

SUPPLEMENTARY MATERIAL

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Differential Lipid Recognition by Mouse versus Human CD300f, Inhibiting Passive Cutaneous Anaphylaxis, Depends on a Single Amino Acid Substitution in its Immunoglobulin-Like Domain

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TO THE EDITOR

CD300 is a paired activating and inhibitory receptor family that regulates immune responses (Chung et al., 2003; Izawa et al., 2007, 2012, 2014, 2017; Maehara et al., 2018; Moshkovits et al., 2014; Takahashi et al., 2019; Takamori et al., 2019; Tian et al., 2014). CD300f is an inhibitory receptor that contains a single immunoglobulin-like domain in the extracellular region. Ceramide is a ligand for mouse CD300f (mCD300f), whereas sphingomyelin and ceramide act as a ligand for human CD300f (hCD300f). The binding of these ligands

to CD300f inhibits IgE-mediated mast cell activation and allergic responses, including passive cutaneous anaphylaxis (PCA) responses (Izawa et al., 2012, 2014). mCD300f also recognizes phosphatidylserine exposed on apoptotic cells (Moshkovits et al., 2014; Tian et al., 2014). In addition, mCD300f, but not hCD300f, was recently identified as an entry receptor for murine norovirus (Haga et al., 2016; Orchard et al., 2016). Thus, both mCD300f and hCD300f share similar ligands and functions, while exhibiting differential ligand specificities. We aimed to elucidate the

underlying molecular mechanisms: we focused on the previous finding that ceramide (d18:1/24:0) binds to both mCD300f and hCD300f, whereas sphingomyelin (d18:1/18:0) only binds to hCD300f (Supplementary Figure S1a) (Izawa et al., 2014). To this end, we used the reporter cell lines 2B4-mCD300f-wild-type (WT)—green fluorescent protein (GFP) and 2B4-hCD300f-WT-GFP, in which the binding of specific ligands to the extracellular domain of mCD300f and hCD300f could induce GFP expression, respectively. In fact, plate-coated ceramide (d18:1/24:0) induced GFP expression in both reporter cell lines, whereas plate-coated sphingomyelin (d18:1/18:0) induced GFP expression only in 2B4-hCD300f-GFP cell lines (Supplementary Figure S1b) (Izawa et al., 2014). One important feature of the CD300f structure is the formation of a

Abbreviations: BMMC, bone marrow–derived mast cell; PCA, passive cutaneous anaphylaxis; WT, wild type

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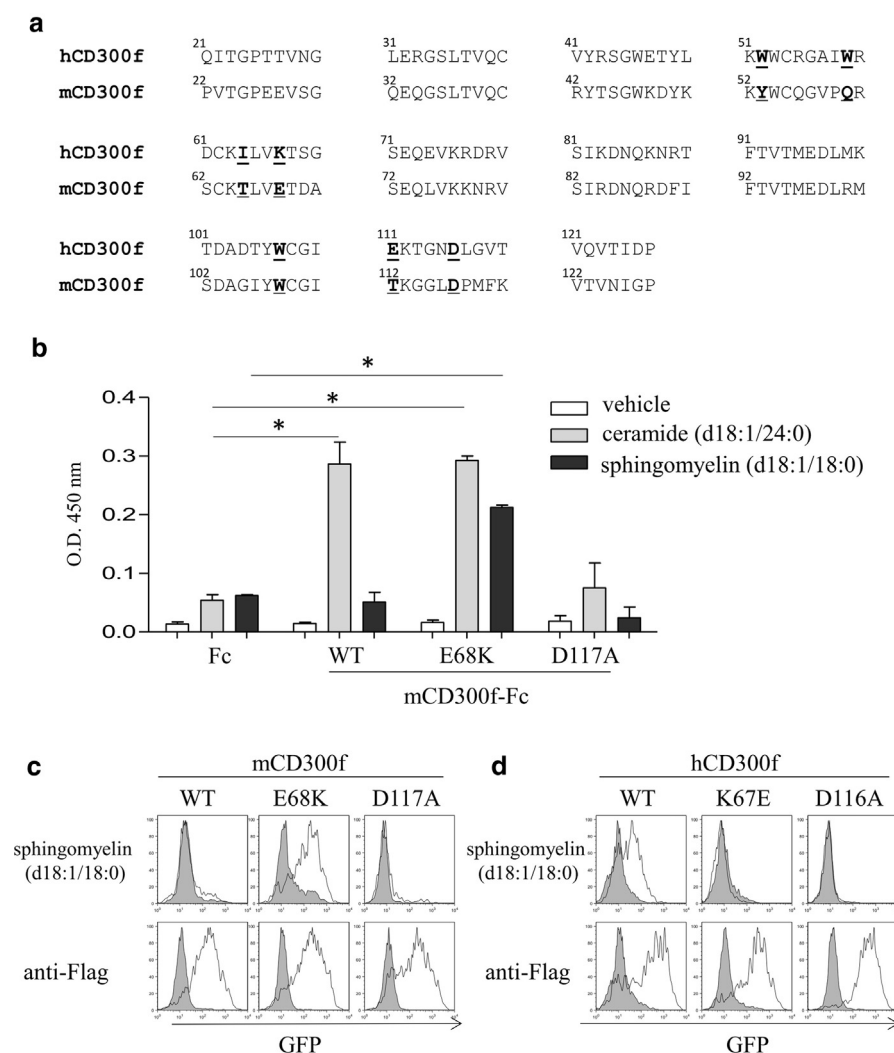


Figure 1. The substitution of E 67 to K renders mCD300f capable of binding to sphingomyelin (d18:1/18:0) in vitro. (a) Amino acid residues of an extracellular domain of mCD300f and hCD300f. The protrusion structure of CD300f involves seven amino acid residues (bold, underline). Numbers indicate the position of each residue. (b) The binding of mCD300f-WT, E67K, or D117A-Fc to plate-coated indicated lipids. * $P < 0.01$. (c, d) Flow cytometry of GFP expression of the indicated reporter cells on plates coated with sphingomyelin (d18:1/18:0) or with anti-Flag antibody. GFP, green fluorescent protein.

prominent protrusion from the immunoglobulin-like domain that has been postulated to recognize the potential ligands (Márquez et al., 2007). This protrusion structure of hCD300f is presumed to be composed of several key amino acids that include tryptophan-52, tryptophan-59, isoleucine-64, lysine (K)-67, tryptophan-107, glutamic acid (E)-111, and aspartate (D)-116. Among them, the corresponding amino acid residues of mCD300f (tyrosine-53, Glutamine-60, Threonine-65, E-68, and threonine-112) were different than those of hCD300f (Figure 1a) (Márquez et al., 2007). The previous finding that the substitution of D-117 to alanine (A) in mCD300f abolishes its binding to apoptotic cells underscores the

significance of this structure in ligand recognition (Tian et al., 2014). On the basis of these concepts, we hypothesized that the oppositely charged residues K-67 of hCD300f versus E-68 of mCD300f would be responsible for the differential ligand specificities between mCD300f and hCD300f. We then generated several Fc fusion proteins: mCD300f-WT-Fc, mCD300f-E68K-Fc, and mCD300f-D117A-Fc. The binding assay showed that mCD300f-WT-Fc bound to ceramide (d18:1/24:0) but not to sphingomyelin (d18:1/18:0), as expected (Figure 1a). mCD300f-D117A-Fc bound to neither ceramide (d18:1/24:0) nor sphingomyelin (d18:1/18:0) (Figure 1b), indicating that the structure involving D-117 is essential for the binding of mCD300f to

ceramide (d18:1/24:0). In addition, mCD300f-E68K-Fc bound to sphingomyelin (d18:1/18:0), as well as ceramide (d18:1/24:0) (Figure 1b), demonstrating that the substitution of E-68 to K rendered mCD300f capable of binding to sphingomyelin (d18:1/18:0). Next, we generated the additional reporter cell lines 2B4-mCD300f-D117A-GFP and 2B4-mCD300f-E68K-GFP. Consistent with the results of the binding assays, sphingomyelin (d18:1/18:0) only induced GFP expression in 2B4-mCD300f-E68K-GFP cell lines (Figure 1c). Moreover, the results using reporter cell lines 2B4-hCD300f-K67E-GFP and 2B4-hCD300f-D116A-GFP showed that the substitution of K-67 to E and of D-116 to A dampened the ability of hCD300f to bind to sphingomyelin (d18:1/18:0) (Figure 1d). Taken together, the protrusion structures involving K67 are essential for the in vitro binding of hCD300f to sphingomyelin (d18:1/18:0).

To next examine the in vivo role of this protrusion structure of CD300f, we used mast cell- and IgE-dependent PCA reactions in mice (Izawa et al., 2014). Bone marrow-derived mast cells (BMMCs) were generated from mCD300f-deficient mice and were transduced with mCD300f-WT, mCD300f-E68K, mCD300f-D117A, or a mock treatment. Equivalent levels of FcεRIα, c-Kit, and Flag-tagged CD300f were found on the surface of these transduced BMMCs (Supplementary Figure S2). Mast cell-deficient *Mcpt5-Cre/R-DTA* mice were reconstituted with these BMMC transfectants on the ear skin before being subjected to PCA. We found similar numbers of mast cells in the ear skin of transduced mast cells-engrafted *Mcpt5-Cre/R-DTA* mice (Supplementary Figure S3). The results showed that mCD300f-E68K in mast cells suppressed the PCA responses at levels comparable with those at which mCD300f-WT did. However, neither CD300f deficiency nor mCD300f-D117A in mast cells failed to do so (Figure 2a). These results indicate that the structure involving D117 is pivotal in the interaction between mCD300f and extracellular lipid ligands, inhibiting IgE-mediated mast cell activation. Furthermore, we generated mCD300f-deficient BMMCs transduced with hCD300f-WT or hCD300f-K67E, expressing equivalent levels of FcεRIα,

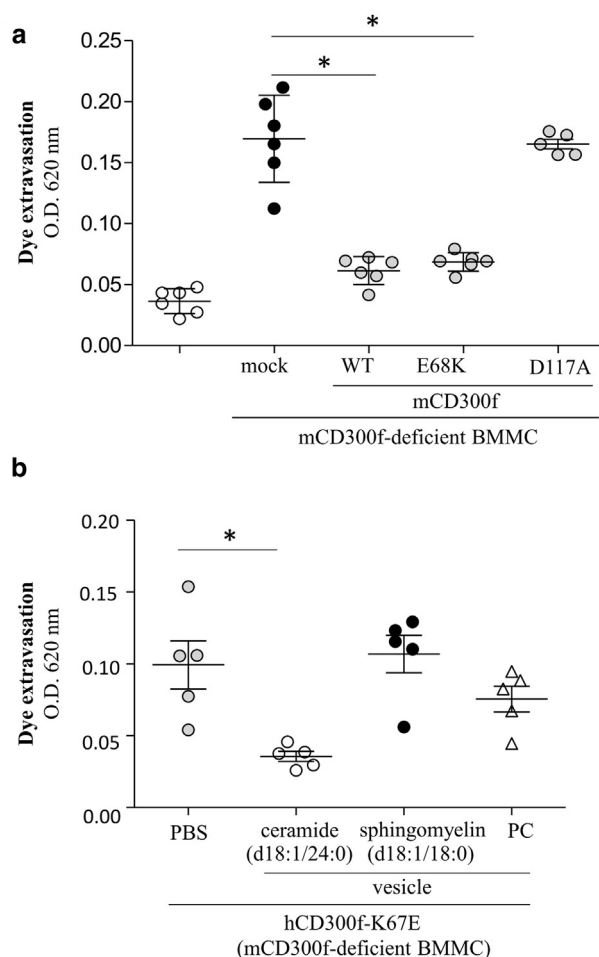


Figure 2. The structure involving K 67 in hCD300f is essential for its binding to sphingomyelin (d18:1/18:0) in vivo. (a, b) PCA responses in *Mcpt5-Cre/R-DTA* mice transplanted with the indicated mast cell transfectants (Figure 2a) or with hCD300f-K67E-transduced mast cells, pretreated with indicated vesicles (Figure 2b) ($n = 5-6$ per group, $\pm$ standard deviation). * $P < 0.01$. PC, phosphatidylcholine; PCA, passive cutaneous anaphylaxis.

c-Kit, or Flag-tagged CD300f on their surfaces (Supplementary Figure S2). *Mcpt5-Cre/R-DTA* mice were reconstituted with these BMMC transfectants, displaying equivalent numbers of mast cells in their ear skin (Supplementary Figure S3). Pretreatment with ceramide (d18:1/24:0)-vesicles or sphingomyelin (d18:1/18:0)-vesicles inhibited the PCA responses in mice reconstituted with the hCD300f-WT-transduced mast cells (Supplementary Figure S4). On the other hand, pretreatment with ceramide (d18:1/24:0)-vesicles suppressed the PCA responses in mice reconstituted with hCD300f-K67E-transduced mast cells; however, sphingomyelin (d18:1/18:0)-vesicles did not suppress the PCA responses in the same mice (Figure 2b). Accordingly, these results indicate that the protrusion structure involving K67 is essential for the in vivo recognition of sphingomyelin (d18:1/18:0) by

hCD300f. Although further structural analysis is needed to completely understand the relevant mechanisms, hCD300f might recognize more diverse ligands as compared with mCD300f via this specific structure. It is also possible to speculate that hCD300f is involved in the entry of certain viruses, which might be prevented by the drugs that specifically bind to hCD300f (Haga et al., 2016; Orchard et al., 2016).

In conclusion, the protrusion structure of human CD300f involving K-67 is pivotal for the recognition of sphingomyelin (d18:1/18:0). A single amino acid substitution of an immunoglobulin-like domain of CD300f determines the lipid ligand-binding specificity between mouse and human, which will help clarify similar or different roles of mouse versus human CD300f in immunity.

All procedures were approved by the Institutional Review Committee of Juntendo University (approval no. 290104, 300132, and 310047).

Data availability statement

Datasets related to this article can be found at <https://www.ncbi.nlm.nih.gov/protein/AAP57942> and <https://www.ncbi.nlm.nih.gov/nuccore/AY457047>.

ORCIDi

Kumi Izawa: <http://orcid.org/0000-0003-4746-7225>
Ayako Kaitani: <http://orcid.org/0000-0003-0660-0480>
Tomoaki Ando: <http://orcid.org/0000-0002-8548-4424>
Akie Maehara: <http://orcid.org/0000-0002-9067-6471>
Masakazu Nagamine: <http://orcid.org/0000-0002-9691-3079>
Hiromichi Yamada: <http://orcid.org/0000-0002-0753-0827>
Taiki Ando: <http://orcid.org/0000-0002-6012-5484>
Takuma Ide: <http://orcid.org/0000-0001-6196-2965>
Moe Matsuzawa: <http://orcid.org/0000-0001-6486-7525>
Yoko Okamoto: <http://orcid.org/0000-0001-7857-0909>
Enzhi Yin: <http://orcid.org/0000-0002-4265-0058>
Saaya Fukase: <http://orcid.org/0000-0002-3643-6004>
Hexing Wang: <http://orcid.org/0000-0001-9215-4821>
Anna Kamei: <http://orcid.org/0000-0001-6309-3337>
Shino Uchida: <http://orcid.org/0000-0001-6428-3773>
Keiko Maeda: <http://orcid.org/0000-0003-2127-4687>
Nobuhiro Nakano: <http://orcid.org/0000-0002-5317-5950>
Koichiro Uchida: <http://orcid.org/0000-0003-0645-8373>
Naoto Tamura: <http://orcid.org/0000-0003-1729-2954>
Katsuhisa Ikeda: <http://orcid.org/0000-0001-8920-9916>
Nobuyuki Ebihara: <http://orcid.org/0000-0002-6772-6549>
Toshiaki Shimizu: <http://orcid.org/0000-0003-0364-0022>
David Voehringer: <http://orcid.org/0000-0001-6650-0639>
Axel Roers: <http://orcid.org/0000-0003-1806-6158>
Hideoki Ogawa: <http://orcid.org/0000-0003-4268-8790>
Ko Okumura: <http://orcid.org/0000-0003-3238-6422>
Jiro Kitaura: <http://orcid.org/0000-0002-5083-8368>

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: K Izawa, A Kaitani, JK; Formal Analysis: K Izawa, A Kaitani, TA, NT, K Ikeda, NE, TS, JK; Funding Acquisition: JK. Investigation: K Izawa, A Kaitani, TA, AM, MN, NN, KU, HY, TI, YO, EY, SF, KM, HW, SU, KM. Methodology: K Izawa, JK. Project Administration: JK. Resources: DV, AR, JK. Supervision: HO, KO, JK. Validation: K Izawa, A Kaitani, NT, K Ikeda, NE, TS, JK. Visualization: K Izawa, A Kaitani, JK. Writing - Original Draft Preparation: K Izawa, A Kaitani, JK. Writing - Review and Editing: K Izawa, A Kaitani, TA, AM, MN, HY, TA, TI, MM, YO, EY, SF, HW, A Kamei, SU, KM, NN, KU, NT, K Ikeda, NE, TX, DV, AR, HO, KO, JK.

**Kumi Izawa^{1,10}, Ayako Kaitani^{1,10},
Tomoaki Ando¹, Akie Maehara¹,
Masakazu Nagamine¹,
Hiromichi Yamada^{1,2}, Taiki Ando^{1,3},
Takuma Ide^{1,4}, Moe Matsuzawa^{1,5},
Yoko Okamoto^{1,6}, Enzhi Yin¹,
Saaya Fukase^{1,5}, Hexing Wang¹,
Anna Kamei¹, Shino Uchida^{1,7},
Keiko Maeda¹, Nobuhiro Nakano¹,
Koichiro Uchida¹, Naoto Tamura^{1,3},
Katsuhisa Ikeda^{1,4},
Nobuyuki Ebihara^{1,5},
Toshiaki Shimizu^{1,2},
David Voehringer⁸, Axel Roers⁹,
Hideoki Ogawa¹, Ko Okumura¹ and
Jiro Kitaura^{1,*}**

¹Atopy Research Center, Juntendo University Graduate School of Medicine, Tokyo, Japan;

²Department of Pediatrics and Adolescent Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan;

³Department of Internal Medicine and Rheumatology, Juntendo University Graduate School of Medicine, Tokyo, Japan;

⁴Department of Otorhinolaryngology, Juntendo University Graduate School of

Medicine, Tokyo, Japan; ⁵Department of Ophthalmology, Juntendo University Urayasu Hospital, Chiba, Japan; ⁶Department of Pediatrics, Graduate School of Medicine, Tokyo University, Tokyo, Japan; ⁷Department of Gastroenterology Immunology, Juntendo University Graduate School of Medicine, Tokyo, Japan; ⁸Department of Infection Biology, University Hospital Erlangen and Friedrich-Alexander University Erlangen-Nuremberg (FAU), Erlangen, Germany; and ⁹Institute for Immunology, Medical Faculty Carl Gustav Carus, TU Dresden, Dresden, Germany

¹⁰These authors contributed equally to this work.

*Corresponding author e-mail: j-kitaura@juntendo.ac.jp

SUPPLEMENTARY MATERIAL

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Low SATB1 Expression Promotes IL-5 and IL-9 Expression in Sézary Syndrome

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TO THE EDITOR

We read with interest the Letter to the Editor by [Gagat et al.\(2019\)](#) on the

ambiguous role of special AT-rich sequence-binding protein-1 (SATB1) expression in malignant tumors. The

Abbreviations: CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; SATB1, special AT-rich sequence-binding protein-1; SS, Sézary syndrome; TCR, T-Cell receptor

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authors make a strong point that miR-155/SATB1 axis contributes to apoptosis resistance not only in malignant T cells but is also critical in regulating cell death in a wide range of cell lines from hematologic malignancies to solid tumors. Among other findings, the authors reported that low SATB1 expression is associated with resistance



SUPPLEMENTARY MATERIAL AND METHODS

Mice

All procedures were approved by the Institutional Review Committee of Junendo University (approval no. 290104, 300132, and 310047). C57BL/6J mice, *CD300f^{-/-}* mice, and *Mcpt5-Cre/R-DTA* mice were used as previously described (Izawa et al., 2012, 2014; Maehara et al., 2018).

DNA constructs

pMXs-IRES-puro^r (pMXs-IP), pMXs-Flag-mCD300f or hCD300f-IP, and pMXs-Flag-mCD300f or hCD300f-CD3 ζ -IP were constructed as previously described (Izawa et al., 2012, 2014). To generate a pMXs-Flag-mCD300f-E68K, Flag-mCD300f-D117A, Flag-hCD300f-K67E-IP and pMXs-Flag-mCD300f-E68K, Flag-mCD300f-D117A, Flag-hCD300f-K67E, or Flag-hCD300f-D116A-CD3 ζ -IP, mCD300f-E68, mCD300f-D117, hCD300f-K67, or hCD300f-D116 was replaced with K, A, E, or A, respectively, using PCR-mediated mutagenesis. pME18S-mCD300f-hlgG1 Fc was previously described (Izawa et al., 2012, 2014). To generate pME18S-mCD300f-E68K or D117A-hlgG1 Fc, an extracellular domain of mCD300f-E68K or mCD300f-D117A was inserted into the cloning sites of the pME18S-hlgG1 Fc vector (H. Arase, Osaka University, Osaka, Japan). All of the constructs were verified by DNA sequencing.

Cells

2B4-GFP (T. Saito, RIKEN Research Center for Allergy and Immunology), 2B4-mCD300f-GFP, and 2B4-hCD300f-GFP cell lines were previously described (Izawa et al., 2012, 2014). 2B4-GFP cells were transduced with mCD300f-E68K-CD3 ζ , mCD300f-D117A-CD3 ζ , hCD300f-K67E-CD3 ζ , or hCD300f-D116A-CD3 ζ to generate 2B4-mCD300f-E68K-GFP, 2B4-mCD300f-D117A-GFP, 2B4-hCD300f-K67E-GFP, or 2B4-hCD300f-D117A-GFP cells, respectively. BMMCs were generated as previously described (Izawa et al., 2012, 2014). BMMC transfectants were generated by retroviral transfection and infection as previously described (Izawa et al., 2012, 2014).

Antibodies and other reagents

The following antibodies were used: Anti-Flag IgG1 Ab (M2), and mouse IgG1 Ab (MOPC21) were purchased from Sigma-Aldrich (St. Louis, MO). R-phycoerythrin-conjugated anti-mouse IgG goat F(ab')₂ Ab was from Beckman Coulter. FITC-conjugated anti-Fc ϵ RI α (MAR-1) and R-phycoerythrin-conjugated anti-c-kit (2B8) were from Thermo Fisher Inc (Waltham, MA). Cytokines were obtained from R&D Systems (Minneapolis, MN). C-24 ceramide (ceramide [d18:1/24:0]; C₄₂H₈₃NO₃) was obtained from Toronto Research Chemicals, Inc (North York, ON, Canada). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (phosphatidylcholine; PC) was obtained from Echelon Biosciences Inc (Salt Lake City, UT). Sphingomyelin (sphingomyelin [d18:1/18:0]; C₄₁H₈₃N₂O₆P) was obtained from ENZO (Farmington, NY).

Preparation of vesicles containing lipids

After 1 mg of dry lipid (ceramide [d18:1/24:0], sphingomyelin [d18:1/18:0], or PC) was hydrated with 1 ml of phosphate buffered saline, the vesicles were generated using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc, Alabaster, AL) according to the manufacturer's instructions as previously described (Izawa et al., 2012, 2014).

Flow cytometry

Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences, San Jose, CA) equipped with CellQuest software and FlowJo software (Tree Star, Ashland, OR).

Binding assay using ELISA

Solid-phase ELISA was also used as previously described (Izawa et al., 2012, 2014). Briefly, the indicated lipids in methanol (10 μ g/ml) or methanol as a control was added to ELISA plates and air-dried. After washing, the plates were incubated with 10 μ M of mCD300f-WT-Fc, mCD300f-E68K-Fc, mCD300f-D117A-Fc, or Fc in the presence of 0.5 mM CaCl₂ for 120 minutes. After washing, the plates were incubated with peroxidase-conjugated

anti-human Ig (Sigma). The absorbance at 450 nm was measured.

Induction of PCA

PCA was performed as previously described (Izawa et al., 2012, 2014). Briefly, mice were sensitized with the intradermal injection of 50 ng anti-2,4-dinitrophenyl IgE (H1-e-26) to each ear. Then 24 hours after the sensitization, the mice were injected intravenously with Evans blue dye (Sigma-Aldrich) containing 250 μ g 2,4-dinitrophenyl-human serum albumin. Thirty minutes after the antigen challenge, the amounts of extravasated dye were measured by measuring the absorbance at 620 nm. In some experiments, 10 μ g of lipid vesicles were injected intradermally in the ears together with anti-2,4-dinitrophenyl IgE (H1-e-26). After the ears were fixed with 10% formaldehyde and embedded in paraffin, the sections were stained.

BMMC reconstitution

The ears of *Mcpt5-Cre/R-DTA* mice were injected intradermally with 1×10^6 of BMMC transfectants 6 weeks before PCA. Mast cells in the ear skin were stained with toluidine blue and quantified as described (Izawa et al., 2012, 2014).

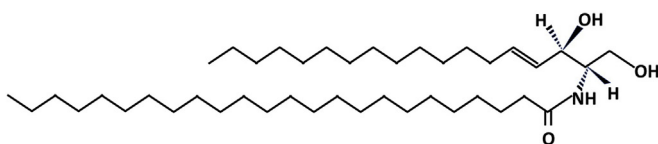
Statistical analysis

Results are expressed as means $\pm$ standard deviation. A two-tailed unpaired Student's *t* test was performed to compare differences between the groups.

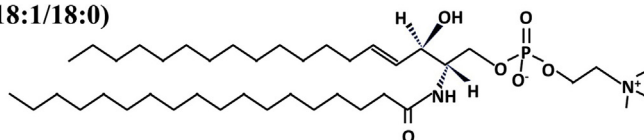
SUPPLEMENTARY REFERENCES

- Izawa K, Isobe M, Matsukawa T, Ito S, Maehara A, Takahashi M, et al. Sphingomyelin and ceramide are physiological ligands for human LMIR3/CD300f, inhibiting Fc ϵ RI-mediated mast cell activation. *J Allergy Clin Immunol* 2014;133:270–3.e1.
- Izawa K, Yamanishi Y, Maehara A, Takahashi M, Isobe M, Ito S, et al. The receptor LMIR3 negatively regulates mast cell activation and allergic responses by binding to extracellular ceramide. *Immunity* 2012;37:827–39.
- Maehara A, Kaitani A, Izawa K, Shiba E, Nagamine M, Takamori A, et al. Role of the ceramide-CD300f interaction in Gram-negative bacterial skin infections. *J Invest Dermatol* 2018;138:1221–4.

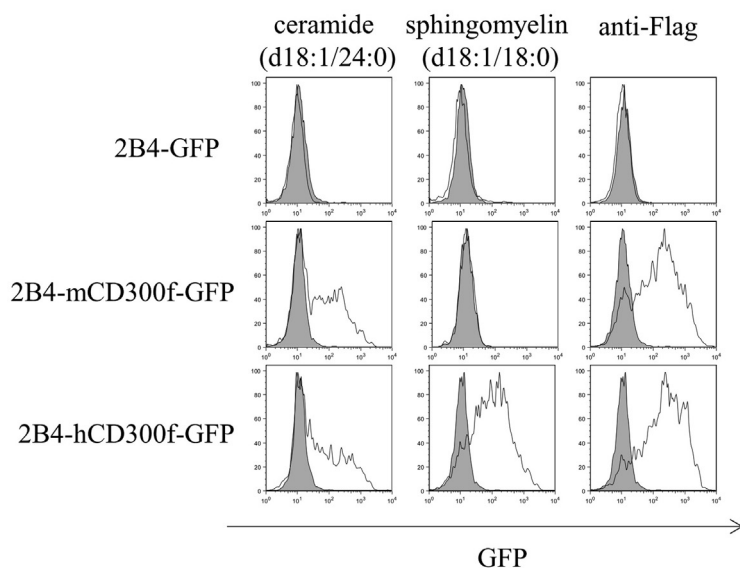
a ceramide (d18:1/24:0)



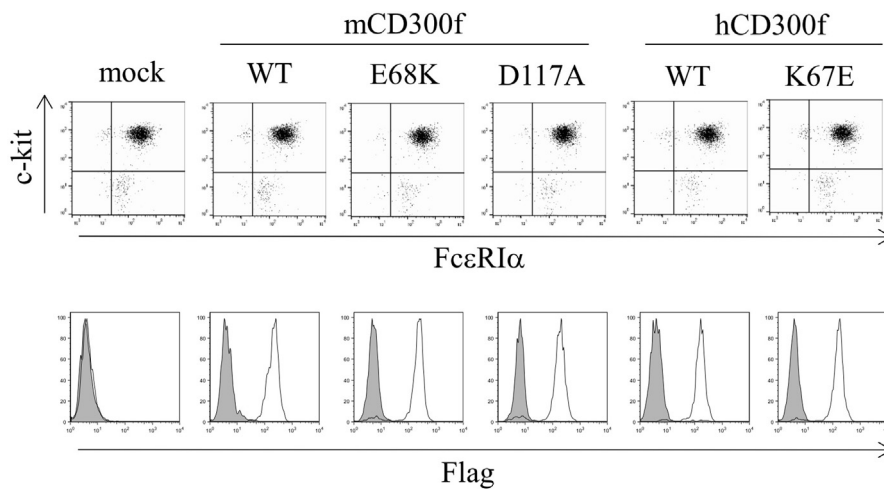
spingomyelin (d18:1/18:0)



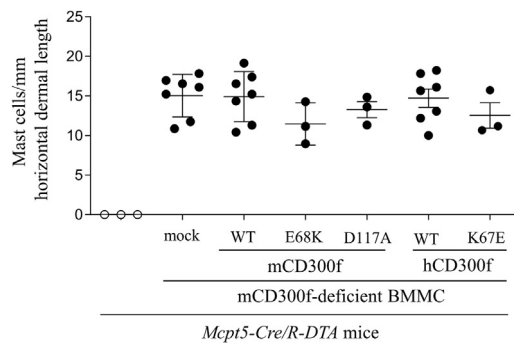
b



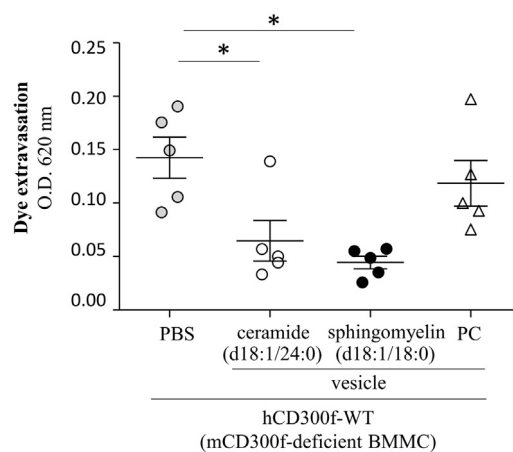
Supplementary Figure S1. The differential lipid ligand specificities of mCD300f versus hCD300f. (a) The structure of ceramide (d18:1/24:0) and sphingomyelin (d18:1/18:0). **(b)** Flow cytometry of GFP expression of the indicated reporter cell lines on plates coated with ceramide (d18:1/24:0), sphingomyelin (d18:1/18:0), or anti-Flag antibody. Representative of three independent experiments. GFP, green fluorescent protein.



Supplementary Figure S2. Surface expression levels of FcεRIα, c-kit, and Flag-tagged CD300f in indicated BMMC transfectants. Representative of two independent experiments. BMMC, bone marrow-derived mast cells.



Supplementary Figure S3. Mast cell numbers in ear skin of *Mcpt5-Cre/R-DTA* mice after intradermal transplantation with indicated transduced BMMCs ($n = 3-7$ per group). BMMC, bone marrow-derived mast cells.



Supplementary Figure S4. PCA responses in *Mcpt5-Cre/R-DTA* mice transplanted with hCD300f-WT-transduced mast cells, pretreated with indicated vesicles ($n = 5$ per group, $\pm$ standard deviation). * $P < 0.01$. PC, phosphatidylcholine; PCA, passive cutaneous anaphylaxis; WT, wild type.