

Cigarette smoke combined with Toll-like receptor 3 signaling triggers exaggerated epithelial regulated upon activation, normal T-cell expressed and secreted/CCL5 expression in chronic rhinosinusitis

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Background: Chronic rhinosinusitis (CRS) is characterized by persistent mucosal inflammation and frequent exacerbations. **Objective:** To determine whether innate epithelial responses to cigarette smoke or bacterial or viral pathogens may be abnormal in CRS leading to an inappropriate inflammatory response.

Methods: Primary nasal epithelial cells (PNECs) were grown from middle turbinate biopsies of 9 healthy controls and 11 patients with CRS. After reaching 80% to 90% confluence, PNECs were exposed to medium or cigarette smoke extract (CSE) 5% (vol/vol) for 1 hour, washed, then stimulated with staphylococcal lipoteichoic acid, LPS, or double-stranded RNA (dsRNA). After 24 hours, gene expression was quantified by RT-PCR.

Results: At baseline, PNECs revealed elevated TNF- α and growth-related oncogene- α (a C-X-C chemokine)/CXCL1 (GRO- α) (4-fold increase, $P = .02$; and 16-fold increase, $P = .004$, respectively) in subjects with CRS compared with controls with normal levels of IL-1 β , IL-6, IL-8/CXCL8, human β -defensin-2, monocyte chemoattractant protein 2/CCL8, monocyte chemoattractant protein 3/CCL7, and regulated upon activation, normal T-cell expressed and secreted (RANTES)/CCL5. Immunostaining of nasal biopsies, however, revealed comparable epithelial staining for TNF- α , GRO- α , and RANTES. There were no differences in mRNA induction by CSE, TNF- α , lipoteichoic acid, LPS, or dsRNA alone. The

combination of CSE+dsRNA induced exaggerated RANTES (12,115-fold vs 1500-fold; $P = .03$) and human β -defensin-2 (1120-fold vs 12.5-fold; $P = .05$) in subjects with CRS. No other genes were differentially induced. Furthermore, CSE+dsRNA induced normal levels of IFN- β , IFN- λ 1, and IFN- λ 2/3 mRNA in subjects with CRS.

Conclusion: Cigarette smoke extract plus dsRNA induces exaggerated epithelial RANTES expression in patients with CRS. We propose that an analogous response to cigarette smoke plus viral infection may contribute to acute exacerbations and eosinophilic mucosal inflammation in CRS. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, epithelial, innate, cigarette smoke, viral, RANTES, exacerbations, eosinophilic

Chronic rhinosinusitis (CRS) is a serious health concern affecting an estimated 14% of the population.¹ At the mucosal level, CRS is described as a chronic inflammatory condition with an increased influx of eosinophils rather than neutrophils.² Microbial colonization, often without pathogenic bacteria, is a common finding.^{3,4} The mechanisms underlying CRS are largely unknown, but systemic immune function is normal in the majority of cases. Patients with CRS experience frequent exacerbations often diagnosed clinically as acute bacterial infection and treated with antibiotics.⁵ The precise etiology of these has not been studied, and the distinction between CRS exacerbations and typical viral upper respiratory infection is not easily made on clinical grounds.

The epithelial innate immune system serves an important function in sensing danger signals from the environment, including conserved molecular motifs from bacteria, fungi, and viruses. We hypothesize that a defect in innate mucosal immunity might underlie CRS, resulting in an increased propensity toward infection or an inappropriate inflammatory response to infection. Several innate components are expressed in nasal and sinus airway epithelial cells.^{6,7} In particular, mRNAs for all 10 known human Toll-like receptors (TLRs) were detected in cultured nasal epithelial cells. Furthermore, TLR-2, TLR-3, and TLR-4 were found to initiate innate epithelial immune responses in nasal epithelial cells.⁸ We therefore sought to examine the function of the TLR-2, TLR-3, and TLR-4 pathways in subjects with CRS.

Cigarette smoke is a known airway irritant that can provoke airway inflammation and adversely affect mucociliary clearance.⁹ Active cigarette smoking has been linked to an increased

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Supported by a grant from the Flight Attendants Medical Research Institute.

Disclosure of potential conflict of interest: D. L. Hamilos has received honoraria from Merck, Critical Therapeutics, Genentech, and Novartis; has consulting arrangements with GlaxoSmithKline, Novartis, Accentia, Schering-Plough, and Sinexus; has received research support from the Flight Attendants Medical Research Institute and Merck; and has received gifts from the GG Monks Foundation to purchase equipment. E. H. Holbrook has received research support from the National Institutes of Health-National Institutes of Deafness and Other Communication Disorders. S. T. Gray has served as a member of the American Academy of Otolaryngology and the American Rhinologic Society. N. Busaba is on the speakers' bureau for Sanofi-Aventis and Schering-Plough. The rest of the authors have declared that they have no conflict of interest.

Received for publication February 19, 2008; revised September 15, 2008; accepted for publication September 19, 2008.

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0091-6749/\$34.00

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doi:10.1016/j.jaci.2008.09.033

Abbreviations used

CRS:	Chronic rhinosinusitis
CSE:	Cigarette smoke extract
dsRNA:	Double-stranded RNA (TLR-3 agonist)
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)
GRO- α :	Growth-related oncogene- α (a C-X-C chemokine)/CXCL1
HBD2:	Human β -defensin-2
HC:	Healthy control
LTA:	Lipoteichoic acid
MCP:	Monocyte chemoattractant protein
RANTES:	Regulated upon activation, normal T-cell expressed and secreted (a C-C chemokine)/CCL5
PNEC:	Primary nasal epithelial cell
TLR:	Toll-like receptor

prevalence of chronic rhinosinusitis^{10,11} and poor outcomes after sinus surgery.¹² Multiple adverse health effects are also linked to secondhand cigarette smoke exposure. Because of the high prevalence of CRS and exposure to secondhand cigarette smoke, we examined whether cigarette smoke triggers abnormal epithelial responses in patients with CRS.

Recent work by Contoli et al supports the concept of deficient epithelial innate immunity in asthma. Specifically, asthmatic airway epithelium was found to have decreased induction of type I (β) and type III ($\lambda 1$ and $\lambda 2/3$) IFN in response to rhinovirus infection.¹³ This offers a potential explanation for the increased susceptibility of patients with asthma to viral upper respiratory infections¹⁴ and the strong association between rhinovirus infection and asthma exacerbations.¹⁵ Because asthma and CRS share similarities at the tissue level and many CRS exacerbations occur during viral season, we hypothesized that CRS might demonstrate similar defects in innate mucosal immunity. This hypothesis was tested by examining the nasal epithelial response to double-stranded RNA (dsRNA), a TLR-3 ligand and surrogate for rhinovirus infection.

The results of our study indicate that subjects with CRS have an abnormal response to cigarette smoke extract (CSE)+dsRNA characterized by exaggerated regulated upon activation, normal T-cell expressed and secreted (RANTES) production. We propose that an analogous response to cigarette smoke and viral infection might contribute to acute exacerbations and eosinophilic mucosal inflammation in CRS.

METHODS

The study was approved by the Institutional Review Board of both Massachusetts General Hospital and the Massachusetts Eye and Ear Institute. Each subject gave informed consent and completed a questionnaire, allergy skin testing, and a rhinoscopic examination. Healthy controls (N = 9) included men and women between the ages of 21 and 70 years in good general health. Exclusion criteria included a history of smoking, nasal or sinus disease, use of nasal or sinus medications, asthma, allergic rhinitis, cystic fibrosis, ongoing exposure to animals, pregnancy, ongoing use of cocaine, or systemic illness. Subjects with CRS without nasal polyps (N = 11) had a history of chronic sinusitis for more than 12 weeks and 2 of the following 3 symptoms: anterior and/or posterior mucopurulent drainage, nasal congestion, and/or facial pain/pressure and evidence of disease on a previous sinus computed tomography scan.¹ Exclusion criteria included active smoking, nasal polyposis, immunodeficiency (ie, >1 case of pneumonia in the past 12 months or known immune deficiency), cystic

fibrosis, Kartagener syndrome, immotile cilia syndrome, or a bleeding disorder.

To minimize their effect on epithelial gene expression, all subjects withheld systemic steroids for 2 weeks, intranasal steroids or anticholinergics for 3 days, antibiotics for 3 days, antihistamines for 1 week, and anticoagulants and/or aspirin or nonsteroidal anti-inflammatory drugs for 3 days before biopsies.

Nasal turbinate biopsies and cotinine measurements

Biopsies (2–3 mm in diameter) were obtained from the middle turbinates under local anesthesia. Patients with CRS were studied during quiescent periods with no acute exacerbations of symptoms for a minimum of 4 weeks. Serum cotinine was measured to assess exposure to secondhand cigarette smoke.

Preparation of primary nasal epithelial cell cultures from nasal turbinate biopsies. One turbinate biopsy was frozen immediately and kept for immunohistochemistry and the other processed to prepare primary epithelial cells (PNECs). The biopsy was dipped in 75% ethanol for 2 to 3 seconds and immediately washed (dipping) 5 times with a large volume of cold sterile medium containing Pen-Strep and Fungizone (GIBCO, Carlsbad, Calif), and put in a tube with 5 mL 0.25% trypsin solution (GIBCO cat. no. 15050-057) for 30 minutes at 37°C, washed 4 to 5 times with cold medium, and put in sterile trypsin neutralizing solution-TNS (Cambrex Bioscience cat. no. 01112711, East Rutherford, NJ) for 15 minutes at room temperature. The TNS was removed, and the biopsy was washed and transferred to a tube with cold medium. We used a 5-mL plastic pipette to disperse individual epithelial cells thoroughly. The cells were washed twice with cold medium (RPMI/F12K), resuspended in LHC-9 medium, and plated in 12-well or 24-well plates that were precoated with human collagen type IV (Sigma cat. no. C5533, St Louis, Mo), 10 $\mu\text{g}/\text{cm}^2$ for 1 to 2 hours. Visual assessment and Pap staining¹⁶ confirmed the epithelial morphology of all cells. The first generation of the plated PNECs when they reached 80% to 90% confluence was used in an effort to avoid the modification of primary cells caused by serial passage. PNECs were trypsinized to remove them from the plastic culture flask and transferred to 24-well plates for stimulation with CSE.

Preparation of CSE

Cigarette smoke extract was prepared as stock solution, fresh on the day of exposure, by bubbling the smoke from 3 cigarettes Code 2R4F (University of Kentucky, Lexington), at a rate of 1 cigarette/10 minutes, via Tygon tubing and a peristaltic pump, to a 50-mL tube containing LHC-9. The concentrate CSE was typically used at 5% vol/vol for a 1-hour pulse in epithelial cell cultures.

Viability assay of PNECs in response to CSE

Primary nasal epithelial cells at 80% to 90% confluence were exposed to fresh CSE (0% to 40% vol/vol) for 1 hour, washed, and fed with fresh LHC-9 medium. After 24 hours, the PNECs were trypsinized and tested for viability using a 0.4% Trypan blue exclusion assay (Sigma cat. no. T8154). Cell viability was expressed as the percentage of viable cells.

PNEC stimulation *in vitro*

Primary nasal epithelial cells were grown in 24-well plates to 80% to 90% confluence, exposed to 5% CSE for a 1-hour pulse, washed, and incubated alone or treated with 1 of the following for 24 hours: lipoteichoic acid (LTA) purified from *Staphylococcus aureus* (a generous gift from Dr Thomas Hartung, University of Konstanz, Germany) at a concentration of 10 $\mu\text{g}/\text{mL}$, LPS from *Salmonella enteritica*, serotype typhimurium (Sigma) at a final concentration of 1 $\mu\text{g}/\text{mL}$, dsRNA (Poly[I]-Poly[C]; Amersham Biosciences, Piscataway, NJ) at a final concentration of 25 $\mu\text{g}/\text{mL}$, or TNF- α at 100 ng/mL. In parallel experiments, PNECs were stimulated for 24 hours with LTA, LPS, dsRNA, or TNF- α without exposure to CSE.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

We used TRIzol Reagent (Invitrogen cat. no. 15596-018, Carlsbad, Calif) to extract total RNA and TaqMan Reverse Transcription Reagents (Applied Biosystems, part no. N808-0234, Carlsbad, Calif) to synthesize cDNA. Quantitative real-time RT-PCR was performed by using the Multiplex MX3000P quantitative RT-PCR system (Stratagene, La Jolla, Calif). In preliminary experiments, housekeeping genes were included in each RT-PCR run, including β_2 -microglobulin, Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene) (GAPDH), and elongation factor 1. GAPDH was found to be representative of the other housekeeping genes. Baseline gene expression and that after culture with CSE or the other stimuli were therefore quantified by RT-PCR relative to GAPDH using the delta-delta Ct ($2^{-\Delta\Delta}$) method¹⁷ and expressed as the ratio of gene expression relative to that in the medium control condition. All primers for RT-PCR were designed by using Primer Express software (<http://pga.mgh.harvard.edu/primerbank>). The primers for IFN- β , IFN- λ 1, and IFN- λ 2/3 were analogous to those used by Contoli et al.¹³

Characterization of the epithelial immune response by RT-PCR

The epithelial immune response was characterized with RT-PCR by measuring mRNA expression of the following species: TNF- α , IL-1 β , IL-6, IL-8, growth-related oncogene- α (a C-X-C chemokine)/CXCL1 (GRO- α), human β -defensin-2 (HBD2), monocyte chemoattractant protein (MCP)-2, MCP-3, RANTES, TLR-3, IL-17 receptor, IFN- β , IFN- λ 1, and IFN- λ 2/3. Forward and reverse primers were selected based on OMIM Blast software (<http://www.ncbi.nlm.nih.gov/pubmed>; see this article's supplementary Methods text in the Online Repository at www.jacionline.org).

Immunohistochemistry

Immunohistochemistry was performed by using frozen nasal tissues sectioned at 5 μ m and fixed with 4% paraformaldehyde or acetone/methanol (50/50 vol/vol) before staining. Endogenous peroxidases were quenched by using 3% H₂O₂, and avidin/biotin was added to block nonspecific binding. Then the tissues were exposed to primary antibody for 45 minutes at 37°C, washed, exposed to biotinylated secondary antibody, washed again, and exposed to avidin-peroxidase complex. Specific immunostaining was produced by addition of NovaRed solution (Fluka, Sigma Aldrich, St Louis, Mo). Nuclei were counterstained with hematoxylin. Primary antibodies recognizing TNF- α , GRO- α , and RANTES were obtained from R&D Systems (Minneapolis, Minn). Secondary biotinylated anti-goat IgG antibody was obtained from Vector Laboratories (Burlingame, Calif). Staining intensity was graded as 0 = absent, 1 = mild, 2 = moderate, 3 = moderately intense and 4 = very intense by 2 independent observers.

Statistical methods

Comparisons between groups were made by using the 2-sided Student *t* test (if data were normally distributed) or the nonparametric Mann-Whitney *U* test. A *P* value of .05 was regarded as statistically significant. Dichotomous variables were compared by using the Fisher exact test. The standard deviation for Ct in 112 different culture conditions studied with 6 to 9 subjects per condition averaged 2.42.

RESULTS

Summary of subject characteristics

The demographics of the healthy controls (HCs) and subjects with CRS are summarized in Table I. HCs were younger (age, 28 $\pm$ 10.8 years vs CRS, 44 $\pm$ 7.4 years) but had a similar sex distribution (female:male ratio, 7:2 and 8:3, respectively). Four subjects with CRS had asthma. Positive allergy skin test results were found in 5 of 9 HCs (despite a negative history of allergic rhinitis) and 7 of 11 subjects with CRS. Serum cotinine

TABLE I. Summary of subject characteristics

	HCs	Subjects with CRS	<i>P</i> value
N	9	11	—
Age (y), mean $\pm$ SD	28 $\pm$ 10.8	44 $\pm$ 7.4	.002
Female:male	7:2	8:3	—
Duration of CRS illness (y)	—	10.6	—
Frequency of CRS exacerbations (in past year)	—	4.8	—
Ongoing asthma*	0	4	.068
Allergy skin testing†			.330
Allergic	5	7	—
Nonallergic	4	4	—
Medication use			
Intranasal steroid	—	8	—
Antihistamine, decongestant, or both	—	6	—
Leukotriene blocker	—	4	—
Intranasal antibiotic rinse	—	0	—
Intranasal antifungal drug	—	1	—
Systemic steroid	—	1	—
Rhinoscopic findings			
Turbinate enlargement‡	1	5	.119
Polypoid thickening	0	2	.289
Mucus accumulation	0	4	.068
Serum cotinine level§ (ng/mL)	0.07	0.02	NS

NS, Not statistically significant.

*Ongoing asthma classified as "asthma for which you used medication during the past 12 months."

†Subjects underwent allergy skin tests with a panel of 23 prick and 15 intradermal allergens used to characterize subjects as allergic or nonallergic.

‡Turbinate enlargement was defined as at least 3+ of either inferior or middle turbinate on a scale of 0 to 4+.

§One CRS subject, who admitted to smoking cigarettes at the time of the nasal biopsy, had a serum cotinine level of 222 ng/mL and was excluded from the cotinine analysis.

(a metabolite of nicotine) levels averaged 0.07 ng/mL in HCs and 0.02 ng/mL in subjects with CRS, consistent with their histories of a low level of secondhand cigarette smoke exposure. (One subject with CRS, who admitted to smoking cigarettes at the time of the nasal biopsy, had a serum cotinine level of 222 ng/mL and was excluded from the cotinine analysis.)

Baseline gene expression and PNECs: HCs versus subjects with CRS

The baseline profile of gene expression in HCs and patients with CRS (*n* = 8 per group) was similar in terms of IL-1 β , IL-6, IL-8, RANTES, MCP-2, MCP-3, and HBD2, as shown in Fig 1. Subjects with CRS showed increased baseline mRNA expression of TNF- α (4-fold increase; *P* = .02) and GRO- α (16-fold increase; *P* = .004).

Expression of TNF- α , GRO- α , and RANTES in nasal turbinates by immunohistochemistry

A portion of each middle turbinate biopsy was frozen and later immunostained for TNF- α , GRO- α , and RANTES (Fig 2). TNF- α and RANTES immunostaining intensity was strongest in the epithelium, whereas GRO- α immunostaining was found in both the epithelium and submucosal glands. Consistent with the baseline increased gene expression for TNF- α , subjects with CRS showed a modest increase in epithelial immunostaining for

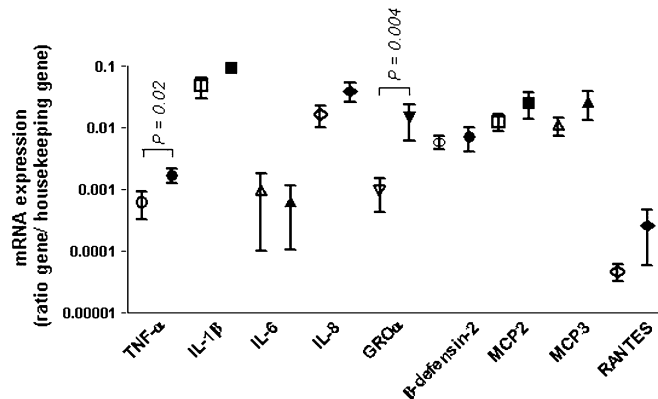


FIG 1. Baseline gene expression in PNECs from HCs and subjects with CRS. Symbols (HC, open symbols; CRS, solid symbols) indicate level of expression relative to the housekeeping gene GAPDH. Subjects with CRS showed increased expression of TNF-α (4-fold increase; $P = .02$) and GRO-α (16-fold increase; $P = .004$) relative to HCs.

TNF-α relative to HC; however, this difference did not reach statistical significance (1.86 ± 1.45 vs 0.94 ± 1.07 ; $P = .10$; Fig 3). The extent of immunostaining for GRO-α and RANTES was comparable in HCs and subjects with CRS with RANTES showing the least baseline immunostaining. The extent of immunostaining for TNF-α, GRO-α, and RANTES was unassociated with the allergic status of subjects in either group (data not shown). This contrasted with our previous finding of increased epithelial immunostaining for eotaxin-3/CCL26 in asymptomatic healthy subjects with positive allergy skin test results.¹⁸

In general, the staining intensity of TNF-α, GRO-α, and RANTES did not correlate strongly with mRNA expression in PNECs (data not shown). This may be a result of the fact that PNECs were cultured for 2 to 3 weeks before harvesting for baseline mRNA expression.

PNEC viability in response to CSE

To evaluate any increased susceptibility of subjects with CRS versus HCs with regard to the toxic effects of CSE, PNEC viability was assessed 24 hours after the 1-hour pulse exposure to CSE (see this article's Fig E1 in Online Repository at www.jacionline.org). PNECs from HCs and patients with CRS ($N = 7$ per group) showed a similar dose-related decrease in viability. Exposure to 5% CSE reduced the viability to 81% at 24 hours and was arbitrarily selected for use in subsequent experiments.

Gene expression after stimulation with CSE, TNF-α, and CSE+TNF-α

To evaluate the response of PNECs from HCs and subjects with CRS to CSE alone and in combination with other inflammatory mediators, gene expression in PNECs was quantified by RT-PCR after stimulation with CSE alone, TNF-α alone, or the combination of CSE+TNF-α (Fig 4). No significant differences were seen in HCs versus subjects with CRS. CSE alone caused modest induction of inflammatory genes ranging from 0-fold to 4.5-fold, with the exception of RANTES, which was induced 35-fold in subjects with CRS but not at all in HC ($P = .31$). TNF-α alone also caused a modest induction of genes ranging from 0-fold to 38-fold with the exception of RANTES, which was induced

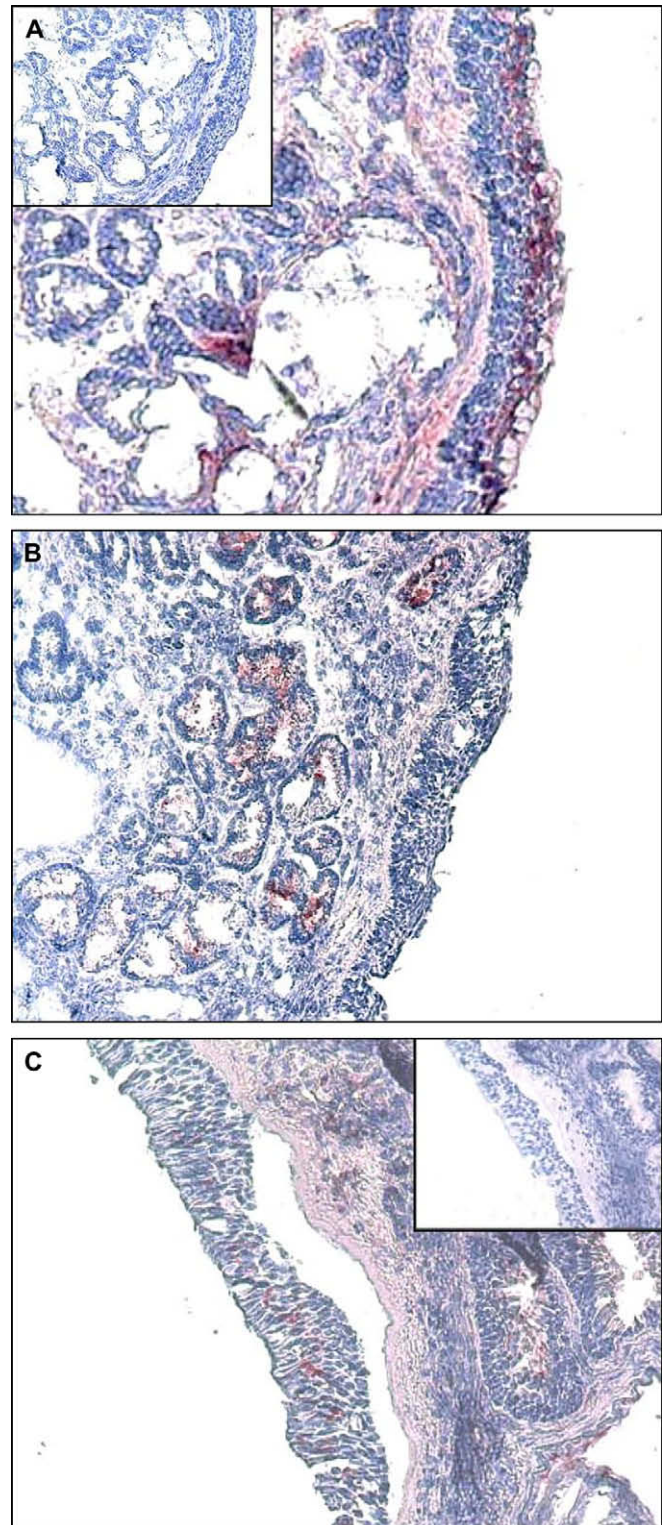


FIG 2. Representative epithelial immunostaining for TNF-α, GRO-α, and RANTES in HCs and subjects with CRS. **A**, Subject with CRS showing predominantly epithelial staining for TNF-α. **B**, Subject with CRS showing epithelial and glandular immunostaining for GRO-α. **C**, Subject with CRS showing epithelial immunostaining for RANTES. Inserts in **A** and **C** show the same tissues immunostained with the secondary antibody alone. Similar patterns of immunostaining were found in HCs. Magnification $\times 200$.

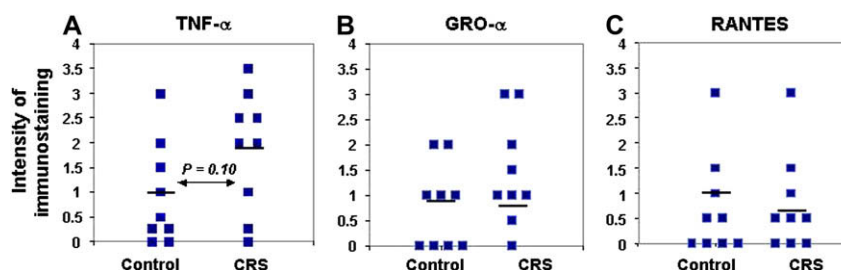


FIG 3. Intensity of PNEC immunostaining in HCs versus subjects with CRS (N = 9 per group) for TNF- α (A), GRO- α (B), and RANTES (C). No statistically significant differences in immunostaining were found in HCs versus subjects with CRS.

64-fold in HCs versus 184-fold in subjects with CRS ($P = .24$). Induction of RANTES by TNF- α was previously reported in cultured fibroblasts.¹⁹

The combination of CSE+TNF- α induced a synergistic increase in IL-1, IL-6, and HBD2 and additive increase in the other genes in both HCs and subjects with CRS.

GRO- α was induced less in subjects with CRS than in HCs in response to CSE and TNF- α , possibly because of the higher baseline expression of GRO- α in subjects with CRS.

Gene expression after stimulation with LTA (TLR-2 agonist) and LPS (TLR-4 agonist)

Lipoteichoic acid induced modest gene expression (maximum 48-fold induction for RANTES; Fig 4). LPS also induced modest gene expression (maximum 16-fold induction for RANTES). No significant differences were seen in HCs versus subjects with CRS in responses to LTA or LPS alone or LTA or LPS combined with CSE (Fig 4) with the exception of RANTES induction in PNECs from subjects with CRS in response to CSE+LTA. The latter was observed despite the higher baseline expression of RANTES in subjects with CRS.

GRO- α was induced less in subjects with CRS than in HCs in response to LTA and LPS, possibly because of the higher baseline expression of GRO- α in subjects with CRS.

Gene expression after stimulation with CSE, dsRNA (TLR-3 ligand), and CSE+dsRNA

Compared with the other stimuli, the TLR-3 ligand dsRNA was the strongest inducer in PNECs, causing >10-fold induction of TNF- α , IL-6, IL-8, GRO- α , and RANTES in HCs (Fig 5). A similar pattern of induction was seen in subjects with CRS, with the exception of GRO- α . GRO- α was induced less in subjects with CRS (3.4-fold in subjects with CRS vs 13.2-fold in HCs; $P = .28$), possibly because of the increased basal GRO- α in subjects with CRS. RANTES was by far the strongest induced gene in both HCs (2760-fold) and subjects with CRS (8980-fold). The difference in RANTES induction by dsRNA alone in HCs versus subjects with CRS was not statistically significant ($P = .18$).

Cigarette smoke extract plus dsRNA was synergistic for induction of IL-1 β , IL-6, and IL-8 in both HCs and subjects with CRS, but also synergistic for induction of TNF- α , MCP-2, and HBD2 in subjects with CRS, suggesting that subjects with CRS were more sensitive to the combined stimulation with CSE+dsRNA. Furthermore, CSE+dsRNA induced exaggerated production of RANTES (12115-fold vs 1500-fold; $P = .03$) and

HBD2 (1117-fold vs 12.5-fold; $P = .05$) in subjects with CRS compared with HCs. None of the other genes showed a differential induction by CSE+dsRNA. We subsequently confirmed in independent experiments that RANTES protein was strongly induced by dsRNA and CSE+dsRNA (see this article's Fig E2 in the Online Repository at www.jacionline.org).

Induction of IFN- β , IFN- λ 1, and IFN- λ 2/3 by CSE, dsRNA, and CSE+dsRNA

Because stimulation of PNECs by dsRNA mimics stimulation by RNA viruses including human rhinovirus, we examined another feature of this response: induction of type I (IFN- β) and type III IFNs (IFN- λ 1 and IFN- λ 2/3; Fig 6).

Baseline expression of IFN- β , IFN- λ 1, and IFN- λ 2/3 was comparable in HCs and subjects with CRS (data not shown). CSE alone induced less IFN- β , IFN- λ 1, and IFN- λ 2/3 in subjects with CRS versus HCs. This was statistically significant for IFN- β (HCs, 4.23-fold induction, vs subjects with CRS, 0.49-fold induction; $P = .009$), but not quite significant for IFN- λ 1 ($P = .055$) and IFN- λ 2/3 ($P = .09$). However, no differences were seen in induction of IFN- β , IFN- λ 1, or IFN- λ 2/3 by dsRNA or the combination of CSE+dsRNA in HCs and subjects with CRS (Fig 6).

Expression of TLR-3 and IL-17R mRNA in HCs versus subjects with CRS and their induction by CSE, dsRNA, and CSE+dsRNA

Because dsRNA is an agonist for TLR-3, we examined whether differences in expression of TLR-3 could account for differences in response to dsRNA in HCs versus subjects with CRS. Likewise, because IL-17A has been shown to augment epithelial innate responses to viral stimuli, including dsRNA, we examined expression of IL-17R in HCs versus subjects with CRS. Baseline expression of TLR-3 and IL-17R was similar in HCs and subjects with CRS (data not shown). No differences were found in induction of TLR3 or IL-17R in response to CSE, dsRNA, or CSE+dsRNA (Fig 6).

DISCUSSION

In this study, we investigated the potential role of the sinonasal epithelium in the pathogenesis of CRS by examining inflammatory gene expression in PNEC cultures. We identified a mild increase in baseline epithelial expression of TNF- α and GRO- α in subjects with CRS. HCs and subjects with CRS responded

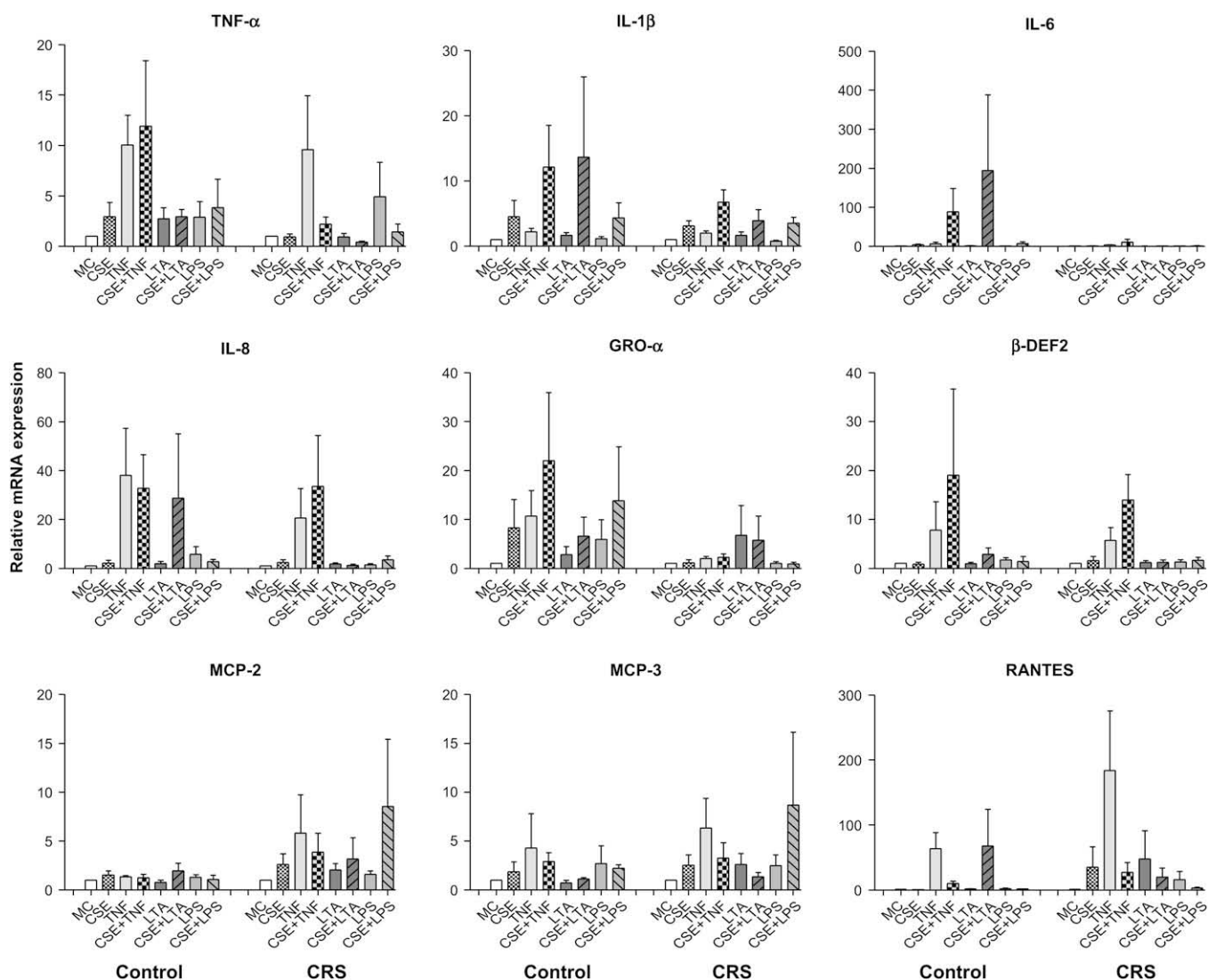


FIG 4. Inflammatory gene induction in HCs and subjects with CRS by CSE, TNF- α , LTA, LPS, CSE+TNF- α , CSE+LTA, and CSE+LPS. The ordinate shows fold increase in gene expression relative to GAPDH housekeeping gene. No statistically significant differences were found between HCs and subjects with CRS except increased GRO- α , which patterned the increased baseline GRO- α in subjects with CRS.

similarly to CSE, TNF- α , LTA (TLR-2 stimulus), and LPS (TLR-4 stimulus) as well as CSE+LTA and CSE+LPS. dsRNA was the strongest stimulus of inflammatory gene expression. Furthermore, patients with CRS responded abnormally to dsRNA+CSE with exaggerated RANTES and HBD-2 expression. None of the responses to CSE, TNF- α , or TLR agonists were deficient in subjects with CRS. These results suggest that exaggerated RANTES production may be a feature of CRS, particularly in response to CSE combined with a TLR-3 agonist. We speculate that exaggerated RANTES production in response to cigarette smoke combined with common viral pathogens may provoke inappropriate inflammation in CRS.

RANTES was the only chemokine induced to a greater degree in subjects with CRS than HCs. The magnitude of RANTES induction was striking and of the same order as the induction of IFN- β , IFN- λ 1, and IFN- λ 2/3, consistent with the known role of RANTES in the epithelial response to viral infection. The role of RANTES in epithelial immunity is incompletely understood. Although there is

minimal expression in healthy controls, increased expression has been found in asthma²⁰ and nasal polyposis.^{21,22} In addition, RANTES is induced by dsRNA and rhinovirus 16 infection *in vitro*,⁸ and elevated levels of RANTES have been associated with increased viral load in patients with asthma.²³ This contrasts with the epithelial response to viral infection *in vitro*, wherein increased production of IFN- β and IFN- λ have been associated with decreased viral load.¹³ Furthermore, IL-17A, a known inducer of epithelial antiviral responses, was found to induce IL-8 and HBD2 expression while simultaneously downregulating RANTES,⁸ suggesting that RANTES may not play an important protective role in the normal epithelial response to rhinovirus infection. In fact, a significant overproduction of RANTES in epithelial cells from patients with asthma was observed despite their increased susceptibility to rhinovirus infection. Increased epithelial RANTES production was found to be a feature of natural asthma exacerbations associated with eosinophil and T-lymphocyte infiltration into the airway.¹⁰ We therefore propose that overproduction of

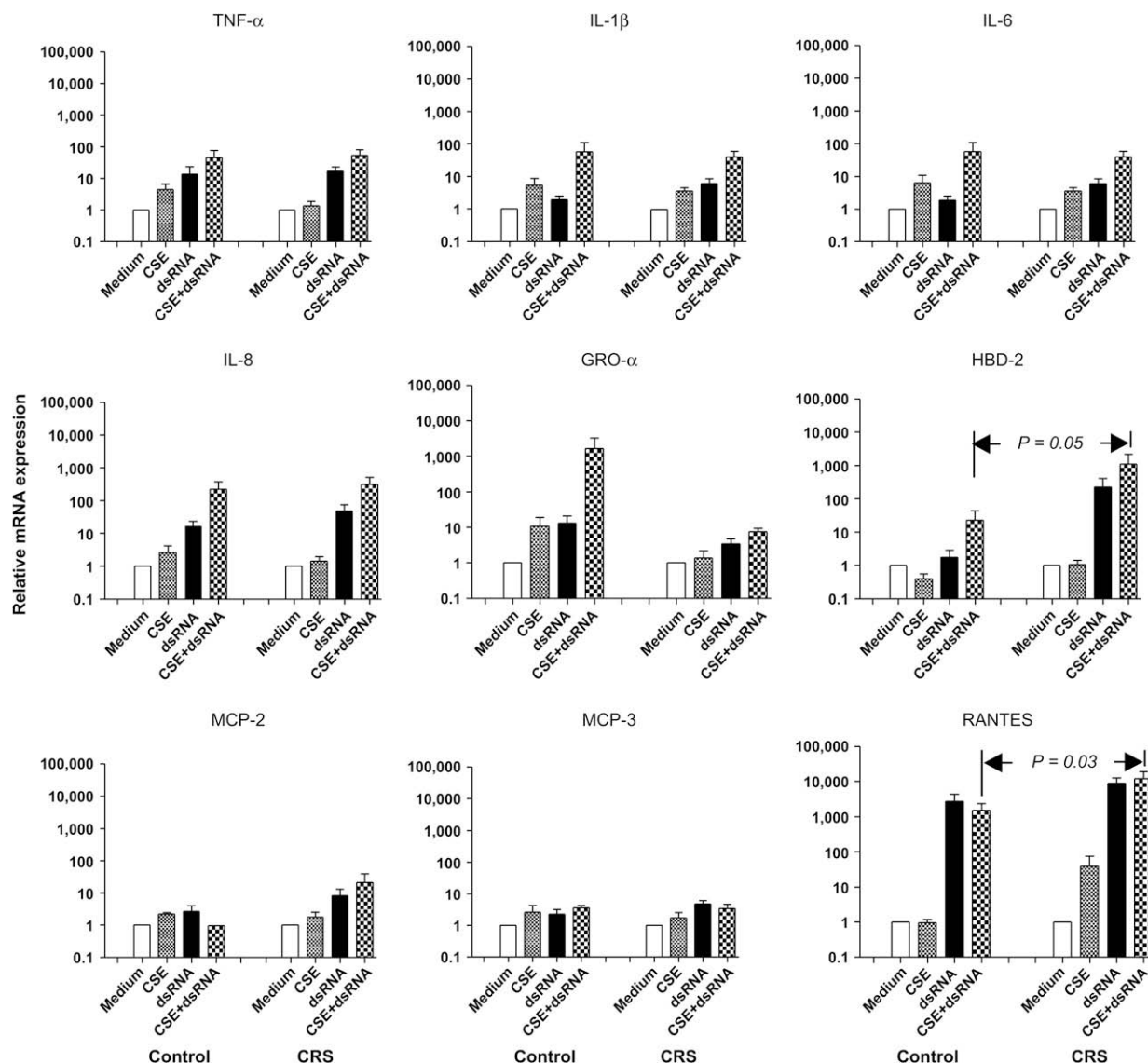


FIG 5. Inflammatory gene induction in HCs and subjects with CRS by CSE, dsRNA, and CSE+dsRNA. The ordinate shows fold increase in gene expression relative to GAPDH housekeeping gene. Statistically significant differences between HCs and subjects with CRS were confined to greater induction of HBD2 and RANTES by CSE+dsRNA, as shown.

RANTES in response to viral infection, especially when coupled with exposure to cigarette smoke, might be a feature of epithelial dysregulation in CRS that promotes eosinophil influx and persistent mucosal inflammation.

We considered whether the increased baseline TNF- α expression in subjects with CRS could account for the exaggerated RANTES response. Indeed, we confirmed that TNF- α induced RANTES expression; however, we did not see much difference between HCs and subjects with CRS when comparing RANTES induction by CSE+TNF- α (HCs, 10.7-fold, vs subjects with CRS, 27.6-fold; $P = .28$).

We considered whether exaggerated RANTES induction could be a result of enhanced signaling through TLR-3. This seems unlikely because the exaggerated response to CSE+dsRNA was selective for RANTES and HBD2. Furthermore, baseline TLR-3 expression was normal in CRS. We also considered whether a defect in IL-17R signaling might account for exaggerated

RANTES production. Recent studies of the role of IL-17A in epithelial immunity elucidated a coordinated chemokine/antimicrobial peptide response that may be key to clearance of infection.^{8,24,25} IL-17A is produced mainly by T lymphocytes.²⁶ Wiehler and Proud⁸ found that IL-17A markedly amplified the epithelial responses to rhinovirus in terms of IL-8 (110-fold increase) and HBD2 (4000-fold increase) while simultaneously decreasing RANTES induction 400-fold. These effects likely facilitate the normal recruitment of neutrophils to sites of epithelial infection. As a first step toward analyzing this pathway, we measured IL-17R mRNA expression and found it to be comparable in HCs and subjects with CRS.

Finally, we considered whether another important component of the epithelial response to dsRNA, induction of type I and type III IFNs, could be defective in CRS. We found no abnormality in expression of IFN- β , IFN- $\lambda 1$, and IFN- $\lambda 2/3$ in response to dsRNA or CSE+dsRNA.

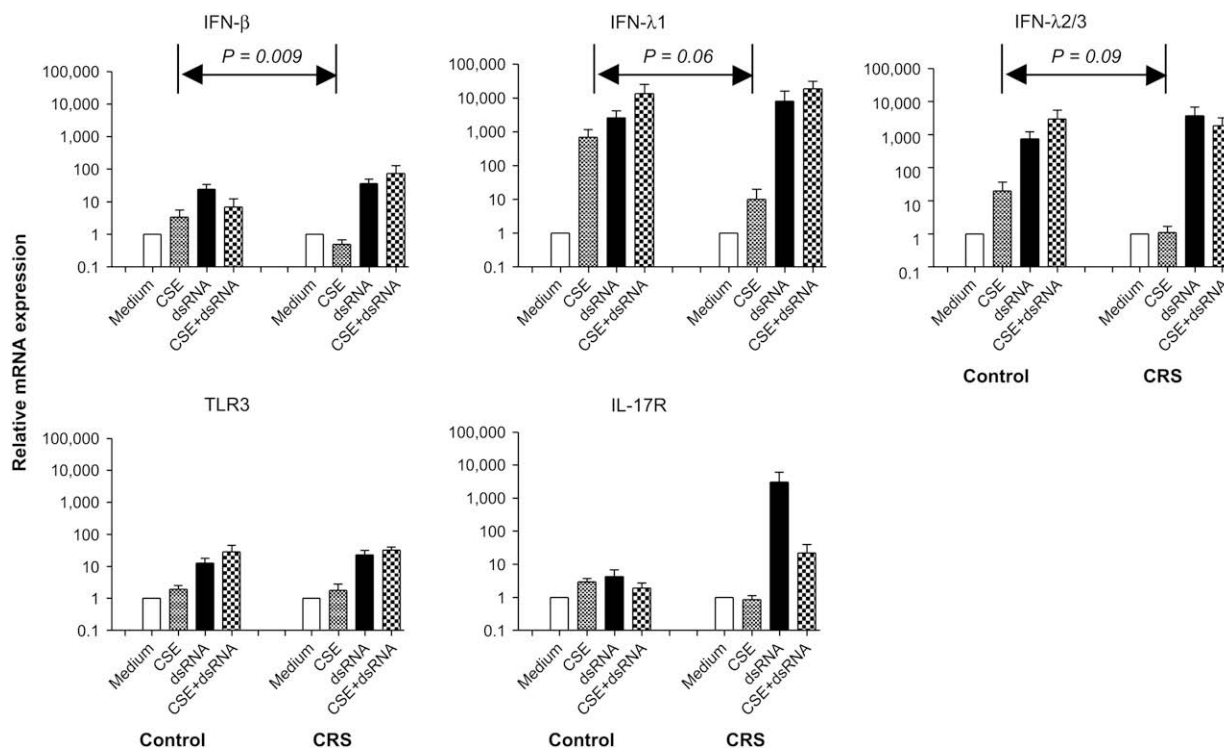


FIG 6. Induction of IFN- β , IFN- λ 1, IFN- λ 2/3, TLR-3, and IL-17R by CSE, dsRNA, and CSE+dsRNA in HCs and subjects with CRS. The ordinate shows fold increase in gene expression relative to GAPDH housekeeping gene. CSE alone suppressed induction of IFN- β in subjects with CRS; however, CSE+dsRNA induced equivalent expression of IFN- β and the other gene studied.

Several theories have been proposed to account for persistent inflammation in CRS, including chronic bacterial infection caused by biofilm formation,²⁷ local IgE production against colonizing *S aureus*-derived exotoxins,²⁸ and host T-lymphocyte-mediated eosinophilic response to ubiquitous airborne colonizing fungi.²⁹ Although each may be involved, it is not clear to what extent they account for acute exacerbations of disease. Furthermore, none of the theories excludes the possibility that the epithelium could be intrinsically defective in CRS, as has been described in asthma.^{30,31} Experimental rhinovirus infection provokes transient mucosal thickening and fluid accumulation in the paranasal sinuses, mimicking acute bacterial infection.³² Rhinovirus infection has been strongly linked to severe exacerbations of asthma.¹⁵ A recent study found that 21% of patients with CRS had evidence of rhinovirus infection despite lacking signs of acute viral upper respiratory infection.³³ Furthermore, patients with asthma who were rhinovirus-positive when hospitalized were more likely to be current smokers, suggesting that cigarette smoke may augment the severity of rhinovirus-induced asthma exacerbations. Our preliminary data suggest that cigarette smoke augments rhinovirus-induced inflammation in nasal epithelium, and we propose that this effect, particularly the exaggerated induction of RANTES, may contribute to acute exacerbations and perhaps also to persistent eosinophilic mucosal inflammation in CRS.

There are limitations to our study. First, the 1-hour period of exposure to 5% CSE may not be optimal to mimic natural secondhand cigarette smoke exposure. Second, we have yet to ascertain the role and relevance of RANTES during acute exacerbations of CRS. Furthermore, the relevance of RANTES

and eosinophils in nonpolypoid versus polypoid CRS is not clear, because eosinophils are more prominent in the latter.^{34,35} Finally, in the context of viral infections, RANTES may not be entirely specific for eosinophils because it has some capacity to promote neutrophil chemotaxis, possibly through CCR1.³⁶ Nonetheless, we feel that further studies are warranted to investigate whether dysregulation of epithelial RANTES production may be involved in the pathogenesis of CRS.

We thank Drs Andrew Luster and Michael T. Wilson for helpful suggestions and critical review of the manuscript.

Key messages

- Innate epithelial responses to noxious stimuli, bacterial or viral pathogens may be involved in the pathogenesis of CRS.
- The combination of CSE+dsRNA selectively induces exaggerated RANTES expression in CRS epithelium.
- An analogous response to cigarette smoke plus viral infection may contribute to acute exacerbations and eosinophilic mucosal inflammation in CRS.

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METHODS

Characterization of the epithelial immune response by RT-PCR

The epithelial immune response was characterized with RT-PCR by measuring mRNA expression of the following species: TNF- α , IL-1 β , IL-6,

IL-8, GRO- α , HBD2, MCP-2, MCP-3, RANTES, TLR-3, IL-17R, IFN- β , IFN- λ 1, and IFN- λ 2/3. Forward and reverse primers were selected based on OMIM Blast software (<http://www.ncbi.nlm.nih.gov/pubmed>) and are summarized here.

	Forward primer (5'-3')	Reverse primer (5'-3')
TNF- α	GGTGCTTGTTTCCTCAGCCTC	GGTGCTTGTTTCCTCAGCCTC
IL-1 β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACCTGAC
IL-6	GACCCAACCACAAATGCCA	GTCATGTCCTGCAGCCACTG
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGCCAAAACCTGCACCTT
GRO- α	CTCTTCCGCTCCTCTCACAG	TCACGTTACACTTTGGATG
HBD2	TGATGCCTCTTCCAGGTGTTT	GGATGACATATGGCTCCACTCTTA
MCP-2	CAATGTGGCTGACTTGACTTCC	GACAGGACTTTCTCATTCCCAG
MCP-3	CAATGTGGCTGACTTGACTTCC	GACAGGACTTTCTCATTCCCAG
RANTES	ATCCTCATTGCTACTGCCCTC	GCCACTGGTGTAAGAAATACTCC
TLR-3	TTGCCTTGATCTACTTTGGGG	GCGGCTGGTAATCTTCTGAGTT
IL-17R	TGCACGGTCAAGAATAGTACCT	GTTGGGTGTGGGCAAAGTG
IFN- β	CAGCAATTTTCAGTGTGAGAAGCT	TCATCCTGTCCTTGAGGCAGT
IFN- λ 1	GCCCCCAAAAAGGAGTCCG	AGGTTCCCATCGGCCACATA
IFN- λ 2/3	CTG CCA CATAGC CCA GTTCA	AGA AGC GACTCTTCT AAGGCA

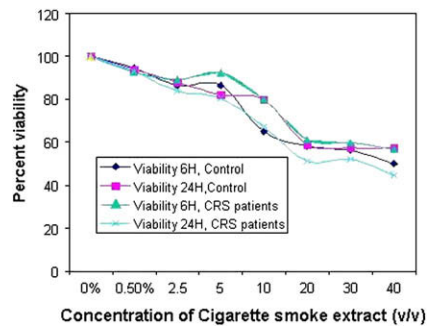


FIG E1. Viability of primary nasal epithelial cells after 1-hour pulse exposure to CSE assessed at 6 hours and 24 hours by Trypan blue dye exclusion. Viability after exposure to 5% CSE was 81% at 24 hours in both normal controls and patients with CRS.

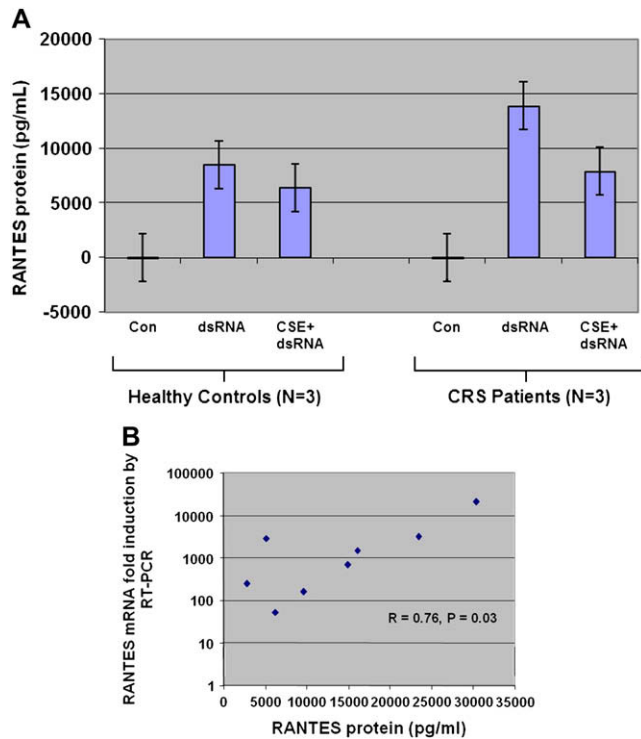


FIG E2. RANTES protein levels and correlation with RANTES mRNA expression in new set of PNEC culture supernatants. **A**, RANTES protein in PNEC culture supernatant measured by ELISA in 3 healthy controls and 3 subjects with CRS. **B**, Dot-plot of RANTES protein in PNEC culture supernatant versus RANTES mRNA relative expression in the same PNEC cultures (N = 8). *Con*, Control, unstimulated condition.