Increased Cytokine Response of Rhinovirus-infected Airway Epithelial Cells in Chronic Obstructive Pulmonary Disease

Dina Schneider[1]*, Shyamala Ganesan[1]*, Adam T. Comstock[1]*, Catherine A. Meldrum[3], Raja Mahidhara[3], Adam M. Goldsmith[1], Jeffrey L. Curtis[3], Fernando J. Martinez[3], Marc B. Hershenson[1,2], and Umadevi Sajjan[1]

[1]Departments of Pediatrics and Communicable Diseases, [2]Molecular and Integrative Physiology, and [3]Internal Medicine, University of Michigan, Ann Arbor, Michigan

Rationale: **Airway inflammation is a central feature of chronic obstructive pulmonary disease (COPD). COPD exacerbations are often triggered by rhinovirus (RV) infection.**

Objectives: **We hypothesized that airway epithelial cells from patients with COPD maintain a proinflammatory phenotype compared with control subjects, leading to greater RV responses.**

Methods: **Cells were isolated from tracheobronchial tissues of 12 patients with COPD and 10 transplant donors. Eight patients with COPD had severe emphysema, three had mild to moderate emphysema, and one had no emphysema. All had moderate to severe airflow obstruction, and six met criteria for chronic bronchitis or had at least one exacerbation the previous year. Cells were grown at air–liquid interface and infected with RV serotype 39. Cytokine and IFN expression was measured by ELISA. Selected genes involved in inflammation, oxidative stress, and proteolysis were assessed by focused gene array and real-time polymerase chain reaction.**

Measurements and Main Results: **Compared with control subjects, cells from patients with COPD demonstrated increased mRNA expression of genes involved in oxidative stress and the response to viral infection, including *NOX1*, *DUOXA2*, *MMP12*, *ICAM1*, *DDX58*/RIG-I, *STAT1*, and *STAT2*. COPD cells showed elevated baseline and RV-stimulated protein levels of IL-6, IL-8/CXCL8, and growth-related oncogene-α/CXCL1. COPD cells demonstrated increased viral titer and copy number after RV infection, despite increased IL-29/IFN-λ1, IL-28A/IFN-λ2, and IFN-inducible protein-10/CXCL10 protein levels. Finally, RV-infected COPD cultures showed increased mRNA expression of *IL28A*/IFNλ2, *IL29*/IFNλ1, *IFIH1*/MDA5, *DDX58*/RIG-I, *DUOX1*, *DUOX2*, *IRF7*, *STAT1*, and *STAT2*.**

Conclusions: **Airway epithelial cells from patients with COPD show higher baseline levels of cytokine expression and increased susceptibility to RV infection, despite an increased IFN response.**

Keywords: C-X-C chemokine; IL-6; IFN; NOX1; retinoic acid inducible gene–I

Airway inflammation is one of the central features of chronic obstructive pulmonary disease (COPD). Exacerbations of COPD are associated with elevated airway levels of proinflammatory mediators, including the neutrophil-attracting C-X-C chemokines, IL-8/CXCL8, growth-related oncogne (GRO)-α/CXCL1, and epithelial cell-derived neutrophil attractant (ENA)-78/CXCL5, and the cytokines, IL-6 and tumor necrosis factor (TNF)–α (1–5). Up to one-half of all COPD exacerbations are caused by viral infections (4, 6–8). Furthermore, respiratory viral infections have been associated with more severe and frequent exacerbations (4).

Rhinovirus (RV) is a positive, single-stranded RNA virus from the *Picornaviridae* family that is responsible for the majority of common colds and virus-induced COPD exacerbations (4, 6–8). Several studies attest to the ability of RV to infect the lower airways, supporting the notion of RV involvement in COPD exacerbations (9–11). RV readily infects airway epithelial cells from normal subjects *in vitro*, inducing the expression of such inflammatory mediators as IL-8, GRO-α, ENA-78, IL-6, and TNF-α (12–15). Nevertheless, little is known about the role that RV-infected epithelial cells play in inciting and maintaining inflammatory responses in COPD. We therefore tested the hypothesis that airway epithelial cells from patients with COPD produce an exaggerated inflammatory response to RV infection.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Recent studies have documented an association between chronic obstructive pulmonary disease (COPD) exacerbations and rhinoviral (RV) infection.

What This Study Adds to the Field

This study demonstrates that primary airway epithelial cells from patients with COPD produce greater levels of proinflammatory cytokines and IFNs in response to RV infection. This *in vitro* human system provides a valuable tool to study the pathogenesis of RV-induced COPD exacerbations.

METHODS

Cell Culture

This study was approved by the University of Michigan Investigational Review Board. Airway epithelial cells were isolated from the tracheas or bronchi of 10 lung transplant donors, the bronchi of 11 patients with COPD who were transplant recipients, and the bronchus of 1 patient with COPD undergoing lobectomy. All patients underwent a standardized evaluation, including postbronchodilator spirometry, detailed assessment of respiratory symptoms and high-resolution computed tomography (www.ltrcpublic.com). The latter allowed a characterization of the population of patients with COPD, including the presence of emphysema versus a chronic bronchitic phenotype. In addition, self-

(*Received in original form November 6, 2009; accepted in final form April 14, 2010*)

Supported by National Institutes of Health grants AT004793 and HL089772 (U.S.), HL82550 and HL81420 (M.B.H), HL082480 (J.L.C), and United States Public Health Services K24 HL04212, Tissue Procurement Core of the University of Michigan Comprehensive Cancer Center grant P30 CA46952, and the Lung Tissue Research Consortium (Clinical Centers) grant N01 HR046162 (F.J.M.).

* These authors contributed equally to the work.

Correspondence and requests for reprints should be addressed to Umadevi Sajjan, Ph.D., University of Michigan, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5688. E-mail: usajjan@umich.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 182. pp 332–340, 2010
Originally Published in Press as DOI: 10.1164/rccm.200911-1673OC on April 15, 2010
Internet address: www.atsjournals.org

TABLE 1. CHARACTERISTICS OF PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND NORMAL DONORS

No.	Age (*Yr*)	Sex	FEV_1 (%)	Smoking History (*Pack-Years*)	Emphysema
COPD					
1	62	Male	25	28	Severe
2	53	Female	13	70	Severe
3	59	Male	13	26	Severe
4	55	Female	56	27	Mild
5	50	Female	17	31	Severe
6	65	Male	15	120	Severe
7	58	Female	25	70	Moderate
8	53	Female	14	37.5	Moderate
9	59	Male	22	70	Severe
10	49	Female	13	136	Severe
11	55	Male	17	25	Severe
12	45	Male	17	31.25	None
Normal donors					
1	54	Female	—	—	—
2	59	Male	—	—	—
3	47	Male	—	—	—
4	48	Male	—	—	—
5	41	Female	—	—	—
6	50	Female	—	—	—
7	40	Male	—	—	—
8	48	Female	—	—	—
9	46	Male	—	—	—
10	51	Male	—	—	—

Definition of abbreviation: COPD, chronic obstructive pulmonary disease.

reported exacerbation history was assessed. Characteristics of the patients with COPD are shown in Table 1. Cells were grown at the air–liquid interface in Transwell inserts (Corning, Lowell, MA) to promote differentiation to a mucociliary phenotype (15, 16). Additional details are provided in the online supplement.

Generation of RV39 Stocks

RV39 was obtained from ATTC (Manassas, VA), and viral stocks generated by infecting HeLa cells, as previously described (15). Additional details for partial purification of the virus are provided in the online supplement. Virus was titered by agarose overlay plaque-forming assay. Briefly, HeLa monolayers were infected with serially diluted RV39 (10^{-5} to 10^{-10} from initial stock), and the number of plaque-forming units per milliliter determined 3 days after infection (17).

RV39 Infection

Primary mucociliary-differentiated airway epithelial cell cultures were shifted to hydrocortisone-free media and incubated for 24 hours. Cells were inoculated apically with 30 μl of either RV39 at a multiplicity of infection of 1.0 or ultraviolet (UV)-irradiated RV and incubated at 33°C for an additional 24–48 hours. Phosphate-buffered saline (PBS)–treated cells served as negative control.

Histology

Differentiated airway epithelial cell cultures were fixed, sectioned, stained with hematoxylin and eosin or periodic acid Schiff (PAS) reagent, and examined by light microscopy. To semiquantify goblet cells, PAS-positive cells in 10 random fields per culture were counted and expressed as the number of positive cells per 100 μM.

ELISA

Conditioned medium was collected and IL-8/CXCL8, IL-6, GRO-α/CXCL1, IFN-inducible protein (IP)-10/CXCL10, IFN-β, IL-29/ IFN-λ1 and IL28A/IFN-λ2 protein levels detected by multiplex immune assay (Bio-Rad, Hercules, CA) or ELISA (R&D Systems, Minneapolis, MN).

Quantitative Polymerase Chain Reaction

The mRNA expression of 40 genes involved in inflammation, oxidative stress, and proteolysis was assessed by focused gene array (SA Biosciences, Frederick, MD; *see* Table E1 in the online supplement). The genes surveyed are listed in Table 2. In addition, quantitative polymerase chain reaction (PCR) was used to determine levels of *TIMP1*, *HDAC2*, *HDAC4*, *SIRT1*, and *NCOR*. All PCR reactions were performed on an Eppendorf Mastercycler (Eppendorf, Westbury, NY) comparing threshold cycle number for target gene with reference gene. The amplification efficiencies of all target genes and the reference gene glyceraldehyde 3-phosphate dehydrogenase were similar.

Viral Load

After infection, positive-strand viral copy number was determined by quantitative PCR, as previously described (18), and the viral titer of basolateral media, cells, and apical mucus was determined. Additional details on this method are provided in the online supplement.

Statistical Analysis

Data are presented as geometric mean and range. Data were compared by an unpaired *t* test, Mann-Whitney test, or nonparametric analysis of variance with Dunn's *post hoc* test, as appropriate.

RESULTS

Features of Patients with COPD and Airway Epithelial Cell Cultures

All 12 patients with COPD had moderate to severe airflow obstruction, 8 had severe emphysema, 2 had moderate emphysema, whereas 2 had mild or no emphysema. Three patients met symptomatic criteria for chronic bronchitis, whereas six had at least one symptomatic exacerbation in the previous year. Epithelial cells were isolated from tracheobronchial segments of 12 patients with COPD and 10 normal donors, and passage 1 cells were cultured at the air–liquid interface. Similar to normal cells, COPD cells polarized and showed a transepithelial resistance between 400 to 800 $\Omega \cdot cm^2$. There was no difference in growth rate between COPD and normal cells. Histological examination of culture cross-sections indicated that both normal and COPD cells differentiated into a mucociliary phenotype. In addition, COPD cultures showed more PAS-positive cells than the normal cell cultures, suggesting goblet cell metaplasia (Figure 1).

Cytokine Expression by Normal and COPD Cultures

COPD is characterized by chronic airway inflammation. To test whether a proinflammatory phenotype is maintained in cultured airway epithelial cells, basolateral media from control and COPD cultures was collected and assayed for cytokines by ELISA. Compared with normal, COPD cultures showed significantly increased levels of IL-6, IL-8/CXCL8, and GRO-α/CXCL1, indicating a persistence of certain proinflammatory changes (Figure 2). A similar pattern was found in the apical washes of these cultures, although the absolute levels of cytokines were lower (data not shown). The cytokine expression of individual cultures was independent of the history of patient-reported exacerbations. In addition, there was no correlation between emphysema score, age, or sex and basal levels of IL-8 protein, our primary outcome variable (data not shown). Finally, there were no changes in the protein secretion of IL-1β, TNF-α, IFN-γ, or IL-18 (data not shown), and IFN-α and -β levels were undetectable in both normal and COPD cultures.

Cytokine Responses of Normal and COPD Cultures to RV Infection

Exacerbations of COPD are associated with elevated levels of proinflammatory cytokines in the airways (1–5), and RV is

TABLE 2. MRNA EXPRESSION OF RHINOVIRUS-INFECTED AIRWAY EPITHELIAL CELL CULTURES

	24 h After Infection				48 h After Infection			
	Normal		COPD		Normal		COPD	
Symbol	UV-RV	RV	UV-RV	RV	UV-RV	RV	UV-RV	RV
IFN genes								
IFNα1	1.48	1.53	1.15	1.30	1.43	1.09	0.78	1.3
IFNβ1	0.84	7.55	1.16	52.20*	1.18	19.21	1.21	139.90*
IFNγ	0.68	0.87	0.89	0.95	0.85	1.31	0.76	0.95
IL28A/IFNλ2	1.24	37.35	1.25	14.49*	1.19	54.37	1.09	253.22*
IL29/IFNλ1	0.80	41.57	1.09	137.85*	1.37	52.30	1.03	311.27*
IFN-stimulated antiviral genes								
NOS2	1.19	1.92	1.43	6.52	0.98	1.22	0.85	7.46*
MX1	0.85	4.75	0.89	12.02*	0.86	6.37	0.75	23.94*
MX2	1.00	7.97	0.84	19.35	0.96	8.35	0.78	48.40*
ISG15	1.06	10.22	1.23	22.88	1.01	15.09	1.01	68.88*
IFIT3	0.82	7.02	0.94	19.10	1.00	10.43	0.83	41.03*
IFI16	0.91	1.61	0.90	2.32	0.85	1.63	0.96	3.48
IFI35	0.89	3.55	0.88	6.83	0.93	7.31	0.88	9.63*
IFI44	0.87	2.80	0.96	4.70	1.02	4.23	0.98	8.60*
IFIT1	0.66	17.41	0.91	57.66*	0.89	25.46	0.64	122.12*
IFIT2	0.84	6.71	0.85	22.30	0.98	9.77	0.79	56.30*
RSAD2	0.45	14.23	0.77	76.77*	0.60	36.61	0.62	180.07*
OAS1	1.14	3.30	1.16	5.17	1.26	4.47	1.03	12.57*
OASL	0.98	41.31	0.89	53.18	0.88	49.01	0.80	124.28*
Double-stranded RNA recognition receptors								
IFIH1/MDA5	1.16	1.19	1.14	4.47	1.13	2.90	1.05	9.76*
DDX58/RIG-I	0.90	3.79	0.89	8.03	1.02	5.29	0.88	13.37*
DHX58	1.20	2.01	1.03	2.47	0.97	1.78	0.80	3.68*
Oxidative stress								
NOX1	1.91	2.28	1.56	1.99	1.53	3.21	1.80	3.49
NOX5	0.91	1.28	0.96	0.79	0.84	1.12	0.59	0.59
DUOX1	1.51	1.93	1.47	2.10	1.57	1.91	1.43	4.18*
DUOXA1	1.02	1.03	0.93	0.97	1.45	1.34	1.01	1.41
DUOX2	1.53	3.59	1.57	3.92	1.89	3.11	2.32	9.67*
DUOXA2	4.16	9.32	3.77	10.85	3.51	10.82	3.62	28.73*
Protease								
MMP12	1.43	2.12	1.61	2.25	1.29	1.03	1.21	1.74
Transcription factors								
IRF3	1.04	1.32	1.02	1.17	1.08	1.13	0.98	0.81
IRF7	1.21	3.48	1.02	5.45	1.16	5.14	0.97	10.54*
STAT1	0.98	2.35	0.97	3.37	1.15	2.93	0.96	7.35*
STAT2	0.91	1.36	0.90	1.48	1.00	1.39	0.80	2.49*
Receptor proteins								
ICAM1	1.78	2.42	1.55	2.11	1.82	2.25	1.26	1.97
TREM1	0.72	1.36	1.01	1.70	1.19	2.03	1.30	2.55
PTAFR	1.45	1.52	1.19	1.49	1.23	1.39	0.93	1.67
NLRP3	0.87	2.26	0.89	3.95	1.11	7.59	0.40	0.52
Other genes								
SOCS1	1.67	3.13	1.77	5.42	2.00	4.18	1.65	8.44
STAB1	1.28	2.15	1.04	0.94	1.84	1.36	2.10	0.66
CASP1	0.89	1.10	0.88	1.24	0.93	1.18	0.88	1.98*
JAK2	0.97	1.30	1.04	1.58	1.33	1.32	1.06	2.24*

Definition of abbreviations: COPD, chronic obstructive pulmonary disease; RV, rhinovirus; UV-RV, ultraviolet-irradiated RV.

mRNA expression was calculated as the fold-increase over the appropriate phosphate-buffered saline control for either the normal or COPD cells. Data represent the geometric mean calculated from 10 normal and 10 COPD cultures.

* Different from normal, $P \leq 0.05$, Mann-Whitney test.

responsible for the majority of virus-induced exacerbations (4, 6–8). We therefore measured the cytokine response of mucociliary-differentiated airway epithelial cell cultures from normal subject and patients with COPD after inoculation with RV39, UV-irradiated, replication-deficient RV39, or PBS. Relative to their respective PBS-treated control cells, both normal and COPD cells showed two- to fourfold increases in IL-8/CXCL-8, IL-6, and GRO-α/CXCL-1 production in response to viral infection (Figure 2). In agreement with data from our laboratory (15, 18, 19) and from others (14, 20–22), UV-irradiated virus also elicited a brisk response, consistent with the notion that binding and endocytosis of the virus are sufficient for a subset of replication-independent RV responses. Finally, after infection, COPD cells showed higher absolute concentrations of all three cytokines than the normal cells (Figure 2).

Viral load was determined by measuring viral copy number by reverse transcriptase–PCR and the amount of infectious virus in cell lysates combined with basolateral media and apical washes. Importantly, COPD cells showed a significantly higher viral load than the normal cells at both time points (Figure 3). To assess whether the increased viral load was due to deficient production of IFN, we measured the levels of IFN-β, IFN-λ1, and IFN-λ2 after infection. Analogous to the cytokine data, both normal and COPD cells showed increased expression of IFN-λ1 and IFN-λ2 at 24 and 48 hours after infection compared with their respective PBS or UV-irradiated RV cells (Figure 4).

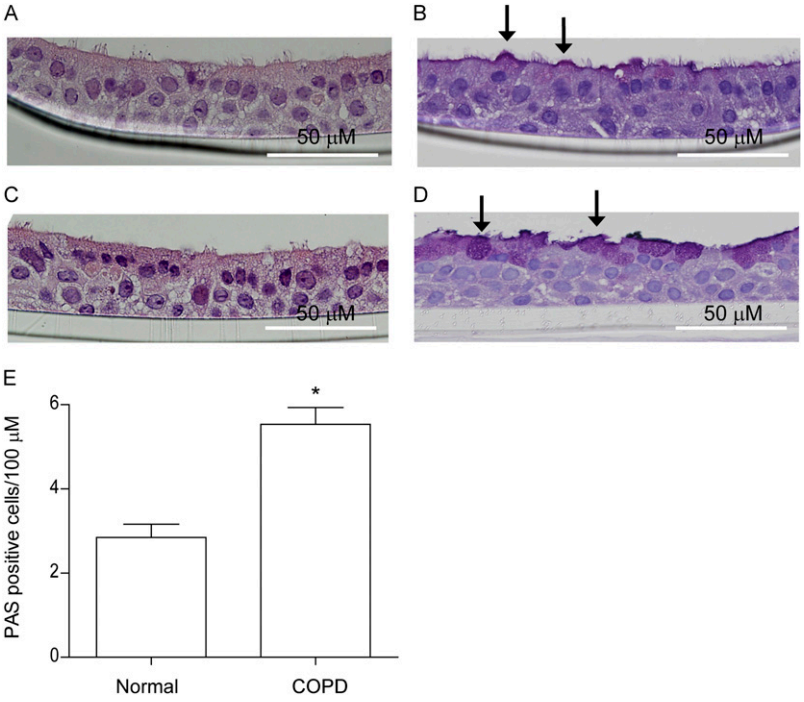

Figure 1. Histology of normal and chronic obstructive pulmonary disease (COPD) airway epithelial cell cultures. Passage one cells from normal lungs (*A* and *B*) or lungs of patients with COPD (*C* and *D*) were grown at air–liquid interface, and cells along with the membrane were fixed and embedded in paraffin. Cross-sections were stained with hematoxylin and eosin (*A* and *C*) or periodic acid Schiff (PAS) reagent (*B* and *D*). Number of PAS-positive cells per 100 μM was counted in five random fields in each culture and expressed as mean (±SEM) (*E*). Both normal cells and COPD cells differentiated into mucociliary phenotype. However, COPD cells show more PAS-positive cells. Images are representative of cultures from 10 normal donors and 12 patients with COPD. *Arrows* point to PAS positive cells. Data in E represent average and SEM (n = 10–12, *different from normal $P < 0.05$, t test).

IFN-β levels, on the other hand, were below the detection limit in both normal and COPD cells. Normal and COPD cultures also showed increased levels of IP-10/CXCL-10 in response to RV infection. In contrast to the IL-6, IL-8/CXCL-8 and GRO-α/CXCL-1 responses, IFN-λ1, IFN-λ2, and IP-10/CXCL-10 responses were replication dependent. Finally, 48 hours after infection, COPD cells showed higher absolute concentrations of IFN-λ1, IFN-λ2, and IP-10/CXCL-10 than the normal cells. These results do not support deficient IFN-λ1 or IFN-λ2 production as a cause of the increased viral load in cells from patients with COPD.

mRNA Levels in Normal and COPD Cultures

Total RNA was isolated from normal and differentiated COPD cultures and subjected to focused gene arrays. Fold changes in basal gene expression between normal and COPD cultures are shown in the online supplement (Table E2). Of the 45 genes tested, COPD cells showed significant increases in the mRNA expression of genes encoding matrix metalloproteinase (MMP) 12 and components of reduced nicotinamide adenine dinucleotide phosphate oxidase, including *Nox1* and *DuoxA2* (Figure 5). At the same time, mRNA expression of *NOS2*, the gene encoding induced nitric oxide (NO) synthase (NOS), was significantly decreased in COPD cells compared with normal cells. This pattern of gene expression is consistent with the increased oxidant and protease stress characteristic of COPD. We also observed increased levels of mRNAs encoding intercellular adhesion molecule (ICAM)–1, triggering receptor expressed on myeloid–1, retinoic acid–inducible gene (RIG)–I, IFN-α, 2′-5′ oligonucleotide synthase–like, and the transcription factors, signal transducer and activator of transcription (STAT)1 and STAT2 (Figure 6).

Increased production of proinflammatory cytokines and MMPs in COPD lungs and alveolar macrophages has been shown to be related to reduced HDAC2 expression (23–25). In cultured mucociliary-differentiated airway epithelial cells, however, there was no decrease in *HDAC2* or *HDAC4* expression at the mRNA level. Nonetheless, we did observe a tendency toward decreased expression of transcription repressors, *SIRT1* and *NCOR* (Figure 7).

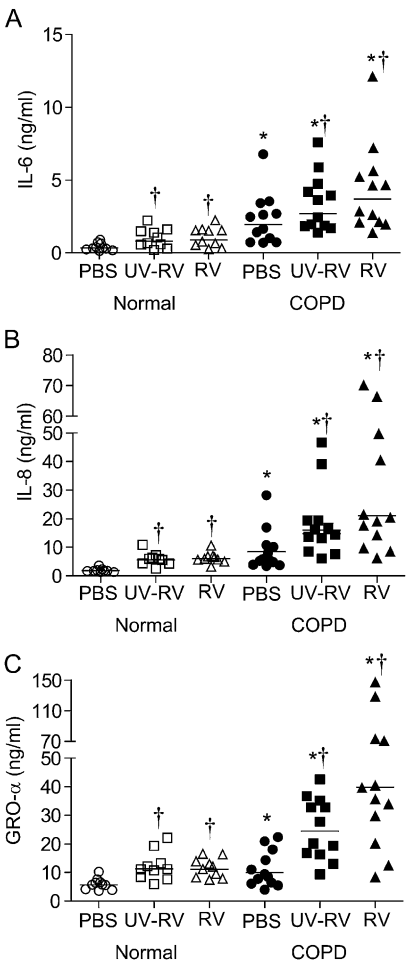

Figure 2. Proinflammatory cytokine production by normal and chronic obstructive pulmonary disease (COPD) airway epithelial cells under basal and stimulated conditions. Normal and COPD cells were grown at air–liquid interface for 3 weeks. Cultures were shifted to fresh hydrocortisone-free media and incubated for 24 hours. Cell cultures were infected apically with rhinovirus (RV) and incubated for another 24 hours at 33°C. Levels of IL-6 (*A*), IL-8/CXCL-8 (*B*), and growth-related oncogene-α/CXCL1 (*C*) present in basolateral media was determined by ELISA. COPD cells showed significantly higher basal levels of all three cytokines, which further increased with RV infection. Data represent the range and geometric mean (n = 10–12; *different from similarly treated normal cells, $P \leq 0.05$, nonparametric analysis of variance with Dunn's *post hoc* test; †different from respective phosphate-buffered saline (PBS)–treated control group, $P \leq 0.05$, Mann-Whitney test). UV-RV = ultraviolet-irradiated RV.

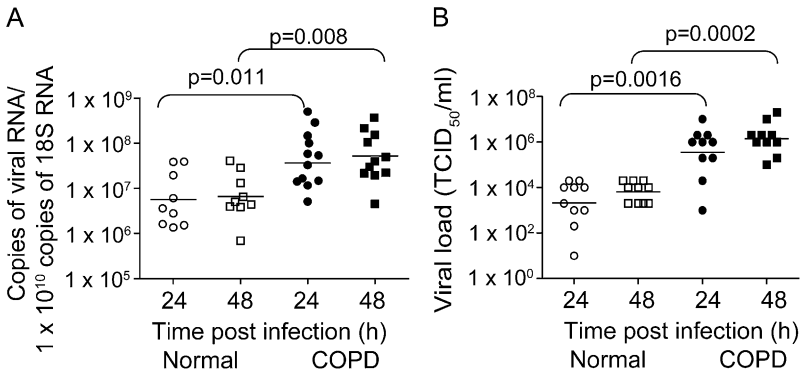

Figure 3. Viral load in rhinovirus (RV)-infected airway epithelial cells. Mucociliary-differentiated normal and chronic obstructive pulmonary disease (COPD) cultures were shifted to hydrocortisone-free media and incubated for 24 hours. Cells were then infected apically with RV or ultraviolet-irradiated RV (UV-RV) and incubated for 24 or 48 hours. The apical surface was rinsed with sterile phosphate-buffered saline and RNA was isolated from the cells. (*A*) Viral RNA copy number was determined by quantitative real-time polymerase chain reaction and normalized to 18S RNA. (*B*) Apical rinses from the cultures were used to determine the load of infectious virus, which was expressed as 50% tissue culture infective dose per milliliter. COPD cells showed a higher viral load than normal cultures at both 24 and 48 hours. *Open circles* = normal 24 h; *open squares* = normal 48 h; *solid circles* = COPD 24 h; *solid squares* = COPD 48 h. Data represent the range and geometric mean (n = 9–12, *$P \leq 0.05$, Mann-Whitney test).

mRNA Responses to RV in Normal and COPD Cultures

Through the use of the focused gene array, we also determined RV-induced changes in the mRNA expression of genes involved with inflammation, oxidative stress, and proteolysis 24 and 48 hours after infection. Both control and COPD cultures demonstrated increased expression of *IFN* and IFN-stimulated genes in response to RV infection, including *IFN*β, *IL28A*/IFNλ2, and *IL29*/IFNλ1 (Table 2). Indeed, compared with normal cells, the response of COPD cultures to RV infection was increased at 48 hours for several genes, including the *IFN*s, *IFIH1*/MDA5, *DDX58*/RIG-I, *Duox1*, *Duox2*, *IRF7*, *STAT1*, and *STAT2*.

DISCUSSION

This analysis of inflammatory mediator expression in cultured mucociliary-differentiated airway epithelial cells from patients with COPD, and their response to RV39 infection, reveals several novel findings. Compared with control cells from lung transplant donors, we found that basal levels of the C-X-C chemokines, IL-8/CXCL8 and GRO-α/CXCL1, as well as the cytokine, IL-6, were elevated in airway epithelial cells from patients with COPD. These findings demonstrate a persistent proinflammatory phenotype in COPD airway epithelial cells, and are consistent with the previously reported increases in IL-8/CXCL8, GRO-α/CXCL1, TNF-α, IL-1β, and MCP-1/

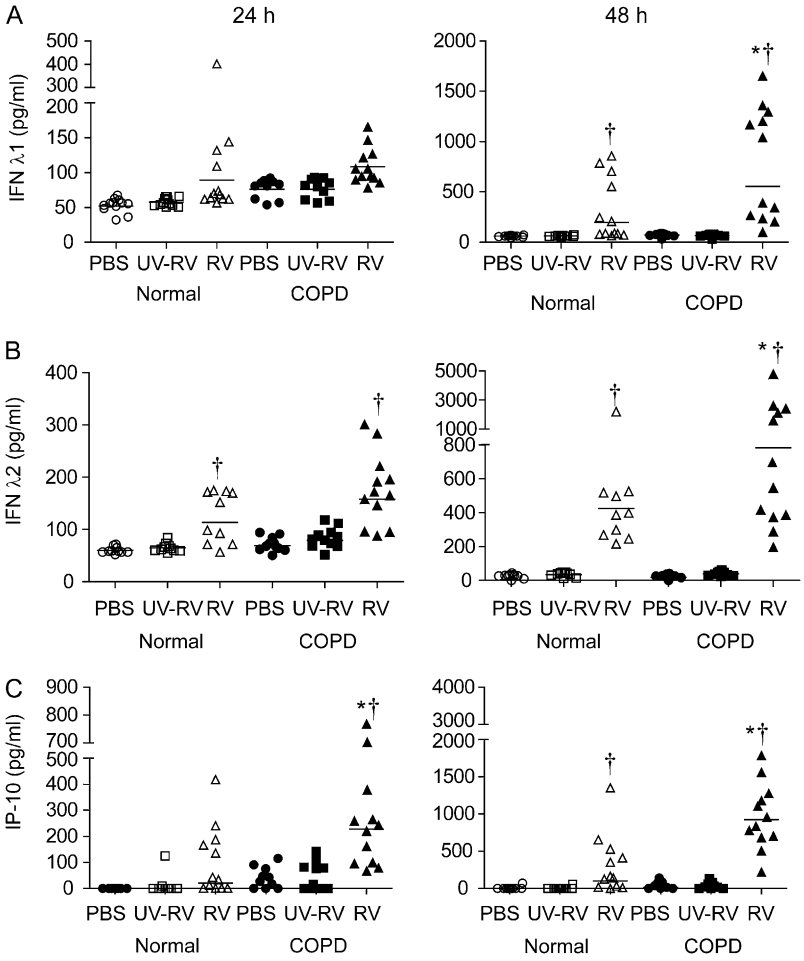

Figure 4. IFN and IP-10 expression in normal and chronic obstructive pulmonary disease (COPD) airway epithelial cells in response to rhinovirus (RV) infection. Cell cultures were infected with RV, as described in Figure 3 legend, and incubated for 24 or 48 hours. Levels of IFN-λ1 (*A*), IFN-λ2 (*B*), and IP-10 (*C*) in the basolateral media were determined by ELISA. Both normal and COPD cells showed increased levels of IFN-λ1, IFN-λ2, and IFN-inducible protein-10 in response to RV infection, although COPD cells showed significantly higher absolute concentrations of both cytokines than normal cells. Data represent range and geometric mean (n = 9–12; *different from normal cells, $P \leq 0.05$, nonparametric analysis of variance with Dunn's *post hoc* test; †different from respective phosphate-buffered saline [PBS]–treated control group, $P \leq 0.05$, Mann-Whitney test).

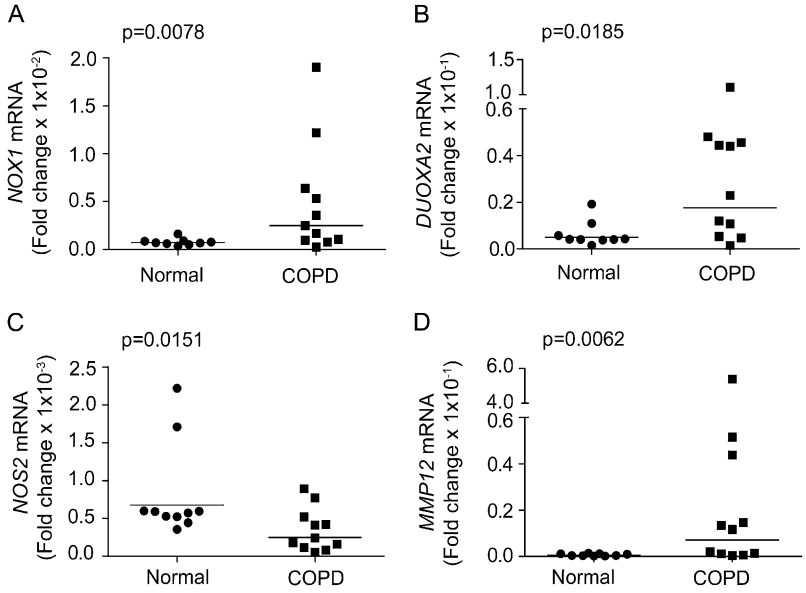

Figure 5. Airway epithelial cell mRNA levels of oxidative stress–related genes, *MMP* and *TIMP*, in normal and chronic obstructive pulmonary disease (COPD) cells under unstimulated conditions. Total RNA was isolated from mucociliary-differentiated cultures and subjected to focused gene arrays. Results are expressed as fold change compared with housekeeping genes. Compared with normal cells, COPD cells showed significant increases in the expression of mRNAs encoding Nox1 (*A*), DuoxA2 (*B*), and matrix metalloproteinase (MMP) 12. (*C*) In contrast, nitric oxide synthase (NOS) 2 mRNA levels were decreased in COPD cells. Data represent the range and geometric mean (n = 9–11; P values calculated by Mann-Whitney test).

CCL2 in the sputum and lung tissue of patients with COPD (26–28). However, we show, for the first time, that this proinflammatory phenotype is maintained in early passage airway epithelial cells. In addition, COPD airway epithelial cells produced increased amounts of the same inflammatory mediators after RV infection. The finding that RV-infected airway epithelial cells from patients with COPD showed increased levels of IL-8/CXCL8, GRO-α/CXCL1, and IL-6 compared with infected control cells is consistent with observed cytokine profiles in patients experiencing COPD exacerbations (1–5). After RV infection, COPD cultures showed higher viral titers and viral RNA copy number, despite greater production of IFN λ1 and IFN-λ2. Finally, mRNA expression of genes involved with oxidative stress and the response to viral infection were significantly elevated in COPD cells. In addition to defining several factors that could contribute to the exaggerated lung inflammation suffered by patients with COPD during RV infection, this cell culture model may facilitate future studies examining mechanisms of airway inflammation *in vivo*.

Oxidative stress appears to be a major factor in the development of airway inflammation in COPD. Exhaled breath hydrogen peroxide levels (29) and plasma lipid peroxidation products (30) are increased in patients with COPD. Oxidative stress, in turn, may activate the inflammatory response via redox regulation of the inhibitor(I)-κB/nuclear factor (NF)–κB signaling pathway. Increased expression and nuclear localization of NF-κB has been noted in the airway epithelium of smokers and patients with COPD (31). In our study, mucociliary-differentiated airway epithelial cells isolated form patients with COPD exhibited elevated mRNA expression of the hydrogen peroxide–generating enzymes, DUOX1 and DUOX2, consistent with the notion that oxidative stress is increased in the

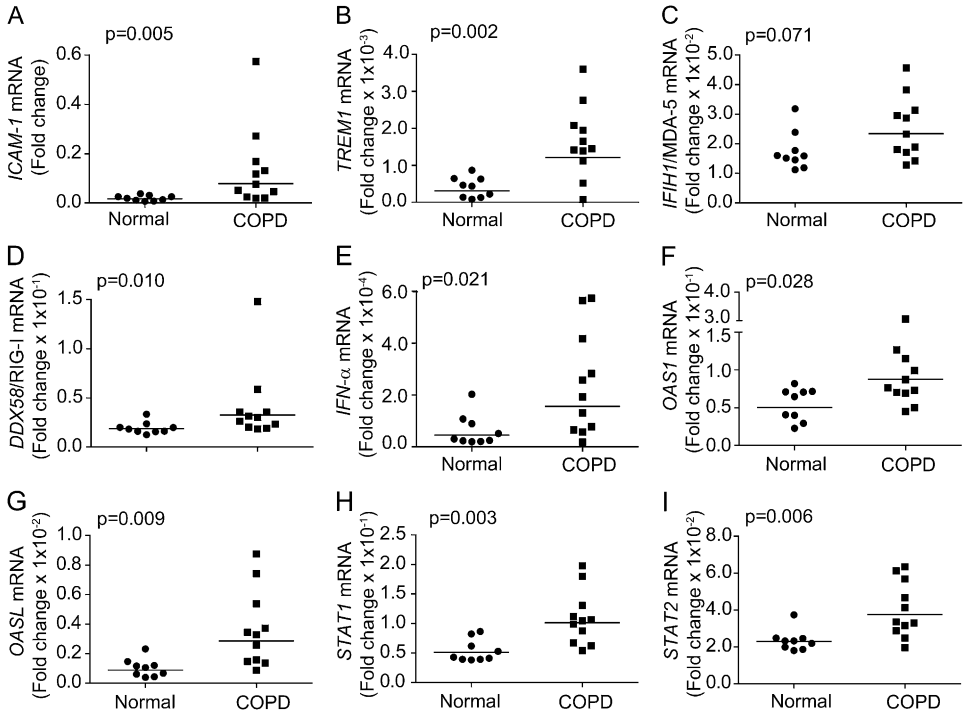

Figure 6. Airway epithelial cell mRNA expression of *ICAM-1*, *TREM1*, *IFIH1*/MDA-5, *DDX58*/RIG-I, and antiviral proteins under basal conditions. RNA isolated from normal and chronic obstructive pulmonary disease (COPD) mucociliary-differentiated cultures was subjected to focused gene arrays, and the results expressed as fold change over housekeeping genes. Compared with normal, COPD cells show significant increases in *ICAM-1* (*A*), *TREM-1* (*B*), *DDX58*/RIG-I (*D*), *IFN-α* (*E*), *OAS* (*F*), *OASL* (*G*), *STAT1* (*H*), and *STAT2* (*I*). Data represent range and geometric mean (n = 9; P values calculated by Mann-Whitney test).

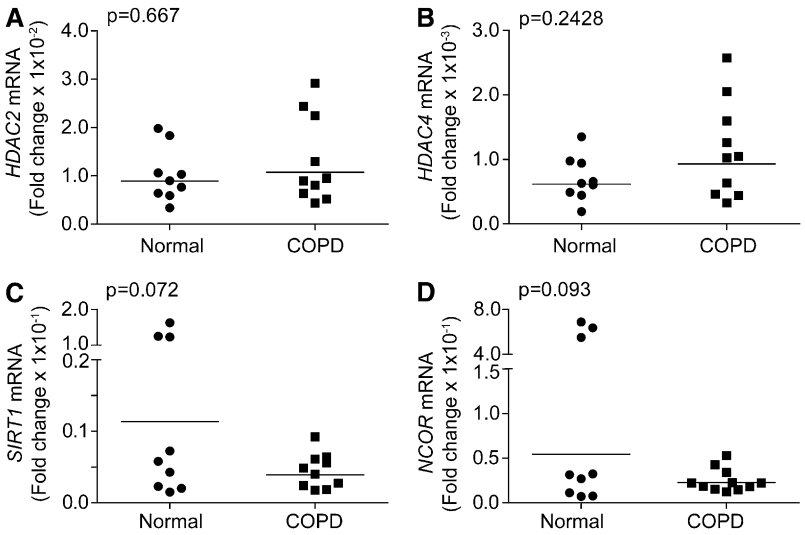

Figure 7. Airway epithelial cell mRNA expression of *HDAC* and gene repressors, *SIRT1* and *NCOR*. Total RNA from normal and chronic obstructive pulmonary disease (COPD) cells was subjected to real-time polymerase chain reaction and the data expressed as fold change over the housekeeping gene, *G3PDH*. mRNA levels of *HDAC2* (*A*) and *HDAC4* (*B*) levels were similar in COPD and normal cells. In contrast, *SIRT1 (C)* and *NCOR (D)* gene expression showed a trend toward a reduction in COPD cells. Data represent range and geometric mean ($n = 9$, P values were calculated by Mann-Whitney test).

airway epithelium of patients with COPD. These enzymes are important sources of hydrogen peroxide production, and play a role in airway host defense. Our results differ from those of a recent study, which demonstrated that mRNA expression of *DUOX1* was down-regulated, and *DUOX2* up-regulated, in current smokers, whereas both were down-regulated in patients with mild/moderate COPD (32). The discrepancy between our results and those of the latter study may be due to differences in disease severity, as the majority of epithelial isolates in our study were taken from patients with severe COPD.

Cigarette smoke has been shown to oxidize and inactivate HDAC2, allowing proinflammatory NF-κB transcriptional complexes to bind to DNA, thereby increasing the transcription of genes encoding proinflammatory cytokines. HDAC2 activity is decreased in bronchoalveolar lavage macrophages and biopsies of smokers and patients with COPD, and HDAC2 levels inversely correlate with inflammatory gene expression (23–25). We did not note differences in *HDAC* expression between airway epithelial cells isolated from control subjects and patients with COPD, perhaps because our patients, all but one of whom underwent lung transplantation, stopped smoking at least 6 months before surgery. However, we also monitored the mRNA expression of another histone deacetylase enzyme, SIRT1, which regulates the expression of proinflammatory genes via the NF-κB pathway, and is reduced in the lungs of patients with COPD (33). There was a trend toward reduced *SIRT1* expression in cultures from patients with COPD.

The elevated *MMP12* mRNA expression in cultures from patients with COPD is noteworthy, given the important role that proteases has been posited to play in the development both of emphysema and of peribronchial fibrosis. MMP12 is increased in the sputum of patients with COPD (34). Although MMP12 is generally considered to be principally a macrophage product, epithelial cells are clearly capable of MMP12 production and secretion (35). In addition, we saw a reduction in expression of the nuclear receptor corepressor (NCoR), NCoR1. NCoR1 has been shown to repress the activation of a battery of AP-1–regulated proinflammatory genes—among them, MMP12 (36).

After RV infection, viral titer and copy number were significantly increased in airway epithelial cells from patients with COPD, suggesting that these cells are somewhat more susceptible to RV infection. At first blush, our data are consistent with findings from airway epithelial cells isolated from patients with asthma, another chronic airways disease. Viral replication was significantly increased in cells from subjects with asthma infected with RV16 *ex vivo* (37, 38). Asthmatic airway epithelial cells also showed reduced levels of IFN-β, -λ1, and -λ2,3 production. In contrast, we found increased IL-29/IFN-λ1 and IL-28/IFN-λ2 responses in the cells of patients with COPD. We also found increased protein expression of IP-10/CXCL10, an IFN-inducible monokine. Taken together, these data suggest that, in airway epithelial cells from patients with COPD, increased viral load leads to enhanced type III IFN responses. Interestingly, two recent reports have shown no difference in IFN response between RV-infected airway epithelial cells from control subjects and those with asthma (39, 40).

Although *IL-29*/IFN-λ1 mRNA and protein levels were increased in RV-infected COPD epithelial cells, we could not detect IFN-β protein in conditioned medium, despite the higher levels of *IFN*β mRNA in COPD cells. One possible explanation for this discrepancy is the inefficient translation of host proteins in RV-infected cells. RV and other picornaviruses have been shown to inhibit or shut down host protein synthesis by cleaving elongation initiation factor 4G in susceptible cells (41, 42).

Another host mediator that regulates RV replication is NO. Expression of induced NOS (iNOS) or NOS2 is up-regulated during RV infection both *in vitro* and *in vivo* (43). Expression of iNOS correlates with exhaled NO levels, which in turn correspond to increased viral clearance and lower symptom scores in experimentally infected subjects (44). In the present study, we found that, although COPD cells expressed significantly less NOS2 mRNA than normal cells under unstimulated conditions, they expressed nearly identical levels of NOS2 after RV infection. Despite similar levels of NOS2 and increased levels of IFN, RV replication was increased in COPD cells compared with normal cells. These data suggest that other antiviral mechanisms, in addition to NOS2 and IFNs, regulate RV replication in infected epithelial cells. In support of this, our recent studies indicate that Toll-like receptor 3 knockout mice clear RV as efficiently as wild-type mice, despite decreased IFN responses (M.B.H., unpublished data). The observed susceptibility of COPD airway epithelial cells may be explained by increased mRNA expression of *ICAM1*, a receptor for binding of most RV isolates. High levels of ICAM-1 expression could also lead to increased cytokine responses (45). Increased ICAM-1 expression has been noted in pulmonary fibroblasts of patients with COPD (46).

A limitation of the current study is the absence of data verifying reduced protein expression of nonsecreted proteins,

such as NOX1, DUOXA2, NOS2, RIG-I, SIRT1, and NCoR1. Samples of airway tissue are extremely difficult to obtain, and limited numbers of passage 1 mucociliary-differentiated cells can be grown from each sample. We were therefore forced to limit our studies to cellular mRNA and supernatant protein levels. We have attempted to verify our results in later-passage, nondifferentiated cells, but the differences were less robust. We are currently defining the culture conditions needed to maintain the altered epithelial cell phenotype. It would not be surprising if the phenotype diminished with repeated passages, as the original stimulus, be it oxidative stress, proteases, or chronic infection, is not maintained *ex vivo*.

An additional limitation of the current study is the sampling of a cohort of patients with COPD with predominantly severe airflow obstruction. However, we found no correlation between emphysema score and basal levels of IL-8 protein (our primary outcome) or RV-induced IL-28 and IL-29A levels, suggesting that the proinflammatory phenotype is not strictly limited to patients with severe disease. On the other hand, it is this population that is generally afflicted with a higher frequency and severity of clinical exacerbations.

Finally, we acknowledge that, in many cases, there was a significant overlap in gene expression between control and COPD cultures. This overlap suggests that the phenotypic changes observed in patients with COPD are not uniform across the patient population. The observed heterogeneity likely results from differences in airway bacterial colonization, exposure to tobacco smoke and other environmental factors, and differences in genetic background.

In conclusion, airway epithelial cells from patients with COPD exhibit a proinflammatory phenotype and altered responses to RV infection. Furthermore, COPD cultures showed increased susceptibility to viral infection, despite increases in IFN expression. This cell culture model may facilitate future studies examining the underlying biochemical mechanisms responsible for airway inflammation in COPD, as well as the susceptibility of these patients to viral-induced exacerbations.

Author Disclosure: D.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.T.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.M.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.L.C. received $1,001–$5,000 from AstraZeneca as an honorarium for nonpromotional lectures, and $10,001–$50,000 from Boehringer Ingelheim in industry-sponsored grants (BI Protocol 205.325). F.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. U.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in copd exacerbations. *Thorax* 2000;55:114–120.
2. Gompertz S, O'Brien C, Bayley DL, Hill SL, Stockley RA. Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. *Eur Respir J* 2001;17:1112–1119.
3. Aaron SD, Angel JB, Lunau M, Wright K, Fex C, Le Saux N, Dales RE. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;163:349–355.
4. Seemungal T, Harper-Owen R, Bhowmik A, Moric I, Sanderson G, Message S, Maccallum P, Meade TW, Jeffries DJ, Johnston SL, *et al.* Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1618–1623.
5. Qiu Y, Zhu J, Bandi V, Atmar RL, Hattotuwa K, Guntupalli KK, Jeffery PK. Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;168:968–975.
6. Greenberg SB, Allen M, Wilson J, Atmar RL. Respiratory viral infections in adults with and without chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;162:167–173.
7. Rohde G, Wiethege A, Borg I, Kauth M, Bauer TT, Gillissen A, Bufe A, Schultze-Werninghaus G. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax* 2003;58:37–42.
8. Hutchinson AF, Ghimire AK, Thompson MA, Black JF, Brand CA, Lowe AJ, Smallwood DM, Vlahos R, Bozinovski S, Brown GV, *et al.* A community-based, time-matched, case-control study of respiratory viruses and exacerbations of COPD. *Respir Med* 2007; 101:2472–2481.
9. Gern JE, Galagan DM, Jarjour NN, Dick EC, Busse WW. Detection of rhinovirus RNA in lower airway cells during experimentally induced infection. *Am J Respir Crit Care Med* 1997;155:1159–1161.
10. Papadopoulos NG, Bates PJ, Bardin PG, Papi A, Leir SH, Fraenkel DJ, Meyer J, Lackie PM, Sanderson G, Holgate ST, *et al.* Rhinoviruses infect the lower airways. *J Infect Dis* 2000;181:1875–1884.
11. Mosser AG, Vrtis R, Burchell L, Lee WM, Dick CR, Weisshaar E, Bock D, Swenson CA, Cornwell RD, Meyer KC, *et al.* Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am J Respir Crit Care Med* 2005;171:645–651.
12. Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* 1995;96:549–557.
13. Schroth MK, Grimm E, Frindt P, Galagan DM, Konno SI, Love R, Gern JE. Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1999;20:1220–1228.
14. Griego SD, Weston CB, Adams JL, Tal-Singer R, Dillon SB. Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J Immunol* 2000;165:5211–5220.
15. Newcomb DC, Sajjan U, Nanua S, Jia Y, Goldsmith AM, Bentley JK, Hershenson MB. Phosphatidylinositol 3-kinase is required for rhinovirus-induced airway epithelial cell interleukin-8 expression. *J Biol Chem* 2005;280:36952–36961.
16. Sajjan U, Keshavjee S, Forstner J. Responses of well-differentiated airway epithelial cell cultures from healthy donors and patients with cystic fibrosis to *Burkholderia cenocepacia* infection. *Infect Immun* 2004;72:4188–4199.
17. Martin S, Casasnovas JM, Staunton DE, Springer TA. Efficient neutralization and disruption of rhinovirus by chimeric ICAM-1/immunoglobulin molecules. *J Virol* 1993;67:3561–3568.
18. Newcomb DC, Sajjan US, Nagarkar DR, Wang Q, Nanua S, Zhou Y, McHenry CL, Hennrick KT, Tsai WC, Bentley JK, *et al.* Human rhinovirus 1B exposure induces phosphatidylinositol 3-kinase–dependent airway inflammation in mice. *Am J Respir Crit Care Med* 2008;177:1111–1121.
19. Newcomb DC, Sajjan US, Nagarkar DR, Goldsmith AM, Bentley JK, Hershenson MB. Cooperative effects of rhinovirus and TNF-{alpha} on airway epithelial cell chemokine expression. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1021–L1028.
20. Grunstein MM, Hakonarson H, Whelan R, Yu Z, Grunstein JS, Chuang S. Rhinovirus elicits proasthmatic changes in airway responsiveness independently of viral infection. *J Allergy Clin Immunol* 2001;108: 997–1004.
21. Kaul P, Biagioli MC, Singh I, Turner RB. Rhinovirus-induced oxidative stress and interleukin-8 elaboration involves p47-phox but is independent of attachment to intercellular adhesion molecule–1 and viral replication. *J Infect Dis* 2000;181:1885–1890.
22. Suzuki T, Yamaya M, Sekizawa K, Hosoda M, Yamada N, Ishizuka S, Nakayama K, Yanai M, Numazaki Y, Sasaki H. Bafilomycin A(1) inhibits rhinovirus infection in human airway epithelium: effects on endosome and ICAM-1. *Am J Physiol* 2001;280:L1115–L1127.
23. Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, *et al.* Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med* 2005; 352:1967–1976.
24. Cosio BG, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF, Adcock IM. Histone acetylase and deacetylase activity in alveolar macrophages

and blood mononocytes in asthma. *Am J Respir Crit Care Med* 2004; 170:141–147.
25. Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, Adcock IM. Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J Exp Med* 2006;203:7–13.
26. de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol* 2000;190: 619–626.
27. Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* 2002;57:590–595.
28. Tomaki M, Sugiura H, Koarai A, Komaki Y, Akita T, Matsumoto T, Nakanishi A, Ogawa H, Hattori T, Ichinose M. Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung. *Pulm Pharmacol Ther* 2007;20:596–605.
29. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL, Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996;154:813–816.
30. Rahman I, MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 1996;21:669–681.
31. Di Stefano A, Caramori G, Oates T, Capelli A, Lusuardi M, Gnemmi I, Ioli F, Chung KF, Donner CF, Barnes PJ, *et al.* Increased expression of nuclear factor-κB in bronchial biopsies from smokers and patients with COPD. *Eur Respir J* 2002;20:556–563.
32. Nagai K, Betsuyaku T, Suzuki M, Nasuhara Y, Kaga K, Kondo S, Nishimura M. Dual oxidase 1 and 2 expression in airway epithelium of smokers and patients with mild/moderate chronic obstructive pulmonary disease. *Antioxid Redox Signal* 2008;10:705–714.
33. Rajendrasozhan S, Yang SR, Kinnula VL, Rahman I. Sirt1, an antiinflammatory and antiaging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008;177:861–870.
34. Demedts IK, Morel-Montero A, Lebecque S, Pacheco Y, Cataldo D, Joos GF, Pauwels RA, Brusselle GG. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax* 2006;61:196–201.
35. Lavigne MC, Thakker P, Gunn J, Wong A, Miyashiro JS, Wasserman AM, Wei SQ, Pelker JW, Kobayashi M, Eppihimer MJ. Human bronchial epithelial cells express and secrete MMP-12. *Biochem Biophys Res Commun* 2004;324:534–546.
36. Ogawa S, Lozach J, Jepsen K, Sawka-Verhelle D, Perissi V, Sasik R, Rose DW, Johnson RS, Rosenfeld MG, Glass CK. A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1–dependent gene networks required for macrophage activation. *Proc Natl Acad Sci USA* 2004;101:14461–14466.
37. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005;201:937–947.
38. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Kebadze T, Mallia P, Stanciu LA, Parker HL, *et al.* Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12:1023–1026.
39. Bochkov YA, Hanson KM, Keles S, Brockman-Schneider RA, Jarjour NN, Gern JE. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. *Mucosal Immunol* 2010;3:69–80.
40. Lopez-Souza N, Favoreto S, Wong H, Ward T, Yagi S, Schnurr D, Finkbeiner WE, Dolganov GM, Widdicombe JH, Boushey HA, *et al.* *In vitro* susceptibility to rhinovirus infection is greater for bronchial than for nasal airway epithelial cells in human subjects. *J Allergy Clin Immunol* 2009;123:1384–1390.
41. Krenn BM, Gaudernak E, Holzer B, Lanke K, Van Kuppeveld FJ, Seipelt J. Antiviral activity of the zinc ionophores pyrithione and hinokitiol against picornavirus infections. *J Virol* 2009;83: 58–64.
42. Svitkin YV, Gradi A, Imataka H, Morino S, Sonenberg N. Eukaryotic initiation factor 4GII (eIF4GII), but not eIF4GI, cleavage correlates with inhibition of host cell protein synthesis after human rhinovirus infection. *J Virol* 1999;73:3467–3472.
43. Sanders SP, Siekierski ES, Richards SM, Porter JD, Imani F, Proud D. Rhinovirus infection induces expression of type 2 nitric oxide synthase in human respiratory epithelial cells *in vitro* and *in vivo*. *J Allergy Clin Immunol* 2001;107:235–243.
44. Sanders SP, Proud D, Permutt S, Siekierski ES, Yachechko R, Liu MC. Role of nasal nitric oxide in the resolution of experimental rhinovirus infection. *J Allergy Clin Immunol* 2004;113:697–702.
45. Sajjan US, Jia Y, Newcomb DC, Bentley JK, Lukacs NW, LiPuma JJ, Hershenson MBH. Influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. *FASEB J* 2006;20:2121–2123.
46. Zandvoort A, van der Geld YM, Jonker MR, Noordhoek JA, Vos JT, Wesseling J, Kauffman HF, Timens W, Postma DS. High ICAM-1 gene expression in pulmonary fibroblasts of COPD patients: a reflection of an enhanced immunological function. *Eur Respir J* 2006;28: 113–122.