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Anti-Siglec-F Antibody Reduces Allergen-Induced Eosinophilic Inflammation and Airway Remodeling[1]

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Siglec-F is a sialic acid-binding Ig superfamily receptor that is highly expressed on eosinophils. We have investigated whether administration of an anti-Siglec-F Ab to OVA-challenged wild-type mice would reduce levels of eosinophilic inflammation and levels of airway remodeling. Mice sensitized to OVA and challenged repetitively with OVA for 1 mo who were administered an anti-Siglec-F Ab had significantly reduced levels of peribronchial eosinophilic inflammation and significantly reduced levels of subepithelial fibrosis as assessed by either trichrome staining or lung collagen levels. The anti-Siglec-F Ab reduced the number of bone marrow, blood, and tissue eosinophils, suggesting that the anti-Siglec-F Ab was reducing the production of eosinophils. Administration of a $F(ab')_2$ fragment of an anti-Siglec-F Ab also significantly reduced levels of eosinophilic inflammation in the lung and blood. FACS analysis demonstrated increased numbers of apoptotic cells (annexin V^+/$CCR3^+$ bronchoalveolar lavage and bone marrow cells) in anti-Siglec-F Ab-treated mice challenged with OVA. The anti-Siglec-F Ab significantly reduced the number of peribronchial major basic protein$^+$/TGF-β^+ cells, suggesting that reduced levels of eosinophil-derived TGF-β in anti-Siglec-F Ab-treated mice contributed to reduced levels of peribronchial fibrosis. Administration of the anti-Siglec-F Ab modestly reduced levels of periodic acid-Schiff-positive mucus cells and the thickness of the smooth muscle layer. Overall, these studies suggest that administration of an anti-Siglec-F Ab can significantly reduce levels of allergen-induced eosinophilic airway inflammation and features of airway remodeling, in particular subepithelial fibrosis, by reducing the production of eosinophils and increasing the number of apoptotic eosinophils in lung and bone marrow. ***The Journal of Immunology,*** **2009, 183: 5333–5341.**

The recruitment of bone marrow-derived eosinophils from the circulation into the airway is a prominent feature of allergic asthma. Important signals mediating the trafficking of eosinophils from the bone marrow to the airway include cytokines such as IL-5 that induce eosinophil proliferation (1), endothelial-induced adhesion molecules such as VCAM-1, P-selectin, and ICAM-1 that localize eosinophils to inflamed tissue sites (2, 3), and CC chemokines such as eotaxin-1 that induce the directed migration of eosinophils in the extracellular matrix (4). Once in the airway the eosinophil may contribute to the proinflammatory response by releasing preformed cytoplasmic granule mediators (i.e., major basic protein (MBP)[3]), newly generated lipid mediators (i.e., leukotriene C4 (LTC_4)), and also transcribe an array of proinflammatory cytokines (1). Although increased levels of eosinophils and eosinophil-derived mediators have been detected in humans with asthma (5, 6), the role of the eosinophil in the pathogenesis of asthma is controversial (7, 8) in part because of results from recent clinical studies with anti-IL-5 that have not demonstrated either reductions in late phase responses to inhalation allergen challenge in mild asthmatics (9), nor improved symptoms and pulmonary function in moderate asthmatics (10). However, in contrast to these studies in which targeting IL-5 in asthma has been ineffective in improving symptoms or lung function, anti-IL-5 has demonstrated effectiveness in reducing levels of airway remodeling in asthma (11). For example, targeting IL-5 reduces both the number of eosinophils in the airway as well as features of airway remodeling in mouse models of allergen-induced airway remodeling (12), and in humans with asthma (11, 13).

One of the strategies to limit the generation of eosinophils is to target receptors expressed by eosinophils that might mediate the resolution of eosinophilic inflammation. One such candidate receptor expressed by eosinophils that mediates the resolution of eosinophilic inflammation is Siglec-F (14, 15). Siglec-F belongs to the CD33-related Siglec family, which is a subclass of Siglecs defined by their mutual sequence similarity (share ~50–80% sequence similarity) and clustered gene localization (chromosome 7 in mice, chromosome 19q in humans) (14). Siglec-F is a transmembrane receptor comprising a ligand-binding V-set domain, three C-2 domains, a transmembrane domain, and a cytoplasmic ITIM domain (16). Of the eight mouse Siglecs and 11 human Siglecs that have been identified, eosinophils are known to express significant levels of Siglec-F in mice (15, 17, 18), as well as its functionally convergent ortholog Siglec-8 in human eosinophils (19–21). Siglec-F, like other CD33-related Siglecs, has a tyrosine-based signal transduction motif in its cytoplasmic tail, including a canonical ITIM motif, which is known to be involved in inhibitory signaling pathways in the immune system (22, 23). Support for inhibitory signaling by the cytoplasmic domain of CD33-related Siglecs has come from studies in which Abs were used to cross-link Siglec cell surface receptors. These studies demonstrated that Ab

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[3] Abbreviations used in this paper: MBP, major basic protein; BAL, bronchoalveolar lavage; IEX, ion exchange chromatography; PAS, periodic acid-Schiff.

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cross-linking of several CD33-related Siglecs results in inhibition of cellular-activation signals, arrest of proliferation, or induction of apoptosis (24–26). Studies of mice deficient in Siglec-F have demonstrated that they have increased levels of allergen-induced airway eosinophilic inflammation, as well as delayed resolution of airway eosinophilic inflammation following acute allergen challenge (15). These studies in Siglec-F-deficient mice suggest that Siglec-F normally functions to down-regulate eosinophilic inflammation. Therefore, in this study we have examined whether administration of an anti-Siglec-F Ab could reduce levels of airway eosinophilic inflammation and, importantly, levels of airway remodeling in a mouse model of chronic allergen-induced airway remodeling.

Materials and Methods

Effect of anti-Siglec-F Ab on levels of lung, bone marrow, and blood eosinophils

Eight to 10-wk-old BALB/c mice (16 mice/group) (The Jackson Laboratory) were immunized s.c. on days 0, 7, 14, and 21 with 25 μg of OVA (grade V; Sigma-Aldrich) adsorbed to 1 mg of alum (Sigma-Aldrich) in 200 μl of normal saline as previously described (27). Intranasal OVA challenges were administered on days 27, 29, and 31 under isoflurane (Vedco) anesthesia. Age- and sex-matched control mice were sensitized but not challenged with OVA. Mice were sacrificed 24 h after the final OVA challenge and blood, bone marrow, bronchoalveolar lavage (BAL) fluid, and lungs were analyzed (27). Peripheral blood was collected from mice by cardiac puncture into EDTA-containing tubes. Erythrocytes were lysed using a 1/10 solution of 100 mM potassium carbonate/1.5 M ammonium chloride. The remaining cells were resuspended in 1 ml of PBS. BAL fluid was collected by lavaging the lung with 1 ml of PBS via a tracheal catheter (27). Bone marrow cells were flushed from femurs with 1 ml of PBS, centrifuged, and resuspended in 1 ml of PBS. Total leukocytes were counted using a hemocytometer. To perform differential cell counts, 200 μl of resuspended BAL cells, peripheral-blood leukocytes, or 20 μl of bone marrow cell suspensions was cytospun onto microscope slides and air-dried (27). Slides were stained with Wright-Giemsa and differential cell counts were performed under a light microscope (27). Lungs from the different experimental groups were processed as a batch for either histologic staining or immunostaining under identical conditions as previously described (12). Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification (×20), gain, camera position, and background illumination. The quantitative histologic and image analyses of all coded slides were performed by research associates blinded to the coding of all the slides. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

Administration of an anti-Siglec-F or control Ab

Different groups of mice ($n = 16$ mice/group) were administered 10 μg of either a rat anti-mouse Siglec-F IgG2a Ab (provided by the Varki Lab) (15) or a control rat IgG2a isotype-matched Ab (BD Biosciences) in 100 μl of PBS by i.p. injection 1 h before each of the three OVA challenges on days 27, 29, and 31. In pilot studies following i.p. injection, the half-life of the anti-Siglec-F Ab in mice was 3–4 days. The dosing regimen of the anti-Siglec-F Ab ensured that serum levels of the anti-Siglec-F Ab were maintained at >4 μg/ml. In pilot studies we demonstrated such levels of anti-Siglec-F Ab were sufficient to bind all eosinophil Siglec-F in blood and bone marrow (data not shown).

Generation and administration of $F(ab')_2$ fragments of anti-Siglec-F Ab

The rat anti-mouse Siglec-F mAbs or the rat IgG2a isotype control mAbs were cleaved individually with pepsin (Fischer Scientific) to produce $F(ab')_2$ and Fc fragments. Since rat IgGs are quite resistant to pepsin digestion, the Abs were initially individually dialyzed against 100 mM Na-acetate buffer (pH 4.0) for 4 h at 4°C before pepsin digestion. Pepsin was equilibrated in Na-acetate buffer (pH 4.0) and added to the individual Abs at a final enzyme to protein ratio of 5% (w/w). The pepsin reaction with Ab was stopped by raising the pH to 7.8 with 2 M Tris base. The Ab pepsin digest was then dialyzed against 25 mM Tris buffer (pH 7.8) for 12 h at 4°C using 50-kDa molecular mass cut-off dialysis tubing (Spectrum Laboratories) to exclude smaller-sized Fc fragments (~26 kDa) and pepsin enzymes (~35 kDa) while retaining undigested Abs (~150 kDa) and $F(ab')_2$ fragments (~105 kDa) to be further purified by ion exchange chromatography (IEX) (28). The $F(ab')_2$ fragments generated by pepsin digestion were purified using a Mono Q 5/50 ion exchange column (GE Healthcare) equilibrated in 25 mM Tris buffer (pH 7.8). All aqueous solutions were prepared with distilled water and were filtered (using 0.22-μm filter), degassed, and equilibrated to 4°C before loading the column. The samples were eluted using a continuous gradient of increasing salt concentration created by a start buffer (25 mM Tris) and elution buffer (25 mM Tris with 1 M NaCl). Flow-through, as well as 500-μl fractions (18–30) corresponding to the peaks in the elution profile, was collected and analyzed on a 10% SDS-polyacrylamide gel under nonreducing and reducing conditions. Fractions 24–30 (containing the $F(ab')_2$ fragments) were pooled together and dialyzed against PBS for in vivo administration. Ten micrograms of $F(ab')_2$ (derived from anti-Siglec-F Ab or control Ab) were administered to mice in 100 μl of PBS by i.p. injection 1 h before each of the three OVA challenges on days 27, 29, and 31. Levels of eosinophilia were quantitated in BAL and blood when mice were sacrificed 24 h after the final OVA challenge on day 31.

Mouse model of OVA-induced airway remodeling

In these experiments, BALB/c mice ($n = 16$/group) were immunized with OVA s.c. as described above, and after receiving intranasal OVA challenges on days 27, 29, and 31 they had the intranasal OVA challenges repeated twice a week for 1 mo (12). Mice were sacrificed 24 h after the final OVA challenge and their BAL fluid and lungs processed as described above. The anti-Siglec-F or control Ab was administered by i.p. injection 1 h before each of the intranasal OVA challenges.

Peribronchial trichrome staining

Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma-Aldrich) to preserve the pulmonary architecture. The area of peribronchial trichrome staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS; Leica Microsystems) attached to an image analysis system (Image-Pro Plus; Media Cybernetics) as previously described (12). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150–200 μm in internal diameter.

Lung collagen assay

The amount of lung collagen was measured as previously described in this laboratory (12) with a collagen assay kit that uses a dye reagent that selectively binds to the [Gly-X-Y]n tripeptide sequence of mammalian collagens (Biocolor). In all experiments, a collagen standard was used to calibrate the assay.

Peribronchial eosinophils and mast cells

Lung sections were processed for MBP immunohistochemistry as described above, using an anti-mouse MBP Ab (provided by Dr. James Lee, Mayo Clinic, Scottsdale, AZ). The numbers of individual cells staining positive for MBP in the peribronchial space were counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive for MBP per bronchiole with 150–200 μm of internal diameter. At least 10 bronchioles were counted in each slide. Similar image analysis methods were used to quantitate mast cells in lung sections stained with chloroacetate esterase and lightly counterstained with hematoxylin as described (29).

Peribronchial TGF-$\beta 1^+$ cells

The numbers of peribronchial TGF-$\beta 1^+$ cells were quantitated by immunohistochemistry using an anti-TGF-$\beta 1$ Ab as previously described in this laboratory (30). In addition to quantitating the total number of TGF-$\beta 1^+$ cells, we also quantitated the number of TGF-$\beta 1^+$ cells that were MBP$^+$ by staining sequential thin sections of lung with an anti-MBP Ab and an anti-TGF-$\beta 1^+$ Ab as previously described in this laboratory (30).

Peribronchial apoptotic cells

The number of peribronchial apoptotic cells was assessed by ultrastructure (cell shrinkage, nuclear chromatin condensation) and TUNEL staining as previously described (12, 31). For TUNEL staining, digoxigenin-labeled nucleotides and TdT were added to 5-μm sections of lung to label the free 3′ DNA ends of apoptotic cells (ApopTag Plus peroxidase in situ apoptosis detection kit; Chemicon). An anti-digoxigenin Ab conjugated to peroxidase was used to label the incorporated digoxigenin-labeled nucleotides and was developed with the substrate supplied by the manufacturer. The sections were counterstained with hematoxylin. The number of apoptotic cells was counted in 10 randomly selected peribronchial regions in each slide using a light microscope attached to the image analysis system as described above.

FACS analysis of BAL fluid and bone marrow for cells expressing annexin V and CCR3

To determine whether the anti-Siglec-F Ab influenced the number of apoptotic eosinophils in the lung or bone marrow, we performed FACS analysis of both BAL cells and bone marrow cells derived from BALB/c mice challenged with OVA (the acute OVA protocol described above) and pretreated with either an anti-Siglec-F Ab or a species- and isotype-matched Ab ($n = 3$ mice/group). BAL or bone marrow cells were initially incubated for 15 min with Fc block (rat anti-mouse CD16/CD32; BD Pharmingen) and then stained for 30 min with the combination of FITC-conjugated anti-CCR3 (R&D Systems) and PE-conjugated annexin V (eBioscience). After being washed, cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences) as previously described (32). Further analyses were performed with FlowJo software (Tree Star).

Peribronchial smooth muscle layer thickness

The thickness of the airway smooth muscle layer was measured with an image analysis system as previously described (12). In brief, the thickness of the smooth muscle layer (the transverse diameter) was measured from the innermost aspect to the outermost aspect of the smooth muscle layer. The smooth muscle layer thickness in at least 10 bronchioles of similar size (150–200 μm) was counted on each slide.

Airway mucus expression

To quantitate the level of mucus expression in the airway, the number of periodic acid-Schiff (PAS)-positive and PAS-negative epithelial cells in individual bronchioles were counted as previously described in this laboratory (12). At least ten bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

Effect of anti-Siglec-F Ab on airway hyperreactivity

Airway hyperresponsiveness to Mch was assessed 24 h after the final chronic OVA in intubated and ventilated mice (flexiVent ventilator; Scireq) as previously described in this laboratory (30). The frequency-independent airway resistance was determined in mice exposed to nebulized PBS and methacholine at 24 mg/ml (30).

Statistical analysis

Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test followed by posttesting using Dunn's multiple comparison of means. All results are presented as means ± SEM. A statistical software package (GraphPad Prism) was used for the analysis. Values of p of <0.05 were considered statistically significant.

Results

Effect of anti-Siglec-F Ab on acute eosinophilic lung inflammation as well as levels of blood and bone marrow eosinophils

Acute OVA challenge significantly increased the numbers of lung ($p = 0.02$; acute OVA vs no OVA) (Fig. 1*A*), bone marrow ($p = 0.02$; acute OVA vs no OVA) (Fig. 1*C*), and blood eosinophils ($p = 0.05$; acute OVA vs no OVA) (Fig. 1*B*). Administration of 10 μg of an anti-Siglec-F Ab significantly reduced the number of lung eosinophils ($p = 0.01$; acute OVA plus control Ab vs acute OVA plus anti-Siglec-F Ab) (Fig. 1*A*), bone marrow eosinophils ($p = 0.05$; acute OVA plus control Ab vs acute OVA plus anti-Siglec-F Ab) (Fig. 1*C*), and blood eosinophils ($p = 0.05$; acute OVA plus control Ab vs acute OVA plus anti-Siglec-F Ab) (Fig. 1*B*). We also examined whether higher doses of the anti-Siglec-F Ab (i.e., 20 or 50 μg) would reduce levels of lung eosinophilic inflammation to a greater extent compared with the 10-μg dose we used in the aforementioned studies. Neither the 20-μg nor the 50-μg anti-Siglec-F Ab dose was more effective than the 10-μg dose in reducing levels of lung eosinophilic inflammation (data not shown).

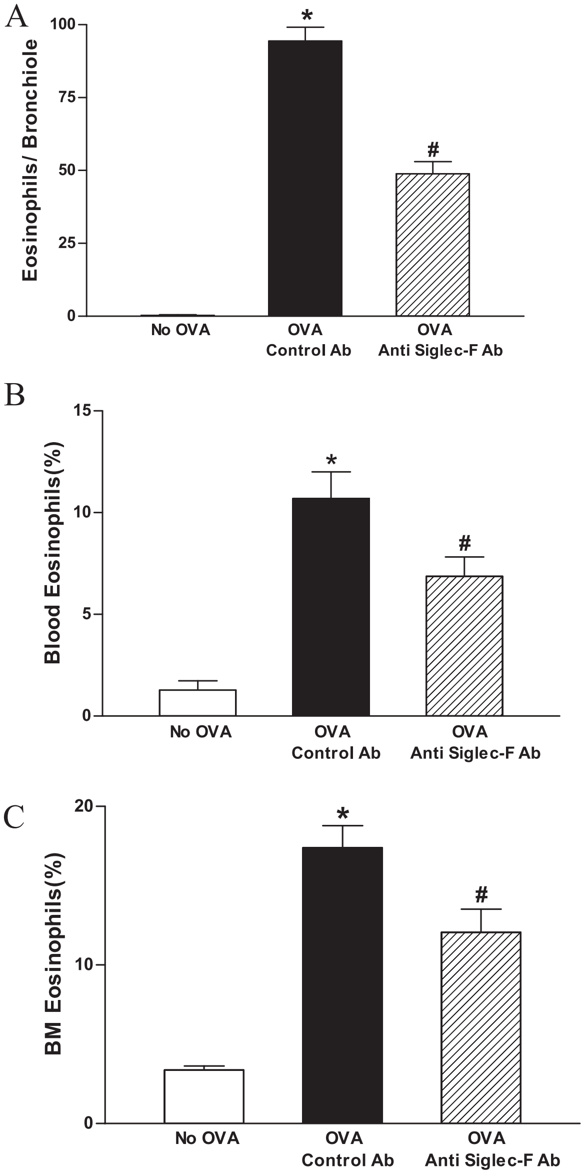

FIGURE 1. Effect of anti-Siglec-F Ab on levels of blood, bone marrow, and lung tissue eosinophils following acute OVA challenge. Different groups of mice received either an anti-Siglec-F Ab or a control Ab i.p. before each acute OVA challenge. Non-OVA-challenged mice served as a control. Eosinophils in blood and bone marrow were quantitated in cytospin slides stained with Wright-Giemsa, whereas eosinophils in lung sections were quantitated by immunostaining with an anti-MBP Ab. Acute OVA challenge significantly increased the number of lung (*, $p = 0.02$) (*A*), blood (*, $p = 0.05$) (*B*), and bone marrow eosinophils (*, $p = 0.02$) (*C*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced levels of eosinophils in lung (*A*) (#, $p = 0.01$), blood (*B*) (#, $p = 0.05$), and bone marrow (*C*) (#, $p = 0.05$) of acute OVA-challenged mice (acute OVA plus anti-Siglec-F Ab vs acute OVA plus control Ab) ($n = 16$ mice/group).

Effect of $F(ab')_2$ fragments of anti-Siglec-F Ab on lung eosinophilic inflammation

To determine whether either the $F(ab')_2$ or the Fc region of the anti-Siglec-F Ab was mediating the inhibition of eosinophilic inflammation in the lung we generated $F(ab')_2$ fragments of the anti-Siglec-F Ab. To obtain $F(ab')_2$ fragments, pepsin was used to

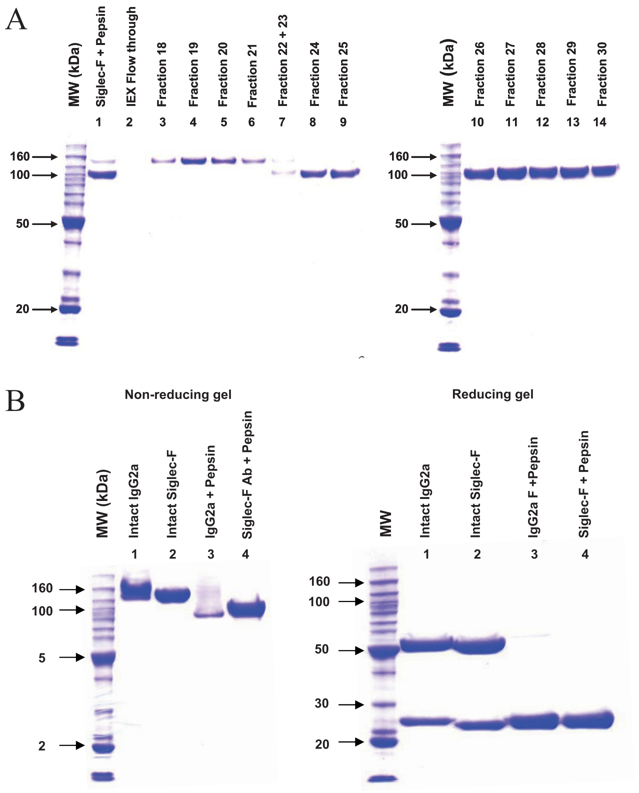

FIGURE 2. Generation of $F(ab')_2$ fragment of anti-Siglec-F Ab. *A*, Pepsin digest of Siglec-F Ab: IEX gradient fractions run on 10% SDS-polyacrylamide gel. To generate $F(ab')_2$ fragments of the anti-Siglec-F Ab, the anti-Siglec-F Ab (or a control rat IgG2a) were incubated with pepsin, dialysed with a 50-kDa dialysis membrane (to excluded smaller sized Fc fragments of ~26 kDa and pepsin enzymes of ~35 kDa), and subjected to IEX. Depicted are the IEX fractions run on a 10% SDS-polyacrylamide gel under nonreducing conditions: *lane 1*, Siglec-F Ab digest products before subjecting to IEX; *lane 2*, IEX flow-through; *lanes 3–6*, fractions 18–21 containing undigested Siglec-F Ab; *lane 7*, fraction 22–23 containing mixture of undigested Siglec-F Ab and Siglec-F Ab $F(ab')_2$fragment; *lanes 8–14*, fractions 24–30 containing $F(ab')_2$ fragments of Siglec-F Ab. The undigested Siglec-F Ab is ~150 kDa, whereas the $F(ab')_2$ fragment is ~105 kDa. *B*, SDS-PAGE 10% nonreducing and reducing gel. Depicted are the undigested control IgG2a Ab (*lane 1*), the undigested anti-Siglec-F Ab (*lane 2*), IEX fractions 24–30 containing the pepsin-cleaved $F(ab')_2$ fragment of the control IgG2a Ab (*lane 3*), and IEX fractions 24–30 containing the pepsin-cleaved $F(ab')_2$ fragment of the anti-Siglec-F Ab (*lane 4*) run on a 10% SDS-polyacrylamide gel under nonreducing as well as reducing conditions. The undigested Siglec-F Ab (and control Ab) are ~150 kDa, whereas the $F(ab')_2$ fragment is ~105 kDa under nonreducing conditions. Under reducing conditions the undigested Siglec-F Ab (and control Ab) is ~50 kDa, whereas the $F(ab')_2$ fragment is ~26 kDa.

cleave either the anti-Siglec-F Ab or a control rat IgG2a. Following dialysis of the pepsin Ab digest with a 50-kDa dialysis membrane (excluded smaller sized Fc fragments of ~26 kDa and pepsin enzymes of ~35 kDa), IEX was utilized to separate the $F(ab')_2$ fragments of the anti-Siglec-F Ab (~105 kDa) from the undigested intact anti-Siglec-F Ab (~150 kDa), each of which appeared as single bands on 10% SDS-polyacrylamide gel under nonreducing conditions (Fig. 2). Under nonreducing conditions, the IEX purified fractions (24 – 30) of $F(ab')_2$ fragments of either control rat IgG2a or anti-Siglec-F Ab appeared as a single band of ~105 kDa (Fig. 2*A*), while under reducing conditions the $F(ab')_2$ fragments dissociated into four fragments of ~26 kDa (Fig. 2*B*) that appear as a single band.

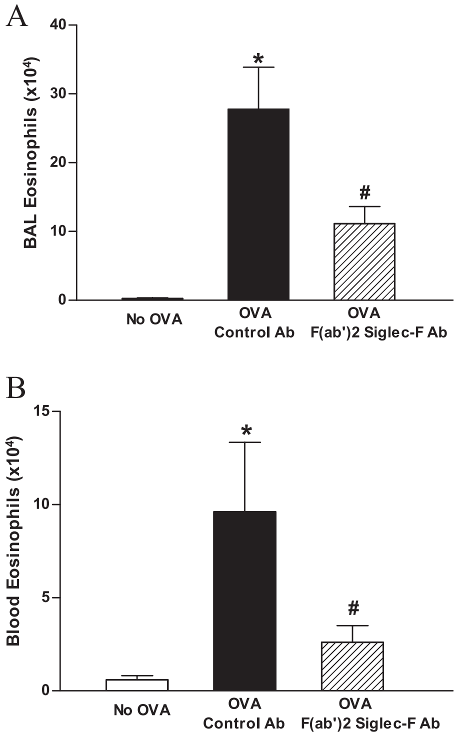

FIGURE 3. Effect of $F(ab')_2$ fragment of anti-Siglec-F Ab on levels of eosinophils following acute OVA challenge. Different groups of mice received either a $F(ab')_2$ fragment of an anti-Siglec-F Ab or a control Ab, i.p. before each acute OVA challenge. Non-OVA-challenged mice served as a control. Eosinophils in BAL fluid (*A*) and blood (*B*) were quantitated in cytospin slides stained with Wright-Giemsa. Acute OVA challenge significantly increased the number of BAL eosinophils (*, $p = 0.002$) (*A*) and blood eosinophils (*, $p = 0.004$) (Fig. 3*B*) compared with non-OVA-challenged mice. Administration of a $F(ab')_2$ fragment of the anti-Siglec-F Ab significantly reduced levels of eosinophils in BAL fluid (*A*) (#, $p = 0.01$) and blood (*B*) (#, $p = 0.05$) of acute OVA-challenged mice (acute OVA plus $F(ab')_2$ of anti-Siglec-F Ab vs acute OVA plus $F(ab')_2$ of control Ab) ($n = 8$ mice/group).

Administration of the $F(ab')_2$ fragments of the anti-Siglec-F Ab to OVA-challenged mice inhibited levels of lung eosinophilic inflammation (Fig. 3*A*) ($p = 0.02$; vs OVA plus control Ab) and blood eosinophils (Fig. 3*B*) ($p = 0.05$; vs OVA plus control Ab) as effectively as the intact anti-Siglec-F Ab.

Effect of anti-Siglec-F Ab on chronic eosinophilic lung inflammation

Immunostained lung sections from chronic OVA-challenged mice showed a significant increase in the number of peribronchial cells expressing MBP, as well as a significant increase in the number of peribronchial cells expressing Siglec-F. Chronic OVA challenge induced a significant increase in the number of BAL eosinophils ($p = 0.001$; OVA vs no OVA) (Fig. 4*A*), as well as a significant increase in the number of peribronchial eosinophils ($p = 0.001$; OVA vs no OVA) (Fig. 4*B*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced the number of BAL eosinophils in chronic OVA-challenged mice (14.5 ± 2.8 vs 6.2 ± 1.2 BAL eosinophils $\times 10^4$; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.003$) (Fig. 4*A*). The anti-Siglec-F Ab also significantly reduced the number of peribronchial eosinophils in chronic OVA-challenged mice (81.3 ± 6.2 vs 39.2 ± 3.0 eosinophils/bronchus; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.002$) (Fig. 4*B*).

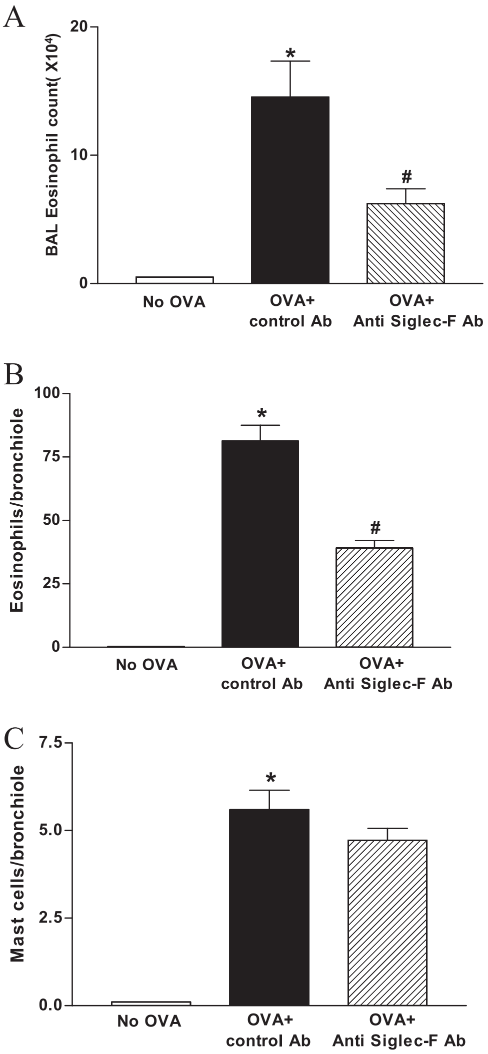

FIGURE 4. Effect of anti-Siglec-F Ab on chronic eosinophilic lung inflammation and levels of peribronchial mast cells. Different groups of mice received either an anti-Siglec-F Ab or a control Ab i.p before each chronic OVA challenge. Non-OVA-challenged mice served as a control. Eosinophils in BAL fluid were quantitated in cytospin slides stained with Wright-Giemsa, whereas eosinophils in lung sections were quantitated by immunostaining with an anti-MBP Ab. Peribronchial mast cells were detected by chloroacetate esterase staining. Chronic OVA challenge induced a significant increase in the number of BAL eosinophils (*, $p = 0.001$) (*A*), peribronchial eosinophils (*, $p = 0.001$) (*B*), and peribronchial mast cells (*, $p = 0.002$) (*C*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced levels of eosinophils in BAL fluid (*A*) (#, $p = 0.003$) and lung (*B*) (#, $p = 0.002$) of chronic OVA-challenged mice (chronic OVA plus anti-Siglec-F Ab vs chronic OVA plus control Ab), but did not influence the number of peribronchial mast cells (p = NS) (*C*) ($n = 16$ mice/group).

Effect of anti-Siglec-F Ab on number of peribronchial mast cells

Chronic OVA challenge also induced a significant increase in the number of peribronchial mast cells compared with non-OVA-challenged mice (5.6 ± 0.6 vs 0 ± 0 mast cells/bronchus; OVA vs no OVA; $p = 0.002$) (Fig. 4*C*). In contrast to its effect on reducing the number of peribronchial eosinophils, the anti-Siglec-F Ab did not significantly reduce the number of peribronchial mast cells in chronic OVA-challenged mice (5.6 ± 0.6 vs 4.7 ± 0.3 mast cells/bronchus; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; p = NS) (Fig. 4*C*).

Effect of the anti-Siglec-F Ab on levels of cells other than eosinophils and mast cells

Anti-Siglec-F Ab administration to OVA-challenged mice did not significantly reduce levels of BAL lymphocytes ($p = 0.64$), BAL neutrophils ($p = 0.53$), or BAL macrophages ($p = 0.15$) as compared with OVA-challenged mice treated with a control Ab (data not shown). Administration of the anti-Siglec-F Ab to OVA-challenged mice reduced peripheral blood eosinophil levels ($p = 0.05$) (Fig. 1*B*) but did not significantly reduce total white blood cell levels ($p = 0.15$) (data not shown).

Effect of anti-Siglec-F Ab on levels of peribronchial fibrosis

Chronic OVA challenge induced a significant increase in levels of peribronchial fibrosis as assessed by either levels of peribronchial trichrome staining ($p = 0.006$; OVA vs no OVA) (Fig. 5*A*) or increases in lung collagen ($p = 0.01$; OVA vs no OVA) (Fig. 5*B*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced the amount of peribronchial trichrome staining in chronic OVA-challenged mice (1.3 ± 0.2 vs 0.6 ± 1.0 $\mu m^2/\mu m$ peribronchial trichrome stained area; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.01$) (Fig. 5*A*), as well as the amount of lung collagen (1717 ± 77 vs 1308 ± 129 μg collagen/lung; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.05$) (Fig. 5*B*), compared with chronic OVA-challenged mice administered a control Ab.

Effect of anti-Siglec-F Ab on number of peribronchial TGF-$\beta 1^+$ cells

As TGF-β has been implicated in peribronchial fibrosis in asthma (33–35), we examined whether administration of the anti-Siglec-F Ab reduced the number of TGF-$\beta 1^+$ peribronchial cells. Chronic OVA challenge induced a significant increase in the number of TGF-$\beta 1^+$ peribronchial cells ($p = 0.0001$; OVA vs no OVA) (Fig. 5*C*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced the number of TGF-$\beta 1^+$ peribronchial cells in chronic OVA-challenged mice (83.3 ± 2.8 vs 54.6 ± 1.7 TGF-$\beta 1^+$ cells/bronchus; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.001$) (Fig. 5*C*) compared with chronic OVA-challenged mice administered a control Ab.

We also investigated whether administration of anti-Siglec-F reduced the number of eosinophils expressing TGF-$\beta 1^+$ cells by quantitating the number of cells expressing MBP and TGF-β1 as previously described in this laboratory (12). These studies demonstrated that chronic OVA challenge significantly increased the number of MBP$^+$/TGF-$\beta 1^+$ peribronchial cells ($p = 0.0001$; OVA vs no OVA) (Fig. 5*D*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced the number of MBP$^+$/TGF-$\beta 1^+$ peribronchial cells in chronic OVA-challenged mice ($p = 0.001$) (Fig. 5*D*) compared with chronic OVA-challenged mice administered a control Ab. Approximately two-thirds of all of the TGF-$\beta 1^+$ peribronchial cells were MBP$^+$.

Effect of anti-Siglec-F Ab on number of peribronchial apoptotic cells

As cross-linking Siglec-F (15) or Siglec-8 (25) in vitro induces eosinophil apoptosis, we examined whether the reduced number of lung eosinophils in anti-Siglec-F Ab-treated mice were associated with increased eosinophil apoptosis. Chronic OVA challenge induced a small but significant increase in the number of TUNEL$^+$ peribronchial cells ($p = 0.002$; OVA vs no OVA) (Fig. 6) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab induced a small but significant increase in the number of TUNEL$^+$ peribronchial cells in chronic OVA-challenged mice

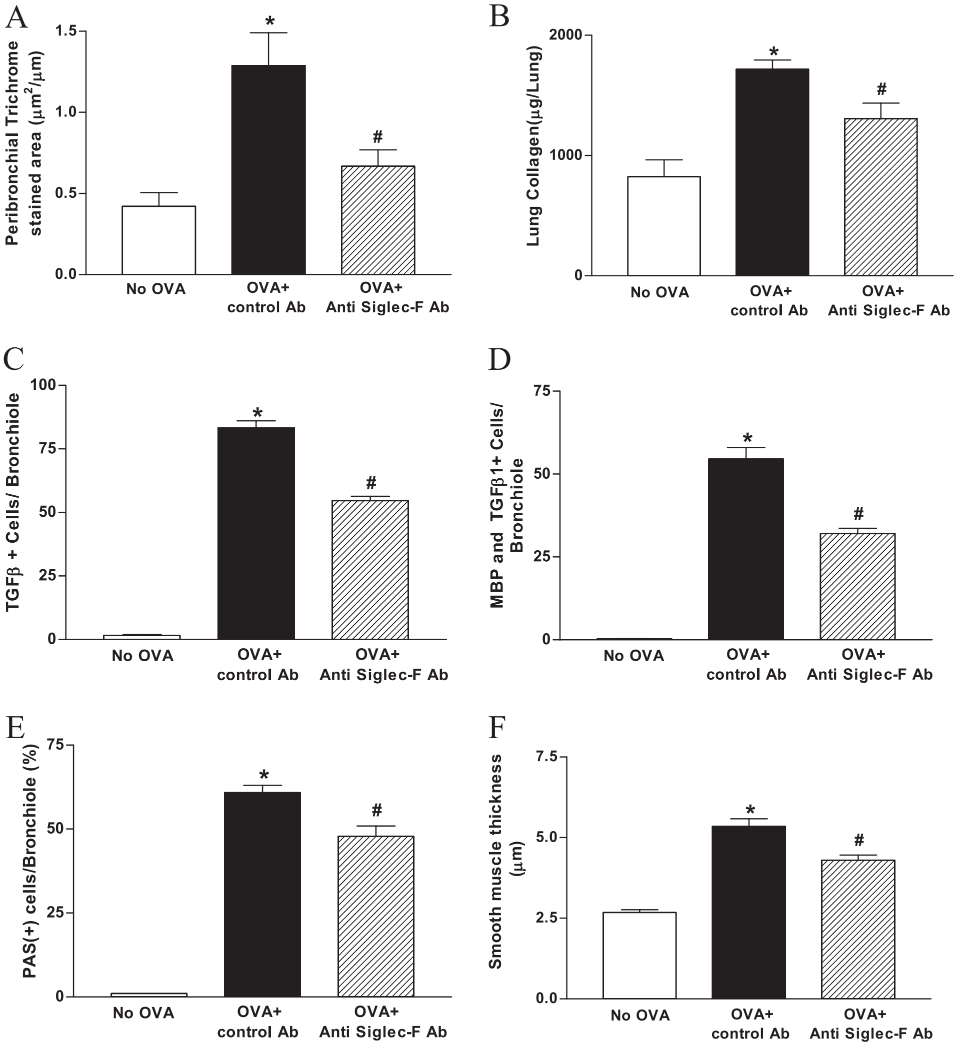

FIGURE 5. Effect of anti-Siglec-F Ab on levels of peribronchial fibrosis and remodeling. Different groups of mice received either an anti-Siglec-F Ab or a control Ab i.p. before each chronic OVA challenge. Non-OVA-challenged mice served as a control. Levels of peribronchial fibrosis were quantitated in lung sections stained with trichrome by image analysis (*A*), as well as by assaying collagen levels in lungs (*B*). The numbers of peribronchial TGF-$\beta1^+$ cells were quantitated by image analysis in lung sections immunostained with an anti-TGF-$\beta1$ Ab (*C*), as well as in lung sections stained with both an anti-TGF-$\beta1$ and anti-MBP Ab (*D*). Levels of mucus expression were quantitated in lung sections stained with PAS (*E*) and the thickness of the smooth muscle layer was quantitated in lung sections by immunohistochemistry (*F*). Chronic OVA challenge induced a significant increase in levels of peribronchial trichrome staining (*, $p = 0.006$) (*A*), lung collagen (*, $p = 0.01$) (*B*), the number of TGF-$\beta1^+$ peribronchial cells (*, $p = 0.0001$) (*C*), as well as the number of MBP$^+$/TGF-$\beta1^+$ peribronchial cells (*, $p = 0.0001$) (*D*) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced levels of peribronchial trichrome staining (*A*) (#, $p = 0.01$), lung collagen levels (*B*) (#, $p = 0.05$), the number of peribronchial TGF-β^+ cells (#, $p = 0.001$) (*C*), the number of MBP$^+$/TGF-$\beta1^+$ peribronchial cells (#, $p = 0.001$) (*D*), the number of PAS$^+$ cells (#, $p = 0.04$) (*E*), and the thickness of the smooth muscle layer (#, $p = 0.04$) (*F*) of chronic OVA-challenged mice (chronic OVA F(ab$'$)$_2$ plus anti-Siglec-F Ab vs chronic OVA plus control Ab) ($n = 16$ mice/group).

(3.7 ± 0.3 vs 5.7 ± 0.5 TUNEL$^+$ cells/bronchus; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.003$) (Fig. 6) compared with chronic OVA-challenged mice administered a control Ab.

Effect of an anti-Siglec-F Ab on the number of annexin V$^+$/CCR3$^+$ cells in BAL fluid and bone marrow

We performed FACS analysis of both BAL cells as well as bone marrow cells using annexin V to detect apoptotic cells, and CCR3 to detect eosinophils. OVA-challenged mice pretreated in vivo with an anti-Siglec-F Ab had a significant increase in the percentage of BAL annexin V$^+$/CCR3$^+$ cells (13.0 ± 1.5 vs 6.6 ± 0.1% apoptotic BAL eosinophils; OVA plus anti-Siglec-F Ab vs OVA plus control Ab; $p < 0.05$) (Fig. 7*A*), as well as a significant increase in the absolute number of annexin V$^+$/CCR3$^+$ cells compared with OVA-challenged mice pretreated with a control Ab (19.4 ± 2.2 vs 9.9 ± 1.9 × 10^2 apoptotic BAL eosinophils; $p < 0.05$) (Fig. 7*B*).

Similarly, OVA-challenged mice pretreated in vivo with an anti-Siglec-F Ab had a significant increase in the percentage of bone marrow annexin V$^+$/CCR3$^+$ cells (38.6 ± 0.3 vs 22.9 ± 1.4% apoptotic bone marrow eosinophils; OVA plus anti-Siglec-F Ab vs OVA plus control Ab; $p < 0.05$) (Fig. 7*A*), as well as a significant increase in the absolute number of bone marrow annexin V$^+$/CCR3$^+$ cells compared with OVA-challenged mice pretreated with a control Ab ($p < 0.05$) (Fig. 7*B*).

Effect of anti-Siglec-F Ab on airway mucus expression

Chronic OVA challenge induced a significant increase in the number of PAS$^+$ mucus cells ($p = 0.002$; OVA vs no OVA) (Fig. 5*E*)

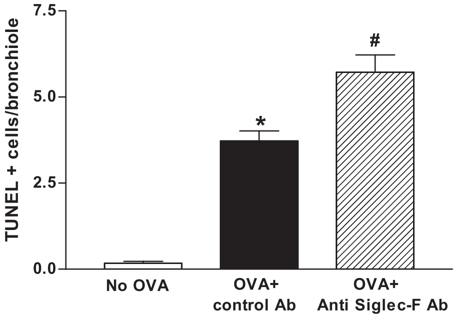

FIGURE 6. Effect of anti-Siglec-F Ab on the number of peribronchial apoptotic cells. Different groups of mice received either an anti-Siglec-F Ab or a control Ab i.p. before each chronic OVA challenge. Non-OVA-challenged mice served as a control. The number of peribronchial apoptotic cells were quantitated in lung sections by TUNEL staining. Administration of an anti-Siglec-F Ab induced a statistically significant but small increase in the number of TUNEL$^+$ cells (#, $p = 0.003$) of chronic OVA-challenged mice (chronic OVA plus anti-Siglec-F Ab vs chronic OVA plus control Ab) (n = 16 mice/group).

Table I. *Airway resistance in anti-Siglec-F Ab-treated mice*[a]

Mouse Group (n = 12)	Airway Resistance	
	Diluent	MCh
No OVA	0.6 ± 0.02	2.1 ± 0.2
Chronic OVA + control Ab	0.6 ± 0.02	6.1 ± 0.7
Chronic OVA + anti-Siglec-F Ab	0.7 ± 0.08	6.4 ± 1.3

[a] Airway resistance ($cmH_2O \cdot s/ml$) was measured in different groups of intubated and ventilated mice following nebulization of either PBS diluent or MCh (24 mg/ml). Chronic OVA challenge induced a significant increase in airway resistance (no OVA vs Chronic OVA + control Ab; $p = 0.01$, 24 mg/ml MCh). The increase in airway resistance following chronic OVA challenge was not statistically different in mice pretreated with an anti-Siglec-F or control Ab (chronic OVA + anti-Siglec-F Ab vs chronic OVA + control Ab; p = NS, 24 mg/ml MCh).

compared with non-OVA-challenged mice. Although the airways of anti-Siglec-F Ab-treated mice had a statistically significant reduction in mucus expression (60.8 ± 2.2 vs 47.8 ± 3.1% PAS$^+$ cells/bronchus; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.04$) (Fig. 5*E*), the reduction in mucus expression induced by the anti-Siglec-F Ab was not as marked as the reduction in peribronchial fibrosis induced by the anti-Siglec-F Ab (Fig. 5*A*).

Effect of anti-Siglec-F Ab on peribronchial smooth muscle layer thickness and airway hyperresponsiveness

Chronic OVA challenge induced a significant increase in the thickness of the peribronchial smooth muscle layer ($p = 0.002$; OVA vs no OVA) (Fig. 5*F*), as well as a significant increase in levels of airway responsiveness to Mch ($p = 0.01$; chronic OVA vs no OVA) (Table I) compared with non-OVA-challenged mice. The anti-Siglec-F Ab-treated mice had a modest but statistically significant reduction in the thickness of the peribronchial smooth muscle layer (5.3 ± 0.2 vs 4.3 ± 0.2 μm smooth muscle layer thickness; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; $p = 0.04$) (Fig. 5*F*). However, the anti-Siglec-F Ab did not reduce airway responsiveness to Mch (Table I).

Discussion

In this study we have demonstrated that administration of an anti-Siglec-F Ab to mice chronically challenged with allergen significantly reduced levels of eosinophilic airway inflammation, as well as levels of airway remodeling (in particular peribronchial fibrosis). The mechanism by which administration of the anti-Siglec-F Ab reduces airway eosinophilic inflammation could theoretically

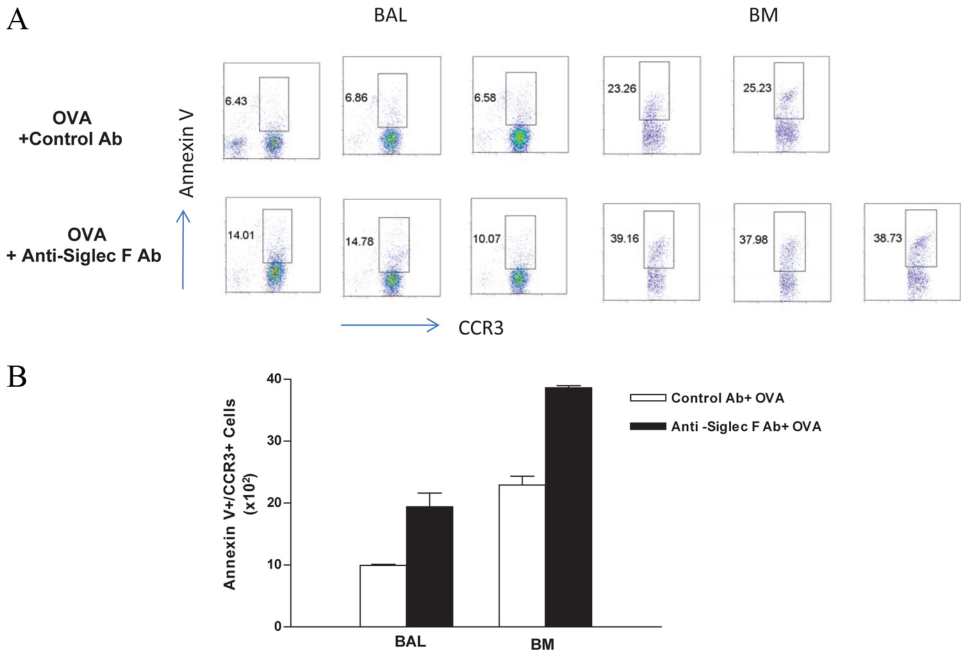

FIGURE 7. Effect of anti-Siglec-F Ab on the number of annexin V$^+$/CCR3$^+$ cells in BAL fluid and bone marrow. FACS analysis was performed on BAL cells as well as bone marrow (BM) cells using annexin V to detect apoptotic cells and CCR3 to detect eosinophils (n = 3 mice/group; one of the mouse bone marrows was not processed). *A*, Percentage of annexin V$^+$/CCR3$^+$ cells in BAL fluid and BM of individual mice challenged with OVA and pretreated with either an anti-Siglec-F or control Ab. *B*, Absolute number of annexin V$^+$/CCR3$^+$ cells in BAL fluid and BM of individual mice challenged with OVA and pretreated with either an anti-Siglec-F or control Ab. OVA-challenged mice pretreated in vivo with an anti-Siglec-F Ab had a significant increase in the percentage of BAL ($p < 0.05$) and BM ($p < 0.05$) annexin V$^+$/CCR3+ cells (*A*), as well as a significant increase in the absolute number of BAL ($p <$ 0.05) and BM ($p < 0.05$) annexin V$^+$/CCR3$^+$ cells compared with OVA-challenged mice pretreated with a control Ab (OVA plus anti-Siglec-F Ab vs OVA plus control Ab) (*B*).

be mediated by decreased trafficking of eosinophils into the lung and/or increased clearance of eosinophils from the lung. Evidence in support of the anti-Siglec-F Ab decreasing trafficking of eosinophils into the lung is derived from our studies demonstrating that anti-Siglec-F Ab-treated mice had significantly reduced numbers of blood and bone marrow eosinophils following allergen challenge, suggesting that reduced numbers of circulating eosinophils were available to traffick into the lung. Additionally, we demonstrated that OVA-challenged mice pretreated with an anti-Siglec-F Ab had increased numbers of annexin V^+/$CCR3^+$ cells in the bone marrow and BAL fluid, suggesting that the anti-Siglec-F Ab was inducing apoptosis of eosinophils in the bone marrow as well as BAL compartments. These effects of the anti-Siglec-F Ab would both decrease the number of eosinophils in the bone marrow released into the circulation as well as increase the numbers of apoptotic cells in the lung. Cross-linking Siglec receptors on purified populations of eosinophils in vitro induces an apoptotic response, as has been demonstrated with Siglec-F in murine eosinophils (15), as well as with Siglec-8 in human eosinophils (25). Our study is also the first study to utilize $F(ab')_2$ fragments of the anti-Siglec-F Ab to investigate the in vivo mechanism by which the anti-Siglec-F Ab reduces levels of eosinophilic inflammation. As administration of the $F(ab')_2$ fragments of the anti-Siglec-F Ab inhibited levels of lung eosinophilic inflammation as effectively as the intact anti-Siglec-F Ab, it is unlikely that eosinophils tagged with the anti-Siglec-F Ab are being more rapidly cleared by the Fc portion of the anti-Siglec-F Ab, or via complement activation.

In addition to reducing levels of eosinophilic inflammation in the airway, the anti-Siglec-F Ab also significantly reduced levels of allergen-induced airway remodeling in particular levels of peribronchial fibrosis. As previous murine (33, 34) and human studies (11, 35) have provided evidence of an important role for eosinophil expression of TGF-β1 in contributing to airway remodeling, we examined whether administration of the anti-Siglec-F Ab was associated with reduced numbers of peribronchial cells expressing TGF-β1. These studies demonstrated that the anti-Siglec-F Ab not only significantly reduced the number of peribronchial eosinophils but also reduced the number of peribronchial eosinophils expressing TGF-β1, suggesting that reductions in TGF-β1 from eosinophils could significantly contribute to the observed decrease in peribronchial fibrosis in anti-Siglec-F Ab-treated mice. The importance of TGF-β1 to airway remodeling in the chronic OVA model is supported by studies in anti-TGF-β-treated mice (34) as well as in SMAD-3-deficient mice that are unable to signal through TGF-β (33), both of which have significant reductions in levels of peribronchial fibrosis when subjected to chronic OVA allergen challenge. In contrast to the significant reductions in levels of peribronchial fibrosis induced by the anti-Siglec-F Ab, the anti-Siglec-F Ab had a statistically significant but more modest effect on reducing levels of mucus expression and the thickness of the smooth muscle layer. The lack of effect of the anti-Siglec-F Ab on reducing levels of airway responsiveness in mice subjected to chronic OVA challenge is similar to our previous observation in Siglec-F-deficient mice challenged acutely with OVA whose levels of airway responsiveness do not differ from wild-type mice (15).

In contrast to reducing levels of peribronchial eosinophilic inflammation, the anti-Siglec-F Ab did not reduce levels of peribronchial mast cell accumulation. As studies using bone marrow-derived murine mast cells demonstrate that these cells do not express Siglec-F (17), the differing responses of eosinophils and mast cells to anti-Siglec-F could be explained on the basis of eosinophils, but not mast cells, expressing Siglec-F. The anti-Siglec-F Ab also did not reduce levels of BAL lymphocytes, neutrophils, or macrophages.

In summary, in this study we have demonstrated that administration of an anti-Siglec-F Ab to allergen-challenged mice significantly reduces levels of eosinophilic airway inflammation and airway remodeling in particular subepithelial fibrosis. Although anti-Siglec-F Ab administration significantly reduces levels of eosinophilic inflammation in different tissue compartments including the airway, the effect is incomplete. The mechanism by which the anti-Siglec-F Ab reduces levels of eosinophilic inflammation in the airway likely involves apoptotic effects in the bone marrow to decrease the numbers of eosinophils available in the circulation to traffick into the lung, as well as increased clearance of eosinophils from the lung by means of increased apoptosis. This study also demonstrated that $F(ab')_2$ fragments of the anti-Siglec-F Ab inhibited levels of eosinophilic inflammation in the lung as effectively as did the intact anti-Siglec-F Ab, suggesting that eosinophils tagged with the anti-Siglec-F Ab are not being cleared through a Fc receptor-mediated mechanism. Recent studies have also demonstrated that administration of anti-Siglec-F Abs reduce levels of eosinophilic inflammation in the gastrointestinal tract (31), as well as in the blood and jejunum in an IL-5 transgenic mouse model of the hypereosinophilic syndrome (36). The reduced number of eosinophils expressing TGF-β1 in the lung in anti-Siglec-F Ab-treated mice is likely to significantly contribute to reduced levels of peribronchial fibrosis. Further studies targeting the functional similar human paralog of Siglec-F (i.e., Siglec-8) are needed to determine whether similar effects will be noted in human subjects with asthma and airway remodeling.

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Disclosures

The authors have no financial conflicts of interest.

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