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Deficiency of Phospholipase A2 Receptor Exacerbates Ovalbumin-Induced Lung Inflammation

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Secretory phospholipase A2 (sPLA2) plays a critical role in the genesis of lung inflammation through proinflammatory eicosanoids. A previous in vitro experiment showed a possible role of cell surface receptor for sPLA2 (PLA2R) in the clearance of extracellular sPLA2. PLA2R and groups IB and X sPLA2 are expressed in the lung. This study examined a pathogenic role of PLA2R in airway inflammation using PLA2R-deficient (PLA2R−/−) mice. Airway inflammation was induced by immunosensitization with OVA. Compared with wild-type (PLA2R+/+) mice, PLA2R−/− mice had a significantly greater infiltration of inflammatory cells around the airways, higher levels of groups IB and X sPLA2, eicosanoids, and Th2 cytokines, and higher numbers of eosinophils and neutrophils in bronchoalveolar lavage fluid after OVA treatment. In PLA2R−/− mice, intratracheally instilled [125I]-labeled sPLA2-IB was cleared much more slowly from bronchoalveolar lavage fluid compared with PLA2R+/+ mice. The degradation of the instilled [125I]-labeled sPLA2-IB, as assessed by trichloroacetic acid-soluble radioactivity in bronchoalveolar lavage fluid after instillation, was lower in PLA2R−/− mice than in PLA2R+/+ mice. In conclusion, PLA2R deficiency increased sPLA2-IB and -X levels in the lung through their impaired clearance from the lung, leading to exaggeration of lung inflammation induced by OVA treatment in a murine model. The Journal of Immunology, 2013, 191: 1021–1028.

It has been shown that eicosanoids, including leukotrienes (LT) and PGs, have crucial roles in the pathogenesis of airway inflammation (1–4). These eicosanoids are 5-lipoxygenase and cyclooxygenase metabolites of arachidonic acids, and the cellular availability of arachidonic acid is tightly controlled by hydrolysis of membrane phospholipids via the catalytic activity of phospholipases A2 (PLA2) (5–10). Previous animal and human studies showed that PLA2 is involved in the genesis of airway inflammation through proinflammatory eicosanoids (11–15).

A number of PLA2 subtypes have been identified and classified into different families based on their biochemical features and primary structures (5–10, 16). Mammalian PLA2 enzymes are classified into three main categories: intracellular cytosolic PLA2 (cPLA2), Ca2+-independent PLA2 (iPLA2), and secretory PLA2 (sPLA2), comprising 6 cPLA2, 9 iPLA2, and 11 sPLA2 (5–10, 16). sPLA2 possess several characteristic features, including a low molecular mass (typically, 13–18 kDa) and an absolute catalytic requirement for millimolar concentrations of Ca2+ (5–10, 16).

Two decades ago, a cell surface receptor for sPLA2-IB (phospholipase A2 receptor 1; PLA2R) was discovered (17). PLA2R, a so-called M-type PLA2 receptor, is a type I transmembrane glycoprotein with a molecular mass of 180 kDa and is composed of a large extracellular portion consisting of an N-terminal cysteine-rich region, a fibronectin-like type II domain, a tandem repeat of eight carbohydrate-recognition domains (CRDs) and, a short intracellular C-terminal region (10, 16, 18, 19). Three of the CRD-like domains (CRDs 3 to 5) are responsible for sPLA2 binding (10, 16, 18, 19). Among sPLA2 isozymes, mouse PLA2R has a high affinity to sPLA2-IB, -IIA, -IIE, -IIF, and -X (18, 19). The intracellular C-terminal region contains a consensus sequence motif of coated pit-mediated-endocytosis that was originally identified in the low-density lipoprotein receptor (18–21). Previous in vitro studies have demonstrated that sPLA2-IB and sPLA2-X are internalized and degraded via the lysosomal pathway after their binding to PLA2R, implicating a possible role of the receptor in the clearance of extracellular sPLA2 (18–22). sPLA2-IB and sPLA2-X are expressed in the lung (11, 12, 23). The animal study using sPLA2−/−deficient mice showed that sPLA2-X produced proinflammatory eicosanoids and Th2 cytokines and played a critical role in genesis of allergen-induced airway inflammation (12). The human study indicated that sPLA2-X could have a role in the generation of proinflammatory eicosanoids in the airways, leading to the development of bronchial hyperresponsiveness (11). In addition, sPLA2−/− was found to elicit the production of proinflammatory eicosanoids in the lung and the contraction of airway smooth muscles (24).

PLA2R is highly expressed in the lung, kidney, and spleen (10, 18, 25); however, the possible pathologic roles of PLA2R remain largely unknown in these organs. Therefore, this study examined...
the pathogenic role of PLA2R in OVA-induced airway inflammation using PLA2R-deficient mice.

Materials and Methods

Materials

Rat monoclonal anti-PLA2R Ab was prepared as described in a previous report (25). Rabbit polyclonal anti-smooth muscle α-actin (αSMA) Ab and rabbit polyclonal anti-cytoketatin Ab were purchased from Abcam (Cambridge, U.K.). Recombinant mouse sPLA2-X was prepared as described previously (22). Recombinant human sPLA2-IB was obtained from R&D systems (Minneapolis, MN). Sodium [125I] iodine (carrier-free, 3.7 GBq/ml) was purchased from Perkin Elmer (Boston, MA). ELISA kits for determination of IL-4 and IL-5 were obtained from R&D systems; the ELISA kit for IFN-γ was obtained from Raybiotech (Norcross, GA); the ELISA kits for LTβR, cysletukotrienes (CysLTs), PGD2, and PGE2 were obtained from Cayman Chemical (Ann Arbor, MI); the ELISA kit for 15-deoxy-Delta 12,14-PGJ2 (15d-PGJ2) (15d-PGJ2) was obtained from Enzo Life Science (Farmingdale, NY); and ELISA kits for sPLA2-IB and sPLA2-X were obtained from Cusabio Biotech (Wuhan, China). Cell culture reagents were obtained from Sigma-Aldrich (Tokyo, Japan) and Invitrogen (Carlsbad, CA). Other chemicals were purchased from Sigma-Aldrich unless indicated otherwise.

Mice

The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (approval reference no. 19-35), and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (8th Edition, 2011). Details regarding the generation and characterization of PLA2R−/− mice (systemically deficient in PLA2R) were described previously (25, 26). PLA2R−/− female mice with a C57BL/6d background from F12 to 15 (6–8 wk old, 14 to 18 g) were used in the current study. The littermates of the wild-type (PLA2R+/+) females served as a control group.

Induction of allergic airway inflammation with OVA treatment

Mice were treated with i.p. injection of 10 μg of chicken egg OVA (Sigma-Aldrich) and 1.125 mg of adjuvant aluminum potassium sulfate (Sigma-Aldrich) emulsified in 0.2 ml of sterile saline at days 0, 7, and 14. On days 21 to 23, mice were treated with intranasal administration of 50 μg of OVA in 0.05 ml saline after anesthesia with i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine. Control groups were treated with saline instead of OVA, otherwise similar to that described above. On day 24, the mice were euthanized by cutting the left renal artery after anesthesia with i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine. Immediately after death, the lungs were lavaged for the experiments for assays of bronchoalveolar lavage fluid (BALF). Thereafter, the lungs were removed for histological analysis. In another set of experiments, the lungs were removed immediately after the euthanasia for biochemical analysis. In some mice, citrated blood was obtained by intracardiac puncture under the anesthesia.

Bronchoalveolar lavage (BAL)

The trachea was exposed and catheterized with a 24-gauge polypropylene endotracheal tube. The lungs were lavaged 8 times with 0.5 ml of PBS to provide 4.0 ml of BALF (23). The BALF was collected by gentle aspiration. For each animal, 90% (3.6 ml) of the total injected volume was consistently recovered. The collected BALF was combined and cooled to 4˚C. The BALF was centrifuged at 300 g for 20 min and the supernatant of the BALF harvested 4 h after instillation was precipitated with 10% trichloroacetic acid (TCA), and the TCA-soluble radioactivity was measured. Supernatants were stored at −80˚C until assayed.

Lung histology

The trachea was exposed and catheterized as described before. Next, paraformaldehyde (4%) in PBS was instilled into the lung via the endotracheal tube with the use of a syringe at low pressure. Upper and lower lobes of the left lung were removed and fixed for 24 h in 4% paraformaldehyde. The tissues were embedded in paraffin, cut in 7-μm sections, and placed on slides. Histologic examination of the lung was determined by staining with H&E and periodic acid–Schiff (PAS). Peribronchial infiltration of inflammatory cells was graded on a semiquantitative scale, from 0 to 4; grade 0 was designated as no detectable inflammation; grade 1 was given when the bronchus was surrounded by a few inflammatory cells; grade 2 was assigned when the bronchus was surrounded by a layer one cell deep; grade 3 was given when the bronchus was surrounded by a layer two to four cells deep; grade 4 was assigned when the bronchus was surrounded by a layer more than four cells deep. The number of PAS-positive mucus-containing cells was determined as the percentage of total airway epithelial cells in each airway examined. For lung morphometry, five airways per section were randomly selected from five sections (from two upper lobes and three lower lobes) per mouse and examined at a magnification of ×400. Each mouse had a score determined by the mean of the individual scores of 25 airways. These histologic measurements were conducted by two investigators (H.M., K.W.) who were blinded to the study protocol for the mice. The interobserver and intraobserver variabilities of repeated measurements of 25 airways were determined and were 4.4 ± 0.5%, respectively, for the grading of peribronchial infiltration of inflammatory cells and 2.6 ± 0.3% and 3.2 ± 0.4%, respectively, for the percentage of PAS-positive mucus-containing cells, indicating high reproducibility in both measurements.

Immunostaining of lungs and cultured cells

The lung was instilled with 50% OCT (Tissue-Tek; Sakura Finetek, Tokyo, Japan) in PBS through the endotracheal tube. Thereafter, lungs were harvested, immediately embedded in OCT, frozen, cut into 5-μm sections and postfixed in acetone. For immunostaining, endogenous peroxidase was inactivated by incubating with 0.3% hydrogen peroxide for 30 min. After washing, the sections were blocked with 10% BSA, and then incubated with the primary Ab followed by a peroxidase-conjugated secondary Ab (Histone Simple Stain; Nichirei Bioscience, Tokyo, Japan). To develop the brown coloration, the sections were treated with 3′-diaminobenzidine tetrahydrochloride, a peroxidase substrate (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. The primary Abs included a rat monoclonal anti-PLA2R Ab (25), a rabbit polyclonal anti-αSMA Ab, and a rabbit polyclonal anti-cytoketatin Ab. Some sections were incubated with normal rabbit, mouse, or goat IgG instead of primary Ab as negative controls. For double immunofluorescence staining of the lung, the sections were incubated with the indicated primary Abs followed by a secondary Ab with Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, Carlsbad, CA) (23).

Cultures of airway smooth muscle (ASM) cells (see below) in eight-well chamber slides were washed with PBS and fixed with methanol for 10 min at −20˚C. Next, the cultured cells were stained as described above. Nuclei were stained with DAPI (Vector Laboratories). Images were acquired with an Olympus Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan) and processed using FV10-ASW software version 1.0 (Olympus).

In vivo clearance of sPLA2 from the lungs

Iodination of sPLA2-IB was performed with PD-10 columns (GE Healthcare, Amersham, U.K.) (25), yielding a specific radioactivity of 500 cpm/fmol. After anesthesia by i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine, the trachea was exposed and catheterized with a polypropylene 24-gauge endotracheal tube. [125I]-Labeled sPLA2-IB (150 ng) was instilled into the lung through the endotracheal tube. After the tracheal instillation, the cervical incision was closed with a 5-0 silk suture, and the mice were returned to their cages. All treated mice recovered rapidly after surgery. After anesthesia with ketamine and xylazine as described earlier, the lungs were lavaged to collect BALF 5, 30, or 60 min or 2 and 4 h after the instillation. BALF was spun at 300 × g for 10 min to pellet cells, and the radioactivity in the cell-free supernatant of the BALF was measured with a γ counter (AccuFLEX γ7000; Aloka, Tokyo, Japan). The cell-free supernatant of the BALF harvested 4 h after instillation was precipitated with 10% trichloroacetic acid (TCA), and the TCA-soluble radioactivity was measured.

Assays of cytokines, eicosanoids, and sPLA2

Cytokines and eicosanoids were measured in BALF supernatants using ELISA kits according to the manufacturer’s instructions. The plasma concentrations of sPLA2-IB and sPLA2-X were also measured. The enzymatic activity of sPLA2 in the culture medium was assayed using the sPLA2 Assay Kit (Cayman Chemical) exactly as recommended by the manufacturer. The eicosanoid levels in the culture medium of ASM cells and A549 cells (DS Pharma Biological, Osaka, Japan), a cell line of human alveolar basal epithelial cells, after incubation with or without sPLA2-IB were measured using ELISA kits. Expression levels of mRNA of cytokines in cultures of ASM cells and A549 cells were measured with real-time PCR.
Real-time PCR

Total RNA was extracted from the lungs or cultures of cells with a Qiagen RNeasy kit and DNase I (Qiagen, Hilden, Germany). The mRNA expression levels were quantified with a two-step real-time PCR assay using SYBR Green I chemistry and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR primers are listed in Supplemental Table I. The GAPDH housekeeping gene was used to normalize gene expression.

Cultures of bone marrow-derived mast cells, Th2 cells, and ASM cells

Bone marrow–derived mast cells (BMMCs) were obtained by cultivation of bone marrow cells in RPMI 1640 medium containing IL-3 (5 ng/ml; Peprotech, Rocky Hill, NJ) and 10% FBS (27). After 3 wk, staining with May-Giemsa and toluidine blue showed that >95% of the cells were BMMCs. Th2 cells were generated from CD4+ T cells (3). CD4+ T cells were incubated with 5 nM [125I]-labeled sPLA2-IB for 2.5 h at 4˚C, for 10 min at 4˚C. After washing with PBS, the cells’ associated radioactivity was treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) containing 10% FBS and 100 g/ml penicillin/streptomycin. The ex-plants were incubated in a 5% CO2 chamber at 37˚C. Explanted tracheas were removed when the outgrowing cells became locally confluent. Once the dish became confluent, the cells were passaged. All examinations were performed on confluent cells at matched passage numbers (3 to 6). The immunohistochemical analysis showed that >95% of these cells were oSMA-positive cells.

Production of PGD2 and Th2 cytokines in cultures of BMMCs and Th2 cells

This study examined whether the constituent cells of the inflamed lung were capable of producing PGD2 and Th2 cytokines in response to sPLA2-IB. BMMCs were stimulated with sPLA2-IB. After 30 min, the levels of PGD2 in the culture medium were measured using ELISA kits (Cayman Chemical). Th2 cells were stimulated by various concentration of sPLA2-IB. After 6 h of culture, Th2 cells were isolated by positive selection using a mouse IL-4 Secretion Assay (Miltenyi Biotec). ASM cells were cultured from explants of tracheas. The entire tracheas were split longitudinally and dissected into 2–3-mm squares. All segments were placed in a sterile Petri dish with DMEM containing 10% FBS and 100 g/ml penicillin/streptomycin. The ex-plants were incubated in a 5% CO2 chamber at 37˚C. Explanted tracheas were removed when the outgrowing cells became locally confluent. Once the dish became confluent, the cells were passaged. All examinations were performed on confluent cells at matched passage numbers (3 to 6). The immunohistochemical analysis showed that >95% of these cells were oSMA-positive cells.

Internalization and degradation of sPLA2-IB in cultured ASM cells

Cultures of ASM cells on 24-well culture plates were incubated for 2.5 h at 37˚C with 5 nM [125I]-labeled sPLA2-IB in binding medium (HBSS containing 0.1% BSA) in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB (22). The supernatants were removed, and the cells were treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) for 10 min at 4˚C. After washing with PBS, the cells’ associated radioactivity was measured after solubilization of the cells by 1 N NaOH by a γ counter. For the assessment of sPLA2-IB degradation, the cells were incubated with 5 nM [125I]-labeled sPLA2-IB for 2.5 h at 4˚C, washed with PBS, resuspended in binding medium, and incubated for the indicated times at 37˚C (22). At the end of incubation, the supernatants were removed and precipitated with 10% TCA, and the TCA-soluble radioactivity was measured with a γ counter. To examine the effects of PLA2-R on the enzymatic activity of sPLA2 added to the culture medium, ASM cells in 24-well culture plates were incubated with mouse sPLA2-X (3 g/ml; US Biochemicals, Cleveland, OH) at 37˚C for the indicated times. After incubation, the supernatant was collected by centrifugation of the culture medium at 1000 x g for 5 min at 4˚C, and the enzymatic activity of sPLA2-X in the supernatant was measured. Among sPLA2:isozymes with a high affinity for PLA2-R, sPLA2-X was selected because of its potent enzymatic activity (6).

Statistical analysis

Unless otherwise stated, all data are expressed as means ± SE. Statistical comparisons of responses were performed using Student t test. When more than two groups were compared, a one-way ANOVA was performed followed by a Sheffe test for post hoc comparison of group means. The serial changes were compared between two genotyped mice using two-way ANOVA for repeated measures followed by post hoc testing with a Scheffe test. The results were accepted as significantly different when p < 0.05.

Results

Histologic analysis of OVA-induced airway inflammation

Compared with saline-treated mice, both PLA2-R−/− and wild-type (PLA2-R+/+ mice after OVA treatment showed an infiltration of inflammatory cells around the airways and blood vessels and hypersecretion of mucus in the airways (Fig. 1A). These histologic changes in the lung after OVA treatment were greater in PLA2-R−/− mice than in PLA2-R+/+ mice (Fig. 1B, 1C).

Expression of PLA2-R in PLA2-R−/− lung

In PLA2-R+/+ mice, the immunohistochemical analysis revealed strong immunoreactivity of PLA2-R in the circumference of airway and fainly in the alveolar wall in the lung after OVA treatment (Fig. 2A–D). The negative control staining had no immunoreactivity to PLA2-R (data not shown). Confocal immunofluorescence microscopy showed that PLA2-R immunoreactivity was colocalized in airway smooth muscle expressing oSMA and in alveolar epithelial cells expressing cytokeratin in PLA2-R+/+ lung (Fig. 2E–L).

Comparison of mRNA expression levels of PLA2-R and sPLA2 in the lungs and the number of inflammatory cells and levels of sPLA2, eicosanoids, and Th2 cytokines in both PLA2-R+/+ and PLA2-R−/− mice

In PLA2-R−/− mice, mRNA levels of PLA2-R in lung tissue were not changed significantly after OVA treatment compared with saline treatment (Fig. 3A). There were no significant differences in the mRNA expression levels of sPLA2-IB, -IIIE, and -X in lung tissue after OVA treatment between the two genotyped mice (Fig. 3B–D). sPLA2-IIIF expression was not detectable in OVA-treated lung in the two genotyped mice (data not shown). Compared with saline treatment, BALF after OVA treatment showed an increase in the number of eosinophils, neutrophils, and lymphocytes, the levels of sPLA2-IB and -X, eicosanoids (LTB4, CysLTs, and PGD2), Th2 cytokines (IL-4 and IL-5) in both PLA2-R+/+ and PLA2-R−/− mice (Fig. 3E–N). These increased levels in BALF after OVA treatment were greater in PLA2-R−/− lungs than in PLA2-R+/+ lungs (Fig. 3E–N). IFN-γ levels in BALF were not changed significantly after OVA treatment, and the IFN-γ levels were similar between PLA2-R−/− mice and PLA2-R+/+ mice after treatment with saline or OVA (Fig. 3O). There was no detectable plasma levels of sPLA2-IB and -X in both PLA2-R−/− and PLA2-R+/+ mice after treatment with either saline or OVA (data not shown).

In vivo clearance of sPLA2-IB instilled into the lungs

To determine the in vivo kinetics of sPLA2 clearance from the lungs, [125I]-labeled sPLA2-IB was instilled intratracheally into the lung. In PLA2-R−/− mice, the instilled [125I]-labeled sPLA2-IB was cleared much more slowly from the BALF, as 50% of [125I]-labeled sPLA2-IB remained in the BALF after 120 min (Fig. 4A). In contrast, in PLA2-R+/+ mice, only 10% of [125I]-labeled sPLA2-IB remained after 120 min. Four hours after instillation, degradation of the instilled [125I]-labeled sPLA2-IB, as assessed by TCA-soluble radioactivity in BALF, was inhibited in PLA2-R−/− mice compared with PLA2-R+/+ mice (Fig. 4B).

In vitro clearance of sPLA2-IB in cultures of ASM cells

Immunohistochemical analysis showed that PLA2-R protein was expressed in cultured ASM cells obtained from PLA2-R+/+ mice but not in PLA2-R−/− mice (data not shown). The levels of cell-associated [125I]-labeled sPLA2-IB 2.5 h after the incubation at 37˚C was significantly lower in cultures of ASM cells from PLA2-R−/− lung than those from PLA2-R+/+ lung (Fig. 4C). The TCA-soluble radioactivity in the culture medium 90 and 120 min after the
incubation at 37˚C was significantly lower in cultures of ASM cells from PLA2R<sup>2</sup>/2 lung compared with those from PLA2R+/+ lung (Fig. 4D). The residual enzymatic activity of sPLA2-X exogenously added to the culture medium gradually decreased in cultures of ASM cells from PLA2R+/+ lung, whereas the sPLA2-X activity was barely changed in those from PLA2R<sup>2</sup>/2 lung (Fig. 4E).

**FIGURE 1.** Comparison of histological changes in the lung after OVA-treatment between PLA2R<sup>2</sup>/2 and PLA2R+/+ mice. (A) Representative pictures of H&E (upper panels) or PAS (lower panels) staining of lungs after OVA treatment. Scale bars in H&E staining, 200 μm; scale bars in PAS staining, 50 μm. Sections are representative of five mice. (B) Semiquantitative analysis of the severity of peribronchial inflammation. Peribronchial inflammation was scored on a scale of 0–4; grade 0 was designated as no detectable inflammation, grade 1 was given when the bronchus was surrounded by a few inflammatory cells; grade 2 was assigned when the bronchus was surrounded by a layer one cell deep; grade 3 was given when the bronchus was surrounded by a layer 2–4 cells deep; grade 4 was assigned when the bronchus was surrounded by a layer more than four cells deep. (C) The number of PAS-positive, mucus-containing cells, expressed as a percentage of total airway epithelial cells in each airway. In each mouse, 25 airways per mouse were randomly selected and examined at a magnification of ×400. n = 7 mice in each experiment. *p < 0.05, †p < 0.05 compared with the respective genotype after saline treatment.+/+ denotes PLA2R+/+ mice and /2 denotes PLA2R<sup>2</sup>/2 mice.

**FIGURE 2.** Expression of PLA2R in the lungs after OVA-treatment. (A–D) Representative immunostaining images of lungs from PLA2R<sup>+/+</sup> mice after treatment with saline (A) or OVA (C) and lung from PLA2R<sup>2</sup>/2 mice after saline (B) or OVA treatment (D) using an Ab against PLA2R (brown). (F–H and J–L) Representative immunofluorescence images of lung from PLA2R<sup>+/+</sup> mice after OVA treatment using Abs against PLA2R (F and J, green), αSMA (G, red), PLA2R and αSMA (H), cytokeratin (K, red) or PLA2R and cytokeratin (L). (E and I) H&E staining of lung (blue rectangular area) corresponding to that in images of F–H and J–L, respectively. Scale bars in panels (A)–(D), (E), (F)–(H), (I), and (J)–(L), were 50, 200, 20, 200, and 20 μm, respectively. Sections are representative of five mice.
Production of PGD2 and Th2 cytokines in BMMCs and Th2 cells

This study examined whether the constituent cells of the inflamed lung were capable of producing PGD2 and Th2 cytokines in response to sPLA2-IB. The addition of sPLA2-IB significantly increased PGD2 levels in the culture medium of BMMCs (Fig. 5A). The addition of PGD2 to Th2 cells significantly increased levels of Th2 cytokines IL-4 and IL-5 levels in the culture medium (Fig. 5B).

Production of eicosanoids and cytokines in cultures of ASM cells and A549 cells in response to sPLA2-IB

This study examined whether there is a difference in production of anti-inflammatory or proinflammatory eicosanoids and cytokines in response to sPLA2-IB in ASM cells through PLA2R-mediated mechanisms. Among eicosanoids and cytokines that we examined (28), there was not significant increase in their levels in ASM cells from either PLA2R+/− mice or PLA2R+/+ mice (Supplemental Figs. 1A, 2A), except that levels of PGD2 and PGE2 increased at 100 nM of human sPLA2-IB in culture of ASM cells from PLA2R+/− mice (Supplemental Fig. 1A). The levels of PGD2 and PGE2 in the culture medium of ASM cells from PLA2R+/− mice did not change in response to the same dose of catalytically inactive human sPLA2-IB pretreated for 4 h with bromophenylacylbromide (1 mM; Sigma-Aldrich), a blocker of sPLA2-IB activity (Supplemental Fig. 1B). Similarly, sPLA2-IB did not induce either eicosanoid or cytokine levels in cultures of A549 cells, a cell line of human alveolar basal epithelial cells that express PLA2R (Supplemental Fig. 1C and Fig. 2B).

Discussion

This study demonstrated that the OVA-induced airway inflammation, characterized by more eosinophils and neutrophils in
with 5 nM \([^{125}I]\)-labeled sPLA2-IB for 2.5 h at 4˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB, washed with PBS, and incubated for the corresponding time point.

The data at each point are expressed as a percentage of that observed 5 min (100%) after the instillation. *\(p < 0.05\) compared with PLA2R\(^{2/2}\) mice at the corresponding time point. \(n = 6\) mice in each experiment. (B) TCA-soluble radioactivity in BALF 4 h after the instillation of \([^{125}I]\)-labeled sPLA2-IB. \(n = 6\) mice in each experiment. *\(p < 0.05\). (C and D) Internalization and degradation of sPLA2-IB in cultured ASM cells. (C) Cell-associated radioactivity after incubation of cultures of ASM cells with 5 nM \([^{125}I]\)-labeled sPLA2-IB for 2.5 h at 37˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB. The specific cell-associated radioactivity is shown after correcting for nonspecific association. *\(p < 0.05\). (D) Cultures of ASM cells were incubated with 5 nM \([^{125}I]\)-labeled sPLA2-IB for 2.5 h at 4˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB, washed with PBS, and incubated for 15, 30, 60, 90, and 120 min at 37˚C. The TCA-soluble radioactivity in the culture medium is shown after correcting for nonspecific degradation. *\(p < 0.05\) compared with PLA2R\(^{2/2}\) ASM cells at the corresponding time point. (E) Serial changes in residual enzymatic activities of sPLA2-X in the culture medium of ASM cells were measured after its addition to the culture medium. *\(p < 0.05\) compared with PLA2R\(^{2/2}\) ASM cells at the corresponding time point. \(n = 6\) mice in each experiment. \(^{+/+}\) denotes PLA2R\(^{+/+}\) mice and \(^{2/2}\) denotes PLA2R\(^{2/2}\) mice.

FIGURE 4. Clearance of instilled sPLA2-IB from the lung in vivo and internalization and degradation of sPLA2-IB in cultured ASM cells. (A and B) \([^{125}I]\)-Labeled sPLA2-IB (150 ng) was instilled into the lung. (A) Radioactivity in BALF 5, 30, 60, and 120 min after instillation of \([^{125}I]\)-labeled sPLA2-IB. The data at each point are expressed as a percentage of that observed 5 min (100%) after the instillation. *\(p < 0.05\) compared with PLA2R\(^{2/2}\) mice at the corresponding time point. \(n = 6\) mice in each experiment. (B) TCA-soluble radioactivity in BALF 4 h after the instillation of \([^{125}I]\)-labeled sPLA2-IB. \(n = 6\) mice in each experiment. *\(p < 0.05\). (C and D) Internalization and degradation of sPLA2-IB in cultured ASM cells. (C) Cell-associated radioactivity after incubation of cultures of ASM cells with 5 nM \([^{125}I]\)-labeled sPLA2-IB for 2.5 h at 37˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB. The specific cell-associated radioactivity is shown after correcting for nonspecific association. *\(p < 0.05\). (D) Cultures of ASM cells were incubated with 5 nM \([^{125}I]\)-labeled sPLA2-IB for 2.5 h at 4˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB, washed with PBS, and incubated for 15, 30, 60, 90, and 120 min at 37˚C. The TCA-soluble radioactivity in the culture medium is shown after correcting for nonspecific degradation. *\(p < 0.05\) compared with PLA2R\(^{2/2}\) ASM cells at the corresponding time point. (E) Serial changes in residual enzymatic activities of sPLA2-X in the culture medium of ASM cells were measured after its addition to the culture medium. *\(p < 0.05\) compared with PLA2R\(^{2/2}\) ASM cells at the corresponding time point. \(n = 6\) mice in each experiment. \(^{+/+}\) denotes PLA2R\(^{+/+}\) mice and \(^{2/2}\) denotes PLA2R\(^{2/2}\) mice.

BALF and inflammatory histologic changes in the airway, was stronger in PLA2R\(^{2/2}\) mice than in PLA2R\(^{+/+}\) mice. PLA2R\(^{2/2}\) mice had higher levels of sPLA2-IB and sPLA2-X in BALF after OVA treatment than did PLA2R\(^{+/+}\) mice. These higher levels were associated with greater levels of eicosanoids and Th2 cytokines in BALF from PLA2R\(^{2/2}\) mice than from PLA2R\(^{+/+}\) mice. Previous animal and human studies showed that sPLA2 have an important role in the genesis of airway inflammation through the production of proinflammatory eicosanoids in a variety of inflammatory cells (11–15). Eicosanoids including CysLTs, LTBa2, and PGD2 cause lung inflammation directly or indirectly through induction of Th2 cytokines (1–4). In line with these previous reports, the present in vitro study showed that sPLA2-IB increased PGD2 in mast cells and that PGD2 induced Th2 cytokines (IL-4 and IL-5) (4). In line with these previous reports, the present in vitro study showed that sPLA2-IB increased PGD2 in mast cells and that PGD2 induced Th2 cytokines (IL-4 and IL-5). Together, the results suggest that the greater increase in sPLA2-IB and sPLA2-X levels can result in higher levels of proinflammatory eicosanoids and Th2 cytokines, leading to enhanced inflammation in the airway after OVA treatment in PLA2R\(^{2/2}\) mice compared with PLA2R\(^{+/+}\) mice.

In agreement with previous reports (10, 18, 19, 21, 22), the current study showed that cultures of ASM cells, expressing PLA2R, internalized and degraded sPLA2-IB. After the exogenous addition of sPLA2-X, the residual enzymatic activity of sPLA2-X decreased more slowly in the culture medium of ASM cells from PLA2R\(^{2/2}\) mice than from PLA2R\(^{+/+}\) mice. In line with these in vitro studies, the present in vivo experiments showed that intratracheally instilled sPLA2-IB decreased much more slowly in BALF of PLA2R\(^{2/2}\) mice than that of PLA2R\(^{+/+}\) mice. In addition, after the instillation of \([^{125}I]\)-labeled sPLA2-IB, TCA-soluble radioactivity in BALF was lower in PLA2R\(^{2/2}\) lungs than PLA2R\(^{+/+}\) lungs, indicating lower rates of degradation of sPLA2-IB in PLA2R\(^{2/2}\) liver than PLA2R\(^{+/+}\) lung. These data suggested that PLA2-R played a role in the clearance of sPLA2 from the lung and that a deficiency of PLA2-R resulted in the increase in PLA2 levels through impaired clearance of sPLA2. That result in turn appears to have led to exacerbation of PLA2-mediated inflammation of the airway in PLA2R\(^{2/2}\) mice. This scenario needs to be confirmed by additional experiments examining whether the increase in the OVA-induced inflammation of lung can be reversed by re-expression of PLA2-R in a PLA2R\(^{2/2}\) lung.

Mouse PLA2-R has a high affinity for sPLA2-IB, -IIA, -IIE, -IFF, and -X (18, 19, 22). A previous report showed that sPLA2-IB and sPLA2-X are expressed in the bronchial epithelium and alveolar epithelial cells or infiltrated inflammatory cells (23). The increase in lung expression of sPLA2-IB and sPLA2-X mRNAs after OVA treatment was similar in PLA2R\(^{+/+}\) and PLA2R\(^{2/2}\) mice. Thus, in

FIGURE 5. PGD2, IL-4, and IL-5 production in BMMCs and Th2 cells. (A) PGD2 levels in culture media of BMMCs 30 min after stimulation with sPLA2-IB. (B) IL-4 and IL-5 levels in culture media of Th2 cells 4 h after stimulation with PGD2. \(n = 6\) in each experiment. *\(p < 0.05\) versus control.
the two genotypes, there might not be a difference in the production of these sPLA2 in OVA-treated lung. The low clearance of sPLA2 (rather than an increase in their production) could partly account for relatively higher levels of these sPLA2 in BALF of PLA2R+/− mice compared with those of PLA2R−/−. It was unlikely that levels of sPLA2-IB and sPLA2-X in BALF were derived from plasma because their levels were not detectable in plasma in both genotyped mice after treatment with either saline or OVA. sPLA2-IE is expressed in lung alveolar cells as reported previously (29). Although lung expression of sPLA2-IE was weak in the current study, we cannot rule out the possibility that sPLA2-IE has a role in the increase in OVA-mediated inflammation in the lungs of PLA2R−/− mice. sPLA2-IEF was not detectable in OVA-treated lungs. sPLA2-IIA is naturally disrupted by a frameshift mutation in C57BL/6J background mice (30). Thus, sPLA2-IIA and IIF were unlikely to contribute to the difference in the lung inflammation between the two genotypes. Although the present and previous in vitro experiments using cultured cells indicated that PLA2R internalizes and degrades sPLA2 (22), a previous in vivo experiment failed to show the clearance effect of PLA2R on sPLA2-IB in systemic circulation (26). In this regard, the current study clearly showed that PLA2R acted as a sink for sPLA2 in the lung and that PLA2R might protect against sPLA2-mediated airway inflammation through clearance of sPLA2 in vivo. Moreover, it is known that PLA2R is expressed in a variety of cells and organs, including the kidney (18, 25, 31). It remains to be determined whether PLA2R also expresses in a variety of cells and organs, including the kidney (32). Cytokines were produced in response to sPLA2-IB in ASM cells in the current study did not find any evidence that eicosanoids and anti-inflammatory molecules or suppression of the production of proinflammatory molecules.

Transduce specific intracellular signals that lead to production of PLA2R-mediated intracellular transduction signals can participate in the cross talk between sPLA2-IB and cPLA2 (37). However, the current study did not find any evidence that eicosanoids and cytokines were produced in response to sPLA2-IB in ASM cells through PLA2R-mediated mechanisms. We cannot rule out the possible effects of a putative signal transduction system on the present findings in PLA2R−/− mice; however, the cytoplasmic tail of PLA2R does not seem to transduce any specific signals other than internalization signals on the basis of its sequence (18–21). The precise mechanisms by which PLA2R inhibited lung inflammation are still unclear. It remains undefined whether PLA2R acts solely as a sink of sPLA2 and whether PLA2R can directly transduce specific intracellular signals that lead to production of anti-inflammatory molecules or suppression of the production of proinflammatory molecules.

In conclusion, in a mouse model of OVA-mediated lung inflammation, PLA2R deficiency increased sPLA2-IB and sPLA2-X levels in the lung through their impaired clearance from the lung, leading to exaggeration of lung inflammation. PLA2R might play a protective role in the pathogenesis of lung inflammation.

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Disclosures
Y.Y. and K.H. are employees of Shionogi Research Laboratories, Shionogi and Co., Ltd. (Osaka, Japan). They contributed to the development of PLA2R mice and an mAb against PLA2R, but they did not contribute to the study design and the data analysis. The remaining authors have no financial conflicts of interest.

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## Supplemental Table 1. Sequences of PCR primers

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The eicosanoids levels in the culture medium of cells after incubation with sPLA2-IB.

(A), The eicosanoids levels in the culture medium of airway smooth muscle (ASM) cells after incubation for 1 h with or without 10 nM or 100 nM of human sPLA2-IB. +/+ denotes ASM cells from PLA2R+/+ mice and -/- denotes ASM cells from PLA2R-/- mice. n = 6-8 mice in each experiment. *p < 0.05; †p < 0.05 compared with control in ASM cells from PLA2R-/- mice.

(B), ASM cells were incubated with same dose of human sPLA2-IB pre-treated for 4 hr with bromophenylacylbromide (1 mM), a blocker of sPLA2-IB activity.

(C), The eicosanoids levels in the culture medium of A549 cells, a cell line of human alveolar basal epithelial cells after incubation with sPLA2-IB.
Supplemental Figure 2

(A), The cytokine mRNA levels in ASM cells after incubation for 6 hr with or without 10 nM or 100 nM of human sPLA₂-IB. +/+ denotes ASM cells from PLA2R<sup>+/+</sup> mice and -/- denotes ASM cells from PLA2R<sup>−/−</sup> mice. n = 6 mice in each experiment. n.d. = not detected. The mRNA levels were normalized to GAPDH mRNA expression and were expressed relative to the control of ASM cells from PLA2R<sup>+/+</sup> mice (= 1).

(B), The cytokine mRNA levels in A549 cells after incubation 6 hr with or without 10 nM or 100 nM of human sPLA₂-IB. n = 6-8 in each experiment. The data were expressed relative to the control (= 1).