The Inhibitory Receptor Siglec-8 Interacts with FcεRI and Globally Inhibits Intracellular Signaling in Primary Mast Cells Upon Activation

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BACKGROUND

- Inhibition of mast cell (MC) activity is warranted in allergic and inflammatory diseases where MCs have a central role in pathogenesis.
- Targeting Siglec-8, an inhibitory receptor on MCs and eosinophils, has shown promising activity in preclinical and clinical studies.
- While the intracellular pathways that regulate Siglec-8 activity in eosinophils have been well studied, the signaling mechanisms that lead to MC inhibition have not been fully elucidated.

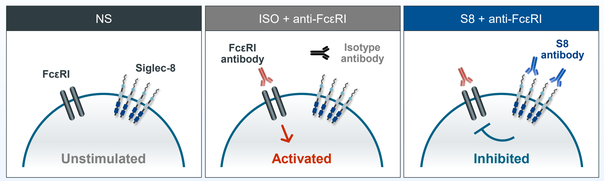
OBJECTIVES

- To investigate the inhibitory effects of Siglec-8 on FceRI intracellular signaling in primary MCs using a Siglec-8 mAb.

METHODS

- Intracellular signaling pathways of Siglec-8-mediated inhibition using an anti-Siglec-8 monoclonal antibody were evaluated in FcεRI-activated primary MCs by phospho-proteomic profiling.
- Biochemical characterization of receptor signaling complexes and confocal imaging of MCs were used to evaluate Siglec-8 complexes.

Figure 1. FcεRI-induced activation and Siglec-8-mediated inhibition of mast cells

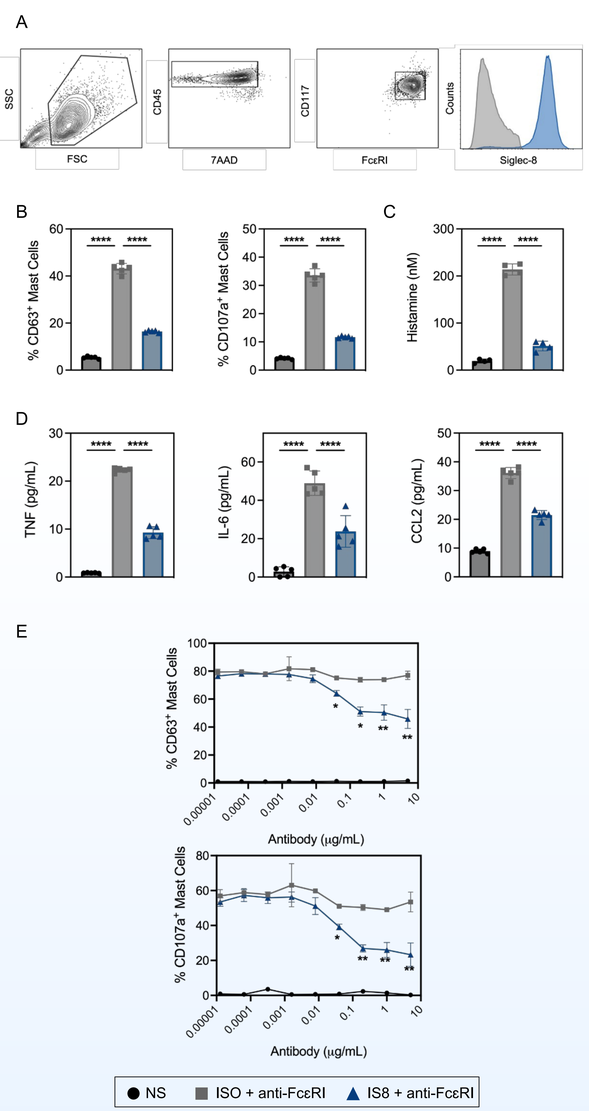


Overview of conditions used to study effects of Siglec-8 mediated MC inhibition. Bone marrow derived mast cells from Siglec-8 transgenic mice (S8-BMMC) were left unstimulated (NS), stimulated with an agonistic anti-FcεRI antibody in the presence of an isotype control antibody (ISO + anti-FcεRI) or an anti-Siglec-8 antibody (S8 + anti-FcεRI).

RESULTS

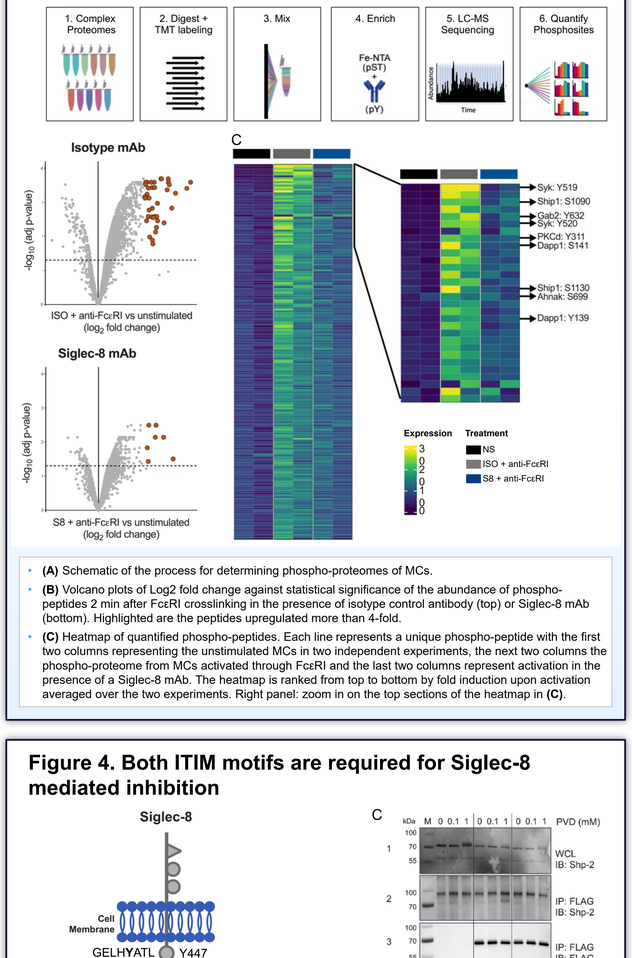
- Siglec-8 mAb-treatment globally inhibited proximal and downstream kinases in the FcεRI signaling cascade, leading to attenuated MC activation and degranulation.
- Siglec-8 inhibition was dependent on both cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that interact with the SH2 containing protein phosphatase Shp-2 upon Siglec-8 phosphorylation.
- Siglec-8 was found to directly interact with FcεRI signaling molecules and co-localized with FcεRI upon Siglec-8 mAb-treatment under conditions of MC activation.

Figure 2. BMMC from transgenic mice respond to FcεRI activation and express functional Siglec-8



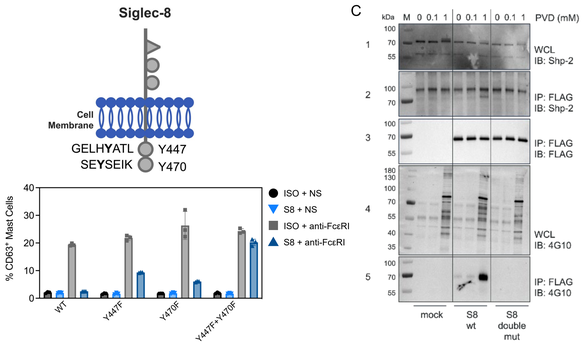
- **(A)** BMMC-S8 flow cytometry gating strategy.
- **(B)** Levels of surrogate degranulation markers CD63 and CD107a on the cell surface of unstimulated MC (NS; black bars), when cross-linked with the anti-FcεRI antibody MAR-1 for 15 min (ISO + anti-FcεRI, grey bars) or co-cross-linked with MAR-1 and Siglec-8 mAb (S8 + anti-FcεRI, blue bars).
- **(C)** Histamine concentrations in the culture medium of MC collected 1 hour after stimulation as in **(B)**.
- **(D)** Cytokine concentrations in the culture medium of MC collected 6 hours after stimulation.
- **(E)** Titration of isotype control mAb and Siglec-8 mAb in combination with anti-FcεRI-mediated (MAR-1) activation. Left panel: % of CD63 positive MC. Right panel: % of CD107a positive MC. Data are plotted as mean ± SEM (n=5) and are representative of 5 experiments.
  *p < 0.05, **p < 0.01, ****p < 0.0001 by one-way ANOVA test **(B–D)** or unpaired t test **(E)**

Figure 3. Phospho-proteome of FcεRI-stimulated and Siglec-8-inhibited S8-BMMCs



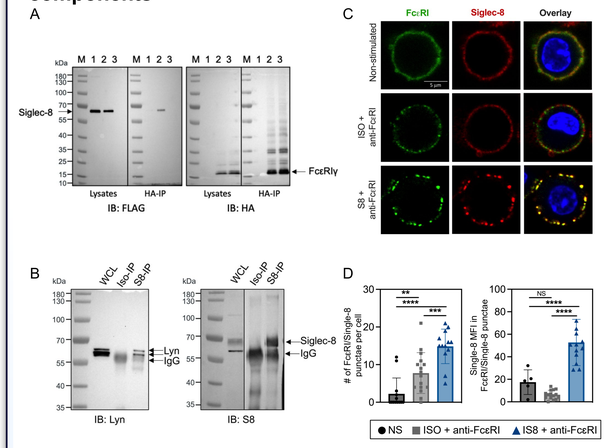
- **(A)** Schematic of the process for determining phospho-proteomes of MCs.
- **(B)** Volcano plots of Log2 fold change against statistical significance of the abundance of phospho-peptides 2 min after FcεRI crosslinking in the presence of isotype control antibody (top) or Siglec-8 mAb (bottom). Highlighted are the peptides upregulated more than 4-fold.
- **(C)** Heatmap of quantified phospho-peptides. Each line represents a unique phospho-peptide with the first two columns representing the unstimulated MCs in two independent experiments, the next two columns the phospho-proteome from MCs activated through FcεRI and the last two columns represent activation in the presence of a Siglec-8 mAb. The heatmap is ranked from top to bottom by fold induction upon activation averaged over the two experiments. Right panel: zoom in on the top sections of the heatmap in **(C)**.

Figure 4. Both ITIM motifs are required for Siglec-8 mediated inhibition



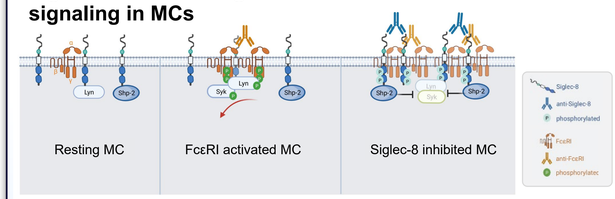
- **(A)** Schematic of the Siglec-8 receptor with its two tyrosine residues in the context of ITIM motifs indicated.
- **(B)** Percent of CD63 positive MC in unstimulated (ISO+NS, S8+NS), stimulated (ISO+anti-FcεRI) and inhibited (S8+anti-FcεRI) MC after transfection of the indicated wild-type or mutant Siglec-8 expression constructs Data are plotted as mean±SEM (n=3) and are representative of 3 experiments.
- **(C)** Western blot analysis of Siglec-8-Shp2 interaction. Immunoblot (IB) of whole cell lysates (WCL) or FLAG immunoprecipitations (IP) from primary MC mock transfected or with expression constructs for wild-type Siglec-8-FLAG or ITIM double mutant Siglec-8-FLAG. Cells were subjected to PVD treatment for two min and WCL were analyzed for presence of Shp-2 (panel 1) and p-Tyr (panel 4). Anti-FLAG IPs were analyzed for presence of Shp-2 (panel 2), Siglec-8-FLAG (panel 3) and p-Tyr (panel 5). Experiments were performed 2-3 times for confirmation and to ensure reproducibility.

Figure 5. Siglec-8 interacts with FcεRI machinery components



- **(A)** Western blot analysis of Siglec-8-FcεRIγ interaction. Immunoblot (IB) of whole cell lysates (WCL) or HA immunoprecipitations (IP) from primary MC transfected with expression constructs for Siglec-8-FLAG (lane 1) or FcεRIγ-HA (lane 3) or both (lane 2). Blots were developed with anti-FLAG (left panel) and anti-HA (right panel).
- **(B)** Western blot analysis of Siglec-8-Lyn interaction. WCL, isotype IP or S8 IP from S8-BMMC were developed using anti-Lyn or anti-Siglec-8 antibodies.
- **(C)** Confocal microscopy imaging of Siglec-8 (red) and FcεRI (green) in S8-BMMC.
- **(D)** Quantification of confocal images. Left: number of FcεRI punctae per cell (PE channel). Right: Siglec-8 median fluorescence intensity within FcεRI/Siglec-8 punctae (AF647 channel). Experiments were performed 2-3 times. NS, not significant, **p < 0.01, ***p < 0.001, ****p < 0.0001 by one-way ANOVA test.

Figure 6. Model of Siglec-8-mediated inhibition of FcεRI signaling in MCs



- Siglec-8 and FcεRI are distributed along the membrane of resting MC while partially colocalizing (left panel).
- Cross-linking with anti-FcεRI results in high density clusters of FcεRI resulting in phosphorylation at the ITAM, subsequent MC activation/degranulation and partial exclusion of Siglec-8 from the activating complexes (middle panel).
- In the presence of Siglec-8 mAb, phosphatases like Shp-2 are recruited to large complexes of activating (FcεRI) and inhibitory (Siglec-8) receptors resulting in potent inhibition of intracellular signaling (right panel).

CONCLUSIONS/DISCUSSION

- These data provide novel mechanistic insight into intracellular signaling pathways and protein interactions that contribute to Siglec-8 mediated MC inhibition.
- Taken together, the data highlight how recruitment of the inhibitory receptor Siglec-8 can affect the complex orchestration of MC activation and inhibition through direct interaction with the activating receptor FcεRI and downstream signaling molecules, thereby regulating the activity of kinases and phosphatases upon stimulation.

Reference: (1) Korver W, et al. Front. Immunol. 2022;13:833728

Presented at the European Academy of Allergy & Clinical Immunology (EAACI) Annual Meeting July 1-3, 2022