The CD300e molecule in mice is an immune-activating receptor

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Masamichi Isobe[‡§], Kumi Izawa[‡§], Masahiro Sugiuchi[§], Tamami Sakanishi[¶], Ayako Kaitani[‡§], Ayako Takamori[‡], Akie Maehara[‡], Toshihiro Matsukawa[§‖], Mariko Takahashi[§], Yoshinori Yamanishi[§], Toshihiko Oki[§], Shino Uchida[‡‡‡], Koichiro Uchida[‡], Tomoaki Ando[‡], Keiko Maeda[‡], Nobuhiro Nakano[‡], Hideo Yagita[§§], Toshiyuki Takai[¶¶], Hideoki Ogawa[‡], Ko Okumura[‡], Toshio Kitamura[§1], and Jiro Kitaura[‡§2]**

From the [‡]Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, the [§]Division of Cellular Therapy/Division of Stem Cell Signaling, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, the [¶]Laboratory of Cell Biology, Research Support Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyoku, Tokyo, the [‖]Department of Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-0808, the [**]Department of Immune Regulation, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, the Departments of [‡‡‡]Gastroenterology Immunology and [§§]Immunology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, and the [¶¶]Department of Experimental Immunology, Institute of Development, Aging, and Cancer, Tohoku University, 4-1 Seiryo, Sendai 980-8575, Japan

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CD300 molecules (CD300s) belong to paired activating and inhibitory receptor families, which mediate immune responses. Human CD300e (hCD300e) is expressed in monocytes and myeloid dendritic cells and transmits an immune-activating signal by interacting with DNAX-activating protein 12 (DAP12). However, the CD300e ortholog in mice (mCD300e) is poorly characterized. Here, we found that mCD300e is also an immune-activating receptor. We found that mCD300e engagement triggers cytokine production in mCD300e-transduced bone marrow–derived mast cells (BMMCs). Loss of DAP12 and another signaling protein, FcRγ, did not affect surface expression of transduced mCD300e, but abrogated mCD300e-mediated cytokine production in the BMMCs. Co-immunoprecipitation experiments revealed that mCD300e physically interacts with both FcRγ and DAP12, suggesting that mCD300e delivers an activating signal via these two proteins. Binding and reporter assays with the mCD300e extracellular domain identified sphingomyelin as a ligand of both mCD300e and hCD300e. Notably, the binding of sphingomyelin to mCD300e stimulated cytokine production in the transduced BMMCs in an FcRγ- and DAP12-dependent manner. Flow cytometric analysis with an mCD300e-specific Ab disclosed that mCD300e expression is highly restricted to CD115^{+}Ly-6C$^{low/int}$ peripheral blood monocytes, corresponding to CD14$^{dim/+}$CD16^{+} human nonclassical and intermediate monocytes. Loss of FcRγ or DAP12 lowered the surface expression of endogenous mCD300e in the CD115^{+}Ly-6C$^{low/int}$ monocytes. Stimulation with sphingomyelin failed to activate the CD115^{+}Ly-6C$^{low/int}$ mouse monocytes, but induced hCD300e-mediated cytokine production in the CD14^{dim}CD16^{+} human monocytes. Taken together, these observations indicate that mCD300e recognizes sphingomyelin and thereby regulates nonclassical and intermediate monocyte functions through FcRγ and DAP12.

A variety of paired activating and inhibitory receptor families, including immunoglobulin-like receptors and C-type lectin-like receptors, regulate immune responses (1–3). CD300 (also called leukocyte mono-immunoglobulin-like receptor (LMIR),[3] CMRF-35-like molecule (CLM), myeloid-associated immunoglobulin-like receptor, or immune receptor expressed by myeloid cell) belongs to paired immunoglobulin-like receptor families (4–13). CD300 members are mainly expressed in myeloid cells and contain a highly homologous single immunoglobulin-like domain in their extracellular regions; CD300a (LMIR1 or CLM-8) and CD300f (LMIR3 or CLM-1) are inhibitory receptors that harbor immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic regions, whereas other CD300 members are thought to be activating receptors that are coupled with adaptor proteins (*e.g.* FcRγ and DNAX-activating protein 12 (DAP12)) bearing immunoreceptor tyrosine-based activating motif (ITAM) (4–13). Multiple studies showed that lipids or lipid-binding proteins act as ligands for several members of the mouse and human CD300 family (14–20). For example, the recognition of ceramide by mouse CD300f or cer-

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[1] To whom correspondence may be addressed. Tel.: 81-3-5449-5759; Fax: 81-3-5449-5453; E-mail: kitamura@ims.u-tokyo.ac.jp.

[2] To whom correspondence may be addressed: Atopy Research Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel.: 81-3-5802-1591; Fax: 81-3-3813-5512; E-mail: j-kitaura@juntendo.ac.jp.

[3] The abbreviations used are: LMIR, leukocyte mono-immunoglobulin-like receptor; mCD300e, mouse CD300e; hCD300e, human CD300e; CLM, CMRF-35-like molecule; ITAM, immunoreceptor tyrosine-based activating motif; BMMC, bone marrow–derived mast cell; BM, bone marrow; PB, peripheral blood; BMmDC, BM-derived myeloid dendritic cell; BMpDC, BM-derived plasmacytoid dendritic cell; BMMϕ, BM-derived macrophage; R-PE, R-phycoerythrin; APC, allophycocyanin; IRES, internal ribosome entry sites; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SLAM, signaling lymphocyte-activating molecule; CNS, central nervous system; Ab, antibody; DAP12, DNAX-activating protein 12.

amide and sphingomyelin by human CD300f induces the suppression of various inflammatory responses (15, 21–24). CD300a, CD300b, CD300c, or CD300f binds to phosphatidylserine, thereby positively or negatively regulating apoptotic cell-mediated immune responses (16, 17, 19, 20). The binding of T-cell immunoglobulin and mucin domain 1 to CD300b accelerates renal ischemia/reperfusion injury (14).

Human CD300e (hereafter referred to as hCD300e) is expressed in monocytes and myeloid dendritic cells. Cross-linking of DAP12-coupled hCD300e with its specific antibody leads to cytokine production in human peripheral blood (PB) monocytes (10, 13). However, the CD300e ortholog in mice (hereafter referred to as mCD300e), also called LMIR6 or CLM-2, remains poorly characterized. In this study, we analyzed mCD300e-transduced bone marrow–derived mast cells (BMMCs) from wild-type (WT), *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice (25, 26), and demonstrate that mCD300e can transmit an activating signal in the transduced BMMCs by interacting with both FcRγ and DAP12. In addition, flow cytometric analysis using a newly generated antibody specific for mCD300e showed that mCD300e expression is highly restricted to CD115^{+}Ly-6C$^{low/int}$ PB monocytes. Moreover, both physical binding and functional reporter assays (15, 27, 28) using the extracellular domain of CD300e identified sphingomyelin as a candidate ligand for mouse and human CD300e.

Sphingomyelin is the most abundant sphingolipid in the cell and lipoprotein components. It is an essential element of plasma membrane that is crucial for cellular function (29, 30). In addition, it is abundant in the central nervous system (CNS); the levels of sphingomyelin in CNS are reported to be associated with the pathologies of CNS diseases, including ischemia/hypoxia, Alzheimer's disease, and Parkinson's disease (30).

Currently, three types of monocytes are classified in humans and mice. In humans, CD14^{+}CD16^{-}, CD14^{dim}CD16^{+}, and CD14^{+}CD16^{+} monocytes are classical, nonclassical (patrolling), and intermediate monocytes, respectively. These three types of monocyte populations correspond to CD115^{+}Ly-6C^{+}, CD115^{+}Ly-6C^{low}, and CD115^{+}Ly-6C^{int} monocytes in mice (31–33). Nonclassical (patrolling) monocytes exhibit unique functions in the vasculature, and play a specialized role in inflammatory diseases and cancer surveillance; they contribute to wound healing and resolution of inflammation in damaged tissues by removing damaged cells and debris (31–36). However, it was also reported that patrolling monocytes produce high levels of proinflammatory cytokines under various contexts (37–39). Accordingly, patrolling monocytes are implicated in the pathogenesis of a variety of inflammatory diseases (*e.g.* atherosclerosis, myocardial infarction, neurological disease, glomerulonephritis, and arthritis) or cancer by either promoting or suppressing the disease progression (31–36, 39–44). Our results together with previous findings (31–36) imply that mCD300e, a novel surface marker of nonclassical and intermediate monocytes, possibly recognize sphingomyelin, thereby controlling vascular and/or tissue inflammation.

Results

mCD300e is an N-glycosylated surface receptor

The full-length cDNA of mCD300e was isolated by PCR from a cDNA library of C57BL/6J mouse-derived bone marrow (BM) cells. The mCD300e protein is composed of an N-terminal signal peptide, extracellular region containing a single V-type immunoglobulin-like domain, transmembrane domain containing a positively-charged amino acid residue lysine, and short cytoplasmic tail without any signaling motif. The immunoglobulin-like domain of mCD300e shares 41% amino acid sequence identity with that of mouse CD300f, which is an inhibitory receptor (Fig. 1*A*). To analyze the function of mCD300e, FLAG-tagged mCD300e was transduced into Ba/F3, a pro-B cell line. Flow cytometric analysis showed that anti-FLAG antibody (Ab) specifically stained FLAG-tagged mCD300e on the surface of the transduced Ba/F3 cells, but not of the mock-transduced cells, demonstrating that mCD300e is a cell-surface receptor (Fig. 1*B*). Western blot analysis showed that FLAG-tagged mCD300e transduced into Ba/F3 cells was detected by anti-FLAG Ab as two discrete bands with differing mobilities (28–34 and 19 kDa) (Fig. 1*C*). Because an *N*-glycosylation site (asparagine at residue 84) is present within the immunoglobulin domain of mCD300e, we pretreated the same cell lysates with *N*-glycosidase F. The results showed that this pretreatment shifted the mobilities of the two forms of FLAG-tagged mCD300e (24–26 and 18 kDa) (Fig. 1*C*). Notably, FLAG-tagged mCD300e-N84Q, in which asparagine (N) at residue 84 was replaced with glutamine (Q), transduced into Ba/F3 cells was detected as two bands (24–26 and 18 kDa), nearly identical to those in the case of FLAG-tagged mCD300e after treatment with *N*-glycosidase F (Fig. 1*C*). In addition, treatment with *N*-glycosidase F no longer influenced the apparent molecular masses of FLAG-tagged mCD300e–N84Q (Fig. 1*C*). Because a positively charged amino acid residue lysine is present at residue 179 (Lys^{179}) within the transmembrane domain of mCD300e, we asked whether mCD300e could be coupled with an ITAM-containing adaptor protein that harbors a negatively charged residue in its transmembrane domain. We further performed co-immunoprecipitation experiments using HEK 293T cells transiently expressing FLAG-tagged mCD300e together with either Myc-tagged FcRγ or DAP12. The results showed that both FcRγ and DAP12 were co-immunoprecipitated with mCD300e (Fig. 1*D*), indicating their roles as candidate adaptor proteins for mCD300e. Collectively, mCD300e is an *N*-glycosylated surface receptor that can be coupled with ITAM-containing adaptor proteins FcRγ and DAP12.

mCD300e can deliver an activating signal in BMMC transfectants in both FcRγ- and DAP12-dependent manner

Because mouse CD300 members are mainly expressed in myeloid cells, FLAG-tagged mCD300e was transduced into BMMCs for further investigation of mCD300e function. Flow cytometric analysis confirmed that BMMCs transduced with FLAG-tagged mCD300e or mock-exhibited equivalent levels of c-Kit and FcεRI, both of which are mast cell-surface markers (7, 45) and that the former transfectants, but not latter, expressed FLAG-tagged mCD300e on the cell surfaces (Fig. 2*A*). Cultur-

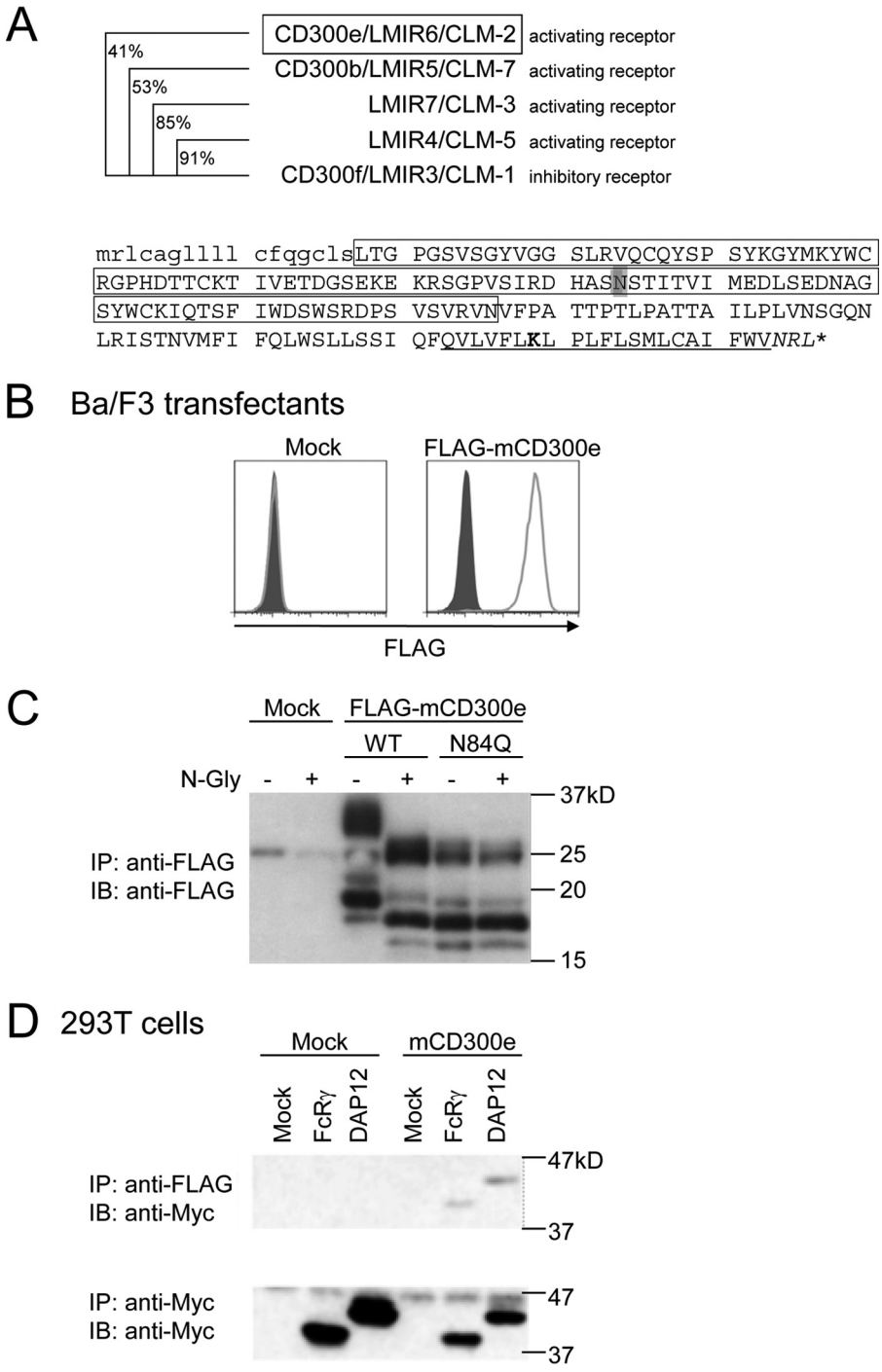

Figure 1. mCD300e is an *N*-glycosylated surface receptor. *A,* the phylogenetic tree of mouse LMIR3 (CLM-1/CD300f), LMIR4 (CLM-5), LMIR5 (CLM-7/CD300b), LMIR6 (CLM-2/CD300e), and LMIR7 (CLM-3) is shown on the basis of homology with the immunoglobulin-like domain (*upper panel*). The percentage of amino acid sequence identity of the immunoglobulin-like domain is indicated. Alignment of amino acid sequences for mCD300e is shown (*lower panel*). The putative signal sequence is shown in *lowercase*. An immunoglobulin-like domain is *boxed*. The transmembrane domain is *underlined*. The potential *N*-linked glycosylation site is *shaded*. The positively charged amino acid residue lysine within the transmembrane domain is shown in *bold*. *B,* Ba/F3 cells were transduced with FLAG-tagged mCD300e or mock. The transfectants were stained with mouse anti-FLAG Ab or mouse IgG1 Ab followed by PE-conjugated anti-mouse IgG goat $F(ab')_2$ Ab. *C,* lysates of Ba/F3 cells expressing FLAG-tagged mCD300e, mCD300e-N84Q, or mock were pretreated with or without *N*-glycosidase F before immunoprecipitation with mouse anti-FLAG Ab and subsequently immunoblotted with rabbit anti-FLAG Ab. *D,* 293T cells were transiently co-transfected with FLAG-tagged mCD300e construct or mock together with Myc-tagged FcRγ or DAP12 construct or mock. Immunoprecipitates of lysates of these transfectants with mouse anti-FLAG Ab were probed with anti-Myc Ab or rabbit anti-FLAG Ab. *B–D,* a representative of three independent experiments is shown. *N-Gly*, *IB*, or *IP* indicates *N*-glycosidase F, immunoblot, or immunoprecipitation, respectively.

ing the two types of BMMC transfectants on plates coated with anti-FLAG Ab revealed robust production of IL-6 in the BMMCs transduced with FLAG-tagged mCD300e, but not in mock transfectants (Fig. 2*B*). In contrast, stimulation with phorbol 12-myristate 13-acetate (PMA) induced comparable levels of IL-6 production in the two types of transfectants (Fig. 2*B*). These results indicate that mCD300e can act as an activating receptor. Next, to identify ITAM-containing adaptor pro-

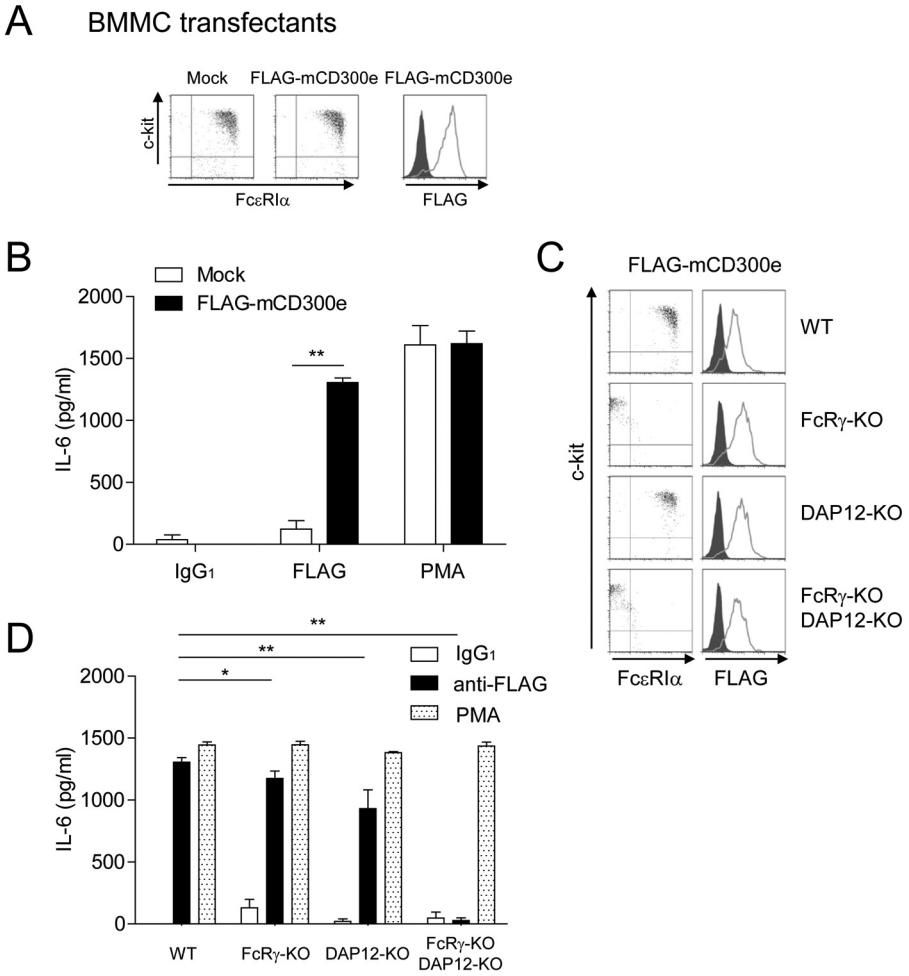

Figure 2. mCD300e delivers an activating signal in BMMC transfectants in both FcRγ- and DAP12-dependent manners. *A,* BMMCs transduced with FLAG-tagged mCD300e or mock were stained with FITC-conjugated anti-FcεRIα Ab and PE-conjugated anti-c-Kit Ab (*left panel*) or with mouse anti-FLAG Ab or mouse IgG1 Ab followed by PE-conjugated anti-mouse IgG goat $F(ab')_2$ Ab (*right panel*). *B,* BMMCs transduced with FLAG-tagged mCD300e or mock were stimulated with plate-coated anti-FLAG Ab or mouse IgG1 as control or with 100 nM PMA for 12 h. IL-6 released into the culture supernatants were measured by ELISA. *C,* FLAG-tagged mCD300e-transduced BMMCs from WT, *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice were stained with FITC-conjugated anti-FcεRIα Ab and PE-conjugated anti-c-Kit Ab (*left panel*) or with mouse anti-FLAG Ab or mouse IgG1 Ab followed by PE-conjugated anti-mouse goat IgG $F(ab')_2$ Ab (*right panel*). *D,* FLAG-tagged mCD300e-transduced BMMCs from WT, *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice were stimulated with plate-coated anti-FLAG Ab or mouse IgG1 Ab as control or with 100 nM PMA for 12 h. IL-6 released into the culture supernatants were measured by ELISA. *A* and *C*, a representative of three independent experiments is shown. *B* and *D*, all data points correspond to the mean ± S.D. of three independent experiments. Statistically significant differences are shown. *, $p < 0.05$ or **, $p < 0.01$ (Student's *t* test).

teins for mCD300e, BMMCs from *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice (25, 26) as well as WT mice were transduced with FLAG-tagged mCD300e. Flow cytometric analysis confirmed equivalent levels of c-Kit in these BMMC transfectants (Fig. 2*C*). Loss of FcRγ dampened the surface expression of FcεRI as expected (7), whereas loss of FcRγ and/or DAP12 failed to influence the surface expression levels of the transduced FLAG-tagged mCD300e (Fig. 2*C*). We then stimulated these BMMC transfectants with plate-coated anti-FLAG Ab or control Ab. The results showed that deficiency of FcRγ or DAP12 significantly lowered mCD300e-mediated IL-6 production (Fig. 2*D*). Remarkably, deficiency of both FcRγ and DAP12 abrogated mCD300e-mediated IL-6 production in BMMC transfectants (Fig. 2*D*). In contrast, stimulation with PMA induced comparable levels of IL-6 production in the transfectants tested (Fig. 2*D*). Collectively, both FcRγ and DAP12 were dispensable for maintaining surface expression of the transduced mCD300e; however, one or the other of the proteins were indispensable for mCD300e-mediated activation of BMMC transfectants.

Generation of specific antibody against mCD300e

To examine the mRNA expression profiles of mCD300e, we performed quantitative real-time PCR analysis using various mouse tissues. We found significantly higher expression levels of mCD300e in PB cells as compared with that in other tissues, although mCD300e expression was detected in the lung, liver, spleen, and BM (Fig. 3*A*). This suggests that mCD300e expression is restricted mainly to circulating PB cells and, to a lesser extent, possibly to tissues with abundant vascularity. To further examine mCD300e protein expression profiles in hematopoietic cells, we generated an Armenian hamster anti-mCD300e monoclonal Ab. To confirm the specificity of this Ab, Ba/F3 cells were transduced with FLAG-tagged mouse LMIR1/CLM-8 (CD300a), LMIR2/CLM-4, LMIR3/CLM-1 (CD300f), LMIR4/CLM-5, LMIR5/CLM-7 (CD300b), LMIR6/CLM-2

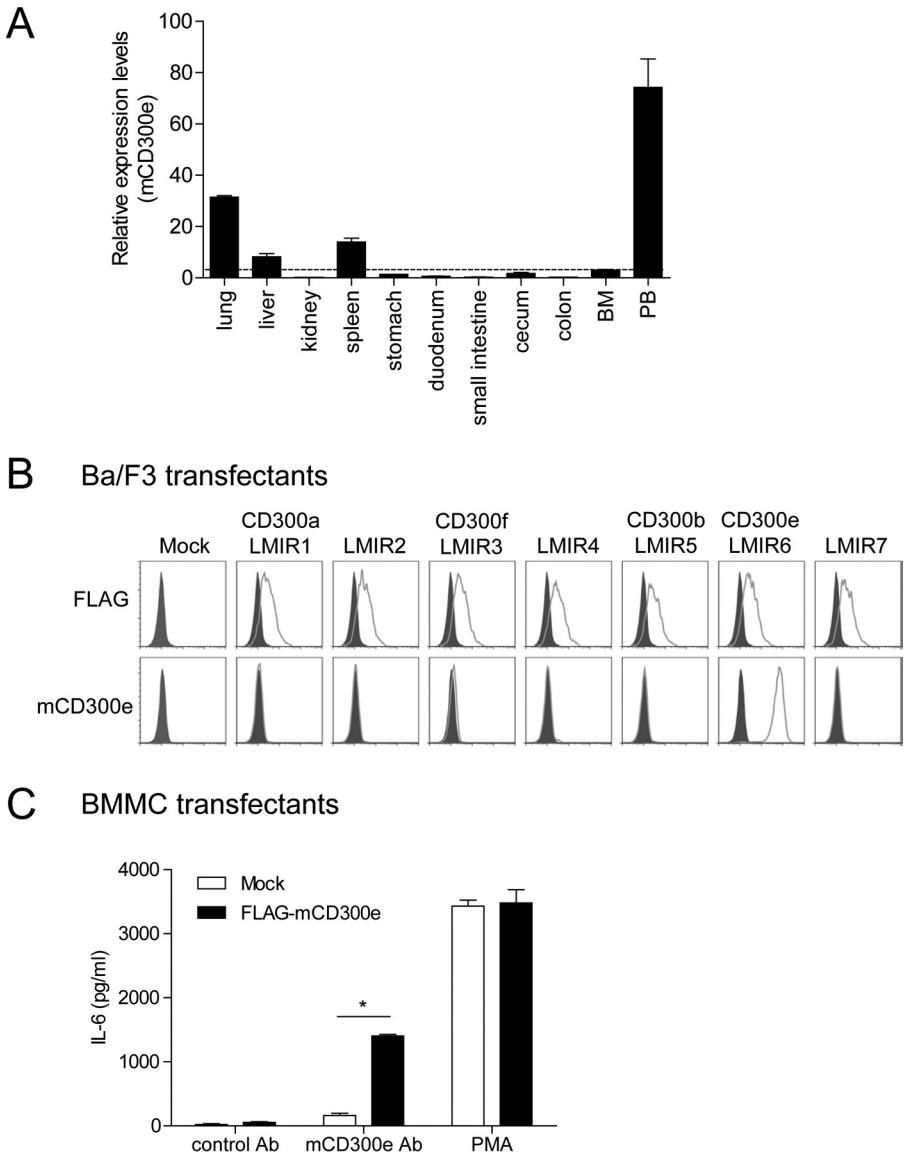

Figure 3. The generation of specific antibody against mCD300e. *A,* relative expression levels of mCD300e in indicated tissues were estimated by real-time PCR. The amount of expression was indicated relative to that in BM. *B,* Ba/F3 cells were transduced with FLAG-tagged mouse LMIR1 (CD300a), LMIR2, LMIR3 (CD300f), LMIR4, LMIR5 (CD300b), LMIR6 (CD300e), LMIR7, or mock. The transfectants were stained either with mouse anti-FLAG Ab or mouse IgG1 Ab followed by PE-conjugated anti-mouse IgG goat $F(ab')_2$ Ab (*upper panel*) or with biotinylated anti-mCD300e Ab or Armenian hamster IgG Ab followed by PE-conjugated streptavidin (*lower panel*). *A* and *B,* data are representative of three independent experiments. *C,* BMMCs transduced with FLAG-tagged mCD300e or mock were stimulated with plate-coated anti-mCD300e Ab or Armenia hamster IgG Ab as control or with 100 ng/ml of PMA for 12 h. IL-6 released into the culture supernatants were measured by ELISA. All data points correspond to the mean ± S.D. of three independent experiments. Statistically significant differences are shown. *, $p < 0.05$ (Student's *t* test).

(CD300e), or LMIR7 (CLM-3) (4–9). We found that monoclonal anti-mCD300e Ab recognized the transduced mCD300e in Ba/F3 cells; however, it did not detect other LMIRs transduced into Ba/F3 cells (Fig. 3*B*). In addition, we found that plate-coated anti-mCD300e Ab remarkably induced IL-6 production in mCD300e-transduced BMMC transfectants, but not in mock transfectants (Fig. 3*C*). Thus, we succeeded in generating a monoclonal Ab specific for mCD300e.

mCD300e is highly expressed in CD115$^+$Ly-6C$^{low/int}$ monocytes

Because high levels of mCD300e mRNA were found in PB cells, we stained PB cells with the monoclonal anti-mCD300e Ab. The results showed that mCD300e was not expressed in CD3$^+$ T cells, CD19$^+$ B cells, or Ly-6G$^+$ neutrophils in PB (Fig. 4*A*). By contrast, mCD300e was selectively expressed in a small subset of cell populations in PB, which was CD11b$^+$CD11c$^+$F4/80$^+$CD80^{high}CD86$^-$MHC-II$^+$, suggesting that mCD300e is expressed in monocyte-lineage cells in PB (Fig. 4*B*). Because mouse PB monocytes are divided into three subpopulations (CD115$^+$Ly-6C^{high}, CD115$^+$Ly-6C^{int}, and CD115$^+$Ly-6C^{low}), we examined the surface expression of mCD300e in these populations, demonstrating that mCD300e is expressed highly in CD115$^+$Ly-6C^{low} nonclassical (patrolling) monocytes and moderately in CD115$^+$Ly-6C^{int} intermediate monocytes, but not in CD115$^+$Ly-6C^{high} classical monocytes (Fig. 4, *C* and *D*) (31–33). In contrast, CD80^{high}mCD300e$^+$ cells were not detectable in the spleen,

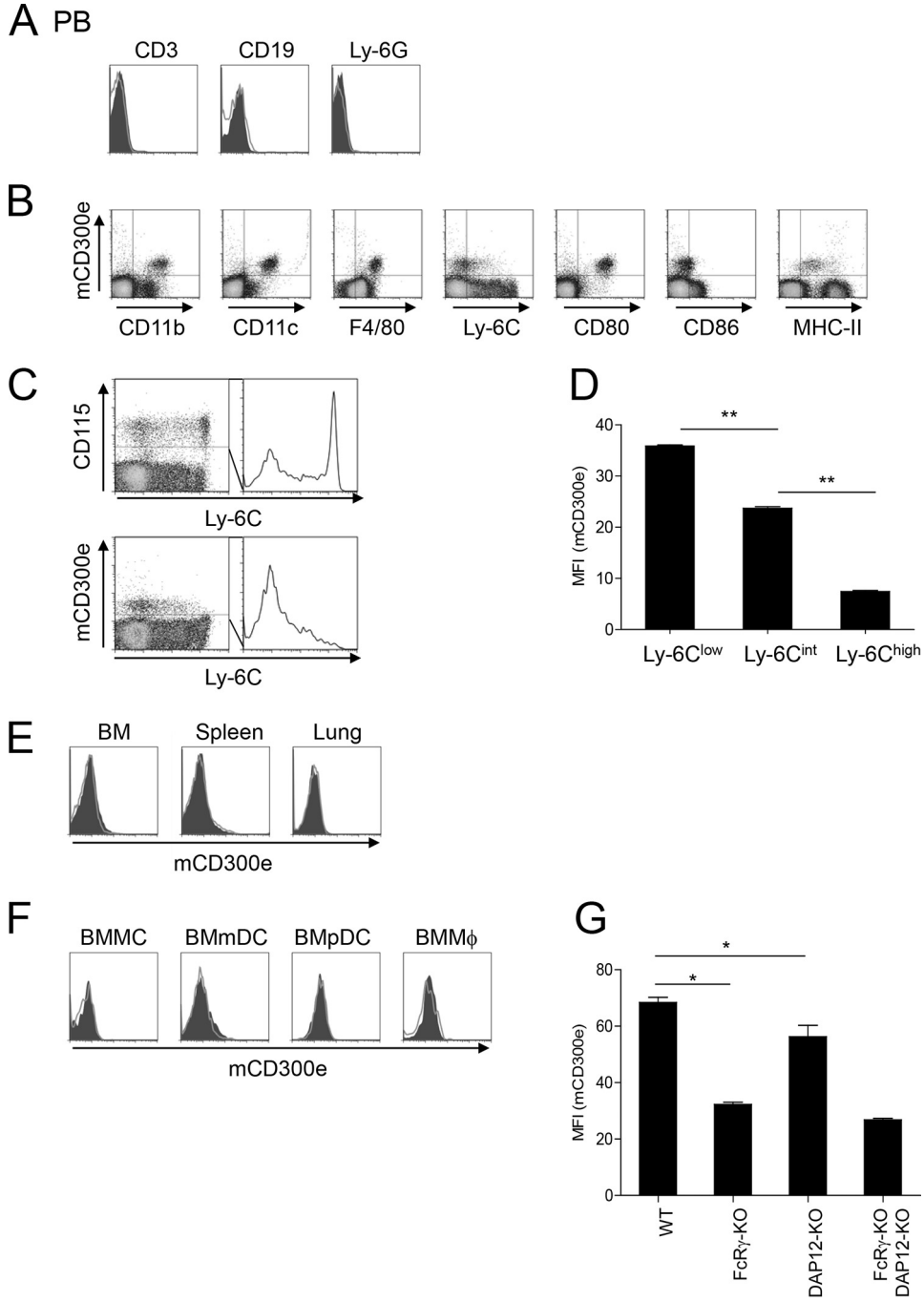

Figure 4. mCD300e is highly expressed in CD115$^+$Ly-6C$^{low/int}$ monocytes. *A,* mouse PB cells were stained with FITC-conjugated anti-CD3, CD19, or Ly-6G Ab and with biotinylated anti-mCD300e Ab or Armenian hamster IgG Ab followed by PE-conjugated streptavidin. Expression of mCD300e in CD3$^+$, CD19$^+$, or Ly-6G$^+$ cells was shown. *B,* mouse PB cells were stained with FITC-conjugated CD11b, CD11c, F4/80, Ly-6C, CD80, CD86, or MHC class II (MHC-II) Ab and with biotinylated anti-mCD300e Ab or Armenian hamster IgG Ab followed by PE-conjugated streptavidin. *C,* mouse PB cells were stained with FITC-conjugated anti-Ly-6C Ab and PE-conjugated anti-CD115 Ab (*upper left panel*) or FITC-conjugated anti-Ly-6C Ab and biotinylated anti-mCD300e Ab or Armenian hamster IgG Ab followed by PE-conjugated streptavidin (*lower left panel*). Histograms showed expression of Ly-6C in CD115$^+$ PB cells (*upper right panel*) or in mCD300e$^+$ PB cells (*lower right panel*). *D,* mean fluorescent intensity (*MFI*) of mCD300e in CD115$^+$Ly-6C^{low}, CD115$^+$Ly-6C^{int}, or CD115$^+$Ly-6C^{high} PB monocytes was measured by flow cytometry. *E,* BM, spleen, or thymus cells were stained with FITC-conjugated anti-CD80 Ab and biotinylated anti-mCD300e Ab or Armenian hamster IgG Ab followed by PE-conjugated streptavidin. *F,* BMMCs, BMmDCs, BMpDCs, or BMMϕ were stained with biotinylated anti-mCD300e Ab or Armenian hamster IgG Ab followed by PE-conjugated streptavidin. *G,* MFI of mCD300e in CD11c$^+$ PB monocytes from WT, *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice were measured by flow cytometry. *A–C, E,* and *F,* data are representative of three independent experiments. *D* and *G*, all data points correspond to the mean ± S.D. of four independent experiments. Statistically significant differences are shown. *, $p < 0.05$ or **, $p < 0.01$ (Student's *t* test).

thymus, or BM (Fig. 4*E*). In addition, we found no detectable levels of mCD300e in BMMCs, BM-derived myeloid dendritic cells (BMmDCs), BM-derived plasmacytoid dendritic cells (BMpDCs), or BM-derived macrophages (BMMϕ) (Fig. 4*F*). Flow cytometric analysis of CD115$^+$Ly-6C$^{low/int}$ PB monocytes from WT, *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice showed that loss of either FcRγ or DAP12 lowered the surface expression levels of endogenous mCD300e, unlike the trans-

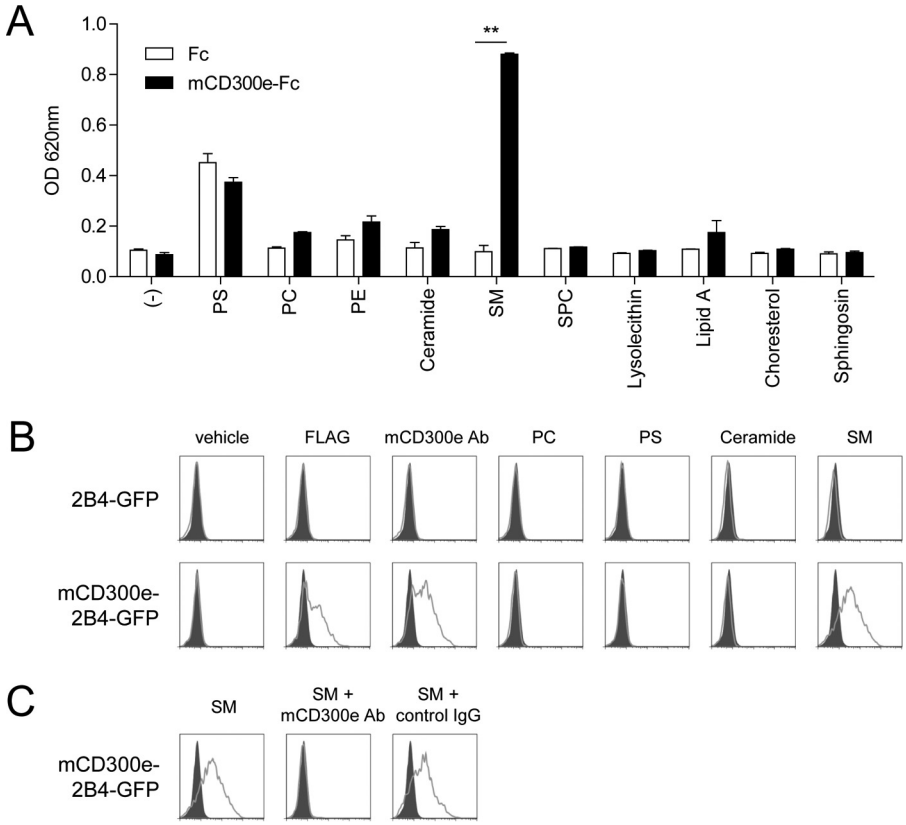

Figure 5. Sphingomyelin is a candidate ligand for mCD300e. *A,* mCD300e–Fc or Fc was incubated on plates coated with the indicated lipids. mCD300e–Fc or Fc bound to the plates was quantified by ELISA. All of the data points correspond to the mean ± S.D. of three independent experiments. Statistically significant differences are shown. **, $p < 0.01$ (Student's *t* test). *PS*, *PC*, *PE*, *SM*, or *SPC* indicates phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, or sphingosylphosphocholine. *B,* flow cytometry of GFP expression of mCD300e–2B4-GFP cells or 2B4-GFP cells that were incubated for 24 h on plates coated with indicated lipids or with anti-FLAG Ab or anti-mCD300e Ab. *C,* mCD300e–2B4-GFP cells were incubated for 24 h on plates coated with sphingomyelin in the presence of 20 μg/ml of either a soluble anti-mCD300e Ab or Armenia hamster IgG Ab as control. *B* and *C,* data are representative of three independent experiments.

duced mCD300e in BMMCs (Fig. 4*G*). Thus, mCD300e expression was highly restricted to CD115$^+$Ly-6C$^{low/int}$ monocytes in PB and possibly to a small subset of the related lineage cells in tissues.

Sphingomyelin is a candidate ligand for mCD300e

To identify the ligand that binds to mCD300e, we performed both binding and reporter assays using the extracellular domain of mCD300e. Because ceramide and sphingolipids have previously been identified as ligands for several CD300 members in mice and humans (15–20), we first asked whether ceramide and sphingolipids could bind to mCD300e-Fc, in which the extracellular domain of mCD300e was fused to the Fc portion of human IgG1. The results showed that mCD300e-Fc, but not control Fc, significantly bound to plated-coated sphingomyelin among the lipids tested (Fig. 5*A*). We then generated reporter cells mCD300e–2B4-GFP, where a chimeric receptor comprised of the extracellular domain of FLAG-tagged mCD300e, the transmembrane domain of mouse CD300f, and the intracellular domain of ITAM-bearing human CD3ζ was transduced into the parental reporter cell line 2B4-GFP (15, 27, 28). Because GFP expression is induced by the activation of nuclear factor of activated T-cells in 2B4-GFP cells, the binding of mCD300e ligands to this chimera receptor would be expected to induce GFP expression in mCD300e–2B4-GFP cells. Notably, plate-coated sphingomyelin as well as anti-FLAG Ab-induced GFP expression in mCD300e–2B4-GFP cells, but not in 2B4-GFP cells (Fig. 5*B*). In contrast, GFP expression was not induced by other plate-coated lipids tested (Fig. 5*B*). In addition, pretreatment with the soluble anti-mCD300e Ab, but not with control Ab, abolished sphingomyelin-mediated GFP expression in mCD300e–2B4-GFP cells (Fig. 5*C*). These results indicate that sphingomyelin is a candidate ligand for mCD300e.

Sphingomyelin is a possible ligand for CD300e

To test whether sphingomyelin acts as a ligand of mCD300e, FLAG-tagged mCD300e-transduced BMMCs were stimulated with plate-coated sphingomyelin or vehicle, and plate-coated sphingomyelin, but not vehicle, significantly induced IL-6 production in the BMMC transfectants (Fig. 6*A*). Pretreatment with the soluble anti-mCD300e Ab, but not control Ab, substantially reduced the cytokine production in mCD300e-transduced BMMCs stimulated by plate-coated sphingomyelin (Fig. 6*A*). Moreover, the deficiency of either FcRγ or DAP12 decreased IL-6 production induced by the sphingomyelin–mCD300e interaction in the BMMC transfectants, whereas the loss of both FcRγ and DAP12 dampened the IL-6 levels (Fig. 6*B*). However, CD115$^+$Ly-6C$^{low/int}$ monocytes sorted from mouse PB failed to produce detectable levels of IL-6 in response

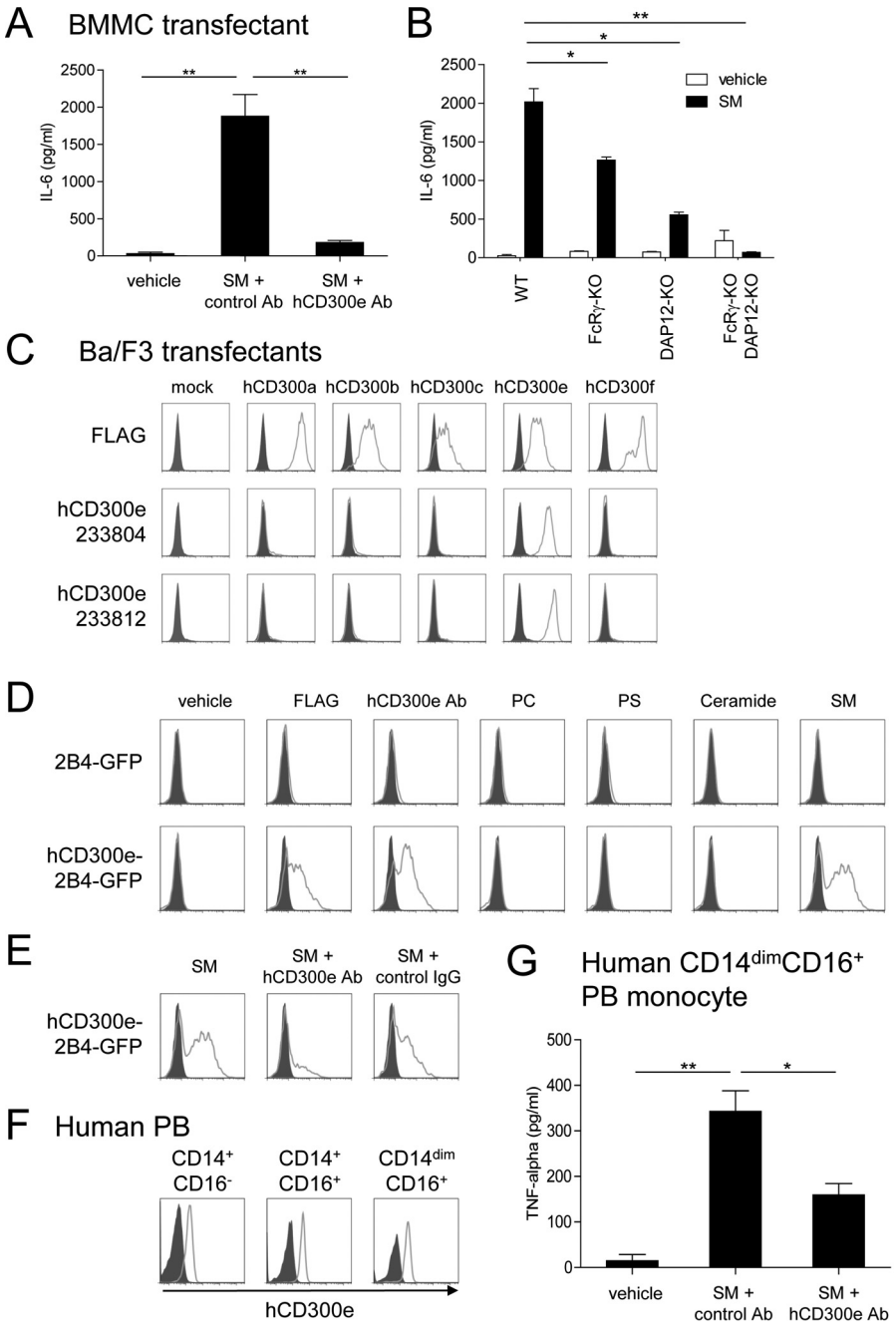

Figure 6. A possible role of the sphingomyelin–CD300e interaction. *A* and *B,* IL-6 released into the culture supernatants were measured by ELISA. All data points correspond to the mean ± S.D. of four independent experiments. Statistically significant differences are shown. *, $p < 0.05$ or **, $p < 0.01$ (Student's *t* test). *A,* BMMCs transduced with FLAG-tagged mCD300e were stimulated with plate-coated sphingomyelin or vehicle in the presence of 20 μg/ml of either soluble anti-mCD300e Ab or Armenia hamster IgG Ab as a control. *B,* FLAG-tagged mCD300e-transduced BMMCs from WT, *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice were stimulated with plate-coated sphingomyelin or vehicle. *C*, Ba/F3 cells were transduced with FLAG-tagged human CD300a, CD300b, CD300c, CD300e, CD300f, or mock. The transfectants were stained either with mouse anti-FLAG Ab or mouse IgG1 Ab followed by PE-conjugated anti-mouse IgG goat F(ab′)$_2$ Ab (*upper panel*) or with anti-hCD300e Ab (233804), anti-hCD300e Ab (233812), or rat IgG2a Ab followed by PE-conjugated anti-rat IgG goat F(ab′)$_2$ Ab (*middle* and *lower panels*). *D*, flow cytometry of GFP expression of hCD300e–2B4-GFP cells or 2B4-GFP cells that were incubated for 24 h on plates coated with indicated lipids or with anti-FLAG Ab or anti-hCD300e Ab (233804). *E*, flow cytometry of GFP expression of hCD300e–2B4-GFP cells that were incubated for 24 h on plates coated with sphingomyelin in the presence of 20 μg/ml of either soluble anti-hCD300e Ab (233812) or rat IgG2a Ab as a control. *F*, human PB cells were stained with APC-conjugated anti-CD14 Ab and FITC-conjugated anti-CD16 Ab and with anti-hCD300e Ab (233812) or rat IgG2a Ab followed by PE-conjugated anti-rat IgG goat F(ab′)$_2$ Ab. Expression of hCD300e in CD14$^+$CD16$^-$, CD14$^+$CD16$^+$, or CD14^{dim}CD16$^+$ PB cells was shown. *C–F*, data are representative of three independent experiments. *G*, CD14^{dim}CD16$^+$ cells sorted from human PB were stimulated with plate-coated sphingomyelin or vehicle in the presence of 20 μg/ml of either a soluble anti-hCD300e Ab (233812) or rat IgG2a Ab as a control. The levels of IL-6 released into the culture supernatants were measured by ELISA. All data points correspond to the mean ± S.D. of three independent experiments. Statistically significant differences are shown. *, $p < 0.05$ or **, $p < 0.01$ (Student's *t* test).

to plate-coated sphingomyelin (data not shown), presumably in part due to reduced viability of these monocytes in culture. Nonetheless, we provided evidence that sphingomyelin could act as a ligand for mCD300e at least in transduced BMMCs. Next, we confirmed that two types of anti-hCD300e Abs (233804 and 233812) specifically recognized hCD300e, but not other members of human CD300 tested, on the transduced Ba/F3 cells (Fig. 6*C*). To further delineate whether sphingomyelin acts as a ligand for hCD300e as well, we generated the new reporter cells hCD300e–2B4-GFP in a similar way as mCD300e–2B4-GFP and stimulated them with plate-coated sphingomyelin. The results showed that plate-coated sphingomyelin or anti-hCD300e Ab (233804), but not other lipids tested, induced GFP expression in hCD300e–2B4-GFP cells like in mCD300e–2B4-GFP cells (Fig. 6*D*). In addition, pretreatment with the soluble anti-hCD300e Ab (233812), but not with control Ab, significantly inhibited sphingomyelin-induced GFP expression in hCD300e–2B4-GFP cells (Fig. 6*E*). Flow cytometric analysis verified that hCD300e was expressed in three subsets of human PB monocytes comprising $CD14^+CD16^-$, $CD14^+CD16^+$, and $CD14^{dim}CD16^+$ monocytes (Fig. 6*F*) (46). Notably, plate-coated sphingomyelin induced TNF-α production in $CD14^{dim}CD16^+$ nonclassical (patrolling) monocytes sorted from human PB (Fig. 6*G*). Pretreatment with the soluble anti-hCD300e Ab (233812) reduced, but not completely, the cytokine production in sphingomyelin-stimulated $CD14^{dim}CD16^+$ monocytes (Fig. 6*G*), suggesting that sphingomyelin can act as a ligand for hCD300e.

Discussion

In the present study, we characterized mCD300e as an immune-activating receptor. Generation of monoclonal anti-mCD300e Ab that specifically recognize mCD300e and not other CD300 members enabled us to uncover expression profiles of mCD300e in mouse hematopoietic cells. Interestingly, mCD300e was highly expressed in $CD115^+$Ly-$6C^{low}$ nonclassical (patrolling) monocytes and moderately in $CD115^+$Ly-$6C^{int}$ intermediate monocytes, but not in $CD115^+$Ly-$6C^{high}$ classical monocytes, in mouse PB. One of the characteristics of $CD115^+$Ly-$6C^{low/int}$ monocytes expressing mCD300e was the high expression levels of CD80, but not CD86. Notably, it was difficult to detect mCD300e expression in cell populations distinct from $CD115^+$Ly-$6C^{low/int}$ monocytes. Accordingly, mCD300e expression might be tightly regulated by transcription factors specific for intermediate and nonclassical (patrolling) monocytes. One such possible candidate is the transcription factor NUR77, also called NR4A1, that is essential for the development of Ly-$6C^{low}$ monocytes (39, 47). The higher mRNA expression levels of mCD300e in the lung or spleen compared with those in BM led us to speculate that $CD115^+$Ly-$6C^{low/int}$ monocytes might preferentially reside in the vasculature of lung or spleen. Alternatively, $CD115^+$Ly-$6C^{low}$ circulating monocytes might differentiate into specialized monocyte/dendritic cell/macrophage lineage cells with mCD300e expression in the spleen or lung (31–33, 48, 49). Notably, CD300e expression profiles in hematopoietic cells differ between humans and mice; hCD300e is expressed in three subsets of monocytes and myeloid dendritic cells, whereas mCD300e expression is restricted to nonclassical (patrolling) and intermediate monocytes in PB and possibly to a small subset of the related lineage cells in tissues. In addition, this selective expression of mCD300e suggested a specialized role of mCD300e in patrolling monocytes.

Analysis of mCD300e-transduced BMMCs definitely demonstrated that similar to hCD300e, mCD300e can transmit an immune-activating signal (12, 13). However, unlike hCD300e that couples with DAP12 (12), mCD300e required both FcRγ and DAP12 as its adaptor proteins to fully transmit an activating signal. Similar to LMIR2 (CLM-4) (50), the relative contribution of FcRγ *versus* DAP12 to the activating signals through mCD300e might be affected by the cellular environments. It is important to note that the loss of these adaptor proteins did not influence the surface expression levels of transduced mCD300e, but reduced those of endogenous mCD300e in the intermediate and nonclassical monocytes. Collectively, these results suggest that FcRγ and DAP12 are required not only for delivering the maximum activating signal but also for maintaining the maximum surface expression of mCD300e in intermediate and nonclassical monocytes.

Importantly, we identified sphingomyelin as a candidate ligand for both mouse and human CD300e by binding and reporter assays. In fact, plate-coated sphingomyelin stimulated significant cytokine production in transduced BMMCs via mCD300e; however, the same treatment did not do so in the case of $CD115^+$Ly-$6C^{low/int}$ monocytes from mouse PB. Different expression levels of mCD300e between transduced BMMCs and $CD115^+$Ly-$6C^{low/int}$ monocytes might account for such different responses. Alternatively, the low viability of $CD115^+$Ly-$6C^{low/int}$ monocytes in the culture possibly masked their activation in response to sphingomyelin. To gain evidence that sphingomyelin acts as a ligand for mCD300e in $CD115^+$Ly-$6C^{low/int}$ monocytes, further examination will be required. On the other hand, it is possible that sphingomyelin-like lipids, either endogenous or exogenous, with stronger affinity to mCD300e might serve as its physiological ligands, which also remain to be further investigated. Sphingomyelin being abundant in lipoproteins or the CNS, the binding of sphingomyelin-containing lipoprotein or sphingomyelin released from injured CNS to mCD300e in patrolling monocytes might regulate vascular inflammation in arteriosclerosis or the pathogenesis of the CNS diseases, respectively (30, 39–43). Moreover, it is interesting to speculate that mCD300e contributes to the engulfment of sphingomyelin-containing debris from damaged tissues by the patrolling monocytes (31–34). Ultimately, analysis of CD300e-deficient mice will be needed to completely understand the physiological significance of the CD300e-sphingomyelin interaction.

In conclusion, mouse CD300e is an activating receptor coupled with FcRγ and DAP12, which is selectively expressed in nonclassical and intermediate monocytes in circulating monocytes, implicating CD300e in the disease pathogenesis involving non-classical monocyte activation.

Materials and methods

Cells and mice

Murine cell lines Ba/F3 and 2B4-GFP (a kind gift from Takashi Saito, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and human cell line HEK 293T were previously used (7, 15, 27, 28). BM, spleen, or thymus cells were isolated from C57BL/6 mice or *FcRγ*$^{-/-}$, *DAP12*$^{-/-}$, or *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice (25, 26). BMMCs were generated and cultured as described (15, 45). All the procedures were approved by the Institutional Review Committee of University of Tokyo and Juntendo University. CD14^{dim}CD16^{+} monocytes were sorted from human PB. 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 and University of Tokyo and Juntendo University.

Antibodies and other reagents

Armenian hamster anti-mouse CD300e monoclonal IgG Ab was generated by immunizing Armenian hamster with mouse CD300e-Fc, as previously described (51, 52). The following antibodies were used in this study: rat anti-human CD300e monoclonal IgG2a Abs (233804 and 233812) (R&D Systems); anti-FLAG Ab (M2) and mouse IgG1 Ab (MOPC21) (Sigma); anti-Myc Ab (9E10) (Roche Diagnostics); Armenian hamster IgG Ab, FITC-conjugated anti-mouse FcεRIα Ab, and R-phycoerythrin (PE)-conjugated anti-mouse c-Kit Ab, CD11b, and B220 Abs or streptavidin (eBioscience); PE-conjugated anti-rat IgG goat F(ab′)$_2$ Ab (Jackson ImmunoResearch); FITC-conjugated anti-human CD16 Ab (Miltenyi Biotech); rat IgG2a and FITC-conjugated anti-mouse CD3, CD19, Ly-6G, CD11b, CD11c, F4/80, Ly-6C, CD80, CD86, and MHC class II Abs (BioLegend); allophycocyanin (APC)-conjugated anti-human CD14 Ab (eBioscience); and PE-conjugated anti-mouse IgG goat F(ab′)$_2$ Ab (Beckman Coulter). Anti-mCD300e Ab and Armenia hamster IgG Ab were biotinylated by sulfo-NHS-LC-biotin (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions. Other reagents used in this study include cytokines (R&D Systems); peptide-*N*-glycosidase F (New England Biolabs); sphingosine, sphingomyelin, and sphingosylphosphorylcholine (BIOMOL); C-24 ceramide (Toronto Research Chemicals, Inc.); lipid A, lysophosphatidylcholine (lysolecithin), and cholesterol (Avanti Polar Lipids, Inc.); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Echelon Biosciences Inc.). All other reagents were from Sigma unless stated otherwise.

Gene expression analysis

Relative expression levels of mouse CD300e and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) among samples were measured by real-time RT-PCR as described (9). The following primers were used: 5′-GTCCATCAGAGACCATGCTTCG-3′ and 5′-ACGTGACCACGAATCCCAG-3′ for mCD300e and 5′-GAAGGTGAAGGTCGGAGTCA-3′ and 5′-GACAAGCTTCCCGTTCTCAG-3′ for GAPDH. Relative gene expression levels were calculated using standard curves generated by serial dilutions of cDNA and normalized to GAPDH expression levels. Product quality was checked by melting curve analysis via LightCycler software (Roche Diagnostics).

Plasmid constructs

cDNAs of mouse CD300e (GenBank™ accession number NM_172050.3) were isolated by PCR from a cDNA library of mouse BM cells. The cDNA fragment of mouse CD300e, lacking the signal sequence, was tagged with a FLAG epitope at the N terminus. The resultant FLAG-tagged mouse CD300e was subcloned into pME vector containing the signaling lymphocyte-activating molecule (SLAM) signal sequence (a gift from Hisashi Arase, Osaka University, Osaka Japan) (53). The resultant SLAM signal sequence–FLAG–mCD300e was subcloned into pMXs-internal ribosome entry sites (IRES)–puromycin^{r} (pMXs–IP) (54) to generate pMXs–FLAG–mCD300e-IP. pMXs–FLAG–mouse LMIR1, LMIR2, LMIR3, LMIR4, LMIR5, or LMIR7-IP was used previously (7–9). pMXs–FLAG– human CD300a, CD300b, CD300c, CD300e, or CD300f-IP was previously used (19). To generate the chimeric receptor mCD300e–CD3ζ, SLAM signal sequence–FLAG–mCD300e, excluding transmembrane and intracellular domains, was fused to the transmembrane domain of LMIR3 followed by the intracellular domain of human CD3ζ (Naoki Matsumoto, The University of Tokyo, Tokyo, Japan). To generate the chimeric receptor hCD300e–CD3ζ, SLAM signal sequence–FLAG– hCD300e, excluding transmembrane and intracellular domains, was fused to the transmembrane domain of human CD300f followed by the intracellular domain of human CD3ζ (Naoki Matsumoto, The University of Tokyo, Tokyo, Japan). mCD300e–CD3ζ or hCD300e–CD3ζ was subcloned into pMXs–IP to generate pMXs–FLAG–mCD300e–CD3ζ or FLAG– hCD300e–CD3ζ–IP (15, 19, 21). To generate the mCD300e–N84Q mutant, two-step PCR mutagenesis was performed using pMXs–FLAG–mCD300e–IP as a template. pMXs–Myc–mouse DAP12 or FcRγ–IRES– blasticidin^{r} (IB) was used as described previously (19). All constructs were verified by DNA sequencing.

Flow cytometry

Cells were stained as described (7–9, 15). Flow cytometric analysis was performed with FACSCalibur (BD Biosciences) equipped with CellQuest software and FlowJo software (Tree Star).

Transfection and infection

Retroviral transfection and infection were performed as described (7–9, 54, 55). Retroviruses were generated by transient transfection of PLAT-E packaging cells (55). Selection with 1 μg/ml of puromycin was started 48 h after infection.

Biochemistry

Western blotting was performed as described (7–9). Equal amounts of cell lysates of Ba/F3 transfectants or HEK 293T cells were immunoprecipitated with mouse anti-FLAG Ab or anti-Myc Ab, and immunoblotted with rabbit anti-FLAG Ab or anti-Myc Ab.

Binding assay using solid-phase ELISA

Solid-phase ELISA was performed to carry out the binding assays (14, 15). The Fc fusion proteins mCD300e–Fc and Fc were purified. The indicated lipids in methanol (50 μg/ml) or methanol as a control was added to ELISA plates and air-dried. After washing, the plates were incubated with 10 μg/ml of mCD300e–Fc or Fc in the presence of 0.5 mM $CaCl_2$ for 120 min before incubating with peroxidase-conjugated anti-human Ig (Sigma). Absorbance at 450 nm was measured.

Measurement of cytokines

To stimulate cells with plate-coated Ab, plates were coated overnight with 20 μg/ml of Ab before stimulation (15). To stimulate cells with plate-coated sphingomyelin, sphingomyelin in methanol (10 μg/ml) or methanol as control was added to plates and air-dried before stimulation (15). $CD14^{dim}CD16^{+}$ monocytes sorted from human PB were stimulated with plate-coated sphingomyelin or vehicle for 24 h in the presence or absence of 10 μg/ml of a soluble anti-hCD300e Ab (233812) or rat IgG2a as control. BMMCs transduced with FLAG-tagged mCD300e were stimulated with plate-coated anti-FLAG Ab or mouse IgG1 Ab as control, plate-coated anti-mCD300e Ab or Armenian hamster IgG Ab as control, or 100 nM PMA for 24 h. Concentrations of human TNFα or mouse IL-6 (R&D Systems) in culture supernatants were measured by ELISA.

Statistical analysis

Data are shown as mean ± S.D., and statistical significance was determined by Student's t test with *, $p < 0.05$ or **, $p < 0.01$ considered to indicate statistical significance.

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