

Lung T-cell responses to nontypeable *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease

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Background: Chronic obstructive pulmonary disease (COPD) is characterized by pulmonary inflammation that persists after the cessation of smoking. T cells have a major role in driving inflammation in patients with COPD and are activated by specific antigens to produce mediators, such as cytokines. The antigens that activate lung T cells have not been clearly defined. Nontypeable *Haemophilus influenzae* (NTHi) is the dominant bacterium isolated from the lungs of patients with COPD.

Objective: We sought to measure the response of lung tissue T cells to stimulation with NTHi.

Methods: We obtained lung tissue from 69 subjects having lobectomies for lung cancer. Of the group, 39 subjects had COPD, and 30 without COPD were classified as control subjects. The lung tissue was dispersed into single-cell suspensions and stimulated with live NTHi. Cells were labeled with antibodies for 5 important inflammatory mediators in patients with COPD and analyzed by using flow cytometry.

Results: NTHi produced strong activation of both T_H cells and cytotoxic T cells in the COPD cohort. The COPD cohort had significantly higher levels of cells producing TNF- α , IL-13, and IL-17 in both T-cell subsets. When control subjects were divided into those with and without a significant smoking history and compared with patients with COPD, there was a progressive increase in the numbers of T cells producing cytokines from nonsmoking control subjects to smoking control subjects to patients with COPD.

Conclusion: NTHi activates lung T cells in patients with COPD. This proinflammatory profibrotic response might be a key cause of inflammation in patients with COPD and has implications for treatment. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: T cell, chronic obstructive pulmonary disease, bacteria, inflammation

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide¹ and is predicted to increase further.² Smoking is the most important risk factor. COPD is characterized by bronchial inflammation, and this inflammatory process persists after the cessation of smoking.³ In addition, smoking impairs bacterial clearance and promotes inflammation in this context.⁴⁻⁶

Hogg et al⁷ described the presence of lymphoid follicles (comprised of T lymphocytes and B lymphocytes) in the small airways. These follicles were present long after the cessation of smoking (the relationship between smoking and follicles was not defined) and were a key factor in airflow obstruction. These bronchial wall lymphocytes produce cytokines and mediators, which can in turn drive the ongoing inflammatory process in patients with COPD.

A key feature of lymphocytes is their specificity for antigen. Each T cell has a surface receptor, which only recognizes 1 type of antigen. Therefore there are a vast number of different types of T cells, which can recognize all the different potential antigens that can be encountered. When a T cell encounters its cognate antigen, it proliferates into an effector cell with the production of mediators, such as cytokines, resulting in an immune response to an antigen (eg, an infectious microorganism). It has been recently recognized that lymphocytes in the lungs of patients with COPD are oligoclonal,^{8,9} and there are probably only a very limited number of antigens in the lung that are responsible for stimulating these cells. The antigens that activate the lymphocytes in patients with COPD have not been defined, although possibilities include (1) autoantigens, (2) embedded chemicals from smoking, and (3) bacterial infection.^{10,11}

Nontypeable *Haemophilus influenzae* (NTHi) is the most common cause of chronic bacterial airway colonization in patients with COPD and also the major bacterial cause of exacerbations (25% to >80% of bacterial exacerbations).¹²⁻¹⁶ In addition, the bacterial load of NTHi in lung airways has been shown to contribute to airway inflammation in patients with stable chronic bronchitis.¹⁷ Colonization with NTHi is associated with more severe exacerbations of COPD.¹⁸ NTHi also has the ability to invade into the lung parenchyma.^{19,20} Our hypothesis is that NTHi is an important antigen activating T lymphocytes to produce inflammatory mediators in patients with COPD.

To test this hypothesis, we obtained lung tissue from subjects undergoing lobectomies for lung cancer. Subjects were divided into those with and without COPD. Lung tissue was converted into single-cell suspensions, stimulated with NTHi, and

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Abbreviations used

APC: Antigen-presenting cell
 COPD: Chronic obstructive pulmonary disease
 CTL: Cytotoxic T lymphocyte
 NTHi: Nontypeable *Haemophilus influenzae*
 SEB: Staphylococcal enterotoxin B

compared with unstimulated samples (as a negative control sample). T-cell responses were assessed with flow cytometry by measuring the production of 5 key inflammatory mediators.

METHODS**Study subjects**

We recruited adults with COPD who were undergoing lobectomies for the treatment of lung cancer at Monash Medical Centre/Southern Health or Cabrini Hospital, Melbourne, Australia. Subjects had COPD defined by using standard criteria (FEV₁/forced vital capacity ratio <0.7 and not fully reversible).²¹ Control subjects did not have airflow obstruction on lung function testing. The study was approved by the Monash Medical Centre/Southern Health and Cabrini Hospital research ethics committee, and all subjects provided written consent.

Clinical measurements

Subjects were interviewed before surgery, and information was obtained about clinical symptoms of lung disease, comorbidities, and medication use. Lung function testing was performed by using standard criteria, and the best postbronchodilator effort of 3 attempts varying by less than 150 mL was recorded for lung function.²¹

Bacteria

A characterized strain of live NTHi was used to stimulate cells.²² The bacterium was grown on chocolate agar and then suspended in broth overnight before being used to stimulate lung tissue the next day.

Lung specimens

Lung tissue was obtained from subjects undergoing a lobectomy at Monash Medical Centre/Southern Health or Cabrini Hospital for the treatment of lung cancer. The obtained lobe was sent fresh to the pathology department, and a section of approximately 2 to 3 cm³ was removed as far from the tumor as possible. The lung tissue was dispersed mechanically into a single-cell suspension in culture medium.

Lymphocyte stimulation assay

Previously described methods were used.^{22,23} Briefly, the lung cell suspension was separated into 3 samples: (1) an unstimulated sample as a negative control, (2) a staphylococcal enterotoxin B (SEB) superantigen-stimulated sample as a positive control, and (3) an NTHi-stimulated sample (to which NTHi was added to cells at a multiplicity of infection of 100:1). Tubes were then incubated with rotation (to facilitate antigen presentation) in a 5% CO₂ incubator at 37°C for 1 hour. Brefeldin A was added after 1 hour, and cells were then incubated overnight to block the Golgi-mediated export of cytokines. The next day, cells were washed and permeabilized before labeling antibodies for surface markers (CD45, CD3, CD4, and CD8) and cytokines/mediators (IL-13, IL-17, TNF- α , IFN- γ , and CD154) were added. Cells were incubated before being washed and analyzed by using flow cytometry. Surface markers were used to distinguish T_H (CD4) cells from cytotoxic T lymphocytes (CTLs; CD8 cells), and the production of the 5 inflammatory mediators was measured for each T-cell subset. Results were expressed as the total percentage of each T-cell subset producing 1 inflammatory marker minus

the percentage of unstimulated cells (negative control) producing 1 inflammatory marker. The gating strategy is shown in Fig E1 in this article's Online Repository at www.jacionline.org.

Bead array

We used a bead array to measure the levels of multiple mediators in supernatants. The supernatants were obtained from the last 40 consecutive patient samples by using both control and NTHi-stimulated specimens. Levels of T-cell cytokines (TNF- α , IL-2, IL-9, IL-10, IL-13, IL-17, and IFN- γ) and innate immune mediators (IL-1 β , IL-6, and IL-12) were measured.

Statistics

Baseline characteristics of the subjects were listed by using descriptive statistics (means and SEs). The T-cell responses that were nonparametrically distributed were expressed as median values with interquartile ranges. The Mann-Whitney test was used for comparisons between pairs of groups. ANOVA was used to assess differences for between-group comparisons, as appropriate. All analyses were 2-sided, and *P* values of less than .05 were considered significant.

Further details about the **Methods** used in this study are available in the **Methods** section in this article's Online Repository at www.jacionline.org.

RESULTS**Characteristics of subjects**

Sixty-nine subjects had specimens obtained that were analyzed for their T-cell responses to stimulation with NTHi. Patients' characteristics are listed in Table I (and see Table E1 in this article's Online Repository at www.jacionline.org).

T-cell response to SEB

Subjects in the control and COPD groups demonstrated clear T-cell mediator/cytokine production to stimulation with SEB, thus demonstrating the validity of our method to measure lymphocyte responses. There were no significant differences in the responses between the 2 groups. Therefore the ability of T cells to respond to maximal stimulation in both groups was similar. The strongest response was in the production of TNF- α and IFN- γ . Median levels of IL-13, IL-17, and CD154 were approximately 1 to 2 logs lower than TNF- α and IFN- γ levels. Results are demonstrated in Fig 1, and examples of the gating strategy used are shown in Figs E1-E4 in this article's Online Repository at www.jacionline.org.

T_H cell response to NTHi

Results were expressed as the percentage of T_H (CD4) cells producing a specific mediator/cytokine after stimulation. NTHi induced very marked T_H cell responses in a number of subjects, with up to 60% of stimulated cells producing 1 cytokine. Overall, the percentages of responding T_H cells were higher in the COPD group (although baseline levels of cytokine production were not different between the 2 groups).

The most clear-cut difference between control subjects and patients with COPD was in the production of TNF- α , with median percentage levels being 2 logs higher in the COPD group (*P* < .001). The percentages of IL-13⁺ cells were close to 2 logs higher in the COPD group (*P* = .02), and the percentages of IL-17⁺ cells were more than 5 times higher (*P* = .03). There were a number of subjects in both the control and COPD groups who had very

TABLE I. Baseline characteristics of the subjects

	Control subjects		Patients with COPD (n = 39)
	Nonsmokers (n = 12)	Smokers (n = 18)	
Sex (no.)			
Male/female	4/8	12/6	24/15
Age (y)	73 ± 4.9	78 ± 1.3	61 ± 1.4
Symptoms			
Chronic bronchitis (patients)	1	6	13
Exacerbations (per year)	0.27	0.45	0.72
Smoking history (pack years)	NA	35 ± 4.6	37 ± 4.7
FEV ₁ (% predicted)	108 ± 10.7	101 ± 3.5	78 ± 2.7
FVC (% predicted)	106 ± 8.7	106 ± 4.1	98 ± 3.3
FEV ₁ /FVC ratio	81 ± 1.5	77 ± 1.5	61 ± 1.4

Values are presented as means ± SEs, where shown. Chronic bronchitis is defined as the presence of chronic productive cough for more than 6 months, and exacerbations represent the number per year.

FVC, Forced vital capacity; NA, not applicable.

strong IFN- γ responses, but overall responses were similar. The weakest response was in the production of CD154, although the COPD group had a significantly higher percentage of CD154⁺ cells than the control group ($P = .04$). Results are shown in Fig 2 (and see Table E2 in this article's Online Repository at www.jacionline.org).

Similar to our previous results in peripheral blood, we found that patients with COPD had a T_H2 response to NTHi, with the percentage of IL-13⁺ cells being significantly higher than the percentage of IFN- γ ⁺ cells ($P = .01$). This result is shown in Fig 3.

Cytotoxic T-cell response to NTHi

Cytotoxic T-cell (CTL/CD8 cell) responses to NTHi were very similar to the T_H cell responses. A number of subjects had very marked cytokine production on stimulation, with overall percentages of responding cells being higher in the COPD group (although baseline levels of cytokine production were not different between the 2 groups).

The median percentage of CTL cells producing TNF- α was 2 logs higher in the COPD group compared with the control group ($P = .002$). Levels of IL-13 and IL-17 were 1 to 2 logs higher in the COPD group ($P = .03$ and $P = .01$, respectively). As with the T_H cell responses, a number of subjects had a strong IFN- γ response, but there was no significant difference between the 2 groups. A relatively low number of CTLs produced CD154 after stimulation with NTHi in both groups. Results are shown in Fig 2 (and see Table E2).

We also used a bead array to measure T-cell cytokine levels in the supernatants. The results obtained were similar to those from the flow cytometer, with TNF- α , IL-13, and IL-17 levels being 1 to 2 logs higher in the patients with COPD, whereas IFN- γ levels were comparable in both the control and COPD groups (see Fig E5 in this article's Online Repository at www.jacionline.org).

T-cell response of subgroups to NTHi

The control group was separated into those who had a significant smoking history (defined as >10 pack years) and those who did not (defined as <2 pack years) to assess the effect of smoking. These 2 groups were compared with the COPD group, and differences in the 3 groups were assessed.

The T_H cell responses demonstrated that there was a progressive increase in the percentage of cells producing cytokines from nonsmoking control subjects to smoking control subjects to smokers with COPD for 3 of the 5 mediators/cytokines. ANOVA demonstrated significant differences in the medians of the 3 groups in the production of TNF- α ($P = .002$), IL-13 ($P = .004$), and IL-17 ($P = .03$). Median levels of IFN- γ were highest in the smoking control subjects, but there were no significant differences. There were no clear differences in the production of CD154 among the 3 groups. Most subjects who were nonsmoking control subjects had no measurable cytokine/mediator responses. Results are shown in Fig 4 (and see Table E3 in this article's Online Repository at www.jacionline.org).

The cytotoxic T-cell responses were similar to the T_H cell responses. There were significant differences in the median percentages of cells producing TNF- α ($P < .001$), IL-13 ($P = .01$), and IL-17 ($P = .004$) among the 3 groups. The percentages were lowest in the nonsmoking control subjects and highest among patients with COPD. Both smoking control subjects and patients with COPD had similar levels of cells producing IFN- γ .

Effect of other mediators on T-cell responses

We assessed 3 key mediators produced by innate immune cells (eg, activated macrophages) that influence T-cell function. IL-1 β , IL-6, and IL-12 levels were similar in both control subjects and patients with COPD (see Fig E6 in this article's Online Repository at www.jacionline.org). In addition, we also measured levels of the T-cell cytokine IL-10 and found them to be similar in both groups (see Fig E7 in this article's Online Repository at www.jacionline.org). The ratio of T cells to other white cells (CD45⁺, CD3⁺) was similar in the 3 subject groups (see Fig E8 in this article's Online Repository at www.jacionline.org). Finally, we assessed levels of IL-2 and IL-9, which have a role in the development of T-cell responses, and found no differences between the control subjects and patients with COPD (see Fig E9 in this article's Online Repository at www.jacionline.org).

DISCUSSION

This study shows that NTHi is an important pathogen that activates lung T cells in patients with COPD. This activation led to the production of cytokines previously been shown to be important in the pathogenesis of COPD.

Airway inflammation persists in patients with COPD despite the cessation of smoking. Hogg et al⁷ described the presence of large numbers of lymphocytes in the small airways, and these cells are thought to have a key role in driving the inflammatory process.²⁴ Sullivan et al⁸ demonstrated that lung T cells are oligoclonal, and there has been great interest in the potential antigens that might activate the lymphocytes. A recent study showed there was an enhanced T-cell response to elastin in patients with emphysema,²⁵ but we are aware of no reports describing a specific response of lung T cells in patients with COPD.

T cells are primarily activated when they recognize their specific antigen presented on the surface of an antigen-presenting cell (APC) in association with MHC. In association with a costimulatory signal, this results in activation and proliferation of the cell and production of inflammatory mediators. We have a longstanding interest in studying T-cell responses to NTHi in patients with obstructive airways disease.^{22,23} This work has

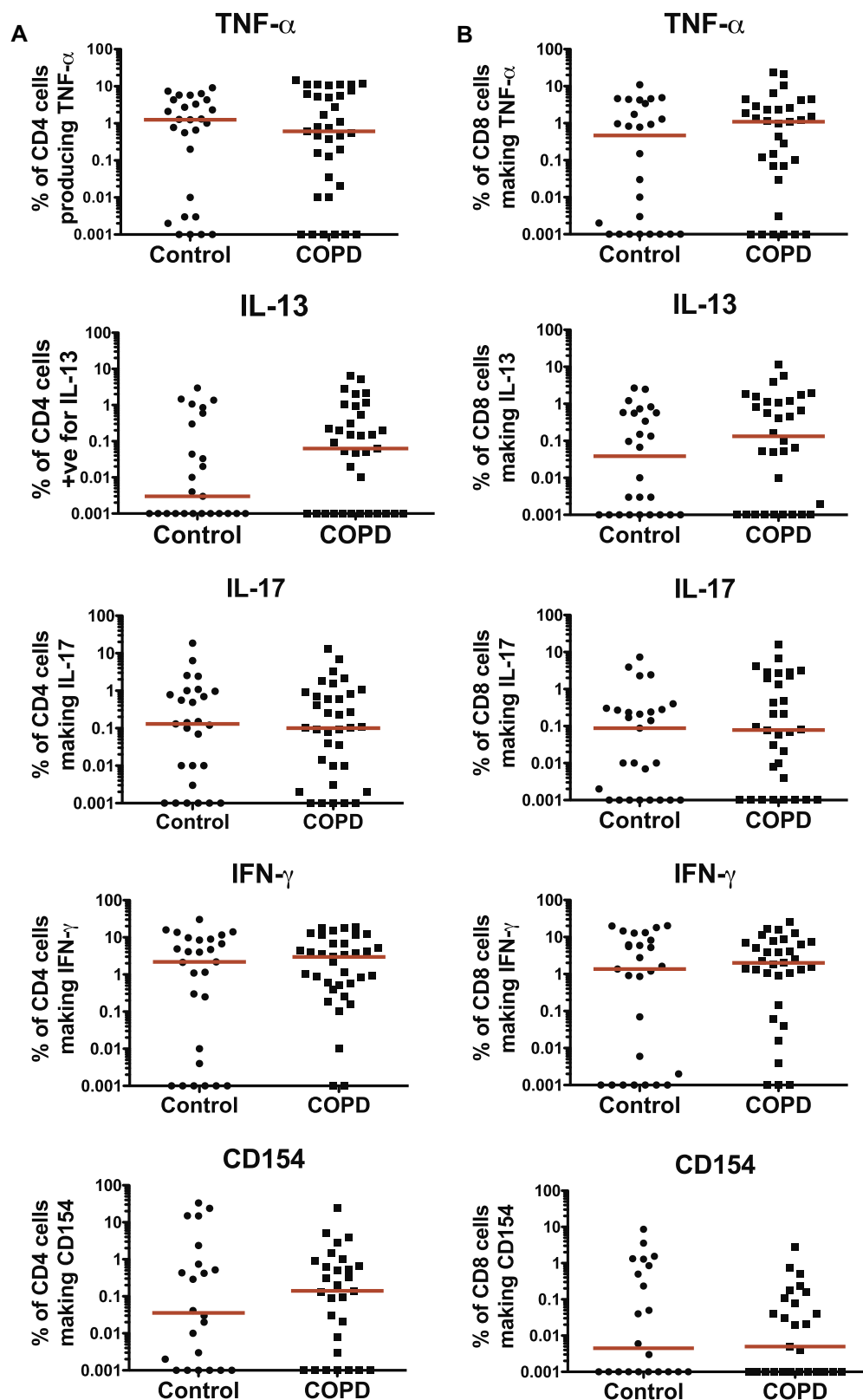


FIG 1. Percentage of T cells expressing the 5 inflammatory mediators after stimulation with SEB in the non-COPD group (control) compared with the COPD group. **A**, T_H cell (CD4) response. **B**, CTL (CD8) response. There was no significant difference in any mediator production between the control and COPD groups.

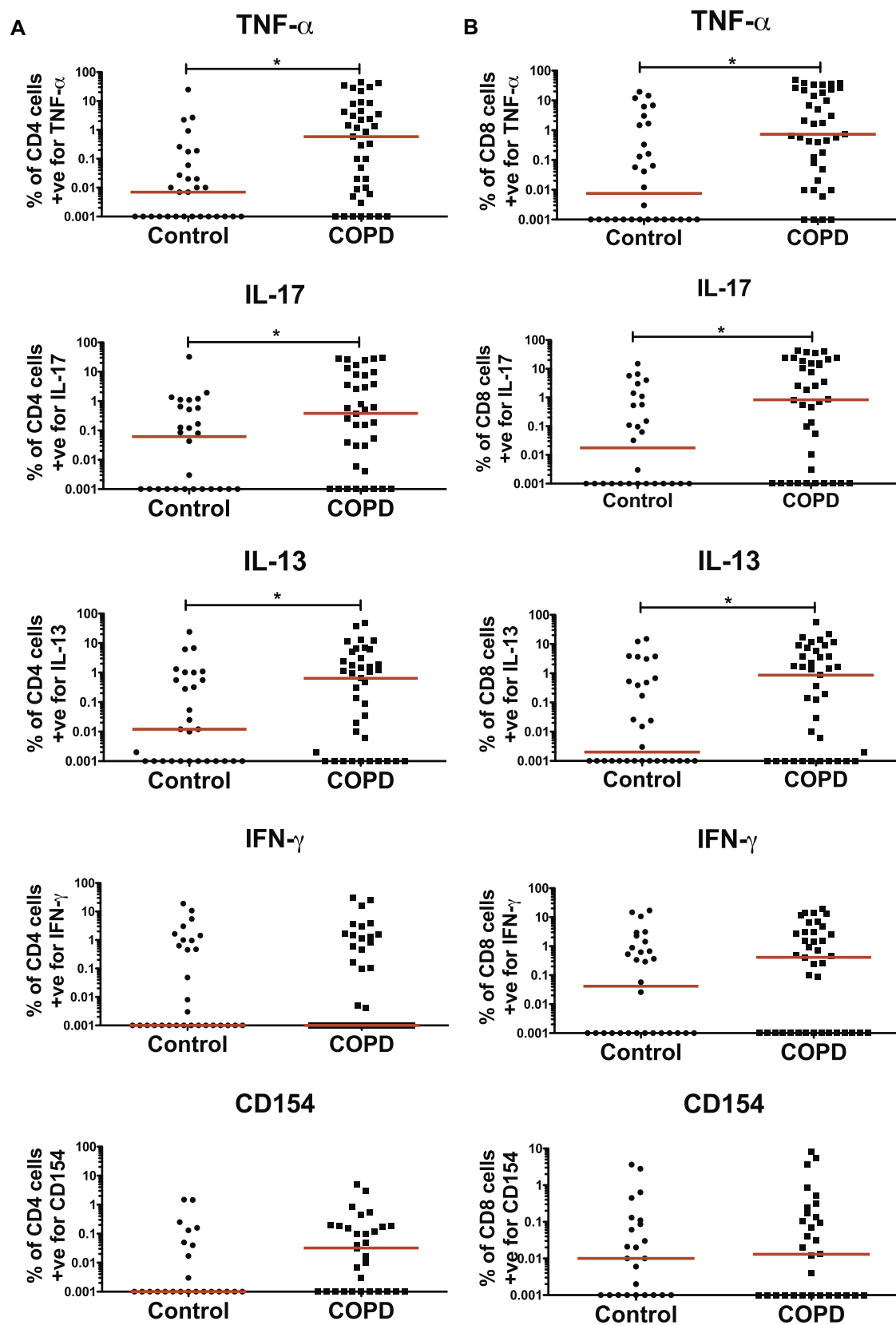


FIG 2. T-cell response to NTHi in the non-COPD group (control) compared with the COPD group. The T_H cell (CD4) response to NTHi stimulation (**A**) and the CTL cell (CD8) response to NTHi stimulation (**B**) in the control and COPD groups by production of 5 inflammatory mediators is shown. The percentage of cells producing TNF- α , IL-13, and IL-17 were significantly higher in the COPD group.

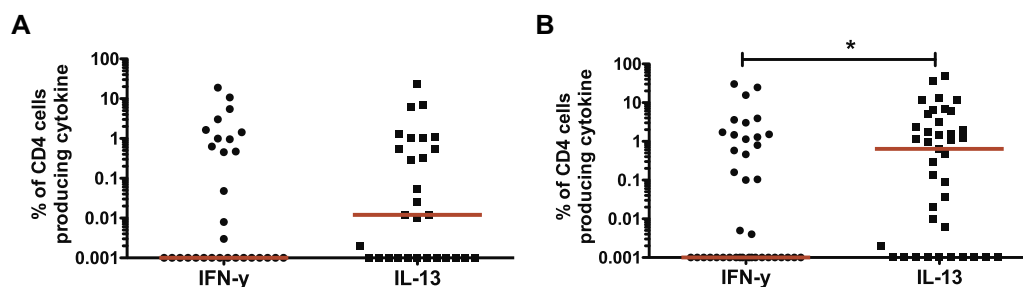


FIG 3. The ratio of the percentage of T_H cells producing IFN- γ compared with the percentage of T_H cells producing IL-13 after stimulation with NTHi in the control (A) and COPD (B) groups is shown. There was no significant difference in control subjects, but IL-13 levels were significantly higher than IFN- γ levels in patients with COPD, which is consistent with a T_H2 response to NTHi.

mainly been done in peripheral blood, in which lymphocytes were activated by NTHi in the presence of APCs. We modified these methods to assess the lung tissue response to NTHi. We initially tried using bronchoscopic samples but found the number of lymphocytes was too low to assess responses, and hence we then studied cells obtained from surgical lung tissue (numbers of lymphocytes were about 2 logs higher in surgical lung tissue compared with bronchoscopic specimens). Using the same methods and bacteria, we found the T-cell responses to NTHi were 1 to 3 logs higher in the lung when compared with our previous results using blood. A number of subjects had very substantial lymphocyte responses, with up to 60% of cells producing 1 cytokine.

The choice of T-cell mediators that we studied was based on previous literature demonstrating their importance in COPD. The cytokine with levels that were most clearly increased in our study was TNF- α . There have been more than 200 articles published on TNF and the pathogenesis of COPD in PubMed.²⁶ TNF- α has a wide variety of effects promoting inflammation and endothelial (particularly microvascular) activation.²⁷

IL-13 levels are increased in lung tissue obtained from patients with COPD²⁶ and thought to be involved in the development of emphysema in a mouse model.²⁸ IL-13 has 3 particular actions that are of relevance to lung disease: (1) it has a potent effect on inhibiting the bactericidal function of macrophages,²⁹ (2) it is an important cause of tissue fibrosis both in liver and lung disease,²⁹ and (3) it has been shown to mediate T_H2 responses and airway hyperreactivity/remodeling.^{30,31} This effect on enhancing airway reactivity has important implications for obstructive airways, particularly the role of bronchodilators. Despite the fact that COPD is classified as not being fully reversible, bronchodilators form the cornerstone of pharmacologic therapy. In our cohort 2 patients had a preoperative clinician's diagnosis of asthma, and 3 of the patients with COPD and 2 of the control subjects had a greater than 12% bronchodilator effect to inhaled β -agonist.

IL-17 was the other cytokine with levels found to be significantly increased in the COPD group (when compared with those in control subjects). This cytokine appears to have a role in the early protection against infection in which it promotes neutrophil function. A role in autoimmunity has also been recognized.³² In the context of COPD, IL-17-secreting cells have been demonstrated to be present in the lung, although their function in this context has still not been well defined.³³

The other 2 inflammatory mediators measured were IFN- γ and CD154. IFN- γ has a primary function in activating the immune response to clear infection, particularly by enhancing macrophage bactericidal function.²⁷ There is a large body of

literature demonstrating the presence of IFN- γ -producing pulmonary T cells in patients with COPD, and also, it can induce emphysema in a mouse model.³⁴⁻³⁶ We found marked T-cell production of IFN- γ to NTHi in a proportion of control subjects who had a significant smoking history and a proportion of patients with COPD, and levels were similar between the groups. The action of IFN- γ is directly opposed by IL-13. In the context of liver fibrosis, it has been suggested that the production of IL-13 might be a default position to a persistent pathogen to downregulate a harmful inflammatory response.²⁹ The lowest level of mediator production was for CD154 (also known as CD40 ligand). CD154 has a key role in activating macrophage killing of bacteria,²⁷ but we found that levels were similar to our previous results in peripheral blood,²³ suggesting that CD154 is not an important mediator in the lung T-cell response to NTHi. The T-cell inflammatory cytokine response is significantly influenced by innate inflammatory mediators, such as the ones we measured (IL-1 β , IL-6, and IL-12), as well as by anti-inflammatory cytokines, such as IL-10. In the current study we were unable to demonstrate any clear effect, but this would be an important area to investigate further. In addition, further defining the exact antigens of NTHi that stimulate lung T-cell responses would also be important.

NTHi colonizes the pharyngeal mucosa of most healthy adults.³⁷ It is a major cause of respiratory tract infection, particularly bronchitis.³⁷ In patients with COPD, NTHi is the dominant pathogen both in terms of exacerbations and colonization of the airways.¹²⁻¹⁵ Recent more sensitive molecular techniques have highlighted the prevalence of bacteria in the lower airways, particularly *Haemophilus* species.³⁸ It also has the ability to invade and live intracellularly in macrophages and epithelial cells.^{39,40} Moller et al¹⁹ demonstrated extensive invasion of lung explants from patients with COPD (and other forms of end-stage lung disease) with *H influenzae*; the organism was present in the epithelium, the submucosa of the bronchi, the bronchioles, the interstitium, and the alveolar epithelium. Dromann et al²⁰ have demonstrated the presence of *H influenzae* in lung tissue in 40% of subjects at all stages of COPD. Finally, smoking leads to permanent damage to the respiratory epithelium¹⁰ and is linked to *H influenzae* infection. This can occur through (1) temporary spread of NTHi into the airway wall from microaspiration of pharyngeal bacteria or from colonization of the airway or (2) permanent NTHi infection in the airway wall/lung parenchyma.

In this study we have demonstrated that NTHi activates lung tissue T cells. Specifically, both lung T_H cells and cytotoxic T cells from patients with COPD respond strongly to stimulation with NTHi, with a distinctive profile of cytokine production that

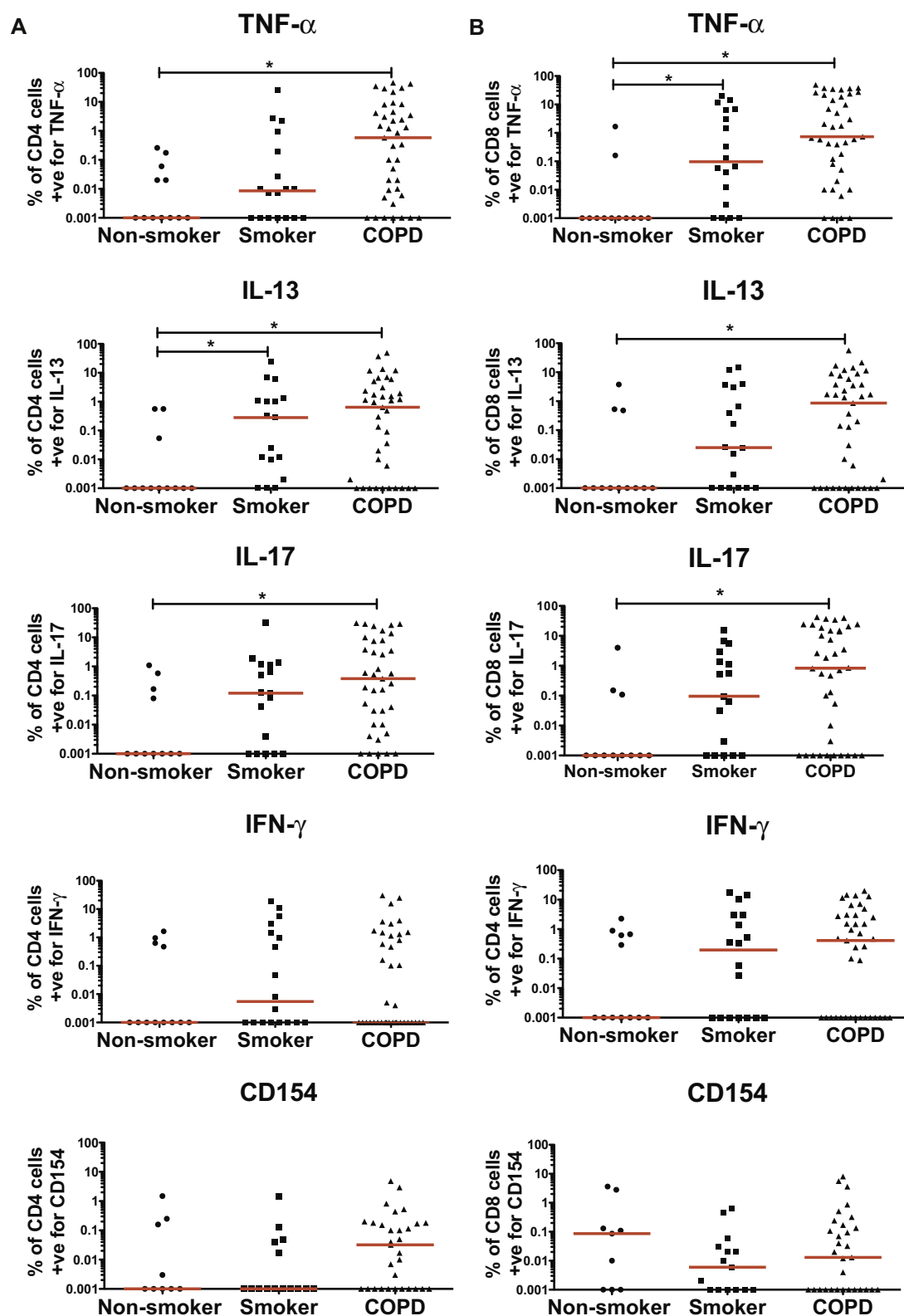


FIG 4. Response of subgroups to NTHi. To measure the effect of smoking, the control group was divided into subgroups of those without a significant smoking history (nonsmoker) and those with a significant smoking history (smoker) and compared with the COPD group. There was a progressive increase in cytokine production for TNF- α , IL-13, and IL-17 in T_H cells (**A**) and CTLs (**B**). Significant differences were mainly between nonsmoking control subjects and patients with COPD.

promotes inflammation and fibrosis while inhibiting bacterial clearance. Thus not only is NTHi very prevalent in patients with COPD, it also causes activation of lung T cells. This observed T-cell response to NTHi might be a key cause of inflammation in patients with COPD and has implications for treatment. For example, the use of long-term azithromycin has recently been shown to be effective in reducing exacerbations of COPD⁴¹; it is possible that this might be partially mediated by reducing the airway prevalence of NTHi and hence lymphocyte-mediated inflammation to this bacterium. There also might be an important role for immune mediators and particularly the development of a vaccine to NTHi in the management of COPD.

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Clinical implications: NTHi stimulates inflammation in patients with COPD. These findings implicate the potential role of antibiotics, immune mediators, and vaccination against NTHi in this condition.

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METHODS

Patients' details

With regard to smoking status, we classified a significant smoking history as being greater than 10 pack years, and this criterion has been widely used previously. The nonsmokers were classified as having a less than 2 pack years history; this classification was arbitrary but reflected that this group had a low level of smoking exposure. The COPD group was more than 10 years younger than the control group; the reason for this was not clear. Age and cytokine responses were correlated by using the Spearman rank coefficient, and there were no significant associations. The nonsmoking control group had a higher proportion of female subjects than the smoking groups. As listed in Table I, most subjects in the COPD group had mild-to-moderate disease with a mean FEV₁ of 78% $\pm$ 2.7% of predicted value. Twenty subjects were in Global Initiative for Chronic Obstructive Lung Disease (GOLD) class 1, and 19 were in GOLD class 2 (there were no subjects who were in GOLD class 3 or 4).

All subjects were living independently, and no subject reported smoking in the month leading up to surgery. The subjects in the COPD group had a much higher use of inhaled corticosteroids (48% vs 23%); all of these subjects had been taking a stable dose of this medication for at least 3 months. There was no significant association between steroid use and T-cell responses. There was a high use of statins in both groups (control group, 27%; COPD group, 23%). The major comorbidities were ischemic heart disease, hypertension, and diabetes. There was a low incidence of comorbid respiratory disease: 1 control subject had nonactive pulmonary fibrosis in the context of rheumatoid arthritis. Four of the subjects in the COPD group were nonsmokers (but did not have a history of asthma). One of the nonsmoking control subjects, an 84-year-old man, had very high lung volumes (eg, FEV₁ was 213% of predicted value), which contributed to the larger SE of lung function in the nonsmoking group. One control subject was taking 5 mg/d prednisone for a low-grade pituitary tumor, and 1 control subject was taking 5 mg/wk methotrexate for rheumatoid arthritis.

Final pathology reports were obtained from the lobectomy specimens. These details are listed in Table E1. The lung cancer type and stage were broadly similar between nonsmoking control subjects, smoking control subjects, and patients with COPD.

Characterization of strains of NTHi

A strain of NTHi from a patient with chronic bronchitis/COPD was used. This strain, which has been designated in a previous study as MU/MMC1,^{E1} had been characterized initially at the Microbiological Diagnostic Unit of the Department of Microbiology and Immunology, University of Melbourne. MU/MMC1 was initially confirmed as *Haemophilus influenzae* by using gram, oxidase, and X and V requirements (the strain was a small gram-negative rod that was oxidase positive and required both X and V factors). Serotyping was performed by using the Phadebact coagglutination test (Bactus AB, Huddinge, Sweden) and standard slide agglutination with Difco (Becton Dickinson, Franklin Lakes, NJ; polyvalent; the strain was found to be nontypeable). PCR was performed on a strain for the *ompP2* gene to confirm the isolate was *H influenzae*.

MU/MMC 1 also was subsequently sequenced. Molecular identification was performed with the MicroSEQ500 16S rDNA Bacterial Identification kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif), as per the manufacturer's instructions. Sequencing was performed on the ABI PRISM 3100 Genetic Analyser by using the BigDye terminator cycle sequencing kit (version 1.0; Perkin-Elmer, Applied Biosystems). Sequence analysis was performed with 489 nucleotides using MicroSEQ 500 software (version 2.1). The isolate had 100% sequence homology with *H influenzae* (American Type Culture Collection 10211). The sequences were also analyzed with BLAST version 2.0 software (<http://www.ncbi.nlm.gov/BLAST>). The sequence analysis revealed a 99% identity with sequences corresponding to *H influenzae* (American Type Culture Collection 9795).

The same procedures were also done for 3 other previously described strains, MU/MMC 2 to 4,^{E1} including sequencing, and all these strains were confirmed to be NTHi.

Preparation of NTHi for experimental assays

For all experiments, the MU/MMC1 strain was used. This strain was chosen because it was well characterized from an appropriate clinical subject and had been shown previously to induce clear T-cell cytokine responses in peripheral blood.^{E1,E2} In our initial studies of T_H cell responses to NTHi, we used a pooled heat-killed NTHi antigen solution.^{E2} When we investigated the response of cytotoxic T cells to this antigen, we found there was minimal response, and it was only when we used live NTHi that we had a clear response.^{E1} The use of live NTHi also resulted in a log increase in T_H cell responses when compared with the killed NTHi. The strain of live NTHi we used has been previously compared with 3 other strains of NTHi (MU/MMC2-4), and comparable cytokine responses were obtained with all 4 specimens,^{E1} confirming that MU/MMC1 was an appropriate strain to use to stimulate the lung T cells. We have also found previously that blocking MHC-I and MHC-II markedly reduces T-cell responses consistent with a T-cell receptor-restricted response.

The maintenance and culture of *H influenzae* was performed according to previously described methods.^{E3,E4} Frozen stocks of NTHi were prepared in Brain Heart Infusion broth (Oxoid, Hampshire, United Kingdom) containing 15% glycerol and stored at -80°C . Bacteria were routinely cultured overnight at 37°C in a 5% CO₂ atmosphere on chocolate agar plates. Before use in infection assays, NTHi from overnight plates was used to inoculate Brain Heart Infusion broth supplemented with both hemin (MP Biomedicals, Santa Ana, Calif) and β -nicotinamide adenine dinucleotide (Sigma-Aldrich, St Louis, Mo) to a final concentration of 10 $\mu\text{g/mL}$ and cultured overnight at 37°C in a 5% CO₂ atmosphere. Bacterial cell density was determined by using spectrophotometric analysis at a wavelength of 600 nm, and the OD was used to determine the bacterial concentration on a growth curve.

Surgery and lung tissue specimens

Lobectomies were performed by the thoracic surgeon for the treatment of lung cancer. When the lobes were resected, the tissue bank service was contacted, and specimens were taken fresh to the Pathology Department at either Monash Medical Centre or Cabrini Hospital.

The pathologist resected a piece of lung tissue at least 2 to 3 cm³ in size. This tissue was taken as far from the primary tumor as possible, with at least a 5-cm margin. Tissue was also not taken from the extreme periphery of the lung. Resected tissue was placed in culture medium and taken fresh to the laboratories of the Department of Medicine, Monash Medical Centre.

Preparation of the lung tissue

We initially digested the tissue using collagenase, but we found that we had poor staining for CD4 and CD8. Therefore we dispersed the tissue mechanically with a Medimachine (Dako, Glostrup, Denmark) and subsequently recorded clear staining for CD4 and CD8 (although there was a moderate decrease in the numbers of cells obtained from the tissue compared with collagenase).

Rather than using isolated mononuclear cells, we used all the cells isolated from the lung tissue. We believed that cells such as fibroblasts and epithelial cells were likely to have a role in antigen presentation and that using all the lung cells most closely reflected the lung environment *in vivo*. The activation of T cells predominantly occurs through presentation of antigen on the surface of APCs in association with MHC.^{E5} When we have previously blocked the MHC (class I and II), we have had at least a 70% reduction in both T_H cell and CTL responses to NTHi stimulation.^{E1,E2} We have also previously found in peripheral blood samples that if we remove the APCs, there is minimal T-cell cytokine production in response to NTHi stimulation (unpublished data).

In some subjects we did not have enough tissue to test for SEB responses, and there were long delays in the arrival of SEB and CD154 antibodies (eg, it took 4 months for replacement SEB to come in because of strict customs requirements), and therefore there were 7 subjects who were not tested for SEB responses and 17 subjects who were not tested for CD154 responses. These subjects who were not tested for these parameters did not affect the overall outcomes of the study.

Fresh lung tissue was resuspended in RPMI-1640 medium supplemented with L-glutamine (2 mmol/L) before lung-derived cells were obtained by

means of mechanical disaggregation. Working aseptically and according to the manufacturer's instructions, tissue was cut into 3- to 5-mm³ sections and placed inside a sterile 50-μm Medicon chamber (BD Biosciences, San Jose, Calif) before being mechanically fragmented in a Medimachine. After tissue disaggregation, red blood cells were lysed with 0.8% ammonium chloride (150 mmol/L NH₄Cl, 1 mmol/L NaHCO₃, and 0.1 mmol/L EDTA). Cells were resuspended by using sterile RPMI before being filtered through a 100-μm sterile nylon mesh (Falcon, BD Biosciences). A viable cell count was performed by using the trypan blue exclusion method, as previously described.^{E6}

NTHi infection assay

Lung cells were resuspended to a final cell concentration of 4×10^6 cells/mL in 3 tubes. One milliliter of suspension was used for the control tube, one milliliter was used for the superantigen SEB tube, and 2 mL was used for the NTHi tube. Cells were infected at a multiplicity of infection of 100 bacteria per cell to the NTHi tube. SEB was added at a concentration of 10 μg/mL to the SEB tube. Cells were placed in a MACSmix tube rotator (Miltenyi Biotec, Bergisch Gladbach, Germany) and left inside a 37°C incubator, gently rotating at 12 rpm. One hour after stimulation, brefeldin A was added to the cell suspensions to a final concentration of 10 μg/mL. The cell suspensions were returned to the incubator for a further 16 to 22 hours on rotation.

Intracellular cytokine staining

The following antibodies were used: CD45-PC7, CD3-PC5.5, CD8-Pacific Blue, CD4-allophycocyanin (all from Beckman Coulter, Fullerton, Calif), CD154-allophycocyanin-eFluor 780 (eBioscience, San Diego, Calif), and IFN-γ-phycoerythrin, TNF-α-AF488, IL-13-phycoerythrin, and IL-17-AF488 (all from BD Biosciences). A Cytofix/Cytoperm kit (BD Biosciences) was used for the fixation, permeabilization, staining, and washing of cells. The cell suspension was washed with PBS containing 1% BSA and 0.01% NaN₃ before cells were fixed and permeabilized. The cell suspension was subsequently divided into 2 aliquots and stained for the specific human lymphocyte cell-surface markers CD45, CD3, CD4, CD8, and CD154 and a combination of the intracellular cytokines IFN-γ and TNF-α or IL-13 and IL-17A. Cells were washed and resuspended in PBS before acquisition on a flow cytometer. Acquisition of samples was performed on a BD FACSCanto II flow cytometer (Becton Dickinson). Data analysis was performed with FlowJo software (version 7.6; TreeStar, Ashland, Ore).

We also compared the ratio of T cells (CD45⁺CD3⁺) with the population of white cells that were CD45⁺ but CD3⁻ (these CD45⁺CD3⁻ cells would comprise most of the classical APCs) among the 3 groups (nonsmoking control subjects, smoking control subjects, and patients with COPD). This result is shown in Fig E8.

Bead array assay

We also saved the supernatants from control and NTHi-stimulated samples for the last 40 consecutive subjects (18 control subjects and 22

patients with COPD). These specimens were used for bead array analysis for (1) T-cell cytokines (TNF-α, IL-13, IL-17, and IFN-γ), (2) innate mediators that affect T-cell function (IL-1β, IL-6, and IL-12), (3) the T-cell anti-inflammatory cytokine IL-10, and (4) IL-2, which is the key cytokine produced by T cells to activate proliferation (and helps drive T_H1 responses), and IL-9, which drives T_H2 responses (and has an important role in asthma).

Cells (5×10^5) were used to seed wells of a 24-well cell-culture plate, with the volume of media adjusted to 500 μL. Cells were then stimulated with NTHi (multiplicity of infection, 100) or without NTHi (control) overnight in a humidified 37°C incubator. After stimulation, the supernatant was collected and frozen at -20°C until ready for analysis of cytokine production by using the FlowCytomix Human kit (Bender Med Systems, Burlingame, Calif). Assaying of the stored supernatant for cytokine production proceeded according to the manufacturer's instructions, with acquisition of bead array data being performed on a BD FACSCanto II flow cytometer (Becton Dickinson). Calculation of results was conducted with BMS FlowCytomix Pro Software (version 2.4), and subsequent data analysis was performed with Graphpad Prism software (GraphPad Software, La Jolla, Calif). Expressed results were for stimulated cells minus control cells.

Statistical analysis

All analyses were 2-tailed. ANOVA has been used to assess whether there was a difference in the medians between nonsmoking control subjects, smoking control subjects, and patients with COPD. We used the Kruskal-Wallis test to detect overall differences and the Dunn multiple comparisons test to look for between-group differences.

Statistical analysis for this study was performed with GraphPad Prism 5 software (GraphPad Software).

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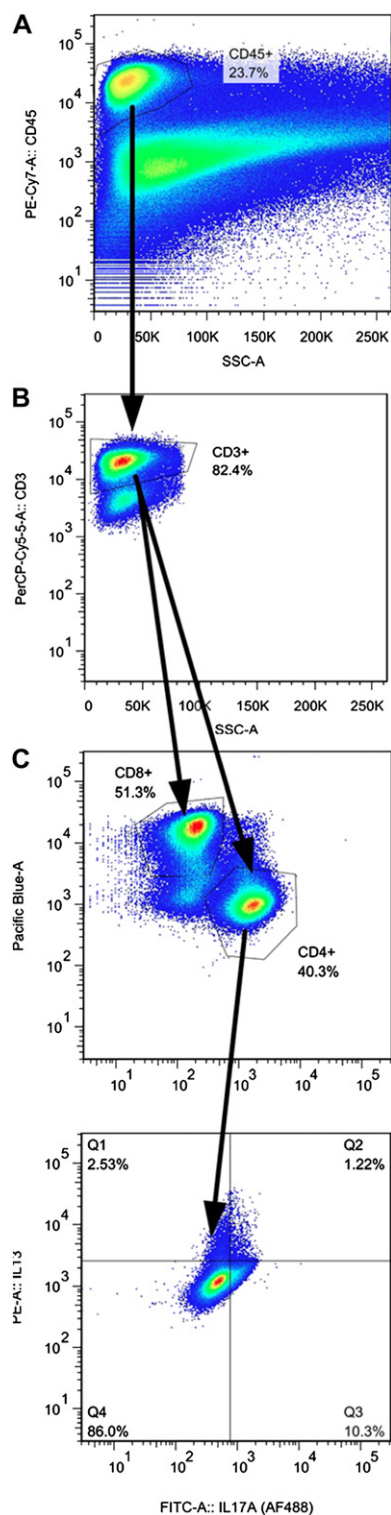


FIG E1. Flow cytometric gating strategy. **A**, Total cell suspension obtained from the lung of a patient with COPD with gating on the cell population expressing CD45. **B**, CD45 cells expressing CD3, which are further analyzed into cells expressing **(C)** either CD4 or CD8. CD4 cells are then further analyzed for their production of IL-13 and IL-17 (after stimulation).

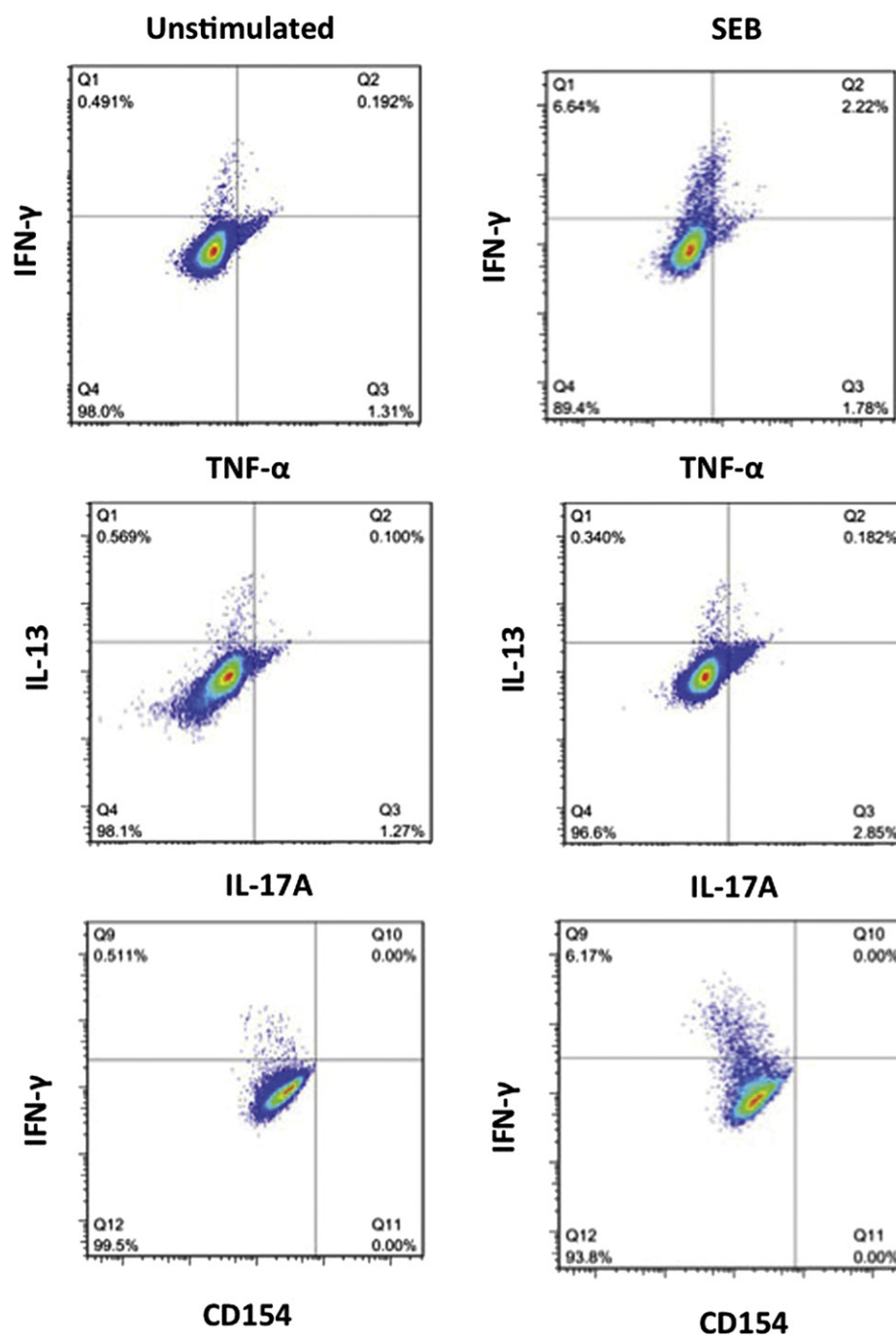


FIG E2. Example of cytokine staining in CD4 cells induced by SEB. The figure shows an example of cytokine staining in T_H cells induced by SEB (same patient with COPD shown in Fig 1, B), demonstrating production of TNF- α , IL-13, IL-17, CD154, and IFN- γ in unstimulated cells (*left column*) and after stimulation with SEB (*right column*).

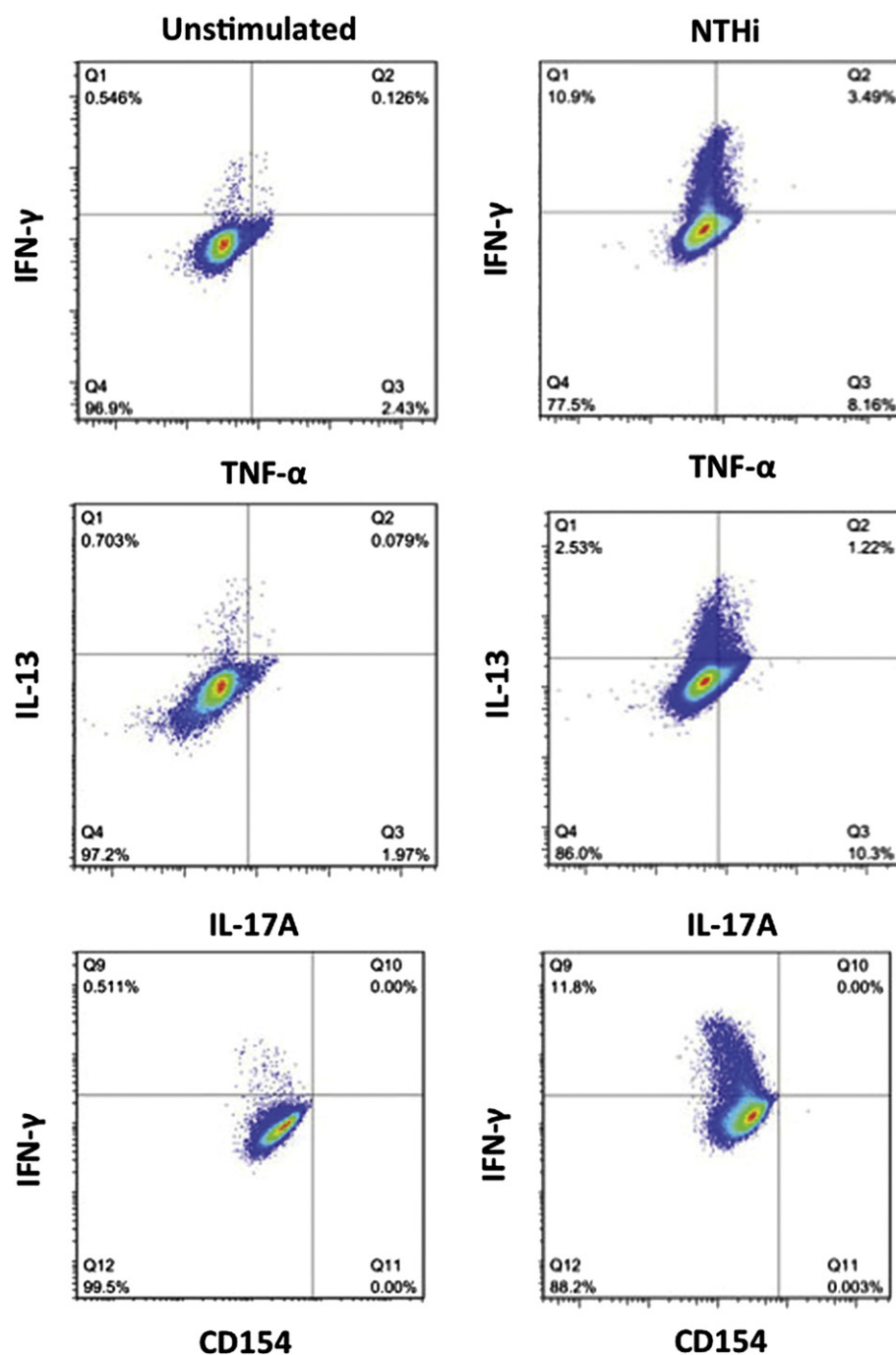


FIG E3. Example of cytokine staining induced in CD4 cells by NTHi. The figure shows an example of cytokine staining in T_H cells induced by NTHi (same subject shown in Fig 1, B), demonstrating production of TNF- α , IL-13, IL-17, CD154, and IFN- γ in unstimulated cells (left column) and after stimulation with NTHi (right column).

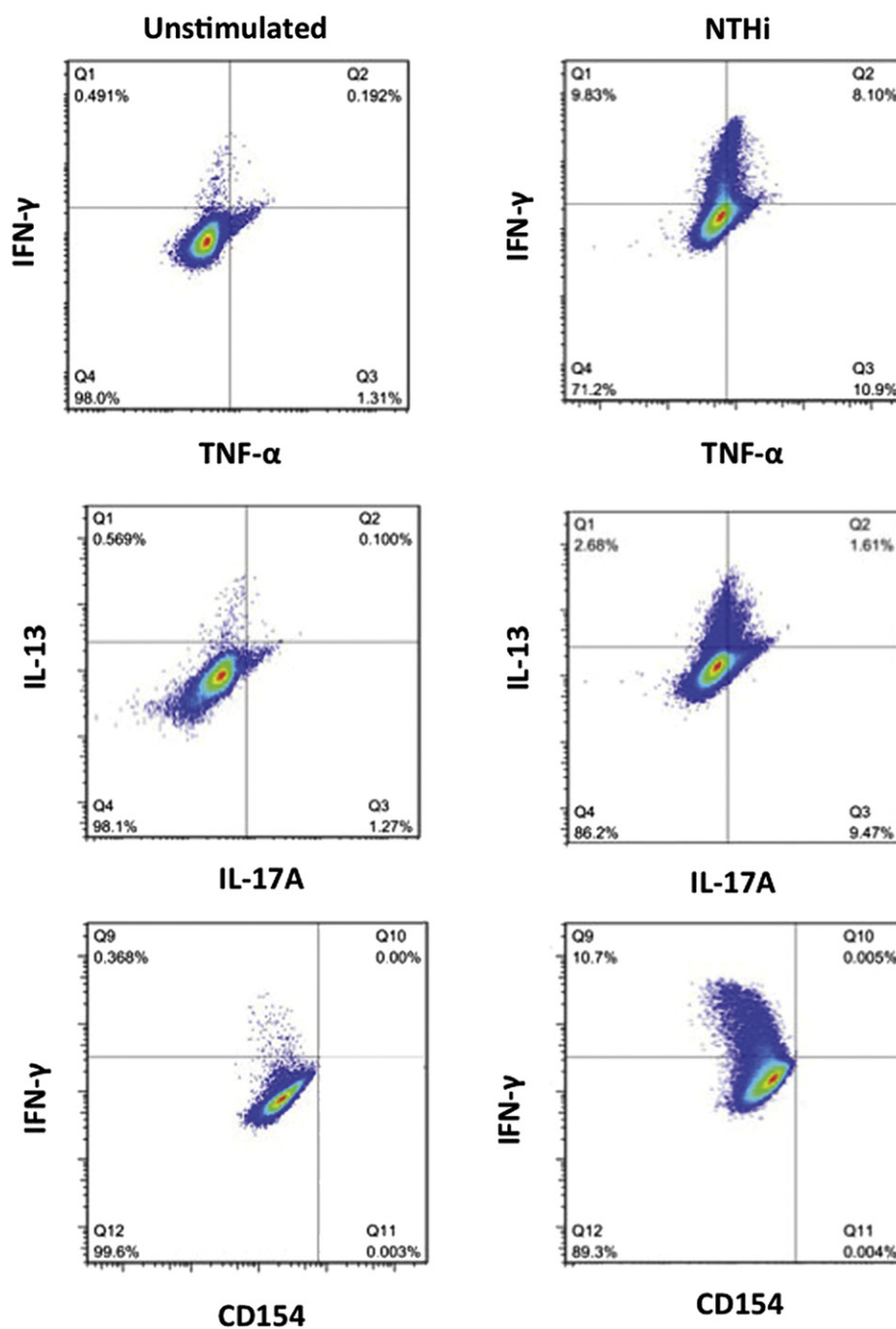


FIG E4. Example of cytokine staining induced in CD8 cells by NTHi. The figure shows an example of cytokine staining in T_H cells induced by NTHi (same subject shown in Fig 1, B), demonstrating production of TNF- α , IL-13, IL-17, CD154, and IFN- γ in unstimulated cells (left column) and after stimulation with NTHi (right column).

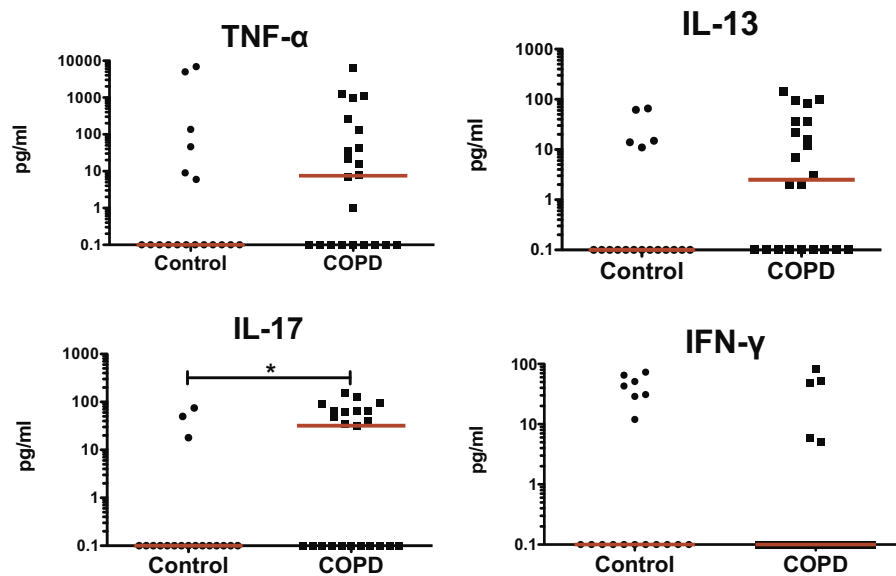


FIG E5. Bead array results for T-cell cytokines from supernatants induced by NTHi stimulation. Cytokine levels were compared between the non-COPD (control) and COPD groups for TNF- α ($P = .16$), IL-13 ($P = .06$), IL-17 ($P = .01$), and IFN- γ ($P = .31$).

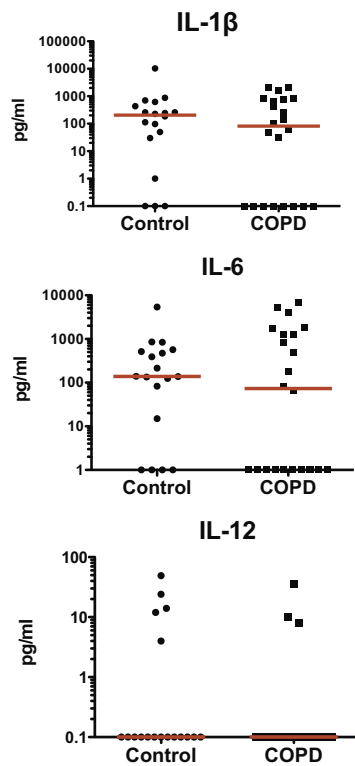
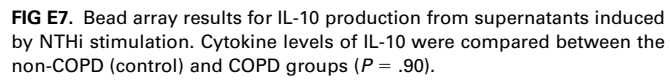


FIG E6. Bead array results for innate immune mediator production from supernatants induced by NTHi stimulation. Cytokine levels were compared between the non-COPD (control) and COPD groups for IL-1 β ($P = .65$), IL-6 ($P = .26$), and IL-12 ($P = .79$).



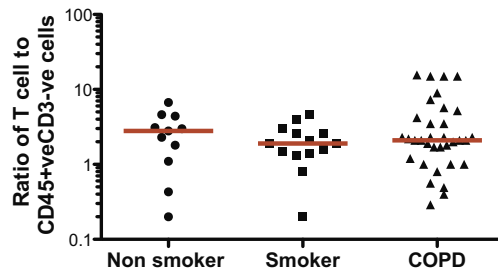


FIG E8. The ratio of T cells ($CD45^{+}$, $CD3^{+}$) to the population of white cells that were $CD45^{+}$ but $CD3^{-}$ (these $CD45^{+}CD3^{-}$ cells would comprise most of the classical APCs) among the 3 groups (nonsmoking control subjects, smoking control subjects, and patients with COPD). ANOVA demonstrated no significant differences ($P = .63$).

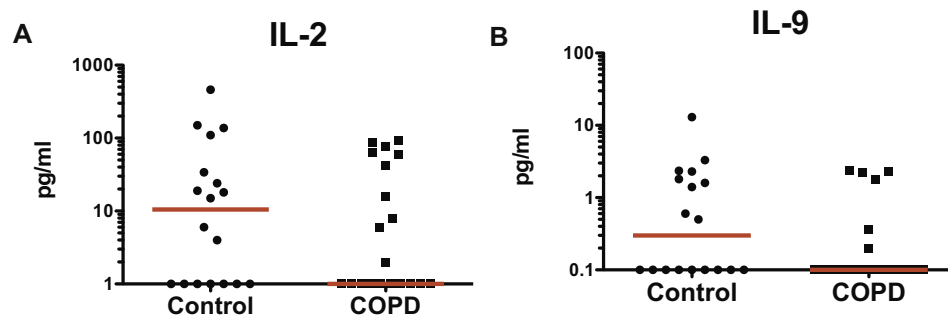


FIG E9. Bead array results for IL-2 and IL-9 production in supernatants induced by NTHi stimulation. Cytokine levels of IL-2 were similar between the non-COPD (control) and COPD groups ($P = .29$), as were IL-9 levels ($P = .24$).

TABLE E1. Histology and stage of lung cancer in subgroups

Control subjects	Histology	Stage	Patients with COPD	Histology	Stage
Nonsmoking control subjects					
1	Adenocarcinoma	T2 N0	1	Adenocarcinoma	T1 N0
2	Adenocarcinoma	T2 N3	2	Adenocarcinoma <i>in situ</i>	T2 N0
3	Adenocarcinoma	T1 N2	3	Large cell	T1 N0
4	Adenocarcinoma	T1 N0	4	Organizing pneumonia	NA
5	Adenocarcinoma	T3 N0	5	Adenocarcinoma	T2 N0
6	Carcinoid	Localized	6	Adenocarcinoma	T1 N0
7	Adenocarcinoma	T1 N0	7	Adenocarcinoma	T2 N2
8	Inflammation/necrosis	NA	8	Adenocarcinoma	T2 N2
9	Adenocarcinoma	T1 N0	9	Adenocarcinoma	T1 N1
10	Melanoma	Metastatic	10	Adenocarcinoma	T2 N2
11	Adenocarcinoma	T3 N2	11	Adenocarcinoma	T1 N0
12	Adenocarcinoma	T1 N0	12	Squamous cell carcinoma	T2 N0
			13	Adenocarcinoma	T1 N0
Smoking control subjects			14	Adenocarcinoma	T2 N0
1	Colonic adenocarcinoma	Metastatic	15	Squamous cell carcinoma	T2 N2
2	Adenocarcinoma	T1 N0	16	Adenocarcinoma	T1 N0
3	Adenocarcinoma	T2 N0	17	Large cell	T1 N0
4	Squamous cell carcinoma	T2 N0	18	Colonic adenocarcinoma	Metastatic
5	Adenocarcinoma	T2 N1	19	Squamous cell carcinoma	T1 N0
6	Squamous cell carcinoma	T1 N0	20	Adenocarcinoma <i>in situ</i>	T1 N0
7	Adenocarcinoma	T1 N1	21	Adenocarcinoma	T2 N1
8	Adenocarcinoma	T1 N0	22	Squamous cell carcinoma	T2 N0
9	Adenocarcinoma	T2 N0	23	Adenocarcinoma	T2 N0
10	Basaloid carcinoma	T1 N0	24	Adenocarcinoma	T2 N2
11	Squamous cell carcinoma	T2 N0	25	Inflammation/necrosis	NA
12	Adenocarcinoma	T2 N0	26	Adenocarcinoma	T1 N0
13	Adenocarcinoma	T1 N0	27	Adenocarcinoma <i>in situ</i>	T2 N0
14	Carcinoid	Localized	28	Adenocarcinoma	T2 N0
15	Adenocarcinoma	T1 N0	29	Squamous cell carcinoma	T2 N0
16	Adenocarcinoma	T2 N0	30	Carcinoid	Localized
17	Adenocarcinoma	T1 N1	31	Adenocarcinoma	T1 N0
18	Adenocarcinoma	T2 N0	32	Squamous cell carcinoma	T2 N1
			33	Adenocarcinoma	T2 N0
			34	Adenocarcinoma	T2 N1
			35	Squamous cell carcinoma	T1 N1
			36	Adenocarcinoma	T1 N0
			37	Adenocarcinoma	T1 N0
			38	Adenocarcinoma	T2 N1
			39	Adenocarcinoma	T1 N1

NA, Not applicable.

TABLE E2. Percentage of T_H cells and CTLs producing mediators after stimulation with NTHi

	Control subjects	Patients with COPD	<i>P</i> value
T _H cell			
TNF- α			
Median	0.007	0.580	<.001
Interquartile range	0.000-0.036	0.006-4.490	
IL-13			
Median	0.012	0.640	.02
Interquartile range	0.000-0.780	0.000-3.100	
IL-17			
Median	0.062	0.380	.03
Interquartile range	0.000-0.633	0.005-7.705	
IFN- γ			
Median	0.000	0.001	.70
Interquartile range	0.000-0.969	0.000-1.290	
CD154			
Median	0.000	0.032	.04
Interquartile range	0.000-0.043	0.000-0.180	
Cytotoxic T cell			
TNF- α			
Median	0.008	0.730	.002
Interquartile range	0.000-1.528	0.050-21.52	
IL-13			
Median	0.002	0.860	.03
Interquartile range	0.000-0.568	0.000-5.790	
IL-17			
Median	0.018	0.833	.01
Interquartile range	0.000-0.965	0.000-15.98	
IFN- γ			
Median	0.042	0.410	.30
Interquartile range	0.000-1.025	0.000-3.00	
CD154			
Median	0.010	0.013	.49
Interquartile range	0.000-0.104	0.000-0.170	

P values are comparisons of the COPD group with the control group by using the Mann-Whitney test. A result of 0.000 means the percentage of antigen-specific T cells producing a cytokine is less than the detectable threshold of 1:100,000.

TABLE E3. T-cell response to NTHi comparing nonsmoking control subjects, smoking control subjects, and patients with COPD

	Nonsmoking control subjects	Smoking control subjects	Patients with COPD	<i>P</i> value
T_H cell				
TNF- α				
Median	0.000	0.004	0.580	.002
Interquartile range	0.000-0.050	0.000-0.373	0.006-4.490	
IL-13				
Median	0.000	0.280	0.640	.004
Interquartile range	0.000-0.041	0.060-1.205	0.005-3.100	
IL-17				
Median	0.000	0.120	0.380	.03
Interquartile range	0.000-0.167	0.000-1.160	0.005-7.705	
IFN- γ				
Median	0.000	0.006	0.001	.51
Interquartile range	0.000-0.590	0.000-1.833	0.000-1.290	
CD154				
Median	0.000	0.000	0.032	.14
Interquartile range	0.000-0.205	0.000-0.040	0.000-0.180	
Cytotoxic T cell				
TNF- α				
Median	0.000	0.097	0.730	<.001
Interquartile range	0.000-0.000	0.000-6.395	0.050-21.52	
IL-13				
Median	0.000	0.025	0.860	.01
Interquartile range	0.000-0.036	0.000-0.324	0.000-5.790	
IL-17				
Median	0.000	0.096	0.833	.004
Interquartile range	0.000-0.000	0.000-0.220	0.000-15.98	
IFN- γ				
Median	0.000	0.198	0.410	.29
Interquartile range	0.000-0.658	0.000-2.985	0.000-3.00	
CD154				
Median	0.086	0.006	0.013	.56
Interquartile range	0.000-1.465	0.000-0.030	0.000-0.170	

P values are comparisons of the COPD group with the smoking control group and the nonsmoking control group by using the Kruskal-Wallis test. Results are expressed as the percentage of T cells producing a specific inflammatory mediator.