SIGLEC-8 LIGANDS IN HUMAN AIRWAY AND AIRWAY SECRETIONS

by
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ABSTRACT

Siglecs – sialic acid binding Ig-like lectins – are regulatory molecules expressed on subsets of immune cells where most inhibit inflammation when engaged by complementary sialoglycan ligands on target tissues. Both eosinophils and mast cells express Siglec-8 on the cell surface, and when Siglec-8 binds to sialoglycan ligands on tissues apoptosis of eosinophils and inhibition of mediator release by mast cells is induced, limiting inflammation. Although Siglec-8 has been shown to bind a synthetic glycan 6'-sulfated sialyl N-acetyllactosamine, the endogenous Siglec-8 ligand in human airways was still unknown.

This study provides evidence of endogenous high molecular weight Siglec-8 ligands in human airway and airway secretions that are sensitive to sialidase and keratanase treatment. Siglec-8 ligands were isolated and identified from normal postmortem human airways or from nasal lavage. Glycoproteins were separated by size exclusion chromatography and resolved by composite agarose-acrylamide gel electrophoresis, blotted, and probed with human Fc-tagged Siglec-8, revealing three binding species (270 kDa, 600 kDa and 1000 kDa) in tracheal extracts and one major binding specie (~900 kDa) in nasal lavage. Ligand-containing fractions were pooled, and ligands were captured by immunoprecipitation using His-tagged pentameric Siglec-8 bound to nickel-Sepharose beads. Siglec-8-precipitated ligands were subjected to mass spectrometric proteomic analysis, revealing the proteoglycan aggrecan as the predominant protein in all three-size species of Siglec-8 ligands extracted from trachea and glycoprotein-340 as the predominant protein in sample purified from nasal lavage. Anti-aggrecan antibody immunoblots of electrophoresed tracheal purified Siglec-8 ligand revealed co-migrating aggrecan immunoreactivity that

was sensitive to aggrecanase treatment. Anti-GP340 antibody immunoblots of electrophoresed nasal lavage purified Siglec-8 ligand revealed co-migrating GP340 immunoreactivity. All Siglec-8 ligands were sensitive to sialidase and keratanase treatment. In conclusion, human airway Siglec-8 ligands are sialylated keratan sulfate chains presented on different proteins dependent on where they are expressed.

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CHAPTER 1: INTRODUCTION

Section 1.1- Siglecs and Immune Regulation

Siglecs (Sialic acid binding ImmunoGlobulin-like LECtins) are cell surface proteins, members of the immunoglobulin gene superfamily that bind sialylated glycans.[1-4] All siglecs are single pass transmembrane proteins with extracellular domains containing one or more Ig-like C2-set domains and an amino terminal variable Ig-like-domain that specifically binds sialic acid[3]. Siglecs are classified in two major subgroups, classic siglecs which are evolutionary conserved across mammalian species and CD33-related siglecs which are not.[3,5-7] Because CD33-related siglecs have evolved rapidly it is hard to determine a homolog of some of them in mouse and therefore, those mouse siglecs were assigned letters instead of numbers (**Fig. 1.1**).[8-10] Unlike other sialic acid binding lectins, siglecs bind to sialic acids with strict specificity for sialic acid linkage.[2,3]

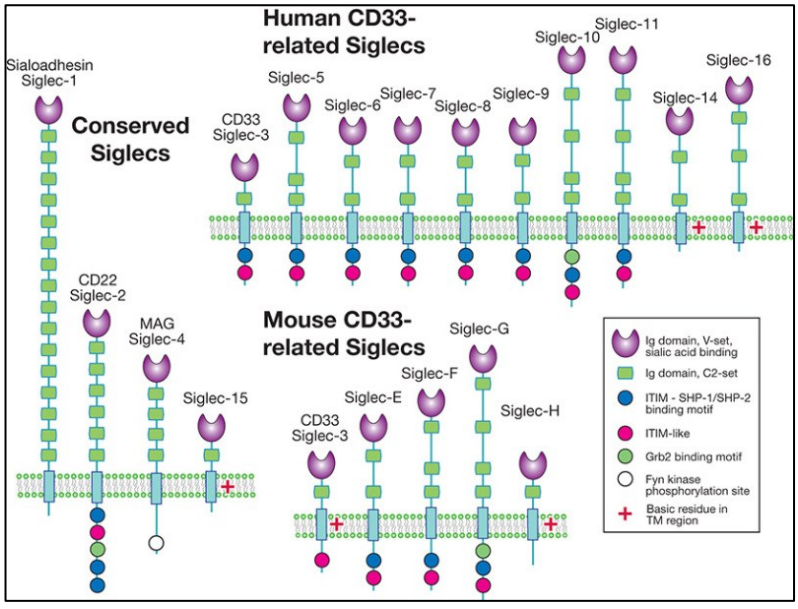


Figure 1.1 Human and mouse Siglecs. To the left, human and mouse siglecs are shown as a cartoon diagram. Sialic acid binding domain in purple, variation in number of C2-set domain in different siglecs and small cytosolic tail containing ITIM and ITIM like motifs.[11]

Interest in siglecs and their natural ligands has increased in the past decade due to their potential for therapeutics and treatment of inflammatory diseases. [8,12,13] There is a constant fight between host and pathogens where a balance between activation and inhibition of immune cells is critical to resolve infection while avoiding hyperinflammation. Many siglecs have a cytosolic tail with immunoreceptor tyrosine-based inhibitory motifs or ITIMs involved in intracellular signaling.[2,3,7,11,14] Most siglecs when engaged to their natural glycan ligand induce downregulation or apoptosis of the cell they are expressed on. [2,3,7,11,14] Selective expression of siglecs on subsets of inflammatory cells (**Fig. 1.2**) and their potential to mediate natural signaling pathways through the ITIM motif makes them an appealing therapeutic target to suppress ongoing inflammation and limit inflammatory tissue damage downstream of immune responses.[12,15-17]

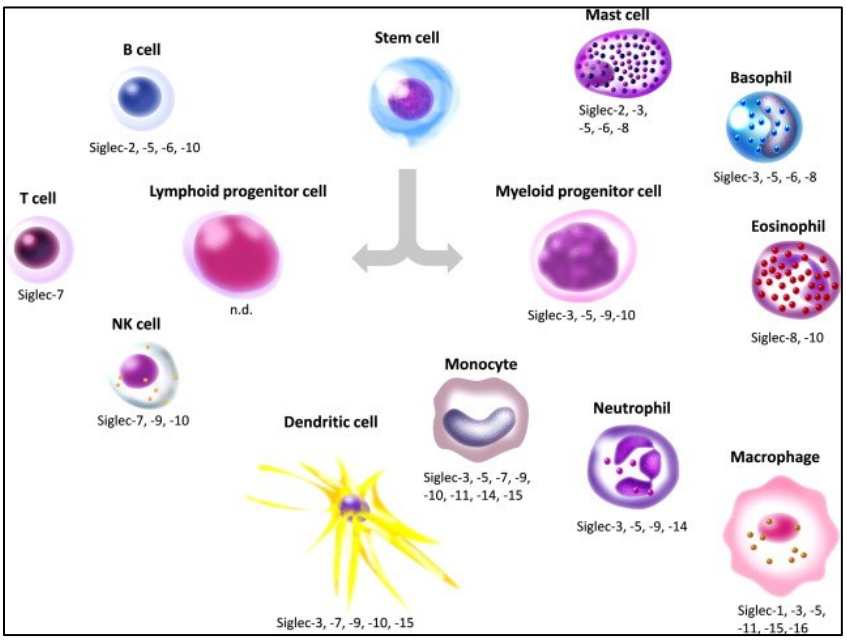


Figure 1.2 Siglecs selective expression in human immune and hematopoietic cells. Each cell type expresses a set of siglecs on the cell surface and some of them share similar siglecs. Siglec-8 is expressed mainly on cells involved in asthma and allergy (mast cells, basophils and eosinophils) whereas Siglec-9 is expressed on neutrophils, monocytes, myeloid progenitor cells and some natural killer cells (NK cells).[12]

Section 1.2- Siglec-8 and Siglec-9

Among the siglecs expressed in human immune cells is Siglec-8. Siglec-8 discovered in 2000 was found to be highly and selectively expressed on eosinophils, basophils and mast cells.[18] Later studies found that Siglec-8 expressed on inflammatory cells associated with asthma and allergy could function as a regulatory molecule.[19] Further analysis of Siglec-8 revealed its high selectivity against alpha-2-3 linked sialic acids and preferential binding to sialyl-Lewis structure 6'-sulfated-sialyl-LewisX [6'-sulfo-sLex; NeuAcα2-3Gal(6-O-SO3) β1-4(Fucα1-3) GlcNAc].[20] A synthetic glycan array of more than 600 glycans, further confirmed its high specificity for the 6'-sulfated-sialyl-LacNAc and 6'-sulfated-sialyl-LewisX.[21] When Siglec-8 was crosslinked on inflammatory cells by antibodies or polyvalent 6'-sulfated-sialyl-LacNAc glycan it induced eosinophil apoptosis and inhibited release of inhibitory mediators from mast cells.[16] This regulatory function was even greater in the presence of proinflammatory mediators.[22] At the time this thesis research was initiated, it was already established that Siglec-8 plays a role in inflammatory regulation, however, the structure of the endogenous human glycan ligand was still unknown. Our hypothesis was that ligands are produced in the lung where they engage inflammatory cells expressing Siglec-8 ligand and control inflammation. Knowledge of the natural ligands for Siglec-8 can provide improved leads for glycomimetic drug development, produce better drugs that halt eosinophilic and mast cell mediated inflammation and improve outcomes of asthma, allergy, and chronic rhinosinusitis.

Siglec-9 is expressed on a broader range of immune cells, neutrophils, monocytes, dendritic cells, and some natural killer cells.[12] Unlike Siglec-8, Siglec-9 has a broader glycan binding specificity. On a custom glycolipid microplate array, Siglec-9 bound to

sialyl-Lewis structure 6-sulfated-sialyl-LewisX [6-sulfo-sLex; NeuAcα2-3Gal β1-4(Fucα1-3) GlcNAc (6-O-SO3)], the 6'-sulfated-sialyl-LacNAc, as well as to the gangliosides GD1a and GT1b.[21] Similar to Siglec-8, when Siglec-9 is crosslinked on neutrophils by antibodies or polyvalent glycans it reduces the presence of proinflammatory mediators.[8] The original intent of this thesis work was to study Siglec-8 and Siglec-9 ligands in human airways but later focused on purifying and identifying Siglec-8 ligands.

Section 1.3- Airway Inflammatory Diseases

Asthma is a lung inflammatory disease that affects about 235 million people estimated by World Health Organization (http://www.who.int/respiratory/asthma/en/). It is an incurable disease clinically diagnosed by three components airflow obstruction, airway hyperresponsiveness and airway inflammation.[23,24] Currently, patients either try to prevent allergen exposure or are treated with drugs that ease symptoms and but do not decrease or halt disease progression.[25-28] Different leukocytes are well established factors in the pathogenesis of asthma.[24,26,29] The disease is characterized by the recruitment of eosinophils, mast cells, and CD4+ T-cells into the lung larger airways.[29-31] Pathogenesis involves airway remodeling characterized by airway wall thickening and enhanced smooth muscle mass.[32,33] Current treatment for asthma includes the use of inhaled corticosteroids that control inflammation through a limited understood mechanism.[31,34] Anti-inflammatory corticosteroids decrease the number of eosinophils in asthmatic patients and remain a key component in asthma management, but prolonged use reduces its effectivity.[35]. Patients with severe asthma have a high number of eosinophils in blood and airway that persist despite treatment with oral and inhaled corticosteroids.[23,36-38] Interleukin-5 (IL-5) is an eosinophil specific growth factor and humanized antibodies against it have been produced,

clinically tested, and shown to reduce asthma exacerbations, further associating eosinophils in the pathophysiology of asthma.[39-41]

COPD is an inflammatory lung disease marked by the influx of neutrophils, macrophages and CD8+ T-cells[42,43]. COPD pathogenesis involves squamous epithelial metaplasia and airway wall fibrosis.[30,43] COPD predominantly affects the smaller airways and lung parenchyma.[43] A marked difference between asthma and COPD is the alveolar destruction characteristic of emphysema.[42-44] Alveolar destruction is most likely due to protease-mediated degradation of connective tissue elements, particularly elastin by neutrophil-derived elastase, and perhaps apoptosis of lung parenchymal cells.[45] Several studies associate neutrophils with the pathophysiology of COPD. COPD patients have increased neutrophils in sputum which is correlated with disease severity.[42-47] Increased levels of elastolytic enzymes, such as neutrophil elastase and several matrix metalloproteinases (MMPs), are present in the lung of COPD patients.[45,46,48] Current therapies primarily treat symptoms but do not achieve disease stabilization, remission, or cure.[25,49] Most COPD patients respond poorly to available drugs, except for prevention and management of acute exacerbations.[50,51] Novel therapeutic targets that limit ongoing inflammatory responses associated with asthma and COPD may help address the need for new therapeutics. Understanding and exploring siglec roles in regulating inflammation may provide better therapeutic leads to make better drugs.

In summary, inflammatory cells expressing Siglec-8 or Siglec-9 on their cell surface are associated with persistent inflammatory diseases and crosslinking of Siglec-8 or Siglec-9 on these cells induce apoptosis.[13,15,18,52-56] Our aim was to identify the natural ligand produced in human airways.

CHAPTER 2: METHODS

Methods described in this section apply to experiments carried out throughout the thesis work. Two types of extraction methods were used to identify Siglec-ligands in human airways and different gel compositions were used to resolve these ligands by electrophoresis. Siglec-8-ligands were further purified using gel chromatography and affinity purification. Enzymatic treatments of Siglec-8 ligands revealed the nature of the ligand glycan structure as well as the protein carrier of these ligands in human airway.

Section 2.1 Tissues and tissue extraction

Tissues

Human lungs or airway were received from organ donors within 24 hours of death. At the time of collection, tissues were flushed and stored in HTK (histidine-tryptophan-ketoglutarate) or UW (University of Wisconsin) solutions used routinely to preserve tissues prior to transplantation. Upon receipt, the trachea, bronchi, smaller airways, and parenchyma were dissected free of surrounding tissue and portions of each separated for histology, extraction, and intact cell isolation.

Tissue extraction

Airway tissue (trachea or bronchus) was placed in Detergent Extract Buffer (Detergent), 1 mL per gram of tissue (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3% CHAPS, 1% NP-40 and protease inhibitor cocktail) and the luminal surface scraped with a cell scraper (Sarstedt Cat# 83.1830) to release the mucosa and submucosa layers. Scraped tissue was homogenized by passing through a 20-gauge syringe needle, centrifuged at 20,000 x g for 5 min, supernatant was collected, stored at -70°C and the pellet discarded. Remaining cartilage after scraping was diced, frozen under liquid nitrogen and pulverized using a cold mortar and pestle. Pulverized tissue was weighed and added at a ratio of 100 mg of pulverized tissue per 1 mL of Guanidinium Extract Buffer (GuHCl) [10 mM sodium phosphate (pH-6.5), 6 M GuHCl (OminPur, EMD Millipore), 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride], incubated with mixing end over end for ≥16 h at 4 °C. After incubation, extract was centrifuged at 22,000 x g for 30 min and the supernatant stored at -20°C. Lung parenchyma was homogenized in Detergent Extract Buffer using a Potter-Elvehjem glass-Teflon homogenizer on ice or pulverized under liquid nitrogen and extracted with Guanidinium Extraction Buffer.

Section 2.2 Dialysis, gel electrophoresis, blotting and detection

Dialysis

Samples in GuHCl buffer were first dialyzed against 1M urea, 20 mM sodium phosphate pH 7.4 using a 0.1 mL Slide-A-Lyzer mini dialysis device, 10K molecular weight cutoff (MWCO), from Thermo Fisher Scientific (Waltham, MA) before loading on a denaturing gel.

Gel electrophoresis

Two types of gels were used to resolve and detect Siglec-ligands. Standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4–12% NuPAGE Bis-Tris precast gels (Thermo Fisher) run in MOPS buffer, at 200V for 1 hour. To resolve larger molecules, composite 2% agarose, 1.5% acrylamide gels were prepared in house. To a 125-ml Erlenmeyer flask was added 10.3 mL of 2 M Tris-HCl (pH 8.1), 8.9 mL of water, 27.5 mL of 8 M urea, and 1.1g agarose (Lonza Seakem Gold #50152). After swirling, the suspension was microwaved for 30-45 seconds and mixed to completely dissolve the agarose. The mixture was placed in an oven preheated to 65°C for 30 minutes then 5.5 mL of glycerol were added, the solution swirled to mix, and 2.8 mL of 30% acrylamide/0.8% bisacrylamide (Biorad cat# 161-0158) and 18.5 μL of N,N,N′,N′-tetramethylethylenediamine (TEMED) was added and the solution thoroughly mixed. The solution was quickly distributed equally into four 15-mL conical tube (~13 mL each). An aliquot (24 μL) of freshly prepared 40% ammonium persulfate (APS) was added to each tube, the solution mixed thoroughly, slowly pippetted into Life Technologies gel cassettes (#NC2015) and sealed with sample combs. After 30 minutes at ambient temperature, cassettes with polymerized gel were transferred to 4 °C for 1 h. Each cassette was wrapped with absorbent paper, moistened with 10 mL running buffer per gel cassette and stored at 4 °C for 24 hours before used. Gels can be stored for up to a month without affecting its properties. Gel electrophoresis was performed using 0.192 M Tris-borate buffer (pH 8.3), 1 mM EDTA, 0.1% SDS. Gels were run at 80 V for 2.5 hours. Samples were prepared 1:1 with NuPAGE LDS Sample Buffer (Thermo Fisher) containing 400 mM dithiothreitol (DTT) before loading on wells.

Blotting and detection

Gels were transferred to polyvinylidene difluoride (PVDF) membranes using a Thermo Fisher first generation iBlot dry transfer device at setting P3. Standard gels were transferred for 10 minutes and composite gels were transferred for 7 minutes. Membranes were blocked with 5% nonfat dry milk in Dulbecco's phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 30 min. During blocking, Siglec-Fc chimeras were precomplexed with secondary antibody in a solution of PBST containing 20 μg/mL of Siglec-8-Fc or Siglec-9-Fc and 14 μg/mL of horseradish peroxidase (HRP)-conjugated anti-human Fc (Sigma-Aldrich). After incubation on ice for ≥30 minutes, the solution was diluted 40-fold with additional PBST. Blots were quickly rinsed once with PBST, overlaid with 6 mL of precomplexed siglec-Fc solution and incubated for 16 h at 4°C. After incubation, membranes were washed 3 times with PBST, 5 minutes each wash and developed using enhanced chemiluminescence (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Pittsburgh, PA).

Molecular weight marker used with standard gels was MagicMark XP Western protein standard (up to 220 kDa, Thermo Fisher). Molecular weight markers used with composite gels included HiMark Pre-stained protein standard (up to 460 kDa, Thermo Fisher) or crosslinked human IgM prepared in house as follows: To 1 mg/mL human-IgM in PBS pH 7.6 (Thermo Fisher Cat# 31146) at ambient temperature was added bis(sulfosuccinimidyl)suberate (BS3, Thermo Fisher Cat# 21580) to a final concentration of 2.5 mM. After 20 minutes the reaction was stopped by adding 1 M TrisHCl pH 7.2 to a final concentration of 91 mM. The marker was stained using Visio real-time stain kit (Advansta Cat# K-11053-B30).

Section 2.3 Size exclusion chromatography

Sample preparation

Samples extracted with GuHCl were dialyzed against 4 M GuHCl, 10 mM sodium phosphate, pH 7.0 buffer using Float-A-Lyzer G2 100 kDa MWCO (G235059, Spectra/Por) dialysis tube to remove smaller molecular weight proteins before loading on the size exclusion column.

Separation, collection, and detection

Siglec-8 ligands in airway extracts were resolved by size exclusion chromatography on a HiPrep 26/60 Sephacryl S-500 HR column (GE-Healthcare Life Sciences) using an AKTA chromatography system (GE-Healthcare Life Sciences). The size exclusion column was equilibrated in 4 M guanidinium hydrochloride, 10 mM sodium phosphate, pH 7.0, then 4.5 mL of sample was injected and the flow rate was set to 0.8 mL/min. After injection, 48 ml (~15% of the total column volume) of eluate was discarded, then 1.8-mL fractions were collected until a full column volume (320 ml) was eluted. Aliquots from each fraction were dotted onto PVDF membranes using a Bio-Dot Microfiltration Apparatus (BioRad), the membrane was blocked and probed as described in Section 2.2. Positive fractions were dialyzed in Urea Buffer (1M urea, 20 mM sodium phosphate pH 7.4) before running samples on composite gels and blotting as described above. Fractions were pooled based on molecular weight (migration on composite gels) for further purification by lectin precipitation.

Section 2.4 Affinity capture of Siglec-8 ligand using pentameric Siglec-8-COMP

Sample preparation

Combined size exclusion fractions were dialyzed against Urea Buffer using a Float-A-Lyzer G2 100kDa MWCO (G235059, Spectra/Por) before loading on an affinity capture column.

Immobilization of Siglec-8-COMP on Nickel beads

To explore natural and synthetic Siglec-8 ligands, a pentameric Siglec-8 chimera was developed. A natural pentamerizing polypeptide domain from cartilage oligomeric matrix protein (COMP) was used to engineer pentameric Siglec-8. Recombinant pentameric Siglec-8 (Siglec-8-COMP) contains the entire extracellular domain of Siglec-8, a BAP biotinylation site, a Factor Xa or TEV cleavage site, Ig-like domains 3 & 4 of CD4 (as a spacer), the COMP pentamerization domain, and a 6-His C-terminal tag (Figure 2.1). The 6-His C-terminal tag was used to capture and purify Siglec-8-COMP using nickel Sepharose beads.

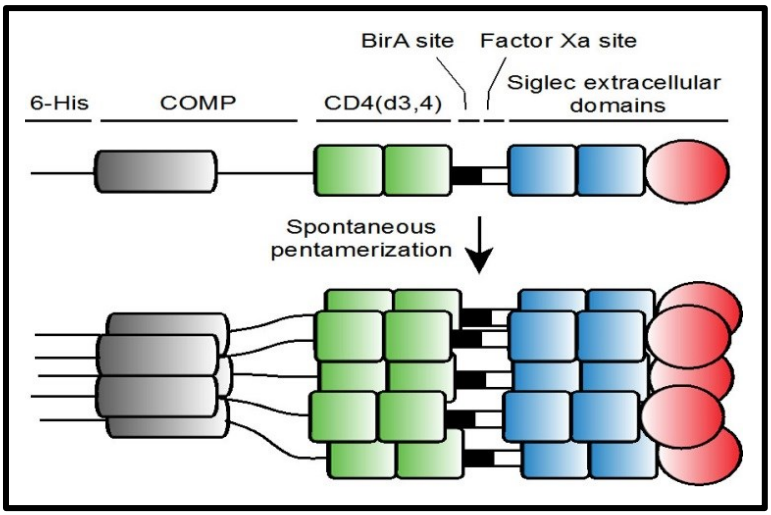


Figure: 2.1 Schematics of recombinant Siglec-8-COMP construct. The tail represents the C-terminal 6-His tag, the gray box represents the pentamerizing domain (COMP), in green the CD4 spacer, black and white boxes the BirA site and Factor Xa site, in blue the two constant Ig-like domains of Siglec-8 and in red the sialic-acid binding domain.

Siglec-8-COMP was transiently expressed in an optimized HEK293 suspension culture system, because the construct had a secretory signal peptide, protein was secreted into media. To immobilize Siglec-8-COMP on nickel beads, a chromatography column (Biorad, Cat# 731-1550) was packed with 1 mL of nickel Sepharose resin (GE Healthcare, Cat# 17-5268-01), the beads washed with 30 mL of binding buffer (500 mM NaCl, 20 mM sodium phosphate, 20 mM imidazole, pH-7.4) and 200 mL (~400 μg) of Siglec-8-COMP media cycled through nickel beads for 24 h using a peristaltic pump at 4 °C. Next day, the column was washed with 30 mL of High Salt Elution Buffer (1M urea, 1M NaCl, 20 mM sodium phosphate, 20 mM imidazole, pH-7.4) and then pre-equilibrated with 30 mL of Urea Buffer (Siglec-8 column). Combined and dialyzed size exclusion fractions (starting material, SM) was pre-cleared by cycling 3 times through 1 mL of underivatized nickel Sepharose resin that had been pre-equilibrated with 30 mL of Urea Buffer. The cleared sample (CL) was then loaded on the Siglec-8-COMP column and cycled through 3 times. The flow-through (FT) was collected and the column was washed with 10 mL of Salt Wash (1M urea, 150 mM NaCl, 20 mM sodium phosphate, 20 mM imidazole, pH-7.4) collecting 2 mL fractions. Siglec-8 ligands were eluted with 4.5 mL of High Salt Elution buffer, collecting one 0.5 mL fraction followed by four 1 mL fractions. These conditions released the Siglec-8 ligands without eluting Siglec-8-COMP from the nickel beads, making it possible to recycle the beads for sequential runs. Equal volumes of each fraction were loaded on composite gels and analyzed as described in Section 2.2.

Section 2.5 Enzymatic treatment of purified Siglec-8 ligands

Ligands were dialyzed against Dulbecco's PBS before enzymatic digestion.

Sialidase

Sialic acid binding specific ligands were detected by treating samples with or without sialidase Siglec-8-Fc binding bands that disappeared after treatment with sialidase were pursued. Sialidase treatment was done as follows: tissue extracts or purified samples were treated with 50-100 mU/mL of *Vibrio cholerae* sialidase and incubated at 37 °C for 1.5 h. Samples were analyzed as described in Section 2.2.

Chondroitinase

Chondroitinase ABC degrades chondroitin sulfate and dermatan sulfate chains into disaccharides containing 4-deoxy-beta-D-gluc-4-enuronosyl groups. To determine if chondroitin sulfate chains were Siglec-8-ligands, purified samples were digested with chondroitinase ABC. A 10x Tris-acetate working buffer (500 mM Tris HCl, 500 mM sodium acetate, pH 8.0) was diluted to 1x with ligand, and Chondroitinase ABC (Seikagaku, Code # 100332) was added to a final concentration of 25-500 mU/mL and incubated at 37 °C for at 16-24 h. Samples were analyzed as described in Section 2.2.

Aggrecanase (ADAMTS4)

Aggrecanase-1 or ADAMTS4 is a protease that selectively cleaves proteoglycans from the lectican family including aggrecan, versican, brevican and neurocan. To determine if aggrecan was a Siglec-8 ligand protein carrier, purified samples were treated with ADAMTS4 as follow: added ADAMTS4 (R&D Cat# 4307-AD-020) was added to purified samples to a final concentration of 0.2-0.5 mU/ml ADAMTS4 and incubated at 37 °C for 16-24 h. Samples were analyzed as described in Section 2.2.

Keratanase I

Keratanase I cleaves keratan sulfate chains at the beta-galactoside linkage where galactose is not sulfated. To determine if keratan sulfate chains were Siglec-8-ligands, purified samples were digested with keratanase I Pseudomonas sp. (Amsbio Cat# 100810-1). A 10x sodium acetate working buffer (100 mM sodium acetate, pH 6.0) was diluted to 1x with ligand, and keratanase I was added to a final concentration of 1-40 mU/mL and incubated at 37 °C for 16-24 h. Samples were analyzed as described in Section 2.2.

Keratanase II

Keratanase II cleaves keratan sulfate chains at the 1,3-β-N-acetylglucosamine linkage where galactose sulfation does not inhibit cleavage. To determine if highly sulfated keratan sulfate chains were Siglec-8-ligands, purified samples were digested with keratanase II from Bacillus sp. (Amsbio Cat# 100812-1). A 10x sodium acetate working buffer (100 mM sodium acetate, pH 6.0) was diluted to 1x with ligand, and keratanase II added to a final concentration of 1-40 mU/mL and incubated at 37 °C for 16-24 h. Samples were analyzed as described in Section 2.2.

CHAPTER 3: DISCOVERY OF SIGLEC-8 AND SIGLEC-9 LIGANDS IN HUMAN LUNG AIRWAYS

Section 3.1 Introduction

The goal of this research project was to identify the endogenous sialylated-glycan-ligands for Siglec-8 and Siglec-9 present in human airways. Immunohistochemistry (IHC) of sections taken from different compartments of human lung revealed that expression of Siglec-ligands was cell specific. Culture of primary human lung epithelial and submucosal gland cells supported the evidence gathered from IHC as well as from tissue extracts. Sialic acid specific ligands were extracted from tissue, treated with sialidase and binding detected with a recombinant Siglec-Fc construct that contained the extracellular domains of Siglec-8 or Siglec-9.

As the project developed, the method of extraction was changed as well as the method to resolve the ligands. The first experiments used a combination of detergents to extract all proteins without denaturing them to avoid affecting further purification methods. However, this method was not successful to extract all siglec ligands, and a harsher denaturant, guanidinium hydrochloride, was used. Analysis by electrophoresis revealed siglec ligands were large molecular weight proteins insufficiently resolved by gel electrophoresis using 4-12% Bis-Tris polyacrylamide gels, therefore, a composite agarose-acrylamide gel was adopted to provide better resolution of ligands extracted. The improved method of extraction and gel electrophoresis was effective in extracting large molecular weight siglec ligands that were sensitive to sialidase treatment. These findings revealed not only that siglec ligands are present in human airways, but also that their expression is restricted to different cell types and compartments within human lung.

Section 3.2 Additional experimental procedures

Siglec overlay histochemistry[21]

Tissues were fixed in neutral 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) at 4°C for 16 h, embedded in paraffin, sectioned to 5 μm and captured on glass slides. Following deparaffinization, the slides were heated briefly in 10 mM sodium citrate (pH 6.0) for antigen retrieval. Subsequent steps were performed at ambient temperature. Slides were incubated in endogenous enzyme Blocking Reagent (Dako North America, Carpinteria, CA) for 10 min, and then in Fc Receptor Blocker (Innovex Biosciences, Richmond, CA) for 30 min. Siglec-8-Fc (160 μg/ml) or Siglec-9-Fc (120 μg/ml) was pre-incubated in PBS with AP-conjugated goat anti-human antibody (16 μg/ml, product 109-055-008, Jackson Immunoresearch, West Grove, PA) for 30 min at ambient temperature. The solution was diluted 8-fold in PBS, then overlaid on blocked slides and incubated 60 min. Slides were washed with PBS, bound lectin conjugate detected with Vector Red AP substrate (Vector Laboratories, Burlingame, CA), slides counterstained with Hematoxylin QS (Vector Laboratories), dehydrated, mounted in Krystalon (EMD Millipore) and imaged using a Nikon Eclipse 90i microscope.

For comparative siglec overlay of mouse and human airway sections, the above procedure was modified as follows. Prior to treatment with blocking reagents, slides were incubated in PBS supplemented with 0.1% Tween-20 and 10 mg/ml BSA (Sigma-Aldrich) for 30 min. Siglec-8-Fc (15 μg/ml), Siglec-9-Fc (15 μg/ml), or Siglec-F-Fc (5 μg/mL) in the same buffer were pre-incubated with AP-conjugated goat anti-human IgG (2 μg/ml, product 109-055-044, Jackson Immunoresearch, West Grove, PA) for 30 min at 4°C. Alternatively, Siglec-E-(mouse Fc) (5 μg/mL) was similarly pre-incubated with AP-

conjugated goat anti-mouse IgG (2 μg/mL, product 115-055-003, Jackson Immunoresearch). Pre-conjugated siglec-Fc chimeras were pipetted onto the washed slides and incubated for 16 h at 4°C. Slides were washed with PBS/0.1% Tween-20, then with 100 mM Tris-HCl (pH 8.3)/0.1% Tween 20 for 10 min prior to conjugate detection, counterstaining and imaging as above.

Primary airway epithelial and submucosal gland cells[21]

Isolation of airway epithelial and submucosal gland cells was performed as described. [57,58] The trachea was rinsed in sterile Ham's F-12 medium, opened longitudinally, and then treated with 0.1% pronase in Ham's F12 medium containing antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone) at 4 °C overnight to release epithelial cells, which were collected as a suspension (see below). Gland-rich submucosal tissue was dissected from the remaining airway and incubated for 24 h in 0.01% dispase/collagenase in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin and 50 μg/ml gentamicin (DMEM-AB). Each strip was scraped after digestion to release the remaining gland cells. Cells were collected by centrifugation, washed in DMEM-AB, and then resuspended in trypsin-EDTA (0.25%, Thermo Fisher) and triturated to dissociate remaining clumps of cells. Digestion was stopped by addition of fetal bovine serum, cells collected by centrifugation and resuspended in DMEM-AB. Gland cells were plated on collagen-coated 12-well plates (BD Biosciences, Billerica, MA) in 1:1 DMEM-AB-Ham's F-12 supplemented with hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), and human EGF (25 ng/ml). Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air.

Airway epithelial cells were plated on collagen-coated T25 flasks and cultured in DMEM/F12 (Thermo Fisher) containing 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone until confluent. Cells were then dissociated with trypsin-EDTA and plated on 6-well Falcon 0.4 μm porous culture inserts (Corning Inc., Corning, NY). The cultures were maintained at an air-liquid interface with medium below the culture insert (DMEM-BEGM (1:1) plus SingleQuots (Lonza Inc, Mapleton, IL)). Culture medium was replaced every third day. Cultures were maintained for 10 days prior to mechanical release, collection by centrifugation and detergent extraction. Protein concentrations in extracts and exudates was determined by Pierce BCA protein assay (Thermo Fisher).

To collect secretion material, intact bronchus and bronchioles were dissected clean of surrounding parenchyma and incubated in RPMI-1640 containing 100 U/mL penicillin and 100 μg/mL streptomycin for 48 h at 4°C. Tissue was removed and soluble exudate analyzed.

Section 3.3 Results and discussion

Siglec-8 and Siglec-9's role in immune regulation of cells associated with airway inflammatory diseases led us to investigate the nature of the endogenous sialoglycan ligands that induce suppression of immune cells. The hypothesis is that upon inflammation, specific cells in surrounding tissue produce these sialoglycan ligands to control inflammation. Two techniques were used, immunohistochemistry staining and western blotting to detect Siglec-8 and Siglec-9 ligands using a recombinant Siglec-Fc construct. Sialidase treatment revealed sialic acid specific interactions. In western blots, bands that did not disappear upon sialidase treatment were not pursued. The datum support the idea

that Siglec-8 and Siglec-9 ligands are being produced by surrounding tissue, however, the nature of these ligands was still to be determined.

Siglec-specific downregulation of inflammation requires Siglecs to interact with their glycan specific ligand. Our hypothesis was that Siglec-8 and Siglec-9 ligands are produced in tissues that they may come in contact with inflammatory cells. A common inflammatory cell involved in asthma and allergy are eosinophils[15,18,23,30,31,37,39,52,53]. Eosinophils are often seen infiltrated in patient's airways but rarely seen in parenchyma tissue[30]. Eosinophils express Siglec-8 on their cell surface and not surprisingly we observed Siglec-8 ligands in trachea but not parenchyma in lung sections overlaid Siglec-8. Siglec-8 intensely stained serous cells in submucosal glands and cartilage but not epithelial cells or connective tissue (**Fig. 3.1**). Tissue extracts showed similar Siglec-8 ligand pattern of expression. Large molecular weight, sialidase sensitive Siglec-8 ligands were detected in trachea and small airway tissue extracts but not lung parenchyma extracts. Most Siglec-8 ligands were extracted with guanidinium extract buffer and small portion was extracted with detergent extract buffer (**Fig. 3.2**).

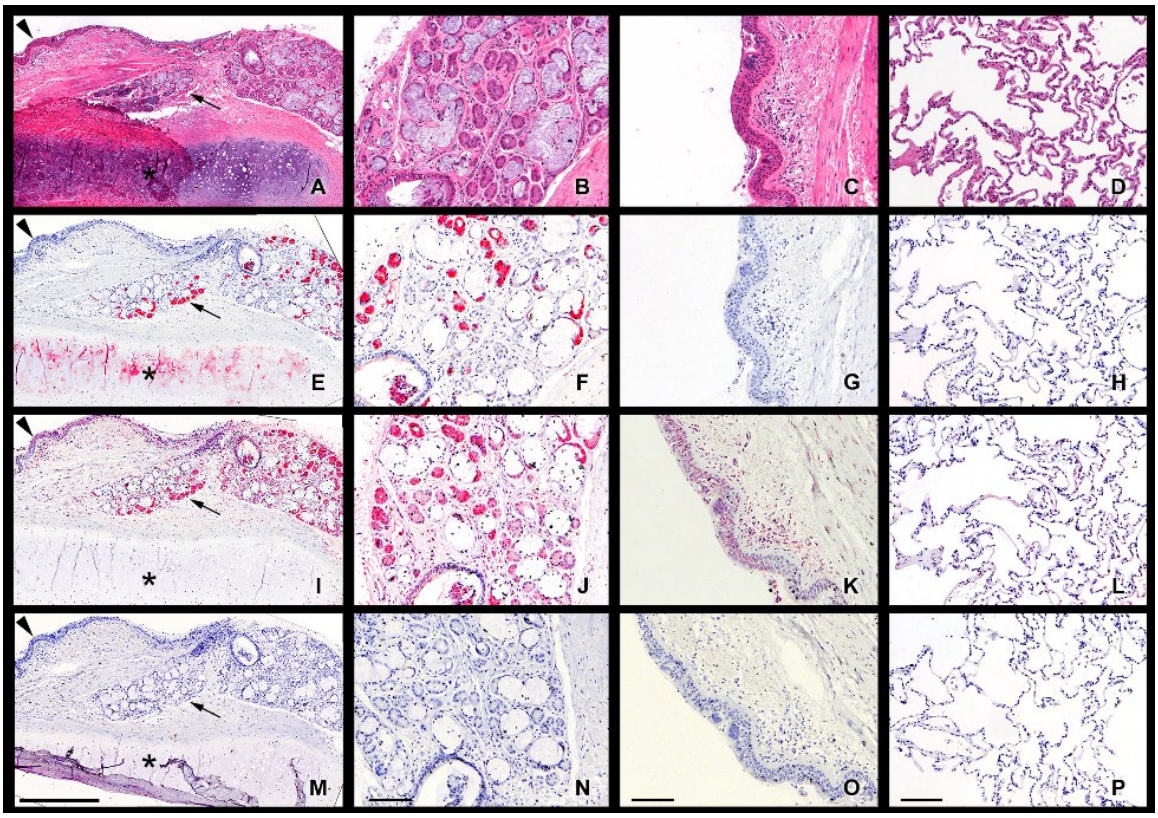


Figure 3.1: Siglec-8 and Siglec-9 ligand distribution in human airways.[21] Serial sections of trachea or lung parenchyma stained with H&E or overlay with siglec-Fcs. Low power microscopic images of trachea (**A, E, I, M**), higher power microscopic images of tracheal submucosal glands (**B, F, J, N**), tracheal airway epithelium (**C, G, K, O**) and lung parenchyma (**D, H, L, P**). Sections stained with H&E (**A-D**), overlaid with precomplexed Siglec-8-Fc (**E-H**), Siglec-9-Fc (**I-L**) or secondary (control AP-conjugated anti-human Fc, **M-P**). Sections were counterstained with Hematoxylin QS and Lectin staining detected with Vector Red AP Stain. Scale bars are 0.5 mm (**A, E, I, M**) and 100 μm for all other panels. Asterisk: Cartilage, arrowhead: epithelium, arrows: submucosal glands.

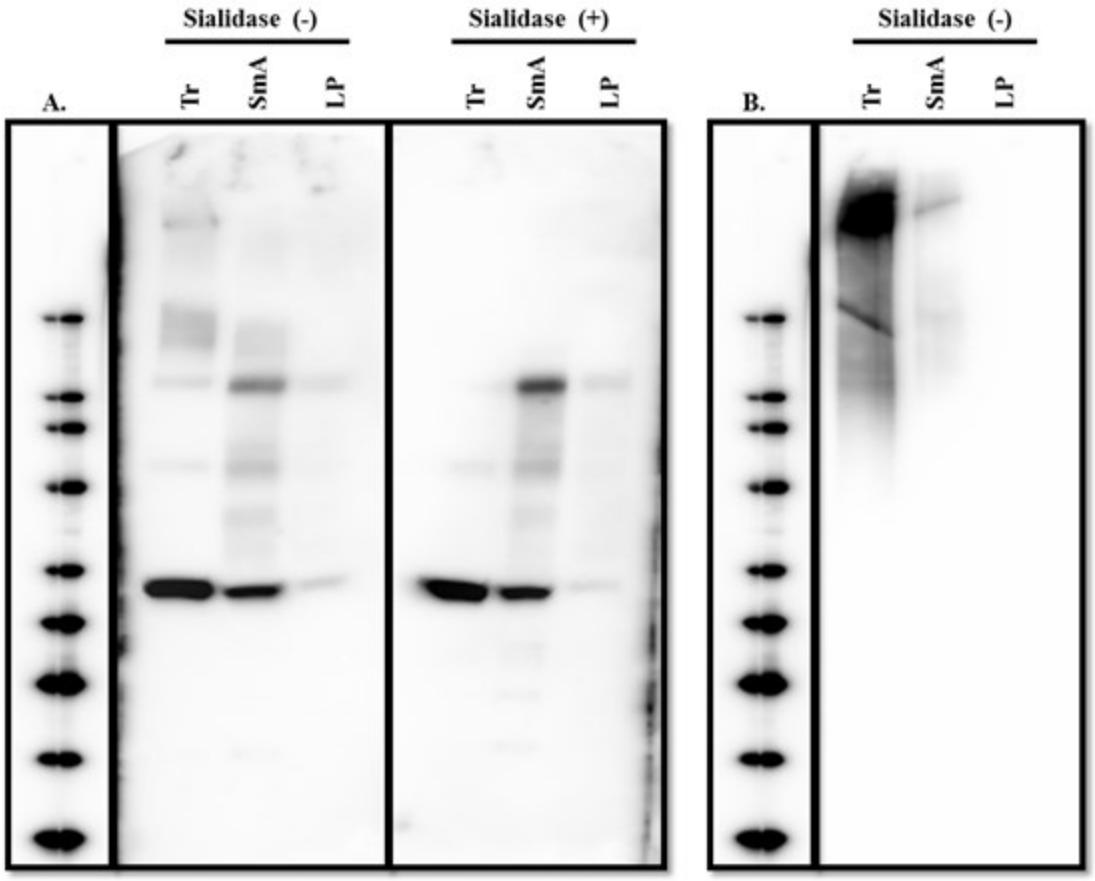


Figure 3.2: Siglec-8 binds predominantly to tracheal and smaller airway extracted with guanidinium but not much to detergent extracts or parenchyma. Trachea (**Tr**), Small Airway (**SmA**) or Lung Parenchyma (**LP**) tissues were extracted with detergent (**A**) or guanidinium (B) extract buffer, treated with or without vibrio cholera sialidase (49 mU/mL). Equal volumes of extracts were loaded on standard gel electrophoresis and blotted with Siglec-8-Fc to detect ligands. Magic Marker's highest molecular weight band is 250 kDa.

Unlike Siglec-8, Siglec-9 has a broader binding specificity and ligand distribution in airways. Siglec-9 ligands, were found to be broadly expressed in epithelial cells, connective tissue, submucosal gland cells, cartilage, and alveolar cells. Siglec-9 strongly bound to epithelial cells and submucosal glands but faintly to cartilage in tracheal sections.

It also bound strongly to parenchyma tissue which Siglec-8 did not (**Fig 3.1**). Tissue extracts showed similar Siglec-9 ligand pattern of expression. Large molecular weight, sialidase sensitive Siglec-9 ligands were detected in trachea, small airway, and parenchyma extracts (**Fig. 3.3**).

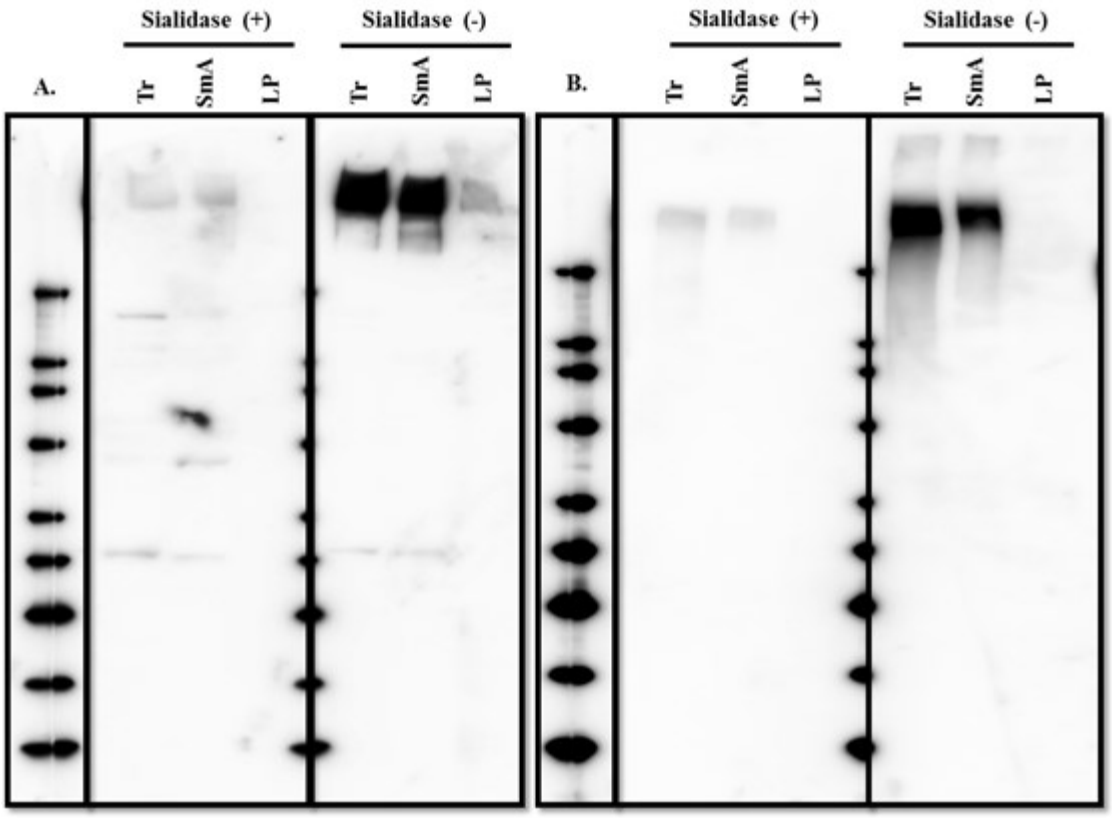


Figure 3.3: Siglec-9 binds equivalently to all tissue extracts. Trachea (Tr), Small Airway (SmA) or Lung Parenchyma (LP) tissues were extracted with detergent (A) or guanidinium (B) extract buffer, treated with or without vibrio cholera sialidase (49 mU/mL). Equal volumes of extracts were loaded on standard gel electrophoresis and blotted with Siglec-9-Fc to detect ligands. Magic Marker's highest molecular weight band is 250 kDa.

Due to the nature of Siglec-8 ligands standard gels insufficiently resolved the different binding species and thus, it appeared as if Siglec ligands were one species. However,

composite gels resolved Siglec-8 binding species and revealed multiple binding large molecular weight ligands with migration comparable to that of mucins. Multiple binding large molecular weight sialoglycoprotein Siglec-8 ligands were present in trachea. It seems like Siglec-8 binding species in trachea extracted with detergent extract buffer were different from those extracted with guanidinium extract buffer. Tracheal detergent extracts Siglec-8 bound to two large molecular weight sialoglycoproteins running at around 900 kDa and 4,000 kDa. On the other hand, guanidinium tracheal extracts showed strong Siglec-8 binding to three large molecular weight sialoglycoproteins running at around 1,000 kDa, 600 kDa and 250 kDa (**Fig. 3.4**).

Opposite to Siglec-8, Siglec-9 bound stronger to trachea extracted with detergent than with guanidinium extract buffer. The amount of Siglec-9 ligand in lung parenchyma extract was small compared to ligands in trachea (**Fig. 3.4**).

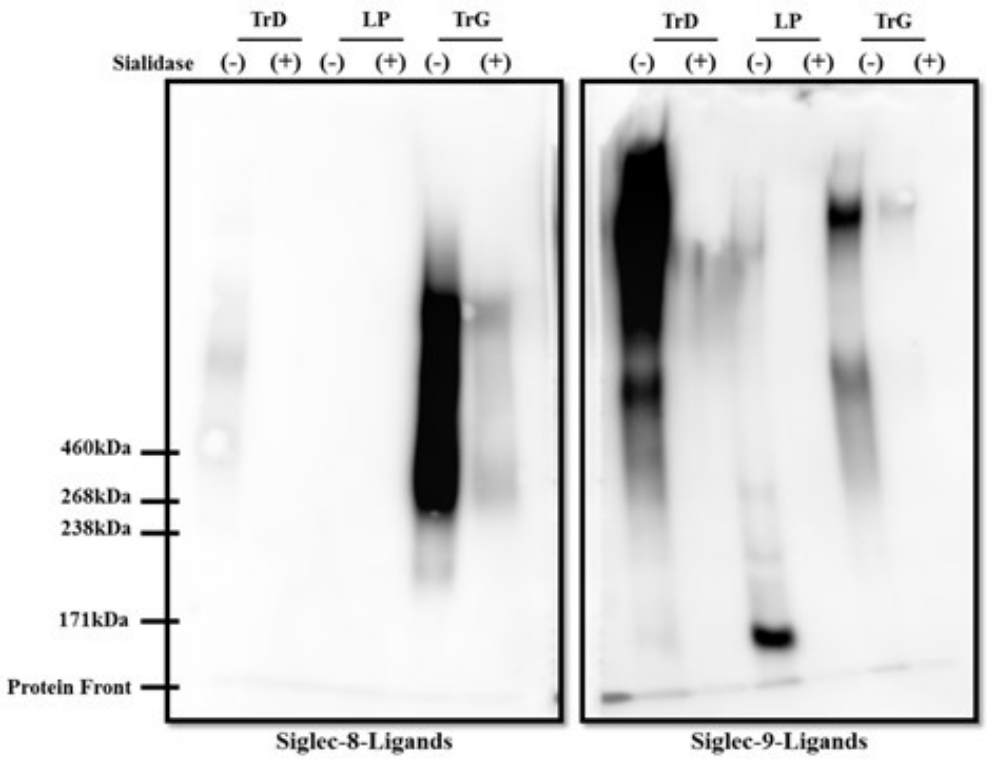


Figure 3.4: Composite gel electrophoresis resolves Siglec-8 and Siglec-9 large molecular weight ligands. Trachea tissue extracted with detergent (**TrD**) or guanidinium extract buffer (**TrG**) and Lung Parenchyma (**LP**) extracted with detergent were treated with (+) or without (-) vibrio cholera sialidase (49 mU/mL). Equal volumes of extracts were loaded on composite gel electrophoresis and blotted with Siglec-8-Fc (Siglec-8 ligands) or Siglec-9-Fc (Siglec-9 ligands) to detect their respective ligands. HiMark molecular weight marker running position shown to the left.

Expression of Siglec-8 ligands in parenchyma tissue was rare. When parenchyma tissue extracts from 7 donors were analyzed only 2 out of 7 donors showed some expression of Siglec-8 ligands very similar to those observed in detergent tracheal extracts (**Fig. 3.5**). Ligands detected in detergent tracheal extracts varied a lot donor to donor, but two binding species were predominant in most extracts (900 kDa and 4M) with donor 1 showing the strongest binding and donors 3 and 7 no binding at all (**Fig. 3.6**). Guanidinium tracheal

extracts on the other hand, showed robust Siglec-8 staining in all donors and consistent binding showing three major binding species (**Fig. 3.7**). All donors were smokers, some even had asthma and although it would be interesting to determine ligand expressions' correlation with disease that was not the focus of this project. Donor demographics are detailed in Table 3.1.

Expression of Siglec-9 ligands in parenchyma tissue was consistent. When parenchyma tissue extracts from 7 donors were analyzed all donors showed expression of a large molecular weight Siglec-9 ligand of about 4,000 kDa (**Fig. 3.5**). The 4,000 kDa binding specie Siglec-9 ligand in detergent tracheal extracts was consistent in all donors, however, there was a 900 kDa binding specie that was not observed in parenchyma and appeared in some donors (**Fig. 3.6**). Guanidinium tracheal extracts also showed robust Siglec-9 staining to all donors and consistent binding showing two major binding species running at around 4,000 kDa and 900 kDa that were observed in other tissue extracts (**Fig. 3.7**).

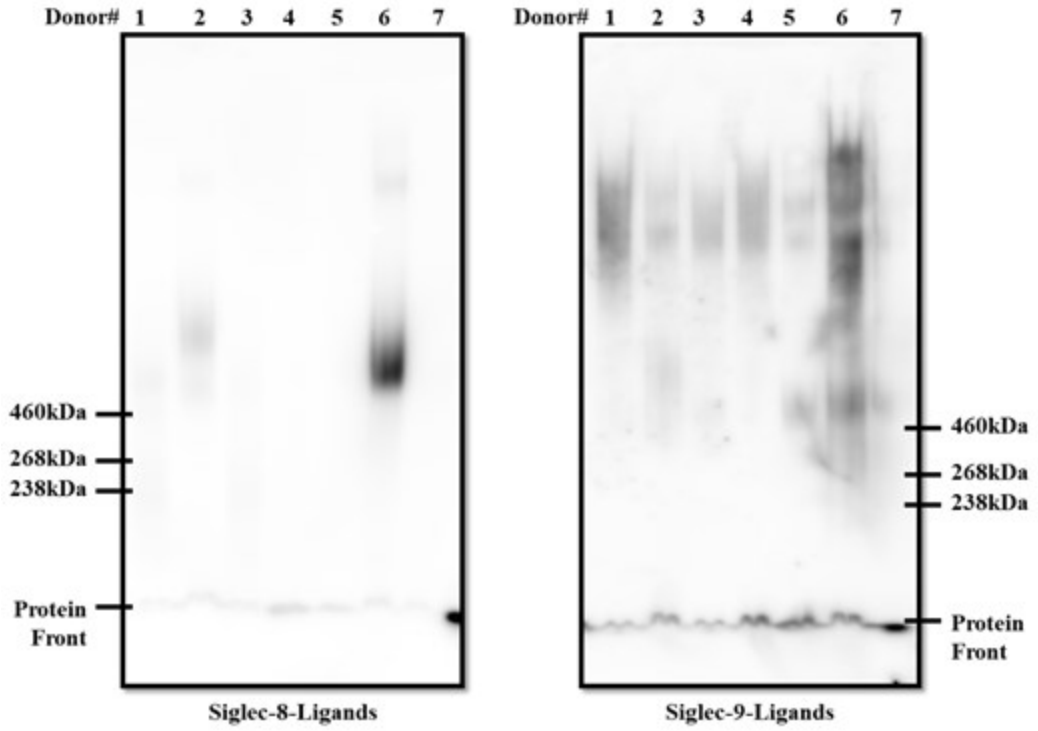


Figure 3.5: Donor variability in Siglec-8 and Siglec-9 ligand expression in lung parenchyma. Lung parenchyma from 7 donors were each extracted with detergent extract buffer, equal protein concentration loaded on composite gel electrophoresis and blotted with Siglec-8-Fc (Siglec-8 ligands) or Siglec-9-Fc (Siglec-9 ligands) to detect their respective ligands. HiMark molecular weight marker running position shown to the left.

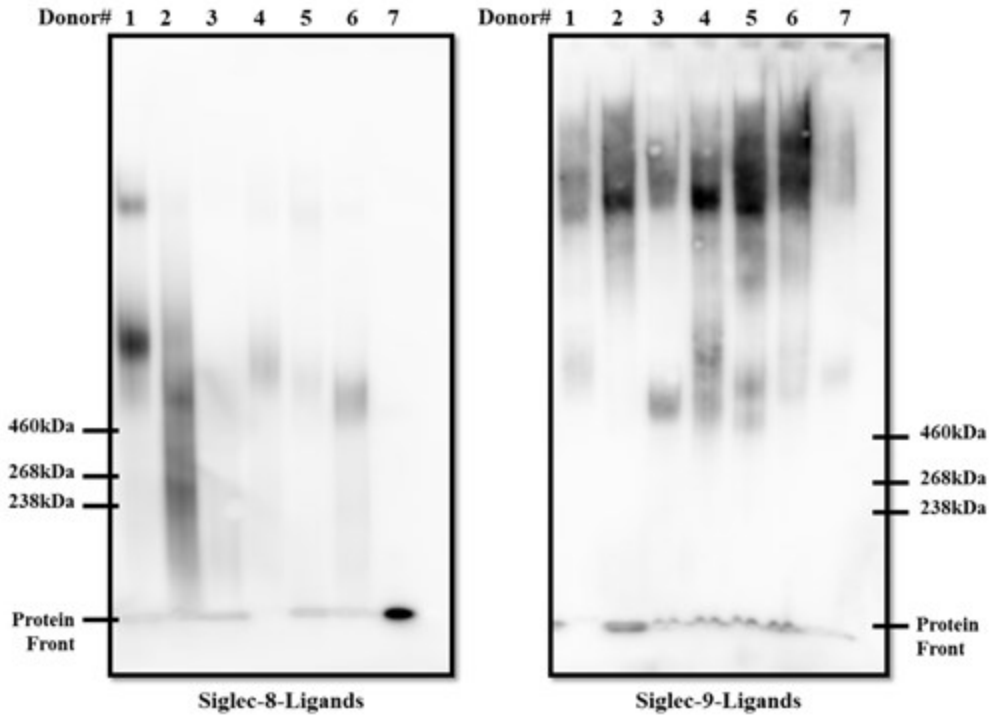


Figure 3.6: Donor variability in Siglec-8 and Siglec-9 ligand in tracheal tissue extracted with detergent extract buffer. Tracheal tissue from 7 donors were extracted with detergent extract buffer, equal protein concentration loaded on composite gel electrophoresis and blotted with Siglec-8-Fc (Siglec-8 ligands) or Siglec-9-Fc (Siglec-9 ligands) to detect their respective ligands. HiMark molecular weight marker running position shown to the left.

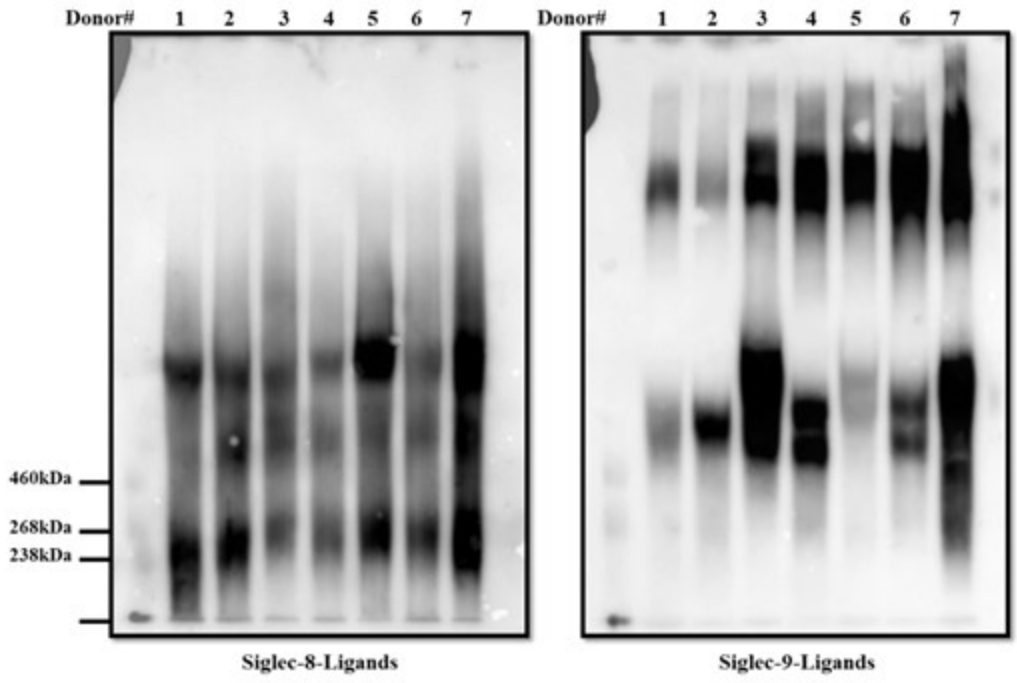


Figure 3.7: Donor variability in Siglec-8 and Siglec-9 ligand in tracheal tissue extracted with guanidinium extract buffer. Tracheal tissue from 7 donors were extracted with guanidinium extract buffer, equal protein concentration loaded on composite gel electrophoresis and blotted with Siglec-8-Fc (Siglec-8 ligands) or Siglec-9-Fc (Siglec-9 ligands) to detect their respective ligands. HiMark molecular weight marker running position shown to the left.

Donor	Gender/ Age	Tobacco	R/L lung	Respiratory disease	Cause of death
1	F/59yrs	1-2 PPD X unknown years	R	No	ICH-Stroke
2	M/32yrs	1 PPD X 15 years	R	Asthma	ICH-Stroke
3	M/27yrs	½ can per day X 3 years	R	No	Head Trauma-2nd to blunt injury
4	F/55yrs	No	L	Asthma	CNS Tumor
5	F/35yrs	1 PPD X 2 yrs	L	No	Anoxia/Aphyxation
6	M/37yrs	1 pack per month X 2 yrs	R	No	Cerebrovasc/Stroke
7	F/38yrs	1 PPD X 26 yrs	R	Asthma	Anoxia 2nd Cardiovascular

Note: ICH: Intracerebral hemorrhage, ppd: packs per day, CVA: cerebrovascular accident, GSW: ballistic trauma or gunshot wound, Heavy smoker: 15 to 20 cigarettes per day.

Table 3.1: Donors demographics[21] Gender, age, tobacco usage, (R) right lung, (L) left lung, respiratory disease and cause of death of each donor is noted in the table above as well as the assigned donor number.

In an effort to produce Siglec-8 ligands in vitro and manipulate cells to determine the nature of Siglec-8 ligands primary tracheal epithelial cells (TEC) and submucosal gland cells (TGC) were culture. Siglec-8 ligands were not detected in TECs extracts but were detected in TGCs (**Fig. 3.8**), however, TGCs that were passaged for a third time did not express ligand (Data not shown) suggesting synthesis of Siglec-8 ligands in airways is complex and cannot be supported in vitro. To obtain Siglec-8 ligands it was necessary to use human tissue to extract ligand, purify and analyze it. Analysis of bronchus exudate suggests Siglec-8 ligand is being secreted into airways, however, amount of ligand present is very small compared to what is extracted from trachea with guanidinium extract buffer. Therefore, ligands extracted from trachea were pursued for identification of glycan ligand and the protein carrier.

Primary epithelial cell culture, reiterated the broad expression of Siglec-9 ligands. Siglec-9 ligands were detected in TECs and TGCs extracts no matter the passaged cells

were able to synthesize Siglec-9 ligands (**Fig. 3.8**). Siglec-9 also bound strongly to bronchus exudate suggesting ligand is secreted into the airway and what function it may exert still needs to be investigated.

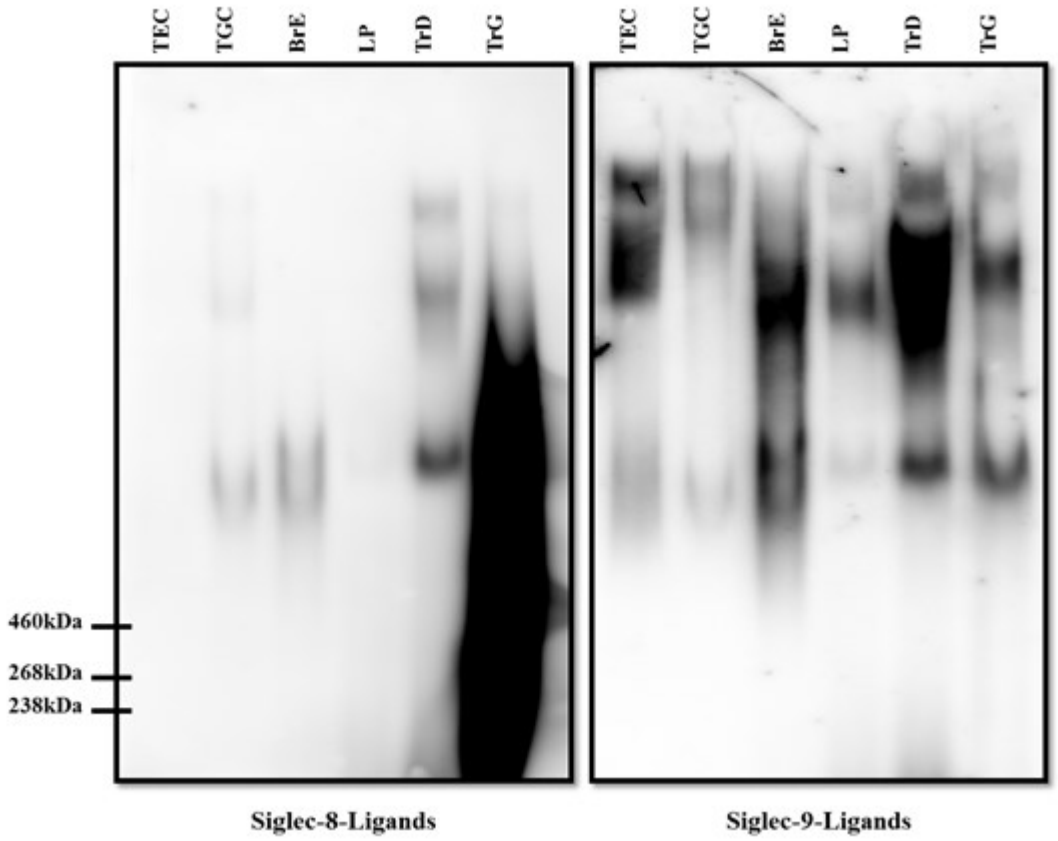


Figure 3.8: Differential Siglec-8 and Siglec-9 ligand expression in primary cell cultures. Loaded equal protein concentration of detergent cell lysates, tracheal epithelial cells (**TEC**), tracheal submucosal gland cells (**TGC**), bronchus exudate (**BrE**), lung parenchyma (**LP**) detergent extract, tracheal tissue detergent extract (**TrD**) and guanidinium extract (**TrG**). HiMark molecular weight marker running position shown to the left.

CHAPTER 4: SIGLEC-8 LIGANDS IN HUMAN TRACHEA AND BRONCHUS

Section 4.1 Introduction

Siglec-8 ligand was shown to be abundantly present in guanidinium tracheal extracts (Chapter 3). Therefore, in this part of the project we pursued to extract, purify, and identify the endogenous glycan structure present in lung as well as the protein carrier of such ligand. Guanidinium extracted trachea was subjected to size exclusion chromatography, fractions collected were dotted and active fractions were analyzed by gel electrophoresis. Siglec-8 binding species running around the same molecular weight were combined and subjected to immunoprecipitation using pentameric Siglec-8-COMP immobilized on nickel sepharose beads. Siglec-8 ligands were eluted with synthetic ligand (as proof of principle) or with buffer containing high concentration of salt. Immunoprecipitated material was sent to our collaborators at CCRC for mass spectrometric analysis. Major species present in the immunoprecipitated material was validated by antibody detection, comigration with Siglec-8 ligand and shift in migration upon treatment enzymatic digestion. A portion of the purified ligands were also sent for eosinophil activity assay to our collaborators at Northwestern University. The data suggest that Siglec-8 ligands are sialylated keratan sulfate chains carried on aggrecan and that it is biologically active in causing human eosinophils apoptosis.

Section 4.2 Additional Experimental Procedures

Siglec-8 endogenous tracheal ligand S8-1M capture and elution with synthetic ligands

Purified Siglec-8-COMP (44 μg) was immobilized on 140 μL magnetic nickel Sepharose beads (GE Healthcare Life Sciences Cat#28967388). Size separated S8-1M, Siglec-8 ligand (200 μL) was incubated with Siglec-8-COMP bound nickel beads overnight mixing end-over-end at 4 °C. Unbound material was collected, the beads were washed 3 times with 500 μL of 100 mM salt wash buffer (100 mM NaCl, 10 mM sodium phosphate, 20 mM imidazole, pH 7.4), then resuspended in 1 mL of Urea Buffer and split equally into 5 microcentrifuge tubes. Urea Buffer was removed and 30 μL of glycan elution solution was added into each tube, comprised of either Urea Buffer (control) or 15 mM of 6'-Sulfo-3'-SLN, 6-Sulfo-3'-SLN, 3'-SLN, or LacNAc in Urea Buffer. After 24 h shaking at 4 °C, the solution was collected and 30 μL Imidazole Elution Buffer (1M urea, 150 mM NaCl, 20 mM sodium phosphate, 500 mM imidazole, pH-7.4) were added to the beads to elute Siglec-8-COMP along with any ligand still bound to it. The remaining beads were boiled in 30 μL of Solubilizing Buffer (NuPAGE LDS Sample Buffer; Thermo Fisher; containing 400 mM dithiothreitol; DTT) to detect any material that was not eluted with imidazole.

Periodate treatment of S8-250k

Purified S8-250k ligand was treated sequentially with periodate and reducing agent (treated) or with reducing agent alone (control, untreated). Freshly prepared periodate solution (100 μL of 120 mM $NaIO_4$) or water (100 μl, control) was added to 2 mL of purified S8-250k ligand and the samples incubated on ice in the dark for 30 minutes. After incubation, 50 μL of glycerol were added to each sample, the samples were mixed, then 100 μL of 200 mM $NaBH_4$ were added and the samples incubated on ice in the dark for

another 30 minutes. After incubation, 2 mL of each sample were dialyzed against RPMI medium supplemented with 2mM GlutaMAX, 25 mM HEPES (Cat # 72400120) and 100 U/mL Penn/Strep using a 10 kDa MWCO dialysis cassette. Samples were dialyzed for 21 hours and media was changed to fresh media and dialyzed an additional 6 hours. Samples were sterilized by passing through a 0.22 μm filter, aliquoted and frozen before sending to our collaborators at Northwestern University for eosinophil apoptosis assay.

Eosinophils apoptosis assay

Freshly isolated eosinophils were primed in medium containing 30 ng/mL IL-5 overnight. Following priming, eosinophils were plated at 200,000 per well, in a 96-well plate. Ligand was thawed shortly before experiment and allowed to come to proper temperature. Cells were incubated for 24 hrs at 37°C with or without 14 ng/μL of treated or untreated S8-250k. Apoptosis was assessed using Annexin V flow cytometry[59].

Mass Spec Analysis

Mass spectrometry was performed by our collaborators at the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens, Georgia. To analyze samples resolved by composite agarose-acrylamide gel electrophoresis, bands of gel at the migration level of ligands were excised, cut into small pieces with a scalpel and placed into a glass tube (13 x 100 mm) with a Teflon-lined screw top. Gel pieces were washed sequentially with 40 mM ammonium bicarbonate and acetonitrile, followed by reduction with 10 mM dithiothreitol (DTT) for 1 h at 55°C and carboxyamidomethylation with 55 mM iodoacetamide (IAD) in the dark for 45 min. After reduction and alkylation, the gel pieces were sequentially washed with 40 mM ammonium bicarbonate and acetonitrile. The washed gel pieces were rehydrated in 50mM ammonium bicarbonate containing Sequence

Grade of a mixture of rLys-C and trypsin (Promega) and incubated at 37°C for 16 h. The resulting peptides were extracted by sequential incubations with 20%, 50% and 80% acetonitrile in 5% formic acid. The washes were combined, dried, and then further purified by C18 zip-tip (Pierce). Peptides were eluted with 60% acetonitrile in 0.1% TFA.

For salt eluted affinity chromatography samples (not electrophoretically resolved), the eluate was desalted on 100K MWCO membrane filter (Amicon, Ultra 0.5ml 100K, Merk-Millipore) reduced with DTT (10mM), carboxyamidomethylated with iodoacetamide (20mM) and digested with rLys-C and trypsin. The peptides were purified by C18 ziptip.

The purified rLys-C/tryptic peptides were reconstituted in 39 µl of mobile phase A (0.1% formic acid in water) and 1 µl of mobile phase B (80% acetonitrile and 0.1% formic acid in water). The peptide solutions were passed through Nanosep MF Centrifugal Devices (0.2 µm) and transferred to autosampler vials capped with septa caps. The sample vial was loaded into the autosampler compartment maintained at 4 °C prior to MS analysis.

The peptide samples were analyzed using an Orbitrap Fusion Lumos tribrid mass spectrometer (Thermo Fisher) equipped with UltiMate3000 RSLCnano liquid chromatograph. The LC system was equipped with a C18 analytical column (Acclaim PepMap 300, 150 mm length × 0.075 mm inner diameter, 5 µm particles, 300 Å pores, Thermo Fisher) and a 20 µL sample loop. Six microliter of the sample solution was used for the analysis.

Peptides were eluted using a multistep gradient at a flow rate of 300 µL/min from 0.1% formic acid in water to 0.1% formic acid in acetonitrile over 90 min. Peptides were fragmented using higher energy collisional dissociation (HCD), electron transfer

dissociation (ETD), and collision-induced dissociation (CID). The electrospray ionization voltage was set to 2.2 kV and the capillary temperature was set to 280 °C. Full-scan mass spectra were acquired in the positive ion mode over the range m/z = 400 to 1600 using the Orbitrap mass analyzer in profile format with a mass resolution setting of 30,000. MS2 scans were collected in the quadrupole or ion trap for the most intense ions in the Top-Speed mode within a 3-s cycle, in centroid format, using Fusion instrument software (version 2.0, Thermo Fisher) with the following parameters: isolation width 4 m/z units, normalized collision energy 30%, charge state 2+ ~ 5+, activation Q 0.25, and activation time 30 ms. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was enabled to preclude reselection of previously analyzed precursor ions, with the following parameters: repeat count 1, exclusion duration 35 s, and mass tolerance within 10ppm.

Mass Spec Data Analysis Procedures (PD analysis)

The raw data was processed using Proteome Discoverer (PD) software (version 1.4.1, Thermo Fisher) and searched against the human-specific SwissProt-reviewed protein database downloaded on October 18, 2017. Indexed databases for rLys-C/tryptic digests were created allowing for up to three missed internal cleavage sites, one fixed modification (carboxyamidomethylcysteine, + 57.021 Da), and variable modifications (methionine oxidation, + 15.995 Da). Precursor ion mass tolerances for spectra acquired using the Orbitrap and linear ion trap (LTQ) were set to 10 ppm. The fragment ion mass tolerance was set to 0.8 Da. High probability assignments were inspected for validity.

Section 4.3 Results and Discussion

Here we present evidence that Siglec-8 binds to sialylated keratan sulfate chains on aggrecan. In guanidinium extracts of human trachea Siglec-8 bound strongly to three large molecular weight species that were separated by size exclusion chromatography (**Figs. 4.1 and 4.2**).

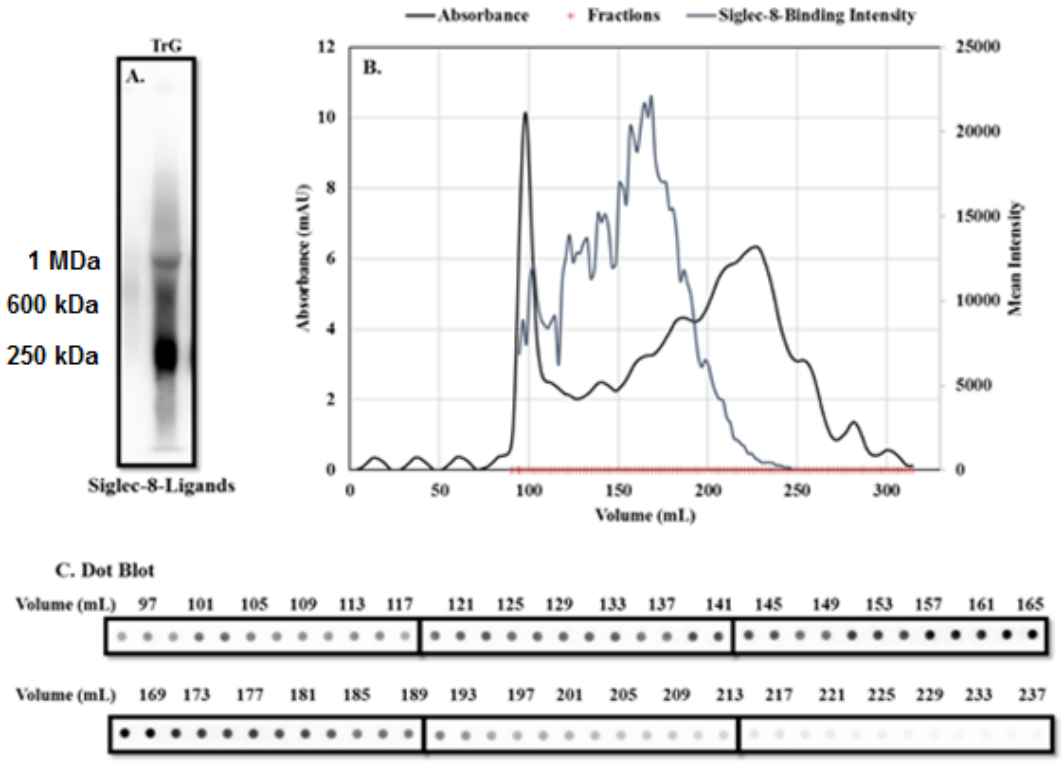


Figure 4.1: Guanidinium extracted tracheal Siglec-8 ligands separated by size exclusion. (**A**) Tracheal guanidinium extract prior to size exclusion (MW marker at left). (**B**) Size exclusion chromatography: A280 (protein, mAU) and relative Siglec-8 binding dot blot. (**C**) Dot blot of fractions at the specified elution volume was blotted with Siglec-8-Fc.

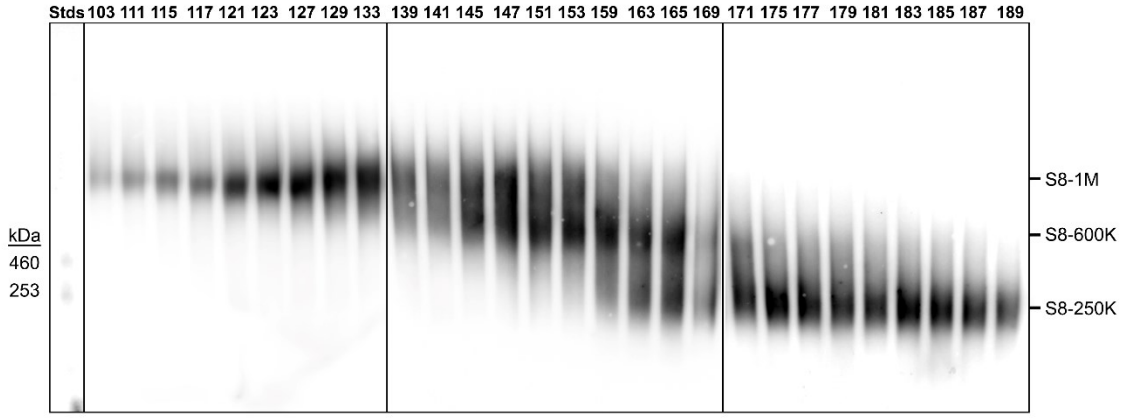


Figure 4.2: Siglec-8 binds to three size classes of sialoglycan ligands. Lung extract was resolved by size column chromatography (Sephacryl S500) and equal volumes of fractions run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands. Three large molecular weight ligands eluted from the size exclusion column. The first large molecular weight ligand runs about 1 million Dalton (S8-1M, first blot), second ligand runs about 600 kilo Daltons (S8-600k, second blot) and third ligand runs about 250 kilo Daltons (S8-250k, third blot).

Three species were named based on the size at which they migrated 1000 kDa (S8-1M), 600 kDa (S8-600k) and 250 kDa (S8-250k). Size resolved Siglec-8 ligands were further purified by immunoprecipitation with Siglec-8-COMP. As a proof of concept, S8-1M ligand was captured on Siglec-8-COMP beads and selectively eluted with the synthetic Siglec-8 sialoglycan ligand (**Table 4.1, Fig. 4.3**).

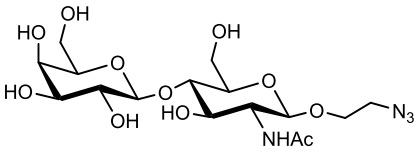
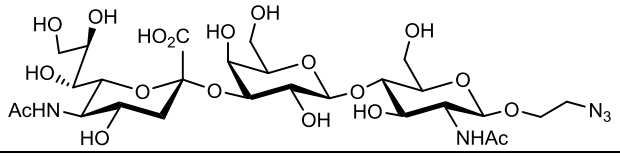
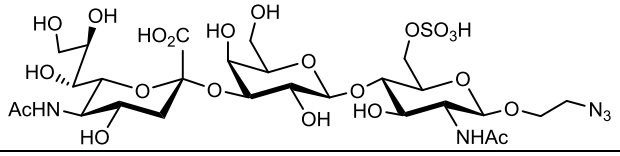
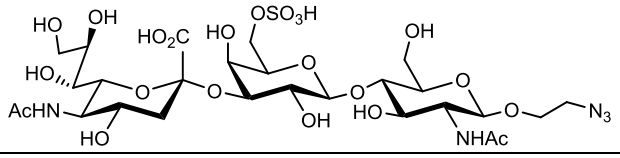
Glycan Abreviation	Glycan Structure	MW
LacNAc		452.42
3'-SLN		743.67
6-Sulfo-3'-SLN		823.73
6'-Sulfo-3'-SLN		823.73

Table: 4.1 Synthetic ligands used for elution of natural ligands captured on Siglec-8 beads. Prepared 50 mM stock solutions of each glycan in water, diluted with 1M Urea buffer to corresponding concentration to elute endogenous human Siglec-8 ligands from Siglec-8-COMP beads.

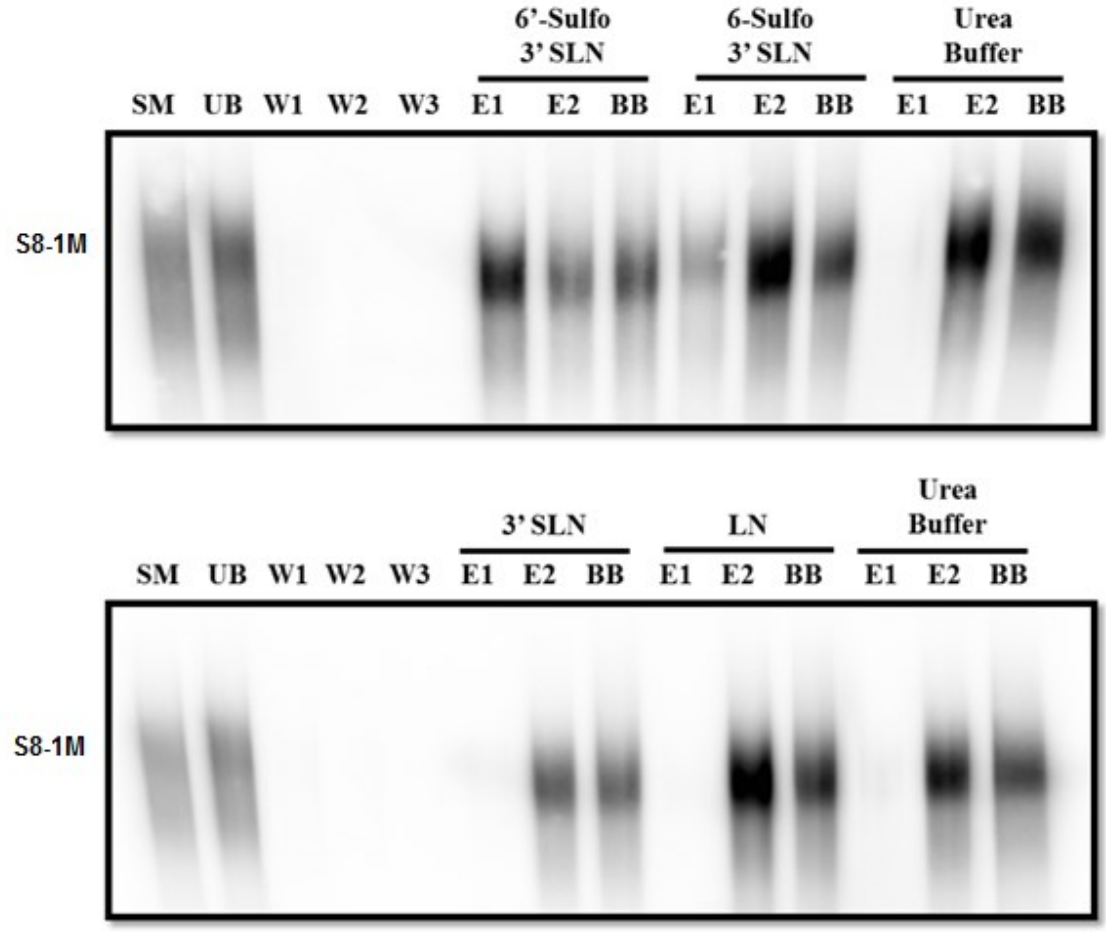


Figure 4.3: S8-1M elution with synthetic glycan ligands. Combined fractions of S8-1M ligand starting material (**SM**) was incubated with Siglec-8-COMP beads overnight and collected sample after incubation (unbound, **UB**). Siglec-8-COMP beads were washed three times (**W1-3**), incubated with synthetic glycan ligand or urea buffer overnight and collected glycan eluted sample (**E1**). Siglec-8-COMP was eluted from beads with imidazole elution buffer (**E2**) and beads were boiled in sample loading buffer (**BB**). Equal volumes of sample were run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands. Respective synthetic glycan ligands used for elution is shown above image.

Since synthetic glycans are monovalent and natural Siglec-8 ligands are likely multivalent, relatively high concentrations of glycan ligands (15 mM) were tested. S8-1M was selectively eluted with Siglec-8 specific sialoside glycan ligand 6'-sulfo-3-sialyl-LacNAc. Only small amounts of ligand were eluted with the structurally similar 6-sulfo-3-sialyl-LacNAc but none with nonsulfated or nonsialylated structures (**Fig. 4.3**). Once the presence of a glycan ligand specific interaction of Siglec-8 with material purified from tracheal extract was confirmed, we pursued to upscale the purification and find another means of elution.

A series of capture and elution trials determined that Siglec-8 binding to its ligands was susceptible to high salt concentrations (data not shown). This strategy helped us elute Siglec-8 ligand without eluting Siglec-8-COMP bound to it. S8-1M, S8-600k and S8-250k were successfully purified and material eluted was subjected to mass spec analysis (**Fig. 4.4**). The predominant species in the purified ligands was aggrecan (**Table 4.2**). Interesting to note is the fact that Siglec-8 or COMP were not detected in mass spec analysis validating that the ligand was eluted without them.

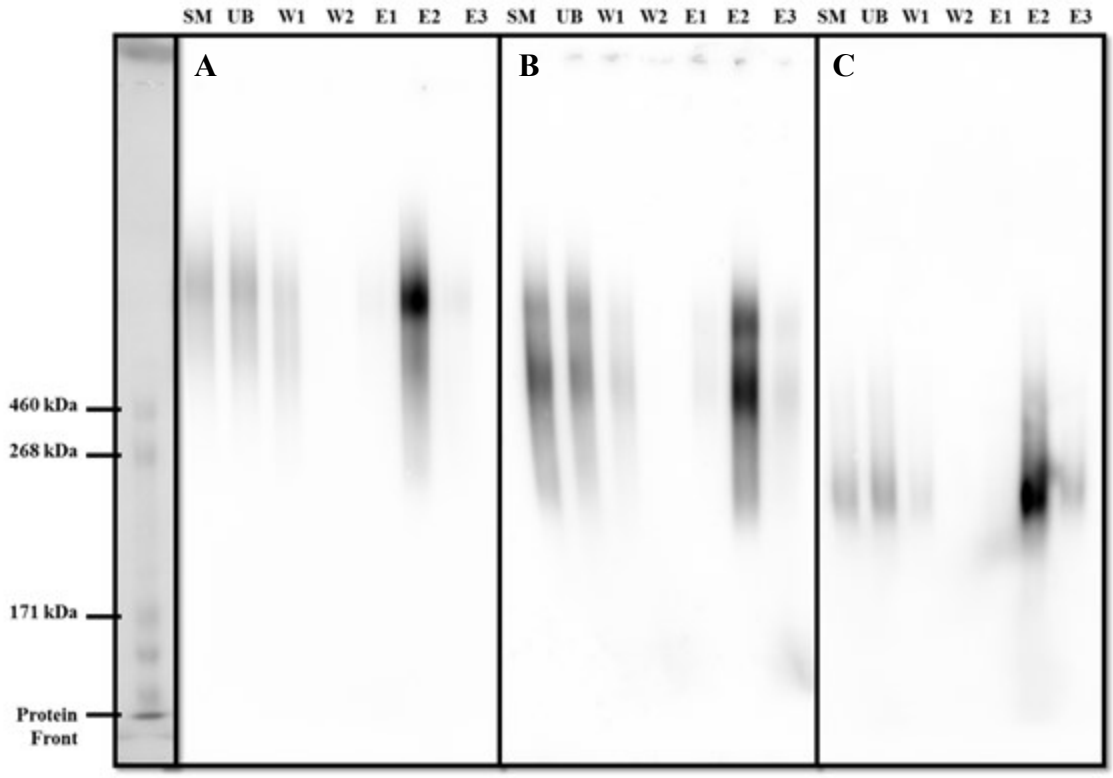


Figure 4.4: Purification of Siglec-8 ligands and specific ligand elution with high salt buffer. Combined fractions of S8-1M (**A**), S8-600k (**B**) or S8-250k (**C**) ligand starting material (**SM**) was incubated with Siglec-8-COMP beads and collected sample after incubation (unbound, **UB**). Siglec-8-COMP beads were washed three times (**W1-3**), eluted ligand in three fractions with high salt elution buffer (**E1-3**). Equal volumes of sample were run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands.

(A) Description	Score	Coverage	Proteins	Unique Peptides	Peptides
Aggrecan core protein	329.01	12.05	7	18	21
Keratin 1	132.56	25.31	15	13	14
YBX1 protein (Fragment)	39.23	24.44	13	4	4
Hyaluronan and proteoglycan link protein	29.36	19.49	10	5	5

(B) Description	Score	Coverage	Proteins	Unique Peptides	Peptides
Aggrecan core protein	946.24	6.96	1	11	16
Keratin, type II cytoskeletal 1	600.67	37.42	2	14	20
Keratin, type I cytoskeletal 9	340.01	33.07	1	10	15
Inactive caspase-12	207.90	6.16	1	1	1

(C) Description	Score	Coverage	Proteins	Unique Peptides	Peptides
Keratin, type II cytoskeletal 1	283.17	32.76	3	21	25
Aggrecan core protein	214.41	6.75	1	12	16
Keratin, type I cytoskeletal 10	145.04	29.62	14	15	18
Keratin, type II cytoskeletal 2 epidermal	123.17	28.33	5	11	17
Keratin, type I cytoskeletal 9	110.96	26.81	1	10	12

Table 4.2: Mass spectrometry proteomic analysis of purified Siglec-8 ligands extracted from trachea. Top protein hits found in purified Siglec-8 ligands S8-1M (**A**), S8-600k (**B**), and S8-250k (**C**). In all cases, keratin is presumed to be an environmental contaminant.

Aggrecan is a large chondroitin sulfate proteoglycan, extensively glycosylated with two known types of glycan chains, chondroitin sulfate and keratan sulfate.[60,61] It is abundant in cartilaginous tissue like trachea. Aggrecan protein core has three globular domains interspaced by linear regions that are highly glycosylated with chondroitin sulfate chains or keratan sulfate chains (**Fig. 4.5**).[60]

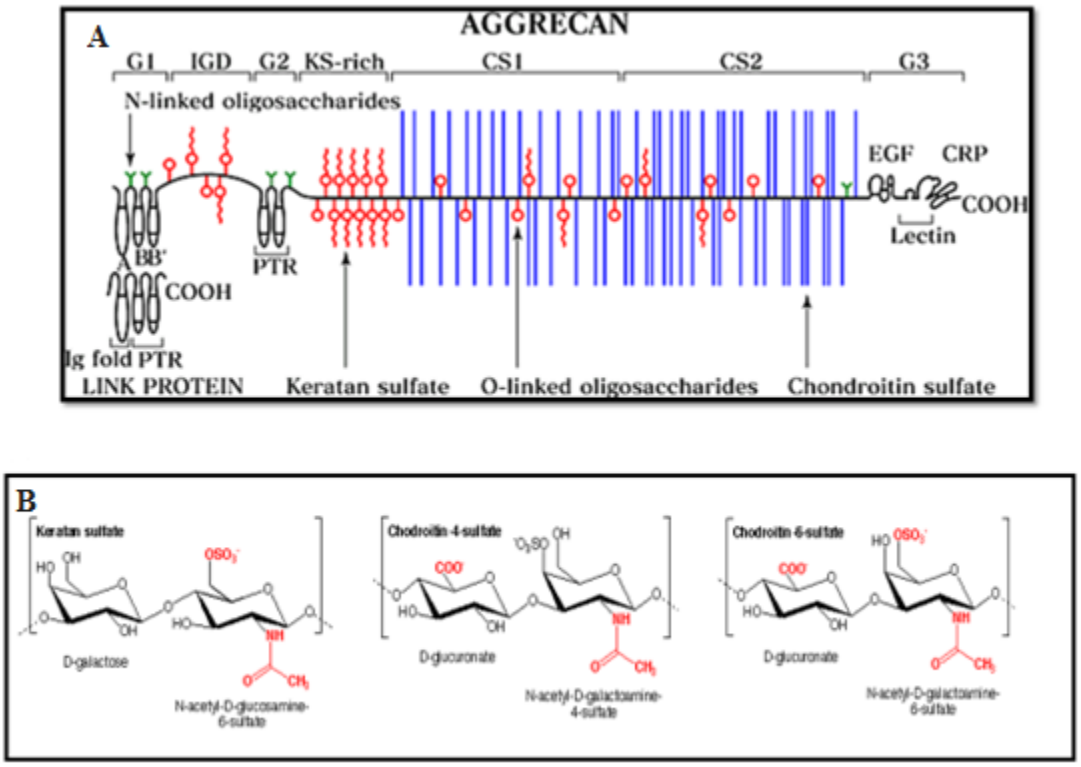


Figure 4.5: Aggrecan protein structure and glycan chains. (**A**)Schematic diagram of Aggrecan structure including globular domains (G1, G2 and G3), interglobular domain (IGD) between G1 and G2, keratan sulfate rich region (KS-rich), and chondroitin sulfate rich regions 1 and 2 (CS-1 and CS-2). Keratan sulfate chains depicted in red squiggly lines and chondroitin sulfate chains in blue. (**B**) Chair conformation of disaccharide units in keratan sulfate and chondroitin sulfate chains.

To validate aggrecan as S8-ligand carrier, purified ligands S8-1M, S8-600k and S8-250k were run on composite gel electrophoresis and overlaid with Siglec-8-Fc or anti-aggrecan antibodies. Two aggrecan antibodies were used to distinguish the length and nature of the aggrecan core protein. One antibody was against the N-terminus globular domains that binds protein containing the G1-IGD-G2 minimum domain. The other antibody is against the C-terminal G3 globular domain, this domain is lost through aggrecanase-mediated degradation of aggrecan commonly seen in osteoarthritis and rheumatoid arthritis patients[62]. Blotting of purified Siglec-8 ligands with Siglec-8-Fc and anti-aggrecan antibodies revealed that S8-1M, S8-600k and S8-250 commigrated in composite gel electrophoresis. All three S8-ligands were detected with aggrecan antibody against the N-terminus of aggrecan but only the largest ligand, S8-1M, was detected with aggrecan antibody against the C-terminus G3 domain (**Fig. 4.6**).

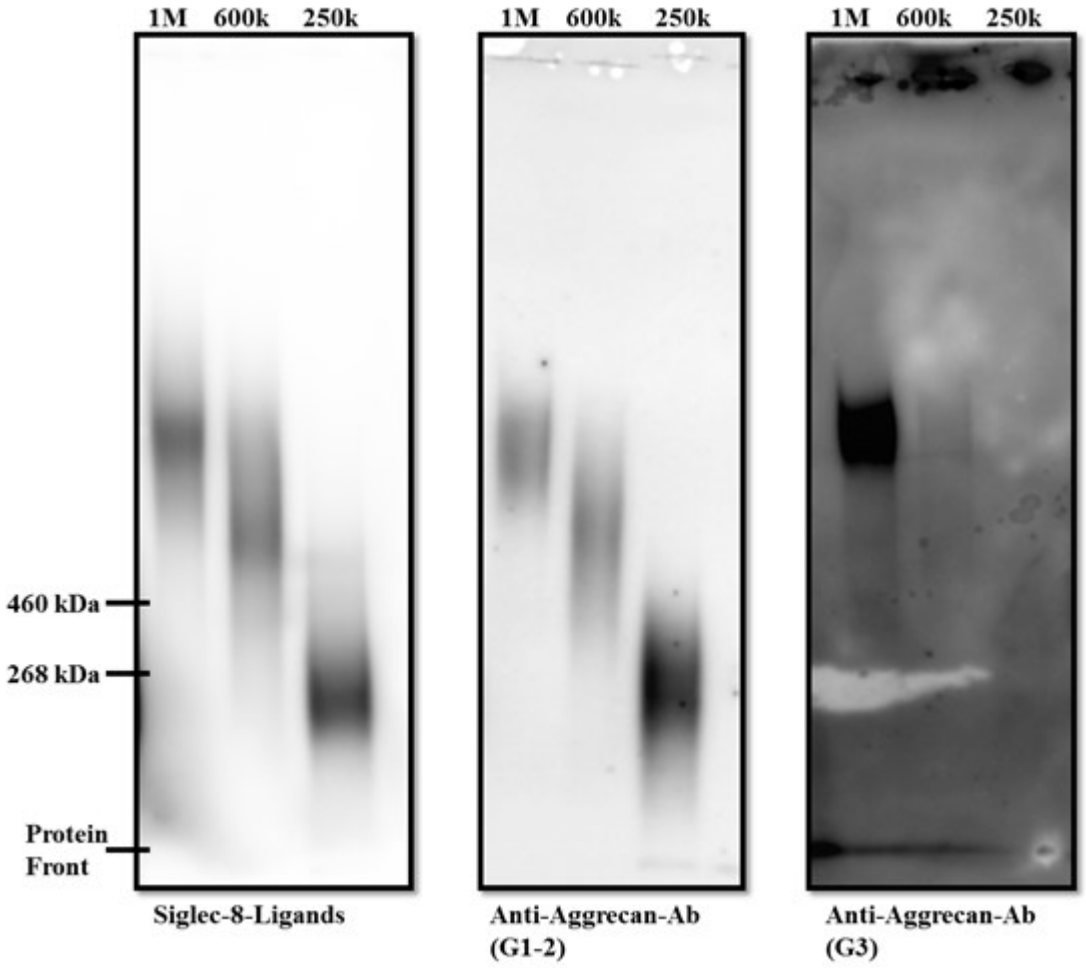


Figure 4.6: Comigration of Siglec-8 ligands with Aggrecan. Equal volumes of S8-1M, S8-600k and S8-250k was run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands (Siglec-8 ligands), anti-Aggrecan-Antibody against the G1-IGD-G2 globular domains of aggrecan (G1-2), or anti-Aggrecan-Antibody against the G3 globular domain of aggrecan.

These findings led us to investigate if aggrecanase treatment of purified S8-ligands would generate the other ligands. Aggrecanse-1, also known as ADAMTS4 (a disintegrin and metalloproteinase with thrombospondin motifs) is a protease that specifically cleaves large chondroitin sulfate hyaluronan-binding proteoglycans (CSPGs) such as aggrecan, brevican, neurocan and versican.[62,63] Purified ligands were treated with ADAMTS4 and

also with chondroitinase ABC to determine if chondroitin sulfate chains were Siglec-8 ligands. Removal of chondroitin sulfate chains by Chondroitinase ABC did not affect Siglec-8 binding to S8-600k or S8-250k and for S8-1M it resulted in increased electrophoretic migration and an increase in Siglec-8-Fc binding (**Fig.4.7 and Fig.4.8**). On the other hand, proteolytic cleavage of aggrecan with ADAMTS4 resulted in a shift of ligand migration as well as reduction in Siglec-8-Fc binding (**Fig.4.7 and Fig.4.8**). Both, S8-ligand and aggrecan comigrated on composite gel electrophoresis even after treatments with chondroitinase and aggrecanase. Tracheal Siglec-8 ligands were indeed carried on aggrecan protein and were not chondroitin sulfate chains. S8-1M, S8-600k and S8-270k Siglec-8 ligands appear to be differential proteolytic cleavage on aggrecan. Chondroitinase treated Siglec-8 ligands that were treated with or without ADAMTS4 showed S8-1M after treatment migrated at about the same position of S8-600k. When the S8-600k was treated with ADAMTS4 it yielded a mixture of molecules, one of which migrated at the S8-270k position (**Fig. 4.9**). To this point, aggrecan was confirmed as a protein carrier of Siglec-8 ligands and chondroitin sulfate chains dismissed as the possible Siglec-8 ligand.

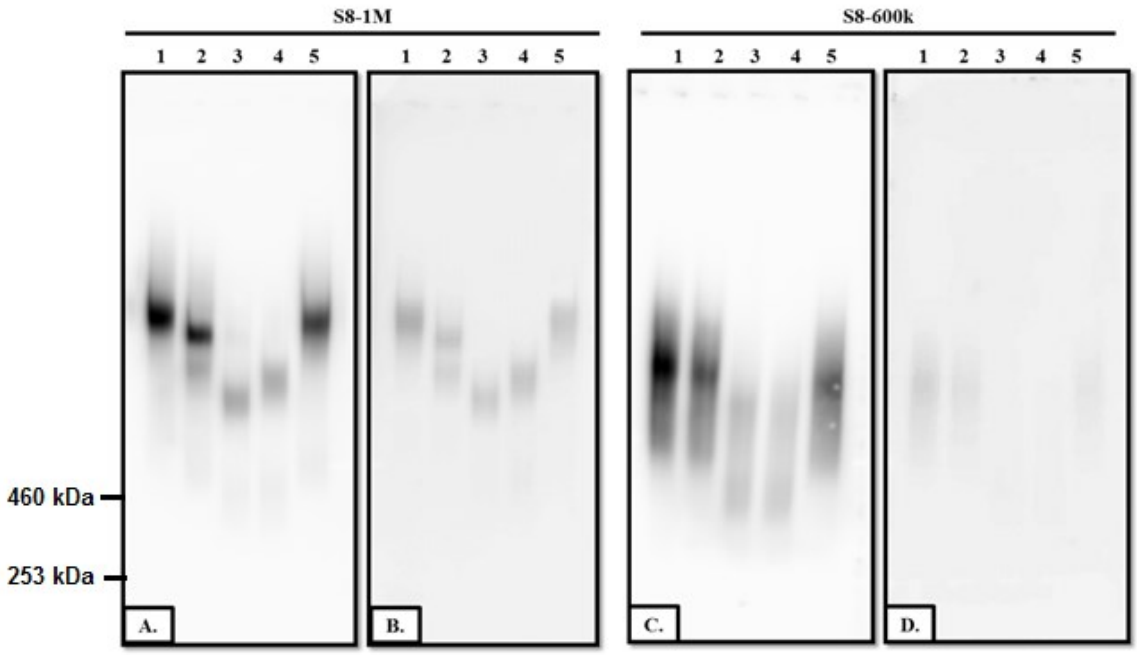


Figure 4.7: Aggrecanase (ADAMTS4) and chondroitinase treatment of purified S8-1M and S8-600k. Purified Siglec 8 ligands were treated with control buffer (**1 and 5**), chondroitinase ABC (**2**), both chondroitinase ABC and ADAMTS4 (**3**), or ADAMTS4 (**4**). Equal volumes of sample were run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands. (**A and C**) or anti-Aggrecan-Antibody G1-2 (**B and D**).

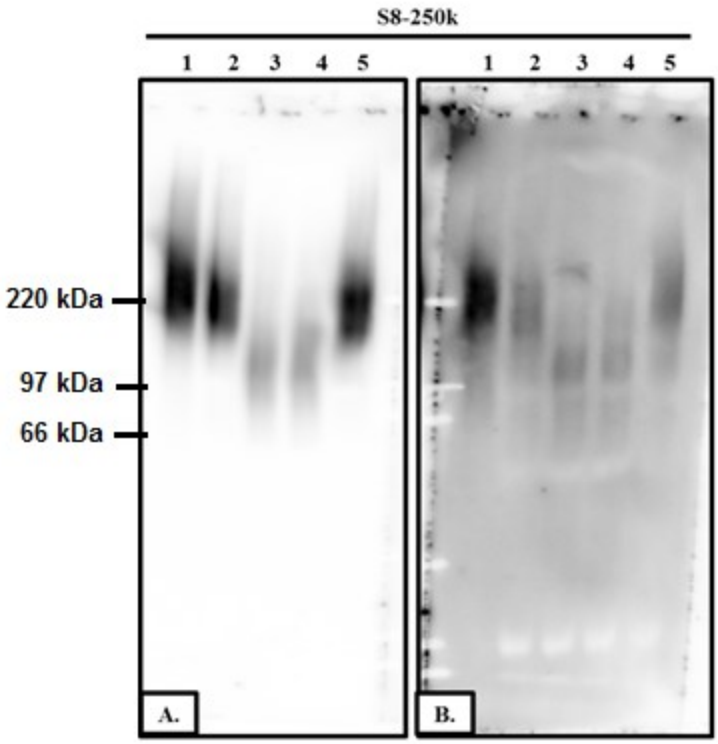


Figure 4.8: Aggrecanase (ADAMTS4) and chondroitinase treatment of purified S8-250k. Purified S8-250k ligand was treated with control buffer (**1 and 5**), chondroitinase ABC (**2**), both chondroitinase ABC and ADAMTS4 (**3**), or ADAMTS4 (**4**). Equal volumes of sample were run on (2% agarose, 3% acrylamide) composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands (**A**) or anti-Aggrecan-Antibody G1-2 (**B**).

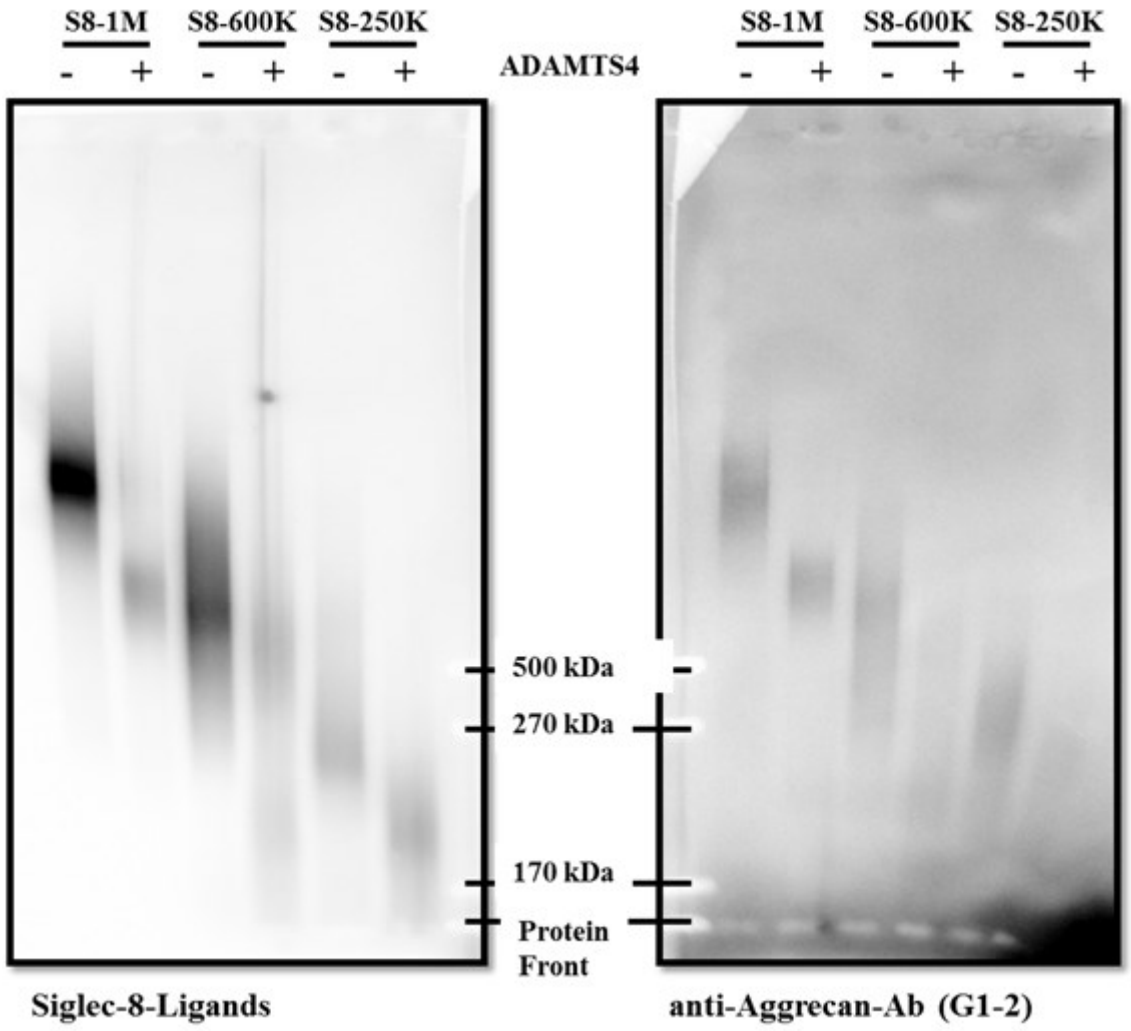


Figure 4.9: Purified Siglec-8-ligands chondroitinase and adamts4 treated. Purified Siglec 8 ligands previously treated with chondroitinase were treated with or without ADAMTS4. Equal volumes were run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands (Siglec-8-ligands) or anti-Aggrecan-Antibody G1-2.

In the literature, chondroitin sulfate chains have not been reported to be terminated with sialic acid but keratan sulfate (KS) chains, have.[61] In keratan sulfate chains extracted from bovine trachea, Lauder et. al. identified a small portion of KS capped with N-acetyl-neuraminic acid α(2-3)-linked to a galactose that may or may not be sulphated (**Fig. 4.10 A**).[61] KS structures previously reported resemble the synthetic Siglec-8 glycan ligand

identified by glycan arrays. A monoclonal IgG1 antibody (5D4) against highly sulfated keratan sulfate chains was used to detect the presence of keratan sulfate chains in purified Siglec-8 ligands (**Fig. 4.10 B**). Keratan sulfate antibody (5D4) binding to Siglec-8 purified ligands treated with chondroitinas ABC and ADAMTS4 comigrated and correlated. Reduction in Siglec-8 binding with ADAMTS4 treated samples also showed reduction in 5D4 binding, suggesting KS chains may be Siglec-8 ligands (**Fig. 4.11**).

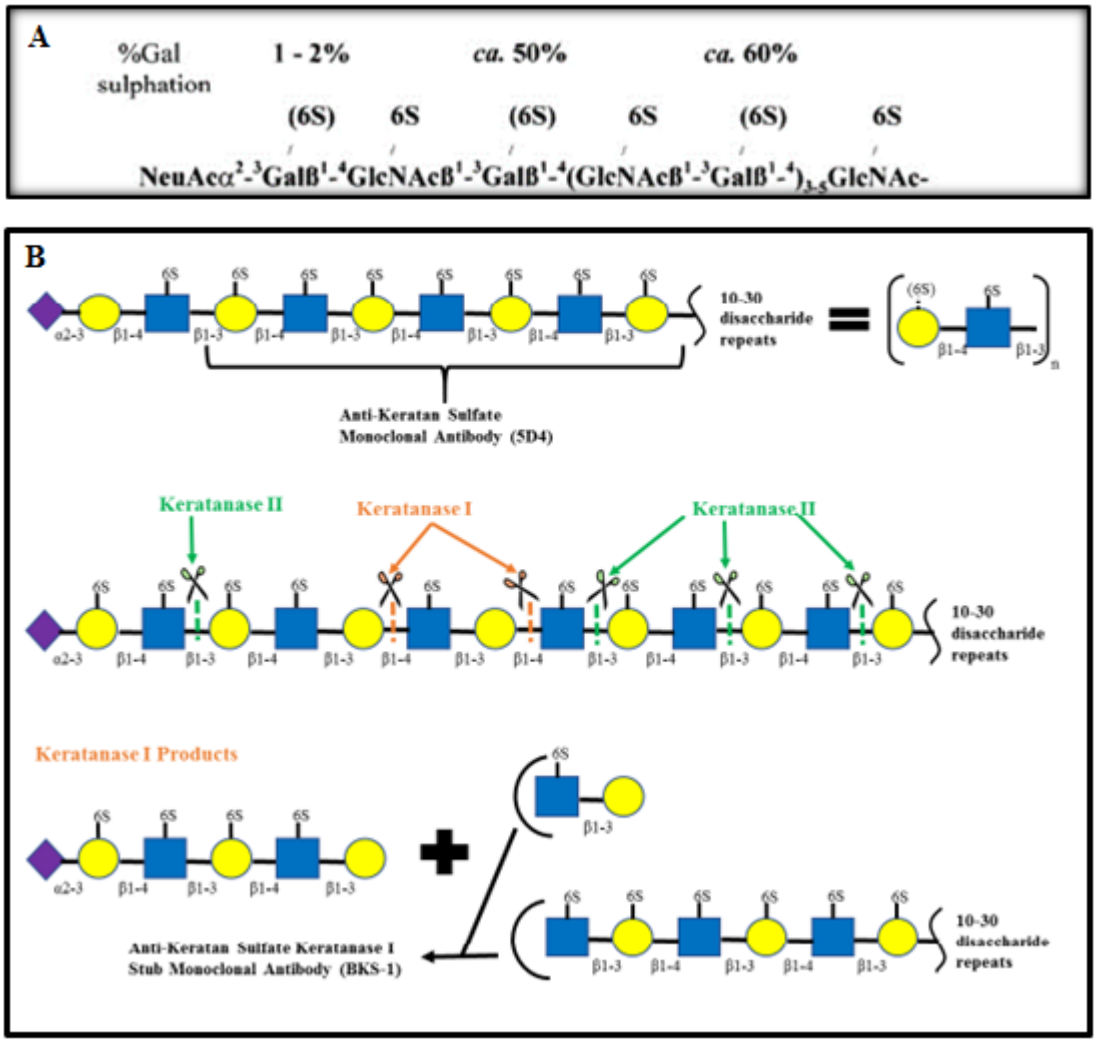


Figure 4.10: Schematic structure of keratan sulfate showing antibody binding motifs and sites of cleavage with different keratanases. (**A**) Galactose sulfation levels of keratan sulfate chains attached to bovine aggrecan.[61] (**B**) Anti-keratan-sulfate antibody 5D4 binds

highly sulfated keratan sulfate chains.[64,65] Keratanase I cleaves the beta-1-3 linkage of the disaccharide units only if the galactose is not sulfated and keratanase II can cleave if the galactose is sulfated.[66,67] Anti-keratan-sulfate stub antibody recognizes keratanase I created stubs after reaction.[68]

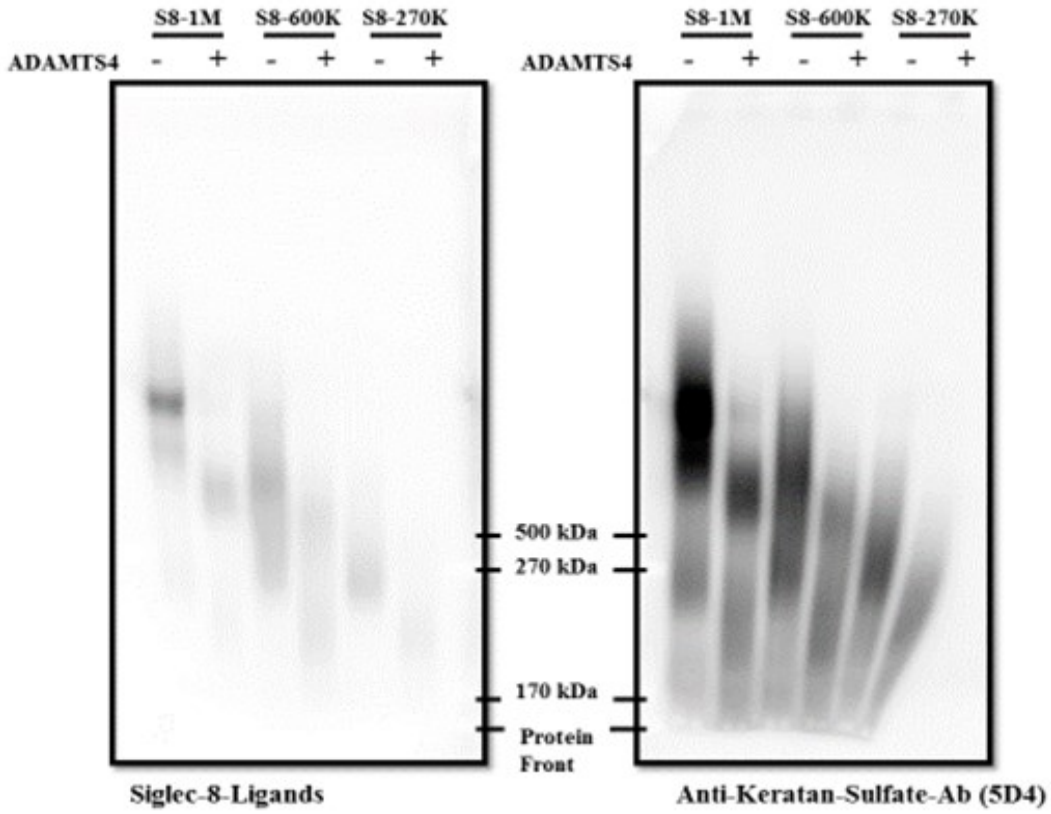


Figure 4.11: Tracheal extracted Siglec-8-ligands co-migrate with antibody staining of highly sulfated keratan sulfate chains (5D4) and binding is shifted and reduced after aggrecanase (ADAMTS4) treatment. Purified Siglec 8 ligands previously treated with chondroitinase were treated with or without ADAMTS4. Equal volumes of treated samples were run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands (Siglec-8-ligands) or anti-Keratan-Sulfate-Antibody (5D4).

To further validate these findings, purified S8-ligands were individually treated with two different endoglycosidases. Keratanase I is an endo-β-galactosidase that cleaves KS chains at the 1,4-β-galactosidic linkage as long as the GlcNAc is sulfated and galactose is not. Anti-keratan sulfate antibody Bks-1 detects the glycosidic product of KS I digested KS chains and was used to show that the reaction occurred. Although, galactose sulfation inhibits keratanase I, it does not inhibit the endo-β-glucosaminidase keratanase II.[66] Keratanase II cleaves at the 1,3-β-GlcNAc linkage and sulfation of galactose or N-acetyl-glucosamine does not affect its activity. Purified Siglec-8 ligands were treated with keratanase I and keratanase II to determine if KS chains were carrying the motif Siglec-8 binds to. Treatment of all purified S8-ligands with either keratanase I or keratanase II eliminated Siglec-8 binding (**Fig. 4.12-14 A**).

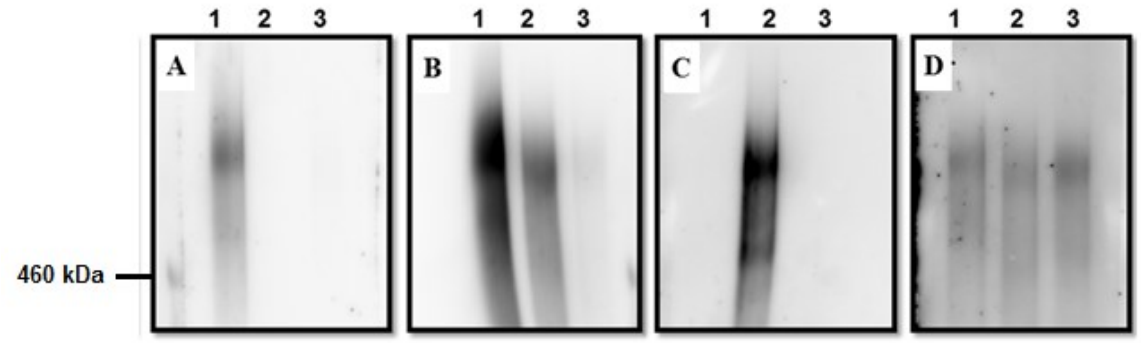


Figure 4.12: Keratanase treatment of purified S8-1M eliminates Siglec-8 binding. Purified S8-1M was treated with buffer control (1), 40.0 mU/mL keratanase I (2), or 40.0 mU/mL keratanase II (3). Equal volumes of treated samples were run on composite gel electrophoresis and blotted with Siglec-8-Fc (**A**), anti-Keratan-Sulfate-Antibody (5D4) (**B**), anti-Keratan-Sulfate-Antibody (Bks-1) (**C**), or anti-Aggrecan-Antibody G1-2 (**D**). Exposure of A-B 15 seconds and C-D 300 seconds.

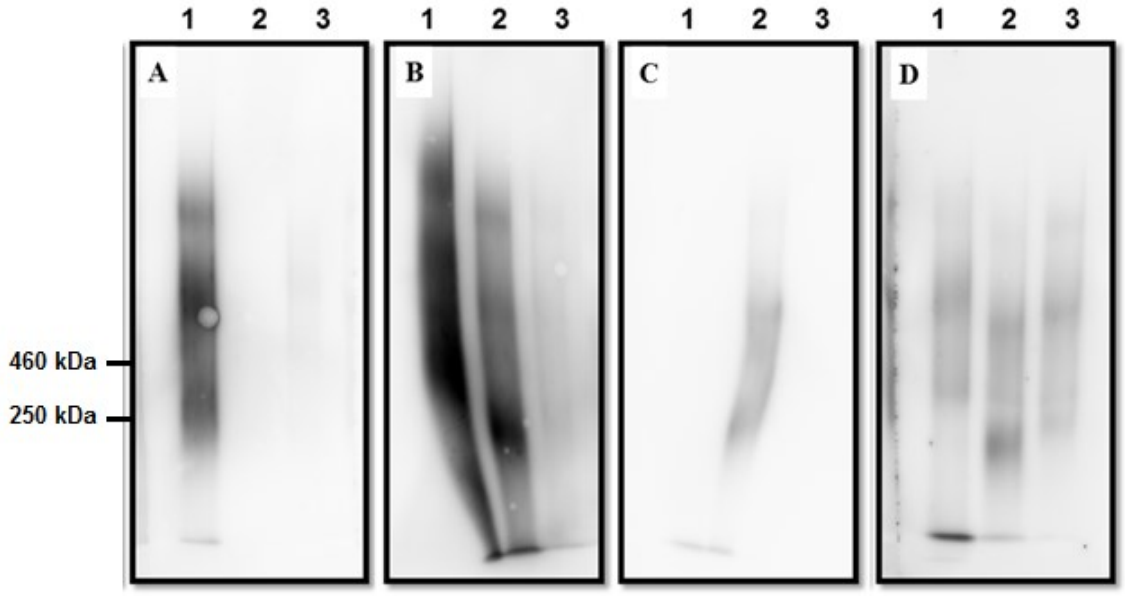


Figure 4.13: Keratanase treatment of purified S8-600k eliminates Siglec-8 binding. Purified S8-600k was treated with buffer control (1), 40.0 mU/mL keratanase I (2), or 40.0 mU/mL keratanase II (3). Equal volumes of treated samples were run on composite gel electrophoresis and blotted with Siglec-8-Fc (**A**), anti-Keratan-Sulfate-Antibody (5D4) (**B**), anti-Keratan-Sulfate-Antibody (Bks-1) (**C**), or anti-Aggrecan-Antibody G1-2 (**D**). Exposure of A-B 15 seconds and C-D 300 seconds.

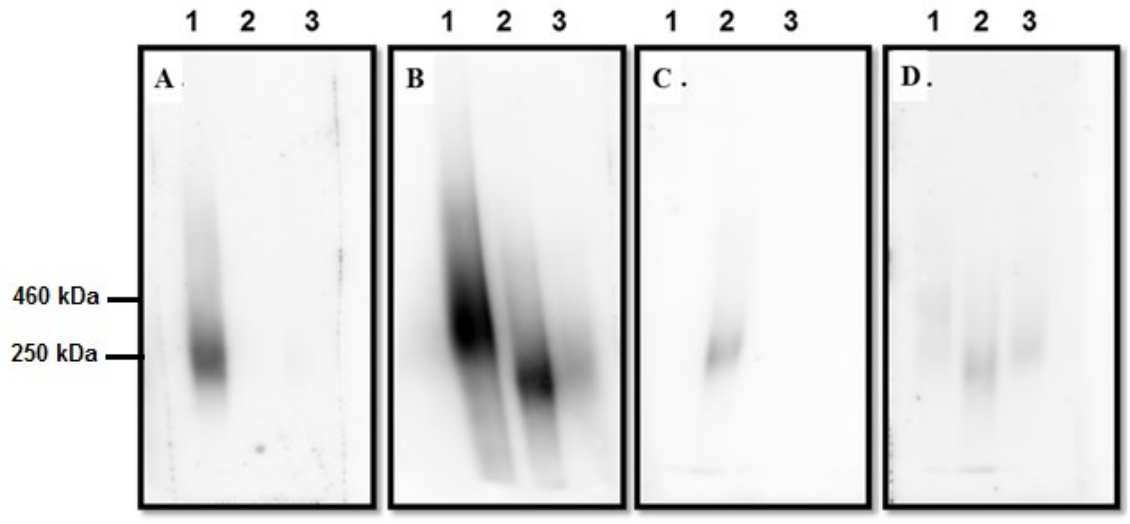


Figure 4.14: Keratanase treatment of purified S8-250k eliminates Siglec-8 binding. Purified S8-250 was treated with buffer control (1), 40.0 mU/mL keratanase I (2), or 40.0 mU/mL keratanase II (3). Equal volumes of treated samples were run on composite gel electrophoresis and blotted with Siglec-8-Fc (**A**), anti-Keratan-Sulfate-Antibody (5D4) (**B**), anti-Keratan-Sulfate-Antibody (Bks-1) (**C**), or anti-Aggrecan-Antibody G1-2 (**D**). Exposure of A-B 15 seconds and C-D 300 seconds.

Keratanase I treatment of purified sample reduced 5D4 staining to samples whereas keratanase II completely eliminated antibody staining confirming digestion of KS chains (**Fig. 4.12-14 B**). Only keratanase I treated samples showed staining with Bks-1 antibody as expected (**Fig. 4.12-14 C**). Loss of Siglec-8 binding to S8-ligands was not due to protease activity in sample because anti-aggrecan antibody showed similar binding to all treated samples (**Fig. 4.12-14 D**). Sialidase and keratanase treatment of purified S8-ligands eliminate S8-binding and reveal KS chains capped with sialic acid are Siglec-8 ligands (**Fig. 4.15**).

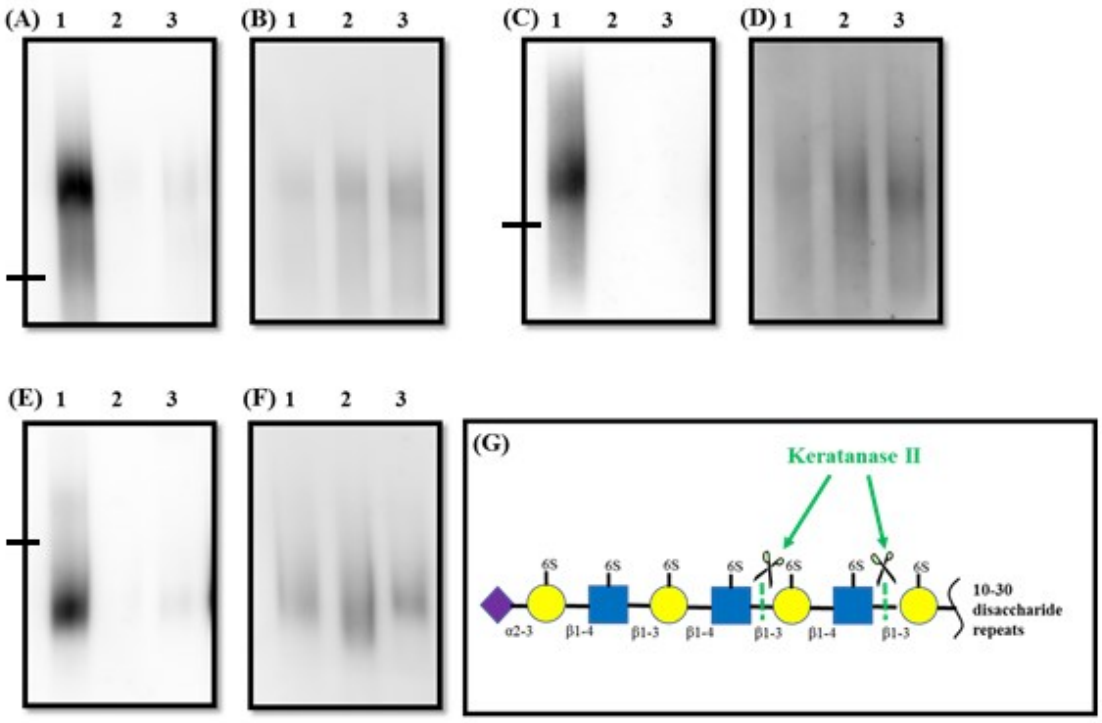


Figure 4.15: Purified Siglec-8-ligands treated with sialidase and keratanase. Purified Siglec 8 ligands S8-1M (**A-B**), S8-600k (**C-D**) and S8-250k (**E-F**) was treated with buffer control (**1**), 67 mU/mL sialidase (**2**), or 6.0 mU/mL keratanase II (**3**). Equal volumes of treated samples were run on composite gel electrophoresis and blotted with Siglec-8-Fc (**A, C and E**) or anti-Aggrecan-Antibody G1-2 (**B, D or F**). Schematic of Siglec-8 ligand keratan sulfate chain sensitive to keratanase II. (**G**). Straight line shows migration of the largest molecular weight band (460 kDa) of HiMark marker.

Siglec-8 binds to KS chains on aggrecan. To confirm it is a functional ligand we needed to show that it induces Siglec-8 mediated cellular responses, such as human eosinophil apoptosis. This was tested using S8-250k, which was relatively abundant and well resolved. S8-250k was oxidized with mild periodate to selectively trim the glycerol side chain of sialic acid[69], which abrogated Siglec-8 binding without affecting the protein carrier (**Fig. 4.16 A**). The use of small chemicals for removal of sialic acid binding specific interaction

was preferred over using sialidase because small chemicals are easily removed whereas enzymes like sialidase or keratanase are not. Amino acids in Siglec-8 binding pocket make specific hydrogen bonding interactions with the glycerol side chain of sialic acid and removal of it reduces affinity for the glycan structure (**Fig. 4.16 B**)[70]. Treatment of eosinophils with periodate-treated S8-250k (no S8-binding) did not significantly increase eosinophil apoptosis above background levels. However, treatment of eosinophils with untreated S8-250k (intense S8-binding) significantly increased eosinophil apoptosis 15% above background levels (**Fig. 4.16 C**). Ligand activity was sialic acid specific since only intact S8-250k induced eosinophil apoptosis, validating the ligand as a functional ligand.

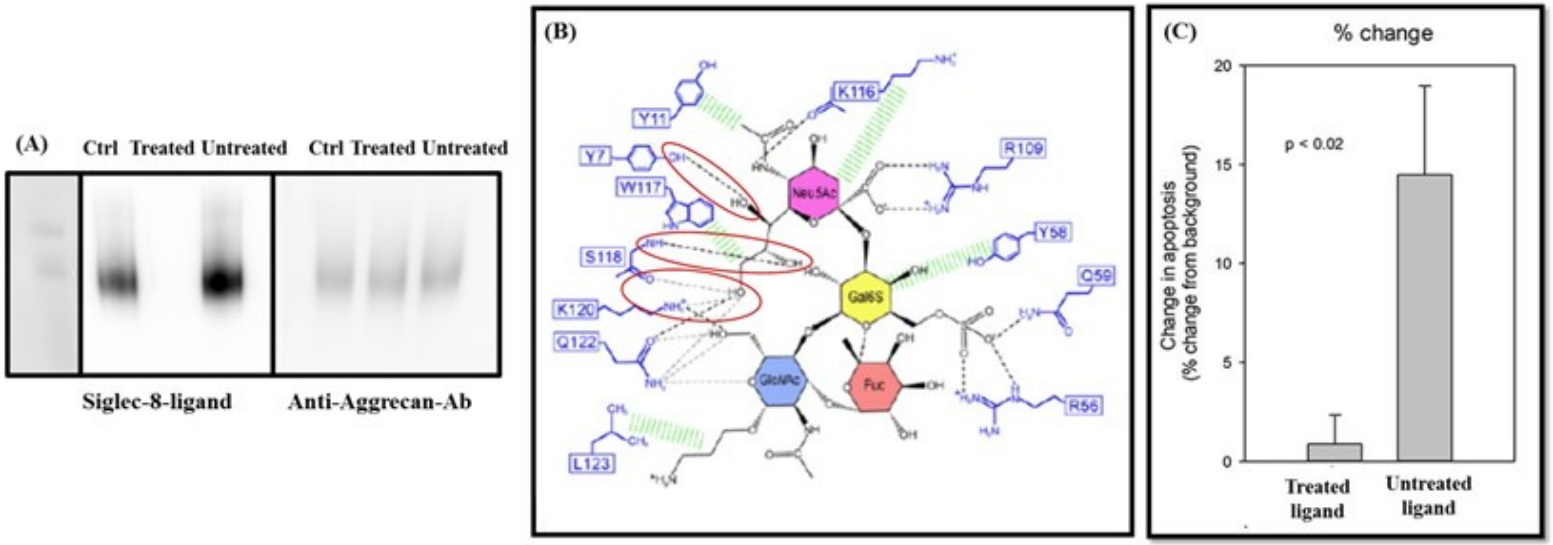


Figure 4.16: Effect of purified S8-250k on eosinophil survival. (**A**) Purified S8-250k ligand (ctrl) was treated with periodate (treated) or with reducing agent only (untreated), equal volumes of treated samples were run on composite gel electrophoresis and blotted with Siglec-8-Fc (Siglec-8 ligands) or with anti-Aggrecan-Antibody G1-2. HiMark loaded on first lane, largest band is 460 kDa and the one bellow is 250 kDa. (**B**) Schematic illustration of Siglec-8 interaction network with synthetic glycan ligand. Circled in red are the hydrogen bond interactions of the glycerol side chain of sialic acid with amino acids in Siglec-8 binding pocket. Modified from Pröpster, et. al.[70] (**C**) Activated eosinophils were incubated with media containing 14 ng/μL of treated or untreated ligand for 24 hours and determined viability of cells staining with Annexin V and DAPI. Percent change in apoptosis from background, $P < 0.02$, paired t test vs control (n=6).

In summary, Siglec-8 binds to sialylated keratan sulfate chains on aggrecan extracted from human tracheal airways. Upon Siglec-8-Fc overlay staining of human tracheal sections we observed strong Siglec-8 binding to cartilage (**Fig. 3.1**) but also to submucosal gland cells. Anti-aggrecan immunohistochemistry detected aggrecan in cartilage, but not in submucosal glands (data not shown) suggesting another protein may carry Siglec-8 ligands in airway secretions. However, Siglec-8 ligand are sialylated keratan sulfate chains because treatment of tracheal sections with keratanases or sialidase completely abrogates Siglec-8 binding (data not shown). What the protein carrier's role is in control of inflammation still needs to be determined as well as what other proteins may carry these modified structures that Siglec-8 binds to.

CHAPTER 5: SIGLEC-8 LIGANDS IN THE HUMAN AIRWAY MUCUS LAYER

Section 5.1 Introduction

Previously, Siglec-8 ligand extracted from human trachea was purified and identified as sialylated keratan sulfate chains on aggrecan. Aggrecan is an extracellular protein widely studied for its function in cartilaginous tissue but less so in other tissues.[71] To this date, there is no evidence that aggrecan is expressed in tracheal gland cells in human airway. If aggrecan is carrying Siglec-8 ligands, how is it coming into contact with inflammatory cells to reduce inflammation? Immunohistochemistry staining of human trachea sections showed aggrecan present abundantly only in cartilage but not in submucosal gland cells where Siglec-8 stained strongly. Therefore, it could be possible Siglec-8 ligands are found on another protein carrier in submucosal gland cells. Our hypothesis is that material produced in submucosal gland cells is secreted into the airway lumen where they come in contact with inflammatory cells. In 2015, we showed expression of Siglec-8 ligands on human inferior turbinate tissue from patients with and without chronic rhinosinusitis.[72] However, we did not analyze secretions from patients and in this project, we pursue to analyze airway secretions for Siglec-8 ligand expression.

Chronic rhinosinusitis (CRS) is diagnosed if a patient suffers persistent upper airway inflammation for more than 12 weeks without resolving[73]. Patients suffer extreme discomfort due to airway congestion making it difficult for them to breathe accompanied by pain and discomfort.[74,75] Sometimes it can be accompanied by abnormal growths in the sinuses called nasal polyps[75]. Treatments available are designed to help patients with symptoms but does not reverse or decrease disease progression.[76,77] Most patients with

nasal polyps undergo surgery to get relief but polyps usually come back within a few years after surgery.[74] These polyps are filled with mucus secretions and inflammatory cells such as eosinophils.[78,79] If Siglec-8 ligands are produced in tissue, eosinophils should undergo apoptosis and inflammation should resolve. If ligands are produced in tissue but are not secreted into the lumen this could explain the high expression of Siglec-8 ligands in inflamed tissue and the of lack inflammation control. To determine if ligands were being secreted into the lumen of healthy patients and diseased patients we investigated the level of expression of Siglec-8 ligands. Ligands were purified and analyzed by mass spec analysis to determine Siglec-8 ligand protein carrier in secretions. Also, samples were treated with keratanases and sialidase to determine if the glycan ligand is similar to that one previously found in tracheal cartilage.

Section 5.2 Additional Experimental Procedures

Siglec-8 endogenous secreted ligand capture with Siglec-8-Fc beads

Nasal lavage from 4 different donors were combined and purified over a size exclusion chromatography column as described in Chapter 2. Combined fractions containing Siglec-8 ligand running at 900 kDa were dialyzed against Urea Buffer before incubating with beads. Dialyzed material was precleared with human IgG-Fc bound protein A/G magnetic beads. Then, incubated with Siglec-8-Fc bound protein A/G magnetic beads (Siglec-8 beads) to capture ligands. Beads were incubated with sample mixing at 4 °C for 2 h. Unbound material was collected, beads washed 5x with Urea Buffer/PBST (4:1), and ligand eluted Siglec-8 with Salt Elution Buffer. Beads were treated with glycine Fc-elution buffer (100 mM glycine, pH-2.7) and then boiled in Solubilizing Buffer.

Section 5.3 Results and Discussion

Here we present evidence that sialylated keratan sulfate Siglec-8 ligands are secreted onto human airways. Siglec-8 ligands in secretions have similar properties to those observed in ligands extracted from human trachea: large molecular weight proteins carrying Siglec-8 ligands sensitive to sialidase and keratanases. Analysis of nasal secretions from more than 20 patients revealed Siglec-8 bound to a protein of ~900 kDa that was detected with anti-keratan sulfate antibody as well (**Fig. 5.1 A-C**). Ligand expression was variable and did not correlate with patients' disease (**Fig. 5.1 E**), however, anti-keratan sulfate detection and Siglec-8 binding intensity correlated with each other, further supporting the presence of sialylated keratan sulfate Siglec-8 ligands (**Fig. 5.1 D**).

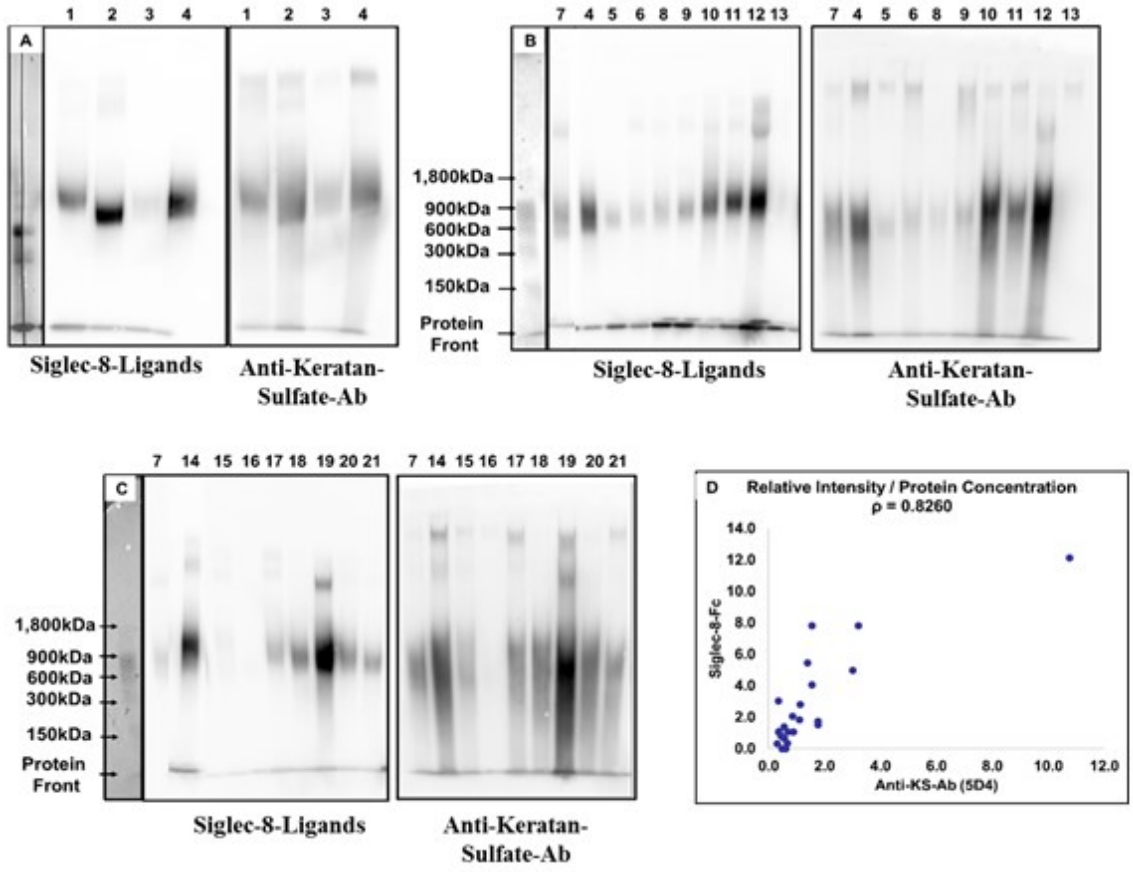


Figure 5.1: Siglec-8-ligand in nasal lavage co-migrate and correlates with antibody staining of highly sulfated keratan sulfate chains (5D4). Equal volumes of aliquoted soluble lavage from 21 patients was loaded per lane. Samples were blotted with soluble Siglec-8-Fc or anti-Keratan-Sulfate antibody (**A-C**). Correlation of Siglec-8 binding intensity with anti-Keratan-Sulfate antibody (**D**). No significant difference observed in Siglec-8 ligand expression in samples from normal patients or diseased patients with (CRSwNP) or without polyp (CRSsNP) (**E**).

First lane in (**A**) shows the HiMark marker migration with the largest band 460 kDa followed by 250 kDa band below.

When nasal lavage secretions were treated with keratanase I or sialidase, Siglec-8 binding to sample was completely abolished. Treatment with keratanase II reduced Siglec-8 binding significantly but was not as profound as keratanase I (**Fig. 5.2 A**). This differs from S8-1M purified from tracheal extracts, which was equally sensitive to both, keratanase I and II (**Fig. 5.2 B**). Enzymatic digestion of the Siglec-8 ligand was effective as seen in blots probed with keratanases. Samples treated with keratanase II lost all binding to anti-keratan sulfate antibody and samples treated with keratanase I were positive with the anti-keratan sulfate stub antibody (**Fig. 5.2 C**). Keratanase II has a higher preference for di-sulfated LacNAc rather than monosulfated and partial decrease of Siglec-8 binding suggests that a portion of Siglec-8 ligands may be at the terminus of chains that contain monosulfated keratan sulfate dimers that are not efficiently cleaved by keratanase II but cleaved by keratanase I.

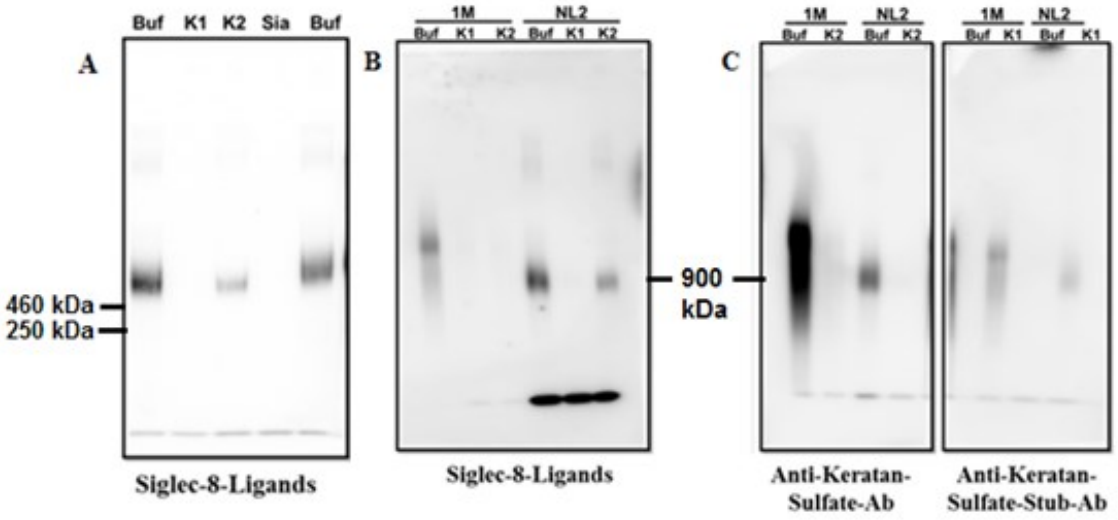


Figure 5.2: Siglec-8-ligand in human nasal lavage is sensitive to keratanase and sialidase treatment. Nasal lavage from donor 2 treated with buffer control (Buf),

keratanase I (K1), keratanase II (K2) or sialidase (Sia), blotted and detected with Siglec-8-Fc (**A**). Siglec-8 ligand extracted from trachea (1M) and nasal lavage treated with keratanases, blotted, and detected with Siglec-8-Fc (**B**) or anti-KS-antibodies (**C**).

Siglec-8 ligands in human airway were further purified by size exclusion chromatography to determine what protein was carrying sialylated keratan sulfate chains in human airway secretions (**Fig. 5.3**). The fractions containing Siglec-8 ligand migrating at 900 kDa on composite gel electrophoresis were combined and further purified over a Siglec-8 column (**Fig. 5.4**). Siglec-8 ligands were efficiently captured and eluted with Siglec-8-Fc affinity beads providing us with enough material for mass spec analysis (**Fig. 5.5**). Mass spec analysis revealed that Siglec-8 ligand in airway secretions was not aggrecan but glycoprotein 340 (**Fig. 5.6**). Glycoprotein 340 (GP340) is a mucin like protein secreted in body fluids and know to have anti-microbial activity.[80-84] A gene expression profile of human nasal polyp tissues identified GP340 as one of four proteins that were upregulated in nasal polyp tissues.[85] The upregulation of GP340 may be an attempt to resolve inflammation without success for some reason. To validate the data obtained from mass spec analysis, fractions collected from size exclusion chromatography column were overlaid with Siglec-8-Fc to detect ligand or with anti-GP340 (**Fig. 5.7**). Both, Siglec-8 ligand and anti-GP340 showed comigration and coelution from the column. To further confirm these findings, Siglec-8 lectin precipitated samples were blotted with Siglec-8-Fc or anti-GP340 antibody (**Fig. 5.8**). Blotting revealed GP340 eluted with Siglec-8 ligand and comigrated at the same position on composite gel electrophoresis.

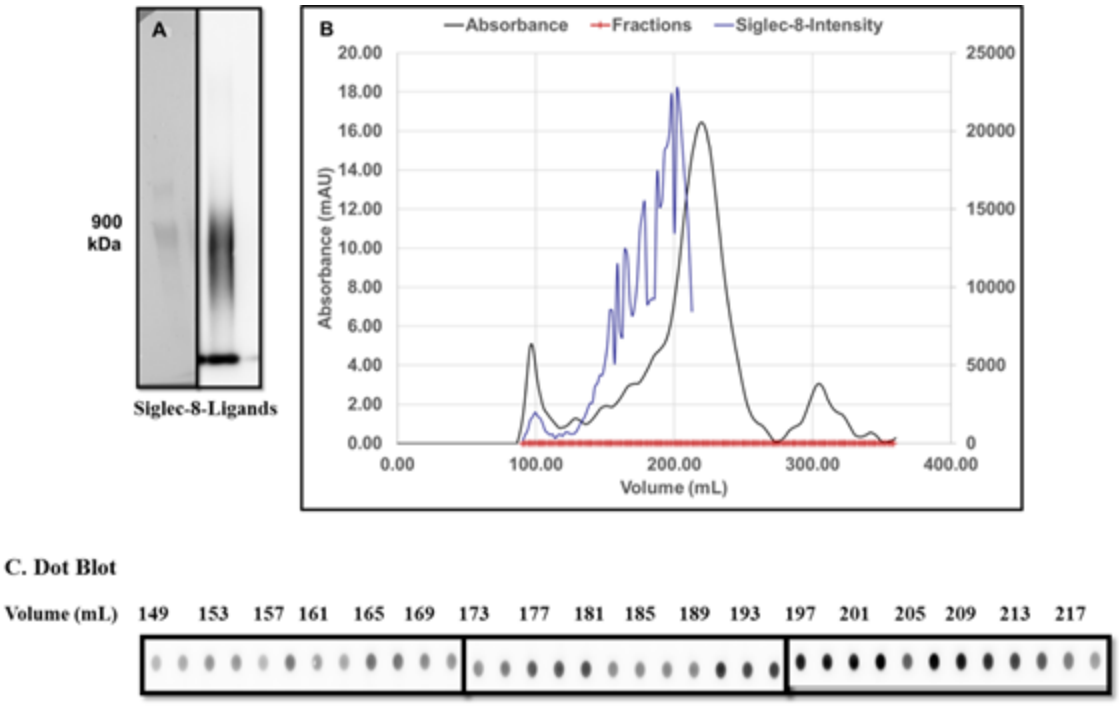


Figure 5.3: Nasal lavage Siglec-8 ligands separated by size exclusion. (**A**) Nasal lavage prior to size exclusion (MW marker at left). (**B**) Size exclusion chromatography: A280 (protein, mAU) and relative Siglec-8 binding dot blot. (**C**) Dot blot of fractions at the specified elution volume overlaid with precomplexed Siglec-8-Fc HRP-conjugated anti-human Fc.

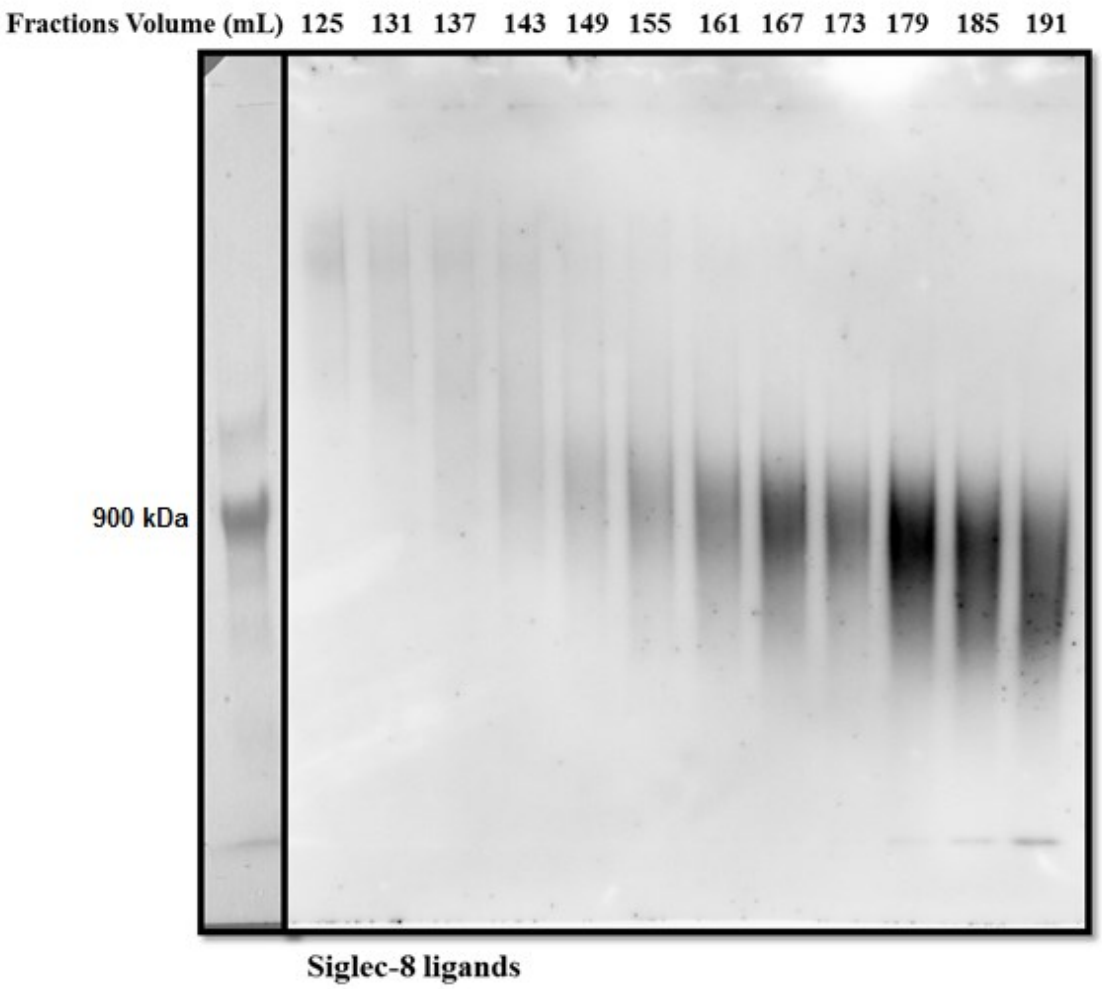


Figure 5.4: In nasal lavage Siglec-8 binds to two size classes of sialoglycan ligands. Nasal lavage was resolved by size column chromatography (Sephacryl S500) and equal volumes of fractions run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands. Major Siglec-8 binding species runs at 900 kDa (S8-900k) and a minor species is observed running above.

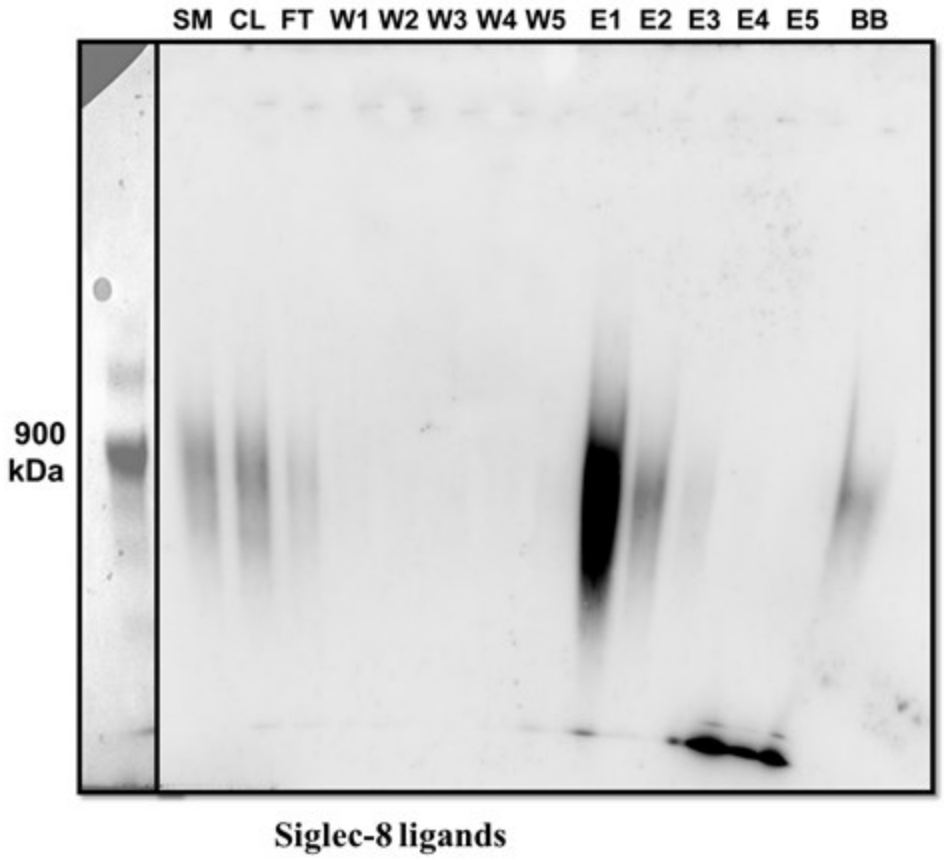
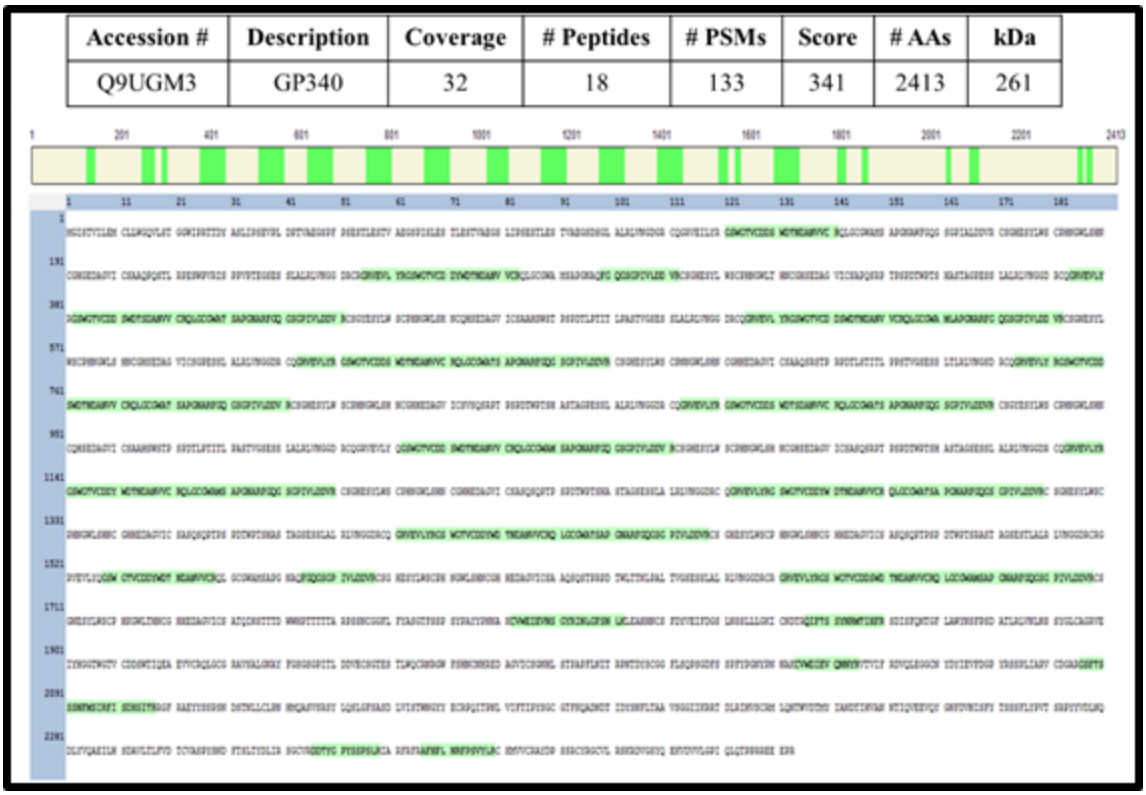


Figure 5.5: Purification of S8-900k major Siglec-8 ligand species in nasal lavage. Fractions containing the major Siglec-8-ligand (S8-900k) were combined (SM), pre-cleared with magnetic-Protein A/G beads (CL) then incubated with magnetic-Protein A/G beads with bound Siglec-8-Fc. Aliquots of flow through (FT), washes (W1-5), salt elutions (E1-3), glycine elutions (E4-5) and boiled beads (BB) were resolved and blotted with Siglec-8-Fc (Siglec-8 ligands).



Accession #	Description	Coverage	# Peptides	# PSMs	Score	# AAs	kDa
Q9UGM3	GP340	32	18	133	341	2413	261

Figure 5.6: Mass spectrometry proteomic analysis of purified S8-900k from nasal lavage. The major hit in proteomic analysis of purified Siglec-8 ligand was glycoprotein 340 (GP340). Schematic of GP340 and unique peptides picked up by mass spec with high confidence and XCorr are highlighted in green.

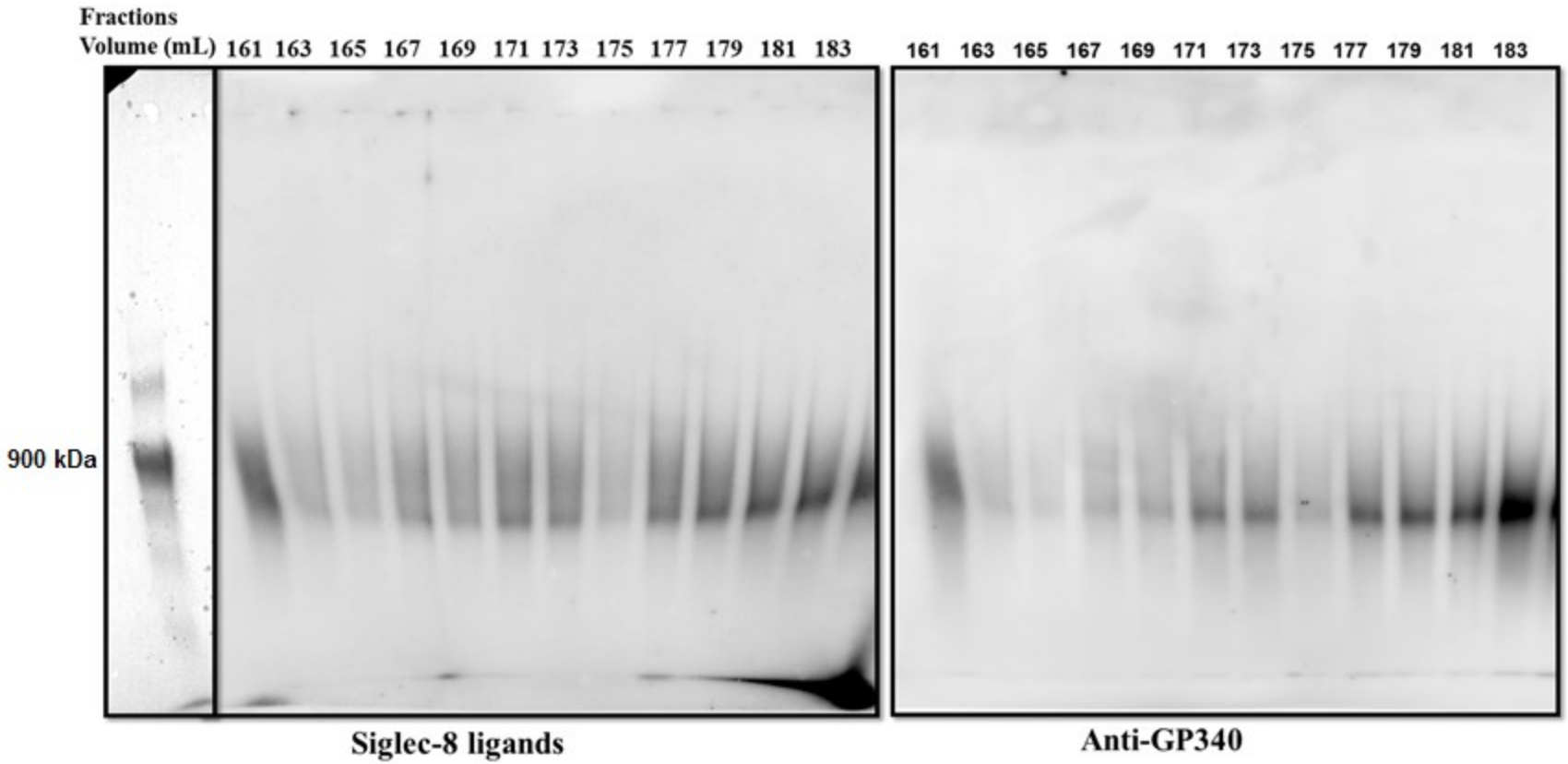


Figure 5.7: Comigration of nasal lavage S8-900k ligand with GP340. Equal volumes of fractions were run on composite gel electrophoresis and blotted with Siglec-8-Fc to detect ligands (Siglec-8 ligands) or anti-GP340 antibody. First lane is marker, 900 kDa band is the darkest band.

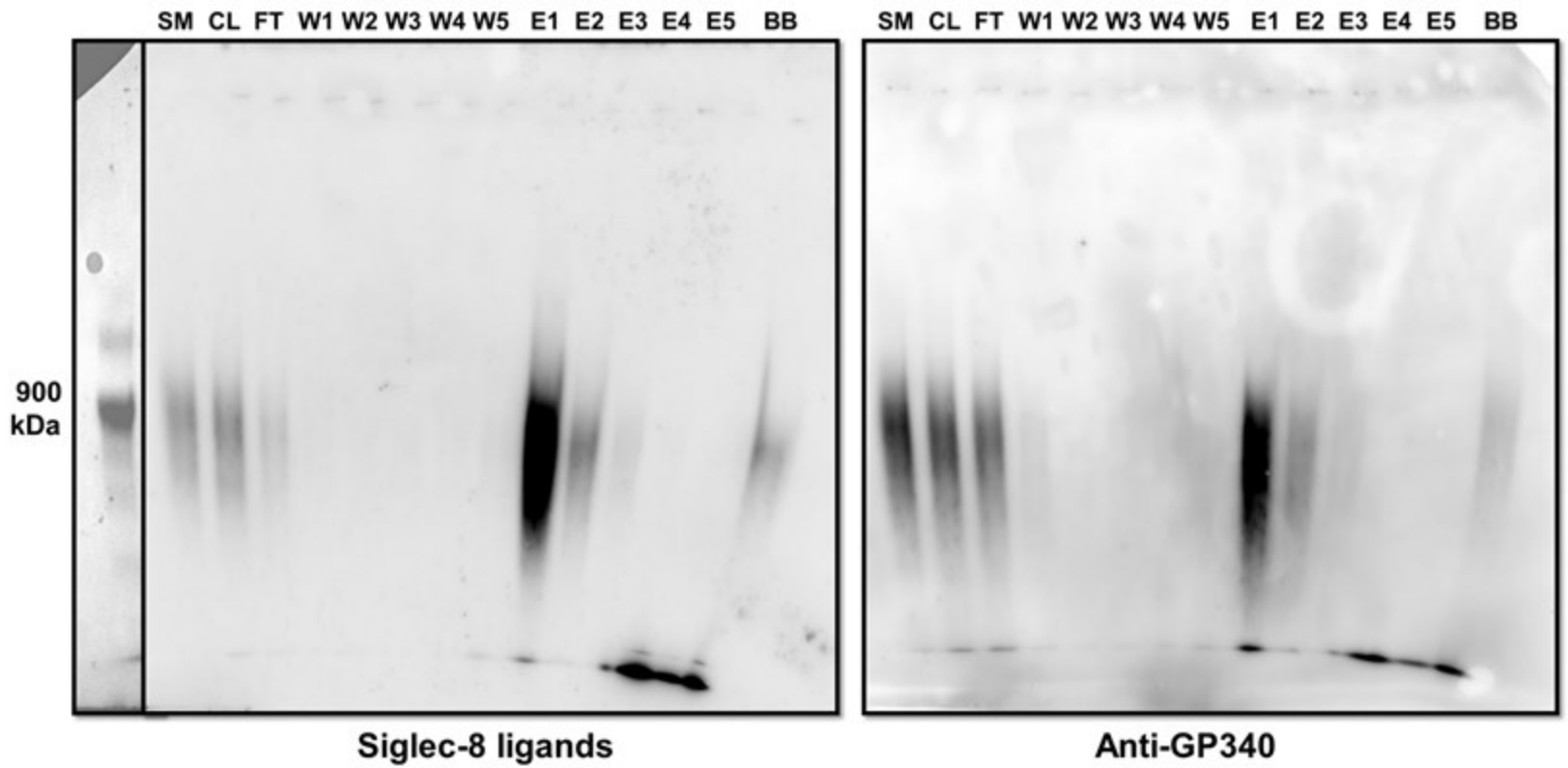


Figure 5.8: Co-precipitation of GP340 with Siglec-8 ligands. Fractions containing the major Siglec-8 ligand were combined (**SM**), pre-cleared with magnetic-Protein A/G beads (**CL**) then incubated with magnetic-Protein A/G beads with bound Siglec-8-Fc. Aliquots of flow through (**FT**), washes (**W1-5**), salt elutions (**E1-3**), glycine elutions (**E4-5**) and boiled beads (**BB**) were resolved and blotted with Siglec-8-Fc to detect ligands (Siglec-8 ligands) or anti-GP340 antibody.

In conclusion, Siglec-8 ligands are sialylated keratan sulfate chains that can be carried on different proteins based on the tissue where it is expressed. In cartilage, Siglec-8 ligands are present on aggrecan but in airway secretions it is present on GP340. It was not possible to definitively determine from what cell type the material secreted into the airway was coming. Nevertheless, based on the presence of Siglec-8-Fc overlay histochemistry, our hypothesis is that this material is secreted by serosal cells in the submucosa layer of the human airway. Further investigation is needed to define the biological function of these ligands purified from airway secretions. We showed in chapter 4, purified aggrecan carrying Siglec-8 ligands was able to increase eosinophil apoptosis above control levels compared to aggrecan that did not have Siglec-8 ligands. A similar approach should be taken with material purified from airway secretions.

Section 5.4 Summary and Future Directions

In conclusion, we identified sialylated keratan sulfate chains as Siglec-8 ligands in human airway tissue and airway secretion. Although the ligands are similar, the protein carriers are different, and many questions remain to be answered. What's the optimal length of keratan sulfate needed to induce biological function? Is the protein carrier necessary to exert a potent anti-inflammatory response? We can hypothesize that in a healthy patient the ligand is added unto the right protein carrier with the correct valency to exert a potent anti-inflammatory response. However, in a patient with inflammatory disease, the process may be accelerated to resolve inflammation resulting in secretion of protein with fewer Siglec-8 ligands and lower valency and decreased ability to resolve inflammation. To test this hypothesis, Siglec-8 ligands from normal and diseased patients need to be purified and

incubated with human eosinophils to determine their effectivity in inducing eosinophil apoptosis. Concurrently, techniques such surface plasmon resonance (SPR) can be used to determine the dissociation constant (Kd) of each purified ligand. Both purified ligands, may have similar biological activity and Kds which would suggest ligand production is not the issue in patients with inflammatory diseases. It is important to understand the underlying mechanism that causes inflammatory diseases to develop better therapeutics with reduced off target effects. These findings take us one step closer to understanding inflammatory diseases involving eosinophils, mast cells and basophils. Once endogenous Siglec-8 ligands are fully characterized and their role in controlling inflammation is further investigated this could lead to state of the art medical treatment for patients suffering from such diseases.

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influenza A viruses. *Am J Physiol Lung Cell Mol Physiol*. 2003;285(5):1066. Accessed Mar 26, 2018. doi: 10.1152/ajplung.00057.2003.

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CURRICULUM VITAE

Johns Hopkins University

Name: Anabel G. Alvarenga
Date of this Version: 03/26/2018

Education

PhD expected	2018	Johns Hopkins University, Baltimore, MD Doctoral Degree in Chemical Biology Chemical Biology Interface Program Mentor: Dr. Ronald L. Schnaar, PhD
M.S.	2013	Johns Hopkins University, Baltimore, MD Master in Science in Chemical Biology Chemical Biology Interface Program Mentor: Dr. Ronald L. Schnaar, PhD
Post-baccalaureate	2011	National Institute on Aging, Baltimore, MD Post-baccalaureate Intramural Research Training Award Program Mentor: Sebastian Fugmann, PhD
B.S.	2010	Florida International University, Miami, FL Deans List All Semesters Major: Chemistry Minors: Education
A.A.	2007	Miami Dade College, Honors Program, Miami, FL Deans List All Semesters Major: Chemistry

Research Experience

2012- 2018: Doctoral Research Dr. Ronald L. Schnaar

Department of Pharmacology and Molecular Science, Johns Hopkins School of Medicine Baltimore, MD

Human Siglec-8 ligands expressed in airway tissue and tissue secretions

- Identified sialylated Siglec-8 ligands in human airway
- Extracted and purified Siglec-8 ligands from tracheal tissue and identified aggrecan as the protein carrier for Siglec-8 ligands
- Identified sialylated keratan sulfate chains as the glycan ligands for Siglec-8
- Identified glycoprotein 340 (GP340) as the carrier of Siglec-8 ligands in human airway secretions
- Experience in protein extraction, western blotting and data analysis. Dissection and isolation of human airways to extract large molecular weight proteins. Primary cell culture, size exclusion chromatography and affinity chromatography. Site directed mutagenesis, DNA electrophoresis and PCR genetic engineering

- Collaborative effort between the laboratories of Dr. Ronald L. Schnaar, Dr. Bruce S. Bochner, Dr. Michael Tiemeyer, and Dr. James Paulson as part of the Lung Inflammatory Disease Program of Excellence in Glycobiology (LIDPEG)

Winter 2007: Rotation Research Dr. Valeria Culotta
Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- Studied the effect of toxic levels of manganese on amino acid uptake, amino acid transporter transcription and expression in yeast.

Fall 2007: Rotation Research Dr. Marc Ostermeier
Department of Chemical and Biomolecular Engineering, Johns Hopkins Whiting School of Engineering, Baltimore, MD

- Developed a series of genetically modified, split HhaI methyltransferases that specifically methylate an 18-base pair target site with low off target methylation.

Summer 2010-2011: Post-baccalaureate Dr. Sebastian Fugmann
Laboratory of Molecular Biology and Immunology, National Institute on Aging, Baltimore, MD

- Examined the biological role of the sea urchin Rag1/Rag2 complex in sea urchin biology by mutagenesis and transfection of designed plasmids to determine their function and similarity to human RAG complex.

Fall 2009-2010: Undergraduate Research Dr. Joong-Ho Moon
Department of Chemistry and Biochemistry, Florida International University, Miami, FL

- Synthesis of novel conjugated polymer nanoparticles (CPNs) for fluorescence labeling and biomolecule detection

Summer 2009: Summer Internship Dr. Amy E. Palmer
Department of Chemistry and Biochemistry, University of Colorado, Coulder, CO

- SMART Program at Colorado University, Boulder Campus
- Genetic manipulation of mammalian calcium sensors to decrease its affinity

Honors and Awards

Fall 2017 Scheinberg Travel Award
Department of Pharmacology, Johns Hopkins School of Medicine

Fall 2017 Chemical Biology Interface Program Travel Award
Department of Chemistry, Johns Hopkins University

Fall 2015 Society for Glycobiology Travel Award
San Francisco, CA

Fall 2015 FASEB MARC Poster/Platform Travel Award
Bethesda, MD

2013 Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship to Promote Diversity in Health-Related Research (Parent F31 - Diversity)
Parent Grant Funded by Lung Inflammatory Disease Program of Excellence in Glycoscience *(P01HL107151)*

2007-2010 Deans List Scholar
Florida International University, Miami, FL

2010 Outstanding Student Award from American Chemical Society Scholar Program
Florida International University, Miami, FL

2010: Received Outstanding Student Award from the Department of Chemistry at Florida International University.
Florida International University, Miami, FL

2009: ABRCMS Travel Award to attend the 9th Annual Biomedical Research Conference for Minority Students in Phoenix, AZ

2009: NSF Scholar Travel Award to attend the 13th Annual Green Chemistry & Engineering Conference in College Park, MD

2008-2010: American Chemical Society Scholar

2005-2007 Miami Dade College (Honors College)
Miami Dade College, Miami, FL

2007: Received The President's Volunteer Service Award for over 100 volunteer hours
Miami Dade College, Miami, FL

2007: Received Excellence Award from the Department of Chemistry, Physics and Earth Sciences
Miami Dade College, Miami, FL

2006: Nominated for the Service-Learning Student of the Year Award
Miami Dade College, Miami, FL

Publications

Siglec-8 and siglec-9 binding specificities and endogenous airway ligand distributions and properties. Glycobiology. 2017;27(7):657-668.

Expression of ligands for Siglec-8 and Siglec-9 in human airways and airway cells. J Allergy Clin Immunol. 2015 Mar;135:799-810

Research Presentations

March 10-11th, 2018: Poster "Glycoprotein 340 carries Siglec-8 ligands (sialylated keratan sulfate chains) in human airway secretions" Programs of Excellence in Glycosciences Inter PEG 2018, San Diego, CA

November 5-8th, 2017: Poster "Siglec-8 ligands in human airway secretions" Society for Glycobiology, Portland, OR (Abstract published in Glycobiology Volume 27 pg.1220-1221)

May 5th, 2017: Poster Presentation "Siglec-8 ligands in human trachea and airway secretions" NIH Glycosciences Day, Bethesda, MD

April 12-13th, 2017 Oral Presentation "Uniquely human Siglec-8 ligands" and Poster Presentation "Siglec-8 ligands in human airway secretions" Inter PEG 2017, Bethesda, MD

April 20-21st, 2016 Oral and Poster Presentation "Aggrecan carries Siglec-8 ligand in human lungs" Inter PEG 2016, Bethesda, MD

March 8th, 2016 Oral Presentation "Siglec-8, Siglec-9 and glycans that control human lung inflammation" Glycobiology Interest Group, Baltimore, MD

December 1-4th, 2015: Poster Presentation "Siglec-8 binds to sialylated keratan sulfate chains on aggrecan extracted from human airways" Society for Glycobiology, San Francisco, CA (Abstract published in Glycobiology Volume 25 pg. 11249)

May 28th, 2015: Poster Presentation "Siglec-8 and Siglec-9 in human airways" NIH Glycosciences Day, Bethesda, MD

April 15-16th, 2015: Oral and Poster Presentation "Siglec ligand expression is upregulated in inflamed human upper airways and human airway (Calu-3) cells" Inter PEG 2015, Bethesda, MD

March 26-28th, 2014: Poster Presentation "Extraction and Analysis of human lung counter-receptors for Siglec-8 and Siglec-9" Inter PEG 2014, Bethesda, MD

November 17-20th, 2013: Poster "Human lung counter-receptors for Siglec-8 and Siglec-9" Society for Glycobiology, St Petersburg, FL (Abstract published in Glycobiology Volume 23 pg.1385)

May 10th, 2013: Poster "Siglec-8 and Siglec-9 specificities for defined glycans and human lung counter-receptors" NIH Glycosciences Day, Bethesda, MD

April 10-12th, 2013: Poster "Siglec-8 and Siglec-9 specificities for defined glycans and human lung counter-receptors" Programs of Excellence in Glycosciences Inter PEG 2013, Cleveland, OH

November 11-14th, 2012: Poster "Comparison of Siglec-8 and Siglec-9 specificities for defined glycans and human lung counter-receptors" Joint Meeting of the American Society for Matrix Biology and the Society for Glycobiology, San Diego, CA (Abstract published in Glycobiology Volume 22 pg. 1543)

June 12th, 2012: Poster "Human lung counter-receptors for Siglec-8 and Siglec-9" NIH Glycosciences Day, Bethesda, MD

April 28th, 2012: Oral Presentation "The effect of Manganese on Amino Acid Transporters in Saccharomyces Cerevisiae" Fifth Annual Frontiers at the

Chemistry-Biology Interface Symposium held at the University of Pennsylvania, PA

May 6th, 2011: Poster Presentation "Comparative Analysis of Sea Urchin and Mammalian RAG1/RAG2 proteins" Post-baccalaureate Fellowship Poster Day National Institute on Aging, Baltimore, MD

August 3rd, 2010: Poster Presentation "Comparative Analysis of Sea Urchin and Mammalian RAG1/RAG2 proteins" Post-baccalaureate Fellowship Poster Day National Institute on Aging, Baltimore, MD

March 21-25th, 2010: Poster presentation of 2009 Summer Research and Biscayne Bay Science Club activities at the 2010 American Chemical Society Spring National Meeting held at San Francisco, CA