Mice deficient in the *St3gal3* gene product α2,3 sialyltransferase (ST3Gal-III) exhibit enhanced allergic eosinophilic airway inflammation

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Background: Sialic acid–binding immunoglobulin-like lectin (Siglec)-F is a proapoptotic receptor on mouse eosinophils, but little is known about its natural tissue ligand.

Objective: We previously reported that the *St3gal3* gene product α2,3 sialyltransferase (ST3Gal-III) is required for constitutive Siglec-F lung ligand synthesis. We therefore hypothesized that attenuation of ST3Gal-III will decrease Siglec-F ligand levels and enhance allergic eosinophilic airway inflammation.

Methods: C57BL/6 wild-type mice and *St3gal3* heterozygous or homozygous deficient (*St3gal3*$^{+/-}$ and *St3gal3*$^{-/-}$) mice were used. Eosinophilic airway inflammation was induced through sensitization to ovalbumin (OVA) and repeated airway OVA challenge. Siglec-F human IgG_1 fusion protein (Siglec-F-Fc) was used to detect Siglec-F ligands. Lung tissue and bronchoalveolar lavage fluid (BALF) were analyzed for inflammation, as well as various cytokines and chemokines. Serum was analyzed for allergen-specific immunoglobulin levels.

Results: Western blotting with Siglec-F-Fc detected approximately 500-kDa and approximately 200-kDa candidate Siglec-F ligands that were less abundant in *St3gal3*$^{+/-}$ lung extracts and nearly absent in *St3gal3*$^{-/-}$ lung extracts. After OVA sensitization and challenge, Siglec-F ligands were increased in wild-type mouse lungs but less so in *St3gal3* mutants, whereas peribronchial and BALF eosinophil numbers were greater in the mutants, with the following rank order: *St3gal3*$^{-/-}$ ≥ *St3gal3*$^{+/-}$ > wild-type mice. Levels of various cytokines and chemokines in BALF were not significantly different among these 3 types of mice, although OVA-specific serum IgG_1 levels were increased in *St3gal3*$^{-/-}$ mice.

Conclusions: After OVA sensitization and challenge, *St3gal3*$^{+/-}$ and *St3gal3*$^{-/-}$ mice have more intense allergic eosinophilic airway inflammation and less sialylated Siglec-F ligands in their airways. One possible explanation for these findings is that levels of sialylated airway ligands for Siglec-F might be diminished in mice with attenuated levels of ST3Gal-III, resulting in a reduction in a natural proapoptotic pathway for controlling airway eosinophilia. (J Allergy Clin Immunol 2014;133:240-7.)

Key words: *Eosinophils, asthma, Siglec-F, 6′-sulfated sialyl Lewis X, 6′-sulfated sialyl N-acetyl-D-lactosamine, apoptosis, glycan ligands, lung, St3gal3*

Sialic acid–binding immunoglobulin-like lectins (Siglecs) are a family of single-pass, transmembrane, cell-surface receptors found predominantly on leukocytes.[1-3] Among them, Siglec-F in mice and Siglec-8 in human subjects are functional paralog inhibitory receptors expressed by eosinophils.[4] Because engagement of Siglec-F or Siglec-8 induces eosinophil apoptosis,[5-10] targeting these molecules might help to fill an unmet need in the treatment of various eosinophilic diseases.[11-13]

Previous studies have reported that both Siglec-F and Siglec-8 uniquely and preferentially recognize the same sialoside ligand 6′-sulfo sialyl Lewis X (6′-su-sLe^x; NeuAcα2-3[6-SO3]Galβ1-4[Fucα1-3]GlcNAc).[14,15] For Siglec-F, constitutive and cytokine-inducible lung epithelial ligands have been detected.[9,16-18] These ligands contain α2,3-linked terminal sialic acid residues and in mouse lung require the *St3gal3* gene product α2,3 sialyltransferase (ST3Gal-III) for their constitutive synthesis.[17,18] We therefore hypothesized that attenuation of ST3Gal-III will decrease Siglec-F airway ligand levels and selectively enhance eosinophilic airway inflammation. Using *St3gal3* heterozygous and homozygous deficient (*St3gal3*$^{+/-}$ and *St3gal3*$^{-/-}$) mice, we found that they exhibit a more intense allergic eosinophilic airway inflammatory reaction and produce less sialylated high molecular weight Siglec-F ligands in their airways.

METHODS

Glycan array analysis of Siglec-F and Siglec-8 ligand binding

The sugar-binding specificities of Siglec-F and Siglec-8 were reanalyzed on the printed glycan microarray, which was developed by the Consortium for Functional Glycomics. Previously reported analyses were carried out with an earlier array format, the microtiter plate array (Version 2.0), which only had 184 biotinylated glycosides and controls immobilized in streptavidin-coated microtiter plates.[14,15] Version 4.1 of the printed glycan microarray was composed of 465 defined, amino-functionalized glycan structures printed as replicates of 6 on an NHS-activated microscope slide. Siglec-F human IgG_1 Fc chimera

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Abbreviations used

BALF: Bronchoalveolar lavage fluid
MBP: Major basic protein
OVA: Ovalbumin
Siglec: Sialic acid–binding immunoglobulin-like lectin
Siglec-8-Fc: Siglec-8 human IgG_1 Fc chimera
Siglec-F-Fc: Siglec-F human IgG_1 Fc chimera
ST3Gal-III: *St3gal3* gene product α2,3 sialyltransferase
6'-su-sLacNAc: 6′-Sulfated sialyl N-acetyl-D-lactosamine
6′-su-sLe^x: 6′-Sulfated sialyl Lewis X
WT: Wild-type

(Siglec-F-Fc; R&D Systems, Minneapolis, Minn) or Siglec-8 human IgG_1 Fc chimera (Siglec-8-Fc)[19] were applied in 70 μL of Tris-buffered saline at several different concentrations to the microarray under a cover slip and incubated at room temperature in a humidified chamber. After 1 hour, the cover slip was carefully removed, and the slides were washed in buffer to remove excess protein. The protein-glycan complexes were detected with an Alexa Fluor 488–conjugated goat anti-human IgG (Invitrogen, Carlsbad, Calif) by using the same procedure. After removing the cover slip, the slides were washed, air dried, and scanned with a PerkinElmer ProScanArray fluorescence scanner (PerkinElmer, Waltham, Mass) set at an excitation of 485 nm and an emission of 535 nm, and the data were processed with Imagene Software (BioDiscovery, El Segundo, Calif). After removing the high and low measurements of each set of replicates, the averages and SDs of the remaining 4 values were used to determine the average relative fluorescence units. Data are reported as a histogram of relative fluorescence units versus glycan structure.

Animals, ovalbumin sensitization, and airway challenge

Mice used in these experiments were 8 to 10 weeks old and included C57BL/6 mice (wild-type [WT]) and St3gal3 heterozygous deficient (*St3gal3*$^{+/-}$) and homozygous deficient (*St3gal3*$^{-/-}$) mice. Breeding sets of *St3gal3*$^{-/-}$ mice were kindly provided by Dr Jamey Marth (University of California, San Diego, Calif), as previously described, but because of breeding limitations of these mutant mice, all *St3gal3*$^{-/-}$ mice used in these studies came from the approximately 25% of *St3gal3*$^{+/-}$ × *St3gal3*$^{+/-}$ mice offspring that were homozygous null.[17,20] Mice were intraperitoneally sensitized with 500 μg of ovalbumin (OVA; Sigma-Aldrich, St Louis, Mo) in 1 mg of alum on days 1 and 8 and then challenged intranasally with 200 μg of OVA on days 17, 19, and 21 to induce allergic airway inflammation. All mice were killed 24 hours after final challenge. Control mice were injected and challenged with PBS. All procedures performed on mice were in accordance with the National Institutes of Health's guidelines for humane treatment and approved by the Johns Hopkins University Institutional Animal Use and Care Committees.

Siglec-F ligand expression

Expression levels of Siglec-F binding protein in lung-derived samples were determined by means of Western blotting with a 4% to 15% polyacrylamide gel (Bio-Rad Laboratories, Hercules, Calif). Protein extracted from lung tissue homogenates was generated as previously described.[17] After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) and incubated overnight with 4% BSA (Sigma-Aldrich). The membranes were incubated with Siglec-F-Fc (0.5 μg/mL, 1 hour, room temperature; R&D Systems). After washing, the membranes were incubated with horseradish peroxidase–linked polyclonal sheep anti-human IgG polyclonal antibody (45 minutes, room temperature; GE Healthcare, Piscataway, NJ). Bands were visualized with the ECL Western blotting detection system (GE Healthcare UK Ltd, Buckinghamshire, United Kingdom). Some of these samples were pretreated with sialidase (*Clostridium perfringens*, 1.6 mU/mL, 2 hours, 37°C; Sigma-Aldrich) to confirm sialidase sensitivity.

Tissue distribution of potential Siglec-F ligands was studied by means of immunohistochemistry, as described previously.[17] Briefly, the 10-μm cryostat sections of lung and tracheal tissues were incubated with Siglec-F-Fc (1 μg/mL, 1 hour, 37°C) and detected by using an alkaline phosphatase imaging method. Isotype-matched humanized IgG_1 mAb (omalizumab, 1 μg/mL, 1 hour, 37°C; Genentech, South San Francisco, Calif) was used as a negative control. In some experiments samples were pretreated with sialidase (10 mU/mL, 24 hours, 37°C; Sigma-Aldrich).

Bronchoalveolar lavage and peripheral blood cell counting

Mouse lungs were lavaged by using 5 repeated instillations of 0.6 mL of saline each through the tracheal cannula. The first 1.2 mL of bronchoalveolar lavage fluid (BALF) was centrifuged, and the supernatant was used for the measurements of various cytokines and chemokines. Cells were counted with a hemocytometer. Differential cell counts in BALF and blood sample smears were performed under standard light microscopy after Diff-Quik staining.

Peribronchial eosinophil enumeration

Major basic protein (MBP) immunohistochemistry was performed on 5-μm paraffin sections, as previously described.[9] Rat anti-mouse MBP mAb was obtained from the laboratory of Dr James J. Lee (Mayo Clinic, Scottsdale, Ariz). Numbers of MBP$^+$ cells in the peribronchial area were counted by a blinded observer to quantitate the severity of eosinophilic airway inflammation, as previously described.[16] The number of cells staining positive was counted in 10 randomly selected peribronchial regions to a depth of 30 μm beneath the epithelium. Results are expressed as the number of cells staining positive for MBP per 150- to 200-μm-sized internal diameter bronchiole.

Measurement of immunoglobulin levels

Serum total IgE (1:100 dilution; detection range, 0.156-20 μg/mL) and OVA-specific IgE (1.56-200 ng/mL), IgG_1 (1:20,000 dilution, 31.3-4000 μg/mL), and IgG_{2a} (1:100 dilution, 0.156-20 μg/mL) levels were determined by using commercial ELISA kits, per the manufacturer's instructions (R&D Systems).

Measurement of cytokines and chemokines

IL-3 (0.2-21,632 pg/mL), IL-4 (2.1-9,372 pg/mL), IL-5 (0.3-13,315 pg/mL), IL-10 (1.0-12,066 pg/mL), IL-17A (0.8-43,337 pg/mL), GM-CSF (5.6-3,401 pg/mL), IFN-γ (1.2-30,164 pg/mL), and TNF-α (1.4-59,626 pg/mL) concentrations in BALF were determined with a Bio-Plex kit (Bio-Rad Laboratories), according to the manufacturer's instructions. IL-13 (1.5-500 pg/mL), IL-33 (14.3-2000 pg/mL), eotaxin (CCL11; 3-1000 pg/mL), and eotaxin-2 (CCL24; 1:4 dilution, 0.0156-12 ng/mL) concentrations were determined by using commercial ELISA kits, according to the manufacturer's instructions (R&D Systems).

Statistical analysis

Data are shown as means ± SEMs or individual dot plots with medians. Statistical significance between groups was evaluated by means of ANOVA and the Tukey multiple comparison test. *P* values of less than .05 were considered statistically significant.

RESULTS

Siglec-F recognizes 6′-su-sLe^x and a closely related nonfucosylated structure, 6′-sulfated sialyl N-acetyl-D-lactosamine

Previous studies have shown that both Siglec-8 and Siglec-F uniquely and specifically recognize the same glycan structure