

Microarray Gene Analysis of Toll-Like Receptor Signaling Elements in Chronic Rhinosinusitis with Nasal Polyps

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Key Words

Toll-like receptors • TLR-9 • Chronic rhinosinusitis • Nasal polyps • Microarray analysis

Abstract

Objective: To identify the regulatory mechanisms of Toll-like receptor (TLR)-associated genes in chronic rhinosinusitis (CRS) with nasal polyps (NP) using gene microarray analyses. **Methods:** We pooled: (1) NP biopsy specimens from 10 nonatopic CRS patients and (2) healthy mucosal tissue from 10 additional nonatopic healthy patients (controls). These pooled samples were evaluated by gene microarrays that included 125 genes for TLRs and associated signaling elements. To validate gene product expressions, 20 NP and 15 normal nasal turbinate tissues were evaluated for TLR-9 expression by immunohistochemical staining and Western blots using samples from gland cells, epithelial cells, and mononuclear cells cytologically identified by HE staining. **Results:** In pooled NP samples compared to pooled controls, 4 genes were upregulated (≥ 2 -fold higher expression) and 19 were downregulated (≤ 0.5 -fold lower expression). TLR-9 was an upregulated gene in NP tissue. Compared to control tissue, there were significantly higher percentages of TLR-9 positively stained NP gland cells, epithelial cells, and mononuclear cells ($p < 0.001$). On Western blots, while both normal

and NP tissues expressed TLR-9 protein, the expression was significantly more pronounced for NP tissue ($p < 0.001$). **Conclusions:** Inflammation associated with CRS may be due to dysregulated innate immune elements, particularly TLR-9 and its associated signal transduction elements, which may impact upon prolonged activation of adaptive immune responses in the sinonasal mucosa.

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Introduction

Chronic rhinosinusitis (CRS), an inflammatory disorder involving the mucosa of the nose and paranasal sinuses, has recently become more prevalent. Quality-of-life surveys have found remarkably large negative impacts due to CRS comparable to those of asthma, angina, chronic pulmonary disease, or chronic lower back pain [1]. The mechanisms underlying CRS inflammation are incompletely understood, although it is becoming clear that both local and systemic factors contribute to this condition.

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The sinonasal cavities are the first line of defense for combating potential pathogens in inspired air by utilizing both innate and adaptive immune processes. The current view hypothesizes that CRS results, at least in part, from an inability of the nasal epithelium to produce appropriate and regulated immune responses to foreign matter. Defects in the mechanical barrier and innate immune response could result in inappropriate recruitment and persistence of adaptive immune responses with development of the clinical symptoms characteristic of CRS [2].

Many studies have demonstrated the presence of adaptive immune elements in CRS, such as infiltrating lymphocytes and their associated proinflammatory cytokines. Studies using cDNA microarray technology have identified differentially expressed genes involved with inflammatory processes on a 'global' level [3, 4] and, in particular, components of adaptive immunity [5–7]. However, innate immune responses in the sinonasal mucosa in CRS have not been extensively studied. Investigations of the innate immune system have led to new insights regarding the pathogenesis of CRS. Much has recently been learned regarding the complex pathways that regulate mucosal innate immunity in the airways [8]. A variety of molecules have been identified that recognize pathogens and lead to their destruction.

Toll-like receptors (TLRs) have recently been recognized as playing major roles in pathogen recognition and innate immunity [9, 10]. To date, 10 TLR family members (TLR1–10) have been identified in the human genome, and different TLRs appear to play crucial roles in the activation of the immune response to distinct pathogen-associated molecular patterns [11, 12]. Further, activation of innate immunity is a critical step for the development of antigen-specific acquired immunity.

Upon activation by specific ligands, TLRs initiate intracellular signaling cascades that culminate in the activation of transcription factors and ultimately lead to changes in proinflammatory gene expressions [13]. It is critical to determine how events that occur on the sinonasal mucosal surface are translated to the immune system via the epithelium in order to understand how dysfunctions of these processes might result in persistent immune activation. A starting point is to determine the patterns of sinonasal TLRs and their signal pathways in healthy and CRS tissues.

CRS with nasal polyps (NP) is characterized by persistent eosinophilic inflammation, edematous mucosa with polyps, and thickened sinonasal secretions. In this study, the possible role of innate immune responses in CRS was explored by examining the microarray gene expressions

of TLRs and their signal pathways in human NP tissue and normal tissue of patients without evidence of atopic disease(s). This may implicate specific TLRs or suggest an innate immune pathway that is abnormally regulated in CRS.

Methods

Patient Specimens

All tissues for microarray analysis were obtained from inpatients seen in our department. Tissues were removed during the normal course of endoscopic surgery. None of the patients had physician-diagnosed asthma and all had negative reactions to skin prick tests for a battery of allergens that included inhalation allergens [feathers, dog hair, pollen, ragweed, date palm (*Phoenix dactylifera*) pollen, dust mites, house dust mites, *Cladosporium*, *Aspergillus fumigatus*, and mugwort] and food allergens (wheat-meal, mutton, milk, shrimp, corn, peanut, fish, eggs, beef, and mango). All patients were taken off steroids and antibiotics 2 weeks before surgery. NP biopsies were obtained from the middle meatus of 10 patients with CRS (5 men and 5 women) aged 16–53 years (mean = 37.4 years). The CRS with NP (CRSwNP) patients fulfilled the diagnostic criteria recommended by Metzler et al. [14]. Control biopsies for microarray assays were of healthy mucosa from the middle meatus of 10 nonatopic patients (9 men and 1 woman) aged 18–56 years (mean = 33.4) during nasal septal reconstruction surgery. After resection, fresh tissues were immediately placed in RNAlater tubes and kept at –20°C. A functional classification array (SuperArray Oligo GEArray®) with TLR signaling pathway genes was used to analyze NP and control tissues.

For the experimental verification of gene product expressions, 20 NP tissues and 15 inferior turbinate tissues were used for immunohistochemistry and Western blot. NP tissues were obtained from another 20 nonatopic patients with CRS (11 men and 9 women; mean age = 39.5 years). Inferior turbinate tissues were obtained from an additional 15 nonatopic patients with nasal septal deviations (8 men and 7 women; mean age = 35.4 years). Tissues were cut into 2 portions. One portion was immediately frozen at –70°C for immunohistochemical staining and another portion was immediately frozen in liquid nitrogen and stored at –70°C for Western blot.

This study was approved by the ethics committee of our institution and written informed consent was obtained from all patients.

RNA Isolation

The 10 NP biopsy specimens from patients with CRSwNP were pooled as an NP sample and the 10 healthy mucosal specimens were pooled as a control. These tissue samples were homogenized in TRIZOL Reagent (Invitrogen Life Technologies) and incubated for 5 min at 15–30°C. Chloroform (0.2 ml) was added per 1 ml of TRIZOL reagent. Following centrifugation, RNA was removed from the aqueous phase. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol and washed once with 75% ethanol. The sample was vortexed and centrifuged at 7,500 g for 5 min at 4°C. RNA was dissolved in RNase-free water, incubated for 10 min at 55–60°C, and then stored at –70°C. The concentration and purity of RNA was assessed by UV absorbance and

loaded onto a denaturing agarose gel for electrophoresis. The concentration of RNA extracted from tissues was valid. The ratio of A260 to A280 values is a measure of RNA purity and should fall in the range of 1.8–2.1. After denaturing agarose electrophoresis, the gel was visualized on a UV transilluminator. The 28S and 18S ribosomal RNA bands showed sharp, intense bands (data not shown).

cDNA Labeling and Synthesis

Each RNA sample was synthesized to cDNA by reverse transcription. cDNA synthesis, labeling, and amplification was performed using a TrueLabeling-AMP™ Linear Amplification Kit. cDNA was labeled with Biotin-16-UTP (Roche). Each chip required a minimum of 4 µg of cDNA. A SuperArray ArrayGrade cDNA Cleanup Kit was used to purify cDNA.

Hybridization

We used human TLR signaling pathway PCR arrays (catalogue No. PAHS-018; SuperArray Bioscience). Each array could evaluate 125 genes for TLRs and associated signaling elements. Oligo GEArrays were hybridized following the manufacturer's protocols (SuperArray Bioscience). Briefly, the array membrane in a hybridization tube was prewet with 5 ml of deionized water and allowed to sit for at least 5 min. GEAhyb Hybridization Solution (SuperArray Bioscience) was warmed to 60°C and the buffer components were dissolved. The deionized water was discarded and the membrane was prehybridized in an oven at 60°C for 1–2 h with continuous agitation at 5–10 rpm. Prewarmed GEAhyb Hybridization Solution (2 ml) was added and vortexed and then 4–20 µg of biotin-labeled cDNA target prepared using the TrueLabeling-AMP kit was added to 0.75 ml of prewarmed GEAhyb Hybridization Solution and mixed well. After discarding the prehybridization solution, a Target Hybridization Mix containing labeled cDNA target was added and hybridized overnight at 60°C with continuous agitation at 5–10 rpm. Each Oligo GEArray was washed separately in Wash Solution 1 (2× SSC, 1% SDS; mix 10 ml 20× SSC, 5 ml 20% SDS, and 85 ml ddH₂O) and Wash Solution 2 (0.1× SSC, 0.5% SDS mix 0.5 ml 20× SSC, 2.5 ml 20% SDS, and 97 ml ddH₂O).

Detection and Data Analysis

After discarding the last wash, 1.5 ml GEA Blocking Solution Q was added and incubated for 40 min with continuous agitation at 30–40 rpm. Binding buffer (2 ml) was added and incubated for 10 min with continuous, gentle agitation. CDP-Star chemiluminescent substrate (1.0 ml) was added and incubated at room temperature for 2 min. The membrane was blotted to remove excess CDP-Star solution and exposed to X-ray film. The chemiluminescent array image was captured from the X-ray film using a flatbed desktop scanner. All images were saved as electronic files in grayscale (8 or 16 bit) in TIFF format or, alternatively, JPEG format. The web-based completely integrated GEArray Expression Analysis Suite was used to complete the data analysis. The analyses of the 2 microarrays, 1 for the pooled NP samples and 1 for the pooled control samples, were performed by KangChen Biotech (Shanghai, China).

Immunohistochemical Staining

Cryosections (4 µm thick) were fixed with acetone for 1 min and then rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4;

3 × 5 min) at room temperature (RT). Sections were blocked for endogenous peroxidase activity using 3% H₂O₂ (10 min at RT). The slides were incubated with rabbit polyclonal antibody TLR-9 (Abcam, Cambridge, UK) diluted 1:200 in 0.01 M PBS for 2 h at 37°C. Negative controls used PBS instead of the primary antibody. Immunohistochemical staining used an SP kit (ZhongShan Company, Beijing, China) for 30 min at RT. Additional controls were evaluated for possible nonspecific staining by secondary antibodies; these results were negative (data not shown). The antigen was visualized by diaminobenzadine. Subsequently, the slides were counterstained with hematoxylin, dehydrated, and mounted. Tissue histology was contrasted by staining with HE to identify gland cells, epithelial cells, and mononuclear cells.

TLR-9 expression was determined as the percentages of cells positive for TLR-9 in each of these cell types. Samples were evaluated by 1 person who was blinded to the patients' health status. Each sample was evaluated for 10 high-power microscopic fields.

Western Blot

Both CRSwNP and control tissues frozen in liquid nitrogen were milled and proteins were extracted. Total protein of a tissue lysate (50 µg) was added to each well of a 1.0-mm-thick gel. Proteins were electroblotted from the gel to a polyvinylidene difluoride membrane (Sigma) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% MeOH). The membrane was blocked with blocking buffer (5% nonfat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, and 0.1% Tween 20). The blots were incubated for 1 h at RT and then treated overnight at 4°C with rabbit polyclonal antibody TLR-9 (Abcam) diluted 1:2,000 and anti-human β-actin (Santa Cruz Biotechnology) diluted 1:750. Blots were washed 3 times and incubated for 1 h at RT with the appropriate secondary antibody (ZhongShan) coupled to horseradish peroxidase. Immunodetection used an ECL detection kit (Amersham Pharmacia Biotech). The expression of TLR-9 on Western blots was normalized to β-actin expression in the samples.

Statistical Analysis

Based on information provided by the company that did the microarray analyses, a 2-fold or greater change indicated a significant change in gene expression based on the microarray manufacturer's recommendations. Continuous variables were all non-normally distributed and given as medians and interquartile ranges (IQR). Statistical comparisons of continuous were conducted using a Mann-Whitney U test. Categorical variables are given as numbers and percentages; Fisher's exact test was used to test their associations. Statistical analyses were set with a significance level of 0.05 and were performed using SPSS 15.0 statistics software (SPSS Inc., Chicago, Ill., USA).

Results

Oligo GEArray Data Analysis

There were 125 genes associated with TLR signaling pathways included on the microarrays. Each of the RNA samples from the pooled NP biopsies and pooled control biopsies were hybridized on 2 different array chips. Comparing normal tissue with polyps, 23 genes were differ-

Table 1. Upregulated and downregulated genes in NP compared to normal mucosa

GeneBank	Description
Downregulated (≤ 0.5-fold) (n = 19)	
NM_001228	CASP8 (caspase 8, apoptosis-related cysteine protease)
NM_002154	HSPA4 (heat shock 70-kDa protein 4)
NM_003639	IKBKG (inhibitor of kappa light polypeptide gene enhancer in B cells, kinase gamma)
NM_000572	IL10 (interleukin 10)
NM_000586	IL2 (interleukin 2)
NM_002228	JUN [V-jun sarcoma virus 17 oncogene homolog (avian)]
NM_002758	MAP2K6 (mitogen-activated protein kinase kinase 6)
XM_042066	MAP3K1 (mitogen-activated protein kinase kinase 1)
NM_002751	MAPK11 (mitogen-activated protein kinase 11)
NM_002969	MAPK12 (mitogen-activated protein kinase 12)
NM_002754	MAPK13 (mitogen-activated protein kinase 13)
NM_139046	MAPK8 (mitogen-activated protein kinase 8)
NM_015133	MAPK8IP3 (mitogen-activated protein kinase 8 interacting protein 3)
NM_003298	NR2C2 (nuclear receptor subfamily 2, group C, member 2)
NM_000963	PTGS2 [prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)]
NM_002908	REL [V-rel reticuloendotheliosis viral oncogene homolog (avian)]
NM_021805	SIGIRR (single Ig IL-1R-related molecule)
NM_016581	SITPEC (likely ortholog of mouse signaling intermediate in Toll pathway, evolutionarily conserved)
NM_006068	TLR6 (Toll-like receptor 6)
Upregulated (≥ 2-fold) (n = 4)	
NM_017442	TLR-9 (Toll-like receptor 9)
NM_002759	EIF2AK2 (eukaryotic translation initiation factor 2-alpha kinase 2)
NM_030956	TLR-10 (Toll-like receptor 10)
NM_015364	LY96 (lymphocyte antigen 96)

entially expressed in NP compared to healthy (control) tissue, with 4 upregulated genes (≥ 2 -fold higher expression) and 19 downregulated genes (≤ 0.5 -fold lower expression). These differentially expressed genes are listed in table 1. Based on the array manufacturer's recommendations, a 2-fold or greater change indicated a significant difference in gene expression.

Immunohistochemical Validation

TLR-9 was included among the genes with increased expressions in NP compared with healthy mucosa. NP tissues from 20 nonatopic patients with CRS and inferior turbinate tissues from 15 nonatopic healthy volunteers were obtained for immunohistochemical validation. The demographic characteristics and TLR-9 expressions for these 35 patients are summarized in table 2.

As summarized in figure 1, TLR-9 expressions differed within the gland cells, epithelium, and mononuclear cells (cytologically identified in tissue samples by HE staining). Results were determined as the percentages of each cell type that expressed TLR-9; statistical comparisons between NP and normal tissues are shown in table 2. TLR-9 expressions were significantly higher in NP tissues than in turbinate tissues for gland cells, epithelial cells, and mononuclear cells (all $p < 0.001$).

Western Blot for TLR-9

By Western blot, TLR-9 showed specific bands in both NP and turbinates. However, the TLR-9 bands for NP were more intense than those for turbinates (fig. 2). The results for TLR-9 expression were normalized to β -actin expression for all samples.

Discussion

Mucosal immunity in sinonasal cavities can be broadly characterized into adaptive and innate immunity. Innate immune elements, such as macrophages, were previously thought to nonspecifically engulf pathogens in order to 'buy time' before an adaptive immune response could be generated [9]. It has recently been shown that the innate immune system has considerable specificity and the ability to discriminate between pathogens and self. Previous studies of NP using cDNA microarray technology have identified differentially expressed genes involved with inflammatory processes on a 'global' level [3, 4] and components of adaptive immunity in particular [5–7]. In contrast with these studies, the aim of this exploratory study was to determine if there were any differentially expressed genes in NP of nonatopic CRS patients that were associated with TLRs and their signaling elements.

Different TLRs appear to play crucial roles in the activation of immune responses to distinct pathogen-associated molecular patterns, such as bacterial lipopeptide, endotoxin, flagellin, double-stranded RNA, or bacterial DNA. Signaling through TLRs activates the expressions

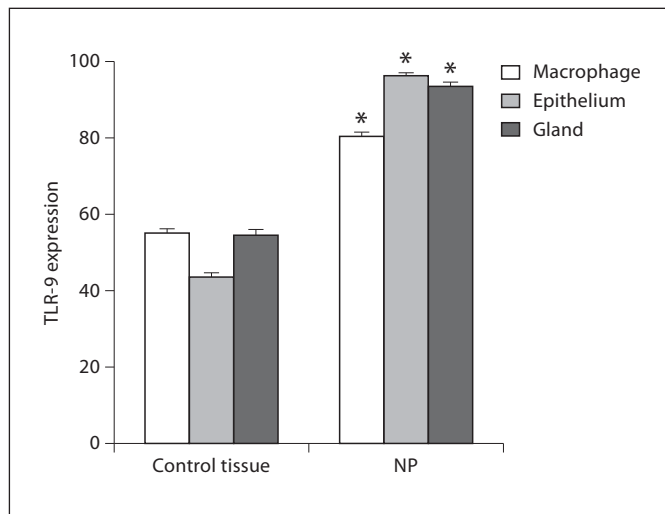


Fig. 1. Summary of the immunohistochemical expressions of TLR-9 in the NP of CRS patients and in the inferior turbinates of healthy tissue. Results are for 20 nonatopic CRS patients and 15 nonatopic control samples. TLR-9 expressions are shown as percentages of mononuclear cells, epithelial cells, and gland cells (identified in tissue sections by HE staining) that were positively stained. Data are means $\pm$ SD. * $p < 0.05$ for NP compared to control (Mann-Whitney U test).

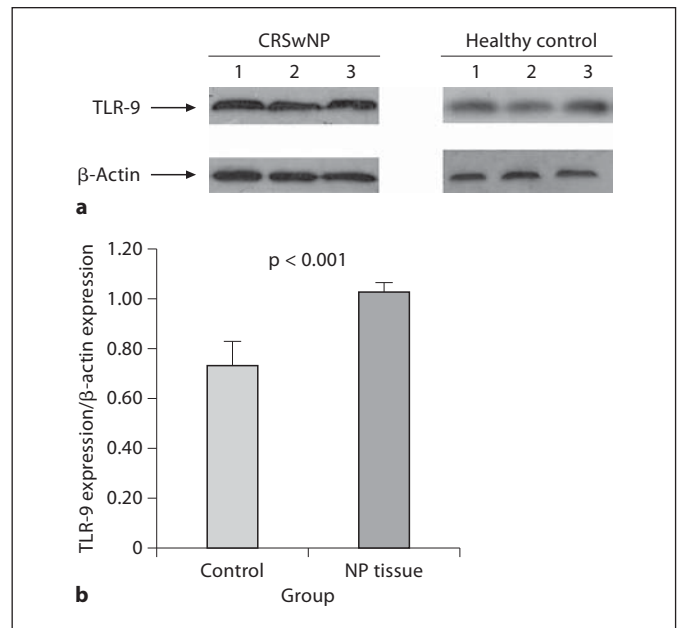


Fig. 2. Representative Western blots for TLR-9 expressions in NP and control turbinates. **a** TLR-9 and β -actin protein expression in tissues from CRSwNP (left) and healthy controls (right) for 3 representative patients. **b** Relative protein expression. The protein expression level was quantified with a densitometer followed by normalization with β -actin protein expression levels. Data are means $\pm$ SD ($n = 10$). * $p < 0.05$ for NP compared to control (Mann-Whitney U test).

Table 2. Demographics and TLR-9 expressions by groups

	Total	Group		p value
		Control tissue (n = 15)	NP (n = 20)	
Age years ¹	36.0 (31.0, 44.0)	36.0 (29.0, 42.0)	37.5 (32.5, 47.0)	0.286
Gender ²				
Female	15 (42.9)	7 (46.7)	8 (40.0)	0.741
Male	20 (57.1)	8 (53.3)	12 (60.0)	
TLR-9 expression in HE staining ^{1,3}				
Gland, %	89.70 (57.30, 94.50)	54.80 (49.80, 58.60)	92.50 (90.55, 97.80)	<0.001
Epithelium, %	93.40 (44.20, 97.50)	42.80 (40.50, 48.10)	97.35 (95.40, 98.15)	<0.001
Mononuclear cells, %	76.50 (56.70, 82.10)	54.90 (52.70, 57.20)	80.90 (77.95, 83.85)	<0.001
TLR-9 expression in Western blot (TLR-9/ β -actin) ¹	0.91 (0.73, 1.02)	0.73 (0.65, 0.82)	1.02 (1.01, 1.06)	<0.001

¹ Results are medians (IQRs) and were compared by the Mann-Whitney U test.

² Results are numbers (percentages) and were compared by Fisher's exact test.

³ TLR-9 as the percentage of cells staining positive.

of a host of cytokines, chemokines, hematopoietic factors, acute phase proteins, and antimicrobial factors. Factors important in the signal transduction pathways of TLRs include MyD88, IL-1 receptor-associated kinase, TNF receptor-associated factor 6, mitogen-activated protein kinases (MAPK), and NF- κ B [15, 16]. The majority of TLR research in respiratory medicine has focused on lower-airway inflammation; very few studies have examined the roles of TLRs in sinonasal disease.

Vandermeer et al. [17] demonstrated that mRNAs for TLRs were expressed in the human sinonasal mucosa of CRS and control patients using real-time PCR. Lane et al. [18] showed increased levels of TLR-2 expression and a variety of inflammatory genes in the sinonasal mucosa of CRS patients compared with controls. Dong et al. [19] demonstrated that epithelial cells from both CRS and controls had upregulated TLR2 and TLR4 mRNA expressions. Similarly, Fransson et al. [20] showed that TLR2, TLR3, and TLR4 expressions are upregulated in patients with seasonal allergic rhinitis. In our study, we found expression differences for TLR signal pathways between NP tissues of CRSwNP and normal nasal mucosa, including TLRs, adaptors, and TLR interacting proteins, effectors, downstream pathways, and target genes, which can regulate adaptive immune responses. Thus, we can determine which alterations of TLR signal pathways may be involved in the pathogenesis of CRS on the whole and provide clues and foundation for further research.

Our study showed 4 upregulated genes in NP. Lymphocyte antigen 96 (LY96), also called MD2, is a secreted glycoprotein that binds to the extracellular domain of TLR-4 and is required for TLR-4 activation by lipopolysaccharides [21]. Eukaryotic translation initiation factor 2- α kinase 2 (EIF2AK2), also called double-stranded RNA-dependent protein kinase (PKR), mediates both antiviral and anticellular activities. EIF2AK2 inhibits translation initiation via phosphorylation of the α subunit of the initiation factor eIF-2 (eIF-2 α) and controls the activation of several transcription factors, such as NF- κ B, p53, and STATs [22].

In our study, TLR-9 and TLR-10 were also shown to be upregulated in CRSwNP. The ligands and possible functions of TLR-10 in humans are currently unknown, although Lazurus et al. [23] postulated that genetic variants of TLR-10 may be associated with asthma susceptibility. Because our patients were nonasthmatic, the clinical relevance of our findings remains uncertain. TLR-9 is a receptor for unmethylated CpG dinucleotides that are found in bacterial and viral, but not in human, DNA. TLR-9 activation induces strong Th1-like immune acti-

vation with the secretion of type-I IFN and activation of natural killer cells and strong CD8 T-cell responses [24]. Expression of TLR-9 has been demonstrated on primary and cultured cells from the human lower airway epithelium and in sinonasal tissue [6, 11, 25]. TLR-9 has also been found on leukocytes like monocytes/macrophages, B cells, neutrophils, and dendritic cells [26, 27].

However, a recent study showed that recalcitrant CRS is associated with decreased levels of sinonasal epithelial cell TLR-9 protein [18]. Similar results were found using primary cultured epithelial cells derived from CRS patients [28]. In another study, TLR-9 expression showed no difference compared to controls in terms of allergic rhinitis [29]. In the BEAS-2B airway epithelial cell line, real-time RT-PCR revealed high expression of TLR-9 mRNA [30].

In our validation experiment, we confirmed high expression of TLR-9 in NP by immunohistochemical staining and Western blots. Distinct expressions of TLR-9 were found in the gland cells, epithelium, and mononuclear cells. In contrast to prior studies, we could find differences of TLR-9 expressions in different cellular compartments. The possible involvement of different cellular compartments has also been highlighted in studies by Zhang et al. [31] and Bachert et al. [32]. Zhang et al. [31] found predominantly Th2-biased T effector cells in polyps of patients with comorbid asthma who were also predominantly whites of Belgian ancestry, while the T effector cells in polyps of Chinese patients without comorbid asthma were predominantly Th1-/Th17-biased. To rule out a possible ethnic association, Bachert et al. [32] found Th2-biased effector cells in both whites (Belgians) and Chinese with comorbid asthma. Because our Chinese patients were nonasthmatic, their NP pathology was presumably Th1-/Th17-biased, and the underlying expression differences of TLR-9 expression in different cell compartments were presumably associated with innate immune differences not associated with asthma or atopic sensitivity.

Another difference between our results and those of previous studies is that our patients were taken off steroids and antibiotics for 2 weeks prior to obtaining samples, which should have minimized any medication effects in our study. A previous study showed that TLR-9 expression is upregulated on epithelial cells when they are exposed to pathogenic bacterial DNA [33]. This may explain the increased TLR-9 expression observed in our study.

In human umbilical vein cells, it was demonstrated that bacterial DNA (CpG DNA) promoted beta2 integrin and

E-selectin-mediated neutrophil adherence, and there was indication of the ability of CpG DNA to initiate and/or maintain an inflammatory response [34]. In our study, the distinct and general expression of TLR-9 in nasal epithelium and in inflammatory cells indicated a broad opportunity for bacterial and viral airway infectious agents to interact directly with an adaptive immune response via expression of CpG-related ligands. This could represent one pathogenic mechanism to explain CRS inflammation.

Our experiments also demonstrated some genes that were downregulated in the TLR signal pathway, which can be classified into several groups. Casp8, Nr2c2 (TAK1), and Sitpec (ECSIT) are effectors. Regarding the downstream pathway and target genes, Ikbkg (IKK-g), Il2, and Il10 belong to the NF- κ B pathway; Jun, Map3k1 (MEKK1), Mapk8 (JNK1), Mapk11 (p38bMAPK), Mapk12 (p38gMAPK), and Mapk13 belong to the JNK/p38 pathway, and PTGES and Ptg2 (Cox-2) belong to the NF/IL6 pathway. TLR-6 was found in decreased levels in CRSwNP in our study. TLR-6 is required for the recognition of diacylated lipoproteins and lipopeptides derived from mycoplasma and activates the NF- κ B signaling cascade in conjunction with TLR-2 [35]. Downregulated TLR-6 in our study could suppress the expression of genes in the NF- κ B signaling pathway. As a cautionary note, many of the downregulated genes in our study only

showed about 0.5-fold changes which may not reflect significant expression changes.

CRSwNP is fundamentally a disease of inflammation rather than infection. The microbial triggers in the nasal cavity are fairly ubiquitous, existing in healthy individuals as well as in CRS patients. Thus, CRS may represent a dysregulation of normal mucosal immune mechanisms. The alterations of the TLR signal pathway in our research may be a preliminary step involved with innate immunity in CRS. Recently, most attention has focused on TLR agonists, which are potential targets for therapeutic interventions against CRS. The TLR-9 agonist CpG has been the most widely explored therapeutic target for cancer, allergy/asthma, and vaccine biology. Although we found differences in TLR-9 expressions in CRSwNP tissue, our results do not provide a mechanistic basis for what these differences imply. The role of TLR-9 in the immunology and etiology of CRS requires further investigation.

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References

- 1 Anand V: Epidemiology and economic impact of rhinosinusitis. *Ann Otol Rhinol Laryngol Suppl* 2004;(suppl)193:3–5.
- 2 Schleimer RP, Kato A, Peters A, Conley D, Kim J, Liu MC, Harris KE, Kuperman DA, Chandra R, Favoreto S Jr, Avila PC, Grammer LC, Kern RC: Epithelium, inflammation, and immunity in the upper airways of humans: studies in chronic rhinosinusitis. *Proc Am Thorac Soc* 2009;6:288–294.
- 3 Liu Z, Kim J, Sypek JP, Wang IM, Horton H, Oppenheim FG, Bochner BS: Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. *J Allergy Clin Immunol* 2004;114:783–790.
- 4 Payne SC, Han JK, Huyett P, Negri J, Kropf EZ, Borish L, Steinke JW: Microarray analysis of distinct gene transcription profiles in non-eosinophilic chronic sinusitis with nasal polyps. *Am J Rhinol* 2008;22:568–581.
- 5 Wang X, Dong Z, Zhu D, Guan B: Expression profile of immune-associated genes in nasal polyps. *Ann Otol Rhinol Laryngol* 2006;155:450–456.
- 6 Figueirido CR, Santos RP, Silva, I, Weckx L: Microarray cDNA to identify inflammatory genes in nasal polyposis. *Am J Rhinol* 2007;21:231–235.
- 7 Sekigawa T, Tajima A, Hasegawa T, Hasegawa Y, Inoue H, Sano Y, Matsune S, Kurono Y, Inoue I: Gene-expression profiles in human nasal polyp tissues and identification of genetic susceptibility in aspirin-intolerant asthma. *Clin Exp Allergy* 2009;39:972–981.
- 8 Wise MT, Hagaman DD: An immunological approach to chronic and recurrent sinusitis. *Curr Opin Otolaryngol Head Neck Surg* 2007;15:10–17.
- 9 Ramanathan M Jr, Lane AP: Innate immunity of the sinonasal cavity and its role in chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 2006;136:348–356.
- 10 Iwamura C, Sakayama T: Toll-like receptors in the respiratory system: their roles in inflammation. *Curr Asthma Allergy Reports* 2008;8:7–13.
- 11 Takeda K, Kaisho T, Akira S: Toll-like receptors. *Annu Rev Immunol* 2003;21:335–376.
- 12 Seitz M: Toll-like receptors: sensors of the innate immune system. *Allergy* 2003;58:1247–1249.
- 13 Greene CM, McElvaney NG: Toll-like receptor expression and function in airway epithelial cells. *Arch Immunol Ther Exp (Warsz)* 2005;53:418–427.
- 14 Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, Adinoff AD, Bachert C, Borish L, Chinchilli VM, Danzig MR, Ferguson BJ, Fokkens WJ, Jenkins SG, Lund VJ, Mafee MF, Naclerio RM, Pawankar R, Ponikau JU, Schubert MS, Slavin RG, Stewart MG, Togias A, Wald ER, Winther B, Rhinosinusitis Initiative: Rhinosinusitis: developing guidance for clinical trials. *J Allergy Clin Immunol* 2006;118(suppl 5):S17–S61.
- 15 Janssens S, Beyaert R: Role of toll-like receptors in pathogen recognition. *Clin Microbiol Rev* 2003;16:637–646.
- 16 Medzhitov R, Preston-Hurlburt P, Janeway CA Jr: A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394–397.
- 17 VanderMeer J, Sha Q, Lane, AP, Schleimer RP: Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and Toll-like receptors. *Arch Otolaryngol Head Neck Surg* 2004;130:1374–1380.

- 18 Lane AP, Truong-Tran Q, Schleimer RP: Altered expression of genes associated with innate immunity and inflammation in recalcitrant rhinosinusitis with polyps. *Am J Rhinol* 2006;20:138–144.
- 19 Dong Z, Yang Z, Wang C: Expression of TLR2 and TLR4 messenger RNA in the epithelial cells of the nasal airway. *Am J Rhinol* 2005;19:236–239.
- 20 Fransson M, Adner M, Erjefält J, Jansson L, Uddman R, Cardell LO: Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. *Respir Res* 2005;6:100–110.
- 21 Mullen GE, Kennedy MN, Visintin A: The role of disulfide bonds in the assembly and function of MD-2. *Proc Natl Acad Sci USA* 2003;100:3919–3924.
- 22 Gil J, Esteban M: Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* 2000;5:107–114.
- 23 Lazurus R, Raby BA, Lange C, Silverman EK, Kwiatkowski DJ, Vercelli D, Klimecki WJ, Martinez FD, Weiss ST: TOLL-like receptor 10 variation is associated with asthma in two independent samples. *Am J Respir Crit Care Med* 2004;170:594–600.
- 24 Krieg AM: Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov* 2006;5:471–484.
- 25 Platz J, Beisswenger C, Dalpke A, Koczulla R, Pinkenburg O, Vogelmeier C, Bals R: Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *J Immunol* 2004;173:1219–1223.
- 26 Saikh KU, Kissner TL, Sultana A, Ruthel G, Ulrich RG: Human monocytes infected with *Yersinia pestis* express cell surface TLR9 and differentiate into dendritic cells. *J Immunol* 2004;173:7426–7434.
- 27 Jozsef L, Khreiss T, El Kebir D, Filep JG: Activation of TLR-9 induces IL-8 secretion through peroxynitrite signaling in human neutrophils. *J Immunol* 2006;176:1195–1202.
- 28 Ramanathan M, Lee W, Dubin MG, Lin S, Spannhake EW, Lane AP: Sinonasal epithelial cell expression of toll-like receptor 9 is decreased in chronic rhinosinusitis with polyps. *Am J Rhinol* 2007;21:110–116.
- 29 Fransson M, Benson M, Erjefält JS, Jansson L, Uddman R, Björnsson S, Cardell LO, Adner M: Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. *Respir Res* 2007;8:17–30.
- 30 Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP: Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* 2004;31:358–364.
- 31 Zhang N, Van Zele T, Perez-Novo C, Van Bruaene N, Holtappels G, DeRuyck N, Van Cauwenberge P, Bachert C: Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus diseases. *J Allergy Clin Immunol* 2008;122:961–968.
- 32 Bachert C, Zhang N, Holtappels G, De Lobel L, Van Cauwenberge P, Liu S, Lin P, Bousquet J, Van Steen K: Presence of IL-5 protein and IgE antibodies to staphylococcal enterotoxins in nasal polyps is associated with comorbid asthma. *J Allergy Clin Immunol* 2010;126:962–968.
- 33 Ewaschuck JB, Backer JL, Churchill TA, Obermeier F, Krause DO, Madsen KL: Surface expression of Toll-like receptor 9 is up-regulated on intestinal epithelial cells in response to pathogenic bacterial DNA. *Infect Immun* 2007;75:2572–2579.
- 34 El Kebir D, József L, Pan W, Wang L, Filep JG: Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. *J Immunol* 2009;182:4386–4394.
- 35 Nakao Y, Funami K, Kikkawa S, Taniguchi M, Nishiguchi M, Fukumori Y, Seya T, Matsumoto M: Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *J Immunol* 2005;174:1566–1573.