

The Receptor LMIR3 Negatively Regulates Mast Cell Activation and Allergic Responses by Binding to Extracellular Ceramide

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SUMMARY

Mast cells (MCs) are key effector cells in allergic reactions. However, the inhibitory mechanism that prevents excessive activation of MCs remains elusive. Here we show that leukocyte mono-immunoglobulin-like receptor 3 (LMIR3; also called CD300f) is a negative regulator of MC activation in vivo. LMIR3 deficiency exacerbated MC-dependent allergic responses in mice, including anaphylaxis, airway inflammation, and dermatitis. Both physical binding and functional reporter assays via an extracellular domain of LMIR3 showed that several extracellular lipids (including ceramide) and lipoproteins were possible ligands for LMIR3. Importantly, MCs were frequently surrounded by extracellular ceramide in vivo. Upon engagement of high-affinity immunoglobulin E receptor, extracellular ceramide-LMIR3 binding inhibited MC activation via immunoreceptor tyrosine-based inhibitory and switch motifs of LMIR3. Moreover, pretreatment with LMIR3-Fc fusion protein or antibody against either ceramide or LMIR3 interfered with this binding in vivo, thereby exacerbating passive cutaneous anaphylaxis. Thus, the interaction between extracellular ceramide and LMIR3 suppressed MC-dependent allergic responses.

INTRODUCTION

Mast cells are key effector cells in immunoglobulin E (IgE)-associated immune responses, including anaphylaxis and allergic disorders. The high-affinity IgE receptor (FcεRI) is expressed in mast cells as a heterotetrameric complex composed of an IgE-binding α chain, a β chain, and two disulfide-linked γ chains. Engagement of IgE-bound FcεRI by multivalent antigens triggers mast cell activation, leading to its degranulation and secretion of preformed chemical mediators such as vasoactive amines. In addition, activated mast cells secrete de novo syn-

thesized lipid mediators, cytokines, and chemokines. An initial step in FcεRI signaling is an activation of β chain-associated Lyn, which phosphorylates immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domains of β and γ chains of FcεRI. Subsequent activation of the kinases Syk and Fyn and the phosphatidylinositol-3-OH kinase are essential for mast cell activation (Blank et al., 1989; Kawakami and Galli, 2002; Parravicini et al., 2002; Kraft and Kinet, 2007; Kalesnikoff and Galli, 2008). Such activation is counterbalanced by immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors (Ravetch and Lanier, 2000): FcγRIIb (Daëron et al., 1995; Ono et al., 1996; Ujike et al., 1999), gp49B1 (Daheshia et al., 2001), PIR-B (Kubagawa et al., 1997; Masuda et al., 2007), and Allergin-1 (Hitomi et al., 2010).

The leukocyte mono-immunoglobulin-like receptor (LMIR; also called CD300, CLM, or MAIR) (Chung et al., 2003; Kumagai et al., 2003; Yotsumoto et al., 2003) family belongs to the paired immune receptors. An inhibitory receptor LMIR3 (also called CD300f, CLM-1, or MAIR-V) is highly expressed in mast cells (Izawa et al., 2007). Coengagement of FcεRI and LMIR3 inhibits FcεRI-mediated activation of mouse bone-marrow-derived mast cells (BMMCs) (Izawa et al., 2009). LMIR3 delivers an inhibitory signal via its two ITIMs and a single immunoreceptor tyrosine-based switch motif (ITSM) that can recruit Src homology 2 domain-containing protein phosphatase-1 (SHP-1) and/or SHP-2 (Izawa et al., 2009). Although LMIR3 had been shown to negatively regulate autoimmune demyelination in the central nervous system (Xi et al., 2010), whether LMIR3 regulates mast cell activation in vivo remained unknown. Here, we found that whereas LMIR3 deficiency failed to influence FcεRI-mediated activation of BMMCs, it profoundly enhanced mast cell-dependent allergic responses in mice. These results suggest that mast cell activation in vivo is suppressed by the interaction between LMIR3 and its unknown ligand surrounded by tissue mast cells.

To identify a ligand for LMIR3, we generated an Fc fusion protein containing an extracellular domain of LMIR3 (LMIR3-Fc). In addition, we used reporter cell lines to show that the binding of an unknown ligand to an extracellular domain of LMIR3 could induce green fluorescent protein (GFP) expression driven by transcription factor NFAT. We have previously identified T cell immunoglobulin mucin 1 (TIM1) as a ligand for an

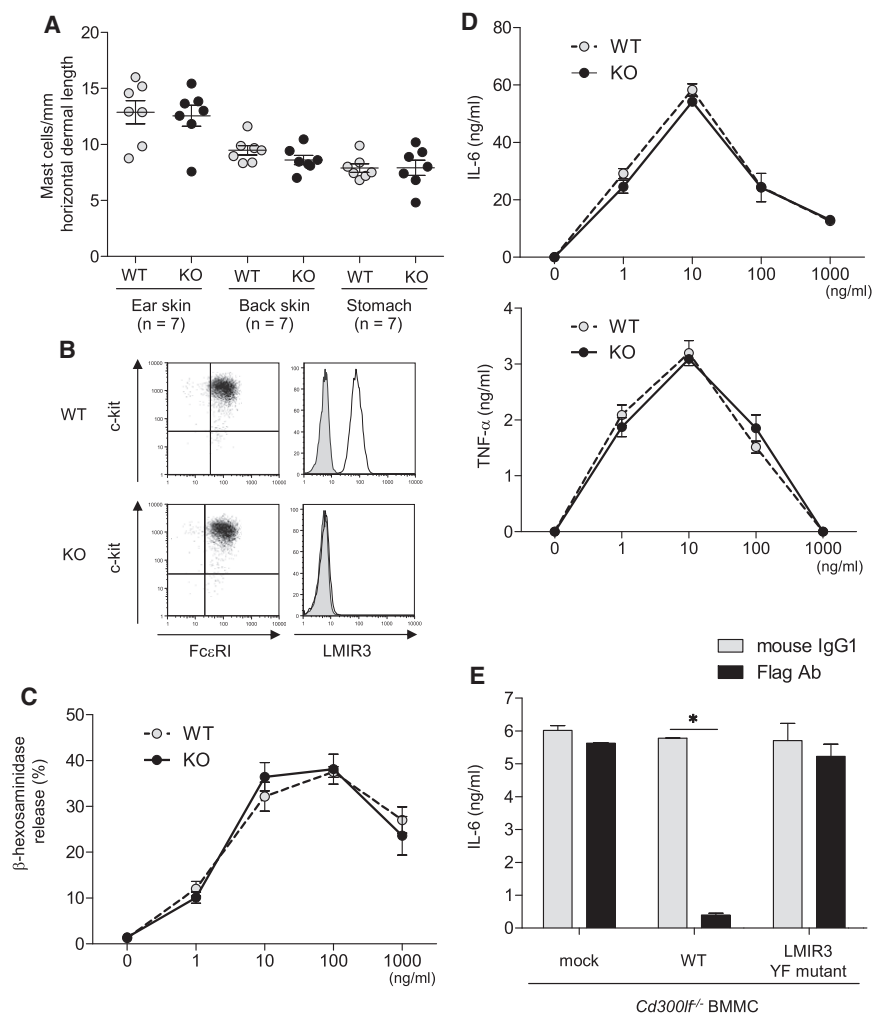


Figure 1. Normal Development of LMIR3-Deficient Mast Cells

(A) Mast cell numbers in ear skin, back skin, and stomach of WT or LMIR3-deficient (*Cd300lf^{-/-}*) (KO) mice (n = 7 per genotype).

(B) Surface expression levels of FcεRI and c-kit (left) or LMIR3 (right) in WT or *Cd300lf^{-/-}* BMMCs. (C and D) Release of β-hexosaminidase (C) or production of IL-6 (top) or TNF-α (bottom) (D) from anti-TNP IgE-sensitized WT or *Cd300lf^{-/-}* BMMCs stimulated with indicated concentrations of TNP-BSA for 8 hr (C) or 1 hr (D).

(E) Production of IL-6 from *Cd300lf^{-/-}* BMMCs transduced with LMIR3 WT, LMIR3(Y241F-Y289F-Y325F) mutant, or mock that were subjected to coengagement of IgE-bound FcεRI and the transduced LMIR3 for 8 hr.

Data are representative of four independent experiments (mean and SD in C–E). *p < 0.01 (Student's t test). See also Figure S1.

decreased in the skin of atopic dermatitis (AD) (Meyer zu Heringdorf et al., 2002; Kawakami et al., 2009). Although intracellular and extracellular ceramide have wide-ranging functions, our results illustrate a role for the extracellular lipid ceramide as an antiallergic tissue component that binds to LMIR3, thereby inhibiting mast cell-dependent allergic responses.

RESULTS

Normal Development of LMIR3-Deficient Mast Cells

To delineate the in vivo function of LMIR3, we generated LMIR3-deficient

activating receptor LMIR5 (also called CLM-7) by retrovirus-mediated expression cloning (Yamanishi et al., 2010). However, LMIR3-Fc failed to bind a variety of cells examined. Therefore, we hypothesized that a ligand for LMIR3 might belong to extracellular proteins or lipids. Interestingly, LMIR3-Fc bound to several lipids, including ceramide, and lipoproteins. Moreover, the binding of these lipids and lipoproteins to an extracellular domain of LMIR3 induced GFP expression in the reporter cell lines. Although it has been recently reported that LMIR3 recognizes phosphatidylserine (PS) and promotes phagocytosis of apoptotic cells (Choi et al., 2011), our results did not provide any evidence that PS worked as a functional ligand for LMIR3. Importantly, FcεRI-mediated activation of wild-type (WT) BMMCs, but not of LMIR3-deficient (*Cd300lf^{-/-}*) BMMCs, was inhibited by plate-coated or liposomal ceramide.

Ceramide is a sphingolipid generated mainly by hydrolysis of sphingomyelin or by de novo synthesis via ceramide synthase. Ceramide regulates a variety of cellular processes, including differentiation, inflammation, proliferation, and apoptosis, in a cell type-dependent manner (Kolesnick, 2002; Meyer zu Heringdorf et al., 2002; Zheng et al., 2006). Recent studies have demonstrated that ceramide is abundant in the epidermis and functions as the epidermal permeability barrier: ceramide content is

(*Cd300lf^{-/-}*) mice (Figures S1A–S1C available online). Consistent with a previous report (Xi et al., 2010), *Cd300lf^{-/-}* mice exhibited no significant differences from WT mice in myeloid and lymphoid development (Figures S1D–S1H). LMIR3 deficiency did not affect mast cell numbers in ear skin, back skin, or stomach in mice (Figure 1A). WT and *Cd300lf^{-/-}* BMMCs displayed equivalent expression levels of c-kit and FcεRI (Figures 1B and S1I) and exhibited comparable growth and apoptosis (Figures S1J and S1K). Surface expression of other LMIR was comparable between WT and *Cd300lf^{-/-}* BMMCs (Figure S1L). Thus, LMIR3 deficiency did not influence mast cell development in vitro or in vivo. When IgE-sensitized BMMCs were stimulated with specific antigens, LMIR3 deficiency did not alter degranulation or cytokine production (Figures 1C and 1D) nor the tyrosine phosphorylation of cellular proteins (Figure S1M). Coengagement of FcεRI and LMIR3 impaired cytokine production in WT BMMCs (Izawa et al., 2009), which was not observed in *Cd300lf^{-/-}* BMMCs (Figure S1N). We transduced *Cd300lf^{-/-}* BMMCs with LMIR3 WT, LMIR3(Y241F-Y289F-Y325F) mutant, or mock, that exhibited equivalent expression levels of both FcεRI and c-kit as well as those of the transduced LMIR3 (Figure S1O). We confirmed that coengagement of FcεRI and LMIR3(Y241F-Y289F-Y325F) mutant failed to impair cytokine

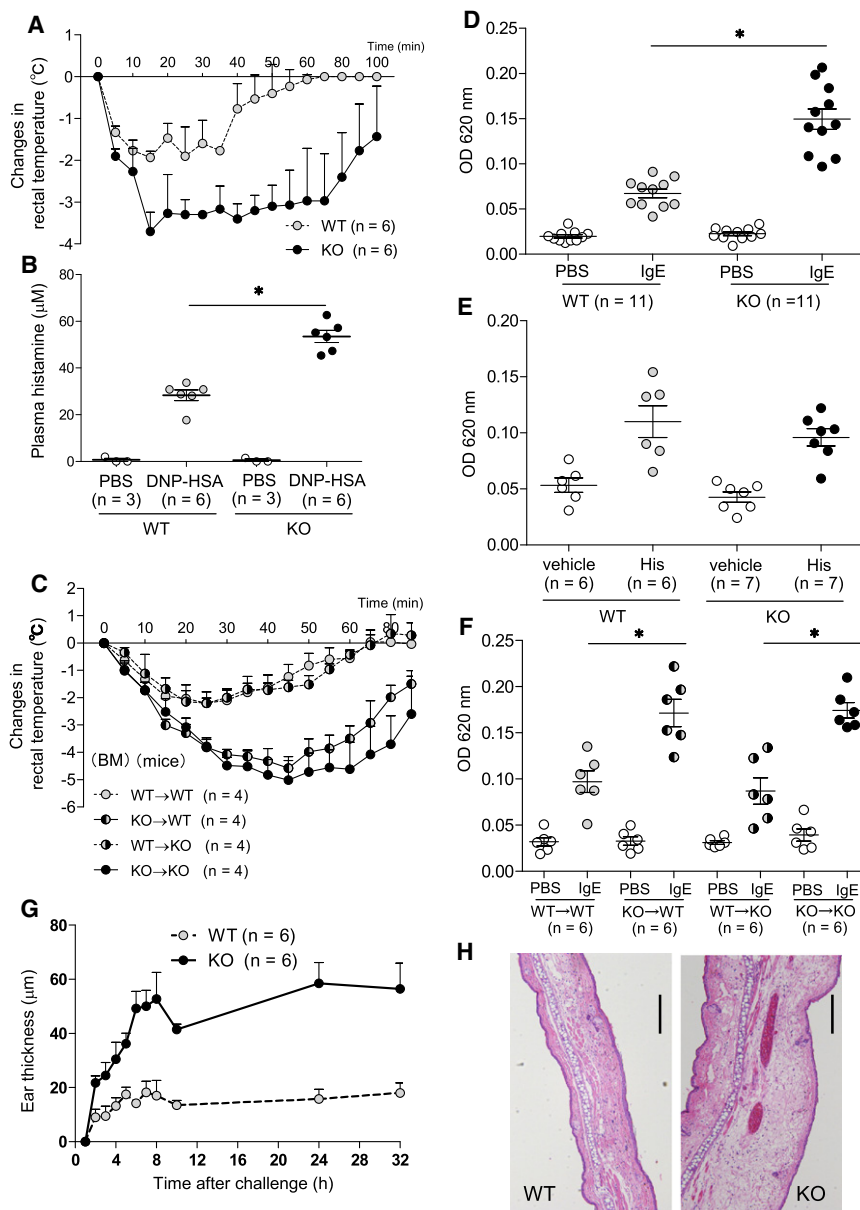


Figure 2. Enhanced Passive Systemic and Cutaneous Anaphylaxis in LMIR3-Deficient Mice

(A–C) PSA reactions.

(A and C) Rectal temperature changes were monitored in (A) WT and LMIR3-deficient (*Cd300lf*^{−/−}) mice (n = 6 per genotype) or (C) WT or *Cd300lf*^{−/−} mice at 2 months after transplantation with WT or *Cd300lf*^{−/−} BM (n = 4 per group).

(B) Serum levels of histamine in WT and *Cd300lf*^{−/−} mice at 3 min before (n = 3 per group) and after (n = 6 per group) antigen challenge.

(D–F) Mice were injected intravenously with (D and F) Evans blue dye containing DNP-HSA after IgE sensitization or (E) Evans blue dye containing histamine. Quantification of Evans blue dye that extravasated into ears at (D, F) 30 min or (E) 3 min after injection.

(D) WT and *Cd300lf*^{−/−} mice (n = 11 per genotype). (E) WT (n = 6) and *Cd300lf*^{−/−} (n = 7) mice.

(F) WT or *Cd300lf*^{−/−} mice at 2 months after transplantation with WT or *Cd300lf*^{−/−} BM (n = 6 per group).

(G and H) Mice were applied with dinitro-fluorobenzene on ears after IgE sensitization.

(G) Ear thickness was measured in WT or *Cd300lf*^{−/−} mice (n = 6 per genotype).

(H) Sections of antigen-challenged ears were stained with hematoxylin and eosin. Scale bars represent 100 μm.

Data are representative of three independent experiments (mean and SD in A–G). *p < 0.01 (Student's t test). See also Figure S2.

production in the transduced *Cd300lf*^{−/−} BMMCs stimulated by FcεRI crosslinking alone (Figure 1E; Izawa et al., 2009). Thus, only when LMIR3 and FcεRI were coengaged in WT BMMC did LMIR3 deliver an inhibitory signal via its ITIMs and ITSM in vitro.

Enhanced Responses of FcεRI-Mediated Passive Anaphylaxis in *Cd300lf*^{−/−} Mice

We first elicited FcεRI-mediated passive systemic anaphylaxis (PSA) reactions (Masuda et al., 2007). WT or *Cd300lf*^{−/−} mice were challenged with dinitrophenyl-conjugated human serum albumin (DNP-HSA) 24 hr after intravenous injection of anti-DNP IgE. Remarkably, the magnitude and duration of body temperature drops were enhanced in *Cd300lf*^{−/−} mice compared to WT mice (Figure 2A), in accordance with the higher amounts of plasma histamine in *Cd300lf*^{−/−} mice at 3 min after antigen challenge (Figure 2B). In both WT and *Cd300lf*^{−/−}

mice, PSA was completely suppressed by pretreatment with cyproheptadine, an antagonist of histamine and serotonin, but not with CV6029, an antagonist of platelet activating factor (PAF) receptor (Figure S2A). We then used WT or *Cd300lf*^{−/−} mice receiving whole WT or *Cd300lf*^{−/−} BM transplants. At 2 months after BM transplantation, LMIR3 was expressed in mast cells residing in ear skin or peritoneal cavity of WT BM-transplanted mice, whereas LMIR3 expression was lacking in those of *Cd300lf*^{−/−} BM-transplanted mice (Figures S2B and S2C). In addition, the numbers of skin mast cells were similar in each group of BM-transplanted mice (Figure S2D). Both WT or *Cd300lf*^{−/−} mice transplanted with *Cd300lf*^{−/−} BM displayed a stronger response of PSA than the mice transplanted with WT BM (Figure 2C).

Next, we elicited FcεRI-mediated passive cutaneous anaphylaxis (PCA) reactions (Hata et al., 1998). Mice were challenged by intravenous injection of DNP-HSA and Evans blue 24 hr after intradermal injection of anti-DNP IgE in their ears. Notably, dye extravasation in IgE-injected sites in *Cd300lf*^{−/−} mice was greater than in that of WT mice (Figure 2D). Pretreatment with cyproheptadine dampened these responses in *Cd300lf*^{−/−} mice as well as in WT mice (Figure S2E). On the other hand, intradermal injection of histamine and Evans blue dye in the ears

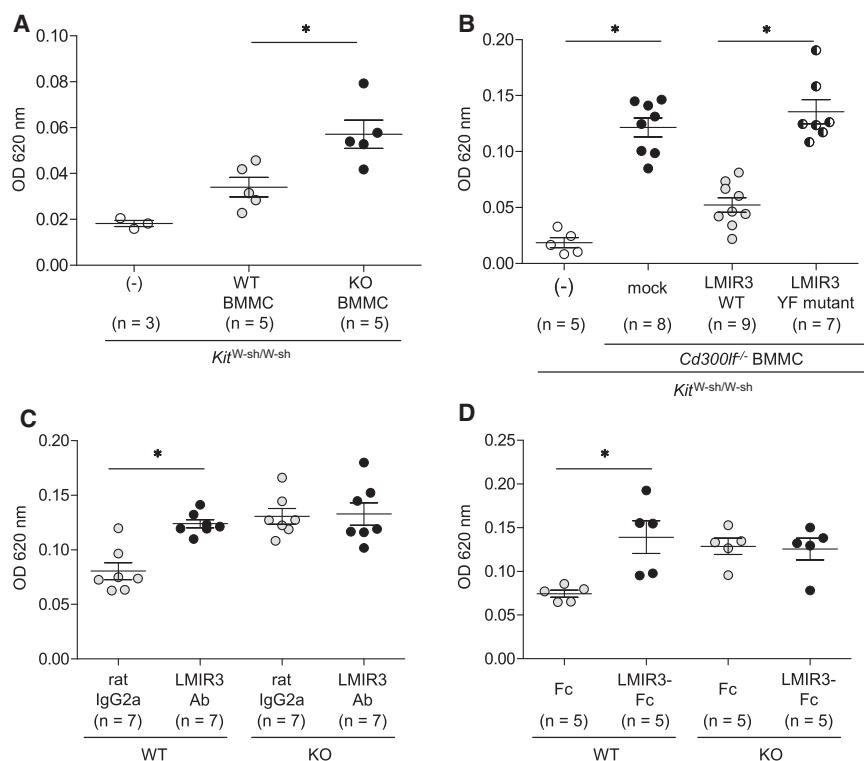


Figure 3. Enhanced PCA Responses in LMIR3-Deficient Mice Were due to the Lack of LMIR3-Mediated Inhibitory Signals in Mast Cells

Mice were injected intravenously with Evans blue dye containing DNP-HSA after IgE sensitization. Quantification of Evans blue dye that had extravasated into ears. Data are representative of three independent experiments (mean and SD). * $p < 0.05$ (Student's t test).

(A) *Klt^{W-sh/W-sh}* mice (n = 3) or *Kd300lf^{-/-}* mice injected intradermally with WT BMMCs (n = 5) or *Kd300lf^{-/-}* BMMCs (n = 5).

(B) *Kd300lf^{-/-}* BMMCs transduced with LMIR3 WT (n = 9), LMIR3(Y241F-Y289F-Y325F) mutant (n = 7), or mock (n = 8).

(C) WT or *Kd300lf^{-/-}* mice injected intradermally with 10 μ g of either LMIR3 Ab (3-14-11) or isotype control Ab together with anti-DNP IgE (n = 7 per group).

(D) WT or *Kd300lf^{-/-}* mice injected intradermally with 10 μ g of either LMIR3-Fc or control Fc together with anti-DNP IgE (n = 5 per group). See also Figure S3.

induced comparable levels of dye extravasation in WT and *Cd300lf^{-/-}* mice (Figure 2E). Analysis of the mice after BM transplantation showed that PCA responses were enhanced in *Cd300lf^{-/-}* BM-transplanted mice as compared to WT BM-transplanted mice (Figure 2F). Moreover, we elicited a different type of Fc ϵ RI-mediated PCA reaction (Hata et al., 1998). Mice were challenged by epicutaneous application of dinitrofluorobenzene on the ears 24 hr after intravenous injection of anti-DNP IgE. Of note, ear swelling with inflammatory edema was greater in *Cd300lf^{-/-}* mice than in WT mice during the entire observation period (Figures 2G and 2H). Early-phase PCA and PSA reactions largely depend on histamine and serotonin secreted by mast cells, whereas late-phase PCA reactions are associated with mast cell-derived cytokines in mice (Hata et al., 1998). Overall, LMIR3 deficiency exacerbated Fc ϵ RI-mediated anaphylactic responses in mice, presumably because LMIR3-deficient mast cells secreted more histamine, serotonin, and cytokines than did WT mast cells in vivo.

LMIR3-Dependent Inhibition of Mast Cell Activation In Vivo

To obtain direct evidence that enhanced responses of PCA in *Cd300lf^{-/-}* mice depended on LMIR3 deficiency in mast cells, we used mast cell-deficient *Klt^{W-sh/W-sh}* mice engrafted intradermally with WT or *Cd300lf^{-/-}* BMMCs. At 8 weeks after transplantation, similar numbers of mast cells were found in ear skin of BMMC-engrafted *Klt^{W-sh/W-sh}* mice (Figure S3A). *Klt^{W-sh/W-sh}* mice did not exhibit significant levels of dye extravasation in response to antigen stimulation (Figure 3A). The amounts of extravasated dye in *Cd300lf^{-/-}* BMMC-transplanted *Klt^{W-sh/W-sh}* mice were higher than those in WT BMMC-transplanted

Klt^{W-sh/W-sh} mice (Figure 3A). Moreover, we induced the same PCA reactions in *Klt^{W-sh/W-sh}* mice engrafted intradermally with *Cd300lf^{-/-}* BMMC transfectants. At 8 weeks after transplantation, similar numbers of mast cells were found in ear skin of each group of the transplanted mice (Figure S3B). The results showed that *Klt^{W-sh/W-sh}* mice engrafted with LMIR3-deficient BMMCs exhibited stronger responses than *Klt^{W-sh/W-sh}* mice engrafted with LMIR3 WT-sufficient BMMCs (Figure 3B). It should be noted that *Klt^{W-sh/W-sh}* mice transplanted with LMIR3(Y241F-Y289F-Y325F) mutant-transduced *Cd300lf^{-/-}* BMMCs exhibited the enhanced responses at levels comparable to those of *Klt^{W-sh/W-sh}* mice transplanted with LMIR3-deficient BMMCs (Figure 3B). Taken together, these results indicated that Fc ϵ RI-mediated PCA responses were negatively regulated by LMIR3-dependent inhibitory signals through its ITIMs and ITSM in mast cells. Interestingly, when pretreated with LMIR3 antibody (Ab), but not with control Ab, WT mice exhibited enhanced PCA responses at levels comparable to those exhibited by *Cd300lf^{-/-}* mice (Figure 3C). Similarly, pretreatment with LMIR3-Fc, but not with control Fc, enhanced the PCA responses in WT mice to a level similar to that observed in *Cd300lf^{-/-}* mice (Figure 3D). Notably, pretreatment with either LMIR3 Ab or LMIR3-Fc failed to further enhance PCA responses in *Cd300lf^{-/-}* mice (Figures 3C and 3D). Collectively, PCA responses in WT mice seemed to be inhibited by the in vivo interaction between LMIR3 and its ligands surrounded by mast cells.

Exacerbation of Mast Cell-Dependent Airway Inflammation and Dermatitis in *Cd300lf^{-/-}* Mice

To clarify the role of LMIR3 in the pathogenesis of other allergic diseases, we used a mast cell-dependent airway inflammation model. According to the established protocol (Williams and Galli, 2000; Taube et al., 2004; Xiao et al., 2011), mice received an

intranasal challenge with ovalbumin (OVA) after the sensitization to OVA without adjuvant. Although OVA-induced chronic airway inflammation, characterized by the accumulation of inflammatory granulocytes and goblet cell hyperplasia, was observed in mast cell-sufficient mice, but not in *Kit^{W-sh/W-sh}* mice, it was drastically enhanced in *Cd300lf^{-/-}* mice (Figures 4A and S4A–S4C). In accordance, OVA-challenged lung weight was considerably higher in *Cd300lf^{-/-}* mice than in WT mice (Figure S4D). LMIR3 deficiency increased the inflammatory cells accumulated in the bronchoalveolar fluids (BALF) of the challenged mice, caused primarily by increased eosinophils (Figure 4B). In addition, *Cd300lf^{-/-}* mice exhibited higher amounts of IL-4, IL-5, and IL-13 in BALF as compared to WT mice (Figure 4C). Then, *Kit^{W-sh/W-sh}* mice intravenously transplanted with WT or *Cd300lf^{-/-}* BMMCs were used 5 months after transplantation. *Cd300lf^{-/-}* BMMC-transplanted *Kit^{W-sh/W-sh}* mice exhibited enhanced airway inflammation with higher numbers of inflammatory cells and levels of cytokines in BALF, when compared with WT BMMC-transplanted *Kit^{W-sh/W-sh}* mice (Figures S4A–S4C).

We next used a mouse AD model, in which AD-like lesions in ear skin were induced by dust mite extract (Kang et al., 2007). WT mice exhibited weak but significant levels of ear thickness at 14 to 19 days from the first application, whereas *Kit^{W-sh/W-sh}* mice did not do so (Figure 4D), implicating mast cells in the pathogenesis of AD-like skin lesions in this model. *Cd300lf^{-/-}* mice displayed enhanced swelling of the ears, where the marked infiltration of leukocytes was observed in the dermis (Figures 4D and 4E). Moreover, we used this model in *Kit^{W-sh/W-sh}* mice engrafted intradermally with *Cd300lf^{-/-}* BMMC transfectants. *Kit^{W-sh/W-sh}* mice engrafted with *Cd300lf^{-/-}* BMMCs transduced with either LMIR3(Y241F-Y289F-Y325F) mutant or mock displayed the greater swelling of ears with AD-like skin lesions when compared with *Kit^{W-sh/W-sh}* mice engrafted with LMIR3 WT-sufficient BMMCs (Figure 4F). Taken together, these results indicated that LMIR3 negatively regulated mast cell-dependent airway inflammation and dermatitis in mice.

Identification of Possible Ligands for LMIR3

As a tool in our ligand search, we used the reporter cell lines (2B4-GFP) (Ohtsuka et al., 2004; Yamasaki et al., 2008). A chimera receptor (LMIR3-CD3 ζ), composed of an extracellular and transmembrane domain of Flag-tagged LMIR3 fused to an intracellular domain of CD3 ζ , was transduced into 2B4-GFP cells to generate 2B4-LMIR3-GFP cells (Figure S5A). The addition of a soluble LMIR3 Ab or Flag Ab to the culture did not induce GFP expression in 2B4-LMIR3-GFP cells (data not shown). However, crosslinking of the chimera receptor (LMIR3-CD3 ζ) by plate-coated LMIR3 Ab strongly induced GFP expression in 2B4-LMIR3-GFP cells but not in 2B4-GFP cells (Figure 5B). This induction of GFP in 2B4-LMIR3-GFP cells was blocked by adding a soluble LMIR3 Ab (Figure 5B). We therefore assumed that GFP expression would be induced in 2B4-LMIR3-GFP cells, but not in 2B4-GFP cells, on plates coated with any ligand for LMIR3. GFP expression was not induced in 2B4-LMIR3-GFP cells on the plates coated with extracellular matrix proteins, such as fibronectin, fibrinogen, vitronectin, or a soluble form of basement membrane including laminin, collagen IV, entactin, and heparin sulfate proteoglycan (Figure S5B). To find a candidate ligand for LMIR3 out of the variety of lipids, we performed

a binding assay prior to a reporter assay. When membranes spotted with many different types of lipids were incubated with LMIR3-Fc in the presence of Ca²⁺, immunoblot analysis showed that LMIR3-Fc strongly bound to ceramide but not to the other lipids tested (Figure 5A). In contrast, neither TIM1-Fc nor control Fc bound to ceramide (Figure 5A). As reported (Kobayashi et al., 2007; Miyanishi et al., 2007), TIM1-Fc bound to phosphatidylserine (PS), whereas LMIR3-Fc did not bind to PS under the same conditions (Figure 5A). None of the lipids on the membranes were bound by control Fc (Figure 5A). To test whether ceramide worked as a ligand for LMIR3, 2B4-LMIR3-GFP cells were incubated on the plates coated with C-24 ceramide. Intriguingly, considerable amounts of GFP expression and IL-2 production were induced by C-24 ceramide in 2B4-LMIR3-GFP cells but not in 2B4-GFP cells (Figures 5B and 5C). In addition, ceramide-induced GFP expression and IL-2 production were both abolished by the addition of a soluble LMIR3 Ab, but not by a control Ab, just after the incubation of 2B4-LMIR3-GFP cells on the ceramide-coated plates (Figures 5B, 5C, and S5C). Other ceramides, C8-ceramide and egg ceramide, also behaved in the same way as C-24 ceramide (Figure 5B), whereas neither glucosylceramide nor alpha-galactosylceramide (α -GalCer) induced GFP expression in 2B4-LMIR3-GFP cells (Figure S5D). In addition, GFP expression was not induced in 2B4-LMIR3-GFP cells on the plates coated with PS, phosphatidylethanolamine (PE), sphingomyelin (SM), sphingosin, cholesterol, lysophosphatidylcholine (lysocleithin), or lipid A (Figure 5D). Although LMIR3-Fc failed to bind both phosphatidylcholine (PC) and sphingosylphosphocholine (SPC) spotted on the membranes (Figure 5A), GFP expression was induced in 2B4-LMIR3-GFP cells on the plates coated with these lipids, which was prevented by the addition of a soluble LMIR3 Ab (Figure 5D). To further test whether LMIR3-Fc was able to directly bind PC or SPC, we used enzyme-linked immunosorbent assay (ELISA) as a different binding assay. LMIR3-Fc, as compared to Fc, significantly bound to PC, SPC, and C-24 ceramide (Figures S5E and S5F). We found no binding of LMIR3-Fc to other lipids tested (Figures S5E and S5F). Intriguingly, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), which were thought to contain the candidate lipid ligands, also bound to an extracellular domain of LMIR3 and induced GFP expression in 2B4-LMIR3-GFP cells, but not in 2B4-GFP cells (Figures 5D and S5E). Alternatively, several types of liposomes were generated. The binding and functional assays with liposome-coated plates confirmed that C-24 ceramide and PC, but not PS, bound to an extracellular domain of LMIR3 and induced GFP expression in 2B4-LMIR3-GFP cells (Figure S5G and data not shown). Notably, the addition of liposomal suspensions of C-24 ceramide and PC, but not of PS, to the culture induced GFP expression in the reporter cells (Figure S5H). Taken together, our results demonstrated that several lipids, including ceramide, and lipoproteins are endogenous ligands for LMIR3.

Upon Fc ϵ RI Engagement, the Extracellular Ceramide-LMIR3 Interaction Inhibited Activation of BMMCs

To clarify the role of the candidate ligands for LMIR3 in mast cells, IgE-sensitized WT or *Cd300lf^{-/-}* BMMCs were stimulated with specific antigens on plates coated with the indicated lipids and lipoproteins. Incubation of BMMCs on the coated plates for

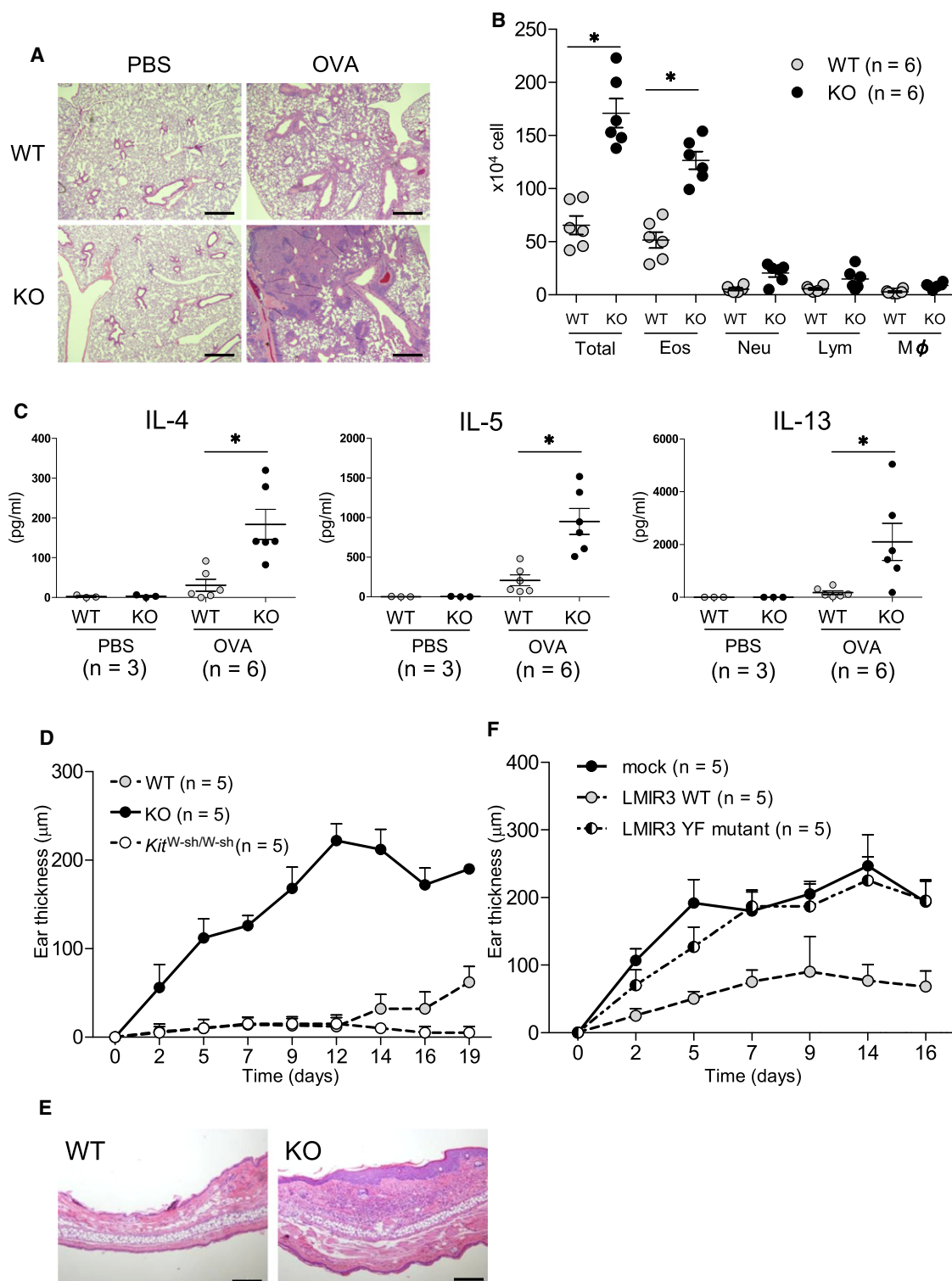


Figure 4. Mast Cell-Dependent Airway Inflammation and Dermatitis Were Exacerbated in LMIR3-Deficient Mice

(A–C) Analysis of WT or *Cd300lf*^{-/-} mice at 48 hr after the last challenge with OVA or PBS in airway inflammation model.

(A) Sections of lung tissues were stained with hematoxylin and eosin. Scale bars represent 500 μm.

(B) Numbers of total cells (Total), eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), or macrophages (Mφ) included in BALF (n = 6 per genotype).

(C) Amounts of IL-4, IL-5, or IL-13 in BALF. OVA, n = 6 per group; PBS, n = 3 per group.

(D–F) AD-like skin lesions were induced by repeated application of dust mite extract.

(D) Ear thickness was measured in WT, *Cd300lf*^{-/-}, and *Kit*^{W-sh/W-sh} mice (n = 5 per genotype) during 19 days after the first treatment.

24 hr did not affect mast cell viability (data not shown). Remarkably, inhibited β -hexosaminidase release was noted in WT BMMCs on the plates coated with ceramide, SPC, HDL, or LDL (Figure 6A). However, this inhibition was not observed in *Cd300lf^{-/-}* BMMCs (Figure 6A). We found no inhibition of β -hexosaminidase release in BMMCs on either the PC- or PS-coated plates (Figure 6A). Similarly, Fc ϵ RI-mediated cytokine production was inhibited in WT, but not *Cd300lf^{-/-}*, BMMCs on the plates coated with ceramide, SPC, HDL, or LDL (Figure 6B). These results indicated that ceramide, SPC, and lipoproteins worked as physiological ligands for LMIR3 in mast cells. Furthermore, similar experiments were conducted in *Cd300lf^{-/-}* BMMC transfectants. Marked inhibition of both degranulation and cytokine production was found in LMIR3 WT-transduced *Cd300lf^{-/-}* BMMCs, but in neither LMIR3(Y241F-Y289F-Y325F) mutant- nor mock-transduced *Cd300lf^{-/-}* BMMCs, on the plates coated with ceramide, SPC, and lipoproteins (Figures 6C and 6D). We did not find any effect of PS or PC on degranulation or cytokine production of BMMC transfectants (Figures 6C and 6D). In addition, the inhibitory effect of plate-coated ceramide on Fc ϵ RI-mediated degranulation of WT BMMCs was dampened by the addition of a soluble LMIR3 Ab before antigen stimulation (Figure S6). Thus, a soluble LMIR3 Ab worked as a blocking Ab to interfere with the extracellular ceramide-LMIR3 interaction. We next examined the tyrosine phosphorylation levels of LMIR3 in WT BMMCs on ceramide- or vehicle-coated plates. The results showed that Fc ϵ RI crosslinking induced the tyrosine phosphorylation of LMIR3 and subsequent recruitment of SHP-1 and SHP-2, but not of SHIP, to tyrosine-phosphorylated LMIR3 in BMMCs only on the ceramide-coated plates (Figure 6E). In accordance, the ceramide-LMIR3 interaction reduced the levels of ERK phosphorylation in BMMCs stimulated by Fc ϵ RI crosslinking (Figure 6E). It should be noted that LMIR3 was not tyrosine phosphorylated in BMMCs on the ceramide-coated plates without Fc ϵ RI engagement (Figure 6E).

Extracellular Ceramide-LMIR3 Interaction Inhibited Fc ϵ RI-Mediated Activation of Mast Cells In Vivo

For further analysis, we used liposomal ceramides that could be administered to mice. Consistently, Fc ϵ RI-mediated degranulation of WT BMMCs, but not of *Cd300lf^{-/-}* BMMCs, was inhibited by the addition of liposomal ceramides to the culture before antigen stimulation. On the other hand, it was not affected by the addition of liposomal PC or liposomal PS to the culture (Figure 7A). We then explored the localization of Fc ϵ RI and LMIR3 in IgE-sensitized BMMCs in the presence of specific antigen and liposomal ceramides. Confocal microscopic analyses were performed with Abs against LMIR3 and IgE. In the absence of antigen and liposomal ceramides, both IgE-bound Fc ϵ RI and LMIR3 were mainly expressed in the surface of BMMCs, but were not frequently colocalized (Figure 7B). The addition of liposomal ceramides alone did not significantly influence the localization patterns (Figure 7B). On the other hand, antigen stimulation alone induced some degree of colocalization of

IgE-bound Fc ϵ RI and LMIR3 in the surface and cytoplasm of BMMCs at 10 min after stimulation (Figure 7B). Remarkably, the addition of both liposomal ceramides and antigen led to the stronger and more frequent colocalization of Fc ϵ RI and LMIR3 than that of antigen alone at the same time point (Figure 7B). These results were compatible with the notion that Fc ϵ RI engagement was required for the substantial tyrosine phosphorylation of LMIR3 through the ceramide-LMIR3 interaction. To test the effect of liposomal ceramides on PCA responses, WT or *Cd300lf^{-/-}* mice were pretreated with liposomal ceramides, PC, or PS in the ears 24 hr before antigen stimulation. Intriguingly, only when pretreated with liposomal ceramides, WT mice, but not *Cd300lf^{-/-}* mice, exhibited the diminished PCA responses (Figure 7C), supporting the hypothesis that extracellular ceramides worked as ligands for LMIR3 in tissue mast cells. We next investigated the distribution of ceramides in vivo, by using ceramide Ab that specifically bound to ceramide (Figure S7A). We confirmed that the addition of a soluble ceramide Ab blocked GFP expression induced by plate-coated ceramide, but not by either SPC or PC, in 2B4-LMIR3-GFP cells (Figure S7B). In addition, HDL- or LDL-induced inhibition of degranulation and cytokine production of BMMCs stimulated by Fc ϵ RI engagement was substantially impaired by the addition of a soluble ceramide Ab (Figure S7C). Confocal microscopic analyses on frozen tissue sections showed that ceramides were distributed not only within cells but also outside DAPI-positive cells in tissues examined (Figure S7D). Expression levels of extracellular ceramides tended to be higher in stomach, skin, or brain (Figure S7D). As previously reported, extracellular ceramides were abundant in epidermis of ear skins, whereas diffuse or patchy expression of extracellular ceramides were also found in dermis, where tryptase-positive mast cells were located (Figure 7D). Extracellular ceramides in tissues were round, linear, or dot-like shaped (Figure 7D). It should be noted that chronic skin and airway inflammation tended to elevate expression of extracellular ceramides (Figure S7E). Importantly, we found frequent contact of extracellular ceramides with mast cells in ear skin (Figure 7D). Furthermore, intradermal injection with ceramide Ab in the ears of WT mice, but not *Cd300lf^{-/-}* mice, led to exacerbated PCA responses (Figure 7E). Collectively, the interaction between extracellular ceramides and LMIR3 negatively regulated Fc ϵ RI-mediated activation of mast cells in vivo.

DISCUSSION

The severity of mast cell-dependent allergic responses depends on the types and amounts of chemical mediators secreted by activated mast cells as the net result of positive and negative signaling events (Kawakami and Galli, 2002; Kraft and Kinet, 2007; Kalesnikoff and Galli, 2008). Negative regulators of mast cell activation prevent spontaneous and excessive activation of mast cells that would be harmful to the host. Mice deficient in an inhibitory receptor, such as PIR-B, gp49B1, Fc γ IIb, or

(E) Hematoxylin and eosin-stained ear sections in WT or *Cd300lf^{-/-}* mice at 19 days from the first application. Scale bars represent 100 μ m.

(F) Ear thickness was measured in *Kit^{W-sh/W-sh}* mice engrafted with *Cd300lf^{-/-}* BMMCs transduced with LMIR3 WT, LMIR3(Y241F-Y289F-Y325F) mutant, or mock (n = 5 per group) during 16 days after the first application.

Data are representative of three independent experiments (mean and SD in B–D, F). *p < 0.01 (Student's t test). See also Figure S4.

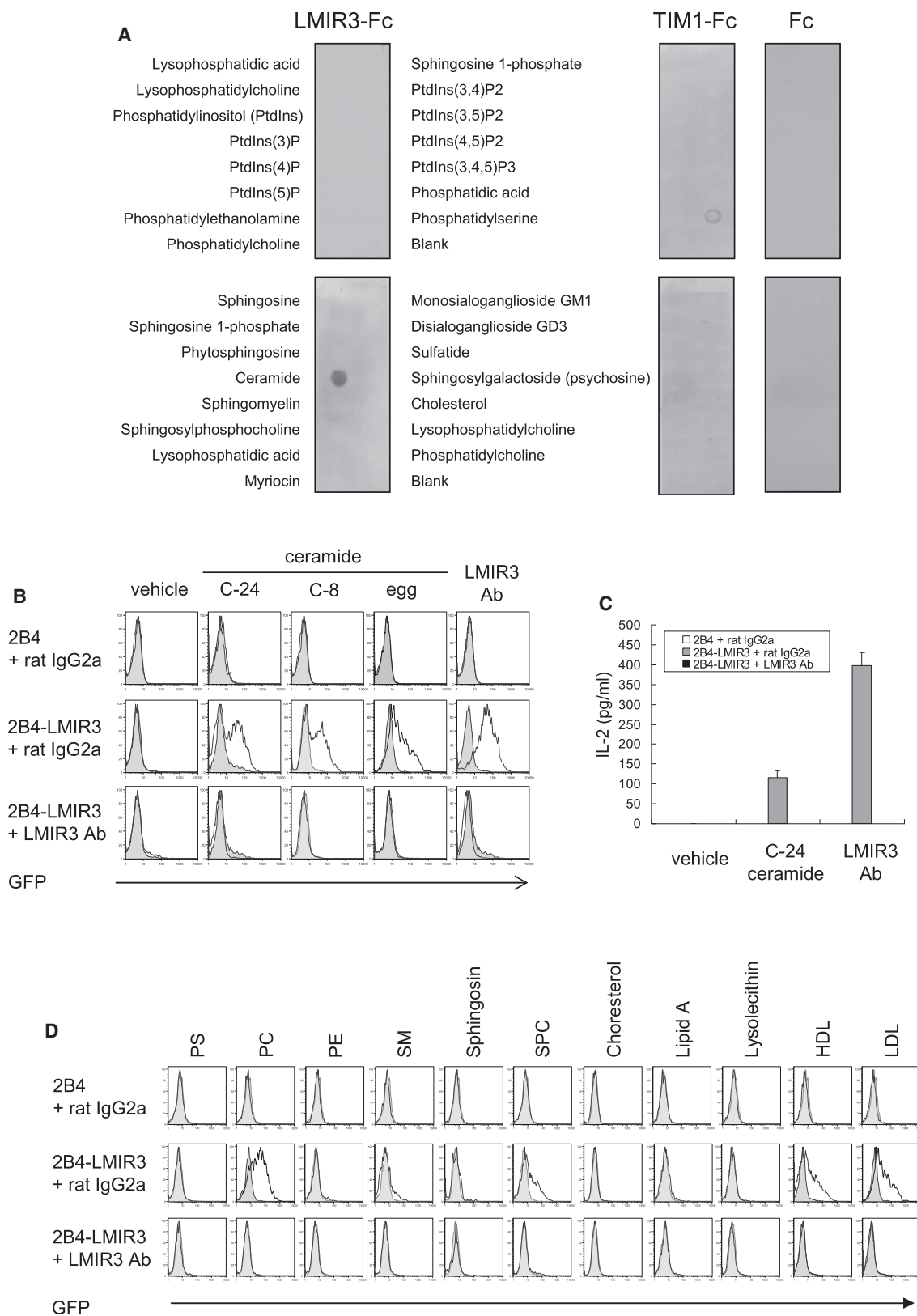


Figure 5. Identification of Possible Ligands for LMIR3

(A) LMIR3-Fc, TIM1-Fc, or Fc were incubated with strips spotted with the indicated lipids and immunoblotted with Ab against human IgG. Data are representative of four independent experiments.

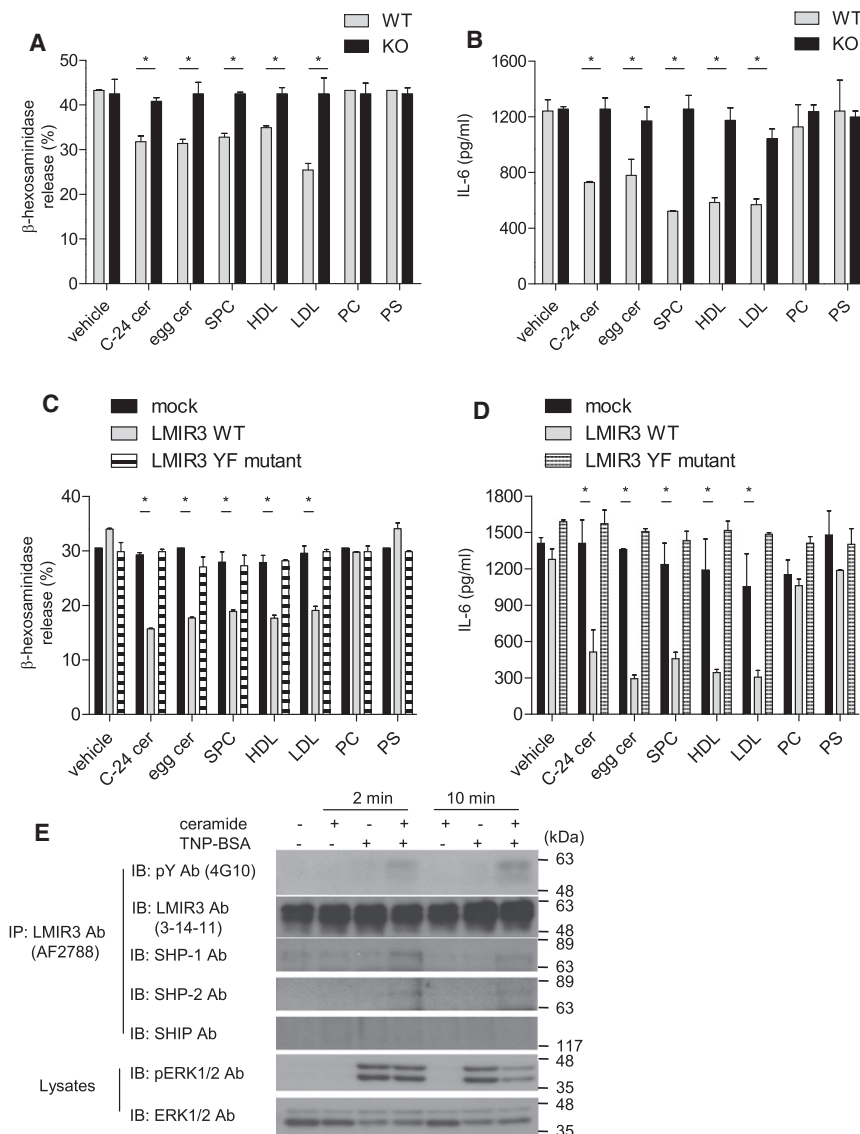


Figure 6. Extracellular Ceramide-LMIR3 Interaction Inhibited FcεRI-Mediated Activation of BMMCs

(A–D) Anti-TNP IgE-sensitized BMMCs were stimulated with 100 ng/ml TNP-BSA for 1 hr (A and C) or 10 ng/ml TNP-BSA for 8 hr (B and D) on plates coated with indicated lipids, lipoproteins, or vehicle. Release of β-hexosaminidase (A and C) or production of IL-6 (B and D) in WT or *Cd300lf^{-/-}* BMMCs (A and B) or *Cd300lf^{-/-}* BMMCs transduced with LMIR3 WT, LMIR3(Y241F-Y289F-Y325F) mutant, or mock (C and D) on each plate. Data are representative of three independent experiments (mean and SD); **p* < 0.01 (Student's *t* test).

(E) Anti-TNP IgE-sensitized WT BMMCs were stimulated with or without 100 ng/ml TNP-BSA for indicated periods on plates coated with either C-24 ceramide or vehicle. Immunoprecipitates of cell lysates with LMIR3 Ab (AF2788) or total cell lysates were immunoblotted with indicated Abs. Data are representative of three independent experiments. See also Figure S6.

PIR-B and MHC class I inhibits FcεRI-mediated activation of BMMCs (Masuda et al., 2007). Thus, whether an inhibitory receptor downregulates mast cell activation in vitro or in vivo depends on the expression profiles of its ligand.

In the present study, we demonstrate that LMIR3 is a critical negative regulator of mast cell activation in vivo. In fact, LMIR3 deficiency considerably augmented mast cell-dependent allergic responses in mice, such as passive anaphylactic reactions, chronic airway inflammation, and AD-like dermatitis. Analysis of mast cell-deficient mice engrafted with BMMCs delineated that LMIR3 deficiency in mast cells was responsible for the enhanced allergic

responses in *Cd300lf^{-/-}* mice. In addition, it was clear that LMIR3 inhibited mast cell activation in vivo through its two ITIMs and/or a single ITSM. Importantly, we identify a ligand for LMIR3. Although lipids have been recently suggested as LMIR ligands (Choi et al., 2011; Cannon et al., 2012), we assume that a functional LMIR3 ligand must fulfill at least two conditions. First, it must be significantly bound by LMIR3-Fc in comparison to control Fc. Second and most critically, its binding to an extracellular domain of LMIR3 must be able to aggregate a chimera receptor LMIR3-CD3ζ, resulting in an ITAM-dependent GFP expression in the 2B4-LMIR3-GFP cells. The observation that LMIR3 deficiency

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(B–D) 2B4-GFP cells or 2B4-LMIR3-GFP cells were incubated for 24 hr on plates coated with the indicated lipids or lipoproteins. 20 μg/ml of either LMIR3 Ab (3-14-11) or isotype control Ab was added to the culture just after the incubation of the reporter cells on the coated plates.

(B and D) Flow cytometry of GFP expression of the reporter cells.

(C) IL-2 production of 2B4-LMIR3-GFP cells or 2B4-GFP cells incubated for 24 hr on plates coated with C-24 ceramide, LMIR3 Ab (3-14-11), or vehicle.

Data are representative of three independent experiments (mean and SD). See also Figure S5.

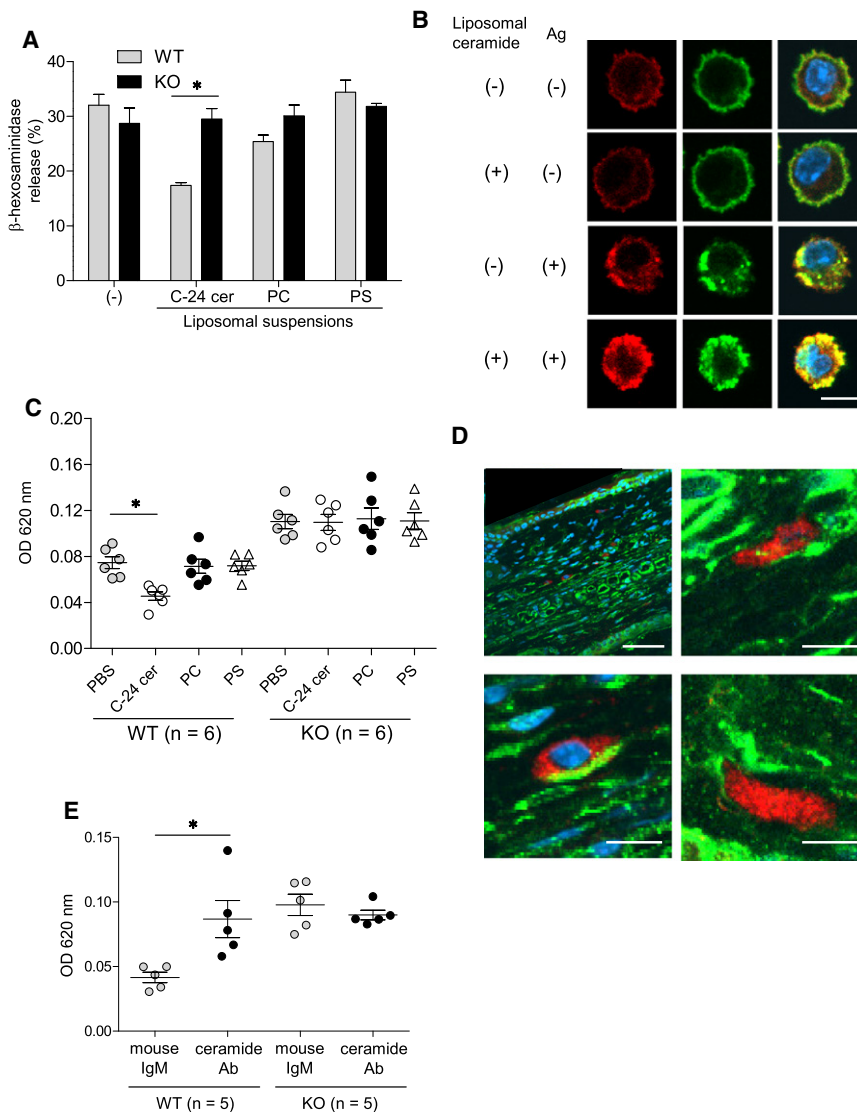


Figure 7. Extracellular Ceramide-LMIR3 Interaction Inhibited Fc ϵ RI-Mediated Activation of Mast Cells In Vivo

(A) Anti-TNP IgE-sensitized WT or *Cd300lf*^{-/-} BMMCs were incubated with 100 ng/ml TNP-BSA and 20 μ g/ml of indicated liposomes for 1 hr to measure the release of β -hexosaminidase. Data are representative of three independent experiments (mean and SD); * $p < 0.01$ (Student's *t* test). (B) Confocal imaging of IgE-bound Fc ϵ RI (green) and LMIR3 (red) in BMMCs. Anti-TNP IgE-sensitized BMMCs were incubated with 100 ng/ml TNP-BSA and/or 20 μ g/ml of liposomal C-24 ceramides for 10 min. After fixation, BMMCs were stained with IgE Ab and LMIR3 Ab (AF2788). Scale bar represents 10 μ m.

(C and E) Mice were injected intravenously with Evans blue dye containing DNP-HSA after IgE sensitization. Quantification of Evans blue dye that had extravasated into ears. Data are representative of three independent experiments (mean and SD). * $p < 0.05$ (Student's *t* test).

(C) WT or *Cd300lf*^{-/-} mice injected intradermally with 20 μ g of liposomes composed of C-24 ceramides, PC, or PS together with anti-DNP IgE (n = 6 per group).

(D) Confocal imaging of frozen sections of ear skin that were stained with ceramide Ab (ceramides; green) and Mast Cell Tryptase Ab (mast cells; red). Scale bars represent 50 μ m (upper left) and 10 μ m (bottom left, upper right, and bottom right).

(B and D) The nuclei were counterstained with DAPI (blue). Data are representative of five independent experiments.

(E) WT or *Cd300lf*^{-/-} mice injected intradermally with 20 μ g of either ceramide Ab (MID 15B4) or isotype control Ab together with anti-DNP IgE (n = 5 per group).

See also Figure S7.

enhanced in vivo mast cell degranulation, an event occurring at the initial phase of anaphylactic responses, led us to speculate that LMIR3 ligands were expressed in the surroundings of tissue mast cells. Extensive ligand screening did not show that cell surface proteins or extracellular matrix proteins are possible ligands for LMIR3. Instead, several extracellular lipids and lipoproteins, including ceramide, SPC, LDL, and HDL, satisfied the necessary conditions for LMIR3 ligands. Interestingly, the binding of these lipids and lipoproteins to LMIR3 inhibited Fc ϵ RI-mediated activation of BMMCs, implicating these lipids and lipoproteins as functional ligands for LMIR3 in mast cells. On the other hand, PC appeared not to be a ligand for LMIR3 in mast cells, notwithstanding that PC worked as a ligand for LMIR3-CD3 ζ in the reporter cells. Although the reason is unknown, it might be in part due to the subtle structural difference of an extracellular domain between endogenous LMIR3 and a chimera receptor LMIR3-CD3 ζ . Alternatively, surface components of mast cells might interfere with the recognition of PC by LMIR3. In addition, our results did not provide any

evidence that PS, including liposomal PS, is a ligand for LMIR3 (Choi et al., 2011), although we cannot exclude the possibility that LMIR3 is involved in the recognition of dead cells via its interaction with certain lipids. It should be noted that our experimental procedures were not the same as those of Choi et al. (2011). Interestingly, LMIR3 ligands ceramide and SPC are generated from SM via the activity of sphingomyelinase and sphingomyelinase, respectively (Kolesnick, 2002; Meyer zu Heringdorf et al., 2002; Zheng et al., 2006), implying that LMIR3 recognizes the specific structure of the lipids. The finding that lipoprotein-induced inhibition of Fc ϵ RI-mediated mast cell activation was substantially impaired by the addition of a soluble ceramide Ab indicated that among the components of lipoproteins, ceramide was a major ligand for LMIR3 in mast cells. In any case, the in vivo role of the candidate lipids for LMIR3 should depend on the extracellular distribution in tissues. Immunohistochemical analyses revealed that extracellular ceramides were expressed in various tissues. The dot-like staining might reflect liposomal structures of ceramides, including exosomes and lipoproteins, whereas round or linear ceramides might be derived from dead cells and bound to structural

proteins of the extracellular matrix. Intriguingly, we found frequent contact of extracellular ceramides with mast cells in the dermis. Because mast cells are located in the surroundings of blood vessels, it is possible that ceramide-containing lipoproteins that circulate in the blood and tissues work as LMIR3 ligands. Importantly, intradermal pretreatment with either LMIR3 Ab, LMIR3-Fc, or ceramide Ab enhanced PCA responses in WT mice but not *Cd300lf^{-/-}* mice. On the other hand, intradermal pretreatment with liposomal ceramides diminished PCA responses in WT mice but not in *Cd300lf^{-/-}* mice. Collectively, these results indicated that the ceramide-LMIR3 interaction was pivotal in LMIR3-mediated inhibition of mast cell activation in vivo. Given that extracellular ceramide levels tended to be elevated in chronically inflamed skin or lungs, it is possible that the extracellular ceramide-LMIR3 interaction plays a prominent role in the negative regulation of mast cell-dependent chronic inflammation. In support of this, extremely severe inflammation was observed in *Cd300lf^{-/-}* mice subjected to mast cell-dependent chronic inflammation models. Therefore, it is also interesting to speculate that the increase or decrease of extracellular ceramides in tissues might influence susceptibility to mast cell-dependent allergic diseases. Although the decrease of ceramide levels in the skin is thought to be associated with the barrier dysfunction that causes AD (Kawakami et al., 2009), it may also accelerate mast cell-dependent inflammation in AD as a result of the lack of ceramide-LMIR3 interaction in the skin. A recent report that AD-like skin lesions were inhibited by topical application of a ceramide derivative appears to support this possibility (Kang et al., 2007).

The molecular mechanism by which the extracellular ceramide-LMIR3 interaction inhibited FcεRI-mediated activation of mast cells in vivo remains to be determined. Taking our observations together, we assume the relevant mechanism to be as follows. In tissues, mast cells are surrounded by extracellular ceramides, either in liposomal forms or in protein-bound forms. The interaction of extracellular ceramides with LMIR3 in mast cells fails to significantly deliver an inhibitory signal without FcεRI engagement. When IgE-sensitized mast cells are encountered with specific antigens, both extracellular ceramide-LMIR3 interaction and FcεRI aggregation cooperatively induce a strong colocalization of LMIR3 and FcεRI in mast cells, where ITIMs and/or ITSM of LMIR3 are considerably tyrosine phosphorylated presumably by activated Src family kinases and/or Syk kinase recruited to β and γ chains of FcεRI. As a result, FcεRI-mediated activation of mast cells is likely to be downregulated by the protein tyrosine phosphatases recruited to LMIR3. Further examination is required to fully understand the precise mechanism of LMIR3-mediated inhibition of FcεRI signaling via colocalization of ceramide-bound LMIR3 and FcεRI in mast cells.

We conclude that the interaction between extracellular ceramide and LMIR3 negatively regulates FcεRI-mediated mast cell activation and MC-related allergic diseases.

EXPERIMENTAL PROCEDURES

Cells

2B4-GFP cell lines (Ohtsuka et al., 2004; Yamasaki et al., 2008) were kind gifts from T. Saito (RIKEN Research Center for Allergy and Immunology). BMMCs

and transfectants were generated as described (Kitaoura et al., 2003; Izawa et al., 2009).

Binding Assay via Solid-Phase ELISA

Solid-phase ELISA was also used (Kobayashi et al., 2007). In brief, the indicated lipids in methanol (10 μg/ml) or methanol as a control was added to ELISA plates and air-dried. Alternatively, plates were coated with 10 μg/ml of protein, PBS as a control, or 20 μg/ml of liposomes. After washing, the plates were incubated with 10 μg/ml of LMIR3-Fc or Fc in the presence of 0.5 mM CaCl₂ for 120 min before incubating with peroxidase-conjugated anti-human Ig (Sigma). Absorbance at 450 nm was measured.

Flow Cytometry

Flow cytometric analysis was performed with FACSCalibur (BD Biosciences) equipped with CellQuest software and FlowJo software (Tree Star) (Yamanishi et al., 2008).

Transfection and Infection

Retroviral transfection was done as described (Morita et al., 2000; Kitamura et al., 2003). Retroviruses were generated by transient transfection of PLAT-E packaging cells (Morita et al., 2000).

BMMC Reconstitution

Kit^{W-sh/W-sh} mice were injected intradermally with either 1 × 10⁶ of WT or *Cd300lf^{-/-}* BMMCs or 1 × 10⁶ of BMMC transfectants 8 weeks before induction of PCA and AD-like model. Alternatively, *Kit^{W-sh/W-sh}* mice were injected intravenously with either 2 × 10⁶ of WT or *Cd300lf^{-/-}* BMMCs 5 months before induction of an airway inflammation model.

Quantification of Mast Cells

Mast cells were stained with toluidine blue and quantified as described (Grimbaldeston et al., 2005).

Induction of PSA

Mice were injected intravenously with 1 mg DNP-HSA 24 hr after the intravenous injection of 5 μg mouse anti-DNP IgE (H1-ε-26). Rectal temperature changes were monitored (Masuda et al., 2007). In some experiments, 50 μg of CV6029 (Santa Cruz Biotechnology), 100 μg of cyproheptadine (Sigma), or vehicle was intraperitoneally injected 1 hr before antigen challenge.

Induction of PCA

Mice were sensitized with the intradermal injection of 50 ng anti-DNP IgE (H1-ε-26) or PBS to each ear. The mice were injected intravenously with 1% Evans blue dye (Sigma) containing 250 μg DNP-HSA 24 hr after the sensitization. The amounts of extravasated dye at 30 min after antigen challenge were measured by absorbance at 450 nm (Hata et al., 1998). In some experiments, CV6029, cyproheptadine, or vehicle was intraperitoneally injected 1 hr before antigen challenge or 10 μg of LMIR3 Ab (3-14-11), ceramide Ab (MID 15B4), isotype control Ab, LMIR3-Fc, Fc, or 20 μg of liposomes was injected intradermally together with anti-DNP IgE (H1-ε-26) in ears. Alternatively, 10 μl of 0.3% dinitrofluorobenzene (Wako) in acetone and olive oil (4:1) was painted on both sides of ears 24 hr after the sensitization. Ear thickness was periodically measured (Hata et al., 1998). After ears were fixed with 10% formaldehyde and embedded in paraffin, sections were stained.

Airway Inflammation

Mice were intraperitoneally sensitized with 10 μg OVA (Sigma) at days 0, 7, 14, 21, 28, and 35. Then the mice were intranasally challenged with 20 μg OVA or PBS at days 40, 43, and 46, and sacrificed 24 hr after the last challenge (Williams and Galli, 2000; Taube et al., 2004; Xiao et al., 2011). All the procedures were approved by an institutional review committee of the University of Tokyo (approval no. 20-8).

Induction of AD-like Skin Lesions

Induction of AD-like skin lesions in mice was performed as described (Kang et al., 2007). In brief, 200 μg of crude extracts of *Dermatophagoides pteronyssinus* (DPE) (Biostir) was painted onto the dorsal surface of each ear three times a week for 19 days. Ear thickness was periodically measured.

Statistical Analyses

Results are expressed as means $\pm$ SD. An unpaired Student's *t* test was used for all the statistical analyses.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2012.08.018>.

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Supplemental Information

The Receptor LMIR3 Negatively Regulates Mast Cell Activation and Allergic Responses by Binding to Extracellular Ceramide

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Supplemental Figures

Figure S1: LMIR3 inhibited FcεRI-mediated activation of BMMCs only when FcεRI and LMIR3 were engaged (related to Figure 1).

Figure S2: Cyproheptadine treatment suppressed both PSA and PCA responses in *Cd300lf^{-/-}* mice as well as in WT mice (related to Figure 2).

Figure S3: Mast cell numbers in ear skin of *Kit^{w-sh/w-sh}* mice after intradermal transplantation with BMMCs or BMMC transfectants (related to Figure 3).

Figure S4: The roles of LMIR3 in a mast-cell-dependent airway inflammation model (related to Figure 4).

Figure S5: Significant binding of LMIR3-Fc to several lipids and lipoproteins by ELISA (related to Figure 5).

Figure S6: A soluble LMIR3 Ab worked as a blocking Ab able to interfere with the extracellular ceramide-LMIR3 interaction (related to Figure 6).

Figure S7. The sensitivity and specificity of ceramide antibody (related to Figure 7).

Supplemental Experimental Procedures

Supplemental references

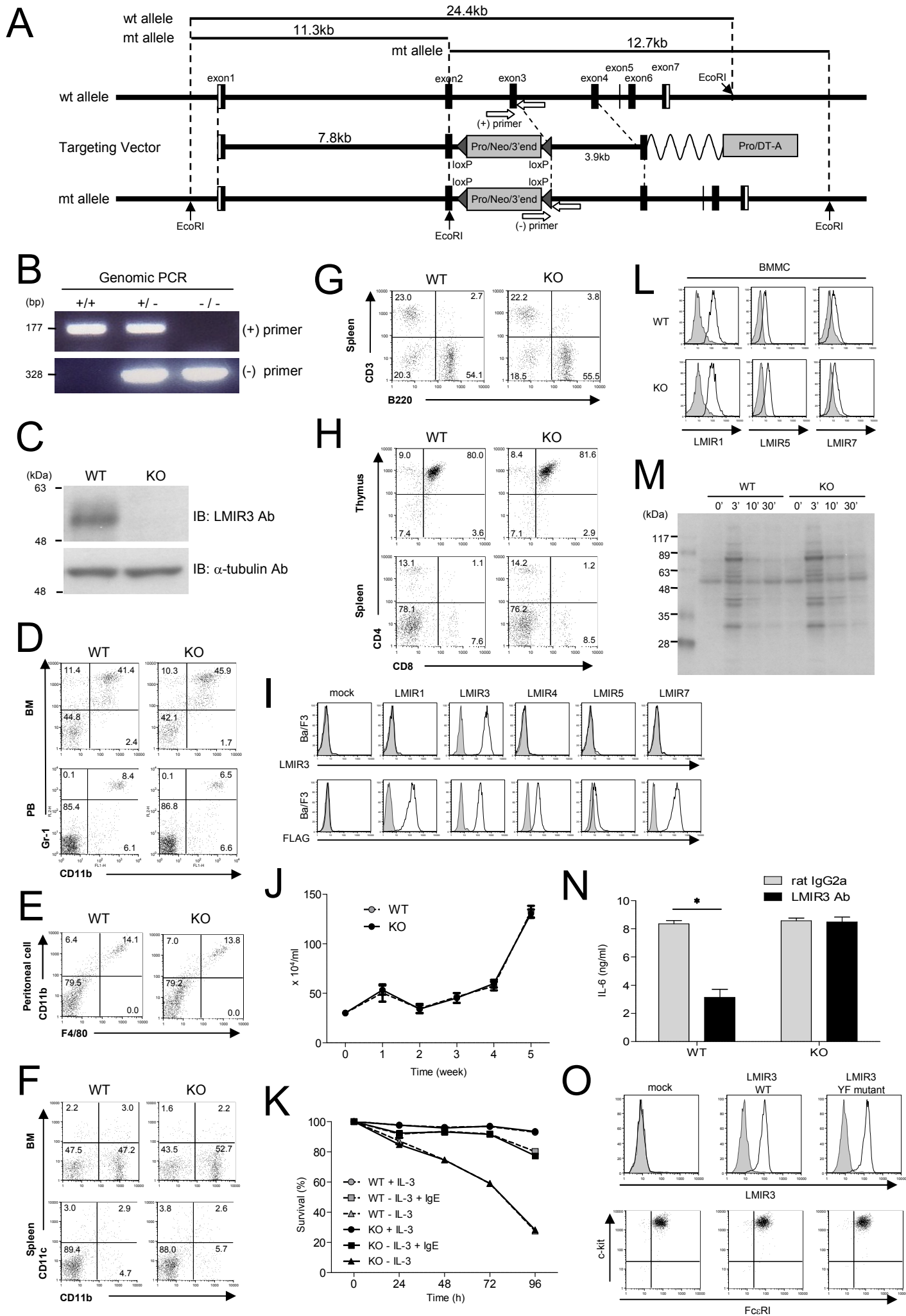


Figure S1. LMIR3 inhibited FcεRI-mediated activation of BMMCs only when FcεRI and LMIR3 were engaged (related to Figure 1).

(A-C) Gene targeting of *Cd300lf*. (A) Schematic representation of *Cd300lf* allele, targeted allele, and targeting vector. Black boxes indicate exons. Pro/DT-A is the diphtheria toxin A fragment gene directed by the MC1 promoter for the negative selection of homologous recombinants, and the zig-zag line indicates the vector-derived sequences. Pro/Neo/3'end is the neomycin-resistant gene directed by the PGK gene promoter; it was flanked by WT loxP sequences at the 5' and 3' ends, respectively. (B) Genomic PCR analysis of the *Cd300lf*-targeted allele. Genomic DNA isolated from +/+, +/-, and -/- mice were amplified with primer pairs for the WT allele (+) or the targeted allele (-), as depicted by the arrows in (A). (C) Protein expression of LMIR3 in WT or *Cd300lf*^{-/-} BM cells. Lysates of WT or *Cd300lf*^{-/-} BM cells were immunoblotted with LMIR3 antibody (3-14-11) or with α-tubulin antibody (B-5-1-2) as control. (B and C) Data are representative of three independent experiments. (D-H) Normal development of myeloid and lymphoid cells in *Cd300lf*^{-/-} mice. BM, peripheral blood (PB), spleen, thymus, or peritoneal cells derived from WT and *Cd300lf*^{-/-} mice were stained with the indicated antibodies. Numbers in the quadrants indicate the percentage of cell populations. Data are representative of five independent experiments. (D) Surface expression of CD11b and Gr-1 in BM or PB cells. (E) Surface expression of F4/80 and CD11b in peritoneal cells. (F) Surface expression of CD11b and CD11c in BM and spleen cells. (G) Surface expression of B220 and CD3 in spleen cells. (H) Surface expression of CD8 and CD4 in thymus and spleen cells. (I) Sensitivity and specificity of LMIR3 Ab (3-14-11). Ba/F3 cells transduced with Flag-tagged LMIR1, LMIR3, LMIR4, LMIR5, or LMIR7 were stained with LMIR3 Ab (3-14-11) (upper) or Flag Ab (M2) (lower). (J) *In vitro* growth curves of bone marrow cells derived from WT or *Cd300lf*^{-/-} mice in the presence of 10 ng/ml IL-3. (K) Survival of WT or *Cd300lf*^{-/-} BMMCs in the absence of IL-3 with or without 5 μg/ml anti-DNP IgE (SPE-7). (L) Surface expression of LMIR1, LMIR5, or LMIR7 in WT or *Cd300lf*^{-/-} BMMCs. (I-L) Data are representative of three independent experiments. Mean and SD in (J and K). (M) WT or *Cd300lf*^{-/-} BMMCs that had been sensitized with anti-TNP IgE were stimulated with 100 ng/ml of TNP-BSA for the indicated time periods. Cell lysates were immunostained with phosphotyrosine antibody (4G10). (N) Anti-TNP IgE-bound FcεRI and LMIR3 in WT or *Cd300lf*^{-/-} BMMCs were co-ligated by 20 μg/ml of LMIR3 Ab (3-14-11) or isotype control Ab on F(ab')₂ Ab against rat IgG (6130-01)-coated plates. IL-6 released into the culture supernatants was measured by ELISA. (M and N) Data are representative of three independent experiments. Mean and SD in (N). **P* < 0.01 (Student's *t*-test). (O) Surface expression levels of FcεRI, c-kit, or the transduced Flag-LMIR3 in *Cd300lf*^{-/-} BMMC transfectants. *Cd300lf*^{-/-} BMMCs transduced with LMIR3 WT, LMIR3(Y241F-Y289F-Y325F) mutant, or mock were stained with c-kit antibody (2B8), FcεRIα antibody (MAR-1), or Flag antibody (M2). Data are representative of three independent experiments.

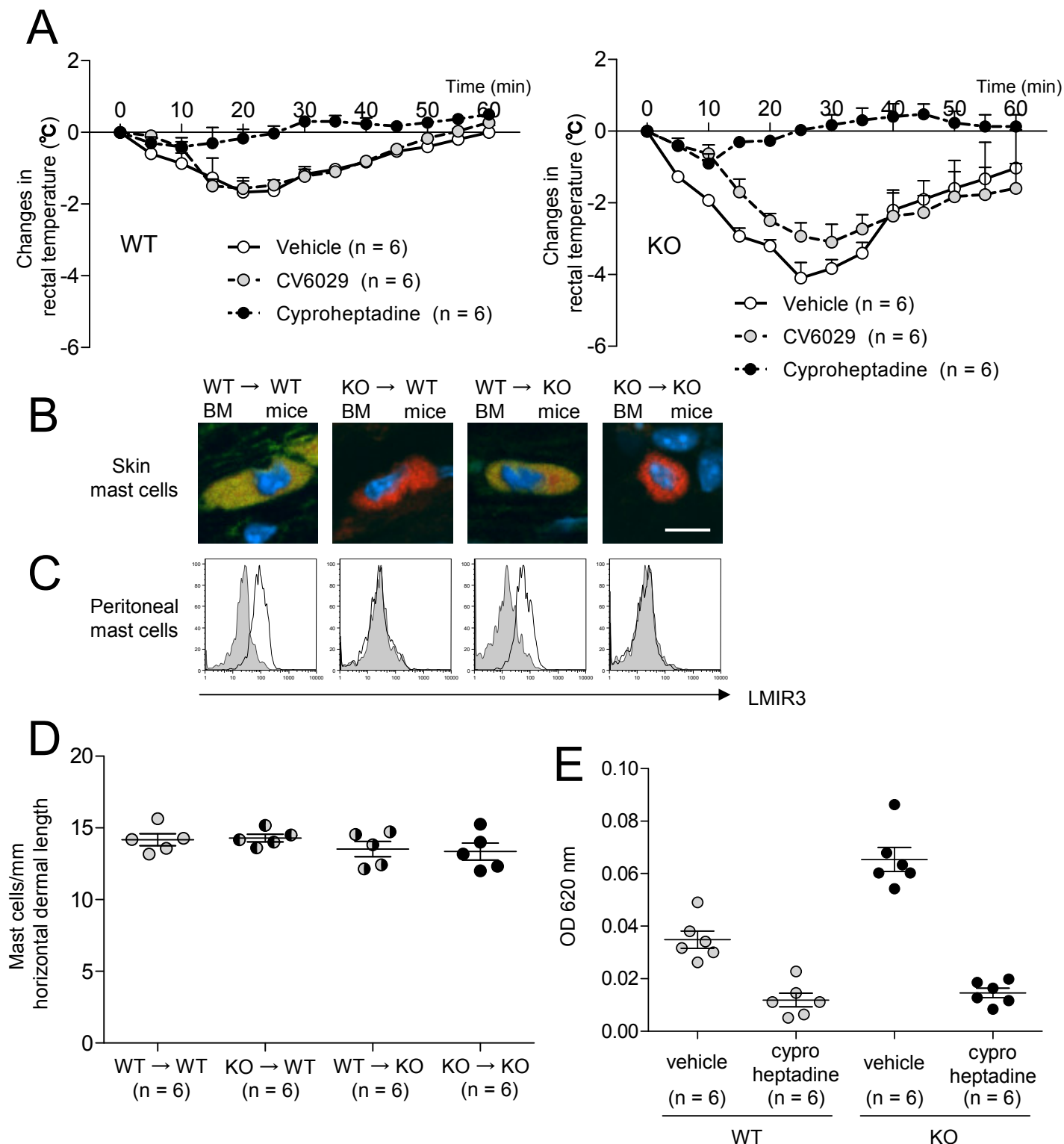


Figure S2. Cyproheptadine treatment suppressed both PSA and PCA responses in *Cd300lf^{-/-}* mice as well as in WT mice (related to Figure 2).

(A) Rectal temperature changes were monitored after PSA. WT or *Cd300lf^{-/-}* mice were injected intraperitoneally with 100 μ g of CV6029, 50 μ g of cyproheptadine, or vehicle 1 h before antigen challenge. (B and C) Expression of LMIR3 in ear skin mast cells (B) or peritoneal mast cells (C) derived from WT or *Cd300lf^{-/-}* mice at 2 months after intravenous transplantation with WT or *Cd300lf^{-/-}* BM. Data are representative of five independent experiments. (B) Mast cells in ear skin of the transplanted mice were detected by staining with Mast Cell Tryptase Ab and Alexa Fluor 546-conjugated goat Ab against rabbit IgG (H+L). Mast cells positive for trptase (red) were stained with goat LMIR3 Ab (AF2788) and Alexa Fluor 488-conjugated Donkey Ab against goat IgG (H+L). LMIR3 was stained green. Mast cells expressing or lacking LMIR3 were stained yellow or red, respectively. (C) Surface expression of LMIR3 in Fc ϵ RI α ⁺c-kit⁺ mast cells included in peritoneal cells was examined by FACS. Staining with LMIR3 Ab (3-14-11) (continuous line histograms). Control staining (shaded histograms). (D) Mast cell numbers in ear skin of WT or *Cd300lf^{-/-}* mice at 2 months after intravenous transplantation with WT or *Cd300lf^{-/-}* BM (n = 5 per group). (E) Dye extravasation after PCA. WT or *Cd300lf^{-/-}* mice were injected intraperitoneally with 100 μ g of CV6029, 50 μ g of cyproheptadine, or vehicle 1 h before antigen challenge. Data are representative of three independent experiments. Mean and SD in (A and E).

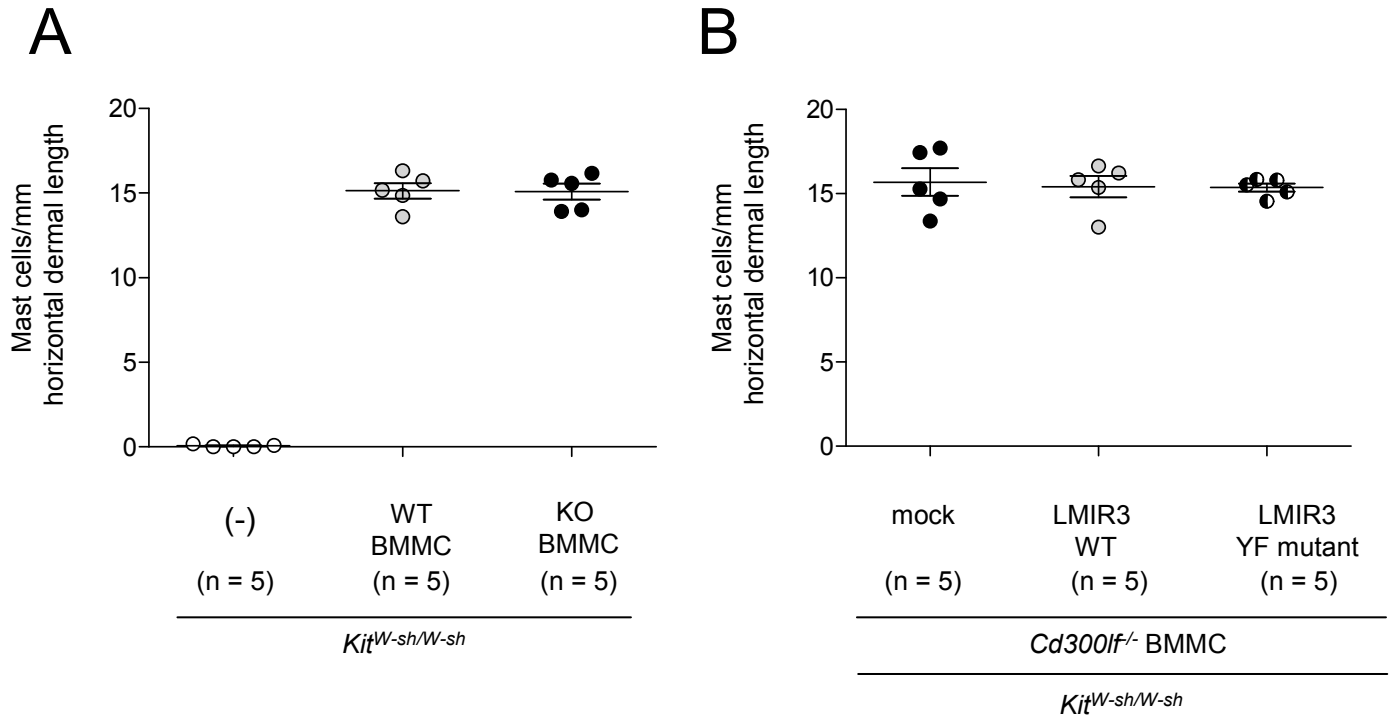


Figure S3. Mast cell numbers in ear skin of *Kit^{W-sh/W-sh}* mice after intradermal transplantation with BMMCs or BMMC transfectants (related to Figure 3).

(A and B) Mast cell numbers in ear skin of (A) *Kit^{W-sh/W-sh}* mice or those mice at 8 weeks after intradermal transplantation with WT or *Cd300lf^{-/-}* BMMCs (n = 5 per group) or (B) *Kit^{W-sh/W-sh}* mice at 8 weeks after intradermal transplantation with *Cd300lf^{-/-}* BMMCs transduced with LMIR3 WT, LMIR3(Y241F-Y289F-Y325F) mutant, or mock (n = 5 per group). Mean and SD (A and B).

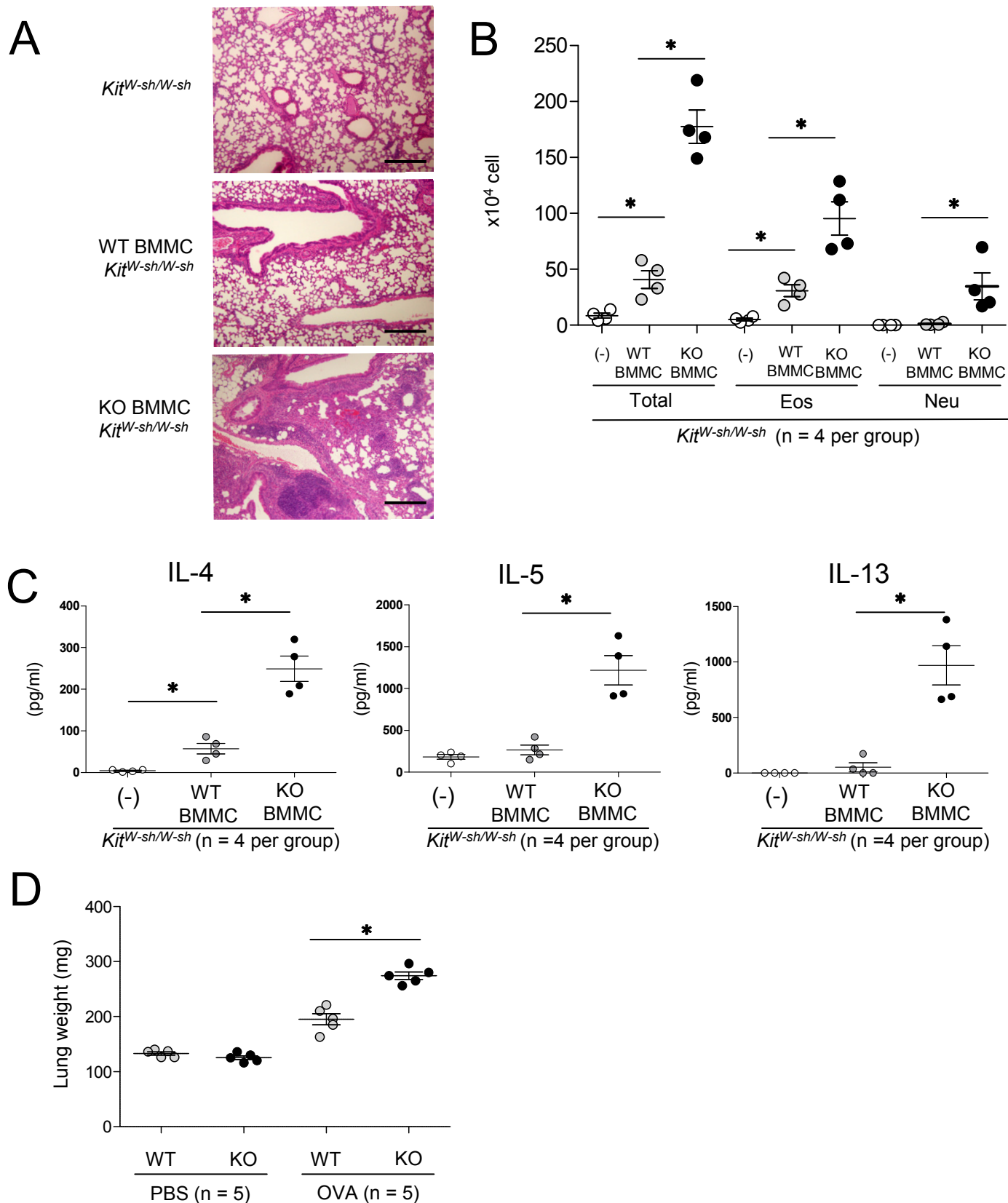


Figure S4. The roles of LMIR3 in a mast-cell-dependent airway inflammation model (related to Figure 4).

(A-C) *Kit^{W-sh/W-sh}* mice or those mice at 5 months after intravenous transplantation with WT or *Cd300lf^{-/-}* BMMCs (n = 4 per group) were used. Analysis of the mice at 24 h after the last challenge with OVA or PBS in an airway inflammation model. Data are representative of two independent experiments (Mean and SD in (B and C)). **P* < 0.05 (Student's *t*-test). (A) Sections of lung tissues were stained with hematoxylin and eosin. Scale bars, 200 μ m. (B) Numbers of total cells (Total), eosinophils (Eos), or neutrophils (Neu) included in BALF. (C) Amounts of IL-4, IL-5, or IL-13 in BALF. (D) Analysis of WT or *Cd300lf^{-/-}* mice at 24 h after the last challenge with OVA or PBS in an airway inflammation model. Weight of lung (n = 5 per group). Data are representative of three independent experiments (Mean and SD). **P* < 0.01 (Student's *t*-test).

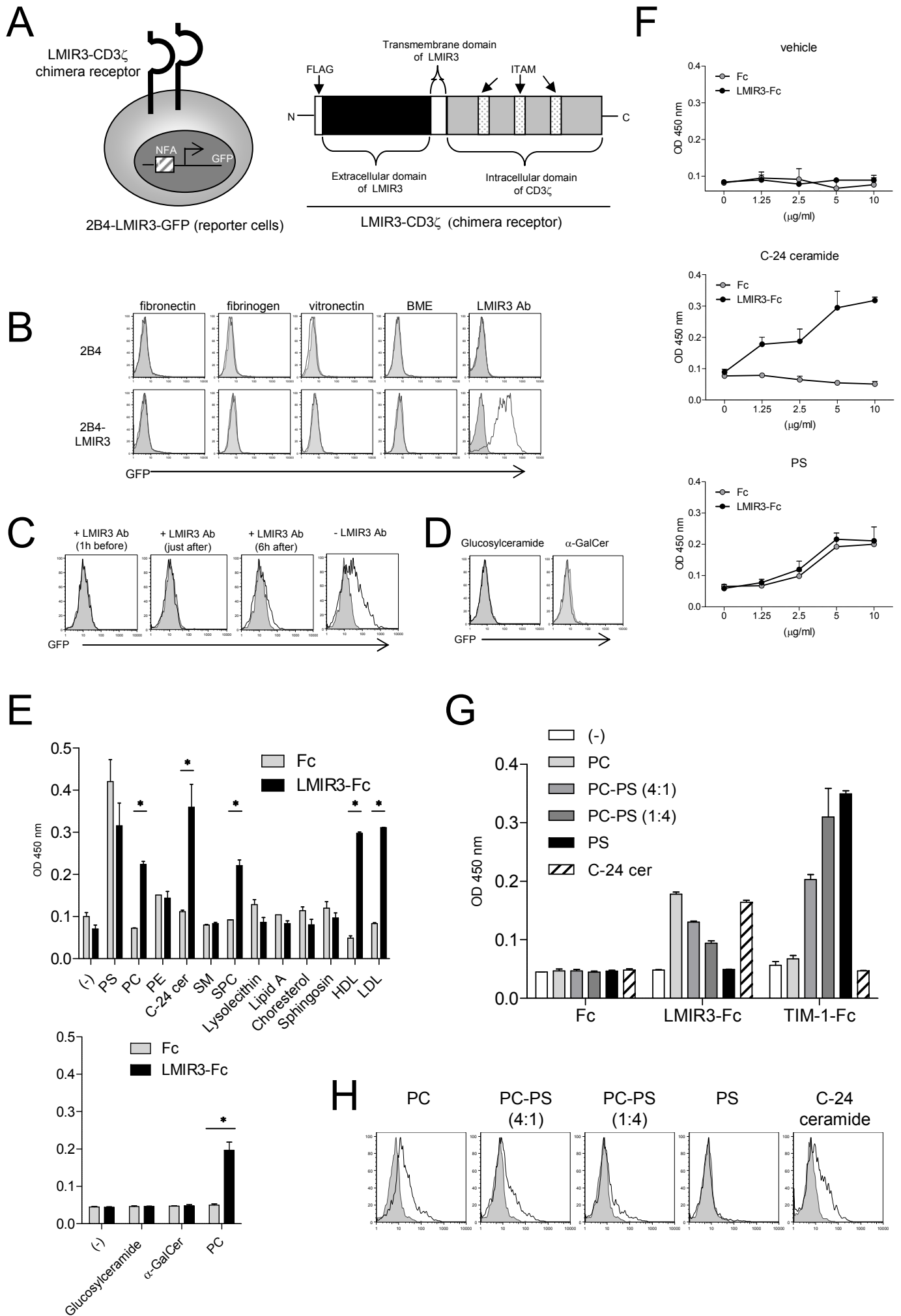


Figure S5. Significant binding of LMIR3-Fc to several lipids and lipoproteins by ELISA (related to Figure 5).

(A) The schematic structure of a chimera receptor (LMIR3-CD3 ζ) expressed in 2B4-LMIR3-GFP cells. (B) The 2B4-LMIR3-GFP or the 2B4-GFP cells were incubated for 24 h on plates coated with fibronectin, fibrinogen, vitronectin, soluble form of basement membrane (BME), or LMIR3 antibody (3-14-11). Flow cytometry of GFP expression in these cells is shown. Data are representative of four independent experiments. (C) Ten $\mu\text{g/ml}$ of a soluble LMIR3 Ab (3-14-11) was added to the culture at 1 h before, just after, or 6 h after the incubation of 2B4-LMIR3-GFP cells on plates coated with C-24 ceramide. Flow cytometry of GFP expression in these cells at 24 h after the incubation is shown. Data are representative of three independent experiments. (D) The 2B4-LMIR3-GFP cells were incubated for 24 h on plates coated with glucosylceramide or alpha-galactosylceramide (α -GalCer). Flow cytometry of GFP expression in these cells is shown. Data are representative of three independent experiments. (E and F) LMIR3-Fc or Fc bound to wells was quantified by ELISA. (E) Ten $\mu\text{g/ml}$ of LMIR3-Fc or control Fc was incubated on plates coated with the indicated lipids, lipoproteins, or vehicle. (F) Indicated concentrations of LMIR3-Fc or Fc was incubated on plates coated with C-24 ceramide, PS, or vehicle. (G) LMIR3-Fc, TIM1-Fc, or Fc bound to wells was quantified by ELISA. Ten $\mu\text{g/ml}$ of LMIR3-Fc, TIM1-Fc, or Fc was incubated on plates coated with the indicated liposomes. PC-PS indicates the mixtures of PC and PS. PC-PS (4:1) or PC-PS (1:4) indicates PC-PS at 4:1 ratio or PC-PS at 1:4 ratio, respectively. (E-G) Data are representative of three independent experiments (Mean and SD) $*P < 0.01$ (Student's *t*-test). (H) The 2B4-LMIR3-GFP cells were incubated for 24 h after the addition of 20 $\mu\text{g/ml}$ of the indicated liposomes to the culture. Flow cytometry of GFP expression in these cells is shown. Data are representative of four independent experiments.

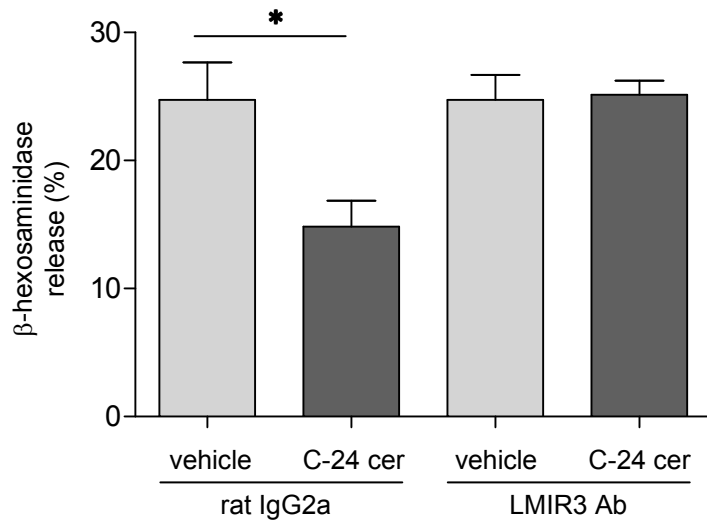


Figure S6. A soluble LMIR3 Ab worked as a blocking Ab able to interfere with the extracellular ceramide-LMIR3 interaction (related to Figure 6).

Anti-TNP IgE-sensitized WT BMDCs were incubated on plates coated with C-24 ceramide or vehicle for 30 min, and then 10 µg/ml of either a soluble LMIR3 Ab (3-14-11) or isotype control Ab was added to the culture medium. After thirty minutes, these cells were stimulated with 100 ng/ml TNP-BSA for 1 h. Release of β-hexosaminidase was measured. Data are representative of three independent experiments (Mean and SD) * $P < 0.01$ (Student's t -test).

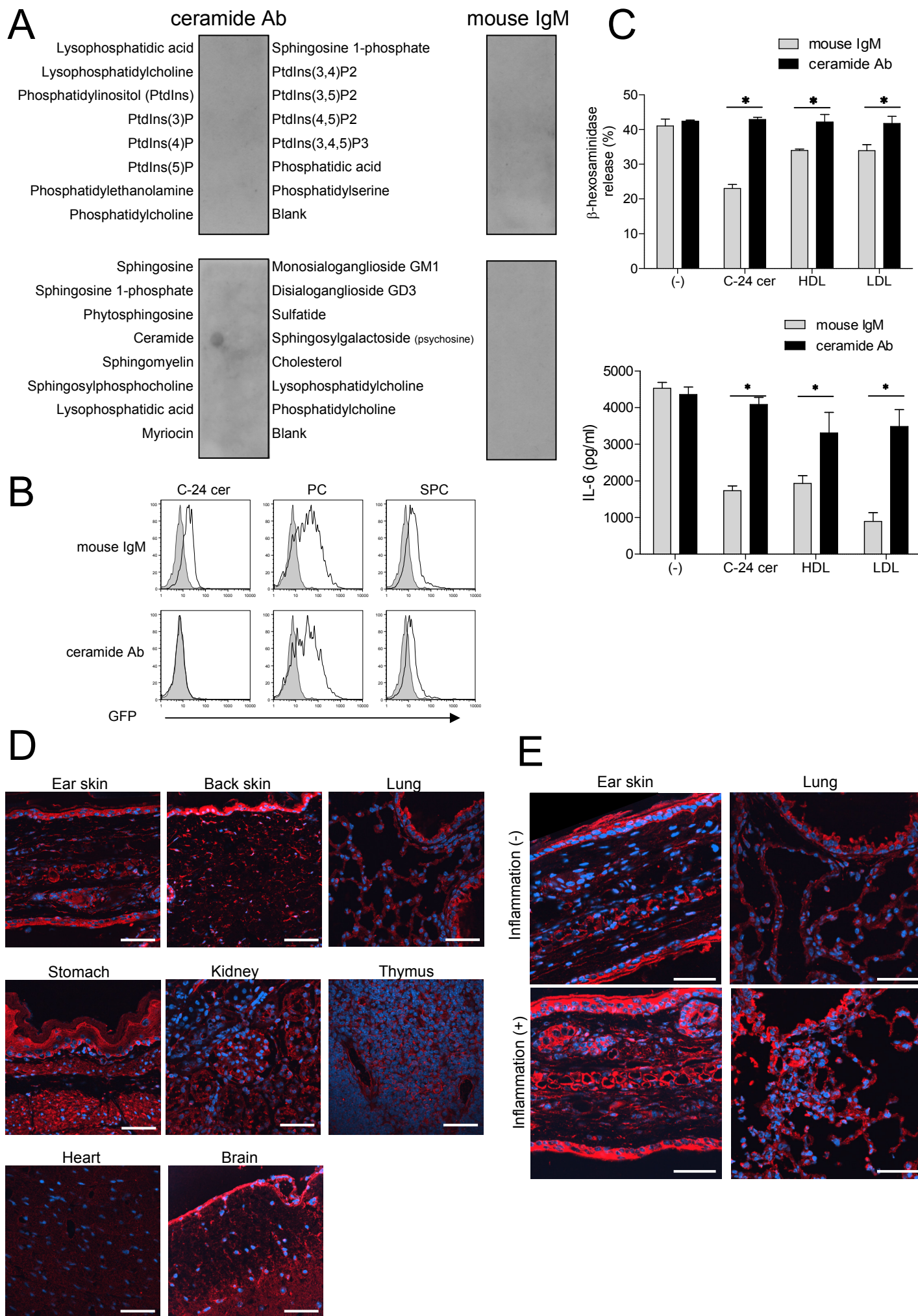


Figure S7. The sensitivity and specificity of ceramide antibody (related to Figure 7).

(A) Flow cytometry of GFP expression in 2B4-LMIR3-GFP cells that had been incubated for 24 h on plates coated with C-24 ceramide, PC, or SPC. Ten $\mu\text{g/ml}$ of either ceramide Ab (MID 15B4) or isotype control Ab was added to the culture just after the incubation of the reporter cells. (B) Ten $\mu\text{g/ml}$ of ceramide Ab (MID 15B4) or isotype control Ab was incubated with strips spotted with the indicated lipids, and immunostained with Ab against mouse Ig. Data are representative of four independent experiments. (C) Anti-TNP IgE-sensitized WT BMMCs were incubated on plates coated with C-24 ceramide, LDL, HDL, or vehicle for 30 min, and then 10 $\mu\text{g/ml}$ of either a soluble ceramide Ab (MID 15B4) or isotype control Ab was added to the culture medium. After 30 minutes, these cells were stimulated with 100 ng/ml TNP-BSA for 1 h (for release of β -hexosaminidase) or with 10 ng/ml TNP-BSA for 8 h (for production of IL-6). Data are representative of three independent experiments (Mean and SD) $*P < 0.01$ (Student's *t*-test). (D) The distribution of ceramide (red) in ear skin, back skin, lung, stomach, kidney, thymus, heart, or cerebrum. Frozen sections were stained with ceramide Ab (MID 15B4), followed by Alex Fluor 488-conjugated goat Ab against mouse IgM. The nuclei were counterstained with DAPI (blue). Scale bars, 50 μm . Data are representative of eight independent experiments. (E) The distribution of ceramide (red) in ear skin of WT mice after repeated application of dust mite extract for 18 days or in lung of WT mice at 24 h after the last challenge with OVA in an airway inflammation model. Frozen sections were stained with ceramide Ab (MID 15B4), followed by Alex Fluor 488-conjugated Ab against mouse IgM. The nuclei were counterstained with DAPI (blue). Scale bars, 50 μm . Data are representative of three independent experiments.

Supplemental Experimental Procedures

Mice

All the procedures were approved by an institutional review committee of the University of Tokyo (approval no 20-8). LMIR3-deficient (*Cd300lf^{-/-}*) mice (Acc. No. CDB0478K: <http://www.cdb.riken.jp/arg/mutant%20mice%20list.html>) were generated as described (<http://www.cdb.riken.jp/arg/Methods.html>); the details of the gene targeting strategy will be provided upon request. We used *Cd300lf^{-/-}* mice that had been backcrossed for eight generations with C57BL/6 mice (Charles River Laboratories Japan). *Kit^{w-sh/w-sh}* mice were provided from H. Suto (Juntendo University).

Antibodies and other reagents

Rat monoclonal antibody against LMIR3 (3-14-11; rat IgG_{2a}) was generated by immunization of rat with Y3Ag transfectants expressing mouse LMIR3 (ACTGen). Anti-DNP IgE (H1-ε-26) was purified as described (Kitaura et al., 2003). Other antibodies used were as follows: LMIR7 Ab (5F7D) was obtained from ACTGen, goat Ab against LMIR3 (AF2788), goat Ab against LMIR5 (AF2580) and LMIR1 Ab (MAB1186) were from R&D Systems (Izawa et al., 2009, Yamanishi et al., 2010, Enomoto et al., 2010), anti-DNP IgE (SPE-7), Flag Ab (M2), mouse IgG1 (MOPC21), α-Tubulin Ab (B-5-1-2) and goat IgG (I9140) were from Sigma-Aldrich, rabbit F(ab')₂ Ab against Rat IgG (6130-01) was from SouthernBiotech, goat Ab against mouse IgG (626500) was from Invitrogen, fluorescein isothiocyanate (FITC)-conjugated Abs

against CD11b (M1/70), F4/80 (BM8), B220 (RA3-6B2), CD8 (53-6.7), or FcεRIα (MAR-1) and phycoerythrin (PE)-conjugated Abs against Gr-1 (RB6-8C5), CD11c (N418), CD4 (GK1.5), CD11b (M1/70), CD3e (145-2C11), or c-kit (2B8), and rat IgG2a (eBR2a) were from eBioscience, ceramide Ab (MID 15B4) was from Enzo Life Sciences, phosphotyrosine Ab (4G10) was from Upstate Biotechnology, Ab against mouse IgM (MOPC-104E) was from BioLegend, anti-TNP IgE (C38-2) was from BD Biosciences, Abs against ERK1 (C-16), ERK2 (C-14), SH-PTP1 (SHP-1) (C-19), SH-PTP2 (SHP-2) (C-18), and SHIP (N-1) were from Santa Cruz Biotechnology, Ab against phospho-p44/42 MAPK (pERK1/2) was from Cell Signaling Technology. Cytokines were from R&D Systems. C-8 ceramide, sphingosine, sphingomyelin (SM), and sphingosylphosphorylcholine (SPC) were from BIOMOL; C-24 ceramide was from Toronto Research Chemicals, Inc.; Egg ceramide, lipid A, lysophosphatidylcholine (lysolecithin), and cholesterol were from Avanti Polar Lipids, Inc.; 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine (PS), and 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE) were from Echelon Biosciences Inc.; Glucosylceramide was from MATREYA LLC; α-Galactosylceramide (α-GalCer) was from Kyowa Hakko Kirin Co. LDL and HDL were from Biomedical technologies, Inc. Fibronectin, fibrinogen, and vitronectin were from Calbiochem. CULTREX Basement Membrane Extract (BME) was from TREVIGEN.

Binding assays using Strips

PIP StripsTM (Echelon Biosciences Inc.) or SphingoStripsTM (Molecular PROBES) were used as described (Yamanishi et al., 2010; Kobayashi et al., 2007). Briefly, Strips were incubated with 10 µg/ml of LMIR3-Fc or Fc in the presence of 0.5 mM CaCl₂ for 120 min before incubating with peroxidase-conjugated Ab against human Ig (Sigma). The binding was detected by enhanced chemiluminescence (ECL).

DNA constructs.

pMXs-IRES-puro^r (pMXs-IP) and pMXs-Flag-LMIR1, LMIR3, LMIR3(Y241/289/325F), LMIR4, LMIR5, or LMIR7-IP were previously described (Kitamura et al., 2003; Izawa et al., 2007; Yamanishi et al., 2008; Enomoto et al., 2010). An extracellular and transmembrane domain of Flag-LMIR3 fused to an intracellular domain of CD3ζ (N. Matsumoto, The University of Tokyo) was inserted into pMXs-IP to generate pMXs-Flag-LMIR3-CD3ζ-IP.

Generation of Fc fusion proteins

cDNA fragments corresponding to the extracellular domain of LMIR3 were inserted into the cloning sites of pME18S-hIgG1 Fc vector (a kind gift from H. Arase, Osaka University) (Shiratori et al., 2004; Izawa et al., 2009; Yamanishi et al., 2010). The Fc fusion proteins were purified as described (Yamanishi et al., 2010). Endotoxin levels of Fc fusion proteins were less than 0.01 ng/µg protein when measured using Limulus Amebocyte Lysate (LAL) (Lonza).

Generation of liposomes

Liposomes were generated by using Avanti Mini-Etruder (Avanti Polar Lipids, Inc) according to the manufacturer's instructions. Briefly, 1 mg of dry lipid mixtures (C-24 ceramide, PC, PS, or the indicated mixtures of PC/PS) was hydrated with 1 ml of PBS. The Extruder stand and heating block were placed on a hot plate. The plunger of the syringe filled with lipid samples was pushed until the lipid solution was completely transferred to the alternate syringe. Total 10 passes through the membrane having a pore size of 100 nm were performed to obtain homogeneous liposomes.

Cell stimulation

When mast cells were sensitized with IgE in the in vitro experiments (Figures 1C-1E, 6A-6E, 7A, 7B, S1M, S1N, and S6), BMMCs or BMMC transfectants were incubated with 0.5 $\mu\text{g/ml}$ anti-TNP IgE for 12 h. Co-engagement of Flag-tagged LMIR3 and Fc ϵ RI in *Cd300lf^{-/-}* BMMC transfectants on the plates was described as previously (Izawa et al., 2009). Briefly, 96-well plates were overnight incubated with F(ab')₂ Ab against mouse IgG (Zymed Laboratories), which recognized mouse IgE as well as mouse IgG1. Anti-TNP IgE-sensitized *Cd300lf^{-/-}* BMMC transfectants were pre-incubated with 20 $\mu\text{g/ml}$ of Flag Ab or mouse IgG1 for 1 h on ice and washed twice before stimulation with plate-coated F(ab')₂ Ab against mouse IgG for 8 h (Figure 1E). Co-engagement of LMIR3 and Fc ϵ RI in BMMCs on the plates was described as previously (Izawa et al., 2009). Briefly, 96-well plates were overnight incubated with

F(ab')₂ Ab against rat IgG (SouthernBiotec), which recognized mouse IgE as well as rat IgG2a. Anti-TNP IgE-sensitized BMMCs were pre-incubated with 20 µg/ml of LMIR3 Ab (3-14-11) or rat IgG2a for 1 h on ice and washed twice before stimulation with plate-coated F(ab')₂ Ab against rat IgG Ab for 8 h (Figure S1N). Alternatively, anti-TNP IgE-sensitized BMMCs or BMMC transfectants were stimulated with indicated concentrations of TNP-BSA for 1h (for β-hexosaminidase release) (Figures 1C, 6A, 6C, S6, and S7C), for 8 h (for cytokine production) (Figures 1D, 6B, 6D, and S7C), or for indicated periods (Figures 6E) on plates coated with lipids, lipoproteins, or not. In some experiments, liposomal suspensions were directly added to the culture just after the addition of 100 ng/ml TNP-BSA, and incubated for 1 h (for β-hexosaminidase release) (Figure 7A) or for 10 min (Figure 7B).

Measurement of cytokines and degranulation assay

Cytokines in BALF or in culture supernatants of BMMCs (R&D Systems) or histamine in serum (MBL) were measured by ELISA (Yamanishi et al., 2008; Izawa et al., 2009). Cytokines released by BMMCs that had been subjected to FcεRI engagement for 8 h in RPMI medium containing 10% FCS were measured, while β-hexosaminidase released by BMMCs that had been subjected to FcεRI engagement for 1 h in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose, 20 mM HEPES, and 1 mg/ml BSA at pH 7.4) was estimated as described (Tam et al., 2004).

Biochemistry

Western blotting was performed as described (Kumagai et al., 2003; Yamanishi et al., 2008). Equal amounts of cell lysates were immunoprecipitated with goat Ab against LMIR3 (AF2788), and were immunoblotted with phosphotyrosine Ab (4G10), SHP-1 Ab, SHP-2 Ab, SHIP Ab, or LMIR3 Ab (3-14-11). Equal amounts of total cell lysates were immunoblotted with phospho-ERK1/2 Ab or ERK1/2 Ab.

Immunohistochemistry

Immunofluorescence staining was performed as previously described (Yamanishi et al., 2010; Kato et al., 2011). For detection of ceramides in tissues, frozen sections of ear skin, back skin, lung, stomach, kidney, thymus, heart, or cerebrum were fixed with 4% paraformaldehyde, and then were permeabilized with 0.1% Nonidet P-40 (NP-40). After blocking with 5% BSA in PBS, sections were stained with ceramide Ab (MID 15B4), and then were stained with Alexa Fluor 488-conjugated goat aAb against mouse IgM (A21042) (Invitrogen) (Figures S7D and S7E). For detection of ceramide and mast cells in ear skin, sections were stained with ceramide Ab (MID 15B4) and Mast Cell Tryptase Ab (FL-275) (Santa Cruz Biotechnology), and then were stained with Alexa Fluor 488-conjugated goat Ab against mouse IgM and Alexa Fluor 546-conjugated goat Ab against rabbit IgG (H+L) (A11035) (Invitrogen), respectively (Figure 7D). For detection of LMIR3 and mast cells in ear skin, sections were stained with goat Ab against LMIR3 (AF2788) and Mast Cell Tryptase Ab (FL-275), and then were stained with Alexa Fluor 488-conjugated donkey Ab against goat IgG (H+L) (A11055) (Invitrogen) and Alexa Fluor 546-conjugated goat Ab against rabbit IgG (H+L), respectively (Figure S2B). For

detection of IgE-bound FcεRI and LMIR3 in BMMCs, BMMCs were fixed and permeabilized. After blocking, cells were stained with Ab against mouse IgE Ab (23G3) (eBioscience) and goat Ab against LMIR3 (AF2788), and were then stained with Alexa Flour 647 goat Ab against rat IgG (H+L) (A21247) (Invitrogen) and Alexa Flour 546 Donkey Ab against goat IgG (H+L) (A11056) (Invitrogen), respectively. All sections were counterstained with DAPI (Invitrogen). Fluorescent images were analyzed on a confocal microscope (FLUOVIEW FV300 scanning laser biological microscope JX70 system; Olympus) equipped with SenSys/0L cold charge-coupled device (CCD) camera (Olympus).

BM transplantation

Mouse BM transplantation was performed as described (Kato et al., 2011). Briefly, one day after lethal γ -irradiation, the recipient mice were intravenously injected with 3×10^6 WT or *Cd300lf^{-/-}* BM cells two months before experiments (Figures 2C, 2F, and S2B-S2D).

Supplemental references

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