aspergillus*-induced TNF and/or IL-6 response developed IA. We next investigated whether the CLR status and functional immune response risk factors could be used to predict the incidence of IA in AML patients within the study. Here, we found no association between AML patients' functional responses and the incidence of IA. Using low Dectin-1 expression as a risk factor stratified 9 patients from the total AML cohort of which 3 (33%) developed IA but this result was not significant.

Proposed Pre-Emptive Stratification System for SCT Patients

Finally, we used the risk factors and associated IA incidence described for SCT patients to propose a strategy that would enable the pre-emptive stratification of SCT patients according to their IA susceptibility (**Figure 3**). We show in the intermediate- and high-risk groups how the risk factors identified in **Table 4** could be used to predict the incidence of IA within the study. We also demonstrate how the risk factors described in this study could be used as exclusion criteria to stratify patients at low risk of IA. Importantly, none of the SCT patients that produced *Aspergillus*-induced TNF and/or IL-6 developed IA.

In this study our investigations into patient's CLR status and functional immune response have led to the identification of novel IA risk factors. We have demonstrated how these risk factors may be applied to stratify patients into low, intermediate, and high-risk cohorts. Crucially, the risk factors identified here would enable the pre-emptive stratification of patients and permit a personalised medicine approach to patient's anti-fungal investigations and treatment.

DISCUSSION

The aims of this pilot study were to identify novel risk factors that could stratify haematology patients according to their IA susceptibility. The development and incorporation of biomarkers to assist in the pre-emptive management of haematology patients at risk of IA have demonstrated significant utility for excluding disease, but even in the presence of multiple positive results the positive predictive value (post-test probability) for confirming IA is not optimal (15). Whilst there are well described IA risk factors, these are frequently encountered in the haematology patient population and IA-associated mortality remains unacceptably high. Therefore, highly drug-interactive, and sometimes ineffective anti-fungal therapy is often empirically administered at the first sign of refractory infection or

TABLE 4 | SCT patients can be stratified according to IA risk using their CLR status and functional immune response results.

Patient group	Risk Factor(s)	Incidence in Patient Cohort	Incidence of IA	Odds Ratio	*p* Value
SCT (23)	No *Aspergillus* TNF response	11/23	5 (45%)	21.15	***0.0137**
SCT (23)	No *Aspergillus* IL-6 response	10/23	5 (50%)	27	***0.0075**
SCT (22)	LPS TNF/IL-6 response + no *Aspergillus* TNF/IL-6 response	8/22	5 (62.5%)	45.57	****0.0021***
SCT (19)	High Mcl expression	9/19	4 (44%)	7.2	0.14
SCT (19)	High Dectin-1 expression	8/19	4 (50%)	10	0.11
SCT (18)	High Mcl expression + LPS TNF/IL-6 response + no *Aspergillus* TNF/IL-6 response	6/18	4 (66%)	45	****0.0049***
SCT (18)	High Dectin-1 expression + LPS TNF/IL-6 response + no *Aspergillus* TNF/IL-6 response	5/18	4 (80%)	81	****0.0016***
SCT (18)	High Dectin-1 expression + High Mcl expression + LPS TNF/IL-6 response + no *Aspergillus* TNF/IL-6 response	5/18	4 (80%)	81	****0.0016***
AML (17)	No *Aspergillus* TNF response	8/17	2 (25%)	1.167	1
AML (17)	No *Aspergillus* IL-6 response	9/17	2 (22%)	0.857	1
AML (17)	LPS TNF/IL-6 response + no *Aspergillus* TNF/IL-6 response	3/17	1 (33%)	0.545	1
AML (18)	Low Dectin-1 expression	9/18	3 (33%)	10.23	0.205

*This data was produced from a contingency multivariate statistical analysis of patient CLR status and PBMC functional assay results as displayed in **Figures 1**, **2**. IA represents probable IA. NEF represents no evidence of fungal disease. Fisher's exact test was used to identify statistical significance. Where two variables were examined, statistical significance was set at *p < 0.05, **p < 0.005; significant values are highlighted bold. Where more than two variables were examined, Bonferroni's correction was applied; significant values are highlighted bold and italic.*

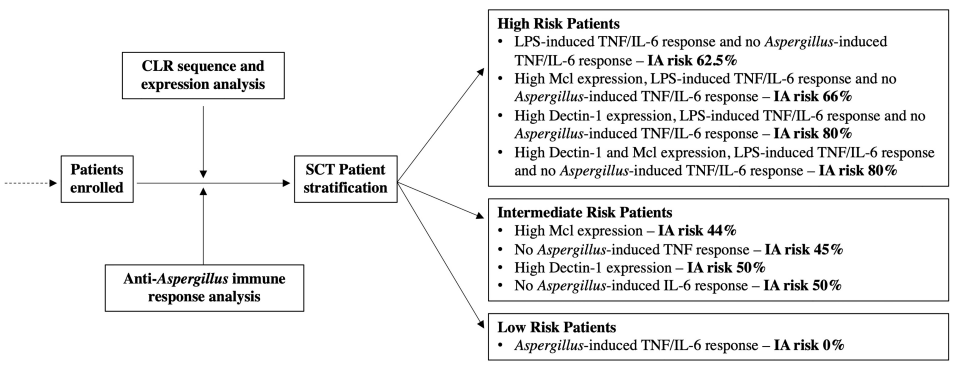

FIGURE 3 | Stratification of SCT patients according to IA susceptibility using risk factors identified in this study. This schematic figure was produced from the results displayed **Figures 1**, **2** and **Table 4**. The proposed strategy utilises the risk factors and incidence of IA from this study and stratifies patients into three groups according to IA susceptibility.

prophylactically administered to asymptomatic patients deemed high risk through host factors or clinical intervention. Ultimately, this results in haematology patients receiving frequent fungal clinical investigations and unnecessary anti-fungal therapy. Identifying patients at low or high risk of infection through host biomarker testing prior to that patient becoming immune suppressed and susceptible to IA would target anti-fungal prophylaxis and allow a personalised medicine approach to managing the haematology patient.

CLR Status and IA

In this study we investigated patient's CLR status as deficiencies in anti-fungal immune components have previously been associated with IA. The first inherited or acquired genetic condition associated with IA susceptibility was identified in CARD9, a downstream adaptor molecule that transduces CLR signalling. CARD9-deficiency resulted in fungal infections without any immune suppression (35, 36, 39). The Dectin-1 Y238X mutation increases IA susceptibility in haematology patients through reduced fungal recognition and immune responses (33). Similarly, the newly identified Dectin-2 N170I mutant was shown to reduce fungal recognition and immune responses (34). Crucially, genetic deficiencies are present prior to the initiation of patient's primary treatment and immune suppression, thereby offering an early indication of a patient's susceptibility to IA. Whilst these risk factors have been associated with IA, they are not yet widely applied in clinical practice (22, 33, 35, 40). Using pre-emptive risk factors to stratify patients has significant promise and has recently been tested. Mutations in Dectin-1 and DC-SIGN, respiratory viral infection, allogeneic

stem cell transplant and *Aspergillus* PCR positivity were used to stratify patients in a predictive disease model. Patients with no risk factors had a 2.4% probability of developing IA whilst patients with four or more risk factors had a 79% probability of developing IA (22). A future study combining the risk factors described by White P. L. et al. and those described in this study may further enhance patient stratification.

In this study, we identified two CLR mutations in our patient cohort and investigated each patient's CLR expression before associating these factors with the incidence of IA. The S32G mutation identified in Mcl has been previously described (rs4304840). This missense mutation results in the substitution of serine to glycine at position 32; this is not thought to have functional consequence. In agreement with this, we identified no association between the incidence of Mcl S32G and IA. Our study also identified a novel N170I mutation in Dectin-2. This mutation resulted in an early stop codon located in the carbohydrate binding domain of the CLR. We recently characterized this mutation and showed that it results in reduced receptor expression and deficient anti-fungal immune responses (34). This mutation was identified in a patient who developed IA and died; however, as this is only one patient the statistical association of this mutation and IA cannot be undertaken.

We determined that high (above the median in this study) Mcl and Dectin-1 expression in SCT patients and low (below the median in this study) Dectin-1 expression in AML patients may be associated with an increased risk of IA. Dectin-1 has been extensively shown to drive protective immune responses against fungal pathogens including *Aspergillus* (28, 41, 42). Therefore, as was found with the Dectin-1 Y238X mutant, it is unsurprising the low/deficient Dectin-1 expression in AML patients may increase IA susceptibility. However, it is not clear why high Dectin-1 expression in SCT patients may be associated with an increased risk of IA. There is limited research describing the functional role of Mcl. Mcl-deficient mice produced defective immune responses against TDM (mycobacterial trehalose dimycolate) (43). A collaborative role for Mcl and the Mincle heterodimerising and enhancing the recognition of carbohydrate and lipids molecules has been identified; however, the functional consequences of this heterodimer CLR complex remain largely unknown (44–46). An explanation for the high Mcl expression identified in SCT patients who developed IA is not clear and requires further investigation.

Functional Response and IA

Previous studies aiming to describe risk factors associated with IA have not investigated patient's functional response against inflammatory stimuli or *Aspergillus*. Patient PBMCs have been used to identify those most receptive to immunotherapy with positive results correlating assay outcome and patient outcome. However, these investigations were completed retrospectively after all patients had received treatment (47, 48). Here, we investigated whether patient's functional immune response could be used to predict IA incidence. Our research describes how the results from a simple assay can be used as a novel IA risk factor that could drive a personalised medicine approach.

In this study we describe how LPS- and *Aspergillus*-induced TNF and IL-6 response can be used to identify the SCT patients most susceptible to IA. PBMCs produce pro-inflammatory cytokines and drive protective immune responses when challenged with *Aspergillus*-extracted chitin or live *Aspergillus* (49, 50). The cytokines IL-6 and TNF play a key role in anti-*Aspergillus* immunity with mice deficient in either cytokine possessing an increased susceptibility to Aspergillosis (37, 38). In agreement, the patients in this study who were unable to produce TNF and/or IL-6 against *Aspergillus* possessed enhanced susceptibility to IA. Similar results were attained with *Candida albicans* where fungal disease was associated with a delayed secretion of cytokines from myeloid cells and T cells (51).

Our functional assay results determined that patients able to produce an LPS-induced response but lacking an *Aspergillus*-induced response possessed the highest IA susceptibility. Anti-*Aspergillus* immune responses are complicated and require collaboration between numerous receptors, signalling molecules and cell types to produce a protective immune response. Patients with haematological malignancies often possess immune defects and have highly variable immune cell counts (52). Here, our functional assay may be able to discriminate between patients with high disruption to their anti-fungal immune response and those with minimal disruption. It is likely those with high disruption possessed deficiencies in anti-fungal immune components that we did not screen for. In contrast to the *Aspergillus*-induced response, the majority of patients were able to produce LPS-induced TNF and/or IL-6. The LPS-induced response requires only TLR4 and CD14 signalling to produce a robust pro-inflammatory response (53) and PBMCs produce large quantities of TNF and IL-6 within 4 h of LPS stimulation (54). Crucially, TLR signalling is retained in patients with a haematological malignancy even after SCT, radiotherapy or chemotherapy and is often responsible for graft versus host disease, gut toxicity and chronic pain (55–57). This maintenance of TLR signalling likely explains why most patients in this study produced LPS-induced responses.

CONCLUSION

Our research is the first to stratify patients at high risk of fungal disease according to their functional anti-*Aspergillus* immune responses and their CLR status. We describe novel risk factors including patient's LPS- and *Aspergillus*-induced TNF and IL-6 PBMC response, and the increased stratification that can be achieved through combining patient's functional responses with their CLR expression levels. We also identified two CLR mutants of which Mcl S32G did not influence IA susceptibility, whilst Dectin-2 N170I likely does increase IA susceptibility. These risk factors were associated with the incidence of IA within our study and resulted in patient stratification into three cohorts. Crucially, within our small patient cohort, we were able to stratify patients

into a 0% risk group (those with an *Aspergillus*-induced TNF and/or IL-6 response), this represents an important step promoting a personalised medicine approach where this cohort's primary disease therapy is prioritised. We were also able to identify a high-risk cohort (those with an LPS- but not *Aspergillus*-induced TNF and/or IL-6 response and high Mcl and/or Dectin-1 expression), this highly susceptible cohort should have a personalised medicine approach that considers their IA susceptibility. Whilst our research was a pilot study that requires further validation in a larger study, we describe novel risk factors and a novel strategy that promotes a personalised medicine approach to haematology patient's fungal disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health and Care Research Wales. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SO, PT, and RB contributed to conception and design of the study. JG undertook experiments and wrote the manuscript. AT, DF, and RP undertook experiments. PW anonymised and provided patient samples and data, and supported study analysis. WI and KW recruited patients and took samples. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.780160/full#supplementary-material

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REVIEW
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The Distinct Roles of Sialyltransferases in Cancer Biology and Onco-Immunology

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Aberrant glycosylation is a key feature of malignant transformation. Hypersialylation, the enhanced expression of sialic acid-terminated glycoconjugates on the cell surface, has been linked to immune evasion and metastatic spread, eventually by interaction with sialoglycan-binding lectins, including Siglecs and selectins. The biosynthesis of tumor-associated sialoglycans involves sialyltransferases, which are differentially expressed in cancer cells. In this review article, we provide an overview of the twenty human sialyltransferases and their roles in cancer biology and immunity. A better understanding of the individual contribution of select sialyltransferases to the tumor sialome may lead to more personalized strategies for the treatment of cancer.

Keywords: tumor glycosylation, sialyltransferases, sialic acid, cancer, tumor immunology

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INTRODUCTION

Cancer remains one of the leading cause of death worldwide (1). During their development, cancer cells undergo important genetic and structural modifications (2). A well-known feature of malignant transformation is aberrant glycosylation (3, 4). Altered tumor glycosylation was initially described in the mid-twentieth century (5–7), and has since been studied in-depth with regard to its role in tumor progression. Tumor-specific glycosylation has been linked to many processes involved in oncogenesis, such as tumor growth and progression, invasion, metastasis, angiogenesis, chemoresistance and tumor immunity (3, 4, 8–12).

Commonly found glycosylation changes in cancer cells include hypersialylation, incomplete synthesis, truncation of O- and N-glycans, altered branching, and even xenoglycosylation (3, 13). Hypersialylation, referring to the increased density of sialic acid-containing glycans (sialoglycans), is one of the most common features of altered tumor glycosylation (3). Overexpressed sialoglycans include sialylated derivatives of Lewis antigens (sialyl-Lewis X [sLeX]), sialyl-Lewis A [sLeA]), which as ligands of selectins are long known to promote tumor metastasis (3, 14). Accumulating evidence suggests that distinct sialoglycans act as glycoimmune checkpoints that suppress anti-tumor immune reactivity by engagement of immunoregulatory Siglec receptors on myeloid and lymphoid immune cells (12, 15–17). Indeed, ligands of Siglecs are broadly expressed on primary human cancer cells and cell lines of different origin (18).

In humans, twenty different sialyltransferases (SiaTs) are involved in the biosynthesis of glycans and each exhibits distinct characteristics and preferences such as for substrates and glycosidic linkages. The expression levels of individual SiaTs varies significantly between different types of

tumors (19), but also within tumors of the same origin (20). While the overexpression of certain sialyltransferases in cancer is associated with tumor hypersialylation and adverse outcome, such positive correlation is not found for all sialyltransferases and may also depend on the type of tumor (see below). Given the significance of distinct sialylation patterns for cancer biology and immunity, in this review article we provide an overview on expression and roles of individual sialyltransferases in cancer.

SIALIC ACIDS AND SIALYLTRANSFERASES

Sialic acids (neuraminic acids) are nine-carbon (C1-9) monosaccharides most commonly found at a terminal position on the outer end of glycoconjugates on many glycoproteins and glycolipids synthesized by living cells (21). Their prominent position on the cell surface glycans of mammalian cells keeps them at the forefront of cellular processes in health, but also in cancer biology and immunity (22–25).

The most prevalent sialic acids in mammals comprise N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) monosaccharides (**Figure 1A**). 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (Kdn) sialic acids are more widespread in lower vertebrates (26) (**Figure 1B**). When one or more hydroxyl groups of Neu5Ac, Neu5Gc or deaminated neuraminic acid (Kdn) are substituted with acetyl, methyl or sulfate residues, more than 50 derivatives with a high diversity are formed (21, 27). As opposed to most mammals, humans do not naturally express Neu5Gc due to the deletion of the *CMAH* (Cytidine monophospho-N-acetylneuraminic acid hydroxylase) gene, which is responsible for the conversion of Neu5Ac into Neu5Gc (28) (**Figure 1A**). It is thought that the deletion of this gene could have provided selective advantages during human evolution and eventually played a role in brain development and running endurance in humans (29, 30). Remarkably, Neu5Gc is often expressed in glycoconjugates of human tumors (13, 31, 32). Due to altered metabolic pathways tumor cells are able to incorporate non-human Neu5Gc (3, 13, 33, 34), which humans can retrieve from foods such as red meat (35, 36).

The sialic acid metabolism involves enzymes that catalyze the biosynthesis and transfer of sialic acid to a glycoconjugate, as well as the removal and degradation of sialic acid (37) (**Figure 2**). Sialic acid biosynthesis starts with UDP-GlcNAc (uridine diphosphate N-acetylglucosamine) produced *via* the hexosamine pathway, which is converted to ManNAC-6-P (N-Acetyl-mannosamine 6-phosphate) by UDP-GlcNAc 2-epimerase/ManNAc-6 (GNE) in a two-step process (38). Then, Neu5Ac synthase (NANS) generates 9-phosphorylated forms of sialic acid (Neu5Ac-9-P), which is then dephosphorylated by Neu5Ac-P-phosphatase (NANP) to generate free sialic acid (Neu5Ac) in the cytoplasm (39). Next, cytosolic Neu5Ac enters the nucleus and is activated by coupling cytidine monophosphate (CMP) *via* the action of cytosine 5'-monophosphate N-acetylneuraminic acid synthetase (CMAS) to produce CMP-Neu5Ac (40). CMP-Neu5Ac is used by sialyltransferases in the Golgi apparatus for sialylation of glycoconjugates. Finally, sialylated glycoproteins and glycolipids are exported to the cell membrane or secreted.

On the other hand, sialic acid can also be released by neuraminidase (also called sialidase) from sialylated glycoconjugates (40). There are 4 mammalian neuraminidases with different cellular localizations: the lysosomal neuraminidase NEU1 (41), the cytosolic neuraminidase NEU2 (42), the plasma membrane-associated neuraminidase NEU3 (43) and the lysosomal or mitochondrial membrane-associated neuraminidase NEU4 (44). The released sialic acids can be reutilized in the biosynthesis pathway (40). Hypersialylation, as occurring in malignancy, is closely associated to an imbalance between sialic acid biosynthesis and desialylation (45).

Human SiaTs comprise a set of 20 glycosyltransferases which all use cytidine monophosphate N-acetylneuraminic acid (CMP-Neu5Ac) as an activated sugar donor for the transfer of sialic acids to the terminal glycosyl group of glycoproteins and glycolipids as acceptor molecules (46). SiaTs catalyze the

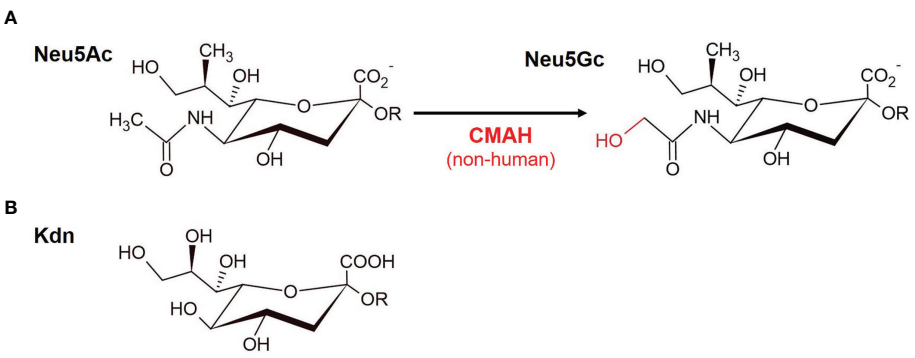


FIGURE 1 | Sialic acids. Sialic acids are nine-carbon monosaccharides. **(A)** The two main mammalian sialic acids N-acetyl neuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) are shown. Neu5Gc is derived from Neu5Ac and differs by one oxygen atom in the N-glycolyl group, which is added by the enzyme cytidine monophosphate N-acetylneuraminic acid hydroxylase (CMAH) in the cytosol. Humans have an inactivating mutation of the CMAH gene and therefore they lack this enzymatic activity. **(B)** Kdn (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid), which is more common among lower vertebrates and bacteria (see text).

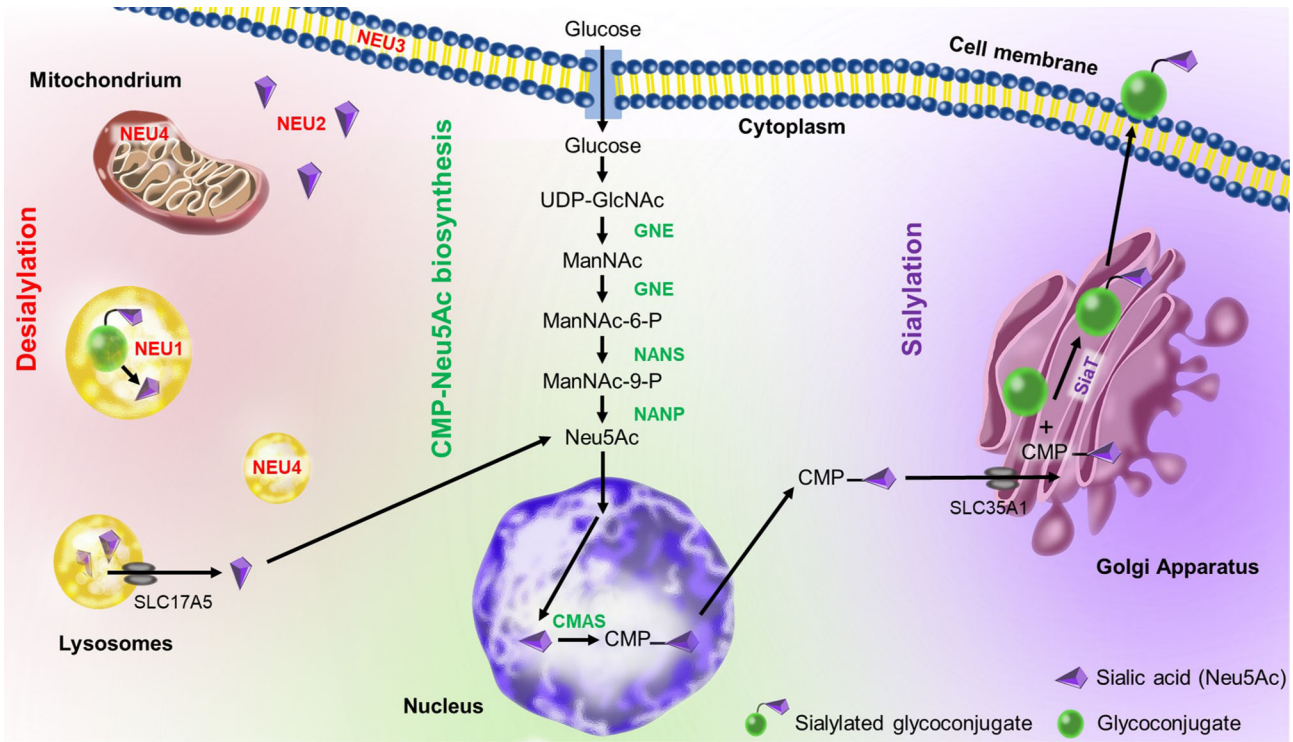


FIGURE 2 | Sialic acid metabolism in humans. CMP-Neu5Ac mostly occurs in the cytoplasm except of the CMP-sialic acid synthase (CMAS)-mediated reaction which takes place in the nucleus. UDP-GlcNAc-2 epimerase (GNE) synthesizes N-acetylmannosamine (ManNAc) in two steps. Then, Neu5Ac synthase (NANS) generates ManNAc-9-P, which is then dephosphorylated by Neu5Ac-P-phosphatase (NANP) to generate free sialic acid in the cytoplasm. The free sialic acid can enter the nucleus to be linked to CMP (cytidine-5'-monophosphate). The CMP-Neu5Ac is transferred to the Golgi apparatus *via* SLC35A1 transporter (solute carrier family 35 member A1), where it is used as a substrate for sialylation by different sialyltransferases (SiaT). Sialylated glycoconjugates are then exported to the cellular membrane or secreted. They can also be broken down by various neuraminidases (NEU1-4) present in different cellular localizations. The released sialic acid can reenter the biosynthesis pathway. Illustration by Aldona von Gunten.

formation of different glycosidic linkages, α2,3-, α2,6-, or α2,8-linkage, and also vary in their acceptor specificities. Accordingly, SiaTs can be grouped into four different families: ST3Gal, ST6Gal, ST6GalNAc, and the ST8Sia (**Figure 3**). Even though SiaTs share the same sugar donors, they present specific substrate specificity, although with some degree of redundancies. Indeed, enzymatic analysis conducted *in vitro* with recombinant enzymes revealed that one linkage can be synthesized by multiple enzymes (47, 48). SiaTs share conserved sialylmotifs, including 'L'- (for long), 'S'- (for short), 'III' (for being third position in sequence), and 'VS'- (for very small) motifs (49). The L-motif is thought to mediate the binding of the donor substrate, the III- and VS-motifs bind the acceptor substrate, and the S-sialylmotif contributes to both binding of donor and acceptor substrates (49). A disulfide bond between the L- and S-motifs bring all sialylmotifs closer together to facilitate interactions with substrates (49).

SiaTs have been shown to be primarily restricted to medial- and trans-cisternae of the Golgi apparatus, with some being present in the trans-Golgi network (50), but some SiaTs are also expressed as post-Golgi and secreted enzymes (51, 52), and SiaT activity was also reported to occur at the cell surface of monocyte-derived dendritic cells (53). Their expression pattern among tissues is diverse, but some SiaTs are preferentially expressed at distinct sites. For specific protein expression of SiaTs the Human Protein Atlas (54) can be consulted (proteinatlas.org).

Increased activity or expression of SiaTs leads to the hypersialylation of cell surfaces which is one of the most common glycosylation changes that occurs in tumors; it entails the enhanced expression of sialic acid-terminated glycoconjugates (3). Many studies show elevated levels of SiaTs in the plasma of cancer patients (55–58). The relative diversity and complexity of sialylation patterns in tumors represents a promising area of research, knowing that each SiaT is involved in the synthesis of various structures, therefore, broadly impacting cancer development in various ways, which will be discussed in the following sections.

ST3Gal FAMILY

Six β-galactoside α2,3-sialyltransferases belong to the ST3Gal family in humans and these enzymes transfer sialic acid residue in an α2,3-linkage to terminal galactose (Gal) residues present on glycolipids or glycoproteins (59, 60). Members of this family are involved in the synthesis of gangliosides (ST3Gal2 and 5), and

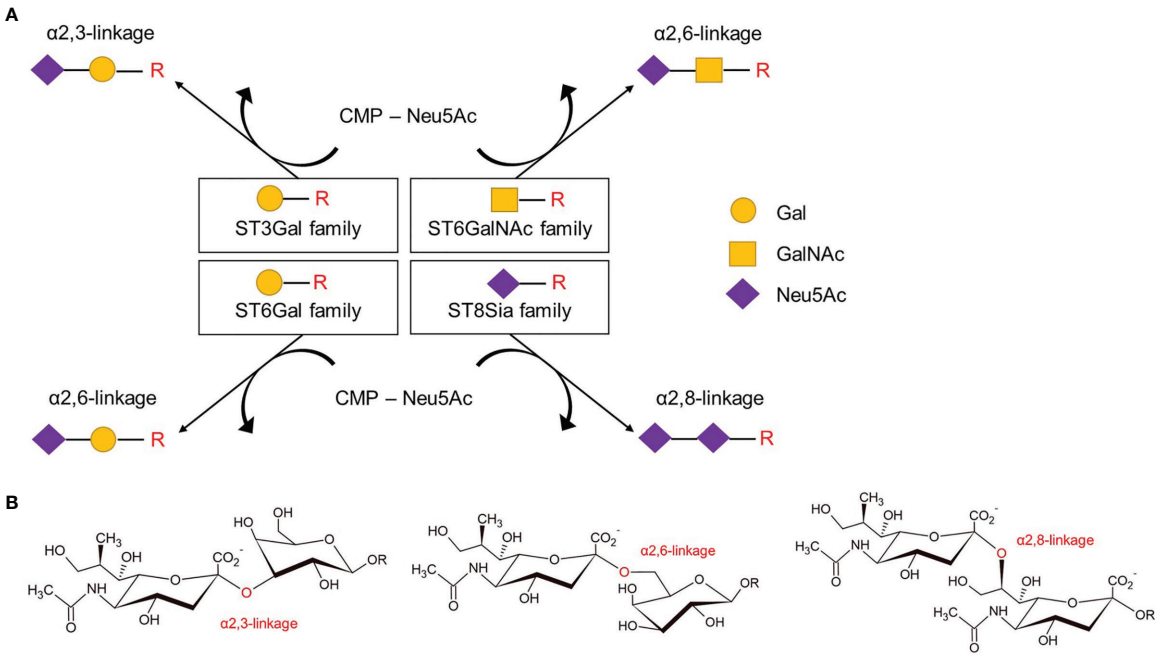


FIGURE 3 | Members of the four families of sialyltransferases catalyze different glycosidic linkages. **(A)** The four families of sialyltransferases as categorized according to restricted glycosidic linkage and acceptor specificity. Indicated are the transfer of activated CMP-Neu5Ac onto Gal, GalNAc or Neu5Ac moieties of carbohydrate chains (-R), such as on glycoproteins or glycolipids. **(B)** Examples of glycosidic α2,3, α2,6, and α2–8 -linkages involving the hydroxyl group at carbon atom 2 of Neu5Ac sialic acid with galactose (left, middle) or another sialic acid (right). CMP, cytidine monophosphate; Neu5Ac, N-acetylneuraminic acid; Gal, galactose; GalNAc, N-acetylgalactosamine.

the tumor-associated sialyl-T (ST) (ST3Gal1) and sialyl-Lewis (ST3Gal3, 4, and 6) antigens (**Figure 4**).

ST3Gal1

ST3Gal1 is known as the major human SiaT to synthesize sialyl-T (ST) antigen from the T antigen Galβ1-3GalNAc. While the T and ST antigens are found on normal *O*-glycans such as in hematopoietic cells (4), ST3Gal1 overexpression is found in different types of malignancies (61–65), and has been linked to poor prognosis (65, 66). MUC1-ST, a glycoform of the mucin MUC1 carrying the ST antigen found in breast cancer patient serum (67), through Siglec-9 engagement, triggers the differentiation of a unique tumor-associated macrophage (TAM) subtype that has been associated with poor prognosis in breast cancer (68). Recently, Rodriguez et al. identified ST3Gal1 as a main contributor to the synthesis of Siglec-7 ligands in pancreatic cancer cells, which by engagement of the sialic acid-Siglec axis may shift TAM differentiation towards a more suppressive phenotype (69). Overexpression of ST3Gal1 has been shown to promote tumor cell migration and metastasis (65, 70–72), which may involve epidermal growth factor receptor (EGFR) signaling (72), or receptor tyrosine kinase AXL dimerization/activation (71). Moreover, ST3Gal1 seems to also play a role in TGF-β1-induced epithelial-mesenchymal transition (EMT) in ovarian cancer cells (70). ST3Gal1 is also enrolled in promoting resistance to anti-cancer effects of agents, such as of adriamycin directed against chronic myeloid leukemia (CML) cell lines (73), paclitaxel against ovarian cancer cells (70), and tamoxifen and/or vandetanib against breast cancer cells (66). The exact mechanisms of ST3Gal1-mediated resistance to chemotherapeutic drugs remain to be deciphered.

ST3Gal2

In vivo genetic experiments showed that ST3Gal2 is a key enzyme mediating α2,3 sialylation of gangliosides in the brain of mice, in particular of GD1a and GT1b, eventually with support of ST3Gal3 (74). *ST3GAL2* mRNA expression was found to be associated with advanced stage and poor clinical outcome in cancer (75, 76). Increased mRNA expression of *ST3GAL2*, as well as *ST3GAL5* and *ST8SIA1*, was also observed in breast cancer stem cells which is eventually linked to increased expression of gangliosides in these cells (77). ST3Gal2 is a rate-limiting enzyme for SSEA-4 (sialyl-glycolipid stage-specific embryonic antigen 4) synthesis (78), which was shown to be limited in normal tissues but highly expressed in glioblastoma cells (79) and has been associated with epithelial-mesenchymal transition (EMT) (76), loss of cell-cell interactions and adaptation of a migratory phenotype (80). Furthermore, a positive correlation between SSEA4 and chemoresistance was reported (76). Notably, gangliosides are differentially recognized by the immunoregulatory receptors Siglec-7 and -9 receptors (81, 82).

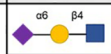
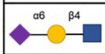
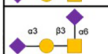
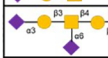
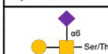
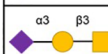
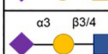
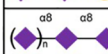
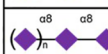
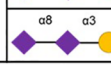
Sialyltransferases	Sialylated oligosaccharide sequence(s) synthesized	Preferred saccharide substrate	Glycan specificity
ST6Gal1		Galβ1,4GlcNac	N-glycan
ST6Gal2		Galβ1,4GlcNac	N-glycan
ST6GalNAc4		Siaα2,3Galβ1,3GalNAc	O-glycan > glycolipid
ST6GalNAc3	 GD1α	Siaα2,3Galβ1,3GalNAc GM1b	Glycolipid > O-glycan
ST6GalNAc5	 (GD1α)	Siaα2,3Galβ1,3GalNAcβ1-4,Galβ1-4 (GM1b)	Glycolipid
ST6GalNAc6	GD1α, GT1aα, GQ1bα	GM1b, GD1a, GT1b	Glycolipid
ST6GalNAc1		GalNAcα1,O-Ser/Thr (Tn Ag)	O-glycan
ST6GalNAc2		Galβ1,3GalNAcα1,O-Ser/Thr (T Ag) > GalNAcα1,O-Ser/Thr (Tn Ag)	O-glycan
ST3Gal1		Galβ1,3GalNAc Galβ1,3GalNAc, O-Ser/Thr (T Ag)	O-glycan
ST3Gal2	 GD1a GT1b	Galβ1,3GalNAc GM1a, GD1b	Glycolipid > O-glycan
ST3Gal4		Galβ1,4(3)GlcNAc	O-glycan, N-glycan
ST3Gal6		Galβ1,4GlcNAc	N-glycan
ST3Gal3		Galβ1,3(4)GlcNAc	O-glycan, N-glycan, glycolipid
ST3Gal5	 (GM3)	Galβ1,4Glc-ceramide	Glycolipid
ST8Sia3		Siaα2,3Galβ1,4GlcNAc	Sialic acid on glycolipids and N-glycan
ST8Sia4		(Siaα2,8)$_n$Siaα2,3Galβ1-R	Sialic acid on N-glycan on NCAM
ST8Sia2		(Siaα2,8)$_n$Siaα2,3Galβ1-R	Sialic acid on N-glycan on NCAM
ST8Sia5	GD1c, GQ1b, GT1a, GT3	GM1b, GT1b, GD1a, GD3	Sialic acid on glycolipid
ST8Sia6		Siaα2,3(6)Gal	Sialic acid on O-glycan > glycolipid
ST8Sia1	 (GD3)	Siaα2,3Galβ1,4Glc-ceramide (GM3)	Sialic acid on glycolipid

FIGURE 4 | Human sialyltransferases. The twenty human sialyltransferases listed according to their homology (60). Select generated oligosaccharides, preferred substrates and glycan specificities of individual sialyltransferases are shown.

ST3Gal3

ST3Gal3 is involved in the synthesis of sLeA (also known as carbohydrate antigen 19-9 [CA19-9]) and sLeX, which are expressed in different types of cancer (83–87), and have been linked to cancer progression and poor prognosis (88), eventually by selectin-mediated invasion and metastasis of tumor cells (14, 89). Indeed, the expression of *ST3GAL3* in breast cancer was found to be associated with poor prognosis (90). ST3Gal3 has also been associated with paclitaxel and cisplatin resistance in ovarian cancer cells (91, 92).

ST3Gal4

ST3Gal4 is involved in the biosynthesis of the tumor-associated antigen sLeX (89, 93). *ST3GAL4* expression correlates with enhanced metastatic potential and poor prognosis in some types of cancer, including pancreatic and gastric cancer (94, 95), which may involve selectin-dependent adhesion through sLeX (87). Recently, ST3Gal4 was found to be responsible for the generation of ligands for the immunoregulatory receptor Siglec-9 in pancreatic cancer cell lines (69), and Siglec-7 and -9 ligand in HEK293 cells (96), indicating its potential role in the generation

of glyco-immune checkpoints. However, overexpression of *ST3GAL4* appears not to be a universal feature of malignancy as downregulation of the enzyme or specific variants has been found for instance in premalignant and malignant cervical tissues (97) and renal cell carcinoma (98). Tissue-specific transcriptional regulation involving alternative splicing and promoter utilization has been described for alpha2,3-sialyltransferases (99), and may explain the differential expression in various types of malignancies.

ST3Gal5/GM3 Synthase

ST3Gal5 initiates the biosynthesis of many downstream gangliosides (100), and is also known by the name "GM3 synthase". GM3, the simplest ganglioside, is involved in various processes such as transmembrane signaling through the regulation of growth receptor activities and in integrin-mediated cell adhesion and motility (101, 102). Furthermore, GM3 has been shown to be recognized by inhibitory Siglec-9 (103). However, ST3Gal5 also mediates the synthesis of GM4 (104). In a breast cancer model, GM3 synthase knockout mice exhibited enhanced tumor growth and angiogenesis (105). In bladder cancer, the downregulation of ST3Gal5 was associated with reduced patient survival (106). Such experimental evidence suggests a beneficial role of GM3 synthase and certain products, such as distinct a-, b- and c-series gangliosides eventually, in at least some tumors. However, given that GM3 synthase acts at an early stage of ganglioside biosynthesis, it remains unclear which ganglioside products and derivatives are effective in such experimental models and differences may exist among different types of tumors.

ST3Gal6

Like ST3Gal3 and ST3Gal4, ST3Gal6 mediates the sialylation of LeX antigen (83). The resulting sLeX antigen interacts with selectins, such as during the initial tethering before extravasation of cells (107). Indeed, ST3Gal6 was shown to have a crucial role in the generation of selectin ligands in mice (108). High expression of ST3Gal6 in multiple myeloma (MM) patients is associated with poor prognosis (109). Knockdown of *ST3GAL6* resulted in a reduced surface expression of α-2,3-linked sialic acid and sLeX on MM cell lines and also reduced the homing and engraftment of malignant cells to the bone marrow niche *in vivo* (109). Furthermore, mice injected with *ST3GAL6* knockdown MM cells demonstrated a decreased tumor burden and prolonged survival. Higher expression of Lewis antigens in neuroblastoma MYCN-amplified cell lines and patient samples could be a consequence of the overexpression of SiaTs, including ST3Gal3/4/6, compared to MYCN-non-amplified counterparts (110). Furthermore, high-grade glioma cell lines exhibit higher expression of terminal sLeX and of the SiaTs ST3Gal3/4/6 compared to low-grade glioma cells (111). ST3Gal6 is also upregulated in human hepatocellular carcinoma (HCC) tissues, and correlates with cell proliferation, migration and invasion ability in HCC cell lines (112). Similar observations were made in urinary bladder cancer with a positive correlation between increased *ST3GAL6* expression and tumor stage, grade as well as poor outcome (113).

ST6Gal FAMILY

ST6Gals preferentially link sialic acids in an α2-6 linkage to galactose residues of Galβ1-4GlcNAc-R on N-glycans (59, 60). This family contains two enzymes ST6Gal1 and ST6Gal2, and is thus the smallest SiaT family.

ST6Gal1

ST6Gal1 is the main sialyltransferase contributing to the addition of α-2,6-linked sialic acid to Galβ4GlcNAc chains, usually present in N-linked chains (59). ST6Gal1 is frequently overexpressed in many solid tumors, such as pancreatic, gastric, cervical, ovarian, brain and colorectal cancers and cancer cell lines (114–120). Indeed, this enzyme has been extensively investigated in regard to cancer research [for a review see (121)]. High expression of *ST6GAL1* in cancer correlates with worse tumor grade (90, 122), advanced stage of disease (120), and poor prognosis (119, 120, 122). While a greater number of experimental studies support an oncogenic role of ST6Gal1 (discussed below), few reports propose an inverse role of this enzyme based on evidence from select *in vitro* and *in vivo* experimental models (123–125). Interestingly, while ST6Gal1 mRNA expression was found to be increased in papillary non-invasive bladder tumors, expression of this enzyme was found to be decreased in muscle-invasive bladder cancer due to epigenetic inactivation of *ST6GAL1* by promoter methylation (126).

Interestingly, ST6Gal1 was shown to protect tumor cells from hypoxic stress, eventually by enhancing the expression of hypoxia-inducible factor-1α (HIF-1α) (127). ST6Gal1 activity has been shown to promote EMT in cell lines of different histological origin (128–130), eventually involving E-cadherin transcription and turnover, as well as PI3K/Akt signaling (128). Silencing of ST6Gal1 in prostate cancer cell lines resulted in decreased expression of components of the PI3K/Akt and β-catenin signaling pathways, resulting in reduced proliferation, migration and invasion (122). Furthermore, ST6Gal1 expression is associated with nonmalignant stem and progenitor cells, but also with stemness in cancer and may drive cancer stem cell (CSC)-like characteristics (131–136). Furthermore, high expression of *ST6GAL1* in CSCs could eventually promote chemo-resistance (137). Indeed, ST6Gal1 has been linked to resistance to a number of agents including gemcitabine (138), cisplatin (139), trastuzumab (140, 141) or gefitinib (142), latter of which appears to involve sialylation and activation of EGFR (142).

Several investigators observed that α2-6 sialylation by ST6Gal1 activity may protect cells from cell death, and eventually block homeostatic epithelial cell apoptosis in cancer (133). ST6Gal1-mediated sialylation prevents apoptosis induced by tumor necrosis factor receptor 1 (TNFR1) (143), eventually by restraining the receptor on the cell surface (144). Similarily, α2-6 sialylation of the death receptor FAS by ST6Gal1 prevents receptor activation by blocking its internalization and the subsequent formation of death-inducing signaling complex and activation of apoptotic caspase-dependent signaling pathways (145). Furthermore, sialylation of β1 integrins by ST6Gal1

conferred protection against galectin-3-induced apoptosis in a cancer cell line (146).

Recently, using gene engineered HEK293 cells, ST6Gal1 was found to be partially responsible for the generation of ligands for the immunoregulatory receptor Siglec-7 (96), indicating its potential role in the generation of glyco-immune checkpoints.

ST6Gal2

ST6GAL2 is predominantly expressed in the adult brain and fetal tissues, and to a lesser extent in the thyroid gland, small intestine, colon, and testis (147, 148). While relatively few studies have investigated the expression and role of ST6GAL2 in tumors, overexpression of this enzyme was found in select types of cancer, including breast cancer (149) and follicular thyroid carcinoma (FTC) (150). In breast cancer ST6GAL2 expression associated with poor prognosis for patients (149). Moreover, silencing of *ST6GAL2* in breast cancer cells resulted in reduced xenograft tumor growth *in vivo* (149). Furthermore, this study revealed that *ST6GAL2* silenced cell lines exhibited reduced adhesion and invasion properties *in vitro*, with downregulation of several focal adhesion molecules (ICAM-1, VCAM-1) and metastasis pathways proteins (MMP2, CXCR4). Similarly, silencing of *ST6GAL2* in FTC reduced tumor growth in an *in vivo* model (150). Findings from this study suggest that the overexpression of *ST6GAL2* leads to the suppression of the Hippo signaling pathway, a tumor suppressor pathway that regulates cellular differentiation and proliferation by inhibiting YAP and TAZ transcription co-activators (151–153).

ST6GalNAc FAMILY

The six SiaTs of the ST6GalNAc family catalyze the glycosidic linkage of sialic acids to N-galactosamine (GalNAc) residues found on *O*-glycosylated proteins or glycolipids in an α2-6 linkage.

ST6GalNAc1

ST6GalNAc1 catalyzes the generation of sialyl-Tn (sTn) antigen from Tn antigen (154). sTn is a well-known tumor-associated carbohydrate antigen (TACA) overexpressed in multiple cancers (155–157), and has been linked to poor prognosis (158–160). Expression of the biosynthetic enzyme ST6GalNAc1 has also been directly associated with poor prognosis (161). Indeed, overexpression of ST6GalNAc1 in gastric, breast, prostate and ovarian cancer cell lines and tissues has shown to induce the expression of the sTn antigen (155, 157, 161–166). The expression of ST6GALNAC1 can also be induced by cytokines, such as IL-13 and CCL17 secreted by M2 macrophages co-cultured with colon cancer cells, which may result in higher expression of sTn antigen including on MUC1 (167).

Downregulation of *ST6GALNAC1 via* hyper-methylation and loss of heterozygosity (LOH) was observed in esophageal carcinoma in tylosis, an inherited epithelial disorder (168). Interestingly, in prostate cancer a splice variant of ST6GalNAc1 is induced by androgens, which consists of a shorter isoform that exhibits sialyltransferase activity yet with slightly different properties (157).

In experimental models, overexpression of ST6GalNac1 reduced cell-cell aggregation and increased extracellular matrix (ECM) adhesion, migration and invasion *in vitro* (163, 166), and promoted tumor growth and metastasis *in vivo* (163, 164) (165). Furthermore, ST6GalNAc1 activity might foster cancer cell stemness, as expression of CSC markers and tumor sphere formation capability were increased in ST6GalNAc1 overexpressing colorectal or ovarian cancer cell lines (161, 164). Stemness through the generation of sTn seems to involve Akt pathway signaling (161, 164), eventually in cooperation with Galectin-3 (161).

The immunoreceptor Siglec-15 was shown to recognize sTn antigen (169, 170), and to depend on ST6GalNac1-mediated biosynthesis (170). Engagement of Siglec-15 by binding to tumor-associated sTn antigen resulted in enhanced TGF-β secretion from monocytes/macrophages following DAP12-Syk signaling (171). Notably, a recent study showed that macrophage-associated Siglec-15 suppressed T cell responses *in vitro* and *in vivo*, eventually establishing a mechanism for immune evasion in the TME (172).

ST6GalNAc2

ST6GalNac2 synthesizes sialyl-6-T antigen from T antigen, and to a lesser extent it sialylates the Tn antigen (154, 166). High transcriptional expression of *ST6GALNAC2* correlated with poor prognosis in colorectal cancer (173), and was found to be associated with higher histological tumor grade, lymph node metastasis, and advanced clinical stage in FTC (174). ST6GalNAc2 has been proposed to enhance invasive properties of cancer cell lines *via* PI3K/Akt pathway signaling (174, 175). However, the role of ST6GalNac2 in cancer appears not to be unequivocally detrimental as Murugaesu and colleagues identified ST6GalNAc2 as a novel metastasis suppressor in mouse and human breast cancer models (176). Indeed, high levels of ST6GALNAC2 expression correlated with increased survival in patients with breast cancer (176). The authors showed that silencing of *ST6GALNAC2* modified the cell surface O-glycome resulting in an increase in unmodified T antigen/core 1 antigen and a reduction in the disialyl core 1 antigen. Such altered glycosylation facilitated the binding of the soluble lectin galectin-3 and resulted in increased tumor cell aggregation, pulmonary tumor cell retention and metastatic burden *in vitro* or *in vivo*.

ST6GalNAc3

ST6GalNAc3 uses α2,3-sialylated ganglioside GM1b as a substrate to synthesize the ganglioside GD1α. In healthy individuals, this enzyme is highly expressed in brain and kidney (177). Aberrant promoter hypermethylation of *ST6GALNAC3* was found in prostate cancer tissue samples (178), but it remains to be shown whether transcriptional silencing of this gene influences the development or progression of prostate cancer. However, ST6GalNAc3 seems to promote the proliferation of A549 non-small cell lung cancer cells through enhanced expression of transferrin receptor protein

1 (TFR1) (179), which is important for cell proliferation and survival (180).

ST6GalNAc4

ST6GalNAc4 mediates the synthesis of disialyl-T antigen from sialyl-T antigen (O-glycan), and also generates the disialyl-lactotetraosyl-ceramide GD1α from sialyl-lactotetraosyl-ceramide GM1b (gangliosides) yet to a lesser degree than ST6GalNAc3 (181, 182). Upregulation of ST6GalNAc4 and downregulation of the core 2 N-acetylglucosaminyltransferase C2GnT2 (*Gcnt3*) were shown to be key in conferring tumor cell glycosylation changes that contribute to metastatic activity in a primary lung cancer model, eventually by preserving presentation of the T-antigen and adherence to galectin 3 (183). In another study, higher expression of *ST6GALNAC4* was observed in FTC tissues compared to transitional tissues and silencing of this enzyme led to decreased invasive ability *in vitro* and *in vivo* (184).

ST6GalNAc5/GD1α Synthase

ST6GalNAc5 transfers a sialic acid residue onto GM1b to form GD1α (185) and this enzyme is also referred to as GD1α synthase (186). Indeed, transfection of the human ST6GalNAc5 cDNA into a breast cancer cell line resulted in the expression of GD1α (187). A study investigating germline single-nucleotide polymorphisms indicates that specific SNPs of *ST6GALNAC5* determine susceptibility for colorectal brain metastasis and overall survival (188). Silencing of *ST6GALNAC5* in breast cancer cells led to decreased metastasis in a murine model *in vivo*, and in an *in vitro* model using human umbilical vein endothelial cells (HUVEC) silenced cells exhibited reduced blood brain barrier (BBB) transmigration activity (189). As opposed, a more recent study showed that ST6GalNac5 overexpression in breast cancer cells leads to a decreased adhesion and no change in transmigration compared to controls in a human BBB model using CD34+ hematopoietic stem cell derived endothelial cells co-cultivated with brain pericytes (190),. The authors of this study suggested that differences in the used BBB models may account for these divergent observations.

ST6GalNAc6

ST6GalNAc6 catalyzes the synthesis of α-series gangliosides, including GD1α, GT1aα and GQ1bα (191), globo-series glycosphingolipids (GSL) (192, 193), and disialyl LeA (194, 195). In humans, ST6GalNAc6 is widely expressed in different organs (193). In human colon cancer ST6GalNAc6 is downregulated compared to nonmalignant epithelium, which is paralleled by a decrease in disialyl LeA expression and a concomitant increase in sialyl LeA (195). Such downregulation of ST6GalNAc6 occurs already in early-stage colon cancer and has been associated with epigenetic silencing (196). The related glycan change from disialyl LeA to sialyl LeA may increase E-selectin binding activity during metastasis and support inflammation-driven carcinogenesis by reduced binding to immunoregulatory Siglec-7 (195). mRNA levels of ST6GalNAc6 have also been found to be reduced in human kidney tumor lesions as compared to healthy tissue from the same patient (193). However, ST6GalNAc6 may also enhance the metastatic capability of tumor cells, as silencing of ST6GalNAc6 in a renal cell carcinoma (RCC) cell line, expressing lower levels of DSGb5, exhibited decreased migration, but not proliferation, *in vitro* (192). Siglec-7 binds to the RCC cell line ACHN in a DSGb5-dependent fashion and silencing of ST6GalNAc6 led to reduced surface binding of a Siglec-Fc chimera protein in these cells (197). These ST6GalNAc6 knockdown cells were more susceptible to cytotoxicity mediated by sialidase-treated NK cells *in vitro*, suggesting that this sialyltransferase has the potential to generate glyco-immune checkpoints at least in some types of tumors.

ST8Sia FAMILY

The ST8Sia family catalyzes the transfer of sialic acid to another sialic acid in an α2,8-linkage (60). Oligosialic acid chains display a chain of 2-7 sialic acids, whereas polysialic acid (polySia) chain exhibit a chain of eight or more polysialic acids (198). ST8Sia2 and 4 are also called polysialyltransferases as they participate in extending linear chains of polysialic acids (60). ST8Sia3 also participate in polysialylation, but with less efficacy than ST8Sia2 and 4 (199). ST8Sia1 (GD3 synthase), ST8Sia3, ST8Sia5 and ST8Sia6 are involved in the synthesis of sialylated glycolipids (60).

ST8Sia1/GD3 Synthase

ST8Sia1 is also known as GD3 synthase (GD3S), as it catalyzes the transfer of a sialic acid residue onto GM3 to give raise to the b-series ganglioside GD3, which can eventually be further processed for the biosynthesis of other b-/c-series gangliosides (59). GD3S expression positively correlates with increasing grades of astrocytomas and is highly expressed in glioblastoma (200). In metastatic melanoma high ST8Sia1 expression is associated with detrimental outcome and higher expression in metastatic lesions, particularly in the brain (201). Recent studies analyzing data from The Cancer Genome Atlas (TCGA) showed an association of high ST8Sia1 expression levels in breast cancer with poor patient survival (202–204), which is eventually linked to epigenetic hypomethylation of the *ST8SIA1* gene (204). As opposed, in another study higher expression of ST8Sia1 mRNA in estrogen receptor (ER) positive breast cancer patients has been associated with higher disease free survival, while no significant difference was found in ER negative patients (205). However, a growing body of evidence supports the notion that ST8Sia1 is associated with tumor growth and progression. In a murine model of glioma, ST8Sia1-deficient mice exhibited attenuated glioma progression, lower-grade pathology and prolonged lifespan (206). Furthermore, in a breast cancer xenograft model silencing of ST8Sia1 led to reduced tumor growth and triptolide-mediated downregulation of ST8Sia1 inhibited tumor growth and prolonged survival (207). ST8Sia1 overexpression has been shown to bypass the need of serum for cell growth and to enhance migratory properties of breast cancer and glioma cell lines (208, 209). Inhibition of ST8Sia1 function by shRNA or

triptolide affected the initiation and maintenance of EMT and ST8Sia1 expression correlated with activation of the c-Met signaling pathway enhancing stemness and metastatic properties (203). The implication of ST8Sia1 in stemness with c-Met signaling downstream of this enzyme was also found in experimental models of glioblastoma (200). ST8Sia1 activity has also been linked to oncogenic signaling through Wnt/b-catenin or Akt, Erk, and Src kinases (206, 210), which eventually may confer chemoresistance (210). GD3 has been identified as a ligand for Siglec-7 (81, 82), and ST8Sia1-transfected P815 cells with high surface expression of GD3 exhibited resistance to NK cell-mediated cytotoxicity due to Siglec-7-dependent inhibition (211).

ST8Sia2/STX

The polysialyltransferase ST8Sia2, also known as sialyltransferase X (STX) is involved in the synthesis of linear polymers of sialic acid, so-called polysialic acid (polySia) chains (212). Polysialic acids are a form of post-translational modifications on different proteins, including the neural cell adhesion molecule (NCAM). Besides expression in healthy neuronal tissues, ST8Sia2 is expressed in neuronal and non-neuronal tumors and expression levels eventually correlate with advanced stage of disease, poor prognosis and risk of relapse (213–215). In an *in vivo* model, ST8Sia2-transfected glioma cells with high expression of polySia exhibited increased tumor invasion within the brain of recipient mice (216). Overexpression of *ST8SIA2* appears to also enhance invasiveness and metastatic capabilities of small cell lung cancer cells *in vitro* (217). Cytidine monophosphate (CMP) was reported to competitively inhibit ST8Sia2 and treatment with CMP led to reduced migration of ST8Sia2-expressing but not non-expressing cell lines in 2D migration assays (218). ST8Sia2 was upregulated in a subset of primary human carcinoma-associated fibroblasts (CAFs), and ST8SIA2 silencing in co-cultured CAFs resulted in decreased lung tumor cells invasion in a 3D model (215).

ST8Sia3

ST8Sia3 is highly expressed in brain and testis and mediates the sialylation of a diversity of glycolipids (GM3, GD3 and α2,3-sialylparagloboside) and select glycoproteins, including striatal glycoproteins (199, 219, 220). ST8Sia3 can also transfer polySia to NCAM, but with a lower efficacy than ST8Sia2 and ST8Sia4 (199). ST8Sia3 was shown to promote survival, proliferation, clonogenicity, and migration of glioblastoma cells based on *ST8SIA3* knockdown experiments *in vitro* (221). Moreover, in the same study it was observed that mice xenografted intracranially with human glioblastoma cell line silenced for ST8Sia3 showed a better overall survival and tumors obtained from these mice demonstrated a lower Ki67 proliferation index.

ST8Sia4/PST

ST8Sia4, also known as polysialyltransferase (PST), synthesizes slightly longer polySia chains compared to ST8Sia2, eventually conferring different molecular properties (222). Both polysialyltransferases are thought to contribute to the polysialylation of NCAM in mammalian cells (223). ST8Sia4 was also reported to be overexpressed in human RCC and breast cancer tissues and to promote cancer progression (224, 225). In these studies, silencing of ST8Sia4 by short-hairpin RNA (shRNA) or specific microRNA (miRNA) reduced cancer cell proliferation and invasion *in vitro*, and decreased tumor growth *in vivo*. High levels of ST8Sia4 expression was observed in chemoresistant leukemic cells (226–228), which may functionally contribute to chemoresistance, eventually by processes involving PI3K/AKT signaling (226, 227). However, in FTC patient tissues, *ST8SIA4* was observed to be downregulated compared to normal thyroid tissue, and ST8Sia4 expression in cell lines inversely correlated with proliferation, migration and invasion *in vitro* or tumor growth *in vivo* (229). Specific miRNAs targeting ST8SIA4 were reported to promote proliferation and invasion capabilities of FTC and oral squamous carcinoma cells (229, 230), and to foster epithelial-to-mesenchymal transition (230).

ST8Sia5

ST8Sia5 exhibits transferase activity of sialic acid moieties onto several gangliosides to synthesize GT3, GD1c, GT1a and GQ1b, respectively (231, 232). Decreased expression of ST8SIA5 from TCGA dataset was linked to a poor survival in patients suffering from colon cancer, and decreased *ST8SIA5* transcript was also observed in a murine model of colitis-associated cancer (233). The reduced expression of ST8Sia5 was linked to gene regulation by forkhead box O3 (FOXO3), the functional deficiency of which may facilitate inflammation-mediated colon cancer growth (233).

ST8Sia6

ST8Sia6 generates disialic acid structures, eventually by transfer of a sialic acid moiety onto a NeuAcα2,3 (6)Gal disaccharide on acceptor substrates, which include glycolipids, but preferentially O-linked glycoproteins (234). Some investigators suggest that ST8SIA6 Antisense RNA 1 (ST8SIA6-AS1) is associated with poor prognosis and enhances the proliferative and metastatic potential of cancer cells (235–240). Furthermore, ST8Sia6 may increase the chemosensitivity of tumor cells at least to certain drugs (226). However, *ST8SIA6* expression was found to be upregulated in several types of cancer and to be associated with a poor prognosis (241). Engineered murine colon and melanoma cancer cell lines expressing ST8Sia6 grew faster and led to a decreased survival *in vivo* and depending on host Siglec-E (241). Also depending on Siglec-E, *ST8SIA6* expression induced an antitumor immune responses characterized by macrophage polarization toward M2 and upregulation of arginase, which required Siglec-E (241). Notably, 2,8-disialic acid structures were shown to be ligands of murine Siglec-E (242), as well as human Siglec-7 and -9 (81, 241), and may thus act as glyco-immune checkpoints in human cancer.

SIALIC ACID-BINDING PROTEINS IN CANCER

Sialyltransferases are involved in the biosynthesis of tumor-associated sialoglycans, which *via* recognition by sialic acid-binding proteins, influence tumor progression and the immune

response of the host. Siglecs and selectins are among the most intensively studied sialic acid-binding lectins, and their implication in cancer will be briefly discussed in this section.

Siglecs

Sialic acid-binding immunoglobulin-type lectins (Siglecs), are a family of I-type lectins that belong to the immunoglobulin superfamily. Siglecs are cell-surface receptors predominantly expressed on leukocytes in a cell-specific and differentiation-dependent manner (243). On the basis of evolutionary conservation and sequence similarity, they are divided into two subsets: the first comprises sialoadhesin (also known as Siglec-1 and CD169), CD22 (also known as Siglec-2), myelin-associated glycoprotein (MAG; also known as Siglec-4) and Siglec-15 (244), and are quite distantly related (~25-30% sequence identity) (245). The other group comprises CD33-related Siglecs (Siglec-3 (CD33), Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-14, and Siglec-16), which have ~50-99% identity and have evolutionary rapidly evolved due to exon shuffling, exon loss, gene conversion and gene duplication (244, 245). Structurally, Siglecs consist of an amino-terminal V-set domain that confers binding specificity for select sialoglycan ligands, which differ across individual family members (246), and between species (245). The V-set domain is followed by a differing number of immunoglobulin-like domains, a transmembrane domain, and the carboxy-terminal cytoplasmic tail that contains inhibitory, or for fewer members activating, signaling motifs (247). It has been proposed that Siglec ligands might serve as self-associated molecular patterns (SAMPs) to avoid autoreactivity of immune cells (248).

Ligands for Siglecs are broadly expressed in different types of human tumors and in a diversity of common cancer cell lines (18). The expression of Siglec-7 and -9 ligands protected tumor cells from NK cell-mediated cytotoxicity *in vitro*, and in a Siglec humanized *in vivo* model (18). In a complementary approach, it was shown that tumor cells decorated with synthetic glycopolymers inhibited NK cell cytotoxicity by engagement of Siglec-7 (249). The body of evidence for Siglec-mediated immune checkpoints in cancer is rapidly growing and indicates that the sialic acid-Siglec axis is relevant for the control of both myeloid and lymphoid immune cells within the tumor microenvironment (12, 16, 17). Interestingly, Siglecs have been shown to be up-regulated on subsets of tumor-infiltrating and circulating cytotoxic T cells in cancer patients (20, 250), in particular on functionally potent effector memory and EMRA T cells (20). While a variety of Siglec-based therapeutic strategies for cancer immunotherapy are currently under investigation (17, 251), a better understanding of the identity and expression not only of tumor-associated sialoside ligands, but also of underlying carrier molecule (252, 253), in specific tumors and patients, may allow for more tailored treatment strategies.

Selectins

Selectins are a family of three calcium-dependent (C-type) lectins comprising E-selectin, L-selectin, and P-selectin, named after their expression on endothelial cells, leukocytes and platelets. In contrast to L-selectin that is constitutively expressed on leukocytes and E-selectin in postcapillary venules of the skin and bone marrow (17), however, E- and P-selectin expression on endothelial cells or platelets are mainly induced following cellular activation (254). The main physiological function of all selectins is to mediate the rolling and adhesion of leukocyte during leukocyte recruitment to sites of inflammation or to lymphoid tissues (254). The carbohydrate-recognition domain (CDR) of all selectins has modest affinity to sLeX and its isomer sLeA (254), which are among the best described ligands for selectins (17). The synthesis of these tetrasaccharides occurs due to the integrated action of α2,3-sialyltransferases with α1,3-fucosyltransferases, β1,4-galactosyltranferases, and *N*-acetyl-β-glucosaminyltransferases (255). As discussed above, ST3Gal3, ST3Gal4 and ST3Gal6 are involved in the synthesis of sLeX, while sLeA is predominantly generated by ST3Gal3.

sLeA and sLeX are known tumor markers and functionally implicated in the malignant behavior of cancer cells (88). Glycosylated proteins carrying sLeX/A moieties, such as PSGL-1, CD24, CD44, ESL-1, and death receptor-3 represent major selectin ligands on cancer cells (14). The overexpression of selectin ligands has been linked to cancer progression and poor prognosis in some cancers (14, 88, 256). *In vivo* studies using selectin knockout or selectin ligand deficient mice highlighted the importance of selectins in metastasis (3). Selectins seems to contribute to metastasis through heterotypic interactions between tumor cells, leukocytes and endothelial cells (14, 256). These interactions may also foster tumor embolus formation with local activation of endothelial cells and increased transendothelial migration of both tumor cells and leukocytes (3). Recruited leukocytes might further enhance vascular permeability and cancer cell extravasation, and also shape the tumor microenvironment (14). While earlier studies on selectin-targeted therapies focused on cardiovascular disease, positive outcomes from clinical trials have raised the interest in strategies targeting selectin receptor-ligand interactions in cancer.

CONCLUSION

In the last decade we have witnessed a significant body of discoveries that highlight the importance of sialic acids in cancer biology and immuno-oncology. As biosynthetic enzymes for sialosides, human SiaTs have long been linked to cancer hypersialylation. However, the twenty SiaTs exhibit different characteristics and their roles in cancer are manyfold and complex, and remain to be fully explored. The expression of SiaTs, sialosides and sialic acid interaction partners (e.g. Siglecs), can vary between different types of tumors, between primary tumor and metastatic lesion, and even between patients (19). Furthermore, controversial observations on the role of a select SiaT may be due to its involvement in the synthesis of multiple glycans, eventually generating various ligands for different glycan-binding proteins. Moreover, limitations of methodological approaches need to be considered, such as missing environmental context for *in vitro* cell cultures or species differences for *in vivo* studies. Functional redundancy may exist between SiaTs, and while specific small-

molecule SiaT inhibitors that bind and block select SiaTs may hold promise for therapeutic and diagnostic use [for recent reviews see (257–259)], combination strategies might be needed in a given context. However, the observation that SiaTs are responsible for the generation of glyco-immune checkpoints has reinvigorated ambitions of researchers to explore the role of individual SiaTs in cancer, which may pave the way for novel immune normalization (260), and more personalized, cancer immunotherapies.

AUTHOR CONTRIBUTIONS

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Glycosylation-Dependent Induction of Programmed Cell Death in Murine Adenocarcinoma Cells

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Altered surface glycosylation is a major hallmark of tumor cells associated with aggressive phenotype and poor prognosis. By recognizing specific carbohydrate motifs, lectins can be applied to distinguish tumor from healthy cells based on the expression of glycosylation-dependent markers. Through their ability to bind to specific carbohydrates, lectins induce cell agglutination and cross-link surface glycoproteins, thereby mediating mitogenic and death-inducing effects in various cell types. The carbohydrate-selective cytotoxic effect of lectins also enables their possible application in therapies targeting cancer cells. To clarify the intracellular pathways mediating cell death induced by a group of plant and fungal lectins, we investigated mouse adenocarcinoma MC-38 cells harboring inactive genes involved in apoptosis, necroptosis and pyroptosis. Treatment of MC-38 cells with wheat germ agglutinin, *Maackia amurensis* lectin I, and *Aleuria aurantia* lectin induced multiple cell death pathways through reactions that relied on the autophagy machinery without depending on caspase activation. Furthermore, inhibition of *de novo* protein synthesis by cycloheximide strongly decreased the cytotoxic response, indicating that the lectins investigated induced cell death *via* effector molecules that are not expressed under normal circumstances and supporting the non-apoptotic nature of cell death. The broad cytotoxic response to lectins can be beneficial for the development of combination therapies targeting tumor cells. Given that tumors acquire resistance to various cytotoxic treatments because of mutations in cell death pathways, compounds inducing broad cytotoxic responses, such as lectins, represent potent sensitizers to promote tumor cell killing.

Keywords: lectin, knockout, apoptosis, necroptosis, pyroptosis, autophagy

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INTRODUCTION

Glycosylation is a complex post-translational modification involved in the regulation of multiple cellular reactions, such as proliferation, adhesion, and trafficking among others. Evidence shows that glycosylation also plays a role in regulating cell death through multiple pathways, including but not limited to prevention of death receptor internalization as in the case of the Fas and TNFR1 receptors, or enhancement of death receptor sensitivity to cognate ligands followed by activation of cell death programs (1–6). Altered glycosylation, such as characterized by increased fucosylation and sialylation of surface glycoproteins, is a hallmark of cancer and is considered as a target for

development of diagnostic and therapeutic tools (7–9). The vast structural diversity of glycosylation is paralleled by a similarly diverse group of carbohydrate-binding proteins, referred to as lectins, which occur in all types of organisms, from bacteria and fungi up to plants and animals. The recognition of specific carbohydrate motifs by lectins enabled their application to distinguish tumor from healthy cells based on the expression of glycosylation-dependent tumor markers (10–14). Several plant and fungal lectins represent promising molecules to target and eliminate various tumors (10, 12, 13, 15).

In addition to their applications as cell markers, lectins exert mitogenic and death-inducing effects on various cell types. For example, concanavalin A and phytohemagglutinin activate T-lymphocytes by crosslinking glycosylated signaling receptors (16). The same concanavalin A induce apoptosis in cancer cells through selective crosslinking and inhibition of receptor tyrosine kinases (17, 18). Because of differences in surface glycosylation between tumor and normal cells, lectins can be applied alone or in a combination with other therapeutic agents to induce cell death selectively in cancer cells while keeping healthy cells intact (12, 19–21). Cell death induced by lectins follow different pathways, such as apoptosis, paraptosis-like death, autophagy and programmed necrosis (10, 19, 20, 22, 23). Specific lectins induce distinct modes of cell death in different type of tumor cells. Wheat germ agglutinin, for example, induces apoptosis in melanoma and leukemic cell (20, 21), whereas it kills cervical carcinoma cells through paraptosis-like cell death (19).

While targeting surface glycans is a promising approach for cancer therapies, lectin-based approaches are still at an early stage of development, as shown in clinical trials with mistletoe lectin (10, 24). The development of efficient therapies based on glycan targeting requires a deep understanding of the mechanisms of cell death induced by lectins on target tumor cells. Using a murine adenocarcinoma cell model, the present study addresses the multiple cell death pathways activated in response to treatment of cells with lectins targeting different glycan structures. Despite targeting different glycan motifs, the lectins tested induced similar pathways of cell death, which were caspase-independent and relied on *de novo* protein synthesis.

MATERIALS AND METHODS

Cell Culture

Mouse colon adenocarcinoma MC-38 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mM non-essential amino acids and 10% FBS. Human embryonic kidney HEK293T cells were cultured in DMEM supplemented with 10% FBS. Young adult mouse colon (YAMC) cells were cultured in DMEM supplemented with 2% FBS, 0.2 μM progesterone, 0.224 μM sodium selenite, 10 μg/ml insulin, 100 μg/ml transferrin, 0.49 μM triiodothyronine, 0.45 μM L-thyroxine, and 5 units/ml mouse interferon-gamma, and incubated at the proliferation permissive temperature of 33°C (25).

CRISPR/Cas9-Mediated Gene Knockout in MC-38 Cells

Single-guide RNAs (sgRNAs) targeting genes of interest in MC-38 cell line were designed using CRISPOR online tool (26). sgRNAs were cloned into either lentiCRISPRv2-puro (Addgene plasmid #52961) or lentiCRISPRv2-neo (Addgene plasmid #98292) (27, 28), lentivirus was produced in HEK293T cells using polyethylenimine "MAX" transfection reagent (Polysciences) followed by transduction of MC-38 cells with lentiviral vectors according to protocols from the Zhang laboratory (27, 29). LentiCRISPRv2-puro transduced MC-38 cells were selected using 10 μg/ml puromycin in DMEM, lentiCRISPRv2-neo transduced cells were selected with 1.25 mg/ml G-418 (Thermo Fischer Scientific) for a time when non-transduced control contained no viable cells. Single clones of transduced cells were obtained by limiting dilutions. Knockouts were validated at the genomic level by PCR and at the protein level by Western blotting (**Supplementary Figure 1**). GNE knockout was validated by genomic PCR followed by Sanger sequencing and by staining with mannose-binding Concanavalin A (ConA) as a control of glycosylation unaffected by GNE knockout, MAL II, and ECL followed by flow cytometry analysis (**Supplementary Figure 2**). The sequences of sgRNAs and corresponding primers for validation of knockouts by PCR are listed in **Table 1**. To avoid

TABLE 1 | sgRNA sequences for targeted gene inactivation in MC-38 using CRISPR/Cas9 system and corresponding primers for knockout verification by PCR.

Gene	Exon	sgRNA sequence	Forward primer	Reverse primer
BAX	3	AGCGAGTGTCTCCGGCGAAT	CTTGGTTCTCAACATTCTGCTCCT	GGATTCTATCTGAGTTGAGTGGAGG
BAK1	3	GGGGCAAGTTGTCCATCTCG	TCATGTGCCAGGACTAACTCTCA	GTAGGGATGAGCATCAGTCAGAGA
	4	GGAACTCTGTGTCGTAGCGC	GAGCCCTATCAGACCTTCAGACA	GAAGTTGGTATGTTCACCCTGACAC
TRADD	2	AGCCGGTCAGAATGGCCACG	ACTTTTTGTTAAAGGCAATGGAGGG	CACAAAGTCCCAGAGTCACTACAC
	3	CCTCCAAGCCTACCGCGAGG	GACTATGGGCTTAGCTTTCTCCTC	ATGTAATTCAAACAGCGCTCTTCAT
FADD	1	TAGATCGTGTCGGCGCAGCG	CGATCTGATGGAGCTCAAGTTCT	GTAAGAAACAAGACCTCCCAGCTT
	2	CCGGACTGGTTAAGGCGCTG	GGCATTTGACATTGTGTGTGACAA	TACATCATGGTGTGATCAAGTCCAC
CASP8	3	CTTCCTAGACTGCAACCGAG	TTTATGCTATTGCTGAAGAACTGGG	TGTATTTAGCCCCTACATTTAGCCC
RIPK3	3	GTGGGACTTCGTGTCCGGGC	CTTCCAGAGCGCAATCCAATTTT	CAGAATGTTAGAGGGCTTGAGGTC
MLKL	2	GCACACGGTTTCCTAGACGC	GATACACAGGGGATTGTGGTATTTC	CATGGAAGAGGATCTTATCATTGCC
	2	GACTTCATCAAAACGGCCCA	GATACACAGGGGATTGTGGTATTTC	CATGGAAGAGGATCTTATCATTGCC
CASP1	5	GAGGGCAAGACGTGTACGAG	AACAAGGTTGGTTTCTTGAAAGGAC	AGAAGTTTTACCAGAGCTGTGAGAT
GSDMD	4	GCAACAGCTTCGGAGTCGTG	ACTTCTCCGTGTTTGAACTTGTCAT	CTTAGTAGAGTCTTCCACCACTGC
GNE	5	AGGAGATGGTTCGAGTGATG	TATCAGCTCTTGGATGAGATGCAG	GTAGGTACCGGTTTCTCTTCCTATC
	9	GATCCAGGAATGGAACTCCG	TTCACTCAGAACTGTCTGATTCCTT	CAGTTCTGGTACACCCTGAAGAAC

clonal effects, two validated clones were used for each knockout. More than one sgRNA sequence per gene listed in **Table 1** indicates that clones were generated using different sgRNAs to minimize potential off-target effects.

Lectin Treatment

MC-38 cells were dissociated with 2 mM EDTA in PBS (pH 7.4) and resuspended in DMEM supplemented with 5% FBS. In total, 1.5 x 10^4 cells in 100 µl of the medium were seeded per well of 96-well plate followed by incubation for 3 h at 37°C to allow cells to adhere to the well surface. Afterward, 50 µl of cell medium in every well were replaced with 50 µl of DMEM containing 2x concentrations of either lectin, in the presence or absence of cycloheximide (Santa Cruz Biotechnology), or corresponding controls followed by incubation at 37°C for 20 h. Final concentrations of MAL I, AAL, WGA were 50 µg/ml, 60 µg/ml, and 4 µg/ml, respectively. Cycloheximide was used at 2.5 µg/ml final concentration. An equivalent amount of lectin resuspension buffer was used as a negative control, recombinant mouse TNF-α (BioLegend) at 20 ng/ml and 80 µM cisplatin (Santa Cruz Biotechnology) were used as positive controls for apoptotic cell death. Cell viability was assessed in a fluorescence-based assay using double staining with Hoechst 33342 (Thermo Fischer Scientific) and propidium iodide (Stemcell Technologies). In experiments, in which cycloheximide was used, cytotoxicity assay based on lactate dehydrogenase (LDH) release was performed instead. All lectins were from Vector Laboratories (Burlingame, California, USA).

Cell Death Measurement

Cytotoxicity was measured either by fluorometric method based on double staining with propidium iodide (PI) and Hoechst 33343 (Hoechst) or by LDH cytotoxicity WST assay (Enzo Life Sciences) according to the manufacturer's protocol. For the fluorometric assay, 11 µl of DMEM containing 10x concentrations of Hoechst (10 µg/ml for MC-38 and 30 µg/ml for YAMC cells) and PI (50 µg/ml) were added to each well of 96-well plate 30 min before the end of treatment. Cells were washed once with 100 µl Dulbecco's phosphate-buffered saline (DPBS). Another 100 µl of DPBS were added per each well followed by fluorescence measurement at 535/617 nm for PI and 360/460 nm for Hoechst using a plate reader (Tecan Infinite® 200 Pro). Before calculating cytotoxicity PI/Hoechst ratio was determined. Cytotoxicity was calculated using the following equation:

$$Cytotoxicity\ (\%) = \frac{PI/Hoechst(test\ sub\tan ce) - PI/Hoechst(low\ control)}{PI/Hoechst(high\ control) - PI/Hoechst(low\ control)} \times 100$$

Positive control values were measured as a mean PI/Hoechst value from three independent experiments by treating MC-38 WT or YAMC cells with 20 ng/ml mouse TNF-α (BioLegend) in combination with 2.5 µg/ml cycloheximide (Santa Cruz Biotechnology). The PI/Hoechst ratio from untreated cells was used as a negative control. For LDH assays, supernatants from lysed cells treated with cycloheximide were used as positive controls.

Flow Cytometry

Totally, 2 x 10^5 cells were stained with 200 µl of 10 µg/ml fluorescein isothiocyanate or biotin-conjugated MAL I, MAL II, SNA, AAL, WGA, and ECL (Vector Laboratories) in lectin staining buffer (PBS, 1% FBS, 0.1 mM $CaCl_2$) for 30 min on ice followed by washing twice with the same buffer. When using biotinylated lectins, additional incubation with streptavidin-FITC (Biolegend) for 20 min on ice was performed. Before the analysis using a FACScanto II flow cytometer (BD Bioscience), cells were washed twice and resuspended in lectin staining buffer.

Western Blotting

Cells were lysed using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and total protein concentration was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). In total, 20 µg of total protein of each sample were separated using 12% SDS-polyacrylamide gel electrophoresis followed by wet transfer to 0.2 µm nitrocellulose membrane for LC3A/B or 0.45 µm membranes for other targets with subsequent incubation with primary and secondary antibodies and detection using ECL reagent (Thermo Fischer Scientific). The following antibodies were used: anti-MLKL (37705S), anti-TRADD (3694S), anti-BAK (3814S), anti-BAX (2772S), anti-caspase-8 (4927S), anti-cleaved caspase-8 (9429S), anti-caspase-3 (9662S), anti-PARP (9542S), anti-caspase-9 (9504S), anti-LC3A/B (4108S) were from Cell Signaling Technology (Danvers, Massachusetts, USA); anti-FADD (ab124812), anti-GSDMD (ab209845), anti-GAPDH (ab9485), goat anti-rabbit IgG (HRP) (ab205718) were from Abcam; anti-RIPK3 (NBP1-77299) was from Novus Biologicals (Littleton, Colorado, USA); anti-caspase 1 (14–9832–82), goat anti-rat IgG2a (HRP) (PA1-84709) were from Thermo Fisher Scientific (Waltham, Massachusetts, USA). For quantitative Western blotting of LC3A/B, protein levels were normalized to total proteins as measured by staining nitrocellulose membranes with Ponceau S (0.2% Ponceau S, 3% trichloroacetic acid, 3% sulfosalicylic acid) for 3 min shortly after the transfer followed by wash with deionized water. All the band intensities were quantified using ImageJ software.

Caspase Activity and Inhibition Assay

Activity of caspases- 3/7 was measured by cleavage of the fluorescently labeled substrate Ac-DEVD-AFC (Sigma-Aldrich). The assay was performed as previously described (30). Briefly, 30 µl of cell lysates was mixed with 30 µl of assay buffer containing 100 µM of respective substrate in black opaque 96-well plate followed by 30 min for caspase-3/7 substrate or 60 min incubation for caspase-8 and -9 substrates at 37°C followed by fluorescence reading at 400/505 nm using a plate reader (Tecan Infinite® 200 Pro). For inhibition assays, 1.5 x 10^4 cells in DMEM, 5% FBS were seeded in wells of 96-well plates 3 h before lectin treatment. The broad-spectrum pan-caspase inhibitor

Q-VD-OPh (Sigma-Aldrich) was added at 20 μM 1 h before lectin treatment and its concentration was maintained at the same level after addition of lectins.

Statistical Analysis

One-way ANOVA followed by Bonferroni's test was used to assess significance between multiple experimental conditions. Unpaired two-tailed t-test was applied to determine significance between two experimental conditions. Differences were considered statistically significant for $p < 0.05$. The error bars in the figures represent the standard deviation (SD). Prism 9.2.0 Software (GraphPad) was used for statistical analysis.

RESULTS

Glycan Ligands and Cell Death Induced by Lectins

The key property of lectins is the ability to recognize specific glycan structures in terms of their monosaccharide composition and glycosidic linkage. In this study, we used a panel of six lectins of plant and fungi origin featuring distinct glycan specificities and containing at least two carbohydrate-recognition domains enabling the crosslinking of cell surface targets. This panel consisted of *Aleuria aurantia* lectin (AAL), which recognizes fucose-containing glycans (31), *Maackia amurensis* lectin I (MAL I), binding to glycans containing β1-4 galactose and α2-3 sialic acid (32), *Maackia amurensis* lectin II (MAL II), which recognizes α2-3-linked sialic acid (32), *Sambuccus nigra* agglutinin (SNA), which recognizes α2-6-linked sialic acid (33), wheat germ agglutinin (WGA), which binds to glycans containing N-acetylglucosamine and sialic acid (34), and *Erythrina crystagalli* lectin (ECL), which preferentially binds to terminal β1-4 galactosylated residues (35). Considering that the abundance of sialic acid-terminated glycans on cancer cells is often associated with a poor prognosis and thus considered to be a prospective target for anticancer therapies, we investigated the impact of MC-38 desialylation on the binding ability and cytotoxic effect of lectins recognizing sialylated and non-sialylated glycan epitopes. For that purpose, we established MC-38 cell line lacking expression of UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), the rate-limiting enzyme of sialic acid biosynthesis.

Among the lectins tested, WGA showed the highest binding levels to MC-38 cells, indicating a wide availability of N-acetylglucosamine ligands exposed at the cell surface. WGA is also known to bind to sialylated glycans (36), yet MC-38 lacking GNE only showed minimally decreased binding for WGA (**Figure 1**). The weaker binding of ECL, AAL, MAL I and MAL II reflected lower densities of cognate carbohydrate ligands. As expected, $GNE^{-/-}$ MC-38 cells showed decreased binding of α2-3-sialic acid-specific MAL II. By contrast, the low binding of SNA was not affected by GNE inactivation, thereby showing that either α2-6-sialylated ligands are largely absent on MC-38 cells or SNA lectin still able to recognize a minimal levels of sialic acids maintained by the lysosomal salvage pathway of sialic acids (37). Furthermore, while SNA is considered as a very specific lectin, non-specific binding is still possible to occur (38). Interestingly, ECL binding was stronger in $GNE^{-/-}$ cells, which expose terminal β1-4 galactose in the absence of sialic acid capping.

The cytotoxic response of the lectins tested largely matched the density of their carbohydrate ligands. MAL I, AAL, WGA in wildtype (WT), and ECL, AAL in $GNE^{-/-}$ cells, induced cell death in a dose-dependent manner with WGA showing the highest cytotoxic effect (**Figure 2**). Despite the minimal decrease in WGA binding observed in $GNE^{-/-}$ cells, the cytotoxic effect of WGA was significantly lower in desialylated cells (**Figure 2A**). This observation is in line with reports on human leukemia cells, which were less sensitive to WGA-mediated cytotoxicity when treated with neuraminidase (20). The cytotoxic effect of MAL I was completely abolished in $GNE^{-/-}$ cells (**Figure 2B**), although MAL I binding was similar between WT and $GNE^{-/-}$ cells (**Figure 1**). This finding underlines the importance of glycan composition for the cytotoxic effect mediated by MAL I, which likely relies on the cross-linking of sialylated glycoproteins by MAL I. The opposite effect was observed for ECL, which was only cytotoxic towards $GNE^{-/-}$ cells (**Figure 2C**). The increase in cytotoxicity matched the increased lectin binding measured in $GNE^{-/-}$ cells (**Figure 1**), suggesting that the higher density of exposed β1-4 galactose was responsible for the sensitivity to ECL-mediated cytotoxicity. AAL showed the lowest toxic effect in MC-38 cells (**Figure 2D**) despite the strong binding of this lectin to cells (**Figure 1**), thus showing that the cross-linking of fucosylated glycoproteins does not significantly induce cell death. Surprisingly, the lack of sialic acids in $GNE^{-/-}$ cells resulted in much stronger cytotoxicity mediated by AAL (**Figure 2D**). The sialic-acid dependent lectins MAL II and SNA, which recognize other sets of sialylated glycans than MAL I, also failed to induce a cytotoxic response in MC-38 cells over the range of concentrations tested (**Figures 2E, F**). Considering the lower cytotoxicity mediated by WGA and MAL I in $GNE^{-/-}$ cells (**Figures 2A, B**), sialylation, however, appears to be critical for the cytotoxic effect of the latter lectins. Accordingly, the lack of cytotoxicity associated with MAL II and SNA indicates that binding to sialylated ligands alone is not sufficient to induce cell death. The activation of cell death pathways probably requires cross-links between differentially glycosylated surface proteins.

Lectins Activate Multiple Cell Death Pathways

To characterize the signaling pathways mediating cell death induced by the cytotoxic lectins WGA, MAL I and AAL, we used a panel of MC-38 cells with knockouts in genes involved in cell death responses. Apoptosis is the most common form of programmed cell death induced in response to various extrinsic and intrinsic stimuli. The extrinsic apoptosis pathway is induced in response to activation of cell death receptors, followed by formation of the death-inducing signaling complex (DISC) that includes Fas-associated *via* death domain protein (FADD) and pro-caspase-8, which cleaves the executioner caspase-3. The intrinsic apoptosis signaling cascade is activated in response to

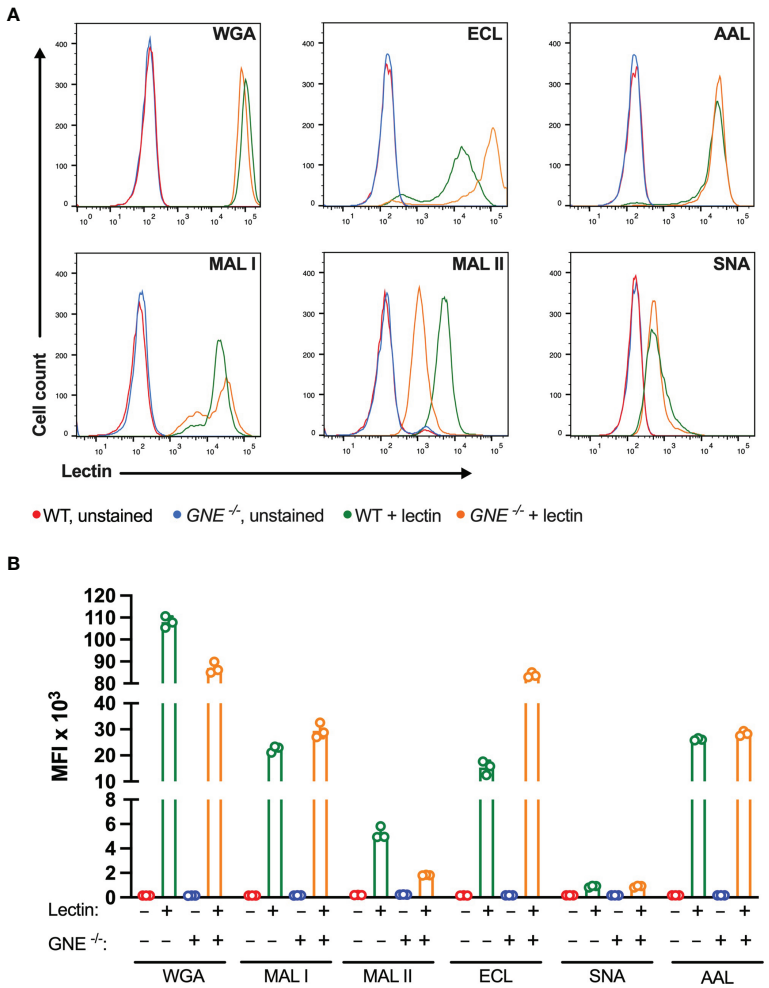

FIGURE 1 | Binding of plant lectins to MC-38 cells. **(A, B)** Binding ability of lectins used in the study to MC-38 cells with unaffected membrane sialylation level (MC-38 WT) and desialylated (MC-38 *GNE* -/-) measured by flow cytometry. Unstained MC-38 WT (red lines), stained MC-38 WT (green lines), unstained MC-38 *GNE* -/- (blue lines), stained MC-38 *GNE* -/- (orange lines). Each lectin was used at 10 μg/ml. Data are presented as mean and standard deviation of three replicates.

various internal cell stress factors, such as DNA damage, and results in the formation of pores in outer mitochondrial membranes. The pro-apoptotic proteins BCL2 antagonist/killer 1 (BAK1) and BCL2 associated X (BAX) form these pores, which lead to the release of cytochrome C and activation of initiator caspase-9, which in turn activates the executioner caspase-3 (39, 40). We first addressed the role of BAX and BAK1, the members of pro-apoptotic BCL-2 protein family mediating intrinsic apoptosis (40). The inactivation of the BAX/BAK1 complex decreased the cytotoxic response induced by WGA, MAL I and AAL treatment (**Figure 3A**). The decrease in cytotoxicity by more than 50% was similar to the effect achieved in cells treated with cisplatin, which is a classical trigger of apoptosis. By contrast, the inactivation of FADD (41), an adaptor protein involved in the extrinsic signaling pathway of apoptosis, did not impacted the cell death mediated by WGA, MAL I and AAL, whereas tumor necrosis factor-α (TNF)-induced cell death was impaired as expected (**Figure 3B**).

Another mode of programmed cell death is necroptosis, which is caspase-independent and is mediated by tumor necrosis factor receptor type 1-associated death domain protein (TRADD), receptor-interacting serine/threonine protein kinases 1 (RIPK1) and 3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL) in response to extrinsic stimuli. MLKL, when phosphorylated by RIPK3, affects cell membrane permeability resulting in membrane rupture and necroptotic morphology (42). The inactivation of TRADD (43), another adaptor molecule required for activation of apoptosis and necroptosis downstream of tumor necrosis factor receptor 1 (TNFR1), decreased cell death in cells treated with MAL I, but not when WGA and AAL was added (**Figure 3C**). This observation suggests that MAL I could potentially mediate its cytotoxic effects *via* crosslinking of TNFR1 or other receptors, such as death receptor 3, that are also known to induce cell death *via* TRADD (44). To assess the ability of the lectins to induce necroptosis, we inactivated MLKL,

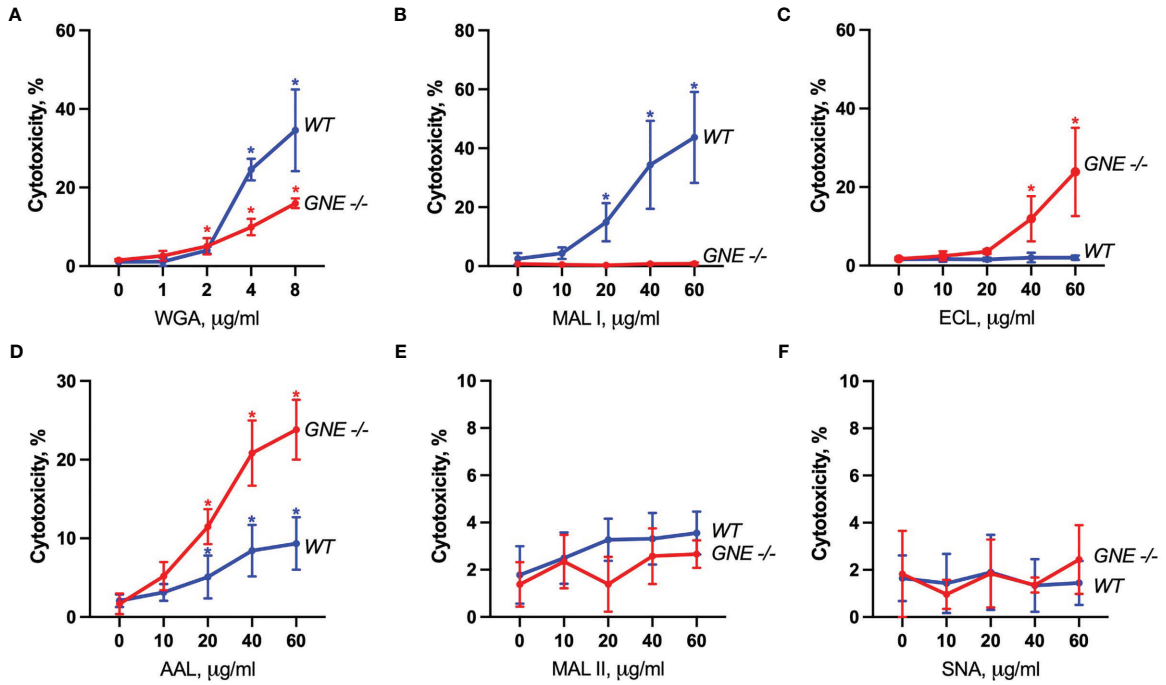

FIGURE 2 | Lectin-induced cell death in MC-38 cells. Cytotoxicity was measured by propidium iodide and Hoechst 33342 staining of cells after treatment with WGA **(A)**, MAL I **(B)**, ECL **(C)**, AAL **(D)**, MAL II **(E)**, and SNA **(F)** lectins in WT and GNE deficient cells for 20 h. Data represent averages and SD of 6 replicates out of 2 independent experiments. *p < 0.05; one-way ANOVA followed by Bonferroni's multiple comparison test.

which is essential in the execution of necroptosis. The loss of MLKL decreased the cytotoxic effect of WGA, MAL I and AAL, indicating the contribution of the necroptosis pathway in cell death induced by these lectins (**Figure 3D**). Of note, loss of MLKL resulted in increased sensitivity to TNF that shows a potential role of MLKL in preventing non-necroptotic forms of cell death induction in response to TNF in MC-38 cells. Given that apoptosis and necroptosis could be activated by similar stimuli, as in case of TNF/TNFR1 signaling, and considering inhibitory effect of caspase-8 on activation of necroptosis that plays a role in switching of apoptotic cell death to necroptosis, we tested the combined role of caspase-8 and RIPK3 in cell death mediated by lectins (40, 42, 45, 46). MC-38 with caspase-8/RIPK3 double knockout showed substantial decrease in cell death in MAL I-treated cells and minor decrease in WGA-treated cells that serves as an indicator of activation of either extrinsic apoptosis or necroptosis in treated cells (**Figure 3E**). Concurrent involvement of MLKL in MAL I and WGA-mediated cell death also confirms a potential activation of necroptosis in response to lectin treatment (**Figures 3D, E**).

The third mode of programmed cell death investigated in this study is pyroptosis. The main pathway of pyroptosis is mediated by caspase-1, which is activated by the inflammasome in response to various microbial infections and non-infectious stimuli. Pyroptosis is central in immune cells, such as macrophages (47), but can also occur in other cell types in response to infection or exposure to chemical compounds (48, 49). The pathway leads to gasdermin D (GSDMD) cleavage, which embeds in the plasma membrane and forms pores that disrupt ionic gradients and facilitate water influx, hence leading to cell swelling and osmotic lysis (47, 50). Considering that some plant lectins are known to induce formation of the NLRP3 inflammasome in immune and cancer cells (51), we addressed the role of pyroptosis in mediating cell death in MC-38 cells treated with WGA, MAL I and AAL. Inactivation of either caspase-1 or GSDMD in MC-38 showed only a minor decrease in MAL I-mediated cytotoxicity, supporting a partial involvement of pyroptosis in response to MAL I treatment (**Figures 3F, G**). Inactivation of caspase-1 resulted in decreased cytotoxicity induced by MAL and also by AAL (**Figure 3F**). A similar effect was observed in TNF-treated cells, in which caspase-1 and GSDMD knockouts reduced cell death, thus pointing to the induction of the pyroptosis response downstream of TNFR1.

Given the dominant apoptosis response induced by lectins, we addressed the activation of key apoptotic caspases and the cleavage of poly (ADP-ribose) polymerase-1 (PARP1), which is a substrate of activated caspase-3 and -7 (52). Indeed, a moderate cleavage of caspases-3, -8 and PARP1 was observed in MC-38 cells after AAL and WGA treatment, while MAL I treatment only resulted in minor cleavage of the same substrates (**Figure 4A**). In comparison to lectin treatment, addition of TNF to MC-38 cells led to significant cleavage of caspases-3, 8 and PARP1 already by 6 h of treatment, while cisplatin treatment showed substantial detection of cleaved forms by 20 h. Caspase-9 cleavage was not detected, neither in cells treated with lectins, nor in cells treated

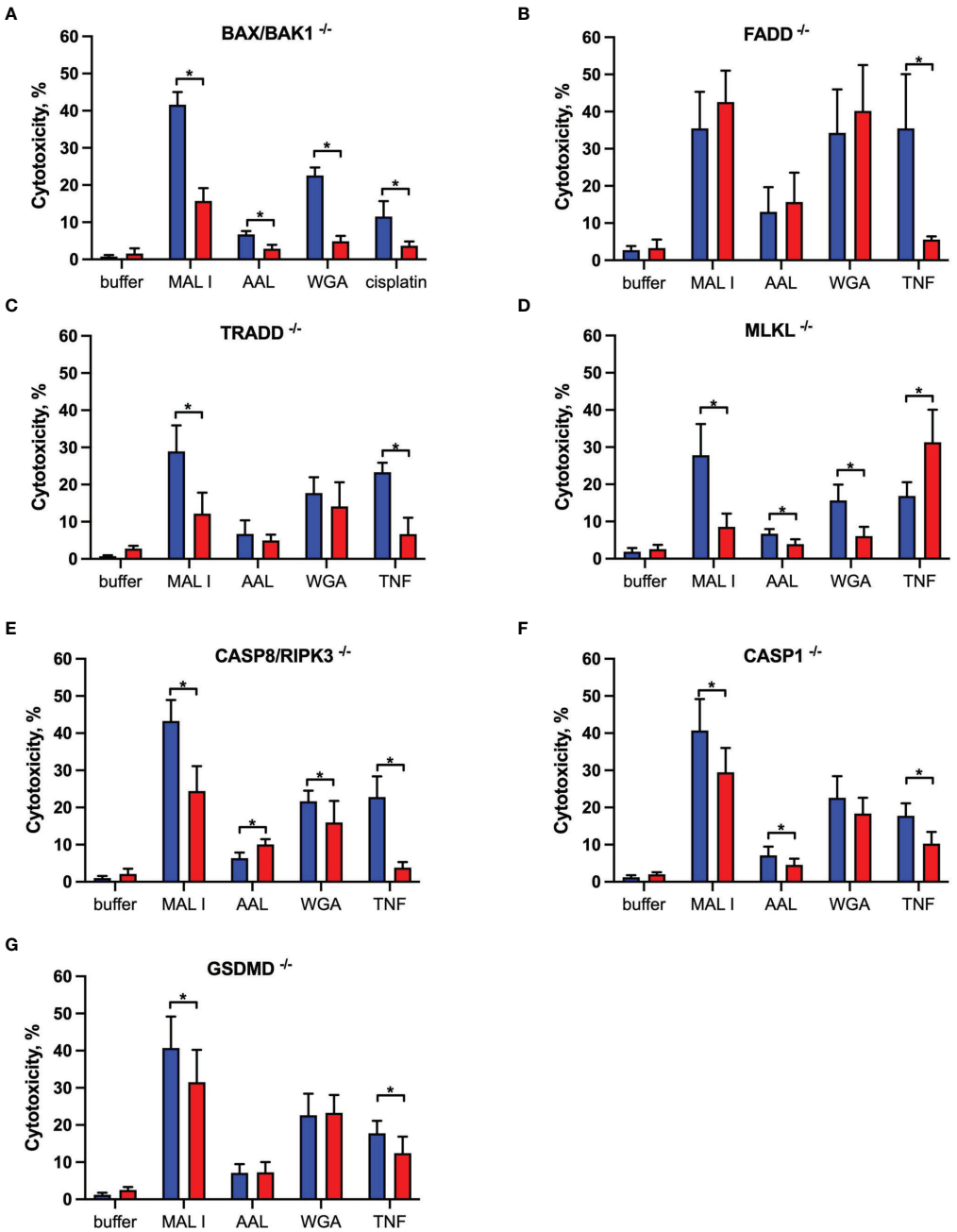

FIGURE 3 | Effects of single gene knockouts on lectin-induced cell death in MC-38 cells. Intrinsic apoptosis was assessed by inactivation of BAK/BAX **(A)**, whereas extrinsic apoptosis and necroptosis pathways were assessed by inactivation of FADD **(B)**, TRADD **(C)**, MLKL **(D)**, and combined caspase 8/RIPK3 **(E)** genes. Pyroptosis was assessed by caspase 1 **(F)** and GSDMD **(G)** gene inactivation. To exclude clonal effects, all experiments were performed in at least two clones for each gene knockout. Data represent 6 replicates from at least 2 independent experiments. WT (blue bars), gene knockouts (red bars). *$p < 0.05$; unpaired two-tailed t-test.

with TNF and cisplatin. To confirm the validity of the anti-caspase-9 antibody applied, we tested lysates of mouse embryonic fibroblasts treated with cytochrome C, which resulted in the detection of caspase-9 cleavage products (**Supplementary Figure 3**). The comparison with TNF demonstrated that AAL and WGA treatments activated apoptotic signaling, yet in a moderate and delayed manner.

In addition to immunoblotting, we measured activation of effector caspases-3/7 using a specific fluorogenic substate. In case of MAL I treatment, no caspase activation was detected, whereas AAL treatment resulted in increased caspases-3/7 activity by 24 h of treatment. WGA treatment also yielded increased caspases-3/7 activation, which was already significant by 14 h (**Figure 4B**). To further validate the role of caspases in lectin-mediated cell death,

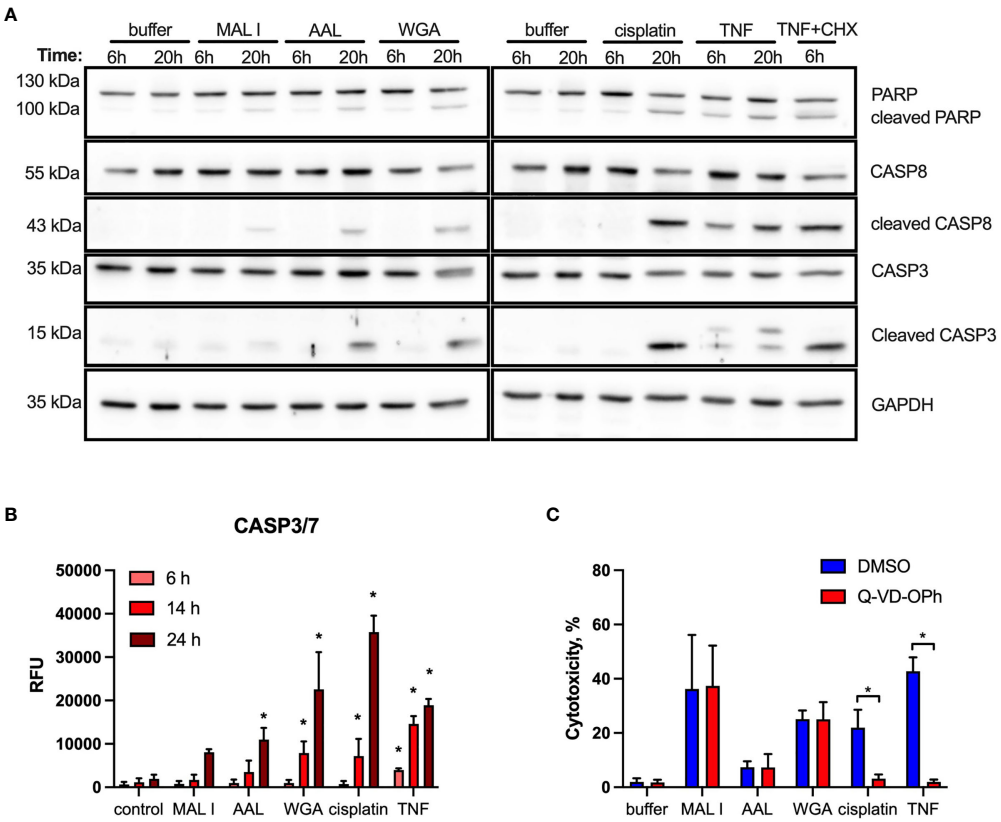

FIGURE 4 | Cleavage of PARP, caspase 8 and caspase 3 induced by lectin treatment of MC-38 cells. **(A)** Immunoblot analysis of poly-ADP-ribose polymerase 1 (PARP), procaspase 8, cleaved caspase 8, caspase 3 and GAPDH as a loading control in MC-38 lysates after treatment with either lectins or cisplatin and TNF controls for 6 and 20 h **(B)** Activation assay for caspases-3,7 (DEVDase activity) using fluorogenic substrate. **(C)** Impact of caspase inhibition on cell death. MC-38 cells were treated either with DMSO or Q-VD-OPH broad spectrum caspase inhibitor in combination with lectins and respective controls. Data represent 6 replicates out of 2 independent experiments. *$p < 0.05$; one-way ANOVA with Bonferroni's multiple comparison test **(B)** or unpaired two-tailed t-test **(C)**.

we applied the pan-caspase inhibitor Q-VD-OPh in cells treated with WGA, MAL I and AAL. Surprisingly, Q-VD-OPh treatment did not affect the cytotoxic response of the three lectins, whereas it completely prevented cell death induced by cisplatin and TNF as expected (**Figure 4C**). This finding showed that, while activated in response to lectins, caspase activities are not essential in mediating the cytotoxic response to WGA, MAL I and AAL.

Lectin Treatment Up-Regulates Autophagy

In addition to the induction of apoptosis, many lectins also up-regulate autophagy, which in some cases results in autophagy-dependent cell death. To address the possible activation of autophagy in MC-38 cells treated with WGA, MAL I and AAL, we measured the cleavage of the autophagy marker LC3. The LC3 protein is cleaved into LC3-I immediately after synthesis and is later conjugated with phosphatidylethanolamine, thereby forming LC3-II, which is associated with autophagosome membranes (53). Given that autophagy is a dynamic process, cellular LC3-II levels reflect the balance between synthesis and degradation resulting from the fusion of autophagosomes with lysosomes. To minimize LC3-II degradation, chloroquine was added to inhibit autolysosome formation and lysosomal protease activity (54, 55). Treatment with MAL I, AAL and WGA up-regulated autophagy in MC-38 cells, as shown by immunoblotting of LC3-II in lysates from cells treated for 6 h with lectins in the absence and presence of chloroquine (**Figures 5A, B**). The increase in steady-state LC3-II levels detected in the absence of chloroquine treatment together with concurrent involvement of BAK1 and BAX, which are known to affect lysosomal and autolysosomal permeability, indicated that cell death induced by MAL I, AAL and WGA was probably initiated through activation of the autophagic/lysosomal response rather than the classical apoptotic mitochondrial pathway (56).

Lectin-Mediated Cell Death Depends on *De Novo* Protein Synthesis

Another cell death pathway reported to be activated in tumor cells in response to WGA is paraptosis, which is caspase-independent and relies on *de novo* protein synthesis (19, 47). Paraptosis can be activated by oxidative stress and other mechanisms resulting in osmotic dysregulation (47, 57). Considering that autophagy also requires *de novo* protein synthesis (39), we tested whether protein synthesis is essential for execution of cell death induced by lectins by treating MC-38

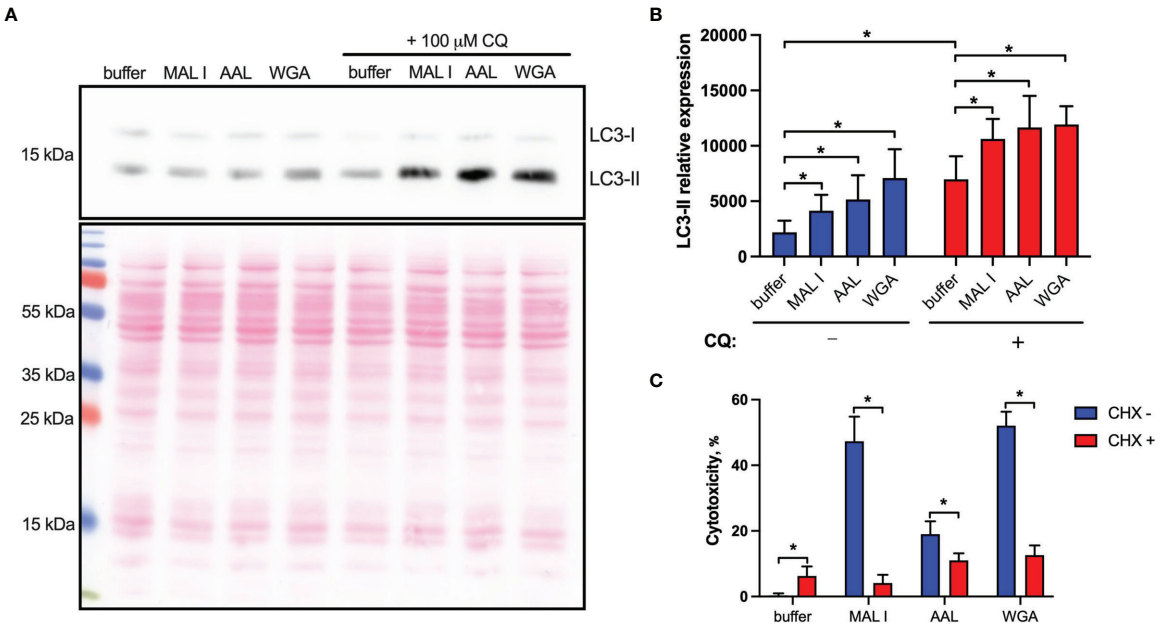

FIGURE 5 | Non-apoptotic effects of lectins in MC-38 cells. **(A)** Induction of autophagy in cells treated with lectins was measured by quantitative immunoblot analysis. MC-38 cells were treated with lectins with or without chloroquine (CQ) (100 μM) for 6 h followed by lysis with RIPA buffer and analysis by immunoblot staining. Chloroquine was added 4 h prior to cell lysis **(B)** Quantification of immunoblot data by densitometry image analysis using ImageJ software. LC3-II levels were quantified and normalized to total protein levels using Ponceau S staining. **(C)** Assessment of *de novo* protein synthesis requirement for cell death induction. MC-38 cells were treated with lectins either alone or in combination with cycloheximide (2.5 μg/ml) for 20 h followed by cytotoxicity measurement using LDH release assay. All data are from at least 6 replicates out of 2 independent experiments with four **(B)** or three **(C)** technical replicates each. *P < 0.05; unpaired two-tailed t-test.

cells with cycloheximide. In addition to inhibiting protein synthesis, cycloheximide is also known to block starvation-induced autophagy *via* activation of mTORC1 signaling (58). The addition of cycloheximide indeed strongly decreased the cytotoxic response of the three lectins investigated (**Figure 5C**). This finding further supported the caspase-independent nature of lectin-mediated cell death induction in MC-38 cells.

Glycan Binding and Cell Death Induced by Lectins in Non-Transformed YAMC Cells

The ability of lectins to bind and induce cell death was also evaluated in matched non-transformed colon YAMC cell line (**Figure 6**). As in MC-38 cell line, all lectins showed binding to YAMC cell surface glycans. The strongest binding was observed for WGA followed by ECL. MAL I, MAL II, AAL and SNA showed weaker binding that reflects various densities of cognate glycan structures. Compared to lectin binding to MC-38 cells, binding of WGA, ECL and SNA was noticeably higher in YAMC cells (**Supplementary Figure 4**).

The evaluation of cytotoxic responses mediated by lectins showed that all lectins used in the study mediated cytotoxic effects in a dose-dependent manner in YAMC cells. While ECL, AAL and SNA showed similar minimal levels of cell death, MAL I, MAL II and WGA showed the highest toxic effects (**Figures 7A–F**). It is unlikely that MAL II-mediated cytotoxicity observed only in YAMC cells was caused by various availability of glycans containing α2-3-linked sialic acids compared to MC-38 cells. The relative abundance of α2-3-linked sialic acid containing glycans was similar in YAMC and MC-38 cells as measured by flow cytometry and, therefore, variability in MAL II-meditated responses could be explained by possible resistance of MC-38 cells to programmed cell death induced by MAL II (**Supplementary Figure 4**). Altogether these results demonstrate that binding ability of all lectins and toxic effects of MAL I, AAL, and WGA are consistent in both MC-38 and YAMC cells.

DISCUSSION

Glycosylation is an important player in the regulation of cells death. Here, we demonstrated that MAL I, AAL and WGA did induce programmed cell death in MC-38 cell line in a caspase-independent manner, with involvement of multiple death signaling pathways, including components of apoptosis, necroptosis, pyroptosis, and autophagy. Furthermore, for MAL I- and WGA-mediated cytotoxicity, we observed the importance of sialic acid in mediation of programmed cell death by these lectins. Sialic acid may increase the binding of the corresponding lectins surface glycoproteins, thereby potentiating their cytotoxic effect. The absence of cytotoxic effects mediated by lectins that recognize sialic acid-containing glycan structures, namely MAL II and SNA, in MC-38 indicates that only glycans with certain linkage of sialic acids and the type of underlying sugars could be

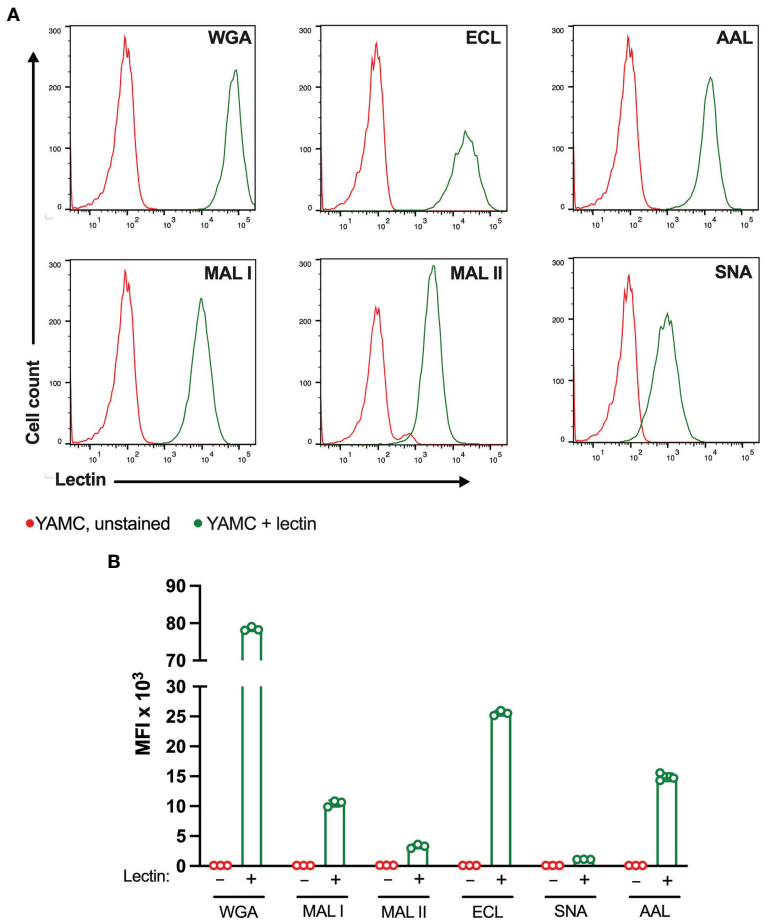

FIGURE 6 | Binding of plant lectins to non-transformed YAMC cells. **(A, B)** Binding ability of lectins used in the study to YAMC cells measured by flow cytometry. Unstained YAMC (red lines), stained YAMC (green lines). Each lectin was used at 10 μg/ml. Data are presented as mean and standard deviation of three replicates.

associated with lectin-mediated cytotoxicity in MC-38 cells. The observation of increased AAL toxicity in desialylated cells may indicate that negatively charged sialic acids may alter cytotoxicity of lectins which recognize other sugars by potentially affecting their ability to cross-link cell surface glycans while not having any negative impact on their binding ability. Moreover, the fact that ECL was only cytotoxic towards desialylated cells underlined the relevance of sialic acid removal, such as achieved by neuraminidase treatment, when targeting tumor cells (59).

One of the main features of cancer cells is their ability to resist to activation of programmed cell death *via* multiple mechanisms, including altered glycosylation and upregulation of pro-survival signaling pathways (60, 61). Compared with MC-38 cells, elevated cytotoxic responses of SNA and ECL correlated with their stronger binding to YAMC cells that indicates that lectin-mediated cytotoxicity could be dependent on the relative lectin binding strength. Among lectins used, WGA showed the highest binding ability in both YAMC and MC-38 cells with the lowest concentrations required for cell death induction that further supports the correlation between the ligand availability and cytotoxic response. Cytotoxicity of MAL II in YAMC was similar to WGA and MAL I cytotoxic responses but it did not correlate with its similar binding abilities in both cell lines which could potentially indicate an acquired resistance of cancerous MC-38 cells toward programmed cell death mode activated by this lectin.

Whereas we found that MAL I, AAL and WGA induced caspase-independent cell death, we also observed that programmed cell death induced in response to lectins relied on the pro-apoptotic BCL2 proteins BAK1 and BAX. These pro-apoptotic BCL2 proteins are known for their involvement in intrinsic apoptosis through formation of pores in the outer mitochondrial membrane (39, 62). In addition to their pore-inducing effect, BAK1 and BAX have also been shown to contribute to the execution of autophagic cell death by affecting permeability of lysosomes and autolysomes (56). Increased LC3-II levels in lectin-treated cells confirmed the increased synthesis of autophagy-related membranes and at the same time reduced degradation of LC3-II located on the inner side of autophagosomal membranes. Reduced LC3-II degradation could result from autophagy inhibition through alteration of lysosomal pH, as achieved by BAK1 and BAX

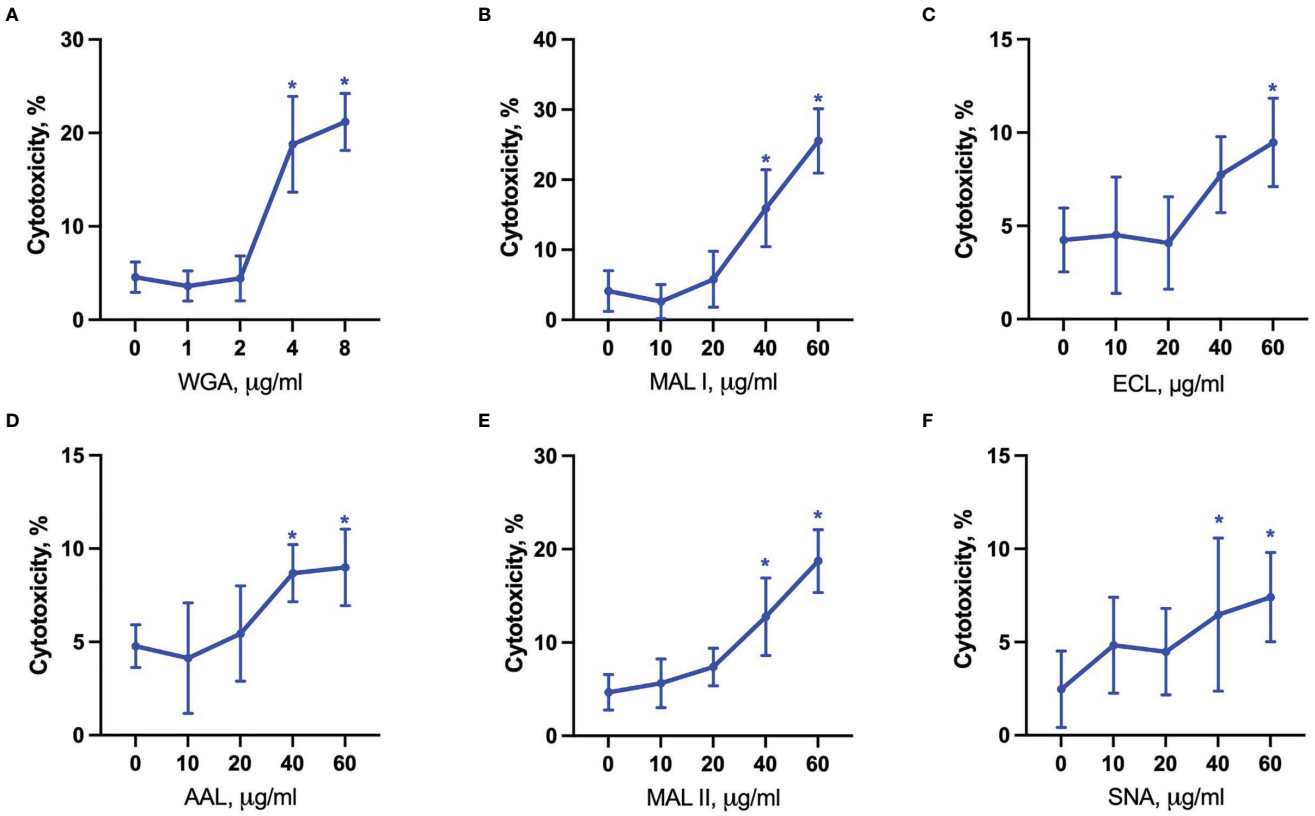

FIGURE 7 | Lectin-induced cell death in YAMC cells. Cytotoxicity was measured by propidium iodide and Hoechst 33342 staining of cells after treatment with WGA **(A)**, MAL I **(B)**, ECL **(C)**, AAL **(D)**, MAL II **(E)**, SNA **(F)** lectins in YAMC for 20 h at 33°C. Data represent averages and SD of 6 replicates out of 2 independent experiments. *$p < 0.05$; one-way ANOVA followed by Bonferroni's multiple comparison test.

ability to affect permeabilization of autolysosomes. The absence of caspase 9 cleavage as an indicator of the activation of intrinsic mitochondrial cell death, which is BAK1/BAX-dependent, further supports the notion that cell death in response to MAL I, AAL and WGA lectins relies on autophagic/lysosomal alterations rather than mitochondrial damage (**Figure 8**).

The involvement of MLKL, a main executioner of necroptosis, in cell death induced by the three lectins suggests a partial contribution of the necroptosis pathway in the process. After activation by RIPK3, MLKL oligomerizes and triggers permeabilization of the cell membrane (42). In addition to its role in necroptosis, MLKL also influences non-necroptotic pathways (63). But considering the inhibitory effect of cycloheximide on the cell death, necroptosis is unlikely a key pathway mediating cell death in lectin-treated cells.

In addition to sharing common features, such as caspase-independence and need for *de novo* protein synthesis, the cell death pathways induced by MAL I, AAL and WGA presented some differences as well. For example, loss of TRADD and caspase-8/RIPK3 only affected the cell death induced by MAL I, which pointed to a prevalence of the extrinsic pathway contributing to the cytotoxic effect of that lectin. Also, the contribution of GSDMD in the cytotoxic response was also unique to MAL I. Previous studies of Maackia amurensis agglutinin, which consists of both MAL I and MAL II, did not discriminate effects of these two lectins (22, 32, 64, 65). In the study, the results obtained showed that only MAL I, and not MAL II, induces cell death in MC-38 cells, while in YAMC cells both lectins showed similar levels of toxicity. The reasons for the lack of cytotoxicity of MAL II in MC-38 remains unknown but may be related to insufficient cross-linking of glycoproteins triggering a cell death response or to the inhibition of key signaling molecules required for the activation of MAL II-mediated cell death.

The lectins applied in this study induced programmed cell death in MC-38 cell line through multiple cell death pathways, which was expected considering the structural diversity of cell surface glycoconjugates able to initiate a cell death response when crosslinked. Whereas several receptor glycoproteins require clustering for activation, membrane glycosphingolipids, known to be essential components of lipid rafts, may also contribute to the cytotoxic effects of lectins as previously reported (66, 67). Considering that tumors often acquire resistance to various cytotoxic treatments because of mutations in cell death pathways or over-expression of survival signaling pathways, compounds inducing broad cytotoxic responses can

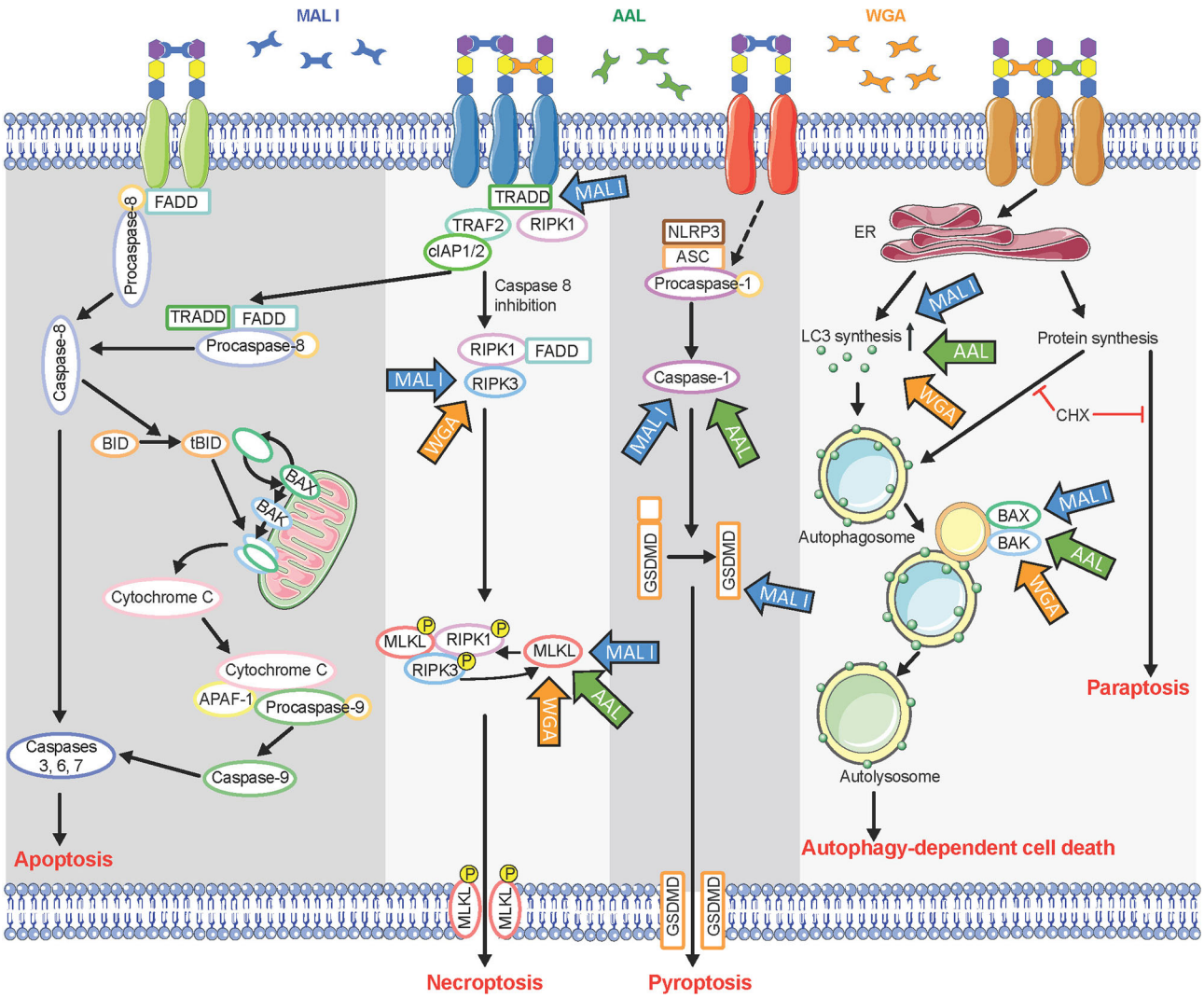

FIGURE 8 | Multiple cell death signaling pathways activated in response to lectin treatment. Glycan chains on surface glycoproteins are represented by colored hexagons. Blue, green, and orange arrows indicate components of cell death signaling pathways involved in the cytotoxic effects of MAL I, AAL, and WGA, respectively. CHX, cycloheximide.

be beneficial for the development of combination therapies. Therefore, lectins could be used as potent sensitizers for killing tumors that acquired resistance to apoptosis and other cell death pathways.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TH designed the study and secured the funding. AP planned and performed the experiments. AP and TH wrote the manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.797759/full#supplementary-material

Supplementary Figure 1 | Validation of gene knockouts in MC-38 by Western blot analysis. Immunoblot analysis of MC-38 lysates harboring knockouts in **(A)** BAX and BAK1, **(B)** FADD, **(C)** TRADD, **(D)** MLKL, **(E)** RIPK3 in mouse embryonic fibroblasts (MEF) and caspase-8 deficient MC-38 cells, **(F)** Caspase-8, **(G)** Caspase-1, and **(H)** GSDMD genes. Red text indicates clones that have been used

in the study. Images were acquired using either LAS-4000 (Fujifilm Life Science, Cambridge, USA) (panels **A, B, D–G)** or Fusion FX7 EDGE (Vilber, Marne-la-Vallée, France) (panels **C, H**) Western blot imaging systems.

Supplementary Figure 2 | Validation of GNE gene knockout in MC-38 by flow cytometry using ConA, MAL II, and ECL. GNE knockout was performed using sgRNAs targeting either exon 5 (clone 1) **(A)**, or exon 9 (clone 2) **(B)**. Each lectin was used at 10 µg/ml.

Supplementary Figure 3 | Verification of caspase 9 cleavage in MC-38 treated with lectins. **(A)** Immunoblot of caspase 9 in MC-38 treated with lectins and the positive controls cisplatin and TNF. **(B)** Validation of caspase 9 cleavage in MC-38 treated with cisplatin, equivalent volume of deionized water and mouse embryonic fibroblasts (MEF) untreated and treated with cytochrome C.

Supplementary Figure 4 | Binding of plant lectins to non-transformed YAMC cells using same flow cytometer settings as for MC-38. **(A)** Binding ability of lectins used in the study to YAMC cells measured by flow cytometry. Unstained YAMC (red lines), stained YAMC (green lines). **(B)** Comparison of lectin mean fluorescence intensities in MC-38 and YAMC. Each lectin was used at 10 µg/ml. Data are presented as mean and standard deviation of three replicates.

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REVIEW
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Polysialic Acid in the Immune System

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Polysialic acid (polySia) is a highly regulated polymer of sialic acid (Sia) with such potent biophysical characteristics that when expressed drastically influences the interaction properties of cells. Although much of what is known of polySia in mammals has been elucidated from the study of its role in the central nervous system (CNS), polySia is also expressed in other tissues, including the immune system where it presents dynamic changes during differentiation, maturation, and activation of different types of immune cells of the innate and adaptive response, being involved in key regulatory mechanisms. At least six polySia protein carriers (CCR7, ESL-1, NCAM, NRP2, ST8Sia 2, and ST8Sia 4) are expressed in different types of immune cells, but there is still much to be explored in regard not only to the regulatory mechanisms that determine their expression and the structure of polySia chains but also to the identification of the *cis*- and *trans*- ligands of polySia that establish signaling networks. This review summarizes the current knowledge on polySia in the immune system, addressing its biosynthesis, its tools for identification and structural characterization, and its functional roles and therapeutic implications.

Keywords: polysialic, glycan, immunity, sialic, glycosylation

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INTRODUCTION

The immune system is the repertoire of processes contained in the innate and adaptive responses that protect our organism from foreign antigens such as microbes, viruses, cancer cells, and toxins (1). In many of these processes, glycosylation is involved. Glycosylation is the biosynthesis and attachment of carbohydrate structures known as glycans to proteins, lipids, and RNA to form glycoconjugates, albeit they can also be found in a soluble form (2, 3). Glycans participate in different key aspects of the immune system, including the recognition of self and non-self. Additionally, the dynamics and richness of the biological information encoded in glycans and the effector functions of glycan binding proteins allow the fine-tuning and control of the immune response (4, 5).

The diversity of glycans results not only from the variety of linkages that are found between monosaccharides but also from different glycosylation pathways that occur in the endoplasmic reticulum/Golgi apparatus through the coordinated action of glycosyltransferases and glycosidases, as well as other types of proteins involved in supporting their function (nucleotide-sugar synthesis and transport, trafficking, organelle pH, etc.) (6).

A common feature of glycans that stands out is the presence of the negatively charged monosaccharide sialic acid (Sia) in the non-reducing terminus. The fine-tuning of the immune response is highly influenced by the presence/absence and type of linkage of Sia (7). Sialic acids are a family of monosaccharides characterized by a nine-carbon structure with a negative charge in the carboxylate (C1). The sialic acids found in mammalian organisms vary in their substituent at C5, which in *N*-glycolylneuraminic acid (Neu5Gc) is a glycolylated amino group, in *N*-acetylneuraminic acid (Neu5Ac) is an acetylated amino group, and in 2-keto-3-deoxy-nonulosonic acid (Kdn) is a hydroxyl group (7). The glycosidic linkage between the C2 of Sia and the underlying monosaccharide may be α2,3- or α2,6- to galactose (Gal) or *N*-acetylgalactosamine (GalNAc), or α2,8- to another Sia forming chains that vary in length as disialic acid (diSia), oligosialic acid (oligoSia), or polysialic acid (polySia) structures with degrees of polymerization (DP) of 2, 3–7, and 8–400, respectively.

The type of Sia, the chemical modifications it can be subject to including acetyl, sulfonyl, lactyl, methyl, and lactone groups, and the configuration of the glycosidic linkage constitute stereospecific biophysical information that cells can dynamically modify and also be sensed by specific endogenous glycan-binding proteins such as selectins, sialic acid-binding immunoglobulin-type lectins (Siglecs), or CD28, thus establishing key functional pathways in the immune response (8, 9).

In mammals, sialic acids are very abundant, and a single cell displays millions of Sia molecules (7). Sia-containing glycans, including polySia glycans, can work as immune checkpoints for differentiation, maturation, migration, tolerance, and activation, also being involved in the pathogenesis of inflammatory disorders and cancer (7, 10).

Mammalian polySia is characterized as a long polymer (8–400) of terminal α2,8-linked Sia that can be found on *N*-linked and *O*-linked glycans of a restricted group of glycoproteins. PolySia presents with a characteristic enormous hydrated volume and negative charge that strongly modulates the repulsion/attraction between cells (7, 11). Three polysialyltransferases (polySTs), ST8Sia 2, ST8Sia 3, and ST8Sia 4 with distinct tissue expression patterns are involved in the synthesis of polySia in the Golgi, using CMP-Sia as donor substrate (12). The ST8Sia 3 is considered a polyST on the basis that it is capable of autopolysialylation; however, other natural acceptors for polysialylation are not known (13, 14).

Aside from autopolysialylation of ST8Sia 2, ST8Sia 3, and ST8Sia 4 (13, 15, 16), eight other polySia protein carriers have been identified in mammals: chemokine receptor CCR7, CD36, E-selectin ligand1 (ESL-1), neural cell adhesion molecule (NCAM), neuropilin-2 (NRP2), megalin, skeletal muscle α-subunit of the voltage-gated sodium channel, and SynCAM 1 (17–24, 26) (**Table 1**). Of these, NCAM is by far the most studied and characterized and much of what is known on polySia has been obtained from understanding the role of polySia-NCAM in the central nervous system (CNS) (75). Nonetheless, polySia has been also identified in the immune system and can be expressed by different types of cancer cells (44, 76–78). As will be addressed in this review, polysialylated proteins expressed by different immune cells include CCR7, ESL-1, NCAM, NRP2, ST8Sia 2, and ST8Sia 4, although data indicate that other protein carriers remain to be identified.

Unlike α2,3- and α2,6-sialylated glycans, which have been easily screened in human cells using lectin panels that use Sia-binding lectins such as *Maackia amurensis* (MAA II; Siaα2,3) and *Sambucus nigra* (SNA; Siaα2,6) and that have been widely used to determine immune glycophenotypes, no lectins are available to detect polySia (79, 80). Additionally, because of its hydrodynamic arrangement, polySia has been difficult to structurally characterize (81). These challenges have lagged the identification of polySia in other tissues; nonetheless, anti-polySia antibodies with differential specificity for the DP are available, easily allowing the identification of polySia (82). Furthermore, there are now many structural techniques that allow characterization of polySia chains.

In this review, we will summarize the current knowledge on polySia in the immune system, addressing its biosynthesis, its tools for identification and structural characterization, and its functional roles and therapeutic implications.

BIOSYNTHESIS OF POLYSIA IN MAMMALS

PolySia is a unique posttranslational modification that consists in linear polymer forms of Sia, joined internally by α2,4, α2,5 *O-glycolyl* α2,8, α2,9, and α2,8/9 linkages (82). In humans, polySia is exclusively formed by the polymeric elongation at position C8 of α2,3- or α2,6-linked Sia, although little is known about the incorporation of dietary Neu5Gc (83).

PolySia was first identified in gram-negative bacterial polysaccharides from pathogens such as *Escherichia coli* K23 and the *Neisseria meningitidis* groups C and B (84, 85). Nonetheless, it is widely expressed in glycoconjugates of the cell surface from bacteria to different types of human cells, although most of its characterization has occurred in CNS tissues (86, 87).

The biosynthesis of polySia in humans requires the synthesis of CMP-Sia that begins with the assembly of monomeric blocks of Sia through several biosynthetic steps (88, 89) (**Figure 1**). The rate-limiting stage occurs during the conversion of UDP-GlcNAc into *N*-acetylmannosamine-6-phosphate (ManNAc6P) by a single- and dual-UDP-GlcNAc-2 epimerase/ManNAc kinase enzyme (GNE). ManNAc6P is then condensed by the sialic acid synthase (NANS) with phosphoenol-pyruvate resulting in *N*-acetyl-9-phosphoneuraminic acid (Sia9P), followed by dephosphorylation catalyzed by Neu5Ac-9-P phosphatase (NANP). Sia is then translocated into the nucleus where the CMP-Sia synthase (CMAS) activates Sia by transferring the CMP moiety from CTP to the β-anomeric hydroxyl group at C2 of Sia in the presence of Mg^{2+} (90, 91). Unlike all other eukaryotic nucleotide sugar synthetases which are expressed in the cytoplasm, the eukaryotic CMAS enzyme is predominantly located in the nucleus (92). CMP-Sia is then transported to the cytosol by an unknown mechanism, and subsequently, the nucleotide sugar is translocated to the Golgi lumen by the action of the CMP-Sia transporter (SLC35A1) where it is used as a donor substrate by sialyltransferases (STs) for subsequent addition to glycoconjugates

TABLE 1 | Mammalian polysialylated proteins.

Protein	Molecular size	PolySia glycan	PolyST	Function	Immune cell expression
CCR7	378 aa	*N*- and *O*-glycans (25)	ST8Sia 4 (25)	Lymphocyte and DC homing to the lymph nodes and intestinal Peyer's patches (27, 28)	Activated B cells, naive T cells, regulatory and memory T cells, NK cells, and DCs (29).
CD36	472 aa	*O*-glycan (18)	n.d.	In milk, it is involved in protection and nutrition during neonatal development (18). Programming cognitive development (30). Exogenous LCFA transmembrane transport in lactating mammary glands (31).	Mononuclear phagocytes (32). Polysialylation of CD36 in these cells has not been determined.
ESL-1	1179 aa	*O*-glycan (24)	ST8Sia 4 (24)	E-selectin ligand in the Leukocyte adhesion cascade (33).	DCs, monocytes, myeloid cells, and neutrophils (33, 34).
Megalin	4655 aa	*O*-glycan (22)	n.d.	Receptor of apolipoprotein E, Ca^{2+}, vitamin B12, polypeptide hormones, and tissue-type plasminogen activator in complex with type-1 inhibitor (22).	n.d.
NCAM	858 aa	*N*-glycan (35, 36)	ST8Sia 4 in immune system (37, 38). ST8Sia 2 and ST8Sia 4 in CNS (39).	Marker for NK cells, high expression in active cytotoxic NK cells (40), mobilization of hematopoietic progenitors (41). Synaptic plasticity, cell adhesion, axon growth and fasciculation in the CNS (42).	DCs, hematopoietic progenitors, microglia, monocytes, neutrophils, NKs (43, 44).
NRP2	931	*O*-glycan (23, 45, 46).	ST8Sia 4 (45, 46).	Receptor for specific isoforms of vascular endothelial growth factors (VEGF) family and for class 3 semaphorins (SEMA3) (47). Angiogenesis (48). Development of selective cranial and sensory nerves, axon guidance, tumorigenesis, vascularization, and cardiovascular development (49–54).	DCs, macrophages and monocytes (23, 43).
Skeletal muscle α-subunit NaV1.4	1836 aa	*N*-glycan (55, 56)	ST8Sia 4 (55, 56)	Generation of action potential in skeletal muscle cells (57).	n.d.
ST8Sia 2	375 aa	*N*-Glycan (58).	ST8Sia 2 (59).	Polysialylation of ST8Sia 2 (autopolysialylation), NCAM in CNS and SynCAM 1 (45).	DCs, hematopoietic precursors, macrophages, monocytes and CD4+ T cells (23, 43, 60).
ST8Sia 3	380 aa	*N*-glycan (13)	ST8Sia 3 (14).	Transfer of polySia and oligoSia to ST8Sia 3 and to NCAM (14, 61). Selective sialylation of several striatum-enriched membrane proteins, adding α2,8-diSia and α2,8-triSia units (62).	n.d.
ST8Sia 4	359 aa	*N*-glycan 16, 63) *O*-glycan	ST8Sia 4 (16, 63)	Transfer of polySia and oligoSia to ST8Sia 4 (autopolysialylation), NCAM, NRP2, ESL-1 and CCR7 (45).	DCs, hematopoietic precursors, macrophages, microglia, monocytes, neutrophils, NK cells, thymocytes, RTEs, CD4+ T cells, CD8+ T cells and B cells (23, 24, 60, 64–67).
SynCAM 1	375 aa	*N*-Glycan (68)	ST8Sia 2 (69)	SynCAM 1 in DCs stimulates IL-22 expression in activated $CD8^+$ T-cells (70). In mast cells, SynCAM 1 along with MITF are essential for development and survival of mast cells *in vivo* (71). Involved in Cell adhesion, epithelial integrity and thymus development (70, 72).	Mast cells and DCs (73, 74). Polysialylation of SynCAM 1 in these cells has not been determined.

aa, amino acids; n.d., not determined.

(**Figure 1**) (15, 93). In vertebrates, STs are classified into four groups (ST6Gal, ST6GalNAc, ST3Gal, and ST8Sia) according to the glycosidic linkage formed and the sugar acceptor specificity (94, 95). It is important to mention that synthetic derivatives of ManNAc (ManNR) or Sia (SiaNR) can be used to metabolically label sialic acid; examples include ManNAz and SiaNAz where *N*-acetyl is replaced with *N*-azidoacetyl (9).

The Polysialyltransferases

The ST8Sia enzymes that synthesize the α2,8 Sia linkage, ST8Sia 1 through 6, on glycoproteins or glycolipids (**Table 2**), belong to the CAZy glycosyltransferase family GT29 that show four consensus motifs called sialylmotifs large (SML), small (SMS), motif III (M3), and very small (SMVS) involved in substrate binding, and catalysis (103, 104). In vertebrates, the ST8Sia enzymes have been characterized in fish, mice, and humans (95, 105, 106). Based on sequence analysis, the ST8Sia enzymes can be grouped in monoSTs (ST8Sia 1, 5, and 6) or oligo- and polySTs (ST8Sia 2, 3, and 4) (107). The acceptor substrate specificities and products for the ST8Sia enzymes are shown in **Table 2**.

The polySTs show a similar structure and are characterized by two motifs likely involved in substrate binding and polySia chain elongation named polysialyltransferase domain (PSTD) of 32 aa located upstream of the SMS, and the polybasic region (PBR) made up of 35 aa that is in the stem region of the enzymes. All the members of the ST8Sia family reside in the Golgi apparatus and possess the catalytic domain tethered to the membrane *via* an N-terminal region and a type II transmembrane domain (107, 108). The amino acid (aa) sequence of the human polyST ST8Sia 4 has 59% identity with that of ST8Sia 2 (109), while the sequence of the human ST8Sia 3 has 33.3% and 34.8% identity with the human ST8Sia 2 and ST8Sia 4, respectively (14).

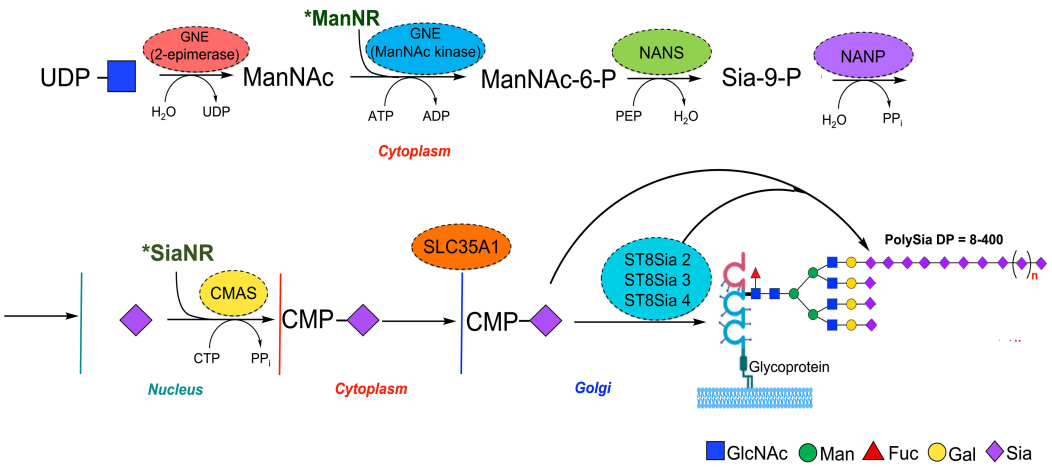

FIGURE 1 | Schematic representation of mammalian polySia biosynthesis. Biosynthesis of PolySia is based on the building block of monomers of Sia through conversion of UDP-GlcNAc into ManNAc by the GNE bifunctional enzyme (epimerase) and subsequently to ManNAc-6-phosphate (kinase). The ManNAc-6-phosphate is then transformed into Sia-9-P by the N-acetylneuraminate synthase (NANS) and dephosphorylated by Sia-9-P-phosphatase (NANP) to yield Sia. After that, Sia is transferred to the nucleus and conjugated with cytidine monophosphate (CMP) by the CMP-Sia synthase (CMAS) and translocated into the Golgi by the CMP-sialic acid transporter SLC35A1, where sialyltransferases, including ST8Sia 2, 3, and 4, use it as donor substrate for polySia synthesis. Synthetic derivatives of ManNAc (ManNR) and Sia (SiaNR) can be used to metabolically label Sia glycans, including polySia.

Regarding NCAM polysialylation in the CNS, it has been observed that both ST8Sia 2 and 4 add polySia to *N*-glycans attached to NCAM more efficiently than to *N*-glycans released from NCAM and that the amount of polySia synthesized by both enzymes is higher than the one obtained by either enzyme alone, exhibiting a synergistic effect (14, 61). Concerning acceptor preferences, ST8Sia 4 is more able with respect to ST8Sia 2 to add polySia to oligosialylated and unpolysialylated antennas in *N*-glycans attached to NCAM, even when polySia is attached to at least one of the other antennas (61). Nonetheless, not all cells express both enzymes and polySia synthesis can be dictated by either ST8Sia 2 or ST8Sia 4. In fact, NCAM polysialylation in immune cells is established by ST8Sia 4 and not by ST8Sia 2 (43).

Autopolysialylation of polySTs is apparently not required to polysialylate NCAM (16, 59, 63, 110). However, ST8Sia 2 and 4 autopolysialylation is required for NRP-2 polysialylation and promotes SynCAM 1 polySia chain elongation (45). Noteworthily, the polyST ST8Sia 3 is also capable of autopolysialylation and presents the PSTD and PBR conserved in ST8Sia 2 and ST8Sia 4 (14, 107, 108). The influence of autopolysialylation in the enzymatic activity of ST8Sia 3 has not been determined.

ST8Sia 3 has not been found to naturally polysialylate NCAM or other known substrates (14, 107, 108). The study of an ST8Sia 3 KO mouse model revealed that ST8Sia 3 is responsible for the selective sialylation of several striatum-enriched membrane proteins, adding α2,8-diSia and α2,8-triSia units to its substrates (62). Nonetheless, these data should be taken cautiously when studying non-neural cells, such as immune cells.

Due to the anti-adhesive properties derived of its large exclusion volume and hydration, polySia can reduce the homophilic or heterophilic interaction in the same membrane (*cis* interaction) or in another cell membrane (*trans* interaction) exhibiting repulsive properties (12). PolySia-repulsive properties are involved in neural cell migration, axonal guidance, fasciculation, myelination, synapse formation, and functional plasticity of the nervous system. In contrast, polySia can also form an attractive field when interacting with soluble molecules such as neurotransmitters, growth factors, and neurotrophic factors directing in many cases binding and release, acting as a reservoir of these molecules on the neural cell surface and as a regulator of the local concentration by condensing them and inhibiting their diffusion (111–113).

It has been shown that polySia binds to brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, forming a complex that allows binding to the BDNF receptor, TrkB, and p75NTR, increasing growth and/or survival of neuroblastoma cells (12). The formation of the BDNF-polySia complex is directly dependent on chain length and requires a DP=12 (12).

Repulsion in polySia-NCAM is differentially regulated by both ST8Sia 2 and ST8Sia 4. Through surface plasmon resonance, it was shown that polySia-NCAM presented different molecule-binding properties depending on the polySTs involved in its synthesis. The polySia-NCAM synthesized by ST8Sia 2 showed a repulsive property toward polySia-NCAM and an attractive field toward BDNF and FGF2 (114). In contrast, polySia-NCAM synthesized by ST8Sia 4 showed only attractive properties toward polySia-NCAM, BDNF, FGF2, and dopamine. This is a consequence of FGF2 and BDNF binding to polySia with DP≥17 and DP≥12, respectively, and as ST8Sia 4 synthesizes larger polySia chains with respect to ST8Sia 2, then polySia synthesized by ST8Sia 4 binds greater amounts of BDNF and FGF2 compared to polySia synthesized by ST8Sia 2 (115). The repulsive field has been observed only in polySia synthesized by ST8Sia 2, but not by

TABLE 2 | Preferred acceptor substrates and products for the human ST8Sia family.

Enzyme	Substrate	Product	Ref.
ST8Sia 1	GM3, GD1a, GT1b gangliosides	GD3, GT1a, and GQ1b gangliosides	(96, 97)
ST8Sia 2	Monosialylated *N*-glycans	DiSia, oligoSia, and polySia (30DP)	(45)
ST8Sia 3	Monosialylated *N*-glycans Keratan sulfate *O*-glycans	DiSia, triSia, oligoSia, and polySia (only autopolysialylation)	(13, 14, 62, 98)
ST8Sia 4	Monosialylated *N*-glycans Oligosialylated *N*-glycans Mucin type *O*-glycans	DiSia, oligoSia, and polySia (50DP)	(39, 46)
ST8Sia 5	GD3, GM1b, GD1a, GT1b, GQ1c	GT3, GD1c, GT1a, GQ1b, GP1c	(99, 100)
ST8Sia 6	α2,3-Sialylated core 1 *O*-glycans	Disia *O*-glycoproteins	(101, 102)

STs from the ST8 family catalyze the transfer of Siaα2,8 to different glycoprotein and glycolipid substrates.
diSia, disialic acid; oligoSia, oligosialic acid; polySia, polysialic acid; triSia, trisialic acid.

ST8Sia 4; however, it is not clear how this homophilic repulsion takes place. This important reservoir function performed by polySia on NCAM has not been explored regarding NCAM-expressing immune cells or other polysialylated proteins.

METHODOLOGIES OF POLYSIA ANALYSIS

The DP of polySia chains has a critical importance in regulating their function. Nonetheless, even with sensitive methods to accurately determine the basic structure, polySia structural characterization, including DP, is still a challenge due to its large size, negative charge, and structural heterogeneity. Some approaches broadly used for polySia analysis may be organized as structural and qualitative, structural and quantitative, quantitative and semiquantitative, or qualitative (**Table 3**) (87, 133).

The structural characterization of polySia requires in most cases a combinational analysis or high-throughput analysis techniques (133). One of the first analytical approaches used for polySia analysis was thin-layer chromatography (TLC) of mild acid hydrolysate of polySia, using resorcinol as a visualization reagent (116, 124, 134, 135). Nowadays, structural and qualitative analyses such as MALDI-TOF and ESI-MS offer many advantages over traditional analytical methods, including low sample consumption and high sensitivity (117, 135).

Furthermore, the structural and quantitative analyses of polySia consists of several steps such as mild acid hydrolysis, derivatization, and HPLC analysis of DMB-polySia derivatives that allow determination of polySia DP and glycosidic linkages. One of the most suitable and recommended approaches to polySia analysis is high-performance anion-exchange chromatography with pulsed amperometry detector (HPAEC-PAD) (136). The major advantage of HPAEC-PAD is that this approach requires no derivatization for sensitive detection and allows the inclusion of different detectors offering diverse ways to detect polySia such as fluorescence (HPAEC-FD), ultraviolet (HPAEC-UV), and corona charged aerosol detection (HPAEC-CAD). HPAEC analysis can characterize poly/oligosaccharides using a pellicular anion-exchange resin and sodium hydroxide phase (87). The Neu5Ac and Neu5Gc residues are stable in alkaline eluents, but sialic acids containing *O*-acyl substituents are unstable, then a gradient of sodium acetate is recommended. Within quantitative methods, periodate fluorometric C7/C9 analysis is available to detect inter Sia residues of oligo and polySia. It consists of periodate oxidation of Sia residues and fluorescent labeling with DMB detecting α2,8-linked oligo/polySia structures using combinatorial platforms such as reverse-phase HPLC with fluorescence detection (RPLC-FD) (116, 117, 136, 137). An additional approach that provides similar results and uses soft acid conditions is mild acid hydrolysis–fluorometric anion-exchange chromatography method (MH-FAEC), recently adapted for oligo-PolySia analysis (138).

The semiquantitative or qualitative analysis represented by Western blot (WB) and quantitative analysis such as ELISA and flow cytometry are mostly based in immunodetection of polySia (138, 139). A disadvantage of ELISA is that it cannot distinguish polySia of different chain lengths and WB only offers relative quantification of polySia based on differential densitometry associated with chemiluminescent and fluorescent signal obtained from blots. Unfortunately, it is frequent in WB to visualize polySia as wide and smeared bands that difficult quantification. Flow cytometry is a rapid high-throughput approach that allows detection and measurement of polySia expression in cells by detecting fluorescence intensity using anti-polySia antibodies conjugated with fluorophores or fusion proteins such as GFP-tagged engineered endoneuraminidase enzyme (EndoN-GFP) that has been modified to bind but not digest polySia (140). Advantages and disadvantages for several methodologies to analyze polySia are described in **Table 3**.

The anti-di/oligo/polySia antibodies can be classified into three groups based on their specificity for chain DP (44, 82) (**Table 4**). The group I antibodies are the "anti-polySia antibodies" that recognize chains of α2,8-linked Sia with ≥DP 8, including fully extended polySia chains. These antibodies recognize the helical conformation formed by Sia residues within the internal region of the polySia chains. The non-reducing terminal residues are not involved in antigen recognition. The group II antibodies recognize

TABLE 3 | Methods for oligoSia/polySia analysis.

Classification	Method	Advantages	Disadvantages	References
Structural and qualitative analyses	TLC	• Resolves oligoSia from polySia chains. • Applied to study the DP of oligoSia composed of different Sia isomers • Easy adaptability and inexpensive.	• Poor resolution of polySia with greater than 10 Sia units. • Requiring at least 1 μg of analyte.	(116)
	MALDI-TOF MS	• Determines exact mass composition and DP of polySia. • Preferential detection of unmodified peptides and partial or complete suppression of glycopeptides. • Low quantities (ng) of polysialylated proteins can be analyzed. • Discerns between α2,9 linked polySia from α2,8 linked polySia. • PolySia of DP up to 100, and 40 Sia units have been successfully detected.	• Poor tolerance to sample impurities. • Remotion of peptides and enrichment of glycopeptides is needed. • The mass accuracy and the resolution of the signals are reduced in the linear mode and did not allow an identification of incompletely lactonized species.	(117)
	ESI-MS	• Determines linkages and structure. • Low quantities from 10 to 20 pmol of the compound with Sia moieties can be analyzed. • Sia dimers, trimers, and tetramers can be detected with higher efficiency.	• Derivatization approach is important to analyze sialylated glycans without losing terminal sia groups.	(118, 119)
Structural and quantitative analyses	HPAEC-PAD	• Allows detection of all non-volatile and most semi-volatile analytes. • It is not necessary to derivatize samples. • PolySia can be quantified by coupling HPAEC with a detector based on amperometry, fluorescence, UV absorbance, or mass spectrometry.	• To detect DP 50 is necessary 10 μg of purified polySia samples. • Epimerization and degradation of carbohydrates. • Unstable baseline, loss of sensitivity, and requirement of a dedicated base compatible HPLC.	(120, 121)
	HPAEC-FD	• Widely employed method. • High sensitivity. • Can detect polySia with DP > 90 • Amount of 200 ng derivatized colominic acid have been analyzed. • Can also detect polySia from tissues with DP ranging from 18 to 60	• Derivatization process with DMB requires acidic conditions and longer periods of incubation. • Cationic charges getting for derivatized polySia are critical for separation.	(122)
	HPAEC-UV	• Quantify-free polySia with a resolution of up to 25 Sia units. • Does not require derivatization.	• Poor selectivity using short UV wavelength (210 nm) by increasing background. • High sample purity is necessary. • Among 10 μg purified polySia for analysis	(123, 124)
	HPAEC-CAD	• CAD separates polySia of higher degree of polymerization >90 colominic acid units. • Can detect DP among 65 and 130 Sia residues. • Does not require derivatization. • Less time than other methods. • Less specific and less sensitive than HPAED-FD. • Lower amount (250 ng)	• Large amount of polySia polymer is necessary. • Restricted to volatile buffers • Decreased resolution by increased salt. • High standards of sample purity, compared to fluorometric detection.	(125–127)
Quantitative analysis	ELISA	• Reproducible and reliable method. • High specificity and sensitivity. • Can analyze very small samples. • Rapid and accurate for quantitation of total polysialylated proteins.	• The method cannot distinguish polySia of different chain lengths.	(128)
	Flow cytometry	• Can detect polySia on the surface of intact cells. • The anti-PolySia antibodies and Endo N-GFP fusion proteins can be used in flow cytometry allowing the analysis of the number of polySia positive cells. • It is selective and sensible.	• To corroborate PolySia antibody specificity, sometimes it is necessary to use the Endo N enzyme. • Specificity is associated with antibodies and controls must be used. • The exact DP is not possible to determine.	(129, 130)
	Fluorometric C7/C9	• Highly sensitive and selective analysis of internal Sia residues of oligo- and polySia. • Internal Sia residues that remain unaffected can be analyzed by HPLC-FD after fluorescence derivatization.	• False positive quantitative results for internal Sia residues of polySia. • The method allows oxidation of α2,9 linked polySia.	(131, 132)

(Continued)

TABLE 3 | Continued

Classification	Method	Advantages	Disadvantages	References
		• Detection of 1-ng amounts of internal Sia residues of oligo- and polySia molecules.		
Semiquantitative or qualitative analysis	Western blotting	• Expression levels of polysialylated-proteins. • Associated chemiluminescent and/or fluorescent signals. • High specificity. • Detects polySia residues with 735 antibody and 12E3 antibody recognizes oligo/polySia.	• Less accurate to quantify polysialylated-proteins. • Smeared bands. • The quantity of polySia determined is lower than with the ELISA method.	(58)

TLC, thin layer chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization mass spectrometry; ESI-MS, electrospray ionisation mass spectrometry; HPAEC, high-performance anion exchange chromatography; PAD, pulsed amperometric detection; FD, fluorometric detection; UV, ultraviolet detection; CAD, charged aerosol detector; ELISA, enzyme-linked immunosorbent assay.

TABLE 4 | Anti-oligoSia/polySia antibodies.

Group	Clone	Organism	Immunoglobulin type	Immunogen	Type of sialic acid recognized	DP specificity
I) Anti-polySia	H.46	Horse	poly, IgM	*Neisseria meningitidis GpB*	Neu5Ac	DP≥8
	735	Mouse	Mono, IgG2a	*Neisseria meningitidis GpB*	Neu5Ac	DP≥11
II) Anti-oligoSia + anti-polySia antibody	12E3	Mouse	Mono, IgM	Embryonic rat forebrain	Neu5Ac	DP≥5
	5A5	Mouse	Mono, IgM	Membrane from embryonic rat spinal cord	Neu5Ac	DP≥3
	2-2B	Mouse	Mono, IgM	*Neisseria meningitidis GpB*	Neu5Ac	DP≥4
	OL.28	Mouse	Mono, IgM	Oligodendrocyte from newborn rat	Neu5Ac	DP≥4
	2-4B	Mouse	Mono, IgM	Oligo/polyNeu5Gc-PE	Neu5Gc	DP≥2
	Kdn8kdn	Mouse	Mono, IgM	KDN-gp	KDN	DP≥2
III) Anti-oligoSia antibody	S2-566	Mouse	Mono, IgM	Human GD3	Neu5Ac	DP=2
	1E6	Mouse	Mono, IgM	(Neu5Ac)2-bearing artificial glycopolymer	Neu5Ac	DP=2
	AC1	Mouse	Mono, IgG3	(Neu5Gc)GD1c	Neu5Gc	DP=2–4
Other	12F8	Rat	Mono, IgM	Mouse membrane fraction	Unknown	Unknown

Poly, polyclonal; mono, monoclonal.

both oligoSia with DP = 2–7 and polySia chains. These antibodies recognize the distal portion of oligo/polySia chains, including the non-reducing terminal. The group III antibodies recognize oligoSia with DP = 2–4, but do not bind to polySia. These antibodies appear to recognize specific conformations of di/oligoSia with DP = 2–4. The combinatory use of antibodies allows estimation of polySia DP. The two most used anti-polySia antibodies are the monoclonal 12E3 antibody (IgM) that recognizes the non-reducing terminal residue of oligoSia/polySia acid structures with DP≥5 and the monoclonal 735 antibody (IgG) that recognizes the internal sialyl residues of polySia structures with DP≥11.

Finally, the use of neuraminidases is a helpful resource to identify the presence or composition of Sia in cells. EndoN is a phage enzyme that specifically degrades α2,8 Sia polymers, diffusing rapidly in tissues and capable of degrading polySia in cultured cells (141). EndoN is highly specific for polySia and requires a minimum DP of 5 to act. Higher DP 150–200 are better substrates for EndoN than oligomers DP 10–20 (142). Treatment with EndoN of purified or whole lysates is indicated to confirm anti-polySia antibody specificity (23). The EndoN-treated proteins can be analyzed by WB to confirm a weight shift related to polySia; alternatively, the liberated glycan product can be subjected to structural analysis. EndoN can also be incubated in cell cultures *in vitro* to evaluate the functional roles of polySia, as well as real-time fluorescence microscopy (143) and flow cytometry (144). Injected EndoN has also been used *in vivo* to evaluate the role of polySia (145).

POLYSIA IN THE INNATE IMMUNE RESPONSE

The innate immune response is the body's first line of defense against pathogens entering the body. The innate immune response uses different mechanisms to stop the spread of infections such as mucous membranes, physical barriers, defense cells, and proteins (4). Sia are essential epitopes which have an important role in self-recognition and regulation of immune system cells (7, 146). The polySia mediates cell–cell interactions and promotes signaling through steric and electrostatic exclusion, making polysialylated glycans key participants in migration and inflammation (8, 82). Additionally, polySia is considered as a recognition pattern and immune regulator in the immune innate response (7). Further research is required to fully elucidate the receptors through which polySia acts, including the known interactions with DC-SIGN and Siglecs. In this regard, the identification of Siglecs as receptors for sialic acid–containing glycans, including polySia, is quite interesting in view of their expression on most white blood

cells and their critical role in immune cell signaling, as well as to distinguish between self and non-self (147). PolySia is also known to bind the myristoylated-alanine-rich C-kinase substrate (MARCKS) expressed in neurons to modulate neuritogenesis (148); interestingly, although MARCKS is also expressed in neutrophils and macrophages, its interaction with polySia in these cells has not been reported (149, 150).

Bone Marrow Hematopoiesis

PolySia has been detected in murine bone marrow (BM) cells and myeloid precursors, using anti-polySia monoclonal antibody (mAb) 735 and a green fluorescent protein (GFP)-fused and inactive endoglycosidase N (EndoN-GFP), which binds specifically to but does not cleave α2,8 polySia. PolySia was detected on the surface of >60% of cells aspirated from the murine BM, with ST8Sia 2 and ST8Sia 4 gene expression being detected in BM cells (43). Nonetheless, ST8Sia 4 was shown to be the one responsible for polySia synthesis of BM resident populations and during myeloid differentiation as was observed when the expression of polySia was conserved in BM cells from ST8Sia $2^{-/-}$ mice and absent in cells from ST8Sia $4^{-/-}$ mice (43, 130) (**Figure 2A**). Further analysis of activity and expression of ST8Sia 2 might help to explain the mechanism that leads to ST8Sia 4 as responsible for polySia synthesis in BM cells.

Murine BM polySia expression has been correlated with receptor tyrosine kinase c-kit (hematopoietic progenitor marker) and defines four BM subsets from a common myeloid lineage: $polySia^{neg}/Kit^{high}$, $polySia^{low}/Kit^{high}$, $polySia^{high}/Kit^{high}$, $polySia^{low}/Kit^{low}$). The $polySia^{neg}/Kit^{high}$ subset contained hematopoietic stem cells ($c\text{-}Kit^{+}$, $Sca\text{-}1^{+}$), the $polySia^{low}/Kit^{high}$ population, committed multipotent progenitors ($CD34^{+}$), and the $polySia^{high}/Kit^{high}$, $polySia^{low}/Kit^{low}$ groups, immature, and mature myeloid cells, respectively (130). In contrast to murine BM cells, human fetal BM cells, myeloid cells, and peripheral myeloid precursors cells do not express polySia. Differences between murine and fetal human BM cells were attributed to the intrinsic polySia expression variability during development (130).

Also, polySia has been detected in murine BM neutrophils and monocytes/macrophages that express the myeloid lineage markers CD11b (Mac-1) and/or Ly6 G/C (Gr-1). PolySia was not detected on the surface of lymphocytes or erythroid cells. PolySia was found to be controlled by expression levels of ST8Sia 4 and

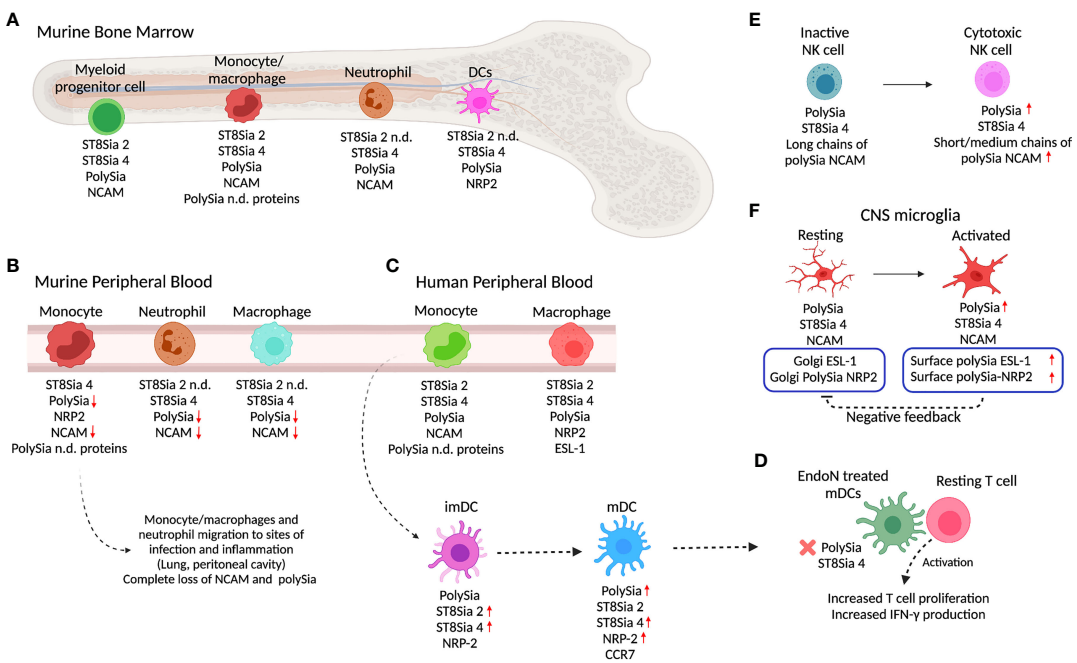

FIGURE 2 | PolySia and polyST expression in innate immune cells. **(A)** In the murine bone marrow (BM), different myeloid progenitor cells, monocytes/macrophages, neutrophils, and DC express polySia, ST8Sia 2, ST8Sia 4, NRP2, NCAM, or non-identified polySia proteins. **(B)** As they migrate into the peripheral blood, murine BM-derived monocytes/macrophages and neutrophils lose polySia-NCAM. As the macrophages and neutrophils arrive at infection or inflammation sites, polySia is depleted from the cell surface. **(C)** In the periphery, human macrophages and monocyte-derived cells express polySia and polySia carriers such as NRP2, NCAM, ESL-1, and unknown proteins. Once monocytes differentiate into imDC, they express polySia-NRP2 which in turn is upregulated during maturation to mDC. **(D)** Depletion of polySia by using EndoN neuraminidase in mDCs promotes increased T cell activation. **(E)** NK cells overexpress polySia and ST8Sia 4 during differentiation into cytotoxic cells. Chain length differentiates between non-cytotoxic and cytotoxic NK cells. **(D)** The depletion of polySia in mDC by using EndoN induces better activation and increased proliferation of T cells, suggesting a role of polySia in regulation of T cell activation. **(F)** In the innate immunity of the CNS, microglia cells also express polySia. The increase in polySia-NRP2 expression in the surface of microglia cells induces negative feedback on Golgi polySia-NRP2 localization and expression. The increase or decrease of polySia expression with respect to the precursor state is indicated with red arrows (up or down, respectively). Created with BioRender.com.

associated with cell surface NCAM, as well as to other unknown polysialylated proteins in certain subpopulations of murine BM monocytes (43).

During migration of murine BM cells, polySia is shed from the surface of monocytes by cleavage of NCAM as they are released from BM into the PB during early stages of monocyte differentiation and completely lost when they localize in pulmonary and peritoneal sites of inflammation (43) (**Figure 2A**). This is probably due to metalloprotease-induced ectodomain shedding that has been described in neurons (151). Additionally, in ST8Sia $4^{-/-}$ mice lacking polySia-NCAM that is required for the mobilization of hematopoietic progenitors from the BM to the thymus with lack of polySia causes retention in the BM and improper access to the thymus for maturation (41).

Differently to murine BM monocytes/macrophages where ST8Sia 2 and ST8Sia 4 are expressed, murine BM neutrophils express only the ST8Sia 4 associated with the biosynthesis of polySia-NCAM which is also progressively lost by protein cleavage as they migrate from the BM to the PB and completely lost when they localize in pulmonary and peritoneal sites of inflammation (**Figures 2A, B)** (43). It has been proposed that the regulation of polySia in monocyte populations might be associated with discrimination of cells on functional grounds or lineage commitment; however, the loss of polySia could also allow migration by increasing the overall negative charge, reducing cell–cell contact, and regulating the differentiation and maturation of different cell subsets.

Dendritic Cells

The dendritic cells (DCs) present antigen to naive T cells at specific intercellular junctions called immunological synapses (152). Monocytes are precursors of peripheral non-lymphoid organ DCs and migratory DCs under inflammatory conditions (153). Maturation of DCs is associated with reprogramming of the glycosylation machinery, especially sialylation (154).

NRP2 is a protein expressed by murine and human DCs and known to be polysialylated (23, 43) (**Figures 2A, C)**. Human peripheral monocytes express both polySTs but do not express polySia-NRP2. The *de novo* NRP2 protein expression and polysialylation occur during differentiation into immature DCs (imDCs) mediated by IL-4 and GM-CSF. The monocyte-derived imDCs upregulate ST8Sia 2 and ST8Sia 4 polyST and increase polySia expression (**Figures 2A, C)** (23). After LPS stimulation to induce maturation of human imDCs into mDCs, ST8Sia 4 is highly overexpressed while ST8Sia 2 remains at the same level as imDCs. In human mDCs, polySia-NRP2 is exclusively polysialylated on *O*-glycans by ST8Sia 4 (23, 46). Similarly, in murine monocytes, the expression of polySia-NRP2 is regulated during stages of differentiation/activation. As occurs in human monocytes, murine BM-derived monocytes do not express polySia-NRP2 that only occurs during their migration from BM to PB (43)(**Figures 2A, B)**. Interestingly, like human mDCs, mouse BM-derived DCs express ST8Sia 4 and polySia-NRP2 (23).

The TLR4 stimulation of monocyte-derived imDCs causes the upregulation of ST8Sia 4 and polySia expression in immunogenic mDCs, similarly to the effect caused by IL-4 and GM-CSF. Prolonged TLR-4 engagement though LPS stimulation is required for the generation of polySia-expressing human monocyte-derived mDCs, which is also required for the CCL21 capture and subsequent CCL21-mediated migration (155). However, unlike monocyte-derived mDCs, monocytes, and monocyte-derived immature mDCs do not overexpress polySia or ST8Sia 4 after prolonged (2 days) LPS stimulation (155). Also, the TLR4 stimulation of human monocyte-derived tolerogenic mDCs does not cause polySia overexpression, although polySia and ST8Sia 4 are expressed by these cells (155).

In NRP2$^{-/-}$ mice, it has been observed that BM monocyte-derived DCs also express polySia in NCAM, as well as other unidentified carrier proteins (**Figure 2A**). Unlike NRP2, which is upregulated during migration from BM to PB, polySia-NCAM is downregulated in BM-derived monocytes (**Figure 2B**) (43).

The blocking of polySia in NRP2 with anti-polySia IgG or digestion with EndoN to remove polySia from the cell surface of DCs enhances their ability to activate T cells, suggesting that polySia-NRP2 regulates the activation of T cells by DCs (23) (**Figure 2D**). Also, the digestion with EndoN showed that stimulation with CCL21 and phosphorylation of Akt and JNK kinases are reduced when polySia is removed, indicating that polySia is required for signaling of CCL21 through CCR7. The depletion of ST8Sia 4 by knockdown in DCs resulted in the reduction of CCL21-mediated migration (156). Similarly, it has been reported that CCR7 and CCL21 contribute to the migratory capacity of DCs within the skin and to the lymph nodes (155).

PolySia also influences migration of imDCs located in the periphery where they capture pathogens and migrate as mDCs to draining lymph nodes to activate T cells. Migration of mDCs to lymph nodes was abrogated in ST8Sia $4^{-/-}$ mice (25).

CCR7 is the central chemokine receptor controlling immune cell trafficking to secondary lymphatic organs. There are data that support that CCR7 is polysialylated in murine BM-derived mDCs and that this modification is essential for CCL21 ligand recognition (25). HEK293 cells transfected to co-express CCR7-GFP fusion protein and ST8Sia 4 showed polySia-CCR7, and mutational analysis demonstrated that both *N*- and *O*- glycans are associated with CCR7 polysialylation. Moreover, flow cytometry by using the anti-polySia 735 monoclonal antibody of murine BM-derived CCR7$^{-/-}$ mDCs showed reduced polysialylation in comparison to control DCs, suggesting that additionally to NRP2, CCR7 is also polysialylated (25). EndoN digestion experiments in human mDCs could be conducted to evaluate if CCR7 is also polysialylated.

Noteworthily, SynCAM 1 (Necl2/CADM1), a member of the immunoglobulin superfamily of transmembrane glycoproteins, mostly known as a neural synaptic adhesion molecule with multiple functions, is also expressed in a specialized subset of murine and human DCs, where it was shown to interact through its extracellular domain with CRTAM (class I MHC-restricted T cell-associated molecule), a receptor expressed in cytotoxic lymphocytes to preserve epithelial integrity and required for proper thymus development (157, 158). SynCAM 1 in DCs was also found to regulate IL-22 expression by activated $CD8^+$ T cells

(70, 72). SynCAM 1 has also been found to be expressed by mast cells where it is involved in driving mast cell-sensory neuron adhesion and promoting the development of a microenvironment in which neurons enhance mast cell responsiveness to antigen (73). SynCAM 1 on tumors has also been shown to interact with CRTAM, promoting cytotoxicity of NK cells and interferon-gamma secretion by $CD8^+$ T cells *in vitro* as well as NK cell-mediated rejection of SynCAM 1-expressing tumors (159). Also, it has been shown that epithelial to mesenchymal transition (EMT) induced SynCAM 1 expression, regulating NK-mediated, metastasis-specific immunosurveillance in balance with E-cadherin (160). However, despite these important functional observations, the polysialylation of SynCAM 1 has not been evaluated in DCs or in the context of interactions with other types of immune cells.

NK Cells

The NCAM protein is used as a marker for NK cells and their subpopulations; however, the function of polySia-NCAM on NK cells is still not clear. Mature human NK cells are divided into 2 subsets based on the relative surface density of the NCAM antigen: $NCAM^{pos}$ cells, predominantly found in secondary lymphoid tissues, and $NCAM^{neg}$ cells, predominant in PB (161) (**Figure 2E**). Differently to the observations in human cells, adult murine NK cells do not express ST8Sia 4 or polySia, with polySia and NCAM expression being restricted to multipotent hematopoietic progenitors and cells derived from the myeloid lineage (130). However, fetal mouse BM-derived NK cells do express polySia, although polyST expression has not been evaluated (130).

In human peripheral NK cells, polySia biosynthesis was found driven by ST8Sia 4 as no expression of ST8Sia 2 was detected (37, 38). The short- (DP 1-10) or medium- (DP 11-140) length polySia chains on NCAM are characteristic of active cytotoxic NK cells, while larger chains (DP 141-370 +) are expressed by cytotoxic inactive NK cells (**Figure 2E**) (130).

Weakly polysialylated NCAM in NK cells binds in trans-homophilic interaction with DC-SIGN and plays a very important role in the fate of DCs. This interaction inhibits homotypic intercellular interactions of $NCAM^{pos}$ cells and protects DC-SIGN-expressing DCs against $NCAM^{pos}$ cell-mediated cytotoxicity (40).

PolySia-NCAM in NK cells also interact with polySia-NCAM expressed by tumor cells. The CRISPR-Cas9 deletion of NCAM in the NK cell line NK-92 showed a reduction in killing of $NCAM^+$ tumor cells (162). Also, the loss of NCAM protein reduced cytotoxicity and lytic granule exocytosis (163). These data strongly support that polySia and NCAM are implicated in the regulation of NK cell cytotoxicity.

Recently, the expression of ST8Sia 4 and polySia expression were evaluated in human peripheral NK cells and different NK cell lines under the activation of IL-2. Upon activation, there was no change in ST8Sia 4 expression; however, polySia expression increased, and this was explained by increased NCAM expression (38, 162) (**Figure 2E**). Despite the relevance of polySia in the function of NCAM in other cells, the specific function of polySia in the NCAM of NK cells has not been evaluated.

Microglia and CNS Immune Response

Under homeostatic conditions, the adaptive immune response of the CNS is very limited, and the innate immune response depends on endogenous brain cells, in particular the microglia. Microglial cells play a pivotal role in brain development, maturation, and homeostasis by responding to infection, trauma, or other pathological conditions, transforming into macrophage-like cells with a professional innate immune defense function that can be regulated by sialylation (164–166). Sialic acids maintain the homeostasis of the CNS innate immune response by inhibiting complement, including microglia *via* Siglecs and other receptors (167).

Polysialylated NCAM has been studied mainly in the development of the nervous system, particularly in neuronal processes such as migration, cytokine response, and differentiation dependent on cell contact (168). Due to these characteristics, it is not surprising that polysialylated molecules also participate in immunological processes (130).

Several studies have used the $NCAM^{-/-}$ mice as a model to evaluate the role of polySia-NCAM in different functions such as neuronal connectivity, plasticity, and migration (169). In the microglia context, the role of polySia in $NCAM^{-/-}$ mice has also been evaluated (170). The NCAM interaction modulates the activation of microglia, and it is responsible for homophilic binding in microglial immune response through production of nitric oxide (NO) and TNFα (171, 172).

Residual polySia signals in the brain of $NCAM^{-/-}$ mice indicated the presence of alternative polySia carriers (173). In murine $NCAM^{-/-}$ microglial cells, polySia was found to be carried by both NRP2 and ESL-1 and synthetized by ST8Sia 4 (**Figure 2F**). In the case of NRP2, it has been identified to be present in *O*-glycans. Golgi-localized polySia-NRP2 and polySia-ESL-1 appeared during injury-induced activation of murine microglia, and inflammatory activation by stimulation with LPS caused their translocation to the cell surface with subsequent depletion by ectodomain shedding (24, 143). The same mechanism was found in the differentiation of human THP-1 monocytic cells into macrophages, where polySia in NCAM disappears, but polySia-ESL-1 and polySia-NRP2 are detected in the Golgi and depleted upon proinflammatory activation with LPS, but not through anti-inflammatory activation induced by IL-4 **(Figure 2F)** (24).

In a model of traumatic brain injury, Golgi retention of polySia proteins was found abrogated by calcium depletion of the Golgi compartment that induced the translocation and rapid depletion of polySia from the Golgi to the cell surface (66). Additionally, depletion of the microglia cell surface polySia occurred through ectodomain shedding induced by metalloproteinase activity, although other reports indicate that it is mediated by exovesicular Neu1 neuraminidase (175). The polySia degradation was shown to cause liberation of the neurotrophic factor BDNF that polySia binds (175). The liberated BDNF from polySia chains would supply this and other neurotrophic factors to injured tissue.

Also, soluble polySia has been proposed to participate in the negative feedback regulation of proinflammatory microglia activation as it attenuates NO production of LPS-induced stem cell-derived microglia and reduced TNFα and IL-1β mRNA levels. LPS-induced NO production of NCAM-deficient microglia increased by the additional deletion of ST8Sia 4, that is, by the inability to produce polysialylated NRP2 or ESL-1 (24). This negative feedback has been proposed to occur through human Siglec-11 interaction or glutamate receptor function (170, 176). In the case of mouse microglia, this had not been observed in view that the inhibiting Siglec-11 has no mouse orthologue. Nonetheless, it was recently shown that this effect was mediated by *trans* interaction with murine Siglec-E, a mouse orthologue of human Siglec-9, expressed in the myeloid lineage (177). CRISPR/Cas9-mediated Siglec-E knockout led to a strong LPS response and failed in preventing inhibition of proinflammatory activation by exogenous polySia (66).

According to a study on Parkinson's disease, the administration of intraperitoneal polySia in humanized Siglec-11 transgenic mice showed neuroprotective properties after repeated injections with LPS, indicating that polySia is a potential drug candidate for preventing Parkinson's disease-associated inflammation and neurodegeneration. Brain transcriptome analysis showed increased levels of immune-related genes that prevent exacerbated immune responses as well as the loss of dopaminergic neurons in the substantia nigra *pars compacta* induced by LPS (178).

Taken together, these data show that polySia has a potential anti-inflammatory function in brain microglia, particularly through interaction with the Siglec-11 receptor, regulating the signaling inflammatory responses and retaining the microglial homeostasis. Nonetheless, these results must be taken cautiously as they are derived from mice that present a more restricted expression of Siglec members, 9 compared to 14 in humans. Specifically, the humanized Siglec-11 transgenic mice do not co-express the human Siglec-16, the paired activating receptor of Siglec-11.

Upon engagement of Sia-containing ligands, inhibitory Siglecs such as Siglec-11 recruit cytoplasmic tyrosine phosphatases SHP1/2 to their ITIM domain to deliver inhibitory signal(s) that modulate and counteract immune responses. Therefore, the available data point out to polySia using Siglecs to establish a negative feedback regulation of the immune response. The human Siglecs known to bind polySia include the inhibitory Siglec-9 and Siglec-11 (179, 180). Other inhibitory Siglecs known to recognize α2,8 Sia glycans in the form of disialic acid include Siglec-5 and Siglec-7, although there are no data regarding their binding of polySia (181).

It is thought that because certain pathogens developed mimicry to evade the immune response by engaging inhibitory Siglecs, Siglecs with activating signaling potential evolved, such as human Siglecs-14 and 16, in which the ITIM/ITIM-like intracellular domains are replaced with an immunoreceptor tyrosine-based activation motif that recruits the activating adapter protein DAP12 (147). Certain inhibiting and activating Siglecs function as paired receptors and are typically expressed together; such is the case of Siglec-5 and Siglec-14 and Siglec-11 and Siglec-16 (182). It is important to note that although no polySia binding has been identified in activating Siglecs, Siglec-14 and 16 are known to recognize α2,8 Sia glycans in the form of disialic acid (183, 184).

Therefore, the role of therapeutic polySia and its signaling through Siglecs must be further analyzed in the context of the inhibiting and activating responses that occur in human physiology, particularly considering that these responses, compared to murine Siglecs, are very probably more complex.

Neutrophils and Macrophages

Neutrophils are polymorphonuclear and phagocytic leukocytes of the innate response that act against pathogens; they are also important effector cells during tissue injury-induced inflammation (185). Murine BM-derived neutrophils have surface expression of polySia-NCAM associated with ST8Sia 4 activity (43). Like murine monocytes, during the exit and migration from BM, neutrophils lose the expression of polySia-NCAM until its complete depletion once they reach the alveolar and peritoneal sites of inflammation (43) (**Figures 2A, B**). The relevance of polySia-NCAM depletion during migration has not been evaluated; however, loss of polySia could have a similar effect to the loss observed when Sia was cleaved by sialidase treatment, improving adhesion and migration of neutrophils (65).

As occurs with the BM-derived monocytes and neutrophils, BM-derived macrophages decrease polySia surface expression during migration. Neutrophils and macrophages lose polySia when they migrate from the BM into the PB and then to pulmonary and peritoneal sites of infection or inflammation (43). When peritoneal macrophages were induced *in vitro* to repolysialylate by inducing a more quiescent state of activation, polySia was re-expressed and found to be carried by NRP2 and other unidentified proteins. Also, EndoN treatment for polySia removal from monocytes, as they mature into macrophages *in vivo* during recruitment to inflammatory sites, improved phagocytic activity of the *Klebsiella pneumoniae* pathogen, indicating that progressive loss of polySia during migration to inflammation sites is necessary for efficient phagocytosis (43).

Murine peritoneal macrophages express CD36 and Siglec-E. In murine macrophages, CD36 is a highly glycosylated protein that mediates the modified low-density lipoprotein (LDL) uptake (186). Both CD36 and Siglec-E interact during oxidized LDL (oxLDL) uptake in macrophages (187). The interaction leads to downregulation of CD36 signaling regulating in the uptake of oxLDL, which in turn promotes foam cell formation that participates in atherogenesis (188). Sialidase treatment showed that Sia is not required for Siglec-E and CD36 interaction but is required for CD36 SHP-1/VAV signaling involved in LDL uptake, through unknown membrane components (187). The CD36 protein is also found in mammalian milk where it is reported to be polysialylated; however, although CD36 in macrophages was found to be predominantly modified by α2,6-linked sialylation, polysialylation has not been evaluated (18, 189).

In the context of infection, the ST8Sia 4-coding gene was found upregulated in human monocyte-derived M2-like-polarized macrophages infected with human rhinovirus (RV), a

single-stranded RNA virus, that causes asthma exacerbation (190). The role of the increased ST8Sia 4 or the presence of polySia has not been investigated during RV infection; however, the authors suggest the potential role of the upregulated genes in the polarization to the M2-like phenotype enhancing the RV-induced type 2 cytokine expression (190).

As mentioned in previous sections, polySia has been found to be a negative feedback regulator of the immune response, a finding with therapeutic implications for diseases such as Alzheimer's disease. The polySia with an average DP of 20 (polySia avDP 20) promoted anti-inflammatory functions in human THP-1 macrophages through its interaction with Siglec-11, inhibiting the LPS-induced gene transcription and protein secretion of TNFSF2 and preventing the oxidative burst associated with phagocytosis of Alzheimer's disease-associated fibrillary amyloid-β_{1-42}. In addition, polySia avDP20 neutralized the LPS-triggered increase in macrophage phagocytosis, showing that polySia DP is relevant for determining the biological effect (191).

Also, the development of polySia-coated nanoparticles demonstrated that polySia binds to histones in NET fibers. The release of histones may be intentionally triggered to the cell surface during apoptosis or to the extracellular fluid during NETosis, a regulated form of neutrophil cell death that contributes to the host defense against pathogens through formation of neutrophil extracellular traps (NETs), which consist of modified chromatin decorated with bactericidal proteins (192, 193). Extracellular histones have cytotoxic properties because they are procoagulant and proinflammatory and are also toxic for mammalian epithelia and endothelia, contributing to the microvascular dysfunction observed in sepsis and autoimmune diseases (194, 195).

PolySia would have a role in protecting endogenous cells against histone-mediated cytotoxicity on the basis that application of polySia decreases the bactericidal function of histones (196). The polySia chains of secreted NCAM were shown to neutralize the cytotoxic activity of extracellular histones as well as DNA/histone-network-containing "neutrophil extracellular traps," which are formed during invasion of microorganisms. The interaction of polySia with histones appears to be improved with increased DP, showing better binding with DP 24–32 and 32–38 in comparison to shorter DP; also, both DP 24–32 and 32–38 improve the migration to distance of histones through binding to polySia, which could impact the cytotoxic function of histones. Similarly, low polySia of DP 15–24 does not influence the migration but does participate in cytotoxicity (197).

NETs are also loaded with lactoferrin that forms a shell around neutrophils, suppressing the release of NETs. Recent evidence suggests that polySia binds not only to histones but also to lactoferrin of NETs and that the expression of polySia regulates the accumulation of external lactoferrin, regulating the formation of NETs by neutrophils (198).

The regeneration of tissue after application of exogenous polySia has been previously studied in the CNS for potential therapeutic use, evaluating the role in axonal growth of polySia (199). Polysia is upregulated in the murine CNS during transplantation of polySia-overexpressing Schwann cells, improving regeneration after spinal cord injury (200). The intravitreal application of polySia with avDP 20 in a macular degeneration laser-damage murine model reduced mononuclear-phagocyte activation and tissue damage as well. PolySia avDP 20 prevented membrane attack complex (MAC) deposition in wild-type and in humanized Siglec-11 transgenic mice. *In vitro*, polySia inhibited the reactivity of mononuclear phagocytes, preventing TNF-α, VEGF-A, and superoxide production, but also *via* Siglec-11 receptors interfering with activation of the alternative complement system and preventing the phagocytosis-associated oxidative burst (201). Siglecs expressed by mononuclear phagocytes recognize Sia as a self-associated molecular pattern (SAMP) functioning as sensors for "self" (202). As mentioned previously, these results derived from humanized transgenic mice must be taken cautiously as they do not include the co-expression of Siglec-16, the paired activating receptor of Siglec-11.

5 POLYSIALIC ACID ROLE IN THE ADAPTIVE IMMUNE RESPONSE

The adaptive immune system takes over if the innate response is not able to clear and destroy pathogens. The adaptive response acts through cytokine mechanisms where effector, cytotoxic, plasmatic, and regulatory cells orchestrate the response. Sia is involved in the regulation of B and T cell maturation, differentiation, migration, and cell survival or cell death fate (203, 204). The presence of polySia has only been characterized in T cells, but not B cells.

Sia is actively involved in pathogen recognition through interaction with glycan-binding proteins and in regulating key pathophysiological steps within T cell biology such as T cell development and thymocyte selection, T cell activity and signaling, and T cell differentiation and proliferation (205). These roles highlight the importance of Sia as a determinant of either self-tolerance or T cell hyperresponsiveness which ultimately might be implicated in the creation of tolerogenic pathways in cancer or loss of immunological tolerance in autoimmunity (202, 203).

Although both ST8Sia 2 and ST8Sia 4 are expressed in BM hematopoietic precursors (and in both primary and secondary human lymphoid organs), the expression and regulation of polySia have been poorly investigated in association with the adaptive immune response. ST8Sia 2 expression has been identified in the adult human thymus, while ST8Sia 4 is abundantly expressed in primary and secondary lymphoid organs such as the placenta, spleen, thymus, intestine, and the PB (206).

The T cell progenitors are produced in the BM and mobilized to the periphery at regular intervals by signals to reach the thymus where they mature. The ST8Sia $4^{-/-}$ mice show a reduction in total thymocytes and a concomitant deficiency in the earliest thymocyte precursors in comparison to multipotent hematopoietic progenitors derived from wild-type ST8Sia 4 mice with normal polySia synthesis (41). *In vivo* reconstitution of polySia expression in ST8Sia $4^{-/-}$ hematopoietic progenitors showed that defective T cell development is caused by improper access to the thymus (**Figure 3A**) (41). These results suggest that the observed defect in

thymocyte development is not due to abnormalities in T cell development, but is related to the inability of these polySia-negative cells to exit the BM and travel to the thymus (41). Because of its increased size, steric hindrance, and negative charges, polySia has been identified as an anti-adhesive molecule (207), modulating the distance between cell–cell interaction of cell–epithelia heterotypical or homotypical interaction. This can explain why BM cells that do not express polySia in ST8Sia 4$^{-/-}$ mice are unable to modulate the interaction in the BM niche, creating an inefficient exit of BM cells and migration to the thymus.

During maturation in the thymus, T cells suffer phenotypic and functional changes derived from thymic environment interaction (208). The interaction of the thymocyte with different surrounding cells determines the fate of their differentiation and maturation, as well as the exit from the thymus. PolySia is a key regulator of cell–cell contact, and as is mentioned above, ST8Sia 4 is responsible for polySia synthesis in hematopoietic precursors. According to the immunological genome project database, ST8Sia 4 is downregulated during the development and maturation of thymocytes, in agreement with observations where polySia is upregulated during differentiation and maturation to generate egress from the BM, requiring downregulation to promote thymic retention (67, 209).

Newly generated peripheral T cells designated as recent thymic emigrants (RTEs) continue post-thymic maturation in secondary lymphoid organs to become long-lived naive T cells (210). The increase in Sia expression on the cell surface glycans after maturation is a signature of thymocyte maturation (**Figure 3B**). Cre-NKAP$^{-/-}$ mice (NKAP is a transcriptional repressor that binds to histone deacetylase 3 required at several points in hematopoiesis) fail to complete T cell maturation

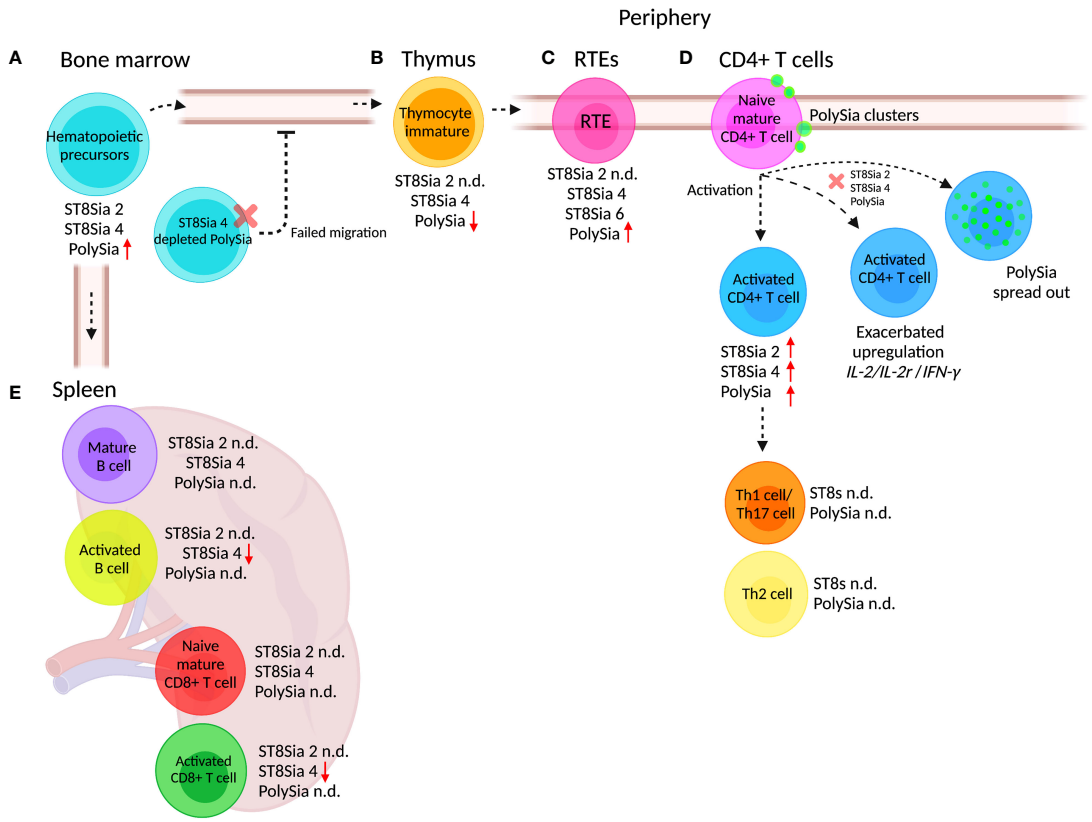

FIGURE 3 | PolySia and polySTs expressed in adaptive immune cells. During differentiation of hematopoietic precursors, maturation, migration, and activation, the cells of the adaptive response suffer changes in the expression of polySia and the polySTs. **(A)** Hematopoietic precursors derived from BM express ST8Sia 4 and ST8Sia 2, but the polySia is synthesized exclusively by ST8Sia 4. In ST8Sia 4$^{-/-}$ mice, the BM hematopoietic progenitors fail to exit, migrate, and access the thymus for maturation into T cells. **(B)** Hematopoietic progenitors mature in the thymus where they differentiate into immature thymocytes which in turn express polySia synthesized by ST8Sia 4. **(C)** After maturation in the thymus, recent thymic emigrants (RTEs) migrate through the circulation to reach the lymph nodes. The RTEs express the ST8Sia 4 and ST8Sia 6 as well as polySia. **(D)** Peripheral naive CD4$^+$ T cells express ST8Sia 2, ST8Sia 4, and polySia, which are overexpressed upon *in vitro* stimulation. When ST8Sia 2 and ST8Sia 4 are downregulated by knockdown (red x) in naive CD4$^+$ T cells, the upregulation of *IL-2*, *IL-2r* (IL-2 receptor), and *IFNγ* genes is exacerbated during activation. **(E)** Activation of mature B cells reduces ST8Sia 4 expression, but polySia expression has not been analyzed. Naive and activated CD8$^+$ T cells express ST8Sia 4; however, such as occurs with B cells, the polySia expression has not been determined (n.d). Different types of lymphocytes express ST8Sia 4, but polySia expression has not been determined (n.d.). The increase or decrease in polySia expression with respect to the precursor state is indicated with red arrows (up or down). Created with BioRender.com.

because RTEs are eliminated by the complement system (211). Defective α2,8 sialylation occurs in NKAP$^{-/-}$ T murine cells because of downregulation of ST8Sia 1, ST8Sia 4, and ST8Sia 6 (**Figure 3C**). Apparently, α2,8 sialylation is critical in RTEs to avoid complement fixation and removal (211).

After maturation, naive T cells need to be activated in the secondary lymphoid organs such as lymph nodes, spleen, and Peyer's patches. During activation, T cells must receive two signals, firstly from an antigen-presenting cell (APC) *via* the immunological synapse, initiated by the TCR recognition of an antigen peptide displayed on the MHC of an APC (212), and secondly from a costimulatory binding mediated by CD28 on T cells and CD80/CD86 ligands on the APC (213). In this context, T cell activation is regulated by the concentration and affinity of antigen, duration of antigen stimulation, and the costimulatory signals and the cytokine environment present at the time of antigen presentation, as well as glycosylation changes, including sialylation (214).

The expression of Sia has been studied in different cell subsets of peripheral T cells and B cells. Previous observations of Sia changes during activation of human peripheral and murine splenocytes showed α2,3 and α2,6 Sia hyposialylation, because of the downregulation of ST3 and ST6 STs (67, 215, 216). This hyposialylation on both CD4$^+$ T and CD8$^+$ T cells has been associated with induction of apoptosis to regulate the homeostasis of these cell populations (217). It is important to remark that murine resting CD8$^+$ T cells express ST8Sia 4 which is downregulated after activation; however, the expression of polySia has not been evaluated in these cells (67).

The hyposialylation of α2,3 and α2,6 Sia linkages during activation of peripheral human naive CD4$^+$ T cells was found to be accompanied by global cell surface sialylation at the expenses of α2,8 Sia (60). During activation of CD4$^+$ T cells, ST8Sia 2 and ST8Sia 4 were found to be upregulated and no expression of ST8Sia 3 was observed (60). The upregulation of ST8Sia 2 and ST8Sia 4 upon activation increased polySia expression in a subpopulation of human peripheral naive CD4$^+$ T cells (**Figure 3D**) (144). In mice, the activation of CD4$^+$ T, CD8$^+$ T cells, and B cells induces the downregulation of ST8Sia 4; however, the expression of polySia has not been evaluated (67).

The expression of polySia in human CD4$^+$ T cells was associated with different unknown protein carriers. Additionally, the cell surface expression of polySia in resting naïve CD4$^+$ T cells occurred in a clustered pattern, which dispersed after activation (144). The knockdown of both ST8Sia 2 and ST8Sia 4 in human resting naïve CD4$^+$ T cells exacerbated the expected overexpression of *IL-2*, *IL-2R*, and *IFN-γ* genes after anti-CD3/CD28 activation, indicating that polySia glycoconjugates participate in the negative regulation of the activation response of human peripheral CD4$^+$ T cells (**Figure 3D**) (144). Differences occur between these observations and reports in murine splenocytes where activation of CD4$^+$ T and CD8$^+$ T cells with anti-CD3 plus IL-2 or of B cells with anti-IgM plus IL-4 causes downregulation of ST8Sia 4 (67) (**Figure 3E**). Furthermore, the presence of polySia in murine T cells or B cells has not been reported, limiting the comparison between these two models.

The expression of NCAM has been detected in γδ T cells. Although these cells represent less than 5% of all T cells, they act as a first line of defense in the skin, gut, and reproductive tract while other lymphocytes are still being developed, performing distinct roles in pathogen clearance, wound healing, autoimmunity, and cancer, supporting the functions of DC, T cells, and NK lymphocytes through both innate and adaptive properties including antigen-presenting capabilities (218). The proportion of NCAM$^+$ γδ T cells appears to be determined by their level of activation. NCAM$^+$ γδ effector T cells produce large amounts of IFN-γ after stimulation and are more resistant to apoptosis. Additionally, NCAM expression is stronger in proliferating cells and gradually disappears with the number of cell divisions. Thus, NCAM expression is considered to define the γδ T cells with the highest antitumor activity (219). The antibody-mediated blocking of NCAM or removal of polySia chains in NCAM$^+$ γδ T cells reduced cell proliferation and caused lower lytic effector activity (220, 221), although further investigation is required to understand the role of polySia in the antitumoral function of NCAM$^+$ γδ T cells.

DISCUSSION

Although most of what we know about the biological roles of polySia has originated from its study in the CNS, and particularly from NCAM, this review shows that different types of cells of the immune system express polySia in NCAM but also in different protein carriers, known and unknown. The accumulated evidence in both innate and adaptive immune cells reveals that polySia is a key regulator of immune cell biology, from hematopoiesis to effector functions, something that is not unexpected from a polymer with such potent biophysical characteristics and that has already been characterized to be dynamically modulated during CNS development.

The use of anti-polySia antibodies has proven very valuable to approach the identification of polySia in cells; nonetheless, it is also important to advance toward a more specific structural characterization of polySia, particularly DP, as length chain also determines the biological information encoded by this glycan polymer. Most of the studies included in this review address the expression of polySia using specific antibodies, but a limited number have determined the DP composition of polySia. It is important to consider that as DP is linked to ST8Sia 2 and/or ST8Sia 4 activity, it would also be subject to dynamic regulation, resulting in heterogeneity at different stages of cellular differentiation and activation that could be part of an additional fine-tuning of the immune response, including differences at the level of the human population. It is therefore important to apply combinatorial techniques for a more precise structural analysis of polySia. Additionally, although ST8Sia 3 has polySia synthesis activity that is apparently limited to autopolysialylation, it is important to evaluate its expression and potential role on polySia synthesis along with the ST8Sia 2 and ST8Sia 4, particularly among the diversity of immune cell types where the role of this enzyme has not been thoroughly studied.

Although the functional relevance of polySia has been clearly demonstrated in all the immune cell types where it has been found, more is needed to identify its *cis*- and *trans*- ligands. The interaction of polySia with members of the Siglec family in different immune cells is very promising to identify signaling pathways as has been shown for microglial cells and macrophages that act through interaction with Siglec-11, inhibiting microglial activation, inflammation, phagocytosis, and oxidative burst (146). Additionally, the identification of the signaling pathways of polySia, particularly involving inhibiting Siglecs, also paves the way to developing polySia-based therapeutics, particularly through its anti-inflammatory properties. However, further research needs to address the net effect of paired inhibiting and activating Siglec receptors to truly model the complexity of Siglec signaling.

Although polySia has been mostly characterized in the cell surface, studies in microglia indicate that intracellular polySia dynamics associated with retention and release is a mechanism linked to changes in ionic concentrations (Ca^{2+}, Mn^{2+}, and Mg^{2+}) that influence polySTs activity. This is very important in understanding polySia dynamics in immune cells where ionic concentration also fluctuate and are of key importance during activation (118, 222). It is therefore critical to address both cell surface and intracellular polySia expression to fully comprehend the involved regulatory mechanisms. This can also be extended to phosphorylation mediated by protein kinase C that regulates signal transduction pathways important for both innate and adaptive immunity and that is known to positively regulate the activity of both ST8Sia 2 and ST8Sia 4 in the polysialylation of murine NCAM in the CNS (223).

A systematic exploration in all human immune cells of the expression and functional role of polySia, as well as its carriers and ligands, promises to reveal potent mechanisms through which this glycan polymer acts throughout the different stages of immune cells. There is a critical mass of information available to extrapolate the different findings on the role of polySia in different types of immune cells, from both mice and humans, to readily accelerate this field of research.

AUTHOR CONTRIBUTIONS

TV-C and IM-D designed this review. All authors contributed equally to the literature revision and manuscript writing. All authors contributed to the article and approved the submitted version.

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Synovial Fibroblast Sialylation Regulates Cell Migration and Activation of Inflammatory Pathways in Arthritogenesis

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Synovial fibroblasts have emerged as critical underlying factors to perpetuate chronic joint inflammation in Rheumatoid Arthritis. Like any other cell, synovial fibroblasts are covered with a complex layer of glycans that can change in response to extracellular signals, such as inflammation. We have previously shown that inflammatory synovial fibroblasts show decreased levels of sialic acid, but our understanding of sialic acid-dependent pathophysiological pathways in these stromal cells is still very limited. In this report, we used *in vivo* and *in vitro* studies with exogenous sialidases and RNA sequencing to investigate the responses of murine synovial fibroblasts upon desialylation. Our results show that hyposialylated fibroblasts present a dysregulated migratory ability and an activated phenotype characterized by the expression of inflammatory mediators, such as cytokines and chemokines, and anti-viral related mechanisms. Removal of surface sialic acid also affected the expression of sialyltransferases, revealing the existence of a positive feedback to sustain reduced sialylation. Moreover, we demonstrate that synovial fibroblasts subsets have distinct sialyltransferase expression profiles, both in healthy and arthritic mice. These findings underline the ability of sialic acid to modulate homeostatic and inflammatory responses in non-immune synovial fibroblasts, suggesting that sialylation plays a key role in perpetuating local inflammation in the arthritic joint.

Keywords: synovial fibroblast (FLS), sialic acid, glycoimmunology, rheumatoid arthritis, inflammation, migration, cytokines

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INTRODUCTION

Rheumatoid Arthritis (RA) is an autoimmune joint condition that causes pain, swelling and stiffness in the joints, the result of an ongoing chronic inflammatory process. Inflammation is however an essential defensive instrument of the human body, starting protective responses and subsequent healing processes to restore tissue homeostasis. Dysregulation of these mechanisms lead to a persistent inflammatory phenotype characteristic of chronic RA, whose primary target is the joint synovium, the soft tissue that lines the inner surfaces of diarthrodial joints. In health, this membrane

nourishes the cartilage and bone tissue. Nonetheless, the synovial membrane becomes noticeably thicker in RA promoting immune cell infiltration and cartilage and bone damage (1). Genetic, epigenetic, and environmental factors can contribute to disease initiation (2) but specific mechanisms are still unclear. Equally importantly, we still do not fully understand why inflammation persists in RA, although recent research in the field of stromal immunology has shown that non-immune cells such as Synovial Fibroblasts (SFs) play a critical role in perpetuation of RA. SFs are a basic part of the synovium and they become activated during inflammatory arthritis, secreting cytokines, such as IL-6, GM-CSF and chemokines like Cxcl10, Ccl2, IL-8 that attract macrophages, neutrophils and lymphocytes (3–6). SFs also support ectopic tertiary lymphoid structures to continue aberrant immune responses in the joint (7). These recruited immune cells increase the local concentration of TNFα, IL-1β or IL-17, that continue to promote SF activation (8–10) generating pathogenic self-perpetuating inflammatory loops. Furthermore, SFs become hyperproliferative, migrate to bone and cartilage inducing tissue damage, secrete matrix-degrading enzymes and RANKL and promote local angiogenesis upon VEGF secretion (11–13). Interestingly, recent findings based on single cell RNA sequencing have demonstrated the existence of distinct SFs subsets with specific anatomical location within the synovium and non-overlapping effector functions (14, 15). For example, FAPα+CD90+ SFs found in the sublining synovium lead the immune effector function, whereas lining FAPα+CD90− SFs drive cartilage destruction (16). Nevertheless, there is still a lack of clinical targets to intervene SFs in the clinic.

Loss of inhibitory or regulatory signalling is a critical mechanism to trigger autoimmunity and chronic inflammation. At the heart of many of these signals is the cell glycome, which comprises the entire pool of glycans found at the cell-cell interface. The outermost monosaccharide decorating glycans in humans is usually a molecule belonging to the Sialic Acid (SA) family. Given its location, negative charge and hydrophilicity, SA modulates a wide variety of pathological processes. An increasing body of evidence supports the hypothesis that SA acts an immune check-point (17), as high SA levels can deliver anti-inflammatory or tolerogenic signals, whereas low concentration of SA is linked to inflammation. Corroborating this theory, multiple cancer cells overexpress α2,3, α2,6, and α2,8 linked SA to evade immune responses, inhibiting NK, T and B cells *via* SA-Siglec signalling (18). On the other hand, deficiency of CD22 (Siglec-2) and Siglec G leads to hyperactivated B cells and autoimmunity (19, 20), including exacerbation of experimental arthritis and lupus (21). Besides, activation of TLR-NFκB-mediated responses in immune cells appears to be associated with a reduction of SA on the cell surface upon sialidase activity (22–25). This agrees with our previous results, where we have shown that TNFα-mediated down-regulation of α2-6 sialylation is a hallmark of activated SFs in experimental arthritis (26). In this report, we first describe the sialylation pathways associated to distinct SFs subsets in healthy and arthritic mice. Second, we investigate the responses of murine SFs upon *in vitro* cell surface desialylation, hypothesising that enzymatic removal of sialic acid in healthy SFs cell surface would trigger intracellular signalling to initiate inflammatory responses. Our data indicate that desialylated SFs rapidly adopt a phenotype reminiscent of the SFs found in the arthritic joint, characterised by enhanced cell migration, activation of NFkB-mediated pathways and antiviral responses. Interestingly, removal of SA also modulates mRNA expression of the sialyltransferases ST6Gal1 and ST3Gal3, suggesting that environmental or temporal changes affecting SA content may be perpetuated in time to consolidate local inflammation.

METHODS

Ex Vivo Culture of SFs

Isolation and *ex vivo* expansion of murine SFs was done as previously described (27). Briefly, paws were harvested from mice, skin and soft tissue were removed, synovial tissue was dissected and digested with type II collagenase (1 mg/ml; Sigma #C6885) for 80 minutes at 37°C. Samples were vortexed vigorously to release cells and centrifuged. For *ex vivo* expansion, cells were resuspended in DMEM (#21969-035) supplemented with 10% fetal calf serum (FCS; #10270106), 1% penicillin and streptavidin (#15140122), 1% L-glutamine (#25030-024) and 1% NEAA (#11140-035, all from Invitrogen, UK) and cultured in 5% CO2 at 37°C for 24 hours, when culture medium was replaced. Media was changed twice a week and the cells were passaged at 90% confluence using trypsin-EDTA (Invitrogen, #25300-054). Prior to experimental setup, expression of Podoplanin (PDPN, Biolegend, #156204) and CD11b (Invitrogen, #11-0112-85) was assessed by flow cytometry. Myeloid CD11b+ cells were labelled with biotinylated anti-CD11b antibody (Biolegend, #101204) and subsequently depleted using Streptavidin magnetic MicroBeads (MACS Miltenyi Biotec, #130-090-485). For *in vitro* cytokine stimulation, recombinant IL-1β was used at the indicated concentrations for 6 hours in cDMEM.

Collagen-Induced Arthritis (CIA) Mouse Model

8-10 weeks male DBA/1 mice were purchased from Envigo (UK) and maintained in the Biological Services Unit of University of Glasgow in according to the Ethics Review Boards (AWERB) of University of Glasgow and the Home Office UK licences PIL IF5AC4409 and PPL P8C60C865. Mice received 100 μg of chicken type II collagen (MD Bioproducts #804002-Sol) emulsified with an equal amount of complete Freund's adjuvant (CFA, MD Bioproducts #501009) on day 0 *via* intradermal injection above the tail base. On day 21, mice were injected intraperitoneally with 100 μg collagen in PBS. Mice were monitored every two days for body weight, paw thickness and clinical scores. Clinical scores were assigned according to clinical signs, using a scale from 0 to 4 for each paw. An overall score exceeding 10 or weight loss exceeding 20%, paw thickness exceeding 4.5 mm or more than three inflamed paws was

considered as an experimental endpoint and the mouse was immediately euthanized.

FACS Sorting of SFs Subsets

Cells from mouse synovium were obtained as described above for cell culture, with the addition of DNase I (1 mg/ml; Sigma #DN25) during collagenase digestion. Cells were then resuspended in red cell lysis buffer for 3 min at room temperature, and red cell lysis was stopped by adding 20 ml of cold PBS. Cells were then centrifuged and stained with flexible viability dye eFluor 780 (Invitrogen #65-0865-14) at 1 µg/ml in PBS for 20 min on ice to discriminate live and dead cells. FC receptor was blocked using CD16/CD32 specific antibody (Invitrogen, #14-0161-85) for 20 min on ice. Cells were then incubated with primary antibodies or isotype controls at 1 µg/ml in FACs buffer (PBS 1%FBS 2 mM EDTA) for 20 min at 4°C. Antibodies used were: anti-CD31PE (Invitrogen, #12-0311-81), anti-CD45-PE (Biolegend, #103106), anti-CD90-FITC (Biolegend, #105316), anti-PDPN-A647 (Biolegend, #156204), anti-rat IgG2b-PE (BD bioscience #25393), anti-rat IgG2a-PE (Biolgend, # 400508), anti-rat IgG2b-FITC (Biolegend #400634) and anti-rat IgG2a-APC (Biolegend, #400512). Cell sorting was performed using FACS Aria III or FACS Aria IIu (all from BD), data were analyzed with FlowJo software 10.7.1.

Desialylation of Synovial Fibroblasts *In Vitro*

To hydrolyse sialic acid, SFs were cultured until reaching 90% confluence. Cells were washed three times with cold PBS and incubated for 1 hour with 100 mU Neuraminidase from Clostridium perfringens (CP, Roche, #11585886001) diluted in sialidase buffer (PBS: RPMI 1640 = 1:1, pH=6.8) or with sialidase buffer only (negative control, NT). Cells were washed with cold PBS prior to assessing the removal of sialic acid by lectin staining, or washed and cultured with cDMEM 10% FCS for further RNA extraction. RNA was isolated as explained below, and used for RNA-Seq or RT-PCR experiments.

RNA Isolation and RT-qPCR

RNA from SFs was isolated using either RNeasy Micro Kit (Qiagen #74004) or EZ-10 RNA Mini-Preps Kit (Bio Basic #BS88136) according to the manufacturer's instructions. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific #4368814) and RT-qPCR was performed using TaqMan™ Gene Expression Assay (Applied Biosystem). The expression of actin mRNA was used as an endogenous control to normalise samples. Taqman predesigned primers (Applied Biosystem) were: Actb/Mm02619580_g1; IL-6/Mm00446190_m1; CCL2/Mm00441242_m1; MMP3/Mm00440295_m1; MMP13/Mm00439491_m1; TNFRSF11b/Mm00435454_m1; TNFSF11/Mm0041906_m1; St6gal1/Mm00486119_m1; St6galnac5/Mm00488855_m1, St3gal1/Mm00501493_m1; St3gal2/Mm00486123_m1; St3gal3/Mm00493353_m1; St3gal4/Mm00501503_m1, St3gal6/Mm00450674_m1; Myd88/Mm00440338_m1 and NFKBIB/Mm01179097_m1.

Cell Migration Assay

SFs (10^4) were seeded in u-dishes (ibidi, #80466) coated with fibronectin (R&D systems #1030-FN) until reaching 90% confluence. U-dishes have a plastic insert that leaves a cell-free gap when removed. Cells were allowed to grow in the gaps after insert removal, and cell-free areas were measured after gap was created (T0) and 24 hours later (T24). Following monitoring of cell cultures, 24 hours was selected as our experimental time point because it allowed sufficient cell migration to observe biological differences without completely covering the cell-free region. Cell migration was quantified by measuring the difference in the width of cell-free region between T0 and T24, calculated with ImageJ software.

Flow Cytometry

For proliferation studies, cells were labelled with 10 µM proliferation dye eFlour 670 (eBiosciences, #65-0840-90) for 10 min on ice. Labelling was stopped by adding 4-5 volumes of complete culture medium for 5 min on ice. Cells were then subjected to flow cytometry analysis (day0) or maintained in culture for additional 5 days prior to analysis by Flow Cytometry. For lectin staining, Peanut Agglutinin (PNA, #B-1075), Sambucus Nigra Lectin (SNA, #B-1305), Aleuria Aurantia Lectin (AAL, #B-1395) and Maackia Amurensis Lectin II (MAA, # B-1265-1), all from vector laboratories, were used. Cells were blocked in carbon-free blocking buffer (vector laboratories, #SP-5040) for 20 min on ice, and then incubated with biotinylated lectins diluted in PBS containing 5% carbon-free blocking buffer. Lectins were then detected with FITC-conjugated streptavidin (Biolegend, #405201), Alexa Flour 647-conjugated streptavidin (Biolegend, #2068269) or PE-conjugated Streptavidin (Biolegend, #410504) in PBS for 20 min at 4°C. To differentiate between live and dead cells, all samples were stained with DAPI (Sigma, #32670, dilution 1:1000) prior to data acquisition. Data were acquired using an LSR II flow cytometer (BD) and analysed using FlowJo version 10.8.0.

RNA-Sequencing (RNA-Seq) and Data Analysis

Total RNA from cultured SFs was isolated, RNA integrity check was performed using the Agilent 2100 Bioanalyzer System and RNA integrity number (RIN) value was > 9 for all samples. Library preparation was done using RNA poly A selection at Glasgow Polyomics (Glasgow, UK). Low sequencing reads were removed using Trimmomatic (28) before mapped to mouse reference genome (GRCM38) using Hisat2 version 2.1.0. Featurecounts version 1.4.6 was used to quantify reads counts. Mouse ENSEMBL gene ID to gene symbol conversion was performed in BioTools (https://www.biotools.fr). Differentially expressed (DE) genes were identified using DESeq2, and Principal component analysis (PCA) were performed using R Bioconductor project DEbrowser (29). Genes passing a threshold of Padj<0.01 and |log2Foldchange| > 1 were considered as differentially expressed. Gene Ontology (GO) Biological Process enrichment and KEGG pathway enrichment were conducted with Metascape (30) and PathfindR (31).

MTS Assay Protocol

The MTS assay kit (Abcam, # ab197010) was used to measure the cellular metabolic activity of SFs according to manufacturer's instructions. Briefly, cells were grown in 96-well plates (10,000 cells/well), medium was removed and 100 µL of cDMEM and 10 µL of MTS solution were added into each well. Plates were incubated for 4 hours at 37°C when absorbance was read at optical density of 590 nm. The same amount of cDMEM and MTS solution without cells was used as an internal control for no metabolomic changes and background absorbance.

Statistical Analysis

Data are presented as the mean ± standard error (SEM). Statistical analysis was performed using Prism 8 software (GraphPad). One-way analysis of variance (ANOVA) was used to test significant differences among multi-groups, and student t-test was used between two groups studies. P values <0.05 were considered significant.

RESULTS

SFs Subsets Show Distinctive Regulation of Sialyltransferases in Arthritic Mice

We have recently shown that inflammatory SFs isolated from mice undergoing experimental Collagen-Induced Arthritis (CIA) show reduced expression of the glycosyltransferase ST6Gal1 and associated α2-6 sialylation (26) but we did not have data about the relative expression of other sialyltransferases in SFs subsets. Therefore, to continue investigating SFs sialylation profile in joint disease, we isolated SFs from healthy and arthritic CIA mice. SFs were identified by flow cytometry as podoplanin+ and CD45-CD31- (to exclude immune and endothelial cells), and expression of CD90 was used to sort lining (CD90-) and sublining (CD90+) SFs (**Figure 1A**), subsets that have shown distinct anatomical locations and pathophysiological roles (14). As expected, we recovered a higher number of cells from the arthritic joints (**Figure 1B**) and the relative proportion of CD90- versus CD90+ SFs was altered (**Figure 1C**). RNA was extracted from naïve and CIA sorted CD90- and CD90+ SFs, and expression of IL-6, Ccl2 and MMP3 was evaluated by RT-PCR (**Figure 1D**). CIA SFs showed elevated expression of these inflammatory markers, corroborating their activated/inflammatory status compared to healthy SFs. In line with previous reports, SFs subsets showed a differential expression pattern for inflammatory cytokines IL-6 and Ccl2, with a more noticeable distinction under non-inflammatory conditions. Next, we quantified the mRNA expression of ST6Gal1, ST3Gal1, ST3Gal2, ST3Gal3, ST3Gal4, ST3Gal6 and ST6GalNAc5, sialyltransferases expressed in murine SFs involved in glycoprotein sialylation (**Figure 1E**). SFs subsets showed a different sialyltransferase expression profile, probably reflecting their different biological role and anatomical location. In healthy synovium, CD90+ SFs presented higher expression of ST6Gal1, ST3Gal1 and ST3Gal2 compared to CD90- cells. However, in inflammatory CIA conditions, CD90+ cells down-regulated expression of ST6Gal1 (which adds α2-6-linked sialic acids to glycoproteins), whereas enzymes involved in α2-3 sialylation remained unaltered or even up-regulated in the case of ST3Gal4. These results corroborate our previous findings (26) and also provide further support to the hypothesis that reduced α2-6 sialylation is an inflammatory checkpoint in CD90+ SFs.

Enzymatic Removal of Sialic Acid Enhances SF Migration

Next, we sought to examine the pathophysiological function(s) of SA in SFs. To this end, cells were expanded *ex vivo* from healthy murine synovium, as cultured SFs maintain most of their epigenetic and phenotypic signatures for several passages (32, 33). Furthermore, cultured SFs express high and homogeneous levels of CD90 (9). Such phenotype is reminiscent of sublining SFs, subset that shows down-regulated ST6Gal1 expression during joint inflammation (**Figure 1E**). Thus, expanded SFs provided a suitable tool for *in vitro* experiments in this context. To mimic the desialylation observed *in vivo* during disease, we treated SFs with *Clostridium perfringens* sialidase. Sialidase treatment reduced levels of both α2-6- and α2-3-linked SA as evidenced by the reduced binding of *Sambucus nigra* agglutinin (SNA) and *Mackia amurensis* agglutinin (MAA) (**Figure 2A**). Cells showed 49.4% (± 0.029) SNA binding and 25.3% (± 0.038) MAA binding after desialylation. The sialidase specificity was further confirmed by an increased Peanut agglutinin (PNA) binding, since the presence of SA inhibits its glycan recognition, and unaffected binding of *Aleuria aurantia* agglutinin (AAL), a fucose specific lectin (**Figure 2A**). SA has been linked to cell migration, with reports showing that both α2-6 and α2-3-linked sialic acid can promote cell migration in various cell types and cancer (34–37). Because SFs adopt a migratory phenotype during RA, we hypothesized that SFs with reduced levels of SA would have an increased migration capacity, similar to the activated cells in RA. To test this, SFs were desialylated with *C. perfringens* sialidase and cell migration was evaluated using wound healing assays on fibronectin coated wells. Results confirmed the proposed hypothesis, since cell migration was significantly increased in desialylated SFs (**Figure 2B**). By contrast, neither cell proliferation (**Figure 2C**), nor cellular metabolomic rate (**Figure 2D**) were affected.

SA Removal Induces Rapid Pro-Inflammatory Transcriptomic Changes in Healthy SFs

We had previously observed that SFs up-regulated IL-6 and Ccl2 mRNA following loss of SA (26), but the functional consequences of SA down-regulation in SFs-mediated immunity were effectively unknown. Therefore, to identify SA-associated pathways and hence further understand the role of sialylation in SFs, non-treated control cells and cells desialylated with *C. perfringens* sialidase were subjected to RNA-Seq analysis. Principal Component Analysis identified that the two groups displayed distinct transcriptome profiles (**Figure 3A**). Thus, we searched for significant differential gene expression [DE fold change > 2, adjp

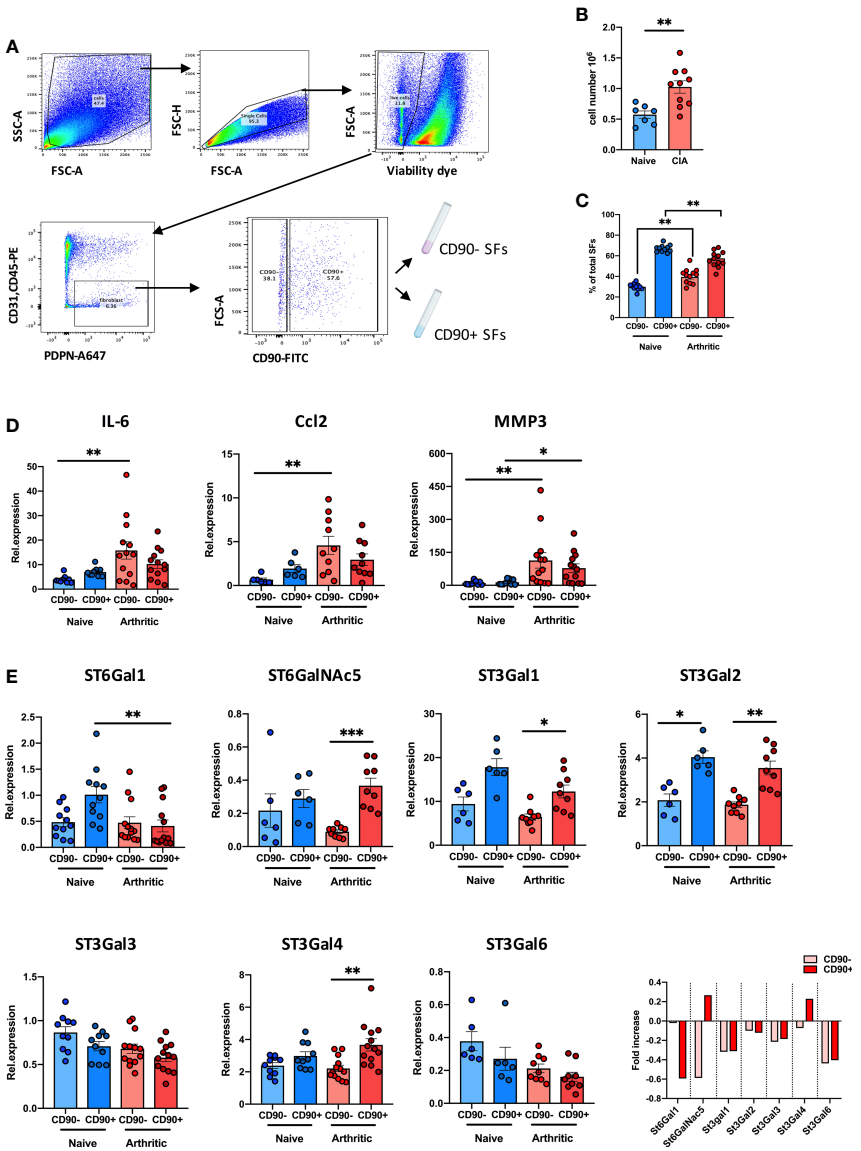


FIGURE 1 | CD90+ SFs show a distinct expression of sialyltransferases in experimental arthritis. **(A)** SFs were isolated from joints of naïve and mice undergoing Collagen-Induced Arthritis. Cells were sorted by FACs gating on low viability dye, CD31-, CD45- and PDPN+ cells. Subsets of SFs were discriminated by expression of CD90. Sorting strategy is shown in the dot plots. **(B)** Total number of isolated cells from paws from naïve (n = 7) and CIA (n = 10) animals. **(C)** Relative proportions of SFs subset (lining: CD90- and sublining: CD90+) in total SFs of naïve (n = 11) and arthritic mice (n = 12) evaluated by flow cytometry. **(D)** Relative expression of IL-6, CCL2 and MMP3 mRNA in SFs subsets were quantified by RT-qPCR (n ≥ 6). **(E)** Relative expression of α2,3- and α2,6-sialyltransferases (St6gal1, St6GalNac5, St3gal1, St3gal2, St3gal3, St3gal4 and St3gal6) were quantified in SFs subsets by RT-PCR (n ≥ 6). Data are represented as mean ± SEM; each dot represents SFs from one individual mouse. **p < 0.01 by Mann-Whitney test in **(B, C)**, *p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal-Wallis test in **(D, E)**.

<0.01] (**Figure 3B**) to identify distinct transcriptomic signatures associated with hyposialylated conditions. This DE gene list (**Supplementary Table 1**) was investigated for pathway enrichment using KEGG database (**Figure 3C**). Remarkably, SA removal induced a clear activated phenotype in SFs, including enriched pathways for Rheumatoid Arthritis, cytokine signaling (TNFα, IL-17, chemokines) and NFkB and TLR signalling (**Figure 3C**). Next, we used the MCODE algorithm *via* the bioinformatics tool Metascape (30) to find functional gene nodes among the differentially expressed genes in desialylated SFs, identifying 10 nodes able to hold significant interconnected protein interactions (**Figure 4**). All nodes could be grouped under the broad immunity label and were connected to a greater or lesser extent. Node 1 covered the larger number of genes, mostly related to the CXCL chemokine signalling and inflammatory mediators, indicating that SFs adopted a distinctive inflammatory stage upon

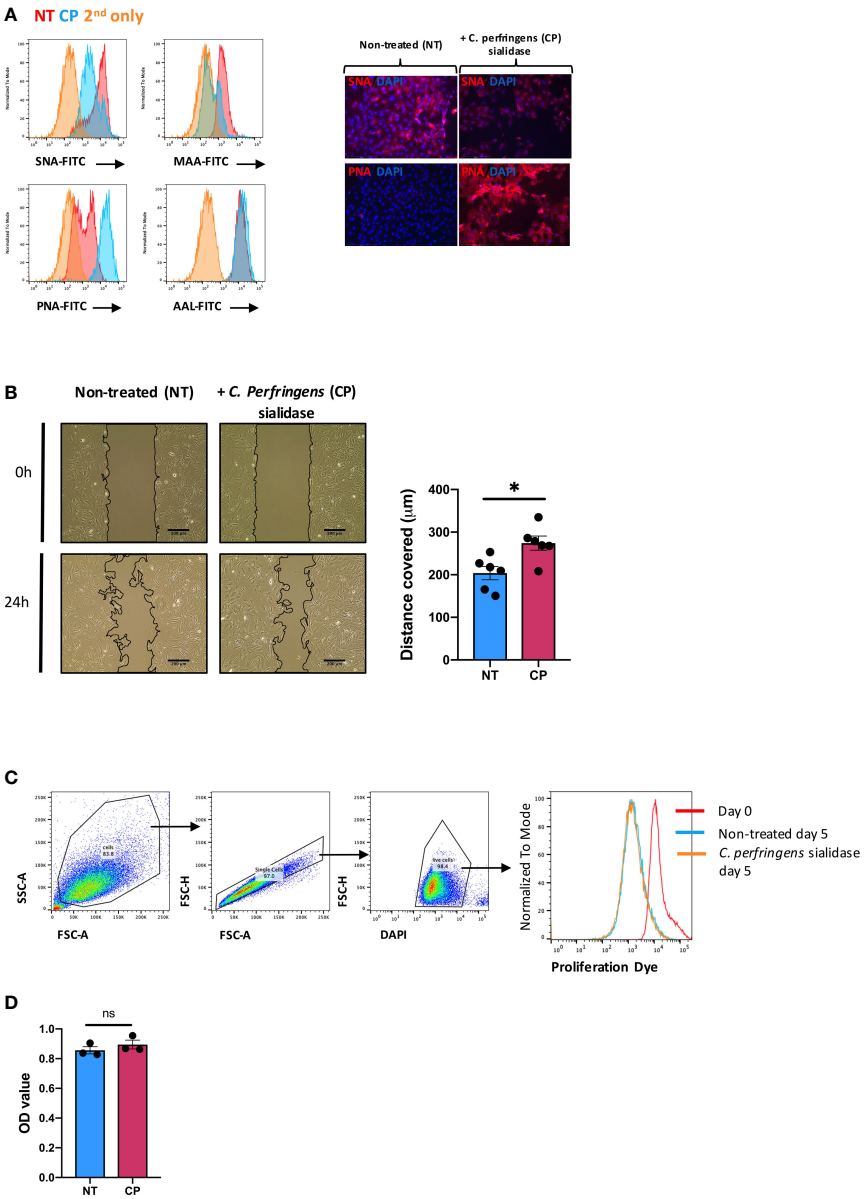


FIGURE 2 | *In vitro* desialylation enhances SFs motility. SFs isolated from naïve mice were cultured and expanded *ex vivo*. Cells were cultured to reach confluency and then treated with 0.1U/ml of *C. perfringens* sialidase (CP) for 1 hour to remove sialic acid from cell surface. **(A)** Control and sialidase-treated cells were stained with biotinylated lectins (SNA, MAA, PNA and AAL), followed by incubation with Alexa-647 streptavidin. Cells were then examined by flow cytometry to quantify lectin binding, as shown in histograms. Images were acquired in an EVOS^{TM} FL Auto microscope, showing SNA and PNA-stained cells. Error bars: 200 µm. **(B)** SFs were seeded in migration chambers and grown until monolayer confluence to conduct migration assays. Images show one representative experiment, including control and sialidase-treated naïve SFs when insert was removed (time 0, T0) and 24 hours after desialylation (T24). Superimposed black lines define the cell-free area, scale bar: 200 µm. Column graph show the mean migration distance of 5 independent experiments, showing non-treated (NT) and sialidase-treated (CP) cells. Error bars represent SEM, each dot represents one independent experiment (n = 5), *p < 0.05 by Mann-Whitney test. **(C)** SFs were labelled with proliferation dye eFluor 670 and analysed directly by flow cytometry (Day0) or treated with sialidase and maintained in culture for 5 days (Day5). Histogram shows one representative experiment. **(D)** Naïve SFs were seeded in 96-well plates, treated with sialidase and maintained in culture condition to assess cell viability and metabolomic activity using a colorimetric MTS assay. Each dot represents one independent experiment (n = 3) analysed in technical triplicate, error bars represent SEM, ns, non-significant, by Mann-Whitney test.

desialylation. In line with this, nodes 2, 3 and 4 comprised genes involved in cytokine-cytokine receptor signalling, NOD-like receptor pathways and NFkB activation. Interestingly, pathways in nodes 1 to 4 resembled a typical SF response during RA (**Figure 4A**), even when cells in this experiment had not been stimulated with any inflammatory factor. In addition, another 5 functional nodes were defined [nodes 5-9], that were directly connected to pathways involved in responses to viruses or

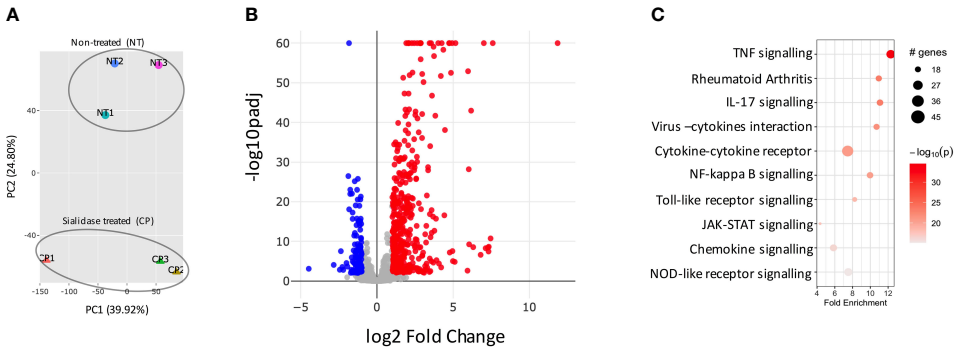


FIGURE 3 | Sialidase-treated SFs show an inflammatory transcriptomic profile. SFs from naive mice were cultured and expanded *ex vivo*, RNA was isolated from non-treated control (NT) and *C. perfringens* sialidase treated (CP) SFs, then subjected to bulk RNA-Seq (75bp paired-end, 30M reads, n = 3). **(A)** Principal component analysis (PCA). **(B)** All detected genes are plotted as a volcano plot. Genes that passed a threshold of padj < 0.01 and |foldChange| > 2 are considered differentially expressed genes (DEGs), comparing sialidase-treated and non-treated SFs. Colour code, red: upregulated, blue: downregulated, in CP treatment. **(C)** Upregulated genes in sialidase treatment identified in **(B)** were used to perform KEGG pathway enrichment analysis. KEGG pathways are plotted in bubble chart with fold enrichment on x-axis and -log10 p-value on the coloured scale. The size of bubble is proportional to the number of DEGs in the given pathway.

associated processes, such as interferon signalling, phagosome formation or MHC-I complexes (**Figure 4B**).

Thus, our RNA-Seq data described in detail how elimination of sialic acid on SF surface acts as a molecular signal to activate an inflammatory and anti-viral programme. Intriguingly, such sialidase-induced inflammation showed common characteristics with the known inflammatory phenotype of activated SFs during chronic RA, albeit some other pathways were unrelated and resembled immune responses to viral infections. To corroborate the inflammatory capacity of desialylated SFs, we selected a representative set of genes associated with inflammatory joint disease whose expression was significantly enhanced in the RNA-Seq dataset to be evaluated in independent experiments by RT-PCR. Specifically, we selected 8 up-regulated genes representative of SF-mediated inflammation, including cytokines (IL-6, Ccl2), matrix metalloproteinases (MMP3, MMP9, MMP13) and NFkB signalling pathways (MyD88, NFκBIB). In addition, we evaluated TNFSF11 (TNFSF11, TNF Superfamily Member 11 or Receptor activator of nuclear factor kappa-B ligand, RANKL) and TNFRSF11B (TNF Receptor Superfamily Member 11b, or Osteoprotegerin, OPG) expression, because disturbed RANKL/OPG ratio promotes osteoclastogenesis and bone damage in RA (38). Corroborating RNA-Seq data (**Figure 5A**), IL-6, Ccl2, MyD88 and all MMPs were up-regulated in desialylated SFs (**Figures 5B-D**). Likewise, RANKL was up-regulated and OPG was down-regulated (**Figure 5E**), matching sequencing results and suggesting an increased osteoclastogenic potential in desialylated SFs.

Overall, our results show that hyposialylated SFs show an inflammatory phenotype reminiscent of arthritic cells, with an increased migratory ability and expression on pro-inflammatory cytokines and chemokines known to play a key role in RA. In fact, when RNA-Seq was used to compare SFs transcriptomic changes upon desialylation with those induced by IL-1β, a well-known inflammatory mediator in RA, we observed 20.7% of overlapping up-regulated genes between both experimental conditions (**Figure 6**). Among these genes, we found inflammatory cytokines and chemokines, like IL-6, Csf3, CXCL and Ccl members and MMPs (**Supplementary Table 2**), which showed significantly enriched pathways for cytokine-receptor signalling, NFκB signalling, and other immune pathways (**Figure 6**), further highlighting the pro-arthritic status of hyposialylated SFs.

Enzymatic Removal of Surface SA Regulates Sialyltransferase Expression

Although the inflammatory signature of hyposialylated SFs shared a significant number of pathways with classical IL-1β stimulation, it also contained a distinctive set of DE 213 genes [fold change > 2, adjp <0.01] only observed in response to sialidase treatment (**Figure 6** and **Supplementary Table 2**). Among these, we still found a clear immune signature, including inflammatory cytokines (IL-15, IL-16), CXC chemokines and TNFα NF-κB signalling pathways. Cellular activation was evidenced by a large presence of P-loop NTPase fold-containing proteins, like Guanylate-binding proteins (Gbp), involved in the hydrolysis of phosphate bond of nucleoside triphosphates like ATP or GTP. This is mechanistically related to oxidative killing, phagolysosomes function and anti-viral responses, some of the other pathways activated only by desialylation. Interestingly, we also observed in our RNA-Seq datasets that the glycosyltranferase genes ST6Gal1, ST3Gal3, Gcnt1, B3Galt1 and Galnt18 were significantly regulated in response to surface desialylation, but not in response to IL-1β (**Figure 7A** and **Supplementary Table 2**). This suggests that exogenous factors inducing a loss of SA might provide a positive feedback to modulate endogenous expression of sialosides, which could represent a link between extracellular factors that modify SA content and consolidation of inflammatory response, perhaps leading to chronic disease. Hence, to evaluate whether SA

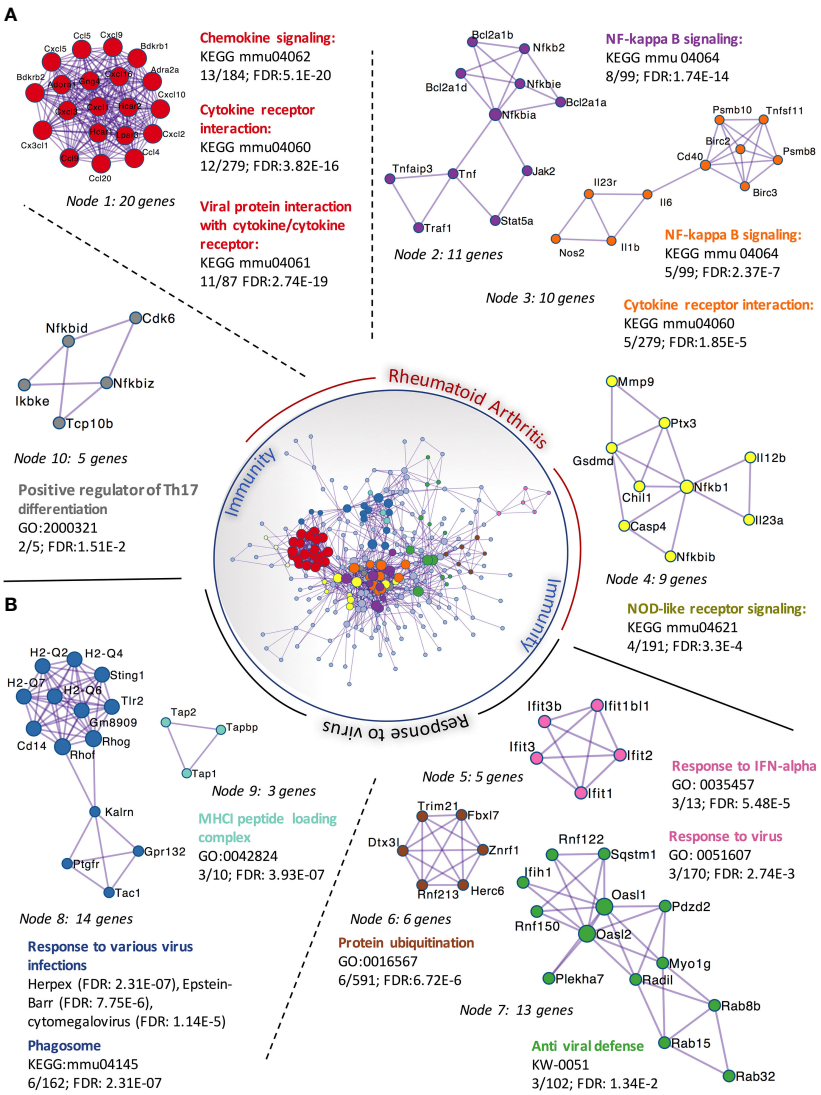


FIGURE 4 | Protein-protein interaction networks of upregulated DEGs in *C. perfringens* sialidase treated SFs. Upregulated DEGs identified in **Figure 3** were used to perform protein-protein interaction enrichment in Metascape (https://metascape.org/). Genes are represented by coloured circles, size is directly proportional to the number of genes in each node. DEGs genes clustered a total of 10 independent nodes based on known protein-protein interactions. Functional pathways significantly represented in each node are shown. Nodes are classified into two main categories based on the functional roles: 'rheumatoid arthritis' **(A)** and 'response to virus' **(B)**.

removal also modulates SF-sialylation pathways, we analysed expression of the two sialyltransferases, St6Gal1 and ST3Gal3, by RT-PCR after *C. perfringens* sialidase treatment. Corroborating the RNA-Seq data, SF desialylation significantly down-regulated St6Gal1 mRNA expression, whilst it up-regulated ST3Gal3 mRNA expression (**Figure 7B**). Moreover, flow cytometry lectin binding experiments showed that CP-treated SFs had a decreased capacity to rebuild surface SA expression upon sialidase treatment, as evidenced by increased PNA-binding and decreased SNA-binding (**Figure 7C**). This could indicate that desialylation implements a molecular mechanism to maintain low levels of α2-6 sialylation even if the cells are no longer exposed to sialidase hydrolytic action.

DISCUSSION

In this study, we report that synovial fibroblasts undertake a highly inflammatory phenotype when SA is removed from the cell surface. This supports our *in vivo* results, showing that inflammatory SFs show lower sialylation than healthy cells, mainly α2-6 linked SA. Notably, sialylation has been involved in cellular processes that are critical for SFs-mediated pathophysiology in RA, like cell migration and immune regulation. SA also determines metastatic potential and migration in cancer as well as tumour aggressiveness and invasiveness (39, 40). Specifically, elevated α2-3 sialylation has been associated with enhanced migration in breast cancer,

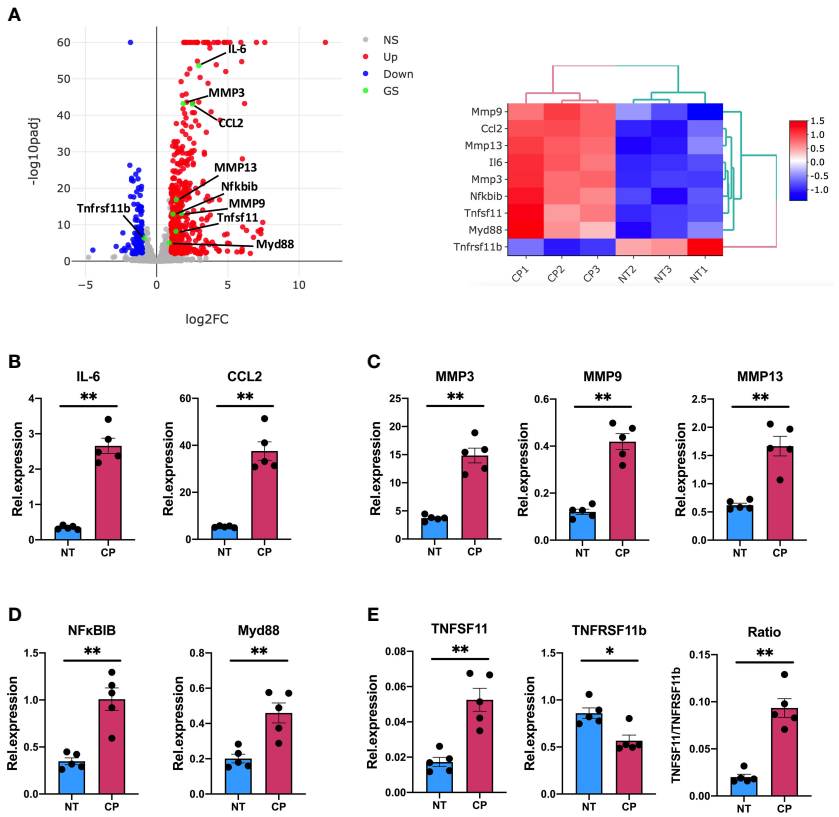


FIGURE 5 | Sialidase-treated synovial fibroblasts show an enhanced inflammatory response. **(A)** mRNA expression as detected by RNA-Seq from **Figure 3** for MMP3, MMP9, MMP13, IL-6, CCL2, Myd88, Nfκbib, Tnfsf11 and Tnfrsf11b. **(B–E)** RNA was isolated from control (NT) and *C. perfringens* sialidase treated (CP) naïve SFs. Relative expression of the genes shown in **(A)** was assessed by RT-qPCR using the ΔΔCt method and actin as housekeeping gene. **(B)** IL-6 and CCL2, **(C)** MMP3, MMP9 and MMP13, **(D)** Myd88 and Nfκbib, and **(E)** Tnfsf11 and Tnfrsf11b. For **(B–E)**, each dot represents one independent experiment, error bars represent SEM (n = 5), *p < 0.05, **p < 0.01 evaluated by Mann-Whitney test. NS, non-significant.

melanoma and pancreatic adenocarcinoma (41–43). Likewise, pathogenic cell migration has also been reported for α2-6 linked SA. Pally et al. described that distinct sialylation levels correlate with migratory phenotypes of epithelial cancer cells in three-dimensional cultures (44) and endometriotic cells show enhanced migration after α2-6 desialylation (45). Similar mechanisms could therefore happen in the arthritic joint to promote SFs migration and invasion of cartilage tissue. However, local factors such as the composition of the extracellular matrix in health and disease will influence cell migration. Thus, comparisons with other cell types or environments should be taken with caution and tailored studies are needed to fully understand how SA might affect SF migration in the context of arthritis.

Our data also revealed a strong link between low sialic acid content and initiation of SFs inflammatory and immune responses, suggesting that sialic acid acts as a molecular switch to control tissue homeostasis and inflammation in the joint synovial space. Our *in vivo* data show that a decreased α2-6/α2-3 SA ratio is responsible of SFs activation, something that *C. perfringens* sialidase recapitulates *in vitro*, proving that hyposialylated SFs become highly inflammatory. In this regard, the pathophysiology of SA in cancer represents the opposite scenario to autoimmune RA, as high SA content provides an advantageous scenario to cancer cells, particularly from the perspective of moderating immune responses. Tumour cells evade immune responses by adopting a hypersialylated phenotype to exploit SA-Siglec immunosuppressive signalling (46–49), whereas highly sialylated SFs may provide the immunosuppressive environment required in joint physiology. These overall results offer strong support to consider SA as an immunoregulatory switch, whose opposite actions in cancer and autoimmunity may represent two sides of the same coin. Considering this, SA is a potential target in cancer immunotherapy, since eliminating SA restraint in the tumour microenvironment could release anti-tumour immunity. Early studies dating back a few decades showed that treatment of a leukaemia cell lines with neuraminidases increased their immunogenicity (50, 51). A more specific approach is the recent development of engineered antibody–sialidases conjugates to target Siglec-dependent binding of NK cells,

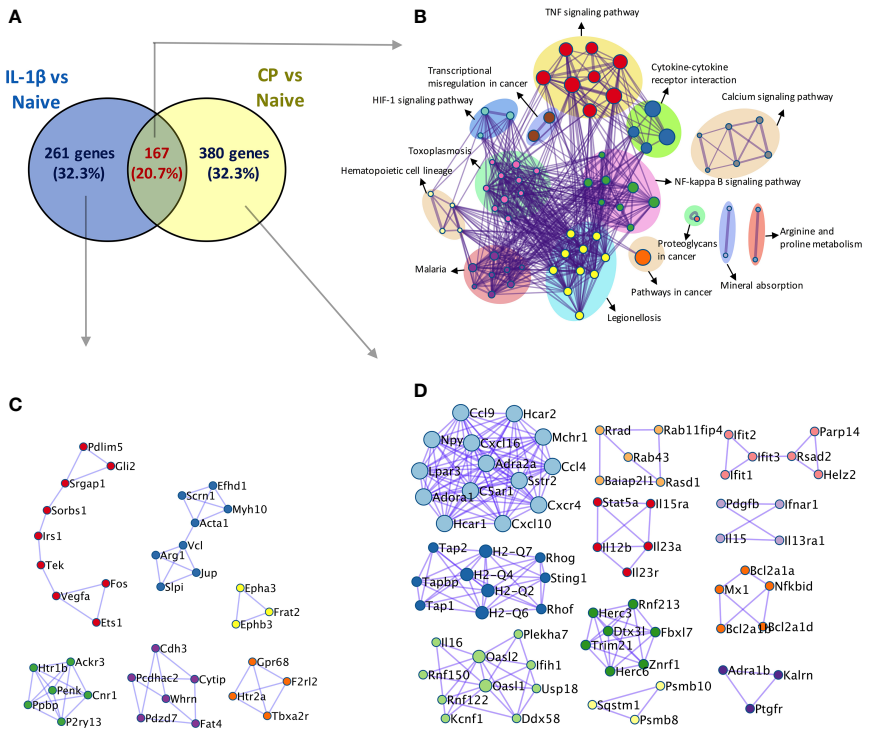
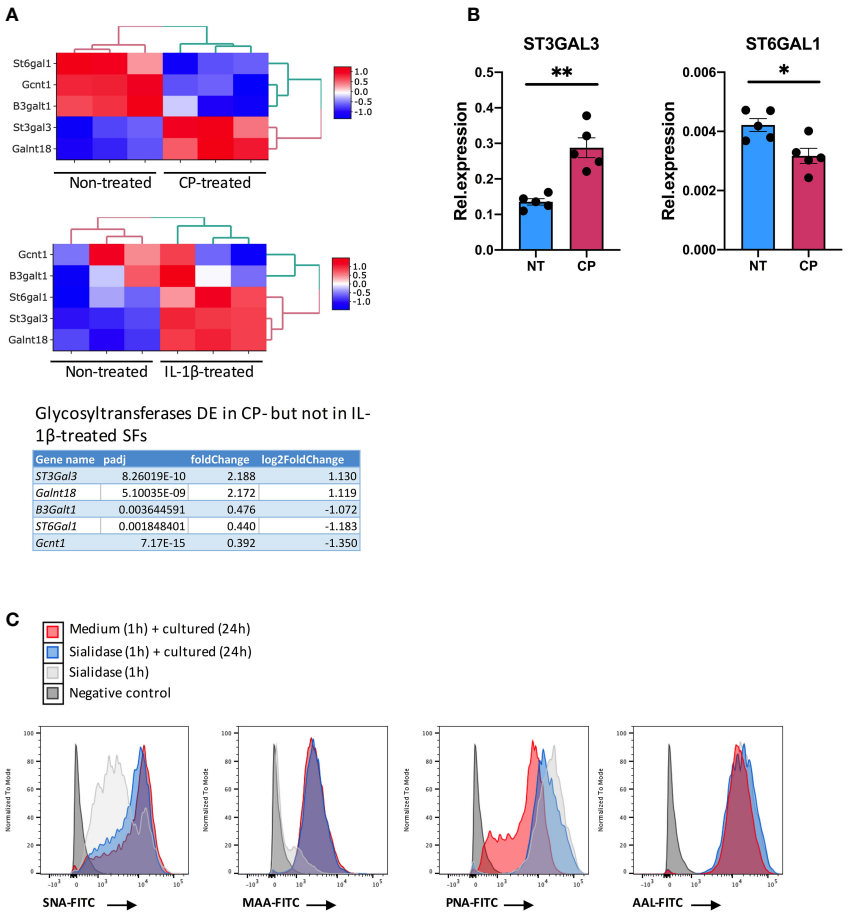


FIGURE 6 | Comparison of transcriptomic activation of desialylated and IL-1β-stimulated SFs. Bulk RNA-Seq (75bp paired-end, 30M reads, n = 3) was performed on i) naïve and IL-1β-stimulated, and ii) non-treated and *C. perfringens*-treated naïve SFs, to identify DEGs (Padj < 0.01 and |foldchange| > 2) in both experimental conditions. **(A)** Venn diagram shows the number of overlapping and unique DEGs identified in the IL-1β vs naïve and CP vs NT comparisons. **(B)** Overlapping genes identified in **(A)** were used to perform pathway enrichment using the bioinformatics tool Metascape. **(C)** Unique genes in IL-1β vs naïve and **(D)** unique genes in CP vs NT identified in **(A)** were used to perform protein-protein interaction enrichment in Metascape.

which makes tumour cells more susceptible to antibody-dependent cell-mediated cytotoxicity (52). However, our results in SFs could perhaps suggest that biologics with sialidase activity could also have the potential of starting off-target inflammation and autoimmunity, although additional work in animal models or clinical studies are required to challenge this hypothesis. Nevertheless, further steps have already been taken to optimise the selectivity of antibody-sialidases conjugates, by assessing several recombinant sialidases (53), but consideration of sialidase-dependent immune effects in long-term therapeutic regimes may be carefully considered for the development of safer and better sialidase-conjugated biologics.

Our study provides a causal link between presence of SA and initiation of immune responses in stromal SFs, but it still presents important limitations that should be addressed in follow-up studies. We used a recombinant sialidase from *C. perfringens* but details about the specific changes in sialylated glycoconjugates are still unknown. Specific SA linkages, membrane distribution, and SA acetylation might be an important aspect of SA-dependent communication and signalling. In fact, studies using CRISPR Cas9 showed that SA acetylation affects Siglec-mediated functions (54). Besides, the predominant sialic acids on murine cells are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), the latter not synthesised in humans because of the loss of the CMAH gene. This evolutionary event caused a rapid adaptation of the Siglec family to the new human glycome dominated by Neu5Ac. Therefore, translation of findings from murine models to human biology may be challenging, especially given that several pathotypes have been described in human RA (55). It is still very unclear how inflammatory mediators modulate the content of SA in human RA to initiate or perpetuate inflammation, and further studies, considering disease heterogeneity, should be conducted. To facilitate these translational findings, it is required to delineate the molecular mechanisms underlying SA-dependent SF activation. Loss of SA would imply a lack of regulatory Siglec signalling, but it may also uncover underlying galactose residues allowing galectin-3-binding and subsequent cell activation, as galectin-3 is a highly inflammatory mediator. In fact, galectin-3 induces a higher expression of pro-inflammatory IL-6, GM-CSF, MMP3 and even TNFα in SFs than in skin fibroblasts (3). A reduction of such inflammatory mediators in the synovium would also reduce local inflammation and cell recruitment, further reducing local TNFα and maintaining high levels of sialylation, since inflammatory TNFα down-regulates ST6Gal1 expression and α2-6 sialylation (26). Therefore, the inflammatory axis TNFα-



Glycosyltransferases DE in CP- but not in IL-1β-treated SFs

Gene name	padj	foldChange	log2FoldChange
ST3Gal3	8.26019E-10	2.188	1.130
Galnt18	5.10035E-09	2.172	1.119
B3Galt1	0.003644591	0.476	-1.072
ST6Gal1	0.001848401	0.440	-1.183
Gcnt1	7.17E-15	0.392	-1.350

FIGURE 7 | Enzymatic removal of sialic acid modulates intracellular sialyltransferases expression. **(A)** mRNA expression in SFs as detected by RNA-Seq from **Figure 6** for ST6Gal1, ST3Gal3, Gcnt1, B3Galt1 and Galnt18, including gene expression following *C. perfringens* sialidase treatment or IL-1β stimulation. Table shows adjp value and fold change of the glycosyltransferase genes, significantly regulated (adjp < 0.01, |log2Foldchange| >1) in *C. perfringens* sialidase treated SFs but not in in IL-1β-stimulated SFs. **(B)** RNA was extracted from control and *C. perfringens* sialidase treated naïve SFs. Relative expression of ST3gal3 and ST6gal1 was evaluated by RT-qPCR. Each dot represents one independent experiment, error bars represent SEM (n = 5), *p < 0.05, **p < 0.01 by Mann-Whitney test. **(C)** The presence of sialic acid on control and *C. perfringens* sialidase treated naive SFs was examined by flow cytometry for the binding of SNA, MAA, PNA and AAL. Experiments were performed after treatment (T0) and 24-hour incubation after treatment (T24).

hyposialylation-galectin-3 could have a stronger impact in the synovium compared with other tissues, such as the skin, perhaps explaining the tissue tropism observed in RA inflammation. Nevertheless, the potential effect of hyposialylated fibroblasts must also be investigated in other tissue more prone to suffer chronic inflammation, like the lung or gut. Interestingly, low sialylation has been linked to other pathways and cell types during RA, for example, activated chondrocytes show reduced levels of α2-3 SA and hyposialylation is also observed in Rheumatoid arthritis (RA)-associated IgG antibodies (56, 57).

Finally, we believe that understanding the factor(s) responsible of SF loss of SA *in vivo* is of high relevance to understand chronic RA. Such factors could have heterogeneous origins including i) cytokine signalling, like the TNFα-mediated downregulation of ST6Gal1 as we have shown before, ii) endogenous sialidases, secreted by SFs or other immune cells in arthritic joint, iii) infections, for example viral sialidases and iv) environmental factors, like diet and environment. For example, cigarette smoke reduces ST6Gal1 and α2-6 sialylation in bronchial epithelial cells leading to IL-6 production (58), similarly to the effects that we have observed in SFs upon desialylation. Similarly, sialidases are secreted by several viruses and other pathogens to modulate SA-dependent actions (59, 60), perhaps indicating that infections can remodel the local glycome to trigger, or favour, the establishment of future inflammatory RA. The fact that desialylated SFs activate anti-viral responses would provide support to such viral infection-chronic inflammation link. Importantly, our results show that removal of surface SA reconfigures expression of some sialyltransferases, suggesting that initial SA loss can lead to chronic inflammatory feedbacks, contributing to perpetuation of disease in RA regardless of the initiating factors.

DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE192488 and GSE196898.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Review Board of the University of Glasgow.

AUTHOR CONTRIBUTIONS

MP conceived and performed experiments, oversaw the project, interpreted the results, and wrote the manuscript with feedback from all authors. YW performed experiments and contributed to design of the experiments, data analysis and manuscript writing. PP, CC, and AK performed experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.847581/full#supplementary-material

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Molecular Basis and Role of Siglec-7 Ligand Expression on Chronic Lymphocytic Leukemia B Cells

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Siglec-7 (sialic acid–binding immunoglobulin-like lectin 7) is an immune checkpoint-like glycan recognition protein on natural killer (NK) cells. Cancer cells often upregulate Siglec ligands to subvert immunosurveillance, but the molecular basis of Siglec ligands has been elusive. In this study, we investigated Siglec-7 ligands on chronic lymphocytic leukemia (CLL) B cells. CLL B cells express higher levels of Siglec-7 ligands compared with healthy donor B cells, and enzymatic removal of sialic acids or sialomucins makes them more sensitive to NK cell cytotoxicity. Gene knockout experiments have revealed that the sialyltransferase ST6GalNAc-IV is responsible for the biosynthesis of disialyl-T (Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcα1–), which is the glycotope recognized by Siglec-7, and that CD162 and CD45 are the major carriers of this glycotope on CLL B cells. Analysis of public transcriptomic datasets indicated that the low expression of *GCNT1* (encoding core 2 GlcNAc transferase, an enzyme that competes against ST6GalNAc-IV) and high expression of *ST6GALNAC4* (encoding ST6GalNAc-IV) in CLL B cells, together enhancing the expression of the disialyl-T glycotope, are associated with poor patient prognosis. Taken together, our results determined the molecular basis of Siglec-7 ligand overexpression that protects CLL B cells from NK cell cytotoxicity and identified disialyl-T as a potential prognostic marker of CLL.

Keywords: chronic lymphocytic leukemia, natural killer cells, Siglec-7, sialomucin, ST6GalNAc-IV, Core 2 GlcNAc transferase

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of hematopoietic malignancy (1, 2). CLL develops over a long period of time by the accumulation of mature clonal B lymphocytes that proliferate in an uncontrolled manner and/or fail to undergo cell death. Clinical outcome of CLL is influenced by many factors, and the mutation status of immunoglobulin heavy chain variable region (*IGHV*), reflecting the differentiation stage of the B cell clone that eventually gives rise to CLL, is a

strong prognostic factor (3, 4). Survival of CLL cells depends on the signaling through B-cell receptor, which may recognize autoantigen or environmental antigen (5–7). Approval of drugs targeting the B-cell receptor signaling pathway (i.e., Btk and PI3Kδ inhibitors) and the anti-apoptotic protein Bcl2 inhibitor has revolutionized the treatment of CLL in the past decade (8). However, drug resistance eventually develops in many patients, necessitating new therapeutic approaches. Recent success in clinical trials of chimeric antigen receptor–transduced T cell and NK cell therapies has marked the beginning of a new era in CLL therapy (9, 10). The identification of factors influencing the success of cell-based CLL therapy is thus of clinical interest.

NK cells are equipped with various germline-encoded receptor proteins working as environmental sensors, and the sum of the inputs from activating and inhibitory receptors determines the cellular response (11–13). A previous study found that genetic polymorphisms determining the ratio between inhibitory and activating killer immunoglobulin-like receptors are associated with susceptibility to CLL (14), suggesting the importance of NK cell–mediated immunosurveillance in CLL. Siglec-7 (sialic acid–binding immunoglobulin-like lectin 7), also known as p75/AIRM-1, is one of the inhibitory receptors on NK cells (15, 16) and is considered to be a potential cancer immunotherapy target (17, 18). Many Siglecs, from a family of glycan recognition proteins expressed on various leukocytes, have immune checkpoint-like properties and contribute to the fine-tuning of immune responses (19, 20). Each Siglec shows a unique expression pattern and its own glycan recognition preference (21, 22). Research has shown that neutralization of Siglec-7 (expressed primarily on NK cells) and Siglec-9 (expressed primarily on myeloid cells but also on cytotoxic T cells in cancer patients) with an antibody can modulate the responses of killer lymphocytes in favor of cancer elimination (18, 23). Removal of sialic acid, a sugar residue recognized by Siglecs, from cancer cells also sensitizes them to cellular cytotoxicity by killer lymphocytes and other mechanisms (17, 24–26).

These previous studies demonstrated that the sialic acid–Siglec axis is a promising target for checkpoint inhibitor–type intervention in cancer treatment. However, our knowledge regarding the identity of Siglec ligands on cancer cells, consisting of the glycan epitope (*glycotope*) recognized by Siglec and the glycoproteins (*counterreceptors*) that exhibit the glycotope, is still limited (27, 28). The inherent difficulties in deciphering glycan-based recognition events include the low affinity of interaction between the glycan recognition protein and cognate glycotope (with the Kd value often being in the order of 10^{-3} M), complexity of glycan structures and biosynthesis pathways, redundancy in counterreceptors (i.e., the same glycotope can be exhibited in multiple glycoproteins), and the membrane-associated nature of functional ligands, among others. Regardless, understanding the molecular basis of Siglec-based immune subversion by cancer is crucial to improving the efficacy of cancer therapy. In this study, we used a combination of approaches to determine the molecular basis of Siglec-7 ligands on CLL B cells and further identified a potential prognostic marker of CLL *via* bioinformatic analysis of public transcriptomic datasets.

MATERIALS AND METHODS

Collection of Donor Blood and Purification of B Cells

The institutional review boards of the National Taiwan University Hospital and Academia Sinica approved this study (approval nos. 201907037RINA and AS-IRB-BM-19043, respectively). Taiwanese CLL patients were recruited at the National Taiwan University Hospital. Informed consent was obtained from each participant before peripheral blood samples were collected. The characteristics of the patients are summarized in **Supplementary Table 1**. Blood samples from healthy donors were obtained from the Taipei Blood Center (Taipei, Taiwan). B cells were purified from the blood samples by density gradient centrifugation using Ficoll-Paque PLUS (cat. no. 17-1440-03; Cytiva, Marlborough, MA, USA) followed by affinity purification with CD19 MicroBeads (cat. no. 130-050-301; Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (29).

Cell Lines

The human CLL cell lines JVM-3, MEC-1, and MEC-2 were obtained from DSMZ–German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). JVM-3 was maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep; Thermo Fisher Scientific, Waltham, MA, USA), whereas MEC-1 and MEC-2 were maintained in IMDM containing 10% FBS and 1% Pen/Strep. The human NK cell line NK-92MI (30) was obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan) and maintained in MEMα containing 12.5% horse serum, 12.5% FBS, 1% Pen/Strep, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, and 0.02 mM folic acid.

Antibodies and Other Reagents

Allophycocyanin-labeled anti-CD43 (clone L10) was obtained from Thermo Fisher Scientific. Phycoerythrin (PE)-labeled anti-CD43 (clone CD43-10G7), PE-labeled anti-CD45 (clone KPL1), and PE-labeled anti-CD162/PSGL-1 (clone 2D1) were purchased from Biolegend (San Diego, CA, USA). Recombinant Siglec–Fcs (consisting of an extracellular lectin domain of Siglec and human immunoglobulin G1 hinge–Fc region, with a FLAG tag in between) were prepared in-house (31). Fluorescein– and Alexa Fluor 647–labeled anti-human immunoglobulin G antibodies were acquired from Jackson ImmunoResearch (West Grove, PA, USA).

Sialidase (neuraminidase) from *Arthrobacter ureafaciens* was purchased from Nacalai (Kyoto, Japan). O-sialoglycoprotein endopeptidase (OSGP-EP) was acquired from Cedarlane Laboratories (Burlington, Ontario, Canada). Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc) and kifunensine were obtained from Millipore Sigma (St. Louis, MO, USA). DL-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Proximity Labeling of JVM-3 Cells With Siglec-7–Fc and Identification of Counterreceptor Candidates

Identification of Siglec-7 counterreceptors was attempted with proximity labeling as previously described (32). In brief, JVM-3 cells (1×10^7) were incubated with Siglec-7–Fc (10 μg) or binding-deficient mutant Siglec-7(R124A)–Fc (10 μg) precomplexed with peroxidase-conjugated anti-FLAG antibody (5 μg; cat. no. A8592; Millipore Sigma), followed by incubation with biotin labeling reagent (10 μM biotin tyramide and 10 mM H_2O_2 in Tris-buffered saline). Biotinylated proteins were purified from cell lysates with Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific), eluted by heat denaturation in sample buffer (Bio-Rad, Hercules, CA, USA), and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and in-gel trypsin digestion. The peptides were analyzed by liquid chromatography with tandem mass spectrometry (LC–MS/MS) using an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific). The raw data were processed using Proteome Discoverer 2.1 (Thermo Fisher Scientific), and peptide identification was performed using Mascot (version 2.3.2) and SEQUEST against the Swiss-Prot human database with a strict false discovery rate of 0.01. Label-free quantification was performed using the peak area of each precursor ion with a mass precision of 2 ppm. Details of the analysis are described in **Supplementary Materials and Methods**. The proteomics data set was deposited to ProteomeXchange *via* the PRIDE database (accession no. PXD024690).

Gene Expression Analysis With Quantitative Real-Time Polymerase Chain Reaction

The transcript levels of the genes of interest were analyzed with quantitative real-time polymerase chain reaction (qRT-PCR) using commercial primer–probe sets (TaqMan Real-Time PCR Assay; Thermo Fisher Scientific; **Supplementary Table 2**), in accordance with the protocols provided by the manufacturer. First-strand complementary DNA was prepared from 1 μg of total RNA extracted from the cells using a SuperScript III First-Strand Synthesis System with random hexamer primers (Thermo Fisher Scientific). The preparation was then used for the qRT-PCR assays with a FastStart Universal Probe Master (Roche, Mannheim, Germany) in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).

Expression of Siglec-7 in NK-92MI Cells

NK-92MI does not express Siglec-7 (33). We thus expressed Siglec-7 by lentiviral transduction as previously described (34). Siglec-7+ cells (NK-92MI/S7) were sorted by fluorescence-activated cell sorting twice. They were later used without further cloning.

Preparation of Gene-Edited JVM-3 and MEC-1 Cells With CRISPR–Cas9

To obtain JVM-3 and MEC-1 sublines lacking the genes of interest, we introduced *Streptococcus pyogenes* Cas9 and single-guide RNA (sgRNA) expression constructs *via* lentiviral transduction. Lentiviruses for the expression of Cas9 (p5w.Cas9.Pbsd) and sgRNAs (pU6-gRNA.Ppuro) were obtained from RNA Technology Platform and Gene Manipulation Core (National Biotechnology Research Park and Academia Sinica, Taipei, Taiwan). Transduced cells were subjected to drug selection and further sorted to select the population that lost the target protein (as revealed by antibody staining) or the target glycotope (as revealed by lectin or antibody staining; **Supplementary Figure 1**). Sorted cells were propagated, and the indels in the target gene were analyzed by genomic PCR and DNA fragment length analysis (with 3730xl DNA Analyzer and GeneMapper Software v4.0, Applied Biosystems/Thermo Fisher Scientific; outsourced to Genomics, New Taipei City, Taiwan; **Supplementary Figure 1**). Sorted cells were used without further cloning. Owing to the pseudo-tetraploid nature of the JVM-3 cell line, sequencing-based genotyping was not conducted. The sequences of the sgRNA and PCR primers used for DNA fragment length analysis are summarized in **Supplementary Table 3**.

NK Cell Cytotoxicity Assay

Target cells were labeled with 5 μM calcein acetoxymethyl ester (Thermo Fisher Scientific) in Dulbecco's PBS, washed three times with 5% FBS in Dulbecco's PBS, and mixed with NK-92MI/S7 at an effector/target ratio in the range of 1:1 to 10:1 in 96-well conical bottom plates (cat. no. 249935, Thermo Fisher Scientific). After a 4-h incubation at 37°C in a CO_2 incubator, the plate was centrifuged (at 600 g, 3 min), the supernatant (150 μL) was transferred to a fresh chimney plate (cat. no. 655096, Greiner Bio-One; Kremsmünster, Austria), and fluorescence intensity (excitation: 485 nm; emission: 535 nm) was measured with a plate reader (SpectraMax Paradigm; Molecular Devices, San Jose, CA, USA). Specific lysis was calculated with the following formula:

$$SpecificLysis(\%) = 100 \times (F_{E+T} - F_T)/(F_{max} - F_T)$$

where F_{E+T}, F_T, and F_{max} represent fluorescence in the supernatant from the effector + target, target alone, and maximum release by detergent lysis, respectively.

Quantitative Analysis of O-Glycans With LC–MS/MS

O-glycans were released from cells by alkaline reductive elimination, permethylated, and subjected to reversed-phase C18 nanoLC–MS/MS analysis as previously described (35). The major O-glycans detected and verified by MS/MS were relatively quantified by the peak areas of their extracted ion chromatograms. Details of the analysis are described in **Supplementary Materials and Methods**.

International Cancer Genome Consortium CLL Transcriptomic Data Analysis

Access to the data sets for CLL patients was granted by the Data Access Compliance Office of the International Cancer Genome Consortium (DACO-1071633). RNA sequencing–based

transcriptomic data sets for CLL patients (EGAD00001000258 and EGAD00001001443) were downloaded and analyzed using a Taiwania 1 supercomputer at the National High-Performance Computing Center (Hsinchu, Taiwan) and GNU Parallel (36). RNA sequencing data of the patients with CLL or small cell lymphoma and with survival status (n = 255 and n = 9, respectively; total n = 264) were included in the analysis. Patient data was obtained as metadata from the International Cancer Genome Consortium, and supplemented with *IGHV* mutation status from (37). Details of the analysis are described in **Supplementary Materials and Methods**.

Statistics

Statistical tests were performed with Prism 8 (GraphPad, San Diego, CA, USA) or with R. *P* value smaller than 0.05 was considered significant. Two-tailed tests were used throughout. For the comparison of two groups, Mann–Whitney test (when the normal distribution of values was not expected; **Figure 1A**) or Student's t test (**Figures 2**, **4A**, **6C**) was used. For the comparison of the means of multiple groups, one-way ANOVA with Dunnett's *post hoc* test (**Figures 1F**, **3C**, **4C**) was used. Association between gene expression and Siglec-7 binding (**Figure 6E**) was analyzed by linear regression, and that between gene expression and patient survival (**Figure 7** and **Table 1**) was analyzed by likelihood ratio test.

RESULTS

B Cells From CLL Patients Express Higher Levels of Siglec-7 Ligands Than Those From Healthy Donors

Differences in the cellular or protein-specific glycosylation patterns between B cells from CLL patients and those from healthy donors have been described in the literature (38–42), but whether these changes alter interactions with Siglecs has not been specifically addressed to date. To compare the glycosylation profiles of B cells from CLL patients with those of B cells from healthy donors in the context of Siglec recognition, we tested the binding of several recombinant Siglecs to these cells by flow cytometry. We chose CD22/Siglec-2, Siglec-7, and Siglec-9 as probes, as these Siglecs showed robust binding to B cells from CLL patients in our preliminary experiments (data not shown). We found that B cells express ligands for several Siglecs and that B cells from CLL patients express higher levels of Siglec-7 ligands compared with those from healthy donors (**Figure 1A**). By contrast, the levels of ligands for CD22/Siglec-2 or Siglec-9 were not significantly different between the two groups (**Figure 1A**). The results for the CD22/Siglec-2 probe are consistent with those we obtained in a previous study, which demonstrated similar degrees of terminal α2–6 sialylation of N-glycans in B cells from CLL patients and healthy donors (42).

Primary Siglec-7 Ligands in CLL B Cells Are O-Glycosylated Proteins

To investigate the molecular basis of Siglec-7 ligands in CLL B cells, we sought a CLL B cell line that resembles B cells from CLL patients in terms of glycan profile. Among the cell lines tested, JVM-3 showed a Siglec binding pattern similar to that of B cells from CLL patients (**Figure 1B**). Thus, we primarily used this cell line for further study.

As expected, sialidase treatment of JVM-3 cells diminished Siglec-7 binding (**Figure 1C**). Among the compounds that interfered with glycan processing, including benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc, mimicking the GalNAc peptide and diverting the O-glycan biosynthesis pathway), kifunensine (blocking N-glycan processing at high mannose–type glycans), and DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (inhibiting glycolipid biosynthesis), only benzyl-α-GalNAc significantly attenuated Siglec-7 binding to JVM-3 cells, suggesting that the glycotope on CLL B cells recognized by Siglec-7 is primarily exhibited on O-glycans (**Figure 1D**). We then treated the cells with O-sialoglycoprotein endopeptidase (OSGP-EP), which selectively digests mucin-like glycoproteins heavily modified with sialylated O-glycans (sialomucins) (43, 44). This treatment diminished Siglec-7 binding to JVM-3 cells (**Figure 1E**), demonstrating that glycoproteins heavily modified with O-glycans are the primary ligands for Siglec-7. Treatment of B cells from CLL patients with sialidase or OSGP-EP also diminished Siglec-7 binding (**Figure 1F**), confirming the observation with JVM-3.

Enzymatic Removal of Sialylated O-Glycans Sensitizes JVM-3 to NK Cell Cytotoxicity

To test whether Siglec-7 ligands protect JVM-3 cells from NK cells, we enzymatically treated JVM-3 cells with sialidase or OSGP-EP and subjected them to NK cell cytotoxicity assay using the NK-92MI cell line expressing Siglec-7 (NK-92MI/S7). We over-expressed Siglec-7, as NK-92MI does not (or only weakly) express Siglec-7 (33).

As expected, both enzymatic treatments sensitized JVM-3 cells to NK cell cytotoxicity (**Figure 2A**). Moreover, the JVM-3 cell culture in the presence of benzyl-α-GalNAc also sensitized the cells to NK cell cytotoxicity (**Figure 2B**). Taken together, these results imply that sialylated glycotopes on heavily O-glycosylated proteins (counterreceptors) protect CLL B cells from NK cell cytotoxicity. We observed a similar enhancement of cytotoxicity by the same treatment of JVM-3 cells when parental NK-92MI cells were used as effector cells (**Supplementary Figure 2**), implying that sialylated and heavily O-glycosylated proteins can protect CLL by a Siglec-7-independent mechanism as well. Although we found Siglec-6 is highly expressed on parental NK-92MI, recombinant Siglec-6 did not show binding to JVM-3 (**Supplementary Figure 3**), excluding the interaction between NK-92MI and JVM-3 cells by way of Siglec-6 and its ligand.

Siglec-7 Counterreceptors on CLL B Cells Include CD43, CD45, and PSGL-1

We used a proximity biotin labeling method (32) to identify the counterreceptors for Siglec-7 and determined CD45 as a candidate (**Supplementary Dataset 1**). CD43, a major sialomucin, was also identified with a single peptide. However,

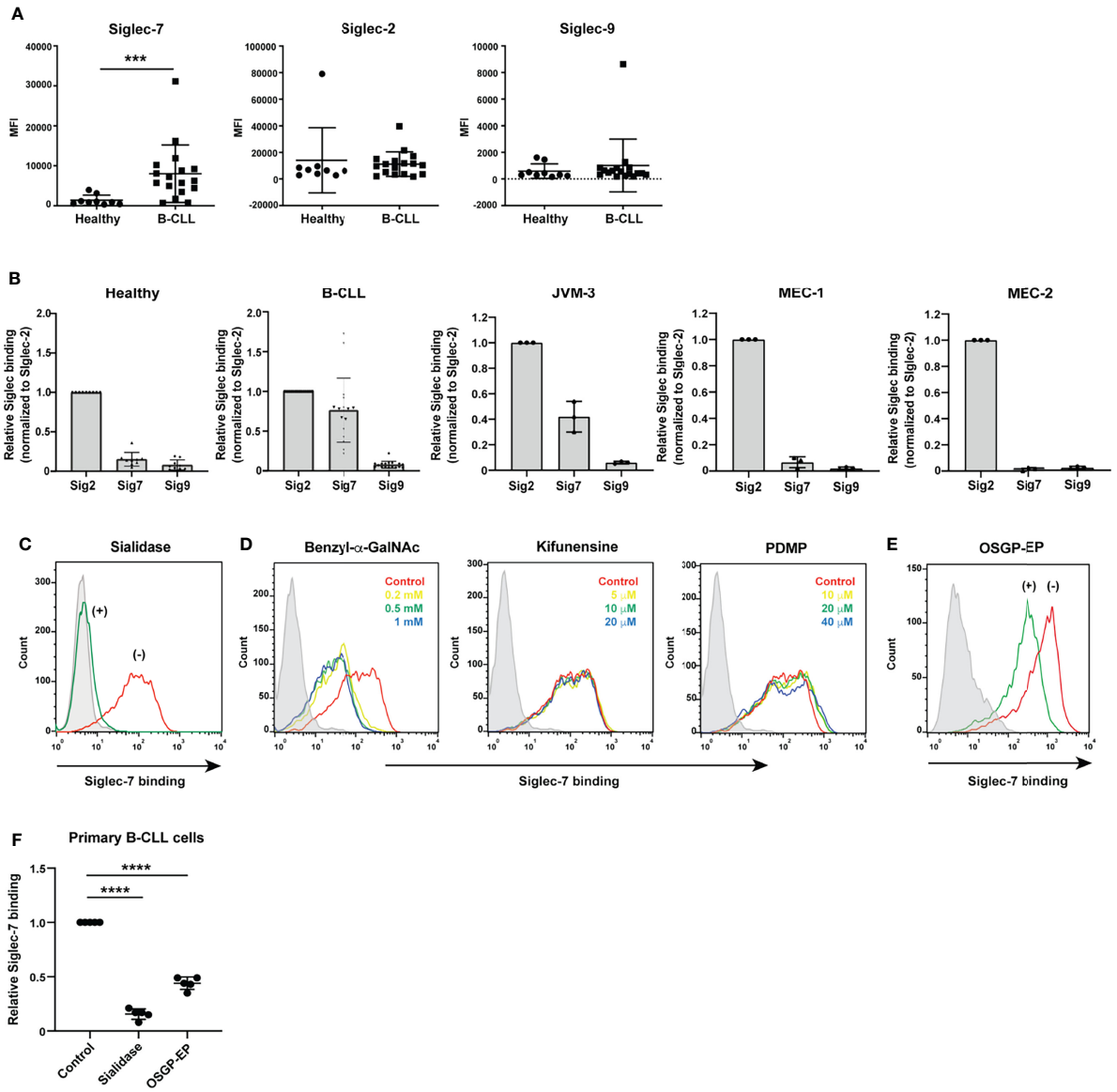

FIGURE 1 | Siglec-7 ligands are highly expressed on B cells from patients with chronic lymphocytic leukemia (CLL). **(A)** Comparison of Siglec ligand levels between B cells from healthy donors and those from CLL patients. The difference in Siglec-7–Fc binding (expressed as median fluorescence intensity [MFI]) to B cells between healthy donors (n = 9) and CLL patients (n = 17) was statistically significant (***$P < 0.001$, Mann–Whitney test), whereas the difference in CD22/Siglec-2–Fc and Siglec-9–Fc binding between the two groups was not ($P = 0.33$ and 0.38, respectively; Mann–Whitney test). Bars represent mean ± SD. **(B)** Siglec ligands in primary B cells and CLL B cell lines. JVM-3, MEC-1, and MEC-2 cells were stained with recombinant CD22/Siglec-2–Fc, Siglec-7–Fc, and Siglec-9–Fc and analyzed with flow cytometry. Siglec–Fc binding signals (in MFI) were normalized to that of CD22/Siglec-2–Fc. Bars represent mean ± SD of three independent experiments. For primary B cells, the data was normalized individually for each donor [healthy donors (n = 9) and CLL patients (n = 17)], using the same dataset as presented in panel **(A)**. JVM-3 most closely resembled CLL B cells in terms of Siglec binding pattern. **(C)** Effect of sialidase treatment on Siglec-7 binding to JVM-3. JVM-3 cells were treated with (green) or without (red) sialidase before probing with recombinant Siglec-7–Fc. Siglec-7–Fc binding was abrogated by treatment of the cells with sialidase. Siglec-7(R124A)–Fc was used as a negative control (gray). **(D)** Effects of glycan processing inhibitors on Siglec-7–Fc binding to JVM-3. Cells were cultured in the presence of benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc; red: control; yellow: 0.2 mM; green: 0.5 mM; blue: 1 mM), kifunensine (red: control; yellow: 5 μM; green: 10 μM; blue: 20 μM), or DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; red: control; yellow: 10 μM; green: 20 μM; blue: 40 μM) for 72 h; stained with recombinant Siglec-7–Fc; and analyzed with flow cytometry. Benzyl-α-GalNAc pretreatment attenuated Siglec-7–Fc binding, whereas neither kifunensine nor PDMP did, implying that O-glycans exhibit the glycan epitope (glycotope) recognized by Siglec-7. **(E)** Effect of O-sialoglycoprotein endopeptidase (OSGP-EP) treatment on Siglec-7–Fc binding to JVM-3. JVM-3 cells were treated with (green) or without (red) OSGP-EP before probing with recombinant Siglec-7–Fc. OSGP-EP treatment of JVM-3 cells attenuated Siglec-7–Fc binding, indicating that glycoproteins heavily modified by sialylated O-glycans (sialomucins) are the major ligands for Siglec-7. **(F)** Effect of enzyme treatment on Siglec-7–Fc binding to B cells from CLL patients (n = 5). Sialidase and OSGP-EP treatment of B cells from CLL patients diminished Siglec-7–Fc binding (****$P <$ 0.0001, one-way ANOVA with Dunnett's *post hoc* test). Bars represent mean ± SD.

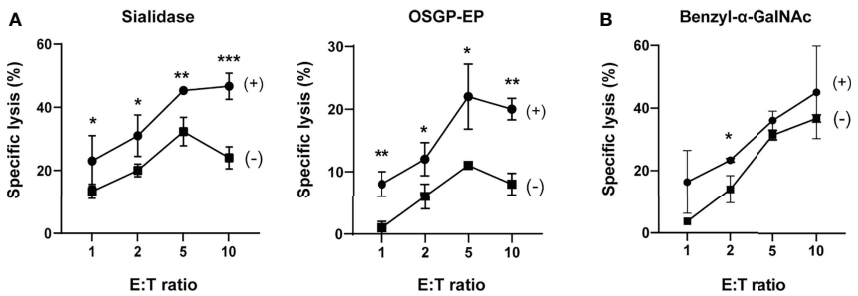

FIGURE 2 | Sialylated O-glycoproteins protect chronic lymphocytic leukemia B cells from NK cell cytotoxicity. **(A)** Effects of sialidase or O-sialoglycoprotein endopeptidase (OSGP-EP) treatment of JVM-3 cells on NK cell cytotoxicity. JVM-3 cells were treated with sialidase or OSGP-EP and subjected to cytotoxicity assay using an NK-92 cell line expressing Siglec-7 (NK-92MI/S7). Both treatments made JVM-3 cells more sensitive to NK cell cytotoxicity. Cytotoxicity assays were conducted in technical triplicate and repeated several times, with consistent results. Representative results are shown (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; Student's t test). Bars represent mean ± SD of technical triplicates. **(B)** Effect of benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc) treatment of JVM-3 on NK cell cytotoxicity. JVM-3 cells cultured in the presence of benzyl-α-GalNAc (72 h) were more sensitive to NK cell cytotoxicity. Cytotoxicity assays were conducted in technical triplicate and repeated several times, with consistent results. Representative results are shown (*$P < 0.05$, Student's t test). Bars represent mean ± SD of technical triplicates.

other sialomucins (e.g., CD162/P-selectin glycoprotein ligand-1 [PSGL-1]) were not identified, likely because these proteins are resistant to proteolysis and inherently difficult to identify by mass spectrometry (45).

Flow cytometry analysis revealed that CD43 and CD162/PSGL-1 are expressed on JVM-3 cells (data not shown). We thus tested whether either of these proteins or CD45 accounts for a major counterreceptor by knocking out each of them. Gene disruption (*SPN* for CD43, *PTPRC* for CD45, and *SELPLG* for CD162/PSGL-1) revealed that none of these proteins alone could account for the Siglec-7 counterreceptor but that depletion of each glycoprotein attenuated the Siglec-7 binding to a small extent (**Figure 3A**).

We then tested whether any of the knockout cells show increased sensitivity to NK cell cytotoxicity. As expected, cells deficient in CD45 or CD162/PSGL-1 were more sensitive to cytolysis by NK-92MI/S7 (**Figure 3B**). Taken together, these results indicate that CD45 and CD162/PSGL-1 are functional Siglec-7 counterreceptors on CLL B cells.

Siglec-7 Glycotope on CLL B Cells Is Synthesized by ST6GalNAc-IV

To gain further insight into the glycan part of Siglec-7 ligands, we sought the sialyltransferase responsible for the biosynthesis of the glycotope recognized by Siglec-7. Siglec-7 preferentially

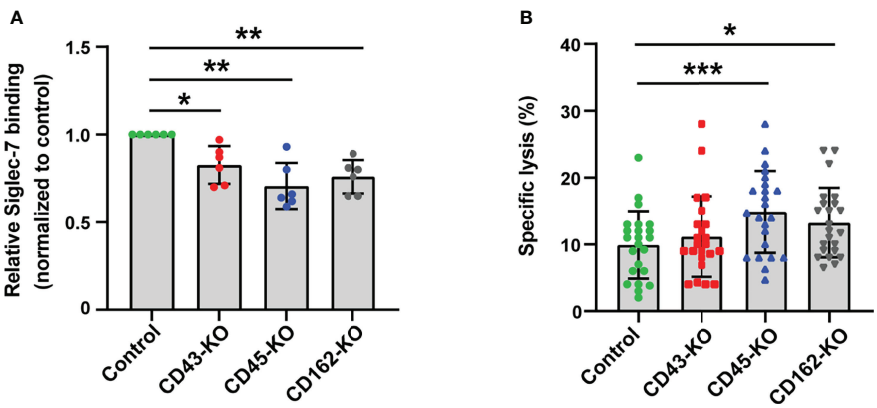

FIGURE 3 | CD43, CD45, and CD162/PSGL-1 are the counterreceptors of Siglec-7. **(A)** Effect of glycoprotein knockout (KO) on Siglec-7–Fc binding. The glycoprotein genes (*SPN*, *PTPRC*, and *SELPLG* – encoding CD43, CD45, and CD162/PSGL-1, respectively) in JVM-3 were disrupted with CRISPR–Cas9 technology, and the cells were subjected to staining with Siglec-7–Fc. The disruption of individual genes led to a small but reproducible reduction in Siglec-7–Fc binding. Data was normalized by the Siglec-7–Fc binding (in MFI) to control JVM-3 cells. *$P < 0.05$, and **$P < 0.01$, one-way ANOVA with Dunnett's *post hoc* test. Bars represent mean ± SD of 6 independent experiments. **(B)** Effect of glycoprotein KO on NK cell cytotoxicity. Glycoprotein KO and control JVM-3 cells were subjected to NK cell cytotoxicity assay. Disruption of CD45 and CD162/PSGL-1 led to increased sensitivity to NK cytotoxicity. A trend toward increased sensitivity of CD43 KO cells to NK cytotoxicity was observed, but it was not statistically significant (*$P < 0.05$ and ***$P < 0.001$, repeated-measures one-way ANOVA with Dunnett's *post hoc* test). Bars represent mean ± SD of 23 independent experiments.

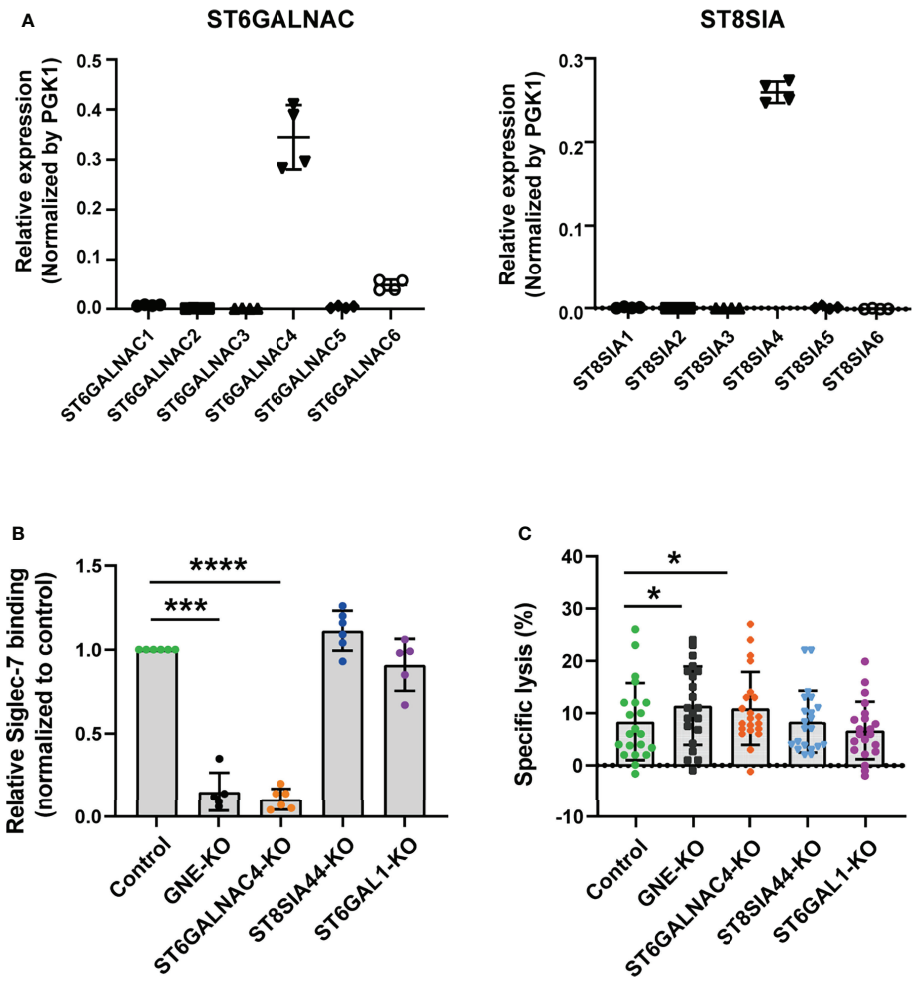

FIGURE 4 | ST6GalNAc-IV is responsible for Siglec-7 ligand glycotope synthesis. **(A)** Sialyltransferases expressed in JVM-3. The transcript level for *ST6GALNAC4* was the highest among *ST6GALNAC*s, whereas that for *ST8SIA4* was the highest among *ST8SIA*s. Bars represent mean ± SD of technical quadruplicates. **(B)** Effect of sialyltransferase KO on Siglec-7–Fc binding. *GNE* and sialyltransferase genes (*ST6GALNAC4*, *ST8SIA4*, and *ST6GAL1*) in JVM-3 were disrupted with CRISPR–Cas9 technology, and the cells were subjected to staining with Siglec-7–Fc. The disruption of *GNE* and *ST6GALNAC4* led to a marked reduction in Siglec-7–Fc binding, whereas the disruption of *ST8SIA4* and *ST6GAL1* did not. Data was normalized by the Siglec-7–Fc binding (in MFI) to control JVM-3 cells. ***$P < 0.001$, and ****$P < 0.0001$, one-way ANOVA with Dunnett's *post hoc* test. Bars represent mean ± SD of 6 independent experiments. **(C)** Effect of sialyltransferase KO on NK cell cytotoxicity. Sialyltransferase KO and control JVM-3 cells were subjected to NK cell cytotoxicity assay. The disruption of *GNE* and *ST6GALNAC4* led to increased sensitivity of JVM-3 cells to NK cytotoxicity, whereas the disruption of *ST8SIA4* and *ST6GAL1* did not (*$P < 0.05$, repeated-measures one-way ANOVA with Dunnett's *post hoc* test). Bars represent mean ± SD of 21 independent experiments.

TABLE 1 | Association of glycosyltransferase expression levels with the survival of patients with chronic lymphocytic leukemia.

Parameter	Hazard ratio (95% CI) by univariate analysis	Hazard ratio (95% CI) by multivariate analysis with age and *IGHV* mutation as covariates
Age (per year)	1.04 (1.01–1.07; $P = 0.008$)	1.04 (1.01–1.07; $P = 0.015$)
IGHV mutation (mutated/unmutated)	0.12 (0.06–0.24; $P < 0.001$)	0.15 (0.07–0.32; $P < 0.001$)
GCNT1 (*G*) and *ST6GALNAC4* (*S*)		
$G^{high}S^{high}/G^{high}S^{low}$	3.25 (1.07–9.91; $P = 0.038$)	1.87 (0.59–5.96; $P = 0.289$)
$G^{low}S^{high}/G^{high}S^{low}$	7.61 (2.60–22.27; $P < 0.001$)	3.59 (1.16–11.12; $P = 0.026$)
$G^{low}S^{low}/G^{high}S^{low}$	4.62 (1.03–20.88; $P = 0.045$)	1.38 (0.27–7.00; $P = 0.701$)

Analyses of the association of GCNT1 and ST6GALNAC4 with mortality, with or without clinical covariates, were performed using International Cancer Genome Consortium data (n = 264) as described in Methods. The cutoff value for sample subgrouping was based on the optimal cutoff for gene expression (3.3 and 10.3 for GCNT1 and ST6GALNAC4, respectively) fitted in the Cox proportional hazards model. P values are based on likelihood ratio test. CI, confidence interval.

recognizes α2–8–linked oligosialic acids ([Neu5Acα2–8]n; n ≧ 2), disialyl N-acetyllactosamine (Neu5Acα2–3Galβ1–4[Neu5Acα2–6]GlcNAcβ1–), and a terminal tetrasaccharide of α-series gangliosides (Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcβ1–) (46–51), which are elaborated by ST8Sia and the ST6GalNAc family of sialyltransferases, respectively. Therefore, we analyzed the expression profiles of these sialyltransferases in the JVM-3 cell line; we found that *ST8SIA4* and *ST6GALNAC4* were highly expressed (**Figure 4A**). As shown in **Figure 4B**, *ST6GALNAC4*-deficient cells showed a clear reduction in Siglec-7 binding, whereas those deficient in *ST8SIA4* or *ST6GAL1* did not. As expected, JVM-3 cells deficient in *GNE* (encoding UDP-GlcNAc 2-epimerase/ManNAc 6-kinase, the first enzyme in the sialic acid biosynthesis pathway) also showed a clear reduction in Siglec-7 binding (**Figure 4B**).

To test whether the JVM-3 cells deficient in Siglec-7 glycotope are more sensitive to NK cell cytotoxicity, we subjected the cells to cytotoxicity assay. As expected, *ST6GALNAC4* and *GNE* deficient cells were more sensitive to NK cell cytotoxicity than the control cells were, whereas *ST8SIA4* and *ST6GAL1* deficient cells were not (**Figure 4C**). Taken together, these results indicate that *ST6GALNAC4* is responsible for the biosynthesis of the glycotope that protects CLL B cells from NK cell cytotoxicity.

The disialyl-T Structure Is the CLL Glycotope Recognized by Siglec-7

To determine the glycotope elaborated by ST6GalNAc-IV, we subjected the control and *ST6GALNAC4*-deficient JVM-3 cells to quantitative O-glycan analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS). As shown in **Figure 5**, control JVM-3 cells predominantly expressed variably sialylated core 1 O-glycan (Galβ1–3GalNAcα1–) structures, with disialyl-T (Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcα1–) being the most abundant. By contrast, *ST6GALNAC4*-deficient JVM-3 cells showed a significant loss of disialyl-T as well as a concomitant increase in monosialyl-T (Neu5Acα2–3Galβ1–3GalNAcα1–) and core 2 O-glycan structures (e.g., Galβ1–3[Galβ1–4GlcNAcβ1–6]GalNAcα1–). These results strongly suggest that disialyl-T is the primary glycotope on CLL B cells recognized by Siglec-7. We noticed that the trisialyl-T structure (Neu5Acα2–3Galβ1–3[Neu5Acα2–8Neu5Acα2–6]GalNAcα1– and/or Neu5Acα2–8Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcα1–) was reduced in *ST6GALNAC4*-deficient JVM-3 cells and further diminished in *ST8SIA4*-deficient cells, suggesting that disialyl-T serves as an acceptor substrate for ST8Sia-IV. Nevertheless, as *ST8SIA4* deficiency neither impaired Siglec-7 binding nor enhanced NK cell cytotoxicity, O-glycans with linear oligosialic acids do not appear to be essential for Siglec-7 binding or the resistance of CLL B cells to NK cell cytotoxicity.

Expression of GCNT1 Interferes With the Biosynthesis of Siglec-7 Ligands

MEC-1 cells are more sensitive to NK cell cytotoxicity compared with JVM-3 cells (**Figure 6A**), which coincided with weaker Siglec-7 binding (**Figure 2A**). Given that (i) the addition of GlcNAc at C6 of GalNAc by core 2 GlcNAc transferase (encoded by *GCNT1*) precludes the sialylation at the same position by ST6GalNAc-IV (**Figure 6B**) and (ii) the expression level of *GCNT1* in MEC-1 is higher than that in JVM-3 (**Figure 6C**), we speculated that the expression of *GCNT1* interferes with the expression of Siglec-7 glycotope in MEC-1 cells. As expected, *GCNT1* disruption in MEC-1 cells enhanced Siglec-7 binding (**Figure 6D**). To confirm the effects of *GCNT1* and *ST6GALNAC4* on Siglec-7 ligand expression, we quantified their transcript levels by qRT-PCR and analyzed their association with Siglec-7 ligand levels on B cells from CLL patients. As expected, high expression of *GCNT1* was associated with weaker Siglec-7 binding, whereas the expression of *ST6GALNAC4* showed a positive correlation with Siglec-7 binding (**Figure 6E**).

High Expression of ST6GALNAC4 and Low Expression of GCNT1 Are Associated With Poor Prognosis in CLL Patients

To test whether the expression levels of *ST6GALNAC4* and *GCNT1* show any association with the prognosis of CLL patients, we analyzed the correlations between the overall survival of CLL patients and the expression levels of these genes using the CLL RNA sequencing data set in the International Cancer Genome Consortium database (37, 53). RNA sequencing data of the patients with CLL or small cell lymphoma and with survival data (n = 255 and n = 9, respectively; total n = 264) were included in the analysis. Our analysis revealed that high *ST6GALNAC4* expression and low *GCNT1* expression are associated with poor prognosis (**Figure 7A, B**, respectively). Moreover, by comparing four groups of patients stratified by *ST6GALNAC4* and *GCNT1* expression levels, we found that the prognosis of $GCNT1^{low}ST6GALNAC4^{high}$ patients is the least favorable (overall, $P = 0.00015$; $GCNT1^{low}ST6GALNAC4^{high}$ vs. $GCNT1^{high}ST6GALNAC4^{low}$ groups, $P < 0.001$; **Figure 7C**). This association remained significant even when age and *IGHV* mutation status (a strong prognostic factor for CLL) were included as covariates ($P = 0.026$; **Table 1**). Taken together, these results suggest that the expression of the disialyl-T structure is associated with poor prognosis in CLL patients, possibly through immunoevasion by engagement of Siglec-7 on NK cells.

DISCUSSION

In this study, we demonstrated that B cells from CLL patients express higher levels of Siglec-7 ligands compared with those from healthy donors and that the ligands protect B cells from NK cell cytotoxicity. The glycotope recognized by Siglec-7 is the disialyl-T (Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcα1–) structure, which was exhibited on various counterreceptors, including CD43, CD45, and CD162/PSGL-1. The glycan epitope was synthesized by ST6GalNAc-IV (encoded by *ST6GALNAC4*), and its synthesis was blocked by core 2 GlcNAc transferase (encoded by *GCNT1*). The expression

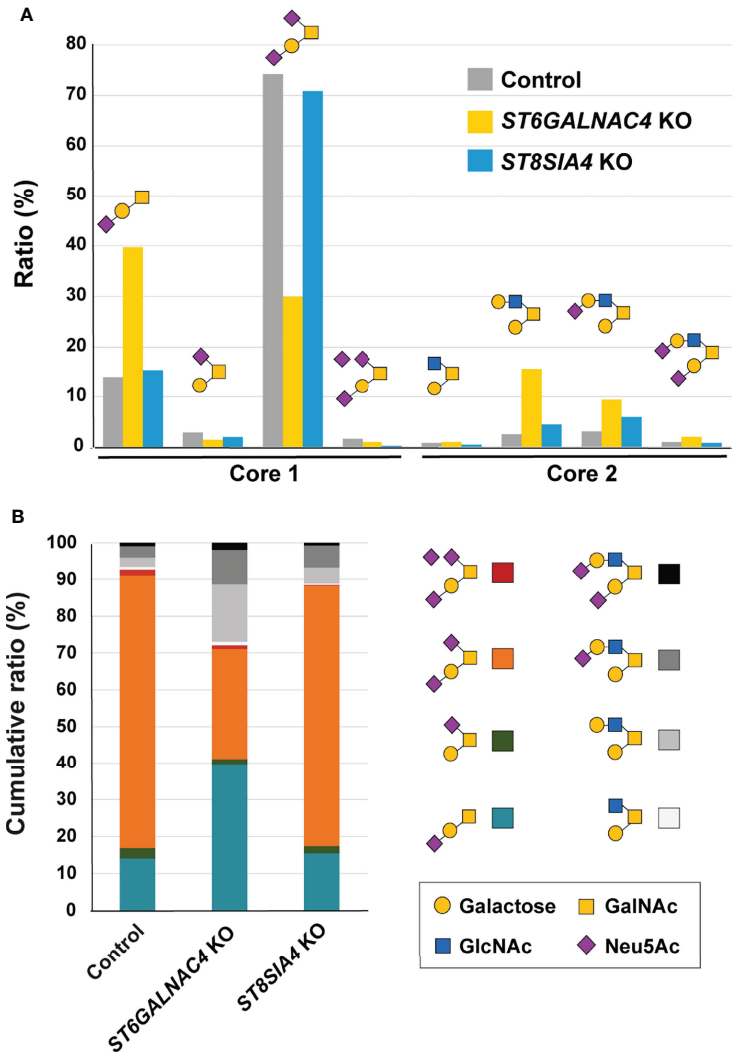

FIGURE 5 | ST6GalNAc-IV is responsible for the biosynthesis of disialyl-T in JVM-3 cells. **(A)** O-glycans were released by reductive elimination from control (gray), *ST6GALNAC4* KO (yellow), and *ST8SIA4* KO (blue) JVM-3 cells; permethylated; and subjected to liquid chromatography with tandem mass spectrometry analysis. Except for monosialyl-T (Neu5Acα2–3Galβ1–3GalNAcα1– or Galβ1–3[Neu5Acα2–6]GalNAcα1–), which could be resolved by liquid chromatography into two distinct isomeric structures, and trisialyl-T, which consisted of two unresolved positional isomers (Neu5Acα2–3Galβ1–3[Neu5Acα2–8Neu5Acα2–6] GalNAcα1– and Neu5Acα2–8Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcα1–), each of the other O-glycans was found to be represented by a single dominating structure, as determined by tandem mass spectrometry and annotated accordingly using the Symbol Nomenclature for Glycans (52). Relative abundance was calculated from the peak areas of extracted ion chromatograms and normalized to the percentage total. Disruption of *ST6GALNAC4* resulted in a reduction in the disialyl-T (Neu5Acα2–3Galβ1–3[Neu5Acα2–6]GalNAcα1–) structure and a concomitant increase in the monosialyl-T (Neu5Acα2–3Galβ1–3GalNAcα1–) and core 2 (e.g., Galβ1–3[Galβ1–3GlcNAcβ1–6]GalNAcα1–) structures. Disruption of *ST8SIA4* resulted in the loss of the trisialyl-T structure. **(B)** Stacked bar chart of the same data shown in panel **(A)**, along with the color code used for each of the eight major O-glycans identified and quantified.

levels of these two glycosyltransferases were associated with the overall survival of CLL patients, and the pattern predictive of high disialyl-T expression (*GCNT1*^{low}*ST6GALNAC4*^{high}) was associated with poor prognosis. These data imply that the mechanism underlying the poor prognosis in *GCNT1*^{low}*ST6GALNAC4*^{high} patients likely involves the high expression of the disialyl-T structure, which may facilitate immunoevasion by engaging Siglec-7 on NK cells.

The O-glycosylation pattern of human B cells has been previously reported to change during differentiation, and a reduction in *GCNT1* expression and a concomitant shortening of O-glycans were observed in the cells that have undergone germinal center reaction (54). Research has also shown that the level of Siglec-7 ligands on human B cells changes during differentiation, with naive and memory cells expressing high levels of Siglec-7 ligands, whereas it decreases temporarily on activated naive cells (55). Therefore, the expression level of Siglec-7 ligands potentially reflects the differentiation stage of the B-cell clone that gave rise to CLL. However, our analysis (data not shown) indicated that *GCNT1* expression is higher in

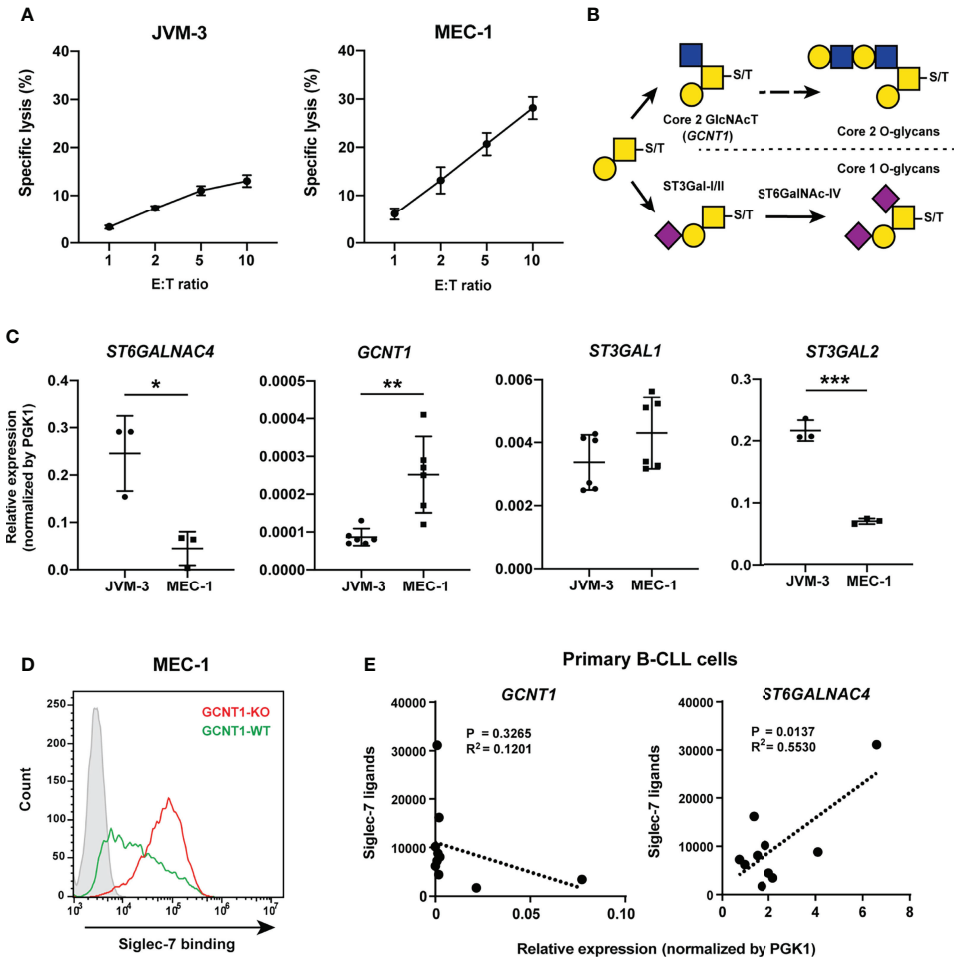

FIGURE 6 | Core 2 GlcNAc transferase interferes with the biosynthesis of the glycotope recognized by Siglec-7. **(A)** NK cell cytotoxicity assay of JVM-3 and MEC-1 cell lines. JVM-3 cells were more resistant than MEC-1 cells to NK cell cytotoxicity. Cytotoxicity assays were conducted in technical triplicate and repeated several times, with consistent results. Representative results are shown. Bars represent mean ± SD of technical triplicates. **(B)** Schematic representation of O-glycan biosynthesis in leukocytes. **(C)** Quantitative real-time polymerase chain reaction analysis of glycosyltransferases in JVM-3 and MEC-1 cells. *ST6GALNAC4* expression was higher in JVM-3, whereas *GCNT1* expression was higher in MEC-1 (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; Student's t test). Bars represent mean ± SD of technical replicates (n = 3–6). **(D)** Effect of *GCNT1* disruption in MEC-1 cells on Siglec-7–Fc binding. **(E)** Correlation of the *GCNT1* and *ST6GALNAC4* transcript levels and Siglec-7–Fc binding (in median fluorescence intensity [MFI]) to B cells from patients with chronic lymphocytic leukemia (n = 10). Association between gene expression and Siglec-7 binding was analyzed by linear regression.

IGHV-mutated CLL (reflecting somatic hypermutation in germinal center), which is opposite of our expectation [i.e., *GCNT1* expression diminishes during B-cell maturation (54)]. Regardless, when the *IGHV* mutation status was included in the multivariate analysis, the association between overall survival and the *ST6GALNAC4* and *GCNT1* expression levels remained significant (**Table 1**). Thus, *ST6GALNAC4* and *GCNT1* transcription and disialyl-T expression levels may serve as independent criteria for the prognosis of CLL patients.

The observed association of *GCNT1* expression with CLL prognosis is incongruent with data reported for solid tumors (e.g., bladder and prostate cancers), in which high expression of *GCNT1* was associated with poor prognosis, presumably through the protection of tumors from NK cells (56–58). We speculate that this discrepancy can be attributed to the difference in the lectins and counterreceptors involved. The extension of polylactosamine on the core 2 O-glycans on major histocompatibility complex class I polypeptide–related sequence A (MICA) was found to reduce its binding with the cognate receptor NK group 2 member D (NKG2D) on NK cells, both directly and by way of binding with galectin-3 (58). The cell lines we used (JVM-3 and MEC-1) expressed low levels of MICA (data not shown). MICA was slightly upregulated on B cells from CLL patients compared with those from healthy donors, whereas high plasma levels of soluble NKG2D ligands (soluble MICA, MICB, and UL16 binding protein 2) were associated with poor treatment-free survival of CLL patients (59), suggesting that soluble NKG2D ligands may compromise NKG2D-mediated

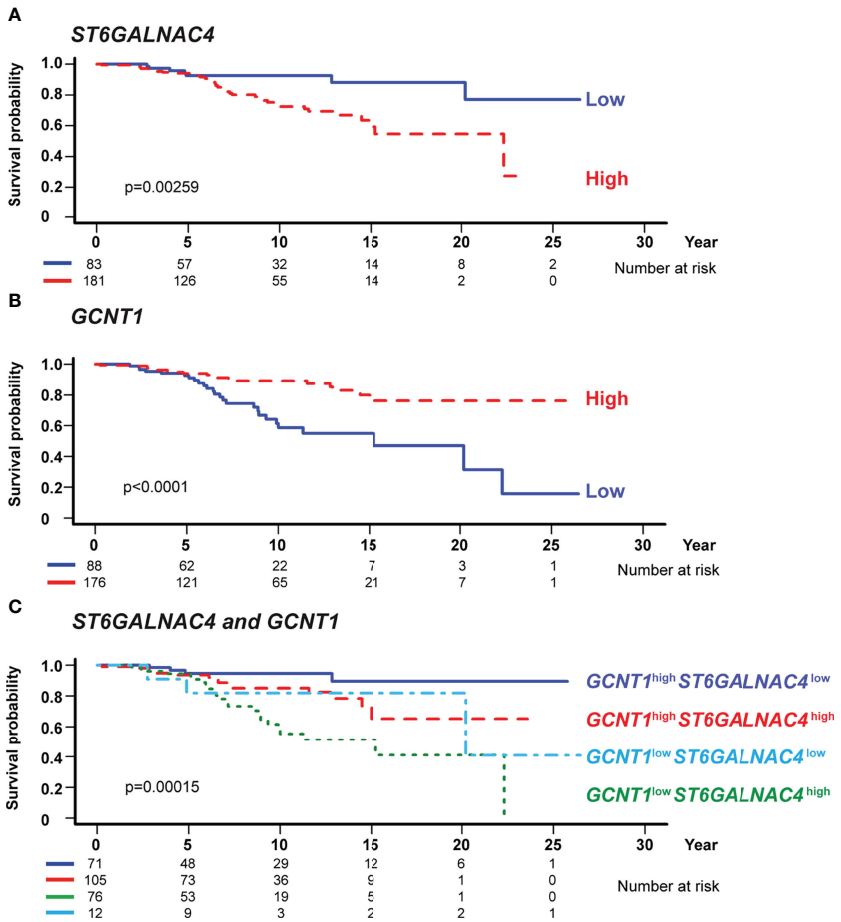

FIGURE 7 | GCNT1 and ST6GALNAC4 expression levels are associated with the prognosis of patients with chronic lymphocytic leukemia (CLL). Shown are the Kaplan–Meier survival plots with logrank test for two subgroups dichotomized with the expression levels of *ST6GALNAC4* **(A)** and *GCNT1* **(B)** as well as for four subgroups with the expression levels of both genes **(C)** using the optimal cutoff fitted in the Cox proportional hazards model. High expression of *ST6GALNAC4* **(A)** and low expression of *GCNT1* **(B)** were associated with poorer prognosis in CLL patients (P = 0.00259 and < 0.0001, respectively; likelihood ratio test). In panel **(C)**, the survival curves of four subgroups are significantly different (P = 0.00015), and the prognosis of *GCNT1*^{low}*ST6GALNAC4*^{high} patients was significantly poorer as compared with that of *GCNT1*^{high}*ST6GALNAC4*^{low} patients (P < 0.001, likelihood ratio test; see also **Table 1**).

NK cell activation in CLL. In addition, as the polylactosamine extension on O-glycans on B cells is limited (data not shown), interruption of the MICA–NKG2D interaction by polylactosamine may not play a major role in CLL. Regardless, the difference in the role of *GCNT1* between solid tumors and CLL underscores the importance of understanding the nature of the glycotope and counterreceptors serving as ligands for Siglecs.

Two recent studies independently identified CD43 as a Siglec-7 counterreceptor on the K562 erythroleukemia cell line, which is often used as a target for NK cytotoxicity assays, and demonstrated that knockout/knockdown of CD43 renders K562 cells more sensitive to NK cytotoxicity (34, 60). By contrast, our analysis revealed that CD43 is not the sole counterreceptor for Siglec-7. The difference between K562 and CLL may be explained by the different repertoires of glycoproteins expressed on these cells. For instance, K562 cells express CD43, but not CD162/PSGL-1, at high levels (data not shown). Another recent study, investigating the resistance of multiple myeloma cells to NK cytotoxicity, revealed that multiple myeloma cells express high level of Siglec-7 ligand, and CD162/PSGL-1 is a major Siglec-7 counterreceptor on multiple myeloma cells (61). Yet another recent study using a genetically manipulated HEK293T cell line demonstrated that *GCNT1* and *ST6GALNAC*s regulate the expression of Siglec-7 glycotope, which the authors deduced to be disialyl-T, and found a strong dependence of Siglec-7 binding on the type of counterreceptor expressed (62).

NK cells in CLL patients have been reported to be functionally impaired (63). Although the subject is beyond the scope of this study, Siglec-7 ligands on CLL B cells can be hypothesized to induce the state of NK cell exhaustion by engaging Siglec-7. If this is true, then blocking the interaction between Siglec-7 on NK cells and its ligands on CLL B cells may restore the cytotoxic activity of NK cells.

Our study has some limitations. Although our analysis of Taiwanese CLL patient samples found correlations between *GCNT1* and *ST6GALNAC4* expression levels and Siglec-7 glycotope (**Figure 6E**), the findings are not definitive because of the limited number of samples. Moreover, the clinical benefit of glycotope testing remains unknown. A prospective study enrolling more patients would address this issue better.

DATA AVAILABILITY STATEMENT

The RNA-sequencing datasets presented in this article are not readily available, because the dataset is International Cancer Genome Consortium (ICGC) Controlled Data, and the Data Access Agreement between ICGC and the PI does not permit the transfer or disclosure of any material derived from the ICGC Controlled Data to anyone not listed in the data access application. Requests to access the datasets should be directed to the Data Access Control Office of ICGC (https://daco.icgc-argo.org). The proteomic data is publicly available in the PRIDE database (accession no. PXD024690).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Taiwan University Hospital and Academia Sinica. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

L-YC, HCT, S-CL, H-YT, C-JT, MS, and Yi-JC performed the experiments and analyzed the data. S-YL analyzed the CLL transcriptomic data. S-JW recruited patients and collected patient blood samples. Yu-JC, K-IL, K-HK, and TA designed the research, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.840388/full#supplementary-material

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