

Mast cells play a key role in the development of late airway hyperresponsiveness through TNF- α in a murine model of asthma

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We have investigated the role of TNF- α in mast cell-mediated late airway hyperresponsiveness (AHR) using mast cell-deficient WBB6F1-W/W^v (W/W^v) mice in a murine model of asthma, which exhibits a biphasic increase in AHR. TNF- α levels in the airway and magnitude of late AHR in response to airway allergen challenge were severely impaired in W/W^v mice compared to their littermates. In addition to TNF- α , cytosolic phospholipase A₂ (cPLA₂) phosphorylation and enzymatic activity in the lungs were also impaired in W/W^v mice. Either anti-TNF- α antibody or an inhibitor of cPLA₂ abolished late AHR in congenic +/+ mice. Intratracheal administration of TNF- α resulted in increases in late AHR, cPLA₂ phosphorylation, cPLA₂ activity, and phosphorylation of mitogen-activated protein kinases. Mast cell replacement restored airway TNF- α level, cPLA₂ phosphorylation and enzymatic activity in the lungs as well as late AHR in W/W^v mice. These data indicate that mast cells play a key role in the development of late AHR through liberation of TNF- α .

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Introduction

Nonspecific airway hyperresponsiveness (AHR) is one of cardinal features of bronchial asthma. When allergic asthmatics are exposed to the antigen, they develop an acute IgE-dependent early AHR, which is often followed by late AHR from 4 to 12 h after the exposure [1, 2]. The early reaction is thought to be caused by many chemical mediators released from pulmonary mast cells activated by an IgE-dependent way [3]. Although the mechanisms leading to the development of the late reaction are not fully understood, the reaction may include several different events: (i) the direct effects of mediators released by mast cells, (ii) infiltration of lymphocytes,

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Abbreviations: AHR: airway hyperresponsiveness · BMMC: bone marrow-derived mast cell · cPLA₂: cytosolic phospholipase A₂ · i.t.: intratracheally · RL: airway resistance · TFMK: arachidonyltrifluoromethyl ketone · W/W^v: mast cell-deficient WBB6F1-W/W^v

eosinophils, basophils, and neutrophils into the airway mediated by chemokines and other cytokines released from the mast cells, and (iii) release of products from the infiltrating cells [4–6].

We have recently described a murine model of asthma, which exhibits a biphasic increase in AHR, in which mice received i.p. immunization twice, followed by two bronchoprovocations with aerosolized OVA with an 1-week interval [7]. The peaks of early and late AHR occur at 1 and 10 h, respectively, after the second airway allergen challenge. Using this protocol, we have found that the second airway allergen challenge results in TNF- α production in the airway, which in turn, promotes the activation of the enzyme, cytosolic phospholipase A₂ (cPLA₂), and the synthesis of cPLA₂ metabolites including platelet-activating factor. This process eventually leads to the development of late AHR [7]. Therefore, elucidation of the cell population representing the source of TNF- α in the airway will help to provide a better understanding of the mechanisms of late AHR.

Some reports have demonstrated that mast cell activation results in a potentiation of late AHR [8–10], and mast cells have been reported to represent a potential source of TNF- α *in vitro* [11, 12]. These findings suggest that TNF- α plays a role in mast cell-mediated late AHR. However, the *in vivo* extent to which the mast cell-mediated late AHR is TNF- α -dependent remains to be elucidated.

In this study, we have investigated the role of TNF- α in mast cell-mediated late AHR using mast cell-deficient WBB6F1-*W/W^v* (*W/W^v*) mice. We found that mast cells are essential in the development of late AHR, because of the liberation of TNF- α , which phosphorylates and activates cPLA₂.

Results

Induction of TNF- α and late AHR are impaired in *W/W^v* mice

We first compared the production of TNF- α in the airway and the development of late AHR after second airway challenge between *W/W^v* mice and their littermates. The levels of TNF- α in bronchoalveolar lavage fluids (BALF) were reduced up to 80% in *W/W^v* mice compared with their littermates (Fig. 1A). The late AHR was determined 10 h after the second airway challenge as a percent increase of airway resistance (RL) in response to increasing doses of methacholine. In OVA-sensitized and -challenged mast cell-sufficient *+/+* mice, the dose-response curve of RL shifted to the left compared with that of control mice (Fig. 1B). In addition, the RL produced by methacholine administration (at doses from 2.5 mg/mL to 50 mg/mL) increased significantly

in the OVA-sensitized and -challenged *+/+* mice compared with the controls. In contrast, the dose-response curve of RL barely shifted to the left in *W/W^v* mice, indicating that little late AHR developed in these mice. Administration of anti-TNF- α Ab showed a dose-response curve of RL that shifted to the right compared with that of OVA-sensitized and -challenged *+/+* mice

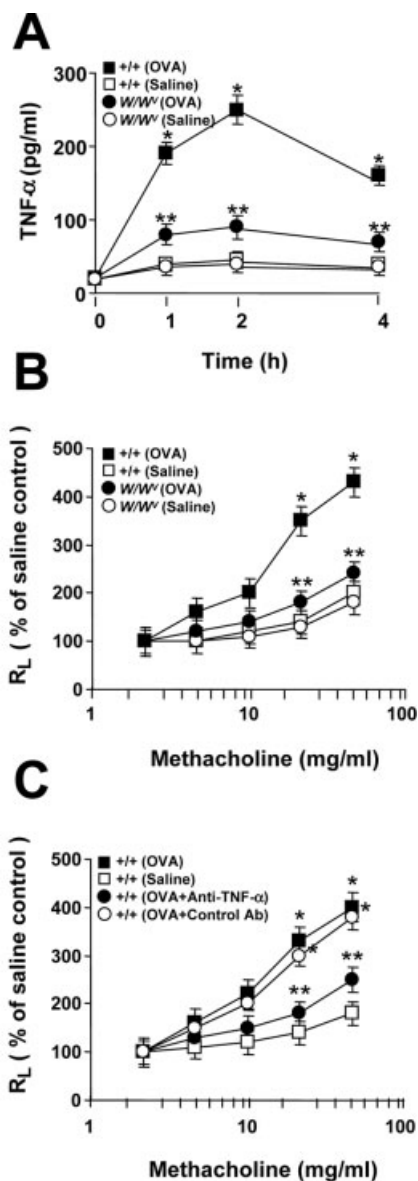


Figure 1. Production of TNF- α in the airways and the development of late AHR are impaired in *W/W^v* mice. (A) After the second airway challenge, TNF- α levels in BALF were measured at the indicated time points. (B) Late AHR was determined 10 h after the second airway challenge. (C) Littermates were injected i.p. with either control or anti-TNF- α Ab (each 2.0 mg/mouse) 10 min before the second airway challenge, and late AHR was determined 10 h after the challenge. Data represent the mean $\pm$ SD of three to five independent experiments ($n = 5$ animals per group). * $p < 0.01$ vs. saline group; ** $p < 0.01$ vs. *+/+* (OVA) group.

(Fig. 1C), indicating that anti-TNF- α Ab treatment reduces OVA-induced late AHR.

cPLA₂ phosphorylation and cPLA₂ enzymatic activity are impaired in W/W^v mice

Based on our previous report, which showed that late AHR is dependent on TNF- α -induced cPLA₂ activation [7], the abilities of W/W^v and +/+ mice to develop cPLA₂ phosphorylation and enzymatic activity were examined. The cPLA₂ phosphorylation (Fig. 2A) and enzymatic activity (Fig. 2B) in the lungs following

airway allergen challenge were significantly diminished in W/W^v mice compared with their littermates. Administration of a specific cPLA₂ inhibitor eliminated an increase in late AHR in +/+ mice (Fig. 2C).

TNF- α induces late AHR, phosphorylation of cPLA₂ and MAPK, and cPLA₂ activity

We examined the direct effects of TNF- α on the development of late AHR and the induction of cPLA₂ phosphorylation and enzymatic activity by administration of TNF- α intratracheally (i.t.) in normal C57BL/6 mice. TNF- α increased not only late AHR in a dose-dependent manner (Fig. 3A), but also cPLA₂ phosphorylation (Fig. 3B) and enzymatic activity (Fig. 3C). The cPLA₂ has a consensus phosphorylation motif (containing Ser-505) for a group of serine/threonine-specific, proline-directed protein kinases, the MAPK family, including ERK1/2, p42/p44, p38 MAPK, and JNK/stress-activated protein kinases [13]. Phosphorylation of cPLA₂ is a key step in the activation of this enzyme and subsequent arachidonic acid release and the generation of biologically important eicosanoids [14, 15]. Thus, we examined the effect of TNF- α on phosphorylation of the MAPK. All three MAPK were significantly phosphorylated in the lungs by i.t. administration of TNF- α (Fig. 3D).

2.4 Reconstitution of bone marrow-derived mast cell restores BALF levels of TNF- α , cPLA₂ activity, and late AHR

To confirm that the lack of mast cells in W/W^v mice is responsible for the failure of late AHR to develop in this strain, we determined the effect of reconstitution of W/W^v mice with bone marrow-derived mast cell (BMMC). Five million BMMC of +/+ mice were transferred into each W/W^v mice via a tail vein, and the recipients were immunized with OVA 18 weeks after the cell transfer. Histological examinations confirmed the presence of mast cells in +/+ (Fig. 4A) and BMMC-reconstituted W/W^v mice (Fig. 4C) in the subepithelial connective tissues of tracheas, whereas mast cells were not detected in W/W^v mice (Fig. 4B). The number of mast cells in BMMC-reconstituted W/W^v mice was about 70% that of +/+ mice (Fig. 4D). However, mast cells were barely observed in the peribronchial and perivascular connective tissues of lungs (less than one cell/mm²) in both +/+ and BMMC-reconstituted W/W^v mice (data not shown). Reconstitution of W/W^v mice with BMMC did not repair their anemia (as determined by hematocrit performed shortly before the date of study), confirming the selectivity of the mast cell reconstitution [16] (data not shown). To verify the selectivity of mast cell transfer further, we examined and compared airway eosinophilia

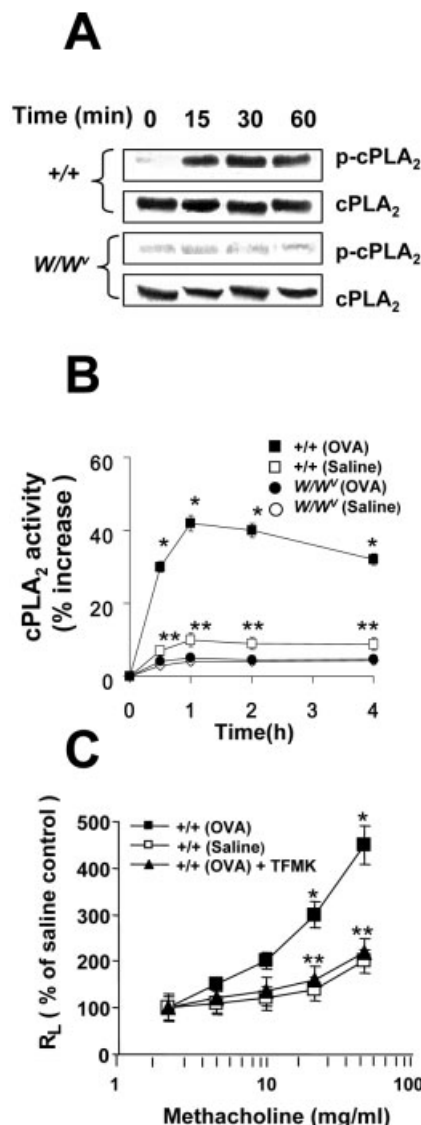


Figure 2. cPLA₂ phosphorylation and enzymatic activity are impaired in W/W^v mice. After the second airway challenge, lungs were removed at the indicated time points to assess (A) cPLA₂ phosphorylation and (B) cPLA₂ enzymatic activity. (C) Littermates were injected i.p. with TFMK (20 mg/kg) 30 min before the second airway challenge. Data represent the mean $\pm$ SD of three to five independent experiments ($n = 5$ animals per group). * $p < 0.01$ vs. saline group; ** $p < 0.01$ vs. +/+ (OVA) group.

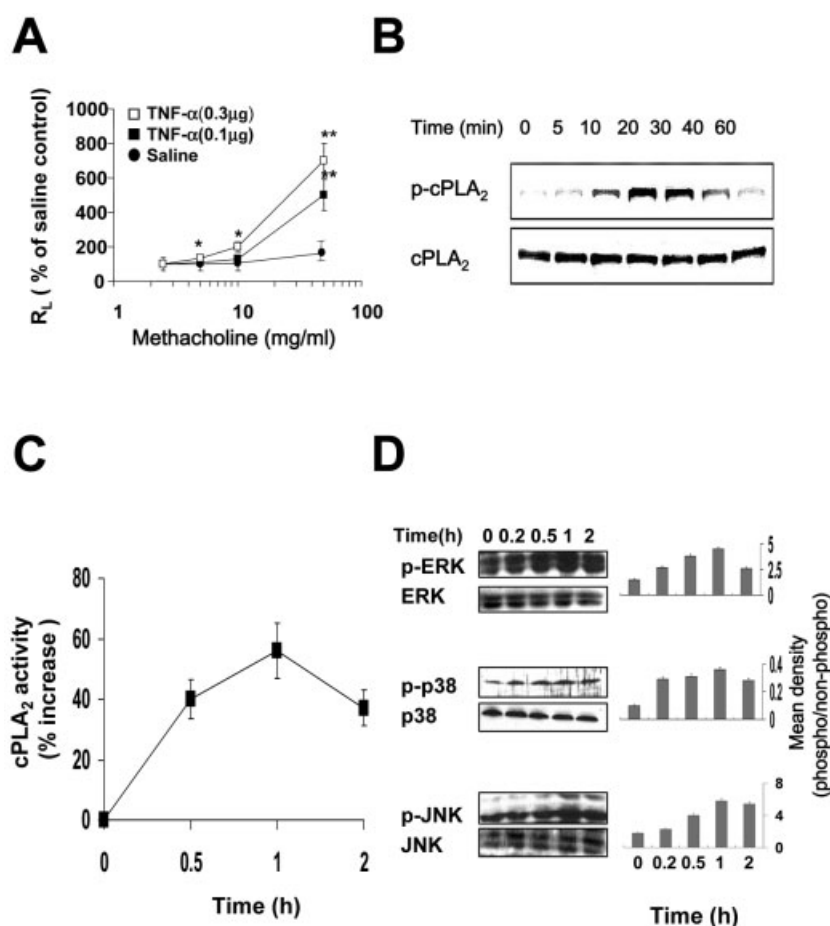


Figure 3. Administration of TNF- α increases late AHR, phosphorylation and activity of cPLA₂, and phosphorylation of MAPK. (A) AHR was determined 10 h after the i.t. administration of 0.1 μ g or 0.3 μ g of TNF- α . After the i.t. administration of 0.3 μ g of TNF- α , lungs were removed at the indicated time points to assess (B) cPLA₂ phosphorylation, (C) cPLA₂ enzymatic activity, and (D) MAPK phosphorylation. The results of the phosphorylation of MAPK were used for quantitation by densitometry (right panels). A representative data of three independent experiments showing similar results are shown. ($n = 3$ –5 animals for each time point). **, $p < 0.01$; *, $p < 0.05$ vs. control.

and the number of eosinophils in the lungs. W/W^v mice reconstituted with BMMC and $+/+$ mice were immunized and then challenged with OVA. Eosinophils were not observed in W/W^v mice, whereas mast cell reconstitution recruited eosinophils to the airways (Fig. 5A) and lungs (Fig. 5B) to the similar levels of those seen in $+/+$ mice. Congo red staining of lung sections demonstrated eosinophil infiltration in the perivascular and peribronchiolar area in OVA-sensitized and -challenged $+/+$ (Fig. 5E) and BMMC-reconstituted W/W^v (Fig. 5F) mice, but not in normal W/W^v (Fig. 5C) and OVA-sensitized and -challenged W/W^v (Fig. 5D) mice.

We finally examined the effects of mast cell reconstitution on TNF- α level and cPLA₂ activity. Reconstitution with BMMC restored TNF- α level in the BALF up to 70–80% of $+/+$ mice (Fig. A) and cPLA₂ phosphorylation (Fig. 6B) and cPLA₂ activity in the lungs (Fig. 6C) to the similar levels of those seen in $+/+$

mice. The reduced value of RL in W/W^v mice was increased significantly after the reconstitution with mast cells (Fig. 6D).

Discussion

Many investigators have studied the role of mast cells in the development of late AHR in murine models of asthma. While some reports have demonstrated that mast cell activation results in a potentiation of late AHR [8–10], others have shown that AHR can be elicited in the absence of IgE Ab [17, 18] or mast cells [19], dependent on the experimental model used to generate the airway response. These data have come from studies that used a protocol that consists of airway antigen challenge for more than 3 consecutive days, and examination of AHR usually 2 days after the last antigen challenge. Therefore, cellular and molecular mechan-

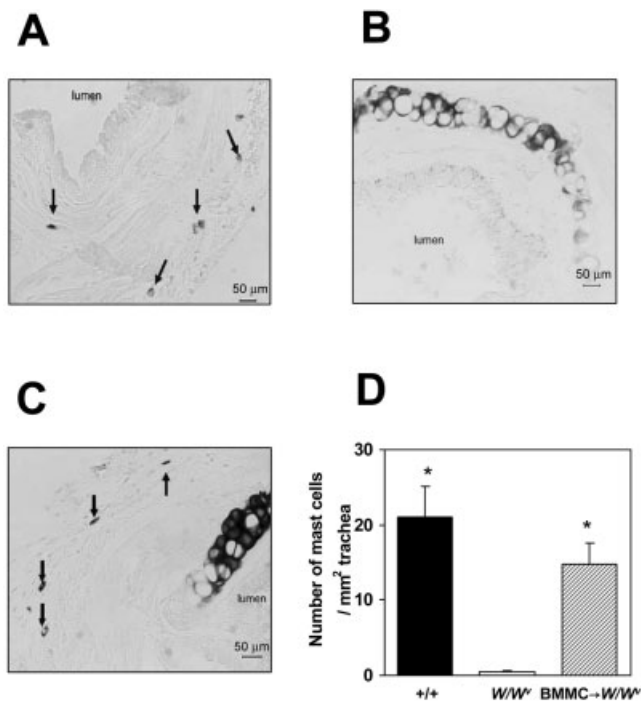


Figure 4. Histological examination of mast cells in the trachea. Mast cells are visualized in the tracheas in (A) +/+, (B) W/W^v, and (C) BMMC → W/W^v mice. The sections were fixed in Carnoy's fixative and stained with toluidine blue. Arrows indicate mast cells. Original magnification: × 400; A representative of two independent experiments is shown. (D) Number of mast cells in the trachea. Data represent the mean ± SD of two independent experiments (*n* = 5 animals per group). **p* < 0.01 vs. W/W^v mice.

isms underlying this AHR should be different from those of the so-called "late AHR" which occurs 4–12 h after initial exposure of the airway to antigen. This makes it potentially difficult to evaluate the role of mast cells in the development of late AHR. Therefore, our development of a murine model of asthma, in which late AHR develops 8–14 h after airway challenge [7], allowed us to investigate the role of mast cells in the development of late AHR.

Using the newly developed protocol, we have previously shown that TNF- α is critical for the development of late AHR occurring 8–14 h after the second airway challenge through cPLA₂ activation. In this process, TNF- α was not involved in the development of early AHR and there was a single peak of TNF- α around 1–4 h during the observation period up to 8 h [7]. In this study, we have demonstrated that (i) mast cells represent the major source of TNF- α in the lungs, (ii) mast cells are critically involved in TNF- α -induced late AHR, and (iii) TNF- α induces late AHR through cPLA₂ activation.

Late AHR was significantly impaired in W/W^v mice undergoing an asthmatic reaction compared with

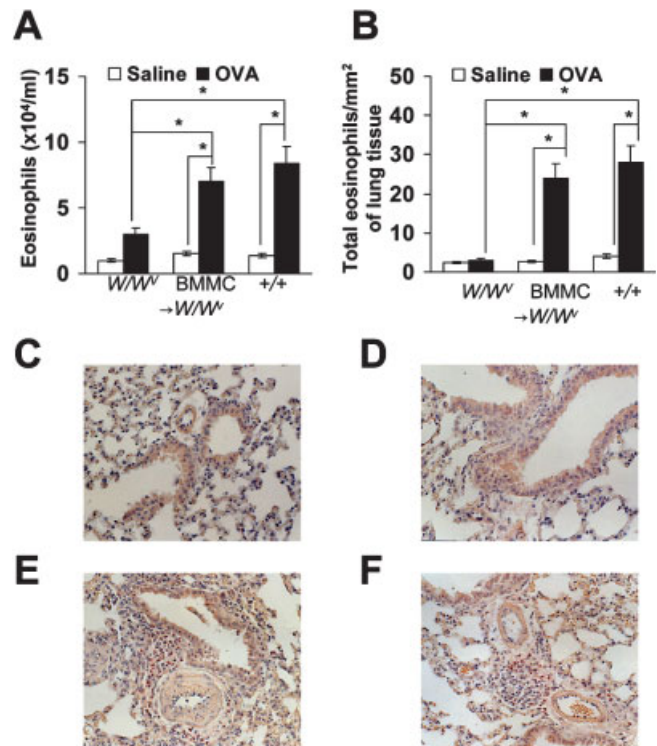


Figure 5. Mast cell reconstitution restores recruitment of eosinophils to the airways and lungs. W/W^v mice reconstituted with BMMC and +/+ mice were immunized and then challenged with OVA. Two days after the second airway challenge, eosinophils were observed in the airway (A) and lungs (B). Data represent the mean ± SD of two independent experiments (*n* = 5 animals per group). **p* < 0.01. Lung sections of normal W/W^v mice (C), OVA-sensitized and -challenged W/W^v (D), +/+ (E), and BMMC-reconstituted W/W^v (F) mice were stained with Congo red solution. Magnification × 200.

congenetic +/+ mice. This impairment of late AHR was associated a marked reduction of TNF- α level in the BALF, cPLA₂ phosphorylation and cPLA₂ enzymatic activity in the lungs. Reconstitution of BMMC restored all of the impaired endpoints in W/W^v mice. These data indicate that mast cells play a key role in the development of late AHR through the liberation of TNF- α , which, in turn, induces cPLA₂ phosphorylation and activation.

A number of studies have suggested a role for TNF- α in the development of AHR. Inhalation of TNF- α in animals [20, 21] and healthy [22] or asthmatic [23] human subjects leads to AHR. In murine models of asthma, either a deficiency in TNF- α receptors [24] or administration [25] or induction of a TNF- α autoantibody [26] resulted in marked attenuation of antigen-induced airway inflammation and AHR. Regarding the role of TNF- α in human asthma, TNF- α has been reported to induce epithelial apoptosis and shedding [27]. Additionally, recent studies have demonstrated that anti-TNF- α therapy in patients with asthma

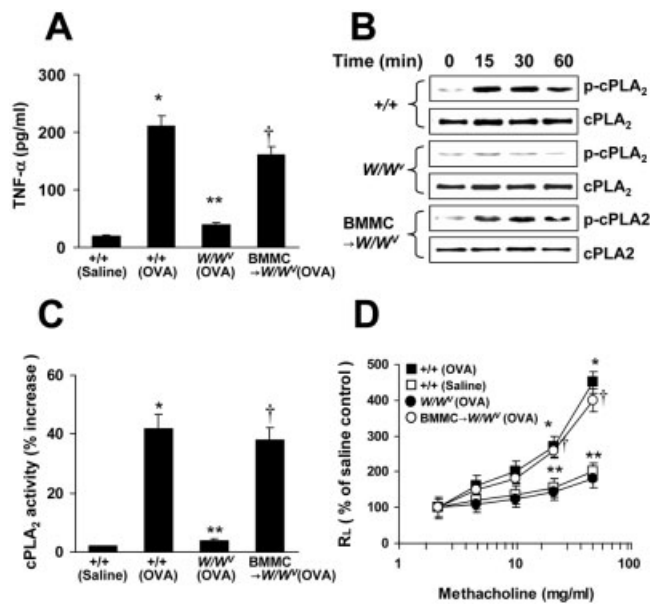


Figure 6. Reconstitution of BMMC restores BALF levels of TNF- α , cPLA₂ phosphorylation and activity, and late AHR. (A) BAL levels of TNF- α were measured 2 h after the second airway challenge. (B) cPLA₂ phosphorylation and (C) cPLA₂ activity were assessed 20 min and 1 h after the second airway challenge, respectively. (D) Late AHR was assessed 10 h after the second airway challenge. Data represent the mean $\pm$ SD of two independent experiments ($n = 5$ animals per group). * $p < 0.01$ vs. saline group; ** $p < 0.01$ vs. +/+ (OVA) group; † $p < 0.01$ vs. W/W^V mice.

markedly improved asthma symptoms, lung function, and AHR, in which the most striking feature of anti-TNF therapy was the improvement in AHR [28, 29], further indicating an important role of TNF- α in the development of AHR.

As a mechanism of TNF- α -induced development of late AHR, we have previously shown a causal relationship between TNF- α and cPLA₂ by demonstrating that (i) cPLA₂ activity and the synthesis of cPLA₂ metabolites such as leukotriene B₄ and platelet-activating factor were increased in asthmatic lungs, which were almost completely abolished by pretreatment with anti-TNF- α Ab or in TNF- α knockout mice, and (ii) a cPLA₂ inhibitor blocked late AHR [7]. In this study, we have confirmed that the development of late AHR is mediated exclusively through a TNF- α -induced cPLA₂-dependent mechanism by demonstrating that (i) i.t. administration of TNF- α resulted in increases in late AHR and cPLA₂ phosphorylation and activity, and (ii) pretreatment of either anti-TNF- α Ab or a specific cPLA₂ inhibitor abolished an increase in late AHR in +/+ mice, further supporting a link between TNF- α and cPLA₂ in the development of late AHR in asthma. In addition to cPLA₂ phosphorylation and activity, TNF- α also increased the phosphorylation of the three MAPK, p38, ERK, and JNK (Fig. 3D). This suggests that TNF- α enhances phosphor-

ylation and enzymatic activity of cPLA₂ through the phosphorylation of MAPK, given that cPLA₂ has a consensus phosphorylation motif (containing Ser-505) for the MAPK family [13]. Although little evidence is existed regarding the MAPK activated by TNF- α *in vivo*, the *in vitro* studies have reported that TNF- α rapidly mediate the phosphorylation of different MAPK depending on the cell types used. For example, activation of JNK and p38 MAPK is a crucial event in TNF- α signaling in HeLa cells [30] and human neutrophils [31], respectively. On the other hand, ERK is activated in mouse macrophages stimulated by TNF- α [32]. Further studies are required to determine which MAPK is importantly involved in TNF- α induced pathologies in asthmatic lungs.

In this study, TNF- α was increased in the BALF after the second airway challenge in +/+ mice, whereas OVA-sensitized and -challenged W/W^V mice showed a significant decrease in TNF- α levels (up to 70%) in their BALF. Furthermore, reconstitution of mast cells significantly restored the TNF- α response. Although these data suggest that mast cells represent the major source of airway TNF- α in mice undergoing an asthmatic reaction, other many different cell types produce TNF- α , but those especially relevant to asthma include T lymphocytes, monocytes, macrophages, eosinophils, and epithelial cells [33]. Our time kinetic study has revealed that TNF- α in the BALF reaches to peak level in 1–2 h after the second airway challenge of allergen, but recruitment of T lymphocytes and other inflammatory cells such as macrophages, neutrophils, and eosinophils into the airway does not take place within 1–2 h. This suggests that the residential cell populations in the airway such as epithelial cells and probably dendritic cells can represent the secondary cell population producing TNF- α . Currently, there are reports showing that NK-T cells alone [34], basophils alone [35], myeloid dendritic cells alone [36], CD4 T cells alone [37] are the only responsible cells in the OVA-induced lung inflammation model, although the relationship between TNF- α and these cells remains to be established.

In the course of this study, we found that OVA itself nonspecifically can induce TNF- α production and induction of AHR (data not shown). Either intratracheal administration or airway challenge of OVA induced not only airway TNF- α production, but also the development of AHR at 10 h. We found that OVA exerted such activity through the activation of NF- κ B in the airway epithelial cells. OVA-induced NF- κ B activation was not due to the contaminated LPS present in commercial OVA, because removal of LPS using an affinity column did not affect the OVA activity. However, given that LPS depleting columns (polymyxin B) do not deplete other TLR ligands such as peptidoglycan, teichoic acid, bacterial DNA etc., it is possible that these TLR ligands with a capacity to

activate NF- κ B may contribute to the nonspecific effects of OVA. Further studies are required to answer this issue.

In summary, we have here demonstrated that mast cells represent the major source of TNF- α in asthmatic lungs, and, therefore, are essential in the development of TNF- α -induced late AHR. This study provides evidence to improve our understanding of the cellular and molecular mechanisms of late AHR, as well as the role of mast cells in the pathogenesis of asthma.

Materials and methods

Animals

Female mast cell-deficient WBB6F1- W/W^v (W/W^v) and mast cell-sufficient control WBB6F1 $+/+$ mice were purchased from the SLC (Shizuoka, Japan). Mice were 7–8 weeks old at the start of each experiment. All experimental animals used in this study were under the protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

Reagents

The cPLA₂ inhibitor, arachidonyltrifluoromethyl ketone (TFMK) (20 mg/kg) purchased from BIOMOL (Plymouth Meeting, PA), was administered i.p. 30 min before the second airway challenge. Anti-TNF- α (MP6-XT22.11, rat IgG1) [7] and control rat IgG1, J4-1 [38], were injected i.p. 10 min before the second airway challenge. These optimal times of administration were determined in the previous study [7].

Immunization and challenge

Mice were immunized and challenged as described previously [7]. In brief, mice were immunized i.p. with 20 μ g of OVA (grade V from Sigma, St. Louis, MO) plus 1.0 mg aluminum hydroxide adjuvant (ImjectR Alum; Pierce, Rockford, IL) on day 0 and OVA alone without alum on day 10. The immunized mice were exposed to aerosolized OVA on days 17 and 24. Aerosolization of OVA was performed using a chamber adapted for mice. Animals were exposed to OVA (2.5% at first aerosolization and 5.0% at second aerosolization) using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan; output 0.8 mL/min) for 20 min. Control animals received the same immunization and the first airway challenge of OVA, but were exposed to aerosolized saline instead of OVA during the second airway challenge.

BAL was performed 48 h after the second airway challenge as described previously [7].

Determination of AHR

AHR was also assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere [39, 40]. Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected i.p. The trachea was exposed by mid-cervical incision. Mice were tracheosto-

mized and an 18-gauge metal needle was then inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with a nominal tidal volume of 10 mL/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that attained during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to a water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol at increasing concentrations (2.5–50 mg/mL in saline). After each methacholine challenge, the RL data were continuously collected. Maximum values of RL were selected to express changes in airway function, which was represented as a percentage change from baseline after aerosol saline challenge.

TNF- α assay

The BALF level of TNF- α was measured using an ELISA kit (R&D Systems, Minneapolis, MN).

Assay of cPLA₂ activity

Mice lungs were minced and sonicated in a buffer (50 mg tissue/mL) containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl and protease inhibitors with tip-type sonicator (Branson sonifier, Branson ultrasonics, CT, 20% amplitude, 3 min), followed by centrifugation for 20 min at 1000 $\times$ g. The supernatants were fractionated by passing with 26G needle for 20 times and ultracentrifuged at 1 000 000 $\times$ g for 1 h. cPLA₂ activity of the supernatants was assayed with sonicated liposomes prepared as described previously. cPLA₂ activity was measured as described previously [7].

Immunoprecipitation and Western blotting

Cell lysates were prepared in radioimmunoprecipitation assay buffer (0.1% SDS, 1% IGEPAL, 0.5% sodium deoxycholate, and 1 mM PMSF). Equal amounts of cell lysate were incubated with mouse anti-cPLA₂ antibody with gentle rocking for 1 h at 4°C, followed by the addition of protein A/G-agarose (Sigma Chemical, St. Louis, MO). The mixtures were incubated overnight at 4°C. After three washes with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 0.5 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT), the pellet was separated on 10% SDS polyacrylamide gel under reducing conditions, and was then transferred onto Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked for 2 h at room temperature in 5% skim milk in TBS, followed by overnight incubation with anti-phospho-cPLA₂ (Ser⁵⁰⁵) (Cell Signaling, Beverly, MA), anti-cPLA₂ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho ERK, anti-ERK, anti-phospho p38, anti-p38, anti-phospho JNK, or anti-JNK. Blots were washed for 15 min with three changes of TBS-0.05% Tween 20 solution, followed by 1 h incubation at room temperature with the HRP-conjugated anti-mouse antibody or anti-rabbit IgG antibody. Blots were washed again for 15 min

and finally developed in the ECL Western detection reagents (Amersham-Pharmacia Biotech, Piscataway, NJ). The signal intensities of specific bands were analyzed quantitatively using a Fluor-STM Imager (Bio-Rad, Munchen, Germany) and were plotted as relative intensity.

Reconstitution of BMMC

Reconstitution of mast cells in W/W^v mice was conducted by the method described elsewhere [8, 41] with slight modification. Bone marrow cells of $+/+$ mice were cultured in RPMI 1640 medium supplemented with 20% WEHI-3 condition medium (20 ng/mL of recombinant murine IL-3) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Culture medium was replaced every 5 days. After 4 weeks of culture, cells were harvested and stained with May-Grünwald-Giemsa solution to confirm mast cells. The staining indicated that more than 99% cells were viable mast cells. Then, 5×10^6 BMMC were injected i.v. twice with 1-week interval into each W/W^v mice via a tail vein. After 18 weeks of the second transfer, the recipients were immunized and challenged with OVA by the method described.

Histological studies

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin for light microscopic examinations. To demonstrate eosinophils, the formalin-fixed sections were stained with hematoxylin-2 (Richard-Allan Scientific, Kalamazoo, MI) for 5 s, then rinsed briefly in distilled water and immersed in alkaline sodium chloride solution (1.0% sodium hydroxide and saturated sodium chloride in 80% ethanol) for 20 min and stained in 0.5% Congo red solution (0.5% Congo red in 50% ethanol) for 20 min and dehydrated and mounted [42].

To examine the presence and distribution of tissue mast cells, lungs were fixed in Carnoy's fixative and sections were stained with toluidine blue.

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical comparison was performed using one-way ANOVA followed by the Fisher test. Significant differences between the groups were determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was accepted as an indication of statistical significance.

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