

Glycogen Synthase Kinase-3 β Inhibition Attenuates Asthma in Mice

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Rationale: Persistent activation of nuclear factor- κ B has been associated with the development of asthma. Glycogen synthase kinase-3 β is known to regulate the activity of nuclear factor- κ B.

Objectives: We hypothesized that inhibition of glycogen synthase kinase-3 β may have anti-inflammatory effects in allergic asthma.

Methods: BALB/c mice sensitized and challenged with ovalbumin developed airway inflammation. Bronchoalveolar lavage fluid was assessed for total and differential cell counts, and for cytokine and chemokine levels. Lung tissues were examined for cell infiltration and mucus hypersecretion, and for the expression of inflammatory biomarkers. Serum immunoglobulin E levels were determined by enzyme-linked immunosorbent assay. Airway hyperresponsiveness was monitored by direct airway resistance analysis.

Measurements and Main Results: Intravenous administration of 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), a selective glycogen synthase kinase-3 β inhibitor, significantly inhibited ovalbumin-induced increases in total cell counts, eosinophil counts, and IL-5, IL-13, and eotaxin levels recovered in bronchoalveolar lavage fluid in a dose-dependent manner. TDZD-8 substantially reduced the serum levels of ovalbumin-specific IgE. Histologic studies showed that TDZD-8 dramatically inhibited ovalbumin-induced lung tissue eosinophilia and airway mucus production. TDZD-8 also markedly suppressed ovalbumin-induced mRNA expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, Muc5ac, and three members of the chitinase family (acidic mammalian chitinase, Ym1, and Ym2). In addition, TDZD-8 significantly reduced ovalbumin-induced airway hyperresponsiveness to inhaled methacholine. Western blot analysis of whole lung lysates revealed that TDZD-8 markedly attenuated the phosphorylation of the nuclear factor- κ B subunit p65 from ovalbumin-challenged mice.

Conclusions: Our findings suggest that inhibition of glycogen synthase kinase-3 β may provide a novel means for the treatment of allergic airway inflammation.

Keywords: bronchoalveolar lavage fluid; ovalbumin; nuclear factor- κ B; eosinophilia; mucus hypersecretion

Allergic asthma is characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) (1). These inflammatory responses are mediated by T-helper type 2 (Th2) cells, together with other inflammatory cells such as mast cells, B cells, and eosinophils, as well as a number of inflammatory cytokines and chemokines (1, 2). Interleukin (IL)-4 plays an important role in the initiation of Th2 inflammatory

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Persistent activation of nuclear factor (NF)- κ B is associated with asthma. Glycogen synthase kinase-3 β (GSK-3 β) is known to regulate NF- κ B activity.

What This Study Adds to the Field

In this study, inhibition of GSK-3 β reduced the severity of ovalbumin-induced allergic asthma.

responses (3). IL-5 is pivotal for the growth, differentiation, recruitment, and survival of eosinophils (4). IL-13 plays a prominent role in the effector phase of Th2 responses such as eosinophilic inflammation, mucus secretion, and AHR (5). In addition, chemokines such as RANTES (regulated on activation, normal T cells expressed and secreted) and eotaxin are central to the delivery of eosinophils to the airways (6). Airway eosinophilia, together with effector cytokines IL-5 and IL-13, may ultimately contribute to AHR in asthma (1, 5, 7).

Glycogen synthase kinase-3 (GSK-3) is a serine-threonine protein kinase that exists in two isoforms in mammalian cells, namely GSK-3 α and GSK-3 β . It was originally discovered based on its ability to phosphorylate and inactivate glycogen synthase, and modulate blood glucose levels (8). Recently, Hoefflich and coworkers (9) reported that GSK-3 β knockout mice resulted in embryonic lethality and displayed a phenotype consistent with mice having dysfunction in nuclear factor- κ B (NF- κ B) subunit p65 at the level of transcriptional complex. This defect was independent of NF- κ B nuclear translocation, inhibitory κ B (I κ B) degradation and NF- κ B-DNA-binding activity (9–11). NF- κ B is a major transcription factor essential for both innate and adaptive immunity. Under resting conditions, NF- κ B is prevented from entering the nucleus by forming a complex with I κ B. Upon stimulation, I κ B becomes phosphorylated by its direct upstream I κ B kinase (IKK) complex. Phosphorylated I κ B dissociates from NF- κ B, followed by degradation, and NF- κ B is then allowed to enter the nucleus to initiate gene transcription (12). Although the exact molecular mechanism of how GSK-3 β activates NF- κ B remains unclear (9), there is some evidence that inhibition of the activity of GSK-3 β may affect multiple steps in the cascade of events leading to the activation of NF- κ B. These include prevention of degradation of I κ B α , inhibition of nuclear translocation of p65, prevention of phosphorylation of p65, and association of CREB with p65 (10, 11, 13–15). Taken together, inhibition of GSK-3 β activity clearly leads to inhibition of NF- κ B activation (9), and this in turn results in potent anti-inflammatory effects in animal models of

(Received in original form September 12, 2006 and in final form June 6, 2007)

Supported by a BioMedical Research Council of Singapore grant BMRC/01/1/21/17/046.

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Am J Respir Crit Care Med Vol 176, pp 431–438, 2007

Originally Published in Press as DOI: 10.1164/rccm.200609-1292OC on June 7, 2007

Internet address: www.atsjournals.org

endotoxemia (16), colitis (17), and collagen-induced arthritis (18).

As asthma is associated with persistent NF- κ B activation (19, 20), we hypothesized that GSK-3 β inhibition may suppress ovalbumin (OVA)-induced Th2-mediated allergic airway inflammation. In this study, we investigated the potential anti-inflammatory effects of 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), a potent and selective small molecule inhibitor of GSK-3 β (21), in an *in vivo* mouse asthma model. Our results clearly indicate that inhibition of GSK-3 β modulates allergic airway inflammation. Some of the results of this study have been previously reported in abstract form (22).

METHODS

Animals

Female BALB/c mice, 6 to 8 weeks old (Interfauna, East Yorkshire, UK), were sensitized with OVA as described previously (23). On Days 22, 23, and 24, animals were challenged by 1% OVA aerosol for 20 minutes. The GSK-3 β inhibitor TDZD-8 (1, 3, and 10 mg/kg; Sigma, St. Louis, MO) or vehicle (10% dimethyl sulfoxide [DMSO]) in 0.1 ml was given by intravenous injection through tail vein 1 hour before each OVA aerosol challenge. Saline aerosol was used as a negative control. Animal experiments were performed according to the Institutional Guidelines for Animal Care and Use Committee of the National University of Singapore.

Bronchoalveolar Lavage Fluid and Serum Analyses

Mice were anesthetized 24 hours after the last aerosol challenge by an intraperitoneal injection of 100 μ l anesthetic mixture (medetomidine and ketamine; Apex Laboratories, Somersby, NSW, Australia). Bronchoalveolar lavage fluid (BALF) collection, total cell counts, and differential cell counts were performed as described previously (23). BALF cytokine and chemokine levels were measured using enzyme-linked immunosorbent assay (ELISA). Mouse IL-4 and IL-5 ELISA were obtained from BD PharMingen (San Diego, CA). Mouse IL-13 and eotaxin ELISA were purchased from R&D systems (Minneapolis, MN). Blood was collected by cardiac puncture, and serum levels of total immunoglobulin (Ig)E and OVA-specific IgE were determined using ELISA as previously described (23).

Fluorescence-activated Cell Sorter Phenotyping of Peripheral Blood Leukocytes

To determine potential cytotoxic effects of TDZD-8 on peripheral blood leukocytes, mice were treated with 10 mg/kg of intravenous TDZD-8 once daily for 3 days. Blood was collected by cardiac puncture. Peripheral blood leukocytes were incubated with 1 μ g of conjugated specific anti-CD3 (PE), anti-CD4 (FITC), anti-CD8 (FITC), anti-B220 (FITC), and anti-NK1.1 (FITC) Abs (BD Biosciences, San Jose, CA), or appropriate isotype controls (BD Biosciences) for 20 minutes at room temperature in the dark, and then analyzed on a FACSCaliber flow cytometer using CellQuest Pro software (Becton Dickinson, Mountain View, CA).

Histologic Examination

Lungs were fixed in 10% neutral formalin, paraffinized, cut into 6- μ m sections, and stained with hematoxylin and eosin for examining cell infiltration and with periodic acid-Schiff stain for measuring mucus production. Quantitative analysis was performed blinded as previously described (23).

Measurements of AHR

AHR was measured as previously described (24). Mice were anesthetized by an intraperitoneal injection of 100 μ l anesthetic mixture (medetomidine and ketamine) and tracheotomy was performed. The internal jugular vein was cannulated and connected to a microsyringe for

intravenous methacholine administration. The mice were placed in a whole-body plethysmograph chamber (Buxco, Sharon, CT) and ventilated mechanically by a ventilator (Hugo Sachs Elektronik, Harvard Apparatus, March-Hugstetten, Germany) at a tidal volume of 200 μ l/breath and a breath rate of 150/minute. Airflow changes in the sealed chamber and pressure changes in the airway were detected by respective transducers, preamplified by MAX and analyzed by the BioSystem XA software (Buxco). Lung resistance (R_L) and dynamic compliance (C_{dyn}) in response to increasing concentrations of methacholine were recorded and expressed as a percentage of the respective basal values in response to phosphate-buffered saline (PBS).

Human Bronchial Epithelial Cell Culture

Normal human bronchial epithelial cells (Cambrex BioScience, Walkersville, MD) were cultured in bronchial epithelial bulletkit medium with supplements (Cambrex BioScience). Cells were pretreated with 10 μ M TDZD-8 or vehicle (0.05% DMSO) 1 hour before stimulation with 10 ng/ml tumor necrosis factor (TNF)- α (BioSource, Camarillo, CA). Total protein and mRNA were extracted from cells 24 hours later.

Immunoblotting and mRNA Expression

Lung lysate proteins and human bronchial epithelial cell lysate proteins (10 μ g per lane) were separated by 10% SDS-PAGE and then transferred into a polyvinylidene difluoride membrane. Immunoblots were probed with either anti-p65 or anti-phospho-p65 (Ser⁵³⁶) antibody (Cell Signaling Technology, Beverly, MA), developed using enhanced chemiluminescence reagent, and quantitated using Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD). Total mRNA was extracted from lung tissues or human bronchial epithelial cells using TriZol reagent (Invitrogen, Carlsbad, CA). Primers for inflammatory biomarkers are shown in Table 1. The PCR products were run in a 2% agarose gel and visualized under ultraviolet light.

Statistical Analysis

Data are presented as means $\pm$ SEM. One-way ANOVA followed by Dunnett's test was used to determine significant differences between treatment groups. Significant levels were set at $P < 0.05$.

RESULTS

TDZD-8 Reduces OVA-induced Eosinophil Recruitment in BALF

BALF was collected 24 hours after the last OVA aerosol challenge, and total and differential cell counts were performed. OVA inhalation significantly ($P < 0.05$) increased total cell, eosinophil, and lymphocyte counts as compared with saline control (Figure 1A). In contrast, macrophage and neutrophil counts were not affected. TDZD-8 (1, 3, and 10 mg/kg) substantially reduced both the eosinophil and lymphocyte counts in BALF in a dose-dependent manner as compared with the DMSO vehicle control (Figure 1B). These results clearly suggest a possible role of GSK-3 β in eosinophil and lymphocyte recruitment during allergic inflammation. Flow cytometric analysis revealed that peripheral blood leukocytes from saline-challenged, OVA-challenged, vehicle control, and TDZD-8-treated mice had similar percentages of CD3⁺, CD4⁺ and CD8⁺ T cells, B cells (B220), NK cells, neutrophils, and monocytes (data not shown). Hence, inhibition of eosinophil and lymphocyte pulmonary recruitment is unlikely due to any potential nonspecific cytotoxic effects of TDZD-8.

TDZD-8 Inhibits OVA-induced Eosinophil Infiltration and Mucus Production

Lung tissue was collected 24 hours after the last OVA challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with saline challenge (Figures 2A and 2B).

TABLE 1. PRIMER SETS FOR REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ANALYSIS

Targets	Sequences	
	Forward	Reverse
VCAM-1 (Mouse)	5'-CAAGGGTGACCAGCTCATGAA-3'	5'-TGTGCAGCCACCTGAGATCC-3'
ICAM-1 (Mouse)	5'-CATCGGGGTGGTGAAGTCTGT-3'	5'-TGTGGGGGAAGTGTGGTC-3'
AMCase (Mouse)	5'-TGGGTTCTGGGCCTACTATG-3'	5'-GCTTGACAATGCTGCTGGTA-3'
Ym1 (Mouse)	5'-CTGGAATTGGTGGCCCTACA-3'	5'-CAAGCATGGTGG TTTTACAGGA-3
Ym2 (Mouse)	5'-CAGAACCCTCAGACATTCATTA-3'	5'-ATGGTCCTTC CAGTAGG TAATA-3'
Muc5ac (Mouse)	5'-GAGTGACATTGCAGGAAGCA-3'	5'-CAGAGGACAGGAAGGTGAGC-3'
IL-6 (Human)	5'-CAGGAGAAGATTCCAAGAT-3'	5'-ACTGGTTCTGTGCCTGCAGC-3'
IL-8 (Human)	5'-ATGACTTCCAAGCTGGCCGTGGCT-3'	5'-TCTCAGCCCTCTTCAAAACTTCTC-3'
RANTES (Human)	5'-ATGAAGGTCTCCGCGGCACGCCT-3'	5'-CTAGCTCATCTCCAAGAGTTG-3'
MCP-1 (Human)	5'-GATCTCAGTGCAGAGGCTCG-3'	5'-TGCTTGTCAGGTGGTCCAT-3'
β -Actin (Both)	5'-TCATGAAGTGTGACGTTGACATCCGT-3'	5'-CCTAGAAGCATTGCGGTGCACGATG-3'

Definition of abbreviations: ICAM-1 = intercellular adhesion molecule-1; IL = interleukin; MCP-1, monocyte chemotactic protein-1; RANTES = regulated on activation, normal T cells expressed and secreted; VCAM-1 = vascular cell adhesion molecule-1.

The majority of the infiltrated inflammatory cells were eosinophils. TDZD-8 (10 mg/kg) markedly attenuated the eosinophil-rich leukocyte infiltration as compared with DMSO control (Figures 2C, 2D, and 2I). On the other hand, OVA-challenged mice, but not saline-challenged mice, developed marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung (Figures 2E and 2F). The OVA-induced mucus secretion was significantly attenuated by TDZD-8 (10 mg/kg) as compared with the DMSO control (Figures 2G, 2H, and 2J).

Effects of TDZD-8 on Cytokine Levels in BALF

As shown in Figure 3, OVA inhalation in sensitized mice induced substantial cytokine release into BALF as compared with

saline control. TDZD-8 significantly ($P < 0.05$) decreased IL-5, IL-13 and, to a lesser extent, eotaxin levels in BALF as compared with DMSO control (Figures 3B–3D). In contrast, TDZD-8 had no effect on IL-4 levels in BALF (Figure 3A).

Effects of TDZD-8 on Serum IgE Levels

To evaluate whether TDZD-8 could modify an ongoing OVA-specific Th2 response *in vivo*, levels of total IgE and OVA-specific IgE were determined using ELISA. Substantial elevation in total IgE and OVA-specific IgE were observed in serum from OVA-sensitized and -challenged mice as compared with saline-challenged mice (Figure 4). TDZD-8 significantly ($P < 0.05$) lowered OVA-specific IgE levels (Figure 4A), but had no effect on the levels of total IgE (Figure 4B), indicating an OVA-specific inhibition on the Th2 response by the GSK-3 β inhibitor.

TDZD-8 Suppresses mRNA Expression of Lung Inflammatory Markers

OVA aerosol challenge markedly up-regulated mRNA levels of adhesion molecules intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which are pivotal for pulmonary recruitment of inflammatory cells such as eosinophils and lymphocytes (25); Muc5ac, which is essential for mucin production (26); and the chitinase family members (AMCase, Ym1, and Ym2), which have recently been shown to play critical roles in airway inflammation and remodeling (27–30). Pretreatment with TDZD-8 (10 mg/kg) demonstrated slight reduction of OVA-induced ICAM-1, VCAM-1, and AMCase mRNA expression, moderate inhibition of Ym1 mRNA levels, and strong suppression of Ym2 and Muc5ac mRNA levels in the allergic airway (Figure 5).

TDZD-8 Reduces OVA-induced AHR in Mice

To investigate the effect of TDZD-8 on AHR in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice. RI is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. As compared with saline-challenged group, OVA-challenged mice developed AHR, which was typically reflected by high RI and low Cdyn (Figure 6). TDZD-8 (10 mg/kg) dramatically prevented AHR in OVA-challenged mice in response to methacholine, suggesting that immune-mediated pathology *in vivo* was modified. DMSO did not show any inhibitory effects on AHR in OVA-challenged mice (Figure 6).

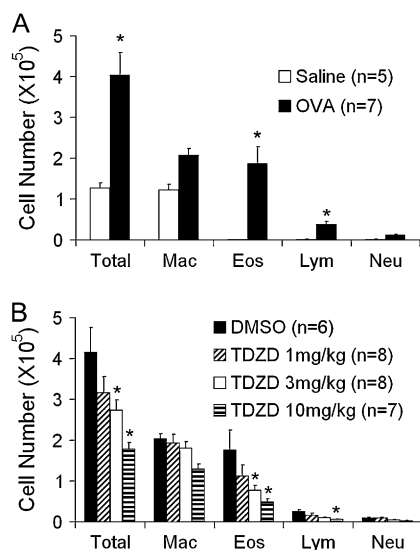
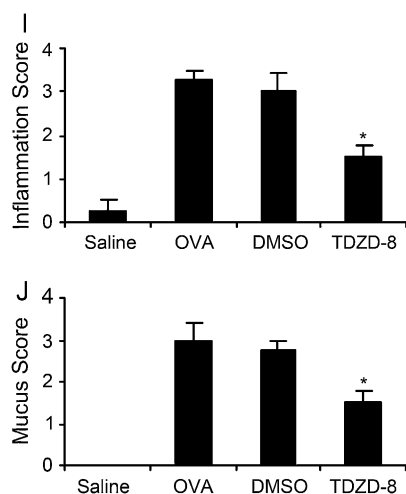
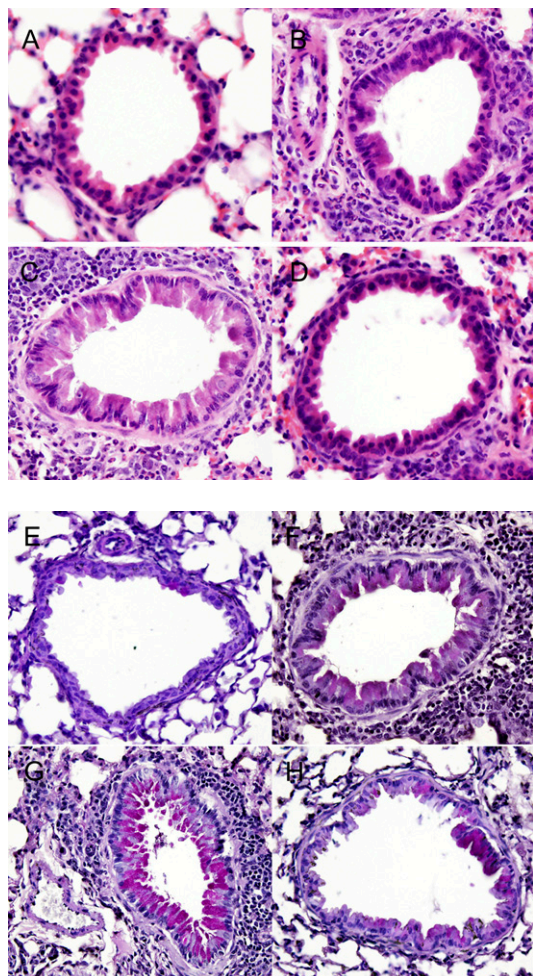


Figure 1. Effects of 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) on bronchoalveolar lavage fluid (BALF) cell infiltration. (A) Inflammatory cell counts in BALF obtained from sensitized mice 24 hours after the last saline aerosol or 10 mg/ml ovalbumin (OVA) aerosol challenge. (B) TDZD-8 reduced OVA-induced inflammatory cell counts in BALF from sensitized mice 24 hours after the last 10 mg/ml OVA aerosol challenge. Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Number of mice tested in each treatment group is shown as n value in parentheses. *Significant difference from dimethyl sulfoxide (DMSO) control, $P < 0.05$.



Immunoblot Analysis of Lung NF- κ B p65

To verify that the inhibitory effects of TDZD-8 on the mouse asthma model were mediated by NF- κ B p65 inhibition, we examined the effects of TDZD-8 on OVA-induced p65 phosphorylation (Ser⁵³⁶) in lung lysates. At 24 hours after the last OVA aerosol challenge, the degree of p65 phosphorylation was similar to the one in saline-challenged control mice (data not shown). However, at 2 hours after the last aerosol challenge, we were able to capture a significant (when compared with saline-

Figure 2. Effects of TDZD-8 on lung tissue eosinophilia and mucus production. Histologic examination of lung tissue eosinophilia (A–D, magnification $\times 400$) and mucus secretion (E–H, magnification $\times 400$) 24 hours after the last challenge of saline aerosol (A, E), OVA aerosol (B, F), OVA aerosol plus DMSO (C, G), or OVA aerosol plus 10 mg/kg TDZD-8 (D, H). Quantitative analyses of inflammatory cell infiltration (I) and mucus production (J) in lung sections were performed as previously described (23). Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5-point scoring system: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells of > 4 cells deep. To determine the extent of mucus production, goblet cell hyperplasia in the airway epithelium was quantified blind using a 5-point grading system: 0, no goblet cells; 1, $< 25\%$; 2, 25–50%; 3, 50–75%; 4, $> 75\%$. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $P < 0.05$.

challenged mice) increase in p65 phosphorylation in the lung lysates from OVA-challenged mice, and this increase in p65 phosphorylation was significantly (when compared with DMSO vehicle control) inhibited by TDZD-8 (Figure 7). Total levels of NF- κ B subunit p65 in lungs were not altered by GSK-3 β inhibition. Equal loadings of proteins were confirmed using β -actin as an internal control.

TDZD-8 Inhibits TNF- α Stimulation of Human Bronchial Epithelial Cells

To directly correlate GSK-3 β inhibition to functional responses in a relevant airway cell type, we studied the effects of TDZD-8 on TNF- α -induced phosphorylation of NF- κ B subunit p65 and cytokine mRNA expression in normal human bronchial epithelial cells. TNF- α plays a critical role in asthma (20, 31) and is a potent stimulator of human airway epithelial cells (32). TDZD-8 markedly blocked TNF- α -induced p65 phosphorylation as well as up-regulation of IL-6, IL-8, MCP-1, and RANTES in bronchial epithelial cells (Figure 8).

DISCUSSION

GSK-3 β is a serine-threonine kinase that is constitutively active in cells and has recently been linked to the regulation of NF- κ B transactivation function in a study using GSK-3 β knockout mice (9). It has been demonstrated that NF- κ B activity is up-regulated in allergic airway inflammation both in human and in animal models of asthma (19, 20, 33–35). Various therapeutic strategies targeted at the NF- κ B signaling pathway such as NF- κ B-specific decoy oligonucleotide (36), p65-specific antisense oligonucleotide (37), and IKK-2-selective small molecule inhibitor (38) have demonstrated beneficial effects in experimental asthma models. Cumulative evidence shows that GSK-3 β can phosphorylate the p65 subunit, particularly the COOH-terminus of p65 at Ser⁵³⁶, to enhance transcriptional responses of NF- κ B (9, 10, 12). Hence, pharmacologic inhibition of GSK-3 β may lead to inhibition of NF- κ B transcriptional activity and offer anti-inflammatory potential. TDZD-8 is a highly specific non-ATP competitive GSK-3 β inhibitor with negligible effect against other protein kinases such as protein kinase A, protein kinase C, casein kinase-II, and cyclin-dependent kinase-1 (21, 39). Our immunoblot analysis revealed a significant inhibition of p65 phosphorylation (Ser⁵³⁶) by TDZD-8 in OVA-challenged lungs and in TNF- α -stimulated human bronchial epithelial cells, confirming that inhibition of GSK-3 β suppressed NF- κ B signaling pathway in inflamed airways and in primary bronchial

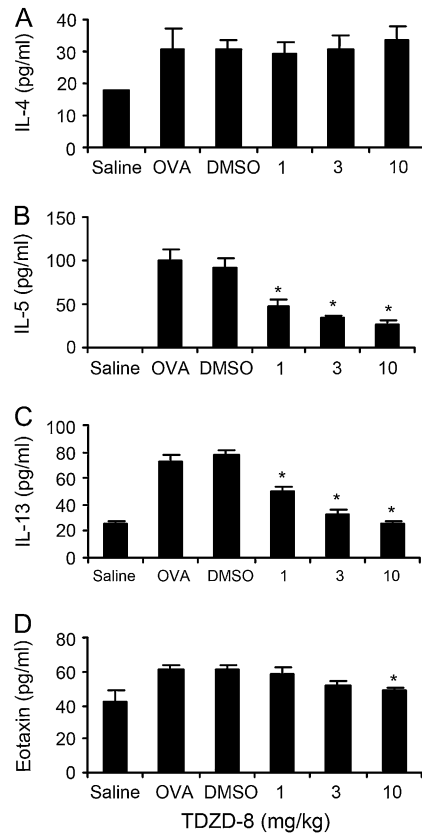


Figure 3. Effects of TDZD-8 on cytokine and chemokine levels in BALF. BAL fluids were collected 24 hours after the last OVA aerosol challenge. Levels of (A) IL-4, (B) IL-5, (C) IL-13, and (D) eotaxin were analyzed using enzyme-linked immunosorbent assay (ELISA) ($n = 5$ mice per group). Values shown are the mean $\pm$ SEM. *Significant difference from DMSO control, $P < 0.05$.

epithelial cell culture. However, our preliminary study using ELISA to measure lung tissue nuclear extract NF- κ B-binding activity to DNA revealed that TDZD-8 had no effect on the binding of NF- κ B to DNA motif (data not shown). Taken together, in our mouse asthma model, TDZD-8 attenuated p65 phosphorylation without affecting NF- κ B to DNA-binding. Our observation is supported by Hoeflich and colleagues (9), Martin and coworkers (15), and Dugo and associates (16), but not by Eto and colleagues (14). The reason for the observed differences is not clear, but may be related to the disease models used or the doses and pharmacokinetics of the inhibitors employed.

Th2 cells play an essential role in the pathogenesis of the allergic airway inflammation (2). IL-4, IL-5, and IL-13 can be produced by various resident cells such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages, as well as by infiltrated inflammatory cells such as lymphocytes and eosinophils. NF- κ B is a critical transcription factor for Th2 cell differentiation (40). Our present results show that TDZD-8 significantly reduced the levels of IL-5, IL-13, and eotaxin in BAL fluids from OVA-challenged mice. These findings are consistent with those from OVA-challenged mice with disrupted NF- κ B function via conditional knockout of IKK β or transgenic I κ B α mutant expression selectively in airway epithelium (34, 35), or via aerosol administration of NF- κ B decoy oligonucleotide (36), suggesting that TDZD-8 may exert its anti-inflammatory action on airway epithelium. In addition, antigen receptor activation in T and B cells and in mast cells has been shown to culminate in NF- κ B activation (13, 41, 42),

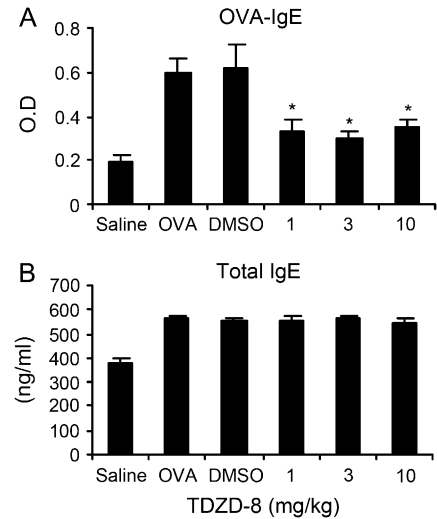


Figure 4. Effects of TDZD-8 on serum IgE production. Mouse serum was collected 24 hours after the last OVA aerosol challenge. The levels of (A) OVA-specific IgE and (B) total IgE were analyzed using ELISA ($n = 6$ mice per group). Values shown are the mean $\pm$ SEM. *Significant difference from DMSO control, $P < 0.05$.

and TDZD-8 may modulate NF- κ B-mediated cytokine production in these inflammatory cells. In addition, airway smooth muscle is another source of proinflammatory cytokines during the airway inflammation (43). Repression of NF- κ B signaling pathway has been shown to block IL-13-induced eotaxin production in cultured human airway smooth muscle cells (38). As such, the observed reduction of IL-5, IL-13, and eotaxin levels in BALF from GSK-3 β inhibitor TDZD-8-treated mice may be due to inhibition of NF- κ B activation in those inflammatory and airway resident cells.

Eosinophils play a central role in the pathogenesis of allergic inflammation (4). Our present findings showed that TDZD-8 prevented eosinophil infiltration into the airways as shown by a significant drop in total cell counts and eosinophil counts in BALF, and in tissue eosinophilia in lung sections. Eosinophil transmigration into the airways is a multistep process that is

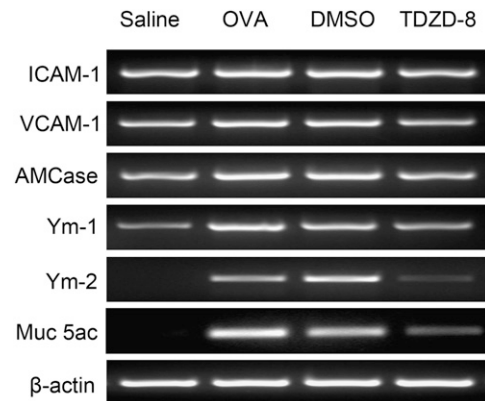


Figure 5. Effects of TDZD-8 on pulmonary mRNA expression of inflammatory markers. Lung tissues were collected 24 hours after the last OVA aerosol challenge. Total mRNA was extracted using TriZol reagent and the PCR products were separated in a 2% agarose gel visualized under ultraviolet light. β -actin was used as an internal control. The experiments were repeated for three times ($n = 3$ mice per group) with similar pattern of results.

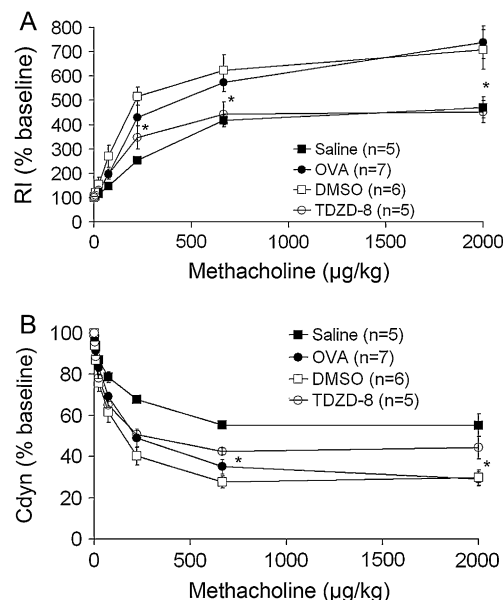


Figure 6. Effects of TDZD-8 on airway hyperresponsiveness (AHR). Airway responsiveness of mechanically ventilated mice in response to intravenous methacholine was measured 24 hours after the last saline aerosol or 10 mg/ml OVA aerosol with pretreatment of either DMSO or 10 mg/kg TDZD-8. AHR is expressed as percentage change from the baseline level of (A) lung resistance (RI) and (B) dynamic compliance (Cdyn). Number of mice tested in each treatment group is shown as n value in parentheses. *Significant difference from DMSO control, $P < 0.05$.

orchestrated by cytokines such as IL-5 and IL-13, and coordinated by specific chemokines such as eotaxin in combination with adhesion molecules such as ICAM-1 and VCAM-1 (4, 6, 25). IL-13 is by far the most potent inducer of eotaxin expression in airway epithelial cells (44). We demonstrated that TDZD-8 slightly down-regulated the expression of VCAM-1 and ICAM-1 mRNA in OVA-challenged lungs. These findings are likely to be due to TDZD-8-mediated NF- κ B inhibition, as the genes for VCAM-1 and ICAM-1 contain the κ B site for NF- κ B within their promoters (14, 45). Taken together, the observed reduction in airway eosinophilia by GSK-3 β inhibitor TDZD-8 may be a result of composite inhibitory effects on IL-13 and eotaxin production, and on VCAM-1 and ICAM-1 expression, secondary to inhibition of NF- κ B activation.

We have also demonstrated a dramatic reduction in mucus production in TDZD-8-treated mice as compared with DMSO control. Cumulative evidence indicates that IL-5 and IL-13 play a critical role in goblet cell hyperplasia and mucin Muc5ac gene and protein expression in mice (26, 46). Interestingly, Muc5ac gene expression is dependent on the transcriptional activity of NF- κ B (47), and selective ablation of NF- κ B function in airway epithelium blocked OVA-induced mucus production in mice (34, 35). We also observed a dramatic drop in Muc5ac mRNA expression by TDZD-8 in OVA-challenged lungs. As such, the marked decrease in mucus production in TDZD-8-treated lungs may be attributed to a substantial drop in IL-5 and IL-13 levels, and a direct inhibitory action on NF- κ B in airway epithelium in asthmatic mice treated with the GSK-3 β inhibitor.

Elevated serum IgE levels are a hallmark of the Th2 immune response. Our data showed that serum levels of OVA-specific IgE were substantially reduced by TDZD-8. NF- κ B plays a crucial role in B cell receptor-induced proliferation of mature B cells (13, 41). In addition, IL-13 is important in directing

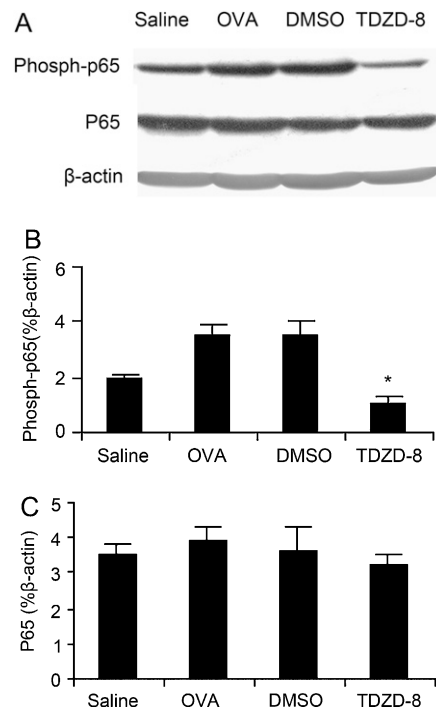


Figure 7. Effects of TDZD-8 on NF- κ B subunit p65 phosphorylation in lung tissue. Western blot analysis of p65 phosphorylation at Ser⁵³⁶ in lung fragments isolated from mice 2 hours after the last saline aerosol or OVA aerosol challenge pretreated with either DMSO or 10 mg/kg TDZD-8. Proteins (10 µg per lane) were separated by SDS-PAGE and probed with anti-p65 or anti-phospho-p65 antibody. The blot was developed by enhanced chemiluminescence reagent and quantitated using Gel-Pro imaging software. β -actin was used as an internal control. Values shown are the mean $\pm$ SEM of three separate experiments. *Significant difference from DMSO control, $P < 0.05$.

B cell growth, differentiation, and secretion of IgE (5, 48). The biological activities of IgE are mediated through its interaction with the high-affinity IgE receptor (Fc ϵ RI) on mast cells and basophils. Cross-linking of Fc ϵ RI initiates multiple signaling cascades leading to NF- κ B activation and production of cytokines and chemokines (42, 49). Therefore, the observed reduction in serum OVA-specific IgE by TDZD-8 in our asthma model may be contributed by its inhibitory effects on B cell activation and on IL-13 production by inflammatory cells such as mast cells, secondary to inhibition of NF- κ B activation.

A family of chitinase proteins including AMCase, Ym1, and Ym2 has recently been found to be markedly elevated in allergic airway inflammation and to play a role in the pathogenesis of asthma (27–29). They are mainly expressed in airway epithelium and alveolar macrophages. A recent study demonstrated an increase in AMCase levels in a mouse asthma model and in subjects with asthma in an IL-13-dependent manner (28). When given intratracheally, IL-13 elevated Ym1 and Ym2 levels in BAL fluid from mice *in vivo* (30). Although the molecular mechanisms of action of chitinases in asthma are largely unknown, they can specifically bind to carbohydrate moieties such as GlcN oligomers and other glycosaminoglycans such as heparin and heparan sulfate to regulate eosinophil chemotaxis, inflammation, and tissue remodeling (28–30). Our data show that TDZD-8 slightly down-regulated AMCase, moderately inhibited Ym1, but dramatically suppressed Ym2 mRNA levels in OVA-challenged lungs. The reduction of chitinases, especially Ym2, by TDZD-8 may be a result of the major drop in IL-13 level in the airways (of animals treated with

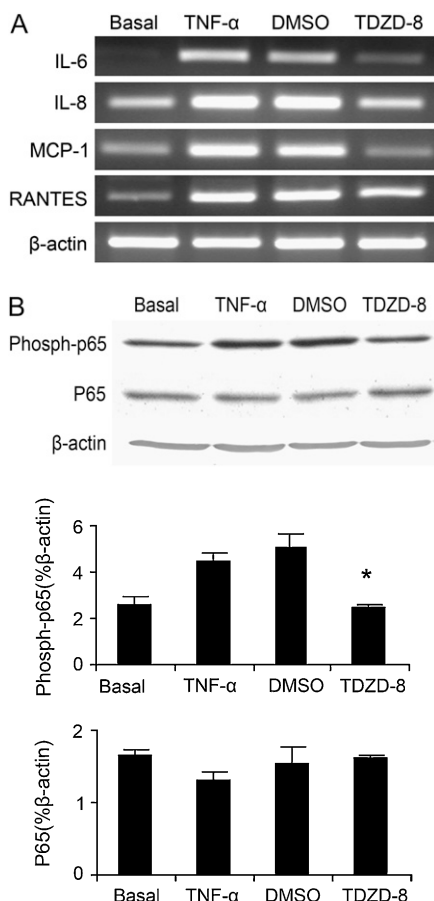


Figure 8. Effects of TDZD-8 on TNF- α stimulation of normal human bronchial epithelial cells. Primary epithelial cells were stimulated with 10 ng/ml TNF- α for 24 hours with and without pretreatment of 10 μ M TDZD-8. (A) Total mRNA was extracted using TriZol reagent and the PCR products were separated in a 2% agarose gel visualized under ultraviolet light. β -actin was used as an internal control. This is a representative gel from three separate experiments with similar pattern of results. (B) Western blot analysis of p65 phosphorylation at Ser⁵³⁶ in human bronchial epithelial cells. Proteins (10 μ g per lane) were separated by SDS-PAGE and probed with anti-p65 or anti-phospho-p65 antibody. The blot was developed by enhanced chemiluminescence reagent and quantitated using Gel-Pro imaging software. β -actin was used as an internal control. Values shown are the mean $\pm$ SEM of three separate experiments. *Significant difference from DMSO control, $P < 0.05$.

TDZD-8) and contribute to the diminished pulmonary eosinophilia.

It is believed that inflammatory mediators released during the allergic inflammation play a critical role in AHR development (1, 50). We report here that TDZD-8 significantly inhibited OVA-induced AHR to increasing concentrations of methacholine. It has been established that IL-5 plays a critical role in AHR by mobilizing and activating eosinophils, leading to the release of pro-inflammatory products such as major basic protein and cysteinyl-leukotrienes, which are closely associated with AHR (4, 51). In addition, IL-13 has been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated in AHR (5, 52). Moreover, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (49). Thus, the observed reduction of AHR by GSK-3 β inhibition may be associated with reduction in Th2 cytokine production, tissue eosinophilia, and serum IgE level by TDZD-8.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report here for the first time that GSK-3 β inhibition effectively reduced OVA-induced IL-5, IL-13 and eotaxin production, pulmonary eosinophilia, serum IgE synthesis, mucus hypersecretion, and AHR in a mouse asthma model. These findings support a potential role for GSK-3 β inhibitor in the treatment of asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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