

Apolipoprotein A1 potentiates lipoxin A4 synthesis and recovery of allergen-induced disrupted tight junctions in the airway epithelium

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Summary

Background Asthma is characterized by chronic airway inflammation triggered by various allergens in the environment. Defects in the bronchial epithelial interface with the external environment are the hallmark of asthma. Apolipoprotein A-1 (ApoA1) or ApoA1 mimetics have demonstrated anti-inflammatory activity and preventive effects in mouse models.

Objective We investigated airway levels of ApoA1 in asthmatics and the possible role of ApoA1 in protection of the bronchial epithelium and in resolution of inflammation in cellular and animal models of asthma.

Methods ApoA1 levels were measured in bronchoalveolar lavage fluid (BALF) from asthmatics and healthy controls. With treatment of ApoA1, mouse model of house dust mite (HDM)-driven asthma and cultured primary bronchial epithelial cells obtained from asthmatics were examined. Tight junction (TJ) expression in the bronchial epithelial cells was assessed by using confocal microscopy and immunoblot.

Results Asthmatics showed significantly lower ApoA1 levels in bronchoalveolar lavage fluid than did healthy controls. Local ApoA1 treatment significantly decreased lung IL-25, IL-33, and thymic stromal lymphopoietin levels in HDM-challenged mice and inhibited allergen-induced production of these cytokines in cultured primary bronchial epithelial cells. ApoA1 promoted recovery of disrupted TJ proteins zonula occludens-1 and occludin in cultured primary bronchial epithelium obtained from asthmatics. ApoA1-induced increases in the TJ proteins were dependent on increased production of lipoxin A4 (LX A₄). **Conclusions and Clinical Relevance** ApoA1 enhances resolution of allergen-induced airway inflammation through promoting recovery of damaged TJs in the bronchial epithelium. ApoA1 could be a therapeutic strategy in chronic airway inflammatory diseases that are associated with a defective epithelial barrier, including asthma.

Keywords apolipoprotein A1, asthma, lipoxin A4, lung, tight junction

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Introduction

Asthma, a disease of chronic airway inflammation characterized by a Th2 cell-mediated immune response, is associated with bronchial hyperresponsiveness,

airflow obstruction, and remodelling [1]. Asthma is a complex disease with a genetic aetiology; however, environmental factors also contribute to its initiation or progression [2]. The airway epithelium acts as an important physical barrier as well as a controller of inflammatory and regenerative responses to allergens, viruses, and environmental pollutants that contribute to asthma pathogenesis of asthma [3]. In response to allergen triggers, the asthmatic airway epithelium

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secretes various cytokines that are strongly implicated in Th2-polarized inflammation, including IL-5, eotaxin, CCL20, and RANTES [4]. Recent studies have found that the airway epithelium releases IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) as important proximal events in the induction and maintenance of Th2 inflammation [5, 6]. The airway epithelium serves as a barrier by means of tight junction (TJ) formation, which seals off the paracellular space, and possesses a gate function that selectively regulates passage of ions and molecules [7, 8]. TJs comprise a series of integral membrane proteins: claudins, occludin, cytoplasmic linker proteins (zonula occludens [Zo] proteins Zo-1 to -3), and transmembrane adhesion proteins [9].

Exposure of epithelial cells to dust mite allergens or ovalbumin inhalation in sensitized mice causes epithelial TJ disruption that allows inhaled allergens to pass more easily into the airway to interact with inflammatory cells [10–12]. In asthmatics, the barrier function of airway epithelium has been reported to be impaired [13–15]. Inflammatory-related lipid mediators are also involved in asthma pathogenesis and contribute to attraction of inflammatory cells to the airway epithelium [16]. Defects in counter-regulatory anti-inflammatory and pro-resolving lipid mediators such as lipoxin present in severe asthma serve to maintain chronic inflammation unresponsiveness to steroids [17]. Thus, identifying new candidate proteins that promote resolution of airway inflammation and restoration of disrupted epithelia could assist development of novel therapies for chronic inflammatory airway diseases, including asthma.

Apolipoprotein A-1 (ApoA1) is a major component of high-density lipoproteins, which possess anti-atherosclerotic properties [18, 19]. ApoA1 also possesses multiple anti-inflammatory properties, including its ability to inhibit expression of inflammatory adhesion molecules at the endothelium and suppress pro-inflammatory signalling pathways such as ERK signalling and NF- κ B activation [20–23]. Based on these effects, ApoA1 or ApoA1 mimetic peptides have been investigated as drugs in sepsis, atherosclerosis, and acute renal injury animal models [24–26]. Inflammation in asthma has been reported to be regulated by ApoA1 in animal models [27–29]. ApoA1-deficient mice exhibit increased pulmonary inflammation and airway hyperresponsiveness (AHR) [28] and treatment with ApoA1 mimetics attenuated allergen induction of airway inflammation in murine models of asthma [27, 29].

The precise mechanism of action by which intranasal delivery of ApoA1 suppresses airway inflammation has not been clearly elucidated. Given the importance of epithelial cells in the pathogenesis of asthma, we hypothesized that ApoA1 acts by suppressing pro-inflammatory pathways in epithelial cells. In addition, we hypothesized that asthma is associated with

changes in airway ApoA1 levels that could be important pathophysiologically, a possibility that has not been addressed by previous reports.

Here, we report that bronchoalveolar lavage (BAL) fluid ApoA1 levels were significantly decreased in asthmatics compared with healthy controls. We also show that ApoA1 abrogated established airway inflammation and promoted repair of disrupted TJs in an allergen challenge model by enhancing production of lipoxin A4 (LX A₄). These findings are related to regulation of the innate immune response, primarily in the bronchial epithelium, and acceleration of the resolution of inflammation.

Methods

Study subjects

The study subjects were recruited from outpatient clinics of Soonchunhyang University Hospital and consisted of asthmatics who had defined mild-to-moderate persistent asthma [1]. None of the asthmatics used anti-asthma drugs, including inhaled corticosteroids, prior to performance of BAL. Normal volunteer subjects were recruited from hospital personnel. Asthmatics and normal volunteers were non-smokers and had been free from respiratory infection for at least 4 weeks prior to the study. Clinical characterization of subjects is listed in Table S1. All subjects provided written informed consent. The study protocols were approved by the human studies review board of the Soonchunhyang University Bucheon Hospital (SCHBC_biobank_2012-003).

Fibre-optic Bronchoscopy

In each subject, BAL and bronchial brushings were performed using fibre-optic bronchoscopy (Olympus B2-10; Olympus, Tokyo, Japan). Lavage was performed by instilling sterile warm saline into the right-middle lobe. Processing of BAL has been described previously [30]. The cell-free BAL fluid was aliquoted and stored at -80°C . Bronchial brushings were obtained using a protected brush (Olympus) to sample bronchial subsegments. The bronchial brushings were performed in accordance with standard guidelines [31], and no complication was identified in any subject during the BAL procedure.

Allergen-induced asthma animal model

The house dust mite (HDM)-induced allergic asthma model used 8-week-old male C57Bl/6 mice (Charles River, Burlington, MA, USA) as described previously, with a modification [32]. HDM-challenged mice (HDM group) were challenged on days 0, 7, and 14 by intratracheal injection of 100- μg *Dermaphagoides*

pteronyssinus extracts (contained 0.01U/ μ L endotoxin; Greer Laboratories, Lenoir, NC, USA). After the last HDM challenge, human ApoA1 (44.7 ng/mouse, ApoA1 group; Calbiochem, San Diego, CA, USA) or mutant ApoA1 control protein (44.7 ng/mouse, Control group) or phosphate-buffered saline (PBS) (100 μ L/mouse, HDM group) was administered intranasally on days 15–16. V156K mutant ApoA1 control protein, known functionally inactive form was prepared by as previously described [33]. Briefly, some of the ApoA1 group mice were co-treated with cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC, a 12/15-LOX inhibitor, 2 μ M per mouse in the ApoA1 + CDC group; Invitrogen, Grand Island, NY, USA). On day 17, AHR was measured, and bronchoalveolar lavage fluid collection, tissue processing, and staining were performed as described previously [34]. To investigate the effect of ApoA1 on dendritic cell (DC) activation and accumulation in the lung, an ovalbumin (OVA)-induced asthma model was used as described previously [34].

Airway hyperresponsiveness

Mice were anesthetized with ketamine–xylazine, tracheostomized, and mechanically ventilated with a Flexi-Vent system (SCIREQ, Flexivent, Scireq Inc., Montreal, Canada) as described previously [34]. Volume changes due to thoracic expansion and alterations in tracheal pressure were measured in response to challenges with increasing concentrations of methacholine (25, 50, and 100 mg/mL) and reported as total lung resistance (R_L).

Measurement of cytokines and the pro-resolution mediator in BAL fluid and the lung

The levels of IL-25, IL-33, TSLP (R&D Systems, Minneapolis, MN USA), and the pro-resolution mediator LX A₄ (Neogen, Lexington, KY, USA) from mouse lung and ApoA1 (Abnova, Walnut, CA, USA) and LX A₄ (Neogen) levels in human BAL fluid were measured using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA).

Air–liquid interface cell culture for human bronchial epithelial cells

Primary normal human bronchial epithelial (NHBE) cells (cat#. CC-2540; Cambrex Bio Science, Baltimore, MD, USA) were prepared as described previously [28]. Frozen passage 1 stocks were thawed and cultured in T-flasks in bronchial epithelial cell growth medium (Cambrex Clonetics, Walkersville, MD, USA) supplemented with bovine pituitary extract, hydrocortisone (0.5 mg/mL), recombinant human epidermal growth factor (0.5 ng/mL), epinephrine (0.5 mg/mL),

transferrin (10 mg/mL), insulin (5 mg/mL), retinoic acid (0.1 ng/mL), triiodothyronine (6.5 ng/mL), gentamicin sulphate (50 mg/mL), and amphotericin B (50 ng/mL). The medium was replaced every 48 h until cells reached 90% confluence. Cells were seeded at 8.25×10^4 /insert on Corning Costar Transwell inserts with 0.4-mm pores (Corning Life Sciences, Lowell, MA, USA). Both the apical (0.5 mL) and basolateral sides (1.5 mL) were filled with differentiation medium containing a 1 : 1 mixture of Dulbecco's modified Eagle's medium (DMEM) and bronchial epithelial cell growth medium with supplements, as above, except that triiodothyronine, gentamicin sulphate, and amphotericin B were omitted, and penicillin, streptomycin, and 50 nM all-*trans* retinoic acid (Sigma-Aldrich, St Louis, MO, USA) were added. Cells were submerged for 7 days in culture, and then the apical medium was removed to establish an air–liquid interface (ALI) for 14 days. The medium was refreshed three times per week. Cells were grown at 37°C in 5% CO₂ in a humidified incubator and protected from light. The apical surface of the cells was rinsed with PBS once weekly to remove accumulated mucus and debris.

Cytokine production and secretion by NHBE cells

After establishment of an ALI, NHBE cells were apically stimulated with *Dermatophagoides pteronyssinus* (Der p1) extract (1 μ g/mL), cockroach extract (50 μ g/mL), lipopolysaccharide (LPS) (100 ng/mL; Sigma-Aldrich), and ionomycin (1 μ M; Sigma-Aldrich) for 6 h. The cockroach extract was a gift from Jung-won Park (Yonsei University). Next, human ApoA1 (1 μ g/mL) was administered apically for 24 h and cell-free supernatants were collected and the IL-33, IL-25, and TSLP levels were measured using ELISA (R&D Systems).

Bronchial epithelial cell culture for investigation of tight junctions

Primary bronchial epithelial cells (BECs) were grown from bronchial brushings (> 95% epithelial cells) from normal or asthmatic subjects. Cell culture was performed as described previously with minor modifications [13]. Briefly, BECs were cultured in flasks pre-coated with collagen I (BD Biosciences, Bedford, MA, USA). Primary cultures were established by seeding freshly collected BECs into hormonally supplemented bronchial epithelial growth medium (Lonza, Walkersville, MD, USA). On the second passage, cells were seeded in bronchial epithelial growth medium onto culture inserts (Transwell, 6.5 mm, 0.4- μ m pore size, Corning Life Sciences) and cultured until almost confluent before being taken to the ALI.

After reaching ALI, cells were starved overnight in Eagle's minimal essential medium without serum. Then,

cells were treated with hApoA1 (1 µg/mL) or mutant ApoA1 (1 µg/mL) with or without CDC (a 12/15-LOX inhibitor 2 µM; Invitrogen, Grand Island). Cells were washed twice with PBS and isolated at 37°C, using a trypsin solution (polyvinylpyrrolidone, 0.2% EGTA, and 0.25% trypsin containing 0.02% EDTA). Epithelial cells were plated at a density of 10^5 to $2 \times 10^5/\text{cm}^2$ on collagen/fibronectin solution (CFN)-coated eight-well Labtek (Nunc) or CFN-coated permeable filters (Transwell filters, 0.4-µm pore size; Corning) or on CFN-coated six-well plates for immunofluorescence experiments, Transepithelial electrical resistance (TER) measurements, and Western blotting. The supernatants were collected and LX A₄ expression levels therein determined by ELISA.

Immunofluorescence

For immunofluorescence experiments, 7 days after plating, cell monolayers were fixed for 15 min in 4% paraformaldehyde in PBS, pH 7.5, followed by a 5-min permeabilization in 0.2% Triton X-100 in PBS, and then incubation in PBS containing 0.1% bovine serum albumin (BSA). Mouse lung tissues and cells were stained using a monoclonal mouse Zo-1 antibody (1 : 200; Invitrogen, Carlsbad, CA, USA) and monoclonal mouse occludin antibody (1 : 200; Invitrogen, Carlsbad) and were visualized with FITC-conjugated rabbit anti-mouse antibody (Zymed Laboratories Inc., San Francisco, CA, USA). Nuclei were counterstained with DAPI (Invitrogen, Grand Island). Confocal laser microscopy was performed using an LSM 510 META with a $\times 40$ oil-immersion objective coupled to a CoolSnap HQ Photometrics camera. Sections were observed using confocal laser scanning microscopy (LSM 510 META) and images were generated using the Zeiss LSM image browser. The images were collected with serial sections at 0.5-µm intervals, and analysed using the Zeiss LSM image browser software (Carl Zeiss, Oberkochen, Germany).

Western blot analysis

Cells were lysed by incubation in radioimmunoprecipitation assay buffer (50 mM Tris-Cl [pH 7.4], 0.1% Na₃, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitor mixture; Calbiochem) for 30 min on ice. A total of 20-µg cell lysate was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk, probed with anti-Zo-1 and anti-occludin, and treated with anti-rabbit horseradish peroxidase-conjugated antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA). Anti-β-actin (Sigma, St. Louis, MO, USA)

was used as a loading control. Immunostained proteins were detected using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Zo-1 and occludin levels were determined by densitometry (image acquisition and analysis software, Ultra-Violet Products, Upland, CA, USA).

Transepithelial electrical resistance measurement

Primary BECs obtained from the subjects were seeded in the upper chamber of a Transwell tissue-culture plate (12-mm diameter, 0.4-µm pore size; Corning) and allowed to reach confluence. ApoA1 (1 µg/mL) or mutant ApoA1 was added to the apical side of the filters when indicated. After 72 h, the TER of cells grown on filters was measured using an epithelial volt-ohm metre (Millicell-ERS; Millipore, Billerica, MA, USA). The background electrical resistance attributed to fluid and a blank Transwell filter was subtracted from the measured TER. All measurements were performed in triplicate, and TER values were normalized using the area of the monolayer and expressed as $\Omega \cdot \text{cm}^2$.

Lung and mediastinal lymph node dendritic cell collection

One-hundred microlitres of FITC-OVA (10 µg/mL; Invitrogen, Grand Island) was administered intratracheally on days 21–23. ApoA1 (44.7 ng/mice) was administered intranasally on days 24 and 25. Control mice received mutant ApoA1 (44.7 ng/mouse). On day 26, lung tissues and mediastinal lymph nodes (MLNs) of mice were minced, and then treated with 0.033% (w/v) of collagenase/complete DMEM solution for 30 min. Single-cell suspensions of the tissues were obtained, and CD11c⁺ cells were positively selected using MACS CD11c microbeads (Myltenyi Biotec, Auburn, CA, USA). Red blood cells were removed by hypotonic lysis. After two washes, the cells were used for experiments.

Flow cytometry and sorting

Antigen-containing DCs were enumerated in the lung and MLNs as CD11c⁺MHCII⁺ cells carrying FITC⁺ material. In all experiments, dead cells were excluded from analysis using propidium iodide. After counting and washing, the cells were stained for 30 min with PE-Cy5 (eBioscience, San Diego, CA)-labelled anti-CD11c (macrophages/DCs; eBioscience), and PE-labelled anti-MHCII (eBioscience) in PBS containing 0.5% BSA and 0.01% sodium azide for determination of the numbers of DCs in the lung and MLN. Differential cell counts were performed by flow cytometry as described previously [35]. The absolute cell number was calculated by multiplying the total leucocyte number by the

percentage of each population of FITC/CD11C/MHCII(+/+) cells. Analyses were performed using a Beckman Coulter Epics XL cytometer (Brea, CA, USA) using EXPO32 ADC software (Beckman Coulter).

Statistical analysis

Data are expressed as means $\pm$ SEM or medians and interquartile ranges. Data were analysed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test with the Bonferroni correction for intergroup comparisons. Correlations between the data were assessed using Spearman's rank test. A value for $P < 0.05$ was deemed to indicate statistical significance.

Results

ApoA1 promotes resolution of HDM-induced airway inflammation

As previous reports have demonstrated the preventive effects of ApoA1 or ApoA1 mimetics in murine asthma models, we determined the effects of ApoA1 on the resolution of established airway inflammation. ApoA1 or

inactive mutant ApoA1 control was administered intranasally for two consecutive days (d15 and d16) after the final HDM challenge (given on day 14) and mice were killed on day 17. ApoA1-treated mice had significantly fewer total BAL fluid cells, including eosinophils and neutrophils, than HDM or mutant ApoA1 control group mice (Fig. 1a). Histopathological analysis also showed markedly less peribronchial, perivascular inflammatory cell infiltration, and lower airway mucus production in the ApoA1-treated lung (Fig. 1b). AHR was measured in the presence of increasing doses of methacholine in the mice. HDM exposure resulted in a trend towards higher baseline airway resistance compared with that of saline challenged controls. ApoA1-treated mice were protected from HDM-induced AHR to methacholine (Fig. 1c). Taken together, these data indicate that ApoA1 increased resolution of HDM-induced airway inflammation, lung pathology, and AHR.

ApoA1 regulates epithelial-derived asthma-related cytokines

To identify the mechanisms by which ApoA1 regulates airway inflammation, we measured cytokines that

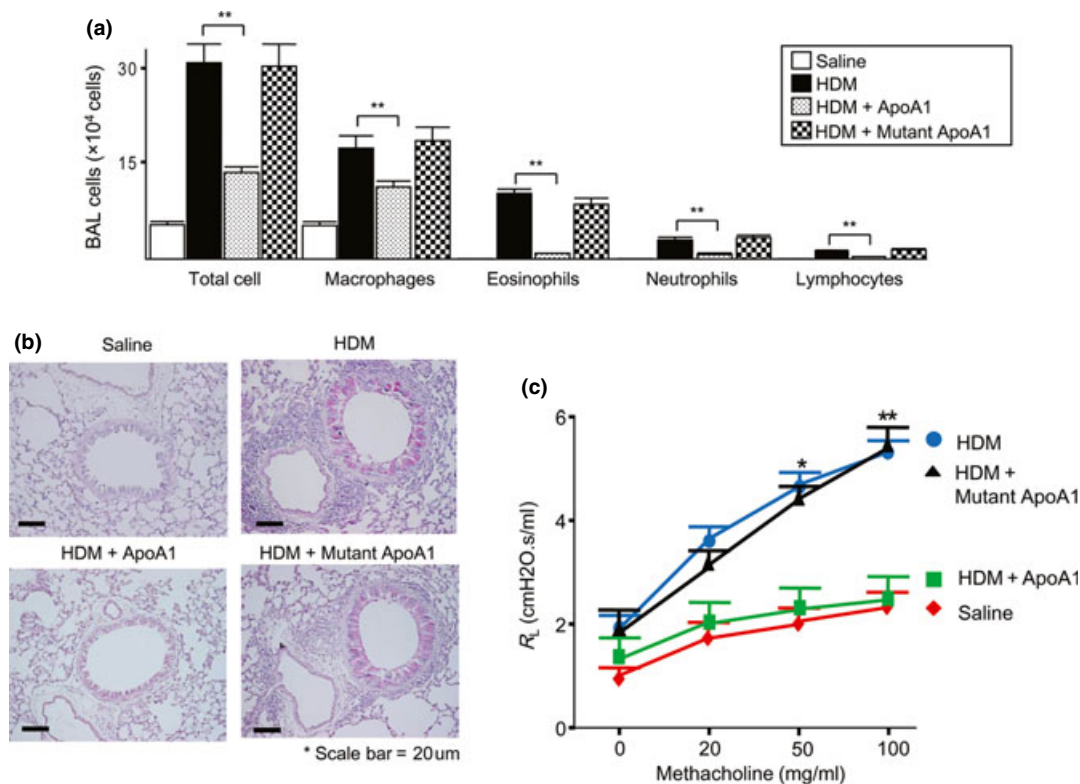


Fig. 1. Apolipoprotein A1 (ApoA1) attenuates established airway inflammation and airway hyperresponsiveness (AHR) in murine allergic asthma. (a) Numbers of bronchoalveolar lavage fluid cells in the four groups are shown as means $\pm$ SEM; $^{***}P < 0.01$. $^{*}P < 0.05$. (b) Fixed lung tissue specimens were sectioned and stained using H&E and periodic acid Schiff. (c) Airway resistance was measured following inhalation of increasing doses of methacholine. Each data point represents the mean $\pm$ SEM of eight mice per group. $^{***}P < 0.01$ and $^{*}P < 0.05$, house dust mite (HDM) vs. HDM + ApoA1, respectively.

potentiated the Th2 response in the bronchial epithelium by ELISA in BAL fluid from vehicle- or mutant ApoA1- or ApoA1-treated mice. ApoA1 treatment resulted in significantly lower IL-25 (IL-17E), IL-33, and TSLP concentrations compared with vehicle- or mutant ApoA1-treated mice (Fig. 2a). These findings indicate that IL-25, IL-33, and TSLP were down-regulated by ApoA1 in established allergic airway inflammation.

ApoA1 regulates IL-25, IL-33, and TSLP release in response to allergens in the human bronchial epithelium

Airway epithelial cells secrete IL-25, IL-33, and TSLP, although both macrophages and granulocytes are also a rich source of these cytokines, which influence development of Th2 immunity [36, 37]. Hence, we determined whether ApoA1 directly inhibits release of IL-25, IL-33, and TSLP at the airway epithelium in response to various environmental stimuli. We incubated NHBE cells with the common allergen extracts Derp1, oriental cockroach extract, or LPS for 6 h, followed by ApoA1 application for 24 h. ApoA1 treatment significantly inhibited Der p1- and cockroach extract-induced IL-25, IL-33, and TSLP production (Fig. 2b).

Effects of ApoA1 on dendritic cell accumulation in the lung

Because TSLP initiates allergic airway inflammation by activation and accumulation of antigen-presenting cells in the lung [38], we evaluated whether local ApoA1 affected DC activation and accumulation in the airways. Intratracheal-injected FITC-OVA was used to identify lung-derived DCs transporting this complex across the epithelial TJ barrier. On days 21–23, mice were instilled intratracheally with FITC-OVA. On days 24–25, ApoA1 was administered intranasally, and then the lungs were removed on day 26. The number of DCs (MHCII^{high}CD11C^{high}) carrying FITC-OVA cargo was markedly decreased by ApoA1 treatment (Figure S1a). Simultaneously, the number of DCs (MHCII^{high}CD11C^{high}) carrying FITC-OVA cargo to MLNs was significantly decreased by ApoA1 (Figure S1b). No difference was noted in the intensity of FITC-OVA staining in lung or lymph node DCs with or without ApoA1, demonstrating that ApoA1 did not interfere with allergen uptake (data not shown). Collectively, the reduction in allergen-containing DCs in the lung and MLNs of ApoA1-treated mice likely resulted mainly from attenuation of lung

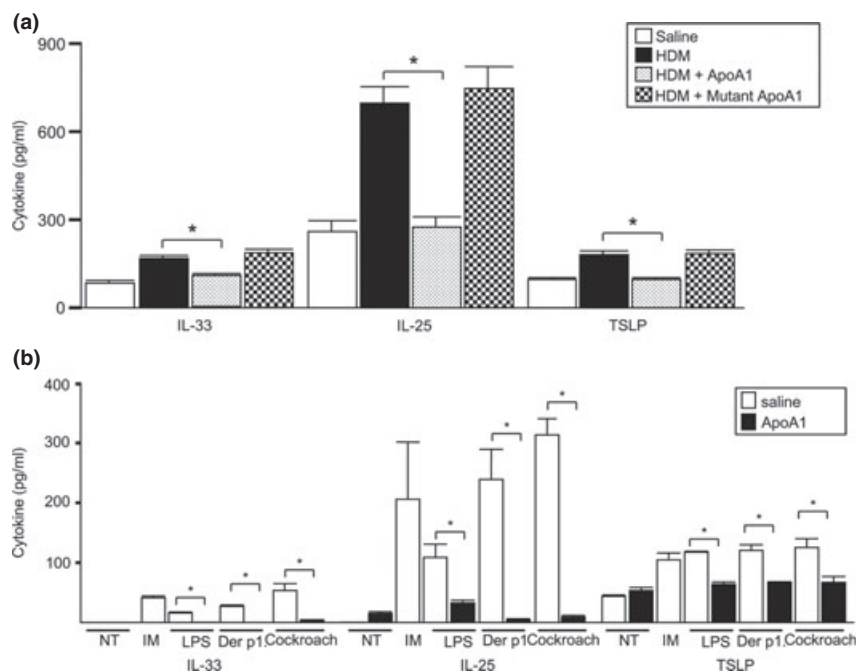


Fig. 2. Apolipoprotein A1 (ApoA1) regulates epithelium-derived Th2 cytokines in the lung of house dust mite-challenged mice and allergen pre-exposed primary bronchial epithelium. (a) IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) levels in the bronchoalveolar lavage fluid were measured using ELISA kits. Data represent the means $\pm$ SEM of eight mice per group. ** $P < 0.01$. * $P < 0.05$. (b) Normal human bronchial epithelium (NHBE) cells cultured at the air–liquid interface for 14 days were washed and stimulated apically with lipopolysaccharide (100 ng/mL), *Dermatophagoides pteronyssinus* (Der p1) extract (10 μ g/mL), cockroach extract (50 μ g/mL), and ionomycin (1 μ M) for 6 h. ApoA1 (1 μ g/mL) or saline vehicle was added to the apical surface for 24 h and cell-free supernatants were harvested. IL-33, IL-25, and TSLP levels in the supernatants were measured using ELISA kits. IM, ionomycin. NT, no treatment. Data are shown as means $\pm$ SEM of four independent experiments performed in triplicate. * $P < 0.05$.

inflammation and the concomitant decrease in DC migration to MLNs.

ApoA1 promotes recovery of disrupted epithelial barriers in bronchial epithelia and is associated with LX A₄ production

Because local ApoA1 treatment reached the bronchial epithelium, we focused on the effect of this treatment on epithelial damage associated with asthma. Allergens cause disruption of the epithelial barrier leading to initiation and maintenance of airway inflammation in asthmatics and in animal models [39, 40]. We investigated expression of the major components of the TJ proteins Zo-1 and occludin in the bronchial epithelium of HDM-challenged mice. HDM-challenged mice demonstrated decreased expression intensity for both proteins in epithelial cells, compared with control mice (Fig. 3a). In the HDM-challenged mice, treatment with ApoA1 dramatically increased the intensity of Zo-1 and occludin expression in the bronchial epithelium compared with those treated with mutant ApoA1 (Fig. 3a). Next, we tested whether ApoA1 restored the damaged epithelial barrier with disruption of TJs in fully differentiated BECs grown from bronchial brushings. Localization and quantification of Zo-1 and occludin were performed by immunofluorescence and immunoblotting, respectively. Confocal microscopy showed that differentiated cultures from healthy subjects ($n = 6$) exhibited strong and continuous TJ expression, whereas cultures from asthmatics ($n = 6$) showed weak and discontinuous expression (Fig. 3b). When ApoA1 (1 $\mu\text{g/mL}$) was administered, disrupted Zo-1 and occludin expression was recovered rapidly and similarly reached the normal range at 72 h (Fig. 3b and Figure S2a, b). Immunoblot analysis also demonstrated that treatment with ApoA1 significantly increased the TJ protein levels (Fig. 3c). The functional integrity of the TJ was evaluated by measurement of the TER of differentiated BECs. The BECs from healthy subjects reached a maximal TER of $920 \pm \Omega\cdot\text{cm}^2$ 7 days after plating. As expected, ApoA1 (1 $\mu\text{g/mL}$) treatment significantly increased TER compared with decreased TER at 72 h with mutant ApoA1 in BECs from asthmatics (Fig. 4). These data suggest that local ApoA1 treatment promotes recovery of disrupted or disassembled TJs in the asthmatic bronchial epithelium.

Recently, Grumbach et al. reported that LX A₄ plays a role in localization and stimulation of Zo-1 expression in human bronchial epithelium [41]. To determine the mechanism of TJ recovery, we measured the levels of the pro-resolution mediator lipoxin A₄ (LX A₄) in supernatants of BECs obtained from the above experiment (Figure S2a,b) that were treated with ApoA1 for 72 h. During the experimental period, the low basal LX

A₄ level was not changed in the mutant ApoA1- or saline-treated group (Fig. 5a). ApoA1 treatment of BECs from asthmatics resulted in a significant increase in LX A₄ levels ($P < 0.01$, Fig. 5a). To determine whether endogenous production of LX A₄ is critical for ApoA1-induced TJ recovery, BECs were co-treated with ApoA1 and CDC, an inhibitor of LX A₄ production. As expected, LX A₄ levels were significantly decreased in the supernatants of CDC-treated cells ($P < 0.01$, Fig. 5a). Immunofluorescence and immunoblot analysis revealed that CDC dramatically decreased TJ protein expression in ApoA1-treated BECs from subjects with asthma (Fig. 6a, b).

Beneficial in vivo effects of ApoA1 are reversed by the LX A₄ inhibitor CDC

We next investigated the role of LX A₄ in the *in vivo* HDM model. ApoA1 treatment markedly increased lung LX A₄ levels, and the increase in LX A₄ was prevented by co-administration of CDC (Fig. 5b). Inhibition of LX A₄ production by CDC dramatically inhibited the beneficial effects of ApoA1, restoring inflammation and AHR to levels similar to those seen in HDM-sensitized and -challenged mice not treated with ApoA1 (Figure S3).

The lung ApoA1 concentration is significantly decreased in patients with asthma compared with normal subjects

ELISA was used to measure the levels of Apo A1 in BAL fluid from 26 asthmatics and 14 normal, control subjects. The Apo A1 concentration normalized to that of total protein was fivefold lower in the asthmatic group compared with that in normal control subjects (Figure S4). These data indicate that patients with mild-to-moderate persistent asthma have lower pulmonary ApoA1 levels.

Discussion

In this study, we show that ApoA1 demonstrates a pro-resolution effect on allergic airway inflammation. ApoA1 resulted in lower concentrations of IL-25, IL-33, TSLP, as well as lower numbers of mature lung DCs. ApoA1 treatment also resulted in increased LX A₄ production in inflamed lungs as well as in primary bronchial epithelial cells. We observed that ApoA1 dramatically recovered defective epithelial TJs, in both a HDM-induced murine asthma model and in primary BECs from asthmatics. Moreover, recovery of TJs is dependent on increased production of endogenous LX A₄. These findings collectively indicate that ApoA1 targets mainly inflamed bronchial epithelium and promotes repair of disrupted TJs, leading to inhibited production of epithelium-derived Th2

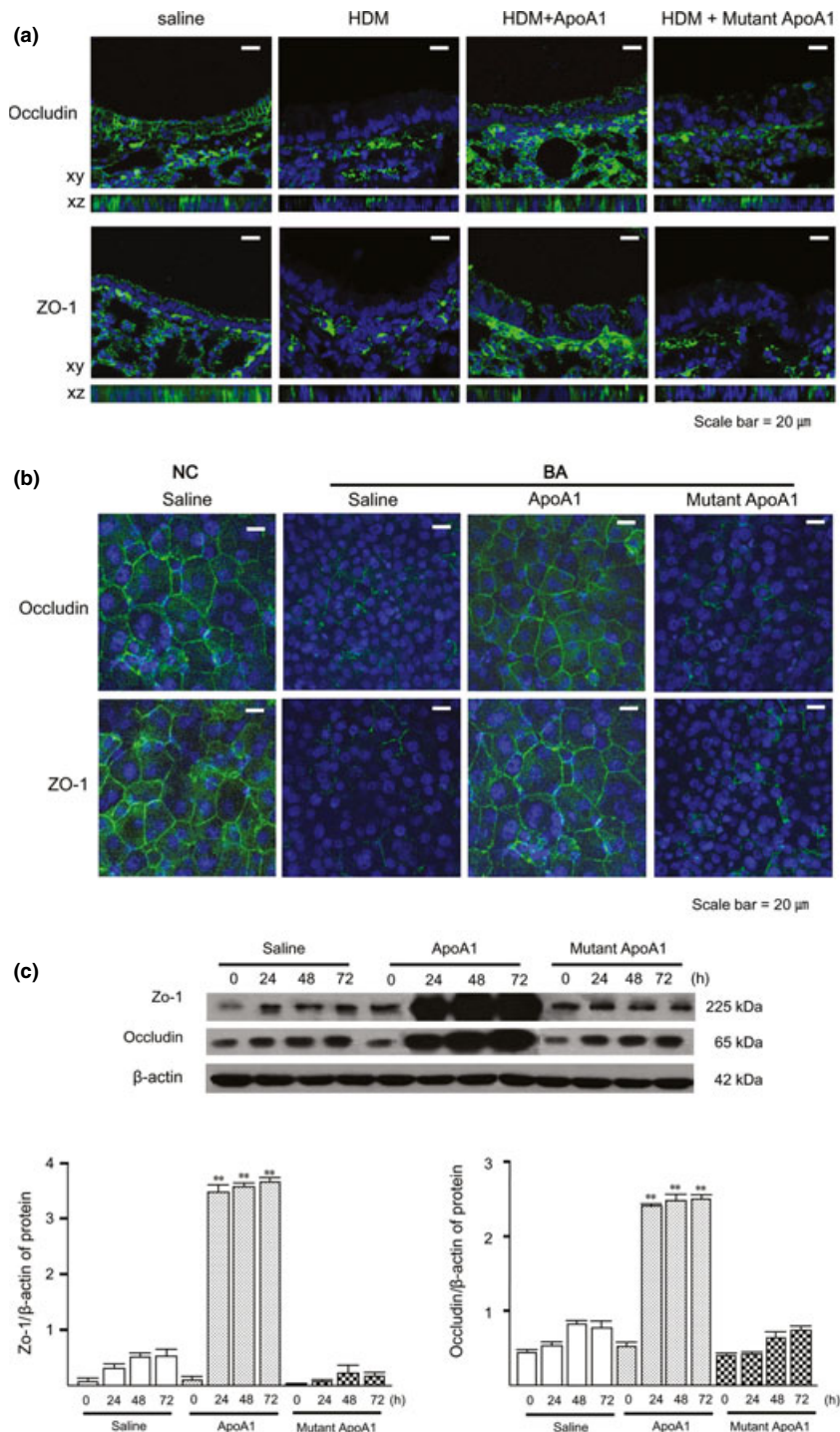


Fig. 3. Apolipoprotein A1 (ApoA1) promotes recovery of allergen-induced disrupted tight junction molecules in the bronchial epithelium. (a) Immunofluorescence staining of epithelial localization of ZO-1 and occludin expression in sections of mouse lungs that were saline treated or house dust mite (HDM) challenged with ApoA1 or mutant ApoA1. ApoA1 was administered intranasally 24 h after the last HDM challenge. Scale bar = 20 μ m. (b) Immunofluorescence of ZO-1 and occludin in cultured primary bronchial epithelial cells (BECs) obtained from healthy controls and asthmatics. Primary BECs from asthmatics were treated with ApoA1 or mutant ApoA1 for 72 h. (c) Immunoblotting and densitometry. Mean normalized densitometries of six immunoblot experiments. Data are shown as means $\pm$ SEM. ** P < 0.01 *vs.* the saline or mutant ApoA1 group at the same time.

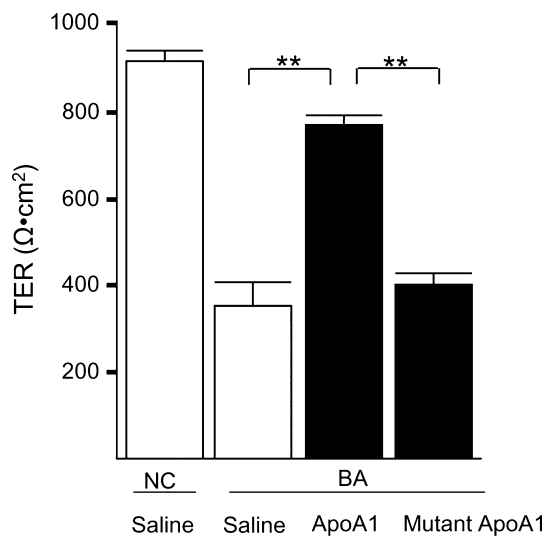


Fig. 4. Assessment of barrier function in primary BEC culture. After ALI was established in primary bronchial epithelial cells (BECs) from asthmatics ($n = 4$) and normal controls ($n = 4$), barrier function was determined by measurement of transepithelial resistance. Data are expressed as means $\pm$ SEM of four independent experiments performed in triplicate $**P < 0.01$.

cytokines. In addition, our findings demonstrate that individuals with mild-to-moderate persistent asthma have diminished BAL fluid ApoA1 concentrations, suggesting that reductions in ApoA1 contribute to asthma pathogenesis.

The bronchial epithelium functions not only as a physical barrier but also as a regulator of innate and adoptive immune responses against foreign stimuli [13]. Studies have revealed the immunologic mechanisms by which the epithelium modulates Th2 responses through production of a group of epithelial-derived Th2-driving cytokines, including IL-25, IL-33, and TSLP [36, 38, 42]. IL-33 is a new member of the IL-1 family that is involved in regulation of innate and adaptive immune responses [43]. The airway epithelium constitutively expresses and stores IL-33, rapid release of which is induced by exposure to an environmental allergen, which drives production of cytokines and chemokines by Th2 cells, mast cells, basophils, eosinophils, and NK T cells [44]. IL-25 (also known as IL-17E), a member of the IL-17 family, has been identified as one of the initiators of the Th2 response [36], and has been shown to be expressed in the bronchial epithelium following exposure to various allergens [45, 46]. TSLP is an IL-7-like cytokine characterized by its ability to promote activation of B cells and DCs to initiate and maintain allergic airway inflammation [38, 47]. We observed that ApoA1 treatment directly inhibits IL-33, IL-25, and TSLP release in response to common allergens such as Der p1 and cockroach extract in primary bronchial epithelium (Fig. 2b). Thus, ApoA1 treatment protects

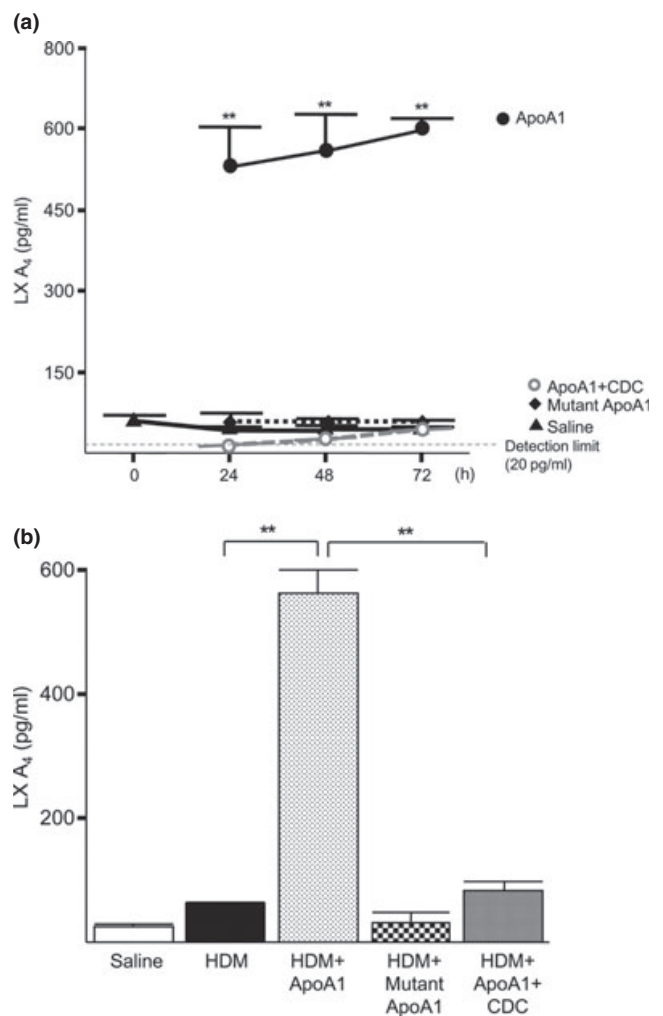


Fig. 5. Apolipoprotein A1 (ApoA1) enhanced the lipoxin A4 (LX A4) secretion in primary cultured bronchial epithelial cells (BECs) from asthmatics and allergen-challenged mouse lungs. (a) BECs were treated with ApoA1 for 72 h. LX A4 levels in the supernatants were measured by ELISA. Data are shown as means $\pm$ SEM of six independent experiments performed in triplicate $**P < 0.01$ vs. ApoA1 + cinnamyl-3,4-dihydroxy- α -cyanocinnamate. (b) The Lung LX A4 levels of house dust mite-challenged mice were measured. Data represent the means $\pm$ SEM of eight mice per group. $**P < 0.01$.

the bronchial epithelium against allergen stimuli by attenuating release of IL-25, IL-33, and TSLP, potent Th2-driven cytokines. Next, we studied the effect of ApoA1 on DC activation or accumulation in the lung following ovalbumin administration. The numbers of allergen-containing mature DCs (CD11c+/MHCII+) in lung tissues were reduced by 50% following ApoA1 treatment (Figure S1a, S1b). The reduction in the number of DCs by 50% in the draining MLN showed that this could result from a reduction in lung inflammation (Figure S1b). To determine whether ApoA1 regulates the antigen-presenting capacity or maturation of DCs to

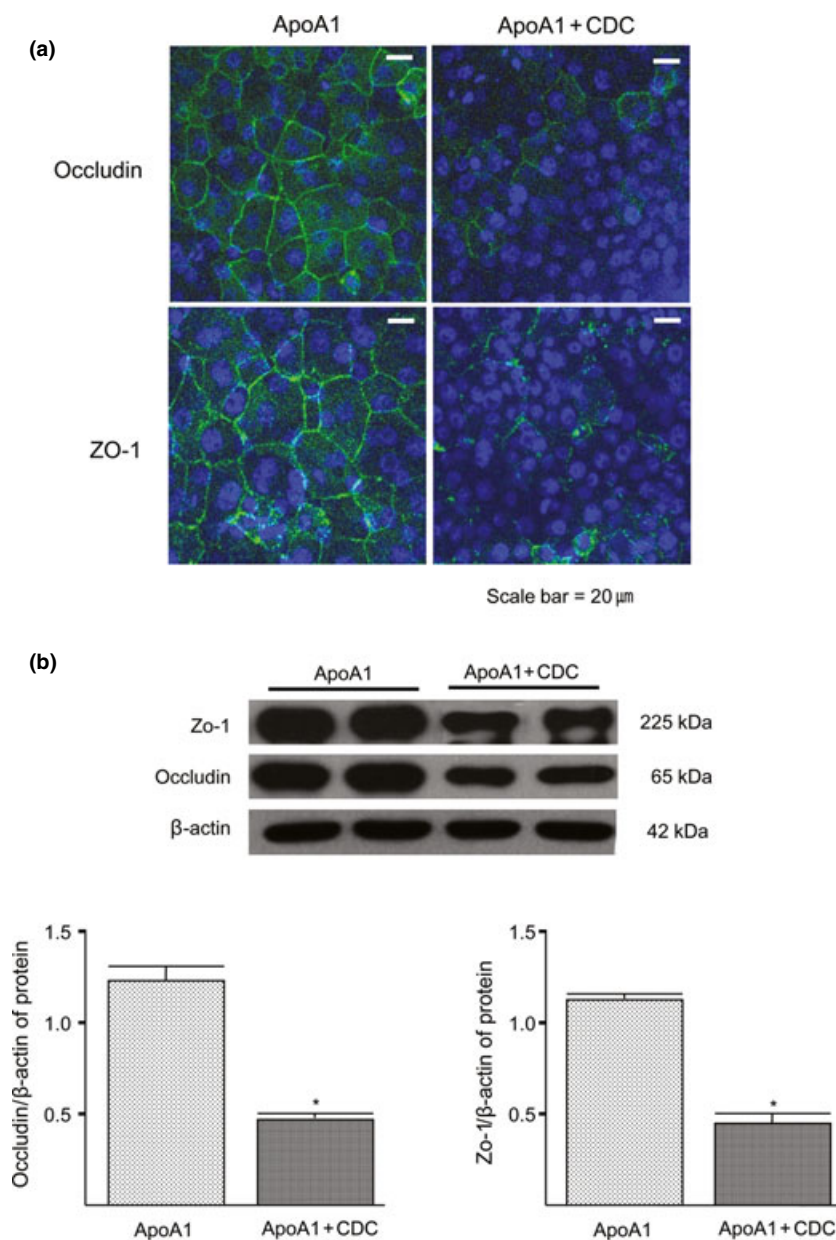


Fig. 6. Enhanced recovery of allergen-induced, disrupted tight junction molecules by Apolipoprotein A1 (ApoA1) depends on endogenous LX A₄ synthesis in the bronchial epithelium. Primary cultured Bronchial epithelial cells from asthmatics were co-treated with ApoA1 (1 μg/mL) and cinnamyl-3,4-dihydroxy-α-cyanocinnamate; 2 μM, 12/15-LOX inhibitor) for 72 h. (a) Immunofluorescence evaluation in Zo-1 and occludin expression. Scale bar = 20 μm. (b) Immunoblots of Zo-1, occludin, and beta-actin. Mean normalized densitometries of six immunoblot experiments. **P* < 0.05 vs. ApoA1.

prime for Th2 cells, *ex vivo* mature DC culture with LPS stimulation and co-culture with effector CD4 T cells were performed. ApoA1 neither modifies DC expression of co-stimulatory molecules CD40, CD83, and CD86 (data not shown) nor Th2 cell stimulation (data not shown). These findings suggest that the inhibitory effect of ApoA1 on reduction in lung DC numbers do not arise from direct effects on lung DCs.

In asthma, the response of the airway epithelium to environmental factors such as respiratory viruses and

proteolytically active allergens is impaired [12, 48]. TJ integrity is maintained by assembled components such as occludin and Zo-1 that stabilize the barrier function through coupling to the perijunctional cytoskeleton [49]. In this study, disruption of TJ proteins was observed in primary BECs obtained from asthmatics. When ApoA1 was added we observed dramatically enhanced restoration of TJ proteins at 24 h, which was maintained for 72 h (Figure S2). These data demonstrate that exogenous ApoA1 promotes restoration of TJ

integrity and contribute to re-establishment of barrier function from already disrupted bronchial epithelium. Inhaled allergens with protease activity compromise epithelial barrier function by degrading TJ proteins, facilitating allergen accessibility to DCs and damaged epithelium release of Th2 cytokines such as IL-33, IL-25, and TSLP [3]. Thus, down-regulation of IL-25, IL-33, and TSLP by ApoA1 could be, at least in part, mediated by TJ restoration. We also demonstrated that ApoA1 significantly increased LX A₄ levels in primary BECs from asthmatics (Fig. 5a). At 24 h after ApoA1 treatment, LX A₄ levels were 11 times higher than those in the mutant ApoA1 in primary BECs supernatants, and the increased LX A₄ level was maintained for 72 h (Fig. 5a). These data demonstrate that ApoA1 significantly potentiates production of endogenous LX A₄ in the inflammatory milieu in the cell. To determine whether LX A₄ is essential for recovery of the TJ proteins that were enhanced by ApoA1 treatment, ApoA1-treated primary BECs from asthmatics were pre-exposed to 12/15-LOX inhibitors. We found that blocking synthesis of LX A₄ inhibited TJ recovery in the presence of ApoA1 in primary BECs (Fig. 6a,b). An *in vivo* study also showed that CDC significantly reversed ApoA1-treated, down-regulated airway inflammation and AHR and recovered TJ proteins in the HDM-induced, murine asthma model (Figure S3). We add these sentences in response to reviewer 1's criticism.→ CDC is not completely specific for blocking LX A₄. CDC could not only decrease LX A₄ but may also affect production of other lipid metabolites such as 12,15-HETE [50, 51] in 12/15 LO pathway. Thus, it may be possible that alterations of other arachidonic acid metabolites are also involved in this *in vivo* experiment. Taken together, ApoA1-induced increased endogenous LX A₄ production is critical for TJs restoration in disrupted bronchial epithelium and promote resolution of allergic airway inflammation.

Lipoxins are biologically active eicosanoids that are synthesized at sites of inflammation, and have anti-inflammatory properties [52, 53]. LX A₄ biosynthesis is mediated by 12/15-lipoxygenase (12/15-LOX) that is expressed in the epithelium, leucocytes, and macrophages, but not in T cells and DCs [54, 55]. Thus, the absence of 12/15-LOX expression in DCs may help explain our finding that ApoA1 does not directly potentiate DC activation or antigen-presenting capacity (data not shown). However, the increased LX A₄ secretion in the ApoA1-treated bronchial epithelium may contribute to down-regulation of DC activation *in vivo* because LX A₄ is known to inhibit both migration of DCs and production of IL-12 [56]. In an experimental allergic asthma model, LX A₄ attenuated both AHR and lung inflammation via the ALX receptor, leading to decreased numbers of eosinophils and lower levels of

IL-5, IL-13, eotaxin, IgE, prostanoids, and cysteinyl leukotrienes [53]. In addition to its anti-inflammatory activity, LX A₄ stimulates neutrophils to clear apoptotic cells by phagocytosis without release of pro-inflammatory cytokines [17, 57, 58], leading to resolution of the inflammation. Planaguma et al. reported that airway LX A₄ levels and expression of lipoxin biosynthetic enzymes and receptors were markedly decreased in severe asthma [17].

Local treatment with ApoA1 mimetics has been reported to prevent allergen-induced airway inflammation by decreasing oxidative stress [27]. Recently, Dai et al. reported that ApoA1 attenuated OVA-induced neutrophilic inflammation in a murine asthma model [59]. We reported previously that ApoA1 concentrations were decreased in the bronchoalveolar lavage fluid by 2D electrophoresis in asthmatics [60]. Compared with those reports, this study has revealed several novel findings. First, we confirmed that the ApoA1 level is decreased in the airways of mild-to-moderate persistent asthmatics. Second, we showed that local ApoA1 treatment regulates the innate immune response by directly inhibiting release of IL-25, IL-33, and TSLP in the airway epithelium, inducing a Th2 response and DC activation in response to allergens. Third, we demonstrated that local ApoA1 treatment potentiates recovery of allergen-induced disrupted TJ proteins through increased production of anti-inflammatory cytokines and the pro-resolution lipid mediator LX A₄ in the bronchial epithelium. Therapeutic use of ApoA1 may be possible in other chronic airway diseases with defective TJs caused by viruses or bacteria, such as cystic fibrosis and chronic obstructive pulmonary disease [61, 62].

In conclusion, the ApoA1 protein level is decreased in the airway of mild-to-moderate persistent asthmatics compared with that in healthy controls. Local administration of ApoA1-attenuated airway inflammation and AHR in established HDM-induced murine asthma. These findings were associated with decreased production of IL-25, IL-33, and TSLP in the airway epithelium and enhanced recovery of impaired TJ proteins caused by increased levels of the pro-resolution mediator LX A₄. Taken together, these data suggest ApoA1 to be a promising therapeutic strategy for repair of defective epithelium in inflammatory lung disease.

Acknowledgement

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Conflicts of Interest

All authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographic characteristics of study subjects.

Figure S1. Intranasal ApoA1 treatment inhibits lung dendritic cell accumulation in OVA-challenged mice. (a) On days 21–23, mice were instilled intratracheally with FITC-OVA. On days 24–25, ApoA1 was administered intranasally, and then the lungs were removed on day 26. Lungs were enzymatically digested and stained for the presence of FITC–MHCII^{high}–CD11C^{high}–DCs, and subjected to flow cytometry. (b) In the same mice, the presence of FITC-migrating DCs in mediastinal lymph nodes was assessed. Results are representative of six mice per group. Experiments were repeated four times with similar results. Data are shown as means $\pm$ SEM. $*P < 0.05$.

Figure S2. Immunofluorescence of occludin (a) and Zo-1 (b) in primary BECs from asthmatics treated with ApoA1, mutant ApoA1, or co-treated with ApoA1 (1 μ g/mL) and cinnamyl-3,4-dihydroxy- α -cyanocinnamate

(CDC; 2 μ M, 12/15-LOX inhibitor) was treated for 72 h. Scale bar = 20 μ m.

Figure S3. Intranasal co-administration of CDC and ApoA1 increased airway inflammation, airway hyperresponsiveness (AHR), and disruption of bronchial TJs in HDM-induced murine asthma. (a) Numbers of bronchoalveolar lavage (BAL) fluid cells in the four groups are shown as means $\pm$ SEM; $**P < 0.01$. (b) Fixed lung tissue specimens were sectioned and stained using H&E and periodic acid Schiff. (c) Airway resistance was measured following inhalation of increasing doses of methacholine. Each data point represents the mean $\pm$ SEM of six mice per group. $*P < 0.05$ HDM + ApoA1 + CDC *vs.* HDM + ApoA1. Data for Saline, HDM, and ApoA1 groups are from the same experiment represented in Fig. 1 and are included here for comparison with the data from the HDM + ApoA1 + CDC group (studied in the same experiments but not shown in Fig. 1).

Figure S4. ApoA1 levels in BAL fluid from normal controls and subjects with asthma. ApoA1 concentration normalized to total protein as determined by ELISA. Data are presented as median values and interquartile ranges. $**P < 0.01$.

Supporting information

Table S1. Demographic characteristics of study subjects.

	Normal controls n= 14	Asthma patients n=26
Age (y) ^a	41.1 ± 4.4	38.8 ± 1.7
Male (%)	5 (35.7%)	10 (38.5%)
FEV1 (%) ^a	110.4 ± 4.2	79 ± 6.1
Atopy (%)	2 (14.3%)	12 (46.2%)
Total IgE (IU) ^b	10.8 (10.8 - 10.8)	159 (27.9 - 2053.0)
Body mass index (kg/m ²) ^a	24.21±1.01	25.15±1.1
PC ₂₀ methacholine (mg/ml) ^b	25 (8 - 25)	1.2 (0.1 - 25)
BALF (%) ^b		
Macrophage	94.5 (81 - 99)	74.5 (28 - 95)
Lymphocyte	1.9 (0 - 8)	7.1 (0 - 38)
Neutrophil	3 (0 - 16)	3.5 (0 - 25)
Eosinophil	0 (0 - 4)	9.4 (0 - 37)

Data are presented as N, percentage or ^a:Mean ± SEM, ^b: median (range)

