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MUC18 regulates IL-13 mediated airway inflammatory response

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Abstract

Objective—To evaluate the effects of MUC18 on IL-13 mediated airway inflammatory responses in human airway epithelial cells and in mice.

Materials—Primary normal human tracheobronchial epithelial (HTBE) cells, wild-type (WT) and Muc18 knockout (KO) mice, and mouse tracheal epithelial cells (mTECs) were utilized.

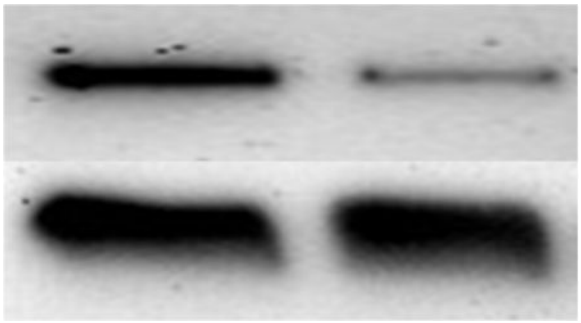
Treatment—Cultured HTBE cells treated with MUC18 siRNA or MUC18 expressing lentivirus were incubated with IL-13 (10 ng/mL) for 24 hours. Mice were intranasally instilled with 500 ng of IL-13 for 3 days. mTECs were treated with IL-13 (10 ng/mL) for 3 days.

Methods—PCR was used to measure mRNA expression. Western Blot and ELISAs were used to quantify protein expression. Cytospins of bronchoalveolar lavage (BAL) cells were used to obtain leukocyte differentials.

Results—MUC18 siRNA reduced IL-13 mediated eotaxin-3 (183 ± 44 pg/mL vs. 380 ± 59 pg/mL, $p<0.05$), while MUC18 overexpression increased IL-13 mediated eotaxin-3 (95 ± 3 pg/mL vs. 58 ± 3 pg/mL, $p<0.05$) in HTBE cells. IL-13 treated Muc18 KO mice had a lower percentage of neutrophils in BAL than WT mice (25 ± 3% vs. 35 ± 3%, $p=0.0565$).

Conclusions—These results implicate MUC18 as a potential enhancer of airway inflammation in a type 2 cytokine (e.g., IL-13) milieu.

Graphical abstract



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Keywords

MUC18/Muc18; inflammation; mucin; airway epithelium

Introduction

Excessive inflammation and mucous goblet cell metaplasia are seen in airways of patients with asthma, a disease that effects millions of people worldwide [1]. Asthma is heterogeneous in nature, with various endotypes characterized by differential responses to treatments, such as inhaled corticosteroids, as well as differing underlying molecular mechanisms [2]. One of the most well characterized endotypes is type 2-high asthma, which is defined by increased type 2 cytokine (e.g., IL-13) expression or signaling [3]. IL-13 has been identified as a key mediator in type 2-high asthma as it is related to two hallmarks of the disease, eosinophilic inflammation [1, 4] and mucus production [5]. IL-13 induces airway epithelial expression of eotaxin (e.g., eotaxin-3), which is a strong chemoattractant for eosinophils [6]. The induction of eotaxin by IL-13 is dependent on the transcription factor signal transducer and activator of transcription 6 (STAT6), which contributes to eosinophil recruitment to the airways [7, 8]. The *in vivo* role of airway epithelial STAT6 in IL-13 mediated effects such as lung eosinophilia and mucus production has been confirmed in mice that expressed STAT6 only in lung epithelium [9]. Several antibodies against IL-13 have been developed as therapeutic agents to combat asthma that is unresponsive to traditional treatments. However, in clinical trials these treatments have proven to be only moderately successful in treating the full range of type 2-high asthma symptoms, necessitating further research into the mechanisms by which IL-13 acts in the airway.

Although not part of the classical type 2 phenotype, excessive neutrophilic inflammation is another characteristic of asthma, which may be driven in part by IL-13 signaling [10]. Indeed, a subset of severe asthmatics present mixed eosinophilic and neutrophilic airway inflammation [11], suggesting that enhanced neutrophilic inflammation is not mutually exclusive with type 2 cytokine-mediated eosinophilia. Furthermore, neutrophil counts in patients across a spectrum of asthma severity correlated with more severe airway obstruction [12], and excessive neutrophilic inflammation has been noted in patients experiencing acute exacerbations of asthma [13]. Thus, in the context of type 2-high asthma, neutrophilic inflammation appears to play a role in increasing disease severity. However, what drives the eosinophilic or neutrophilic inflammation in the type 2 cytokine milieu remains uncertain.

In this study, we explored the role of MUC18 in IL-13-mediated production of pro-inflammatory cytokines involved in eosinophilic or neutrophilic inflammation. MUC18 is a transmembrane protein normally expressed in vascular endothelial cells and airway smooth muscle cells. MUC18 was originally found to correlate with metastasis of melanoma [14]. However, MUC18/Muc18 (human/mouse) has recently been shown to regulate airway immunity. Specifically, we have shown that MUC18 is overexpressed in airway epithelium of patients with mild to moderate asthma and is induced by IL-13 in human airway epithelial cells [15]. Furthermore, MUC18 has been shown to enhance IL-8 production in response to Toll-like receptor (TLR) agonists, decrease antiviral gene expression, and promote bacterial

adherence [15, 16]. Here, we hypothesized that MUC18/Muc18 plays a distinct role in eosinophilic and neutrophilic inflammation in response to IL-13 stimulation. We used primary airway epithelial cells from both humans and mice, as wells as a Muc18 knockout mouse model of IL-13 treatment, to test our hypothesis.

Methods

Ethics Statement

Experimental animals (i.e., mice) used in this study were covered by a protocol approved by Institutional Animal Care and Use Committee (IACUC) of National Jewish Health. All experimental procedures were carried out to minimize animal discomfort, distress, and pain by following the American Veterinary Medical Association Guidelines.

Mouse model of airway IL-13 treatment

Muc18 knockout (KO) mice and control littermates were bred at National Jewish Health Biological Resources Center as we previously reported [17]. Recombinant mouse IL-13 protein (R&D Systems, Minneapolis, MN) was reconstituted in 0.1% BSA and stored at −80°C. Age (8 to 12 weeks old)- and gender-matched Muc18 KO and control (wild-type) mice were intranasally inoculated with 50 μL of IL-13 (10 ng/μL) diluted in 0.1% BSA or 0.1% BSA alone as a control. IL-13 or BSA treatment was administered once daily for three consecutive days. Mice were sacrificed at 24 hours after final IL-13 treatment.

Mouse lungs were lavaged with 1 ml of sterile saline. Cell-free bronchoalveolar lavage (BAL) fluid was stored at −80°C for cytokine analysis. Cytospins of BAL cells were stained with Diff-Quick Kit (IMEB INC., San Marcos, CA), and leukocyte differentials were determined as percentage of 500 counted leukocytes.

The left lung lobes were collected in RNAlater (Thermo-Fisher Scientific, Waltham, MA) and stored for 24 hours at 4°C. RNAlater was then removed and lungs were frozen at −80°C. Frozen lung lobes were homogenized in TRIzol reagent (Thermo-Fisher Scientific, Waltham, MA) and used to extract RNA.

MUC18 knockdown (KD) in human tracheobronchial epithelial (HTBE) cells

We obtained de-identified donor lungs through the International Institute for the Advancement of Medicine (Edison, NJ) and the National Disease Research Interchange (Philadelphia, PA). These lungs were not suitable for transplantation and donated for medical research. The donors we chose for the current study did not have history of lung disease, and were non-smokers or ex-smokers who had quit smoking for at least 10 years. Cell collection was approved by the Institutional Review Board (IRB) at National Jewish Health.

To isolate HTBE cells, tracheal and main bronchial tissue was digested with 0.1% protease in DMEM (GE Life Sciences, Logan, Utah) overnight at 4°C, and processed as previously described [18].

HTBE cells were grown in submerged culture, as a previous study has shown selective induction of eotaxin-3 by IL-13 in submerged human airway epithelial cells, and a positive correlation between eotaxin-3 levels and airway eosinophil counts in asthma patients [19]. At passage 2, HTBE cells were seeded onto 12 well plates at 1.2×10^5 cells/well in bronchial epithelial cell growth medium (BEGM) (Lonza, Walkersville, MD). The cells were then transfected with either MUC18 siRNA or scrambled control siRNA with no homology to any known gene sequence for 24 hours as per the manufacturer's protocol (Santa-Cruz, Dallas, TX). After the transfection, medium was removed and replaced with BEGM. Three hours later, cells were either left untreated or treated with 10 ng/mL of recombinant human IL-13 (R&D Systems, Minneapolis, MN) for 24 hours. Cell supernatants and lysates were collected for ELISA and lysates were harvested in RLT (Qiagen, Hilden, Germany) or RIPA Western lysis buffer (Thermo-Fisher Scientific, Waltham, MA) for RT-PCR and Western Blot, respectively.

MUC18 overexpression (OE) in HTBE cells

A lentivirus system was utilized to generate a MUC18-overexpressing HTBE cell line as previously described [16]. Briefly, HTBE cells were seeded into collagen coated 60 mm dishes with BEGM and grown to 80% confluence. The cells were transduced with MUC18 expressing or control (GFP) lentivirus by centrifugation at 2000 rpm for 1 hr at 25 °C, followed by a full medium change. One day later the cells were either passaged onto 12 well plates for IL-13 treatment as described above, or frozen down for future cell expansion and experiments.

Mouse tracheal epithelial cell isolation and culture

Mouse tracheal epithelial cells (mTECs) were isolated from Muc18 KO and WT mice by incubating excised mouse tracheas in DMEM with 0.1% protease and 50 μg/mL amphotericin B overnight at 4°C, then washed and collected in DMEM with 2% FBS. The cells were then dissociated, washed, and resuspended in F media with Y-27632 at 10 μM and 125 ng/mL of mouse epidermal growth factor (mEGF) before being plated and expanded onto 100 mm dishes coated with irradiated 3T3 fibroblasts [20]. After expansion, the cells were trypsinized and seeded onto collagen coated 12-well transwell plates with PneumaCult complete air-liquid interface (ALI) culture medium (Stem Cell Vancouver, BC) with 10 μM of Y-27632 and 37.5 ng/mL mEGF (PeproTech, Rocky Hill, NJ). Cells in transwells were grown for 6 days in submerged conditions and then shifted to ALI for 14 days. The cells were continuously treated with 10 ng/mL of rmIL-13 in the final 3 days of ALI. On day 14 of ALI, cultured cells were lysed using RLT lysis buffer and used to quantify mucin mRNA expression by RT-PCR.

Quantitative *real-time* PCR

TaqMan gene expression assays by Applied Biosystems (Life Technologies, Foster City CA) were used to measure mRNA levels of human MUC18, eotaxin-3 and GAPDH, and mouse eotaxin-1, eotaxin-2, Muc5AC and 18S rRNA. Target gene expression was normalized to GAPDH and 18S rRNA for human and mouse genes, respectively. The comparative threshold cycle method was used to determine relative gene expression levels.

Western Blot

Equal amounts of cell lysate proteins were loaded into an 8% polyacrylimide gel and separated by electrophoresis at 100V. The separated proteins were transferred onto PVDF or nitrocellulose membranes, blocked for one hour, and probed for STAT6 and p-STAT6 (Cell Signaling Technologies, Danvers, MA) in 5% BSA, and MUC18 (Abcam, Cambridge, MA), β-actin or GAPDH (Santa Cruz Biotechnology, Dallas, TX) in 5% non-fat milk. Densitometry was performed to quantify protein expression levels.

ELISA

ELISA kits from R&D systems (Minneapolis, MN) were used to measure human eotaxin-3 and IL-8 in cell supernatants, and mouse eotaxin-2 in BAL fluid.

Statistical analysis

One-way analysis of variance (ANOVA) was used for multiple group (≥3 groups) comparisons, and a Tukey's post hoc test was then applied for pairwise comparisons. Student's *t* test was used when only two groups were compared. A p value < 0.05 was considered significant.

Results

MUC18 promotes eotaxin-3, but not IL-8 production in cultured primary human tracheobronchial epithelial (HTBE) cells stimulated with IL-13

We used both MUC18 KD and OE approaches to investigate the role of MUC18 in IL-13 mediated inflammatory response in HTBE cells.

MUC18 siRNA was shown to significantly reduce MUC18 protein expression (Figure 1A and 1B). IL-13 trended to increase neutrophil chemoattractant IL-8 in both control (scrambled siRNA) and MUC18 KD cells (Figure 1C). However, IL-8 levels were not significantly different between control and MUC18 KD cells after 24 hours of IL-13 treatment.

Next, we investigated the effect of MUC18 KD on levels of the eosinophil chemoattractant eotaxin-3. As previously reported, IL-13 markedly ($p < 0.05$) increased eotaxin-3 expression (Figure 1D and 1E) in control cells. Notably, MUC18 siRNA significantly reduced IL-13 induced eotaxin-3 expression at protein level, and trended to reduce eotaxin-3 at the mRNA level (7,570 ± 1,813 vs. 4,797 ± 1,405).

To extend our findings in MUC18 KD cells, a lentivirus-mediated MUC18 OE approach was utilized. Cells transduced with MUC18 expressing lentivirus as compared to control cells (GFP expressing lentivirus) showed greater MUC18 protein expression (Figure 2A and 2B). β-actin was used in place of GAPDH as a loading control in MUC18 OE Western Blot experiments. We observed that GAPDH levels were consistently low and varied greatly between conditions despite the fact that we had loaded equivalent amounts of protein. This suggests that GAPDH does not serve as a good protein loading control in the MUC18 OE cells, which may be due to effects of MUC18 OE cells expanded on irradiated fibroblasts

and the use of lentivirus system. We therefore decided to change the housekeeping gene to β-actin, a popular control for protein loading in Western Blot experiment, for our MUC18 OE cells.

IL-13 significantly increased levels of IL-8 protein in both GFP control and MUC18 OE cells, however MUC18 OE had no significant effect on IL-13 induced IL-8 production (Figure 2C). Eotaxin-3 protein and mRNA levels were significantly higher in MUC18 OE cells than the control cells after 24 hours of IL-13 treatment (Figure 2D and 2E).

MUC18 up-regulates IL-13-mediated eotaxin-3 in part through STAT6 activation in HTBE cells

How MUC18 regulates human airway epithelial eotaxin expression in the context of IL-13 stimulation remains poorly understood. IL-13 has been shown to induce eotaxin-3 at least in part through activating the transcription factor STAT6 [7, 21]. We knocked down STAT6 (Figure 3A) using siRNA to confirm the role of STAT6 in IL-13-mediated eotaxin-3 expression. STAT6 KD had similar effects to MUC18 KD on eotaxin-3 production (Figure 3B). We demonstrated STAT6 activation ($p < 0.05$) following IL-13 treatment in cultured HTBE cells with control siRNA (Figure 4A and 4B). Importantly, the ratio of phosphorylated STAT6 (p-STAT6) to GAPDH in IL-13 treated HTBE cells transfected with MUC18 siRNA was significantly reduced when compared to cells transfected with control siRNA. However, the ratio of p-STAT6 to total STAT6 was similar between the two groups (densitometry data not shown). Likewise, STAT6 phosphorylation level in IL-13-treated MUC18 OE cells, as indicated p-STAT6/β-actin, trended to increase with respect to the control cells at 24 hours after treatment (Figure 5A and 5B).

Muc18 increases neutrophilic inflammation and KC protein levels in IL-13 treated mice

To reveal the *in vivo* role of Muc18 in modulating IL-13-mediated lung inflammation, we measured neutrophils and eosinophils in BAL fluid. At 24 hours after IL-13 treatment, both WT and KO mice showed an increase in the percentage of neutrophils as well as the total number of neutrophils in BAL compared to their BSA control treated counterparts (Figure 6A and 6B). Muc18 KO mice treated with IL-13 had near significant reduction in the percentage of neutrophils found in BAL compared to WT mice treated with IL-13 ($25.4 \pm 3.1\%$ vs. $35.1 \pm 2.6\%$, $p = 0.0565$) (Figure 6A), which was accompanied by significantly lower levels of KC in BAL fluid of Muc18 KO mice (Figure 6C).

In contrast to neutrophilic inflammation, lung eosinophilic inflammation was similar between Muc18 KO and WT mice. After 24 hours, IL-13 treatment increased BAL eosinophils in both WT and Muc18 KO mice (Figure 7A and 7B). However, there was no significant difference in BAL eosinophils between Muc18 KO and WT mice. In keeping with BAL eosinophil data, BAL eotaxin-2 protein (Figure 7C) and lung tissue eotaxin-2 mRNA (data not shown) levels were similar between the two groups of mice.

Muc18 promotes Muc5AC expression in mouse lungs

To further determine the role of Muc18 in type 2 cytokine exposed airways, we measured the lung tissue gel-forming mucin Muc5AC, as it is up-regulated by IL-13. Understanding

MUC18's broader impact on lung pathobiology would likely provide a better strategy to target MUC18 in human lung diseases where IL-13/MUC18 axis is up-regulated.

IL-13 significantly increased Muc5AC mRNA levels in lungs of WT mice after 24 hours of IL-13 treatment (Figure 8A). Notably, following IL-13 treatment, Muc18 KO mouse lungs showed significantly less Muc5AC mRNA expression than the WT mice.

To examine the direct effect of Muc18 on Muc5AC expression, mouse tracheal epithelial cells were cultured under ALI conditions in the presence or absence of IL-13 for 3 days to mimic our mouse model of IL-13 treatment. IL-13 treatment increased Muc5AC expression in WT cells, but not in Muc18 KO cells. After IL-13 treatment, Muc5AC expression trends to be lower in Muc18 KO cells than WT cells (Figure 8B).

Discussion

Our current study, for the first time, suggests that in a type 2 cytokine milieu, MUC18/Muc18 likely promotes airway eosinophilic or neutrophilic inflammation as well as gel-forming mucin expression. Airway eosinophilic inflammation is one of the major features in asthma, which exists even with corticosteroid treatment. Understanding the mechanisms of persistent eosinophilic inflammation in asthma is critical to finding more effective therapies. Eosinophils are recruited to the site of inflammation by a number of mediators, including eotaxin-3 produced by airway epithelial cells [22]. The mechanisms by which MUC18 up-regulates eotaxin-3 expression remain unclear. Our human airway epithelial cell culture experiments using the MUC18 KD and OE approaches strongly suggest that MUC18 may contribute to eosinophilic inflammation in type 2 cytokine-exposed human airways by promoting the production of eotaxin-3, one of the major eotaxins expressed by airway epithelial cells. We have shown that MUC18 in human airway epithelial cells, upon IL-13 stimulation, further increases STAT6 activation, which is critical to IL-13-induced eotaxin expression. Our finding of MUC18's contribution to eotaxin-3 expression in human airway epithelium may provide an alternative approach (e.g., airway MUC18 targeting) to ameliorate eosinophilic inflammation in type 2-high asthma.

To determine the *in vivo* contribution of Muc18 to lung eosinophilic inflammation, we utilized the Muc18 KO mouse model of IL-13 treatment. To our surprise, we did not observe significant differences in eosinophils or eotaxins between wild-type and Muc18 knockout mice. The discrepancy of MUC18/Muc18's role in regulating eosinophilic inflammation between humans and mice is not clear. We speculate that regulation of eosinophil influx *in vivo* involves multiple mechanisms that ultimately decide the predominance of an inflammatory cell type such as eosinophils over other immune cells. Likewise, *in vivo*, MUC18/Muc18 can be expressed by multiple cell types, including airway epithelial cells, alveolar macrophages and smooth muscle cells. It is unclear whether MUC18/Muc18 has similar or even opposite effects on IL-13-induced eotaxin expression in different cell types. Another plausible explanation is that eosinophil recruitment in mice, unlike humans, does not utilize eotaxin-3 [23]. We also measured eotaxin-1 mRNA in mouse lungs, and did not find any significant differences between Muc18 KO and wild-type mice. Therefore,

MUC18's enhancing effect on IL-13-mediated eosinophilic inflammation may only occur in humans due to the specific effect of MUC18 on eotaxin-3 induction.

In contrast to human airway epithelial cell culture data, results from our mouse model of IL-13 treatment suggest that Muc18 promotes IL-13 mediated lung neutrophilic inflammation. This finding further extended our early studies in mouse models of bacterial and viral infection [15–17]. Intriguingly, our human airway epithelial cell culture model does not indicate a role of MUC18 in promoting IL-8 production under IL-13 stimulation. However, our previous studies suggest that under stimulation of TLR agonists that mimic bacterial or viral infection, MUC18 enhances IL-8 production [16, 20]. Together, MUC18 in humans may promote eosinophilic and/or neutrophilic inflammation by promoting eotaxin-3 and/or IL-8 production depending on the nature of stimulants. Future experiments will be designed in both human airway epithelial cells and mouse models to test the role of MUC18/Muc18 in the context of both IL-13 and pathogens or their mimics.

Airway obstruction caused by excessive mucus production is another hallmark of type 2-high asthma. It has been shown that IL-13 increases expression of Muc5AC in mouse models [24]. Our discovery that Muc18 enhances IL-13 induced Muc5AC production in mouse lungs presents the possibility of novel treatment options for asthmatic airway obstruction caused by mucus overproduction. How Muc18 effects the expression of gel-forming mucin Muc5Ac has not been explored. By using the air-liquid interface culture of mouse tracheal epithelial cells from wild-type and Muc18 KO mice, we demonstrated that Muc18 deficiency trends to decrease IL-13-mediated Muc5AC expression, suggesting additional mechanisms underlying the enhancing effects of Muc18 on Muc5AC expression. One of the other potential mechanisms for Muc18 to enhance Muc5AC expression *in vivo* may be related to our observation that Muc18 promotes lung neutrophilic inflammation in response to IL-13, as neutrophil elastase is able to up-regulate Muc5AC production in the airways [25]. As MUC18, in our current study, was shown to amplify IL-13-induced STAT6 activation in human airway epithelial cells, and STAT6 contributes to IL-13-indcued MUC5AC expression [26], the pro-Muc5AC function of Muc18 in mice may act in part through the STAT6 signaling pathway, but this warrants further investigation.

Our study does come with limitations. First, although our submerged culture model of human airway epithelial cells showed significant and reproducible data with respect to MUC18's enhancing effect on IL-13 induced eotaxin-3 protein, we may need to use air-liquid interface culture model to extend our initial findings as well as to study the role of MUC18 in MUC5AC expression. Second, we have studied the role of Muc18 in the model of acute IL-13 stimulation, but we have not explored its role in the broader context of either chronic type 2 inflammation or within the framework of asthmatic exacerbations by known allergic stimuli such as house dust mite. Finally, we realize that IL-13 induced eotaxin-3 level is higher in cells treated with control siRNA than cells treated with control GFP lentivirus. While we do not know the exact mechanisms for this discrepancy, we propose that two factors may be involved. First, GFP lentivirus control airway epithelial cells, but not the control siRNA cells, were expanded on irradiated 3T3 fibroblasts. The difference in expansion techniques could account for differences in IL-13-induced eotaxin-3 production. Second, the lentivirus and siRNA we used to achieve MUC18 OE or KD may also affect

eotaxin-3 production. Despite the differences in techniques for cell culture and MUC18 protein manipulation, our conclusion that MUC18 promotes IL-13 induced eotaxin-3 protein remains unchanged. These limitations provide the immediate basis to further define the role of MUC18 in airway inflammation under different conditions that effect asthma patients.

In summary we have shown that MUC18/Muc18 may serve as a regulator of airway inflammation and mucus overproduction, two important features of type 2-high asthma. Our data suggest that MUC18/Muc18 or its downstream signaling mediators may be targets for potential therapeutic agents.

Acknowledgments

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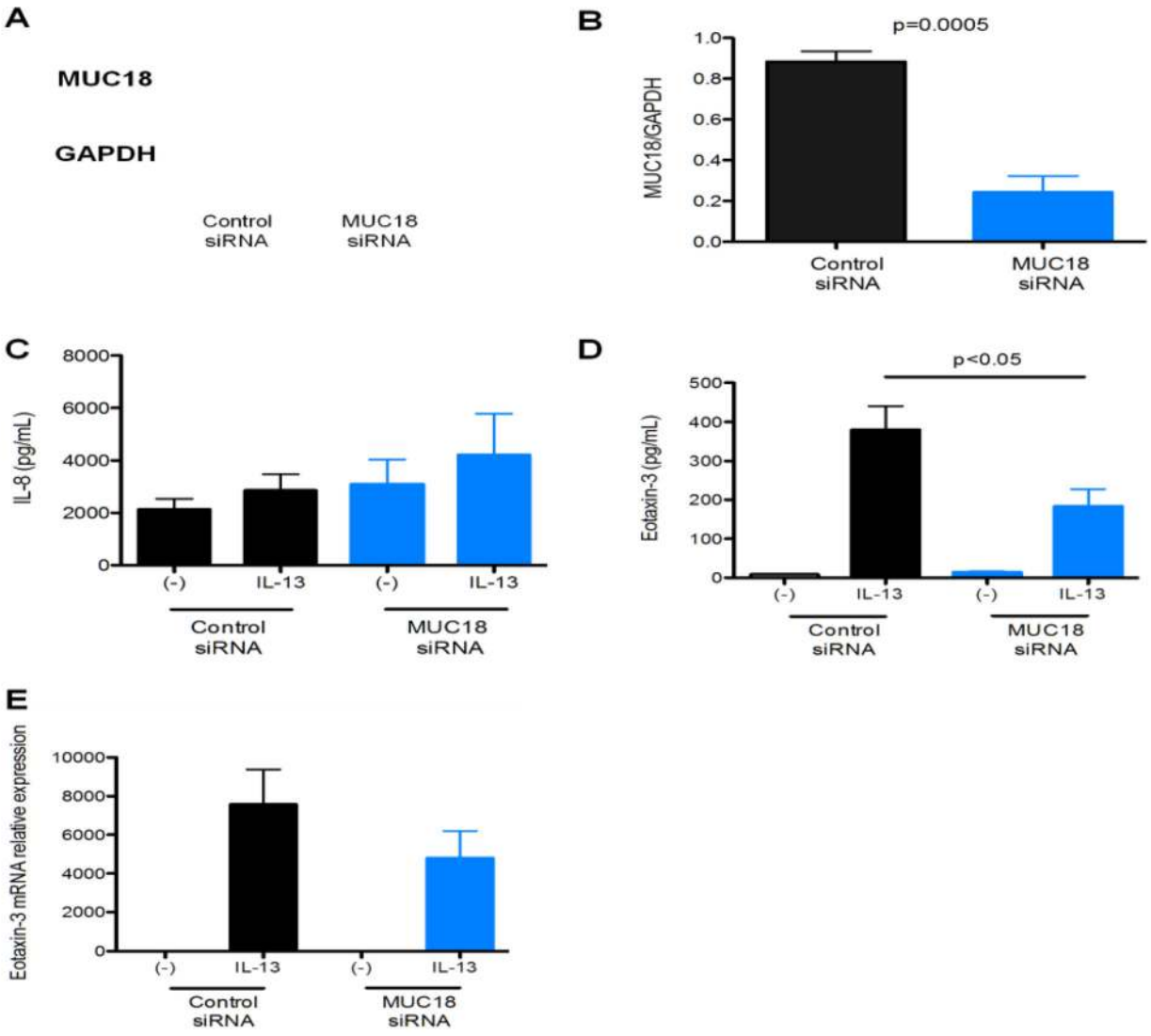


Figure 1. Effect of MUC18 knockdown on IL-8 and eotaxin-3 production in IL-13 treated primary human tracheobronchial epithelial (HTBE) cells

HTBE cells (n = 4 subjects) were transfected with either scrambled control siRNA or MUC18 siRNA. MUC18 was detected by Western Blot (**A**), and quantified using densitometry (**B**). Cells transfected with either scrambled control siRNA or MUC18 siRNA were treated with IL-13 for 24 hours. Cell supernatants were used to measure IL-8 (**C**) and eotaxin-3 (**D**) protein, while lysates were used to measure eotaxin-3 mRNA (**E**). Data are presented as Mean ± SEM. Statistical analysis was performed using the Student's *t* test (**B**) or ANOVA with a Tukey's post hoc test (**C – E**).

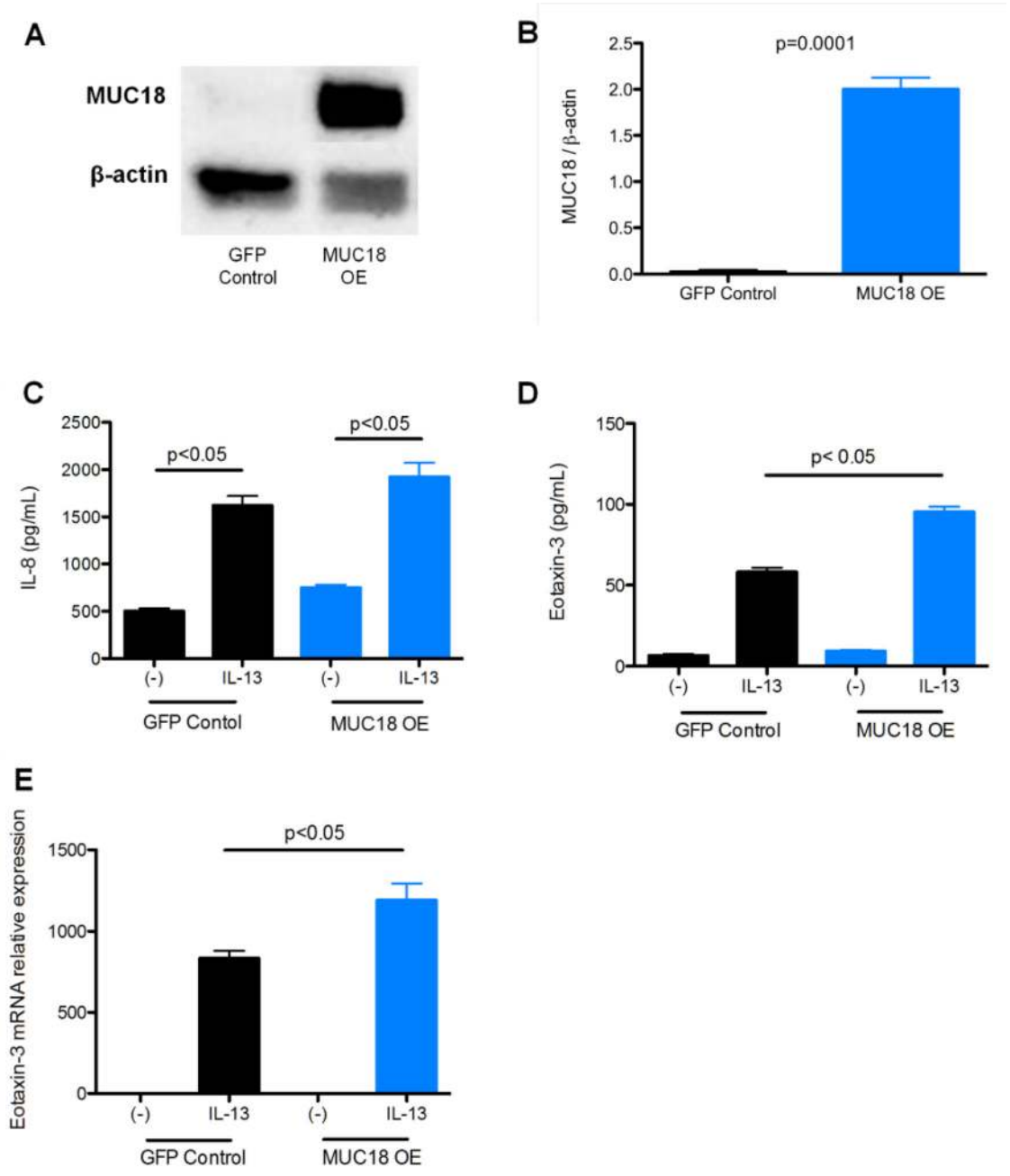
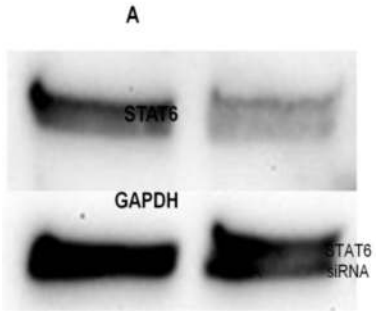


Figure 2. Effect of MUC18 overexpression (OE) on IL-8 and eotaxin-3 production in IL-13 treated primary human tracheobronchial epithelial (HTBE) cells

A HTBE cell line expressing either MUC18 or GFP was generated using a lentivirus system. MUC18 was detected by Western Blot (**A**) and quantified using densitometry (**B**). After 24 hours of IL-13 treatment, cell supernatants were used to measure IL-8 (**C**) and eotaxin-3 (**D**) protein, while lysates were used to measure eotaxin-3 mRNA (**E**). Data are presented as Mean ± SEM. Statistical analysis was performed using the Student's *t* test (**B**) or ANOVA with a Tukey's post hoc test (**C – E**). N = 6 replicates.



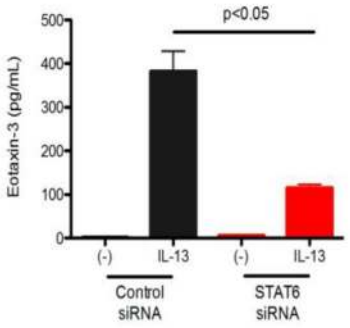


Figure 3. Effect of STAT6 knockdown on IL-13 mediated eotaxin-3 production in primary human tracheobronchial epithelial (HTBE) cells

HTBE cells were transfected with either scrambled control siRNA or STAT6 siRNA. Total STAT6 was detected by Western Blot (**A**). After 24 hours of IL-13 treatment, STAT6 siRNA-treated cells demonstrated lower levels of eotaxin-3 protein than the control cells (**B**). Data are presented as Mean ± SEM. Statistical analysis was performed using ANOVA with a Tukey's post hoc test. N = 6 replicates.

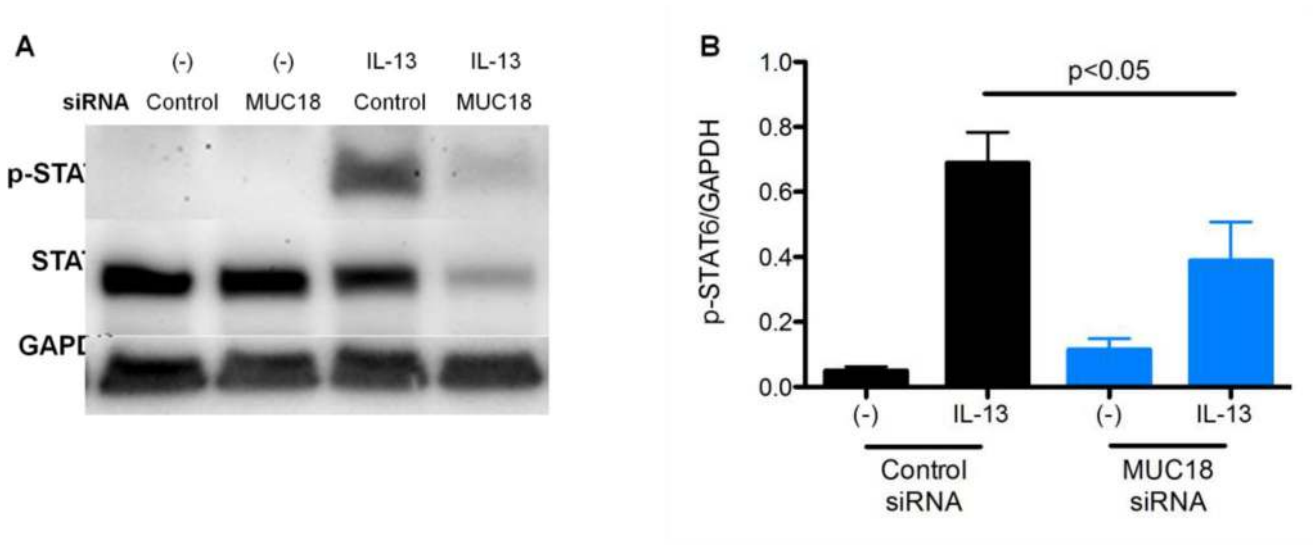


Figure 4. MUC18 knockdown in primary human tracheobronchial epithelial (HTBE) cells attenuates IL-13 induced STAT6 activation

Cell lysates (n = 4) from MUC18 siRNA or control siRNA treated HTBE cells with or without IL-13 treatment for 24 hours were subjected for Western Blot of total STAT6, phosphorylated STAT6, and GAPDH as a loading control (**A**). STAT6 was quantified using densitometry (**B**). Data are presented as Mean ± SEM. Statistical analysis was performed using ANOVA with a Tukey's post hoc test.

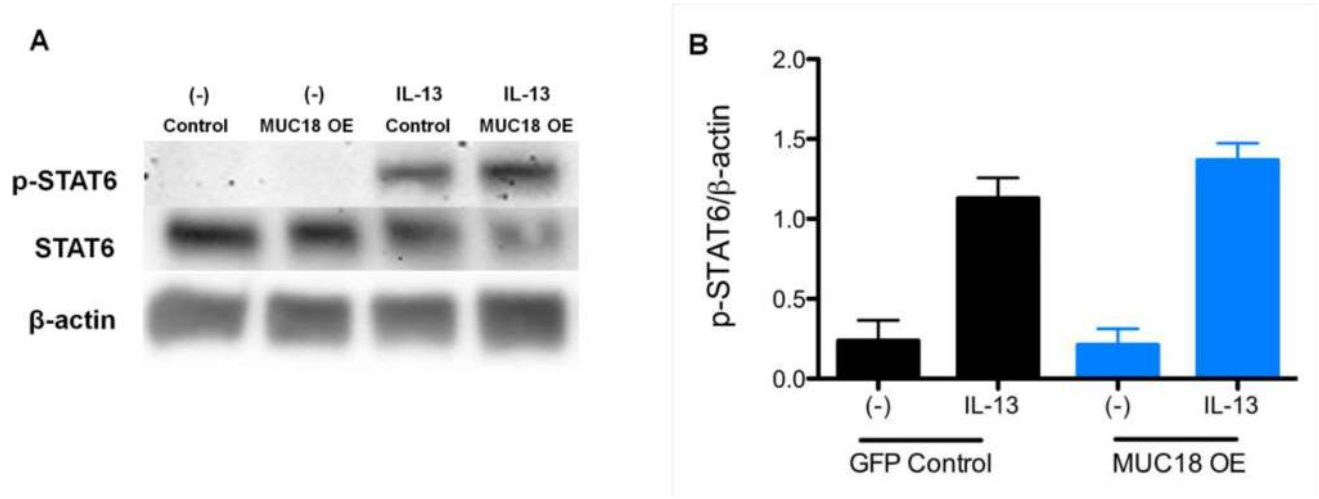


Figure 5. Effect of MUC18 overexpression (OE) on IL-13 induced STAT6 activation in primary human tracheobronchial epithelial (HTBE) cells

Cell lysates (n = 4) from MUC18 overexpressing or control HTBE cells with or without IL-13 treatment for 24 hours were subjected for Western Blot of total STAT6, phosphorylated STAT6, and GAPDH as a loading control (**A**). STAT6 was quantified using densitometry (**B**). Data are presented as Mean ± SEM. Statistical analysis was performed using ANOVA with a Tukey's post hoc test.

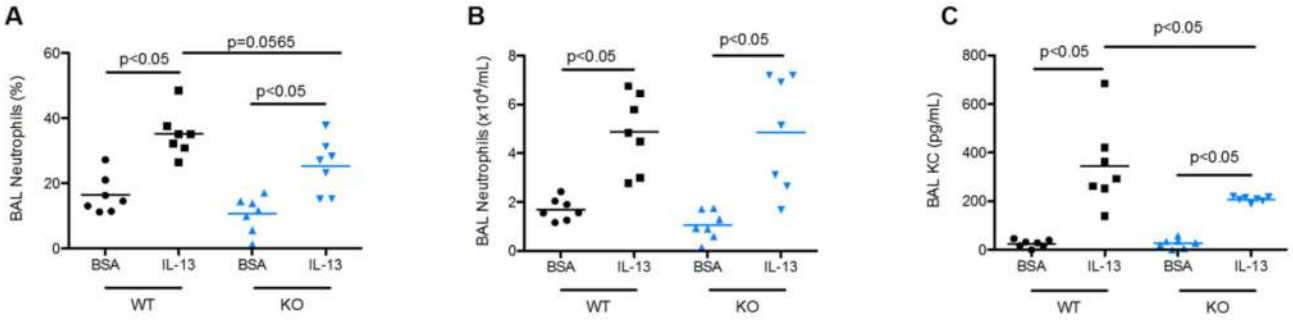


Figure 6. Effect of Muc18 on lung neutrophilic inflammation in IL-13 treated mice

Wild-type (WT) and Muc18 knockout (KO) mice (n = 7 mice/group) were treated with IL-13 and sacrificed at 24 hours after the final treatment. Bronchoalveolar lavage (BAL) fluid was collected to quantify neutrophils (**A** and **B**), and neutrophil chemokine KC (**C**). Horizontal bars indicate the Mean of the data, and statistical analysis was performed using ANOVA with a Tukey's post hoc test.

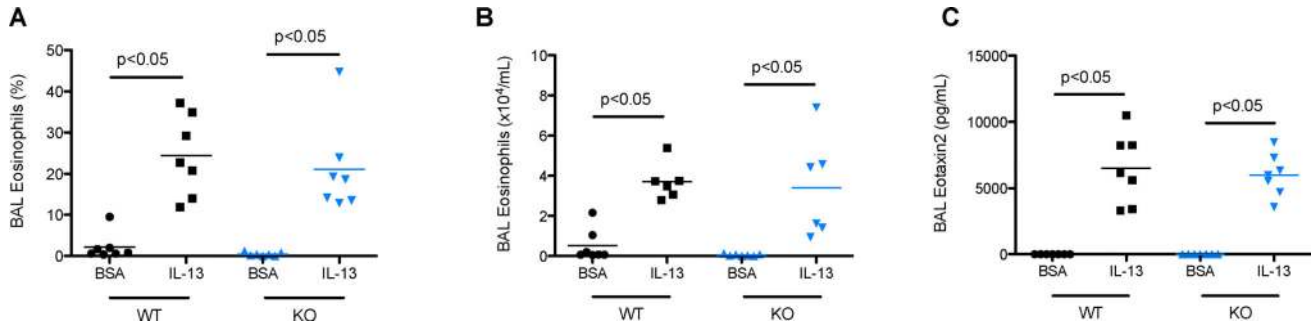


Figure 7. Effect of Muc18 on lung eosinophilic inflammation in IL-13 treated mice

Wild-type (WT) and Muc18 knockout (KO) mice (n = 7 mice/group) were treated with IL-13 and sacrificed at 24 hours after the final treatment. Bronchoalveolar lavage (BAL) fluid was collected to quantify eosinophils (**A** and **B**), and eosninophil chemokine eotaxin2 (**C**). Horizontal bars indicate the Mean of the data, and statistical analysis was performed using ANOVA with a Tukey's post hoc test.

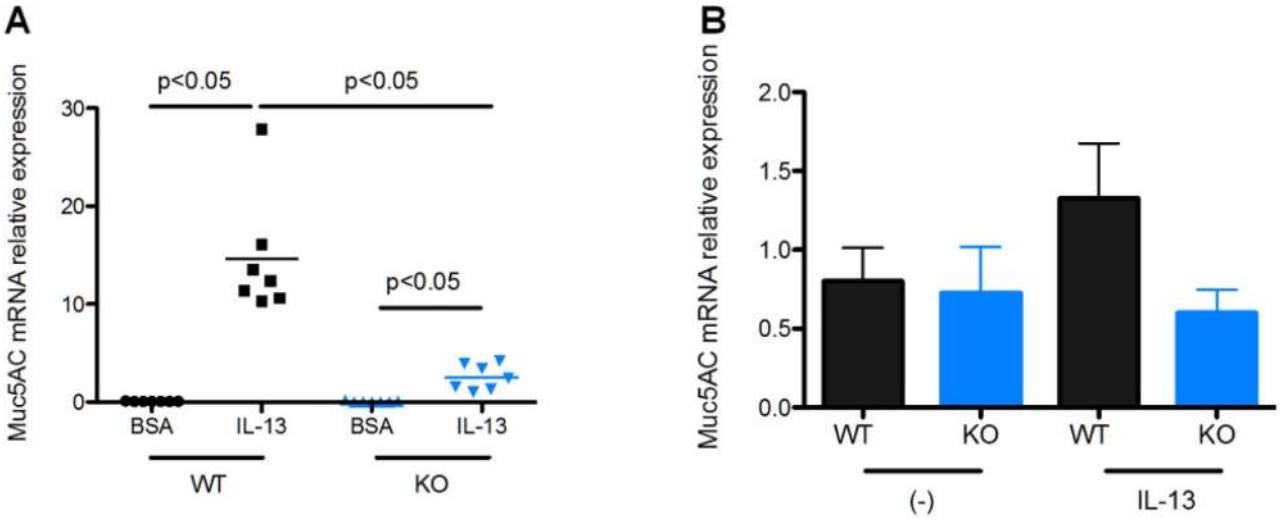


Figure 8. Effect of Muc18 on lung Muc5AC expression in mice

Wild-type (WT) and Muc18 knockout (KO) mice (n = 7 mice/group) were treated with IL-13 and sacrificed at 24 hours after the final treatment. Left lung tissues (**A**) were used to quantify Muc5AC mRNA expression. Tracheal epithelial cells from WT and Muc18 KO mice were isolated and cultured under air-liquid interface (ALI) conditions for 14 days. IL-13 (10 ng/mL) was added for 3 days (day 12 to day 14 of ALI culture). Tracheal epithelial cells (**B**) were used to quantify Muc5AC mRNA expression. Data are presented as the Mean (horizontal bars, A) or Mean ± SEM (B). Statistical analysis was performed using ANOVA with a Tukey's post hoc test.