Haemophilus influenzae Lysate Induces Aspects of the Chronic Obstructive Pulmonary Disease Phenotype

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Nontypeable *Haemophilus influenzae* (NTHi) commonly colonizes the lower airways of patients with chronic obstructive pulmonary disease (COPD). Whether it contributes to COPD progression is unknown. Here, we determined which aspects of the COPD phenotype can be induced by repetitive exposure to NTHi products. Mice were exposed weekly to an aerosolized NTHi lysate, and inflammation was evaluated by measurement of cells and cytokines in bronchoalveolar lavage fluid (BALF) and immunohistochemical staining; structural changes were evaluated histochemically by periodic acid fluorescent Schiff's reagent, Masson's trichrome, and Picrosirius red staining; mucin gene expression was measured by quantitative RT-PCR; and the role of TNF- α was examined by transgenic airway overexpression and use of an inhibitory antibody. NTHi lysate induced rapid activation of NF- κ B in airway cells and increases of inflammatory cytokines and neutrophils in BALF. Repetitive exposure induced infiltration of macrophages, CD8⁺ T cells, and B cells around airways and blood vessels, and collagen deposition in airway and alveolar walls, but airway mucin staining and gel-forming mucin transcripts were not increased. Transgenic overexpression of TNF- α caused BALF neutrophilia and inflammatory cell infiltration around airways, but not fibrosis, and TNF- α neutralization did not reduce BALF neutrophilia in response to NTHi lysate. In conclusion, NTHi products elicit airway inflammation in mice with a cellular and cytokine profile similar to that in COPD, and cause airway wall fibrosis but not mucous metaplasia. TNF- α is neither required for inflammatory cell recruitment nor sufficient for airway fibrosis. Colonization by NTHi may contribute to the pathogenesis of small airways disease in patients with COPD.

Keywords: pulmonary disease, chronic obstructive; *Haemophilus influenzae*; bronchitis; inflammation; fibrosis

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible (1–4). COPD is thought to be caused by inflammation induced by inhaled smoke and particulates, and possibly by infecting pathogens as well, leading to the structural changes in airways and alveoli that result in airflow limitation. At the level of the conducting airways, there is metaplasia of the airway epithelium to a mucus hypersecreting phenotype that causes luminal obstruction, thickening of the airway wall from increased deposition of matrix molecules and proliferation of mesenchy-

CLINICAL RELEVANCE

Nontypeable *Haemophilus influenzae* (NTHi) commonly colonizes the airways of patients with chronic obstructive pulmonary disease (COPD). Whether NTHi colonization contributes to COPD progression is unknown. Our findings define which aspects of COPD might be induced by repetitive exposure to NTHi to help future clinical research.

mal cells, and narrowing of the airway from fibrosis. In the peripheral lung, there is destruction of alveolar walls leading to a reduction in the radial tethering that normally helps to hold conducting airways open and an enlargement of distal airspaces (5–8).

In histopathologic specimens of distal lung and in bronchoalveolar lavage fluid (BALF) from patients with COPD, macrophages, neutrophils, and CD8⁺ T cells are prominent (9–11). This cellular inflammation is accompanied by increased levels of inflammatory mediators, notably TNF- α , IL-6, IFN- γ , and the chemokine IL-8 (12–14). A striking feature of COPD is that even after withdrawal of the usual inciting stimulus, cigarette smoke, inflammation persists and lung function continues to deteriorate (15). Several possibilities have been proposed to explain the persistent inflammation: self-perpetuation of the immune response by autoantigens resulting from inflammatory and oxidative lung injury, persistent or recurrent infection of damaged airways as a co-stimulator, or antigenic mimicry or as a polyclonal activator, which could provide a persisting antigenic stimulus and maintain the inflammatory process (16, 17).

Nontypeable (unencapsulated) *Haemophilus influenzae* (NTHi) is present frequently in the airways of adults with COPD (18–21). In addition to colonization during clinically stable periods, acquisition of new strains of NTHi is an important cause of lower respiratory tract infection resulting in exacerbations of COPD (22–25). Incubation of cultured human bronchial epithelial cells with endotoxin from NTHi leads to markedly increased expression and release of proinflammatory mediators, including IL-6, IL-8, and TNF- α (26). Together, these findings suggest that persistent or repetitive exposure of the airway to NTHi products may contribute to airway inflammation in COPD (22).

Animal studies have been critical in shaping contemporary views of the pathogenesis of asthma and COPD. So far, animal models of experimentally induced COPD have included inhalation of noxious agents (cigarette smoke, SO₂, NO₂, and ozone), instillation of elastase, and generation of genetic models that mimic particular aspects of the complex pathogenesis of this disease (27). To help determine which aspects of the COPD phenotype can be ascribed to exposure to NTHi products, we established a mouse model of repetitive exposure to an aerosolized NTHi lysate and characterized the inflammatory and

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structural responses for comparison to published descriptions of airway changes in patients with COPD.

MATERIALS AND METHODS

Animals

Female, specific pathogen-free, 5- to 6-week-old C57BL/6 mice were purchased from Harlan (Indianapolis, IN) for the NTHi exposure experiments. To generate CCSP-TNF- α mice, the 3.7-kb transgene was excised as a linear fragment and injected into the male pronucleus of C57BL/6 fertilized eggs in the MD Anderson Cancer Center Genetically Engineered Mouse Facility. All mice were housed in specific pathogen-free conditions, and handled in accordance with the Institutional Animal Care and Use Committee of MD Anderson Cancer Center. For killing, mice were first anesthetized by intraperitoneal injection (5 ml/kg) of a mixture of ketamine (37.5 mg/ml), xylazine (1.9 mg/ml), and acepromazine (0.37 mg/ml), then exsanguinated by transection of the abdominal aorta.

Aerosol NTHi Lysate Exposure

A clinical isolate of NTHi strain 12 (28), which is one of the most common strains during COPD exacerbations and otitis media infections (28–30), was stored as frozen stock (1×10^7 colony-forming units/ml). The identity of the stock was confirmed using a 16S rDNA PCR analysis that distinguishes NTHi from *Haemophilus haemolyticus*, as described previously (31). NTHi was grown on chocolate agar at 300 μ l per 10-cm plate (Remel, Lenexa, KS) for 24 hours at 37°C in 5% CO₂, then harvested and incubated for 16 hours in 1 L brain-heart infusion broth (Acumedia, Lansing, MI) supplemented with 3.5 μ g/ml NAD (Sigma-Aldrich, St. Louis, MO). The culture was centrifuged at $2,500 \times g$ for 10 minutes at 4°C, washed and resuspended in 20 ml PBS, ultraviolet irradiated in a 100-mm Petri dish at 3,000 μ J/cm², then sonicated three times for 30 seconds each in a 50-ml conical plastic tube (Sonic Dismembrator 50; Fisher Scientific, Pittsburgh, PA). Protein concentration was adjusted to 2.5 mg/ml in PBS by bicinchoninic assay (Pierce, Rockford, IL), and the lysate frozen in 10 ml aliquots at –80°C. For exposure to mice, a thawed aliquot was placed in an AeroMist CA-209 nebulizer (CIS-US) driven by 10 L/minute 5% CO₂ in air for 20 minutes. This resulted in aerosolization of 4 ml of lysate, with the protein concentration in residual lysate confirmed at 2.5 mg/ml.

Measurement of Inflammatory Cells and Mediators in BALF

Mice were deeply anesthetized, then tracheotomized with a luer stub adapter cannula, and BALF was obtained by sequentially instilling and collecting two aliquots of 1 ml each of PBS. Total leukocyte count was determined with a hemacytometer, and differential count by cytocentrifugation of 300 μ l of BALF at $300 \times g$ (2,000 rpm) for 5 minutes, followed by Wright-Giemsa staining. The remaining BALF (~1,400 μ l) was centrifuged at $1,250 \times g$ for 10 minutes, and supernatants were collected and stored at –70°C. Cytokine concentrations were measured in duplicate by multiplexed sandwich enzyme-linked immunosorbent assay using SearchLight Proteome Arrays (Pierce). To inhibit TNF- α , 1 mg of rat/mouse chimeric monoclonal antibody cV1q (Centocor, Horsham, PA) in 100 μ l PBS was injected intraperitoneally 24 hours before exposure to aerosolized NTHi lysate or 600 ng intratracheally injected recombinant mouse TNF- α (PeproTech, Rocky Hill, NJ).

Histologic Analyses

Mice were killed, their lungs were perfused *in situ* with PBS via the right cardiac ventricle, then the lungs were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) infused through a tracheal cannula at 10 to 15 cm pressure at 21°C. The lungs were removed from the thoracic cavity and further fixed overnight at 4°C, then embedded in paraffin and cut into 5- μ m sections. Slides were dewaxed and rehydrated, and tissues were stained with Harris' hematoxylin and eosin (H&E) to examine cellular elements, Masson's trichrome stain (MTC) or Picrosirius red (PSR) to examine collagen (32, 33), and periodic acid fluorescent Schiff's (PAFS) reagent to examine intracellular mucin (34).

Morphometric Analyses

H&E sections were chosen from three to five animals per group. Five randomly selected microscopic fields from peripheral and central

regions of the lungs were photographed, and stereologic analysis was performed as described previously (35–37). Briefly, color images were converted to black and white and saved as bitmap files using Photoshop CS2 (Adobe, San Jose, CA). Tissues and airspaces were separately highlighted, and the selected layers of these images were overlaid on a vertical line grid of $1,280 \times 1,024$ pixels with 22 vertical lines, a line width of 3 pixels, and interval between lines of 60 pixels (20 μ m at the magnification of analysis). Selections superimposed upon extrapulmonary space, nonalveolar airways, vascular walls, and airway or vascular lumens were deleted, and the areas of the remaining gridlines were recorded as total alveolar area, A(T). Morphometric parameters were then measured using Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD). For mean chord length (MCL), selections transferred from highlighted tissues were deleted and the average lengths of the remaining vertical line segments were tallied. For alveolar tissue thickness, A(t), selections transferred from highlighted airspaces (the inverse of those for MCL) were deleted and the average areas of the remaining vertical line segments were tallied. To normalize alveolar thickness data among images and groups, results are represented as a ratio of A(t) to A(T).

Collagen Measurement

Collagen in fibrous tissue was quantified by examination of linear polarized light birefringence in PSR-stained airway, alveolar, and vascular regions using randomly selected microscopic fields from peripheral and central regions of the lungs using the same grid system as for morphometry, but instead of deleting selections superimposed upon nonalveolar airways and vessels, fractions of nonalveolar airway, alveolar, and vascular fibrosis were calculated separately. For example, selections transferred from highlighted nonalveolar airways that intersected vertical grid lines were tallied and measured by Image-Pro Plus 5.1.

Immunohistochemical Analysis

Tissue sections were heated in a pressure cooker in 25 mM citrate buffer (pH 6.0) for 6 minutes, then cooled and treated for 10 minutes with 3% H₂O₂ and blocked with serum-free proteins (Dako, Glostrup, Denmark). Six complete sets of contiguous slides from each mouse were labeled separately for 3 hours at room temperature with antibodies against macrophages (rabbit α -CD68; Santa Cruz Biotechnology, Santa Cruz, CA), T-cell subsets (goat α -CD4 and rabbit α -CD8; Santa Cruz), B cell (goat α -CD20; Santa Cruz), neutrophil (rat α -p40, SeroTec, Raleigh, NC), and NF- κ B (rabbit α -p65; Abcam, Cambridge, MA). Slides were then incubated with biotin-labeled secondary antibodies, exposed to horseradish peroxidase-labeled streptavidin, developed with diaminobenzidine, and counterstained with Meyer's hematoxylin (Dako). The number of labeled submucosal cells were quantitated as a fraction of total nuclei per high power field in five fields from three mice.

Mucin Gene Expression Analysis

Mice were anesthetized, and their lungs were removed and frozen in liquid nitrogen. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), and quantitative real-time RT-PCR for airway mucin transcripts was performed as described (38). For the ovalbumin allergic inflammation control studies, mice were sensitized and challenged as described previously (34).

Statistical Methods

Summary statistics for cell counts in BALF and nuclear translocation of NF- κ B in airway epithelium were computed within time groups, and analysis of variance with adjustment for multiple comparisons was performed to examine the differences between the mean cell counts of the control group and each of the NTHi treatment groups. For immunohistochemical staining of leukocytes and morphometric analyses in NTHi-treated mice, comparisons of groups were made using Student's *t* test. Differences were considered significant for *P* < 0.05.

RESULTS

Acute Inflammatory Response to NTHi Products

Exposing mice to the aerosolized NTHi lysate resulted in a rapid and marked influx of neutrophils into pulmonary airspaces

Figure 1. Inflammatory cell populations in bronchoalveolar lavage fluid (BALF) after nontypeable *Haemophilus influenzae* (NTHi) lysate aerosol exposure. Mice were exposed to aerosols containing 2.5 mg/ml NTHi lysate in PBS for 20 minutes, then killed in groups of five to six to recover BALF for counting cell populations by cyto-centrifugation and Wright-Geimsa staining. (A) BALF cell counts at various times after a single NTHi lysate exposure. (B) BALF cell counts at various times after the eighth weekly NTHi exposure. (C) BALF cell counts 1 day after a single NTHi lysate exposure (1 w) or 1 day after multiple weekly exposures (8 w, 25 w, 50 w). *Different from control, $P < 0.05$; #different from W1-D1, $P < 0.05$.

(Figure 1A), with BALF neutrophils increasing from $4.0 \pm 2.0 \times 10^2$ at baseline to $5.3 \pm 4.7 \times 10^5$ at 4 hours. The number of neutrophils peaked on the second day after exposure, declined markedly on the third day, and returned to baseline by the seventh day. The numbers of macrophages and lymphocytes increased significantly on the second day after exposure, and remained elevated several-fold on the seventh day (Figure 1A). Macrophage numbers remained slightly elevated 14 days after exposure, but had returned to baseline by 28 days (not shown). A similar pattern, but with lesser acute increases in BALF

inflammatory cell numbers and with longer persistence of neutrophils, was observed with successive weekly exposures to the aerosolized NTHi lysate (Figures 1B and 1C).

Leukocyte recruitment was accompanied by a marked increase in cytokines in BALF at 4 hours (Table 1), both after the first exposure and after eight weekly exposures. In particular, the inflammatory cytokines TNF- α and IL-6 increased thousands-fold, whereas Th1 (IFN- γ and IL-12) and Th2 (IL-4 and IL-13) immunoregulatory cytokines increased only tens-fold (Table 1). The levels of most cytokines returned to baseline during the first day after exposure. Consistent with the rapid rise and fall in the levels of inflammatory cytokines, the p65 subunit of NF- κ B had already translocated from the cytoplasm to the nucleus of most airway epithelial cells upon completion of the 20-minute exposure to the aerosolized NTHi lysate, and 60 minutes later the number of positively stained nuclei had nearly declined to baseline (Figure 2).

Chronic Leukocyte Infiltration in Response to NTHi Products

The lungs of mice 24 hours after their eighth weekly exposure to NTHi lysate showed dense leukocyte infiltration around bronchial and bronchiolar airways and around blood vessels (Figure 3B). Rounded lymphoid aggregates were also occasionally seen (Figure 3B), usually adjacent to bronchi. Smaller numbers of leukocytes were also seen in alveolar airspaces. The peribronchial and perivascular leukocyte infiltrates did not change appreciably after 25 and 50 exposures to the NTHi lysate, but greater leukocyte infiltration of alveolar septae was observed (Figures 3C and 3D).

The identities of infiltrating leukocytes were determined by immunohistochemical labeling with lineage-specific antibodies (Figure 4). The most abundant leukocyte population was macrophages, and this was followed by B cells, cytolytic T cells, neutrophils, and helper T cells (Table 2). These leukocyte lineage markers represent 82.8% of extravascular submucosal nuclei, and the remaining 17.2% of cells could represent other leukocyte lineages or resident mesenchymal cells.

Lung Remodeling after Chronic Exposure to NTHi Products

Up-regulated lung mucin gene expression in response to bacterial products and neutrophils has been previously reported (39–41). However, no increase in PAFS staining for mucins was seen after 1, 8, 25, or 50 weekly exposures of mice to the aerosolized NTHi lysate (Figure 5B). In contrast, there was a dramatic increase in PAFS staining after a single exposure to aerosolized ovalbumin in

TABLE 1. CYTOKINE LEVELS IN BALF AFTER NTHI EXPOSURE

Cytokine	First NTHi Exposure			Eighth NTHi Exposure				
	0	4 h	Increase*	0	4 h	1 d	7 d	Increase*
Inflammatory								
TNF- α	4.1 $\pm$ 1.2	2,827 $\pm$ 1,974	689.5	11.2 $\pm$ 2.5	14,545.8 $\pm$ 3,257	20.9 $\pm$ 5.7	5.9 $\pm$ 2.1	1,299
IL-6	9.1 $\pm$ 1.0	19,678 $\pm$ 3,073	2,162	10.8 $\pm$ 1.9	59,873.7 $\pm$ 13,094	318.3 $\pm$ 114.6	25.2 $\pm$ 3.2	5,544
IL-1 β	8.4 $\pm$ 3.5	111 $\pm$ 48.4	13.2	18.2 $\pm$ 11	41.4 $\pm$ 5.4	2.9 $\pm$ 0.6	8.7 $\pm$ 4.8	2.3
Th1								
IFN- γ	21.9 $\pm$ 4.6	68 $\pm$ 7.8	3.1	84.1 $\pm$ 8.2	541.4 $\pm$ 66.1	240.8 $\pm$ 206.4	54.3 $\pm$ 15.9	6.4
IL-12	2.4 $\pm$ 0.4	5.6 $\pm$ 1.1	2.3	0.4 $\pm$ 0.1	49.4 $\pm$ 1.1	0.6 $\pm$ 0.3	3.2 $\pm$ 0.7	123.5
Th2								
IL-4	0.5 $\pm$ 0.2	5.4 $\pm$ 1	10.8	0.6 $\pm$ 0.1	10.2 $\pm$ 3.8	0.5 $\pm$ 0.2	1.4 $\pm$ 0.7	17
IL-13	7.6 $\pm$ 5.3	17.5 $\pm$ 1.4	2.3	8.9 $\pm$ 2.9	83.3 $\pm$ 16.3	8.5 $\pm$ 5.8	14.1 $\pm$ 5.4	9.3
Chemokine								
KC	7.1 $\pm$ 1.9	1,846 $\pm$ 1,034	260	9.6 $\pm$ 0.7	111.9 $\pm$ 34.5	3.8 $\pm$ 0.6	26.3 $\pm$ 9.7	11.6
Eotaxin	38.4 $\pm$ 17.6	932.3 $\pm$ 123.5	24.3	41.4 $\pm$ 40	1,374.8 $\pm$ 1,047.4	142.8 $\pm$ 51.8	217.3 $\pm$ 160	33.2

Definition of abbreviations: BALF, bronchoalveolar lavage fluid; KC, keratinocyte-derived chemokine; NTHi, nontypeable *Haemophilus influenzae*; Th1 and Th2, T helper cells type 1 or 2.

All data are the mean $\pm$ SEM, and are expressed as pg/ml.

* Fold increase at 4 h compared with zero time point.

Figure 2. NF- κ B activation after NTHi lysate aerosol exposure. Mice were exposed or not to NTHi lysate for 20 minutes, then killed at various times and their lungs processed for immunohistochemical staining with antibody against the p65 subunit of NF- κ B. (A) Control mouse lung not exposed to NTHi lysate that does not show nuclear staining. (B) Mouse lung 5 minutes after treatment with NTHi lysate showing intense nuclear staining. Scale bar = 8 μ m. (C) Percentage of stained nuclei of airway epithelial cells in mice not exposed to the NTHi lysate (data point on the ordinate) and at various times after exposure to NTHi lysate for 20 minutes. *Different from unexposed lungs, $P < 0.05$.

sensitized mice (Figure 5C), as we have described previously (34). To further assess expression of the secreted gel-forming mucins, transcripts for Muc2, Muc5ac, Muc5b, Muc6, and Muc19 were measured in lung and trachea by RT-PCR. There was no increase in any of the mucin transcripts after a single exposure to NTHi lysate (Figure 5D), though there was a robust increase in lung and tracheal Muc5ac and a lesser increase in Muc5b in the ovalbumin model (data not shown) (38). Similarly, there was no increase in transcripts for any of the lung secreted gel-forming mucins—Muc2, Muc5ac, and Muc5b—with repetitive exposure to the NTHi lysate (Figure 5E). We next performed quantitative RT-PCR of lung secreted mucins at baseline, 3 days after the first exposure, and 1 day after 8 and 25 weekly exposures. As we have found previously (38), Muc5b was the most abundant mucin transcript at baseline (1.9×10^{-3} pg/pg 18S RNA), Muc5ac was also present (4.8×10^{-5} pg/pg 18S RNA), and Muc2 was not detectable. At no time after NTHi lysate exposure was there any measurable increase in mucin transcripts (data not shown).

To assess structural changes in airway walls, mouse lungs were stained with MTC after 8, 25, and 50 weekly exposures to the aerosolized NTHi lysate. Blue-stained collagen around blood vessels was obvious at baseline (Figure 6A, a) and in age-matched control animals (not shown), but was not apparent around airways. After eight weekly exposures to the NTHi lysate, there was no observable increase in collagen staining. However, after 25 weekly exposures, blue staining was apparent in airway walls (Figure 6A, c), and further increased after 50 weekly exposures (Figure 6A, d). PSR staining was used to quantitate collagen

Figure 3. Histopathologic changes in lungs after repetitive NTHi lysate aerosol exposure. Mice were exposed to NTHi lysate weekly for various numbers of weeks, then killed 1 day after the last exposure and their lungs processed for light microscopy with hematoxylin and eosin staining. (A) Lung tissue from a control mouse not exposed to NTHi lysate. (B) Lung tissue from a mouse exposed weekly for 8 weeks to the NTHi lysate, showing cellular infiltrates around bronchioles (arrow) and blood vessels (arrowhead), and a nearby lymphoid aggregate (open arrow). (C) Lung tissue from a mouse exposed weekly for 25 weeks to the NTHi lysate, showing cellular infiltrates around bronchi. Scale bar = 50 μ m. (D) Lung tissue from a mouse exposed weekly for 50 weeks to the NTHi lysate, showing cellular infiltrates around airways (arrow), blood vessels (arrowhead), and extending into alveolar walls. (E) Alveolar tissue area as a fraction of total lung parenchymal tissue area was measured at 8, 25, and 50 weeks as described in MATERIALS AND METHODS. *Different from control, $P < 0.05$.

deposition in airway walls (Figure 6B), and showed no increase compared with age-matched controls at 8 weeks (Figure 6B, a and b), but was visible after 25 weeks (Figure 6B, c), and had increased significantly (5-fold) at 50 weeks (Figures 6B, d, and 6C, a).

Increased alveolar wall thickness by H&E staining (Figure 3D) and increased alveolar MTC staining (Figure 6A, d) were also noted after 50 weeks. To further assess structural changes in the lung parenchyma, alveolar septal thickness and alveolar airspace mean chord length were measured. There was no apparent change in either parameter after 8 NTHi lysate exposures, but after 25 exposures there was a significant increase in alveolar wall thickness that increased further after 50 weeks (Figure 3E). Increased alveolar septal staining for collagen with PSR was also apparent after 50 weeks (Figure 6C, b).

CCSP-TNF- α Transgenic Mice

Since TNF- α levels are prominently increased in the airway lining fluid and sputum of patients with COPD (12, 42) and the BALF of

Figure 4. Immunohistochemical identification of leukocytes infiltrating the lungs after repetitive NTHi lysate aerosol exposure. Mice were exposed weekly for 8 weeks to the aerosolized NTHi lysate, then killed 1 day after the last exposure and their lungs processed for light microscopy by immunohistochemical labeling with lineage-specific antibodies and hematoxylin counterstaining. (A–C) Labeling with α -CD68 to detect macrophages. (E–G) Labeling with α -CD20 to detect B cells. (I–K) Labeling with α -CD8 to detect cytolytic T cells. (M–O) Labeling with α -CD4 to detect helper T cells. (Q–S) Labeling with α -p40 to detect neutrophils. (A, E, I, M, Q) Control lungs exposed to PBS alone rather than NTHi to detect leukocytes present at baseline without NTHi exposure. (D, H, L, P, T) Control antibody to detect nonspecific labeling in NTHi-treated lungs. Scale bar = 100 μ m for all panels except C, G, K, and O, where it = 20 μ m.

mice exposed to NTHi lysate (Table 1), we assessed the contribution of TNF- α to airway inflammatory and structural changes by transgenically expressing TNF- α in airway epithelial cells using the CCSP promoter (Figure 7). PCR analysis of genomic DNA from tail biopsies of pups identified seven transgene-positive lines. The strength of transgene expression was assessed by RT-PCR analysis of TNF- α transcripts in lung mRNA, and the two lines with the highest expression were maintained by mating to C57BL/6 mice. TNF- α protein levels in BALF of transgenic mice at 8 weeks of age were increased 165-fold compared to transgene negative littermate controls (1.6 versus 263.7 pg/ml) (Figure 7B). Since MMP-9 has been shown to play an important role in lung remodeling in COPD and COPD-like animal models (43), we also measured MMP-9 levels and found these increased 38-fold (31.2 versus 1,174.8 pg/ml) (Figure 7C). At 8 weeks of age, there was a 1.6-fold increase in total BALF inflammatory cells in transgenic mice (4.13 versus 6.75×10^4) (Figure 7D), which reflected a 50-fold increase in neutrophils (0.12 versus 5.45×10^4) but 3.6-fold decrease in macrophages (3.85 versus 1.07×10^4), with little change in lymphocytes (0.14 versus 0.22×10^4). Similar

cell count distributions persisted at 25 and 50 weeks of age (data not shown).

Staining with H&E showed prominent cellular infiltration around airway walls of transgenic mice at 8, 25, and 50 weeks of age (Figures 8A and 8B). This infiltrate was composed of a mixture of macrophages, lymphocytes, and neutrophils by immunohistochemical staining, similar in numbers to those in BALF by histochemical staining (data not shown). Leukocytes were not seen in increased numbers in alveolar walls, nor were there obvious changes in alveolar structure (Figures 8A and 8B). PAFS staining for intracellular mucin showed no increase compared with wild-type at 8 weeks of age, but a slight increase at 25 and 50 weeks (approximately 1/10 that seen with ovalbumin sensitization and challenge; data not shown). Neither MTC nor PSR staining showed increased collagen deposition in airway or alveolar walls at 8, 25, or 50 weeks of age (Figure 8C–8F).

To assess the contribution of TNF- α to leukocyte recruitment in response to NTHi products, we administered an inhibitory monoclonal antibody by intraperitoneal injection 24 hours before airway challenge. This inhibited neutrophil influx 24 hours after intratracheal injection of TNF- α by 88.5% (data not shown), indicating effective TNF- α inhibition. However, there was no measureable effect of the antibody on neutrophil influx 24 hours after exposure to the aerosolized NTHi lysate (data not shown), indicating that even though TNF- α is capable of inducing neutrophil recruitment to airways, it is not required for neutrophil recruitment in response to NTHi products.

DISCUSSION

The principal goal of our study was to determine which aspects of the COPD phenotype could be attributed to repetitive or persistent exposure of the intrapulmonary airways of patients to NTHi products. We found that weekly exposure of mice to an aerosolized NTHi lysate induces lung inflammation with a profile

TABLE 2. AIRWAY WALL LEUKOCYTE POPULATIONS AFTER 8 WK OF NTHi EXPOSURE

Cell Type	Marker	Frequency (%)
Macrophage	CD68	32.0 ± 5.3
B cell	CD20	25.3 ± 7.5
T cytolytic	CD8	13.5 ± 6.7
Neutrophil	p40	9.7 ± 1.7
T helper	CD4	2.3 ± 0.3
Total		82.8

Definition of abbreviation: NTHi, nontypeable *Haemophilus influenzae*. Immunohistochemical markers associated with nuclei are recorded as percentage $\pm$ SEM of total nuclei in the airway wall other than those of epithelial and endothelial cells.

Figure 5. Mucin detection in lungs of mice exposed to aerosolized NTHi lysate or ovalbumin. (A–C) Periodic acid fluorescent Schiff's reagent staining for mucin in lungs of (A) control mice exposed to PBS alone, (B) mice exposed weekly for 8 weeks to aerosolized NTHi lysate and killed 1 day after the last exposure, and (C) ovalbumin-sensitized mice exposed to a single ovalbumin aerosol and killed 3 days later. Scale bar = 20 μm. (D) RT-PCR of gel-forming mucins from RNA extracted from lungs and trachea of mice at various times expressed in days after a single exposure to aerosolized NTHi lysate. Controls were intestine for Muc2, stomach for Muc5ac and Muc6, and tongue for Muc5b and Muc19. (E) RT-PCR of airway gel-forming mucins from RNA extracted from lungs of mice exposed weekly for 8 or 25 weeks to aerosolized NTHi lysate, then killed 1 day after the last exposure. Controls are as for D.

of mediators and inflammatory cells similar to that observed in patients with COPD, and it causes airway wall fibrosis but not airway epithelial mucous metaplasia. These experiments isolate the effects of exposure to NTHi products from other environmental stimuli, such as particulates and volatile chemicals in cigarette smoke and air pollution, that also contribute to COPD pathogenesis. Isolating the effects of NTHi exposure in mice should help in defining the relation between NTHi infection and COPD progression in future clinical studies. One limitation of our study is that mice were only exposed to the products of dead bacteria, and these had been grown in laboratory medium under favorable environmental conditions. In contrast, the airways of patients with COPD are also exposed to live bacteria, and microbial growth in a host alters microbial gene expression. Whether these factors might lead to differences in host responses to NTHi products is not known.

Lung Inflammation

The nature of lung inflammation in patients with COPD has been characterized by measurement of cells and mediators in BALF, expectorated sputum, and pathologic specimens. Unlike inflammation in patients with asthma, in which eosinophils, Th2-deviated CD4⁺ lymphocytes, mast cells, and Th2 cytokines predominate, inflammation in patients with COPD is dominated by increases in neutrophils, macrophages, CD8⁺ lymphocytes, and inflammatory cytokines such as TNF- α and IL-6 (9–14). These same inflammatory cell types and mediators predominate in the BALF and lung tissue of mice exposed repetitively to NTHi products by aerosol. Neutrophils are seen more prominently in BALF in our model than in lung tissue; conversely, lymphocytes are less prominent in BALF than in lung tissue (Figures 1 and 4, Table 2). Lymphoid aggregates were seen surrounding airways and blood vessels in our model (Figures 2 and 4), similar to what has been observed in patients with COPD (1) and other inflammatory lung diseases (44, 45). These aggregates suggest that chronic exposure to NTHi evokes an adaptive immune response. In support of this, antibodies against NTHi have been found in the serum of patients with COPD, with the appearance of strain-specific antibodies often following the isolation of a new NTHi strain from the airway (46, 47). Of note, the rise in BALF neutrophils became less with repeated administration (Figure 1). This may be due to cell-autonomous silencing of inflammatory genes by chromatin modification (48), or to suppression of innate immune responses by adaptive immune cells (49).

Airway Fibrosis

Fibrosis of the airway wall is one of the most widespread structural changes in COPD, and it is thought to be a critical determinant of chronic airflow obstruction (5, 6). Our mouse model of repetitive exposure to aerosolized NTHi products leads to progressive airway wall fibrosis that is apparent after 25 weeks (Figure 6A), and to a 5-fold increase in airway wall collagen by 50 weeks (Figure 6C). This finding indicates that persistent or repetitive infection of the intrapulmonary airways of patients with COPD by NTHi could contribute to airway wall fibrosis, so clinical studies examining such an association should be considered. If this association were confirmed, then further studies aimed at preventing the progression of airway wall fibrosis by antimicrobial therapy might be undertaken.

Alveolar Fibrosis

Our mice also showed alveolar wall thickening and collagen deposition (Figures 3 and 6), in contrast to COPD, in which loss of alveolar wall matrix proteins and alveolar wall destruction are more prominent. This might reflect an artifact of the delivery of NTHi products to the alveolar level in our aerosol model, in contrast to the probable confinement of NTHi colonization to conducting airways in patients with COPD (46). Thus, at the alveolar level, our NTHi lysate aerosol might be a better model of hypersensitivity pneumonitis than COPD. On the other hand, alveolar fibrosis coexists with alveolar wall destruction in COPD (50, 51), so the induction of alveolar fibrosis by NTHi products in our model may have some relevance to COPD.

Lack of Mucous Metaplasia due to NTHi Products or Neutrophils

One prominent structural feature of COPD not induced by exposure of mice to NTHi lysate was mucous metaplasia of the surface airway epithelium (Figure 5). This is surprising because airway infection with organisms such as NTHi and *Pseudomonas aeruginosa* is widely believed to induce mucus hypersecretion in airway diseases such as cystic fibrosis and COPD (46, 52). Mucous metaplasia in these settings is thought to be induced both directly by the interaction of NTHi products with airway epithelial cells (28, 53), as well as indirectly by recruited neutrophils (54–56). The promoter for the major inducible gel-forming mucin of the airway epithelium, Muc5ac, is activated *in vitro* by products of NTHi, and signal transduction pathways leading

Figure 6. Histochemical staining for connective tissue in mice repetitively exposed to aerosolized NTHi lysate. Mice were exposed weekly to NTHi lysate for various numbers of weeks, then killed 1 day after the last exposure and their lungs processed for light microscopy with (A) Masson's trichrome staining or (B) Picosirius Red staining. For both A and B, lung tissue is from (a) control mice not exposed to NTHi lysate, (b) mice exposed weekly for 8 weeks to NTHi lysate, (c) mice exposed weekly for 25 weeks to NTHi lysate, (d) mice exposed weekly for 50 weeks to NTHi lysate. (C) The area of airway (a) and alveolar (b) tissue stained by Picosirius Red as a fraction of the area of total lung tissue is plotted for mice exposed weekly for 50 weeks to NTHi lysate compared with unexposed control mice. *Different from control, $P < 0.05$.

to promoter activation have been characterized (28, 57). Nonetheless, neither single nor multiple exposures to the NTHi lysate aerosol resulted in any increase in intracellular epithelial mucin measurable by histochemical staining for glycoconjugates or

RT-PCR for gel-forming mucin transcripts (Figure 5), consistent with similar *in vivo* results with *Staphylococcus aureus* and *P. aeruginosa* (58). It is possible that products of NTHi do not provide a sufficient stimulus to induce mucin production in the presence of tonic mucous cell nonautonomous inhibitory signals *in vivo*, that they simultaneously activate nonautonomous pathways *in vivo* that suppress mucin production, or that the reporter assays used *in vitro* are sensitive to faint stimuli that are insufficient to induce mucin production *in vivo*. Similarly, neutrophils and neutrophil products such as elastase have been found to increase mucin expression in airway epithelial cells and cell lines *in vitro* (59–61), but do not induce polymeric mucin production in our model. Neutrophil elastase did increase airway Muc5ac expression *in vivo* (54), but in that study human neutrophil elastase was instilled into the airways of mice and eosinophil numbers were elevated, suggesting an allergic inflammatory response to a foreign protein. Supporting this, mouse pancreatic elastase instilled into the airways of mice did not induce mucous metaplasia (personal communication, Dr. W. Michael Foster, Duke University).

Minimal Mucous Metaplasia due to TNF- α

TNF- α has also been reported to increase Muc5ac expression *in vitro* (62, 63) and induce mucous metaplasia *in vivo* (64). However, TNF- α levels in our NTHi model were transiently elevated more than 1,000-fold without mucous metaplasia and in our CCSP-TNF- α transgenic model were persistently elevated 165-fold with only slight mucous metaplasia. As above, it is possible that stimulation sufficient to induce detectable mucin promoter activation *in vitro* is insufficient to induce metaplasia *in vivo*, that a contaminating molecule or unrecognized property of the stimulating reagent accounts for the reported gene activation, or that a mucin-suppressive activity is induced in our *in vivo* models. No functional NF- κ B response element has been found in the mouse Muc5ac promoter (38), making direct induction of the Muc5ac gene unlikely. Also epithelial expression of NF- κ B is not required for the induction of Muc5ac expression and goblet cell metaplasia under conditions of allergic inflammation *in vivo*, since goblet cell metaplasia increases in NF- κ B-deficient p50 $^{-/-}$ mice receiving antigen-specific Th2 cells by adoptive transfer and subsequently exposed to antigen (65). However, activation of NF- κ B serves as a co-stimulus that can augment allergic inflammation (66), so TNF- α might promote mucous metaplasia in the context of inflammatory stimuli associated with Muc5ac induction such as allergy, fungal infection, viral infection, or acrolein exposure (67).

Inflammation but No Airway Fibrosis due to TNF- α

TNF- α is prominently elevated in tissues and lung lining fluid of patients with COPD, and it is thought to play a pathogenic role (12, 42). We found that even though TNF- α is capable of recruiting neutrophils into BALF of mice when instilled intratracheally or expressed transgenically in the airway, TNF- α is not required for neutrophil recruitment in response to aerosolized NTHi lysate (Figure 7). Further, while chronic airway overexpression of TNF- α induces peribronchial and peribronchiolar inflammatory cell infiltrates, it does not induce airway wall fibrosis (Figure 8). These findings are consistent with the lack of clinical benefit of TNF- α antagonism in a randomized controlled trial (68), despite an association between TNF- α antagonism and reduced hospitalization for COPD in an observational study of patients with rheumatoid arthritis (69). Similarly, no association between TNF- α polymorphisms and COPD susceptibility or progression has been found in most well-controlled studies (70, 71). In contrast to our transgenic model in which TNF- α was

Figure 7. Structure and inflammatory effects of the CCSP-TNF- α transgene. (A) The 3.7-kb transgene consists of the 709-bp murine TNF- α cDNA (74) cloned into a Clara cell targeting construct containing a 2.1-kb fragment of the mouse Clara cell secretory protein (CCSP) promoter, 640-bp fragment of the rabbit β -globin intron, and 260-bp fragment of the bovine growth hormone polyadenylation signal (75). Restriction sites used to assemble the construct and excise it from the plasmid vector are indicated below the transgene. (B) TNF- α and (C) MMP-9 levels in BALF from 8-week-old CCSP-TNF- α transgenic mice and littermate controls. (D) Inflammatory cell counts in BALF from 8-week-old CCSP-TNF- α transgenic mice and littermate controls. *Different from control, $P < 0.05$.

expressed in the airway under control of the CCSP promoter, transgenic expression of TNF- α in alveoli under control of the SP-C promoter leads to lymphocytic alveolar inflammation and varying degrees of emphysema and alveolar fibrosis depending on whether the transgene is expressed constitutively or conditionally (72, 73).

Conclusions

We established a model of repetitive exposure of mice to an aerosolized NTHi lysate to determine which aspects of the

COPD phenotype might be caused by persistent or repetitive exposure of the airways of patients with COPD to NTHi products. We found inflammation dominated by neutrophils, macrophages, CD8⁺ lymphocytes, and inflammatory cytokines, similar to that seen in patients with COPD. Repetitive exposure also led to airway wall fibrosis, a structural change that is prominent in patients with COPD, but surprisingly did not lead to airway epithelial mucous metaplasia. TNF- α was prominently elevated in the lungs of NTHi-exposed mice but was not required for neutrophil recruitment, and when transgenically expressed was not sufficient to induce airway wall fibrosis. These findings help clarify the possible roles of NTHi infection and elevated TNF- α expression in COPD progression, and can help guide more focused clinical studies in the future.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Figure 8. Lung histopathologic changes in CCSP-TNF- α transgenic mice. (A) H&E staining of the lungs from 25-week-old CCSP-TNF- α transgenic mice revealed marked inflammatory cell infiltration of the airway wall and peribronchovascular lymphoid aggregates compared with age-matched littermate controls. (B) MTC staining and (C) PSR staining of lungs from CCSP-TNF- α transgenic mice did not show a significant increase in airway fibrosis tissue compared to age-matched littermate controls. Scale bar = 100 μ m.

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