

Patients with cystic fibrosis have inducible IL-17⁺IL-22⁺ memory cells in lung draining lymph nodes

Yvonne R. Chan, MD,^a Kong Chen, PhD,^b Steven R. Duncan, MD,^a Kira L. Lathrop, MAMS,^c Joseph D. Latoche, BS,^a Alison J. Logar, BS,^b Derek A. Pociask, PhD,^b Brendon J. Wahlberg, MS,^a Prabir Ray, PhD,^a Anuradha Ray, PhD,^a Joseph M. Pilewski, MD,^a and Jay K. Kolls, MD^b Pittsburgh, Pa

Background: IL-17 is an important cytokine signature of the T_H differentiation pathway T_H17. This T-cell subset is crucial in mediating autoimmune disease or antimicrobial immunity in animal models, but its presence and role in human disease remain to be completely characterized.

Objective: We set out to determine the frequency of T_H17 cells in patients with cystic fibrosis (CF), a disease in which there is recurrent infection with known pathogens.

Methods: Explanted lungs from patients undergoing transplantation or organ donors (CF samples = 18; non-CF, nonbronchiectatic samples = 10) were collected. Hilar nodes and parenchymal lung tissue were processed and examined for T_H17 signature by using immunofluorescence and quantitative real-time PCR. T cells were isolated and stimulated with antigens from *Pseudomonas aeruginosa* and *Aspergillus* species. Cytokine profiles and staining with flow cytometry were used to assess the reactivity of these cells to antigen stimulation.

Results: We found a strong IL-17 phenotype in patients with CF compared with that seen in control subjects without CF. Within this tissue, we found pathogenic antigen-responsive CD4⁺IL-17⁺ cells. There were double-positive IL-17⁺IL-22⁺ cells [T_H17(22)], and the IL-22⁺ population had a higher proportion of memory characteristics. Antigen-specific T_H17 responses were stronger in the draining lymph nodes compared with those seen in matched parenchymal lungs.

Conclusion: Inducible proliferation of T_H17(22) with memory cell characteristics is seen in the lungs of patients with CF. The

function of these individual subpopulations will require further study regarding their development. T cells are likely not the exclusive producers of IL-17 and IL-22, and this will require further characterization. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: T_H17, T_H22, IL-17, IL-22, cystic fibrosis, *Pseudomonas* species, *Aspergillus* species, chronic infectious disease, memory T-cell response, lung transplant

The discovery of the effector cytokine IL-17 has caused a major paradigm shift from our formerly dichotomous understanding of T_H1/T_H2 immune inflammation. It has emerged as a crucial regulator of mucosal immunity against infection in the lung, skin, and gut. Moreover, the identification of subsets of memory T cells expressing IL-17 has led to the recognition of a distinct arm of immune response called T_H17. However, as studies on the regulation of IL-17 demonstrate, the T_H17 immune response walks a fine line between protective and destructive tissue inflammation.

At the height of the HIV epidemic, intense focus on CD4⁺ T cells led to the discovery of 2 classes of T_H cells: T_H1 and T_H2.¹ T_H1 cells are characterized by IFN-γ production, whereas T_H2 cells produce IL-4, IL-5, and IL-13. This model of T_H cell development went far to explain immune defense against many intracellular pathogens (T_H1) and parasites (T_H2). However, murine models lacking either class of T_H response were still able to mount a protective response against *Pneumocystis* species infection.² Therefore the simple T_H1/T_H2 polarization model was insufficient to explain the rampant susceptibility to opportunistic bacterial and fungal infection associated with CD4⁺ deficiency.

Further studies on lymphotropic viruses found that they often orchestrate infection by producing homologues of host cytokines, and IL-17 was identified in a T-cell hybridoma cDNA library in a search for viral protein homologues.³⁻⁶ Subsequent work has shown that IL-17 mediates neutrophil recruitment to sites of infection⁶⁻⁸ and unequivocally established its protective host defense role in mucocutaneous bacterial and fungal infections.⁹⁻¹⁵

On the flip side of the inflammatory coin, IL-17 pathways have been shown to exacerbate autoimmune disease. Murine models of rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, and psoriasis have subsequently highlighted the importance of the T_H17 subtype in the maintenance of inflammation, and this has been extensively reviewed elsewhere.¹⁶ Whether it is infection or self-antigen that incites the initial inflammatory response, a sustained favorable cytokine milieu leads to the development of memory T_H17 cells.^{14,17-20} These cells mediate the crucial recall response to bacterial invaders but are disastrously destructive in autoimmunity. Although this is well understood in murine models of disease, the identification and characterization of T_H17 cells in human subjects is still a developing frontier.

From ^athe Division of Pulmonary, Allergy and Critical Care Medicine and ^cthe Eye and Ear Institute, University of Pittsburgh, and ^bthe Richard King Mellon Foundation Institute for Pediatric Research, Children's Hospital of Pittsburgh.

Supported by research grants from the National Heart, Lung, and Blood Institute, National Institutes of Health (K08 HL089189 to Y.R.C. and P50 HL084932 to J.K.K. and J.M.P.), the Howard Hughes Medical Institute (Early Career Physician Scientist Award to Y.R.C.), the CF Foundation (to J.M.P.), and Novartis (to J.K.K. and J.M.P.). Also supported by the confocal imaging resources of the Core Grant for Vision Research at the University of Pittsburgh (NIH P30-EY08098).

Disclosure of potential conflict of interest: Y. R. Chan has received research support from the Howard Hughes Medical Institute and the National Institutes of Health (NIH) and has received payment from Novartis for contract performance of experiments. S. R. Duncan and K. Lathrop have received research support from the NIH. J. M. Pilewski has received research support from the Cystic Fibrosis Foundation and the NIH. J. K. Kolls has received research support from the NIH, is chair of the board for MiniVax, is coinventor on a patent submitted by Louisiana State University Health Sciences Center, and has received travel compensation from MiniVax. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication July 8, 2011; revised May 1, 2012; accepted for publication May 31, 2012.

Corresponding author: Yvonne R. Chan, MD, Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, 3459 Fifth Avenue, NW 628 MUH, Pittsburgh, PA 15213. E-mail: chany3@upmc.edu.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2012.05.036>

Abbreviations used

Asp: *Aspergillus* mitogilin
 CF: Cystic fibrosis
 Con A: Concanavalin A
 COPD: Chronic obstructive pulmonary disease
 CORE: Center for Organ Recovery and Education
 DLN: Draining lymph node cell
 NK: Natural killer
 Pa(EL): *Pseudomonas aeruginosa* early log-phase sonicate antigen
 Pa(LL): *Pseudomonas aeruginosa* late log-phase filtrate antigen
 PLC: Parenchymal lung leukocyte
 ROR: Retinoic acid receptor (RAR)-related orphan receptor
 TCR: T-cell receptor

In human disease the crucial role of IL-17 pathways in host defense is seen in patients with Job syndrome, a disorder involving a signal transducer and activator of transcription 3 defect that results in impaired differentiation of T_H17 cells. These patients present with hyper-IgE syndrome, as well as recurrent bacterial and fungal infections of the skin and respiratory tract, particularly with *Staphylococcus aureus*, as well as oropharyngeal candidiasis. Furthermore, patients with autoimmune polyendocrine syndrome type I who have chronic mucocutaneous candidiasis were found to have autoantibodies against IL-17 and IL-22.²¹ Conversely, increased IL-17A and IL-17F levels are detected in asthmatic patients,^{22,23} in bronchoalveolar lavage fluid of patients with cystic fibrosis (CF) undergoing exacerbation associated with pseudomonal colonization,²⁴ and in patients with chronic obstructive pulmonary disease (COPD).²⁵

As important as T_H17 responses are in host defense, it is conceivable that diseases involving anatomic or physiologic defects permissive of chronic infection could mimic the persistent T_H17 inflammatory response seen in autoimmune models.²⁶ Although T_H17 cells mediate memory adaptive immunity against infection, IL-17 also elicits robust innate inflammatory responses by recruiting neutrophils^{7,9,27} and upregulating antimicrobial effectors.^{15,28-35} Thus it presents a potential mechanistic target for remediation of the tissue destruction seen in patients with chronic infection.

The human lung is a unique organ for examining T_H17 development in this setting because it is the only organ with direct environmental contact but is not known to have substantial normal flora. Investigation can be focused on the host memory response to a limited number of organisms in the lung. Indeed, there is evidence that lower respiratory tract colonization with select classes of organisms is associated with functional decrease in patients with COPD,³⁶⁻⁴⁰ as extensively reviewed by Morris et al.⁴¹ Moreover, murine models of COPD demonstrate that matrix metalloproteinases play a role in mediating tissue destruction.^{42,43}

For the current study, our group has chosen to examine CF as the model of chronic infection and inflammation. We previously found increased levels of IL-17 in bronchoalveolar lavage fluid during exacerbations associated with positive *Pseudomonas* species sputum cultures.²⁴ Subsequently, we showed higher levels of IL-17 in the draining lymph nodes of patients with CF undergoing transplantation.⁴⁴ In the current work we now identify a source of IL-17 in these patients as CD4⁺ cells in the draining lymph nodes. Furthermore, we demonstrate that these cells have an antigen-specific response, producing a T_H17 cytokine response to bacterial and fungal pathogens with which the patients were colonized.

This is a breakthrough in the isolation of T_H17 cells that can be physiologically tested and warrants prospective studies of its potential as a prognostic tool in transplant recipient outcome. Moreover, our finding that IL-17 levels can be augmented by simultaneous suppression of T_H1 (IFN- γ) and T_H2 (IL-4 or IL-13) cytokines in human subjects, an effect mediated by many standard therapies for CF or airways hyperreactivity, has therapeutic implications that warrant further exploration.

METHODS**Ethics statement**

All patients' samples were collected after obtaining informed consent and were deidentified as approved by an Institutional Review Board at the University of Pittsburgh (Institutional Review Board no. REN10070105).

Collection of explanted lungs and tissue bank specimens

Explanted lungs were collected from patients undergoing lung transplantation at the University of Pittsburgh after approval by the Institutional Review Board. Controls were lungs from patients without CF who died of trauma and were not ultimately used for transplantation under the Center for Organ Recovery and Education (CORE) or patients with non-CF, nonbronchiectatic end-stage lung disease undergoing transplantation. Samples from 18 patients with CF and 10 patients without CF were used. Sample size was determined based on the volume of transplantations done at the University of Pittsburgh and the availability of tissue for processing. For draining lymph node cells (DLNs), hilar lymph nodes were dissected from the specimens and dispersed into single-cell suspensions per a protocol adapted from murine mononuclear cell preparation.⁴⁵ For parenchymal lung leukocytes (PLCs), peripheral lung tissue was processed, as previously described.⁴⁶ Lung tissue was frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, Calif) compound for immunofluorescence staining and RNA analysis.

Antigen preparation and testing

Several antigens were tested for their ability to stimulate proliferation by using bromodeoxyuridine incorporation in DLC cultures (FITC BrdU Flow Kit; BD Biosciences, San Jose, Calif). Early log-phase and late log-phase *Pseudomonas aeruginosa* (PA01) cultures grown in Luria Broth were pelleted and subsequently either sonicated and sterile filtered or heat killed. In addition, the supernatant from the pelleted culture was also sterile filtered and tested. All samples were compared with fresh tryptic soy broth. From these studies, we found the *Pseudomonas aeruginosa* early log-phase sonicate antigen (Pa [EL]) sonicated pellet and the *Pseudomonas aeruginosa* late log-phase sonicate antigen (Pa[LL]) culture filtrate had the highest activity and subsequently used these fractions. *Aspergillus* mitogilin (Asp; INDOOR Biotechnologies, Charlottesville, Va) was used at 1 μ g/mL, as titrated previously.⁴⁷ Concanavalin A (Con A; Sigma, St Louis, Mo) was used at 5 μ g/mL, and *Candida* species antigen (Hollister-Stier, Spokane, Wash) and tetanus toxoid (adsorbed injectable solution; Aventis, Bridgewater, NJ) both were tested at 1:10, 1:100, and 1:1000 dilution.

Culture conditions and antigen stimulation

DLNs and PLCs were resuspended at 5×10^6 cells/mL in RPMI 1640, 2 mmol/L L-glutamine, 25 mmol/L HEPES, 100 U/mL penicillin/streptomycin, 50 μ mol/L β -mercaptoethanol, 10% FCS, and 100 U/mL IL-2 (Roche, Mannheim, Germany) for stimulation. DLN and PLC cultures were stimulated in triplicate with 1 μ g/mL Pa(EL), 10 μ g/mL Pa(LL), 1 μ g/mL Asp, or 5 μ g/mL Con A (see Fig E1 in this article's Online Repository at www.jacionline.org). For antibody neutralizations, anti-IL-4, anti-IFN- γ , anti-IL-13, or appropriate isotype control (all from R&D Systems, Minneapolis, Minn) were added to wells at 10 μ g/mL per antibody. Cultures were incubated for 5 to 7 days, collected, and washed for flow cytometric staining and analysis, and supernatants were collected for cytokine analysis assays.

Immunofluorescence

Slides from the OCT-embedded tissue described above were fixed in 4% paraformaldehyde, washed with PBS, and blocked with 5% secondary antibody source animal serum. They were stained with anti-CD4 (AF-379-NA, R&D Systems), anti-Zo-1 (617300; Invitrogen, Carlsbad, Calif), anti-IL-17A (MAB3171, R&D Systems), anti-IL-17F (MAB13351, R&D Systems), anti-CD56 (559049, BD Biosciences), and/or anti-IL-22 (AF782, R&D Systems). They were counterstained with appropriate anti-isotype Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen). Isotype controls were also used to assess the level of nonspecific binding (see Fig E2 in this article's Online Repository at www.jacionline.org). Confocal microscopy was done on an Olympus FluoView 1000 inverted laser scanning confocal microscope (Olympus, Center Valley, Pa) with a $\times 20$ oil objective (numeric aperture, 0.85) and reviewed by an experienced microscopist (K. Lathrop) who was blinded to the identity of the slides and selected 6 to 8 representative fields on each slide for image quantitation. Images were acquired in 5 channels: 4 immunofluorescent and 1 differential interference contrast brightfield channels. For each fluorescent channel, images were thresholded to segment fluorescent signal above background levels and then binarized. Area measurements were read out into Excel software (Microsoft, Redmond, Wash), and the binary images were saved. Image arithmetic was used to determine the total pixel area of CD4, Zo-1, and IL-17 individually, as well as the area of CD4 that was not coincident with Zo-1 but was coincident with IL-17. Three-dimensional image reconstructions of confocal stacks were also done (see Videos E1 and E2 in this article's Online Repository at www.jacionline.org). All digital image processing was done with Metamorph 7.7.6 software (Molecular Devices, Sunnyvale, Calif), and statistical analysis was done with GraphPad Prism software (GraphPad Software, La Jolla, Calif) by using 2-way ANOVA with the Bonferroni posttest comparison.

Quantitative real-time PCR analysis

Serial sections from the OCT-embedded tissue blocks that were cut for immunofluorescence staining were placed in TRIzol (Invitrogen). Total RNA was isolated per the manufacturer's protocol and repurified by using a cleanup procedure (RNeasy mini Kit; Qiagen, Hilden, Germany). Total RNA was reverse transcribed with Superscript II and random primers (Invitrogen Life Technologies). The cDNAs were amplified with specific primers and probes from Applied Biosystems (Foster City, Calif) by using custom-designed 384-well arrays (Micro Fluidic Card) and according to the Applied Biosystems protocol. Level of mRNA expression was first normalized to human T-box transcription factor (*TBX21*), fold change was calculated according to the $\Delta\Delta$ cycle threshold, and data were analyzed in GraphPad Prism software by using 2-way ANOVA with the Bonferroni posttest comparison.

Multiplex cytokine analysis and ELISA

Supernatants from the cultures were examined for multiple cytokine levels by using a bead-based cytokine array. Specifically, DLN- and PLC-stimulated culture supernatants were processed with the Luminex (now Millipore, Temecula, Calif) Human 30-plex cytokine kit. Results were read with a Bio-Rad Bio-Plex instrument (Bio-Rad Laboratories, Hercules, Calif). Data were then exported to Microsoft Excel and GraphPad Prism software for analysis by using 2-way ANOVA with the Bonferroni posttest comparison.

Flow cytometric analysis of cell populations

Before harvest, DLN and PLC cultures intended for flow cytometry were restimulated with the antigen to which they were initially exposed, and monensin was added to 5 $\mu\text{mol/L}$ in the wells for 6 hours. Cells were blocked with Flow Cytometry Staining Buffer (00-4222; eBioscience, San Diego, Calif) with 5% murine IgG. Then cells were surface labeled with conjugated antibodies directed against CD3, CD4, CD45RO, and T-cell receptor (TCR) $\alpha\beta$ (all from BD Biosciences). Intracellular cytokine expression for DLN and PLC cultures was also analyzed by using flow cytometry. Intracellular staining for IL-17 (12-7179, eBioscience) and IL-22 (IC7821A, R&D Systems) was carried out by using reagents from a commercially available intracellular

staining kit per their instructions (00-5523, eBioscience). Samples were run on a BD FACSARIA cell sorter, and data were analyzed with FACSDiVa (BD Biosciences) or FlowJo (TreeStar, Ashland, Ore) software. Acquisition gates were first set by size (forward scatter) and granularity (side scatter) characteristics. Total live cells were further analyzed after doublet discrimination. Gate statistics for all samples were then exported to a spreadsheet and analyzed in GraphPad Prism software by using 2-way ANOVA with the Bonferroni posttest comparison.

RESULTS

Patients' demographics

Patients with CF (Table I) were evenly distributed by sex (9 male and 9 female patients). The average age at transplantation among patients with CF was 29 ± 6.9 (SD) years. Culture results for a majority of patients revealed a history of polymicrobial colonization or infection before transplantation. Ten of 18 patients had positive cultures for *P aeruginosa* before transplantation. Four patients were receiving oral prednisone at the time of transplantation, whereas 9 patients were receiving an inhaled corticosteroid. Patients without CF included in the comparison (Table II) were a more heterogeneous group among the patients for whom medical information was available. The average age of patients with available medical information was 48 ± 20 (SD) years. Unpaired 2-tailed *t* tests revealed a statistically significant difference in the age of the populations for whom medical information was known. Patients who underwent transplantation for nonbronchiectatic or nonobstructive lung diseases (5 patients) were included, as well as lungs from CORE that were not used for transplantation (5 patients). Medical information was available for 1 CORE patient because of that lung originating at our transplantation center. Of the transplant recipients without CF, 3 of 5 were receiving inhaled corticosteroids and oral prednisone at the time of transplantation. Thus the proportion of inhaled steroid use was similar in recipient patients (with CF, 0.5; without CF, 0.6), whereas the proportion of oral steroid use was similar among patients with CF compared with all control subjects without CF (CF, 0.22; non-CF, 0.30).

T_H17 cytokines levels are increased in lungs of patients with CF

We examined the status of IL-17 in banked lung tissues from patients with CF who had undergone transplantation and compared them with CORE samples or patients undergoing transplantation for nonbronchiectatic end-stage lung disease (primary pulmonary hypertension, idiopathic pulmonary fibrosis, and sarcoidosis). Tissues from patients with CF had many patches of dramatic staining for IL-17A and IL-17F (Fig 1). Examination of the airways revealed sheets of inflammatory cells in patients with CF, whereas this was usually not present in non-CF genotypes. Quantitative analysis of IL-17F fluorescence area revealed that the overall signal area of IL-17F trended higher for patients with CF and the signal area of colocalized CD4 and IL-17F was significantly higher in patients with CF (Fig 2).

Higher levels of T_H17-related transcripts were also found in the CF samples compared with the non-CF samples in serial sections taken from the same blocks used for immunofluorescent analysis (Fig 2). Notably, *IL17A*, *IL17F*, and *IL22* expression was significantly higher in CF tissues compared with that seen in non-CF control tissues. Consistent with these findings, expression of RAR-related orphan receptor alpha (*RORA*), a second

TABLE I. Characteristics of patients with CF

Age (y)	Sex	Immunomodulators	Pathogen status	Studies
25	M	None	<i>Pseudomonas aeruginosa</i> , <i>Torulopsis glabrata</i>	F, C
36	F	Fluticasone/salmeterol inhaled	<i>Candida parapsilosis</i> , <i>Candida tropicalis</i> , <i>Candida albicans</i>	F, C
34	M	Fluticasone/salmeterol inhaled Prednisone, 15 mg/d	<i>Stenotrophomonas maltophilia</i> , coagulase-negative <i>Staphylococcus</i> , <i>Candida albicans</i>	F, C
26	F	Prednisone, 15 mg/d	<i>Pseudomonas aeruginosa</i> , <i>Achromobacter xylosoxidans</i> , <i>Aspergillus fumigatus</i> , yeast, <i>Staphylococcus aureus</i>	F, C
36	M	Prednisone, 20 mg/d	<i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Serratia marcescens</i>	F, C, IF, R
49	M	None	<i>Pseudomonas aeruginosa</i> , <i>Moraxella</i> species, <i>Streptococcus viridans</i> , coagulase-negative <i>Staphylococcus</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas putida</i> , <i>Neisseria</i> species, <i>Candida albicans</i>	F, C, IF, R
25	F	None	<i>Candida albicans</i>	IF, R
25	F	Fluticasone/salmeterol inhaled	<i>Staphylococcus aureus</i> , <i>Stenotrophomonas maltophilia</i> , <i>Enterobacter aerogenes</i>	F, C
29	M	Fluticasone/salmeterol inhaled Prednisone, 10 mg/d	<i>Pseudomonas aeruginosa</i>	C
26	F	Fluticasone/salmeterol inhaled	<i>Pseudomonas aeruginosa</i> , <i>Streptococcus viridans</i>	F, C IF, R
23	F	None	<i>Pseudomonas aeruginosa</i> , <i>Aspergillus fumigatus</i>	F, C IF, R
19	M	Fluticasone/salmeterol inhaled	<i>Burkholderia cepacia</i> , <i>Achromobacter xylosoxidans</i> , <i>Streptococcus viridans</i> , <i>Candida albicans</i>	IF, R
36	F	Fluticasone/salmeterol inhaled	<i>Pseudomonas aeruginosa</i> , <i>Streptococcus viridans</i> , <i>Neisseria</i> species	IF, R
27	M	Prednisone, 10 mg/d	<i>Burkholderia cepacia</i>	IF, R
29	F	Mometasone inhaled	<i>Staphylococcus aureus</i> , <i>Streptococcus viridans</i> , <i>Enterobacter cloacae</i> , <i>Neisseria</i> species, coagulase-negative <i>Staphylococcus</i> , yeast	IF, R
29	M	None	<i>Burkholderia cepacia</i> , <i>Streptococcus viridans</i> , coagulase-negative <i>Staphylococcus</i> , <i>Neisseria</i> species, <i>Candida albicans</i>	IF, R
24	F	Prednisone, 10 mg/d	<i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , yeast	IF, R
30	M	Fluticasone/salmeterol inhaled Prednisone, 5 mg/d	<i>Pseudomonas aeruginosa</i> , <i>Streptococcus</i> β -hemolytic group C, <i>Streptococcus viridans</i> , <i>Candida albicans</i> , <i>Exophiala jeikei</i>	IF, R

Demographic information on patients with CF from whom lungs were collected is shown along with a key describing the studies each sample underwent: C, cell stimulation, antibody neutralization, and multiplex cytokine analysis; F, cell stimulation and flow cytometric analysis; IF, immunofluorescence studies on OCT-embedded tissue blocks; R, real-time PCR on serial sections from OCT-embedded blocks.

F, Female; M, male.

TABLE II. Characteristics of patients without CF

Age (y)	Sex	Diagnosis	Immunomodulators	Pathogen status	Studies
18	M	CORE	None	None	F, C, IF, R
U	U	CORE	Unknown	Unknown	F, C
U	U	CORE	Unknown	Unknown	F, C, IF, R
U	U	CORE	Unknown	Unknown	F, C, IF, R
U	U	CORE	Unknown	Unknown	IF, R
64	M	IPF	Fluticasone inhaled Prednisone, 12.5 mg/d	<i>Streptococcus viridans</i> , <i>Neisseria</i> species	IF, R
66	M	IPF	Fluticasone/salmeterol inhaled Prednisone, 20 mg/d	None	IF, R
30	M	PPH	None	<i>Streptococcus viridans</i> , <i>Micrococcus</i> species, <i>Neisseria</i> species	IF, R
58	F	PPH	None	<i>Streptococcus viridans</i> , <i>Streptococcus</i> β -hemolytic group F, <i>Staphylococcus aureus</i> , <i>Neisseria</i> species	IF, R
54	M	Sarcoidosis	Fluticasone/salmeterol inhaled Prednisone, 30 mg taper	<i>Candida albicans</i>	IF, R

Demographic information on control subjects without CF from whom lungs were collected is shown along with a key describing the studies each sample underwent: C, cell stimulation, antibody neutralization, and multiplex cytokine analysis; F, cell stimulation and flow cytometric analysis; IF, immunofluorescence studies on OCT-embedded tissue blocks; R, real-time PCR on serial sections from OCT-embedded blocks.

F, Female; IPF, idiopathic pulmonary fibrosis; M, male; PPH, primary pulmonary hypertension; U, unknown.

T_H17-specific transcription factor,⁴⁸ also trended higher in patients with CF, whereas *RORC* expression was not significantly different. IL-4 and IL-13 did trend higher in patients with CF, but this was not statistically significant. In contraposition, expression of *IFNG* and forkhead box protein 3 (*FOXP3*), a key regulatory T-cell transcription activator and simultaneous inhibitor of T_H17, trended lower in patients with CF. This provided evidence that T_H17 pathways were indeed significant in patients with CF.

Antigen-specific IL-17 response is more dominant in draining lymph nodes from patients with CF than in those from control subjects without CF

We next determined whether there was a functional and physiologically relevant IL-17 response of live leukocytes derived from lung tissue. Most patients with CF in this study had previous positive sputum cultures for *P aeruginosa* and some for *Aspergillus* species. Therefore we reasoned that there would likely be a

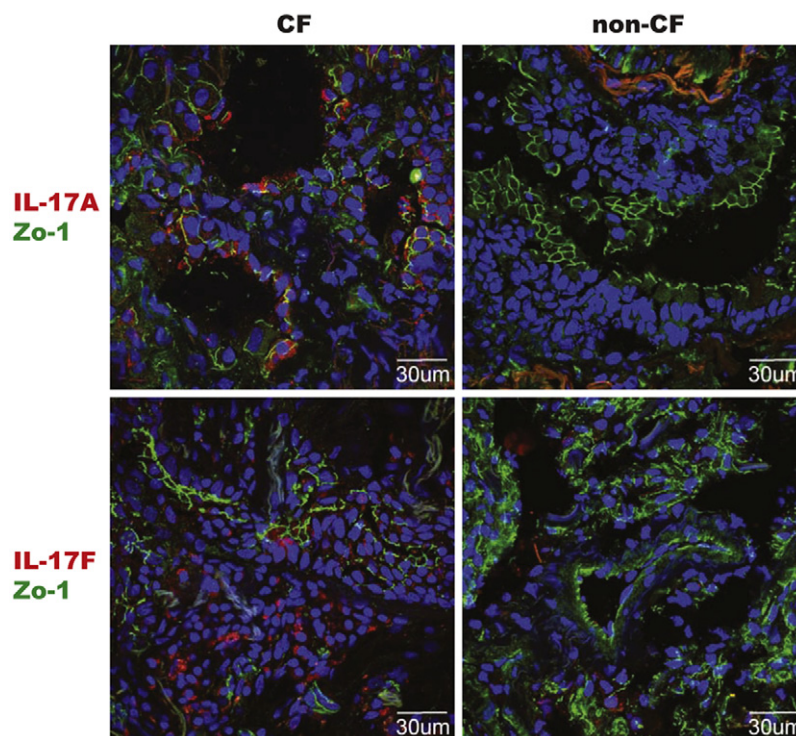


FIG 1. IL-17⁺ cells are prominent in lung tissue from patients with CF. Parenchymal lung tissue stained for IL-17A (red, top panels) or IL-17F (red, bottom panels) and costained with tight junction protein 1 (ZO-1, green, all panels) to delineate anatomic structure. 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI) counterstaining (blue) was done to highlight nuclei. Representative images from patients with CF (left panels) and control subjects without CF (right panels, patients undergoing transplantation for primary pulmonary hypertension and idiopathic pulmonary fibrosis) are shown.

memory T_H17 response at least to these organisms. *Pseudomonas* species antigens from different phases of culture were generated as previously described and tested against DLN and PLC cultures from patients early in the study. This was done to capture the diversity of antigens and virulence factors elaborated by *Pseudomonas* species in their different phases of growth. The fractions eliciting the most robust proliferation, as measured by using bromodeoxyuridine incorporation, Pa(EL) and Pa(LL), were used for all subsequent patients (Fig 3, A). In addition, Asp and a positive mitogenic control, Con A (not shown), were used in separate cell-culture stimulations for comparison. Examination of the unstimulated culture supernatant results for selected cytokines reassuringly revealed that unstimulated IL-17 levels were not significantly different between cohorts for either the DLN or PLC cultures (Fig 3, B). Also, prestimulated DLN and PLC population composition demonstrated the presence of potential antigen-presenting cells and T cells (Fig 3, C). Stimulated cells were first plotted by using forward scatter and side scatter, and gated live cells were further analyzed for CD3, CD4, IL-17, and IL-22 status (Fig 4, A and B). Although patients with CF started with higher baseline numbers of CD3⁺CD4⁺ cells, which persisted after antigen stimulation (Fig 4, C), the percentage of IL-17⁺ T cells clearly increased relative to that seen in non-CF samples after antigen stimulation (Fig 4, D, top). Notably, there are CD3[−]IL-17⁺ cells in both patients without and those with CF (Fig 4, D, bottom) that are not inducible and could reflect a population of natural killer (NK) cells. Further subdivision by CD4 status reveals a strong T-cell IL-17 response in patients with CF that might be equally divided among T_H and other

CD3⁺ T-cell subsets. Note the inducible fraction of CD4[−]IL-17⁺ cells that might reflect CD8⁺ or TCRγδ cells (Fig 4, E). Patients with CF had more IL-17⁺IL-22⁺ double-positive cells in the live cell gate after stimulation (Fig 4, F, top). Examination of the back gating and ancestry reveals that some of these cells appear in population R2 outside the lymphocyte gate R1, even though they are CD3⁺. IL-22⁺ cells made up a larger proportion of CD3⁺IL-17⁺ cells regardless of CD4 status in both CF and non-CF control tissues (Fig 4, F, bottom).

IL-22-expressing CD4⁺IL-17⁺ cells demonstrate higher proportions of memory cell markers

Further characterization of CD4⁺IL-17⁺ cells from patients with CF yielded intriguing results; when gated on their IL-22 status and then analyzed for CD45RO and TCRαβ expression, IL-22⁺ cells had a higher proportion of CD45RO⁺ cells (Fig 5). IL-22⁺CD45RO⁺TCRαβ⁺ cells made up 7.770% (SEM, 0.210%) to 10.58% (SEM, 0.729%) in DLNs from patients with CF across all pathogenic stimuli, whereas IL-22[−] cells had only 1.553% (SEM, 0.074%) to 1.840% (SEM, 0.132%) CD45RO⁺TCRαβ⁺ cells. These data endorse a potential role for IL-22 in maturation of the memory T_H response to pathogenic antigens.

Alternative T_H response might explain lower levels of T_H17 cells in lung parenchyma

In contrast to these findings, we found a comparatively diminished T_H17 response to antigen in PLCs (Fig 6). The

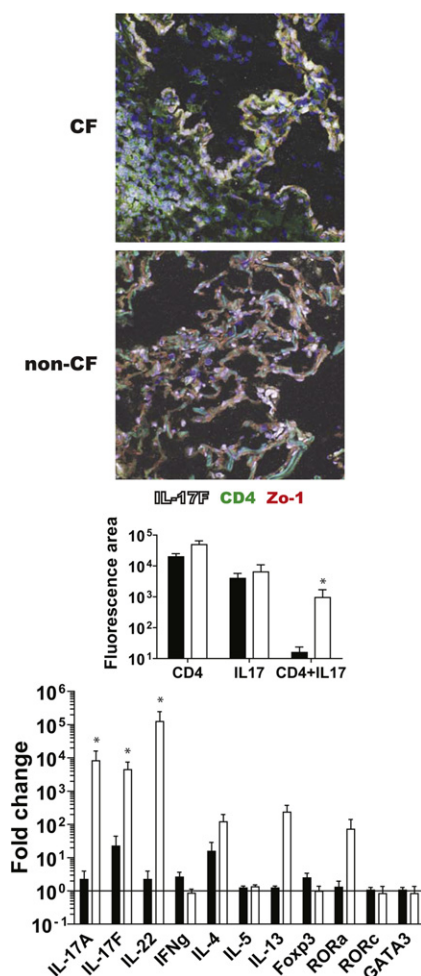


FIG 2. Lungs from patients with CF demonstrate higher numbers of dual-positive CD4⁺IL-17⁺ cells and have stronger T_H17 cytokine message profile. *Top photomicrographs*, Representative images of parenchymal lung tissue stained for IL-17F (white) and costained with anti-CD4 (green) and anti-Zo-1 (red). *Middle graph*, Quantitative confocal analysis of all image files obtained compared by genotype control (black columns, n = 9) versus CF (white columns, n = 12). *Bottom graph*, Quantitative real-time PCR reveals increased T_H17 transcripts in lungs from patients with CF. $\Delta\Delta$ Cycle threshold values for control subjects (black columns, n = 9) and patients with CF (white columns, n = 12) are shown. IL-17A, IL-17F, and IL-22 expression is upregulated in lung tissue from patients without CF, whereas expression of the related transcript *RORA* also trends higher in patients with CF. **P* < .05.

stimulated PLC CD3⁺ population was remarkably smaller than that in DLNs. The proportion of IL-17⁺IL-22⁺ cells in the live cell gate after stimulation was similar to that of unstimulated DLNs. Although there are many potential explanations for this, it is known that suppression of T_H1 and T_H2 pathways can foster the independent development of the T_H17 lineage.^{18,19} Therefore the lack of a PLC T_H17 response could possibly be from strong peripheral T_H1, T_H2, or regulatory T-cell signatures. We examined these cytokine levels from the multiplex analysis in antigen-stimulated PLC cultures from patients with CF and plotted their average normalized values relative to the DLN result (Fig 7, A). Here we note that IL-5, IL-10, IL-13, and RANTES were among the cytokines with expression that trended higher in PLCs relative to DLNs, suggesting that stronger presence of regulatory T-cell or T_H2 factors in the periphery could regulate

the parenchymal T_H17 response. Real-time PCR of T_H2 cytokines comparing the lymph node with the parenchyma also showed a trend toward higher IL-4 and IL-13 levels in the parenchyma (Fig 7, B).

We reasoned that the T_H17 response in the human lung could be further amplified by means of suppression of T_H1 and T_H2 cytokines, such as IL-4 and IFN- γ , which is analogous to findings in the murine model.^{18,49} Furthermore, recent evidence shows the presence of IL-13 receptors on T_H17 cells, which can attenuate IL-17A production.⁵⁰ Because of the preceding work showing that T_H17 could be potently inhibited by IFN- γ , IL-4, and IL-13, we proceeded to neutralize them in culture. Because of sparse recovery of T cells in PLCs, we stimulated DLNs in the presence of neutralizing antibodies to IL-4 and IFN- γ or IL-13 and IFN- γ to see whether the IL-17A response in these cells could be amplified. After stimulation, supernatants were collected and analyzed for IL-17 cytokine levels. Although the IL-17 gain was modest with IL-4 and IFN- γ neutralization (Fig 8, A), a more dramatic magnification of the IL-17 response was found with neutralization of IL-13 and IFN- γ (Fig 8, B). This effect was not observed with neutralization of each of the cytokines individually, except for *Aspergillus* species stimulation, for which IL-17 responses were significantly unmasked with IL-13 neutralization alone (data not shown).

DISCUSSION

This work is an important advance in our understanding of IL-17's role in human disease. First, we have shown that T_H17 cells are isolable from draining lymph nodes of the lung in human CF. Moreover, these T_H17 cells demonstrate an antigen-specific response rather than a merely nonspecific mitogen-induced IL-17 release. We further find that the CD4⁺IL-17⁺ population expressing IL-22 has a higher proportion of CD45RO⁺TCR $\alpha\beta$ ⁺ cells compared with IL-22⁻ cells, suggesting a role for IL-22 in maturation of the adaptive response to pathogens. However, there are sparse data in the literature to help judge whether this is an adequate memory T-cell fraction. Much of the current literature focuses on viral vaccination, which might elicit a different immune response. It would also be difficult to compare our findings because vaccination constitutes a discrete exposure in contrast to CF, whereas bacterial colonization leads to chronic exposure. One study examining the human memory response to meningococcal vaccine suggests that our results are comparable with a 1-time administration of vaccine or persistence 2 years after completion of a 3-dose prime and boost series.⁵¹ However, the dramatic shift of T cells into memory effector T-cell populations in a murine model of chronic antigen exposure suggests that the response we present in this work might be inadequate or less than expected.^{52,53} This presents evidence that there might be acute generation of a T_H17 response to an antigen to which the host has had long-term exposure. Given the high frequency of chronic colonization of patients with late-stage CF with *Pseudomonas* species, our findings might represent an ineffective or inefficient memory response in patients with CF. This would also be consistent with recent findings that the antecedent and primary defect in patients with CF is impaired host defense.⁵⁴

Our findings help further characterize the nature of the host defense defect in patients with CF. Although the IL-17 cytokine response is robust, it might be the balance of IL-17's differing roles in early innate defense versus adaptive immune

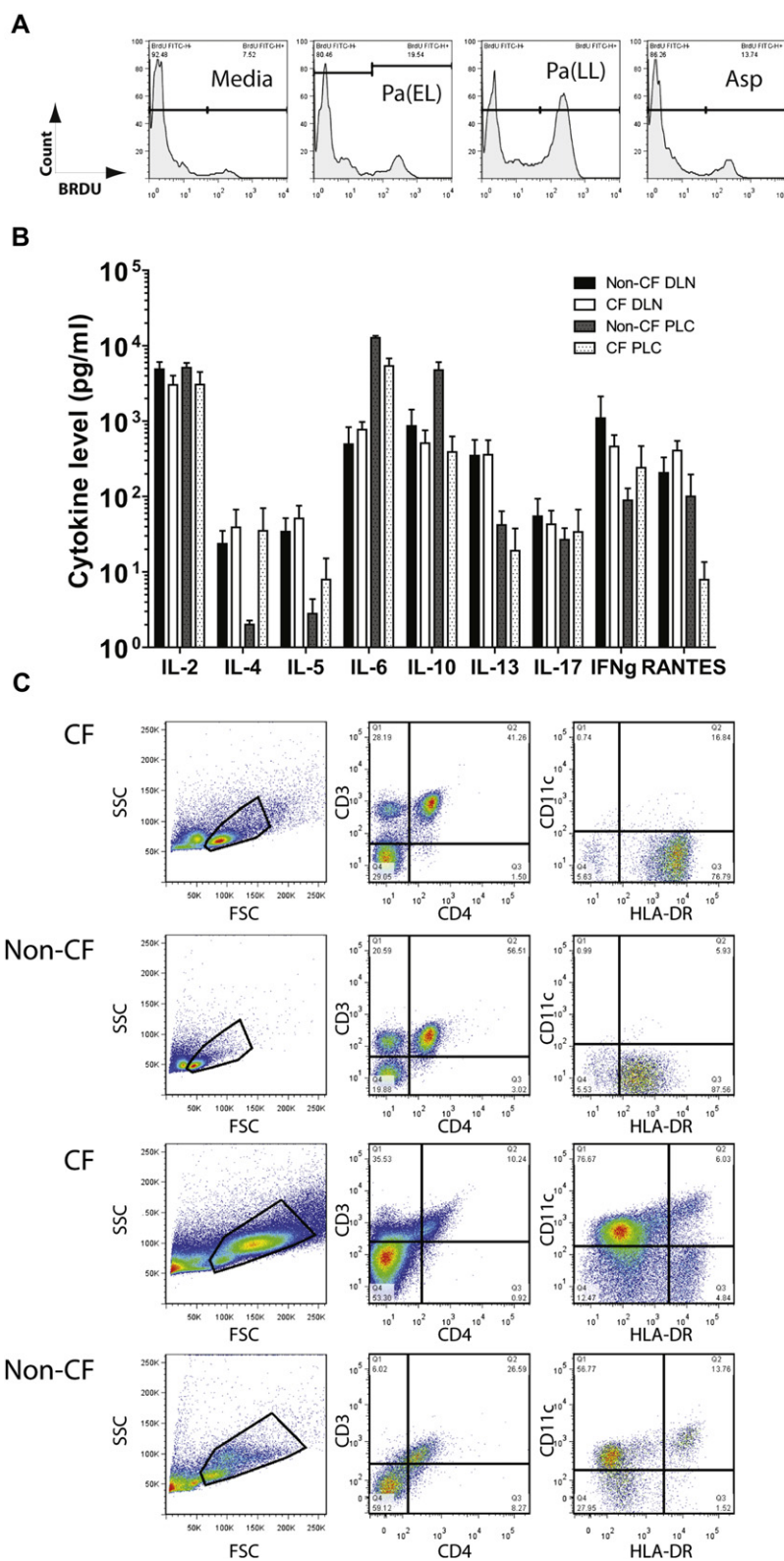


FIG 3. Antigen stimulation preparations and baseline characteristics of CF and non-CF samples. **A**, DLNs from patients with CF were stimulated as described, and bromodeoxyuridine (*BrdU*) histograms from antigen concentrations that were chosen are shown. **B**, Cultured DLN and PLC supernatants were assayed with the multiplex cytokine bead array, IL-17 mean absolute values with SEMs for each cohort and tissue sample are graphed. IL-17 levels are not remarkably different among all CF ($n = 10$) or control ($n = 4$) samples before stimulation. Culture media included IL-2. **C**, DLNs (top 2 rows of plots) and PLC (bottom 2 rows of plots) are assessed before culture with antigen stimulation. Representative dot plots are shown. Total events examined by size (forward scatter [*FSC*]) versus granularity (side scatter [*SSC*]) were first plotted, and a live cell gate was drawn. This live cell gate was subsequently analyzed for CD3 versus CD4 and CD11c versus HLA-DR.

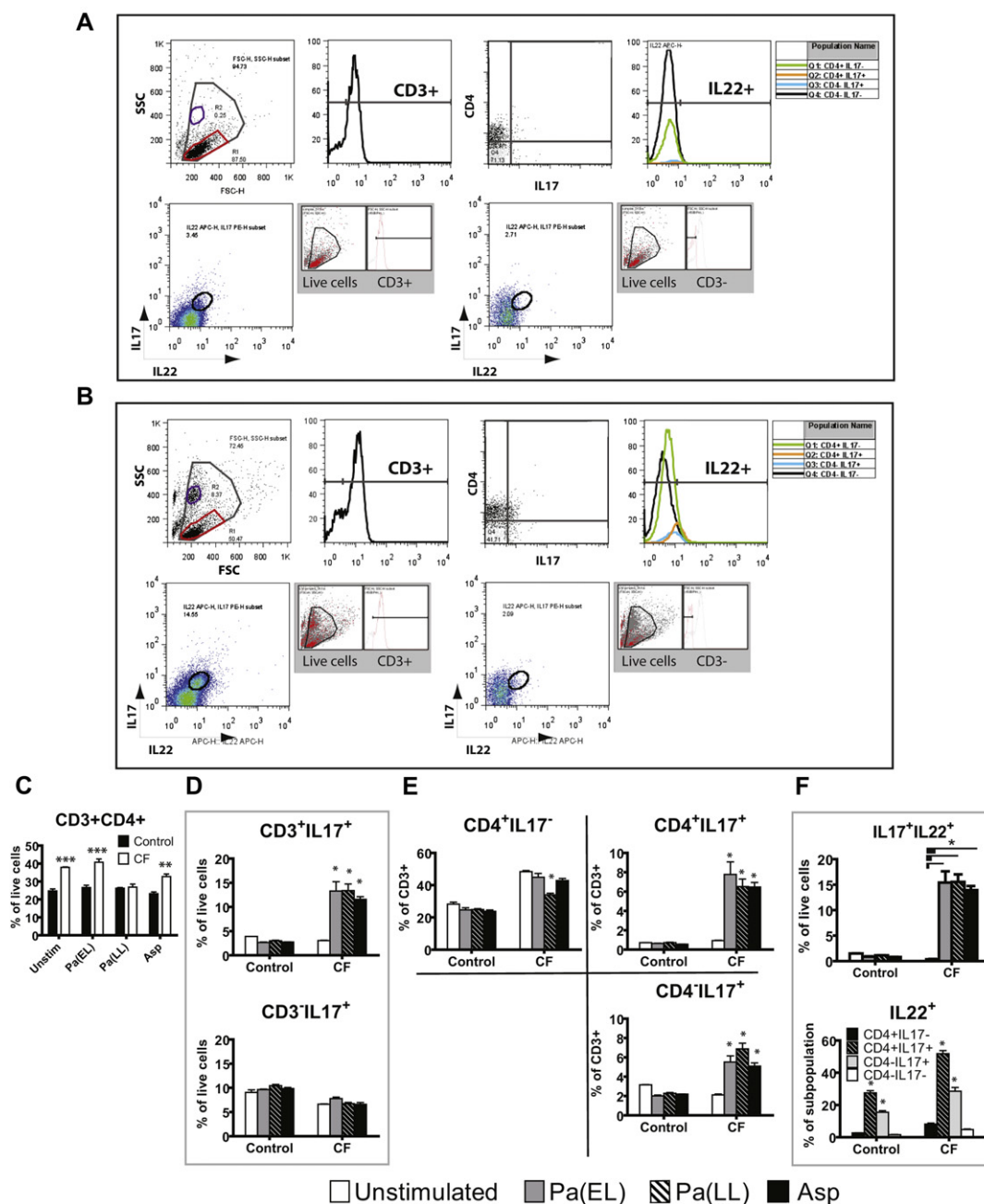


FIG 4. IL-17⁺ and IL-22⁺ T-cell numbers are increased in patients with CF. **A** and **B**, A Representative gating strategy for control subjects without CF (Fig 4, A) and patients with CF (Fig 4, B) is shown on a Pa(EL)-stimulated sample. Total events were analyzed based on size (forward scatter [FSC]) versus granularity (side scatter [SSC]), and a live cell gate (largest gray gate) was drawn. This gate was further examined by using univariate histogram analysis of CD3 status (top row, second from left). Gated CD3⁺ cells were then analyzed based on CD4 versus IL-17 (top row, third from left) and proportion of IL-22⁺ cells in each quadrant, as graphed in the second histogram. The second row in each lettered section shows all live cells that were CD3⁺ (first bivariate plot) and CD3⁻ (second bivariate plot) analyzed for IL-17 versus IL-22. An IL-17⁺IL-22⁺ double-positive gate was drawn, and back gating to show ancestry is shown in the gray inset. **C**, Control subjects without CF (black columns, n = 4) and patients with CF (open columns, n = 7) population results of bivariate analysis of the live cell gate by CD3 versus CD4. **D**, Cohort statistics by antigen stimulation of CD3⁺ and CD3⁻ cells that are IL-17⁺. **E**, Breakdown of quadrant statistics in the CD4 versus IL-17 plot by genotype and antigen stimulation. **F**, Top, Cohort statistics of the IL-17⁺IL-22⁺ double-positive gate by genotype and antigen stimulation. Bottom, IL-22⁺ statistics for each quadrant in the CD4 versus IL-17 analysis for each genotype and antigen stimulation. Legends for Fig 4, D, E, and F, are shown at the bottom of the figure, unless otherwise noted for Fig 4, F, bottom panel. *P < .05, **P < 0.001, and ***P < .0001.

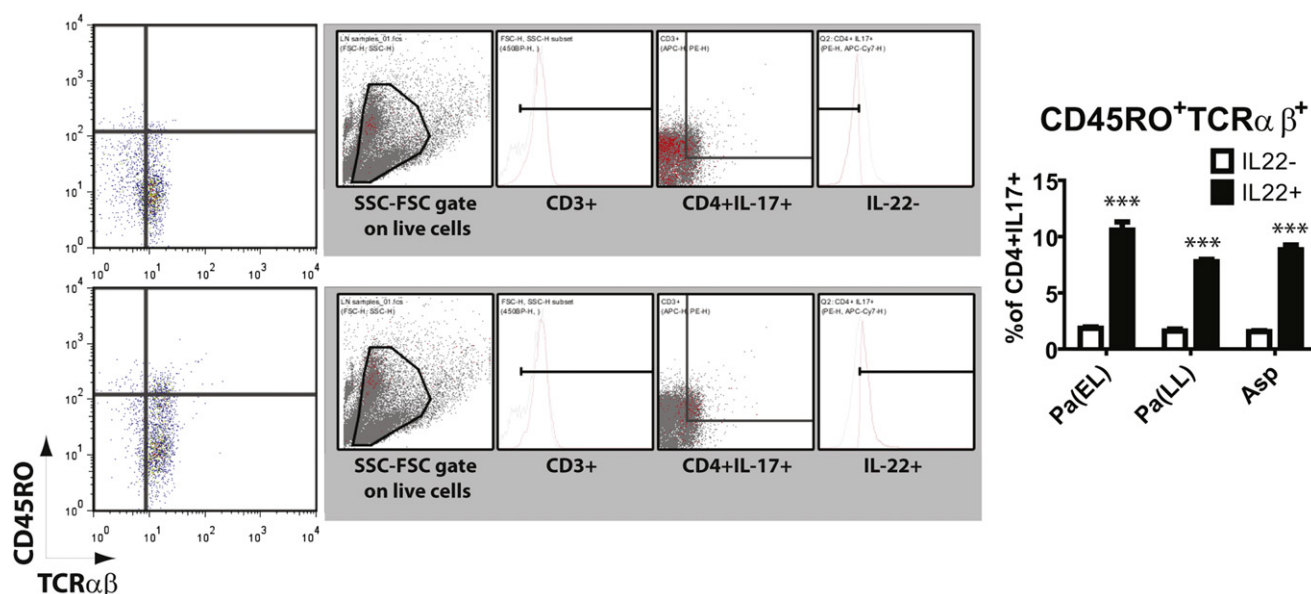


FIG 5. IL-22⁺ status correlates with memory T-cell characteristics. IL-22⁺ and IL-22⁻ cells in the live cell gate were analyzed for CD45RO versus TCRαβ expression. Representative plots, with back gating and ancestry shown in the gray inset next to each bivariate plot, for Pa(EL)-stimulated DLNs from patients with CF are shown. IL-22 positivity in CD4⁺IL-17⁺ cells correlated with CD45RO⁺TCRαβ⁺ status (right graph). **P* < .05, ***P* < .001, and ****P* < .0001. FSC, Forward scatter; SSC, side scatter.

development that ultimately determines effective bacterial eradication. Robust *de novo* T_H17 responses might keep a pathogen at bay and prevent septic dissemination, but the memory response ultimately eliminates it. On the other hand, this T_H17 response might represent unchecked tissue-damaging inflammation in the setting of ineffective innate antimicrobial mechanisms, which is similar to what has been shown in a murine model of *Aspergillus* species infection in patients with chronic granulomatous disease.⁵⁵ In the case of CF, impaired epithelial bicarbonate secretion,⁵⁶ which is required for killing activity of antimicrobial peptides,^{32,57} leads to a persistent IL-23/IL-17-mediated inflammatory response.^{55,58,59} The interaction of IL-17 with IL-22 might be an important switch point in immune development from danger signal-initiated tissue-damaging inflammation to a focused adaptive memory response. Further clinical studies characterizing the IL-17⁺ cell subsets identified in this study are certainly indicated.

Additional work characterizing the proliferation and clonality of the T_H17+IL-22⁺ population, as well as the status of the other memory T-cell markers CD62 ligand and CCR7, would be fruitful future work on prospective patients undergoing transplantation. If T_H17 cells prove to be clonally expanded after stimulation, this would open the possibility of following the status of these cells through the natural course of disease at earlier stages before end-stage CF by using bronchoscopic lavage or endobronchial ultrasound biopsy. If CD4⁺IL-17⁺IL-22⁻ and CD4⁺IL-17⁺IL-22⁺ cells represent T_H cells along the same developmental pathway at different stages, then understanding their function or plasticity along this pathway might provide a key therapeutic target in remediating tissue inflammation in patients with CF.

The striking difference between draining lymph nodes and the parenchymal lung also provides clues that the T_H17 response is defective or absent in areas of bacterial colonization. Although

the draining lymph nodes appear readily T_H17 responsive to *Pseudomonas* and *Aspergillus* species stimulation, parenchymal leukocytes were inert. This appears to be at odds with our findings that the parenchyma is simultaneously positive for IL-17 protein, as determined based on results of immunofluorescence staining and T_H17 pattern gene expression. A potential explanation for this is that IL-17 cytokine is likely elaborated by a variety of cell types in the parenchyma that were not purified in our preparation for flow cytometric and cytokine analysis.

Because of findings by Cella et al⁶⁰ that NK cells were a likely source of IL-22 in human subjects, we also examined lung sections for IL-22⁺ NK cells by examining dual positivity for CD56 and IL-22 (see Fig E3 in this article's Online Repository at www.jacionline.org). Although we did not replicate their findings as dramatically, this serves to illustrate the diversity of the innate immune response as it encounters infection from the upper airway to the lower respiratory tract.

Alveolar epithelium has also been shown to be a significant source of IL-22 message,⁶¹ and the lack of *RORC* upregulation (Fig 2) might point to a cell type other than T cells producing IL-17 in the parenchyma.

Finally, our own data showing inducible CD3⁺ but CD4⁻IL-17-producing cells speaks to the possibility of CD8 and TCRγδ IL-17 responders in various regions of the lung. The extent of parenchymal destruction in the lungs of patients with end-stage CF makes this difficult to discern and compare with others' results in more readily accessed and architecturally preserved tissue,⁶² despite extensive 3-dimensional reconstruction of confocal images (see Video E1). However, consistent with others' results in COPD,⁶³ we found that IL-17 might be in the parenchymal subepithelial tissue when examining more architecturally preserved lungs of patients with COPD (see Fig E4 and Video E1 in this article's Online Repository at www.jacionline.org). An alternative explanation to the paucity

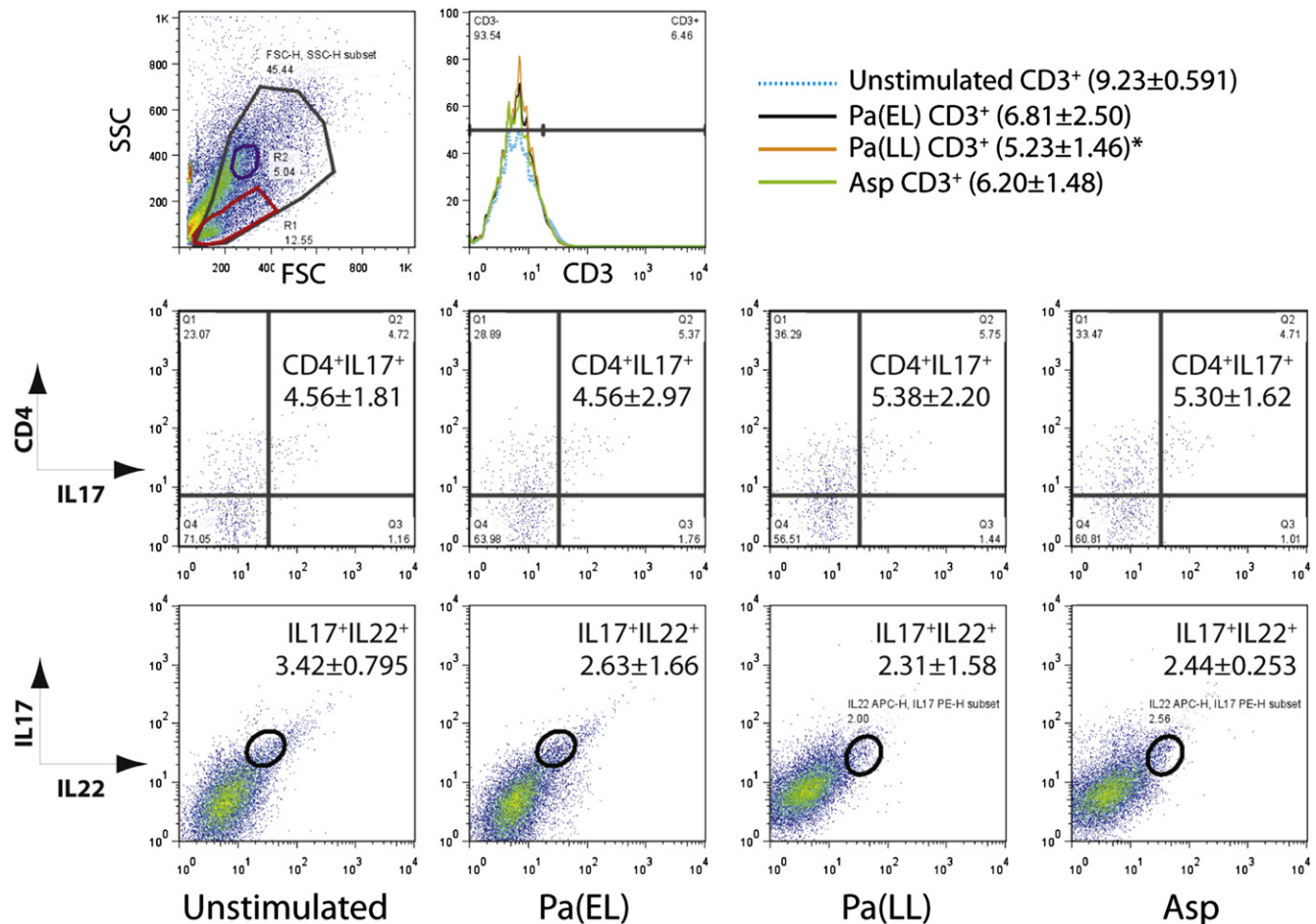


FIG 6. PLCs show minimal to modest IL-17 and IL-22 responses to antigen stimulation. Representative plots on a PLC sample from a patient with CF are shown. *Top row*, Live cell gate in the side scatter (SSC) versus forward scatter (FSC) plot was examined for CD3 status (*histogram*). Statistics for each stimulation are shown in the histogram legend. One stimulation condition resulted in a statistically significant reduction in the number of CD3⁺ cells relative to the unstimulated sample (Pa(LL)). The live cell gate was then examined for CD4 versus IL-17 (*second row of plots*), with statistics for the CD4⁺IL17⁺ quadrant shown for each antigen stimulation. Similarly, the live cell gate was examined for IL-17 versus IL-22 in each antigen stimulation, and statistics are shown for the IL-17⁺IL22⁺ quadrant (*bottom row of plots*). **P* < .05.

of IL-17⁺ cells in the parenchyma is that these cells are more apoptotic and sensitive to ischemic time after removal of the lung from the patient. Future studies devoted to characterizing apoptosis markers early on in this population would be important.

In light of work that supports a more mutually exclusive developmental pathway for T_H17 cells in the setting of T_H1 and T_H2 suppression,^{18,64} we report a similar result in human subjects. In contrast to murine models, however, we find that IL-13 plays a greater role than IL-4 and is consistent with work published by Newcomb et al.⁵⁰ This might explain the weak IL-17 signal in *Pseudomonas* species-stimulated parenchymal leukocytes because these samples had higher normalized poststimulation levels of IL-10, IL-13, and RANTES, a T_H1-polarizing dendritic cells cytokine.⁶⁵ However, we found that concurrent neutralization of IFN-γ was necessary for demonstrable IL-17 augmentation in *Pseudomonas* but not *Aspergillus* specie stimulation. The higher magnitude of IFN-γ even before stimulation (Fig 3, B) might be the reason for this, showing the powerful inhibition of T_H17 that can be mediated by IFN-γ. Conversely, IFN-γ neutralization

alone did not augment IL-17 in any cases. Further future studies examining the status of IL-13 receptor on both DLNs and PLCs in this population of patients are indicated. This finding also advises greater scrutiny of the current state of medical management in patients with obstructive lung diseases because it has been shown that combined therapy with prednisone and leukotriene inhibitors decrease IL-13 and IFN-γ secretion.⁶⁶ Potentially unopposed effects of IL-17 should be examined in this population. Issues that might confound this interpretation of the data are age differences and immunomodulating medication use in the 2 cohorts. Of the patients without CF for whom demographic data were available, there is clearly a difference in average age. It is not known whether the IL-17 response changes with age independently of pathogen exposure. The best comparison population to test for age effects would be patients with COPD with bronchiectasis who have also been colonized with *Pseudomonas* species. The data in our supplemental results on COPD samples suggests that the findings might trend similarly with CF, and this would need to be explored further. Presuming that CORE lungs came from patients that did not have a reason to receive long-term

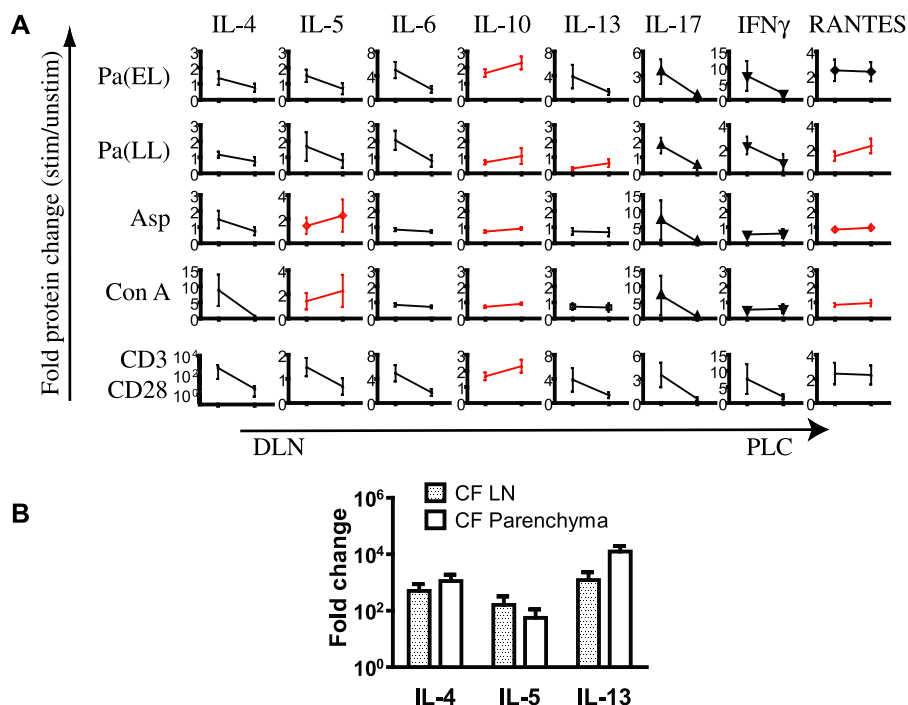


FIG 7. Presence of T_H2 or regulatory T-cell cytokines in the parenchyma might blunt the IL-17 response therein. **A**, Average normalized antigen-stimulated cytokine values for DLNs and PLCs from patients with CF were juxtaposed. Cytokines in which PLC numbers were greater than DLN numbers are in red. Among the cytokines exhibiting an increase in PLC numbers relative to DLN numbers are IL-10, IL-13, and RANTES for *Pseudomonas* antigen stimulation and IL-5, IL-10, and IL-13 for Asp stimulation. **B**, Quantitative real-time PCR of T_H2 cytokines in lymph nodes (LN) and parenchyma from patients with CF show a trend toward higher signals in the parenchyma.

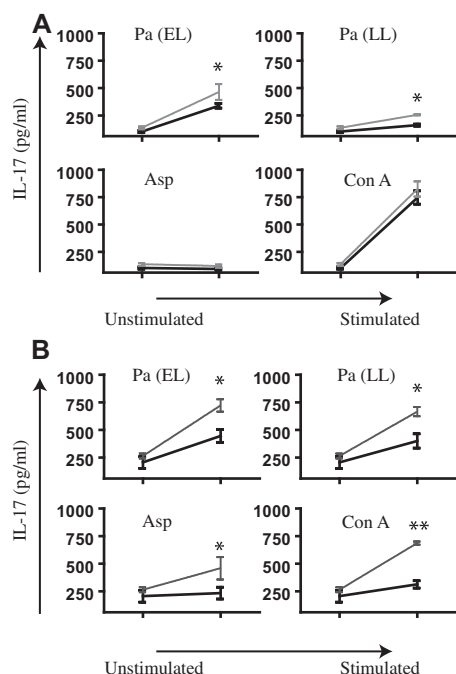


FIG 8. Neutralization of T_H1 and T_H2 cytokines augments the IL-17 response. **A**, DLNs from patients with CF ($n = 3$) were cultured with indicated antigen along with neutralizing antibodies to IL-4 and IFN- γ together (gray lines) and compared with no neutralization (black lines) with 20 μ g/mL IgG. **B**, IL-13 and IFN- γ were also neutralized (gray lines, $n = 3$) and compared with no neutralization (black lines). * $P < .05$ and ** $P < .001$.

systemic steroids, the rates of systemic steroid use were similar in both populations when comparing immunofluorescence and RNA data. However, our flow cytometric and cytokine comparisons were between lungs from patients with CF and CORE patients, presenting the possibility that systemic steroid use might have primed CF samples for a more robust IL-17 response. This again reinforces the need to evaluate the effects of routine clinical practice on lung immunologic response.

It would be interesting to further fractionate and explore the individual antigenic components of microbial extracts specifically leading to T_H17 development because we arbitrarily chose *Pseudomonas* species in the early and late log phase of growth for our studies. It is possible that antigens created from *Pseudomonas* species growing as a biofilm might more accurately reflect the true growth phase in live patients and that the antigens we used simulate CF “exacerbation” as the organism transforms from planktonic growth and disseminates. Further studies with clinical isolates of *Pseudomonas* and *Aspergillus* species from the same patients might provide more information about how the immune system is primed against these organisms.

Proliferation assays were used as a starting point to determine antigen stimulation, and it is recognized that measurement of proliferation and IL-17 release might only approximate T_H17 's physiologic effects. Furthermore, we separately measured IL-17F and IL-22 because these cytokines were not included in the 30-plex array. DLN cultures from patients with CF trended toward a higher antigen-specific IL-17F and IL-22 response, but the absolute magnitude of this response is comparatively larger than that for IL-17A (data not shown). This suggests that assays for

IL-17F and IL-22 might have greater sensitivity for detecting T_H17 memory responses in patients with CF.

Finally, this work establishes a streamlined protocol for isolating T_H17 cells from a population of patients undergoing transplantation. T_H1/T_H17 cells have been implicated in solid organ transplant rejection,⁶⁷⁻⁷¹ and these cells might have a role in predicting transplantation outcome.⁷² Thus we present a possible tool for use and study in prognostication. Prospective studies examining the presence of these cells and ultimate transplantation outcome are indicated.

Clinical implications: This work begins to elucidate the antimicrobial defect in patients with CF. Examining lung T_H17 cells is a potential tool for transplantation follow-up because T_H17 has been implicated in transplant rejection.

REFERENCES

- Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348-57.
- Garvy BA, Wiley JA, Gigliotti F, Harmsen AG. Protection against *Pneumocystis carinii* pneumonia by antibodies generated from either T helper 1 or T helper 2 responses. *Infect Immun* 1997;65:5052-6.
- Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, et al. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 1995;3:811-21.
- Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, et al. Human IL-17: a novel cytokine derived from T cells. *J Immunol* 1995;155:5483-6.
- Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus Saimiri gene. *J Immunol* 1993;150:5445-56.
- Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 1996;183:2593-603.
- Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 1999;162:2347-52.
- Laan M, Prause O, Miyamoto M, Sjostrand M, Hytonen AM, Kaneko T, et al. A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF- α . *Eur Respir J* 2003;21:387-93.
- Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 2001;194:519-27.
- Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, et al. Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. *Am J Respir Cell Mol Biol* 2001;25:335-40.
- Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ, et al. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J Exp Med* 2005;202:761-9.
- Raffatellu M, Santos RL, Verhoeven DE, George MD, Wilson RP, Winter SE, et al. Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes *Salmonella* dissemination from the gut. *Nat Med* 2008;14:421-8.
- Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor- β induces development of the T(H)17 lineage. *Nature* 2006;441:231-4.
- Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17F in host defense against mucocutaneous bacterial infection and allergic responses. *Immunity* 2009;30:108-19.
- Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* 2009;206:299-311.
- Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* 2010;129:311-21.
- Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235-8.
- Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;6:1123-32.
- Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005;6:1133-41.
- Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006;24:179-89.
- Puel A, Doffinger R, Natividad A, Chrabieh M, Barcenas-Morales G, Picard C, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med* 2010;207:291-7.
- Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003;97:726-33.
- Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β , IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111:1293-8.
- McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, et al. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene- α and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J Immunol* 2005;175:404-12.
- Di Stefano A, Caramori G, Gnemmi I, Contoli M, Vicari C, Capelli A, et al. T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol* 2009;157:316-24.
- Sethi S. Bacterial infection and the pathogenesis of COPD. *Chest* 2000;117(suppl):286S-91S.
- Yu JJ, Ruddy MJ, Wong GC, Sfintescu C, Baker PJ, Smith JB, et al. An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. *Blood* 2007;109:3794-802.
- Chan YR, Liu JS, Pociask DA, Zheng M, Mietzner TA, Berger T, et al. Lipocalin 2 is required for pulmonary host defense against *Klebsiella* infection. *J Immunol* 2009;182:4947-56.
- Kao C-Y, Chen Y, Thai P, Wachi S, Huang F, Kim C, et al. IL-17 Markedly up-regulates β -defensin-2 expression in human airway epithelium via JAK and NF- κ B signaling pathways. *J Immunol* 2004;173:3482-91.
- Karlsen JR, Borregaard N, Cowland JB. Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor- α is controlled by IkappaB-zeta but neither by C/EBP-beta nor C/EBP-delta. *J Biol Chem* 2010;285:14088-100.
- Kolls JK, McCray PB Jr, Chan YR. Cytokine-mediated regulation of antimicrobial proteins. *Nat Rev Immunol* 2008;8:829-35.
- Kreindler JL, Bertrand CA, Lee RJ, Karasic T, Aujla S, Pilewski JM, et al. Interleukin-17A induces bicarbonate secretion in normal human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L257-66.
- Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006;203:2271-9.
- Peric M, Koglin S, Kim SM, Morizane S, Besch R, Prinz JC, et al. IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J Immunol* 2008;181:8504-12.
- Shen F, Ruddy MJ, Plamondon P, Gaffen SL. Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF- α -induced genes in bone cells. *J Leukoc Biol* 2005;77:388-99.
- Meshi B, Vitalis TZ, Ionescu D, Elliott WM, Liu C, Wang XD, et al. Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. *Am J Respir Cell Mol Biol* 2002;26:52-7.
- Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, et al. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am J Respir Crit Care Med* 2006;173:1114-21.
- Patel IS, Seemungal TA, Wilks M, Lloyd-Owen SJ, Donaldson GC, Wedzicha JA. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax* 2002;57:759-64.
- Wilkinson TM, Patel IS, Wilks M, Donaldson GC, Wedzicha JA. Airway bacterial load and FEV1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;167:1090-5.
- Morris A, Sciurba FC, Lebedeva IP, Githaiga A, Elliott WM, Hogg JC, et al. Association of chronic obstructive pulmonary disease severity and *Pneumocystis* colonization. *Am J Respir Crit Care Med* 2004;170:408-13.

41. Morris A, Sciruba FC, Norris KA. *Pneumocystis*: a novel pathogen in chronic obstructive pulmonary disease? COPD 2008;5:43-51.
42. Elkington PT, Friedland JS. Matrix metalloproteinases in destructive pulmonary pathology. Thorax 2006;61:259-66.
43. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 2004;4:617-29.
44. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against gram-negative bacterial pneumonia. Nat Med 2008;14:275-81.
45. Kruisbeek A. Isolation of mouse mononuclear cells. In: Current protocols in immunology. New York: Wiley; 2001. p. 3.1.-3.1.5.
46. Lefrançois L, Lycke N. Isolation of mouse small intestinal intraepithelial lymphocytes, peyer's patch, and lamina propria cells. In: Current protocols in immunology. New York: Wiley; 2001. p. 3.19.
47. Kreindler JL, Steele C, Nguyen N, Chan YR, Pilewski JM, Alcorn JF, et al. Vitamin D3 attenuates Th2 responses to *Aspergillus fumigatus* mounted by CD4+ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. J Clin Invest 2010;120:3242-54.
48. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity 2008;28:29-39.
49. Newcomb DC, Zhou W, Moore ML, Goleniewska K, Hershey GK, Kolls JK, et al. A functional IL-13 receptor is expressed on polarized murine CD4+ Th17 cells and IL-13 signaling attenuates Th17 cytokine production. J Immunol 2009;182:5317-21.
50. Newcomb DC, Boswell MG, Zhou W, Huckabee MM, Goleniewska K, Sevin CM, et al. Human TH17 cells express a functional IL-13 receptor and IL-13 attenuates IL-17A production. Journal Allergy Clin Immunol 2011;127:1006-13, e1-4.
51. Naess LM, Oftung F, Aase A, Wetzler LM, Sandin R, Michaelsen TE. Human T-cell responses after vaccination with the Norwegian group B meningococcal outer membrane vesicle vaccine. Infect Immun 1998;66:959-65.
52. Arce F, Rowe HM, Chain B, Lopes L, Collins MK. Lentiviral vectors transduce proliferating dendritic cell precursors leading to persistent antigen presentation and immunization. Mol Ther 2009;17:1643-50.
53. Rowe HM, Lopes L, Ikeda Y, Bailey R, Barde I, Zenke M, et al. Immunization with a lentiviral vector stimulates both CD4 and CD8 T cell responses to an ovalbumin transgene. Mol Ther 2006;13:310-9.
54. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, et al. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. Sci Transl Med 2010;2:29ra31.
55. Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, et al. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. Nature 2008;451:211-5.
56. Coakley RD, Grubb BR, Paradiso AM, Gatzky JT, Johnson LG, Kreda SM, et al. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. Proc Natl Acad Sci U S A 2003;100:16083-8.
57. Dorschner RA, Lopez-Garcia B, Peschel A, Kraus D, Morikawa K, Nizet V, et al. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. FASEB J 2006;20:35-42.
58. Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, et al. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 2007;37:2695-706.
59. Dubin PJ, Kolls JK. IL-23 mediates inflammatory responses to mucoid *Pseudomonas aeruginosa* lung infection in mice. Am J Physiol Lung Cell Mol Physiol 2007;292:L519-28.
60. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 2009;457:722-5.
61. Whittington HA, Armstrong L, Uppington KM, Millar AB. Interleukin-22: a potential immunomodulatory molecule in the lung. Am J Respir Cell Mol Biol 2004;31:220-6.
62. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest 2009;119:3573-85.
63. Shan M, Cheng HF, Song LZ, Roberts L, Green L, Hacken-Bitar J, et al. Lung myeloid dendritic cells coordinately induce TH1 and TH17 responses in human emphysema. Sci Transl Med 2009;1:4ra10.
64. Walsh KP, Brady MT, Finlay CM, Boon L, Mills KH. Infection with a helminth parasite attenuates autoimmunity through TGF-beta-mediated suppression of Th17 and Th1 responses. J Immunol 2009;183:1577-86.
65. Lebre MC, Burwell T, Vieira PL, Lora J, Coyle AJ, Kapsenberg ML, et al. Differential expression of inflammatory chemokines by Th1- and Th2-cell promoting dendritic cells: a role for different mature dendritic cell populations in attracting appropriate effector cells to peripheral sites of inflammation. Immunol Cell Biol 2005;83:525-35.
66. Gibbons FK, Israel E, Deykin A, Schaub B, He HZ, Perkins DL, et al. The combined effects of zafirlukast, prednisone, and inhaled budesonide on IL-13 and IFN-gamma secretion. J Clin Immunol 2005;25:437-44.
67. Braun RK, Molitor-Dart M, Wigfield C, Xiang Z, Fain SB, Jankowska-Gan E, et al. Transfer of tolerance to collagen type V suppresses T-helper-cell-17 lymphocyte-mediated acute lung transplant rejection. Transplantation 2009;88:1341-8.
68. Deteix C, Attuili-Audenis V, Duthey A, Patey N, McGregor B, Dubois V, et al. Intragraft Th17 infiltrate promotes lymphoid neogenesis and hastens clinical chronic rejection. J Immunol 2010;184:5344-51.
69. Vokaer B, Van Rompaey N, Lemaitre PH, Lhomme F, Kubjak C, Benghiat FS, et al. Critical role of regulatory T cells in Th17-mediated minor antigen-disparate rejection. J Immunol 2010;185:3417-25.
70. Wang S, Li J, Xie A, Wang G, Xia N, Ye P, et al. Dynamic changes in Th1, Th17, and FoxP3(+) T cells in patients with acute cellular rejection after cardiac transplantation. Clin Transplant 2011;25:E177-86.
71. Xie A, Wang S, Zhang K, Wang G, Ye P, Li J, et al. Treatment With interleukin-12/23p40 antibody attenuates acute cardiac allograft rejection. Transplantation 2011;91:27-34.
72. Carvalho A, Cunha C, Di Ianni M, Pitzurra L, Aloisi T, Falzetti F, et al. Prognostic significance of genetic variants in the IL-23/Th17 pathway for the outcome of T cell-depleted allogeneic stem cell transplantation. Bone Marrow Transplant 2010;45:1645-52.

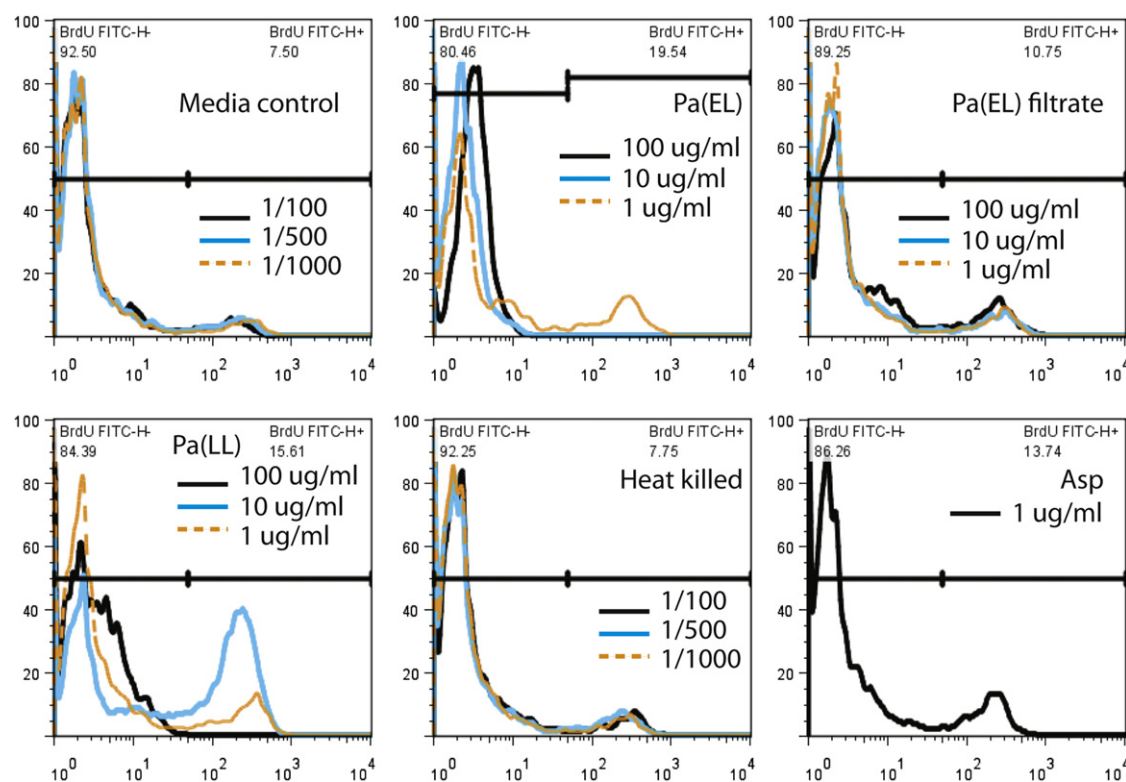


FIG E1. Antigen panel testing. DLN cultures were stimulated in triplicate with a panel of microbially derived antigens in the presence of bromodeoxyuridine (*BrdU*), which was then detected with fluorescein isothiocyanate (*FITC*)-labeled anti-*BrdU*. Proliferation was assessed by using single-channel flow cytometric analysis of total cell cultures for *FITC* incorporation. Representative graphs are shown. *Heat killed*, Heat-killed *Pseudomonas aeruginosa*; *Media control*, tryptic soy broth; *Pa(EL)*, early log-phase sonicate of *P aeruginosa*; *Pa(EL) filtrate*, filtrate of early log-phase *P aeruginosa* culture; *Pa(LL)*, filtrate of stationary-phase *P aeruginosa* culture.

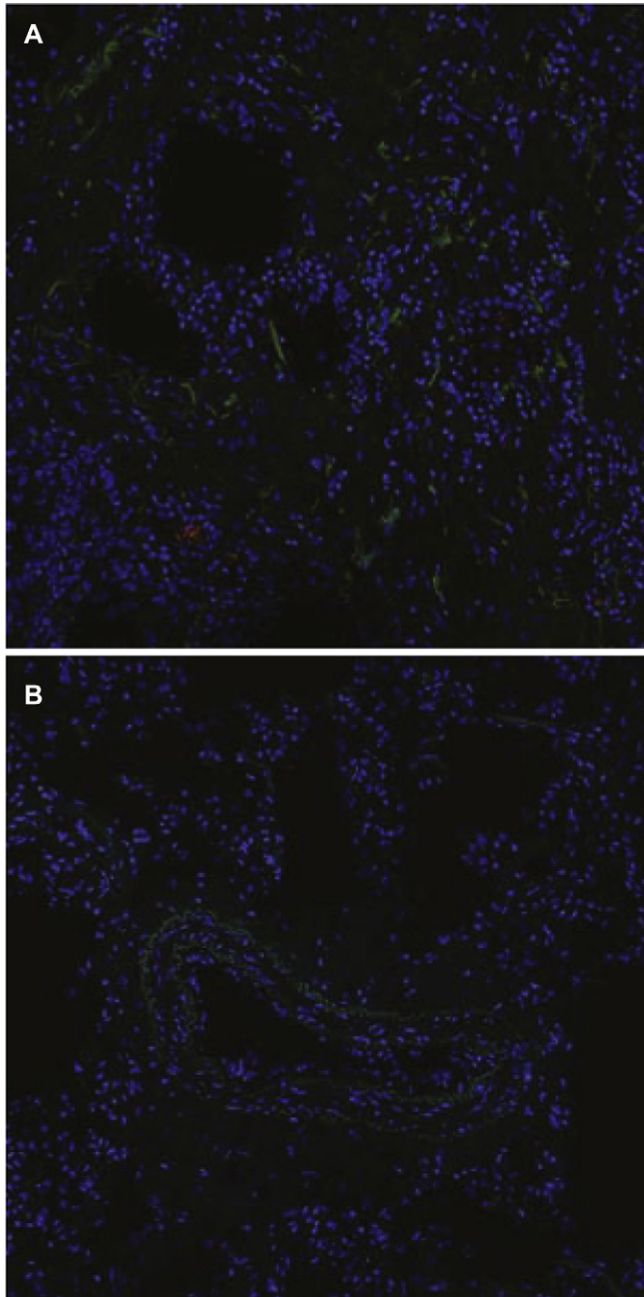


FIG E2. Isotype antibody controls. CF (*top photomicrograph*) and non-CF (*bottom photomicrograph*) samples were treated with goat, rabbit, and mouse IgG followed by donkey anti-isotype fluorescent secondary antibodies. Photos shown were taken at $\times 20$ magnification and demonstrate minimal nonspecific staining related to species interactivity.

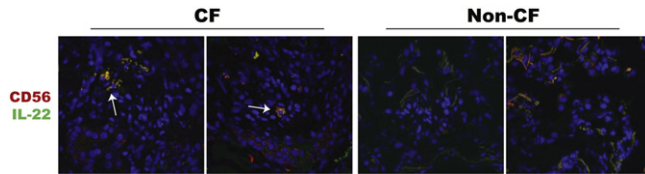


FIG E3. IL-22⁺ NK cells in patients with CF. OCT-embedded tissues were fixed and stained as described, and the *left 2 panels* show 2 patients with CF and the presence of IL-22⁺ cells that costain with CD56 (*white arrows*, yellow merge). These cells are not present in non-CF control samples (*right 2 panels*).

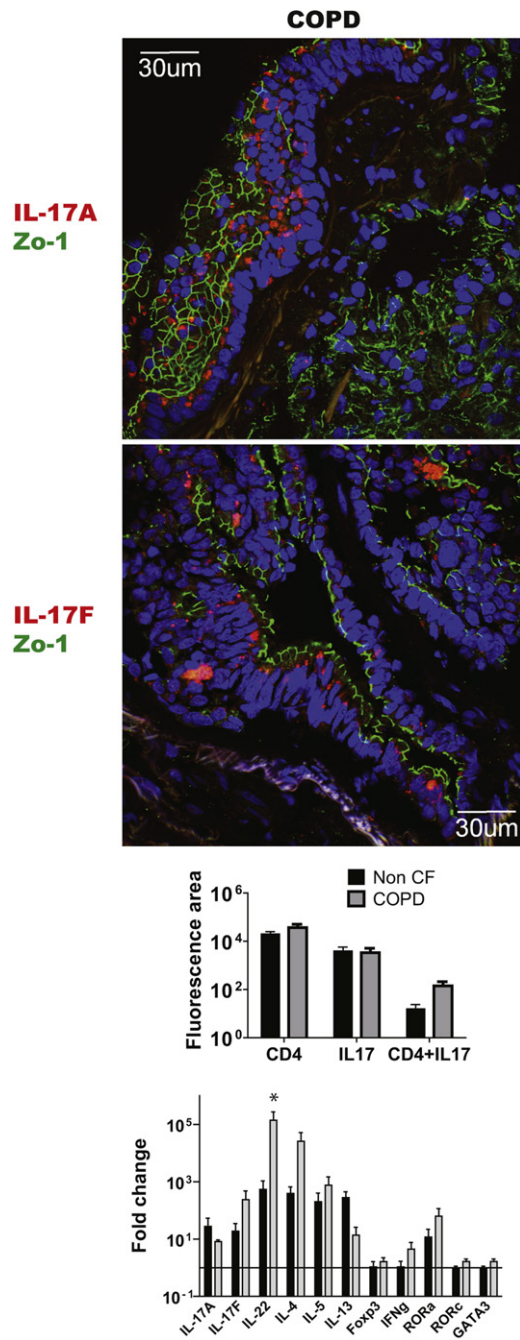


FIG E4. IL-17 in tissues from patients with COPD. OCT-embedded tissues were sectioned and stained with antibodies against IL-17A and IL-17F. Patients with COPD are similarly positive, but the distribution of staining differs from the more widely distributed and diffuse submucosal pattern in patients with CF. Noted here is a subepithelial focal pattern of staining. Patients with COPD also trend higher in CD4⁺IL-17⁺ pixel area on quantitative confocal analysis and demonstrate high levels of IL-22 message in the parenchyma (Non-CF, n = 9; COPD, n = 5).

TABLE E1. Demographic characteristics of patients with COPD

Age	Sex	Immunomodulators	Pathogen status	Studies
68	F	None	<i>Streptococcus viridans</i> , <i>Neisseria</i> species, yeast	IF, R
68	M	Fluticasone/salmeterol inhaled Prednisone, 5 mg/d	<i>Pseudomonas aeruginosa</i> , <i>Streptococcus viridans</i> , <i>Neisseria</i> species, <i>Propionibacterium</i> species, <i>Micrococcus</i> species	IF, R
48	M	Fluticasone/salmeterol inhaled Prednisone, 5 mg/d	<i>Streptococcus viridans</i> , <i>Neisseria</i> species	IF, R
50	F	Fluticasone/salmeterol inhaled Prednisone, 10 mg/d	<i>Streptococcus viridans</i> , <i>Neisseria</i> species, yeast	IF, R
63	F	Prednisone, 20 mg/d	<i>Staphylococcus aureus</i>	IF, R

Demographic information on patients with COPD from whom lungs were collected is shown along with a key describing the studies each sample underwent: *IF*, immunofluorescence studies on OCT-embedded tissue blocks; *R*, real-time PCR on serial sections of OCT-embedded blocks.

F, Female; *M*, male.