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Research Article

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Hanbin Lee [1,2], Chigusa Nakahashi-Oda [1,3,*], Wenxin Lyu [1,2], Mamoru Tanaka [1,4], Akiyoshi Rai [1,5], Yoichi Muramoto [1,5], Yaqiu Wang [1,2], Seiya Mizuno [6], Kazuko Shibuya [1,3], and Akira Shibuya [1,3,7,*]

[1]Department of Immunology, Institute of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan
[2]Ph.D. Program in Human Biology, Graduate School of Comprehensive Human Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan
[3]R&D Center for Innovative Drug Discovery, University of Tsukuba, Tsukuba, Ibaraki, Japan
[4]Master's Program in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan
[5]School of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan
[6]Laboratory Animal Resource Center, Institute of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan
[7]Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), Tsukuba, Ibaraki, Japan

*Corresponding authors: Department of Immunology, Institute of Medicine, University of Tsukuba, 1-1-1, Tennohdai, Tsukuba, 305-8575 Japan. Email: chigusano@md.tsukuba.ac.jp (Chigusa Nakahashi-Oda) and Email: ashibuya@md.tsukuba.ac.jp (Akira Shibuya).

Abstract

Mast cells (MCs) play a central role in allergic immune responses. MC activation is regulated by several inhibitory immunoreceptors. The CD300 family members CD300a and CD300lf recognize phospholipid ligands and inhibit the FcεRI-mediated activating signal in MCs. While CD300a binds to phosphatidylserine (PS) to inhibit MCs activation, CD300lf function is less clear due to its ability to bind with ceramide and PS. Moreover, it also remains blurring whether CD300a and CD300lf function independently, cooperatively, or by interfering with each other in regulating MC activation. Using imaging and flow cytometric analyses of bone marrow-derived cultured MCs (BMMCs) from wild-type (WT), *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice, we show that CD300lf and CD300a colocalized with PS externalized to the outer leaflet of the plasma membrane with a polar formation upon activation, and CD300lf cooperates with CD300a to inhibit BMMCs activation. CD300lf also colocalized with extracellular ceramide in addition to the internal PS on the cell surface, which results in stronger inhibition of MC activation than CD300lf binding to PS alone. Similarly, although both *Cd300a*$^{-/-}$ and *Cd300lf*$^{-/-}$ mice showed decreased rectal temperatures compared with WT mice in the model of passive systemic anaphylaxis, *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice showed lower rectal temperature than either *Cd300a*$^{-/-}$ or *Cd300lf*$^{-/-}$ mice. Our results demonstrate the cooperativity of multiple inhibitory receptors expressed on MCs and their regulatory functions upon binding to respective ligands.

Keywords: CD300a, CD300lf, ceramide, mast cells, phosphatidylserine

Introduction

Mast cells (MCs) play a central role in allergic immune responses.[1] They express the high-affinity Fc receptor for immunoglobulin E (IgE) (FcεRI), which is a tetramer consisting of α, β, and γ subunit.[2] Upon binding to an IgE-allergen immune complex, FcεRI initiates cytoplasmic signaling through an immunoreceptor tyrosine-based activation motif (ITAM) in the FcεRIγ subunit, which is phosphorylated by the Src family protein tyrosine kinase Lyn, which in turn phosphorylates the Syk tyrosine kinase and activates downstream signaling pathways, Grb2, PLC-γ1, and SLP-76.[3] The FcεRI-mediated signaling pathway is strongly induced by multivalent antigens, causing degranulation and the release of mediators contained in the granules to elicit allergic symptoms.

The FcεRI-mediated signaling for MC activation is regulated by inhibitory immunoreceptors containing immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic portion. The phosphorylated tyrosine in the ITIM provides a docking site for Src homology 2 (SH2)-containing cytoplasmic phosphatases, including SHP-1, SHP-2, and SHIP, which dephosphorylate intracellular substrates in the proximal steps of activation signaling pathways,[4] resulting in the suppression of MC activation. Several inhibitory immunoreceptors on MCs have been identified, including CD200R,[5] Sialic acid-binding immunoglobulin-like lectin (Siglec)-6,[6] Siglec-8,[7] Allergin-1,[8] FcγRIIb,[9] CD300a,[10] CD300lf,[11] and others.

The CD300 family molecules are encoded by eight genes in humans and 12 genes in mice[12] and recognize phospholipids.[13] They are preferentially expressed on myeloid cells, such as granulocytes, monocytes, macrophages, dendritic cells, eosinophils and MCs,[12] and mediate either activating or inhibitory signals. CD300a and CD300lf transmit inhibitory signals via the ITIM in the cytoplasmic region among the family members and inhibit MC activation in vitro and in vivo.[14] The ligand for CD300a is phosphatidylserine (PS) exposed on the outer leaflet of plasma membrane of dead cells.[15] Moreover, we previously reported that CD300 also interacts with the cell-intrinsic PS exposed on the plasma membrane of activated, rather than dead, MCs in cis-form and inhibits MC activation in vitro and in vivo.[16] On the other hand, CD300lf is able to bind to PS and extracellular ceramide.[11,17] Although the interaction of

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CD300lf with extracellular ceramide inhibits MC activation,[11] whether CD300lf interaction with PS regulates MC activation remains undetermined. In addition, the functional relationship between CD300a and CD300lf in MC activation remains unknown.

Here, we generated mice deficient in either CD300a, CD300lf, or both. Using these mice and bone marrow-derived cultured MCs (BMMCs), we analyzed the role of the CD300a and CD300lf interactions with their respective ligands in MC activation *in vitro* and *in vivo*. We show that CD300lf, as well as CD300a, binds to PS in cis-form and cooperates with PS-bound CD300a to inhibit MC activation. Additional extracellular ceramide binding with CD300lf in trans-form results in stronger inhibition of MC activation. Our results demonstrate that CD300a and CD300lf cooperatively suppressed MC activation upon binding to their respective ligands, exemplifying a regulatory mode of multiple inhibitory receptors expressed on MCs that can be therapeutically leveraged.

Materials and methods

Mice

C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). *Cd300a*$^{-/-}$ mice on the C57BL/6J genetic background were generated, as described previously.[18] *Cd300lf*$^{-/-}$ and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice on the C57BL/6J genetic background were generated by deleting 991 base pairs of the Cd300lf gene from the WT and *Cd300a*$^{-/-}$ mice, respectively, using the CRISPR/Cas9 system. PCR genotyping and DNA sequencing were performed to confirm the deletion. Humanized mice were purchased from In-Vivo Science Inc. (Tokyo, Japan), in which these mice were generated from NOG mice expressing transgenic human GM-CSF and IL-3 (NOG-EXL) by transferring human cord blood CD34$^+$ cells. The chimerism of human cells in the peripheral blood was 39% to57%. Four- to 12-week-old female mice were used for each in vitro and in vivo experiment. All mice were housed and maintained under the specific-pathogen-free conditions, and all procedures were approved by the animal ethics committee of the University of Tsukuba (approval number 22-154) and performed at the laboratory animal resource centers of the University of Tsukuba in accordance with the guidelines of the institutional animal ethics committees.

Mast cells

Bone marrow-derived cultured mast cells (BMMCs) were generated from bone marrow cells collected from the femur and tibial bones by culture for 4 to 8 weeks in RPMI 1640 (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) containing 10% FBS (Biosera, Tokyo, Japan), 10 ng/ml stem cell factor (455-MC/CF, R&D systems, Minneapolis, USA)), and 4 ng/ml IL-3 (403-ML, R&D system), as previously described.[16] For establishment of *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs transfectants expressing DSRed-tagged CD300a or DSRed-tagged CD300lf (CD300a-DSRed BMMCs and CD300lf-DSRed BMMCs, respectively) and *Cd300lf*$^{-/-}$BMMCs transfectants expressing DSRed-tagged CD300lf (CD300lf-DSRed *Cd300lf*$^{-/-}$BMMCs), HEK293gp cells were transfected with *Cd300a*- or *Cd300lf*-inserted *DsRed* Monomer-N1 vector (Clontech, Tokyo, Japan), and BMMCs were transduced with the retroviruses, as described previously.[16]

Human MCs, which were identified on the gate of mouse CD45-, human CD45$^+$, and human C-kit$^+$ cells by flow cytometry (LSRFortessa, BD Biosciences, USA), were isolated from the peritoneal cavity of the humanized mice described above.

Antibodies and flow cytometry

BMMCs were incubated with CD16/32 Fc blocker (2.4G2, BD Biosciences) for 10 min in a staining medium (PBS supplemented with 2% fetal bovine serum (FBS) and 0.05% sodium azide (NaN_3)) and stained with PE-Cy7-conjugated anti-mouse C-kit (2B8, Biolegend, San Diego, California, USA), FITC-conjugated anti-mouse FcεRIα (MAR-1, Biolegend) mAb, and propidium iodide (PI) (Sigma-Aldrich, StLouis, MO, USA) for 30 min at 4°C. MCs were identified on the gate of PI$^-$ C-kit$^+$ FcεRIα$^+$ cells. For the analysis of human MCs, cells collected from the peritoneal cavity of the humanized mice were stained with FITC-conjugated anti-mouse CD45 (30-F11, Biolegend), BV510-conjugated anti-human CD45 (HL30, Biolegend), PE-Cy7-conjugated anti-human C-kit (104D2, BD biosciences) and APC-conjugated anti-human CD107a (H4A3, Biolegend) mAbs, and human MCs were identified on the gate of mouse CD45$^-$, human CD45$^+$, human C-kit$^+$, and human CD107a$^+$ cells. For the analysis of CD300a, CD300lf, CD300A, and CD300LF expressions, anti-mouse CD300a mAb (EX42 generated in our laboratory), anti-mouse CD300lf mAb (TX73 generated in our laboratory), anti-human CD300A mAb (TX113), and anti-human CD300f/LMIR3 mAb (R&D systems) were used, respectively.

Passive systemic anaphylaxis

WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice were sensitized by i.v. injection of 10 ug of mouse anti-trinitrophenol (TNP) IgE (BD biosciences). One day later, these mice were i.v. challenged with 20ug of TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA, and rectal temperatures were monitored before and every 10 min after the challenge using a digital thermometer.

Degranulation assay and pSyk analysis

WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$ and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs were seeded at 2×10^6 cells/mL and sensitized overnight with mouse anti-TNP IgE at a final concentration of 0.5 ug/ml in the presence or absence of biotinylated ceramide liposomes for 24 h, washed twice with Tyrode's buffer, and plated in Tyrode's buffer at 2×10^5 cells/well in 96 well round culture plates (Costar®). BMMCs were then stimulated with TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA (Sigma-Aldrich) (final concentration of 1 ng/ml) for 30 min at 37°C, then stained with APC-conjugated anti-mouse CD107a mAb (1D4B, Biolegend) for 30 min at 4°C, and analyzed for CD107a expression by flow cytometry. For p-Syk analysis, BMMCs after stimulation with either multivalent TNP-OVA were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton X-100 for 5 min, and incubated with 5% BSA-PBST for 60 min. These transfectants were then stained with rabbit anti-phospholyrated Syk (Tyr525/526) polyclonal antibody (dilution at 1:1000) (#2711s, Cell signaling, Danvers, Massachusetts, USA) overnight at 4°C, washed with PBS three times, and stained with anti-rabbit IgG Alexa 647, FITC-conjugated streptavidin, and 4',6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories,

Burlingame, California, USA) for 2 h at RT, and analyzed by laser scanning confocal microscopy using the FV-ASW software.

For human MCs degranulation, cells were seeded at 3 × 10^6/mL and sensitized with 0.5 ug/ml of human IgE (Sigma-Aldrich) for 1 h, washed with Tyrode's buffer, and plated at 1 × 10^5 cells/well in a 96-well plate. Human MCs were then stimulated with 300 ng/mL of anti-human IgE (E124.2.8, Beckman coulter, Brea, CA, USA) for 30 min at 37°C, washed twice with cold Tyrode's buffer to stop the reaction, and analyzed for CD107a expression by flow cytometry.

Imaging analysis

For the analysis of the interaction between CD300a or CD300lf and PS on MCs, CD300a-DSRed BMMCs and CD300lf-DSRed BMMCs were plated in the FD10300 integrated film bottom dish (Matsunami, Tokyo, Japan). These transfectants were sensitized with anti-TNP IgE at a final concentration of 0.5 ug/mL, washed twice with Tyrode's buffer, and stimulated with 10 ng/mL of TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA in the presence of biotinylated ceramide-liposomes for 20 min, stained with PSVue480 (Molecular Targeting Technologies, West Chester, Pennsylvania, USA) and APC-conjugated streptavidin. To stain intracellular ceramide, transfectants were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.3% triton X-100 for 5 min, and incubated with 5% BSA-PBST for 60 min. After, cells were stained with biotin-conjugated anti-Ceramide mAb (MID15B4, Enzo Life Sciences, Farmingdale, New York, USA), followed by APC-conjugated streptavidin, and analyzed by a laser scanning confocal microscopy (FV10i, Olympus, Tokyo, Japan) using the FV-ASW software (Olympus). Timelapse analysis was performed to observe the dynamic interaction between CD300lf and PS. CD300lf-DsRed BMMCs were sensitized with an anti-TNP IgE mAb and stained with PSVue 480. Immediately after adding PSVue 480, timelapse imaging was started by laser scanning confocal microscopy. Images were captured every 30 sec for a total duration of approximately 25 min. To simultaneously analyze the CD300a and CD300lf interaction with PS, CD300lf-DsRed *Cd300lf*$^{-/-}$ BMMCs were stained with biotinylated anti-CD300a (TX73), sensitized with anti-TNP IgE antibody at a final concentration of 0.5 ug/ml, washed twice with Tyrode's buffer, and stimulated with 10 ng/mL of TNP_6-OVA for 20 min, stained with PSVue 480 and APC-conjugated streptavidin, and analyzed by laser scanning confocal microscopy.

Affinity assay

For generation of biotinylated PS-liposomes and ceramide-liposomes, phosphatidylcholine (PC) (Sigma-Aldrich, St Louis, Missouri, USA), cholesterol (Sigma-Aldrich), either PS (Sigma-Aldrich) or N-Palmitoyl-D-erythro-Sphingosine (C16 Ceramide), and distearoylphosphatidylethanolamine (DSPE)-polyethylene glycol (PEG) (2000) (Avanti Polar Lipids, Alabaster, AL, USA) were mixtured at a molar ratio of 60: 30: 5: 5, respectively[22] and added into a round-bottomed glass tube (Pyrex, Corning, New York, USA). Each mixture was dried under a vacuum until a lipid film was visible. To rehydrate the lipid film, 1 ml of TBS was added, and the mixture was incubated at 37°C for 30 minutes. After incubation, the mixture was repeatedly heated to 70°C until the lipids were fully dissolved and a white liposome mixture was formed. To analyze the binding affinity of CD300a and CD300lf with PS and ceramide, biotinylated PEG-containing PS and ceramide liposomes were coated on streptavidin biosensors (Sartorius, Geottingen, Germany). After coating, CD300a-Fc or CD300lf-Fc (50, 25 or 10 ug/mL) was measured with the coated-biotinylated PS or ceramide liposomes. CD300a-Fc or CD300lf-Fc was generated by using HEK293F cells (Gibco, Grand Island, New York, USA) with plasmid. All samples were diluted with TBS buffer. Data analysis of all samples was performed with BLItz Pro 1.2 software. Data were analyzed using global fitting (1:1) for curve fitting and display of results.

Statistical analysis

The unpaired Student's *t*-test and 1-way or 2-way ANOVA were used to compare the data by using GraphPad Prism 9 software (GraphPad Software, San Diego, California, USA). $P < 0.05$ was considered statistically significant.

Results

Generation of mice deficient in CD300a and/or CD300lf

To clarify how CD300a and CD300lf regulate MC activation, we generated *Cd300lf*$^{-/-}$ mice using the CRISPR/Cas9 system (Fig. 1A). Because *Cd300a* and *Cd300lf* genes are mapped at the close positions in the same chromosome (11E2), mating *Cd300a*$^{-/-}$ mice with *Cd300lf*$^{-/-}$ mice is ineffective to generate *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice. Therefore, we targeted *Cd300lf*$^{-/-}$ under *Cd300a*$^{-/-}$ background[18] and successfully generated *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice (Fig. 1A). BMMCs from wild-type (WT), *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice were cultured and their respective deficiency for CD300a and CD300lf were confirmed (Fig. 1B).

CD300lf colocalized with the exposed PS and extracellular ceramide on the cell surface of activated MCs

The ligand for CD300a is PS,[10,16] while those of CD300lf are both PS and ceramide.[11,17] To determine the binding affinity of CD300a and CD300lf with their ligands, we generated the chimeric proteins consisting of the extracellular portions of CD300a or CD300lf fused with the Fc portion of human IgG (CD300a-Fc and CD300lf-Fc, respectively) and the liposomes conjugated with PS (PS-liposome) or ceramide (ceramide-liposome). The affinity of PS-liposome to CD300a-Fc was greater than that to CD300lf-Fc, as determined by the biolayer interferometry (Fig. 2A). In contrast, while CD300lf bound to ceramide-liposome with a lower affinity than to PS-liposome, no interaction was observed between CD300a-Fc and ceramide-liposome (Fig. 2B).

To investigate how the inhibitory functions of CD300a and CD300lf are regulated in MC activation, we analyzed the interaction between these receptors and PS and ceramide before and after MC activation by laser scanning confocal microscopy. For this purpose, we established the transfectants of *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs expressing CD300a or CD300lf tagged with DS-Red fluorescent protein (CD300a-DSRed BMMCs and CD300lf-DSRed BMMCs, respectively) (Supplemental Fig. 1A). These transfectants were sensitized with an anti-TNP IgE and challenged with either bivalent, quadrivalent, or hexavalent TNP antigens (TNP_2-OVA,

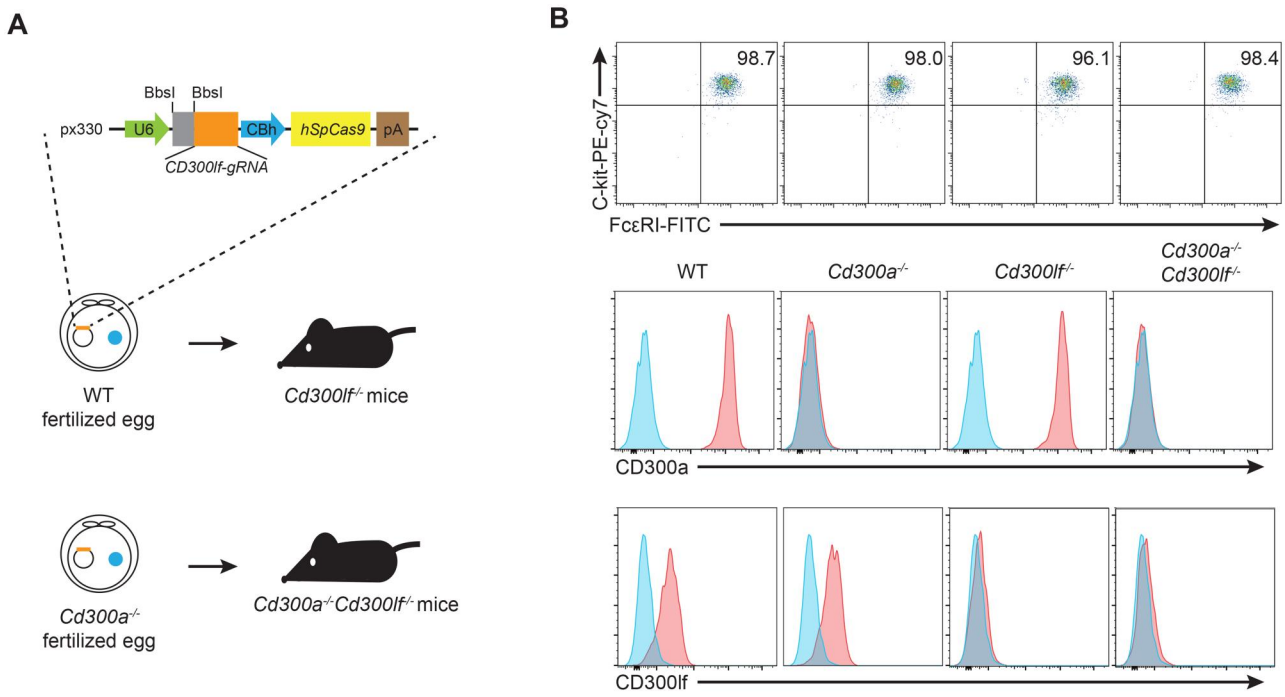

Figure 1. Generation of *Cd300a*$^{-/-}$ and/or *Cd300lf*$^{-/-}$ mice. (A) Generation of *Cd300lf*$^{-/-}$ and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice using the CRISPR/Cas9 system. The *Cd300lf-gRNA* gene was injected into fertilized eggs of WT and *Cd300a*$^{-/-}$ mice to produce *Cd300lf*$^{-/-}$ and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice, respectively. (B) Flow cytometric analysis of the expression of FcεRI and c-kit on WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs.

TNP_4-OVA, and TNP_6-OVA, respectively) in the presence of biotinylated ceramide-liposomes. CD300a-DSRed and CD300lf-DSRed BMMCs were then stained with PSVue-480, a probe specifically binds to PS, together with APC-conjugated streptavidin for detecting ceramide, and analyzed for the interaction of CD300a and CD300lf with PS and ceramide by laser scanning confocal microscopy. While both phospholipids were not detected by staining with PSVue-480 and incubation with ceramide-liposomes on the cell surface of these transfectants before the antigen challenge, PS appeared in a polar formation on the cell surface and colocalized with CD300a after the challenge of any multivalent of the TNP-OVA antigens (Fig. 2C, 2D), consistent with our previous observation that CD300a binds to PS exposed on MCs in cis-form after stimulation with an IgE immune complex.[16] In contrast, ceramide was not detected on CD300a-DSRed BMMCs even after antigen stimulation (Fig. 2C). However, we found that ceramide, as well as PS, colocalized with CD300lf on the cell surface of CD300lf-DSRed BMMCs after the challenge of any multivalent of the TNP-OVA antigens (Fig. 2D, E, Video 1, Video 2, Video 3). By contrast, although cell intrinsic ceramide was detected at the plasma membrane of CD300lf-DSRed BMMCs by staining with anti-ceramide mAb, we did not observe the colocalization of CD300lf with ceramide on these BMMCs in the absence of ceramide-liposome (Fig. S1B). These results suggest that CD300lf binds to extracellular, but not cell intrinsic, ceramide in trans-form. Region of interest analysis demonstrated that CD300a and CD300lf simultaneously colocalized with PS on the cell surface of *Cd300lf*$^{-/-}$ BMMCs expressing CD300lf-DSRed after stimulation with the TNP_6-OVA antigens (Fig. 2F). Together, these results suggest that, while CD300a binds to the PS in cis-form, CD300lf not only binds to the PS in cis-form but also to ceramide in trans-form on the cell surface of activating MCs.

CD300a and CD300lf cooperate to suppress IgE-mediated MC activation

We next examined how the interaction of CD300a and CD300lf with their ligands regulate MCs activation. WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs were sensitized with an anti-TNP IgE, followed by challenging with either TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA in the presence of either ceramide-liposome or PC-liposome, as a negative control, and analyzed for degranulation by CD107a externalization by flow cytometry. CD107a expression was not detected on any of the genotypes of BMMCs without any stimulations, but it was upregulated after stimulation with antigens on all those genotypes of BMMCs (Fig. 3A, Fig. S2A). Although the CD107a expression was comparable between *Cd300a*$^{-/-}$ and *Cd300lf*$^{-/-}$ BMMCs after stimulation with any multivalent TNP-OVA antigens in the presence of PC-liposome, both *Cd300a*$^{-/-}$ and *Cd300lf*$^{-/-}$ BMMCs showed a larger proportion of CD107a^{+} cells than did WT BMMCs. In addition, *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs showed higher percentage of CD107a^{+} cells than either *Cd300lf*$^{-/-}$ or *Cd300lf*$^{-/-}$ BMMCs when these BMMCs were challenged with TNP_2-OVA or TNP_4-OVA. However, an additive effect of CD300a and CD300lf deficiencies on CD107a expression was not observed in BMMCs after stimulation with TNP_6-OVA. Together, these results suggest that CD300a and CD300lf additively contribute to the suppression of MC degranulation via cis-interaction with PS after stimulation with lower antigen stimulation.

To explore the role of CD300lf-ceramide binding in regulating MC activation, BMMC were pretreated with exogenous ceramide-liposome. The addition of ceramide-liposome decreased the CD107a expression of WT and *Cd300a*$^{-/-}$, but not *Cd300lf*$^{-/-}$ or *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$, BMMCs compared with the addition of PC-liposome after stimulation with TNP_2-OVA or TNP_4-OVA (Fig. 3A, Fig. S2B). Moreover,

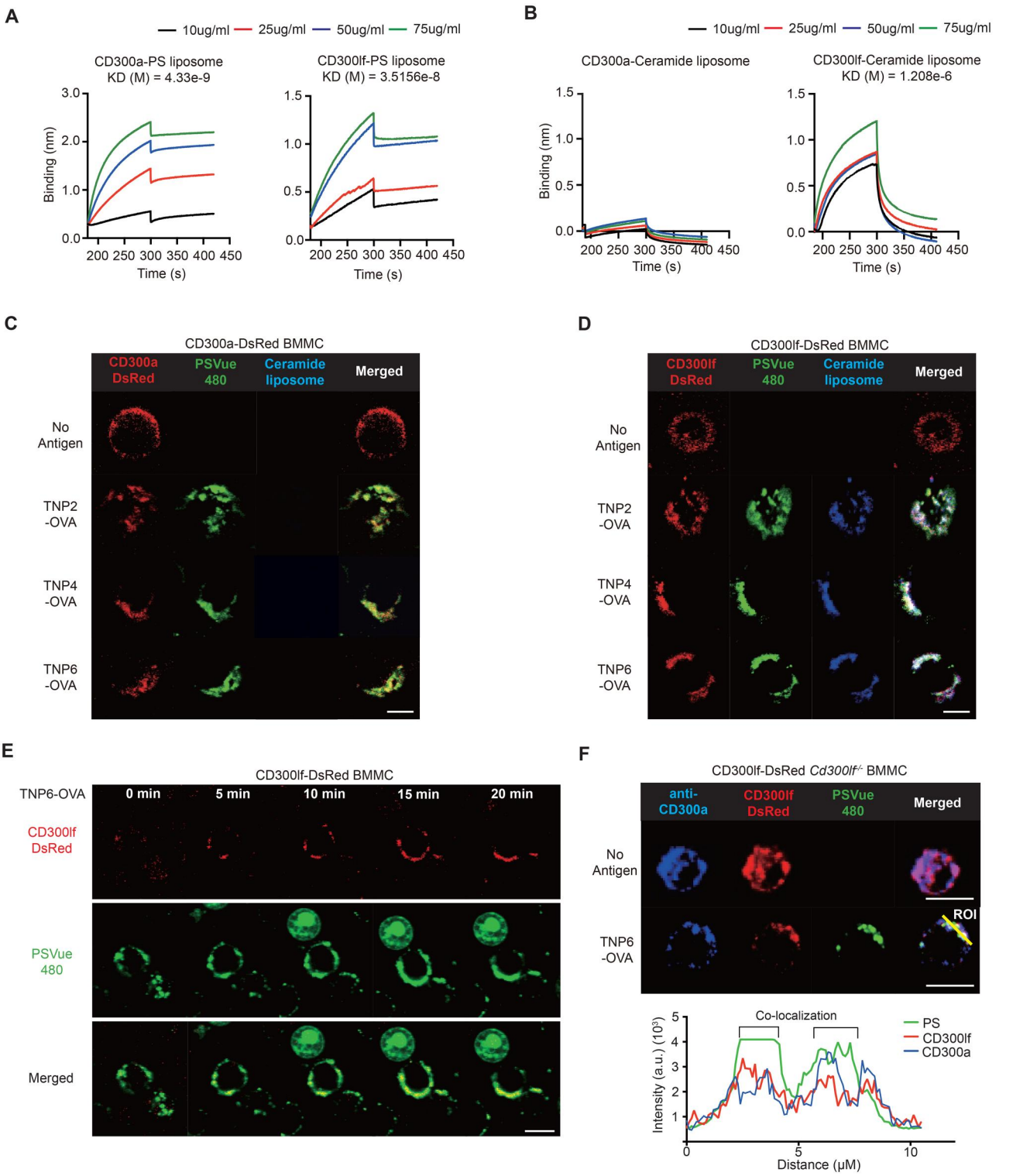

Figure 2. Interaction of CD300a and CD300lf with PS and ceramide. (A and B) Bio-layer interferometry analysis for the binding affinities of CD300a-Fc and CD300lf-Fc to PS-liposome (A) and ceramide-liposome (B). (C–E) Laser scanning confocal microscopy analysis of the colocalization of CD300a and CD300lf with PS and ceramide in *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs transfectants expressing CD300a or CD300lf tagged with DSRed (CD300a-DSRed BMMCs and CD300lf-DSRed BMMCs, respectively). These transfectants were sensitized with an anti-TNP IgE, challenged with either TNP_2-OVA, TNP_4-OVA, and TNP_6-OVA in the presence of biotinylated ceramide-liposomes, and then stained with PSVue-480 together with APC-conjugated streptavidin. Time-lapse imaging of CD300lf and PS accumulation. (F) ROI analysis of intensity of CD300a and CD300lf with PS. Scale bars indicate 10 μm (C–F).

Cd300lf$^{-/-}$ BMMCs had more CD107a^{+} cells than did *Cd300a*$^{-/-}$ BMMCs in the presence of ceramide-liposome after the challenge with any multivalent TNP-OVA antigens (Fig. 3A, Fig. S2B). These results suggest that CD300lf binds to both ceramide and PS and thus has a stronger suppressive function than CD300a in the presence of exogenous ceramide.

To investigate the mechanisms underline CD300a- and CD300lf-mediated inhibition, we examined spleen tyrosine kinase (Syk) phosphorylation, a proximal downstream

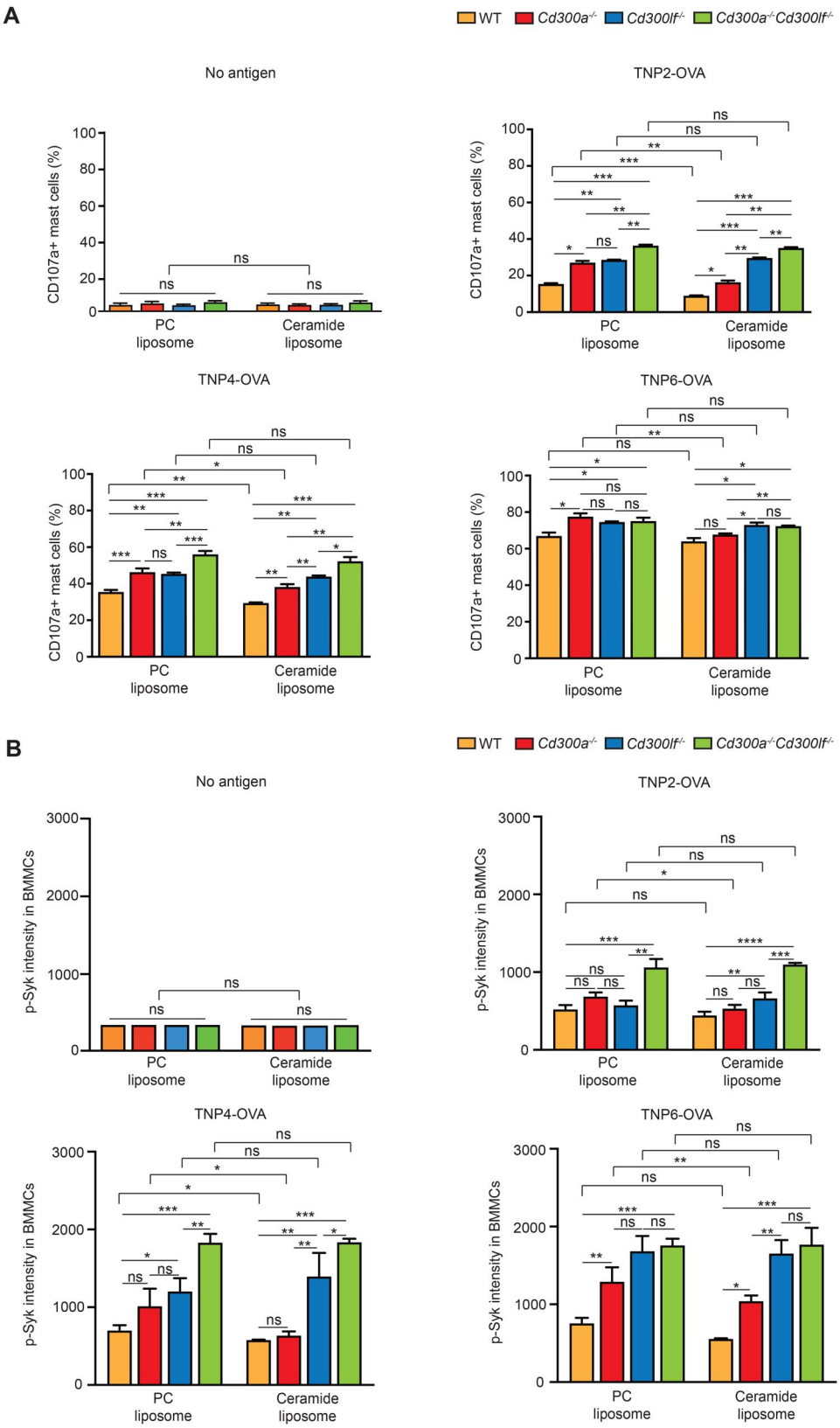

Figure 3. The role of CD300a and CD300lf in activation of BMMCs. WT (n = 3), *Cd300a*$^{-/-}$ (n = 3), *Cd300lf*$^{-/-}$ (n = 3), and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ (n = 3) BMMCs were sensitized with an anti-TNP IgE in the presence of PC-liposome or ceramide-liposome, followed by challenge with TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA, and analyzed for CD107a expression by flow cytometry (A) or for intensity of phosphorylated Syk (p-Syk) by laser scanning confocal microscopy (B). Data are representative of three independent experiments and presented as means ± SEM. Statistical analyses were performed by two-way ANOVA followed by Dunnett's multiple comparisons test. ns, not significant, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

signaling event of FcεRI.[19] WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs were sensitized with an anti-TNP IgE and challenged with TNP_2-OVA or TNP_4-OVA, or TNP_6-OVA in the presence of either PC-liposome or ceramide-liposome, and analyzed pSyk using an anti-phosphorylated Syk (pSyk) mAb by laser scanning confocal microscopy. While pSyk signaling was not detected in non-activated WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs, *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ BMMCs showed a greater signal of pSyk than either *Cd300a*$^{-/-}$ or *Cd300lf*$^{-/-}$ BMMCs in the presence of PC-liposome after stimulation with the IgE immune complex (Fig. 3B, Fig. S2C). Moreover, the addition of ceramide-liposome decreased pSyk in *Cd300a*$^{-/-}$, but neither *Cd300lf*$^{-/-}$ nor *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$, BMMCs compared with that of PC-liposome. These results were consistent with those in the degranulation assay.

CD300a and CD300lf cooperate to ameliorate IgE-mediated anaphylaxis

To examine whether CD300a and CD300lf cooperate to suppress allergic responses *in vivo*, we used a passive systemic anaphylaxis (PSA) model. WT, *Cd300a*$^{-/-}$, *Cd300lf*$^{-/-}$, and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice were i.v. injected with an anti-TNP IgE and then i.v. challenged with TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA. The rectal temperature of *Cd300a*$^{-/-}$ and *Cd300lf*$^{-/-}$ mice were lower than that of WT mice after challenge with TNP_2-OVA or TNP_4-OVA, consistent with previous reports.[11,16] However, *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ mice showed further lower rectal temperature than did *Cd300a*$^{-/-}$ and *Cd300lf*$^{-/-}$ mice after challenge with TNP_2-OVA or TNP_4-OVA (Fig. 4), indicating that CD300a and CD300lf cooperate to suppress the PSA. However, *Cd300lf*$^{-/-}$ mice showed lower rectal temperature than did *Cd300a*$^{-/-}$ mice after challenge with TNP_4-OVA or TNP_6-OVA (Fig. 4), in agreement with the results of in vitro analyses of CD107a expression and pSyk of *Cd300a*$^{-/-}$ and *Cd300lf*$^{-/-}$ mice after the challenge with any multivalent TNP-OVA antigens in the presence of ceramide-liposome (Fig. 3A, B). Taken all together, these results suggest that although CD300a and CD300lf additively contribute to the suppression of PSA induced by a lower antigen stimulation, CD300lf, rather than CD300a, plays a major role in regulating PSA induced by a stronger antigen stimulation.

CD300A and CD300LF interact with PS and suppresses CD107A expression on human mast cells

To elucidate whether CD300A and CD300LF also have inhibitory functions in human MC (HuMCs) degranulation, we isolated HuMCs from the peritoneal cavity of humanized mice, which were generated by transplantation of human $CD34^+$ hematopoietic stem cells into NOD SCID mice deficient in the common γ chain of the interleukin (IL)-2 receptor (NOG mice) with transgenic expression of human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (named NOG-EXL mice). We confirmed the expression of CD300A and CD300LF on the population expressing human CD45 and human c-Kit, which contains human MCs (Fig. S3). To analyze the interaction of CD300A and CD300LF with PS in activated MCs, HuMCs were sensitized with a human IgE and then stimulated with an anti-IgE antibody, then stained with PSVue together with either anti-CD300A mAb or anti-CD300LF mAb, and analyzed for the localization of CD300A and CD300LF with PS by laser scanning confocal microscopy. In agreement with our previous observation,[16] CD300A colocalized with PS on HuMCs after FcεRI stimulation (Fig. 5A). We also found that CD300LF colocalized with PS on activated HuMCs (Fig. 5A), suggesting that CD300LF as well as CD300A bind to PS in cis-form on the cell surface of activated HuMC, as observed in mouse MCs.

We next investigated the role of CD300A and CD300LF in the regulation of HuMCs activation by flow cytometry. HuMCs were stimulated with an anti-FcεRI mAb in the presence of a neutralizing anti-CD300A mAb, anti-CD300LF mAb, both of which are able to interfere with PS binding,[20,21] respectively. Treatment with neutralizing anti-CD300A and anti-CD300LF mAbs upregulated the CD107a expression on HuMCs compared to control antibody treatment upon stimulation with anti-FcεRI mAb (Fig. 5B). However, the proportion of CD107a-expressing HuMCs in the presence of anti-CD300LF mAb was larger than that in the presence of anti-CD300A mAb but comparable to that in the presence of both anti-CD300A and anti-CD300LF mAbs (Fig. 5B), suggesting a stronger inhibitory effect of CD300LF-PS interaction than that of CD300a in the regulation of HuMCs degranulation.

Discussion

The CD300 family members CD300a and CD300lf can both bind to PS and CD300lf can also interact with ceramide, and inhibit MC activation *in vitro*[16,17,21] and suppress allergic responses such as PSA *in vivo*.[16,21] In the present study, we demonstrated that CD300lf, binds to PS externalized on the cell surface in cis-form and cooperates with PS-bound CD300a to inhibit MC activation induced by antigens with lower valence. In addition, CD300lf simultaneously binds to extracellular ceramide in trans-form, which results in stronger inhibition of MC activation than CD300lf binding to PS alone. However, the additive inhibitory effect of CD300a on MC activation to CD300lf was not observed when MCs were stimulated with TNP_6-OVA antigen. These results suggest that CD300lf, rather than CD300a, plays a major role in regulating MC activation induced by a stronger antigen stimulation via the interaction with both PS and ceramide, and CD300a may fine-tune MC activation in collaboration with CD300lf. These results of *in vitro* analysis were indeed in agreement with those of the in vivo study of PSA.

We showed that CD300a and CD300lf colocalized with PS in cis-form on the cell surface of activated, but not steady state of, MCs, consistent with our previous report.[16] We confirmed the direct binding of CD300a with PS on the cell surface by the analysis of fluorescence resonance energy transfer.[16] Although we did not perform such studies of CD300lf with PS in the current study, it is likely that CD300lf is also the case as in CD300a, because CD300lf colocalized with CD300a and PS, and its deficiency enhanced MC activation in the absence of ceramide-liposome. We also demonstrated that CD300A and CD300LF colocalized with PS on the cell surface of HuMCs, and both neutralizing mAbs against CD300A and CD300LF upregulated degranulation from HuMCs, suggesting that the interaction of CD300A and CD300LF with PS inhibits HuMCs activation. However,

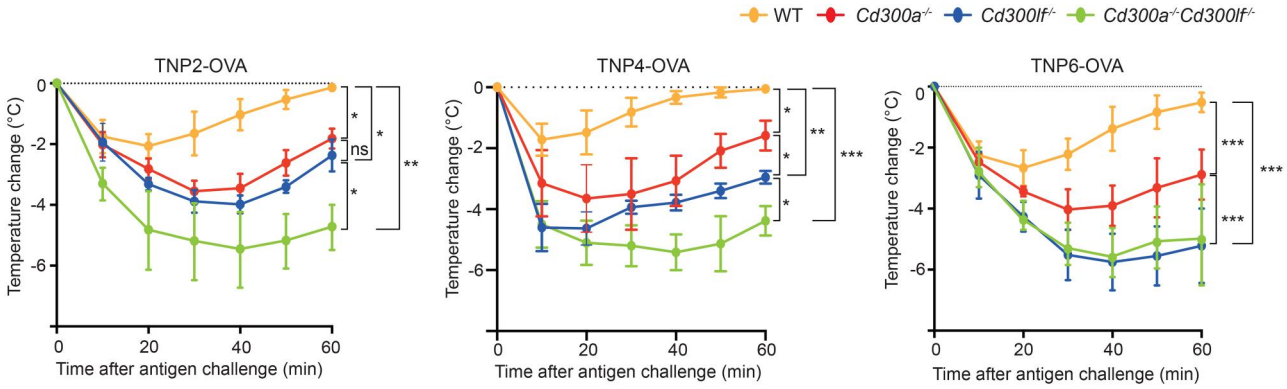

Figure 4. The role of CD300a and CD300lf in passive systemic anaphylaxis. WT(n = 6), *Cd300a*$^{-/-}$ (n = 6), *Cd300lf*$^{-/-}$ (n = 6), and *Cd300a*$^{-/-}$*Cd300lf*$^{-/-}$ (n = 6) mice were i.v. injected with a TNP-specific IgE, followed by i.v. challenge with TNP_2-OVA, TNP_4-OVA, or TNP_6-OVA, and rectal temperature was measured. Data are pooled from three independent experiments and presented as means ± SEM. Statistical analyses were performed by two-way ANOVA, followed by Tukey's multiple comparisons test. ns, not significant, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

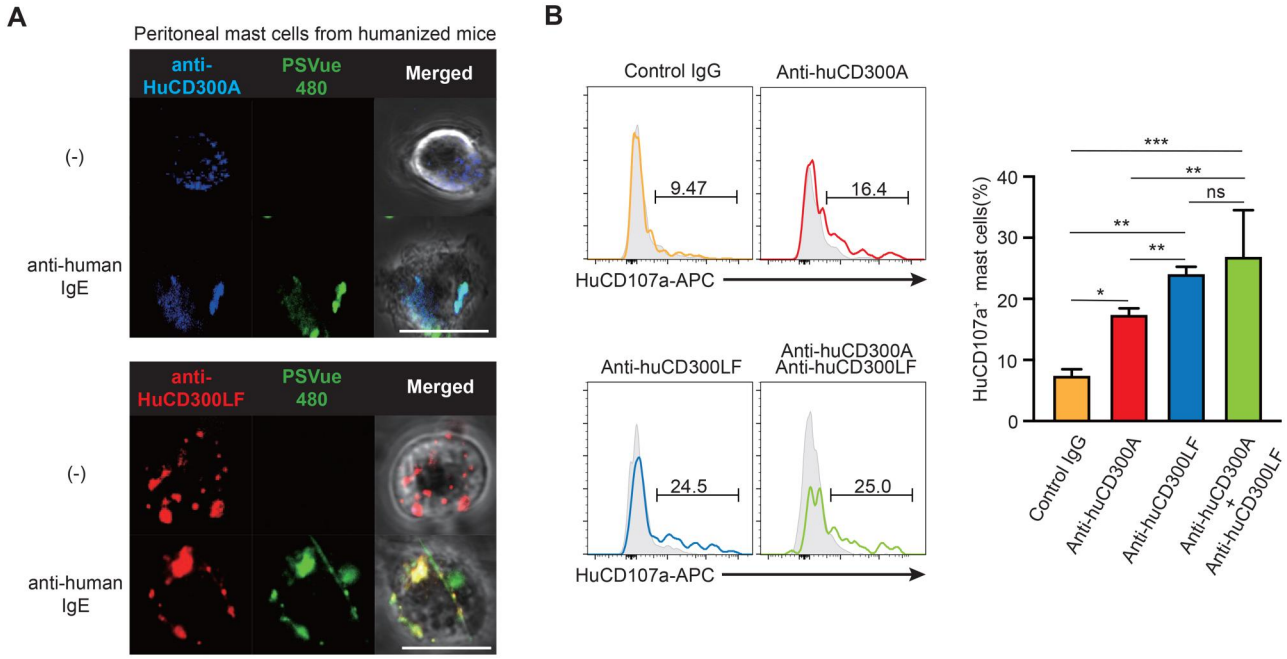

Figure 5. Colocalization of CD300A and CD300LF with PS on human mast cells. (A) Human MCs from the peritoneal cavity of humanized mice were sensitized with human IgE, stimulated with an anti-IgE mAb, then stained with PSVue together with either anti-CD300A mAb or anti-CD300LF mAb, and analyzed by laser scanning confocal microscopy. The images were shown before and 20 min after stimulation with anti-human IgE. Scale bars indicate 10 μm. (B) Human MCs were sensitized with human IgE, treated with a neutralizing anti-CD300A and/or anti-CD300LF mAb, then stimulated with an anti-human IgE mAb, and stained with anti-CD107A mAb or control mAb. Data are representative of 2 independent experiments and presented as means ± SEM. Statistical analyses were performed by two-way ANOVA, followed by Dunnett's multiple comparisons test. ns, not significant, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

we did not have a chance to examine the role of interaction between CD300LF and ceramide. This is a remaining issue to be clarified in the future.

Previous reports demonstrated that the PS exposure on the plasma membrane of MCs is reversible and associated with the exocytosis of MC granules from the cytoplasm,[23] in which the granules fuse with the plasma membrane of MCs. It is unclear at present whether the exposed PS is derived from either the plasma membrane or MC granules membrane after the fusion of these membranes upon degranulation.[24] In the study of chromaffin granules, PS expression is limited on the outer leaflet (cytoplasmic monolayer) of the granules,[25] suggesting that even if this is also the case of MC granules, the granule-derived PS cannot be externalized onto the outer leaflet of the plasma membrane upon the fusion of secreting MC granules and plasma membranes. Therefore, it is supposed that the PS may be externalized from the internal plasma membrane likely due to the redistribution of membrane lipids forced by extensive fusion events taking place in stimulated mast cells. Nonetheless, CD300a and CD300lf encounter the externalized PS, leading to suppression of MCs activity to restore homeostasis. By contrast, the current study showed that, unlike PS, CD300lf was colocalized with extracellular, but not intracellular, ceramide. Previous studies have

indicated that CD300lf interacts with extracellular ceramide from lipoprotein during IgE-mediated allergic responses in mice.[21] Although sphingosine1-phosphate and ceramide 1-phosphate are activated in MCs to enhance IgE-mediated degranulation,[26] we showed that the intracellular ceramide was not associated with CD300lf probably due to the lack of the ceramide distribution on the outer leaflet of the plasma membrane.

MCs also express several inhibitory immunoreceptors in addition to CD300a and CD300lf, including CD200R,[5] Siglec-6,[6] Siglec-8,[7] Allergin-1,[8] FcγRIIb,[9] and others. These inhibitory immunoreceptors contain the ITIM in the cytoplasmic portion, which is phosphorylated upon binding to respective extracellular ligands. Thus, the regulation of MC activation by these inhibitory immunoreceptors depends on the chance of encountering each ligand in different spatiotemporal conditions. In this respect, CD300a and CD300lf are unique, because both receptors bind to cell intrinsic PS that is always externalized on the cell surface upon MC degranulation, suggesting that CD300a and CD300lf play a fundamental role in regulating MC activation. Understating the functional cooperativity between CD300a and CD300lf as well as the effect of their respective ligands recognition demonstrated the significance of multiple inhibitory receptors expressed on MCs and informed new MCs-centric strategy for allergy treatment.

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Supplementary material

Supplementary material is available at *The Journal of Immunology* online.

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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