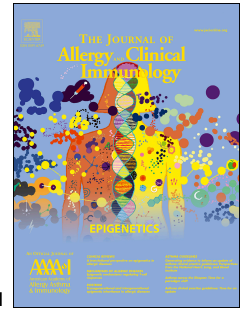


Accepted Manuscript

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Identification of inhibitory mechanisms in pseudo-allergy involving Mrgprb2/MRGPRX2-mediated mast cell activation

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A short summary

Ceramide-CD300f binding inhibits mouse Mrgprb2 (human MrgprX2)-mediated mast cell activation, thereby suppressing pseudo-allergic reactions. CD300f could be a therapeutic target against IgE-independent pseudo-allergy as well as IgE-dependent allergy.

Key words

pseudo-allergy; mast cell; Mrgprb2/MRGPRX2; CD300f; ceramide

Abbreviations

BMMCs, bone marrow-derived mast cells; CTMCs, connective tissue mast cells; FcεRI, high affinity IgE receptor; FDA, US Food and Drug Administration; LMIR3, leukocyte mono-immunoglobulin-like receptor 3; MCs, mast cells; MC_{TC}, tryptase and chymase-expressing mast cells; MRGPRX2, Mas-related G-protein coupled receptor X2; WT, wild-type.

To the editor

Anaphylaxis is a life-threatening systemic allergic reaction that can develop rapidly. Classically, anaphylaxis is thought to be induced by the crosslinking of high affinity IgE receptor (FcεRI) in the mast cells (MCs) with specific antigens; FcεRI engagement leads to the MC degranulation causing the rapid release of preformed granule constituents such as vasoactive amine histamine. However, the pseudo-allergic drug reaction, a representative of IgE-independent anaphylaxis, is triggered by the first intake of drugs: MCs are directly activated by basic secretagogues (e.g., compound 48/80) or an array of US Food and Drug Administration (FDA)-approved drugs (e.g., cationic peptidergic drugs and fluoroquinolone antibiotics), leading to MC degranulation. These drugs activate human tryptase and chymase-expressing MCs (MC_{TC}) via the Mas-related G-protein coupled receptor X2 (MRGPRX2) or the mouse connective tissue MCs (CTMCs) via Mrgprb2, the ortholog of human MRGPRX2.¹⁻³ An inhibitory receptor CD300f, also known as leukocyte mono-immunoglobulin-like receptor 3 (LMIR3), suppresses IgE- and MC-dependent anaphylactic responses by binding to its physiological ligand ceramide.⁴⁻⁶ In addition, ceramide-CD300f interactions inhibit ATP- or LPS-induced MC activation.⁷⁻⁹ However, unlike the inhibitory mechanisms seen in IgE-dependent allergy, those in the IgE-independent pseudo-allergy have not been clarified. Therefore, we asked whether CD300f regulates IgE-independent pseudo-allergic reactions.

To examine whether CD300f regulates Mrgprb2-mediated MC activation, peritoneal MCs were first isolated as mouse CTMCs (see Fig E1). We then confirmed their

stimulation with compound 48/80 that induces degranulation of peritoneal MCs, but not of bone marrow-derived MCs (BMMCs) (see Fig E2).² Real-time RT-PCR analysis showed that peritoneal MCs had higher levels of mRNA of Mrgprb2 and Mrgprb1 than did the BMMCs (see Fig E3). Knockdown of Mrgprb2 but not of Mrgprb1 by specific small interfering RNA (siRNA) dampened the compound 48/80-stimulated peritoneal MC degranulation. This confirms the activation of the mouse peritoneal MC via Mrgprb2 by compound 48/80 (Fig 1, A and see Fig E4).² We then isolated the peritoneal MCs from the wild-type (WT) and the *CD300f*^{-/-} mice, both expressing equivalent levels of FcεRI and c-Kit (see Fig E5). To test if the binding of ceramide to CD300f inhibits Mrgprb2-mediated degranulation in CTMCs, WT or CD300f-deficient peritoneal MCs were stimulated with compound 48/80 in plates coated with either ceramide, phosphatidylcholine, or a vehicle.⁵ Notably, CD300f deficiency did not influence the compound 48/80-mediated degranulation of peritoneal MCs in plates coated with the vehicle (Fig 1, B). However, the compound 48/80-stimulated degranulation was inhibited only in WT and not in the CD300f-deficient peritoneal MCs in ceramide-coated plates (Fig 1, B). This indicates suppression of Mrgprb2-mediated degranulation in mouse peritoneal MCs *in vitro* due to ceramide-CD300f binding. We then asked if the interactions between human CD300f and its ligands, ceramide and sphingomyelin, inhibit MRGPRX2-mediated degranulation in human MCs.⁶ We found that both CD300f and MRGPRX2 were highly expressed in the human MC line LAD2 (see Fig E6). When LAD2 cells were stimulated with compound 48/80 in plates coated with ceramide, sphingomyelin, phosphatidylserine, or a vehicle, the suppression of

degranulation in LAD2 cells was noted only in ceramide- or sphingomyelin-coated plates (Fig 1, C).⁶ However, pretreatment with a soluble anti-human CD300f antibody neutralized the ceramide- or sphingomyelin-mediated suppression of LAD2 MC degranulation (Fig 1, C). Similar results were obtained in human peripheral blood-derived mast cells (see Fig E7). These results indicate that the interactions of human CD300f and its lipid ligands inhibit MRGPRX2-mediated degranulation in human MCs.

To address the *in vivo* role of CD300f in Mrgprb2-mediated pseudo-allergy, we intradermally injected compound 48/80 or the vehicle in the ears of WT or *CD300f*^{-/-} mice just prior to the intravenous injection of Evans blue dye. Evaluation of extravasated dye amounts in the ears 0.5 h after injection showed that *CD300f*^{-/-} mice had higher amounts of dye extravasation in compound 48/80-stimulated ears than did its WT counterparts (Fig 2, A). In addition, *CD300f*^{-/-} mice had higher activity of β -hexosaminidase released from mast cells via degranulation in compound 48/80-stimulated ears than did its WT counterparts (see Fig E8). These results indicated that CD300f deficiency enhanced the compound 48/80-stimulated increase in vascular permeability via mast cell degranulation. Similarly, the *CD300f*^{-/-} mice exhibited higher amounts of dye extravasation with ciprofloxacin (fluoroquinolone antibiotic)- or icatibant (cationic peptidergic drug known as bradykinin B₂ receptor antagonist)-stimulated ears than did its WT counterparts (see Fig E9).^{2,3} Given these compounds directly activate CTMCs via Mrgprb2,^{2,3} it was possible to conclude that CD300f inhibits Mrgprb2-mediated pseudo-allergic reactions in mice. To further clarify

the role of ceramide-CD300f interactions in pseudo-allergic reactions, WT or *CD300f*^{-/-} mice were treated with ceramide-containing vesicles along with compound 48/80 in the ears. The results showed that the treatment with ceramide-containing vesicles suppressed compound 48/80-stimulated dye extravasation in the ears of WT mice but not in those of *CD300f*^{-/-} mice (Fig 2, B). By contrast, treatment with anti-ceramide antibody increased the amounts of compound 48/80-stimulated dye extravasation at levels comparable to those seen in *CD300f*^{-/-} mice (Fig 2, C). In addition, the same treatments did not influence the compound 48/80-stimulated increase of vascular permeability in *CD300f*^{-/-} mice (Fig 2, B and C). Taken together, these results indicate that the ceramide-CD300f binding inhibits Mrgprb2-mediated degranulation in CTMCs, thereby suppressing pseudo-allergic reactions in mice. To finally examine the role of human CD300f in pseudo-allergic reactions, CD300f-deficient BMMCs were transduced with mouse CD300f, human CD300f, or mock.^{5,6} These transduced MCs were engrafted into the ears of MC-deficient *Kit*^{W-sh/W-sh} mice prior to measuring the amount of extravasated dye in the ears injected with compound 48/80. The results reveal that similar to mouse CD300f, the human CD300f also suppressed compound 48/80-stimulated increase in vascular permeability (see Fig E10). In addition, these results suggested that transduced BMMCs engrafted in the ears differentiated into CTMCs expressing Mrgprb2. It is speculated that CD300f inhibits pseudo-allergic reactions in humans and mice. Considering that the degree of pseudo-allergic reactions varies by person, differential surface expression of MRGPRX2 and/or CD300f in MCs may be involved in the relevant mechanisms. Moreover, because MRGPRX2 also

recognizes host defense peptides and neuropeptides,³ it is possible that CD300f regulates the related host defense responses and neuro-inflammatory responses as well.

In conclusion, we provide evidence that CD300f inhibits IgE-independent pseudo-allergic responses via MRGPRX2/Mrgprb2 as well as the IgE-dependent allergic responses via FcεRI, suggesting that CD300f could be a potential therapeutic target for MC-dependent anaphylaxis.⁵⁻⁹

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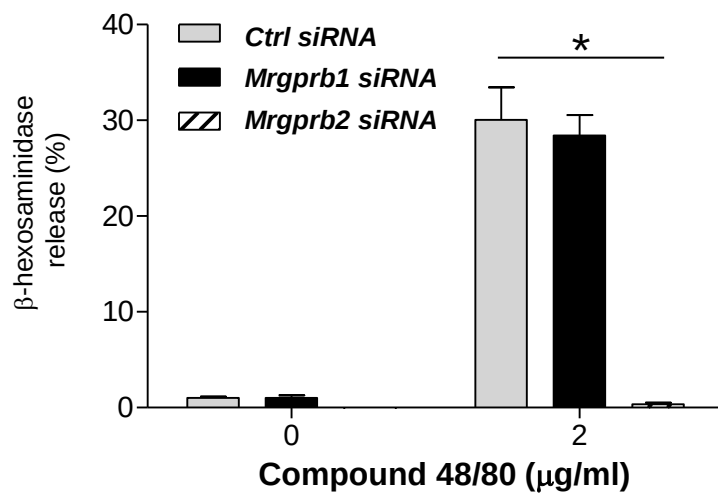
Figure legends

FIG1. Ceramide-CD300f binding inhibits Mrgprb2-mediated degranulation in peritoneal MCs. **A–C**, The degranulation ratio of compound 48/80-stimulated WT peritoneal MCs transfected with *Mrgprb2* siRNA (Fig 1, A), WT or *CD300f*^{-/-} peritoneal MCs (Fig 1, B) or LAD2 cells (Fig 1, C) in plates coated with indicated lipids (n = 3; ± SD. *p < 0.01).

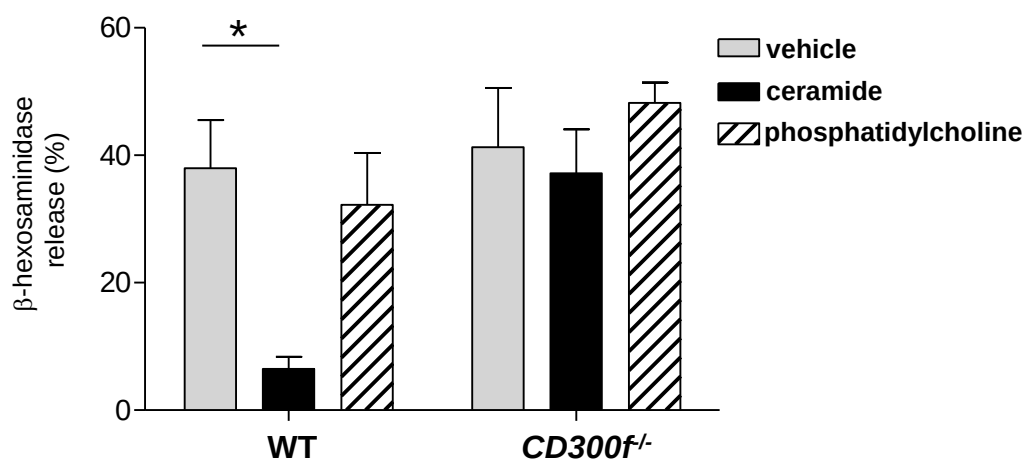
FIG2. Ceramide-CD300f binding inhibits Mrgprb2-mediated pseudoallergy. **A–C**, Dye extravasation (representative picture) in the ears of WT or *CD300f*^{-/-} mice injected intradermally with a vehicle or compound 48/80 (Fig 1, A) or those, pretreated with PBS or ceramide vesicles (Fig 1, B) or with control or ceramide antibody (Fig 1, C). n = 4-10 per group; ± SD. *p < 0.01.

Figure 1

A



B



C

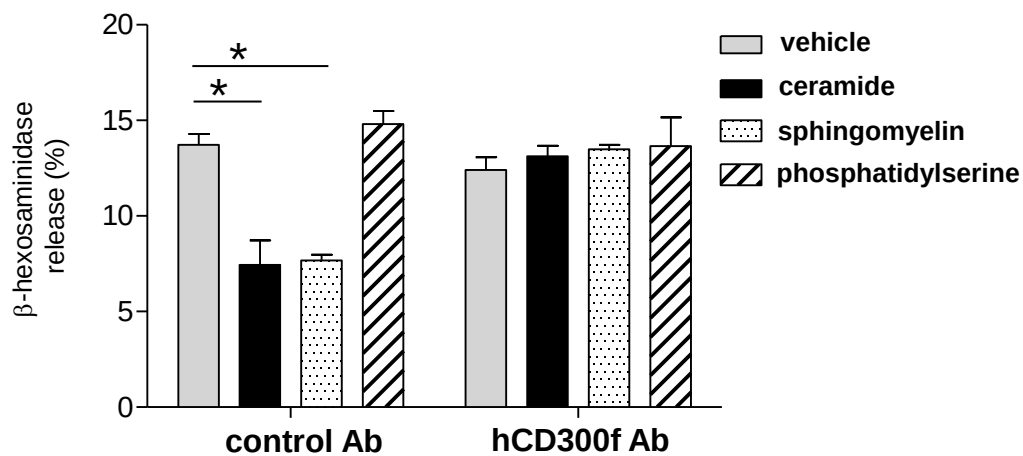
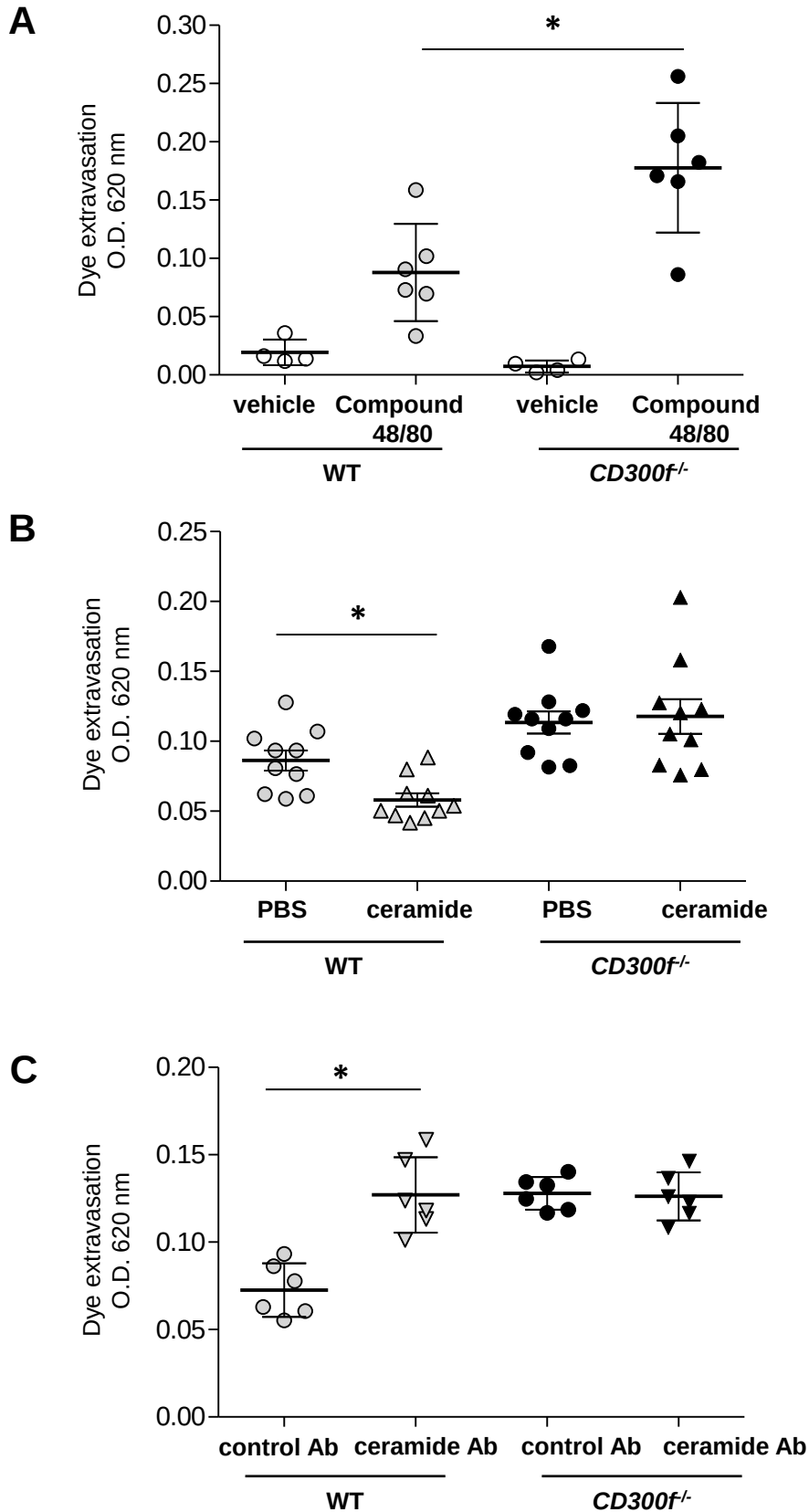


Figure 2



Methods

Mice

All procedures were approved by an Institutional Review Committee of Juntendo University (approval no 290104 and 300132). C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). *CD300^{f/-}* mice and *Kit^{W-sh/W-sh}* mice were used as described.⁵

Cells

BMMCs were prepared following methods previously described.⁵ LAD2 cells were cultured as described.⁶ Peritoneal MCs were purified and used as described with some modifications.^{E1} Briefly, peritoneal cells were collected from mice injected intraperitoneally with PBS supplemented with 2% fetal calf serum (FCS), and cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS, 10 ng/mL recombinant mouse IL-3, and 10 ng/mL recombinant mouse SCF for 10-14 days. Floating cells were used as peritoneal MCs. Human peripheral blood-derived mast cells were generated as previously described.^{E2,E3} Briefly, human peripheral blood CD34⁺ cells (STEMCELL Technologies) were cultured in serum-free Iscove's methylcellulose medium (STEMCELL Technologies) containing SCF, IL-6, and IL-3 for 2 weeks and in that containing SCF and IL-6 without IMDM for 2 weeks and then with IMDM containing SCF and IL-6 for 3 weeks. The collected cells were used. The study was approved by the Ethics Committee of Juntendo University.

Antibodies and other reagents

The following antibodies were used: Rat anti-CD300f monoclonal antibody (3-14-11; rat IgG_{2a}) (ACTGen), fluorescein isothiocyanate (FITC)-conjugated anti-FcεRIα (MAR-1) and human FcεRIα (AEP-37), phycoerythrin (PE)-conjugated anti-c-kit (2B8) and rat IgG2a (eBR2a) (eBioscience), Allophycocyanin (APC)-conjugated anti-human c-Kit (104D2) (BioLegend), anti-ceramide (MID 15B4) (Enzo Life Sciences), anti-mouse IgM (MOPC-104E) (BioLegend), goat anti-human CD300f (AF2774) (R&D Systems), and PE-conjugated F(ab')₂ anti-goat IgG (Jackson ImmunoResearch). Cytokines were obtained from R&D Systems. C-24 ceramide was obtained from Toronto Research Chemicals, Inc. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (PC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine (PS) were obtained from Echelon Biosciences Inc. Sphingomyelin (SM) was obtained from BIOMOL. Ciprofloxacin and icatibant were from Sigma-Aldrich.

DNA constructs, transfection, and infection

pMXs-IRES-puro^r (pMXs-IP) and pMXs-Flag-mLMIR3 or hLMIR3-IP were previously used.^{5,6} Retroviral transfection and infection were done as previously described.^{5,6}

Preparation of vesicles containing lipids

After 1 mg of dry lipid (C-24 ceramide, PC, PS, or SM) was hydrated with 1 mL of PBS, the vesicles were generated using an Avanti Mini-Extruder (Avanti Polar Lipids,

Inc) according to the manufacturer's instructions, and as previously described.^{5,6}

Cell stimulation

BMMCs or peritoneal MCs were stimulated with indicated concentrations of compound 48/80 (Fig 1, A and Fig E2). Alternatively, lipids were diluted to a concentration of 50 µg/mL in methanol. 96-well EIA/RIA plates (Corning Costar 3590) were coated with 50 µL of each solution, air-dried, and washed twice with PBS, as previously described.^{5,6} Peritoneal MCs were pre-incubated in lipid-coated plates for 0.5 h before stimulation with 2 µg/mL compound 48/80 for 0.5 h in plates coated with indicated lipids or vehicle (Fig 1, B). LAD2 cells (Fig 1, C) or human peripheral blood-derived mast cells (Fig E7) were stimulated with 2 µg/mL compound 48/80 for 0.5 h in plates coated with the indicated lipids or vehicle. Ten µg/mL of either rat anti-human CD300f antibody or control antibody was added to the culture just before stimulating with compound 48/80.

Degranulation assay

β-hexosaminidase released by the peritoneal mast cells that had been stimulated with compound 48/80 for 1 h in Tyrode's buffer was estimated as described.⁵

Flow cytometry

Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences) equipped with CellQuest software and FlowJo software (Tree Star).^{5,6}

67

68 **BMMC reconstitution**

69 Ears of *Kit*^{W-sh/W-sh} mice were intradermally injected with 1×10^6 of transduced BMMCs
70 6 weeks prior to injecting compound 48/80 (Fig E10).^{5,6}

71

72 **Pseudo-allergic reactions in the mouse ear**

73 Mice were intradermally injected with 50 ng of compound 48/80 (Fig 2, A), 10 µg of
74 ciprofloxacin (Fig E9, A), 1.75 µg of icatibant (Fig E9, B), or PBS in each ear just
75 before intravenous injection with 1% Evans blue dye (Sigma). The amount of
76 extravasated dye 30 min after injection was measured by absorbance at 620 nm.^{5,6,8} In
77 some experiments, 10 µg of ceramide vesicles or PBS (Fig 2, B) or 10 µg of
78 anti-ceramide antibody (MID 15B4) or isotype control antibody (Fig 2, C) together with
79 compound 48/80 was injected intradermally to each ear of the mice. Alternatively, mice
80 were intradermally injected with 50 ng of compound 48/80 or PBS in each ear (Fig E8).
81 Each ear sample was homogenized in 350 µl of PBS with 5% bovine serum albumin
82 using a BioMasher II (Nippi, Tokyo, Japan). Tissue homogenates were incubated with 1
83 mM of p-nitrophenyl N-acetyl-D glucosaminide (Sigma-Aldrich) in 0.05M citrate
84 buffer (pH 4.5) for 1 h at 37 degree in the dark and quenched with 0.05M sodium
85 carbonate buffer (pH10.0). The absorbance was read at 405 nm.^{E4}

86

87 **RNA interference**

88 Peritoneal MCs were transfected with 50 nM of mouse *Mrgprb1* siRNA

(ON-TARGETplus Mouse Mrgpb1 (233231) siRNA – SMARTpool) (Dharmacon), mouse Mrgprb2 siRNA (ON-TARGETplus Mouse Mrgpb2 (243979) siRNA – SMARTpool) (Dharmacon), or control siRNA (ON-TARGETplus Non-targeting Pool) (Dharmacon), according to the manufacturer's instructions. Forty-eight hours after transfection, the peritoneal MCs were used for further experiments.

Real-time RT-PCR

Real-time RT-PCR was performed with the Applied Biosystems StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific) by using TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression products for mouse Mrgpb1 (Mm01701895_s1) or mouse Mrgprb2 (Mm01956240_s1) (Applied Biosystems). The mRNA expression levels were quantified by using the comparative method with StepOne software and normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (Mm99999915_g1) (Applied Biosystems) expression levels.

Statistical analysis

Results are expressed as means $\pm$ s.d. A two-tailed unpaired Student's t test or ANOVA with Dunnett's multiple comparison was performed to compare differences between groups. Percentage data were transformed by logit transformation before statistical analysis.

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Figure legends

FIG E1. Surface expression levels of c-kit and Fc ϵ RI α in BMMC or peritoneal MC.

Representative of four independent experiments.

FIG E2. The percentages of β -hexosaminidase released in BMMC or peritoneal MC in response to compound 48/80. $n = 3$ per group; $\pm$ SD. Representative of three independent experiments.

FIG E3. Relative expression levels of *Mrgprb1* or *Mrgprb2* in BMMC versus peritoneal MC. $n = 3$ per group; $\pm$ SD. Representative of three independent experiments.

FIG E4. Relative expression levels of *Mrgprb1* or *Mrgprb2* in peritoneal MC transfected with *Mrgprb1*, *Mrgprb2*, or control siRNA. $n = 3$ per group; $\pm$ SD. Representative of three independent experiments.

FIG E5. Surface expression levels of c-kit, Fc ϵ RI α , or CD300f in WT or CD300f^{-/-} peritoneal MC. Representative of four independent experiments.

FIG E6. Surface expression levels of human CD300f or MRGPRX2 in LAD2 cells. Representative of four independent experiments.

FIG E7. Ceramide or sphingomyelin-CD300f binding inhibits MRGPRX2-mediated degranulation in human peripheral blood-derived mast cells. **A**, Surface expression levels of Fc ϵ RI α , c-Kit, CD300f, or MRGPRX2 in human mast cells. **B**, The

degranulation ratio of compound 48/80-stimulated human mast cells in plates coated with indicated lipids, pretreated with a soluble anti-human CD300f antibody or a control antibody. $n = 4$ per group; $\pm$ SD. $*p < 0.01$. Representative of three independent experiments.

FIG E8. $CD300f^{-/-}$ mice had higher activity of β -hexosaminidase in compound 48/80-stimulated ears than did its WT counterparts. The activity of β -hexosaminidase in the ears of WT or $CD300f^{-/-}$ mice injected intradermally with vehicle or compound 48/80. $n = 4-6$ per group; $\pm$ SD. $*p < 0.01$. Representative of two independent experiments.

FIG E9. Dye extravasation in the ears of WT or $CD300f^{-/-}$ mice injected intradermally with vehicle or ciprofloxacin (Fig E9, A) or icatibant (Fig E9, B). $n = 5$ per group; $\pm$ SD. $*p < 0.01$. Representative of two independent experiments.

FIG E10. Dye extravasation in the ears of $Kit^{W-sh/W-sh}$ mice reconstituted with CD300f-deficient BMMC transduced with mouse or human CD300f or mock, injected intradermally with compound 48/80. $n = 4$ per group; $\pm$ SD. $*p < 0.01$. Representative of two independent experiments.

Figure E1

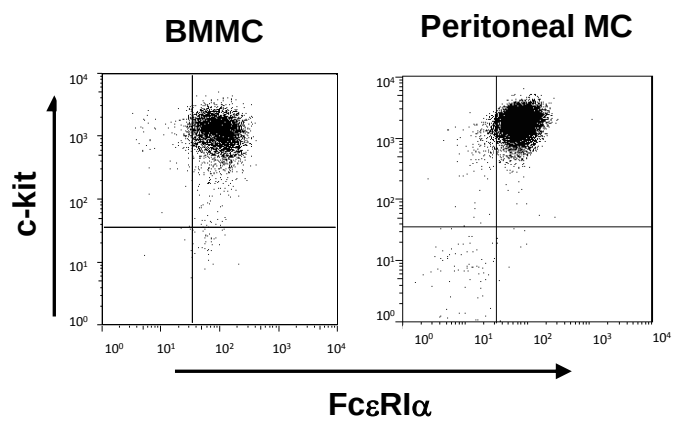


Figure E2

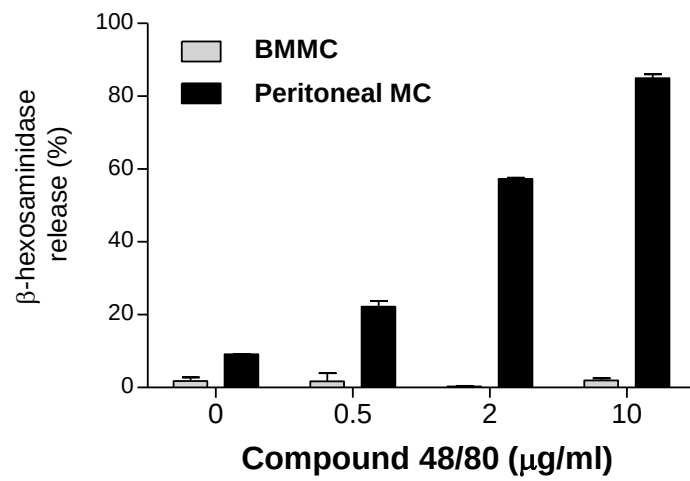


Figure E3

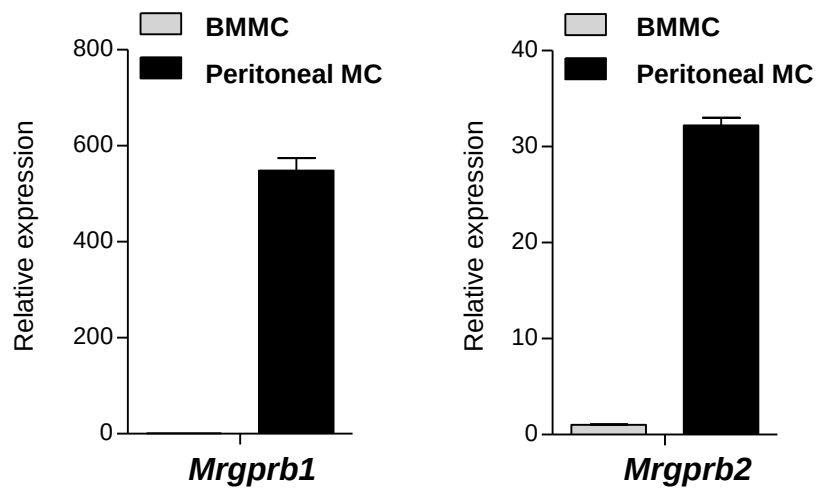


Figure E4

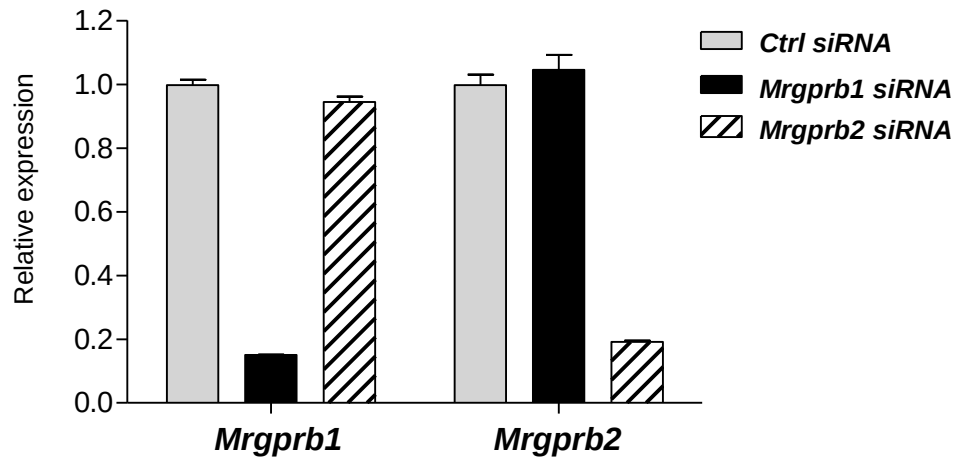


Figure E5

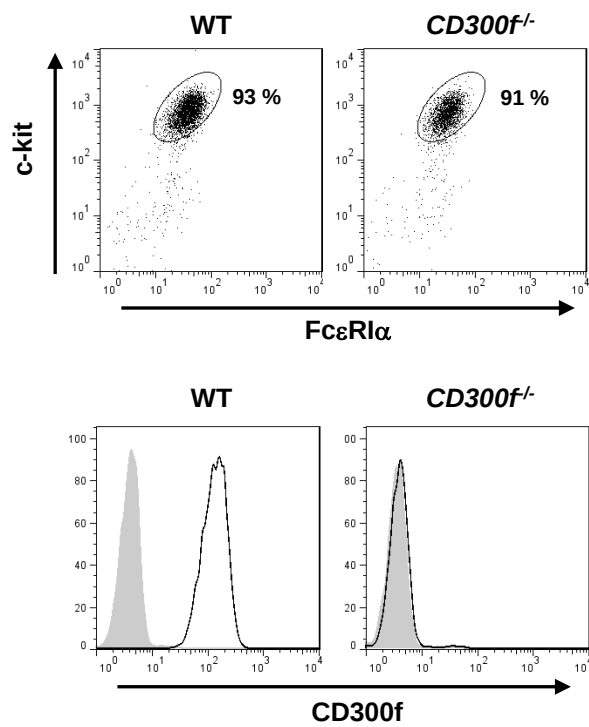


Figure E6

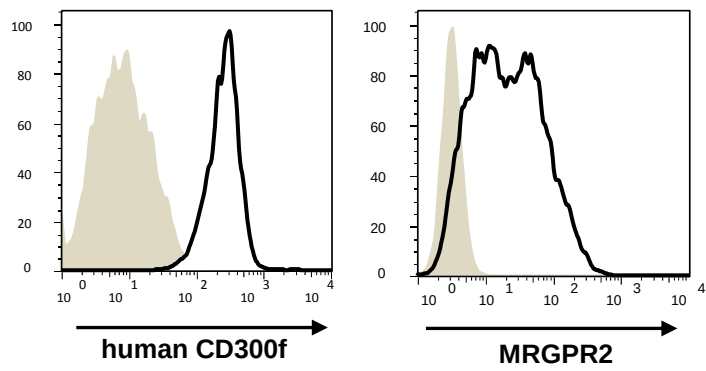
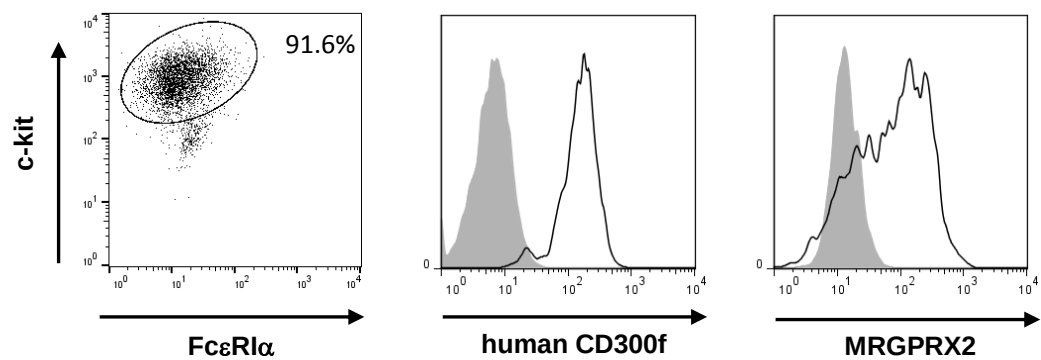


Figure E7

A



B

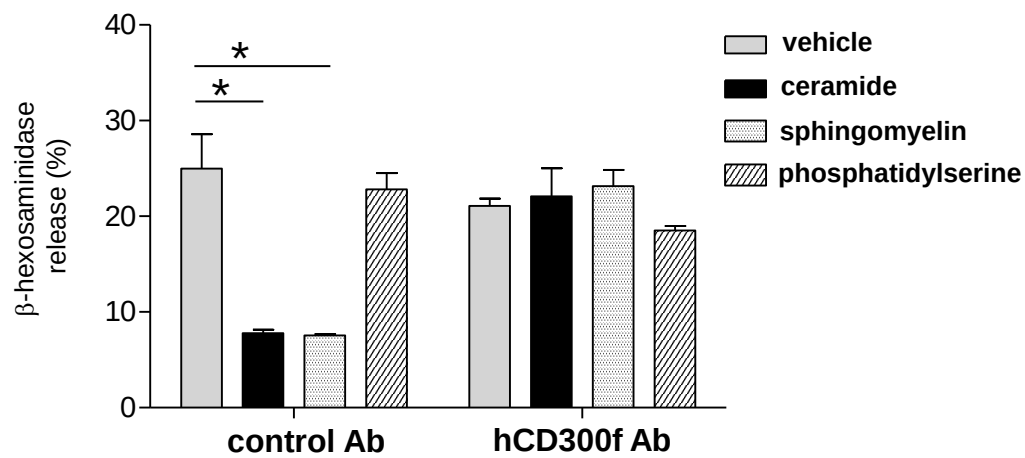


Figure E8

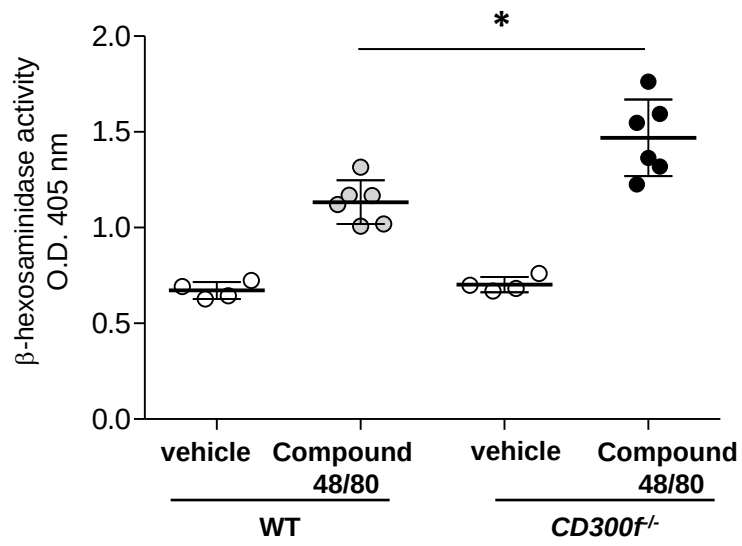


Figure E9

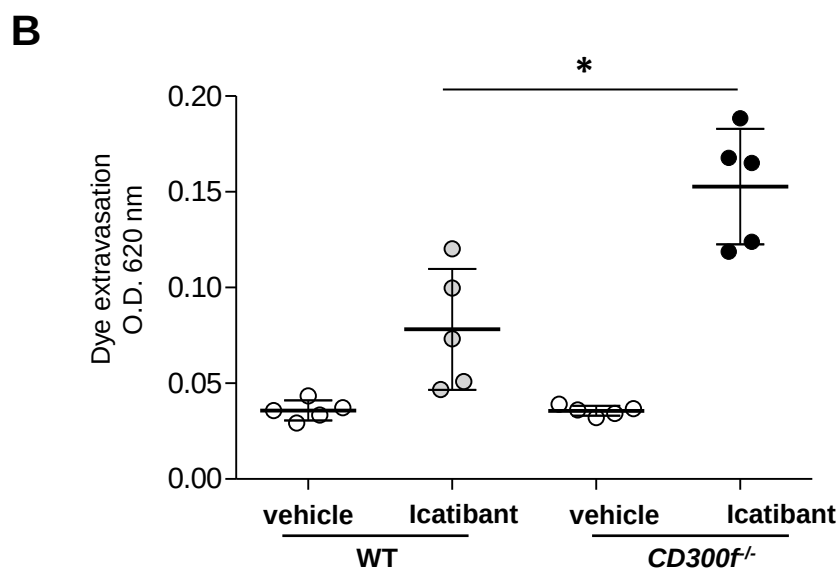
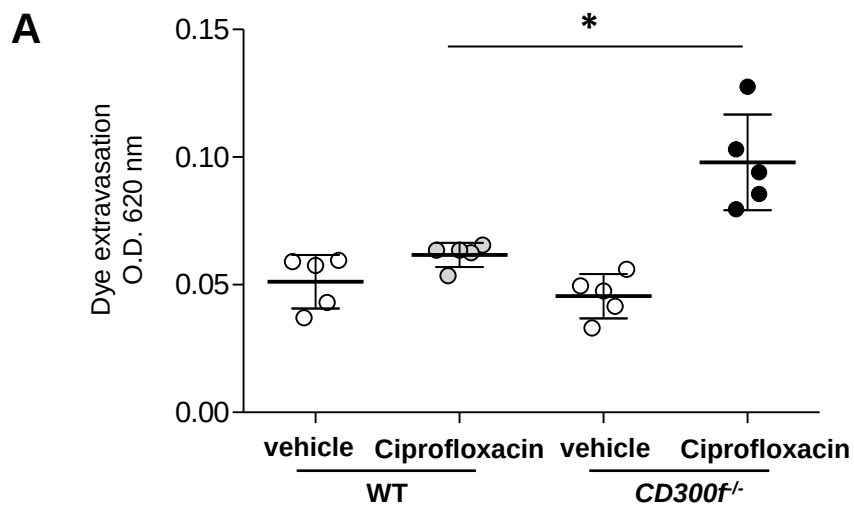


Figure E10

