

IL-4 Receptor Signaling in Clara Cells Is Required for Allergen-Induced Mucus Production¹

Douglas A. Kuperman,^{2*‡} Xiaozhu Huang,* Louis Nguyenvu,* Christoph Hölscher,[§] Frank Brombacher,[¶] and David J. Erle^{*†}

Excessive mucus production is an important pathological feature of asthma. The Th2 cytokines IL-4 and IL-13 have both been implicated in allergen-induced mucus production, inflammation, and airway hyperreactivity. Both of these cytokines use receptors that contain the IL-4R α subunit, and these receptors are expressed on many cell types in the lung. It has been difficult to determine whether allergen-induced mucus production is strictly dependent on direct effects of IL-4 and IL-13 on epithelial cells or whether other independent mechanisms exist. To address this question, we used a cell type-specific inducible gene-targeting strategy to selectively disrupt the IL-4R α gene in Clara cells, an airway epithelial cell population that gives rise to mucus-producing goblet cells. Clara cell-specific IL-4R α -deficient mice and control mice developed similar elevations in serum IgE levels, airway inflammatory cell numbers, Th2 cytokine production, and airway reactivity following OVA sensitization and challenge. However, compared with control mice, Clara cell-specific IL-4R α -deficient mice were nearly completely protected from allergen-induced mucus production. Because only IL-13 and IL-4 are thought to signal via IL-4R α , we conclude that direct effects of IL-4 and/or IL-13 on Clara cells are required for allergen-induced mucus production in the airway epithelium. *The Journal of Immunology*, 2005, 175: 3746–3752.

Asthma is characterized by exaggerated airway narrowing in response to specific and nonspecific stimuli (airway hyperreactivity), chronic eosinophilic inflammation, elevated serum IgE levels, and excessive mucus accumulation in the airways (1). Excessive mucus production has an important impact on asthma symptoms (2). Increased sputum production is a marker of asthma instability, and some deaths due to asthma have been attributed to mucus plugging. Mucus is produced by cells in submucosal glands and by goblet cells at the luminal surface of the airway. Gland hypertrophy occurs in moderate to severe asthma (3), but goblet cell hyperplasia is present even in people with mild asthma (4).

Th2 cells orchestrate allergic responses and are generally considered central to asthma pathogenesis (5). Mast cells, eosinophils, and basophils may also contribute to increased type 2 cytokine production in asthma (6–9). Transgenic overexpression of several type 2 cytokines, including IL-4, IL-5, IL-9, and IL-13, in the lungs of mice produces many of the pathophysiological abnormalities seen in asthma (10–13). IL-13 is thought to be particularly critical because blockade of just IL-13 in the airways is sufficient to prevent inflammation, airway hyperreactivity, and mucus pro-

duction induced by allergen challenge (14, 15), and IL-13 neutralization protects mice from effects of transgenic overexpression of IL-9, IL-10, and IL-25 in the lung (12, 16, 17). IL-13 and IL-4 have similar effects because they share a common receptor, the type II IL-4R, which is composed of the IL-4R α and IL-13R α 1 subunits. The type II receptor is broadly expressed, although T cells and murine B cells lack this receptor (18). Lymphocytes and some other cells express a second receptor, the type I IL-4R, which is composed of the IL-4R α subunit and the common γ -chain and binds IL-4, but not IL-13. Both IL-4R types signal by activating STAT6 and insulin receptor substrate pathways.

It is not clear whether allergen-induced mucus production results from IL-4R signaling in airway epithelial cells or from other effects involving other cells or mediators. We hypothesized that allergen-induced mucus production depends upon IL-4R signaling in Clara cells. Clara cells are nonciliated secretory airway epithelial cells that can give rise to goblet cells, a process called mucus metaplasia (19). We previously produced mice that expressed STAT6 only in Clara cells and used these mice to show that IL-13 can act directly on these cells to induce mucus metaplasia (20). In those studies, IL-13 was produced by transgenic overexpression in Clara cells, which does not mimic the site or amount of cytokine production seen in allergic airways disease. Furthermore, the possible contributions of other pathways activated by allergen challenge could not be evaluated using that model. In this study, we describe a new model in which the effects of IL-4 and IL-13 on Clara cells were evaluated by selectively disrupting *Il4ra*, the gene encoding IL-4R α , in these cells. We found that Clara cell-specific *Il4ra* disruption did not inhibit the development of allergic inflammatory responses in the lung, but almost completely protected mice from allergen-induced mucus metaplasia.

Materials and Methods

Mice

These studies conformed to the principles of laboratory animal research outlined by the National Institutes of Health and to University of California institutional guidelines. Two lines of transgenic mice generously provided

*Lung Biology Center, Department of Medicine, and [†]Program in Immunology, University of California, San Francisco, CA 94143; [‡]Allergy-Immunology Division, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [§]Research Center Borstel, University of Lübeck, Lübeck, Germany; and [¶]Division of Immunology, Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

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² Address correspondence and reprint requests to Dr. Douglas A. Kuperman, Division of Allergy-Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, 240 East Huron Street, McGaw 2317, Chicago, IL 60611. E-mail address: d-kuperman@northwestern.edu

by J. Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) were used to generate mice with doxycycline-inducible expression of Cre recombinase in Clara cells, as previously described (21). One line carried the rat Clara cell secretory protein (CCSP)³ promoter 5' to the open reading frame for the reverse tetracycline *trans* activator (rtTA) fusion protein (CCSP-rtTA). The other line carried an array of seven tetracycline operon (TetO) promoter sequences 5' to the open reading frame for Cre recombinase (TetO-Cre). The production and characterization of BALB/c embryonic stem cells and mice carrying an IL-4R α gene (*Il4ra*) with loxP sites flanking exons 7–9 have been described previously (22, 23). In the absence of Cre recombinase, the *Il4ra*^{loxP} allele produces functional IL-4R α . Cre-mediated recombination of *Il4ra*^{loxP/loxP} produces the *Il4ra*^{-/-} allele. The *Il4ra*^{-/-} allele was shown to be a null allele by demonstrating that membrane-bound and soluble IL-4R α were undetectable in *Il4ra*^{-/-} cells and that *Il4ra*^{-/-} cells did not respond to IL-4 or IL-13 (22, 23). We used CCSP-rtTA⁺, TetO-Cre⁺, *Il4ra*^{loxP/loxP} mice to assess the performance of the inducible Cre recombinase system. To maximize the efficiency of *Il4ra* deletion for allergen challenge experiments, we generated transgenic mice with one floxed allele and one null allele, as follows. CCSP-rtTA⁺ transgenic mice were backcrossed to *Il4ra*^{loxP/loxP} BALB/c mice for six generations to produce CCSP-rtTA⁺, *Il4ra*^{loxP/loxP} mice. TetO-Cre⁺ transgenic mice were backcrossed to *Il4ra*^{-/-} BALB/c mice for six generations to produce TetO-Cre⁺, *Il4ra*^{-/-} mice. The CCSP-rtTA⁺, *Il4ra*^{loxP/loxP} and TetO-Cre⁺, *Il4ra*^{-/-} mice were mated to produce CCSP-rtTA⁺, TetO-Cre⁺, *Il4ra*^{loxP/-} mice (used for Clara cell-specific deletion of IL-4R α). Littermate mice that were also *Il4ra*^{loxP/-}, but lacked the CCSP-rtTA and/or TetO-Cre transgenes, were used as controls. All mice were housed under laminar flow in an environmentally controlled pathogen-free animal facility and given free access to tap water or doxycycline-treated water and OVA-free rodent chow.

Analysis of *Il4ra* deletion in transgenic mice

CCSP-rtTA⁺, TetO-Cre⁺, *Il4ra*^{loxP/loxP}, and control mice were maintained on water containing doxycycline (2 mg/ml, protected from light and changed twice weekly) for 30 days. Mice were sacrificed, and genomic DNA from lungs, spleens, and tracheas was isolated using Qiagen's DNeasy kit. DNA was assayed for *Il4ra* null alleles by PCR using published primer sequences and conditions (22).

Ag sensitization and challenge

CCSP-rtTA⁺, TetO-Cre⁺, *Il4ra*^{loxP/-}, and littermate control mice used for allergic studies received doxycycline (2 mg/ml; Sigma-Aldrich) in their drinking water starting 1 wk before the first sensitization. Mice were sensitized by i.p. injection of 50 μ g of OVA (grade V; Sigma-Aldrich) in 0.2 ml of PBS/AIOH three times at 1-wk intervals. One week after the last sensitization, mice were anesthetized with isoflurane and challenged by aspiration with 50 μ l of a 2% OVA solution once daily for 3 days. Mice in the sham groups were treated identically, except that OVA was omitted from the solutions used for immunization and challenge. Mice were studied 1 day after the final allergen challenge.

Assessment of airway inflammation

Lungs were lavaged once with 1 ml of PBS with 1% FCS. After centrifugation (1000 rpm, 5 min), the supernatant was removed for cytokine analysis. The cell pellet was resuspended in normal saline after a brief hypotonic exposure to lyse RBC. Total cells were counted with a hemacytometer. Cytospin preparations were prepared and stained with Diff-Quik (Baxter), and bronchoalveolar lavage (BAL) fluid cell differential percentages were determined based on light microscopic evaluation of >500 cells/slide.

IgE and cytokine assays

Serum total IgE level was determined by a sandwich ELISA using complementary Ab pairs for mouse IgE (clones R35-72 and R35-118; BD Pharmingen) and used according to the manufacturer's instructions. Color development was achieved using streptavidin-conjugated HRP (BD Pharmingen), followed by addition of HRP substrate (tetramethylbenzidine; BD Pharmingen). IL-4 and IL-13 protein levels were measured in 200 μ l of unconcentrated BAL fluid from each animal by ELISA (R&D Systems), according to the manufacturer's instructions. The limit of detection of each assay was 15 pg/ml.

Quantitative RT-PCR

Total RNA was extracted from mouse lungs using TRIzol (Invitrogen Life Technologies), according to the manufacturer's protocol. Synthesis of cDNA and quantitative real-time PCR were performed, as described previously (24). Primers and probes for Muc5ac, Muc5b, Clca3, AMCase, MCP-1, and cathepsin K (Ctsk) have been described previously (24). IL-4 primers were 5'-CGCCATGCACGGAGATG-3' and 5'-ACGAGCTC ACTCTCTGTGGTGT-3', and the IL-4 probe was 5'-FAM-TGCCAAA CGTCCTCACAGCAACG-BHQ3'. IL-5 primers were 5'-TGCCTGGAG CAGCTGGAT-3' and 5'-GTGGCTGGCTCTCATTACACA-3', and the IL-5 probe was 5'-FAM-TGCAAGGAGATGTCCCTTTTCTTTTCCA BHQ3'. IL-13 primers were 5'-ACTGCTCAGCTACACAAAGCA ACT-3' and 5'-TGAGATGCCCCAGGGATGGT-3', and the IL-13 probe was 5'-FAM-TCGCCACGGCCCTTCTAATGA-BHQ3'. IFN- γ primers were 5'-AGCTCATCCGAGTGGTCCAC-3' and 5'-AGCAGCGACTC CTTTTCG-3', and the IFN- γ probe was 5'-FAM-AGCTGTTG CCGGAATCCAGCCTC-BHQ3'.

Airway reactivity measurements

Airway reactivity measurements were performed on paralyzed and anesthetized mice, as described previously (20). Airway reactivity was measured by determining the dose of i.v. acetylcholine required to produce a 200% increase in respiratory system resistance over baseline (PC₂₀₀).

Quantification of airway epithelial mucus volume

The volume of mucus contained within the airway epithelium was measured using a quantitative stereology approach. Lungs were inflated to 20 cm of H₂O with 10% Formalin. Three days later, fixed lungs were dehydrated in serially increasing concentrations of ethanol and embedded in paraffin with a known orientation. We produced vertical uniform random sections using a random number generator and a 360° template. Embedded lungs were cut transversely into three equal-sized pieces. Each piece was rotated orthogonally and randomly oriented on the 360° template. Each piece was then cut again along the 0° axis, and the resulting pieces were rotated orthogonally again before being re-embedded in a paraffin block with other randomized pieces from the same lung. A random 5- μ m section from each block was stained with periodic acid-Schiff for detection of mucus. A blinded operator measured the total epithelial cell volume and the volume of epithelial cell occupied by mucus by point counting of randomly sampled microscopic fields at $\times 40$ magnification, as described (4).

Data analysis

Data are reported as mean $\pm$ SEM. Significance testing was performed by ANOVA and Tukey-Kramer posttest for multiple comparisons. Values of $p < 0.05$ were considered significant.

Results

The model system confers specific *Il4ra* gene disruption

We used a previously characterized transgenic system to selectively disrupt gene expression in Clara cells (21). The system relies on two transgenes: CCSP-rtTA, which drives expression of the rtTA selectively in Clara cells, and TetO-Cre, which produces the recombinase Cre in cells in which rtTA is expressed and when doxycycline is present. We analyzed genomic DNA from mice of various genotypes to determine whether this system could be used to selectively disrupt *Il4ra* gene expression. As a positive control, we analyzed mice with constitutive null alleles (*Il4ra*^{-/-} mice). As expected, null alleles were detected following 25–30 cycles of PCR in each of the three organs we studied (Fig. 1a). When we analyzed doxycycline-treated mice that carried both the CCSP-rtTA and TetO-Cre transgenes and were homozygous for a conditional floxed *IL4R α* gene (*Il4ra*^{loxP/loxP}), we detected disrupted (null) alleles in the lung and the trachea after 25–30 cycles of PCR (Fig. 1b). However, the null allele was not detected in the spleen, even after 35 cycles of PCR. Null alleles were not detected in *Il4ra*^{loxP/loxP} mice lacking one or both of the CCSP-rtTA and TetO-Cre transgenes (Fig. 1c, and data not shown). These results indicate that the inducible transgenic system produced selective disruption of the *Il4ra* gene in organs containing Clara cells (lung and trachea), but not in the diverse variety of immune cells that are

³ Abbreviations used in this paper: CCSP, Clara cell secretory protein; BAL, bronchoalveolar lavage; Ctsk, cathepsin K; PC₂₀₀, provocative concentration of acetylcholine required to produce a 200% increase in respiratory system resistance; rtTA, reverse tetracycline *trans* activator; TetO, tetracycline operon.

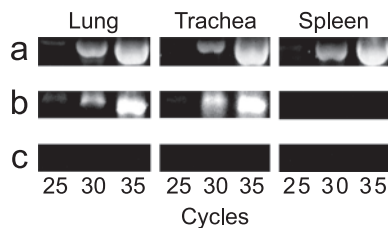


FIGURE 1. *Il4ra* deletion in the lung and trachea. Genomic DNA from lung, trachea, or spleen of *Il4ra*^{-/-} (a), CCSP-rtTA⁺, TetO-Cre⁺, *Il4ra*^{loxP/loxP} (b), and *Il4ra*^{loxP/loxP} (c) mice was analyzed by PCR using primers specific for the disrupted allele (*Il4ra*⁻). Reactions were stopped after 25, 30, or 35 cycles, and products were visualized by agarose gel electrophoresis and EtBr staining. All mice had been treated with doxycycline (used to induce Cre expression in Clara cells in mice carrying the CCSP-rtTA and TetO-Cre transgenes).

represented in the spleen. To maximize the efficiency of *Il4ra* disruption in Clara cells, mice carrying one copy of the floxed allele and one copy of the null allele (*Il4ra*^{loxP/-}) were used for all subsequent experiments involving the response of mice to allergen.

Loss of Clara cell IL-4Rα does not impair allergen-induced IgE production

Mice with Clara cell-specific disruption of *Il4ra* and appropriate control mice were sensitized and then challenged by intranasal administration of OVA to produce an allergic response in the lung and airways. To determine whether loss of IL-4Rα in Clara cells affected the ability of these mice to mount an allergic humoral response, we measured serum IgE levels (Fig. 2). OVA administration induced increases in IgE production in mice with Clara cell-specific disruption of *Il4ra*. These increases were similar to those seen in OVA-treated littermate control mice that did not have Clara cell-specific disruption of *Il4ra* (because one or both of the CCSP-rtTA and TetO-Cre transgenes were absent). IL-4Rα signaling in both T cells and B cells is critical for IgE production (18). The preserved IgE response (along with the absence of detectable *Il4ra* disruption in spleen cells) indicates that the disruption of *Il4ra* alleles induced by the transgenic system did not affect critical immune cells important for IgE production.

Loss of Clara cell IL-4Rα does not impair allergen-induced inflammation

BAL was performed to assess the effects of allergen sensitization and challenge on inflammatory cell recruitment. Cells retrieved

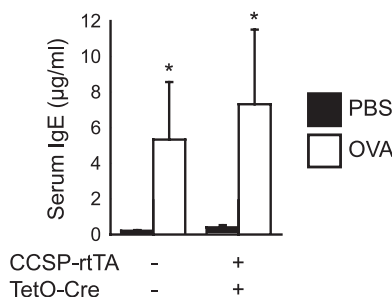


FIGURE 2. Atopic sensitization is not prevented by loss of IL-4Rα in Clara cells. Control mice (lacking the CCSP-rtTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS or OVA, and serum was analyzed for IgE content by ELISA. Values represent mean ± SEM for five to seven mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge.

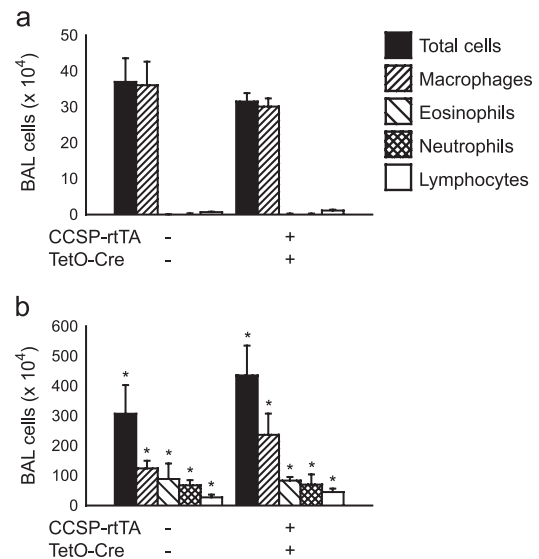


FIGURE 3. Airway inflammation is not reduced by loss of IL-4Rα in Clara cells. Control mice (lacking the CCSP-rtTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS (a) or OVA (b), and BAL was performed. Total cell counts and counts for macrophages, eosinophils, neutrophils, and lymphocytes are shown. Note that the scale is different in the two panels. Values represent mean ± SEM for five to seven mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge.

from sham (PBS)-sensitized and -challenged mice were mostly macrophages (Fig. 3a). OVA-sensitized and -challenged mice developed large increases in macrophages, neutrophils, eosinophils, and lymphocytes (Fig. 3b). Mice with Clara cell-specific disruption of *Il4ra* had inflammatory responses that were similar to those seen in control mice. This result indicates that the transgenic system did not disturb the ability of mice to mount an eosinophilic inflammatory response to OVA.

Loss of Clara cell IL-4Rα does not impair allergen-induced cytokine production

BAL fluid supernatant was assayed for IL-4 and IL-13 by ELISA. We found significant allergen-induced increases in IL-4 in control mice and mice with Clara cell-specific disruption of *Il4ra* (Fig. 4a). There was a trend toward small allergen-induced increases in IL-13 in both groups of mice, although this did not reach statistical

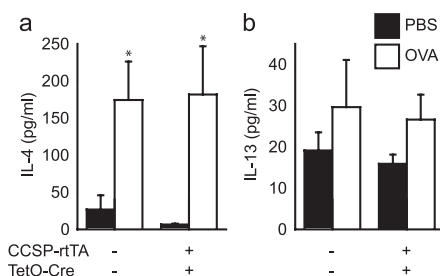


FIGURE 4. BAL fluid cytokine levels are not altered by loss of IL-4Rα in Clara cells. Control mice (lacking the CCSP-rtTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS or OVA, and BAL fluid was analyzed for IL-4 (a) and IL-13 (b) by ELISA. Values represent mean ± SEM for five to seven mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge.

significance (Fig. 4*b*). Quantitative RT-PCR analysis of RNA prepared from whole lungs showed large Ag-induced increases in IL-4 and IL-13 transcripts in both groups of mice (Fig. 5). IL-5 and IFN- γ transcripts were also increased, but to a lesser extent. These results indicate that disruption of *Il4ra* in Clara cells did not interfere with allergen-induced cytokine production.

Loss of Clara cell IL-4R α does not prevent the development of allergen-induced airway hyperreactivity

Airway reactivity was measured in sedated and paralyzed, mechanically ventilated mice after allergen or sham challenge. The PC₂₀₀ was decreased in allergen-challenged mice, indicating the development of airway hyperreactivity (Fig. 6). Similar decreases in PC₂₀₀ were seen in mice with Clara cell-specific disruption of *Il4ra* and in control mice.

Clara cell IL-4R α signaling is required for allergen-induced mucus production

We used computer-assisted stereology to quantify allergen-induced mucus production in mice with Clara cell-specific disruption of *Il4ra* and in control mice (Fig. 7). Sham-challenged mice had little, if any, mucus visible in the airways. OVA-challenged control mice produced substantial amounts of mucus, occupying ~8–10% of the volume of the epithelium. Mice that inherited both the CCSP-rTA and TetO-Cre transgenes were dramatically protected from allergen-induced increases in mucus production. To exclude the possibility that expression of rTA or Cre had nonspecific effects that suppressed mucus production, we also examined mucus production in littermate controls expressing just one of the two transgenes. Mice expressing only the CCSP-rTA transgene or only the TetO-Cre transgene had high levels of mucus production, similar to levels seen in mice lacking both transgenes. This indicates that the reduction in mucus production seen in mice carrying

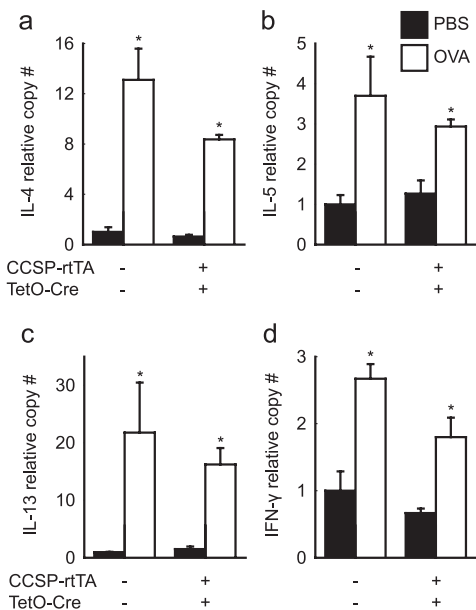


FIGURE 5. Induction of lung cytokine mRNAs is not prevented by loss of IL-4R α in Clara cells. Control mice (lacking the CCSP-rTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS or OVA, and lung RNA was analyzed for IL-4 (*a*), IL-5 (*b*), IL-13 (*c*), and IFN- γ (*d*) by quantitative RT-PCR. Values represent mean $\pm$ SEM for three mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge.

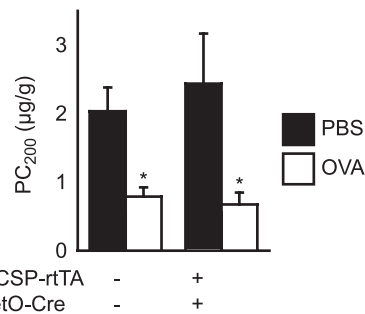


FIGURE 6. Induction of airway hyperreactivity is not prevented by loss of IL-4R α in Clara cells. Control mice (lacking the CCSP-rTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS or OVA, and airway reactivity was measured in sedated, paralyzed, and mechanically ventilated mice. Reactivity is represented as the provocative concentration of acetylcholine (per gram mouse body weight) required to produce a 200% increase in respiratory system resistance (PC₂₀₀). Values represent mean $\pm$ SEM for five to seven mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge.

both transgenes is not attributable to nonspecific effects of expressing rTA or Cre in Clara cells.

We used quantitative RT-PCR to measure the expression of mucins and other genes that have been shown to be induced by IL-13 (20, 24). The gel-forming mucins Muc5ac and Muc5b are large glycoproteins that are major constituents of airway mucus (2). Muc5ac and Muc5b were both highly induced by allergen treatment of control mice, but induction of these transcripts was markedly impaired in mice with Clara cell-specific disruption of *Il4ra* (Fig. 8, *a* and *b*). The chitinase, AMCase, and the calcium-activated chloride channel Clca3 (also known as Gob-5) have both been implicated in asthma pathogenesis (25, 26), and both can be induced by direct effects of IL-13 on Clara cells (20, 24). We found that the induction of AMCase and Clca3 transcripts was markedly reduced in mice with Clara cell-specific disruption of *Il4ra* (Fig. 8, *c* and *d*). We also analyzed the expression of the chemokine Ccl2 (MCP-1) and the proteinase Ctsk, which are both induced by the effects of IL-13 on cells other than Clara cells (20, 24). MCP-1 and Ctsk transcripts were both induced by allergen, and the degree of induction was not reduced in mice with Clara cell-specific disruption of *Il4ra* (Fig. 8, *e* and *f*). The marked impairment of mucus

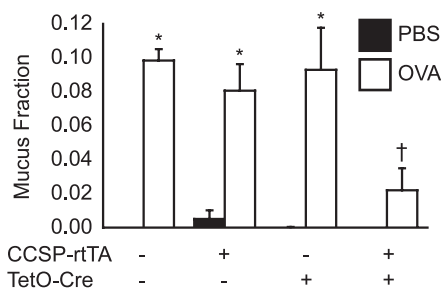


FIGURE 7. Induction of mucus is markedly reduced by loss of IL-4R α in Clara cells. Control mice (lacking one or both of the CCSP-rTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS or OVA, and epithelial mucus content was measured by stereology. Mucus content is represented as the volume of mucus divided by the total volume of the epithelium. Bars are not visible for some of the PBS groups because no mucus was seen in those groups. Values represent mean $\pm$ SEM for five to seven mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge. †, $p < 0.05$ as compared with other OVA groups.

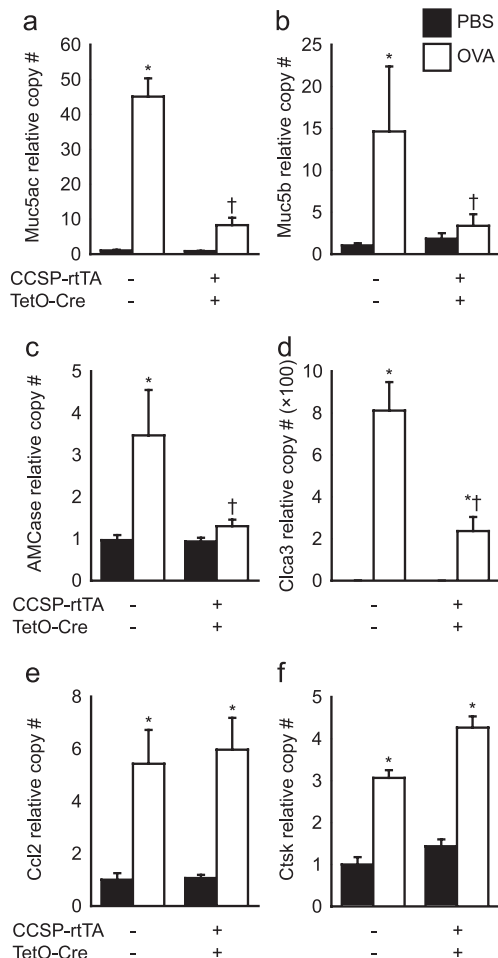


FIGURE 8. Effects of loss of IL-4R α in Clara cells on allergen induction of mucins and other genes. Control mice (lacking the CCSP-rtTA and TetO-Cre transgenes) and mice with Clara cell-specific disruption of *Il4ra* induced by these transgenes were sensitized and challenged with PBS or OVA, and lung RNA was analyzed for mucins 5ac (a) and 5b (b), acidic mammalian chitinase (c), calcium-activated chloride channel 3 (d), Ccl2 (MCP-1) (e), and Ctks (f) by quantitative RT-PCR. Values represent mean $\pm$ SEM for three mice per group. *, $p < 0.05$ as compared with PBS sensitization and challenge. †, $p < 0.05$ as compared with OVA-challenged control mice.

production and mucin gene induction in mice with Clara cell-specific disruption of *Il4ra* indicates that direct effects of IL-4 and/or IL-13 on Clara cells are required for allergen-induced mucus production in the airway.

Discussion

We investigated the contribution of direct effects of IL-4 and/or IL-13 on Clara cells to the development of experimental allergic asthma using transgenic mice with a selective loss of IL-4R α expression in these cells. Because the system used to drive *Il4ra* disruption relied upon a promoter (CCSP) that is active selectively in Clara cells, other cells, including immune cells, remained responsive to IL-4 and IL-13. Mice with Clara cell-specific disruption of *Il4ra* responded to allergen by developing atopy (elevated IgE), airway inflammation with eosinophilia, increased Th2 cytokine production, and airway hyperreactivity to a similar extent as allergen-challenged control mice. In contrast, mucus metaplasia as measured by the volume of mucus contained in the epithelium and by expression of mucin genes was dramatically reduced in mice with a loss of IL-4R α in Clara cells. Because IL-4 and IL-13 both

bind to receptors containing the IL-4R α subunit and both IL-4 and IL-13 are expressed at increased levels in lungs of allergic mice, we conclude that the direct effects of IL-4 and/or IL-13 on Clara cells are required for mucus metaplasia in experimental allergic asthma.

The studies reported in this work build on previous studies that have examined the role of IL-4 and IL-13 in mucus metaplasia. Key clues about the importance of these cytokines came from demonstrations that IL-13, IL-4R α , and STAT6 were required for mucus metaplasia in allergic mice and that airway administration or overexpression of IL-4 or IL-13 induced mucus production in nonallergic mice (11, 13–15, 27–29). Because IL-4/IL-13Rs are expressed on a wide range of cell types, subsequent studies have attempted to determine whether these cytokines act directly on epithelial cells to induce mucus metaplasia. Some studies showed IL-4 or IL-13 treatment of cultured airway epithelial cells increased mucus production and/or expression of transcripts encoding the major airway mucin Muc5ac (30–34), but others did not (35–40). IL-4 and IL-13 might also induce mucus production indirectly, via their ability to recruit and activate inflammatory cells such as neutrophils and eosinophils. Neutrophil elastase can stimulate mucus production in some systems (41, 42). Recent studies with eosinophil-deficient mice produced differing conclusions about whether eosinophils are involved in mucus production (43, 44). Eosinophils have been reported to be an important source of IL-4 and IL-13 in the airways of people with asthma (6, 45) and might therefore contribute to mucus production by releasing these cytokines.

It has been difficult to determine whether allergen-induced mucus metaplasia in vivo is principally attributable to direct effects of IL-4 and IL-13 on epithelial cells or to other mechanisms. Cohn et al. (46) used a system involving adoptive transfer of Th2 cells to show that mucus production depended upon expression of IL-4R α in recipient mice, but was independent of IL-5-induced eosinophilia. Using mice that expressed STAT6 only in Clara cells, we previously showed that IL-13 overexpression resulted in mucus metaplasia in the absence of inflammation, indicating that direct effects of IL-13 on Clara cells can be sufficient to cause mucus production (20). In this study, we used a complementary system (Clara cell-specific disruption of *Il4ra*) that allows us to reach two new major conclusions. First, we can now conclude that our earlier findings from the transgenic IL-13 overexpression system are relevant to allergic airway disease. Second, we can now conclude that direct effects of IL-4 and/or IL-13 on Clara cells are not only sufficient, but also necessary for mucus production, because mucus is dramatically attenuated by reducing IL-4R α expression selectively in these cells. There was a small amount of residual allergen-induced mucus production in mice with Clara cell-specific disruption of *Il4ra*, although epithelial mucus content and mucin gene expression in these mice were not significantly higher than in sham (PBS)-treated mice. Residual mucus production may be attributable to a minor population of Clara cells with persistent IL-4R α expression, because inducible cell type-specific systems such as the one we used can be highly effective, but might not result in gene disruption in every targeted cell. Although we cannot exclude the possibility that a small amount of mucus can be induced by independent mechanisms, our findings clearly demonstrate that the major pathway leading to allergen-induced mucus production depends upon direct effects of IL-4 and/or IL-13 on Clara cells.

Airway epithelial cells may make important contributions to allergic airway inflammation and hyperreactivity. In some systems, cytokines produced in allergic responses stimulate epithelial cells to produce chemokines that might contribute to the recruitment of inflammatory cells (47–49). In a transgenic model, direct effects of

IL-13 on Clara cells did not induce chemokine expression or inflammatory cell recruitment (20, 24). Consistent with this result, the studies reported in this work indicate that loss of IL-4R α expression on Clara cells did not reduce airway inflammation or prevent induction of the chemokine MCP-1. Taken together, these studies indicate that direct effects of IL-4 and IL-13 on Clara cells are not necessary or sufficient for chemokine induction or inflammation. It remains possible that airway epithelial cells contribute to the inflammatory response by other mechanisms. Although IL-13 effects on Clara cells did not produce inflammation, these effects were sufficient to induce airway hyperreactivity in a transgenic model (20). In this study, we found that Clara cell-specific disruption of *Il4ra* did not prevent the development of allergen-induced hyperreactivity. We cannot exclude the possibility that a small population of Clara cells with persistent IL-4R α expression is responsible for this hyperreactivity. Alternatively, it may be that IL-4R α signaling in multiple cell types, including Clara cells, other epithelial cells, and smooth muscle cells, promotes hyperreactivity by various mechanisms.

We did find that loss of IL-4R α expression on Clara cells inhibited allergen-induced increases of Clca3 and AMCase, which have each been implicated in the pathogenesis of various manifestations of allergic airway disease (25, 26). We previously showed that expression of Clca3 (also known as Gob-5) was dramatically increased by IL-13-induced activation of STAT6 in Clara cells (20). It has been reported that Clca3 plays a critical role in mucus production and mucin gene expression in vivo and in vitro (26), although a recent report demonstrates that allergen- and IL-13-induced mucus production and mucin gene induction are unimpaired in Clca3-deficient mice (50). In this study, we report that Clca3 induction was reduced by 71% in mice with Clara cell-specific disruption of *Il4ra*, indicating that Clca3 induction by allergen is largely dependent on direct effects of IL-4 and/or IL-13 on these cells. The residual induction of Clca3 might be due to incomplete disruption of IL-4R α expression in Clara cells, to IL-4R α -dependent induction of Clca3 in other cells, or conceivably to IL-4R α -independent mechanisms. Inhibitors of the chitinase AMCase were reported to attenuate airway inflammation and hyperreactivity, but the effects of these inhibitors on mucus production were not reported (25). We found that AMCase induction was almost completely prevented by Clara cell-specific disruption of *Il4ra*, although this did not result in decreased inflammation or hyperreactivity in our model system. Further studies are warranted to determine how effects of IL-4 and IL-13 on gene expression in Clara cells and other airway epithelial cells contribute to various aspects of asthma pathogenesis other than mucus production.

The approach that we used in this study does not allow us to distinguish between effects of IL-4 and IL-13 because both cytokines act via the IL-4R α subunit. We found that BAL fluid IL-4 protein and lung IL-4 mRNA transcript levels were significantly elevated after allergen challenge. We found only a modest, statistically insignificant increase in BAL fluid IL-13, but much larger (~20-fold) increases in IL-13 transcripts in whole lung. The difference between IL-13 BAL fluid protein and lung mRNA results may relate to the site of IL-13 protein production or to the kinetics of IL-13 in the BAL fluid. Although our results could be explained by a requirement for either IL-4 or IL-13, previous studies indicate that IL-13 is required for mucus induction in allergic models, but IL-4 is not (14, 15, 28, 29). In any event, the results reported in this work indicate a central role for Clara cell IL-4R α signaling in mucus production induced by allergen and suggest that this pathway is a promising target for asthma therapy.

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Disclosures

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