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# Nanoparticles Displaying Allergen and Siglec-8 Ligands Suppress IgE-FcεRI-Mediated Anaphylaxis and Desensitize Mast Cells to Subsequent Antigen Challenge

Shiteng Duan,\* Britni M. Arlian,\* Corwin M. Nycholat,\* Yadong Wei,<sup>†</sup> Hiroaki Tateno,\* Scott A. Smith,<sup>‡</sup> Matthew S. Macauley,\* Zhou Zhu,<sup>§</sup> Bruce S. Bochner,<sup>¶</sup> and James C. Paulson\*

Siglec-8 is an inhibitory receptor expressed on eosinophils and mast cells. In this study, we took advantage of a novel Siglec-8 transgenic mouse model to assess the impact of modulating IgE-dependent mast cell degranulation and anaphylaxis using a liposomal platform to display an allergen with or without a synthetic glycan ligand for Siglec-8 (Sig8L). The hypothesis is that recruitment of Siglec-8 to the IgE-FcεRI receptor complex will inhibit allergen-induced mast cell degranulation. Codisplay of both allergen and Sig8L on liposomes profoundly suppresses IgE-mediated degranulation of mouse bone marrow–derived mast cells or rat basophilic leukemia cells expressing Siglec-8. In contrast, liposomes displaying only Sig8L have no significant suppression of antigenic liposome-induced degranulation, demonstrating that the inhibitory activity by Siglec-8 occurs only when Ag and Sig8L are on the same particle. In mouse models of anaphylaxis, display of Sig8L on antigenic liposomes completely suppresses IgE-mediated anaphylaxis in transgenic mice with mast cells expressing Siglec-8 but has no protection in mice that do not express Siglec-8. Furthermore, mice protected from anaphylaxis remain desensitized to subsequent allergen challenge because of loss of Ag-specific IgE from the cell surface and accelerated clearance of IgE from the blood. Thus, although expression of human Siglec-8 on murine mast cells does not by itself modulate IgE-FcεRI-mediated cell activation, the enforced recruitment of Siglec-8 to the FcεRI receptor by Sig8L-decorated antigenic liposomes results in inhibition of degranulation and desensitization to subsequent Ag exposure. *The Journal of Immunology*, 2021, 206: 1–11.

The rate-limiting step of current allergen immunotherapies, both injection-based and oral, is to safely build up to a therapeutic dose of allergen because of the risk of serious adverse events, including anaphylaxis (1, 2). Although strategies, such as alternative routes of allergen administration (i.e., sublingual), have been implemented to mitigate safety issues in some contexts of allergen immunotherapies, novel approaches to further prevent undesired allergic reactions and improve the safety of allergen immunotherapy are needed (3).

Mast cells express the FcεRI, which strongly binds allergen-specific IgE. Allergen cross-linking of the IgE-FcεRI complex results in phosphorylation of kinases that initiate a signaling cascade, leading to mast cell degranulation, which causes the signs and symptoms of allergies from mild itching or sneezing to life-threatening anaphylaxis. Mast cells also express inhibitory receptors with ITIMs in their cytoplasmic domains that, when phosphorylated, can recruit tyrosine phosphatases, such as

SHIP1, SHP-1, or SHP-2 (4). Enforced recruitment of ITIM-containing receptors to IgE-FcεRI through chimeric proteins that bind both receptors have been shown to suppress allergen-induced IgE-mediated mast cell degranulation and prevent anaphylaxis in mouse models (5–7).

Human mast cells express several inhibitory receptors that are members of the sialic acid-binding Ig-like lectin (Siglec) family, including CD33 (Siglec-3), CD22 (Siglec-2), Siglec-5, Siglec-7, and Siglec-8 (Sig8) (8–10). We recently showed that an antigenic liposome containing a glycan ligand of CD33 can recruit CD33 to the IgE-FcεRI complex and inhibit Ag-induced mast cell degranulation (8). Moreover, exposure to liposomal nanoparticles displaying both Ag and CD33 ligand (CD33L) desensitized mast cells to subsequent allergen challenges. In principle, this modular platform could be adapted to other Siglecs by substituting a specific glycan ligand to test the possibility that recruitment of other inhibitory Siglecs on mast cells could also inhibit signaling of the FcεRI.

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S.D. and J.C.P. designed the experiments, analyzed the data, and interpreted the results. S.D. performed the majority of the experiments, and B.M.A. performed some of the

in vivo experiments. C.M.N., M.S.M. and S.A.S. provided key reagents for liposome formulation and anaphylaxis experiments. H.T. transfected the Siglec-8 RBL cells. Y.W., Z.Z., and B.S.B. generated the *Rosa26-Stop<sup>fl/fl</sup>*-Siglec-8 transgenic mice. S.D. and J.C.P. wrote the initial draft of the manuscript. All authors participated in editing the manuscript.

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Abbreviations used in this article: AF647, Alexa Fluor 647; BMMC, bone marrow–derived mast cell; CD33L, CD33 ligand; hFcεRIα, human FcεRIα; hIgE, human IgE; LP-Sig8L, liposome with Sig8L; OVA-LP, OVA liposome; OVA-LP-Sig8L, OVA-LP with Sig8L; PBS-T, PBS containing 0.05% Tween-20; PEG-DSPE, polyethylene glycol-distearoyl phosphoethanolamine; PSA, passive systemic anaphylaxis; RBL, rat basophilic leukemia; RT, room temperature; Sig8, Siglec-8; Sig8L, Siglec-8 ligand; Siglec, sialic acid-binding Ig-like lectin; Sig8-RBL, Siglec-8–expressing RBL cell; TNP, trinitrophenol; TNP-LP, TNP liposome; TNP-LP-Sig8L, TNP and Sig8L on the same liposome.

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Siglec-8 is uniquely expressed on eosinophils and mast cells and has been proposed as a target for modulating immune responses (11–13). Like CD33, Siglec-8 contains an ITIM in its cytoplasmic tail that could participate in negative regulation of mast cell functions. In this regard, anti-Siglec-8 Abs alone have been shown to partially inhibit FcεRI-mediated mast cell activation, an activity that was ITIM dependent (14), and have been shown to inhibit anaphylaxis in humanized mice when given prophylactically 2 d before allergen challenge (13). But signaling by Siglec-8 is clearly more complex because anti-Siglec-8 induces death in eosinophils that is enhanced by IL-5 priming and occurs by an activatory mechanism involving integrins and production of reactive oxygen species (12, 15, 16).

We have recently reported a novel synthetic glycan ligand of Siglec-8 that enables the production of antigenic liposomal particles to investigate the potential for enforced ligand-mediated recruitment of Siglec-8 to IgE–FcεRI to suppress mast cell activation (17). Analysis of the impact of the nanoparticles on mast cell functions in vivo is greatly facilitated using the recently reported transgenic mouse line expressing Siglec-8 on mast cells (18, 19). In this study, we show that expression of Siglec-8 on mast cells of transgenic mice has little impact on IgE-mediated mast cell activation. However, enforced recruitment to the IgE–FcεRI complex using liposomes displaying Ag and Siglec-8 ligand (Sig8L) causes a striking suppression of activation and desensitizes mast cells to the subsequent allergen challenge. We show that the enforced recruitment of Siglec-8 is required for suppression of IgE-mediated mast cell degranulation in this context because ligation of Siglec-8 by anti-Siglec-8 or Sig8 liposomes has no effect on allergen-mediated mast cell degranulation.

## Materials and Methods

### Mice

*Rosa26-Stop<sup>fl/fl</sup>-Sig8* and *Mcpt5-Cre* on C57BL/6 background were generated and genotyped as previously described (18–20). In brief, cDNA encoding full-length Siglec-8 was inserted into *Rosa26* locus, and its translation was controlled by a floxed-STOP codon. To enable translation of Siglec-8 only in connective tissue mast cells, the *Rosa26-Stop<sup>fl/fl</sup>-Sig8* were mated to *Mcpt5-Cre* mice, which constitutively express Cre recombinase in connective tissue mast cells. Cre recombinase deletes the floxed-STOP codon and enables translation of Siglec-8 only in connective tissue mast cells. Mice bearing a *Mcpt5-Cre<sup>+/+</sup>-R26-Sig8<sup>+/+</sup>* genotype are abbreviated as Sig8-Tg. Unless stated otherwise, *Mcpt5-Cre<sup>+/+</sup>-R26-Sig8<sup>+/+</sup>* littermates were used as controls (control-Tg). For the experiment in Fig. 4I, the Sig8-Tg mice were further bred to the human FcεRI-α<sup>+</sup> and mouse FcεRI-α-deficient mice on a C57BL/6 background as previously described (21), and the resulting mice had a genotype of *Mcpt5-Cre<sup>+/+</sup>-R26-Sig8<sup>+/+</sup> hFcεRIα<sup>+</sup> mFcεRIα<sup>-/-</sup>*. The resulting mice lack the endogenous mFcεRIα receptor but express human FcεRIα (hFcεRIα) of their mast cell surface.

### Abs and flow cytometry

All Abs were ordered from BioLegend unless otherwise noted and included Abs to mouse c-Kit (clone 2B8, rat IgG2b labeled with allophycocyanin-Cy7), mouse FcεRIα (clone MAR-1, hamster IgG labeled with PE/Cy7), mouse IgE (clone RME-1 rat IgG1 labeled with FITC), mouse CD45 (clone 30-F11, rat IgG2b labeled with BV605), Fc blocker (clone 93), and mouse Siglec-8 (clone 7C9, mouse IgG1 unconjugated or labeled with either PE or allophycocyanin-Cy7). Siglec-8 mAb (clone 2C4, unconjugated) was generated as previously described (22). Cells were suspended in buffer (HBSS containing 2 mM EDTA and 0.1% BSA) containing 1 μg/ml of Abs (on ice for 20 min). Cells were washed and suspended in excess FACS buffer and analyzed by flow cytometry (LSR II; BD Bioscience). The anti-OVA human IgE (hIgE) (clone 11B6) was produced from a B cell hybridoma immortalized from the blood of an OVA-allergic patient using methods previously described (23).

### Siglec-8 rat basophilic leukemia cells

Full-length Siglec-8 was subcloned into a p3xFLAG-CMV-14 vector and transfected into rat basophilic leukemia (RBL) 2H3 cells as previously described (14). Transfected RBL cells were cultured and selected in DMEM

(Life Technologies, 11965092) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml of streptomycin and selected with geneticin (G418, 1 mg/ml, Invitrogen). The Siglec-8-transfected RBL cells were sorted by FACSARIA (BD Biosciences) based on anti-Siglec-8 staining (1 μg/ml, no. 347104, BioLegend). Sorted cells were expanded and cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin without geneticin.

### Bone marrow-derived mast cells

Bone marrow-derived mast cells (BMMCs) were cultured as previously described (24). To prepare IL-3-conditioned media, WEHI-3B cells were expanded in culture media, which is RPMI-1640 supplemented with 10% heat-inactivated FBS, 1 mmol/L sodium pyruvate, 100 U/ml penicillin, 100 μg/ml of streptomycin, 1% MEM nonessential amino acids, 55 μmol/L 2-ME, 10 mmol/L HEPES, and 2 mmol/L L-glutamine (Life Technologies). Femurs from the Sig8-Tg mice were flushed with ice-cold RPMI-1640 under sterile conditions. Collected cells were then suspended in culture media mixed with IL-3-conditioned media (50:50). Nonadherent cells were transferred to a new flask with new media twice per wk. By 4 wk of culture, 90% of the cells are c-Kit<sup>+</sup>FcεRI<sup>+</sup>, determined by flow cytometry.

For in vitro experiments, Siglec-8<sup>+</sup> BMMCs (20 × 10<sup>6</sup> cells/ml) were stained with anti-Siglec-8 (1 μg/ml, no. 347104, BioLegend) and positively sorted by FACSARIA (8–10 × 10<sup>6</sup> cells/ml, BD Biosciences). Sorted cells were expanded in culture media for at least overnight prior to experiments. After overnight culture, sorted Siglec-8<sup>+</sup> BMMCs regained surface Siglec-8 expression to levels comparable to those prior to sorting cells, as analyzed by flow cytometry (see Supplemental Fig. 2).

### Liposomes

All liposomes were composed of a 57:38:5 molar ratio of distearoyl phosphatidylcholine (Avanti Polar Lipids), cholesterol (Sigma-Aldrich), and polyethylene glycol-distearoyl phosphoethanolamine (PEG-DSPE; NOF). Trinitrophenol (TNP) and Sig8L were linked to PEG-DSPE as previously described (17). When TNP-PEG-DSPE or Sig8L-PEG-DSPE are included in the formulation, there is a proportionate reduction in PEG-DSPE such that the total of the PEG-DSPE is kept at 5%. To make TNP liposomes (TNP-LP), 0.1% or 0.4% of TNP-PEG-DSPE was added to lipid mixture. To make liposomes with Sig8L only (LP-Sig8L), 2–3% Sig8L-PEG-DSPE was added to the lipid mixture. To copresent TNP and Sig8L on the same liposomes (TNP-LP-Sig8L), both TNP-PEG-DSPE (0.1% or 0.4%) and Sig8L-PEG-DSPE (2–3%) were added to the lipid mixture prior to lyophilization. To make fluorescently labeled liposomes, 0.1% of Alexa Fluor 647 (AF647)-PEG-DSPE was added to lipid mixture. Liposomes were lyophilized, reconstituted in PBS, and extruded as previously described (25). For OVA liposomes (OVA-LP), OVA (Worthington Biochemical, LS003054) was coupled to PEG-DSPE as previously described (26). OVA-PEG-DSPE was added to PBS-hydrated lipid prior to extrusion.

In Figs. 1 and 2 and Supplemental Fig. 2, liposomes containing 0.4% TNP-PEG-DSPE were used. In Figs. 3, 4A–H, 5B, and 6 and Supplemental Figs. 3 and 4, liposomes containing 0.1% TNP-PEG-DSPE were used. In Figs. 4I and 5D, liposomes containing 0.1% OVA-PEG-DSPE were used. In all experiments, liposome concentrations are expressed as the molar amount of total lipids in the solution, so the Sig8L and allergen/protein concentrations will represent the relevant percentage of that concentration.

### Liposome binding

RBL, BMMCs (0.5–1 × 10<sup>5</sup> cells), or peritoneal cells (1–2 × 10<sup>6</sup> cells) were incubated with 20 μM liposomes in 100 μl culture media at 37°C for 1 h. Cells were then washed with FACS buffer and stained with Abs if needed.

### Degranulation assays

Assessment of degranulation was performed using existing protocols with minor modification (24). RBL, Siglec-8–RBL, or Siglec-8<sup>+</sup> BMMCs (2–3 × 10<sup>4</sup> cells per 96-well) were sensitized with anti-TNP-IgE (1 μg/ml, clone MEB38, BioLegend) in culturing media (100 μl/well) overnight. The next day, cells were washed and stimulated with the indicated liposomes in HEPES buffer (HBSS supplemented with 20 mM HEPES, 0.2 mg/ml CaCl<sub>2</sub>, 0.2 mg/ml MgSO<sub>4</sub>, and 0.4 mg/ml BSA) for 60 min at 37°C. Degranulation was quantified by the release of β-hexosaminidase and quantified by the digestion of its substrate 4-nitrophenyl-N-acetyl-β-glucosaminide (0.35 mg/ml in PBS supplemented with 8 mg/ml sodium citrate [pH 4.5]). Percentage of degranulation was determined by dividing the β-hexosaminidase activity in the supernatant by total activities from both the cell pellet lysate and the supernatant (24). Cells receiving buffer only were used to determine the background for each experiment, which was consistently less than 10%.

In Fig. 1G, cells were first treated with isotype or anti-Siglec-8 (4 μg/ml in 50 μl at 37°C for 1 h). TNP-LP or TNP-LP-Sig8L were then added to cells in the presence of Abs (60 μM of liposome in 50 μl at 37°C for 1 h),

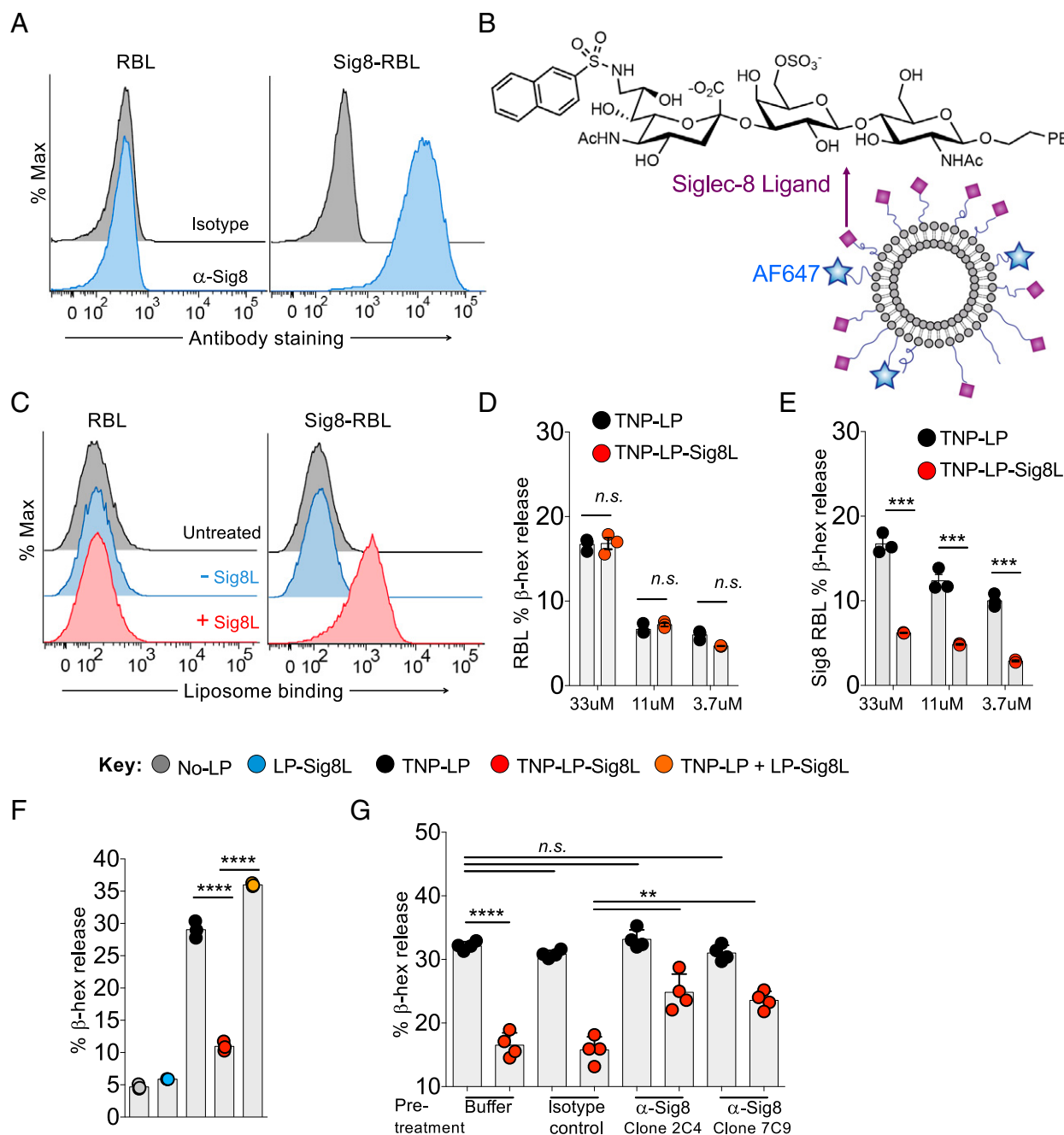


**FIGURE 1.** Copresentation of Sig8L on TNP-LP suppresses degranulation of anti-TNP-IgE-sensitized RBL cells expressing Siglec-8. Anti-TNP-IgE-sensitized RBL cells and RBL cells expressing Siglec-8 (Sig8-RBL) are compared for FcεRI-mediated degranulation by TNP-LP with and without copresentation with Sig8L (TNP-LP-Sig8L). **(A)** Expression of Siglec-8 on native RBL cells (left) or Siglec-8-transfected RBL cells (Sig8-RBL) (right) assessed by flow cytometry using anti-Siglec-8 Ab (allophycocyanin-Cy7). **(B)** Chemical structure of the Sig8L and a schematic representation of a liposome containing Sig8L and a lipid linked fluorophore (AF647). **(C)** Binding of fluorescent liposomes (20 μM) with or without Sig8L to RBL cells (left) and Sig8-RBL cells (right). **(D)** and **(E)** Degranulation of RBL cells (**D**) or Sig8-RBL cells (**E**) induced by TNP-LP or TNP-LP-Sig8L. **(F)** Degranulation of Sig8-RBL cells induced by LP-Sig8L, TNP-LP, TNP-LP-Sig8L (30 μM), or a mixture of TNP-LP with LP-Sig8L (30 μM each). **(G)** Degranulation of Sig8-RBL cells induced by TNP-LP or TNP-LP-Sig8L (30 μM) in the presence of isotype control or anti-Siglec-8 Abs (2 μg/ml). Results shown are representative of three (A and C–E) or two (F–G) independent experiments. Each dot in (D)–(G) represents one technical replicate from one independent experiment. Statistical analysis for (D) and (E) was done using unpaired two-tailed Student *t* test, and data in (F) and (G) were analyzed by one-way ANOVA followed by Tukey test: \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001. n.s., not significant (*p* > 0.05).

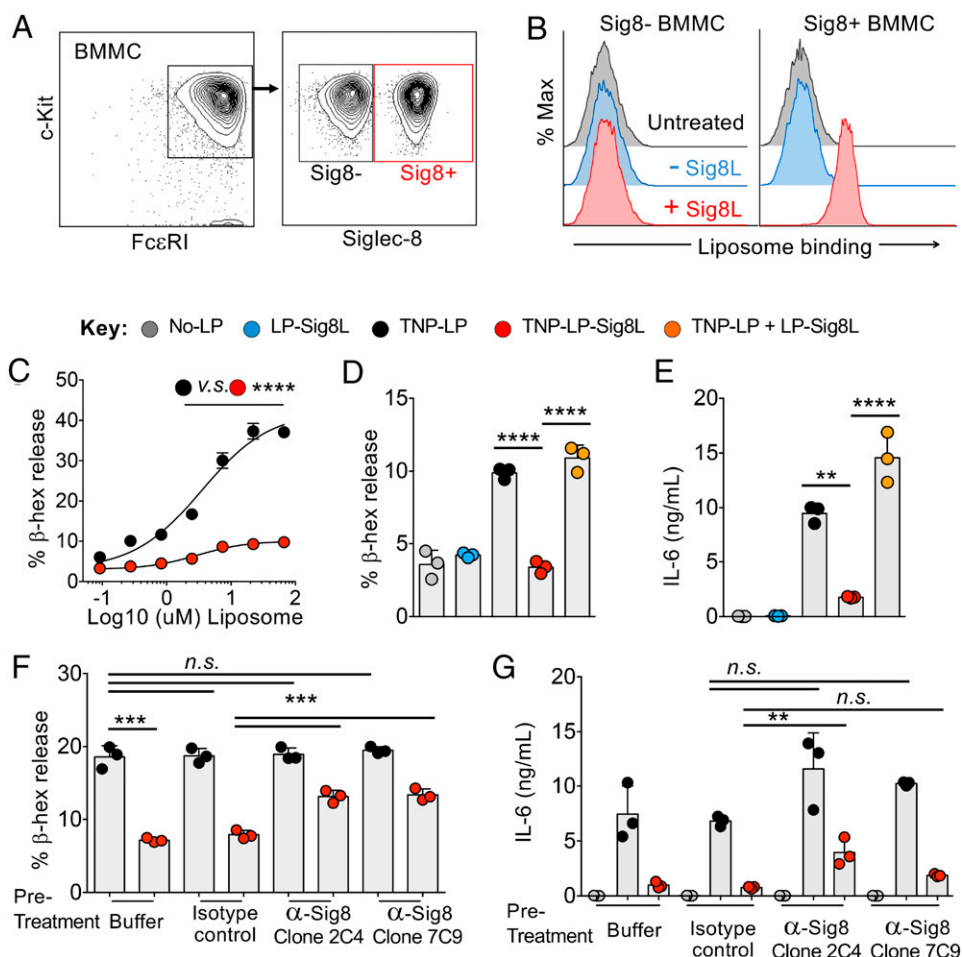
yielding a final Ab concentration per well in 100 μl of 2 μg/ml and a final liposome concentration of 30 μM.

In Fig. 2F, cells were first treated with isotype or anti-Siglec-8 (2 μg/ml in 50 μl at 37°C for 1 h). TNP-LP or TNP-LP-Sig8L were then added to cells in the presence of Abs (60 μM of liposome in 50 μl at 37°C for 1 h), yielding a final Ab concentration at 1 μg/ml and final liposome concentration at 30 μM in 100 μl per well.

#### Cytokine ELISA

Cytokine ELISAs were performed using existing protocols (24). Sorted Siglec-8<sup>+</sup> BMMCs (10<sup>5</sup> cells/100 μl of IL-3-conditioned media) were sensitized with anti-TNP-IgE (MEB38, BioLegend, 1 μg/ml) overnight. The next day, cells were washed with IL-3-free culture media and stimulated with the indicated liposomes (20 μM at 37°C for 5 h). IL-6 was measured following

**FIGURE 2.** Demonstration that Siglec-8 is an inhibitory receptor in BMMCs generated from Siglec-8-Tg mice. BMMCs were generated to assess the function of Siglec-8 in murine mast cells. **(A)** Flow cytometry analysis of BMMCs derived from the Siglec-8-Tg mice. **(B)** Binding of fluorescent (AF647) liposomes (20  $\mu$ M) with or without Sig8L to BMMCs gated on Siglec-8<sup>−</sup> (left) or Siglec-8<sup>+</sup> (right) populations. **(C)** Assessment of degranulation of Sig8<sup>+</sup> BMMCs induced by TNP-LP or TNP-LP-Sig8L ( $n = 3$  per condition; values plotted as means  $\pm$  SD). **(D)** Degranulation of Siglec-8<sup>+</sup> BMMCs. **(E)** IL-6 released following exposure to LP-Sig8L, TNP-LP, TNP-LP-Sig8L (20  $\mu$ M), or a mixture of TNP-LP with LP-Sig8L (20  $\mu$ M each). **(F)** Impact of anti-Siglec-8 (clone 7C9) or isotype Abs (1  $\mu$ g/ml) on degranulation of Siglec-8<sup>+</sup> BMMCs induced by TNP-LP or TNP-LP-Sig8L (30  $\mu$ M). **(G)** Impact of anti-Siglec-8 (clone 7C9) or isotype Abs (1  $\mu$ g/ml) on IL-6 release by Siglec-8<sup>+</sup> BMMCs following exposure to TNP-LP or TNP-LP-Sig8L (20  $\mu$ M). Results shown are representative of three (B–E) or two (F–G) independent experiments. Each dot in (D)–(G) represents one technical replicate from one independent experiment. Degranulation at each concentration of liposome in (C) was analyzed by unpaired two-tailed Student  $t$  test; (D)–(G) were analyzed by one-way ANOVA followed by Tukey test. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . n.s., not significant ( $p > 0.05$ ).



the manufacturer's protocol (no. 431301, BioLegend). Capture and detection Abs were used at 1  $\mu$ g/ml.

In Fig. 2F, Siglec-8<sup>+</sup> BMMCs first treated with isotype or anti-Siglec-8 (2  $\mu$ g/ml in 50  $\mu$ l at 37°C for 1 h). TNP-LP or TNP-LP-Sig8L were then added to cells in the presence of Abs (40  $\mu$ M of liposome in 50  $\mu$ l at 37°C for 1 h), yielding a final Ab concentration at 1  $\mu$ g/ml and final liposome concentration at 20  $\mu$ M in 100  $\mu$ l per well.

#### Western blotting

Siglec-8<sup>+</sup> BMMCs were sensitized with anti-TNP-IgE (1  $\mu$ g/ml, MEB38, BioLegend) overnight in culture media. Cells were washed and suspended in culture media ( $3 \times 10^6$  cells condition in 500  $\mu$ l of culture media) and rewarmed at 37°C for at least 10 min. Cells were stimulated with the indicated liposomes (2  $\mu$ M liposomes at 37°C for 10 min). To quench the experiments, cells were centrifuged (15,800 g for 15 s), suspended in cold PBS, centrifuged (15,800 g for 15 s), and suspended in lysis buffer (150  $\mu$ l at 4°C for 10 min, no. 9803, Cell Signaling Technology).

All Abs for Western blotting were purchased from Cell Signaling Technology. Phospho-PLC $\gamma$ 1 (Tyr<sup>183</sup>, no. 2821), phospho-PLC $\gamma$ 2 (Tyr<sup>1217</sup>, no. 3871), phospho-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>, no. 4668), phospho-p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>, no. 4511), and phospho-Akt (Ser<sup>473</sup>, no. 4060) were used at a 1:2000-fold dilution. Phospho-Erk (Thr<sup>202</sup>/Tyr<sup>204</sup>, no. 4370), Erk (no. 4695), and Akt (no. 4691) were used at a 1:5000-fold dilution. HRP-linked anti-rabbit IgG (no. 7074) was used at a 1:2000-fold dilution. In Fig. 3B and 3C, when probed using phospho-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>, no. 4668 at a 1:2000 dilution), three bands were observed. The top two bands marked with arrows are JNK related as identified using anti-JNK (Fig. 3C). The bottom band is likely cross-reactivity of the Ab to phosphorylated p44/42 or p38 kinases, as described by the provider Cell Signaling Technology. Intended phospho-JNK bands were marked with arrows.

#### Passive cutaneous anaphylaxis

Ears were injected intradermally with 25  $\mu$ l of PBS (left ears) or 125 ng of anti-TNP-IgE (MEB38, BioLegend) in 25  $\mu$ l of PBS (right ears). The next day, TNP-LP or TNP-LP-Sig8L were given i.v. in 200  $\mu$ l of PBS containing

1% Evans blue. All mice were euthanized 60 min after injection. The ears were excised into 4–6 pieces and dissolved in 500  $\mu$ l of DMF (68-12-2, Thermo Fisher Scientific) with constant shaking (37°C overnight or 50°C at 3 h). Vascular leakage of the dye was quantified by measured A650 with a plate reader (200  $\mu$ l per 96-well). Data are compiled from 10 independent experiments. Genotypes were determined by PCR after the experiments.

In Fig. 4A and 4B, 50  $\mu$ g (200  $\mu$ l of 0.33 mM liposome) of TNP-LP or TNP-LP-Sig8L was used. In Supplemental Fig. 3, 150  $\mu$ g (200  $\mu$ l of 1 mM liposome) of TNP-LP or TNP-LP-Sig8L was used.

#### Passive systemic anaphylaxis

Control-Tg or Siglec-8-Tg mice were i.v. sensitized with anti-TNP-IgE (MEB38, BioLegend). The next day, after baseline rectal temperatures were measured, mice were i.v. given TNP-LP or TNP-LP-Sig8L. Rectal temperatures were measured every 10 min for the next 50 min.

**Dose of IgE.** All sensitizations were performed with an i.v. injection of IgE in 200  $\mu$ l of PBS. Mice were sensitized with 10  $\mu$ g of anti-TNP-IgE in Fig. 4C–H and 5  $\mu$ g of anti-TNP-IgE in Figs. 5B and 6. In Fig. 4I, mice were sensitized with 20  $\mu$ g of anti-OVA-IgE (clone 11B6). In Fig. 5C–E, mice were sensitized with 20  $\mu$ g of anti-OVA-IgE (10  $\mu$ g of EC1, Chondrex; 10  $\mu$ g of PMP68, Bio-Rad Laboratories).

**Dose of liposomes.** Liposome doses are based on total lipid content. For Fig. 4C and 4D, 60  $\mu$ g of TNP-LP or TNP-LP-Sig8L (200  $\mu$ l of 0.4 mM liposome) was used. For Figs. 5B and 6, 230  $\mu$ g of TNP-LP-Sig8L (200  $\mu$ l of 1.5 mM liposomes) was used in the treatment step. For Fig. 5B, 50  $\mu$ g of TNP-LP (200  $\mu$ l of 0.33 mM liposome) was used in the challenge step. For Fig. 5C–E, 159  $\mu$ g of OVA-LP  $\pm$  Sig8L (200  $\mu$ l of 1 mM liposome containing 150  $\mu$ g of total lipid with 9  $\mu$ g of OVA-PEG-DSPE) was used per mouse.

In Fig. 4E–H, mice were first i.p. given 100  $\mu$ g of isotype control or anti-Siglec-8 (clone 2C4) in 200  $\mu$ l of PBS. One hour later, mice were i.v. treated with 50  $\mu$ g of TNP-LP or TNP-LP-Sig8L (200  $\mu$ l of 0.33 mM

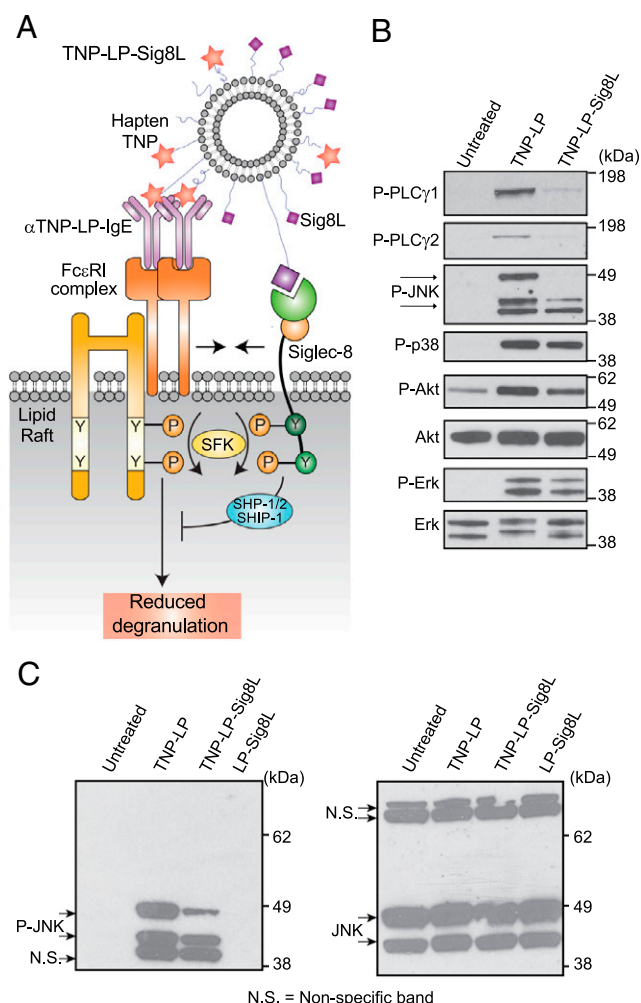


**FIGURE 3.** Copresentation of Ag and Sig8L reduces FcεRI-induced phosphorylation of downstream signaling molecules. **(A)** Proposed model for Siglec-8-mediated inhibition of mast cell degranulation. Antigenic liposomes (TNP-LP) stabilize IgE-FcεRI in lipid rafts enriched with Src kinase activities. Antigenic TNP-LP-Sig8L recruits Siglec-8 to the same lipid raft as IgE-FcεRI and subjects the cytoplasmic ITIM of Siglec-8 to be phosphorylated by local SRC family kinase. The phosphorylated ITIM of Siglec-8 can then recruit phosphatases such as SHP-1/2 that dephosphorylate downstream kinases or SHIP-1 that dephosphorylates phosphatidylinositol-3,4,5-triphosphate, preventing amplification of the signaling pathways induced by ligation of the IgE/FcεRI complex. **(B and C)** Phosphorylation of representative kinases in the signaling cascades induced by FcεRI ligation in Siglec-8<sup>+</sup> BMMCs cells after 10-min stimulation using liposomes with and without Ag (TNP) and with and without Sig8L. Signaling lipases and kinases evaluated included the following: **(B)** PLCγ1, PLCγ2, JNK, p38, Akt, Erk, and **(C)** JNK. Western blotting was conducted using Abs specific for the phosphorylated lipases or kinases to assess the level of phosphorylation induced and Abs specific for the proteins with and without phosphorylation (Akt, Erk, and JNK) to control for the amount of lysate added to the well. Liposomal preparations (TNP-LP, TNP-LP-Sig8L, or LP-Sig8L) were provided at a final concentration of 2 μM based on the total molar lipid content.

liposome) in 200 μl of PBS. In Fig. 4I, 200 μl of 2mM liposome containing 300 μg total lipid with 18 μg of OVA-PEG-DSPE was used.

#### Anti-TNP-IgE ELISA

The half-life of anti-TNP-IgE was determined as previously described (8). Microplates (Greiner Bio-One, no. 655081) were coated with TNP<sup>31</sup>BSA (Biosearch Technologies, 10 μg/ml in 50 μl of PBS/well overnight). The next day, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T) (v/v) five times, blocked with PBS containing 1% BSA (w/v, >2 h,

room temperature [RT]), and washed with PBS-T five times. Mice were bled prior to treatment and 6 or 24 h after treatment. Serum was serially diluted in PBS containing 1% BSA and loaded onto plates at 4°C overnight. Serially diluted anti-TNP-IgE (MEB38, BioLegend) was loaded as a standard. The next day, after the plates were washed five times with PBS-T, plates were incubated with biotin anti-mouse IgE (clone RME-1, at 2 μg/ml in 50 μl, RT, >1 h) and streptavidin-HRP (BioLegend, no. 405210, 1 μg/ml, >30 min, RT). Plates were then washed with PBS-T five times. All ELISAs were developed using TMB Peroxidase Substrate (75 μl/well, Rockland Immunochemicals), quenched with 2M H<sub>2</sub>SO<sub>4</sub> (75 μl/well), and A450 measured using a plate reader (Synergy H1, BioTek Instruments).

#### Statistical analysis

Statistical significance between two groups was determined by unpaired two-tailed Student *t* test. Significance among more than two conditions was determined by one-way ANOVA followed by Tukey test. Temperature curves in systemic anaphylaxis were analyzed by repeated measures two-way ANOVA followed by Tukey test if needed. All *p* values were determined using Prism (version 6.0f). A *p* value <0.05 was considered significant.

#### Study approval

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute.

## Results

### Ligand-mediated recruitment of Siglec-8 inhibits degranulation of Siglec-8 RBL cells

To test the ability of the Sig8L to inhibit IgE-FcεRI signaling, we transfected RBL cell line with full-length Siglec-8 and generated cell lines heterogeneously expressing Siglec-8 as previously described (14). The Siglec-8-expressing RBL cells (Sig8-RBL) were sorted by flow cytometry to obtain a homogeneous stable cell line (Fig. 1A). Fluorescently labeled liposomes with Sig8L (LP-Sig8L) (Fig. 1B) strongly bound to Sig8-RBL cells but did not bind untransfected RBL cells (Fig. 1C). To test whether incorporation of Sig8L into antigenic liposomes inhibits IgE-mediated basophil degranulation, RBL or Sig8-RBL cells were sensitized with anti-TNP-IgE and stimulated with liposomes formulated with TNP only as TNP-LP or TNP-LP-Sig8L. Using untransfected RBL cells, TNP-LP and TNP-LP-Sig8L induced a similar degree of degranulation as quantified by the percentage of release of β-hexosaminidase (Fig. 1D). Using Sig8-RBL cells, TNP-LP induced degranulation, but in contrast, incorporation of the Sig8L into TNP liposomes (TNP-LP-Sig8L) suppressed degranulation, strongly suggesting that the Sig8L recruits Siglec-8 to the IgE-FcεRI complex (Fig. 1E).

To test if liposomes bearing Sig8L alone (LP-Sig8L) had an impact on degranulation, we formulated LP-Sig8L. LP-Sig8L by itself did not cause degranulation of Sig8-RBL cells (Fig. 1F). When added together with TNP-LP, LP-Sig8L did not inhibit but instead modestly enhanced degranulation induced by TNP-LP (*p* < 0.001, Fig. 1F). Thus, Sig8L only suppressed degranulation when displayed on the same liposome as the Ag, which we presume is a result of the recruitment of Siglec-8 to the FcεRI-IgE complex.

Monoclonal anti-Siglec-8 Abs have been shown to partially inhibit anti-FcεRI-induced mast cell mediator release and calcium flux in human mast cells generated from CD34<sup>+</sup> cells. Moreover, strong inhibition of degranulation and calcium flux was observed in Siglec-8-transfected RBL cells when anti-Siglec-8 Abs were cross-linked to the IgE-FcεRI complex with the secondary Ab (14). To assess the impact of anti-Siglec-8 on degranulation induced by antigenic liposomes, Sig8-RBL cells were preincubated with anti-Siglec-8 prior to the addition of TNP-LP or TNP-LP-Sig8L. Remarkably, anti-Siglec-8 had no significant impact on degranulation induced by TNP-LP and partially restored degranulation induced by TNP-LP-Sig8L (Fig. 1G). Thus, in RBL cells, neither

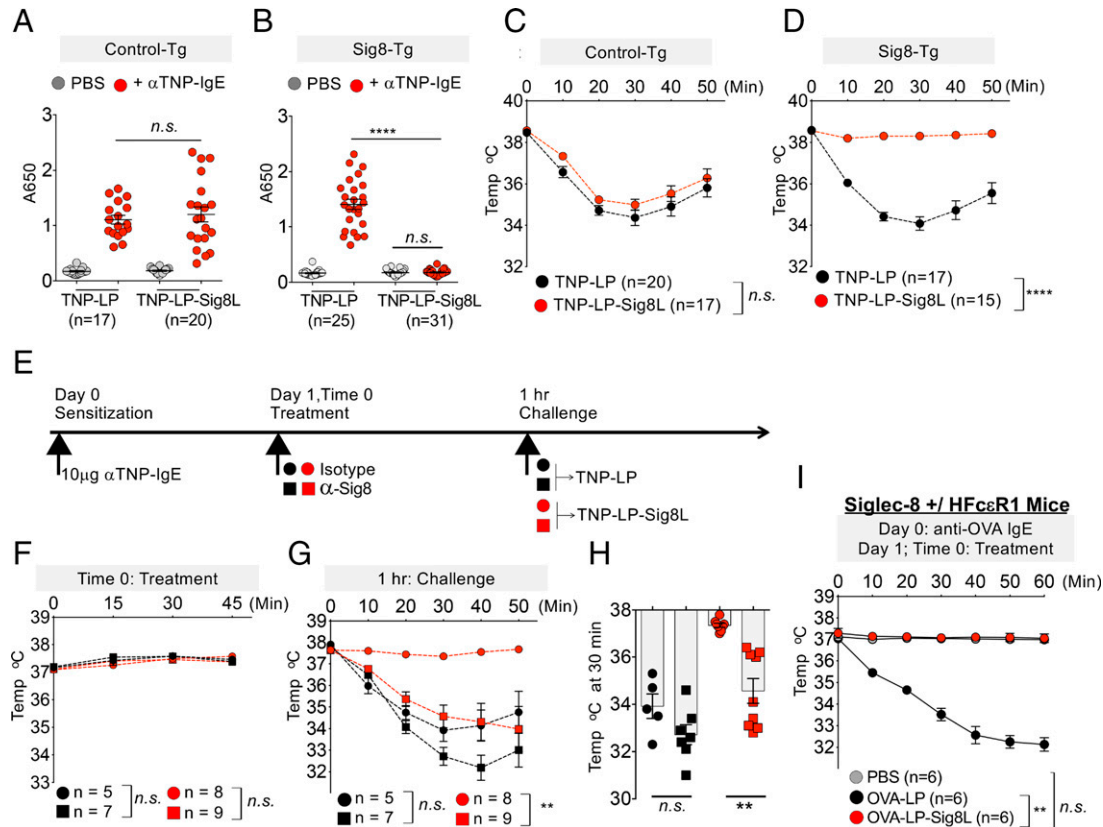


**FIGURE 4.** Ligand-mediated recruitment of Siglec-8 by Sig8L copresented on liposomal allergen inhibits IgE-mediated anaphylaxis in Siglec-8-Tg and Siglec-8/hFcεRI mice. The impact of copresentation of Sig8L with allergen (TNP or OVA) on induction of IgE-mediated anaphylaxis was investigated in models of passive cutaneous anaphylaxis (**A** and **B**) and systemic anaphylaxis (PSA) (**C**–**I**) in transgenic mice with mast cells expressing Siglec-8 (**B**, **D**–**H**) or human Siglec-8 and human FcεRI on a murine FcεRI-null background (Siglec-8/hFcεRI mice) (**I**). Detailed procedures are described in methods. For passive cutaneous anaphylaxis, one ear of control-Tg mice (**A**) and Sig8-Tg mice (**B**) was injected with anti-TNP (125 ng) and the ear with PBS as a control. One day later, mice were injected i.v. with TNP-LP or TNP-LP-Sig8L (50 μg) and Evans blue dye, and 1 h later, dye was extracted from ears of euthanized mice to quantify vascular leak (A650). Results from control-Tg mice (**A**) and Sig8-Tg mice (**B**) show that Sig8L blocks IgE-mediated anaphylaxis only when mast cells are expressing Siglec-8. For PSA, control-Tg (**C**) and Siglec-8-Tg (**D**) mice were sensitized with anti-TNP IgE (10 μg) systemically and, 1 d later, were challenged with TNP-LP or TNP-LP-Sig8L. Results show that Sig8L ligand protects mice if mast cells are expressing Siglec-8. Blockade of Siglec-8 with anti-Siglec-8 (clone 2C4) prevents Sig8L-mediated protection from PSA (**E**–**H**). As illustrated in the injection scheme (**E**), 1 h prior to challenge with TNP-LP or TNP-LP-Sig8L, mice were administered anti-Siglec-8 or isotype control, resulting in no anaphylaxis (**F**). Upon challenge, mice receiving isotype control Ab were protected from anaphylaxis by copresentation of TNP with Sig8L, whereas blockade with anti-Sig8L prevented protection by Sig8L as measured by rectal temperature over time (**G**) or rectal temperature at 30 min (**H**). PSA was also performed in Siglec-8/hFcεRI mice following sensitization with human anti-OVA IgE (clone 11B6) and challenge with OVA-LP or OVA-LP-Sig8L, showing protection from anaphylaxis by copresentation of OVA with Sig8L (**I**). Data were compiled from ten (**A** and **B**), four (**C** and **D**), or three (**F**–**I**) sets of experiments. For (**C**)–(**I**), values were plotted as means ± SEM at the indicated time points. Statistical analysis was conducted using one-way ANOVA followed by Tukey test (**A**, **B**, **H**). (**C**)–(**I**) or repeated measures two-way ANOVA followed by Tukey test (**C**, **D**, **F**, **G**, **I**): \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ . n.s., not significant ( $p > 0.05$ ).

ligation of Siglec-8 with anti-Siglec-8 or Sig8L liposomes is able to suppress degranulation, in contrast to the inhibition of degranulation observed by the display of Sig8L with Ag on liposomes, underscoring the need in this model to recruit Siglec-8 to the IgE–FcεRI complex. Moreover, we attribute the ability of anti-Siglec-8 to relieve the inhibition of degranulation caused by TNP-LP-Sig8L to blocking the interaction of Siglec-8 with the Sig8L, preventing its recruitment to the IgE–FcεRI.

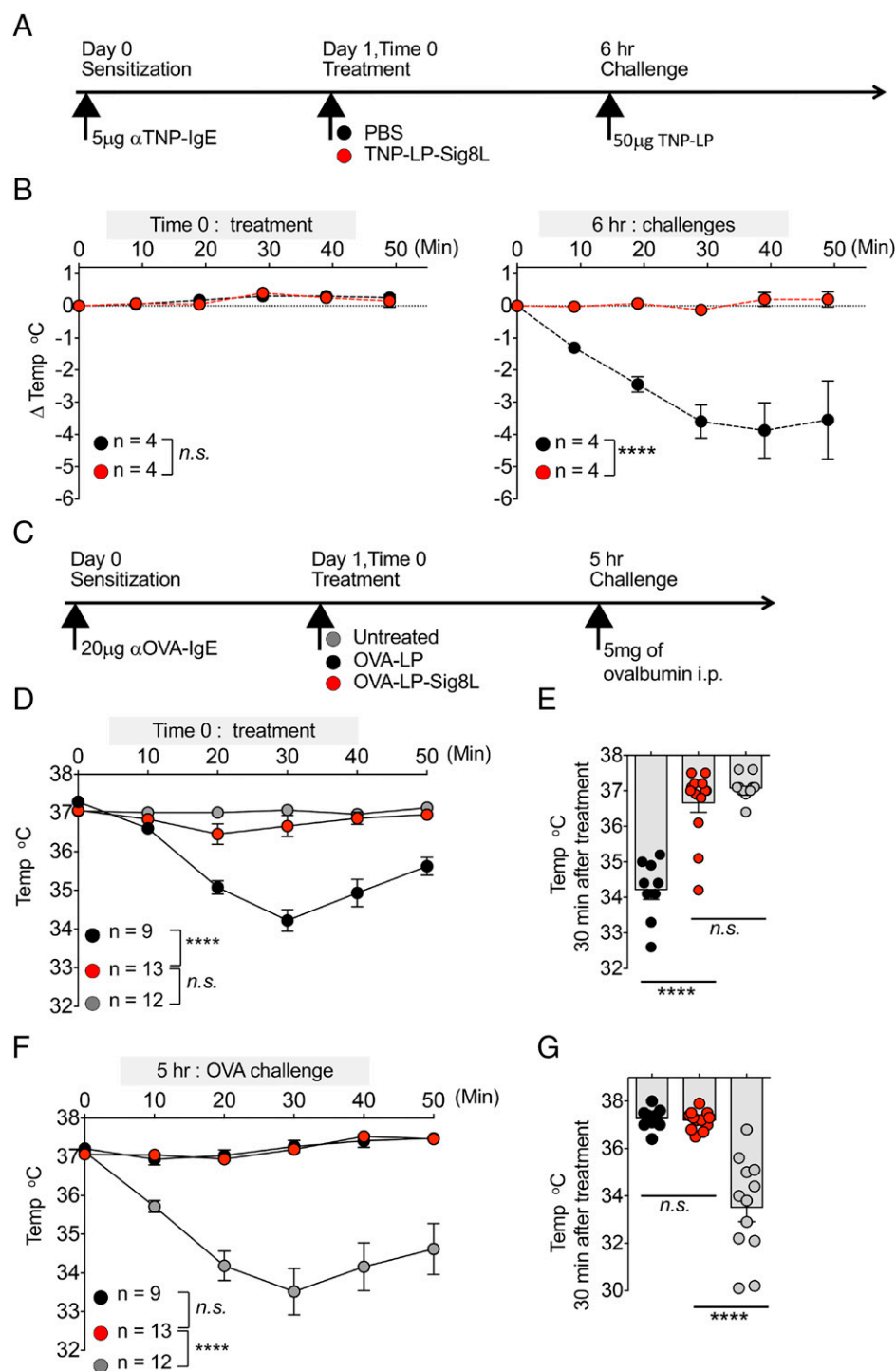
#### Human Siglec-8 functions as an inhibitory receptor in mast cells of transgenic mice

With a goal of studying the role of Siglec-8 in mouse models of anaphylaxis, we used the recently reported Siglec-8 transgenic mice (*Rosa26-Stop<sup>fl/fl</sup>-Siglec-8*) that express Siglec-8 only when cells express the Cre recombinase to remove the floxed-STOP codon. When mated with *Mcpt5-Cre* mice (*Mcpt5-Cre<sup>+</sup>Rosa26-Stop<sup>fl/fl</sup>-Siglec-8<sup>+/+</sup>*), Siglec-8 is expressed only in connective tissue mast cells, referred to as Siglec-8-Tg mice (19, 20). Littermates that do

not express the Cre recombinase, bearing the genotype *Mcpt5-Cre<sup>+</sup>Rosa26-Stop<sup>fl/fl</sup>-Siglec-8<sup>+/+</sup>*, do not express Siglec-8, serving as negative controls (control-Tg) (Supplemental Fig. 1A, 1B). Fluorescent LP-Sig8L did not bind peritoneal mast cells harvested from the control-Tg mice but strongly bound to mast cells harvested from the Siglec-8-Tg (Supplemental Fig. 1C). The results demonstrate that LP-Sig8L bind to murine peritoneal mast cells through Siglec-8.

To determine if Siglec-8 expressed on murine mast cells could inhibit degranulation mediated by mFcεRI, we derived BMMCs from the Siglec-8-Tg mice. By 4 wk of culture, >90% of the cells were c-Kit<sup>+</sup>FcεRI<sup>+</sup>, and 20–50% of the cells were Siglec-8<sup>+</sup> population (Fig. 2A). Fluorescent LP-Sig8L did not bind Siglec-8<sup>−</sup> BMMCs but strongly bound to Siglec-8<sup>+</sup> BMMCs (Fig. 2B). Because the Sig8L is known to also bind the murine paralog Siglec-F, it is important to note that neither murine peritoneal nor BMMCs express detectable Siglec-F, consistent with the lack of binding of LP-Sig8L to Siglec-8<sup>−</sup> BMMCs (Fig. 2B) (8, 17).

**FIGURE 5.** Antigenic LP-Sig8L desensitize mast cells in vivo. Experiments were done to test if liposomes presenting TNP (A and B) or OVA (C–G) Ags with Sig8L also desensitize mice to subsequent allergen challenge. (A) Injection scheme used to desensitize mice against anti-TNP-IgE (5  $\mu$ g per mouse, i.v.). (B) Rectal temperature upon initial treatment with PBS or TNP-LP-Sig8L (200  $\mu$ l of 1.5 mM liposome, left), and challenge 6 h later with TNP-LP (200  $\mu$ l of 0.33 mM, right). (C) Injection scheme used to desensitize mice against anti-OVA-IgE. Assessment of anaphylaxis as measured by rectal temperature over time (D) or at 30 min (E) following treatment with OVA-LP (black) or OVA-LP-Sig8L (red) or untreated (gray) (200  $\mu$ l of 1 mM liposome). Assessment of desensitization to challenge with 5 mg of soluble OVA as measured by rectal temperature over time (F) or at 30 min (G). Values for (B), (D), and (F) were plotted as the means  $\pm$  SEM. Statistical analysis was performed by repeated measures two-way ANOVA followed by Tukey test for (B), (D), and (F) and by one-way ANOVA followed by Tukey test for (E and G). \*\*\*\* $p$  < 0.0001. n.s., not significant ( $p$  > 0.05).



For degranulation experiments, Siglec-8<sup>+</sup> BMMCs were positively sorted by anti-Siglec-8 staining. After overnight culture, sorted cells regained cell surface Siglec-8 expression (Supplemental Fig. 2A), and binding of Sig8L liposomes was confirmed by flow cytometry (Supplemental Fig. 2B). Siglec-8<sup>+</sup> BMMCs were expanded and sensitized with anti-TNP-IgE. Similar to what was observed with Siglec-8-transfected RBL cells, TNP-LP without Sig8L induced strong degranulation and IL-6 production, but the corresponding LP-Sig8L (TNP-LP-Sig8L) potently suppressed degranulation and IL-6 production (Fig. 2C–E). In contrast, when LP-Sig8L were added simultaneously with TNP-LP, there was no inhibition, and in fact, there was a slight enhancement of degranulation and IL-6

production (Fig. 2D, 2E, Supplemental Fig. 2C). As expected, liposomes containing Sig8L (LP-Sig8L) when added by themselves did not cause degranulation or IL-6 production of BMMCs (Fig. 2D, 2E).

We also tested the impact of two clones of anti-Siglec-8 Abs on degranulation and IL-6 production. As seen with Siglec-8 RBL cells, neither clone of anti-Siglec-8 significantly enhanced or inhibited Siglec-8<sup>+</sup> BMMC degranulation induced by TNP-LP but partially reversed the inhibition of degranulation caused by Sig8L (Fig. 2F), presumably by interfering with recruitment of Siglec-8 by Sig8L. At a higher concentration, clone 2C4 was able to abolish the inhibition mediated by the Sig8L while causing no significant inhibition of degranulation induced by TNP-LP (Supplemental Fig. 2D).

Both clones of anti-Siglec-8 Abs weakly enhanced IL-6 production induced by TNP-LP and TNP-LP-Sig8L, but this did not reach statistical significance (Fig. 2G). Taken together, these data support the hypothesis that liposomes copresenting both Ag and Sig8L can recruit Siglec-8, leading to suppression of Ag-mediated degranulation, and in this context, ligation of Siglec-8 with Abs or Sig8L liposomes (Sig8L-LP) produce little or no inhibition of degranulation.

#### *Recruitment of Siglec-8 inhibits FcεRI signaling*

The impact of Sig8L-mediated recruitment of Siglec-8 on FcεRI signaling was examined by Western blots based on the presumed mechanism of Siglec-8 recruitment to the IgE-FcεRI complex, as illustrated in Fig. 3A. Siglec-8<sup>+</sup> BMMCs sensitized with anti-TNP-IgE were stimulated with TNP-LP or TNP-LP-Sig8L for 10 min, and cells were assessed for phosphorylation of lipases involved in calcium mobilization (PLCγ1 and PLCγ2) and kinases involved in cytokine synthesis (Erk, p38, Akt, and JNK) (27–29). Ligand-mediated recruitment of Siglec-8 (TNP-LP-Sig8L) resulted in a reduction of phosphorylation of all these kinases (Fig. 3B).

Because it was earlier shown that anti-Siglec-8 could induce phosphorylation of JNK1 in IL-5-primed human eosinophils (15), we assessed if liposomes displaying only Sig8L (LP-Sig8L) could induce JNK phosphorylation. As shown in Fig. 3C, LP-Sig8L did not induce phosphorylation of either JNK1 or JNK2, suggesting that in the context of FcεRI-mediated mast cell activation, Siglec-8 is primarily a negative regulator. We presume, in analogy with what has been shown with Siglecs in other contexts, that recruitment of Siglec-8 to the IgE-FcεRI activation complex results in phosphorylation of Siglec ITIM and recruitment of phosphatases (e.g., SHP-1, SHP-2, or SHIP1) that effect dephosphorylation of kinases (8, 30, 31), but the specific phosphatases involved remain to be established.

#### *Ligand-mediated recruitment of Siglec-8 inhibits IgE-mediated anaphylaxis*

We next assessed if ligand-mediated recruitment of Siglec-8 can suppress mast cell activation in passive cutaneous and passive systemic anaphylaxis (PSA) models. In the passive cutaneous anaphylaxis model, one ear was given an intradermal injection of PBS as control, whereas the other ear received 125 ng of anti-TNP-IgE. The next day, all mice were i.v. given TNP-LP or TNP-LP-Sig8L (200 μl of 0.33 mM liposome) in PBS containing 1% Evans blue dye. In control-Tg mice (*Mcpt5-Cre<sup>+</sup>Siglec-8<sup>+/+</sup>*), which do not express Siglec-8 on their mast cells, both TNP-LP and TNP-LP-Sig8L induced vascular leakage in the sensitized ear (Fig. 4A). In Siglec-8-Tg mice (*Mcpt5-Cre<sup>+</sup>Siglec-8<sup>+/+</sup>*), TNP-LP induced vascular leakage in the sensitized ear, suggesting that expressing Siglec-8 in the mast cell compartment did not impair their activity (Fig. 4B). By contrast, TNP-LP-Sig8L did not induce significant vascular leakage in the sensitized ear relative to the mock sensitized ear (Fig. 4B). Potent suppression of anaphylaxis was also seen with copresentation of Sig8L when the dose of the liposome was increased to 200 μl of 1 mM liposome per mouse ( $p < 0.0001$ ), although significant vascular leakage was seen in 4 of 17 mice, suggesting a breakthrough with this higher dose (Supplemental Fig. 3). Taken together, the results strongly suggest that ligand-mediated recruitment of Siglec-8 potently suppressed cutaneous mast cell activation in Siglec-8-Tg mice.

To assess generalized mast cell activation, we used a PSA model. Control or Siglec-8-Tg mice were i.v. sensitized with anti-TNP-IgE. The next day, mice were i.v. given 60 μg of TNP-LP or TNP-LP-Sig8L (200 μl of 0.4 mM liposome). In control-Tg and Siglec-8-Tg mice, TNP-LP induced a similar degree of systemic anaphylaxis, as quantified in the decrease of rectal temperatures (Fig. 4C). However, when mice were injected with TNP-LP containing Sig8L (TNP-LP-

Sig8L), control-Tg mice showed robust anaphylaxis (Fig. 4D), whereas Siglec-8-Tg mice showed no significant rectal temperature drop (Fig. 4D). The results strongly support the conclusion that Sig8L-mediated recruitment of Siglec-8 to the IgE-FcεRI complex prevents IgE-mediated systemic anaphylaxis.

We next examined the impact of anti-Siglec-8 on the induction of systemic anaphylaxis with antigenic liposomes. Siglec-8-Tg mice sensitized with anti-TNP-IgE were first treated with anti-Siglec-8 (clone 2C4) or an isotype control Ab followed by TNP-LP or TNP-LP-Sig8L (Fig. 4E). Anti-Siglec-8 nor the isotype control Ab by themselves did not induce detectable changes in rectal temperature (Fig. 4F). One hour after administration of Ab, challenge with TNP-LP induced similar degrees of anaphylaxis in mice treated with the isotype control Ab or anti-Siglec-8. In contrast, challenge with TNP-LP-Sig8L induced anaphylaxis in mice treated with anti-Siglec-8 but no anaphylaxis in mice treated with the isotype control Ab (Fig. 4G, 4H). These results suggest that anti-Siglec-8 either remained bound to Siglec-8 on the mast cell surface or induced Siglec-8 internalization (12) such that Siglec-8 was no longer available to be recruited by Sig8L to the anti-TNP-IgE-FcεRI for suppression of anaphylaxis.

Last, we tested if ligand-mediated recruitment of Siglec-8 can inhibit anaphylaxis caused by hIgE and hFcεRIα using Sig8-Tg mice on a *mFcεRIα<sup>-/-</sup>hFcεRIα<sup>+</sup>* background. These animals were passively sensitized with human anti-OVA IgE (clone 11B6) and i.v. challenged with PBS and OVA-LP or OVA-LP with Sig8L (OVA-LP-Sig8L) the next day (Fig. 4I). OVA-LP-induced robust systemic anaphylaxis suggested that the hIgE and hFcεRIα were functional in these animals. By contrast, OVA-LP-Sig8L did not induce a significant decrease in rectal temperature compared with PBS injection controls. Therefore, we conclude that ligand-mediated recruitment of Siglec-8 to the hIgE-hFcεRIα also suppresses mast cell activation caused by the corresponding allergen.

#### *Antigenic LP-Sig8L desensitize mice to the subsequent allergen challenge*

We previously demonstrated that antigenic liposomes with CD33L desensitized mice from the subsequent Ag challenge (8). In this study, we ask if antigenic LP-Sig8L can also achieve desensitization without causing anaphylaxis. Siglec-8-Tg mice sensitized with anti-TNP-IgE were treated with PBS, TNP-LP, or TNP-LP-Sig8L followed by an i.v. challenge of TNP-LP (Fig. 5A, Supplemental Fig. 4). As expected, a treatment using TNP-LP induced systemic anaphylaxis (Supplemental Fig. 4A). After resting the mice for 24 h, when challenged using TNP-LP, mice previously treated with TNP-LP did not develop systemic anaphylaxis, presumably because their mast cells had not yet recovered (Supplemental Fig. 4B) (32). By contrast, in mice sensitized with 5 μg of anti-TNP-IgE, TNP-LP-Sig8L did not cause a significant rectal temperature drop compared with PBS treatment (Fig. 5B, left). After resting the mice for 6 h, all mice were challenged with 50 μg of TNP-LP. PBS-treated mice developed anaphylaxis upon challenge, but TNP-LP-Sig8L-treated mice were protected from the challenge (Fig. 5B, right). Analogous desensitization using TNP-LP-Sig8L was also achieved in mice sensitized with 10 μg of anti-TNP-IgE (Supplemental Fig. 4C–E), similar to what we observed in the human CD33 transgenic mice (8).

We also assessed the ability of antigenic LP-Sig8L to desensitize mice against OVA, an allergen in egg allergy (33). Mice that were already passively sensitized with two clones of mouse anti-OVA-IgEs were i.v. treated with OVA-LP or OVA-LP-Sig8L followed by an i.p. challenge of soluble OVA (Fig. 5C). OVA-LP containing 9 μg of OVA-PEG-DSPE induced moderate to severe anaphylaxis in nine out of nine mice. In OVA-LP-Sig8L-treated mice, 2 out of 13

mice developed mild to moderate anaphylaxis, whereas 11 out of 13 mice did not develop significant rectal temperature changes (Fig. 5D, 5E). After resting the mice for 5 h, all mice were rechallenged i.p. with 5 mg of soluble OVA. Untreated mice developed systemic anaphylaxis upon challenge. Similar to what we observed with TNP-LP (Supplemental Fig. 4A–B), OVA-LP-treated mice did not develop anaphylaxis upon challenge (Fig. 5F, 5G). None of the OVA-LP-Sig8L-treated mice developed anaphylaxis upon challenge, even though only two mice previously exhibited a response in the initial treatment (Fig. 5F, 5G). These results suggest that antigenic LP-Sig8L can desensitize mast cells without triggering anaphylaxis.

To further investigate the mechanism of TNP-LP-Sig8L-mediated desensitization, mice were treated with PBS or TNP-LP-Sig8L. After 6 h, peritoneal cells were harvested and incubated with fluorescent TNP-LP *in vitro* followed by flow cytometry analysis (Fig. 6A). Mast cell frequencies (c-Kit<sup>+</sup>/CD45<sup>+</sup>PI<sup>−</sup>) from TNP-LP-Sig8L treated mice were unaltered (Fig. 6B), but binding of TNP-LP was dramatically reduced (Fig. 6C, 6D). This result suggests that the treatment of mice with TNP-LP-Sig8L blocks subsequent binding of Ag (TNP) either by physically occupying the Ag-specific IgE or by causing endocytosis of anti-TNP-IgE-FcεRI complexes from the cell surface (32, 34).

We also assessed the impact of TNP-LP-Sig8L on serum anti-TNP-IgE. Prior to treatment, mice had similar levels of anti-TNP-IgE (Fig. 6E). After resting for 6 h, serum anti-TNP-IgE in PBS-treated mice decreased by ~50% (Fig. 6F), consistent with previously reported half-life of infused IgEs (35). TNP-LP-Sig8L further decreased circulating anti-TNP-IgE levels to less than 10% (Fig. 6F). Taken together, the results suggest that TNP-LP-Sig8L administration desensitizes mast cells in this passive sensitization model by blocking subsequent Ag binding to anti-TNP-IgE-FcεRI complexes on mast cells by reducing circulating Ag-specific IgE and by preventing resensitization of mast cells.

## Discussion

Allergy is a growing concern worldwide, but effective therapeutic options are limited (36, 37). The standard of care for common food allergies is strict dietary avoidance and timely administration of life-rescuing agents, such as epinephrine upon accidental exposures (3). Allergen desensitization is one of few treatment options that aims to reduce sensitivity against allergen by administering gradually increasing doses of allergens over time, providing protection against lethal reactions caused by accidental allergen exposures (38). However, this treatment itself can trigger adverse events including anaphylaxis, and it must be conducted under close medical supervision monitoring for signs of anaphylaxis (1, 39), resulting in its discontinuation (40, 41).

Current approaches that seek to improve the safety of allergen desensitization focus on reducing allergic reactions to the treatment (32, 42–44). Omalizumab, an Ab that blocks circulating IgE from binding to mast cells, has been shown effective in reducing adverse events caused by oral immunotherapy (45, 46). Although omalizumab reduces free IgE in the blood, it does not reduce it completely, nor does it remove IgE bound to the FcεRI on mast cells, which remain responsive to the allergen for a prolonged period because of the long half-life of tissue-resident mast cells and slow turnover of IgE-FcεRI complexes. Another approach to suppress mast cell activation is to inhibit kinases involved in FcεRI signaling of mast cells using pharmacologic inhibitors (47–49). However, the risk versus benefit offered by these kinases inhibitors for allergic patients remains to be determined (4).

Another approach relevant to this report is to exploit other endogenous inhibitory receptors on mast cells to suppress FcεRI signaling (4). Notably, FcγRIIB, a low-affinity IgG receptor with ITIM in its cytoplasmic domain, has the ability to suppress signaling when recruited to the IgE-FcεRI complex. Bispecific Abs that ligate the inhibitory FcγRIIB with IgE or FcεRI (7) or chimeric allergen-Fc fusion proteins (5, 6) were efficacious in mouse models of allergy. Antiallergen IgGs suppressed IgE-mediated hypersensitivity via FcγRIIB in mouse models of food allergy (50). One challenge in translating molecular targets from murine models to humans is the heterogeneity of mast cells and the inhibitory receptors they express. For example, FcγRIIB is expressed on human intestinal mast cells but not skin mast cells (51).

Human mast cells express a number of ITIM-containing Siglecs, including CD22 (Siglec-2), CD33 (Siglec-3), Siglec-5, Siglec-6, and Siglec-7, which are potential targets for suppressing mast cell activation (8–10). We show, in this study, that Siglec-8 profoundly suppresses mast cell activation when recruited to the IgE-FcεRI complex by antigenic liposomes bearing high affinity ligands for Siglec-8 (Sig8L). When liposomes containing only Sig8L were mixed with antigenic liposomes, no inhibition of Ag-induced signaling was observed, demonstrating the requirement of having Sig8L on the same nanoparticle as the Ag. We suggest that the display of Ag and Sig8L on the same particle results in the close physical association of Siglec-8 with the IgE-FcεRI complex, leading to phosphorylation of the ITIM of Siglec-8 by SRC family kinases and recruitment of phosphatases that directly (SHP-1 and SHP-2) or indirectly (SHIP1) (52) dephosphorylate the downstream kinases involved in FcεRI signaling (Fig. 3A). This mechanism is supported by analogous results we obtained for the suppression of mast cell activation by human CD33 using antigenic liposomes with ligands for CD33 (8). For human CD33, it was shown that CD33-mediated suppression of mast cell activation depended on SHP-1 because murine mast cells that express human CD33 but lack SHP-1 were not susceptible to the suppression of activation by antigenic liposomes with CD33L on the same particle. Whether Siglec-8 also inhibits IgE-mediated mast cell activation through SHP-1 or another phosphatase remains to be tested.

Previously, we showed that anti-Siglec-8 Abs could partially inhibit mast cell mediator release and calcium flux induced by anti-human FcεRI Ab in human CD34<sup>+</sup> cell-derived mast cells and inhibit degranulation and calcium flux in Siglec-8-transfected RBL cells when cross-linked to the FcεRI receptor using secondary Abs (14). Anti-Siglec-8 Abs administered prophylactically to humanized mice also protected against PSA upon Ag challenge 1 d later (12). However, in the context of acute antigenic liposome-induced mast cell activation in this study, we saw no inhibition resulting from ligation using anti-Siglec-8 or liposomes containing Sig8L only (Figs. 1, 2). Moreover, anti-Siglec-8 partially reversed the inhibition caused by the Sig8L formulated into the antigenic liposomes, which we attribute to preventing ligand-mediated recruitment of Siglec-8 to the antigenic synapse formed between the liposome and the IgE-FcεRI complex. Although these results may appear inconsistent, we believe that they reflect the context-dependent conditions of the experiments. In this report, highly multivalent antigenic liposomes, which extensively cross-link IgE-FcεRI, could provide a stronger activatory signal than anti-FcεRI Ab used in previous work (14), requiring stronger recruitment of Siglec-8 to the activation complex to induce suppression. In the experiments with humanized mice, sensitized mice were treated with anti-Siglec-8 2 d before challenge (12). Thus, differences in the impact of anti-Siglec-8 in this context could reflect the prolonged exposure to anti-Siglec-8 *in vivo* prior to allergic challenge. Alternatively, there could be differences in the microdomain localizations or intrinsic signaling aspects of Siglec-8 and

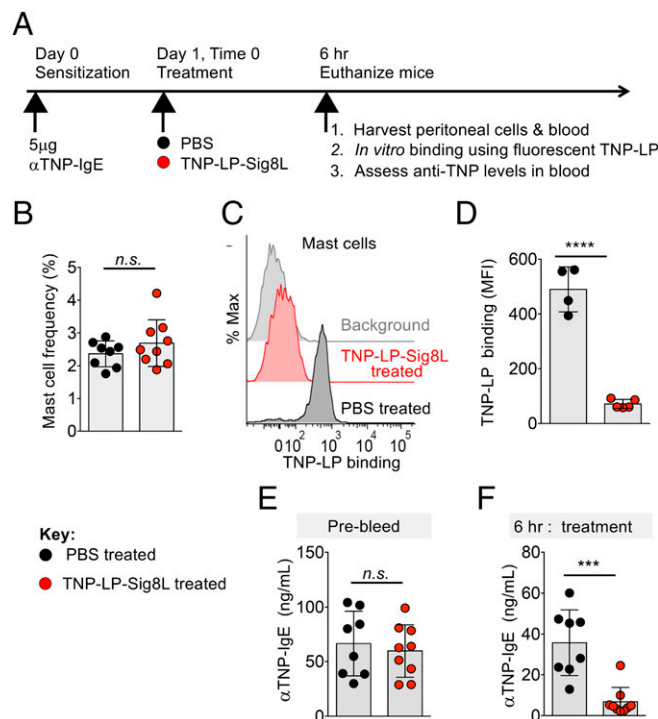


**FIGURE 6.** Impact of antigenic LP-Sig8L on the subsequent binding of TNP-LP to peritoneal mast cells and serum anti-TNP-IgE clearance in sensitized Siglec-8 Tg mice. Experiments were performed to assess the accessibility of anti-TNP-IgE/FcεRI on the surface of mast cells and the levels of anti-TNP-IgEs in the blood following treatment with TNP-LP-Sig8L as a basis for desensitizing mice to challenge. **(A)** Scheme for treatment and characterization of treated mice. **(B)** Quantitation of peritoneal mast cells posttreatment with PBS or TNP-LP-Sig8L. **(C and D)** Binding of fluorescent TNP-LP (20 μM) to peritoneal mast cells posttreatment with PBS or TNP-LP-Sig8L. **(E and F)** Anti-TNP-IgE in blood of mice before (E) or 6 h after (F) treatment with PBS or TNP-LP-Sig8L (200 μl of 1.5 mM liposome) determined by ELISA, as described in *Materials and Methods*. Results in (B), (E), and (F) were compiled from two independent experiments. Statistical analysis was conducted using unpaired two-tailed Student *t* test. \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001. n.s., not significant (*p* > 0.05).

the FcεRI receptor in human mast cells, mast cells of Siglec-8 transgenic mice, and RBL cells used in these experiments that account for differences between experiments. Although additional work on operative mechanisms is needed to understand differences in these experimental settings, in this study, we have provided strong evidence for enhancement of Siglec-8-mediated inhibition of mast cell activation and degranulation by enforced recruitment to the FcεRI activation complex.

In this study, we report that recruitment of Siglec-8 to IgE-FcεRI strongly inhibits Ag-induced mast cell degranulation in vitro and IgE-mediated anaphylaxis in vivo. These observations combined with our previous demonstration that CD33 similarly suppresses mast cell activation suggest a general mechanism for Siglecs on mast cells as checkpoint receptors and that other ITIM-containing Siglecs on mast cells, including CD22, Siglec-5, -6, -7, and -10, could potentially be exploited as targets for controlling allergen-induced IgE-mediated reactions including anaphylaxis (10).

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## Disclosures

S.D., C.M.N., M.S.M., B.M.A., and J.C.P. are listed as coinventors on patent applications covering aspects of this work that have been filed with the

United States Patent and Trademark Office and in the event the patents issue may be entitled to a share of royalties received by the Scripps Research Institute during development and potential sales of such products. B.S.B. receives remuneration for serving on the scientific advisory board of Allakos, owns stock in Allakos, and receives publication-related royalty payments from Elsevier and UpToDate. B.S.B. is also a coinventor on existing Siglec-8-related patents and thus may be entitled to a share of royalties received by Johns Hopkins University during development and potential sales of such products. B.S.B. is also a cofounder of Allakos, which makes him subject to certain restrictions under university policy. The terms of this arrangement are being managed by the Johns Hopkins University and Northwestern University in accordance with their conflict of interest policies. The other authors have no financial conflicts of interest.

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Supporting information:

**Figure S1**

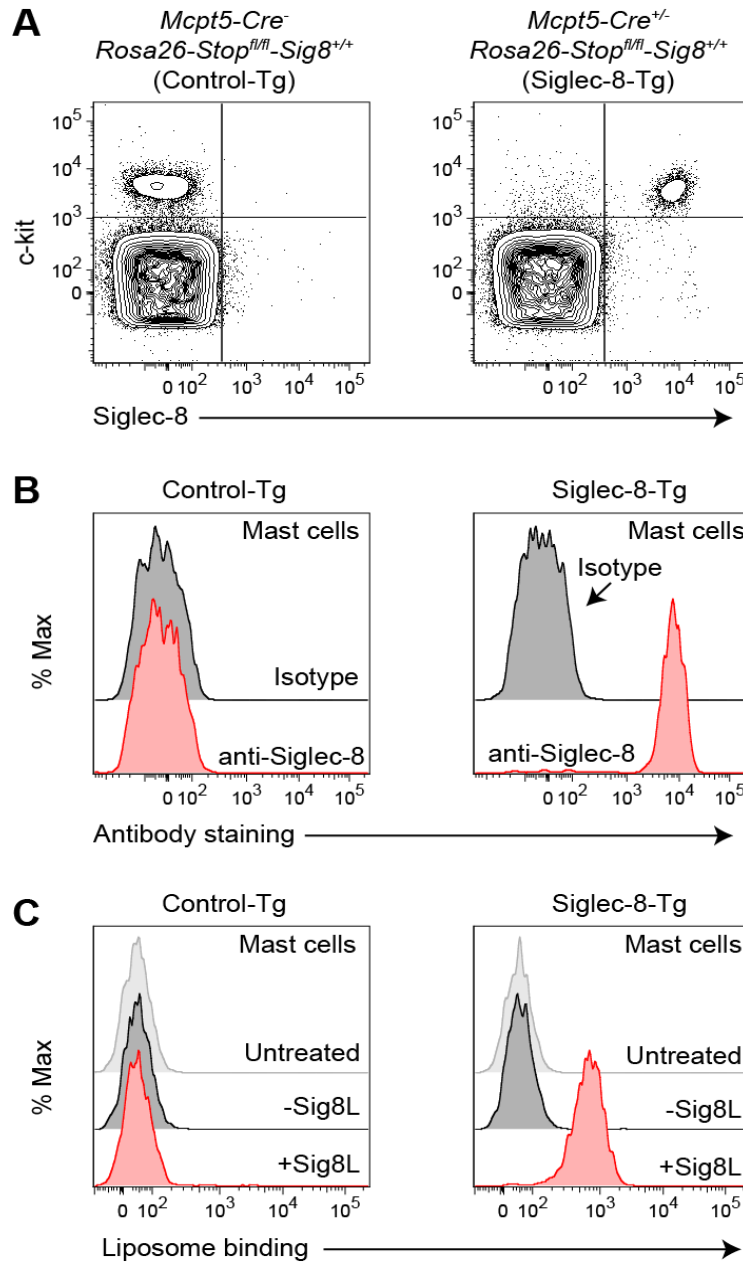


**Figure S1. Characterization of Siglec-8 transgenic mice and liposome binding to peritoneal mast cells.**

(A) Anti-Siglec-8 staining of peritoneal cells harvested from Control (left) or Siglec-8-Tg mice (right).

(B) Staining of an isotype control or anti-Siglec-8 antibody to peritoneal mast cells (c-Kit<sup>+</sup>FcεRI<sup>+</sup>) from Control (left) or Siglec-8-Tg mice (right).

(C) In vitro binding of fluorescent liposomes (20 μM) with or without Sig8L to peritoneal mast cells (c-Kit<sup>+</sup>FcεRI<sup>+</sup>) from Control (left) or Siglec-8-Tg mice (right).

**Figure S2**

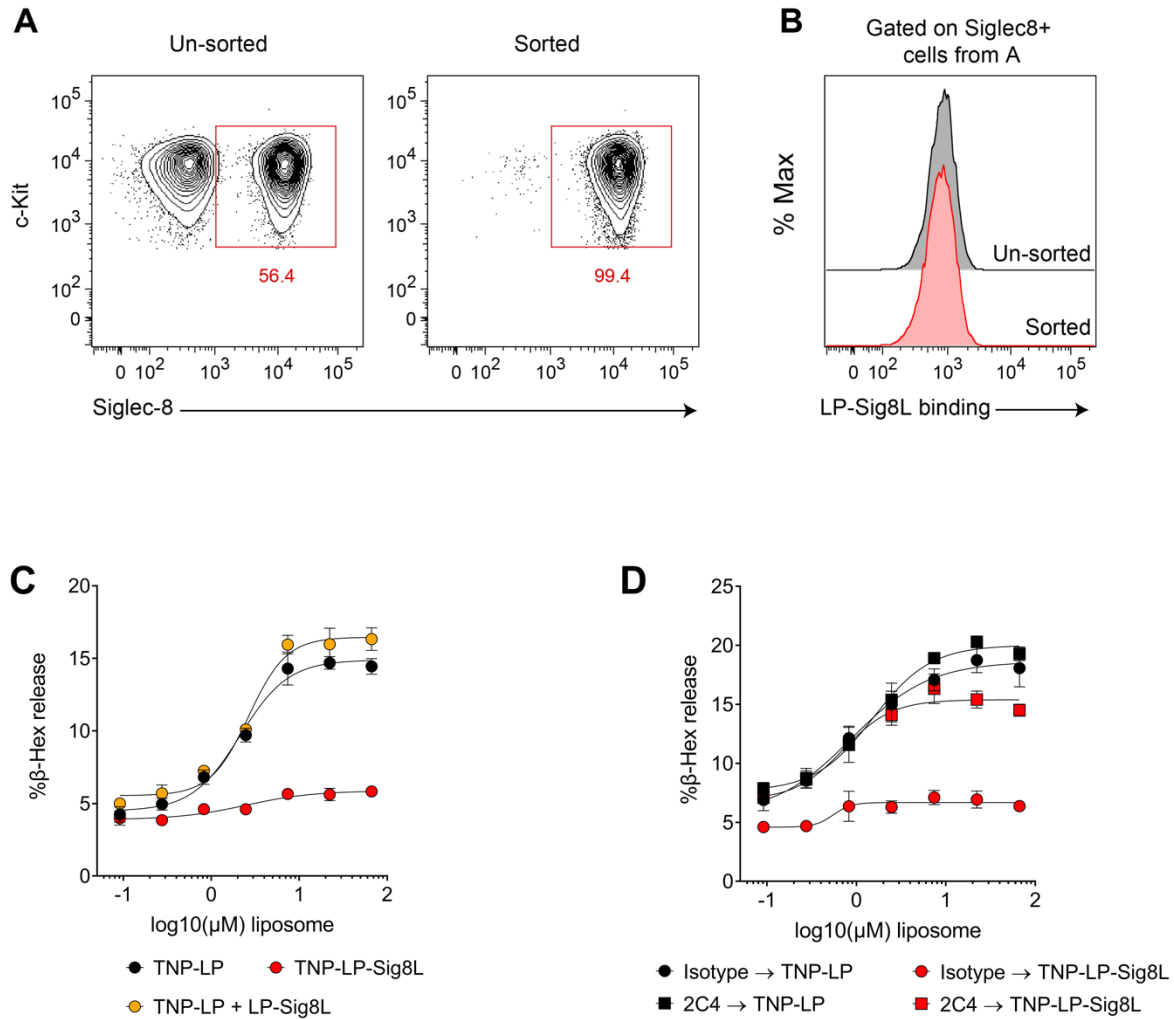


**Figure S2. Recruitment of Siglec-8 to the IgE-FcεRI complex is required for Sig8L to inhibit degranulation of Siglec-8+ BMMCs.**

For in vitro experiments, Siglec-8+ BMMCs were positively sorted based on Siglec-8 staining by flow cytometry.

(A) After overnight culture, anti-Siglec-8 staining of un-sorted or sorted cells was analyzed by flow cytometry. Siglec-8+ gate was determined using un-sorted culture.

(B) Binding of LP-Sig8L (20 μM) to sorted or un-sorted Siglec-8+ BMMCs. The binding of liposomes without Sig8L was analogous to what is shown in Fig 2.

(C) Degranulation of Siglec-8+ BMMC induced by titrated amounts of TNP-LP, TNP-LP-Sig8L, or a mixture of TNP-LP with LP-Sig8L. N=4 per condition. Data were plotted as means ± S.D.

(D) Siglec-8+ BMMCs were first treated with isotype control or anti-Siglec-8 antibody (clone 2C4, 10 μg/mL in 50 μL, 1 hour at 37°C). Titrated amount of TNP-LP or TNP-LP-Sig8L (in 50 μL) were then directed added to cells for another hour at 37°C followed by assaying β-hexosaminidase activity in the supernatant versus cell pellet. Final antibody concentration is 5 μg/mL in 100 μL per 96 well. N=3 per condition. Data were plotted as means ± S.D.

**Figure S3**

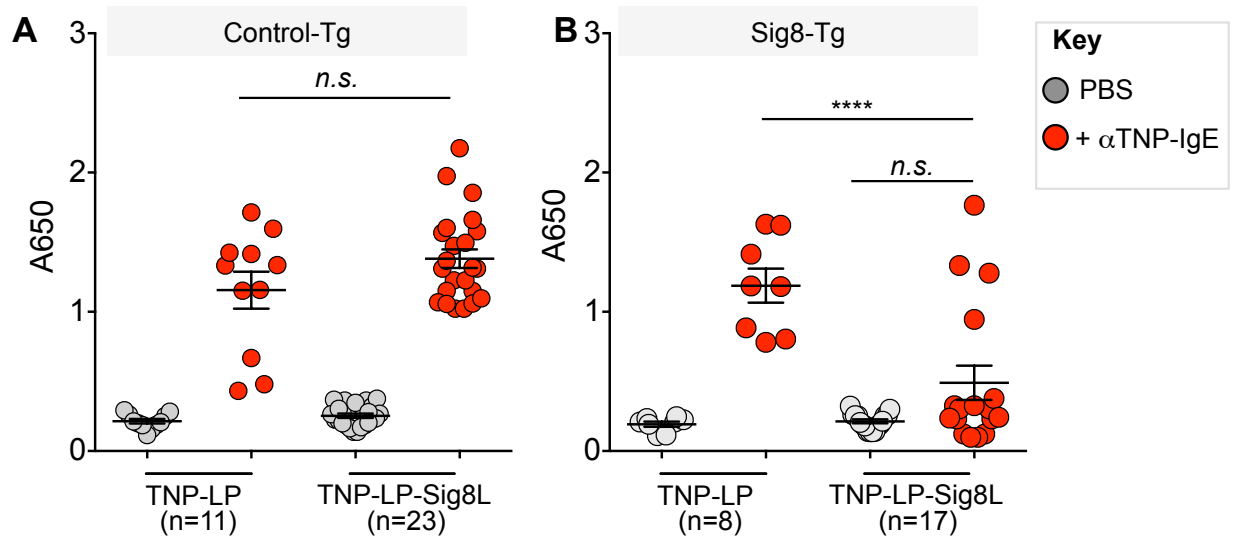


**Figure S3. Siglec-8 ligand blunts antigen-induced passive cutaneous anaphylaxis**

Quantification of skin mast cell activation (dye extravasation from vascular leak as detected as absorbance at 650 nm) induced by 150  $\mu$ g (200  $\mu$ L of 1mM) TNP-LP or TNP-LP-Sig8L in Control (A) or Siglec-8-Tg (B) mice. Data were analyzed by 1-way ANOVA followed by Tukey's test. Data were compiled from five experiments. Genotypes of the mice were determined by PCR. Bars represent means  $\pm$  S.D.

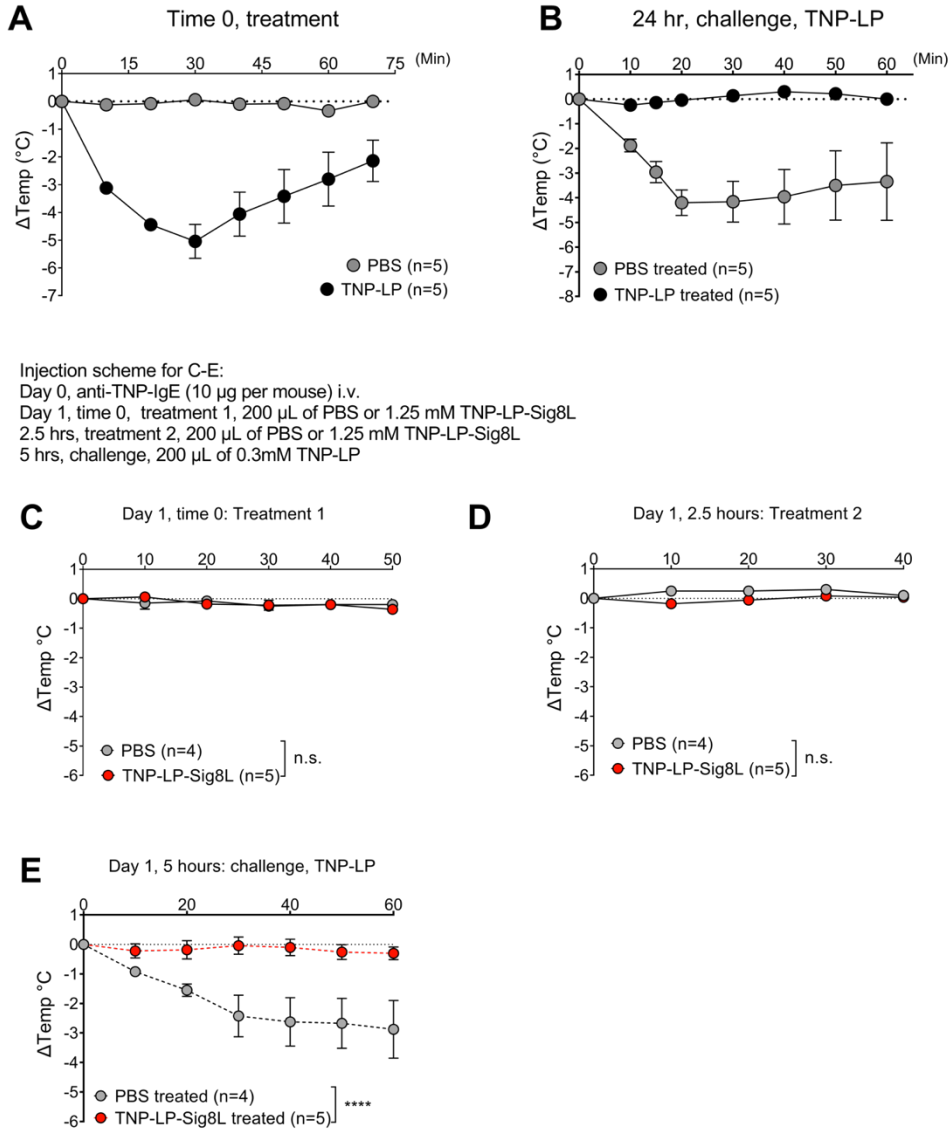
**Figure S4**

Injection scheme for A and B:

Day 0, anti-TNP-IgE (10  $\mu$ g per mouse) i.v.

Day 1, time 0, treatment, 200  $\mu$ L of PBS or 0.5 mM TNP-LP i.v.

Day 2 (24 hour), challenge, 200  $\mu$ L of 0.5 mM TNP-LP i.v.



Injection scheme for C-E:

Day 0, anti-TNP-IgE (10  $\mu$ g per mouse) i.v.

Day 1, time 0, treatment 1, 200  $\mu$ L of PBS or 1.25 mM TNP-LP-Sig8L

2.5 hrs, treatment 2, 200  $\mu$ L of PBS or 1.25 mM TNP-LP-Sig8L

5 hrs, challenge, 200  $\mu$ L of 0.3mM TNP-LP

**Figure S4. Impact of TNP-LP or TNP-LP-Sig8L on subsequent challenge of TNP-LP.**

(A) Change of rectal temperature induced by PBS or TNP-LP treatment.

(B) Change of rectal temperature induced by TNP-LP challenge 24 hours after treatment. 200  $\mu$ L of 0.5 mM TNP-LP (0.1% TNP) were used in both treatment and challenge step.

(C, D) Change of rectal temperature induced by 200  $\mu$ L of PBS or 1.25 mM TNP-LP-Sig8L (0.1% TNP and 3% Sig8L) i.v. (E) Change of rectal temperature upon challenge with TNP-LP (200  $\mu$ L of 0.3 mM liposome). Values were plotted by means  $\pm$  s.e.m. Interaction p values were determined by repeated measures 2-way ANOVA: \*\*\*\* p < 0.0001; n.s., not significant (p > 0.05).