

Allergic rhinitis is associated with decreased expression of Toll-like receptor 9 by sinonasal epithelial cells

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Background: Toll-like receptors (TLRs) are important in sinonasal mucosal innate immunity. Previous studies demonstrate that sinonasal epithelial cell (SNEC) TLR9 expression is reduced in T-helper 2 (Th2) cytokine-predominant chronic rhinosinusitis with polyps, and with the in vitro application of Th2 cytokines. To further investigate in vivo modulation of TLR9 by the local cytokine environment, this study examines TLR9 expression in freshly isolated SNECs from subjects with and without active allergic rhinitis (AR).

Methods: SNECs were gathered via endoscopic-guided middle meatal brushings from 9 AR subjects who were skin-prick test (SPT)-positive to environmental allergens in season at the time of study, and 8 controls. Flow cytometry was utilized to compare SNEC TLR9 expression in the 2 groups.

Results: TLR9 expression by SNEC in the AR group was significantly reduced compared to normals ($35\% \pm 26\%$ vs $76\% \pm 10\%$, $p = 0.002$).

Conclusion: Similar to observations in eosinophilic chronic rhinosinusitis, this study shows that active AR is associated with decreased SNEC TLR9 expression. These findings are consistent with the concept that Th2 cytokines suppress expression of TLR9 and other innate immune genes. Multiple endogenous and microbial factors likely modulate sinonasal innate immunity to maintain homeostasis and prevent infection in AR. © 2011 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; innate immunity; sinonasal epithelial; Toll-like receptor; allergy

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Allergic rhinitis (AR) is a chronic disorder secondary to immunoglobulin E (IgE)-mediated hypersensitivity of the nasal mucosa. It affects 10% to 20% of Americans and leads to health care costs of \$2 billion to \$5 billion dollars per year.¹ There has been a steady rise in atopic diseases including AR. AR has also been shown to negatively impact quality of life. Poor sleep quality is associated with AR, and daytime sleepiness can result from antihistamine medications.² Decreased concentration in school children has been demonstrated,³ and studies have shown that AR

can lead to decreased productivity. AR is linked to other disease states and is 4 to 6 more times likely to develop in those with asthma supporting the link between the upper and lower airways in the unified airway concept.⁴ AR, although frequently not life-threatening, can have significant impact on overall health status and daily life of those affected individuals.

The innate immune system comprises our first-line defense against microbes and pathogens. It was once thought to be nonspecific; however, advances in understanding Toll-like receptors (TLRs) supports marked specificity in the innate immune system. TLRs were first discovered as immunomodulators of the innate immune response and can affect the balance between T-helper 1 (Th1) or T-helper 2 (Th2) immune responses. TLRs are pattern recognition receptors (PRRs) of the human innate immune system that bind to pathogen-associated molecular patterns (PAMPs), which are structural components of microbes.⁵ Examples of PAMPs are lipopolysaccharides (LPS), a component of endotoxin, and muramic acid, a component of peptidoglycan that is part of the cell wall of most bacteria. TLR2, which recognizes peptidoglycan of gram-positive bacteria,

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and TLR4, which binds LPS, have been implicated in the development of allergic disease. Polymorphisms in the genes for TLR4 and TLR2 have been shown to interact with these environments. The observation that the peripheral blood leukocytes of farmers' children, who live in an environment with a high load of endotoxin, express higher levels of TLR2 than those of less exposed children supports a modulation of the innate immune response by microbial compounds even in the absence of infection.⁶

All TLRs have been shown to be expressed in sinonasal epithelial cells (SNECs).⁷ Specifically, TLR9 is expressed by innate immune cells in the nose⁸ and binds with great specificity to cytosine phosphorothioate guanosine (CpG) oligonucleotides, a noncoding 6-basepair sequence of deoxyribonucleic acid (DNA).^{9,10} TLR9 is of interest because its PAMP, CpG oligodeoxynucleotides, has proven to be a potent inhibitor of atopic responses by suppressing Th2 cytokines and reducing airway eosinophilia and systemic levels of IgE.^{11,12} There is a growing body of evidence that TLR9 stimulation via CpG motifs, as potent Th1 activators, can be therapeutic against allergic disease by driving Th2 toward Th1 responses.¹¹⁻¹⁴ To further elucidate the significance of the innate immune system in AR, this study examines the TLR9 expression in AR compared with normal controls using flow cytometric techniques.

Patients and methods

Patients and sample collection

Samples from 17 adult patients from the Johns Hopkins Otolaryngology–Head and Neck Surgery clinic were collected. Samples were divided into an AR group ($n = 9$) and a normal control group ($n = 8$). This research protocol has been approved through the Johns Hopkins Institutional Review Board (NA_00008593) and informed consent was acquired from each research subject prior to enrollment into study.

Inclusion criteria for normal subjects included being skin-prick test (SPT)-negative for common local allergens, whereas the AR group was SPT-positive for allergens while being “in-season.” All allergic subjects were sampled during a period of exposure to their corresponding sensitive allergen(s), during which time subjects also exhibited active allergic symptoms. Subjects from the AR group who were SPT-positive also had clinical symptoms of corresponding seasonal or perennial allergies. Positivity on SPT was defined by a wheal greater than or equal to 3 mm compared with negative controls after 15 minutes. Negative control was glycerin and positive control was histamine.

A medical history with special attention to medications and rhinitis symptoms was performed. None of the study subjects had history of sinusitis nor were these individuals on systemic steroids. Study subjects underwent rigid nasal endoscopy after topical nasal oxymetazoline and pontocaine were applied. A cytology brush was introduced in

the middle meatus to collect SNECs. Brushed cells were immediately placed in normal saline.

Flow cytometry

The preparation, flow cytometry protocol, and analysis were performed as previously described.¹⁵ Immunofluorescent markers used included anti-TLR9 antibody conjugated to phycoerythrin (PE) (eBioscience, San Diego, CA) and anti-epithelial cell antigen conjugated to fluorescein (fluorescein isothiocyanate [FITC]) (Dako, Carpinteria, CA). During flow cytometric analysis, cell population gating was carried out over similar cell populations across all samples.

Within 4 hours, the collected cells were then stained with immunofluorescent markers and analyzed using flow cytometry the day of collection. Cells were transferred into microcentrifuge tubes at the concentration of 200,000 cells per tube. The cells were first permeabilized and then incubated with anti-TLR9 antibody conjugated to PE and anti-epithelial cell antigen conjugated to FITC. Cells were centrifuged, washed, and fixed. Analysis was performed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). Each subject's cell sample was divided into unstained, rat immunoglobulin G (IgG) isotype control (eBioscience), single-stain controls, and double stained samples, each of which underwent flow cytometric analysis. The cell surface proteins were expressed as the increase in mean fluorescence over background intensity (Fig. 1).

Statistical analysis

Data were represented using means and their corresponding standard deviations (SDs). Raw data were analyzed using spreadsheet software (Excel; Microsoft, Redmond, WA). Statistical analysis was performed using the Mann-Whitney U test assuming nonparametric data due to small sample size.

Results

The group of healthy normals was comprised of 1 man and 7 women (average age, 42 years old). The AR group had 2 men and 7 women (average age, 41 years old). SPT positives in the AR included Bermuda grass, birch, candida, cat, cottonwood, *D. farinae*, *D. pteronyssinus*, dog, elm, English plantain, feather mix, hickory, hormodendrum, Johnson grass, lamb's quarters, oak, pigweed, ragweed, sycamore, and Timothy grass. The averages and SDs of TLR9 expression in normal and allergic SNECs were measured as: $76\% \pm 10\%$ and $34\% \pm 26\%$, respectively (Fig. 2). This difference was found to be significantly different ($p = 0.002$).

Discussion

In this study, we have demonstrated decreased baseline expression of TLR9 by SNEC derived from allergic patients when compared to normal controls. Among PRRs, TLR9

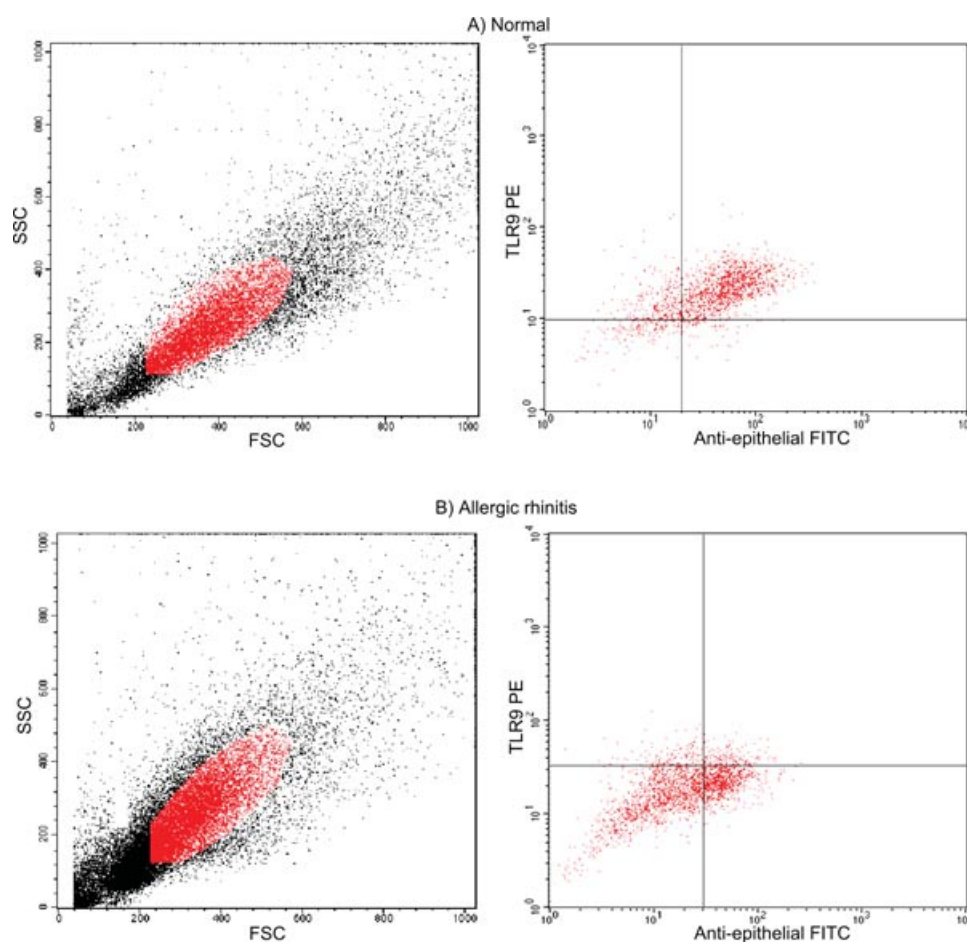


FIGURE 1. Examples of flow cytometry dot-plots from (A) a normal and (B) an AR subject. The left column shows forward-scatter and side-scatter plots and the gating (red) used for analysis among the background of all cells collected (black + red dots). The right column shows double-stained SNEC with TLR9 and epithelial cell antibodies, in which the upper right corner represents cells that stained positively for epithelial and TLR9. AR = allergic rhinitis; SNEC = sinonasal epithelial cell; TLR9 = Toll-like receptor 9.

is of particular interest because its ligand, CpG, drives the immune system toward the Th1 pathway and antagonizes Th2-like responses.^{11,13,14,16} Previous work has demonstrated decreased TLR9 expression by SNEC in chronic rhinosinusitis with nasal polyps (CRSwNP),¹⁷ and elevated

TLR9 expression in AR with recurrent acute rhinosinusitis when compared to subjects with AR only.¹⁵ These findings support the concept that the cytokine milieu in the nasal cavity modulates epithelial TLR9 expression and impacts sinonasal host innate antimicrobial responses.

The mucosal surface of the sinonasal tract maintains a defensive barrier through a combination of innate and adaptive immune mechanisms. Epithelial cells at the junction of the environment and the host must interact with external microbial factors as well as endogenous cytokines to achieve a homeostatic balance that prevents infection without inciting persistent inflammation. Under normal conditions, stimulation of TLR9 by bacterial products activates Th1-like inflammatory pathways that promote an appropriate antimicrobial response. The relationship between AR and rhinosinusitis has been frequently highlighted, however, there are conflicting data regarding the level IgE-mediated disease implicated in rhinosinusitis.¹⁸ In CRSwNP, where the inflammation is Th2-dominant, expression of TLR9 and innate immune effectors is blunted, despite frequent colonization with bacteria.¹⁷ The current study demonstrates that TLR9 expression is similarly reduced in AR, another Th2-skewed nasal disorder.

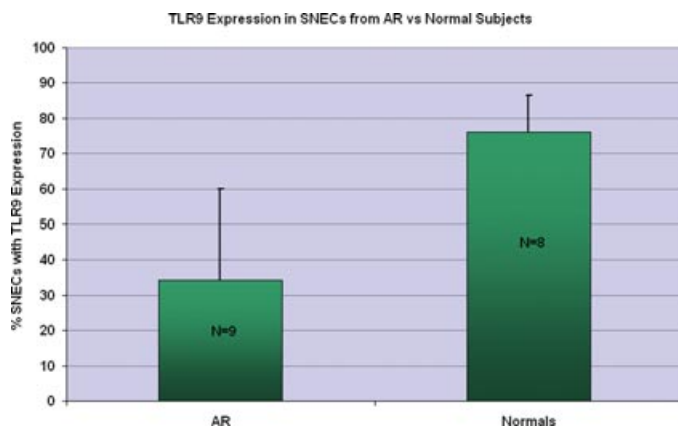


FIGURE 2. The mean and standard deviation TLR9 expressing SNEC for both AR and normal subjects; $p = 0.002$. AR = allergic rhinitis; SNEC = sinonasal epithelial cell; TLR9 = Toll-like receptor 9.

However, unlike CRSwNP, bacterial infection in AR leads to increased TLR9 expression.¹⁵ Thus, while the epithelium in AR successfully balances infectious threats against the underlying Th2 inflammatory tone, epithelial cells in CRSwNP fail to upregulate antimicrobial innate immunity in the setting of profound eosinophilic inflammation, which may contribute to the persistence of bacteria and fungi in the sinuses.

The development of therapeutic approaches to treat cancers, allergic disease, and infections via stimulation of TLRs continues to emerge.¹⁹ With respect to sinonasal inflammatory disease, clinical studies support TLR9 vaccination against allergic disease. CpG conjugated to allergen, as opposed to separate administration of allergen and CpG, immunotherapy appears to offer even greater immune responses.¹² There are early studies showing efficacy when CpG conjugated with ragweed allergen is administered to AR subjects who are undergoing immunotherapy.¹¹ Additionally, CpG oligodeoxynucleotides are being fitted as effective adjuvants to sublingual immunotherapy in Europe,¹⁴ as opposed to subcutaneous immunotherapy, which is used more commonly in the United States. It is important to note that the normal response to TLR9 activation is proinflammatory, and hence treatment with CpG could well exacerbate rhinosinusitis instead of opposing it. To that point, it has recently been shown that while intranasal CpG leads to improvement in patients with high levels of allergic inflammation, it causes measurably increased Th1 cytokine expression and inflammation in non-allergic subjects.¹⁶ As a whole, initial results are encour-

aging and support a potential immunomodulatory role for TLR9 stimulation to drive the sinonasal immune response from Th2-like allergic responses to Th1-like immune responses. A better understanding of the role of TLR9 and the broader interaction between mucosal innate and adaptive immune pathways is necessary to make immunotherapeutic advancements for the treatment of AR and other chronic sinonasal inflammatory conditions.

Conclusion

Similar to observations in chronic rhinosinusitis with eosinophilic polyps, this study shows that active AR is associated with decreased TLR9 expression by SNECs. This finding suggests that Th2 cytokines may suppress expression of TLR9 and other innate immune genes. The marked degree and persistence of eosinophilic inflammation in chronic polypoid sinusitis as compared to AR may explain the clinically apparent difference in innate immune dysfunction between these conditions. Maintenance of homeostasis between endogenous cytokine signals and external microbial factors is an important function of the sinonasal epithelium that may become dysregulated in chronic disease states. However, the inflammatory systems regulating TLR 9 expression are complex and not fully elucidated, and further studies will shed further insight into this topic. 4

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References

1. Reed SD, Lee TA, McCrory DC. The economic burden of allergic rhinitis: a critical evaluation of the literature. *Pharmacoeconomics*. 2004;22:345–361.
2. Santos CB, Pratt EL, Hanks C, et al. Allergic rhinitis and its effect on sleep, fatigue, and daytime somnolence. *Ann Allergy Asthma Immunol*. 2006;97:579–586.
3. Blaiss MS. Allergic rhinitis and impairment issues in school children: a consensus report. *Curr Med Res Opin*. 2004;20:1937–1952.
4. Krouse JH, Altman KW. Rhinogenic laryngitis, cough, and the unified airway. *Otolaryngol Clin North Am*. 2010;43:111–21, ix–x.
5. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197–216.
6. Lauener RP, Birchler T, Adamski J, et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. *Lancet*. 2002;360:465–466.
7. 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.
8. Fransson M, Benson M, Erjefält JS, et al. Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. *Respir Res*. 2007;8:17.
9. Horner AA, Redecke V, Raz E. Toll-like receptor ligands: hygiene, atopy and therapeutic implications. *Curr Opin Allergy Clin Immunol*. 2004;4:555–561.
10. Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov*. 2006;5:471–484.
11. Fonseca DEM, Kline JN. Use of CPG oligonucleotides in treatment of asthma and allergic disease. *Adv Drug Deliv Rev*. 2009;61:256–262.
12. Shirota H, Sano K, Hirasawa N, et al. Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. *J Immunol*. 2001;167:66–74.
13. Broide DH. Immunomodulation of allergic disease. *Annu Rev Med*. 2009;60:279–291.
14. Marcucci F, Duse M, Frati F, Incorvaia C, Marsegli GL, La Rosa M. The future of sublingual immunotherapy. *Int J Immunopathol Pharmacol*. 2009;22(4 Suppl):31–33.
15. Melvin TA, Lane AP, Nguyen MT, Lin S. Allergic rhinitis patients with recurrent acute sinusitis have increased sinonasal epithelial cell TLR9 expression. *Otolaryngol Head Neck Surg*. 2010;142:659–664.
16. Mansson A, Bachar O, Adner M, Cardell LO. Nasal CPG oligodeoxynucleotide administration induces a local inflammatory response in nonallergic individuals. *Allergy*. 2009;64:1292–1300.
17. Ramanathan M Jr, Lee WK, 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.
18. Pant H, Ferguson BJ, Macardle PJ. The role of allergy in rhinosinusitis. *Curr Opin Otolaryngol Head Neck Surg*. 2009;17:232–238.
19. Sims P, Coffman RL, Hessel EM. Biomarkers measuring the activity of Toll-like receptor ligands in clinical development programs. *Methods Mol Biol*. 2009;517:415–440.