Ceramide-CD300f binding inhibits lipopolysaccharide-induced skin inflammation

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Running Title: The role of CD300f in lipopolysaccharide responses

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Keywords: receptor, mast cell, neutrophil, lipopolysaccharide (LPS), inflammation

ABSTRACT

Lipopolysaccharide (LPS) triggers inflammatory responses; however, the negative regulation of LPS responses in vivo remains poorly understood. CD300f is an inhibitory receptor among the CD300 family of paired activating and inhibitory receptors. We have previously identified ceramide as a ligand for CD300f and shown that the binding of ceramide to CD300f inhibits IgE-mediated mast cell activation and allergic responses in mouse models. Here we identify the critical role of CD300f in inhibiting LPS-induced skin inflammation. CD300f deficiency remarkably enhanced LPS-induced skin edema and neutrophil recruitment in mice. Higher levels of factors that increase vascular permeability and of factors that induce neutrophil recruitment were detected in LPS-injected skin pouch exudates of CD300f-/- mice compared to wild-type mice. CD300f was highly expressed in mast cells and recruited neutrophils, but not in macrophages, among skin myeloid cells. CD300f deficiency failed to influence the intrinsic migratory ability of neutrophils. Ceramide-CD300f binding suppressed the release of chemical mediators from mast cells and from neutrophils in response to LPS. Adoptive transfer experiments indicated that mast cells mediated enhanced edema in LPS-stimulated skin of CD300f-/- mice, whereas mast cells together with recruited neutrophils mediated robust neutrophil accumulation. Importantly, administering a ceramide antibody or ceramide-containing vesicles enhanced or suppressed LPS-induced skin inflammation of wild-type mice, respectively. Thus, ceramide-CD300f binding inhibits LPS-induced skin inflammation, implicating CD300f as a negative regulator of Toll-like receptor 4 (TLR4) signaling in vivo.

INTRODUCTION

The CD300, also known as leukocyte mono-immunoglobulin-like receptor (LMIR), CMRF35-like molecule (CLM), or myeloid-associated immunoglobulin-like receptor (MAIR), members modulate immune cell responses via their paired activating and inhibitory receptor functions (1-7). CD300f, also known as LMIR3 or CLM-1, is an inhibitory receptor that
contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and a single immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic region (4, 7-9). CD300f is expressed in myeloid cells, including mast cells and neutrophils. We have recently identified ceramide as a ligand for CD300f and demonstrated that the binding of extracellular ceramide to CD300f inhibits IgE- or ATP-mediated mast cell activation via its ITIMs and ITSM, in allergic responses or colitis, respectively, in mouse models (9, 10). Tian et al. demonstrated that CD300f regulates the clearance of apoptotic cells by binding to surface-exposed phosphatidylserine (11). Engagement of CD300f with its specific antibody inhibits both myeloid differentiation factor 88 (MyD88) and toll-interleukin 1 receptor-domain-containing adaptor-inducing interferon-β (TRIF)-mediated Toll-like receptor (TLR) signaling in human monocyte/macrophage cell lines (12-14), while it augments TLR4 signaling in mouse bone marrow-derived mast cell (BMMC) (8). However, the in vivo role of CD300f in innate immune responses remains poorly understood. Therefore we examined whether CD300f regulated in vivo responses to lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, which activates myeloid cells through TLR4 (15). Accumulated studies show that TLR4 plays an important role not only in infectious inflammation characterized by gram-negative bacterial infection and sepsis, but also in non-infectious inflammation such as ischemia/reperfusion injury and neurodegenerative/neurological diseases (16, 17).

In the present study, we use LPS-induced skin inflammation models in WT and CD300f−/− mice, demonstrating that CD300f deficiency remarkably enhances edema and neutrophil accumulation in LPS-stimulated skin. In general, tissue-resident mast cells and macrophages initiate neutrophil recruitment by releasing factors that induce neutrophil recruitment (e.g., macrophage inflammatory protein 2 (MIP2), keratinocyte-derived chemokine (KC), leukotriene B4 (LTB4), and mast cell proteases) in response to specific stimuli. Moreover, neutrophils recruit further neutrophils to the tissue by producing LTB4 and chemokines MIP2 and KC. On the other hand, mast cells play an important role in edema formation by releasing factors that increase vascular permeability (e.g., histamine and LTC4) (18-21). Here we describe the molecular mechanisms by which CD300f suppresses LPS-induced skin inflammation.

RESULTS

LPS-induced skin inflammation was profoundly enhanced in CD300f−/− mice as compared with wild-type (WT) mice-LPS was intradermally injected into the ears of WT or CD300f−/− mice. In histological examinations of ear sections, severe skin edema was evident 1.5 h after LPS injection in CD300f−/− mice, but not WT mice (Fig. 1A) (9). Consistently, intravenous injection of Evans blue dye resulted in a massive extravasation of dye in LPS-injected ears of CD300f−/− mice, but not of WT mice 1 h after LPS injection (Fig. 1B). We next tested dorsal air pouch models of LPS-induced inflammation in WT or CD300f−/− mice (22). We found a remarkable increase in the number of neutrophils in skin pouch exudates of CD300f−/− mice but not of WT mice 4 h after LPS injection (Fig. 1C). Thus, CD300f deficiency enhanced edema and neutrophil recruitment in LPS-stimulated skin in mice.

Higher levels of chemical mediators were detected in LPS-stimulated skin pouch exudates of CD300f−/− mice as compared with WT mice-We then measured levels of factors that increase vascular permeability (e.g., histamine and cysteinyl leukotrienes (LTs)) and neutrophil chemoattractants (e.g., MIP2, KC, and LTB4) in LPS-injected skin pouch exudates of WT or CD300f−/− mice (18-21). Levels of histamine, cysteinyl LTs, LTB4, MIP2, or KC were higher in CD300f−/− mice than in WT mice, whereas those of complements C3a and C5a were not different (Fig. 2, A and B). Histological analysis showed that mast cells were frequently degranulated in LPS-stimulated skin sections.
of CD300f<sup>−/−</sup> mice but not of WT mice (Fig. 2C) (18, 20, 21). CD300f<sup>−/−</sup> deficiency enhanced edema and neutrophil accumulation in skin treated with LPS, presumably due to local increases in factors that increase vascular permeability and neutrophil chemoattractants, respectively.

Mast cells and neutrophils contributed to enhanced inflammation in LPS-induced skin of CD300f<sup>−/−</sup> mice-To identify cell populations in CD300f<sup>−/−</sup> mice that mediate the enhanced inflammatory responses in LPS-stimulated skin, we examined the surface expression of CD300f in ear skin myeloid cells. Flow cytometric analysis revealed that mast cells and neutrophils expressed high levels of CD300f, whereas other resident myeloid cells, including macrophages, expressed low or undetectable levels (Fig. 3A). We therefore focused on the role of CD300f in mast cells and neutrophils in LPS-induced skin inflammatory responses. To this end, we used the skin inflammation models in mast cell-deficient Kit<sup>W-sh/W-sh</sup> mice transplanted with WT or CD300f<sup>−/−</sup> BMMC with equivalent expression levels of FcεRI and c-kit on the surface (Fig. 3B) (9, 10). Dye extravasation in LPS-stimulated ear skin of Kit<sup>W-sh/W-sh</sup> mice was enhanced by the adoptive transfer of CD300f-deficient BMMC but not of WT BMMC (Fig. 3C). The following mutations in the cytoplasmic region of CD300f, Y241F, Y289F, and Y325F, abolish two ITIM and a single ITSM. Vascular permeability was enhanced in Kit<sup>W-sh/W-sh</sup> mice transplanted with CD300f<sup>−/−</sup> BMMC, transduced to express CD300f<sup>−/−</sup> BMMC transduced to express CD300f-Y241/289/325/F, at levels comparable to those of Kit<sup>W-sh/W-sh</sup> mice transplanted with CD300f-deficient BMMC (Fig. 3, D and E) (9, 10), indicating the critical importance of the ITIM and ITSM to LPS-induced skin edema. In addition, neutrophil recruitment to LPS-stimulated skin pouches of Kit<sup>W-sh/W-sh</sup> mice was enhanced by the adoptive transfer of CD300f<sup>−/−</sup> BMMC as compared with WT BMMC (Fig. 3F). On the other hand, WT or CD300f<sup>−/−</sup> neutrophils with equivalent expression levels of CD11b and Gr-1 on the surface were injected into skin pouches of WT mice before LPS stimulation (Fig. 3G). Adoptive transfer of CD300f<sup>−/−</sup> neutrophils, but not of WT neutrophils, also enhanced host-derived neutrophils recruited to LPS-stimulated skin pouches of WT mice (Fig. 3H). Taken together, these results indicate that CD300f-deficient mast cells play a major role in edema formation, and CD300f-deficient mast cells together with recruited neutrophils contribute to neutrophil accumulation in LPS-stimulated skin.

CD300f deficiency did not influence the intrinsic migratoty ability of neutrophils-Transwell migration assays demonstrated that more neutrophils were attracted to LPS-stimulated skin pouch exudates of CD300f<sup>−/−</sup> mice, and that equivalent numbers of WT or CD300f<sup>−/−</sup> neutrophils migrated into the same exudate (Fig. 4A) (22). To next examine the in vivo migration of WT vs CD300f<sup>−/−</sup> neutrophils to LPS-stimulated skin pouches, we used dorsal air pouch models in mixed chimera mice that received CD300f<sup>−/−</sup> (Ly5.2<sup>+</sup>) bone marrow (BM) mixed in the ratio of 1:4, 1:1, or 4:1 with WT (Ly5.2<sup>+</sup>) BM. We then measured accumulation of these two types of neutrophils in this model. The proportions of CD300f<sup>−/−</sup> cells among the recruited neutrophils were similar to their proportions among BM neutrophils in all the mice 4 h after LPS injection (Fig. 4B). The total numbers of migrating neutrophils in the chimeric mice (CD300f<sup>−/−</sup>:WT=1:1) were lower or higher than those in the chimeric mice (CD300f<sup>−/−</sup>:WT=4:1) or (CD300f<sup>−/−</sup>:WT=1:4), respectively (Fig. 4C). Thus, we found equivalent chemotactic abilities of WT and CD300f<sup>−/−</sup> neutrophils in LPS-induced skin inflammation model (22, 23). Therefore, the enhancement of neutrophil accumulation in LPS-stimulated skin pouches of CD300f<sup>−/−</sup> mice likely depends on neutrophil chemoattractants released by both CD300f<sup>−/−</sup> mast cells and recruited neutrophils rather than on the intrinsic migratory ability of CD300f<sup>−/−</sup> neutrophils.

Ceramide-CD300f binding inhibited the release of chemical mediators from LPS-stimulated mast cells and neutrophils in vitro-Next, we examined the effect of
ceramide-CD300f binding on the release of chemical mediators from mast cells or neutrophils in response to LPS. In the absence of plate-coated ceramide, CD300f deficiency failed to influence the release of chemical mediators from BMMC or neutrophils in response to LPS. However, the binding of plate-coated ceramide to CD300f inhibited the release of MIP2 and LTC4 from LPS-stimulated BMMC (Fig. 5A) and of MIP2 and LTB4 from LPS-stimulated neutrophils (Fig. 5B) (9). Thus, ceramide-CD300f binding inhibited the release of MIP2 and LTC4 from LPS-stimulated BMMC (Fig. 5A) and of MIP2 and LTB4 from LPS-stimulated neutrophils (Fig. 5B) (9). Thus, ceramide-CD300f binding inhibited the release of chemical mediators from LPS-stimulated mast cells and neutrophils, implying a significant role of ceramide-CD300f interactions in innate immune responses.

Ceramide-CD300f binding inhibited LPS-induced skin inflammation—To next address the role of ceramide-CD300f interactions in LPS-induced skin inflammation, we disrupted ceramide-CD300f binding in vivo with either a fusion protein, CD300f-Fc, in which the extracellular domain of CD300f was fused to the Fc domain of human IgG1, or an antibody against ceramide (9). Conversely, we increased the concentration of CD300f ligands in vivo by administering vesicles containing ceramide (9). Disrupting ceramide-CD300f interactions by pretreating with CD300f-Fc or ceramide antibody increased the vascular permeability of LPS-injected ear skin (Fig. 6, A and B) and the recruitment of neutrophils to LPS-stimulated skin pouches of WT mice at levels comparable to those observed in CD300f−/− mice (Fig. 6C). These effects were not observed when pretreating with control Fc or antibody. In addition, pretreatment of CD300f−/− mice with CD300f-Fc or ceramide antibody did not affect LPS-stimulated vascular permeability or neutrophil recruitment responses in skin (Fig. 6, A-C). Conversely, pretreatment with vesicles containing ceramide, but not with vesicles lacking ceramide, decreased neutrophil recruitment in LPS-stimulated skin of WT mice (Fig. 6D). Taken together, LPS-induced skin inflammation was suppressed by ceramide-CD300f binding in resident mast cells and recruited neutrophils.

DISCUSSION

In this study, we provide several lines of evidence that ceramide-CD300f interactions normally suppress LPS-induced skin inflammation (characterized by edema and neutrophil accumulation) by inhibiting the release of chemical mediators in LPS-stimulated skin; CD300f deficiency elevated levels of factors that increase vascular permeability and of factors that induce neutrophil recruitment in LPS-stimulated skin and remarkably enhanced skin inflammation; administering a ceramide antibody or ceramide-containing vesicles enhanced or inhibited, respectively, LPS-induced skin inflammation of wild-type mice, while the same treatment did not influence that of CD300f−/− mice; CD300f deficiency failed to influence the intrinsic migratory ability of neutrophils in vitro and in vivo.

There are several conflicting reports regarding the association between ceramide and LPS responses in vitro; ceramide acts as a TLR4 agonist in human epithelial cells (24); ceramide negatively regulates TNF-α production in mouse macrophages (25). In most cases, soluble short-chained ceramide was used as an exogenous ceramide. On the other hand, plate-coated long-chained ceramide or vesicles containing long-chained ceramide were used in our experiments. Different ceramide species might exert differing effects on LPS responses in a variety of cells.

In accordance with the finding that CD300f was highly expressed in mast cells and neutrophils, but not in macrophages among skin myeloid cells, ceramide-CD300f binding inhibited the release of chemical mediators from mast cells and from neutrophils in response to LPS in vitro. Given that a CD300f antibody, coated on plates, enhanced LPS-induced cytokine production in BMMC (8), the strength of CD300f aggregation induced by its ligand ceramide or by a specific antibody appears to be associated with the negative or positive
regulation of LPS signaling in BMMC. In any case, adoptive transfer of CD300f-/− mast cells, but not of WT counterparts, enhanced LPS-induced skin edema and neutrophil recruitment in mast cell-deficient mice. Because LPS administration induces mast cell degranulation in vivo although LPS stimulation fails to do so in vitro (18, 20), it is possible that ceramide-CD300f binding suppresses LPS-induced mast cell degranulation in vivo. On the other hand, transfusion of CD300f−/− neutrophils, but not of WT counterparts, enhanced LPS-induced recruitment of recipient neutrophils in WT mice. Collectively, these results indicated that mast cells play an important role in edema formation, while mast cells together with recruited neutrophils contribute to neutrophil accumulation in LPS-stimulated skin of CD300f−/− mice. Moreover, it is possible to speculate that CD300f inhibits mast cell degranulation, leading to the release of histamine and mast cell proteases, as well as its production of cytokines, chemokines, and lipid mediators in LPS-stimulated skin, whereas CD300f inhibits neutrophil release of chemical mediators, including neutrophil chemoattractants. However, it should be noted that a contributory role of other CD300f−/− myeloid cells cannot be ruled out. Thus, on the basis of previous in vitro studies (12-14), we clarified a novel role of ceramide-CD300f binding in LPS signaling in vivo.

Our in vivo results suggest that disrupting ceramide-CD300f interactions could promote the local recruitment of neutrophils to skin infected by Gram-negative bacteria (19). Since human CD300f binds both ceramide and sphingomyelin (26), a novel drug specifically disrupting these interactions might be a promising treatment for bacterial skin infections. Since CD300f deficiency also enhances neutrophil accumulation induced by intraperitoneal injection of LPS (data not shown), treatment with ceramide-containing vesicles might improve TLR4-dependent inflammation not only in skin but also in other tissues. However, further examination will be required to delineate the role of CD300f in human relevant diseases.

In conclusion, ceramide-CD300f interactions inhibit LPS-induced skin edema and neutrophil accumulation, implicating CD300f as a negative regulator of TLR4 signaling in myeloid cells in vivo which is involved in a variety of TLR4-dependent non-infectious inflammatory diseases as well as infectious diseases.

**Experimental Procedures**

**Mice**-All procedures were approved by an institutional review committee of the University of Tokyo (approval no 20-8) and Juntendo University (approval no 270015). C57BL/6 mice (Ly-5.1 and Ly-5.2) (Charles River Laboratories Japan), CD300f−/− mice and KitWsh/Wsh mice were used as described (8, 9, 27).

**Cells**-BMMC and transduced BMMC (more than 90% of living cells expressed both c-Kit and FcεRI) were prepared following methods as previously described (8, 28). Neutrophils (more than 90% of living cells expressed both CD11b and Gr-1) were isolated from BM using a three-layer gradient as previously described (7, 22).

**Antibodies and other reagents**-The following antibodies were used: Rat anti-CD300f monoclonal antibody (3-14-11; rat IgG2a) (ACTGen), fluorescein isothiocyanate (FITC)-conjugated CD11b (M1/70), F4/80 (BM8), FcεRIα (MAR-1), and Ly5.1(A20), phycocerythrin (PE)-conjugated Gr-1 (RB6-8C5), CD11b (M1/70), c-kit (2B8), Ly5.2 (104), and rat IgG2a (eBR2a) (eBioscience), PE-conjugated Ly-6G (1A8) and streptavidin-allophycocyanin (APC) (BioLegend), anti-ceramide (MID 15B4) (Enzo Life Sciences), and anti-mouse IgM (MOPC-104E) (BioLegend). Cytokines were obtained from R&D Systems and C-24 ceramide was obtained from Toronto Research Chemicals, Inc. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (PC) was obtained from Echelon Biosciences Inc.

**Generation of Fc fusion proteins**-cDNA fragments corresponding to the extracellular domain of CD300f were inserted into the cloning sites of the pME18S-hIgG1 Fc vector.
Fc fusion proteins were purified as previously described. Endotoxin levels of Fc fusion proteins, measured using Limulus Amebocyte Lysate (Lonza), were less than 0.01 ng/µg protein (9).

Preparation of vesicles containing lipids—After 1 mg of dry lipid (C-24 ceramide or PC) was hydrated with 1 mL of PBS, vesicles were generated using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc.) according to the manufacturer's instructions, as previously described (9). The Extruder stand and heating block were placed on a hot plate. The plunger of the syringe containing lipid samples was pushed through the membrane having a pore size of 100 nm until the lipid solution was completely transferred to the alternate syringe. At least total 10 passes were performed to obtain homogeneous vesicles containing indicated lipids.

Cell treatments—Lipids (C-24 ceramide or PC) were diluted to a concentration of 20 µg/mL in methanol. MaxiSorp 96-well plates (Nunc, catalog no. 430341) were coated with 50 µL of each solution, air-dried, and washed twice with medium, as previously described (9). BMMC or neutrophils were pre-incubated on lipid-coated plates for 1 h before stimulation with 100 ng/mL LPS for 6 h.

Measurement of chemokines, histamine, complement proteins, or LTs—ELISA kits for KC, MIP-2, C5a, and LTB4 (R&D Systems), histamine (MBL), C3a (BD Biosciences), or cysteinyl LTs (Cayman Chemical Company) were used (9).

Flow cytometry—Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences) equipped with CellQuest software and FlowJo software (Tree Star) (8).

DNA constructs—The construction of pMXs-internal ribosome entry sites (IRES)-puromycin (pMXs-IP) and pMXs-Flag-CD300f or CD300f-Y241/289/325IP was previously described (9, 30).

Transfection and infection—Retroviral transfections were performed as previously described (9, 30). Retroviruses were generated by transient transfection of PLAT-E packaging cells (31).

BM transplantation—BM transplantation in mice was performed as previously described (9). Briefly, one day after lethal γ-irradiation and 8 weeks before experiments, recipient mice (Ly5.1+) were intravenously injected with a total of 3 x 10⁶ cells mixed at indicated ratios of WT versus CD300f⁻⁻ BM (Ly5.2⁻) cells 8 weeks before experiments. In vivo chemotactic ability of WT versus CD300f⁻⁻ neutrophils was assessed as previously described (23).

BMMC reconstitution and neutrophil transfusion—Kitw-sh/w-sh mice were injected into dorsal pouches with either 1 x 10⁶ of WT or CD300f⁻⁻ BMMCs 6 weeks before LPS administration 1 h before LPS administration. WT mice were injected into dorsal pouches with either 1 x 10⁶ of WT or CD300f⁻⁻ neutrophils 1 h before LPS administration (9). Ears of Kitw-sh/w-sh mice were intradermally injected with either 1 x 10⁶ of WT or CD300f⁻⁻ BMMC or with 1 x 10⁶ of transduced BMMC 6 weeks before LPS administration (9).

LPS-induced ear skin inflammation model—Mice were intradermally injected with 10 µg of LPS or PBS to each ear 30 min before intravenous injection with 1% Evans blue dye (Sigma). The amount of extravasated dye 1 h after LPS administration was measured by absorbance at 620 nm (9). In some experiments, 10 µg of anti-ceramide (MID 15B4), isotype control, CD300f-Fc, or Fc was injected intradermally in ears 1 h before LPS administration. The doses of antibodies or Fc proteins were chosen based on previous results in mouse models of passive cutaneous anaphylaxis (9). After ears were fixed with 10% formaldehyde and embedded in paraffin, sections were stained.

Dorsal skin air pouch model—Air pouches were formed on the dorsal skin of mice following methods as previously described (22). Briefly, 5 mL of sterile air was injected subcutaneously into the dorsal skin on days 0 and 3. On day 6, 10 µg of LPS was injected into the air pouches. At a given time after injection, the air pouches were lavaged with 1 mL of PBS. Total cells in PLF were...
counted 4 h after LPS administration, and the percentages of CD11b^Gr-1^{high} (or CD11b^Ly-6G^+) neutrophils were estimated by FACS. The concentrations of chemical mediators in PLF were measured by ELISA 1 h after LPS administration. In some experiments, 10 µg of anti-ceramide (MID 15B4), isotype control, or 100 µg of vesicles containing indicated lipids was injected into skin pouches 1 h before LPS administration. The doses of antibodies or vesicles were chosen based on previous results in mouse models of passive cutaneous anaphylaxis or colitis (9, 10).

**Quantification of mast cells**-Mast cells were stained with toluidine blue and quantified as described (9). Quantification of mast cell degranulation was classified as extensively degranulated (>50%), moderately degranulated (10% to 50%), not degranulated (<10%), as previously described (32).

**Transwell migration assays**-Migration assays were performed using transwell filters with 3 µm pores (BD Falcon), as previously described (22). Briefly, the upper wells were seeded with 1.5 x 10^6 cells in 0.2 mL medium and the lower wells were filled with 0.6 mL of LPS-stimulated dorsal pouch exudates. After a 1 h incubation, the number of neutrophils that had migrated into the lower wells were counted.

**Statistical analyses**-Results are expressed as means ± standard deviation. An unpaired Student’s t test was performed to compare differences between groups.

**Acknowledgments**: We thank Dr. Hisashi Arase for providing plasmids.

**Conflict of interest**: The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions**: E. S. performed all the experiments and participated in writing the manuscript. K. I., A. K., M. I., A. M., K. O., K. M., and N. N. assisted with the experiments. H. O., K. O., and T. S. analyzed the data. T. K. and J. K. conceived the project, analyzed the data, and actively participated in manuscript writing.

**REFERENCES**


FOOTNOTES

This work was supported by JSPS KAKENHI Grant Number 23390257 and 26293231.

The abbreviations used are: LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; LMIR, leukocyte mono-immunoglobulin-like receptor; CLM, CMRF-35-like molecule; MAIR, myeloid-associated Ig-like receptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; MyD88, myeloid differentiation factor 88, TRIF, toll-interleukin 1 receptor-domain-containing adaptor-inducing interferon-β; BMMC, bone marrow-derived mast cell; MIP2, macrophage inflammatory protein 2; KC, keratinocyte-derived chemokine; LT, leukotriene; wild-type, WT; LTs, leukotrienes; BM, bone marrow; PC, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin; APC, allophycocyanin; IRES, internal ribosome entry sites.

FIGURE LEGENDS

FIGURE 1. LPS-induced skin inflammation was profoundly enhanced in *CD300f<sup>c</sup>* mice as compared with WT mice. A, Hematoxylin/eosin staining of ear sections of WT and *CD300f<sup>c</sup>* mice 1.5 h after intradermal injection with LPS (scale bars, 100 µm). B, Dye extravasation in ears of WT and *CD300f<sup>c</sup>* mice before or 1 h after intradermal injection with LPS or PBS as a
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FIGURE 2. Higher levels of chemical mediators were detected in LPS-stimulated skin pouch exudates of CD300f−/− mice as compared with WT mice. A and B, Levels of (A) histamine, LTB4 or cysteinyll LTs or (B) MIP2, KC, C3a, or C4a in skin pouch exudates of WT and CD300f−/− mice 0.5 h (for histamine) or 1 h (for LTB4, cysteinyll LTs, MIP2, KC, C3a, or C4a) after LPS injection. C, Quantification of mast cell degranulation in LPS-stimulated dorsal skin sections of WT and CD300f−/− mice 4 h after LPS injection was determined by classification in 3 categories: not degranulated (none: 1st column), moderately degranulated (mod: 2nd column), and extensively degranulated (ext: 3rd column). Percentages of mast cells belonging to each category are shown. Insets show (not, moderately, extensively) degranulated mast cells in toluidine blue-stained sections (scale bars, 10 µm). Data are representative of two independent experiments. Means ± standard deviations are plotted. *P < 0.01 (Student’s t-test).

FIGURE 3. Mast cells and neutrophils contributed to enhanced inflammation in LPS-induced skin of CD300f−/− mice. A, Surface expression of CD300f in skin myeloid cells. B, Surface expression of FceRI, c-kit, or CD300f in WT or CD300f−/− BMMC. C, Dye extravasation in ears of KitW−sh/W−sh mice or KitW−sh/W−sh mice transplanted with 1 x 10⁶ of either WT or CD300f−/− BMMC 1 h after LPS stimulation. D, Surface expression of FceRI, c-kit, or CD300f in CD300f−/− BMMC transduced with CD300f-WT, CD300f-Y241/289/325F mutant, or mock. E, Dye extravasation in ears of KitW−sh/W−sh mice transplanted with 1 x 10⁶ of CD300f−/− BMMC transduced with CD300f-WT, CD300f-Y241/289/325F mutant, or mock 1 h after LPS stimulation. F, The number of neutrophils recruited to skin pouches of KitW−sh/W−sh mice or KitW−sh/W−sh mice transplanted with 1 x 10⁶ of either WT or CD300f−/− BMMC 4 h after LPS stimulation. G, Surface expression of CD11b, Gr-1, or CD300f in WT or CD300f−/− neutrophils. H, The number of neutrophils (Ly5.1+) recruited to skin pouches of WT mice (Ly5.1+) transplanted with 1 x 10⁶ of either WT or CD300f−/− neutrophils (Ly5.2+) 4 h after LPS stimulation. Data are representative of three (A, B, D, G) or two (C, E, F, H) independent experiments. Means ± standard deviations are plotted. *P < 0.01, **P < 0.05 (Student’s t-test).

FIGURE 4. CD300f deficiency did not influence the intrinsic migratory ability of neutrophils. A, Numbers of WT or CD300f−/− neutrophils migrated into the lower wells containing dorsal pouch exudates derived from either WT or CD300f−/− mice 4 h after an intradermal injection of LPS. Data are representative of two independent experiments. B, The ratio of CD300f−/− neutrophils in total neutrophils included in BM or dorsal pouch exudates from the mixed chimera mice (WT: CD300f−/−=4:1, 1:1, or 1:4) (n = 5) 4 h after an intradermal injection of LPS. Values for the X and Y axes represent the percentage in BM and dorsal pouch exudates, respectively. C, Numbers of total neutrophils in dorsal pouch exudates from WT BM-transplanted mice (n = 5), the mixed chimera mice (n = 5), or LMIR3−/− BM-transplanted mice (n = 5) 4 h after an intradermal injection of LPS. (B and C) Representative of two independent experiments. Means ± standard deviations are plotted.

FIGURE 5. Ceramide-CD300f binding inhibited the release of chemical mediators from LPS-stimulated mast cells and neutrophils in vitro. A and B, The levels of MIP2 or LTC4 released from WT or CD300f−/− BMMC (A) or neutrophils (B) stimulated with 100 ng/mL LPS for 6 h. Cells were pre-incubated on plates coated with ceramide, phosphatidylcholine (PC), or vehicle for 1 h before stimulation. Data are representative of three independent experiments. Means ± standard deviations are plotted. *P < 0.01 (Student’s t-test).
FIGURE 6. Ceramide-CD300f binding inhibits LPS-induced skin inflammation. A and B, Dye extravasation in ears of WT and CD300f−/− mice 1 h after intradermal injection with LPS. C and D, The number of neutrophils recruited to skin pouches of WT and CD300f−/− mice 4 h after intradermal injection with LPS. A–D, Mice intradermally injected with CD300f-Fc or control Fc (A), ceramide antibody or control antibody (B, C), or vesicles containing ceramide, PC, or vehicle (D) 1 h before LPS injection. Data are representative of two independent experiments. Means ± standard deviations are plotted. *P < 0.01 (Student’s t-test).
FIGURE 1

A

WT

CD300f⁻⁻

1.5 h

B

Dye extravasation

OD 620 nm

1 h

* p < 0.01

C

CD11b⁺Gr-1high cells

(X10⁶ cells/pouch)

4 h

* p < 0.01

PBS LPS

WT

CD300f⁻⁻
FIGURE 2

A

**Histamine (nM/pouch)**

*WT vs. CD300f−/−, *p < 0.01*

**LTB4 (pg/ml)**

*WT vs. CD300f−/−, *p < 0.01*

**Cysteiny LT (pg/ml)**

*WT vs. CD300f−/−, *p < 0.01*

B

**MIP-2 (pg/ml)**

*WT vs. CD300f−/−, *p < 0.01*

**KC (pg/ml)**

*WT vs. CD300f−/−, *p < 0.01*

**C3a (pg/ml)**

*WT vs. CD300f−/−, *p < 0.01*

**C5a (pg/ml)**

*WT vs. CD300f−/−, *p < 0.01*

C

**MC degranulation (%)**

*WT vs. CD300f−/−, *p < 0.01*

Legend:

- none
- mod
- ext

Scale bars: 50 μm
FIGURE 3

A

FcεRI+c-kit+ mast cells

CD11b+Gr-1^high neutrophils

CD11b+F4/80+ macrophages

CD300f

B

BMMC

WT

CD300f^/-

c-kit

FcεRI

CD300f

C

Dye extravasation

O.D.620

1 h

Kit^W-sh/W-sh

Kit^W-sh/W-sh + WT BMMC

Kit^W-sh/W-sh + CD300f^/- BMMC

(n = 5)

E

Dye extravasation

O.D.620

1 h

mock

CD300f-WT

CD300f-YF

Kit^W-sh/W-sh

(n = 5)

F

CD11b+Gr-1^high cells

(×10^6 cells/pouch)

Kit^W-sh/W-sh

Kit^W-sh/W-sh + WT BMMC

Kit^W-sh/W-sh + CD300f^/- BMMC

CD300f

G

neutrophil

WT

CD300f^/-

Gr-1

97.7%

97.6%

CD11b

H

CD11b+Gr-1^high cells

(×10^6 cells/pouch)

PBS

WT neutrophil

CD300f^/- neutrophil

WT (n = 5)
**Figure 4**

**A**

Bar graph showing the migrated neutrophils (x10⁴) from pouch exudates. The x-axis represents WT neutrophils and CD300f⁻/⁻ neutrophils. The y-axis shows the migrated neutrophils. The graph indicates that CD300f⁻/⁻ neutrophils migrate more than WT neutrophils, as indicated by the asterisks.

**B**

Graph showing the linear relationship between the migrated neutrophils (WT/CD300f⁻) and the BM (WT/CD300f⁻). The y-axis represents migrated neutrophils, and the x-axis represents BM. The data points are connected by a line, showing a positive correlation.

**C**

Graph showing the number of CD11b⁺Gr-1⁺h cells (X10⁶ cells/pouch) in WT and CD300f⁻/⁻ mice after BM transplantation. The x-axis represents different BM ratios (WT/CD300f⁻) and WT/CD300f⁻/⁻. The y-axis shows the number of cells. The data points are represented by different symbols, indicating variations in cell counts across different BM ratios.
FIGURE 5

A

WT BMMC

CD300f−/− BMMC

B

WT neutrophil

CD300f−/− neutrophil
FIGURE 6

A

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<th></th>
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1 h

Dye extravasation (O.D.620)

```
* p < 0.01
```

B

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<tr>
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1 h

Dye extravasation (O.D.620)

```
* p < 0.01
```

C

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<tr>
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4 h

CD11b+Ly6G+ cells (X10⁶ cells/pouch)

```
* p < 0.01
```

D

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</table>
```

4 h

CD11b+Ly6G+ cells (X10⁶ cells/pouch)

```
* p < 0.01
```