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Blockade of β -catenin signaling attenuates toluene diisocyanate-induced experimental asthma

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Abstract

Aberrant activation of β -catenin signaling by both WNT-dependent and -independent pathways has been demonstrated in asthmatic airways, which is thought to contribute critically in remodeling of the airways. Yet the exact role of β -catenin in asthma is very poorly defined. As we've previously reported abnormal expression of β -catenin in a toluene diisocyanate (TDI)-induced asthma model, in this study we evaluated the therapeutic efficacy of two small molecules XAV-939 and ICG-001 in TDI-asthmatic male BALB/c mice, which selectively block β -catenin mediated transcription. TDI exposure led to a significantly increased activity of β -catenin, which was then confirmed by a luciferase assay in 16HBE transfected with the TOPFlash reporter plasmid. Treatment with either XAV-939 or ICG-001 effectively inhibited activation of β -catenin and downregulated mRNA expression of β -catenin targeted genes in TDI-asthmatic mice, paralleled by dramatically attenuated TDI-induced hyperresponsiveness and inflammation of the airway, alleviated airway goblet cell metaplasia and collagen deposition, decreased Th2 inflammation, as well as lower levels

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of TGF β 1, VEGF, HMGB1 and IL1 β . The results showed that β -catenin is a principal mediator of TDI-induced asthma, proposing β -catenin as a promising therapeutic target in asthma.

Key words

asthma; airway inflammation; airway remodeling; β -catenin; toluene diisocyanate

Introduction

β -catenin is an evolutionary conserved molecule that exerts dual roles in cellular signaling. As a membrane-bound protein, β -catenin constitutes a key component of adherens junctions where it interacts with the cadherins to connect them to the cytoskeleton (1). Besides, cytosolic and nuclear β -catenin is the pivotal effector of the WNT signaling. The canonical WNT/ β -catenin pathway initiates a signaling cascade fundamental in regulating various biologic processes such as organ development, tissue homeostasis and pathogenesis of human diseases (2). The hallmark of this pathway is that it activates the transcriptional role of β -catenin. In the absence of Wnt signals, a multiprotein destruction complex including casein kinase I (CKI), glycogen synthase kinase 3 β (GSK3 β), axin and the adenomatous polyposis coli (APC) phosphorylates β -catenin at serine residues in the N terminus to promote its ubiquitination and proteosomal destruction. Activation of Wnt signaling inhibits this degradative process by binding to Frizzled receptors and signaling through the associated low-density lipoprotein-related proteins, LRP5/6, thereby allowing β -catenin to accumulate in the cytosol (active) and enter the nucleus, where it binds to members of the T cell factor (TCF)/lymphoid enhancer-binding factor family of transcription factors (3, 4). Nuclear β -catenin/TCF then assembles a transcriptionally active complex by recruiting the transcriptional coactivators cAMP response element-binding protein (CBP) or its closely related protein p300, as well as other components of the basal transcription machinery, to stimulate transcription of various target genes (5).

Activation of β -catenin signaling has been demonstrated in airway smooth muscle cells and airway epithelium in both in vivo and in vitro models of asthma (6–8). Recently, β -catenin has been proposed as a regulator and therapeutic target for asthmatic airway remodeling (9).

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Using small interfering RNA targeting β -catenin not only decreased smooth muscle hyperplasia, subepithelial fibrosis and eosinophil numbers in bronchoalveolar lavage fluid (BALF) in an ovalbumin (OVA)-induced chronic asthma model (10), but also inhibited LPS/lipoteichoic acid-induced upregulation of IL-6, IL-8 and IL-1 β in airway epithelial cell lines (11, 12), suggesting critical roles for β -catenin in asthma pathogenesis. In a previous study, we've demonstrated aberrant activation of β -catenin signaling in a toluene diisocyanate (TDI)-induced asthma model (13). Yet how β -catenin signaling contributes to the pathology of TDI asthma still remains unclear.

Significant advances have been made in the generation of small-molecule inhibitors that target β -catenin directly or indirectly. Recent studies have identified ICG-001, a peptidomimetic small molecule that selectively inhibits β -catenin signaling in a CBP-dependent fashion. Mechanistically, ICG-001 competes with β -catenin for binding to CBP to prevent β -catenin/CBP complex formation, thereby inhibiting β -catenin-mediated gene expression (14–17). Another example is XAV-939. Pharmacologic inhibition of β -catenin signaling by XAV-939 stabilizes the β -catenin destruction complex, decreased cytoplasmic retention and nuclear import of β -catenin, and efficiently inhibited β -catenin targeted gene transcription (18). These two offer almost perfect approaches to selectively disrupt the signaling role of β -catenin without affecting its adhesive function. Both have proved great capacity to suppress organ fibrosis in animal models (19–22). Therefore, in this study, we intended to examine the therapeutic effects of ICG-001 and XAV-939 in our asthma model to investigate deeper mechanisms for β -catenin signaling in TDI-induced asthma.

Methods

Reagents

TDI (toluene-2,4-diisocyanate, $\geq 98.0\%$) was obtained from Sigma-Aldrich (Shanghai, China). The vehicle (AOO) used to dissolve TDI consists of a mixture of 2 volumes of acetone and 3 volumes of olive oil for sensitization, and 1 volume of acetone and 4 volumes of olive oil for the challenge. ELISA kit for total IgE, as well as multiplex immunoassay kit for detecting IFN- γ , IL-4, IL-5, IL-13, VEGF and IL-25, was from eBioscience (San Diego, US). Rabbit anti- β -catenin and anti-IL1 β antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-non-phospho- β -catenin (Ser33/37/Thr41) antibody was from Cell

Signaling Technology. Anti-HMGB1, anti-VEGF, and anti-TGF β 1 antibodies were bought from ABclonal Biotech (Beijing, China).

Animals and in vivo treatment with β -catenin signaling inhibitors

Male BALB/c mice (6~8 weeks) were purchased from Southern Medical University. The mice were housed in a SPF facility with 12-h dark/light cycles and fed with sterile water and irradiated food ad libitum. All studies were conducted in accordance with the committee of Southern Medical University on the use and care of animals. The protocols were approved by the Animal Subjects Committee of Nanfang Hospital. In the first protocol, mice were randomized to the following groups: (1) AOO-sensitized, AOO-challenged, and DMSO-treated (AOO/AOO group); (2) TDI-sensitized, TDI-challenged, and DMSO-treated (TDI/TDI group); (3) TDI-sensitized, TDI-challenged, and XAV-939-treated (TDI/TDI+XAV-939 group); (4) TDI-sensitized, TDI-challenged, and ICG-001-treated (TDI/TDI+ICG-001 group). In a second protocol, to explore the role of β -catenin in the toxic response of TDI, mice were allocated to the paralleled four groups: (1) none-sensitized, AOO-challenged, and DMSO-treated (-/AOO group); (2) none-sensitized, TDI-challenged, and DMSO-treated (-/TDI group); (3) none-sensitized, TDI-challenged, and XAV-939-treated (-/TDI+XAV-939 group); (4) none-sensitized, TDI-challenged, and ICG-001-treated (-/TDI+ICG-001 group). Animal models were generated as described elsewhere (13). Briefly, on days 1 and 8, mice were dermally sensitized with 0.3% TDI on the dorsum of both ears (20 μ L per ear). On days 15, 18, and 21 the mice were placed in a horizontal rectangle chamber and challenged for 3h each time with 3% TDI through compressed air nebulization (NE-C28; Omron, Tokyo, Japan). As a control, mice were sensitized and challenged by the same procedures with the same amount of AOO. The none-sensitized mice only underwent airway challenge twice on days 15 and 18. XAV-939 (2.5mg/kg, i.p.; Selleck Chem, Shanghai, China) (22) and ICG-001 (5mg/kg, i.p.; Selleck) (19) dissolved in DMSO and diluted with PBS (pH=7.4) were respectively given to the mice once daily, beginning immediately after the first challenge to the last day of challenge. Sham mice received the same volume of vehicle by comparison.

Assessment of airway hyperresponsiveness (AHR)

Airway reactivity to methacholine was assessed on day 22. Measurements of lung resistance (R_L) were performed on anaesthetized, intubated and mechanically ventilated (Buxco Electronics, Troy, NY) mice in response to increasing doses of aerosolized methacholine (Mch; 3.12, 6.25, 12.5 and 25 mg/mL). Measurements of R_L were performed every 5 minutes following each nebulization step.

Quantification of serum IgE, preparation of lymphocyte supernatants, and analysis of bronchoalveolar lavage fluid

One day after measuring airway parameters, mice were sacrificed with overdose of pentobarbital, then blood samples were immediately taken, rest at room temperature for 2 hours, then centrifuged (3000 $\times$ g, 20min) and supernatants were harvested for detection of total IgE by ELISA according to the manufacturers' instructions.

As soon as blood taken, cervical lymph nodes were dissected and processed and supernatants of cultured lymphocytes were obtained as previously described (23), as well as bronchoalveolar lavage fluid (BALF). Total cells in BALF were counted, and a cytospin sample was prepared and stained with hematoxylin and eosin (H&E) for blinded assessment of differential cell percentages in BALF. Then the remaining fluids were centrifuged (1000 $\times$ g, 10min) and supernatants were stored at -80°C. VEGF, IL-25 in supernatants of BALF and IFN- γ , IL-4, IL-5, IL-13 in supernatants of cultured lymphocytes were detected by a multiplex immunoassay kit according to procedures recommended by the manufacturer.

Histopathological examination of the lung

Left lungs were isolated and fixed with 4% neutral formalin, paraffin-embedded, cut in 4 μ m sections, and stained with H&E, periodic acid-Schiff (PAS), and Masson's trichrome for blinded histopathologic assessment. Lung inflammation and airway goblet cell metaplasia were semiquantified as previously (23). The presence of collagen fibril deposition with Masson's staining was analyzed by Image-Pro Plus software. Scoring was performed at a magnification of 200 $\times$ by examining at least 40 image fields of 20 slices from 8 mice per group. Sections were assigned a random code to blind the examiner to the identity of each specimen.

Western blot and Immunohistochemistry

Pulmonary expression of non-phospho (active) β -catenin (Ser33/37/Thr41), HMGB1, TGF β 1 and IL1 β was detected by western blot. Whole lung extracts mixed with 5 $\times$ SDS loading buffer were separated by 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were then probed with anti- β -catenin, anti-non-phospho- β -catenin (Ser33/37/Thr41), anti-TGF β 1, anti-HMGB1 and anti-IL1 β antibodies with indicated dilutions. After incubation with a IRDye® 680WC-conjugated secondary antibody (LI-COR Biosciences), immunoreactive bands were exposed to Odyssey® CLx Imager for image capture. Data analysis was done with Odyssey Software.

For immunohistochemistry of β -catenin and VEGF, lung sections were deparaffinized, then subject to 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- β -catenin and anti-VEGF antibodies. After washing with PBS, slices were incubated with a secondary antibody for 20 min at room temperature. Signals were visualized with DAB.

Treatment of mouse lung for qPCR

Expression of MMP-2, MMP-9, fibronectin, TGF β 1 and VEGF was detected by the SYBR green-based PCR technique. Total lung RNA was isolated using an RNAiso Plus kit (Takara, Guangzhou, China). RNA samples were then reverse transcribed into first-strand cDNA using the PrimeScript™ RT reagent kit from Takara. A model LightCycler480 Fast Real-Time PCR System was used. Primers for amplifying GAPDH cDNA were 5'-AAGAGGGATGCTGCCCTTAC-3' (forward) and 5'-CCAATACGGCCAAATCCGTTC-3' (reverse); primers for MMP-2 were 5'-TGCAGGAGACAAGTTCTGGAG-3' (forward) and 5'-GTAGCTATGACCACCACCCTG-3' (reverse); primers for MMP-9 were 5'-CGTGTCTGGAGATTTCGACTTGA-3' (forward) and 5'-TGGTTCACCTCATGGTCCAC-3' (reverse); primers for fibronectin were 5'-GAAATCTGCAGCCTGGGTCT-3' (forward) and 5'-ACACCCAGCTTGAAGCCAAT-3' (reverse); primers for TGF β 1 were 5'-ACGTCACTGGAGTTGTACGG-3' (forward) and 5'-GGGGCTGATCCCGTTGATT-3' (reverse); primers for VEGF were 5'-CACTGGACCCTGGCTTTACT-3' (forward) and 5'-ACTTGATCACTTCATGGGACTTCT-3' (reverse). The amplification protocol were set as

follows: 95°C denaturation for 30s followed by 40 cycles of 15s denaturation at 95°C, 1 min of annealing/extension, and data collection at 60°C.

Cell culture and TOPflash Luciferase Assay

This part of methods was shown in the online Supplement Material.

Statistics

Statistical analysis was performed using SPSS version 20.0. Data were expressed as mean $\pm$ Standard Error (SE) and comparisons among groups were analyzed by one-way analysis of variance (ANOVA) accompanied by Bonferonni post hoc test (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc tests for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

XAV-939 and ICG-001 inhibited activation of β -catenin

Consistent with our previous study (13), TDI exposure leads to aberrant distribution of β -catenin in the cytoplasm and nucleus, which is most prominent in bronchial epithelial cells (Fig. 1A), while β -catenin in the alveolar region showed no significant alterations (data not shown). In line with this is an increased level of non-phospho- β -catenin (Ser33/37/Thr41) in the asthmatic lung. (Fig. 1B). Next, we performed transient transfection reporter assay with the well documented TOPFlash reporter in the bronchial epithelial cell line 16HBE. As can be seen in Fig.1D, TDI-HSA induced a significant increased luciferase activity in 16HBE. Together, these in vivo and in vitro experiments supported an active β -catenin signaling in TDI asthma, which can be suppressed by treatment with either XAV-939 or ICG-001.

XAV-939 and ICG-001 blocked β -catenin-mediated gene expression and attenuated airway remodeling

Quantitative PCR (qPCR) demonstrated that TDI challenge following dermal sensitization significantly increased the pulmonary expression of a number of well-documented β -catenin target genes also associated with remodeling (MMP-2, MMP-9, fibronectin, TGF β 1 and VEGF) [http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes]. The increased expression of these genes was almost eliminated in TDI-treated mice given XAV-939 or ICG-001 (Fig. 1C). Protein expression of TGF β 1, a critical mediator involved in asthmatic airway remodeling (24), was also upregulated in the lungs of TDI-inhaled mice, but reduced

by XAV-939 and ICG-001 (Fig. 2E).

We next investigated whether treatment with XAV-939 or ICG-001 could prevent airway remodeling induced by TDI. As seen by histopathology and morphometry, inhalation of 3% TDI 3 hours daily on days 15, 18 and 21 following sensitization induced extensive deposition of collagen and pronounced goblet cell metaplasia in the airway compared with vehicle control. Administration of XAV-939 (2.5 mg/kg per day) or ICG-001 (5 mg/kg per day), beginning the first day of TDI inhalation and continuing for a week, significantly reduced collagen deposition around the airways and epithelial goblet cell hyperplasia as assessed by Masson's trichrome and PAS staining (Fig. 2A-D).

XAV-939 and ICG-001 decreased AHR and airway inflammation induced by TDI

Mice underwent TDI sensitization and challenge displayed increased reactivity to methacholine when compared with those AOO-treated (Fig. 3F), together with a dense accumulation of inflammatory cells around the airway, and numerous neutrophils and eosinophils infiltrating into the airway as shown by histology and BALF analysis (Fig. 3A-E).

Daily intraperitoneal injection with either XAV-939 or ICG-001 since the first day of challenge dramatically decreased TDI-induced AHR and airway inflammation. Higher total serum IgE, a marker of sensitization, was also detected in TDI asthmatic mice, but was not affected by XAV-939 nor ICG-001 (Fig. 3G).

XAV-939 and ICG-001 diminished Th1/Th2 responses

Dysregulation of Th1 and Th2 responses was observed in TDI sensitized and challenged mice, with raised levels of IFN- γ (Th1-related) and IL-4, IL-5, IL-13 (Th2-related) in supernatants of cultured lymphocytes, which was greatly recovered by XAV-939 and ICG-001 (Fig. 3H).

Yet we failed to detect IL-25 in BALF, which is a typical epithelia-derived Th2 cytokine.

XAV-939 and ICG-001 downregulated expression of VEGF and HMGB1, IL1 β in TDI asthma

VEGF is a canonical β -catenin targeted gene that participates critically in TDI-induced asthma (25). Immunohistochemistry revealed relatively faint staining in AOO-exposed lung, but abundant expression after TDI treatment, with robust immunoreactivity in the airway epithelia, airway smooth muscles and the infiltrating inflammatory cells (Fig. 4A). In line with this, there's a robust increase of VEGF in BALF after TDI inhalation (Fig. 4B).

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Blockade of β -catenin targeted transcriptional signaling with XAV-939 and ICG-001 effectively inhibited VEGF secretion into BALF as well as its expression in the lung. Similar results were obtained when pulmonary HMGB1 and IL-1 β were detected by western blot (Fig. 4C).

XAV-939/ICG-001 ameliorated TDI-challenged airway inflammation without dermal sensitization

To investigate the effect of signaling inhibition on the toxic response of TDI, another group of mice underwent TDI challenge without prior dermal sensitization were treated with XAV-939 or ICG-001 respectively. As with previously reported (13), inhalation of 3% TDI 3 hours daily on days 15 and 18 without dermal sensitization was not sufficient to generate asthma-like responses. Indeed, only slight inflammatory infiltration around the airway accompanied by mild epithelial hyperplasia was seen in this group of mice, which was completely abolished by daily injection with XAV-939 (2.5mg/kg) or ICG-001 (5mg/kg) (Fig5.A). In line with this, immunohistochemistry showed that treatment with XAV-939/ICG-001 also ameliorated the diffused expression of β -catenin in the airway epithelium (Fig5.B). Both inhibitors (with aforementioned doses daily for a week) had no effects on normal BALB/c mice aged 6~8 weeks (data not shown).

Discussion

In this study, we showed that β -catenin signaling is activated in a murine model of TDI-induced asthma. Selective blockade of β -catenin-mediated transcriptional signaling with XAV-939 and ICG-001 significantly relieved TDI-induced airway remodeling, and for the first time we demonstrated that β -catenin mediates airway hyperresponsiveness and inflammation, dysregulated Th1/Th2 responses and upregulation of TGF β 1, VEGF, HMGB1, IL1 β in asthma.

It is well established that aberrant Wnt/ β -catenin signaling underlines a wide range of human pathologies, including cancer, metabolic, inflammatory and fibrotic diseases (26). Recent studies have suggested β -catenin as a therapeutic target in airway remodeling of asthma due to its preeminent roles in cell proliferation, survival, repair and regeneration (9). Interrupting β -catenin by siRNA technique in an OVA-induced asthma model decreased smooth muscle hyperplasia and subepithelial fibrosis (10), which are key features of airway remodeling. As

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we've previously demonstrated abnormal expression of β -catenin in TDI-induced asthma model, we hypothesized that β -catenin signal might be important for TDI-induced inflammation and remodeling. In the present study, an increased level of non-phospho- (active) β -catenin (Ser33/37/Thr41) was found in lungs of TDI-treated mice, along with diffused expression in the cytoplasm and nucleus mainly in the airway epithelia, implying activated β -catenin signaling in vivo. Subsequent luciferase assay in 16HBE fueled our hypothesis that TDI induces bona fide β -catenin signaling activation in airway epithelia. Animals treated with the tankyrase inhibitor XAV-939 had enhanced β -catenin phosphorylation at Ser33/37/Thr41, which triggers ubiquitination and degradation of the protein (3). Though the direct mechanistic function of ICG-001 is not to degrade cytoplasmic β -catenin to inhibit activation, different from that of XAV-939, ICG-001 still downregulated non-phospho- β -catenin and rescued the abnormal immunostaining of β -catenin in the airway. This could be explained by a positive feedback response induced by ICG-001-targeted genes. For instance, TGF β 1, whose mRNA and protein levels were both suppressed by ICG-001 in our asthma model, was known to activate β -catenin signaling in airway smooth muscles and bronchial epithelial cells (7, 27). As with the study of Kwak and coworkers (10), we found that blockade of β -catenin-mediated transcriptional signaling with XAV-939 or ICG-001 significantly inhibited TDI-induced airway remodeling, manifested by down-regulated gene expression associated with remodeling and fibrosis, decreased collagen deposition around the airway and slighter airway goblet cell hyperplasia.

In addition to remodeling, another central feature of the airways in TDI-induced asthma is inflammation, which is dominated by a marked infiltration of neutrophils, a slight infiltration of eosinophils into the airways, and a mixed Th1/Th2 response (28). Yet currently there are limited evidence suggesting a direct link between β -catenin and airway inflammation. In 2014, researchers from Korea found for the first time that using siRNA downregulating β -catenin inhibited activation of NF κ B and expression of IL-6, IL-8, MCP-1 and TNF- α induced by house dust mite (HDM) in THP-1 (29). Subsequently, the same research team reported that β -catenin is responsible for LPS-induced NF κ B activation and inflammatory cytokine expression in bronchial epithelia (11). Their contributions shed light on a probably underestimate function of β -catenin in inflammation. In this study, both inhibitors displayed

high capacity to blunt TDI-induced airway inflammation, with smaller numbers of neutrophils and eosinophils recruited into the airway, decreased levels of inflammatory cytokines such as VEGF, HMGB1 and IL1 β , and a compromised Th1/Th2 response, indicating that activation of β -catenin signaling is critically engaged in TDI-induced inflammation. In contrast, Trischler J and colleagues found that genetic loss of Wnt10b led to augmented inflammation in an HDM asthma model (30). Their finding seems opposite to ours given the central role of β -catenin in Wnt signaling. Yet it should be noted that ligation of Wnt ligands to coreceptors would result in not only the canonical Wnt/ β -catenin signaling but also pathways independent of β -catenin (31). So it's easy to understand that inhibition of one Wnt ligand may result in almost opposite outcomes from inhibition of Wnt/ β -catenin signaling.

Though β -catenin signaling is proposed to contribute to TDI-induced airway inflammation, the underlying mechanisms still remains obscure. Vascular endothelial growth factor (VEGF) has long been identified as a prototypical target gene of β -catenin (32). It is one of the most potent proangiogenic cytokines, and occupies a central role in the process of angiogenesis as well as tissue remodeling (33). Besides, studies have demonstrated that VEGF also contributes critically to airway inflammation, airway remodeling and Th2 inflammation in the lung (34, 35). Increased expression of VEGF was found in patients with TDI-induced asthma as well as animal models (25, 36). Blocking VEGF signaling with VEGFR inhibitors reduced airway hyperresponsiveness and inflammation induced by TDI in a murine model (25). And we found increased expression of VEGF induced by TDI-HSA conjugate in our previous study (37). These studies suggest that VEGF is one of the major determinants of TDI-induced asthma. In consistent with these findings, we detected higher gene and protein levels of VEGF in TDI sensitized and challenged mice when compared with the AOO-treated. Of note, decreased bronchial hyperresponsiveness, lung neutrophilic, eosinophilic and Th2 inflammation were coupled with lower levels of VEGF when mice were treated with XAV939 or ICG-001, suggesting that β -catenin signaling might modulate TDI-induced airway inflammation through a VEGF-dependent mechanism, which needs to be further confirmed in our future studies.

There are other important mediators underlying the etiology of TDI-induced asthma. Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in extracellular matrix (ECM) turnover that play an essential role in physiological tissue remodeling and repair (38). Members of this family include MMP-2 and MMP-9. Both are well known downstream target genes of β -catenin signaling [http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes], and potent chemotactic factors that mediate transmigration of neutrophils and eosinophils to the sites of inflammation (38, 39). In agreement with the results of Lee et al. (39), we detected fold increases in mRNA expression of MMP-2 and MMP-9 in TDI asthmatic mice, which were extensively suppressed by XAV-939 and ICG-001, indicating a possible β -catenin/MMPs-mediated neutrophilic and eosinophilic airway inflammation pathway initiated by TDI. Besides, we also found that XAV-939 or ICG-001 decreased protein expression of HMGB1 and IL1 β in lungs of mice subjected to TDI sensitization of challenge, both of which are critical regulators in TDI-induced asthmatic responses (23, 40). This suggests that β -catenin might mediate airway inflammation through modulation of HMGB1 and IL1 β .

In summary, our studies demonstrated that blocking β -catenin signaling attenuates airway hyperresponsiveness, inflammation and remodeling in a TDI-induced murine asthma model, providing novel evidence for targeting β -catenin in asthma.

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Author contributions

C. S., Y. L., Z. H. and T. H. designed the experiment and wrote the manuscript. Y. L. Z. H. and T. H. performed the experiments and analyzed the data. X. J., Z. W. helped with the experiments. L. L., D. H. and Z. F. helped with data analysis and revised 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.

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Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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Figure legends

Fig1. XAV-939 and ICG-001 inhibited activation of β -catenin and downregulated β -catenin target genes associated with remodeling. A. Representative immunohistochemical staining of β -catenin showed that treatment with XAV-939 or ICG-001 leads to better arrangement of β -catenin in airway epithelia, with less immunoreactivity in the cytoplasm and nucleus. Original magnification was 1000 $\times$. B. Western blot analysis of non-phospho- (active) β -catenin (Ser33/37/Thr41) with whole lung extracts. n=5~6. C. Quantitative PCR was performed using RNA extracted from TDI-treated mice given either vehicle or XAV-939/ICG-001. By qPCR, the significantly increased expression of VEGF, TGF- β 1, fibronectin, MMP-9, MMP-2 in lungs of TDI asthmatic mice was inhibited by 2.5mg/kg per day XAV-939 or 5 mg/kg per day ICG-001. n=5~6. *p<0.05 compared with AOO/AOO group; #p<0.05 compared with TDI/TDI group. D. 16HBE cells cultured in DMEM medium supplemented with 10% calf serum were grown to 70~80% confluent and then cotransfected using Lipofectamine 2000 reagent with M50 (1 μ g) plus Rellina (1 μ g) as an internal control. After transfection, cells were treated with HSA (40 μ g/mL), TDI-HSA (40 μ g/mL), ICG-001 (10 μ M) and XAV-939 (10 μ M) for 12h and then collected for luciferase assay. Luciferase assay was performed using the Dual Luciferase Assay System kit according to the manufacturer's protocols (Promega). Relative luciferase activity (arbitrary unit) was reported as fold-induction over controls after normalizing for transfection efficiency. The result is representative of 3 independent experiments. *p<0.05 compared with empty control, #p<0.05 compared with TDI-HSA-treated.

Fig2. XAV-939 and ICG-001 ameliorated TDI-induced asthmatic airway remodeling. A. Representative masson trichrome-stained lung sections and subsequent semiquantification (C) showed less collagen deposition around the airway after mice were treated by XAV/939/ICG-001. Original magnification was 200 $\times$. n=8~10. B. Representative PAS--stained lung sections and subsequent semiquantification (D) showed the TDI-induced

goblet cell metaplasia was significantly reduced by XAV-939/ICG-001. The red arrows indicate PAS-stained positive epithelia. Original magnification was 200 $\times$. n=8~10. E. Level of TGF β 1 in the whole lung detected by western blot. n=5~6. *p<0.05 compared with AOO/AOO group; #p<0.05 compared with TDI/TDI group.

Fig3. XAV-939 and ICG-001 decreased TDI-induced airway inflammation, airway hyperresponsiveness, as well as Th1/Th2 response. A. Representative H&E-stained lung sections showed the dense inflammatory infiltration around the airway induced by TDI sensitization and challenge was decreased by treatment with XAV-939 or ICG-001. Original magnification was 200 $\times$. B. Representative H&E-stained cytospin samples from BALF showed that TDI challenge following sensitization induced numerous neutrophils and eosinophils into the airway lumen, which was decreased by XAV-939/ICG-001. Black arrows indicate neutrophils, and red arrows indicate eosinophils. Original magnification was 400 $\times$. C. Semiquantification of airway inflammation in H&E stained lung sections. n=8~10. D. Total inflammatory cells in BALF were counted. n=8~10. E. Differential cell percentages in BALF. Results are expressed as percentages of total cells by counting a total of 200 cells in H&E-stained cytospin samples. n=8~10. F. Airway hyperresponsiveness was measured by lung resistance (R_L). Results are expressed as percentage of baseline value for each concentration of methacholine. n=6. G. Total serum IgE quantified by ELISA. n=8~10. H. Levels of IFN γ (Th1-related) and IL-4, IL-5, IL-13 (Th2-related) in supernatants of cultured lymphocytes. n=8~10. *p<0.05 compared with AOO/AOO group; #p<0.05 compared with TDI/TDI group.

Fig4. Blockade of β -catenin signaling downregulated pulmonary expression of VEGF, HMGB1 and IL1 β . A. Representative immunohistochemistry of VEGF in lung revealed much more staining after TDI sensitization and challenge when compared with the AOO-exposed, which is most prominent in airway epithelia and the infiltrating inflammatory cells. Treatment with XAV-939 or ICG-001 significantly inhibited VEGF expression. The lower panel showed magnified view of different cells expressing VEGF in TDI-exposed mice. Original magnification was 400 $\times$. B. Level of VEGF in BALF. n=8~10. C. Protein expression of HMGB1 and IL1 β in lung homogenates was detected by western blot and densitometric analysis was performed. Fig.4C and Fig.2E share the same loading control (β -actin). n=6.

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*p<0.05 compared with AOO/AOO group; #p<0.05 compared with TDI/TDI group.

Fig5. Effects of XAV-939/ICG-001 on TDI-induced toxic responses in mice challenged with TDI without dermal sensitization. BALB/c mice underwent airway challenge through inhalation on days 15 and 18 without prior dermal sensitization. XAV-939 (2.5mg/kg per day) or ICG-001 (5mg/kg per day) was given to the mice every 24 hours for a total of four times. A. Representative H&E-stained lung sections showed that TDI inhalation without dermal sensitization leads to slight inflammatory infiltration around the airway accompanied by mild epithelial hyperplasia, which was abolished by XAV-939/ICG-001. Original magnification was 200×. B. Representative immunohistochemical staining of β -catenin. Original magnification was 1000×.







