Human CD300C Delivers an Fc Receptor-γ-dependent Activating Signal in Mast Cells and Monocytes and Differs from CD300A in Ligand Recognition* [S]

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Background: Human CD300C is not fully characterized because of the unavailability of its specific antibody.

Results: Stimulation with a specific CD300C antibody activates human monocytes and mast cells that express high levels of CD300C.

Conclusion: Specific engagement of CD300C, but not its co-engagement with CD300A, delivers an Fc receptor-γ-dependent activating signal.

Significance: The activating function of CD300C is associated with its ligand specificity.

CD300C is highly homologous with an inhibitory receptor CD300A in an immunoglobulin-like domain among the human CD300 family of paired immune receptors. To clarify the precise expression and function of CD300C, we generated antibodies discriminating between CD300A and CD300C, which recognized a unique epitope involving amino acid residues CD300A(F56-L57) and CD300C(L63-R64). Notably, CD300C was highly expressed in human monocytes and mast cells. Cross-linking of CD300C by its specific antibody caused cytokine/chemokine production of human monocytes and mast cells. Fc receptor γ was indispensable for both efficient surface expression and activating functions of CD300C. To identify a ligand for CD300A or CD300C, we used reporter cell lines expressing a chimera receptor harboring extracellular CD300A or CD300C and intracellular CD3ζ, in which its unknown ligand induced GFP expression. Our results indicated that phosphatidyethanolamine (PE) among the lipids tested and apoptotic cells would be possible ligands for both CD300C and CD300A. PE and apoptotic cells more strongly induced GFP expression in the reporter cells through binding to extracellular CD300A as compared with CD300C. Differential recognition of PE by extracellular CD300A and CD300C depended on different amino acid residues CD300A(F56-L57) and CD300C(L63-R64). Interestingly, GFP expression induced by extracellular CD300C-PE binding in the reporter cells was dampened by co-expression of full-length CD300A, indicating the predominance of CD300A over CD300C in PE recognition/signaling. PE consistently failed to stimulate cytokine production in monocytes expressing CD300C with CD300A. In conclusion, specific engagement of CD300C led to Fc receptor γ-dependent activation of mast cells and monocytes.

The CD300 family of paired immune receptors consists of several activating and inhibitory receptors harboring a single Ig-like domain (1–5). CD300 is also called leukocyte mono-Ig-like receptor (LMIR)3 CLM (CMRF-35-like molecule) or MAIR (myeloid-associated Ig-like receptor) in mice (6–12) and IREM (immune receptor expressed by myeloid cells) in humans (13, 14). The CD300 family of genes is clustered on human chromosome 17. CD300A is highly homologous with CD300C in an Ig-like domain. CD300A contains immunoreceptor tyrosine-based inhibitory motifs in the cytoplasmic region, whereas CD300C has no signaling motif in the short cytoplasmic tail (15–17). Accumulated studies show that CD300A functions as an inhibitory receptor and that CD300A is expressed in a variety of hematopoietic cells, including mast cells, neutrophils, and macrophages. CD300A, like the FcεRI, is involved in IgE-mediated mast cell activation and does not support T cell activation. By contrast, CD300C can act as an activating receptor on mast cells and monocytes. CD300C also shares a conserved domain with the CD200 receptor and the FcεRI, which can be engaged by the FcεRα chain. CD300C binds to the FcεRα chain and delivers a signal activating phosphorylation of Igα and Igβ, indicating a potential role of CD300C in activating mast cells and monocytes.

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3 The abbreviations used are: LMIR, leukocyte mono-immunoglobulin-like receptor; PDC, plasmacytoid dendritic cell; NK, natural killer; ITAM, immunoreceptor tyrosine-based activation motif; FcγR, Fc receptor-γ; TIM, T cell immunoglobulin mucin; PS, phosphatidylserine; PE, phosphatidylethanolamine; FcεRα, high affinity IgE receptor I; Ab, antibody; BMMC, bone marrow-derived mast cell; PB, peripheral blood; Mφ, macrophage; DC, dendritic cell; R-PE, R-phycoerythrin; TREM-1, triggering receptor expressed on myeloid cells-1; CCL1, chemokine (C-C motif) ligand 1; SLAM, signaling lymphocyte-activating molecule; PMA, phorbol 12-myristate 13-acetate.
eosinophils, plasmacytoid dendritic cells (PDCs), natural killer (NK) cells, and a certain subset of T cells and B cells (16–25). On the other hand, a recent study using CD300C-transduced cell lines indicates that CD300C is an activating receptor that is coupled to an immunoreceptor tyrosine-based activating motif (ITAM)-containing Fc receptor-γ (FcRγ) (15). However, the exact expression and function of human CD300C in primary hematopoietic cells remain unclear because attempts to generate an antibody (Ab) specific for CD300C have been so far unsuccessful.

Identifying the ligands for the CD300/LMIR family is essential for clarifying its biological functions. We have previously identified T cell Ig mucin 1 (TIM-1) and TIM-4 as ligands for mouse CD300B/LMIR5, using retrovirus-mediated expression cloning (26). According to recent studies, both mouse and human CD300A recognized apoptotic cells: phosphatidylserine (PS) was a ligand for mouse CD300A, whereas PS and phosphatidylethanolamine (PE) were ligands for human CD300A (27–29). In addition, the interaction between CD300A and apoptotic cells suppressed mast cell inflammatory responses in mice (28), whereas it suppressed macrophage in phagocytosing apoptotic cells in humans (29). Moreover, we have recently identified ceramide as a physiological ligand for mouse CD300f/LMIR3, using reporter cells in which the interaction between an extracellular domain of LMIR3 and its ligand induced GFP expression driven by NFAT (nuclear factor of activated T cells) (30). Extracellular ceramide-LMIR3 binding was shown to inhibit high affinity IgE receptor I (FceRI)-mediated activation of mast cells in vitro and in vivo (30). The structural homology of an Ig-like domain between CD300A and CD300C implied that CD300C shared a similar or the same ligand with CD300A; however, a ligand for human CD300C remained to be identified.

In the present study, we generate Abs discriminating between CD300A and CD300C and clarify expression profiles and biological functions of CD300C in human primary cells. Functional reporter assays suggest that PE and apoptotic cells are possible ligands for CD300C and CD300A; however, CD300A more strongly recognizes such potential ligands than does CD300C. Our results indicate that specific engagement of CD300C by an unknown ligand, but not co-engagement of CD300C with CD300A, induces an FcRγ-dependent activation of human mast cells and monocytes.

**EXPERIMENTAL PROCEDURES**

**Cells and Mice**—Murine cell lines used in this study were as follows: Ba/F3, NIH3T3, and 2B4-GFP (a kind gift from Takashi Saito, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) (26, 30–32). Mouse bone marrow cells were isolated from C57BL/6 mice (Charles River Laboratories Japan) or FcRγ−/− mice (a kind gift from Toshiyuki Takai, Tohoku University, Sendai, Japan) (33). Bone marrow-derived mast cells (BMMCs) were generated and cultured as described (7, 34). All of the procedures were approved by an institutional review committee of the University of Tokyo. Human hematopoietic cell lines used in this study were as follows: MOLM13, THP-1, U-937, HL-60, HMC-1, UT-7, TF-1, HEL, K562, Jurkat, ATL-1K, NALM-6, and RAMOS (35). Human peripheral blood (PB)-derived mast cells were generated as described (36). Briefly, lineage-negative mononuclear cells isolated from human PB mononuclear cells were cultured in serum-free Iscove’s methylocellulose medium (Stem Cell Technologies) and Iscove’s modified Dulbecco’s medium containing stem cell factor and IL-6. After 42 days of culture, methylcellulose was dissolved in PBS, and the collected cells were cultured in Iscove’s modified Dulbecco’s medium containing 2% FCS, stem cell factor, and IL-6. To isolate human PB-derived monocytes, granulocytes, B cells, T cells, NK cells, PDCs, basophils, eosinophils, neutrophils, MicroBeads, and cell isolation kits (Miltenyi Biotech) were used according to the manufacturer’s protocol. Human PB monocytes isolated by using CD14 MicroBeads (Miltenyi Biotech) were cultured in RPMI 1640 medium containing 10% FCS. To generate macrophage-1 (MΦ-1) or MΦ-2, human PB monocytes were cultured for 6 days in RPMI 1640 medium containing 10% FCS in the presence of 10 ng/ml GM-CSF or 50 ng/ml macrophage colony-stimulating factor, respectively (37). To generate monocyte-derived dendritic cells (DCs), human PB monocytes were cultured for 6 days in RPMI 1640 medium containing 10% FCS in the presence of 10 ng/ml IL-4 and 50 ng/ml GM-CSF (37). All human subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medicine Association. The study was approved by the Ethics Committee of Nihon University and the University of Tokyo.

**Abs and Other Reagents**—Rat anti-CD300A IgG2a mAb (6–2A) and mouse anti-CD300C IgG1 mAb (1E7/D) were generated from ACTGen. Anti-FLAG mAb (M2), FITC-conjugated anti-FLAG mAb (M2), mouse IgG1 mAb (MOPC21), mouse anti-dinitrophenyl IgE mAb (SPE-7), and LPS (Escherichia coli 0111:B4) were from Sigma-Aldrich. Anti-Myc mAb (9E10) was from Roche Applied Science. FITC-conjugated anti-mouse FcεRIα mAb, R-phycocerythrin (R-PE)-conjugated anti-mouse c-KIT mAb or streptavidin, and rat IgG2a were from eBioscience. R-PE-conjugated anti-human blood dendritic cell antigen-2 mAb and FITC-conjugated CD16 or CD123 mAbs were from Miltenyi Biotech. Anti-human triggering receptor expressed on myeloid cells-1 (TREM-1) mAb was from R&D Systems. FITC-conjugated anti-human CD3, CD19, or CD56 mAbs, R-PE-conjugated anti-human CD11b, CD14, CD80, CD83, CD86, or HLA-DR mAbs, and allophycocyanin-conjugated anti-human CD14 mAb were from eBioscience. Anti-ERK1 and ERK2 Abs were from Santa Cruz Biotechnology. Anti-phospho-p44/42 MAPK (pERK1/2) Ab was from Cell Signaling Technology. Anti-CD300A mAb, mouse IgG1 mAb, anti-CD300C mAb, and rat IgG2a mAbs were biotinylated by sulfo-NHS-LC-biotin (Pierce) according to the manufacturer’s instructions. The NK cell isolation kit, basophil isolation kit, eosinophil isolation kit, CD304 (blood cell antigen-4) MicroBead kit, and the CD14 MicroBeads were from Miltenyi Biotech. Cytokines were from R&D Systems. Sphingomyelin and sphingosylphosphorylcholine were from BIOMOL; C-24 ceramide was from Toronto Research Chemicals, Inc. Egg ceramide and cholesterol were from Avanti Polar Lipids, Inc. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE) were from Echelon Biosciences Inc.
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LDL and HDL were from Biomedical Technologies, Inc. Human RNA derived from a variety of tissues was from Clontech. All other reagents were from Sigma-Aldrich unless stated otherwise.

Gene Expression Analysis—Relative expression levels of human CD300C, GAPDH, and chemokine (C-C motif) ligand 1 (CCL1) among samples were measured by real time RT-PCR as described (7, 8). The following primers were used: 5′-AGAAA-GGACAGCCCGCCGAAC-3′ and 5′-CTAGAGCTTCTTCTGAG-GTCTG-3′ for CD300C; 5′-GGGAAATGTTGACAGCAA-G-3′ and 5′-CATTGGGACAGATGGAGC-3′ for CCL1; and 5′-GAAAGGTGAAGGTCGGAC-3′ for GAPDH. Relative gene expression levels were calculated using standard curves generated by serial dilutions of cDNA and normalized by a GAPDH expression level. Gene expression analysis was performed using real time RT-PCR as described (7, 8).

Plasmid Constructs—cDNA of mouse CD300A, CD300B, CD300C, CD300D, CD300E, CD300F, GAPDH, and chemokine (C-C motif) ligand 1 was isolated by PCR from a cDNA library of human peripheral mononuclear cells. The cDNA fragment of each CD300 family member, lacking the signal sequence, was tagged with a Myc epitope at the N terminus. Site-directed mutagenesis was also performed by using pMXs-FLAG-CD300A-IP or pMXs-FLAG-CD300C-IP, respectively, as a template. To generate CD300A(F56L-L57R)-CD3γ mutant or CD300C(L63F-R64L)-CD3γ mutant, two-step PCR mutagenesis was also performed by using pMXs-FLAG-CD300A-CD3γ-IP or pMXs-FLAG-CD300C-CD3γ-IP, respectively, as a template. All of the constructs were verified by DNA sequencing.

Generation of Fc Fusion Proteins—The Fc fusion proteins, CD300A-Fc, CD300C-Fc, and Fc were purified as described (26, 30).

Flow Cytometry—Cells were stained as described (7–9). Flow cytometric analysis was performed with FACS Calibur (BD Biosciences) equipped with CellQuest software and FlowJo software (TreeStar). To monitor apoptosis, cells were incubated with R-PE-conjugated annexin V (BD Biosciences) at room temperature for 20 min.

Transfection and Infection—Retroviral transfection and infection were performed as described (39, 41). Retroviruses were generated by transient transfection of PLAT-E packaging cells (41). Selection with 1 μg/ml puromycin or 10 μg/ml blasticidin was started 48 h after infection. 2B4-GFP cells were transduced with CD300A-CD3γ, CD300C-CD3γ, CD300A(F56L-L57R)-CD3γ, or CD300C(L63F-R64L)-CD3γ to generate 2B4-CD300A, CD300C, CD300A(F56L-L57R), or CD300C(L63F-R64L)-GFP cells, respectively. 2B4-CD300C-GFP cells were further transduced with CD300A or mock.

Biochemistry—Western blotting was performed as described (7–9). Equal amounts of cell lysates of Ba/F3 transfectants were immunoprecipitated with anti-FLAG mAb and were immunoblotted with anti-Myc mAb. Equal amounts of total cell lysates of Ba/F3 or BMMC transfectants were immunoblotted with anti-FLAG mAb, anti-Myc mAb, anti-phospho-ERK1/2 Ab, or anti-ERK1/2 Ab.

Binding Assay Using Solid Phase ELISA—Solid phase ELISA was used (26, 30). Briefly, the indicated lipids in methanol (50 μg/ml) or methanol as a control was added to ELISA plates and air-dried. Alternatively, plates were coated with 20 μg/ml of protein or PBS as a control. After washing, the plates were incubated with 5, 10, or 20 μg/ml of CD300A-Fc, CD300C-Fc, or Fc in the presence of 0.5 mM CaCl2 for 120 min before incubating with peroxidase-conjugated anti-human Ig (Sigma-Aldrich). Absorbance at 450 nm was measured.

Measurement of Cytokines—Human PB-derived mast cells or monocytes were stimulated with plate-coated anti-CD300C mAb, anti-TREM-1 mAb, or mouse IgG1 as a control in the presence or absence of 10 ng/ml LPS for 24 h. Plates were coated overnight with 20 μg/ml of each Ab. Alternatively, human monocyes were stimulated with plate-coated PE for 24 h. PE in methanol (10 μg/ml) or methanol as a control was added to plates and air-dried (30). BMMCs transduced with FLAG-tagged CD300C were stimulated with 10 μg/ml of either...
anti-FLAG mAb or mouse IgG1 mAb as a control, 5 μg/ml SPE-7 IgE, or 100 nm phorbol 12-myristate 13-acetate (PMA) for 24 h. Concentrations of human TNFα (R&D Systems) or IL-8 (BD Biosciences) or mouse IL-6 or TNFα (R&D Systems) in culture supernatants were measured using ELISA, as described (7–9).

Phagocytosis Assay—Phagocytosis assay was performed as previously described (26, 42, 43). Carboxyfluorescein diacetate succinimidyl ester–labeled live or apoptotic U937 cells were co-incubated for 60 min with NIH3T3 transfectants. The percentage of carboxyfluorescein diacetate succinimidyl ester–positive cells was measured by flow cytometric analysis.

Statistical Analysis—The data are shown as the means plus or minus S.D., and statistical significance was determined by the Student’s t test.

RESULTS

Generation of Abs Discriminating between CD300A and CD300C—To clarify CD300C expression profiles in human, we performed real time RT-PCR analysis using RNA samples derived from a variety of human tissues. The results showed that CD300C was more highly expressed in PB than in other tissues (Fig. 1A). We then investigated the expression patterns of CD300C in human hematopoietic cell lines. Real time RT-PCR analysis showed that CD300C was expressed in human myeloid cell lines, including HMC-1 mast cell lines, but not in lymphoid cell lines (Fig. 1B). We compared CD300C transcript levels among different lineages of PB cells displaying relatively high levels of CD300C expression in CD14-positive monocytes (Fig. 1C). Next, to examine surface expression of CD300C in human hematopoietic cells, we generated Abs specific for CD300A or CD300C. To test the sensitivity and specificity of these two Abs, Ba/F3 cells were transduced with FLAG-tagged CD300A, CD300B, CD300C, CD300D, CD300E, or CD300F. Flow cytometric analysis using anti-FLAG mAb confirmed surface expression of FLAG-tagged CD300A, B, C, E, or F in Ba/F3 transfectants. Consistent with a previous report, surface expression of CD300D was undetectable in FLAG-tagged CD300D-transduced Ba/F3 cells (44). Staining with either anti-CD300A or anti-CD300C mAb demonstrated that an extracellular domain of CD300A or CD300C was specifically recognized by anti-CD300A mAb or anti-CD300C mAb, respectively (Fig. 1D). Thus, we generated Abs discriminating between CD300A and CD300C, which were highly homologous in an extracellular domain.

CD300C Was Highly Expressed in Human Monocytes and Mast Cells—To investigate surface expression of CD300A or CD300C in primary hematopoietic cells, different cell fractions, including T cells, B cells, monocytes, NK cells, PDCs, basophils, neutrophils, and eosinophils, were isolated from PB cells and stained with mAb against CD300A or CD300C. In accordance with previous reports, CD300A was highly expressed in several types of cells, including monocytes, NK cells, PDCs, and basophils (Fig. 2A) (16–25). Consistent with results by real time RT-PCR analysis, flow cytometric analysis displayed relatively high levels of surface CD300C expression in CD14–positive monocytes. Weak levels of surface CD300C expression were also found in a small population of NK cells or PDCs. We found no significant expression of surface CD300C in the cells we tested from other lineages (Fig. 2A). We then asked whether CD300C was expressed in Mφ or DCs. To this end, CD11b+CD11c+CD14+HLA-DR+Mφ-1, CD11b+CD11c+CD14+HLA-DR+Mφ-2, and CD80+CD83lowCD86+DCs were generated from CD14–positive monocytes (Fig. 2, B and C). Interestingly, CD300C was weakly expressed in both Mφ-1 and Mφ-2, whereas CD300A was expressed only in Mφ-2 (Fig. 2B). Notably, neither CD300A nor CD300C was expressed in monocyte–derived DCs (Fig. 2C). Whereas stimulation with LPS or TNFα up-regulated CD80, CD83, or CD86 in monocyte–derived DCs, it induced expression of neither CD300A nor CD300C (Fig. 2C). Alternatively, we generated human PB–derived mast cells to test whether CD300C was expressed in human mast cells. The results showed that CD300C, as well as CD300A, was highly expressed in human mast cells (Fig. 2D). Taken together, our results indicated that CD300C is highly expressed in human monocytes and mast cells.

FcRγ Was Required for the Activating Functions of CD300C in Mast Cells—To ascertain whether CD300C functions as an activating receptor in human mast cells, human PB–derived mast cells were stimulated with plate-coated anti-CD300C mAb or mouse IgG1 mAb as a control. Remarkably, human mast cells stimulated by CD300C cross-linking produced significant levels of IL-8 protein or CCL1 mRNA (Fig. 3, A and B), implicating CD300C as an activating receptor in human mast cells. To investigate whether CD300C interacted with adaptor molecules containing ITAM and the related activating motif, Ba/F3 cells were transduced with FLAG-tagged CD300C or mock together with Myc-tagged mouse DAP10, DAP12, FcRγ, CD3ζ, or mock. Co-immunoprecipitation experiments demonstrated that CD300C bound only mouse FcRγ (Fig. 3C). Similar experiments using human adaptor molecules confirmed that CD300C physically interacted only with human FcRγ as well (data not shown). We then generated WT or FcRγ-deficient BMMCs transduced with FLAG-tagged CD300C. Flow cytometric analysis showed that the surface expression levels of c-Kit were comparable between these two types of BMMC transfectants, whereas FcεRI expression were undetectable in FcRγ-deficient BMMC transfectants (Fig. 3D, left panel) (7). FcRγ-deficient BMMC transfectants exhibited detectable but lower levels of surface CD300C expression as compared with WT BMMC transfectants, indicating that FcRγ was required for the efficient surface expression of CD300C (Fig. 3D, right panel). We then examined the effect of FcRγ deficiency on the CD300C–mediated activation signal. To this end, WT or FcRγ-deficient BMMCs expressing FLAG-tagged CD300C were stimulated with anti-FLAG mAb or mouse IgG1 as a control. Immunoblot analysis showed that stimulation with anti-FLAG mAb, but not a control mAb, led to the efficient phosphorylation of ERK1/2 in FcRγ–sufficient BMMC transfectants. On the other hand, FcRγ deficiency abolished ERK1/2 phosphorylation in BMMC transfectants stimulated by anti-FLAG mAb (Fig. 3E). Consistent with these results, FcRγ deficiency dampened cytokine production of FLAG-tagged CD300C–transduced BMMCs stimulated by anti-FLAG mAb (Fig. 3F). We confirmed that irrespective of FcRγ expression, these BMMC
transfectants released comparable levels of cytokines in response to PMA stimulation (Fig. 3). We also found that stimulation with anti-FLAG mAb rendered FcRγ-sufficient, but not FcRγ-deficient, BMMCs expressing FLAG-tagged CD300C resistant to apoptosis induced by the withdrawal of IL-3 (Fig. 3G) (8, 34). Taken together, these results suggested that FcRγ was indispensable for both the efficient surface expression and activating functions of CD300C. To further test whether ITAM of FcRγ was essential for activating functions of CD300C, FcRγ-deficient BMMCs were transduced with hFcRγ WT, hFcRγ(Y65F-Y76F) mutant, or mock together with FLAG-tagged CD300C or mock. We found equivalent levels of surface c-Kit expression among these BMMC transfectants. Whereas surface expression levels of FceRI were comparable among BMMCs transduced with hFcRγ WT or hFcRγ(Y65F-Y76F) mutant, surface expression of FceRI was undetectable in FcRγ-deficient BMMC transfectants (Fig. 4A, upper panel) (7). Surface expression levels of CD300C were comparable between BMMCs transfectants expressing FLAG-tagged CD300C with either hFcRγ WT or hFcRγ(Y65F-Y76F) mutant (Fig. 4A, lower panel).
When these BMMC transfectants expressing FLAG-tagged CD300C were stimulated with anti-FLAG mAb, ERK phosphorylation was found only in BMMC transfectants expressing hFcR/H9253 WT, but not in those expressing hFcR/H9253 (Y65F-Y76F) mutant (Fig. 4B). We confirmed that stem cell factor stimulation caused equivalent levels of ERK phosphorylation among these transfectants (Fig. 4B). In accordance with these results, stimulation with anti-FLAG mAb led to cytokine production only in BMMCs expressing hFcRy WT, but not in those expressing hFcRy(Y65F-Y76F) mutant (Fig. 4B). We confirmed that stem cell factor stimulation caused equivalent levels of ERK phosphorylation among these transfectants (Fig. 4B). In accordance with these results, stimulation with anti-FLAG mAb led to cytokine production only in BMMCs expressing hFcRy WT, but not in those expressing hFcRy(Y65F-Y76F) mutant, whereas PMA stimulation caused equivalent levels of cytokine production among the BMMC transfectants tested (Fig. 4C). Collectively, our findings suggest that CD300C delivers an activating signal that depends on an ITAM of FcRy.

**CD300C Functioned as an Activating Receptor in Human Monocytes**—To examine whether CD300C delivered an activating signal in human monocytes as well as in mast cells, CD14-positive monocytes were isolated from human PB. Human monocytes were stimulated with plate-coated anti-CD300C mAb, anti-TREM-1 mAb, or mouse IgG1 mAb as a control. As reported, stimulation with anti-TREM-1 mAb induced robust cytokine production in human monocytes.
FIGURE 3. Specific engagement of CD300C activated human mast cells. A and B, human PB-derived mast cells were incubated for 24 h on plates coated with anti-CD300C mAb, mouse IgG1 mAb, or PBS. IL-8 released into the culture supernatant was measured by ELISA (A). Relative gene expression levels of CCL1 were estimated by real time PCR. The amount of expression was indicated relative to that in nonstimulated cells (B). All of the data points correspond to the means ± S.D. of three independent experiments.

FIGURE 4. An ITAM of Fcγ was required for CD300C-mediated activation of mast cells. A, Fcγ-deficient BMMCs were transduced with hFcγ WT, hFcγ(Y65F-Y76F) mutant, or mock together with FLAG-tagged CD300C or mock. Surface expression levels of FLAG-tagged CD300C (lower panel) or c-Kit and FcɛRI (upper panel) in BMMC transfectants were measured by flow cytometry. B, Fcγ-deficient BMMCs expressing hFcγ WT, hFcγ(Y65F-Y76F) mutant, or mock together with FLAG-tagged CD300C were stimulated with 10 μg/ml anti-FLAG mAb or 10 ng/ml stem cell factor (SCF) for the indicated time. Cell lysates were immunoblotted (IB) with anti-pERK1/2 Ab or anti-ERK1/2 Ab. C, Fcγ-deficient BMMCs expressing hFcγ WT, hFcγ(Y65F-Y76F) mutant, or mock together with FLAG-tagged CD300C or mock were stimulated for 24 h with 10 μg/ml of either anti-FLAG mAb or mouse IgG1 mAb, 5 μg/ml SPE-7 IgE, or 100 nM PMA. IL-6 released into the culture supernatants was measured by ELISA. All of the data points correspond to the means ± S.D. of three independent experiments.

* p < 0.05.
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FIGURE 5. Specific engagement of CD300C activated human monocytes. A and B, human PB-derived monocytes were stimulated for 24 h with plate-coated anti-CD300C mAb, anti-TREM-1 mAb, or mouse IgG1 mAb. TNFα released into the culture supernatants was measured by ELISA (A). The cells were stained with R-PE-conjugated anti-CD83 or CD86 mAb. The results of control staining are shown as filled histograms (B). C, human PB-derived monocytes were stimulated for 24 h with plate-coated anti-CD300C mAb, anti-TREM-1 mAb, or mouse IgG1 mAb in the presence or absence of 10 ng/ml LPS. TNFα released into the culture supernatants was measured by ELISA. A and C, all of the data points correspond to the means ± S.D. of three independent experiments. Statistically significant differences are shown. *, p < 0.05.

(2, 45). Specific engagement of CD300C caused cytokine production at levels comparable with that of TREM-1 in human monocytes (Fig. 5A). In agreement with these findings, engagement of CD300C as well as that of TREM-1 up-regulated surface expression of both CD83 and CD86 in human monocytes (Fig. 5B). Plate-coated control Ab induced neither cytokine production nor up-regulation of costimulatory molecules in human monocytes (Fig. 5, A and B). It should be noted that plate-coated anti-CD300C mAb failed to induce cytokine production in both Mb-1 and Mb-2 that are CD300C (data not shown). To further examine whether the CD300C signal synergized with the Toll-like receptor 4 signal, human monocytes were stimulated with plate-coated anti-CD300C mAb, anti-TREM-1 mAb, or mouse IgG1 in the presence or absence of LPS. Like TREM-1 cross-linking, CD300C cross-linking synergistically enhanced the cytokine production of human monocytes stimulated by LPS (Fig. 5C) (2, 45). Thus, CD300C delivered an activation signal in human monocytes.

PE More Strongly Induced GFP Expression in 2B4-CD300A-GFP Cells than in 2B4-CD300C-GFP Cells—To identify a ligand for CD300A or CD300C, we used the reporter cell lines (2B4-GFP) (30–32). A chimera receptor CD300A-CD3ζ or CD300C-CD3ζ was transduced into 2B4-GFP cells to generate 2B4-CD300A-GFP cells or 2B4-CD300C-GFP cells, respectively (Fig. 6A). The addition of a soluble anti-CD300A mAb or anti-CD300C mAb to the culture did not induce GFP expression in 2B4-CD300A-GFP cells or 2B4-CD300C-GFP cells, respectively (data not shown). However, plate-coated anti-FLAG mAb induced GFP expression in both 2B4-CD300A-GFP cells and 2B4-CD300C-GFP cells, but not in parental 2B4-GFP cells (Fig. 6B and data not shown). To find out whether lipid or lipoprotein worked as a ligand for CD300A or CD300C, reporter cell lines were incubated on plates coated with several types of lipids, including ceramide, phosphatidylcholine, PE, PS, sphingomyelin, sphingosylphosphorylcholine, cholesterol, LDL or HDL. Notably, GFP expression was induced only by PE among lipids and lipoproteins tested in both 2B4-CD300A-GFP cells and 2B4-CD300C-GFP cells but not in 2B4-GFP cells (Fig. 6B and data not shown). It should be noted that PE-induced GFP expression levels were higher in 2B4-CD300A-GFP cells than in 2B4-CD300C-GFP cells (Fig. 6B). Importantly, PE-induced GFP expression in 2B4-CD300A-GFP cells or 2B4-CD300C-GFP cells was abolished by pretreatment with a soluble anti-CD300A mAb or anti-CD300C mAb, respectively (Fig. 6C). These results indicated that the specific interaction between PE and an extracellular domain of CD300A or CD300C induced GFP expression in the reporter cells. In accordance with these results, physical binding assay using ELISA showed that CD300A-Fc and CD300C-Fc, as compared with control Fc, significantly bound to PE. In addition, CD300A-Fc more strongly bound to PE than did CD300C-Fc (Fig. 6D, upper panel). Although PS failed to induce GFP expression in the reporter cells, CD300A-Fc and CD300C-Fc, as compared with control Fc, significantly bound to PS (Fig. 6D, lower panel). The binding affinity of CD300A-Fc to PS was also stronger than that of CD300C-Fc to PS (Fig. 6D, lower panel). In any case, PE may work as a possible ligand for CD300C as well as CD300A in vitro.

Differential Recognition of PE by CD300A and CD300C Depended on Different Amino Acid Residues CD300A(F56-L57) and CD300C(L63-R64)—Because the amino acid sequences of an extracellular domain were highly homologous between CD300A and CD300C, a mAb specific for CD300A or CD300C was expected to discriminate among subtle differences in an extracellular domain between them (supplemental Fig. S1). We paid attention to amino acid residues Phe-56 and Leu-57 in CD300A and L63-R64 in CD300C. For further analysis, FLAG-tagged CD300A(F56L-L57R) or CD300C(L63F-R64L) was transduced into Ba/F3 cells. Staining using anti-FLAG mAb verified surface expression of FLAG-tagged CD300A, CD300A(F56L-L57R), CD300C, or CD300C(L63F-R64L) in the Ba/F3 transfectants. Surface expression levels of CD300A WT or mutant were found to be higher than those of CD300C WT or mutant (Fig. 7A, top row). Interestingly, anti-CD300A mAb detected surface expression of CD300C(L63F-R64L) as well as CD300A (Fig. 7A, middle row), whereas anti-CD300C mAb detected CD300A(F56L-L57R) as well as CD300C (Fig. 7A, bottom row). Anti-CD300A mAb recognized neither CD300C nor CD300A(F56L-L57R), whereas anti-CD300C mAb recognized neither CD300A nor CD300C(L63F-R64L) (Fig. 7A, middle and bottom rows). Collectively, these results indicated that anti-CD300A mAb and anti-CD300C mAb detected the specific structures depending on amino acid residues CD300A(F56L57) and CD300C(L63-R64), respectively. We next asked
whether the differential recognition of PE by an extracellular domain of CD300A or CD300C was also associated with the different amino acid residues CD300A(F56-L57) and CD300C(L63-R64). For this purpose, we generated 2B4-CD300A(F56L-L57R)-GFP cells and 2B4-CD300C(L63F-R64L)-GFP cells as additional reporter cells. Plate-coated anti-FLAG mAb induced equivalent levels of GFP expression in 2B4-CD300A-GFP, 2B4-CD300A(F56L-L57R)-GFP, 2B4-CD300C-GFP, or 2B4-CD300C(L63F-R64L)-GFP cells (Fig. 7B, second row). Stimulation with PMA and ionomycin nicely induced GFP expression in the reporter cells tested, including the parental 2B4-GFP cells (Fig. 7B, third row). Consistent with the specific recognition of amino acid residues CD300A(F56-L57) and CD300C(L63-R64) by anti-CD300A mAb and anti-CD300C mAb, respectively, plate-coated anti-CD300A mAb induced GFP expression only in 2B4-CD300C(L63F-R64L)-GFP cells, as well as 2B4-CD300A-GFP cells (Fig. 7B, fourth row). In addition, plate-coated anti-CD300C mAb induced GFP expression only in 2B4-CD300A(F56L-L57R)-GFP cells, as well as 2B4-CD300C-GFP cells (Fig. 7B, fifth row). Importantly, substitution of Phe-56 and Leu-57 with Leu-56 and Arg-57 in an extracellular domain of CD300A reduced GFP expression.

**FIGURE 6.** Plate-coated PE induced GFP expression more strongly in 2B4-CD300A-GFP cells than in 2B4-CD300C-GFP cells. A, the schematic structure of the chimera receptor CD300A-CD3/CD300C chimera receptor expressed in 2B4-CD300A-GFP cells or the chimera receptor CD300C-CD3/CD300A chimera receptor expressed in 2B4-CD300C-GFP cells. B and C, flow cytometry of GFP expression of the reporter cells. 2B4-CD300A-GFP cells or 2B4-CD300C-GFP cells were incubated for 24 h on plates coated with C-24 ceramide, egg ceramide, phosphatidylcholine (PC), PE, PS, sphingomyelin (SM), sphingosylphosphorylcholine (SPC), cholesterol, LDL, HDL, or anti-FLAG mAb (B). 2B4-CD300A-GFP cells or 2B4-CD300C-GFP cells were incubated for 24 h on plates coated with PE. After incubating the reporter cells, 20 μg/ml of either anti-CD300A mAb or rat IgG2a mAb or 20 μg/ml of either anti-CD300C mAb or mouse IgG1 mAb was added to the culture of 2B4-CD300A-GFP cells (upper panel) or 2B4-CD300C-GFP cells (lower panel), respectively (C). D, CD300A-Fc, CD300C-Fc, or Fc bound to wells was quantified by ELISA. Indicated concentrations of CD300A-Fc, CD300C-Fc, or Fc were incubated on plates coated with PE (upper panel) or PS (lower panel). All of the data points correspond to the means ± S.D. of three independent experiments. Statistically significant differences are shown. *, p < 0.05.
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FIGURE 7. The differential recognition of PE by CD300A and CD300C depended on the specific structure involving amino acid residues CD300A(F56-L57) and CD300C(L63-R64). A, Ba/F3 cells were transduced with FLAG-tagged CD300A, CD300A(F56-L57R), CD300C, CD300C(L63F-R64L), or mock. The cells were stained with anti-FLAG mAb (first panel), anti-CD300A mAb (second panel), or anti-CD300C mAb (third panel). The results of control staining are shown as filled histograms. B and C, flow cytometry of GFP expression of the reporter cells. The data are representative of three independent experiments. B, 2B4-GFP cells, 2B4-CD300A-GFP cells, 2B4-CD300A(F56L-L57R)-GFP cells, 2B4-CD300C-GFP cells, or 2B4-CD300C(L63F-R64L)-GFP cells were incubated for 24 h in the presence of 100 nM PMA plus 1 μM ionomycin (third row), live U937 cells (sixth row), or apoptotic U937 cells (seventh row) or on plates coated with PE (first row), anti-FLAG mAb (second row), anti-CD300A mAb (fourth row), or anti-CD300C mAb (fifth row). C, 2B4-GFP cells or 2B4-CD300C-GFP cells transduced with Myc-tagged CD300A or mock were incubated for 24 h in the presence of 100 nM PMA plus 1 μM ionomycin (second row) or on plates coated with PE (first row).

induced by plate-coated PE in 2B4-CD300A-GFP cells, whereas substitution of Leu-63 and Arg-64 with Phe-63 and Leu-64 in an extracellular domain of CD300C increased that in 2B4-CD300C-GFP cells (Fig. 7B, first row). Thus, higher levels of GFP expression induced by PE in 2B4-CD300A-GFP cells compared with 2B4-CD300C-GFP cells were explained partly by the different amino acid residues CD300A(F56-L57) and CD300C(L63-R64). To mimic the effect of PE on the cells expressing CD300C with CD300A, 2B4-GFP or 2B4-CD300C-GFP cells were further transduced with full-length CD300A or mock. Stimulation with PMA plus ionomycin efficiently induced GFP expression in the transduced reporter cells tested (Fig. 7C, lower row). Interestingly, PE-induced GFP expression in 2B4-CD300C-GFP cells was dampened by co-expression of CD300A (Fig. 7C, upper row). These results indicated that PE-CD300A binding inhibited activating signal induced by the interaction between PE and an extracellular domain of CD300C in the reporter cells. Because it was recently reported that apoptotic cells were recognized by mouse and human CD300A (28, 29), we tested whether apoptotic cells worked as functional ligands for CD300C. Live U937 cells failed to induce GFP expression in the reporter cells tested, whereas apoptotic U937 cells did induce GFP expression in both 2B4-CD300A-GFP cells and 2B4-CD300C-GFP cells (Fig. 7B, sixth and seventh rows). Like PE-induced GFP expression, that induced by apoptotic cells was stronger in 2B4-CD300A-GFP cells than in 2B4-CD300C-GFP cells (Fig. 7B, sixth and seventh rows). However, the same replacement of amino acid residues in an extracellular domain of CD300A or CD300C did not affect GFP expression induced by apoptotic cells in the reporter cells (Fig. 7B, sixth and seventh rows). These results indicated that the differential recognition of apoptotic cells by CD300A and CD300C was independent of the structural differences involving amino acid residues CD300A(F56-L57) and CD300C(L63-R64). We asked finally whether PE and apoptotic cells worked a functional ligand for CD300C in human monocytes. PB-deprived monocytes were incubated on the plates coated with PE or with apoptotic U937 cells. Notably, neither plated-coated PE nor apoptotic cells stimulated cytokine production of human monocytes expressing both CD300A and CD300C (supplemental Fig. S2). Collectively, specific engagement of CD300C, but not co-engagement of CD300C with CD300A, delivered an activating signal in CD300C<sup>high</sup> cells.

DISCUSSION

It is widely accepted that human CD300A is broadly expressed in hematopoietic cells and delivers an inhibitory signal via immunoreceptor tyrosine-based inhibitory motifs in the cytoplasmic region (16–25). On the other hand, CD300C, a putative counterpart of CD300A, has been difficult to fully characterize because of the unavailability of a specific CD300C Ab. Here, we succeeded in generating mAbs discriminating between CD300A and CD300C. Intriguingly, these two mAbs recognized the specific structure of CD300A or CD300C, involving amino acid sequences CD300A(F56-L57) or CD300C(L63-R64), respectively. By using a specific CD300C mAb, we provided the first demonstration that CD300C was highly expressed in human monocytes and mast cells, whereas
it was weakly expressed in Mϕ and a small population of NK cells or PDCs. Importantly, engagement of CD300C with its specific mAb led to cytokine/chemokine production in CD300<sup>high</sup> cells such as monocytes and mast cells, but not in CD300<sup>low</sup> Mϕ. The finding that CD300C signal synergized with Toll-like receptor 4 signal in monocytes implicated an activating CD300C in innate immune responses. On the other hand, low levels of CD300C expression in Mϕ, NK cells, or PDCs implied a limited role of CD300C in these cells. Although CD300C signaling was reported to be partially mediated by its interaction with FcRγ (15), our results demonstrated that CD300C physically interacted with an ITAM-bearing adaptor protein FcRγ, thereby delivering an activating signal in an FcRγ ITAM-dependent manner. In addition, FcRγ was shown to be required for the efficient cell surface expression of CD300C. We concluded that FcRγ was essential for activating functions of CD300C in mast cells and monocytes.

Our results showed that CD300A was highly expressed in the cells expressing CD300C, except for Mϕ-2, which were CD300A<sup>−</sup>CD300C<sup>low</sup>. Accordingly, if CD300A and CD300C had a similar or the same ligand, CD300C-mediated activating signal might be counterbalanced by CD300A-mediated inhibitory signal in mast cells and monocytes. It is widely accepted that the output signal in such situations is likely determined by the affinity for and the prevalence of ligands, as well as the expression levels of receptors. In any case, identifying a ligand for CD300A as well as CD300C is necessary to clarify the physiological role of CD300C. In accordance with recent findings that human and mouse CD300A recognized apoptotic cells (28, 29), our results demonstrated that CD300C high cells, but not CD300C low cells, recognized apoptotic cells. The causal relationship between the binding of CD300A to PE and/or PS exposed on the cell surface of apoptotic cells and the following CD300A signal warrants careful examination (46–50).

Intriguingly, a reporter assay using an extracellular domain of CD300C showed that PE and apoptotic cells were possible ligands for CD300C, as well as CD300A. However, levels of GFP expression induced by PE or apoptotic cells in 2B4-CD300C-GFP cells were lower than those in 2B4-CD300A-GFP cells, indicating that PE and apoptotic cells were more strongly recognized by extracellular CD300A in comparison with CD300C. Analyses of 2B4-CD300A, CD300A(F56L-L57R), CD300C, or CD300C(L63F-R64L)-GFP cells indicated that the different amino acid residues CD300A(F56-L57) and CD300C(L63-R64) were responsible for the differential recognition of PE, but not of apoptotic cells, by CD300A and CD300C. According to the results from Simhadri <i>et al.</i> (29), amino acid residues 109–112 (WLRD), 115 (D), and 56 (F) of CD300A were pivotal for the binding of CD300A to apoptotic cells. However, because amino acid residues 109–115 of CD300A and 116–122 of CD300C were the same (WLRFHFD), these amino acid residues seemed not be involved in the differential recognition of apoptotic cells by CD300A and CD300C. In any case, stronger recognition of PE and apoptotic cells by CD300A compared with CD300C led us to speculate that the CD300A-mediated inhibitory signal dominated the CD300C-mediated activating signal when CD300A<sup>high</sup>CD300C<sup>high</sup> cells were stimulated with apoptotic cells or PE. In support of this, GFP expression induced by PE or apoptotic cells in 2B4-CD300C-GFP cells was abolished by co-expression of CD300A. In addition, stimulation with PE or apoptotic cells failed to activate CD300A<sup>high</sup>CD300C<sup>high</sup> monocytes. However, if CD300A was down-regulated under certain conditions such as infection, these ligands might be able to activate the CD300C<sup>high</sup> cells. Because stimulation with a specific CD300C mAb efficiently activated CD300C<sup>high</sup> cells, human monocytes or mast cells would be activated if stimulated with an unknown ligand specific for CD300C. Considering the accumulated research suggesting a role of the CD300 family as lipid sensors (28–30, 51), it is plausible to assume that CD300C-specific ligand is a physiological lipid as well. Lipids derived from apoptotic/necrotic cells or released from inflammatory cells might be candidate ligands for CD300C. Further extensive screening using our reporter assay will be useful for identifying an endogenous or exogenous ligand for CD300C. In conclusion, specific engagement of CD300C, but not co-engagement of CD300C with CD300A, activated human mast cells and monocytes in an FcRγ-dependent manner.

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