The role of E-cadherin/β-catenin signalling in the development of an asthmatic airway epithelial phenotype

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CHAPTER 6

Inhibition of β-catenin/CREB binding protein signaling attenuates house dust mite-induced goblet cell metaplasia in mice

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Abstract

Excessive mucus production is a major feature of allergic asthma. Disruption of epithelial junctions by allergens such as house dust mite (HDM) results in the activation of β-catenin signaling, which has been reported to stimulate goblet cell differentiation. β-catenin interacts with various co-activators including CREB binding protein (CBP) and p300, thereby regulating the expression of genes involved in cell proliferation and differentiation, respectively. We specifically investigated the role of the β-catenin/CBP signaling pathway in goblet cell metaplasia in a HDM-induced allergic airway disease model in mice using a small molecule inhibitor ICG-001. Female 6-8-week-old BALB/c mice were sensitized to HDM/saline on days 0, 1 and 2, followed by intranasal challenge with HDM/saline with or without subcutaneous ICG-001/vehicle treatment from days 14-17, and samples harvested 24 hours after the last challenge/treatment. Differential inflammatory cells in bronchoalveolar lavage (BAL) fluid were enumerated. Periodic acid–Schiff (PAS)/alcian blue (AB) staining was used to identify goblet cells/mucus production, and airway hyperresponsiveness (AHR) was assessed using invasive plethysmography. Exposure to HDM induced airway inflammation, goblet cell metaplasia and increased AHR, with increased airway resistance in response to the non-specific spasmogen methacholine. Inhibition of the β-catenin/CBP pathway using treatment with ICG-001 significantly attenuated the HDM-induced goblet cell metaplasia and infiltration of macrophages, but had no effect on eosinophils, neutrophils, lymphocytes or AHR. Increased β-catenin/CBP signaling may promote HDM-induced goblet cell metaplasia in mice.

Key words: asthma, β-catenin, goblet cell metaplasia, ICG-001

Introduction

Asthma is broadly characterized by chronic inflammation and remodeling of the airways, excessive airway mucus production, and reversible airflow obstruction with loss of lung function (1). The airway epithelium is the first line of defense against inhaled allergens and is more susceptible to damage by allergens such as house dust mite (HDM) in asthma. This results in the deterioration of cellular junctions (2), increased epithelial growth factor receptor (EGFR) signaling (3), extracellular matrix proteins (4) and expression of mesenchymal markers such as α-smooth muscle actin (α-SMA), fibronectin and vimentin (5), and airway hyperresponsiveness (AHR) (4,6). Damaged epithelial cells release pro-inflammatory

cytokines like Chemokine (C-C motif) ligand 20 (CCL20), CCL17, interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP), which activate and attract immune cells like dendritic cells (DCs) and macrophages, promoting the differentiation of type-2 cells (3,7,8). T helper 2 (Th2) cells and innate lymphoid cells (ILCs) release various cytokines including IL-4, IL-5 and IL-13, which activate B-lymphocytes, induce the infiltration of eosinophils, and enhance the differentiation of goblet cells (9–11). Besides IL-13, EGF also enhances goblet cell differentiation (12,13). Goblet cells are secretory cells that produce mucus, which consists of polypeptides, water, DNA, enzymes and high molecular weight glycoproteins called mucins (14). MUC5AC and MUC5B are the major mucins secreted by goblet cells, and their expression is increased in asthma and is associated with goblet cell metaplasia (15,16). MUC5AC production is regulated by the transcription factors SAM pointed domain containing ETS transcription factor (SPDEF) and forkhead box a2 (FOXA2), which activate and repress the expression of *MUC5AC*, respectively (15,17). In the asthmatic airway, goblet cell hyperplasia is accompanied by loss of ciliated cells concomitant with higher expression of basal cell markers cytokeratin 5 and p63 compared to healthy individuals, resembling undifferentiated epithelium undergoing aberrant repair responses that is unable to re-differentiate into a pseudostratified layer (18).

Adherens junctions, which mainly constitute the transmembrane protein E-cadherin, help in maintaining the integrity and function of the airway epithelium. Reduced expression of E-cadherin has been observed in airway epithelial cells from asthmatic donors along with decreased epithelial barrier function (19). We have previously shown that allergens like HDM disrupt E-cadherin at the cell junctions and induce goblet cell metaplasia in mice (20). In mice, embryonic knock-out of E-cadherin in airway epithelial cells induced club cell hyperplasia (21) and excessive mucus production (22). The loss of E-cadherin leads to the delocalization of its intracellular binding partner β-catenin into the cytoplasm, where its levels are regulated by a destruction complex consisting of axin, adenomatous polyposis coli APC and GSK-3β. Activation of Wnt and/or growth factor signaling prevents phosphorylation and degradation of β-catenin by the destruction complex in the cytoplasm. The non-phosphorylated β-catenin, which is the transcriptionally active form of β-catenin, translocates to the nucleus and acts as a transcription factor to regulate the expression of various genes involved in cellular development and regulate cell fate decisions (23). Increased transcriptional activity of β-catenin has been shown to induce goblet cell

metaplasia in mice (24). The regulation of a variety of genes by β-catenin is possible because of its ability to bind to various transcriptional coactivators including CREB binding protein (CBP) and p300 leading to divergent cellular processes of cell migration and proliferation versus differentiation, respectively (25). Specifically, β-catenin/CBP signaling has been shown to regulate epithelial-to-mesenchymal transition of primary airway epithelial cells, leading to loss of epithelial markers such as E-cadherin, possibly through transcriptional regulation of E-cadherin repressors such as SNAIL and SLUG in addition to EGFR, whose downstream signaling results in the loss of E-cadherin (26,27). In addition, inhibition of β-catenin/CBP pathway by the small molecule inhibitor ICG-001 improved airway epithelial barrier function *in vitro* upon Ca^{+2} signaling-induced damage by stabilizing E-cadherin at cell junctions (28). We hypothesized that inhibition of the β-catenin/CBP pathway upon HDM-induced E-cadherin loss using ICG-001 would attenuate HDM-induced goblet cell metaplasia and AHR in a mouse model of allergic asthma.

Materials and Methods

Mice

6-8 week-old female BALB/c mice were obtained from the University of Newcastle's central animal house and housed at the Hunter Medical Research Institute animal facility in individually ventilated cages in an SPF PC2 facility. Mice were provided standard rodent chow *ad libitum*. All protocols were approved by the University of Newcastle Animal Care and Ethics Committee.

HDM model of allergic airway disease

Mice (n=6-8/group) were sensitized to HDM (*Dermatophagoides pteronyssinus*) extract (intranasal (i.n.): days 0, 1 and 2; 50 µg; Greer Labs, North Carolina, USA) in sterile saline (50 µl) and challenged with HDM (i.n.: day 14–17; 5 µg in 50 µl saline) with/without ICG-001 (subcutaneous: day 14–17; 5mg/Kg) under isoflurane anesthesia and euthanized on day 18 (Figure 1A) as previously described (6,29).

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from lung homogenates using TRIZOL (Sigma, Missouri, USA) as previously described (30–32). Synthesis of cDNA was performed using high-capacity cDNA reverse transcription kit with RNase inhibitor (Thermo Fisher Scientific,

Massachusetts, USA) and T100 Thermal Cycler (Bio-Rad Laboratories, California, USA). Quantitative real time PCR (qRT-PCR) targeting *Cdh1* (Mm01247357_m1, Thermo Fisher Scientific), *Muc5ac* (Mm01276718_m1, Thermo Fisher Scientific), *Muc5b* (Mm00466391_m1, Thermo Fisher Scientific), *Spdef* (Hs00171942_m1, Thermo Fisher Scientific), and *Foxa2* (Mm01976556_s1, Thermo Fisher Scientific) was performed on individual biological replicates, which were normalized to *Hprt* (Mm03024075_m1, Thermo Fisher Scientific) and presented as gene expression ($2^{-\Delta Ct}$) as previously described (33).

Histological analysis

Lung sections of 5 µm thickness were obtained for all the experimental mice and stained for goblet cells/mucins using standard Alcian blue pH 2.5 (AB) followed by periodic acid and Schiff's reagent (PAS) as previously described (30,34,35). A minimum of 8 different airways per section were imaged at 10x magnification and quantified using color deconvolution algorithm in ImageJ (National institute of Health) as previously described (30,34,35). Each data point represents the percentage of total staining intensity of mucus in the whole section per total area of the airway epithelial cells in that section. After importing the AB/PAS staining image of the section into ImageJ, the airway epithelial cells were selected by drawing a region of interest (ROI) using the 'free hand selection tool'. The area outside the airway(s) was deleted using 'clear outside' function. The 'Threshold color' was adjusted to select all airway epithelial cells and the total area of the airway epithelial cells was measured using the 'Measure' function, which was exported to an excel file. Next, by selecting the "color deconvolution" function with H-PAS vector, the image was split into three different color channels. The blue/magenta color channel was selected to quantify the AB/PAS staining intensity using the 'Threshold' function. By changing the value of the 'threshold' function, the AB/PAS staining area was selected. Setting a very high threshold value results in an increase of incorrect selection of unstained areas in the image and setting a very low threshold may not sufficiently select the AB/PAS staining area. So, the threshold was manually adjusted to a value which best covers the whole area of the AB/PAS staining. Importantly, the same threshold value was used for the selection of AB/PAS staining for all the images for consistency. After selecting the area of AB/PAS staining, the image was 'flattened' and 'inverted'. The area of the AB/PAS staining was selected using 'Threshold color' and the staining intensity was measured using 'Measure' function, which were also exported to the excel file. All the values of AB/PAS staining intensities and area of airway epithelial cells

from different airways of a single image section were separately added to get the total AB/PAS staining intensity and total area of airway epithelial cells, respectively. Finally, the % AB/PAS staining was calculated using the following equation: (total AB/PAS staining intensity/total area of airway epithelial cells) x 100%. Each data point represents the % AB/PAS staining of mucins per total area of the airway epithelial cells in a section from a single mouse.

Immune cell quantification

BAL fluid (2 ml) was prepared, and total cell numbers were determined with a hemocytometer. Cells prepared by cytocentrifugation (Shandon Cytospin; Thermo Fisher Scientific, Waltham, MA) were stained with May-Grünwald-Giemsa and leukocytes were enumerated on the basis of morphologic criteria (200 cells by light microscopy [×40]) as previously described (30,36,37). Further, eosinophils, neutrophils, lymphocytes and macrophages were calculated and represented as the percentage of the total leukocytes in BAL fluid.

Airway hyperresponsiveness (AHR)

Mice were anesthetized (ketamine and xylazine [80–100 and 10 mg/kg, respectively]; Troy Laboratories, Smithfield, Australia) and the tracheas were cannulated. Each cannula was connected to an inline aerosol administrator and ventilator, which were attached to a preamplifier and computer (Buxco, Wilmington, NC) to analyze pressure and flow waveforms and to determine airway resistance and dynamic compliance. Mice were nebulized with saline followed by increasing doses of methacholine (Sigma) as previously described (30,32,38).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (Graphpad software, San Diego, USA). Non-parametric Mann–Whitney U test was performed to assess for significant differences in gene expression, staining quantification, and the infiltration of immune cells between different groups. For AHR, two-way analysis of variance (ANOVA) was used to compare different groups and multiple comparisons were done by uncorrected Dunn's test. $P < 0.05$ was considered statistically significant. Outliers were identified by Grubb's test. We detected one outlier in the HDM + ICG-001 group for the *Spdef* gene expression analysis

(Figure 1F) and one outlier in the HDM + ICG-001 group for the AB/PAS staining quantification (Figure 1H) and removed these from the specific analyses. In addition, we also detected and removed one outlier each in the Saline + ICG-001 group for the measurement of total leucocytes, eosinophils, neutrophils, lymphocytes, and macrophages (Figure 2A-E and Figure S1A-E), which are from a single mouse.

Results

ICG-001 decreases HDM-induced goblet cell metaplasia in vivo

Sensitization of mice for 3 days from day 0 to 3 followed by exposure to HDM for 4 days from day 14-17 showed a strong trend (p=0.06) towards a decrease in the mRNA expression of E-cadherin (*Cdh1*) compared to PBS-sensitized and PBS-challenged mice. In mice treated with ICG-001, no differences were observed in the *Cdh1* mRNA expression levels after HDM exposure (Figure 1B). HDM significantly increased the mRNA expression of secretory mucins *Muc5ac* and *Muc5b* (Figure 1C, D). Treatment of mice with ICG-001 resulted in a trend (p=0.09) towards the suppression of HDM-induced mRNA expression of *Muc5ac*, but had no effect on *Muc5b* expression (Figure 1C, D). The *Muc5ac* transcriptional activator *Spdef* was significantly increased (Figure 1E) and the *Muc5ac* transcriptional repressor *Foxa2* was significantly decreased (Figure 1F) following HDM exposure, which was not affected by ICG-001 treatment (Figure 1E, F). The increase in *Muc5ac* and *Muc5b* expression was validated at the protein level by AB/PAS staining, revealing that HDM exposure promoted goblet cell metaplasia and mucus production. Notably, HDM-induced goblet cell metaplasia and mucus production was significantly repressed by ICG-001 treatment (Figure 1G, H).

ICG-001 inhibits HDM-induced infiltration of macrophages, but does not have an effect on HDM-induced AHR

Airway inflammation with increases in leukocytes and notably eosinophils is a hallmark of the HDM- induced asthma model in mice. HDM significantly increased the total leukocyte counts. The majority of leukocytes in the BAL fluid of PBS exposed mice were macrophages (98.3 ± 1.6 %) and the remaining fraction included eosinophils, neutrophils and lymphocytes (Figure S1A-E). Exposure to HDM significantly increased the total number of eosinophils, neutrophils, lymphocytes, and to a lesser extent, macrophages in BAL fluid (Figure 2A-E), resulting in a decrease in the percentage of macrophages (Figure S1E). ICG-001 did not have

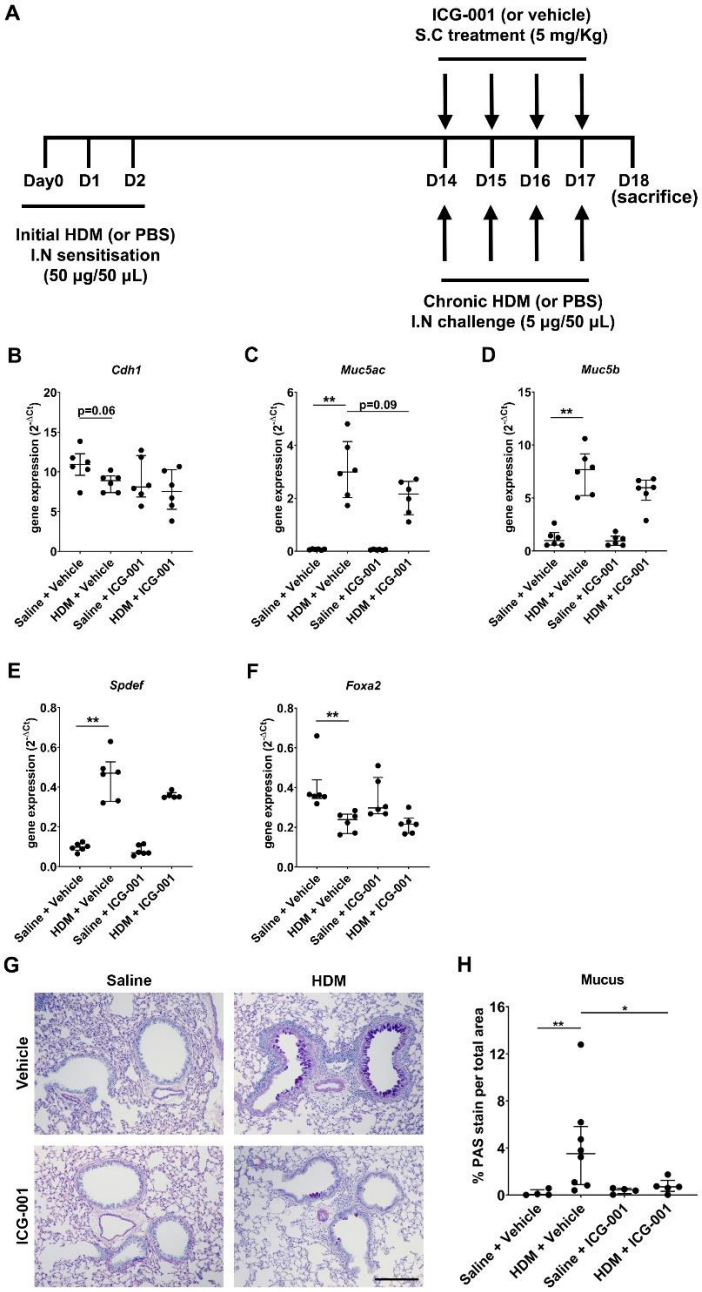

Figure 1: ICG-001 decreases HDM-induced goblet cell metaplasia. (A) HDM-induced allergic airway disease model in mice treated with the ICG-001 inhibitor. **(B-F)** Gene expression of *Cdh1*, *Muc5ac*, *Muc5b*, *Spdef* and *Foxa2* normalized to *Hprt*. **(G)** Alcian Blue (AB)/ Periodic acid–Schiff (PAS) staining of lung sections from mice for mucus, scale = 100 µm. **(H)** Semi-quantification of AB-PAS staining for mucus using color deconvolution. Data is presented as median ± IQR; n=4-8, *p<0.05, **p<0.01, Mann–Whitney U test.

any effect on HDM-induced increases in the absolute number of total leukocytes including eosinophils, neutrophils, and lymphocytes (Figure 2A-D). ICG-001 treatment did result in a significant reduction in HDM-induced macrophages (Figure 2E). Methacholine induced a dose-dependent increase in airway resistance, which was significantly elevated by HDM sensitization and challenge (Figure 2F). In line with the lack of effect on inflammation, ICG-001 treatment did not suppress HDM-induced increase in the airway resistance (Figure 2F).

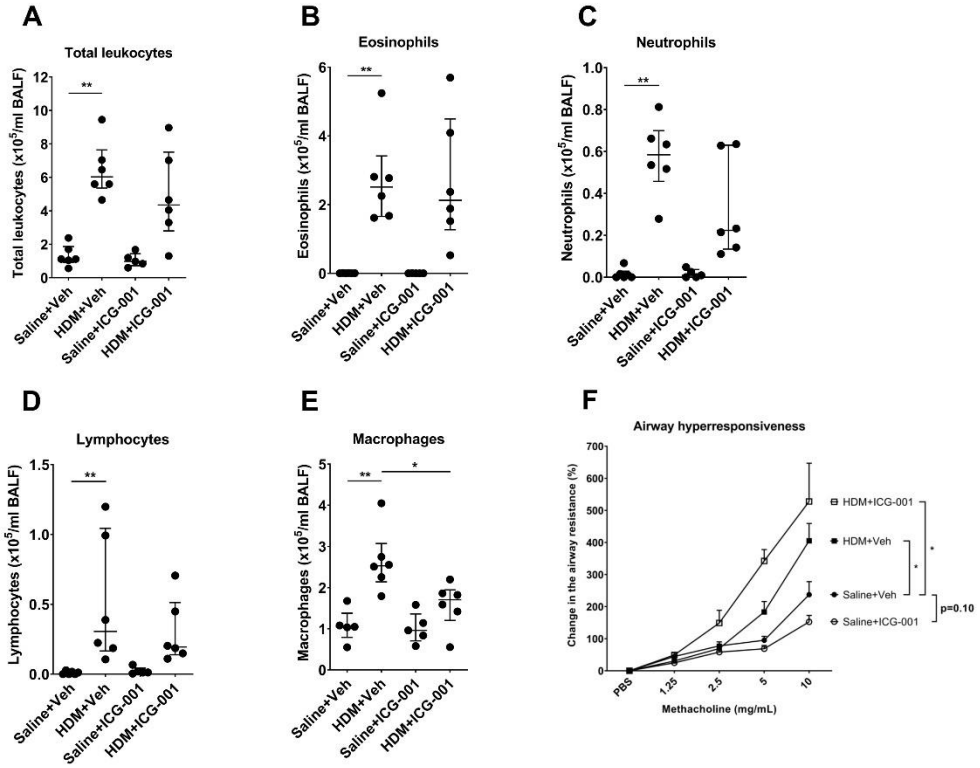

Figure 2: ***ICG-001 inhibits HDM-induced infiltration of macrophages, but does not have an effect on HDM-induced AHR*****.** Numbers of **(A)** total leukocytes, **(B)** eosinophils, **(C)** neutrophils, **(D)** lymphocytes, and **(E)** macrophages present in BAL fluid of mice. Data is presented as median ± IQR; *p<0.05, **p<0.01, Mann–Whitney U test. **(F)** Change in the methacholine-induced airway resistance (%) in mice following exposure to HDM and treated with ICG-001 or vehicle-treated controls. Data is presented as median ± IQR; n=5-6, *p<0.05, two-way ANOVA.

Discussion

In this study, we investigated the role of β-catenin/CBP signaling in allergen-induced manifestations of asthma. HDM exposure showed a trend towards a decrease in E-cadherin

expression, which was accompanied by increases in *Muc5ac*, *Muc5b* and *Spdef* expression, decreased *Foxa2* expression, the induction of goblet cell metaplasia and the infiltration of inflammatory cells, thereby representing the phenotype of asthmatic airway epithelium. Specific inhibition of β-catenin/CBP signaling using ICG-001 significantly decreased goblet cell metaplasia and mucus production. This effect of ICG-001 on mucus production was in line with a trend towards a decrease in HDM-induced *Muc5ac* expression with ICG-001 treatment, which likely failed to reach statistical significance due to high variation between mice. Our findings are in line with a previous study in which ICG-001 was shown to inhibit goblet cell metaplasia in a toluene diisocyanate treated mouse model of asthma (39). Also, we have recently shown that ICG-001 significantly inhibited IL-13-induced MUC5AC gene expression and production in an air-liquid interface (ALI) culture model of human primary bronchial epithelial cells (Chapter 5), which was accompanied by higher *FOXA2* expression, while leaving *SPDEF* expression unaffected. The discrepancy of the effects of ICG-001 on *MUC5AC* expression between *in vitro* and *in vivo* models could be due to differences in the cellular composition of airway epithelium between human (our *in vitro* data) and mice (this study). Indeed, lineage tracing studies revealed that IL-13-induced goblet cells were derived from FOXJ1-expressing ciliated cells in human primary airway epithelial cells in ALI (40), but this was not demonstrated *in vivo* in an Ovalbumin-induced mouse model of allergic airway disease (41). Newly differentiated goblet cells generated in response to HDM in mice may originate predominantly from club cells that are present in the pseudostratified layer of airway epithelium (42). Also, RNA analysis of lung tissue exhibits greater within-group variance than PAS staining, as the former is dependent on the relative proportion of RNA coming from the airway epithelial cells in the total lung tissue RNA pool. Furthermore, the design of our study may explain the discrepancy between the effect of ICG-001 on HDM-induced Muc5ac expression and production. We treated mice with ICG-001 after the initial HDM sensitization and we cannot be sure that the changes in *Muc5ac* gene expression were already induced during the initial sensitization phase.

ICG-001 treatment also had no effect on the gene expression of *Foxa2*, which was previously identified to be regulated by the β-catenin pathway (24). Of note, *Foxa2* is a transcriptional activator of E-cadherin (43,44), and the HDM-induced downregulation of *Foxa2* may have resulted in reduced expression of E-cadherin. In line with the lack of effect on *Foxa2*, we did not observe an effect of ICG-001 on E-cadherin expression. ICG-001 was able to

significantly decrease the HDM-induced infiltration of macrophages, suggesting the important role of β-catenin/CBP signaling in HDM-induced pro-inflammatory responses in mice. Indeed, we have previously shown that ICG-001 inhibits the HDM-induced granulocyte macrophage-colony stimulating factor (GM-CSF) (28), a cytokine that stimulates the production of macrophages. Alternatively, ICG-001 was not able to prevent the infiltration of eosinophils, neutrophils, and lymphocytes. This is in contrast to our *in vitro* data where we showed that ICG-001 reduced HDM-induced CCL20 levels in primary bronchial epithelial cells (28), as neutrophils and T cells (predominantly Th17) are known to express CCL20 receptor CCR6. Moreover, ICG-001 was previously shown to be able to significantly decrease eosinophil and neutrophil influx into the airways induced by toluene diisocyanate in mice (39). These contrasting findings could be due to the difference in the mechanism of action of HDM and toluene diisocyanate. HDM activates pathogen recognition receptors (PRRs) like dectin-1 and Toll-like receptor (TLR)-4 resulting in the release of pro-inflammatory cytokines like CCL17, CCL20, IL-5 and IL-13 (45,46), whereas toluene diisocyanate acts on transient receptor potential melastatin 8 (TRPM8) resulting in the release of IL-25, IL-4 and IL-13 (47). This suggests that TRPM8, but not PRR activation is regulated by β-catenin/CBP signaling. As HDM is a major allergen responsible for airway inflammation in Type-2 driven, atopic asthma, our model may be mainly relevant for better understanding of the role of β-catenin/CBP signaling in allergic airway inflammation.

Here, we show that ICG-001 attenuates HDM-induced goblet cell metaplasia independent of HDM-induced airway inflammation. Inhibition of β-catenin/CBP pathway could be an alternative strategy to regulate mucus hypersecretion in asthma. Future studies should be directed towards more targeted delivery of the ICG-001 to specific airway epithelial cell types for increased efficiency of the drug.

Conflicts of interest

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Supplementary Data

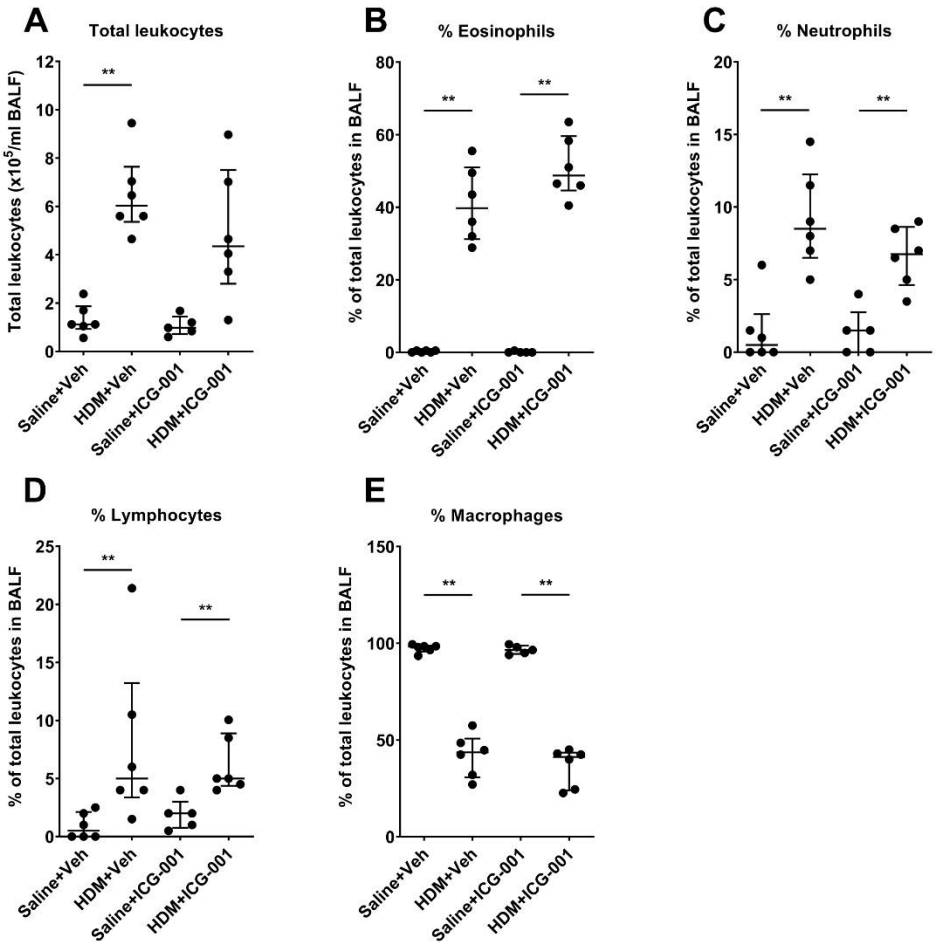

Figure S1: Effect of ICG-001 on HDM-induced airway inflammation and AHR in mice. Numbers of (A) total leukocytes, and percentage of (B) eosinophils, (C) neutrophils, (D) lymphocytes, and (E) macrophages present in BAL fluid of mice. Data is presented as median ± IQR; n=5-6, **p<0.01, Mann–Whitney U test.