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Increased epithelial galectin-13 associates with eosinophilic airway inflammation in asthma

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Short title: The role of galectin-13 in asthma airway eosinophilia

Key messages list:

Increased galectin-13 expression associates with airway eosinophilic inflammation in asthma.

Epithelial galectin-13 expression associates with epithelial gene expression signature for type 2

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status.

Plasma galectin-13 level reflects airway response to asthma therapy.

Galectin-13 may contribute to allergic airway eosinophilic inflammation by regulating the expression of MCP-1 and eotaxin-1.

ABSTRACT:

Background: Airway eosinophilic inflammation is a central feature in asthma which is mainly driven by type 2 response. The expression of galectin-13 was up-regulated in a parasitic infection model which is also characterized by type 2 immune response. We hypothesized that galectin-13 may be involved in airway eosinophilic inflammation in asthma.

Objective: To unveil the role of galectin-13 in asthma airway inflammation.

Methods: We measured galectin-13 expressions in bronchial brushings, sputum, serum of asthma patients ($n = 54$) and healthy controls ($n = 15$), analyzed the correlations between galectin-13 expression and airway eosinophilia. We use human bronchial epithelial (HBE) cells to investigate the possible mechanism by which galectin-13 participates in eosinophilic inflammation.

Results: The expression of galectin-13 was markedly increased in subjects with asthma compared to control. Epithelial galectin-13 mRNA levels in asthmatic subjects were strongly correlated with eosinophilic airway inflammation (the percentage of sputum eosinophils, the number of eosinophils in bronchial submucosa, and FeNO) and Th2 signature genes (*CLCA1*, *POSTN* and *SERPINB2*). Inhaled corticosteroid (ICS) treatment reduced galectin-13 expression, and plasma galectin-13 level reflects airway response to asthma therapy. In cultured human bronchial epithelial cells, knockdown of galectin-13 suppressed IL-13-stimulated MCP-1 and eotaxin-1 by inhibiting the activation of EGFR and ERK.

Conclusions & Clinical Relevance: Galectin-13 is a novel marker for airway eosinophilia in asthma, and may contribute to allergic airway eosinophilic inflammation by regulating the expression of MCP-1 and eotaxin-1. Plasma galectin-13 level may be useful for predicting responses to asthma therapy.

Keywords galectin-13, asthma, airway eosinophilia, airway epithelial cell

INTRODUCTION

Asthma is an increasingly common disease and there are more than 300 million asthma patients worldwide[1]. Asthma is characterized by airway eosinophilic inflammation, mucus overproduction, airway hyperreactivity (AHR), and peribronchial fibrosis[2]. Airway eosinophilic inflammation is a cardinal feature of allergic asthma [3]. Airway epithelial cells promote airway eosinophilia by producing cytokines such as monocyte chemotactic protein (MCP-1) (chemokine ligand, CCL-2) and eotaxin-1 (chemokine ligand, CCL11). Both MCP-1 and eotaxin-1 are important mediators of inflammatory response and play important roles in allergic inflammation[4, 5].

Lectins are a group of molecules that have important roles in the innate immune response to pathogens[6]. In our previous study, we found that intelectin, one of the members of lectins, contributes to allergen-induced IL-25, IL-33, and TSLP expression in asthma and atopic dermatitis[7]. Galectins are a family of β -galactoside-binding animal lectins that regulate diverse biological processes, such as cell activation, cell adhesion, migration, cell growth, phagocytosis, and apoptosis[8-10]. Today, we know of 15 members of the galectin family, of them, galectin-1, 3, 9, 10 are involved in eosinophilic inflammation[11].

Galectin-13 is a member of the galectin family which was first isolated from the human placenta and characterized by Bohn et al. in 1983[12]. Immunohistochemistry studies have pointed to its predominant localization in the placental syncytiotrophoblast layer and blood vessels[13]. A growing number of studies have shown that galectin-13 is associated with the development of severe pregnancy complications with a robust immune component[14-16]. 3D computational modeling of galectin-13 revealed that it's proposed structure homology to other galectins, especially to human eosinophil Charcot-Leyden crystal protein/galectin-10[17]. In a Holstein bull calf cooperia oncophora infection model, the expression of galectin-13 was strongly up-regulated starting at 14 days and peaked at 28 days (65.52-fold)[18]. Cooperia oncophora infection elicits a Th2-like immune response, characterized by the up-regulation of IL-4 and the involvement of both eosinophils and mucosal IgA[19]. Therefore, we hypothesized that galectin-13 may contribute to airway eosinophilic inflammation in asthma.

To test our hypothesis, we detected the expression of galectin-13 in bronchial epithelial brushings, sputum and serum of untreated, newly diagnosed subjects with asthma and healthy controls. The location of galectin-13 was confirmed by immunohistochemistry using tissues of endobronchial biopsies. Correlation assays between epithelial galectin-13 expression and the measures of airway eosinophilia were performed. The role of galectin-13 in epithelial cytokine expression and its possible mechanisms were examined in an in vitro system.

METHODS

Subjects

We recruited 16 healthy control subjects and 54 subjects with asthma. All participants are Chinese and were recruited from Tongji Hospital. Asthmatic subjects were diagnosed by a physician based on the clinical presentation and the results of pulmonary function tests; had symptoms of episodic cough, wheeze, and/or breathlessness; and had accumulated dosage of methacholine provoking 20% fall of force expiratory volume in the first second (FEV₁PD₂₀) <2.505mg and or ≥12% increase in FEV₁ following inhalation of 200μg salbutamol. Healthy control subjects had no respiratory symptoms, normal spirometric values and methacholine PD₂₀ ≥2.505mg. None of the subjects had ever smoked or received inhaled or oral corticosteroid or leukotriene antagonist. After baseline evaluation, subjects initiated ICS prescribed by physicians. All subjects provided written informed consent prior to study onset, and all experiments were approved by the ethics committee of Tongji Hospital, Huazhong University of Science and Technology (ID: TJ-IRB20210117).

Sample Acquisition

We recorded demographic information, collected blood, and performed spirometry. We also performed bronchoscopy with brushing and endobronchial biopsies. We brushed 10 sites within the subsegmental bronchi of the right middle and lower lobes (10 gentles upward and downward strokes per site). We used forceps to biopsy left lower lobe carinae and fixed samples in polyoxymethylene. Written informed consent was obtained from all the subjects.

Sputum Eosinophil Percentages

All subjects underwent sputum induction with the inhalation of hypertonic saline (4.5%) through an ultrasonic nebulizer. The samples were processed according to the method of Simpson[20]. Briefly, sputum was selected and weighed; four-times the volume of 0.1% dithiothreitol (DTT) was added, after agitated for 15 minutes on ice, an equal volume of PBS was added. The suspension was filtered through a 40µm cell filter, viable cell counted, and centrifuged at 4°C, 980 rpm, for 10 minutes. The supernatant was stored at -80°C for future galectin-13 detecting. Cells were resuspended in PBS at 1×10^6 cells/ml and stained with Wright Giemsa. A minimum of 300 cells were counted, and the percentage of eosinophils

Immunohistochemistry

Four µm sections were cut from paraffin-embedded tissues of endobronchial biopsies. Sections were incubated with citric acid buffer (PH6.0) to expose antigens. After inhibition of endogenous peroxidases with 3% H₂O₂, unspecific antibody binding was blocked with 3% goat serum. Sections were incubated overnight at 4°C with monoclonal anti-galectin-13 antibody (1:200) or an isotype matched control rabbit IgG (1:2000; Abmart Shanghai, China) in PBS. After washing, the second incubation was performed with HRP-conjugated anti-rabbit IgG. Antibodies were detected using the DAB kit (Zhongshan Goldenbridge Biotechnology, China) as directed by the manufacturer.

Cell culture and treatment

HBE cells (American Type Culture Collection) were cultured in DMED / high glucose (HyClone) in 12-well plates. When cells were 90% confluent, 3.8µl galectin-13 siRNA (10µM) or scrambled siRNA(10µM) in 75µl Transfection Buffer was mixed with 3.3µl GenMute Reagent (SignaGen Laboratories, USA) and added to each cell. Six hours later, the media were substituted with DMEM with or without IL-13 (20ng/ml). After 48 hours of treatment, cells were harvested for quantitative RT-PCR and Western Blots, and the media were collected for ELISA. Human recombinant galectin-13 was also used to stimulate HBE cells. After different time and concentration culturing, the cells and media were collected for quantitative RT-PCR and ELISA.

Quantitative RT-PCR

We isolated total RNA from bronchial epithelial brushings and HBE cells using TRIzol

(Invitrogen, USA) and synthesized first-strand cDNA using the PrimeScript RT reagent kit (Takara, Japan). The transcript levels of human galectin-13 in brushing samples, and galectin-13, MCP-1, eotaxin-1 in HBE cells were determined by using the Takara Perfect Real-Time PCR kit, Takara SYBR PremixExTaq polymerase, and an ABI Prism 7500 PCR System. The cycle threshold (Ct) of each gene transcript was normalized to the mean Ct of β -actin. Fold differences were determined by the $2^{-\Delta\Delta C_t}$ method.

Western Blots

The protein concentrations of sputum supernatant samples and HBE cells were measured using the BCA protein assay. Fifty μ g protein samples were resolved on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred and immuno-probed with rabbit-anti-human galectin-13 antibody (1:1000). The reaction was detected by Enhanced Chemiluminescence (ECL) (GE Healthcare, Buckinghamshire, UK). The optical density of the bands was measured using ImageJ (Rasband, National Institutes of Health). For phosphorylated EGFR, total EGFR, phosphorylated ERK1/2, total ERK1/2, phosphorylated JNK, total JNK, phosphorylated p38-MAPK and total p38-MAPK western blotting, antibodies from Cell Signaling Technology (Danvers, MA, USA) were used.

Cytokine assay in cell supernatants

Human MCP-1 (RayBiotech, USA) and eotaxin-1 (RayBiotech, USA) in cell culture medium, human galectin-13 (BioVendor, Czech) were determined using commercially available ELISA sets. ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Statistics Analysis

Prism version 6 (GraphPad Software, USA) and SPSS version 19 (SPSS Inc., USA) were used to analyze the data. For normally distributed data we calculated means $\pm$ standard deviation (SD) and used parametric tests (one-way analysis of variance with Tukey correction or unpaired t-test) to compare across groups. For non-normally distributed data we calculated medians (with interquartile ranges) and used non-parametric tests (Kruskal-Wallis test with Dunn intergroup comparison or Mann-Whitney test). Correlations were evaluated by Spearman's rank-order

correlation.

RESULTS

Galectin-13 expression is increased in the airway epithelial cells of subjects with asthma.

We recruited 15 healthy controls and 54 subjects with asthma. Subject characteristics are summarized in Table 1. Compared with healthy controls, subjects with asthma had lower FEV₁ and methacholine PD₂₀, higher serum IgE levels, higher blood eosinophil numbers, and higher sputum eosinophil counts (Table 1).

We examined galectin-13 transcript levels in bronchial brushings from healthy controls and subjects with asthma by quantitative PCR. Galectin-13 transcript levels were markedly increased in subjects with asthma when compared with healthy controls (Fig.1A).

We determined the localization of galectin-13 protein in human airway biopsy samples. We performed immunohistochemical staining and found that galectin-13-positive cells were mainly airway epithelial cells and submucosal gland cells in subjects with asthma. We could not detect staining for galectin-13 in healthy controls. Our data indicates that galectin-13 is mainly expressed in airway epithelial cells and submucosal gland cells (Fig.1B).

Increased epithelial galectin-13 expression associates with airway eosinophilic inflammation in asthma

To investigate the association between galectin-13 expression and eosinophilic airway inflammation, we analyzed the correlation between epithelial galectin-13 levels and the percentage of sputum eosinophilia, number of eosinophils in bronchial submucosa, FeNO and the percentage of peripheral blood eosinophilia in all subjects. We found that epithelial galectin-13 levels were significantly correlated with the percentage of sputum eosinophils ($r_s = 0.65$, $p < 0.0001$, $n = 48$ for all subjects; $r_s = 0.63$, $p < 0.0001$, $n = 42$ for asthma; Fig 2A), the number of eosinophils in bronchial submucosa ($r_s = 0.64$, $p < 0.0001$, $n = 68$ for all subjects; $r_s = 0.53$, $p < 0.0001$, $n = 54$ for asthma; Fig 2D), FeNO ($r_s = 0.55$, $p < 0.0001$, $n = 56$ for all subjects; $r_s = 0.49$, $p = 0.0003$, $n = 50$ for asthma; Fig 2C) and the percentage of peripheral blood eosinophilia ($r_s = 0.57$, $p < 0.0001$, $n = 67$ for all subjects; $r_s = 0.47$, $p = 0.0005$, $n = 52$ for asthma; Fig 2F). Moreover, a previously described cut-off to classify

the sputum eosinophil status[21] and bronchial eosinophil status[22] were used to evaluate the association between airway eosinophilia and epithelial galectin-13 expression (Fig 2B, E). Our data indicate that epithelial galectin-13 is associated with airway eosinophilic inflammation in asthma.

To find a non-invasive method of measuring galectin-13, sputum samples from subjects with asthma and controls were used to detect the levels of galectin-13 protein by western blotting and ELISA. Consistent with our findings in bronchial epithelial brushing samples and airway biopsy samples, the levels of galectin-13 in sputum from subjects with asthma were greater than healthy controls (Fig.3).

Epithelial galectin-13 expression associates with epithelial gene expression signature for type 2 status

Airway eosinophilic inflammation is a typical feature in type 2-high asthma. Chloride channel regulator 1 (*CLCA1*), periostin (*POSTN*), and *SERPINB2* are epithelial genes that used as gene signature to identify type 2-high asthma[23-25]. We next examined whether epithelial galectin-13 expression is associated with type 2 status. These three genes in bronchial brushings were determined by quantitative PCR in our included subjects. We found that *CLCA1*, *POSTN*, and *SERPINB2* were markedly increased in subjects with asthma compared to controls (Fig.4A, C, E). In addition, epithelial galectin-13 expression was significantly correlated with *CLCA1* ($r_s = 0.6, p < 0.0001, n = 68$ for all subjects; $r_s = 0.45, p = 0.0007, n = 52$ for asthma; Fig 4B), *POSTN* ($r_s = 0.53, p < 0.0001, n = 68$ for all subjects; $r_s = 0.41, p = 0.002, n = 52$ for asthma; Fig 4D), *SERPINB2* ($r_s = 0.58, p < 0.0001, n = 68$ for all subjects; $r_s = 0.38, p = 0.005, n = 52$ for asthma; Fig 4F) and the three-gene-mean ($r_s = 0.61, p < 0.0001, n = 68$ for all subjects; $r_s = 0.44, p = 0.0009, n = 52$ for asthma; Fig 4G) expression. Our data suggest that galectin-13 is upregulated in type 2-high asthma and may be a candidate signature gene for type 2 status.

Plasma galectin-13 level reflects airway response to asthma therapy

As galectin-13 expression in bronchoscopic samples has limited application, we examined galectin-13 levels in peripheral blood by ELISA. Consistent with our findings in bronchial epithelial brushing samples, plasma galectin-13 levels were significantly increased in asthma subjects compared to healthy controls (Fig 5A). Elevated plasma galectin-13 levels decreased after

corticosteroid inhalation. Plasma galectin-13 levels were correlated with the expression of epithelial galectin-13 levels ($r_s = 0.615$, $p < 0.0001$, Fig 5B). This finding suggests that plasma galectin-13 may be a surrogate for airway epithelial galectin-13 expression.

We examined the relationship between plasma galectin-13 and pulmonary function (FEV1). There was a weak correlation between plasma galectin-13 and FEV1 in asthma subjects at baseline ($r_s = 0.44$, $p = 0.005$, Fig 5C). To determine whether baseline galectin-13 expression is associated with ICS responsiveness in subjects with asthma, we measured FEV1 after 4 weeks of treatment with ICS. There was significant association between the change in FEV1 and baseline plasma galectin-13 level ($r_s = 0.61$, $p = 0.0001$, Fig 5D). These data indicate that plasma galectin-13 level may reflect airway response to asthma therapy during treatment with ICS.

Galectin-13 contributes to IL-13-induced MCP-1 and eotaxin-1 expression

We next explored the possible mechanism by which galectin-13 participates in airway eosinophilic inflammation. HBE airway epithelial cells were stimulated with IL-13, a key type 2 cytokine. We found that IL-13 significantly increased galectin-13 expression in HBE cells. After transfection with galectin-13 siRNA, the basal and IL-13-induced galectin-13 transcript levels were significantly reduced in HBE cells (Fig.1C).

Since MCP-1 and eotaxin-1 are secreted by airway epithelial cells and contribute to eosinophilic inflammatory diseases, we next examined whether galectin-13 is involved in the expression of these chemokines in airway epithelial cells. We found that IL-13 markedly increased the transcript levels of MCP-1 and eotaxin-1 in HBE cells. IL-13-induced MCP-1 and eotaxin-1 expressions were suppressed after transfection with galectin-13 siRNA in HBE cells. Using ELISA, we confirmed that IL-13 significantly increased MCP-1 and eotaxin-1 protein levels in the media, and IL-13-induced MCP-1 and eotaxin-1 expression were also inhibited by galectin-13 siRNA (Fig.6A-D).

To investigate whether galectin-13 is sufficient for MCP-1 and eotaxin-1 expression, we used human recombinant galectin-13 to stimulate HBE cells at different time points and concentrations. We found that human recombinant galectin-13 (100ng/ml) significantly increased MCP-1 and eotaxin-1 transcript levels. Moreover, galectin-13 at high concentration (200ng/ml) further

increased the expression of MCP-1 and eotaxin-1 Human recombinant galectin-13 (100ng/ml) also increased the protein levels of MCP-1 and eotaxin-1 in culture medium (Fig.S1).

Galectin-13 expression contributes to IL-13-induced EGFR and ERK1/2 activation

As galectin-13 overexpression promoted apoptosis presumably by activating the p38-mitogen-activated protein kinase (p38-MAPK) and c-Jun N-terminal Kinase (JNK) pathways and by suppressing phosphatidylinositol-3-kinase (PI-3K)-Akt and extracellular signal-regulated kinase (ERK1/2) pathways[26], we examined whether the epidermal growth factor receptor (EGFR), ERK1/2, p38-MAPK, JNK are involved in IL-13-induced MCP-1 and eotaxin-1 expression in human airway epithelial cells.

We found that the EGFR inhibitors ZD1839 and the ERK1/2 inhibitor U0126 each blocked IL-13-induced MCP-1 and eotaxin-1 expression in HBE cells (Fig.6E-H, S2). Furthermore, IL-13 stimulation increased ERK1/2 phosphorylation at 24h and 48h, increased EGFR phosphorylation at 24h, and suppressed p38-MAPK phosphorylation at 48h. After transfecting with galectin-13 siRNA, IL-13-induced ERK1/2 and EGFR phosphorylation were inhibited (Fig.7). However, there was no significant effect on the p38-MAPK phosphorylation.

Our data indicates that galectin-13 is required and sufficient for IL-13-induced MCP-1 and eotaxin-1 expression in human airway epithelial cells and that galectin-13 expression contributes to IL-13-induced EGFR and ERK1/2 activation.

DISCUSSION

In the present study, we found that airway epithelial, sputum and serum galectin-13 expressions were increased in subjects with asthma compared with healthy controls. The galectin-13 protein is mainly localized in human airway epithelial cells and submucosal gland cells. Epithelial galectin-13 expression was significantly correlated with airway eosinophilia and markedly associated with epithelial gene expression signature for type 2 status in subjects with asthma. Besides, plasma galectin-13 level may reflect airway response to asthma therapy during treatment with ICS. Galectin-13 is required and sufficient for IL-13-induced MCP-1 and eotaxin-1 expression in airway epithelial cells, and it might participate in IL-13-induced MCP-1 and

eotaxin-1 expression by regulating the activation of EGFR and ERK1/2 pathways.

Galectins are a group of proteins that bind β -galactosidase. These proteins are highly conserved and are expressed by diverse cell types, including monocytes, DCs, macrophages, MCs, and B and T cells[27]. In this study, we demonstrated that galectin-13 expression is located in airway epithelial cells and submucosal gland cells. Galectins participate in the signaling pathway, gene expression, and vesicular trafficking, et al[28]. Several galectins, particularly galectin -3, galectin -9, and galectin -10, have been shown to play an important role in modulating allergic inflammatory responses[11]. Among them, galectin-3 showed the capacity to bind IgE[29]. A mouse model of asthma found that OVA-sensitized Gal-3^{-/-} mice demonstrate diminished eosinophil recruitment and reduced airways hyperreactivity[30]. Further study indicated that galectin-3 functions as a cell surface adhesion molecule to support eosinophil rolling and adhesion, a crucial step in transit to the lungs[31]. Galectin-9 demonstrated the therapeutic effect by showing reduced AHR, as well as Th2-associated airway inflammation[32].

Galectin-13, also known as Placental Protein 13 (PP13) and LGALS13, was first purified and cloned from the placenta and is predominantly located in placental syncytiotrophoblast layer, placental blood vessels, and specific sites within the placental bed[33]. Its ability to induce apoptosis of activated T cells in vitro, to divert and kill T cells as well as macrophages, and to regulate the activity of neutrophils in the placenta, suggests important immune functions[16, 34]. The proposed structure of galectin-13 is similar to human eosinophil Charcot-Leyden crystal protein/galectin-10, and it was strongly up-regulated in a Holstein bull calves cooperia oncophora infection model[19]. These researches suggest that galectin-13 may partly contribute to allergic airway inflammation.

In our study, we demonstrated that airway epithelial galectin-13 was increased in subjects with asthma. This is the seventh galectins which has an association with asthma[11]. Except galectin-7, other five galectins(galectin-1, 3, 9, 10 and 14) all play important roles in eosinophil recruitment to airway inflammation[35, 36]. Although galectin-13 has a similar structure to galectin-10, we have not yet found its expression in eosinophils. Instead, we found that galectin-13 is mainly localized in human airway epithelial cells and submucosal gland cells. Our data

demonstrated that epithelial galectin-13 expression was significantly correlated with airway eosinophilia (the percentage of sputum eosinophilia, number of eosinophils in bronchial submucosa, FeNO) and epithelial gene expression signature for type 2 status (*CLCA1*, *POSTN*, and *SERPINB2*) in subjects with asthma. A study conducted by *Chua et al* found that galectin-10 in sputum has a strong correlation with sputum eosinophil percentages[37]. In our study, we found that galectin-13 in sputum has a greater median protein concentration in asthmatic samples when compared to healthy controls.

As epithelial gene expression is not available in every subject, we use less invasive methods to detect the expression of galectin-13. We found that plasma galectin-13 correlated with airway epithelial galectin-13 expression in our study. Elevated plasma galectin-13 levels normalized after corticosteroid inhalation. More importantly, elevated plasma galectin-13 revealed better improvement in lung function. Our work suggests plasma galectin-13 level may reflect airway response to asthma therapy and could help to identify appropriate cohorts for testing treatments in asthma.

Since galectin-13 and the chemokines are produced by airway epithelial cells, we use HBE cells to investigate the role of galectin-13 in IL-13-induced chemokine expression. In some research, we found that both MCP-1 and eotaxin-1 play significant roles in allergen-induced eosinophilic airway inflammation. In our study, we found that IL-13 treatment increased galectin-13, MCP-1 and eotaxin-1 expression in HBE cells. When we use galectin-13 siRNA, an inhibitor of galectin-13, we found that MCP-1 and eotaxin-1 were decreased. Human recombinant galectin-13 elevates the expression of MCP-1 and eotaxin-1 in HBE cells.

As p38-MAPK, JNK, ERK1/2 pathways have been previously implicated in galectin-13 induced apoptosis, we examined the possibility that galectin-13 might participate in IL-13-induced MCP-1 and eotaxin-1 expression via effects on these pathways. We found that EGFR and ERK1/2 phosphorylation were inhibited by galectin-13 siRNA. EGFR inhibitors and ERK1/2 inhibitors each can block IL-13-induced MCP-1 and eotaxin-1 expression. Therefore, we concluded that galectin-13 may contribute to eosinophilic airway inflammation by regulating cytokines MCP-1 and eotaxin-1 expression, at least in part, by contributing to the activation of EGFR and ERK1/2

activation. The mechanism by which galectin-13 contributes to ERK1/2 and EGFR pathways activation is not yet known.

Our study has some limitations. First, the mechanism of galectin-13 participated in airway eosinophil inflammation still needs to be researched more deeply. Second, the sample size is relatively small. Future study with a larger sample size is warranted to validate the role of galectin-13 in airway eosinophilia.

In summary, our findings indicate that galectin-13 is mainly expressed in airway epithelial cells and submucosal gland cells, and epithelial galectin-13 are potential biomarkers for airway eosinophilia in asthma. Galectin-13 may participate in eosinophilic airway inflammation by regulating the expression of MCP-1 and eotaxin-1 in airway epithelium. Plasma galectin-13 level may be useful for predicting responses to asthma therapy.

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Disclosures

The authors declare no conflict of interest.

Author contributions

L.Y. and G.Z. conceptualized the study design. L.Y., S.Z., Y.F., W.W., C.C., D.C., and S.C. collected demographic, clinical, and laboratory data. L.Y., S.Z. and J.Z. analyzed the data. L.Y. and S.Z. interpreted the results. L.Y. and G.Z. wrote the manuscript with all authors providing feedback for revision. The authors read and approved the final report.

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Table 1 Subjects characteristics

	Healthy control subjects	Subjects with asthma	p-value
Number	15	54	
Age, year	31 ± 9.49	39 ± 11.15	0.27
Sex, M: F (%F)	3 : 12	13 : 41	0.11
FEV1, % predicted	98.89 ± 7.06	84.69 ± 16.19	0.006
Methacholine PD20, mg	2.505 ± 0.00	0.24 ± 0.41	0.009
Blood eosinophils, ×10 ⁹	0.08 ± 0.07, n=15	0.24 ± 0.41, n=52	<0.0001

Sputum eosinophils, %	0.94 ± 0.77, n=6	14.75 ± 20.09, n=42	0.003
Biopsy eosinophils, number/mm ²	0.49 ± 0.89, n=14	12.78 ± 11.92, n=54	0.0004
FeNO, ppm	28.75 ± 21.34, n=6	93.06 ± 66.22, n=50	0.005

Definition of abbreviation: PD20 = provocative dosage required to cause a 20% decline in FEV1. The minimal and maximal provocative dosages were 0.01 and 2.505 mg, respectively.

Figure Legends:

Figure 1. Galectin-13 expression is increased in the airway epithelial cells of subjects with asthma. (A) The transcript levels of galectin-13 in bronchial brushings of subjects with asthma (n = 53) and healthy controls (n = 15) were determined by quantitative PCR. Values were relative to the median value of healthy controls and were log2 transformed. (B) Immunohistochemistry of galectin-13 in epithelium and submucosal gland in representative sections of bronchial biopsies from subjects with asthma and healthy controls. Original magnification, ×400. (C) HBE cells were treated with IL-13 or both galectin-13 shRNA and IL-13. Forty-eight hours after treatment, cells were harvested for galectin-13 expression by quantitative RT-PCR. Data are mean ± SEM. *, P<0.05; ***, P<0.001 vs. controls.

Figure 2. Increased epithelial galectin-13 expression associates with airway eosinophilic inflammation in asthma. (A) The association between epithelial galectin-13 levels and the percentage of sputum eosinophils (B) Epithelial galectin-13 levels in subjects with asthma with and without sputum eosinophilia. (C) The association between epithelial galectin-13 levels and FeNO. (D) The association between epithelial galectin-13 levels and the number of eosinophils in bronchial submucosa. (E) Epithelial galectin-13 levels in subjects with asthma with and without submucosal eosinophilia in bronchial biopsies. (F) The association between epithelial galectin-13 levels and the percentage of eosinophils in peripheral blood.

Figure 3. The protein levels of galectin-13 in induced sputum of subjects with asthma and healthy controls. Galectin-13 protein levels in sputum were determined by Western blotting (A) and ELISA (C). Densitometry analysis of galectin-13 protein was normalized by β-actin (B). Data

are mean $\pm$ SEM. *, $P < 0.05$ vs. healthy controls.

Figure 4. Epithelial galectin-13 expression is correlated with the expression of signature genes for type 2 status. The transcript levels of *CLCA1* (A), *POSTN* (C) and *SERPINB2* (E) in bronchial brushings of subjects with asthma ($n = 52$) and healthy controls ($n = 15$). Spearman's rank order correlation assays between epithelial galectin-13 transcript levels and epithelial *CLCA1* (B), *POSTN* (D) and *SERPINB2* (F), three-gene-mean (G) in subjects with asthma ($n=52$, presented as closed circles) and healthy controls ($n=15$, presented as open circles).

Figure 5. Plasma galectin-13 levels are associated with epithelial galectin-13 expression and reflects airway response to asthma therapy. (A) The protein levels of galectin-13 in serum of subjects with asthma ($n = 54$) and healthy controls ($n = 15$), and asthmatic patients after corticosteroid inhalation treatment ($n=15$ at 4 weeks; $n=16$ at 8weeks). (B) Correlation assay between epithelial galectin-13 transcript levels and plasma galectin-13 levels. (C) Correlation assay between plasma galectin-13 levels and FEV1 in subjects with asthma at the baseline ($n=39$). (D) Correlation assay between plasma galectin-13 levels and the changes in FEV1 after ICS treatment at 4weeks in asthma subjects($n=33$).

Figure 6. EGFR and ERK1/2 pathways are required for IL-13-induced MCP-1 and eotaxin-1 expression in human airway epithelial cells. HBE cells were treated with IL-13 or both galectin-13 siRNA and IL-13. Forty-eight hours after treatment, cells were harvested for MCP-1 and eotaxin-1 expression by quantitative RT-PCR and ELISA (A-D). HBE cells were treated with IL-13, both EGFR inhibitors ZD1839 and IL-13, or both ERK1/2 inhibitor U0126 and IL-13. Forty-eight hours after treatment, cells were harvested for MCP-1 and eotaxin-1 expression by quantitative RT-PCR (E-H). Data are mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$. The data are representative of three independent experiments.

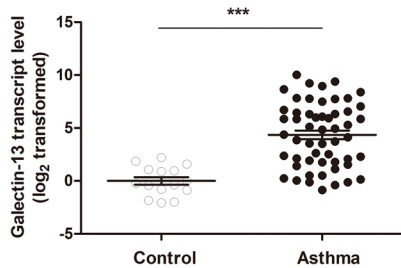
Figure 7. Galectin-13 expression contributes to IL-13-induced ERK1/2 and EGFR activation. (A) IL-13 stimulating increased ERK1/2 and EGFR phosphorylation, suppressed p38-MAPK phosphorylation, but galectin-13 siRNA transfection inhibited IL-13-induced ERK1/2 and EGFR phosphorylation. Densitometry of phosphor-JNK relative to total JNK (B),

phosphor-ERK1/2 relative to total ERK1/2 (C), phosphor-p38-MAPK relative to total p38-MAPK (D), and phospho-EGFR relative to total EGFR (E). Data are mean $\pm$ SEM. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. The data are representative of three independent experiments.

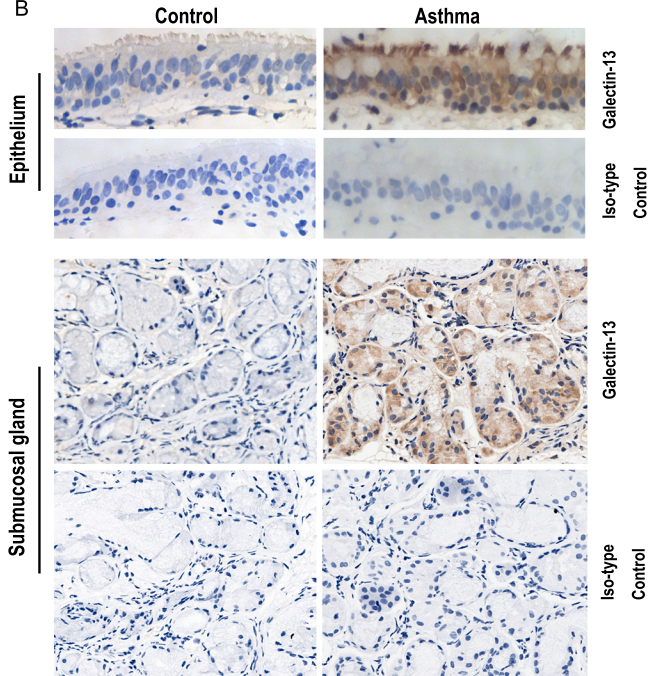
Figure S1. Galectin-13 is sufficient for MCP-1 and eotaxin-1 expression in human airway epithelial cells. HBE cells were treated with human recombinant galectin-13 (100ng/ml). After treated for 6 hours, 24 hours, and 48 hours, cells and culture supernatants were harvested for MCP-1 and and eotaxin-1 expression by quantitative RT-PCR and ELISA(A,C,E,G). 1HBE cells were treated with human recombinant galectin-13 for different concentrations (0, 50, 100, 200ng/ml). Twenty-four hours after treatment, cells and culture supernatants were harvested for MCP-1 and and eotaxin-1 expression by quantitative RT-PCR and ELISA(B,D,F,H). Data are mean $\pm$ SEM. *, $P<0.05$; **, $P<0.01$. The data are representative of three independent experiments.

Figure S2. EGFR and ERK1/2 pathways are required for IL-13-induced MCP-1 and eotaxin-1 expression in human airway epithelial cells. HBE cells were treated with IL-13, both EGFR inhibitors ZD1839 and IL-13, or both ERK1/2 inhibitor U0126 and IL-13. Forty-eight hours after treatment, the cell culture supernatants were collected for MCP-1 and eotaxin-1 expression by ELISA. Data are mean $\pm$ SEM. *, $P<0.05$; **, $P<0.01$. The data are representative of three independent experiments.

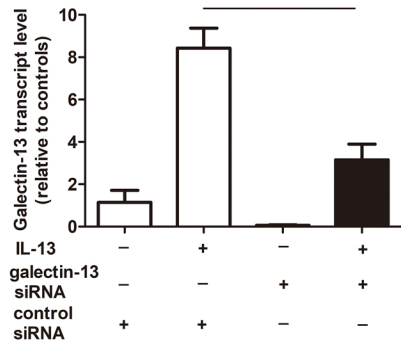
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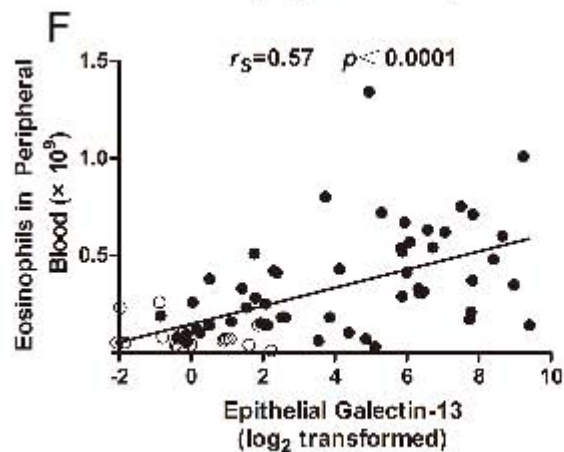
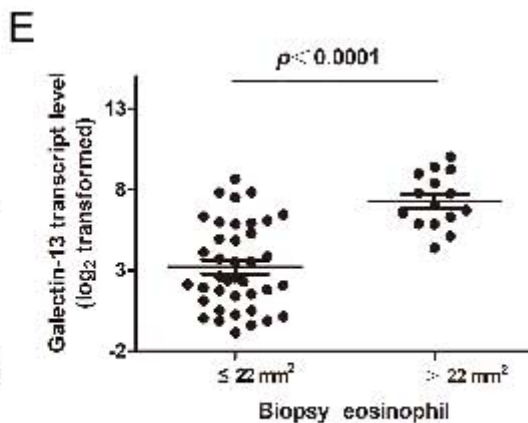
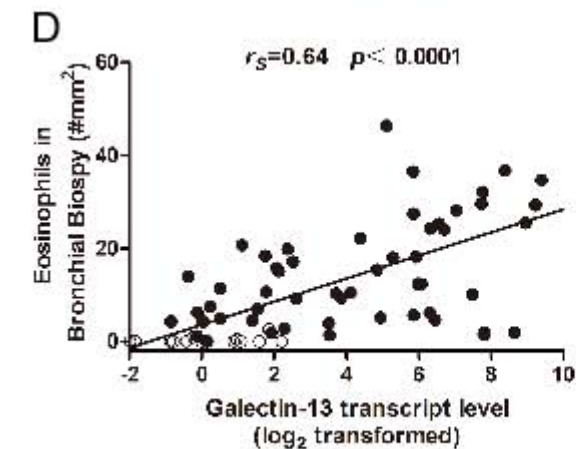
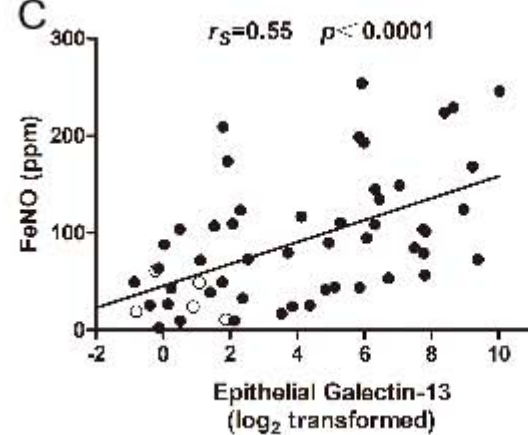
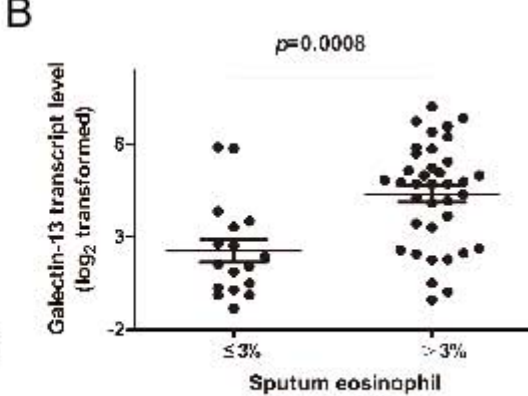
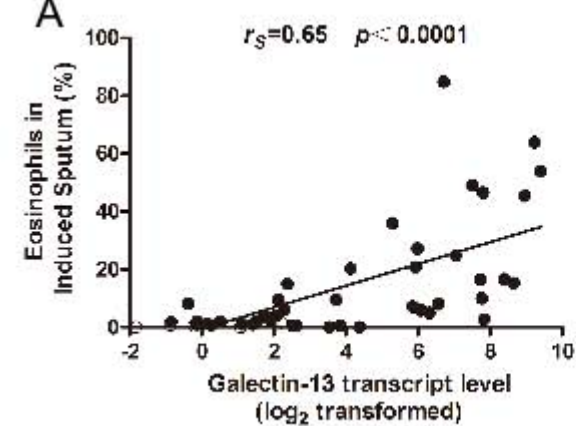


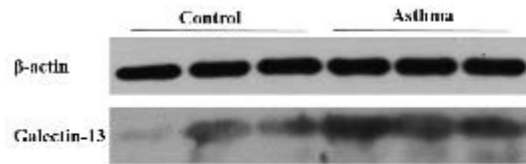
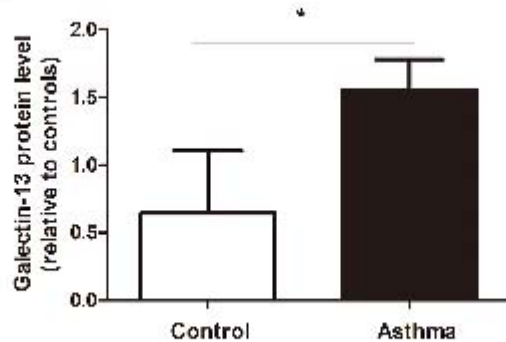
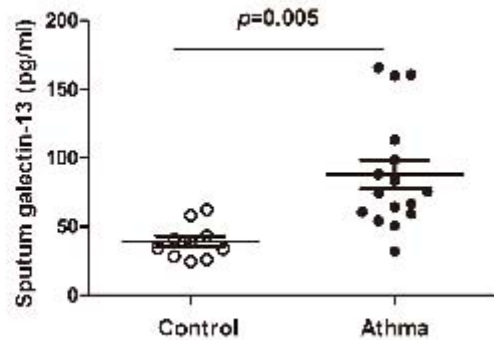
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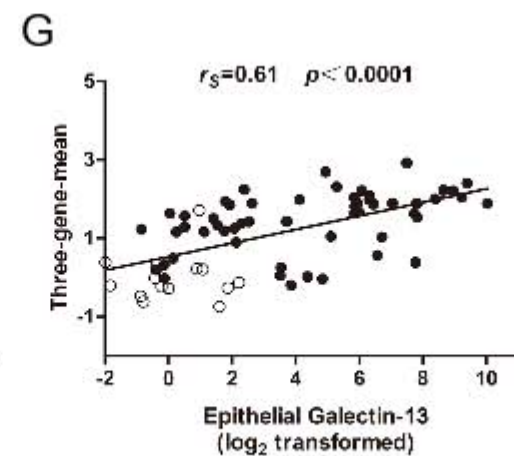
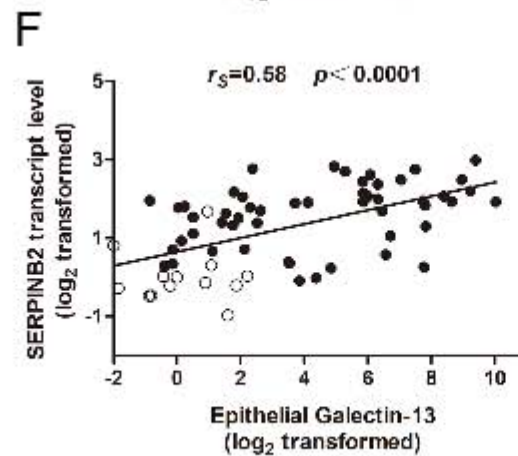
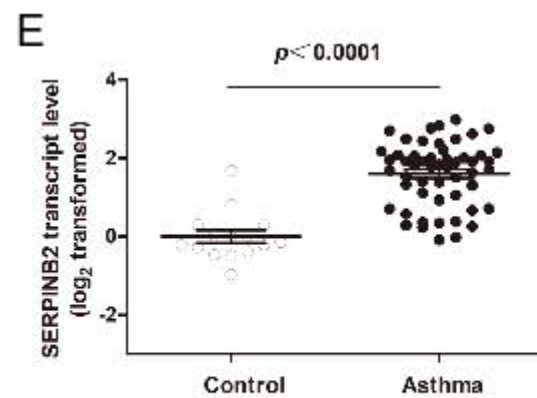
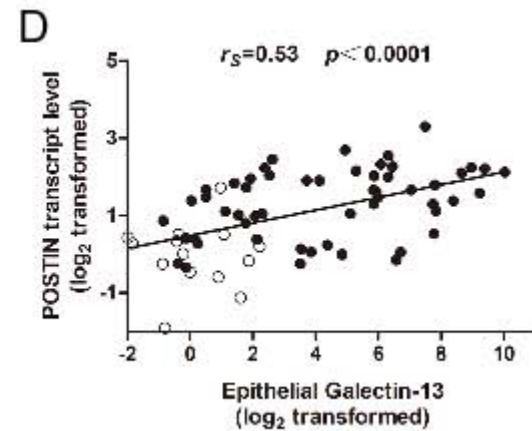
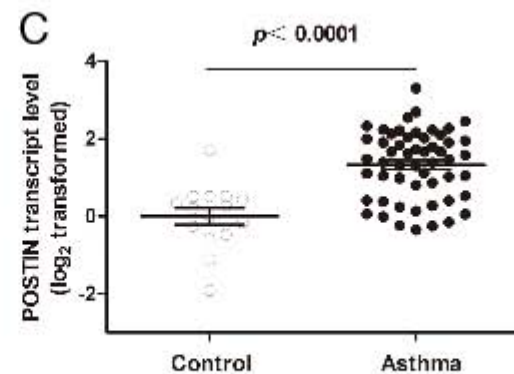
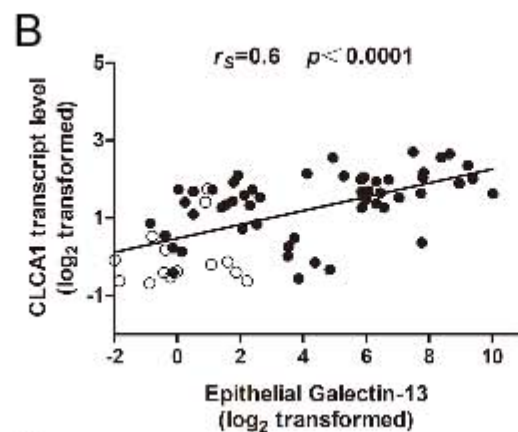
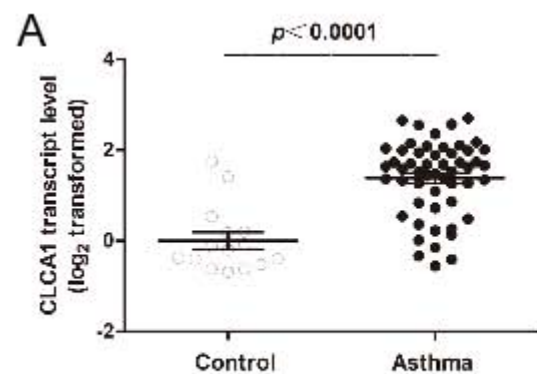


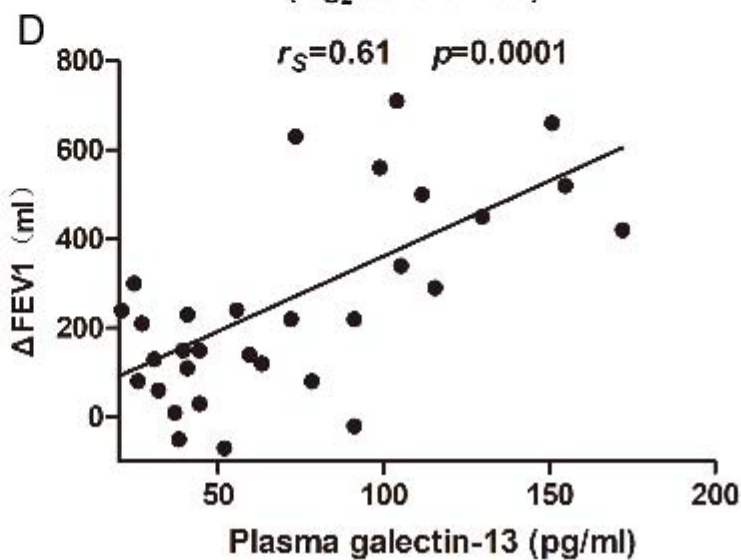
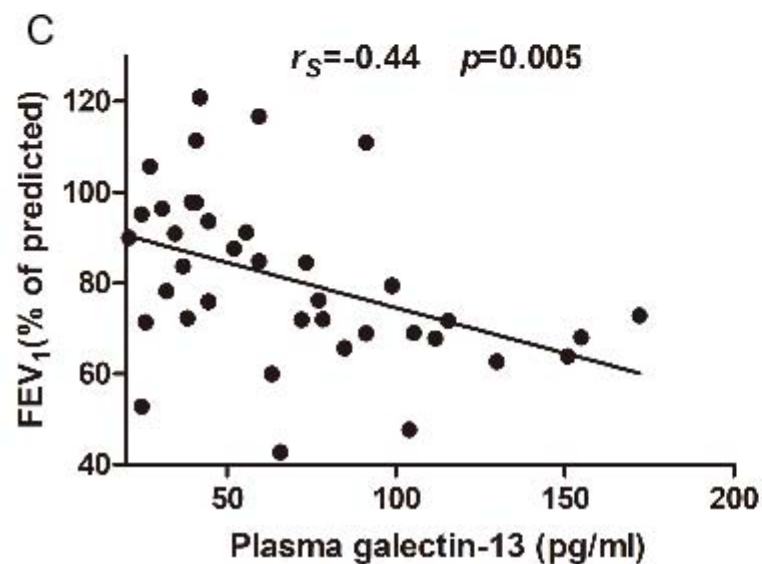
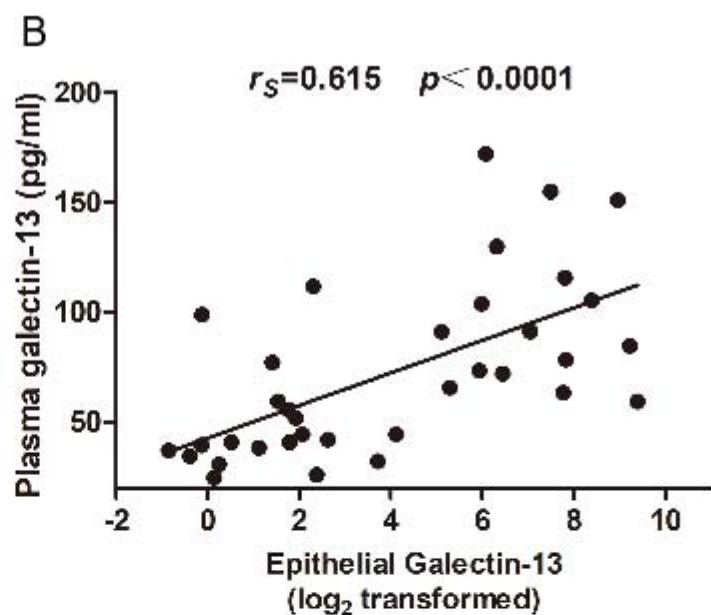
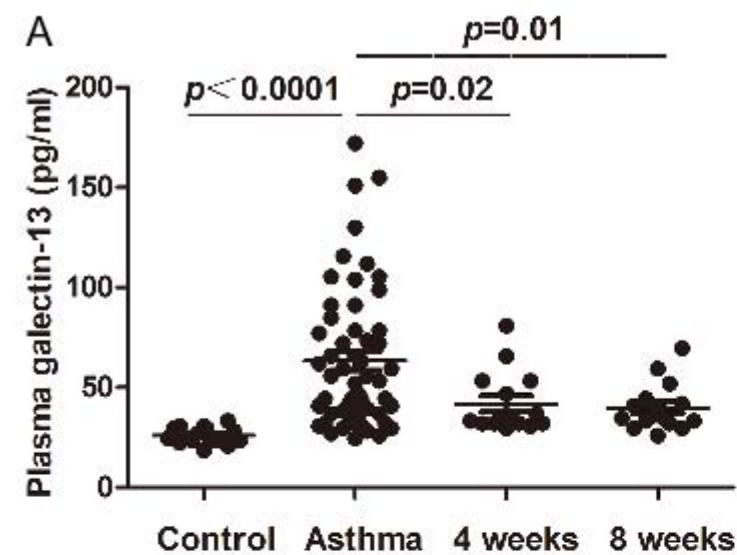
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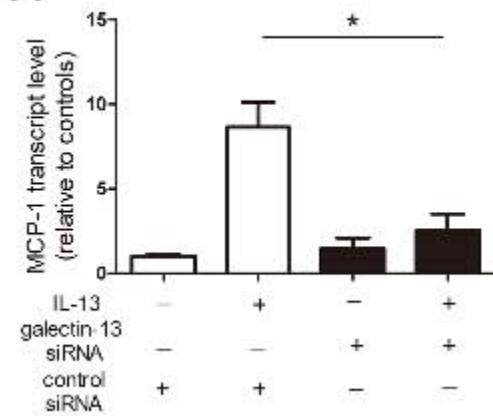


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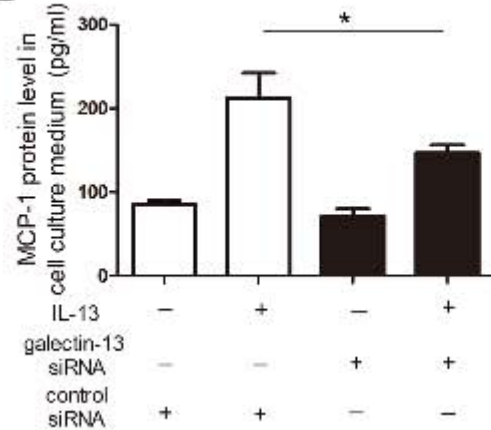




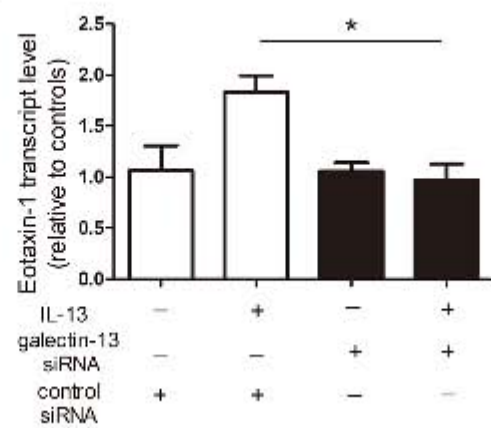
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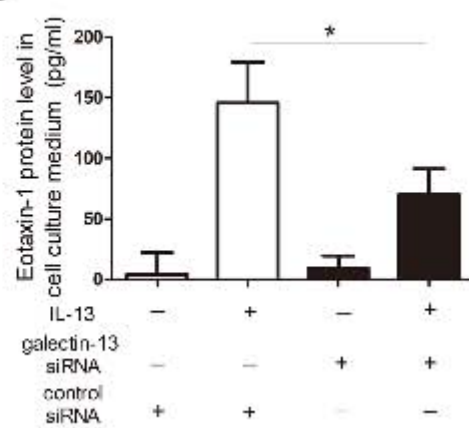
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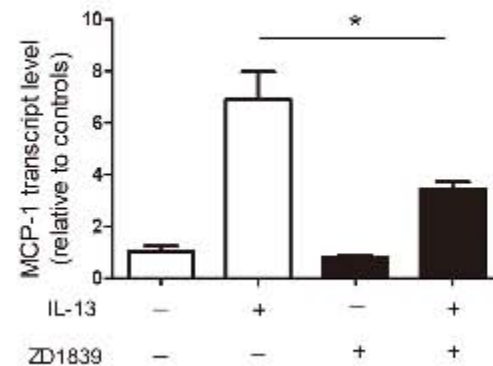
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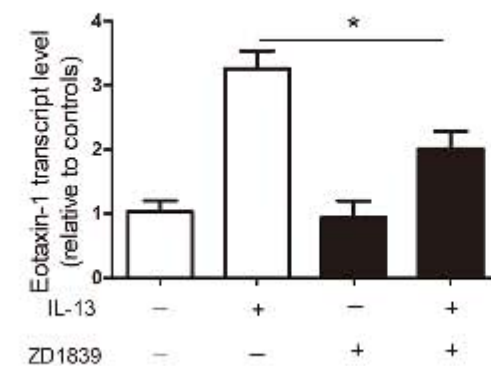
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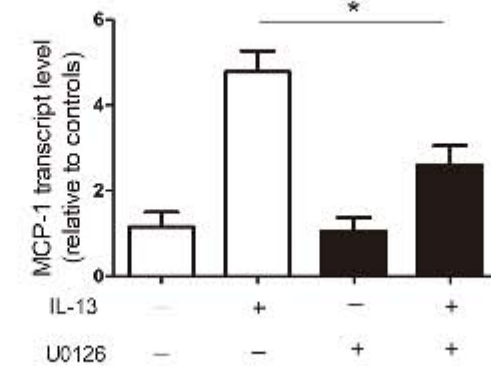
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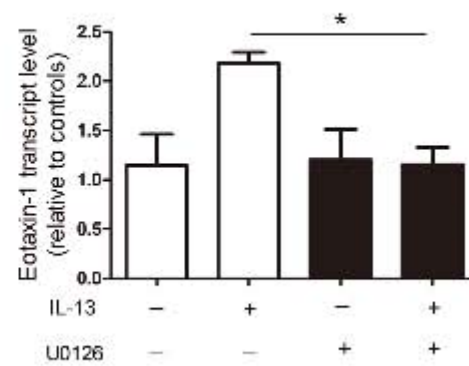
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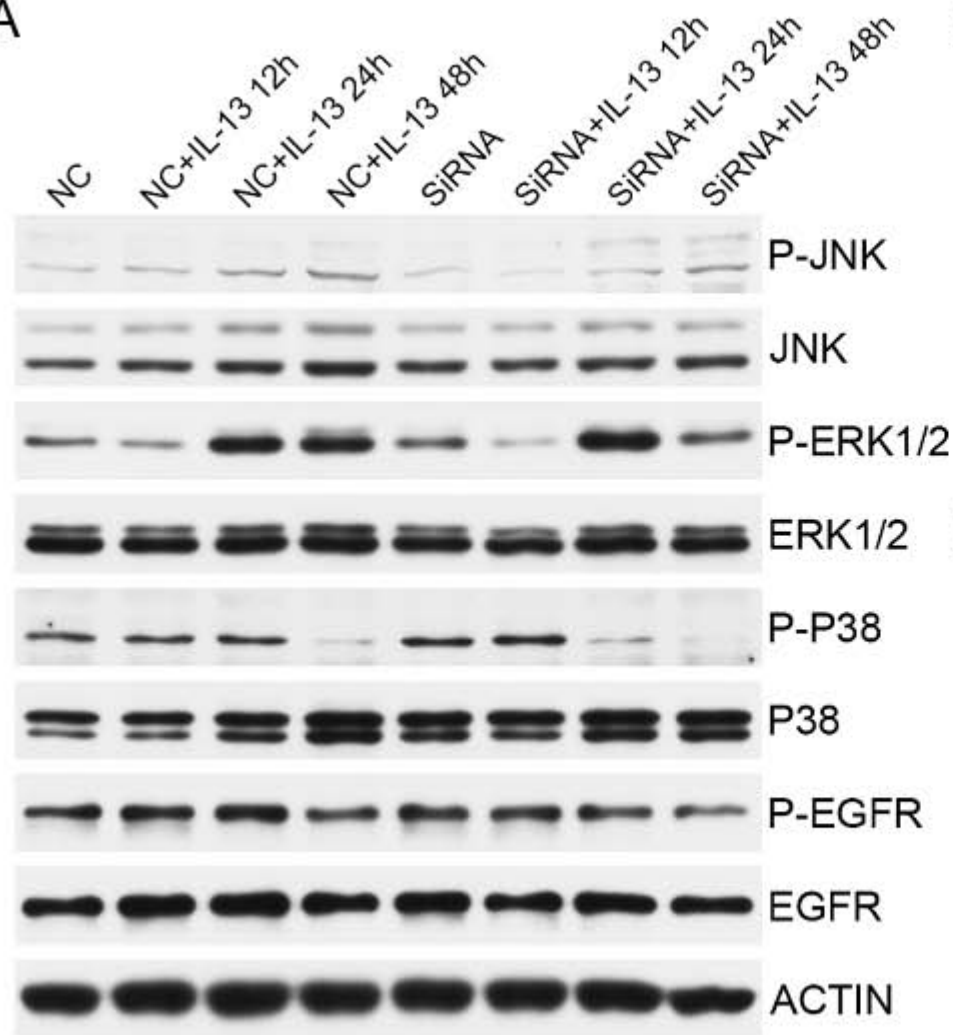
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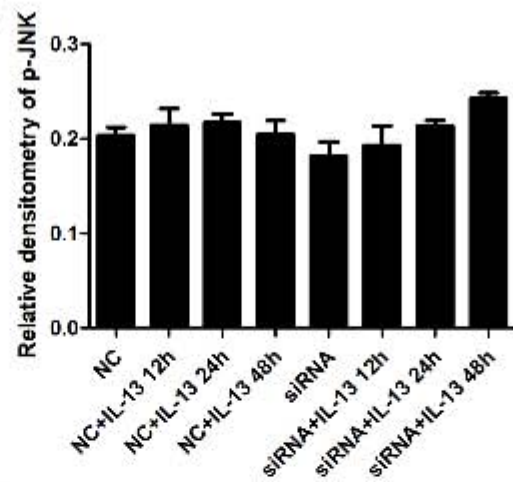
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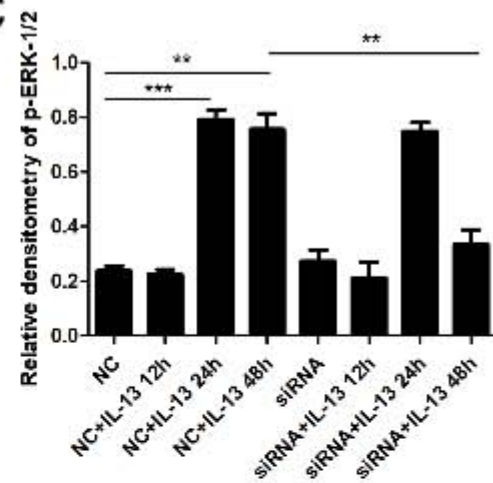
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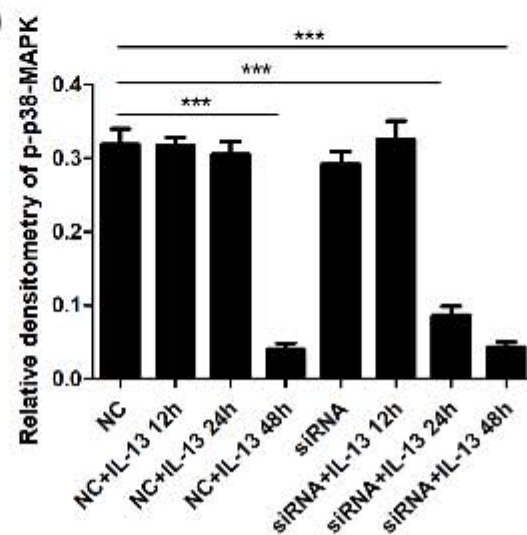
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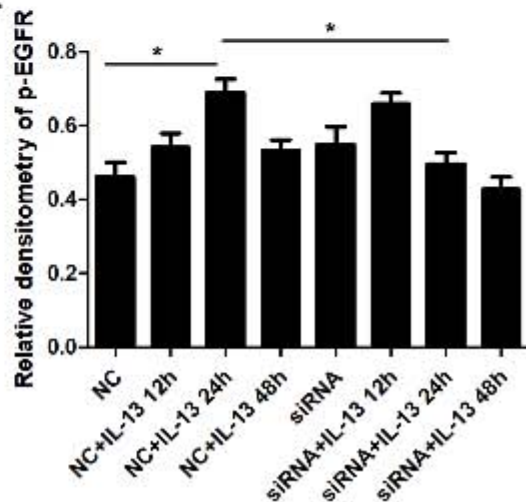
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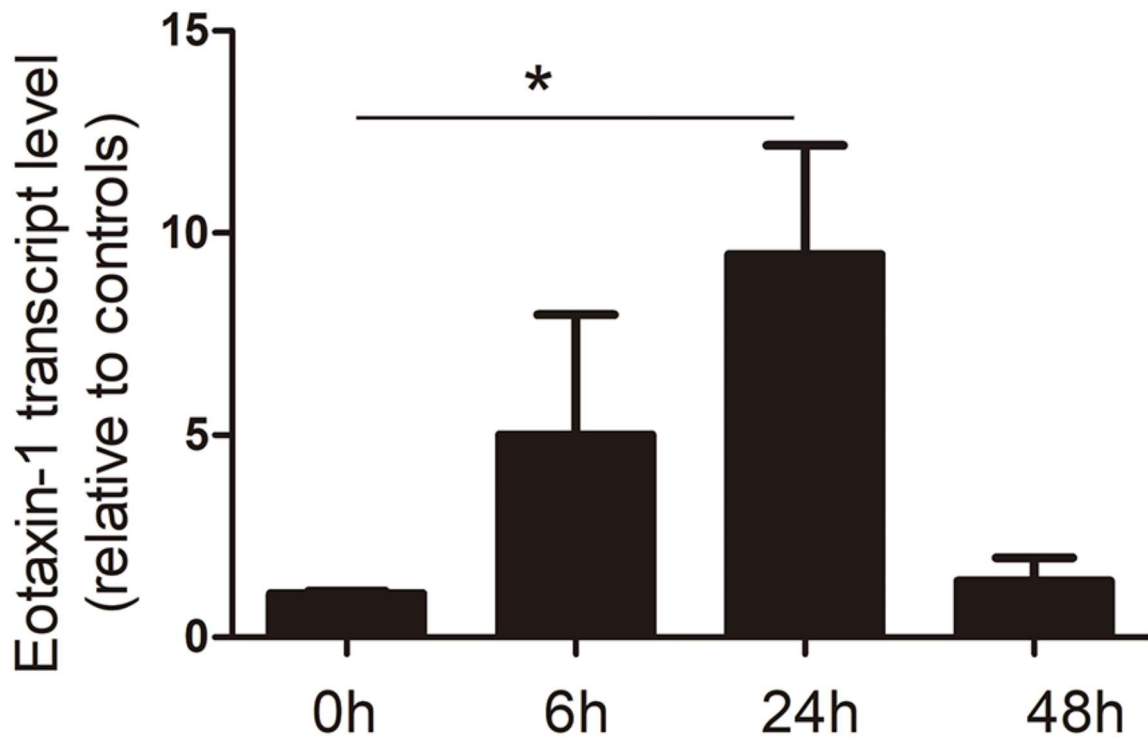


E



A

Human recombinant galectin-13 (100ng/ml)



B

Human recombinant galectin-13 (100ng/ml)

