

PTEN Down-Regulates IL-17 Expression in a Murine Model of Toluene Diisocyanate-Induced Airway Disease¹

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Toluene diisocyanate (TDI)-induced airway disease is a disorder characterized by chronic airway inflammation and airway remodeling. A recently discovered group of cytokines is the IL-17 family, which has been introduced as an important regulator of immune and inflammatory responses, including airway inflammation. Recently, we have reported that phosphatase and tensin homologue deleted on chromosome 10 (PTEN) plays a pivotal role in the pathogenesis of bronchial asthma. However, there are no available data for the effects of PTEN or IL-17 on TDI-induced airway disease and the relationship between PTEN and IL-17. We used a murine model to determine the role of PTEN in the pathogenesis of TDI-induced airway disease and the regulation of IL-17 production. These mice developed the typical pathophysiological features of TDI-induced airway disease and increased IL-17 expression in the lungs. Administration of phosphoinositide 3-kinase inhibitors or adenoviruses carrying PTEN cDNA (AdPTEN) reduced the pathophysiological features of TDI-induced airway disease and decreased the increased levels of IL-17 expression. Our results also showed that PI3K inhibitors or AdPTEN down-regulated a transcription factor, NF- κ B activity, and BAY 11-7085 substantially reduced the increased levels of IL-17 after TDI inhalation. We also found that inhibition of IL-17 activity with an anti-IL-17 Ab reduced airway inflammation and airway hyperresponsiveness. These results suggest that PTEN plays a protective role in the pathogenesis of TDI-induced airway disease, at least in part through the regulation of IL-17 expression. Thus, PTEN may be a useful target for treating TDI-induced airway disease by modulating IL-17 expression. *The Journal of Immunology*, 2007, 179: 6820–6829.

Toluene diisocyanate (TDI),⁴ a low molecular weight compound widely used in the production of polyurethane foams, automobile paints, varnishes, and related products, is a leading cause of occupational asthma (1). Although considerable controversy remains regarding its pathogenesis, TDI-induced airway disease is characterized by hyperresponsiveness, inflammation, and remodeling of the airways (1–3). Recent studies have shown that IL-17, a new family of cytokines that reveals a distinct

ligand-receptor signaling system, plays an important role in the regulation of immune responses. IL-17 has been shown to be proinflammatory in nature and this proinflammatory activity is exemplified by its involvement in pulmonary inflammatory responses (4–6). In addition, IL-17 levels are increased in serum and tissues in rheumatoid arthritis, inflammatory bowel disease, asthma, and other chronic diseases (7–9). However, a recent study has reported a negative regulatory function of IL-17 in allergen-induced airway inflammation and bronchial hyperresponsiveness of OVA-inhaled mice, showing that IL-17 is an essential player during Ag sensitization and that in sensitized mice IL-17 attenuates the allergic response by inhibiting dendritic cells and chemokine synthesis (10).

PI3K is a signal transduction enzyme that phosphorylates the D3 position of the inositol ring of phosphoinositide and its phosphorylated derivatives (11). Studies have suggested that PI3K contributes to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells (12, 13). PI3K plays a key role in the induction of the Th2 cell responses (12–15). Recently, Kim et al. (16) have shown that the PI3K/Akt signaling pathway is involved in the overproduction of the key inflammatory cytokine IL-17. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) functions primarily as a lipid phosphatase and has been implicated in regulating cell survival signaling through the PI3K/Akt signaling pathway (17). PTEN blocks the action of PI3K by dephosphorylating the signal lipid phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3, produced by PI3K following activation by receptor tyrosine kinases, activated Ras, or G proteins, leads to the stimulation of several downstream targets including the serine/threonine protein kinase Akt (18). PTEN is also known to play a protective role in Th2-mediated inflammation and airway hyperresponsiveness (19). However, the effects of PTEN or

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Received for publication May 27, 2007. Accepted for publication September 11, 2007.

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¹ This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF) through the National Research Laboratory. The program was funded by the Ministry of Science and Technology Grant R0A-2005-000-10052-0 (2007), Korea Research Foundation Grant KRF-2005-201-E00014 funded by the Korean Government (MOEHRD, Basic Research Promotion Fund), Korea Health 21 Research and Development Project Grant A060169 from the Ministry of Health and Welfare, Republic of Korea, and also Korea Health 21 Research and Development Project Grant 0412-CR03-0704-0001).

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⁴ Abbreviations used in this paper: TDI, toluene diisocyanate; AdPTEN, adenovirus gene transfer vector expressing PTEN cDNA; BAL, bronchoalveolar lavage; p-Akt, phosphorylated Akt; PAS, periodic acid-Schiff; PIP3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; R_L, airway resistance.

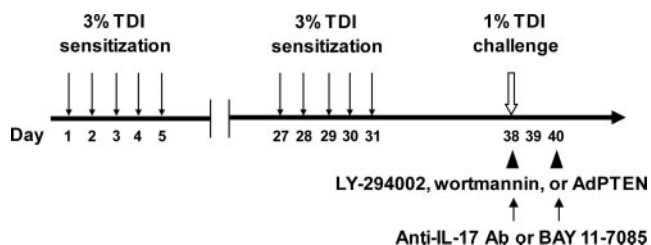


FIGURE 1. Schematic diagram of the experimental protocol. Mice were sensitized twice by two courses of intranasal administration of 3% TDI once a day for 5 consecutive days with a 3-wk interval. Seven days after the second course of sensitization, mice were challenged via the airways with 1% TDI for 10 min by ultrasonic nebulization. In the case of treatment with wortmannin, LY-294002, or adenoviral vector, it was administered intratracheally two times to each treated animal, once on day 38 (1 h before the airway challenge with TDI) and the second time on day 40 (48 h after the airway challenge with TDI). In the case of treatment with anti-IL-17 Ab, isotype control mAb, or BAY 11-7085, it was administered i.p. two times to each animal under the same administration schedule described above.

IL-17 on TDI-induced airway disease and their related signaling pathways are unknown.

In the present study, we used a murine model of TDI-induced airway disease to determine the effect of PTEN in the pathogenesis of TDI-induced airway disease and in the regulation of IL-17 production.

Materials and Methods

Animals and experimental protocol

Female BALB/c mice, 8–10 wk of age and free of murine-specific pathogens, were obtained from Orientbio, housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School (Jeonju, South Korea). Mice were sensitized by the intranasal administration of 20 μ l of 3% TDI dissolved in ethyl acetate:olive oil (1:4) under light anesthesia (sodium pentobarbitone, 30 mg/kg, i.p.) once daily for 5 consecutive days according to the method of Scheerens and colleagues (20, 21) with some modifications. Animals were kept in a supine position for 10 min after each sensitization. After 3 wk, these animals were further sensitized with the same reagent for 5 consecutive days. At 7 days after the second course of sensitization (day 38), mice were individually placed in a horizontal cylindrical chamber and challenged via the airways with 1% TDI dissolved in ethyl acetate:olive oil (1:4) by ultrasonic nebulization (NE-U12 nebulizer; Omron) (Fig. 1). As a control, mice were sensitized and challenged using the same protocol but using only the solvent, ethyl acetate:olive oil (1:4). Bronchoalveolar lavage (BAL) was performed 72 h after the challenge. At the time of lavage, the mice were sacrificed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg per kg of body weight). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution (800 μ l) was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. Part of each pool was then centrifuged and the supernatants were kept at –70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron). The smears were stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico) to examine the cell differentials.

Vectors

The E1/E3-deleted, replication-deficient, recombinant adenovirus was made using the AdEasy system (Quantum Biotechnologies) described by He et al. (22). To create an adenovirus gene transfer vector expressing PTEN cDNA (AdPTEN), *KpnI-XhoI* restriction fragments from pcDNA3/wild-type PTEN cDNA were ligated into *KpnI-XhoI*-digested pShuttleCMV as previously described (19).

Administration of wortmannin, LY-294002, adenoviral vectors, BAY 11-7085, anti-IL-17 Ab, and isotype control mAb

Wortmannin (100 μ g per kg of body weight per day; Calbiochem-Novabiochem) or LY-294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (1.5 mg per kg of body weight per day; BIOMOL Research Laboratories) dissolved in DMSO and diluted with 0.9% NaCl was administered in a volume of 50 μ l as described previously (19, 23). Wortmannin or LY-294002 was administered intratracheally two times to each treated animal, once on day 38 (1 h before the airway challenge with TDI) and the second time on day 40 (48 h after the airway challenge with TDI). The vehicle was 0.9% NaCl containing DMSO. AdPTEN or AdLacZ (10^9 plaque-forming units) was administered intratracheally two times to each animal under the same administration schedule described above. An inhibitor of NF- κ B activation, BAY 11-7085 (20 mg per kg of body weight per day; BIOMOL Research Laboratories), dissolved in DMSO and diluted with 0.9% NaCl, was administered by i.p. injection two times to each animal, once on day 38 and the second time on day 40. Anti-IL-17 Ab or isotype control mAb (5 mg per kg of body weight per day; R&D Systems) was administered i.p. two times to each animal in accordance with the schedule described above (Fig. 1).

Western blot analysis

Protein expression levels were analyzed by means of Western blot analysis as described previously (19). The blots were incubated with an anti-PTEN Ab (Santa Cruz Biotechnology), anti-IL-17 Ab (R&D Systems), anti-Akt Ab (Cell Signaling Technology), or anti-phosphorylated Akt (p-Akt) Ab (Cell Signaling Technology), overnight at 4°C.

RNA isolation and RT-PCR

Levels of mRNA expression were analyzed by RT-PCR assay using total RNA isolated from lung tissues by a rapid extraction method (TRI-Reagent; Sigma-Aldrich) as previously described (24). The primers used were as follows: IL-17, 5'-TCTCATCCAGCAAGAGATCC-3' (sense) and 5'-AGTTTGGGACCCCTTTACAC-3' (antisense); and GAPDH, 5'-GC CATCAACGACCCCTTCATTGAC-3' (sense) and 5'-ACGGAAGGC CATGCCAGTGAGCTT-3' (antisense). PCR was performed in a thermocycler (GeneAmp PCR System 2400).

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed using the LightCycler FastStart DNA Master SYBR Green I system (Roche Diagnostics). The sequences of primers used were as follows: IL-17, 5'-TCTCATCCAG CAAGAGATCC-3' (sense) and 5'-AGTTTGGGACCCCTTTACAC-3' (antisense); and β -actin, 5'-CAGATCATGTTTGAGACCTTC-3' (sense) and 5'-ACTTCATGATGGAATTGAATG-3' (antisense). Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol.

Measurement of PTEN activity

PTEN activities were measured using the PTEN malachite green assay kit according to the protocol provided by the manufacturer (Upstate Biotechnology).

Measurement of PI3K enzyme activity in lung tissues

PI3K enzyme activity was measured as described previously (19, 25). The amount of PIP3 produced was quantified by PIP3 competition enzyme immunoassays (Echelon).

Cytosolic or nuclear protein extractions for analysis of NF- κ B p65

Cytosolic or nuclear extraction was performed as described previously (26, 27). The levels of NF- κ B p65 were analyzed by Western blotting using Ab against NF- κ B p65 (Upstate Biotechnology).

Determination of airway responsiveness to methacholine

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways as described elsewhere (28). Each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/ml in saline). After each methacholine challenge, the data of airway resistance (R_L) was continuously collected. Maximum values of R_L were selected to express changes in airway function, which was represented as a percentage change from baseline after administration of the saline aerosol.

Processing of lungs for histologic and image analysis

At 72 h after the last challenge, lungs were removed from the mice after sacrifice. The specimens were dehydrated and embedded in paraffin. After sectioning of the specimens, they were placed on slides, deparaffinized, and stained sequentially with H&E (Richard-Allan Scientific) or periodic acid-Schiff (PAS). All stained slides were quantified under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination (29).

Histology

For histological examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems). The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (30).

Quantitation of airway mucus expression

To quantitate the level of mucus expression in the airway, the number of PAS-positive and PAS-negative epithelial cells in individual bronchioles were counted as described previously (29, 31). Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchiole divided by the total number of epithelial cells of each bronchiole.

Densitometric analyses and statistics

All immunoreactive and phosphorylative signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad). Data are expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffé's test. Significant differences between groups were determined using the unpaired Student's *t* test. Statistical significance was set at $p < 0.05$.

Results

IL-17 protein levels and mRNA expression increased in TDI-sensitized and -challenged mice

Western blot analysis revealed that IL-17 protein levels in lung tissues were increased approximately 1.4-, 1.5-, 1.7-, 3.4-, 5.9-, and 2.8-fold at 6, 12, 24, 48, 72, and 96 h after challenge with TDI, respectively, compared with the prechallenge period (Fig. 2, A and B). In contrast, no significant changes in the IL-17 protein level were observed after vehicle inhalation. Real-time RT-PCR analysis revealed that IL-17 mRNA expression had increased approximately 1.1-, 1.4-, 1.7-, 2.3-, 2.5-, and 2.0-fold at 6, 12, 24, 48, 72, and 96 h after challenge with TDI, respectively, compared with the prechallenge period (Fig. 2, C and D). In contrast, no significant changes in IL-17 mRNA expression were observed after vehicle inhalation.

Effect of wortmannin, LY-294002, or AdPTEN on expression of IL-17 protein and mRNA in lung tissues of TDI-sensitized and -challenged mice

To investigate whether the IL-17 expression in lung tissues is regulated by the PI3K or the PTEN signaling pathway in TDI-inhaled mice, we used the PI3K inhibitors, wortmannin and LY-294002, and AdPTEN. The increased IL-17 levels at 72 h after TDI inhalation were decreased significantly by the administration of wortmannin, LY-294002, or AdPTEN (Fig. 3, A and B). RT-PCR and real-time RT-PCR analyses showed that the increased IL-17 mRNA expression after TDI inhalation was significantly reduced by the administration of wortmannin, LY-294002, or AdPTEN (Fig. 3, C and D).

Effect of AdPTEN on IL-17 protein in BAL fluids of TDI-sensitized and -challenged mice

To measure extracellular IL-17 protein, BAL fluids were obtained from the trachea of mice and centrifuged to remove cells. Each supernatant was recovered and quantified using the Bradford reagent (Bio-Rad). The levels of IL-17 protein were analyzed by

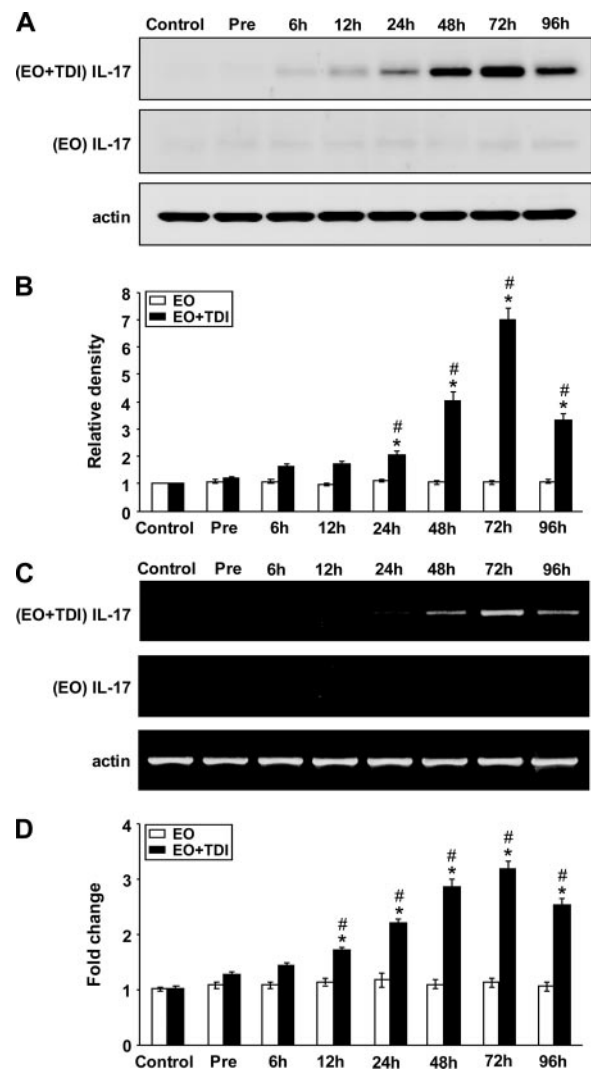


FIGURE 2. Expression of IL-17 protein and mRNA in lung tissues of TDI-sensitized and -challenged mice. Sampling was performed in lung tissues from sensitized mice challenged with TDI or vehicle control only. A, Western blot analyses of IL-17 protein. B, Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of control mice is arbitrarily presented as 1. C, Representative RT-PCR analysis of IL-17 mRNA expression. D, Quantitative analysis of IL-17 mRNA expression by means of real-time RT-PCR. Data represent mean $\pm$ SEM from eight mice per group, and 6, 12, 24, 48, 72, and 96 h are time periods after the challenge. Control, No treatment; Pre, 1 h before the challenge; EO, vehicle control (ethyl acetate plus olive oil); EO+TDI, TDI-sensitized and -challenged mice; #, $p < 0.05$ vs Pre; *, $p < 0.05$ vs EO.

Western blotting. Western blot analysis revealed that levels of IL-17 protein in BAL fluids were increased at 72 h after TDI inhalation compared with the levels in vehicle control mice administered saline (Fig. 4). The increased IL-17 protein levels after TDI inhalation were decreased by the administration of AdPTEN.

PTEN protein levels and PTEN activities in lung tissues of TDI-sensitized and -challenged mice

Western blot analysis revealed that PTEN protein levels were decreased significantly 72 h after TDI inhalation compared with the levels in the vehicle control mice administered saline (Fig. 5, A and B). The decreased PTEN levels after TDI inhalation were increased substantially by the administration of AdPTEN. Consistent

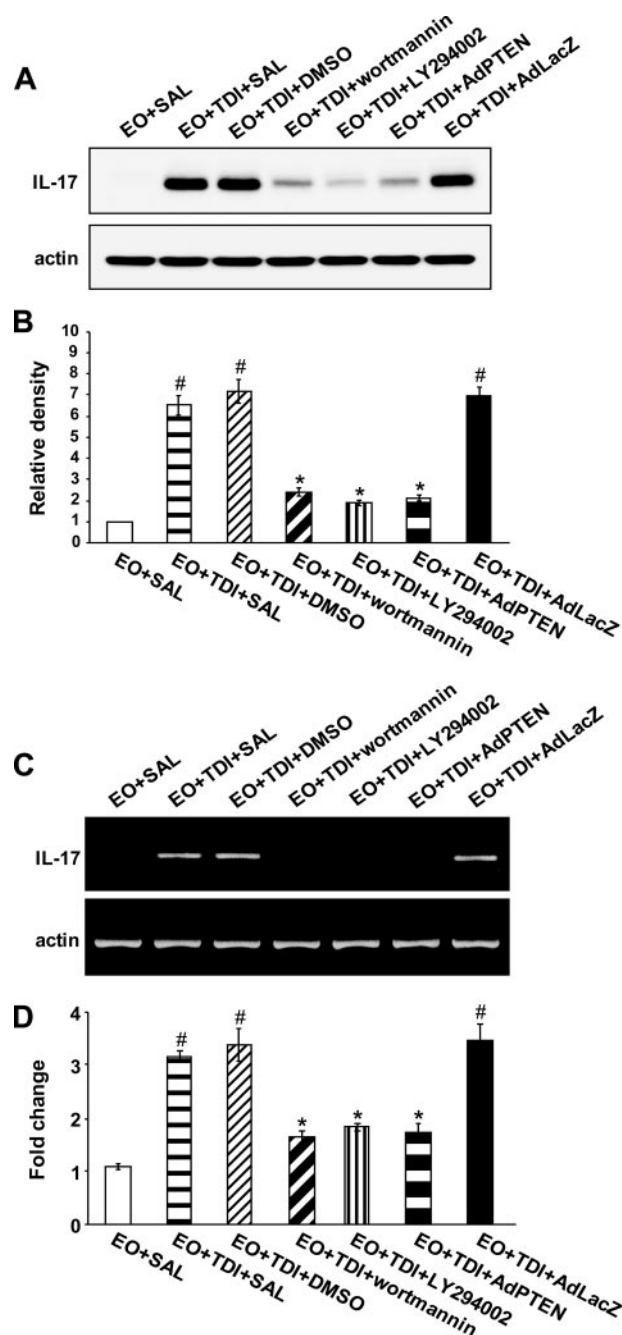


FIGURE 3. Effect of wortmannin, LY-294002, or AdPTEN on IL-17 protein and mRNA in lung tissues of TDI-sensitized and -challenged mice. *A*, Western blot analysis of IL-17. Sampling was performed 72 h after the challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (0.05% DMSO) (EO+TDI+DMSO), TDI-inhaled mice administered wortmannin (EO+TDI+wortmannin), TDI-inhaled mice administered LY-294002 (EO+TDI+LY294002), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ). *B*, Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of EO+SAL is arbitrarily presented as 1. *C*, Representative RT-PCR analysis of IL-17 mRNA expression. *D*, Quantitative analysis of IL-17 mRNA expression by means of real-time RT-PCR. Data represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL.

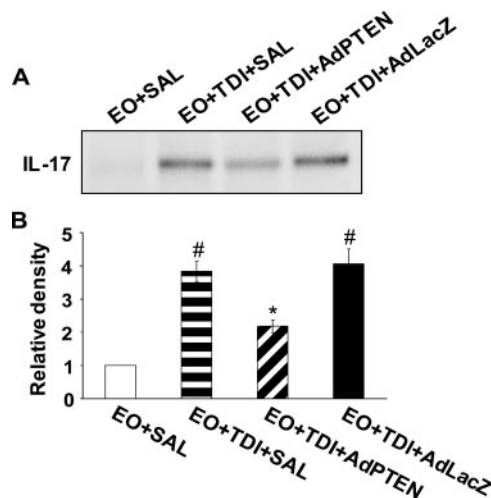


FIGURE 4. Effect of AdPTEN on IL-17 protein in BAL fluids of TDI-sensitized and -challenged mice. *A*, Western blot analysis of IL-17. Sampling was performed 72 h after the last challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ). *B*, Densitometric analyses are presented as the relative ratio of IL-17 levels in all groups to those in EO+SAL. The IL-17 protein level in BAL fluids of EO+SAL is arbitrarily presented as 1. Bars represent mean $\pm$ SEM from five independent experiments. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL.

with these results obtained from Western blot analysis, PTEN enzyme assays revealed that PTEN activity was decreased significantly after TDI inhalation compared with the levels in the vehicle control mice administered saline (Fig. 5C). The decreased PTEN activity was also increased by the administration of AdPTEN.

Determination of Akt phosphorylation and PI3K enzyme activity in lung tissues of TDI-sensitized and -challenged mice

To determine an involvement of the PI3K/Akt pathway in TDI-inhaled mice, we evaluated the effects of PI3K inhibitors and AdPTEN on p-Akt levels and PI3K enzyme activity. Levels of p-Akt protein in the lung tissues were significantly increased 72 h after TDI inhalation compared with the levels in the vehicle control mice administered saline (Fig. 6, *A* and *B*). However, no significant changes in Akt protein levels were observed in any of the groups tested. The increased p-Akt, but not Akt, levels in the lung tissues after TDI inhalation were significantly reduced by the administration of wortmannin, LY-294002, or AdPTEN. Consistent with these results, PIP3 levels in the lung tissues were increased after TDI inhalation compared with the vehicle control mice administered saline (Fig. 6C). The increased PIP3 levels in the lung tissues were significantly decreased by the administration of wortmannin, LY-294002, or AdPTEN.

Effect of wortmannin, LY-294002, or AdPTEN on NF- κ B p65 protein levels in lung tissues of TDI-sensitized and -challenged mice

We evaluated the effect of wortmannin, LY-294002, or AdPTEN on NF- κ B p65 protein levels in lung tissues of TDI-inhaled mice to determine whether PI3K inhibitors or AdPTEN down-regulates NF- κ B activity. Western blot analysis revealed that levels of NF- κ B p65 in nuclear protein extracts from lung tissues were increased 72 h after TDI inhalation compared with the levels in the vehicle control mice administered saline (Fig. 7). The increased

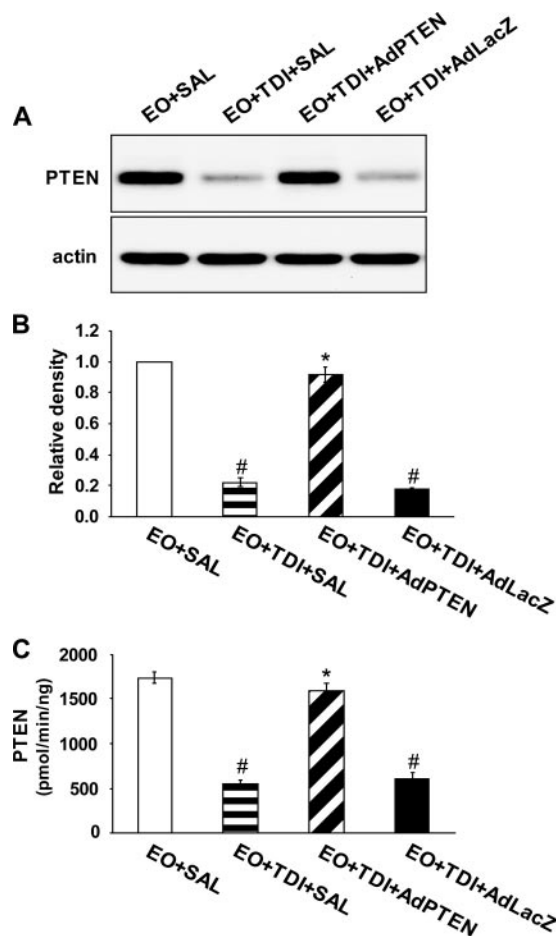


FIGURE 5. Effect of AdPTEN on PTEN protein expression and PTEN activity in lung tissues of TDI-sensitized and -challenged mice. **A**, Western blotting of PTEN. PTEN protein expression was measured 72 h after the challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+Sal), TDI-inhaled mice administered saline (EO+TDI+Sal), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ). **B**, Densitometric analyses are presented as the relative ratio of PTEN to actin. The relative ratio of PTEN in the lung tissues of EO+Sal is arbitrarily presented as 1. **C**, PTEN activity. Data represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+Sal; *, $p < 0.05$ vs EO+TDI+Sal.

NF- κ B p65 levels after TDI inhalation were decreased by the administration of wortmannin, LY-294002, or AdPTEN. In contrast, the levels of NF- κ B p65 in cytosolic protein extracts from lung tissues were decreased after TDI inhalation compared with the levels in the vehicle control mice administered saline (Fig. 7). The decreased NF- κ B p65 levels in cytosolic preparations were increased by the administration of wortmannin, LY-294002, or AdPTEN. These results suggest that a PI3K inhibitor or AdPTEN inhibits NF- κ B activity by preventing the translocation of this transcription factor into the nucleus.

Effect of BAY 11-7085 on IL-17 levels in lung tissues of TDI-sensitized and -challenged mice

To elucidate whether the IL-17 production in lung tissues is regulated by NF- κ B activation in TDI-inhaled mice, we used an NF- κ B inhibitor, BAY 11-7085. Western blot analysis showed that IL-17 protein levels in lung tissues were increased significantly 72 h after TDI inhalation compared with the levels in the vehicle control mice administered saline (Fig. 8). The increased IL-17 lev-

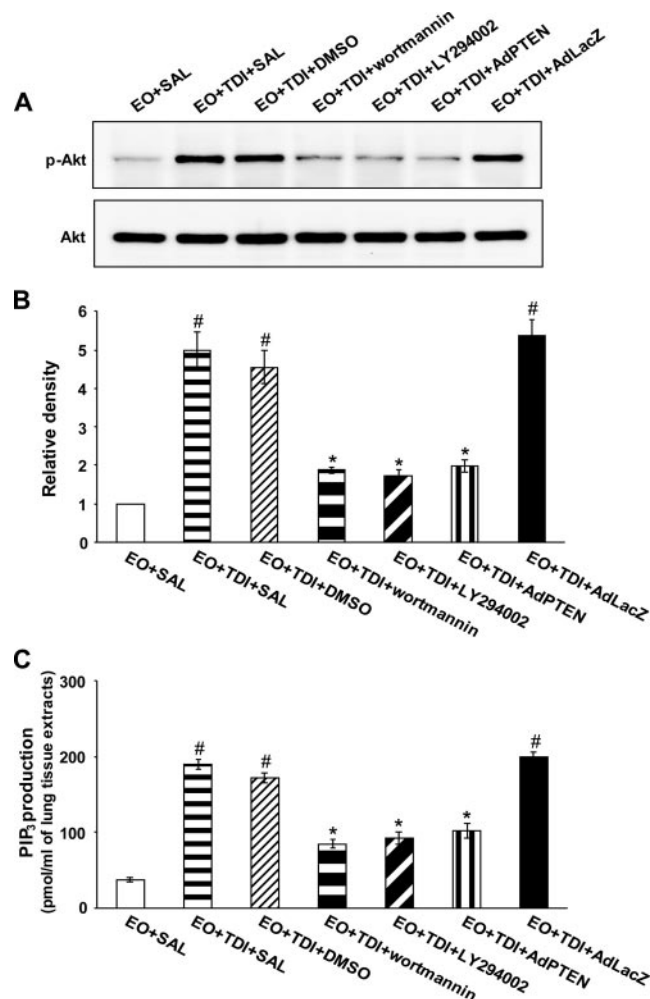


FIGURE 6. Effect of wortmannin, LY-294002, or AdPTEN on p-Akt, Akt protein levels, and PI3K enzyme activity. Sampling was performed 72 h after the challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+Sal), TDI-inhaled mice administered saline (EO+TDI+Sal), TDI-inhaled mice administered drug vehicle (EO+TDI+DMSO), TDI-inhaled mice administered wortmannin (EO+TDI+wortmannin), TDI-inhaled mice administered LY-294002 (EO+TDI+LY294002), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ). **A**, Western blotting of p-Akt and Akt in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of p-Akt to Akt. The relative ratio of p-Akt in the lung tissues of EO+Sal is arbitrarily presented as 1. **C**, Enzyme immunoassay of PIP₃ generation by PI3Ks in lung tissue extracts. Bars represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+Sal; *, $p < 0.05$ vs EO+TDI+Sal.

els after TDI inhalation were significantly reduced by the administration of BAY 11-7085. These results suggest that NF- κ B activation regulates the expression of IL-17.

Effect of wortmannin, LY-294002, AdPTEN, or anti-IL-17 Ab on cellular changes in BAL fluids

To determine whether PTEN or inhibition of the PI3K signaling pathway reduces airway inflammation, we analyzed the changes in cell numbers in BAL fluids after the administration of wortmannin, LY-294002, or AdPTEN to TDI-inhaled mice. Additionally, we examined the cellular changes after the administration of the anti-IL-17 Ab to investigate whether inhibition of IL-17 activity reduces airway inflammation. Numbers of total cells, lymphocytes,

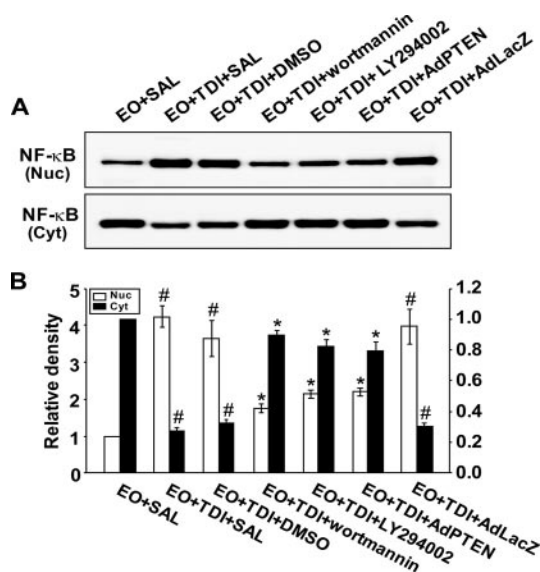


FIGURE 7. Effect of wortmannin, LY-294002, or AdPTEN on NF-κB p65 expression in lung tissues of TDI-sensitized and -challenged mice. *A*, Western blotting of NF-κB p65 in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. *B*, Densitometric analyses are presented as the relative ratio of NF-κB p65 levels in all groups to those in vehicle control (ethyl acetate plus olive oil (EO)) administered saline (SAL) (EO+SAL). The NF-κB p65 level in nuclear protein extracts of EO+SAL is arbitrarily presented as 1. Bars represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL.

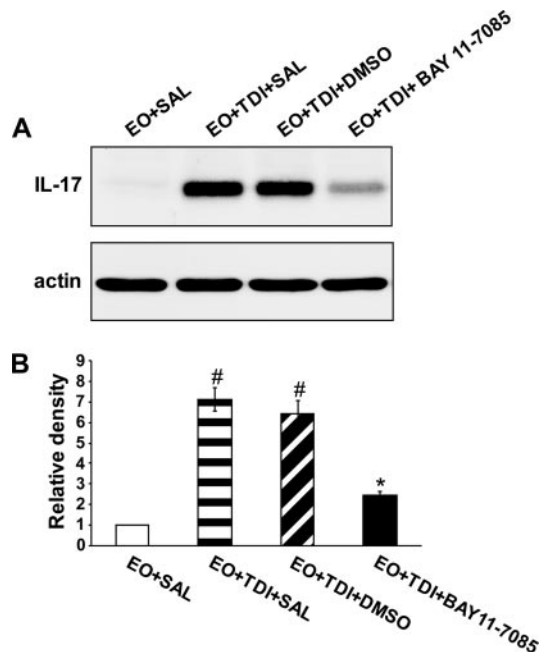


FIGURE 8. Effects of BAY 11-7085 on IL-17 expression in lung tissues of TDI-sensitized and -challenged mice. Sampling was performed at 72 h after the challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (EO+TDI+DMSO), and TDI-inhaled mice administered BAY 11-7085 (EO+TDI+BAY 11-7085). *A*, Western blotting of IL-17 in lung tissues. *B*, Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of EO+SAL is arbitrarily presented as 1. Bars represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL.

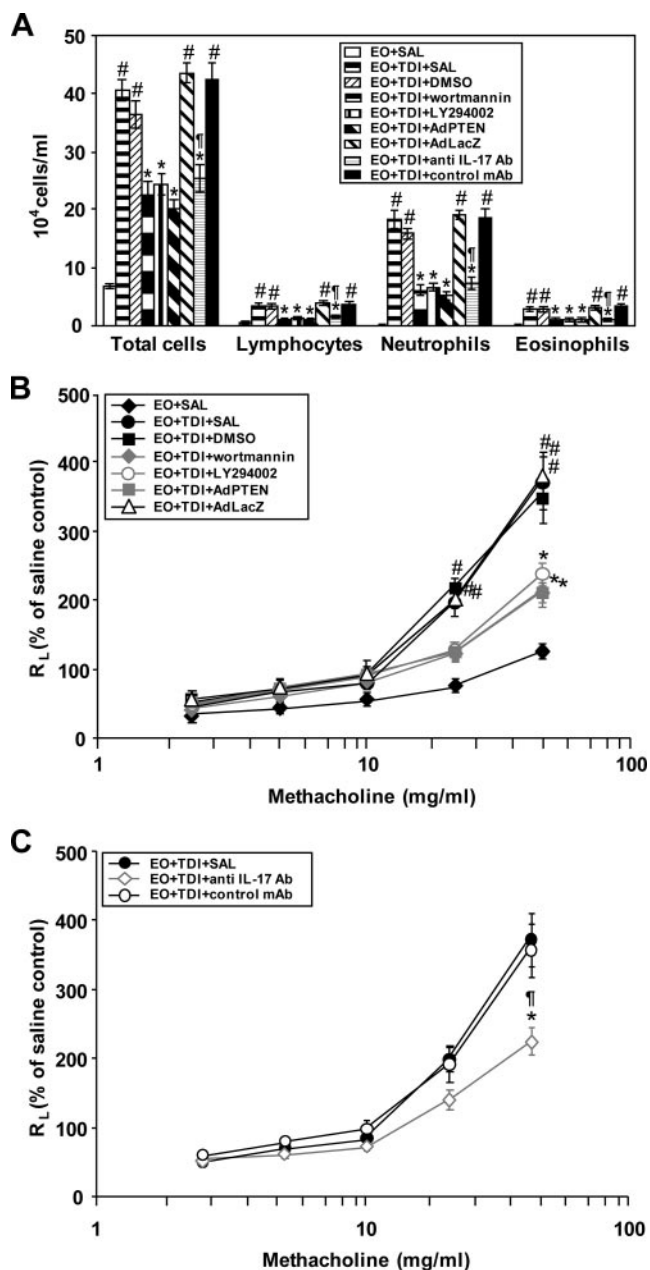


FIGURE 9. Effects of wortmannin, LY-294002, AdPTEN, or anti-IL-17 Ab on total cells and differential cellular components in BAL fluids (*A*) and on airway responsiveness (*B* and *C*) of TDI-sensitized and -challenged mice. The number of each component of BAL cells and airway responsiveness were measured at 72 h after the challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (EO+TDI+DMSO), TDI-inhaled mice administered wortmannin (EO+TDI+wortmannin), TDI-inhaled mice administered LY-294002 (EO+TDI+LY294002), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ), TDI-inhaled mice administered anti-IL-17 Ab (EO+TDI+anti IL-17 Ab), and TDI-inhaled mice administered isotype control mAb (EO+TDI+control mAb) as described in *Materials and Methods*. Data represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; ¶, $p < 0.05$ vs EO+TDI+control mAb.

neutrophils, and eosinophils in BAL fluids were increased significantly at 72 h after TDI inhalation compared with the numbers in the vehicle control mice administered saline (Fig. 9A). The

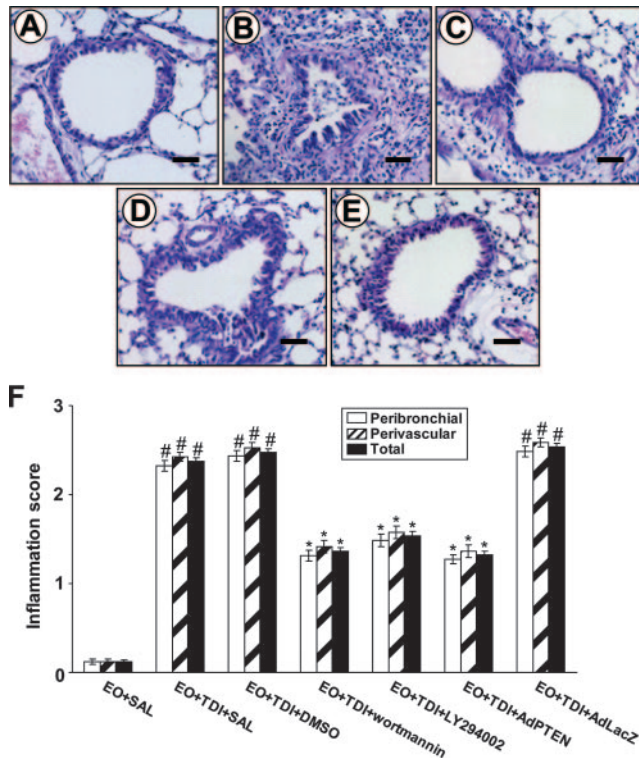


FIGURE 10. Effects of wortmannin, LY-294002, or AdPTEN on pathologic changes in lung tissues of TDI-sensitized and -challenged mice. *A–E*, Representative H&E-stained sections of the lungs. Sampling was performed 72 h after the challenge in vehicle control mice administered saline (*A*), TDI-inhaled mice administered saline (*B*), TDI-inhaled mice administered wortmannin (*C*), TDI-inhaled mice administered LY-294002 (*D*), and TDI-inhaled mice administered AdPTEN (*E*). Bars indicate scale of 50 μ m. *F*, Peribronchial lung inflammation and perivascular lung inflammation were measured in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (EO+TDI+DMSO), TDI-inhaled mice administered wortmannin (EO+TDI+wortmannin), TDI-inhaled mice administered LY-294002 (EO+TDI+LY294002), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ). Results are represented as mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL.

increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of wortmannin, LY-294002, or AdPTEN. However, no significant changes were observed in TDI-inhaled mice administered AdLacZ. In addition, the administration of the anti-IL-17 Ab decreased the increased numbers of total cells, lymphocytes, neutrophils, and eosinophils compared with the numbers in TDI-inhaled mice administered saline or an isotype control mAb.

PI3K inhibitors, AdPTEN, or anti-IL-17 Ab reduced TDI-induced airway hyperresponsiveness

To investigate whether airway hyperresponsiveness is decreased by PTEN or inhibition of PI3K the signaling pathway, we measured R_L after the administration of wortmannin, LY-294002, or AdPTEN to TDI-inhaled mice. Additionally, we examined the changes in airway responsiveness after the administration of anti-IL-17 Ab to determine whether the inhibition of IL-17 activity reduces TDI-induced airway hyperresponsiveness. Airway responsiveness was assessed as a percentage increase of R_L in response to increasing doses of methacholine. In TDI-inhaled mice, the

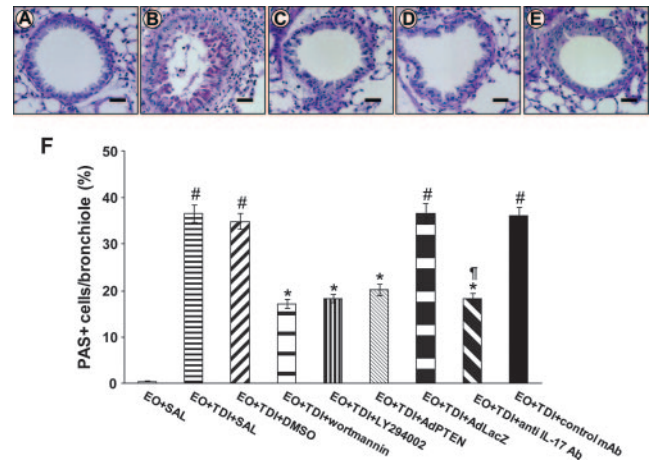


FIGURE 11. Effect of wortmannin, LY-294002, AdPTEN, or anti-IL-17 Ab on airway mucus expression in TDI-sensitized and -challenged mice. *A–E*, Representative PAS-stained sections of the lungs. Sampling was performed 72 h after the challenge in vehicle control (ethyl acetate plus olive oil (EO)) mice administered saline (SAL) (EO+SAL) (*A*), TDI-inhaled mice administered saline (EO+TDI+SAL) (*B*), TDI-inhaled mice administered wortmannin (EO+TDI+wortmannin) (*C*), TDI-inhaled mice administered AdPTEN (EO+TDI+AdPTEN) (*D*), and TDI-inhaled mice administered anti-IL-17 Ab (EO+TDI+anti IL-17 Ab) (*E*). The violet color indicates PAS-positive mucus expression. Bars indicate 50 μ m. *F*, Quantitation of airway mucus expression. Results are expressed as the percentage of PAS-positive cells per bronchiole, which is described in the *Materials and Methods*. Bars represent mean $\pm$ SEM from eight mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; ¶, $p < 0.05$ vs EO+TDI+control mAb.

dose-response curve of R_L shifted to the left compared with that of the vehicle control mice administered saline (Fig. 9, *B* and *C*). In addition, the percentage of R_L produced by methacholine administration (at doses from 25 mg/ml to 50 mg/ml) increased significantly in the TDI-inhaled mice compared with the vehicle control mice administered saline. TDI-inhaled mice administered wortmannin, LY-294002, or AdPTEN showed a dose-response curve of the percentage of R_L that shifted to the right compared with that of TDI-inhaled mice administered saline. In addition, the administration of anti-IL-17 Ab also shifted a dose-response curve of the percentage of R_L to the right compared with that in TDI-inhaled mice administered saline or isotype control mAb. These results indicate that the PI3K inhibitors, AdPTEN, or the anti-IL-17 Ab treatment reduces TDI-induced airway hyperresponsiveness.

Effect of wortmannin, LY-294002, or AdPTEN on pathological changes of TDI-induced airway disease

To assess the pathological changes in the lungs of TDI-inhaled mice by the administration of PI3K inhibitors and AdPTEN, histologic analyses were performed 72 h after TDI inhalation. Histologic analyses revealed typical pathologic features of TDI-induced airway disease. Numerous inflammatory cells infiltrated around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Fig. 10*B*) as compared with the control (Fig. 10*A*). Mice treated with wortmannin (Fig. 10*C*), LY-294002 (Fig. 10*D*), or AdPTEN (Fig. 10*E*) showed marked reductions in the thickening of airway epithelium, the infiltration of inflammatory cells in the peribronchiolar region, the number of inflammatory cells, and the amount of debris in the airway lumen. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly after TDI inhalation compared with the scores of the vehicle control mice administered saline (Fig. 10*F*). The increased peribronchial, perivascular, and total lung inflammation was significantly reduced by the

administration of wortmannin, LY-294002, or AdPTEN. These results suggest that PI3K inhibitors and AdPTEN inhibit Ag-induced inflammation in the lungs.

Effect of wortmannin, LY-294002, AdPTEN, or anti-IL-17 Ab on airway mucus expression

For the contention that airway mucus production is reduced by PTEN or the inhibition of PI3K signaling pathway, we measured the level of mucus expression in the airway after the administration of wortmannin, LY-294002, or AdPTEN to TDI-inhaled mice. We also used anti-IL-17 Ab to determine whether the inhibition of IL-17 activity decreases TDI-induced airway mucus expression. The percentage of cells stained with PAS in airway epithelium of TDI-inhaled mice (Fig. 11, *B* and *F*) was significantly greater than the levels in the vehicle control mice administered saline (Fig. 11, *A* and *F*). The administration of wortmannin (Fig. 11, *C* and *F*), LY-294002 (Fig. 11*F*), or AdPTEN (Fig. 11, *D* and *F*) to TDI-inhaled mice reduced substantially the percentage of cells stained with PAS in the airway epithelium compared with the levels in TDI-inhaled mice administered saline, whereas the administration of AdLacZ to TDI-inhaled mice did not. In addition, TDI-inhaled mice administered anti-IL-17 Ab (Fig. 11, *E* and *F*) showed a significant decrease of the percentage of cells stained with PAS in the airway epithelium compared with the levels in TDI-inhaled mice administered saline or isotype control mAb.

Discussion

Airway inflammation and remodeling are prominent features of TDI-induced airway disease and are connected by complex signaling networks. PTEN and IL-17 are thought to contribute to the pathogenesis of airway disease, but the interrelationship between these proteins in airway inflammation and remodeling has not been clarified. Our present study with the TDI-induced murine model of airway disease has revealed that the numbers of inflammatory cells of the airways, airway hyperresponsiveness, airway mucus production, and the levels of IL-17 protein and mRNA expression were increased. Administration of PI3K inhibitors or AdPTEN reduced the pathophysiological features of TDI-induced airway disease and decreased the increased levels of IL-17 protein and mRNA. Our results also showed that PI3K inhibitors or AdPTEN down-regulated a transcription factor, NF- κ B activity, and the inhibition of NF- κ B activation reduced the increase of IL-17 protein after TDI inhalation. In addition, our data showed that the inhibition of IL-17 activity with an anti-IL-17 Ab reduced airway inflammation and airway hyperresponsiveness. These findings suggest that a protective role of PTEN in the pathogenesis of TDI-induced airway disease is mediated in part through an IL-17-dependent mechanism.

IL-17 is a recently discovered cytokine family, notably characterized by three members IL-17A, IL-17E, and IL-17F, and known to play a role in tissue inflammation by inducing the release of proinflammatory and neutrophil-mobilizing cytokines (4). T lymphocyte-derived cytokine IL-17 causes an accumulation of neutrophils in the airways in part via the release of CXC chemokines (32, 33). Intratracheal stimulation with IL-17 also increases the concentration of the neutrophil-derived enzymes neutrophil elastase and myeloperoxidase in rat airways in vivo (34). Interestingly, the concentration of IL-17 may also be increased in the airways of patients with acute severe asthma (35, 36) and in healthy volunteers with severe airway inflammation induced by exposure to organic dust (37). In addition, a blockade of endogenous IL-17 also inhibits the endotoxin-induced accumulation of neutrophils in rodent airways in vivo (38, 39). Thus, it has been proposed that IL-17 plays a central role in mobilizing neutrophils in the airways and

lungs (39). IL-17A is able to induce mucin gene expression in vitro (40) and to enhance it in vivo (41). A recent study of IL-17A-deficient mice has demonstrated that IL-17A is involved in the activation of allergen-specific T cells. In those mice, decreased levels of IL-4 and IL-5, but not IFN- γ , were seen that are associated with a reduced level of airway hypersensitivity (42). Transgenic overexpression of IL-17E results in the induction of airway hyperresponsiveness, mucus hypersecretion, airway eosinophilia, and an increase in serum levels of IL-5, IL-13, and IgE (43, 44). Consistent with these observations, in this study we have found that IL-17 expression was up-regulated in TDI-induced airway disease. Interestingly, anti-IL-17 Ab blocked the airway inflammation, airway hyperresponsiveness, and increased airway mucus production. These findings suggest that IL-17 plays an important role in the induction and maintenance of the airway inflammatory and remodeling responses in TDI-induced airway disorders.

TDI-induced airway disease is an inflammatory disease characterized by airway obstruction and bronchial hyperresponsiveness. Many inflammatory mediators attract and activate inflammatory cells via signal transductions involving PI3K (14, 15, 19, 23, 25, 45, 46). Recent studies have demonstrated that PI3K plays a key role in the pathogenesis of the asthma phenotype such as airway inflammation, mucus hypersecretion, and hyperresponsiveness (12–15, 19). Consistent with these observations, we have found that PI3K activities were increased significantly in a murine model of TDI-induced airway disease. Administration of wortmannin or LY-294002, which are potent and selective PI3K inhibitors, reduced the airway inflammation, airway hyperresponsiveness, and increased airway mucus production. In addition, previous studies have demonstrated that PI3K acts downstream of IL-17 in synovial cells and airway smooth muscle cells (47–50) but not in other local tissues such as bronchial epithelial cells (51–53), suggesting that the involvement of PI3K downstream of IL-17 may be cell type dependent. In contrast, recent studies have reported that PI3K acts upstream of IL-17, indicating that IL-17 production is mediated via activation of the PI3K/Akt pathway (16, 54, 55). In this present study, the administration of PI3K inhibitors inhibited the increased level of IL-17 after TDI inhalation. The use of PI3K inhibitors has revealed that PI3K may be involved upstream of IL-17 production as well as in the transduction of activating signals generated by many inflammatory mediators in inflammatory cells, although a possibility that PI3K acts downstream of IL-17 could not be excluded. Therefore, we conclude that PI3K may play an important role in the induction and maintenance of TDI-induced airway disease.

PTEN is one of the most frequently mutated tumor suppressors in human cancer. It is also essential for embryonic development, cell migration, and apoptosis (17, 56, 57). PTEN has been implicated in regulating cell survival signaling through the PI3K/Akt pathway (17, 18, 58–60). PTEN dephosphorylates the D3 position of the key lipid second messenger PIP3 (17). In addition, PTEN has weak protein tyrosine phosphatase activity, which may target focal adhesion kinase and/or Shc and thereby modulate other complex pathways. However, the major function of PTEN appears to be down-regulation of PIP3 produced by PI3K. Recently, we have reported that PTEN plays a pivotal role in the pathogenesis of the allergic airway disease in mice (19). A previous study has revealed that somatic mutation or deletion of PTEN was observed in the epithelium of patients of chronic airway inflammatory disease (61). Additionally, our preliminary analysis of BAL fluids from asthmatic human patients also showed decreased PTEN expression compared with normal individuals (our unpublished data). In this study, we have observed that the expression of PTEN protein and

PTEN activity were decreased in TDI-induced airway disease. Intratracheal administration of AdPTEN reduced the airway inflammation, bronchial hyperresponsiveness, increased airway mucus production, and increased IL-17 expression.

NF- κ B is present in most cell types and is known to play a critical role in immune and inflammatory responses, including asthma (62–67). As expected, the NF- κ B p65 protein level in nuclear protein extracts of lung tissues was substantially increased in the TDI-induced model of airway disease used for the present study. It is known that activation of this transcription factor induces a variety of inflammatory genes that are abnormally expressed in asthma. These genes include cytokines (e.g., *IL-4*, *IL-5*, *IL-9*, *IL-11*, *IL-15*, *IL-17*, and *TNF- α*), chemokines (e.g., *RANTES*, *eotaxin*, and *MCP-3*), and adhesion molecules (e.g., *ICAM-1* and *VCAM-1*) (16, 68, 69). In addition, recent studies have found that IL-17 production in CD4⁺ T cells is mediated via the activation of Jak2, PI3K/Akt, STAT3, and NF- κ B (16, 54, 55). These experiments were performed using CD4⁺ T cells from peripheral human blood (16, 55) or from murine spleen (54). Studies with PI3K inhibitor and NF- κ B inhibitor have shown that the PI3K and NF- κ B signaling pathway is required for IL-17 production in CD4⁺ T cells. These in vitro data were consistent with our present in vivo data. Thus, the results from our present study have revealed that the expression of IL-17 was increased significantly in a murine model of TDI-induced airway disease. Administration of PI3K inhibitors or AdPTEN resulted in a significant reduction of NF- κ B activity as well as IL-17 expression. In addition, the increased IL-17 protein levels after TDI inhalation were decreased by the administration of an inhibitor of NF- κ B activation, BAY 11-7085. Therefore, these results suggest that PI3K inhibitors or AdPTEN reduces IL-17 expression in TDI-induced airway disease and that NF- κ B may be one of the signaling molecules related to IL-17 expression mediated by PTEN in TDI-induced airway disease.

In summary, we have examined the effect of the PI3K inhibitors or AdPTEN on the regulation of IL-17 expression in a murine model of TDI-induced airway disease. Administration of PI3K inhibitors or AdPTEN reduced the pathophysiological features of TDI-induced airway disease and decreased the increased levels of IL-17 protein and mRNA expression. Moreover, our results also showed that PI3K inhibitors or AdPTEN down-regulated NF- κ B activity. In addition, inhibition of NF- κ B activation decreased the increase of IL-17 after TDI inhalation. These findings suggest that a protective role for PTEN in the pathogenesis of TDI-induced airway disease is mediated through an IL-17-dependent mechanism. Our findings may also assist a strategy for the treatment of airway inflammation and remodeling in occupational airway disease.

Acknowledgment

We thank Prof. Mie-Jae Im for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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