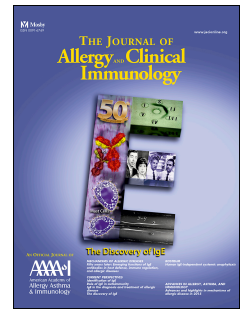


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Leveraging Siglec-8 endocytic mechanisms to kill human eosinophils and malignant mast cells

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Additional information

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ABSTRACT

Background: Sialic acid-binding immunoglobulin-like lectin (Siglec)-8 is a cell-surface protein expressed selectively on human eosinophils, mast cells, and basophils, making it an ideal target for the treatment of diseases involving these cell types. However, the effective delivery of therapeutic agents to these cells requires an understanding of the dynamics of Siglec-8 surface expression.

Objective: To determine whether Siglec-8 is endocytosed in human eosinophils and malignant mast cells, identify mechanisms underlying its endocytosis, and demonstrate whether a toxin can be targeted to Siglec-8-bearing cells to kill these cells.

Methods: Siglec-8 surface dynamics were examined by flow cytometry using peripheral blood eosinophils, mast cell lines, and Siglec-8-transduced cells in the presence of inhibitors targeting components of endocytic pathways. Siglec-8 intracellular trafficking was followed by confocal microscopy. The ribosome-inhibiting protein saporin was conjugated to a Siglec-8-specific antibody to examine targeting of an agent to these cells through Siglec-8 endocytosis.

Results: Siglec-8 endocytosis required actin rearrangement, tyrosine kinase and PKC activities, and both clathrin and lipid rafts. Internalized Siglec-8 localized to the lysosomal compartment. Maximal endocytosis in Siglec-8-transduced HEK293T cells required an intact immunoreceptor tyrosine-based inhibitory motif. Siglec-8 was also shuttled to the surface via a distinct pathway. Sialidase treatment of eosinophils revealed that Siglec-8 is partially masked by sialylated *cis*

ligands. Targeting saporin to Siglec-8 consistently caused extensive cell death in eosinophils and the human mast cell leukemia cell line HMC-1.2.

Conclusion: Therapeutic payloads can be targeted selectively to eosinophils and malignant mast cells by exploiting this endocytic pathway.

Key messages:

- Siglec-8 is internalized in human eosinophils and mast cells and trafficked to the lysosomal compartment
- The intracellular ITIM, actin rearrangement, tyrosine kinase and PKC activities, clathrin, lipid rafts, and sialylated cis ligands control Siglec-8 endocytosis
- Antibody-toxin targeting via Siglec-8 effectively kills human eosinophils and mast cell leukemia cells in vitro

Capsule summary: Our findings demonstrate that Siglec-8 can be exploited as an endocytic receptor to deliver drugs or toxins to kill human eosinophils and mast cells. This approach holds promise to treat diseases involving these cell types.

Key words: Siglec-8, endocytosis, eosinophil, mast cell, allergic diseases, chronic eosinophilic leukemia, systemic mastocytosis, immunotoxin, targeting.

Abbreviations used

BIM: bisindolylmaleimide I

88	BCR:	B cell receptor
89	CME:	clathrin-mediated endocytosis
90	ITIM:	immunoreceptor tyrosine-based inhibitory motif
91	ITSM:	immunoreceptor tyrosine-based switch motif
92	PI3K:	phosphatidylinositol 3-kinase
93	PTP:	protein tyrosine phosphatase
94	SAMP:	self-associated molecular pattern
95	SAP:	saporin
96	SFK:	Src family kinase
97	siglec:	sialic acid-binding immunoglobulin-type lectin
98	SR-A:	scavenger receptor A
99	RIP:	ribosome-inactivating protein
100	WT:	wild-type

INTRODUCTION

Eosinophils, basophils and mast cells contribute to host defense responses against parasitic infections, but are also involved in allergic disorders including asthma and certain myeloproliferative disorders.^{1, 2} Currently, there are no therapies that selectively and directly target these cells. Sialic acid-binding immunoglobulin-type lectin (Siglec)-8 is a human glycan-binding receptor expressed selectively on eosinophils, mast cells, and basophils.³ Notably, Siglec-8 is expressed at normal levels on eosinophils and basophils from patients diagnosed with hypereosinophilic syndrome, chronic eosinophilic leukemia, or chronic myeloid leukemia as well as on bone marrow mast cells from patients diagnosed with indolent systemic mastocytosis.⁴ Consistent with the presence of a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) in the protein,⁵ Siglec-8 acts as an inhibitory receptor. Ligation of Siglec-8 on eosinophils using a primary antibody and a secondary crosslinking antibody leads to cell death.⁶ Priming eosinophils with IL-5, GM-CSF, or IL-33 potentiates this cell death function of Siglec-8 and eliminates the requirement for a secondary crosslinking antibody to achieve consistently high levels of cell death.^{6, 7} In contrast, Siglec-8 ligation on mast cells does not cause cell death but rather inhibits FcεRI-mediated Ca²⁺ flux and prostaglandin D₂ and histamine release.⁸ There are no published data regarding the function of Siglec-8 on basophils. No specific endogenous ligand for Siglec-8 has yet been defined, but high molecular weight O-linked Siglec-8 sialoglycoproteins ligands have been detected in extracts of human trachea and cultured tracheal gland cells.⁹

In addition to its ligand-induced inhibitory functions, Siglec-8 may represent a target via which to deliver toxins, chemotherapeutic agents, corticosteroids or other drugs selectively into Siglec-8-expressing cells to treat a variety of diseases involving eosinophils, mast cells, and basophils

and achieve the selectivity that is currently lacking. However, the delivery of therapeutic payloads to these cell types requires knowledge of the endocytosis of Siglec-8, which has not been determined.

In this report, we examine the endocytosis of Siglec-8, identify the pathway and proteins involved, and determine whether this process can be leveraged to deliver a toxic payload to kill human eosinophils and malignant mast cells. Our findings reveal unique ways in which Siglec-8 endocytosis may be modulated and leveraged to selectively target eosinophils and mast cells. Finally, we demonstrate that the dynamics of Siglec-8 cell surface expression are well suited for the targeting of therapeutic payloads to Siglec-8-expressing cells.

METHODS

Human eosinophil isolation and culture

Written informed consent for blood donation was obtained using an institutional review board–approved protocol. Eosinophils from blood donors were purified from peripheral blood using density gradient centrifugation, erythrocyte hypotonic lysis, and immunomagnetic negative selection with CD16 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described.¹⁰ Purity and viability were consistently greater than 95% as determined by Siglec-8 staining and DAPI exclusion. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (all from Gibco, Grand Island, NY) as well as up to 30 ng/ml rhIL-5 (R&D Systems, Minneapolis, MN) as previously described.⁶ Experiments were performed with eosinophils only from a single donor; samples were not pooled for any analysis.

Mast cell lines and culture conditions

The human mast cell leukemia cell line HMC-1.2 was generously provided by P. Valent of the Medical University of Vienna (Vienna, Austria).¹¹ HMC-1.2 cells were cultured in IMDM medium supplemented with 25 mM HEPES, 3.024 g/L sodium bicarbonate, 10% FCS, 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% β -mercaptoethanol (all from Gibco). The LUVA human mast cell line was generously provided by J. Steinke and L. Borish of The University of Virginia School of Medicine (Charlottesville, VA).¹² LUVA cells were cultured in StemPro-34 serum-free medium supplemented with the StemPro-34 nutrient supplement, 1% L-glutamine, and 1% penicillin/streptomycin.

Flow cytometric analysis of Siglec-8 surface expression

The expression and internalization of Siglec-8 was assessed with a delayed secondary staining method, whereby the Siglec-8 initially present on the cell surface was bound by an unlabeled mouse IgG1 anti-Siglec-8 mAb (clone 2C4 or 1H10, the latter generously provided by Dr. Nenad Tomasevic, Allakos, Inc., San Carlos, CA) or isotype-matched control mAb (clone MOPC-21; Tonbo Biosciences, San Diego, CA) at 2.5 µg/ml at 4°C, the excess unbound antibody was washed off, and the cells were incubated at 37°C for various durations to permit internalization. The antibody remaining at the surface was detected by addition of a FITC-labeled secondary anti-mouse IgG1 antibody (BioLegend, San Diego, CA). Siglec-8 that had been shuttled or recycled to the surface during the incubation at 37°C was detected with a directly Alexa Fluor 647–conjugated 2C4 mAb after staining with the secondary antibody and extensive washing. To address the possibility of Siglec-8 shedding, surface Siglec-8 was bound by Alexa Fluor 647–conjugated 2C4 mAb, and fluorescence was detected after the cells were incubated at 37°C for various durations to permit internalization. This method was designed to measure both surface and intracellular Siglec-8; loss of fluorescence would indicate shedding of Siglec-8. Cells were analyzed using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA), and FlowJo software version 10 (Tree Star, Ashland, OR). Mean fluorescence intensity (MFI) of isotype control samples was subtracted from experimental MFIs to obtain adjusted MFIs, which were used in further calculations. The calculation of loss of Siglec-8 from the cell surface was based on the loss of fluorescence relative to fresh cells kept on ice that had not been incubated at 37°C (t=0): $\%_{loss\ at\ x\ min} = \frac{AdjMFI_{t=0} - AdjMFI_{t=x}}{AdjMFI_{t=0}}$. The same formula was used to assess shedding of Siglec-8 from the cell surface.

Assessing the effects of Siglec-8 intracellular motifs on endocytosis in Siglec-8-transduced

HEK293T cells

Tyrosine residues in the cytoplasmic signaling motifs (Y447 of the ITIM, Y470 of the ITSM) of Siglec-8 were mutated to phenylalanine residues using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Full-length Siglec-8 and the mutant versions were separately cloned into the MCS of a pCDH-CMV-EF1-GFP-PURO lentiviral vector and lentiviral particles were produced by the DNA/RNA Delivery Core at Northwestern University. The lentiviral particles were used to transduce HEK293T cells generously provided by N. Lu of Northwestern University (Chicago, IL). Total loss of Siglec-8 from the cell surface and any potential shedding of Siglec-8 at 120 min were measured by flow cytometry following delayed secondary staining and, in parallel, detection of Siglec-8 with an Alexa Fluor 647-conjugated anti-Siglec-8 mAb (2C4) in the transduced (GFP⁺) population as described above. Endocytosis calculations accounted for the loss of Siglec-8 from the cell surface due to shedding after normalization to initial levels of surface Siglec-8:

$$\%Siglec8_{internalized} = \%Siglec8_{lost\ from\ surface} - \%Siglec8_{shed}.$$

Confocal microscopy

The cells were incubated in the presence or absence of DQ Green BSA, a self-quenched dye conjugate of BSA that fluoresces when the BSA is cleaved by proteases and the quenching is relieved (10 µg/ml; Thermo Fisher Scientific, Carlsbad, CA) for 120 min at 37°C to mark lysosomes either prior to labeling of Siglec-8 on the cell surface or following Siglec-8 labeling that would lead to the visualization of internalized Siglec-8. Eosinophils were incubated with Alexa Fluor 647-conjugated anti-Siglec-8 mAb (2C4) or Alexa Fluor 647-conjugated isotype-

matched control mAb (MOPC-21; both at 2.5 µg/ml) during a 20-minute incubation at 4°C. The eosinophils were cytospun onto glass slides and fixed with 4% paraformaldehyde (Affymetrix, Santa Clara, CA) for 15 min at room temperature. Slides were mounted using Fluoromount-G mounting medium (Electron Microscopy Services, Hatfield, PA) and imaged using a Nikon A1R+ confocal laser microscope (Tokyo, Japan) using a 100×/1.45 NA oil-immersion objective lens and 488- and 640-nm lasers with a 1.5-AU pinhole. Images were acquired and analyzed using Nikon Elements software.

Siglec-8 targeting and assessment of cell death

Eosinophil cell death was assessed after 18–24 h of incubation with 2C4 mAb or isotype control (MOPC-21; both at 2.5 µg/ml) or equimolar concentrations of saporin-conjugated 2C4 or MOPC-21 (produced for us by Advanced Targeting Systems, San Diego, CA) in the presence or absence of 15 ng/ml rhIL-5. Cell death was assessed with annexin V (BD Biosciences)/DAPI (Thermo Fisher Scientific) staining. Cell death induction in the HMC-1.2 human mast cell line was assessed after 7–13 d (unless otherwise indicated) of culture in medium containing 0.78 µg/ml of the non-ionic surfactant saponin (Sigma) following a 10-min incubation at 4°C with 1.25 µg/ml of 2C4 or MOPC-21 mAbs or equimolar concentrations of their saporin-conjugated counterparts and extensive washing.

Statistical analysis

Data are presented as means ± standard deviations unless otherwise indicated. Statistical significance was determined by ANOVA and either a Dunnett or Tukey multiple comparisons test, as appropriate, using GraphPad Prism version 6.0e for Mac OS X (La Jolla, CA). When

227 appropriate, single outliers were identified and removed from the data using a Grubbs' outlier
228 test. Statistical differences were considered significant at $p < 0.05$.

RESULTS

Siglec-8 is internalized in human eosinophils and mast cell lines

Because Siglec-8 endocytosis has not been studied previously, we first sought to confirm that Siglec-8, like other siglecs, is indeed internalized following its engagement. To measure Siglec-8 endocytosis, cell-surface Siglec-8 was bound by an unlabeled anti-Siglec-8 mAb and, following incubation at 37°C to permit endocytosis, any mAb remaining at the cell surface was detected using a labeled secondary antibody. Upon antibody engagement of the receptor on primary human eosinophils, Siglec-8 was slowly internalized—about half of the initial pool of Siglec-8 surface molecules was internalized by about 90 min—and about 20% of this pool remained even after extended incubations (Fig 1, A). Because soluble forms of Siglec-8 have been detected in patient sera,¹³ the possibility that Siglec-8 shedding from the cell surface contributed to these measures of Siglec-8 endocytosis was examined. In order to assess Siglec-8 shedding, we detected Siglec-8 directly with a fluorescently labeled mAb and incubated the cells at 37°C as before. This staining protocol measures total cell-associated Siglec-8 (i.e., both cell-surface and internalized). No loss of signal was detected throughout this time course, demonstrating that the loss of signal in the previous experiment was indeed due to receptor endocytosis rather than shedding from the cell surface (Fig 1, B). Internalization of surface Siglec-8 was also measured on the human mast cell leukemia cell line HMC-1.2 in a similar fashion (Fig 1, C). As with the eosinophils, no Siglec-8 shedding from the HMC-1.2 cells was detected (Fig 1, D), indicating that the loss from the cell surface was the result of endocytosis in these cells as well. To extend these findings, the internalization of Siglec-8 was also confirmed in the LUVA human mast cell line (Fig E1). To explore the possibility that the clone of anti-Siglec-8 mAb used in these experiments (2C4) is unique in its ability to induce endocytosis, we examined the ability of

another clone (1H10) recognizing a distinct epitope on a different extracellular domain (unpublished observations) to induce Siglec-8 endocytosis. As shown, both clones cause Siglec-8 endocytosis on eosinophils (Fig E2).

Mechanisms of Siglec-8 endocytosis

Receptors may be internalized via a variety of pathways, including those mediated by clathrin or lipid rafts/caveolae as well as phagocytosis. The dependence of Siglec-8 internalization on each of these endocytic pathways was investigated on the basis of the sensitivities of each of these pathways to pharmacological or chemical inhibition. Hypertonic sucrose has been shown to impede clathrin-coated pit formation and has been used to disrupt clathrin-mediated endocytosis (CME).¹⁴⁻¹⁶ Solutions made hypertonic by the addition of sucrose (at 500 mM, but not at 250 mM or lower concentrations) significantly prevented Siglec-8 endocytosis (Fig 2, A). However, hypertonic sucrose solutions may also disrupt other endocytic pathways.¹⁷ Therefore, more selective inhibitors of each of the endocytic pathways were utilized. Amantadine inhibits the formation of clathrin-coated vesicles and thereby prevents CME.¹⁸⁻²¹ Treatment of eosinophils with 500–1000 μ M amantadine significantly decreased Siglec-8 endocytosis (Fig 2, B). Dynasore is a noncompetitive inhibitor of dynamin activity, which is required for clathrin-coated pit formation and CME.^{22, 23} Treatment with dynasore also inhibited Siglec-8 endocytosis, suggesting a role for dynamin in this process (Fig 2, C). The polyene antibiotic nystatin has been shown to inhibit lipid raft/caveolae-dependent endocytosis by sequestering cholesterol from these membrane domains.^{24, 25} Interestingly, 50 μ M nystatin treatment decreased Siglec-8 endocytosis, suggesting that Siglec-8 may be internalized via a hybrid pathway or parallel pathways (Fig 2, D). However, treatment of eosinophils with LY2940002, a PI3K inhibitor

shown to prevent both pinocytosis and phagocytosis,^{25, 26} exerted no effect on Siglec-8 internalization, indicating that these pathways play no role in the internalization of the receptor (Fig 2, E).

The Siglec-8 ITIM is necessary for maximal Siglec-8 endocytosis

It is presumed that Siglec-8 signaling through its intracellular immunoreceptor tyrosine-based inhibitory and switch motifs (ITIM and ITSM, respectively) leads to the internalization of the receptor. However, the motif and signaling molecules that are necessary for this process have not been identified. In order to examine the contributions of each motif on receptor endocytosis, we introduced Siglec-8 or mutated versions in which the tyrosine residues in the motifs have been replaced by phenylalanine residues into the HEK293T cell line by transduction (Fig 3, A). We observed that the replacement of the tyrosine in the ITIM (Y447F) significantly reduced Siglec-8 endocytosis (Fig 3, B; Y447F vs. WT, Y447/470F vs. Y470F). However, there was no effect on Siglec-8 endocytosis associated with replacement of the tyrosine in the ITSM (Y470F; Y470F vs. WT, Y447/470F vs. Y447F). Together, these data suggest that the ITIM—and not the ITSM—is responsible for receptor endocytosis upon ligation.

Siglec-8 is shuttled to the cell surface via a pathway distinct from that underlying its endocytosis

By employing pharmacological inhibitors and disruptors of various signaling molecules and cytoskeletal elements, we investigated the roles of these molecules in Siglec-8 surface expression dynamics. Treatment with the actin monomer-sequestering agent latrunculin B or the actin polymerization-inducing agent jasplakinolide potently inhibited Siglec-8 endocytosis, indicating

that Siglec-8 is internalized in a manner dependent on actin rearrangement (Fig 4, A). In contrast, nocodazole, which prevents microtubule polymerization, exerted no inhibitory effect. To identify signaling molecules that may be involved in this endocytic pathway, we pharmacologically inhibited PKC, tyrosine kinases, Src family kinases (SFKs), and protein tyrosine phosphatases (PTPs). Treatment with the PKC inhibitor bisindolylmaleimide I (BIM) also prevented endocytosis of the receptor. In addition, treatment with the tyrosine kinase inhibitor genistein significantly blocked Siglec-8 endocytosis. Based on the canonical molecular mediators of ITIM signaling,²⁷ we expected to find that Src family kinases (SFKs) and protein tyrosine phosphatases (PTPs) were involved in Siglec-8 endocytosis; however, no effect was observed with the SFK inhibitor PP1 or the PTP inhibitor sodium orthovanadate.

Because this method of tracking Siglec-8 endocytosis only labels the pool of Siglec-8 molecules initially on the cell surface, we employed an additional sequential labeling step to detect Siglec-8 newly expressed on the cell surface during the incubation. This technique involved using unlabeled anti-Siglec-8 mAb to ligate the receptor to induce and measure endocytosis and only then using directly labeled antibody of the same clone to measure newly expressed surface Siglec-8. Indeed, this technique indicates the expression of low levels of unbound Siglec-8 at the cell surface after 120 min and after as little as 5 min of incubation at 37°C (Fig E3 and data not shown). As expected, the binding of directly conjugated anti-Siglec-8 to cells that had been preincubated with unconjugated mAb and maintained at 4°C to prevent endocytosis and shuttling did not significantly differ from staining with an isotype control antibody (Fig E3). To explore the mechanisms involved in shuttling Siglec-8 to the cell surface, we again relied on pharmacological inhibition. Shuttling of Siglec-8 to the cell surface occurred independently of

cycloheximide treatment, indicating that this was not Siglec-8 synthesized *de novo*, and was dependent on microtubule assembly, actin rearrangement, dynamin function, and the activities of tyrosine kinases and PI3K (Fig 4, *B*). While these data do not rule out recycling of Siglec-8 back to the surface following disengagement from the unlabeled antibody in an intracellular compartment, the inhibition of endocytosis and concurrent promotion of shuttling to the cell surface observed with hypertonic sucrose solution (an average increase in Siglec-8 surface expression of 25% at 120 min with 500 mM sucrose) or BIM treatment (an average increase of 13–18% at 30–180 min) indicate that the recycling pathway is not likely to be the sole contributor to this pool of Siglec-8 on the cell surface (Fig 4, *C* and *D*).

Siglec-8 is internalized to the lysosome within human eosinophils

Following endocytosis, internalized receptors may follow one of several pathways into distinct intracellular compartments or back to the cell surface via a recycling pathway. The compartment to which Siglec-8 localizes was investigated by confocal microscopy. Surface Siglec-8 was first labeled with a fluorophore-conjugated mAb. Lysosomes were labeled with DQ Green BSA (DQ BSA), a self-quenched dye conjugate of BSA that fluoresces when the BSA is cleaved by proteases and the quenching is relieved. Siglec-8 was observed at the surface of cells in the absence of endocytosis and, to a small degree, at or near the surface of cells in which endocytosis was allowed to occur (Fig 5, asterisks). Co-localization of the internalized Siglec-8 signal with DQ BSA was observed, indicative of trafficking to the lysosomal compartment (Fig 5, arrows).

Sialylated cis ligands on the eosinophil surface partially mask Siglec-8 from interaction with a high-avidity ligand

Many siglecs are known to interact in *cis* with sialylated glycans at the cell surface that block interactions with lower affinity ligands, thereby setting a threshold for productive siglec signaling.²⁸⁻³² However, no such interaction had been demonstrated for Siglec-8. In order to investigate the possible interaction of Siglec-8 with masking *cis* ligands, a high-avidity synthetic ligand (biotinylated 1-MDa polyacrylamide decorated with 6'-O-sulfo-3'-sialyl-LacNAc) was used to bind Siglec-8 through its ligand-binding domain.³³ In addition, the ligand, eosinophils, both, or neither were pretreated with sialidase to remove sialic acid groups necessary for siglec binding. The amount of biotinylated synthetic ligand on the cell surface initially or following incubations of various durations at 37°C was measured by flow cytometry using fluorophore-conjugated streptavidin. Sialidase pretreatment of the ligand decreased surface binding, as expected, while sialidase treatment of the eosinophil surface increased synthetic ligand binding by approximately 50%, demonstrating that at least one-third of the surface Siglec-8 pool is prevented from interacting with this high-avidity ligand by sialylated *cis* ligands (Fig E4, A). In addition, internalization of Siglec-8-bound synthetic ligand is significantly greater in sialidase-treated eosinophils than in untreated eosinophils (Fig E4, B) but is proportional to the initial amount of ligand bound (Fig E4, C).

Targeting of an immunotoxin to Siglec-8 eliminates the need for cytokine priming for induction of death in eosinophils and allows effective killing of malignant mast cells

Signaling through Siglec-8 is well known to induce cell death in eosinophils, especially those that have been primed with IL-5, GM-CSF, or IL-33.^{6,7} However, in the absence of priming or a secondary cross-linking antibody, anti-Siglec-8 mAb fails to consistently induce substantial eosinophil cell death.^{6,7} Thus, we sought to develop an agent capable of more consistently

causing eosinophil cell death even in the absence of cellular preactivation. To this end, we chose to deliver the ribosome-inactivating protein saporin to eosinophils via Siglec-8 endocytosis. Eosinophils were incubated in the presence or absence of IL-5 and anti-Siglec-8 mAb (2C4) or isotype control with or without saporin conjugation. These treatments were compared to eosinophils that were incubated in the absence of any antibody. After 18–24 h, cell death was assessed using annexin V/DAPI staining. As expected, no statistically significant improvement was achieved in the presence of IL-5 between unconjugated versus saporin-conjugated 2C4 due to the consistently high levels of cell death seen with both antibodies (Fig 6, A and B). However, saporin-conjugated 2C4 was significantly and more consistently effective at causing eosinophil cell death in the absence of IL-5. Importantly, this was not due to a non-specific effect of the saporin because the saporin-conjugated isotype mAb did not induce cell death in the absence of IL-5. In addition, saporin-conjugated anti-Siglec-8 exhibited no off-target cell toxicity when tested separately on donor-matched neutrophils (Fig E5).

In mast cells, Siglec-8 signaling does not lead to cell death but rather inhibits IgE receptor signaling-dependent mediator release⁸. However, the selective targeting of a toxin to malignant mast cells via Siglec-8 may provide a means to eliminate such cells. Thus, we examined whether this immunotoxin could also induce the death of human mast cell leukemia HMC-1.2 cells. Following a brief preincubation with the antibodies with or without saporin conjugation, the cells were cultured and regularly assessed for viability and surface Siglec-8 levels as an indicator of protein synthesis. After one day of incubation, saporin-mediated inhibition of protein synthesis in the immunotoxin treatment group was already highly significant (Fig E6, A). Loss of cell viability in this treatment group was slightly delayed and became significant on day 2 (Fig E6,

B). To examine whether rare live populations of cells would eventually overcome the treatment effect, we assessed cell viability after at least a week of incubation. However, even at that time point, the vast majority of malignant mast cells treated with the immunotoxin were apoptotic, and there was no evidence of cells overcoming the treatment (Fig 6, *C* and *D*).

DISCUSSION

Siglec-8 is considered a potential therapeutic target for allergic disease due to its expression on eosinophils, mast cells, and basophils and its roles in inducing cell death in activated eosinophils and preventing FcεRI signaling-induced mediator release in mast cells.³⁴ However, its potential as a target via which to deliver toxins, chemotherapeutic agents, or drugs had not been explored previously and is especially relevant for mast cells and non-cytokine-primed eosinophils that do not consistently undergo apoptosis with Siglec-8 mAb exposure alone. In this study, it was determined using primary human cells and cell lines that Siglec-8 is internalized and trafficked into the lysosomal compartment via both clathrin-mediated and lipid raft/caveolae-mediated pathways that are dependent on actin cytoskeletal rearrangement and the activities of tyrosine kinases and PKC (Fig 7). Although Siglec-8 endocytosis was found to be relatively slow and endocytosis was partially hindered by the presence of sialylated masking *cis* ligands on the surface of eosinophils, this endocytic pathway was found to be suitable for targeting an immunotoxin to eosinophils and malignant mast cells. This approach effectively and consistently induced extensive cell death of eosinophils *in vitro* without prior cellular activation. Notably, for the first time, this approach has been successfully employed to target and kill mast cell leukemia cells.

Murine Siglec-F is a functionally similar paralog to Siglec-8 in that it also binds preferentially to the glycan 6'-sulfo-sialyl Lewis X,³⁵ has a similar but distinct pattern of expression (on eosinophils, alveolar macrophages, and intestinal tuft cells, rather than eosinophils, mast cells, and basophils),^{36, 37} and promotes eosinophil cell death.³⁸⁻⁴⁰ Because of this, it is often used as a surrogate for Siglec-8 in the mouse. The endocytic pathway employed by Siglec-F has been

studied,⁴¹ and one key similarity between that pathway and the one characterized here for Siglec-8 is that both siglecs are internalized slowly. In fact, relatively slow endocytosis seems to be a feature of many CD33-related siglecs. For example, CD33, Siglec-5, Siglec-F, and Siglec-9 are all internalized slowly when cross-linked with antibody, and, similar to our findings regarding Siglec-8, this endocytosis is dependent on the ITIM in the cytoplasmic domains of CD33, Siglec-F, and Siglec-9.⁴¹⁻⁴⁴ While CD33 endocytosis is promoted by treatment with a PTP inhibitor and diminished by treatment with a SFK inhibitor,⁴⁴ suggesting that tyrosine phosphorylation of the ITIM controls its endocytosis, no significant effect on Siglec-8 endocytosis was seen with similar inhibitor treatments. SFK inhibition also failed to diminish Siglec-F internalization, although tyrosine kinase inhibition prevented its endocytosis,⁴¹ as was seen with Siglec-8 as well (Fig 4, A).

These findings highlight yet another key difference between Siglec-8 and Siglec-F that may limit the usefulness of Siglec-F as a surrogate to study Siglec-8 biology. Siglec-F is internalized in a clathrin- and dynamin-independent manner,⁴¹ whereas Siglec-8 endocytosis is dependent on both of these components but is also internalized in a lipid raft/caveolae-dependent manner. While there is abundant evidence linking endocytosis to the organization of signaling events,⁴⁵ it remains to be determined whether the endocytosis of Siglec-8 via each of these pathways leads to distinct signaling pathways or functional consequences. Indeed, it remains unknown whether Siglec-8 endocytosis is necessary to achieve some of its physiologic functions, such as apoptosis induction.

It is important to note that two splice variants of Siglec-8 exist and are expressed by human eosinophils, one of which has a truncated cytoplasmic domain that lacks the ITIM and ITSM found in the full-length form of the receptor.^{5, 46} Critically, both the antibody and synthetic ligand used in this study would bind both variants of Siglec-8. Although we found that not all surface Siglec-8 is internalized in response to ligation, it is possible that some or all of the remaining surface Siglec-8 is the truncated form of the receptor that lacks the cytoplasmic signaling motifs that may be necessary for endocytosis.

Because Siglec-8 measurement and ligation were inextricably linked by using an antibody or synthetic ligand, it remains possible that Siglec-8 is constitutively internalized with the kinetics and trafficking we observed. Consistent with this possibility, the siglec CD22 undergoes constitutive endocytosis that is not affected by binding with a multivalent glycan ligand.⁴⁷ However, the fact that total surface expression of Siglec-8 did not remain constant following antibody ligation indicates that endocytosis of Siglec-8 was ligation-dependent rather than constitutive (Fig 4, C and D). The precise signaling mechanism leading to Siglec-8 endocytosis remains incompletely defined. However, genistein and BIM both prevent endocytosis of Siglec-8, implicating tyrosine kinases and PKC in this effect. Actin cytoskeletal rearrangement also played a key role in endocytosis, as demonstrated by treatment with the actin monomer-sequestering and actin filament-stabilizing agents latrunculin B and jasplakinolide. Detecting surface Siglec-8 after various periods of incubation that was not bound by the initial detecting antibody allowed the determination of how much Siglec-8 was shuttled to the surface. While some components were involved in both endocytosis and shuttling (actin rearrangement and tyrosine kinases), certain components were only involved in shuttling (microtubule

polymerization and PI3K) or endocytosis (PKC), indicating that these two processes are regulated independently. It is important to note that, although off-target effects have been noted for several of these inhibitors at higher concentrations, they were used here at concentrations that were appropriate for their intended protein targets. More uncertainty surrounds the specificity of BIM in this process, however. The IC_{50} of BIM on internalization was approximately 5 μ M (data not shown), suggesting it may be acting on an atypical PKC isoform, although effects on other PKC isoforms, PKG, or myosin light chain kinase cannot be ruled out.

Frequently, other siglecs interact in *cis* with sialylated ligands that can prevent their interaction with ligands in *trans*.²⁸⁻³² These *cis* interactions may be overcome with a higher-affinity *trans* ligand or by unmasking the siglec, e.g., through cellular activation.⁴⁸ Data in Figure E4 show for the first time that Siglec-8 is partially masked by such *cis* ligands. Although unmasking with sialidase is not essential for Siglec-8 to interact with the polymeric synthetic glycan ligand used in this study, determining the nature of this interaction and disrupting it may potentiate the effects of targeting strategies and enable less high-avidity synthetic ligands to be utilized for this purpose in the future. Given the strong preference of Siglec-8 to recognize specific sulfated α 2,3-linked sialylated structures, such *cis* ligands are likely to contain similar features, although none have yet been detected.⁴⁹⁻⁵¹

There remains a clinical need for improved, targeted therapies for diseases involving eosinophils and mast cells, such as chronic eosinophilic leukemia, hypereosinophilic syndrome, and systemic mastocytosis. While some therapeutic monoclonal antibodies, such as benralizumab, an antibody that targets IL-5R α , have shown promise in depleting eosinophils and basophils,⁵² none of these

therapies also target mast cells. In addition, there is no curative therapy for systemic mastocytosis.^{2, 53} Therefore, it was of interest to determine whether Siglec-8 endocytosis could be leveraged therapeutically in these cell types. In order to examine the efficacy of delivering a toxic payload to human eosinophils and mast cell leukemic cells through Siglec-8, a saporin-conjugated anti-Siglec-8 mAb was tested in vitro. Saporin is a type-I ribosome-inactivating protein (RIP) that lacks a B chain responsible for binding to a target cell surface for internalization that is found in type-II RIPs like ricin.⁵⁴ However, conjugation of saporin to an antibody or ligand for an endocytic receptor allows saporin to be internalized to exert its function. Therefore, a saporin anti-Siglec-8 conjugate seemed to be an ideal candidate to induce the cell death of Siglec-8-expressing cells that do not otherwise undergo Siglec-8-induced apoptosis, such as non-cytokine-primed eosinophils and mast cells.^{6, 8} Indeed, we found that the saporin-antibody conjugate caused consistently high levels of cell death in eosinophils that had not been primed with IL-5, whereas few of these cells would undergo cell death with the anti-Siglec-8 mAb alone (Fig 6, A and B). Furthermore, the saporin-antibody conjugate induced high levels of cell death in the HMC-1.2 human mast cell leukemia cell line (Fig 6, C and D). An additional concern is the type of cell death that these cells undergo. The induction of necrosis or other non-apoptotic cell death of mast cells might cause a dangerous release of pre-formed mediators in vivo. In contrast, this should not occur during apoptotic cell death. The detection of phosphatidylserine on the outer membrane leaflet by annexin V staining well before loss of membrane integrity by DAPI nuclear staining suggests that HMC-1.2 cells treated with the saporin-antibody conjugate underwent apoptotic cell death. This is consistent with prior publications using other saporin-conjugated antibodies and cell types where apoptotic cell death was observed.⁵⁵⁻⁵⁷ Brentuximab vedotin, a formulation that links the microtubule-disrupting

agent monomethyl auristatin E to a mAb specific for the CD30 protein expressed by a majority of neoplastic mast cells, has been investigated in a small number of patients with systemic mastocytosis.⁵⁸ Encouragingly, there was no evidence of mast cell mediator release upon treatment in this study. This represents the first time that Siglec-8 has been leveraged to selectively induce mast cell death. This demonstrates that Siglec-8 may be a suitable therapeutic target on eosinophils and mast cells in a range of diseases that involve these cell types. Future efforts will include the use of transgenic mice expressing Siglec-8 in appropriate cellular compartments to explore the effectiveness of Siglec-8 targeting in preclinical models of eosinophil- and mast cell-associated diseases.

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Figure legends

Figure 1. *Kinetic analysis of Siglec-8 endocytosis versus shedding.*

Siglec-8 loss from the cell surface of human primary eosinophils (**A**) and HMC-1.2 mast cells (**C**) was measured by the loss of anti-Siglec-8 mAb from the cell surface. Data represent the means $\pm$ standard deviations of 4 independent experiments. Surface Siglec-8 that remained cell-associated (intracellular or remaining on the cell surface) in eosinophils (**B**) and HMC-1.2 cells (**D**) was measured using a fluorophore-conjugated anti-Siglec-8 antibody. Remaining fluorescence at times 0 min (normal line), 30 min (dashed line), 60 min (bold line), 90 min (dotted line), and 120 min (grey filled histogram) of incubation at 37°C was detected by flow cytometry. The black histogram represents labeling with the fluorophore-conjugated mouse IgG1 control. Data are representative of results from 3 and 4 independent experiments, respectively.

Figure 2. *Siglec-8 endocytosis occurs via a clathrin- and dynamin-dependent pathway.*

Siglec-8 surface expression on eosinophils was measured as in Fig 1 after 120 min of incubation at 37°C after preincubation with the indicated concentrations of (**A**) sucrose, (**B**) amantadine, (**C**) dynasore, (**D**) nystatin, or (**E**) LY294002 and is expressed as a percentage of surface expression at 0 min. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data represent the means $\pm$ standard deviations of 3 (**A**, **B**, **C**, and **D**) or 5 (**E**) independent experiments.

Figure 3. *The ITIM but not the ITSM is necessary for maximal Siglec-8 endocytosis.*

(**A**) Schematic representations of WT and mutant forms of Siglec-8 in which the tyrosine residues in the ITIM (residues 445–450) and/or the ITSM (residues 468–473) have been mutated

to phenylalanine residues. **(B)** Siglec-8 endocytosis was examined in HEK293T cells transduced with WT or mutant forms of Siglec-8. Siglec-8 endocytosis is expressed as a percentage of that observed in HEK293T cells transduced with WT Siglec-8. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data represent the means $\pm$ standard deviations of 5 independent experiments.

Figure 4. *Siglec-8 is internalized and shuttled to the cell surface via independent pathways.* Eosinophils were pretreated with a panel of inhibitors at the indicated concentrations. Their effects on Siglec-8 endocytosis **(A)** or Siglec-8 shuttling to the cell surface **(B)** were assessed at 120 min. Siglec-8 shuttling was measured by detecting surface Siglec-8 that was not bound by the unconjugated primary antibody initially. **(C)** Eosinophils were pretreated with the indicated concentrations of sucrose as in Figure 2. Total surface Siglec-8 was divided into an original surface pool and a shuttled pool at 120 min and are represented in a stacked column, normalized to total Siglec-8 surface levels at 0 min. **(D)** Stacked columns of the original surface pool of Siglec-8 and the shuttled pool at various durations of incubation at 37°C in the presence or absence of LY294002 and BIM (both at 10 μ M). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ relative to untreated eosinophils. Data represent the means $\pm$ standard deviations of ≥ 3 independent experiments.

Figure 5. *Internalized Siglec-8 localizes to the lysosome.*

Siglec-8 at the eosinophil cell surface was labeled red with an Alexa Fluor 647-conjugated mAb, and endocytosis of the receptor was permitted at 37°C (120 min) or prevented with no incubation at 37°C (0 min). A matched fluorophore-conjugated isotype-matched mAb was used as a negative staining control in the same manner (middle panel). DQ Green BSA (DQ BSA) was

taken up by the cells during a 120-min incubation to selectively label the proteolytically active lysosomal compartment in green. An incubation with DQ BSA at 4°C only (DQ BSA [0 min]) was used as a negative staining control (top panel). Colocalization of internalized Siglec-8 and the lysosomal marker (bottom panel) is indicated by yellow fluorescence signal in the merged image (arrows). Siglec-8 at or near the cell surface and distinct from the lysosomal compartment is observed at both 0 min and 120 min (asterisks). Images are representative of 3 independent experiments. Scale bar, 5 μ m.

Figure 6. *A toxin-conjugated mAb targeted to Siglec-8 causes cell death of eosinophils and a malignant mast cell line in vitro.*

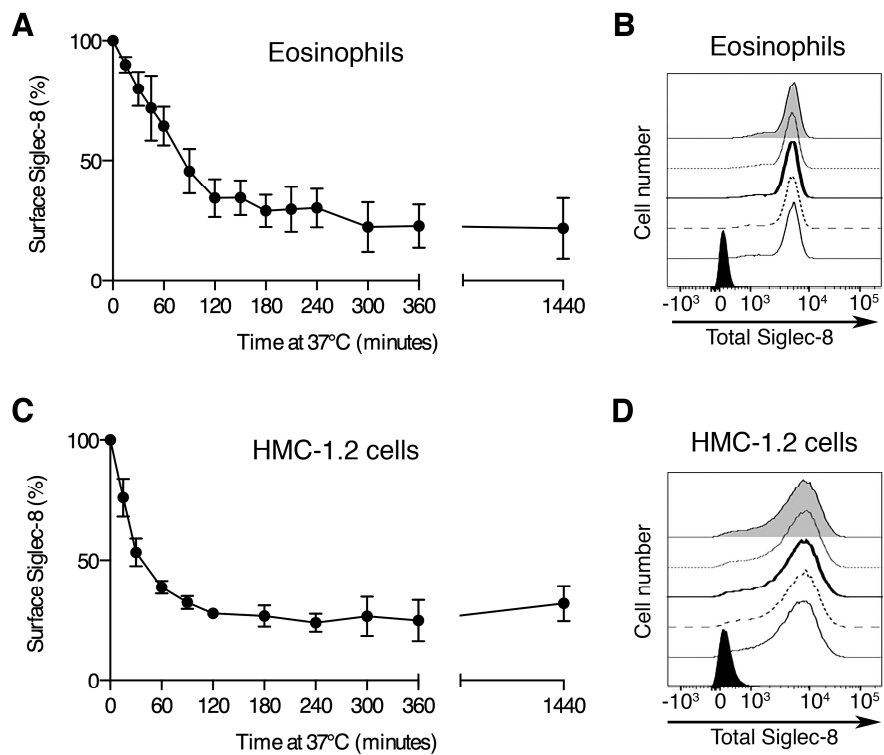
(A, B) Eosinophils were primed with 15 ng/ml IL-5 or left unprimed and concurrently treated with equimolar amounts of anti-Siglec-8 mAb (Anti-Sig8), isotype control mAb, saporin (SAP)-conjugated 2C4, or saporin-conjugated isotype control mAb or were left untreated for 18–24 h.

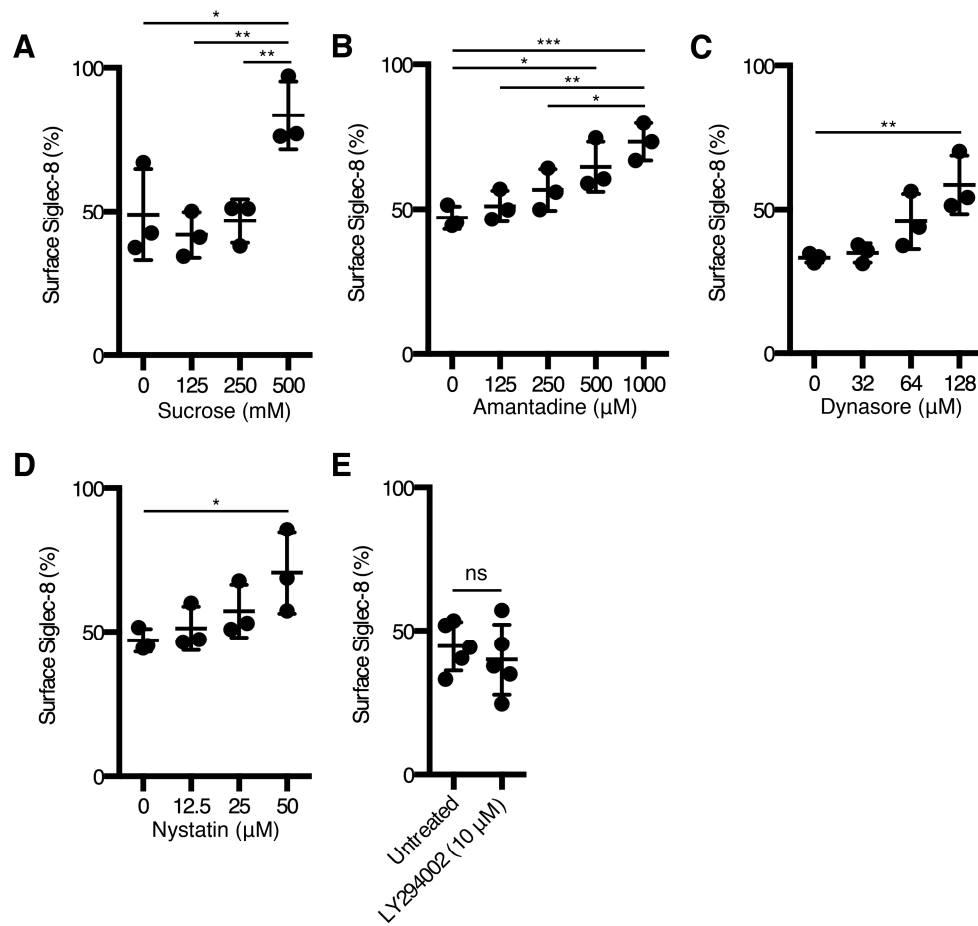
(C, D) HMC-1.2 cells were preincubated with the indicated mAbs or conjugates, washed extensively, and incubated for 7–13 d. Cell death was measured using FITC-labeled annexin V and DAPI staining and flow cytometry. **(A, C)** Representative flow plots for each treatment. Percentages of live cells are shown. **(B, D)** Percentages of viable (annexin V–negative, DAPI–negative) cells for each treatment normalized to the untreated group for each priming condition.

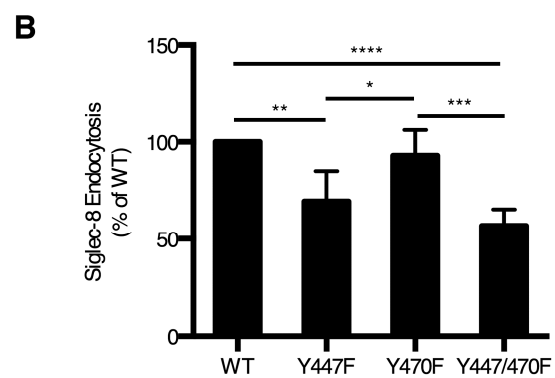
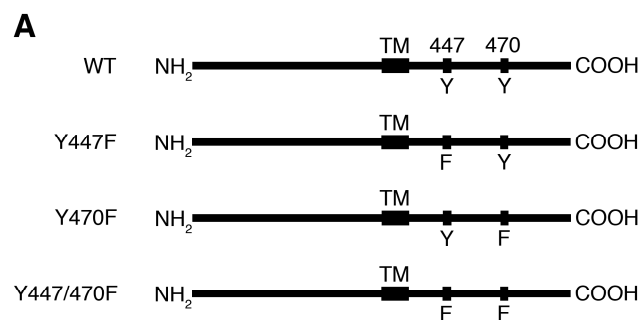
*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$. Error bars represent the means $\pm$ standard deviations **(B, D)** of 12 and 8 independent experiments, respectively.

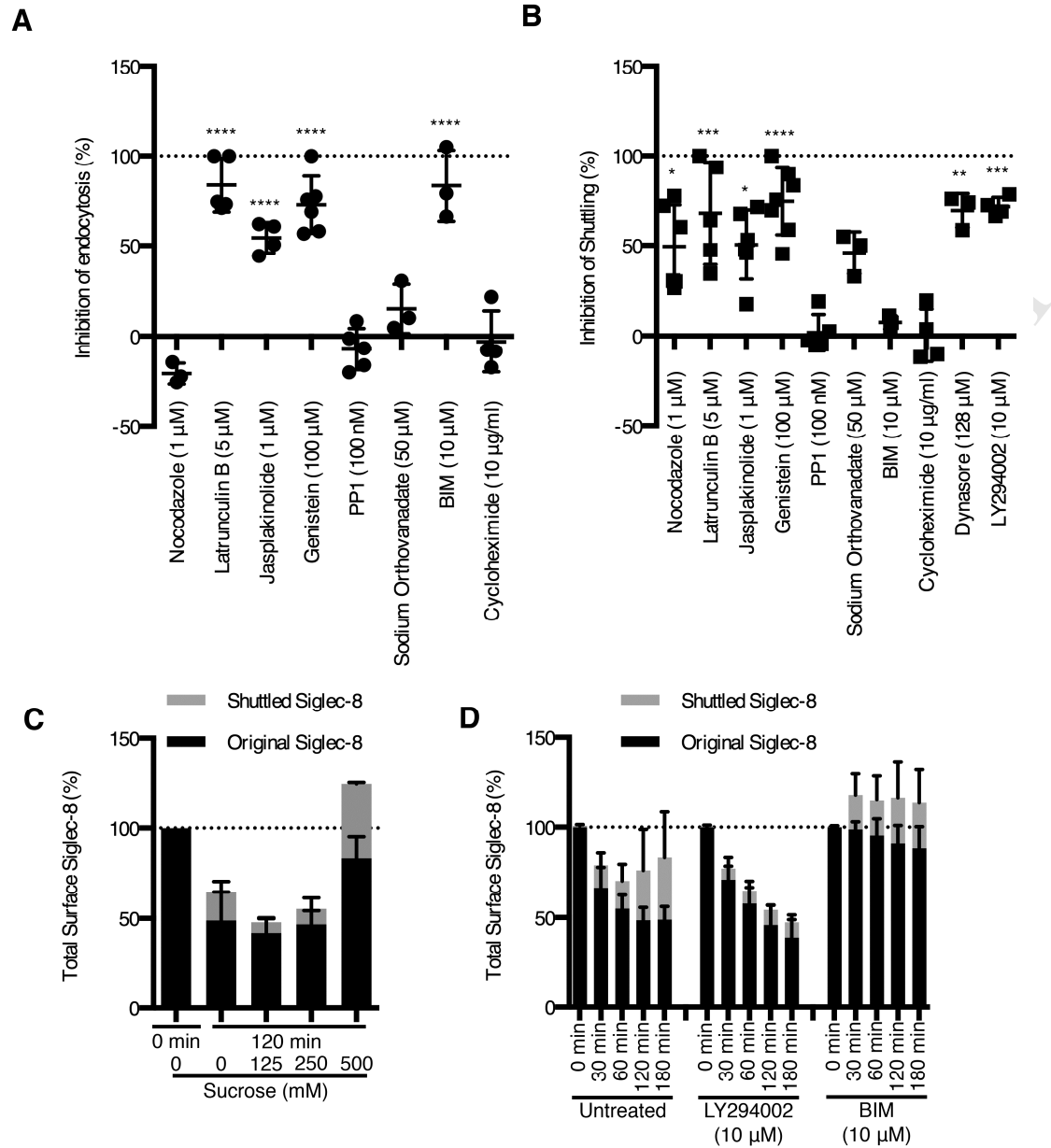
Figure 7. Schematic diagram showing a summary of mechanisms and pathways by which a toxic payload can be delivered into an eosinophil via binding to and inducing the internalization of surface Siglec-8.

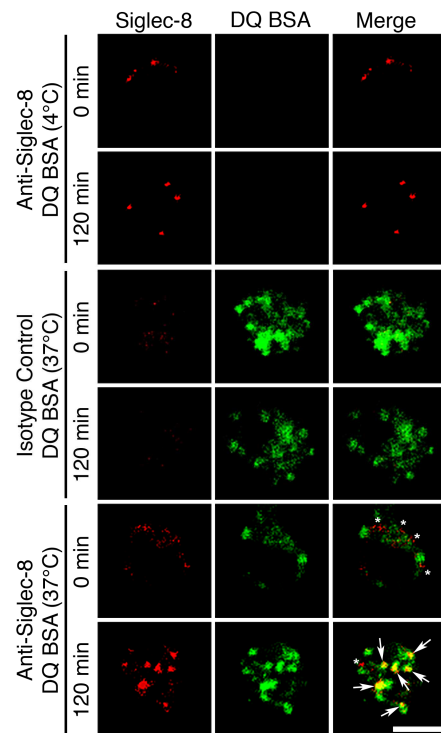
(1) At baseline, Siglec-8 interacts with sialylated *cis* ligands on the surface of eosinophils that prevent interaction with *trans* activating ligands. (2) Antibody ligation of Siglec-8 activates signaling from the cytoplasmic motifs of the receptor. Signaling from the ITIM is involved in the induction of endocytosis, which involves actin rearrangement and the activities of tyrosine kinases and PKC. (3) Siglec-8 endocytosis proceeds via both clathrin-mediated and lipid raft/caveolae-mediated pathways. (4) Internalized Siglec-8 localizes to the lysosomal compartment. (5) The linker between the cargo and the anti-Siglec-8 antibody is cleaved, and the cargo is released within the eosinophil to exert its effects.

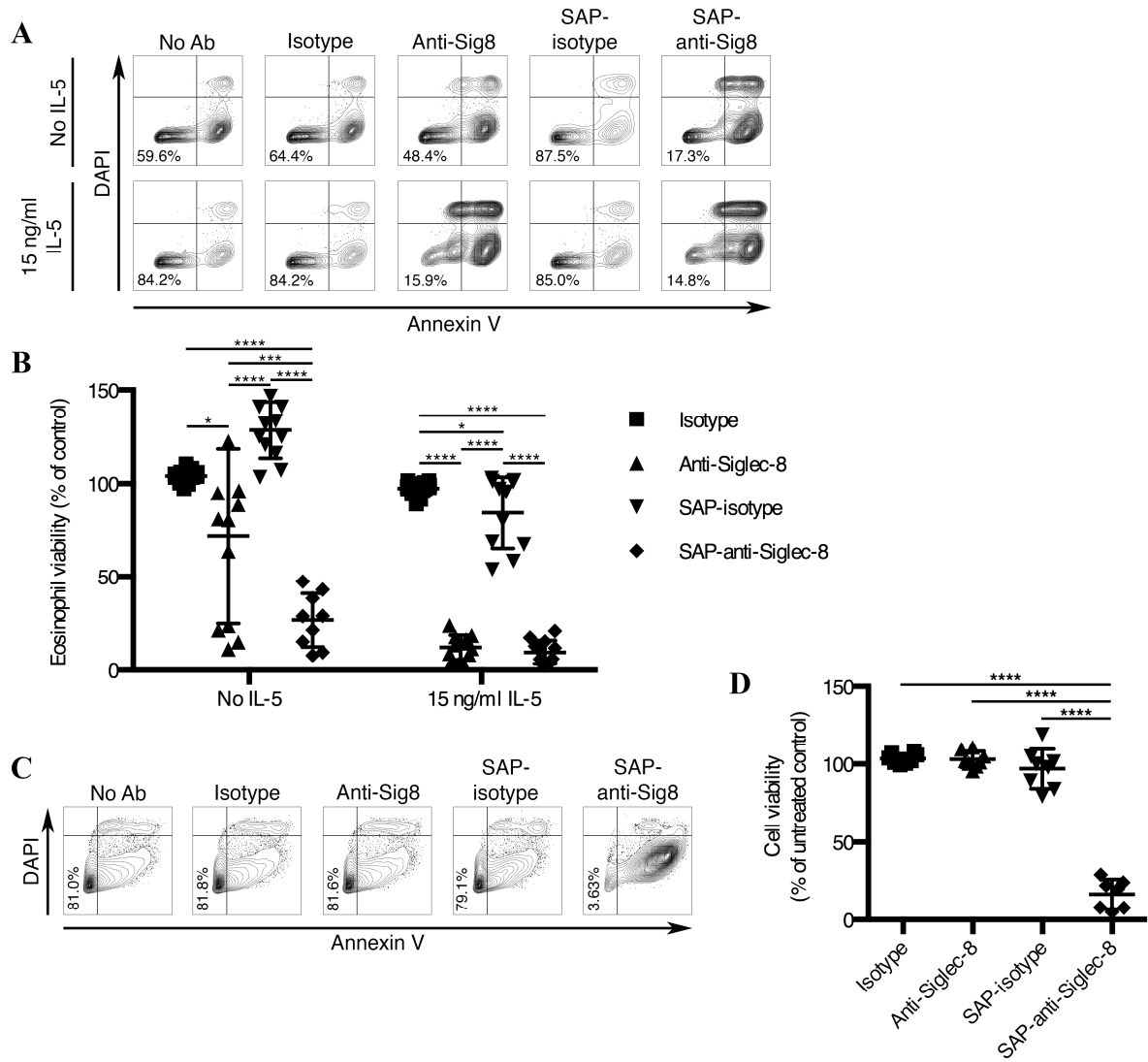


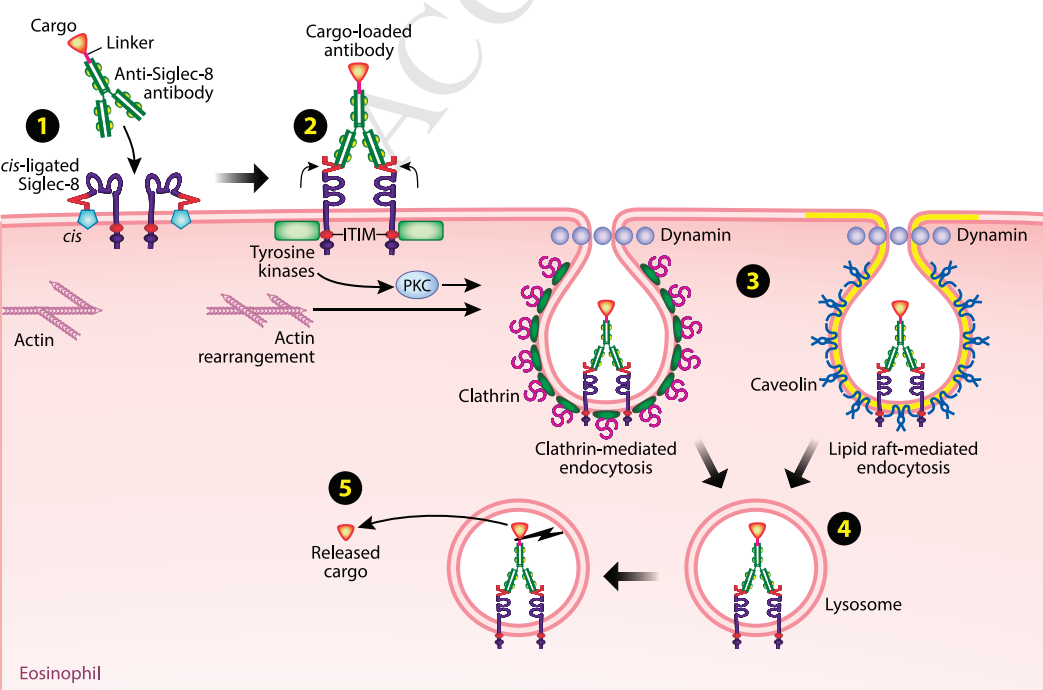












Leveraging Siglec-8 endocytic mechanisms to kill human eosinophils and malignant mast cells

Online supplement

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ONLINE SUPPLEMENTAL MATERIALS

Eosinophil inhibitor treatments

To assess the involvement of various proteins in Siglec-8 endocytosis and shuttling to the cell surface, eosinophils were treated with the following compounds at the indicated concentrations: sucrose, amantadine, nystatin, nocodazole, latrunculin B, genistein, sodium orthovanadate (all from Sigma, St. Louis, MO), dynasore, cycloheximide (both from Santa Cruz Biotechnology, Santa Cruz, CA), jasplakinolide (Calbiochem), LY294002, PP1, and bisindolylmaleimide I (all from Selleckchem, Houston, TX). Cells were pretreated with the agents for 30 min at 37°C prior to Siglec-8 ligation and maintained in the presence of these agents thereafter. Percent inhibition of endocytosis or shuttling was calculated relative to the untreated control sample: $\%_{inhibition} =$

$$\frac{\%internalization_{untreated} - \%internalization_{treated}}{\%internalization_{untreated}}.$$

Siglec-8 synthetic glycan ligand binding to eosinophils and sialidase treatments

The 6'-O-sulfo-3'-sialyl-N-acetyl-D-lactosamine (LacNAc)-polyacrylamide-biotin probe was generously provided by Prof. Nicolai Bovin (Russian Academy of Sciences, Moscow, Russia).¹ To demonstrate the necessity of the sialylation of the ligand and as a control for sialidase treatment of cells, the ligand was treated with 50 mU/ml of sialidase from *V. cholerae* generously provided by R. Schnaar of the Johns Hopkins School of Medicine (Baltimore, MD) for 60 min at 37°C. Eosinophils were incubated with the sialidase-treated or untreated synthetic ligand at 5 µg/ml at 4°C for 20 min to allow binding, then the excess ligand was washed off. After an incubation at 37°C to permit

internalization, the remaining synthetic ligand on the cell surface was detected with APC-conjugated streptavidin (2 μ g/ml; BD Biosciences). To eliminate sialylation on any masking *cis* ligands, eosinophils were pretreated with 50 mU/ml sialidase for 60 min at 37°C and washed prior to testing synthetic ligand binding.

REFERENCES

1. Hudson SA, Bovin NV, Schnaar RL, Crocker PR, Bochner BS.
Eosinophil-selective binding and proapoptotic effect in vitro of a synthetic Siglec-
8 ligand, polymeric 6'-sulfated sialyl Lewis x. J Pharmacol Exp Ther 2009;
330:608-12.

SUPPLEMENTAL FIGURE LEGENDS

Figure E1. Loss of Siglec-8 from the surface of HMC-1.2 and LUVA mast cells. The percentage of Siglec-8⁺ HMC-1.2 (A) and LUVA (B) cells was assessed by flow cytometry after antibody ligation of the receptor and incubation at 37°C. The Siglec-8⁺ gate was positioned on the basis of negative isotype staining. Data represent the means $\pm$ standard deviations of 4 independent experiments.

Figure E2. Ligation-induced Siglec-8 endocytosis is not unique to the 2C4 mAb clone. Eosinophils were incubated with anti-Siglec-8 (either clone 2C4 [black columns] or 1H10 [white columns]) for 20 minutes at 4°C. Excess antibody was washed off, and the cells were incubated at 37°C for the indicated durations to permit endocytosis. Antibody remaining at the surface of cell was detected with a secondary anti-mouse IgG1 antibody. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ relative to eosinophils incubated with 2C4 mAb without any incubation at 37°C. ##, $p < 0.01$; ###, $p < 0.001$; ####, $p < 0.0001$ relative to eosinophils incubated with 1H10 mAb without any incubation at 37°C. Data represent the means $\pm$ standard deviations of 4 independent experiments.

Figure E3. Siglec-8 shuttled to the cell surface can be detected using a sequential labeling step. Eosinophils were labeled with unconjugated anti-Siglec-8 mAb (2C4) and incubated at 37°C to permit endocytosis as in Fig. 1. Following detection of remaining anti-Siglec-8 at the cell surface with a FITC-conjugated secondary mAb ("Original"), Siglec-8 newly expressed at the cell surface ("Shuttled") was detected using anti-Siglec-8 (2C4) directly

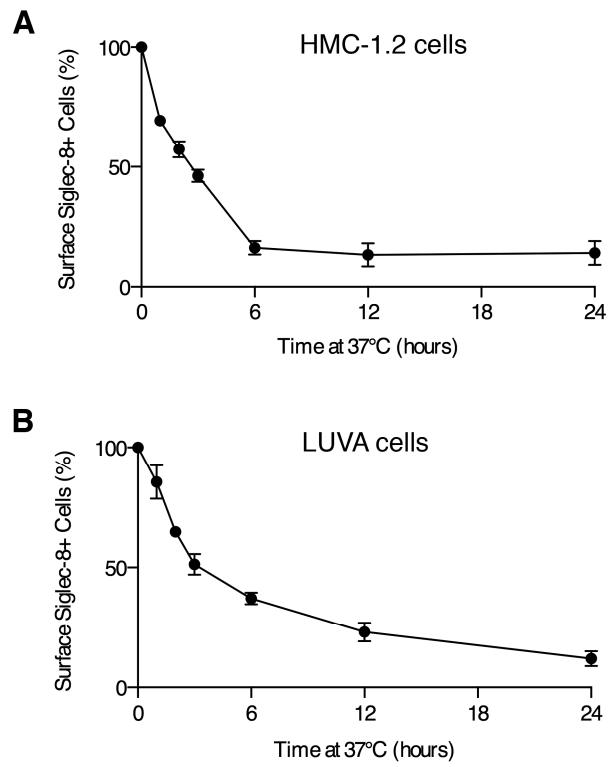
conjugated to Alexa Fluor 647. Control antibodies of the same isotype (iso) are included as negative staining controls. Loss of FITC signal and gain of Alexa Fluor 647 signal at 120 min when using anti-Siglec-8 mAbs in both channels represent receptor endocytosis and shuttling to the cell surface. Effective blockade of Alexa Fluor 647-conjugated anti-Siglec-8 binding can be seen at 0 min when anti-Siglec-8 mAbs are used for both channels (no additional Alexa Fluor 647 signal).

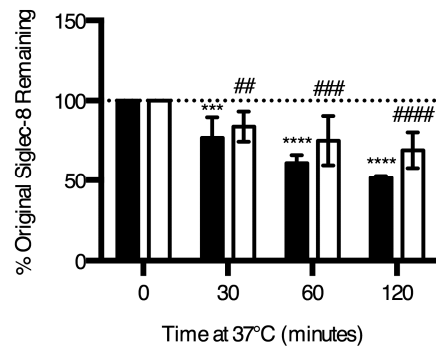
Figure E4. Sialylated *cis* ligands on eosinophils limits binding and internalization of an artificial Siglec-8 glycan ligand. A biotinylated synthetic Siglec-8 ligand-decorated polyacrylamide polymer was used to label Siglec-8 through its ligand-binding domain. (A) Biotinylated 1-MDa polyacrylamide decorated with 6'-O-sulfo-3'-sialyl-LacNAc binding to the cell surface after various durations of incubation at 37°C was detected by flow cytometry using fluorophore-conjugated streptavidin. Sialidase from *V. cholerae* was used to desialylate the ligand (dark gray columns), the eosinophil cell surface (black columns), or both (light gray columns) prior to binding of Siglec-8 with the artificial ligand. Untreated cells and ligand are represented by the white columns. (B) Endocytosis of the Siglec-8 ligand in untreated (unfilled circles) or sialidase-treated (filled squares) eosinophils was calculated as the loss of surface-bound ligand normalized to the amount of ligand bound by untreated eosinophils at time 0. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ relative to untreated eosinophils incubated for the same duration at 37°C. (C) Endocytosis of the Siglec-8 ligand in untreated (unfilled circles) or sialidase-treated (filled squares) eosinophils was calculated as the loss of surface-bound ligand normalized

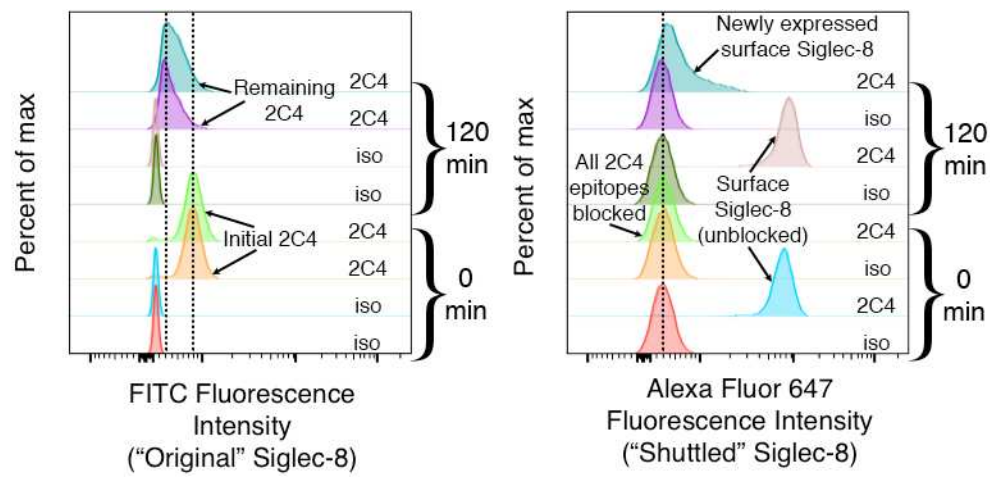
to the amount of ligand bound at time 0 by eosinophils within each treatment group. Data represent the means $\pm$ standard deviations of 8 independent experiments.

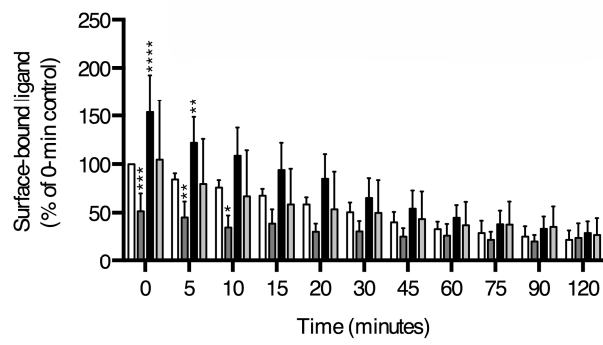
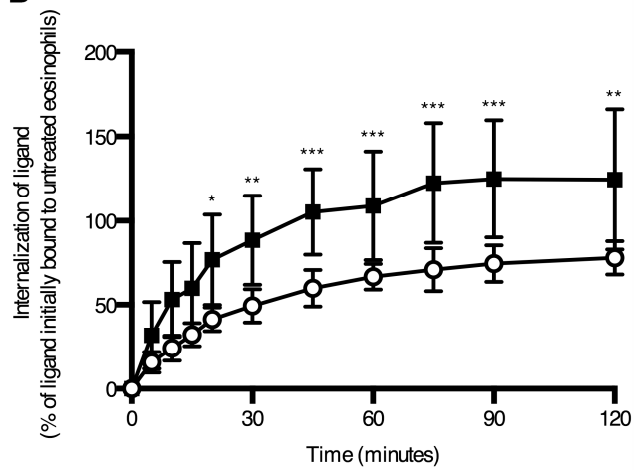
Figure E5. Anti-Siglec-8 and saporin-conjugated anti-Siglec-8 induce cell death in eosinophils but not in neutrophils. Eosinophils and neutrophils were isolated from the same donors. Eosinophils were incubated in 30 ng/ml IL-5. Neutrophils were incubated in 25 ng/ml GM-CSF. Eosinophil (white columns) and neutrophil (black columns) viability was assessed following treatment with anti-Siglec-8 with or without saporin (SAP) conjugation, isotype controls, or anti-Siglec-9 (clone K8, also mouse IgG1) as a positive control for siglec-mediated neutrophil cell death. ****, $p < 0.0001$ relative to isotype-treated eosinophils. #####, $p < 0.0001$ relative to isotype-treated neutrophils.

Figure E6. Timeline of the effects of saporin-conjugated anti-Siglec-8 on HMC-1.2 cells. Following treatment with anti-Siglec-8 with (unfilled circles) or without (filled circles) saporin conjugation or isotype control mAb with (unfilled squares) or without (filled squares) saporin conjugation, surface Siglec-8 expression (A) and viability (B) were assessed daily. Cell viability was assessed with annexin V and DAPI staining. ****, $p < 0.0001$ relative to anti-Siglec-8; #####, $p < 0.0001$ relative to saporin-conjugated isotype control; §, $p < 0.05$; §§, $p < 0.01$; §§§, $p < 0.001$; §§§§, $p < 0.0001$ relative to isotype control. Data represent the means $\pm$ standard deviations of 4 independent experiments.







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