Asthma and nasal polyposis: a mechanistic paradigm in asthma

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The Nose and Sinuses in Respiratory Disorders

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Nasal polyposis and asthma: a mechanistic paradigm

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Nasal polyposis and asthma: a mechanistic paradigm

Nasal polypsis and late-onset asthma are characterized by a Th2 immune response, which, although mostly non-allergic, involves increased IgE levels. IgE antibodies found in these patients are predominantly antibodies to *Staphylococcus aureus* enterotoxins, but not inhalant allergens; their expression indicates more severe upper and lower airway disease and airway co-morbidity. *S. aureus* is a frequent colonizer of nasal polyps, but also has been shown to reside intra-mucosally, even intracellularly, and releases secreted proteins which have been demonstrated in the mucosa and correlate with type-2 cytokine production. Apart from enterotoxins, known for their superantigen activity, other secreted proteins such as serine protease-like proteins (Spl's) have been identified as allergens. The secreted proteins are able to bias the immune response to type-2 inflammation, which results in severe impairment of the epithelial barrier and innate immune functions, which consequently eases the survival of *S. aureus*. A better understanding of the role of this germ in airway disease highly warrented.

Key words: Nasal polyposis, CRSwNP, asthma, Staphylococcus aureus, enterotoxins, Spl's, IgE, epithelial barrier, EETs, DAMPs

Abbreviations

Interleukin	IL
Staphylococcus aureus	*S. aureus*
CRSwNP	chronic rhinosinusitis with nasal polyps (nasal polyposis)
Spl	serine protease like
SE	staphylococcal enterotoxin
PNA-FISH	peptide nucleic acid–fluorescence in situ hybridization
ILC2s	innate lymphocytes type-2
TSST-1	toxin shock syndrome toxin 1
RAG	recombination activating genes
DCs	dendritic cells
EETs	eosinophil extracellular traps
RAGE	receptor for advanced glycation end products
Tregs	T regulatory cells
TJ, AJC	tight junction, apical junctional complex
DAMP	Danger-associated molecular pattern

Introduction

The link between the upper and lower airways has long been of interest, derived from epidemiologic observations (1, 2), but mainly focused on allergic rhinitis and asthma. Local tissue factors such as the airway epithelium and microbial stimuli and infections, but also systemic inflammatory mechanisms have been suggested to play a role in the clinical expression of the united allergic airway syndrome (3, 4). Mechanistic observations on this link between the airways boosted the discussion further: When non-asthmatic allergic rhinitis patients were undergoing a segmental bronchial provocation, basophils from the peripheral blood were reduced in numbers, whereas they increased in the bronchi, but also in the nasal mucosa (5). Furthermore, serum interleukin (IL)-5 and blood eosinophils increased in allergic patients after challenge of any compartment of the airways (6), which locally upregulated adhesion molecules to attract those cells from the blood; this indicated that the bloodstream might link the different organs.

The finding that progenitor cells also could use the blood stream to reach active allergic areas of the airways furthermore introduced the bone marrow into the equation (7). The bone marrow was shown to release CD34(+) progenitor cells that finally differentiate into mast cells, basophils and eosinophils at the organ sites of the allergic reaction (8). Circulating CD34(+) cells expressed receptors for TSLP and IL-33, possibly derived from innate lymphocytes type-2 (ILC2s) and responded to these cytokines by rapidly releasing high levels of IL-5 and IL-13. Activated CD34(+) cells could be detected in the sputum of allergic asthmatics, and numbers increased in response to specific allergen inhalation. Thus, these cells distributing over the blood stream and attracted by the release of local chemokines are likely to contribute to the manifestation of allergic inflammation in different compartments of the airways. Although not specifically demonstrated for other type-2 inflammatory diseases of the airways such as nasal polyposis and late-onset athma, the same mechanisms very likely also apply here.

Recently, the available epidemiological observations on the so-called « asthma co-morbidity » in chronic rhinosinusitis (CRS) without (sNP) or with nasal polyposis (wNP) have been summarized (9); however, a differentiation between those two major phenotypes of CRS was insufficiently done. Today, asthma comorbidity in CRSwNP patients has been reported be as low as 7% (e.g. in parts of China) to as high as 45% and more (in Europe, the US, Australia), in parallel with the expression of a type-2 inflammatory inflammation within the polyp tissue (10). In Europe, there

is a clear association between asthma and (not further defined) CRS, as shown in the Global Allergy and Asthma Network of Excellence (GA2LEN) study involving 52000 subjects aged 18–75 years. A postal questionnaire in representative samples of adults living in Europe was used to estimate CRS prevalence as 11.9% of the European population; furthermore, there was a strong association of asthma with CRS at all ages. CRS in the absence of nasal allergies was positively associated with late-onset asthma (11). Of great interest, we also observed this phenomenon in a recent europe-wide GA2LEN sinusitis cohort study (unpublished); again, nasal polyposis patients suffered typically from late-onset often non-allergic asthma, with either of them diagnosed first, whereas allergic rhinitis subjects suffered from early-onset allergic asthma. However, also early onset asthma and allergy can be observed in CRSwNP, but rather as an independent disease, not impacting on the inflammatory profile of the upper airways.

The question therefore arises what else but inhalant allergens may drive this comorbidity of CRSwNP with asthma, obviously able to induce even higher amounts of mucosal IgE and an often severe eosinophilic "type-2" inflammation?

Staphylococcus aureus left its signature in CRSwNP and asthma

With the support of Gunnar Johansson, who developed a specific tool to discover IgE to several *Staphylococcus aureus* enterotoxins (SE-IgEs), we investigated a small group of clinical CRSwNP samples and found SE-IgEs in about half of the patients (12). The SE-IgE positive group had high total IgE concentrations in the tissue, a severe eosinophilic inflammatory reaction, and frequent asthma co-morbidity. From this first observation in airway disease to the hypothesis of *S. aureus* as initiator of severe inflammation in CRSwNP (13), a wide range of epidemiologic as well as mechanistic studies have been performed by us and others (14), confirming the potential causal role of *S. aureus* and its secreted proteins not only in CRSwNP, but also in asthma.

Colonisation and intramucosal presence of the germ and its secreted proteins

It was observed early that nasal polyps were frequently colonized by S. aureus (15), whereas this was not obvious for the bronchi in asthma; however, in the upper as well as the lower airways, it was noted that macrophages were insufficiently clearing the airways from *S. aureus* (16, 17). In

an unpublished bronchial biopsy study (P. Howarth, Southampton), we observed macrophages with intracellular *S. aureus* in healthy, but extracellular bio-film forming germs in severe asthma subjects. Of note, it does take only a few *S. aureus* germs to induce and maintain an immune reaction, as we will discuss later.

S. aureus has repeatedly been found intramucosally, even intracellularly in macrophages, mast cells and epithelial cells in CRSwNP, but not other tissues (18-20); we recently also succeeded in demonstrating that secreted proteins from the immune proteom of *S. aureus* can be fould in CRSwNP tissues (21). In total, more than 600 *S. aureus* proteins were identified by high resolution mass spectrometry or multiple reaction monitoring in nasal polyp tissues directly collected from the patients. For 115 *S. aureus* proteins, IgA- and IgG specific antibody signals were also profiled. Strong antibody signals were predominantly found for surface or secreted proteins, among those were also antibodies against superantigens such as SEA, SEB, SEC, TSST-1, SEK and others. It is obvious that the local immune response in this chronic inflammatory reaction does not eliminate intra-cellular persisting *S. aureus* nor does it abolish *S. aureus* carriage. The antibodies will exert their biological effector functions upon binding to their specific bacterial antigens, which will induce proinflammatory processes such as complement activation; evidence for that has been provided (22). Moreover, local T cell responses may be triggered by superantigens and other antigens leading to cytokine release of Th1/Th17 but also Th2 cells, depending on the nature of the antigen and the predisposition of the host. In fact, the frequency of carriage is strongly increased in nasal polyp patients, as discussed before (15). It therefore is likely that *S. aureus* and the innate and adaptive immune responses directed against it contribute to the chronic recalcitrant inflammation of the airways. This notion is supported by the strong relationship between the presence of IgE-immune responses to staphylococcal proteins and the severity of disease in upper and also lower airway diseases. Similarly, for atopic dermatitis, *S. aureus* density on lesional vs. nonlesional skin appears to be associated with disease severity (23).

S. aureus drives IL-5 production in polyp tissue

In a further step to demonstrate that *S. aureus* residing within nasal polyp tissue in CRSwNP patients does impact inflammation, we have analyzed CRSwNP tissue freshly obtained during routine surgery, which did or did not contain cultivatable *S. aureus*, and studied the spontaneous

IL-5 production by nasal polyp tissue over 24 and 72h in tissue culture. In *S. aureus*-positive samples we interfered by killing the bacteria using antibiotics or *S. aureus* specific ISP bacteriophages, active or heat-inactivated. Finally, phage-neutralizing antibodies were used to demonstrate the specificity of the phage-mediated effects. We monitored the number of colony-forming units of *S. aureus* and identified secreted *S. aureus* proteins by mass spectrometry (24). For the first time we demonstrated that cultivatable *S. aureus* may be found in type-2 inflamed nasal polyps; the pathogen is replicating within 24h and secretes proteins, including superantigens and serine protease like proteins (see later). The presence of *S. aureus* was associated with a significantly higher spontaneous release of IL-5. Killing of *S. aureus* by antibiotics or specific bacteriophages significantly reduced or abolished the IL-5 release. The suppressive activity of the bacteriophage on IL-5 production could be abolished by heat inactivation or anti-phage antibodies, demonstrating the specificity of the effect of the bacteriophage. These experiments undoubtly indicate a direct role of *S. aureus* in the regulation of IL-5 production in nasal polyps and suggest the involvement of bacterial proteins detected in the tissues.

Classical superantigens and IgE formation

The classical superantigens released from the intra-mucosal germs are known to induce a "cytokine storm"; however, as they are released in low quantities, they contribute to the chronic inflammation, but do not induce a shock as it has been described with nasal or vaginal tamponage. Exposing normal nasal mucosa, but specifically nasal polyp tissue, to classical superantigens elicits a strong cytokine production including Th1, Th17 and Th2 cytokines (25). Specifically IL-4 and IL-13 may contribute to the strong polyclonal IgE formation that is observed in nasal polyps; extra-nodal lymph follicles have been demonstrated in nasal polyp tissues, as well as all necessary ingredients to locally switch to IgE formation such as AID, BAFF and recombination activating genes RAG1 and RAG2 (26-29). Of interest, switch circle transcripts revealed ongoing local class switch recombination to IgE, and the expression of RAG1 and -2, required for receptor revision, correlated with the magnitude of inflammation and the presence of SE-IgE in the nasal polyp mucosa (29). It recently has been elegantly demonstrated that indeed superantigens can activate basophils (and mast cells) by simultaneously binding as antigens in the conventional manner and as superantigens to framework regions of

anti-SEE IgE in anti-SEE IgE-FcεRI complexes (30). SEB-IgE as well as allergen-specific IgE present in the polyp tissue is functional and thus contributes to the chronic activation of mast cells (31). The presence of SE-IgE in tissue or serum furthermore may serve as proof for the former contact of the patient with *S. aureus* enterotoxins, and may therefore be used as a marker of interaction with superantigens and possibly severe inflammation. As diagnostic tool, we and others have therefore used it in airway diseases.

SE-IgE as a diagnostic marker in tissue and serum

With the first description of the SE-IgE CAP used in tissues of nasal polyp patients (12), a tool was created that allowed studies in various populations of airway disease patients in tissues and sera.

SE-IgE in nasal polyp tissue predicts asthma

In nasal polyp tissues from 70 European and 93 Chinese patients, 34% and 8% with comorbid asthma, possible biomarkers of asthma comorbidity were analyzed in nasal polyp tissues (32). A classification tree evaluation identified IL-5 as the main positive determinant; 83% of the European and 16% of the Chinese polyp tissues expressed the type-2 cytokine. SE-IgE in tissue was associated with significantly increased total IgE and eosinophil cationic protein concentrations in both populations. Expression of SE-IgE within the tissue; total tissue IgE of greater than 1,440 kU/L; and eosinophil cationic protein of greater than 17,109 mg/L in samples with a total IgE concentration of greater than 246 kU/L significantly predicted asthma (odds ratio 5.8-13). SE-IgE was not only a marker for severe inflammation in nasal polyps, it also predicted asthma comorbidity in CRSwNP subjects and could serve as a link between the upper and lower airway diseases. These findings were in agreement with the findings of a bronchial and nasal biopsies study, demonstrating significantly higher concentrations of Th2 cytokines in nasal polyps compared to inferior turbinate and bronchial biopsies (33). At the same time, the inflammatory profile of nasal polyps and bronchial biopsies correlated significantly ($p<0.01$), indicating that the sinuses may control the inflammation in the entire airways.

SE-IgE in asthma cohortsand asthma severity

Making use of adult cohorts of 69 control subjects, 152 patients with nonsevere asthma, and 166 patients with severe asthma (defined as inadequately controlled disease despite high-dose inhaled corticosteroids plus at least 2 other controller therapies, including oral steroids) were analysed for blood biomarkers (34). Serum SE-IgE positivity was significantly greater in patients with severe asthma (59.6%) than in healthy control subjects (13%, P < .001). Logistic regression analyses demonstrated significantly increased risks for SE-IgE positive subjects to have any asthma (OR, 7.25; 95% CI, 2.7-19.1) or severe asthma (OR, 11.09; 95% CI, 4.1-29.6) versus SE-IgE negative subjects. The presence of grass pollen or house dust mite IgE antibodies was not associated with either risk. Oral steroid use and hospitalizations were significantly increased in patients with SE-IgE and in nonatopic asthma subjects. SE-IgE was also associated with a lower long function measured as FEV1 percent predicted value. SE-IgE, but not IgE against inhalant allergens, was thus identified as risk factor for asthma severity in both, the allergic and the non-allergic populations. Again, the presence of SE-IgE in serum indicated the involvement of staphylococcal superantigens in the pathophysiology of severe airway disease.

The GA2LEN survey in Europe described above (11) was used again to study a random sample of 2908 subjects by skin prick tests for common aeroallergens and blood total IgE and SE-IgE measurements (35). Prevalence of positive SE-IgE was 29% and more common in smokers (<15 pack-year: OR 1.11, P = 0.079, ≥15 pack-year: OR 1.70, P < 0.001). SE-IgE was associated with asthma (OR 2.10, P = 0.001) in a concentration-dependent manner. This study for the first time demonstrated that SE-IgE was significantly and independently associated with asthma in the European population.

Similar findings were made in Korean urban and rural populations (36) consisting of 1080 adults (mean age 60.2 years). Positive SE-IgE had a prevalence of 27.0% in serum, risk factors were identified as male sex, current smoking, advanced age (≥ 61 years), and inhalant allergen sensitization. Current asthma was mostly adult onset (≥ 18 years old) and showed independent associations with high SE-IgE levels in multivariate logistic regression tests. These findings were confirmed in an independent even older population of 249 patients with late-onset asthma and 98 controls above the age of 65 years (37). Elderly asthma patients with high SE-IgE levels were characterized by more severe asthma, sputum eosinophilia and CRS, compared to those with lower SE-IgE levels. The association between serum SE-IgE concentrations and severe asthma was significant and independent of covariables in multivariate logistic regression analysis

(odds ratio 7.47, P = 0.005) in SE-IgE-high (≥ 0.35 kU/L) vs. negative (< 0.10 kU/L) patients). Multiple correspondence analyses also showed that high serum SE-IgE level had close relationships with severe asthma, CRS and sputum eosinophilia. This paper prompted an Editorial by K.F.Chung, Imperial College London, entitled ”Staphylococcal enterotoxin-specific IgE: a biomarker for a distinct phenotype of severe asthma?” which indicated the possible role of SE-IgE as a predictor of response to Omalizumab in non-atopic late-onset asthmatics, which needs further study (38). As mentioned above, SE-IgE was identified as risk factor for asthma severity in both, the allergic and the non-allergic populations (34), and these patients are likely of responding well to Omalizumab (39).

S. aureus is an active immune modulator to type-2 inflammation

S. aureus is a versatile germ frequently found colonizing patients with type-2 biased diseases such as atopic dermatitis and CRSwNP. Compelling evidence exists showing that *S. aureus* enterotoxins act as modifying agents in the inflammatory response in upper and lower airway diseases (40, 41). *S. aureus* actively manipulates the host immune response by releasing proteins that facilitate bacterial invasion, colonization, and have immunomodulatory properties (Figure 1) (42, 43). As *S. aureus* has been identified as a major cofactor in atopic disease, it is important to understand the interplay between *S. aureus* and its products with the immune response of the host and elucidate its role in the initiation and persistence of chronic airway disease.

Spls induce type 2 immune response

Six *S. aureus* protease-like proteins (A-F) (abbreviated Spls) belong to a small subfamily S1B, encompassing staphylococcal V8 protease, epidermolytic toxins, and Spl proteases. They share high sequence similarity and contain putative 35- 36-amino-acid signal peptides (44). Eighty four percent of *S. aureus* strains contain at least one Spl-protease-encoding gene (45). Stentzel et al. demonstrated that healthy carriers have increased levels of IgG1 and IgG4 antibodies compared to all Spls in the blood (46). Importantly, the levels of IgE antibodies to all Spls, except for SplC, were significantly higher in sera of asthmatic patients compared to controls, demonstrating a strong link between the immune response to Spls and atopic status. SplD induces type 2 immune response with typical features of asthma such as airway hyperreactivity, goblet cells hyperplasia, Th2 cytokine production, and IgE antibody response in mice (47). Likewise, SplF, which has 95

% sequence homology with SplD, induces strong type 2 biased airway inflammation (47). Inhalation of allergens with proteolytic capacities such as house dust mite (HDM) leads to asthma via initiation of the TLR4 signaling pathway (48). In contrast to HDM-induced allergic asthma, SplD induces airway eosinophilia and specific IgE production in a TLR4-independent manner; the mechanisms of Spl's sensing are not entirely clear and need further investigation. The SplD-induced allergic features could be antagonized by soluble ST2 intratracheal treatment, suppressing the IL-33 mediated activation of eosinophils, innate lymphoid cells type 2, production of type 2 cytokine in the local lymph nodes, and reduced airway hyperreactivity, thereby attenuating allergic asthma symptoms in mice (47). In addition to aggravating allergic airway inflammation, SplD alters epithelial airway integrity, facilitating the access of other allergens and pollutants to the innate immune system.

It is important to understand the interplay between the immune proteome of *S. aureus* and the immune response of the host and elucidate its role in the initiation and persistence of chronic airway diseases. Apart from acting as an allergen, SplD is capable of inducing specific IgE production to an inert protein, thus acting as an adjuvant responsible for the broadening of allergic immune response (47). This observation supports epidemiological studies that the exposure of some *S. aureus* secretome proteins, including enterotoxins or Spls concurrent with allergen exposure, could be a common factor leading to asthma susceptibility. A similar action was ascribed to *S. aureus* enterotoxins, which were shown to break tolerance to inert proteins and induce type 2 biased allergic immune response in mice (48, 49).

S. aureus-induced tolerogenic DCs and Tregs

Accumulating data suggests that *S. aureus* could produce immunomodulatory molecules promoting either immune tolerance (anti-inflammatory response) or pro-inflammatory responses. A pro-Th2 anti-inflammatory response provides a distinct advantage for *S. aureus* survival, thus facilitating bacterial persistence and chronicity of disease. The *S. aureus* secretome was shown to promote Th2 inflammation and inhibit the modulatory function of resident Tregs *in vitro*, while *S. epidermididis* was able to induce competent Treg production and induction of Th1 cytokines in the inflammation, therefore counteracting type 2 immune response (50). *S. aureus* phenol-soluble modulin peptide toxins induce tolerogenic DCs producing IL-10 with impaired capacity to induce activation and proliferation of CD4(+) T cells, while increasing the numbers of Tregs (51). *S.*

aureus enterotoxin B also induced DCs maturation and promoted Th2 polarization in vitro (52). Along with the generation of suppressive antigen-presenting cells, *S. aureus* secreted proteins, such as *S. aureus* enterotoxin A possesses the ability to convert neonatal conventional T cells into Tregs and to support Th2 polarization (53-55). Furthermore, S. *aureus*-derived lipoteichoic acid supresses T cell cytokine production (56).

It has been estimated that the *S. aureus*-specific T-cell pool may comprise up to 3.6% of T-cells in healthy individuals (57). *S.aureus*-specific T cells produce Th1, Th17, and Th2 cytokines upon *S. aureus* antigen re-stimulation, therefore affecting the course of *S. aureus* infection. Several *S. aureus* virulence factors such as δ-toxin, delta-hemolysin (Hld), and phenol soluble modulins (PSMs) PSMα1 and PSMα3 promote type 2 immune responses by inducing a dose-dependent activation of mast cells and exacerbating allergic disease (58-60). Mast cells in the nasal mucosa of CRSwNP patients were shown to contain intracellular *S.aureus*, therefore protecting the bacteria from antimicrobial actions and establishing an intracellular reservoir that contributed to the chronic carriage in CRSwNP (20). These findings provide new insights on the role of the mucosal microbime in the airways, and its interplay with mucosal immune cells, which could modulate the inflammatory response towards type-2 biased inflammation.

Eosinophil extracellular traps (EETs) in response to *S. aureus*

Eosinophils are abundantly present in Th2 biased upper airway pathologies such as asthma and CRSwNP. They are terminally differentiated granulocytes, playing a role in innate host defense against pathogens through the release of toxic granule proteins and reactive oxygen species. By the release of their toxic contents, they can damage both pathogenic and host cells. For this reason, eosinophils have long been considered as harmful cells and, unsurprisingly, their presence in tissue or airway secretions is positively associated with the severity of both asthma and CRSwNP (61, 62). Beside their well-known cytotoxic activities, it is now clear that they play a role in both innate and adaptive immunity via different effector mechanisms. In addition, eosinophils can contribute to antibacterial defense by releasing DNA in association with granule proteins. These so called eosinophil extracellular traps (EETs) are able to bind and kill bacteria (63). *In vitro*, eosinophils are triggered to form EETs after stimulation with IL-5, TSLP, eotaxin and C5a (64). Interestingly, co-culture of eosinophils with *S. aureus* causes a rapid generation of EETs in the absence of any additional stimuli. Under these *in vitro* conditions, EETs are able to

entrap *S. aureus* and inhibit its growth *in vitro* (63, 65). Moreover, the exposure of eosinophils to TSLP, reported as an important regulator in both CRSwNP and asthma pathogenesis, also results in the release of EETs *in vitro.* TSLP-evoked EET generation seems to be dependent on CD18 and CD11b adhesion (65, 66).

The presence of extracellular nucleases as a defense strategy against DNA traps in several pathogenic bacteria, including *S. aureus, Clostridium perfringens*, and *S. pyogenes* provides further evidence for the pathophysiological relevance of EETs in several pathogenic conditions. In addition, a streptococcal DNase has previously been implicated in disease progression (67). The presence of EETs has been shown in multiple eosinophilic diseases, including secretions from eosinophilic asthma and CRSwNP patients (68, 69). Recently EETs were shown to contribute to the properties of highly viscous eosinophilic mucin and impairment of its clearance in chronic rhinosinusitis; similar viscous mucin is also typical for asthma (69, 70). We recently demonstrated presence of EETs targeting *S. aureus* at the epithelial layer, esp. at epithelial barrier defects in CRSwNP; EETs can also be found above the epithelium within nasal secretions (71). When eosinophils are brushed from the epithelium and exposed to S. aureus, they rigorously release EETs (Figure 2). In nasal polyps, we showed a positive correlation between EETs and periostin in the tissue, the involvement of reactive oxygen species in EET generation *in situ*, the active migration of eosinophils towards and the entrapment of *S. aureus* in situ within a 2 hour time frame (71). These findings yield new insights into the possible role of EETs, and by extension of eosinophils, in human airways, and link *S. aureus* to eosinophilic airway disease such as CRSwNP and co-morbid asthma. In conclusion, the formation of these EETs could be viewed as a double edged sword providing an important mechanism of host immunity against infections and simultaneously causing damage to cells and tissue.

Damage associated molecular patterns and their dysregulation in the airways

It became recently apparent that some endogenous intracellular and/or extracellular matrix components from the host can be released into the extracellular space, subsequently gaining pro-inflammatory functions contrasting their regulatory functions during normal physiology. In analogy to “Pathogen Associated Molecular Patterns” (PAMPs) originating frm outside, these host-self molecules are collectively classified as “Damage Associated Molecular Patterns” (DAMPs) (72). DAMPs often execute their effect via activation of one or more “Toll-like

receptors" (TLRs) but also via other members of the pattern recognition receptor (PRR) family including "scavenger receptors" (eg CD36) and/or specialized receptors such as "the receptor for advanced glycation end products" (RAGE) (73).

A group of molecules possessing the characteristics of DAMPs are β-defensins. These are small antimicrobial peptides produced in response to microbial infection of mucosal tissues and skin (74); β-defensins may also modulate the functional responses of inflammatory cells. Indeed, β-defensin 2 is reported to activate dendritic cells via binding to TLR4 (75), and β-defensin 3 similarly can activate the professional antigen-presenting DC's and monocytes by interacting with TLR1 and -2 (76). Cultured sinonasal epithelial cells show decreased expression of β-defensin 2 compared to control subjects (77). These levels were further reduced in the presence of the Th2 cytokines IL-4 or IL-13 while the TLR9 ligand CpG DNA increased the levels of β-defensin 2 after the upregulation of TLR9 expression by the Th1 cytokine IFN-γ. The low β-defensin 2 levels in nasal polyp tissues are therefore in agreement with CRSwNP being a Th2-skewed disease for which reduced (78) or absent (79) expression of TLR9 was reported. Reduced levels of β-defensin 2 and -3 would result in reduced antimicrobial activity against known pathogens such as *S. aureus* (80) which play an important role in the pathophysiology of CRSwNP.

The calcium-binding proteins S100A7 (psoriasin), S100A8 (MRP8 or calgranulin A), S100A9 (MRP14 or calgranulin B), and the latters' heterodimeric form S100A8/A9 (calprotectin) are reported to have antimicrobial effects (81-83). S100 proteins also show the typical activities of DAMPs when released to the extracellular milieu; they activate TLR4 (84, 85) and RAGE (86). The inflammatory and remodeling characteristics allow increased retention of S100A8, S100A9 and S100A8/A9 proteins in the extracellular matrix. Upon release, the S100 proteins act as a local danger signal inducing inflammatory mediators, predominantly via TLR-4 activation (84).

Similar to the lungs (87), RAGE is expressed in the human upper airways under normal physiologic conditions, but dysregulated in chronic inflammatory conditions; in CRSwNP, both tissue soluble sRAGE and membrane bound mRAGE protein levels are reduced (88). Reduced levels of mRAGE protein are associated with an increased proteolytic cleavage of the full length mRAGE protein which is converted to sRAGE. An imbalance between MMP's and their natural "tissue inhibitors of metalloproteinases (TIMPs)" was previously reported to occur in CRSwNP (89), resulting in increased proteolytic cleavage. Finally, an increased colonization rate of *S.*

aureus in the airways (90) may further contribute to the overall reduction in sRAGE, as *S. aureus* induces the release of sRAGE from the ECM (88). High levels of eosinophil cationic protein (ECP) will break non-ECM associated sRAGE down, further reducing the levels. As RAGE is involved in the differentiation of T cells towards a Th1 phenotype, and RAGE deficiency decreases production of Th1-cytokines while increasing the production of Th2 cytokines (91), its deficit will prevent the redirection of T helper cells into the Th1 type. This has been confirmed in ex-vivo experiments showing that treatment with recombinant sRAGE significantly increased the release of IL-1ß and TNF-a, but decreased the levels of IL-5 in type-2 biased tissue (84).

The epithelial barrier function and its impairment in Th2 inflammation

Epithelial cells lining the airway mucosa forms not only a physical and chemical barrier to the outside world but also functions as an interface between innate and adaptive immune responses to the abundant particles, microbes, viruses and allergens that are inhaled throughout life.

In conjunction with submucosal glands, the secretory goblet cells produce mucus, a fluid containing hydrated gel-forming mucins and a range of host defense and cytoprotective molecules, including antimicrobial molecules, antiproteases, and antioxidants that act as a "chemical" barrier (92). The properties of the mucus are dictated in large part by the oligomeric secreted mucins MUC5AC and MUC5B, multifunctional glycoproteins that provide the structural framework of the mucous barrier (93). The mucus traps and inactivates inhaled agents and facilitates their clearance via the mucociliary escalator. It is a testament to the effectiveness of the chemical and physical barriers of the bronchial epithelium that most environmental challenges are largely overcome without the need to develop an inflammatory response. This is well illustrated in recent studies on the importance of Muc5b for defending the airways and for maintaining immune homeostasis. Muc5b deficiency caused accumulation of materials in upper and lower airways and led to chronic infection by multiple bacterial species, including Staphylococcus aureus, and to inflammation that failed to resolve normally (94). An impaired mucociliary clearance is reported in both CRSwNP and AR and might cause an accumulation of antigens contributing to inflammation (95). This impairment mostly is acquired as a result of inflammatory or environmental stimuli such as allergens and microbial products (95).

The function of the respiratory mucosa barrier is largely regulated by the apical junctional complex between neighboring epithelial cells. Apical junctional complexes consist of tight and adherens junctions and link to the cellular cytoskeleton via numerous adaptor proteins (96). Accumulating evidence indicates that epithelial permeability or barrier dysfunction is a hallmark of airway inflammation and mucosal immunity. Leaky epithelium may result in greater penetration of inhaled allergens, bacteria, and viruses in the subepithelial space, facilitating antigen uptake and activating epithelial signal transduction and innate and adaptive immune responses. Inflammatory cytokines are known to disrupt barrier function and AJC's in intestinal epithelial cells. For example, both Th1 (IFN-γ), Th2 (IL-13) and innate immune (TNF-α) cytokines can disrupt the structure and function of intestinal epithelial AJC's via distinct mechanisms (97) IL-4 disrupts the airway epithelial barrier by altering normal structure of epithelial AJs and TJs via mechanisms not involving changes in junction protein expression (98). CRSwNP patients have a defective epithelial barrier mainly caused by secreted pro-inflammatory cytokines, such as IFN-c, IL-4, and oncostatin M (99).

Allergens such as house dust mite, pollen grains, and fungi with cysteine proteinase activity, but likely also staphylococcal proteins with enzymatic activity, can degrade occludin and directly disrupt the TJ integrity (100). Endogenous protease inhibitors cystatin A and SPINK5 have been shown in nasal and bronchial epithelial cells; they serve to protect the airway epithelium from exogenous proteases (101). Compared with controls and patients with non-eosinophilic CRS, patients with eosinophilic CRS showed significantly lower protein and mRNA expression of cystatin A and SPINK5 in the nasal epithelium. Allergen-induced production of IL-25, IL-33, and TSLP in normal human bronchial epithelial cells, favoring type-2 inflammation, was inhibited by treatment with recombinant cystatin A or SPINK5, preventing the protease activity of the allergens (101).

The response of the nasal epithelium to bacteria and viruses has been described to be impaired, inducing an inadequate cytokine and interferon release (102) and allowing the invasion of pathogens into the subepithelial space. When healthy mucosa is exposed to frequent viral intruders such as human rhinovirus or herpes simplex virus, a rigorous response from the epithelium with release of IFN-c and IL-17 can be expected within 24–72 h. However, in a Th2-biased disease such as CRSwNP, this response is largely lacking; instead, significantly more pro-inflammatory cytokines including IL-1b and TNFa are released (102). These findings were

associated with a significantly higher viral invasion at 48 and 72 h in CRSwNP compared to healthy mucosa, indicating a functional deficit in the defense against viruses. IL-4 and IL-13, through inhibition of TLR-3 expression and signaling (IRF3), may impair this immune response to viral infection (103). IL-4 and IL-13 were induced by rhinovirus in the asthmatic airway in vivo and relate to exacerbation severity; this induction was entirely dependent on IL-33 release from epithelial cells (104). IL-4 and IL-13-induced barrier dysfunction was accompanied by reduced expression of membrane AJC components. This study indicates that IL-4 and IL-13 have disruptive effect on airway epithelial barrier function. Th2-cytokine induced epithelial barrier dysfunction may contribute to airway inflammation in allergic asthma.

Also impacted by Th2 cytokines, macrophages are alternatively activated, which renders them inadequate to phagocytize and kill bacteria, once invaded into the mucosa (105). Bacteria are frequently cultured from samples of severe asthma patients suggesting a defect in bacterial clearance from the airway. Persistence of bacteria in the lower airway may result partly from a reduced phagocytic capacity of macrophages for bacteria (17). Macrophages produce a variety of cytokines and mediators that are vital for immune and inflammatory responses in their response with external agents. It has been shown that there was a reduction in phagocytosis of *H. influenzae* and *S. aureus* by monocyte-derived macrophages from patients with severe asthma (104). We found a significantly lower percentage of phagocytosis of *S. aureus* by CD206+ cells in CRSwNP as compared to control inferior turbinate tissue. The clearance of non-opsonized bacteria by macrophages depends on their innate immune receptors for detection, binding and internalization of pathogens (105). In summary, we propose that the increased presence of *S. aureus* and other germs in CRSwNP can be partly explained by inefficiency of the phagocytic system in the sino- mucosal tissue of these patients. In line with our study, defects in phagocytosis of bacteria and apoptotic cells and increased bacterial colonization have been described for several lower airway pathologies, including asthma, cystic fibrosis and chronic obstructive pulmonary disease (106).

Conclusion

In the majority of patients, inflammation in the upper and the lower airways is characterized by a type-2 inflammation. Presence of this type of inflammation increases the risk for co-morbidity in the other part of the airways. In both airways, there also is severe inflammatory disease, with the

expression of SE-IgE as a hallmark. There is accumulating evidence that SE-IgE antibodies can be used as a biomarker for severe airway disease. It is yet to clarify whether *S. aureus* profits from the type-2 inflammation and its consequences such as impairment of the epithelial barrier, innate and adaptive immunity and secondarily modifies a pre-existing inflammation, aggravating the disease, or whether it may be causal in inducing an inflammation leading to disease under certain circumstances. It is clear today that *S. aureus* can reside intramucosaly, release secreted proteins into the mucosa, and by this regulates Th2 cytokines. It also is clear that eosinophils in airway disease do have a job to do defending the airways against germs such as *S. aureus* by EETs, apparantly after other defense mechanisms failed. Type-2 inflammation, impairment of epithelial defense mechanisms, eosinophils and EETs, and finally *S. aureus* and its products unite the airways in disease. It remains to clarify which targets are finally optimal to treat severe airway disease, the restauration of the epithelial barrier and immune defense, the suppression of adaptive immune regulators such as IL-5, IL-4, IL-13 or IgE and eosinophils, the germ and its products or a combination thereof.

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Figures:

Figure 1: **Possible strategies of *S. aureus* to affect type 2-biased inflammation.** Several secreted components of *S. aureus* may affect type 2 inflammation. This includes induction of airway hyperreactivity, stimulation of type 2 cytokine production by airway epithelium, Th2 cells and innate lymphoid cells type 2 (ILC2s), production of inmmunoglobulin E (IgE) and degranulation of mast cells, induction of tolerogenic dendritic cell subtypes (DCs) and T regulatory cells. *S. aureus* protease like proteins (Spls).

Figure 2: **EET formation upon S. aureus exposure.** DNA stain with DAPI showing an eosinophil (EOS) from a CRSwNP patient responding with massive EET formation (indicated by dashed lines) to S. aureus (some germs indicated by *) in vitro. (Scale bar = 5 µm)

SpIs

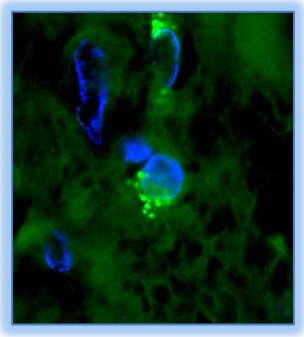
- IL-33
- Th2 and ILC2s cells activation
- IgE production
- AHR

Superantigens

- Th2 cells activation
- IgE production
- AHR
- tolerogenic DCs subtypes

α-hemolysin

- Cell death
- Epithelial barrier integrity



S. aureus

δ-toxin

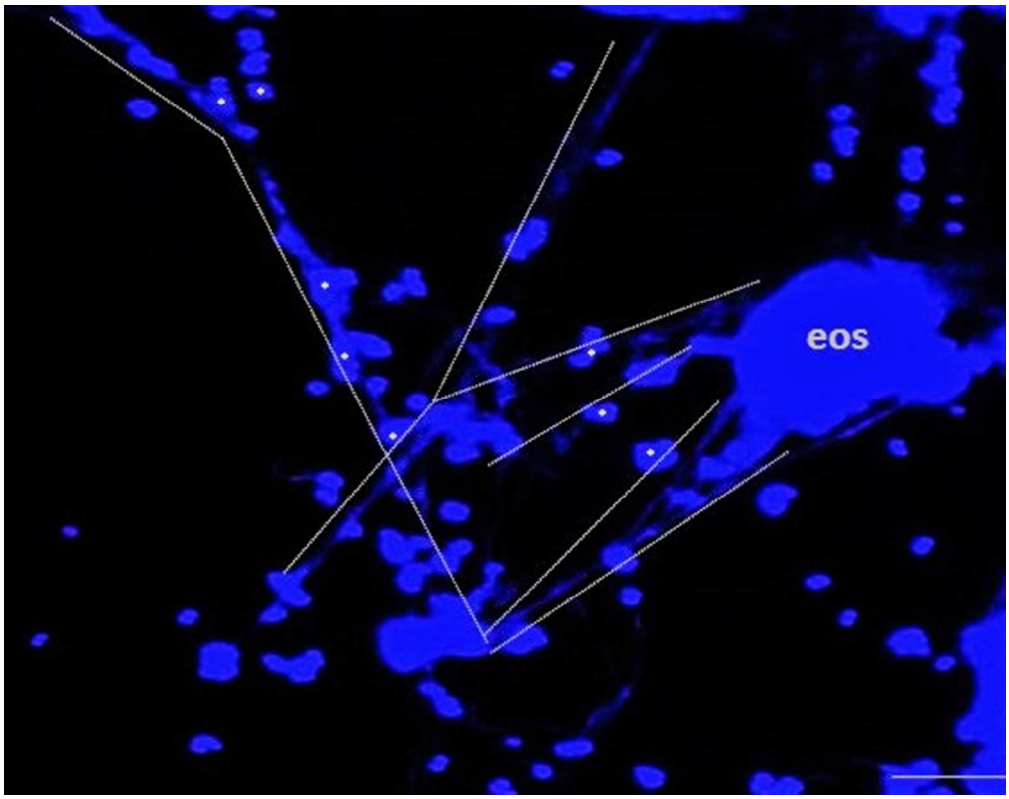
- mast cells degranulation
- IgE production

Protein A

- decreased phagocytosis
- B-cell apoptosis

Phenol-soluble modulins

- tolerogenic DCs subtypes
- T regulatory cells



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