Siglec-8 antibody reduces eosinophil and mast cell infiltration in a transgenic mouse model of eosinophilic gastroenteritis

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Aberrant accumulation and activation of eosinophils and potentially mast cells (MCs) contribute to the pathogenesis of eosinophilic gastrointestinal diseases (EGIDs), including eosinophilic esophagitis (EoE), gastritis (EG), and gastroenteritis (EGE). Current treatment options such as diet restriction and corticosteroids have limited efficacy and are often inappropriate for chronic use. One promising new approach is to deplete eosinophils and inhibit MCs with a monoclonal antibody (mAb) against Siglec-8, an inhibitory receptor selectively expressed on MCs and eosinophils. Here, we characterize MCs and eosinophils from human EG and EoE biopsies using flow cytometry and evaluate the effects of an anti-Siglec-8 mAb using a novel Siglec-8 transgenic mouse model in which EG/EGE was induced by ovalbumin sensitization and intragastric challenge. Mast cells and eosinophils were significantly increased and activated in human EG and EoE biopsies compared to healthy controls. Similar observations were made in EG/EGE mice. In Siglec-8 transgenic mice, anti-Siglec-8 mAb administration significantly reduced eosinophils and MCs in the stomach, small intestine, and mesenteric lymph nodes, and decreased levels of inflammatory mediators. In summary, these findings suggest a role for both MCs and eosinophils in EGID pathogenesis and support the evaluation of anti-Siglec-8 as a therapeutic approach that targets both eosinophils and MCs.

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Aberrant accumulation and activation of eosinophils and potentially mast cells (MCs) contribute to the pathogenesis of eosinophilic gastrointestinal diseases (EGIDs), including eosinophilic esophagitis (EoE), gastritis (EG), and gastroenteritis (EGE). Current treatment options such as diet restriction and corticosteroids have limited efficacy and are often inappropriate for chronic use. One promising new approach is to deplete eosinophils and inhibit MCs with a monoclonal antibody (mAb) against Siglec-8, an inhibitory receptor selectively expressed on MCs and eosinophils. Here, we characterize MCs and eosinophils from human EG and EoE biopsies using flow cytometry and evaluate the effects of an anti-Siglec-8 mAb using a novel Siglec-8 transgenic mouse model in which EG/EGE was induced by ovalbumin sensitization and intragastric challenge. Mast cells and eosinophils were significantly increased and activated in human EG and EoE biopsies compared to healthy controls. Similar observations were made in EG/EGE mice. In Siglec-8 transgenic mice, anti-Siglec-8 mAb administration significantly reduced eosinophils and MCs in the stomach, small intestine, and mesenteric lymph nodes, and decreased levels of inflammatory mediators. In summary, these findings suggest a role for both MCs and eosinophils in EGID pathogenesis and support the evaluation of anti-Siglec-8 as a therapeutic approach that targets both eosinophils and MCs.

Key Words: Siglec-8, mouse, eosinophils, mast cells, eosinophilic esophagitis, eosinophilic gastritis, eosinophilic gastroenteritis, eosinophilic gastrointestinal disease, AK002, ex vivo, degranulation
Introduction

Eosinophilic gastritis (EG) and gastroenteritis (EGE) are rare diseases of the gastrointestinal (GI) tract, affecting minimally 20 in 100,000 people worldwide (1). Prevalence has likely been underestimated due to the absence of a standard diagnosis and medical education (2, 3), but is reported to be highest in children and adults ages 20-50 years, with a slightly higher incidence in men (1). These conditions belong to a larger spectrum of GI disorders known as eosinophilic gastrointestinal disorders (EGIDs), which are characterized by eosinophilic infiltration of the GI tract and debilitating GI symptoms (i.e., abdominal pain, nausea, vomiting, diarrhea, and dysphagia) (1). Current EGID treatment options such as diet restriction and corticosteroids have limited efficacy and are often inappropriate for chronic use. Thus, there is a significant medical unmet need for novel targeted therapies.

In EG/EGE and other EGIDs, eosinophils are believed to be one of the principal effector cells responsible for GI symptoms and disease pathogenesis (1). While mast cells have been reported to be elevated in eosinophilic esophagitis (EoE), their role in EG/EGE is less well understood, despite evidence that EGIDs are atopic in nature (4–6). Development of EG and EGE is associated with a history of allergy, food sensitivity, eczema, and seasonal asthma, implicating involvement of mast cells (1). Furthermore, mast cells and genes associated with the mast cell transcriptome are increased in EG patient tissue (7). Despite elevated numbers in EoE and EG, no further characterization of the role of the mast cell has been performed in EGIDs.

Sialic acid-binding immunoglobulin-like lectin (Siglec)-8 is a cell surface receptor that has emerged as a promising therapeutic target for treatment of allergic and inflammatory diseases. Siglec-8 is an inhibitory receptor that is found selectively on human eosinophils and mast cells (and to a lesser degree, on basophils). Binding of a monoclonal antibody (mAb) to Siglec-8 induces apoptosis to activated eosinophils and inhibits mast cell activation (8–10). Only one Siglec-8 targeted therapy, AK002, is currently in clinical development for the treatment of allergic, inflammatory, and proliferative diseases.
involving eosinophils and mast cells. AK002 is a humanized, non-fucosylated IgG1 anti-Siglec-8 mAb that has been shown to deplete eosinophils via antibody-dependent cellular cytotoxicity and apoptosis, and inhibit mast cell function (8).

Given the putative roles of eosinophils and mast cells in EGIDs, we sought to characterize these cell types in EG patient tissue and to develop a mouse model of EG and EGE wherein the effect of an anti-Siglec-8 mAb could be evaluated. Here we show that mast cells and eosinophils are significantly increased and activated in EG and EoE patient tissue biopsics compared to non-diseased tissue. Moreover, the increase in mast cell numbers in these EGID biopsies was similar in degree to that of eosinophils, suggesting that both of these cell types are involved in the pathology of EGIDs. To evaluate the potential activity of an anti-Siglec-8 mAb in a mouse model of EG and EGE, we developed a novel transgenic mouse that selectively expresses human Siglec-8 on mast cells, eosinophils and basophils, as is the case in humans (11). Systemic sensitization and intragastric challenge with ovalbumin (OVA) induced significant infiltration of eosinophils and mast cells in the stomach, small intestine and mesenteric lymph nodes (MLNs), similar to human EG and EGE disease. Therapeutic administration of an anti-Siglec-8 mAb significantly and selectively reduced eosinophils, mast cells, and inflammation in all of these GI tissues. These data provide evidence that both mast cells and eosinophils contribute to EGID pathogenesis and demonstrate that targeting of Siglec-8 suppresses eosinophil and mast cell involvement in an EG and EGE mouse model, thus supporting further clinical investigation of this receptor as a therapeutic target in EGIDs.

Results

EG and EoE tissues from patients have elevated numbers of both mast cells and eosinophils

Of the EGIDs, EoE is the most prevalent and well characterized, whereas much less is known about EG and EGE. To gain greater understanding of the biology driving EG, we procured fresh gastric tissue from patients with EG or non-diseased control subjects without a history of gastrointestinal disease.
(Supplemental Table 1). Tissues were digested into single cell suspensions and immune cells were
characterized by flow cytometry (Figure 1, A). To confirm that mast cells and eosinophils were correctly
identified in our gating strategy, we examined known surface markers for these cells within the individual
gating windows. Mast cells (CD45⁺ 7AAD⁻ SSC⁺ CD24⁻ CD16⁻) expressed Siglec-8 and CD117
(Supplemental Figure 1, A) and eosinophils (CD45⁺ 7AAD⁻ SSC⁺ CD24⁺ CD16⁻) expressed Siglec-8,
CCR3, and CD11b (Supplemental Figure 1, A). To further validate the gating strategy, we sorted mast
cells and eosinophils from their respective windows, followed by May-Grunwald Giemsa and H&E
staining, respectively. Cells sorted from the mast cell window routinely contained metachromatic granules
that were characteristic of mast cells (Supplemental Figure 1, B). Similarly, >95% of cells sorted from the
eosinophil window displayed a bilobed nucleus and eosin-pink granules that resembled human
eosinophils (Supplemental Figure 1, B). These data demonstrate that the gating strategy used in Figure 1A
correctly identifies mast cells and eosinophils in GI tissue.

As expected, EG tissues had significantly increased numbers of eosinophils compared to non-diseased
tissue (Figure 1, B). In addition, we found that mast cells were increased by approximately 5-fold in EG
tissue compared to non-diseased tissue (Figure 1, C). These data were confirmed in EG tissue using
additional flow cytometry surface markers for mast cells and eosinophils (Supplemental Figure 1, C-E).
Interestingly, mast cells were elevated to a similar extent as eosinophils in EG patient tissues (8.9% vs.
9.3% of all CD45⁺ cells, respectively). In contrast, the percentage of neutrophils and monocytes was
reduced in EG tissue compared to non-diseased control tissue, whereas other immune cells, including T
cells remained unchanged (Figure 1, D).

To determine whether these observations could be extended to other EGIDs, we processed and
characterized esophageal tissue from EoE patients. Consistent with findings in EG tissue, as well as
previously published findings, mast cells and eosinophils were significantly increased to a similar extent
compared to non-diseased esophageal tissue (Figure 1, E and F) (12–16). These data demonstrate that
both eosinophils and mast cells are markedly and proportionally elevated in both EG and EoE patient
tissue and validate our flow cytometry-based approach to quantitatively assess immune cells in fresh human tissue.

**Eosinophils and mast cells in EG and EoE tissue are in an activated state**

To characterize the state of activation of eosinophils and mast cells from EG tissue, we examined the expression of surface markers associated with activation by flow cytometry (Figure 2, A and B, Supplemental Figure 2, A). As anticipated, Siglec-8 was selectively expressed on mast cells and eosinophils from both EG and non-diseased stomach tissues (Figure 2, C and D; Supplemental Figure 2, B). In contrast to Siglec-8, IL-5Rα was minimally expressed on EG and non-diseased tissue eosinophils and mast cells (Figure 2, C and Supplemental Figure 2, C). In addition, tissue eosinophils from EG patients displayed significantly higher expression of the activation markers, CD11b and CD49d, compared to non-diseased tissue eosinophils, consistent with an increased activation state (Figure 2, C) (17–19). Moreover, mast cells from EG patients displayed significantly increased expression of the degranulation and activation markers CD63 (LAMP3) and CD107a (LAMP1) suggesting an activated and degranulating state (Figure 2, D) (20). Consistent with atopy and high serum IgE levels reported for EG patients (21), EG tissue mast cells also displayed significantly higher levels of surface IgE and FceRI (Figure 2, D, Supplemental Figure 2, D).

Lastly, to determine if increased mast cell activation occurred in other EGIDs, we examined the same markers on mast cells from EoE patient tissue. Compared to non-diseased esophageal mast cells, EoE mast cells also had significantly higher expression of CD107a, CD63, and IgE (Supplemental Figure 2, E). These data demonstrate that eosinophils and mast cells are found in elevated numbers and in an activated state in EG patient tissue. Furthermore, we show that mast cells from EoE patient tissues are also activated and display increased levels of cell surface markers associated with degranulation, such as CD107a. These findings suggest that increased mast cell activation may be a shared feature across EGIDs and supporting a role for activated eosinophils and mast cells in EGID disease pathogenesis.
Siglec-8 transgenic mice express Siglec-8 on eosinophils, mast cells and basophils

Based on the data above demonstrating elevation and activation of eosinophils and mast cells in EG patient tissue, we set out to evaluate the activity of an anti-Siglec-8 mAb in a murine disease model of EG and EGE. Like other members of the CD33-related Siglecs, Siglec-8 appears to have evolved recently, with close homologs found only in some primate species (11, 22). Therefore, to examine the in vivo activity of anti-Siglec-8 mAb, we generated a human Siglec-8-expressing transgenic mouse, distinct from previously generated mice (e.g., ROSA26-Siglec-8 knock-in mice) (23, 24). Instead, Siglec-8 transgenic founder mice were generated via the pronuclear microinjection of DNA (Supplemental Figure 3, A) as described in the methods. Transmission of the Siglec-8 transgene was successful in two of the chimeric founders’ progeny (lines 335 and 307) and the transgenic mouse line selected (335) contained a single-copy insertion of the Siglec-8 gene (Supplemental Figure 3, B-D). Siglec-8 transgenic mice did not display differences between males or females compared to wildtype (WT) littermates in body weight, weights of major organs, absolute or relative percentages of blood cell types, blood chemistries, or coagulation (Supplemental Figure 3, E). Examination of peripheral blood leukocytes (PBL) and peritoneal lavage (PL) cells from these mice showed high levels of Siglec-8 on the cell surface of mast cells, eosinophils, and basophils in PBL and PL (Supplemental Figure 4, A-C), consistent with the selective expression of Siglec-8 on human immune cells (8, 11, 25). Siglec-8 was also found on eosinophils and mast cells in other tissues, including GI, lung and skin (data not shown), demonstrating expression on both connective tissue and mucosal mast cells. In contrast, Siglec-8 was not detected on lymphocyte, neutrophil, monocyte, or macrophage cell populations (Supplemental Figure 4, A-C).

To determine if certain functional properties of Siglec-8 were preserved in the transgenic mice, such as ligand-induced internalization (26), we engaged the Siglec-8 receptor in vivo by dosing with AK002-G4 (hIgG4 anti-Siglec-8 mAb (8)) or isotype-matched control mAb and examined the extent of receptor internalization. Compared to isotype control mAb-treated mice, anti-Siglec-8 mAb treatment induced
Siglec-8 internalization, but not Siglec-F internalization on peripheral blood eosinophils as expected
(Supplemental Figure 4, D and E). Finally, insertion of the Siglec-8 gene in these transgenic mice had no
effect on endogenous levels of Siglec-F expression on eosinophils (Supplemental Figure 4, E, and data
not shown).

OVA sensitization and intragastric challenge induces EG and EGE in Siglec-8 transgenic mice

Next, we adapted a previously published experimental study design to create a Siglec-8-expressing
murine model of EG and EGE (27, 51). Eosinophils were identified in GI tissue preparations as viable,
CD45+, Lin−, Ly6G−, CD11b+, CCR3+ and Siglec-F+ cells by flow cytometry (Supplemental Figure 5, A).
GI eosinophils displayed robust Siglec-8 expression, consistent with transgene expression (Supplemental
Figure 5, A). Systemic sensitization and repeated intragastric challenge with OVA (Figure 3, A) induced
significant eosinophilic infiltration into the stomach, small intestine and MLNs compared to sham-treated
mice at study takedown on day 39 that resembled EG and EGE (Figure 3, B-G). Consistent with an
allergen-specific response, OVA-sensitized and -challenged mice had increased serum levels of OVA-
specific IgE and IgG1 compared to sham-treated mice (Figure 3, H and I).

Anti-Siglec-8 mAb reduces OVA-induced eosinophilic infiltration in the stomach and intestinal tissues

To determine the timing for therapeutic treatment with an anti-Siglec-8 antibody in our EG and EGE
mouse, we examined eosinophils in the periphery, stomach, small intestine and MLNs after the third OVA
challenge on day 32 (Supplemental Figure 6). OVA-sensitized and -challenged mice had significantly
increased eosinophils in the stomach, MLNs, and blood compared to sham-treated mice on day 32
(Supplemental Figure 6, A-C). Surprisingly, OVA-exposed mice did not have significantly increased
eosinophils in the small intestine at this time point (Supplemental Figure 6, D), suggesting the stomach
and MLNs are the primary sites of eosinophil infiltration, while the small intestine is secondary. Having
established EG and EGE-like disease on day 32, we selected this timepoint for therapeutic dosing of the anti-Siglec-8 mAb.

Therapeutic administration of an anti-Siglec-8 mAb (mIgG2a) on day 32 led to a significant reduction of eosinophils in the stomach, small intestine, and MLNs at study takedown on day 39, compared to isotype control mAb-treated mice (Figure 4, A-D, Supplemental Figure 7, A-C). Eosinophil numbers in anti-Siglec-8 mAb-treated mice were not completely eliminated in the tissue, but rather reduced to levels seen in sham-treated mice. In addition to a reduction in tissue eosinophils, anti-Siglec-8 mAb-treated mice had a significant decrease in peripheral blood eosinophils, consistent with the known ADCC-activity of this antibody isotype and subclass (28), compared to sham- and control mAb-treated mice (Figure 4, E, Supplemental Figure 7, D). To confirm the OVA-induced intestinal eosinophilia seen by flow cytometry, we quantified the mRNA levels of major basic protein (MBP), an eosinophil granule protein, in the small intestine. Anti-Siglec-8 mAb-treated mice had decreased expression of MBP down to background levels too, similar to the pattern seen with eosinophils in the small intestine compared to control mAb-treated mice (Supplemental Figure 8, A). These data demonstrate that anti-Siglec-8 mAb treatment reduced OVA-induced intestinal tissue eosinophilia in our EG and EGE mouse model.

Anti-Siglec-8 mAb reduces OVA-induced mast cell accumulation in the stomach and intestinal tissues

We also examined mast cell infiltration in the EG and EGE mouse model. Mast cells were identified in GI preparations as viable, CD45⁺, Lin⁻, CD117⁺, FcεRI⁺ cells by flow cytometry and expressed Siglec-8 (Supplemental Figure 5, B). As seen with stomach tissue eosinophils, significantly increased mast cells were found in the stomach, but not small intestine by day 32 in OVA-administered mice compared to sham mice (Supplemental Figure 6, E and F). On day 39, significantly increased mast cell numbers were seen in the stomach, small intestine, and MLNs in OVA-sensitized and challenged mice compared to sham treated mice (Figure 5, A-D; black vs gray). Therapeutic treatment with an anti-Siglec-8 mAb led to a significant reduction in the percentage of mast cells in the stomach and small intestine on day 39.
compared to control mAb-treated mice (Figure 5, A-C, Supplemental Figure 7, E-F), albeit not quite back
to baseline levels. Similar effects were seen with MLN mast cells, but at very low overall cell counts
(Figure 5, D, Supplemental Figure 7, G). To confirm the differences in OVA-induced mast cell
infiltration seen by flow cytometry, we quantified the mRNA levels of mast cell protease 1 (MCPT1) in
the small intestine. Anti-Siglec-8 mAb-treated mice had significantly reduced expression of MCPT1 in a
pattern similar to changes in mast cell levels, confirming decreased mast cell numbers in the small
intestine compared to control mAb-treated mice (Supplemental Figure 8, B).

Stomach eosinophils and mast cells are differentially reduced after anti-Siglec-8 mAb treatment

The cell-specific activity of Siglec-8 on eosinophils and mast cells has been well characterized in vitro
and ex vivo (8–10). To evaluate if the reduction of mast cells and eosinophils in Siglec-8 mAb-treated
mice reflected the known activity of Siglec-8 in vivo, we analyzed stomach tissue on day 32 (before mAb
treatment), day 34 (2-days post mAb treatment), and day 39 in our EG and EGE mouse model. As was
seen previously, OVA-challenged mice displayed elevated mast cells and eosinophils in the stomach on
day 32 compared to sham-treated mice (Figure 5, E-F). On day 34, 2 days post mAb treatment, mice
dosed with an anti-Siglec-8 mAb had significantly decreased eosinophils in the stomach compared to
isotype control-mAb treated mice, whereas mast cells decreased only nominally (Figure 5, E-F). On day
39, both stomach eosinophils and mast cells were significantly reduced in anti-Siglec-8 mAb-treated
mice, however the magnitude of decrease seen with eosinophils was greater than that of mast cells (Figure
5, E-F). These data suggest that anti-Siglec-8 mAb treatment differentially reduces eosinophils and mast
cells in the GI tract, consistent with the unique cell-specific activities of Siglec-8 in eosinophils and mast
cells.

The faster and more extensive reduction of tissue eosinophils seen in anti-Siglec-8 mAb-treated mice
compared to mast cells on days 34 and 39 suggest that Siglec-8 mAb treatment may directly decrease
eosinophils in GI tissue. To evaluate this, we collected and cultured dissociated ex vivo stomach tissue
from OVA-challenged mice on day 39 overnight in the presence of either an anti-Siglec-8 mAb or isotype control mAb, followed by analysis of eosinophils by flow cytometry. Anti-Siglec-8 mAb treatment of dissociated stomach tissue led to significantly fewer eosinophils compared to isotype control-mAb-treated tissue (Supplemental Figure 8, C-E). Similarly, AK002 directly reduced human tissue eosinophils in ex vivo lung tissue (8). These data suggest that Siglec-8 mAb treatment directly reduces GI tissue eosinophils, consistent with the known apoptotic activity of Siglec-8.

Anti-Siglec-8 mAb reduces OVA-induced inflammation in the intestine and serum

Upon activation with IgE and allergen, mast cells and subsequently eosinophils elicit inflammatory allergic effects via production of chemokines that drive a type 2 immune response. To evaluate these responses and effects of anti-Siglec-8 mAb treatment in the EG and EGE mouse model, we quantified the mRNA expression of known mediators implicated in driving type 2 inflammation in the small intestine. We did not observe increased gene expression of the eosinophil recruiting chemokine, CCL11, in OVA-challenged mice or detectable expression of the canonical Th2 mediators, IL-4, IL-5, and IL-13, in the intestine (Supplemental Figure 9, A). However, the expression of CCL17 (TARC), CCL2 (MCP1) and CCL5 (RANTES) were increased upon OVA challenge in the intestine and decreased with anti-Siglec-8 mAb treatment compared to isotype control treated mice (Figure 6, A-C). Furthermore, we measured MLN weight as a surrogate for intestinal inflammation (29). Mice sensitized and challenged with OVA had increased MLN weight compared to sham-treated mice on day 39 and, consistent with the decreased inflammatory signature in the intestine, mice treated with an anti-Siglec-8 mAb had significantly reduced MLN weights compared to isotype control-treated mice (Supplemental Figure 9, B).

To evaluate systemic changes in mice challenged with intragastric OVA, we examined the expression of cytokines and chemokines in the serum throughout the challenge phase on days 28, 32, 34, and 39. Serum levels of known eosinophil chemokines and cytokines, such as CCL11 and IL-5, were similar in OVA-challenged and sham mice (Supplemental Figure 9, C-D). In contrast, the levels of CCL2, IL-9, and...
CXCL1 increased throughout the challenge phase in mice exposed to OVA (Figure 6, D-E and Supplemental Figure 9, E). Consistent with the reduction of mast cells and eosinophils in GI tissues, serum levels of CCL2, CXCL1 and IL-9 in OVA-challenged mice on day 39 were significantly reduced with anti-Siglec-8 mAb therapeutic treatment (Figure 6, F-H). These data demonstrate that the anti-Siglec-8 mAb reduced the expression of several OVA-induced inflammatory mediators associated with eosinophil and mast cell-driven inflammation.

**Discussion**

EGIDs, including EG and EGE, are orphan diseases that are not well managed by current therapies. These studies yielded two findings of clinical significance: they provide evidence that in addition to eosinophils, mast cells are elevated in number, express Siglec-8, and display increased levels of degranulation markers within human EG tissue; and that a mouse anti-Siglec-8 mAb can reduce allergen-induced intestinal inflammation in a transgenic mouse model of EG/EGE.

Indeed, because of its selective expression on eosinophils and mast cells, Siglec-8 has emerged as a promising therapeutic target for allergic and inflammatory diseases. Mechanism of action studies in humans have demonstrated depletion of blood and tissue eosinophils and inhibition of mast cells with a humanized, non-fucosylated IgG1 anti-Siglec-8 mAb, AK002 (8). Early and ongoing clinical studies with AK002 have yielded promising results, demonstrating rapid depletion of blood eosinophils in healthy volunteers with acceptable safety and tolerability (30). Clinical trials in multiple mast cell and eosinophil-driven diseases are ongoing.

While aberrantly high numbers of gastric eosinophils in patients are well documented and serve as the diagnostic criterion for EG and EGE, the involvement of mast cells has not been fully established. A role for mast cells driving EGID pathogenesis is supported by the association of EGIDs with atopic diseases, the effectiveness of dietary therapy, and induction of immediate and late phase allergic reactions upon mast cell activation via high-affinity IgE receptors (12, 13, 31). Earlier studies in EoE have shown that
mast cells are elevated and correlate with clinical symptoms as well as endoscopic and histologic findings (14). To better define the role of mast cells and eosinophils in EG, we used flow cytometry to characterize fresh gastric tissue from EG and non-diseased subjects at the single cell level and found that both eosinophils and mast cells were selectively and significantly increased in EG gastric tissue. In addition, we found significantly elevated levels of mast cells and eosinophils in tissue from EoE patients, consistent with previously published findings (15, 16, 32, 33). Interestingly, we show that mast cells are elevated by a similar magnitude as eosinophils in both EG and EoE tissue, a finding not previously reported. Surprisingly, we found that the frequencies of neutrophils and monocytes were reduced in EG tissue compared to non-diseased gastric tissue. While this observation could reflect differences in the type of tissue examined (biopsy vs. resected tissue), it could also be related to the strong Th2 response associated with EG. These hypotheses will be addressed in future studies.

Further evaluation of mast cells and eosinophils in human EG tissue showed that eosinophils from EG tissue were activated, based on the expression of CD11b and CD49d (as previously described with EoE) (17–19, 34). Additionally, mast cells from EG and EoE patient tissues displayed significantly increased degranulation markers (CD107a and CD63) and surface-bound IgE, consistent with an atopic state. To our knowledge, this is the first evidence demonstrating that mast cells in EG/EGE tissue are activated and apparently undergoing degranulation, based on changes in surface marker expression. These results suggest that, in addition to eosinophils, mast cells are key effector cells in EGID disease pathogenesis, and that therapies that only affect one of these cell types may not fully address EGID pathogenesis.

Our analysis confirmed that Siglec-8 was highly expressed on human tissue eosinophils and mast cells, which stands in contrast to the low expression levels of IL-5Rα. To explore the therapeutic potential of Siglec-8, we used an EG and EGE disease model in Siglec-8 transgenic mice. Development of a transgenic mouse was necessary due to the disparities between human Siglec-8 and its functional paralog in mice, Siglec-F, most notably, differences in Siglec-8 and Siglec-F function and a lack of expression of Siglec-F on murine mast cells (9). Sensitization and intragastric challenge with OVA led to a significant
increase in eosinophils and mast cells in stomach, intestinal, and MLN tissue, correlating with the human ex vivo data described above, further implicating both eosinophils and mast cells in EG pathogenesis.

Treatment with an anti-Siglec-8 antibody reduced mouse eosinophils and mast cells across all GI tissues assessed, as well as decreased blood eosinophils. In this model, GI tissue eosinophils were reduced to sham (baseline) levels in anti-Siglec-8 mAb treated mice. Interestingly, a similar pattern of eosinophil reduction to sham levels was also seen after anti-Siglec-F antibody treatment in two different EGID mouse models (27, 35). These data suggest, but do not prove, that anti-Siglec-F/8 treatment may preferentially reduce inflammatory eosinophils over the non-inflammatory, tissue-resident eosinophil populations in the mouse. Given that the lifespan of murine intestinal eosinophils is estimated to be greater than 7 days (36, 37), our data also suggests that anti-Siglec-8 mAb may elicit direct effects against existing tissue eosinophils, rather than simply on their production. Indeed, ex vivo treatment with an anti-Siglec-8 mAb significantly reduced murine stomach tissue eosinophils compared to isotype control mAb-treated cells, consistent with our findings of AK002’s effect on human tissue eosinophils (8). The reduction of tissue eosinophils by anti-Siglec-8 mAb or AK002 in ex vivo tissue, where accessory cells are absent, is most likely due to the direct, ADCC-independent induction of death of primed eosinophils. Anti-Siglec-8 mAb treatment also significantly reduced the number of mast cells in stomach and small intestinal tissue. As anti-Siglec-8 mAbs are known to have an inhibitory, rather than apoptotic, effect on mast cells, the mechanism of reduction of mature mast cells in the intestine in our model is likely due to the decreased recruitment of mast cell progenitors through reduced production of chemokines, including CCL5 and CCL2 (8, 10). Indeed, the CCL2/CCR2 axis has been shown to be important in the recruitment of mast cell progenitors in allergic airway inflammation (38), suggesting that CCL2 plays a role in the recruitment of mast cell progenitors to inflamed GI tissue. Alternatively, anti-Siglec-8 mAb treatment could directly decrease mast cell progenitors or inhibit pro-survival signaling pathways in mature mast cells, resulting in reduced mature mast cell numbers over time. However, Siglec-8 expression is restricted to mature mast cells and the turn-over of mast cells is thought to be in the range of months, suggesting...
that mice would need to be dosed with an anti-Siglec-8 mAb for a longer period of time to allow for mast cell turn-over (10, 39). The exact mechanism of Siglec-8 mediated mast cell reduction will be addressed in future studies. Consistent with the apoptotic vs. inhibitory activity of Siglec-8 on eosinophils and mast cells, respectively, we found that eosinophils were reduced faster and to a greater extent than mast cells in anti-Siglec-8 mAb treated mice. These data may reflect the apoptotic activity of Siglec-8 on eosinophils, and an inhibitory effect on mast cells. In support of Siglec-8 mediated mast cell inhibition in vivo, anti-Siglec-8 mAb treatment has recently been shown to inhibit IgE-mediated passive systemic anaphylaxis in humanized mice (8).

Treatment with the anti-Siglec-8 mAb also reduced the expression of inflammatory cytokines and chemokines in intestinal tissues and serum. We did not observe increased expression of the canonical Th2 mediators, IL-4, IL-13 and CCL11, despite significant production of OVA-specific IgE and IgG. The lack of change in these Th2-driving cytokines may be attributed to the C57BL/6 background of the transgenic mice (40, 41). Mice sensitized and challenged with OVA displayed increased expression of other inflammatory cytokines and chemokines associated with type 2 inflammation, including CCL2, CCL5, CCL17, IL-9 and CXCL1, in the intestine and serum. Many of these mediators have been implicated in driving eosinophilic and mast cell infiltration in allergic asthma and food allergy models (42–44). The OVA-mediated increase of cytokines and chemokines was significantly reduced by anti-Siglec-8 mAb, suggesting that mast cells and/or eosinophils may drive their expression. Indeed, CCL2, CCL5 and IL-9 have been shown to be produced by mast cells as well as promote eosinophil recruitment (42, 45–49); however, the role of these specific mediators in the stomach and small intestine remains to be elucidated. Consistent with an overall reduction in allergen-induced GI inflammation, Siglec-8 mAb treated mice also had decreased MLN weights compared isotype-treated mice.

In summary, we show that mast cells, as well as eosinophils, are significantly elevated and activated in gastric tissue from patients with EG. Consistent with human disease, our mouse model of EG and EGE also displayed infiltration of eosinophils and mast cells in GI tissues. Therapeutic treatment with an anti-


Siglec-8 mAb significantly suppressed eosinophil and mast cell accumulation and intestinal inflammation. Collectively, these findings suggest a role for both mast cells and eosinophils in EGID pathogenesis and further support the evaluation of Siglec-8 as a new therapeutic approach to treat EGIDs.

**Methods**

**Generation of Siglec-8 transgenic mice**

Siglec-8 transgenic founder mice were generated via the pronuclear microinjection of DNA containing the hSiglec-8 gene and the flanking regions containing the putative native promoter and regulatory elements into (C57BL/6J X SJL/J) F2-fertilized murine eggs and transferred to pseudopregnant recipients using standard methodology at the University of Michigan transgenic animal model core (50). At 2 weeks of age, viable pups were assayed by PCR for the hSiglec-8 gene using specific primers (forward primer 5’-AATCAGGTCCCGCCAATAGGAAAAATAATG-3’; reverse primer 5’-CGTGATATAAATCCCCAAGCAACTCCAAT-3’). Transmission of the Siglec-8 transgene was successful in 2 of the chimeric founders’ progeny. Siglec-8 transgenic lines 307 and 335 were established and further characterized. Mouse genotypes from tail snips were determined using real-time PCR with specific probes designed for Siglec-8 gene by Transnetyx (Cordova, TN). Raw signal intensity is reported after correcting for the housekeeping gene, c-June. ARQ Genetics (Bastrop, TX) was contracted to determine the copy number of Siglec-8 in gDNA from Siglec-8 transgenic lines compared with normal human genomic DNA. gDNA was isolated from tail biopsies of transgenic mice, non-transgenic littermate controls, and human blood. Primers within exon 1 of Siglec-8 were designed using Primer Express 3.0 from Applied Biosystems (Grand Island, NY) (forward primer 5’-GGGCCTGTGTGTCCATGTG-3’; reverse primer 5’-CCATGAACTGGGTCAGAGTCAGT-3’). A FAM-labeled Siglec-8 probe (5’-CCTGCTCCTTCTCCTACCCCCAGGA-3’) was used for qPCR (Applied Biosystems). Amplification curve threshold cycle of the human controls, having a copy number of two, were set to a relative quantity (RQ) level of 1.00; the mouse line samples were quantified relative...
to human. A mouse line with one copy per genome is consistent with an RQ of 0.5 in this assay. The 335
line was selected and back-crossed 6 times to C57BL/6 by Taconic Biosciences (Rensselaer, New York)
under murine pathogenic free (MPF) health standards prior to any analysis. Age-matched C57BL/6 WT
control mice were purchased from Taconic Laboratories and Charles River Laboratories (CRL) was
contracted to perform comparisons of age and sex-matched WT littermates to Siglec-8 transgenic mice.
Blood chemistries, coagulation, and hematology were performed by Charles River RADS bioassay
services.

**Mouse model of OVA-induced eosinophilic gastritis with gastroenteritis**

EGE was induced as previously described (27, 51), in which the induction of EG was a novel finding
from the same study design. Briefly, Siglec-8 transgenic mice (8-10 weeks of age) were systemically
sensitized with OVA (Sigma) in 1mg aluminum hydroxide adjuvant (alum) on day zero and day 14.
Beginning on day 28, mice received a total of 6 intragastric OVA challenges every 2 days. Mice received
a single intraperitoneal (IP) dose of 5 mg/kg of either an anti-Siglec-8 mAb (mIgG2a, Allakos, Redwood
City, CA) or isotype-matched control mAb (mIgG2a, Biolegend) on day 32. Sham-treated mice were
systemically sensitized with OVA + alum but intragastrically challenged with phosphate-buffered saline
(PBS; Sigma). Mice were harvested on day 39 or as otherwise indicated.

**Siglec-8 in vivo internalization study**

Siglec-8 transgenic mice (8-10 weeks of age) were systemically dosed (IP) with 100 µg isotype control
(hIgG4, Eureka Therapeutics) or AK002-G4 (humanized hIgG4 anti-Siglec-8 mAb that does not have
ADCC activity) (8). After 48 hours, peripheral blood was collected and transgenic Siglec-8 and
endogenous Siglec-F expression was analyzed on eosinophils using flow cytometry. Conjugated
antibodies to non-cross-reactive epitopes were used to detect any remaining Siglec-8 (Allakos, Inc,
Redwood City, CA) or Siglec-F on the cell surface (Biolegend).

**Human Tissue Collection**
EG and EoE were diagnosed as ≥30 eosinophils/HPF in 5 HPFs in the gastric mucosa and ≥15 eosinophils/HPF in the esophagus without any other cause for the gastric/duodenal eosinophilia (e.g., parasitic or other infection or malignancy). EGID tissue was collected and placed into Roswell Park Memorial Institute (RPMI) with Penicillin Streptomycin (Sigma) at the University of Utah and shipped overnight to Allakos on wet ice for flow cytometry analysis. Human esophageal and stomach tissue were provided by the NCI Cooperative Human Tissue Network (CHTN) from subjects who expired from non-gastrointestinal diseases. Other investigators may have received specimens from the same tissue specimens. All tissues were processed and analyzed approximately 24 hours after collection.

**Blood and tissue sample processing**

EDTA-treated anti-coagulated blood was collected by cardiac, tail, or submandibular vein bleeding. Peritoneal cells were harvested in PBS. Both were obtained using an IACUC approved animal protocol. Blood was processed by lysing red blood cells (RBCs) with ammonium-chloride-potassium buffer and washed in PBS (Thermo Fisher Scientific, Waltham, MA) to obtain PBL. PBL were resuspended in RPMI 1640 medium + 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA). Human and murine intestinal tissue was enzymatically and mechanically dissociated using the gentleMACs™ Dissociator system (Miltenyi Biotec, Germany), according to the manufacturer's protocol. Cells derived from tissue were then treated with RBC lysing buffer, washed in PBS and resuspended in RPMI 1640+10% low IgG FBS. Immediately after digestion, cell viability was examined using flow cytometry. From biological fluids and dissociated tissues, only single-cell suspensions that had at least 70% viability were used in subsequent experiments.

**Flow cytometric analysis of blood and tissue**

PBL from murine blood and single cells derived from tissue were obtained as described above. Approximately 0.1–0.5 x 10^6 cells were stained/well in 96-well plates with mouse or human Fc blocking reagents (BD Biosciences, Franklin Lakes, NJ) for 10 minutes at 4°C followed by incubation with
conjugated antibodies for 10 minutes at 4°C. Cells were then washed with fluorescence-activated cell sorting buffer (0.1% bovine serum albumin in PBS), resuspended in fixative (1% paraformaldehyde in PBS) and analyzed by flow cytometry on a NovoCyte flow cytometer (Acea Biosciences, San Diego, CA). Antibodies used for identifying immune cell populations in murine tissue included: Fc Block (BD Biosciences, Franklin Lakes, NJ), CD45 BV785 (BioLegend, 103149), CD3e Biotin (BD, 553060), CD4 Biotin (BD, 553728), CD8a Biotin (BD, 553029), CD19 Biotin (BD, 553784), TER-119 Biotin (BD, 553672), CD5 Biotin (BD, 553019), CCR3 PE (BioLegend, 310714), Ly6G PECy7 (BioLegend, 127618), CD11b APCCy7 (BD, 101257), Siglec-F BV421 (BD, 562681), Streptavadin BV605 (BioLegend, 405229), FceRI PECy7 (BioLegend, 334620), CD49b APCCy7 (BioLegend, 108918), CD117 SB436 (eBioscience, 62117182), Siglec-8 AF647 (Allakos, Redwood City, CA). Live/dead cells were identified by 7AAD (BD, 559925). ΔMFI was determined by subtracting median fluorescence intensity (MFI) for a Fluorescence Minus One (FMO) control sample from the MFI for cells stained with a conjugated antibody. Gating strategy for cells from mouse tissues was as follows: Mast cells: CD45+ Lineage (CD3, CD4, CD8, CD19, CD19, TER-119, CD5)- IgER+ CD117+; Eosinophils: CD45+ Lineage- CD11b+ Ly6G- Siglec-F+ CCR3+. The gating strategy for identifying human immune cells was directly adapted from Yu et al (52). Antibodies used for identifying immune cell populations in human tissue included: CD45 BV785 (BD, 368528), CD14 BV605 (BioLegend, 301834), CD123 BV421 (BioLegend, 563362), CD16 PeCy7 (BD, 557744), CD24 APC Cy7 (BioLegend, 311132), HLA-DR APC (BD, 340549), CD11c PE (BioLegend, 301606), IgER FITC (Miltenyi, 130095978), CD117 PE/APC (Miltenyi, 130091733), CD11b PE (BioLegend, 101257), CCR3 PE (BD, 310714), and CD123 BV421 (BD, 563362). Live/dead cells were identified by 7AAD (BD, 559925). IL5Rα PE (BD, 555902), and Siglec-8 PE (R&D Systems, FAB7975P). Gating strategy for cells from human GI tissue in Figure 1A was as follows: Mast cells: CD45+ SSChi CD16- CD24-; Eosinophils: CD45+ SSChi CD16- CD24+; Neutrophils: CD45+ SSChi CD16+ CD24+; Monocytes: CD45+ SSClo CD14+ CD24+; Basophils: CD45+ SSClo CD14- CD123+ HLA-DR-; B and T cells: CD45+ SSClo CD14- CD123- CD11c- CD16-; NK cells: CD45+ SSClo CD14- CD123- CD11c- CD16+. 
Human GI tissue was processed into single cells as described above. Following digestion, cells were stained with 7AAD, CD45 BV421, CD16 FITC, and CD24 APCCy7 to identify mast cells and eosinophils for flow cytometry. Mast cells (CD45+ 7AAD- SSCHi CD16- CD24-) and eosinophils (CD45+ 7AAD- SSCHi CD16- CD24+) were sorted by FACS (Sony Biotechnology) into RPMI + 10% FBS (Gibco). Approximately 80K mast cells and 100K eosinophils were sorted with greater than 95% purity. Following sorting, cells were centrifuged for 5 min at 1200 RPM to create cell pellets and resuspended for cytopsin evaluation using a Shandon Cytospin 3 instrument. For May-Grunwald Giemsa staining, the slides were then fixed in methanol for 90 seconds and stained with May-Grunwald Giemsa stain (Newcomer Supply part #s 1210A and 1121A) according to the manufacturer’s instructions. For H&E staining, the slides were stained with Gill III hematoxylin, transferred to 4.25% acetic acid, and dipped in eosin. Slides were evaluated by a pathologist, using an Olympus BX51 microscope, equipped with a DP72 camera. Representative images were captured using CellSensEntry, v 1.18 (Olympus) at an original magnification of 400x.

Cytokine, chemokine and OVA-specific IgE and IgG1 analysis in serum and tissue

RNA was isolated from homogenized mouse small intestinal tissue according to methods provided by the manufacturer (Qiagen). cDNA was synthesized according to manufacturer’s protocol (Thermo Fisher) and qPCR was performed using SYBR green (Thermo Fisher) and pre-designed gene specific primers for GAPDH, MCPT1, MBP, CCL17, CCL2, CCL5, CCL11, CCL24, IL4, IL5, and IL-13 (IDT). Relative gene expression was calculated using the ΔΔCt method. Levels of cytokines and chemokines in mouse serum were quantified using Luminex technology (Millipore). OVA-specific mouse IgE and IgG was quantified by ELISA according to manufacturer’s procedures (MD Bioproducts, St. Paul, MN).

Statistics
To determine statistical significance, nonparametric Mann Whitney U test, unpaired 2-tailed Student’s t test, 2-tailed t test with Sidak’s post-test, or one-way ANOVA with Tukey’s post-test for multiple comparisons were performed using GraphPad Prism (GraphPad Software). A $P$ value of 0.05 or less was considered significant.

*Study approval*

The collection of human EGID biopsy tissue was approved by the University of Utah Institutional Review Board (IRB# 00110127). The IRB approved protocol did not include collection of personal information and medical history from patients besides meeting diagnostic criteria. All animal experiments were done at Aragen Biosciences (Morgan Hill, CA) under an Institutional Animal Care and Use committee protocol. For all studies, animals were randomly selected without formal pre-randomization, and quantitative measurements were done without the opportunity for bias.

*Author Contributions*

This work was performed by employees of Allakos, Inc. BAY, ECB, RF, HSN, and NT designed the experiments, BAY, ECB, RF, JL conducted the experiments, BAY, ECB, RF acquired data, BAY, ECB, RF analyzed the data, JL, KP provided reagents, and BAY, BSB, ECB, NT wrote the manuscript.

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Figure Legends

Figure 1. EG and EoE patient tissue have significantly increased numbers of eosinophils and mast cells compared to non-diseased control tissue. (A) Flow cytometry gating strategy used to identify immune cells, including eosinophils and mast cells in human stomach tissue from EG patient. Percentage of (B) eosinophils (CD45+ 7AAD- SSCHi CD16- CD24+) and (C) mast cells (CD45+ 7AAD- SSCHi CD16- CD24-) present in non-diseased (black) or EG (gray) stomach tissue identified using the gating strategy in panel A. (D) Percentage of neutrophils, T cells, monocytes, DCs, and other (B cells, NK cells, macrophages, basophils) in non-diseased (black) or EG (gray) stomach tissue using gating strategy shown in panel A. Percentage of (E) eosinophils or (F) mast cells present in non-diseased (black) or EoE (gray) esophageal tissue identified using the gating strategy in panel A. The percentage of cells was derived from the CD45+, viable population. Data are plotted as mean ± SEM for n=7 non-diseased stomach tissue and n=4 non-diseased esophageal tissue; n=4 EG, n=3 EG+EoE, and n=3 EoE patients. *P<0.05, **P<0.01 by Mann Whitney U test.

Figure 2. Mast cells and eosinophils from EG and EoE patient tissues are highly activated compared to non-diseased control cells. (A) Dot plot of eosinophils in EG patient tissue identified by CD45+ 7AAD- CD117- CD16- CCR3+ SSCHi cells. Histogram of EG eosinophils labeled for analysis of surface expression of Siglec-8, IL-5Ra, CD11b, or CD49d or an FMO negative control (gray). (B) Dot plot of mast cells in EG patient tissue identified by CD45+ 7AAD- CD117+ FcεRI+ cells. Histogram of EG mast cells labeled for analysis of surface expression of Siglec-8, CD107a, CD63, or IgE or an FMO negative control (gray). (C) Expression as shown by ΔMFI of Siglec-8, IL-5Ra, CD11b, and CD49d on stomach eosinophils from non-diseased controls (black) or EG patients (gray). (D) Expression as shown by ΔMFI of the mast cell activation and degranulation markers, CD63, CD107a, and IgE on stomach mast cells from non-diseased controls (black) or EG patients (gray). Data are plotted as mean ± SD for n=5-6 non-diseased stomach tissue; n=2 EG and n=3 EG+EoE. *P<0.05; **P<0.01 by Mann Whitney U test.

Figure 3. Systemic sensitization and intragastric challenge with OVA induces EG and EGE in Siglec-8 transgenic mice. (A) Schematic of EG and EGE mouse model in Siglec-8 transgenic mice. Mice were systemically sensitized with OVA in alum on days 0 and 14, followed by six intragastric OVA challenges starting on day 28 until day 39. (B) Representative flow cytometry contour plots of stomach eosinophils and (C) the percentage of eosinophils in the stomach in sham or OVA-administered mice on day 39 quantified by flow cytometry. (D) Representative flow cytometry contour plots of duodenal eosinophils and (E) the percentage of eosinophils in the duodenum in sham- or OVA-administered mice on day 39 quantified by flow cytometry. (F) Representative flow cytometry contour plots of MLN eosinophils and (G) the percentage of eosinophils in the MLN in sham or OVA-administered mice on day 39 quantified by flow cytometry. (H and I) Serum levels of OVA-specific IgE or IgG1 in sham-treated mice (black) or mice sensitized and challenged.
with OVA (gray) on day 39. The percentage of eosinophils is derived from the CD45^+, viable cell population. Data are plotted as mean ± SEM (n=8-10 mice/group) and are representative of 3 experiments. *P<0.05; **P<0.01 by Mann Whitney U test.

**Figure 4. Administration of an anti-Siglec-8 mAb reduces eosinophils in gastrointestinal tissues in mice with EG and EGE.** (A) Representative flow cytometry dot plots of stomach tissue eosinophils in sham, OVA+ isotype control mAb or OVA + anti-Siglec-8 mAb treated mice. The percentage of eosinophils on day 39 in the (B) stomach, (C) duodenum, (D) MLN and (E) peripheral blood quantified by flow cytometry in sham mice (black) or mice sensitized and challenged with OVA and dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue). The percentage of eosinophils is derived from the CD45^+, viable cell population. Data are plotted as mean ± SEM (n= 6-7 mice/group) and are representative of 3 experiments. *P<0.05; **P<0.01 by one-way ANOVA with Tukey’s multiple comparisons test.

**Figure 5. Administration of an anti-Siglec-8 mAb reduces mast cells in gastrointestinal tissues in mice with EG and EGE.** (A) Representative flow cytometry dot plots of stomach tissue mast cells in sham, OVA+ isotype control mAb or OVA + anti-Siglec-8 mAb treated mice. The percentage of mast cells on day 39 in the (B) stomach, (C) duodenum, and (D) MLN quantified by flow cytometry in sham mice (black) or mice sensitized and challenged with OVA and dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue). The percentage of stomach (E) eosinophils or (F) mast cells on day 32, 34, and 39 in sham (black), OVA+ isotype control mAb (gray) or OVA + anti-Siglec-8 mAb (blue) treated mice quantified by flow cytometry. The percentage of mast cells is derived from the CD45, viable cell population. Data are plotted as mean ± SEM (n= 6-7 mice/group for B-D and n=4-6 mice/group for E-F) and are representative of 3 experiments. *P<0.05; **P<0.01 by one-way ANOVA with Tukey’s multiple comparisons test (B-D) or two-tailed t test with Sidak’s post-test (E-F).

**Figure 6: Siglec-8 mAb treated mice display reduced expression of OVA-induced type 2 immune-associated inflammatory cytokines and chemokines in intestinal tissue and serum.** qPCR gene expression analysis of (A) CCL17 (B) CCL2 and (C) CCL5 in the duodenum at day 39 of study in sham mice (black) or mice sensitized and challenged with OVA and dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue). (D and E) CCL2 and IL-9 levels in serum in sham (black) or OVA (gray) treated mice on day 28 (before first OVA challenge) and days 32, 34, and 39. (F-H) CCL2, IL-9, and CXCL1 levels in serum in sham (black), OVA + isotype control mAb (gray) and OVA + anti-Siglec-8 mAb (blue) treated mice. (n=5 mice/group). Graphs are plotted as mean ± SEM (n= 6-8 mice/group) and are representative of 3 experiments. *P<0.05; **P<0.01 by one-way ANOVA with Tukey’s multiple comparisons test (A-C) or two-tailed t test with Sidak’s post-test (D-E).
Figure 1
Figure 2
Figure 3
Figure 6
Supplemental Figure 1: EG patient tissue has significantly increased eosinophils and mast cells compared to non-diseased control tissue. (A) Flow cytometry gating strategy used in Figure 1A to identify mast cells (CD45+ 7AAD- SSC^hi CD16- CD24^) and eosinophils (CD45+ 7AAD- SSC^hi CD16- CD24^) in human GI tissue. Histogram of gated mast cells (blue) labeled for analysis of surface expression of Siglec-8 and CD117 or an FMO negative control (gray). Histogram of gated eosinophils (black) labeled for analysis of surface expression of Siglec-8, CCR3, and CD11b or an FMO negative control (gray). (B) Representative images of FACS-sorted mast cells stained with May-Grunwald Giemsa and eosinophils stained with H&E from their respective windows (40x magnification; scale bar is 10 µM). (C) Alternative flow cytometry gating strategy used to identify mast cells (CD45+ 7AAD- CD117^FcεRI^) and eosinophils (CD45+ 7AAD- CD16- SSC^hi CCR3^) in human GI tissue. Percentage of (D) eosinophils or (E) mast cells present in non-diseased (black) or EG (gray) tissue identified using the gating strategy in panel C. Data are plotted as mean ± SEM for n=7 non-diseased stomach tissue; n=2 EG, n=4 EG+EoE. *P<0.05, **P<0.01 by Mann Whitney U test.
Supplemental Figure 2: Siglec-8 is selectively expressed on mast cells and eosinophils in human stomach tissue and mast cells from EoE patient tissue are highly activated. (A) Histogram of EG mast cells stained with either IL-5Rα or an FMO negative control (gray). (B) Flow cytometry dot plots of stomach eosinophils (red) and mast cells (blue) from (left) non-diseased control or (right) EG patient tissue stained with a Siglec-8 mAb. Mast cells and eosinophils were identified by flow cytometry as described in Supplemental Figure 1, C. (C and D) Expression as shown by ΔMFI of IL-5Rα and FcεRI on stomach mast cells from non-diseased controls (black) or EG patients (gray). (E) Expression as shown by ΔMFI of Siglec-8, CD63, CD107a, and IgE on esophageal mast cells from non-diseased controls (black) or EoE patients (gray). Data are plotted as mean ± SEM for n=7 non-diseased stomach tissue and n=4 non-diseased esophageal tissue; n=2 EG, n=3 EG+EoE, and n=2 EoE patients. *P<0.05, ** P<0.01 by Mann Whitney U test.
Supplemental Figure 3: Genotyping and Phenotyping of Siglec-8 Transgenic Mice. (A) Schematic of the human Siglec-8 DNA fragment used to generate Siglec-8 transgenic mice. Arrow represents the native putative promoter. (B) Tail biopsy genotyping of Siglec-8 transgenic mice or non-transgenic WT littermates by real-time PCR with specific probes designed to detect the hSiglec-8 gene. Siglec-8 raw signal data for each of the progeny derived from Siglec-8 transgenic lines 335 (n=263), 307 (n=30), and non-transgenic WT littermates (negative, n=228) are plotted. (C) Siglec-8 gDNA copy number evaluation in tail biopsies using qPCR. The presence of Siglec-8 DNA was quantified by qPCR and human values were defined as a relative quantity of 1. Values of 0.5 are consistent with 1 copy per genome. (D) cDNA from two Siglec-8 transgenic murine lines, littermate controls (negative), or a normal human donor were generated from RNA extracted from mouse or human blood. The expected 1591 bp mRNA product supports the presence of the full-length Siglec-8 coding sequence. (E) Body weights of Siglec-8 transgenic mice or WT littermates at 11 weeks of age from each sex were evaluated (n=15/group). Age-matched Siglec-8 transgenic mice or WT littermates were evaluated for fibrinogen, PTT, or PT in plasma (n=5/group). Age matched Siglec-8 transgenic mice or WT littermates were evaluated for serum chemistry for Na, ALT, K, total protein, albumin, and creatinine in serum (n=5/group). Anticoagulated blood from age-matched Siglec-8 transgenic mice or WT littermates were evaluated for PLT count, HCT, HGB, RBC count, and WBC count.
Supplemental Figure 4: Siglec-8 expression is restricted to eosinophils, mast cells, and basophils in Siglec-8 transgenic mice. (A) Immune cells in PBL and PL were identified by flow cytometry. Representative histograms for the immune cells stained with either an anti-Siglec-8 mAb (blue) or isotype control mAb (gray). Siglec-8 expression plotted as $\Delta$ MFI on (B) PBL or (C) PL from panel A (mean ± SD n=4 mice). (D) Siglec-8 and (E) Siglec-F MFI on blood eosinophils in Siglec-8 transgenic mice dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue). Graphs are plotted as mean ± SEM (n= 9-10 mice/group). *$P<0.05$, **$P<0.01$ by Mann Whitney U test.
Supplemental Figure 5. Gating strategy for murine eosinophils and mast cells in stomach tissue.
(A) Eosinophils were identified as CD45+ 7AAD− Lineage− CD11b+ Siglec-F+ CCR3+. Histogram of Siglec-8 (blue) or FMO control (gray) MFI on eosinophils (B) Mast cells were identified as CD45+ 7AAD− Lineage− IgER+ CD117+. Histogram of Siglec-8 (blue) or FMO control (gray) MFI on mast cells. Lineage markers: CD3, CD4, CD8, CD19, TER119, CD5.
Supplemental Figure 6. Siglec-8 transgenic mice sensitized and intragastrically challenged with OVA have elevated stomach eosinophils and mast cells on day 32. Percentage of eosinophils on day 32 in the (A) stomach, (B) MLN, (C) peripheral blood and (D) duodenum in sham-treated mice (black) or mice sensitized and challenged with OVA (gray). Percentage of mast cells on day 32 in the (E) stomach and (F) duodenum in sham mice (black) or mice sensitized and challenged with OVA (gray). The percentage of mast cells and eosinophils are derived from the CD45⁺, viable cell population. Data are plotted as mean ± SEM (n=4-5 mice/group). *P<0.05, ** P<0.01 by Mann Whitney U test.
Supplemental Figure 7. Administration of an anti-Siglec-8 mAb reduces eosinophil and mast cell numbers in gastrointestinal tissues in mice with EG and EGE. The absolute counts of eosinophils per tissue on day 39 in the (A) stomach, (B) small intestine, (C) MLN and (D) peripheral blood quantified by flow cytometry in sham mice (black) or mice sensitized and challenged with OVA and dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue). The absolute counts of mast cells per tissue on day 39 in the (E) stomach, (F) duodenum, and (G) MLN quantified by flow cytometry in sham mice (black) or mice sensitized and challenged with OVA and dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue). Data are plotted as mean ± SEM (n= 6-7 mice/group) and are representative of 3 experiments. *P<0.05; **P<0.01 by one-way ANOVA with Tukey’s multiple comparisons test.
Supplemental Figure 8. Siglec-8 mAb treated mice display reduced expression of mast cell and eosinophil specific genes in intestinal tissue and Siglec-8 mAb directly decreased stomach eosinophils ex vivo. qPCR based gene expression of (A) *Mbp* and (B) *Mcpt1* in the duodenum on day 39 of in sham mice (black) or mice sensitized and challenged with OVA and dosed with either an isotype control mAb (gray) or anti-Siglec-8 mAb (blue) (mean ± SEM; n= 6-8 mice/group). (C) Representative dot plots of murine stomach eosinophils after overnight treatment with an isotype control-mAb (gray) or anti-Siglec-8 mAb (blue). (D) The percentage of stomach eosinophils remaining or (E) absolute eosinophil counts after overnight treatment with either 1µg/mL isotype control mAb (gray) or anti-Siglec-8 mAb (blue) in ex vivo stomach tissue (mean ± SEM; n= 6 mice/group). *P<0.05; **P<0.01 by one-way ANOVA with Tukey’s multiple comparisons test (A and B) or by Mann Whitney U test (D and E). The percentage of eosinophils remaining was calculated by normalizing the number of eosinophils in the isotype control-treated group to 100 percent.
Supplemental Figure 9. Siglec-8 mAb treated mice display reduced expression of OVA-induced type 2 immune-associated inflammatory cytokines and chemokines in serum. (A) qPCR-based gene expression of CCL11 in the duodenum on day 39 of in sham mice (black) or mice sensitized and challenged with OVA (gray). (B) MLN weights on day 39. (C, D, and E) CCL11, IL-5, and CXCL1 levels in serum in sham (black) or OVA (gray) treated mice on day 28 (before first OVA challenge) and days 32, 34, and 39. (n=6-7 mice/group). Graphs are plotted as mean ± SEM (n= 6-8 mice/group) *P<0.05; **P<0.01 by one-way ANOVA with Tukey’s multiple comparisons test (B) or two-tailed t test with Sidak’s post-test (C-E).
| Subject/Disease | Tissue   | GI Disease |
|---------------|----------|------------|
| EG 1          | Stomach  | EGID       |
| EG 2          | Stomach  | EGID       |
| EG 3          | Stomach  | EGID       |
| EoE/EG 4      | Eso/Stomach | EGID   |
| EoE/EG 5      | Eso/Stomach | EGID   |
| EoE/EG 6      | Eso/Stomach | EGID   |
| EoE 7         | Esophagus | EGID       |
| EoE 8         | Esophagus | EGID       |
| EoE 9         | Esophagus | EGID       |
| Control 1     | Stomach  | Normal     |
| Control 2     | Stomach  | Normal     |
| Control 3     | Stomach  | Normal     |
| Control 4     | Stomach  | Normal     |
| Control 5     | Stomach  | Normal     |
| Control 6     | Stomach  | Normal     |
| Control 7     | Stomach  | Normal     |
| Control 8     | Esophagus | Normal     |
| Control 9     | Esophagus | Normal     |
| Control 10    | Esophagus | Normal     |
| Control 11    | Esophagus | Normal     |

**Supplemental Table 1. EGID patients and non-diseased, normal tissue subjects.**

EG, eosinophilic gastritis
EoE, eosinophilic esophagitis
EGID, eosinophilic gastrointestinal disease