



Involvement of CD300a Phosphatidylserine Immunoreceptor in Aluminum Salt Adjuvant –Induced Th2 Responses

This information is current as
of May 3, 2015.

Haruka Miki, Chigusa Nakahashi-Oda, Takayuki Sumida
and Akira Shibuya

J Immunol published online 24 April 2015

<http://www.jimmunol.org/content/early/2015/04/24/jimmunol.1402915>

Supplementary Material	http://www.jimmunol.org/content/suppl/2015/04/24/jimmunol.1402915.DCSupplemental.html
Subscriptions	Information about subscribing to <i>The Journal of Immunology</i> is online at: http://jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at: http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/cgi/alerts/etoc

Involvement of CD300a Phosphatidylserine Immunoreceptor in Aluminum Salt Adjuvant–Induced Th2 Responses

Haruka Miki,^{*,†} Chigusa Nakahashi-Oda,^{*} Takayuki Sumida,[†] and Akira Shibuya^{*,†,§}

Aluminum salt (alum) has been widely used for vaccinations as an adjuvant. Alum not only enhances immunogenicity but also induces Th2 cell immune responses. However, the mechanisms of how alum enhances Th2 cell immune responses have been controversial. In an experimental allergic airway inflammation model, in which alum in conjunction with OVA Ag was i.p. injected for immunization, we found that apoptotic cells and inflammatory dendritic cells (iDC) expressing CD300a, an inhibitory immunoreceptor for phosphatidylserine (PS), significantly increased in number in the peritoneal cavity after the immunization. In contrast, apoptotic cells and iDCs were scarcely observed in the peritoneal cavity after injection of OVA alone. In CD300a-deficient mice, eosinophil infiltration in bronchoalveolar lavage fluid, serum IgE levels, and airway hyperreactivity were significantly decreased after immunization with alum plus OVA compared with wild-type mice. In vitro, iDCs purified from CD300a-deficient mice after the immunization induced significantly less IL-4 production from OT-II naive CD4⁺ T cells after coculture with OVA Ag. CD300a expressed on iDCs bound PS on apoptotic cells in the peritoneal cavity after injection of OVA plus alum. Blocking CD300a interaction with PS by injection of a neutralizing anti-CD300a Ab resulted in inhibition of the development of allergic airway inflammation. These results suggest that CD300a is involved in alum-induced Th2 skewing. *The Journal of Immunology*, 2015, 194: 000–000.

Vaccines have made a stronger impact on public health than any other advance in medicine. Immunogenicity of vaccines is strongly augmented by treatment with adjuvants in conjunction with Ags such as live or killed organisms or their purified subunits. Aluminum salt (alum) has been most widely used for vaccinations as an adjuvant (1). However, alum adjuvant not only enhances immunogenicity but also induces Th2 immune responses (1). Although the mechanisms of how alum enhances Th2 immune responses have been controversial, it is acknowledged that innate immune responses play an important role in the skewing to Th2 responses. It was reported that alum adjuvant directly or indirectly stimulates APCs, in which NLRP3 inflammasome-dependent (2, 3) or Syk- and p38 MAPK-dependent pathways are involved (4). Recent evidences also

suggested that alum causes cell death; the subsequent release of danger signals such as DNA or uric acid acts as a potent endogenous immunostimulatory signal, which induces Th2 responses through Irf3-dependent and Irf3-independent pathways in monocyte-derived inflammatory dendritic cells (iDCs) (3, 5). However, the precise mechanisms of Th2 skewing by alum adjuvant that lead to Th2 responses have been incompletely understood.

CD300a, also named MAIR-I (6, 7), LMIR1 (8), and CLM-8 (9) in mice and IRp60 (10) and CMRF-35H (11) in humans, is an Ig-like receptor preferentially expressed on myeloid cells (including macrophages, neutrophils, and dendritic cells [DCs]) and mast cells (12, 13). CD300a contains cytoplasmic ITIMs; consequently, it mediates inhibitory signals. Specifically, human and murine CD300a inhibits FcεRI-mediated signals in mast cells and basophils, resulting in the suppression of their degranulation (6, 7, 14–16). Recently, phosphatidylserine (PS), which is exposed on the outer leaflet of the plasma membrane of apoptotic cells, was identified as a functional ligand for CD300a (17, 18). When CD300a on mast cells binds PS on apoptotic cells, it inhibits the mast cell cytokine and chemokine production that resulted from LPS stimulation (16, 17).

Allergy is a representative phenotype of Th2 responses. In allergic airway responses, mast cells are central in the effector phase, including the development of airway hyperreactivity (AHR) and acute airway constriction (19). In contrast, the initiation of a Th2 response requires Ag presentation by DCs in the presence of IL-4 to activate GATA3, a master regulator of Th2 cells (20). Because many apoptotic cells are generated at the site of allergic inflammation, CD300a expressed on myeloid cells (including both mast cells and DCs) may modulate allergic inflammation. However, it is unclear what role CD300a plays with apoptotic cells in Th2 induction and the development of allergic airway inflammation.

In this study, we investigate the role of CD300a in the Th2 responses and pathogenesis of allergic airway inflammation induced by OVA Ag emulsified with alum adjuvant. We show that iDCs express CD300a, which interacts with PS on alum-induced apoptotic cells. These DCs play an important role in Th2 induction and the development of allergic airway inflammation.

^{*}Department of Immunology, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; [†]Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; [‡]Life Science Center of Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; and [§]Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

Received for publication November 20, 2014. Accepted for publication March 26, 2015.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

H.M. performed the experiments and wrote the manuscript; C.N.-O. contributed to the study design and performed the experiments; T.S. contributed to the study design; and A.S. supervised the overall project and wrote the manuscript.

Address correspondence and reprint requests to Prof. Akira Shibuya, Institute of Basic Medical Sciences, University of Tsukuba, T1-1-1, Ten-nohdai, Sukuba, Ibaraki, 305-8575 Japan. E-mail address: ashibuya@md.tsukuba.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperreactivity; Alum, aluminum salt; BAL, bronchoalveolar lavage; DC, dendritic cell; D89E MFG-E8, MFG-E8 mutated at residue 89; iDC, inflammatory dendritic cell; LN, lymph node; Med LN, mediastinal LN; MFG-E8, milk fat globule EGF factor 8; MHC-II, MHC class II; PAS, periodic acid–Schiff; PEC, peritoneal cavity cell; PS, phosphatidylserine; WT, wild type.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/\$25.00

Materials and Methods

Mice

CD300a-deficient (*Cd300a*^{-/-}) mice with a BALB/c background were generated in our laboratory as previously described (21) and backcrossed onto the C57BL/6J genetic background for 12 generations. Wild-type (WT) C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). CD11c-EGFP mice on the C57BL/6J genetic background were purchased by Jackson Laboratories (Bar Harbor, ME). OT-II mice were originally constructed as described by Barnden et al. (22). All experiments were performed by using 6- to 8-wk-old male mice. All experiments were performed in accordance with the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center.

Allergic airway inflammation

Mice were sensitized on days 0, 7, and 14 by i.p. injection of 100 µg OVA (chicken egg protein grade V; Sigma-Aldrich, St. Louis, MO) or OVA free from endotoxin (EndoGrade, Hyglos GmbH, Germany) emulsified with 100 µl Alhydrogel 2% (VacciGrade; InvivoGen, San Diego, CA) and challenged with an aerosol of 10% OVA on 3 consecutive days (days 21–23) for 30 min by ultrasonic nebulizer (NE-U17; Omron, Kyoto, Japan), and analyzed on day 25. For induction of papain-induced airway inflammation, mice were intranasally administrated with papain (150 µg in PBS per mouse; Wako Pure Chemical) three times on days 0–2 and analyzed for bronchoalveolar lavage (BAL) fluid on day 3.

BAL fluid was collected with 1 ml PBS containing 2% FBS three times and analyzed for differential cell count by flow cytometry. For histologic analysis, whole lung lobes were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E and periodic acid–Schiff (PAS) and analyzed by light microscopy (×40). We randomly selected five sections stained with H&E per lung and scored peribronchial inflammatory cell infiltration, as described previously (23). The population of PAS-stained goblet cells per total epithelial cells was calculated by using hybrid cell count BZ-H2C software (KEYENCE, Osaka, Japan).

Measurement of AHR

Airway reactivity to different doses of acetylcholine aerosol was measured by the computer-controlled piston ventilator system (FlexiVent, Montreal, QC) in accordance with the manufacturer's instructions. In brief, mice were intubated intratracheally and exposed to an aerosol of PBS (basal readings) and then to methacholine in PBS at 0.75, 1.5, 3, 6, and 12 mg/ml. Respiratory resistances were determined by percentage change from the baseline index.

i.m. immunization

Mice were sensitized on days 0 and 7 by i.m. injection of 10 µg OVA emulsified with 10 µl Alhydrogel 2% into the right quadriceps, and analyzed on day 14. Inguinal lymph nodes (LNs) were collected and prepared for single-cell suspensions.

ELISA

For quantitation of the levels of IgE in plasma, 96-well ELISA plates (NUNC Thermo Scientific, Yokohama, Japan) were coated with rat anti-mouse IgE (R35-72; BD Biosciences) diluted in 50 mM carbonate B buffer (pH 9.6) as a capture Ab. Total IgE was detected with biotinylated anti-mouse IgE (R35-118; BD Biosciences) and streptavidin conjugated with HRP (GE Healthcare Biosciences, Little Chalfont, U.K.). For the IgE standard, we used purified mouse IgE (C38-2; BD Biosciences). Data are expressed as relative IgE OD at 405 nm. OVA-specific IgE was detected with an enzyme immunoassay kit (Shibayagi, Gunma, Japan). IgG1 was detected with an enzyme immunoassay kit (Cayman, Ann Arbor, MI), and IgG2b, IgG2c, and IgG3 were detected by mouse ELISA Quantitation Set (Bethyl, Montgomery, TX). IL-4 and IFN-γ were analyzed with Optia EIA kit (BD Biosciences). All these assays were performed in accordance with the manufacturers' instructions.

Cytospin

Cells in the peritoneal cavity lavage fluid were centrifuged (100 × g, 5 min) onto slides and stained with Wright–Giemsa for 8 min or with toluidine blue for 3 min.

Quantitative RT-PCR analysis

Total RNA was extracted from whole mediastinal LN (Med LN) cells or sorted DCs. RT was performed with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) by

using an ABI 7500 sequence detector (Applied Biosystems). The PCR primers are as follows: *Il4*-F, 5'-ATC ATC GGC ATT TTG AAC GAG G-3'; *Il4*-R, 5'-TGC AGC TCC ATG AGA ACA CTA-3'; *Ifng*-F, 5'-ACA GCA AGG CGA AAA AGG ATG-3'; *Ifng*-R, 5'-TGG TGG ACC ACT CGG ATG A-3'; *Il17*-F, 5'-TTT AAC TCC CTT GGC GCA AAA-3'; *Il17*-R, 5'-CTT TCC CTC CGC ATT GAC AC-3'; *Il12p35*-F, 5'-TGC CTT GGT AGC ATC TAT GAG G-3'; *Il12p35*-R, 5'-CGC AGA GTC TCG CCA TTA TGA T-3'; *Il12/23p40*-F, 5'-GGAGACCCTGCCCATTAAGACT-3'; *Il12/23p40*-R, 5'-CAACGTTGCATCCTAGGATCG-3'. Values were normalized according to the expression of the housekeeping gene β-actin.

Cytokine production from CD4⁺ T cells in the Med LNs

Mice were immunized with i.p. injection of 100 µg OVA emulsified with 100 µl alum in total volume of 200 µl. Five days later, CD4⁺ T cells were sorted from Med LNs by flow cytometry and cultured at 1 × 10⁵ cells per 200 µl complete RPMI 1640 medium (Sigma-Aldrich) containing 10% FBS in a 96-well, round-bottom plate with PMA (50 ng/ml; Sigma-Aldrich) and Ionomycin (500 ng/ml; Sigma-Aldrich). Supernatants were collected 30 h later and analyzed for cytokines by ELISA.

Coculture of OT-II naive CD4⁺ T cells with iDCs

CD4⁺ T cells were prepared from spleens of OT-II mice with a magnetic bead-based cell sorting system (Miltenyi, Bergisch Gladbach, Germany) by using anti-mouse CD4 Ab. Naive CD4⁺ T (CD4⁺CD44^{lo}CD62^{high}) cells were sorted by using a FACSAria II cell sorter (BD Biosciences). The purity of naive CD4⁺ T cells was >98%. Naive CD4⁺ T cells (2 × 10⁴ or 5 × 10⁴/well) were cultured with Ly6C^{high}CCR2⁺CD11b^{high}CD11c⁺iDCs (2 × 10⁴/well), which were purified from the peritoneal cavity, in 96-well round-bottom plates in the presence of OVA_{323–339} peptide (Operon Biotechnologies, Tokyo, Japan) for 5 d.

Analysis of intracellular cytokines

Cells were stimulated with PMA at 50 ng/ml plus ionomycin at 500 ng/ml for 3 h. Brefeldin A (Sigma) was added at 10 µg/ml 2 h before cell harvest. Intracellular cytokines were stained by using Fix and Perm kit (Invitrogen) and analyzed by flow cytometry.

Confocal scanning laser microscopy analysis

Peritoneal cavity cells (PECs; 5.0 × 10⁵) from CD11c-GFP mice were stained with PSVue (Molecular Targeting Technology, West Chester, PA). The imaging was performed by using FV10i FLUOVIEW (Olympus, Tokyo, Japan). Milk fat globule EGF factor 8 (MFG-E8) mutated at residue 89 (D89E MFG-E8) was generated in our laboratory, as previously described (24). D89E MFG-E8 (100 µg in 200 µl PBS) or anti-CD300a (TX41; 300 µg in 200 µl PBS) was administrated i.p. to the mice 24 h after i.p. injection of OVA plus alum.

Abs and flow cytometry

Anti-mouse CD3 (145-2C11), CD4 (RM4-5), CD62L (MEL-14), CD44 (IM7), Siglec-F (E50-2440), CD11b (M1/70), CD11c (HL3), Gr-1 (RB6), Ly6C (AL-21), MHC class II (MHC-II; AF6-120.1), CD80 (16-10A1), CD86 (GL1), CD40 (HM40-3), IL-4 (11B11), and IFN-γ (XMGI.2) were purchased from BD Biosciences. Anti-mouse F4/80 (MCA497A647) was purchased from BioRad (Hercules, CA). Anti-mouse CCR2 (475301) was purchased from R&D (Minneapolis, MN). Anti-PS (1H6) was purchased from Merck Millipore (Darmstadt, Germany). Anti-CD300a mAb (TX41) was generated in our laboratory, as described previously (7). Data from all samples were acquired with a Fortessa flow cytometer (BD) and analyzed by using FlowJo software (Tree Star, Ashland, OR).

Statistical analyses

Statistical analyses were performed by using the unpaired Student *t* test or one-way ANOVA followed by Bonferroni's multiple comparison test as for posttest. A *p* value <0.05 was considered statistically significant.

Results

Allergic airway inflammation is attenuated in CD300a-deficient mice

To explore the physiological role of CD300a in allergic responses, we injected WT and CD300a-deficient C57BL/6J mice i.p. with OVA plus alum each week for 3 consecutive weeks and then challenged with 10% OVA on days 21, 22, and 23 (Fig. 1A). Because CD300a contains cytoplasmic ITIMs and inhibits FcεRI-

mediated signals in mast cells in vitro (6, 7, 14), we hypothesized that allergic responses would be exacerbated in CD300a-deficient mice compared with WT mice. Unexpectedly, allergic airway inflammation was attenuated in CD300a-deficient mice. The numbers of total cells and eosinophils in the BAL fluid were significantly decreased in CD300a-deficient mice compared with WT mice (Fig. 1B). By histology, CD300a-deficient mice had decreased cell infiltration (stained by H&E) and mucous production (by PAS) after Ag challenge (Fig. 1C). In addition, AHR was attenuated in CD300a-deficient mice compared with WT mice (Fig. 1D). Serum total IgE and OVA-specific IgE levels were significantly lower in CD300a-deficient mice than WT mice on days 14 and 25 after immunizations with OVA (Fig. 1E). We also observed significantly increased IgG2c, but not IgG1, IgG2b, and

IgG3, in the sera of CD300a-deficient mice compared with WT mice (Fig. 1F). The decreased Th2 responses in CD300a-deficient mice were similarly observed when we used OVA free from endotoxin (Supplemental Fig. 1A, 1B).

To investigate whether CD300a participates in shifting CD4⁺ T cells toward a Th2 phenotype, we sorted those cells from Med LNs of WT and CD300a-deficient mice 5 d after immunization with OVA plus Alum by flow cytometry. IL-4 concentration in the culture supernatants of CD4⁺ T cells sorted from CD300a-deficient mice in the presence of PMA and ionomycin was significantly lower than that from WT mice (Fig. 1G). In contrast, IFN- γ production was comparable from CD4⁺ T cells between two genotypes of mice (Fig. 1G). When we immunized WT and CD300a-deficient mice by i.m. injection with OVA and alum at a dose corresponding to that used for human vac-

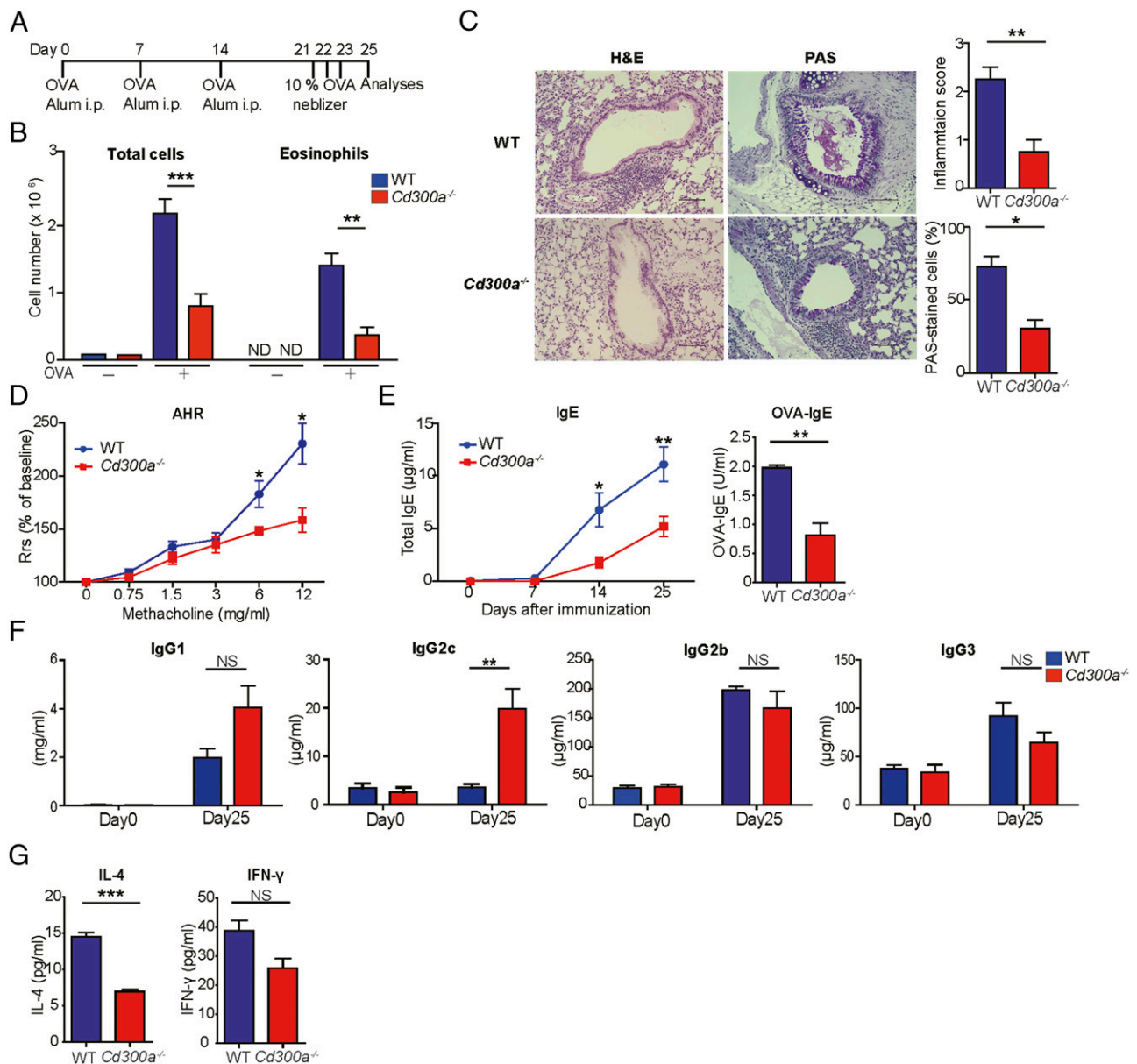


FIGURE 1. CD300-deficient mice showed attenuated allergic airway inflammation. (A) WT ($n = 6$) and CD300a-deficient ($Cd300a^{-/-}$; $n = 6$) mice were immunized and challenged with OVA with or without alum, as shown in the protocol. (B–E) Numbers of total cells and eosinophils in the BAL fluid of mice treated with PBS (–) ($n = 2$) or OVA (+) ($n = 4$ –5) (B). Lung histology stained with H&E or PAS, the scores of inflammatory cell infiltration, and the percentages of PAS⁺ cells per total epithelial cells. Scale bars, 100 μ m (C). AHR index in response to methacholine (D), and serum total IgE or IgE specific to OVA on day 14 after first sensitization (E). (F) Serum levels of Ig subclasses on days 0 and 25 after the first immunization ($n = 4$). (G) Cytokine production from CD4⁺ T cells sorted from Med LNs 5 d after immunization ($n = 4$). Data are representative of more than three independent experiments. Error bars indicate SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ND, not detectable.

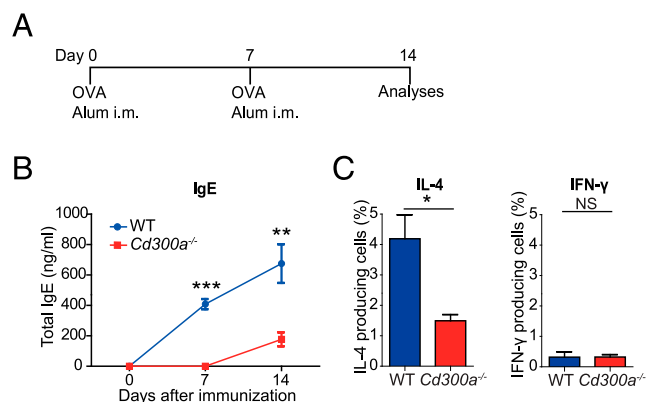


FIGURE 2. Th2 responses were decreased in *Cd300a*^{-/-} mice after i.m. immunization with OVA and Alum. WT ($n = 5$) or *Cd300a*^{-/-} ($n = 6$) mice were sensitized on days 0 and 7 by i.m. injection of 10 μ g OVA emulsified with 10 μ l Alhydrogel 2% into the right quadriceps as shown in (A). On day 14, total IgE was measured by ELISA (B) and intracellular cytokines were analyzed in CD4⁺ T cells of inguinal LNs (C). Data are representative of three experiments. Error bars indicate SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

cination (Fig. 2A), Th2 responses, such as eosinophil infiltration and IgE concentration in the BAL fluid, were again lower in CD300a-

deficient mice compared with WT mice (Fig. 2B, 2C). These results suggest that CD300a is involved in alum-induced Th2 polarization.

We also examined the role of CD300a in the development of allergic airway inflammation induced by intranasal treatment with papain (Supplemental Fig. 1C). In contrast with the model induced by i.p. injection with OVA plus alum, there was no difference in eosinophil infiltration and IL-4 concentration in BAL fluid between WT and CD300a-deficient mice (Supplemental Fig. 1D, 1E). Taken together, these results suggest that CD300a is specifically involved in alum-induced Th2 responses.

CD300a is expressed on iDCs

Next, we analyzed the expression of CD300a on immune cells in the peritoneal cavity. Flow-cytometry analyses demonstrated that CD300a was expressed on mast cells from the peritoneal cavity in naive mice and mice 24 h after administration of OVA alone (Fig. 3A, Supplemental Fig. 2A). However, mast cells and macrophages were not detected in the peritoneal cavity after i.p. injection of OVA emulsified with alum (Fig. 3B, Supplemental Fig. 2B). Instead, CD300a expression was upregulated on Ly6C^{high} CD11b^{high} DCs and neutrophils after immunization with OVA plus alum (Fig. 2A). In agreement with a previous report (3), these cells increased in number 24 h after peritoneal immunization with OVA plus alum (Fig. 3B, Supplemental Fig. 2A). Further-

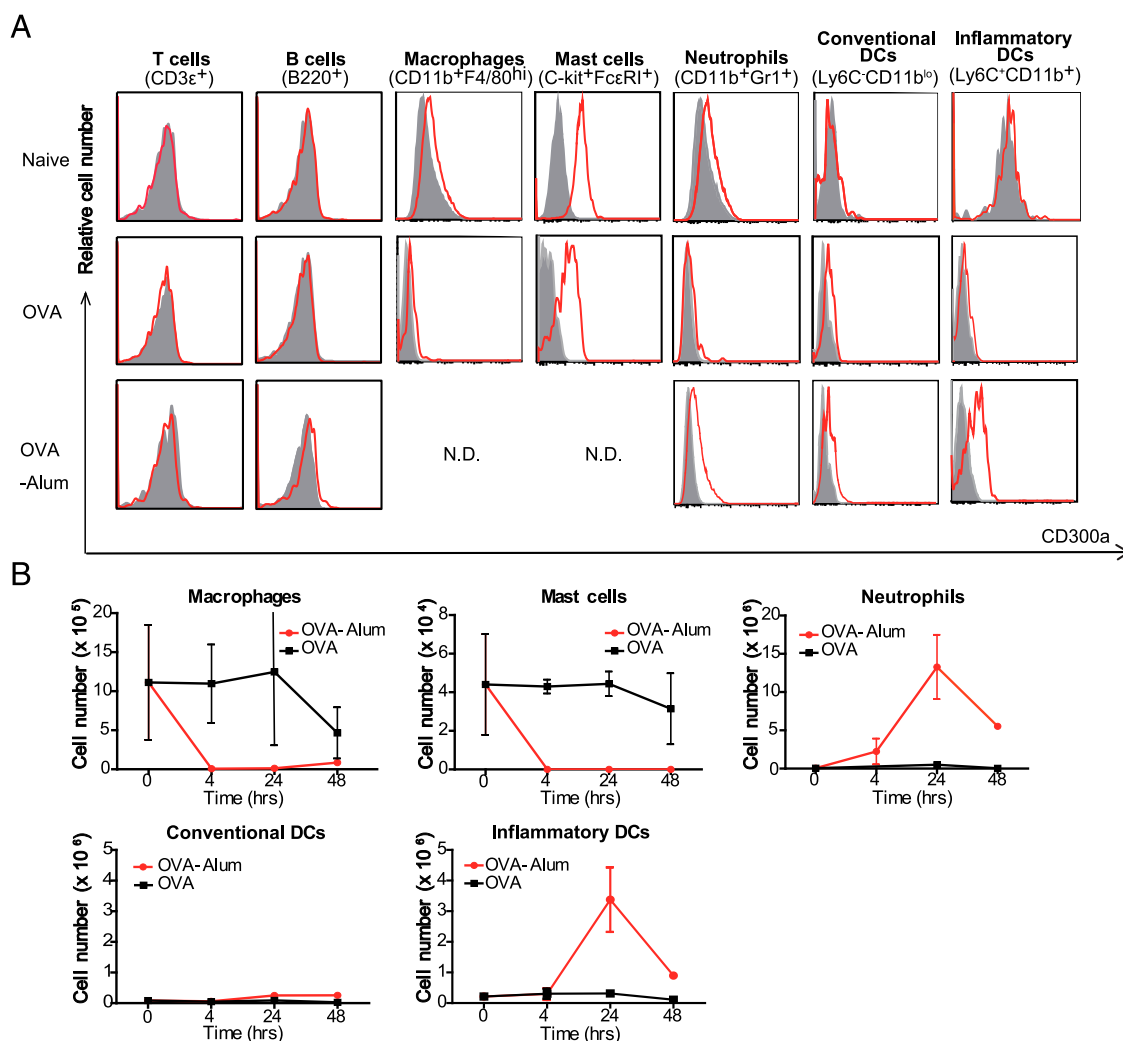


FIGURE 3. CD300a-expressing iDCs are increased in the peritoneal cavity after alum administration. (A) CD300a expression on cells in the peritoneal cavity before or 24 h after OVA alone or OVA plus alum i.p. administration. (B) Number of PECs after i.p. administration of OVA alone or OVA plus alum. Data are representative of three independent experiments (A and B). Error bars indicate SD. N.D., not detected.

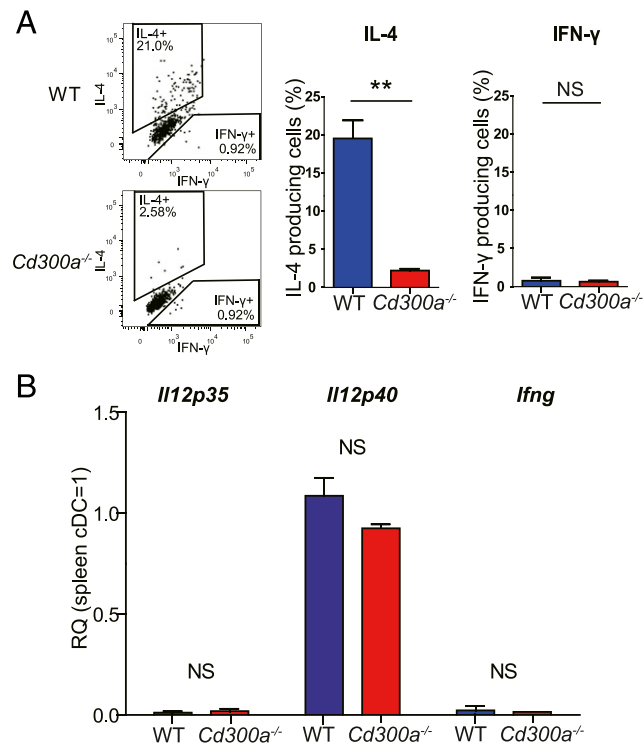


FIGURE 4. CD300a on iDCs promotes Th2 responses in vitro. **(A)** Naive CD4⁺ T cells sorted from the spleen of OT-II mice were cocultured with iDCs purified from the peritoneal cavity of WT or *Cd300a*^{-/-} mice 48 h in the presence of OVA peptide. Intracellular cytokines of CD4⁺ T cells were analyzed 5 d after the start of culture. **(B)** iDCs sorted from the PECs of WT or *Cd300a*^{-/-} mice 48 h after i.p. administration of OVA plus alum. Relative mRNA expression of each cytokine of iDCs was determined compared with splenic conventional DCs sorted from naive WT mice as a control (respiratory quotient = 1). Data are representative of three experiments. Error bars indicate SD. ***p* < 0.01.

more, Ly6C^{high} CD11b^{high} CD11c⁺ DCs expressed CCR2, but not CX3CR1 (Supplemental Fig. 2C), consistent with the iDC phenotype (25). iDCs are activated by alum (3, 5) and migrate to the draining LNs in a CCR2-dependent manner after immunization (26, 27). Indeed, CD11b^{high} CD11c⁺ DCs were increased in Med LNs after immunization with OVA plus alum, and a subpopulation of these DCs expressed CCR2, as well as Ly6C^{high}, MHC-II^{high} and CD300a (Supplemental Fig. 3A, 3B). The Ly6C^{high} CD11b^{high} CD11c⁺ iDC populations in the peritoneal cavity were comparable between WT and CD300a-deficient mice 24 h after administration of OVA plus alum (Supplemental Fig. 4A). In addition, the expression of MHC-II and costimulatory molecules (CD80, CD86, and CD40) by Ly6C^{high} CD11b^{high} CD11c⁺ iDCs was comparable between WT and CD300a-deficient mice (Supplemental Fig. 4B). Nonetheless, these results suggest that CD300a on iDCs participate in T cell polarization toward a Th2 phenotype.

CD300a on iDCs promotes Th2 polarization in vitro

To further investigate the involvement of CD300a on iDCs in Th2 polarization, we sorted peritoneal iDCs (Ly6C^{high} CCR2⁺ CD11b^{high} CD11c⁺) from WT or CD300a-deficient mice 48 h after OVA plus alum injection. We then cocultured them with naive CD4⁺ T cells (CD4⁺CD44L^{lo}CD62^{high}) from OT-II mice in the presence of OVA peptide for 5 d. We did not add artificially induced apoptotic cells for the ligand of CD300a because many T cells undergo physiological apoptosis under the process of proliferation in coculture system. The population of IL-4-producing cells generated from naive CD4⁺ T cells cocultured with CD300a-deficient iDCs was significantly lower than that cocul-

tured with WT iDCs (Fig. 4A). However, there were few IFN-γ-producing cells in both of the cultures and no difference in the population between two genotypes of mice (Fig. 4A). Moreover, the mRNA levels of *Il12p35*, *Il12p40*, and *Ifng* in iDCs sorted from the PECs 48 h after i.p. administration of OVA plus alum were comparable between WT and CD300a-deficient mice (Fig. 4B). These results suggest that CD300a on iDCs is not involved in Th1 differentiation, but rather promotes Th2 responses after immunization with OVA and alum.

Blocking CD300a interaction with PS attenuates allergic airway inflammation

Because CD300a binds PS on apoptotic cells, we examined the interaction of CD300a on iDCs with PS on apoptotic cells in the peritoneal cavity. By flow cytometry, we gated out PI⁺ cells to exclude late apoptotic cells and necrotic cells, and detected early apoptotic cells by anti-PS Ab. Peritoneal PI⁺PS⁺ cells were scarcely detected before and 4 h after OVA plus alum injection, but they increased in number 24 h after the injection (Fig. 5A). Because OVA alone did not generate PI⁺PS⁺ cells by 48 h after

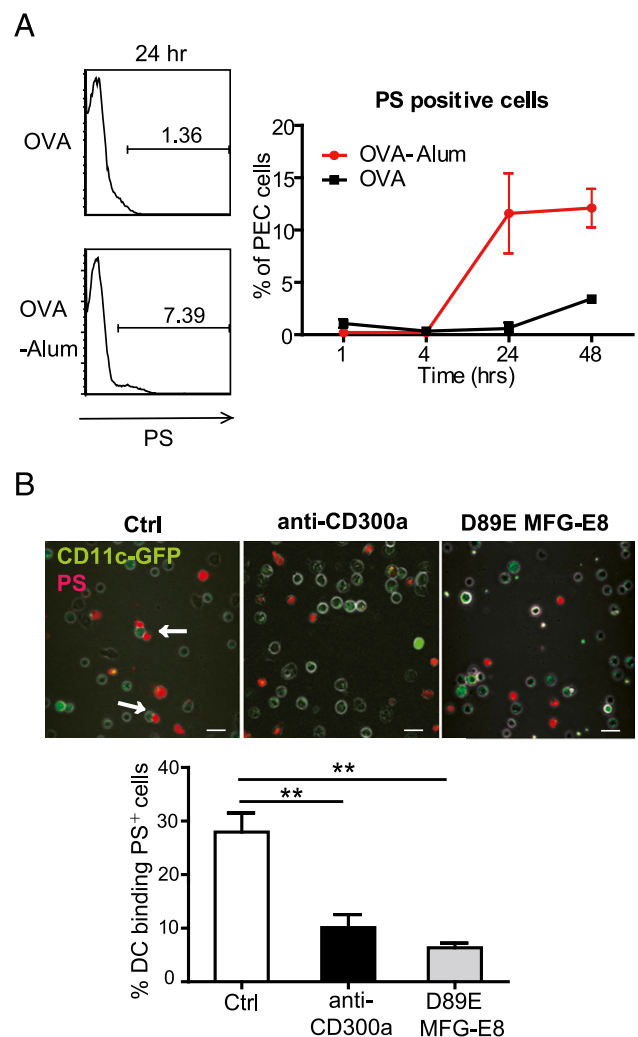


FIGURE 5. Apoptotic cells interact with iDCs via CD300a in peritoneal cavity. **(A)** Flow-cytometry analyses of peritoneal cells stained with anti-PS Ab and the population of PS⁺ apoptotic cells after immunization with OVA alone or OVA plus alum. **(B)** Peritoneal cells derived from CD11c-GFP mice stained with PSVue and analyzed by confocal microscopy. Anti-CD300a neutralizing Ab or D89E MFG-E8 was i.p. injected 24 h after immunization with OVA plus alum. Scale bars, 20 μm. Data are representative of three experiments. ***p* < 0.01. Error bars indicate SD.

the injection, alum is likely involved in the induction of apoptotic cell death as previously revealed (3, 5, 28).

To examine the direct interaction between iDCs and alum-induced apoptotic cells in the peritoneal cavity, we collected cells from the peritoneal cavity of CD11c-EGFP mice 48 h after administration of OVA plus alum, stained them with PSVue, and analyzed them by laser scanning confocal microscopy. A significant number of DCs, most of which were iDCs (Fig. 2B), directly bound to one or two PS⁺ cells (Fig. 5B). However, i.p. injection of a neutralizing anti-CD300a Ab significantly decreased the number of DCs binding to PS⁺ cells (Fig. 5B).

MFG-E8 is a PS binding protein that bridges $\alpha_v\beta_3$ integrin on macrophages and mediates phagocytosis of apoptotic cells (24). In contrast, MFG-E8 containing a point mutation (D89E) in the RGD motif (D89E MFG-E8) is able to bind PS but is not able to bind $\alpha_v\beta_3$ integrin (24). Therefore, D89E MFG-E8 is able to block only the binding of CD300a to PS without enhancement of phagocytosis of apoptotic cells. Injection of D89E MFG-E8 also significantly decreased the number of DCs binding to PS⁺ cells (Fig. 5B). Together, these results indicated that the binding of iDCs to PS⁺ cells in the peritoneal cavity after immunization with OVA plus alum was mediated by the interaction between CD300a and PS.

Next, we examined whether the interaction between CD300a and PS is involved in the pathogenesis of allergic airway inflammation induced by OVA and alum. Mice were injected i.p. with a neutralizing anti-CD300a Ab on the days of injection of OVA and alum and 3 d after each injection (Fig. 6A). We then analyzed their BAL fluid and serum IgE levels. Mice treated with anti-CD300a Ab had

significantly lower numbers of total cells and eosinophils in the BAL fluid and serum total and OVA-specific IgE levels than did mice treated with a control Ab (Fig. 6B, 6C). IL-4 production from CD4⁺ T cells sorted from Med LNs of mice treated with anti-CD300a Ab was significantly lower than that of mice treated with control Ab (Fig. 6D). However, IFN- γ was comparable between two genotypes of mice.

These results suggest that a direct interaction of CD300a on iDCs with dead cells exacerbates allergic airway inflammation.

Discussion

In this study, we investigated the role of CD300a in Th2 responses and allergic airway inflammation induced by alum in conjunction with OVA. We demonstrated that injecting mice with alum was involved in the generation of dead cells in the peritoneal cavity and upregulated CD300a expression on iDCs. These iDCs bound dead cells via the interaction between CD300a and PS, resulting in augmented Th2 responses, such as IL-4 production from CD4⁺ T cells and IgE production, and enhanced allergic airway inflammation. Blocking the interaction of CD300a and PS with an anti-CD300a neutralizing Ab suppressed Th2 responses; therefore, CD300a-mediated signaling in iDCs was involved in Th2 responses induced by dead cells. Our results demonstrate an as yet undetermined mechanism, other than danger signals by DNA or uric acid, by which alum-induced dead cells enhanced Th2 responses via CD300a, PS immunoreceptor on iDCs. However, it is also possible that recognition of PS on dead cells by CD300a on iDCs may be an early step, which is followed by recognition of danger signals from the dead cells.

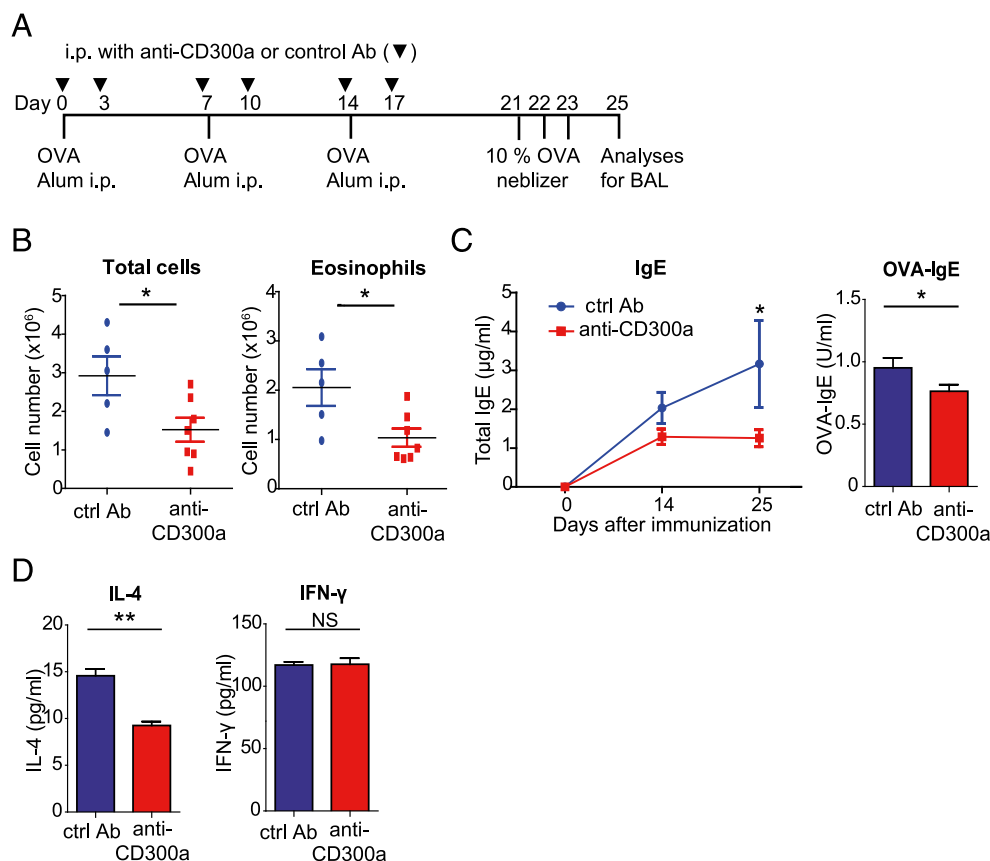


FIGURE 6. Blocking the interaction between CD300a and PS attenuates allergic airway inflammation. **(A)** Protocol for anti-CD300a Ab treatment during the induction of airway inflammation. **(B and C)** Number of total cells and eosinophils in the BAL fluid of mice treated with anti-CD300a ($n = 7$) or control Ab ($n = 5$) (B). Serum total IgE or IgE specific to OVA on day 7 after first sensitization in mice treated with anti-CD300a Ab ($n = 4$) or control Ab ($n = 4$) (C). Data are representative of three experiments (B and C). **(D)** Cytokine production from CD4⁺ T cells sorted from mediastinum LNs 7 d after immunization ($n = 4$). * $p < 0.05$, ** $p < 0.01$. Error bars indicate SD.

Nonetheless, the CD300a-mediated signaling pathway in iDCs for Th2 skewing has still remained undetermined. Because DCs do not produce IL-4, an important cytokine for Th2 differentiation, we assumed that Th2 suppression in CD300a-deficient mice might result from increased production of cytokines, such as IL-12 and IFN- γ , which promote Th1 differentiation and/or proliferation (29–31). Indeed, recent reports have shown the possibility that alum inhibits production of IL-12p35 or IL-12p40 by DCs, resulting in promoted Th2 responses (5, 32). However, we found that *Il12p35*, *Il12p40*, and *Ifng* mRNA expressions were not up-regulated in CD300a-deficient iDCs. CD300a may inhibit a signal that leads to production of other negative regulator(s) (e.g., immunosuppressive cytokines IL-10 or TGF- β) in iDCs for Th2 induction. Recent evidences demonstrated that alum binds membrane lipids on DCs and promotes lipid sorting in the plasma membrane, triggering activation of the immunoreceptor tyrosine-based activating motif-containing molecules and subsequent signaling molecules such as Syk and PI3K (33). Further studies are required for clarification of CD300a-mediated signaling in iDCs.

Clearance of apoptotic cells by phagocytes is likely important for preventing the development of asthma (34, 35). Airway epithelial cells also recently have been shown to engulf apoptotic epithelial cells and secrete anti-inflammatory cytokines, resulting in attenuated airway inflammation (36). Indeed, patients with asthma have increased apoptotic cells in their airways because their bronchial epithelial cells are more susceptible to oxidative stress than are healthy controls (37, 38). Therefore, our results suggest CD300a in iDCs might contribute to asthma severity. Future studies are required to determine whether apoptotic cells stimulate iDCs in the airway via CD300a and induce Th2 responses in humans.

Previous studies on CD300a focused on its role in the function of human and murine mast cells (16, 21, 39, 40). In this study, we show that CD300a is also expressed on iDCs in mice. CD300a-deficient iDCs induced IL-4 production from naive CD4⁺ T cells significantly less than did WT iDCs after coculture in the presence of a cognate Ag. In contrast, IFN- γ production was significantly increased in the coculture of naive CD4⁺ T cells with CD300a-deficient iDCs. Currently, it remains undetermined how CD300a regulates iDC function that is involved in CD4⁺ T cell differentiation. Furthermore, it is still controversial how Th2 differentiation from naive CD4⁺ T cells is triggered. Mice genetically depleted of CD11c⁺ DCs show dramatically impaired priming of Th2 responses against the parasite helminth *Schistosoma mansoni* (41). In addition, iDCs—but not cDCs, basophils, and eosinophils—are necessary and sufficient for induction of Th2 immunity to household dust-mite allergen (29). These reports support the idea that CD300a-mediated signaling augments iDC function for Th2 induction.

Using a neutralizing anti-CD300a Ab to block apoptotic cell interaction with CD300a attenuated the development of allergic airway inflammation and IgE production. Because a significant number of apoptotic cells are generated throughout the body under both physiologic and pathologic (such as inflammation) conditions, it is intriguing whether CD300a interaction with PS on apoptotic cells is involved in allergic and nonallergic immune responses. Furthermore, blocking this interaction has therapeutic applications for the prevention or treatment of vaccine-involved pathologic conditions and allergic airway inflammation induced by CD300a-mediated signaling in iDCs.

Acknowledgments

We thank F. Abe and R. Hirochika for technical assistance and S. Mitsuishi and Y. Nomura for secretarial assistance.

Disclosures

The authors have no financial conflicts of interest.

References

- Marrack, P., A. S. McKee, and M. W. Munks. 2009. Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Immunol.* 9: 287–293.
- Demento, S. L., S. C. Eisenbarth, H. G. Foellmer, C. Platt, M. J. Caplan, W. Mark Saltzman, I. Mellman, M. Ledizet, E. Fikrig, R. A. Flavell, and T. M. Fahmy. 2009. Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy. *Vaccine* 27: 3013–3021.
- Kool, M., T. Soullie, M. van Nimwegen, M. A. Willart, F. Muskens, S. Jung, H. C. Hoogsteden, H. Hammad, and B. N. Lambrecht. 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* 205: 869–882.
- Kuroda, E., K. J. Ishii, S. Uematsu, K. Ohata, C. Coban, S. Akira, K. Aritake, Y. Urade, and Y. Morimoto. 2011. Silica crystals and aluminum salts regulate the production of prostaglandin in macrophages via NALP3 inflammasome-independent mechanisms. *Immunity* 34: 514–526.
- Marichal, T., K. Ohata, D. Bedoret, C. Mesnil, C. Sabatel, K. Kobiyama, P. Lekeux, C. Coban, S. Akira, K. J. Ishii, et al. 2011. DNA released from dying host cells mediates aluminum adjuvant activity. *Nat. Med.* 17: 996–1002.
- Okoshi, Y., S. Tahara-Hanaoka, C. Nakahashi, S. Honda, A. Miyamoto, H. Kojima, T. Nagasawa, K. Shibuya, and A. Shibuya. 2005. Requirement of the tyrosines at residues 258 and 270 of MAIR-I in inhibitory effect on degranulation from basophilic leukemia RBL-2H3. *Int. Immunol.* 17: 65–72.
- Yotsumoto, K., Y. Okoshi, K. Shibuya, S. Yamazaki, S. Tahara-Hanaoka, S. Honda, M. Osawa, A. Kuroiwa, Y. Matsuda, D. G. Tenen, et al. 2003. Paired activating and inhibitory immunoglobulin-like receptors, MAIR-I and MAIR-II, regulate mast cell and macrophage activation. *J. Exp. Med.* 198: 223–233.
- Kumagai, H., T. Oki, K. Tamitsu, S. Z. Feng, M. Ono, H. Nakajima, Y. C. Bao, Y. Kawakami, K. Nagayoshi, N. G. Copeland, et al. 2003. Identification and characterization of a new pair of immunoglobulin-like receptors LMIR1 and 2 derived from murine bone marrow-derived mast cells. *Biochem. Biophys. Res. Commun.* 307: 719–729.
- Chung, D. H., M. B. Humphrey, M. C. Nakamura, D. G. Gininger, W. E. Seaman, and M. R. Daws. 2003. CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. *J. Immunol.* 171: 6541–6548.
- Cantoni, C., C. Bottino, R. Augugliaro, L. Morelli, E. Marcenaro, R. Castriconi, M. Vitale, D. Pende, S. Sivori, R. Millo, et al. 1999. Molecular and functional characterization of IRp60, a member of the immunoglobulin superfamily that functions as an inhibitory receptor in human NK cells. *Eur. J. Immunol.* 29: 3148–3159.
- Clark, G. J., B. J. Green, and D. N. Hart. 2000. The CMRF-35H gene structure predicts for an independently expressed member of an ITIM/ITAM pair of molecules localized to human chromosome 17. *Tissue Antigens* 55: 101–109.
- Shibuya, A., C. Nakahashi-Oda, and S. Tahara-Hanaoka. 2009. Regulation of immune responses by the activating and inhibitory myeloid-associated immunoglobulin-like receptors (MAIR) (CD300). *Immune New.* 9: 41–45.
- Borrego, F. 2013. The CD300 molecules: an emerging family of regulators of the immune system. *Blood* 121: 1951–1960.
- Munitz, A., I. Bachelet, and F. Levi-Schaffer. 2006. Reversal of airway inflammation and remodeling in asthma by a bispecific antibody fragment linking CCR3 to CD300a. *J. Allergy Clin. Immunol.* 118: 1082–1089.
- Karra, L., B. Berent-Maoz, M. Ben-Zimra, and F. Levi-Schaffer. 2009. Are we ready to downregulate mast cells? *Curr. Opin. Immunol.* 21: 708–714.
- Sabato, V., M. Boita, S. Shubber, C. H. Brits, A. Shibuya, L. S. De Clerck, F. H. Falcone, and D. G. Ebo. 2014. Mechanism of phosphatidylserine inhibition of IgE/Fc ϵ RI-dependent anaphylactic human basophil degranulation via CD300a. *J. Allergy Clin. Immunol.* 134: 734–737.e3.
- Nakahashi-Oda, C., S. Tahara-Hanaoka, S. Honda, K. Shibuya, and A. Shibuya. 2012. Identification of phosphatidylserine as a ligand for the CD300a immunoreceptor. *Biochem. Biophys. Res. Commun.* 417: 646–650.
- Simhadri, V. R., J. F. Andersen, E. Calvo, S. C. Choi, J. E. Coligan, and F. Borrego. 2012. Human CD300a binds to phosphatidylethanolamine and phosphatidylserine, and modulates the phagocytosis of dead cells. *Blood* 119: 2799–2809.
- Holgate, S. T. 2012. Innate and adaptive immune responses in asthma. *Nat. Med.* 18: 673–683.
- Das, J., C. H. Chen, L. Yang, L. Cohn, P. Ray, and A. Ray. 2001. A critical role for NF- κ B in GATA3 expression and Th2 differentiation in allergic airway inflammation. *Nat. Immunol.* 2: 45–50.
- Nakahashi-Oda, C., S. Tahara-Hanaoka, M. Shoji, Y. Okoshi, T. Nakano-Yokomizo, N. Ohkohchi, T. Yasui, H. Kikutani, S. Honda, K. Shibuya, et al. 2012. Apoptotic cells suppress mast cell inflammatory responses via the CD300a immunoreceptor. *J. Exp. Med.* 209: 1493–1503.
- Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
- Doherty, T. A., P. Soroosh, D. H. Broide, and M. Croft. 2009. CD4⁺ cells are required for chronic eosinophilic lung inflammation but not airway remodeling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 296: L229–L235.
- Hanayama, R., M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu, and S. Nagata. 2002. Identification of a factor that links apoptotic cells to phagocytes. *Nature* 417: 182–187.

25. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71–82.
26. Domínguez, P. M., and C. Ardavín. 2010. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. *Immunol. Rev.* 234: 90–104.
27. Serbina, N. V., T. Jia, T. M. Hohl, and E. G. Pamer. 2008. Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* 26: 421–452.
28. Goto, N., H. Kato, J. Maeyama, M. Shibano, T. Saito, J. Yamaguchi, and S. Yoshihara. 1997. Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties. *Vaccine* 15: 1364–1371.
29. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 207: 2097–2111.
30. Jankovic, D., M. C. Kullberg, P. Caspar, and A. Sher. 2004. Parasite-induced Th2 polarization is associated with down-regulated dendritic cell responsiveness to Th1 stimuli and a transient delay in T lymphocyte cycling. *J. Immunol.* 173: 2419–2427.
31. Trinchieri, G., S. Pflanz, and R. A. Kastelein. 2003. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19: 641–644.
32. Mori, A., E. Oleszycka, F. A. Sharp, M. Coleman, Y. Ozasa, M. Singh, D. T. O'Hagan, L. Tajber, O. I. Corrigan, E. A. McNeela, and E. C. Lavelle. 2012. The vaccine adjuvant alum inhibits IL-12 by promoting PI3 kinase signaling while chitosan does not inhibit IL-12 and enhances Th1 and Th17 responses. *Eur. J. Immunol.* 42: 2709–2719.
33. Flach, T. L., G. Ng, A. Hari, M. D. Desrosiers, P. Zhang, S. M. Ward, M. E. Seamone, A. Vilaysane, A. D. Mucsi, Y. Fong, et al. 2011. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. *Nat. Med.* 17: 479–487.
34. Vandivier, R. W., P. M. Henson, and I. S. Douglas. 2006. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest* 129: 1673–1682.
35. McCubrey, A. L., and J. L. Curtis. 2013. Efferocytosis and lung disease. *Chest* 143: 1750–1757.
36. Juncadella, I. J., A. Kadl, A. K. Sharma, Y. M. Shim, A. Hochreiter-Hufford, L. Borish, and K. S. Ravichandran. 2013. Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. *Nature* 493: 547–551.
37. Knight, D. A., and S. T. Holgate. 2003. The airway epithelium: structural and functional properties in health and disease. *Respirology* 8: 432–446.
38. Bucchieri, F., S. M. Puddicombe, J. L. Lordan, A. Richter, D. Buchanan, S. J. Wilson, J. Ward, G. Zummo, P. H. Howarth, R. Djukanović, et al. 2002. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am. J. Respir. Cell Mol. Biol.* 27: 179–185.
39. Bachelet, I., A. Munitz, and F. Levi-Schaffer. 2006. Abrogation of allergic reactions by a bispecific antibody fragment linking IgE to CD300a. *J. Allergy Clin. Immunol.* 117: 1314–1320.
40. Bachelet, I., A. Munitz, A. Moretta, L. Moretta, and F. Levi-Schaffer. 2005. The inhibitory receptor IRp60 (CD300a) is expressed and functional on human mast cells. *J. Immunol.* 175: 7989–7995.
41. Phythian-Adams, A. T., P. C. Cook, R. J. Lundie, L. H. Jones, K. A. Smith, T. A. Barr, K. Hochweller, S. M. Anderton, G. J. Hammerling, R. M. Maizels, and A. S. MacDonald. 2010. CD11c depletion severely disrupts Th2 induction and development in vivo. *J. Exp. Med.* 207: 2089–2096.

Supplementary Figure and Legends

Involvement of CD300a phosphatidylserine immunoreceptor in alum adjuvant-induced Th2 responses

Short title: Involvement of CD300a in alum-induced Th2 responses

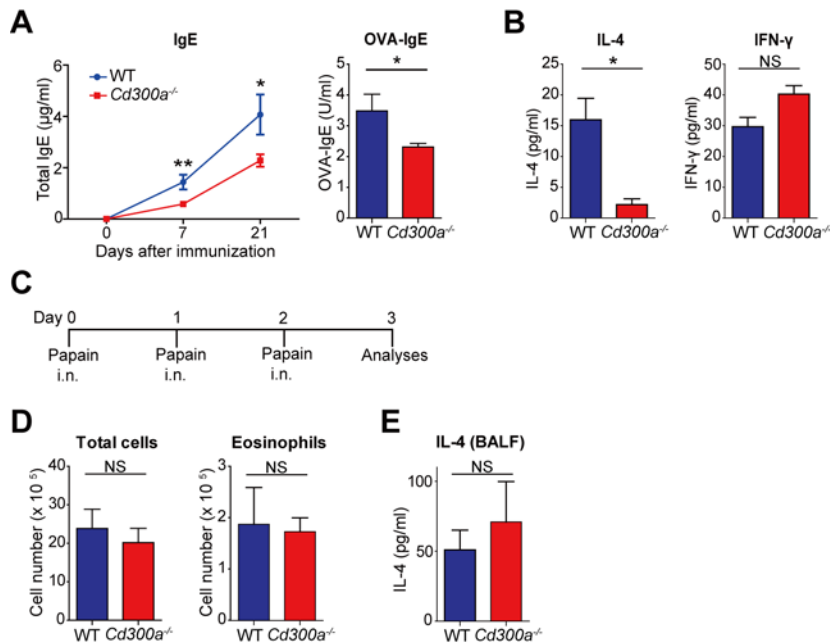
Haruka Miki^{1, 2}, Chigusa Nakahashi-Oda¹, Takayuki Sumida², Akira Shibuya^{1, 3, 4}

Department of ¹Immunology and ²Internal Medicine, Faculty of Medicine, ³Life Science Center of Tsukuba Advanced Research Alliance (TARA), ⁴Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), University of Tsukuba, 1-1-1, Ten-nohdai, Tsukuba, Ibaraki 305-8575, Japan.

Correspondence should be addressed to Akira Shibuya, MD, PhD, e-mail:

ashibuya@md.tsukuba.ac.jp, FAX: 81-29-853-3410, Phone: 81-29-853-3281

Supplemental Figure1

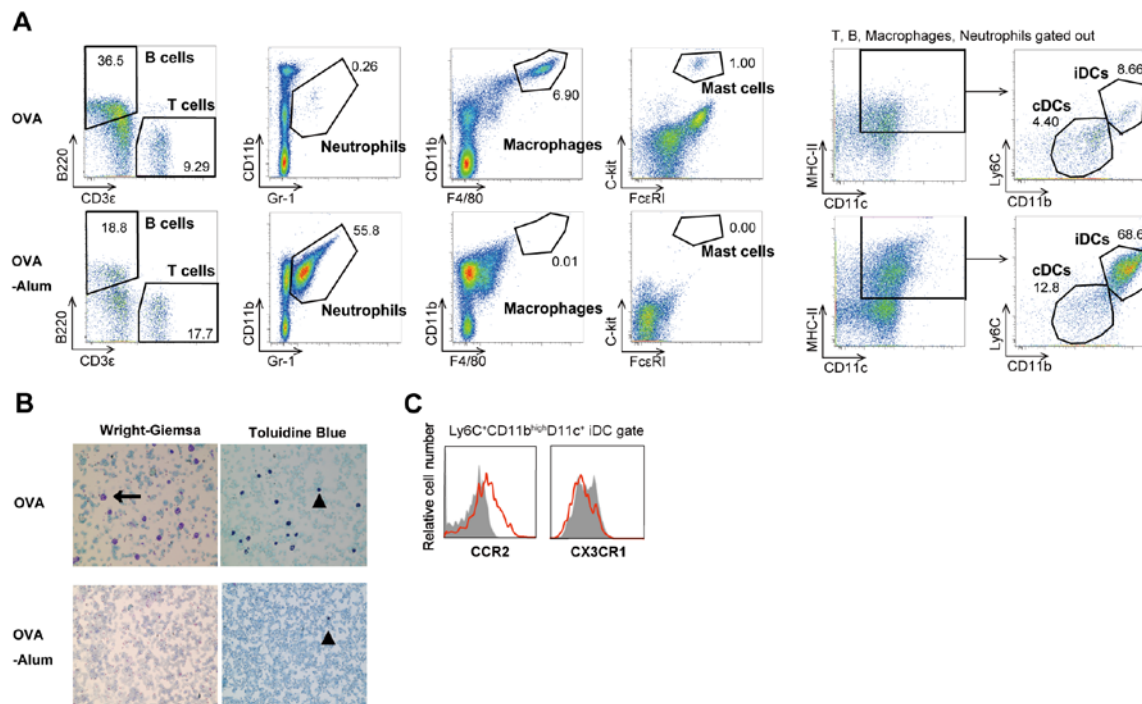


Supplemental Figure 1. Th2 responses after immunization with endotoxin-free OVA plus Alum or with papain.

A, B, WT (n=5) or *Cd300a*^{-/-} (n=5) mice were immunized with endotoxin-free OVA plus Alum on day 0, 7 and day14. Serum total IgE or OVA-specific IgE on day21 after the first sensitization were analyzed (A), Cytokine production from sorted CD4⁺ T cells in mediastinal LNs 5d after immunization (B).

C-E. WT (n=4) or *Cd300a*^{-/-} (n=4) mice were immunized intranasally with papain, as shown in (C). On day 3, numbers of total cells and eosinophils (D) and L-4 concentration (E) in the BAL fluid were analyzed. Data are representative of three independent experiments (A-E). *, $P < 0.05$; **, $P < 0.01$. Error bars indicate SD. NS, not significant.

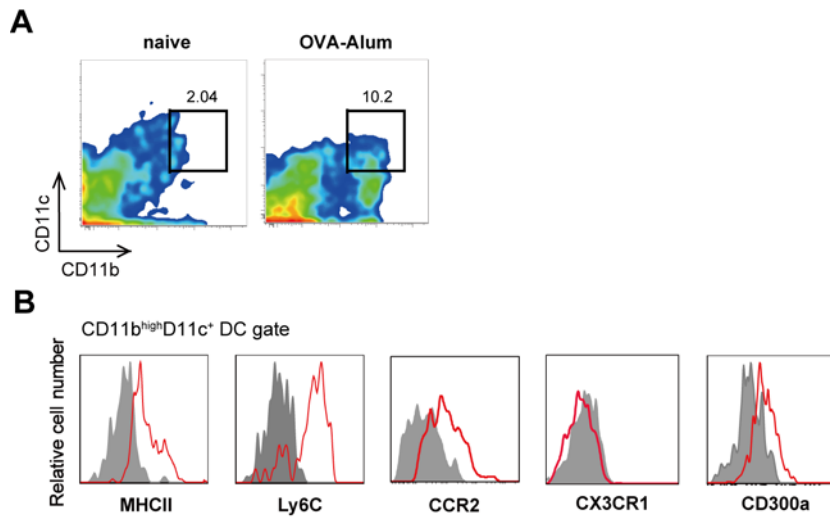
Supplemental Figure2



Supplemental Figure 2. Flow cytometry analyses of peritoneal cells.

A, Peritoneal cells were collected 24 h after i.p. administration of OVA alone or OVA plus alum and simultaneously stained with PI, anti-CD45.2 and antibodies indicated. CD45.2⁺ PI⁻ cells gated were analyzed by flow cytometry. **B**, Morphology (H&E, Hematoxylin and Eosin and PAS, Periodic acid–Schiff) of PEC cells 4 h after OVA alone or OVA plus alum i.p. administration. Arrows indicate macrophages and arrow heads indicate mast cells, respectively. **C**, Flow cytometry of chemokine expression of Ly6C^{high}CD11b^{high} iDCs. Data are representative of three experiments (A-C).

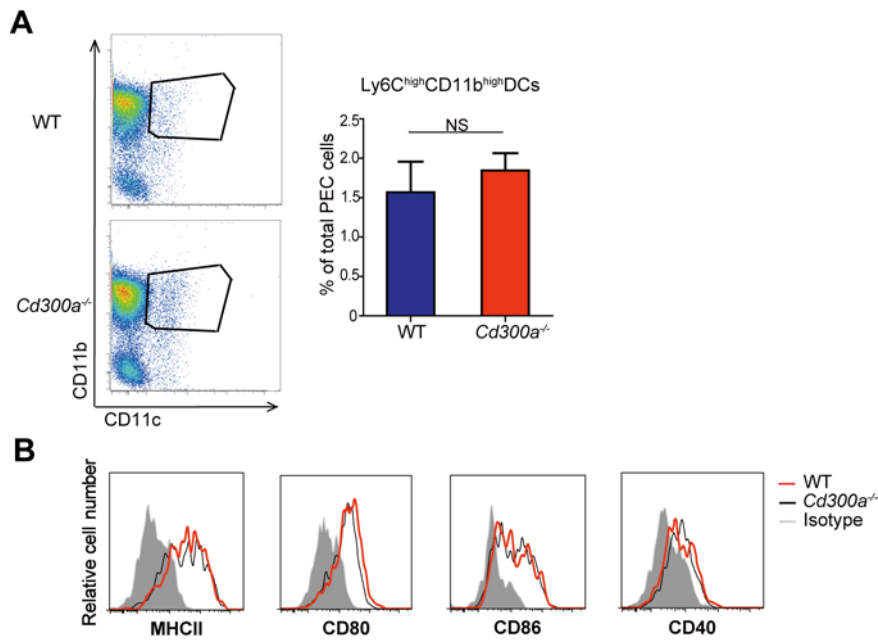
Supplemental Figure3



Supplemental Figure 3. Flow cytometry analyses of inflammatory DCs in mediastinal LN.

A, B, Med LN cells were collected from naïve mice or 72 h after OVA plus alum immunization and simultaneously stained with PI, anti-CD45.2 and antibodies indicated. CD45.2⁺ PI⁻ cells gated were analyzed by flow cytometry (**A**). The population of inflammatory DCs (CD11b^{high}CD11c⁺ cells) gated as shown in (**A**) was analyzed for expression of each molecule (**B**). Data are representative of three experiments (A, B).

Supplemental Figure4



Supplemental Figure 4. Flow cytometry analyses of peritoneal inflammatory DCs.

A, The population of Ly6C^{high}CD11b^{high}CD11c⁺ inflammatory DCs in the peritoneal cells of WT or *Cd300a^{-/-}* mice 24 h after immunization with OVA alone or OVA plus alum. **B**, Expression of each molecule on peritoneal inflammatory DCs of WT or *Cd300a^{-/-}* mice 24 h after immunization with OVA plus alum. NS, not significant. Data are representative of three experiments (A, B). n=4 mice per group