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Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation

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Homer, Robert J., Zhou Zhu, Lauren Cohn, Chun Gun Lee, Wendy I. White, Suping Chen, and Jack A. Elias. Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation. *Am J Physiol Lung Cell Mol Physiol* 291: L502–L511, 2006. First published March 23, 2006; doi:10.1152/ajplung.00364.2005.—The mammalian chitinase family includes members both with and without enzymatic activity against chitin, a product of fungal cell walls, exoskeletons of crustaceans and insects, and the microfilarial sheaths of parasitic nematodes. Two members of that family, Ym1 and acidic mammalian chitinase (AMCase), are strongly upregulated in pulmonary T helper (Th) 2 inflammation but not in Th1 inflammation. The sites of expression of these products are incompletely known. We show here that, in two different models of Th2 inflammation, Ym1 and AMCase are mutually exclusively expressed in proximal vs. distal airway epithelium, respectively, whereas both are expressed in alveolar macrophages. This regional difference along the airway corresponds to the previously noted distinction between mucus positive proximal cells and mucus negative distal cells under the same conditions. Among distal cells, AMCase colocalizes with epithelial cells expressing the Clara cell marker Clara cell secretory protein. These AMCase-expressing cells retain expression of FOXA2, a transcription factor whose down-regulation in association with IL-13 signaling has previously been associated with production of mucus in proximal airway epithelial cells. These results provide evidence that secretory cells of proximal and distal airways undergo fundamentally different gene expression programs in response to allergic inflammation. Furthermore, AMCase provides the first positive molecular marker of distal Clara cell secretory protein-expressing cells under these conditions.

airway epithelial differentiation; mucus; airway remodeling

ASTHMA IS A CHRONIC INFLAMMATORY DISEASE that affects millions of people worldwide. Current concepts of pathogenesis center on the notion that asthma is the result of exaggerated Th2 airway inflammation (15). A major component of asthma is so-called airways remodeling in which various components of the airway undergo various pathological alterations in response to this chronic inflammation, including alterations of the airway epithelium (20). In the lungs of normal mice and humans, the airways are lined by a mixed population of ciliated and nonciliated epithelial cells. Normal murine airways are lined by roughly equal portions of ciliated cells and Clara cells, whereas mucus-producing cells are scarce or absent. In humans, Clara cells are predominantly found in distal airways and only rarely in central airways. In both human and murine asthma, there is marked increase in the number of mucus-

producing cells that line the airways. The increased production of mucus is a major factor in airflow obstruction in asthma. Fatal asthma is virtually always associated with mucus plugging of the airways. In murine asthma models, this increase in mucus production is limited to the surface epithelium, since no submucosal glands exist in the mouse lung proper, although submucosal glands do exist in the proximal trachea near the thyroid. Mucus production in mice is due to acquisition of a mucus phenotype in secretory cells expressing the Clara cell marker Clara cell secretory protein (CCSP) rather than to proliferation of existing mucus-producing cells (16, 40). Strikingly, in mice, only the Clara cells of the proximal airway undergo this change, whereas those of the distal airway do not. This finding suggests that there is a fundamental difference in the phenotypic response of these cell populations to a common stimulus.

It is believed that T helper (Th) 2 inflammation originally evolved to deal with parasites, whereas allergy and atopic asthma arise as a consequence of poorly controlled Th2 responses elicited independently of parasitic infection (15). Chitin, the second most abundant polysaccharide in nature, is found in fungal cell walls, in the exoskeletons of crustaceans and insects, and in the microfilarial sheaths of parasitic nematodes (5, 44, 45). Chitinase production is a common feature of antiparasite responses of lower life forms against chitin-containing organisms (19, 31). Paradoxically, although chitin and chitin synthase do not exist in mammals, human chitinase family members such as acidic mammalian chitinase (AMCase) have recently been described (5). Our laboratory and others have previously shown that AMCase and Ym1 are specifically upregulated in response to Th2 inflammation in the lung (43, 59, 60). Furthermore, inhibition of AMCase by a variety of means inhibits this inflammation (59). In the course of that work, we noticed a striking heterogeneity in the staining pattern of AMCase among airway epithelial cells, a major site for expression of AMCase. We now report that AMCase is expressed by nonmucus-producing CCSP-expressing cells of the distal but not proximal airway, whereas Ym1 is expressed by the mucus-producing cells of the proximal airways. AMCase is the first molecular marker that defines this distal airway secretory cell population under these conditions.

METHODS

Mice. Studies involving mice conformed to the standards prescribed by the National Institutes of Health on the experimental use of

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animals and were approved by the Yale University Institutional Animal Care and Use Committee. In the aeroallergen experiments, we utilized 8-wk-old female C57BL/6 mice that were purchased from Jackson Laboratory (Bar Harbor, ME). Transgenic mice in which IL-13 was constitutively overexpressed in a lung-specific manner using the Clara cell-specific protein promoter were generated and maintained by our laboratory as previously described and are referred to here as IL-13 transgenic mice (58). All the mice were housed in the Yale Animal Resource Center in a specific pathogen-free environment. Experiments using transgenic mice were initiated when the mice were 4–6 wk old.

Ovalbumin sensitization and challenge. Six- to 8-wk-old wild-type C57BL/6 mice received intraperitoneal injections of chicken ovalbumin (20 mg) (Sigma, St. Louis, MO) complexed to alum (Resorptar, Indergen, New York, NY). This process was repeated 5 days later. After an additional 7 days, the animals received aerosol challenge with ovalbumin (1% wt/vol) in endotoxin-free PBS. This was accomplished in a closed 27 × 20 × 10-cm plastic aerosol chamber in which the mouse was placed for 40 min. The aerosol was generated with an Omron NE-U07 ultrasonic nebulizer (Omron Healthcare, Vernon Hills, IL).

Histological analysis. Mice were anesthetized, a median sternotomy was performed, and the trachea was dissected free and cannulated. The pulmonary vascular tree was then perfused with calcium- and magnesium-free PBS (pH 7.40) with a catheter in the right heart, and the lungs were inflated to 25 cmH₂O with either Streck's tissue fixative (Streck Laboratories, Omaha, NE) or 10% formalin in PBS (pH 7.40). The lungs were then removed and maintained in same fixative for 24 h. The tissues were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and diastase-periodic acid-Schiff. The stains were performed in the Department of Pathology of Yale University School of Medicine.

In situ hybridization. In situ hybridization assays were undertaken as previously described (59). For Ym1, the coding region was cloned into the vector pBS KS II with T3 and T7 promoters. For IL-13, the template was derived from the same construct as was used to make the transgenic mice. Sense and antisense probes were transcribed in vitro and labeled using a digoxigenin RNA labeling kit (Roche Bioscience).

Immunohistochemistry and immunofluorescence. Immunohistochemistry of AMCase was performed as described by our laboratory (59), using a polyclonal rabbit anti-AMCase as previously described or a mouse monoclonal antibody developed by us (171.204), with specificity for AMCase. The antibody was applied to the lung sections at a 1/100 dilution. To verify the specificity of the reactions, the rabbit antibody was incubated with AMCase-specific peptide (amino acids 428–446) at a 1:1 ratio for 2 h before being applied to the tissues. The mouse monoclonal antibody specificity was determined by ELISA against other murine chitinase family members (chitotriosidase, Ym1, Ym2, YKL-40) and by comparison of staining pattern to the rabbit antibody. In all cases, Dako pH 6 antigen retrieval solution was used for 20 min. Other antibodies/reagents used include rabbit anti-CCSP (gift of William Philbrick, Yale University), sheep anti-Foxa2 (Upstate Biotechnology, Albany, NY), and mouse anti-acetylated alpha tubulin (clone 6-11B-1, Sigma). For two-color fluorescence, secondary reagents included anti-mouse, anti-rabbit, or anti-sheep either biotinylated or directly conjugated with Alexa-488, Alexa-546, or Alexa-647 (Molecular Probes). Tissues were mounted using Prolong Gold (Molecular Probes). For combined tubulin-AMCase staining, biotinylated anti-mouse and streptavidin Alexa-647 was combined with rabbit anti-AMCase and anti-rabbit Alexa-546. For combined CCSP-AMCase staining, anti-rabbit Alexa-546 was combined with anti-mouse AMCase, biotinylated anti-mouse, and either streptavidin Alexa-647 or streptavidin Alexa-488. In the latter case, nuclei were counterstained with Topro-3. Immunohistochemistry was developed either with horseradish peroxidase/diaminobenzadine or alkaline phosphatase/Vector Red (Vector Laboratories). Conventional fluorescence was performed with an Olympus BH-2 microscope. Confocal

microscopy was performed with a Zeiss LSM 510 meta with an optical thickness of either 5.0 or 1.0 μ m.

RESULTS

AMCase is expressed in distal airway epithelial cells in which mucus is not expressed. We have previously described that airway epithelial cells express AMCase in response to either allergic inflammation or IL-13 alone (59). We have also shown that mucus is produced in airway epithelium under the same circumstances (9, 58). To determine whether the same or different cell populations express these products, we performed serial section analysis of both ovalbumin challenged and IL-13 transgenic mice for mucus and AMCase (Figs. 1 and 2). Mucus is only identified in the large central airway (Fig. 1A) and in the proximal portions of intermediate airways (Fig. 1B, arrow), whereas there is no mucus in the most distal airways (Fig. 1C, first column) as has previously been reported (16, 40). AMCase expression is a mirror image of mucus expression in which central airways are negative (Fig. 1A), transitional airways are positive in the most distal portion (Fig. 1B), and the terminal airways is diffusely and strongly positive (Fig. 1C). The same finding can be seen in the IL-13 mice (Fig. 2). This indicates that IL-13 alone is capable of inducing this response.

Staining pattern for AMCase is not a general feature of chitinase family members. Both Ym1 and Ym2 proteins, related members of the chitinase family, are dramatically upregulated in the presence of Th2 inflammation (43, 52, 60). To determine whether the staining pattern for AMCase is typical of all members of the chitinase family, we performed in situ hybridization for Ym1 in ovalbumin-challenged and IL-13 transgenic mice (Figs. 1 and 3). Unlike AMCase, Ym1 is expressed only in proximal but not distal airway epithelium (compare Fig. 1, A–C, and Fig. 3, A and B). As seen in Fig. 1, Ym1 colocalizes to airways that express mucus (Fig. 1A) but not to distal airways (Fig. 1C). Intermediate airways show Ym1 colocalization with mucus (arrows in Fig. 1B). In IL-13 mice, expression is particularly intense in central airways in which mucus expression is expected (Fig. 3A, arrow). Distal airways show expression not greater than sense or wild-type control (compare Fig. 3B with Fig. 3, C–E). Intense expression of Ym1 is identified in macrophages in both the antigen challenged (Fig. 1C, arrow) and IL-13 transgenic mice (Fig. 3B, arrow), consistent with previous results (38).

Most airways can be readily characterized as either large and central or small and peripheral, e.g., Fig. 3, box A vs. box B. However, some smaller airways branch directly off of large central airways and open directly into alveoli (Fig. 3, asterisks). The epithelial cells that line these small airways in some cases have the phenotype of the adjacent larger airway with expression of mucus and Ym1 but not AMCase (Fig. 3, and data not shown). Importantly, however, the key observation remains that, along a given airway, AMCase is always expressed more distally than Ym1 or mucus.

AMCase is expressed in secretory but not ciliated cells. The predominant cell types of proximal mouse airways are ciliated cells, secretory (Clara) cells and basal cells, whereas secretory and ciliated cells but not basal cells are found more distally. Given the heterogeneity of expression we have reported, we were interested in determining which of these cell populations expressed AMCase. To examine this issue, we performed

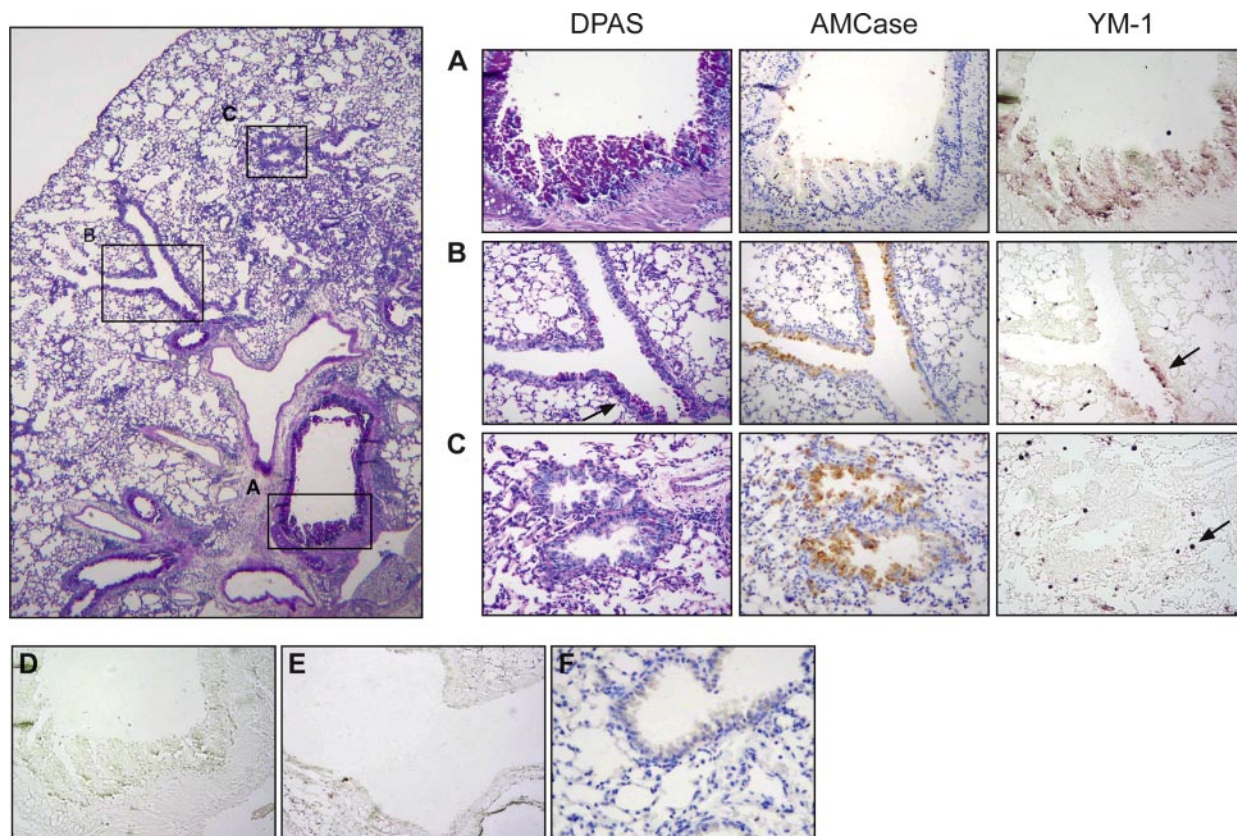
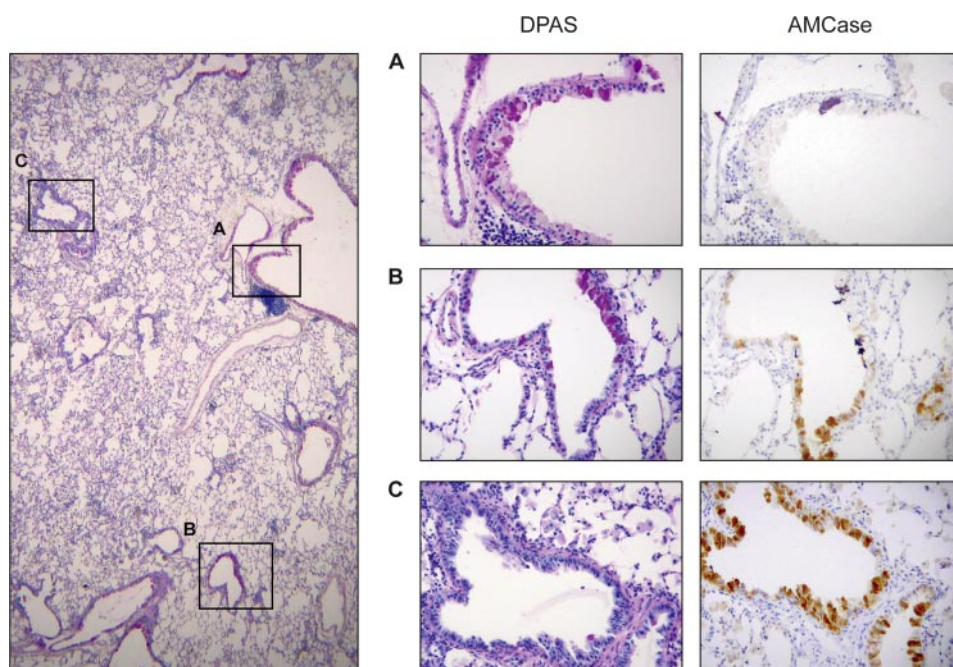


Fig. 1. Expression of mucus and chitinase family members acidic mammalian chitinase and Ym1 in ovalbumin challenged mouse lung. Serial sections were stained for mucus (DPAS), acidic mammalian chitinase (AMCase) (immunohistochemistry) and Ym1 (in situ hybridization). A low-power image of ovalbumin-primed and -challenged mouse lung is included for orientation (*top left*). The boxes highlight the areas enlarged to the side. Mucus and Ym1 is present only in central airways (A) and in proximal portions of mid-airways (B) with no expression in distal airways (C). The expression of AMCase is a mirror image of mucus and Ym1 expression. D–F: relevant negative controls. Sense probe hybridization for Ym1 is negative proximally in the ovalbumin challenged mouse (D). Anti-sense for Ym1 is also negative in a central airway of an unchallenged mouse (E). Immunostain for AMCase is negative in the distal airway of an unchallenged mouse (F). Low-power orientation, $\times 2$ original objective. A, B, D, and E: $\times 10$ original objective. C and F: $\times 40$ original objective.

Fig. 2. Acidic mammalian chitinase and mucus expression in airways of IL-13 transgenic mice. Serial sections of central and peripheral airways were stained for DPAS or AMCase. A low-power view is provided for orientation (*left*). There is abundant mucus in a central airway with no AMCase expression (A) and no mucus and abundant AMCase in the peripheral airway (C). Intermediate airways show a transition with striking segregation of AMCase and mucus expression (B). B, *right*: dark material at 1 to 3 o'clock of airway is nonspecific debris. Low-power view is $\times 2$ original objective; $\times 40$ original objective for A–C.



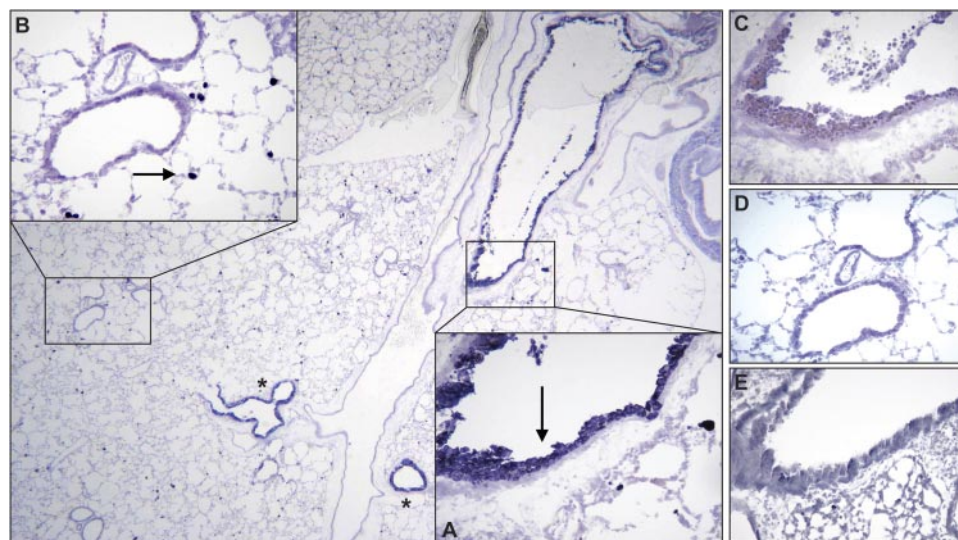


Fig. 3. In situ hybridization analysis of Ym1 expression in IL-13 transgenic mice. The low-power image is provided for orientation, with the *insets* shown at higher power. A and B: antisense for Ym1. There is strong expression in airway epithelium of central (A, arrow) but not peripheral airways (B; compare with sense probe in D). Note that macrophages are strongly positive in the region of the distal airway as an internal control (arrow in B). Smaller airways on either side of the large artery in the center of the figure are also positive for Ym1 (asterisks). The airway on the left leads directly into alveoli. Note that these latter airways are branching off of the continuation of the large airway seen at the top of the figure but which is out of the plane of section at the bottom of the figure. Controls include sense probe in central airways of IL-13 mice (C), sense probe in distal airways of IL-13 mice (D), and anti-sense for Ym1 in central airway of wild-type littermates (E).

two-color staining of AMCase with CCSP (a marker for secretory cells), and acetylated alpha tubulin (a marker for ciliated cells) (16). Basal cells are only present in proximal airways in which AMCase is not expressed, so these cells were not examined. As can be seen in Figs. 4 and 5, a subset of CCSP-expressing cells express AMCase in ovalbumin and IL-13 transgenic mice. On the other hand, two-color staining with acetylated tubulin in IL-13 transgenic mice shows essentially no overlap with AMCase. Thus AMCase is expressed by a subset of CCSP-expressing cells but not ciliated cells.

At higher power, AMCase and CCSP both show a distinctly granular intracytoplasmic staining pattern (Fig. 5). However, within the cytoplasm of individual cells, there is only partial overlap of CCSP and AMCase granules (Fig. 5, C and F, arrows). For example, at the cell indicated by the arrow in Fig. 5C, the expression of CCSP is markedly peripheral, as has been noted above. In this cell, the AMCase granules are more widely distributed within the cell. On the other hand, as seen in Fig. 5F, there is also striking heterogeneity of expression of AMCase among CCSP-expressing cells. The cell at the arrow, despite being strongly CCSP positive, only expresses a few AMCase granules. The immediately adjacent cell shows marked expression of AMCase.

Relationship of CCSP, *Foxa2*, and AMCase expression in response to allergen and IL-13. The distinction between proximal and distal airway epithelial cells could represent a fundamental difference in the expression program of these cells in response to allergic stimulation. On the other hand, it could be argued that the ovalbumin model shows proximal but not distal mucus due to proximal deposition of antigen and rate-limiting local production of IL-13. However, the inflammatory infiltrate in the distal airways is comparable to that seen in the proximal airways. Previous work has shown that this pattern holds despite multiple administrations of antigen (16, 40). Finally, this explanation would only apply to the transgenic mice if there were a limiting amount of IL-13 in the distal airway of these animals as well. It is important to note that the expression of AMCase in the distal airways in both of our models shows that this lack of mucus production is not a null phenotype but actually represents an alternative activation pathway to IL-13.

We directly address this issue by performing in situ hybridization for IL-13 in the IL-13 mice. An airway is shown in Fig. 6 that corresponds to the mid-portion of the lung as seen in Figs. 1B and 2B. The proximal portion of the airway, which has hyperplastic epithelium indicative of mucus production, is negative for IL-13 mRNA (Fig. 6A). The distal portion of the same airway, which lacks hyperplastic changes and is thus lacking in mucus, shows moderate staining for IL-13 in the bronchiolar epithelium (Fig. 6B). In addition, there is also staining in parenchymal cells (Fig. 6C) presumably alveolar type II cells, which is a known leak of this promoter (33). We conclude, based on this data, that IL-13 levels in the IL-13 mice are likely to be at least as high if not higher in the distal as the proximal airway. Therefore, a lower level of IL-13 in the distal part of the airway cannot account for the differential expression of mucus and AMCase that we see in both the IL-13 mice and the allergen-challenged mice. This finding supports the concept that the differences we documented between proximal and distal cells are not simply a response to differential amounts of IL-13.

The promoter we have used in these mice has previously been shown to reliably direct expression to Clara cells throughout the airway (48). The differential expression in proximal and distal airway that we see in the IL-13 mice therefore strongly suggests that there has been downregulation of this promoter due to the microenvironment. To address the mechanism whereby IL-13 is differentially expressed in the airways of these mice and to understand the relationship of AMCase expression to other regulatory factors important in airway differentiation, we analyzed *Foxa2* expression in the ovalbumin-challenged and IL-13 transgenic mice. It has previously been shown that STAT-6 signaling and IL-13 are required for AMCase expression (60). Thus IL-13 signaling is clearly taking place in the AMCase-producing distal cells. However, IL-13 signaling in airway epithelial cells has previously been reported to downregulate expression of *Foxa2*, a member of the forkhead transcription factor family (50). This downregulation of *Foxa2* has been reported to lead to mucus production (50). We wondered therefore whether *Foxa2* downregulation was occurring in all airway epithelial cells, including AMCase-producing cells, or was limited to the proximal mucus produc-

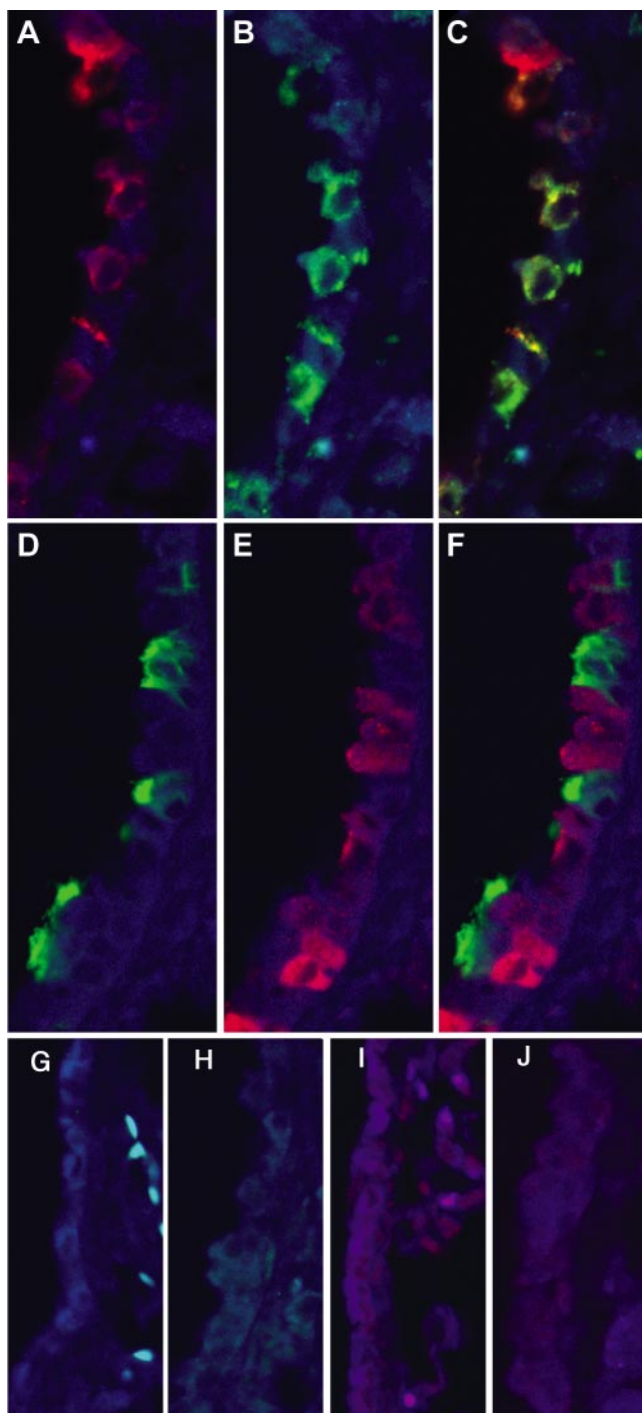


Fig. 4. Coexpression of AMCase with markers of airway epithelial cell differentiation in IL-13 transgenic mice. An airway of an IL-13 transgenic mouse that corresponds to that seen in Fig. 2B is stained for AMCase and CCSP (Clara cell marker) or AMCase and acetylated tubulin (a ciliated cell marker). A–C: CCSP (red), AMCase (green), and merge. There is excellent colocalization of CCSP and AMCase. D–F: tubulin (green), AMCase (red), and merge. There is no correlation of tubulin staining with AMCase staining. Although some overlap appears (bottom of F), optical sectioning shows this to be an artifact due to pseudostratification of epithelium. Background autofluorescence in FITC channel is pseudo-colored blue and used for tissue identification. G–J: negative controls [G: autofluorescence in Cy5 channel (control for mouse anti-AMCase) of IL-13 transgenic mouse; H: stain with mouse anti-AMCase on nontransgenic mouse; I: autofluorescence in Cy3 channel (control for rabbit anti-AMCase) of IL-13 mouse; J: stain with rabbit anti-AMCase on nontransgenic mouse]. Confocal microscopy with original magnification $\times 63$, and optical thickness is 5 μm .

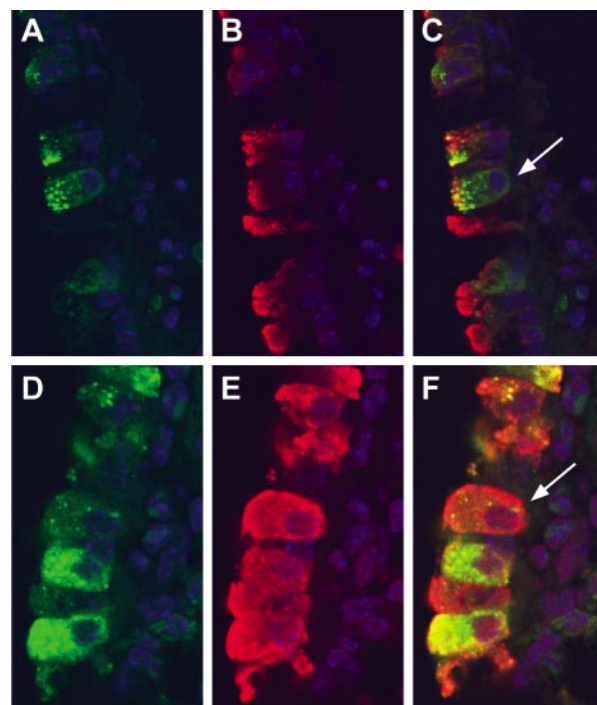


Fig. 5. Localization of AMCase and CCSP granules in airway epithelial cells of ovalbumin-challenged mice. Ovalbumin-challenged mouse double stained for AMCase (green) and CCSP (red). The arrow in C highlights a cell with peripheral expression of CCSP and abundant AMCase containing granules. There is only slight colocalization of CCSP and AMCase in the same granule. The arrow in F shows a CCSP-positive cell with only a few AMCase granules adjacent to other CCSP-positive cells that are strongly AMCase positive. Topro3 is used for nuclear localization. Confocal microscopy with original magnification $\times 63$ objective and digitally enlarged. Optical thickness is 1 μm .

ing cells. As previously reported, we see expression of *Foxa2* in airway epithelial cells and type II cells in wild-type mice (data not shown). We confirm the previous observation that Th2 inflammation and IL-13 leads to downregulation of *Foxa2* in mucus-producing cells by seeing loss of *Foxa2* in proximal airway cells (Fig. 7, E and F). However, cells that express AMCase retain their expression of *Foxa2* (Fig. 7, B, C, H, and I). These findings support the previous observation relating downregulation of *Foxa2* to mucus production.

Foxa2 is also thought to control CCSP expression (10). We therefore asked whether CCSP production correlates with *Foxa2* expression in the IL-13 mice. As can be seen in Fig. 8, CCSP is produced at similar levels in both transgenic and wild-type mice in distal airway, whereas CCSP is expressed at reduced levels in the mucus-producing proximal cells of the IL-13 mice compared with wild-type mice. This result is consistent with the reported control of CCSP by *Foxa2*. Furthermore, since the CCSP promoter drives IL-13 expression in our mice, this result explains the differential expression of IL-13 noted in Fig. 6.

DISCUSSION

The present study shows that two members of the chitinase family define molecularly distinct subsets of secretory cells within the murine-conducting airway epithelium under conditions of allergic inflammation. IL-13 signaling in proximal airway cells leads to downregulation of *Foxa2* and CCSP,

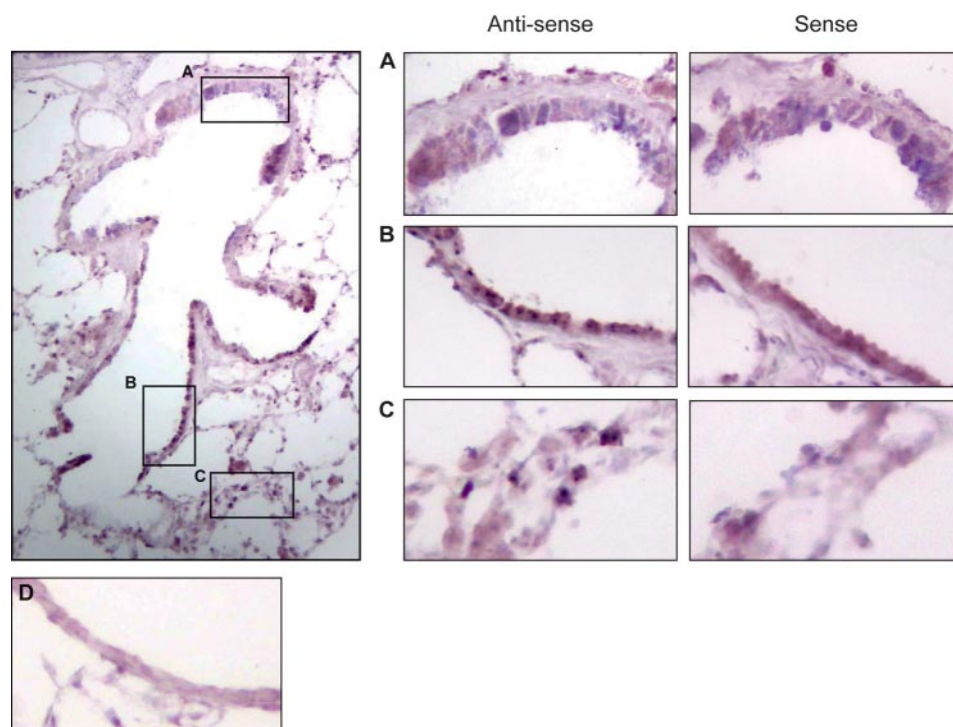


Fig. 6. In situ hybridization for IL-13 in IL-13 transgenic mice. The low-power image shows a transitional airway comparable to those shown in Figs. 1B and 2B. A: lack of expression of IL-13 in the more proximal portion of this airway. Note the airway cell hyperplasia indicative of mucus differentiation. B: staining for IL-13 in the distal portion of this airway. Note that these cells lack hyperplasia consistent with lack of mucus production. C: staining in parenchymal cells, presumably type II cells. For comparison, anti-sense of wild-type mice [terminal airways and parenchyma (D)] shows no staining. Low-power original magnification, $\times 10$ objective; high power, $\times 40$ objective.

upregulation of Ym1 and mucus, and no effect on AMCase. In distal airway cells, IL-13 signaling does not affect Foxa2, CCSP, Ym1, or mucus but does upregulate AMCase. In the IL-13 transgenic mice, this leads to reduced expression of IL-13 in proximal airways cells, since it is regulated by the CCSP promoter, whereas IL-13 is expressed in distal airway and alveolar cells.

Previous work has suggested identifiable morphological, biochemical, and functional differences between proximal and distal airway secretory cells (35, 36). Expression of CCSP is initially restricted to bronchi in the early pseudoglandular period and only extends to the entire airway 24 h later (54). Within the murine airway, bronchial Clara cells exhibit a columnar-to-low cuboidal morphology and have relatively

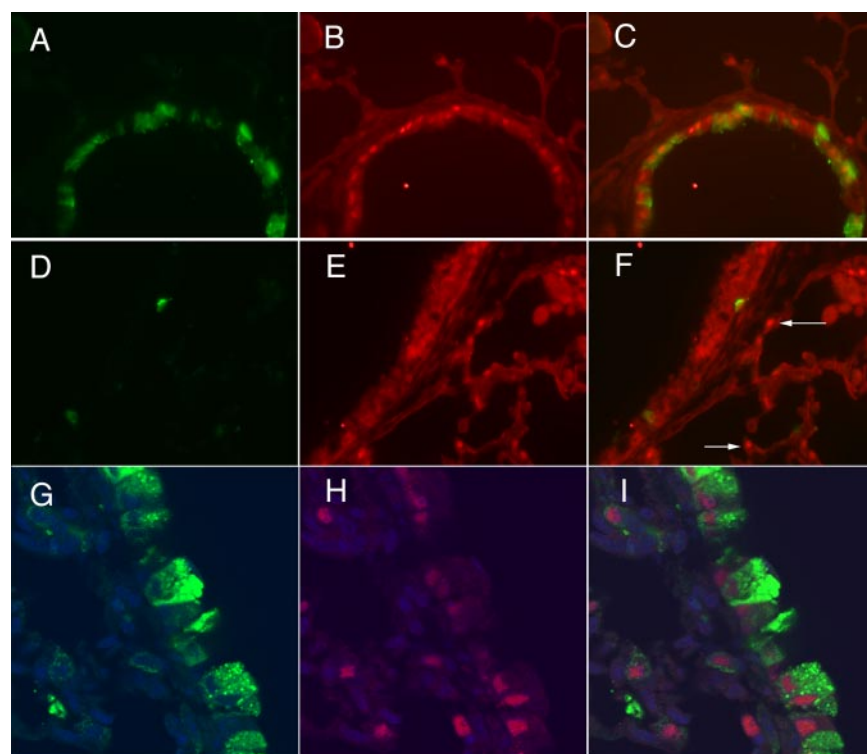


Fig. 7. Foxa2 expression in IL-13 transgenic and ovalbumin-challenged mice. A–C: distal airway of an IL-13 transgenic mouse stained for AMCase (A), Foxa2 (B), or merge (C). Cells that express AMCase also express Foxa2. Foxa2 staining is nuclear. D–F: central airway of an IL-13 transgenic mouse stained for AMCase (D), Foxa2 (E), or merge (F). Expression of Foxa2 is reduced in central airways, which undergo mucus metaplasia. Arrows show retained expression of Foxa2 in type II cells as an internal control. G–I: distal airway of ovalbumin-challenged mice stained for AMCase (G), Foxa2 (H), or merge (I) analyzed via confocal microscopy. There is abundant expression of AMCase in cells that also express Foxa2. Type II cells are also notably Foxa2 positive. A–C: original magnification, $\times 40$; D–F: original magnification $\times 20$; G–I: original magnification $\times 63$. Confocal microscopy with Topro3 as a nuclear counterstain, optical thickness of 1 μm .

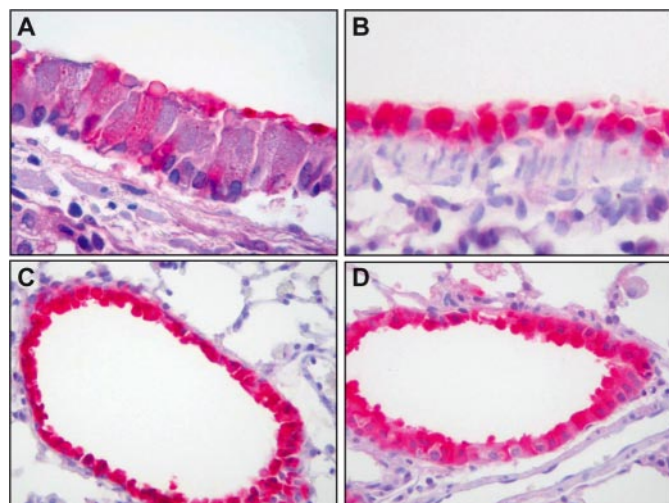


Fig. 8. CCSP expression in IL-13 transgenic mice. Central airways (comparable with Fig. 1A) show strong staining in wild-type mouse (B). Note that there is staining of roughly one-half of the cells present, consistent with the known percentage of Clara cells there. In comparison, central airways of IL-13 transgenic mice show reduced expression of CCSP (A). Distal airways of both transgenic (C) and wild-type mice have abundant CCSP (D). (Alkaline phosphatase with Vector Red immunohistochemistry, original objective $\times 40$.)

more granular endoplasmic reticulum and less smooth endoplasmic reticulum than their tall columnar counterparts in the bronchioles (35). Regionally specialized secretory activity is suggested by variation in the number, density, and morphological characteristics of secretory granules and granular endoplasmic reticulum, differences in cytoplasmic and granular CCSP concentration, and in resynthesis of CCSP after induced secretion (3, 12, 13, 35, 51). In addition to quantitative differences in CCSP expression, qualitative molecular differences exist as well, since secretoglobulin 3A1, a gene product related to CCSP, is expressed by proximal but not distal airway secretory cells (42). Differences in CYP450 enzymatic activity, cellular glutathione pool size, and glutathione regenerative capacity further distinguish these cells and may contribute to variation in pollutant sensitivity (14, 17, 34, 37, 55, 56). The local stem cells that repopulate the airway epithelium also differ between proximal and distal airways (18, 23). Progenitor cells with the potential to contribute to airway repair include secretory, neuroendocrine, and basal cell populations. The individual contributions of each progenitor cell may depend on the nature of the injury. However, it is noteworthy that basal cells only exist in the proximal airway and thus can only contribute to proximal airway epithelial repair, whereas local stem cells for distal airway cells include so-called variant Clara cells, cells that express CCSP but not CYP-2F2 (22, 23, 41). Finally, it is known that the proximal but not distal secretory cells undergo mucus metaplasia in response to allergic inflammation, whereas distal cells undergo relatively more proliferation under the same stimulus (16, 40). Thus much previous data suggest both qualitative and quantitative differences between proximal and distal CCSP-expressing secretory cells. The present study reinforces this concept and extends it to expression of members of the chitinase gene family in allergic inflammation. Furthermore, before this study, no positive molecular marker had yet been established that uniquely defined these distal secretory cells. Unfortunately, AMCase is only

expressed under conditions of allergic inflammation, so it cannot be used to identify this population in the resting state.

The role of the chitinase family in allergic inflammation is only beginning to be investigated. The expression pattern of these chitinases may provide some clues to the function of these molecules. It is striking that, although Ym1 and AMCase are closely related and both expressed by airway epithelium and macrophages, they have distinctly different expression patterns within the epithelium. It is difficult to speculate on the functional significance of this finding since so little is known about the biology of these molecules. Both molecules have been associated with induction of inflammation and proposed to be involved in tissue remodeling. Inhibition of AMCase activity, either via inhibition of enzymic activity via a small molecule inhibitor or through antibody-mediated inhibition, reduces inflammation downstream of IL-13, IL-13 receptors, and STAT6 (59). AMCase has also been suggested to be involved in remodeling of the extracellular matrix (43). Ym1 has been associated with alternatively activated macrophages and has weakly active eosinophil recruiting activity (38, 52). Ym1 binds saccharides with a free amine group, including *n*-glucosamine and their oligosaccharides, as well as heparin, suggesting a role in binding to both extracellular matrix and parasite eggs (8, 49). It has therefore been suggested that Ym1 is involved in tissue remodeling involving changes in the extracellular matrix (52). If both AMCase and Ym1 are involved in tissue remodeling, this would imply that the mechanism by which tissue remodeling occurs may be different between proximal and distal airways. In humans, it is known that there are significant differences among various clinical groups of asthmatics in the degree of airway remodeling between proximal and distal airways (20). Further work is in progress to examine the effect of ectopic expression of AMCase in airways.

Despite lack of mucus production, the distal Clara cells are clearly responding to IL-13 via the production of AMCase. Furthermore, the production of AMCase, Ym1, and mucus in response to IL-13 is STAT6 dependent (27, 53, 60). Difference in an IL-13-induced phenotype despite similar signaling pathways has previously been shown in cells of different lineages as well as in airway cells under different culture conditions (25, 29). Qualitative differences in response to quantitatively differing doses of IL-13 have also been described (1). Previous work on mucus induction in proximal airway cells was unable to address the issue of differences in the local production of mediators being responsible for the effects seen (16, 40). Although we could not directly measure concentrations of IL-13 along the airway, our *in situ* hybridization result indicates that differences in local amounts of IL-13 cannot account for production of mucus in proximal airways. In particular, although in any given model an IL-13 gradient might account for the differences, an IL-13 gradient cannot account for the range of models in which these differences are seen. Thus, although the ovalbumin challenge model could be argued to have more IL-13 proximally and the IL-13 transgenic mice could be argued to have more IL-13 distally, the same distinction of proximal mucus and distal AMCase is found. Finally, it appears that the vast majority of the *in vitro* work done with murine and human airway cell lines show induction of mucus in response to IL-13 (1, 7, 11, 25, 26). The results here suggest that these cell lines are representative of proximal but not distal

airway epithelial secretory cells. No cell line has currently been shown to have the phenotype of these distal Clara cells.

Since Foxa2 had previously been reported to be downregulated by IL-13 in airway epithelial cells and this downregulation was proposed to be important in induction of mucus in those cells (50), we examined the AMCase-expressing cells to determine their Foxa2 status. We confirmed the observation that Foxa2 is downregulated in proximal cells while noting that Foxa2 expression is retained distally. This supports the relationship of loss of Foxa2 with mucus production. Furthermore, CCSP expression correlates with Foxa2 expression in that central airways show less CCSP expression than distal airways. This is consistent with previous data that Foxa2 regulates CCSP expression (10). Since IL-13 in our mice is driven by the CCSP promoter, these results explain the differential expression of IL-13 noted. CCSP has previously been reported to be upregulated in response to IL-13 in vivo (26). However, that study only looked at short-term effects. Work in humans is consistent with our observations that CCSP is downregulated in asthma and in areas of mucus metaplasia (2, 24, 32, 46, 47). It is interesting that, although IL-13 downregulates CCSP, interferon gamma is known to upregulate CCSP (39).

The differential expression of chitinase and Ym1 occurs both with allergen-challenged mice and mice that overexpress IL-13. This result shows that IL-13 alone is sufficient for the induction of the differential secretory program of proximal and distal cells. We have not examined other Th2 cytokines to see whether they are also capable of inducing this program as well. However, at least some Th2 cytokines such as IL-9 and IL-10 appear to mediate their mucus induction through IL-13 (28, 57). The IL-13 mice have shown that IL-13 can induce a remarkable range of mediators and pathological alterations. The airways of these mice show markedly increased mucus, subepithelial fibrosis, and inflammation. Functionally, these mice exhibit airway hyperresponsiveness, although the airway hyperresponsiveness is not dependent on the inflammation or the fibrosis (27, 58). The parenchyma undergoes a number of changes, including protease-dependent emphysema, TGF- β 1-dependent pulmonary fibrosis, lipoproteinosis with surfactant lipid and protein accumulation, and type II cell hypertrophy (20, 21). Both STAT6 and MAPK pathways of intracellular signaling have been implicated in these abnormalities (27, 30).

It has recently been shown that, in humans, chitinase polymorphisms correlate with asthma severity, supporting a role for these molecules in human disease (4). Our laboratory previously showed expression of AMCase in human asthmatic airway epithelium via in situ hybridization but do not have antibodies suitable for the kind of experiments performed here in mice (59). Furthermore, Ym1 does not appear to be encoded in humans, and there is a marked difference in expression patterns of AMCase at baseline between humans and mice (6). Thus further work is needed to assess the direct relevance of the present work to humans.

The basis for the difference in intracellular staining pattern for CCSP and AMCase is not clear. CCSP is known to be expressed in both endoplasmic reticulum as well as peripheral secretory granules. (13). Although the pattern of staining of AMCase is distinctly granular, suggesting expression in secretory granules, there appears to be a difference in the pattern from that of the CCSP granules. AMCase has a pH optimum of 2, suggesting that it is active in lysosomes in addition to or

instead of in secretory granules or endoplasmic reticulum. Work to perform ultrastructural localization of AMCase is in progress.

In conclusion, we have shown that AMCase and Ym1 are markers for mutually exclusive subsets of distal airway secretory cells that respond to allergic inflammation. Identification of the factors that control spatially restricted expression of Ym1 and AMCase may lead to further insights into cellular and cell/matrix interactions leading to regional specialization of the airway epithelium. Further investigation into contributions made by Ym1 and AMCase in regional regulation of inflammatory and immunological responses may yield critical new insights into mechanisms of airway dysfunction that accompany chronic lung disease.

NOTE ADDED IN PROOF

Arora et al. (1a) have shown that Ym1 in dendritic cells enhances Th2 differentiation.

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