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Anti-Siglec-F antibody inhibits oral egg allergen induced intestinal eosinophilic inflammation in a mouse model

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KEYWORDS

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Abstract Siglec-F is a sialic acid binding immunoglobulin-superfamily receptor that is highly expressed on eosinophils. We have used a mouse model of oral egg ovalbumin (OVA)-induced eosinophilic inflammation of the gastro-intestinal mucosa associated with diarrhea and weight loss to determine whether administering an anti-Siglec-F antibody would reduce levels of intestinal mucosal eosinophilic inflammation. Mice administered the anti-Siglec-F antibody had significantly lower levels of intestinal eosinophilic inflammation, and this was associated with reduced intestinal permeability changes, normalization of intestinal villous crypt height, and restoration of weight gain. The reduced numbers of intestinal eosinophils in anti-Siglec-F antibody treated mice was associated with significantly reduced numbers of bone marrow and peripheral blood eosinophils, but was not associated with significant changes in the numbers of proliferating or apoptotic jejunal eosinophils. In addition, the anti-Siglec-F Ab reduced Th2 cytokines and IgE levels. Overall, these studies demonstrate that administration of an anti-Siglec-F antibody significantly reduces levels of eosinophilic inflammation in the intestinal mucosa and that this was associated with reduced intestinal permeability changes, normalization of intestinal villous crypt height, and restoration of weight gain.

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Introduction

Eosinophilic gastro-intestinal diseases (EGIDs) is a group of diseases (eosinophilic esophagitis, eosinophilic gastritis,

eosinophilic gastroenteritis, eosinophilic colitis) characterized by gastro-intestinal symptoms and mucosal eosinophilia [1]. Although the etiology of these diseases is currently unknown, food allergy appears to play a role [1], as approximately 75% of patients with EGIDs are atopic [2], and EGIDs can be reversed in some individuals by institution of an allergen free diet [2,3].

Mouse models of allergen induced gastro-intestinal eosinophilic inflammation have shed important light on the

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mechanisms of eosinophil trafficking to the gastro-intestinal mucosa [4,5]. In particular, studies with mice deficient in either IL-5 or eotaxin-1 have demonstrated an important role for IL-5 in the bone marrow generation of eosinophils that migrate to the intestinal mucosa [4,5], and for eotaxin-1 in the chemoattraction of eosinophils into the gastro-intestinal mucosa [4,5]. In mice, eosinophil trafficking to the gastro-intestinal mucosa is both constitutive [4,5], and can also be further up-regulated by exposure to allergen [4,5].

While much is known about the pathways that induce eosinophilic inflammation in the gastro-intestinal tract, there is more limited information regarding the pathways which mediate resolution of eosinophilic inflammation in the gastro-intestinal tract. One such candidate molecule is Siglec-F. Siglec-F is highly expressed by eosinophils [6], and in vivo studies demonstrate that Siglec-F deficient mice challenged by inhalation with allergen have enhanced and prolonged eosinophilic airway inflammation [7] suggesting that activation of Siglec-F normally functions to down-regulate eosinophilic inflammation.

Siglecs (sialic-acid-binding immunoglobulin-superfamily lectins) are a homologous group of thirteen human and nine mouse molecules involved in cell-cell interactions and signaling functions in the haemopoietic, immune and nervous systems [8]. Siglecs are single pass type I transmembrane receptors characterized by an extracellular N terminal V-set domain important in binding to sialic acid-containing ligands, a variable number of extracellular immunoglobulin like C2-set domains, and a cytosolic domain that often has tyrosine-based signaling motifs. Siglec-F, contains a cytoplasmic ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif) domain (a characteristic of inhibitory receptors) and this domain may thus be important in down-regulating eosinophil activation during immune and inflammatory responses [9].

Eosinophils express high levels of Siglec-8 in humans [10], and high levels of Siglec-F in mice [6]. Although not direct orthologs, Siglec-8 and Siglec-F are functional equivalents as both are highly expressed on eosinophils and preferentially bind to the same ligand 6' sulfo sLe^x [11]. *In vitro* studies of eosinophils incubated with anti-Siglec-8 antibodies have demonstrated that cross-linking this molecule on eosinophils induces an apoptotic signal [12]. Interestingly, neither IL-5 nor GM-CSF (eosinophil survival-promoting cytokines) is able to counteract this ability of Siglec-8 cross-linking to induce eosinophil apoptosis [12]. Based on pharmacologic studies with a pan-caspase inhibitor, caspases were shown to be involved in Siglec-8 mediated eosinophil apoptosis [13]. In particular, Caspase-3-like activity (an important mediator of apoptosis or programmed cell death) is induced in eosinophils by Siglec-8 cross-linking.

Because mouse Siglec-F shares many properties with human Siglec-8, including predominant expression on eosinophils, and shared unique ligand specificity [6,11], studies of mouse Siglec-F have provided insight into the potential role of Siglec-8 in human allergic disease. In previous studies using Siglec-F deficient mice we have demonstrated an important role for Siglec-F in mediating the resolution of eosinophilic inflammation in the airway following allergen challenge [7]. Siglec-F deficient mice challenged with inhaled allergen have enhanced levels of eosinophilic airway inflammation as well as delayed resolution of eosinophilic airway inflammation suggesting that Siglec-F normally

functions to down-regulate eosinophilic inflammation. Here, we have used a mouse model of oral food (i.e. OVA) allergen induced gastro-intestinal eosinophilic inflammation to determine whether targeting Siglec-F could be used as a potential therapeutic intervention in EGIDs.

Materials and methods

Oral OVA allergen induced gastro-intestinal eosinophilic inflammation

Eight to 10 week old BALB/c mice (8 mice/group; The Jackson Laboratory, Bar Harbor, ME) were sensitized intraperitoneally on day 0 and day 14 (50 µg of OVA adsorbed to 1 mg of aluminum hydroxide adjuvant) (Sigma-Aldrich, St. Louis, MO) and challenged intragastrically on six occasions with ovalbumin (OVA; grade V; Sigma-Aldrich) on days 28, 30, 32, 35, 37, and 39 to induce gastro-intestinal eosinophilic inflammation [14] (Fig. 1). The intragastric OVA challenges (50 mg of OVA suspended in 250 µL of phosphate buffered saline (PBS)) were administered through an intragastric feeding needle (22-gauge, 1.5-inch, 1.25-mm ball; Fisher Scientific, Pittsburgh, PA). Control age- and sex-matched BALB/c mice were sensitized and challenged with PBS instead of OVA. Intestinal epithelial permeability was assessed 1 h after the final OVA or PBS challenge on day 39 (as described below), and mice were then sacrificed and their gastro-intestinal tract processed for immunohistology using a light microscope (Leica DMLS; Leica Microsystems Inc., Depew, NY) and an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, Maryland, USA). For immunohistology, the jejunum was removed 10 cm distal to the stomach, fixed with 4% paraformaldehyde solution (Sigma) for 24 h, embedded in paraffin, and 5-µm tissue sections prepared for analysis. In each experiment in which stained and immunostained slides were quantified by image analysis, identical light microscope conditions, magnification, gain, camera position, and background illumination were utilized. The analysis of slides was performed by an

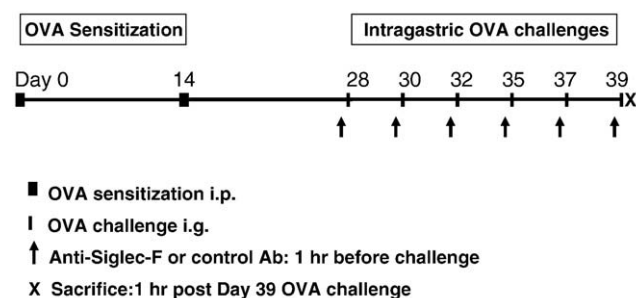


Figure 1 Experimental food allergy protocol. Mice were sensitized intraperitoneally on day 0 and 14 with OVA. Intra-gastric OVA challenges were performed on day 28, 30, 32, 35, 37, and 39. Age- and sex-matched control mice were sensitized but not challenged with OVA. 1 hr before each OVA challenge, an anti-Siglec-F or isotype control antibody was administered intraperitoneally (arrows). Mice were sacrificed 1 hr after the final OVA challenge and their jejunums were analyzed.

investigator blinded to the study group. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

Therapeutic intervention with anti-Siglec-F antibody

Different groups of mice were administered with 10 μg of either a rat anti-mouse Siglec-F IgG2a antibody, or a control rat IgG2a isotype matched antibody, in 100 μl PBS by intraperitoneal injection 1 h before each of the six OVA intragastric challenges (Fig. 1). In pilot studies following intraperitoneal injection, the half-life of the anti-Siglec-F Ab in mice was 3–4 days. The dosing regimen of the anti-Siglec-F antibody ensured that serum levels of the anti-Siglec-F antibody were maintained at $>4 \mu\text{g}/\text{mL}$. In pilot studies we demonstrated such levels of anti-Siglec-F antibody were sufficient to bind all eosinophil Siglec-F in blood and bone marrow (data not shown).

Intestinal eosinophil and mast cell quantitation

Eosinophils were detected in jejunal tissue by immunohistology using an anti-mouse Major Basic Protein (MBP) antibody (kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, AZ) as previously described [15]. The tissue sections were also stained for mucosal mast cells using chloroacetate esterase activity as described [16] and lightly counterstained with hematoxylin. Quantification of eosinophils and mast cells was performed using a light microscope attached to an image-analysis system. Results are expressed as the number of eosinophils or mast cells per mm^2 of lamina propria. At least ten randomly selected areas of jejunal mucosa were counted in each slide at $\times 20$ magnification.

Blood and bone marrow eosinophil quantitation

Peripheral blood was collected from different groups of mice by cardiac puncture into EDTA-containing tubes as previously described [7]. 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 PBS. Bone marrow cells were flushed from femurs with 1 mL PBS, centrifuged, and resuspended in 1 mL PBS. Total leukocytes were counted using a hemocytometer. To perform differential cell counts, 200 μL peripheral-blood leukocytes, or 20 μL bone marrow cell suspensions was cytospun onto microscope slides and air-dried [7]. Slides were stained with Wright-Giemsa and differential cell counts were performed under a light microscope [7].

Measurement of intestinal epithelial permeability

Jejunal intestinal epithelial permeability was assessed with Evans Blue dye (EB; Sigma) using a previously described technique with modifications [17]. One hour after the final intragastric OVA challenge, mice were anesthetized (pentobarbital sodium 60–70 mg/kg intraperitoneally) and the jejunum of each mouse was cannulated with a polyethylene tube which was positioned in the jejunum approximately 10 cm distal to the stomach. After removal of the luminal

contents from the jejunum with PBS, 0.3 mL of 2% EB dye in PBS containing 4% bovine serum albumin was injected through the cannula into the jejunum. After 3 min equilibration of the EB dye in the jejunum, the EB dye was gently washed out for 10 min. A 5 cm length of jejunum 10–12 cm distal to the stomach was then removed after which the mouse was sacrificed. The removed jejunum was opened and rinsed with 6 mM *N*-acetylcysteine in PBS to clear mucus as well as to remove any absorbed EB in the mucus. After being weighed, the jejunal tissue samples were extracted in 5 mL *N,N*-dimethylformamide (DMF) for 24 h at room temperature. The jejunal tissue extract was collected and centrifuged at 3000 rpm for 15 min. The jejunal tissue extract supernatant was used to evaluate the amount of EB dye in the jejunal tissue by spectrophotometry at a wavelength of 655 nm.

Analysis of intestinal mucosal epithelial cell proliferation

5'-bromodeoxyuridine (5'-BrdU) (Zymed Laboratories Inc., South San Francisco, CA) incorporation analysis into intestinal mucosal epithelial cells was performed using previously reported methods [18]. In brief, saline or OVA-challenged mice were injected intraperitoneally with 0.25 mL 5'-BrdU 3 h before sacrifice. Immunohistochemical detection of BrdU in intestinal mucosal epithelial cells was performed by incubating tissue sections with a biotinylated anti-BrdU antibody for 60 min at room temperature (Chemicon, Temecula, CA). The slides were then incubated with streptavidin-HRP conjugate followed by peroxidase reagent and 3,3'-diaminobenzidine chromogen, and hematoxylin counterstain. Positive controls were provided by the manufacturer and omitting the primary antibody served as a negative control. Epithelial cell proliferation was quantitated in each group of mice as the percentage of BrdU labeled cells per 500 crypt cells assessed in well-oriented crypt-villous units.

Intestinal villous/crypt morphologic changes

Measurement of villous height and crypt depth in the jejunum was performed using a light microscope (Leica DMLS) attached to an image-analysis system (Image-Pro Plus). Jejunal eosinophilic inflammation is associated with decreased villous height, increased crypt depth, and a reduced villous crypt ratio [19]. Ten well-oriented crypt-villous units from hematoxylin–eosin stained sections were randomly chosen for villous height and crypt depth measurement at $\times 10$ magnification. Results are expressed as the villous height or crypt depth in μm .

Detection of apoptotic cells in intestinal mucosa

The number of apoptotic cells in the intestinal mucosa was assessed by ultrastructure (cell shrinkage, nuclear chromatin condensation) and TUNEL staining as previously described [20]. For TUNEL staining, digoxigenin-labeled nucleotides and terminal deoxynucleotidyl transferase (TdT) were added to 5- μm sections of intestinal mucosa to label the free 3' DNA ends of apoptotic cells (ApopTag Plus Peroxidase In Situ

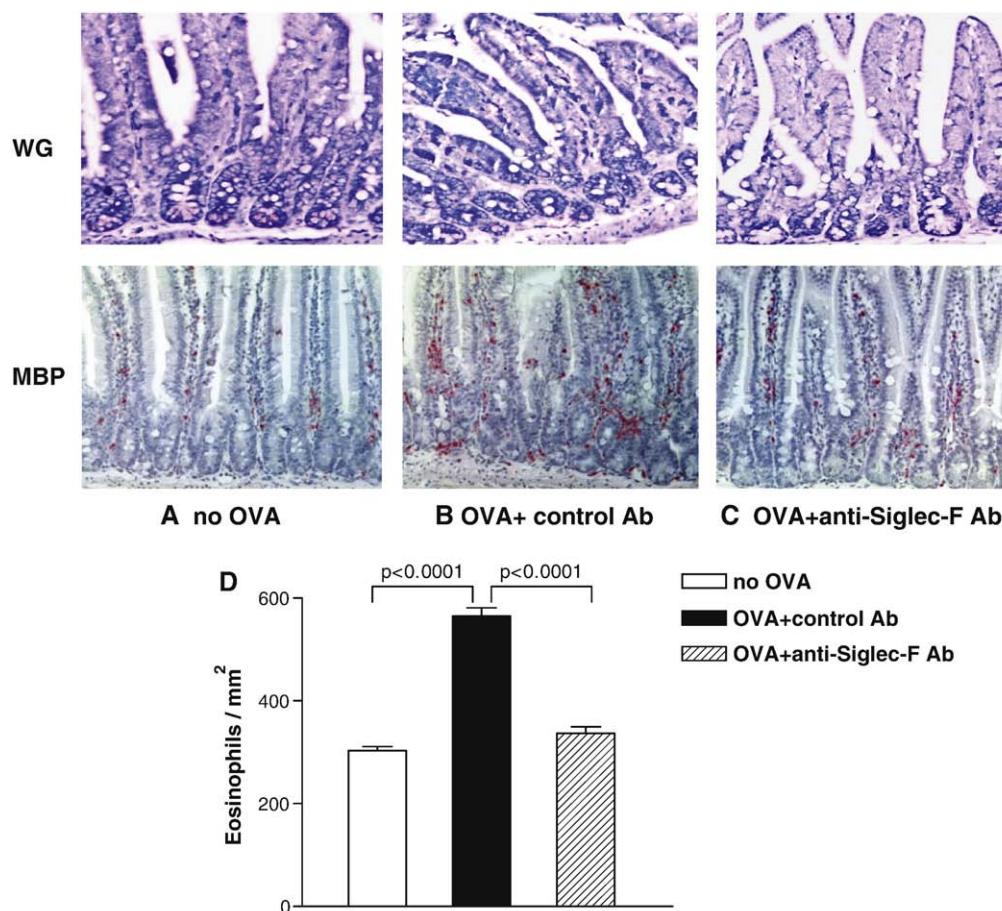


Figure 2 Eosinophils in jejunum. A–C. Wright-Giemsa (WG) and anti-MBP immunostain of jejunum (A, no OVA; B, OVA+control Ab; C, OVA+anti-Siglec-F Ab). The number of eosinophils per mm² of jejunal lamina propria was quantitated (D). Intra-gastric OVA challenge (OVA+control Ab) induced a significant accumulation of eosinophils in jejunal mucosa (vs. no OVA, $p < 0.0001$). Treatment with an anti-Siglec-F antibody significantly reduced the number of eosinophils (vs. OVA+control Ab, $p < 0.0001$) ($n = 8$ mice/group).

Apoptosis Detection Kit; Chemicon, Temecula, CA). An anti-digoxigenin antibody conjugated to peroxidase was used to label the incorporated digoxigenin-labeled nucleotides and developed with the substrate supplied by the manufacturer. The sections were counterstained with hematoxylin. The number of apoptotic cells was counted in ten randomly selected sections in intestinal mucosa in each slide using a light microscope attached to the image-analysis system as described above.

Effect of anti-Siglec-F antibody on mast cell apoptosis *in vitro*

We have previously demonstrated that culturing mouse eosinophils *in vitro* with an anti-Siglec-F antibody and a cross-linking secondary antibody induces eosinophil apoptosis [7]. To determine whether incubating mast cells *in vitro* with an anti-Siglec-F antibody induced mast cell apoptosis, we purified mast cells from the peritoneal cavity (>93% purity) of naïve Balb/c mice as previously described [21]. The purified mast cells were incubated for 24 h at 37 °C either in media alone, or with an anti-Siglec-F antibody (2.5 µg/mL),

or with an anti-Siglec-F antibody and a secondary cross-linking anti-rat IgG1/2a antibody (2.5 µg/mL; BD Biosciences Pharmingen). Apoptotic mast cells were then assessed as the percentage of TUNEL-positive cells per 400 cells on cytospin slide preparations.

Detection of proliferating and/or apoptotic eosinophils in intestinal mucosa

To determine whether anti-Siglec-F influenced the number of proliferating and/or apoptotic eosinophils in the intestinal mucosa we performed double label immunofluorescence microscopy with either an anti-MBP Ab combined with BrdU labeling (to detect proliferating eosinophils), or an anti-MBP Ab combined with TUNEL staining (to detect apoptotic eosinophils) as previously described in this laboratory [22]. The two primary antibodies were detected with two different horseradish peroxidase (HRP) enzyme-labeled secondary antibodies with signal amplification using tyramide signal amplification (Molecular Probes) according to the manufacturer's instructions. The anti-MBP primary antibody was detected with an HRP-labeled secondary

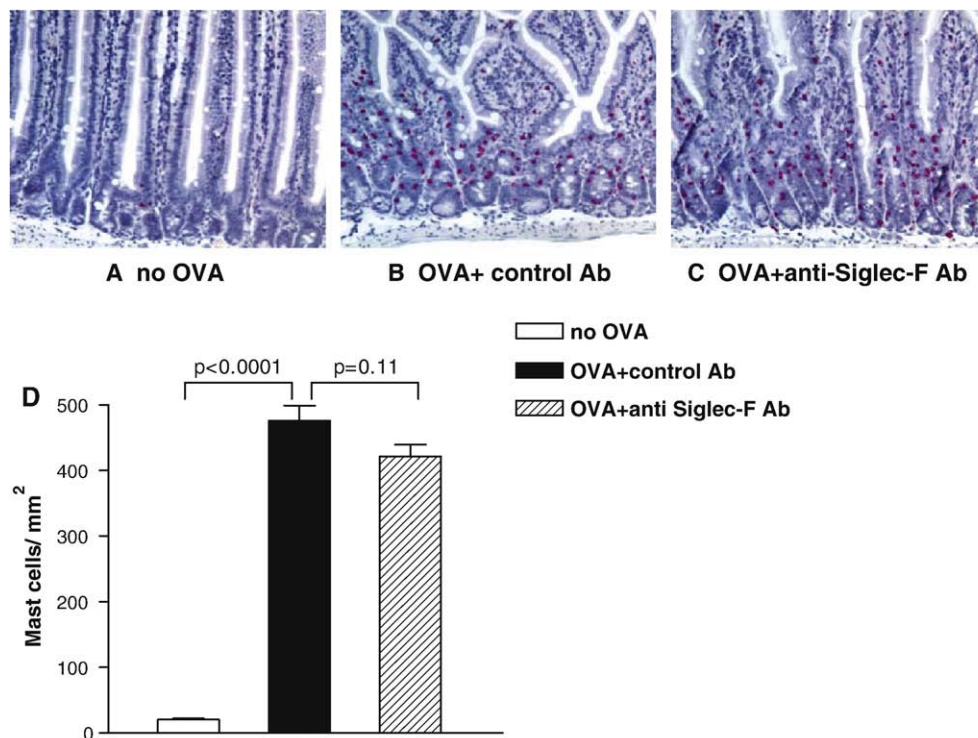


Figure 3 Mast cells in jejunum. Mast cells were detected in jejunal mucosa using chloroacetate esterase staining (red color) (A, no OVA; B, OVA+control Ab; C, OVA+anti Siglec-F Ab). The number of mast cells per mm² of lamina propria was quantitated (D). Intragastric OVA challenge (OVA+control Ab) induced a significant accumulation of mast cells in jejunal mucosa (vs. no OVA, $p < 0.0001$). Treatment with an anti-Siglec-F antibody did not inhibit the accumulation of mast cells (vs. OVA+control Ab, $p = \text{NS}$) ($n = 8$ mice/group).

antibody (Alexa 555, red color), while the anti-digoxigenin primary antibody (to detect apoptotic cells) was detected with a different HRP-labeled secondary antibody (Alexa 488, green color). Cells co-expressing MBP and TUNEL have a yellow color. To detect MBP+ cells expressing BrdU, the anti-MBP primary antibody was detected with an HRP-labeled secondary antibody (Alexa 555, red color), while the anti-BrdU primary antibody was detected with a different HRP-labeled secondary antibody (Alexa 488, green color).

Measurement of intestinal eotaxin-1 levels

Eotaxin-1 levels were measured in intestinal mucosa by ELISA. Intestinal tissue was homogenized in 2.0 mL PBS (pH 7.4) and supernatants were obtained by centrifugation (1800 rpm for 10 min) and frozen at -80°C in polypropylene tubes until assayed [21]. Eotaxin-1 levels were quantitated by ELISA (R&D Systems, Minneapolis, MN) with a sensitivity of 5 pg/mL. Intestinal tissue protein levels were quantitated using a BCA protein assay (Pierce, Rockford, IL). Results are expressed as pg eotaxin-1/mg intestinal mucosa protein.

Measurement of splenocyte Th2 cytokines

Spleens were harvested from non-OVA and OVA challenged mice (treated with either anti-Siglec-F Ab or a control Ab) to

determine splenocyte Th2 cytokine production *in vitro*. A single cell suspension of splenocytes was obtained by mincing spleens and using a 70- μm nylon cell strainer (BD Falcon Biosciences, Bedford, MA). Spleen cells were cultured in 96-well plates (1×10^6 cells/well) with or without 100 $\mu\text{g}/\text{mL}$ OVA (Sigma, St. Louis, MO) for 72 h at 37°C . Supernatants were collected and stored at -80°C for cytokine ELISA assays. Levels of mouse IL-4, IL-5, and IL-13 were assayed in splenocyte cell culture supernatants using an ELISA (R&D Systems, Minneapolis, MN). The sensitivity of the Elisa assays were 15.6 pg/mL (IL-4), 31.25 pg/mL (IL-5), and 31.25 pg/mL (IL-13), respectively.

Measurement of OVA specific IgE

Serum OVA-specific IgE concentrations were determined by ELISA. Ninety six well plates were pre-coated with 100 $\mu\text{g}/\text{mL}$ OVA, blocked with 10% FBS. Mouse serum samples diluted 1:5 and 1:10 were added to the OVA coated wells. After 2 h incubation at 37°C , the plates were washed and biotinylated anti-mouse IgE (Pharmingen, San Jose, CA) was added. The OD was read at 490 nm within 30 min.

Weight gain

As intestinal symptoms associated with food allergy such as diarrhea are difficult to quantitate in mice, we measured

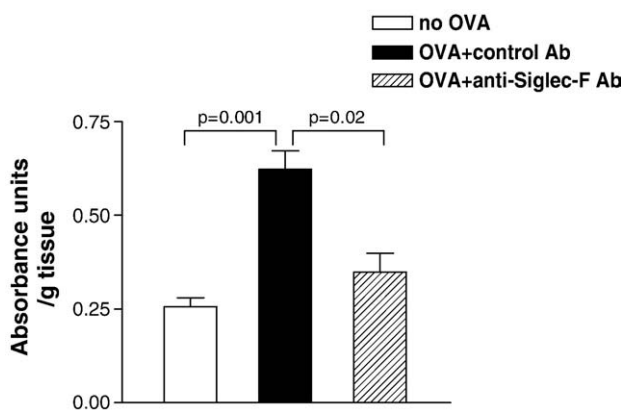


Figure 4 Jejunal epithelial protein permeability. Intra-gastric OVA challenge (OVA+control Ab) induced a significant increase in jejunal epithelial permeability to Evans Blue-albumin (vs. no OVA, $p=0.0001$). Treatment with an anti-Siglec-F antibody significantly inhibited the OVA-induced permeability change (vs. OVA+control Ab, $p=0.02$) ($n=8$ mice/group).

differences in weight gain as a surrogate end-point for diarrhea. Mice were weighed in grams using a TR-104 Balance (Denver Instrument Company, Denver, CO) on day 0 and day 39. The % weight gain over the 39 day study was assessed in the different groups of mice. We also determined liquid stool scores by placing mice on paper toweling in a small clear plastic chamber for 1.5 h on day 0, day 35, and day 39. After 1.5 h the paper towels were scored as dry (assigned score 0) or wet (assigned score 1).

Statistical analyses

Results from the different groups were compared by a Mann-Whitney test using a statistical software package (Graph Pad Prism, San Diego, CA). P values <0.05 were considered statistically significant. All results are presented as mean \pm SEM.

Results

Effect of anti-Siglec-F antibody on the number of eosinophils and mast cells in the intestinal mucosa

The number of eosinophils in the intestinal mucosa was significantly increased in the mice challenged with oral OVA compared to the non-OVA challenged mice (565 ± 16 vs. 303 ± 80 eosinophils/ mm^2 ; $p < 0.0001$; Fig. 2). Administration of an anti-Siglec-F antibody to oral OVA-challenged mice significantly reduced levels of intestinal eosinophils compared to oral OVA challenged mice administered a control antibody. (336 ± 13 vs. 565 ± 16 eosinophil/ mm^2 ; $p < 0.0001$; Fig. 2). Levels of eosinophils in the intestine of oral OVA challenged mice treated with an anti-Siglec-F antibody were reduced to levels similar to that noted in non-OVA challenged mice (Fig. 2).

The number of intestinal mast cells were also significantly higher in the oral OVA-challenged mice compared with non-OVA challenged control mice (476 ± 23 vs. 21 ± 2 mast cells/ mm^2 ; $p < 0.001$; Fig. 3). In contrast to the inhibitory effect of the anti-Siglec-F antibody on the increased accumulation of

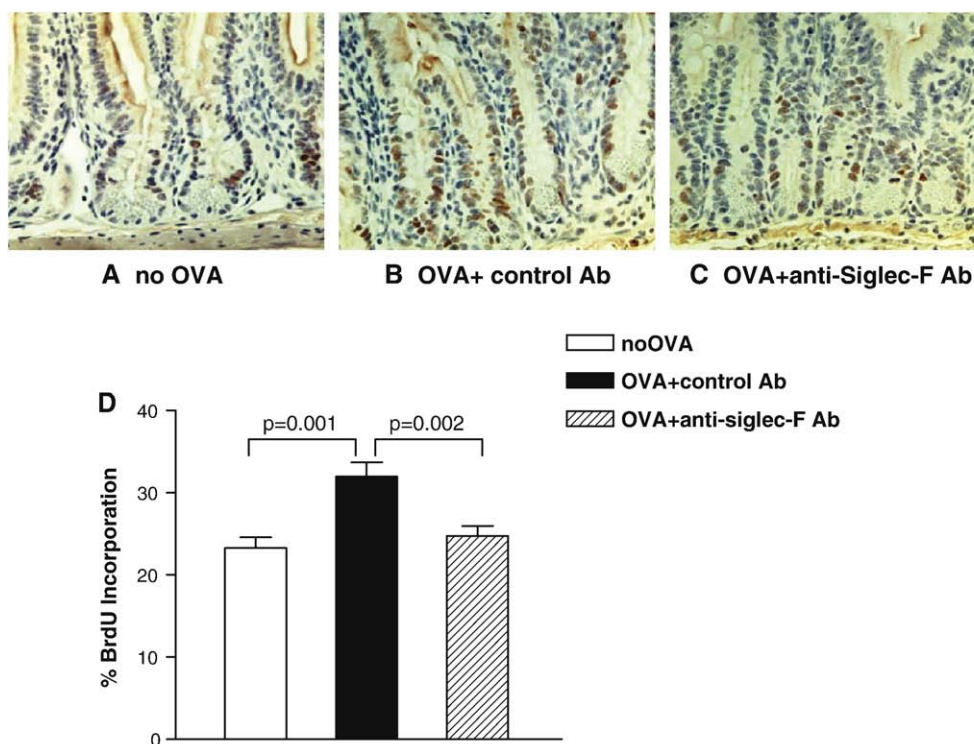


Figure 5 Jejunal cellular proliferation. The percentage of BrdU labeled cells per 500 crypt cells was quantitated. Intra-gastric OVA challenge (OVA+control Ab) induced a significant increase in the percentage of BrdU-labeled cells (vs. no OVA, $p=0.001$). Treatment with an anti-Siglec-F antibody significantly reduced BrdU incorporation (vs. OVA+control Ab, $p=0.002$) ($n=8$ mice/group).

intestinal eosinophils in oral OVA challenged mice, the anti-Siglec-F antibody did not inhibit the increase in the number of intestinal mast cells observed in OVA-challenged mice (Fig. 3).

Effect of anti-Siglec-F antibody on intestinal mucosal epithelial permeability

Intestinal epithelial permeability to EB-albumin was significantly increased in oral OVA-challenged mice compared with non-OVA challenged control mice (0.62 ± 0.05 vs. 0.26 ± 0.02 AU/gm intestine, $p=0.001$; Fig. 4). Administration of an anti-Siglec-F antibody to oral OVA challenged mice significantly reduced levels of intestinal permeability compared to a control antibody administered to oral OVA challenged mice (0.62 ± 0.05 vs. 0.37 ± 0.06 AU/gm intestine, $p=0.02$; Fig. 4).

Effect of anti-Siglec-F antibody on intestinal mucosal epithelial cell proliferation

To investigate the effect of Siglec-F on intestinal epithelial cell proliferation, BrdU incorporation of jejunal epithelial cells was measured in the different groups of mice. The percentage of BrdU-labeled jejunal epithelial cells in oral OVA-challenged mice was significantly increased compared with non-OVA challenged control mice (31.4 ± 1.4 vs. $23.3 \pm 1.3\%$ BrdU+ intestinal epithelial cells; $p=0.001$; Fig. 5). Administration of an anti-Siglec-F antibody to oral OVA challenged mice significantly reduced the numbers of BrdU+ intestinal epithelial cells compared to oral OVA challenged mice administered a control antibody (31.4 ± 1.4 vs. $23.7 \pm 1.2\%$ BrdU+ intestinal epithelial cells; $p=0.002$; Fig. 5).

Effect of anti-Siglec-F antibody on intestinal mucosal villous atrophy and crypt hyperplasia

The villous height of the intestinal mucosa of oral OVA-challenged mice was significantly reduced compared to non-OVA challenged control mice (272.3 ± 10.3 vs. 339.4 ± 11.6 μm intestinal villous height; $p<0.0001$; Fig. 6). Administration of an anti-Siglec-F antibody to oral OVA challenged mice significantly increased levels of villous height compared to a control antibody administered to oral OVA challenged mice (342.4 ± 11.1 vs. 272.3 ± 10.3 μm intestinal villous height; $p<0.0001$; Fig. 6).

The crypt depth of the intestinal mucosa of the oral OVA-challenged mice was significantly increased compared with the non-OVA challenged control mice (83.1 ± 2.3 vs. 50.3 ± 1.5 μm intestinal crypt depth; $p<0.0001$; Fig. 6). Administration of an anti-Siglec-F antibody to oral OVA challenged mice significantly reduced levels of crypt depth compared to that with a control antibody (70.3 ± 1.9 vs. 83.1 ± 2.3 μm intestinal crypt depth; $p<0.0001$; Fig. 6).

The villous/crypt ratio of the intestinal mucosa of the oral OVA-challenged mice was also significantly reduced compared with the non-OVA challenged control mice (3.4 ± 0.1 vs. 6.9 ± 0.2 ; $p<0.0001$; Fig. 6). Administration of an anti-Siglec-F antibody to oral OVA challenged mice partially reversed the villous/crypt ratio levels of crypt depth compared

to that with a control antibody (5.0 ± 0.2 vs. 3.4 ± 0.1 ; $p<0.0001$; Fig. 6).

Effect of anti-Siglec-F antibody on number of apoptotic cells in the intestinal mucosa

As studies of eosinophils have demonstrated that cross linking Siglec-8 receptors on human eosinophils induces apoptosis [12], we quantitated levels of apoptosis in the intestinal mucosa using a TUNEL assay. OVA challenge did not induce an increased number of TUNEL+ apoptotic cells in the intestinal mucosa compared to non-OVA challenged mice (Fig. 7). However, the number of TUNEL+ cells was significantly higher

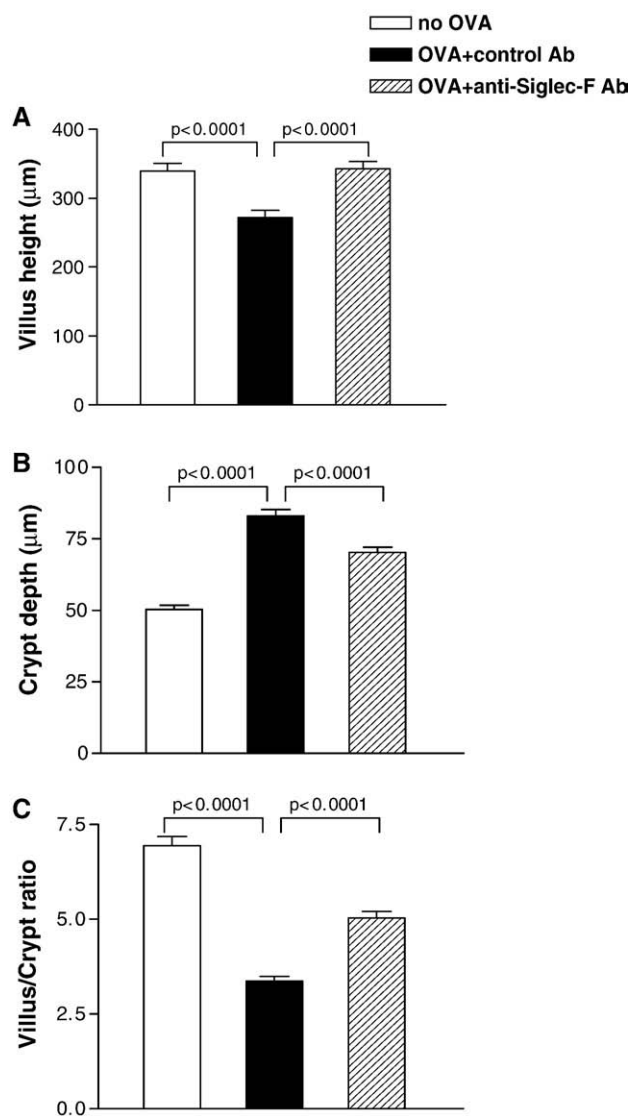


Figure 6 Villous/crypt jejunal morphologic changes. Intra-gastric OVA challenge (OVA+control Ab) induced a significant decrease in villous height (vs. no OVA, $p<0.0001$) (A), an increase in crypt depth (vs. no OVA, $p<0.0001$) (B), and a decrease in villous/crypt ratio (vs. no OVA, $p<0.0001$) (C). Treatment with an anti-Siglec-F antibody significantly inhibited the OVA-induced morphologic changes (vs. OVA+control Ab, $p<0.0001$) ($n=8$ mice/group).

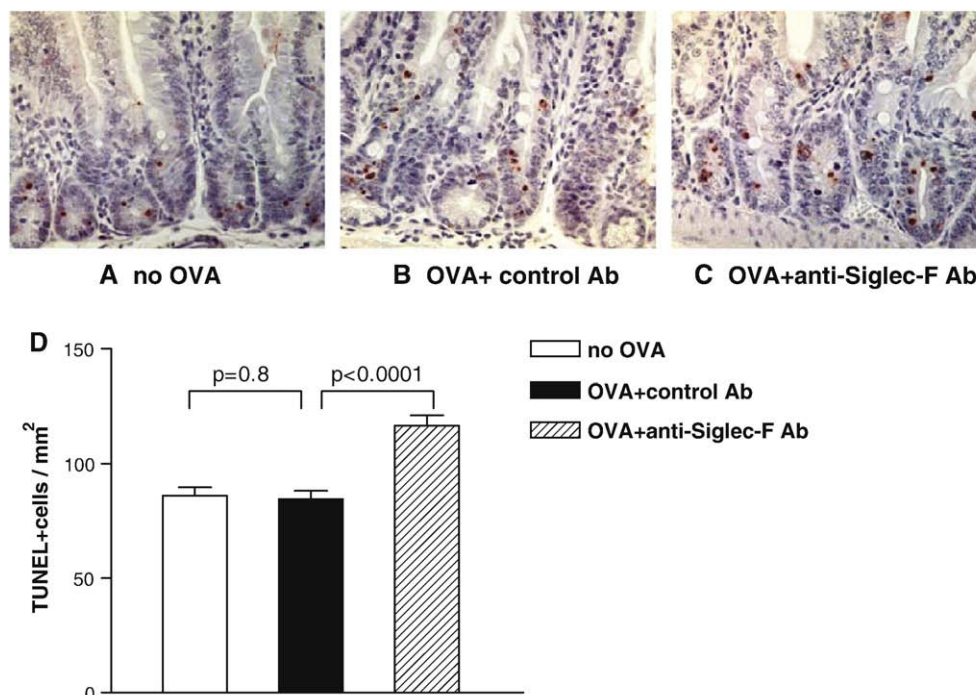


Figure 7 Jejunal apoptotic cells. A TUNEL assay was used to detect apoptotic cells in jejunal mucosa (brown color) (A, no OVA; B, OVA+ control Ab; C, OVA+anti-Siglec-F Ab). The number of apoptotic cells per square millimeter of lamina propria was quantitated (D). Treatment with an anti-Siglec-F antibody increased the number of apoptotic cells in jejunal mucosa compared with no OVA control mice (vs. no OVA, $p<0.0001$), and compared with control antibody treated mice (vs. OVA+control Ab, $p<0.0001$) ($n=8$ mice/group).

in oral OVA challenged mice administered an anti-Siglec-F antibody compared with OVA challenged mice administered a control antibody (116.5 ± 4.6 vs. 84.6 ± 3.7 TUNEL+ cells/mm²; $p<0.0001$; Fig. 7).

Effect of anti-Siglec-F antibody on apoptosis and proliferation of eosinophils in the intestinal mucosa

In double label experiments the vast majority of TUNEL+ cells in the intestinal mucosa were not MBP+ (Fig. 8). TUNEL+ cells were detected within the epithelium as well as beneath the epithelium. The lack of MBP/TUNEL double staining could either mean that the apoptotic cells are not eosinophils, or alternatively that the

apoptotic cells no longer express levels of MBP detectable by immunohistochemistry.

We have also performed double label experiments with MBP and BrdU. The vast majority of BrdU+ cells are epithelial cells (as well as other non-MBP+ cells) (Fig. 9). Thus, eosinophils are not significantly proliferating in the mucosa, and anti-Siglec-F is not playing a role in modulating this minimal proliferation.

Effect of anti-Siglec-F antibody on bone marrow and blood eosinophils

The number of eosinophils in the bone marrow was significantly increased in the mice challenged with oral

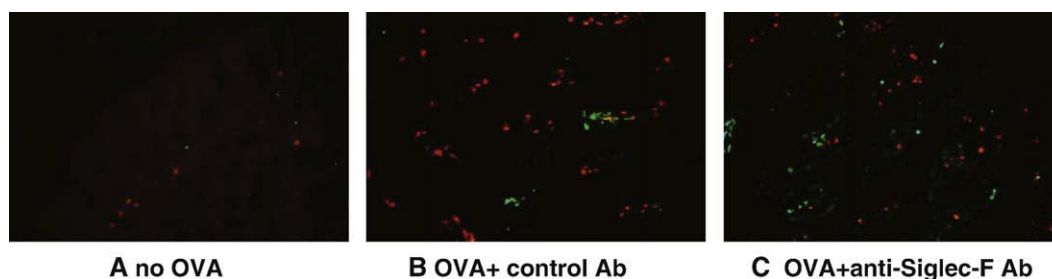


Figure 8 MBP and TUNEL co-localization in jejunum. Jejunal tissue was stained with both anti-MBP (Alexa 555; red color) and TUNEL (Alexa 488; green color) with merged colors being yellow. (A) No OVA challenged mice; (B) OVA+ control Ab; (C) OVA+ anti-Siglec-F Ab).

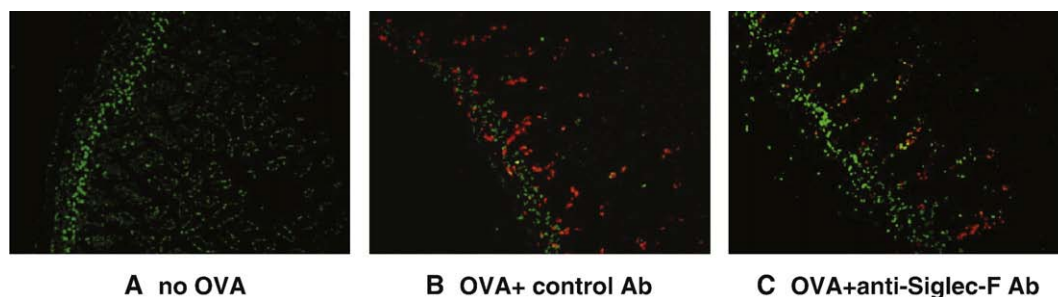


Figure 9 MBP and BrdU co-localization in jejunum. Jejunal tissue was stained with both anti-MBP (Alexa 555; red color) and BrdU (Alexa 488; green color) with merged colors being yellow. (A) No OVA challenged mice; (B) OVA + control Ab; (C) OVA + anti-Siglec-F Ab.

OVA compared to the non-OVA challenged mice (14.4 ± 0.6 vs. $5.4 \pm 0.3\%$ eosinophils; $p < 0.0001$; Fig. 10A). Administration of an anti-Siglec-F antibody to oral OVA-challenged mice significantly reduced levels of bone marrow eosinophils compared to oral OVA challenged mice administered a control antibody (9.3 ± 0.9 vs. $14.4 \pm 0.6\%$ eosinophils; $p < 0.003$; Fig. 10A).

The number of eosinophils in peripheral blood was also significantly increased in the mice challenged with oral OVA compared to the non-OVA challenged mice (10.7 ± 1.3 vs. $1.3 \pm 0.5\%$ eosinophils; $p < 0.006$; Fig. 10B). Administration of an anti-Siglec-F antibody to oral OVA-challenged mice significantly reduced levels of blood eosinophils compared to oral OVA challenged mice administered a control antibody (6.9 ± 0.9 vs. $10.7 \pm 1.3\%$ eosinophils; $p < 0.04$; Fig. 10B).

Effect of anti-Siglec-F antibody on intestinal eotaxin-1 expression

As eotaxin-1 is important in eosinophil trafficking to intestinal mucosa [4,5], we evaluated the effect of anti-Siglec-F antibody administration on levels of intestinal eotaxin-1 expression. The level of eotaxin-1 expression in the intestinal mucosa of the oral OVA-challenged mice was significantly increased compared with non-OVA challenged mice (677.2 ± 220.1 vs. 49.8 ± 7.6 pg eotaxin-1/mg intestinal mucosa protein; $p = 0.01$; Fig. 11). There was no difference in levels of intestinal mucosal eotaxin-1 in oral OVA challenged mice administered an anti-Siglec-F antibody compared to OVA challenged mice administered a control antibody (Fig. 11).

Effect of anti-Siglec-F antibody on mast cell apoptosis *in vitro*

Mast cells cultured *in vitro* with the anti-Siglec-F antibody did not have increased levels of apoptosis compared to mast cells cultured in media alone (4.4 ± 0.8 vs. $4.9 \pm 0.7\%$ TUNEL+ mast cells; $p = \text{NS}$). Similarly, mast cells cultured with an anti-Siglec-F antibody and a secondary cross-linking antibody did not have increased levels of apoptosis compared to mast cells cultured in media alone (4.7 ± 0.5 vs. $4.9 \pm 0.7\%$ TUNEL+ mast cells; $p = \text{NS}$).

Effect of anti-Siglec-F antibody on OVA stimulated splenocyte Th2 cytokine production *in vitro*

Supernatants derived from OVA stimulated splenocytes had significantly increased levels of Th2 cytokines including IL-5 ($p = 0.03$; vs. non-OVA stimulated splenocytes) (Fig. 12A), IL-13 ($p = 0.02$; vs. non-OVA stimulated splenocytes) (Fig. 12B), and IL-4 ($p = 0.03$; vs. non-OVA stimulated splenocytes) (Fig. 12C). Splenocyte supernatants derived from mice administered an anti-Siglec-F Ab (OVA + anti-Siglec-F Ab) had significantly reduced levels of IL-5 ($p = 0.03$; vs. OVA + control Ab) (Fig. 12A), and IL-13 ($p = 0.01$; vs. OVA + control Ab) (Fig. 12B), while the trend for decreased IL-4 ($p = 0.14$; vs. OVA + control Ab) (Fig. 12C) was not significant.

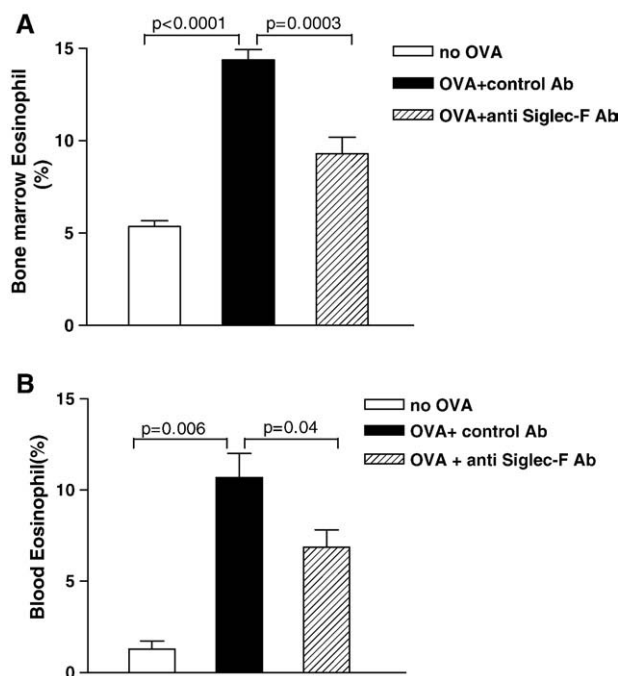


Figure 10 Eosinophils in bone marrow and blood. The number of eosinophils was quantitated in Wright-Giemsa stained bone marrow (A) and blood (B). Treatment with an anti-Siglec-F antibody significantly reduced the number of eosinophils in the bone marrow (vs. OVA + control Ab, $p < 0.0003$), and blood (vs. OVA + control Ab, $p < 0.04$) ($n = 8$ mice/group).

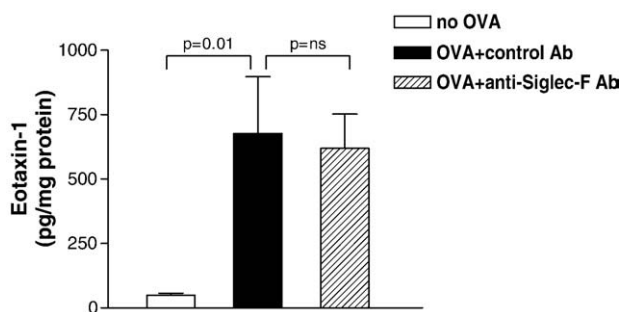


Figure 11 Jejunal eotaxin-1 expression. Eotaxin-1 was measured by ELISA. Intra-gastric OVA challenge (OVA+control Ab) induced a significantly higher level of eotaxin-1 expression (vs. no OVA, $p=0.01$). Treatment with an anti-Siglec-F antibody did not inhibit eotaxin-1 expression (vs. OVA+control Ab, $p=NS$) ($n=8$ mice/group).

Effect of anti-Siglec-F antibody on OVA specific IgE

OVA specific IgE was significantly increased in OVA challenged mice ($p<0.0001$ vs. no OVA) (Fig. 13). Administration of an anti-Siglec-F antibody to oral OVA-challenged mice significantly reduced levels of OVA specific IgE ($p=0.002$; vs. OVA+control Ab) (Fig. 13).

Effect of anti-Siglec-F antibody on weight gain

After six consecutive oral OVA challenges, OVA-sensitized mice developed liquid stools in contrast to the normal, well-formed fecal pellets noted in control non-OVA challenged mice. As accurately quantitating the volume of liquid stool in mice is difficult, the severity of diarrhea was indirectly assessed by monitoring body weight during the entire study period. The average weight of the mice used in this study was

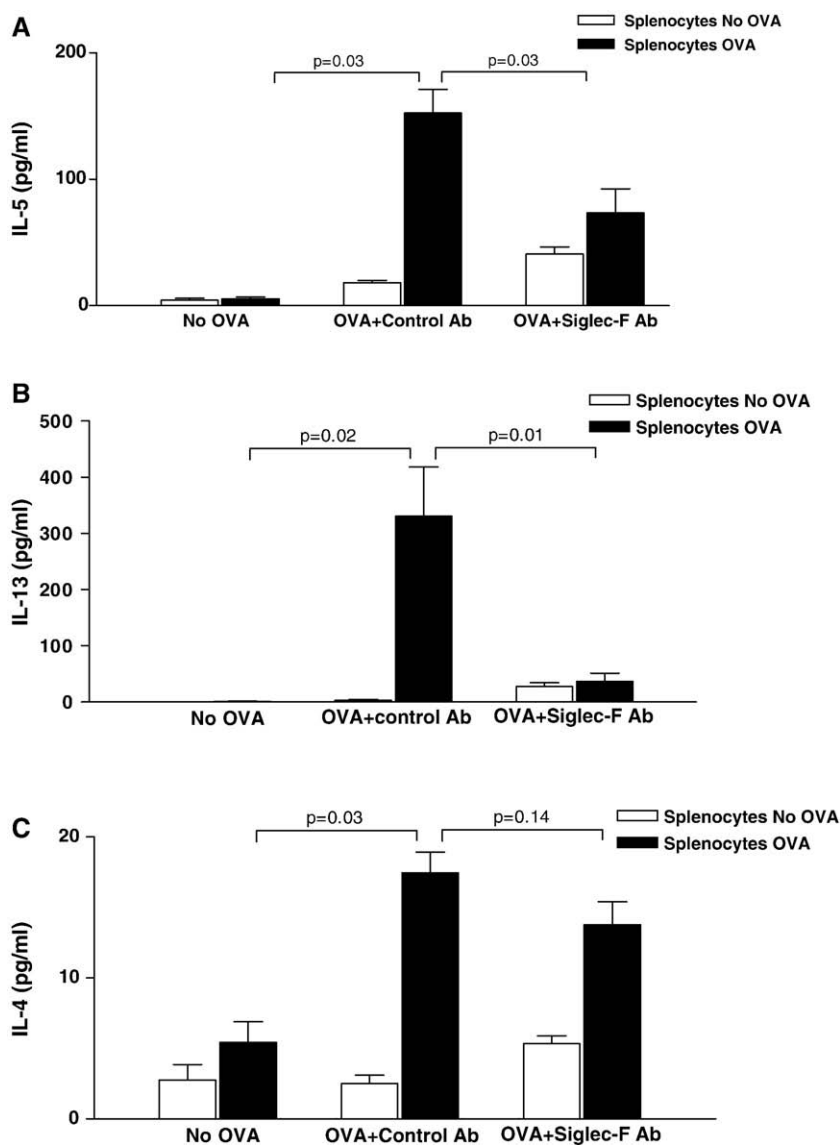


Figure 12 Effect of anti-Siglec-F Ab on splenocyte Th2 cytokines. Splenocytes were obtained from three groups of mice (No OVA challenged mice; OVA + control Ab; OVA + anti-Siglec-F Ab). The splenocytes were incubated *in vitro* for 72 h with either No OVA or OVA and supernatants assayed by Elisa for IL-5 (A), IL-13 (B), and IL-4 (C) ($n=8$ mice/group).

16.8±0.2 g at day 0, with no difference in baseline body weight between the experimental groups. During the course of the study, the control non-OVA challenged mice showed a gradual increase in body weight, whereas oral OVA-challenged mice with diarrhea showed a significantly smaller weight gain. The level of weight gain from the start to the end of the study in the oral OVA-challenged mice was significantly reduced compared with non-OVA challenged mice (10.6±0.8 vs. 17.0±2.3% weight gain, $p=0.03$; Fig. 14). Administration of an anti-Siglec-F antibody to oral OVA challenged mice significantly improved weight gain compared to OVA-challenged mice administered a control antibody (15.2±1.7 vs. 10.6±0.9% weight gain, $p=0.03$; Fig. 14).

We also determined whether administration of an anti-Siglec-F Ab reduced levels of liquid stools. On day 0 none of the mice had liquid stools and their liquid stool score was 0. The liquid stool score increased in OVA challenged mice from 0 (on day 0) to 0.88±0.13 on day 35 ($p<0.001$; vs. No OVA), and to 0.75±0.16 on day 39 ($p<0.005$ vs. no OVA). Administration of an anti-Siglec-F Ab to OVA challenged mice significantly reduced the number of liquid stools at day 35 (0.13±0.13 vs. 0.88±0.13) ($p=0.01$; vs. OVA+control Ab) with a statistically insignificant trend for reducing the number of liquid stools at day 39 (0.38±0.18 vs. 0.75±0.16) ($p=0.2$; vs. OVA+control Ab).

Discussion

In this study we have utilized a mouse model of food allergen induced gastro-intestinal eosinophilic inflammation to demonstrate that administration of an anti-Siglec-F antibody significantly reduces levels of eosinophilic inflammation in the intestinal mucosa and that this was associated with significantly reduced intestinal permeability changes, normalization of intestinal villous crypt height, and restoration of weight gain. Eosinophil accumulation in the gastro-intestinal tract is a common feature of EGIDs. Although the role of eosinophils in EGIDs is not fully understood, they are believed to be one of the principal effector cells inducing gastro-intestinal tissue injury and disease pathogenesis through the release of various toxic granule proteins, lipid mediators, and pro-inflammatory cytokines [23]. In this

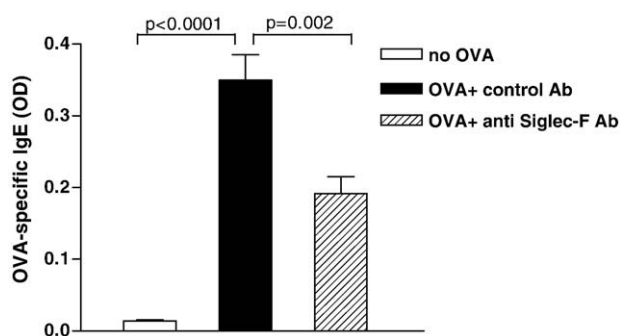


Figure 13 OVA specific IgE. OVA specific IgE levels were measured by ELISA on day 39 in the three groups of mice (no OVA challenged mice; OVA+control Ab; OVA+anti Siglec-F Ab) ($n=8$ mice/group).

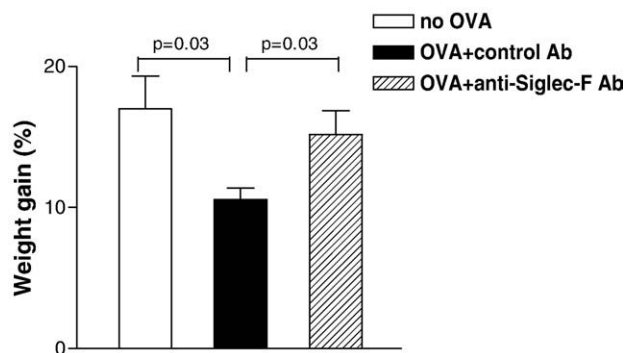


Figure 14 Weight change. The % change from baseline body weight to day 39 body weight was calculated. Intra-gastric OVA challenge (OVA+control Ab) significantly reduced weight gain (vs. no OVA, $p=0.03$). Treatment with an anti-Siglec-F antibody significantly attenuated the effect of OVA challenge on weight gain (vs. OVA+control Ab, $p=0.03$) ($n=8$ mice/group).

study, we demonstrate that repetitive intra-gastric OVA challenge can induce significant accumulation of eosinophils in the gastro-intestinal mucosa with concomitant mucosal damage including villous atrophy, crypt hyperplasia, and increased permeability. The fact that eosinophils highly express Siglec-F, and that an anti-Siglec-F antibody significantly reduces both the number of gastro-intestinal eosinophils and levels of gastro-intestinal mucosal damage, supports the notion that Siglec-F positive cells play a key role in mucosal damage associated with EGIDs. However, as mast cells contribute to oral allergen induced gastro-intestinal mucosal changes, it is not possible to determine from this study which Siglec-F positive cell (eosinophil and/or mast cell) is being targeted by the anti-Siglec-F antibody to reduce levels gastro-intestinal mucosal damage. Nevertheless, the utility of targeting Siglec-F on reducing levels of eosinophilic inflammation and gastro-intestinal mucosal damage is clearly demonstrated.

The numbers of eosinophils in the gastro-intestinal mucosa can theoretically be reduced by either inhibiting trafficking of eosinophils into the intestinal mucosa and/or alternatively by increasing the clearance of eosinophils from the gastro-intestinal mucosa. Overall our studies suggest that anti-Siglec-F decreased jejunal eosinophilic inflammation predominantly by decreasing bone marrow production of eosinophils, rather than by decreasing eosinophil proliferation, or increasing eosinophil apoptosis. In particular we noted that administration of the anti-Siglec-F Ab reduced bone marrow production of eosinophils and the number of circulating eosinophils, thus reducing the numbers of eosinophils that could be recruited from the circulation into the jejunum. Studies using double immunofluorescence labeling did not identify significant numbers of MBP+ eosinophils that were TUNEL+ in the jejunal mucosa, nor did we identify that MBP+ cells were proliferating in the jejuna mucosa. Thus anti-Siglec-F Ab did not significantly influence eosinophil proliferation or apoptosis in the jejunum.

As Siglec-8 [24] and Siglec-F are also expressed on mast cells, we examined whether the anti-Siglec-F Ab influenced mast cell trafficking to the gastro-intestinal mucosa. OVA

challenge significantly increased the number of mast cells in the gastro-intestinal mucosa. However, in contrast to the effect of the anti-Siglec-F antibody in reducing eosinophil accumulation in the gastro-intestinal mucosa, the antibody did not inhibit mast cell accumulation in the gastro-intestinal mucosa. In contrast to our *in vitro* studies demonstrating that cross-linking Siglec-F on murine eosinophils induces apoptosis [7], our studies with murine mast cells demonstrated that cross-linking Siglec-F on this cell type does not induce apoptosis. Previous *in vitro* studies using culture-derived human mast cells have also demonstrated that antibody cross-linking of Siglec-8 inhibits Fc ϵ RI-dependent histamine release but has minimal effects on mast cell viability [25,26]. Thus, it is possible that *in vivo* the anti-Siglec-F antibody had an inhibitory effect on mast cell functional responses (i.e. permeability changes in the gastro-intestinal tract), but did not have an inhibitory effect on mast cell numbers.

We also demonstrated that anti-Siglec-F reduced levels of Th2 cytokines and reduced OVA specific IgE levels. The reduction in Th2 cytokines may be mediated by Siglec-F expressed on CD4+ cells. We have previously demonstrated that OVA stimulated CD4+ cells express Siglec-F [7], and thus the reduced levels of Th2 cytokines in splenocytes derived from anti-Siglec-F treated mice may be mediated by Siglec-F expressed on CD4+ cells. In addition to reduced Th2 cytokines, anti-Siglec-F treated mice also had reduced levels of serum OVA specific IgE. The reduced levels of OVA specific IgE in mice treated with the anti-Siglec-F Ab could be due to effects of anti-Siglec-F on reducing levels of cytokines such as IL-13 which contribute to IgE synthesis. No Siglec-F has been detected on B cells suggesting that the effect of anti-Siglec-F on B cell IgE production is unlikely to be a direct one.

In summary, in this study we have demonstrated in a mouse model of food allergen induced gastro-intestinal eosinophilic inflammation that an anti-Siglec-F antibody can significantly reduce the severity of oral allergen-induced eosinophilic inflammation, villous atrophy, crypt hyperplasia, epithelial permeability changes, and associated symptoms of weight loss associated with diarrhea. These effects of the anti-Siglec-F Ab were not associated with increased numbers of apoptotic eosinophils suggesting that the anti-Siglec-F Ab may not be inhibiting gastro-intestinal eosinophilic inflammation by increasing clearance of eosinophils from the gastro-intestinal mucosa. Since human Siglec-8 is a functional paralog of mouse Siglec-F, targeting of Siglec-8 may be a novel therapeutic approach for EGIDs and other eosinophil-mediated diseases.

Acknowledgments

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