

CD1d expressed in mast cell surface enhances IgE production in B cells by up-regulating CD40L expression and mediator release in allergic asthma in mice

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ABSTRACT

Mast cells play important roles via FcεRI-mediated activation in allergic asthma. A nonpolymorphic MHC I-like molecule CD1d, which is mainly expressed in APCs, presents glycolipid Ag to iTCR on iNKT cells and modulates allergic responses. This study aimed to investigate the role of CD1d on IgE production and mast cell activation related to allergic asthma. Bone marrow-derived mast cells (BMMCs) from C57BL/6 Wild type (WT) or KO (CD1d^{−/−}) mice were activated with Ag/Ab (refer to WT-act-BMMCs and KO-act-BMMCs, respectively) or α-Galactosylceramide (WT-αGal-BMMCs, KO-αGal-BMMCs) in the presence of iNKT cells. WT, KO or BMMC-transferred KO mice were sensitized and/or challenged by OVA or α-Gal to induce asthma. KO-act-BMMCs reduced intracellular Ca²⁺ levels, expression of signaling molecules (Ras, Rac1/2, PLA₂, COX-2, NF-κB/AP-1), mediator release (histamines, leukotrienes and cytokines/chemokines), and total IgE levels versus the corresponding WT-BMMCs. KO mice reduced total and OVA-specific serum IgE levels, number of mast cells, recruiting molecules (CCR2/CCL2, VCAM-1, PECAM-1), expression of tryptase, c-kit, CD40L and cytokine mRNA, co-localization of c-kit and CD1d or iNKT cells in BAL cells or lung tissues, and PCA responses, compared with the corresponding WT mice. BMMC-transferred KO-both mice showed the restoration of all allergic responses versus KO-both mice (Ag/Ab reaction plus α-Gal). KO-αGal-BMMCs or KO-αGal mice did not show any responses. Our data suggest that CD1d-expressed mast cells may function as APC cells for iNKT cells and exacerbate airway inflammation and remodeling through up-regulating IgE production via B cell Ig class switching and mediator release in mast cells of OVA-challenged mice.

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1. Introduction

Mast cells derived from hematopoietic progenitor cells migrate into all vascularized tissues, which complete their maturation [1]. They are activated by cross-linking of Ag-specific IgE bound to FcεRI on their membrane, followed by secretion of a variety of mediators [2], which contribute to airway inflammation and remodeling in allergic asthma [3].

CD1d as a nonpolymorphic MHC class I-like molecule among CD1 family [4] is constitutively expressed in most cell types including APCs [4,5]. Expression of CD1d is enhanced by cytokines (IFN-γ and TNF-α) and Toll-like receptor (TLR) 2/4 ligands [6]. A glycolipid Ag isolated

from marine sponge (α-galactosylceramide, α-Gal) is presented to CD1d, and then recognized by TCR (iTCR) on the iNKT surface [5,7]. Inflammation-associated lysophospholipids, which are produced from cellular phospholipids via phospholipase A₂ (PLA₂), can also act as a ligand for CD1d-restricted NKT cells in human cancer [8]. Engagement of the iNKT cells by CD1d-lipid complexes can direct the differentiation of native T cells toward the development of a Th2 phenotype and increases total serum IgE level [9], which leads to the prompt production of Th1 and Th2-like cytokines and B cell maturation into plasma cells [10,11], and activates and helps B cells to produce both IgG and IgE through a CD1d-dependent mechanism in IL-4-producing CD4⁺ iNKT cells [12,13].

CD1d-restricted iNKT cells play an important role in the pathogenesis of allergic asthma [14] independent of conventional CD4⁺ T cells [15], induce α-Gal-induced airway eosinophilia [16], induce airborne lipid Ag- or environmental Ag-challenged airway inflammation [17–19], and promote immune complex-induced acute lung injury by producing MIP-1α and proinflammatory cytokines [18,20]. There is a report that NKT cells play a role in the development of an acute asthma model, but are dispensable in the development of a chronic asthma model [21]. However, the relationship between CD1d and mast cells is

Abbreviations: α-Gal, α-galactosylceramide; APC, antigen presenting cells; BAL, Bronchoalveolar lavage; BMMCs, Bone marrow-derived mast cells; CCL2, Chemokine ligand 2; CCR2, Chemokine receptor 2; CD1d^{−/−}, CD1d KO; LTs, leukotrienes; MMPs, Matrix metalloproteinases; OVA, Ovalbumin; PAS, Periodic acid-Schiff; PCA, Passive cutaneous anaphylaxis; PLA₂, Phospholipase A₂; Rx, Ag/Ab reaction; TIMPs, Tissue inhibitor metalloproteinases; TLR, Toll like receptor.

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not clarified yet excepting that IL-9 and CD1d-restricted NKT cells increase the number of Ag-induced pulmonary mast cell progenitors (MCp) [22].

Based on the reports above that mast cells or CD1d play a key role in the allergic asthma, we hypothesized that mast cells express CD1d on their surfaces via the Ag/Ab reaction, and the expressed CD1d is ligated with glycolipid-Ag and engaged by iTCR on iNKT cells, which is followed by exacerbation of allergic asthma symptoms through the release of more mediators. We observed that CD1d which had originally existed in mast cell surface was enhanced in the mast cells activated by Ag/Ab reaction, and it enhanced the release/secretion of more mediators by up-regulating the signaling molecules in the downstream of Ras in FcεRI-mediated responses, and CD1d-mediated mast cells enhanced IgE production, which is more produced in the engagement of iNKT cells by CD1d-lipid-pulsed B cells, through B cell Ig class switching via up-regulating the CD40L expression and cytokines.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice weighing 20 g (8 wks old) were obtained from ORIENT BIO Company (Seongnam, Korea). CD1d^{-/-} mice (CD1d-deficient mice backcrossed with C57BL/6) were generated by homologous recombination as previously described [23]. All animals were bred and maintained in specific pathogen-free conditions at the Laboratory Animal Research Center of Sungkyunkwan University (Suwon, Korea). The mice were treated in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all animal experiments were approved by the Institutional Review Board in the Laboratory Animal Research Center of Sungkyunkwan University.

2.2. Culture and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells flushed from femurs and tibias of WT and TGase^{-/-} mice were cultured for 5 wks in RPMI-1640 containing IL-3 [24]. The purity for BMMCs using May-Grünwald-Giemsa staining was more than 95% of total cells (see Supplementary data).

BMMCs (1×10^6 cells) were sensitized with 0.1 µg/mL anti-DNP IgE Ab overnight at 37 °C. The cells were washed twice and then challenged 5 ng/mL DNP-HSA (Ag, refer to act-BMMCs) (Sigma-Aldrich, St. Louis, MO) for 5 h or the indicated time periods at 37 °C [24]. BMMCs (1×10^6 cells) were directly stimulated with 100 ng/mL α-Gal (refer to αgal-BMMCs) (Enzo Life Sciences, Farmingdale, NY) for 5 h or time periods indicated. In both stimulations (both-BMMCs), DNP-HSA and α-Gal were simultaneously added in the BMMCs sensitized with anti-DNP IgE Ab. All BMMCs were activated in the presence of iNKT cells (DN32.D3 cell line) because the cells with iNKT cells were increased in all responses versus those without iNKT cells, and the optimal ratio for BMMCs (1×10^6 cells) or B cells (1×10^6 cells) and iNKT cells (5×10^6 cells) was 1:5. We selected the Ag dose that was less than the maximum Ag dose for yielding the maximum responses in the combination stimulation. Thus, the responses for Ag alone are lower than those in the maximum responses. Optimal dose and time for the Ag/Ab reaction or α-Gal stimulation were yielded in the preliminary experiments.

2.3. Western blot analysis

Immunoblotting was performed for each protein extract from homogenized BMMCs (1×10^6 cells), BAL cells (1×10^6 cells/50 µL) or lung tissues (50 mg/500 µL) using a previously described method [25] (see Supplementary data).

2.4. CD1d expression by flow cytometry

Act-BMMCs or gal-BMMCs were stained with CD1d Ab. The cells were incubated with FACS buffer (2% BSA and 0.1% sodium azide in PBS) and washed twice. The cells were subsequently stained with 5 µg/mL PE-conjugated anti-CD1d Ab (BD Biosciences, San Jose, CA). Flow cytometric analysis was conducted on FACS Canto II (BD Biosciences, Franklin Lakes, NJ) and analyzed by a flow cytometer Flowjo software (Treestar Inc., San Carlos, CA) [8].

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BMMCs (1×10^6 cells), BAL cells (1×10^6 cells) or lung tissues (50 mg/500 µL) using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). RT-PCR was performed in a final volume of 50 µL using an amfiRivert One-step RT-PCR kit (GenDEPOT, Barker) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR was performed for 35 cycles [25] (see Supplementary data).

2.6. GST effector pull-down assay

Small GTPase protein activities in act-BMMCs (1×10^6 cells) were assayed using EZ-Detect protein activation kits (Upstate Biotechnology, Lake Placid, NY). The active form of small GTPase proteins was obtained from the supernatants by affinity precipitation using Pak-1 protein binding domain (PBD), which was fused to GST, and visualized by Western blot analysis with anti-rabbit Rac1/2, cdc42 (1:1,000; PIERCE, Rockford, IL) [24] (see Supplementary data).

2.7. Measurement of intracellular Ca^{2+} [Ca^{2+}]_i level

BMMCs (1×10^6 cells) were incubated for 30 min after adding Fluo-3 AM (5 µM) and placed on a glass slide treated with poly-L-lysine. The [Ca^{2+}]_i levels in act-BMMCs were quantified with fluorescence intensity using a LSM 510 laser scanning microscope (Carl Zeiss, Oberkochen, Germany) [24]. Fluorescence intensity (F_0) for control was adjusted to 1 ($F_0 = 1$).

2.8. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BMMCs (1×10^6 cells), BAL cells (1×10^6 cells) or lung tissues (50 mg/500 µL), and then EMSA was performed as described previously [25] (see Supplementary data).

2.9. Histamine assay

The histamine in supernatants (100 µL) isolated from act-BMMCs (1×10^6 cells), gal-BMMCs or both-BMMCs activated for 30 min were determined using an ELISA kit (Neogen Corp., Lexington, KY). The concentrations of histamine were calculated using the standard curves generated by specific histamine standards. The results are expressed in ng/mL (1×10^6 cells) [24]. The lowest detection limit for LTs mounts was 2.5 ng/mL.

2.10. Measurements of leukotrienes (LTs)

The LT contents in supernatants (50 µL) isolated from act-BMMCs, gal-BMMCs or both-BMMCs activated for 1 h were determined using an ELISA kit (Cayman Chemical, Ann Arbor, MI). The results are expressed in pg/mL (1×10^6 cells) [24]. The lowest detection limit for LTs mounts was 7.8 pg/mL (see Supplementary data).

2.11. Mast cell migration by bioluminescence images in vivo

To investigate the migration of mast cells in vivo, BMMCs were stained with 200 $\mu\text{g}/\text{mL}$ magnetic nanoparticle (MNP) for 24 h at 37 °C using the Neo-Live™ Magnoxide 675 (Biterials, Seoul, Korea). KO mice were nasally stimulated with α -Gal right after sensitization and nebulization with OVA as the same method as induction of allergic asthma one day after transplantation of MNP-labeled BMMCs (1×10^7 cells/100 mL) to mice through tail vein. All anesthetized mice were analyzed through the images using the IVIS Lumina XR imaging system (Xenogen Corp., Alameda, CA) [27]. Photon flux was measured at base line by drawing a circle in lung area using the Living Image software (Xenogen Corp., Alameda, CA). Values were converted from total photon (p) flux to steradian ($\text{p/s}/\text{cm}^2/\text{sr}$) per lung area ($\mu\text{W}/\text{cm}^2$). The sr means steradian, s means sec, and μW means μ watt (see supplementary data).

To confirm mast cell migration in lung tissues, lung tissues were fixed for paraffin section right after imaging analysis, and cut at 3 μm . The slides were mounted using aqueous mounting medium, and then BMMCs in lung tissues were analyzed using a LSM 5 EXCITER confocal microscope (Carl Zeiss, Oberkochen, Germany). MNP-labeled BMMCs were quantified at 5 sites of $200 \times 200 \mu\text{m}$ areas (5 sites/each slide $\times$ 6 mice = 30 sites) under a microscope [25].

2.12. OVA-challenged allergic asthma model

The experimental mice were divided into nine groups (8 mice/group). NC (Negative control for WT mice) or WT-OVA, WT mice sensitized and nebulized with PBS and OVA, respectively; Con (Negative control for KO mice) or KO-OVA, KO ($\text{CD1d}^{-/-}$) mice sensitized and nebulized with PBS and OVA, respectively; WT- α Gal or KO- α Gal, WT and KO mice, respectively, intranasal stimulated with α -Gal; WT-both or KO-both, WT and KO mice, respectively, intranasal stimulated with α -Gal right after sensitization and nebulization with OVA; and KO-both + BMMCs (BM+), KO-both mice received normal BMMCs (1×10^7 cells) by intravenous injection 1 day before OVA sensitization. The reason that the BMMCs were injected 1 day before the OVA sensitization was because they were delivered in lung tissues 1 day after the BMCM injection using the IVIS Lumina XR imaging system (Xenogen Corp., Alameda, CA).

Mice were sensitized with 20 $\mu\text{g}/200 \mu\text{L}$ OVA (Grade V) (Sigma-Aldrich, St. Louis, MO) adsorbed in 1 mg/50 μL of alum (aluminum hydroxide, 2% Alhydrogel) (Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection on days 1 and 15 in all mice except the control. After 5 days, the mice were nebulized with 5% OVA in PBS for 30 min once daily for consecutive days from days 21 to 23 (OVA challenge) using a nebulizer (Mega Medical, Seoul, Korea). Control mice were nebulized by PBS at the same time. All animals were euthanized at day 24 [28].

α -Gal was dissolved in 0.5 mg/mL in PBS. Mice received 10 $\mu\text{g}/20 \mu\text{L}$ α -gal by intranasal injection [16] on day 23 for the same experimental condition. All mice were sacrificed on day 24. The data for KO- α Gal mice (KO mice intranasal stimulated with α -Gal) were omitted in all figures of text to avoid complexity because this group did not show any effects on all allergic responses, but they were only shown in Supplementary Fig. 5.

2.13. Isolation of Bronchoalveolar lavage (BAL) fluid

BAL fluid was collected, stained and counted using previously described Materials and methods [26]. Infiltrated inflammatory cells stained with Diff-Quick or mast cells stained with May-Grünwald-Giemsa were quantified under light microscopy [26] (see Supplementary data).

2.14. Lung tissue histology

Lungs tissue sections from the mice embedded in paraffin (3 μm) were stained with H&E to observe the general morphology, PAS for goblet cells, and Masson's trichrome for collagen deposition [26]. The amount of collagen was quantified by a Sircol collagen assay [25] (see Supplementary data).

2.15. Preparation of mast cells isolated from lung tissues

After removing large airways and blood vessels from isolated-lung tissues, the lungs were minced with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd, Surrey, UK). Small sections of lung tissues were treated two times with Hanks' balanced salt solution (HBSS) containing 25 mM HEPES, 20% FBS, 5 U/mL collagenase and 5 U/mL elastase (Gibco, Grand Island, NY) using a rotor apparatus. The time of consecutive exposure of lung tissues to the enzymes was 30 min. Cells were filtered through metal and nytex mesh (100 μm) [29]. The filtered cells were blocked with 1% BSA in PBS for 1 h and incubated with the Ab against TEXAS-Red-conjugated Fc ϵ RI and FITC-conjugated tryptase (diluted 1:25) in blocking solution for 1 h. The cells were sorted using FACS Aria II fluorescence-activated cell sorting system (BD Biosciences, Franklin Lakes, NJ). The purity of mast cells was more than 96%, and these mast cells were used for determining the expression of surface markers and migration/adhesion molecules.

2.16. Measurement of zymography

Activities of matrix metalloproteinases (MMP-2/9) in BAL fluid (35 μL of first BAL fluid samples) were analyzed as described previously [30]. Gelatinolytic activity was detected as a clear band against a blue background. Human MMP-2 and MMP-9 (Chemicon, Temecula, CA) were used as gelatinase zymography standard markers (see Supplementary data).

2.17. Isolation and activation of B cells or co-cultured with mast cells

B cells were isolated from the spleens of C57BL/6 mice using the MagCelect mouse B cell isolation kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's protocol. The purity of B cells was more than 94% of total cells.

BMMCs (1×10^6 cells) were activated with Ag/Ab for 5 h as presented above. B cells (1×10^6 cells) were incubated with 0.5 $\mu\text{g}/\text{mL}$ anti-mouse IgM Ab (refer to act-B cells) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 48 h [25]. The act-B cells (1×10^6 cells) were added on non-act-BMMCs (BMMCs alone without Ag/Ab reaction) or act-BMMCs (1×10^6 cells) in the presence of iNKT cells (5×10^6 cells), and co-cultured for 72 h. The optimal incubation time and ratio (1:1:5) for co-culture of mast cell, B cell and iNKT cells were yielded in preliminary experiments. Total IgE secreted in the culture media was determined by the ELISA method [25].

2.18. Measurement of total and OVA-specific serum IgE or IL-2

Total or OVA-specific IgE amounts in the supernatants (200 μL) isolated from act-B cells with or without act-BMMCs in the presence of iNKT cells or sera were analyzed using ELISA [25] (see Supplementary data).

Amounts of IL-2 were determined using an ELISA kit (Enzo Life Sciences, Farmingdale, NY). The lowest detection limit for total and OVA-specific IgE, and for IL-2 was better than 2.1 ng/mL, 1.5 ng/mL and 3.12–1,000 pg/mL, respectively [25].

2.19. Co-culture of BMMCs and NKT cells

BMMCs (1×10^6 cells) activated with 5 ng/mL DNP-HSA and 0.1 μ g/mL anti-DNP IgE Ab (act-BMMCs) or with 100 ng/mL α -gal (gal-BMMCs) were co-cultured with NKT cells (DN32.D3, TCB11 or IC8.DC1, 5×10^6 cells) in RPMI media. The optimal incubation time (15 h) and ratio (BMMCs: NKT cells = 1:5) were yielded in preliminary experiments (data not shown). IL-2 amounts secreted in culture media were quantified using ELISA (Enzo Life Sciences, Farmingdale, NY) [28].

2.20. Immunofluorescence

Lung sections (3 μ m) were deparaffinized with xylene and washed in ethanol. Slides were blocked with 1% BSA in PBS for 1 h and incubated with the Ab against c-kit or NK1.1 and CD1d (diluted 1:25) in a blocking solution for overnight. The slides were treated with the corresponding FITC or TEXAS-Red-conjugated anti-IgG (Zymed Laboratory Inc. San Francisco, CA) or cy5.5-conjugate anti-IgG for 1 h at room temperature. Washing between steps in each stage was performed for 2–3 min. After washing, the slides were mounted using an aqueous mounting medium (Thermo Shandon), and examined using a LSM 5 EXCITER confocal microscope (Carl Zeiss, Oberkochen, Germany) [25].

2.21. Passive cutaneous anaphylaxis (PCA) analysis

An anti-DNP-specific IgE Ab (0.5 μ g) was injected intradermally into one ear of each mouse group (ten mice/group). After 24 h, the mice were challenged with an intravenous injection of DNP-HSA (200 μ g) in 200 μ L of PBS containing 3% Evans blue, and 2 h after, the treated ear was excised to measure the amount of dye that had extravasated in response to Ag. The dye was extracted using formamide at 63 °C. The dye intensity was read at 620 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) [25].

2.22. Statistical analysis

Experimental data are shown as mean $\pm$ SEM ($n = 4$). The unpaired Student's *t* test was used to compare the two groups. Multiple group comparisons were performed using a one-way ANOVA followed by Scheffe's post hoc test, using SPSS (SPSS Inc., Chicago, IL). *p* values < 0.05 were considered as significant. The densitometry analysis of Western blots, RT-PCR and EMSA was performed with Quantity One version 4.6.3 (Bio-RAD, Hercules, CA) and are indicated as the mean $\pm$ SEM ($n = 4$) obtained from the ratio of each band density versus those in the control and loading control of four independent experiments.

3. Results

3.1. CD1d expression

As mast cells [3] and CD1d [14] play important roles in the development of allergic asthma, we first observed that BMMCs originally have CD1d, and BMMCs, which were activated with Ag/Ab reaction (refer to WT-act-BMMCs and KO-act-BMMCs, respectively), with α -Gal (refer to WT- α Gal-BMMCs and KO- α Gal-BMMCs, respectively), or with both (Ag/Ab and α -Gal; WT-both-BMMCs and KO-both-BMMCs, respectively), enhanced the CD1d expression in the presence of iNKT versus those in NC (negative control), and WT-both-BMMCs enhanced its expression more than that in WT-act-BMMCs. However, KO-act-BMMCs, KO- α Gal-BMMCs or KO-both-BMMCs did not express it (Fig. 1A; Supplementary Fig. 1A and B). CD1d was also expressed in WT-act-, WT- α Gal- and WT-both-BMMCs in the absence of iNKT cells (Supplementary Fig. 1C). We

confirmed that CD1d which originally existed in mast cell surface is enhanced in WT-act- or WT- α Gal-BMMCs with iNKT cells using flow cytometry (Fig. 1B). IgG Ab as a positive control instead of anti-CD1d Ab did not affect its expression. Thus, we used BMMCs co-cultured with iNKT cells because BMMCs originally had CD1d, although act-BMMCs with iNKT cells weakly enhanced CD1d expression versus those without iNKT cells. Hereafter, the use of iNKT cells was not shown in the text because they were used in all experiments.

3.2. Effects of CD1d^{-/-} on expression of Fc ϵ RI and signal molecules

Binding of IgE to Fc ϵ RI on the mast cell surface can enhance the expression of Fc ϵ RI, and increase sensitivity to Ag [31]. Thus, we observed that expression of Fc ϵ RI and activities of Lyn/Syk in KO-act-BMMCs were increased similar to those in WT-act-BMMCs, but were not increased in WT- α Gal- and KO- α Gal-BMMCs. The data show that CD1d deficiency does not impair Fc ϵ RI expression, and binding of iNKT cells to CD1d- α -Gal complexes does not influence Fc ϵ RI expression and Lyn/Syk activities (Fig. 1C and D).

However, Ras expression and Rac1/2 activities, which activate intracellular kinase cascades to modulate gene transcription in mast cell activation [24], reached a maximum at 20 s and 40 s, respectively, in WT- α Gal- or WT-act-BMMCs (Supplementary Fig. 1D), and were enhanced in WT-both-BMMCs versus those in WT-act- or WT- α Gal-BMMCs (Fig. 1E and F). In KO-act-BMMCs, their expression and activities were reduced similar to KO-both-BMMCs, but were reduced more than those in WT-act-BMMCs. As these phenomena, that KO-both-BMMCs reduced all responses similar to KO-act-BMMCs, KO-act-BMMCs reduced more than those in WT-act-BMMCs, and WT-both-BMMCs enhanced all responses versus WT-act-BMMCs, showed similar effects in this experiment, hereafter, we did not explain these effects any more.

3.3. Effects of CD1d^{-/-} on intracellular calcium ([Ca²⁺]_i) level, signaling molecules and cytokine expression

Increments of [Ca²⁺]_i level are necessary for the release of mediators in mast cell activation [24]. WT-act-BMMCs initiated an increase in [Ca²⁺]_i level at 2–5 s, and this reached a plateau at 2 min, whereas KO-act-BMMCs reduced the [Ca²⁺]_i level versus the WT-act-BMMCs. WT- α Gal-BMMCs weakly increased [Ca²⁺]_i level versus the WT-act-BMMCs, whereas KO- α Gal-BMMCs did not affect [Ca²⁺]_i level (Fig. 2A).

To investigate the order of signaling molecules for α -Gal, we used specific inhibitors in WT-both-BMMCs. Ras expression was not blocked by pretreatment with Rac inhibitor (100 μ M NCS) or Ca²⁺ blocker (50 μ M SKF) in WT-both-BMMCs, whereas Rac1/2 was inhibited by a Ras inhibitor (75 μ M FTS) as well as its inhibitor, but was not inhibited by a Ca²⁺ blocker (Supplementary Fig. 1E). The [Ca²⁺]_i level was blocked by all inhibitors used (Supplementary Fig. 1F).

Activities of downstream signaling molecules of [Ca²⁺]_i such as PKCs, MAPKs, PLA₂ and inducible cyclooxygenase-2 (COX-2) reached a maximum at 3 min, 5 min, 10 min, and 10 min, respectively (data not shown). Expression of PLA₂ and COX2 was increased in WT-act- or WT- α Gal-BMMCs (Fig. 2B), and this is followed by release of histamine (Fig. 2C) and newly synthesized LTs (Fig. 2D). KO-act-BMMCs reduced the phosphorylation of PKCs and MAPKs (data not shown), and expression of PLA₂ and Cox-2 versus WT-act-BMMCs, but KO- α Gal-BMMCs remarkably reduced their activities and mediator release versus those increased in WT- α Gal-BMMCs. Constitutive COX-1 had no expression either in the absence/presence of CD1d or stimuli (Ag/Ab or α -Gal) (Fig. 2B).

WT-act-BMMCs had enhanced activities of NF- κ B/AP-1 and expression of cytokine mRNA (IL-1 β , -4, -5, -8, -13, TNF- α and TGF- β) (Fig. 2E and F), whereas KO-act-BMMCs reduced their activities and

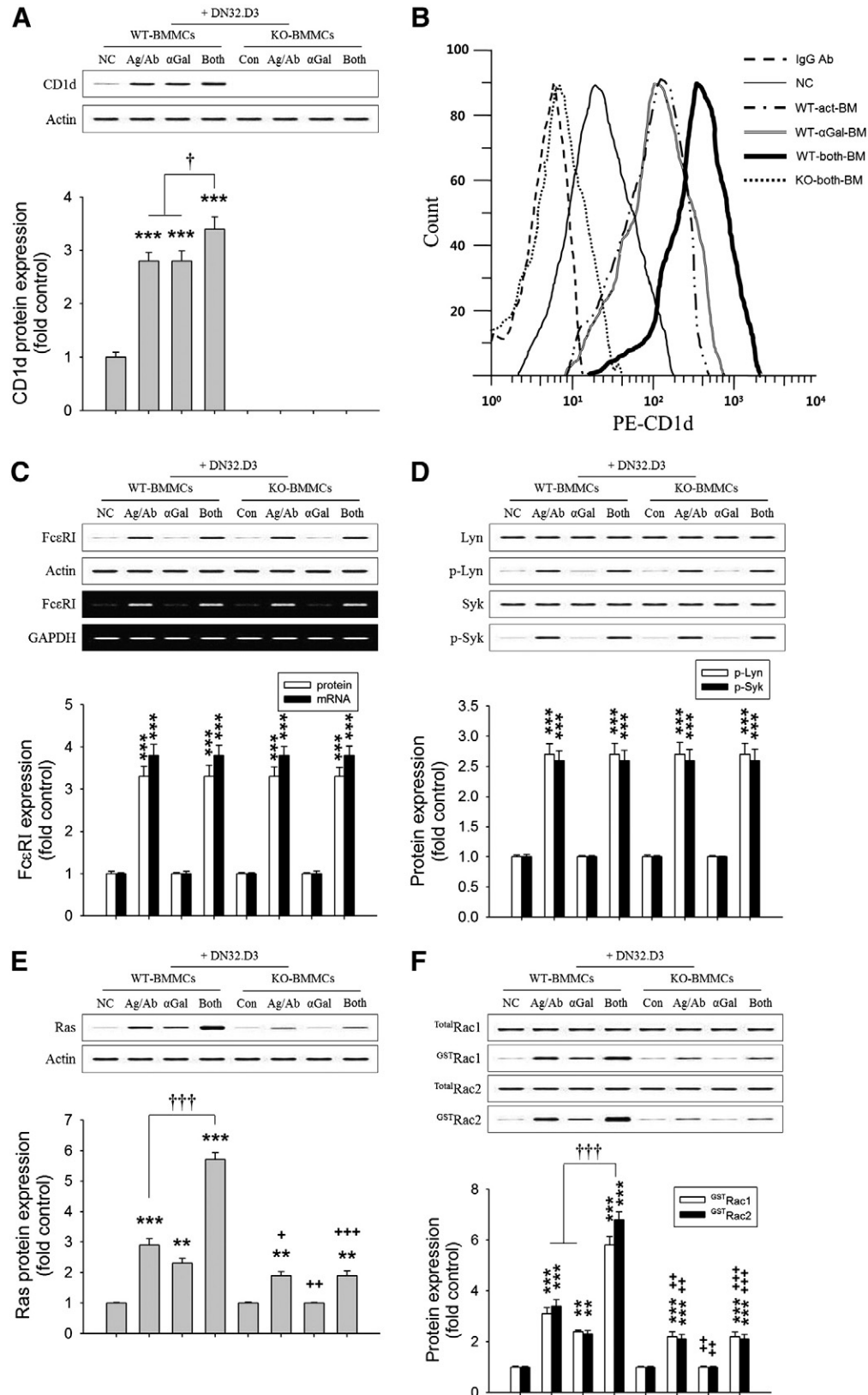


Fig. 1. Effects of CD1d^{-/-} on the expressions of CD1d, FcεRI and Ras or activities of Lyn, Syk and Rac1/2 in the act-BMMCs in the presence of iNKT cells. BMMCs (1 × 10⁶ cells) from WT or KO (CD1d^{-/-}) mice were activated with 5 ng/mL DNP-HSA/0.1 μg/mL anti-DNP IgE Ab (Ag/Ab) (WT-act-BMMCs or KO-act-BMMCs) or 100 ng/mL α-Gal (WT-αGal-BMMCs or KO-αGal-BMMCs) or both (Ag/Ab reaction plus α-Gal; WT-both-BMMCs or KO-both-BMMCs) in the presence of iNKT cells for the indicated time periods (20 s for Ras; 40 s for Rac1/2; 5 h for CD1d). NC or Con, non-activated BMMCs from the WT and KO mice, respectively. Expression of CD1d, FcεRI and Ras or activities of Lyn/Syk and Rac were determined in the protein or mRNA extracts from BMMCs using Western blot, RT-PCR and GST-pull-down assay, respectively. (A) Expression of CD1d in BMMCs. (B) CD1d expressed in the BMMC (BM) surface using a FACS analyzer. IgG Ab as a positive control for anti-CD1d Ab. (C) Expression of FcεRI protein and mRNA. (D) Phosphorylation of Lyn and Syk. (E and F) Expression of Ras and Rac1/2, respectively. The histogram from the densitometry analysis for each band image shows the mean ± SEM (n = 4) obtained from the ratio of each band density versus those of the control and actin, GAPDH or total protein from four independent experiments. **p < 0.01; ***p < 0.001 versus NC or Con. †p < 0.05; ††p < 0.01; †††p < 0.001 versus the corresponding WT-BMMCs. ‡p < 0.05; ‡‡p < 0.01 versus WT-act-BMMCs.

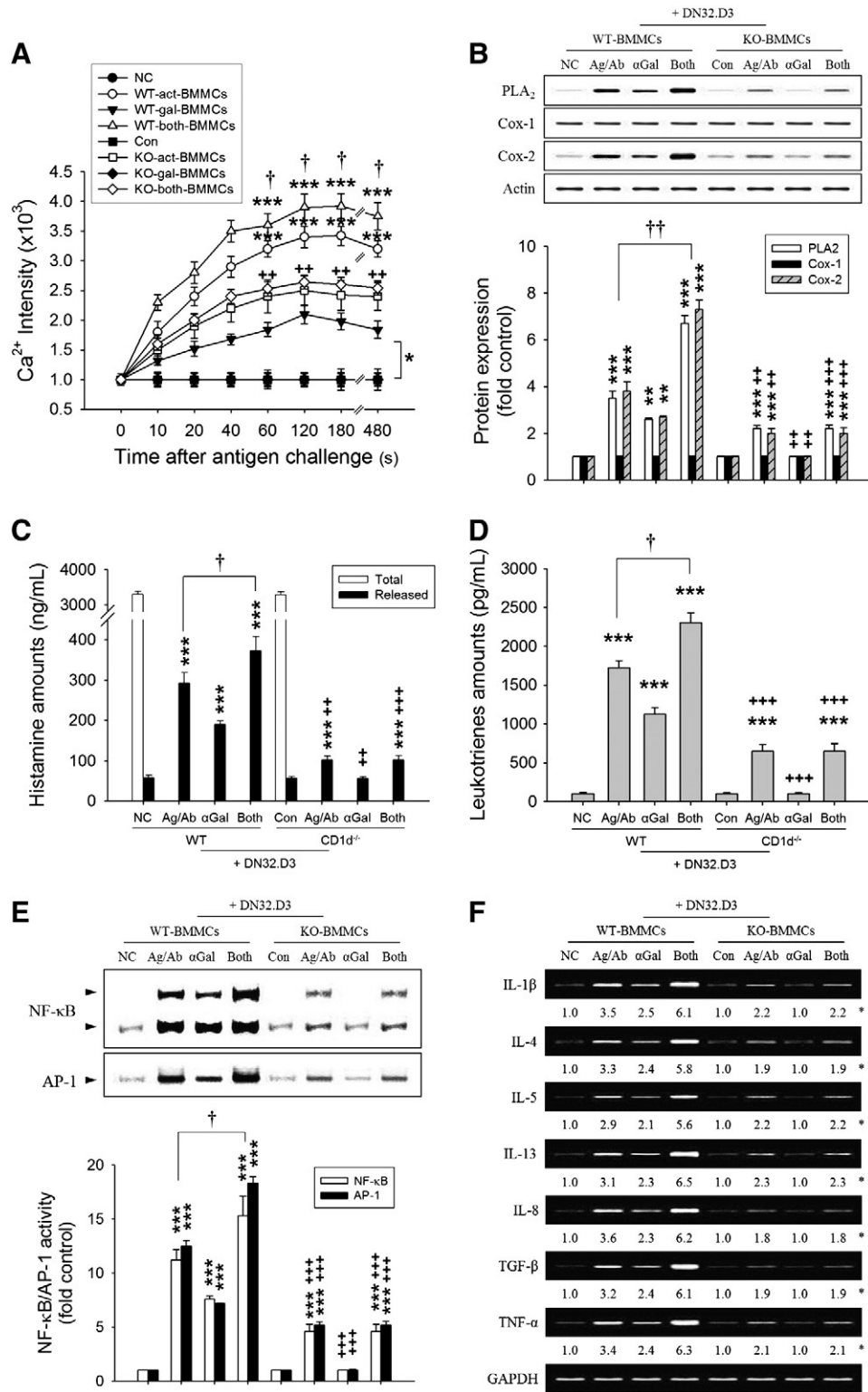


Fig. 2. Effects of CD1d^{-/-} on Ca²⁺ influx, the expression of signaling molecules, the amounts of mediators, activities of transcription factors or expression of cytokines in the BMMCs in the presence of iNKT cells. Experimental details for BMMC activation, and definition of all symbols and abbreviations were described in Fig. 1 legend. Fluo-3-AM (5 μM) was added to BMMCs and incubated for 30 min. The [Ca²⁺]_i level was analyzed by confocal microscopy. Expression of PLA₂ and COX-1/COX-2 was determined in the protein extracts isolated from BMMCs using Western blot. Amounts of mediators were determined in supernatants from BMMCs (n = 8) using ELISA, and activities of NF-κB/AP-1 or expression of cytokine mRNA using EMSA and RT-PCR, respectively. (A) Time courses of [Ca²⁺]_i level expressed as the mean ± SEM of eight independent experiments (n = 8). (B) Expression of PLA₂ and COX-1/2. (C and D) Amounts of histamine and LTs, respectively. (E) Activities of NF-κB and AP-1. (F) Expression of cytokine mRNA. The histogram from the densitometry analysis for each band image was obtained as described in Fig. 1 legend (n = 4). *, Numbers below bands show the mean (n = 4) for values expressed from the ratio of band density of each cytokine mRNA versus those of the control and GAPDH mRNA from four independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 versus NC or Con. ++p < 0.01; +++p < 0.001 versus the corresponding WT-BMMCs. †p < 0.05; ††p < 0.01 versus WT-act-BMMCs.

expression versus WT-act-BMMCs, but KO- α Gal-BMMCs did not affect them.

3.4. Effects of $CD1d^{-/-}$ on the recruitment of inflammatory cells and remodeling molecules into BAL fluid or lung tissues in OVA-challenged mice

We investigated whether the events for BMMCs in vitro occur in a mouse model in vivo using KO ($CD1d^{-/-}$) mice and for $CD1d$ deficiency were restored in BMBC-transferred KO-both mice (refer to BM + KO-both mice). We first observed that MNP-labeled BMMCs were migrated into whole body lung areas in KO mice (Supplementary Fig. 2A, upper column), and into lung tissues of KO-both mice (Supplementary Fig. 2A lower column) sufficiently one day after tail vein injection.

$CD1d$ was not expressed in the bronchoalveolar lavage (BAL) cells and lung tissues of KO-OVA mice versus the WT-OVA mice, and it was recovered in BM + KO-both mice, although not completely (Supplementary Fig. 2B). As these phenomena, that KO-both mice reduced all responses similar to KO-OVA mice, KO-OVA mice reduced more than those in WT-OVA mice, and WT-both mice enhanced all responses versus WT-OVA mice, showed similar effects in this experiment, we did not explain the effects any more.

The number of mast cells in BAL fluid was significantly increased in WT-OVA mice (from $1 \pm 0.1 \times 10^3$ cells to $32 \pm 3.5 \times 10^3$ cells) and WT- α Gal mice (to $21 \pm 3.0 \times 10^3$ cells) versus the NC mice, whereas KO-OVA mice (to $11 \pm 1.9 \times 10^3$ cells) showed a reduced number of mast cells versus the WT-OVA mice in BAL cells (Fig. 3A). BM + KO-both mice ($26 \pm 2.1 \times 10^3$ cells) showed restored number of mast cells by approximately 57% versus KO-both mice. The number of mast cells in lung tissues of KO-OVA mice showed similar reduction to those in BAL cells (Fig. 3B).

Surface marker c-kit and granular content tryptase in mast cells, and recruitment molecules (CCL2/CCR2, VCAM-1 and PECAM-1) expressed in the mast cells were reduced in BAL cells or mast cells isolated from lung tissues of KO-OVA mice compared with WT-OVA mice (Fig. 3C and D), and BM + KO-both mice recovered their expression.

KO-OVA mice reduced the number of total inflammatory cells including eosinophils, neutrophils, lymphocytes and macrophages in BALF, and the number of inflammatory cells, goblet cell hyperplasia and collagen deposition in the surrounding peribronchial and perivascular area of lung tissues (Supplementary Fig. 2C–F), expression of eosinophil major basic protein (EMBP) that is over-expressed in eosinophilia, and Muc5ac mRNA expression that is predominantly secreted during goblet cell hyperplasia [26], collagen levels, the gelatinolytic activities, imbalance of MMP2/9 and TIMP1/2 (Supplementary Fig. 3A–E), phosphorylation of PKCs and MAPKs (data not shown), activities of NF- κ B/AP-1 and expression of cytokine mRNA (Supplementary Fig. 4A and B), which was increased in the BAL cells and lung tissues of WT-OVA mice. WT- α Gal mice weakly increased all responses than those increased in WT-OVA mice. BM + KO-both mice showed a recovery in all responses above versus KO-both mice.

3.5. Effects of $CD1d^{-/-}$ on the total and serum OVA-specific IgE level

As $CD1d$ -restricted iNKT cells activate B cells to produce IgE [12,13], we observed that total IgE (323 ± 12.5 ng/mL) or OVA-specific Serum IgE (245 ± 18.8 ng/mL) level were elevated in WT-OVA mice versus NC mice (total, 20 ± 5.4 ng/mL; specific, 0.8 ± 0.1 ng/mL). KO-OVA (total, 68 ± 9.2 ng/mL; specific, 30 ± 1.4 ng/mL) reduced both IgE level by approximately 80–90% versus OVA mice (Table 1). However, WT- α Gal mice had weakly increased total IgE levels (total, 68 ± 9.2 ng/mL; specific, 30 ± 1.4 ng/mL). BM + KO-both mice (total, 188 ± 7.3 ng/mL; specific, 154 ± 5.3 ng/mL) showed recovered total and OVA-specific IgE level versus KO-both mice (total, 70 ± 8.4 ng/mL; specific, 32 ± 2.0 ng/kg) or KO- α Gal mice (total, 20 ± 4.6 ng/mL; specific, 0.9 ± 0.3 ng/kg).

3.6. Effects of $CD1d^{-/-}$ on the IgE production in interaction of B cells with BMMCs

In vitro, act-B cells co-cultured with KO-act-BMMCs (from 4.1 ± 0.416 ng/mL to 27.1 ± 2.80 ng/mL) or with KO- α Gal-BMMCs (to 6.8 ± 1.20 ng/mL) in the presence of iNKT cells reduced the total IgE level secreted in the co-cultured media compared with those enhanced in act-B cells with WT-act-BMMCs (from 4.2 ± 0.18 ng/mL to 52.3 ± 5.42 ng/mL) and with WT- α Gal-BMMCs (to 35.0 ± 0.88 ng/mL), respectively (Fig. 4A). Non-act-B cells co-cultured with the corresponding KO-stimulated BMMCs also reduced total IgE versus those enhanced in non-act-B cells with the corresponding WT-stimulated BMMCs.

3.7. Effects of $CD1d^{-/-}$ on the CD40/CD40L expression in BMMCs

As the total and OVA-specific serum IgE level (in vivo) was reduced in KO mice more than those in other allergic responses observed in this experiment, we inferred that engagement of iNKT cell by $CD1d$ - α -Gal-pulsed B cells triggers IgE production through B cell Ig class switching via up-regulating CD40 in BMMCs in the presence of iNKT cells. WT- α Gal-BMMCs increased CD40L expression less than that in WT-act-BMMCs (Fig. 4B). KO- α Gal-BMMCs did not affect CD40L expression, KO-act-BMMCs remarkably reduced CD40L expression versus the WT-act-BMMCs. However, CD40 expression did not affect WT- or KO-BMMCs activated with each stimulus.

Next, using a co-culture system, non-act-B cells or act-B cells co-cultured with KO-act-BMMCs or with KO- α Gal-BMMCs reduced the CD40L expression compared with the enhancement seen in non-act-B cells and act-B cells co-cultured with the corresponding WT mice, respectively (Fig. 4C).

As $CD1d$ is expressed and has functional activity in BMMCs, we examined whether α -Gal presented to $CD1d$ on mast cell surface could interact with NKT cells through the secretion of IL-2. WT-act-BMMCs (from 54 ± 1.2 pg/mL to 112 ± 7.5 pg/mL) or WT- α Gal-BMMCs (to 125 ± 8.2 pg/mL) co-cultured with iNKT cell line (DN32.D3) increased IL-2 secreted into media, whereas KO-act-BMMCs (from 55 ± 1.2 pg/mL to 69 ± 2.7 pg/mL), KO- α Gal-BMMCs (to 52 ± 1.9 pg/mL) or KO-both-BMMCs (to 70 ± 5.7 pg/mL) co-cultured with iNKT remarkably reduced IL-2 secretion (Fig. 4D). However, all act-BMMCs co-cultured with variable NKT cells (TCB11 and IC8.DC1 cell lines) did not affect IL-2 secretion, and all WT-act-BMMCs cultured without iNKT cells weakly secreted IL-2 (data not shown). Co-culture cell ratio or optimal time in BMMCs and NKT was 1:5 and 15 h, respectively.

3.8. Effects of $CD1d^{-/-}$ on the co-localization of $CD1d$ and mast cells

Now, we examined whether $CD1d$ molecule was co-localized with mast cells in lung tissues by double staining. WT-OVA or WT- α Gal mice had an increase in the number of cells (merge, yellow color) expressing the c-kit (green color) and $CD1d$ (red color), whereas KO-OVA mice did not show any cells expressing them (Fig. 5A).

WT-OVA or WT- α Gal mice increased the co-localization of the c-kit and iNKT cells in lung tissues versus NC mice, but KO-OVA (Fig. 5B) or KO- α Gal mice (Supplementary Fig. 5) did not show any co-localization. iNKT (NK1.1) cells and $CD1d$ were also co-localized in the lung tissues of individual WT mice (Supplementary Fig. 4C). And, BM + KO-both mice restored the co-localization of c-kit/ $CD1d$, c-kit/iNKT or iNKT/ $CD1d$.

3.9. Relationship between mast cells and $CD1d$ in allergic asthma

As the number and the mediator release of mast cells, and IgE production were reduced in KO-BMMCs (in vitro) and KO mice (in vivo), we focused on the relationship between $CD1d$ and mast cells using the PCA test. KO-OVA mice suppressed the Fc ϵ RI-mediated

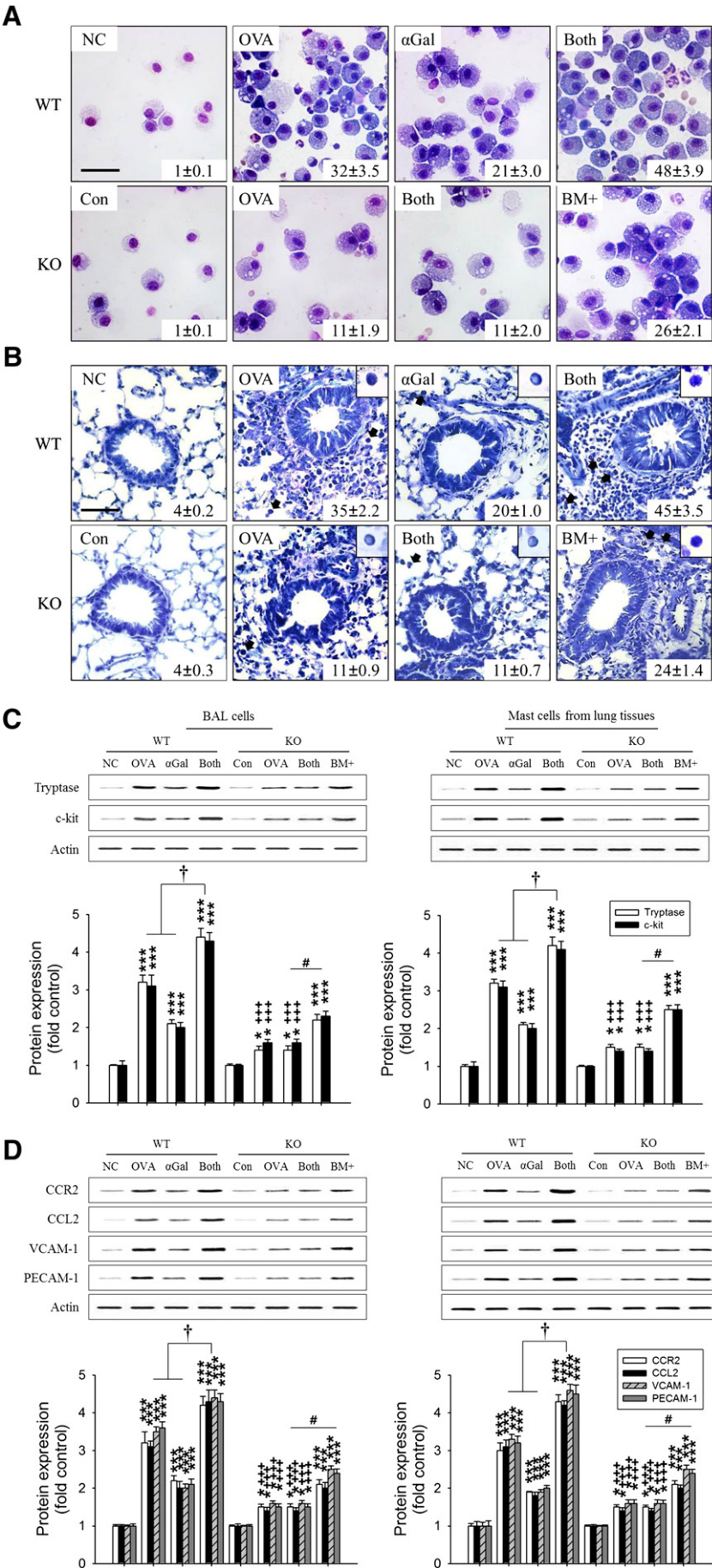


Table 1
Effect of CD1d^{-/-} on the total and OVA-specific serum IgE levels in OVA-challenged mice^a.

	Group	Total IgE Ab (ng/mL)	OVA-specific IgE Ab (ng/mL)
WT	NC	020 ± 5.4	.08 ± 0.1
	OVA	323 ± 12.5 ^{***}	245 ± 18.8 ^{***}
	αGal	070 ± 10.2 ^{***}	.13 ± 0.5
	Both	418 ± 22.3 ^{***,†}	270 ± 10.9 ^{***}
KO	Con	019 ± 3.2	.07 ± 0.1
	OVA	068 ± 9.2 ^{***,+++}	030 ± 1.4 ^{***,+++}
	αGal	020 ± 4.6 ⁺⁺⁺	.09 ± 0.3
	Both	070 ± 8.4 ^{***,+++}	032 ± 2.0 ^{***,+++}
	BM +	188 ± 7.3 ^{***,###}	154 ± 5.3 ^{***,###}

^a C57BL/6 mice were sensitized and/or challenged with Ag. NC or Con, WT and KO mice, respectively, sensitized and nebulized with PBS; WT-OVA or KO-OVA, WT and KO mice, respectively, sensitized and nebulized with OVA; WT-αGal or KO-αGal, WT and KO mice, respectively, nasally stimulated with α-Gal; WT-both or KO-both, WT and KO mice, respectively, nasally stimulated once with α-Gal right after nebulization with OVA; BM +, KO-both mice adaptively transferred with normal BMMCs 1 day before OVA sensitization. Total and OVA-specific serum IgE levels were determined using ELISA. Data are presented as the mean ± SEM (n = 8).

*** p < 0.001 versus NC or Con mice.

+++ p < 0.001 versus the corresponding WT mice.

† p < 0.05 versus WT-OVA mice.

p < 0.001 versus KO-both-mice.

reaction by 55% versus the WT-OVA mice (Fig. 5C). WT-αGal mice weakly increased the PCA reaction. BM + KO-both mice recovered the PCA reaction versus KO-both mice.

3.10. Responses in KO mice nasally stimulated with α-Gal

In order to avoid complexity and to reduce space in the figures, the data for KO-αGal mice were omitted in Fig. 2A and B, Fig. 5; Supplementary Fig. 2B–F; Supplementary Figs. 3 and 4 of text. However, we showed only the important data, such as population of mast cells in BAL cells and lung tissues, and infiltration of inflammatory cells, goblet cell hyperplasia, collagen deposition, co-localization of mast cells and c-kit or relationship between mast cells and CD1d (Supplementary Fig. 5), in BAL cells or lung tissues of KO-αGal mice. KO-αGal mice did not show any effects on all allergic responses checked above.

3.11. Effects of CD1d^{-/-} on the expression of TLR2 mRNA

As mast cells play an important role in host defense against pathogens via TLRs [32], we observed that TLR2 mRNA expression in KO-BMMCs or BAL cells of KO-OVA mice increased the same as those enhanced in WT-BMMCs and BAL cells of WT-OVA mice, respectively (Supplementary Fig. 4D). This implies that CD1d deficiency does not impair host defense against pathogens in mast cells.

4. Discussion

We demonstrated that CD1d expression which originally existed in mast cell surface is enhanced in FcεRI-derived responses, glycolipid Ag (α-Gal) is presented to CD1d (CD1d-lipid complexes) on the surface of mast cells or B cells, and then recognized by iTCR on the iNKT cells, and then CD1d-restricted iNKT cells on the mast cell surface is activated through Ras downstream signaling cascades, followed by the release of

granular histamine and newly synthesized LTs via PKCs and PLA₂ activities, and by expression of inflammatory cytokines, various remodeling and surface molecules via NF-κB/AP-1 activities, and that CD1d-restricted iNKT cells on the B cell surface enhance total and OVA-specific serum IgE production through B cell Ig class switching via up-regulating CD40L expressed in mast cell surface and cytokines produced in a variety cells of OVA-challenged mice, whereas KO (CD1d^{-/-})-BMMCs and KO mice or BMMC-transferred KO-both mice reduce and restore, respectively, all events. Therefore, our data suggest that CD1d may induce airway inflammation and tissue remodeling more than those caused by mast cells activated in OVA-challenged allergic responses.

CD1d-restricted iNKT cells play an important role in regulating the development of allergic asthma [14,33]. Mast cells, which are known as effector cells in the innate immune system [34,35] and as APCs for intrinsic Ag [36–38], activate a variety of kinases in IgE-mediated FcεRI signaling pathways [38,39]. However, there are no reports yet on the expression and a role of CD1d in mast cells.

Transcription factors NF-κB, which is degraded via I-κB phosphorylation and activated in the nucleus, and AP-1, which is activated by MAPKs [40,41], increase the expression of multiple inflammatory genes in mast cells and allergic asthma [25], followed by up-regulating the expression of inflammatory cytokines [25,41], adhesion molecules [42], and imbalance of MMPs/TIMPs [30,43]. However, there are no reports that CD1d is expressed via NF-κB and AP-1 activation in mast cell activation, except in glial cells [28]. Thus, our data suggest that CD1d expression may be enhanced by up-regulation of NF-κB/AP-1 in FcεRI-mediated mast cell activation. Our data also suggest that engagement of iNKT cells by CD1d/lipid-pulsed mast cells may enhance airway inflammatory and tissue remodeling responses, such as recruitment of inflammatory cells, goblet cell hyperplasia, collagen deposition and imbalance of MMPs and TIMPs, through enhancing the release of mediators (histamine, LTs and cytokines) via the activation of Ras downstream signaling molecules.

iNKT cells activated by α-Gal-pulsed B cells promote IgE production [13], and CD40L, which is expressed in mast cells [24,25,44], induces IgE production through B cell Ig isotype class switching via CD40/CD40L interaction in the presence of IL-4, IL-5, IL-9 and IL-3 [25,45–48]. Thus, our data suggest that CD1d may enhance IgE production, which is produced in the engagement of iNKT cells by CD1d-lipid complexes, through B cell Ig class switching via up-regulating CD40L expressed in the mast cell surface and cytokines (IL-4, IL-5 and IL-13), which are secreted in various inflammatory cells.

Migration of inflammatory cells from the blood into the airway occurs through the binding to specific adhesion molecules like VCAM-1 and chemokines such as RANTES, CCL5 and IL-8 [26,49]. Circulating mast cell progenitors (MCp) released from the bone marrow migrate into lung tissues via CD1d-restricted iNKT and become matured during allergic asthma development [22] or that mast cells generally migrate via chemokines (CCR2/CCL2 and IL-8) [50] and adhesion molecules (VCAM-1 and PECAM-1) [51]. And, we confirmed that mature BMMCs migrated into lung tissues one day after mouse tail vein injection (Supplementary Fig. 2A). Our data suggest that CD1d may induce migration of mast cells and other inflammatory cells into lung tissues via up-regulating the expression of chemokines and adhesion molecules, as demonstrated by the data that KO-BMMCs or KO mice

Fig. 3. Effects of CD1d^{-/-} on the population of mast cells, expression of mast cell surface molecules, chemokines and cell adhesion molecules in the BAL cells or lung tissues of OVA-challenged mice. C57BL/6 mice were sensitized and challenged with Ag. NC or Con, (negative control), WT or KO mice sensitized and nebulized with PBS; WT-OVA or KO-OVA, WT and KO mice, respectively, sensitized and nebulized with OVA; WT-αGal or KO-αGal, WT and KO mice, respectively, intranasal injected with α-Gal; WT-both or KO-both mice, WT and KO mice, respectively, intranasal injected once with α-Gal right after nebulization with OVA; BM + (BM + KO-both mice), KO-both mice adaptively transferred with normal BMMCs (1 × 10⁷ cells) 1 day before OVA sensitization. The data for KO-αGal mice were omitted in Fig. 3 because this group did not show any responses, but they were shown in Supplementary Fig. 5. Number of mast cells stained with May-Grünwald-Giemsa (magnification: ×400) were quantified at 5 sites of 200 μm × 200 μm areas under a microscope (5 sites/each slide × 8 mice = 40 sites), and then values were shown in the box. Bar in NC indicated 50 μm. Expression of mast cell markers, adhesion molecules was determined in the protein extracts from BAL cells or mast cells isolated from lung tissues using Western blot. (A and B) Population of mast cells in BAL cells and lung tissues, respectively (n = 8). (C) Expression of tryptase and c-kit. (D) Expression of chemokines or adhesion molecules. The histogram from the densitometry analysis for each band image was obtained as described in Fig. 1 legend (n = 4). *p < 0.05; ***p < 0.001 versus NC or Con mice. +++p < 0.001 versus the corresponding WT mice. †p < 0.05; versus WT-act-BMMCs. #p < 0.05 versus KO-both mice.

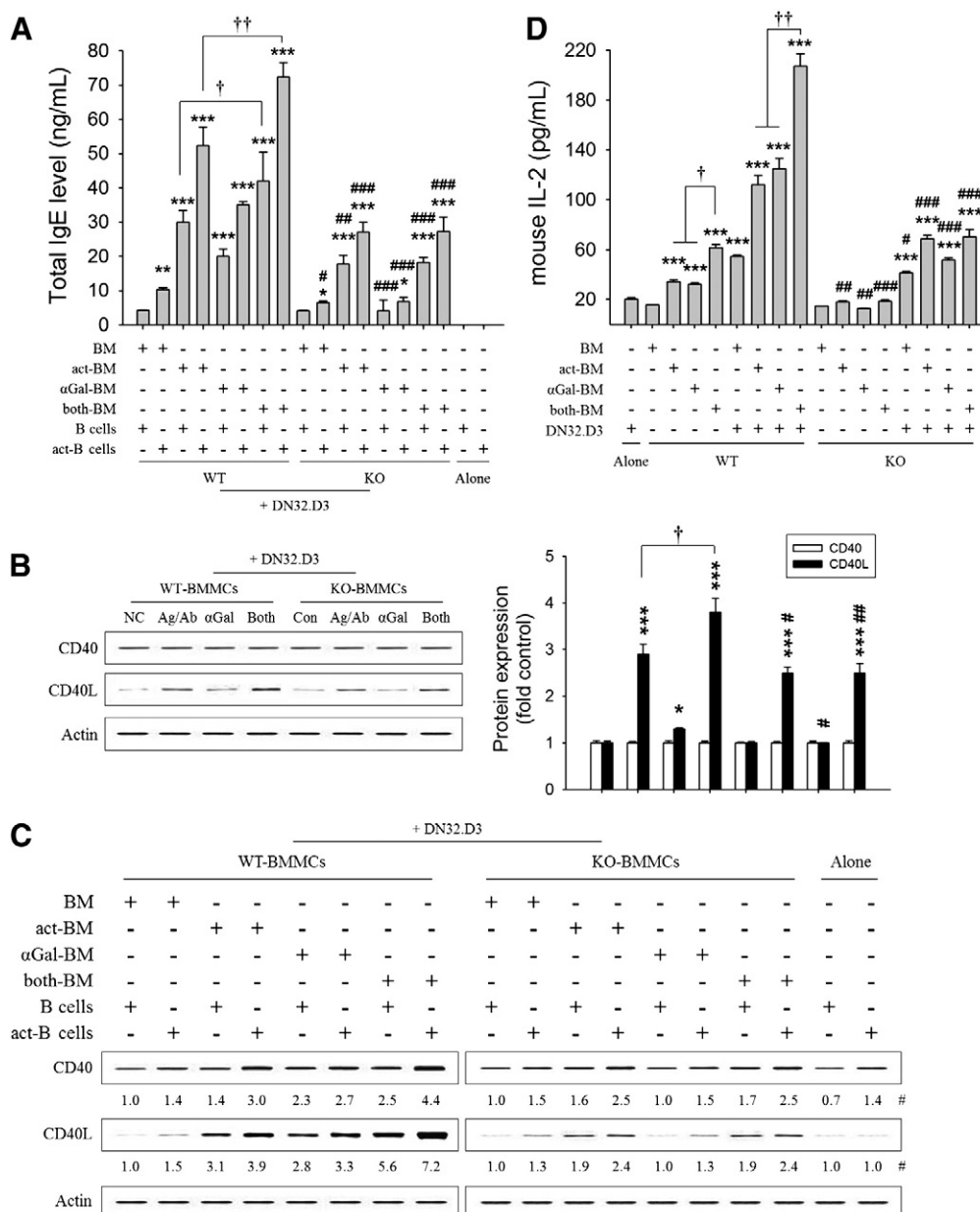


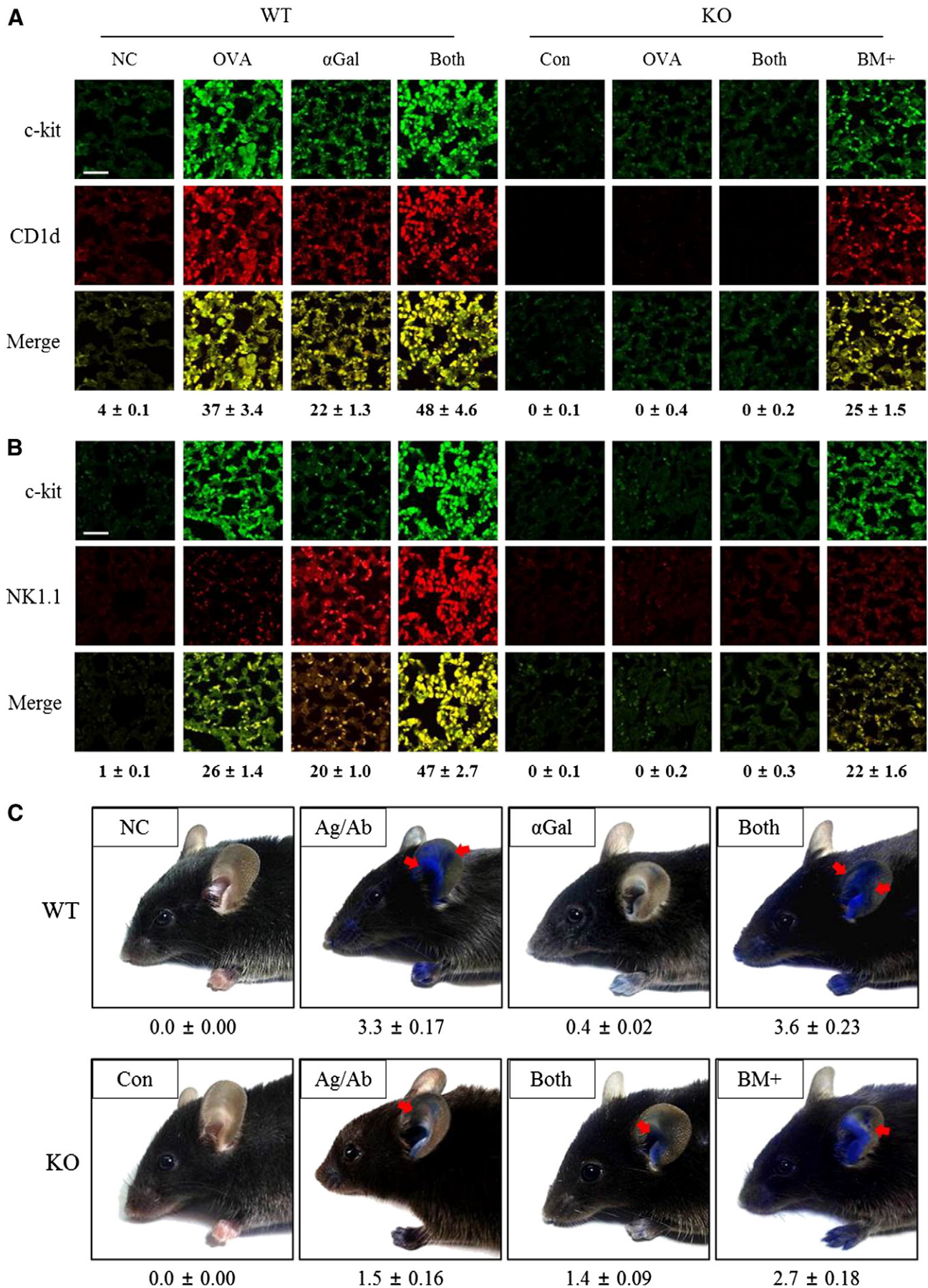
Fig. 4. Effects of CD1d^{-/-} on the total IgE Ab levels, expression of CD40 and CD40L or IL-2 secretion in B cells co-cultured with BMMCs in the presence of iNKT cells. Experimental details for BMMC activations, and definition of all symbols and abbreviations were described in Fig. 1 legend. B cells were co-cultured with act-BMMCs (activation for 72 h) for 15 h in the presence of iNKT cells (DN32.D3). Expression of CD40/CD40L was determined in the protein extracts from BMMCs using Western blot, and total IgE levels or IL-2 using ELISA. (A) Total IgE Ab levels produced in B cells co-cultured with BMMCs ($n = 8$). (B) Expression of CD40/CD40L. The histogram from the densitometry analysis for each band image was obtained as described in Fig. 1 legend ($n = 4$). (C) Expression of CD40/CD40L in B cell co-cultured with BMMCs. #, Numbers below bands show the mean ($n = 4$) for values obtained from the ratio of band density of each CD40 and CD40L versus those of control and actin from four independent experiments. (D) IL-2 levels secreted in iNKT cells co-cultured with BMMCs ($n = 8$). Co-culture cell ratio or optimal time in BMMCs and iNKT cells was 1:5 and 15 h, respectively. – or +, without and with the cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus NC or Con. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ versus the corresponding WT-BMMCs. † $p < 0.05$; †† $p < 0.001$ versus WT-act-BMMCs with non-act-/act-B cells or without/with iNKT.

reduced the expression of cytokines/chemokines (Fig. 2F; Supplementary Fig. 4B) and adhesion molecules (Fig. 3D) in BAL cells and mast cells from lung tissues.

Based on our observation that CD1d was enhanced in Fc ϵ R1-mediated responses, that iNKT cells co-cultured with WT- α Gal-BMMCs increased

IL-2 secretion, that KO mice dramatically reduced PCA reaction caused by Fc ϵ R1-derived mast cell activation [25,52] (Fig. 5C), and that all allergic responses were restored in the BMMC-transferred KO-both mice, our data can be inferred that CD1d expression enhanced in the surface of mast cells activated by the Ag/Ab reaction may act as APCs for intrinsic

Fig. 5. Effects of CD1d^{-/-} on co-localization of mast cells and CD1d in the lung tissues of OVA-challenged mice, and passive cutaneous anaphylaxis (PCA) test. Experimental details for the sensitization of WT or KO mice and definition of all symbols and abbreviations were described in Fig. 3 legend. Co-localization for c-kit and CD1d or iNKT (NK 1.1) cells in lung tissues was determined by immunofluorescence. (A) Co-localization (yellow color) of c-kit (green) and CD1d (red) in the lung tissues. The degree of Immunofluorescence color (yellow color) developed by co-localization was quantified by number of cells at 5 sites of 200 μ m $\times$ 200 μ m areas under a microscopy (5 areas/slide $\times$ 8 mice/group = 40 areas), and then the mean $\pm$ SEM for 40 areas was indicated as the values in the merge box below. Bar in group NC indicates 50 μ m. (B) Co-localization (yellow color) of c-kit (green) and iNKT cells (red) in lung tissues. (C) PCA test in WT or KO mice intravenously injected with 200 μ g of Ag (DNP-HSA) containing 3% Evans blue 24 h after intradermal injection of the ear with anti-DNP IgE Ab (0.5 μ g). Values in the box below show the intensity of the dye (blue color) extravasated in response to Ag (red colored-arrows). Data are presented as the mean $\pm$ SEM ($n = 8$). The data for KO- α Gal mice were omitted in Fig. 5 because this group did not show any responses, but they were shown in Supplementary Fig. 5.



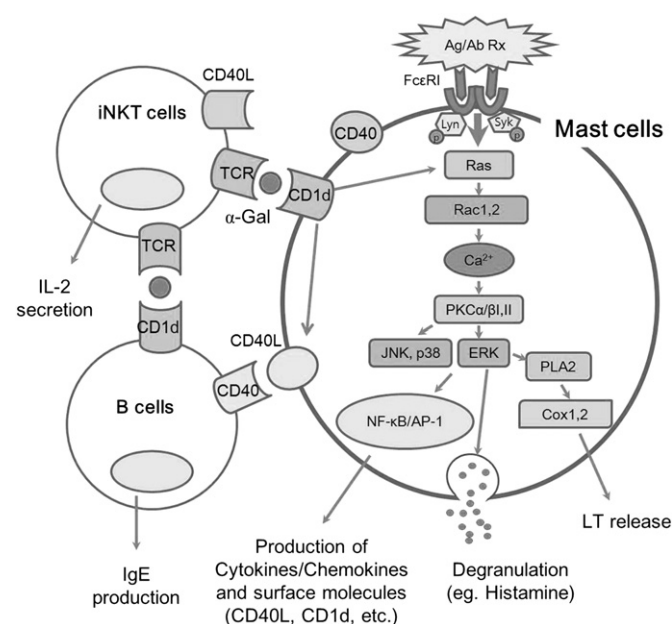


Fig. 6. A schematic diagram for signaling pathways in mast cells activated with the Ag/Ab reaction or α -Gal in the presence of iNKT cells in vivo and in vitro. The data suggest that mast cells activated with the Ag/Ab reaction induce the release/production of mediators (histamine, LTs and cytokines) and expression of surface molecules through downstream signaling pathways of Fc ϵ RI-associated Lyn/Syk kinases, such as Ras/Rac/Ca²⁺/PKCs/MAPKs/PLA₂/COX₂/NF- κ B-AP-1, in accordance with general signals. The data also show that the ligation of lipid Ag (α -Gal) to CD1d expressed in mast cells or B cells (glycolipid Ag/CD1d complexes) binds to iTCR on the iNKT cells, and then engagement of iNKT cells by these complexes may enhance IgE production, which is produced in the interaction of CD1d-restricted iNKT cells–B cells, through B cell Ig class switching via up-regulating the CD40L and the release/secretion of more mediators through signaling pathways in Ras downstream in Fc ϵ RI-mediated responses, and that these engagements may activate iNKT cells and secrete cytokine like IL-2. Therefore, it can be inferred that CD1d expressed in the surface of mast cells, which are known as a APCs of intrinsic Ags, may enhance airway inflammation and tissue remodeling via interaction with glycolipids, which may be secreted from various cell types, in OVA-induced allergic asthma in mice. However, which cell types secrete the various kinds of glycolipids needs to be further investigated.

Ag like iGb3 [53] or lysophospholipids [8], which can be secreted in inflammatory sites, and then the lipid Ag/CD1d ligation may be recognized by TCR on iNKT cells, and both cells (iNKT and mast cells) may induce bi-directional cross-talk, after which, the iNKT cells secrete IL-2, and mast cell activation via Ras, which is early kinase in Fc ϵ RI-mediated responses [39,54], may be activated more than Fc ϵ RI-mediated responses, followed by more secretion of mediators (histamine, LTs and cytokines) related to the development of allergic asthma. However, it needs to be studied more to investigate the interaction between mast cell-mediated CD1d and iNKT.

In conclusion, this report is the first study to demonstrate that binding of iNKT cells by CD1d- α -Gal complexes on the mast cell surface may enhance IgE production and the expression/activities of signaling molecules in the Fc ϵ RI-mediated responses. Our data suggest that CD1d expression on mast cell surface is enhanced via various signaling cascades in mast cells activated with the Ag/Ab reaction, α -Gal presented in CD1d is recognized by TCR on the iNKT cell membranes, and then the CD1d/ α -Gal/TCR ligation induces the release of IL-2, and that the CD1d/ α -Gal/iTCR ligation may enhance IgE production in B cells Ig class switching by up-regulating CD40L expression and the release of mediators through Ras downstream signaling cascades caused by Fc ϵ RI-mediated mast cell activation (Fig. 6). Thus, as CD1d may contribute to the pathogenesis of airway inflammation and tissue remodeling in OVA-induced mice, this finding suggests that a therapeutic strategy for allergic asthma that targets CD1d may work well. However, it needs to be investigated more which cell types secrete the different kinds of glycolipids.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cellsig.2014.01.029>.

Conflict of interest statement

The authors have no financial conflicts of interest.

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Supplementary data

CD1d expressed in mast cell surface enhances IgE production in B cells by up-regulating CD40L expression and mediator release in allergic asthma in mice

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Highlights: A role of CD1d on mast cells in allergic asthma

Key words: allergic asthma, CD1d, IgE, iNKT cells, mast cells.

Abbreviations: α -Gal, α -galactosylceramide; APC, antigen presenting cells; BAL, Bronchoalveolar lavage; BMMCs, Bone marrow-derived mast cells; CCL2, Chemokine ligand 2; CCR2, Chemokine receptor 2; CD1d^{-/-}, CD1d KO; LTs, leukotrienes; MMPs, Matrix metalloproteinases; OVA, Ovalbumin; PAS, Periodic acid-Schiff; PCA, Passive cutaneous anaphylaxis; PLA₂, Phospholipase A₂; Rx, Ag/Ab reaction; TIMPs, Tissue inhibitor metalloproteinases; TLR, Toll like receptor.

Materials and methods

Culture and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells obtained from femurs and tibias of WT and CD1d^{-/-} mice were cultured in RPMI-1640 supplemented 50% WEHI-3B conditioned media for 5 wks. The medium was changed every 2~3 days during cultured periods, and the cells were transferred to new plates to separate them from other adherent cells [24]. The purity for BMMCs using May-Grünwald-Giemsa staining was more than 95% of total cells.

Western blot analysis

BMMCs (1×10^6 cells), BAL cells (1×10^6 cells) or lung tissues (50 mg/50 μ L) were suspended in a low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μ g/mL aprotinin, 2 μ g/mL leupeptin] and allowed to swell on ice for 30 min. The cells or tissues were then homogenized using a Polytron homogenizer (Kinematica, Littau, Switzerland). After centrifugation, supernatants obtained from cells or tissue extracts were analyzed by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed with PBS containing 0.1% Tween 20 (PBST), and then blocked for 1 h in 5% skim milk in PBST. After the membranes were washed with PBST, they were incubated for 1 h at room temperature with primary Abs against actin, CD1d, Fc ϵ RI, Rho family, Ras, PKCs, MAPKs, PLA₂, COX-1, COX-2, EMBP, CD40, CD40L (Santa Cruz Biotechnology, Santa Cruz, CA), Lyn, Syk, CCR2, CCL2, PECAM-1, VCAM-1 (Abcam, Cambridge, MA) as well as p-Lyn, p-Syk, p-PKCs (α , β I and β II), p-ERK, p-JNK, p-p38 (Cell Signaling Technology, Beverly, MA) diluted with PBST (1:1,000). Membranes were washed with PBST and treated with HRP-conjugated goat anti-mouse or HRP-conjugated

rabbit anti-goat IgG or HRP-conjugated goat anti-rabbit IgG (diluted to 1:5,000~1:10,000) (Bethyl Laboratories, Montgomery, TX) in PBST for 1 h. After washing, the protein bands were visualized by ECL using a chemiluminometer (Amersham Biosciences, Buckinghamshire, UK) [25].

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BMMCs (1×10^6 cells), BAL cells (1×10^6 cells) or lung tissues (50 mg/500 μ L) using Trizol reagent (Invitrogen, Carlsbad, CA). RT-PCR was performed in a final volume of 50 μ L using an amfiRivert one-step RT-PCR kit (GenDEPOT, Barker, TX) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR assays were performed for 35 cycles. PCR products were analyzed using 1% agarose gel containing ethidium bromide (EtBr) (Sigma-Aldrich, St. Louis, MO) [25].

The primer sequences used were as follows: Fc ϵ RI sense, 5'-CTT AGG ATG TGG GTT CAG AAG CA-3; Fc ϵ RI anti-sense, 5'-GAC AGT GGA GAA TAC AAA TGT CA-3; Muc5ac sense, 5'-AAA GAC ACC AGT AGT CAC TCA GCA AA-3; Muc5ac anti-sense, 5'-CTG GGA AGT CAG TGT CAA ACC A-3; IL-1 β sense, 5'-TGG TGT GTG ACG TTC CCA TT-3; IL-1 β anti-sense, 5'-CCG ACA GCA ACG AGG CTT TT-3; IL-4 sense, 5'-TCG GCA TTT TGA ACG AGG TC-3; IL-4 anti-sense, 5'-GAA AAG CCC GAA AGA GTC TC-3; IL-5 sense, 5'-ATG GAG ATT CCC ATG AGC AC-3; IL-5 anti-sense, 5'-GTC TCT CCT CGC CAC ACT TC-3; IL-8 sense, 5'-CAA ACC TTT CCA CCC CAA AT-3; IL-8 anti-sense, 5'-ATT GCA TCT GGC AAC CCT AC-3; IL-13 sense, 5'-CAG CTC CCT GGT TCT CTC AC-3; IL-13 anti-sense, 5'-CCA CAC TCC ATA CCA TGC TG-3; TGF- β sense, 5'-CTC TCC ACC TGC AAG ACC AT-3; TGF- β anti-sense, 5'-CTG CCG TAC AAT TCC AGT GA-3; TNF- α sense, 5'-TTA TCT CTC AGC TCC ACG CC-3; TNF- α anti-sense, 5'-TGC

GCA CTG AAA GCA TGA TC-3; MMP2 sense, CAC ACC AGG TGA ACC ATG TG; MMP2 anti-sense, AGG GCT GCA TTG CAA ATA TC; MMP9 sense, GTA TGG TCG TGG CTC TAA GC; MMP9 anti-sense, AAA ACC CTC TTG GTC TGC GG; TIMP1 sense, CAC CAC CTT ATA CCA GCG TT; TIMP1 anti-sense, GTC ACT CTC CAG TTT GCA AG; TIMP2 sense, CCA GGT CCT TTT CAT CCT GA; TIMP2 anti-sense, TCC ATT CGC TGA AGT CTG TG; GAPDH sense, 5-GAT GCA GGG ATG ATG TTC TG-3; GAPDH anti-sense, 5-GTG AAG GTC GGT AAC GG-3.

GST effector pull-down assay

Small GTPase protein activities were assayed using EZ-Detect protein activation kits (Upstate Biotechnology, Lake Placid, NY). BMMCs (1×10^6 cells) were suspended in 0.5 mL of a lysis buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1% NP-40, 1 mM DTT, 5% glycerol, 1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin] for 30 min on ice, and supernatants were obtained by centrifugation ($13,000 \times g$ for 20 min). The active form of small GTPase proteins were obtained from the supernatants by affinity precipitation using Pak-1 protein binding domain (PBD), which was fused to GST, and visualized by Western blot analysis with anti-rabbit Rac1/2, cdc42 (1:1,000; PIERCE, Rockford, IL) [24].

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BMMCs (1×10^6 cells), BAL cells (1×10^6 cells) or lung tissues (50 mg/500 µL), and then EMSA were performed as described previously [25].

Ten µL of a mixture of NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5') or AP-1 (5'-CGC TTG ATG AGT CAG CCG

GAA-3', 3'-GCG AAC TAC TCA GTC GGC CTT-5') oligonucleotide (1.75 pmol/ μ L) (Promega Corporation), T4 polynucleotide kinase 10 X buffer, [α - ^{32} P]ATP (10 μ Ci; 3,000 Ci/mmol), nuclear-free water, and T4 polynucleotide kinase (5-10 U/ μ L) were incubated at 37 °C for 30 min. After adding 89 μ L Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column (Amersham Biosciences, Buckinghamshire, UK). The nuclear extract (4 μ L) and gel shift binding 5X buffer (6 μ L) [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50mM Tris-HCl (pH 7.5), 0.25 mg/mL poly (dI-dC)] were incubated at room temperature for 10 min, and then 20-30 fmol of ^{32} P-labeled NF- κ B or AP-1 oligonucleotide (9 μ L) was added, and incubated at room temperature for 20 min. After stopping the reaction, 1 μ L of 10 X gel loading buffer was added to each reaction. Reaction mixtures (20 μ L) were electrophoresed on 6% polyacrylamide gels, and the gels were analyzed using FLA-2000 (FUJIFILM, Tokyo, Japan) [25].

Measurements of leukotrienes (LTs)

The LTs contents in supernatants (50 μ L) isolated from act-BMMCs, gal-BMMCs or both-BMMCs activated for 1 h were determined using an ELISA kit (Cayman Chemical, Ann Arbor, MI). The results are expressed in pg/mL (1×10^6 cells). The concentrations of LTs were then calculated using the standard curves generated by specific LTs standards and analyzed using analysis tools on the Cayman Chemical website (<http://www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z>) [24]. The lowest detection limits for LTs mounts was 7.8 pg/mL.

Mast cell migration by bioluminescence images in vivo

To investigate the migration of mast cells in vivo, BMMCs were stained with 200 $\mu\text{g/mL}$ magnetic nanoparticle (MNP) for 24 h at 37°C using the Neo-LiveTM Magnoxide 675 (Biterials, Seoul, Korea), and then KO mice was nasally stimulated with α -Gal right after sensitization and nebulization with OVA as the same method as induction of allergic asthma one day after transplantation of MNP-labeled BMMCs (1×10^7 cells/100 mL) to mice through tail vein. As the transferred-BMMCs are migrated in lung tissues sufficiently, allergic asthma model in mice were prepared one day after injection. All mice were anesthetized with isoflurane (JW Pharm., Seoul, Korea), and then analyzed the images using the IVIS Lumina XR imaging system (Xenogen Corp., Alameda, CA) [27]. Photon flux was measured at base line by drawing a circle in lung area using the Living Image software (Xenogen Corp., Alameda, CA). Values were converted from total photon (p) flux to steradian ($\text{p/s/cm}^2/\text{sr}$) per lung area ($\mu\text{W/cm}^2$). The sr means steradian, s means sec, and μW means μwatt .

To confirm mast cell migration in lung tissues, lung tissues were fixed for paraffin section right after imaging analysis, and cut at 3 μm . The slides were mounted using aqueous mounting medium, and then BMMCs in lung tissues were analyzed using a LSM 5 EXCITER confocal microscope (Carl Zeiss, Oberkochen, Germany). MNP-labeled BMMCs were quantified at 5 sites of $200 \times 200 \mu\text{m}$ areas (5 sites/each slide $\times$ 6 mice = 30 sites) under a microscope [25].

Isolation of Bronchoalveolar lavage (BAL) fluid

Immediately after blood collection, thoracic cavities were carefully opened. After tracheas were exposed, BLA fluid was collected by cannulating the upper part of the trachea by lavaging twice with 1 mL and then 0.8 mL PBS (85–90% of the lavage volume was recovered). Lavaged samples from each mouse were kept on ice, and were centrifuged at 400

$\times g$ for 5 min at 4 °C. After centrifugation, the supernatants were separated for measurement of MMP 2/9 using zymography, pellets were resuspended in 100 μ L PBS, and total viable cell numbers were counted by Trypan blue exclusion using a hemocytometer. BAL cells were adjusted to a concentration of 5×10^4 cells/mL in PBS. For cytospin preparations, cells were centrifuged at $400 \times g$ for 3 min using a Cytospin III (Shandon, Pittsburgh, PA), and were stained with Diff-Quik (Sysmex corporation, Kobe, Japan) for different cell counting using standard morphological criteria [26].

To confirm numbers of mast cells, BAL cells were obtained from Cytospin, and lung tissues were fixed in 4% paraformaldehyde embedded in paraffin, and cut at 3 μ m. BAL cells or lung sections were stained with May-Grünwald-Giemsa solution (Merck, Darmstadt, Germany). Briefly, BAL cells or lung sections fixed in methanol for 2-3 min were stained with May-Grünwald solution for 15 min, and then dipped in Giemsa solution (Merck, Darmstadt, Germany) for 10 min. After washing with running tap water, dried slides were covered with mounting solution (Fisher Chemicals, Fair Lawn, NJ) and confirmed under a microscope. The number of mast cells were quantified at 5 sites of $200 \times 200 \mu$ m areas (5 areas/each slide $\times$ 8 mice/each group = 40 areas) [26].

Lung tissue histology

Lungs were perfused with 5 mL PBS via the right ventricle to wash out blood in lung tissues of mice. Left lung tissues were removed from the chest cavity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 μ m. Tissue sections were stained by H&E (Sigma-Aldrich, St. Louis, MO) to examine changes in general histology of lung tissues, by periodic acid-Schiff (PAS) (Sigma-Aldrich, St. Louis, MO) to examine goblet cell hyperplasia in the bronchial epithelium, by Masson's trichrome (Sigma-Aldrich, St. Louis,

MO) to examine collagen deposition, by May-Grünwald Giemsa (Merck, Darmstadt, Germany) to investigate the number of mast cells [26].

Histological changes for H&E and collagen, and the percentage of PAS-positive cells were performed as described previously [26].

Total Collagen measurement

Collagen levels in lung tissue homogenates were assessed by the Sircol collagen assay (Biocolor Ltd., Carrickfergus, UK) according to the manufacturer's instructions. Absolute collagen content was calculated by comparing the sample values to a pertinent standard curve [25].

Measurement of zymography

Activities of matrix metalloproteinases (MMP-2/9) in BALF were analyzed as described previously [30]. The first BALF samples (35 μ L) collected were loaded onto 7.5% polyacrylamide gels containing 10 mg/mL gelatin (Sigma-Aldrich, St. Louis, MO), in the presence of SDS under non-reducing conditions. After electrophoresis, gels were washed three times for each 15 min in 20 mM Tris/HCl (pH 7.8) containing 2.5% Triton X-100, and then repeatedly washed twice for 15 min in developing buffer [20 mM Tris/HCl (pH 7.8), 1% Triton X-100, 10mM CaCl_2 , and 5 μ M ZnCl_2]. After washing, gels were incubated in developing buffer at 37 °C for 18 h, stained with 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA), destained in a solution of 10% acetic acid containing 40% methanol, dried, and analyzed with a LAS1000 Image Reader (Fuji film, Tokyo, Japan). Gelatinolytic activity was detected as a clear band against a blue background. Human MMP-2

and MMP-9 (Chemicon, Temecula, CA) were used as gelatinase zymography standard markers.

Measurement of total and OVA-specific serum IgE or IL-2

The supernatants (200 μ L) were isolated from act-B cells co-cultured with or without act-BMMCs in the presence of iNKT cells. To obtain serum, blood samples collected by cardiac puncture immediately after anesthesia were centrifuged at $550 \times g$ for 30 min after clotting at room temperature. IgE amounts in the supernatants or sera were analyzed using ELISA [25]. Briefly, for total IgE or OVA-specific IgE in supernatants or serum, microplates were coated with purified rat anti-mouse IgE, and then treated with supernatants or mouse sera followed by total IgE (Bethy Laboratories, Montgomery, TX) or biotinylated OVA which is prepared with a peroxidase-conjugated goat anti-biotin Ab, respectively, which was used as secondary Ab. Mouse IgE (Bethy Laboratories, Montgomery, TX) was used for calibrating the assay. Reactions were read using an ELISA plate reader at 450 nm.

Supplementary Figure Legends

Supplementary Fig. 1. Dose responses or time courses for expression of CD1d, signaling molecules and Ca^{2+} influx in WT- α Gal- or WT-act-BMMCs in the presence of iNKT cells. BMMCs (1×10^6 cells) from WT mice were activated with 100 ng/mL α -Gal or 5.0 ng/mL DNP-HSA and 0.1 $\mu\text{g/mL}$ anti-DNP IgE Ab (Ag/Ab) (refer to WT- α Gal-BMMCs and WT-act-BMMCs, respectively) for the indicated time periods and the doses. Expression of CD1d and intracellular signaling molecules was determined in the protein extracts isolated from WT- α Gal- or WT-act-BMMCs using Western blots. $[\text{Ca}^{2+}]_i$ level was analyzed by confocal microscopy. Specific Ras inhibitor (75 μM Farnesylthiosalicylate, FTS), Rac inhibitor (100 μM NSC23766, NSC) or Ca^{2+} channel blocker (50 μM SKF96365, SKF) was pretreated 10 min before α -Gal stimulation or Ag challenge. (A) Dose- and time-dependent expression of CD1d in WT-gal-BMMCs. (B) Dose- and time-dependent expression of CD1d in WT-act-BMMCs or WT-both-BMMCs. (C) Expression of CD1d in WT-BMMCs without iNKT cells. (D) Time-dependent expression for Ras and Rac1/2 in WT-act- or WT- α Gal-BMMCs. (E) Expression of Ras and Rac1/2 after individual inhibitor pretreatment. (F) $[\text{Ca}^{2+}]_i$ level after individual inhibitor pretreatment in WT-both-BMMCs. NC, negative control; Rx, Ag/Ab reaction; α Gal, α -galactosylceramide. *, Numbers below bands show the mean ($n = 4$) for values expressed from the ratio of band density of each CD1d, Ras or Rac1/2 versus those of the control and actin or total protein from four independent experiments. *** $p < 0.001$ versus NC; +++ $p < 0.001$ versus WT-both-BMMCs.

Supplementary Fig. 2. Effects of CD1d^{-/-} on the mast cell migration, expression of CD1d, recruitment of inflammatory cells into BAL fluid, and histopathological changes in lung tissues of OVA-challenged mice. Experimental details for the sensitization of WT or KO mice,

and definition of symbols and abbreviations were described in the Fig. 3 legend. The MNP-labeled BMMCs (1×10^7 cells/100 μ L) migrated in lung tissues was analyzed in whole mice one day after injection using Bioluminescence imaging, and then mast cells migrated into lung tissues (section 3 μ m) were isolated and analyzed using Confocal microscope (5 sites/each slide $\times$ 6 mice = 30 sites). Values (p/s/cm²/sr) for representative image of mice and the number of mast cells (magnification, $\times$ 400) were shown in box ($n = 6$). Bar in Con image indicates 50 μ m, and white color arrow indicates mast cells. The expression of CD1d was determined in the protein extracts from BAL cells and lung tissues using Western blot. BAL cells separated using cytopsin were stained with Diff-Quick for counting by standard morphological criteria. Lung tissues were stained with H&E (magnification: $\times$ 200) for recruitment of inflammatory cells, with PAS (magnification: $\times$ 200) for goblet cell hyperplasia, with Masson's trichrome (magnification: $\times$ 200) for collagen deposition. Histological appearances for inflammatory cells (black dot) or collagen deposition (blue color) were scored for the presence of peribronchial and perivascular inflammation. The number of goblet hyperplasia were quantified as the percentage of PAS-positive cells/total cells at 5 sites of 200 μ m $\times$ 200 μ m areas (5 sites/each slide $\times$ 8 mice/group = 40 sites). Arrow in figure indicates goblet cells (pink color). (A) Bioluminescence image (upper column, circle in figure) for BMMCs migrated in the lung areas of whole body KO mice injected with MNP-labeled BMMCs, and the number of mast cells migrated in lung tissues (lower column). (B) CD1d expression. *, Numbers below bands were obtained as described in the Supplementary Fig. 1 legend ($n = 4$). (C) Numbers of individual inflammatory cells recruited in BAL fluid. (D)-(F) H&E, PAS and Masson's trichrome staining, respectively. Bar in NC indicates 100 μ m. Data are presented as the mean $\pm$ SEM ($n = 8$). ** $p < 0.01$; *** $p < 0.001$ versus NC or Con mice. + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$ versus the corresponding WT mice, respectively. † $p < 0.05$ versus WT-OVA mice. # $p < 0.05$ versus KO-both mice.

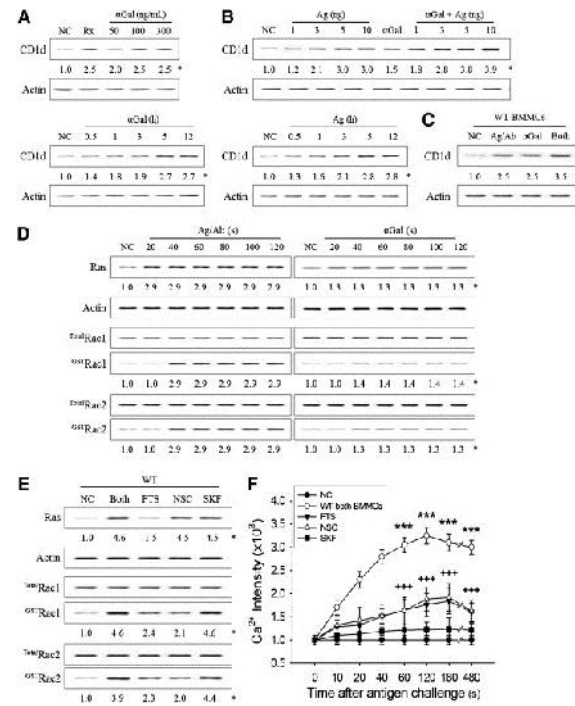
Supplementary Fig. 3. Effects of CD1d^{-/-} on the expression of EMBP, Muc5ac and MMPs/TIMPs, total collagen amounts in BAL cells or lung tissues of OVA-challenged mice. Experimental details for the sensitization of WT or KO mice, and definition of all symbols and abbreviations were described in the Fig. 3 legend. The expression of EMBP, Muc5ac mRNA or MMPs/TIMPs mRNA was determined in the protein or mRNA extracts isolated from the BAL cells and lung tissues using Western blot and RT-PCR, respectively. Collagen amounts in lung tissue homogenates were determined by the Sircol collagen assay. Gelatinolytic MMP 2/9 activities were detected as a clear band against a blue background. (A) Expression of EMBP. (B) Expression of Muc5ac mRNA. *, Numbers below bands were obtained as described in the Supplementary Fig. 1 legend (*n* = 4). (C) Total collagen amounts in lung tissues. (D) Gelatinolytic MMP 2/9 activities in BAL fluid. * and **, Numbers below bands were obtained as described in the Supplementary Fig. 1 legend (*n* = 4). (E) Expression of MMP 2/9 and TIMP 1/2 mRNA, respectively. The histogram from densitometry analysis shows the mean ± SEM (*n* = 4) expressed from ratio of each band density versus those of control and GAPDH from four independent experiments. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 versus NC or Con mice. [†]*p* < 0.05; ⁺⁺⁺*p* < 0.001 versus the corresponding WT mice, respectively. [†]*p* < 0.05 versus WT-OVA mice. [#]*p* < 0.05; ^{##}*p* < 0.01 versus KO-both mice.

Supplementary Fig. 4. Effects of CD1d^{-/-} on the activities of transcription factors, expression of cytokine mRNA or co-localization of iNKT and CD1d in the BAL cells or lung tissues of OVA-challenged mice. Experimental details for the sensitization of WT or CD1d^{-/-} mice, BMDCs stimulation and definition of all symbols and abbreviations were described in the Fig. 3 and Fig. 1 legends. Activities of transcription factors and expression of cytokine and

TLR2 mRNA were determined in the nuclear or mRNA extracts isolated from BAL cells and lung tissues using EMSA and RT-PCR respectively. (A) Activities of NF- κ B and AP-1. *, Numbers below bands were obtained as described in the Supplementary Fig. 1 legend ($n = 4$). (B) Expression of cytokine mRNA. (C) Co-localization (yellow color) of iNKT cells (green) and CD1d (red) in lung tissues. The degree of Immunofluorescence color (yellow) developed by co-localization (iNKT and CD1d) was quantified by numbers of cells at 5 sites of $200\ \mu\text{m} \times 200\ \mu\text{m}$ areas under a microscope (5 areas/each slide $\times$ 8 mice/each group = 40 areas), and then the mean $\pm$ SEM for 40 areas was indicated as values in the merge box below. Bar in group NC indicates $50\ \mu\text{m}$. (D) Expression of TLR2 in the individual BMMCs (left panel) and BAL cells (right panel).

Supplementary Fig. 5. The integrated responses in KO mice injected with α -Gal. Experimental details for the sensitization of KO mice, number of mast cells, H&E staining, goblet cell hyperplasia, collagen deposition, co-localization of mast cells and CD1d or NK1.1, or relationship between mast cells and CD1d in BAL cells or lung tissues of allergic mice were described in Fig. 3 legend and Supplementary Fig. 2 legend. (A) Population of mast cells in BAL cells and lung tissues, respectively. Bar in group NC indicates $50\ \mu\text{m}$. (B-D) H&E, PAS and Masson's trichrome staining, respectively. Bar in group NC indicates $100\ \mu\text{m}$. (E) PCA test in KO- α Gal mice intravenously injected with $200\ \mu\text{g}$ of Ag (DNP-HSA) containing 3% Evans blue 24 h after intradermal injection of the ear with anti-DNP IgE Ab ($0.5\ \mu\text{g}$). Values in the box show the intensity of the dye (blue color) extravasated in response to Ag, but not show any color. (F and G) Co-localization (yellow color) of c-kit (red color) and CD1d (green color) or NK1.1 (green color) in lung tissues, respectively. The degree of Immunofluorescence color developed by co-localization was quantified by numbers of cells

at 5 sites of 200 μm x 200 μm areas under a microscopy (5 areas/each slide x 8 mice/each group = 40 areas), and then the mean $\pm$ SEM for 40 areas was indicated as the values in the merge box below. Bar in group NC indicates 50 μm . Data are presented as the mean $\pm$ SEM ($n = 8$). KO- αGal mice did not show any effects on allergic symptoms. Thus, the data were shown in Supplementary figure to avoid complexity and to reduce space.



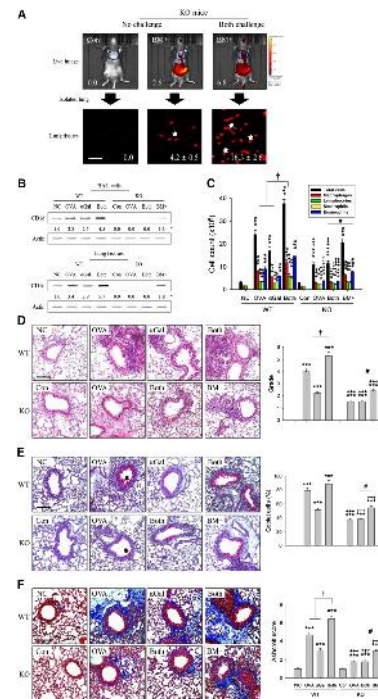
Supplementary Fig. 1. Dose responses or time courses for expression of CD1d, signaling molecules and Ca²⁺ influx in WT-αGal- or WT-act-BMMCs in the presence of iNKT cells. BMMCs (1 × 10⁶ cells) from WT mice were activated with 100 ng/mL α-Gal or 5.0 ng/mL DNP...

Gwan Ui Hong, Nam Goo Kim, Tae Jin Kim, Jai Youl Ro

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Cellular Signalling, Volume 26, Issue 5, 2014, 1105–1117

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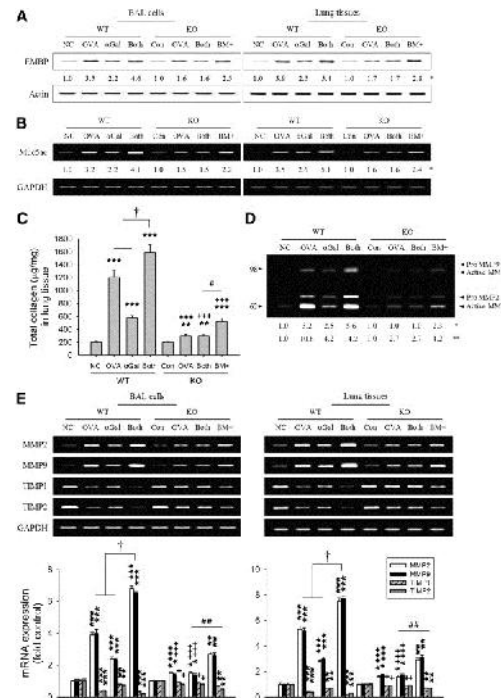
Supplementary Fig. 2. Effects of CD1d^{-/-} on the mast cell migration, expression of CD1d, recruitment of inflammatory cells into BAL fluid, and histopathological changes in lung tissues of OVA-challenged mice. Experimental details for the sensitization of WT or...

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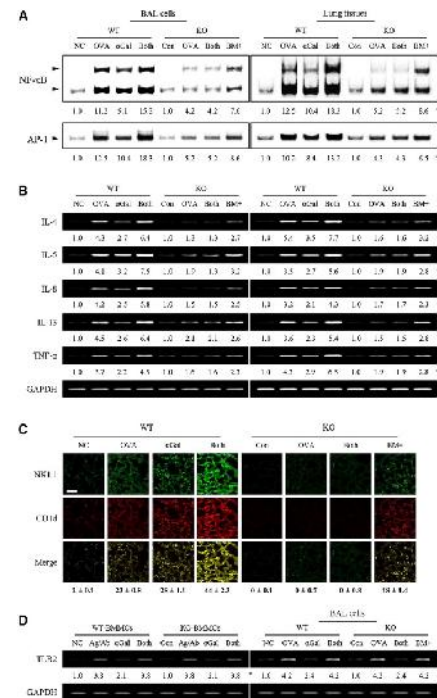
Supplementary Fig. 3. Effects of CD1d^{-/-} on the expression of EMBP, Muc5ac and MMPs/TIMPs, total collagen amounts in BAL cells or lung tissues of OVA-challenged mice. Experimental details for the sensitization of WT or KO mice, and definition of all symbols an...

Gwan Ui Hong, Nam Goo Kim, Tae Jin Kim, Jai Youl Ro

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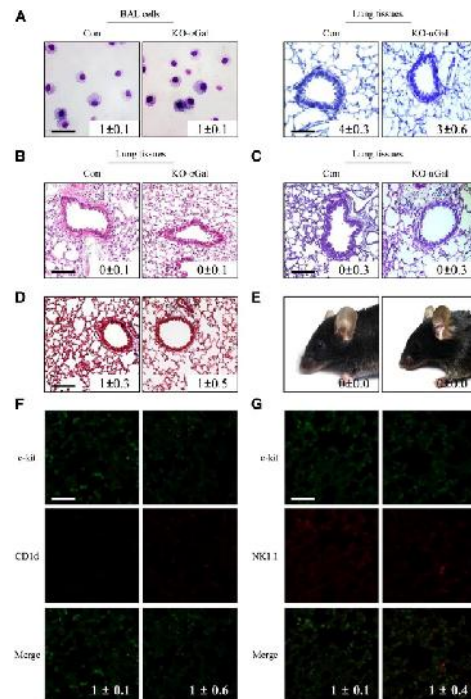
Supplementary Fig. 4. Effects of CD1d^{-/-} on the activities of transcription factors, expression of cytokine mRNA or co-localization of iNKT and CD1d in the BAL cells or lung tissues of OVA-challenged mice. Experimental details for the sensitization of WT or CD...

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