Phosphoinositide 3-kinase δ inhibitor suppresses IL-17 expression in a murine asthma model


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ABSTRACT

Phosphoinositide 3-kinases (PI3Ks) contribute to the pathogenesis of asthma by regulating the activation of inflammatory mediators, inflammatory cell recruitment, and immune cell function. Recent findings have indicated that PI3Ks also regulate the expression of interleukin (IL)-17, which has been introduced as an important cytokine involved in airway inflammation.

In the present study, we investigated a role of PI3Kδ in the regulation of IL-17 expression in allergic airway disease using a murine model of asthma.

After ovalbumin (OVA) inhalation, administration of a selective p110δ inhibitor, IC87114, significantly attenuated airway infiltration of total cells, lymphocytes, neutrophils, and eosinophils as well as airway hyperresponsiveness and decreased the increase in IL-17 protein and mRNA expression. Moreover, IC87114 reduced levels of IL-4, IL-5, and IL-13, expression of keratinocyte chemoattractant protein and mRNA, and nuclear factor-κB (NF-κB) activity. In addition, a NF-κB inhibitor, BAY 11-7085 substantially reduced the increase in IL-17 protein levels. Our results also showed that inhibition of IL-17 activity with an anti-IL-17 antibody remarkably reduced airway inflammation and hyperresponsiveness.

These findings suggest that inhibition of the p110δ signalling pathway suppresses IL-17 expression through regulation of NF-κB activity and thus has therapeutic potential in asthma.
KEYWORDS: Asthma, interleukin-17, nuclear factor-κB, phosphoinositide 3-kinase, p110δ
INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by reversible airway obstruction and airway hyperresponsiveness [1]. Airway inflammation in asthma increases an expression of various components of the inflammatory cascade, which include cytokines, chemokines, adhesion molecules, growth factors, and enzymes. Central to this inflammatory cascade is a complex interaction between receptor signalling and downstream lipid and protein kinases. In particular, phosphoinositide 3-kinase (PI3K) is an important kinase involved in the inflammatory process.

The PI3K is a large family of signalling kinases that generate lipid second messengers responsible for regulating a number of cellular activities. PI3Ks mediate key signal transduction reactions during immune and inflammatory responses and hence represent an attractive target for therapeutic development in various inflammatory diseases [2, 3]. Research conducted over the last few years has highlighted the importance of PI3Ks in bronchial asthma. Our previous study with a murine model of asthma has shown that PI3K activity increases after ovalbumin (OVA) challenge compared with pre-challenge period [4]. In addition, administration of PI3K inhibitor, wortmannin or LY-294002 reduces T-helper 2 (Th2) cytokine production, pulmonary eosinophilia, airway inflammation, and bronchial hyperresponsiveness [4]. There is increasing evidence that PI3Ks contribute to the pathogenesis of asthma by regulating the expression and activation of inflammatory
mediators, inflammatory cell recruitment, and immune cell function [2, 4]. Recent work has begun to explore specific roles of different PI3K isoforms, and p110δ and p110γ have been suggested to be the dominant isoforms involved in the PI3K-mediated immune responses [5]. Two commercially available PI3K inhibitors, wortmannin and LY-294002, used to investigate the biological roles of PI3Ks and to analyze PI3K-driven pathways, are poorly selective among four members of the class I PI3K. Therefore, development of PI3K isoform-specific inhibitors has been an area of considerable interest. A selective inhibitor of p110δ catalytic activity, IC87114, has recently been synthesized and used to explore the role of PI3Kδ in neutrophil migration [6, 7]. In addition, previous studies using IC87114 have suggested that p110δ plays an important role in the pathogenesis of allergic asthma [8, 9].

The interleukin (IL)-17 family is a recently described group of cytokines and consists of six members, namely IL-17 (also called IL-17A), IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F [10]. IL-17, the most investigated member in this family, has been implicated in many immune and inflammatory responses primarily as a pro-inflammatory regulator, inducing expression of many inflammatory mediators such as cytokines, chemokines, adhesion molecules, and growth factors [11, 12]. IL-17 mRNA is up-regulated in inflamed airways after allergen inhalation in a mouse model of allergic asthma [13]. Consistent with the animal study, IL-17 mRNA and/or protein expression is increased in sputum, bronchoalveolar lavage (BAL) fluids, and peripheral blood in asthmatics [14-18]. Moreover, the levels of IL-17
expression in airways correlate with the severity of airway hypersensitivity in asthmatic patients [14]. These findings have indicated the potential role of IL-17 in the pathogenesis of asthma. Recent studies, including our own, have found that IL-17 production is mediated by the activation of PI3K/Akt pathway [12, 19-21]. However, the contributions of individual PI3K isoforms to the up-regulation of IL-17 expression have not been examined. In the present study, we investigated an involvement of PI3Kδ in IL-17 expression and its molecular mechanism using a murine model of asthma.
MATERIALS AND METHODS

A more detailed methodology is available in the online supplementary material.

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine specific pathogens, were obtained from Orientbio Inc. (Seoungnam, Korea). 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. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 µg OVA (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) in a total volume of 200 µL, as described previously [8, 9]. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (weight/volume) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). BAL was performed at 48 h after the last challenge. At the time of lavage, mice were sacrificed with an overdose of pentobarbital sodium (100 mg·kg⁻¹ of body weight, administered intraperitoneally).

Administration of IC87114, LY-294002, anti-IL-17 antibody (Ab), isotype control

monoclonal Ab (mAb), Akt inhibitor, or BAY 11-7085
IC87114 (0.1 or 1 mg·kg\(^{-1}\) body weight·day\(^{-1}\)), LY-294002 (a selective inhibitor of PI3K; 1.5 mg·kg\(^{-1}\) body weight·day\(^{-1}\); BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA), or vehicle control (dimethylsulfoxide (DMSO)) diluted with 0.9% NaCl, was administered in a volume of 50 µL by intratracheal instillation two times to each animal, once on day 21 (1 h before the first OVA challenge) and the second time on day 23 (3 h after the last OVA challenge) [8, 9]. Anti-IL-17 Ab or isotype control mAb (5 mg·kg\(^{-1}\) body weight·day\(^{-1}\); R&D Systems, Inc., Minneapolis, MN, USA) was administered intraperitoneally two times to each animal, once on day 21 and the second time on day 24 (24 h after the last OVA challenge). An Akt inhibitor, 1L6-hydroxymethyl-chiro-inositol-2-(R)-2-\(O\)-methyl-3-\(O\)-octadecyl-\(sn\)-glycerocarbonate (3 mg·kg\(^{-1}\) body weight·day\(^{-1}\); Calbiochem, Darmstadt, Germany) dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection two times to each animal, once on day 21 and the second time on day 24. An inhibitor of nuclear factor-κB (NF-κB) activation, BAY 11-7085 (20 mg·kg\(^{-1}\) body weight·day\(^{-1}\); BIOMOL Research Laboratories Inc.) dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection two times to each animal, once on day 21 and the second time on day 24.

*Western blot analysis*
Protein expression levels were analyzed by means of Western blot analysis, as described previously [8]. The blots were incubated with an anti-IL-17 Ab (R&D Systems, Inc.), anti-IL-4 Ab (Serotec Ltd, Oxford, UK), anti-IL-5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IL-13 Ab (R&D Systems, Inc.), biotinylated anti-mouse keratinocyte chemoattractant (KC) Ab (R&D Systems, Inc.), anti-Akt Ab (Cell Signaling Technology Inc., Beverly, MA, USA), anti-phosphorylated Akt (p-Akt) Ab (Cell Signaling Technology Inc.), or anti-inhibitor of κBα (IκBα) Ab (Santa Cruz Biotechnology).

**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 described previously [12].

**Quantitative real-time RT-PCR**

Quantitative real-time RT-PCR analysis was performed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) as described previously [12].

**Measurement of Th2 cytokines**
Levels of IL-4, IL-5, and IL-13 were quantified by enzyme immunoassays (R&D Systems, Inc.).

_Cytosolic or nuclear protein extractions for analysis of NF-κB p65_

Cytosolic or nuclear extractions were performed as described previously [9].

_Measurement of PI3K enzyme activity in lung tissues_

PI3K enzyme activity in lung tissues was measured as described previously [9]. Amounts of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) were quantified by a PIP3 competition enzyme immunoassay (Echelon, Inc., Salt Lake City, UT, USA).

_Determination of airway responsiveness_

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine, as described previously [9].

_Densitometric analyses and statistics_

All immunoreactive and phosphorylative signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories Inc., Hercules, CA, USA). Data were expressed as mean
± SEM. Statistical comparisons were performed using one-way ANOVA followed by Scheffe’s test. Statistical significance was set at p<0.05.
RESULTS

Effect of IC87114 on IL-17 protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that the levels of IL-17 protein in lung tissues were significantly increased at 48 h after the last inhalation of OVA compared with those in the control mice (figs 1a and 1b). The increase in the IL-17 protein levels after OVA inhalation was decreased significantly by the administration of IC87114. RT-PCR and real-time RT-PCR analysis showed that the increase in the IL-17 mRNA expression after OVA inhalation was significantly reduced by the administration of IC87114 (figs 1c and 1d).

Effect of IC87114 on IL-17 protein levels in BAL fluids of OVA-sensitized and -challenged mice

Western blot analysis revealed that the levels of IL-17 protein in BAL fluids were significantly increased at 48 h after OVA inhalation compared with those in the control mice (fig. 2). The increase in the IL-17 protein levels after OVA inhalation was significantly inhibited by the administration of IC87114.

Effect of IC87114 or anti-IL-17 Ab on cellular changes in BAL fluids
Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 48 h after OVA inhalation compared with the numbers after saline inhalation (fig. 3a). The increases in the numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of IC87114. In addition, administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice also significantly decreased the increases in the numbers of total cells, lymphocytes, neutrophils, and eosinophils compared with the numbers in OVA-inhaled mice administered drug vehicle or isotype control mAb.

**Effect of IC87114 or anti-IL-17 Ab on pathological changes of OVA-induced asthma**

Histologic analysis revealed the typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells infiltrated around the bronchioles and mucus and debris had accumulated in the lumens of bronchioles (fig. 3c) compared with the control (fig. 3b). Mice treated with IC87114 (fig. 3d) or anti-IL-17 Ab (fig. 3e) showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar region and in the amount of debris in the airway lumens.

**Effect of IC87114 or anti-IL-17 Ab on airway hyperresponsiveness**
The airway responsiveness was assessed as a percent increase of respiratory system resistance (Rrs) in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent Rrs shifted to the left compared with that of the control mice (fig. 3f). In addition, the percent Rrs produced by methacholine administration (at dose of 50 mg·mL⁻¹) increased significantly in the OVA-inhaled mice compared with the control mice. OVA-sensitized and -challenged mice treated with IC87114 showed the dose-response curve of percent Rrs that shifted to the right and a significant reduction in the percent Rrs produced by methacholine at 50 mg·mL⁻¹ dose compared with those of untreated mice. In addition, the administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice also decreased significantly the percent Rrs produced by methacholine at 50 mg·mL⁻¹ dose compared with that of OVA-inhaled mice administered drug vehicle or isotype control mAb. These results indicate that treatment of IC87114 or anti-IL-17 Ab reduces OVA-induced airway hyperresponsiveness.

**Effect of IC87114 on levels of IL-4, IL-5, and IL-13 in lung tissues and BAL fluids of OVA-sensitized and -challenged mice**

Western blot analysis revealed that the protein levels of IL-4, IL-5, and IL-13 in lung tissues were increased at 48 h after OVA inhalation compared with those in the control mice (fig. 1Sa in the supplementary data). The increases in these cytokine levels were reduced by the
administration of IC87114. Consistent with the results obtained from the Western blot analysis results, an enzyme immunoassay of IL-4, IL-5, or IL-13 showed that the levels of these cytokines in BAL fluids were significantly reduced by the administration of IC87114 (fig. 1Sb in the supplementary data).

**Effect of IC87114 on KC protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice**

Western blot analysis revealed that the levels of KC protein in lung tissues were significantly increased at 48 h after OVA inhalation compared with those in the control mice (figs 4a and 4b). The increase in the KC protein levels after OVA inhalation was decreased significantly by the administration of IC87114. RT-PCR and real-time RT-PCR analysis showed that the increase in the KC mRNA expression after OVA inhalation was significantly reduced by the administration of IC87114 (figs 4c and 4d).

**Effect of anti-IL-17 Ab on KC protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice**

Western blot analysis revealed that administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice significantly decreased the levels of KC protein in lung tissues compared with the levels in OVA-inhaled mice administered isotype control mAb (figs 4e and 4f). In
addition, RT-PCR and real-time RT-PCR analysis showed that the administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice significantly decreased the KC mRNA expression compared with that in OVA-inhaled mice administered isotype control mAb (figs 4g and 4h).

**Effect of LY-294002 or IC87114 on p-Akt and Akt protein levels and PI3K enzyme activity in lung tissues of OVA-sensitized and -challenged mice**

The levels of p-Akt protein in the lung tissues were significantly increased at 48 h after OVA inhalation compared with levels in the control mice (figs 5a and 5b). However, no significant changes in the Akt protein levels were observed in any of the groups tested. The increase of p-Akt but not Akt protein levels in the lung tissues at 48 h after OVA inhalation was significantly reduced by the administration of LY-294002 or IC87114. Consistent with these results, PIP3 levels in the lung tissues were significantly increased at 48 h after OVA inhalation compared with those in the control mice (fig. 5c). The increase of the PIP3 levels in the lung tissues at 48 h after OVA inhalation was significantly reduced by the administration of LY-294002 or IC87114.

**Effect of LY-294002 or IC87114 on IκBα protein levels in lung tissues of OVA-sensitized and -challenged mice**
Western blot analysis revealed that the IκBα protein levels in the lung tissues were significantly decreased at 48 h after OVA inhalation compared with those in the control mice (figs 5d and 5e). The decrease in the IκBα protein levels after OVA inhalation was significantly inhibited by the administration of LY-294002 or IC87114.

Effect of Akt inhibitor on IκBα and NF-κB p65 protein levels in lung tissues of OVA-sensitized and -challenged mice

The IκBα protein levels decreased after OVA inhalation was significantly increased by the administration of Akt inhibitor (figs 6a and 6b). Western blot analysis revealed that the levels of NF-κB p65 in nuclear protein extracts from the lung tissues were increased at 48 h after OVA inhalation compared with the levels in the control mice administered saline (figs 6c and 6d). The administration of Akt inhibitor significantly reduced the increase in the NF-κB p65 levels in nuclear protein extracts after OVA inhalation. In contrast, the levels of NF-κB p65 in cytosolic protein fractions from the lung tissues were decreased after OVA inhalation compared with the levels in the control mice administered saline. The decrease in the NF-κB p65 levels in cytosolic protein fractions was substantially inhibited by the administration of Akt inhibitor.
**Effect of IC87114 on NF-κB p65 protein levels in lung tissues of OVA-sensitized and -challenged mice**

The increase in the NF-κB p65 levels in nuclear protein extracts of the lung tissues after OVA inhalation was significantly reduced by the administration of IC87114 (fig. 7). In contrast, the decrease in the NF-κB p65 levels in cytosolic protein fractions of the lung tissues was substantially inhibited by the administration of IC87114.

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

Western blot analysis showed that the IL-17 protein levels increased after OVA inhalation were significantly reduced by the administration of BAY 11-7085, an inhibitor of NF-κB activation (fig. 8).
DISCUSSION

In this study, we demonstrated that inhibition of the p110δ signalling pathway suppressed IL-17 protein and mRNA expression in the lungs and attenuated allergen-induced airway inflammation and airway hyperresponsiveness. Our OVA-induced model of asthma used in this study revealed the following typical pathological features of asthma: increased numbers of inflammatory cells in airways, airway hyperresponsiveness, and increased levels of Th2 cytokines (IL-4, IL-5, and IL-13), chemokine KC, and nuclear NF-κB. In addition, the expression of IL-17 protein and mRNA in the lungs was increased after OVA inhalation. The administration of a selective PI3Kδ inhibitor, IC87114 significantly attenuated airway infiltration of total cells, lymphocytes, neutrophils, and eosinophils as well as airway hyperresponsiveness, and also decreased IL-17 protein and mRNA expression. Moreover, IC87114 reduced levels of Th2 cytokines, expression of KC protein and mRNA, Akt phosphorylation, IκBα degradation, and NF-κB activity. Supporting the results, an Akt inhibitor also increased IκBα protein levels with reducing NF-κB activity, and an NF-κB inhibitor, BAY 11-7085 substantially reduced the increase in IL-17 protein levels after OVA inhalation. In addition, the inhibition of IL-17 activity with an anti-IL-17 Ab remarkably reduced bronchial inflammation and airway hyperresponsiveness. These results suggest that the therapeutic effect of a PI3Kδ inhibitor in asthma is partly mediated by suppressing IL-17.
expression via modulation of Akt-mediated NF-κB activation. To the best of our knowledge, this is the first study clarifying a role of PI3Kδ in regulation of IL-17 expression.

The PI3K family is divided into class I, II, and III PI3Ks, and the class I PI3K is further subdivided into class IA (PI3Kα, PI3Kβ, and PI3Kδ isoforms) and class IB PI3K (PI3Kγ isoform) on the basis of structures and substrate specificities [22]. Structurally, the class IA PI3K consists of a heterodimer composed of a 110-kd (p110α, β, or δ) catalytic subunit and a regulatory subunit (p85α, p85β, p55α, p55γ, or p50α) [23]. These differences allow PI3K isoforms to mediate distinct functions. Of these isoforms of PI3K, p110δ isoform has been implicated to play a key role in immune responses and inflammation [24]. Recent studies have demonstrated a novel biological role of p110δ signalling in allergic airway inflammation and emphasized the importance of PI3Kδ isoform as a potential therapeutic target in asthma [8, 9]. The genetic inactivation of p110δ has resulted in reduction of antigen receptor function, Th2 cytokine production, and Th2 cell-mediated inflammatory reactions [25]. In the present study, the results showed that administration of a specific inhibitor of PI3Kδ substantially reduced the pathological features of asthma, including airway inflammation, airway hyperresponsiveness, and the increases in Th2 cytokine levels. These results are consistent with the findings of previous studies [8, 9, 25], confirming the therapeutic potential of the PI3Kδ inhibitor in asthma.
IL-17 is a new cytokine that has recently captured attention of many scientists due to its involvement in immune and inflammatory responses [26]. IL-17 in airways is most likely produced by a unique T helper lineage called Th17 cells [27]. Recent studies have indicated that eosinophils, neutrophils, and monocytes are also sources of IL-17 [15, 26]. The biological function of IL-17 appears to be associated with neutrophil-dominated inflammation, as a promoter of granulopoiesis, neutrophil accumulation, and neutrophil activation in the lung [11, 26]. Intratracheal administration of IL-17 has been shown to increase the absolute number of neutrophils in BAL fluids [28]. In addition, IL-17 increases the expression of IL-8 mRNA in bronchial epithelial cells, and the neutrophil chemotactic effect of IL-17 is blocked by an anti-IL-8 Ab [29]. Therefore, it is likely that IL-17 is able to recruit neutrophils selectively into airways by inducing the release of the potent neutrophil-directed chemokine IL-8 [29, 30]. In this study, we evaluated an effect of an anti-IL-17 Ab on the expression of KC, which is a functional murine homolog of IL-8. Consistent with previous studies, we found that inhibiting IL-17 activity with the anti-IL-17 Ab remarkably reduced the increases in airway infiltration of neutrophils and KC protein and mRNA expression after allergen inhalation. These results suggest that IL-17 orchestrates neutrophilic influx into airways by inducing the release of IL-8 in our model of allergic airway inflammation. It is well established that the pathognomonic features of asthma are mediated by eosinophils, mast cells, and Th2 cells as well as their cytokines [31]. On the other hand, it
has been reported that the numbers of neutrophils in airways are increased in severe asthma [32]. Neutrophils in asthma potentially contribute to airway gland hypersecretion, airway obstruction, and airway remodelling [17, 33]. Our data from the present study support the notion that IL-17-induced neutrophil recruitment into airways is implicated in the pathogenesis of asthma.

Interestingly, a recent study has demonstrated that IL-17 receptor gene-deficient mice show a reduced recruitment of not only neutrophils but also eosinophils into airways upon antigen challenge [34]. In addition, eosinophil peroxidase activity in lung tissues and OVA-specific serum IgE concentrations are reduced in the absence of IL-17 receptor signalling [35]. Furthermore, an involvement of IL-17 in the activation of allergen-specific T cells has also been reported [36]. IL-17-deficient or IL-17 receptor-deficient mice have revealed the decreases in Th2 cytokine levels, which are associated with reduced airway hypersensitivity [34, 35]. A more recent study has shown that the IL-17 mRNA levels correlate positively with the IL-5 mRNA levels in sputum from asthmatic patients [18]. Therefore, IL-17 seems to contribute to Th2 cell-mediated and eosinophilic inflammation in asthma. Consistent with these observations, our present data showed that inhibition of IL-17 activity with an anti-IL-17 Ab remarkably reduced antigen-induced airway infiltration of eosinophils and airway hyperresponsiveness. We also observed that inhibition of IL-17 activity significantly reduced the increases in Th2 cytokine levels in BAL fluids. Taken together, these findings indicate
that IL-17 plays a crucial role in the pathogenesis of asthma, contributing to neutrophilic inflammation as well as Th2 cell-mediated eosinophilic inflammation.

IL-17 production is mediated by activation of PI3K/Akt pathway [23, 19-21]. It has been reported that LY-294002 inhibits IL-17 production and mRNA expression in CD4+ T cells [20]. Consistent with these *in vitro* data, we have previously reported that administration of wortmannin or LY-294002 reduces the increase in IL-17 levels after allergen inhalation in a murine model of asthma [12]. However, these inhibitors do not differentiate the four isoforms of class I PI3K. Our present study found that pharmacological blockade of p110δ activity with IC87114 substantially decreased the expression of IL-17 protein and mRNA in the OVA-inflamed lungs. In addition, IC87114 was able to reduce the levels of Th2 cytokines as well as the expression of KC protein and mRNA, which are associated with IL-17 expression. PI3Kδ has been shown to play a prominent role in the accumulation and activation of neutrophils in inflamed tissues [6, 7]. However, the signalling pathways for the contribution of PI3Kδ to neutrophil chemotaxis have not been well defined. The results of the present study suggest that IL-17 is a key regulator underlying in neutrophilic inflammation mediated by PI3Kδ. Taken together, these findings suggest that the therapeutic effect of the PI3Kδ inhibitor in the asthma is exerted by down-regulating IL-17 expression, thereby attenuating Th2 cell-mediated eosinophilic inflammation and neutrophilic inflammation.
A transcription factor, NF-κB plays an essential role in immune and inflammatory responses and is consequently associated with the pathogenesis of asthma. As expected, nuclear NF-κB protein levels were substantially increased in our present OVA-induced asthma model, indicating that NF-κB is activated. Activation of this transcription factor induces many inflammatory genes encoding cytokines (tumor necrosis factor-α, IL-4, IL-5, IL-6, IL-9, and IL-13), chemokines (RANTES, eotaxin, and IL-8), adhesion molecules (intracellular cell adhesion molecule-1 and vascular cell adhesion molecule-1), growth factors, and receptors, which are potentially relevant to the pathogenesis of asthma [37]. Furthermore, \textit{in vitro} and \textit{in vivo} studies have shown that inhibition of NF-κB activation reduces IL-17 production [13, 19-21]. These findings suggest that NF-κB is an important regulator of IL-17 expression.

Activation of PI3K/Akt pathway leads to a pronounced augmentation of NF-κB activity through IkBα degradation [37, 38]. However, no data are available for the role of PI3K catalytic subunit p110δ in the activation of NF-κB. Our present study showed that administration of a PI3Kδ inhibitor substantially decreased allergen-induced Akt phosphorylation with a significant reduction of IkBα degradation and NF-κB activity in lung tissues. Consistent with the published data [38], we also found that an Akt inhibitor increased IkBα protein levels with reducing NF-κB activity in lung tissues of OVA-challenged mice. In addition, the increase in IL-17 protein and mRNA expression after OVA inhalation was decreased by administration of an inhibitor of NF-κB activation, BAY 11-7085 or an Akt
inhibitor (fig. 2S in the supplementary data). These findings suggest that p110δ activates Akt and thereby enhances IκBα degradation and subsequent NF-κB activation, which regulates IL-17 expression in allergic airway inflammation.

In conclusion, our results have demonstrated that a specific inhibitor of PI3Kδ, IC87114, reverses all pathological features of asthma, including airway infiltration of inflammatory cells, Th2 cytokine levels, and KC expression. In addition, IC87114 decreased the expression of IL-17 protein and mRNA in lungs increased by OVA challenge. These results suggest that IC87114 down-regulates IL-17 expression and attenuates Th2 cell-mediated eosinophilic inflammation and neutrophilic inflammation. In addition, we have also found that this PI3Kδ inhibitor inhibits NF-κB activity and that NF-κB inhibition reduces the increase in IL-17 expression. Based on these observations, we suggest that inhibition of the p110δ signalling pathway has therapeutic potential in asthma by down-regulating NF-κB pathway and resultant suppressing IL-17 expression. Accordingly, the present study provides an important mechanism for the use of PI3Kδ inhibitor to prevent and/or treat asthma and other allergic airway diseases.
SUPPORT STATEMENT

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STATEMENT OF INTEREST

Kamal D. Puri is employed by Calistoga Pharmaceuticals. The rest of the authors have declared that they have no conflict of interest.

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FIGURE LEGENDS

FIGURE 1. Effect of IC87114 on interleukin (IL)-17 protein levels and mRNA expression in lung tissues of ovalbumin (OVA)-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 mg·kg⁻¹ (OVA+IC87114 0.1 mg·kg⁻¹), and OVA-inhaled mice administered IC87114 1.0 mg·kg⁻¹ (OVA+IC87114 1.0 mg·kg⁻¹). 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 SAL+VEH is arbitrarily presented as 1. c) Representative RT-PCR analyses of IL-17 mRNA expression. d) Quantitative analyses of IL-17 mRNA expression by means of real-time RT-PCR. Data represent mean ± SEM from 7 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH.
FIGURE 1
FIGURE 2. Effect of IC87114 on interleukin (IL)-17 protein levels in bronchoalveolar lavage (BAL) fluids of ovalbumin (OVA)-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 mg·kg⁻¹ (OVA+IC87114 mg·kg⁻¹), and OVA-inhaled mice administered IC87114 1.0 mg·kg⁻¹ (OVA+IC87114 1.0 mg·kg⁻¹). a) Western blot analyses of IL-17 protein. b) Densitometric analyses are presented as the relative ratio of IL-17 levels in OVA+VEH, OVA+IC87114 mg·kg⁻¹, or OVA+IC87114 1.0 mg·kg⁻¹ to those in SAL+VEH. The IL-17 protein level in BAL fluids of SAL+VEH is arbitrarily presented as 1. Data represent mean ± SEM from 7 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH.
FIGURE 3. a) Effect of IC87114 or anti-interleukin-17 antibody (anti-IL-17 Ab) on total and differential cell counts in bronchoalveolar lavage (BAL) fluids of ovalbumin (OVA)-sensitized and -challenged mice. Differential cell counts in BAL fluids from saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 mg·kg⁻¹ (OVA+IC87114 0.1 mg·kg⁻¹), OVA-inhaled mice administered IC87114 1.0 mg·kg⁻¹ (OVA+IC87114 1.0 mg·kg⁻¹), OVA-inhaled mice administered anti-IL-17 Ab (OVA+anti-IL-17 Ab), and OVA-inhaled mice administered isotype control monoclonal Ab (mAb) (OVA+control mAb). b-e) Effect of IC87114 or anti-IL-17 Ab in lung tissues of OVA-sensitized and -challenged mice. Representative hematoxylin and eosin-stained sections of the lungs. Sampling was performed.
at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (b), OVA-inhaled mice administered drug vehicle (c), OVA-inhaled mice administered IC87114 1.0 mg·kg⁻¹ (d, and OVA-inhaled mice administered anti-IL-17 Ab (e). Bars indicate scale of 20 µm. Results were similar in seven mice per group. f) Effect of IC87114 or anti-IL-17 Ab on airway responsiveness in OVA-sensitized and -challenged mice. Airway responsiveness was measured at 48 h after the last challenge in SAL+VEH, OVA+VEH, OVA+IC87114 0.1 mg·kg⁻¹, OVA+IC87114 1.0 mg·kg⁻¹, OVA+anti-IL-17 Ab, and OVA+control mAb. Data represent mean ± SEM from 7 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH; §, p<0.05 versus OVA+control mAb.
FIGURE 4. Effect of IC87114 or anti-interleukin-17 antibody (anti-IL-17 Ab) on keratinocyte chemoattractant (KC) protein levels and mRNA expression in lung tissues of ovalbumin (OVA)-sensitized and -challenged mice. a-d) Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-
inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 mg·kg⁻¹ (OVA+IC87114 0.1 mg·kg⁻¹), and OVA-inhaled mice administered IC87114 1.0 mg·kg⁻¹ (OVA+IC87114 1.0 mg·kg⁻¹). e-h) Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), OVA-inhaled mice administered isotype control monoclonal Ab (mAb) (OVA+control mAb), and OVA-inhaled mice administered anti-IL-17 Ab (OVA+anti-IL-17 Ab). a and e) Western blot analyses of KC protein. b and f) Densitometric analyses are presented as the relative ratio of KC to actin. The relative ratio of KC in the lung tissues of SAL+VEH (b) or SAL+SAL (f) is arbitrarily presented as 1. c and g) Representative RT-PCR analyses of KC mRNA expression. d and h) Quantitative analyses of KC mRNA expression by means of real-time RT-PCR. Data represent mean ± SEM from 7 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH; §, p<0.05 versus SAL+SAL; ¶, p<0.05 versus OVA+control mAb.
FIGURE 4
**FIGURE 5.** Effect of LY-294002 or IC87114 on phosphorylated Akt (p-Akt) and Akt protein levels, phosphoinositide 3-kinase (PI3K) enzyme activity, and inhibitor of IκBα (IκBα) protein levels in lung tissues of ovalbumin (OVA)-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered LY-294002 (OVA+LY-294002), and OVA-inhaled mice administered IC87114 1.0 mg·kg$^{-1}$ (OVA+IC87114). a) Western blotting of p-Akt and Akt protein. 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 SAL+VEH is arbitrarily presented as 1. c) Enzyme immunoassay of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) generation by PI3Ks in lung tissues. d) Western blotting of IκBα protein. e) Densitometric analyses are presented as the relative ratio of IκBα to actin. The relative ratio of IκBα in the lung tissues of SAL+VEH is arbitrarily presented as 1. Data represent mean ± SEM from 6 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH.
FIGURE 6. Effect of Akt inhibitor on inhibitor of κBα (IκBα) and nuclear factor-κB (NF-κB) p65 protein levels in lung tissues of ovalbumin (OVA)-sensitized and challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), and OVA-inhaled mice administered Akt inhibitor (OVA+Akt inhibitor). a)
Western blotting of IκBα protein. b) Densitometric analyses are presented as the relative ratio of IκBα to actin. The relative ratio of IκBα in the lung tissues of SAL+VEH is arbitrarily presented as 1. c) Western blot analyses of NF-κB p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. d) Densitometric analyses are presented as the relative ratio of NF-κB p65 levels in OVA+VEH or OVA+Akt inhibitor to those in SAL+VEH. The relative ratio of NF-κB in nuclear protein extracts from the lung tissues of SAL+VEH is arbitrarily presented as 1. Data represent mean ± SEM from 6 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH.
FIGURE 6
FIGURE 7. Effect of IC87114 on nuclear factor-κB (NF-κB) p65 protein levels in lung tissues of ovalbumin (OVA)-sensitized and -challenged mice. Levels of NF-κB p65 were measured at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 mg·kg⁻¹ (OVA+IC87114 0.1 mg·kg⁻¹), and OVA-inhaled mice administered IC87114 1.0 mg·kg⁻¹ (OVA+IC87114 1.0 mg·kg⁻¹). a) Western blot analyses of NF-κB p65 levels 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 OVA+VEH, OVA+IC87114 0.1 mg·kg⁻¹, or OVA+IC87114 1.0 mg·kg⁻¹ to those in SAL+VEH. The relative ratio of NF-κB in nuclear protein extracts from the lung tissues of SAL+VEH is arbitrarily presented as 1. Data represent mean ± SEM from 7 mice per group.

#, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH.
FIGURE 8. Effect of BAY 11-7085 on interleukin (IL)-17 protein levels in lung tissues of ovalbumin (OVA)-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), and OVA-inhaled mice administered BAY 11-7085 (OVA+BAY 11). a) Western blotting of IL-17. 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 SAL+VEH is arbitrarily presented as 1. Data represent mean ± SEM from 7 mice per group. #, p<0.05 versus SAL+VEH; *, p<0.05 versus OVA+VEH.

FIGURE 8