ROLE OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN B

IN THE AGGRAVATION OF AIRWAY INFLAMMATION

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Cover picture: Ribbon diagram of the SEB-TCR complex - ‘Artist’s impression’ by Herwig Beyaert

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LIST OF PUBLICATIONS

This thesis is based on the following articles accepted or published in international peer reviewed journals:


LIST OF ABBREVIATIONS

ACTB beta-actin
AIAR aspirin intolerant asthma/rhinitis
APC antigen-presenting cell
ASRS aspirin sensitive rhinosinusitis
BAL bronchoalveolar lavage
BHR bronchial hyperreactivity
CRSsNP chronic rhinosinusitis without nasal polyposis
CRSwNP chronic rhinosinusitis with nasal polyposis
COX cyclooxygenase
COPD chronic obstructive pulmonary disease
CS cigarette smoke
DC dendritic cell
ECP eosinophil cationic protein
ELISA enzyme-linked immunosorbent assay
ESS endoscopic sinus surgery
FoxP3 forkhead box P3
GCP-2 granulocyte chemotactic protein 2
G-CSF granulocyte colony stimulating factor
HBD2 human beta defensin 2
HLA human leukocyte antigen
HNEC human nasal epithelial cell
i.t. intratracheal
IHC immunohistochemistry
IL interleukin
IP-10 interferon-inducible protein-10
KC keratinocyte chemoattractant
kDa kiloDalton
LN lymph node
LPS Lipopolysaccharide
MCP-1 monocyte chemotactic protein-1
MHC major histocompatibility complex
MIG monokine induced by interferon-γ
MIP-3α macrophage inflammatory protein-3 alpha
MLN mediastinal lymph node
MMR macrophage mannose receptor
NSAID non-steroidal anti-inflammatory drug
OVA ovalbumin
RANTES regulated upon activation, normal T cell expressed and secreted
RORe orphan nuclear receptor RORgammat
S. aureus Staphylococcus aureus
SAE Staphylococcus aureus Enterotoxin
SAg superantigen
SE staphylococcal enterotoxin
SEB Staphylococcus aureus enterotoxin B
TCR T cell receptor
TGF-β1 transforming growth factor beta 1
Th T helper
TLR toll-like receptor
TNF-α tumor necrosis factor-alpha
Treg T regulatory cell
TSST-1 toxic shock syndrome toxin-1
**SUMMARY**

*Staphylococcus aureus* is a common human pathogen, which is regularly part of the normal microflora found in the nose and skin. It represents a significant threat to human health, not in the least because of its capability to produce exotoxins, which have superantigenic properties. These exotoxins, in particular the staphylococcal enterotoxins (SEs), are involved in the aggravation of airway inflammation. Indeed, recent studies show an important impact of SEs on the natural course of allergic rhinitis, nasal polyposis, asthma and COPD. This thesis started from these observations in human airway disease, and we aimed to confirm the hypothesis that SEs significantly contribute to the modulation and aggravation of airway inflammation.

In the first model, we have demonstrated that concomitant airway exposure to allergen ovalbumin and *Staphylococcus aureus* enterotoxin B (SEB) has lead to sensitization to inhaled allergen, in a mouse model of experimental asthma. SEB was able to overcome the primary tolerance to ovalbumin (OVA) allergen, leading to OVA-specific IgE production, increased dendritic cell maturation and migration to the draining lymph nodes, as well as an increased allergen-specific T cell proliferation. Moreover, bronchial inflammation with influx of eosinophils and lymphocytes was demonstrated in OVA/SEB mice, together with bronchial hyperresponsiveness and production of IL-4, IL-5, IL-10 and IL-13 by draining lymph nodes stimulated with OVA. Interestingly, the sole application of either OVA or SEB did not lead to significant airway disease. This novel model of experimental asthma, using airway exposure of allergen and SEB closely mimics the human situation of allergic sensitization, and confirms that staphylococcal enterotoxins play an important role in this process.

We have demonstrated in this thesis that SEB exerts also a direct pro-inflammatory effect on epithelial cells, besides the above described effects on dendritic cells and T cells. In a novel model of freshly isolated nasal epithelial cells, SEB was able to induce a significant release of chemokines like IP-10, MIG, RANTES, MCP-1 and G-CSF. These chemokines augmented the granulocyte migration and survival, indicating an important role for the epithelium in the induction and orchestration of SE-induced and granulocyte-dominated airway inflammation.
The disease-modifying effects of SEB are also demonstrated in this thesis in a different inflammatory background, such as cigarette smoke-induced airway inflammation. In a mouse model of subacute and chronic CS exposure, we have demonstrated a clearly different regulation of airway inflammation in upper versus lower airways, in particular upon chronic CS exposure. CS-induced inflammation in upper airways appeared to be adequately suppressed, in particular compared to lower airway inflammation, which was clearly present and ongoing. Furthermore, we evaluated in this model the effect of combined CS and SEB exposure. Interestingly, CS/SEB mice displayed a raised number of lymphocytes and neutrophils in BAL fluid, as well as increased numbers of CD8+ T lymphocytes and granulocytes in lung tissue, compared to sole CS or SEB exposure. Moreover, concomitant CS/SEB exposure induced both IL-13 mRNA expression in lungs and goblet cell hyperplasia in the airway wall. In addition, combined CS/SEB exposure stimulated the formation of dense, organized aggregates of B and T lymphocytes in lungs, as well as significant higher CXCL-13 (protein, mRNA) and CCL19 (mRNA) levels in lungs. These findings confirm the hypothesis derived from human observations, which suggest that S. aureus could influence the pathogenesis of COPD.

In summary, we have demonstrated that Staphylococcus aureus enterotoxin B is a potent inducer and modulator of airway inflammation. The findings described in this work further unravel the pathophysiology of airway inflammatory conditions, and may indicate new possible therapeutic strategies in their prevention and treatment.
SAMENVATTING

*Staphylococcus aureus* is een frequent voorkomend humaan pathogeen, dat regelmatig wordt gevonden in de normale microflora in de neus en op de huid. De kiem vormt een belangrijke bedreiging voor de menselijke gezondheid, niet het minst door de mogelijkheid om exotoxines te produceren, die kunnen functioneren als superantigen. Deze exotoxines, meer in het bijzonder de *Staphylococcus enterotoxines* (SEs), zijn betrokken in de aggravatie van luchtweg ontsteking. Recente studies tonen inderdaad aan dat er een belangrijke impact is van SEs op het natuurlijke verloop van allergische rhinitis, nasale polyposis, astma en COPD. Dit proefschrift vertrok van de observaties in humane luchtwegaandoeningen, en het doel was de hypothese te bevestigen dat SEs significant bijdragen aan de modulatie en aggravatie van luchtweg ontsteking.

In het eerste model hebben we aangetoond dat gelijktijdige blootstelling van de luchtweg aan het allergene ovalbumine (OVA) en *Staphylococcus aureus* enterotoxine B (SEB) leidt tot sensitisatie aan dit allergene, in een muismodel van experimenteel astma. SEB kon de primaire tolerantie tegenover ovalbumine allergene doorbreken, wat leidde tot OVA-specifieke IgE productie, toegenomen maturatie en migratie van dendritische cellen naar de drainerende lymfeklieren, alsook een toename van de allergene-specifieke T cel proliferatie. Daarenboven konden we ook bronchiale inflammatie met instroom van eosinofielen en lymfocyten aantonen in OVA/SEB muizen, tezamen met bronchiale hyperreactiviteit en productie van IL-4, IL-5, IL-10 en IL-13 door de drainerende lymfeknopen na OVA stimulatie. Belangwekkend is dat toediening van OVA of SEB alleen niet leidt tot luchtwegaandoeningen. Dit nieuwe model van experimenteel astma maakt gebruik van luchtwegblootstelling aan allergen en SEB en weerspiegelt daardoor heel precies de humane situatie van allergische sensitisatie. Dit bevestigt dat *Staphylococcus enterotoxines* een belangrijke rol spelen in dit proces.

We hebben aangetoond in dit proefschrift dat SEB een pro-inflammatoir effect uitoefent op epitheliale cellen, naast het hierboven beschreven effect op dendritische cellen en T cellen. In een nieuw model van vers geïsoleerde nasale epitheliale cellen, was SEB in staat een significante vrijstelling van chemokines te bewerkstelligen, waaronder IP-10, MIG, RANTES, MCP-1 en G-CSF. Deze
chemokines zorgden voor een toename in de migratie en overleving van granulocyten, wat duidt op een belangrijke rol voor het epitheel in de inductie en orkestratie van SE-geïnduceerde en granulocyt-gedomineerde luchtweginflammatie.

De ziekte-modifiërende effecten van SEB worden in dit proefschrift ook aangetoond in een andere ontstekingsachtergrond, met name bij sigarettenrook (SR) geïnduceerde luchtweg ontsteking. In een muismodel van subacute en chronische SR blootstelling hebben we duidelijke verschillen aangetoond in de regulatie van luchtweginflammatie in de bovenste luchtwegen ten opzichte van de onderste luchtwegen, in het bijzonder bij chronische blootstelling. SR-geïnduceerde luchtweginflammatie in de bovenste luchtwegen blijkt adequaat onderdrukt te zijn, terwijl de ontsteking de onderste luchtwegen duidelijk aanwezig is. Vervolgens hebben we in dit model het effect van gecombineerde SEB en SR blootstelling bestudeerd. SR/SEB muizen vertoonden een toename van het aantal lymfocyten en neutrofielen in het BAL vocht, alsook een toename in CD8+ T lymfocyten en granulocyten in het longweefsel, in vergelijking met zuivere SR of SEB blootstelling alleen. Daarenboven induceerde concomitante SR/SEB blootstelling zowel IL-13 mRNA expressie in de long als slijmbekercel hyperplasie in de luchtwegwand. Verder toonden we aan dat gecombineerde SR/SEB blootstelling de vorming stimuleert van dense, georganiseerde aggregaten van B en T lymfocyten in de long, alsook significant hogere niveaus van CXCL-13 (eiwit, mRNA) en CCL19 (mRNA) in de longen. Deze bevindingen bevestigen de hypothese die uitgaat van de humane observaties, die suggereert dat \textit{S. aureus} de pathogenese van COPD kan beïnvloeden.

Samengevat kunnen we stellen dat dit proefschrift aantoont dat \textit{Staphylococcus aureus} enterotoxine B een krachtige inductor en modulator is van luchtweg inflammatie. De resultaten die beschreven zijn in dit werk ontrafelen verder de pathofysiologie van luchtweg ontsteking, en wijzen nieuwe therapeutische strategieën aan voor de preventie en behandeling van deze ziektes.
Chapter 1

Chronic upper airway disease
Chapter 1: Chronic upper airway disease

CHRONIC RHINOSINUSITIS WITH AND WITHOUT NASAL POLYPS: WHAT IS THE DIFFERENCE

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INTRODUCTION

Rhinosinusitis is a considerable and increasing health problem causing in a large financial burden for the society\(^1\). Its high prevalence has recently led to an increasing interest in the pathophysiology of different forms of rhinosinusitis, in order to develop better treatment modalities. Because sinusitis usually coexists with rhinitis, and isolated sinusitis is rare, the more correct term to use now is rhinosinusitis.

Rhinosinusitis is a group of disorders defined as inflammation of the nose and paranasal sinuses, characterized by nasal blockage/obstruction/congestion, nasal discharge, facial pain/pressure and/or reduction or loss of smell. The diagnosis is based upon these symptoms and their duration, clinical findings, nasal endoscopy and CT scan. Acute rhinosinusitis – defined as a duration of < 12 weeks, with complete resolution of symptoms – in most cases is of viral origin, but rarely may also be caused by bacterial infections. In these cases, the disease is generally more severe and may lead to complications. It remains speculative whether recurrent acute rhinosinusitis might be a prerequisite for the development of chronic rhinosinusitis – defined as a duration of > 12 weeks, without complete resolution of symptoms – and possibly would result in persistent obstruction of the ostiomeatal complex\(^4\).\(^6\).

Chronic upper airway disease, or chronic rhinosinusitis (CRS), is an ill defined group of sinus diseases, when used as an umbrella covering different disease entities. CRS, defined as having ‘sinus trouble’ for more than 3 months in the year before the interview, is estimated to affect 15.5% of the total population in the United States\(^7\). A recent European survey reports a prevalence of 10.8% of
CRS. Chronic rhinosinusitis with nasal polyposis (CRSwNP) might be difficult to differentiate from chronic rhinosinusitis without nasal polyposis (CRSsNP) without nasal endoscopy. Therefore, both groups are often taken together as ‘chronic upper airway disease’ by non-specialists. However, it is unclear why in some patients polyps develop and in others not. Moreover, nasal polyps (CRSwNP) have a strong tendency to recur after sinus surgery, even when aeration is improved. This reflects a distinct property of those mucosae vs. CRSsNP patients.

In specific conditions like cystic fibrosis and allergic fungal sinusitis, polyp formation can be differentiated into disease entities based on genetic defects in cystic fibrosis and specific immunoglobulin E immune responses to fungi in allergic fungal sinusitis respectively. Moreover, development of appropriate disease markers have recently opened new possibilities and facilitated disease classification. These markers might be manifold: inflammatory cells and their products, T cell differentiation markers, markers of the remodeling processes, or markers derived from innate or adaptive immunity products. This disease differentiation might help to ameliorate the management of sinusitis by introducing new diagnostic and therapeutic strategies specifically targeted and adapted to the diagnosed disease entity.

A clear epidemiological and clinical association has been demonstrated between upper and lower airway diseases like allergic rhinitis and asthma, leading to the ‘united airways’ concept. Interestingly, this concept holds also true beyond the scope of allergic asthma, as patients with allergic and nonallergic asthma and COPD show increased nasal symptoms and more nasal inflammation. Moreover, in CRSwNP up to 70% of patients suffer from asthma, and there is evidence of bronchial hyperreactivity in CRSwNP patients without a history of asthma, in particular in non-atopic patients. The link of upper and lower airways is again reflected by the fact that medical or surgical treatment of nasal polyposis may have an impact on the control of asthma in those subjects. Until now, the link between upper airway disease and co-morbid lower airway pathology is not fully understood, possibly due to the ill-defined upper airway disease entities.
PREVALENCE AND PATHOPHYSIOLOGY

Prevalence

When reviewing the literature, it becomes clear that the estimation of the prevalence of CRS remains speculative, because of the heterogeneity of this group of disorders and the diagnostic uncertainties. As mentioned above, CRS has a prevalence of 15.5% in the United States, ranking this condition second among all chronic conditions. This high prevalence of CRS has been confirmed by another survey suggesting that 16% of the adult US population has CRS. In a Canadian study, the prevalence increased with age, with a mean of 2.7% in the group of 20-29 years and 6.6% in the group of 50-59 years. After the age of 60, prevalence levels of CRS leveled off to 4.7%. In Belgium, Gordts et al. reported that 6% of the subjects in the general population suffered from chronic nasal discharge. However, a recent population based survey found 16% of the adult population suffering from signs of CRS (unpublished data).

Until now, epidemiological studies exploring the prevalence and incidence of CRSsNP and CRSwNP are scarce, but increasing data is now becoming available from postal questionnaires or personal interviews. These data have demonstrated that CRS affects approximately 5–15% of the general population both in Europe and the USA. However, the prevalence of doctor-diagnosed CRS was 2–4%.

The prevalence of CRSwNP in the general population is commonly considered to be low. Valid epidemiological studies are greatly missing, and the fact that an endoscopic examination would be necessary for diagnosis further complicates such approaches. A postal questionnaire survey of a population-based random sample of 4300 adult women and men aged 18-65 years was performed in southern Finland. The prevalence of CRSwNP was 4.3%, and nasal polyposis and aspirin sensitivity were associated with an increased risk of asthma. The prevalence of doctor-diagnosed aspirin sensitivity was 5.7%. The incidence was higher in men than in women and significantly increases after the age of 40 years.
Pathophysiology

There is a distinct pathophysiology proposed for the different CRS subgroups. CRSsNP displays a predominantly neutrophilic inflammation, while in contrast, CRSwNP is in approximately 80% of all cases dominated by eosinophilic inflammatory mechanisms, and may be accompanied by aspirin sensitivity and asthma. Recently, the role of bacterial infection in CRSsNP has been challenged, whereas a modifying role of colonizing *Staphylococcus aureus* in CRS with nasal polyps has been introduced.

Fungal disease

Fungi have been increasingly recognized as important pathogens in sinusitis. Fungal infection, mainly by moulds, can impose a severe acute and chronic sinusitis in the immunocompromised host. In contrast, fungi are regarded as frequent innocent bystanders when cultured from the respiratory tract of immunocompetent hosts. The concept of fungi involvement in chronic rhinosinusitis should be that the ubiquitous airborne fungi become entrapped in sinonasal mucus, are attacked by eosinophils, and cause, via the release of toxic granules from eosinophils, secondary mucosal inflammation in susceptible individuals. If true, fungal eradication by using intranasal antifungals should improve the course of the disease. However in a double-blind, placebo-controlled, multicenter trial no additional benefit of amphotericin B nasal lavages to intranasal steroids and irrigations in patients with CRS with or without NP with a previous history of ESS was shown. Amphotericin B nasal lavages is therefore not suited to reduce clinical signs and symptoms in patients with CRS. These results might indicate that extramucosal fungi are probably innocent bystanders in the upper respiratory tract and playing no demonstrable role in the pathophysiology of CRS in immunocompetent patients.
**Cystic fibrosis**

In case of children suffering from nasal polyp formation, systemic diseases such as cystic fibrosis (CF) have to be considered. Cystic fibrosis is the most common fatal inherited disease among Caucasians, affecting approximately 1 of 2000 live births. The basic metabolic derangement is related to a mutation in the gene regulating the chloride transport in epithelial cells. Although bacterial infection is widely accepted to be a major factor in the pathogenesis of acute exacerbations and chronic progression of lung disease in CF, it remains unclear if the CF-specific sinonasal pathogens, of which *Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenza* and anaerobes are the most common, play a particular role in the pathogenesis of CF-NP. Because of the ubiquitous and persistent nature of the disease and the often transient effect of surgery, sinus surgery should only be performed in case of sufficient symptoms or before lung transplantation. The development of functional endoscopic sinus surgery has decreased the morbidity of sinus surgery and reduced the recurrence of nasal polyposis in cystic fibrosis. A careful postoperative follow-up is mandatory, but often difficult in young patients. Nasal irrigations with saline solution may help to clean the cavities after surgery.

**Aspirin sensitivity, or AERD (aspirin-exacerbated respiratory disease)**

The symptom triad consisting of aspirin sensitivity, steroid-depend asthma and nasal polyposis (rhinosinusitis) described by Widal in 1922, was made known by Samter and Beers later. Aspirin-sensitive rhinosinusitis (ASRS) is characterized by increased eosinophils in the nasal and bronchial mucosa, and elevated cysteinyl-leukotriene concentrations in the tissue and urine, which further increases after aspirin exposure. Initially, the symptoms mostly develop after a prolonged common cold episode in the third or fourth decade of life with nasal congestion, rhinorrhea, post-nasal drip and hyposmia, based on persistent mucosal inflammation. Within a few years, nasal polyposis and bronchial asthma develop, until aspirin sensitivity is suspected due to a typical respiratory reaction and eventually is diagnosed by oral provocation test. Asthma and rhinitis attacks are caused by ingestion
of aspirin and other non-steroidal anti-inflammatory drugs that share the ability to inhibit cyclooxygenase enzymes (COX-1,-2). About 15% of patients with aspirin-inducible asthma and rhinitis are unaware of aspirin sensitivity, indicating that aspirin challenge is necessary to fully diagnose the disease. About 50% of patients need systemic steroid treatment on top of inhaled corticosteroids, emphasizing the severity of the disease in the upper and lower respiratory tract. Interestingly, the course of disease is independent from aspirin intake, indicating that the disease is driven by so far unknown agents and with few exceptions, aspirin sensitivity remains life-long. Until now, no validated laboratory test is available, and the diagnosis is based on oral, bronchial or nasal provocation tests33.

Although ASRS often is associated with allergy and highly elevated local IgE levels34, an IgE-mediated mechanism has not been demonstrated, and atopy does not seem to influence the risk to develop aspirin sensitivity32. However, alterations in arachidonic acid metabolism, resulting in an alteration of the cellular response to aspirin, have been suggested30. Blockage of COX-2 reduces asthma symptoms and CysLTs release, in contrast to COX-1 inhibition which precipitates asthma attacks35. Additionally, it has been found that COX-2 expression is down regulated in nasal mucosa of aspirin-intolerant patients. COX-2 mRNA expression is regulated by cytokines, which activate the nuclear factor kappa B (NF-kappa B) transcription factor. Further studies are needed to explain whether an alteration in the NF-kappa B and/or other regulatory mechanism is responsible for the abnormal expression of COX-2 mRNA in patients with AIAR36. The LTC4-synthase has been demonstrated to be overexpressed in eosinophils and mast cells37, resulting in an overproduction of cys-leukotrienes, which may be released into the airways after aspirin challenge, causing typical symptoms (nasal congestion, rhinorrhea, bronchoconstriction), as well as constantly, inducing eosinophilia. In a subgroup of nasal polyp patients with the clinical history of asthma and aspirin sensitivity, a marked tissue eosinophilia, increased IL-5 and eotaxin expression as well as leukotriene C4-E4 overproduction have been linked to an immune reaction to Staphylococcus aureus enterotoxins, also inducing a local multiclonal IgE response34. However, the mechanisms of direct impact of
Staphylococcus aureus on the arachidonic metabolism still need to be defined. It seems that aspirin sensitivity and immune reactions to SAEs are independently related to eosinophilic inflammation. Conservative treatment possibilities consist of 1) avoidance of aspirin and other NSAIDs, which does prevent exacerbations, but does not prevent progression of disease 2) oral and/or topical glucocorticosteroids, 3) eventually leukotriene receptor antagonists or synthesis inhibitors, and 4) in selected cases, aspirin desensitisation. To prevent exacerbations, the ingestion of aspirin and COX-inhibiting NSAIDs has to be avoided, while acetaminophen, nimesulide (dose-dependently) and selective COX-2 inhibitors (celecoxib, rofecoxib) may be tolerated. Whereas systemic steroids have been proven effective, but may cause side effects in long-term usage, anti-leukotriene drugs deserve further trials to find their place in the treatment regimen. Aspirin desensitisation consists of administering incremental oral doses, to reach a maintenance dose of > 650 mg daily, inducing a refractory period of a few days. Continuous treatment over years may lead to a significant reduction in numbers of sinus infections per year, hospitalizations for treatment of asthma per year, improvement in olfaction, and reduction in use of systemic corticosteroids. Furthermore, numbers of sinus operations per year were significantly reduced. However, due to gastro-intestinal side effects of aspirin and a relapse of risk in case of non-compliance, this therapy is not widely accepted. Furthermore, aspirin desensitization does not seem to change the long-term course of the disease.

Signs and symptoms

The pattern of symptoms and signs caused by CRS is somewhat overlapping in all patients with chronic sinus inflammation, with most of the symptoms occurring in both CRSsNP and CRSwNP. However, looking more in detail to symptom profile, patients suffering from CRSwNP have a more pronounced nasal obstruction and loss of smell, while CRSsNP patients complain more of headache and postnasal drip. In particular the partial or complete loss of smell with a subsequent effect on taste and the loss to discriminate subtleties of flavour is a typical feature of CRSwNP. Even with obstructed sinuses, CRSwNP patients without prior surgery rarely complain from facial pain or headache,
although these patients have more extensive opacifications on CT scan compared with CRSsNP patients, as scored with the Lund-Mackay score: this scoring system consists of a scale of 0-2 dependent on the absence, partial or complete opacification of the sinus system and the ostiomeatal complex, with a maximum score of 12 per side. Lund Mackay scoring system is the most widely used grading system for CRS.

**CYTOKINES, MEDIATORS AND CELLULAR PROFILES**

In order to differentiate more clearly between the different disease entities that compose chronic sinus disease, identification of inflammatory cells and the array of cytokines and mediators released by them may proof very useful. It has been demonstrated that CRSsNP, CRSwNP and cystic fibrosis patients with nasal polyps (CF-NP) are dissociated disease entities with distinct cytokine, mediator and cellular profiles[^40^], as demonstrated in Figure 1. These groups share a T cell mediated immune response, although different T lymphocyte subsets contribute to it. When analysing diseased tissue from CRSsNP and CRSwNP patients, a clear Th1/Th2 polarisation becomes clear with IFN-γ (Th1 related) characterising CRSsNP, whereas IL-5 (Th2 related) is emblematic for CRSwNP. Moreover, inflammation in CRSwNP is accompanied by abundant eosinophils and IgE formation, again stressing the Th2 bias in this disease. Eosinophilic inflammation is a key feature of CRSwNP, with highly increased concentrations of ECP as marker of eosinophil activation, and of eotaxin, a CC chemokine, which co-operates with IL-5 to recruit and activate eosinophils[^41^]. Interestingly, these markers of eosinophilic inflammation, which are increased in CRSwNP vs. control patients, but also compared to CRSsNP patients, are further increased in polyps with IgE antibodies to *S. aureus* enterotoxins, pointing towards a modifying role of these bacterial superantigens in the pathophysiology of CRSwNP: staphylococcal superantigens[^34^, ^42^] induce a polyclonal T cell and B cell activation with multyclonal IgE formation and T cell activation, severely amplifying the eosinophilic inflammation. In contrast, specific IgE to enterotoxins and multyclonal IgE formation in tissue is a rare finding in
CRSsNP and CF-NP\textsuperscript{43}, although \textit{S. aureus} belongs to the usual germ flora, in particular in upper airway manifestations of cystic fibrosis\textsuperscript{44}.

\section*{A \hspace{2cm} CRSsNP}

\begin{itemize}
  \item Adequate FOXP-3 expression
  \item TGF-\(\beta\)1 \(\uparrow\)
  \item Fibrosis
  \item TIMP-1 \(\uparrow\)
  \item MMP-9 \(\uparrow\)
  \item Higher collagen deposition
\end{itemize}

\begin{itemize}
  \item Macrophages
  \item Th1 lymphocyte activation
  \item TNF-\(\alpha\) \(\uparrow\)
  \item T-bet \(\uparrow\)
  \item IFN-\(\gamma\) \(\uparrow\)
\end{itemize}

\section*{B \hspace{2cm} CRSwNP}

\begin{itemize}
  \item Staphylococcal colonisation and enterotoxin release
  \item B-lymphocyte activation
  \item Class switch recombination in plasmacells
  \item specIgE \(\uparrow\)
  \item IgE \(\uparrow\)
  \item IgG1 \(\uparrow\)
  \item Decreased Immunoregulatory function
\end{itemize}

\begin{itemize}
  \item Th2 lymphocyte activation
  \item IL-5 \(\uparrow\)
  \item Decreased FOXP-3 expression
  \item TGF-\(\beta\)1 \(\downarrow\)
  \item Destruction of ECM
  \item TIMP-1 \(\downarrow\)
  \item MMP-9 \(\uparrow\)
  \item Oedema
\end{itemize}

\begin{itemize}
  \item Eosinophils
  \item Increased expression of MMR
  \item Increased recruitment and activation
  \item IL-5 \(\uparrow\)
  \item ECP \(\uparrow\)
  \item Eotaxin \(\uparrow\)
\end{itemize}

\begin{itemize}
  \item Macrophages
\end{itemize}

\textbf{Figure 1.} Chronic rhinosinusitis without (A) and with (B) nasal polyps. Distinct cytokine, mediator and cellular profiles differentiate chronic sinonasal disease. In Caucasians, CRSsNP is characterized by a Th1 profile (high IFN-\(\gamma\)), adequate FoxP3 expression, high levels of TGF-\(\beta\)1 with excessive tissue repair and fibrosis. Conversely, a Th2 profile (high IL-5) is found in CRSwNP, decreased FoxP3 levels, low amounts of TGF-\(\beta\)1 and lack of tissue repair resulting in loose connective tissue and oedema formation.
Nasal polyps in cystic fibrosis patients also show oedema formation and matrix disruption, but display a prominent neutrophilic instead of eosinophilic inflammation, and a significant lower tissue ECP concentration compared to CRSwNP, suggesting that the typical oedema formation in CRSwNP may not be exclusively dependent on tissue eosinophilia and their activation. The apparent neutrophilic inflammation in CF-NP is characterized by high IL-8 (a CXC chemokine), MPO (released by neutrophil granulocytes), and pro-inflammatory mediator IL-1β concentrations.

CRSwNP and CF-NP not only differ in terms of inflammatory cell and cytokine patterns, but also in terms of expression of innate markers: CF-NP is associated with the up-regulation of both human beta defensin 2 (HBD2) and Toll-like receptor 2 (TLR2), while the expression of the macrophage mannose receptor (MMR) dominates the innate defense in non-CF-NP. These apparent differences point towards a variable inflammatory background in CRSwNP and nasal polyps in cystic fibrosis.

**T cell biology**

T cell polarization of CRSSNP (Th1) and CRSwNP (Th2) is controlled by intracellular mechanisms, which initiate differentiation of naïve T cells towards the distinct T cell subtypes, based on the expression of certain transcription factors. T-box transcription factor (T-bet) involves commitment toward Th1 cells; GATA-3 is critical for commitment toward Th2 cells and controls the expression of IL-4 and IL-5. T regulatory (Treg) cells – another T cell subset, characterised by the transcription factor forkhead box P3 (FoxP3) – control the balance between Th1 and Th2 cells and limit chronic inflammation. Interestingly, we recently showed a decreased expression of FoxP3 mRNA and protein (IHC) in CRSwNP tissue, which reflects deficiency of Treg cells in this often persistent, severely inflamed sinus mucosa. Consequently, lower TGF-β1 protein levels were found in this group, which led to a defective suppression of Th1 and Th2 transcription signals in CRSwNP. In contrast, adequate expression of FoxP3 with increased TGF-β1 protein levels were found in CRSSNP, which resulted in maintained control over T-bet and GATA-3 expression, suggesting adequate Treg cell function in this
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sinus disease subgroup. These differences in T cell biology again clearly distinguish CRSwNP from CRSsNP entities.

Besides Th1/Th2/Treg cells, other T cell subsets like the recently described Th17 cells can be used to further divide and characterise disease entities within the CRSwNP group. Although clinical appearance, mucosal oedema formation, T effector cell activation and T regulatory cell impairment (decreased FoxP3 expression) are shared by European and Asian nasal polyps, the pattern of inflammation is remarkably different between the disease groups, with a Th1/Th17 dominance in South Chinese and a Th2 dominance in Belgian polyps\(^46\). This Th1/Th17 effector cell polarization in South Chinese CRSwNP was characterized by T-bet expression and IFN-\(\gamma\) protein formation, IL-17 and related IL-1\(\beta\) and IL-6 protein synthesis in tissue homogenates, whereas Belgian CRSwNP demonstrated increased GATA-3 expression with consequently raised IL-5 protein levels compared to control tissue. Moreover, these differences in T cell biology are reflected by a distinguished neutrophil and eosinophil granulocyte activation bias in South Chinese and Belgian CRSwNP respectively, which is reflected in a significantly lower ECP/MPO ratio in South Chinese CRSwNP compared to Belgian CRSwNP. These findings largely affect treatment approaches in CRSwNP, as the focus of the treatment in daily practice in Europe and US is on eosinophils, with the use of topical and systemic corticosteroids and humanized anti-IL-5 monoclonal antibodies becoming a treatment option in the future. It is very unlikely that the anti-IL-5 concept will be appropriate in neutrophilic IL-17 biased CRSwNP in Asia, or CF nasal polyps in Europe\(^46\).

**REMODELING**

Chronic inflammation in the nose and paranasal sinuses eventually results in tissue destruction and remodeling processes within the mucosa, which are characterised by changes in the extracellular matrix (ECM) protein deposition and tissue structure. A major role in airway remodeling in general, and chronic sinus disease in particular, is played by TGF-\(\beta\)\(^47\), a pleiotropic and multifunctional growth factor, with important immunomodulatory and fibrogenic characteristics. Interestingly, the distinct
disease entities within the group of chronic sinus disease display significantly different levels of TGF-β1, with high levels in CRSsNP and conversely low TGF-β1 levels in CRSwNP. These low TGF-β1 levels may reflect the decreased T regulatory cell function in CRSwNP, which was confirmed by the low FoxP3 levels. The high TGF-β1 levels and adequate expression of FoxP3 in CRSsNP on the other hand, mirror the adequate control of inflammation. Moreover, recent immunohistochemistry findings confirm these data, and show increased TGF-β1 receptor expression and increased active intracellular signal in CRSsNP.

Besides its immunomodulatory effect, TGF-β plays a crucial role in the extracellular matrix metabolism, as it acts as a master switch in the induction of fibrosis. TGF-β counteracts tissue destruction that can result from inflammation by inducing fibrogenesis. Moreover, it induces the expression of ECM proteins in mesenchymal cells, and stimulates the production of TIMP-1, a tissue inhibitor of metalloproteinases that prevent enzymatic breakdown of the ECM. In CRSsNP MMP-9 and TIMP-1 are found upregulated, whereas in CRSwNP, MMP-9, but not TIMP-1, is up-regulated, which is in line with the observed edema formation in CRSwNP. The observed lack of upregulation of TIMP-1 can be related to the low TGF-β1 levels in CRSwNP. Furthermore, the extracellular matrix remodeling pattern in CRSwNP is characterized by low amount of collagen compared to control tissue, without thick collagen fibers. The lack of TGF-β1 in CRSwNP can be interpreted as a lack of tissue repair, reflected by loose connective tissue and oedema formation in a severely inflamed tissue. In contrast, remodeling in CRSsNP results in a higher collagen content compared to controls. This is indicative for excessive tissue repair and fibrosis formation in CRSsNP. These differences in TGF-β signaling clearly support the distinction between CRSsNP and CRSwNP as separate disease entities.
ROLE OF SAE

The finding of IgE antibodies to *S. aureus* enterotoxins in CRSwNP tissue homogenates indicates that superantigens could be involved in the pathogenesis of this disease.\(^5^3\). We previously reported an increased colonization rate of *S. aureus* in CRSwNP, but not CRSsNP patients.\(^4^2\) Colonization with *S. aureus* was present in more than 60% of patients with CRSwNP, with rates as high as 87% in the subgroup with asthma and aspirin sensitivity, which were significantly higher than in control individuals and patients with CRSsNP (33% and 27%, respectively).

IgE antibodies to *S. aureus* enterotoxins were present in 28% in polyp samples, with rates as high as 80% in the subgroup with asthma and aspirin sensitivity, compared to 15% in control individuals and 6% in patients with CRSsNP, respectively. The presence of specific IgE against *S. aureus* enterotoxins in nasal polyp tissue also coincided with higher levels of IL-5, eotaxin and eosinophil cationic protein (ECP). Moreover, an increased number of T cells expressing the T cell receptor β-chain variable region known to be induced by microbial superantigens was detected in CRSwNP and correlated with the presence of specific IgE against *S. aureus* enterotoxin.\(^5^4\) These findings confirm the role played by *S. aureus* enterotoxins as disease modifiers specifically in CRSwNP. Interestingly, stimulation of CRSwNP tissue with SEB results in significantly higher release of proinflammatory cytokines compared to controls. Besides induction of T cell activation, SEB introduced a bias towards Th2 cytokines, as IL-4 and IL-5 production was favored.\(^5^5\)

The strikingly high correlation between IL-5 and IgE antibody concentrations in CRSwNP homogenates support the hypothesis that *S. aureus* enterotoxins, apart from T cells, also modify B and plasma cells.\(^4^1\) In fact, there is accumulating evidence that *S. aureus* enterotoxins can directly affect the frequency and activation of the B cell repertoire. We recently described a markedly increased number of plasma cells in sinonasal mucosal tissue samples from CRSwNP patients as compared with those from CRSsNP patients and control individuals. We have recently extended these findings with *S. aureus* specific data. In a follow-up study, high concentrations of total IgE, IgA and IgG were measured in CRSwNP homogenates, and these concentrations were significantly greater in CRSwNP.
than in CRSsNP patients and control individuals. These changes were not reflected in the serum of patients; the presence of *S. aureus* enterotoxin–IgE antibodies in tissue or serum did not influence immunoglobulin concentrations in serum, confirming the notion of a local impact of superantigens – via direct action on B cells or indirectly via T cell derived cytokines – on immunoglobulin synthesis.\(^6\)

The functional role played by local IgE antibodies in CRSwNP is currently being investigated by our group, and others have called it into question by observations in ragweed-sensitive polyp patients, who do not exhibit specific seasonal changes in symptoms or mediators.\(^7\) In laboratory experiments in which basophils armed with specific IgE to enterotoxin B were exposed to the superantigen, however, the basophils degranulated rapidly.\(^8\) Thus, enterotoxin specific IgE antibodies could potentially contribute to the disease via degranulation of mast cells in polyp tissue, as well as other IgE antibodies with specificities against inhalant allergens. Indeed, because of the multiclonality, hundreds of allergens could possibly induce a constant degranulation of those mast cells, a condition that has actually been observed in polyp tissue.\(^9\) Based on these observations, anti-IgE treatment could be expected to suppress the IgE-mediated inflammatory cascade in a nonallergic disease such as CRSwNP, similar to its activity in allergic respiratory disorders. A proof-of-concept study is currently being performed at the Ear, Nose and Throat Department of the Ghent University Hospital.

These findings provide increasing evidence that *S. aureus* derived enterotoxins play a role as modifier of inflammation in CRSwNP, but not in CRSsNP.

**TREATMENT**

Medical treatment consisting of nasal corticosteroids (sprays/drops) and antibiotics with anti-inflammatory activities is the first step in treatment, but surgery is indicated for CRSs/wNP in the case of failure. Clear diagnosis and management schemes for both CRSsNP and CRSwNP for GPs, non-ENT specialists and ENT specialists are published in the [EP3OS position paper](#), summarizing the current knowledge. Nowadays, functional endoscopic sinus surgery (FESS) has become the standard procedure to restore sinus ventilation and drainage by opening the key areas while preserving sinus
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mucosa. An overall success rate of 85% is reported in primary FESS, with a 2-24% failure rate because of recurrence of disease or poor healing due to persistent inflammation and/or bacterial colonization\(^{59, 60}\). In approximately 18% of the patients, poor healing is linked to abnormal scarring, super-infection and fibrosis formation, with these complications potentially leading to revision surgery\(^{61}\). The healing prognosis in CRSwNP patients is worse compared with patients with CRSsNP, especially if CRSwNP is associated with asthma and/or aspirin sensitivity. Previous data from our group demonstrated that both pre- and postoperative levels of matrix metalloproteinase-9 (MMP-9) are significantly and independently predictive for the healing outcome\(^{62}\). This was clinically linked to the diagnosis CRSwNP rather than CRSsNP, as well as to previous surgery. Indeed, patients with high concentrations of MMP-9 (and probably other MMPs, not balanced by TIMPs) in the preoperative and late postoperative period suffered from poor healing. Moreover, recent data indeed support the hypothesis that MMP-9 can serve as a target for therapeutic intervention to achieve better healing quality\(^{63}\).

Consistent with the current knowledge on the pathophysiology of CRSwNP, new therapeutic approaches could focus on eosinophilic inflammation, eosinophil recruitment, the T cell as the orchestrating cell and IgE antibodies, as well as on tissue destruction and remodelling processes. Recently, the introduction of monoclonal humanised antibodies opened new perspectives, and these are currently evaluated in clinical studies. An interleukin-5 antagonist, reslizumab, induced a reduction of blood eosinophil numbers and concentrations of eosinophil cationic protein up to 8 weeks after treatment in serum and nasal secretions\(^{64}\). Individual nasal polyp scores improved only in half of the verum-treated patients for up to 4 weeks. When carefully analyzing responders and nonresponders in a post-hoc analysis, only those CRSwNP patients with increased baseline levels of IL-5 (>40 pg/mL) in nasal secretions seemed to benefit from the anti–IL-5 treatment. Furthermore, nasal IL-5 levels decreased only in the responders, whereas they increased in the nonresponders. These data show that at least in 50% of the CRSwNP, IL-5 and eosinophils play a key role (IL-5 dependent) in sustaining polyp size, whereas in others eosinophilia might be dependent on other factors (IL-5 independent) or lacking\(^{64}\).
As mentioned above, our group is currently evaluating the principle of IgE antagonism in CRSwNP, in order to suppress the IgE-mediated inflammatory cascade. Preliminary data confirm the concept that anti-IgE is an effective treatment option in patients with CRSwNP and comorbid asthma. In this study patients were selected based on the presence of nasal polyps and asthma irrespective of the presence of allergy. Nevertheless, anti-IgE was equally efficacious in both allergic and non-allergic patients with nasal polyps and asthma.

Other therapies focus on neutralization of CCR3 or eotaxin and their role in the regulation of eosinophil, basophil and potentially T helper type 2 and mast cell recruitment. Moreover, future therapies in CRSwNP might consist of anti-IL-4, anti-IL-13, matrix metalloproteinase inhibitors or immunosuppression with for example ciclosporin. However, until now, none of these therapies has been evaluated in randomized, placebo-controlled clinical trials in CRSwNP.

We have demonstrated the microbial involvement in CRSwNP by several studies. In that perspective, doxycycline was evaluated as systemic treatment, based on its antimicrobial and anti-inflammatory effects. In a double blind, placebo controlled trial, doxycycline showed an effect on bilateral CRSwNP, as it reduced polyp size and post nasal drip, and increased peak nasal inspiratory flow. Furthermore, doxycycline was able to significantly reduce local inflammation in terms of ECP, IgE, MPO and MMP-9. The reduction of ECP reflects a down-regulation of eosinophil activation, and together with the decrease in IgE may reflect the anti-staphylococcal activity, whereas the drop in MPO reflects a diminished neutrophilic activity. The MMP-9 suppressive effect, which represents a specific activity of tetracyclines, favors a beneficial remodeling of the polyp tissue, leading to a reduction of matrix degradation and oedema formation. The observed decrease in polyp size might therefore require both the antimicrobial and anti-inflammatory effect of doxycycline.

Another anti-inflammatory approach is the long-term treatment with macrolide antibiotics. A number of clinical reports indeed have stated that long-term low-dose macrolide antibiotics are effective in treating chronic rhinosinusitis incurable by surgery or glucocorticosteroid treatment, with an improvement in symptoms varying between 60 and 80 %. The exact mechanism of action is
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currently unknown, but may involve downregulation of the local host immune response as well as
downgrading the virulence of the colonizing bacteria. However, placebo-controlled studies are
necessary to clarify if macrolide therapy is acceptable as evidence-based medicine in chronic sinus
disease.

CONCLUSION

Chronic rhinosinusitis is a heterogeneous group of inflammatory conditions in the nose and paranasal
cavities and differentiation between disease entities is mandatory to achieve progress in the diagnosis
and treatment of CRSwNP and CRSsNP. The orchestrating T effector cells, the remodeling patterns,
and the type of inflammation clearly differ between groups. The usage of disease markers, which
might be manifold, will allow clear differentiation and accurate diagnosis, resulting in more precise
and focused treatment. This principle might increase the success rate of both medicinal and surgical
treatment, as therapies could be modified according to the specific disease entity. Patient phenotyping,
based on clinical, but also biological markers, will be indicated in order to recommend an optimal,
individualized treatment of CRS patients in the near future.

The involvement of staphylococcal enterotoxins in the pathogenesis of chronic upper airway disease
like CRSwNP, is becoming increasingly clear. Furthermore, these enterotoxins are known to modulate
inflammation in other diseases like atopic dermatitis, allergic rhinitis, severe late-onset asthma or
chronic obstructive pulmonary disease. In chapter 2, we focus on these staphylococcal superantigens,
and their role in airway pathology.
REFERENCES


Role of Staphylococcal superantigens in airway disease
INTRODUCTION

Superantigens represent a growing family of bacterial and viral proteins that share the capacity of inducing massive activation of the immune system. Marrack and colleagues first described the concept of superantigens already in 1989, pointing out the ability of *Staphylococcus aureus* enterotoxin B to induce a remarkable expansion of T cells expressing T cell receptors with a specific subset of the T cell receptor β-chain variable region. Superantigens (SAgs) are able to bind to the major histocompatibility complex (MHC) class II out-side the peptide-binding groove, and cross-link to the T cell receptor (TCR) via the variable region of the TCR β-chain (Figure 1).

This leads to a potent polyclonal activation of up to 25% of an individual’s T cell population. Superantigens differ from conventional antigens because they’re not processed by antigen-presenting cells (APC) and presented as short peptides. These conventional peptide antigens then require recognition by all five variable elements of the TCR (Vβ, Dβ, Jβ, Vα, Jα), resulting in a stimulation of about 0.001-0.0001% of naïve T cells.

*Figure 1: Superantigen cross-linking of MHC class II receptor on antigen presenting cell (APC) and T cell receptor (TCR) on T lymphocyte. Left: conventional antigen presentation with processing and presentation by the MHC II within the peptide binding groove. Right: Superantigen cross-linking MHC II and TCR, without being processed.*
Since the number of different Vβ regions in the human T cell repertoire is restricted to less than 50, and since most SAgs can bind more than one Vβ, a far more compelling activation of circulating T cells is achieved. Consequently, each SAg is associated with a characteristic Vβ signature that is independent of the MHC polymorphism, as is shown in Table 1.

This immune system stimulation of T cells and antigen-presenting cells by superantigens initiates a cascade of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1b), and T cell mediators, such as IL-2, leading to fever and shock. Concentrations of less than 0.1 pg/ml of a bacterial SAg are sufficient to stimulate T cells in an uncontrolled manner, making SAgs the most powerful T cell mitogens ever discovered.

Enterotoxin-producing *Staphylococcus aureus* (*S. aureus*) is probably the best studied source of superantigens. However, other bacteria like *Streptococcus pyogenes*⁴, *Yersinia pseudotuberculosis*⁴, or *Mycoplasma arthritis mitogen*⁵ have known superantigen activities. Similarly, superantigen formation is linked to some viruses like Rabies⁶, Epstein Barr virus⁷, Cytomegalo virus⁸ or HIV⁹.
### Table 1: List of frequently studied bacterial SAgs. The major TCR Vβ targets are underlined[^10].

<table>
<thead>
<tr>
<th>Superantigen</th>
<th>MW(kDa)</th>
<th>Organism</th>
<th>Human TCR Vβ specificity</th>
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<td>S. pyogenes</td>
<td>6.9, 9.1, 18.1, 22</td>
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[^10]: Links to the primary source or specific references.
Chapter 2: Role of staphylococcal superantigens in airway disease

**T CELL DIFFERENTIATION**

T cells or T lymphocytes play a central role in cell-mediated immunity. They are named T cells because they mature in the thymus, where they differentiate into several subsets: T helper cells, cytotoxic T cells, memory T cells, regulatory T cell, natural killer T cells or γδ T cells. Each of these subtypes have a distinct function. In this thesis, we mainly focus on T helper cells, which express the CD4+ protein on their surface. T helper (Th) cells become activated after the encounter of the innate immune system with antigen, when Th cells are presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen presenting cells. Once activated, differentiation into different effector T cell lineages, Th1, Th2, Th17 and T regulatory cells is initiated as depicted in Figure 2. Tight management of this adaptive immune response is essential for host function and survival, maintaining a balance between antigen clearance and immune pathology, while tolerance to all components of self as well as many harmless antigens needs to be preserved.

Th1, Th2, Treg, and Th17 are characterized by their synthesis of specific cytokines and their immunoregulatory functions. IFNγ is the signature cytokine produced by Th1 cells, while IL-4, IL-5 and IL-13 are major cytokines produced by Th2 cells. Th17 cells produce IL-17A (IL-17), IL-17F, IL-21, and IL-22 as major cytokines, while Treg cells synthesize IL-10 and TGF-β.

T helper and Treg cells play a critical role in several inflammatory responses, including adaptive immune responses to various pathogens. Host defense is coordinated by the proinflammatory Th1, Th2, and Th17 cells, while Treg cells are involved in the down regulation and contraction of an immune inflammatory response. Th17 cells are believed to be the major proinflammatory cells involved in autoimmunity, while Treg cells protect against autoimmunity.

Th1 cells appear to drive cell-mediated immune responses leading to tissue damage, as well as antibody-mediated responses in certain subclasses of the G isotype of immunoglobulin antibody, specifically termed IgG2a. In contrast, Th2 cells drive certain antibody-mediated responses, particularly those that are involved in allergy dominated by the IgE isotype.
Th cell differentiation upon activation, involves the activation of distinct signaling cascades and transcription factors and the synthesis of other cyto/chemokines and cyto/chemokine receptors that are part of additional positive and negative feedback loops. For example, induction of Th1 cells by IL-12 involves amongst others the transcription factors T-bet, while differentiation along the Th2 lineage in response to IL-4 requires GATA3. Interestingly, there is an important plasticity of effector and regulatory T cells and differentiation different subsets is often reciprocal, involving several positive and negative regulatory networks that favor one or the other lineage.

**Figure 2: T helper cell differentiation and regulation (adapted from [11]).**

**OVALBUMIN**

In this thesis, we use ovalbumin as allergen in order to study allergic sensitization (Chapter 3). Ovalbumine (OVA) is the main protein found in egg white, making up 60-65% of the total protein. The proteins of hen’s egg white, like ovalbumin (OVA), frequently induce hypersensitivity symptoms among egg allergic individuals. OVA is the most dominant ingredient of the five major allergens of egg white and is universally used as the main allergen in establishing different animal models of
asthma, food and skin allergy. The ovalbumin protein of chickens is made up of 385 amino acids, and its relative molecular mass is 45 kDa. It is a glycoprotein with 4 sites of glycosylation\textsuperscript{15}.

In mouse models of allergic asthma, sensitization is classically achieved after intraperitoneal injections with OVA, often using adjuvants like alum to boost the immune response. In contrast, we have obtained allergic sensitization applying OVA endonasally, more mimicking the natural way of mucosal contact with allergen. The purity of OVA used in research is of uttermost importance, in particular the endotoxin content, which was monitored in all our experiments in order not to interfere with the natural immune response after allergen contact.

**STAPHYLOCOCCAL SUPERANTIGENS**

*Staphylococcus aureus* is one of the most significant infectious threats to human health. It is a facultative anaerobic, Gram-positive coccus, which is frequently part of the normal microflora found in the nose and on skin. Carriage of *S. aureus* appears to play a key role in the epidemiology and pathogenesis of infection. In healthy subjects, the anterior nares are most frequently colonized with *S. aureus*, and over time, three patterns of carriage can be distinguished: about 20% of people are persistent carriers, 60% are intermittent carriers, and approximately 20% almost never carry *S. aureus*\textsuperscript{16}. This carriage is a major risk factor for infection, as the colonizing strains may serve as endogenous reservoirs for overt clinical infections or may spread to other patients. Indeed, the elimination of carriage in the anterior nares, the principal reservoirs of *S. aureus*, reduces the incidence of *S. aureus* infections\textsuperscript{17}.

*S. aureus* is responsible for an array of diseases ranging from minor skin and soft tissue infections, such as pimples, impetigo, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to more invasive and life-threatening infections, such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. The ability of *S. aureus* to cause such diverse diseases is due primarily to an arsenal of virulence factors encoded in the staphylococcal genome. Amongst these bacterial pathogenicity or virulence factors, there are products whose role in
the disease process is either clearly demonstrable, like toxins, or more or less obvious on the basis of biological properties, e.g. enzymes that degrade tissue components\textsuperscript{18}. \textit{S. aureus} is known for its production of potent toxins. Once introduced into a host's system, these toxins can act to profoundly stimulate the immune system. The proteins are known to act on host systems in three distinct ways: as enterotoxins, they induce emesis and diarrhea in humans and nonhuman primates\textsuperscript{19}; as exotoxins, they have been implicated in induction of toxic shock\textsuperscript{20}; and as superantigens, they induce extensive V\textbeta- specific T cell stimulation followed by anergy and apoptosis, which results in immunosuppression\textsuperscript{21}. For a long time, the production of SAgs by \textit{S. aureus} was thought to be a rare phenomenon, linked to the occurrence of staphylococcal toxemia and therefore restricted to isolates responsible for such syndromes. However, because of the structural and biological similarities that these staphylococcal toxins share, it is no surprise that phylogenetic analysis has identified an enterotoxin gene cluster (egc) in \textit{S. aureus}, which was probably generated from an ancestral gene through gene duplication and variation\textsuperscript{22}. This is shown in Figure 3. In fact, SAg production by \textit{S. aureus} isolates appeared more frequent than initially expected, and it is more common for \textit{S. aureus} to possess a SAg than not. Moreover, most of the strains harbor several SAg genes as illustrated in genome sequences of \textit{S. aureus} strains\textsuperscript{23}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Reconstruction of phylogenetic tree of staphylococcal enterotoxin genes (A) and toxins (B)\textsuperscript{22}.}
\end{figure}
STAPHYLOCOCCAL ENTEROTOXINS

Enterotoxins are short, secreted proteins (from 194 to 245 amino acids long) of approximately 27kDa molecular mass. They share common biological and structural properties. Remarkably resistant to heat, the potency of these toxins can only be gradually degraded by prolonged boiling or autoclaving. Except for toxic shock syndrome toxin-1 (TSST-1), they are highly stable and resistant to most proteolytic enzymes, such as pepsin and trypsin, which explains the retention of their activity in the digestive tract after ingestion.

Staphylococcal enterotoxins (SEs) are able to induce high fever similar to bacterial endotoxin induction, lethal shock in animals resulting from excessive intravenous doses, enhanced host susceptibility to endotoxin lethality, cytokine production, and the typical superantigenic feature of polyclonal T cell proliferation\(^ {24}\). SEs can bind directly and unprocessed to MHC class II molecules on APCs. Figure 4 depicts this binding of the \(\beta\)-barrel of the SE domain A to the MHC II at a region distinct from the peptide-binding groove\(^ {25}\). SEs are able to bind a wide variety of molecules, although there is some preference depending on the toxin involved: SEC prefers HLA-DQ and SEA, SEB, SED, SEE, SEH and TSST-1 prefer HLA-DR\(^ {26}\). Subsequently, the \(\alpha\)-helices of domain A can co-bind and facilitate ligation between MHC II molecules of APCs and certain TCRs. As described above, this binding is not dependent on T cell antigenic specificity, but occurs as a function of the variable region of the TCR \(\beta\)- or \(\alpha\)-chain\(^ {27}\). Indeed, each SAg interacts with a defined TCR repertoire, resulting in massive cell activation of both cell types and cytokine release.

The exact reason why bacteria produce superantigens, or what superantigens are actually doing for the bacteria remains elusive. Most likely, it is not the purpose to induce systemic toxic shock in the host. Superantigens are probably rather inducing local T cell activation in the early stages of infection. This local T cell activation might result in cytokine production such as IL-2, IFN-\(\gamma\) and TNF-\(\alpha\), which suppress local inflammation\(^ {10}\).
Figure 4: Ribbon diagram of SEB as representative of SEs. The MHC class II and TCR recognition sites are indicated.

EFFECTS ON OTHER CELLS

Besides activation of T cells, increasing evidence shows the ability of staphylococcal enterotoxins to interact with other cells, such as B cells, eosinophils, mast cells and epithelial cells. Similarly to T cell binding, this unconventional type of B cell ligands can, in principle, trigger all B cells bearing the appropriate variable region, regardless of the other junctional and diversity segments. Interestingly, the concentrations of SEs and the surrounding cytokines determine the effect of SEs on B cells. Cross-linking of the MHC class II molecule on B cells with TCR on T cells results in IgE production, when low amounts of IFN-γ are combined with low SE concentration. However, IgE production is inhibited by IFN-γ upon high SE concentration. Interestingly, the effect of SEs on B cell stimulation also depends on the type of superantigen. SEA and SEB fail to induce proliferation of B cells in the absence of T cells. Survival of B cells on the other hand is enhanced by SEB.

Eosinophils are mostly studied as actors in the effector limb of the immune response, because of their origin in the myelocytic cell series and biochemical similarities to neutrophils. However, several biological features of human eosinophils suggest they may also perform functions in the afferent limb of the immune response. Human eosinophils become hypodense and express class II major
Chapter 2: Role of staphylococcal superantigens in airway disease

Histocompatibility (MHC) molecules when activated by granulocyte-macrophage colony-stimulating factor (GM-CSF) in vitro or in vivo in pathological conditions such as allergic disorders. Studies have therefore been undertaken to examine the capacity of class II MHC-expressing eosinophils to serve as antigen-presenting cells (APC) for resting and activated CD4+ T cells. Preincubated eosinophils induced resting T cells to proliferate in response to SEs (SEA, SEB and SEE). Furthermore, superantigen-induced T cell proliferation correlated with the proportion of eosinophils expressing class II MHC molecules.

**Figure 5: Effects of staphylococcal enterotoxins on different cell types.**

Compared to macrophages however, eosinophils are not as efficient in acting as accessory cells for SE-induced T cell proliferation. The function of macrophages as accessory cells for SE-induced T cell proliferation has already extensively been studied in several research lines. On the other hand, direct effects of SE on macrophage activity have been documented, in particular on the production of cytokines. Interleukin 8 is synthesized and secreted by human alveolar macrophages upon incubation with SEA. Furthermore, stimulation of macrophages with SEB causes release of chemotactic protein...
leading to neutrophil migration by a mechanism mediated by platelet-activating factor, histamine H(2) receptors, lipoxygenase products and substance P.

Similar to eosinophils and macrophages, mast cells can also be either directly activated by SEs, but also act as accessory cells for T cell activation. Staphylococcal protein A is capable of cross-linking IgE molecules on mast cells. Mast cells isolated from human heart tissue are able to release histamine, tryptase en leuktrien C4, when incubated with staphylococcal protein A.

The effect of SEs, in particular SEB, on epithelial cells is extensively elaborated in Chapter 4. Briefly, SEB can activate the immune system after contact with epithelial cells by MHC class II binding and cross-linking. In this case, epithelial cells act as accessory cells for superantigen-induced T cell activation. However, involvement of non MHC class II receptors has also been demonstrated. Moreover, SEB is known to activate APCs like dendritic cells via Toll-like receptor (TLR)2, a receptor which plays an important role in pathogen recognition and innate immunity. Interestingly, these TLRs are also present on nonprofessional antigen presenting cells like these epithelial cells.

Our findings on the effect of SEB on human nasal epithelial cells, with chemokine release and subsequent increase in granulocyte migration and survival are described in Chapter 4.

ROLE OF STAPHYLOCOCCAL ENTEROTOXINS IN DISEASE

Atopic dermatitis

Atopic dermatitis (AD) is a chronically relapsing inflammatory disease of still unknown aetiopathogenesis. A typical characteristic of AD is the infiltration of T cells, monocytes/macrophages, and eosinophils into the skin lesions. AD patients show an enhanced susceptibility to cutaneous infections with certain viral, fungal and microbial pathogens. The first evidence that S. aureus might play a role in AD has been given already 30 years ago. In this regard, the prominent role of S. aureus skin colonization as a factor contributing to the exacerbation of AD has been well established. In AD patients colonization with S. aureus is found significantly more (80-
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100%) compared to healthy controls (5-30%)\textsuperscript{42}. This is likely the result of a combination of host factors including skin barrier dysfunction as well as impaired host immune responses in AD\textsuperscript{43}. The majority of these \textit{S. aureus} isolates from AD skin secrete identifiable \textit{S. aureus} enterotoxin A, SEB and toxic shock syndrome toxin (TSST)-1, suggesting a direct involvement of SAg in the disease mechanism. Furthermore, IgE to these toxins was found in the serum of 57\% of these patients\textsuperscript{44}, and these IgE antisuperantigens correlate with skin disease severity. This study also showed an increased numbers of T cell bearing a superantigen-specific TCR V\textbeta repertoire in diseased skin of AD patients, further supporting the role of SAgs in AD pathophysiology.

Superantigens have been demonstrated to induce corticosteroid resistance of T cells in vitro\textsuperscript{45}. This could contribute to difficulty in management of AD, because topical corticosteroids are the most common medication used for treatment of atopic dermatitis. Indeed, \textit{S. aureus} isolates from patients with steroid-resistant atopic dermatitis showed the ability to produce large numbers of superantigen types per organism, significantly higher than those produced by other skin isolates. Furthermore, \textit{S. aureus} isolates from patients with steroid-resistant atopic dermatitis have been selected for their production of greater numbers of superantigens than those produced by isolates from a general population of patients with atopic dermatitis. Thirdly, in addition to having the potential to make more types of superantigens, \textit{S. aureus} isolates from patients with steroid-resistant atopic dermatitis also have dysregulated production of superantigens and produce unusual combinations of superantigens\textsuperscript{46}.

Mouse models of atopic dermatitis have been developed, using epicutaneous immunization with SEB. This elicits allergic skin inflammation accompanied by a systemic Th2 response to the superantigen, suggesting that superantigens might play a role in the pathogenesis of AD\textsuperscript{47}. Indeed, epicutaneous exposure to SEB elicited a local, cutaneous, inflammatory response characterized by dermal infiltration with eosinophils and mononuclear cells and increased mRNA expression of the Th2 cytokine IL-4, sharing several features with skin lesions in AD. In another model, topical SEB treatment provokes epidermal accumulation of CD8+ T cells, a mixed Th1/Th2 type dermatitis and vigorous production of SEB-specific IgE and IgG2a antibodies, as well as significantly increased the
production of OVA-specific IgE and IgG2a antibodies\textsuperscript{48}. These findings can be related to the chronic phase of atopic skin inflammation.

**Food allergy**

SEs have an established association with several atopic conditions. *S. aureus* contamination is one of the most prevalent causes of food poisoning, and SEs are most frequently found in foods, such as milk, dairy products, eggs, meats and fish, all food allergens\textsuperscript{49}. As described above, SEB is resistant to breakdown by stomach acid and is transcytosed across the intestinal epithelium. This led to the development of a new mouse model of food allergy to oral antigen, using concomitant ovalbumin (OVA) and SEB application. In this model SEB administered though oral route, was able to elicit sensitization to low doses OVA, promoting a dominant Th2 response\textsuperscript{50}. Although this was only the case when SEB was administered together with the antigen, as SEB alone did not show any response. The authors hypothesize that SEB exposure leads to allergic sensitization to food allergens, perhaps through breakthrough of oral tolerance. The development of Th2 immunity to oral antigen in this model likely occurred as a result of a loss in immune suppression, with decreased levels of FoxP3 mRNA and TGF-β levels in OVA/SEB mice.

**Mouse models**

Other experimental animal models have been described using SEB in the study for colitis\textsuperscript{51}. In this model, the authors demonstrate alteration of Treg development by SEB, which contributes to the activation of effector T cells in a dysregulated environment. The observation that mucosal exposure to SEs activated the development of intestinal inflammation in immune deficient hosts may explain how gut flora and bacterium-derived products could lead to the development of chronic IBD in immune-dysregulated intestinal mucosa.
One other interesting experimental model from Rajagopalan et al. studies the superantigenic capacity of SEs in the field of toxic shock syndrome (TSS), a serious systemic illness. TSS is caused by SAgs which cause a massive T cell activation. The T cells activated by SAg rapidly produce large amounts of cytokines and chemokines resulting in a sudden surge in the systemic levels of these biological mediators. This process, called systemic inflammatory response syndrome, may lead to multiple organ dysfunction syndrome, wherein several vital organs within the body fail to perform their physiological functions. The usage of mouse models to study TSS is necessary in order to find new therapeutical strategies for this possible life-threatening disease, caused by the superantigen exotoxins of \textit{S. aureus}.

\textbf{ROLE OF STAPHYLOCOCCAL ENTEROTOXINS IN AIRWAY DISEASE}

\textit{Staphylococcus aureus} is an ubiquitous common human pathogen, which is most frequently found in the anterior nares of the nose. Producing enterotoxins with superantigenic properties, \textit{S. aureus} is known to modulate airway disease. Most likely, it is not the purpose of these superantigens to induce systemic toxic shock in the host, but rather act locally, probably suppressing local immune responses. However, transcytosis of SEB, a prototypic staphylococcal superantigen, has been described in intestinal epithelium\textsuperscript{53, 54}. Furthermore, SEB can elicit robust systemic immune activation following exposure through non-enteric portals of entry such as the nasal, conjunctival or vaginal routes\textsuperscript{55}. In order to study the systemic levels of SEB after endonasal application in our studies, we have demonstrated a time-dependent increase in serum levels of SEB after endonasal application, demonstrating the systemic absorption after local airway application.
Chapter 2: Role of staphylococcal superantigens in airway disease

Figure 6: time-dependent increase in serum levels of SEB after endonasal application. Local upper airway application of SEB results in systemic absorption.

Allergic rhinitis

Similarly to atopic dermatitis, a role for staphylococcal enterotoxins has been suggested in allergic rhinitis. It has been reported that the nasal carriage of S. aureus was higher in patients with perennial allergic rhinitis (44%) than in control subjects (20%). There was an even more significant association with enterotoxin-producing S. aureus (22% versus 6.7% in control subjects)\textsuperscript{56}. Rossi\textsuperscript{57} analyzed the prevalence of serum-specific IgE towards SEA, SEB, SEC, SED, and TSST-1 in dust mite-allergic patients with allergic rhinitis and found an increase in serum eosinophil cationic protein in patients with anti-enterotoxin IgE compared with IgE-negative patients. This observation pointed to an involvement of SEs in local IgE production and a Th2-type biased eosinophil inflammation. Further evidence was obtained from animal experiments with BALB/c mice that were intranasally sensitized with Schistosoma mansoni egg antigen (SmEA) in the presence or absence of SEB. Nasal exposure to SEB resulted in enhanced development of allergic rhinitis in SmEA-sensitized mice, shown by SmEA-specific IgE production, nasal eosinophilia, and IL-4 and IL-5 production by nasal mononuclear cells after antigen challenge\textsuperscript{58}. 

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Nasal Polyposis

Nasal polyps, also referred to as chronic rhinosinusitis with nasal polyps (CRSwNP), are characterized by an eosinophilic type of inflammation, driven by interleukin-5 and eotaxin, which together orchestrate the chemotaxis, activation and survival of eosinophils. An increased colonization rate with *S. aureus* has been described in 60% of CRSwNP patients, and 87% of CRSwNP with co-morbid asthma and aspirin sensitivity, vs. 33% in control patients. Furthermore, IgE antibodies to SEs were present in 28% in polyp samples, with rates as high as 80% in the subgroup with asthma and aspirin sensitivity, as compared with 15% in control individuals. Moreover, an increased number of T cells expressing the TCR Vβ known to be induced by microbial superantigens was detected in CRSwNP and correlated with the presence of specific IgE against SEs. This SE-specific IgE serves as an indicator for superantigen impact on the mucosal inflammation. These specific IgE antibody level in the tissue is associated with high total IgE titers, which is polyclonal. Interestingly, this polyclonal IgE from mucosal tissue appears to be functional, and may induce mast cell degranulation to numerous inhalant allergens. Together with the fact that SEB may serve as an allergen, these findings may contribute to the persistent inflammation by continuously activating mast cells. These findings confirm the role played by *S. aureus* enterotoxins as disease modifiers in CRSwNP.

Asthma

The relation between SE-specific IgE and severity of asthma has been suggested from the above mentioned data in CRSwNP patients. Interestingly, it has been shown that serum from asthmatic patients contains more often SE-specific IgE compared to serum from controls and. Furthermore, within the group of asthmatic patients, severe asthmatics have more often SE-specific IgE than those with mild asthma. A pathophysiologic link between SE-specific IgE and bronchial inflammation has therefore been suggested. In mice, SEB enhanced the allergen-induced bronchial inflammation in experimental asthma, as reflected by more eosinophilic inflammation in the airway lumen and in bronchial tissue. Aggravation of experimental asthma correlated with higher expression of mRNA for...
IL-5, IL-4, IFN-γ, IL-12 p40, eotaxin-1 and TGF-β in bronchi. In addition, nasal SEB elevated concentrations of IL-4, IL-5 and IFN-γ in serum and bronchial SEB increased titres of OVA-specific and total IgE in serum.\textsuperscript{66}

Staphylococcal enterotoxins cause a Th2 biased inflammation and amplification in CRSwNP patients. In addition, in a specific phenotype of CRSwNP patients, namely the ones with high IL-5 expression, SEs significantly modify the severity of upper airway inflammation. Interestingly, these patients carry a considerable risk for comorbid asthma.\textsuperscript{67}

Recently, a new hypothesis was formulated, questioning the role of superantigens in the development of intrinsic asthma.\textsuperscript{68} S. aureus is able to invade bronchial epithelial cells and release its enterotoxins. Subsequent stimulation of T and B cell proliferation may follow, as well as induction of class-switching to IgE and the production of allergen-specific IgE in mucosal B cells. Furthermore, S. aureus is also capable of invading mast cells and causing the release of cytokines. It is therefore possible that patients with intrinsic asthma may in some way be susceptible to colonization of the lower airways with S. aureus, which, through the local released of superantigens, drives an allergic inflammatory response and local IgE formation, which is associated with more severe asthma as the anti-inflammatory response to corticosteroids is reduced.\textsuperscript{68} These findings correlate with murine data, where SEB induces lymphocytic inflammation and eosinophilia in the lungs with increased production of IL-4, together with airway hyperresponsiveness in both IgE-and non-IgE-producing strains, demonstrating the capacity of superantigens to induce allergic inflammation independent of any allergen.\textsuperscript{69}

In early childhood, SEs are also potential modifiers of childhood wheeze and eczema. The levels of SE-specific IgE in children with eczema and wheeze is significantly increased compared to healthy 5-year-old children. Furthermore, the proportion of patients sensitized to SEs increases with increasing symptoms severity.\textsuperscript{70}

In another cohort of 1380 teenagers, we were able to demonstrate a positive association between type 2 immunity to S. aureus and asthma phenotypes.\textsuperscript{71} This association reflects IgE-mediated effector cell
activation of SEs, which are secreted in soluble form. In this study, we could demonstrate significant higher SE-specific IgE levels in atopic individuals, compared to non-atopic individuals. Moreover, in univariate models SE-specific IgE titre was a significant risk factor for asthma and BHR, particularly amongst atopics (Figure 7).

In non-atopics, SE-specific IgE appeared to function as an independent risk factor for BHR, implying that despite its low titre this IgE may contribute to airway inflammation in subjects in whom significant IgE of other specificities is extremely rare. Interestingly, we found a contrasting benign nature of IgE to *H. influenzae* and *S. pneumoniae* antigens: HI- and SP-specific IgE is inversely associated with asthma risk in healthy atopic teenagers despite the fact that respective mean production levels are higher than corresponding responses to SEs which are positively associated with risk. This may reflect their lower availability in soluble forms that can crosslink IgE receptors. We theorize that instead they may be processed by antigen presenting cells and presented to type-2 memory cells leading to mucosal secretion of IL-4/IL-13, a mechanism widely recognized in other tissues to attenuate T-helper-1 associated bacterial-induced inflammation.

The role of SEs in the pathophysiology of asthma is further elaborated in Chapter 3.
COPD

Staphylococcal enterotoxins can modulate airway inflammation in COPD patients, similar to unstable asthma. This was studied in smokers, stable COPD, exacerbated COPD, and healthy controls. The authors found a significant increase in serum SE-specific IgE levels in COPD patients compared to controls. This indeed reflects the superantigen activity on B and T cells of these SEs, which challenge the lower airways locally. This hypothesis is reinforced by the fact that bacterial colonization in the lower airways is increased in COPD patients, including *S. aureus*. Moreover, during exacerbations of COPD, *S. aureus* has been found as a pathogen. Altogether, these findings indicate a possible role for SEs in the pathogenesis of COPD, similar to that in severe asthma. This hypothesis is further elaborated in Chapter 6.
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Aims of the studies
AIMS OF THE STUDIES

The aims of the studies described in this thesis are to investigate the role of *Staphylococcus aureus* enterotoxin B as inducer and modulator of airway inflammation. For this purpose, we took advantage of mouse models of allergic asthma and cigarette smoke-induced inflammation, as well as in vitro models. Furthermore, a comparison was made between upper and lower airway inflammation in CS-exposed mice.

Specific aims of this thesis are:

1. To investigate the role of SEB in the induction of allergic sensitization upon combined application with inhaled allergen. We aimed to unravel the mechanisms involved in the development of allergic sensitization in this model, in particular the role of dendritic cells and T cells. *(Chapter 3)*

2. To study the immunologic interaction between SEB and airway epithelial cells. We used a novel technique of freshly isolated and purified human nasal epithelial cells to evaluate chemokine production after SEB application. In addition, the chemotactic activity of the supernatant for granulocytes was evaluated. *(Chapter 4)*

3. To investigate and compare the effects of CS on upper and lower airways, in a mouse model of subacute and chronic CS exposure. We obtained bronchoalveolar lavage fluid and tissue cryosections from nasal turbinates for staining of neutrophils and T cells. Furthermore, we evaluated cytokines and chemokines in nasal turbinates and lungs by RT-PCR. *(Chapter 5)*

4. To study the effect of SEB on cigarette smoke-induced inflammation, in a mouse model of COPD. We evaluated the aggravating effects of endonasal SEB application upon concomitant CS-exposure in C57/Bl6 mice. *(Chapter 6)*
Chapter 3

**SEB facilitates allergic sensitization**

**in experimental asthma**
STAPHYLOCOCCUS AUREUS ENTEROTOXIN B FACILITATES ALLERGIC SENSITIZATION IN EXPERIMENTAL ASTHMA

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ABSTRACT

Background: Staphylococcus aureus Enterotoxin B (SEB) has immunomodulatory effects in allergic airway disease. The potential contribution of SEB to the sensitization process to allergens remains obscure.

Objective: We evaluated the effect of concomitant airway exposure to the allergen ovalbumin (OVA) and different bacterial derived toxins on the induction of experimental allergic asthma.

Methods: Nasal applications of OVA and Saline, SEA, SEB, TSST-1, Protein A or LPS were performed on alternate days from day 0 till 12. On day 14, mice were sacrificed for evaluation of OVA-specific IgE, cytokine production by mediastinal lymph node (MLN) cells and bronchial hyperreactivity (BHR) to inhaled metacholine. The effect of SEB on dendritic cell (DC) migration and maturation, and on T cell proliferation was evaluated.

Results: Concomitant endonasal application of OVA and SEB resulted in OVA-specific IgE production, whereas this was not found with SEA, TSST-1, Protein A or LPS. Increased DC maturation and migration to the draining lymph nodes was observed in OVA/SEB mice, as well as an increased T cell proliferation. Bronchial inflammation with influx of eosinophils and lymphocytes was demonstrated in OVA/SEB mice, together with bronchial hyperresponsiveness and production of IL-4, IL-5, IL-10 and IL-13 by MLN stimulated with OVA.
Conclusions: Our data demonstrate that SEB facilitates sensitization to OVA and consecutive bronchial inflammation with features of allergic asthma. This is likely due to augmentation of DC migration and maturation, as well as the allergen-specific T cell proliferation upon concomitant OVA and SEB application.

INTRODUCTION

Epidemiologic data provide strong evidence for a steady increase in the prevalence of allergic diseases in the Western world over the past three decades, reaching epidemic proportions\(^1\)\(^-\)\(^3\). As the reason for the steep rise in prevalence of allergic rhinitis and asthma remains speculative, several hypotheses have been put forward to explain this phenomenon. Among others, environmental factors may interfere with the sensitization process and lead to the development of allergic inflammation\(^4\). These exogenous factors provide immunostimulatory signals that are necessary to overcome immunological tolerance and induce allergic sensitization\(^5\)\(^-\)\(^12\). Eventually, these immunostimulatory signals result in dendritic cell (DC) activation, leading to functional DC differentiation and priming of Th2 effector cells\(^13\).

Enterotoxins of *Staphylococcus aureus* have been implicated in the pathology of chronic airway inflammation, as IgE directed against *Staphylococcus aureus* enterotoxins (SAE) was found in chronic upper airway inflammation\(^14\)\(^-\)\(^16\). These SAE act as superantigens as they potentially activate T cells via linkage of the β chain of the T cell receptor to MHC class II molecules on antigen presenting cells (APC) outside the peptide-binding groove area\(^17\).

Recent evidence suggests a putative role of these enterotoxins in allergic diseases. In patients suffering from the atopic eczema/dermatitis syndrome (AEDS), colonization with *S. aureus* is found more frequently (80-100%) compared to healthy controls (5-30%)\(^18\), and *S. aureus* isolates secrete identifiable *S. aureus* enterotoxins A (SEA), SEB and toxic shock syndrome toxin (TSST)-1. IgE to these toxins was found in the serum of 57% of AEDS patients\(^19\), indicating immune responses against these bacterial products. Beside AEDS, 25% of allergic rhinitis (AR) patients have detectable serum
IgE levels to SAE, whereas this is only a minority of healthy controls (6.3%). In AR, the presence of SAE-specific IgE was associated with the highest titer of total serum IgE\textsuperscript{14,20}. IgE against SEA and SEB was also found in nasal polyps (NP)\textsuperscript{21} and levels of SAE-specific IgE in NP correlated with markers of eosinophil activation and recruitment\textsuperscript{22,23}. In mice, Herz et al\textsuperscript{24} reported lymphocyte dependent bronchial inflammation with increased bronchial responsiveness by repeated nasal application of SEB. Furthermore, epicutaneous sensitization of mice with SEB elicited an immune response with Th2 cell activation, allergic skin inflammation and increased IgE titres in serum\textsuperscript{25}. Recently, a new mouse model of food allergy was described, demonstrating that SEB impairs the oral tolerance\textsuperscript{26}. Finally, we previously reported aggravation of experimental allergic asthma by application of SEB, characterized by higher of IL-4 production and allergen-specific IgE\textsuperscript{27}.

Until now, the contribution of SAE to the sensitization to inhaled allergens remains unclear. Therefore, we took advantage of a mouse model of repeated endonasal applications of enterotoxins of Staphylococcus aureus in order to study which of the enterotoxins alters the sensitization to allergens, and whether this process involves the activity of sensitized CD4\textsuperscript{+} cells. Evaluating the involved mechanisms in this model, we investigated the behavior of dendritic cells and allergen-specific T cells.

**MATERIALS AND METHODS**

**Mice**

Male inbred BALB/c mice were obtained from Harlan CBP (Horst, the Netherlands) and kept under conventional relative pathogen-free conditions. 6 to 8 week old mice were used in all experiments, with 5 to 6 mice per group. OVA-TCR transgenic mice (DO11.10) were purchased from the Jackson Laboratory (ME, USA).
**Experimental protocol**

Nasal application of 50 µL of pyrogen-free saline, SEB, SEA, TSST-1, protein A (all 10 µg/mL) or LPS (0.1 µg/mL) combined with OVA at 10 mg/mL was performed by means of placing this volume at the nostrils of spontaneously breathing mice in a supine position. Seven applications were performed on alternate days from day 0 till day 12, as depicted in Fig. 1A. In subsequent experiments, 50 µL of pyrogen-free saline, SEB 10µg/mL, Sigma-Aldrich, LPS content: 34.82 pg/mL) and/or OVA (10 mg/mL, grade V, Sigma-Aldrich, Saint Louis, USA, LPS content: 0.18 µg/mL) was endonasally applied in an identical schedule (Fig. 2A).

![Figure 1](image.png)

**Figure 1**: (A) Experimental protocol: Nasal application of 50 µL of saline, SEB, SEA, TSST-1, protein A (all 10 µg/mL) or LPS (0.1 µg/mL) combined with OVA (10 mg/mL) was performed 7 times on alternate days from day 0 till day 12.

The dose of SEB was chosen according to dosing experiments in naïve\(^{24}\) and in sensitized mice\(^{27}\), where it was shown that 500 ng of SEB potently altered bronchial inflammation without clinical signs of wasting disease. The same dose was chosen for the other toxins. The chosen dose of LPS has been described to induce Th2 responses in a mouse model of allergic asthma\(^{9}\). Mice were sacrificed on day 14, i.e. 48h after the last application. All experimental procedures were approved by the local Ethical Committee.
Assessment of bronchial responsiveness (BHR)

Airway responses to inhaled methacholine (MCh) was measured using the forced ventilation technique (FlexiVent, SCIREQ, Montreal, Canada) as reported previously. 48h after the last endonasal application, airway resistance and compliance to incremental doses of MCh was assessed. To this purpose, mice were anaesthetized with sodium pentobarbital (70mg/kg i.p.). After exposure of the trachea, a tracheotomy was performed and a 19-gauge metal needle inserted into the bronchus. The mice were connected to a computer-controlled small animal ventilator and were quasi-sinusoidally ventilated with a tidal volume of 10 mL/kg at frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H$_2$O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of baseline values, each mouse was exposed to an aerosol, generated with an in-line nebulizer and administered directly through the ventilator for 5 seconds, containing increasing concentrations of MCh (0, 0.625, 1.25, 2.5, 5 and 10 mg/mL). Airway resistance (R) was measured using a “snapshot” protocol each 20 seconds for 2 minutes. For each concentration of MCh, the mean of six values of R was calculated. For each mouse, R was plotted against the MCh concentration (from 0 to 10 mg/mL) and the area under the curve (AUC) was calculated.

Measurement of OVA-specific and total IgE levels

After anaesthesia with sodium pentobarbital, a retro-orbital bleed was performed on day 14 (i.e. 48h after the last exposure) and serum was frozen until analysis. For measurement of OVA-specific IgG2a, 96-well plates were first coated overnight with rat anti-mouse IgG2a (20 µg in 100 µL PBS, PharMingen, San Diego, CA, USA). Remaining binding sites were blocked and plates were incubated with 100 µL of diluted serum (1:10). After washing, following substances were sequentially added, incubated and washed: OVA (1 µg/100 µL, Sigma), peroxidase-labeled rabbit anti-OVA IgG (240 ng/100 µL, Rockland, Gilbertsville, PA, USA) and buffer containing tetramethylbenzidin dihydrochloridhydrate (1 µL/100 µL, ACROS, New Jersey, NJ, USA) and H2O2 (1 µL/100 µL). Then, H$_2$SO$_4$ was added and the optical density measured at 450 nm.
For measurement of OVA-specific and total IgE, commercially available ELISA kits were purchased and samples were processed according to the manufacturer’s guidelines (OVA-specific IgE: MD Biosciences, Zürich, Switzerland – total IgE: Pharmingen). Data were expressed as ng/mL (OVA-specific IgE) or as optical density values at 450 nm (total IgE, OVA-specific IgG2a).

**Analysis of broncho-alveolar lavage fluid**

On day 14, mice were lethally anaesthetized with sodium pentobarbital. A polyethylene catheter (0.85 mm) connected to a syringe was gently inserted into the trachea. Then, the bronchoalveolar tree was lavaged five times with 1 mL aliquots of pyrogen-free phosphate-buffered saline (PBS) supplemented with 5% of bovine serum albumin (BSA; Sigma, Bornem, Belgium) at 37°C through a polyethylene tracheal catheter (0.85 mm). The first lavage was performed with 1 mL PBS/BSA 5%, centrifuged (1400 × g, 5 min) and the supernatant was stored at −20°C until analysis. The cellular pellet was added to the subsequent four lavages, each one performed with 1 mL of PBS. The bronchoalveolar lavage (BAL) fluid was centrifuged (1400 × g, 5 min) and the pellet washed and resuspended in 100 µL of PBS. Ten microlitres of cell suspension from BAL fluid were added to 90 µL of Türk's solution (Merck Diagnostica, Darmstadt, Germany), and the total number of cells was counted in a Bürker–Türk chamber. For differential cell counts, cytospin preparations were stained according to the May–Grünwald–Giemsa method and 300 cells differentiated into eosinophils, neutrophils, monocytes/macrophages and lymphocytes according to standard morphological criteria.
Histologic analysis

After performing BAL, the bronchoalveolar tree was removed for histological examination and fixed overnight in buffered formalin. After dehydration and embedding in paraffin, 3 µm sections were stained with hematoxylin and eosin (H&E) to demonstrate inflammatory infiltrates, or Congo red to highlight eosinophils. Quantification of eosinophilic influx was performed on Congo red staining by counting the number of eosinophils per airway wall at magnification x 400, for 10 representative airways with perimeter of basement membrane around 800 µm.

Cytokine production by draining lymph node (LN) cells

At the time of analysis, submandibular and peribronchial lymph nodes (LN) were dissected and homogenized using a cell strainer (Falcon®, Becton Dickinson, Franklin Lakes, NJ, USA) and suspended in RPMI (Biowhittaker, Walkersville, MD, USA) supplemented with FCS (5%). The homogenates were washed twice with PBS/FCS and centrifuged at 1500 rpm for 10 min. In parallel, spleen homogenates were incubated for 1 min with 5 mL of lysis buffer containing NH₄Cl, KHCO₃ and Na₂EDTA, and then washed with PBS/FCS. Cells were counted using a Coulter Counter (Analis, Ghent, Belgium).

For analysis of cytokine production, 1×10⁶ LN cells were incubated with splenocytes from naive mice, as a source of antigen-presenting cells, in the presence of OVA, BSA (as negative control) or SEB in 1 mL of culture medium (RPMI with 10% FCS supplemented with penicillin, streptomycin, L-glutamine and 0.1% of 2-mercaptoethanol). Pilot studies showed that 1 ng/mL and 10 µg/mL were the optimal doses for SEB and OVA/BSA respectively for further experiments (data not shown). Cells were incubated for 5 days at 37°C. The supernatants were stored at –20°C until cytokine measurement.

To determine levels of IL-4, IL-5, IL-13, IL-10, IL-12 and IFN-γ, a Cytometric Bead Array Flex set (BD Biosciences, Erembodegem, Belgium) was used. IL-17 (R&D) and TGF-β (BD Biosciences) were measured by ELISA according to the manufacturer’s specifications.
Anti-CD4 mAb treatment

To study the role of CD4+ cells in the SEB-mediated effects on sensitization to OVA, 50 µg of GK1.5 rat anti-mouse CD4 blocking antibody (Biolegend, San Diego, CA, USA) was administered i.p. in a volume of 0.5 mL on day 0 and day 6, which is during the phase of exposure of mice to OVA and SEB from day 0 until 12. Rat IgG (50 µg in 0.5 mL, Rockland, Gilbertsville, PA, USA) was injected i.p. on day 0 and 6 as control. The antibody preparations were centrifuged to remove aggregates (30000 x g, 20 min) as reported previously. Groups consisted of 5 mice and were exposed endonasally to both OVA and SEB from day 0 until 12 on alternate days.

Reduction of CD4+ cells was assessed in blood and draining lymph nodes. To this purpose, blood was collected in 0.5M EDTA by retro-orbital bleed. Subsequently, draining lymph nodes were taken and put through a cell strainer. Samples were incubated with antibodies against CD3 (rat anti-mouse clone RM4-5), CD8 (rat anti-mouse Ly-2 Clone 53–67) and CD4 (rat anti-mouse clone 17A2, all BD Biosciences). The red blood cells were lysed and all cells were fixed with 4% paraformaldehyde. 10 x 10^3 lymphocytes were analyzed using a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA). Data were analyzed using CellQuest Software.

Analysis of DC and T cell behavior

DC migration was evaluated in vivo 24h after i.t. application of 500µg OVA-FITC. For that, mice were slightly anaesthetized with isoflurane. Mediastinal lymph node (MLN) cells were harvested and stained with antibodies against CD11c and MHC class II. DAPI was used to exclude dead cells. Cells were analyzed by FACS Aria II (BD Biosciences).
DC maturation was investigated in bone marrow–derived dendritic cell cultures from naive WT BALB/c mice. Bone marrow cells were collected, depleted of red blood cells using RBC-lysis buffer (0.15M NH₄Cl, 1mM KHCO₃, 0.1mM Na₂EDTA), and grown in RPMI culture medium containing 5% FCS and GM-CSF for 8 days. At day 8, DCs were pulsed in vitro with TCM, OVA (100 µg/ml), SEB (100 ng/ml) or OVA/SEB. After 24h, cells were collected and stained for MHC class II, CD11c, CD86 or isotype control before analysis by FACS Aria II.

To study T cell proliferation in vivo, cell suspensions of pooled peripheral lymph nodes and spleen from naive OVA T-cell receptor transgenic DO11.10 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). Then, 5 x 10⁶ CFSE+ T cells were transferred intravenously into naive WT mice. After 24h, mice received OVA (100 µg) +/- SEB (500 ng) in 55 µl PBS intratracheally. Seventy-two hours after i.t. injection, MLN were analyzed for the proliferation of CFSE-labeled OVA-specific TCR Tg T cells. As a control, non–lung-draining LNs were used. Transferred OVA-specific TCR transgenic CD4+ T cells were recognized as CD4+CFSE+ cells expressing the clonotypic TCR. DAPI was used to exclude dead cells. T cell divisions were measured by flow cytometry. The percentage of cells recruited into each cell division was calculated by dividing the number of individual cells by CFSE content, after correction for the multiplying effect of division, as describe previously.

**Statistical analysis**

Statistical analysis was performed with Medcalc software 9.2.0.1 (F. Schoonjans, Belgium; http://www.medcalc.be). Data are expressed as mean with error bars expressing standard error of the mean. All outcome variables were compared using non-parametrical tests (Kruskal-Wallis; Mann Whitney U test for unpaired data). The significance level was set at α = 0.05.
RESULTS

Effects of different toxins on OVA-specific and total IgE levels

In order to study the effects of staphylococcal derived toxins on the sensitization to ovalbumin and induction of allergic airway inflammation, we have combined the nasal application of OVA with different toxins. Interestingly, only the combination of OVA and *Staphylococcus aureus* enterotoxin B resulted in increased serum levels of OVA-specific IgE compared to OVA/Sal, whereas this was not the case for other proteins (Fig. 1B). Moreover, levels of total IgE were also significantly higher in OVA/SEB mice (OD 0.69 ± 0.1) compared to OVA/Sal (OD 0.33 ± 0.02, p < 0.0001). Concurrent application of OVA/Prot A also resulted in modestly raised levels of total IgE (OD 0.47 ± 0.07, p < 0.05). The combined application of OVA and LPS did not result in raised OVA-specific or total IgE levels in our model (Fig. 1B-C).

Consequently, the same protocol was used to study the effect of SEB on the immune response to OVA (Fig. 2A), and we confirmed that repeated and concomitant exposure to OVA and SEB resulted in significantly higher titers of OVA-specific IgE, compared to the three control groups Sal/Sal, Sal/SEB or OVA/Sal (Fig. 2B). In addition, OVA-specific IgG2a levels were also significantly higher in the OVA/SEB group (OD 43.59 ± 4.56) compared to all other groups (OD 0.18 ± 13 in Sal/Sal, 7.68 ± 7.65 in Sal/SEB and 19.44 ± 4.53 in OVA/Sal, p < 0.05, Fig. 2C).
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Figure 1: (B) Experimental protocol: on day 14, OVA-specific IgE levels in serum were evaluated. Interestingly, only the combination of OVA and Staphylococcus aureus enterotoxin B resulted in increased serum levels of OVA-specific IgE, whereas this was not the case for other proteins. (C) Moreover, levels of total IgE were also significantly higher in OVA/SEB mice compared to OVA/Sal, and concurrent application of OVA/Prot A also resulted in modestly raised levels of total IgE. *p < 0.05, ***p < 0.001 compared to OVA/Sal.

Effects of SEB on the OVA-induced bronchial allergic inflammation

Mice that were sensitized to OVA following OVA/SEB application developed features of allergic asthma. A significant influx of eosinophils and lymphocytes counts in the broncho-alveolar lumen was seen in mice that were simultaneously exposed to OVA and SEB (p < 0.01, Fig. 2D). Interestingly, neither Sal/SEB nor OVA/Sal exposure caused any bronchial influx of eosinophils. Sal/SEB application resulted in raised BAL lymphocyte and neutrophil number compared to controls (p < 0.05, Fig. 2D). The total number of cells in BAL fluid did not differ between groups (data not shown).

Simultaneous application of OVA and SEB resulted in a significant increase in bronchial responsiveness (BHR, measured as described above) to incremental doses of nebulized methacholine (p < 0.01, Fig. 2E) compared to all other conditions, while OVA or SEB application alone did not cause any BHR compared to control mice.
The BAL fluid findings were in line with the histologic analyses, revealing that concomitant application of OVA and SEB induced bronchial inflammation in the OVA/SEB group with minor inflammation around bronchi in the 3 control groups (Fig. 3, A-D). Detailed analysis of the cellular infiltrate revealed that simultaneous application of OVA and SEB caused a significant higher number of eosinophils in the lung parenchyma of OVA/SEB (2.825 ± 0.66) mice compared to the 3 other groups (0.32 ± 0.06, 0.43 ± 0.24 and 0.87 ± 0.49, p < 0.05), as was shown by Congo red staining.

Figure 2: (A) Experimental protocol. (B) Concomitant application of OVA and SEB resulted in increased levels of OVA-specific IgE levels, (C) OVA-specific IgG2a levels, (D) BAL eosinophils and lymphocytes and (E) bronchial hyperreactivity measured as resistance (R), compared to control conditions. One representative experiment out of 2 is shown. *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.01 vs. all others (eosinophils) and vs. OVA/Sal and Sal/Sal (lymphocytes). §p < 0.01 vs. Sal/Sal (neutrophils). ●p < 0.05 vs. Sal/Sal (lymphocytes).
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Figure 3: Histologic analysis revealed that concomitant application of OVA and SEB induced bronchial inflammation primarily in the OVA/SEB group (D) with minor inflammation around bronchi in the 3 control groups: Sal/Sal (A), Sal/SEB (B) and OVA/Sal (C). H&E, x200. Representative bronchial sections are presented.

Cytokine profile analysis of SEB mediated effects on OVA sensitization

Submandibular and peribronchial draining lymph nodes were harvested for IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, TGF-β and IL-17 cytokine production, after stimulation with OVA, BSA or SEB. LN cells of OVA/SEB mice showed an increase in IL-4, IL-5, IL-13 and IL-10 production upon stimulation with OVA compared to BSA (p < 0.05, Fig. 4, A-D). In vitro stimulation of OVA/SEB LN cells with OVA induced higher IL-5 production compared to OVA stimulation of control LN cells (Fig. 4B). This trend was also seen for IL-4, IL-10 and IL-13 although significance was not reached for these cytokines. In contrast to the Th2 cytokines, the production of IFN-γ, IL-12, TGF-β or IL-17 was not altered in the OVA/SEB group upon stimulation with OVA (data not shown).
Intraperitoneal injection of blocking anti-CD4 mAb significantly altered the relative CD3⁺CD4⁺ and CD3⁺CD8⁺ cell percentages in blood and in draining lymph nodes (Fig. 5A). Furthermore, anti-CD4 mAb caused a significant reduction in OVA-specific IgE production in OVA/SEB mice (Fig. 5B) compared to control IgG treated OVA/SEB mice (p < 0.05, Fig. 5B), as well as a reduction of total IgE levels (OD 0.86 ± 0.18 in anti-CD4 vs. 0.29 ± 0.003 in control IgG, p < 0.01). Anti-CD4 treated OVA/SEB mice showed a reduced number of eosinophils and lymphocytes in BAL fluid compared to control IgG treated OVA/SEB mice (Fig. 5C). In addition, levels of BHR were reduced to baseline values in anti-CD4 treated mice (Fig. 5D), reaching levels of naive (Sal/Sal) mice (Fig. 2D). This was not the case in the control group where BHR consistently developed upon OVA/SEB administration (p < 0.01, Fig. 5D).
SEB augments OVA-induced DC migration, maturation and T cell proliferation

After antigen recognition, lung DCs are known to migrate to the MLN. We evaluated the DC migration process in vivo 24h after i.t. OVA-FITC +/- SEB administration. As shown in Fig. 6A, the number of OVA-FITC+ DCs in the MLN is augmented in mice receiving both SEB and OVA-FITC, compared to mice receiving only OVA-FITC. Subsequently, we investigated the dendritic cell maturation in this model, and demonstrated that DCs exposed to both OVA and SEB had a higher expression of the CD86 maturation marker in vitro, compared to control conditions (Fig. 6B), which is consistent with their involvement in the induction of allergic sensitization in this model.

Figure 5: (A) Change in the relative CD3+CD4+ and CD3+CD8+ cell percentages in blood and in draining lymph nodes by i.p. injection of anti-CD4 mAb on day 0 and 6 of the experimental protocol. This has resulted in decreased levels of (B) OVA-specific IgE and (C) total IgE, (D) decreased BAL eosinophils and lymphocytes and (E) bronchial hyperreactivity measured as resistance (R), compared to control IgG treatment. All mice received endonasally OVA/SEB. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control IgG.
In addition to the effect of SEB on DCs, we also investigated its effects on T cell proliferation in vivo, through adoptive transfer of CFSE-labeled DO11.10 OVA-specific T cells into WT BALB/c mice. 24h after i.v. injection of DO11.10 T cells, mice were i.t. challenged with OVA +/- SEB or SEB. Fig. 7 demonstrates the significant increase in proliferation index of DO11.10 OVA-specific T cells in the MLN of mice that were concomitantly exposed to OVA and SEB, compared to mice exposed to OVA (p < 0.001) or SEB (p < 0.05) alone.

**DISCUSSION**

We here demonstrate that SEB facilitates the sensitization of CD4$^+$ cells to nasally applied allergen resulting in subsequent development of experimental asthma. Concomitant endonasal application of OVA and enterotoxin B resulted in an immune response to otherwise inert allergen, characterized by the production of allergen-specific IgE, increased production of IL-4, IL-5 and IL-13, bronchial influx of eosinophils and development of BHR. Moreover, these findings can be explained by the observed increase in DC migration and maturation in these mice, which occurs in parallel to the SEB mediated augmentation of allergen-specific T cell proliferation.
Mouse models of allergic asthma classically use artificial Th2 inducing stimuli or adjuvants such as aluminum hydroxide acting mainly on DC activation\textsuperscript{31}, to obtain allergic sensitization. Alternatively, additional danger signals can be used to induce Th2 priming. Interestingly, we could induce the allergic inflammation in this model by applying the allergen and the enterotoxin endonasally, instead of using intraperitoneal injections. Moreover, combination of both was critical to induce asthma, as neither substance could do so itself. Asthmatic features in this model consisted among others of a marked bronchial influx of eosinophils. This influx may largely be attributed to the increased IL-5 production by T lymphocytes within the lung-draining lymph nodes. Besides eosinophils, SEB also

\begin{figure}[h]
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\caption{(A) 24h after OVA-FITC +/- SEB application, the number of OVA-FITC+ DCs in the MLN is augmented in mice receiving both SEB and OVA-FITC, compared to mice receiving only OVA-FITC. (B) DCs exposed to both OVA and SEB had a higher expression of the CD86 maturation marker compared to control conditions, which is consistent with their involvement in the induction of allergic sensitization in this model. One out of 2 representative experiments is shown.}
\end{figure}
induced a minor increase in neutrophils and lymphocytes in BAL fluid. The increase in neutrophils in Sal/SEB and OVA/SEB mice may be induced by the stimulation of macrophages which in response release chemotactic proteins for neutrophils\textsuperscript{32}. In addition, SEB-induced IL-8 secretion by endothelial cells has also been described in \textit{in vitro} studies\textsuperscript{33}. A similar increase in SEB treated mice was seen in lymphocyte counts, likely due to the superantigen activity of this enterotoxin, activating large numbers of lymphocytes.

![Graph](image1)

\textbf{Figure 7:} 24 h after i.v. injection of CFSE-labeled DO11.10 T cells, mice were i.t. challenged with OVA +/- SEB or SEB. After 3 days, MLN were harvested and analyzed for proliferation of OVA-specific TCR Tg T cells. Proliferation index was calculated, and appeared to be significantly higher in OVA/SEB mice compared to mice receiving OVA or SEB alone. One out of 2 representative experiments is shown. *p < 0.05, **p < 0.001.

We demonstrate a clear increase of total serum IgE in OVA/SEB mice, indicating the activation of B lymphocytes by SEB. Besides the induction of total IgE production, SEB also induced the production of OVA-specific IgE, suggestive of an important role for SEB in the process of sensitization to allergens. Of note, the facilitating effects on OVA sensitization appear to be mouse strain independent, as we have observed a similar influx of BAL eosinophils and induction of OVA-specific IgE in C57BL/6 mice using the same experimental protocol (unpublished observations). In our model, OVA or SEB application alone did not primarily alter the OVA-specific IgE production, whereas concomitant exposure of the nasal mucosa to OVA and SEB did lead to OVA-specific IgE production. We suspect that enhanced production of IL-4 and/or IL-13 at the level of the LNs may be responsible for these observations. Among the enterotoxins studied here in BALB/c mice, only SEB influenced the sensitization process to OVA. The fact that SEA, TSST-1 or Prot A did not induce sensitization to OVA may be related to the V\textbeta chain profile of the TCR. It has been shown that particularly SEB has a
high affinity for Vβ8+ compared to Vβ6+ T cells, and mucosal contact with SEB selectively induces the expansion of Vβ8+ T cells\textsuperscript{24-26}. The fact that we could not repeat previous reported data on the enhancing effects of LPS on OVA sensitization, might be due to differences in experimental protocol related to the route of administration, doses of allergen and toxin used and/or genetic background.

To demonstrate the Th2 priming by concomitant mucosal contact with OVA/SEB, cytokines IL-4, IL-5 and IL-13 were measured in lymph node cell cultures after stimulation with OVA or BSA. As expected, we demonstrated a rise in levels of IL-4, consistent with the increase in total and OVA-specific IgE observed in OVA/SEB mice\textsuperscript{34,35}. Moreover, OVA stimulation in OVA/SEB mice caused a significant increase in IL-5 production compared to OVA stimulation in naive (Sal/Sal) mice, explaining the observed BAL eosinophilia in this group\textsuperscript{36}. However, increased titers of OVA-specific IgG2a in OVA/SEB mice, which are classically found in Th1 immune responses, suggest a B cell expansion involving both IgE and IgG2a production\textsuperscript{37,38}. The described Th2 priming in the draining lymph nodes did not alter Th1 cytokine production (IFN-γ and IL-12), as allergen stimulation did not result in increased levels compared to BSA stimulation.

In this model, we could induce one of the major characteristics of human asthma, namely bronchial hyperresponsiveness. The increase in bronchial responsiveness to metacholine as observed in the OVA/SEB group may be attributed to activation of different cell types among which are CD4\textsuperscript{+} cells. Moreover, BHR in these mice may be explained by the increased production of IL-13 and IL-10 by these T lymphocytes in OVA/SEB mice, as both cytokines have been associated with BHR in experimental asthma\textsuperscript{39,40}. Interestingly, the secretion of the regulatory cytokine IL-10 was also augmented in OVA/SEB mice upon allergen stimulation \textit{in vitro}, probably representing an inhibitory signal for the further rise in Th2 activity\textsuperscript{41}.

Treatment with anti-CD4 mAb abrogated the development of allergic disease in OVA/SEB mice, indicating the critical involvement of CD4\textsuperscript{+} cells in the SEB-mediated effects. Prevention of SEB-induced T cell activation by anti-CD4 mAb averted the production of OVA-specific and total IgE, the development of BHR and the influx of eosinophils in the BAL fluid. As CD4\textsuperscript{+} cells are primarily
responsible for the different features of asthma through their cytokine profile\textsuperscript{42-44}, it is no surprise to find that a reduction of IL-4, IL-5 and IL-13 production by anti-CD4 mAb treatment resulted in abrogation of the development of experimental asthma.

Mechanistically, we were interested whether and how dendritic cells (DCs) were involved in the facilitation of sensitization in this model, as DCs are pivotal in the induction of Th2 sensitization. Upon contact with antigens, and in the presence of some form of activation, DCs evolve towards their mature state and migrate out of the antigen-exposed site towards the draining LNs. There, antigen-specific naive T lymphocytes are activated by DCs for proliferation and differentiation. We observed an increased migration of OVA-FITC loaded dendritic cells towards the draining lymph nodes upon concomitant SEB application. Moreover, DCs from OVA/SEB mice appeared to express higher levels of the CD86 maturation marker. As DC migration and maturation are pivotal in the induction of Th2 sensitization, these findings can explain the observed induction of allergic sensitization. In parallel, we demonstrated the SEB-driven significant increase in allergen-specific T cell proliferation, possibly due to the superantigen activity of this enterotoxin, contributing to the described features of allergic asthma in this mouse model. Altogether, these findings provide arguments for a direct effect of SEB on DCs. However, SEB might also activate DCs via TLRs on epithelial cells producing chemokines and DC growth/differentiation factors\textsuperscript{13}. In addition, these processes might be reinforced by the polyclonal T cell stimulation of the SEB superantigen.

In conclusion, we show that \textit{Staphylococcus aureus} Enterotoxin B facilitates the sensitization process to allergens like ovalbumin, thereby inducing allergic airway disease. The SEB-mediated effects on sensitization to OVA and the consecutive bronchial inflammation and hyperreactivity appeared to be CD4\textsuperscript{+} cell dependent, and rely on increased DC migration and maturation upon SEB exposure in the presence of allergen, in parallel to augmented allergen-specific T cell proliferation. These combined pathways result in the allergic asthma phenotype in this mouse model of nasal applied allergen and enterotoxin.
REFERENCES


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Chapter 4

SEB activates airway epithelial cells to augment granulocyte migration and survival
Chapter 4: SEB activates airway epithelial cells to augment granulocyte migration and survival

**STAPHYLOCOCCUS AUREUS ENTEROTOXIN B AUGMENTS GRANULOCYTE MIGRATION AND SURVIVAL VIA AIRWAY EPITHELIAL CELL ACTIVATION**

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**ABSTRACT**

Background: *Staphylococcus aureus* enterotoxin B (SEB) has recently been postulated to be involved in the pathology of granulocyte-dominated disease. Studying the immunologic interaction between SEB and airway epithelial cells in immortalized cell lines or long term epithelial cell cultures has obvious disadvantages.

Methods: We used a novel technique of freshly isolated and purified human nasal epithelial cells from healthy, non-allergic individuals, which were incubated for 24 h without/with SEB at different concentrations. Chemokine production was evaluated in the supernatant using Cytometric Bead Array. The chemotactic activity of the supernatant was studied *in vitro* using a Boyden chamber. Survival was evaluated with flow cytometry, using propidium iodide to identify dead cells.

Results: SEB showed a dose-dependent induction of IP-10, MIG, RANTES, MCP-1 and G-CSF production by epithelial cells *in vitro*. The supernatant of epithelial cells had chemotactic activity for granulocytes *in vitro*, which was enhanced in the supernatant of SEB-stimulated epithelial cells. Reduced number of propidium iodide positive granulocytes was found in the conditions where supernatant of SEB-stimulated epithelial cells was applied.

Conclusion: *Staphylococcus aureus* enterotoxin B exerts a direct pro-inflammatory effect on human nasal epithelial cells, with induction of chemokine and growth factor release, resulting in the migration and prolonged survival of granulocytes *in vitro*. 
INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is a common human pathogen which is often found as part of the normal microflora in the nasal cavity. The anterior nares of the nose are the most frequent carriage site for *S. aureus*, although multiple sites can be colonized (e.g. skin, pharynx and perineum). Colonization with *S. aureus* may represent a major source of superantigens as a set of toxins are being produced including staphylococcal enterotoxins SAEs and toxic shock syndrome toxin-1 (TSST-1) which cause food poisoning and toxic shock syndrome respectively. These toxins activate up to 20% of all T cells in the body by binding the human leukocyte antigen (HLA) class II molecules on antigen-presenting cells (APCs) and specific V beta regions of the T cell receptor. Between 50 and 80% of *S. aureus* isolates are positive for at least one superantigen gene, and close to 50% of these isolates show superantigen production and toxin activity. The pathophysiologic role of enterotoxin producing *S. aureus* in human disease has recently been recognized. SAEs have immune-modulatory and pro-inflammatory effects in several granulocyte-dominated diseases like atopic dermatitis, allergic rhinitis and asthma, nasal polyposis or chronic obstructive pulmonary disease (COPD). Studies have shown a putative role for SAEs in patients suffering from the atopic eczema/dermatitis syndrome (AEDS), where colonization with *S. aureus* is found more frequently (80-100%) compared to healthy controls (5-30%). *S. aureus* isolates secrete identifiable enterotoxins like *Staphylococcus aureus* enterotoxin (SEA), SEB and toxic shock syndrome toxin (TSST)-1. IgE to these toxins was found in the serum of 57% of AEDS patients, indicating immune responses against these bacterial products. Moreover, in 25% of allergic rhinitis (AR) patients detectable serum IgE levels to SAE are found, whereas this is only found in a minority of non-allergic patients (6.3%). In AR, the presence of SAE-specific IgE was associated with the highest titer of total serum IgE. Furthermore, SAEs are thought to play a major role in the pathogenesis of nasal polyp (NP) disease, as IgE against SEA and SEB has been demonstrated in nasal polyps and levels of SAE-specific IgE in NP correlated with markers of eosinophil activation and recruitment. Moreover, in COPD patients a significantly elevated IgE to SAE was found, pointing to a possible disease modifying role in COPD, similar to that in severe asthma. In murine research, the role of SAEs as disease modifier has been demonstrated by in models of
airway disease\textsuperscript{15}, atopic dermatitis\textsuperscript{16} and food allergy\textsuperscript{17}. In addition, we have previously reported aggravation of experimental allergic asthma by application of SEB, characterized by higher IL-4 production and allergen-specific IgE\textsuperscript{18}. These findings highlight the important pathological consequences of SAE exposure, as these superantigens not only cause massive T-cell stimulation, but also lead to activation of B-cells and other pro-inflammatory cells like eosinophils, macrophages and mast cells\textsuperscript{19}. Moreover, SAEs are known inducers of chemokine production in epithelial cells\textsuperscript{20}.

The involvement of the epithelium in the pathogenesis of airway disease is increasingly acknowledged. The airway epithelium is not only a physiological barrier, but is actively involved in the immune response as a major source of inflammatory cytokines and mediators\textsuperscript{21}, relevant to the ongoing inflammatory responses dominated, amongst other features, by abundant granulocytes. Activated epithelial cells are potent sources of haematopoietic cytokines such as granulocyte(-macrophage) colony stimulating factor (G(M)-CSF), and chemokines like regulated upon activation normal T cell expressed and secreted (RANTES), eotaxin, monokine induced by interferon-\(\gamma\) (MIG), interferon-inducible protein-10 (IP-10), interleukin (IL)-8\textsuperscript{22} and monocyte chemoattractant protein 1 (MCP-1)\textsuperscript{23}.

Because of the above mentioned association between SAEs and granulocyte-dominated inflammatory disease, we have investigated the effect of \textit{S. aureus} enterotoxin B – a prototypic staphylococcal superantigen – on chemokine production of human nasal epithelial cells (HNEC). Until now, the immunologic interaction between SEB and epithelial cells has mostly been studied in immortalized cell lines. Here, a novel technique of pure and freshly isolated epithelial cells is being elaborated and presented. Furthermore, we investigated the effects of these epithelial derived mediators \textit{in vitro} in a granulocytic chemotaxis and survival assay.

**METHODS**

**Human nasal epithelial cell isolation procedure**

Nasal inferior turbinates were obtained from patients (\(n=10\)) undergoing nasal surgery for non-mucosal anatomical abnormalities causing nasal obstruction. Exclusion criteria were smoking, occupational exposure to irritants, IgE-mediated hypersensitivity to a panel of frequent inhalant
allergens demonstrated with a standardized skin prick test, or use of intranasal corticosteroid spray 6 weeks before the surgery. Inferior turbinates were harvested at the end of the septoplasty or rhinoplasty procedure, and immediately placed in a sterile saline solution, washed with saline and incubated for 24h at 4°C with 0.1% sterile pronase solution for dissociation of the epithelial cell layer (Sigma, Bornem, Belgium, Fig. 2). After 24h, large tissue pieces were removed with a sterile pincet. FCS (Sigma) was added to the solution in order to stop the pronase reaction. Cells were washed three times in culture medium (HBSS supplemented with 0.05% BSA). Next, supernatant was discarded and the pellet was resolved in culture medium, transferred in a cell culture flask and incubated for 90min at 37°C in order to let fibroblasts attach to the wall.

**Figure 1: Sagittal view of the lateral nasal wall, with indication of inferior, middle and superior nasal turbinate.**

In a next step, nasal epithelial cells were negatively selected using MACS cell separation columns (Miltenyi, Utrecht, The Netherlands). Therefore, magnetic beads coated with anti-CD45 were added to the cell suspension, incubated for 20min and put in a column before transferring the supernatant. This procedure was repeated with anti-CD15 coated beads and the supernatant was transferred into a cell culture flask. Viability was evaluated using Trypan blue, and May-Grünwald-Giemsa (MGG) staining of the cytospins was performed to confirm cell culture purity. This epithelial cell isolation procedure using 2 negative selections resulted in an epithelial cell population with 98% purity and viability.
Evaluation of SEB-induced chemokine secretion

Pilot studies showed that $3 \times 10^5$ Human Nasal Epithelial Cells (HNEC) was the optimal number of cells for stimulation studies and analyses of supernatants (data not shown). HNEC were incubated with 0.1, 1 or 10 µg of SEB in a total volume of 1mL culture medium for 24h. Incubation with IL-1beta was used as positive control condition. Supernatant was evaluated for IP-10, G-CSF, GM-CSF, MCP-1, MIG, RANTES, Eotaxin-1/2, IL-3, IL-4 and IL-8 using cytometric bead array Flex (BD Biosciences, Erembodegem, Belgium) according to the manufacturers’ instructions. Samples were acquired with the FACS Array (BD Biosciences).

Stimulation index was calculated by dividing the values from the experimental conditions (SEB or IL-1beta) by the respective values from the control condition.

Granulocyte isolation

For migration and survival assays on granulocytes \textit{in vitro}, blood cells were obtained from house dust mite allergic donors ($n=5$) suffering from allergic rhinitis which presented at the Outpatient clinic of the Department of Otorhinolaryngology of the University Hospital Leuven, Belgium. Patients with a
positive skin prick test to house dust mite allergen (>5 mm – GA²LEN pan-European skin prick test) were included in the study for blood sampling. All patients included in this study completed written informed consent and the local ethical committee approved the study.

Blood was obtained and diluted with an equal volume of PBS. Then, 40 mL of this suspension was added to 10 mL Lymphoprep (Axis-Shield, Oslo, Norway) and tubes were centrifuged for 30 min at 800 x g. Plasma and peripheral blood mononuclear cells (PBMCs) were discarded and tubes were supplemented with an equal volume of Plasmasteril (Bad Homburg, Germany) and PBS. Tubes were incubated for 30 min at 37°C before centrifuging (10 min – 218 x g). The pellet was washed with PBS and residual red blood cells were lysed with hypotonic shock.

**Granulocyte migration assay**

In order to evaluate the chemotactic activity of SEB-stimulated HNEC supernatant, we used a Boyden chamber-based cell migration assay, as reported previously. Briefly, granulocytes (50 µL – 1 x 10⁶ per mL) from house dust mite allergic donors were placed in the upper compartment and were allowed to migrate through 5 µm pore size poly(vinylpyrrolidone)-free (PVPF) polycarbonate filters (Nuclepore, Pleasanton, CA) for 45 min at 37°C. The lower compartment contained supernatant of unstimulated HNEC (SN Medium), SEB-stimulated HNEC (SN SEB) or control medium. IL-8 (10 µg/ml) was used as positive control condition. After 45 min incubation, the membrane was subjected to MGG staining, and its lower side was evaluated for number of migrated granulocytes, by counting 10 high power fields per sample. According to standard morphological criteria of the cell nucleus and cytoplasmic granules, cells were further categorized into neutrophilic and eosinophilic granulocytes. The chemotactic index was calculated by dividing the number of cells migrated under the experimental condition (SN SEB) by the number of cells migrated under control condition (SN Medium). A chemotactic index of >2 was considered to be a positive index of chemotaxis.

**Granulocyte survival assay**

Granulocytes (2 x 10⁶ cells per mL) were incubated for 24 h and 48 h with SN Medium, SN SEB or Medium. At time of analysis, cells were evaluated with flow cytometry (FACS Array, BD
Biosciences), where propidium iodide was used to identify dead cells. Granulocytes were sorted with MACS into neutrophils and eosinophils using a CD16 monoclonal antibody (Miltenyi).

**Statistical analysis**

Statistical analysis was performed with Medcalc software 9.2.0.1 (F. Schoonjans, Belgium; http://www.medcalc.be). All outcome variables were compared using non-parametrical tests. When comparisons were made between groups, the Kruskal-Wallis test was used to establish the significant inter-group variability. The Mann Whitney-U test was then used for between-group comparison. The significance level was set at $\alpha = 0.05$. Data are expressed as mean with error bars expressing standard error of the mean.

![Figure 3: HNEC incubation for 24h with increasing doses of SEB resulted in a significant increase in chemokine secretion of IP-10, G-CSF, MCP-1, MIG and RANTES, showing a dose-dependent induction. IP-10 (A) and MIG (B) were already increased at the lowest (0.1 µg) SEB concentration, whereas G-CSF (C) only raised above control at SEB 1µg. Finally, RANTES (D) and MCP-1 (E) were significantly increased just at the highest SEB concentration (10 µg). Incubation with IL-1beta as a general pro-inflammatory stimulus resulted in a significant increase in G-CSF (C) and IL-8 (F) secretion by nasal epithelial cells. **p < 0.01 vs. control, ***p < 0.001 vs. control, §§p < 0.01 vs. control, §§§p < 0.001 vs. control.]
Chapter 4: SEB activates airway epithelial cells to augment granulocyte migration and survival

RESULTS

Effect of SEB on human nasal epithelial cells (HNEC)

HNEC (n=6) incubation for 24h with increasing doses of SEB resulted in a significant increase in chemokine secretion of IP-10, G-CSF, MCP-1, MIG and RANTES, showing a dose-dependent induction as depicted on Fig. 3 A-F. Interestingly, IP-10 and MIG were already increased at the lowest (0.1 µg/mL) SEB concentration, whereas G-CSF only raised above control at SEB 1µg/mL. Finally, RANTES and MCP-1 were significantly increased just at the highest SEB concentration (10 µg/ml). Incubation with IL-1beta as a general pro-inflammatory stimulus resulted in a significant increase in G-CSF and IL-8 secretion by nasal epithelial cells. Levels of eotaxin-1/2 and GM-CSF were below detection limit (data not shown).

Figure 4: Boyden chamber-based cell migration assay. Granulocytes from house dust mite allergic donors were allowed to migrate through 5 µm pore size poly(vinylpyrrolidone)-free (PVPF) polycarbonate filters for 45min at 37°C. At 45min after incubation, the membrane was subjected to May-Grünwald-Giemsa staining, and its lower side was evaluated for number of migrated granulocytes, by counting 10 high power fields per sample. (A) Control: Granulocytes incubated with medium. (B) SN Medium: Granulocytes incubated with supernatant from medium-stimulated epithelial cells. (C) SN SEB: Granulocytes incubated with supernatant from SEB –stimulated epithelial cells. (magnification x400).
Effects of SEB-stimulated epithelial cell mediators on the granulocyte migration in vitro

A Boyden chamber was utilized for the evaluation of migration of granulocytes in vitro through a semipermeable membrane. We evaluated the level of granulocyte migration after 45min incubation with supernatant from medium-stimulated (SN Medium) or SEB-stimulated HNEC (SN SEB, n=4) and demonstrate that the chemotactic index of SN SEB is significantly higher (2.62 ± 0.30) compared to SN Medium which was set to 1 (p < 0.001).

The supernatant from SEB-stimulated HNEC (SN SEB) appeared to be particularly chemotactically active for neutrophils. As shown in Figure 5A, the number of migrated neutrophils was significantly higher upon SN SEB incubation (10.0 ± 0.6 cells per field) compared to SN Medium (7.4 ± 0.6 cells per field, p < 0.001) and Medium (3.6 ± 0.4, p < 0.001). Interestingly, the supernatant of epithelial cells itself (SN Medium) had chemotactic activity for neutrophils (Medium, p < 0.05, Fig. 5A). As expected, IL-8 was the most potent chemo-attractant in this assay (18.0 ± 1.8, p < 0.001 vs. Medium).

Looking to the chemoattraction of eosinophils, we could demonstrate a significant decrease in chemotactic activity in SN SEB (4.8 ± 0.4) compared to SN Medium (6.9 ± 0.6, p < 0.01) and Medium (9.3 ± 1.1, p < 0.001, Fig. 5B).

![Figure 5](image-url)

**Figure 5:** (A) Effects of SEB-stimulated epithelial cell mediators on the granulocyte migration in vitro. The number of migrated neutrophils was significantly higher upon SN SEB incubation compared to SN Medium. Interestingly, control experiments revealed a significantly increased number of migrated neutrophils upon SEB incubation, compared to control medium. (B) The chemoattraction of eosinophils did not differ upon incubation with SN Medium or SN SEB. Surprisingly, a significant lower number of eosinophils was found upon SEB incubation. *p < 0.05, ***p < 0.001.
Effects of SEB stimulated epithelial cell-derived mediators on granulocyte survival in vitro

Modulation of granulocyte survival by supernatant from SEB-stimulated HNEC (SN SEB) was evaluated after 1 and 2 days of co-incubation. As can be appreciated from Fig. 6A, neutrophil survival was not significantly altered upon SN SEB incubation after 24h or 48h. However, SN SEB caused a significant increase in eosinophil survival after 24h (62.76 ± 9.70 %), compared to medium (30.63 ± 3.83 % living eosinophils, p < 0.001, Fig. 6B). After 48h incubation with SN SEB 27.99 ± 4.14 % of eosinophils were alive, versus 9.90 ± 4.22 % in the Medium group (p < 0.05). Interestingly, at that time point SN Medium incubation resulted only in 14.16 ± 1.97 % living eosinophils, which is significantly lower compared to SN SEB incubation (p < 0.01, Fig. 6B).

DISCUSSION

The experimental approach of using freshly isolated and purified epithelial cells from healthy donors allowed us to study epithelial cell immunology more accurately compared to the usage of cell lines. By means of magnetic cell sorting we obtained a cell population consisting of uncontaminated, naïve nasal epithelial cells, in which we could study the effect of exogenous signals. Specifically, the role of S. aureus enterotoxin B in the activation of human nasal epithelial cells (HNEC) for chemokine secretion with subsequent granulocyte migration and survival was evaluated.

![Figure 6: Effects of SEB stimulated epithelial cell-derived mediators on granulocyte survival in vitro. (A) Neutrophil survival was not significantly altered upon SN SEB incubation after 24h or 48h. (B) However, SN SEB caused a significant increase in eosinophil survival after 24h and 48h. * p < 0.05, ** p < 0.01, *** p < 0.001.](image-url)
Stimulation of HNEC with SEB has resulted in significantly increased, dose-dependent chemokine production of IP-10, G-CSF, MCP-1, MIG and RANTES, as was measured in the supernatant. Interestingly, compared to control medium this supernatant was significantly more chemotactically active for granulocytes, in particular for neutrophils. Moreover, granulocyte survival analysis revealed a significantly prolonged survival of eosinophilic granulocytes, when incubated with supernatant from SEB-stimulated HNEC. These results indicate the importance of the epithelium in the orchestration of granulocyte-dominated inflammation, and demonstrate the significant role of SEB as disease modifier. Previous studies using either SEB or S. aureus itself confirm the role of the innate immune activation through epithelial cells secreting cytokines and chemokines, which lead to a secondary influx of inflammatory cells. However, this is the first study evaluating the effect of the prototypic staphylococcal superantigen SEB on a highly purified human nasal epithelial cell population. Interestingly, HNEC are capable of secreting IFN-gamma-inducible protein-10 (IP-10) and monokine induced by IFN-gamma (MIG) upon SEB stimulation. Both chemokines have been shown to be functional agonists of CXC chemokine receptor 3 (CXCR3), and they largely act on natural killer (NK) cells and activated T cells. However, CXCR3-expression is also found on eosinophils and neutrophils, in particular in inflammatory microenvironments. Furthermore, increased levels of RANTES and MCP-1 – both chemoattractant for mononuclear cells – after SEB stimulation are in line with previous data, and increased levels of G-CSF – causing granulocyte proliferation and differentiation – are reported after S. aureus stimulation of epithelial cells.

Pretreatment of HNEC with IFN-gamma has resulted in increased levels of interleukin 8 (IL-8) upon SEB stimulation. However, we and others did not find increased IL-8 levels in untreated SEB-stimulated HNEC. Altogether, these data stress the major role of airway epithelial cells in the immune response to SEB, therefore actively participating in the pathogenesis of granulocyte-dominated diseases linked to SAEs like asthma, nasal polyposis or allergic rhinitis. A potential drawback of the used approach however, might be the loss of epithelial cell polarization, allowing SEB to reach the apical and basal side of epithelial cells equally.
The mechanism via which SEB activates HNEC to produce chemokines has been linked to MHC class II binding and crosslinking, although involvement of non-MHC class II receptors has also been demonstrated. Moreover, SEB is known to activate antigen presenting cells (APCs) like dendritic cells via Toll-like receptor (TLR)2, a receptor which plays an important role in pathogen recognition and innate immunity. Interestingly, these TLRs are also present on nonprofessional antigen presenting cells like epithelial cells. However, we were not able to block SEB-induced chemokine secretion in our study using TLR2 or TLR4 mAb (data not shown). The typing and contribution of receptors involved in the SEB-induced chemokine secretion therefore clearly merits further investigation.

Chemokines present in the supernatant of SEB-stimulated HNEC (SN SEB) were able to increase granulocyte chemotaxis, in particular neutrophilic chemotaxis (Fig. 5A), probably due to the increase in G-CSF. Interestingly, eosinophilic chemotaxis was downregulated by SN SEB, a finding possibly related to raised levels of MIG, which has a known negative regulatory effect on eosinophil recruitment.

Granulocyte survival has been evaluated upon incubation with SN SEB. Interestingly, only eosinophilic survival was significantly increased after 1 and 2 days. Although we could not demonstrate an increase in individual factors directly linked to eosinophil survival like GM-CSF or eotaxin1/2, it is tempting to speculate that their synergistic effect has contributed to the observed increase in survival, as was shown by others. Alternatively, other yet unidentified factors, which are also under the tight control of the nuclear factor (NF)-κB complex, could be responsible for the observed effects. Surprisingly, neutrophilic survival was not altered by SN SEB, although epithelial derived G-CSF has been described to prolong neutrophil survival in cystic fibrosis (CF) airways. Altogether, we could speculate that the presence of neutrophils in SEB-mediated granulocyte-dominated disease might be due to increased chemotaxis, whereas increased number of eosinophilic granulocytes could be linked to augmented survival. However, this hypothesis needs to be confirmed in further studies.
In summary, we hereby demonstrate that stimulation of human nasal epithelial cells with the superantigen SEB leads to production of cytokines and chemokines, important in the chemotaxis of T cells, monocytes and granulocytes. Moreover, in vitro analysis of these factors confirmed their involvement in the pathogenesis of granulocyte-dominated disease, as they significantly increased granulocyte migration and survival. These findings contribute to the understanding of SAE modulation of airway disease, and stress the opportunity to target epithelial cells for therapeutic intervention.
REFERENCES


Chapter 5

Different regulation of CS-induced inflammation in upper versus lower airways
DIFFERENT REGULATION OF CIGARETTE SMOKE INDUCED INFLAMMATION IN UPPER VERSUS LOWER AIRWAYS

Wouter Huvenne, Claudina Pérez-Novo, Lara Derycke, Natalie De Ruyck, Olga Krysko, Tania Maes, Nele Pauwels, Lander Robays, Ken R. Bracke, Guy Joos, Guy Brusselle, Claus Bachert

Respir Res 2010; 11:100.

ABSTRACT

Background: Cigarette smoke (CS) is known to initiate a cascade of mediator release and accumulation of immune and inflammatory cells in the lower airways. We investigated and compared the effects of CS on upper and lower airways, in a mouse model of subacute and chronic CS exposure.

Methods: C57BL/6 mice were whole-body exposed to mainstream CS or air, for 2, 4 and 24 weeks. Bronchoalveolar lavage fluid (BAL) was obtained and tissue cryosections from nasal turbinates were stained for neutrophils and T cells. Furthermore, we evaluated GCP-2, KC, MCP-1, MIP-3α, RORc, IL-17, FoxP3, and TGF-β1 in nasal turbinates and lungs by RT-PCR.

Results: In both upper and lower airways, subacute CS-exposure induced the expression of GCP-2, MCP-1, MIP-3α and resulted in a neutrophilic influx. However, after chronic CS-exposure, there was a significant downregulation of inflammation in the upper airways, while on the contrary, lower airway inflammation remained present. Whereas nasal FoxP3 mRNA levels already increased after 2 weeks, lung FoxP3 mRNA increased only after 4 weeks, suggesting that mechanisms to suppress inflammation occur earlier and are more efficient in nose than in lungs.

Conclusions: Altogether, these data demonstrate that CS induced inflammation may be differently regulated in the upper versus lower airways in mice. Furthermore, these data may help to identify new therapeutic targets in this disease model.
INTRODUCTION

Tobacco smoking can induce bronchial inflammation and structural changes, and is one of the major causes of Chronic Obstructive Pulmonary Disease (COPD), which is characterized by a slowly progressive development of airflow limitation that is not fully reversible\(^1\). There is growing evidence that the disease process is not confined to the lower airways, which is perhaps not surprising given the fact that the entire airway is exposed to tobacco smoke. Epidemiological data suggest that 75% of the COPD patients have concomitant nasal symptoms and more than 1/3 of patients with sinusitis also have lower airway symptoms of asthma or COPD\(^2\). These arguments stress the significant sinonasal inflammation in patients with lower airway complaints, beyond the scope of allergic inflammation\(^3-5\).

We know from human and murine research that both inflammatory and structural cells actively participate in the inflammatory response that characterizes COPD. An accumulation of inflammatory cells such as neutrophils, macrophages, dendritic cells and CD8+ T lymphocytes is seen, although the cellular and molecular pathways behind this increased cellular influx are still incompletely unraveled. However, CC-chemokines (MIP-1alpha, MIP-3alpha, RANTES and MCP-1)\(^6\) and CXC-chemokines (IL-8, GCP-2)\(^7\), binding to their respective receptors play an important role. Moreover, the role of lymphocytes in the development of COPD is demonstrated by the fact that chronic cigarette smoke (CS) exposure leads to an increase in peribronchial lymphoid follicles in both mice and humans\(^8, 9\), although the importance of these lymphoid follicles remains unclear\(^10\).

COPD is frequently considered a Th1/Tc1 disease\(^11\), although recent developments in cytokine biology imply that COPD might be better explained by the pro-inflammatory T helper 17 (Th17) phenotype\(^12\), therefore suggesting a role of the interleukin (IL)-17 family members in COPD\(^13\). Alternatively, T regulatory cells which are widely investigated in the pathogenesis of asthma, might be involved in a possible autoimmune base of COPD\(^14\). These cells, expressing the transcription factor FoxP3, are involved in the interplay between lymphocyte subpopulations in order to control the cigarette smoke induced inflammation, including the activity of autoreactive lymphocytes\(^15\).

Compared to lungs, the direct effect of CS on upper airways is less extensively studied, although the link between upper and lower airway smoke induced inflammation is illustrated by increased nasal IL-
8 concentrations correlating with IL-8 in sputum of COPD patients. Moreover, these patients report a high prevalence of nasal symptoms and sinusitis, and nasal and bronchial inflammation coexist in smokers and is characterized by infiltration of CD8+ T lymphocytes. In upper airways, CS may act as a local irritant, influencing the local inflammatory process. It has been described that nicotine has an effect on the nasal epithelium, regulating physiological processes and influencing cell transport systems, although an individual variability in response has been reported. CS can increase nasal resistance, and the direct use of tobacco could also be linked to an increased prevalence of sinusitis. In addition, a correlation between duration of secondhand smoke exposure and sinusitis has recently been described.

Also in mice, obligatory nose breathers, little knowledge has been gathered on the effects of CS on upper airways, especially in comparison to the lower airways. We therefore aimed to investigate the inflammatory response of the upper airways in a murine model of COPD in comparison to the lower airway response after exposure to mainstream cigarette smoke.

**METHODS**

**Mouse model of Cigarette Smoke exposure**

Groups of 8 Male C57BL/6 mice, 6–8-week old were exposed to the tobacco smoke of five cigarettes (Reference Cigarette 2R4F without filter; University of Kentucky, Lexington, KY, USA) four times per day with 30 min smoke-free intervals as described previously. The animals were exposed to mainstream cigarette smoke by whole body exposure, 5 days per week for 2 weeks, 4 weeks and 24 weeks. The control groups (8 age-matched male C57BL/6 mice) were exposed to air. All experimental procedures were approved by the local ethical committee for animal experiments (Faculty of Medicine and Health Sciences, Ghent University).
**Bronchoalveolar lavage**

Twenty-four hours after the last exposure, mice were weighed and sacrificed with an overdose of pentobarbital (Sanofi-Synthelabo), and a tracheal cannula was inserted. A total of 3 x 300 µl, followed by 3 x 1 ml of HBSS, free of ionized calcium and magnesium, but supplemented with 0.05 mM sodium EDTA, was instilled via the tracheal cannula and recovered by gentle manual aspiration. The six lavage fractions were pooled and centrifuged, and the cell pellet was washed twice and finally resuspended in 1 ml of HBSS. A total cell count was performed in a Bürcker chamber, and the differential cell counts (on at least 400 cells) were performed on cytocentrifuged preparations using standard morphologic criteria after May-Grünwald-Giemsa staining.

**Quantitative real time PCR**

*RNA and cDNA synthesis*

Total RNA was isolated from mouse inferior turbinate or lung tissue by using the Aurum Total RNA Mini Kit (BioRad Laboratories, CA, USA). Single stranded cDNA was then synthesized from 2 µg of total RNA with the iScript cDNA Synthesis Kit (BioRad Laboratories, CA, USA). Primer sequences are listed in table 1.

*PCR amplifications using SYBR Green*

PCR reactions contained 30 ng cDNA (total RNA equivalent) of each sample in duplicate, 1x SYBR Green I Master mix (BioRad laboratories, CA, USA) and 250 nM of specific primer pairs (table 1) in a final volume of 20 µl. Real time amplifications were performed on the iQ5 Real-Time PCR Detection System (BioRad laboratories, CA, USA) with a protocol consisting of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 62°C for 1 minute. At the end of each PCR run, a melting curve analysis to control for unspecific amplification was performed by increasing the temperature by 0.4°C for 10 seconds starting from 62°C until 95°C.
PCR amplifications using TaqMan probes

PCR reactions contained 30 ng cDNA (total RNA equivalent) of each sample in duplicate, 1x TaqMan Master mix (BioRad laboratories, CA, USA), 100 nM of TaqMan probe and 250 nM of specific primer pairs (table 1) in a final volume of 20 µl. Real time amplifications were performed on the iQ5 Real-Time PCR Detection System (BioRad laboratories, CA, USA) with a protocol consisting of 1 cycle at 95°C for 90 seconds followed by 50 cycles at 95°C for 15 seconds, 62°C for 1 minute and 72°C for 1 minute.

PCR amplifications using Assay on demand kits

PCR reactions contained 30 ng cDNA (total RNA equivalent) of each sample in duplicate and 1x TaqMan Master mix (BioRad laboratories, CA, USA). Primers were obtained from Applied Biosystems inventoried TaqMan Gene Expression Assay (table 1). Real time amplifications were performed on the iQ5 Real-Time PCR Detection System (BioRad laboratories, CA, USA) with a protocol consisting of 1 cycle at 95°C for 90 seconds followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Normalization and data analysis

Quantification cycles (Cq) values were selected and analyzed using the iQ5 Real-Time PCR software (BioRad laboratories, CA, USA). Then, the relative expression of each gene was calculated with the qBase software (version 1.3.5, University of Ghent, Belgium). Results (expressed as relative expression units / 30 ng cDNA) were then normalized to the quantities of gene beta-actin (ACTB) to correct for transcription and amplification variations among samples.
Table 1: Primer sequences used for real time PCR amplification

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<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>TaqMan probe (5’-6-FAM →TAMRA -3’)</th>
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<th>GA number</th>
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RORc: Applied Biosystems – TaqMan Gene Expression Assays - Mm00441139_m1
KC (CXCL1): Applied Biosystems – TaqMan Gene Expression Assays - Mm00433859_m1
FoxP3: Applied Biosystems – TaqMan Gene Expression Assays - Mm00475156_m1
IL-17: Applied Biosystems – TaqMan Gene Expression Assays - Mm00439619_m1

Immunohistochemistry

Presence of lymphoid follicles

To evaluate the presence of lymphoid infiltrates in lung tissues, sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to an immunohistological CD3/B220 double-staining, as described previously.

Inferior turbinate stainings

After removal of the palate, nasal turbinates were obtained, snap frozen and stored at -80°C until analysis. Cryosections were prepared (3-5 µm) and mounted on SuperFrost Plus glass slides (Menzel Glaeser, Braunschweig, Germany), packed in aluminum paper and stored at -20°C until staining.

Sections were fixed in acetone and incubated with peroxidase blocking reagent. Then, primary biotinylated antibodies (anti-CD3 (DakoCytomation, CA, USA) and neutrophil 7/4 clone (Serenoc, Düsseldorf, Germany)) or isotype control were added, followed by anti-rabbit polymer HRP (DakoCytomation). Finally, ready-to-use AEC+ substrate-chromogen-solution was added, sections were counterstained with hematoxylin and coverslips were mounted with aquatex. Slides were evaluated by light microscopy (Olympus CX40) at magnification of x400 for the number of positive cells per field, and this was done for the entire surface of the tissue cryosection by two independent observers (on average, $12.43 \pm 1.00$ number of fields were counted per mouse).
Nasal epithelial cell isolation

Nasal epithelial cells were isolated in order to determine their contribution to the overall nasal FoxP3 expression. Therefore, pooled inferior turbinates were incubated in collagenase/DNAsel solution for 30min at 37°C. Then, mechanical digestion was performed, and supernatant was discarded. The pellet was washed and incubated for 30min at 4°C with Fc blocking solution. Next, Dynabeads (sheep anti-mouse IgG, Dynal, Invitrogen, Belgium) coated with anti-pan cytokeratin (catalog nr C 1801, Sigma, Belgium) were for 30min at 4°C during gentle rotation and tubes were placed in the magnet for 2min. The two fractions containing epithelial and subepithelial cells respectively, were resuspended in 75µl RNA lysis buffer (Qiagen, Venlo, The Netherlands) in separate tubes. Finally, tubes containing subepithelial cells were centrifuged, and tubes containing epithelial cells were put again in the magnet. Supernatant was taken to store at -80°C.

In order to isolate total RNA from nasal epithelial cells and subepithelial cells, we used the RNeasy Micro kit (Qiagen) according to the manufacturer’s specifications. Single stranded cDNA was then synthesized from 2 µg of total RNA with the iScript cDNA Synthesis Kit (BioRad Laboratories).

Statistical analysis

Statistical analysis was performed with the Medcalc software 9.2.0.1 (F. Schoonjans, Belgium, http://www.medcalc.be). Data are expressed as mean with error bars expressing standard error of the mean. All outcome variables were compared using non-parametrical tests (Kruskal-Wallis; Mann Whitney U test for unpaired data). The significance level was set at $\alpha = 0.05$. A Bonferoni correction was used in case of multiple statistical comparisons.
RESULTS

BAL fluid analysis

2-wk, 4-wk and 24-wk CS exposure caused a significant increase in the absolute numbers of total cells, lymphocytes and neutrophils in the BAL fluid (table 2). Significant increase in alveolar macrophages was seen at 4-wk and 24-wk CS exposure.

<table>
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<th>4-wk Air</th>
<th>4-wk Smoke</th>
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<th>24-wk Smoke</th>
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<td>410.00 ± 144.12</td>
<td>1046.00 ± 154.98†</td>
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<td>598.55 ± 38.90</td>
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<td>408.71 ± 142.94</td>
<td>797.55 ± 103.16†</td>
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<td>8.32 ± 1.42†</td>
<td>1.29 ± 1.21</td>
<td>47.82 ± 8.19‡</td>
<td>2.46 ± 0.32</td>
<td>26.15 ± 8.42‡</td>
</tr>
<tr>
<td>Eosinophils, (x10³)</td>
<td>1.46 ± 1.46</td>
<td>2.96 ± 1.49</td>
<td>0.00 ± 0.00</td>
<td>0.39 ± 0.26</td>
<td>0.27 ± 0.18</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Table 2: Bronchoalveolar lavage analysis. Subacute (4-wk) and chronic (24-wk) CS exposure caused a significant increase in the absolute numbers of total cells, alveolar macrophages, lymphocytes and neutrophils in the BAL fluid, compared to air exposed littermates. Values are reported as mean ± SEM; n = 8 mice/group, *p < 0.05 versus Air, †p < 0.01 versus Air, ‡p < 0.001 versus Air.

Immunohistochemistry

CS induced neutrophilic inflammation in upper airways

We analyzed the presence of neutrophils in the nasal turbinate tissue of subacute (2-wk and 4-wk) and chronic (24-wk) CS exposed mice by immunohistochemistry, evaluating the average number of neutrophils per high power field, for the entire section. The increase in neutrophils was seen only after 4-wk CS exposure, compared to air exposed littermates (Fig. 1B). Interestingly, the number of neutrophils in the nasal turbinate decreased when the mice were chronically (24-wk) exposed, resulting in a significant lower amount of neutrophils per field in the CS exposed group compared to the air exposed group (Fig. 1C).
Chapter 5: Different regulation of CS-induced inflammation in upper vs. lower airways

Figure 1: Average number of neutrophils in nasal turbinate sections. Increase in number of neutrophils after CS exposure was not seen after 2-wk, compared to air exposed littermates (Fig. 1A), but only after 4-wk (Fig. 1B). Interestingly, the number of neutrophils in the nasal turbinate decreased when the mice were chronically (24-wk) exposed, resulting in a significant lower amount of neutrophils per field in the CS exposed group compared to the air exposed group (Fig. 1C). n = 8 mice/group, * p < 0.05.

Scattered CD3+ T cells in nasal turbinates versus (CS-induced) lymphoid follicles in lungs

The presence of peribronchial lymphoid follicles has been shown both in mice after chronic CS exposure and patients with severe COPD. We could demonstrate the presence of these lymphoid follicles in lungs after chronic CS exposure, using a CD3/B220 double staining (Fig. 2A). Lymphoid aggregates, absent in the bronchovascular lung regions of air-exposed mice, were strongly induced upon chronic CS exposure. In nasal turbinate tissue on the other hand, the number of CD3+ cells did not differ at any time point when air and smoke exposed mice were compared (Fig. 3). Moreover, CD3+ cells were not organized in lymphoid follicles – in contrast to findings in lower airways upon chronic exposure – but were scattered throughout the tissue section (Fig. 2B).
Figure 2: CD3+ cells. Lymphoid follicles were demonstrated in lungs after chronic CS exposure, using CD3(brown)/B220(blue) doublestaining (Fig. 2A, x200). In nose however, no increased number of CD3+ cells in inferior turbinate, or lymphoid follicle neogenesis was found at that time point (Fig. 2B, x400).

Figure 3: CD3+ staining. Nasal turbinate sections were evaluated for the presence of CD3+ cells, within lymphoid follicles. Number of CD3+ cells per field did not differ between air and CS exposed group at any time point (Fig. 3 A-C). n = 8 mice/group.
Real time Quantitative PCR analysis

In order to evaluate the immune response after CS exposure in detail, we analyzed a panel of genes. Neutrophilic chemoattraction was evaluated by measurements of Granulocyte Chemotactic Protein (GCP)-2 and keratinocyte chemoattractant (KC), which is the mouse homologue of IL-8.

Because of the role of chemokines like MCP-1 and MIP-3α in the recruitment of inflammatory cells like dendritic cells, monocytes or T lymphocytes after CS-exposure, these parameters were also included in the panel. Finally, FoxP3 and TGF-β1 were evaluated, in order to estimate the effect on T regulatory cells, and RORc and IL-17 as parameters for Th17 behavior. These parameters are increasingly studied in the perspective of CS-induced inflammation, as mentioned in the introduction.

Gene expression analysis in nasal turbinate

Neutrophilic chemoattraction related genes

In the nasal turbinates, no significant difference could be found in GCP-2 and KC levels after 2-wk CS exposure (Fig. 4A). Continued exposure (4-wk) however resulted in significant up-regulation of GCP-2 representing the neutrophilic chemoattractant signal in the CS group compared to the air group, since levels of KC did not differ between groups (Fig. 4B). This increase in GCP-2 expression disappeared at chronic (24-wk) CS exposure; moreover KC levels were significant lower in the CS group at that time point (Fig. 4C).

Monocyte/Macrophage chemoattraction related genes

We also found an interesting kinetics in the levels of MCP-1 and MIP-3α. At 2-wk, a significant up-regulation of MCP-1 mRNA in the CS-exposed group and a similar tendency for MIP-3α was seen (p = 0.08, Fig. 4A). This increase disappeared on continued exposure at 4-wk, both for MCP-1 and MIP-3α (Fig. 4B). Moreover, a significant lower expression of MCP-1 and a similar tendency for MIP-3α were noticed at chronic (24-wk) CS exposure (Fig. 4C).
Figure 4: Gene expression analysis in nasal turbinate. 2-wk CS exposure resulted in increased levels of MCP-1 and FoxP3. Levels of RORc and subsequent IL-17 were significantly down-regulated at this time point (Fig. 4A). At 4-wk, GCP-2, but not KC, levels are increased. Moreover, FoxP3 is significantly higher in the CS exposed group (Fig. 4B). 24-wk CS exposure results in significant down-regulation of nasal MCP-1, MIP-3α, and TGF-β1 (Fig. 4C). n = 8 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001.
T cell related genes

Interestingly, FoxP3 was already significantly increased after 2-wk and 4-wk CS exposure – although this was not the case for TGF-β1 – but not after 24-wk.

Levels of RORc and subsequent IL-17 were significantly down-regulated after 2-wk CS exposure (Fig. 4A), but this finding disappeared when CS exposure was prolonged.

Gene expression analysis in lung

Neutrophilic chemoattraction related genes

Significant up-regulation of both GCP-2 and KC in the CS group remained consistent throughout the entire study, representing the neutrophilic chemoattractant signal triggered by CS exposure (Fig. 5 A-C).

Monocyte/Macrophage chemoattraction related genes

Both MCP-1 and MIP-3α were significantly increased in the CS group at every time point (except for MIP-3α at 24wk, p = 0.05) (Fig. 5 A-C).

T cell related genes

In contrast to the nose, 2-wk CS exposure did not result in increased FoxP3 expression in the lungs (Fig. 5A). At 4-wk and 24-wk however, significantly higher FoxP3 levels were found in the CS exposed groups although we could only find higher TGF-β1 levels at 4-wk (Fig. 5B and C).

Although levels of RORc did not differ between experimental groups, IL-17 mRNA levels were significantly increased at 2-wk and 4-wk CS exposure, correlating with the neutrophilic chemoattraction signals.
Figure 5: Gene expression analysis in lung. Pulmonary levels of GCP-2, KC, MCP-1, MIP-3α and IL-17, but not FoxP3 were significantly increased after 2-wk CS exposure (Fig. 5A). After 4-wk CS exposure, all markers of neutrophilic and monocyte/macrophage chemoattraction are significantly increased, as well as FoxP3 and TGF-β1 (Fig. 5B). Chronic CS exposure caused an increase in levels of GCP-2, KC, MCP-1 and FoxP3 levels (MIP-3α p = 0.05) (Fig. 5C). n = 8 mice/group, *p < 0.05, **p < 0.01, ***p < 0.001.
Analysis of FoxP3 expression in epithelium vs. subepithelium of nasal turbinates

Recently, FoxP3 expression in epithelial cells has been described\(^2\). In order to determine the source of FoxP3 expression in whole nasal turbinate, we isolated nasal epithelial cells and subepithelial cells by magnetic cell sorting. The mRNA expression of FoxP3 however was not altered in the nasal epithelium after 4-wk CS exposure (Air 0.3453 ± 0.0084 versus Smoke 0.2894 ± 0.0084 normalized relative expression units). On the contrary, we demonstrated a nearly 5-fold increase in subepithelial FoxP3 expression in nasal turbinates upon 4-wk CS exposure, possibly due to infiltrating T regulatory cells (Air 1.0432 ± 0.0723 versus Smoke 5.1730 ± 0.9323).

DISCUSSION

In this study we aimed to investigate the effects of cigarette smoke (CS) on upper airways and lower airways, in a mouse model of subacute and chronic CS exposure. We here demonstrate for the first time that the inflammatory response upon CS exposure clearly differs between nose and lungs in mice. The nature and kinetics of both the neutrophil and monocyte/macrophage inflammation differ in both airways compartments. This indicates the involvement of different regulatory mechanisms, which is reflected by the observed differences in FoxP3 increase after CS exposure. The suppressive mechanisms arise earlier and appear to be more efficient in nose than in lungs. Although increased levels of MCP-1, MIP-3\(\alpha\) and GCP-2 are found both in nose and lungs after subacute CS exposure, the neutrophilic influx and increase in neutrophilic chemoattraction signals are transient in upper airways while they remain constant in lower airways. Consequently, chronic upper airway CS exposure results in a non-inflammatory status with a significant downregulation of inflammation, while lower airway inflammation is clearly present and ongoing.

Neutrophilic inflammation in the nasal turbinate tissue was not present after 2-wk CS exposure, likely due to the absence of a neutrophilic chemoattraction signal, as both GCP-2 and KC levels were not increased in the CS group. However, prolonged (4-wk) exposure caused a significant GCP-2 increase in the CS group, which correlates with the immunohistochemistry, showing a higher number of
neutrophils per field in the CS group compared to the air group, but only after 4-wk. To our surprise, chronic (24-wk) CS exposure did not cause a further increase in neutrophil accumulation in the nasal turbinate tissue. Moreover, GCP-2 levels and KC levels in the CS group did not differ and were significantly down regulated from controls respectively. This was again confirmed by IHC, where we found a significant decrease in the number of neutrophils per field in the CS group compared to controls. This may be interpreted as a clear sign of down-regulation of the neutrophilic inflammatory long-term response in the nasal turbinates. Evaluation of neutrophilic inflammation in upper airways was done in nasal turbinate tissue, because nasal lavage did not yield sufficient cells allowing a reliable cell differentiation. As a consequence, compartmentalization of inflammation in both upper and lower airways may influence the interpretation of these findings. Indeed, cigarette smoke causes an increase of neutrophil numbers in BAL (mouse studies), or sputum (human studies), whereas its effect in lung tissue or biopsies is less pronounced.

Our findings on neutrophilic inflammation in upper airways are in sharp contrast with the data obtained from experiments in the lung, where CS exposure resulted in a significant increase in both GCP-2 and KC at all time points, accounting for the observed influx of neutrophils in the BAL fluid of these mice\textsuperscript{23}.

We have shown a remarkable change over time in the nasal mRNA MCP-1 levels of CS exposed mice, showing an initial increase, followed by a significant decrease in MCP-1 levels in the nasal turbinate upon chronic exposure. In the lungs of these mice however, we detected a consistent increase in MCP-1 levels in CS exposed mice on each time point\textsuperscript{23}. This is another sign of the different inflammatory response to CS in the upper airway.

The role of pro-inflammatory T helper 17 phenotype in the pathogenesis of COPD is increasingly studied, and it is suggested that COPD might be better explained by the Th17 phenotype\textsuperscript{12}. These Th17 cells, which require the up-regulation of the orphan nuclear receptor RORgammat (encoded by RORc) for differentiation from naïve T cells\textsuperscript{24}, account for the production of several members of the IL-17 family of cytokines, which have proven abilities to recruit and activate neutrophils\textsuperscript{25}. Here, nasal
mRNA levels of RORc and IL-17 in the nose were significantly down-regulated after 2-wk CS exposure, but not upon longer (4-wk and 24-wk) exposure. In lungs however, the response of Th17 cells appears to be opposite, as 2-wk and 4-wk CS exposure resulted in a significant up-regulation of IL-17, and chronic (24-wk) exposure showed a similar tendency. These differences in IL-17 levels between nose and lungs, can explain the observed differences in neutrophil accumulation, as described above.

Table 3: Effect of CS-exposure in nose and lungs at 2-wk, 4-wk and 24-wk.

<table>
<thead>
<tr>
<th>Duration</th>
<th>2-wk</th>
<th>4-wk</th>
<th>24-wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nose</td>
<td>↑ MCP-1</td>
<td>↑ GCP-2</td>
<td>↓ KC</td>
</tr>
<tr>
<td></td>
<td>↑ FoxP3</td>
<td>↑ FoxP3</td>
<td>↓ MCP-1</td>
</tr>
<tr>
<td></td>
<td>↓ RORc</td>
<td>↓ FoxP3</td>
<td>↓ TGF-β1</td>
</tr>
<tr>
<td></td>
<td>↓ IL 17</td>
<td>↓ FoxP3</td>
<td>↓ GCP-2</td>
</tr>
<tr>
<td>Lung</td>
<td>↑ GCP-2</td>
<td>↑ GCP-2</td>
<td>↑ GCP-2</td>
</tr>
<tr>
<td></td>
<td>↑ KC</td>
<td>↑ KC</td>
<td>↑ KC</td>
</tr>
<tr>
<td></td>
<td>↑ MCP-1</td>
<td>↑ MCP-1</td>
<td>↑ MCP-1</td>
</tr>
<tr>
<td></td>
<td>↑ MIP-3α</td>
<td>↑ MIP-3α</td>
<td>↑ MIP-3α</td>
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<td></td>
<td>↑ IL 17</td>
<td>↑ IL 17</td>
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<td></td>
<td>↑ FoxP3</td>
<td>↑ FoxP3</td>
<td>↑ FoxP3</td>
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<td></td>
<td>↑ TGF-β1</td>
<td>↑ TGF-β1</td>
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</table>

T regulatory cells expressing FoxP3 are thought to play a role in controlling CS induced inflammation, amongst others via the immunomodulatory cytokine TGF-β1. In nose, FoxP3 mRNA expression was increased already after 2-wk, and was mainly found – at least at 4-wk – in the subepithelium, possibly due to invading Tregs expressing FoxP3. In lungs, FoxP3 was only increased after 4-wk, which is in line with increased Tregs in lungs after CS exposure in humans. Interestingly, these infiltrating Tregs in lungs are thought to have a weak functionality, as they are unable to control inflammation in lungs. It is tempting to speculate that Tregs act early and adequately in nose to suppress CS-induced inflammation, but that they invade later and have weak functionality in lungs, allowing inflammation to persist. Alternatively, the CS exposure of the nose might be higher in mice – obligatory nose-breathing animals – compared to lungs, allowing tolerazation or change in cell
populations to occur earlier. Indeed, upon 24-wk CS exposure the number of neutrophils shows a decreasing tendency compared to 4-wk CS exposed mice.

Although *in vivo* cigarette smoke-exposed mice can offer valuable information on several aspects of the pathogenesis of COPD, such as the time course of upper and lower airway inflammation, there are also limitations that need to be taken into account. Firstly, a number of anatomical and physiological differences exist between the respiratory tract of mice and humans. For example, mice are obligate nose breathers that filter tobacco smoke inefficiently, and they have less branching of the bronchial tree. Furthermore, the profile of inflammatory mediators is also slightly different in the mouse. And lastly, there is no mouse model that mimics all the hallmarks of COPD pathology, including exacerbations and extrathoracic manifestations.

Another possible limitation to this study is the fact that not only T cells are able to produce either IL-17, TGF-β or FoxP3, but a number of other cells like neutrophils or epithelial cells can do so. Furthermore, the suppressive capacity of the FoxP3 producing Tregs in upper airways stills remains to be elucidated.

Although the inflammatory answer of nose and lungs is clearly different upon CS exposure, possible confounding factors might influence the data interpretation in this model. Above, we have described the issue of compartmentalization of inflammation, and the relative dosage exposure, with higher deposition of CS in the nose vs. lungs. Furthermore, physiologic temporal changes are seen in the inflammatory readouts: levels of inflammatory cells and mediators of unexposed control mice vary over time, as shown in Fig. 3-5. By using age-matched control mice in our experiments, we have corrected for these physiologic temporal changes. Altogether, the above mentioned limitations of this model remain to be elucidated.
CONCLUSIONS

In conclusion, we have demonstrated that cigarette smoke-induced inflammation differs between nose and lungs in this mouse model. After CS exposure, inflammatory markers were upregulated in lungs at all time points. However, this was not the case in the nose, where particularly upon chronic CS exposure, nasal inflammatory markers were significantly lower than the control (air) conditions. It is possible that infiltrating FoxP3 expressing Tregs might account for these observed differences, although further investigation is necessary to identify possible differences in their suppressive functionality in both airway compartments.
REFERENCES

Chapter 6

Exacerbation of CS-induced pulmonary inflammation

by SEB in mice
EXACERBATION OF CIGARETTE SMOKE-INDUCED PULMONARY INFLAMMATION BY

STAPHYLOCOCCUS AUREUS ENTEROTOXIN B IN MICE

Wouter Huvenne*, Ellen Lanckacker*, Olga Krysko, Ken Bracke, Tine Demoor, Peter Hellings, Guy Brusselle, Guy Joos, Claus Bachert, Tania Maes

* Equal contribution


ABSTRACT

Background: Cigarette smoke (CS) is a major risk factor for the development of COPD. CS exposure is associated with an increased risk of bacterial colonization and respiratory tract infection, because of suppressed antibacterial activities of the immune system and delayed clearance of microbial agents from the lungs. Colonization with Staphylococcus aureus results in release of virulent enterotoxins, with superantigen activity which causes T cell activation.

Objective: To study the effect of Staphylococcus aureus enterotoxin B (SEB) on CS-induced inflammation, in a mouse model of COPD.

Methods: C57/Bl6 mice were exposed to CS or air for 4 weeks (5 cigarettes/exposure, 4x/day, 5 days/week). Endonasal SEB (10µg/ml) or saline was concomitantly applied starting from week 3, on alternate days. 24h after the last CS and SEB exposure, mice were sacrificed and bronchoalveolar lavage (BAL) fluid and lung tissue were collected.

Results: Combined exposure to CS and SEB resulted in a raised number of lymphocytes and neutrophils in BAL, as well as increased numbers of CD8+ T lymphocytes and granulocytes in lung tissue, compared to sole CS or SEB exposure. Moreover, concomitant CS/SEB exposure induced both IL-13 mRNA expression in lungs and goblet cell hyperplasia in the airway wall. In addition, combined
Chapter 6: Exacerbation of CS-induced pulmonary inflammation by SEB in mice

CS/SEB exposure stimulated the formation of dense, organized aggregates of B- and T- lymphocytes in lungs, as well as significant higher CXCL-13 (protein, mRNA) and CCL19 (mRNA) levels in lungs.

Conclusions: Combined CS and SEB exposure aggravates CS-induced inflammation in mice, suggesting that *S. aureus* could influence the pathogenesis of COPD.

INTRODUCTION

Cigarette smoking is associated with an increased risk of bacterial colonization and respiratory tract infection, because of suppressed antibacterial activities of the immune system and delayed clearance of microbial agents from the lungs\(^1\). This is particular relevant in COPD patients, where bacterial colonization in the lower respiratory tract has been shown\(^2\). These bacteria are implicated both in stable COPD and during exacerbations, where most commonly pneumococci, *Haemophilus influenza*, *Moraxella catarrhalis* and *Staphylococcus aureus (S. aureus)* are found\(^3\). Interestingly, colonization with *S. aureus* may embody a major source of superantigens as a set of toxins are being produced including *S. aureus* enterotoxins (SAEs)\(^4\). These toxins activate up to 20% of all T cells in the body by binding the human leukocyte antigen (HLA) class II molecules on antigen-presenting cells (APCs) and specific V beta regions of the T cell receptor\(^5\). Between 50 and 80% of *S. aureus* isolates are positive for at least one superantigen gene, and close to 50% of these isolates show superantigen production and toxin activity\(^6\).

During the last few years, it became increasingly clear that SAEs are known to modify airway disease\(^7\), like allergic rhinitis\(^8\), nasal polyposis\(^9\) and asthma\(^10\). Furthermore, studies have shown a putative role for SAEs in patients suffering from the atopic eczema/dermatitis syndrome (AEDS), where colonization with *S. aureus* is found more frequently (80-100%) compared to healthy controls (5-30%)\(^11\), and *S. aureus* isolates secrete identifiable enterotoxins like *Staphylococcus aureus* enterotoxin A and B (SEA, SEB) and toxic shock syndrome toxin (TSST)-1. Until now, evidence for SAE involvement in the pathogenesis of upper airway disease like chronic rhinosinusitis with nasal polyposis (CRSwNP), arises from the finding that IgE against SEA and SEB has been demonstrated...
in nasal polyps\textsuperscript{12} and levels of SAE-specific IgE in NP correlated with markers of eosinophil activation and recruitment\textsuperscript{13}. Similarly, in COPD patients, a significantly elevated IgE to SAE was found, pointing to a possible disease modifying role in COPD, similar to that in severe asthma\textsuperscript{14}. Moreover, we have recently demonstrated the pro-inflammatory effect of SEB on human nasal epithelial cells \textit{in vitro}, resulting in augmented granulocyte migration and survival\textsuperscript{15}.

In murine research, the role of SAEs as inducer and modifier of disease has been demonstrated in models of airway disease\textsuperscript{16, 17}, allergic asthma\textsuperscript{18}, atopic dermatitis\textsuperscript{19} and food allergy\textsuperscript{20}. These findings highlight the important pathological consequences of SAE exposure, as these superantigens not only cause massive T-cell stimulation, but also lead to activation of B-cells and other pro-inflammatory cells like neutrophils, eosinophils, macrophages and mast cells\textsuperscript{21}.

To date, the exact pathomechanisms of COPD are not yet elucidated. Cigarette smoking is a primary risk factor for the development of COPD, but only 20\% of smokers actually develop the disease, suggesting that genetic predisposition plays a role\textsuperscript{22}. However, understanding the impact of toxin-producing bacteria on cigarette-smoke induced inflammation might provide novel insights into the pathogenesis of smoking-related disease such as COPD. Therefore, we investigated the effects of concomitant \textit{Staphylococcus aureus} Enterotoxin B (SEB) application on a well established mouse model of cigarette-smoke (CS) induced inflammation\textsuperscript{23}. We evaluated inflammatory cells and their mediators in bronchoalveolar lavage (BAL) fluid and lung tissue, looked at systemic effects by measuring serum immunoglobulins, and evaluated goblet cell hyperplasia and lymphoid neogenesis.

**METHODS**

**Experimental protocol**

Male C57BL/6 mice (n=8), 6–8-week old were purchased from Charles River Laboratories (Brussels, Belgium). Mice were exposed to the tobacco smoke of five cigarettes (Reference Cigarette 2R4F without filter, University of Kentucky, Lexington, KY, USA) four times per day with 30 min smoke-
free intervals. The animals were exposed to mainstream cigarette smoke (CS) by whole body exposure, 5 days per week for 4 weeks. Control groups (8 age-matched male C57BL/6 mice) were exposed to air. Starting from day 14 of the CS exposure, mice received concomitant endonasal application of SEB (50 µL – 10 µg/mL - Sigma-Aldrich, LPS content below detection limit) or Saline, on alternate days. For this, mice were slightly anaesthetized with isoflurane, and six applications were performed as depicted in Fig. 1. All experimental procedures were approved by the local ethical committee for animal experiments (Faculty of Medicine and Health Sciences, Ghent University). The results section contains data from one representative experiment out of three independent experiments.

Figure 1: Experimental protocol. Male C57BL/6 mice (n=8) were exposed to the tobacco smoke of five cigarettes four times per day with 30 min smoke-free intervals. Controls were exposed to air. Starting from day 14 of the CS exposure, mice received concomitant endonasal application of SEB (50 µL – 10 µg/mL) or saline, on alternate days.

Bronchoalveolar lavage and cytospins

Twenty-four hours after the last cigarette smoke (CS) exposure and endonasal application, mice were sacrificed by a lethal dose of pentobarbital (Sanofi-Synthelabo). A cannula was inserted in the trachea, and BAL was performed by instillation of 3 x 300 µl of HBSS supplemented with BSA for cytokine measurements. Three additional instillations with 1 ml of HBSS plus EDTA were performed to achieve maximal recovery of BAL cells. A total cell count was performed in a Bürker chamber. Approximately fifty thousand BAL cells were processed for cytospins and were stained with May-Grünwald-Giemsa for differential cell counting. The remaining cells were used for FACS analysis.
Preparation of lung single-cell suspensions

Blood was collected via retro-orbital bleeding. Then, the pulmonary and systemic circulation was rinsed to remove contaminating blood cells. Lungs were taken and digested as described previously\textsuperscript{24}. Briefly, minced lung pieces were incubated with 1 mg/ml collagenase and 20 µg/ml DNase I for 45 min at 37°C. Red blood cells were lysed using ammonium chloride buffer. Finally, cell suspensions were filtered through a 50-µm nylon mesh to remove undigested organ fragments.

Flow cytometry

All staining procedures were conducted in calcium- and magnesium-free PBS containing 10 mM EDTA, 1% BSA (Dade Behring), and 0.1% sodium azide. Cells were preincubated with anti-CD16/CD32 (2.4G2) to block Fc receptors. Antibodies used to identify mouse DC populations were anti-CD11c-allophycocyanin (APC; HL3) and anti-I-Ab-phycoerythrin (PE; AF6-120.1). The following mAbs were used to stain mouse T-cell subpopulations: anti-CD4-fluorescein isothiocyanate (FITC; GK1.5), anti-CD8-FITC (53-6.7), anti-CD3-APC (145-2C11) and anti-CD69-PE (H1.2F3). + anti-Gr-1-PE (RB6-8C5).

As a last step before analysis, cells were incubated with 7-aminoactinomycin D (or viaprobe; BD Pharmingen) for dead cell exclusion. All labeling reactions were performed on ice in FACS-EDTA buffer. Flow cytometry data acquisition was performed on a FACScalibur\textsuperscript{TM} running CellQuest\textsuperscript{TM} software (BD Biosciences, San Jose, CA, USA).

Measurement of Immunoglobulins

Retro-orbital blood was drawn for measurement of total IgE, IgG, IgM and IgA with ELISA. Commercially available ELISA kits were used to determine serum and BAL titers of IgG (ZeptoMetrix, Buffalo, NY, USA), IgM (ZeptoMetrix, Buffalo, NY, USA) and IgA (Alpha Diagnostic International, San Antonio, TX, USA). For the measurement of total IgE, a two-side in-house
sandwich ELISA was used, with two monoclonal rat anti-mouse IgE antibodies reacting with different epitopes on the epsilon heavy chain (H. Bazin, Experimental Immunology Unit, UCL, Brussels, Belgium). The second antibody was biotinylated and detected colorimetrically after adding horseradish peroxidase-streptavidine conjugate. Absorbance values, read at 492 nm (Labsystems Multiscan RC, Labsystems b.v., Brussels, Belgium) were converted to concentrations in serum and BAL fluid by comparison with a standard curve obtained with mouse IgE of known concentration (H. Bazin).

**Goblet cell analysis**

Left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Transversal sections of 3 µm were stained with periodic acid-Schiff (PAS) to identify goblet cells. Quantitative measurements of goblet cells were performed in the airways with a perimeter of basement membrane (Pbm) ranging from 800 to 2000 µm. Results are expressed as the number of goblet cells per millimeter of basement membrane.

**Morphometric quantification of lymphoid neogenesis**

To evaluate the presence of lymphoid infiltrates in lung tissues, sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to an immunohistological CD3/B220 double-staining as described previously. Infiltrates in the proximity of airways and blood vessels were counted. Accumulations of ≥50 cells were defined as lymphoid aggregates. Counts were normalized for the number of bronchovascular bundles per lung section.
RT-PCR analysis

Total lung RNA was extracted with the Rneasy Mini kit (Qiagen, Hilden, Germany). Expression of CXCL-13, CCL19 and IL-13 mRNA relative to HPRT mRNA\textsuperscript{25}, were performed with Assay-on-demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA). Real-time RT PCR for CCL21-leucine and CCL21-serine started from 25 ng of cDNA. Primers and FAM/TAMRA probes were synthesized on demand (Sigma-Proligo). Primer/probe sequences and PCR conditions were performed as described previously\textsuperscript{26, 27}.

Protein measurement in BAL

IL-13 and CXCL13 protein levels in BAL SN were determined using a commercially available ELISA (R&D Systems, Abingdon, UK). Cytometric Bead Array (BD Biosciences, San Jose, CA, USA) was used to detect the cytokines KC, MCP-1, IL-17A and IFN-gamma in the supernatant of BAL fluid.

Statistical analysis

Reported values are expressed as mean ± SEM. Statistical analysis was performed with SPSS software (version 16.0) using nonparametric tests (Kruskal-Wallis and Mann-Whitney \textit{U} test). The significance level was set at $\alpha = 0.05$. (\textsuperscript{*}p < 0.05; \textsuperscript{**}p < 0.01; \textsuperscript{***}p < 0.001).

RESULTS

SEB aggravates the CS-induced pulmonary inflammation

To evaluate the effects of \textit{Staphylococcus aureus} enterotoxin B (SEB) on cigarette smoke (CS)-induced pulmonary inflammation, C57Bl6 mice were exposed to CS for 4 weeks, with a concomitant SEB exposure during the last 2 weeks (Fig. 1).
Chapter 6: Exacerbation of CS-induced pulmonary inflammation by SEB in mice

Figure 2: BAL fluid analysis. In BAL fluid, sole endonasal SEB application and sole CS-exposure resulted in increased numbers of total cells, alveolar macrophages, dendritic cells (DCs), lymphocytes and neutrophils, compared to air/saline exposed animals. Interestingly, the combination of CS exposure and SEB significantly increased BAL neutrophil numbers compared to sole CS or SEB exposure. Also BAL lymphocyte numbers in smoke-exposed mice were significantly increased upon SEB application. Results are expressed as mean±SEM, n=8 animals/group, *p<0.05, **p<0.01, ***p<0.001.

In BAL fluid, sole endonasal SEB application and sole CS-exposure resulted in increased numbers of total cells, alveolar macrophages, dendritic cells (DCs), lymphocytes and neutrophils, compared to air/saline exposed animals (Fig. 2). However, these increases in cell numbers were much more pronounced upon SEB application compared to CS-exposure. Also a modest eosinophilic inflammation was observed in the SEB-exposed groups.

Interestingly, the combination of CS exposure and SEB significantly increased BAL neutrophil numbers compared to sole CS or SEB exposure (Fig. 2). Also BAL lymphocyte numbers in smoke-exposed mice were significantly increased upon SEB application.

In lung single cell suspensions, SEB solely induced an increase in DCs, CD3+ T cells and macrophages, whereas CS exposure caused increased DCs and CD3+ T cells in lung tissue (Fig. 3).
Interestingly, combined CS and SEB exposure caused a further increase in CD3+ T cells, and more specifically CD8+ T-cells, compared to CS or SEB alone (Fig. 3). Also DC, CD4+ T-cells and GR1+ cells tended to be higher in the combined CS/SEB group versus sole CS or SEB application.

![Figure 3: Lung cell differentiation. In lung single cell suspensions, SEB solely induced an increase in DCs, CD3+ T cells and macrophages, whereas CS exposure caused increased DCs and CD3+ T cells in lung tissue. Interestingly, combined CS and SEB exposure caused a further increase in CD3+ T cells, and more specifically CD8+ T-cells, compared to CS or SEB alone. Results are expressed as mean±SEM, n=8 animals/group, *p<0.05, **p<0.01, ***p<0.001.](image)

**Increased IL-17A in BAL upon combined SEB and CS exposure**

As previously described, 4-wk CS-exposure clearly induced high levels of KC (mouse homolog for IL-8) and MCP-1 in BAL (Fig. 4). In contrast sole SEB application induced a modest increase in KC, and very low levels of IFN-gamma and IL-17A. Whereas the CS-induced KC and MCP-1 levels in BAL were not affected by an additional SEB exposure, the combined CS and SEB exposure did induce IL-17A levels in BAL, compared to single CS or SEB exposure (Fig. 4). Also IFN-gamma levels tended to be highest in the combined CS/SEB group.

mRNA levels of MIP-3a were increased after both CS or SEB exposure. Combined CS/SEB exposure did not cause a further MIP-3a increase (Fig. 4).
Chapter 6: Exacerbation of CS-induced pulmonary inflammation by SEB in mice

Figure 4: Protein measurement in BAL. 4-wk CS-exposure clearly induced high levels of KC and MCP-1 in BAL. In contrast, sole SEB application induced a modest increase in KC, and very low levels of IFN-gamma and IL-17A. Combined CS and SEB exposure significantly increased IL-17A levels in BAL, compared to single CS or SEB exposure. Also IFN-gamma levels tended to be highest in the combined CS/SEB group. Levels of MIP-3a mRNA were significantly increased after both CS or SEB exposure, but combined CS/SEB exposure did not cause a further MIP-3a increase. Results are expressed as mean±SEM, n=8 animals/group, *p<0.05, **p<0.01, ***p<0.001.

Figure 5: BAL immunoglobulin levels. In BAL, CS exposure induced a small but significant increase in IgA. Both IgA and IgM levels in BAL were increased upon SEB-exposure. IgE in BAL was below the detection limit. Results are expressed as mean±SEM, n=8 animals/group, *p<0.05, **p<0.01, ***p<0.001.
**SEB induces IgA and IgM levels in BAL**

Systemic effects of either CS or SEB, or both were evaluated in serum, but no significant differences in total IgG, IgM, IgA or IgE levels were detected between the experimental groups. In BAL, CS exposure induced a small but significant increase in IgA. Both IgA and IgM levels in BAL were increased upon SEB-exposure (Fig. 5). IgE in BAL was below the detection limit, IgG was not measured in BAL.

**Combined CS/SEB exposure affects epithelial remodeling**

Epithelial remodeling was evaluated by counting the number of PAS-positive goblet cells per millimeter of basement membrane. A strong tendency towards increased numbers of goblet cells in the CS/SEB mice was observed, compared to all other conditions (Fig. 6A,B). This finding correlated nicely with a significant increase in IL-13 mRNA expression in total lung in CS/SEB mice (Fig. 6C).

**Combined CS/SEB induces the formation of dense lymphoid aggregates in lung tissue**

Previously, our group has demonstrated increased lymphoid neogenesis after 6 months of CS-exposure\(^\text{25}\). As earlier shown in the CS-model, subacute CS-exposure as such did not result in lymphoid neogenesis. Interestingly however, already after 4-wk CS-exposure, dense, organized lymphoid aggregates could be demonstrated in the combined CS/SEB group whereas air/SEB mice displayed mainly loose, non-organized lymphoid aggregates (Fig. 7).

Since CXCL13, CCL19 and CCL21 are chemokines involved in the homeostatic trafficking of leukocytes, mainly lymphocytes, to the secondary and tertiary lymphoid tissues, their expression was also evaluated in this model. The increase in dense lymphoid aggregates in CS/SEB mice correlated nicely with significant increases in CXCL13 (protein levels in BAL fluid, mRNA levels in total lung) and CCL19 (mRNA levels) expression in CS/SEB mice compared to all other groups (Fig. 8). CCL21 mRNA levels (both isoforms CCL21-Ser and CCL21-Leu) decreased upon CS exposure, confirming
previous findings of CCL21 downregulation upon subacute CS exposure\textsuperscript{26} and decreased even further in the CS/SEB group. Intriguingly, the CCL21 mRNA levels of both isoforms tended to increase upon sole SEB exposure.

Figure 6: Epithelial remodeling. A strong tendency towards increased numbers of goblet cells in the CS/SEB mice was observed, compared to all other conditions. This finding collated nicely with a significant increase in IL-13 mRNA expression in total lung in CS/SEB mice. Results are expressed as mean±SEM, n=8 animals/group, *\textit{p}<0.05, **\textit{p}<0.01, ***\textit{p}<0.001.
DISCUSSION

We hereby describe a novel mouse model of combined *Staphylococcus aureus* enterotoxin B (SEB) application and cigarette smoke exposure, which results in a significant aggravation of key features of CS-induced pulmonary inflammation, such as neutrophils and CD8+ T cells in BAL and lung. Furthermore, levels of IL-17A in BAL were significantly increased upon concomitant SEB and CS exposure, compared to sole exposures of SEB or CS. In addition, tendencies of increased goblet cell hyperplasia, IL-13 mRNA expression and lymphoid neogenesis in smoke/SEB mice have been demonstrated, as well as increased expression of the relevant chemokines CXCL13 and CCL19. Altogether, these findings point to a possible disease-modifying role for SEB in CS-induced inflammation in this mouse model of subacute CS exposure.

![Figure 7: Evaluation of lymphoid aggregates in lung tissue. Subacute CS-exposure as such did not result in lymphoid neogenesis. Interestingly however, already after 4-wk CS-exposure, dense, organized lymphoid aggregates could be demonstrated in the combined CS/SEB group whereas air/SEB mice displayed mainly loose, non-organized lymphoid aggregates. Results are expressed as mean, n=8 animals/group, *p<0.05, **p<0.01, ***p<0.001.](image)
Increasing evidence from human and murine research suggests that *Staphylococcus aureus* enterotoxin B is able to aggravate underlying disease. Moreover, SEB itself is also able to induce inflammation, depending on the dosage and timing of the experimental protocol\textsuperscript{16, 19}. Interestingly, these findings are not confined to SEB, as other staphylococcal superantigens demonstrate similar effects upon mucosal contact\textsuperscript{28,29}. In line with previously reported findings, in our model sole endonasal SEB application caused an increase in total BAL cell number, lymphocytes and neutrophils\textsuperscript{16}. Moreover, we could demonstrate raised numbers of macrophages and dendritic cells, a finding previously reported after *S. aureus* enterotoxin A exposure\textsuperscript{28,29}. In the latter studies however, the authors could not demonstrate increased eosinophils, which was the case in our model. The superantigen effect of SEB caused the expected lymphocyte accumulation in BAL, which appeared to be non-specific, as both CD4+ and CD8+ T cells were increased. These data stress the potency of staphylococcal superantigens of initiating a massive immune response.

Concomitant CS/SEB exposure lead to a remarkable increase in neutrophil number, compared to CS or SEB exposure alone. Although the findings for neutrophils in lung (measured with granulocyte marker GR-1) were less convincing than in BAL, the combined CS/SEB group showed the highest number of GR-1+ cells. Interestingly, also the CD8+ T cell fraction in lung single cell suspensions, was significantly upregulated when smoke and SEB were combined. The potential clinical relevance of increased neutrophil and CD8+ T-cell numbers lays in the fact that neutrophilic inflammation in the airways in smokers correlates with an accelerated decline in lung function\textsuperscript{30}, and increased T-cell numbers correlate with the amount of alveolar destruction and the severity of airflow obstruction\textsuperscript{31}. 


We confirm an increased MIP-3a expression in lungs after CS exposure leading to an accumulation of dendritic cells in this model\(^\text{24}\). Interestingly, this increase in MIP-3a is also seen after SEB exposure, with raised DCs in BAL and airway parenchyma in these groups.

As previously demonstrated in the subacute CS-model, we have observed an increase in levels of KC, MCP-1 and IFN-gamma after 4-wk CS exposure\(^\text{24}\), explaining the accumulation of inflammatory cells in BAL and lung. Sole SEB application on the other hand resulted in raised levels of KC, IFN-gamma and IL-17A, but not MCP-1. Interestingly, the combined exposure of smoke and SEB further...
increased the IL-17A levels, which might explain the exacerbated BAL neutrophilia in CS/SEB mice. Indeed, IL-17 is known to be important in neutrophil maturation, migration and function in the lung tissue and airways. Furthermore, IL-17 induction of neutrophil activation and migration is important in defense against organisms infecting the lung\(^{32}\). Interestingly, IL-17 can also induce eosinophilic accumulation, in particular circumstances\(^{33}\).

IL-17 is normally produced by CD4+ T cells, although it might also arise from CD8+ T cells and in some cases even from macrophages, neutrophils or eosinophils\(^{34}\), as a necessary step in the normal immunity against bacterial infections in the airways. However, IL-17 has been linked to unfavorable outcome to infection, in particular in the presence of IFN-gamma\(^{35}\), resulting a high inflammatory pathology and tissue destruction. Increasing evidence dedicates a role to exaggerated recruitment and activation of neutrophils in the clinical course of airway diseases like COPD. Therefore, it is tempting to speculate on a role for SEB in the induction of IL-17 release, leading to the aggravation of cigarette smoke-induced inflammation, with increased number and activation of neutrophils, which causes amplification of tissue destruction and subsequent disease progression.

In addition, we could observe already after 4-wks an increase in the number of dense lymphoid aggregates in CS/SEB mice, linked to increased levels of CXCL13 and CCL19, which are attractants for B- and T-cells respectively. Moreover, it has been described that the respective receptors for these chemokines – CXCR5 and CCR7 – are also expressed on Th17 cells migrating into inflamed tissue\(^{36}\), indicating a potential contribution of IL17-producing Th17 cells in this model of early COPD. The finding that lymphoid aggregates and the chemokines responsible for their neogenesis and organization\(^{25}\) are already upregulated after 4-wk CS/SEB exposure, stresses the clinical relevance of this novel model of combined CS and enterotoxin exposure.

Staphylococcal superantigens are able to cause massive polyclonal T and B cell proliferation. Upon local application, as is done in this model, this leads to the mucosal synthesis of immunoglobulins, explaining the observed increase in BAL IgA and IgM. In humans, it is thought that continuous
microbial stimulation leads to B cell turnover and plasma cell formation in nasal polyp disease, leading to an overproduction of immunoglobulins\textsuperscript{37}.

In this mouse model of early stage COPD with goblet cell hyperplasia and increased number of lymphoid follicles, endonasal SEB application has resulted in augmented CS-induced lower airway inflammation. CS and subsequent bacterial colonization are, amongst others, factors believed to determine both progression of COPD, as well as the frequency and severity of COPD exacerbations\textsuperscript{38}. Therefore, mouse models of CS and bacterial co-exposure have been used in the past, mainly using \textit{Haemophilus influenzae}\textsuperscript{39}. Bacterial colonization and infection is rare in lower airways, but not in upper airways. Local carriage of enterotoxin-producing \textit{S. aureus} in the nasal cavity is common, although multiple sites can be colonized (e.g. skin, pharynx and perineum)\textsuperscript{40}. These toxins, like toxic shock syndrome toxin-1 (TSST-1), are known superantigens causing systemic diseases like food poisoning and toxic shock syndrome \textsuperscript{4}. In nasal polyp disease, these toxins are believed to drive the local immunoglobulin production in response to enterotoxin-producing \textit{Staphylococcus aureus}.

Usage of a single toxin instead of \textit{S. aureus} in this model is both a strength and a limitation, since it simplifies the interpretation on one hand, but is not the real life situation on the other hand. Another limitation is that we cannot rule out endotoxin related effects in our model, although the LPS content of our SEB was below detection limit. In addition, SEB on itself has resulted in pronounced inflammation in BAL and lungs, as it is a known superantigen. Finally, another possible limitation of this model is the short term (4-wk) CS exposure, whereas COPD is a chronic disease.

Despite these limitations, altogether our findings indicate the importance of bacterial toxins present in the upper airways, affecting lower airway inflammation. The possible disease-modifying role for SAEs in COPD that has been described in humans in the past\textsuperscript{14}, combined with our findings stress the potential role of airway colonizing and toxin-producing \textit{Staphylococcus aureus}, in the pathophysiology of COPD\textsuperscript{3}. 
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Chapter 7

Discussion
DISCUSSION

Enterotoxin-producing *Staphylococcus aureus* are one of the most intensively studied pathogens in relation to inflammatory airway disease. The ubiquitous nature of this bacteria and its ability to secrete potent exotoxins, explain the fact that staphylococcal enterotoxins are possibly involved in the aggravation or modulation of numerous inflammatory conditions. Starting from the initial observations in chronic upper airway disease, where SEs are known modulators of CRSwNP disease, we have developed new mouse models and *in vitro* models, in which we have demonstrated that the prototypic staphylococcal enterotoxin B is able to initiate pronounced immune activation, leading to clinically significant disease. This proof of concept clearly dedicates an important role for staphylococcal enterotoxins in general, and SEB in particular, in the pathogenesis of airway disease.

SEB FACILITATES ALLERGIC SENSITIZATION

In chapter 3 we have investigated whether the immunostimulatory signal provided by SEB, is able to overcome immunological tolerance, which is the normal immune answer upon contact of the airway with an allergen. In collaboration with P. Holt we started from an observation in humans that in a cohort of 1380 14-year old teenagers, levels of SE-specific IgE were significantly higher in atopic individuals compared to non-atopics\(^1\). Moreover, SE-specific IgE was an independent risk factor for asthma and bronchial hyperreactivity (BHR) amongst these atopics. SEs may therefore play a role in allergic sensitization, and the development of BHR. This hypothesis was tested in a mouse model of repeated endonasal application of SEB, combined with the commonly used allergen OVA. Increasing data confirms on the role of microbial stimuli as adjuvant activity of microbial stimuli became recently available\(^2, 3\). These findings confirm our hypothesis that SEB is able to break primary tolerance to inhaled allergen\(^4\), possibly involving CD98 expression on nasal epithelial cells\(^5\).

Concomitant endonasal application of OVA and enterotoxin B resulted in an immune response to an otherwise inert allergen, characterized by the production of OVA-specific IgE, increased production of IL-4, IL-5 and IL-13, bronchial influx of eosinophils and development of BHR. Moreover, these
findings can be explained by the increase in DC migration and maturation observed in these mice, which occur in parallel to the SEB-mediated augmentation of allergen-specific T cell proliferation. This mouse model displays several interesting features mimicking more closely the human situation of allergic sensitization. First of all, both SEB and allergen are applied endonasally, which is the natural way of airway mucosal contact with these substances. Mouse models of allergic asthma, however classically use intraperitoneal injections with artificial Th2-inducing stimuli or adjuvants such as aluminium hydroxide to obtain allergic sensitization. Secondly, the combination of both the allergen and the enterotoxin appeared to be critical to induce sensitization and features of allergic asthma. Neither substance could induce these features on itself, which is indeed the case in the human situation, where airway contact with allergens normally results in tolerance. Thirdly, the presence of *S. aureus* in the upper airways has been demonstrated before, serving as a possible reservoir for SE exposure to the airways. Testing of normal adolescent sera indeed reveals widespread seroconversion to many of the common staphylococcal SAgs, indicating that exposure to a range of strains begins very early in life. Altogether, these findings strongly indicate a role for SEs in the development of allergic sensitization to common inhaled allergens.

**Perspectives**

In parallel with the demonstration of the capacity of SEB to break immune tolerance and cause sensitization to the allergen OVA in Balb/c mice, we showed that other enterotoxins of the *Staphylococcus aureus* were not capable of inducing similar immune effects. It may well be that other staphylococcal toxins can induce sensitization to OVA in difference mouse strains with another genetic repertoire like TCR VB isotypes or TLR expression profiles. In addition, toxins of other bacteria like *Streptococcus, Yersinia, …* or viruses may be involved in the immune responses to OVA, and can help in understanding the pathophysiology of sensitization and chronic airway inflammation.

Furthermore, the pivotal role of dendritic cells in this model cannot be underestimated. As SEB is known not only to bind on MHC class receptors, but also on Toll-like receptors, the specific role of these receptors on the antigen presenting cell must be elucidated. The role of the specific DC subsets...
involved in the facilitation of allergic sensitization in this model will be investigated. Testing whether other, more relevant allergens like grass pollen or house dust mite also result in sensitization upon concomitant application with SEB, will confirm the clinical relevance of this model. Finally, SEB might be able to break the secondary tolerance, which establishes in mouse models of allergic asthma after repeated allergen challenge. This repeated challenge results in the establishment of a state of inhalational tolerance, which can be delayed upon concomitant cigarette smoke exposure\(^7\). As SEB is able to break the primary tolerance in our model, it might also be able to achieve similar results.

**SEB-INDUCED CHEMOKINES AND GRANULOCYTE MIGRATION AND SURVIVAL**

Functioning as superantigens, SEs cause massive T and B cell stimulation. Other cell types however are also activated upon superantigenic contact: eosinophils, macrophages, mast cells, dendritic cells and epithelial cells. Mostly, these cells act as accessory cells for T or B cell activation, although direct activation of these pro-inflammatory cells is also possible. In this study, we evaluated the effect of SEB on human nasal epithelial cells. The epithelium is increasingly acknowledged as an important player in the pathogenesis of inflammatory airway disease, like CRSwNP as demonstrated recently\(^8\). The airway epithelium is not only a physiological barrier, but actively participates in the inflammatory response, because it is a major source of inflammatory cytokines and mediators. The fact that SEs are secreted from *S. aureus* which resides in this epithelium, contributes to the clinical relevance of this question.

The innate immune activation which was achieved by applying the prototypic superantigen SEB, was characterized by increased levels of IP-10, MIG, G-CSF, RANTES and MCP-1. These data stress the important role of the epithelium in chemokine production after contact with SEB, therefore actively participating in the pathogenesis of inflammatory diseases. Recently, investigators have demonstrated increase in IL-19 release by nasal epithelial cells from CRSwNP patients, indicating the importance of the TLR4/MyD88 pathway after microbial encounter\(^8\).
Interestingly, in our studies the supernatant containing the epithelial secreted chemokines, significantly altered granulocyte biology. Augmented migration and survival of granulocytes was induced upon co-incubation of this supernatant. As epidemiological data suggest a role for SEs in granulocyte-dominated disease like atopic dermatitis, allergic rhinitis, asthma, nasal polyposis or COPD, our data might contribute to the understanding of the granulocyte biology in these diseases.

The mechanism via which SEB switches on the epithelium to release chemokines, has been shown to rely on both MHC II receptors as well as non-MHC II receptors. Furthermore, SEB can bind Toll-like receptors, which are also present on epithelial cells. In this model however, we were not able to suppress SEB-induced epithelial cell activation using anti-TLR2/4 mAbs.

**Perspectives**

Mechanistically, the role of TLRs in the initiation of SEB-induced inflammation clearly merits further investigation. The pathway via which SEB activates the epithelial cells to secrete chemokines will be further investigated, in order to identify possible therapeutical targets. Furthermore, this novel model of freshly isolated nasal epithelial cells can be used to study the interaction of the epithelium with other agents, like allergens, pollutants, viruses, etc. This will provide further insight in the specific role of the epithelium in the pathogenesis of specific inflammatory airway diseases, and may contribute to the development of new therapeutic strategies using specific monoclonal antibodies in order to prevent the initiation of SEB-induced inflammation.
In a mouse model of subacute and chronic cigarette smoke (CS) exposure, which is described in chapter 5, we have investigated the regulation of inflammation in upper and lower airways. The findings of this model were relevant for the development of the protocol used in the experiments in chapter 6. Until now, little data has been published on the inflammatory response of the upper airways upon CS exposure, especially in comparison to the lower airways. However, human data suggest that 75% of the COPD patients have concomitant nasal symptoms and more than 1/3 of patients with sinusitis also have lower airway symptoms of asthma or COPD. Moreover, the link between upper and lower airway smoke induced inflammation is illustrated by increased nasal IL-8 concentrations correlating with IL-8 in sputum of COPD patients. In addition, these patients report a high prevalence of nasal symptoms and sinusitis, and nasal and bronchial inflammation coexist in smokers and is characterized by infiltration of CD8+ T lymphocytes. In this mouse model, we have demonstrated
that cigarette-smoke induced inflammation differs between nose and lungs. After CS exposure, inflammatory markers were upregulated in lungs at all time points. However, this was not the case in the nose, where particularly upon chronic CS exposure nasal inflammatory markers were significantly lower than the control (air) conditions. These findings are recently confirmed in the human situation, where authors demonstrate a divergent response in nasal vs. bronchial epithelial cells after CS extract exposure. Interest in the behavior of upper airways after CS exposure is growing, in particular in the context of chronic infectious disease. Other investigators evaluate upper airway inflammation after CS exposure in the scope of sudden infant death syndrome, indicating the important contribution of the upper airways in the inflammatory answer of the respiratory tract after CS exposure.

The nature and kinetics of both the neutrophil and monocyte/macrophage inflammation are clearly different in both airways compartments. This indicates the involvement of different regulatory mechanisms, which is reflected by the observed differences in FoxP3 increase after CS exposure. The suppressive mechanisms arise earlier and appear to be more efficient in nose than in lungs. Although increased levels of MCP-1, MIP-3α and GCP-2 are found both in nose and lungs after subacute CS exposure, the neutrophilic influx and increase in neutrophilic chemoattraction signals are transient in upper airways while they remain constant in lower airways. Consequently, chronic upper airway CS exposure results in a non-inflammatory status with a significant downregulation of inflammation, while lower airway inflammation is clearly present and ongoing. The regulation of the CS-induced inflammation was assessed in both airway compartments by measuring levels of FoxP3 mRNA. This is a marker for regulatory T cells, which are thought to play a role in controlling CS induced inflammation. In nose, we have demonstrated a fast and pronounced increase in FoxP3, mirroring a fast and adequate control of inflammation. In lungs however, FoxP3 increase occurred later and appeared to be inadequate, as inflammatory markers are increased despite high levels of T regulatory cells. Interestingly, in humans the functionality of infiltrating Tregs in lungs after CS exposure is thought to be weak. It is tempting to speculate that Tregs act early and adequately in the nose to suppress CS-induced inflammation, but invade later and have weaker functionality in lungs, allowing inflammation to persist.
Perspectives

The differences in regulation of inflammation between upper and lower airways are possibly due to differences in functionality in Tregs in both airway compartments. Further studies actually demonstrating these differences in Treg functionality could confirm this interesting hypothesis. Therefore, Tregs isolated from inferior turbinates can be co-incubated with naïve T cells, in order to study the suppressive capacity of these nasal Tregs. As a proof of concept, this suppressive capacity can be compared to the suppressive capacity of the pulmonary Tregs after CS-exposure. In addition, transfer of Tregs from upper to lower airways in order to suppress lower airway inflammation could prove the principle of adequate control of inflammation. Furthermore, unraveling the mechanisms responsible for adequate disease control in upper airways could lead to new therapeutic strategies for the optimization of disease control in lower airways.

SEB EXACERBATES CIGARETTE SMOKE-INDUCED INFLAMMATION

Similar to severe asthma, significantly higher levels of sIgE to SEs were found in COPD patients, indicating a disease-modifying role for enterotoxins in this particular condition. This hypothesis formed the basis for the experiments described in chapter 6. Here, we tested whether endonasally applied SEB could aggravate CS-induced inflammation, and expedite disease progression towards more severe stage COPD-like features. Therefore we took advantage of a mouse model of subacute CS exposure and developed a protocol of concomitant SEB application. Interestingly, combined Staphylococcus aureus enterotoxin B (SEB) application and cigarette smoke exposure resulted in a significant aggravation of key features of CS-induced pulmonary inflammation, such as neutrophils and CD8+ T cells in BAL and lung. Furthermore, levels of IL-17A in BAL were significantly increased upon concomitant SEB and CS exposure, compared to sole exposure to SEB or CS. In addition, tendencies of increased goblet cell hyperplasia, IL-13 mRNA expression and lymphoid neogenesis in smoke/SEB mice have been demonstrated, as well as increased expression of the relevant chemokines CXCL13 and CCL19.
An interesting feature of this model was the presence of dense lymphoid aggregates already after 4 weeks, in the mice receiving both CS and SEB. Indeed, the clinical relevance of this model exist in the fact that it confirms the initial hypothesis that COPD patients had a significant higher immune answer to SEs, indicating a possible role for these SEs in the pathogenesis of COPD. Moreover, lower airways COPD patients are more frequently colonized with bacteria, amongst which enterotoxin-producing *S. aureus*. In addition, upper airways are the most frequent region in the human body where *S. aureus* colonization is found. Altogether, these findings indicate that the pathophysiology of COPD is possibly influenced by *S. aureus* which colonizes upper and/or lower airways and is able to produce disease-modifying enterotoxins.

![Diagram](image)

**Figure 2**: CS exposure induced a remarkably different alteration in secretion of inflammatory mediators in nose vs. lungs at different time points (right). Combined CS/SEB exposure (left) aggravated CS induced inflammation, leading to activation of B cells, Th17 cells and neutrophils. Increased goblet cell hyperplasia and lymphoid neogenesis were also observed.
Perspectives

We could confirm the initial hypothesis of SAE involvement in the pathogenesis of COPD. Therefore, returning to the human situation and confirming the presence of increased number of lymphoid follicles in the COPD patients with higher SE-specific IgE levels, would prove the critical role of these SEs in this human diseases. In addition, we will elaborate more on the mechanisms behind the aggravating effects of SEB on CS-induced inflammation, in order to find new preventative strategies which could prevent the disease progression towards more severe stages of COPD. Therapeutic interventions like specific eradication of enterotoxin-producing S. aureus, or usage of enterotoxin-specific antibodies to prevent superantigen-induced immune activation could contribute to better disease control in severe COPD patients.
Chapter 7: Discussion

GENERAL CONCLUSIONS AND PERSPECTIVES

This thesis for the first time demonstrates the promotion of sensitization to inhalational allergens in mice, when these are co-administrated with \textit{Staphylococcus aureus} enterotoxin B. Furthermore, concomitant allergen and SEB application to the airways augmented dendritic cell migration and maturation, which results in effector T cell activation and the induction of airway inflammation and bronchial hyperresponsiveness. In another in vitro model, this pro-inflammatory role of SEB was confirmed: we have demonstrated that the incubation of nasal epithelial cells with SEB has resulted in chemokine secretion by the epithelium, which activates granulocytes for increased migration and survival. For the first time, freshly isolated nasal epithelial cells were used for such experiments which is a valuable protocol for future research purposes.

Regulation of cigarette smoke induced inflammation is different in upper versus lower airways. This thesis demonstrates the early and adequate control of inflammation in upper airways, which is indicated by an increase in FoxP3 mRNA. In lower airways, this increase begins later and appears to be inadequate, as inflammation in the lower airways is still present and ongoing, despite high FoxP3 levels. In this same mouse model of subacute CS exposure, combined CS and SEB exposure has led to hallmarks of early COPD, displaying lymphoid neogenesis already after 4 weeks. Furthermore, CS and SEB appeared to function synergistic, and not just additive. These findings confirm human observations, where a disease-modifying role for SEs in COPD has been suggested.

In summary, this thesis shows that \textit{Staphylococcus aureus} enterotoxin B is potent inducer and modulator of airway inflammation. Derived from observations in human airway disease where SEs are known to modulate ongoing inflammation, new experimental models have been developed, closely mimicking human airway disease. The key role that SEB plays in these models, by inducing and aggravating airway inflammation, opens possibilities for new therapeutic strategies in the prevention and treatment of airway inflammatory conditions. Specific eradication of enterotoxin producing \textit{Staphylococcus aureus} in patients with early airway disease like asthma or COPD in order to prevent disease progression, may offer better disease control in these patient. Furthermore, antagonism of SEB
using monoclonal antibodies targeting SEB is another possible therapeutic tool. Our findings may contribute also to the prevention of allergic sensitization, where eradication of SAE again offers a possible therapeutic tool against the allergic march. Clinical studies confirming this hypothesis therefore are certainly worthwhile undertaking.
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  - XXV EAACI congress, Vienna, 10-14 June 2006
- Upper airway inflammation in chronic smoke exposed mice:
  - 5th EAACI GA²LEN Davos Meeting, Davos, 1-4 February 2007
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• SEB facilitates allergic sensitization. Second Annual Scientific IAP-Meeting – AIReWAY study consortium, Leuven, 3 september 2008

• Staphylococcus aureus Enterotoxin B facilitates allergic sensitization. American Academy of Asthma Allergy and Immunology (AAAAI) Annual meeting, Washington DC, USA, 14 maart 2009

• Doxycycline releasing frontal sinus stents ameliorate postoperative healing quality after sinus surgery. Scientific Meeting of Royal Belgian ENT Society, Brussels, 20 maart 2010

• Exacerbation of cigarette smoke-induced pulmonary inflammation by Staphylococcus aureus Enterotoxin B in mice. First ERAM-SERIN Meeting, Brussels, 6 november 2010

Beurzen

• The SERIN – EAACI (ENT Section) Travel Grant for the poster presentation titled: Rabbit anatomy of the nasal and paranasal cavities in experimental work, Barcelona, 9-11 February 2006

• EAACI JMA Travel Grant for the presentation: Staphylococcus aureus Enterotoxin B breaks primary tolerance to ovalbumin. XXVII EAACI congress, Barcelona, 7-11 June 2008

• The SERIN – EAACI (ENT Section) Travel Grant for the presentation: Staphylococcus aureus Enterotoxin B facilitates allergic sensitization. 7th Symposium on Experimental Rhinology and Immunology of the Nose (SERIN), Dubrovnik, 13-15 November 2008

• 2009 FIT International Travel Grant Scholarship. American Academy of Asthma Allergy and Immunology (AAAAI) Annual meeting, Washington DC, USA. 13 – 17 maart 2009

Prijzen en onderscheidingen

• Glaxo Smith Kline scientific ENT award. Wetenschappelijke Vergadering van de Koninklijke Belgische Vereniging voor Otorhinolaryngologie, Gelaat- en Halschirurgie (2008)

• Glaxo Smith Kline clinical ENT award. Wetenschappelijke Vergadering van de Koninklijke Belgische Vereniging voor Otorhinolaryngologie, Gelaat- en Halschirurgie (2010)
Varia

- EAACI Newsletter contributor (2005-2009)
- Medewerker GA²LEN management Office – Global Allergy and Asthma European Network
- VA-NKO (Vereniging Assistenten NKO) bestuurslid (2007-2009)
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