

TNF- α induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A₂ activation

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Background: Late-phase airway hyperresponsiveness (AHR) in asthma is considered the event leading to persistent inflammation in the lungs, but the molecular mechanisms involved in this process are poorly understood.

Objective: To examine the role of TNF- α in the development of a late AHR and airway inflammation in asthma.

Methods: We established a murine model of asthma with not only biphasic AHR to methacholine but also airway eosinophilia. The effect of TNF- α blockade was determined by using anti-TNF- α antibody and TNF- α knockout mice. Cytosolic phospholipase A₂ (cPLA₂) mRNA expression and activity were assessed by using RT-PCR and 1-stearoyl-2-[1-¹⁴C] arachidonyl-sn-glycero-3-phosphocholine as the substrate, respectively.

Results: TNF- α blockade resulted in significant inhibition of the late AHR without affecting the early AHR, and reduction in airway eosinophilia and inflammation. cPLA₂ activity was increased in asthmatic lungs in a TNF- α -dependent way, and cPLA₂ inhibitor blocked late AHR and airway eosinophilia. TNF- α also stimulated the synthesis of cPLA₂ metabolites such as leukotriene B₄ and platelet-activating factor in the airway. Specific inhibitors of cPLA₂ metabolites inhibited the late AHR and airway eosinophilia.

Conclusions: TNF- α is the proximal key cytokine capable of developing late-phase AHR and subsequent airway

inflammation through expression/activation of cPLA₂. (J Allergy Clin Immunol 2005;116:537-43.)

Key words: Asthma, late airway response, TNF- α , cytosolic phospholipase A₂, airway inflammation, cyclooxygenase, 5-lipoxygenase, platelet-activating factor

Bronchial asthma is characterized by reversible airway obstruction in response to allergen, chronic eosinophilic airway inflammation, and nonspecific airway hyperresponsiveness (AHR). When allergic patients with asthma are exposed to the antigen, they develop an acute IgE-dependent early response, which is often followed by a late AHR from 4 to 12 hours after the exposure.^{1,2} The early reaction is thought to be caused by many chemical mediators, such as histamine, platelet-activating factor (PAF), and leukotrienes released from the lung mast cells activated in an IgE-dependent way.³ The late reactions may include several different events: (1) the direct effects of mediators released by mast cells, (2) infiltration of lymphocytes, eosinophils, basophils, and neutrophils into the airway mediated by chemokines and other cytokines released from the mast cells, and (3) release of products from the infiltrating cells.⁴⁻⁶ Because these late reactions are considered events leading to persistent inflammation in the lungs, it is important to delineate the precise molecular mechanisms involved in the late reaction.

We have recently shown that TNF- α produced by a PAF-mediated nuclear factor (NF)- κ B-dependent way plays an essential role in the development of a late-phase anaphylactic reaction.⁷ Several studies have suggested that TNF- α plays an important role in the pathogenesis of asthma. TNF- α mRNA and protein levels are increased in the sputum or serum of patients with asthma,⁸⁻¹⁰ and TNF- α induces aggravation of AHR in animals^{11,12} and human beings¹³ as well as recruitment of inflammatory cells into the airways.^{12,14} However, the precise role of TNF- α in the pathogenesis of asthma remains to be elucidated. In this study, we investigated the role of TNF- α in the development of AHR and airway inflammation by establishing a murine asthma model, which displays both the early and late phase of AHR and airway

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Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
cPLA ₂ :	Cytosolic phospholipase A ₂
LTB ₄ :	Leukotriene B ₄
PAF:	Platelet-activating factor
Penh:	Enhanced pause
PTPBS:	4-[5-phenyl-3-(trifluoromethyl)-1H-pyrazole-1-yl] benzenesulfonamide
TFMK:	Arachidonyltrifluoromethyl ketone

eosinophilia. The results indicated that TNF- α plays an essential role in the development of the late AHR and subsequent airway inflammation through expression/activation of the enzyme cytosolic phospholipase A₂ (cPLA₂).

METHODS

Immunization and challenge

Specific pathogen-free male BALB/c mice were obtained from the Korean Institute of Chemistry Technology (Daejeon), housed in a laminar flow cabinet, and maintained on standard laboratory chow ad libitum. TNF- α knockout mice (TNF- $\alpha^{-/-}$) derived from breeder pairs of B6; 129S-Tnf α 1Gk1 stock and the original wild-type strain (B6129SF2/J) were obtained from the Jackson Laboratory (Bar Harbor, Me). Mice were 7 to 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.

Mice were immunized intraperitoneally with 20 μ g ovalbumin (grade V; Sigma, St Louis, Mo) plus 1.0 mg aluminum hydroxide adjuvant (ImjectR Alum; Pierce, Rockford, Ill) on day 0 and ovalbumin alone without alum on day 10. The immunized mice were exposed to aerosolized ovalbumin on days 17 and 24. Aerosolization of ovalbumin was performed by using a chamber adapted for mice. Animals were exposed to ovalbumin (2.5% at first aerosolization and 5.0% at second aerosolization) by using ultrasonic nebulizer (NE-U12; output 0.8 mL/min; Omron, Tokyo, Japan) for 20 minutes in a Plexiglas (Rohm & Haas, Philadelphia, Pa) exposure chamber (24.5 cm $\times$ 40.5 cm $\times$ 15.0 cm). Control animals received the same immunization and first airway challenge of ovalbumin, but were exposed to aerosolized saline instead of ovalbumin at the second airway challenge.

Determination of AHR to methacholine

Airway hyperresponsiveness was measured in unrestrained, conscious mice as described elsewhere.¹⁵ Enhanced pause (Penh) is used as a measure of the airway responsiveness to methacholine.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed 48 hours after the second airway challenge, unless otherwise stated, as described previously.¹⁶

PAF, histamine, TNF- α , and leukotriene B₄ assay in BAL fluids

Platelet-activating factor and histamine were measured as previously described.¹⁷ TNF- α was measured by using an ELISA kit

(R&D Systems, Minneapolis, Minn). Leukotriene B₄ (LTB₄) assay was performed by using the Biotrak Enzymeimmunoassay System (Amersham Biosciences, Piscataway, NJ).

RT-PCR and assay of cPLA₂ activity

For RT-PCR, total RNA was prepared at different time points from the lungs by a single extraction on the basis of the guanidine isothiocyanate extraction method as described previously.¹⁷ cDNA (15 ng) was amplified by PCR in a thermal cycler (denaturation for 30 seconds at 95°C, annealing for 30 seconds at 62°C, and elongation for 30 seconds at 72°C; Perkin Elmer, Norwalk, Conn) by using cPLA₂ (33 cycles) and β -actin (23 cycles) primers. The primers used in this analysis are as follows: cPLA₂, 5'-ATGTCTTTCATAGATCCTTATCAG-3' and 5'-TGAACAACTTCCAGAGACAT TTC-3'; and β -actin, 5'-GGGTCAGAACTCCTATG-3' and 5'-TTGACCTCAGCGCT GATGTG-3'.

For the assay of cPLA₂ activity, mice lungs were minced and sonicated in a buffer (50 mg tissue/mL) containing 50 mmol/L Tris-Cl, pH 7.5; 1 mmol/L EDTA; 150 mmol/L NaCl; and protease inhibitors with tip-type sonicator (20% amplitude, 3 minutes; Branson Sonifier, Branson Ultrasonics Corp, Danbury, Conn), followed by centrifugation for 20 minutes at 1000g. The supernatants were fractionated by passing with a 26G needle for 20 times and ultracentrifuged at 1,000,000g for 1 hour. cPLA₂ activity of the supernatants was assayed with sonicated liposomes prepared as described previously.¹⁸

Effects of various inhibitors on asthmatic reactions

The PAF antagonist CV-6209 (15.0 mg/kg, WAKO, Kyoto, Japan) was administered intraperitoneally either 10 minutes before (to examine the early AHR) or 6 hours after (to examine the late AHR) the second airway challenge of ovalbumin. The cPLA₂ inhibitor arachidonyltrifluoromethyl ketone (TFMK; 20 mg/kg; Biomol, Plymouth Meeting, Pa), the 5-lipoxygenase inhibitor pirioprost (40 mg/kg; Cayman, Ann Arbor, Mich), and the COX-2 inhibitor 4-[5-phenyl-3-(trifluoromethyl)-1H-pyrazole-1-yl] benzenesulfonamide (PTPBS) (20 mg/kg; Calbiochem, San Diego, Calif) were administered intraperitoneally 30 minutes before the second airway challenge. Two milligrams anti-TNF- α (MP6-XT22.11)⁷ and control rat IgG1, J4-1,¹⁹ were injected intraperitoneally 10 minutes before the second airway challenge.

Lung histology

The lungs were removed and fixed in 10% neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- μ m sections of fixed, embedded tissues were cut on a microtome, placed on glass slide, deparaffinized, and stained sequentially with hematoxylin 2 and eosin.

Statistical analysis

Data were expressed as means $\pm$ SDs. Statistical comparison was performed by using 1-way ANOVA followed by the Fisher test. Significant differences between the groups were determined by using the unpaired Student *t* test. A value of *P* < .05 was accepted as an indication of statistical significance.

RESULTS

Biphasic AHR in a murine model of asthma

We first developed a murine model of asthma, which exhibited biphasic increase in AHR as well as airway

inflammation in response to the airway allergen challenge. This protocol included intraperitoneal immunization twice (the first with 20 μ g ovalbumin plus 1.0 mg alum on day 0 and the second with ovalbumin alone without alum on day 10) and airway challenge with ovalbumin twice (the first with 0.5% ovalbumin for 20 minutes on day 17 and the second with 1.0% ovalbumin for 20 minutes on day 24). After the second airway challenge with ovalbumin, the specific airway resistance increased 5-fold to 7-fold at 1 hour, followed by the late-phase response being larger and broader in resistance at 7 to 14 hours, peaking at 10 hours with a 3.5-fold to 4.5-fold increase (Fig 1, A). Moreover, the second antigen challenge resulted in an increase of the total cell number in the BAL fluids from $2.0 \pm 0.3 \times 10^4$ /mL before the challenge to $21 \pm 3.2 \times 10^4$ /mL at 48 hours (Fig 1, B). Eosinophils were the predominant cell population, which accounted for 60% to 70% of the total cells. Histological examination of the lungs showed that antigen challenge induced a marked inflammatory cell infiltration (largely eosinophils) in the perivascular and peribronchiolar area and accumulation of mucus and debris in the lumen of bronchioles (Fig 1, C). Such AHR and airway inflammation were not observed in saline-exposed control animals (Fig 1, C). Single-airway antigen challenge with 1% ovalbumin for 20 minutes, however, elicited little AHR and eosinophil influx into the airway, irrespective of the frequency of immunization (once or twice with alum or 10 times without alum), immunizing doses of ovalbumin (from 10 μ g to 500 μ g), and intervals between the second immunization and airway challenge (from 1 to 6 weeks; data not shown).

TNF- α blockade inhibits late AHR and airway inflammation

We next examined the role of TNF- α in the development of AHR and airway inflammation by blocking TNF- α action by using anti-TNF- α antibody or TNF- $\alpha^{-/-}$ mice. Pretreatment of anti-TNF- α antibody resulted in 80% to 90% inhibition of the late AHR without affecting the early AHR (Fig 2, A), 80% to 90% reduction in the number of eosinophils in BAL fluid (Fig 2, B), and a weak development of the lung inflammation (Fig 2, C). Control antibody failed to show any inhibition. A similar magnitude of inhibition in the AHR (Fig 2, C), airway eosinophilia (Fig 2, D), and lung inflammation (Fig 2, E) was also observed in TNF- $\alpha^{-/-}$ mice. These data indicate that TNF- α is the key cytokine involved in the development of the late-phase AHR and the airway inflammation. We verified the involvement of TNF- α by measuring this cytokine in BAL fluid, and kinetics of TNF- α levels were compared with those of histamine levels. After the airway challenge, an immediate single peak of histamine was observed at 20 to 30 minutes. In contrast, TNF- α levels were gradually increased and peaked at 2 hours, slowly declining thereafter (Fig 2, F). TNF- α was not detected in BAL fluids in TNF- $\alpha^{-/-}$ mice. These findings suggest that TNF- α in BAL fluid does not come from preformed stores in the mast cells but is synthesized.

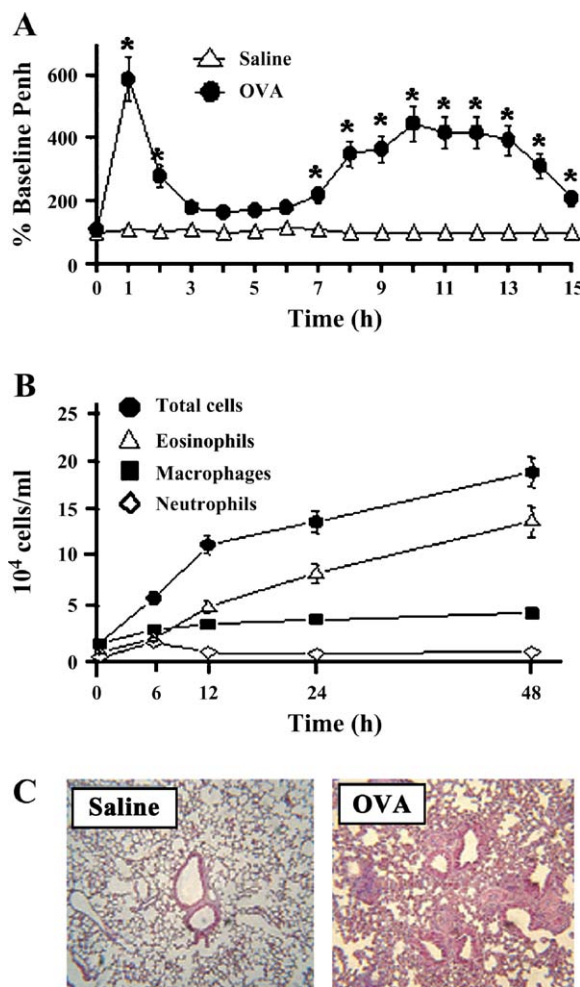


FIG 1. Development of murine model of asthma with biphasic AHR and airway inflammation. BALB/c mice exhibit biphasic AHR (A), cell infiltration in the airway (B), and lung inflammation (C) in response to the second airway ovalbumin (OVA) challenge. Data represent the means $\pm$ SDs of 3 to 7 separate experiments ($n = 5$ per group). * $P < .05$ vs saline.

TNF- α induces the late AHR through activation of cPLA₂

Cytosolic phospholipase A₂ mRNA expression was observed in the asthmatic lungs with the peak levels at 3 hours, but its expression was not observed in mice pretreated with anti-TNF- α antibody or in TNF- $\alpha^{-/-}$ mice (Fig 3, A). cPLA₂ activity increased by about 82% in BALB/c (Fig 3, B) and 60% in TNF- $\alpha^{+/+}$ mice (Fig 3, C) at 3 hours, and TNF- α blockade completely abrogated the activity (Fig 3, B and C), indicating that TNF- α is responsible for the cPLA₂ activation. The selective cPLA₂ inhibitor, TFMK, inhibited the late AHR measured 10 hours after second allergen challenge (Fig 3, D) and airway eosinophilia (Fig 3, E) by more than 80%, respectively. These data indicate that TNF- α induces the late AHR and airway eosinophilia through cPLA₂ activation. We further verified the TNF- α dependency of cPLA₂ activation by

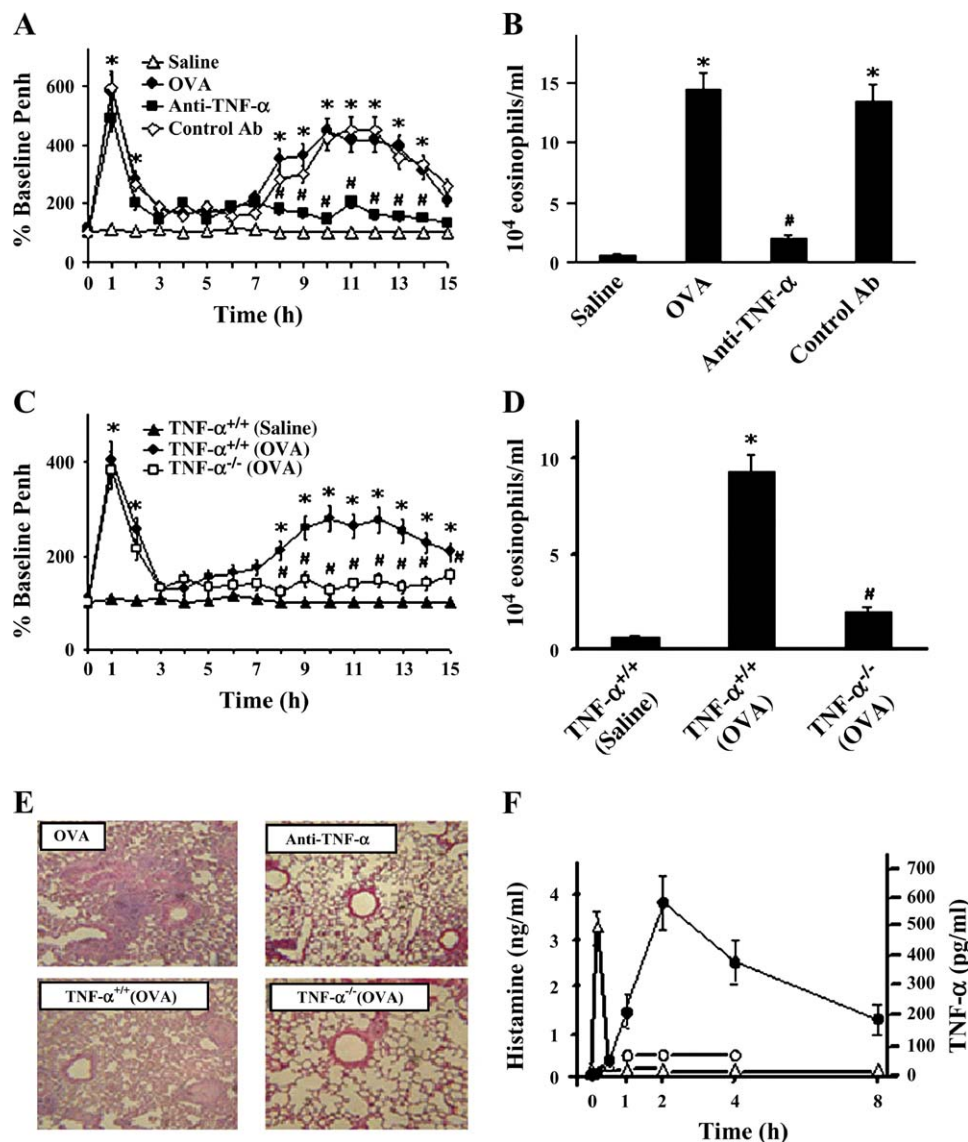


FIG 2. Anti-TNF- α antibody blocks the late AHR (A), airway eosinophilia (B), and lung inflammation (E) in BALB/c mice. Late AHR (C), airway eosinophilia (D), and lung inflammation in *TNF- α ^{-/-}* mice. F, BAL fluid levels of histamine in BALB/c mice and TNF- α levels in BALB/c (●) and *TNF- α ^{-/-}* (○) mice. Data represent the means $\pm$ SDs of 3 to 5 separate experiments ($n = 5$ per group). * $P < .05$ vs saline; # $P < .05$ vs ovalbumin.

determining whether TNF- α also regulated the synthesis of the cPLA₂ metabolites such as LTB₄ and PAF in the airway. The levels of LTB₄ and PAF were increased in BAL fluid 10 hours after second allergen challenge, which were significantly reduced (more than 75%) in TNF- α -blockaded mice (Fig 4, A and B). We finally assessed the effects of various eicosanoids produced by cPLA₂ activation on the development of the late-phase AHR and airway eosinophilia by using specific inhibitors. The PAF antagonist CV-6209 almost completely blocked both the late AHR and BAL eosinophilia (Fig 4, C and D). The 5-lipoxygenase inhibitor piriprost significantly reduced BAL eosinophilia but had no effect on the late AHR,

whereas the COX-2 inhibitor PTPBS attenuated neither the late AHR nor BAL eosinophilia (Fig 4, C and D).

DISCUSSION

Many protocols have been used to study asthma in mice. Usually, the protocols include sensitization twice with antigen mixed with adjuvant, such as alum, followed by airway antigen challenge for more than 3 consecutive days, and examination of AHR and lung inflammation 1 to 3 days after the last antigen challenge.²⁰⁻²⁴ However, these protocols do not allow examination of early events

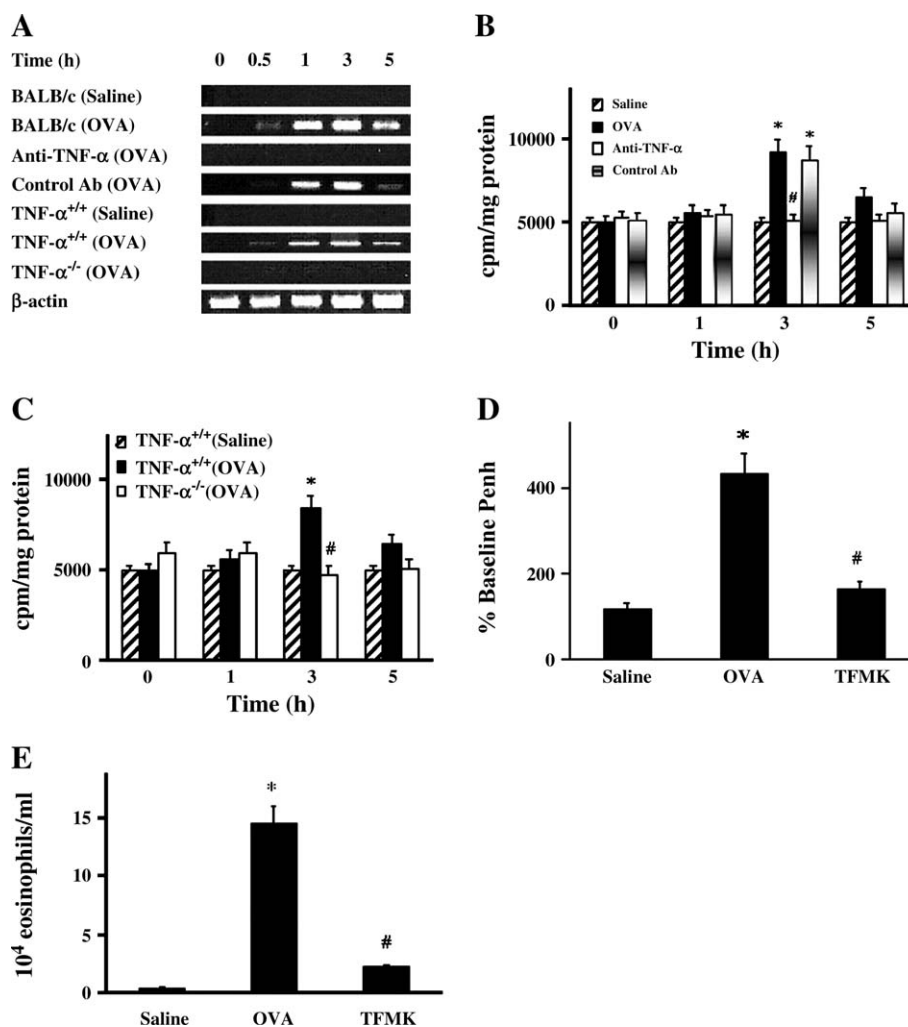


FIG 3. TNF- α dependency of cPLA₂ activation. Expression of mRNA (**A**) and activity (**B** and **C**) of cPLA₂ in the asthmatic lungs and their abrogation by TNF- α blockade. cPLA₂ inhibitor, TFMK, attenuates the late AHR (**D**) and airway eosinophilia (**E**). Data represent the means $\pm$ SDs of 3 to 5 separate experiments ($n = 5$ per group). * $P < .05$ vs saline; # $P < .05$ vs ovalbumin.

occurring after the initial airway exposure to antigen because the early and the late responses overlap and become almost indistinguishable as the response expands. In this study, we were able to elicit both biphasic AHR to methacholine and airway eosinophilia ($1-2 \times 10^5$ cells/mL) in mice that had received twice intraperitoneal immunization, followed by bronchoprovocation twice with aerosolized ovalbumin with a 1-week interval. This murine model of biphasic AHR response is highly reproducible. Thus, this model seems to be a suitable tool to identify the potential contribution of inflammatory molecules and cells involved in the early phase of an asthmatic reaction, but it does not seem to be relevant to chronic asthma.

Using the newly developed protocol, we have here demonstrated that TNF- α plays a critical role in the development of the late-phase AHR and airway eosinophilic inflammation through cPLA₂ activation. Although

an association of TNF- α with pathogenesis of asthma has been suggested,⁸⁻¹⁴ little is known about the role of TNF- α in the development of late AHR. Keatings et al¹⁰ have suggested the association of TNF- α with late response to allergen by showing that the concentration of TNF- α was significantly elevated in induced sputum after allergen challenge in atopic patients with asthma. Moreover, our data showing that TNF- α blockade using anti-TNF- α antibody or TNF- $\alpha^{-/-}$ mice resulted in significant inhibition in the late AHR and airway eosinophilia have demonstrated for the first time the role of TNF- α as a key cytokine in the development of late AHR and subsequent airway inflammation. Consistent with the findings,¹⁰ we have also observed an elevated TNF- α level in BAL fluid after allergen challenge. It has been reported that mast cells represent potential sources of TNF- α , which can be released either rapidly from preformed stores or, with more prolonged kinetics, from transcripts produced in

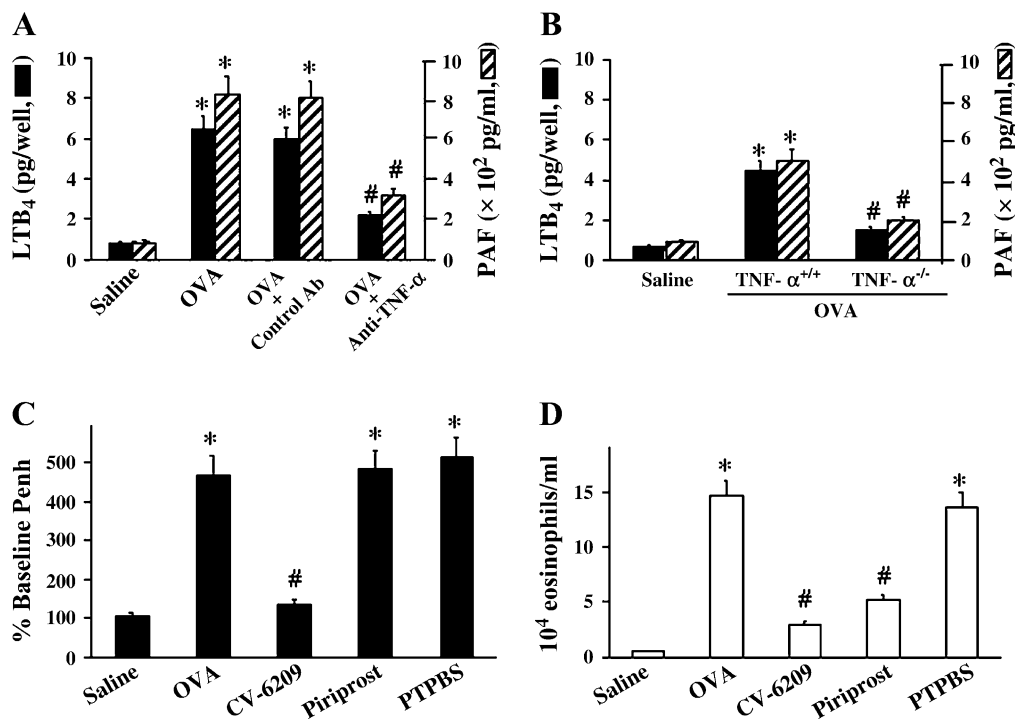


FIG 4. TNF- α dependency of cPLA₂ metabolites (LTB₄ and PAF) synthesis (**A and B**) and effects of PAF antagonist (CV 6209), 5-lipoxygenase inhibitor (piriprost), and COX-2 inhibitor (PTPBS) on the late AHR (**C**) and airway eosinophilia (**D**). Data represent the means $\pm$ SDs of 3 to 5 separate experiments ($n = 5$ per group). * $P < .05$ vs saline; # $P < .05$ vs ovalbumin.

response to appropriate activation of the cell.²⁵ However, our kinetic study has suggested that TNF- α is not released from preformed stores in mast cells but is synthesized in the airway.

Cytosolic phospholipase A₂ plays a key role in many signal transduction cascades by hydrolyzing arachidonic acid from membrane phospholipids. Tight control of cPLA₂ activity by several regulatory mechanisms is essential to its cellular function. cPLA₂ has been reported to be importantly implicated in the pathogenesis of asthma.^{26,27} The physiological effect of the enzyme is probably attributed to its capability to generate various eicosanoids, such as leukotrienes, prostaglandins, and PAF. These metabolites have been shown to induce airway eosinophilia and bronchoconstriction.²⁷⁻³⁰ However, the mechanism for the activation of cPLA₂ in asthmatic lungs remains to be elucidated. Our results have indicated that TNF- α is responsible for the activation of cPLA₂. This conclusion comes from the observations that cPLA₂ activation took place in the asthmatic lungs, which was almost completely abolished by pretreatment with anti-TNF- α antibody or in TNF- α ^{-/-} mice. Studies have indicated that proinflammatory properties of TNF- α are associated with its capacity to activate cPLA₂.³¹⁻³³ In addition, a report showing that TNF- α can induce cPLA₂ activation in bronchial epithelial cells³⁴ further supports the causal relationship between TNF- α and cPLA₂ in the pathogenesis of asthma. Among the cPLA₂ inhibitors tested in this study, PAF antagonist was effective to inhibit both late AHR and

airway eosinophilia. However, PAF antagonists have proved to be very disappointing for the treatment of human asthma, producing minor or no effects, even during chronic treatment.^{35,36} This may be because (1) there is a species difference in the role of PAF in the pathogenesis of asthma, (2) PAF does not play an important role in chronic asthma, or (3) the dosage of PAF antagonist is not sufficient to completely block PAF activity in the human study.

Taken together, we have identified TNF- α -induced cPLA₂ activation as an important mechanism for the development of the late AHR in asthma. However, the relationship does not seem to be restricted to asthma. Recently, we also demonstrated that TNF- α develops late-phase anaphylaxis through activation of cPLA₂ (Unpublished data, HK Lee et al, September 2004). Thus, the cascade involving TNF- α -induced cPLA₂ activation and a development of late-phase reaction by subsequent generation of eicosanoids appears to be the initial underlying mechanism common to allergic diseases.

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