Biochemical and Biophysical Research Communications 417 (2012) 646–650

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of phosphatidylserine as a ligand for the CD300a immunoreceptor

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ARTICLE INFO

Article history:
Received 16 November 2011
Available online 11 December 2011

Keywords:
CD300a
Apoptosis
Phosphatidylserine receptor
Inhibitory receptor

ABSTRACT

CD300a is a member of CD300 family molecules consisting of seven genes on human chromosome 17 and nine genes in mouse chromosome 11. CD300a has a long cytoplasmic region containing the consensus immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence. Upon crosslinking with antibodies against CD300a, CD300a mediates an inhibitory signal in myeloid cells. However, the ligand for CD300a has not been identified and the physiological role of CD300a remained unclear. Here, we demonstrate that the chimeric fusion protein of CD300a extracellular domain with the Fc portion of human IgG specifically bound phosphatidylserine (PS), which is exposed on the outer leaflet of the plasma membrane of apoptotic cells. PS binding to CD300a induced SHP-1 recruitment by CD300a in mast cells in response to LPS. These results indicated that CD300a is a new PS receptor.

1. Introduction

CD300 is a multigene family consisting of seven genes on human chromosome 17 [1,2]. CD300 molecules are member of immunoglobulin (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) [3–8]/CMRF-35-like molecule (CLM) [9–11]/leukocyte mono-Ig-like receptor (LMIR) [12–14]/DIgR [15,16], were encoded by nine genes on a small segment of mouse chromosome 11, syntenic region of human chromosome 17 [9,17]. CD300 molecules are preferentially expressed on myeloid cells, including macrophages, neutrophils, dendritic cells (DCs) and/or mast cells, and may regulate activation of these cells. One of the CD300 molecules, CD300a (also called MAIR-I [3], LMIR1 [12], or CLM-8 [9] in mice and IRp60 [18] or CMRF-35H [1] in human), has a long cytoplasmic region containing the consensus immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence. Upon crosslinking with monoclonal antibodies, CD300a inhibits FcεRI-mediated signals, resulting in the suppression of degranulation from human and mouse mast cells in vitro [3,4,19,20]. In addition, CD300a modulates inflammatory responses by myeloid cells [21,22]. CD300a is also expressed on human, but not mouse, NK cells and involved in cytotoxic function [18,23]. However, the ligand for CD300a has not been identified and the physiological role of CD300a remained unclear.

In this study, we identified phosphatidylserine (PS), which is exposed on the outer leaflet of the plasma membrane of apoptotic cells, as a ligand for CD300a.

2. Materials and methods

2.1. Antibodies, CD300-Fc and MFG-E8

The human and mouse CD300a-specific monoclonal antibodies TX49 (mouse IgG1) and TX41 (rat IgG2a), respectively, were generated in our laboratory, as described previously [3]. The fusion proteins of CD300a and CD300d with the Fc portion of human IgG (CD300a-Fc and CD300d-Fc, respectively) were generated from chimeric cDNA containing the entire extracellular domain of each CD300 and the human IgG1Fc, as described [24]. Milk fat globule EGF factor 8 (MFG-E8) and D89E MFG-E8 containing a point mutation (D89E) in the RGD motif were provided by Masato Tanaka (RCAI, Yokohama, Japan) and Shigekazu Nagata (Kyoto University, Kyoto, Japan) and also generated in our laboratory, as described [25].

2.2. Cells

Ba/F3 cells, Jurkat cells and PLB-985 cells are pro-B cell line, T cell line and myeloid cell lines, respectively. PLB-985 cells were provided by Hiraoka (Meiji University, Tokyo). For preparation of apoptotic cells, thymocytes from C57BL/6 were incubated with 10 μM dexamethasone (Sigma, St. Louis, MO) in RPMI 1640 medium for 5 h. Ba/F3, Jurkat and PLB-985 cells suspended in RPMI

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doi:10.1016/j.bbrc.2011.12.025

1640 medium were irradiated with UV for 60 min. Induction of apoptosis was determined by labeling PS expressed on the plasma membrane with APC-conjugated annexin V (BD Pharmingen, Franklin Lakes, NJ) and propidium iodide (Sigma). Bone marrow-derived cultured mast cells (BMMCs) were generated by culture of bone marrow cells in the presence of IL-3 and stem cell factor, as described [3].

2.3. Binding assay

Cells were stained with CD300-Fc or control human IgG in PBS buffer containing 2% FBS in the presence or absence of 1 mM $CaCl_2$ for 30 min, washed twice with the same buffer, and then incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti-human IgG. For staining with annexin V, cells were incubated at room temperature for 15 min with APC-conjugated annexin V in 10 mM HEPES-NaOH buffer containing 140 mM NaCl and 2.5 mM $CaCl_2$. For the blocking assay, cells were preincubated with a monoclonal antibodies against mouse or human CD300a (TX41 or TX49, respectively), an isotype control antibody, or MFG-E8 for 30 min and then stained with CD300a-Fc, as described above. To assay binding of CD300a-Fc to phospholipids, a PIP-strip assay (Echelon Bioscience, Salt Lake City, UT) was performed in accordance with the manufacturer's instructions. For solid phase ELISA for PS binding, PS (100 μl, 2.5 μg/ml) in methanol was added to 96-well plates and air-dried. After blocking non-specific binding by treatment with 2% BSA in PBS, CD300a-Fc, CD300d-Fc, or human IgG were added to the wells, and incubated at room temperature for 30 min. After washing with PBS containing 0.05% Tween 20, the bound proteins were quantified by ELISA.

2.4. Biochemistry

BMMCs were stimulated with LPS (1 μg/ml) for 4 h, or left unstimulated, and then immunoprecipitated with an antibody against CD300a (TX41) in the presence or absence of D89E MFG-E8 (5 μg/ml). The immunoprecipitates were immunoblotted with antibodies against SHP-1 or CD300a (TX41), as described [4].

3. Results

3.1. CD300a binds dead cells

To identify the ligand for CD300a, we generated a chimeric fusion protein of the extracellular portion of CD300a with the Fc portion of human IgG (CD300a-Fc). We used the CD300a-Fc to screen for the expression of a CD300a ligand on hematopoietic cells and tumor cell lines by using flow cytometry. Although the CD300a-Fc did not stain any viable cells tested, we found that CD300a-Fc bound a small population of dead cells. To confirm this observation, Ba/F3 cells were irradiated with UV for 30 min and then stained with PI and CD300a-Fc. CD300a-Fc bound the PI^+, but not PI^-, population of Ba/F3 cells (Fig. 1A). These results suggest that CD300a ligand is expressed on dead cells.

3.2. CD300a binds phosphatidylserine on apoptotic cells in both human and mice

To examine whether CD300a-Fc binds apoptotic cells, apoptosis was induced in mouse thymocytes by treatment with dexamethasone. The cells were stained simultaneously with allophycocyanin (APC)-conjugated annexin V and CD300a-Fc, followed by staining

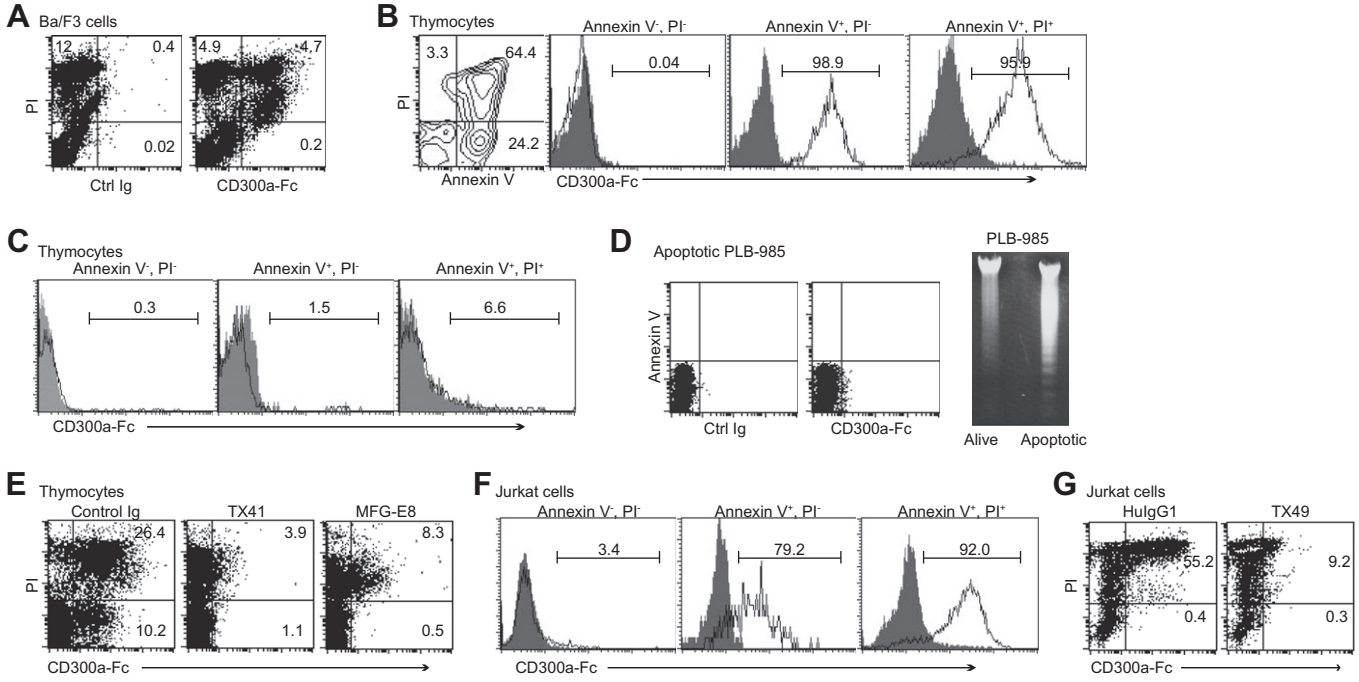

Fig. 1. CD300a-Fc binds phosphatidylserine on apoptotic cells. UV-irradiated BaF/3 cells were stained with either CD300a-Fc or control human IgG, followed by an FITC-conjugated antibody against human IgG and propidium iodide (PI), and analyzed by flow cytometry. (B and C) Dexamethasone-treated mouse thymocytes were stained with APC-conjugated annexin V and mouse CD300a-Fc followed by an FITC-conjugated antibody against human IgG and PI, in the presence (B) or absence (C) of $CaCl_2$, and analyzed by flow cytometry. (D) UV-irradiated PLB-985 cells were stained with either CD300a-Fc or control human IgG, followed by an FITC-conjugated antibody against human IgG and APC-conjugated annexin V, and analyzed by flow cytometry (left panel). Induction of apoptotosis in PLB-985 cells were validated by genomic DNA fragmentation (right panel). (E) Dexamethasone-treated mouse thymocytes were incubated with human IgG (Control Ig), anti-mouse CD300a (TX41) or MFG-E8, together with mouse CD300a-Fc, followed by an FITC-conjugated antibody against human IgG and PI, in the presence of $CaCl_2$, and analyzed by flow cytometry. (F, G) UV-irradiated Jurkat cells were stained with APC-conjugated annexin V and human CD300a-Fc, followed by an FITC-conjugated antibody against human IgG and PI, in the presence of $CaCl_2$, and analyzed by flow cytometry (F). UV-irradiated Jurkat cells were incubated with human IgG (Control Ig) or anti-human CD300a (TX49) together with human CD300a-Fc, followed by an FITC-conjugated antibody against human IgG and PI, in the presence of $CaCl_2$, and analyzed by flow cytometry.

with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG and PI. Whereas CD300a-Fc did not stain annexin V^- thymocytes, annexin V^+ (apoptotic) thymocytes bound to CD300a-Fc in the presence of $CaCl_2$ (Fig. 1B). However, this binding was not observed in Ca^{2+}-free medium (Fig. 1C). This Ca^{2+}-dependent binding of CD300a-Fc to apoptotic thymocytes suggested that CD300a binds phosphatidylserine (PS) exposed on the outer leaflet of plasma membrane of apoptotic cells. In fact, CD300a-Fc did not bind to the apoptotic PLB-985 cells induced by the irradiation with UV, a human myeloid cell line that does not expose PS on the outer leaflet of plasma membrane [26] (Fig. 1D).

To examine the specific binding of CD300a to PS, apoptotic thymocytes was stained with CD300a-Fc in the presence of a neutralizing monoclonal antibody (mAb) against mouse CD300a (TX41). TX41 mAb specifically inhibited the CD300a-Fc binding to apoptotic cells (Fig. 1E). MFG-E8 binds both PS and αVβ3 integrin and bridges between apoptotic cells and αVβ3-expressing phagocytes [25]. We observed that MFG-E8 also inhibited the CD300a-Fc binding to apoptotic cells (Fig. 1E). Together, these results suggested that CD300a-Fc specifically bound PS. Similarly, human CD300a-Fc bound annexin V^+ Jurkat human T cells that had been irradiated with UV, and this binding was inhibited by pretreatment with an antibody against human CD300a (TX49) (Fig. 1F, G), suggesting that human CD300a also bound PS.

3.3. CD300a directly binds phosphatidylserine

We next examined whether CD300a-Fc directly bound PS. An immunoblotting study showed that CD300a-Fc specifically bound PS, but did not bind either phosphatidylethanolamine (PE) or phosphatidylcholine (PC) (Fig. 2A). Solid phase ELISA demonstrated that CD300a bound PS in a CD300a dose-dependent manner. In contrast, although CD300d, a member of the CD300 family, contains the Ig-like domain in the extracellular portion with more than 90% homology with that of CD300a [3], it did not bind PS (Fig. 2B). These results demonstrated the direct binding of CD300a-Fc to PS.

3.4. Phosphatidylserine delivers a signal via CD300a in mast cells

CD300a contains immunoreceptor-tyrosine based inhibitory motif (ITIM) in the cytoplasmic portion and recruits SHP-1 upon cross-linking with an antibody against CD300a on mast cells [4]. SHP-1 recruitment was also observed when BMMC were co-cultured with apoptotic cells in response to LPS (Fig. 3). However, in the presence of the D89E MFG-E8, which is able to bind PS but is not able to bind αVβ3 [25], CD300a did not recruit SHP-1 (Fig. 3), indicating that PS binding was required for SHP-1 recruitment by CD300a in response to LPS.

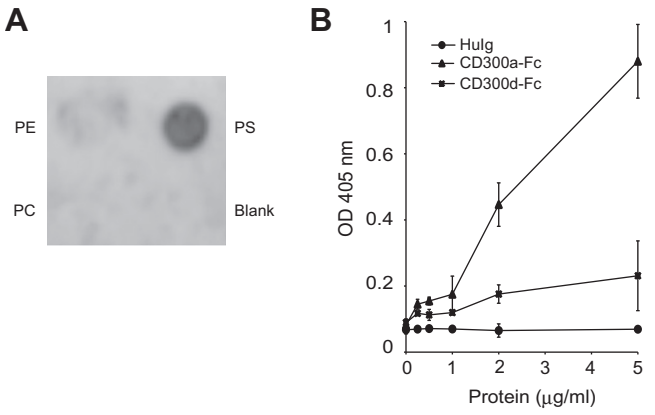

Fig. 2. CD300a-Fc directly binds phosphatidylserine. (A) A membrane spotted with phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) was incubated with CD300a-Fc, followed by a horse raddish peroxidase (HRP)-conjugated antibody against human IgG. A representative results from three independent experiments is shown. (B) Microtiter plates coated with PS were incubated with increasing concentrations of CD300a, CD300d, or human IgG, and PS binding was analyzed by ELISA. A representative result from three independent experiments is shown. Error bars show SD.

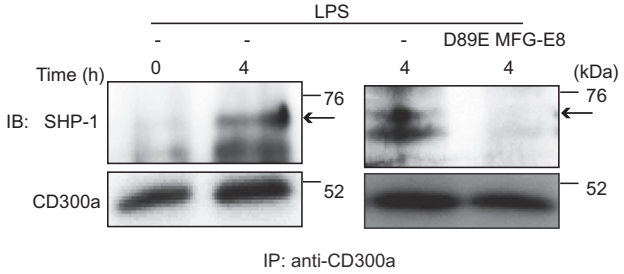

Fig. 3. Interaction of CD300a with phosphatidylserine induces SHP-1 recruitment. Bone marrow-derived cultured mast cells were mixed with apoptotic cells at a ratio of 1:0.1 and stimulated with LPS (1 μg/ml) for 4 h in the presence or absence of D89E MFG-E8 or left unstimulated, and then immunoprecipitated with an antibody to CD300a and immunoblotted with antibodies against SHP-1 or CD300a. The arrows indicate SHP-1. A representative result from three independent experiments is shown.

4. Discussion

Several receptors for PS are expressed on phagocytes and are involved in clearing apoptotic cells [27–29]. For example, T cell immunoglobulin- and mucin-domain-containing molecules 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 [30,31]. Brain-specific angiogenesis inhibitor 1 (BAI1) [32] and stabilin-2 [33], which are expressed on neuron [34] and the sinusoidal endothelial cells of the spleen, lymph nodes, and bone marrow [35], respectively, were also reported to be PS receptors for apoptotic cells. Engulfment of apoptotic cells also involves the bridging molecules that recognize PS. MFG-E8, which is expressed by tingible-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 αvβ3 or αvβ5 integrin on the phagocytes, stimulating the engulfment of apoptotic cells [25]. Similarly, growth arrest-specific 6 (Gas6) and protein S that are abundant in the plasma and binds PS and TAM family members (Tyro3, Axl, and Mer) is also a bridging molecules between apoptotic cells and phagocytes [36,37].

The CD300 family consists of seven and nine members in human and mouse, respectively. The extracellular portions are conserved each other in the family. In particular, the Ig-like domain of CD300a shows 92% amino acid identity to that of CD300d [3,6], which associates with DAP12 or FcεRIγ and mediates activation and inhibitory signals in myeloid cells and B cells, respectively [5,8]. Despite this similarity of CD300a with CD300d in amino acid structure in the extracellular portion, CD300d-Fc did not bind to apoptotic cells, suggesting that CD300d has a different ligand. A recent report demonstrated that CD300b, which mediates an activating signal via an adaptor protein DAP12, bound to TIM-1 and TIM-4. While TIM-1 and TIM-4 bind PS, CD300b did not bind PS [14]. Very recently, Choi et al. has just reported that CD300f also recognizes PS and exogenous expression of CD300f enhanced phagocytosis of apoptotic cells by fibroblast cell line L929 cells [38]. Like CD300a, CD300f contains ITIM in the cytoplasmic portion. Upon crosslinking with antibody, the ITIM of CD300f is phosphorylated, recruits SHP-1 and SHP-2 and mediates an inhibitory signal in

myeloid cells [39]. It is unclear, however, how CD300f-mediated signaling involves phagocytosis of apoptotic cells. To address whether CD300f on primary myeloid cells is involved in phagocytosis of apoptotic cells, in vitro and in vivo studies using CD300f-deficient mice should be required. The CD300 molecules, other than CD300a, CD300b and CD300f, have still remained to be orphan receptors and identification of ligands for these CD300 molecules should be important for understanding immune responses by this receptor family.

In this study, we demonstrated that PS directly binds CD300a and phosphorylates ITIM in the cytoplasmic portion of CD300a on mast cells, indicating that CD300a is a new PS receptor. CD300a is expressed on a variety of cell lineages, including mast cells, granulocytes, dendritic cells and macrophages. It is an exclusively important issue to explore the functional role of CD300a as a PS receptor in regulation of immune responses.

Acknowledgments

We thank S. Mitsuishi for secretarial assistances. 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.

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