

# Aspirin attenuates the anti-inflammatory effects of theophylline via inhibition of cAMP production in mice with non-eosinophilic asthma

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Abbreviations: AERD, ASA-exacerbated respiratory tract disease; ASA, acetylsalicylic acid; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; LN, lymph node; SA, salicylic acid; Th1 cell, type 1 T helper cell; WT, wild type

## Abstract

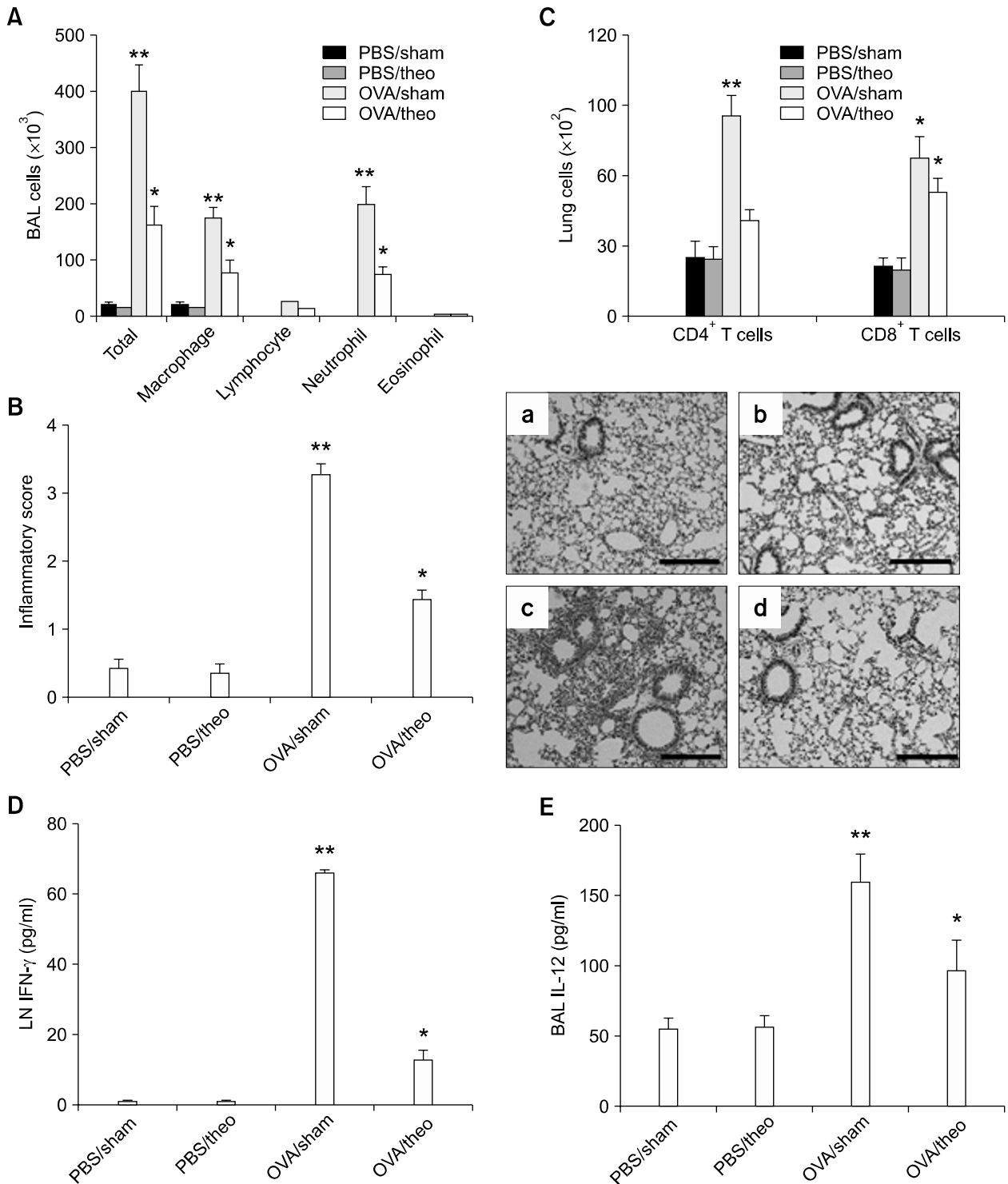
Theophylline is commonly used to treat severe asthma and chronic obstructive pulmonary disease (COPD) characterized by non-eosinophilic inflammation. Acetyl salicylic acid (ASA) is one of the most widely used medications worldwide, but up to 20% of patients with asthma experience aggravated respiratory symptoms after taking ASA. Here we evaluated the adverse effect of ASA on the therapeutic effect of theophylline in mice with non-eosinophilic asthma. A non-eosinophilic asthma mouse model was induced by airway sensitization with lipopolysaccharide-containing allergen and then challenged with allergen alone. Therapeutic intervention was performed during allergen challenge. Theophylline inhibited lung inflammation partly induced by Th1 immune response. ASA attenuated the beneficial effects of theophylline. However, co-administration of the ASA metabolite salicylic acid (SA) showed no attenuating effect on theophylline treatment. The therapeutic effect of theophylline was associated with increase in cAMP levels, which was

blocked by co-treatment of theophylline and ASA. ASA co-treatment also attenuated the anti-inflammatory effects of a specific phosphodiesterase 4 inhibitor. These results demonstrate that ASA reverses anti-inflammatory effects of theophylline, and that ASA exerts its adverse effects through the inhibition of cAMP production. Our data suggest that ASA reverses lung inflammation in patients taking theophylline, although clinical evidence will be needed.

**Keywords:** adverse effect; aspirin; asthma, aspirin-induced; cyclic AMP; cyclic nucleotide phosphodiesterases, type 4; drug toxicity; pneumonia; theophylline

## Introduction

Asthma is a complex syndrome with many clinical phenotypes in both adults and children. Its major characteristics include a variable degree of airflow obstruction, airway hyperresponsiveness, and eosinophilic or non-eosinophilic inflammation (Busse and Lemanske, 2001). Current asthma treatment protocols for those with severe symptoms or in need of frequent relief (more than twice weekly) recommend the use of preventative medications such as inhaled corticosteroids, which suppress inflammation and reduce swelling of the airway lining (Bateman *et al.*, 2008). If symptoms persist, additional anti-inflammatory drugs, such as theophylline, are added until the condition is controlled (Busse and Lemanske, 2007; Bateman *et al.*, 2008). Persistent asthma may be associated with significant neutrophilic lung inflammation (Gibson *et al.*, 2001), and in patients with difficult-to-control asthma, neutrophils rather than eosinophils often predominate (Green *et al.*, 2002). Meanwhile, chronic obstructive pulmonary disease (COPD) is a severe clinical condition that is characterized by irreversible airflow limitations (Scanlon *et al.*, 2000). The pathophysiology of irreversible airway obstruction in COPD is multifactorial and, similar to severe asthma, involves neutrophilic inflammation (Keatings *et al.*, 1996). Theophylline is also one of commonly used drugs for the treatment of COPD (Celli and MacNee, 2004).



**Figure 1.** Theophylline shows therapeutic effects on neutrophilic inflammation induced by lipopolysaccharide-containing allergens. Evaluation ( $n = 5$  each group) was performed after allergen challenge. PBS, mice sensitized with ovalbumin and then challenged with PBS; OVA, mice sensitized with ovalbumin + lipopolysaccharide and then challenged with ovalbumin; \* $P < 0.05$  compared to the PBS sensitized groups; \*\* $P < 0.05$  compared to other groups. (A) BAL cellularity 48 h after the final allergen challenge. (B) Lung inflammatory scores based on histology (left panel) and representative lung histological findings 48 h after the final allergen challenge. a: PBS\_sham; b: PBS\_theo; c: OVA\_sham; d: OVA\_theo (H&E stain,  $\times 200$ ). Magnification bar = 200  $\mu\text{m}$ . (C) Number of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in lung tissues 6 h after allergen challenge on day 21. (D) IFN- $\gamma$  levels in supernatants prepared from T-cells collected 6 h after anti-CD3 and anti-CD28 stimulation. T-cells were isolated from lung draining lymph nodes (LNs) 6 h after allergen challenge on day 21. (E) BAL IL-12p40 levels 48 h after the final allergen challenge.

Theophylline, also known as dimethylxanthine and marketed under a variety of brand names, has been conventionally used as a bronchodilator to relieve airway obstruction (Pauwels *et al.*, 1985); recently, its anti-inflammatory properties have been demonstrated to be more effective for the long-term control of asthma (Ward *et al.*, 1993). Although theophylline has been in clinical use for more than 60 years, its exact pharmacologic mechanism(s) of action remains uncertain, although proposed mechanisms include non-specific inhibition of adenosine receptor signalling (Cushley *et al.*, 1984) and increase of cAMP production due to phosphodiesterase inhibition (Rabe *et al.*, 1995).

Acetylsalicylic acid (ASA), also known as aspirin, is one of the most widely used medications in the world, with an estimated 40,000 metric tons consumed each year (Warner and Mitchell, 2002). ASA is often used as an analgesic to relieve pains, as an antipyretic to reduce fever, and as an anti-inflammatory medication; this drug also has an anti-platelet or anti-clotting effect and is commonly used to prevent heart attacks, strokes, and blood clot formation (Lewis *et al.*, 1983). However, ASA use may also lead to ASA-exacerbated respiratory tract disease (AERD), which is a common and often under-diagnosed disease that affects up to 20% of the adult asthmatic population (Hamad *et al.*, 2004). This condition is associated with more severe asthma requiring theophylline treatment in addition to inhaled corticosteroids (Hamad *et al.*, 2004; Morwood *et al.*, 2005). A number of hypotheses regarding the pathogenesis of AERD have been proposed (Hamad *et al.*, 2004). However, to the best of our knowledge, there are no data regarding pharmacodynamic interaction between ASA and theophylline, in the treatment of non-eosinophilic lung inflammation characterized by severe asthma and COPD. In the present study, we hypothesized that ASA would antagonize lung inflammation during treatment with theophylline.

## Results

### Therapeutic effect of theophylline against non-eosinophilic lung inflammation induced by lipopolysaccharide-containing allergens

Recently, we reported non-eosinophilic asthma phenotypes induced by airway sensitization with lipopolysaccharide-containing allergens, which are induced by allergen-specific type 1 T helper cell (Th1) response (Kim *et al.*, 2007). We first evaluated the therapeutic effects of theophylline treatment on the development of lung inflammation in the lipopolysaccharide asthma model. To test

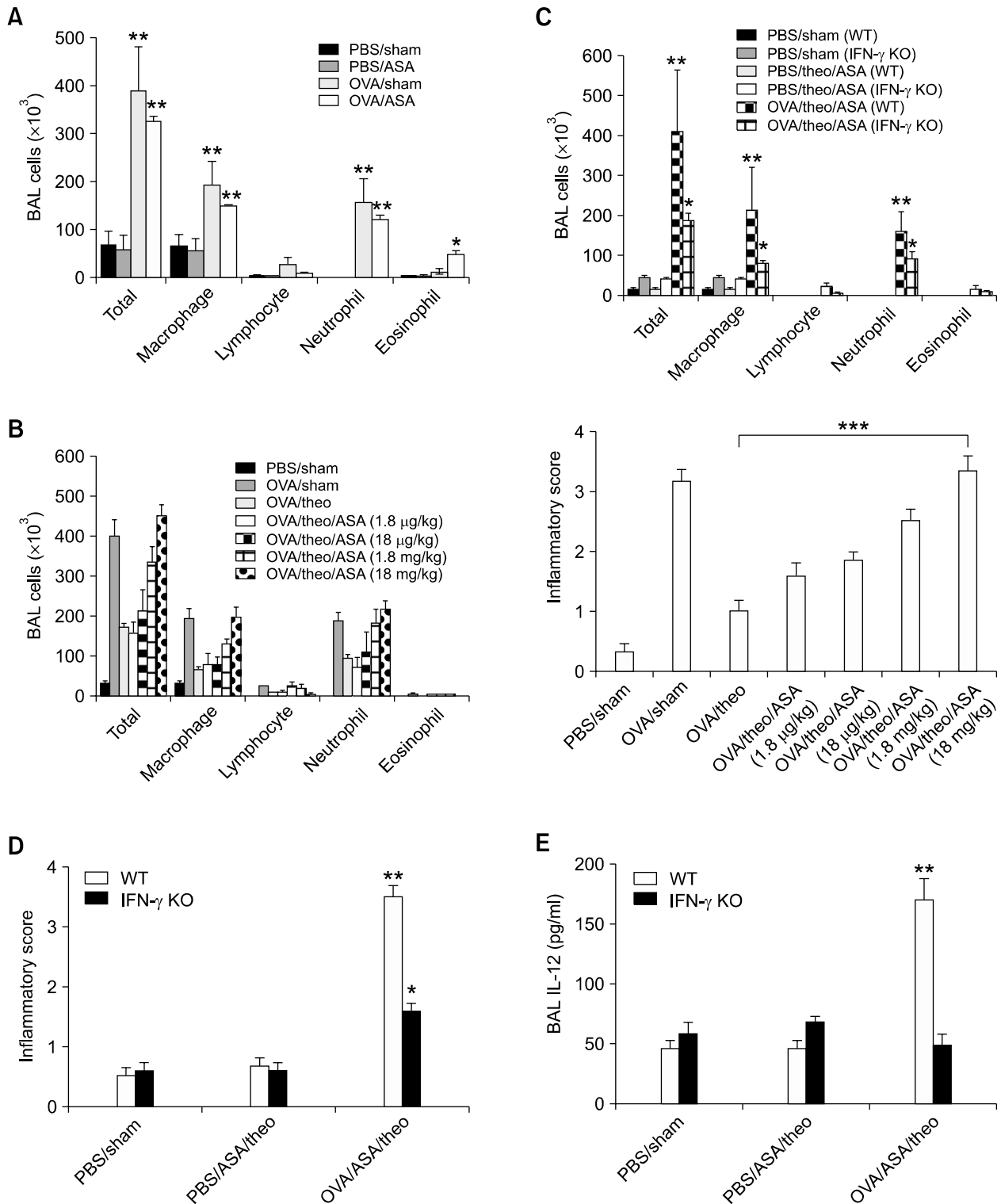
this, theophylline (10 mg/kg) was administered intraperitoneally during allergen challenge on days 14, 15, 21, and 22. Theophylline reduced the number of inflammatory cells (especially neutrophils) in BAL fluids, the production of which increased after sensitization with lipopolysaccharide-containing allergen (ovalbumin/lipopolysaccharide) (Figure 1A). Histological analysis of the lung showed that peribronchiolar and perivascular inflammatory cell infiltration (as indicated by inflammatory scores) also decreased significantly in ovalbumin/lipopolysaccharide-sensitized mice treated with theophylline, compared to sham-treated ovalbumin/lipopolysaccharide-sensitized mice (Figure 1B). In addition, theophylline treatment reduced CD4<sup>+</sup> T-cell recruitment into the lung after allergen challenge (Figure 1C), and significantly inhibited IFN- $\gamma$  production by T-cells in lung-draining LNs after stimulation with anti-CD3 and anti-CD28 (Figure 1D). Similarly, the levels of IL-12 (a downstream cytokine of IFN- $\gamma$ ) were decreased significantly in BAL fluids collected from theophylline-treated, ovalbumin/lipopolysaccharide-sensitized mice compared to sham-treated mice sensitized in the same manner (Figure 1E). Taken together, these findings suggest that theophylline treatment reduces lung inflammation primarily through inhibition of allergen-specific Th1 immune response.

### Adverse effects of ASA on the anti-inflammatory effects of theophylline

Based on the results that theophylline inhibits lung inflammation, we then assessed the adverse effects of ASA when co-administered with theophylline. WT C57BL/6 mice were treated with theophylline and ASA in combination during allergen challenge in the lipopolysaccharide asthma model. This study showed that ASA treatment did not inhibit lung inflammation induced by ovalbumin/lipopolysaccharide sensitization (Figure 2A). However, co-treatment with theophylline and ASA attenuated theophylline-reduced BAL cellularity in a dose-dependent manner; in addition, the recruitment of inflammatory cells into the lung (assessed by lung inflammatory score), which was blocked by theophylline, was re-initiated by ASA in a dose-dependent manner (Figure 2B). To further analyze the adverse effect of ASA on the anti-inflammatory effect of theophylline, we administered ASA at 18 mg/kg.

### The role of IFN- $\gamma$ on the adverse effect of ASA on the anti-inflammatory effects of theophylline

Because sensitization with lipopolysaccharide-con-



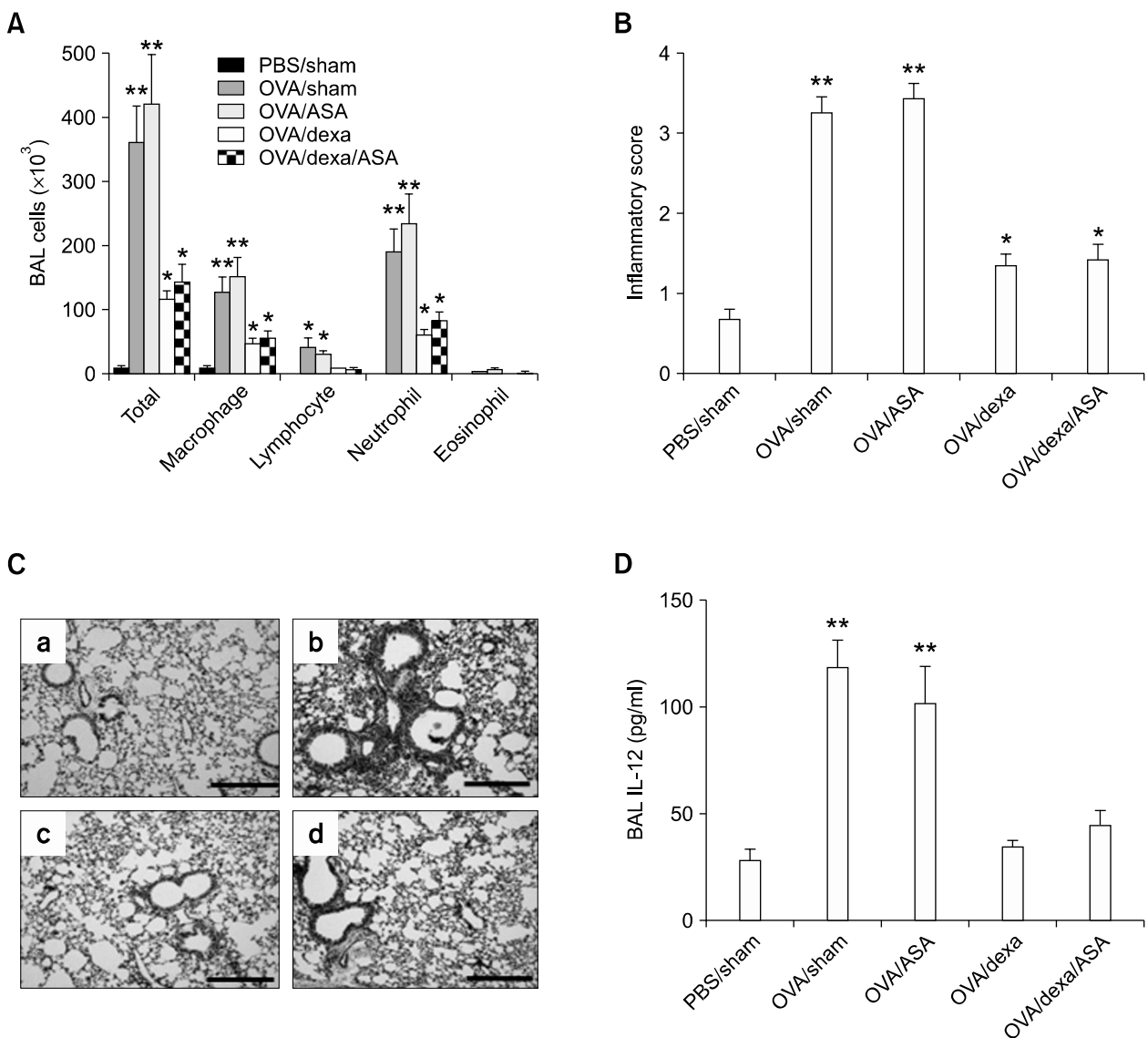
**Figure 2.** Acetylsalicylic acid (ASA) attenuates the therapeutic effects of theophylline. Evaluation ( $n = 5$  each group) was performed 48 h after the final allergen challenge. PBS, mice challenged with PBS; OVA, mice sensitized with ovalbumin + lipopolysaccharide and then challenged with ovalbumin; \* $P < 0.05$  compared to the PBS groups; \*\* $P < 0.05$  compared to other groups; \*\*\* $P < 0.05$  by ANOVA linearity testing. (A) BAL cellularity in response to ASA (18 mg/kg) in wild type (WT) mice. (B) BAL cellularity (left panel) and lung inflammatory scores based on histology (right panel) in response to different doses of ASA. (C) BAL cellularity in IFN- $\gamma^{-/-}$  and WT control mice in response to theophylline with or without ASA (18 mg/kg). (D) Lung inflammatory score based on histology in IFN- $\gamma^{-/-}$  and WT control mice. (E) BAL IL-12 levels in IFN- $\gamma^{-/-}$  and WT control mice.

taminated allergens induces lung inflammation mainly via the Th1 immune response (Kim *et al.*, 2007), we evaluated the role of IFN- $\gamma$  in ASA-exacerbated inflammation using IFN- $\gamma$  KO and WT (both C57BL/6 background) mice. Theophylline + ASA co-treatment significantly reduced BAL cellularity in ovalbumin/lipopolysaccharide-sensitized IFN- $\gamma$  KO mice compared to WT mice sensitized in the same manner (Figure 2C). Similarly, inflammatory cell recruitment into the lung (assessed by inflammatory score) was significantly lower in the former group compared to the latter (Figure 2D). In

addition, ovalbumin/lipopolysaccharide sensitization-induced IL-12 production was not detected in IFN- $\gamma$  KO mice (Figure 2E). These findings suggest that ASA reverses the anti-inflammatory effects of theophylline primarily via the Th1 immune response.

**Adverse effects of ASA on the anti-inflammatory effects of systemic corticosteroid treatment**

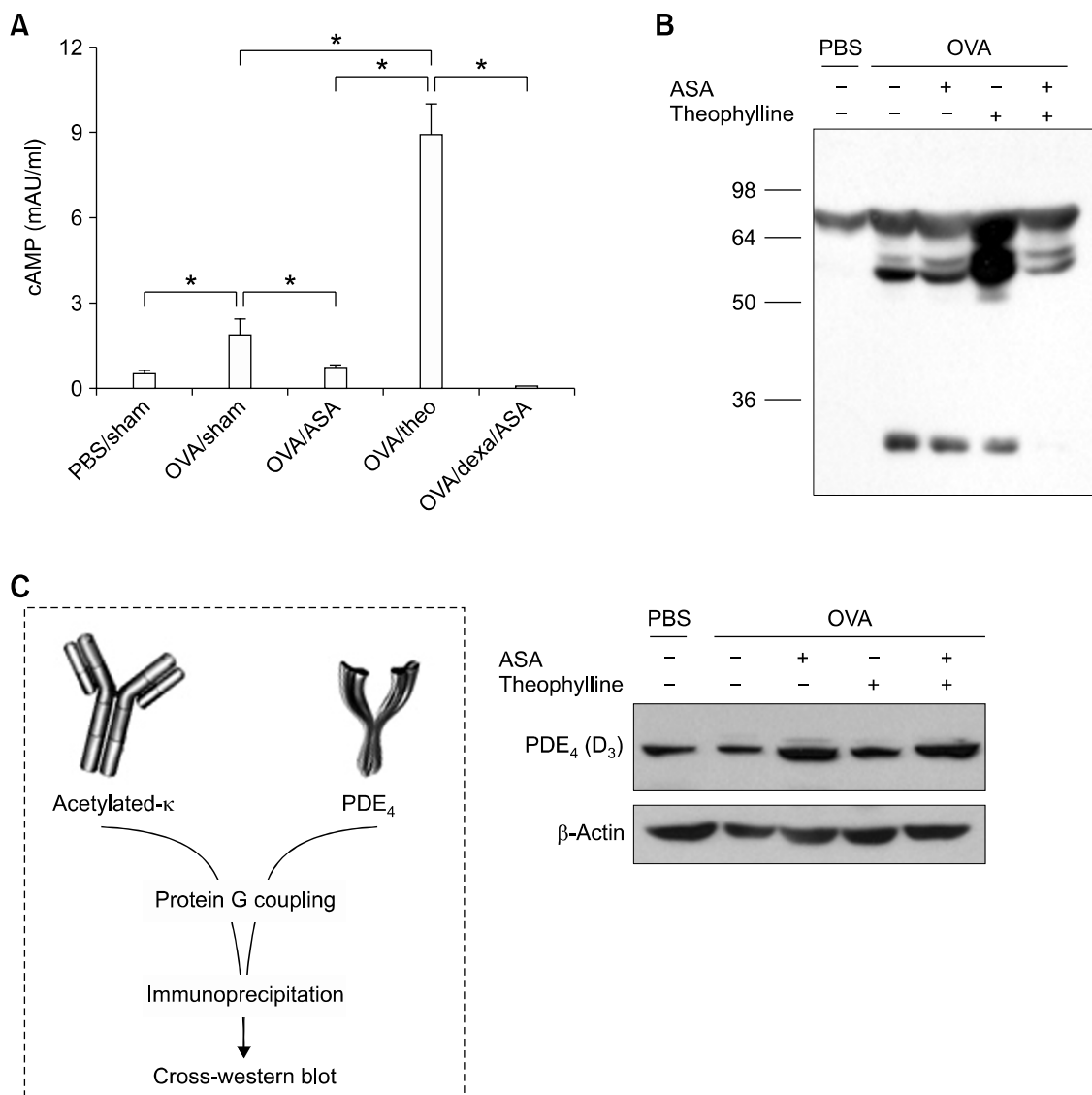
Corticosteroids are potent anti-inflammatory drugs against lung inflammation in asthma and COPD patients. We evaluated the effects of systemic



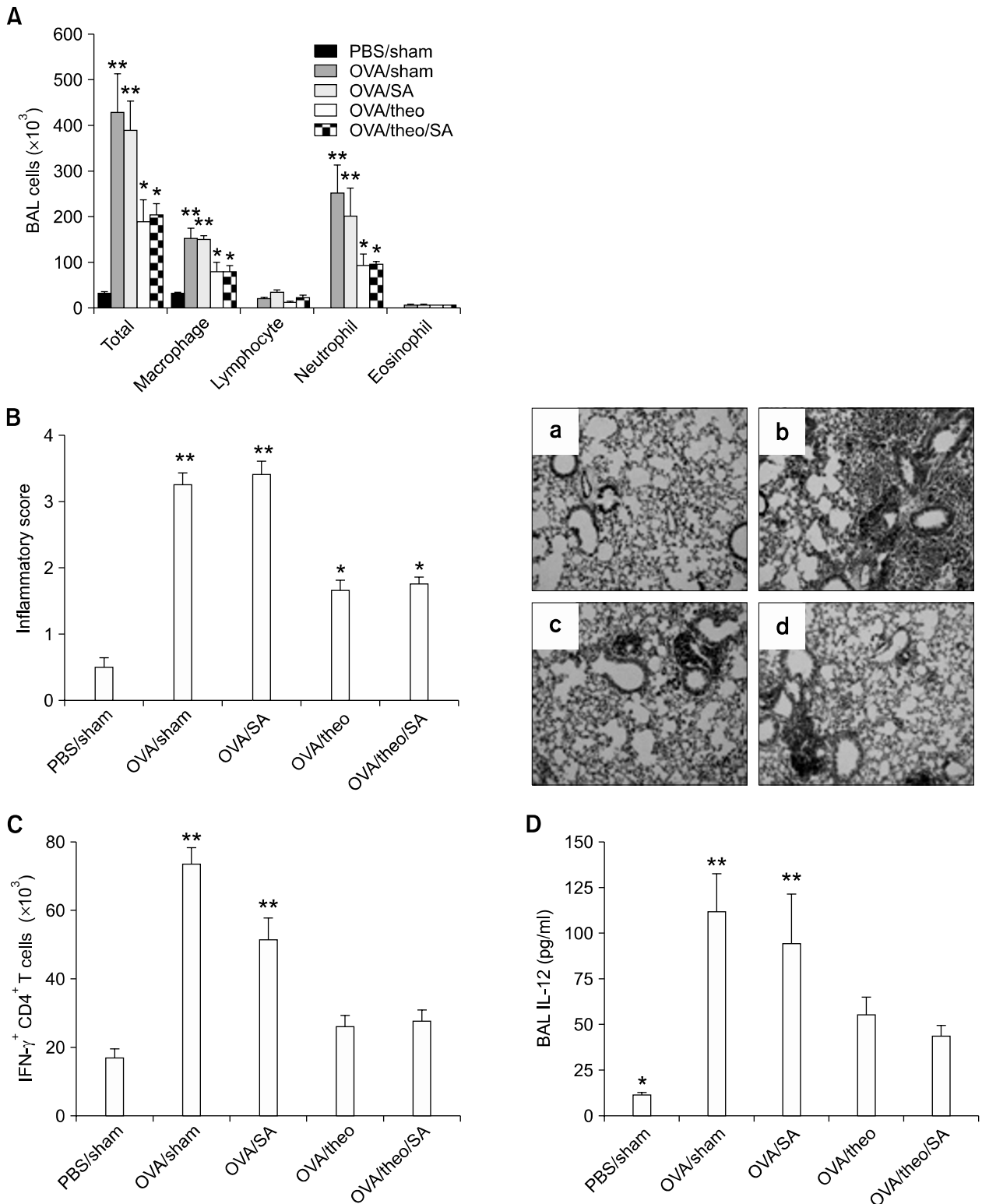
**Figure 3.** ASA shows no adverse effect on the anti-inflammatory properties of corticosteroid (dexamethasone). For all figures, evaluation ( $n = 5$  each group) was performed 48 h after the last allergen challenge; PBS, mice challenged with PBS; OVA, mice sensitized with ovalbumin + lipopolysaccharide and then challenged with OVA; \* $P < 0.05$  compared to the PBS\_sham group; \*\* $P < 0.05$  compared to the other groups. (A) BAL cellularity. (B) Lung inflammatory scores based on histology. (C) Representative lung histological findings. a: PBS\_sham; b: OVA\_ASA; c: OVA\_dexa; d: OVA\_dexa/ASA (H&E stain,  $\times 200$ ). Magnification bar = 200  $\mu\text{m}$ . (D) IL-12 levels in BAL fluids.

corticosteroid treatment, alone or in combination with ASA, on the treatment of neutrophilic lung inflammation. WT C57BL/6 mice were treated intraperitoneally with a corticosteroid (dexamethasone, 10 mg/kg) with or without ASA (18 mg/kg) during allergen challenge in the lipopolysaccharide asthma model. BAL cellularity, which increased in response to ovalbumin/lipopolysaccharide sensitization, was significantly inhibited by dexamethasone treatment alone, and ASA co-treatment did not attenuate this decrease in BAL cellularity (Figure 3A). Similarly, the ovalbumin/lipopolysaccharide sensitization-

duced recruitment of inflammatory cells (lung inflammatory score) was decreased significantly after dexamethasone treatment alone; this effect was not attenuated by ASA co-treatment (Figure 3B); moreover, this beneficial effect was evident from lung histological findings (Figure 3D). Moreover, ovalbumin/lipopolysaccharide sensitization-induced IL-12 production was decreased significantly after both treatment of dexamethasone alone and co-treatment with dexamethasone and ASA (Figure 3D). These findings indicate that systemic corticosteroid treatment inhibits lung inflammation,



**Figure 4.** cAMP levels is negatively correlated with acetylated phosphodiesterase (PDE)4 in lung tissues. Evaluation was performed 6 h after the final allergen challenge; PBS, mice challenged with PBS; OVA, mice sensitized with ovalbumin + lipopolysaccharide and then challenged with ovalbumin. (A) Cyclic AMP levels in lung tissues. \* $P < 0.05$ . Data from four mice in each group are shown. (B) Acetylation status of proteins isolated from lung tissues. (C) The protocol (left panel) and the levels of acetylated phosphodiesterase 4 (D<sub>3</sub>) in lung tissues (right panel) assessed by immunoprecipitation and cross-western blotting.



**Figure 5.** Salicylic acid (SA) does not attenuate the anti-inflammatory properties of theophylline. Evaluation ( $n = 5$  each group) was performed after allergen challenge. PBS, mice challenged with PBS; OVA, mice sensitized with ovalbumin + lipopolysaccharide and then challenged with ovalbumin; \* $P < 0.05$  compared to the PBS\_sham group; \*\* $P < 0.05$  compared to the other groups. (A) BAL cellularity 48 h after the final allergen challenge. (B) Lung inflammatory scores based on histology 48 h after the final allergen challenge (left panel) and representative lung histological findings (right panel) obtained 48 h after the final allergen challenge (a: PBS; b: OVA\_SA; c: OVA\_theo; d: OVA\_theo/SA, H&E stain, 200 $\times$ ). (C) Number of IFN- $\mu$ -producing CD4<sup>+</sup> T-cells in lung tissues 6 h after allergen challenge on day 21. (D) IL-12 in BAL fluids collected 48 h after the final allergen challenge.

and that ASA does not attenuate this therapeutic effect.

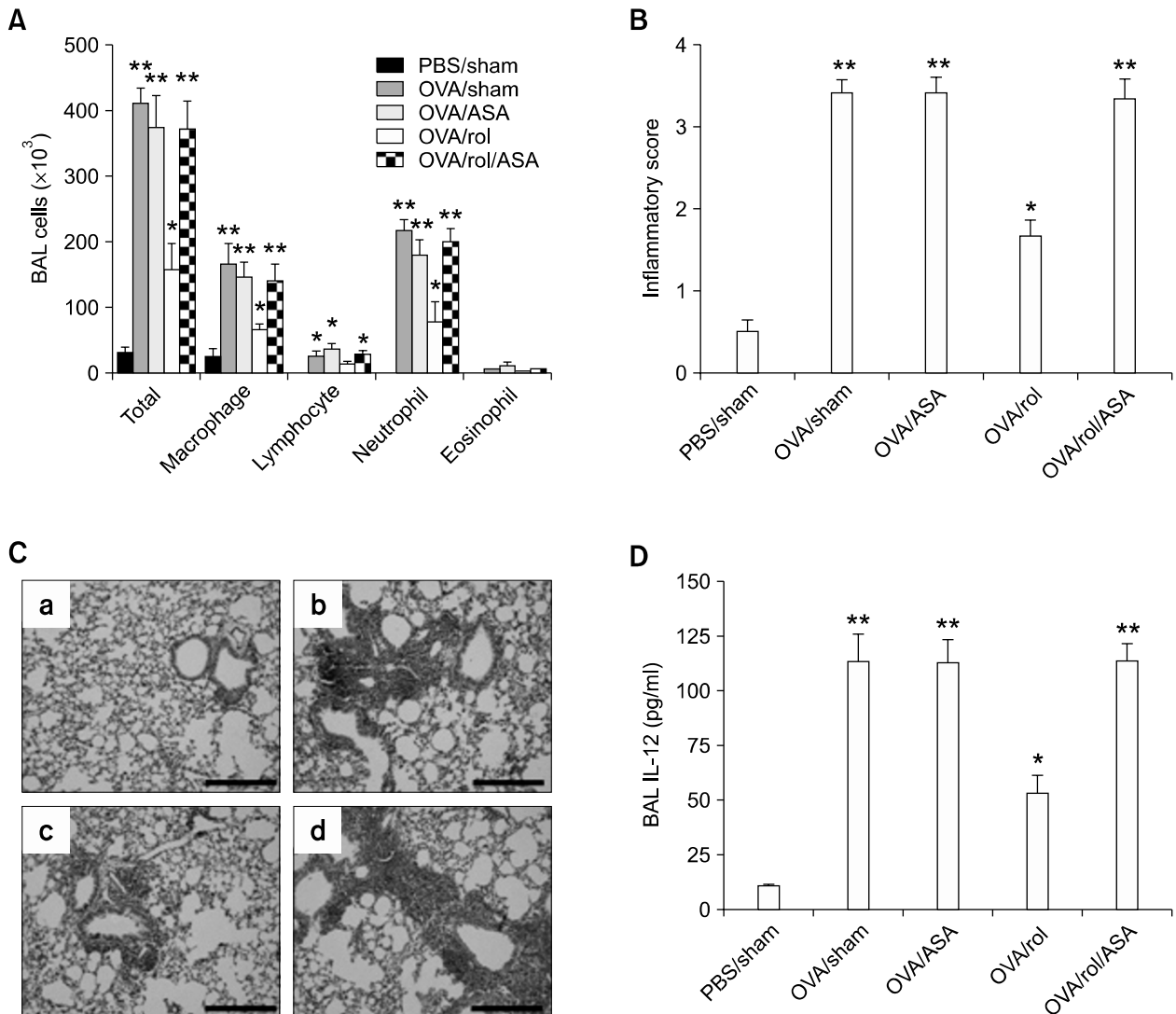
**The effects of ASA on the production of cAMP**

One of the proposed action mechanisms for theophylline is the non-specific inhibition of phosphodiesterases, which results in increased intracellular cAMP levels (Rabe *et al.*, 1995; Hidi *et al.*, 2000). We found that lung cAMP levels was enhanced by ovalbumin/lipopolysaccharide sensitization compared to negative control mice; theophylline treatment significantly increased cAMP

levels in the lungs of ovalbumin/lipopolysaccharide-sensitized mice compared to those treated with sham or ASA alone; however, the theophylline-enhanced cAMP levels were blocked by ASA co-treatment (Figure 4A). These findings suggest that theophylline treatment induces its beneficial effect via up-regulation of cAMP.

**The effects of ASA on the expression of phosphodiesterase 4**

The present study showed that acetylation status of proteins isolated from lung tissues after allergen



**Figure 6.** ASA attenuates the therapeutic properties of the phosphodiesterase 4 inhibitor rolipram. Evaluation ( $n = 5$  each group) was performed 48 h after the final allergen challenge; PBS, mice challenged with PBS; OVA, mice sensitized with ovalbumin + lipopolysaccharide and then challenged with ovalbumin. \* $P < 0.05$  compared to the PBS\_sham group; \*\* $P < 0.05$  compared to the other groups. (A) BAL cellularity. (B) Lung inflammatory scores based on histology. (C) Representative lung histological findings. a: PBS; b: OVA\_ASA; c: OVA\_rol; d: OVA\_rol/ASA (H&E stain, 200 $\times$ ). Magnification bar = 200  $\mu$ m. (D) IL-12 levels in BAL fluids.



challenge was different between PBS and ovalbumin/lipopolysaccharide sensitized mice; however, the acetylation status changed by ovalbumin/lipopolysaccharide sensitization was unaffected by treatment of ASA (Figure 4B). In contrast, acetylation status of lung proteins in mice sensitized with ovalbumin/lipopolysaccharide and treated with theophylline was altered by co-treatment of ASA (Figure 4B). These findings suggest that the adverse effect of ASA on the anti-inflammatory effect of theophylline may be related with acetylation of lung proteins altered by ASA. Among phosphodiesterases, the phosphodiesterase 4 subtypes form the predominant family in lung inflammatory cells (Dent *et al.*, 1991; Hidi *et al.*, 2000). This evidence led us to evaluate pharmacodynamic interactions on the phosphodiesterase 4 enzyme between ASA and theophylline in the lipopolysaccharide asthma model. As shown in Figure 4C, the amount of acetylated phosphodiesterase 4 (D3) subtype in lung tissues 6 h after allergen challenge was enhanced in ovalbumin/lipopolysaccharide-sensitized mice treated with ASA alone compared to ovalbumin/lipopolysaccharide-sensitized mice treated with sham. In contrast, phosphodiesterase 4 was decreased in ovalbumin/lipopolysaccharide-sensitized mice treated with theophylline alone compared to ovalbumin/lipopolysaccharide-sensitized mice treated with ASA alone. However, the amount of phosphodiesterase 4 was increased in ovalbumin/lipopolysaccharide-sensitized mice treated with ASA + theophylline compared to ovalbumin/lipopolysaccharide-sensitized mice treated with theophylline alone. Taken together, these findings indicate that ASA enhances acetylated phosphodiesterase 4 in lung tissues, which may inhibit cAMP production.

#### **Adverse effects of SA on the anti-inflammatory effects of theophylline**

SA is produced when ASA is hydrolyzed by esterases in the liver and used as an anti-pyretic and anti-inflammatory drug (Rainsford and Whitehouse, 1980). To evaluate the potential adverse effects of SA moiety of ASA on theophylline therapy, WT C57BL/6 mice were treated with theophylline (10 mg/kg) with or without SA (10 mg/kg) during allergen challenge in the lipopolysaccharide asthma model. BAL cellularity was not decreased in response to SA treatment alone or SA + theophylline co-treatment in ovalbumin/lipopolysaccharide-sensitized mice (Figure 5A). Similarly, ovalbumin/lipopolysaccharide sensitization-induced lung inflammation indicated by inflammatory scores was not decreased after SA

treatment alone or SA + theophylline co-treatment; lung histology also showed that the beneficial effect of theophylline was not attenuated by SA co-treatment (Figure 5B). Moreover, the recruitment of IFN- $\gamma$ -producing CD4<sup>+</sup> T-cells into the lung was similar in ovalbumin/lipopolysaccharide-sensitized mice treated with theophylline alone and those co-treated with theophylline + SA (Figure 5C). In addition, theophylline-induced IL-12 down-regulation in ovalbumin/lipopolysaccharide-sensitized mice was unaffected by SA co-treatment (Figure 5D). These findings indicate that the adverse effect of ASA on theophylline-induced benefits is not related with the SA moiety of ASA.

#### **Adverse effects of ASA on the anti-inflammatory effects of a specific phosphodiesterase 4 inhibitor**

Among phosphodiesterases, phosphodiesterase 4 is the major cAMP-metabolizing enzyme, and phosphodiesterase 4 inhibitors are potential anti-inflammatory drugs, especially in inflammatory respiratory diseases such as asthma and COPD (Torphy *et al.*, 1999). This evidence led us to evaluate the adverse effect of ASA on the therapeutic effect of a specific phosphodiesterase 4 inhibitor. WT C57BL/6 mice were treated with the specific phosphodiesterase 4 inhibitor rolipram (20 mg/kg) with or without ASA (18 mg/kg) during allergen challenge in the lipopolysaccharide asthma model. The present study showed that BAL cellularity was significantly lower in ovalbumin/lipopolysaccharide-sensitized mice treated with rolipram than in ovalbumin/lipopolysaccharide-sensitized mice treated with sham; however, this beneficial effect of rolipram was attenuated by ASA co-treatment (Figure 6A). Similarly, ovalbumin/lipopolysaccharide sensitization-induced lung inflammation, as indicated by increased inflammatory scores, was decreased significantly in response to treatment with rolipram alone, but this effect was also attenuated by the addition of ASA (Figures 6B and 6C). Likewise, ovalbumin/lipopolysaccharide sensitization-induced IL-12 up-regulation was inhibited by treatment with rolipram alone; moreover, the rolipram-induced IL-12 down-regulation was reversed by ASA co-treatment (Figure 6D). These findings indicate that ASA reverses the anti-inflammatory effect due to phosphodiesterase 4 inhibition.

## **Discussion**

ASA treatment is a cornerstone therapy for anti-pyretic treatment (400-500 mg/day) and pre-

vention of atherosclerosis (75-325 mg/day), with an event-reduction rate of up to 33% (2002). However, some patients are unable to tolerate ASA, leading to AERD (Stevenson, 2004), this condition is more common in severe asthma (Hamad *et al.*, 2004; Morwood *et al.*, 2005), requiring theophylline treatment in addition to the regular use of inhaled corticosteroids (Bateman *et al.*, 2008). Given the prevalence of atherosclerosis and chronic inflammatory lung diseases such as asthma and COPD, it is important to evaluate the adverse effects of ASA on non-eosinophilic lung inflammation (the underlying pathology of severe asthma and COPD) in patients undergoing theophylline therapy. To the best of our knowledge, we are the first to demonstrate that ASA reverses anti-inflammatory effects of theophylline and rolipram when ASA was simultaneously administered. Moreover, we found that the attenuating effect of ASA on theophylline-inhibited inflammation was related with decrease of cAMP production. These findings suggest that ASA reverses anti-inflammatory effects of theophylline by inhibition of cAMP production.

Although theophylline has been in use for several decades, its pharmacologic mode of action in asthma and COPD remains unclear. Theophylline has a bronchodilatory effect and effectively attenuates lung inflammation in asthma or COPD patients (Sullivan *et al.*, 1994). In patients with nocturnal asthma, theophylline inhibits the influx of neutrophils and, to a lesser extent, eosinophils (Kraft *et al.*, 1996). It also inhibits neutrophilic inflammation in patients with severe asthma (Wenzel *et al.*, 1997; Jatakanon, *et al.*, 1999; Kim *et al.*, 2007), and COPD (Culpitt *et al.*, 2002). *In vitro*, theophylline has inhibitory effects on inflammatory mediators released from neutrophils and on neutrophil chemotaxis (Nielson *et al.*, 1988; Yasui *et al.*, 2000). Similarly, our *in vivo* data indicate that theophylline inhibits neutrophilic lung inflammation induced by lipopolysaccharide-containing allergens. Taken together, these findings suggest that theophylline effectively inhibits non-eosinophilic lung inflammation in patients with asthma or COPD.

Theophylline exhibits several immunomodulatory properties. It reduces the recruitment of activated T-cells into the lungs of asthmatic individuals and decreases the number of activated T-cells in BAL fluids collected after allergen challenge (Kidney *et al.*, 1995; Jaffar *et al.*, 1996). Our previous data indicate that neutrophilic lung inflammation due to lipopolysaccharide-containing allergen sensitization occurs primarily through the Th1 immune response (Kim *et al.*, 2007). Our present *in vivo* data show

that theophylline inhibits the recruitment of Th1 cells into the lung after allergen challenge, thus reducing neutrophilic inflammation. Taken together, these findings suggest that theophylline reduces neutrophilic inflammation primarily through inhibition of the Th1 immune response.

Several action mechanisms of theophylline have been proposed. For example, at therapeutic concentrations, theophylline treatment is associated with the non-specific inhibition of adenosine receptors (Pauwels and Joos, 1995), which in turn result in its therapeutic effects including immune modulation (Zidek, 1999). However, some evidence indicates that extracellular adenosine attenuates IL-12 production in dendritic cells and inhibits the initiation of the Th1 immune response (Ia Sala *et al.*, 2001). Previous studies also showed that the *in vivo* activation of adenosine receptors in Th1 cells directly inhibited the expansion of Th1 cells (Erdmann *et al.*, 2005). Our present data demonstrate that theophylline-induced inhibition of the Th1 immune response is positively associated with cAMP enhancement. Along with the evidence that adenosine is a metabolite of cAMP (Jackson *et al.*, 1997), these findings suggest that the cAMP-adenosine pathway is a negative regulator of the Th1 immune response, and that the immune modulatory effect of theophylline is mediated by mechanism(s) other than its adenosine receptor antagonism.

Phosphodiesterases hydrolyze the phosphodiester bond present in cyclic second messengers, such as cAMP and cGMP; thus, treatment with the non-specific phosphodiesterase inhibitor theophylline results in increased cAMP and cGMP levels (Lugnier *et al.*, 1986). Indeed, our present data showed that theophylline treatment enhanced cAMP production in lung tissues. The phosphodiesterase 4 subtypes form the predominant family of phosphodiesterases in inflammatory cells, including T-lymphocytes, neutrophils, eosinophils, mast cells, and macrophages (Dent *et al.*, 1991; Hidi *et al.*, 2000). *In vitro* data have indicated that phosphodiesterase 4 inhibitors reduce release of mediators from human neutrophils (Au *et al.*, 1998). Our *in vivo* data also showed that rolipram, a phosphodiesterase 4 inhibitor, reduced neutrophilic inflammation after an allergen challenge. These findings suggest that phosphodiesterase 4 inhibitors may be useful in the treatment of severe asthma and COPD, which may involve neutrophilic inflammation. Moreover, phosphodiesterase 4 inhibitors preferentially block the production of Th1 cytokines *in vitro* (Claveau *et al.*, 2004). Here, we showed that rolipram blocked the Th1 immune response. Taken together, these findings suggest

that the immunomodulatory and anti-inflammatory effects of theophylline in the asthma model are related with phosphodiesterase 4 inhibition.

SA is a hydrolytic metabolite of ASA that lacks the acetyl moiety present on ASA. Although metabolic drug interactions involving ASA are theoretically possible, no conclusive data exist to demonstrate that ASA affects the metabolism of co-administered drugs. ASA blocks the biosynthesis of prostaglandins from arachidonic acid (Roth *et al.*, 1975), which is mediated by the incorporation of the acetyl moiety of ASA into cyclooxygenase (Roth *et al.*, 1975). ASA selectively acetylates the Ser residue of cyclooxygenase-1, thereby inactivating cyclooxygenase activity of the enzyme without affecting peroxidase activity (Shimokawa and Smith, 1992). Furthermore, PGE2 is one of cyclooxygenase metabolite that controls inflammation in the lung (Vancheri *et al.*, 2004). We have no experimental evidence whether this PGE2 may participate in reverse effects of ASA or not and SA has poor ability to block cyclooxygenase activity. However, like cyclooxygenase inhibition, ASA may inhibit phosphodiesterase 4 enzymatic activity through acetylates phosphodiesterase 4 certain residues because SA did not represent reverse effects of theophylline. Our present data indicate that the anti-inflammatory effects of theophylline are not inhibited by SA, suggesting that the acetyl moiety of ASA is responsible for the adverse effects of ASA on theophylline treatment. In the present study, we found that expression of phosphodiesterase 4 in lung tissues in the non-eosinophilic asthma model was enhanced by treatment of ASA. Moreover, we demonstrated that ASA attenuated the anti-inflammatory effect induced by phosphodiesterase 4 inhibition. Taken together, these findings suggest that the anti-inflammatory effects of theophylline via phosphodiesterase 4 inhibition appear to be attenuated via ASA that has possibility of phosphodiesterase 4 acetylation.

In summary, our data demonstrate that ASA attenuates the anti-inflammatory effects of theophylline when concomitantly administered with theophylline. Our present study suggest that ASA prescription is avoided in patients with asthma and COPD patients taking theophylline alone and combinatorial therapy with corticosteroids, although clinical evidence will be needed.

## Methods

### Mice

Wild type (WT) and IFN- $\gamma$  knockout (KO) (both C57BL/6

background) mice were purchased from Jackson Laboratories (Bar Harbor, ME), and were bred in an animal facility at Pohang University of Science and Technology (POSTECH) in Pohang, Republic of Korea. Animal study protocols were approved by the Institutional Animal Care and Use Committee of POSTECH Biotech Center (PBC).

### Chemicals and drugs

To generate a non-eosinophilic asthma mouse model, lipopolysaccharide and ovalbumin was purchased from Calbiochem (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO), respectively. Theophylline (aminophylline) was purchased from Daewon Pharmaceutical Co. Ltd. (Seoul, Korea), ASA (aspirin-lysine) from Ilyang Pharmaceutical Co. (Seoul, Korea), salicylic acid (SA) from Sigma-Aldrich (St. Louis, MO), and dexamethasone and rolipram from Calbiochem (La Jolla, CA). Lipopolysaccharide, ovalbumin, theophylline, ASA, and SA were dissolved in PBS and rolipram and dexamethasone in DMSO, respectively.

### Protocols for generation of a non-eosinophilic asthma mouse model and for pharmacologic intervention

Lipopolysaccharide was depleted from ovalbumin as previously described (Kim *et al.*, 2007). A mouse model with non-eosinophilic (or neutrophilic) inflammation was generated as previously described (Kim *et al.*, 2007). Briefly, 6-week-old mice were sensitized intranasally with 75  $\mu$ g ovalbumin and 10  $\mu$ g lipopolysaccharide on days 0, 1, 2, and 7, and then challenged four times intranasally with 50  $\mu$ g ovalbumin on days 14, 15, 21, and 22. As a negative control, mice were sensitized with ovalbumin (75  $\mu$ g) alone and then challenged with PBS (30 ml). Drugs (theophylline, ASA, dexamethasone, SA, and rolipram) were administered intraperitoneally on days 14, 15, 21, and 22. To evaluate the therapeutic and adverse effects of these drugs, ovalbumin-specific adaptive immune response and lung inflammation were evaluated 6 h after the ovalbumin challenge on day 21 and 48 h after the final ovalbumin challenge on day 22, respectively.

### Cellularity in bronchoalveolar lavage (BAL) fluids

BAL fluids were obtained as previously described (Kim *et al.*, 2007). Briefly, mice were anesthetized with ketamin and trachea was isolated by blunt dissection. A small caliber tube was inserted and secured in the airway. Two successive volumes of 0.75 ml of PBS were instilled and gently aspirated and these two volumes were pooled. Each BAL sample was centrifuged and the supernatants were stored at -70°C until use. The total numbers of inflammatory cells were counted after dilution of cell pellets with 100  $\mu$ l of PBS. After Diff-Quick staining (Dade Behring) of BAL pellets in a cytospin preparation, types of inflammatory cells were determined by counting 300 cells, which were classified as macrophages, lymphocytes, neutrophils, or eosinophils. And then percentages of differential cell counts were re-calculated with total cell number.

### Histological evaluation of lung inflammation

Lung tissues were obtained as previously described (Kim *et al.*, 2007). Briefly, the lung was perfused with cold PBS through the right ventricle until the pulmonary vasculature was clean. The whole lung was then inflated with histological fixative. Hematoxylin and eosin staining was performed after pressure fixation with Streck solution (Streck Laboratories, Omaha, NE). For the purposes of comparison, the same magnification was used for all sample slides. Furthermore, lung inflammation was scored according to the degree of peribronchiol and perivascular inflammation using a previously described subjective scale (Park *et al.*, 2001). Briefly, values were assigned as follows: 0, no infiltration was detectable; 1, occasional cuffing with inflammatory cells; 2, most bronchi or vessels surrounded by a thin layer (one to five cells) of inflammatory cells; and 3, most bronchi or vessels surrounded by a thick layer (more than five cells deep) of inflammatory cells. The inflammatory score was determined by summing the peribronchiolar and perivascular scores.

### Evaluation of immunological parameters

To identify T-cells recruited into the lung, FACS analysis was performed using antibodies for T-cell surface markers, including anti-CD3, anti-CD4, and anti-CD8. After preparing single-cell suspensions from the lung tissue, cells were counted as previously described (Kim *et al.*, 2007). To identify individual cells of interest,  $1 \times 10^6$  isolated cells were aliquoted into tubes and stained with fluorescein isothiocyanate (FITC)-conjugated cell surface markers (BD Biosciences Pharmingen, San Diego, CA). To determine intracellular cytokine levels, isolated T-cells were incubated at 37°C for 3 h in RPMI medium containing 10% FBS and 2 µg/ml brefeldin A (Sigma-Aldrich), washed, and immunostained on ice for 30 min. Cells were then washed in PBS containing 3% FBS and 0.1% Na<sub>3</sub>, followed by fixation in PBS containing 4% formaldehyde for 20 min. After washing, the cells were permeabilized with 0.5% saponin (Sigma-Aldrich) in PBS for 10 min, centrifuged, re-suspended in 50 µl of the same solution, and stained with anti-IFN-γ antibodies for 30 min. The cells were analyzed using a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ), and the results were processed using CellQuest software (BD Biosciences). The number of cells in the lung was determined by multiplying total lung cells by the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T-cells. To measure cytokine expression in lung draining lymph nodes (LNs), isolated T-cells ( $1 \times 10^6$  cells) were stimulated with a nonspecific stimulus (anti-CD3 and anti-CD28) for 6 h and then the cytokines in the supernatants were measured via ELISA.

### ELISA

IFN-γ and IL-12 (p40 subunit) levels were quantified via ELISA as previously described (Kim *et al.*, 2007).

### Evaluation of cAMP levels in lung tissues

To determine cAMP levels in lung cells, samples were processed as previously described (Resta *et al.*, 1997). In

short, mice were sacrificed at 6 h after the ovalbumin challenge on day 21 and the lungs were frozen in liquid nitrogen. Later lung samples were homogenized in 0.85 ml of ice-cold 0.4 N HClO<sub>4</sub>. The samples were placed on ice for 15 min and then centrifuged for 2 min at 4°C. The supernatants were transferred to a clean tube and 0.7 ml of the supernatant were neutralized with 0.35 ml of 0.6 M KHCO<sub>3</sub>/0.72 M KOH. The samples were clarified by centrifugation for 1-2 min at full speed in a microcentrifuge at 4°C and then analyzed by HPLC using a 0.50-ml injection volume. The levels of cAMP were calculated from the areas under the respective peaks using authentic standards (cAMP purchased from Sigma-Aldrich) and an internal peak to correct for variations in extraction efficiency and sample loading.

### Western blot analysis and immunoprecipitation assay

The protein concentrations of the whole cell lysate (WCL) using a DC protein assay kit (BioRad, Hercules, CA) with bovine serum albumin as the standard. The proteins in the WCLs (30 µg) were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 3% non-fat skim milk in TBS with 0.05% tween-20 for 1 h at room temperature, the membranes were incubated for 2 h at room temperature with antibodies against phosphodiesterase 4 (Abcam, Cambridge, United Kingdom). Then the blots were washed and incubated with a secondary antibody conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The immunoreactive bands were visualized using a chemiluminescent substrate.

For immunoprecipitation assay, phosphodiesterase 4 and acetylated lysine antibodies were coupled to Protein G Magnetic Agarose Resin (Elpis biotech, South Korea). Antibodies coupling reaction was conducted in 1 mM disuccinimidyl suberate solution (Pierce, Rockford, IL) and quenched by 50 mM ethanolamine. Beads were washed with PBS and 1 M glycine (pH 3) to remove unlinked antibodies. Beads were stored in PBS with 0.02% sodium azide until use. WCL (100 µg) was incubated with antibodies coupled beads for overnight, washed with cell lysis buffer containing 50 mM Tris-Cl (pH 7.5), 1% NP-40, 0.25% Na-deoxycholate, 100 mM NaCl, 1 mM EDTA and protease inhibitors and eluted in 2 × SDS loading dye.

### Statistic analysis

Significant differences among treatment groups were assessed using Analysis of variance (ANOVA), Student's *t*-test, or the Wilcoxon rank-sum test. ANOVA was initially used for multiple comparisons; if significant differences were found, individual *t*-tests or the Wilcoxon rank-sum test were used to distinguish between groups.

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