Adaptive immune responses in *Staphylococcus aureus* biofilm–associated chronic rhinosinusitis


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Although chronic rhinosinusitis (CRS) is a common chronic health condition, affecting 10–15% of the European and US population in recent epidemiologic studies (1, 2), its underlying pathogenetic mechanisms remain unclear. CRS probably represents a heterogenous group of diseases resulting from a multifaceted interaction between the host and the environment. The microorganisms colonizing the airways have been identified as sources of signals for the innate, as well as adaptive, mucosal immune response. Variations such as skewing of T-cell populations and polarized cytokine patterns may modulate an abnormal response to the presence of environmental triggers within the upper airway (3, 4). Research into the staphylococcal superantigens elegantly reflects the interplay between microorganisms and the local immune system, and their role as disease modifiers in nasal polyp disease (CRSwNP) is now well established (4). Biofilms are a relatively new concept in the CRS literature, but do have several features that might be relevant when considering the impact of bacteria such as *Staphylococci* (5).

CRSwNP and CRSsNP (without nasal polyps) are increasingly recognized as distinct disease entities based on cytokine, mediator, and cellular profiles (6). CRSsNP is largely a fibrotic, remodeling disease driven by T-helper1 (Th1) cytokines such as IFN-γ with normal T-regulatory cell function (7, 8). Conversely, polyp formation of the mucosa in CRSwNP ensues following escape from the inhibitory function of T-regulatory

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Background: The etiopathogenesis of chronic rhinosinusitis (CRS) is currently an area of intense debate. Recently, biofilms have been proposed as a potential environmental trigger in this disease. In particular, *Staphylococcus aureus* biofilms appear to be a predictor of severe disease recalcitrant to current treatment paradigms. However, direct causal links between biofilms and host immune activation are currently lacking. This study aimed to document both the adaptive immune responses that characterize *S. aureus* biofilm–associated CRS and the relative contributions of staphylococcal superantigens and *S. aureus* biofilms in the inflammatory make-up of this disease.

Methods: A total of 53 disease subjects and 15 controls were recruited. Sinonasal mucosa was collected for the determination of *S. aureus* and *Haemophilus influenzae* biofilms and presence of total and superantigen-specific IgE and for the measurement of cytokines that characterize the T-helper pathways.

Results: *Staphylococcus aureus* biofilms and superantigens are significantly associated in CRS patients, suggesting the biofilm may be a nidus for superantigen-eluting bacteria. The presence of *S. aureus* biofilms is associated with eosinophilic inflammation, across the spectrum of CRS, on the back of a T-helper2 skewing of the host’s adaptive immune response (elevated Eosinophilic Cationic Protein and IL-5). This can be distinguished from the superantigenic effect resulting in the induction of IgE.

Conclusion: This study provides novel evidence of a link between *S. aureus* biofilms and skewing of the T-cell response toward the T-helper2 pathway that is independent of superantigen activities. Further research is required to confirm the cause–effect relationship of this association.
Allergy cells (evidenced by a reduction in FoxP3 and TGF-β), enabling the T-helper2 (Th2) cells to predominate and their cytokines, in particular IL-5, to recruit and activate eosinophils (9). The presence of superantigens has been consistently demonstrated in 20–50% of Caucasian patients with CRSwNP, but rarely in CRSsNP or control subjects. The Th2-biased cytokine patterns are further exaggerated in this subgroup of CRSwNP, linking comorbid asthma to nasal polyposis (10, 11). However, the genesis of the eosinophilic and occasional neutrophilic responses in the remaining nasal polyposis patients is still elusive, underlining both the heterogeneity of this condition and the limitations of our current knowledge.

A biofilm is defined as a community of bacteria that are encased in an exopolysaccharide matrix they have produced and are irreversibly attached to a surface (12). Biofilm bacteria exhibit unique characteristics with respect to both growth and metabolism. Resulting biofilm-mediated diseases share common features, being chronic diseases with repeated acute exacerbations, variable culture rates, and extreme antibiotic resistance. These are characteristics that are commonly seen in our CRS population; hence, the biofilm hypothesis has recently been applied to CRS. The existence of biofilms in CRS has now been well established (13–16). Recent work from our department has outlined the polymicrobial nature of CRS biofilms (17), with *Staphylococcus aureus* and *H. influenzae* featuring prominently (17–19). The presence of biofilms has been associated with poor evolution following sinus surgery (20, 21); *S. aureus* biofilms are linked to more severe and surgically recalcitrant disease, whereas *H. influenzae* biofilms are generally seen in mild disease that is highly responsive to current management paradigms (22).

A direct role for biofilms in CRS disease initiation remains circumstantial. By evaluating both the cytokine patterns associated with staphylococcal biofilms and their coexistence with superantigen-specific IgE within the sinuses, we hope to provide insights into the adaptive immune responses that characterize *S. aureus* biofilm–associated CRS and possibly further clarify their pathogenic role in this disease.

**Methods**

**Study design**

This study was conducted as a prospective investigation accessing patients from the tertiary rhinology practice of one of the senior authors (P.J.W.). Institutional review board approval was obtained, and all patients gave their informed consent to participate. A total of 53 patients who met the criteria for diagnosis of CRS outlined by the American Rhinosinusitis Taskforce (23) and were undergoing endoscopic sinus surgery after failing maximal medical therapy were recruited. Additionally, 15 patients without clinical or radiological evidence of chronic sinus disease undergoing trans-sphenoidal removal of pituitary adenomas served as control patients. Patients were excluded if they were under the age of 18, were immunocompromised, had a ciliary dyskinesia, or had taken antibiotics or steroids in the three weeks prior to surgery. All patients had baseline clinical and radiological assessments. Symptom scores were patient-derived and physician-recorded on a scale of 1–5 for the five common sinonasal symptoms – nasal obstruction, rhinorrhea, facial pain/headache, postnasal drip, and altered sense of smell. Radiological assessment utilized the Lund–MacKay system.

**Sinus mucosa acquisition and preparation**

At the time of endoscopic sinus surgery, sinus mucosa and mucus were harvested from the middle meatus and ethmoid cavity. For control patients, tissue was harvested from the ethmoid during pituitary gland surgery. The mucosal biopsies were transported on ice in Dulbecco’s modified Eagle medium (Gibco, Invitrogen Corp., Grand island, NY, USA) until stored at –80°C. This tissue was used for both biofilm characterization and cytokine analysis.

**Biofilm characterization**

Biofilm characterization was performed by an investigator (A.F.) blinded to both the clinical presentation and cytokine results of the patient, using a previously described Fluorescence in situ Hybridization (FISH) protocol (17). FISH probes for *S. aureus* and a universal bacterial probe were obtained from AdvanDx (Woburn, MA, USA) and used according to the manufacturer’s directions. An *H. influenzae* FISH protocol developed in our laboratory was employed. Posthybridization slides were evaluated using the Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany). Image acquisition and data analysis were performed using the Leica Application Suite Advanced Fluorescence. The biofilm definitions were outlined previously (24). While there are many ways to image biofilms on sinus mucosa, FISH was selected because of its ability to characterize the species within the biofilm. FISH has previously demonstrated comparable specificity and sensitivity to BacLight LIVE/DEAD staining (24), which is superior to scanning electron microscopy and transmission electron microscopy (25). Representative images are presented in Fig. 1.

**Cytokine and mediator analysis**

Measurement of cytokines that characterize the Th1, Th2, and T-h17 pathways along with total IgE, *S. aureus* enterotoxin–specific IgE, and ECP were performed as previously described (10). The cytokine and mediator assays were performed by an investigator (G.H.) blinded to both the clinical phenotype and biofilm status of the patients. All samples were assayed for IL-5, IFN-γ, IL-1b, IL-6, IL-17, TGF-β1, and Myeloperoxidase (MPO). IFN-γ, TGF-β1, and MPO were analyzed using commercially available ELISA kits (IFN-γ, TGF-β1: R&D Systems Quantikine ELISA, Minneapolis, MN, USA; MPO: BioCheck Inc, Foster City, CA, USA). IL-5, IL-1b, IL-6, and IL-17 were analyzed using the Luminex xMAP Technology with commercially available Fluorokine Map kits (R&D Systems) and measured on a Luminex Platform (BioRad, Hercules, CA, USA). IgE and IgE antibodies to *S. aureus* enterotoxins, as well as ECP,
were measured by the UNICAP system (Phadia, Uppsala, Sweden) according to manufacturer’s guidelines.

Statistical analysis

Results were analyzed using GRAPHPAD PRISM 5.0 software (GraphPad Software, San Diego, CA, USA). Significance values of $P \leq 0.05$ were used. As data are nonparametric, median and interquartile range (IQR) were reported. Chi-squared test or Fisher’s exact test was used for dichotomous data, with a two-tailed Kruskal–Wallis test, followed by a Mann–Whitney $U$ test, used for the analysis of ordinal data. Bonferroni corrections were applied for the analysis of multiple comparators. The linear discriminant analysis and construction of comparison trees were performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). A linear discriminant analysis reduces the variability of numerous measures into a biplot graph. A vector represents each mediator, and the patients, according to subgroup, are plotted on the graph with their position determined by the relative contribution of each mediator to their inflammatory make-up. A comparison tree analyses all variables simultaneously and determines which measures differentiate two groups of interest (e.g., biofilm positive and negative). A computer-generated cut-off point is determined, at which the greatest separation of the two groups occurs. Finally, the differentiating variables are then organized in sequence starting with the most distinguishing variable, a feature that is determined by the mean decrease in Gini index.

Results

Demographic data

53 CRS patients (27 males, 26 females) and 15 control subjects [seven men and eight women, median age 54 years (IQR 46–63)] were enrolled in this study. Based on nasal endoscopic findings, the CRS patients were divided into 33 with nasal polyps [median age 55 years (IQR 47–58) and 20 without [59 years (IQR 48–68)]. A total of 33/53 patients (62%) had undergone one or more previous surgeries for CRS, reflecting the tertiary nature of this practice and the overall disease severity. A total of 30/53 patients (57%) had allergies to one or more of the common inhalant allergens, without differences between groups. Symptom scores were significantly greater (Mann–Whitney $U$ test, $P = 0.017$) in the CRSwNP group (median = 17/25, IQR 15–20) compared with the CRSsNP group (median = 15/25, IQR 14–17). Lund–MacKay scores were also significantly greater (Mann–Whitney $U$ test, $P < 0.001$) in the CRSwNP group (median 15, IQR 13–20) than in the CRSsNP (median 10, IQR 8–14). A total of 14/33 (42%) of CRSwNP vs 9/20 (45%) of CRSsNP patients had asthma. This difference was not significant.

Biofilm prevalence and distribution

No control subjects had evidence of biofilms on their sinus mucosa. Seventy percent of the CRS patients had biofilms present (universal bacterial probe). One patient’s biofilm speciation could not be ascertained; $H. influenzae$ was present in 35% of CRS patients (Fig. 1A), most commonly in association with $S. aureus$, rather than in a unimicrobial biofilm.

$Staphylococcus aureus$ was observed in 26/53 (49%) CRS patients (Fig. 1B). $S. aureus$ biofilms were significantly more prevalent in the CRSwNP than in the CRSsNP group (chi-squared test, $P = 0.047$). The presence of $S. aureus$ biofilms was associated with more severe symptoms (median 18, IQR 15–20 vs median 16, IQR 14–17, Mann–Whitney $U$ test, $P = 0.005$) and greater radiological disease burden (median 15, IQR 13–20 vs median 12, IQR 9–15, Mann–Whitney $U$ test, $P = 0.003$).

Cytokine and mediator results based on polyp status

The median and IQRs for all cytokines and mediators, based on polyp status, are summarized in Table 1. The CRSwNP group was characterized by polarization of the T-cell response toward the Th2 pathway (Table 1 and Fig. 2A). Cytokines associated with the CRSsNP phenotype, such as IFN-$\gamma$ and TGF-$\beta$, were not significantly different between the subgroups.

Superantigen-specific IgE data

The CRSwNP group contained 17/33 (52%) enterotoxin-specific IgE-positive patients, whereas none of the 20 CRSsNP patients had detectable enterotoxin-specific IgE (Table 2, Fisher’s exact test, $P < 0.001$). Two of the control patients had enterotoxin-specific IgE present. A total of 12/26 $S. aureus$ biofilm–positive polyp patients also had detectable enterotoxin-specific IgE vs only 5 of 27 $S. aureus$ biofilm–negative CRS patients (Table 2, Fisher exact test, $P = 0.042$).

Cytokine and mediator results based on superantigen status

The presence of superantigen-specific IgE was associated with significantly elevated total IgE, IL-5, and ECP concentrations.
Table 1  Median and interquartile range of all mediators assessed in this study with division of patients into subgroups based on evidence of nasal polyps (columns 2 and 3) and the presence or absence of *Staphylococcus aureus* biofilms (column 5 and 6). Control patients are represented in column 4. (All control patients are biofilm negative)

<table>
<thead>
<tr>
<th>Mediator</th>
<th>CRSwNP</th>
<th>CRSsNP</th>
<th>Control</th>
<th>Biofilm positive</th>
<th>Biofilm negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE (kU/l)</td>
<td>321.8 (138.6–1045)</td>
<td>68.2 (28.6–154)</td>
<td>22.44 (10.12–128.2)</td>
<td>317.4 (82.77–1238)</td>
<td>23.10 (1.9–140.8)</td>
</tr>
<tr>
<td>ECP (mg/l)</td>
<td>46.15 (2456–13 118)</td>
<td>841.5 (541.2–3222)</td>
<td>9842 (119.9–558.8)</td>
<td>9756 (2540–13 241)</td>
<td>6905 (660–4615)</td>
</tr>
<tr>
<td>TGF-β (pg/ml)</td>
<td>7270 (5185–10 728)</td>
<td>77.3 (6241–13 488)</td>
<td>39.1 (5643–19 038)</td>
<td>167.7 (5922–12 759)</td>
<td>66 (5082–9882)</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>160.2 (31.4–398.4)</td>
<td>73.3 (49.5–294.3)</td>
<td>10 (10–79)</td>
<td>43.85 (34–325.9)</td>
<td>34 (34–414)</td>
</tr>
<tr>
<td>IL-5 (pg/ml)</td>
<td>113.1 (6.5–320.9)</td>
<td>6.5 (6.5–52.6)</td>
<td>6.5 (6.5–6.5)</td>
<td>113.1 (6.5–6.5)</td>
<td>6.5 (6.5–99.9)</td>
</tr>
<tr>
<td>IL-17 (pg/ml)</td>
<td>12.5 (12.5–63.0)</td>
<td>12.5 (12.5–28.7)</td>
<td>12.5 (12.5–65.0)</td>
<td>12.5 (12.5–28.7)</td>
<td>12.5 (12.5–28.7)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>209.2 (71.8–791.8)</td>
<td>60 (18.2–154.8)</td>
<td>18.2 (18.2–68.9)</td>
<td>205.1 (80.85–790.8)</td>
<td>71.1 (18.2–334.7)</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>42.9 (42.9–98.25)</td>
<td>42.9 (42.9–208.8)</td>
<td>42.9 (42.9–42.9)</td>
<td>42.9 (42.9–42.9)</td>
<td>42.9 (42.9–188.5)</td>
</tr>
<tr>
<td>MPO (ng/ml)</td>
<td>2356 (1212–6364)</td>
<td>1606 (793.3–2789)</td>
<td>791.5 (618.0–1451)</td>
<td>1769 (1142–4841)</td>
<td>1850 (727.0–4368)</td>
</tr>
</tbody>
</table>

Table 2  Presence of staphylococcal superantigen specific-IgE in the subgroups of nasal polyps. Superantigens are significantly associated with the CRSwNP phenotype (Fisher’s exact test, \( P < 0.001 \)) as well as with *Staphylococcus aureus* biofilms (Fisher’s exact test, \( P = 0.041 \))

<table>
<thead>
<tr>
<th></th>
<th>CRSwNP</th>
<th>CRSsNP</th>
<th>Total</th>
<th>Biofilm positive</th>
<th>Biofilm negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAE-IgE-positive patients</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>12</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>SAE-IgE-negative patients</td>
<td>16</td>
<td>20</td>
<td>36</td>
<td>14</td>
<td>22</td>
<td>36</td>
</tr>
<tr>
<td>Total patients</td>
<td>33</td>
<td>20</td>
<td>53</td>
<td>26</td>
<td>27</td>
<td>53</td>
</tr>
</tbody>
</table>

Figure 2  IL-5, IL-6, ECP, and total IgE results, based on nasal polyp status and *Staphylococcus aureus* biofilm status. (A) IL-5, IL-6, total IgE, and ECP are significantly elevated in the CRSwNP patients, reflecting the TH2 bias of this phenotype. The TH1 cytokines are not significantly different between groups. (B) Significantly elevated TH2 cytokines across the spectrum of chronic rhinosinusitis are dependent on the presence or absence of *S. aureus* biofilms, whereas the TH1 cytokines are not significantly different between the biofilm subgroups.
Table 3 Cytokine and mediator results based on Staphylococcus aureus biofilm status and superantigen status. The presence of S. aureus biofilms correlates with a Th2 bias of the T-cell response regardless of the polyp status of the patient. Furthermore, the presence of S. aureus biofilms is associated with eosinophilic inflammation, as evidenced by the elevated ECP levels in this subgroup when compared to both non-biofilm CRS and controls. Superantigen-specific IgE-positive CRS (SAE+) is associated with significantly elevated total IgE, IL-5, and ECP when compared to both superantigen-specific IgE-negative CRS (SAE−) and controls.

|----------|---------------------|---------------------| |----------|---------------------|---------------------|
|          | Biofilm+ vs Control | Biofilm+ vs Biofilm− | Biofilm− vs Control | SAE+ vs Control | SAE+ vs SAE− | SAE− vs Control |
| IL-5     | <0.001              | 0.003               | NS                  | 0.033          | 0.02          | 0.02          | NS |
| IL-6     | <0.001              | <0.001              | 0.01                | NS             | 0.05          | NS            | NS |
| ECP      | <0.001              | <0.001              | <0.001              | <0.001         | <0.001        | <0.001        | NS |
| IL-17    | 0.02                | 0.006               | NS                  | NS             | NS            | NS            | NS |
| IFN-β    | NS                  | NS                  | NS                  | NS             | NS            | NS            | NS |
| MPO      | 0.02                | 0.004               | NS                  | NS             | 0.02          | NS            | NS |

NS, not significant.

(Table 3). This confirms the Th2-bias associated with staphylococcal superantigens as well as the ability of superantigens to induce IgE formation.

Cytokine and mediator results based on biofilm status

Staphylococcus aureus biofilm–associated CRS demonstrated significantly higher levels of IL-5 (P < 0.001), IL-6 (P < 0.001), and ECP (P < 0.001) than the remaining CRS patients and controls (Table 3 and Fig. 2B). The remaining cytokines were not significantly different between groups. There was no significant difference between groups based on H. influenzae biofilm status.

Interrelation of biofilm and superantigen status

Further subgroup analysis was undertaken, based on S. aureus biofilm status. Examination of the CRSwNP subgroup (n = 33) alone revealed significantly higher levels of IL-5 (P = 0.03) and ECP (P = 0.04) in the S. aureus biofilm group, with IL-6 (P = 0.06) approaching significance. Analysis of the CRSsNP subgroup (n = 20) alone demonstrated elevated IL-5 (P = 0.03) and IL-6 (P = 0.03) in the S. aureus biofilm-positive group. Finally, all CRS patients without detectable superantigen-specific IgE (n = 34) were investigated. IL-5 (P = 0.007), IL-6 (P = 0.03), and ECP (P = 0.03) were all significantly higher in the S. aureus biofilm-positive group.

Linear discriminant analysis of Staphylococcus aureus biofilms and superantigen-specific IgE

Subjects were divided into four groups based on their superantigen and S. aureus biofilm status, and the relationship of each group to the different cytokine vectors are displayed in Fig. 3. Numbers grouped more closely to a vector have a stronger relationship with that mediator.

Classification tree analysis of Staphylococcus aureus biofilms and superantigen-specific IgE

Classification tree analysis was performed to support the discriminant analysis in distinguishing the independent effects of biofilms and superantigens (Fig. 4). Using computer-generated high-low points, the predictors of S. aureus biofilm-positive CRS in order of importance are ECP, IL-5, and TGF-β. Total IgE is the most important predictor of superantigen status because 41/42 patients with a low total IgE were superantigen negative. In combination with total IgE, elevated MPO is a secondary distinguisher of superantigen presence.

Discussion

The results of this study not only confirm previous findings of a skewed cytokine profile in CRS patients with nasal polyposis and the presence and impact of staphylococcal superantigens on the mucosal inflammation but also demonstrate a polarized immune response in the presence of S. aureus biofilms. Despite circumstantial evidence, little published research has specifically examined the immune consequences of biofilms in CRS. Our discovery of an association between S. aureus biofilms and an eosinophilic, Th2-polarized inflammation in CRS, irrespective of polyp status and independent of the superantigen pathway, implies a direct link between microorganism and host. This may finally allow definitive conclusions on the pathogenic role of biofilms in this poorly understood disease.

The role of S. aureus in CRS is expanding as its importance as a pathogen is increasingly recognized. Superantigens released by S. aureus have a well-defined role in the pathogenesis of CRS, acting as disease modifiers in the CRSwNP phenotypic subgroup, a fact confirmed for an Australian population by the current results (26). S. aureus is known to be able to reside intracellularly and intramucosally in the...
sinonasal mucosa (27–29), and nasal colonization rates with *S. aureus* exceed 60% in the CRSwNP subgroup (11). These are both potential reservoirs for superantigen release in the sinuses. Alternatively, bacteria existing in the biofilm form might act as a nidus for planktonic bacteria dispersing into the mucosa, a theory that was confirmed in the current study. Methodologically, we utilized FISH because of its ability to identify individual biofilm-forming species (24). *S. aureus* is the most common biofilm-forming organism in the CRS population (17), and its presence is a predictor of more severe disease (22). In contrast, *H. influenzae* biofilms are associated with a favorable disease course (22). Our data on *H. influenzae* biofilm did not demonstrate an impact on the mucosal response, and this confirmed the clinical observation.

The results of this investigation associate the presence of *S. aureus* biofilms in polyp patients skewing toward the T-helper2 pathway with a resultant eosinophilic inflammatory milieu. This may occur both dependent and independent of superantigens (22–24). The separation of group 1 from both groups 2 and 3 suggests the effects of *Staphylococcus aureus* biofilms and staphylococcal superantigens are distinct.
Within the CRSwNP subgroup, the CRSwNP and CRSsNP subgroups when analyzed separately. We were also able to replicate similar findings in both the patients are associated with moderately higher T-helper2 cytokines IL-5 and ECP, suggesting a further delineation of what is already known to be an eosinophilic disease. Interest-ingly, within the CRSsNP subgroup, the biofilm-positive biofilms do indeed interact with the host in CRS. To differentiate the effects of S. aureus biofilms and superantigen, a number of different analyses were carried out. Firstly, when the non-superantigen CRS patients (both with and without polyps) were analyzed, IL-5, IL-6, and ECP were all significantly elevated in the biofilm-positive group. Thus, the release of superantigens is not required for a skewing of the T-helper2 host response in the presence of S. aureus biofilms. We were also able to replicate similar findings in both the CRSwNP and CRSsNP subgroups when analyzed separately. Within the CRSwNP subgroup, the S. aureus biofilm group has higher IL-5 and ECP, suggesting a further delineation of which is already known to be an eosinophilic disease. Interest-ingly, within the CRSsNP subgroup, the biofilm-positive patients are associated with moderately higher T-helper2 cytokines IL-5 and IL-6 but not a raised ECP. CRSwNP is generally thought not to be eosinophilic in nature, and this may in part explain this result. Alternatively, this may be a type 2 statistical error owing to insufficient numbers. Only further patient recruitment and adaptive immune evaluation will answer this.

Perhaps most importantly though, in attempting to differentiate the effects of S. aureus biofilms from staphylococcal superantigens, is the results of the linear discriminant analysis (Fig. 3). A linear discriminant analysis allows the variation in the nine cytokines to be statistically reduced into two components that permit visualization on a biplot graph in which each cytokine is represented by a vector. In Fig. 3, the T-helper2 cytokines trend toward the left and downwards. The T-helper1 cytokines are toward the right. Importantly, the effect of S. aureus biofilms, independent of superantigen release, can be differentiated on the basis of how much they contribute to the vectors of IL-5, IL-6, TGFB-1, and particularly ECP. Patients with S. aureus biofilms but without superantigens (group 3) shares a stronger relationship with all three of these cytokines than does the superantigen IgE–positive, biofilm-negative patients (group 2). This implies that the link between S. aureus biofilms and both the T-helper2 pathway and an eosino-philic inflammatory response is independent of the effect of superantigens, reinforcing the results of the subgroup analysis discussed above which first suggested this independent association. Conversely, the presence of superantigens alone correlates with IgE levels, and the presence of both superantigens and S. aureus biofilms (group 1) produces an amplified T-helper2 response in CRSwNP, perhaps advancing our understand-ing of the pathomechanics of the CRSwNP subgroup of this disease. This is important, new information because despite the clear evidence of eosinophilic inflammation in CRSwNP, not all of these patients have detectable superantigen IgE. There must be another mechanism, and S. aureus biofilms may be one of the answers. The link between the innate and adaptive immune response appears to be important, yet not understood in CRS. The data presented in our comparison trees (Fig. 4) confirm the hypothesis that the mode of action of superantigens and S. aureus biofilms may be different. Elevated total IgE and MPO distinguish the presence of superantigen IgE, whereas the presence of S. aureus biofilms associates with elevated ECP and IL-5 in particular, in addition to TGFB-1. The combined elevation of both ECP and IL-5 is 100% predictive of S. aureus biofilm presence, whereas the combined elevation of total IgE and MPO is 100% predictive of superantigen-specific IgE in the sinusosal mucosa. Thus, the classification tree analysis supports the discriminant analysis in distinguishing the effects of Staphylococcus aureus biofilms and superantigen.

A link between biofilms and T-helper2 polarization has been found before. Chronic periodontitis is a classical biofilm-mediated disease in which their role has been extensively researched over the last 15 years. Porphyromonas gingivalis is associated with an exuberant T-helper2 response that subsequently dictates disease initiation and progression (30, 31). P. gingivalis is thought to incite a poor innate immune response leading to polyclonal B-cell activation and a T-helper2 response. Ultimately, nonprotective antibodies are produced and a chronic infection established. In contrast, Aggregatibacter actinomycetemcomitans and Prevotella intermedia are biofilm-forming organisms that do not associate with a T-helper2 response (32). These observations seem plausible at another host–environment interface, namely the sino-nasal mucosa, where the differential pathogenicity of S. aureus and H. influenzae may influence disease characteris-tics. It is interesting to note that the only other paper in the literature to examine cytokine profile of biofilm-positive CRS patients had contrasting results. In a study of 19 CRS patients, the presence of biofilms on the sinus mucosa was associated with a skewing toward the T-helper1 pathway. In contrast to the present study, however, the biofilm-forming species were not determined by Hekiert et al. (33).

Despite the evidence produced by this study, a number of questions remain. The interrelation between the T-helper2-biased mucosal immune response and S. aureus biofilm formation needs to be clarified. Profiting from a pre-existing T-helper2 bias within the mucosa, S. aureus may switch to the biofilm phenotype and establish itself within the damaged mucosa. Alternatively, S. aureus biofilms may induce the T-helper2 bias, which initiates the specific mucosal changes already described. Host lactoferrin is reduced in biofilm-associated CRS (34), and alternatively activated M2 macrophages display deficient microbial activity against a range of microorganisms, including S. aureus (35), associated with bacterial persistence (36). Recent data suggest an increase in M2 macrophages in CRSwNP with reduced phagocytosis and killing abilities (37). The sequence of these events still needs further study.

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