Expression of ligands for Siglec-8 and Siglec-9 in human airways and airway cells

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Background: Balanced activation and inhibition of the immune system ensures pathogen clearance while avoiding hyperinflammation. Siglecs, sialic acid–binding proteins found on subsets of immune cells, often inhibit inflammation: Siglec-8 on eosinophils and Siglec-9 on neutrophils engage sialoglycan ligands on airways to diminish ongoing inflammation. The identities of human siglec ligands and their expression during inflammation are largely unknown.

Objective: The histologic distribution, expression, and molecular characteristics of siglec ligands were explored in healthy and inflamed human upper airways and in a cellular model of airway inflammation.

Methods: Normal and chronically inflamed upper airway tissues were stained for siglec ligands. The ligands were extracted from normal and inflamed tissues and from human Calu-3 cells for quantitative analysis by means of siglec blotting and isolation by means of siglec capture.

Results: Siglec-8 ligands were expressed on a subpopulation of submucosal gland cells of human inferior turbinate, whereas Siglec-9 ligands were expressed more broadly (submucosal glands, epithelium, and connective tissue); both were significantly upregulated in patients with chronic rhinosinusitis. Human airway (Calu-3) cells expressed Siglec-9 ligands on mucin 5B (MUC5B) under inflammatory control through the nuclear factor κB pathway, and MUC5B carried sialoglycan ligands of Siglec-9 on human upper airway tissue.

Conclusion: Inflammation results in upregulation of immune-inhibitory Siglec-8 and Siglec-9 sialoglycan ligands on human airways. Siglec-9 ligands are upregulated through the nuclear factor κB pathway, resulting in their enhanced expression on MUC5B. Siglec sialoglycan ligand expression in inflamed cells and tissues may contribute to the control of airway inflammation. (J Allergy Clin Immunol 2015;135:799-810.)

Key words: *Chronic rhinosinusitis, inflammation, submucosal gland cells, siglecs, glycobiology, mucin 5B, nuclear factor κB*

An appropriate balance between positive and negative inflammatory signals is essential to the effective clearance of pathogens while avoiding autoimmune and hyperinflammatory syndromes.[1] Among the molecules that regulate the level of inflammation are members of the siglec family, cell-surface molecules on leukocytes that bind to sialylated glycans and translate glycan binding into changes in immune cell function.[2] There are 14 human siglecs, most of which are expressed on specific subsets of cells in the innate and adaptive immune systems. Siglecs are single-pass membrane proteins containing multiple extracellular immunoglobulin-like domains that mediate glycan binding and short cytoplasmic tails, most of which express immunoreceptor tyrosine-based inhibitor motifs (ITIMs) that negatively regulate the immune cells on which they are expressed. Human Siglec-8 is expressed on eosinophils, mast cells, and basophils, where ligation results in eosinophil apoptosis and inhibition of mast cell mediator release.[3-5] In models of allergic inflammation, mice lacking the paralog of Siglec-8 (Siglec-F) display exacerbated eosinophilic infiltration.[6,7] Siglec-9 is expressed on neutrophils, monocytes, dendritic cells, and natural killer cells. Its ligation on neutrophils results in their apoptotic death.[8] Mice lacking the homolog of Siglec-9 (Siglec-E) display exaggerated neutrophil recruitment to the lung in an acute inflammation model (aerosolized LPS).[9] The effects of Siglec-8 on eosinophils and Siglec-9 on neutrophils are more pronounced when the cells are activated,[8,10] implicating siglec function in the downregulation of ongoing inflammatory responses.

In the airways Siglec-8 and Siglec-9 might be involved in the regulation of allergic and nonallergic inflammation in diseases such as asthma and chronic obstructive pulmonary disease, which involve activated eosinophils and neutrophils. During an ongoing immune response, the activated inflammatory cells come into

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Abbreviations used

Benzyl-α-GalNAc:	Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside
CRS:	Chronic rhinosinusitis
ECL:	Enhanced chemiluminescence
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HRP:	Horseradish peroxidase
ITIM:	Immunoreceptor tyrosine-based inhibitor motif
MUC5B:	Mucin 5B
Neu5Ac2en:	N-acetyl-2,3-dehydro-2-deoxyneuraminic acid
NF-κB:	Nuclear factor κB
PBS:	Dulbecco PBS
PBSTr:	PBS supplemented with 0.1% Triton X-100
PBSTw:	PBS supplemented with 0.1% Tween 20
PVDF:	Polyvinylidene fluoride
siRNA:	Small interfering RNA

contact with the appropriate siglec ligands on airways, resulting in the inflammatory response subsiding. Knowing the molecular characteristics of human siglec ligands and control of their expression in human airways during inflammation might provide additional insight into this pathway of immune system regulation in health and disease.[2] We report here the tissue distribution and molecular characteristics of Siglec-8 and Siglec-9 ligands in normal and inflamed human upper airways.

Chronic rhinosinusitis (CRS) is a common disorder defined by inflammation of the upper airways and sinuses with symptoms persisting longer than 12 weeks, even with medical intervention.[11] Its cause is heterogeneous, might be related to allergies or infections, and is believed to result in hyperinflammation of different types.[12] Histologic data from its 2 major clinical subclassifications, with or without nasal polyps, reveal both eosinophilic and neutrophilic infiltration, which depends on the stage, cause, and geography of the affected populations.[12,13] As a clinically accessible source of human inflamed airway tissue, we focused the current study on the expression of Siglec-8 and Siglec-9 ligands in patients with CRS.

METHODS

Patients and biopsy specimens

Patients with CRS (see Table E1 in this article's Online Repository at www.jacionline.org) were recruited from the Allergy-Immunology and Otolaryngology Clinics of the Northwestern Medical Faculty Foundation and the Northwestern Sinus Center at the Northwestern Medical Faculty Foundation. Sinonasal tissues were obtained during routine functional endoscopic sinus surgery in patients with CRS. All patients met the criteria for CRS, as defined by the American Academy of Otolaryngology–Head and Neck Surgery's Chronic Rhinosinusitis Task Force. Unless otherwise indicated, CRS in this article refers to patients with CRS without nasal polyps. Patients with an established immunodeficiency, pregnancy, coagulation disorder, diagnosis of classic allergic fungal sinusitis, Samter triad, Churg-Strauss syndrome, or cystic fibrosis did not participate in the study. All subjects signed informed consent forms, and the protocol and consent forms governing procedures for the study were approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine.

Siglec-8 and Siglec-9 ligand histochemistry

Siglec ligands were detected by overlaying upper airway histologic sections with Siglec-8 or Siglec-9 chimeras consisting of the complete extracellular domain of each siglec fused to human Fc. Expression plasmids for the chimeras were cloned behind an elongation factor 1α promoter by replacing the green fluorescent protein sequence in pEF-GFP (Addgene, Cambridge, Mass) with the extracellular sequence of each siglec followed in frame with human IgG Fc. Plasmids were transiently transfected into HEK293T cells and soluble chimeras purified from the supernatant by means of affinity chromatography on Protein G Sepharose (HiTrap; GE Healthcare, Pittsburgh, Pa). Eluted purified protein was dialyzed against Dulbecco PBS (PBS) and quantified by using a bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, Ill).

Nasal tissue was dehydrated, paraffin embedded, and sectioned at 3 μm onto glass slides with a Leica RM2245 Cryostat (Leica Microsystems, Buffalo Grove, Ill). After xylene deparaffinization and rehydration, slides were immersed in 10 mmol/L sodium citrate, pH 6.0, at 95°C to 100°C for 15 minutes and then blocked with Dako enzyme block (Dako, Carpinteria, Calif) and Fc receptor blocker (Innovex, Richmond, Calif). Siglec-8-Fc (20 μg/mL) or Siglec-9-Fc (15 μg/mL) were precomplexed by means of incubation with 2 μg/mL alkaline phosphatase–conjugated goat anti-human Fcγ (Jackson Immunoresearch, West Grove, Pa) in ice-cold PBS supplemented with 0.1% Triton X-100 (PBSTr) for 30 minutes. Blocked slides were washed in PBSTr and overlaid with 200 μL of the precomplexed siglec for 2 hours at ambient temperature. After washing 3 times with PBSTr, bound siglec-Fc chimeras were detected by using Vector Red Substrate (SK-5100; Vector Laboratories, Burlingame, Calif). For detection of mucin 5B (MUC5B), slides were blocked and washed as above and then overlaid with 1:200 rabbit anti-MUC5B primary antibody (Santa Cruz Biotechnology, Dallas, Tex) in PBSTr for 2 hours at ambient temperature, followed by alkaline phosphatase–conjugated goat anti-rabbit IgG (1:1000 in PBSTr) for 1 hour. Binding was detected as above. As indicated, control slides were treated with 80 mU/mL of *Vibrio cholerae* sialidase[14] for 2.5 hours at 37°C and then washed with PBSTr before probing with siglec-Fc chimeras.

Images of stained sections were captured with a Nikon Eclipse 90i automated research microscope (Nikon Instruments, Melville, NY). Siglec ligand and MUC5B expression levels were quantified in randomly chosen well-demarcated submucosal glands containing at least 50 gland cells. The positively stained (red pixel) area was determined relative to the total area of the submucosal glands by using NIS-Elements image analysis software (Nikon).

Inferior turbinate protein extracts

Freshly obtained tissue specimens were weighed, and 1 mL of PBS supplemented with 0.05% Tween 20 and protease inhibitor cocktail (Sigma-Aldrich, St Louis, Mo) was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averil Park, NY) at setting 7 for 8 minutes at 4°C. After homogenization, the suspension was centrifuged at 2000*g* for 20 minutes at 4°C, and supernatants were stored at −80°C until analyzed.

Human tracheobronchial gland cell and Calu-3 cell culture, inflammatory mediators, and glycan inhibitors

Human tracheobronchial submucosal gland cells were prepared as described, with modifications.[15,16] The trachea and main bronchi from organ donors were opened and digested with 0.1% protease in Ham F12 medium at 4°C overnight to remove epithelial cells. Submucosal tissue was then dissected and incubated for 24 hours in 0.01% dispase/collagenase (Roche, Indianapolis, Ind) in Dulbecco modified Eagle medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin, and 50 μg/mL gentamicin. Gland cells were recovered mechanically, washed in the same medium, and then resuspended in 0.25% trypsin-EDTA (Life Technologies, Grand Island, NY) and triturated to dissociate the clumps of gland cells. Digestion was stopped by addition of FBS, and cells were collected and resuspended in gland cell medium comprised of a 1:1 mixture of Dulbecco modified Eagle medium and Ham F12 medium supplemented with 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 10 μL/mL transferrin, 0.5 μg/mL epinephrine, 6.5 ng/mL triiodothyronine, and 25 ng/mL human epidermal growth factor. Cells were plated on collagen-coated dishes and