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Chenodeoxycholic acid attenuates ovalbumin-induced airway inflammation in murine model of asthma by inhibiting the T_H2 cytokines

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ABSTRACT

Asthma is a complex highly prevalent airway disease that is a major public health problem for which current treatment options are inadequate. Recently, farnesoid X receptor (FXR) has been shown to exert anti-inflammatory actions in various disease conditions, but there have been no reported investigations of Chenodeoxycholic acid (CDCA), a natural FXR agonist, in allergic airway inflammation. To test the CDCA effectiveness in airway inflammation, ovalbumin (OVA)-induced acute murine asthma model was established. We found that lung tissue express FXR and CDCA administration reduced the severity of the murine allergic airway disease as assessed by pathological and molecular markers associated with the disease. CDCA treatment resulted in fewer infiltrations of cells into the airspace and peribronchial areas, and decreased goblet cell hyperplasia, mucus secretion and serum IgE levels which was increased in mice with OVA-induced allergic asthma. The CDCA treatment further blocked the secretion of T_H2 cytokines (IL-4, IL-5 and IL-13) and proinflammatory cytokine TNF-α indicate that the FXR and its agonists may have potential for treating allergic asthma.

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1. Introduction

Asthma is a disease characterized by reversible airway obstruction, airway inflammation, that results in narrowing of airways leading to shortness of breath, chest tightness, coughing and wheezing. Prominent characteristics of allergic asthma is overproduction of antigen-specific IgE, extreme immunological responses, and consequent inflammation and clinical symptoms include excessive mucus secretion and bronchospasm [1]. Asthma may affect nearly 334 million people all-over the world according to the Global asthma report 2014, prevalence continuing to increase with tremendous socioeconomic impact in developing countries. Current medications targeting specific and crucial pathways have shown some beneficial effects, but the standard therapy is continuous use of potent anti-inflammatory glucocorticoids, but associated side effects limit enthusiasm for their use [2]. Furthermore, they fail to suppress significant aspects of asthma pathology and are ineffective in some patients [3,4], hence alternative, efficacious and safe anti-inflammatory strategies are required to combat this disease.

Nuclear receptors with anti-inflammatory properties have recently begun to attract attention to develop potential novel treatments, for better understanding and management of asthma [5,6]. Several nuclear hormone receptors, including peroxisome proliferator-activated receptor-γ (PPAR-γ) and farnesoid X receptor (FXR), have been shown to exert anti-inflammatory and anti-fibrotic activity [7,8]. The role of PPAR-γ in asthma and its potential as a therapeutic target is currently under active investigation, but there have been no reported investigations of FXR in this context. We accordingly propose to study the ability of FXR activation to reduce airway inflammation in a murine model of asthma generated by ovalbumin (OVA).

FXR is mainly expressed in liver, intestine, kidney and adrenal glands [9,10]. Recently, it is also found to be expressed in endothelial cells of lungs [11]. Chenodeoxycholic acid (CDCA), lithocholic acid, deoxycholic acid, and ursodeoxycholic acid (UDCA) are the natural FXR ligands [12–17]. FXR acts as a multipurpose

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nuclear receptor as it plays crucial roles in controlling bile acid homeostasis, lipoprotein and glucose metabolism. Thus its agonists proved useful in the treatment of various disorders including inflammation [10,18]. FXR has been proved to down-regulate the genes involved in inflammation. $FXR^{-/-}$ mice studies revealed that FXR deficiency as a significant risk factor in the development of various inflammatory diseases [19,20]. To assess whether CDCA, a natural agonist of FXR, might have therapeutic potential in asthma, we tested the effects of CDCA on inflammation and asthma-relevant pathophysiological markers in a murine model of allergic airway disease.

2. Materials and methods

2.1. Animals and experimental protocol

Female Balb/C mice at 6–7 weeks of age (22–24 g), free of murine specific pathogens, were obtained from National Institute of Nutrition, Hyderabad, India, and maintained on a 12-h light and 12-h dark cycle and fed with standard rodent chow and water *ad libitum*. All procedures were approved by the institutional animal ethics committee for the care and use of laboratory animals. Mice were randomly divided into three groups, namely control, ovalbumin (OVA) and OVA-CDCA group. To induce asthma mouse was sensitised and challenged as described previously with little modifications [21]. Briefly, mice of OVA and OVA-CDCA groups were sensitized by intraperitoneal injection of 100 μg of chicken egg white OVA (Grade V, sigma, USA) adsorbed to 1 mg of adjuvant aluminium hydroxide (alum) (IMject Alum; Thermoscientific, Rockford) in 100 μl of sterile PBS to each mouse on days 0, 10 and the control group mouse were injected with 200 μl PBS alone. The response was localised to airways by intranasally, on days 19–24, the anaesthetized mice of OVA group and OVA-CDCA group were challenged with OVA by intranasally with 1% OVA in 25 μl of sterile PBS and for control group with 25 μl of sterile PBS alone (Fig. 1A). After 24 h of final OVA challenge the mice were sacrificed for measurement of various markers associated with airway inflammation. To evaluate the therapeutic effects of CDCA, OVA-CDCA group received 100 mg/kg body weight by oral gavage [22] one day before intranasal OVA challenge and continued till the end of the study. CDCA (Sigma Aldrich–India) was dissolved in 0.5% carboxymethyl cellulose (Sigma) as a vehicle.

2.2. RNA isolation and RT-PCR

Total lung RNA was extracted by using TRIZOL reagent (Sigma) according to the manufacturer's instructions [23]. RNA was quantified by measuring absorption at 260 nm and stored at −80 °C unitl use. The total RNA was reverse-transcribed to cDNA using the first-strand cDNA synthesis system (Takara Clontech, India). Then these first strand cDNAs were used for PCR amplification of FXR and GAPDH. The primers used were as follows:

FXR-forward, TGGGCTCCGAATCCTCTTAGA,
FXR-reverse, CTCGTCCGTAGCCTGTAAATGGG and
GAPDH-forward, CTGAGTATGTCGTGGAGTCTAC,
GAPDH-reverse, GTTGGTGGTGCAGGATGCATTG.

2.3. Isolation of bronchoalveolar lavage (BAL) fluid cells and counting

Twenty-four hours after final intranasal challenge, mice were anesthetized and BAL fluid was collected as described previously [24]. Briefly, the trachea was cannulated with an 18-gauge needle, and the lungs were lavaged twice with 1 ml PBS. The lavage fluid was pooled and centrifuged at 1500 rpm for 10 min at 4 °C, and stained with trypan blue, and the cells were counted using a hemocytometer. For differential counts, cells were spun onto glass slides using a cytocentrifuge and stained with Diff-Quick (Fisher Scientific International).

2.4. Measurement of total and OVA-specific IgE

After 24 h following the final OVA challenge, the blood was collected from right ventricle and centrifuged at 2500 × *g* for 10 min. The serum was then separated and stored at −80 °C until use. Concentrations of total serum IgE (BD Biosciences) and OVA-specific IgE (Cayman chemicals) levels were determined according to the manufacturer's instructions. Concentrations were calculated using a standard curve generated with the kit's IgE standard.

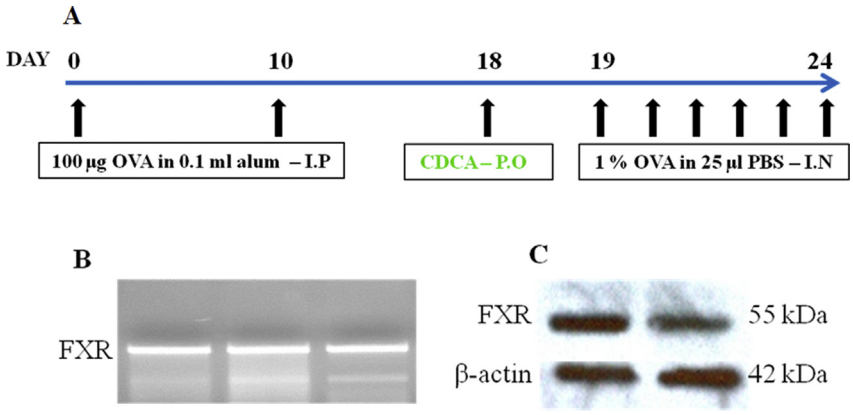

Fig. 1. A. Schematic representation of the acute ovalbumin (OVA)-induced asthma. Mice were immunized intraperitoneally (I.P) on days 0, and 10 with OVA. Intranasal (I.N) OVA challenges were administered on days 19–24. Control mice were sensitized and challenged with PBS alone. Mice were sacrificed 24 h after the final OVA challenge. Chenodeoxycholic acid (CDCA, 100 mg/kg body weight) was administered by oral gavage (P.O) starting on day 18, one day before the first I.N OVA challenge and then continued daily till the end of the study. B. Reverse transcriptase-PCR analysis of FXR mRNA of control mouse lung. C. Western blot analysis shows FXR is constitutively expressed in control mice lung at 45 kDa. Experiments were repeated three times and representative data was shown.

2.5. Histology

Lungs from euthanized animals were inflated with 10% neutral buffered formalin and fixed overnight. They were then dehydrated in 70% ethanol and embedded in paraffin and 5 μm sections were cut on a microtome and placed on a glass slides, deparaffinised and stained with Haematoxylin and Eosin. Other sections were stained with periodic acid Schiff's (PAS) which stains mucus producing goblet cells.

2.6. Western blot analyses

Equal amount of protein samples were run on a 12% SDS-PAGE and transferred to 0.2 micron nitrocellulose membrane. The membrane was then blocked with 5% non-fat dry milk powder with 0.1% Tween 20 in Tris-buffered saline (TBST), the blots were then incubated overnight with anti-FXR antibody (Santacruz -sc-13063) and anti-β-actin antibody at 4 °C at a dilution of 1:1000 and HRP-labelled secondary antibody at a dilution of 1:10,000 (Thermo-scientific-31460) at room temperature for 60 min. Both antibodies were diluted in the 5% non-fat dry milk in TBST. Corresponding secondary antibody was treated with ECL chemiluminescent substrate and emitted signal was detected with X-ray film.

2.7. Murine cytokine ELISA

Lung tissue was homogenized in 1 ml of PBS (containing 0.05% Triton X-100 and protease inhibitors) on ice for 30 s with a tissue tearor (Biospec Products, Bartlesville, OK). The homogenate was centrifuged at 10,000 × *g* for 15 min and the resulting supernatant was separated into fresh tube and stored at −80 °C until further use. The murine ELISAs were set up using standardized, specific IL-4, IL-5, IL-13 and TNF-α antibody (R&D Systems, Minneapolis, MN) that detect protein at concentrations greater than 10 pg/ml and do not crossreact with any other cytokines and final concentrations were calculated per lung using the standard plot according to the manufacture's protocol.

2.8. Statistical analysis

Each experiment was repeated three times with n = 5 animals per group. Values were expressed as means ± SEMs. Data were analyzed with the unpaired *t* tests using Prism 5 software (GraphPad Software, San Diego, CA). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Mouse lung tissue express FXR

To determine the expression of FXR in lung tissue RT-PCR and Western blot were performed. RT-PCR analysis of RNA from control lung confirmed constitutive expression of transcript for FXR (Fig. 1B). As shown in Fig. 1C, similar results were obtained by Western blot analysis of lung protein and FXR expression was observed at 55 kDa.

3.2. CDCA inhibits infiltration of inflammatory cells into the airspace

The cells from BAL fluid was collected from control, OVA, and CDCA treated groups after 24 h of final OVA challenge were counted using hemocytometer. Total number of cells in BAL fluid was increased significantly in OVA challenged group compared with control mice ($P < 0.05$). In OVA-challenged, CDCA treated mice the total number of cells was significantly low as compared to OVA-challenged mice (Fig. 2A). The differential counts revealed that eosinophil, neutrophil, and lymphocyte numbers in BAL fluid were increased in OVA challenged mice, and macrophages did not change significantly compared to the control mice. Administration of FXR ligand CDCA reduced eosinophil, neutrophil, lymphocyte and macrophages significantly ($P < 0.05$) compared to vehicle treated mice (Fig. 2B).

3.3. Effect of CDCA on pathophysiological changes of OVA-induced asthma

As CDCA inhibited the influx of inflammatory cells into the airspace of OVA-induced mice, we next examined the effect of CDCA in lung tissue of asthmatic mouse. Histologically, asthma is characterised by peribronchial tissue infiltration of inflammatory cells particularly eosinophils. The H& E stained sections from lungs of OVA-challenged mice shows edema, inflammatory cell infiltrate with numerous eosinophils, hypertrophy of the bronchial wall musculature and loss of parenchymal architecture, haemorrhages, and congestion, thickened airway epithelium compared with the vehicle treated control (Fig. 3A). Mice administered with CDCA, a natural FXR agonist, showed marked reductions in the infiltration of inflammatory cells and various other pathological features seen in the OVA-challenged mice (Fig. 3A). In control group, there was no significant change in lung tissues observed. Moreover, morphometric analysis also demonstrated a significant decrease ($P < 0.05$) in the number of eosinophils present following CDCA treatment when compared to the vehicle-treated animals (Fig. 3B). Asthma is linked with mucus over secretion, resulting in narrowing of airways and obstructive lung disease. We examined the PAS-stained lung sections for goblet cell hyperplasia and mucin secretion. OVA-challenged lung sections show goblet cell hyperplasia and higher mucin production and much less staining was observed in OVA-challenged CDCA treated lung sections (Fig. 3C). No positive PAS staining was seen in control sections (data not shown). These results suggest that the activation of FXR by its ligand inhibits OVA-induced airway inflammation and mucin production in the murine model of asthma.

3.4. CDCA inhibits the serum IgE and lung T_H2 cytokines

IgE plays a critical role in the development of asthma [25]. In typical allergic asthma, excessive levels of IgE in serum are directed against the sensitizing antigen, with an inappropriate exuberant cell-mediated response to further exposure of same antigen. We aimed to test whether activation of the FXR suppress the expression of IgE in OVA-induced asthma. CDCA treated mice show significantly decreased expression of total and OVA-specific IgE ($P < 0.05$) when compared to OVA-challenged mice (Fig. 2C, D). Whereas both types of serum IgE levels were found to be very low in control mice (Fig. 2C, D).

Cytokines and TNF-α protein levels in lung homogenate were measured by ELISA after 24 h of final OVA challenge. OVA-challenged mouse shows increased T_H2 cytokines (IL-4, IL-5 and IL-13) whose functions include IgE production, eosinophil activation and mucus production. In each case, CDCA-treated lungs show significantly reduced levels ($P < 0.05$) of IL-4, IL-5 and IL-13 when compared to the vehicle treated OVA-challenged mice lung (Fig. 4) which are in consistent with the IgE levels. Increased levels of tumor necrosis factor-α (TNF-α; $P < 0.05$) was observed which promotes inflammation, mucus secretion, and airway hyperresponsiveness in OVA-challenged mice compared to control, however, CDCA-treated animal shows reduced levels of TNF-α ($P < 0.05$) when compared to the OVA-challenged and vehicle

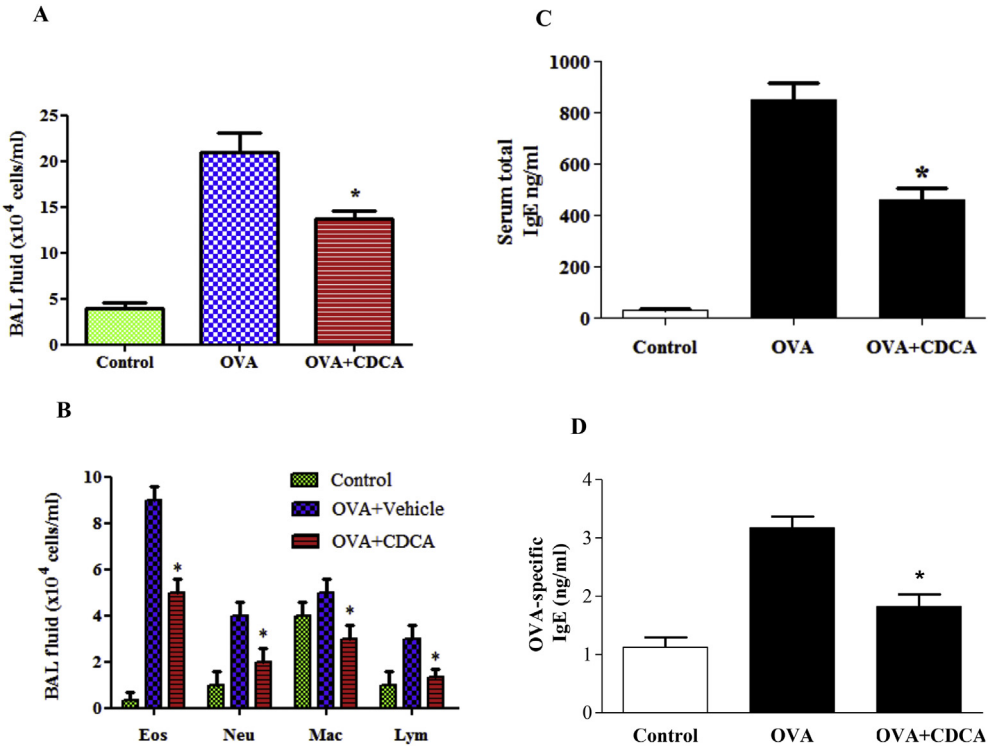

Fig. 2. BAL fluid cell count. Twenty-four hours following the last intranasal OVA challenge, BAL cells (4 mice/group) were collected and total cells were counted as described in the protocol (A). Cytospin slides were prepared and stained with Diff-Quick for differential counts (B). Eos-eosinophils; Neu-neutrophils; Mac-macrophages; Lym-lymphocytes. *$P > 0.05$ compares to OVA and vehicle treatment. Measurement of total and OVA-specific IgE levels in serum by ELISA. Total IgE levels (C), Ova-specific IgE (D). CDCA treated group (5 mice/group) showing significant decrease in total IgE and OVA-specific IgE (*$P > 0.05$) compared to OVA-induced group.

treated lung homogenate (Fig. 4). These results indicate that FXR ligand is inhibiting the secretions of T_H2 cytokines and TNF-α, the important markers in murine model of asthma.

4. Discussion

Asthma is a complex clinical complication of persistent inflammation of the airway and subsequent airway hyperresponsiveness. We show, for the first time that farnesoid X receptor natural agonist, CDCA, treatment significantly reduce or alleviate allergen-induced responses in a murine model of acute asthma induced by OVA sensitization and challenge. We propose that FXR suppresses airway inflammation through its anti-inflammatory actions [10].

FXR was initially identified as a regulator of liver metabolism but has recently been shown to exhibit anti-inflammatory [19,26–28] and anti-fibrotic [26,29,30] effects. A possible role of FXR in airway inflammation or pulmonary disease has not previously been investigated. Here we show the expression of FXR mRNA and protein in lung tissue (Fig. 1B, C) of mouse and it has been shown to express in rat and human pulmonary endothelial cells [31] suggesting that FXR play a role in lung physiology. Endothelin (ET)-1 is produced predominantly by endothelial cells, and the lung represents a primary target for ET-1 effects. The ET-1 has been implicated in the pathophysiology of asthma. Activation of FXR in endothelial cells downregulates the expression of ET-1, and this indicates that FXR might play an anti-inflammatory role and essential for the resolution of airway inflammation [11,20,32].

The airway inflammation in this model is characterised by excessive airway eosinophil and inflammatory cell infiltration, goblet cell hyperplasia with excessive airway mucus and elevated IgE and T_H2 cytokines [21,33,34]. In this study specifically, we observed significant decrease in peribronchial infiltration of inflammatory cells, BAL fluid cells, lung T_H2 cytokine levels and TNF-α with FXR ligand, CDCA treatment. Similar reductions were seen in goblet cell hyperplasia and mucin production along with serum levels of total and OVA-specific IgE. Our study with natural ligand of FXR is consistent with the UDCA a secondary bile acid formed from the primary bile acid CDCA, suppresses eosinophilic airway inflammation by inhibiting the function of dendritic cells through the farnesoid X receptor [17,35] in OVA-induced airway inflammation.

This also is a first study to demonstrate CDCA beneficial effects on airway inflammation in OVA-induced model. The anti-inflammatory actions of CDCA are by blocking the inflammatory cell infiltration into the airspace, in turn by inhibiting the secretions of T_H2 cytokines IL-4, IL-5 and IL-13 that are mainly responsible for the IgE secretion, eosinophil activation and mucus production respectively [36,37]. In previous studies it has been reported that FXR significantly decrease various cytokines involved in inflammation [38,39] and FXR ligand UDCA has been shown to inhibit these actions in OVA-sensitized mouse asthma model [17], thus our results confirm previous observations.

Nuclear factor kappa B (NF-κB) is a pleotropic transcription factor that plays a central role in asthma and induces the recruitment of inflammatory cells and drive antigen-induced inflammation that is important contributor to adaptive immune response [40,41]. Activation of FXR has been shown to inhibit hepatic inflammation by antagonizing the NF-κB signalling and target gene expression *in vivo* [19]. Here we show that CDCA treated animal revealed decreased levels of TNF-α in response to OVA-challenge. The increased levels of lung TNF-α, IFNγ, IL-6, iNOS, and MCP1 was seen in $FXR^{-/-}$ mice compared to wild

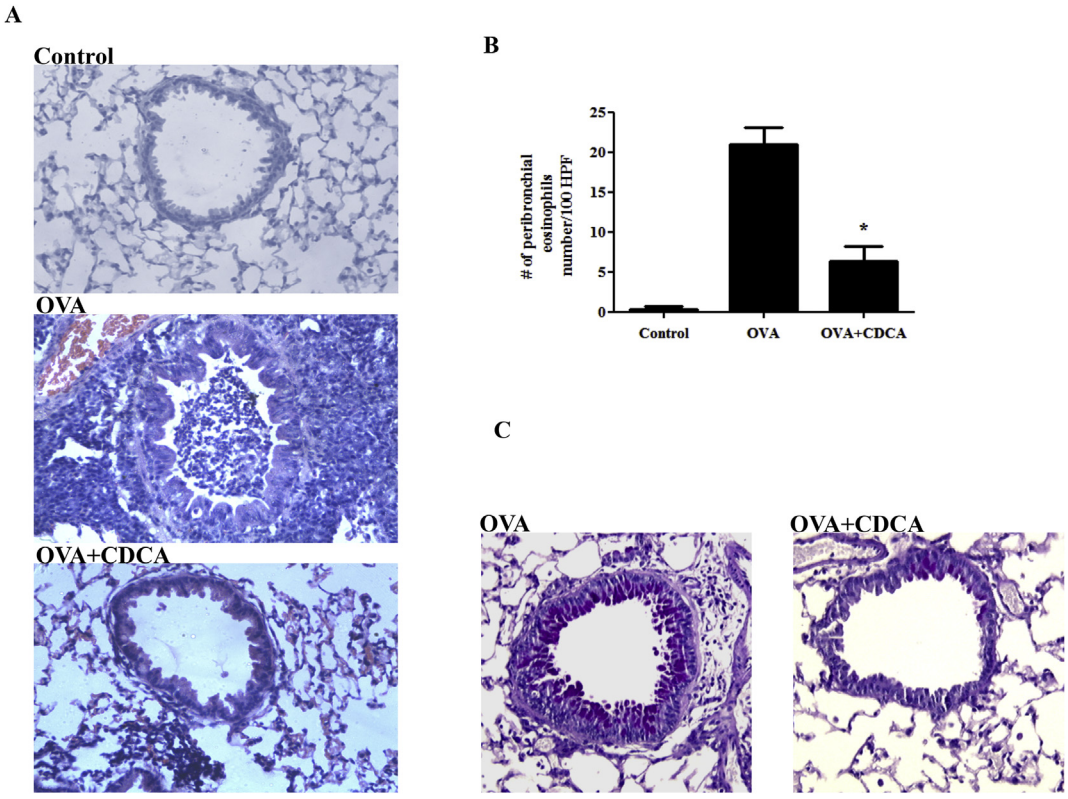

Fig. 3. CDCA inhibits inflammatory cell infiltration into the lungs. All measurements (5 mice/group) were performed after 24 h of final OVA challenge. A. Peribronchial infiltration of inflammatory cells was visualized by H& E staining. Representative H& E stained photographic lung section shown are from control, ovalbumin (OVA) and OVA-CDCA. B. Eosinophil infiltration was quantified by counting 100 high power fields per lung. *$P > 0.05$ compares to OVA and vehicle treatment. C. Lung tissue (5 mice/group) was fixed and stained with periodic acid Schiff's (PAS) to examine the extent of mucin production.

type mice after LPS treatment [20]. This anti-inflammatory response of FXR ligand may be in part by antagonising the NF-κB activity and suggest that FXR play an important role in allergen-induced airway inflammation.

The bile acid 'CDCA' dissolves cholesterol gallstones and is used for the management of hypertriglyceremia, cerebrotendinous xanthomatosis, rheumatoid arthritis, congenital liver diseases, and constipation [42]. It is enticing to speculate that some of these

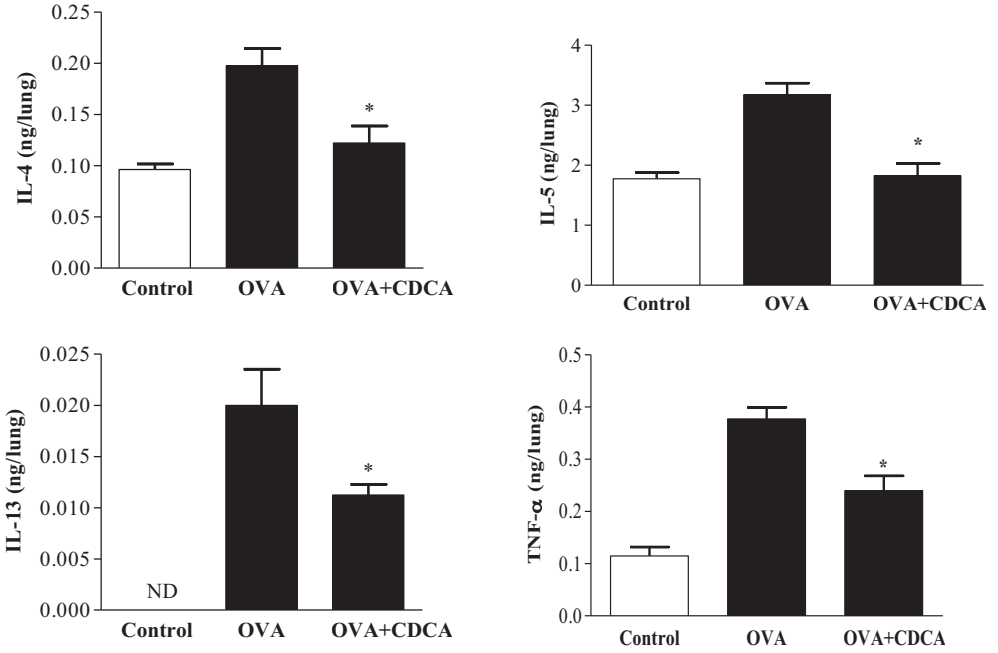

Fig. 4. Measurement of T_H2 cytokines and TNF-α in lung homogenate by ELISA after final OVA challenge. FXR agonist CDCA treated lung group (5 mice/group) showing lower level of IL-4, IL-5, IL-13 and pro inflammatory cytokine TNF-α compared to OVA alone (*$P > 0.05$).

effects are mediated by FXR. FXR ligands have been proposed as possible drugs to target various metabolic and inflammatory conditions and CDCA has been demonstrated the hepatoprotective role on familial hypertriglyceridemia and familial combined hyperlipidemia (ClinicalTrials.gov:http://www.clinicaltrials.gov NCT00465751). CDCA under the brand name Chenodiol has been proved to accelerate colonic transit time in irritable bowel syndrome (ClinicalTrials.gov: http://www.clinicaltrials.gov NCT00912301) and ulcerative colitis [43].

In conclusion, our results demonstrate that FXR may reduce airway inflammation via the suppression of T_H2 cytokines (IL-4, IL-5, IL-13), and TNF-α. Since the clinical safety of various FXR ligands have been evaluated in clinical trials, and as our results indicate that CDCA effectiveness in a murine model of asthma, we suggest that it should be investigated as a potential therapy for human allergic airway diseases.

Acknowledgements

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