**Full title: Distinct roles of PI3Kδ and PI3Kγ in a toluene diisocyanate-induced steroid insensitive murine asthma model**

**Running title: PI3Kδ and PI3Kγ function differently in TDI asthma**

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## Key message

PI3Kδ contributes to TDI-induced airway neutrophilic and eosinophilic inflammation, while PI3Kγ offers protection against TDI-induced asthma.

## Abstract

**Background:** TDI-induced asthma is characterized by neutrophil-dominated airway inflammation and often associated with poor responsiveness to steroid treatment. Both PI3Kδ and PI3Kγ have been demonstrated to play important proinflammatory roles in ovalbumin-induced asthma. We've already

reported that blocking pan PI3K effectively attenuated TDI-induced allergic airway inflammation. Yet the specific functions of PI3Kδ and PI3Kγ in TDI-induced asthma are still unclear.

**Methods:** Male BALB/c mice were first dermally sensitized and then challenged with TDI to generate an asthma model. Sellective inhibitors of PI3Kδ (IC-87114, AMG319) and PI3Kγ (AS252424, AS605240) as well as prednisone were respectively given to the mice after each airway challenge.

**Results:** Treatment with IC-87114 or AMG319 after TDI exposure led to significantly decreased airway hyperresponsiveness (AHR), less neutrophil and eosinophil accumulation, attenuated airway smooth muscle (ASM) thickening, less M1 and M2 macrophages in lung, as well as lower levels of IL-4, IL-5, IL-6 and IL-18 in bronchoalveolar lavage fluid (BALF) and recovered IL-10 production. While mice treated with AS252424 or AS605240 had increased AHR, more severe ASM thickening, larger numbers of neutrophils and eosinophils, more M1 but less M2 macrophages, and higher BALF levels of IL-4, IL-5, IL-6, IL-10, IL-12, IL-18 when compared with those treated with vehicle.

**Conclusion:** These data revealed that pharmacological inhibition of PI3Kδ attenuates TDI-induced steroid insensitive airway inflammation while PI3Kγ inhibition exacerbates TDI-induced asthma, indicating distinct biological functions of PI3Kδ and PI3Kγ in TDI-induced asthma.

**Key words**

asthma; PI3Kδ; PI3Kγ; steroid insensitive; toluene diisocyanate

## Introduction

As one of the most commonly reported causes of asthma, toluene diisocyanate (TDI) induces airway inflammation characterized by a large number of neutrophils and a smaller number of eosinophils [1]. Upon a long-term exposure to TDI, the incidence of asthma increases to nearly 15% and an accelerated decline in lung function can be observed [2]. Evidence from human and animal models revealed that TDI-induced asthma responds poorly to steroid treatment and complete removal from exposure is often the only effective approach to prevent asthma attacks [3-5]. Therefore, elucidating of the pathogenesis of TDI-induced asthma is necessary for prevention and treatment.

The family of lipid kinases termed phosphoinositide-3-kinase (PI3K) is known to contribute at multiple levels to innate and adaptive immune responses, and is hence an attractive target for drug discovery in inflammatory and autoimmune diseases. PI3Ks can be divided into 3 classes according

to their structures and functions: class I, II, and III. Among these, class I is the most widely studied, which is subdivided into class Ia (p110α, p110β and p110δ isoforms) and class Ib (p110γ only) [6, 7]. The ubiquitously expressed p110α and p110β catalytic subunits make it particularly difficult to study them individually, because mice deficient of p110α or p110β have been proved to be lethal at the embryonic stage, suggesting central roles for these isoforms in cell proliferation during development [8, 9], while p110δ and p110γ are expressed predominantly (but not exclusively) in leucocytes, leading to the speculation that they are the dominant isoforms involved in PI3K-mediated innate and adaptive immune responses [10]. Recent studies supported that both p110δ and p110γ are critically involved in asthma pathogenesis. Genetic knockout or inhibition of either p110δ or p110γ dramatically decreased ovalbumin (OVA)-induced airway hyperreactivity and inflammation [11-15]. Moreover, treatment with a dual PI3Kδ/γ inhibitor also led to a significant reduction of allergic symptoms in OVA-exposed rats [16]. We've previously reported that blocking pan PI3K effectively attenuated TDI-induced steroid-insensitive airway inflammation [17, 18]. Yet, the separate functions of different isoforms of PI3K have not been assessed. Therefore in this study we intended to investigate the roles of PI3Kδ and PI3Kγ in TDI-induced asthma model.

## Methods

### Ethics statement

All animal experiments described here complied with the guidelines of the Committee of Guangzhou Medical University on the use and care of animals and were approved by the Animal Subjects Committee of Guangzhou Medical University.

### Mice, allergen and treatments

6~8-week-old male BALB/c mice purchased from Guangdong Medical Laboratory Animal Center were housed under specific pathogen-free conditions and had free access to food and water on a 12-hour light-dark cycle. Mice were randomized to the following groups: (1) vehicle-sensitized, vehicle-challenged, and DMSO-treated (control group); (2) TD-sensitized, TDI-challenged, and DMSO-treated (TDI group); (3) TD-sensitized, TDI-challenged, and prednisone-treated (TDI+Pred group); (4) TD-sensitized, TDI-challenged, and IC87114-treated (TDI+IC87114 group); (5) TD-sensitized, TDI-challenged, and AMG319-treated (TDI+AMG319 group); (6) TD-sensitized, TDI-challenged, and AS252424-treated (TDI+AS252424 group); (7) TD-sensitized, TDI-challenged, and AS605240-treated (TDI+AS605240 group).

A TDI-induced asthma model was prepared based on our previous work [18]. In short, BALB/c mice were dermally sensitized with 0.3% TDI on the dorsum of both ears (20 μL per ear) on days 1 and 8. On days 15, 18 and 21, the mice were placed in a horizontal rectangle chamber and challenged for 3 h each time with 3% TDI through compressed air nebulization (NE-C28, Omron). TDI was dissolved in a mixture of 3 volumes of olive oil and 2 volumes of acetone for sensitization and 4 volumes of olive oil and 1 volume of acetone for challenge. Control mice were sensitized and challenged with the same amount of vehicle. IC-87114 (PI3Kδ inhibitor, Selleck, Shanghai, China), AMG319 (PI3Kδ inhibitor, Selleck), AS252424 (PI3Kγ inhibitor, Selleck), or AS605240 (PI3Kγ inhibitor, Selleck) was dissolved in sterile 2% DMSO in PBS with 0.05% Tween-80. IC-87114 (1mg/kg) [19, 20] or AMG319 (2mg/kg) [21] was administered intratracheally (i.t.) in mice lightly anesthetized with isofluorane. Prednisone (Pred, SigmaAldrich, 5 mg/kg), AS252424 (10mg/kg) [22], or AS605240 (10mg/kg) [22] was administered separately via the intraperitoneal (i.p.) route. All inhibitors were given once immediately after each inhalation, while Pred were administrated once daily beginning after the first challenge to the last day of challenge for a consecutive of one week.

**Airway responsiveness measurements**

As previously described [18], one day after the last inhalation, airway responsiveness was assessed by lung resistance ($R_L$) measurement (Buxco Electronics, Troy, NY, USA) in mice receiving increasing doses of aerosolized methacholine (6.25, 12.5, 25 and 50 mg/mL). $R_L$ was recorded every five minutes following each nebulisation step until a plateau phase was reached. Results were expressed as percentage of baseline value (value at 0 mg/mL methacholine) for each concentration of methacholine.

**Analysis of bronchoalveolar lavage fluid (BALF)**

After measuring airway parameters, the lungs were lavaged in situ, twice with 0.8 mL prewarmed normal saline, and the recovered fluid was pooled. BALF total cells were counted. As soon as the fluids were centrifuged (1000× g, 5 min), the cell pellets were used for cytospin preparation, which was then stained with haematoxylin and eosin (H&E) for blinded assessment of differential cell percentages, while the supernatants were stored for further detection of IL-4, IL-5, IL-6, IL-18, IL-10 and IL-12 using multiplex immunoassay or ELISA kits (eBioscience) according to the manufacturer's instructions.

**Histopathological analysis**

The left lung lobes were harvested, inflated/fixed overnight in 4% neutral formalin, embedded in paraffin, and then sectioned. Prepared lung slides (4 μm) were subjected to H&E staining to show morphological changes and inflammation. Airway inflammation and cellular infiltrates were scored by a blinded observer, and were semi-quantified as previously described [23]. Epithelial denudation was measured by assessing the percentage of the denuded area in the entire circumference of the bronchus [23]. Thickness of airway smooth muscle and epithelia was measured as previously reported [18]. 20~24 image fields of eight sections from 8~10 mice per group were analyzed.

**Immunohistochemistry**

For immunohistochemistry of p110δ and p110γ, deparaffinized lung sections (4 μm) were submerged in citrate buffer (pH 6.0) for antigen retrieval. Samples were treated with H2O2 for 15 min to block endogenous peroxidase, and then incubated overnight at 4°C in recommended dilutions of anti-p110δ and anti-p110γ antibodies. After washing with PBS, slices were incubated with a secondary antibody for 30 min at room temperature. Signals were visualized with a diaminobenzidine (DAB) peroxidase substrate kit (Zhongshan Jinqiao, Beijing, China).

For staining of different macrophage subsets (M1/M2), a general macrophage marker Mac3 (rat anti-Mac3, BD Biosciences) was used, in combination with phenotype-specifific markers by standard immunohistochemical procedures. To visualize Mac3, an immunoalkaline phosphatase procedure was used with Fast red (Zhongshanjinqiao, Beijing, China) as chromogen. M1-dominant macrophages were determined by double staining of Mac3 and IRF5 (rabbit anti-IRF5; Proteintech, Manchester, UK); M2-dominant macrophages were determined by double staining of Mac3 and ECFL [goat anti mouse eosinophil chemotactic factor (ECFL), R&D Systems]; IRF5 and ECFL were visualized with DAB as chromogen.

**Statistics**

Data are expressed as mean ± SD. Results were interpreted using one-way ANOVA and Bonferroni's difference post hoc test with SPSS 22.0. Differences were considered statistically significant when $p < 0.05$.

**Results**

**Pulmonary expression of p110δ and p110γ in TDI-indued asthma**

Pulmonary expression of p110δ and p110γ was analysed in TDI asthmatic mice. Immunohistochemistry revealed that expression of p110δ and p110γ in the airway epithelia varies

among different treatment groups (Fig1). TDI exposure dramatically increased airway epithelial expression of both p110δ and p110γ, paralleled by recruited inflammatory cells with rich staining of p110δ and p110γ, together contributing to their increased total levels in lung. However, there were no significant changes in p110δ and p110γ expression in the alveolar region, for all groups.

**PI3Kδ inhibitors prevented TDI-induced AHR and pathological changes**

As previously reported [4], TDI-induced airway responses was insensitive to steroid treatment (supplementary Fig2 and 3). Fortunately, we found that these can be suppressed by PI3Kδ inhibition. As shown in Fig2, treatment with IC-87114 (1mg/kg) or AMG319 (2mg/kg) per time after each TDI challenge for a total of 3 times resulted in dramatically decreased airway inflammation and AHR, extensively compromised epithelial injury, ASM and epithelial thickening, as well as significantly smaller numbers of neutrophils and eosinophils in BALF (Fig2). At the same time, treatment with either IC-87114 or AMG319 also inhibited the release of IL-4, IL-5, IL-6, IL-10 and IL-18 in BALF (Fig3). The effects of PI3Kδ inhibitors on naive mice were shown in supplementary Fig1.

**PI3Kγ inhibitors excerbated TDI-induced allergic airway inflammation**

Compared with PI3Kδ inhibition, blocking PI3Kγ is proved to be detrimental in TDI-induced asthma. Both antagonists used (AS252424 and AS605240) exacerbated TDI-induced airway hyperresponsiveness and inflammation, led to more severe epithelial injury and remodeling, and drove greater numbers of neutrophils and eosinophils into the airway lumen (Fig4), coupled with markedly enhanced secretion of IL-4, IL-6, IL-10, IL-12 and IL-18 in BALF (Fig5). These results suggest that PI3Kγ is restraining TDI-induced airway hyperreactivity and allergic responses. The effects of PI3Kγ inhibitors on naive mice were shown in supplementary Fig1.

**Blocking PI3Kδ and PI3Kγ showed different effects on M1 and M2 macrophages in TDI-exposed mice**

M1 is believed to be the major effector macrophages in non-allergic asthma and linked with the pathophysiology of severe steroid-insensitive asthma, whereas M2 predominates in allergic asthma [24, 25]. To assess the presence of M1 and M2 macrophages in lung tissue after TDI exposure, lung sections were stained with a general macrophage marker (MAC3) in combination with more specific markers for macrophage subsets. M1 was identifified as IRF5 MAC3+; M2 was identifified as ECFL MAC3+. As can be seen in Fig6, compared with control, mice sensitized and challenged with TDI had greater numbers of M1 and M2 cells in both the airway and alveolar regions, which were

significantly suppressed by treatment with IC87114 or AMG319. Interestingly, selective blockade of PI3Kγ with AS252424 or AS605240 in TDI-exposed mice led to an increased number of M1 macrophages but less M2 macrophages in the lung.

**Discussion**

Although eosinophilic airway inflammation is recognized as a hallmark feature of most patients with chronic, stable asthma, evidence indicates that neutrophils also play an important role, especially in steroid resistant asthma [26]. As one of the most commonly reported causes of occupational asthma [27], TDI drives neutrophil dominated airway inflammation that often responds poorly to steroid treatment, and is associated with poor prognosis even after cessation of the exposure [28]. As previously reported [4], we prepared a TDI-induced asthma model that is neutrophil predominant and steroid insensitive. Blocking neutrophils by antibodies can prevent TDI-induced airway hyperresponsiveness (AHR) and lung epithelial injury, and dramatically reduce airway inflammation [29], supporting that neutrophils may be a therapeutic target in TDI-induced asthma.

Members of the PI3K family, including PI3Kδ and PI3Kγ, are known to have a preeminent role in neutrophil migration and activation [30]. They facilitate neutrophil chemotaxis by catalyzing the synthesis of phosphatidylinositol (3,4,5) trisphosphate (PIP3), which is required for asymmetric F-actin synthesis and cell polarization [30]. Blocking PI3Kδ significantly inhibited polarized morphology of neutrophils, fMLP-stimulated PIP3 production and chemotaxis [31]. We've already demonstrated an important role of PI3K in TDI-induced asthma [17]. In the present study, we used selective pharmacological inhibitors to study the independent functions of PI3Kδ and PI3Kγ. As expected, treatment with PI3Kδ inhibitors markedly decreased TDI-induced neutrophil and eosinophil aggregation in the airway, AHR and Th2/Th17 responses, as well as epithelial injury and thickening. Intriguingly, we detected a higher level of IL-12 in TDI sensitized and challenged mice that can not be suppressed by PI3Kδ inhibition, which is in disagreement with the finding that atopic asthmatic patients usually have lower IL-12 compared with healthy volunteers [32]. It is generally thought that TDI-induced asthma is mediated by a mixed Th1, Th2 and Th17 response [4]. Enhanced cytokines specifically responsible for Th1 differentiation and expression (including IL-12, TNF-α and IFN-γ) were also found to be enhanced in neutrophilic asthma [33]. Likewise, an increased number of CD4 IFNγ+ cell in TDI-induced asthma was reported previously [4]. Therefore it's reasonable that the TDI-exposed mice had higher IL-12. Yet IC87114 or AMG319 had no effects on

BALF IL-12 production. These data suggest that PI3Kδ contributes to TDI-induced neutrophilc and eosinophilic airway inflammation as well as other pathological changes.

In contrast to the effects of PI3Kδ inhibition, blocking PI3Kγ with AS252424 or AS605240 revealed completely different outcomes. As mentioned above, PI3Kγ is also capable of driving neutrophilc inflammation [30]. Loss, inhibition or mutation of p110γ would impair PIP3 production, Akt phosphorylation, and therefore hamper neutrophil migration in response to GPCR activation [34]. Giovanna B et al. reported that PI3Kγ is engaged in Th17 cell differentiation [35], a critical process involved in neutrophilic asthma [4]. In the airway, genetic and pharmaceutical PI3Kγ inhibition significantly limited neutrophil recruitment and lung injury induced by different insults [36-38]. Here, we detected an increased expression of p110γ in the airway after mice were sensitized and challenged with TDI. Yet, to our surprise, treatment with PI3Kγ antagonists AS252424 or AS605240 not only failed to confer protection for TDI-exposed mice, but also augmented airway neutrophil and eosinophil infiltration, exacerbated epithelial injury and airway remodeling, and enhanced the production of IL-4 and IL-6 despite the recovered IL-10 in BALF. These were opposite to the findings in OVA-induced asthma [15], suggesting that PI3Kγ functions to counteract TDI-induced allergic airway inflammation.

Though a large list of studies support that PI3Kγ is responsible for neutrophil migration, there's also increasing evidence demonstrating its antagonistic role in inflammation. Early investigations revealed that mice lack of p110γ exhibit greater numbers of neutrophil in response to *S. pneumoniae* or *E. coli* infection and more severe lung injury compared with wild-type [39, 40]. Additionally, in 2017 Bucher K and colleagues discovered that double deficiency of p110δ/γ in mice would result in significantly spontaneous neutrophilia in blood, spleen and lung [41]. These findings indicate that PI3Kγ functions to limit TDI-induced airway inflammation partly through interrupting neutrophil invasion.

Besides modulating neutrophils, PI3Kγ also controls macrophage switch between immune stimulation and suppression. Researchers observed that mice lacking p110γ mounted exaggerated, macrophage-mediated pro-inflammatory responses upon exposure to pathogenic stimuli [42]; in vitro, p110γ deficient macrophages produced elevated IL-12 and IL-23 upon TLR stimulation [43]. Indeed, macrophages are an extremely heterogeneous population, displaying a combination of inflammatory and anti-inflammatory functions that are represented by the classically activated (M1) and

alternatively activated (M2) phenotypes [24]. In asthma, macrophage polarization is thought to have a profound impact [44]. M1 acts as the major effector macrophages in non-allergic asthma and participates in the pathophysiology of severe steroid-insensitive asthma, whereas M2 predominates in allergic asthma [24, 25]. In our study, both M1 and M2 macrophages were increased in TDI-exposed mice, coupled with pronounced Th1 (IL-12) and Th2 responses. Sellective PI3Kγ inhibition increased the number of M1 macrophage and IL-12 production in TDI-exposed mice but decreased M2 macrophage count, implying that PI3Kγ restrains TDI-induced airway inflammation by orchestrating macrophage polarization.

In conclusion, we found that PI3Kγ restricts TDI-induced steroid-insensitive airway inflammation through regulating neutrophil migration macrophage polarization, while PI3Kδ contributes to TDI-induced airway neutrophilic inflammation and bronchial hyperresponsiveness, which might be effective therapeutic targets for severe asthma in the future.

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**Author contributions**

Y.L. and T.H. conceived the study and designed the experiments. X.C., C.S., D.Y. and S.J. performed the experiments. Y.L., T.H., X.C. and S.J. analyzed the results and wrote the manuscript. L.J, C.X. and C.P. helped with the experiment and data analysis. X.C., C.S. and S.J. helped to revise the manuscript. All the authors agreed that the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

**References**

1. Pollaris L, Devos F, De Vooght V, Seys S, Nemery B, Hoet PH. Toluene diisocyanate and methylene diphenyl diisocyanate: asthmatic response and cross-reactivity in a mouse model. Arch Toxicol 2016;90(7):1709-17.

2. Ott MG, Diller WF, Jolly AT. Respiratory effects of toluene diisocyanate in the workplace: a discussion of exposure-response relationships. Crit Rev Toxicol 2003;33(1):1-59.

3. Paggiaro PL, Vagaggini B, Bacci E, Bancalari L, Carrara M, Di Franco A, Giannini D, Dente FL, Giuntini C. Prognosis of occupational asthma. Eur Respir J 1994 Apr;7(4):761-7.

4. Chen R, Zhang Q, Chen S, Tang H, Huang P, Wei S, Liang Z, Chen X, Tao A, Yao L. IL-17F, rather than IL-17A, underlies airway inflammation in a steroid-insensitive toluene diisocyanate-induced asthma model. Eur Respir J 2019;53(4):1801510.

5. Lau A, Tarlo SM. Update on the management of occupational asthma and work exacerbated asthma. Allergy Asthma Immunol Res 2019;11(2):188-200.

6. Fruman DA, Chiu H, Hopkins B.D, Bagrodia S, Cantley LC, Abraham RT. The PI3K pathway in human disease. Cell 2017; 170(4): 605-635.

7. Bilanges B, Posor Y, Vanhaesebroeck B. PI3K isoforms in cell signalling and vesicle trafficking. Nat Rev Mol Cell Biol 2019;20(9):515-534.

8. Bi L, Okabe I, Bernard DJ, Wynshaw-Boris A, Nussbaum RL. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. J Biol Chem. 1999;274(16):10963-8.

9. Bi L, Okabe I, Bernard DJ, Nussbaum RL. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. Mamm Genome 2002;13(3):169-72.

10. Chantry D, Vojtek A, Kashishian A, Holtzman DA, Wood C, Gray PW, Cooper JA, Hoekstra MF. p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. J Biol Chem 1997;272(31):19236-41.

11. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. FASEB J 2006;20(3):455-65.

12. Kim JS, Jeong JS, Kwon SH, Kim SR, Lee YC. Roles of PI3K pan-inhibitors and PI3K-δ inhibitors in allergic lung inflammation: a systematic review and meta-analysis. Sci Rep 2020;10(1):7608.

13. Takeda M, Ito W, Tanabe M, Ueki S, Kato H, Kihara J, et al. Allergic airway hyperresponsiveness, inflammation, and remodeling do not develop in phosphoinositide 3-kinase gamma–defificient mice. J Allergy Clin Immunol 2009;123(4):805-12.

14. Thomas M, Edwards MJ, Sawicka E, Duggan N, Hirsch E, Wymann MP, et al. Essential role of phosphoinositide 3-kinase gamma in eosinophil chemotaxis within acute pulmonary inflammation. Immunology 2009;126(3):413-22.

15. Lim DH, Cho JY, Song DJ, Lee SY, Miller M, Broide DH. PI3Kgamma-defificient mice have reduced levels of

allergen-induced eosinophilic inflammation and airway remodeling. Am J Physiol Lung Cell Mol Physiol 2009;296(2):L210-9.

16. Doukas J, Eide L, Stebbins K, Racanelli-Layton A, Dellamary L, Martin M, et al. Aerosolized phosphoinositide 3-kinase gamma/delta inhibitor TG100-115 [3-[2,4-diamino-6-(3-hydroxyphenyl)pteridin-7-yl]phenol] as a therapeutic candidate for asthma and chronic obstructive pulmonary disease. J Pharmacol Exp Ther 2009;328(3):758-65.

17. Yao L, Zhao H, Tang H, Song J, Dong H, Zou F, et al. Phosphatidylinositol 3-kinase mediates β-catenin dysfunction of airway epithelium in a toluene diisocyanate-induced murine asthma model. Toxicol Sci 2015;147(1):168-77.

18. Yao L, Zhao H, Tang H, Liang J, Liu L, Dong H, Zou F, et al. The receptor for advanced glycation end products is required for β-catenin stabilization in a chemical-induced asthma model. Br J Pharmacol 2016;173(17):2600-13.

19. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. FASEB J 2006;20(3):455-65.

20. Lee KS, Jeong JS, Kim SR, Cho SH, Kolliputi N, Ko YH, et al. Phosphoinositide 3-kinase-δ regulates fungus-induced allergic lung inflammation through endoplasmic reticulum stress. Thorax 2016;71(1):52-63.

21. Cushing TD, Hao X, Shin Y, Andrews K, Brown M, Cardozo M, et al. Discovery and in vivo evaluation of (S)-N-(1-(7-fluoro-2-(pyridin-2-yl)quinolin-3-yl)ethyl)-9H-purin-6-amine (AMG319) and related PI3Kδ inhibitors for inflammation and autoimmune disease. J Med Chem 2015;58(1):480-511.

22. Huang L, Sherchan P, Wang Y, Reis C, Applegate RL 2nd, Tang J, et al. Phosphoinositide 3-kinase gamma contributes to neuroinflammation in a rat model of surgical brain injury. J Neurosci 2015;35(29):10390-401.

23. Yao L, Chen S, Tang H, Huang P, Wei S, Liang Z, et al. Transient receptor potential ion channels mediate adherens junctions dysfunction in a toluene diisocyanate-induced murine asthma model. Toxicol Sci 2019;168(1):160-170.

24. Robbe P, Draijer C, Borg TR, Luinge M, Timens W, Wouters IM, et al. Distinct macrophage phenotypes in allergic and nonallergic lung inflammation. Am J Physiol Lung Cell Mol Physiol 2015;308(4):L358-67.

25. Oriss TB, Raundhal M, Morse C, Huff RE, Das S, Hannum R, et al. IRF5 distinguishes severe asthma in humans and drives Th1 phenotype and airway hyperreactivity in mice. JCI Insight 2017;2(10):e91019.

26. Ray A, Kolls JK. Neutrophilic Inflammation in Asthma and Association with Disease Severity. Trends Immunol 2017;38(12):942-954.

27. Gautrin D, Newman-Taylor AJ, Nordman H, Malo JL. Controversies in epidemiology of occupational asthma.

Eur Respir J 2003;22(3):551-9.

28. Paggiaro PL, Vagaggini B, Bacci E, Bancalari L, Carrara M, Di Franco A, et al. Prognosis of occupational asthma. Eur Respir J 1994;7(4):761-7.

29. Vooght VD, Smulders S, Haenen S, Belmans J, Opdenakker G, Verbeken E, et al. Neutrophil and eosinophil granulocytes as key players in a mouse model of chemical-induced asthma. Toxicol Sci 2013;131(2):406-18.

30. Gambardella L, Vermeren S. Molecular players in neutrophil chemotaxis--focus on PI3K and small GTPases. J Leukoc Biol 2013 Oct;94(4):603-12.

31. Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. J Immunol 2003;170(5):2647-54.

32. Plummeridge MJ, Armstrong L, Birchall MA, Millar AB. Reduced production of interleukin 12 by interferon gamma primed alveolar macrophages from atopic asthmatic subjects. Thorax 2000;55(10):842-7.

33. Steinke JW, Lawrence MG, Teague WG, Braciale TJ, Patrie JT, Borish L. Bronchoalveolar lavage cytokine patterns in children with severe neutrophilic and paucigranulocytic asthma. J Allergy Clin Immunol 2020;S0091-6749(20)30809-5.

34. Deladeriere A, Gambardella L, Pan D, Anderson KE, Hawkins PT, Stephens LR. The regulatory subunits of PI3Kγ control distinct neutrophil responses. Sci Signal 2015;8(360):ra8.

35. Bergamini G, Bell K, Shimamura S, Werner T, Cansfield A, Müller K, et al. A selective inhibitor reveals PI3Kγ dependence of T(H)17 cell differentiation. Nat Chem Biol 2012;8(6):576-82.

36. Martin EL, Souza DG, Fagundes CT, Amaral FA, Assenzio B, Puntorieri V, et al. Phosphoinositide-3 kinase gamma activity contributes to sepsis and organ damage by altering neutrophil recruitment. Am J Respir Crit Care Med 2010;182(6):762-73.

37. Galluzzo M, Ciraolo E, Lucattelli M, Hoxha E, Ulrich M, Campa CC, et al. Genetic Deletion and Pharmacological Inhibition of PI3Kγ Reduces Neutrophilic Airway Inflammation and Lung Damage in Mice with Cystic Fibrosis-Like Lung Disease. Mediators Inflamm 2015;2015:545417.

38. Mothes B, Bucher K, Ammon-Treiber S, Schwab M, Piekorz RP, Hirsch E, et al. p110γ/δ Double-Deficiency Induces Eosinophilia and IgE Production but Protects from OVA-Induced Airway Inflammation. PLoS One 2016;11(7):e0159310.

39. Ong E, Gao XP, Predescu D, Broman M, Malik AB. Role of phosphatidylinositol 3-kinase-gamma in mediating lung neutrophil sequestration and vascular injury induced by E. coli sepsis. Am J Physiol Lung Cell Mol Physiol 2005;289(6):L1094-103.

40. Maus UA, Backi M, Winter C, Srivastava M, Schwarz MK, Rückle T, et al. Importance of phosphoinositide 3-kinase gamma in the host defense against pneumococcal infection. Am J Respir Crit Care Med 2007;175(9):958-66.

41. Bucher K, Schmitt F, Mothes B, Blumendeller C, Schäll D, Piekorz R, et al. Deficiency of PI3-Kinase catalytic isoforms p110γ and p110δ in mice enhances the IL-17/G-CSF axis and induces neutrophilia. Cell Commun Signal 2017;15(1):28.

42. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kγ is a molecular switch that controls immune suppression. Nature 2016;539(7629):437-442.

43. Takeda AJ, Maher TJ, Zhang Y, Lanahan SM, Bucklin ML, Compton SR, et al. Human PI3Kγ deficiency and its microbiota-dependent mouse model reveal immunodeficiency and tissue immunopathology. Nat Commun 2019;10(1):4364.

44. Saradna A, Do DC, Kumar S, Fu QL, Peisong Gao P. Macrophage polarization and allergic asthma. Transl Res 2018;191:1-14.

## FIGURES

**Fig1.** Pulmonary expression of p110δ, p110γ. Representative immunohistochemical staining of p110δ (**A**) and p110γ (**B**) in the bronchial and alveolar region of mice from different treatment groups. Scale bar = 50 μm.

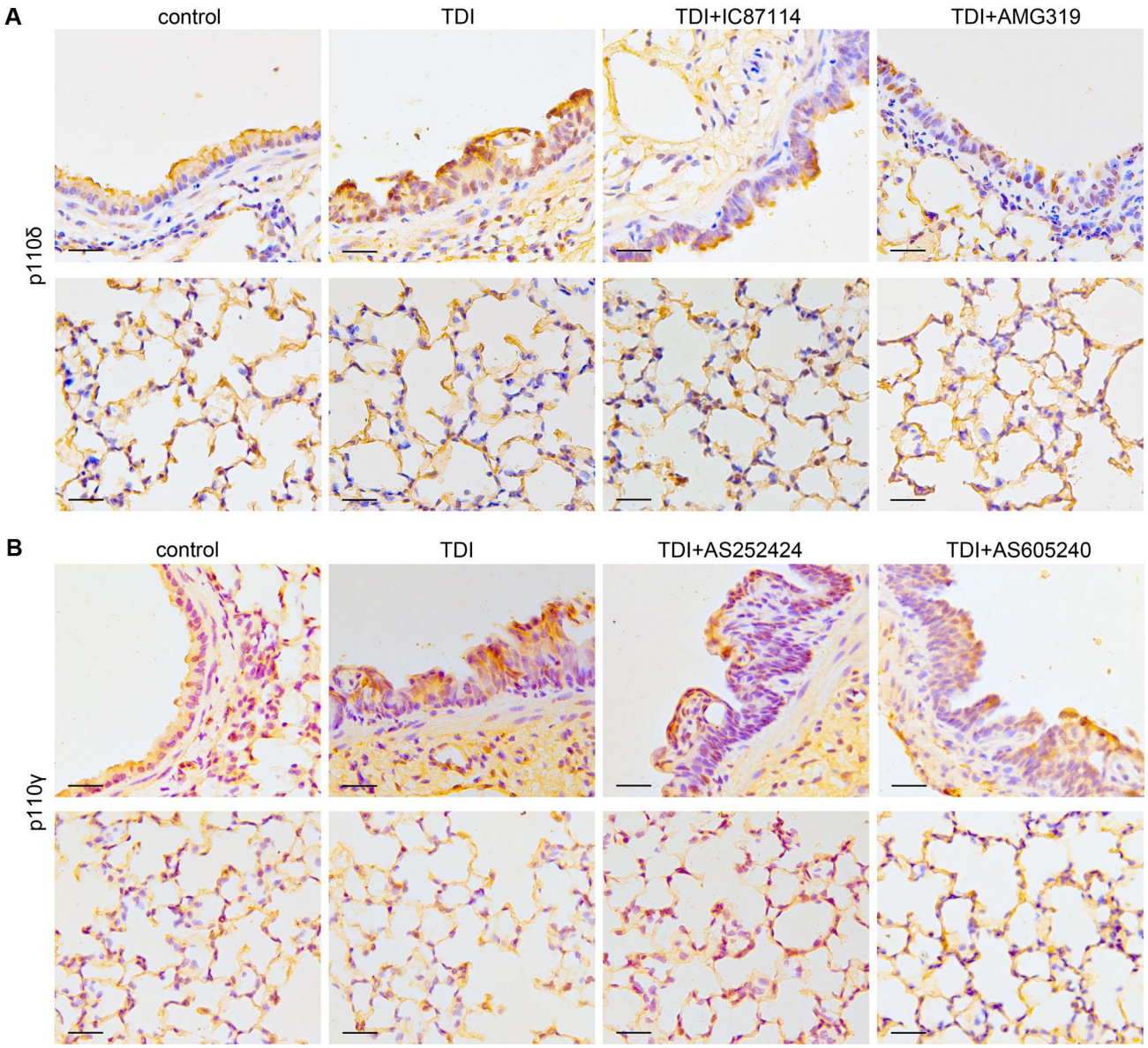

**Fig2.** PI3Kδ inhibition attenuated TDI-induced airway hyperresponsiveness (AHR) and airway inflammation. **A,** AHR was measured by lung resistance ($R_L$). Results were shown as percentage of baseline value. n=4~5. **B-F,** Representative H&E-stained lung sections, and semiquantification of peribronchial inflammation and epithelial denudation, as well as analysis of airway smooth muscle and epithelial thickness. Scale bar = 100 μm. n=8~10. **G-I,** Total cell counts, as well as the numbers of neutrophils and eosinophils in BALF. n=8~10. *p<0.05; **p<0.01; ***p<0.001. *NS* represents none significant differences.

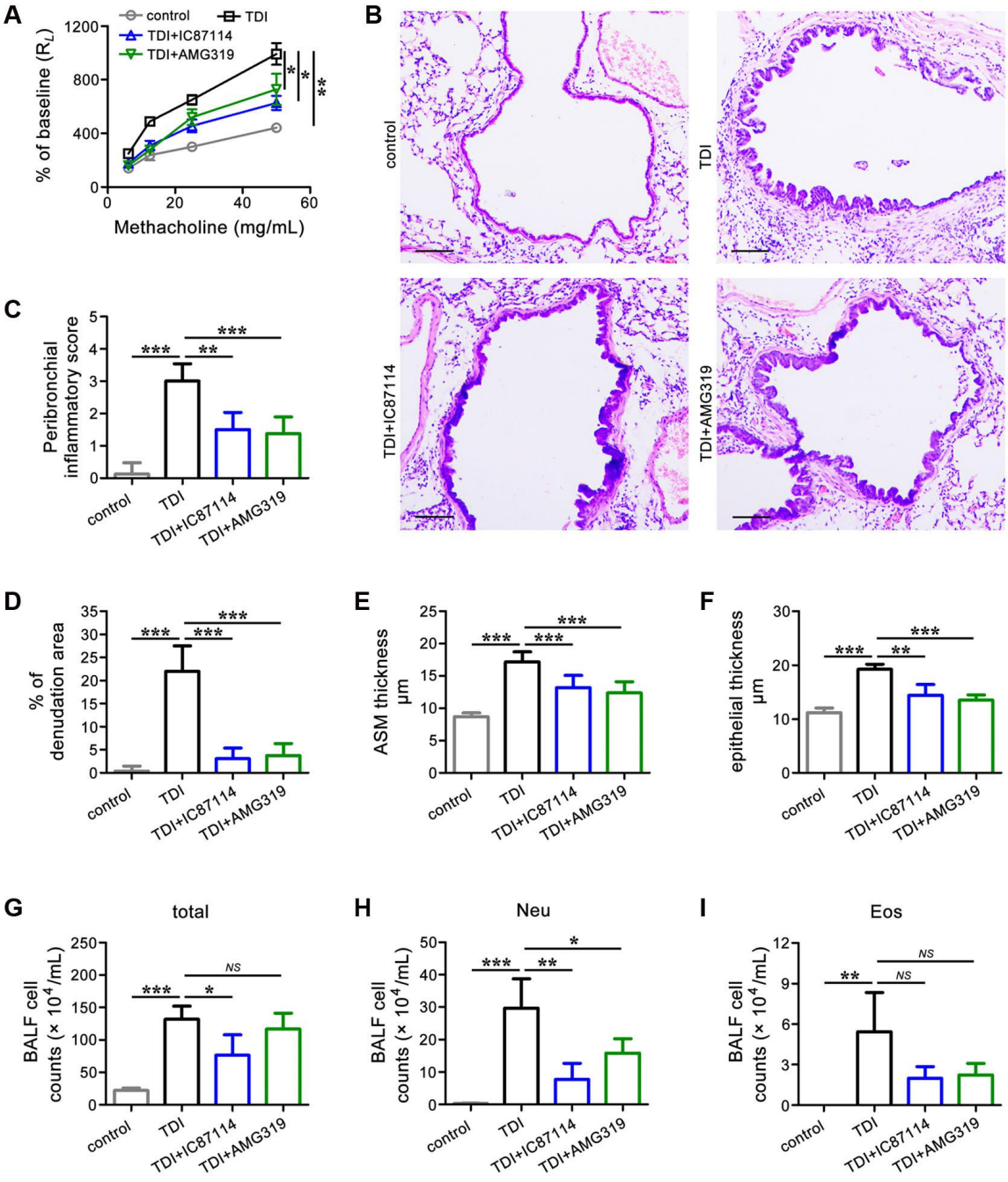

**Fig3.** The effects of PI3Kδ inhibition on TDI-induced cytokine secretion in BALF. Levels of IL-4, IL-5, IL-6, IL-10, IL-12 and IL-18 in BALF. n=8~10. *p<0.05; **p<0.01; ***p<0.001. *NS* represents none significant differences.

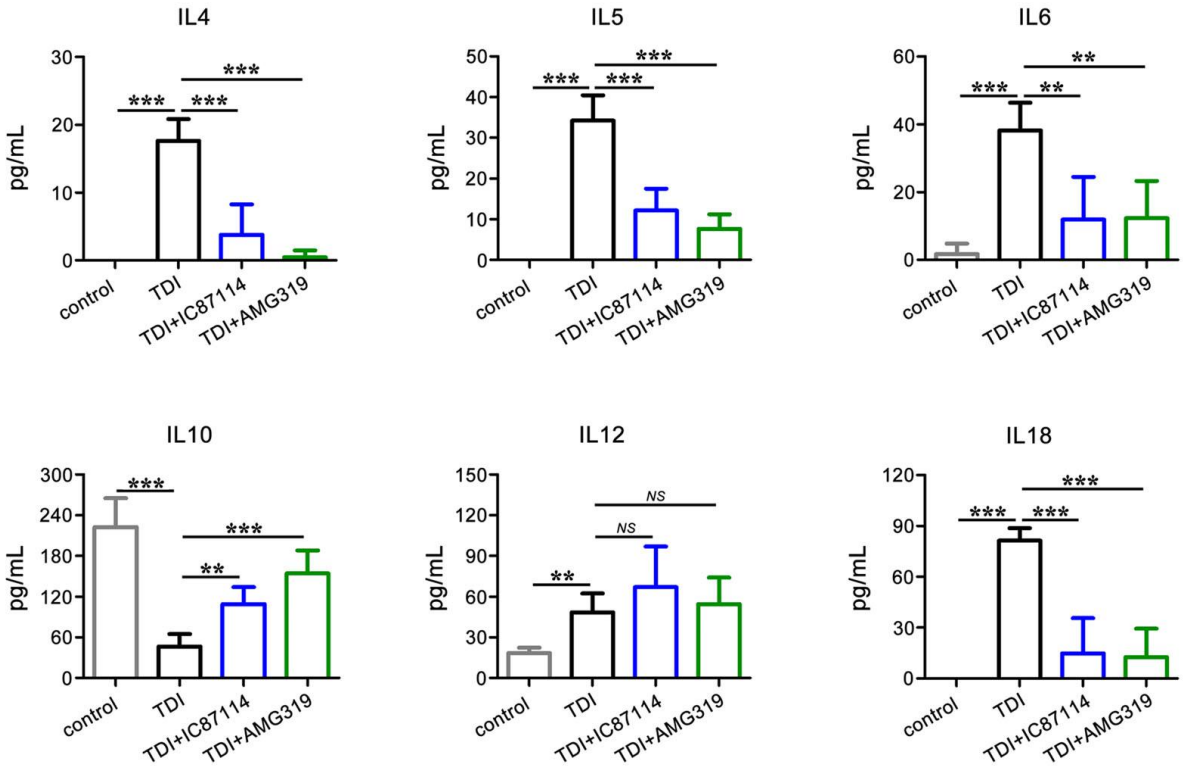

**Fig4.** PI3Kγ inhibition exacerbated TDI-induced AHR and airway inflammation. **A,** AHR was measured by lung resistance ($R_L$). Results were shown as percentage of baseline value. n=4~5. **B-F,** Representative H&E-stained lung sections, and semiquantification of peribronchial inflammation and epithelial denudation, as well as analysis of airway smooth muscle and epithelial thickness. Scale bar = 100 μm. n=8~10. **G-I,** Total cell counts, as well as the numbers of neutrophils and eosinophils in BALF. n=8~10. *p<0.05; **p<0.01; ***p<0.001. *NS* represents none significant differences.

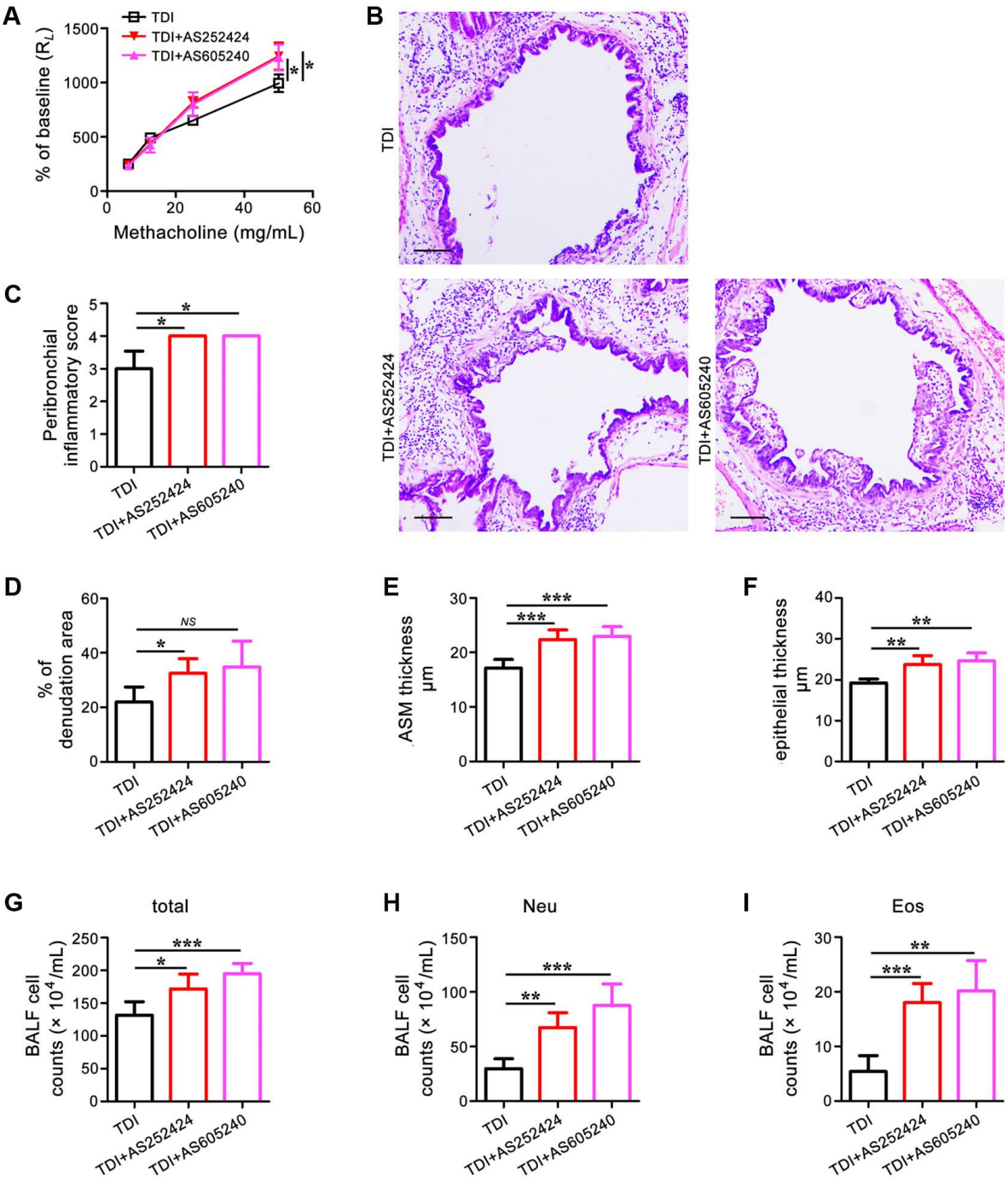

**Fig5.** The effects of PI3Kγ inhibition on TDI-induced cytokine secretion in BALF. Levels of IL-4, IL-5, IL-6, IL-10, IL-12 and IL-18 in BALF. n=8~10. *p<0.05; **p<0.01; ***p<0.001. *NS* represents none significant differences.

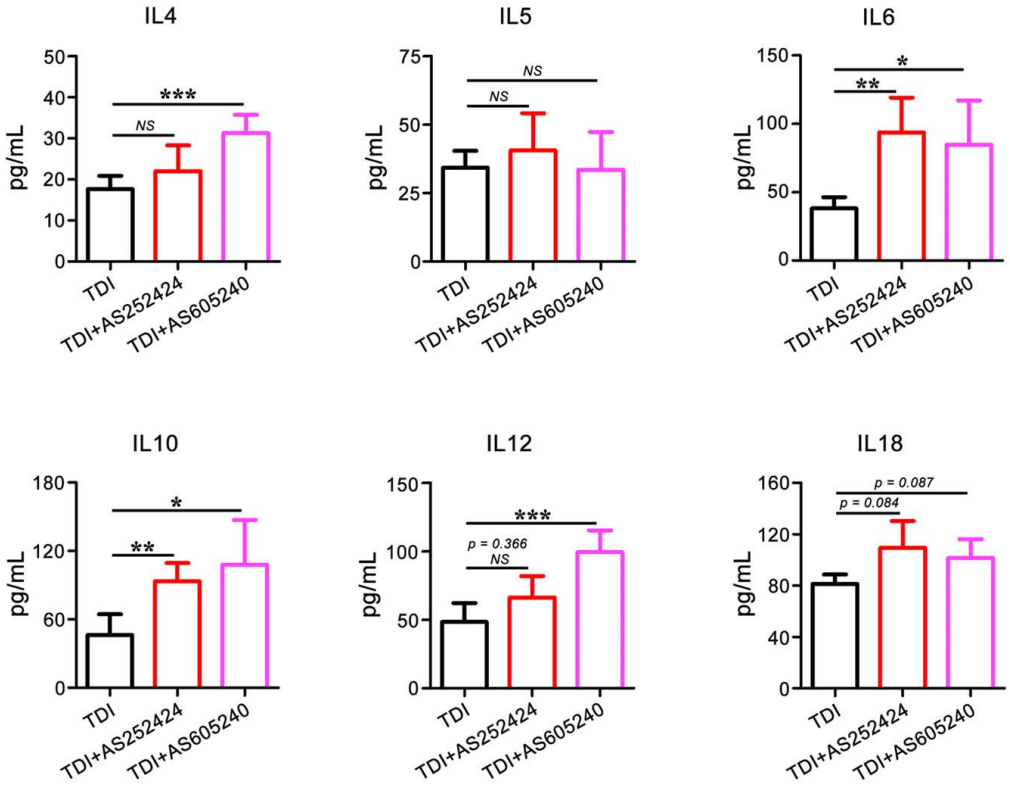

**Fig6.** Representative immunohistochemical staining of M1 [IRF5 (brown) + MAC3 (red)] (**A**) and M2 [ECFL (brown) + MAC3 (red)] (**B**) in the bronchial and alveolar region of mice from different treatment groups. Scale bar = 50 μm.