

Apoptotic cells suppress mast cell inflammatory responses via the CD300a immunoreceptor

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When a cell undergoes apoptosis, phosphatidylserine (PS) is exposed on the outer leaflet of the plasma membrane. PS acts as an "eat-me" signal to direct phagocytes expressing PS receptors to engulf the apoptotic cell. We recently reported that the immunoreceptor CD300a, which is expressed on myeloid cells, is a PS receptor. We show that CD300a does not facilitate macrophage phagocytosis of apoptotic cells. Instead, CD300a delivers an inhibitory signal in mast cells to suppress production of LPS-induced inflammatory cytokines and chemokines. After cecal ligation and puncture (CLP), when a large number of cells undergo apoptosis in the peritoneal cavity, CD300a-deficient peritoneal mast cells produced more chemoattractant and recruited more neutrophils than did wild-type (WT) mast cells. As a result, CD300a-deficient mice showed increased neutrophil recruitment and improved bacterial clearance in the peritoneal cavity, and survived longer than WT mice. Antibody blockade of CD300a-PS interactions improved bacterial clearance and extended survival of WT mice subjected to CLP. These results indicated that CD300a is a nonphagocytic PS receptor that regulates mast cell inflammatory responses to microbial infections.

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Abbreviations used: BMMC, BM-derived mast cell; *CAD*, caspase-activated DNase; CLP, cecal ligation and puncture; ITIM, immunoreceptor tyrosine-based inhibition motif; MFG-E8, milk fat globule EGF factor 8; PS, phosphatidylserine; TIM-4, T cell immunoglobulin- and mucin domain-containing molecule 4.

Apoptosis, referred to as programmed cell death, is one of the most important events in the cell fate. Several cells undergo apoptosis every day in physiological and pathological settings in the body. When a cell undergoes apoptosis, phosphatidylserine (PS) is exposed on the outer leaflet of the plasma membrane and signals phagocytes to engulf the apoptotic cells (Ravichandran and Lorenz, 2007). Several receptors for PS are expressed on phagocytes and are involved in clearing apoptotic cells (Ravichandran and Lorenz, 2007; Zhou, 2007; Nagata et al., 2010). For example, T cell immunoglobulin- and mucin domain-containing molecule 4 (TIM-4), TIM-1, and TIM-3 are expressed on macrophages and/or dendritic cells and mediate engulfment of apoptotic cells upon binding PS (Kobayashi et al., 2007; Miyaniishi et al., 2007). BAI1 (brain-specific angiogenesis inhibitor 1; Park et al., 2007) and stabilin-2

(Park et al., 2008), which are expressed on neuron (Mori et al., 2002) and the sinusoidal endothelial cells of the spleen, lymph nodes, and BM (Harris et al., 2007), respectively, were also reported to be PS receptors for apoptotic cells. Engulfment of apoptotic cells also involves the bridging molecules that recognize PS. Milk fat globule EGF factor 8 (MFG-E8), which is expressed by tingibile body macrophages and follicular dendritic cells at the germinal centers in the spleen and lymph nodes—intermediates between apoptotic cells and phagocytes—by binding both PS and $\alpha\beta 3$ or $\alpha\beta 5$ integrin on the phagocytes, stimulating the engulfment of apoptotic cells (Hanayama et al., 2002). Similarly,

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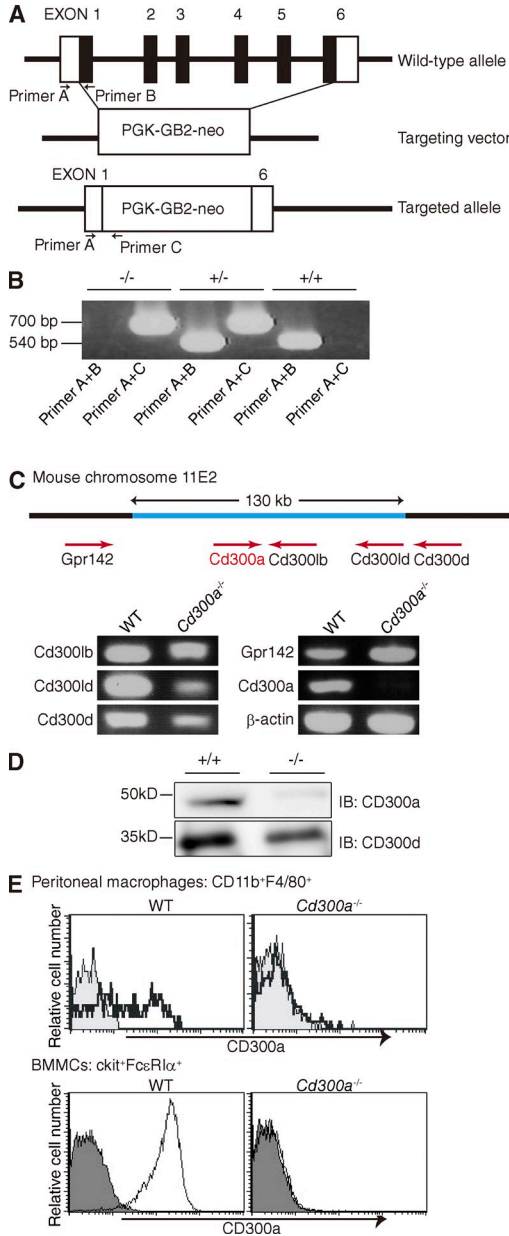


Figure 1. Generation of *Cd300a*^{-/-} mice. (A) The WT *Cd300a* allele, the targeting vector, the targeted allele, and the binding sites of the PCR primers are shown. (B) Genomic DNA from the tails of *Cd300a*^{-/-} (-/-), *Cd300a*^{+/-} (+/-), and WT (+/+) mice were analyzed for WT and mutant alleles by PCR. (C) mRNA expression of the genes neighboring *Cd300a*, including *Cd300b*, *Cd300l*, *Cd300d*, and *Gpr142* in the spleen of WT and *Cd300a*^{-/-} mice was determined by PCR. β -Actin, loading control. The blue line indicates the BAC clone containing the targeted allele. (D) Western blot analysis of BM cells from WT and *Cd300a*^{-/-} mice. Cells were lysed with 1% NP-40 lysis buffer and immunoblotted with an antibody to CD300a (or CD300d as a loading control). (E) Flow cytometric analysis of peritoneal resident macrophages and cultured BMMCs. Peritoneal exudate cells from WT and *Cd300a*^{-/-} mice were stained with FITC-conjugated anti-CD11b, APC-conjugated F4/80 antibodies, and biotin-conjugated anti-CD300a, followed by PE-conjugated streptavidin. BMMCs from WT and *Cd300a*^{-/-} mice were stained with FITC-conjugated anti-Fc ϵ RI, APC-conjugated anti-c-Kit, and biotin-conjugated anti-CD300a, followed by PE-conjugated

Gas6 (growth arrest-specific 6) and protein S, which are abundant in the plasma and bind PS and TAM family members (Tyro3, Axl, and Mer), are also bridging molecules between apoptotic cells and phagocytes (Nakano et al., 1997; Scott et al., 2001). However, whether PS receptors deliver signals that lead to cellular responses other than phagocytosis is unclear.

Activation of immune cells is regulated by positive and negative signals triggered by activating and inhibitory cell surface immunoreceptors, respectively. These immunoreceptors play important roles in regulation of immune responses (Ravetch and Lanier, 2000; Lanier, 2001). Inhibitory receptors are characterized by the immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domains. The prototype 6-aa sequence for ITIM is (I/V/L/S)-x-Y-x-x-(L/V) (x denotes any amino acid), whose tyrosine is phosphorylated upon ligand binding, providing a docking site for the recruitment of SH2 (Src homology 2)-containing cytoplasmic phosphatases (Malbec et al., 1998; Smith et al., 1998) and shutting down activation signals by dephosphorylation of intracellular substrates at the earliest steps of the activation response. The ITIM-bearing cell surface immunoreceptors, including certain NK receptors, Fc receptors (Fc γ RIIb), and others, play a central role in mediating negative signals in both lymphoid and myeloid cells (Daëron et al., 2008).

CD300 is a multigene family consisting of seven genes on human chromosome 17 (Clark et al., 2000, 2001). CD300 molecules are member of Ig super family bearing one Ig-like domain in the extracellular portion. The mouse counterparts of CD300 molecules, which were reported to be as myeloid-associated Ig-like receptor (MAIR; Yotsumoto et al., 2003; Okoshi et al., 2005; Nakahashi et al., 2007; Can et al., 2008; Nakano et al., 2008; Nakano-Yokomizo et al., 2011)/CMRF-35-like molecule (CLM; Chung et al., 2003; Fujimoto et al., 2006; Xi et al., 2010)/leukocyte mono-Ig-like receptor (LMIR; Kumagai et al., 2003; Izawa et al., 2007; Enomoto et al., 2010)/DIgR (Luo et al., 2001; Shi et al., 2006), were encoded by nine genes on a small segment of mouse chromosome 11, the syntenic region of human chromosome 17 (Chung et al., 2003; Nakano et al., 2007). CD300 molecules are preferentially expressed on myeloid cells, including macrophages, neutrophils, DCs, and/or mast cells, and may regulate activation of these cells. One of the CD300 molecules, CD300a (also called MAIR-I [Yotsumoto et al., 2003], LMIR1 [Kumagai et al., 2003], or CLM-8 [Chung et al., 2003] in mice, and IRp60 [Cantoni et al., 1999] or CMRF-35H [Clark et al., 2000] in human), has a long cytoplasmic region containing the consensus ITIM sequence. Upon cross-linking with monoclonal antibodies, CD300a inhibits Fc ϵ RI-mediated

streptavidin. These cells were then analyzed by flow cytometry. The open histograms show CD300a expression on the gates of CD11b⁺F4/80⁺ and c-Kit⁺Fc ϵ RI⁺ cells. The closed histograms show isotype control. Experiments were performed three times with similar results. The results of one experiment are shown (B-E).

signals, resulting in the suppression of degranulation from human and mouse mast cells in vitro (Yotsumoto et al., 2003; Okoshi et al., 2005; Munitz et al., 2006; Karra et al., 2009). In addition, CD300a modulates inflammatory responses by myeloid cells (Alvarez et al., 2008; Ju et al., 2008). CD300a is also expressed on human—but not mouse—NK cells and involved in cytotoxic function (Cantoni et al., 1999; Lankry et al., 2010). We have recently identified CD300a as a new PS receptor (Nakahashi-Oda et al., 2012). However, the physiological role of CD300a remained undetermined.

To explore the physiological role of CD300a, we generated mice deficient in *Cd300a*. We describe that CD300a does not stimulate engulfment of apoptotic cells by phagocytes but, rather, inhibits inflammatory cytokine and chemokine production from mast cells upon binding to PS in vitro and in vivo.

RESULTS

CD300a does not mediate phagocytosis

Several receptors for PS are expressed on phagocytes and are involved in clearing apoptotic cells in physiological and pathological settings (Scott et al., 2001; Hanayama et al., 2002; Kobayashi et al., 2007; Miyanishi et al., 2007; Park et al., 2007, 2008). Because PS mediates an “eat-me” signal to phagocytes such as macrophages (Fadok et al., 1992; Savill et al., 2002), we generated CD300a-deficient (*Cd300a*^{-/-}) mice (Fig. 1). *Cd300a*^{-/-} mice lacked the expression of *Cd300a* mRNA and CD300a protein but showed normal expression of neighboring genes to *Cd300a* in the *Cd300* family gene cluster on chromosome 11 (Fig. 1), indicating that *Cd300a*^{-/-} mice were correctly generated. We examined whether CD300a is involved in phagocytosis of apoptotic cells by using macrophages from *Cd300a*^{-/-} mice and apoptotic thymocytes from *caspase-activated DNase* (*CAD*)^{-/-} mice. *CAD*^{-/-} cells do not undergo apoptotic DNA fragmentation, but their DNA is degraded in phagocytes after they are engulfed (Nagata, 2005). WT or *Cd300a*^{-/-} peritoneal resident macrophages were cocultured with dexamethasone-induced apoptotic *CAD*^{-/-} thymocytes for 2 h, stained with TUNEL, and analyzed by laser-scanning confocal microscopy. The frequencies of WT and *Cd300a*^{-/-} peritoneal macrophages carrying a given number of apoptotic cells were comparable (Fig. 2 A). Peritoneal resident macrophages also express several receptors, including TIM-1, TIM-4, and $\alpha_v\beta_3$ (Kobayashi et al., 2007; Miyanishi et al., 2007; Fig. 3 A), that directly or indirectly bind PS and are involved in engulfment of apoptotic cells (Kuchroo et al., 2008). To examine whether these PS receptors had functional redundancy that masked the CD300a phagocytic function, we established NIH 3T3 transfectants stably expressing either CD300a or TIM-4 (Fig. 2 B). We labeled apoptotic thymocytes with pHrodo succinimidyl ester, a pH-sensitive fluorescent dye, which can be detected when they are engulfed as a result of the increased post-phagocytic light emission (Miksa et al., 2009). The NIH 3T3 transfectants or parental NIH 3T3 cells were cocultured with pHrodo dye-labeled apoptotic thymocytes and analyzed by

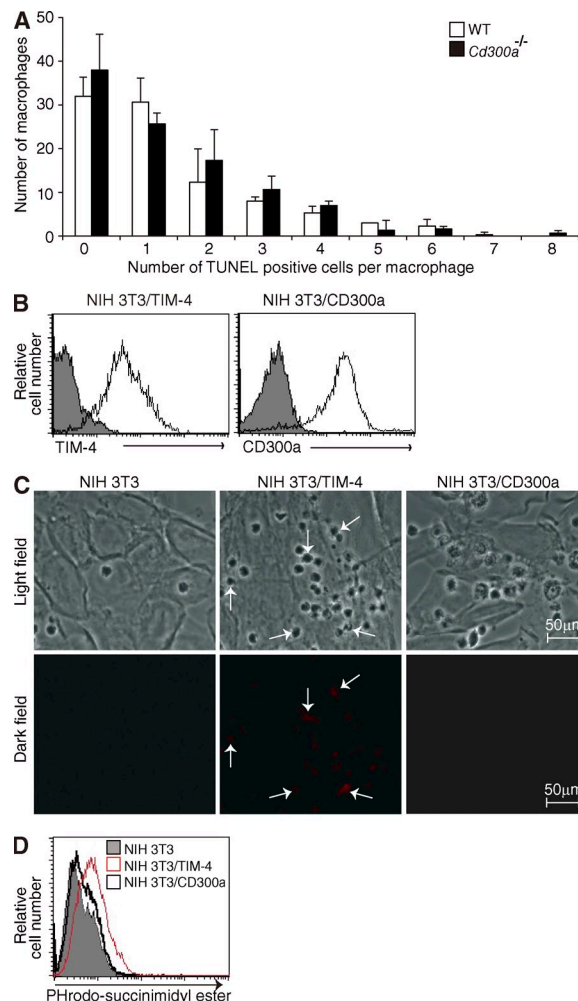


Figure 2. CD300a is not involved in phagocytosis of apoptotic cells by macrophages. (A) Apoptotic *CAD*^{-/-} thymocytes were cocultured with peritoneal resident macrophages from *Cd300a*^{-/-} or WT mice and stained using TUNEL. More than 100 macrophages were randomly analyzed by using laser-scanning confocal microscopy, and the number of TUNEL-positive cells in each macrophage was determined. Error bars indicate SD. (B–D) NIH 3T3 transfectants expressing CD300a and TIM-4 were stained with FITC-conjugated anti-CD300a and anti-TIM-4 (open histogram), respectively, or FITC-conjugated control antibody (closed histogram), and analyzed by flow cytometry (B). These transfectants were cocultured with pHrodo succinimidyl ester-labeled apoptotic thymocytes for 1 h, washed with PBS, fixed with paraformaldehyde, and then analyzed by laser-scanning confocal microscopy (C). The round cells under the light-field illumination indicate apoptotic thymocytes (C, top) and the pHrodo red signal under the dark-field illumination indicates engulfed apoptotic thymocytes (C, bottom). The arrows indicate the transfectants engulfing apoptotic cells (C). NIH 3T3 or NIH 3T3 transfectants were also analyzed by flow cytometry (D). Data are representative of more than three independent experiments (A–D).

laser-scanning confocal microscopy. In contrast to the parental NIH 3T3 cells, both CD300a and TIM-4 transfectants bound to apoptotic cells (Fig. 2 C). However, although TIM-4 transfectant expressed a pHrodo signal, we could not detect any signals from CD300a transfectant (Fig. 2 C). Flow cytometry

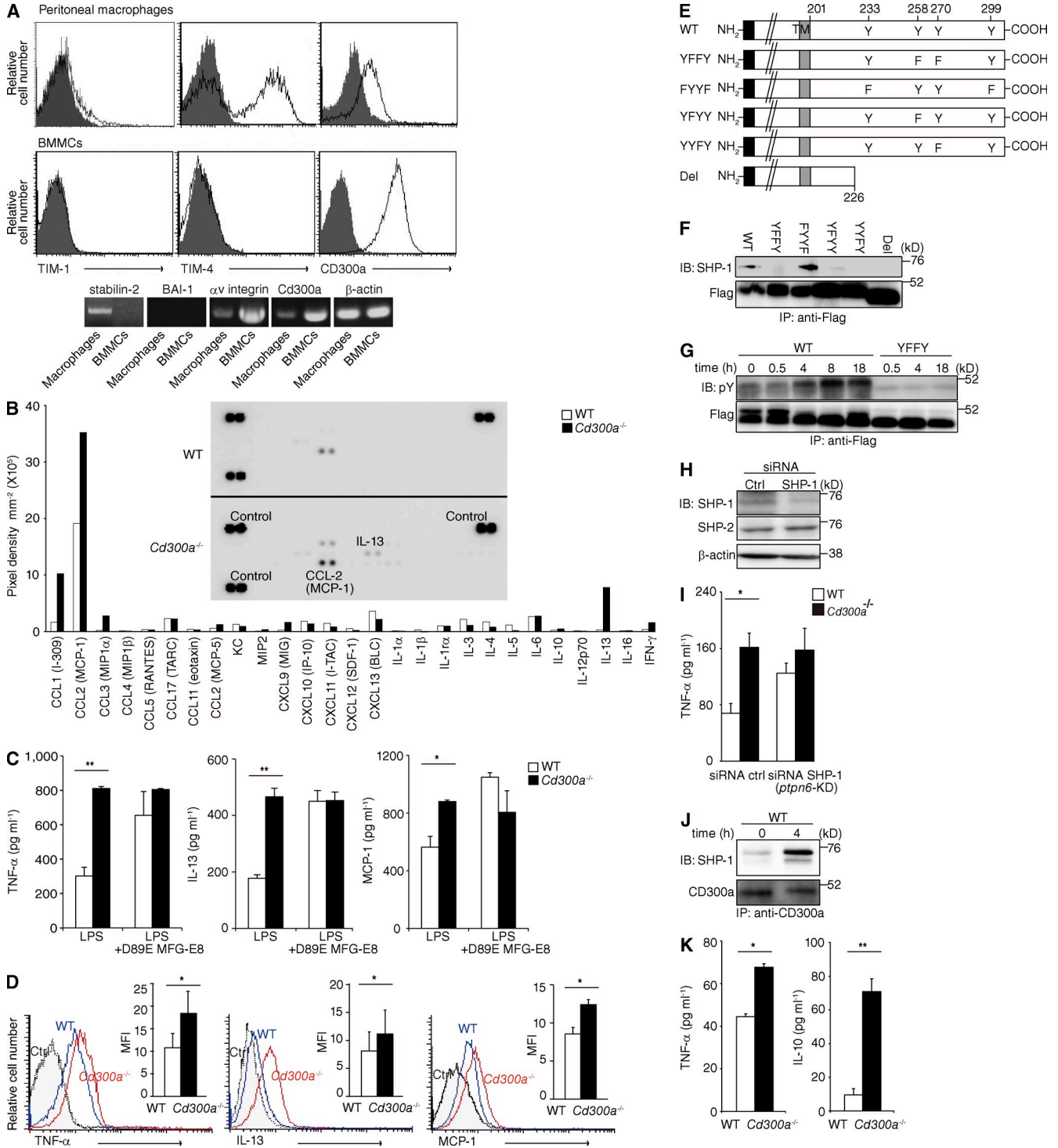


Figure 3. CD300a inhibits LPS-induced cytokine secretion from mast cells. (A) Expression of PS receptors. Peritoneal resident macrophages and BMMCs were stained with a biotin-conjugated monoclonal antibody to TIM-1 followed by PE-conjugated streptavidin, a PE-conjugated monoclonal antibody to TIM-4, and an Alexa Fluor 647-conjugated monoclonal antibody to mouse CD300a (open histograms), and analyzed by flow cytometry. The closed histograms show isotype control. These cells were also subjected to RT-PCR to analyze expression of mRNA indicated. (B–D) WT or *Cd300a*^{-/-} BMMCs mixed with apoptotic cells at a ratio of 1:0.1 were stimulated with 1 μ g/ml LPS for 4 h. The culture supernatant was subjected to proteome analyses (B) or ELISA (C; $n = 6$) in the absence (B and C) or presence (C) of D89E MFG-E8. After culture, intracellular cytokine staining was also performed in BMMCs (D; $n = 6$). (E–G) RBL-2H3 transfectants expressing Flag-tagged WT or mutated CD300a, in which the tyrosines Y²³³, Y²⁵⁸, Y²⁷⁰, and/or Y²⁹⁹ were replaced with phenylalanine or the cytoplasmic region was deleted (Del; E), were cocultured with apoptotic cells at a ratio of 1:0.1 in the presence of 100 ng/ml LPS for 4 h (F) or the indicated times (G). Cells were immunoprecipitated with anti-Flag and immunoblotted with anti-SHP-1 (F), anti-phosphotyrosine

analyses also demonstrated that a pHrodo signal was detected only in TIM-4, but not CD300a, transfectant (Fig. 2 D). Together, these results indicated that CD300a does not mediate phagocytosis of apoptotic cells by macrophages.

PS delivers an inhibitory signal in mast cells via CD300a

CD300a is also expressed on mast cells. To address whether apoptotic cells affect mast cell activation via CD300a, we generated BM-derived mast cells (BMMCs) from *Cd300a*^{-/-} or WT mice. Unlike macrophages, mast cells do not express the PS receptors TIM-1, TIM-4, and stabilin-2, although they do express CD300a and α_v integrin (Fig. 3 A). We examined whether PS binding to CD300a has any effect on LPS-induced cytokine or chemokine production from BMMCs by proteome analyses. WT or *Cd300a*^{-/-} BMMCs were cocultured with apoptotic cells at a ratio of 1:0.1 in the presence of LPS for 4 h. *Cd300a*^{-/-} BMMCs produced greater amounts of several chemokines and cytokines, including MCP-1 and IL-13, compared with WT BMMCs (Fig. 3 B). We therefore performed quantitative analysis of MCP-1 and IL-13 in addition to a proinflammatory cytokine TNF by ELISA. Although we did not detect any cytokines or chemokines in the culture supernatants in the absence of stimuli, stimulation with 1 μ g/ml LPS induced both WT and *Cd300a*^{-/-} BMMCs to produce TNF, IL-13, and MCP-1; however, *Cd300a*^{-/-} BMMCs produced them at significantly higher concentrations (Fig. 3 C). Flow cytometry analyses also detected significantly more of these cytokines and chemokines in the cytoplasm of *Cd300a*^{-/-} BMMCs than in the cytoplasm of WT BMMCs 4 h after stimulation with LPS (Fig. 3 D). However, in the presence of a variant of MFG-E8 containing a point mutation (D89E) in the RGD motif (D89E MFG-E8), which is able to bind PS but is not able to bind $\alpha_v\beta_3$ integrin (Hanayama et al., 2002), the levels of these cytokines and chemokines became comparable in WT and *Cd300a*^{-/-} BMMCs (Fig. 3 C), suggesting that CD300a-PS interaction inhibits these cytokines' and chemokines' production from BMMCs.

CD300a contains the five tyrosines at residues 233, 258, 270, 299, and 313 in the cytoplasmic domain, and Y²⁵⁸ and Y²⁷⁰ consist of the consensus ITIM sequence ((I/V/L/S)xYxx(L/V)). We previously reported that Y²⁵⁸ and Y²⁷⁰ are responsible for phosphorylation of CD300a and recruitment of SHP-1 in a rat basophilic leukemia cell line (RBL-2H3) upon stimulation with pervanadate (Okoshi et al., 2005). We found that Y²⁵⁸ and/or Y²⁷⁰ were phosphorylated and involved in SHP-1 recruitment in RBL-2H3 transfectants expressing WT or mutated CD300a when these cells were cocultured with apoptotic cells in the presence of LPS (Fig. 3, E-G). We have recently reported that CD300a also recruited SHP-1 in

BMMCs when BMMCs were cocultured with apoptotic cells in the presence of LPS (Nakahashi-Oda et al., 2012). To examine whether SHP-1 is involved in CD300a-mediated signaling, we knocked down *Ptpn6* with a small interfering RNA (siRNA) to create SHP-1-deficient (*Ptpn6*-KD) WT and *Cd300a*^{-/-} BMMCs (Fig. 3 H). Whereas the control *Cd300a*^{-/-} BMMCs produced significantly more TNF than did WT BMMCs after stimulation with LPS for 4 h, there was no significant difference in TNF production between *Ptpn6*-KD WT and *Ptpn6*-KD *Cd300a*^{-/-} BMMCs (Fig. 3 I). Collectively, these results indicate that upon binding to PS, CD300a recruits SHP-1 and mediates an inhibitory signal in BMMCs, resulting in the suppression of cytokines and chemokines secretion.

We next examined whether CD300a also recruits SHP-1 and delivers an inhibitory signal in macrophages upon binding to PS. WT or *Cd300a*^{-/-} peritoneal resident macrophages were cocultured with apoptotic thymocytes at 1:0.1 ratios in the presence of LPS, demonstrating that CD300a also recruited SHP-1 in peritoneal resident macrophages (Fig. 3 J). Moreover, the production of TNF and IL-10 by *Cd300a*^{-/-} peritoneal macrophages was significantly greater than that by WT peritoneal macrophages (Fig. 3 K), indicating that PS binding of CD300a inhibits cytokine production by macrophages as well as mast cells.

CD300a-deficient mice show longer survival after cecal ligation and puncture (CLP)

TNF, IL-13, and MCP-1 produced by mast cells are chemoattractants for neutrophils and play an important role in bacterial clearance in a CLP peritonitis model in mice (Echtenacher et al., 1996; Malaviya et al., 1996; Baumhofer et al., 1998; Matsukawa et al., 1999; Marshall, 2004). Therefore, we hypothesized that CD300a affects immune regulation by mast cells at the site of peritonitis, where large numbers of cells undergo apoptosis in the peritoneal cavity (Hotchkiss and Nicholson, 2006). In fact, we observed that >10% of cells in the peritoneal cavity 4 h after CLP were apoptotic cells, as determined by staining with Annexin V (Fig. 4 A). To test the hypothesis, we subjected WT and *Cd300a*^{-/-} mice to CLP and observed that *Cd300a*^{-/-} mice survived significantly longer than did WT mice after CLP (Fig. 4 B).

To elucidate the mechanism responsible for the longer survival advantage of the *Cd300a*^{-/-} mice in this model, we measured bacterial content in the peritoneal cavity 4 h after CLP. *Cd300a*^{-/-} mice had significantly fewer bacterial colony-forming units (CFUs) than did WT mice (Fig. 4 C). Because we did not observe any differences in the number and type of commensal bacteria between the two mouse genotypes

(G), or anti-Flag (F and G). (H and I) BMMCs were transfected with control siRNA (Ctrl) or SHP-1 siRNA and analyzed by immunoblotting with antibodies indicated (H). The mixture of these BMMCs and apoptotic cells were stimulated with 1 μ g/ml LPS for 4 h and analyzed for TNF production by ELISA (I; $n = 3$). (J and K) *Cd300a*^{-/-} or WT peritoneal resident macrophages were cocultured with apoptotic cells at a ratio of 1:0.1 in the presence of LPS for 4 h, immunoprecipitated with anti-CD300a, and immunoblotted with anti-SHP-1 or anti-CD300a (J). TNF and IL-10 in the culture supernatants were analyzed by ELISA ($n = 3$; K). Data are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test. Error bars show SD.

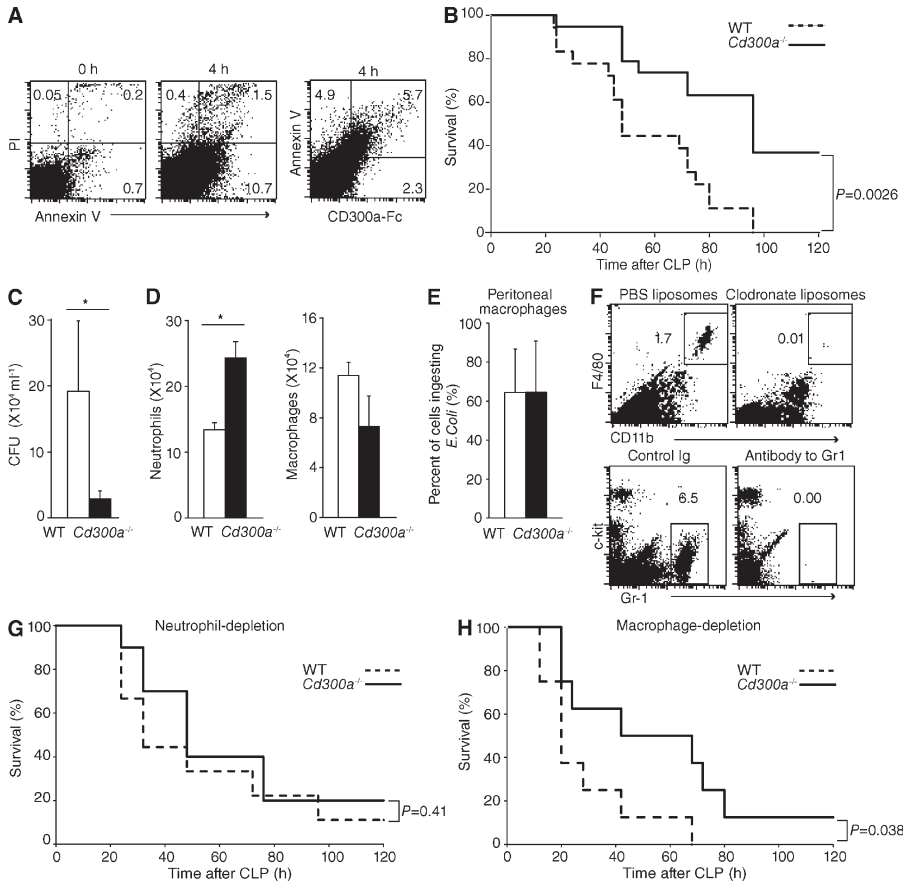


Figure 4. CD300a-deficient mice showed longer survival after CLP. (A–D) WT and *Cd300a*^{−/−} mice were subjected to CLP. Before and 4 h after CLP, cells in the peritoneal lavage fluid of WT mice were stained with APC-conjugated Annexin V and CD300a-Fc, followed by FITC-conjugated anti-human IgG and PI, and analyzed by flow cytometry (A). Data are representative from three mice. (B) The survival rate after CLP is shown (*n* = 20 WT mice and *n* = 21 *Cd300a*^{−/−} mice. Data were pooled from two independent experiments.). Bacterial CFUs (C) and the numbers of neutrophils and macrophages (D) in the peritoneal lavage fluid of WT and *Cd300a*^{−/−} mice (*n* = 5 in each group) were determined 4 h after CLP. (E) Peritoneal resident macrophages from *Cd300a*^{−/−} and WT mice (*n* = 5 in each group) were cocultured with fluorescein-labeled *Escherichia coli* for 1 h in a 24-well plate and analyzed by flow cytometry. The frequencies of macrophages ingesting *Escherichia coli* are shown. (F–H) *Cd300a*^{−/−} and WT mice were injected intraperitoneally with clodronate liposomes to deplete macrophages or an antibody to Gr-1 to deplete neutrophils. The respective controls were injected with PBS liposomes or control Ig. 24 h later, peritoneal lavage fluid cells were stained with the antibodies indicated and analyzed by flow cytometry. Data are representative of three mice in each group of WT mice, and similar results were obtained in each group of *Cd300a*^{−/−} mice (F). The survival rate after CLP was shown (*n* = 9 in each group. Data were pooled from two independent experiments; G and H). *, *P* < 0.05, Student's *t* test. Error bars show SD.

(unpublished data), these results suggested that *Cd300a*^{−/−} mice had more efficient bacterial clearance. We therefore examined the number of phagocytes in the peritoneal cavity after CLP. *Cd300a*^{−/−} mice had significantly greater numbers of neutrophils than did WT mice 4 h after CLP (Fig. 4 D). However, macrophage numbers were comparable between the two genotypes (Fig. 4 D). Because *Cd300a*^{−/−} peritoneal resident macrophages did not differ in the phagocytic function of *Escherichia coli* from those from WT mice (Fig. 4 E), these results suggested that the increased numbers of neutrophils in *Cd300a*^{−/−} mice contributed to the enhanced bacterial clearance, which in turn led to the decreased mortality rates after CLP.

To verify the important role of neutrophils, but not macrophages, in the lower mortality rates in *Cd300a*^{−/−} mice, we generated WT and *Cd300a*^{−/−} mice lacking neutrophils as a result of injection with anti-Gr-1 monoclonal antibody or fewer macrophages as a result of injection with clodronate liposomes. Flow cytometric analyses demonstrated that the neutrophils or macrophages were efficiently depleted in these mice (Fig. 4 F). Depletion of neutrophils rendered the survival rates of both mouse genotypes comparable (Fig. 4 G), indicating that neutrophils played an important role in improving the mortality rate of *Cd300a*^{−/−} mice after CLP. In contrast, there was still a significant difference in survival duration between WT and *Cd300a*^{−/−} mice in which macrophages were depleted (Fig. 4 H). These

results indicated that neutrophils play an important role in the survival of mice after CLP.

CD300a on mast cells is critical for survival of mice after CLP

In the early phase of the CLP peritonitis model, mast cells play an important role in recruitment of neutrophils into the peritoneal cavity by secretion of chemoattractants for neutrophils, such as TNF, and prolong the survival (Echtenacher et al., 1996; Malaviya et al., 1996). We have demonstrated that *Cd300a*^{−/−} BMMCs produced more chemoattractants for neutrophils—including TNF, IL-13, and MCP-1—in response to LPS than WT BMMCs in vitro (Fig. 3, C and D). To examine whether CD300a on mast cells is involved in neutrophil recruitment and longer survival, WT and *Cd300a*^{−/−} BMMCs were or were not transferred into the peritoneal cavities of *Kit*^{W^{sh}/W^{sh} mice—which are deficient in mast cells (Grimbaldeston et al., 2005)—before CLP. After CLP, *Kit*^{W^{sh}/W^{sh} mice that received WT BMMCs survived significantly longer than those that did not receive any BMMCs (Fig. 5 A), which is consistent with previous studies (Echtenacher et al., 1996; Malaviya et al., 1996). However, *Kit*^{W^{sh}/W^{sh} mice that received *Cd300a*^{−/−} BMMCs showed significantly longer survival after CLP and significantly greater bacterial clearance than}}}

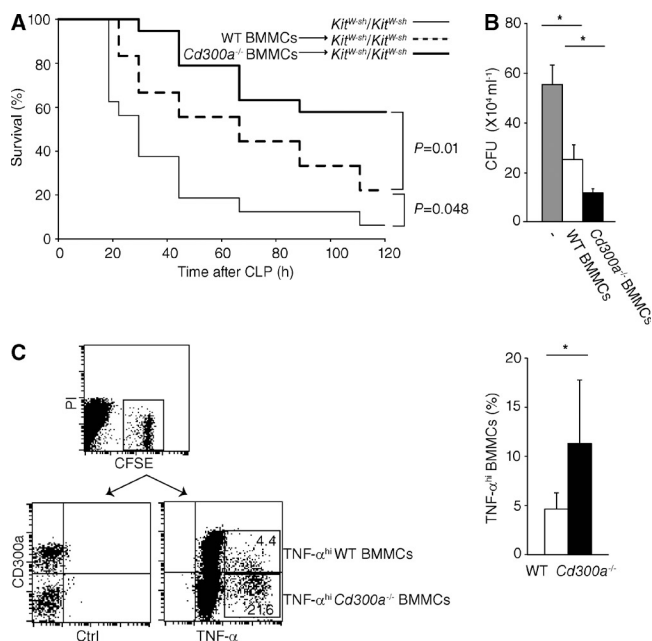
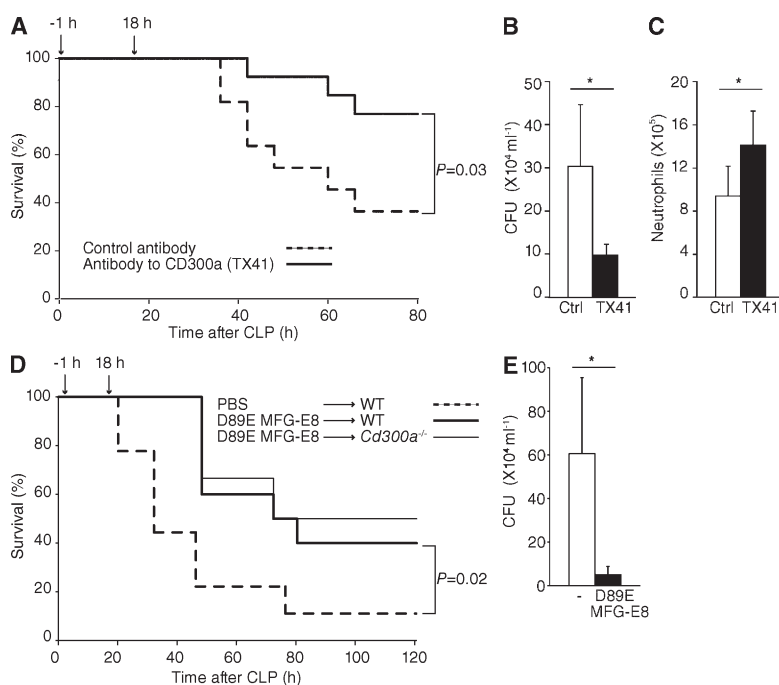


Figure 5. CD300a on mast cells is critical for survival after CLP. (A and B) *Kit^{W-sh/W-sh}* mice were injected i.p., or not injected, with BMMCs from WT or *Cd300a^{-/-}* mice, and then 28 d later, CLP was performed ($n = 20$ in each group, data were pooled from two independent experiments) and the survival rate was shown (A). Bacterial CFUs in the peritoneal lavage fluid of mice transferred with *Cd300a^{-/-}* ($n = 4$) and WT ($n = 4$) BMMCs were assayed 4 h after CLP (B). (C) *Kit^{W-sh/W-sh}* mice were injected i.p. with a 1:1 mixture of CFSE-labeled *Cd300a^{-/-}* and WT BMMCs 24 h before CLP. CFSE-labeled cells in the peritoneal lavage fluid of mice 4 h after CLP were analyzed by flow cytometry. Data are representative of three independent experiments. *, $P < 0.05$, Student's t test. Error bars show SD.



those that received WT BMMCs (Fig. 5, A and B), suggesting that *Cd300a^{-/-}* BMMCs produced a greater amount of chemoattractants for neutrophils than did WT BMMCs in the peritoneal cavity after CLP. To directly confirm this idea, *Kit^{W-sh/W-sh}* mice received a 1:1 mixture of CFSE-labeled WT and *Cd300a^{-/-}* BMMCs via the peritoneal cavity and were subjected to CLP. Although both WT and *Cd300a^{-/-}* BMMCs transferred into the peritoneal cavity showed a low level of TNF expression, *Cd300a^{-/-}* BMMCs, defined as $CD300a^-CFSE^+$ cells, showed significantly higher frequency in the BMMCs producing a high level of TNF than did WT ($CD300a^+CFSE^+$) BMMCs (Fig. 5 C). Together, these results indicate that CD300a inhibits production of chemoattractants for neutrophils from BMMCs in the peritoneal cavity after CLP. Although BMMC transfer into *Kit^{W-sh/W-sh}* mice does not completely reconstitute the immune system in *Kit^{W-sh/W-sh}* mice (Dudeck et al., 2011), our results suggested that CD300a on mast cells was primarily responsible for the phenotype of prolonged survival of *Cd300a^{-/-}* mice after CLP.

Blockade of CD300a-PS interaction prolonged survival of mice after CLP

Because *Cd300a^{-/-}* mice survived longer after CLP, we examined whether an antibody against mouse CD300a (TX41) could have a prophylactic effect on CLP-induced sepsis. TX41 does not deplete myeloid cells, including mast cells. Intraperitoneal injection of mice with TX41 1 h before and 18 h after CLP significantly increased neutrophil numbers in the peritoneal cavity, improved bacterial clearance 4 h after CLP, and prolonged survival compared with treatment with a control antibody (Fig. 6, A–C). Similarly, intraperitoneal injection with D89E MFG-E8 also prolonged survival of WT mice compared with treatment with PBS. In contrast, D89E MFG-E8 did not change the survival of *Cd300a^{-/-}* mice, indicating that D89E MFG-E8 blocked the interaction between PS and CD300a, but not other PS receptors (Fig. 6 D). Moreover, D89E MFG-E8 improved bacterial clearance of WT mice (Fig. 6 E). These results provided the formal evidence that PS on apoptotic cells affected

Figure 6. The blockade of the CD300a-PS interaction prolongs survival of mice after CLP.

(A–C) WT mice were injected i.p. with 500 μ g of control antibody ($n = 11$) or anti-CD300a monoclonal antibody (TX41; $n = 13$), 1 h before and 18 h after CLP, and the survival rate is shown (A). Bacterial CFUs (B) and the numbers of neutrophils (C) in the peritoneal lavage fluid of mice ($n = 5$ in each group) were determined 4 h after CLP, as described. (D and E) WT or *Cd300a^{-/-}* mice were injected i.p. with 50 μ g D89E MFG-E8 ($n = 10$ and 8, respectively) or PBS ($n = 9$), 1 h before and 18 h after CLP, and the survival rate is shown (D). Bacterial CFUs in the peritoneal lavage fluid of mice ($n = 4$ in each group) were determined 4 h after CLP, as described (E). *, $P < 0.05$, Student's t test. Error bars show SD. Data in A and D were each pooled from two independent experiments.

the bacterial clearance. Thus, blocking the interaction between PS and its receptor CD300a is potentially a useful therapy for prophylaxis against peritonitis-induced sepsis.

DISCUSSION

A large number of cells undergo apoptosis every day in physiological and pathological settings. PS receptors play a central role in engulfment of apoptotic cells and are crucial to avoiding the development of autoimmune diseases (Hanayama et al., 2004). Pathological conditions such as microbial infections significantly augment cell death by apoptosis (Hotchkiss and Nicholson, 2006) and trigger the first line of inflammatory responses by mast cells through receptors for pathogen-associated molecular patterns such as Toll-like receptors (Marshall, 2004; Bischoff, 2007; Abraham and St John, 2010). Mast cells therefore play an important role in the early phase of immunity against microbial pathogens. Our results indicate that PS not only delivers “eat-me” signals to phagocytes via PS receptors but also suppresses inflammatory responses by mast cells and macrophages via CD300a.

Although CD300a is expressed on professional phagocytes, such as macrophages and dendritic cells (Yotsumoto et al., 2003), we did not observe CD300a-mediated phagocytosis of apoptotic cells by macrophages. Because CD300a associates with SHP-1 upon ligand binding and inhibits activation signals, CD300a may not have a functional property of phagocytosis. However, it cannot be excluded that the CD300a-mediated signal suppresses, rather than augments, phagocytosis by other PS receptors such as TIM-4 (Kobayashi et al., 2007), if PS binding could be shared by CD300a and TIM-4 on a macrophage. This may depend on the binding affinity of these receptors to PS. At present, the affinity of CD300a binding to PS has remained undetermined. Very recently, Choi et al. (2011) have reported that CD300f also recognizes PS and exogenous expression of CD300f enhanced phagocytosis of apoptotic cells by fibroblast cell line L929 cells. Like CD300a, CD300f contains ITIM in the cytoplasmic portion. Upon cross-linking with antibody, the ITIM of CD300f is phosphorylated, recruits SHP-1 and SHP-2, and mediates an inhibitory signal in myeloid cells (Izawa et al., 2009). It is unclear, however, how CD300f-mediated signaling involves phagocytosis of apoptotic cells.

One of the key biological features of apoptotic cell engulfment by phagocytes is the noninflammatory nature (Fadok et al., 1998; Ravichandran and Lorenz, 2007). The molecular mechanism under this feature has still remained unclear. The suppressive effect of CD300a on the proinflammatory cytokine and chemokine production by mast cells does not necessarily account for this feature because mast cells are not phagocytes and their activation is induced by pathological conditions such as microbial infections. However, it may contribute to avoiding excessive inflammations by suppressing mast cell-mediated triggering of inflammation. Our data showed that CD300a deficiency also affected the production of both the proinflammatory and antiinflammatory cytokines TNF and IL-10, respectively, without selectivity by peritoneal resident macrophages, when

these cells were cocultured with apoptotic cells in the presence of LPS. However, because SHP-1 recruitment by CD300a that is essential for CD300a-mediated inhibitory signal requires both PS binding and LPS stimulation in macrophages as well as mast cells, it is unlikely that CD300a on macrophages is involved in the noninflammatory nature of phagocytosis of apoptotic cells mediated by PS receptors other than CD300a in the physiological conditions. Further studies should be required to clarify the regulatory role of CD300a in activation of myeloid cells, including dendritic cells and neutrophils as well as mast cells and macrophages. Nonetheless, CD300a is the first PS receptor identified on mast cells and suppresses inflammatory responses by mast cells.

Sepsis, the systemic inflammatory syndrome induced by severe infections, remains a leading cause of death worldwide (Angus and Wax, 2001; Cohen, 2002). Despite extensive basic and clinical research, the pathophysiology of sepsis is still poorly understood, and trials of several therapies have failed to improve the prognosis of septic patients in large multicenter clinical trials (Riedemann et al., 2003; Russell, 2006). The best established mouse model of sepsis, CLP, has shown that mast cells play essential roles in host defense against CLP (Malaviya et al., 1996). Here, we provide compelling experimental evidence supporting the efficacy of anti-CD300a monoclonal antibody and D89E MFG-E8 adjunctive therapy for polymicrobial peritonitis-induced sepsis. Both anti-CD300a and D89E MFG-E8 blocked the binding of PS to CD300a, resulting in the increased production of chemoattractants for neutrophils from mast cells induced by LPS in the peritoneal cavity. Long-term treatment with D89E MFG-E8 leads to the development of autoimmune diseases as a result of the defect of apoptotic cell clearance (Hanayama et al., 2004). However, we injected D89E MFG-E8 only twice before CLP and 18 h after CLP, which was enough for the prophylaxis of peritonitis-induced sepsis. Mast cell activation is important in the very early phase after infection, only when the blocking CD300a-mediated signaling by D89E MFG-E8 or anti-CD300a antibody should be required.

The classical function of mast cells is involved in allergic responses, in which Fc ϵ RI-mediated signals lead to degranulation from mast cells (Gilfillan and Tkaczyk, 2006). In vitro analyses demonstrated that cross-linking CD300a with monoclonal antibody suppressed Fc ϵ RI-mediated signals, resulting in the decreased degranulation in human and mice (Yotsumoto et al., 2003; Okoshi et al., 2005; Munitz et al., 2006; Karra et al., 2009). Because many cells undergo apoptosis at the site of allergic inflammation, CD300a may play an important role in allergic responses by mast cells and may be a potential prophylaxis and/or therapeutic target for treatment of allergic diseases.

MATERIALS AND METHODS

Mice. To construct the CD300a targeting vector, we replaced exon 1 to exon 6 of the *Cd300a* gene with a neomycin-resistance gene cassette (PGK-GB2-neo) using the bacterial artificial chromosome (BAC) system (Yang and Seed, 2003). BALB/c embryonic stem cells were electroporated with linearized *Cd300a* targeting vector, and cells were selected for G418 antibiotic resistance.

Correctly targeted embryonic stem cell clones with normal karyotypes were injected into 3.5-d-old BALB/c blastocysts to create chimeric mice (*Cd300a^{+/-}*). *Cd300a^{+/-}* mice were intercrossed to generate *Cd300a^{-/-}* mice. Homozygous mice were obtained at the expected Mendelian frequencies and developed normally. *Cd300a^{-/-}* mice and control WT mice were bred under specific pathogen-free conditions in the same room of our animal facility. CD300a-deficient (*Cd300a^{-/-}*) mice in the BALB/c background were backcrossed onto the C57BL/6J genetic background for 12 generations. C57BL/6J-*Kit^{W-sh/W-sh}* mice were provided by Riken BioResource Center (Tsukuba, Japan). *CAD*-deficient mice have been previously described (Nagata, 2005). C57BL/6J mice were purchased from Clea Japan. All experiments were performed in accordance with the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center.

Generation of CD300a-Fc and MFG-E8. The fusion protein of CD300a with the Fc portion of human IgG (CD300a-Fc) was generated from chimeric cDNA containing the entire extracellular domain of CD300a and the human IgG1Fc, as previously described (Tahara-Hanaoka et al., 2004). MFG-E8 and D89E MFG-E8 were provided by M. Tanaka (Research Center for Allergy and Immunology, Yokohama, Japan) and also generated in our laboratory, as previously described (Hanayama et al., 2002).

Antibodies and cells. Control rat antibodies and antibodies to CD11b (M1/70), F4/80, c-Kit (2B8), Gr-1 (RB6), FcεRI (MAR-1), and TNF were purchased from BD. The CD300a-specific monoclonal antibody TX41 (rat IgG2a) was generated in our laboratory, as described previously (Yotsumoto et al., 2003). The antibody to MCP-1 was purchased from BioLegend, and the antibody to IL-13 was purchased from eBioscience. Antibodies to SHP-1 (C-19) and SHP-2 (C-18) were purchased from EMD Millipore. To generate BMMCs, 2×10^6 BM cells were cultured in a 10-cm dish in complete RPMI 1640 medium containing 10% FBS in the presence of 10 ng/ml stem cell factor and 4 ng/ml IL-3 for >5 wk. Cells were passaged with fresh medium every week. Flow cytometry analyses showed that >99% of the cultured cells were c-kit⁺FcεRI⁺ cells. NIH 3T3 cells were transfected with a pMX-neo retrovirus vector plasmid containing Flag-tagged CD300a cDNA. An NIH 3T3 transfectant stably expressing TIM-4 was provided by T. Kitamura (University of Tokyo; Yamanishi et al., 2010). RBL-2H3 transfectants expressing WT CD300a and mutated CD300a tagged with Flag at the N terminus, in which the tyrosines were replaced with phenylalanine or the cytoplasmic region was deleted, were previously described (Okoshi et al., 2005).

Preparation of apoptotic cells and phagocytosis assay. Thymocytes from C57BL/6 or *CAD*-deficient mice were incubated with 10 μM dexamethasone (Sigma-Aldrich) in RPMI medium for 6 h. Induction of apoptosis was determined by labeling PS expressed on the plasma membrane with APC-conjugated annexin V (BD) and propidium iodide (Sigma-Aldrich).

2×10^5 thioglycollate-elicited peritoneal macrophages were cocultured with apoptotic thymocytes at a ratio of 1:5 for 1 h at 37°C in 8-well Laboratory-Tek II chamber slides (Thermo Fisher Scientific). The cells were then washed with cold PBS and fixed with 1% paraformaldehyde, subjected to TUNEL staining with FITC-labeled dUTP (Roche), and then analyzed by laser-scanning confocal microscopy, as described previously (Miyamishi et al., 2007; Yamanishi et al., 2010).

Apoptotic thymocytes were labeled with pHrodo succinimidyl ester (Invitrogen), as described previously (Miksa et al., 2009). In brief, 10^6 thymocytes were incubated at room temperature in pHrodo succinimidyl ester solution at a concentration of 20 ng/ml for 30 min, washed twice with PBS, and resuspended in OPTI-MEM 1. 6×10^4 NIH 3T3 transfectants expressing CD300a or TIM-4 were cocultured with pHrodo succinimidyl ester-labeled apoptotic thymocytes at a ratio of 1:5 for 1 h at 37°C in 8-well Laboratory-Tek II chamber slides, washed with PBS and fixed as described in the previous paragraph, and then analyzed by laser-scanning confocal microscopy or flow cytometry.

CLP. CLP was performed as previously described (Echtenacher et al., 1996). In brief, the cecum was exposed by a 1- to 2-cm midline incision on the

ventral abdomen, ligated at its distal portion, and punctured twice with a 27-gauge needle in the ligated segment. We then returned the cecum to the abdomen, injected 1 ml of sterile saline subcutaneously for rehydration, and closed the incision by suture.

Measurement of cytokines and chemokines. The concentrations of cytokines and chemokines were measured in triplicate using ELISA kits from BD (TNF) and R&D Systems (MCP-1 and IL-13). Intracellular cytokines and chemokines were analyzed by flow cytometry after fixation and permeabilization using a Fix & Perm cell fixation and permeabilization kit (Invitrogen). The supernatant of the co-culture of BMMC with apoptotic cells was subjected to cytokine and chemokine proteome analyses with a Proteome Profiler Array (R&D Systems) in accordance with the manufacturer's instructions.

Measurement of CFUs of aerobic bacteria. Aerobic bacterial CFUs were quantified by plating serial dilutions of peritoneal lavage fluid and culturing them at 37°C for 48 h on brain-heart-infusion agar plates. CFUs were then enumerated by calculating the number of colonies in 1 ml peritoneal lavage fluid, as previously described (Plitas et al., 2008).

In vivo depletion of macrophages and neutrophils. For macrophage depletion, clodronate liposomes and control PBS liposomes (Encapsula NanoSciences) were prepared as previously described (Van Rooijen, 1989). Mice were injected i.p. with 0.5 ml liposomes 24 h before CLP. For neutrophil depletion, mice were injected i.p. with 5 μg anti-Gr-1, 24 h before CLP. WT or *Cd300a^{-/-}* neutrophils (1×10^6 /mice) purified from the BM were injected into some neutrophil-depleted mice just after CLP.

RNA interference. To target SHP-1 in BMMCs, 0.5 mM SHP-1 siRNA (siGENOME SMARTpool; Thermo Fisher Scientific) was mixed with 1 μl X-tremeGENE siRNA transfection reagent (Roche) and transfected into 5×10^5 BMMCs, as previously described (An et al., 2006). After 48 h, the supernatant was removed, and fresh medium and 0.3 mM SHP-1 siRNA were added. The cells were cultured for another 16 h before further experiments.

Statistical analyses. The Kaplan-Meier log-rank test was used for survival analyses. All other statistical analyses were performed using an unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

We thank S. Mitsuishi and F. Abe for secretarial and technical assistances, respectively.

This research was supported in part by grants provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation (NIBIO), Japan.

The authors have no competing financial interests.

Submitted: 13 January 2012

Accepted: 2 July 2012

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