

Letter to the Editor

Sphingomyelin and ceramide are physiological ligands for human LMIR3/CD300f, inhibiting FcεRI-mediated mast-cell activation

To the Editor:

Leukocyte mono-immunoglobulin-like receptor (LMIR) belongs to a paired activating and inhibitory receptor family.¹⁻³ LMIR is also known to be a CMRF-35-like molecule, a myeloid-associated immunoglobulin-like receptor, an immune receptor expressed on myeloid cells, or CD300.⁴⁻⁷ Mouse LMIR3 (mLMIR3)/CD300f is an inhibitory receptor containing 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and a single immunoreceptor tyrosine-based switch motif (ITSM).^{1,2} We have recently demonstrated that extracellular ceramide is a ligand for mLMIR3/CD300f, which is a negative regulator of high-affinity IgE receptor (FcεRI)-mediated activation of mast cells (MCs).² Recent studies have implied that lipids or lipid-binding proteins are ligands for LMIR/CD300^{2,3,5,7,8}; T-cell immunoglobulin mucin 1 and 4, phosphatidylserine-binding proteins, are ligands for mLMIR5/CD300b,³ phosphatidylserine is a ligand for mouse CD300a,⁵ and phosphatidylserine and phosphatidylethanolamine are ligands for human CD300a and CD300c.⁷ Although human LMIR3 (hLMIR3)/CD300f was suggested to inhibit FcεRI-mediated activation of MCs via 2 ITIMs and a single ITSM,^{1,2,6} its physiological ligands remained elusive.

To clarify the role of hLMIR3 in MCs, we elicited MC- and FcεRI-dependent passive cutaneous anaphylaxis (PCA) reactions in MC-deficient *Kit^{W-sh/W-sh}* mice engrafted intradermally with mLMIR3-deficient bone-marrow-derived MCs (BMMCs) transduced with mLMIR3-wild-type (WT), hLMIR3-WT, hLMIR3(Y205F-Y249F-Y284F) mutant, or mock. These transfectants exhibited equivalent expression levels of both FcεRI and c-kit as well as those of the transduced LMIR3 (see Fig E1 in this article's Online Repository at www.jacionline.org). Similar numbers of MCs were found in ear skin of *Kit^{W-sh/W-sh}* mice at 8 weeks after transplantation (data not shown). We found diminished PCA responses in *Kit^{W-sh/W-sh}* mice with either mLMIR3- or hLMIR3-sufficient MCs than in those with mLMIR3-deficient MCs (detailed information on methods is provided in the Methods section in this article's Online Repository at www.jacionline.org and Fig 1, A).² In addition, disrupting ITIMs and ITSM in hLMIR3 abrogated hLMIR3-mediated suppression of the PCA responses (Fig 1, A). Thus, hLMIR3 acted as a substitute for mLMIR3 in suppressing the PCA responses via its ITIMs and ITSM.

As the overall amino acid homology is 55% in an extracellular domain of hLMIR3 and mLMIR3, it is possible to speculate that like mLMIR3, hLMIR3 recognizes ceramide. To test this possibility, we performed a binding assay by using hLMIR3-Fc. Interestingly, hLMIR3-Fc bound to sphingomyelin as well as to ceramide (Fig 1, B). We next used the reporter cell lines (2B4-hLMIR3-GFP) to show that the binding of an unknown ligand to an extracellular domain of hLMIR3 could induce green fluorescent protein (GFP) expression.² Plate-coated anti-hLMIR3 antibody induced GFP expression in 2B4-hLMIR3-GFP cells, which was dampened by the addition of a soluble anti-hLMIR3 antibody (see Fig E2 in this article's Online Repository at www.jacionline.org). Among the lipids tested, plate-coated

sphingomyelin, ceramide, or phosphatidylcholine induced GFP expression in 2B4-hLMIR3-GFP cells, which was prevented by adding a soluble anti-hLMIR3 antibody (see Fig E2). Although hLMIR3-Fc failed to bind phosphatidylcholine spotted on the membranes, ELISA verified the binding of hLMIR3-Fc to plate-coated phosphatidylcholine (see Fig E3 in this article's Online Repository at www.jacionline.org). Taken together, these results indicated that ceramide, sphingomyelin, and phosphatidylcholine were candidate ligands for hLMIR3.

To test whether candidate lipids acted as functional ligands for hLMIR3 in MCs, we used mLMIR3-deficient BMMC transfectants that were sensitized with IgE and stimulated with specific antigens on plates coated with C-24 ceramide, sphingomyelin, phosphatidylcholine, phosphatidylserine, or vehicle. Inhibited β-hexosaminidase release was noted in BMMC transfectants expressing hLMIR3-WT, but not in those expressing the hLMIR3(Y205F-Y249F-Y284F) mutant, on the plates coated with either ceramide or sphingomyelin (data not shown). Similarly, liposomal ceramide or sphingomyelin inhibited the degranulation of BMMC transfectants expressing hLMIR3 WT (Fig 2, A). We next used a human MC line LAD2 expressing FcεRI, c-kit, and endogenous hLMIR3 (Fig 2, B). The inhibition of FcεRI-mediated degranulation was observed in LAD2 cells on plates coated with ceramide or sphingomyelin, but not with phosphatidylcholine (Fig 2, C). In addition, the inhibitory effect of ceramide or sphingomyelin on degranulation was canceled by adding the anti-hLMIR3 antibody, but not the control antibody (Fig 2, C, and see Fig E4 in this article's Online Repository at www.jacionline.org). Consistently, FcεRI crosslinking augmented tyrosine-phosphorylation of hLMIR3 in LAD2 cells on ceramide- or sphingomyelin-coated plates, but not on vehicle-coated plates, which increased the recruitment of SHP-1 to hLMIR3 (see Fig E5 in this article's Online Repository at www.jacionline.org). Collectively, these results indicated that ceramide- or sphingomyelin-hLMIR3 binding inhibited FcεRI-mediated activation of MCs *in vitro*.

To examine the distribution of extracellular sphingomyelin in dermis in which the MCs were located, we used lysenin, a pore-forming toxin that specifically binds sphingomyelin (Fig 1, B).⁹ Staining of WT mouse ear skin sections by lysenin displayed a patchy or dot-like distribution of extracellular sphingomyelin in dermis (Fig 2, D, and see Fig E6 in this article's Online Repository at www.jacionline.org). Moreover, MCs, irrespective of hLMIR3 expression, were frequently surrounded by extracellular sphingomyelin (Fig 2, D). These results implied that both ceramide and sphingomyelin could bind to hLMIR3 in tissue MCs. In accordance, hLMIR3-mediated suppression of the PCA response was partially canceled by intradermal injection with the anti-ceramide antibody in the ears of *Kit^{W-sh/W-sh}* mice with hLMIR3-expressing MCs (Fig 2, E). Furthermore, only when pretreated with liposomal ceramide or sphingomyelin did *Kit^{W-sh/W-sh}* mice with MC-expressing hLMIR3 exhibit diminished PCA response (Fig 2, F). The same pretreatment did not influence the PCA response in *Kit^{W-sh/W-sh}* mice, with MCs expressing neither mLMIR3 nor hLMIR3 (Fig 2, F). These results indicated that extracellular ceramide and sphingomyelin worked as ligands for hLMIR3 in tissue MCs. On the basis of our previous findings² on mLMIR3, we assume

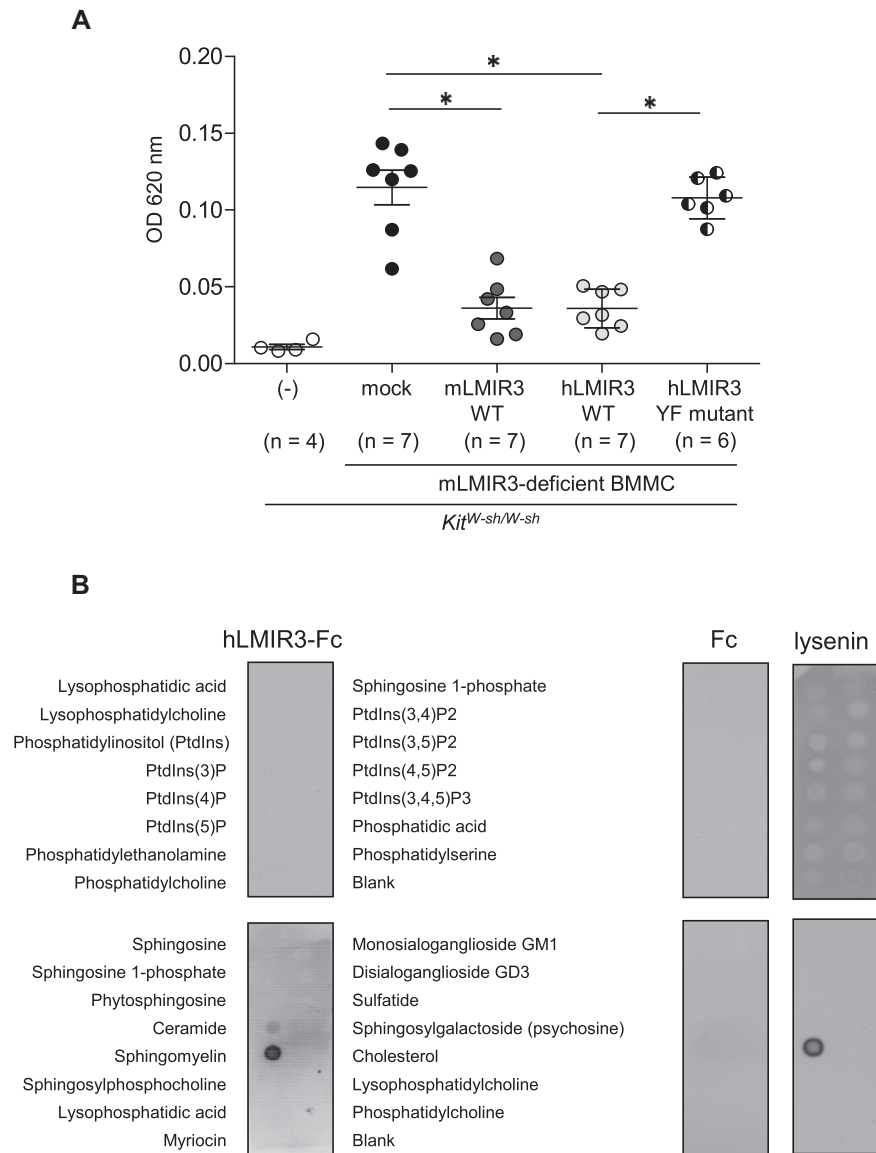


FIG 1. Possible ligands for hLMIR3. **A**, PCA responses in *Kit^{W-sh/W-sh}* mice harboring LMIR3-deficient BMMCs transduced with mLMIR3-WT, hLMIR3-WT, hLMIR3(Y205F-Y249F-Y284F), or mock (n = 6-7 per group; $\pm$ SD). * $P < .05$. **B**, The binding of hLMIR3-Fc, Fc, or lysenin to the lipids.

that sphingomyelin and ceramide bind to hLMIR3 in tissue MCs; however, ITIMs and ITSM of hLMIR3 are not tyrosine-phosphorylated without Fc ϵ RI engagement. Sphingomyelin and ceramide-bound hLMIR3 delivers an inhibitory signal only after Fc ϵ RI engagement. Further examination is required to completely understand the relevant mechanism in human beings.

In conclusion, although ceramide is a major ligand for mLMIR3, both sphingomyelin and ceramide act as physiological ligands for hLMIR3 in MCs, thereby inhibiting Fc ϵ RI-mediated activation of MCs. These results will help understand the intrinsic mechanism by which excessive activation of MCs is prevented in human beings, leading to the development of novel therapeutic strategies against allergic diseases.

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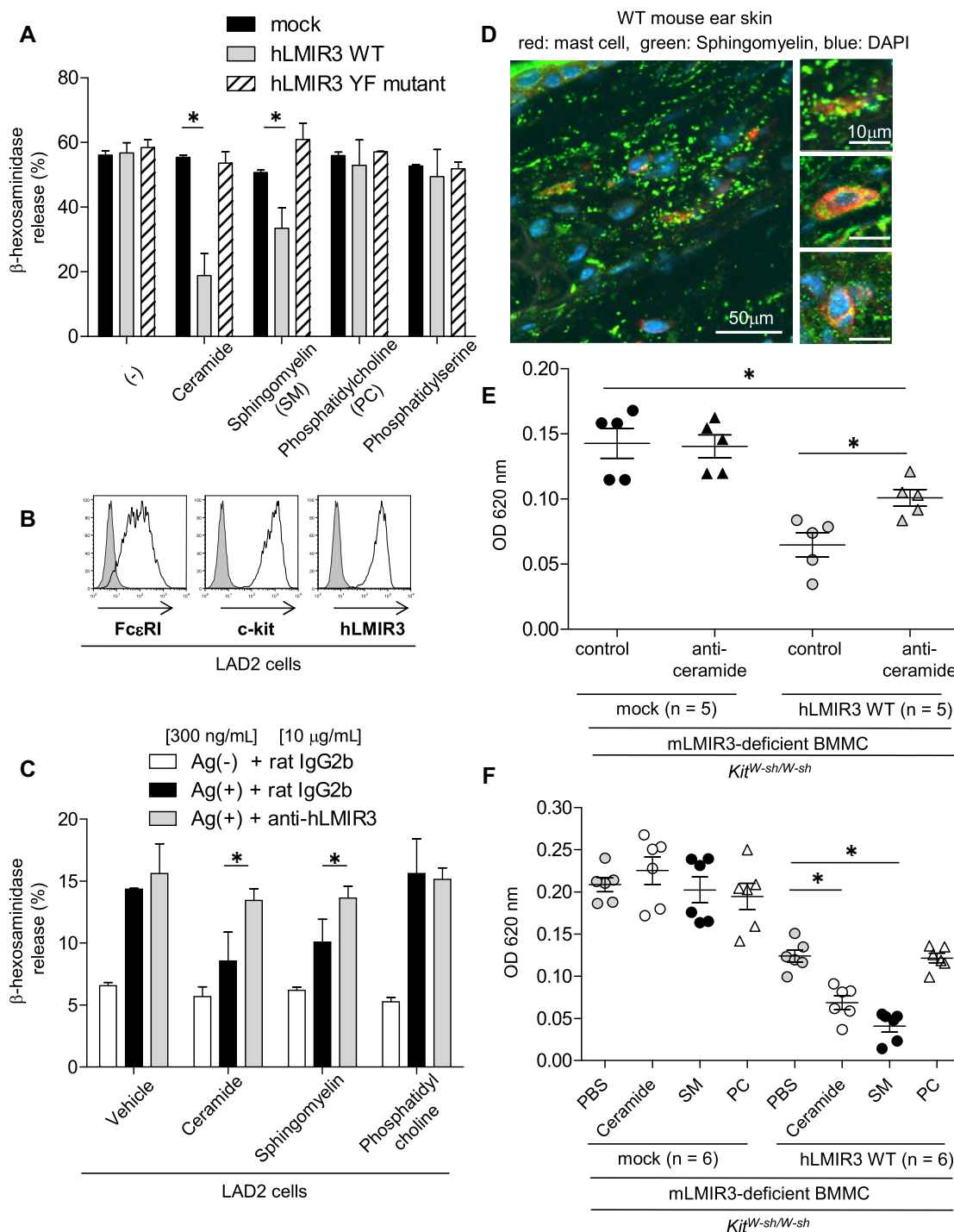


FIG 2. Functional ligands for hLMIR3. **A** and **C**, β -Hexosaminidase release ($n = 4$, $\pm$ SD). * $P < .05$. BMMC transfectants or LAD2 cells treated with liposomes (Fig 2, A) or plated-coated lipids/antibodies (Fig 2, C), respectively. **B**, Surface receptor expression. **D**, Confocal imaging. **E** and **F**, PCA responses in the transplanted *Kit^{W-sh/W-sh}* mice, pretreated with antibodies (Fig 2, E) or liposomes (Fig 2, F) ($n = 5$ -6 per group, $\pm$ SD). * $P < .05$.

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REFERENCES

- Izawa K, Kitaura J, Yamanishi Y, Matsuoka T, Kaitani A, Sugiuchi M, et al. An activating and inhibitory signal from an inhibitory receptor

- LMIR3/CLM-1: LMIR3 augments lipopolysaccharide response through association with FcRgamma in mast cells. *J Immunol* 2009;183:925-36.
2. 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.
 3. Yamanishi Y, Kitauro J, Izawa K, Kaitani A, Komeno Y, Nakamura M, et al. TIM1 is an endogenous ligand for LMIR5/CD300b: LMIR5 deficiency ameliorates mouse kidney ischemia/reperfusion injury. *J Exp Med* 2010;207:1501-11.
 4. Chung DH, Humphrey MB, Nakamura MC, Ginzing DG, Seaman WE, Daws MR. CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. *J Immunol* 2003;171:6541-8.
 5. Nakahashi-Oda C, Tahara-Hanaoka S, Shoji M, Okoshi Y, Nakano-Yokomizo T, Ohkohchi N, et al. Apoptotic cells suppress mast cell inflammatory responses via the CD300a immunoreceptor. *J Exp Med* 2012;209:1493-503.
 6. Alvarez-Errico D, Sayós J, López-Botet M. The IREM-1 (CD300f) inhibitory receptor associates with the p85alpha subunit of phosphoinositide 3-kinase. *J Immunol* 2007;178:808-16.
 7. Simhadri VR, Andersen JF, Calvo E, Choi SC, Coligan JE, Borrego F. Human CD300a binds to phosphatidylethanolamine and phosphatidylserine, and modulates the phagocytosis of dead cells. *Blood* 2012;119:2799-809.
 8. Cannon JP, O'Driscoll M, Litman GW. Specific lipid recognition is a general feature of CD300 and TREM molecules. *Immunogenetics* 2012;64:39-47.
 9. Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, et al. Lysenin, a novel sphingomyelin-specific binding protein. *J Biol Chem* 1998;273:5300-6.

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METHODS

Cells and mice

2B4-GFP cells (T. Saito, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan)^{E1} were transduced with hLMIR3-CD3 ζ to generate 2B4-hLMIR3-GFP cells.^{E2,E3} LAD2 cells were cultured as described.^{E4} Mice used were previously described.^{E2} Experimental procedures in mice were approved by an institutional review committee of the University of Tokyo (approval no. 20-8).

Antibodies and other reagents

Some antibodies and reagents were previously described.^{E2} Other antibodies were as follows: goat anti-human LMIR3 antibody (AF2774) and rat anti-human LMIR3 antibody (MAB2774) (R&D Systems, Minneapolis, Minn), phycoerythrin-conjugated rat IgG_{2b} (eB149/10H5) (eBioscience, San Diego, Calif), and anti-NP chimeric human IgE (MCA333S) (AbD Serotec, Oxford, United Kingdom).

DNA constructs

pMXs-IRES-puro^r (pMXs-IP) and pMXs-Flag-mLMIR3 or hLMIR3-IP were previously described.^{E2,E3,E5,E6} To generate a Flag-hLMIR3(Y241F-Y289F-Y325F) mutant in which 3 tyrosine residues were replaced with phenylalanine, a 2-step PCR mutagenesis was performed. An extracellular and transmembrane domain of Flag-hLMIR3 fused to an intracellular domain of CD3 ζ (N. Matsumoto, The University of Tokyo, Tokyo, Japan) was inserted into pMXs-IP to generate pMXs-Flag-hLMIR3-CD3 ζ -IP.^{E2} An extracellular domain of hLMIR3 was inserted into the cloning sites of pME18S-hIgG1 Fc vector (H. Arase, Osaka University, Osaka, Japan). All the constructs were verified by DNA sequencing.

Induction of PCA

Induction of PCA in *Kit^{w-sh/w-sh}* mice engrafted with BMDC transfectants was previously described.^{E2}

Cell stimulation

LMIR3-deficient BMDC transfectants were sensitized with 0.5 μ g/mL antitrinitrophenyl IgE for 12 hours and then stimulated with 100 ng/mL trinitrophenyl-BSA and 20 μ g/mL C-24 ceramide, sphingomyelin, or phosphatidylcholine liposomes for 1 hour (Fig 2, A). LAD2 cells were sensitized with 0.5 μ g/mL anti-4-Hydroxy-3-nitrophenylacetyl (NP) chimeric human IgE for 24 hours and then stimulated with 300 ng/mL NP-BSA for 1 hour on plates coated with C-24 ceramide, sphingomyelin, phosphatidylcholine, or vehicle (Fig 2, C) or with 100 or 300 ng/mL NP-BSA for 1 hour on

plates coated with C-24 ceramide or sphingomyelin (Fig E4). A total of 10 μ g/mL (Fig 2, C) or 2 or 20 μ g/mL (Fig E4) of either the anti-hLMIR3 antibody or the control antibody was added to the culture just before stimulating with antigens.

Immunohistochemistry

Immunofluorescence staining was performed as previously described.^{E2} To detect sphingomyelin and MCs in WT (Fig 2, D) or *Kit^{Wsh/Wsh}* (Fig E6) mouse ear skin, sections were first stained with lysenin (Peptide Institute, Osaka, Japan), then with rabbit antilysenin serum (Peptide Institute), and finally with Alexa Flour 488-conjugated goat anti-rabbit IgG (H+L) antibody (Invitrogen, Carlsbad, Calif).^{E7} The same sections were stained first with biotinylated anti-MC tryptase antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and then with Alexa Flour 546-conjugated streptavidin (Invitrogen). All sections were counterstained with 4'-6-diamidino-2-phenylindole, dihydrochloride (Invitrogen). Fluorescent images were analyzed on a confocal microscope (FLUOVIEW FV300 scanning laser biological microscope JX70 system; Olympus, Albertslund, Denmark) equipped with SenSys/OL cold charge-coupled device camera (Olympus).

Statistical analyses

Results are expressed as means $\pm$ SD. An unpaired Student *t* test was used for all the statistical analyses.

REFERENCES

- E1. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat Immunol* 2008;9:1179-88.
- E2. 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.
- E3. Takahashi M, Izawa K, Kashiwakura J, Yamanishi Y, Enomoto Y, Kaitani A, et al. Human CD300C delivers an Fc receptor- γ -dependent activating signal in mast cells and monocytes and differs from CD300A in ligand recognition. *J Biol Chem* 2013;288:7662-75.
- E4. Gao ZG, Wei Q, Jayasekara MP, Jacobson KA. The role of P2Y₁₄ and other P2Y receptors in degranulation of human LAD2 mast cells. *Purinergic Signal* 2013;9:31-40.
- E5. Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, et al. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* 2003;31:1007-14.
- E6. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000;7:1063-6.
- E7. Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, et al. Lysenin, a novel sphingomyelin-specific binding protein. *J Biol Chem* 1998;273:5300-6.

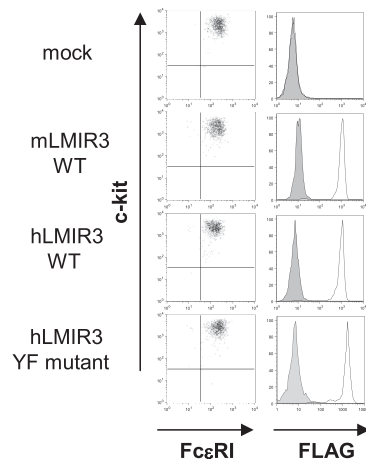


FIG E1. Representative surface expression levels of FcεRI and c-kit (*left panel*) or Flag-tagged LMIR3 (*right panel*) in LMIR3-deficient BMMCs transduced with Flag-tagged mLMIR3-WT, hLMIR3-WT, hLMIR3(Y205F-Y249F-Y284F) mutant, or mock.

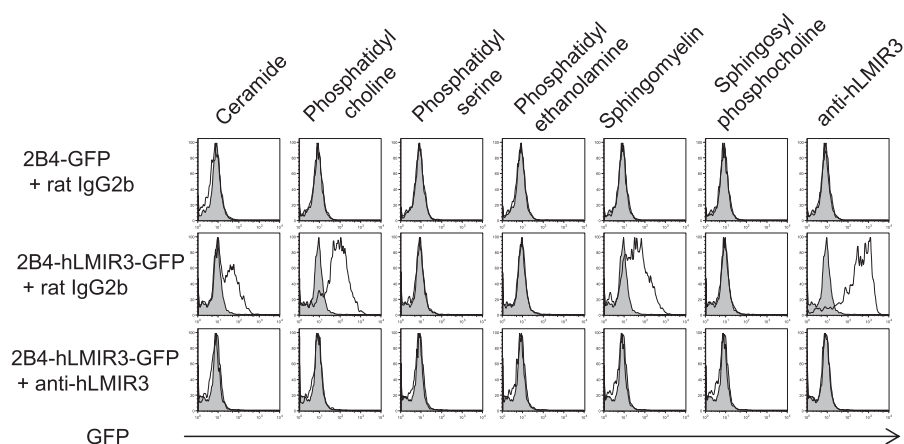


FIG E2. 2B4-GFP cells and 2B4-hLMIR3-GFP cells were incubated for 24 hours on plates coated with the anti-hLMIR3 antibody or lipids such as C-24 ceramide, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, sphingosylphosphocholine, or vehicle. A total of 20 mg/mL of either the hLMIR3 antibody or the isotype control antibody was added to the culture just after the incubation of the reporter cells on the coated plates. Flow cytometry of GFP expression of the reporter cells. Data are representative of 4 independent experiments.

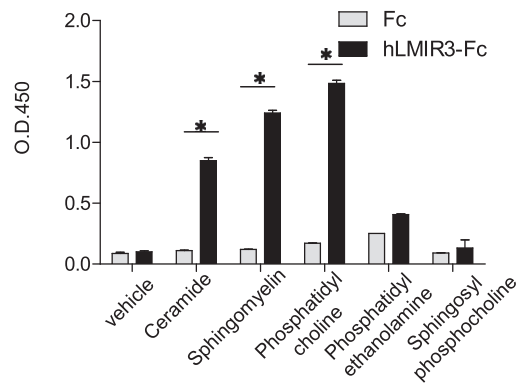


FIG E3. The binding of hLMIR3-Fc or Fc to plate-coated lipids was quantified by using ELISA. A total of 10 mg/mL of hLMIR3-Fc or Fc was incubated on plates coated with C-24 ceramide, sphingomyelin, phosphatidylcholine, sphingosylphosphocholine, or vehicle. Data are representative of 4 independent experiments.

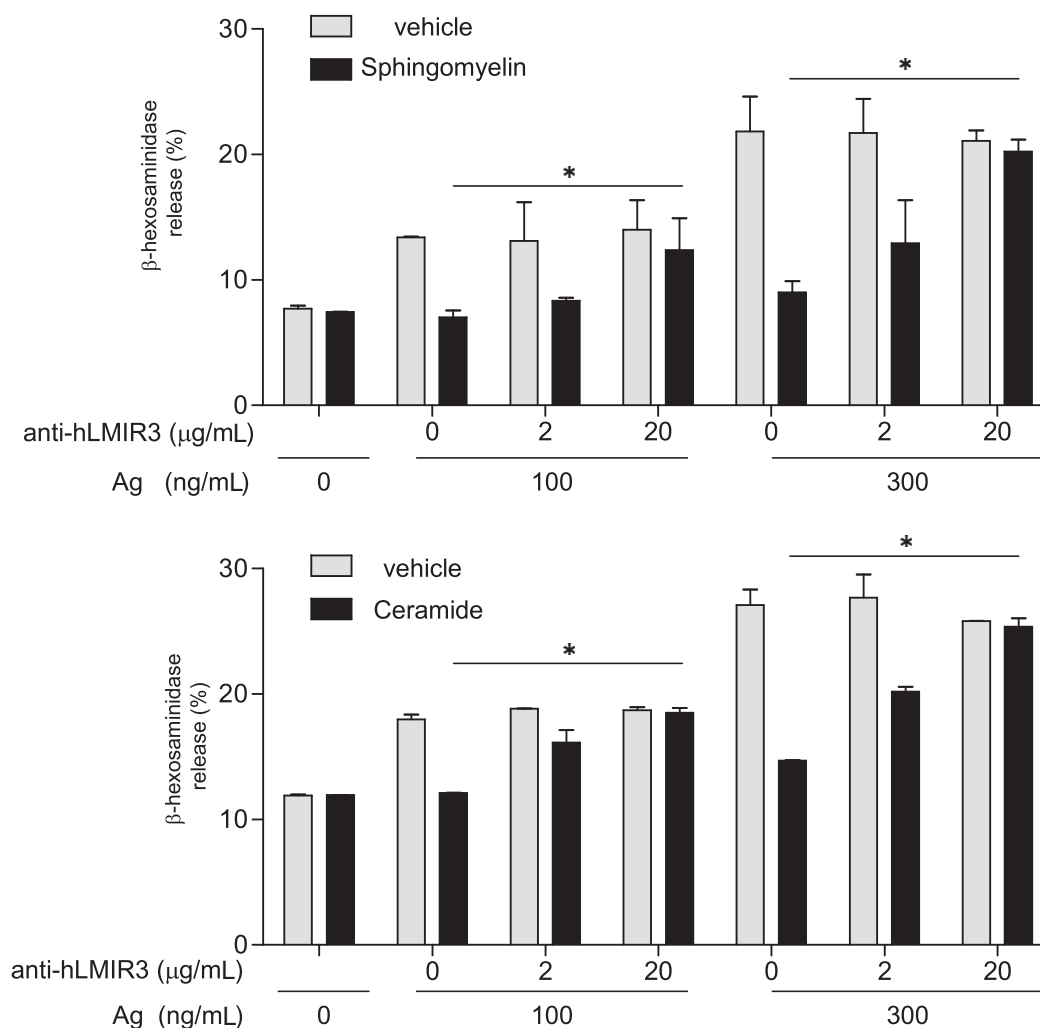


FIG E4. β -Hexosaminidase release ($n = 4$, $\pm$ SD). $*P < .05$. LAD2 cells stimulated with IgE/antigen plus plated-coated sphingomyelin or ceramide. A total of 2 or 20 μ g/mL of either the anti-hLMIR3 antibody or the control antibody was added to the culture just before stimulating with antigens.

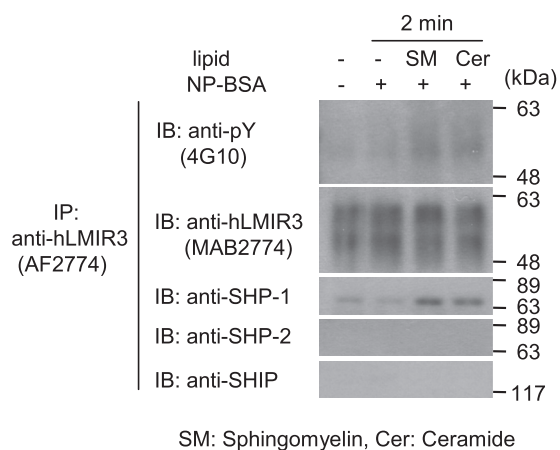


FIG E5. Anti-NP IgE-sensitized LAD2 cells were stimulated with or without 300 ng/mL NP-BSA for 2 minutes on plates coated with C-24 ceramide, sphingomyelin, or vehicle. Immunoprecipitates of cell lysates with the anti-hLMIR3 antibody were immunoblotted with indicated antibodies. Data are representative of 3 independent experiments. *Cer*, Ceramide; *SM*, sphingomyelin.

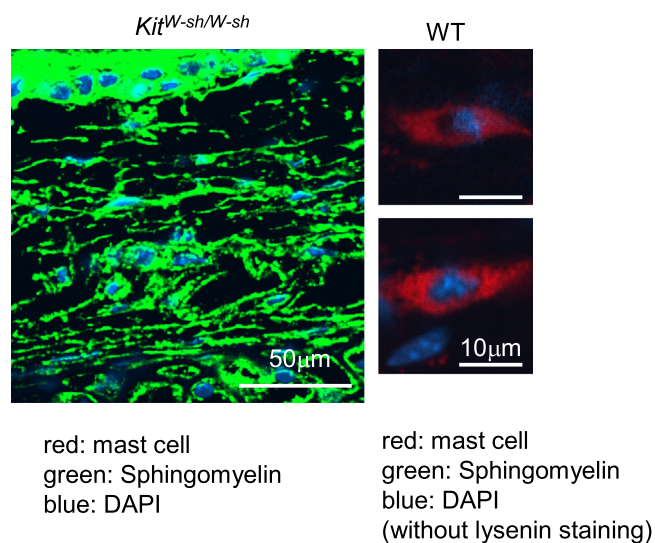


FIG E6. Confocal imaging of mouse ear-skin sections. Ear skin sections of *Kit^{W-sh/W-sh}* mice were first stained with lysenin, then with rabbit antilysenin serum, and finally with Alexa Fluor 488-conjugated secondary antibody (*left panel*). Ear skin sections of WT mice were not stained with lysenin, and were stained first with rabbit antilysenin serum and finally with Alexa Fluor 488-conjugated secondary antibody (*right panel*). DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride.