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*J Immunol* 2009; 183:5333-5341; Prepublished online 25 September 2009;
doi: 10.4049/jimmunol.0801421
http://www.jimmunol.org/content/183/8/5333

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

Dae Jin Song,*† Jae Youn Cho,* Sang Yeub Lee,*† Marina Miller,* Peter Rosenthal,* Pejman Soroosh,* Michael Croft,* Mai Zhang,* Ajit Varki,* and David H. Broide2*

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+/CD33+) 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.

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Received for publication May 2, 2008. Accepted for publication August 7, 2009.

Abstract

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 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 (LTC4)), 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...
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 mouse 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 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 and were 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 cytosponed onto microscope slides and air-dried (27). Slides were stained with Wright-Giemsma 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 (derived from 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 are 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 from 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 collagen (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 bronchiolus 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-β1+ cells

The numbers of peribronchial TGF-β1+ cells were quantitated by immunochemistry using an anti-TGF-β1 Ab as previously described in this laboratory (30). In addition to quantitating the total number of TGF-β1+ cells, we also quantitated the number of TGF-β1+ cells that were positive for MBP per bronchiolus with 150–200 μm of internal diameter. By guest on April 14, 2017 http://www.jimmunol.org/ Downloaded from
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 bronchiole 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; Sci-req) 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. 1A), bone marrow (p = 0.02; acute OVA vs no OVA) (Fig. 1C), and blood eosinophils (p = 0.05; acute OVA vs no OVA) (Fig. 1B). 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. 1A), bone marrow eosinophils (p = 0.05; acute OVA plus control Ab vs acute OVA plus anti-Siglec-F Ab) (Fig. 1C), and blood eosinophils (p = 0.05; acute OVA plus control Ab vs acute OVA plus anti-Siglec-F Ab) (Fig. 1B). 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).

**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
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 Siglec-F Ab digest products before each acute OVA challenge. Non-OVA-challenged mice served as a control. Eosinophils in BAL fluid (A) and blood (B) were quantitated in cytopsin slides stained with Wright-Giemsa. Acute OVA challenge significantly increased the number of BAL eosinophils (\( p = 0.002 \)) and blood eosinophils (\( p = 0.004 \)) (Fig. 3B) 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).

**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. 4A), as well as a significant increase in the number of peribronchial eosinophils (\( p = 0.001; \) OVA vs no OVA) (Fig. 4B) 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 × 10^4; OVA plus control Ab vs OVA plus anti-Siglec-F Ab; \( p = 0.003 \)) (Fig. 4A). 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. 4B).
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. 1B) 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 \((p = 0.006; \text{OVA vs no OVA})\) (Fig. 5A) or increases in lung collagen \((p = 0.01; \text{OVA vs no OVA})\) (Fig. 5B) 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 \((1.3 \pm 0.2 \text{ vs } 0.6 \pm 1.0 \mu \text{m}^2 / \mu \text{m peribronchial trichrome stained area})\); OVA plus control Ab vs OVA plus anti-Siglec-F Ab; \(p = 0.01\) (Fig. 5A), as well as the amount of lung collagen \((1717 \pm 77 \text{ vs } 1308 \pm 129 \mu \text{g collagen/lung})\); OVA plus control Ab vs OVA plus anti-Siglec-F Ab; \(p = 0.05\) (Fig. 5B), compared with chronic OVA-challenged mice administered a control Ab.

**Effect of anti-Siglec-F Ab on number of peribronchial TGF-β1+ 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-β1+ peribronchial cells. Chronic OVA challenge induced a significant increase in the number of TGF-β1+ peribronchial cells \((p = 0.0001; \text{OVA vs no OVA})\) (Fig. 5C) compared with non-OVA-challenged mice. Administration of an anti-Siglec-F Ab significantly reduced the number of TGF-β1+ peribronchial cells in chronic OVA-challenged mice \((83.3 \pm 2.8 \text{ vs } 54.6 \pm 1.7 \text{TGF-β1+ cells/bronchus})\); OVA plus control Ab vs OVA plus anti-Siglec-F Ab; \(p = 0.001\) (Fig. 5C) 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-β1+ cells by quantitating the number of cells expressing MBP and TGF-β1. 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 was associated with increased eosinophil apoptosis. Chronic OVA challenge induced a small but significant increase in the number of TUNEL+ peribronchial cells \((p = 0.002; \text{OVA vs no OVA})\) (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.

**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 was associated with increased eosinophil apoptosis. Chronic OVA challenge induced a small but significant increase in the number of TUNEL+ peribronchial cells \((p = 0.002; \text{OVA vs no OVA})\) (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.

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

Chronic OVA challenge also induced a significant increase in the number of peribronchial mast cells compared with non-OVA-challenged mice \((5.6 \pm 0.6 \text{ vs } 0 \pm 0 \text{ mast cells/bronchus})\); OVA vs no OVA; \(p = 0.002\) (Fig. 4C). 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 \((5.6 \pm 0.6 \text{ vs } 4.7 \pm 0.3 \text{ mast cells/bronchus})\); OVA plus control Ab vs OVA plus anti-Siglec-F Ab; \(p = 0.64\) (Fig. 4C).
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. 7A), 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. 7B).

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. 7A), 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. 7B).

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. 5E)
The percentage of BAL (A, %) and BM (B, %) cells stained with annexin V and CCR3 was quantitated in lung sections by TUNEL staining. Administration of an anti-Siglec-F Ab induced a statistically significant increase in the number of annexin V+ and CCR3+ cells compared with OVA-challenged mice pretreated with a control Ab (chronic OVA + anti-Siglec-F Ab vs chronic OVA + control Ab; p = 0.01; 24 mg/ml MCh) (Table I). The increase in airway responsiveness to Mch 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).

**Discussions**

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 be explained by the observation that the anti-Siglec-F Ab reduces airway eosinophilic inflammation could theoretically reduce the number of eosinophils in the airway and reduce the production of pro-inflammatory cytokines. The reduction in eosinophilic inflammation could theoretically lead to a decrease in the production of pro-inflammatory cytokines, which could in turn reduce the number of eosinophils in the airway. This could explain the observed reduction in airway eosinophilic inflammation following administration of the anti-Siglec-F Ab.
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 traffic 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 Ab 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 traffic 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.

Acknowledgments
The authors acknowledge the contribution of Cristina Pop (Burnham Institute for Medical Research, La Jolla, CA) in assisting with the generation of the F(ab')2 Abs.

Disclosures
The authors have no financial conflicts of interest.

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