

# RESEARCH PAPER

## The receptor for advanced glycation end products is required for $\beta$ -catenin stabilization in a chemical-induced asthma model

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### BACKGROUND AND PURPOSE

Cytoplasmic retention of  $\beta$ -catenin will lead to its nuclear translocation and subsequent interaction with the transcription factor TCF/LEF that regulates target gene expression. We have previously demonstrated aberrant expression of  $\beta$ -catenin in a model of asthma induced by toluene diisocyanate (TDI). The aim of this study was to examine whether the receptor for advanced glycation end products (RAGE) can regulate  $\beta$ -catenin expression in TDI-induced asthma.

### EXPERIMENTAL APPROACH

Male BALB/c mice were sensitized and challenged with TDI to generate a chemically-induced asthma model. Inhibitors of RAGE, FPS-ZM1 and the RAGE antagonist peptide (RAP), were injected i.p. after each challenge. Airway resistance was measured *in vivo* and bronchoalveolar lavage fluid was analysed. Lungs were examined by histology and immunohistochemistry. Western blotting and quantitative PCR were also used.

### KEY RESULTS

Expression of RAGE and of its ligands HMGB1, S100A12, S100B, HSP70 was increased in TDI-exposed lungs. These increases were inhibited by FPS-ZM1 or RAP. Either antagonist blunted airway reactivity, airway inflammation and goblet cell metaplasia, and decreased release of Th2 cytokines. TDI exposure decreased level of membrane  $\beta$ -catenin, phosphorylated Akt (Ser<sup>473</sup>), inactivated GSK3 $\beta$  (Ser<sup>9</sup>), dephosphorylated  $\beta$ -catenin at Ser<sup>33/37</sup>/Thr<sup>41</sup>, which controls its cytoplasmic degradation, increased phosphorylated  $\beta$ -catenin at Ser<sup>552</sup>, raised cytoplasmic and nuclear levels of  $\beta$ -catenin and up-regulated its targeted gene expression (MMP2, MMP7, MMP9, VEGF, cyclin D1, fibronectin), all of which were reversed by RAGE inhibition.

### CONCLUSION AND IMPLICATIONS

RAGE was required for stabilization of  $\beta$ -catenin in TDI-induced asthma, identifying protective effects of RAGE blockade in this model.

### Abbreviations

BALF, bronchoalveolar lavage fluid; GSK, glycogen synthase kinase; RAGE, receptor for advanced glycation end products; TDI, toluene diisocyanate

## Tables of Links

| TARGETS                                           |
|---------------------------------------------------|
| <b>Enzymes<sup>a</sup></b>                        |
| Akt                                               |
| GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ |
| MMP2                                              |
| MMP7                                              |
| MMP9                                              |
| <b>Other protein targets<sup>b</sup></b>          |
| HSP70                                             |
| RAGE                                              |

| LIGANDS          |
|------------------|
| $\beta$ -Catenin |
| Fibronectin      |
| IL-4             |
| IL-5             |
| IL-13            |
| Methacholine     |
| VEGF             |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (<sup>a</sup>Alexander *et al.*, 2015a,b).

## Introduction

The plasma membrane-associated protein  $\beta$ -catenin plays a dual role in cellular signalling. It is involved in both cadherin-mediated intercellular adhesion and transcriptional coactivation downstream of the Wnt signalling pathway (Sineva and Pospelov, 2014). Corresponding to its dual functions in cells,  $\beta$ -catenin is located in two cellular pools. In mature epithelial cells, the majority of the protein is anchored to the cell membrane, where it binds tightly to the cytoplasmic domain of type I cadherins and plays an essential role in the structural organization and function of cadherins by linking cadherins through  $\alpha$ -catenin to the cytoskeleton (Gumbiner, 2000; Jamora and Fuchs, 2002; Nawijn *et al.*, 2011). A smaller pool of  $\beta$ -catenin is found in both the nucleus and cytoplasm. In the absence of Wnt signalling,  $\beta$ -catenin is usually bound to a multiprotein destruction complex composed of casein kinase I, glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), adenomatous polyposis coli gene product, diversin and axin, which phosphorylates  $\beta$ -catenin on N-terminal Ser and Thr residues (S45, S33, S37, T41), thus priming it for ubiquitination and proteasomal degradation (Monga, 2015), maintaining very low levels in the cytosol. Binding of an extracellular Wnt ligand to the Frizzled LRP5/6 co-receptors rescues  $\beta$ -catenin from this destructive complex and allows its cytosolic accumulation and nuclear translocation. When targeted to the nucleus,  $\beta$ -catenin promotes transcription of TCF/LEF-dependent genes, which include cell cycle regulatory proteins, such as cyclin D1, growth factors such as VEGF, matrix proteins such as fibronectin and versican, proteases such as MMP-2, -7 and -9, and pro-inflammatory enzymes and mediators, such as COX-2 and IL-8 (Brabletz *et al.*, 1999; Howe *et al.*, 1999; Tetsu and McCormick, 1999; Zhang *et al.*, 2001; De Langhe *et al.*, 2005; Masckauchan *et al.*, 2005; Rahmani *et al.*, 2005)].

Impaired airway epithelial integrity and loss of junctional proteins such as E-cadherin were found in patients with asthma and in animal models (Hackett *et al.*, 2013; Yao *et al.*, 2015), which would promote  $\beta$ -catenin release and lead to an increase in its cytoplasmic level thereby activating

$\beta$ -catenin (Nelson and Nusse, 2004; Valenta *et al.*, 2012). Recently,  $\beta$ -catenin promoter polymorphism has been associated with asthma risk in Korean subjects (Bae *et al.*, 2012). In asthma, WNT-dependent activation of  $\beta$ -catenin signalling has already been shown (Cohen *et al.*, 2009; Moheimani *et al.*, 2015). Aberrant activation of  $\beta$ -catenin would contribute considerably to airway remodelling due to its central role in cell proliferation, survival, repair and regeneration (Kumawat *et al.*, 2014). These effects would suggest  $\beta$ -catenin as an important regulator and therapeutic target in asthma. We have also demonstrated aberrant expression of  $\beta$ -catenin in a model of asthma induced by toluene diisocyanate (TDI) (Yao *et al.*, 2015). However, there are currently few studies addressing the upstream mechanisms involving in  $\beta$ -catenin stabilization in asthma.

The receptor for advanced glycation end products (RAGE) is a multi-ligand pattern recognition receptor that belongs to the immunoglobulin gene superfamily. It senses a diverse range of signalling molecules like advanced glycation end products, HMGB1, serum amyloid A, HSP70, complement 3a (C3a), S100B and  $\beta$ -amyloid (Schmidt *et al.*, 2001). Ligation of RAGE induces the activation of many signalling pathways, which include GSK-3 $\beta$  (Sukkar *et al.*, 2012), a critical member of the destruction complex of  $\beta$ -catenin that negatively orchestrates  $\beta$ -catenin activity by promoting its degradation. So, we inferred that RAGE might also mediate  $\beta$ -catenin signalling. Here, we have examined whether RAGE is required for airway epithelial  $\beta$ -catenin activation in a TDI-induced asthma model.

## Methods

### Animals

All animal care and experimental procedures studies complied with the guidelines of the Committee of Southern Medical University on the use and care of animals and were approved by the Animal Subjects Committee of Nanfang Hospital. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Specific pathogen-free adult male BALB/c mice (20–22 g) aged 6–8 weeks were purchased from Southern Medical University. The mice were housed in a specific pathogen-free facility with 12 h dark/light cycles [temperature  $23 \pm 2^\circ\text{C}$ , humidity range 40–70%, 12 h light/dark cycle (lighting: 7:00–19:00h)] and with sterile water and irradiated food available *ad libitum*.

### Experimental procedures

A total of 120 mice were used ( $n = 15$  for each experimental group). The vehicle (AOO) used to dissolve TDI consisted of a mixture of two volumes of acetone and three volumes of olive oil for sensitization, and one volume of acetone and four volumes of olive oil for the challenge. Two antagonists of the RAGE were used, FPS-ZM1 and the RAGE antagonist peptide (RAP).

In the first protocol, mice were randomized to the following four groups: (i) AOO-sensitized, AOO-challenged and DMSO-treated (AOO/AOO group); (ii) TDI-sensitized, TDI-challenged and DMSO-treated (TDI/TDI group); (iii) TDI-sensitized, TDI-challenged and FPS-ZM1-treated (TDI/TDI + FPS-ZM1 group); and (iv) TDI-sensitized, TDI-challenged and RAP-treated (TDI/TDI + RAP group). In a second protocol, to explore the role of RAGE in the toxic response to TDI, mice were allocated to another four groups: (i) non-sensitized, AOO-challenged and DMSO-treated (–/AOO group); (ii) non-sensitized, TDI-challenged and DMSO-treated (–/TDI group); (iii) non-sensitized, TDI-challenged and FPS-ZM1-treated (–/TDI + FPS-ZM1 group); and (iv) non-sensitized, TDI-challenged and RAP-treated (–/TDI + RAP group).

Animal models were generated as described elsewhere (Yao *et al.*, 2015). Briefly, on days 1 and 8, the mice were dermally treated with 0.3% TDI on the dorsum of both ears (20  $\mu\text{L}$  per ear). On days 15, 18 and 21, the mice were placed in a horizontal rectangle chamber and challenged for 3 h each time via the airways with 3% TDI by means of 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 non-sensitized mice were challenged only twice on days 15 and 18. FPS-ZM1 (1.5  $\text{mg}\cdot\text{kg}^{-1}$ ; i.p.) and RAP (4  $\text{mg}\cdot\text{kg}^{-1}$ ; i.p.) dissolved in DMSO and diluted with PBS (pH = 7.4) were given to the mice immediately after each airway challenge. Sham mice received the same volume of vehicle for comparison.

### Assessment of airway hyperresponsiveness

Twenty-four hours after the last inhalation, measurements of lung resistance ( $R_L$ ) were performed on anaesthetized, intubated and mechanically ventilated (Buxco Electronics, Troy, NY, USA) mice in response to increasing doses of nebulised methacholine (3.12, 6.25, 12.5 and 25  $\text{mg}\cdot\text{mL}^{-1}$ ). Measurements of  $R_L$  were performed every 5 min following each nebulisation step until a plateau phase was reached. Results were expressed as percentage of baseline  $R_L$  value for each concentration of methacholine.

### Quantification of serum IgE, and analysis of bronchoalveolar lavage fluid

As previously described (Tang *et al.*, 2014), mice were killed with pentobarbital (100  $\text{mg}\cdot\text{kg}^{-1}$ , i.p.) 1 day after the last airway challenge. Blood samples were first taken from the retro-

orbital plexus/sinus, allowed to rest for 1 h at room temperature, then centrifuged (3000 $\times$  g, 20 min), and supernatants were harvested for detection of total IgE. Next, the lungs were lavaged *in situ*, twice with 0.8 mL sterile saline (0.9% NaCl, prewarmed), and the recovered fluid was pooled. Total cells in bronchoalveolar lavage fluid (BALF) were counted, and a cytospin sample was prepared and stained with haematoxylin and eosin (H&E) (JianChen, Nanjing, China) for blinded assessment of differential cell percentages in BALF. Then the remaining fluids were centrifuged (1000 $\times$  g, 10 min), and supernatants were stored for further analysis of IFN- $\gamma$  and IL-4, IL-5 and IL-13, according to the manufacturer's instructions.

### Pulmonary histological examination

Left lungs were gently infused with 4% neutral formalin to fully inflate all lobes (judged visually) and immersed in formalin for at least 24 h, then fixed, paraffin-embedded, cut in 4  $\mu\text{m}$  sections and stained with H&E for histopathological assessment, which was carried out without knowledge of the treatments (blinded). Lung inflammation was quantified as previously described (Tang *et al.*, 2014). Briefly, two criteria were scored to quantify the pulmonary inflammation: peribronchial inflammation and perivascular inflammation. A value of 0 was allocated when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by one layer (1 to 10 cells) of inflammatory cells, a value of 3 for most bronchi or vessels surrounded by 2 layers (10 to 20 cells) of inflammatory cells and a value of 4 for most bronchi or vessels surrounded by more than two layers (more than 20 cells) of inflammatory cells. 16–20 image fields of eight sections from 8–10 mice per group were scored.

To visualize airway goblet cell metaplasia, lung sections were stained with periodic acid-Schiff base (PAS), and semi-quantitative scoring was used to compare groups. PAS-stained tissues were examined under light microscopy for PAS-positive staining in the upper airway epithelium in fields at 200 $\times$  magnification. PAS-positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups. The 8–10 image fields of eight sections from 8–10 mice per group were analysed.

### Western blot analysis and immunohistochemistry

Lung tissues obtained from the experimental mice were snapfrozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Tissue samples were ground to a fine powder under liquid nitrogen and lysates subjected to SDS-PAGE and Western blotting for detection of the following antigens: RAGE (ABclonal Biotech Co., Ltd.), HMGB1 (ABclonal), S100 A12 (ABclonal), S100B (ABclonal), HSP70,  $\beta$ -catenin (Santa Cruz, CA, USA), non-phospho- (active)  $\beta$ -catenin (Ser<sup>33/37</sup>/Thr<sup>41</sup>), phospho- $\beta$ -catenin (Ser<sup>33/37</sup>/Thr<sup>41</sup>), Akt, phospho-Akt (Ser<sup>473</sup>), phospho- $\beta$ -catenin (Ser<sup>552</sup>), GSK3 $\beta$ , phospho-GSK3 $\beta$  (Ser<sup>9</sup>), VEGF (ABclonal) and MMP9 (ABclonal). After incubation with an IRDye® 680WC-conjugated secondary antibody (LI-COR Biosciences), immunoreactive bands were exposed to Odyssey® CLx Imager for image capture. Data analysis was performed with Odyssey Software.

For immunohistochemistry of RAGE, HMGB1, S100A12, S100B, HSP70, E-cadherin,  $\beta$ -catenin, p-GSK3 $\beta$  and VEGF, sections were deparaffinized, then submerged in citrate buffer (pH 6.0) for antigen retrieval. Samples were treated with H<sub>2</sub>O<sub>2</sub> for 15 min to block endogenous peroxidase, and then incubated overnight at 4°C in recommended dilutions of anti-RAGE and anti- $\beta$ -catenin 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).

### Preparation and analysis of subcellular fractions

Frozen lung tissues were homogenized for subcellular protein isolation. Nuclear and cytoplasmic extractions were isolated by NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (#78833, Thermo Fisher Scientific, Pierce, Rockford, IL, USA), and membrane fractions isolated by Membrane Protein Extraction Kit (#89842, Thermo Fisher Scientific) according to the manufacturer's protocols. All fractionated samples were boiled with 5 $\times$  sample buffer for 10 min, and then loaded on 10% SDS-PAGE for immunoblotting. Na/K ATPase (Abcam, ab76020),  $\beta$ -actin (Abclonal) and Histone H3 (Abclonal) were used as membrane, cytosolic and nuclear markers respectively.

### Quantitative PCR

Expression of MMP2, MMP7, MMP9, VEGF, cyclin D1 and fibronectin 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were 5'-AAGAGGGATGCTGCCCTTAC-3' (forward) and 5'-CCAATACGGCCAAATCCGTTC-3' (reverse); primers for MMP2 were 5'-TGCAGGAGACAAGTTCTGGAG-3' (forward) and 5'-GTAGCTATGACCACCACCTG-3' (reverse); primers for MMP7 were 5'-GGCTTCGCAAGGAGAGATCAT-3' (forward) and 5'-GCCAAATTCATGGGTGGCAG-3' (reverse); primers for MMP-9 were 5'-CGTGTCTGGAGATTCGACTTGA-3' (forward) and 5'-TGTTTCACCTCATGGTCCAC-3' (reverse); primers for VEGF were 5'-CACTGGACCCTGGCTTTACT-3' (forward) and 5'-ACTTGATCACTTCATGGGACTTCT-3' (reverse); primers for fibronectin were 5'-GAAATCTGCAGCCTGGGTCT-3' (forward) and 5'-ACACCCAGCTTGAAGCCAAT-3' (reverse). The amplification protocol was set as follows: 95°C denaturation for 30 s followed by 40 cycles of 15 s denaturation at 95°C, 1 min of annealing/extension and data collection at 60°C.

### Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are expressed as mean  $\pm$  SEM and were normally distributed. Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, Illinois, US). Comparisons among groups were analysed by one-way ANOVA, followed by Bonferonni *post hoc* test for

multiple comparisons.  $P < 0.05$  was considered statistically significant.

### Materials

Methacholine and TDI were obtained from Sigma-Aldrich (Shanghai, China). The RAGE inhibitors, FPS-ZM1 and RAGE antagonist peptide (RAP), were purchased from Millipore (Billerica, MA, USA). ELISA kit for IgE was from eBioscience (San Diego, CA, USA), and kits for detecting IFN- $\gamma$ , IL-4, IL-5 and IL-13 were from R&D system. Primary antibodies were bought from Cell Signaling Technology unless otherwise indicated.

### Results

#### *RAGE inhibition leads to decreased airway hyperreactivity and airway inflammation in TDI asthma*

TDI-induced asthmatic mice had increased airway reactivity, numerous neutrophils and eosinophils infiltrating into the airway, pronounced bronchial epithelial hyperplasia, higher serum IgE, as well as elevated levels of IL-4, IL-5 and IL-13 in BALF (Figure 1). Treatment with both antagonists of RAGE almost abolished those responses, except for the raised IgE titers, indicating central roles for the receptor in the pathogenesis of TDI asthma. At the same time, pulmonary expression of RAGE was analysed in TDI asthmatic mice. Levels of RAGE protein in the whole lung were up-regulated after TDI sensitization and challenge and these increased levels were suppressed by treatment with FPS-ZM1 or RAP (Figure 2C). Immunohistochemistry showed that in the lungs of vehicle (AOO)-exposed mice, RAGE was most abundantly expressed in alveolar epithelia, much less in airway smooth muscle and very faint in airway epithelia (Figure 2A and B). However, after TDI exposure, the airway epithelia exhibited more immunoreactivity of RAGE when compared with the AOO/AOO group, paralleled by recruited inflammatory cells with rich staining of RAGE (Figure 2C), together contributing to the increased total RAGE levels in lung. However, there were no significant changes in RAGE expression in the alveolar region or airway smooth muscle, for all groups.

#### *Expression of RAGE ligands in TDI-exposed lung*

Expression of RAGE ligands (HMGB1, S100A12, S100B, HSP70) was also determined in the asthmatic lungs. As can be seen in Figures 3 and 4, all of these proteins were up-regulated after TDI exposure. Immunohistochemistry showed that in AOO-treated mice, HMGB1 was abundantly expressed in the nucleus of all cell types, and the other three were most predominantly expressed in the alveolar macrophages with little in the epithelia. Following the TDI challenge in sensitized mice, there was nucleocytoplasmic translocation of HMGB1 in airway epithelia, alveolar epithelia and the infiltrating inflammatory cells. In line with this, there was increased staining of S100A12, S100B, HSP70 in the airway epithelia and inflammatory cells of TDI asthmatic mice. However, TDI treatment did not alter the alveolar expression of S100A12, S100B, HSP70. Blocking RAGE signalling with FPS-ZM1 or RAP restored the TDI-induced changes for all these ligands.

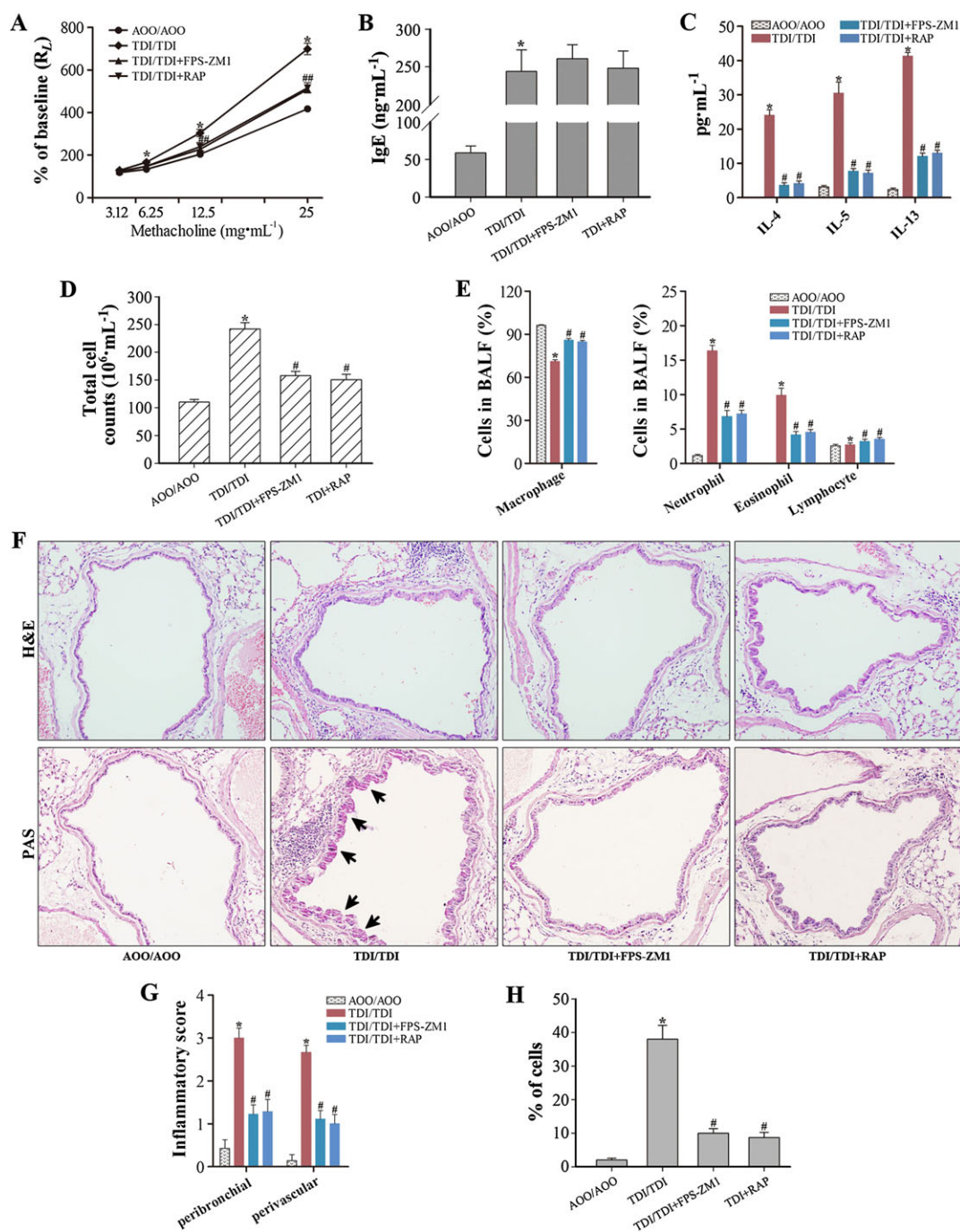


**Figure 1**

Effects of RAGE antagonists on TDI-induced asthmatic responses. (A) Airway hyperresponsiveness was measured by lung resistance (RL). Results were shown as percentage of baseline value.  $n = 6$ . (B–E) IgE in the serum (B), IL-4, IL-5, IL-13 in bronchoalveolar lavage fluid (BALF) (C), and total (D) and differential inflammatory cells (E) in BALF in AOO-treated group ( $n = 8$ ) and TDI-treated groups ( $n = 10$ ). Percentages of different inflammatory cells were calculated by counting a total of 200 cells in H&E-stained cytospin samples. (F) Representative H&E-stained and PAS stained lung sections of different groups. The black arrows in the lower panel indicate PAS-positive epithelial cells in the airway. Original magnification was 200 $\times$ . Semiquantification of airway inflammation (G) and PAS staining (H) was performed in AOO-treated group ( $n = 8$ ) and TDI-treated groups ( $n = 10$ ). Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells.  $*P < 0.05$ , significantly different from the AOO/AOO group;  $\#P < 0.05$ , significantly different from the TDI/TDI group.

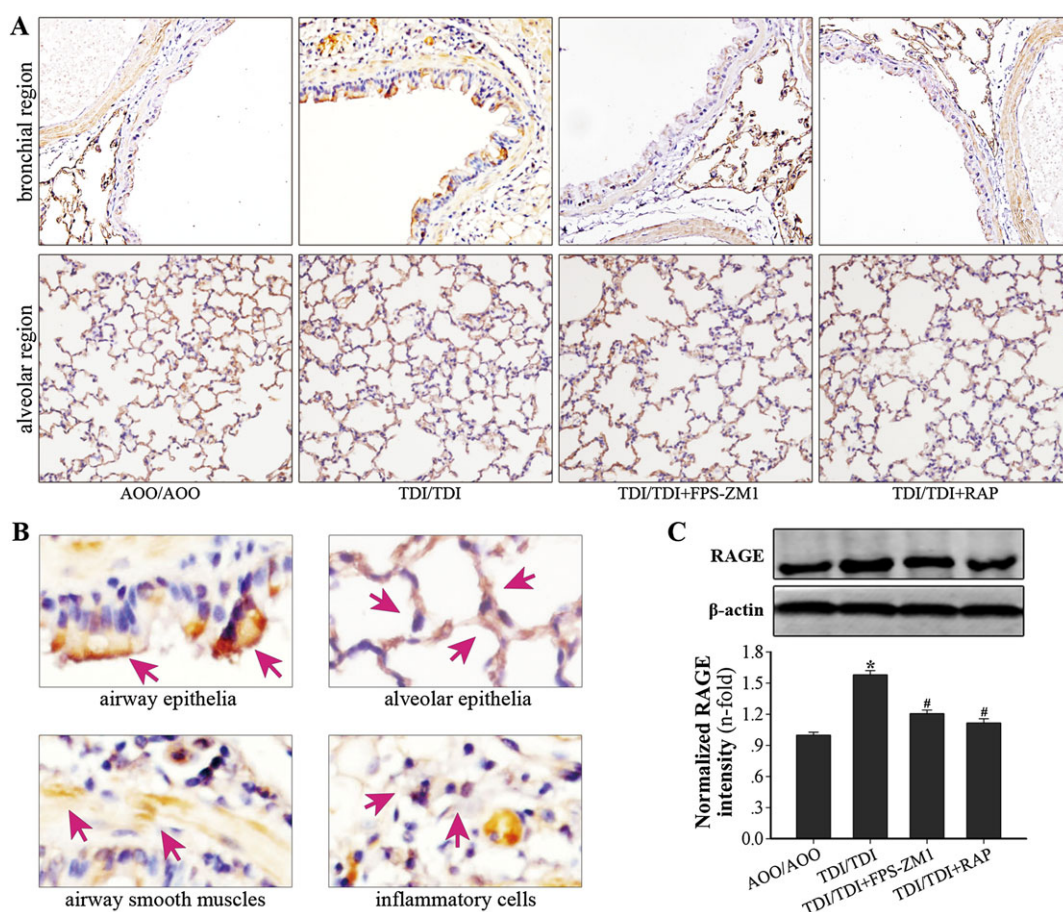


**Figure 2**

Pulmonary expression of RAGE. (A) Representative immunohistochemical staining of RAGE in AOO-exposed ( $n = 8$ ) and TDI-exposed ( $n = 10$ ) lungs. Original magnification was 400 $\times$ . (B) Amplified view of RAGE staining in different cell types of TDI-sensitized and challenged mice. (C) Western blot analysis of RAGE in whole lung and subsequent densitometric analysis of the blots ( $n = 6$ ). \* $P < 0.05$ , significantly different from the AOO/AOO group; # $P < 0.05$ , significantly different from the TDI/TDI group. Original magnification was 400 $\times$  ( $n = 8$ ).

### *RAGE inhibition ameliorated redistribution of $\beta$ -catenin*

Western blot analysis showed a mildly increased total expression of  $\beta$ -catenin in sensitized lung after challenge with TDI (Figure 5C and D). Further analysis with immunohistochemical staining disclosed abundant  $\beta$ -catenin expression in the airway epithelia, less in the alveolar region, with very weak immunoreactivity found in smooth muscle. TDI challenge following dermal sensitization leads to aberrant distribution of  $\beta$ -catenin, which is most prominent in airway epithelial cells, with much immunostaining in the cytoplasm and nucleus, while few changes in the alveolar epithelia and smooth muscle were seen (Figure 5B).

### *RAGE is required for $\beta$ -catenin stabilization and mediates its translocation to the nucleus*

Phosphorylation of  $\beta$ -catenin at Ser<sup>33/37</sup>/Thr<sup>41</sup> triggers its degradation by proteasomes in the cytoplasm (Monga, 2015). So, decreased serine and threonine phosphorylation of  $\beta$ -catenin would lead to its cytoplasmic retention and activation, namely stabilization. In this model, we found

that TDI induced a dramatically decreased level of phospho- $\beta$ -catenin at Ser<sup>33/37</sup>/Thr<sup>41</sup>, Ser<sup>552</sup> and activation of  $\beta$ -catenin. Both were reversed by blockade of RAGE (Figure 6A).

To identify the effect of RAGE inhibition on different pools of  $\beta$ -catenin, we analysed subcellular fractions isolated from lung tissues of asthmatic mice. Purity of the subcellular extracts was first verified by immunoblotting (Figure 6B). As can be seen, TDI dermal sensitization followed by airway challenge resulted in decreased membrane expression of  $\beta$ -catenin and E-cadherin (Figure 6C), along with increased phosphorylation of Tyr<sup>654</sup> (Figure 6A), which controls its binding with E-cadherin (Nelson and Nusse, 2004), indicating breached epithelial integrity. On the other hand, extensively increased cytoplasmic and nuclear levels of  $\beta$ -catenin were detected (Figure 6C), indicating cytoplasmic accumulation as well as nuclear translocation of  $\beta$ -catenin. In line with the results of immunohistochemistry, blockade of RAGE signalling by FPS-ZM1 or RAP dramatically suppressed the cytosolic and nuclear accumulation of  $\beta$ -catenin, inhibited tyrosine phosphorylation at Tyr<sup>654</sup> and restored its membrane level.

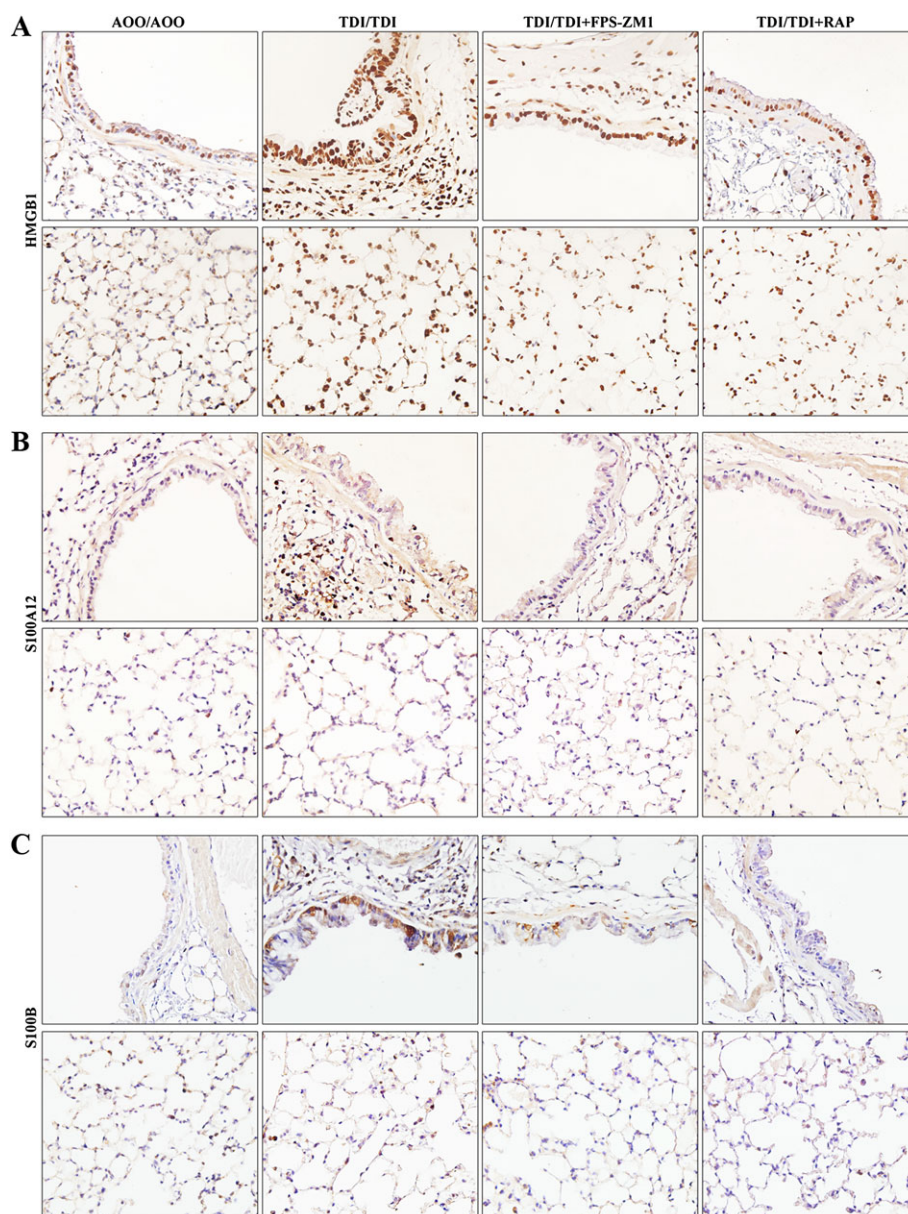


**Figure 3**

Pulmonary expression of RAGE ligands. Representative immunohistochemistry of HMGB1 (A), S100A12 (B) and S100B (C) in the bronchial regions (upper panel) and alveolar regions (lower panel).

### *RAGE increases phosphorylation of GSK3 $\beta$ and Akt*

Phosphorylation of GSK3 $\beta$  at Ser<sup>9</sup> inactivates this kinase, thereby compromising the function of the destruction complex, inhibits phosphorylation and ubiquitination of  $\beta$ -catenin and thus promotes  $\beta$ -catenin stabilization (Monga, 2015). As with our previous study (Yao *et al.*, 2015), phosphorylation of GSK3 $\beta$  at Ser<sup>9</sup> was increased after TDI exposure (Figure 5B–D). Immunohistochemical staining showed that the TDI-induced serine phosphorylation of GSK3 $\beta$  was not confined to the airway epithelia. Airway smooth muscles and alveolar epithelia also displayed significantly increased immunoreactive phospho-GSK3 $\beta$ . We also detected phospho-Akt (Ser<sup>473</sup>), an upstream component of the GSK3 $\beta$

pathway (Yao *et al.*, 2015). As expected, a significantly higher level of phospho-Akt (Ser<sup>473</sup>) was seen in lungs of TDI asthmatic mice, compared with those in the lungs of the AOO-treated mice. Increased phosphorylation of both GSK3 $\beta$  (Ser<sup>9</sup>) and Akt (Ser<sup>473</sup>) induced by TDI was reversed by RAGE blockade (Figure 5B–D).

### *Blocking RAGE inhibited TDI-induced up-regulation of $\beta$ -catenin targeted genes*

We next investigated whether RAGE inhibition affected the expression of  $\beta$ -catenin targeted genes. Quantitative PCR revealed that TDI sensitization and challenge dramatically up-regulated mRNA expression of a list of well-documented  $\beta$ -catenin targeted genes, which was then inhibited by

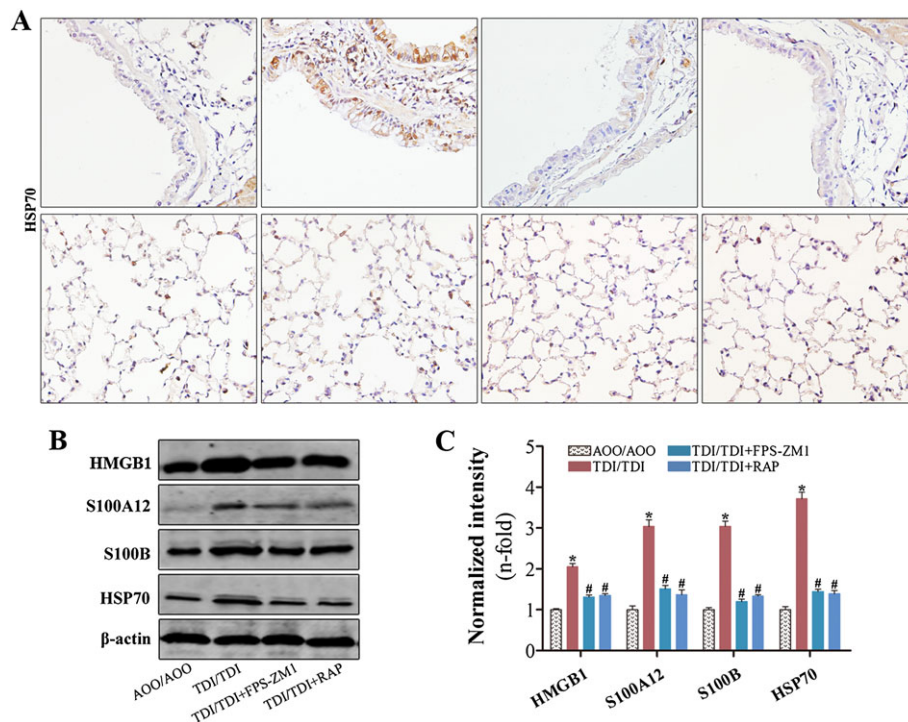


**Figure 4**

Pulmonary expression of RAGE ligands. (A) Representative immunohistochemical staining of HSP70 in the bronchial regions (upper panel) and alveolar regions (lower panel). Original magnification was  $400\times$  ( $n = 8$ ). (B) Western blot analysis of HMGB1, S100A12, S100B and HSP70 with whole lung extracts and densitometric analysis (C) ( $n = 6$ ). \* $P < 0.05$ , significantly different from the AOO/AOO group; # $P < 0.05$ , significantly different from the TDI/TDI group.

treatment of the mice with FPS-ZM1 ( $1.5 \text{ mg}\cdot\text{kg}^{-1}$ ) or RAP ( $4 \text{ mg}\cdot\text{kg}^{-1}$ ) (Figure 7D).

VEGF and MMP9 are two critical mediators involved in TDI-induced allergic airway inflammation (Lee *et al.*, 2002, 2003). By immunohistochemistry, we observed increased VEGF and MMP9 staining in bronchial epithelia, airway smooth muscles and recruited inflammatory cells in mice sensitized and challenged with TDI (Figure 7A and B). In line with this, Western blot analysis demonstrated elevated protein expression of VEGF and MMP9 in the asthmatic lung (Figure 7C). As expected, both were reduced by treatment with FPS-ZM1 or RAP.

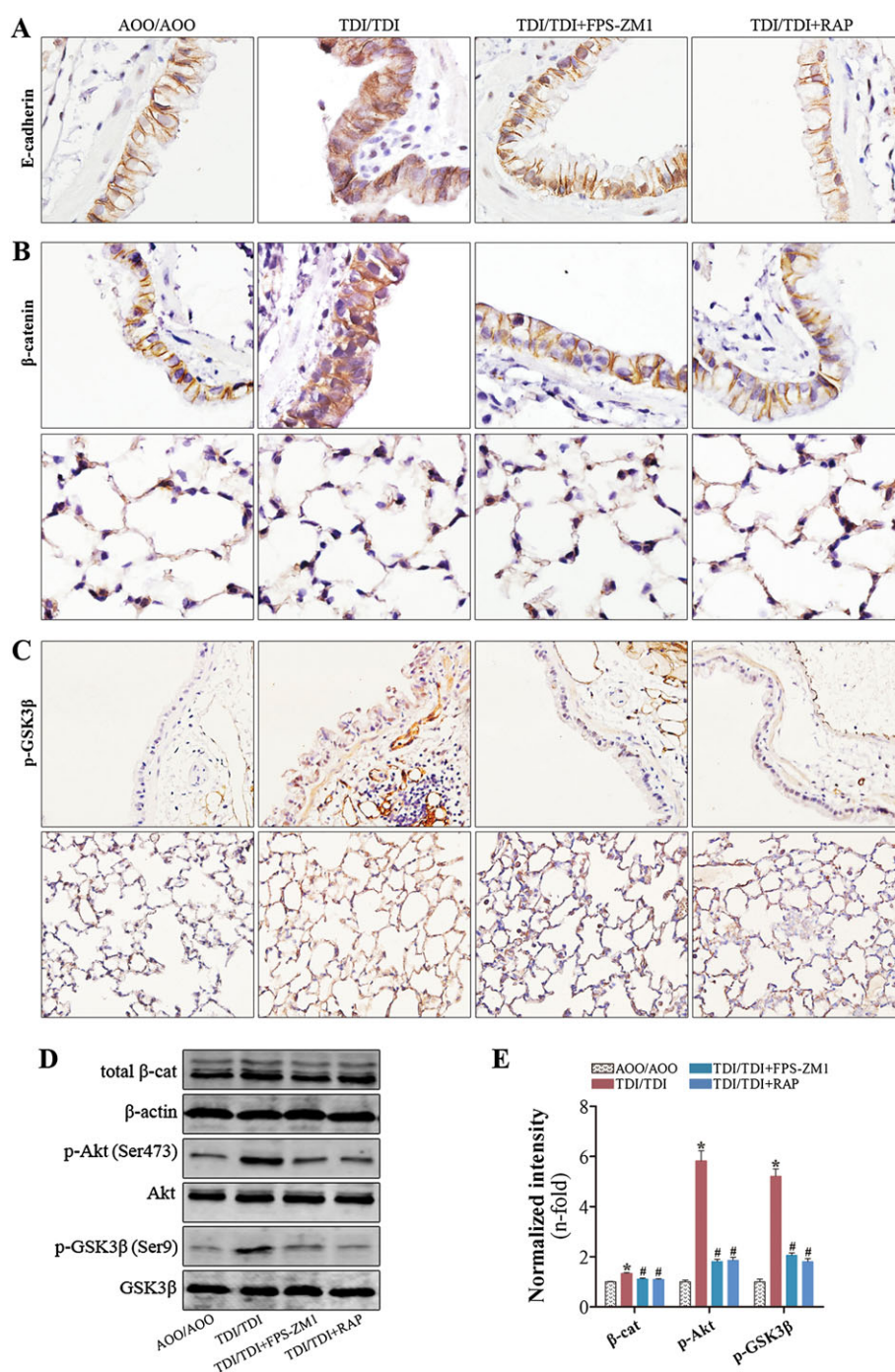
### *RAGE inhibition has no effect on $\beta$ -catenin expression in TDI-induced cytotoxic response*

To investigate the role of RAGE in the toxic response to TDI, another group of mice were challenged with TDI, without prior dermal sensitization. This airway challenge with TDI had no effects on total protein expression of RAGE and  $\beta$ -catenin, but elicited sporadic distribution of  $\beta$ -catenin in the cytosol and nuclei. GSK3 $\beta$  also showed increased phosphorylation at Ser<sup>9</sup>, and larger amounts of active  $\beta$ -catenin were detected. RAGE inhibition had no effect on TDI-elicited epithelial responses, under these conditions. Moreover, mice treated with RAGE antagonists exhibited no alterations of RAGE, total  $\beta$ -catenin, p-GSK3 $\beta$  (Ser<sup>9</sup>) or active  $\beta$ -catenin (Figure 8).

## Discussion

In this study, for the first time, we demonstrated active  $\beta$ -catenin signalling in TDI-induced asthma. Blockade of the RAGE axis with specific antagonists not only attenuated TDI-induced airway inflammation but also blunted the activation of  $\beta$ -catenin signalling and protected against the loss of membrane expression of  $\beta$ -catenin, suggesting a critical role for RAGE in the regulation of  $\beta$ -catenin.

We prepared the asthma model using the method described previously (Yao *et al.*, 2015). The experimental mice developed typical asthmatic features induced by TDI: airway hyperreactivity, airway inflammation characterized by neutrophil and eosinophil aggregation, epithelial goblet cell metaplasia and imbalanced Th1/Th2 responses. As expected, higher amounts of cytoplasmic and nuclear  $\beta$ -catenin were detected in TDI asthmatic mice, coupled with decreased levels in the cell membrane. We also observed a slightly increased total level of  $\beta$ -catenin, in contrast to the decrease in our previous study (Yao *et al.*, 2015). A reasonable explanation is that, as an invasive method for drug delivery to the lung, intratracheal instillation before challenge (in the previous study) may exert local pain to mice that would lead to accelerated breathing rates and therefore inhalation of larger doses of TDI. We also observed more severe airway inflammation in our earlier experiments, which may have caused greater damage to epithelial barrier function, with larger amounts of adhesive proteins, including  $\beta$ -catenin, liberated from the membrane.



## Figure 5

Effects of RAGE inhibition on  $\beta$ -catenin and p-GSK3 $\beta$  in the lung. Representative immunohistochemical staining of E-cadherin (A, 1000 $\times$ ),  $\beta$ -catenin (B, 1000 $\times$ ) and p-GSK3 $\beta$  (C, 400 $\times$ ) in lungs of AOO-treated ( $n = 8$ ) and TDI-treated ( $n = 10$ ) mice. (D) Western blot analysis of  $\beta$ -catenin, p-GSK3 $\beta$  and p-Akt (Ser<sup>473</sup>) in whole lung and subsequent densitometric analysis of the blots (E) ( $n = 6$ ). Figure 5D shares the same loading control blot ( $\beta$ -actin) with Figure 4B. \* $P < 0.05$ , significantly different from the AOO/AOO group; # $P < 0.05$ , significantly different from the TDI/TDI group.

RAGE is a multi-ligand receptor of the immunoglobulin superfamily involved in a number of physiological and pathological events characterized by inflammatory responses (Sims *et al.*, 2010). Mice deficient in RAGE developed significantly attenuated airway inflammation in response to

different asthrogens (Milutinovic *et al.*, 2012; Ullah *et al.*, 2014), indicating central roles for RAGE in asthma pathogenesis. FPS-ZM1 is a tertiary amide compound that shows high affinity for the V domain of RAGE. RAP, on the other hand, is an S100P-derived peptide that competitively antagonizes

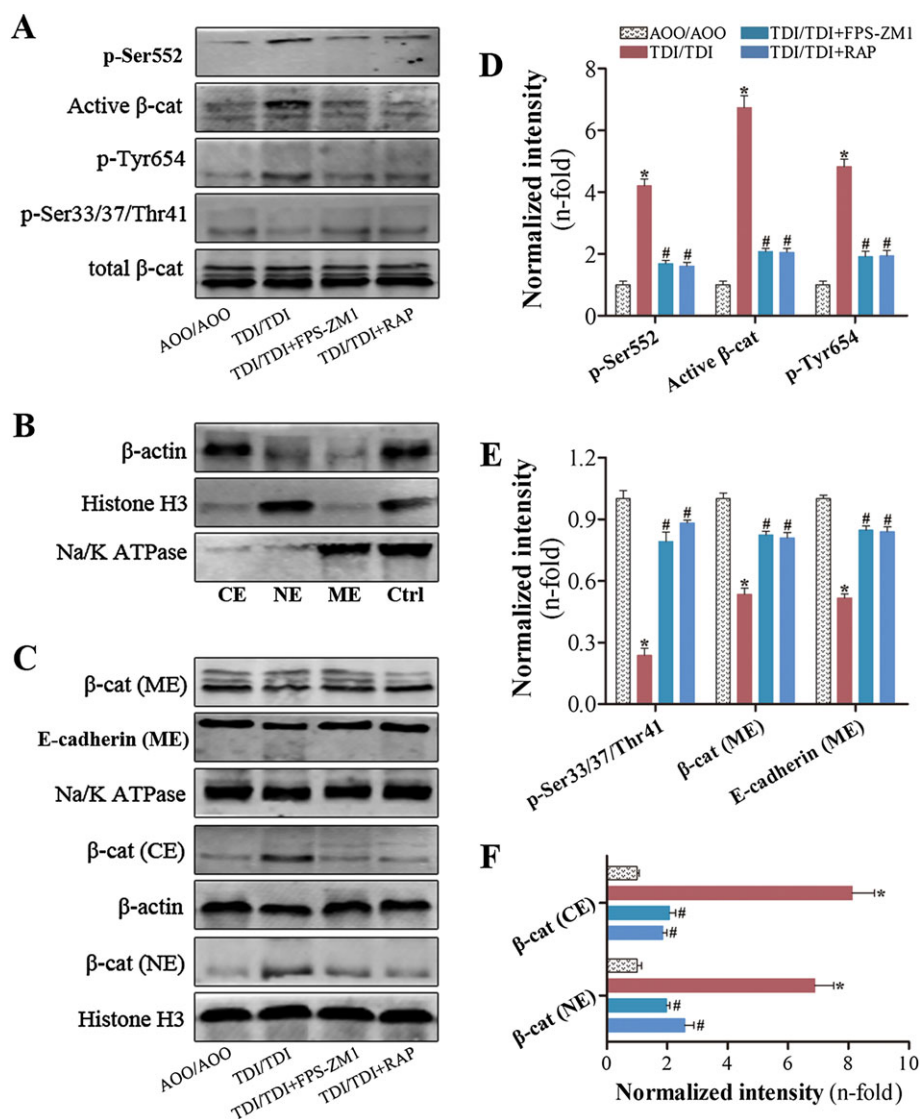


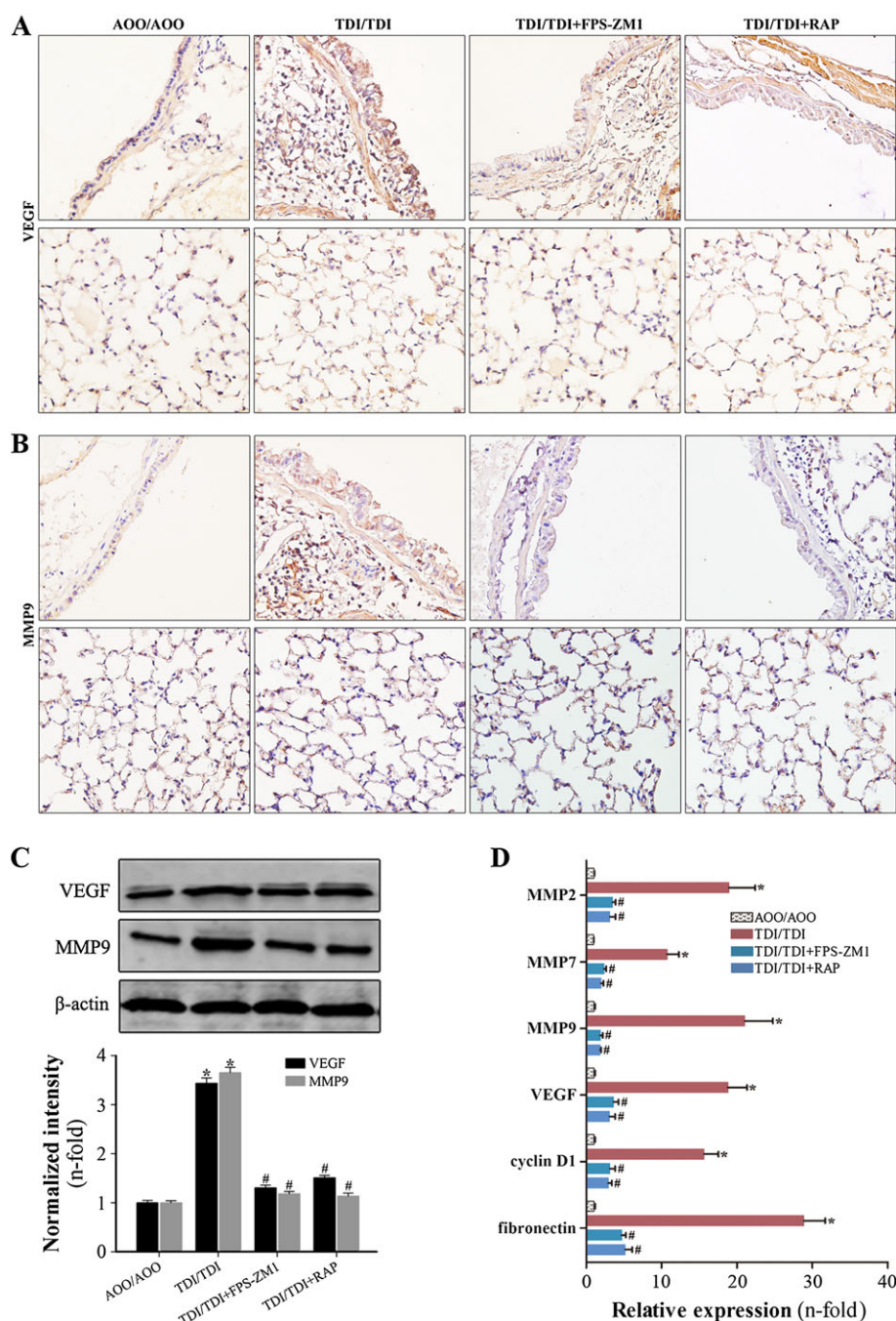
**Figure 6**

Effects of RAGE inhibition on subcellular location and phosphorylation of  $\beta$ -catenin. (A) Western blot analysis of active  $\beta$ -catenin, phospho- $\beta$ -catenin (Tyr<sup>654</sup>, Ser<sup>552</sup>) and phospho- $\beta$ -catenin (Ser<sup>33/37</sup>/Thr<sup>41</sup>) in whole lung homogenates. (B) Purity of membrane extracts (MEs), cytoplasmic extracts (CEs) and nuclear extracts (NEs) was confirmed by immunoblotting with antibodies against  $\beta$ -actin, Histone H3 and Na/K ATPase. Lysates of whole lung were used as positive control (Ctrl). (C)  $\beta$ -catenin level in the MEs, CE and NEs, as well as membrane E-cadherin of all groups. (D–F) Densitometric analysis of the immunoreactive bands in A and C ( $n = 6$ ). \* $P < 0.05$ , significantly different from the AOO/AOO group; # $P < 0.05$ , significantly different from the TDI/TDI group.

RAGE. Either of them can act as a decoy, binding specifically to the V domain of RAGE and therefore interfering with the binding of various RAGE ligands and thus they can inhibit subsequent signalling pathways. *In vivo* and *in vitro* studies have already verified the safety and effectiveness of these two antagonists in other disease models (Arumugam *et al.*, 2012; Carnevale *et al.*, 2012; Deane *et al.*, 2012; Yue *et al.*, 2015). Both are non-toxic and safe at the doses used in this study. Treatment with either FPS-ZM1 or RAP not only attenuated TDI-induced airway inflammation and hyperresponsiveness but also reduced total, cytoplasmic and nuclear levels of  $\beta$ -catenin, enhanced  $\beta$ -catenin phosphorylation at Ser<sup>33/37</sup>/Thr<sup>41</sup>, which triggers ubiquitination, down-

regulated expression of  $\beta$ -catenin targeted genes, and tended to keep  $\beta$ -catenin at the cytomembrane, shifting  $\beta$ -catenin from a signalling active pattern to an adhesive function. This was in agreement with what was found in endothelial cells (Xiong *et al.*, 2011), but in disagreement with the data of Li *et al.* in osteoblastic cells, where there was a reduction of total  $\beta$ -catenin level in response to Wnt3a-CM after RAGE overexpression (Li *et al.*, 2012). This could be attributed to the different cell types studied.

Our results suggest that RAGE contributes to both  $\beta$ -catenin signalling activation and to airway inflammation in TDI asthmatic mice, although it is not responsible for the activation of  $\beta$ -catenin in TDI-challenged mice, without prior



## Figure 7

Effects of RAGE inhibition on expression of VEGF, MMP9 and other  $\beta$ -catenin targeted genes. Representative immunohistochemical staining of VEGF (A) and MMP9 (B) as well as Western blot analysis of whole lung extracts (C,  $n = 6$ ) showed that both were up-regulated after TDI sensitization and challenge, but were then inhibited by treatment with FPS-ZM1 or RAP. Original magnification of immunohistochemistry was 400 $\times$  ( $n = 8$ ). Figure 7C shares the same loading control blot ( $\beta$ -actin) with Figure 4B. (D) Quantitative PCR was performed using lung RNA extracts. The significantly increased expression of MMP2, MMP7, MMP9, VEGF, cyclin D1 and fibronectin was inhibited by RAGE blockade with FPS-ZM1 or RAP ( $n = 6$ ). \* $P < 0.05$ , significantly different from the AOO/AOO group; # $P < 0.05$ , significantly different from the TDI/TDI group.

sensitization. Although currently there is little evidence for a direct relationship between  $\beta$ -catenin stabilization and inflammation, there is substantial literature demonstrating that some of the downstream genes of  $\beta$ -catenin are closely related to allergic inflammation in the lung. VEGF, for instance, has long been confirmed to contribute critically to allergen

induced airway inflammation, airway remodelling and Th2 inflammation (Lee *et al.*, 2004; Takyar *et al.*, 2013). Similarly, MMP9, as a potent chemotactic factor that mediates transmigration of neutrophils and eosinophils (Demedts *et al.*, 2005), occupies a pivotal role in TDI-induced airway inflammation (Lee *et al.*, 2003). In our study, their mRNA and protein

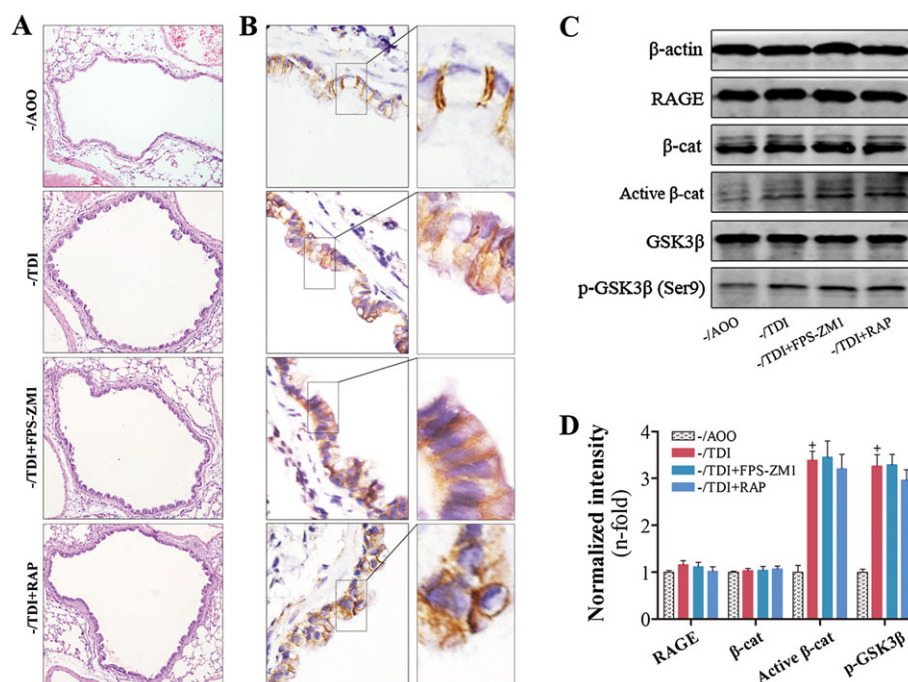


**Figure 8**

Effects of RAGE inhibition on  $\beta$ -catenin signalling in lungs of non-sensitized mice challenged with TDI. (A) Representative H&E-stained lung sections showed FPS-ZM1, and RAP had no effects on epithelial responses to TDI challenge. Images were at 200 $\times$  magnification. (B) Representative immunohistochemical staining of  $\beta$ -catenin showed TDI challenge without sensitization induced breached expression in cell–cell contacts and diffused expression in the cytosol and nucleus. RAGE blockade had no effect on this redistribution of  $\beta$ -catenin. Images were at 1000 $\times$  magnification. (C) Western blot analysis of whole lung homogenates to quantify pulmonary expression of RAGE,  $\beta$ -catenin, active  $\beta$ -catenin and p-GSK3 $\beta$  and subsequent densitometric analysis (D) ( $n = 6$ ).  $+P < 0.05$ , significantly different from the -/AOO group.

expression were both up-regulated by TDI and then inhibited by RAGE antagonists, helping to bridge the gap between stabilization of  $\beta$ -catenin and inflammation. However, the mechanisms by which RAGE regulates  $\beta$ -catenin are still open to speculation.

It is well established that RAGE engagement could activate a complex array of signalling pathways depending on the ligand, cell and tissue micro-environment (Schmidt *et al.*, 2001). Blockade of RAGE would therefore interfere with downstream signals. Here, we found that RAGE inhibition modified the phosphorylation of GSK3 $\beta$  (Ser<sup>9</sup>) in lung. Unlike most kinases, GSK3 $\beta$  is constitutively active in its unphosphorylated form. Phosphorylation of GSK3 $\beta$  at Ser<sup>9</sup> inhibits its activity and consequently induces perturbation of the ubiquitin-proteasome system, leading to protein stability (Kim and Kimmel, 2000). Increased phosphorylation of GSK3 $\beta$  (Ser<sup>9</sup>) following RAGE ligation in vascular smooth muscle cells was reported by Toure *et al.*, (2012). Similarly, inhibited phosphorylation of GSK3 $\beta$  (Ser<sup>9</sup>) in response to TDI was seen after RAGE blockade in our study. As an important member of the destruction complex of  $\beta$ -catenin, GSK3 $\beta$  captures  $\beta$ -catenin for proteasomal degradation by phosphorylating it at Ser<sup>33/37</sup> and Thr<sup>41</sup> (Doble and Woodgett, 2003; Clevers and Nusse, 2012; Anastas and Moon, 2013). Therefore, when GSK3 $\beta$  is phosphorylated, active  $\beta$ -catenin (not phosphorylated at Ser<sup>33/37</sup>/Thr<sup>41</sup>) was detected. Our data suggest that RAGE could increase  $\beta$ -catenin stabilization through modulation of GSK3 $\beta$  activity.

PI3K/Akt is another well-documented downstream pathway of RAGE (Sukkar *et al.*, 2012; Xie *et al.*, 2013). Interestingly, Akt can phosphorylate GSK3 (both  $\alpha$  and  $\beta$  isoforms) to turn off the activity of this enzyme (Cantley, 2002), leading to increased level of  $\beta$ -catenin. We have demonstrated in our previous study that PI3K/Akt could facilitate GSK3 $\beta$  phosphorylation at Ser<sup>9</sup> and induce intracellular diffusion of  $\beta$ -catenin (Yao *et al.*, 2015). Here, we observed blunted Akt activity after RAGE inhibition, implying the possibility of activation of  $\beta$ -catenin through a RAGE/PI3K/Akt/GSK3 $\beta$ -dependent process. In addition, Akt can directly phosphorylate  $\beta$ -catenin at Ser<sup>552</sup> to promote its nuclear translocation and transcriptional activity (Fang *et al.*, 2007). So, we also detected p- $\beta$ -catenin (Ser<sup>552</sup>). As expected, the protein change of p- $\beta$ -catenin (Ser<sup>552</sup>) agreed with that of p-Akt (Ser<sup>473</sup>), suggesting another possibility of RAGE/Akt-mediated  $\beta$ -catenin translocation.

Adhesive  $\beta$ -catenin might also contribute to its signalling pool when epithelial integrity is impaired. As an important member of adherent junctions,  $\beta$ -catenin links E-cadherin, whose extracellular domain forms homotypic calcium-dependent adhesions between epithelial cells, to  $\alpha$ -catenin to connect the actin cytoskeleton (Nawijn *et al.*, 2011). So cadherins function as a membrane trap for a part of free  $\beta$ -catenin. Loss of cadherin-mediated cell adhesion can promote  $\beta$ -catenin release, thus favouring  $\beta$ -catenin to act in a signalling role (Heuberger and Birchmeier, 2010). Besides, binding of  $\beta$ -catenin to E-cadherin is controlled by phosphorylation of  $\beta$ -catenin at Tyr<sup>654</sup> (Nelson and Nusse, 2004).

Phosphorylation of  $\beta$ -catenin at Tyr<sup>654</sup> will interfere with its binding to the cytoplasmic domain of E-cadherin (Taddei *et al.*, 2002; Brembeck *et al.*, 2006). In the present study, coupled with loss of junctional  $\beta$ -catenin, TDI also induced a decreased level of E-cadherin in the membrane, as well as increasing phosphorylation of  $\beta$ -catenin at Tyr<sup>654</sup>. Both effects were reversed by RAGE inhibition. These results indicate that RAGE might promote  $\beta$ -catenin activation through interruption of E-cadherin and its adhesive pool.

In conclusion, our results demonstrated that RAGE is required for  $\beta$ -catenin stabilization in TDI-induced asthma. Our findings suggest that RAGE antagonists should be assessed for potential therapeutic use in the prevention and treatment of TDI-induced airway inflammation, which has important implications for patients with TDI-induced 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 analysed the data. L.J., 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.

## Conflict of interest

The authors declare no conflicts of interest.

## Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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