Differential expression of innate immunity genes in chronic rhinosinusitis

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ABSTRACT

Background: Prior research has identified several components of the innate immune system that may play a significant role in chronic rhinosinusitis (CRS), but the role of innate immunity in patients with CRS is poorly understood. The objective of this study was to determine differential expression of innate immunity genes in the mucosa of patients with CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSsNP) when compared with controls.

Methods: Control patients ($n = 9$) and patients with CRS ($n = 36$) who failed medical management were prospectively enrolled. Ethmoid mucosa samples were harvested during surgery and quantitative real-time polymerase chain reaction was used to determine levels of mRNA expression of Toll-like receptor (TLR) 2 and TLR9 and interleukin-22 receptor (IL-22R). The average change in crossover threshold and fold change were calculated and differences between controls, CRSwNP, and CRSsNP were compared. Statistical analysis was performed using the Kruskal–Wallis and adjusted Mann–Whitney U tests.

Results: Patients with CRSwNP ($n = 16$) and CRSsNP ($n = 20$) showed lower mean expression of TLR2 ($p < 0.05$) compared with controls. Patients with CRSsNP showed significantly higher mean expression of IL-22R ($p < 0.05$) than controls.

Conclusion: The sinonasal innate immune system may have a significant role in the development of CRS. We found differential expression of innate immune mediators between patients with and without nasal polyposis. These results provide further evidence of disruption of innate immunity at the mucosal level in CRS and highlight differences between polyp- and non-polyp-forming CRS phenotypes at the molecular level. In addition to our knowledge, this is the first report of altered IL-22R expression in CRSsNP patients. This study was a part of the clinical trial NCT01332136 registered in www.clinicaltrials.gov.

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Despite the wide prevalence of chronic rhinosinusitis (CRS) and its significant deleterious effect on health-related quality of life and health-care expenditure, the pathogenesis of this disease is surprisingly poorly understood. Although it is clear that CRS is a disease of chronic and uncontrolled inflammation, we are still struggling to understand the underlying mechanisms leading to the development of this inflammation. Inflammation is regulated by the immune system, which may be conceptually divided into two components: adaptive and innate. Although these systems use different pathways, there is bidirectional communication between these two systems, enabling the innate immune system to activate the adaptive immune response. Although much of the research, to date, has focused on the imbalance in the adaptive immune system,^{1–3} it is increasingly clear that the adaptive immune system is not the only factor in the development of CRS and that the innate immune system may play a significant role in pathophysiology of CRS.^{4–8} Illuminating the role of the primary in-

nate immune response in CRS is necessary and could lead to more effective disease treatment.

Prior research has identified several components of the innate immune system that may play a significant role in CRS, including Toll-like receptor (TLR) 2, TLR9, and interleukin-22 receptor (IL-22R).^{9–14} There is a paucity of evidence, however, regarding these innate immunity mediators and their relationship to CRS. In the current study, we sought to further investigate these innate immunity mediators in patients with CRS and to elucidate the differential expression between patients with CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSsNP) when compared with controls.

MATERIALS AND METHODS

Study Population and Inclusion Criteria

Patients presenting to the Oregon Sinus Center (Oregon Health and Science University, Portland, OR) for surgical treatment of CRS were offered prospective subject enrollment. Patients without a clinical diagnosis of CRS who were undergoing endoscopic nasal surgery for concha bullosa resection or transsphenoidal approach to the pituitary were prospectively enrolled as control subjects. Exclusion criteria included a history of recurrent acute sinusitis, cystic fibrosis, and/or immunodeficiency. The exclusion criteria were developed in the interest of creating a relatively homogeneous study population. All patients with CRS met diagnostic criteria for CRS as described by the 2007 Multi-Disciplinary Sinusitis Guidelines.¹⁵ All subjects elected to pursue endoscopic sinus surgery (ESS) after symptoms persisted after initial medical management consisting of a 3-week course of culture-directed or broad-spectrum antibiotics, a tapering course of oral prednisone, nasal saline irrigations, and an 8-week course of topical nasal steroid spray. Subjects undergoing both primary and revision ESS were included in this study. All study subjects were placed on peri-operative steroids and antibiotics 1 week before surgery. Voluntary informed consent was obtained from all subjects and the Institutional Review Board at Oregon Health and Science University provided approval and oversight for this research.

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Table 1 Demographic and comorbid characteristics of the control patients, patients with CRSsNP, and patients with CRSwNP

Characteristics	Controls (n = 9) Mean (SD)	n (%)	CRSsNP (n = 19) Mean (SD)	n (%)	CRSwNP (n = 17) Mean (SD)	n (%)	p Value
Age (yr)	37.1 (15.3)		47.2 (13.7)		52.4 (17.2)		0.120
Male		3 (33.3)		7 (36.8)		8 (47.1)	
Female		6 (66.7)		12 (63.2)		9 (52.9)	0.741
White		8 (88.9)		17 (89.5)		15 (88.2)	
African American		1 (11.1)		1 (5.3)		1 (5.9)	
Asian		—		1 (5.3)		—	
American Indian/Alaska native		—		—		1 (5.9)	0.763
Hispanic/Latino		—		1 (5.3)		1 (5.9)	0.767
Prior sinus surgery		—		6 (31.6)		12 (70.6)	0.001
Allergy (test confirmed)		1 (11.1)		6 (31.6)		7 (41.2)	0.289
Asthma		1 (11.1)		7 (36.8)		10 (58.8)	0.057
ASA Intolerant		—		—		2 (11.8)	0.178

CRSsNP = chronic rhinosinusitis without nasal polyposis; CRSwNP = chronic rhinosinusitis with nasal polyposis; ASA = aspirin.

Specimen Collection

Mucosal biopsy specimens were taken intraoperatively from the ethmoid bulla or anterior ethmoid mucosa in the region of the former bulla in revision cases of study subjects and concha bullosa or sphenoid face of control subjects. The mucosa was separated from the bone and the tissues were preserved immediately in 1.0 mL of RNAlater (Applied Biosystems, Carlsbad, CA). The tissue samples were subsequently removed from RNAlater and cryogenically stored (Nalge Nunc International/Thermo Fisher Scientific, Rochester, NY) at -80°C until they were processed for RNA extraction.

Reverse-Transcription Polymerase Chain Reaction

RNA for reverse-transcription polymerase chain reaction (RT-PCR) was extracted from mucosa using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA). A total of 5 μg of mRNA was used to synthesize a cDNA probe using the RT² first strand kit (SABiosciences, Frederick, MD) for hybridization to a custom-made human gene array membrane including TLR-2, TLR-9, and IL-22R with GAPDH as a housekeeping control (Applied Biosystems). The method of semiquantitative real-time RT-PCR was performed using an ABI StepOnePlus system (Applied Biosystems, Inc.). Using this system, the parameter C_t (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the cDNA probe passes a fixed threshold above baseline. ΔC_t was calculated for each subject specimen as the difference between C_t values of each target gene of interest and the GAPDH gene and averaged, and higher mean ΔC_t values represent less gene expression.

The Kruskal–Wallis test for nonparametric distributions and χ^2 -testing was used to evaluate differences in patient characteristics across CRS subgroups and control values. The Kruskal–Wallis and adjusted Mann–Whitney U nonparametric tests were used to determine if the mean ΔC_t for all patients with CRS and patient subgroups of CRSwNP and CRSsNP were significantly different from control subjects. Means, SDs, and associated p values are reported where appropriate. Significance was determined by the conventional 0.05 α -level.

RESULTS

Patient Population

A total of 45 surgical patients with CRS were prospectively enrolled between July 2011 and April 2012. Study patients with a current diagnosis of recurrent acute sinusitis ($n = 7$) and comorbid cystic fibrosis ($n = 2$) were excluded from the final study cohort ($n = 36$).

Nineteen patients had CRSsNP and 17 patients had CRSwNP with a male/female ratio of 15:21. A total of nine control study patients were also enrolled during the same investigational period. Demographic and comorbid characteristics were compared across all three subgroups and reported in Table 1.

Reverse-Transcription Polymerase Chain Reaction

Toll-like Receptor 2. When evaluating subgroups of CRSwNP and CRSsNP, both patients with CRSwNP (mean $\Delta C_t = 6.73$ [0.58]) and CRSsNP (mean $\Delta C_t = 6.40$ [0.33]) exhibited significant decreased mean expression of TLR2 compared with controls ($p \leq 0.006$; Fig. 1).

Toll-like Receptor 9. There were no significant differences in expression of TLR9 between controls (mean $\Delta C_t = 11.57$ [0.88]) and patients with CRSwNP (mean $\Delta C_t = 11.79$ [1.81]) or CRSsNP (mean $\Delta C_t = 12.04$ [1.18]; $p = 0.532$).

Interleukin-22 Receptor. Patients with CRSsNP showed significantly higher expression of IL-22R (mean $\Delta C_t = 6.96$ [0.86]) compared with controls ($p = 0.033$). Patients with CRSwNP did not show a significant difference in mean expression of IL-22R (mean $\Delta C_t = 7.85$ [1.05]) compared with controls ($p = 0.958$; Fig. 2).

DISCUSSION

Although the exact mechanisms are unknown, modern theories of CRS pathogenesis favor a multifactorial and heterogeneous disease process resulting in chronic and uncontrolled mucosal inflammation. Active inquiry into the etiology of this inflammation is ongoing, and the innate immune system is an attractive potential candidate. The sinonasal mucosa is constantly exposed to a variety of microbes, and the innate immune system provides the first line of defense against these pathogens. Innate immunity consists of genetically encoded defense mechanisms that can rapidly respond to pathogens. Examples of innate immunity include barrier mechanisms, mucociliary clearance, antimicrobial peptides, and pattern recognition receptors such as TLRs. In contrast, adaptive immunity is a learned response to specific environmental antigens carried out by B and T lymphocytes and provides memory to the immune system. These systems work in conjunction and the adaptive immune system may be activated by various components of the innate immune system.^{4–6} Alterations in innate immune gene expression have been shown in patients with CRSwNP,^{6,10,12,14,16,17} but there is less literature regarding the role of the innate immune system in CRSsNP. Our objective was to expand the current literature by investigating both patients with and without NP. We showed that there is differential expression of innate immunity genes in both patients with CRSsNP and CRSwNP, supporting the theory that the innate immune system may be a factor in the pathophysiology of CRS.

Figure 1. Toll-like receptor (TLR) 2 ΔC_t values for control patients, patients with chronic rhinosinusitis with nasal polyposis (CRSwNP), and patients with chronic rhinosinusitis without nasal polyposis (CRSsNP). *Significance with $p < 0.05$.

Figure 2. Interleukin 22 receptor (IL-22R) ΔC_t values for control patients, patients with chronic rhinosinusitis with nasal polyposis (CRSwNP), and patients with chronic rhinosinusitis without nasal polyposis (CRSsNP). *Significance with $p < 0.05$.

TLR2 recognizes a wide array of microbial molecules such as peptidoglycan and lipoteichoic acid and mediates the immune response to numerous bacteria and fungi. TLR2 expression has been found to be significantly up-regulated in patients with CRSwNP, but significantly down-regulated specifically in those patients with recalcitrant CRSwNP as defined by those patients who had a recurrence or persistence of polyps within 6 months after surgery.¹⁰ TLR2 polymorphisms have also been associated with CRS in Korean adults.¹¹ Data regarding TLR2 expression in CRSsNP patients, however, is lacking. Our data found decreased expression of TLR2 in both patients with CRSwNP and CRSsNP, and to our knowledge, this is the first study to show TLR2 dysregulation in CRSsNP patients. It is possible the patient population we studied suffered from more recalcitrant forms of CRS, evidenced by the fact that >50% of our patients were undergoing at least their second ESS, which is consistent with prior studies showing TLR2 down-regulation in patients with recalcitrant disease.

TLR9 recognizes that unmethylated CpG sequences in bacterial DNA has been shown in prior studies to be both over- and underexpressed in patients with CRSwNP.^{12,14} We did not find, however, a significant difference in expression between controls and patients with CRSwNP or CRSsNP. This may reflect the heterogeneous nature of the disease and perhaps that the categories of CRSsNP and CRSwNP are too broad to determine differential expression of TLR9. In addition, different adaptive immune profiles have been found between Chinese and Belgian patients with CRSwNP,⁷ and it is possible that ethnic differences in innate immunity exist as well. The study showing increased TLR9 expression was in Chinese patients and may not be comparable with our patient data set given our predominantly white population.

IL-22R is associated with the IL-10 family of inflammatory cytokines. The IL-22R activates the Jak-STAT signal transduction pathway and, when activated, helps regulate inflammation, immunosurveillance, and homeostasis at mucosal barrier surfaces. IL-22R activation results in up-regulation of multiple genes involved in antimicrobial host defense and increases innate immune activity through production of antimicrobial peptides such as β -defensins.^{16,18} Abnormal activation and dysregulation of the IL-22 pathway is associated with inflammatory diseases such as psoriasis, atopic dermatitis, and inflammatory bowel disease.^{18,19} Decreased IL-22R expression has been associated with recalcitrant CRSwNP,¹⁶ and polymorphisms in this gene have been associated with severe CRS.⁹ Our data revealed increased IL-22R expression in patients with CRSsNP. We believe this is the first report of increased IL-22R expression in patients with CRS. Our data lends support to the theory that dysregulation of mucosal barrier immunity may be a starting point in the pathogenesis of CRS.

Alterations in innate immunity may reflect an underlying imbalance of the mucosal barrier in patients with CRS. Dysfunction of the innate immune system could lead to an initial altered response to pathogens culminating in chronic sinonasal inflammation and perhaps account for why only certain people develop CRS, despite the general population's exposure to the same environmental pathogens. Dysregulation of the innate immune system could affect patients' ability to mount a sufficient initial immune response to bacteria or fungi and allow for colonization and continued inflammation. This argument is supported by Pitzurra *et al.* who showed decreased TLR2 expression in patients with CRSwNP who were colonized with bacteria and fungi.²⁰ Alternatively, aberrant innate immune signaling may alter the response of the adaptive immune system and prevent Th-1/Th-2 homeostasis subsequently leading to CRS. In addition, a dysfunctional innate immune system may explain why our current treatments are inadequate for certain patients. There are drugs in development targeting the innate immune system in allergy and asthma,²¹ and it is possible similar medications could play a role in the treatment of select patients with CRS in the future.

Our data support the role of disruption of the innate immune system in the pathophysiology of CRS and add several novel findings related to differential gene expression in patients with CRSsNP. Innate immunity dysfunction is a potential contributor to the overall pathophysiology of CRS and elucidating its role will lead to a better understanding of the disease process and, ultimately, improved patient care. Dysregulation of the innate immune system in CRSwNP has been previously established, and our data augment the current literature and enhance it by evaluating those patients without nasal polyps; however, there are potential limitations to our study. The medical treatment of our patients with oral and topical corticosteroids before surgical management could theoretically alter innate immunity gene expression. Glucocorticoid treatment has been shown to enhance TLR2 expression in human keratinocytes, but we are unaware of any direct effects of steroids on sinonasal mucosal expression of TLR2, TLR4, or IL-22R.²² This potential limitation is difficult to overcome in our current observational study design; however, we are in the process of specifically identifying and evaluating patients who are not exposed to these perioperative medications. In our current clinical practice, we are no longer routinely treating patients with perioperative oral steroids, which has allowed for enrollment of an additional cohort of patients without a history of oral corticosteroid treatment for at least 6 weeks before surgery. Additional study of this additional cohort is ongoing.

CONCLUSION

We found that TLR2 and IL-22R are differentially expressed in CRS compared with controls. In our population, TLR2 is underexpressed in patients with both CRSsNP and CRSwNP. Only patients with CRSsNP show increased expression of IL-22R. Our data augment the

current literature and suggest that innate immune system dysregulation may contribute to CRS.

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