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Epithelial Folliculin Enhances Airway Inflammation in Aspirin-Exacerbated Respiratory Disease

Running title: Folliculin in Aspirin-exacerbated Respiratory Disease

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Abstract

Background

Clinical features of aspirin-exacerbated respiratory disease (AERD) are characterized by overproduction of cysteinyl leukotrienes (LT) and eosinophil activation, in which epithelial cells contribute to eosinophilic airway inflammation. Folliculin (FLCN) helps maintain the integrity of epithelial barrier, but little is known about FLCN in AERD.

Objective

We investigated the role of FLCN in the pathogenic mechanisms of AERD.

Methods

We recruited 178 subjects with AERD, 276 subjects with aspirin tolerant asthma (ATA) and 71 normal healthy controls (NC) at Ajou Medical Center. Levels of FLCN and interleukin (IL)-8 in sera and supernatants were measured by ELISA. Peripheral blood eosinophils isolated from asthmatic patients were cocultured with human airway epithelial cells (HAECs) pretreated with LTE₄, dexamethasone and montelukast. The intracellular expressions of FLCN, tight (TJ) (occludins, claudin-1) and adherens (AJ) junctions (E-cadherin) were analyzed by Western blotting. shRNA was used to down-regulate *FLCN* (*shFLCN*) in HAECs.

Results

Serum FLCN levels were significantly higher in AERD group than in ATA and NC groups (all $P < 0.001$). The cutoff value of 56.6 pg/mL was used to define the high FLCN phenotype (highFLCN). Asthmatic patients with highFLCN were associated with increased airway hyperresponsiveness to methacholine ($P = 0.015$). The serum FLCN level could discriminate AERD group from NC group with 82% sensitivity (AUC = 0.793, $P < 0.001$). When HAECs were exposed to LTE_4 , FLCN release was increased significantly ($P < 0.05$), which were amplified along with disruption of TJ and AJ expressions when HAECs were co-cultured with eosinophils and LTE_4 (all $P < 0.05$); these effects were suppressed by dexamethasone and montelukast. *FLCN* knockdown reduced IL-8 release and occludin expression from *shFLCN* HAECs.

Conclusions

Our findings suggest that high LT and airway eosinophilia increased FLCN release from HAECs, which enhance epithelial activation and disruption. Modulation of FLCN may be a potential target for AERD.

Key words: FLCN, epithelial cells, eosinophils, cysteinyl leukotrienes, AERD

Introduction

Aspirin-exacerbated respiratory disease (AERD) is a major phenotype of non-steroidal anti-inflammatory drug (NSAID) hypersensitivity. Patients with AERD present moderate-to-severe upper and lower respiratory symptoms exacerbated by NSAID exposure and chronic rhinosinusitis (CRS) with nasal polyps (NPs) as common comorbid conditions.[1] AERD is found in 7%-20% of adult asthmatic patients.[2] There have been convincing data on pathological characteristics of AERD including release of proinflammatory lipid mediators, such as cysteinyl leukotrienes from effector cells, as well as involvement of eosinophilia.[3-5] Overproduction of leukotrienes (LT) and LT synthetic enzymes in the resting state and in response to aspirin was observed in AERD.[6] Inhalation of LT E₄ enhances migration of inflammatory cells to the lung and nasal mucosa. In addition, eosinophils from AERD show an increased number as well as a highly activated state in nasal mucosa.[6]

Airway epithelial layer functions in the innate immunity by providing the first barrier against environmental pathogens and internal stimuli such as LTs and cytokines.[7] Impairment of the epithelial barrier function is relevant in asthma and AERD, including epithelial apoptosis and changes of junctional complexes.[7] Apoptosis of epithelial cells may result in the loss of the epithelial barrier, consequent alveolar flooding and penetration of the pathogens.[8] In subjects with AERD and CRS accompanied by NPs, the epithelial barrier is disrupted due to detachment of epithelial cells from the basement membrane and damage to intercellular junctions.[9] The close interplay between epithelial cells and eosinophils induces mucus production as well as release of interleukin (IL)-8 and transforming growth factor (TGF- β)-1 in the supernatant.[10, 11] Folliculin (FLCN) is an intracellular protein, located in the cytoplasm of various cell types including alveolar epithelial cells.[12-15] Over the past few years, attention has been paid to FLCN due to its intersection with various cellular signaling pathways. Through these pathways, FLCN contributes to activating epithelial cells and

maintaining the integrity of the epithelial barrier by regulating cell-cell adhesion and expression of cell-cell junctions.[16, 17]

In patients with asthma and AERD, the role of FLCN is not clearly understood. In subjects who were exposed to toluene-diisocyanate (TDI), higher serum FLCN levels were noted in association with epithelial cell activation.[18] Hence, this study was aimed to investigate the role of FLCN in airway epithelial cells in AERD. We hypothesized that FLCN may be associated with activation and integrity of epithelial cells, thereby contributing to AERD pathogenesis.

Methods

Study subjects

Three study groups including 178 subjects with AERD, 276 subjects with ATA and 71 normal healthy controls (NC) were recruited from the Department of Allergy and Clinical Immunology, Ajou University Medical Center, Suwon, South Korea. All subjects were investigated for clinical history, chest radiography and pulmonary function tests. The diagnosis of asthma was established by recurrent episodes of wheezing, dyspnea, cough and airway hyperresponsiveness to methacholine as indicated in the Global Initiative for Asthma guideline.[19] Subjects with stable asthma and baseline forced expiratory volume in 1s (FEV_1) $\geq 50\%$ predicted values were evaluated for responsiveness to methacholine (Mch) challenge in accordance with American Thoracic Society Standards.[20] In this assay, subjects were indicated to inhale aerosolized normal saline, followed by aerosolized saline containing Mch with increasing doses from 0.03 to 25.0 mg/mL. Mch was administered until either FEV_1 fell by 20% of baseline or until the final dose. The test was considered positive if the subject experienced a drop in $FEV_1 \geq 20\%$ compared to baseline before or immediately

after administration of the final dose. The concentrations of MCh required to cause a 20% production in FEV₁ (MChPC₂₀) were recorded. CRS was defined as an inflammatory condition consisting of paranasal sinusitis and/or NP that lasts 12 weeks or longer along with compatible radiologic findings. Serum total IgE levels were measured using the ImmunoCAP system (ThermoFisher Scientific, Waltham, MA, USA), ranging: between 2 and 5000 kU/L.

Determination of asthma phenotype

AERD is defined as a clinical condition which develops typical reactions including upper/lower respiratory tract symptoms after ingestion of aspirin (ASA) or cross-reacting NSAIDs with or without CRS and/or NP. The confirmative diagnosis of aspirin hypersensitivity was established by the aspirin-lysine bronchoprovocation test (L-ASA-BPT) after the patients were asked to stop all anti-asthmatic drugs, including leukotriene modifiers, for at least 3 days prior to the test.[21] The L-ASA-BPT was performed with increasing doses of L-ASA solution (up to 300 mg/mL) as previously described, and the test was considered “positive” when FEV₁% dropped by more than 15% after the challenge. Asthma severity was evaluated according to the American Thoracic Society Standards workshop on severe asthma.[20] All of the participants provided written informed consent, and the study protocol was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). All methods were performed in accordance with the relevant guidelines and regulations.

Measurement of inflammatory cytokines

Commercial ELISA kits were applied to measure the levels of FLCN (CUSA Biotech, Wuhan, Hubei Province, China), IL-8 (Endogen, Woburn, MA, USA) and TGF- β 1 (R&D Systems, Inc, Minneapolis, MN, USA), in sera or cell-free supernatants, in accordance with the manufacturer's instructions.

Antibodies, chemicals and reagents

Antibodies to detect FLCN (Cell Signaling Technology, Danvers, MA, USA), tight junction (TJ) proteins (occludin, [ThermoFisher Scientific], claudin-1 [Invitrogen, Waltham, MA, USA]) and adherens junction (AJ) protein (E-cadherin; Santa Cruz Biotechnology, Inc, Dallas, Texas, USA) were used for Western blotting.

Isolation of eosinophils

Primary blood eosinophils (PBE) were isolated from 5 patients with asthma as previously described.[22] Briefly, blood was layered on the Lymphoprep (Axis-Shield, Oslo, Norway), followed by density-gradient centrifugation at 2,000 rpm at 20°C for 25 minutes without brakes. The granulocyte fraction was lysed for red blood cells by cold distilled water for 30 seconds. Subsequently, eosinophils were isolated from the above fraction using the Eosinophil Isolation Kit (Miltenyl Biotec Inc., Auburn, CA, USA). Cell viability (>98%) and cell purity (>95%) were assessed by trypan blue staining (Sigma Aldrich) and flow cytometry by labeling with Siglec-8 antibody, respectively.

Human airway epithelial cell (HAEC) culture and treatment

The human lung carcinoma cell line (A549) and primary small airway epithelial cells (SAECs) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). A549 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium and 100 µg/mL streptomycin sulfate (all from Gibco, Grand Island, NY, USA) and maintained at 37°C with 5% CO₂ in humidified air. SAECs were cultured within 3 to 8 passages in airway epithelial cell basal medium supplemented with the Bronchial Epithelial Cell Growth Kit (all from ATCC). A549 (2x10⁵ cells) and SAECs (1x10⁵ cells) were seeded onto a 12 well-plate (TPP, Trasadingen, Switzerland) until the cells reached around 80% of confluency. In some experiments, cells were stimulated with LTE₄ (50, 100 and 200 nM) in serum-free RPMI medium for 24 hours. Cells were pretreated with dexamethasone (1 µM) and montelukast (1 µM) for 1 hour prior to experiments. HAECs were co-cultured with PBE at ratios of HAECs to PBE of 1:0.5 and 1:2.5 for 16 hours. Supernatants were harvested for further ELISA analysis and the cell lysates were kept for Western blotting. Ethanol was added to the negative control well as a solvent control. Mock-treated cells were used as the negative control.

Knockdown system

In order to evaluate for the role of FLCN in airway epithelial cells, we performed knockdown using short hairpin (sh) RNA against FLCN. Bacteria (*Escherichia coli*) stocks containing shRNA against FLCN (*shFLCN*), p-CMV-VSV-G, a gift from Bob Weinberg (Addgene plasmid # 8454) and psPAX2, a gift from Didier Trono (Addgene plasmid #12260), were cloned into a PLKO.1 vector. Non target (*shControl*) shRNA was used as a control. FLCN shRNA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Briefly, FLCN shRNA was

co-transfected with the p-CMV-VSV-G vector and the psPAX2 vector into HEK293TN cells (System Biosciences, Mountain View, CA, USA) using Lipofectamine[®] 2000 Transfection Reagent (ThermoFisher Scientifics) to obtain pseudo-lentivirus particles. After 48 hours, virus particles were harvested, with which A549 cells were infected in the culture medium supplemented with hexadimethrine bromide (8 µg/mL). Forty-eight hours later, lentivirus-infected A549 cells underwent selection by puromycin (2 µg/mL) to produce stable *FLCN*-knockdown cell lines. The knockdown level of *FLCN* was confirmed by Western blotting (>70%).

Statistical analysis

Data are presented as mean ± SD for continuous variables and percentage for categorical variables. In order to correct the skewed distribution, serologic data were log-transformed prior to analysis. The cutoff value of serum FLCN was chosen by mean + 2 SD of serum FLCN levels in the NC group. *P* values were analyzed by the Mann-Whitney *U* test for continuous variables or Pearson's Chi square test for categorical variables. Comparisons were made by one way ANOVA with the Tukey *post hoc* test between the experimental groups. Correlations among continuous clinical variables were calculated by Spearman's rank correlation coefficient. To evaluate the diagnostic value of serum FLCN in the discrimination between AERD and other phenotypes, a receiver operating characteristic (ROC) curve analysis was performed. A *P* value of <0.05 was considered significant. All statistical analyses were performed with SPSS statistical software version 23 (SPSS Inc, Chicago, IL, USA).

Results

Clinical characteristics

The number of females was larger in subjects with AERD (69.66%) than in those with ATA (58.55%), with statistical significance ($P = 0.017$). The prevalences of CRS and severe asthma were significantly higher in the AERD than in the ATA group (CRS: 60.67% vs. 22.1%, severe asthma: 14.1% vs. 13.62%) ($P < 0.05$ for each). In addition, the AERD group showed lower levels of FEV₁% predicted values and MChPC20, and a greater fall in FEV₁ after the L-ASA BPT ($P < 0.05$ for each). There were no differences in age, atopic status or total IgE level between the groups.

Comparison of serum FLCN levels in the 3 study groups

Serum FLCN levels were significantly higher in the AERD group (1.6 ± 0.33 pg/mL) than in ATA (1.51 ± 0.27 pg/mL) and NC groups (1.31 ± 0.21 pg/mL) ($P < 0.05$ for each) (Fig. 1).

When serum FLCN level was higher than the cut-off value which was defined as mean + 2SD (56.6 pg/mL; 1.75) of the serum FLCN level from NC group, high FLCN phenotype (highFLCN) was classified, while low FLCN phenotypes (lowFLCN) had lower FCN levels less than the cutoff value. The prevalence of highFLCN was higher in the AERD group (34.3%) than in the ATA (18.5%) and NC (4.2%) groups ($P < 0.001$ for all). In order to evaluate the diagnostic value of serum FLCN level, we applied the ROC curve to choose the optimal cutoff level (Fig. 2). The serum FLCN level was able to differentiate the AERD group from the ATA group at the optimal cutoff value of 28.1 pg/mL with 64% sensitivity and 50.4% specificity (AUC = 0.619; 95% CI = 0.567-0.671, $P < 0.001$) (Fig. 2A). In addition, to differentiate the AERD group from the NC group, the optimal cutoff value was at

10.16 pg/mL, with 82% sensitivity and 67.6% specificity (AUC = 0.793; 95%CI = 0.732-0.854, $P < 0.001$) (Fig. 2B).

Association of serum FLCN and clinical variables

Asthmatic subjects with highFLCN had a significantly lower MChPC₂₀ level ($P = 0.015$) (Fig. 3A). Within the AERD group (Fig. 3B), subjects with highFLCN had a significantly higher serum TGF- β 1 level ($P = 0.029$). We could not find any significant differences in the prevalence of CRS, severe asthma, atopy, FEV1% predicted values, total IgE or eosinophil count between subjects with highFLCN and lowFLCN (data not shown).

Effects of LTE₄ with or without eosinophils on FLCN production from HAECs

We aimed to investigate the mechanisms by which FLCN is produced from HAECs in AERD. Considering that AERD is characterized by eosinophilic inflammation and increase in LTE₄ as *in vitro* assays, LTE₄ was incubated with 2 HAECs, A549 cells and SAECs. When HAECs were stimulated by LTE₄ alone, significant releases of FLCN were noted from A549 and SAECs ($P < 0.05$ for each) as shown in Figs. 4 and 5. However, we could not find a significant increase in IL-8 from HAECs. Furthermore, when the A549 cells were primed with LTE₄ (200 nM) for 1 hour prior to the experiments and were incubated with PBE from asthmatic patients, FLCN production was further increased along with a significant increase in IL-8 ($P < 0.05$) compared to conditions of LTE₄- or PBE-stimulated cells (Fig. 4 A-C, Fig. S1). Similarly, LTE₄-stimulated SAECs with PBE significantly enhanced release FLCN and IL-8 compared to LTE₄-stimulated SAECs without PBE (Fig. 5A-C, Fig. S1). A significant positive correlation was noted between FLCN and IL-8 levels released from A549 cells ($r =$

0.53; $P < 0.001$) (Fig. 4D), which was suppressed by pretreatment with dexamethasone and montelukast.

Effects of LTE_4 and PBE on epithelial junctional proteins

Stimulation of HAECs with LTE_4 significantly induced epithelial apoptosis, which was attenuated by dexamethasone and montelukast significantly ($P < 0.05$ for each) (Fig. S2).

However, there were no significant effects of LTE_4 on the expression of TJ or AJ. In the co-culture of LTE_4 -primed A549 with PBE, the higher number of PBE downregulated the expression of E-cadherin, occludin and claudin-1, which were restored significantly by the treatment with dexamethasone and montelukast (Fig. 6A). Co-culture of SAECs with PBE or LTE_4 disrupted the expression of occludin and claudin-1 (even more robustly) which were recovered by montelukast (Fig. 6B). The expression of E-cadherin was not detected in SAECs.

Role of FLCN in IL-8 release and dysintegrity of epithelial junctional proteins

To confirm the role of FLCN in epithelial integrity, *shFLCN* A549 cells were prepared.

Knockdown of FLCN in the *shFLCN* A549 cells significantly led to the down-regulation of occludin ($P < 0.05$), but increased the expression of claudin-1 ($P < 0.05$) compared to the *shControl* A549 cells. No significant down-regulation was observed for E-cadherin (Fig. 7A).

In contrast, to the *shControl* A549 cells *shFLCN* A549 cells incubated with LTE_4 and PBE from asthmatics did not show IL-8 release (Fig. 7B). Pre-incubation of the *shControl* A549 cells with anti-FLCN antibody (0.5 $\mu\text{g/mL}$) down-regulated IL-8 release from airway epithelial cells co-cultured with PBE. These findings suggest that intracellular and

extracellular FLCN may share the same role in the IL-8 release pathway. Co-culture of *shFLCN* A549 cells with PBE and LTE_4 remarkably increased the claudin-1 expression ($P < 0.05$) and did not interfere with E-cadherin or occludin (Fig. 7C).

Discussion

To the best of our knowledge, this is the first study to investigate FLCN in AERD and to demonstrate a significantly higher serum FLCN level in AERD subjects compared to ATA and NC subjects. In addition, the serum FLCN level can differentiate the AERD group from the ATA and NC groups, suggesting that serum FLCN may be a potential serum biomarker for differentiating the phenotypes of AERD. In *in vitro* assay, when HAECs were exposed to LTE_4 and PBE, more release of FLCN and IL-8 and more disruption of epithelial cell integrity were noted. Furthermore, knockdown of FLCN in HAECs suppressed epithelial cell activation and maintained epithelial integrity. These findings implicated a novel link between FLCN and the epithelial cell role in the airway inflammation of AERD.

Previous work has generally focused on the role of FLCN in the Birt-Hogg-Dubé syndrome and pneumothorax in adults,[12, 14, 15, 23, 24] ; however, there is only 1 report showing the association of FLCN in TDI-induced occupational asthma.[18] In the present study, the asthmatic patients with highFLCN were associated with airway hyperresponsiveness to MCh. However, among the patients with AERD, highFLCN was not directly associated with the features of AERD such as severe asthma, airway hyperresponsiveness and CRS/NPs, indicating that serum FLCN may not be directly associated with key clinical features, but contribute to aggravating the severity of airway inflammation in AERD, as AERD subjects with highFLCN had a higher serum TGF- β 1 level. TGF- β 1 is produced from various cells, in

which eosinophil-derived TGF- β 1 is the major source for provoking the epithelial to mesenchymal transition, hence worsening airway inflammation and remodeling.[25, 26]

We targeted epithelial cells, an abundant source of FLCN in order to evaluate its role in AERD.[17] The effects of LTE₄ on epithelial apoptosis and FLCN, but not IL-8, indicated other mechanisms for epithelial activation. Several studies have advocated that eosinophil-epithelial cell interactions induce activation of epithelial cells and IL-8 production.[22, 27] Similarly, we found increased IL-8 levels from HAECs, when PBE were co-cultured with epithelial cells. Moreover, PBE from asthmatic patients increased the production and release of FLCN from HAECs, which was further enhanced when primed with LTE₄. These findings suggest that high LTE₄ environment in the airway of AERD patients (in both conditions, at baseline and when challenged by aspirin/NSAIDs) may prime epithelial cells to be activated by eosinophils. IL-8 is responsible for the activation of neutrophils and eosinophils in asthmatic airways, as well as the migration of mast cell [28-30], thereby amplifying airway inflammatory responses in AERD. The decreased IL-8 level by blocking FLCN in HAEs indicates that FLCN is involved in epithelial cell activation, and thus the epithelial-eosinophil interaction may enhance eosinophil activation in the airway mucosa of AERD.

The role of FLCN in maintaining the integrity of epithelial barrier has been extensively studied.[12, 16, 17, 24] The integrity of the epithelial barrier helps maintain airway permeability in AERD.[31, 32] Emerging evidence reveals the increased level of E-cadherin containing microparticles as a hallmark of epithelial injury in AERD.[9] When the epithelial layer is disturbed, uptake of allergens and viruses may trigger either allergic inflammation or toll-like receptor-dependent pathways.[33] In addition, signal transduction cascades in HAECs are more likely to be dysregulated, affecting activation and differentiation of epithelial cells.[7, 34] In the present study, we demonstrated that eosinophils disrupted the

expression of AJ and TJ in HAECs, which are comparable to the results of previous reports.[26, 35] In addition, LTE_4 was able to aggravate eosinophil-induced epithelial disruption in AJ and TJ. Interestingly, in *shFLCN* A549 cells, LTE_4 and PBE failed to induce IL-8 release and to perturb E-cadherin, occludin and claudin-1 levels. There are 2 plausible explanations, including the effect of FLCN on expression of TJ and AJ proteins, and IL-8 release. The lowered expression levels of occludin and E-cadherin in *shFLCN* A549 cells may not be further suppressed upon exposure to LTE_4 and eosinophils. Additionally, the remarkably increase of claudin-1 could protect HAECs from the effects of LTE_4 and eosinophils, since claudin-1 is more relevant in TJ-strand formation [36] and permeability in cell layers.[37] IL-8 is documented to down-regulate the expression of TJ proteins [28]; therefore, IL-8 decrease in *shFLCN* A549 cells incubated with LTE_4 and PBE may maintain the integrity of TJ and AJ proteins. These data reinforced the close association between FLCN/IL-8 and epithelial activation dysintegrity in asthmatic airway of AERD. Increased FLCN in sera of AERD patients may up-regulate HAECs to be more activated and disrupted, thereby facilitating airway inflammation in AERD. In addition, the release of TGF- β 1 from HAECs being co-cultured with PBE and LTE_4 was elevated significantly (Fig. S3). These results imply that patients whose airway epithelial cells are highly activated may have the up-regulation of TGF- β 1, which contributes to airway remodeling to some extent.

Inhaled steroid and leukotriene modifiers are commonly prescribed medications in the long-term management of asthma and AERD. In the present study, montelukast and dexamethasone was able to suppress the release of FLCN and IL-8 from HAECs as well as recover the expression of AJ and TJs. Dexamethasone and montelukast were effective in attenuating epithelial cell apoptosis mediated by LTE_4 (Fig. S2), which may improve airway permeability and epithelial injury.[8] Steroids, potent anti-inflammatory drugs, were found to induce eosinophil apoptosis and to suppress the granulocyte-macrophage colony-stimulating

factor (GM-CSF) released from epithelial cells, which enhances eosinophil survival.[37, 38] Steroids also inhibited the *IL-8* gene expression in HAECs possibly through the inhibiting the phosphatidylinositol 3-kinase/Akt pathway,[39, 40] which is implicated in FLCN signaling cascades; thus, the blocking effect of dexamethasone on FLCN may be exerted through this pathway, although further studies are warranted.[14] Similarly, montelukast, a cysteinyl LT receptor-1 antagonist, may elicit its effects by inhibiting the release of GM-CSF and eosinophil survival.[41] Montelukast was able to inhibit IL-8 release from nasal epithelial cells in patients with asthma and allergic rhinitis.[42] More importantly, convincing evidence showed that montelukast was able to reverse airway remodeling induced by eosinophils,[26, 35] which should be further elucidated by recovery of TJ and AJ proteins as shown in the present study. Taken together, dexamethasone and montelukast are involved in restoring epithelial activation and dysintegrity induced by LTE₄ and eosinophils in AERD.

In conclusion, high cysLT and airway eosinophilia increase FLCN release from HAECs, which enhances epithelial activation and disruption. Modulation of FLCN may be a potential therapeutic target for AERD.

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Conflict of interests

There is no conflict of interests related with this study.

Author contributions

HKTT performed the experiments, analyzed data and wrote the manuscript. DLP assisted the experiments and data analysis. YWC, SHK assisted the experimental design and data interpretation. HMK supported the experiments with human serum samples. HSP supervised the study design and progress, revised the first and subsequent drafts of manuscript.

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Table. Clinical characteristics of the study subjects

	AERD	ATA	Non atopic NCs	<i>P value</i>		
	(n = 178)	(n = 276)	(n = 71)	AERD vs. ATA	AERD vs. NC	ATA vs. NC
Age (yr)	42.23 ± 13.17	44.53 ± 15.17	39.54 ± 13.86	0.094	0.152	0.012
Gender (female, %)	124 (69.66%)	161 (58.55%)	45 (63.38%)	0.017	0.338	0.459
Atopy (positive, %)	81/173 (46.82%)	125/251 (49.8%)	NA	0.549	NA	NA
Smokers (%)	64/137 (46.71%)	90/115 (70.31%)	NA	<0.001	NA	NA
CRS (presence)	108 (60.67%)	39 (14.1%)	NA	0.023	NA	NA
Severe asthma (%)	35/159 (22.01%)	32/235 (13.62%)	NA	0.003	NA	NA
FEV ₁ (% predicted)	85.17 ± 20.56	90.20 ± 20.32	NA	0.023	NA	NA
Percentage decrease of FEV ₁	23.16 ± 11.63	6.73 ± 4.57	NA	<0.001	NA	NA

after Lys-ASA-BPT (%)

MChPC ₂₀ (mg/mL)	6.02 ± 10.46	12.42 ± 12.04	NA	<0.001	NA	NA
Serum total IgE (IU/L)	382.70 ± 615.19	405.25 ± 682.13	NA	0.729	NA	NA

AERD, aspirin-exacerbated respiratory disease; ATA, aspirin- tolerant asthma; CRS, chronic rhinosinusitis; FEV₁, forced expiratory volume in 1 s; MChPC₂₀, concentration of methacholine required to produce a 20% decrease in FEV₁; N, number of patients; NA, not available; NC, normal healthy controls. The continuous data are presented as the means ± SD. The dichotomous data are presented as numbers (%). Data were analyzed with the *t* test for continuous variables and the χ^2 test for categorical variables. Values in bold indicate statistical significance.

Figure Legends

Fig. 1. Comparison of serum FLCN levels between subjects with aspirin-exacerbated respiratory disease (AERD), those with aspirin tolerant asthma (ATA) and normal healthy controls (NC). Data are presented as means $\pm$ SEM. Log-transformed data are shown to correct the skewed distribution of the serum FLCN level. *P* values were analyzed by the Mann-Whitney *U* test.

AERD, aspirin-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; NC, normal healthy controls.

Fig. 2. Utility of the serum FLCN level to discriminate the phenotypes of AERD. The receiver operating characteristic curve (ROC) analysis of serum FLCN levels was performed to determine the cutoff for the diagnosis of AERD. (A) Discrimination between the AERD and ATA group; (B) diagnosis of AERD from NC groups. Abbreviations as in Fig. 1.

Fig. 3. Association of the highFLCN phenotype with clinical characteristics. (A) MChPC₂₀ values in asthmatic patients with highFLCN or lowFLCN; (B) comparison of serum levels of TGF- β 1 between AERD patients with highFLCN and lowFLCN phenotype. The cutoff value was determined by the means +2 SD. *P* values were analyzed by the Mann-Whitney *U* test.

Fig. 4. Eosinophil-induced release of FLCN and IL-8 from A549. Cells (2×10^5) were seeded onto a 12 well- plate. Some cells were pretreated with LTE₄ (200 nM), dexamethasone (1 μ M) and montelukast (1 μ M) for 1 hour prior to the experiments. PBE isolated from asthmatic patients and co-cultured with A549 cells. (A and B). Increased FLCN release and reduced local expression of FLCN from A549 cells. (C) Increased release of IL-8 from A549. *P* values were analyzed by using one-way ANOVA with the Tukey *post hoc* test. (D) Correlation of FLCN and IL-8 release from A549. *P*

values were analyzed by the Pearson coefficient correlation test. Data are presented as means $\pm$ SD at least 3 to 5 independent experiments.

, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the negative control; # $P < 0.05$ and ## $P < 0.01$ between the indicated groups. Dex, dexamethasone; FLCN, folliculin; IL-8, interleukin-8; Mon, montelukast.

Fig. 5. Eosinophil-induced release of FLCN and IL-8 from SAEC. Cells (2×10^5) were seeded onto a 12-well plate. Some cells were pretreated with LTE_4 (200 nM), dexamethasone (1 μM), montelukast (1 μM) for 1 hour prior to the experiments. PBE isolated from asthmatic patients and co-cultured with SAECs. (A and B) Increased FLCN release and reduced local expression of FLCN from SAECs. (C) Increased release of IL-8 from SAECs. P values were analyzed by one-way ANOVA with the Tukey *post hoc* test. (D) Correlation of FLCN and IL-8 release from SAEC. P values were analyzed by the Pearson coefficient correlation test. Data are presented as means $\pm$ SD from at least 3 to 4 independent experiments.

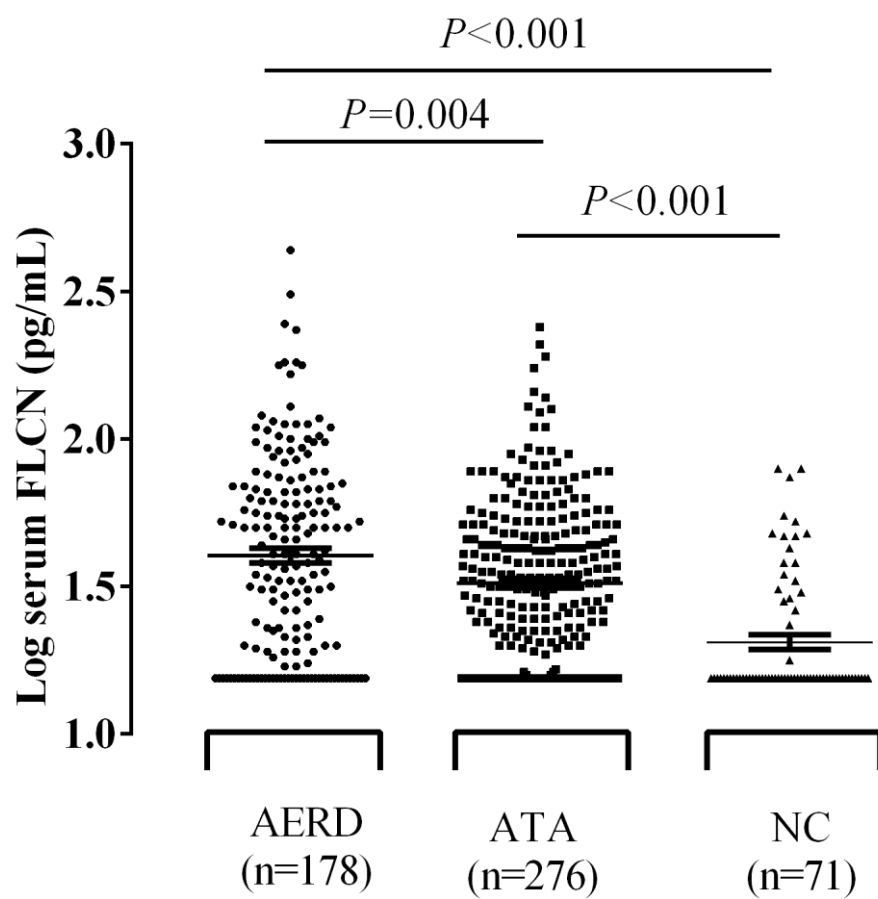
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the negative control; # $P < 0.05$ compared between the indicated groups. Abbreviations were used as in Fig. 3.

Fig. 6. Effects of eosinophils on cell-to-cell junctions of epithelial cells. Cell lysates were kept to investigate the expression levels of E-cadherin, occludin and claudin-1 using Western blot analysis. Expression levels of cell junctions in (A) A549 and (B) SAEC are displayed. The relative densities of cell junctions compared to β -actin were calculated. Representative images from at least 3 to 5 independent experiments are shown. P values were analyzed by one-way ANOVA with the Tukey *post hoc* test.

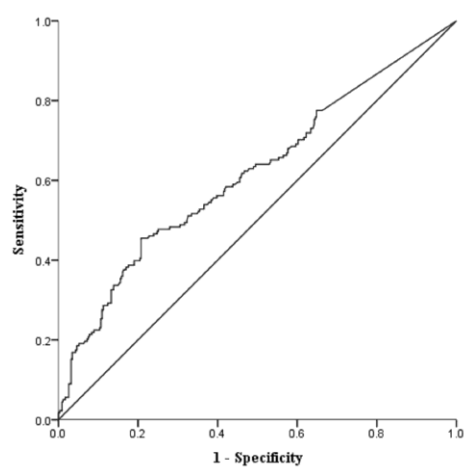
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the negative control; # $P < 0.05$ compared between the indicated groups. Abbreviations were used as in Fig. 3.

Fig. 7. FLCN required for epithelial function and integrity in AERD. (A) The knockdown level of *FLCN* was evaluated by Western blotting. (B) IL-8 release from *shFLCN* A549 cells co-cultured with PBE compared with *shControl* A549 cells. Some *shControl* A549 cells were pretreated with anti-*FLCN* antibody. (C) The expression levels of E-cadherin, occluding and claudin-1 in the *shFLCN* A549 cells co-cultured with PBE. Data are shown as results from three independent experiments.

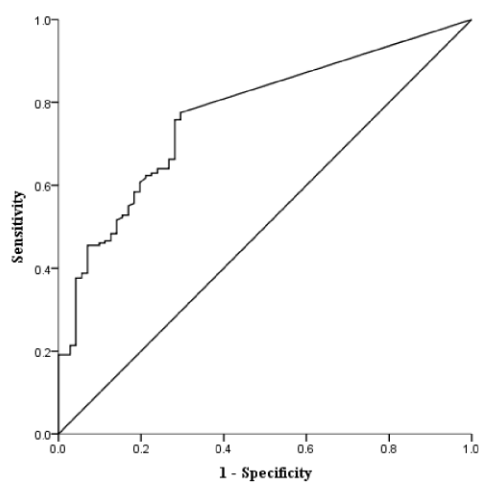
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the negative control; # $P < 0.05$ compared to the stimulated group. Abbreviations were used as in Figure 3.



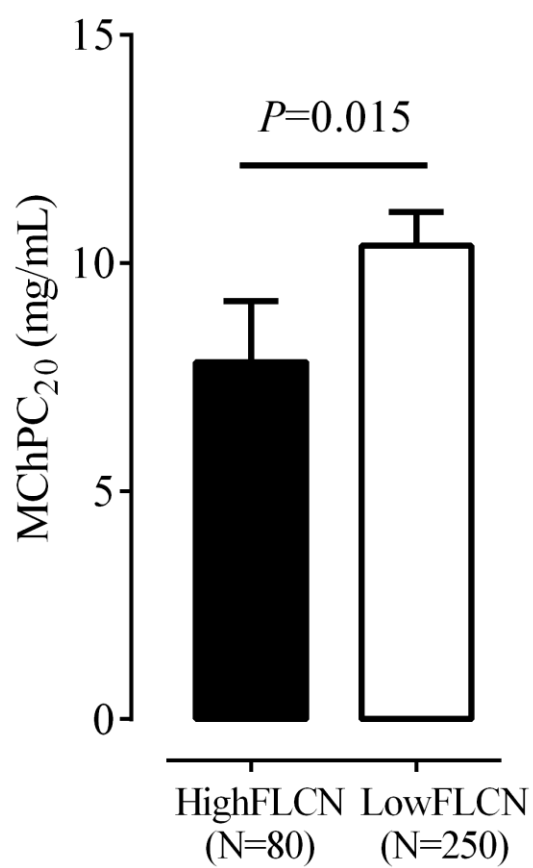
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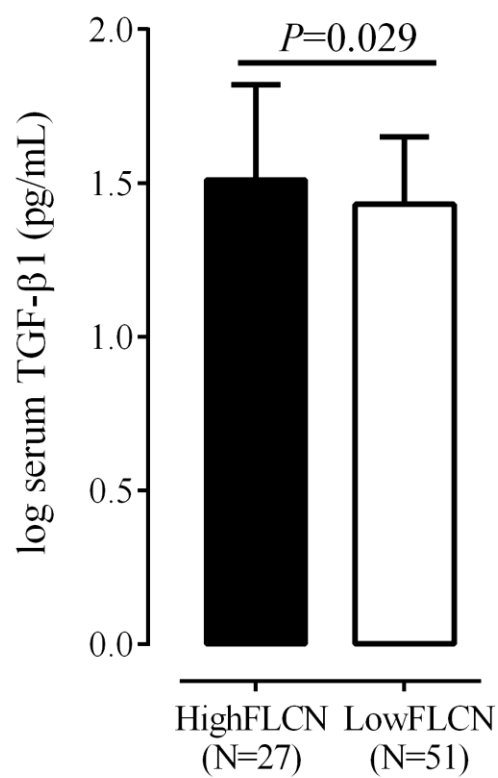
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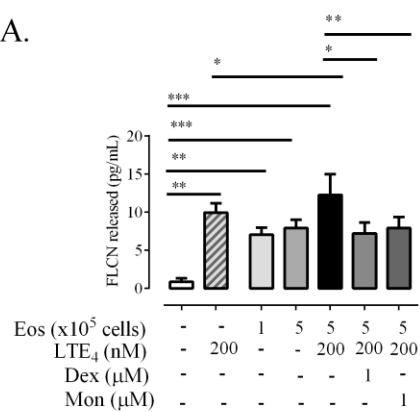
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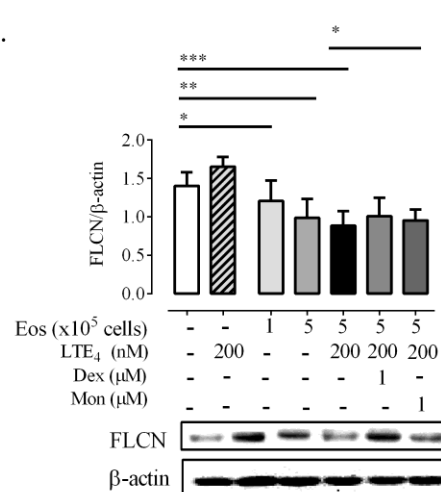
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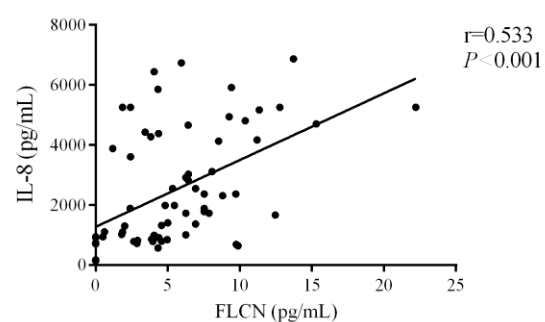
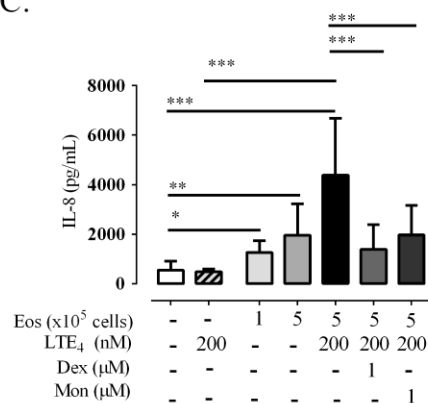
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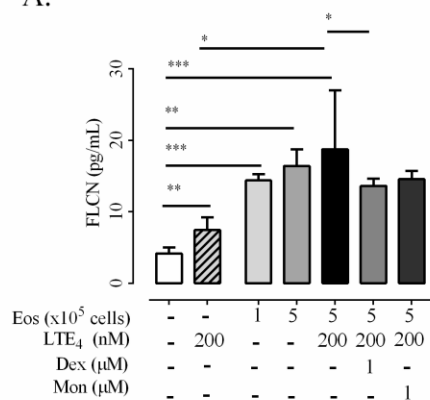
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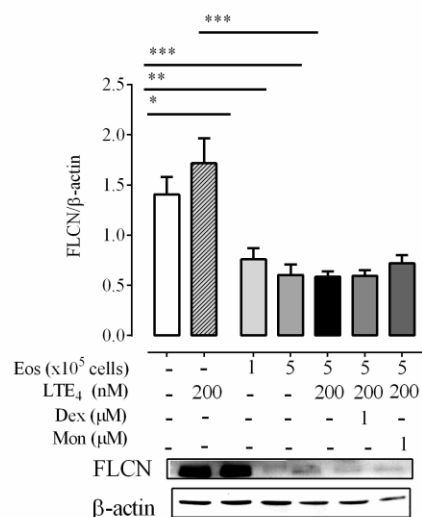
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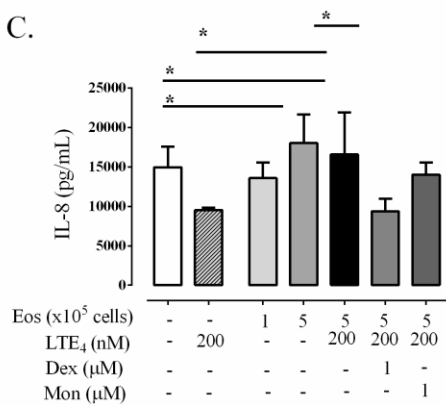
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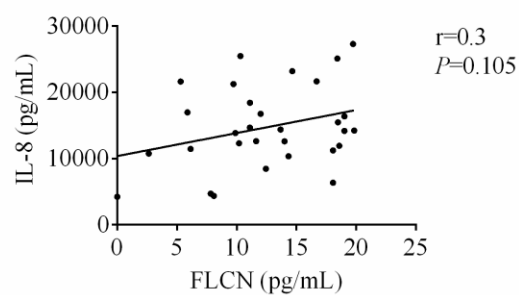
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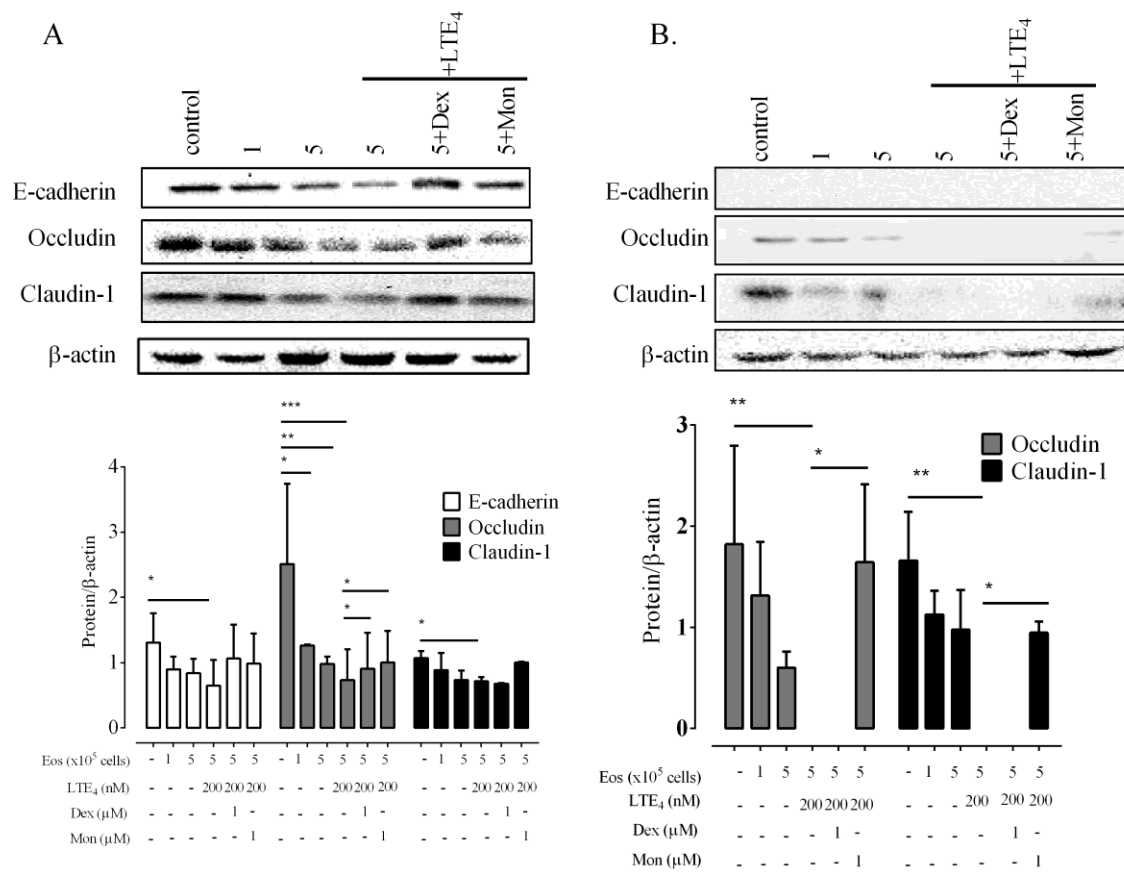


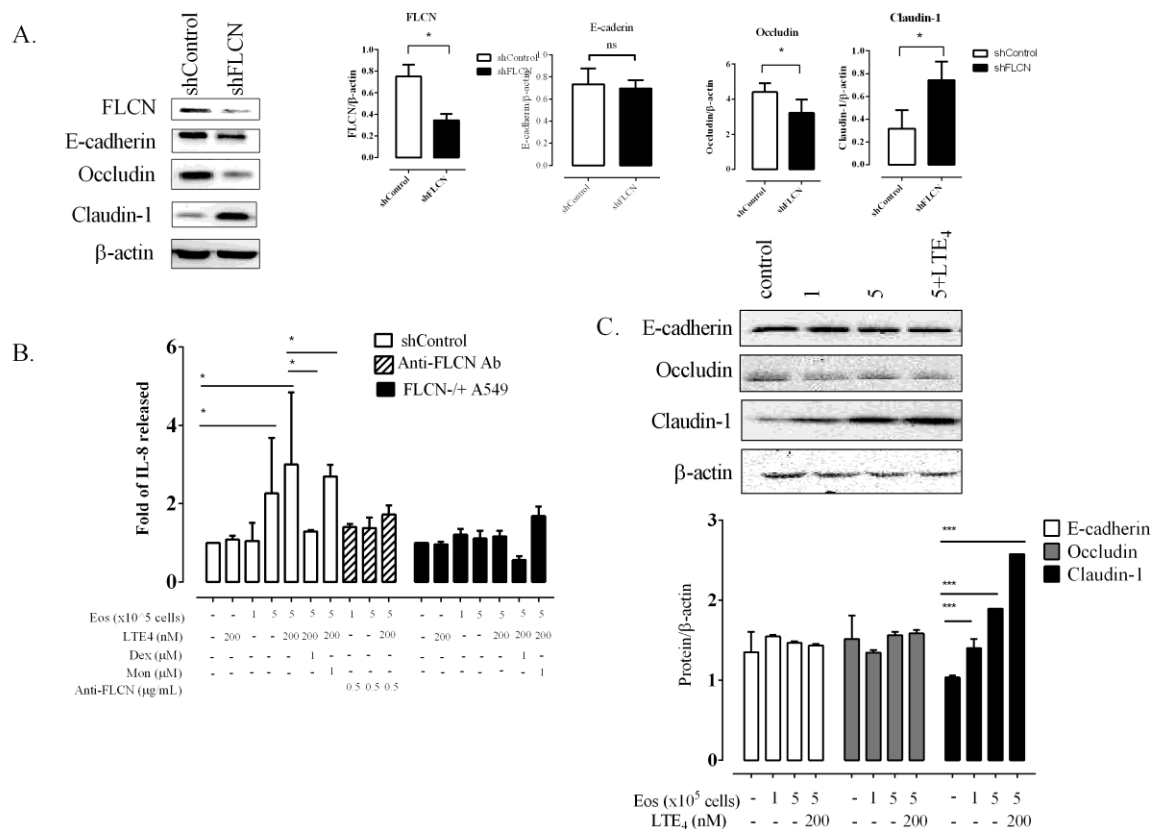
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D.

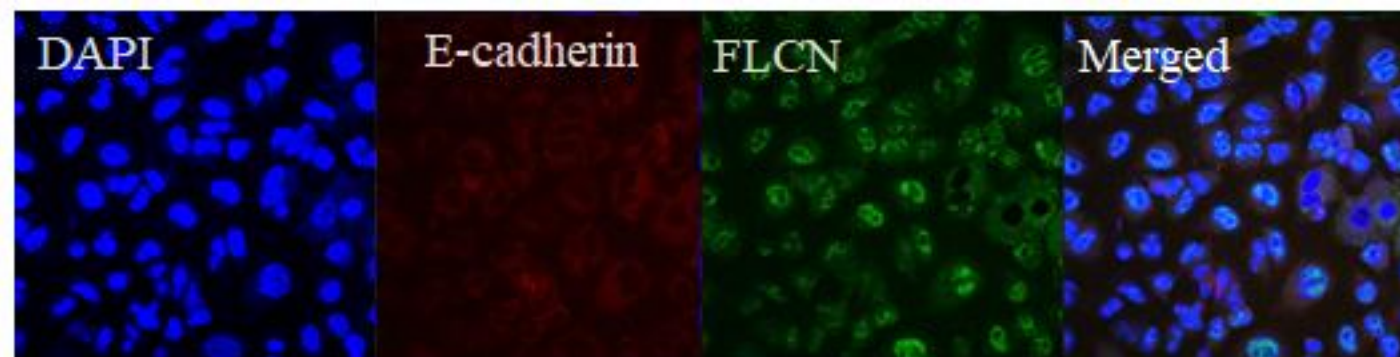




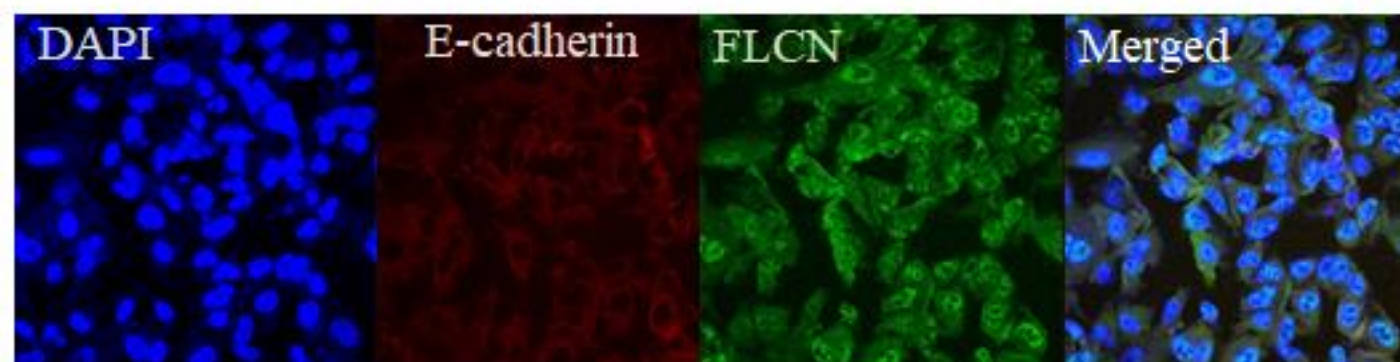
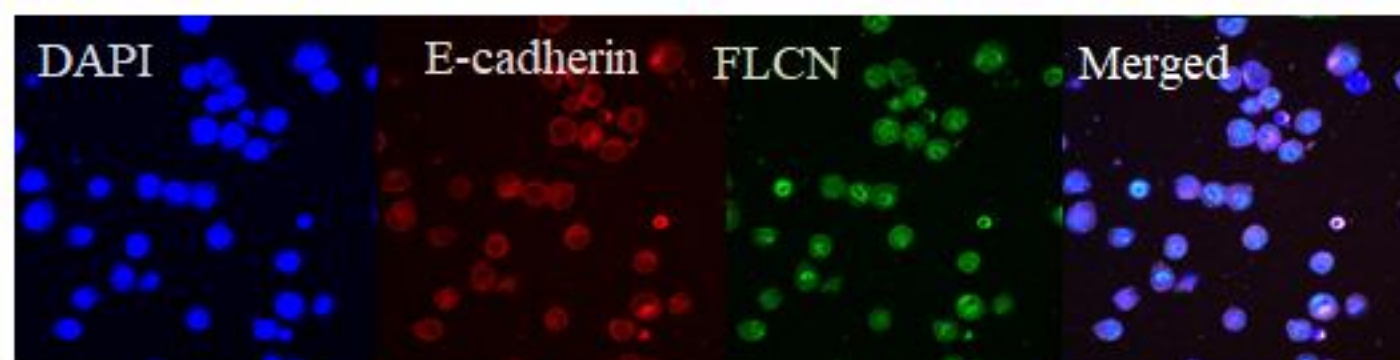


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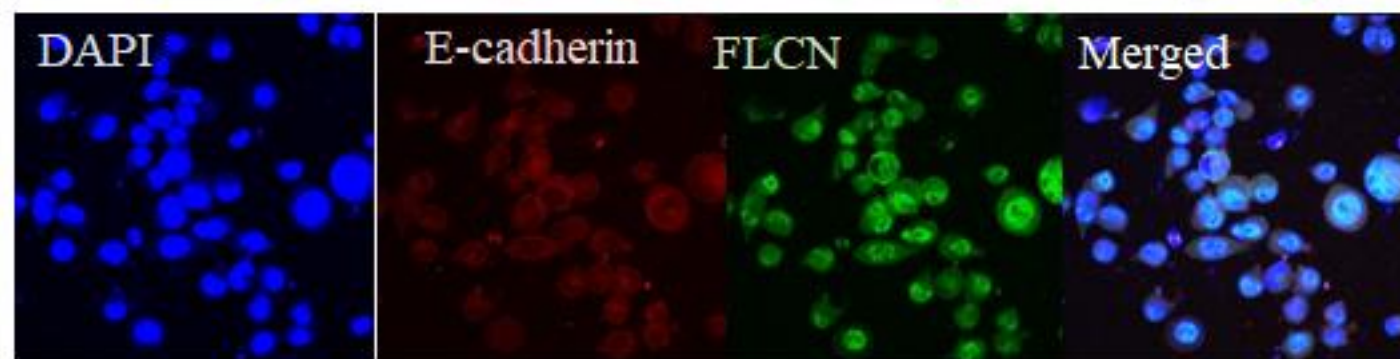
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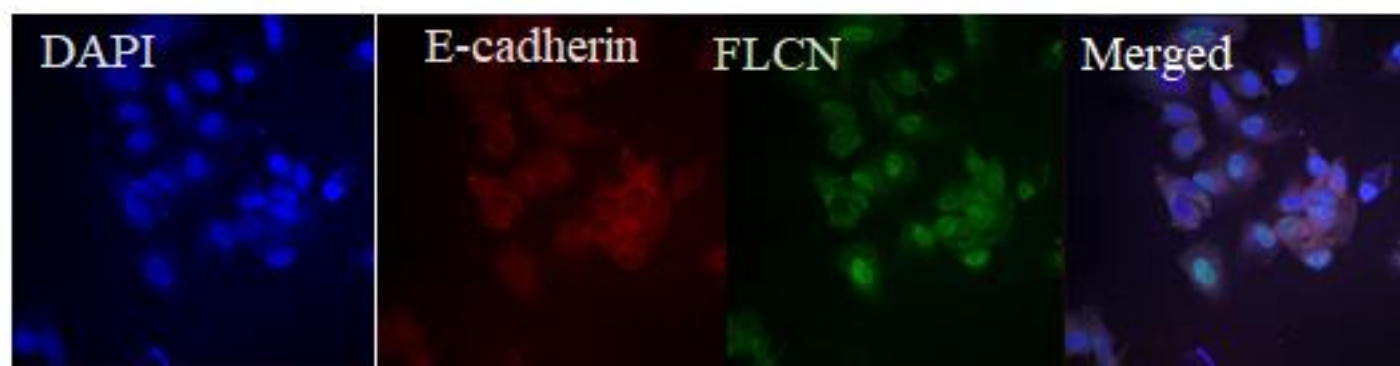
A549+PBE

A549+PBE
+LTE₄

Dex

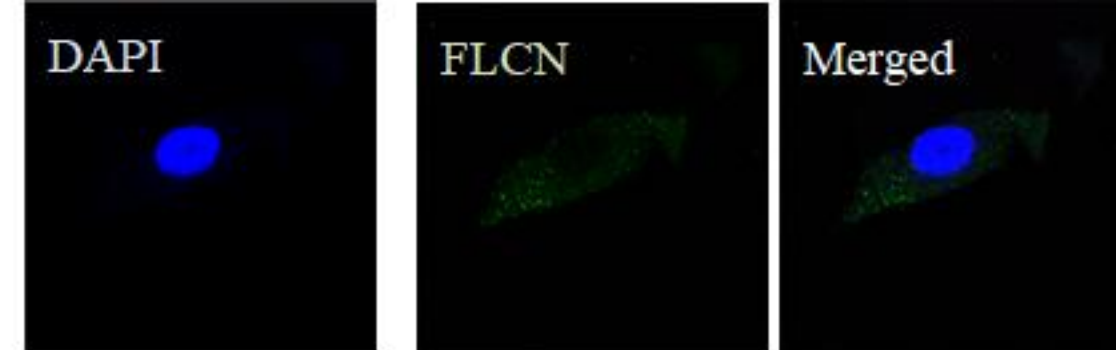


Mon

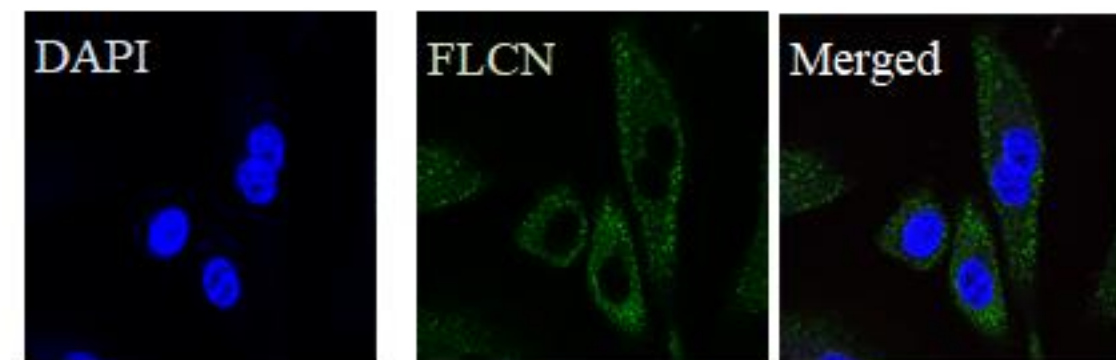
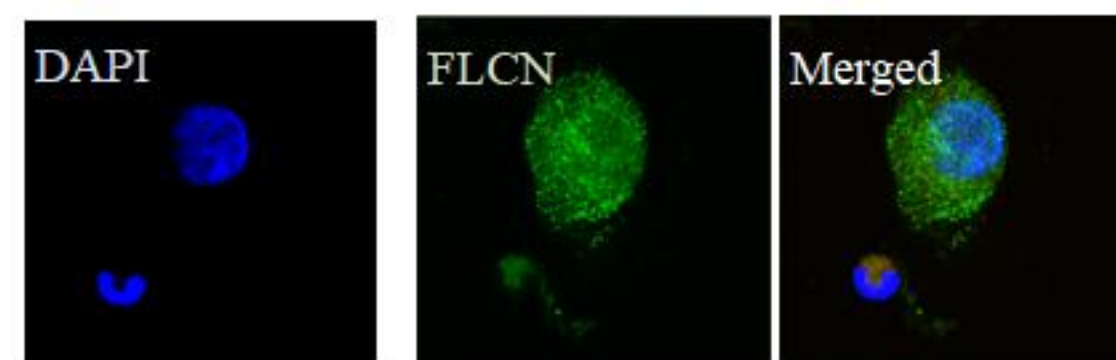


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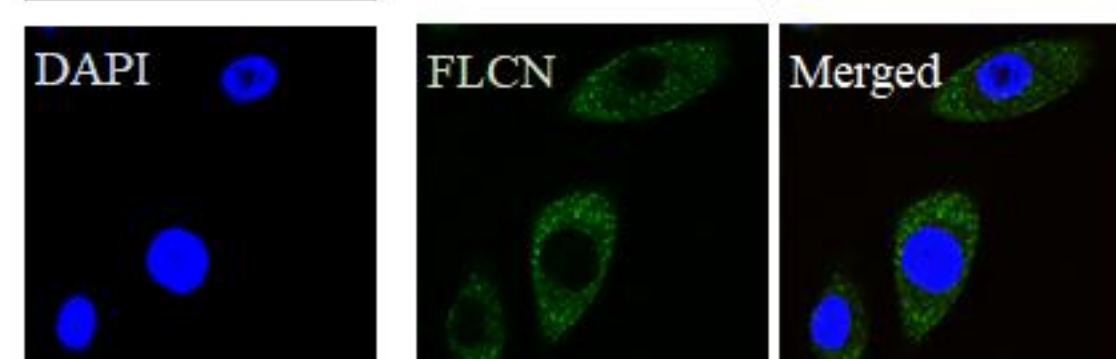
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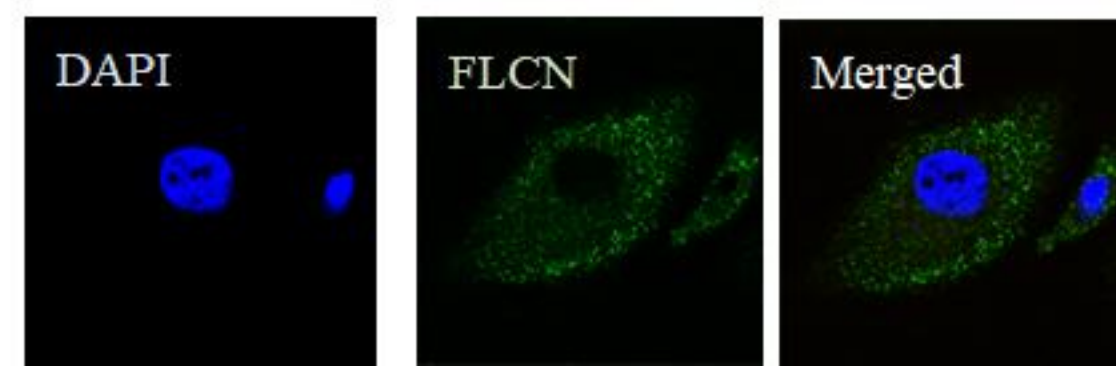
SAEC+PBE

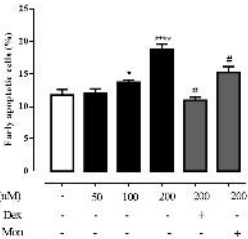
SAEC+PBE
+LTE₄

Dex

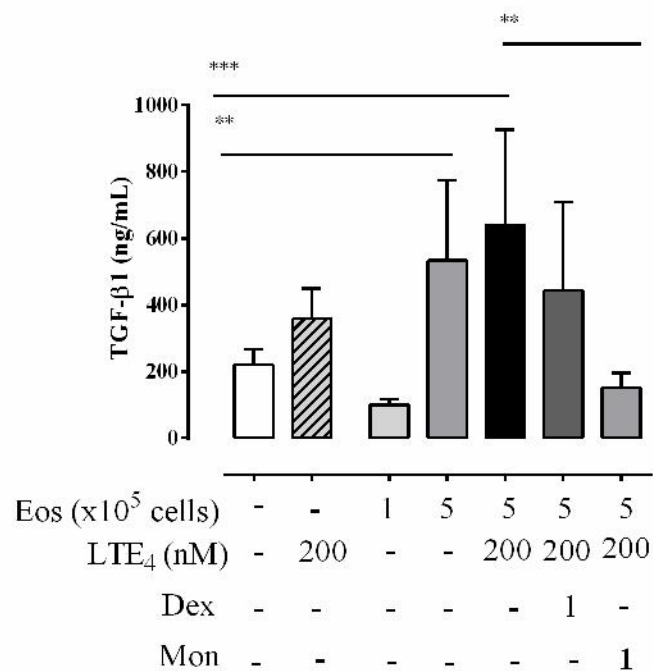


Mon

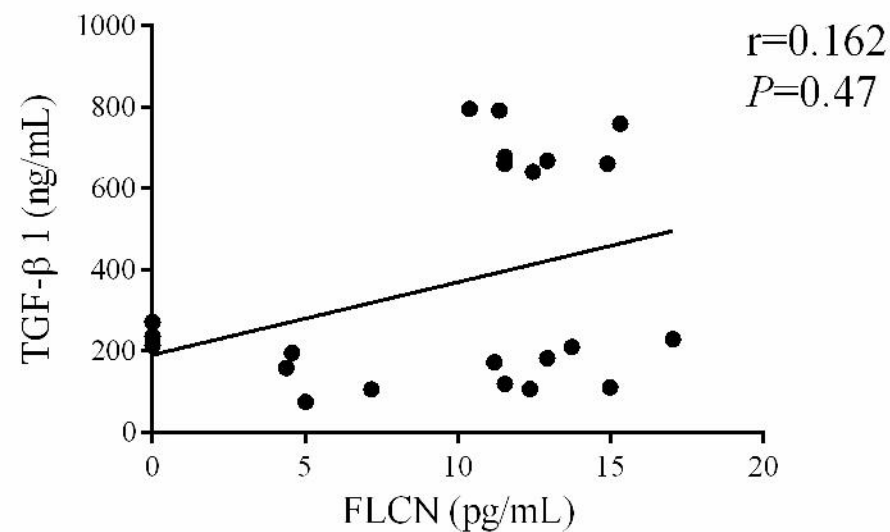




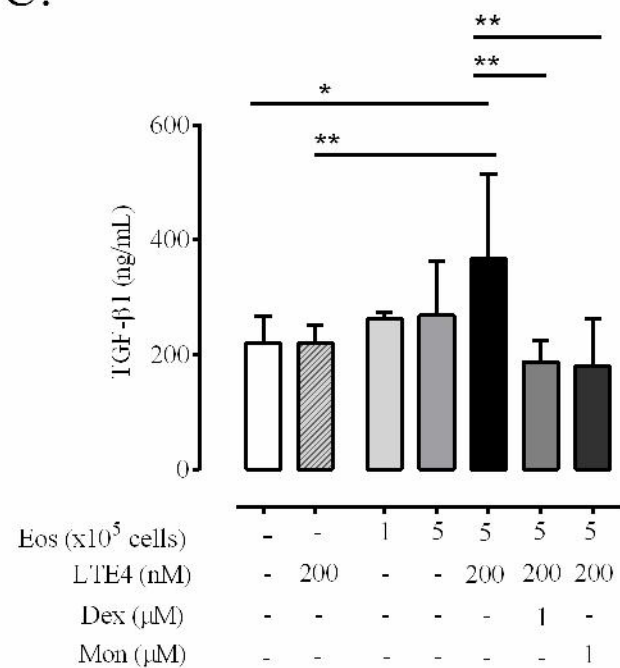
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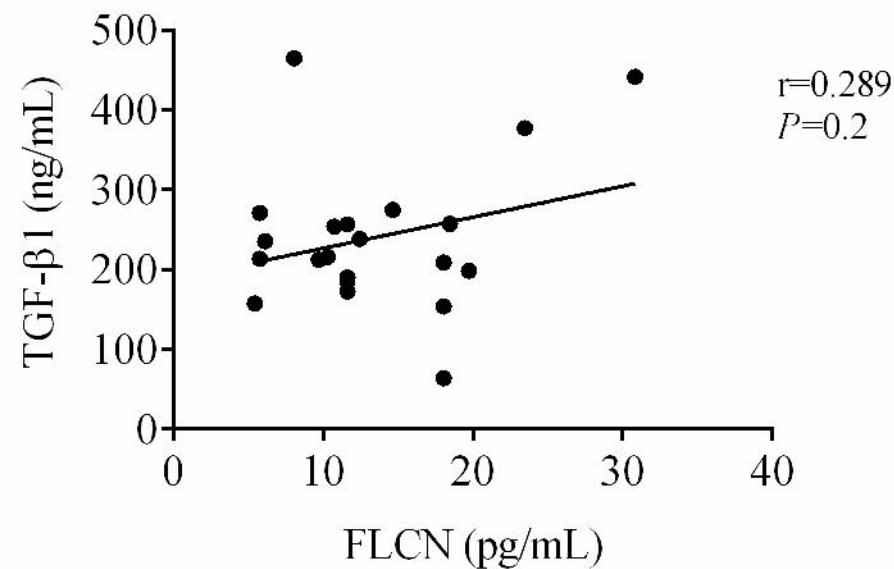
B.



C.



D.



Epithelial Folliculin Enhances Airway Inflammation in Aspirin-Exacerbated Respiratory Disease

(Running title: Folliculin in Aspirin-exacerbated Respiratory Disease)

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Supplementary methods

Apoptosis assay

A549 cells were labeled with FITC Annexin V and Propidium Iodine (PI) following the manufacturer's instructions (BD BioSciences, San Jose, CA, USA). Apoptotic cells were defined as Annexin^{high}/PI^{low} and analyzed by BD FACSCanto II (BD BioSciences), at least, 10,000 events were recorded for each condition.

Immunocytochemistry

Cells (5×10^4 cells) were suspended onto Poly-L-Lysine-coated coverslips and cultured until the confluency has been reached. Cells were stimulated with LTE₄ 200 nM, dexamethasone and montelukast for 1 hour and subsequently cocultured with PBE for 16 hours. After incubation, cells were fixed with 4% paraformaldehyde for 15 minutes, followed by washing briefly with 1X PBS and kept until further experiments. Cells were blocked with 1% bovine serum albumin in 1X PBS, incubated with the antibodies against E-cadherin and FLCN overnight at 4°C. Subsequently, cells were incubated with the respective secondary antibodies conjugated with fluorescence for 1 hour at room temperature (RT). Cells were counterstained with 4',6-diamidino-2-phenylindole (1 µg/mL) for 10 minutes at RT and mounted with the Gel/Mount mounting solution (Biomedica Corporation, CA, USA). Signals were visualized by ZEN Microscopy software.

Measurement of TGF-β1 release from the HAECs

HAECs were stimulated and co-cultured as previously described in the Materials & Methods section, the level of TGF-β1 in the cell-free supernatants was measured by ELISA (R&D systems).

SUPPLEMENTARY FIGURES

Figure S1. Co-culture of HAECs with PBE from asthmatics leads to co-localization of FLCN.

Representative images are from 3 independent experiments. After being cocultured, cells were washed to remove unbound PBE. Cells were fixed, permeabilized and incubated with respective primary and secondary antibodies. Signals were visualized using confocal microscopy.

Figure S2. LTE₄-induced apoptosis of HAECs. A549 cells were seeded at 0.5×10^5 in 24 well plate.

Cells were stimulated with LTE₄ at 50, 100, 200 nM in serum free media for 72 hours. Cells were treated with dexamethasone (1 μ M) and montelukast (100 nM). Cells were labeled with Annexin V-FITC/PI and analyzed with flow cytometry. *P* values were analyzed by one way ANOVA with the Tukey post hoc test.

*, *P* < 0.05; ****, *P* < 0.0001 comparing to control; #, *P* < 0.05, ###, *P* < 0.001 comparing to LTE₄-treated A549 at respective doses.

Figure S3. Increased levels of TGF- β 1 from the HAECs cocultured with PBE and LTE₄. HAECs

were stimulated with LTE₄ 200 nM, Dex 1 μ M, Mon 1 μ M and cocultured with PBE (1, 5×10^5 cells).

Supernatants were harvested and measured for TGF- β 1 release from (A) A549 cells and (B) SAECs. . *P* values were analyzed by one way ANOVA with the Tukey post hoc test. *, **, *P* < 0.05, 0.01 compared between the indicated groups. Correlations of TGF- β 1 with FLCN released from (A) A549 and (B) SAEC, analyzed by Spearman's rank correlation coefficient.