Staphylococcus aureus Induces a Mucosal Type 2 Immune Response via Epithelial Cell-derived Cytokines

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Abstract

Rationale: Chronic rhinosinusitis with nasal polyps is characterized by a T-helper cell type 2-skewed upper airway inflammation. Mucosal *Staphylococcus aureus* colonization is found in the majority of patients with nasal polyps. *S. aureus* is known to induce type 2 cytokine release via enterotoxins.

Objectives: To investigate the impact of non-enterotoxin-producing *S. aureus* on type 2 cytokine release.

Methods: TSLP (thymic stromal lymphopoietin), IL-33, and type 2 cytokines were assessed in a human mucosal tissue model upon *S. aureus* infection.

Measurements and Main Results: *S. aureus* exposure increased the expression of IL-33, TSLP, IL-5, and IL-13 in nasal polyp tissue, accompanied by elevated expression levels of TSLP and IL-33

receptors, predominantly on CD3⁺ T cells. *S. aureus* infection led to the release of TSLP, but not IL-33, IL-5, or IL-13, from healthy inferior turbinate tissue. In contrast, *S. epidermidis* did not induce any epithelial cell-derived cytokines in nasal polyp or healthy tissue. *S. aureus* infection also increased the release of IL-33 and TSLP in BEAS-2B epithelial cells, accompanied by activation of NF- κ B (nuclear factor κ B) pathways. Incubation with CU-CPT22, a specific Toll-like receptor 2 antagonist, significantly reduced the *S. aureus*-induced release of TSLP and IL-33, and the activity of the NF- κ B signal in BEAS-2B cells.

Conclusions: This study demonstrates for the first time that *S. aureus* can directly induce epithelial cell-derived cytokine release via binding to Toll-like receptor 2, and may thereby propagate type 2 cytokine expression in nasal polyp tissue.

Keywords: chronic rhinosinusitis with nasal polyps; IL-33; *Staphylococcus aureus*; Th2; thymic stromal lymphopoietin

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory condition in the upper airways. CRSwNP is responsible for serious morbidity and is

often accompanied by comorbid asthma. About 85% of European patients with CRSwNP show T-helper cell type 2 (Th2)-biased and eosinophilic inflammation (1–5).

An increase in colonization with *Staphylococcus aureus* and the presence of *S. aureus* enterotoxin-specific IgE antibodies have been demonstrated in the

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At a Glance Commentary

Scientific Knowledge on the

Subject: *Staphylococcus aureus* has been considered important in the pathomechanisms of chronic rhinosinusitis with nasal polyps (CRSwNP). An increase in colonization of *S. aureus* and the presence of *S. aureus* enterotoxin-specific IgE are also observed in CRSwNP. It also has been identified that the staphylococcal enterotoxin B of *S. aureus* induces T-helper cell type 2 (Th2)-associated cytokines in human nasal tissue. However, there is no evidence of the direct impact of non-enterotoxin-producing *S. aureus* on Th2-mediated inflammation in CRSwNP. We hypothesized that *S. aureus* plays a vital role in the pathomechanisms of CRSwNP independently of enterotoxin production.

What This Study Adds to the

Field: Here we demonstrate, for the first time, that non-enterotoxin-producing *S. aureus* can directly induce epithelial cell-derived cytokine IL-33 and TSLP (thymic stromal lymphopoietin) release via binding to Toll-like receptor 2, thereby propagating type 2 cytokine expression in CRSwNP tissue. Thus, this study confirms our hypothesis that *S. aureus* has a direct role in the pathomechanisms of CRSwNP, and further suggests that eradication of *S. aureus* might be a useful strategy to alleviate persistent Th2-biased inflammation in the upper airway.

mucosa of subjects with CRSwNP in comparison with control subjects or patients with chronic rhinosinusitis without nasal polyps (6, 7), and even more so in patients with CRSwNP and asthma comorbidity or aspirin sensitivity. Critically, staphylococcal enterotoxin B (SEB) of *S. aureus* induces Th2-associated cytokines, including IL-4, IL-5, and IL-13, and also enhances eosinophilic inflammation in human nasal tissues and mouse bronchial tissues (8–11). However, a link between *S. aureus* biofilms and skewing of the Th2 response has also been

demonstrated independently of enterotoxin activities (12). Thus, *S. aureus* may impact Th2 responses not only via enterotoxins but also via other pathologic mechanisms, which are presently unresolved.

Epithelial cells act as the first physical defense barrier against infection. Epithelial cell-derived cytokines such as IL-33 and TSLP (thymic stromal lymphopoietin), potentially released by various viral and bacterial stimuli, are critical in orchestrating a Th2 immune response (13, 14). Recently, Nagarkar and colleagues (15) showed that the biological activity of cleaved TSLP derived from full-length TSLP by endogenous tissue proteases is increased in CRSwNP, enhancing IL-5 production from mast cells. Besides the classical Th2 cells, a new group of innate lymphoid cells (ILC2s) have also been shown to produce type 2 cytokines, including IL-5, IL-9, and IL-13, in response to IL-25 and IL-33 (16). These findings identify a close relationship between epithelial cell-derived cytokines and type 2-skewed airway diseases.

Importantly, TSLP can be induced by rhinovirus or double-stranded RNA in human airway epithelial cells (17). IL-33 was found to be related to the severity of virus-induced asthma exacerbations, and rhinovirus-induced IL-33 was able to strongly induce type 2 cytokines in human T cells and ILC2s (18). Increased levels of IL-33 were shown in an atopic dermatitis mouse model after SEB exposure (19). However, the role of *S. aureus*-induced type 2 immune responses independent of enterotoxins in human chronic airway disease via epithelial cell-derived cytokines has not been elucidated.

Making use of previously described human nasal tissue models (20), we developed an *S. aureus*-infected human mucosal tissue model to investigate the

possible role of *S. aureus* in a Th2 response in chronic upper airway disease. We specifically analyzed epithelial cell-derived cytokine release in CRSwNP tissue induced by an *S. aureus* strain that is unable to secrete classical enterotoxins such as SEB, and studied the type 2 cytokines that were subsequently expressed.

Methods

Patients

Nasal tissues were obtained from 20 patients with CRSwNP and 21 control subjects at the Department of Otorhinolaryngology, Ghent University Hospital. None of the patients had taken oral or nasal corticosteroids for 4 weeks or antibiotics for 2 weeks before surgery. The diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2007 guidelines (21). The inferior turbinates of patients with septal deviation were used as controls, particularly because inferior turbinates have been shown to reflect the type 2 inflammatory mediator profiles of sinuses in patients with CRSwNP (22). The clinical data for all of the patients are shown in Table 1. The ethics committees of Ghent University Hospital approved the study and informed consent was obtained from all of the patients before samples were collected.

Human Airway Infection Model

Details regarding *S. aureus* and *S. epidermidis* and the techniques used to culture these bacteria are provided in the online supplement. CRSwNP tissues or control tissues were cut into small cubes approximately 25 mm² in size and washed three times with tissue culture medium (TCM) containing 50 IU/ml penicillin (Invitrogen) and 50 µg/ml streptomycin

Table 1. Clinical Data of the Included Patients

	Subjects with CRSwNP	Control Subjects
Number of subjects	20	21
Age, median (range)	47.8 (30–65)	31.2 (15–58)
Sex, female/male	9/11	10/11
Atopy	7/20	3/21
Asthma	13/20	0/21
AERD	5/20	0/21

Definition of abbreviations: AERD = aspirin-exacerbated respiratory disease; CRSwNP = chronic rhinosinusitis with nasal polyps.

(Invitrogen). The washed tissue was then placed on a triangle metal mesh in a 6-well plate over culture medium as the air-liquid interface culture condition and incubated overnight at 37°C in humidified air with 5% CO₂. The nasal tissues were then incubated with *S. aureus* or *S. epidermidis* at a concentration of 2.0×10^7 cfu/well (20), and at 2 hours after *S. aureus* or *S. epidermidis* infection, the tissues were transferred back onto the triangle metal mesh in a 6-well plate and cultured for another 24 or 48 hours, as previously. The dose of *S. aureus* and incubation time used in the present study were chosen based on preliminary studies investigating the release of IL-6 and IL-1 β from nasal tissue without prominent cell damage after infection with different doses of *S. aureus* (see Figure E1 in the online supplement). Uninfected tissues were used as the control group.

Details regarding cultures of the human bronchial epithelial cell line BEAS-2B, the BEAS-2B cell infection model, and the novel Toll-like receptor 2 (TLR2) blocking experiment on BEAS-2B cells during infection are provided in the online supplement.

Stimulation of Nasal Single-Cell Suspensions

Single-cell suspensions of human CRSwNP mucosa were prepared as described previously (23). Then, 5×10^6 human nasal single cells per well were stimulated with recombinant human TSLP (rhTSLP), rhIL-33 (both R&D Systems), or both cytokines together at different doses for 24 hours. Finally, recombinant TSLP and IL-33 were used at a concentration of 100 ng/ml for the induction of IL-5 release. Cytokines were measured in the supernatants of cell suspensions after 24 hours of incubation.

qRT-PCR

RNA isolation and PCR were performed as described previously (24). The primer

sequences for all target genes are shown in Table 2. To normalize for transcription and amplification variations among the samples, two housekeeping genes (*SDHA* [succinate dehydrogenase complex] and *EF-1* [elongation factor 1]) were used. The relative quantities expressed were calculated with the commercially available qBasePlus software (Bio-Rad) (25).

Cytokine Measurements

IL-4, IL-5, IL-13, TSLP, and IL-33 measurements were performed in the supernatants of BEAS-2B cells, as well as in supernatants or homogenates of tissue from the human airway infection model, using commercially available cytokine kits as described in detail in the online supplement. Tissue homogenates were prepared as described previously (26).

Immunofluorescence Staining

IL-33, TSLPR (TSLP receptor), and the IL-33 receptor ST2 were localized by immunofluorescence staining for IL-33/terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), CD3/TSLPR, and CD3/ST2 using cryosections of CRSwNP tissue as described in detail in the online supplement. Leica confocal software was used for image capture and analysis.

Western Blotting

Total protein was extracted from CRSwNP tissue and BEAS-2B cells after *S. aureus* infection. Details regarding the antibodies used in the Western blotting are provided in the online supplement. The relative band densities of the target protein to GAPDH (Invitrogen) were estimated with the use of Lab Image Analysis software (Bio-Rad).

Flow Cytometry

CRSwNP tissue cubes infected with *S. aureus* for 24 hours ($n = 3$ in quadruplicate) were collected for preparation

of single-cell suspensions as described above. To measure the expression of TSLPR, the single cells were stained with anti-CD3-Vioblu anti-TSLPR-APC antibodies for 30 minutes. The IL-33 receptor ST2 is internalized after binding to IL-33, thus reducing IL-33-induced cytokine release (27); therefore, after incubation with anti-CD3-Vioblu, the single cells from the *S. aureus* infection model were stained by anti-ST2-PE (R&D) after fixation and permeabilization. Data were acquired on a BD FACS Canto II using FACS Diva software (BD Bioscience) and FlowJo software (Tree Star, Inc.) for analysis.

Statistical Analyses

The data were analyzed using GraphPad Prism 6 software (GraphPad Software Inc.). Results are presented as median and interquartile range, and the Mann-Whitney *U* test was used for between-group comparisons. For multiple-group comparisons, the Kruskal-Wallis test with Bonferroni correction was used. A *P* value <0.05 was considered statistically significant.

Results

TSLP and IL-33 Were Induced in CRSwNP Tissue in the Presence of *S. aureus*, but Not *S. epidermidis*

RT-PCR analysis demonstrated two human transcript variants of TSLP in *S. aureus*-infected tissue. Although the mRNA level of long-form TSLP (*lftTSLP*), an active form of TSLP, increased in CRSwNP and healthy control tissues 24 hours after *S. aureus* infection, the expression of short-form TSLP (*sfTSLP*) decreased in CRSwNP and healthy control tissues 24 and 48 hours after *S. aureus* infection (Figure 1A). TSLP protein levels were significantly higher in the

Table 2. Primer Sequences Used for qRT-PCR

Targets	Forward (5'–3')	Reverse (5'–3')	Amplicon Size (bp)	Accession Number
<i>IL-33</i>	TGAATCAGGTGACGGTGTGATGG	TGAAGGACAAAGAAGGCCTGGTC	150	NM_033439.3
<i>lftTSLP</i>	GGGCTGGTGTTAACTTACGACTTCA	ACTCGGTACTTTTGGTCCCACTCA	118	NM_033035.4
<i>sfTSLP</i>	CGTAAACTTTGCCGCCTATGA	TTCTTCATTGCCTGAGTAGCATTTAT	186	NM_138551.4
<i>IL-5</i>	AACTCTGCTGATAGCCAATGAGAC	ACTCTCCAGTGTGCCTATTCCC	106	NM_000879.2
<i>IL-4</i>	ACAGCCTCACAGAGCAGAAGAC	TCTCATGGTGGCTGTAGAAGTGC	138	NM_000589.3
<i>IL-13</i>	CAACATCACCCAGAACCAGAAGGC	AGGTTGATGCTCCATACCATGCTG	63	NM_002188.2

Definition of abbreviations: lftTSLP = long-form TSLP; sfTSLP = short-form TSLP; TSLP = thymic stromal lymphopoietin.

Figure 1. Epithelial cell-derived cytokines TSLP (thymic stromal lymphopoietin) and IL-33 in chronic rhinosinusitis with nasal polyps (CRSwNP) tissue after *Staphylococcus aureus* infection. (A) Transcript levels of long-form TSLP (IfTSLP), short-form TSLP (sfTSLP), and IL-33 in CRSwNP ($n = 7$) and healthy inferior turbinate (IT) tissues ($n = 8$) after *S. aureus* infection for 24 or 48 hours. Uninfected tissues incubated with tissue culture medium were used as controls ($*P < 0.05$). (B) Protein levels of TSLP and IL-33 CRSwNP and IT tissues after *S. aureus* infection for 24 or 48 hours ($*P < 0.05$). (C) IL-33 was

supernatants of CRSwNP and healthy control tissue after 48 hours of exposure to *S. aureus* than in the supernatants of unexposed tissue (Figure 1B).

S. aureus infection did not significantly increase IL-33 mRNA in CRSwNP or healthy control tissue (Figure 1A). However, IL-33 protein was increased in CRSwNP tissue homogenates, but not in control tissue, 24 hours after *S. aureus* infection (Figure 1B). Because knowing the location of IL-33 in *S. aureus*-infected tissue helps one evaluate its functional status, IL-33 immunofluorescence staining and TUNEL assay were performed. Abundant IL-33 staining was observed in the extracellular space between epithelial cells of CRSwNP, where TUNEL-positive cells were found after *S. aureus* stimulation after 24 hours (Figure 1C). Similarly, IL-33 was located in the nuclei of basal cells in uninfected tissues.

Increased phosphorylation of MLKL (mixed lineage kinase domain-like protein), a mediator of necroptosis, was observed in CRSwNP tissue 24 hours after *S. aureus* infection, which was compatible with immunofluorescence staining (Figure 1D). Assessment of TSLP and IL-33 separated by Tris-Tricine gel demonstrated that the band intensities of full-length IL-33 and lftTSLP were dramatically increased in CRSwNP tissue after *S. aureus* exposure (Figure 1E). Recombinant N-terminally processed human IL-33 protein, human lftTSLP, and human salivary gland protein extracts were used as positive controls. As the recombinant lftTSLP proteins were produced by prokaryotic cells, which do not carry a post-translational modification with a sugar group, the molecular weight of lftTSLP carrying an N-glycosylation from the infection model shown in the Figure 1E was higher than that of the recombinants from prokaryotic cells.

S. epidermidis, which is of the same *Staphylococcus* genus as *S. aureus*, was used in the human mucosal tissue infection model as a control to demonstrate the specificity of the effect of *S. aureus* on epithelial cell-derived cytokine release. Although *S. epidermidis* demonstrated a more prominent growth compared with *S. aureus* over the course of 24 or 48 hours of culture (Figure E2), *S. epidermidis* did not induce TSLP or IL-33 proteins in the CRSwNP tissues (Figure 2A). Furthermore, a low basal epithelial expression of IL-33 without any release was found in CRSwNP

Figure 1. (Continued). observed in the extracellular space of epithelial cells in CRSwNP tissue after *S. aureus* infection for 24 hours. Low expression of IL-33 was found in control conditions without any infection. Pink fluorescence indicates the presence of IL-33, red fluorescence indicates TUNEL-positive cells, and green fluorescence indicates the presence of *S. aureus* (SA) in the nasal mucosa; isotype control staining for IL-33 was completely negative (not presented here). (D) Western blot of MLKL (mixed lineage kinase domain-like protein), a necroptosis mediator protein, in CRSwNP tissue after *S. aureus* infection for 24 and 48 hours. (E) Full-length IL-33, processed IL-33, lftTSLP, and sftTSLP were separated by Western blot. Re. = recombinant; TCM = tissue culture medium; TUNEL = terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Figure 2. Expression of epithelial cell-derived cytokines in chronic rhinosinusitis with nasal polyps (CRSwNP) tissue after *Staphylococcus epidermidis* infection. (A) The protein levels of TSLP (thymic stromal lymphopoietin) and IL-33 were measured in supernatants of CRSwNP tissue at 24 or 48 hours after *S. epidermidis* infection. (B) Immunofluorescence staining for IL-33 expression in CRSwNP tissue was assessed after *S. epidermidis* infection for 24 hours. Uninfected tissues incubated with tissue culture medium were used as controls. Blue fluorescence indicates the nucleus of the cell, and pink fluorescence indicates the presence of IL-33. TCM = tissue culture medium.

tissue 24 hours after *S. epidermidis* infection (Figure 2B), indicating that IL-33 release in CRSwNP tissue in this study was specifically due to *S. aureus*.

CD3⁺TSLPR⁺ and CD3⁺ST2⁺ Cells Increased in CRSwNP Tissue after *S. aureus* Infection

To further unveil TSLP and IL-33 signaling pathways in the human airway infection model, we evaluated the expression and localization of TSLPR and ST2 upon *S. aureus* exposure by immunofluorescence staining, Western blot, and flow cytometry. Increased levels of TSLPR and ST2 were observed in the epithelial layer of CRSwNP mucosa 24 hours after *S. aureus* infection (Figures 3A and 3B). TSLPR and

ST2 were predominantly expressed on CD3⁺ T cells in the epithelial and subepithelial layers. In line with the staining results, the expression of TSLPR and ST2 proteins was also significantly upregulated 24 hours after *S. aureus* infection compared with that in noninfected mucosal tissue, as shown by the Western blot assay (Figures 3C and 3D). Additionally, the number of CD3⁺ST2⁺ and CD3⁺TSLPR⁺ cells in tissue increased in response to *S. aureus* exposure (Figure 3E). ST2 and TSLPR were also expressed on CD3⁻ T cells; however, the number of CD3⁻ST2⁺ and CD3⁻TSLPR⁺ cells increased only marginally after *S. aureus* infection (Figure 3F).

Effect of *S. aureus* Infection on Induction of the Th2 Response in CRSwNP Tissue and Healthy Control Tissue

To determine the contribution of TSLP and IL-33 to type 2 cytokine release in this human airway infection model, we measured the release of IL-4, IL-5, and IL-13. *IL-5* and *IL-13* mRNA expression and IL-5 protein release were upregulated in CRSwNP tissue 48 hours after *S. aureus* infection, but not in control tissue (Figure 4A). Similarly, single-cell suspensions of nasal cells from patients with CRSwNP that were stimulated with rhTSLP and rhIL-33, both individually and together, demonstrated that rhIL-33 alone induced IL-5 release individually, and that IL-5 release was significantly further increased by the combination of rhTSLP and rhIL-33 (Figure 4B).

TLR2 Antagonism Inhibits *S. aureus*-induced IL-33 and TSLP Release

The observed effects of *S. aureus* in the human mucosal tissue infection model were confirmed by the use of epithelial BEAS-2B cell cultures infected with *S. aureus*. As demonstrated in the nasal tissue model, *S. aureus* induced TSLP and IL-33 release from BEAS-2B cell cultures in a dose-dependent fashion (Figure 5A). The full-length IL-33, processed IL-33, and lTSLP increased markedly in cell protein extracts of BEAS-2B after *S. aureus* exposure (Figure 5B). Assessment of the relevant NF-κB signaling pathways activated in epithelial cell cytokine production demonstrated an increase in phosphorylation of p50 and p65 at early time points in BEAS-2B cells after exposure to *S. aureus* (Figure 5C). In contrast, no induction of TSLP or IL-33 expression or upregulation of NF-κB in BEAS-2B cells was observed after *S. epidermidis* infection (data not shown). To investigate whether induction of TSLP and IL-33 was dependent on TLR2-mediated NF-κB activation, BEAS-2B cells were infected with *S. aureus* in the presence of the TLR2 antagonist CU-CPT22. The TLR2 antagonist CU-CPT22 inhibited the phosphorylation of p50 and p65 in BEAS-2B cells 10 hours after *S. aureus* infection (Figure 5F). Consequently, the downstream induction of IL-33 and TSLP expression was also decreased in BEAS-2B cells in a

Figure 3. Number of CD3⁺TSLPR⁺ and CD3⁺ST2⁺ cells in CRSwNP tissue after *Staphylococcus aureus* infection. (A and B) Immunofluorescence staining of TSLPR⁺ T cell (white arrow) and IL-33 receptor (ST2)⁺ T cell (white arrow) in CRSwNP tissue after *S. aureus* infection for 48 hours. Uninfected tissues incubated with TCM were used as controls. Yellow fluorescence indicates the presence of receptors, and red fluorescence indicates the presence of CD3⁺ cells in the nasal mucosa; isotype control staining for TSLPR and ST2 was completely negative (not presented here).

Figure 3. (Continued). (C) Western blot of TSLPR and ST2 in CRSwNP tissue after *S. aureus* infection for 48 hours. (D) Quantification of TSLPR and ST2 proteins in CRSwNP tissues with *S. aureus* infection ($*P < 0.05$). (E) Expression of $CD3^{+}ST2^{+}$ and $CD3^{-}ST2^{+}$ cells in CRSwNP tissue with or without exposure to *S. aureus* ($n = 3$). (F) Expression of $CD3^{+}TSLPR^{+}$ and $CD3^{-}TSLPR^{+}$ cells in CRSwNP tissue with or without exposure to *S. aureus* ($n = 3$). APC = allophycocyanin; Comp = compensation; CRSwNP = chronic rhinosinusitis with nasal polyps; PE = phycoerythrin; SA = *S. aureus*; SSC = side scatter; TCM = tissue culture medium; TSLPR = thymic stromal lymphopoietin receptor.

dose-dependent manner in the presence of the TLR2 antagonist CU-CPT22 (Figures 5D and 5E).

Discussion

To our knowledge, this study is the first to demonstrate that *S. aureus* can directly induce the release of the epithelial cell-derived cytokines lftTSLP, full-length IL-33, and processed IL-33 from human nasal tissue from patients with CRSwNP. Furthermore, this cytokine release is accompanied by the upregulation of receptor-positive T cells ($CD3^{+}TSLPR^{+}$ and $CD3^{+}ST2^{+}$), *IL-5* and *IL-13* mRNA expression, and IL-5 protein release in CRSwNP tissue, but not control tissue. Necrosis and NF- κ B signaling are involved in the production of TSLP and IL-33 after binding of *S. aureus* to TLR2. In contrast, *S. epidermidis* infection did not induce the release of any cytokines, indicating a potentially selective pathologic role of *S. aureus* in CRSwNP.

In the present study, we used human nasal tissue explants to serve as a valid model to investigate the role of human epithelial cells in the etiology of human airway disease,

and specifically focused on the pathogenic role of *S. aureus* based on the detailed information available on its immune proteome, including enterotoxins and newly discovered serin protease-like proteins (28). Because enterotoxins are known to work as superantigens and induce the release of large amounts of Th2 and other cytokines, as well as induce IgE formation in CRSwNP tissue (29), we specifically used a strain of *S. aureus* that does not produce enterotoxins to evaluate whether *S. aureus*-induced inflammatory cytokine release is also mediated independently of enterotoxin secretion. Our study demonstrated that *S. aureus* can directly induce a Th2 response in CRSwNP tissue, and that this appears to be specific to *S. aureus*, as *S. epidermidis* was unable to induce the release of any cytokines from CRSwNP tissue.

The downstream activities of epithelial cell-derived cytokines are currently the focus of interest, but the factors that affect epithelial cell-derived cytokine release are only partially understood. Rhinovirus exposure appears to trigger TSLP expression in the lungs of mice with allergic airway inflammation (30). Similarly, mouse epithelial MLE-15 cells and human alveolar

A549 epithelial cells infected with influenza virus have also been shown to release increased levels of IL-33 (31). TSLP has also been shown to be produced by murine dendritic cells in response to certain microbial pathogen products, such as lipopolysaccharide, flagellin, FLS-1, and pam3csk4 (32), and *S. aureus* membrane and diacylated lipopeptides can induce TSLP release in human keratinocytes via the TLR2/TLR6 heterodimer (33). Heat-inactivated *S. aureus* infection was shown to increase IL-33 in human and murine epidermal keratinocytes and murine dermal macrophages (34). Our study further demonstrates that non-enterotoxin-producing *S. aureus* also can induce the release of both TSLP and IL-33 in human CRSwNP tissue and human bronchial epithelial cells. We also measured IL-25, another epithelial cell-derived cytokine, in the human *S. aureus* infection model. However, IL-25 was undetectable in both CRSwNP and control tissues and supernatants at the gene and protein levels in this model.

Although the predominant form of TSLP in humans remains controversial, two different TSLP forms have been described: lftTSLP is considered the functional human

Figure 4. Th2 response in chronic rhinosinusitis with nasal polyps (CRSwNP) tissue in the presence of *Staphylococcus aureus*. (A) Expression of IL-4, IL-5, and IL-13 in CRSwNP tissues after *S. aureus* infection for 24 or 48 hours. Uninfected tissues incubated with tissue culture medium were used as controls ($P < 0.05$). (B) IL-5 cytokine increased in single-cell suspensions of CRSwNP tissue after stimulation with 100 ng/ml rhTSLP and rhIL-33 for 24 hours ($P < 0.05$). SA = *S. aureus*; TCM = tissue culture medium; SCS = single-cell suspensions; rhIL-33 = recombinant human IL-33; rhTSLP = recombinant human thymic stromal lymphopoietin; Th2 = T-helper cell type 2.

TSLP (35), whereas human sTSLP exhibits a markedly stronger antibacterial activity in skin and salivary glands (36). Our study demonstrated that lTSLP increased at the mRNA and protein levels in CRSwNP tissue after *S. aureus* infection to propagate a Th2 immune response, whereas sTSLP decreased in CRSwNP with *S. aureus* infection, further decreasing the mucosal defense against infection.

IL-33 is a chromatin-associated nuclear cytokine from the IL-1 family that functions as an “alarmin.” It has been shown to be constitutively expressed in the nuclei of epithelial cells or to be released into the extracellular space after cell necrosis to elicit innate immune responses (37, 38). In the present study, immunofluorescence staining indicated that after *S. aureus* infection, a large amount of IL-33 was present in the extracellular space between epithelial cells, which was

accompanied by an increase in phosphorylation of MLKL, a necroptosis marker. In contrast, IL-33 was preferentially located in the nuclei of nasal basal cells in CRSwNP tissue without *S. aureus* infection or after *S. epidermidis* infection, but was not released and thus did not impact the mucosal immune response. Full-length IL-33 and processed IL-33 reportedly have biological activities that promote Th2 responses (38, 39). In the present study, both processed IL-33 and full-length IL-33 were observed in CRSwNP tissue after *S. aureus* infection, indicating that induced IL-33 was functional in this model of nasal infection. Furthermore, these findings suggest that the necroptosis process of nasal epithelial cells upon *S. aureus* infection may contribute to IL-33 production. As blocking of TLR2 reduced NF- κ B signaling together with a

reduction in the downstream induction of TSLP and IL-33, these findings additionally suggest that both the NF- κ B pathway and necroptosis may be involved in the production of TSLP and IL-33 exposure to *S. aureus*.

Besides the release of TSLP and IL-33, *S. aureus* infection can also increase the expression of their receptors. The TSLP and ST2 receptors were mainly expressed on CD3⁺ T cells in CRSwNP tissue infected with *S. aureus*. Thus, it is likely that CD3⁺ T cells contributed to the IL-5 production observed after *S. aureus* infection, whereas mast cells and ILC2s were unlikely to be involved, as the numbers of CD3⁺TSLPR⁺ and CD3⁺ST2⁺ increased only marginally after *S. aureus* infection. Interestingly, IL-5 protein release was propagated in CRSwNP tissue upon *S. aureus* infection, but not in control tissue, indicating that the typical Th2 response predominates in CRSwNP. The findings of the present study, however,

Figure 5. Expression of TSLP (thymic stromal lymphopoietin) and IL-33 in supernatants of bronchial epithelial BEAS-2B cell cultures infected with *Staphylococcus aureus*; possible induction pathway. (A) Expression of TSLP and IL-33 in supernatants of bronchial epithelial BEAS-2B cells after exposure to different concentrations of *S. aureus* for 10 hours. (B) The expression of long-form TSLP (IfTSLP), full-length IL-33, and processed IL-33 was evaluated in BEAS-2B cell protein extracts after exposure to different concentrations of *S. aureus*. (C) The phosphorylation of p50 and p65 increased in BEAS-2B cells with *S. aureus* infection in a time-dependent manner ($P < 0.05$). (D) The TLR2 antagonist CU-CPT22 partially inhibited the downstream expression of IL-33 and TSLP in BEAS-2B cells after 10 hours of *S. aureus* infection ($P < 0.05$). (E) TLR2 antagonist CU-CPT22 preincubation inhibited IfTSLP, full-length IL-33, and processed IL-33 expression in BEAS-2B cells after *S. aureus* exposure. (F) The TLR2 antagonist CU-CPT22 inhibited the phosphorylation of p50 and p65 in BEAS-2B cell after 10 hours of *S. aureus* infection. SA = *S. aureus*; TCM = tissue culture medium; TLR2 = Toll-like receptor 2.

are in keeping with other findings from our group. This is even more relevant in view of the evidence that 1) a defective epithelial barrier in CRSwNP tissue may be insufficient to effectively remove bacterial colonization (20); 2) in contrast to healthy controls, patients with CRSwNP are unable to upregulate expression of the tight junction to strengthen the epithelial barrier during *S. aureus* infection (40); 3) defense

mechanisms against *S. aureus* may be impaired in CRSwNP, with a high rate of alternatively activated macrophages in CRSwNP mucosa compared with healthy mucosa, which are not suited to phagocytose and kill *S. aureus* efficiently (41); and 4) CRSwNP tissue has been reported to have a deficit of regulatory T cells, which makes it unable to restore the balance of Th1, Th2, and Th17 responses (2).

This study is somewhat limited in that as TSLP and IL33 release was not totally blocked by CU-CPT22, the specific TLR2 antagonist, the possibility that other TLRs played a role in the induction of TSLP/IL-33 during *S. aureus* infection cannot be excluded. In the present study, we selected *S. aureus* for investigation because this is a common bacterium found in CRSwNP, and *S. epidermidis* because it is frequently found

in healthy control subjects. It is noteworthy, however, that other bacteria, such as *H. influenzae* and *S. pneumoniae*, are also potential candidates for similar experiments; this needs to be investigated in future studies.

In summary, this study demonstrates for the first time that *S. aureus*, but not

S. epidermidis, induces a type 2 response in CRSwNP tissue via the epithelial cell-derived cytokines TSLP and IL-33. Given the frequent colonization of CRSwNP tissue by *S. aureus*, this likely contributes to the initiation or persistence of the Th2-biased inflammation in airway diseases. ■

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