Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

Allergy

ORIGINAL ARTICLE AIRWAY DISEASES

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

A. Foreman[1], G. Holtappels[2], A. J. Psaltis[1], J. Jervis-Bardy[1], J. Field[3], P. -J. Wormald[1] & C. Bachert[2]

[1]Department of Surgery-Otorhinolaryngology, Head and Neck Surgery, University of Adelaide and Flinders University, Adelaide, Australia; [2]Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University Hospital, Ghent, Belgium; [3]Faculty of Health Sciences, University of Adelaide, Adelaide, Australia

To cite this article: Foreman A., Holtappels G., Psaltis A.J., Jervis-Bardy J., Field J., Wormald P.-J., Bachert C. Adaptive immune responses in *Staphylococcus aureus* biofilm–associated chronic rhinosinusitis. *Allergy* 2011; **66**: 1449–1456.

Keywords
biofilm; chronic rhinosinusitis; cytokine; *Staphylococcus aureus*; superantigen.

Correspondence
Prof. Peter-John Wormald, Department of Otorhinolaryngology, Head and Neck Surgery, The Queen Elizabeth Hospital, 28, Woodville Road, Woodville, Adelaide, SA 5011, Australia.
Tel.: +61 88222 7158
Fax: +61 88222 7419
E-mail: peterj.wormald@adelaide.edu.au

Accepted for publication 3 July 2011

Edited by: Reto Crameri

DOI:10.1111/j.1398-9995.2011.02678.x

Abstract

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-helper$_2$ 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-helper$_2$ pathway that is independent of superantigen activities. Further research is required to confirm the cause–effect relationship of this association.

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-helper$_1$ (Th$_1$) 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 cells (evidenced by a reduction in FoxP3 and TGF-β), enabling the T-helper$_2$ (Th$_2$) 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 Th$_2$-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 Th$_1$, Th$_2$, and T-h$_{17}$ 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-1ß, 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-1ß, 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,

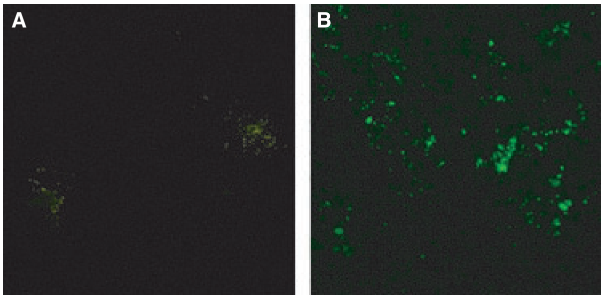


Figure 1 Representative images of bacterial biofilms on sinus mucosa using a FISH protocol, imaged on the confocal scanning laser microscope. Both images demonstrate brightly fluorescing bacterial-sized dots surrounded by a less-intense fluorescing blush, thought to represent the matrix. (A) *H. influenza* FISH probe tagged with Cy3 fluorophore. (B) *Staphylococcus aureus* FISH probe tagged with Alexa488 fluorophore.

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 Th_2 pathway (Table 1 and Fig. 2A). Cytokines associated with the CRSsNP phenotype, such as IFN-γ and TGF-β, 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)

Mediator	CRSwNP	CRSsNP	Control	Biofilm positive	Biofilm negative
IgE (kU/l)	321.8	68.2	22.44	317.4	23.10
	(138.6–1045)	(28.6–154)	(10.12–128.2)	(82.77–1238)	(1.9–140.8)
ECP (mg/l)	4615	841.5	190.3	6160	1639
	(2456–13 118)	(541.2–3322)	(119.9–558.8)	(2540–13 241)	(660–4615)
TGF-β (pg/ml)	7270	9545	9842	9756	6905
	(5185–10 728)	(6241–13 488)	(5643–19 038)	(5922–12 759)	(5082–9882)
IL-1β (pg/ml)	160.2	77.3	39.1	167.7	66
	(31.4–398.4)	(49.5–294.3)	(10–79)	(43.85–325.9)	(34–414)
IL-5 (pg/ml)	113.1	6.5	6.5	113.1	6.5
	(6.5–320.9)	(6.5–52.6)	(6.5–6.5)	(26.83–530.8)	(6.5–99.9)
IL-17 (pg/ml)	12.5	12.5	12.5	12.5	12.5
	(12.5–63.0)	(12.5–28.7)	(12.5–12.5)	(12.5–65.08)	(12.5–28.7)
IL-6 (pg/ml)	209.2	60	18.2	205.1	71.1
	(71.8–791.8)	(18.2–154.8)	(18.2–68.9)	(80.85–790.8)	(18.2–334.7)
IFN- (pg/ml)	42.9	42.9	42.9	42.9	42.9
	(42.9–98.25)	(42.9–208.8)	(42.9–42.9)	(42.9–96.9)	(42.9–188.5)
MPO (ng/ml)	2356	1606	791.5	1769	1850
	(1212–6364)	(793.3–2789)	(618.0–1451)	(1142–4841)	(727.0–4368)

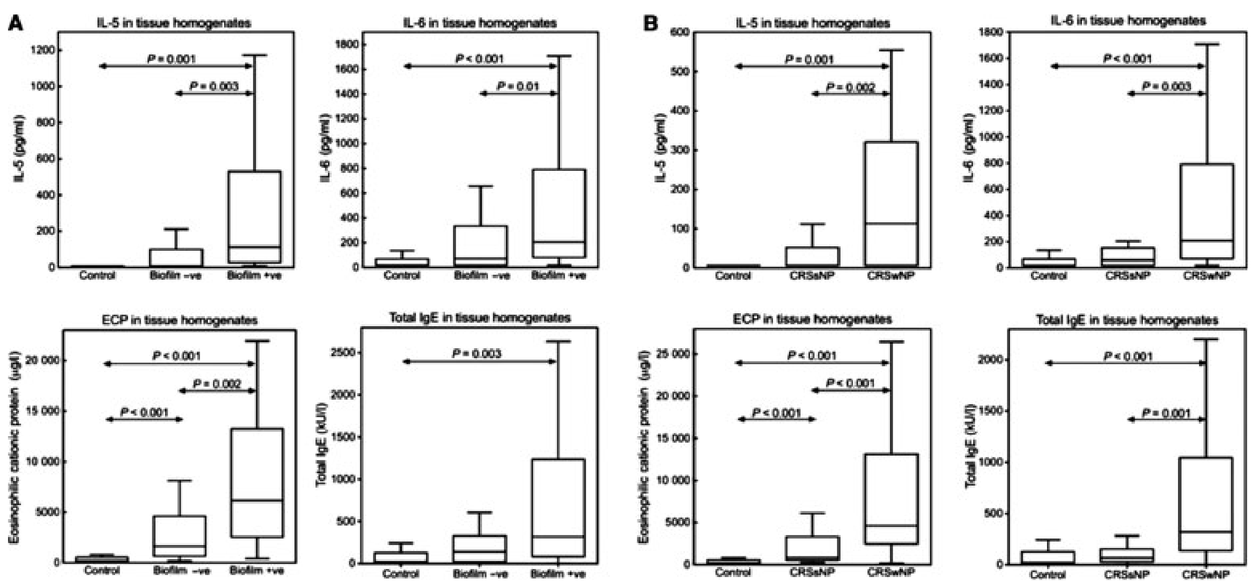


Figure 2 IL-5, IL-6, ECP, and total IgE results, based on nasal polyp status and *Staphylococcus aureus* biofilm status. (A) IL-5, IL-6, total IgE, and ECP are significantly elevated in the CRSwNP patients, reflecting the Th_2 bias of this phenotype. The Th_1 cytokines are not significantly different between groups. (B) Significantly elevated Th_2 cytokines across the spectrum of chronic rhinosinusitis are dependent on the presence or absence of *S. aureus* biofilms, whereas the Th_1 cytokines are not significantly different between the biofilm subgroups.

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, $P = 0.041$)

	CRSwNP	CRSsNP	Total	Biofilm positive	Biofilm negative	Total
SAE-IgE-positive patients	17	0	17	12	5	17
SAE-IgE-negative patients	16	20	36	14	22	36
Total patients	33	20	53	26	27	53

Table 3 Cytokine and mediator results based on *Staphylococcus aureus* biofilm status and superantigen status. The presence of *S. aureus* biofilms correlates with a Th_2 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

		Mann–Whitney *U* test				Mann–Whitney *U* test		
Mediator	Kruskal–Wallis test	Biofilm+ *vs* Control	Biofilm+ *vs* Biofilm −	Biofilm− *vs* Control	Kruskal–Wallis test	SAE+ *vs* Control	SAE+ *vs* SAE −	SAE− *vs* Control
IL-5	<0.001	0.001	0.003	NS	0.033	0.02	0.02	NS
IL-6	<0.001	<0.001	0.01	NS	0.05	0.05	NS	NS
ECP	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	0.08	<0.001
IL-17	0.02	0.006	NS	<0.001	0.05	NS	NS	NS
IL-1β	0.02	0.007	NS	0.04	NS	NS	NS	NS
TGF-β	NS	NS	NS	NS	NS	NS	NS	NS
IFN-	NS	NS	NS	NS	NS	NS	NS	NS
MPO	0.02	0.004	NS	NS	0.03	0.02	NS	NS

NS, not significant.

(Table 3). This confirms the Th_2-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.

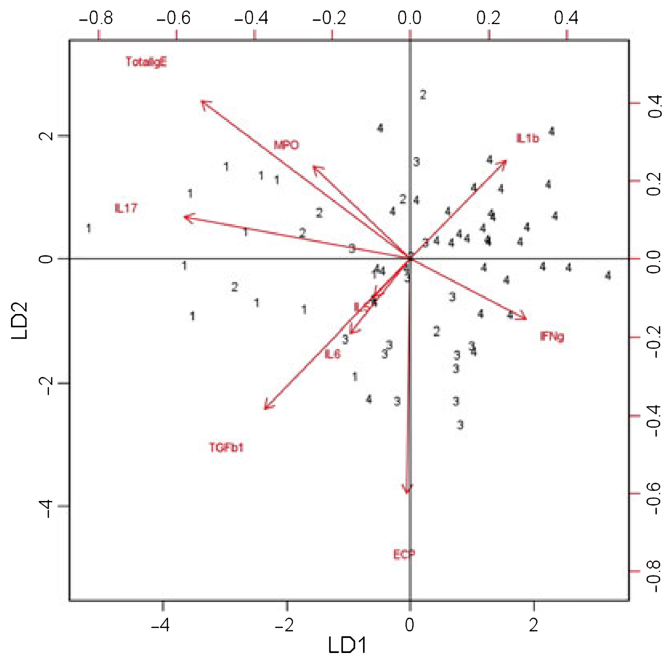
Classification tree analysis of *Staphylococcus aureus* biofilms and superantigen-specific IgE

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, Th_2-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



Group	Superantigen	Biofilm
1	Present	Present
2	Present	Absent
3	Absent	Present
4	Absent	Absent

Figure 3 Linear discriminant analysis of the nine cytokines and mediators measured in this study. Group 3 is more closely associated with the vectors for IL-5, IL-6, and particularly ECP than group 2, which is more closely aligned with total IgE and MPO. The separation of group 1 from both groups 2 and 3 suggests the effects of *Staphylococcus aureus* biofilms and staphylococcal superantigens are distinct.

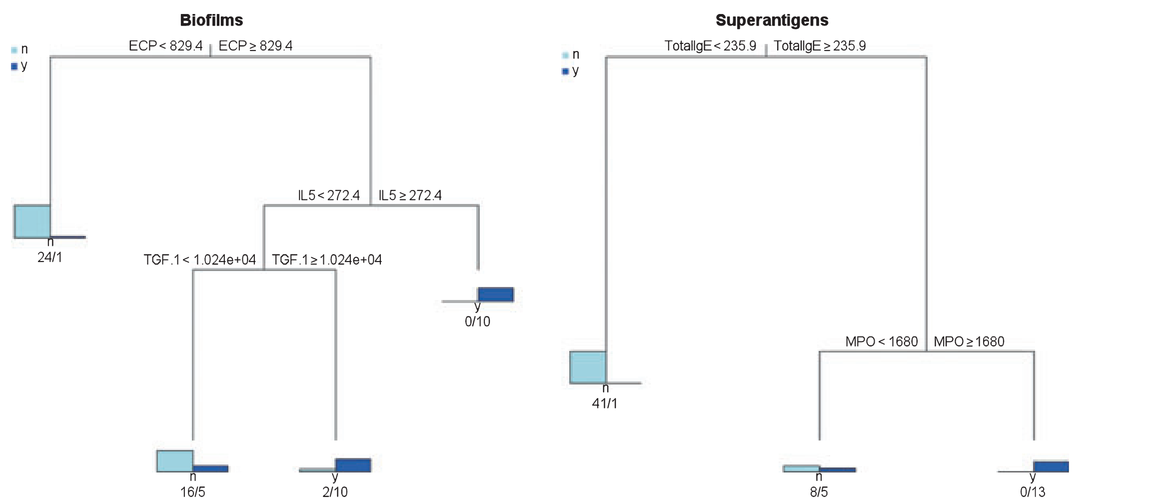


Figure 4 Comparison trees based on the presence or absence of *Staphylococcus aureus* biofilms and superantigens. ECP, IL-5, and TGF-β_1 identify the biofilm-positive group. Contrastingly, total IgE is the primary discriminating mediator between those with and without detectable mucosal superantigen-specific IgE. MPO is a secondary determinant of the superantigen-positive patients.

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-helper$_2$ pathway with a resultant eosinophilic inflammatory milieu. This may occur both dependent and independent of the superantigen pathway. The statistically significant association between biofilms and superantigens suggests that *S. aureus* biofilms may act as a nidus from which planktonic *S. aureus* and superantigens are released into the paranasal sinuses. Given the combined presence of *S. aureus* biofilms and superantigen-specific IgE was only observed in 12/26 CRSwNP patients, other mechanisms must be at play if *S. aureus* biofilms do indeed interact with the host in CRS. To differentiate the effects of *S. aureus* biofilms and superantigens, 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-helper$_2$ 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 what is already known to be an eosinophilic disease. Interestingly, within the CRSsNP subgroup, the biofilm-positive patients are associated with moderately higher T-helper$_2$ cytokines IL-5 and IL-6 but not a raised ECP. CRSsNP 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 to permit visualization on a biplot graph in which each cytokine is represented by a vector. In Fig. 3, the T-helper$_2$ cytokines trend toward the left and downwards. The T-helper$_1$ 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, TGF-β_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-helper$_2$ pathway and an eosinophilic 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-helper$_2$ response in CRSwNP, perhaps advancing our understanding 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 TGF-β_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 sinonasal 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-helper$_2$ 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-helper$_2$ 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-helper$_2$ 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-helper$_2$ response (32). These observations seem plausible at another host–environment interface, namely the sinonasal mucosa, where the differential pathogenicity of *S. aureus* and *H. influenzae* may influence disease characteristics. It is interesting to note that the only other paper in the literature to examine cytokine profile of biofilm-positive CRS patients had contrastingly different results. In a study of 19 CRS patients, the presence of biofilms on the sinus mucosa was associated with a skewing toward the T-helper$_1$ 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-helper$_2$-biased mucosal immune response and *S. aureus* biofilm formation needs to be clarified. Profiting from a pre-existing T-helper$_2$ 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-helper$_2$ 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 microbicidal 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.

Acknowledgments

This work was supported by grants to Andrew Foreman from the Garnett Passe and Rodney Williams Memorial

Foundation and to Claus Bachert from the Flemish Scientific Research Board, FWO, Nr. A12/5-HB-KH3 and G.0436.04, and the Global Allergy and Asthma European Network. (GA^{2}LEN).

Conflict of interest

None to declare.

References

1. Fokkens W, Lund V, Mullol J. EP3OS 2007: European position paper on rhinosinusitis and nasal polyps 2007. A summary for otorhinolaryngologists. *Rhinology* 2007;**45**:97–101.
2. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA et al. Rhinosinusitis: developing guidance for clinical trials. *J Allergy Clin Immunol* 2006;**118**:S17–S61.
3. Bachert C, Gevaert P, Holtappels G, Cuvelier C, van Cauwenberge P. Nasal polyposis: from cytokines to growth. *Am J Rhinol* 2000;**14**:279–290.
4. Tripathi A, Kern R, Conley DB, Seiberling K, Klemens JC, Harris KE et al. Staphylococcal exotoxins and nasal polyposis: analysis of systemic and local responses. *Am J Rhinol* 2005;**19**:327–333.
5. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999;**284**:1318–1322.
6. Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy* 2006;**61**:1280–1289.
7. Van Bruaene N, Derycke L, Perez-Novo CA, Gevaert P, Holtappels G, De Ruyck N et al. TGF-beta signaling and collagen deposition in chronic rhinosinusitis. *J Allergy Clin Immunol* 2009;**124**:253–259.
8. Ebbens FA, Scadding GK, Badia L, Hellings PW, Jorissen M, Mullol J et al. Amphotericin B nasal lavages: not a solution for patients with chronic rhinosinusitis. *J Allergy Clin Immunol* 2006;**118**:1149–1156.
9. Van Bruaene N, Perez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N et al. T-cell regulation in chronic paranasal sinus disease. *J Allergy Clin Immunol* 2008;**121**:1435–1441, 1441 e1431–1433.
10. Bachert C, Zhang N, Holtappels G, De Lobel L, van Cauwenberge P, Liu S et al. Presence of IL-5 protein and IgE antibodies to staphylococcal enterotoxins in nasal polyps is associated with comorbid asthma. *J Allergy Clin Immunol* 2010;**126**:962–968, 968 e961–966.
11. Van Zele T, Gevaert P, Watelet JB, Claeys G, Holtappels G, Claeys C et al. *Staphylococcus aureus* colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. *J Allergy Clin Immunol* 2004;**114**:981–983.
12. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002;**15**:167–193.
13. Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. *ORL J Otorhinolaryngol Relat Spec* 2004;**66**:155–158.
14. Ferguson BJ, Stolz DB. Demonstration of biofilm in human bacterial chronic rhinosinusitis. *Am J Rhinol* 2005;**19**:452–457.
15. Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. *Laryngoscope* 2007;**117**:1302–1306.
16. Sanclement JA, Webster P, Thomas J, Ramadan HH. Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis. *Laryngoscope* 2005;**115**:578–582.
17. Foreman A, Psaltis AJ, Tan LW, Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. *Am J Rhinol Allergy* 2009;**23**:556–561.
18. Healy DY, Leid JG, Sanderson AR, Hunsaker DH. Biofilms with fungi in chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 2008;**138**:641–647.
19. Sanderson AR, Leid JG, Hunsaker D. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. *Laryngoscope* 2006;**116**:1121–1126.
20. Singhal D, Psaltis AJ, Foreman A, Wormald PJ. The impact of biofilms on outcomes after endoscopic sinus surgery. *Am J Rhinol Allergy* 2010;**24**:169–174.
21. Psaltis AJ, Weitzel EK, Ha KR, Wormald PJ. The effect of bacterial biofilms on post-sinus surgical outcomes. *Am J Rhinol* 2008;**22**:1–6.
22. Foreman A, Wormald P. Different biofilms, different disease? A clinical outcomes study *Laryngoscope* 2010;**120**:1301–1306.
23. Benninger MS, Ferguson BJ, Hadley JA, Hamilos DL, Jacobs M, Kennedy DW et al. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. *Otolaryngol Head Neck Surg* 2003;**129**:S1–S32.
24. Foreman A, Singhal D, Psaltis AJ, Wormald PJ. Targeted imaging modality selection for bacterial biofilms in chronic rhinosinusitis. *Laryngoscope* 2010;**120**:427–431.
25. Ha KR, Psaltis AJ, Tan L, Wormald PJ. A sheep model for the study of biofilms in rhinosinusitis. *Am J Rhinol* 2007;**21**:339–345.
26. Patou J, Gevaert P, Van Zele T, Holtappels G, van Cauwenberge P, Bachert C. *Staphylococcus aureus* enterotoxin B, protein A, and lipoteichoic acid stimulations in nasal polyps. *J Allergy Clin Immunol* 2008;**121**:110–115.
27. Plouin-Gaudon I, Clement S, Huggler E, Chaponnier C, François P, Lew D et al. Intracellular residency is frequently associated with recurrent *Staphylococcus aureus* rhinosinusitis. *Rhinology* 2006;**44**:249–254.
28. Corriveau MN, Zhang N, Holtappels G, Van Roy N, Bachert C. Detection of *Staphylococcus aureus* in nasal tissue with peptide nucleic acid-fluorescence in situ hybridization. *Am J Rhinol Allergy* 2009;**23**:461–465.
29. Sachse F, Becker K, von Eiff C, Metze D, Rudack C. *Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. *Allergy* 2010;**65**:1430–1437.
30. Seymour GJ, Gemmell E, Reinhardt RA, Eastcott J, Taubman MA. Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *J Periodontal Res* 1993;**28**:478–486.
31. Berglundh T, Donati M. Aspects of adaptive host response in periodontitis. *J Clin Periodontol* 2005;**32**(Suppl 6):87–107.
32. Cullinan MP, Hamlet SM, Westerman B, Palmer JE, Faddy MJ, Seymour GJ. Acquisition and loss of Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Prevotella intermedia over a 5-year period: effect of a triclosan/copolymer dentifrice. *J Clin Periodontol* 2003;**30**:532–541.
33. Hekiert AM, Kofonow JM, Doghramji L, Kennedy DW, Chiu AG, Palmer JN et al. Biofilms correlate with TH1 inflammation in the sinonasal tissue of patients with chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 2009;**141**:448–453.
34. Psaltis AJ, Wormald PJ, Ha KR, Tan LW. Reduced levels of lactoferrin in biofilm-associated chronic rhinosinusitis. *Laryngoscope* 2008;**118**:895–901.
35. Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J Immunol* 2008;**181**:3733–3739.
36. Brubaker RR. Interleukin-10 and inhibition of innate immunity to Yersiniae: roles of Yops and LcrV (V antigen). *Infect Immun* 2003;**71**:3673–3681.
37. Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L et al. Alternatively activated macrophages and impaired phagocytosis of S. aureus in chronic rhinosinusitis. *Allergy* 2011;**66**:396–403.