Autonomous regulation of immunoglobulin E-mediated mast cell degranulation and immediate hypersensitivity reaction by an inhibitory receptor CD300a

Yaqiu Wang, *BSc*[a, b, c] Chigusa Nakahashi-Oda, *MD, PhD*[b,*]
Yoshimichi Okayama, *MD, PhD*[d], and Akira Shibuya, *MD, PhD*[b, c,*]

[a]Ph.D. Program in Human Biology, School of Integrative and Global Majors, and [b]Department of Immunology, Faculty of Medicine, and [c]Life Science Center of Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.
[d]Allergy and Immunology Project Team, Center for Institutional Research and Medical Education, Nihon University School of Medicine, 30-1 Oyaguchi Kamicho, Itabashi-Ku, Tokyo 173-8610, Japan

[*]Address correspondence and reprint requests to Akira Shibuya (ashibuya@md.tsukuba.ac.jp) or Chigusa Nakahashi-Oda (chigusano@md.tsukuba.ac.jp).
Department of Immunology, Faculty of Medicine, and Life Science Center of Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.
Phone; (+81)29853-3281, Fax; (+81)29853-3410

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Capsule Summary

Cis-interaction of an inhibitory receptor CD300a with its ligand phosphatidylserine exposed on the cell surface of degranulating mast cells mediates the self-regulation of MC degranulation.

To the editor:

Although phosphatidylserine (PS) confined to the inner leaflet of plasma membrane is exposed on the cell surface when cells undergo apoptosis, viable cells, including mast cells (MCs), also externalize PS in certain cellular states[1,2, E1-E3]. However, the pathophysiological significance of PS exposure on viable cells remains elusive. To address the role of PS externalization on the cell surface of viable MCs, we monitored PS surface exposure on bone marrow-derived cultured MCs (BMMCs) by confocal microscopy after stimulation with TNP-specific IgE and TNP-OVA in the presence of PSVue 643, a fluorescent dye with rapid binding capacity for PS. The dye began to accumulate on the cell surface of live BMMCs within 600 s after FcεRI stimulation, whereas the non-stimulated BMMCs remained negative for the staining (Fig 1, *A*, see Video E1 in the Online Repository), indicating that PS is externalized within 10 min after activation.

MCs abundantly express CD300a, an inhibitory immunoreceptor for PS[3–5]. By imaging flow cytometry analyses, we found that both PS and CD300a showed a polarization and colocalization on the cell surface of mouse BMMCs (Annexin V stained) and human cultured synovial MCs (hMCs) 15 min after FcεRI stimulation (Fig 1, *B, See* Fig E1, *A, B,* in this article's Online Repository). Fluorescence resonance energy transfer (FRET) analyses using NBD-PS as a donor and a non-neutralizing anti-CD300a antibody (TX10) conjugated with Alexa546 as an acceptor demonstrated increased FRET efficiency calculated by sensitized emission

between CD300a and PS during degranulation (Fig 1, *C*). Treatment with a neutralizing anti-CD300a antibody (EX42) that interferes the binding of CD300a with PS dramatically decreased the FRET+ cell number during degranulation (*See* Fig E1, *C, D,* in this article's Online Repository), demonstrating the direct cis-interaction between CD300a and PS in the colocalized region of degranulating MCs. PS also showed a colocalization with CD107a on the cell surface of degranulating (i.e., $CD107a^+$ Annexin-V^+) BMMCs (see Fig E1, *E,* in this article's Online Repository), suggesting that PS exposure on the cell surface of MCs is associated with degranulation. Indeed, treatment of BMMCs with the MC degranulating stimuli ATP and ionomycin induced the exposure of PS as well as CD107a on the cell surface. In contrast, neither CD107a nor PS were observed on the cell surface of BMMCs after stimulation with either MC activators LPS or IL-33 [7] (see Fig E1, *F* in this article's Online Repository). Unlike FcεRI stimulation, however, stimulation with neither ATP nor ionomycin induced the polarization of CD300a and PS on the cell surface of MCs (see Fig E1, *G* in this article's Online Repository). Together, these data demonstrate that PS is promptly exposed together with CD107a during degranulation of MCs and colocalized with CD300a.

We next examined the degranulation of WT and *Cd300a*$^{-/-}$ BMMCs following stimulation of FcεRI by addition of TNP-specific IgE and TNP–OVA. Flow cytometry analyses showed that the size of the $CD107a^+$ population was significantly larger in *Cd300a*$^{-/-}$ BMMCs than in WT BMMCs from 8 min (480 s) to 30 min (1,800

s) after stimulation (Fig 2, *A* and *B*). To confirm the cell-intrinsic effect of CD300a on degranulation, equal numbers of WT and *Cd300a*$^{-/-}$ BMMCs were mixed and stimulated with TNP-specific IgE and TNP–OVA. The CD107a^{+} population was again observed to be larger in *Cd300a*$^{-/-}$ BMMCs than in WT BMMCs (see Fig E2, *A* and *B* in the Online Repository). Moreover, *Cd300a*$^{-/-}$ BMMCs released a larger amount of β-hexosaminidase than did WT BMMCs when they were analyzed 30 min after stimulation (Fig 2, *C*). We found that the population of PI+ dead cells was comparable between the cultures of WT and *Cd300a*$^{-/-}$ BMMCs before and after stimulation of FcεRI (see Fig E2*, C,* in the Online Repository). Moreover, even in the low concentrations of BMMCs in the culture, in which trans-interactions of CD300a with PS were unlikely, *Cd300a*$^{-/-}$ BMMCs still showed increased degranulation compared with WT BMMCs (see Fig E2, *D and E,* in the Online Repository). Treatment with a neutralizing anti-CD300a antibody (EX42) increased CD107a expression on WT BMMCs to a level comparable to that of *Cd300a*$^{-/-}$ BMMCs (Fig 2, *D*). Similarly, a neutralizing anti-human CD300a antibody increased the CD107a expression during FcεRI-mediated degranulation of hMCs (Fig. 2, *E*). Together, these results further support the involvement of *cis*-interaction, rather than *trans*-interaction, of CD300a with PS in the suppression of MCs degranulation. In contrast, CD107a expression was comparable between WT and *Cd300a*$^{-/-}$ BMMCs stimulated with either ATP or ionomycin (see Fig E2, *F* in the Online Repository). Interestingly, FcεRI stimulation also induced polarization of FcεRI and colocalization of FcεRI with CD300a and PS

(see Fig E2, *G, H,* in the Online Repository). Thus, the same spatio-temporal localization of CD300a, PS and FcεRI on MCs might cause CD300a-mediated suppression of FcεRI-mediated, but not ATP- or ionomycin-mediated, CD107a expression during degranulation. Together, these imaging and functional analyses suggest that the *cis*-interaction of CD300a with PS specifically suppresses FcεRI-mediated signaling for degranulation of MCs. Indeed, Syk phosphorylation was higher in *Cd300a*$^{-/-}$ BMMCs than in WT BMMCs after antigen challenge at 10 min (600 s) after FcεRI stimulation, but was comparable between WT and *Cd300a*$^{-/-}$ BMMCs at 2 min (120 s) after stimulation (see Fig E3, in this article's Online Repository), consistent with our observation that degranulation was significantly higher in *Cd300a*$^{-/-}$ than WT BMMCs at 8 min (480 s), but not 2 min (120 s), after FcεRI stimulation (Fig 2, *B*).

We further analyzed the role of CD300a in the pathogenesis of passive systemic anaphylaxis. WT and *Cd300a*$^{-/-}$ mice were i.v. injected with TNP-specific IgE, followed by i.v. challenge with TNP–OVA. Although rectal temperature of WT and *Cd300a*$^{-/-}$ mice decreased to a similar level by 20 min (1,200 s) after the challenge, the recovery of the rectal temperature after this period was slower in *Cd300a*$^{-/-}$ mice than in WT mice (Fig 2, *F*). Interestingly, this seems to correlate to the time course of the self-regulation of MCs degranulation in vitro by CD300a. Similar results were also observed when mice were i.p. injected with a neutralizing anti-CD300a antibody (EX42) (Fig 2, *G*), suggesting that the CD300a-PS interaction suppressed PSA.

Moreover, polarized PS was detected only in MCs, rather than at regions surrounding MCs, in the ear tissue of mice after injection with Flag-tagged MFG-E8-D89E together with TNP–OVA (Fig. E4). These results suggest that the *cis*-interaction regulated MCs degranulation in vivo as well as in vitro.

In conclusion, by combining imaging and functional analyses of MCs degranulation, we revealed the physical and functional associations of externalized PS with CD300a via *cis*-interaction on viable MCs during degranulation (Fig. E5). Given that MCs are widely distributed in many tissues in a scattered manner without contact with each other[7] and apoptotic cells[8], such self-regulation of MC degranulation represents a novel strategy that MCs evolved to control their own activation, adding another layer of regulation in allergic responses.

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Yaqiu Wang, *BSc*[a, b, c]

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from: [a]Ph.D. Program in Human Biology, School of Integrative and Global Majors,

and [b]Department of Immunology, Faculty of Medicine, and [c]Life Science Center of Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.

[d]Allergy and Immunology Project Team, Center for Institutional Research and Medical Education, Nihon University School of Medicine, 30-1 Oyaguchi Kamicho, Itabashi-Ku, Tokyo 173-8610, Japan

Email: ashibuya@md.tsukuba.ac.jp or chigusano@md.tsukuba.ac.jp

[*]These authors contributed equally to this work.

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Figure Legends

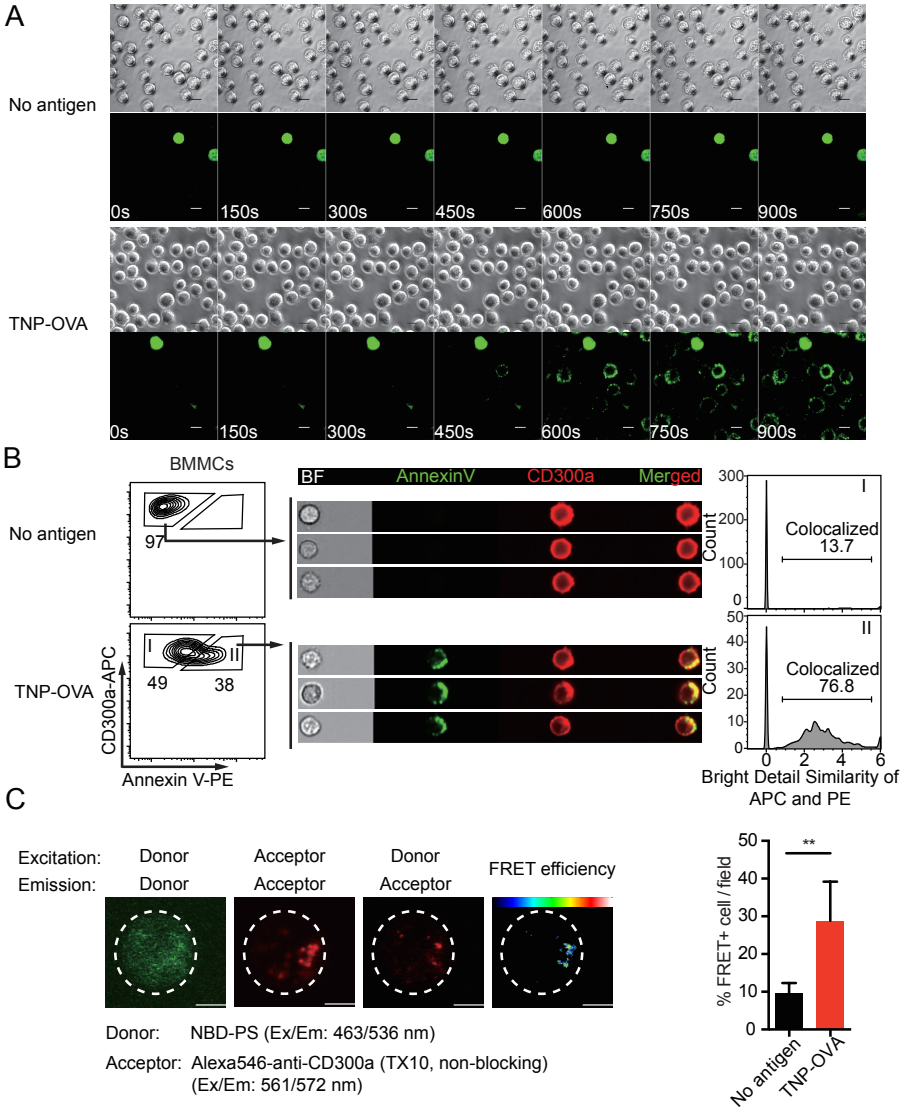
Figure 1. Imaging analyses of PS and CD300a on BMMCs during degranulation

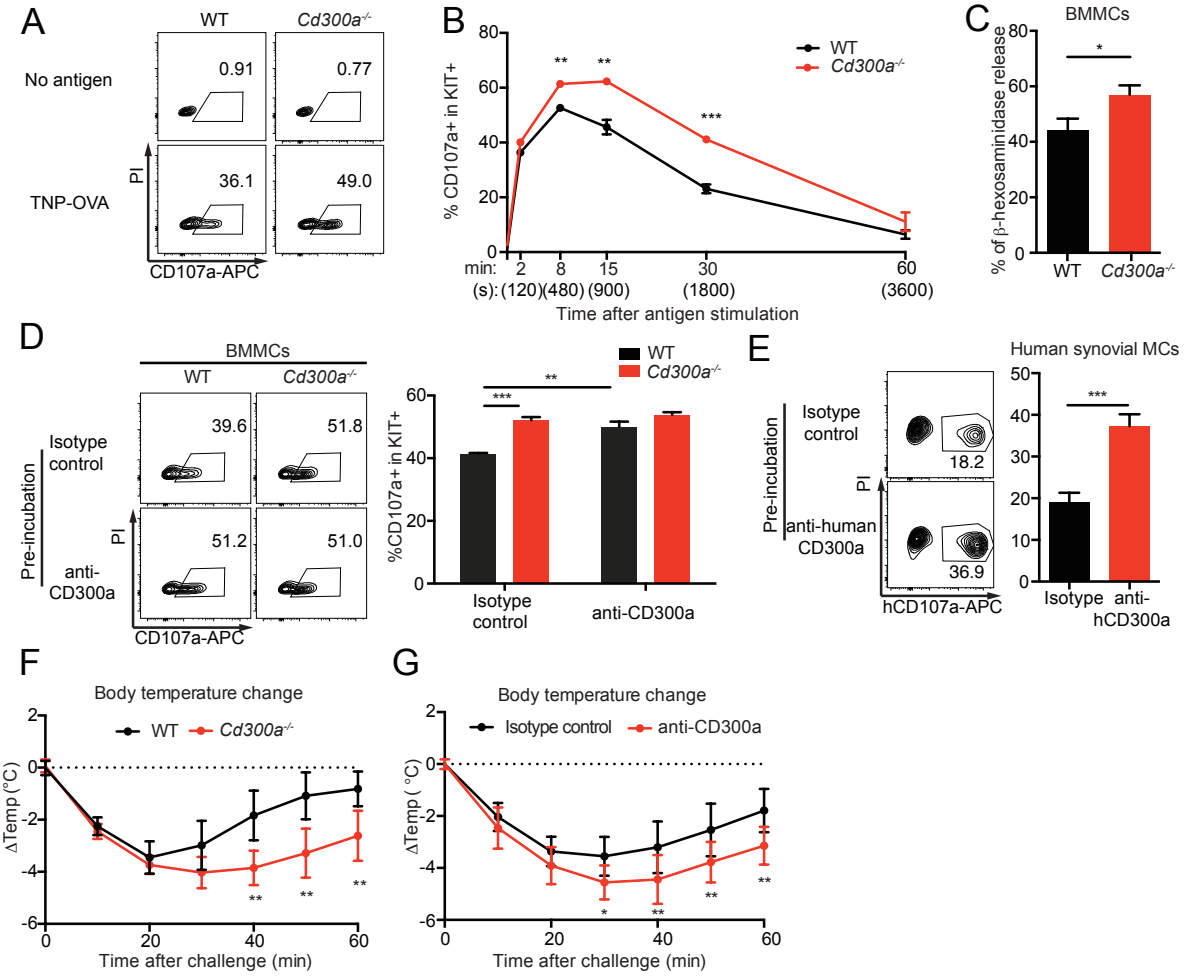
(**A**) Time-lapse montage of BMMCs degranulation in the presence of PSVue643 by confocal microscopy. Dead cells were used as a PSVue-643 staining positive control. Scale bars, 10 μm. See also extended Video 1. (**B**) Imaging flow cytometry of degranulated BMMCs stained with anti-CD300a and Annexin V. Representative gating (left); single cell images (center); and colocalization of CD300a and PS analyzed using bright detail similarity R3 based on images of single cells (see *Extended methods*) in the indicated gate (right). (**C**) FRET analysis between CD300a and PS. Representative confocal pictures (left) and FRET+ cell quantification (right) (see *Extended methods*). Scale bars, 5 μm. Data are representative of more than three independent experiments. Error bars indicate SD. $^{**}P < 0.01$

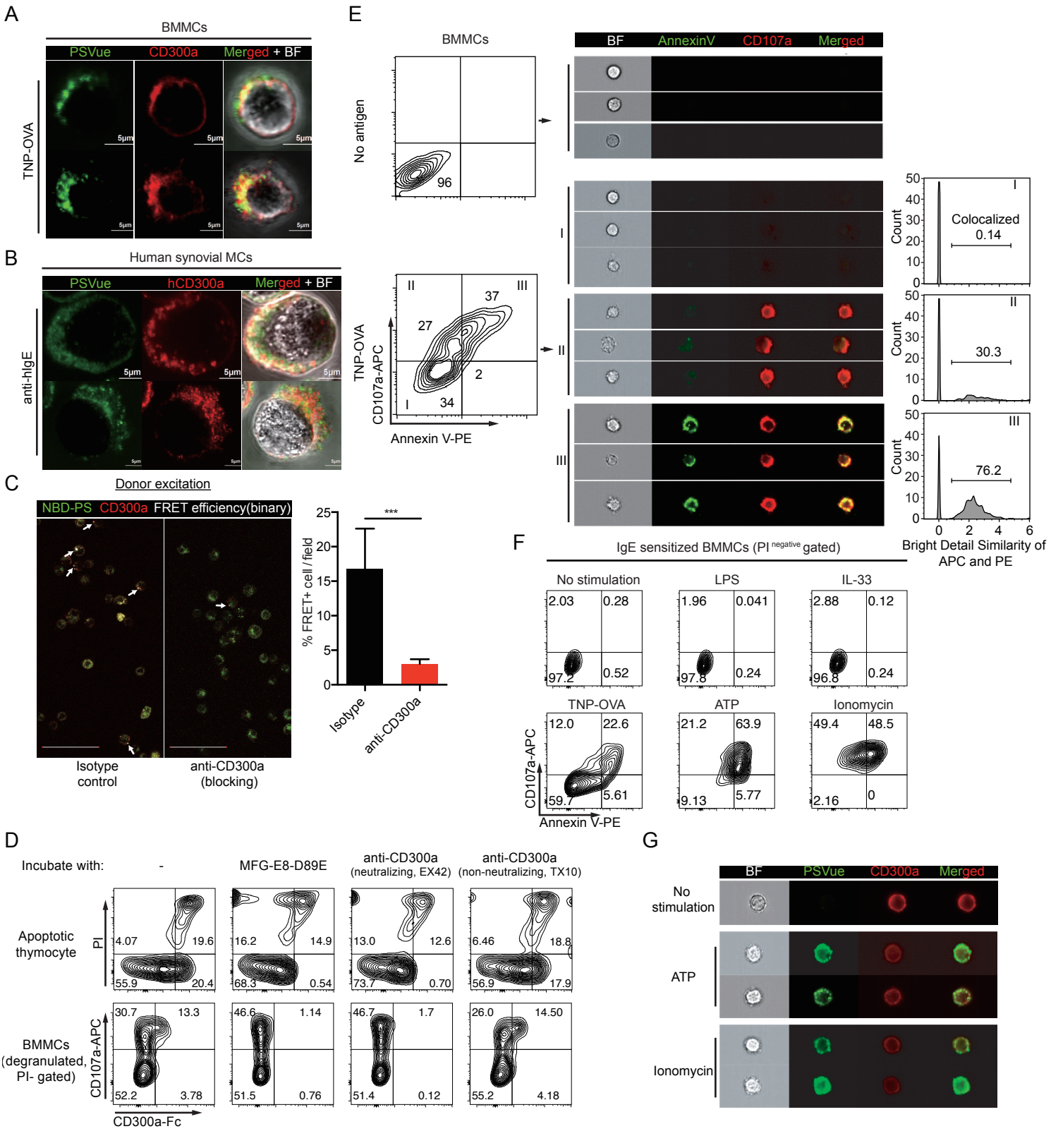
Figure 2. Functional interaction between CD300a and PS externalized during degranulation of MCs.

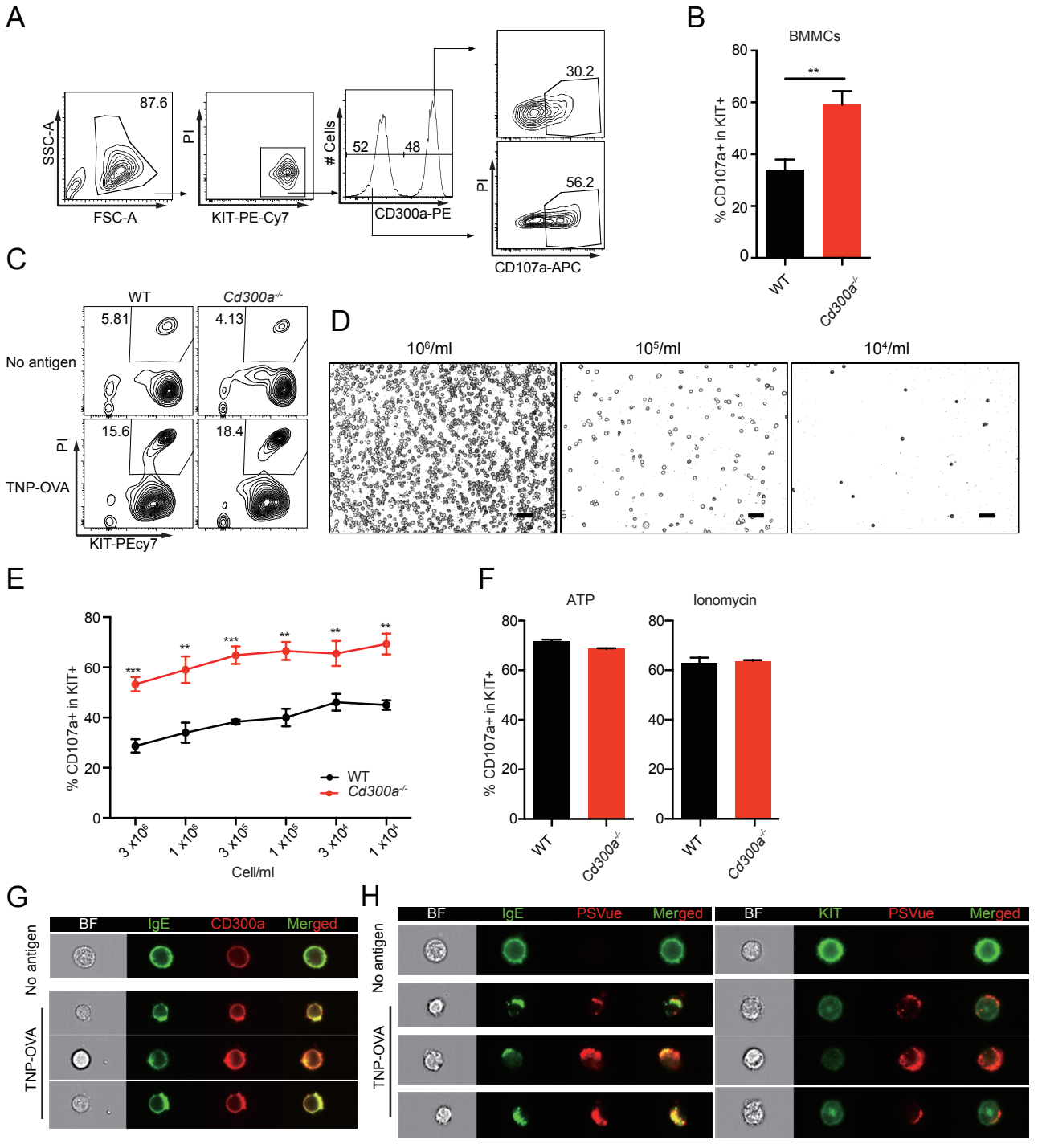
(A) Representative plots showing CD107a expression of antigen stimulated WT or *Cd300a*$^{-/-}$ BMMCs gated on the PI^{-} c-Kit^{+} cells. (**B**) Kinetics of CD107a expression after antigen stimulation. (**C**) β-hexosaminidase release in the culture after degranulation. (**D, E**) Effect of anti-mouse CD300a or anti-human CD300a neutralizing antibodies on degranulation of WT and *Cd300a*$^{-/-}$ BMMCs (D) and cultured human synovial MCs (E) gated on the PI^{-} c-Kit^{+} cells. (**F, G**) Change in

intrarectal temperature in WT (n = 6) and *Cd300a*$^{-/-}$ (n = 6) mice (**F**) or WT mice that had been injected with control (n = 8) or anti-CD300a (n = 9) antibody (**G**) after i.v. sensitization with TNP-specific IgE, followed by i.v. challenge with TNP–OVA. Data are representative of three independent experiments (**A-E**). Data are pooled from two to three experiments (**F, G**). Error bars indicate SD. **P* < 0.05, ***P* <0.01, ****P* < 0.001.


A
No antigen
TNP-OVA
0s
150s
300s
450s
600s
750s
900s
B
BMMCs
No antigen
TNP-OVA
97
49
38
I
II
CD300a-APC
Annexin V-PE
BF
AnnexinV
CD300a
Merged
Count
Colocalized
13.7
Colocalized
76.8
Bright Detail Similarity of
APC and PE
C
Excitation:
Emission:
Donor
Donor
Acceptor
Acceptor
Donor
Acceptor
FRET efficiency
Donor: NBD-PS (Ex/Em: 463/536 nm)
Acceptor: Alexa546-anti-CD300a (TX10, non-blocking)
(Ex/Em: 561/572 nm)
% FRET+ cell / field
**
No antigen
TNP-OVA


A
WT
Cd300a-/-
No antigen
TNP-OVA
0.91
0.77
36.1
49.0
PI
CD107a-APC
B
% CD107a+ in KIT+
WT
Cd300a-/-
min: 2 8 15 30 60
(s): (120) (480) (900) (1800) (3600)
Time after antigen stimulation
C
BMMCs
% of β-hexosaminidase release
WT
Cd300a-/-
D
BMMCs
WT
Cd300a-/-
Isotype control
anti-CD300a
Pre-incubation
39.6
51.8
51.2
51.0
PI
CD107a-APC
%CD107a+ in KIT+
Isotype control
anti-CD300a
E
Human synovial MCs
Isotype control
anti-human CD300a
Pre-incubation
18.2
36.9
PI
hCD107a-APC
Isotype
anti-hCD300a
F
Body temperature change
WT
Cd300a-/-
ΔTemp (°C)
Time after challenge (min)
G
Body temperature change
Isotype control
anti-CD300a
ΔTemp (°C)
Time after challenge (min)


A
BMMCs
PSVue
CD300a
Merged + BF
TNP-OVA
5μm
B
Human synovial MCs
PSVue
hCD300a
Merged + BF
anti-hIgE
C
Donor excitation
NBD-PS CD300a FRET efficiency(binary)
Isotype control
anti-CD300a (blocking)
% FRET+ cell / field
***
Isotype
anti-CD300a
D
Incubate with:
-
MFG-E8-D89E
anti-CD300a (neutralizing, EX42)
anti-CD300a (non-neutralizing, TX10)
Apoptotic thymocyte
BMMCs (degranulated, PI- gated)
PI
CD107a-APC
CD300a-Fc
4.07
19.6
55.9
20.4
16.2
14.9
68.3
0.54
13.0
12.6
73.7
0.70
6.46
18.8
56.9
17.9
30.7
13.3
52.2
3.78
46.6
1.14
51.5
0.76
46.7
1.7
51.4
0.12
26.0
14.50
55.2
4.18
E
BMMCs
No antigen
96
BF
AnnexinV
CD107a
Merged
TNP-OVA
CD107a-APC
Annexin V-PE
II
III
I
37
27
2
34
Count
Colocalized
0.14
30.3
76.2
Bright Detail Similarity of APC and PE
F
IgE sensitized BMMCs (PI negative gated)
No stimulation
LPS
IL-33
TNP-OVA
ATP
Ionomycin
2.03
0.28
97.2
0.52
1.96
0.041
97.8
0.24
2.88
0.12
96.8
0.24
12.0
22.6
59.7
5.61
21.2
63.9
9.13
5.77
49.4
48.5
2.16
0
CD107a-APC
Annexin V-PE
G
BF
PSVue
CD300a
Merged
No stimulation
ATP
Ionomycin


A
87.6
SSC-A
FSC-A
PI
KIT-PE-Cy7
Cells
52
48
CD300a-PE
30.2
56.2
PI
CD107a-APC
B
BMMCs
**
% CD107a+ in KIT+
WT
Cd300a-/-
C
WT
Cd300a-/-
No antigen
TNP-OVA
5.81
4.13
15.6
18.4
PI
KIT-PEcy7
D
10⁶/ml
10⁵/ml
10⁴/ml
E
% CD107a+ in KIT+
WT
Cd300a-/-
3 x10⁶
1 x10⁶
3 x10⁵
1 x10⁵
3 x10⁴
1 x10⁴
Cell/ml
F
ATP
Ionomycin
% CD107a+ in KIT+
WT
Cd300a-/-
G
BF
IgE
CD300a
Merged
No antigen
TNP-OVA
H
BF
IgE
PSVue
Merged
BF
KIT
PSVue
Merged
No antigen
TNP-OVA

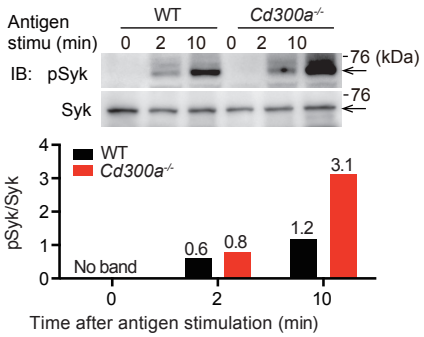
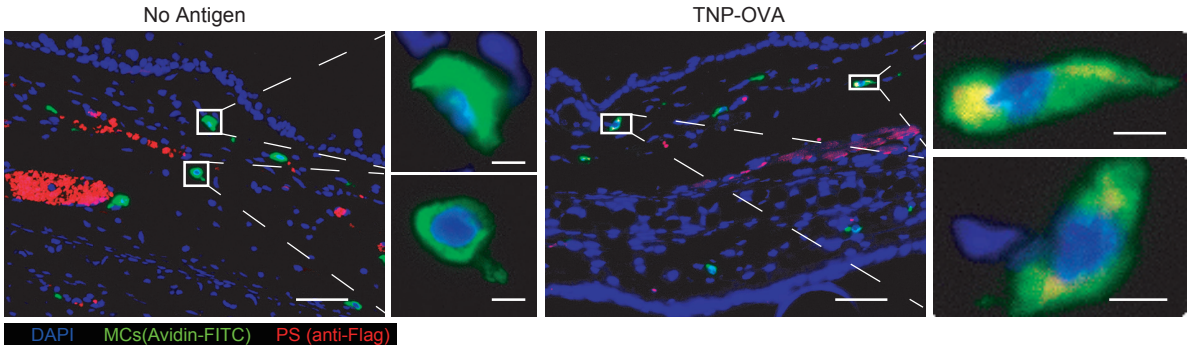
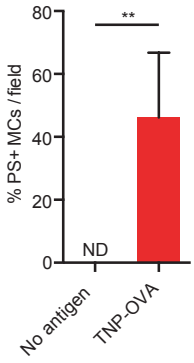

Antigen
stimu (min)
WT
Cd300a-/-
0 2 10 0 2 10
IB: pSyk
Syk
76 (kDa)
76
WT
Cd300a-/-
pSyk/Syk
No band
0.6
0.8
1.2
3.1
0 2 10
Time after antigen stimulation (min)

FIGURE.E4


No Antigen
TNP-OVA
DAPI
MCs(Avidin-FITC)
PS (anti-Flag)


80
60
40
20
0
% PS+ MCs / field
**
ND
No antigen
TNP-OVA

Extended Materials

Autonomous regulation of immunoglobulin E-mediated mast cell degranulation and immediate hypersensitivity reaction by an inhibitory receptor CD300a

Yaqiu Wang, *BSc*[a, b, c] Chigusa Nakahashi-Oda, *MD, PhD*[b,*]

Yoshimichi Okayama, *MD, PhD*[d], and Akira Shibuya, *MD, PhD*[b, c,*]

[a]Ph.D. Program in Human Biology, School of Integrative and Global Majors, and [b]Department of Immunology, Faculty of Medicine, and [c]Life Science Center of Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.

[d]Allergy and Immunology Project Team, Center for Institutional Research and Medical Education, Nihon University School of Medicine, 30-1 Oyaguchi Kamicho, Itabashi-Ku, Tokyo 173-8610, Japan

Extended Materials and Methods

Cells

Bone marrow (BM)-derived cultured MCs (BMMCs) were generated by culturing wild-type (WT) and *Cd300a*$^{-/-}$ mouse BM cells in the presence of 10 ng/ml stem cell factor (SCF) (455-MC/CF, R&D Systems) and 4 ng/ml IL-3 (403-ML, R&D Systems) as previously described. [6] Briefly, weekly passages were performed by seeding 2 × 10^6 cells in 10 ml medium. Cells were cultured for 5 to 8 weeks before use. *Cd300a*$^{-/-}$ mice were described previously.[6] Cultured human synovial MCs were prepared and maintained as previously reported. [E4] All mice experiments were conducted in accordance with the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center. The human mast cell study was approved by the Ethics Committee of the Nihon University School of Medicine (RK-160112-2), and all the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Antibodies, other reagents, and flow cytometry

Anti-mouse CD107a (1D4B), anti-mouse c-Kit (2B8), anti-mouse IgE (RME-1), mouse IgG1 (MOPC-21), anti-human CD107a (H4A3), anti-Flag (L5) antibodies and FITC-Avidin were purchased from Biolegend. Human IgE Myeloma (401152) was purchased from CALBIOCHEM. Anti-human IgE (Dε2) (E124.2.8) was purchased from BECKMAN COULTER. Annexin V, Trinitrophenyl (TNP)-specific mouse IgE (C38-2) was purchased from BD Bioscience. Anti-Syk (#2712) and anti-pSyk (#2711) antibodies were purchased from Cell Signaling Technology. TNP–ovalbumin (OVA), MFG-E8-D89E, MFG-E8-EPT, neutralizing and non-neutralizing anti-mouse CD300a antibodies (EX42 and TX10, respectively), CD300a-human Fc protein and anti-human CD300a (mouse IgG1, TX49) were made in our laboratory, as previously described. [3,4]
1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoserine (NBD-PS) was from Avanti. Lipopolysaccharide (LPS; L2880) derived from *Escherichia coli* O55:B5, ATP (A26209), Ionomycin (I9657) were purchased from Sigma-Aldrich. PSVue480 and PSVue643 were purchased from Molecular Targeting Technologies. Recombinant mouse IL-33 was purchased from R&D (3626-ML).

CD300a-Fc blocking experiment was done with either apoptotic cell induced by dexamethasone or degranulated BMMCs. Briefly, 10^5 cells were incubated with CD300a-Fc together with 50ng anti-CD300a antibody (TX10 or EX42) or 100ng MFG-E8-D89E on ice for 15min, and further stained by anti-CD107a (for BMMCs only) and anti-human IgG Fc-PE (M1310G05, Biolegend) and PI.
Flow cytometry analyses were performed using a LSRFortessa system (BD Bioscience), and data were analyzed by using FlowJo software (BD).

Degranulation and other stimulations

For mouse *in vitro* stimulations, MCs were sensitized with 1 μg/ml TNP-specific IgE overnight, washed twice with Tyrode's buffer, pre-incubated with reagents as indicated for 30min, and then challenged with 1ng/ml TNP–OVA for 30min or indicated time point. β-Hexosaminidase activity was measured as previously reported.[E5] Briefly, 50 μl of the culture supernatant was mixed with 50 μl of 4-nitorophenyl-N-acetyl-β-D-glucosaminide (1.3 mg/ml; Sigma, N9376) in substrate buffer (0.4 M citric acid, 0.2 M NaH_2PO_4, pH 4.5) and incubated at 37°C for 3 h. The reaction was stopped by adding 100 μl of glycine (0.2 M, pH 10.7), the solution was thoroughly mixed, and then absorbance of 415 nm was determined. CD107a was measured by flow cytometry after staining with anti-cKit and anti-CD107a antibodies and PI. Apoptotic thymocytes were induced in 2.5μM dexamethasone with 10^7 cell/ml RPMI medium for 12hr. Cell density pictures were acquired under 20X objective lens under bright field and converted into binary by ImageJ.

For human MCs stimulation, cultured human synovial MCs were sensitized with 500ng/ml human IgE, stained with 50ug/ml anti-human CD300a or isotype control antibody, challenged with 300ng/ml anti-human IgE antibody for 30min, followed by staining with anti-human CD107a and PI, and analyzed by flow cytometry.

For measuring PS exposure of BMMCs after stimulations by LPS (1 μg/ml), IL-33 (150 ng/ml), TNP-OVA (10 ng/ml), ATP (0.5 mM), or ionomycin (2500 ng/ml), the cells were incubated with indicated reagents for 20 min after IgE sensitization and then stained with antibody against CD107a, annexin V and PI. For measuring degranulation after ATP (0.5mM) or ionomycin (500ng/ml) stimulation, the stimulation time was 30min.

Live imaging

For time lapse-imaging, BMMCs were sensitized as mentioned above and then incubated in 500 μl of Tyrode's buffer containing 1 mM PSVue-643 fluorescent probe (Polysciences, Inc.) in a glass-bottom dish (CELLview, Greiner Bio-One) for 30 min before gently adding 10 μl of TNP–OVA (100 ng/ml). Cells were monitored under a laser scanning confocal microscope (Olympus FV10i) at 10 s intervals under 60× optical magnification. Data were analyzed and exported by FV10-ASW (Olympus). The video and montage were generated by ImageJ software with 10 frames/s. The confocal images of single mast cells were also collected and analyzed on the same platform with PS stained by PSVue480 and CD300a by Alexa647 conjugated anti-CD300a.

For imaging flow cytometry, the Image Stream Mark II system (Amnis) was used to observe single cells after 15min degranulation and staining. Data were acquired and analyzed by using the Inspire and Ideas software packages (Merck), respectively. The

Bright Detail Similarity R3 Feature (based on Pearson correlation coefficient) was adopted as the localization measurement according to the manufacturer's instructions. Briefly, the Bright Detail Similarity R3 Feature value was calculated with a customized imaging mask to identify aggregation of the molecule of interest (CD107a or PS) on the cell surface. To detect colocalization of CD107a and PS, the mask was set on the CD107a channel with the following parameters: Threshold (M05, Ch05, 73) & Peak (M05, Ch05, Bright 10). To detect colocalization of CD300a and PS, the mask was set on the PS channel with following parameters: Threshold (M05, Ch05, 60) & Peak (M05, Ch05, Bright 4). The data were exported as an FCS (Flow Cytometry Standard) file and analyzed by FlowJo (TreeStar).

FRET analysis was used as direct evidence of *cis*-interaction between other receptors[E6]. To analyze the *cis*-interaction between CD300a and PS, TNP-specific IgE sensitized BMMCs was firstly stained by non-blocking anti-CD300a antibody TX10 (Alexa546 conjugated) for 15min and then labeled by 500nM NBD-PS in Hank's Balanced Salt Solution (HBSS) with 1mM $CaCl_2$ for 8min in room temperature. The cells were immediately washed by 5 mg/ml fatty acid-free Bovine Serum Albumin (BSA) in HBSS with 1mM $CaCl_2$. The stained cells were challenged by TNP-OVA and observed under live imaging conditions with laser set 471nm/559nm and filter set 490nm-540nm and 570nm-620nm.

FRET efficiency was calculated by sensitized emission according to the FRET package instructions in FV10-ASW (Olympus).

$$\text{Efficiency (E)} = 1 - \frac{e}{e + PFRET * \left(\frac{\Psi_{dd}}{\Psi_{aa}}\right) * \left(\frac{Q_d}{Q_a}\right)}$$

Ψ_{dd}, Ψ_{aa}: collection efficiency in donor and acceptor channel;

Q_d, Q_a :Quantum yield of the donor and acceptor;

$$PFRET = f - DSBT - ASBT$$

f: Acceptor with donor excitation

$$DSBT = \left(\frac{b}{a}\right) * e \qquad ASBT = \left(\frac{c}{d}\right) * g$$

e: Donor with donor excitation

g: Acceptor with acceptor excitation

e, f and g were obtained using donor and acceptor double stained samples (FRET samples).

DSBT(donor spectral bleedthrough) and ASBT (acceptor spectral bleedthrough) were calculated by images from the donor/acceptor single stained sample excited by designated laser according to the package instruction to obtain the value *a*, *b c* and *d*. FRET+ cell percentage was calculated by counting the FRET+ cells in each field.

Passive systemic anaphylaxis

WT and *Cd300a*$^{-/-}$ mice (age-matched 8- to 14-week-old females) were sensitized by

intravenous (i.v.) administration of 5 μg of TNP-specific IgE (BD Biosciences, C38-2) for 24 h and then i.v. challenged with 40 μg TNP–OVA. Body temperature was measured intrarectally at the indicated time points. For antibody blocking, a neutralizing anti-CD300a or isotype (400μg/mice) was injected i.p. 5 h before antigen challenge.
For staining ear tissue sections, 50 μg/mice MFG-E8-D89E (Flag-taged) were i.v injected together with or without 40μg/mice antigen. Ear tissue was harvested 10min after injection and fixed with formalin. Paraffin sections were deparaffinized, antigen retrieval was done by using AR6 buffer (PerkinElmer), staining for MCs by using FITC-Avidin (Biolegend) and PS by using PE-anti-Flag (L5, Biolegend).

Western blot analysis

One hundred thousand BMMCs were degranulated as described above and immediately washed with ice-cold PBS at the indicated time points. Cells were stimulated with 1 mM sodium orthovanadate and then lysed with 1% (w/v) NP40. The lysates were immunoblotted with antibodies against Syk or phosphorylated Syk.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software). For comparing between two groups, statistical significance was determined by 2-tailed unpaired Student *t* test with or without correction by Holm-Sidak method. For comparing more than two groups, statistic significance was determined by 2way-ANOVA multiple comparisons with Bonferroni's or Sidak's test. Error bars indicate SEM except for elsewhere mentioned.

Extended reference

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Extended legends

Figure E1. PS exposure of degranulating MCs.

(**A, B**) Confocal images of degranulated BMMCs (A) and human synovial MCs (B) stained with anti-mouse and anti-human CD300a, respectively, and analyzed in the presence of PSVue480. (**C**) FRET analysis of the CD300a-PS interaction on degranulated BMMCs pretreated with either isotype or anti-CD300a neutralizing antibody. Representative merged image (left) and FRET+ cell quantification (right). White arrows indicate FRET+ cells. (**D**) Flow cytometry analysis of apoptotic mouse thymocytes or degranulating BMMCs incubated with MFG-E8-D89E, neutralizing anti-mouse CD300a (EX42), or non-neutralizing anti-mouse CD300a (TX10) together with mouse CD300a-Fc in the presence of $CaCl_2$, followed by an PE-conjugated antibody against human IgG and PI. (**E**) Imaging flow cytometry analysis of degranulated BMMCs stained by anti-CD107a and annexin V. Gating (left), images (middle) and colocalization of CD107a and PS (right) of single cells from indicated gates. (see ***Extended methods***). (**F**) Flow cytometry analysis of BMMCs after indicated stimulations and staining with anti-CD107a, annexin V and PI. **(G)** Imaging flow cytometry analysis of ATP or ionomycin stimulated BMMCs stained with anti-CD300a antibody and PSVue643. Data are representative of two (**C**, **D, F, G**) to three (**A**-**B, E**) independent experiments. Scale: A, B 5μm; C 50 μm.

Figure E2. (**A, B**) WT and *Cd300a*$^{-/-}$ BMMCs were equally mixed and degranulated by IgE-antigen complex and analyzed by flow cytometry. Data are representative plots (**A**) and the mean CD107a expression (**B**) in each genotype. (**C**) PI+ cell before and after degranulation in WT and *Cd300a*$^{-/-}$ BMMCs. (**D, E**) WT and *Cd300a*$^{-/-}$ BMMCs were diluted into different density (scale 50μm) (**D**), and then CD107a+ cells were measured after degranulation in PI- gated cells (**E**). (**F**) WT and *Cd300a*$^{-/-}$ BMMCs were stimulated with 0.5 mM ATP or 500ng/ml ionomycin and analyzed by flow cytometry. (**G, H**) Imaging flow cytometry of naïve or antigen stimulated BMMCs stained with anti-IgE and anti-CD300a antibodies (**E**), or PSVue643 plus either anti-IgE or anti-cKit (**F**), and analyzed by. Data are representative of two (**D-H**) to three (**A-C**) independent experiments.

Figure E3. Western blot analysis of Syk phosphorylation in whole cell lysates of degranulated WT and *Cd300a*$^{-/-}$ BMMCs at indicated time points. Data are representative of two independent experiments.

Figure E4. Immunohistochemistry analysis of PS exposure in MCs during PSA. WT mice were sensitized with TNP-specific IgE 24hr before injection of 50 μg MFG-E8-D89E (Flag-taged) together with or without 40μg TNP-OVA antigen. Ten min after the injection, ear tissue sections were stained for MCs by FITC-conjugated Avidin and for PS by PE-conjugated anti-Flag antibody. Scale bar: 50μm (tissue view) and 5μm (enlarged cell view).

Figure E5. Graphical summary of this study.

Extended Video 1. Time-lapse video of Figure 1, A.

BMMCs were sensitized by anti-TNP IgE, seeded in Tyrode's buffer containing PSVue 643 with gentle addition of TNP–OVA under the monitoring by confocal microscopy (the images at 10 s intervals). The speed of the video is 10 frames/s. Scale 10μm.