



Differential short palate, lung, and nasal epithelial clone 1 suppression in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyps: implications for pathogenesis and treatment

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Purpose of review

Short palate, lung, and nasal epithelial clone 1 (SPLUNC1) is an epithelium-secreted protein that is involved in innate immunity. A protective role for SPLUNC1 in lower respiratory inflammation and chronic rhinosinusitis (CRS) has recently been recognized.

Recent findings

An impaired epithelial immune barrier has been proposed to play a critical role in the pathogenesis of CRS. Recent research has demonstrated that SPLUNC1 is profoundly reduced in polyp tissues of CRS with nasal polyps (CRSwNP) compared with control tissues. Studies investigating the differential expression of SPLUNC1 in eosinophilic and noneosinophilic CRSwNP have been published. Nasal SPLUNC1 expression was inhibited by Th2 cytokines (IL-4 and IL-13) but was stimulated by toll-like receptor (TLR) agonists and glucocorticoids. Decreased SPLUNC1 expression in the sinus mucosa is associated with positive *Pseudomonas aeruginosa* bacterial colonization and poor surgical outcomes in CRS patients.

Summary

These studies identify the role of SPLUNC1 in sinonasal innate immunity and the pathogenesis of CRS. Defective expression of SPLUNC1 in CRSwNP patients may lead to insufficient maintenance of the epithelial barrier function and enhanced bacterial colonization. The use of SPLUNC1 as a therapeutic target for CRSwNP remains to be determined.

Keywords

chronic rhinosinusitis, innate immunity, nasal polyps, SPLUNC1, toll-like receptor

INTRODUCTION

Chronic rhinosinusitis (CRS) can generally be classified into 2 subtypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) [1]. The phenotypic differences between CRSwNP and CRSsNP may signify distinct pathogenetic mechanisms. The tissue inflammatory response in CRSwNP patients is characterized by prominent eosinophilia with a Th2 skewed response [2], which will be the focus of discussion in this review.

Until now, the pathophysiology of CRSwNP has not been fully understood. Recent investigations have highlighted environmental factors, such as fungal or bacterial colonization, the presence of biofilms or superantigens, osteitis, and allergen exposure in the pathogenesis of CRSwNP [2,3], and an 'immune barrier hypothesis', which suggests

that a multitude of potential defects in the mechanical (epithelial) and immunologic (innate and adaptive) barriers contribute to the development of CRSwNP, has been proposed [4]. Generally, it is believed that the dysregulated immune regulation in CRSwNP reflects a complex interplay between the

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KEY POINTS

- There is growing evidence that eosinophilic and noneosinophilic inflammations are relatively distinct pathologic processes in CRSwNP patients.
- SPLUNC1 has been localized to large airway epithelium in humans and mice may contribute to the host defense as an epithelial barrier. It is an epithelium-secreted protein with an antimicrobial effect and antibiofilm activity.
- SPLUNC1 expression is differentially suppressed in eosinophilic and noneosinophilic CRSwNP. Decreased SPLUNC1 expression in sinusitis may be associated with poor surgical outcomes.

host defense and environmental factors that trigger these responses, including bacterial, viral, and fungal exposures. Therefore, aberrant host defenses, indicated by the defective epithelial barrier and dysregulated innate immunity, have been suggested to play a pivotal role in initiating and perpetuating inflammatory responses in CRSwNP patients [5].

The goal of this article is to review important and recent findings relating to the pathophysiology and endotype of CRSwNP and to discuss the possible implication of the antimicrobial peptide short palate, lung, and nasal epithelial clone 1 (SPLUNC1) in the pathogenesis of eosinophilic CRSwNP.

INNATE IMMUNITY, THE EPITHELIAL IMMUNE BARRIER AND CHRONIC RHINOSINUSITIS

The sinonasal tract functions are a complex physical barrier that defends against extrinsic stimuli, such as inhaled irritants, aero-allergens, and microbial pathogens [6]. Structurally, the sinonasal mucosa consists of an epithelial layer of ciliated, pseudostratified, columnar cells joined by tight junctions, interspersed with goblet cells. Disturbed tight junctions can lead to the entrance of pathogens and environmental antigens into the organism. Some recent studies [7,8] with nasal airway epithelial cells have indicated that the physical epithelial barrier might be compromised in CRSwNP patients. For example, Soyka *et al.* [7] reported a decreased trans-tissue resistance in biopsy specimens from CRSwNP patients along with irregular, patchy, and decreased expression of the tight junction molecules occludin and zonula occludens 1. Similarly, our group observed decreased occludin mRNA and protein expression in polyp tissues, which were significantly upregulated after

corticosteroid administration, and demonstrated that corticosteroids significantly increased occludin expression *in vitro* in a MKP-1-dependent manner [8]. Functional studies [9,10] have also suggested that the epithelial barrier is more permeable in polyp tissues. Additionally, goblet cell hyperplasia, increased mucin expression and mucus hypersecretion were also demonstrated to be important pathological characteristics of both CRSsNP and CRSwNP, which may also represent promising targets for the management of CRSwNP [11–14]. Together, these studies suggest that mucociliary dysfunction may play a role in the pathogenesis of CRS, while a defective physical barrier has been more closely linked to CRSwNP.

In addition to constituting the physical barrier, sinonasal epithelial cells have been shown to play crucial roles in initiating and augmenting host defense mechanisms upon activation. Environmental stimuli exposed to the mechanical epithelial barrier may trigger a protective immune response through the production of functional molecules and via physical interactions with cells of the immune system (e.g. macrophages and dendritic cells) [15]. Two distinct but integrated immune responses to microbial entities and foreign proteins have been described: innate and acquired. The innate immune response is the first line of host defense and is responsible for the immediate recognition and control of microbial invasion, which secondarily induces the recruitment of immune cells into the epithelium to initiate adaptive immunity. The innate immune response relies on evolutionarily ancient germline-encoded receptors, the pattern-recognition receptors (PRRs), which recognize highly conserved microbial structures. Toll-like receptors (TLRs) are one of the best-characterized classes of PRRs, which were originally identified as homologues of *Drosophila Toll*. To date, more than 10 human TLRs have been identified. TLR signaling can lead to the activation of several transcription factors, including Nuclear factor kappa-light-chain-enhancer of activated B cells and interferon regulatory factors [16]. Airway epithelial cells have been shown to mainly express TLR2–4 [17]. These particular TLRs are thus very strategically positioned on epithelial cells to enable the recognition of organisms that are frequently encountered on the mucosal surface [18]. There is increasing evidence indicating that airway epithelial cells are central participants in the innate and adaptive immune responses [19,20]. However, the potential derangement of TLR signaling in the development of CRSwNP is inconsistent. Lane *et al.* [21] previously demonstrated significantly higher expressions of TLR2 and granulocyte-macrophage colony-

stimulating factor in CRS patients when compared with controls. Zhang *et al.* [22] reported that TLR2, TLR4, and TLR7 expression were significantly increased in CRSwNP patients when compared with either CRSsNP patients or control study participants. In contrast, our group recently demonstrated a decreased expression of TLR2, TLR-3, and TLR4 in CRSwNP patients, which was associated with the eosinophilic phenotype and less submucosal glandular formation [23²⁴].

Upon PRR stimulation, sinonasal epithelial cells produce a series of antimicrobial and antiviral proteins, along with proinflammatory cytokines, that activate other mucosal innate immune cells. Epithelial host defense molecules include small peptides that lyse bacteria, such as defensins and cathelicidins, as well as enzymes and larger proteins, such as lysozyme, lactoferrin, and secretory leukocyte proteinase inhibitor [24–26]. Cathelicidin is an antimicrobial peptide that contributes to the innate immune defense against invasive bacterial infection [27,28]. Because CRSwNP has been proposed as a disease characterized by the dysfunctional host-environment interaction of the sinonasal mucosa, it has been postulated that the decreased expression of cathelicidin may be associated with CRSwNP [27,29]. Earlier studies of CRSwNP did not demonstrate consistent alterations in the expression of these antimicrobial molecules, with increased levels reported in some molecules [30,31], whereas others are reportedly decreased [32]. Theoretically, defects in immune barrier function could serve as a primary etiologic mechanism in CRSwNP pathogenesis by rendering the patient susceptible to microbial colonization, invasion, or both, leading to a heightened inflammatory response. Some investigators thus suggest the hypothesis, namely the ‘immune barrier hypothesis’ [33], in which a primary sinonasal innate immune defect may contribute to local microbial proliferation and a dysregulated immune response, thereby fostering the development of CRSwNP.

ENDOTYPES OF CHRONIC RHINOSINUSITIS: EOSINOPHILIC VERSUS NONEOSINOPHILIC

The pathogenesis of CRS is attributable to a multifactorial inflammatory disorder resulting from a dysfunctional host-environment interaction involving various exogenous agents and changes in the sinonasal mucosa [34]. The current literature broadly divides CRS into CRSsNP and CRSwNP based on the existence of nasal polyps or not. It has been demonstrated that CRSsNP may be a Th1-pre-dominant immune response with a relatively greater

proportion of neutrophils, and a cytokine profile characterized by increased IFN- γ . In contrast, CRSwNP is characterized by excessive Th2 inflammation, prominent eosinophilic infiltration and decreased Treg function. The cytokine profile is characterized by increased IL-5 and eosinophilic cationic protein. The majority of CRSwNP cases have a strong tendency to recur after surgery and show a higher incidence of concomitant asthma [35]. Moreover, it should be noted that CRS is a clinical diagnosis that actually encompasses a variety of conditions with heterogeneous inflammation patterns. However, the current distinction between subtypes (CRSsNP vs. CRSwNP) is simply clinically based. This subclassification is convenient for use in clinical studies and clinical practice, but it simply focuses on phenotype rather than the underlying histopathology or serum markers (endotype) [36³⁷]. Because certain forms of CRS might be more likely to produce nasal polyps, and nasal polyps can develop as a complication of any form of CRS, this simple subclassification system thus confers less use for predicting disease severity and prognosis. Therefore, the opposing expert view is that the existence of nasal polyps should not be used as the exclusive basis for CRS diagnosis or treatment recommendations.

Despite several pathophysiological hypotheses that have been suggested, the initiating event that triggers the formation of nasal polyps remains unknown [37]. Histologically, the mucosal surface of nasal polyps consists of respiratory epithelium and an edematous stroma infiltrated by inflammatory cells. Polyp mucosa exhibits a markedly greater inflammatory infiltrate and a lower density of mucous glands than inferior turbinate mucosa [38]. In the United States and Europe, nasal polyps are accompanied by an eosinophilic infiltrate in more than 80% of cases [39]. Eosinophilic damage to the sinonasal mucosa was believed to be the central pathophysiologic mechanism of CRSwNP and the hallmark of this disorder. A substantial body of evidence, including clinical studies and animal models, has demonstrated a causal role for eosinophils in CRSwNP pathogenesis. Significantly, the degree of tissue eosinophilia in polyp tissues is correlated with objective disease severity and comorbid asthma [35]. However, although the data demonstrating that eosinophilia is more likely to be associated with the presence of nasal polyps are compelling, both the presence and extent of eosinophilia in polyp tissues can be quite variable, and a large subset of nasal polyps observed in CRSwNP patients does not demonstrate significant eosinophilia [40]. For example, neutrophilic inflammation is also a consistent finding in the nasal polyps found

in cystic fibrosis. Except for in cystic fibrosis, the relationship between CRSwNP and the mucosal eosinophilia is not always maintained in polyp tissues obtained from some Asian populations, in addition to a demonstrable minority of the Western/Caucasian population. Specifically, in some regions of Asia, it has recently been shown that some CRSwNP cases (nearly 50%) are more likely to be associated with a noneosinophilic or neutrophilic infiltrate and Th1/17 cytokine skewing [41–44]. In correlation with reduced eosinophilic inflammation, we also observed a very low rate of concomitant asthma and Aspirin-exacerbated respiratory disease in Chinese CRSwNP patients [45,46]. It is also interesting to note that over a 12-year period (from 1999 to 2011), a shift from predominantly neutrophilic to eosinophilic inflammation was recently reported in Asian (Thai) patients with CRSwNPs in association with an increase in the intramucosal presence of *Staphylococcus aureus* [47]. With advances in our understanding of the inflammatory processes, many investigators have recommended endotyping patients into eosinophilic versus noneosinophilic subsets using histopathological classification to improve the pathophysiology understanding and personalized management of CRSwNP [34,36,40].

While many studies have tried to subclassify CRSwNP into eosinophilic and noneosinophilic subtypes, clear criteria for subclassifying eosinophilic and noneosinophilic remain elusive [48]. Most of the literature defines eosinophilic inflammation by examining the number or ratio of eosinophils in polyp tissues [49–52]. The current cutoffs proposed in the literature to define Eosinophilic chronic rhinosinusitis include a tissue eosinophil amount to more than 5% of all leukocytes in five visual fields or a tissue eosinophil count of more than five cells/High power field (HPF). Recently, Soler *et al.* [53] proposed an optimal cut-point of more than 10 eosinophils/HPF because this reflects the largest absolute difference in score changes on the Rhinosinusitis Disability Index after sinus surgery. In our recent study [41], we established an optimal cut-point of more than eight eosinophils/HPF for eosinophilic CRSwNP based on patient-based tissue examination with larger-size samples, and reported an eosinophilic phenotype in 76% of CRSwNP patients. Because it is not routine to conduct histological examinations in CRSwNP patients, many authors have proposed the use of a surrogate marker for the diagnosis of eosinophilic CRSwNP in clinical practice. The utility of serum eosinophilia for predicting tissue eosinophilia has recently been addressed. Sakuma *et al.* [50] proposed the cut-point of at least 6% eosinophils of the total

leukocytes for predicting eosinophilic CRSwNP with a sensitivity of 97.4% and a specificity of 70.7%. In our previous study, we found that the blood eosinophil number had a sensitivity of 84.9% and a specificity of 84.4% at the cutoff level of $0.16 \times 10^9/l$, and the blood eosinophil ratio had a sensitivity of 89.0% and a specificity of 84.4% at the cutoff level of 2.05% for the diagnosis of eosinophilic CRSwNP in a Chinese cohort [54]. Moreover, radiological findings such as computed tomography (CT) scans may be used as a surrogate marker for eosinophilic CRSwNP patients. For example, Ishitoya *et al.* [35] proposed that eosinophilic patients displayed ethmoid predominance in early stages, whereas noneosinophilic patients showed maxillary predominance in early stages as assessed by CT scans. Based on these findings, Sakuma *et al.* [50] established a set of CT parameters (olfactory cleft score >1 and posterior ethmoid score >1), in addition to blood examination, to differentiate eosinophilic from noneosinophilic cases in regular outpatient clinics.

In clinical practice, eosinophilic CRSwNP is different from noneosinophilic CRSwNP in terms of many features: symptom appearance, the occurrence site of nasal polyps, CT scan findings, the histology of the nasal polyps, blood examination findings, clinical course after surgery, and comorbid asthma [35]. Currently, there is no official gold standard diagnostic test for eosinophilic CRSwNP. Sakuma *et al.* [50] proposed a clinical diagnostic indicator for eosinophilic CRSwNP on the basis of clinical features, CT scan findings (using the Lund–Mackay scoring system), peripheral blood eosinophil counts above the normal range and the postoperative course. The differential diagnosis of eosinophilic and noneosinophilic CRSwNP has significant implications for treatment. For example, the efficacy of macrolides is greatest in neutrophilic diseases because of the immunomodulatory response. Macrolide therapy is effective in noneosinophilic patients by its suppression of IL-8 production and neutrophilic inflammation but is not likely to be an effective treatment modality for eosinophilic patients [55–57]. Importantly, the presence of severe tissue eosinophilia in some CRSwNP patients may explain the spectacular failure of some patients by correcting the ostiomeatal complex (OMC) occlusion through endoscopic sinus surgery [58]. It is highly likely that such a surgical patient subgroup will go on to develop recurrent nasal polyps over time, and the early administration of either systemic or aggressive high dose topical or systemic steroid therapy in these patients may greatly improve their outcomes.

THE IMPLICATIONS OF SHORT PALATE, LUNG, AND NASAL EPITHELIAL CLONE 1 IN THE PATHOGENESIS AND TREATMENT OF EOSINOPHILIC CHRONIC RHINOSINUSITIS WITH NASAL POLYPS

As to noneosinophilic inflammation, it has been well recognized that the anatomical obstruction of the OMC compromises ventilation and the drainage of dependent sinuses. These effects may cause a hypoxic state and enhanced bacterial colonization in the paranasal sinuses, which would activate endothelial cells and fibroblasts to release inflammatory mediators, leading to the accumulation of inflammatory cells (e.g. neutrophils), tissue remodeling and polyp formation. However, OMC occlusion may not be the fundamental predisposing factor for the development of eosinophilic inflammation [58]. Any interventions focused on correcting the OMC occlusion may not provide a significant modification of chronic mucosal inflammation in eosinophilic CRSwNP cases. Currently, the exact reason for high eosinophil infiltration accompanied with a shift to a more Th2-typed response in eosinophilic CRSwNP patients is not fully understood. IL-5 and eotaxin are traditionally considered the key cytokines for the accumulation, survival and activation of eosinophils in polyp tissues [59,60]. Recently, our group, and others, demonstrated that osteopontin, a phosphorylated acidic glycoprotein, plays a critical role in the chemotaxis of eosinophil in eosinophilic CRSwNP [61,62]. In addition, many other inflammatory mediators have been reported to be increased in eosinophilic polyp tissues [63–67], and more than one mechanism has been proposed to contribute to the responses.

The upstream mechanisms that incite the Th2 response may hold the key to understanding how extrinsic factors influence the pathogenesis of eosinophilic CRSwNP [68,69]. It has been shown that the respiratory epithelium mediates the Th2 response through producing a series of epithelium-derived cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin [70]. Interestingly, IL-25, IL-33, and thymic stromal lymphopoietin were significantly increased in eosinophilic CRSwNP cases compared with noneosinophilic counterparts, and they were recently found to play an essential role in regulating both the innate immunity and adaptive immunity in sinus mucosa [71–73]. They are able to activate both pro-Th2-skewed dendritic cells and group 2 innate lymphoid cells. Their overexpression in polyp tissues is significantly associated with the degree of tissue eosinophilia and overall inflammatory severity [74,75]. Thus, these epithelium-derived cytokines may represent a novel therapeutic target for

CRSwNP and lower airway inflammation in future research.

Alterations in epithelial barrier function and host defense response have also been implicated in the pathogenesis of CRSwNP. The immune barrier hypothesis suggests that a multitude of potential defects in mechanical (epithelial) and immunologic (innate and adaptive) barriers contribute to the inflammatory response in CRSwNP [5]. Among these defects is the decreased level of sinonasal epithelium-derived antimicrobial proteins, which may cause increased microbial colonization, accentuated barrier damage, and a compensatory and damaging immune response. This hypothesis has been supported by a series of novel findings. For example, Tieu *et al.* [76] demonstrated the presence of psoriasin and calprotectin at the epithelial interface and showed that levels of these proteins are significantly diminished in CRSwNP patients, and Seshadri *et al.* [77] observed decreased SPLUNC1 protein in polyp tissues of CRSwNP patients when compared with uncinate tissue from control study participants. Similarly, in our recent study [23²²], we observed that SPLUNC1 levels were significantly decreased in the polyp tissues of CRSwNP patients when compared with the control study participants. More importantly, we also found more significant suppression of SPLUNC1 and TLR signaling in eosinophilic CRSwNP patients compared with their noneosinophilic counterparts, indicating that an inferior innate immunity is associated with tissue eosinophilia in eosinophilic CRSwNP.

SPLUNC1 is an epithelium-secreted protein with an antimicrobial effect and antibiofilm activity. Specifically, it is predominately produced by mucous cells and submucosal glands, although expression by epithelial cells of the upper airway has also been reported [78]. SPLUNC1 is likely to promote the health of the airway through bactericidal and nonbactericidal mechanisms [79]. For example, purified recombinant protein can kill bacteria and also reduce biofilm formation by *Pseudomonas aeruginosa in vitro*, supporting the assertion that this molecule functions in host defense [80]. In addition, SPLUNC1 is a multifunctional protein that serves to protect the sodium channel protein Epithelial sodium channel, which regulates the airway surface liquid volume, from proteolytic cleavage [81]. The effective maintenance of mucosal liquid volume, mediated by the appropriate expression of SPLUNC1, is thought to promote the mucociliary clearance of microorganisms in the airway.

The expression and regulation of SPLUNC1 in airway inflammation have recently attracted much attention. SPLUNC1 was upregulated in chronic

obstructive pulmonary disease, cystic fibrosis, and after olfactory bulbectomy but was found to be decreased in seasonal allergic rhinitis and nasal polyps [82]. Seshadri *et al.* [77] found that reduced levels of SPLUNC1 in polyp tissues is associated with a profound decrease in the number of submucosal glands, and suspected that decreased glands may lead to a localized defect in the production and release of glandular innate defense molecules. Reduced SPLUNC1 in polyp tissues may also be associated with the response and tissue eosinophilia. For example, Thaikoottathil *et al.* [83] showed that SPLUNC1 in the bronchoalveolar lavage fluid of Ovabumin (OVA)-challenged wild-type mice was significantly reduced, which was negatively correlated with the levels of lung eosinophilic inflammation, and SPLUNC1 Knockout mice demonstrated significantly higher numbers of eosinophils in the lung after OVA challenges than wild-type mice. Yeh *et al.* [84] examined the regulation of SPLUNC1 in nasal epithelial cells, and demonstrated that IL-13 is able to significantly inhibit SPLUNC1 expression. Consistently with this observation, our study [23²²] demonstrated that SPLUNC1 was differentially suppressed in eosinophilic and noneosinophilic CRSwNP. We found Th2 cytokines significantly inhibited the epithelial SPLUNC1 expression *in vitro*. Conversely, TLR signaling, which was impaired in eosinophilic CRSwNP, significantly increased the epithelial SPLUNC1 expression *in vitro*. As a consequence, decreased SPLUNC1 expression in CRS patients may cause positive *P. aeruginosa* bacterial colonization, which is shown to be associated with poor surgical outcomes [85²³]. Because eosinophilic CRSwNPs is a disorder characterized by predominant Th2 inflammation, it is reasonable to postulate that a skewed Th2 response may subsequently impair the mucosal innate immunity, including SPLUNC1 expression, to drive the inflammation progression in the pathogenesis of CRSwNP in a vicious cycle.

Although the use of SPLUNC1 as a therapeutic target for CRSwNP remains to be determined, we can postulate the clinical implications of SPLUNC1 in the management of eosinophilic CRSwNP. First, we observe worse innate immunity in eosinophilic CRSwNP cases compared with noneosinophilic cases, and thus, enhancing the innate immunity (e.g. SPLUNC1) with immunomodulatory reagents (e.g. probiotics and bacterial extracts) may represent a novel strategy for the optimal control of the inflammatory response in eosinophilic CRSwNP. Additionally, although glucocorticoid treatment is recommended as the primary treatment of choice for CRSwNP patients by extensively inhibiting the

Th2-mediated inflammatory response, our results suggest that glucocorticoid was able to upregulate SPLUNC1 expression *in vitro* and *in vivo*, in parallel with nasal symptom improvement in eosinophilic CRSwNP patients. Because decreased SPLUNC1 expression in CRS patients is associated with poor surgical outcomes, this newly established mechanism thus expands our understanding of the importance of glucocorticoids and helps establish an optimal strategy for the control of eosinophilic inflammation.

CONCLUSION

There is growing evidence that eosinophilic and noneosinophilic inflammations are relatively distinct pathologic processes. Reduced SPLUNC1 in polyp tissues may also be associated with the impaired antimicrobial response and tissue eosinophilia. An improved understanding of the defective immune barrier is likely to have tremendous potential to reveal novel strategies for the improved management of different subtypes of CRSwNP.

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Conflicts of interest

There are no conflicts of interest.

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- of special interest
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