Abnormal Nasal Nitric Oxide Production, Ciliary Beat Frequency, and Toll-like Receptor Response in Pulmonary Nontuberculous Mycobacterial Disease Epithelium

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Rationale: **Pulmonary nontuberculous mycobacterial (PNTM) disease has increased over the past several decades, especially in older women. Despite extensive investigation, no consistent immunological abnormalities have been found. Using evidence from diseases such as cystic fibrosis and primary ciliary dyskinesia, in which mucociliary dysfunction predisposes subjects to high rates of nontuberculous mycobacterial disease that increase with age, we investigated correlates of mucociliary function in subjects with PNTM infections and healthy control subjects.**

Objectives: **To define *ex vivo* characteristics of PNTM disease.**

Methods: **From 2009 to 2012, 58 subjects with PNTM infections and 40 control subjects were recruited. Nasal nitric oxide (nNO) was determined at the time of respiratory epithelial collection. Ciliary beat frequency at rest and in response to Toll-like receptor (TLR) and other agonists was determined using high-speed video microscopy.**

Measurements and Main Results: **We found decreased nNO production, abnormally low resting ciliary beat frequency, and abnormal responses to agonists of TLR2, -3, -5, -7/8, and -9 in subjects with PNTM compared with healthy control subjects. The low ciliary beat frequency in subjects with PNTM was normalized *ex vivo* by augmentation of the NO–cyclic guanosine monophosphate pathway without normalization of their TLR agonist responses.**

Conclusions: **Impaired nNO, ciliary beat frequency, and TLR responses in PNTM disease epithelium identify possible underlying susceptibility mechanisms as well as possible avenues for directed investigation and therapy.**

Keywords: nontuberculous mycobacteria infections; cilia beat frequency; nasal nitric oxide; Toll-like receptors

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pulmonary nontuberculous mycobacterial disease has increased over the past several decades, especially in older women. Despite a distinctive endomorphotype, no consistent immunological abnormalities have been found.

What This Study Adds to the Field

This study describes functional cellular characteristics that distinguish subjects with pulmonary nontuberculous mycobacterial disease from control subjects that are suggestive of a genetic predisposition. These differences also provide a possible therapeutic intervention.

Nontuberculous mycobacteria (NTM) are widely distributed in the environment but rarely cause disease (1). Disseminated disease occurs only in the setting of relatively rare specific host immune defects affecting IFN-γ or IL-12 activity (2–4). Pulmonary NTM (PNTM) disease occurs in conditions with underlying structural lung damage or impaired mucociliary clearance, such as cystic fibrosis (CF) or primary ciliary dyskinesia (PCD). The rate of PNTM in the general population has been increasing over the past few decades, especially in elderly women (5–8).

PNTM infection in postmenopausal women without recognized preexisting conditions was described in 1989 (9). The clinical phenotype includes lean and tall women whose onset of symptoms occurs in the sixth decade (10–14). These women also have high rates of scoliosis (51%), pectus excavatum (11%), mitral valve prolapse (9%), and mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (36%) (15). The likely role of genetic factor(s) contributing to PNTM susceptibility is evidenced by a case-control study that favors a "susceptible persons" model of PNTM disease (16, 17) and familial clustering of PNTM disease (18, 19). Despite the importance of immune control for disseminated NTM disease, patients with PNTM have relatively normal lymphocyte subsets and intact IL-12/IFN -γ axes (15, 20–22).

Mucociliary clearance is a critical determinant of PNTM infections, as evinced by the elevated rates of disease in CF and PCD (23). In both of these groups, immune function appears to be relatively normal despite the fact that the rate of PNTM is high and increases dramatically with age (24, 25). Therefore, considering the importance of respiratory epithelial cells in mucociliary clearance and their impairment in CF and

PCD, we hypothesized that respiratory epithelium might be critical to PNTM disease predisposition. Mucociliary function reflects the composite and dynamic interaction of ciliary beat frequency (CBF), ciliary waveform, and mucus viscosity. Mucus clearance is linearly dependent on CBF (26, 27), and nitric oxide (NO) (28) is known to be involved in regulating CBF. NO is released from the sinus respiratory epithelium and is thought to affect tissue repair and pulmonary arterial pressures and has a direct toxic effect on certain pathogens (29).

We found reductions in nasal NO (nNO) production and CBF in patients with PNTM. Patients with PNTM also had decreased CBF responses to selected Toll-like receptor (TLR) agonists. Most importantly, PNTM patient epithelial cell CBF could be normalized *ex vivo* by therapeutic augmentation of the NO–cyclic guanosine monophosphate (cGMP) pathway. These data provide a mechanistic basis for susceptibility to PNTM disease that can be targeted. Some preliminary results from this study were previously reported in the form of conference abstracts (30).

METHODS

A detailed description of the methods is provided in the online supplement.

Patient Recruitment

Recruitment of patients with PNTM, control subjects with disease, and healthy control subjects occurred from November 2009 to August 2012 at the Clinical Center, National Institutes of Health, Bethesda, Maryland. All patients and normal volunteers provided informed consent under approved National Institutes of Health protocols. All patients with PNTM had microbiologic and radiographic evidence of PNTM infection and fulfilled the American Thoracic Society criteria for PNTM disease (10, 15, 18). Patients with PNTM did not meet diagnostic criteria for CF or PCD. Control patients with CF had abnormal sweat chloride tests and known disease-causing mutations in CFTR. Patients diagnosed with PCD met published criteria (24, 31). CFTR status was determined by full gene sequence of CFTR (Ambry Genetics, Aliso Viejo, CA).

Collection and Analysis of Respiratory Epithelium

Primary human respiratory epithelial cells were collected as described in the online supplement and as previously described (24). Primary patient epithelial samples and normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) were visualized at time of collection (day of collection [DOC]) and after mucociliary differentiation at the air–liquid interface (ALI).

Symptoms of acute upper respiratory tract infection at the time of collection were associated with decreased CBF when compared with the individual's baseline (data not shown). We included only CBF measurements taken in the absence of symptomatic upper respiratory tract infection and without evidence of inflammation by visual inspection.

Measurement of nNO

nNO was measured by direct sampling through a NO analyzer (model 280i; Sievers Instrument, Boulder, CO) and reported as steady-state production of NO in nl/min (24).

Epithelial Stimulation

TLR location and reactivity differ between lung epithelium and leukocytes (32). Stimulating TLRs in airway epithelial cells requires higher agonist concentrations than for phagocytic cells (33). Samples were stimulated with single doses of TLR agonists (Invivogen, San Diego, CA): TLR2, 10 μg/ml Pam3CSK4; TLR3, 25 μg/ml poly(I:C) high molecular weight; TLR4, 5 μg/ml ultrapure *Escherichia coli* K12–derived LPS; TLR5, 100 ng/ml recombinant Flagellin; TLR7/8, 5 μg/ml imidazoquinoline compound R848 derivative CL097; TLR9, 4 μg/ml ODN 2006 Type B CpG oligonucleotide. Samples were also stimulated with 1 mM 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP) (Sigma, St. Louis, MO), sildenafil citrate (10 μM) (Tocris, Bristol, UK), or 1 mM *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (Sigma). Clinical strains of *Mycobacterium avium* (MAC) and *Mycobacterium abscessus* (MAB) were cultured as previously described (34). Respiratory epithelial infection experiments were done in antibiotic/antifungal-free ALI media.

Cytokine Measurement

Supernatants from select healthy control and PNTM ALI cultures were examined for IL-6, IL-8, TNF-α, G-CSF, IFN-γ–inducible protein 10, and monocyte chemoattractant protein-1 in duplicate using bioluminescent beads (Bio-Rad, Hercules, CA) as previously described (15).

Statistical Analysis

Data, including figures, are expressed as mean ± SD. Statistical testing was done using two-tailed Student's *t* test with Welch's correction and one-way ANOVA with Bonferroni correction.

RESULTS

Demographics

Fifty-eight patients with PNTM, eight patients with PCD, five patients with CF, one IRAK4-deficient patient (35), and 41 healthy control subjects seen at the National Institutes of Health from 2009 to 2012 were recruited. The PNTM cohort has been previously described (15) (Tables 1 and 2 and *see* the online supplement).

Patients with PNTM Have Reduced CBF

To determine CBF in PNTM patient airway epithelium, we examined cells on DOC (Figure 1a). Healthy control subjects ($n = 41$) had a mean CBF of 10.03 ± 0.97 Hz, comparable to published values (36). Patients with PCD ($n = 8$) were studied as a control group known to have decreased CBF (5.0 ± 5.5 Hz, similar to published reports [37]). Patients with CF ($n = 5$; CBF, 10.8 ± 1.1 Hz) were included as a control population that has chronic infections. Patients with PNTM had a CBF of 7.97 ± 1.09 Hz, significantly different from healthy subjects, patients with PCD, and control subjects with CF. CF CBF was similar to healthy control CBF as previously reported (38).

The differences between healthy control and PNTM CBF were present when measured at room temperature (22°C) and at physiological temperature (37°C) (*see* Figure E1a in the online supplement) (39). Similar to prior reports (40), CBF did not vary greatly by age in healthy control subjects or in patients with PNTM (Figure 1b). CBF on DOC cells was repeatedly measured in one subject throughout the study and did not significantly change (Figure E1b). To control for confounding factors not related to ciliary function (e.g., host inflammatory mediators) that might influence DOC samples, we differentiated a subset of healthy control and PNTM patient cells at the ALI (Figure 1c). In contrast, in subjects with chronic sinusitis the depressed CBF is transient and is suspected to be due to host inflammatory response (41) because the decreased CBF quickly normalizes when the samples are cultured *ex vivo*.

Patients with PNTM Have Normal Cilia Length

Because cilia length could affect ciliary function (42), we examined it in our patient population. Cilia length was the same for healthy control subjects (6.08 ± 0.85 μm) and patients with PNTM (6.14 ± 1.04 μm) (Figure E1c).

TABLE 1. POPULATION CHARACTERISTICS AND BACTERIAL ORGANISMS

Characteristics	Patients with PNTM (*n* = 58)	PCD (*n* = 8)	CF (*n* = 5)	Healthy Control Subjects (*n* = 41)
Mean ± SD age, yr	63 ± 9	36 ± 18	46 ± 14	40 ± 15
Female gender	51 (88)*	7 (88)	2 (40)	36 (88)
Ethnicity				
White	54 (93)	5 (62)	5 (100)	26 (63)
Asian	4 (7)	1 (13)	0 (0)	3 (7)
Never smokers	33 (57)	8 (100)	5 (100)	nd
CFTR carriers	14 (24)	3 (38)	5 (100)	nd
Scoliosis	30 (52)	nd	nd	nd
Joint hypermobility	12 (21)	nd	nd	nd
Positive thumb–wrist sign	12 (21)	nd	nd	nd
Mitral valve prolapse	4 (7)	0 (0)	nd	nd
Cough	55 (95)	8 (100)	5 (100)	nd
Bronchiectasis	58 (100)	8 (100)	5 (100)	nd
NTM positive sputum at collection	31 (53)	5 (63)	3 (60)	nd
On NTM therapy at collection	31 (53)	6 (75)	5 (100)	nd
Elevated inflammatory markers at collection	39 (67)	6 (75)	2 (40)	nd
Macrolide exposure at collection	24 (47)	4 (50)	5 (100)	nd
Immunomodulatory drugs	4 (7)	0 (0)	2 (40)	nd
BMI ± SD	21.7 ± 4.7	22.3 ± 8.3	26.3 ± 6.5	nd
Mycobacteria				
MAC	41 (71)	3 (60)	1 (33)	
MAB	6 (10)	2 (40)	2 (67)	
MAC and MAB	10 (17)			
Mycobacterium xenopi	1 (2)			

Definition of abbreviations: BMI = body mass index; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator; MAC = clinical strain of *Mycobacterium avium*; MAB = clinical strain of *Mycobacterium abscessus*; nd = not detected; NTM = nontuberculous mycobacteria; PCD = primary ciliary dyskinesia; PNTM = pulmonary nontuberculous mycobacteria.

*Values are n (%) unless otherwise noted.

CFTR Mutations Do Not Influence CBF

There is a high prevalence of CFTR mutations in patients with PNTM. We examined whether the presence of a CFTR mutation correlated with CBF and found that patients with PNTM had lower CBF regardless of CFTR mutation status (Figure 1d). Therefore, CFTR mutations do not seem to cause reduced CBF in patients with PNTM. No other PNTM characteristic segregated with CBF.

Mycobacterial Infection Does Not Reduce CBF in Patients with PNTM

Because patients with PNTM harbor mycobacteria, bacterial growth *per se* could account for depression of PNTM patient CBF. To test if NTM infection played a role in the cultures, PNTM and healthy control ALI cultures were tested for NTM infection. No organisms were recovered after 6 weeks of culture (data not shown). To further assess whether mycobacterial infection could inhibit CBF, NHBE cells were cultured at the ALI and exposed to MAB or MAC every 3 days over an 8-day course (multiplicity of infection, 5:1). A significant increase in CBF was observed in NHBE cultures exposed to MAB or MAC compared with uninfected cultures (Figure 1e). The increase in CBF was similar in MAB- and MAC-infected cultures. These data suggest that infection does not account for the reduction in CBF seen in patients with PNTM.

Released Soluble Factors Do Not Account for the Lowered CBF in Patients with PNTM

Soluble factors can modify ciliary motility (43). To determine if PNTM epithelial cells were producing soluble factors that reduced CBF, we transferred conditioned media from PNTM or NHBE cultures onto uninfected NHBE cultures over the course of 2 weeks. PNTM-conditioned media did not alter CBF in NHBE cultures, suggesting that a soluble factor from PNTM epithelial cells is not responsible for the decreased CBF seen in PNTM patient cells (Figure 1f).

Although conditioned media from unstimulated PNTM epithelial cells did not induce lowered CBF in NHBE cultures, we wondered whether differential induction of soluble factors after stimulation could explain the altered CBF. To assess this, we used TLR agonists to stimulate respiratory epithelial cells differentiated at ALI from healthy volunteers and patients with PNTM. No significant differences were noted between patients

TABLE 2. ORGANISMS RECOVERED IN THE PREVIOUS 2 YEARS

Infection History	Patients with PNTM (*n* = 58)	PCD (*n* = 8)	CF (*n* = 5)
Bacteria	*P. aeruginosa*, n = 18	*P. aeruginosa*, n = 6	*P. aeruginosa*, n = 2
	S. maltophilia, n = 6	*S. pneumonia*, n = 2	*Staphylococcus* species, n = 2
	Achromobacter species, n = 4	*Staphylococcus* aureus, n = 1	*Burkholderia gladioli*, n = 2
	Other bacteria, n = 12		Other bacteria, n = 2
Fungi	*Aspergillus* species, n = 25	*Penicillium* species, n = 3	*Aspergillus* species, n = 3
	Penicillium species, n = 18	*Aspergillus* species, n = 2	*Penicillium* species, n = 3
	Dactylaria gallopava, n = 5	*Fusarium* species n = 2	*Fusarium* species n = 1
	Other fungi, n = 90	Other fungi, n = 19	Other fungi, n = 5

Definition of abbreviations: CF = cystic fibrosis; PCD = primary ciliary dyskinesia; PNTM = pulmonary nontuberculous mycobacteria.

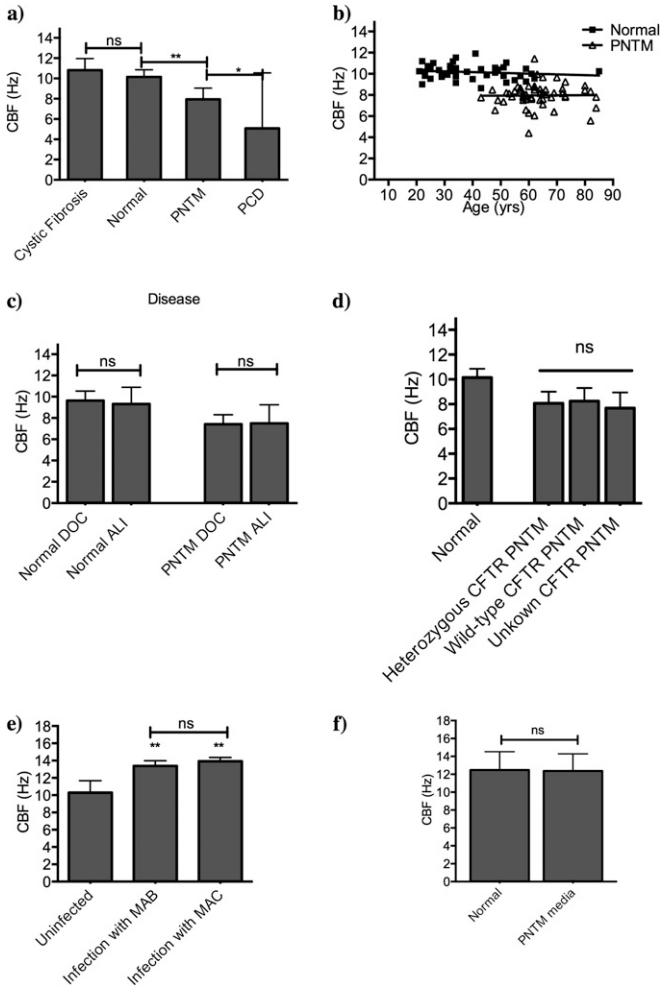

Figure 1. Determinants of ciliary beat frequency (CBF) in patients and healthy control subjects. (*a*) Absolute CBF on day of collection (DOC) for cystic fibrosis (CF) (n = 5; 10.8 ± 1.1 Hz), healthy control subjects (n = 41; 10.03 ± 0.96 Hz), pulmonary nontuberculous mycobacteria (PNTM) (n = 58; 7.97 ± 1.09 Hz), and primary ciliary dyskinesia (PCD) (n = 8; 5.0 ± 5.5 Hz). PNTM CBF is significantly different from healthy, PCD, and CF control CBF ($P < 0.0001$, $P < 0.04$, and $P < 0.001$, respectively; two-tailed *t* test). CF CBF is not significantly different from healthy control CBF. (*b*) Age (yr) versus CBF (Hz) for healthy control subjects (n = 41) and patients with PNTM (n = 58). Linear regression for healthy control subjects ($y = -0.007x + 10.46$ Hz) and patients with PNTM ($y = -0.002x + 8.11$ Hz). (*c*) Comparison of healthy control (n = 6) and PNTM patient (n = 6) CBF at time of collection and after isolation and growth at the air–liquid interface (ALI). No significant differences in CBF were found in healthy control subjects or patients with PNTM. (*d*) Effect of CFTR mutation status on CBF. Measurement of absolute CBF on DOC for healthy control subjects and patients with PNTM. Patients with PNTM were separated into three groups based on CFTR mutation status: heterozygous for CFTR mutation (n = 17), no mutation detected (n = 21), and unknown mutation status (n = 23). CFTR status had no effect on PNTM CBF. (*e*) Normal human bronchial epithelial (NHBE) cultures grown at ALI were exposed to two rounds of infection of *Mycobacterium abscessus* (n = 4) or *Mycobacterium avium* (n = 3) at a multiplicity of infection of 5:1 over the course of 8 days. CBF was measured after 8 days. Significant increases in CBF were seen in NHBE cultures exposed to MAB or MAC compared with uninfected cultures ($P < 0.002$, ANOVA). (*f*) NHBE cultures grown at ALI were exposed to media from PNTM epithelial cultures grown at ALI for 2 weeks (n = 6). No significant differences were seen in the CBF of cells exposed to PNTM patient conditioned media compared with NHBE cultures not exposed to PNTM patient conditioned media. Data are presented as means; *error bars* show SD. *$P < 0.05$; **$P < 0.005$.

and healthy control subjects for IL-6, IL-8, TNF-α, IFN-γ–inducible protein 10, G-CSF, and monocyte chemoattractant protein-1 responses (data not shown). Epithelial cell cytokine responses to TLR agonist stimulation were also not altered in patients with PNTM.

CBF Response to TLR Agonists in Respiratory Epithelial Cells

We also examined the CBF response to TLR agonists. TLR agonists differentially regulate respiratory epithelial CBF. In healthy control subjects, the bacterial TLR2 (Pam3CSK4), TLR4 (LPS), TLR5 (Flagellin), and TLR9 (CpG) agonists increase absolute CBF, whereas agonists to viral TLRs, TLR3 [poly(I:C)], and TLR7/8 (CL097) led to decreased absolute CBF (Figure 2a). Patients with PNTM, in contrast, had a depressed response to TLR4 (LPS), TLR9 (CpG), TLR2 (Pam3CSK4), and TLR5 (Flagellin) agonists but still had a significant increase in absolute CBF (Figure 2b). Viral TLR agonists TLR3 [poly(I:C)] and TLR7/8 (CL097) did not affect CBF in patients with PNTM. The absence of response to viral TLR agonist is significant because those agonists decrease CBF in healthy control subjects.

To explore the role of innate immune signaling, the respiratory epithelial cells of an IRAK4-deficient patient (35) were examined. The patient does not have a depressed baseline CBF, and the CBF response to TLR agonists was as expected because the only significant response was to the TLR3 [poly(I:C)] agonist (Figure 2c).

The absolute CBF values, baseline and in response to TLR agonists, are important; it is also informative to consider the percent change from baseline CBF induced by TLR agonists. Patients with PNTM are less responsive to all TLR agonists tested except for TLR4 (LPS) (Figure 2d). PNTM respiratory epithelial cells are more responsive to TLR4 (LPS) agonist than cells from healthy control subjects. Similar CBF responses to TLR agonists were seen in ALI cultures (data not shown).

CBF Response to TLR Agonists after Mycobacterial Infection

To examine whether mycobacterial infection influenced the TLR response profile of epithelial cells, NHBE ALI cultures were infected with two rounds of MAB or MAC (multiplicity of infection, 5:1) for 8 days and then challenged with TLR agonists (Figure 2e; Figures E1d and E1e). Infection with either mycobacterial strain induced a TLR response profile that was not observed in uninfected NHBE cultures or in PNTM cells (Figure 2e). When infected NHBE ALI cultures were stimulated with TLR agonists, a further increase in CBF was observed with all TLR agonists (Figures E1d and E1e). This was most dramatic with the viral TLR (TLR3 and TLR7/8) agonists.

Patients with PNTM Have Reduced nNO Production

nNO has been shown to be positively correlated with CBF and to influence CBF (44). We found that healthy individuals had a mean nNO of 316 ± 76 nl/min, similar to prior reports (45). Patients with known PCD who are known to have dysregulated NO had mean nNO levels of 26 ± 24 nl/min. Patients with PNTM had mean nNO levels of 237 ± 77 nl/min, significantly different from healthy and PCD control subjects (Figure 3a). As previously reported, intercohort CBF and exhaled nNO production were positively correlated for all patients (36) (Figure 3b).

Ex Vivo Modification of Baseline CBF through the NO–cGMP Pathway

Decreased nNO at the population level correlated with reduced baseline CBF in patients with PNTM. This correlation warranted

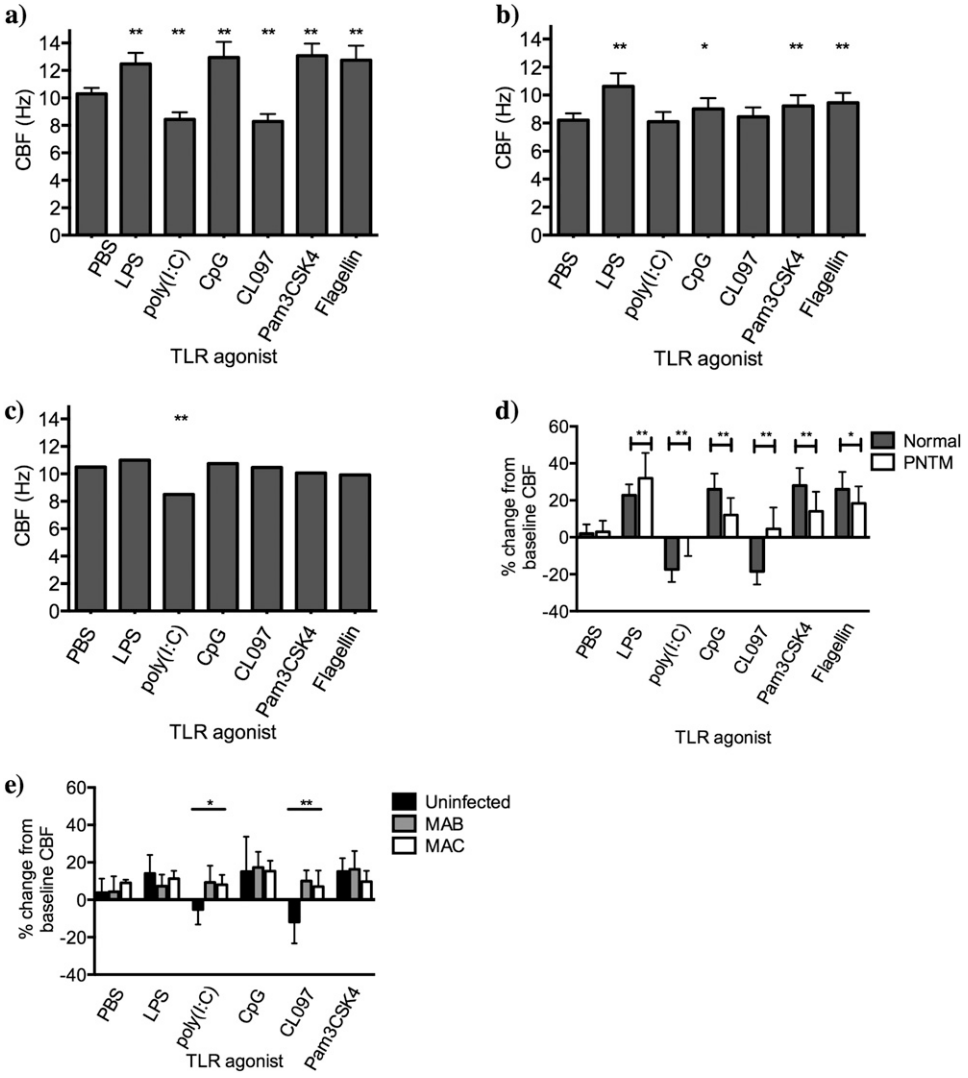

Figure 2. Ciliary beat frequency (CBF) response to TLR agonists in day of collection (DOC) and air–liquid interface cultures. (*a*) Absolute CBF on DOC. Healthy control respiratory samples CBF was measured after 8 hours of exposure to no agonist (PBS; n = 10), Toll-like receptor (TLR4) (LPS; n = 9), TLR3 [poly (I:C); n = 12), TLR9 (CpG; n = 13), TLR7/8 (CL097; n = 11), TLR2 (Pam3CSK4; n = 12), and TLR5 (Flagellin; n = 10]. Healthy control subjects significantly increase CBF in response to the TLR4 (LPS), TLR9 (CpG), TLR2 (Pam3CSK4), and TLR5 (Flagellin) agonists; there is a significant decrease in CBF in response to the TLR3 [poly(I:C)] and the TLR7/8 (CL097) agonists ($P < 0.0001$ for all agonists, ANOVA). (*b*) Absolute CBF on DOC. Pulmonary nontuberculous mycobacteria (PNTM) respiratory samples CBF was measured after 8 hours of exposure to no agonist (PBS; n = 13), TLR4 (LPS; n = 20), TLR3 [poly(I:C); n = 18], TLR9 (CpG; n = 26), TLR7/8 (CL097; n = 22), TLR2 (Pam3CSK4; n = 22), and TLR5 (Flagellin; n = 11). Patients with PNTM have significant responses to the TLR4 (LPS), TLR9 (CpG), TLR2 (Pam3CSK4), and TLR5 (Flagellin) agonists ($P < 0.0001$, $P < 0.009$, $P < 0.0006$, $P < 0.0005$, respectively; ANOVA). (*c*) Absolute CBF on DOC. PNTM respiratory samples CBF was measured after 8 hours of exposure to no agonist (PBS), TLR4 (LPS), TLR3 [poly(I:C)], TLR9 (CpG), TLR7/8 (CL097), TLR2 (Pam3CSK4), and TLR5 (Flagellin) agonists. The TLR3 [poly(I:C)] agonist significantly decreased CBF ($P < 0.002$, ANOVA). (*d*) Percent change from preexposure baseline CBF for healthy control subjects and patients with PNTM exposed to 8 hours of TLR agonists TLR4 (LPS), TLR3 [poly(I:C)], TLR9 (CpG), TLR7/8 (CL097), TLR2 (Pam3CSK4), and TLR5 (Flagellin). PNTM were significantly different from healthy control subjects with respect to TLR2 (Pam3CSK4), TLR3 [poly (I:C)], TLR4 (LPS), TLR5 (Flagellin), TLR7/8 (CL097), and TLR9 (CpG) ($P < 0.0006$, $P < 0.0001$, $P < 0.007$, $P < 0.03$, $P < 0.0001$, $P < 0.0001$, respectively; two-tailed *t* test). (*e*) Comparison of normal human bronchial epithelial (NHBE) cultures' response to TLR agonists in the presence of clinical concentrations of *Mycobacterium abscessus* (MAB) (n = 4), clinical concentrations of *Mycobacterium avium* (MAC) (n = 3), or uninfected (n = 9). NHBE cultures were infected (multiplicity of infection, 5:1) with MAB or MAC for 8 days before TLR stimulation. Data are expressed as percent change from preagonist CBF (uninfected or infected) to 8-hour post-TLR agonists: TLR4 (LPS), TLR3 [poly(I:C)], TLR9 (CpG), TLR7/8 (CL097), and TLR2 (Pam3CSK4). Significant change in response (i.e., reversal of response) was seen with poly(I:C) and CL097 ($P < 0.01$ and $P < 0.005$, respectively; ANOVA). Data are presented as means; *error bars* show SD. *$P < 0.05$; **$P < 0.005$.

further exploration because estrogen is involved in the regulation of the NO–cGMP pathway. Therefore, DOC cells from healthy control subjects and patients with PNTM were exposed to 8Br-cGMP, a cGMP analog that increases CBF through the NO–cGMP pathway (46). Stimulation led to significant increase in CBF in healthy control subjects and patients with PNTM. A significant increase above preexposure baseline CBF continued to be observed after 8 hours in patients with PNTM but not in healthy control subjects (Figure 4a). Increases in CBF were similar for healthy control subjects and patients with PNTM across a broad dose range (Figure 4b). This increase in CBF confirmed that baseline CBF was not maximal in either group and that modulation of the NO–cGMP pathway could increase CBF in healthy control subjects and in patients with PNTM. To further explore the NO–cGMP pathway, healthy control and PNTM patient cells were exposed to SNAP, a compound that releases NO upon photoactivation. SNAP addition led to a 30% increase in CBF in PNTM patient cells but no increase in CBF in cells from normal control subjects (Figure 4c).

To determine whether pharmacologic manipulation of the NO–cGMP pathway could improve CBF in PNTM cells, the selective inhibitor of cGMP-specific phosphodiesterase type 5, sildenafil, was used. A 40% increase from preexposure baseline CBF was seen in the PNTM patient cells, whereas the healthy control cells had no change in CBF. PNTM CBF was still significantly elevated compared with preexposure baseline at 8 hours (Figure 4d). The dose-response curve to sildenafil in PNTM patient cells had a broad plateau (Figure 4e). Although sildenafil increased PNTM cell resting CBF, it did not alter PNTM cell CBF response to TLR agonists (Figure 4f).

DISCUSSION

PNTM infection is not universal, even in diseases with well-known mucociliary clearance defects and pulmonary manifestations. In

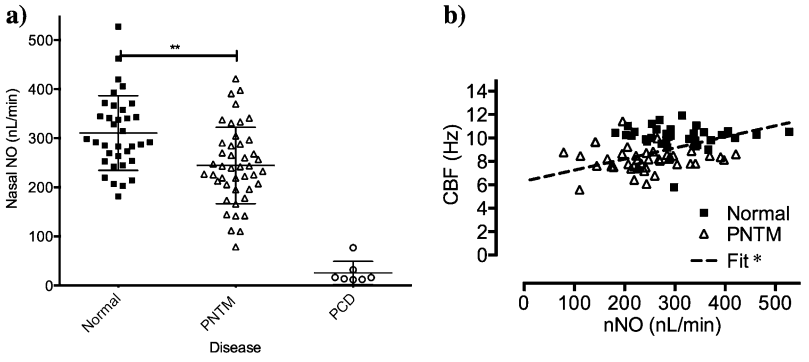

Figure 3. Nasal nitric oxide (nNO) and ciliary beat frequency (CBF) association in patients and healthy control subjects. (*a*) nNO was measured in healthy control subjects (310 ± 76 nl/min; n = 35), patients with pulmonary nontuberculous mycobacteria (PNTM) (237 ± 74 nl/min; n = 52), and patients with primary ciliary dyskinesia (PCD) (26 ± 24 nl/min; n = 7). Patients with PNTM had significantly lower nNO than healthy control subjects but not in the range of PCD ($P < 0.0001$; ANOVA). (*b*) CBF (Hz) is plotted as a function of nNO concentration (nl/min). A positive correlation was found between CBF and nNO concentration (0.31 Spearman's rho, $P < 0.01$; linear regression, $y = 0.005x + 7.63$ Hz) for all patients. Data are presented as means; *error bars* show SD. $^{**}P < 0.005$.

CF and PCD, the overall prevalence rate of PNTM infections is 11 to 13% (24, 25). This overall rate of PNTM infections is misleading because there is a striking increase seen in PNTM infections with increasing age. In patients with PCD, the PNTM rate is very low in children and increases to over 10% in adults over 30 years of age (24). In patients with CF who are over 40 years old, PNTM rates can reach 40% (25). The increasing prevalence of PNTM infection with increasing age is also seen in the general population (47). It is therefore very informative to study a population in which PNTM disease is a primary clinical manifestation.

Patients with PNTM disease have consistently low respiratory epithelial CBF (Figure 1a). The depressed baseline CBF is not associated with mycobacteria infection (Figures 1e and 2e; Figures E1d and E1e) or with factors associated with the respiratory epithelial cells (Figure 1b; Figures E1a and E1b) or secondary to inflammatory mediators produced by the patient's

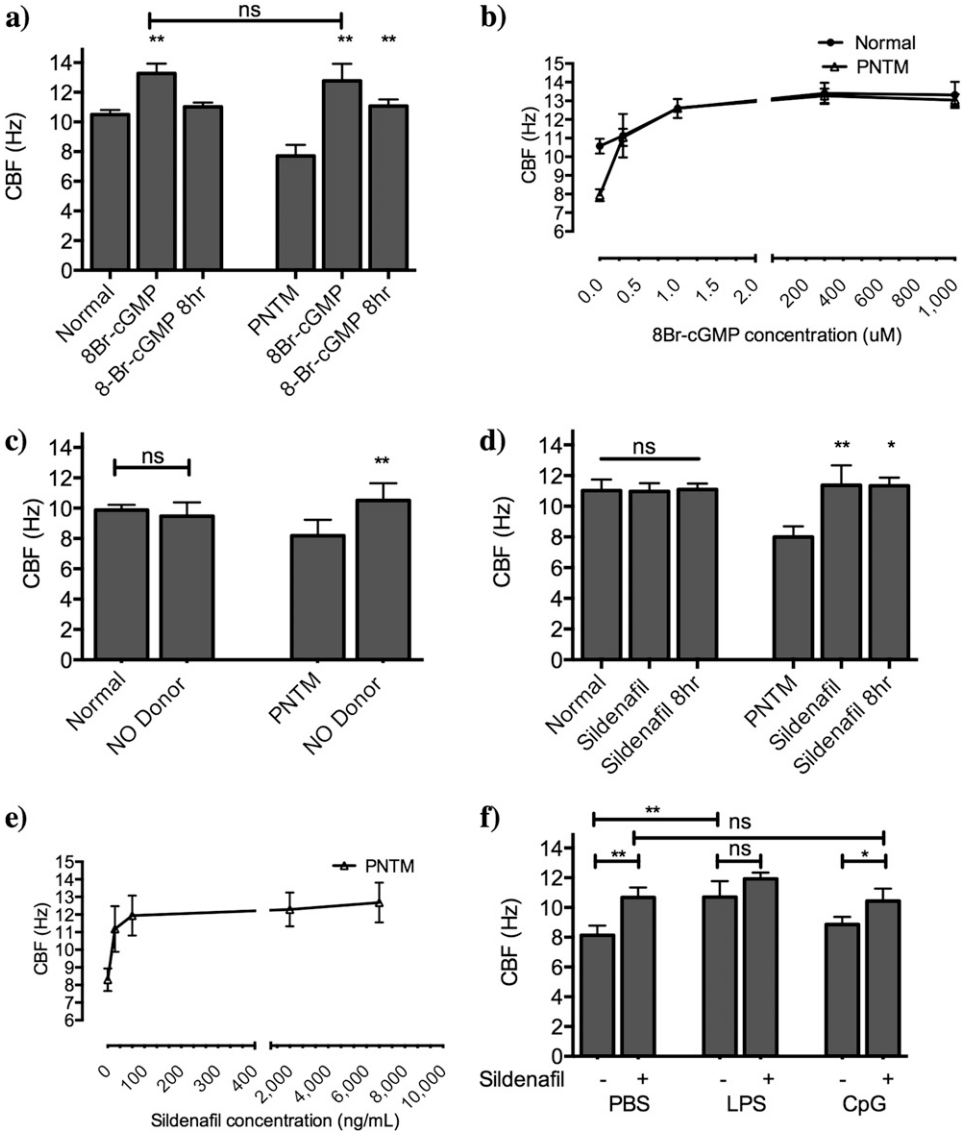

Figure 4. Modulating ciliary beat frequency (CBF) through the NO–cyclic guanosine monophosphate (cGMP) pathway on day of collection. (*a*) CBF before and after addition of 1 mM 8Br-cGMP in normal subjects (n = 8) and in patients with pulmonary nontuberculous mycobacteria (PNTM) (n = 8) ($P <$ 0.0001 compared with baseline, two-tailed *t* test). A prolonged 8-hour increase in CBF was only seen in patients with PNTM ($P < 0.0032$, two-tailed *t* test). (*b*) 8Br-cGMP dose–response curve in healthy control subjects (n = 4) and in subjects with PNTM (n = 4). (*c*) CBF before and after addition of 1 mM *S*-nitroso-*N*-acetyl-D,L-penicillamine in normal subjects (n = 9) and in patients with PNTM (n = 9) ($P < 0.003$ compared with baseline, two-tailed *t* test). (*d*) CBF before and after addition of 10 μM sildenafil in normal subjects (n = 10) and in patients with PNTM (n = 10) ($P <$ 0.0001 compared with baseline, two-tailed *t* test). The 8-hour response was only seen in patients with PNTM ($P <$ 0.009, two-tailed *t* test). (*e*) PNTM (n = 5) sildenafil dose-response curve. (*f*) CBF of PNTM respiratory epithelium (n = 4) before and after addition of sildenafil (30 nM) and Toll-like receptor (TLR)4 (LPS) or TLR9 (CpG) agonists for 8 hours. CBF was significantly increased with sildenafil, TLR4 agonist, and when comparing TLR9 agonist and TLR9 agonist with sildenafil ($P < 0.002$, $P < 0.002$, and $P =$ 0.05, respectively; ANOVA). Data are presented as means. *Error bars* show SD. $^{**}P < 0.005$.

immune system (Figures 1c and 1f). The depressed baseline CBF in patients with PNTM was unaffected by CFTR gene mutation status (Figure 1d), suggesting that the high rate of CFTR mutations in these patients does not cause their diminished CBF, even though it may contribute to disease susceptibility or severity in other ways.

These findings are important because they align PNTM disease with a group of diseases that have defects in mucociliary clearance, such as PCD and CF, and not chronic sinusitis (41). A deficiency in mucociliary clearance in patients with PNTM may also help to explain why the infection predisposition is specific to PNTM manifestations and why the patients do not present with disseminated NTM disease (48).

The critical role of TLRs in airway innate immunity is well established. We found abnormalities in the PNTM patient epithelial CBF responses to the TLR2, TLR3, TLR5, TLR7/8, and TLR9 agonists. A depressed CBF response to TLR agonists in PNTM epithelial cells was not present with all TLR agonists. CBF in response to the TLR4 agonist LPS was increased in PNTM respiratory epithelial cells when compared with healthy control respiratory cells (Figure 2d), suggesting that changes in the CBF responses to TLR agonists are unlikely to be due to mutations in the TLR signaling pathway, such as with the MyD88, IRAK4, or NEMO deficiencies (49). The CBF response to TLR agonists in an IRAK4-deficient subject, a protein that is required for all MyD88-dependent TLR signaling, showed no depression of baseline CBF, and the CBF response to TLR agonists was as expected. Changes in CBF in IRAK4-deficient respiratory epithelial cells were only seen in response to the TLR3 agonist poly(I:C) (Figure 2c). TLR3 signals through TRIF and not MyD88. This suggests that disruption of MyD88-dependent signaling could not account for the *ex vivo* phenotype observed in patients with PNTM. Additionally, cytokine production is similar between patients with PNTM and healthy control subjects.

We found a modest but significant decrease in nNO in patients with PNTM compared with healthy control subjects. The relative importance of this and its cause are under evaluation. The decrease in nNO did suggest that studying the NO–cGMP pathway might be informative. Administration of a cGMP analog, 8Br-cGMP, confirmed that CBF could be increased in patients with PNTM and control subjects. The swiftness of the response and the ability of both patient populations to respond to CBF indicated that the differences in CBF between patients with PNTM and healthy control subjects were unlikely to be due to altered cilia structure. Surprisingly, sildenafil exerted a selective CBF increase in PNTM patient cells *ex vivo* (Figure 4d). The normalization of CBF by sildenafil without normalization of PNTM patient TLR responses suggests that, although the ciliary and TLR functional defects may be etiologically linked, both responses do not normalize with NO augmentation, at least not at the doses and durations explored here.

Although we are still searching for the specific gene or genes responsible for this syndrome, decreased baseline CBF is a robust and consistent marker that offers a partial mechanistic basis for susceptibility to PNTM. The ability of NO donors to rapidly increase mucociliary activity *in vivo* has been demonstrated (44), indicating that modulation of the NO–cGMP pathway might be of therapeutic value. Furthermore, agents to supply or manipulate the NO–cGMP pathway are abundant, and increasing CBF may have direct clinical benefit.

Conclusions

These data suggest a defect in CBF in patients with PNTM that probably predisposes them to this infection. Identification of this defect in the respiratory epithelium may help to explain the lack of extrapulmonary infections in patients with PNTM and the previous failures to find robust immune dysfunction associated with the common entity of idiopathic PNTM. Familial clusters of PNTM disease are consistent with the hypothesis that this predisposition is genetically determined and likely precedes the development of PNTM infection. The existence of this predisposition is supported by the demonstration of a specific morphotype consisting of leanness and tallness that predates the onset of disease. The relative modesty of the abnormality we identify here aligns with the fact that most aspects of life are normal (e.g., development, fertility, activity) in patients with PNTM and that onset of PNTM disease is delayed until the sixth decade. The recognition of NO-cGMP pathway impairment in patients with PNTM offers immediate avenues for further mechanistic and therapeutic investigation.

Author disclosures are available with the text of this article at www.atsjournals.org.

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