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Distal airways are protected from goblet cell metaplasia by diminished expression of IL-13 signaling components

Running title: Mucus regulation in distal airways

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Abstract

Background: Increased mucus production is a critical factor impairing lung function in patients suffering from bronchial asthma, the most common chronic inflammatory lung disease worldwide.

Objective: This study aimed at investigating whether goblet cell (GC) metaplasia and mucus production are differentially regulated in proximal and distal airways.

Methods: Female Balb/c mice were sensitized to ovalbumin (OVA) and challenged with an OVA-aerosol on two consecutive days for one week (acute) or twelve weeks (chronic). Real-time RT-PCR analysis was applied on microdissected airways.

Results: In acutely and chronically OVA-challenged mice GC metaplasia and mucus production was observed in proximal but not in distal airways. In contrast, inflammation

reflected by the infiltration of eosinophils and expression of the TH2-type cytokines IL-4 and IL-13 was increased in both, proximal and distal airways. Abundance of IL-13Rα1 was lower in distal airways of healthy control mice. Under acute and chronic OVA-exposure**,** activation of IL-13Rα1-dependent signaling cascade, reflected by Spdef and Foxo3A transcription factors, was attenuated in distal compared to proximal airways.

Conclusion & Clinical Relevance: These data indicate that distal airways might be less sensitive to IL-13 induced GC metaplasia and mucus production through lower expression of IL-13Rα1 and attenuated activation of downstream signaling. This might represent a protective strategy in order to prevent mucus plugging of distal airways and thus, impaired ventilation of attached alveoli.

Introduction

Mucus is an integral part of the airway epithelial barrier and protects the lung by trapping inhaled particles including pathogens, toxins and allergens. In contrast, excessive mucus production also contributes to the pathogenesis of many airway diseases including allergic bronchial asthma. Thus, submucosal gland hypertrophy and goblet cell (GC) metaplasia are important components of airway remodeling observed in asthma and are associated with mucus hypersecretion as well as dysfunction [1]. Overproduction of mucus has in turn marked impact on lung function. Thus, luminal obstruction with mucus together with concentric contraction of airway smooth muscle, e.g. in response to allergen inhalation, leads to diffuse airway narrowing and, thus, to enhanced airway resistance [2]. Furthermore, airway occlusion by mucus plugs causes local atelectasis, especially in patients suffering from acute asthma exacerbations. Ultimately, patients that died from fatal asthma show wide-spread mucus plugging of the airway lumen affecting large as well as peripheral distal airways [3;4].

"Small" or "distal" airways are defined as the terminal bronchioles located at the end of the conducting zone of the airway tree and they have been specified by their internal diameter, which is below 2 mm [5]. These airways divide to form the respiratory bronchioles, followed by generations of alveolar ducts. Interestingly, these distal airways were for some decades believed to be the "quiet zone" of the lung [6] not contributing to functional lung parameters in healthy humans.

However, distal airways are much more vulnerable to mucus obstruction and plugging than proximal airways since the resistance to airflow varies inversely with the fourth power of the airway radius [7]. Consequently, bronchoscopic studies revealed distal airways to be the major site of airflow limitation in asthma [8-10]. Moreover, distal airways seem also to be more prone to the infiltration of inflammatory cells. In resected lung specimens of asthmatics, the total number of CD3 positive T-cells and eosinophils was increased in proximal as well as in distal airways, however, greater numbers of activated eosinophils were identified in distal airways indicating a higher grade of inflammation [11]. In line with this, more IL-4 and IL-5 mRNA positive cells such as T cells, eosinophils, and mast cells could be detected in distal airways of asthmatic patients [12-15]. Based on these observations, deciphering the regulation of mucus production in these airway regions is a key towards understanding asthma pathophysiology. Besides these obvious features distal airways of course differ from "proximal" or "large" airways with respect to tissue structure and composition. Thus, distal airways do not possess cartilage and the tunica adventitia as well as the tunica muscularis are less distinct [16]. Furthermore, this airway region completely lacks submucosal glands, so that goblet cells represent the only production site of mucus [17]. Goblet cells in turn may develop from Clara cells, which are more prominent in distal then in proximal airways [18],

suggesting that not only mucus production but also its regulation in distal airways might differ from that in proximal airways.

Mouse models of acute experimental asthma have been utilized to investigate asthma pathogenesis since the mid of the 1990s. However, due to their acute character they reflected hallmarks of human bronchial asthma like allergic airway inflammation and goblet cell metaplasia only in proximal airways and lacked signs of chronicity such as airway remodeling and stable airflow limitation [19]. As previously reported, these limitations are overcome by establishing a mouse model of chronic experimental asthma, where the entire airway tree is infiltrated by eosinophils and T helper 2 (TH2) cells and displays subepithelial fibrosis, smooth muscle layer thickening and GC metaplasia [20] Using this model, it was the aim of the present study to investigate the region-specific regulation of mucus production in proximal and distal airways.

Material and methods

Animals

Pathogen-free female wildtype Balb/c mice (Charles River, Sulzfeld, Germany), aged 6 to 8 weeks, were housed under standard conditions. Mice had free access to water and rodent laboratory chow. All experimental and animal handling procedures were in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics Board of the Ministry of Environment, Kiel, Germany.

Asthma mouse models

Mice were sensitized to ovalbumin (OVA) by three intraperitoneal injections of 10 µg OVA grade VI (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg $Al(OH)_3$ (Pierce, Rockford, IL, USA) diluted in 200 µL phosphate-buffered saline (PBS) on days one, 14 and 21. Subsequently, mice were challenged with OVA (grade V) aerosol (1 % wt/vol in PBS) via the airways twice a week on two consecutive days for one (acute experimental asthma) or twelve weeks (chronic experimental asthma) [20]. Sham sensitization and challenges were carried out with sterile PBS. All analyses were performed 24 hours after the last allergen challenge.

Lung histology

Lungs were fixed ex-situ with 4% (wt/vol) phosphate-buffered paraformaldehyde via the trachea, removed and stored overnight at 4°C in paraformaldehyde. Lungs were embedded into paraffin, and 2 µm thick lung sections were stained with periodic acid-Schiff (PAS) as well as Congo red as previously described [20;21]. Lung sections from IL-13 transgenic (tg) mice [22] were also stained with PAS.

Immunohistochemistry

Immunohistochemistry was performed against Spdef (1:2500, generated in the lab of J. A. Whitsett [23]). Antibody-antigen complexes were detected using biotinylated secondary antibody followed by avidin-biotin peroxidase (Vectastain Elite kit, Vector Laboratories, Burlingame, USA) and DAB substrate (Sigma-Aldrich, Steinheim, Germany). Sections were counterstained with Mayer's hematoxylin (Merck Millipore, Darmstadt, Germany). Unless stated otherwise, all steps were performed at room temperature.

Quantitative morphology

Design-based stereology was applied to paraffin sections stained with PAS or Congo Red using a point and intercept-counting technique in order to quantify goblet cell metaplasia, total volume of stored mucus in airway epithelium, and total volume of eosinophils. A distal airway was defined as the segment of a terminal bronchiolus that, starting at the bronchoalveolar duct transition, extended up to five alveoli along the proximal direction [20]. Thus, quantification of mucus and eosinophils was performed according to the orientator technique [24] and modified based on a previously described method [25]. The volume of PAS-stained epithelial mucus (Vmucus) per surface area of airway epithelial basal membrane (Sep) was determined by means of point and intersection counting using a computer-assisted stereology tool box [26] (CASTGrid 2.0, Olympus, Kobenhavn, Denmark) and subsequent calculation according to the formula: $V_{mucus}/Sep = L_p\Sigma\ P_{mucus}/2\Sigma I_{ep}$ with ΣP_{mucus} = sum of all points hitting mucus, ΣI_{ep} = sum of all intersections of test lines with epithelial basal membrane, and L_p = test-line length at final magnification. Volume of eosinophils was quantified accordingly.

Microdissection of the airways (MdA)

The trachea of the mice was cannulated and the lungs were inflated with 1.5 ml RNAlater (Qiagen, Hilden, Germany). Under a stereomicroscope the parenchyma was removed in order to microdissect the distal and proximal airways for subsequent RNA isolation [27;28].

Laser-capture microdissection (LCM) from snap-frozen lung tissue

The trachea of the mice was cannulated and the lungs were inflated with cryoprotective Tissue-Tek O.C.T. solution (50 % wt/vol in PBS) (Sakura, Leiden, Netherlands). Subsequently, the lung was removed, snap-frozen in liquid nitrogen and stored at -80°C. On a cryotome, 7 µm thick cryo-sections were prepared, fixed on PALM membrane slides (Zeiss,

Jena, Germany) with 70% ethanol for 1 min and stained with cresyl violet solution (0.5 % wt/vol in EtOH) for 1 min (Merck Millipore, Darmstadt, Germany). The area of interest was marked and isolated using the PALM Mircobeam microdissection system (Zeiss, Jena, Germany) and collected in RLT- buffer (Qiagen, Hilden, Germany).

RNA isolation

After initial disruption of the tissue (ULTRA-TURRAX, IKA, Staufen, Germany), isolation of RNA from microdissected airways was performed with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction . RNA was quantified spectrophotometrically (NanoDrop, ThermoScientific, Wilmington, USA). For cDNA synthesis the Omniscript kit (Qiagen, Hilden, Germany) and the Maxima First Strand cDNA Synthesis kit (ThermoScientific, Wilmington, USA) was used according to the manufacturer's instruction. cDNA was diluted 1:5 in DEPC-treated water for PCR analysis.

Real-time RT-PCR analysis

Real-time RT-PCR was performed on a LightCycler 480 II instrument (Roche Applied Science, Mannheim, Germany). Each real-time RT-PCR reaction was done in triplicate according to the manufacturer's instruction with the Light Cycler 480 Sybr Green I Master (Roche Applied Science, Mannheim, Germany) in a total volume of 10 µl. Cycling conditions were: 45 cycles at 95°C for 10 s, touch-down annealing temperature (63 to 58°C, temperature reduction 0.5°C per cycle) for 4s and 72°C for 10 s. For each primer pair a standard curve was established by serial cDNA dilutions. GAPDH and RPL-32 were used as housekeeping genes. Primers are listed in the online supplement (Tables S2). Data were analyzed employing the "Advanced Relative Quantification" and "Standard Curve Method". A calibrator cDNA was included in each run in order to correct for run-to-run differences. Two negative controls (-reverse transcriptase/-RNA template) were also included to detect possible contaminations.

Western Blotting

Microdissected airways were disrupted and then lysed in 150 µl RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1 % NP-40, 0.5 % Na-desoxycholate, 0.1 % SDS and 15 µl Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Homogenates were then incubated for 20 min at 4°C before centrifugation at 14.000 rpm for 20 min. After protein estimation using the BCA assay (ThermoScientific, Rockford, USA), samples were suspended in NuPage LDS sample buffer (Life Technologies, Darmstadt, Germany) and denaturated at 70°C for 10 min. Samples were loaded on a polyacrylamide gel and transferred by electroblotting to a PVDF membrane (Roth, Karlsruhe, Germany). For immunodetection membranes were probed with primary antibodies against IL-13Rα1 (1:250, Santa Cruz Biotechnology, Santa Cruz, USA) and ß-Actin (1:5000, Sigma-Aldrich, Saint Louis, USA) followed by horseradish peroxidase-coupled secondary antibody (1:2000). Immunoreactive bands were visualized using enhanced chemiluminescent substrate (GE Healthcare, Freiburg, Germany). Band intensity was quantified with ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical analysis

Results are presented as mean values ± SD. One-way ANOVA test with Tukey post-testing or Student's unpaired *t*-test were used to determine the significance of differences between groups. A p value of $p<0.05$ was considered significant.

Results

Goblet cell metaplasia and airway inflammation

We quantified the total amount of GC, reflected by increased surface fraction of epithelial basement membrane (EBM) covered by GC, and the volume of stored mucus per E.B.M. in

proximal and distal airways in healthy mice and mice with acute or chronic experimental asthma in order to investigate region-specific mucus production in health and disease.

Healthy animals did not show any mucus producing GC in the airway epithelium, whereas mice with acute or chronic asthma had markedly enhanced GC metaplasia and mucus production (Fig. 1A, B). However, GC metaplasia and mucus production were restricted to proximal airways and were not observed in distal airways in mice with acute or chronic experimental asthma.

Since GC metaplasia and mucus production in asthmatic patients and mouse models of experimental asthma are strongly associated with the infiltration of inflammatory cells, the number of eosinophils was quantified in proximal and distal airways of healthy mice and mice with acute or chronic experimental asthma (Figure 2) [29;30]. Therefore, Congo-Red stained lung sections were analysed by stereological measurements. While healthy controls rarely showed single eosinophils in airway tissues, mice with acute as well as chronic experimental asthma displayed dramatically increased amounts of airway tissue infiltrating eosinophils along the entire airway tree. Neither in acute nor in chronic experimental asthma eosinophil infiltration was different in proximal and distal airways (Fig. 2).

Thus, although mice with acute or chronic experimental asthma display marked allergic airway inflammation with high amounts of infiltrating eosinophils along the entire airway tree, GC metaplasia and mucus production were restricted to proximal airways and nearly absent in distal airways.

Trigger of GC metaplasia and mucus production

In allergic bronchial asthma**,** GC metaplasia and mucus production are induced by various pro-inflammatory mediators such as the TH2-type cytokines interleukin (IL)-4, IL-9, IL-13 as well as by pro-inflammatory cytokines like tumor necrosis factor (TNF) or IL-1ß. To determine whether these factors might contribute to region-specific GC metaplasia and mucus production observed in acute or chronic experimental asthma, the expression levels of these mediators were assessed in microdissected proximal and distal airways by Real-time RT-PCR (Figure 3). Expression of TNF and IL-1ß was detected in airways from healthy mice and mice with acute or chronic experimental asthma without marked differences between the groups or airway regions (Fig. 3A, B). The TH2-type cytokines IL-4 and IL-13 were not detectable in healthy animals, but expression was strongly induced in airways of animals with acute or chronic experimental asthma. However, no airway-region-specific differences could be observed (Fig. 3C, D). IL-9 could not be detected in microdissected airways, neither in PBS nor in mice with acute or chronic experimental asthma.

Neither TNF nor IL-1ß show significant enhanced expression in mice with acute or chronic experimental asthma. Moreover, no differences in expression levels could be observed between airway-regions. Thus, TNF and IL-1ß expression levels are not related with airway inflammation and with mucus production in mice with acute or chronic experimental asthma. IL-4 and IL-13 as surrogates for TH2-cell activity were up-regulated in mice with acute or chronic experimental asthma and thus, correlated with airway inflammation. However, the increased IL-4 and IL-13 expression did not differ between proximal and distal airway-region in mice with acute or chronic experimental asthma.

GC metaplasia and mucus production in IL-13 transgenic mice

In order to proof whether the observed discrepancy between IL-13 production on the one hand and lack of mucus production in distal airways on the other hand could depend on the applied models of experimental asthma, we used the well established IL-13 transgenic mouse and assessed GC metaplasia and mucus production in the airways. These mice constitutively express IL-13, which is the main functional cytokine in allergic bronchial asthma [31], under the control of the Clara-cell specific promoter along the entire airway tree [22;32]. In accordance with results from the acute and chronic models of experimental asthma, prominent PAS-staining was observed in proximal airways of IL-13 transgenic mice indicating GC metaplasia and enhanced mucus production, whereas distal airways were free of mucus producing GCs (Fig.S1).

Similar to the applied models of experimental asthma, GC metaplasia and mucus production were restricted to proximal airways, although IL-13 is markedly expressed along the entire airway tree. This let us to further investigate, whether distal airways might possess less precursor cells suitable for GC differentiation.

CC10 expression

Clara cells are known to be the progenitor cell type for GC metaplasia [33]. Thus, we assessed the expression of the Clara cell specific protein 10 (CC10) as marker for the abundance of Clara cells. Highest CC-10 expression can be observed in healthy animals compared to animals with acute or chronic experimental asthma. However, we found around 1.5-fold higher expression of CC10 in microdissected distal airways compared to proximal airways derived from healthy control animals as well as animals with acute or chronic experimental asthma (Fig. 4). Hence, precursor cells for GC metaplasia are even more abundant in distal

airways compared to proximal airways. Dependent on the cellular composition and the presence of an allergic inflammation, distal airways might be predisposed to the development of GC metaplasia and increased mucus production. However, mucus-producing GCs are absent in distal airways in mice with acute or chronic experimental asthma. This let us to investigate the IL-13 dependent signaling cascade in more detail.

IL-13 signaling cascade

In order to elucidate whether the absence of GC metaplasia and the lack of increased mucus production in distal airways is related to an impaired IL-13 dependent signaling cascade, the expression of the IL-13 receptor components as well as down-stream signaling proteins was investigated comprehensively. IL-13 signals via the IL-13 receptor (IL-13R) formed by the IL-4Rα and the IL-13Rα1 chains. Moreover, the decoy receptor IL-13Rα2 sequesters IL-13, thus preventing IL-13 signaling [31]. The decoy receptor IL-13Rα2 was not detectable in airways of healthy mice, whereas IL-13Rα2 was up-regulated in mice with acute or chronic experimental asthma (Fig. S2A). No differences were observed between proximal and distal airways. Next, expression levels of IL-4Rα and IL-13Rα1 were investigated in microdissected airways. The IL-4Rα chain is expressed in healthy animals and mice with acute or chronic experimental asthma, but mRNA levels are not different between the groups or airway regions (Fig. S2B). Transcript levels of IL-13Rα1 are slightly lower in distal airways compared to proximal airways in healthy animals and in mice with acute or chronic experimental asthma (Fig. S1C). This was also confirmed on the protein level by immunoblotting (Fig. 5A, B). Since the IL-4Rα and IL-13Rα1 subunits are not solely expressed in the airway epithelium, but are also present in endothelial cells, airway smooth muscle cells or fibroblasts [31], laser capture microdissection (LCM) was applied. This method enabled us to quantify IL-4Rα and IL-13Rα1 expression exclusively in epithelial cells of proximal and distal airways. Real-time

RT-PCR analysis revealed similar mRNA levels in the epithelium of proximal and distal airways for IL-4Rα (Fig. 5C), whereas IL-13Rα1 mRNA levels were by a factor of 2 lower in epithelial cells of distal airways compared to those of proximal airways of healthy mice (Fig. 5D).

Thus, lower expression and subsequent lower activation of IL-13Rα1 in distal airways might be responsible for the absence of GC metaplasia and the lack of increased mucus production observed in mice with acute or chronic experimental asthma. In order to proof this hypothesis, the expression of down-stream signaling molecules of IL-13Rα1 was investigated (Fig. 6).

The transcription factors SAM-pointed domain-containing Ets-like factor (Spdef) and Forkhead box A3 (FoxA3) are major regulators of GC metaplasia and mucus production and both are up-regulated in the airway epithelium after local application of IL-13 and in allergen-induced asthma mouse models [23;34]. In vivo expression of Spdef is sufficient to induce extensive GC metaplasia and mucus production [34]. Contrarily, FoxA2 and NK2 homeobox 1 (Nkx2-1) are important transcriptional regulators of genes expressed selectively in Clara cells and are known to repress GC metaplasia and thus, mucus production. Furthermore, both are negatively regulated by Spdef [23;35].

Both Spdef and FoxA3 are weakly expressed in healthy animals. Consistent with the described role in GC metaplasia and mucus production Spdef and FoxA3 become strongly induced in proximal airways in mice with acute or chronic experimental asthma. In contrast, distal airways revealed only slightly but not statistically significant induced expression levels of Spdef as well as FoxA3 in mice with acute or chronic experimental asthma (Fig. 6B, C). Immunohistochemical staining confirmed profound induction of Spdef expression in proximal airways in mice with acute or chronic experimental asthma (Fig. S3).

Next, the expression levels of FoxA2 and Nkx2-1 were assessed in microdissected airways of healthy animals and mice with acute or chronic experimental asthma by Real-time RT-PCR. Consistent with their role as negative regulators of GC metaplasia and mucus production, FoxA2 and Nkx2-1 displayed a significant higher expression in distal airways compared to proximal airways in healthy animals. Similar results were observed in mice with acute and chronic experimental asthma, although this was not statistically significant and the differences were not as distinct as it was suggested by the decreased Spdef expression. (Fig. 6D, C). .

Taken together, these results demonstrate that lower expression of IL-13Rα1 in distal airways might be responsible for the markedly attenuated induction of Spdef and FoxA3 in distal airways in mice with acute and chronic experimental asthma, thereby contributing to the absence of GC metaplasia and mucus production in distal airways.

Discussion

Mucus hyperproduction is one hallmark of allergic bronchial asthma and is closely associated with allergic airway inflammation in both, human patients and mouse models of experimental asthma. Increased mucus production contributes to airway obstruction and, especially in distal airways, may lead to mucus plugging with perilous impact on lung physiology as ultimately seen in patients that died from status asthmaticus [36;37]. These patients display a marked increase of GC metaplasia in distal airways together with excessive peripheral mucus plugging [37]. Over the last decades progress has been made in the understanding of the molecular mechanisms regulating goblet cell differentiation and mucus production. Differences between proximal and distal airways with respect to GC metaplasia and mucus production have been found, however, an explanation for these observations is still missing

[32;38]. In this study, we have examined the airway region-specific expression of those cytokines and their respective receptor systems regulating GC differentiation and mucus production in both, healthy animals and well established mouse models of experimental asthma (19). We found that despite allergic inflammation of the entire airway tree, GC metaplasia and increased mucus production is largely restricted to the proximal airways and nearly absent in distal airways. Thus, neither the infiltration of inflammatory eosinophils nor the expression of the TH2-type cytokines IL-4 and IL-13 did correlate with the distribution of GC metaplasia and mucus production along the airway tree. Although distal airways are equipped with more Clara cells as a source of GC metaplasia than proximal airways, even in transgenic mice overexpressing IL-13 in the airway epithelium distal airway did not display GC metaplasia or markedly increased mucus production. Hence, we could demonstrate that the divergence of region-specific GC metaplasia and mucus production might be related to different epithelial signatures concerning the IL-13 signaling cascade. Accordingly, the observed lower abundance of the IL-13Rα1 in distal airways might also impact the activation of down-stream signaling mediators as reflected by weak expression of Spdef and FoxA3 in distal airways. Herewith, we suggest that distal airways might be less sensitive to IL-13 dependent induction of GC metaplasia and increased mucus production, which could protect these airway regions from obstruction by mucus plugging.

In humans GC metaplasia and mucus production are closely associated with the infiltration of inflammatory cells such as eosinophils into the airways. Thus, it was observed that in human asthmatics eosinophil numbers in broncho-alveolar lavage (BAL) fluids correlates with mucus hypersecretion [39;40] as well as disease severity [11;41]. In line with this, we observed massive infiltration of eosinophils in proximal as well as distal airways in mice with acute or chronic experimental asthma, mimicking the disease in humans [11]. However, in distal airways inflammation did not correlate with the distribution of mucus-producing goblet cells.

In allergic asthma, metaplasia of GC and mucus production are induced by a plethora of mediators, such as the TH2-type cytokines IL-4, IL-9 and IL-13 as well as the pro-inflammatory cytokines IL-1ß and TNF. Each of these mediators is capable to induce the expression of mucin genes in airway epithelial cells [42-44]. In our study, both IL-4 and IL-13 were significantly up-regulated in mice with acute or chronic experimental asthma yet in proximal and distal airways. IL-4 and IL-13 are closely related cytokines sharing some but not all biological functions [45]. Whereas IL-4 is not exclusively required for GC metaplasia [46], IL-13 is the most prominent inducer of GC metaplasia and mucus production in allergic asthma as demonstrated by IL-13 transgenic over-expression in mice [22]. In accordance with our data histological examination of lungs derived from IL-13 transgenic mice revealed absence of GC metaplasia and mucus production is distal airways. From the concurrent finding that distal airways are almost free of GC metaplasia and mucus we speculated, that a) distal airways might possess less progenitor cells for GC metaplasia or b) epithelial cells of distal airways might be less sensitive towards IL-13 mediated signaling. Concerning the variability of proximal and distal airways by points of structural and cellular composition, the expression of Clara-cell specific protein 10 was assessed. Clara cells represent the original cell type capable to differentiate into GC after allergen contact [33]. There are species-related differences concerning the distributing of Clara cells along the airway-tree. Whereas, Clara cells in mice are ubiquitous throughout the conducting airways, they are restricted to distal airways in humans [47]. In healthy humans goblet cells are also immunoreactive for CC10, supporting for the fact that Clara cells are also a source for GC metaplasia in humans [48]. In accordance to others, we confirmed higher abundance of Clara cells in distal airways of healthy mice [49] and even in mice with experimental asthma. Thus, inadequate cellular composition of distal airways is unlikely to be causative for the lack of GC metaplasia and mucus production in healthy and asthmatic mice.

This suggests that distal airways might be less sensitive towards the effects of IL-13. This cytokine binds to two cognate receptors, IL-13Rα1 and IL-13Rα2, which are both family members of the type 1 cytokine receptor family [50]. IL-13Rα2 was shown to exist in a large intracellular pool and is described to act mainly as decoy receptor for IL-13 in its soluble form, thereby limiting IL-13 actions [51-53]. As already observed by others [54], IL-13Rα2 was not detected in healthy animals. Since IL-13Rα2 expression was increased equally in proximal and distal airways in acute or chronic models of experimental asthma, the significance of IL-13Rα2 for airway-region specific regulation of GC metaplasia and mucus production might be negligible. Though, effects of IL-13 on GC metaplasia and mucus production are attributable to the IL-13R, a heterodimeric receptor composed of the IL-4Rα and IL-13Rα1 chains. Munitz et al. [55] clearly demonstrated that the IL-13Rα1 chain is substantial for IL-13 induced GC metaplasia, mucus production, AHR, and fibrosis in a classical OVA model of experimental asthma. Consistently, IL-13 and allergen-dependent induction of mucus was totally absent in IL-13Rα1 knock-out mice [55;56]. Both, the IL-4Rα and the IL-13Rα1 receptor chains are broadly expressed reflecting versatile IL-13 actions on a wide variety of cell types [57;58]. This let us to investigate receptor expression in laser-microdissected airway epithelial cells. In this study, we showed for the first time less abundant IL-13Rα1 expression in distal airway epithelial cells of healthy animals, whereas IL-4Rα was equally expressed. One can now speculate that the diminished abundance of IL-13Rα1 in distal airways might also have impact on down-stream signaling mediators. Thus, ligation of the IL-13Rα1/IL-4Rα receptor complex by IL-13 results in the activation of a complex network of transcription factors regulating the differentiation of airway epithelial cells towards mucus-producing GC [59]. Components of this cascade are depicted in figure 6. In brief, Spdef and FoxA3 become activated and by this drive GC metaplasia and mucus production [23;34;60;61]. This is in accordance with our work, where Spdef and FoxA3 are significantly up-regulated in proximal airways in mice with acute or chronic asthma.

Moreover, the low abundance of Spdef and FoxA3 in distal airways on the one side and markedly diminished capability of IL-13 mediated induction on the other side might contribute to distal airway protection from GC metaplasia and mucus production in experimental asthma. Beside, activated Spdef inhibits the Clara cell specific transcription factors FoxA2 and Nkx2-1, since both are known to negatively regulate GC metaplasia and mucus production [35;60]. Concordantly, FoxA2 and Nkx2-1 were more abundant in distal airways of healthy mice and mice with acute or chronic experimental asthma. Interestingly, the differences in FoxA2 and Nkx2-1 expression were not as distinct as it could be expected by the dramatically low expression of its negative regulator Spdef, which suggests that additional factors could be involved in the regulation of these two genes. Nevertheless, the significantly higher abundance of FoxA2 and Nkx2-1 in distal airways might be additive in the protection of distal airways from GC metaplasia and mucus production.

In summary, our study demonstrates airway region-specific regulation of GC and mucus production in healthy mice and three mouse models of experimental asthma. Since the expression of factors stimulating the mucus production revealed no region-specific differences, we postulate that this is dependent on the lower expression of IL-13Rα1 in distal airways, which is the major receptor for IL-13 dependent GC metaplasia and mucus production. Additionally, IL-13Rα1-dependent activation of the down-stream transcription factors Spdef and FoxA3, which are functionally associated with GC and mucus production, was diminished in distal airways. Thus, we provide a regulatory mechanism that makes distal airways less sensitive to IL-13 induced GC metaplasia and mucus production and, thus, could protect from mucus plugging and impaired ventilation of the attached alveoli.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Quantification goblet cell hyperplasia and mucus production in airways of acute and chronic experimental asthma. Goblet cell numbers (A) and stored mucus (B) were quantified stereologically in periodic-acid Schiff stained whole lung sections with the CAST system (n=3-8 each time point). White bars represent proximal airways and black bars distal airways. The significance between experimental groups was analyzed using one-way

ANOVA with Tukey post-testing (*$p \leq 0.05$; [#]significantly different from PBS prox). Data are presented as mean ± SD.

Figure 2. Quantification of eosinophils in airways of acute and chronic experimental asthma. Numbers of eosinophils was quantified stereologically in Congo-Red stained whole lung sections with the CAST system (n=6-7 each time point). White bars represent proximal airways and black bars distal airways. The significance between experimental groups was analyzed using one-way ANOVA with Tukey post-testing (*$p \leq 0.05$; [#]significantly different from PBS prox; [‡]significantly different from PBS dis). Data are presented as mean ± SD.

Figure 3. Expression analysis of factors triggering goblet cell hyperplasia and mucus production. Gene expression levels for TNFα (A), IL-1ß (B), IL-4 (C), IL-13 (D), were quantified in microdissected airways of PBS and acutely and chronically OVA-treated mice by Real-time RT-PCR (n=6-8 each time point). White bars represent proximal airways and black bars distal airways. The significance between experimental groups was analyzed using one-way ANOVA with Tukey post-testing (*$p \leq 0.05$; [#]significantly different from PBS prox; [‡]significantly different from PBS dis; [+]significantly different from chronic prox; [§]significantly different from chronic dis). Data are presented as mean ± SD.

Figure 4. Expression analysis of CC10 in proximal and distal airways. Expression of CC10 was assessed by Real-time RT-PCR in microdissected airways of PBS and acutely and chronically OVA-treated mice by Real-time RT-PCR (n=6 each time point). White bars represent proximal airways and black bars distal airways. The significance between experimental groups was analyzed using one-way ANOVA with Tukey post-testing (*$p \leq 0.05$; [#]significantly different from PBS prox; [‡]significantly different from PBS dis). Data are presented as mean ± SD.

Figure 5. Expression analysis of IL-4Rα and IL-13Rα1 in airway epithelial cells Detection of IL-13Rα1 was done by western blotting from microdissected airways of PBS mice (n=3) (A). Densitometric quantification was performed and ß-Actin served as a loading control. Expression levels of IL-4Rα (C) and IL-13Rα1 (D) receptor subunits were assessed by Real-time RT-PCR from laser-microdissected airway epithelial cells of PBS mice (n=5). The significance between groups was analyzed using Student's unpaired t-test (*$p \leq 0.05$). Data are presented as mean ± SD.

Figure 6. Expression analysis of IL-13 dependent signaling cascade in airways of acute and chronic experimental asthma. A) IL-13 signals via a complex receptor system including the decoy receptor IL-13Rα2 and active heterodimeric IL-13 receptor build by the IL-4α chain and the IL-13Rα1 chain. Since the IL-13Rα2 lacks the intracellular signaling domain, it does not contribute to IL-13 signaling. However, secreted IL-13Rα2 can bind IL-13 and attenuate IL-13 mediated actions. After binding of IL-13 towards the IL-13Rα1/IL-4Rα receptor complex a signaling cascade becomes activated with the phosphorylation of the JAK1 and Tyk-2 kinases. Subsequent activation of STAT6 by phosphorylation and nuclear translocation results in gene expression of the transcription factors Spdef and FoxA3, both promoting Muc5AC expression and thus goblet cell metaplasia. Concurrent Spdef-mediated repression of Nkx2-1 and FoxA2 furthermore enhances Muc5AC expression and goblet cell metaplasia. Spdef (B), FoxA3 (C), FoxA2 (D) and Nkx2-1 (E) mRNA levels were detected by Real-time RT-PCR in microdissected airways of PBS and acutely and chronically OVA-treated mice (n=6-8 each time point). White bars represent proximal airways and black bars distal airways. The significance between experimental groups was analyzed one-way ANOVA with Tukey post-testing (*$p \leq 0.05$; $^{\#}$significantly different from PBS prox; ‡significantly different from PBS dis; $^{+}$significantly different from chronic prox). Data are presented as mean ± SD.

Table 1: Primer sequences for Real-Time RT-PCR

Gene name	Accession		Sequence	Amplicon
murine				
RPL-32	NM_172086	for rev	AAAATTAAGCGAAACTGGCG ATTGTGGACCAGGAACTTGC	156 bp
GAPDH	NM_008084	for rev	AATGGTGAAGGTCGGTGTGAAC GAAGATGGTGATGGGCTTCC	226 bp
IL-13Rα1	NM_133990	for rev	AATAATACTCAAACCGACCGAC CTTCACTCCAATCACTCCAC	204 bp
IL-13Rα2	NM_008356	for rev	GGACTCATCAGACTATAAAGA GTGTGCTCCATTTCATTCTA	176 bp
IL-4Rα	NM_001008700	for rev	CAGTGGTAATGTGAAGCCCC TGAATTCTGCAGGGTTGTCC	176 bp
TNF	NM_013693	for rev	TCGTAGCAAACCACCAAGTG AGATAGCAAATCGGCTGACG	207 bp
IL-1β	NM_008361	for rev	GAGCCCATCCTCTGTGACTC AGCTCATATGGGTCCGACAG	131 bp
IL-9	NM_008373	for rev	ACACCGTGCTACAGGGAGGGA TCGCAGGAAAAGGACGGACACG	134 bp
CC10	NM_011681	for rev	GCTCAGCTTCTTCGGACATC GTATCCACCAGTCTCTTCAGC	155 bp
Quantitect (Qiagen) primer assays for Real-Time RT-PCR				
IL-13	NM_008355	QT00099554		90 bp
Spdef	NM_013891	QT00107191		109 bp
FoxA3	NM_008260	QT01657705		122 bp
FoxA2	NM_010446	QT00242809		115 bp
Nkx2-1	NM_009385	QT00131264		134 bp
Muc5AC	NM_010844	QT01744575		78 bp

Figure 1

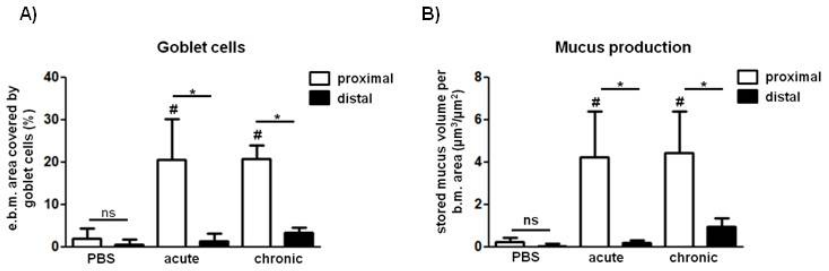

Figure 2

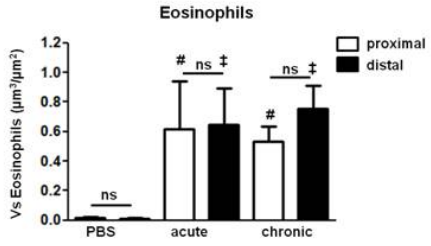

Figure 3

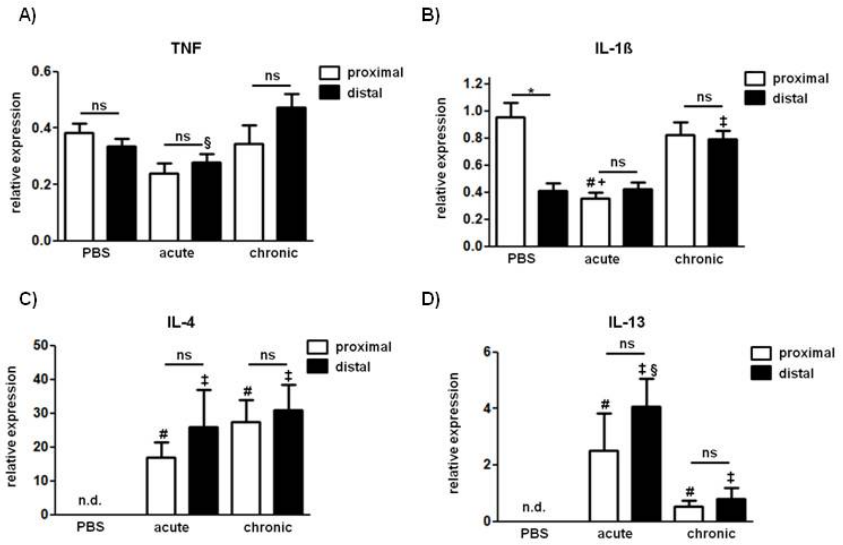

Figure 4

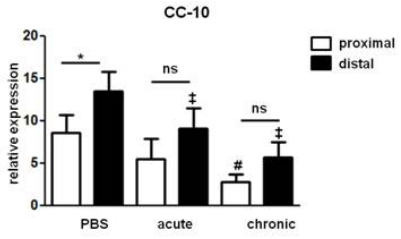

Figure 5

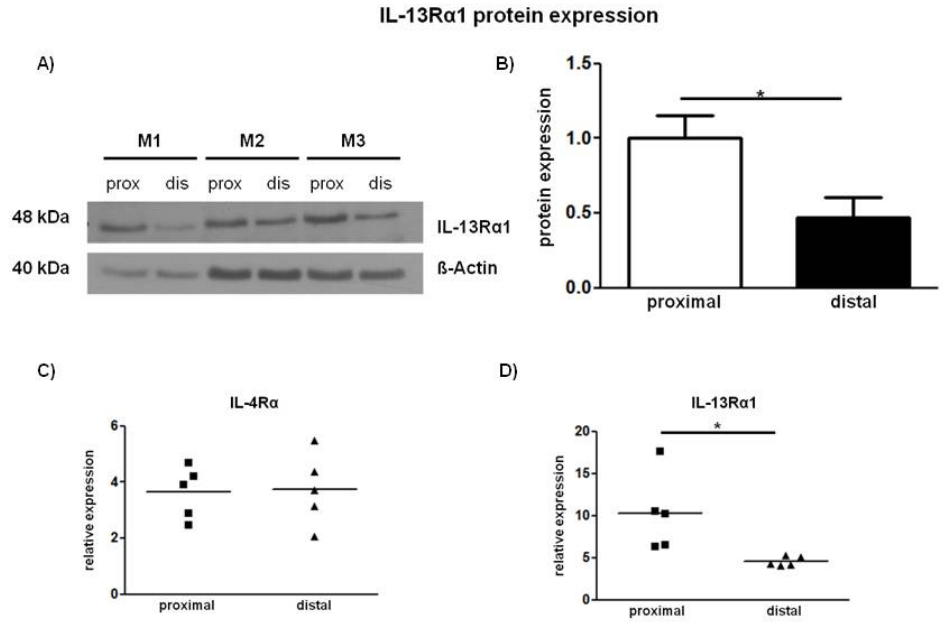

Figure 6