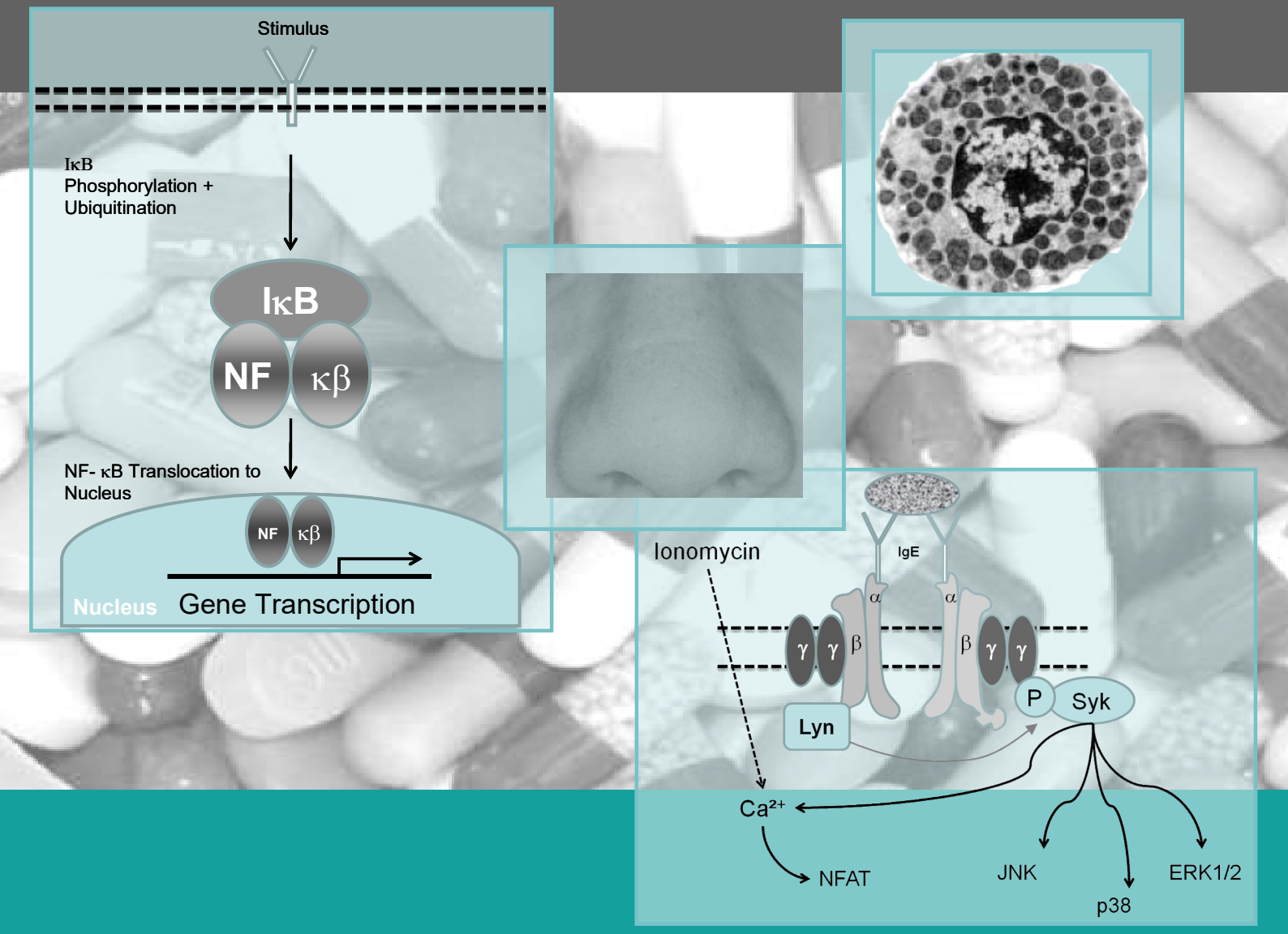
EX-VIVO MODEL TO STIMULATE NASAL TISSUE AND NEW THERAPEUTIC TARGETS



Joke Patou

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Promotors:

Prof. Dr. Claus Bachert

Prof. Dr. Philippe Gevaert

UNIVERSITEIT GENT

Faculty of Medicine and Health Sciences
Upper Airways Research Laboratory
Department of Otorhinolaryngology & Head and Neck Surgery

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Patou Joke

Promotor:
Prof. Dr. Claus Bachert

Co-promotor:
Prof. Dr. Philippe Gevaert

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LIST OF PUBLICATIONS

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

Patou J, Holtappels G, Affleck K, Gevaert P, Perez-Novo C, Van Cauwenberge P, Bachert C. Enhanced release of IgE-dependent early phase mediators from nasal polyp tissue. J Inflamm. 2009 Apr; 20:6-11.

Patou J, Gevaert P, Van Zele T, Holtappels G, Van Cauwenberge P, Bachert C. Staphylococcus aureus enterotoxin B, protein A and lipoteichoic acid stimulations in nasal polyps. J Allergy Clin Immunol. 2008 Jan; 121:110-5.

Patou J, Holtappels G, Affleck K, Van Cauwenberge P, Bachert C. Syk-kinase inhibition prevents mast cell activation in nasal polyps. Rhinology. 2011 March; 49:100-6.

Patou J, Holtappels G, Affleck K, Van Crombruggen K, Gevaert P, Bachert C. IKK2 inhibition in nasal polyps. *Submitted to Allergy*

LIST OF ABBREVIATIONS

AP-1 :	Activator protein 1
APC :	Antigen presenting cell
ASO:	Antisense oligonucleotides
CD :	Cluster of differentiation
COX-2:	Cyclooxygenase-2
ECP:	Eosinophil cationic protein
EPO^{3}S:	European position paper on rhinosinusitis and nasal polyps
FcεRI:	IgE receptor I
Foxp3:	Forkhead box P3
GATA3:	GATA-binding protein-3
GM-CSF:	Granulocyte macrophage-colony stimulating factor
GR:	Glucocorticoid receptor
HE:	Haematoxylin
HLA:	Human leucocyte antigen
ICAM:	Intercellular adhesion molecule
IFN-γ:	Interferon gamma
IgE:	Immunoglobulin E
IκB:	Inhibitor of NF-κB
IKK-2:	Inhibitor of kappaB kinase-2
IL:	Interleukin
IP3:	Inositol triphosphate
ITAM:	Immunoreceptor tyrosine-based activation motif
LAT:	Linker for activator of T cells
LTA:	Lipoteichoic acid
$LTC_4/D_4/E_4$:	Leukotrienes $C_4/D_4/E_4$
MAP:	Mitogen activated protein
MCP-1:	Monocyte chemotactic protein-1
MHC:	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein

MMP:	Matrix metalloproteinase
MPO:	Myeloperoxidase
NEMO:	NF-κB essential modulator
NFAT:	Nuclear factor of activated T cells
NF-κB:	Nuclear Factor -κB
NP:	Nasal polyposis
PGD_2:	Prostaglandin D_2
PLA2:	Phospholipase A2
PLCγ:	Phospolipase Cγ
qPCR:	Quantitative real-time polymerase chain reaction
RANTES:	Regulated upon activation, normal T expressed and secreted
SAEs:	*S. aureus* superantigens
S. *aureus*:	Staphylococcus aureus
SCF :	Stem cell factor
SEB:	Staphylococcus enterotoxin B
SOS:	Son of Sevenless
SpA :	Staphylococcal protein A
SPT :	Skin prick test
Syk:	Spleen tyrosine kinase
TAK-1	TGF-β activated protein kinase 1
T-bet:	T-box transcription factor
TBS:	Tris buffered saline
TCR :	T-cell receptor
TGF-β :	Transforming Growth Factor beta
Th :	T-helper lymphocyte
TNF-α:	Tumor necrosis factor alpha
TSST-1:	Toxic shock syndrome toxin 1
Vav:	Vasopressinase-altered-vasopressin
VCAM:	Vascular cell adhesion molecule

SUMMARY

Allergic rhinitis and nasal polyposis are both chronic nasal inflammatory diseases. The inflammatory response shows a lot of similarities such as elevated IgE, elevated IL-5 and eosinophilia. The magnitude of inflammation is however a lot more prominent in nasal polyposis. Current treatment is often unsatisfactory. Human tissue models may be helpful to accelerate testing of new pharmaceutical molecules.

First of all, in both nasal polyps and inferior turbinates, we established a whole tissue nasal mucosal stimulation model which can be used to mimic the early phase of an allergic reaction. By using whole tissue preparations without enzymatic digestion, the cells remained in their natural environment, and unchanged surface receptor expression was maintained, thus closely mimicking the *in vivo* situation. The induced concentration-dependent release of histamine, $LTC_4/D_4/E_4$ and PGD_2 after 30 minutes' stimulation with anti-IgE, was significantly higher in the nasal polyp group compared to the inferior turbinate group**,** although tryptase, FcεRIα positive cells and FcεRIα-chain transcripts were equally present in both groups.
Secondly, we established a nasal polyp tissue stimulation model with SEB for late-phase release of numerous immunoregulatory and proinflammatory cytokines. SEB stimulation for 24 hours (late-phase) resulted in a significant increase of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, TNF-α and IL-1β in nasal polyps and inferior turbinates. Furthermore, in this study we elucidated the disease-modifying role of *S. aureus* derived proteins SEB, SpA and LTA in nasal polyps. SpA induced mast cell degranulation, whereas SEB induced the release of numerous immunoregulatory and pro-inflammatory cytokines, favouring Th2-cytokines and disfavouring IL-10 and TGF-β1 in nasal polyps, supporting the modifying role of superantigens in the development of this inflammatory disease.

These models allowed us to test new compounds in human disease relevant tissue in a pre-safety and pre-clinical setting. Drugs targeting kinases have become the focus of a large number of drug discovery programs in the pharmaceutical and biotech industry. We tested two kinase inhibitors, namely a Syk inhibitor and an IKK-2 inhibitor. In cord blood-derived mast cells the Syk inhibitor significantly prevented the degranulation, assessed by measurement of histamine release, and the production of $LTC_4/LTD_4/LTE_4$ and PGD_2. The

Syk inhibitor was similarly able to significantly inhibit histamine, LTC_4/LTD_4/LTE_4 and PGD_2 in nasal polyp tissue in a dose dependent manner. Syk inhibitors might provide a new therapeutic possibility in the treatment of upper airway diseases with mast cell involvement, such as allergic rhinitis.

IKK-2 inhibition can result in the blockage of transcription of multiple inflammatory mediators, such as TNF-α, interleukins, cell adhesion molecules, etc. We determined, in nasal polyposis, the impact on the release of several Th1, Th2 and pro-inflammatory cytokines when inhibiting IKK-2 in comparison to the impact of the topical corticosteroid fluticasone propionate. Our results suggest that the IKK-2 inhibitor seemed, in certain aspects, to possess a more comprehensive anti-inflammatory profile than that of fluticasone propionate in nasal polyposis.

SAMENVATTING

Allergische rhinitis en nasale polyposis zijn beiden chronische nasale inflammatoire aandoeningen. Hun inflammatoir patroon vertoont vele gelijkenissen zoals gestegen IgE, gestegen IL-5 en eosinophilie. De ernst van de aandoening en de grootte van inflammatie zijn echter prominenter in nasale polyposis. De huidige behandelingen schieten af en toe tekort met daarom de nood aan het op punt stellen van weefselmodellen waarop nieuwe farmaceutische moleculen kunnen worden uitgetest.

Wij konden twee modellen op punt stellen. Ten eerste konden we een stimulatiemodel op punt stellen, dat de vroege fase van een allergische reactie nabootst, zowel in nasale poliepen als in concha inferiores. Er werden weefselfragmentjes gebruikt zonder enzymatische digestie. Op die manier bleven de cellen in hun natuurlijke omgeving, bleven de celreceptoren bewaard en kon de *in vivo* situatie zo goed mogelijk nagebootst worden. Het bleek dat de geïnduceerde concentratie-afhankelijke vrijstelling van histamine, $LTC_4/D_4/E_4$ en PGD_2 na 30 minuten stimulatie met anti-IgE significant hoger was in de neuspoliep groep in vergelijking met de concha inferior groep. Echter, tryptase-, FcεRIα positieve cellen en FcεRIα-chain transcripts waren evenveel aanwezig in beide groepen.
Ten tweede, konden we een "late-fase" model op punt stellen, waarbij neuspoliepweefsel met SEB gestimuleerd werd en waarbij talrijke immunoregulatoire en pro-inflammatoire cytokines vrijkwamen. Stimulatie met SEB gedurende 24 uur (late fase) resulteerde in het significant vrijgeven van IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, TNF-α en IL-1β in neuspoliepen en concha inferiores. Vervolgens konden we in deze studie de rol van *S. aureus*- afgeleide eiwitten zoals SEB, SpA en LTA verder toelichten. SpA induceerde mastcel degranulatie en SEB was verantwoordelijk voor het vrijgeven van talrijke immunoregulatoire en pro-inflammatoire cytokines, met een voorkeur voor het vrijgeven van Th-2 cytokines en met een minder vrijkomen van IL-10 en TGF-β1. Deze resultaten ondersteunen de rol van superantigenen in de ontwikkeling van nasale polyposis.

Deze modellen maakten het mogelijk om nieuwe moleculen te testen op humaan ziekte-relevant weefsel in een pre-safety en pre-clinical setting. Heel wat onderzoek in de farmaceutische en biotechnische industrie is momenteel gefocust op moleculen gericht tegen

kinases. Wij hebben twee kinase-inhibitoren uitgetest, namelijk een Syk-inhibitor en een IKK-2 inhibitor. De Syk-inhibitor kon het vrijkomen van histamine, LTC_4/LTD_4/LTE_4 en PGD_2 significant inhiberen in mastcellen gekweekt uit navelstrengbloed. Gelijklopend kon de Syk-inhibitor histamine, LTC_4/LTD_4/LTE_4 en PGD_2 significant inhiberen in nasale poliepen. Syk-inbibitoren zouden in de toekomst een nieuwe therapie kunnen vormen in de behandeling van bovenste luchtweg infecties, waarbij de mast cel een belangrijke rol speelt, zoals allergische rhinitis.

IKK-2 inhibitie kan leiden tot het blokkeren van de transcriptie van verscheidene inflammatoire mediatoren, zoals TNF-α, interleukines, cel-adhesie moleculen enz. In neuspoliepen werd de impact van een IKK-2 inhibitor op het vrijgeven van verschillende Th1, Th2 en pro-inflammatoire cytokines bepaald en deze impact werd vergeleken met fluticasone propionaat. Onze resultaten toonden aan dat de IKK-2 inhibitor op bepaalde vlakken een uitgebreider anti-inflammatoir profiel heeft in vergelijking met fluticasone propionaat.

RESUME

La rhinite allergique et la polypose naso-sinusienne sont toutes les deux des affections inflammatoires chroniques. Leur réponse inflammatoire présente de nombreuses similitudes comme des taux d'IgE et d'IL-5 ainsi qu'une éosinophilie élevés. Cependant, la gravité de l'affection et l'importance de l'inflammation sont de plus grande ampleur pour la polypose. Les traitements actuels ne sont pas toujours à la hauteur d'où la nécessité de mettre en place un modèle tissulaire sur lequel de nouvelles molécules pharmaceutiques pourraient être testées.

Premièrement, nous avons pu établir un modèle de stimulation aussi bien pour les polypes nasaux que pour le cornet nasal inférieur, pour simuler la phase initiale d'une réaction allergique. Des fragments tissulaires sans digestion enzymatique ont dans cette optique été utilisés. De cette façon, les cellules restent dans leur environnement naturel, les récepteurs cellulaires sont conservés et la situation *in vivo* a pu être simulée de la meilleure façon possible. La libération d'histamine, $LTC_4/D_4/E_4$ et PGD_2 concentration-dépendante après 30 minutes de stimulation avec anti-IgE, était significativement plus élevée dans le groupe des polypes nasaux que dans celui du groupe du cornet inférieur. Néanmoins, les cellules tryptase ou FcεRIα positives et les transcriptions de chaînes FcεRIα étaient présentes dans les deux groupes.

Deuxièmement, nous avons pu établir un modèle d'une "phase tardive" au cours de laquelle les tissus des polypes nasaux ont été stimulés avec SEB et pour laquelle quantité de cytokines immunorégulatrices et proinflammatoires ont été libérées. La stimulation pendant 24 heures (phase tardive) avec SEB a entraîné la libération significative d'IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, TNF-α et IL-1β pour les polypes nasaux et le cornet inférieur. Ensuite, nous avons pu expliquer plus largement dans cette étude le rôle des protéines dérivées de S. aureus comme les SEB, SpA et LTA. La SpA a engendré une dégranulation des mastocytes et SEB fut responsable de la libération de nombreuses cytokines immunorégulatrices et proinflammatoires, avec une prédominance notée pour la libération des cytokines Th-2 et une libération moindre d'IL-10 et TGF-β1. Ces résultats confirment le rôle des superantigènes dans le développement des polypes nasaux.

Ces modèles nous ont permis de tester de nouvelles molécules sur des tissus humains sensibles à la maladie dans des conditions précliniques avant étude de tolérance. Beaucoup de recherches dans l'industrie pharmaceutique et biotechnique sont actuellement ciblées sur des molécules détruisant les kinases.

Nous avons testés deux inhibiteurs de kinase, soit un inhibiteur de Syk kinase et un inhibiteur IKK-2. L'inhibiteur de Syk kinase a pu inhiber la libération d'histamine, de $LTC_4/LTD_4/LTE_4$ et PGD_2 de façon significative dans des mastocytes cultivées à partir de sang provenant de cordon ombilical.

Au même niveau, l'inhibiteur de Syk kinase a pu significativement inhiber l'histamine, les $LTC_4/LTD_4/LTE_4$ et PGD_2 dans le cas des polypes nasaux. Les inhibiteurs de Syk kinase devraient dans le futur former un traitement pour les infections des voies respiratoires supérieures, auprès duquel les mastocytes jouent un rôle important, comme la rhinite allergique.

L'inhibition IKK-2 peut conduire au blocage de la transcription de différents médiateurs inflammatoires, comme les interleukines TNF-α, les molécules d'adhésion cellulaire, etc... Au niveau des polypes nasaux l'impact de l'inhibiteur IKK-2 sur la libération de différents Th1, Th2 et cytokines pro-inflammatoires a été analysé et cet impact a été comparé à celui du propionate de fluticasone.

Nos résultats démontrèrent qu'à certains niveaux l'inhibiteur IKK-2 a un profil anti-inflammatoire plus développé en comparaison à celui du fluticasone propionate.

PART I : INTRODUCTION

CHAPTER I:
NASAL INFLAMMATORY DISEASES

Allergic rhinitis and nasal polyposis are both chronic nasal inflammatory diseases. The inflammatory response shows many similarities such as elevated IgE, elevated IL-5 and eosinophilia. However the magnitude of inflammation is much more prominent in nasal polyposis. Current treatment is sometimes unsatisfactory.
Drugs targeting kinases, have become the focus of a large number of drug discovery programs in the pharmaceutical and biotech industry. In laboratory settings, new molecules are primarily tested in animals or on cell lines. However, responses in animals or *in vitro* may differ from responses in human tissue. To avoid misleading conclusions, new pharmaceutical molecules should be tested in *ex vivo* models of human disease relevant tissue before testing such molecules in clinical settings.

Kinases, important in the signal transduction pathways in allergic rhinitis and nasal polyposis have been the target of interest. Mast cells are crucial effector cells in the allergic cascade. After the cross-linking of the high affinity IgE receptor (FcεRI) they release and produce a wide array of mediators important in the early and late phase of allergic rhinitis.
An early - and late phase model on inferior turbinate tissue and nasal polyp tissue would allow us to test these kinds of drugs. Furthermore the usability of nasal polyp tissue versus inferior turbinate tissue would be of great importance as polyp tissue is easier to obtain in larger quantities.

In this thesis, tissue of both disease identities was used in ex-*vivo* stimulation models. Two kinase inhibitors were tested, namely a Syk inhibitor and an IKK2 inhibitor.
Syk kinase is positioned upstream of the IgE receptor signal transducing pathway and IKK2 helps activating the NF-κB transcription factor and therefore they may represent important targets for the blockage of early- and late phase release of mediators.

ALLERGIC RHINITIS

In the general population in Europe, the prevalence of allergic rhinitis is found to be about 25%. It ranges from 17% (Italy) to 28,5 % (Belgium)[1, 2]. The ISAAC study (The International Study on Asthma and Allergy in Childhood) demonstrates the prevalence of asthma and rhinitis symptoms in children throughout the world. The prevalence of allergic rhinitis varies across centres from 0.8% to 14.9% in 6-7 year olds and from 1.4% to 39.7% in 13-14 year olds. It is likely that environmental factors are responsible for the major differences among countries[3, 4]. The prevalence of allergic rhinitis is higher in urban than in rural areas[5]. An increase in the prevalence of allergic rhinitis has been observed over the past 40 years of the last millennium[4, 6, 7]. In countries with high prevalence, rates are now plateauing or decreasing[4].

Definition

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced after allergen exposure by an IgE-mediated inflammation[8]. Symptoms of allergic rhinitis include rhinorrhea, nasal obstruction[9], nasal itching and sneezing which are reversible spontaneously or with therapy[10, 11].

Previously allergic rhinitis was subdivided based on the time of exposure into seasonal, perennial and occupational[12, 13]. Perennial allergic rhinitis is most frequently caused by indoor allergens such as house dust mite, molds, insects and animal danders. Seasonal allergic rhinitis is related to a wide variety of outdoor allergens such as pollens and molds. This classification was not entirely satisfactory f.e. in certain areas pollen are perennial allergens[14] or symptoms of perennial allergens may not be present all year round[15], and the majority of patients are sensitized to many different allergens and therefore exposed throughout the year. In a lot of patients, perennial symptoms are often present and patients experience seasonal exacerbations when exposed to pollens or molds[11, 16].

In 2001 a major change in the classification of allergic rhinitis was proposed in the ARIA (allergic rhinitis and its impact on asthma) document[10]. Nowadays, allergic rhinitis is subdivided into intermittent allergic rhinitis or persistent allergic rhinitis. The severity of allergic rhinitis can be classified as “mild” or “moderate/severe” (table 1).

Table 1. Classification of allergic rhinitis according to ARIA[10]

-"Intermittent" means that the symptoms are present:
<4 days a week
Or for <4 consecutive weeks.
-"Persistent" means that the symptoms are present:
More than 4 days a week,
And for more than 4 consecutive weeks.
-"Mild" means that none of the following items are present:
Sleep disturbance,
Impairment of daily activities, leisure and/or sport,
Impairment of school or work,
Symptoms present but not troublesome.
-"Moderate/severe" means that one or more of the above items are present

The diagnosis of allergic rhinitis is based upon the concordance between a typical history of allergic symptoms and diagnostic tests. Objective tests for the diagnosis of IgE-mediated allergy are the skin prick test and the measurement of serum specific IgE with RAST (radioallergosorbent test) [17, 18].

Comorbidities

Asthma

The majority of patients with asthma experience rhinitis symptoms, suggesting the concept of "one airway disease" [19, 20]. The presence of allergic rhinitis commonly exacerbates asthma, increasing the risk of asthma attacks, emergency visits and hospitalizations for asthma[21].

However, not all patients with rhinitis have asthma. The prevalence of asthma in patients with allergic rhinitis varies from 10% to 40% [19, 22]. Patients with moderate/severe persistent allergic rhinitis are more likely to suffer from asthma than those with intermittent allergic rhinitis and/or a milder form of the disease[23].

Allergic conjunctivitis

Ocular symptoms occur in a large proportion of patients with rhinitis. Allergic conjunctivitis is more common with outdoor allergens than with indoor allergens. In some studies on pollen allergy, conjunctivitis is sometimes present in over 75% of patients suffering from rhinitis. However, the prevalence of the association between rhinitis and conjunctivitis cannot easily

be defined, because conjunctival symptoms are often considered to be of minor importance and are possibly not spontaneously reported by patients. Accordingly, the association between rhinitis and conjunctivitis is probably largely underestimated in epidemiologic studies[24].

Rhinosinusitis and nasal polyposis

It has been speculated that nasal inflammation induced by IgE-mediated mechanisms favours the development of acute and/or chronic sinus disease. Sinus involvement has been observed by CT-scans in allergic patients[25] and total serum IgE correlates with the thickness of sinusal mucosa observed on CT-scans[26]. However, epidemiologic studies are inconclusive and are not conclusive to estimate that allergic rhinitis may predispose to the development of rhinosinusitis. There are no prospective studies on the incidence of rhinosinusitis in patients with and without allergic rhinitis. However, there is a high prevalence of sensitization to allergens in patients with acute and chronic rhinosinusitis[27].

Both allergic rhinitis and nasal polyposis are characterized by an inflammatory response that shows many similarities, however, until now, no clear data support a role of allergy in nasal polyposis.

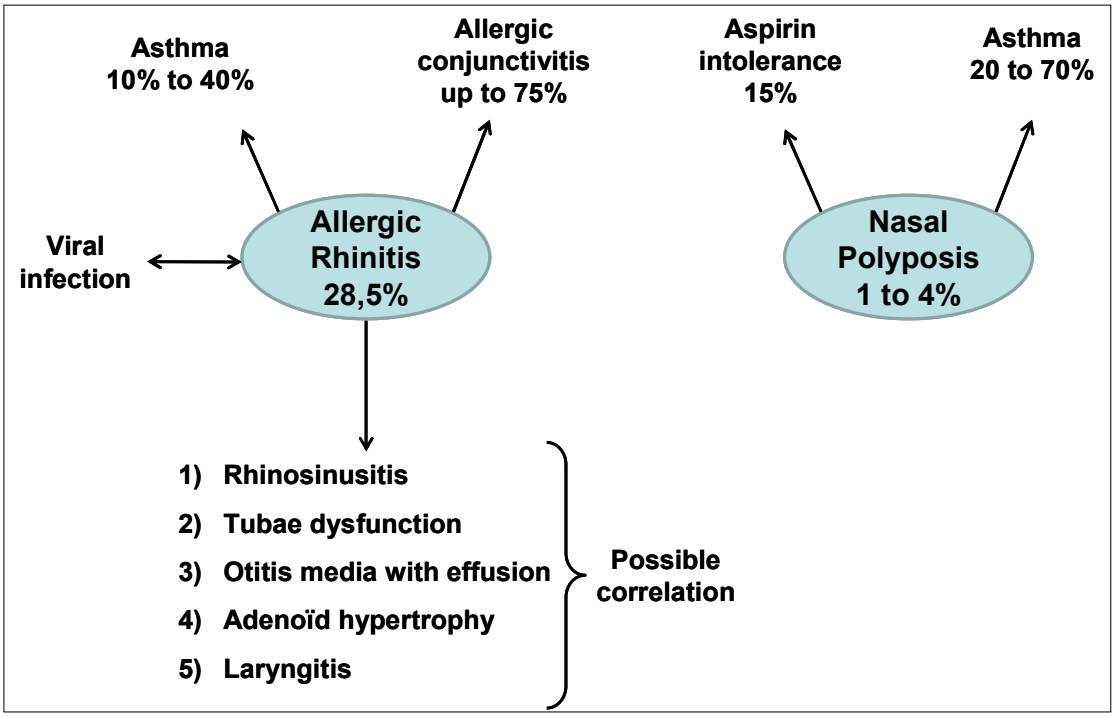

Fig.:Comorbidities of allergic rhinitis and nasal polyposis. Adapted from ARIA[8] and EPO3S[28]

Otitis media with effusion (OME)

In view of the concept of global airway allergy, it may be expected that an allergic inflammatory response may also take place in the middle ear. Allergic rhinitis patients have a higher risk of Eustachian tube dysfunction than non allergic subjects, particularly during childhood[29]. Eustachian tube dysfunction may lead to OME. The middle-ear fluid of atopic patients with OME contains more eosinophils and IL-4 and IL-5 mRNA-positive cells compared to nonatopic patients with OME, suggesting a role of allergic inflammation in OME[30]. However, the role of allergic rhinitis in OME is the subject of much controversy[31].

Pathophysiology

Histomorphologically, the nasal mucosa displays structural alterations in response to the allergic inflammation. The epithelium shows thickening and hyperplasia with degeneration. Initially there is an increase of goblet cell numbers and later a decrease. Furthermore, in the lamina propria, blood vessels are dilated and thickened and glands show multiple changes: hyperplasia and distension of acini with secretions, degeneration and obstruction of the acini and ducts and periglandular cell infiltration of the superficial glands. In addition, there is compression and atrophy of the deeper glands. Furthermore, neo-glandular formation has been demonstrated in the allergic nasal mucosa. In addition, infiltration with eosinophilic, mononuclear and lymphoid cells are evident [32].

Allergic rhinitis is classically considered to result from a sustained overproduction of IgE in response to common environmental antigens, associated with nasal inflammation of variable intensity. When an antigen presenting cell has picked up an antigen, it will present the antigen peptide through the MHC complex. In allergic rhinitis naïve T cells differentiate more to Th2 cells than to Th1 cells after the encounter with this activated antigen presenting cell (priming). Cytokines produced by Th2 cells (IL-3, IL-4, IL-5, IL-6, IL-8, IL-13) and a downregulation of T-regulatory cell responses drive the B cell to produce IgE and stimulate the recruitment, maturation, survival and effector function of accessory cells such as eosinophils, basophils and mast cells [33]. IgE is produced in the local lymphoid tissues and locally in the nasal mucosa[34, 35]. Persistent IgE synthesis takes place in the nasal mucosa during and just after the pollen season[36]. Allergens drive class switching to IgE transcripts in the nasal mucosa in allergic rhinitis[37].

Early/immediate allergic response. Within minutes of inhalation of allergen in sensitized subjects, deposited allergens are recognized by allergen specific IgE antibody and this

complex becomes fixed to the IgE receptor (FcεRI). Crosslinking of the receptor bound to mast cells and basophils, causes degranulation and release of preformed mediators, such as histamine and tryptase, and the rapid *de novo* generation of mediators such as leukotrienes C_4, D_4 and E_4 and prostaglandin D_2[38]. Mediators cause plasma leakage from blood vessels and dilation of arteriovenous anastomoses, with consequent oedema, pooling of blood in cavernous sinusoids and occlusion of nasal passages. Furthermore secretion of mucus from glandular and globet cells is stimulated. Histamine provokes itching, rhinorrhea and sneezing, whereas other mediators are more responsible for nasal congestion. Stimulation of sensory nerves results in the perception of nasal congestion and itching and may provoke systemic reflexes such as sneezing[39].

Late phase response. Mediators and cytokines released during the early phase set off a cascade of events over the following 4 to 8 hours that lead to an inflammatory response called the late response. Symptoms are similar to those of the immediate reaction, but nasal congestion is more prominent. The expression of adhesion molecules such as E-selectin and VCAM-1 is promoted and these give adherence of circulating leukocytes to endothelial cells. Mainly eosinophils but also neutrophils, basophils, Th2 lymphocytes and macrophages infiltrate the lamina propria of the nasal mucosa[39, 40]. These cells become activated and release more mediators which in turn activate more proinflammatory reactions.

During the pollen season there is an increase in various inflammatory cells, correlating with severity of symptoms[41]. Immunohistochemical staining of nasal biopsies with antibodies against mast cell tryptase shows an increase in mast cells within the airway epithelium and submucosa in both intermittent and persistent allergic rhinitis when compared to biopsy findings in non-atopic subjects[42, 43, 44, 45]. Epithelial mast cells are in an activated state in symptomatic allergic rhinitis with evidence of degranulation on electron microscopy[46] and with elevated levels of histamine, leukotrienes and tryptase in nasal lavage fluid[47, 48].

Eosinophils are found in the mucosa between epithelial cells, in the submucosa and in nasal secretions, and they are present especially in the late-phase allergic response[42]. They have the capacity to produce cytotoxic proteins such as major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin and ECP and are proposed to cause desquamation of the airways in vivo. In nasal secretion of rhinitis patients increased ECP is found[49].

Significantly more basophils are found in nasal allergic mucosa and are shown to increase in asymptomatic intermittent rhinitis patients following nasal allergen challenge[50]. Moreover an increase in circulating basophils is observed in rhinitis[51].

An increase in the T-lymphocyte cell population has been described in both intermittent and persistent allergic rhinitis. Furthermore, following nasal allergen challenge, an increase in IL-4, IL-5, and GM-CSF mRNA positive cells has been described in association with mucosal eosinophilia, which is consistent with Th2-lymphocyte activation[52]. Moreover, the role of Treg cells in controlling allergic diseases became apparent. In nasal tissue of allergic rhinitis patients, a reduced number of FOXP3-positive cells is reported compared to controls[53].
Also Langerhans cells, as well as other dendritic cells, are found in the nasal mucosa. They form a network of antigen presenting cells, especially in the epithelial surface. Dendritic cell recruitment to the nasal mucosa has been observed during natural seasonal and allergen provocation[54, 55].

Treatment

Topical corticosteroids and oral antihistamines are currently the two most important groups to treat allergic rhinitis. The principle of pharmacological treatment is a stepwise approach according to the severity and duration. In resistant patients, the effects of allergenspecific immunotherapy can be both complementary and synergistic but careful patient selection is absolutely crucial to its success.

Allergen avoidance

The 2008 ARIA guidelines have reported that there is a lack for the effectiveness of the avoidance of house dust mites or pet animal dander[8].
However, for occupational allergic rhinitis strict avoidance of offending allergens is the safest and most effective treatment[56].

Oral antihistamines

H1-blockers or H1-antihistamines are drugs with blocking activity at the H1 histamine receptor level. Most of the new oral H1-antihistamines have a fast onset of action (20 minutes to 2 hours) and duration of effect that lasts up to 24 hours. They are effective in the treatment of rhinorrhea, sneezing, nasal itching and eye symptoms. They are usually less effective against nasal obstruction because other mediators, and histamine acting through the H3 receptor, impact this symptom[57].
First-generation antihistamines, which have been used since the early 1940s, have some side effects such as sedation, memory impairment and psychomotor dysfunction. In contrast, second-generation antihistamines penetrate the blood-brain barrier far less than first-

generation antihistamines, and consequently have fewer side effects on the central nervous system[58]. Moreover, in addition to their antagonistic action on histamine receptors, antihistamines possess potent anti-inflammatory properties[59].Therefore, the use of second-generation antihistamines is recommended[8].

Intranasal corticosteroids

Intranasal corticosteroids inhibit both early and late reactions and reduce IgE production and eosinophilia by inhibiting the secretion of cytokines including IL-4, IL-5 and IL-13. They are effective in all allergic rhinitis symptoms, especially nasal obstruction and eye symptoms[60]. The therapeutic effect of intranasal corticosteroids is encountered 7 hours after administration and reaches the maximal level after 2 weeks[61]. For a better choice of topical steroids, their pharmacological characteristics should be considered. Most of the different drugs have similar clinical effects, but their systemic absorption is different. Mometasone furoate (Nasonex) and fluticasone propionate (Flixonase aqua) have very low absorption rates (<0.1% and <2%) and are considered safe for children[62, 63].

Leukotriene receptor antagonists (LTRAs)

In 2008 ARIA guidelines re-evaluated the role of LTRAs and it was recommended that they may be used in all types of severity. Montelukast (Singulair) (LTRA) is effective in reducing nasal and eye symptoms in patients with seasonal allergic rhinitis and improves nasal obstruction comparable to loratadine (antihistamine). The additive or synergic effect of montelukast and loratadine is controversial[64, 65]. When montelukast and cetirizine were administered 6 weeks before the pollen season, the exacerbation of seasonal allergic rhinitis symptoms was effectively prevented[66]. The additive effect of LTRAs and antihistamines requires more investigations. Up to now, the pharmacological effects of LTRAs are estimated to be similar to those of antihistamines but less than those of intranasal corticosteroids in patients with seasonal allergic rhinitis[8].

Decongestants

Decongestant drugs cause vasoconstriction by their action on α-adrenergic receptors, and may be administered intranasally or orally. In the short term, these agents are effective in the treatment of nasal obstruction in allergic rhinitis. However, these agents do not improve other symptoms of rhinitis, such as nasal itching, sneezing or rhinorrhea. A prolonged use may lead to rebound swelling of the nasal mucosa (rhinitis medicamentosa).

Anti-IgE antibody

Omalizumab, an anti-IgE recombinant humanized monoclonal antibody, interferes with the interactions between mast cells/eosinophils and IgE by binding to free IgE and hence lowers serum free IgE. It also suppresses inflammatory reactions in blood or nasal mucosa[67], and suppresses the expression of FcεRI located on the surface of mast cells or eosinophils[68]. While anti-IgE antibody therapy appears to be helpful in severe asthma, it is controversial whether anti-IgE therapy is suitable as a treatment option for allergic rhinitis, due to anaphylactic risk and high costs.

Topical anticholinergics

Parasympatic stimulation of the nasal glands results in vasodilatation of the blood vessels that supply them and in an associated watery secretion. The muscarinic receptors can be blocked by the anticholinergic drug ipratropium bromide, which is available as a nasal spray. Its use can be combined with intranasal corticosteroids or antihistamines when rhinorrhea is the predominant symptom. Ipratropium bromide may also be of specific interest for use in elderly patients with isolated rhinorrhea, or in patients with rhinorrhea secondary to contact with cold air.

Immunotherapy

Allergen-specific immunotherapy, performed under controlled conditions with immediate access to resuscitative equipment, has a prominent role in the treatment of severely symptomatic patients with allergic rhinitis who have failed to respond to conventional treatment. Extracts of offending allergens are injected subcutaneously with increasing doses until a maintenance dose is reached. Recently, sublingual immunotherapy starts to replace subcutaneous immunotherapy. Regulation of antigen-specific responses (an increase in the IgG4/IgE ratio), inhibition of recruitment/activation of inflammatory cells, shift of Th2 to Th1 responses and activation of regulatory T cells are the main mechanisms of immunotherapy.

NASAL POLYPOSIS

The prevalence of nasal polyposis varies between 1% and 4% of the general population[69, 70]. In nasal polyposis the male: female ratio is 2: 1 and it is more likely to occur in elderly

patients[69]. The average onset is approximately at 42 years old[71]. Under the age of 20, nasal polyps are uncommon and are more related to cystic fibrosis[72, 73].

Definition

According to the European position paper on rhinosinusitis(EPO3S), nasal polyposis is a disease which is included in the current clinical definition of rhinosinusitis. Rhinosinusitis is defined as an inflammation of the nose and the paranasal sinuses characterized by two or more symptoms including nasal blockage, anterior or posterior nasal drip, facial pain or pressure, and reduction in or loss of smell. Furthermore, together with the symptoms, there must be endoscopic signs and/or changes identifiable using computed tomography (CT). Endoscopic signs include the presence of polyps, mucopurulent discharge from the middle meatus and/or oedema or mucosal obstruction primarily in the middle meatus. The CT changes are mucosal swelling within the ostiomeatal complex and/or sinuses. Severity of sinus disease can be divided in mild, moderate or severe, based on a severity visual analogue scale. Based upon duration of symptoms there is the difference between acute rhinosinusitis, when symptoms resolve completely within 12 weeks, and chronic rhinosinusitis, when symptoms last longer than 12 weeks. For general practice, however, diagnostic tools are limited to symptoms.

For research purposes nasal polyposis is considered as a subgroup of chronic rhinosinusitis and the differentiation must be based upon endoscopic evaluation with presence of polyps or not[28, 74].

To determine the severity of the disease a score is given from 0 to 3[75] or from 0 to 4[76]depending on the endoscopic size of the polyps. The scoring system from 0 to 4 has proved to be the most reliable[77].To examine the anatomical extent of nasal polyps, a CT scan is performed. Here the grading score of Lund Mackay is used and recommended[77]. Nor the endoscopic size[78], nor the CT scores[79] correlate well with patient's symptom scores.

Comorbidities

Asthma

In asthma, 7% of patients have nasal polyposis[80]. The proportion is higher in patients with nonatopic asthma (13%) than in those with atopic asthma (5%)[81]. Late-onset asthma is associated with development of nasal polyposis in 10-15%[80].

In patients with nasal polyposis, asthma is present in 20 to 70%[82, 83] and 15% have aspirin-intolerance[84]. In approximately 69% of patients with both asthma and nasal polyposis, asthma

is the first disease to develop, and nasal polyposis takes between 9 and 13 years to be diagnosed. In only 10% of patients with both asthma and nasal polyposis both diseases do develop simultaneously, and in the remaining patients polyps develop first, followed 2-12 years later by asthma[81]. In nasal polyps the male: female ratio is 2: 1. Women with nasal polyposis, however, are 1.6 times more likely to be asthmatic and 2.7 times more likely to have allergic rhinitis than are men[85].

Aspirin-intolerance

The triad of asthma, nasal polyps and intolerance to aspirin and aspirin-like medications is known as the Samter's triad[86]. Alterations in arachidonic acid metabolism have been suggested as one of the main factors driving to aspirin-intolerance[87-89].

Of patients with aspirin-intolerance, 36-96% have nasal polyps and aspirin-intolerance is present in 5-10% of asthmatic patients[86, 87].

Patients with asthma, nasal polyposis and aspirin sensitivity are usually nonatopic and the prevalence increases in those older than 40 years. When parents have asthma, nasal polyposis and aspirin sensitivity, their children more commonly suffer from nasal polyposis and rhinosinusitis than do control children[90]. Of 500 patients with aspirin-induced asthma , nasal polyposis was diagnosed in 62% of them[87].

Allergy

Due to similarities in cytokine measurements in allergic rhinitis patients and nasal polyposis (elevated IgE, elevated IL-5, eosinophilia), a link was postulated between allergy and nasal polyposis. However, only 2.8% of allergic rhinitis patients have nasal polyposis while nasal polyps are found in 5.2% in non-allergic rhinitis patients[80]. Furthermore, allergen exposure in nasal polyp patients with a positive skin-prick test to ragweed, does not induce elevation of inflammatory parameters[91] and moreover, local and specific IgE in nasal polyps correlates with local eosinophilic inflammation but does not correlate with skin prick test results[92].

Pathophysiology

Nasal polyps appear as grape-like structures in the upper nasal cavity and are found in the middle nasal meatus or arise from the middle turbinates. Histomorphologically, the epithelium is mostly respiratory ciliary pseudo-stratified epithelium with less ciliated and more globet cells compared to normal healthy tissue[93]. Common features in bilateral polyps are epithelial damage, a thickened basement membrane, loose connective tissue with a reduced number of

blood vessels and glandular structures and no nervous structures[94]. In the stroma of nasal polyps often edema and pseudocystic formations are found. Albumine and other plasma proteins have been found within those pseudocysts. Furthermore, in patients from Western countries more than 70% of polyps exhibit abundant eosinophils, and these are located around the vessels, glands and directly beneath the mucosal epithelium[95]. The eosinophils are activated and are believed to play a central role in polyp formation and growth of nasal polyps[96]. Other inflammatory cells seen in nasal polyps are lymphocytes and mast cells. Mast cells can be detected in the epithelium as well as in the stroma of nasal polyps. It is well described that the majority of degranulated mast cells are localized in the deep stroma of nasal polyps [97, 98]. In most nasal polyps follicular structures characterized by B and T cells, and lymphoid accumulations with diffuse plasma cell infiltration have been found, suggesting a local production of immunoglobulines[99].

An increased production of chemokines/cytokines have been found in nasal polyps. Increased IL-5, RANTES and eotaxin induces eosinophil chemotaxis, migration, activation and prolonged survival [96, 100-102]. Regulation of eosinophils in polyps is partially understood; in-vitro treatment of eosinophil infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody resulted in eosinophil apoptosis and decreased tissue eosinophilia[103] and anti-IL-5 treatment in vivo resulted in reduction of eosinophils and ECP in serum and nasal secretions[104]. Furthermore, increased concentrations of eosinophilic toxins like ECP and MBP are found in nasal polyps and the eosinophilic marker eotaxin is also more often found in nasal polyps compared to controls[105]. IL-5 expression has been found to correlate with ECP in polyp tissue suggesting its autocrine role in the activation of eosinophils[92].
This T-cell activation with Th-2 polarisation pattern and eosinophilic inflammation, goes together with a decreased expression of FOXP3 (transcription factor for T regulatory cells), reflecting a deficiency or dysfunction of Treg cells in an often persistent, severely inflamed sinus disease. In line with low FOXP3 expression, low levels of TGF-β1 protein expression and an upregulation of transcription signals for Th1 (T-bet) and Th2 (GATA-3) subpopulations were found, pointing to a defective suppression[106].
Next to the high IL-5, another typical finding in nasal polyp tissue is the high local total amount of IgE. It mostly doesn't correlate with the serum IgE[92]. Furthermore polyclonal IgE is present in nasal polyp homogenates and again it doesn't correlate with specific serum IgE or results of skin prick test. This polyclonal type is associated with high IgE concentrations and specific IgE antibodies against *S. aureus* enterotoxins[99]. Not only high concentrations of

IgE can be found in nasal polyp homogenates, but also significantly higher IgA, total IgG and IgG subclasses are found compared to controls. This observation could not be found in serum. Furthermore, naïve B cells and plasma cells were significantly higher in nasal polyp tissue, suggesting a local production of immunoglobulins[107].

Another finding in nasal polyp tissue is the increase of leukotriene C4 synthase mRNA, 5-lipoxygenase mRNA and proinflammatory cysteinyl leukotrienes and the decrease of others such as cyclo-oxygenase 2 mRNA and prostaglandin E2 in parallel with the severity of disease[89]. Prostaglandin E2 can be considered an anti-inflammatory metabolite as it may induce FOXP3 expression and thus influence the formation of T-regulatory cells[108].

Consistent with the oedema formation and extracellular matrix destruction in nasal polyps, an upregulation of metalloproteinases (MMP)-7 and (MMP)-9, but not of the tissue inhibitor of metalloproteases (TIMP)-1 has been described[109], which is in line with a lack of upregulation of TGF-β1, a TIMP-1 inducing and pro-fibrotic growth factor[105]. Additionally a low number of pSmad-2 positive cells, a decreased expression of TGF-β R II and a lack of collagen was found in nasal polyps, all indicating a low level of TGF-β signalling[110].

In general, chronic rhinosinusitis with nasal polyps is characterized by a predominant Th2-biased eosinophilic inflammation with high levels of IL-5, ECP and eotaxin; high levels of local IgE; and low levels of TGF-β1 and they consist of albumin accumulation and edema formation within the extracellular matrix (pseudocyst formation).

All these studies were mainly performed on European and US nasal polyps. Recently, a difference has been demonstrated in inflammatory cells and cytokine pattern between Caucasian and Asian nasal polyps. Asian polyps demonstrated more Th1 and Th17 signals, including T-bet (transcription factor for Th1 cells) expression and IFN-γ protein formation, and IL-17 and the related cytokines IL-1β and IL-6 protein synthesis in tissue homogenates[111]. This new view on the type and role of inflammation in airway disease needs to be elaborated.

S. aureus superantigens as disease modifiers

Enterotoxins derived from *Staphylococcus aureus*, a gram positive bacterium, have been implicated in the pathophysiology of nasal polyps as disease-modifying factors. These S. aureus -derived enterotoxins (SAEs) are called “superantigens” as they have the ability to crosslink the class II major histocomptability complex of antigen-presenting cells and the T-cell receptor (TCR) β-chain variable regions. This crosslinking takes place outside the conventional antigen-binding grove. This may lead to the stimulation of up to 20-25% of the

T-cell population in a non-specific way, compared with stimulation of only about 0.1% via the conventional allergen-specific way[112]. Once activated, T-cells may produce interleukins including IL-4, IL-5, IL-13, eotaxin and many others, which may lead to an eosinophilic inflammation and local IgE-production. Specific IgE against SAEs is found more frequently in nasal polyp tissue versus controls and correlates with higher levels of IL-5, eotaxin, and ECP[92]. Moreover, an increased number of T cells expressing the TCR β-chain variable region, known to be induced by microbial superantigens, was detected in NP and correlated with the presence of IgE against SAEs[113]. Furthermore, in polyp homogenates positive for sIgE against SAEs, a greater fraction of IgG4 was observed and this correlated with IgE and the number of plasma cells[107]. Additionally, in polyp homogenates positive for sIgE against SAEs, production of leukotrienes and lipoxin A4 is upregulated[114].

Colonization with *S. aureus* was present in more than 60% of patients with polyps, 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 chronic rhinosinusitis without nasal polyps (33% and 27%, respectively)[115]. However, the presence of *S. aureus* in the submucosa of nasal tissue did not correlate with the amplification of the Th2-related inflammation typically found in nasal polyps, but this reaction is dependent on the formation of specific IgE against SAEs[116]. In nasal polyps versus controls, similar percentages of enterotoxin genes are present; however, in nasal polyps a higher number of strains belonged to agr I or II, which are associated with strains causing enterotoxin-mediated disease[117].

Treatment

The primary goal of treatment for patients with nasal polyposis is the relief of symptoms such as nasal blockage, hyposmia, rhinorrhea or postnasal drip. Oral and topical corticosteroids represent the major treatment strategies, followed by surgical interventions. However, recurrences are frequent regardless of treatment, making a combination of repeated surgical interventions and a long-term drug treatment necessary[118].

Corticosteroids

The biological action of glucocorticoids is mediated through activation of intracellular glucocorticoid receptors (GR) expressed in many tissues and cells[119]. Two human isoforms of the receptor have been identified, GRα and GRβ. Upon hormone binding, GRα enhances anti-inflammatory or represses pro-inflammatory gene transcription. This is mediated by protein-protein interactions between GR and transcription factors such as AP-1 and NF-κB[119].

Topical corticosteroids sprays have a documented effect on the size of bilateral nasal polyps and also on symptoms associated with nasal polyposis such as nasal blockage, secretion and sneezing but the effect on the sense of smell is not high[28]. Several studies underline the effect of topical corticosteroids to reduce nasal polyp size, reduce complaints of nasal obstruction and increase nasal airflow[120-122]. Efficacy of treatment of nasal polyps with topical glucocorticosteroids is partly associated with their ability to reduce eosinophil viability and activation[123, 124], and to reduce the secretion of chemotactic cytokines[124, 125]. Intranasal corticosteroid treatment postoperative gives long-term improvement in nasal symptoms and reduces polyp recurrence[126]. Topical application of corticosteroids reduces side effects; however nasal sprays cannot reach the middle meatus where polyps originate. Adverse effects with topical corticosteroids are rare, but may consist of dry nose, crusting and eventually mild bleeding.

Based on clinical experience systemic corticosteroids remain a cornerstone for the treatment of nasal polyps and are indicated to initiate or enforce local treatment, mainly as a 2 to 3 weeks' course. According to experts opinion such treatment may be given up to four times a year if no contra-indications are present or no side-effects occur[127]. In a Cochrane review of 2007[128] only one small randomized controlled trial was found that suggests a short-term effect of oral steroids in patients with multiple nasal polyps[129]. In a more recent, double-blind placebo controlled multicenter study nasal polyposis patients were treated with placebo or methylprednisolone in decreasing doses or doxycycline, and patients were followed for 12 weeks. Until month 2 the methylprednisolone group had a significant reduction in polyp score, with a maximal reduction in polyp size after 2 weeks. This group reported decrease in nasal congestion, post-nasal drip symptoms and better smell after a few weeks. No effect on rhinorrhea was described. Methylprednisolone significantly reduced levels of ECP, IL5 and IgE in nasal secretions[130].

Antibiotics

As a new approach, antibiotics with anti-inflammatory effect are being used to treat nasal polyposis. Several studies have shown the effect of macrolide antibiotics in patients leading to a reduction in polyp size together with a decrease of IL-8 in nasal lavage[131, 132]. Long term treatment with these drugs may induce bacterial resistance, which may limit this approach. Placebo-controlled studies with macrolides in nasal polyps are clearly needed before final conclusions.

As *S. aureus* enterotoxins may be considered as disease modifiers in nasal polyposis, in a double-blind placebo controlled multicenter study nasal polyposis patients were treated with placebo, or methylprednisolone in decreasing doses, or doxycycline, and patients were followed for 12 weeks. Doxycycline was used because of its antibacterial activity against *S. aureus* as well as its anti-inflammatory properties. It significantly reduced polyp size, starting at week 2, and this effect remained for up to 12 weeks after dosing. Furthermore it reduced postnasal drip symptom scores and induced a reduction in rhinorrhea. It also reduced levels of myeloperoxidase, ECP an MMP9 in nasal secretions[130].

Anti-IL-5

IL-5 concentrations are increased in nasal polyps and activate and prolong survival of eosinophils. One double-blind placebo-controlled study showed that administration of a humanized anti-IL-5 is a safe and well-tolerated therapy. However, only 50% of the patients showed a clinical response with reduction of polyp size. Subgroup analysis demonstrated that high local IL-5 concentrations in nasal secretions predicted the positive response[104].

Surgery

Functional endoscopic sinus surgery (FESS) is now widely accepted as the surgical treatment of nasal polyposis. Surgery should be considered only after appropriate pharmaceutical therapy. However, nasal polyposis is a chronic inflammatory disease with a high rate of recurrence. The recurrence rate after surgery is linked to previous surgery, asthma and allergy[133]. In nasal polyp patients with co-morbid asthma and aspirin sensitivity, surgery improves asthma symptoms for relatively long periods of time. Especially in the first year after surgery emergency department visits, hospitalizations and asthma attacks decreased in about 90% of the patients[134].

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CHAPTER II:
HUMAN MAST CELLS: ORCHESTRATORS OF NASAL INFLAMMATORY DISEASES?

From bone marrow to nasal tissue

It has been more than a century (1878) since the discovery of mast cells whom Paul Ehrlich is credited for[1]. Since then, subsequent developments have started about the immune response, immunoglobuline E and the function of mast cells in various pathological states.

In vivo, mast cells may only be identified when they are totally matured in tissue and then consequently express their characteristic granules and high-affinity receptor for IgE (FcεRI). Human mast cells are believed to derive from pluripotential CD34+c-kit+CD13+ progenitors [2, 3] . These progenitors arise from the uncommitted hematopoietic stem cells in bone marrow[4] and are present in bone marrow, peripheral blood, human fetal liver[5] and cord blood. The interactions between the membrane receptor c-kit and its ligand, stem cell factor (SCF), and the subsequent signalling that follows, are crucial for the growth and the development of mast cells. SCF is expressed in endothelial cells, fibroblast and other stromal cells[6]. SCF has multiple biological effects on mast cells, including growth, differentiation and homing, prolonging viability, inducing mast cells hyperplasia, and enhancing mediator production[7, 8]. Furthermore, IL-6, eotaxin, and nerve growth factor (NGF) enhance mast cell development from hematopoietic stem cells[9, 10]

After tissue localisation, mast cells can undergo further differentiation into distinct subsets. In terms of granule constituents, human mast cells have been divided into three phenotypes: those only positive for tryptase (MC_T), those positive for tryptase and chymase (MC_{TC}) and those only positive for chymase (MC_C). All three subtypes are present in human nasal tissue[11]. MC_{TC} is more present in connective tissue, whether MC_T is mainly localized into mucosal surfaces, often in close proximity to Th2 cells. This subset usually is noticed in increased numbers infiltrating the mucosa in patients suffering from allergic disease[12].

In vitro, it is possible to grow human mast cells from bone marrow, peripheral blood mononuclear cells and cord blood mononuclear cells[13, 14].

Morphological characteristics of human mast cells

Mature human mast cells are large, mononuclear cells filled with membrane-bound secretory granules. The nucleus has a partially condensed chromatin pattern. The secretory granules exhibit metachromasia when stained with toluidine blue. Other cytoplasmatic features include small Golgi structures and different amounts of lipid bodies. They extrude membrane-free granules either into newly formed degranulation channels in the cytoplasm or individually through pores in the plasma membrane to the exterior environment, when appropriately stimulated[15].

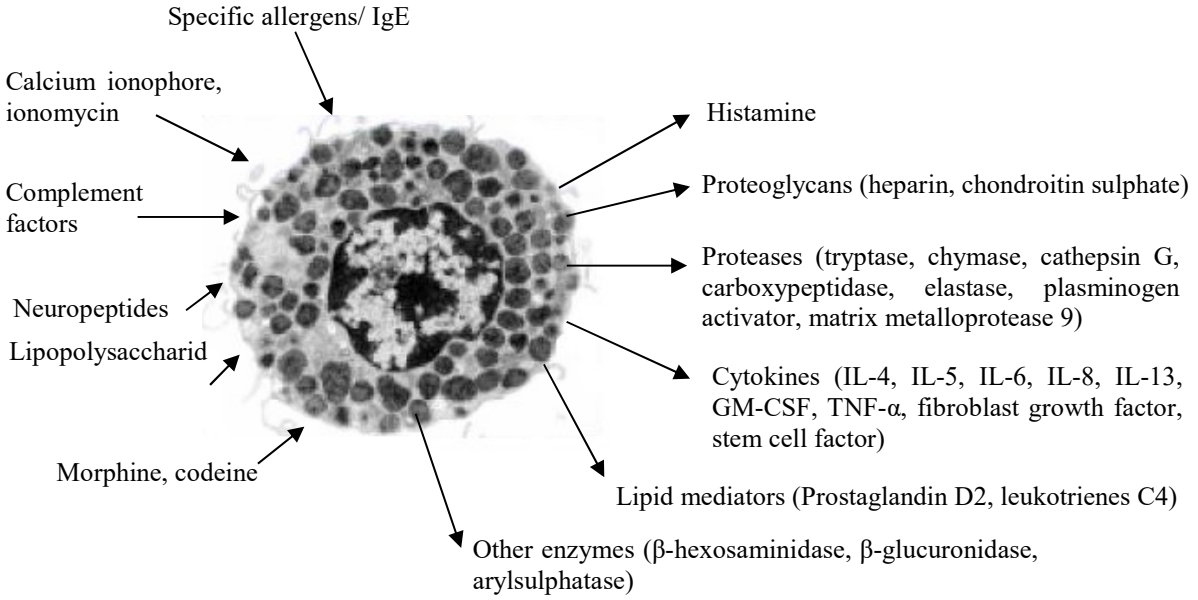
Activators and mediators

In a typical allergic reaction, antigen/ allergen crosslinks two IgE molecules occupying FcεRI, resulting in a cascade of molecular events (see next chapter), and leading to degranulation and elaboration of mediators[16]. Furthermore, a variety of stimuli can activate and degranulate mast cells: opiates, complement factors C3a and C5a, neuropeptides (substance P, vasoactive intestinal peptide, calcitonin gene-related protein), histamine releasing factors (a heterogeneous group of cytokines eg. IL-1, IL-3, IL-8, GM-CSF, TNF-α)[17], chemokines, bacteria, parasites or changes in osmolality. Consequently a lot of membrane receptors are present on human mast cells. They express FcεRI, FcγRI, and the c-kit receptor[18]. They also express Toll-like receptors (TLR2-7 and TLR9)[19] and $CysLTR_1$ and $CysLTR_2$[20]. Mast cells express at least four chemokine receptors (CCR3, CXCR1, CXCR3 and CXCR4)[21] and cultured mast cells express low levels of IL-4Rα, IL-5Rα and integrins and P-selectin ligand to bind to adhesion molecules[6]. Furthermore, they express the histamine H_4 receptor[22].

Mast cells produce a wide array of mediators and cell signalling molecules, and this variety may account for the unique features of the mast cell in the immune system. Human mast cells synthesize histamine, which is stored in secretory granules as a preformed mediator. These granules also contain a variety of proteolytic enzymes (α and β tryptase, chymase, carboxypeptidase A and cathepsin G) and proteoglycans (heparin and chondroitin sulphate E)[23]. Activation of mast cells leads to the synthesis of PGD_2 and cysLTs (LTB_4, LTC_4, LTD_4 and LTE_4). There are wide varieties in the cysLTs production amount among different human tissue mast cells suggesting that this biochemical property is modulated by tissue specific factors. In contrast, the apparent consistency in PGD_2 production of all human mast cells suggests that this is an innate characteristic. After allergenic stimulation, mast cells

immediately release histamine, which is preformed. Leukotrienes and prostaglandins need to be metabolized and are released about 10 minutes later.

Furthermore they are a major source of a wide spectrum of cytokines (IL-3, IL-5, IL-6, IL-13, IL-16, IL-18, IL-25, TGF-β, GM-CSF, TNF-α) and chemokines (IL-8, MIP-1α, MCP1,…). Interestingly, SCF, the principal growth, differentiating and chemotactic factor for human mast cells is present and released by mast cells, which may represent an autocrine factor that sustains mast cell hyperplasia in allergic diseases[24].



Mast cells in allergic rhinitis

Immunohistochemical staining of nasal biopsies with monoclonal antibodies directed against mast cell tryptase shows an increase in mast cells in both intermittent and persistent allergic rhinitis as compared to biopsy findings in non-atopic and non-rhinitis subjects[25, 26]. In symptomatic allergic rhinitis epithelial mast cells are in an activated state with evidence of degranulation[27]. Morphologically, mast cells in the nasal epithelium and the superficial lamina propria resemble MC_T and those in the deep lamina propria resemble MC_{TC}[28]. Several mast cell derived mediators like histamine[29], PGD_2[29], cysLTs[30] and tryptase[31] are elevated in nasal lavage fluid after allergen challenge and during the season in naturally occurring rhinitis[32].

It is well known that mast cell numbers (MC_T) are increased in the epithelium of the allergic nasal mucosa[33]. The mechanism for this accumulation is not completely clear. It has been suggested that the accumulation of mast cells in nasal epithelium was due to the cytokine

SCF, produced from nasal epithelial cells[34]. In human allergic nasal mucosa CD34+, c-kit receptor+, tryptase negative and IgE negative cells were detected and were presumed to be mast cell progenitor cells and may contribute to the increase of fully matured mast cells in the epithelium and subepithelial layer of allergic nasal mucosa[35].

Not only Th2 cells can induce IgE synthesis in B cells but also mast cells can. Nasal mast cells from perennial allergic rhinitis patients express significantly greater levels of FcepsilonRI and CD40L compared to controls and after allergen challenge they can induce IgE synthesis in B cells[36].

Mast cells in nasal polyps

Literature reports show contradictory findings concerning the number of mast cells in nasal polyps; it has been described that the number of epithelial mast cells in nasal polyps is elevated compared to controls[37, 38], or that there is no difference in the number of epithelial mast cells compared to controls[39-41]. The number of tryptase positive cells is not significantly different between atopic nasal polyps and non atopic nasal polyps, demonstrating that the presence of atopy does not determine the type of extent of mast cell infiltration in nasal polyps[42]. There is a significant expression of SCF mRNA and protein in cultured nasal polyp epithelial cells and fibroblasts[43]. Furthermore, the number of polypectomies correlate with expression of SCF mRNA, SCF protein in nasal polyp epithelial cell supernatants and the density of mast cells in the epithelial layer and the stromal layer[44].

It is well described that mast cells in nasal polyps are mostly located in the stroma and are more degranulated compared to inferior turbinate mast cells[45, 46]. Furthermore, stromal mast cells of dispersed nasal polyp tissue release higher amounts of histamine after anti-IgE stimulation compared to epithelial mast cells of the same tissue[47]. This could underline the heterogeneity of mast cells in different tissue localizations, and could point to a more activated status of polyp versus turbinate mast cells, and a higher sensitivity to external triggers. Levels of mast cell-derived mediators such as histamine and tryptase in nasal fluids from patients with nasal polyps are significantly higher than those observed in patients without nasal polyps[48].

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CHAPTER III:
NEW THERAPIES IN INFLAMMATORY DISEASES: KINASE INHIBITORS

The discovery of novel drugs targeting kinases has become the focus of a large number of drug discovery programs in the pharmaceutical and biotech industry.

Kinases are an important class of intracellular enzymes that play a critical role in signal transduction pathways controlling a variety of cellular functions. The role of a protein kinase in the signal transduction is to transfer the terminal phosphate group of ATP to the hydroxyl group of a serine, threonine or tyrosine residue on the target protein, leading to the activation of the substrate for its role in the next step of the signalling cascade[1]. The substrate is often another kinase or a transcription factor.

The protein kinases are classified as serine/threonine or tyrosine substrate kinases based on the receiving amino acid of their substrates. Analysis of the human genome showed that there are 518 protein kinases, of which only 90 are tyrosine kinases, and these include 58 receptor tyrosine kinases[2].

A large majority of kinase inhibitors are designed to inhibit the enzyme, by binding them at or near the ATP-binding site. An inhibitor of one kinase is often found to inhibit other structurally related or unrelated kinases. Therefore, extensive profiling is necessary.

In the present century, protein kinases, especially the tyrosine protein kinases, have become prime targets for cancer intervention. Until the late 1990s, anti-cancer drugs were directed towards metabolic enzymes (e.g. methotrexate), to DNA (e.g. cisplatin), to hormonal signalling pathways (via nuclear hormone receptors for breast (e.g. tamoxifen) and prostate cancer (e.g. flutamide)),... Nowadays, as an anti-cancer therapy, always in combination with other drugs, there are 10 protein kinase inhibitors that are approved and in clinical use, as well as many more compounds in clinical trials[1].

Protein kinases are also targets for treatment of inflammatory and autoimmune diseases. Progress has been made in these areas and some drugs are in different phases of clinical trials (phase I/II)[3], but to our knowledge none of these inibitors have reached the clinical stage of drug development, possibly because of the complexity of the signalling pathways that

involves a lot off cross-talk between pathways. One of the reasons for this lack of success for the treatment of patients with inflammation or autoimmune disorders, has been the high hurdle for safety required for the chronic treatment of patients whose life expectancy is usually significantly longer than that of cancer patients[4].

A large number of kinases from different signal transduction pathways have been the targets of interest for the treatment of inflammatory disorders. Examples of drugs targeting such kinases are inhibitors of Syk (spleen tyrosine kinase), IKK-2 (IκB kinase 2), MAP (mitogen-activated protein kinases), Lck (lymphocyte-specific kinase), JAK3 (Janus kinase 3), …[3, 4]

A majority of kinase inhibitors have inhibitory activity for one kinase and are found to be potent inhibitors of other kinases.

The kinase inhibitors we tested are a Syk inhibitor and an IKK-2 inhibitor and they were profiled for a particular kinase. Extensive profiling has already been done in inflammatory and autoimmune disorders such as asthma and rheumatic arthritis, less research is done in the field of allergic rhinitis. No studies were published yet about the efficacy in treating nasal polyposis. Practically all studies used animal models so far.

SYK INHIBITION

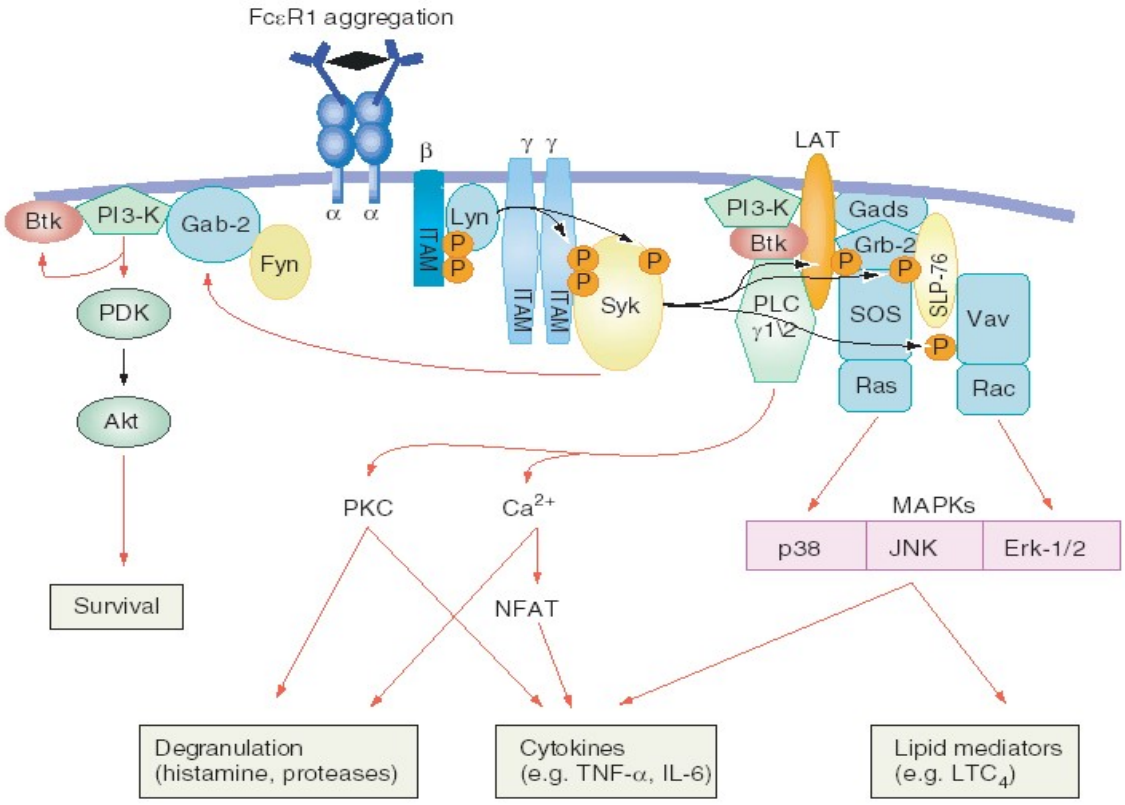
Spleen tyrosine kinase (Syk) is a cytosolic protein tyrosine kinase that plays a crucial role in the IgE (FcεRI) and IgG (Fcγ) receptor-mediated signalling in mast cells[5, 6] and basophils[7].

Mast cells are multifunctional effector cells of the immune system and they play a crucial role in the allergic response.

The IgE/ allergen –mediated stimulation through the cross-linking of the high-affinity receptor for IgE (FcεRI) remains a very important step in the activation of the mast cell. The FcεRI expressed on mast cells and basophils is a tetrameric receptor comprised of the IgE-binding α chain, the signal-amplifying β chain[8] and the two signal transducing γ chains[9].

The transmission of the signals is dependent on the specific sequence of the immunoreceptor tyrosine-based activation motif (ITAM) present on the β and γ chains. Following subsequent allergen exposure, receptor crosslinking results in the recruitment and the activation of cytoplasmatic kinase Lyn. Lyn causes tyrosine phosphorylation of ITAM in the cytoplasmatic domains of β- and γ-chains. Phosphorylated ITAM in the γ-chains serve as binding sites for Syk SH2 domains. As a result of its binding to ITAM, Syk becomes activated[10-13]. Activated Syk regulates the phosphorylation of a host of substrates that form signalling complexes,

which are necessary for downstream signal transductions that result in mediator release and production. It phosphorylates the membrane proximal adaptor linker for activator of T cells (LAT) that recruits and activates signalling proteins like phospholipase Cγ (PLCγ) and it phosphorylates the exchange factors VAV and SOS, which in turn activate the RAS-RAF-MAP kinase pathway. Degranulation is the result of sustained calcium mobilization induced by IP3 released by PLCγ activity. Eicosanoids, including leukotrienes and PGD_2, are generated by conversion arachidonic acid. Arachidonic acid itself is released by the action of cytosolic phospholipase A2 (PLA2) that is also activated by the MAPK pathway. Cytokine production is dependent on both the calcium signal and the MAPK pathway that activate transcription factors including NFAT, AP1 and NF-κB. Therefore most, if not all, mediator release and production is controlled by the Lyn-Syk activation leading to calcium mobilization and the MAPK pathways[10, 14-16].



Adapted from Wong et al.[17]

Syk is essential for mast cell signalling, as a variant of rat basophilic leukemia RBL-2H3 mast cells that lacks the expression of Syk, fails to degranulate after FcεRI aggregation, although the cells still release histamine when stimulated with calcium ionophore[18]. Furthermore, in

bone marrow derived mast cells isolated from Syk -/- mice, there is no FcεRI-induced Ca^{2+} mobilization or secretion[12].

As Syk is positioned upstream of the IgE receptor signal transducing pathway, it may represent an important target for the treatment of allergic rhinitis.

Commonly used antihistamines or leukotriene receptor antagonists target only a single mediator, whereas the mast cell, upon activation by allergen, is able to produce a large number of inflammatory mediators whose activities will be unaffected by such drugs.

Syk inhibition during an allergic response will block three mast cell functions: the release of preformed mediators such as histamine, the production of lipid mediators such as leukotrienes and prostaglandins and the secretion of cytokines.

There is a growing body of literature on the importance of Syk in the development of allergic inflammation in the lower airways. In animal asthma models, aerosolized Syk antisense oligonucleotides (ASO) inhibited many of the central components (f.e. the level of eosinophils, tumour necrosis factor) of allergic asthma[19], and a Syk-selective tyrosine kinase inhibitor prevented mast cell degranulation and airway hyperresponsiveness[20, 21]. In human lung mast cells piceatannol prevented histamine release[22]. Piceatannol was originally described as a syk-specific inhibitor but it inhibits other kinases as well[23] and applied at concentrations inhibiting IgE-induced mediator release from basophils, it doesn't act on syk[24].

Syk inhibitor NVP-QAB205 is considered selective for its respective kinase as it doesn't inhibit other signalling steps thought to be downstream of Syk kinase such as ras-ERK pathway elements. Furthermore, it inhibits histamine release in human basophils and cultured CD 34+ mast cells and it inhibits bronchial smooth muscle contraction in human isolated bronchial preparations [25, 26].

Supplementary with the limited findings of Syk inhibition in the human bronchial biopsies, a clinical study, where another specific Syk inhibitor (R112) was applied, demonstrated the reduction of symptoms in seasonal allergic rhinitis patients [27].

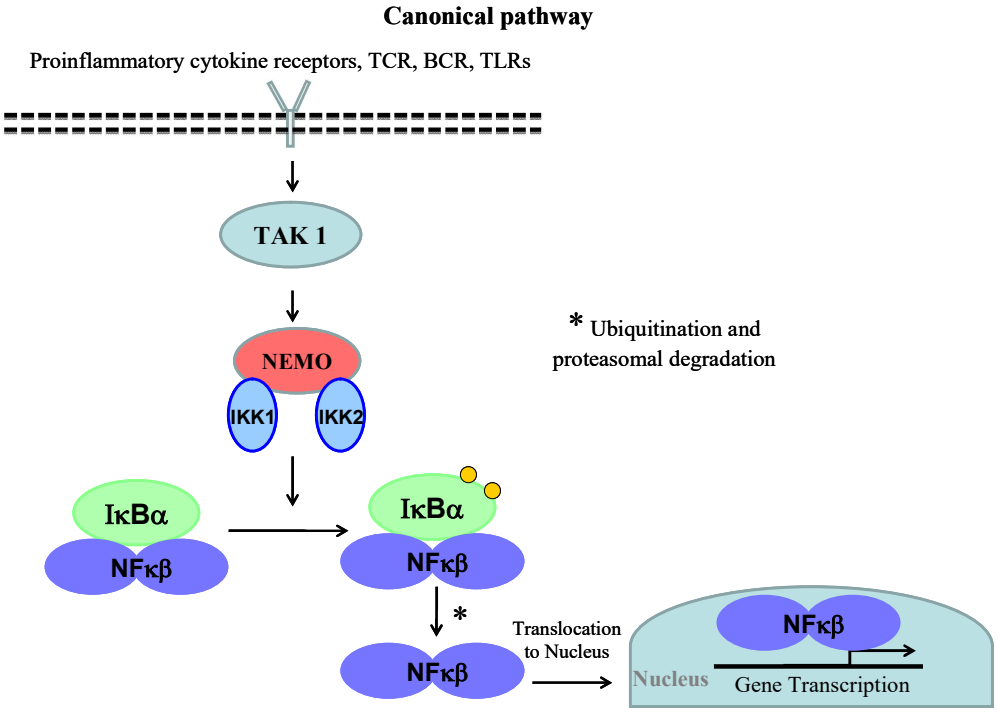
Syk is not only expressed in mast cells and basophils, but also in eosinophils[28], neutrophils[29], macrophages[30], dendritic cells[31] and B-cells[32]. Therefore the contribution of Syk is not only limited to Fc receptors. Unlike many other tyrosine kinases Syk has tumorsuppressing properties. Increased expression of Syk in neoplastic cells from breast-cancer tumors suppresses tumor growth[33]. Moreover, reduced expression of Syk has been found in patients with breast cancer, suggesting possible severe side effects whit longterm using of Syk

inhibitors. In a recent phase 2 study, where the efficacy and safety of a Syk inhibitor was evaluated in patients with rheumatoid arthritis, some adverse events were reported such as diarrhea, hypertension and neutropenia[34].

In summary, syk activation is an important upstream event in the pathways activated through FcεRI and may therefore control the synthesis and release of a whole range of mediators of both the early and late allergic responses. In future, specific syk inhibitors might provide a new therapeutic possibility in the treatment of upper airway disease with mast cell involvement such as allergic rhinitis.

IKK-2 INHIBITION

Nuclear factor (NF)-κB is major family of transcription factors that regulates multiple cell functions. Five mammalian NF-κB proteins have been identified including NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA/p65, RelB and cRel. The predominant form of NF-κB in many cell types is a p65:p50 heterodimer[35]. In unstimulated cells, this transcription factor is found in the cytoplasm in an inactive form because of its binding to the inhibitory protein, IκB (inhibitor of NF-κB)[36, 37]. Upon activation of the cell following the stimulation of receptors such as the TNF receptor, Toll-like receptors or the T-cell receptor (canonical pathway), a signal transduction cascade unravels that leads to the activation of the serine-threonine kinase complex (IKK-complex). The IKK complex comprises two catalytic subunits, IKK-1 and IKK-2, and a regulatory subunit termed NF-κB essential modulator (NEMO)[35, 38]. The IKK-2 subunit seems to have the dominant role in the canonical pathway; it phosphorylates IκB bound to NF-κB. The phosphorylated complex is ubiquitinated and degraded to generate the active NF-κB. The transcription factor then translocates to the nucleus and its active fraction induces the transcription of cytokines, chemokines and adhesion molecules such as IL-1β, TNF-α, IFN-γ, eotaxin, GM-CSF, ICAM-1 and VCAM-1[39, 40].



Because both IL1-β and TNF-α may in turn activate NF-κB, the perpetuation of the inflammatory process in inflammatory diseases may be explained easily.

IKK-2 inhibitors might prove useful in inflammatory and autoimmune disorders. They are expected to be efficacious in rheumatoid arthritis by affecting multiple mechanisms and cell types. In the murine collagen induced arthritis model they could inhibit disease severity as well as NF-κB related cytokines (IL-1β, IL-6, TNF-α and IFN-γ)[41-43]. Furthermore, in inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, characterized by chronic relapsing inflammation, IKK-2 inhibitors ameliorated inflammatory responses in mice models. They inhibited clinical endpoints such as weight loss and colon thickening. Moreover, histological evaluation showed pronounced reduction in the severity of inflammation, in addition to diminished disruption of the mucosal epithelium and loss of glandular crypts [44]. Additionally, IKK-2 inhibition could be beneficial in patients with type 2 diabetes because the activation of NF-κB through IKK-2 induces inflammatory mediators that cause insuline resistance[45]. During insulin resistance tests in mice, IKK-2 inhibition dosed intraperitoneally significantly decreased plasma glucose levels[46].

Furthermore NF-κB has an essential role in the production of chemokines, cytokines and cell adhesion molecules in allergic asthma[47]. In mice, IKK-2 inhibition suppressed allergen-induced airway inflammation and hyperreactivity, with inhibition of airway eosinophilia, IL-4, IL-5 and IL-12[48]. Similar results were reported in an antigen-driven model of airway

inflammation in rats and in human airway smooth muscle[49, 50]; IKK-2 inhibition decreased inflammatory cell recruitment, TNF-α, IL-1, IL-4, IL-5, IL-13 and eotaxin.

To our knowledge, there are no reports on IKK-2 inhibition in allergic rhinitis or nasal polyposis. However, the NF-κB pathway may play an important role in those inflammatory diseases. There is a higher gene and protein expression of NF-κB in nasal polyps than in control mucosa, whereas transcription factor AP-1 does not seem to have a significant role in this pathological process[51] and the low expression of COX-2 mRNA in nasal polyps from aspirin-sensitive patients is associated with a down-regulation of NF-κB activity[52]. In nasal cultured epithelium cells from healthy individuals there is an increased expression of the transcription factor NF-κB and AP-1 related genes in the presence of a house dust mite stimulus, and cultured epithelium cells from allergic individuals show already an activated NF-κB regulatory pathway and upon exposure to house dust mite allergen the AP-1 pathway is downregulated[53].

There are concerns that inhibition of NF-κB may cause side effects such as an increased susceptibility to infection, which has been observed in gene disruption studies when components of NF-κB are inhibited[54, 55]. Furthermore, it is suggested that IKK-2 may serve additional functions beyond regulating the NF-κB pathway: the possibility has been raised that the IKK family may be involved in control of normal mitosis [56, 57]. In addition, IKK-2 knockout mice do not survive due to liver apoptosis. Teratogenicity and susceptibility to infection could be problematic and therefore more information will become available as structurally distinct IKK-2 inhibitors progress through pre-clinical safety studies.

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CHAPTER IV : AIMS OF THE STUDY

The main objective of this thesis was to develop *ex-vivo* models to stimulate nasal tissue, closely mimicking the natural environment and to use these models to test new pharmaceutical molecules.

Chapter V: to set up an *ex-vivo* model for early-phase release of mast cell mediators in inferior turbinate tissue and nasal polyp tissue. To look for similarities or differences in the response of both type of tissues.

Chapter VI: to set up an *ex-vivo* model for late-phase release of immunoregulatory and proinflammatory cytokines. To stimulate nasal polyps with *S. aureus* derived proteins to further analyse the disease-modifying role of *S. aureus* in nasal polyps.

Chapter VII: to study the usefulness of a specific syk-kinase inhibitor in inhibiting the release of early phase mediators and to compare with the response of *in vitro* cord blood derived mast cells.

Chapter VIII: to study the usefulness of a specific IKK-2 inhibitor in decreasing SEB-induced release of cytokines in nasal polyps and to compare with the effect of fluticasone propionate.

PART II: NASAL TISSUE STIMULATIONS

CHAPTER V:

ENHANCED RELEASE OF IGE-DEPENDENT EARLY PHASE MEDIATORS FROM NASAL POLYP TISSUE.

Joke Patou[1*], Gabriele Holtappels[1], Karen Affleck[2], Philippe Gevaert[1], Claudina Perez-Novo[1], Paul Van Cauwenberge[1], Claus Bachert[1]

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[1]Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University, Ghent, Belgium
[2]GSK, Stevenage, United Kingdom

ABSTRACT

Background: The mast cell is a crucial effector cell in allergic rhinitis and other inflammatory diseases. During the acute allergic reaction preformed mediators such as histamine, but also *de novo* produced mediators such as leukotrienes ($LTC_4/D_4/E_4$) and prostaglandins (PGD_2) are released. Mast cells represent targets for therapeutic intervention, and thus a human ex-vivo model to stimulate mast cells taken from mucosal sites would be instrumental for drug intervention studies. We have aimed to activate mast cells within ex-vivo human nasal tissue by IgE/ anti-IgE specific (ε chain specific) stimulations and in this respect to test the usability of nasal polyps versus inferior turbinates

Methods: Biopsy samples were collected from patients with nasal polyps and inferior turbinates from patients who underwent sinus or septal surgery. Tissue fragments were primed with IgE 1µg/ml for 60 minutes and then stimulated for 30 minutes with tissue culture medium (negative control), anti-IgE 10µg/ml, anti-IgE 30µg/ml and ionomycin 10µM (positive control). Histamine, leukotrienes and PGD_2 were measured in supernatants. To help provide an understanding of the extent of the response, the number of tryptase and FcεRIα positive cells was evaluated by means of immunohistochemistry and the FcεRIα-chain was measured by means of quantitative PCR in the nasal polyp and inferior turbinate tissues. Finally, the correlation between IgE concentrations in the nasal tissue and the release of mediators was analysed.

Results: Stimulations with anti-IgE on IgE-primed nasal tissue fragments lead to a concentration-dependent release of histamine, leukotrienes and PGD_2. The release of these early phase mediators was significantly higher in nasal polyps compared to inferior turbinates,

although tryptase, FcεRIα positive cells and FcεRIα-chain transcripts were equally present in both groups. No correlation was found between baseline concentrations of IgE, and the release of histamine, LTC_4/LTD_4/LTE_4 and PGD_2 after stimulation.
Conclusions: This human nasal challenge model mimics the allergic early phase reaction. The release of histamine, cys-leukotrienes and PGD_2 was significantly higher in nasal polyps versus inferior turbinates, however, this observation could not be explained by differences in mast cell or FcεRI+ cell numbers.

BACKGROUND

Mast cells play a crucial role in allergic rhinitis and other inflammatory responses. Positioned at mucosal surfaces, these cells are situated to be among the first to encounter antigens that elicit allergic reactions. Interaction of multivalent allergens with cell-bound specific immunoglobuline E (IgE) leads to cross-linking of the high affinity IgE receptor (FcεRI), which is primarily expressed on mast cells and basophils. First, this results in the immediate release of the content of mast cell secretory granules, which includes preformed mediators such as histamine, neutral proteases and proteoglycans and second, it results in the *de novo* synthesis of mediators including the products of the arachidonic acid metabolism, such as prostaglandin D_2 (PGD_2) and sulfidopeptidyl leukotrienes C_4/D_4/E_4, and the production of several cytokines (i.e. IL-4, IL-5, IL-6, TNF-α, IL-13) [1, 2]. During the acute allergic reaction mainly preformed mediators such as histamine, but also newly produced mediators such as leukotrienes (LTC_4/D_4/E_4) and PGD_2 are released [3]. These mediators initiate rapid vascular permeability, leading to plasma extravasation and tissue edema, mucous overproduction and leukocyte recruitment.

Most early studies of mast cells rely on the use of transformed mast cells from murine mastocytoma cells [4, 5]. Currently, it is possible to grow human mast cells *in vitro*. Interleukin (IL)-3, IL-6 and stem cell factor (SCF) may act on hematopoietic stem cells present in bone marrow, umbilical cord blood, fetal liver or peripheral blood and make it possible to grow large numbers of committed mast cell precursors. These cells express high levels of c-kit receptor and FcεRI [6]. Furthermore, several mast cell lines such as HMC-1 [7] or LAD-1/2 [8] are available to study mast cell biology. The use of murine cells, the addition of several factors to grow human mast cells, or the use of human mast cell lines may induce responses different from primary *in vivo* tissue mast cells.

Considerable difficulties exist to isolate and stimulate mast cells from nasal tissue; especially the limited amount of tissue extracted after surgery (turbinotomy) and the low number of mast cells isolated from nasal tissue, may give problems to stimulate nasal mast cells directly [9]. To study nasal mast cells, stimulations have been done in enzymatic dispersed nasal polyp tissue [10, 11]. Accessibility of nasal polyp tissue allows for easy assessment of interaction between different cell types in an inflammatory environment; however, enzymatic digestion of tissue may possible damage receptors and the comparability of results obtained from nasal polyp stimulations to inferior turbinate stimulations is not clear.

We therefore aimed to study mast cells and basophils in their tissue environment by using IgE/ anti-IgE driven (ε chain specific) stimulations in human nasal tissue explants without enzymatic digestion to closely mimic the *in vivo* situation. Second we wanted to test the usability of nasal polyps versus inferior turbinates in this respect, as polyp tissue is easier to obtain in larger quantities. Finally, we aimed to explain differences in the response between tissues, and studied tryptase and FcεRIα+ cell numbers, as well as baseline concentrations of IgE in relation to mast cell responses [12].

METHODS

Patients

Nasal tissue was obtained from 8 polyp patients and 8 control patients at the Department of Otorhinolaryngology of the University Hospital of Ghent. The ethical committee of the Ghent University Hospital approved the study and all patients gave their written informed consent prior to inclusion in the study.

None of the subjects received intranasal corticosteroids, anti-histamines or anti-leukotrienes, oral and intranasal decongestants or intranasal anticholinergics within 1 week prior to surgery and none of the subjects received oral and/or intramuscular corticosteroids within 4 weeks prior to surgery. For female subjects pregnancy or lactation was excluded.

The control group was composed of samples collected from the inferior turbinates from patients undergoing septal surgery and/or turbinotomy because of nasal obstruction, unrelated to this study.

Nasal polyp samples were collected during functional endoscopic sinus surgery. Nasal polyposis was diagnosed based on symptoms, clinical examination, nasal endoscopy, and sinus computed tomography (CT) scan according to the EP^{3}OS guidelines [13].

The atopic status of all patients was evaluated by skin prick tests with a standard panel of 14 inhalant allergens, including negative (NaCl solution) and positive controls (10 mg/ml histamine solution). The reaction to a skin prick test was considered positive if the wheal area caused by the allergen was greater than 7 mm² (diameter >3 mm). Patient characteristics are displayed in table 1.

	Inferior turbinates	Nasal polyps
N	8	8
Age (median, range)	36.5 (17-47)	38.5 (18-54)
Female/ male	2/6	4/4
Asthma in history	1/8	0/8
Skin prick test-positive	0/8	2/8
Aspirin intolerance	0/8	0/8
Smoking	1/8	1/8

Table 1. Patient characteristics

The nasal tissue collected during surgery was immediately transported to the laboratory, partly snap frozen in liquid nitrogen, and stored at –80°C until analysis for immunohistochemistry, IgE measurement and PCR. The remaining tissue was used for the ex-vivo stimulations.

Mechanical disruption and stimulations of human nasal tissue

The human nasal mucosa and submucosa was cut thoroughly in tissue culture medium consisting of RPMI 1640 (Sigma-Aldrich, Bornem, Belgium), containing 2mM L-Glutamine (Invitrogen, Merelbeke, Belgium), antibiotics (50 IU/ml penicillin and 50µg/ml streptomycin) (Invitrogen) and 0.1% BSA (Bovine Serum Albumin, Sigma). The tissue was passed through a mesh to achieve comparable fragments. The tissue fragments (+/- 0.9 mm^3) were weighed and resuspended as 0.04g tissue/ 1 ml tissue culture medium. The tissue was preincubated for 1 hour at 37°C, 5% CO_2 with 1µg/ml human myeloma IgE (Calbiochem, VWR International, Leuven, Belgium). After 3 washing steps the tissue fragments were resuspended in the appropriate amount of culture medium and 0.5 ml of this fragment suspension was dispensed per well of a 48 well plate. (BD Falcon, VWR, Leuven, Belgium). The fragment suspensions

were stimulated with either culture medium (negative control), ε-chain specific anti-human IgE antibody (Dako Belgium N.V., Heverlee, Belgium), at 10 or 30 µg/ml (Dako Belgium N.V., Heverlee, Belgium), or 10 µM ionomycin (Calbiochem) for 30 minutes.
Supernatants were separated by centrifugation and stored immediately at -20°C until analysis of histamine, $LTC_4/D_4/E_4$ and PGD_2.

Measurements of mediators in supernatants of stimulated tissue fragments

Concentrations of histamine, $LTC_4/D_4/E_4$ and PGD_2 were measured in tissue supernatants obtained after the stimulations using ELISA kits for Histamine (IBL Hamburg, Germany), $LTC_4/D_4/E_4$ (Oxford Biomedical Research, Nuclilab BV, Ede, The Netherlands) and PGD_2 (Cayman Chemicals, Ann Arbor, Michigan) following the instructions of the manufacture.

Immunohistochemistry

Cryostat sections were prepared (6 µm) and mounted on SuperFrost Plus glass slides (Menzel Glaeser, Braunschweig, Germany), packed in aluminium paper and stored at -30°C until staining. Sections were immunohistochemically stained with the following antibodies: mouse anti human mast cell tryptase (clone G3, Chemicon International, Biognost, Heule, Belgium) and mouse anti human FcεRIα (clone CRA1, Gentaur, Brussels, Belgium). For immunohistochemical staining, specimens were fixed in Carnoy's Fluid (60% ethanol, 30% chloroform, 10% glacial acetic acid). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in TBS (Tris-buffered-Saline) containing 0.1% sodium azide for 20 minutes. The primary antibody or the negative control, consisting of the corresponding isotype control, was incubated for 1 hour and signal was detected using the LSAB+ technique conjugated with peroxidase according to the manufacturer's instructions (labelled streptavidin-biotin; Dako). The peroxidase activity was detected using AEC Substrate chromogen (Dako), which results in a red-stained precipitate. Finally the sections were counterstained with hematoxylin and mounted.
The number of positive cells was analysed using a magnification of 400× and scored by two independent observers who did not know the diagnosis and clinical data. The analyses included 10 relevant fields of the biopsy, and for each sample, the sum of positive cells/10 fields were scored.

RNA preparation and real-time RT-PCR

Snap frozen tissue samples were placed in liquid nitrogen and thoroughly ground with a mortar and pestle and homogenized with Lysis Buffer (Bio-Rad Laboratories, CA, USA). Total RNA was purified using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, CA, USA) following manufacture's intructions. One microgram of total RNA was than reverse transcribed to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) as instructed by the supplier. Expression of the IgERα-chain was determined using real-time PCR performed on an iCycler Real Time Detection System (Bio-Rad Laboratories, CA, USA). Primers and probes were purchased from Invitrogen (Merelbeke, Belgium) and contained the following sequences: IgERα (sense): 5′-TCTTCAGTGACTGGCTGCTCC-3′, IgERα (antisense): 5′-GCTGGCCCTCCATCACC-3′, IgERα-probe: FAM- 5′-TCAGGCCTCTGCTGAG-3′-TAMRA [14]. PCR reaction contained 20ng of cDNA, 300 nM of specific primers, 100nM of TaqMan probe and 1X TaqMan Master mix (Bio-Rad Laboratories, CA, USA) in a final volume of 0.02ml. Amplification program consisted in 1 cycle at 95°C for 10 min followed by 40 cycles at 60°C for 1 min and 95°C for 15 seconds. The expression of two housekeeping genes: Beta actin (ACTB) and Hydroxymethyl-bilane synthase (HMBS) was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software as described previously [15]. The relative expression of the receptor was calculated with the qBase program (version 1.3.5, UGent, Belgium) based on the delta-C_T relative quantification method. Results are shown as relative expression units per 20 ng cDNA (RNA based).

Measurement of IgE in tissue homogenates

Snap frozen tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added per every 0.1 g tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun, Melsungen, Germany) at 1000 rpm for 5 min on ice as described previously [16]. After homogenization, the suspension was centrifuged at 3000 rpm for 10 min at 4°C and the supernatants separated and stored at -80°C until analysis. Immunoglobuline E was measured by the UNICAP system (Phadia, Uppsala, Sweden).

Statistical analysis

Statistical analysis was performed using the Wilcoxon test (for paired comparisons). The Mann-Whitney U test was used for between-group (unpaired) comparisons. P values of less

than .05 were considered as statistically significant. Correlations were made by using the Spearman rank correlation analysis.

RESULTS

Mediator release after ex-vivo stimulations

A stimulation model was set up to stimulate inferior turbinate tissue (n=8) and in larger quantities obtainable nasal polyp tissue (n=8). IgE-primed nasal tissue fragments were stimulated with anti-IgE (10µg/ml and 30µg/ml) or ionomycin (10µM) for 30 minutes. Stimulation resulted in a significant release and production of histamine, leukotrienes and PGD_2 measured in the supernatants by ELISA. These mediators were released in a concentration-dependent manner, except for $LTC_4/D_4/E_4$ in the inferior turbinate group (Table 2), where the difference between 10 and 30µg/ml was not statistically significant.

After 30 minutes culture in medium alone, the spontaneous release of histamine and leukotrienes was significantly higher in nasal polyps compared to inferior turbinates ($p<0.01$ and $p=0.03$ respectively). However, the spontaneous release of PGD_2 was not different between the two groups ($p=0.1$). After correction for spontaneous release, the induced release of histamine, $LTC_4/D_4/E_4$ and PGD_2 was significantly higher in the nasal polyp group compared to the inferior turbinate group, both after stimulation with anti-IgE 10 µg/ml and anti-IgE 30 µg/ml (Fig 1).

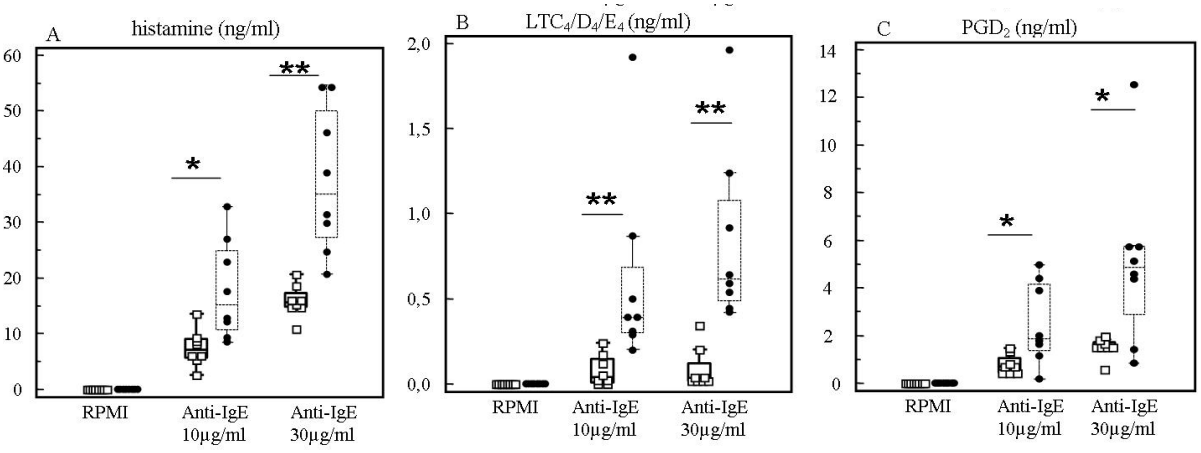

*Fig 1. Histamine (ng/ml) (A), $LTC_4/D_4/E_4$ (ng/ml) (B) and PGD_2 (ng/ml) (C) release after 30 minutes anti-IgE (10µg/ml and 30µg/ml) stimulation. Comparison between nasal polyps (n=8) and inferior turbinates (n=8) after correction for baseline. The box-and-whisker plot represents the median, the lower to upper quartile, and the minimum to the maximum value, excluding outside and far out values, which are displayed as separate points. Statistical analyses were performed by using the Mann-Whitney U test. * p≤0.05, ** p≤0.001.*
——— *= inferior turbinates,* ---------- *= nasal polyps.*

Immunohistochemistry

In an attempt to explain the stronger response upon stimulation in nasal polyps versus inferior turbinates, mast cells and basophils were stained for tryptase and counted (Fig 2A), but no difference in the total numbers of mast cells in the nasal polyp group compared to the inferior turbinate group was detected. Furthermore, staining for FcεRIα showed no differences between the numbers of positive cells in both groups (Fig 2B). Representative stainings are shown in Fig 3.

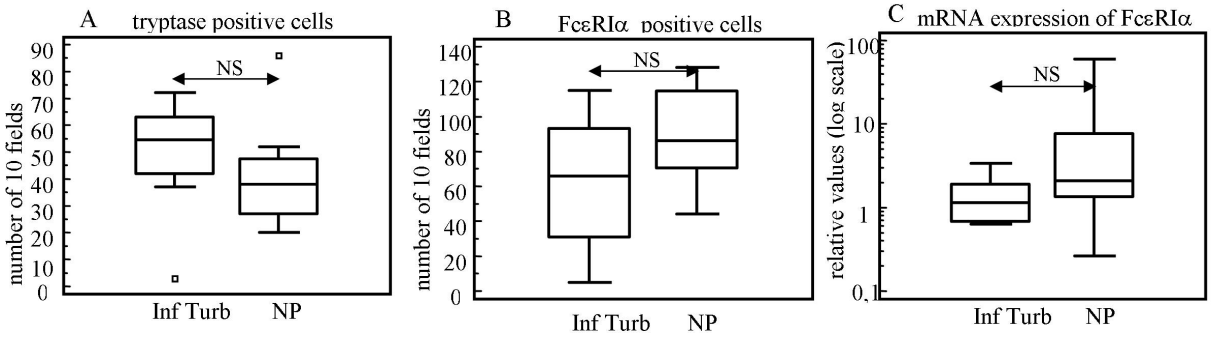

Fig 2. Numbers of tryptase positive cells (A) and FcεRIα positive cells (B) in the inferior turbinate group (Inf Turb) (n=8) and the nasal polyp group (NP) (n=8), expressed as 10 scored fields (×400). The mRNA expression of FcεRIα in the inferior turbinate group and the nasal polyp group (C). The box-and-whisker plot represents the median, the lower to upper quartile, and the minimum to the maximum value, excluding outside and far out values, which are displayed as separate points. Statistical analyses were performed by using the Mann-Whitney U test. NS= Not Significant

FcεRIα-chain mRNA

To study the expression of the high affinity IgE receptor, the amount of FcεRIα mRNA was quantified by RT-PCR in the nasal polyp and inferior turbinate groups. Equivalent FcεRIα mRNA levels were found in nasal polyps compared to inferior turbinates (Fig 2C).

IgE in tissue homogenates

As it is described that the concentration of IgE is related[12] to the surface expression of FcεRI, and IgE concentrations are significantly higher in nasal polyps compared to controls [17], we studied the correlation between the IgE levels in tissue homogenates, and the release of histamine, $LTC_4/LTD_4/LTE_4$ and PGD_2 after anti-IgE challenge. Confirming earlier results, the concentrations of IgE were significantly higher in nasal polyps [97.6 (55.3-190.1) kUA/l] [median (IQR)] compared to inferior turbinates [10.3 (9.4-30.7) kUA/l] (p=0.02). However, we were not able to demonstrate any correlation between the concentrations of IgE in nasal polyp homogenates and the amount of histamine release (r=0.05, p=0.9) (r=0.1, p=0.8),

leukotriene release (r=0.3, p=0.4) (r=0.4, p=0.3) and PGD2 release (r=0.3, p=0.4) (r=0.2, p=0.5) after anti-IgE 10µg/ml and anti-IgE 30µg/ml stimulation respectively. Furthermore no correlation could be found between the concentrations of IgE in inferior turbinate homogenates and the amount of histamine release (r=0.1, p=0.7) (r=0.5, p=0.2), leukotriene release (r=0.01, p=1.0) (r=0.5, p=0.2) and PGD2 release (r=0.02, p=1.0) (r=0.8, p=0.1) after anti-IgE 10µg/ml and anti-IgE 30µg/ml stimulation respectively.

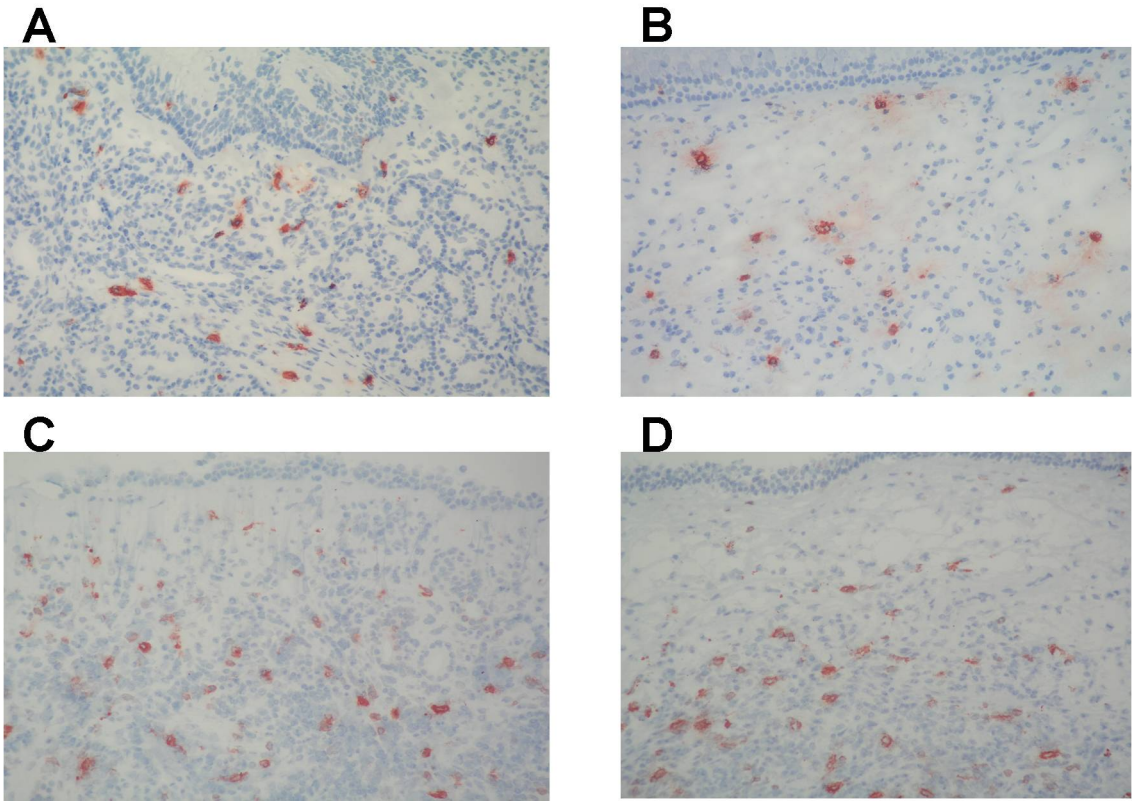

Fig 3. Representative staining of tryptase positive cells in inferior turbinate tissue (A) and in nasal polyp tissue (B). Representative staining of FcεRIα positive cells in inferior turbinate tissue (C) and in nasal polyp tissue (D) (× 200).

DISCUSSION

Until recently, cell systems used for exploration of mast cell biology have mainly been of rodent origin (the rat basophilic leukaemia cell line RBL-2H3, mouse bone marrow derived mast cells). The only human cell line available (HMC-1) [7] has been of limited usefulness due to the cells' stem cell factor independence, and inconsistent degranulation to IgE-dependent signals, presumably due to variable expression of the FcεRIα-subunit [7, 18]. Other cell cultures, designated LAD 1 and 2, derived from bone marrow aspirates from a patient with mast cell sarcoma/ leukemia, resemble CD34+-derived human mast cells with functional FcεRI and FcγRI receptors [8]. The use of bone marrow derived mast cells [19], umbilical cord blood derived

mast cells [20], and foetal liver [21] or peripheral blood derived mast cells [19] have improved the models for studying mast cell biology [22]. Here, addition of certain interleukins such as IL-3, IL-6 or SCF to CD34+ progenitor cells made it possible to grow large numbers of committed mast cell precursors. However, mast cells display phenotypic heterogeneity depending on their tissue localisation, and any of those surrogate cell systems may prove not to represent the mast cells in a diseased tissue. It is therefore advantageous to study mast cells derived from human nasal mucosal, especially diseased tissue.

Pawankar et al [9] were able to isolate mast cells from inferior turbinates and study the IgE receptor, however, the number of mast cells remaining after stimulation is too little to perform meaningful mast cell activation and mediator release. Several studies [10, 11] have made use of mast cells within digested nasal polyp tissue for stimulation, however these cells did not release histamine upon IgE receptor stimulation [23].

In this study we stimulated *ex-vivo* nasal tissue with anti-IgE to study mast cell activation and to compare the response in inferior turbinates and nasal polyps. By using whole tissue preparations, the cells remained in their natural environment, and unchanged surface receptor expression was maintained by omitting enzymatic digestion, thus closely mimicking the *in vivo* situation.

The stimulation with anti-IgE 10 µg/ml and anti-IgE 30 µg/ml resulted in a significantly higher production and release of mediators such as histamine, $LTC_4/D_4/E_4$ and PGD_2 compared to baseline, and these mediators were released in a concentration-dependent manner.

Although we measured mediators which are relatively restricted to mast cells such as histamine, PGD_2 and $LTC_4/D_4/E_4$, we could not totally exclude that other cells, which have been reported to express the IgE receptor, such as dendritic cells [24] and eosinophils [25], may also have been activated during this process. However, dendritic cells do not produce and release histamine, $LTC_4/D_4/E_4$ or PGD_2, and it is generally accepted that eosinophils are not a source of histamine and PGD_2. Moreover, it has been shown that stimulation with human IgE and anti-IgE does not cause production of leukotriene C4 in eosinophils [26], demonstrating only mast cell activation in this setting.

Theoretically, basophils could contribute to the responses demonstrated here. It is, however difficult to discriminate between basophils and mast cells as effector cells. There are no reports about the number of basophils in nasal polyps in literature, suggesting a minor role of

those cells in nasal polyps. Secondly, in the lamina propria of inferior turbinates of allergic patients, at baseline, the number of mast cells is at a median of 88%, with the percentages of basophils being as low as 3%. Only after allergen provocation, in the early phase, numbers of mast cells diminish sharply to a median percentage of 27% and basophils increase to 23% [27]. However, in the setting used here, mast cells most probably are the major contributors, as an influx of basophils in this *ex-vivo* model is impossible. Moreover, studies measuring mediators in nasal lavage fluid in an allergen-induced late-phase reaction revealed high levels of histamine but relatively low levels of products such as PGD_2. Since histamine is released by mast cells and basophils, but prostaglandin D_2 is not produced by basophils, these findings have implicated the basophils as an important contributor to histamine release in the late phase but not in the early phase[28, 29]. In the here presented model, we thus most likely restrict the stimulation to mast cells.

Accessibility of nasal polyp tissue allows for easy assessment of interaction between different cell types in an inflammatory environment; however, the comparability of results obtained from nasal polyp stimulations to inferior turbinates was not studied so far. We therefore investigated the comparability of release of early mediators in nasal polyps versus inferior turbinates. We here demonstrate that the production and release of histamine, $LTC_4/D_4/E_4$ and PGD_2 was significantly and consistently higher in nasal polyps compared to inferior turbinates, both after stimulation with anti-IgE 10 µg/ml and anti-IgE 30 µg/ml.

The increased release of early phase mast cell mediators in nasal polyps could be due to the presence of a higher number of mast cells in nasal polyps. However, no difference in the total number of tryptase-positive cells in inferior turbinates compared to nasal polyps could be found by tryptase staining. Literature reports show contradictory findings; it is described that the number of epithelial mast cells in nasal polyps is elevated compared to controls[30, 31] or that there is no difference in number of epithelial mast cells compared to controls[17, 32]. In line with our findings, a recent study couldn't find any difference in the total number of mast cells between nasal polyps and inferior turbinates[33].

It is well described that mast cells in nasal polyps are mostly located in the stroma and are more degranulated compared to inferior turbinate mast cells [34, 35]. Furthermore, stromal mast cells of dispersed nasal polyp tissue release higher amounts of histamine after anti-IgE stimulation compared to epithelial mast cells of the same tissue[36]. This underlines the heterogeneity of mast cells in different tissues and could point to a more activated status of

polyp versus turbinate mast cells, and a higher sensitivity to external triggers. In line with our findings, levels of mast cell-derived mediators such as histamine and tryptase in nasal fluids from patients with nasal polyps are significantly higher than those observed in patients without nasal polyps [37]. Here we show that mast cells, even if partially degranulated in polyp tissue, still can produce and release higher amounts of mediators compared to the non-degranulated mast cells in inferior turbinates.

Having shown that the number of mast cells present was similar between polyp and turbinate tissue, we investigated whether the number of FcεRIα-positive cells was different between the two tissue types, but no difference was shown. The number of FcεRIα positive cells was higher than the number of tryptase positive cells, in both nasal polyps and inferior turbinates, which may be explained by the staining of other than mast cells, such as basophils, eosinophils [25] and dendritic cells [24].

Moreover, the FcεRIα chain expression at mRNA level did not demonstrate any difference in relative expression in nasal polyps compared to inferior turbinates. In the past, our group and others have described significantly higher levels of IgE in nasal polyp homogenates compared to controls [17, 38]. As IgE levels may control cell surface levels of FcεRI [39], we expected higher levels of FcεRIα mRNA in the nasal polyps, which then could explain the increased release of mediators. However, in line with our results, other studies demonstrated that the presence or absence of IgE has no influence on the levels of mRNA for either alpha, beta, or gamma subunits of FcεRI [40, 41].

In cord blood derived human mast cells, pre-incubation of mast cells for 4 days with IgE resulted in an enhancement of the IgE-binding ability of cells, and this was reflected by an increased surface expression of FcεRI. Moreover, this resulted in the elevated release of histamine, LTC_4 and PGD_2 in response to anti-IgE challenge[12]. However, we were not able to demonstrate a correlation between baseline IgE levels in nasal polyp and inferior turbinate homogenates and the amount of histamine, LTC_4/LTD_4/LTE_4 or PGD_2 release upon stimulation. Moreover, the release of mediators also was significantly different in polyp versus turbinate tissue after ionomycin stimulation, suggesting that the higher release in nasal polyps might be unrelated to the surface expression of FcεRI. Further studies need to clarify the mechanism behind this phenomenon.

	Histamine (ng/ml)	$LTC_4/LTD_4/LTE_4$ (ng/ml)	PGD_2 (pg/ml)
Nasal polyps			
RPMI	24.1 (15.1-32.6)	0.0815 (0.048-0.11)	109 (66.5-221)
Versus	*p<0.01*	*p<0.01*	*p<0.01*
Anti-igE 10µg/ml	43.2 (28.1-55.5)	0.469 (0.348-0.816)	1960 (1518-4544)
Versus	*p<0.01*	*p<0.01*	*p<0.01*
Anti-IgE 30µg/ml	63.6 (44.8-75.5)	0.675 (0.561-1.21)	4949 (2991-6152)
Ionomycin 10µM	130 (77.5-135)	3.40 (1.80-5.37)	2717 (1364-4298)
Versus baseline	*p<0.01*	*p<0.01*	*p<0.01*
Inferior turbinates			
RPMI	8.5 (5.6-12.9)	0.036 (0.016-0.0395)	58.6 (40.2-88.2)
Versus	*p<0.01*	*p<0.01*	*p<0.01*
Anti-igE 10µg/ml	16.2 (12.0-20.2)	0.0655 (0.038-0.181)	840 (492-1269)
Versus	*p<0.05*	*p=0.44*	*p<0.05*
Anti-IgE 30µg/ml (n=6)	28.1 (21.8-31.7)	0.0715 (0.058-0.331)	1669 (1311-1732)
Ionomycin 10µM	27.6 (22.3-45.5)	0.361(0.24-0.525)	967 (548-1373)
Versus baseline	*p<0.01*	*p<0.01*	*p<0.01*

Table 2. Overview of anti-IgE and ionomycin-induced release of histamine (ng/ml), $LTC_4/LTD_4/LTE_4$ (ng/ml) and PGD_2 (pg/ml) after 30 minutes in the nasal polyp (n=8) and inferior turbinate group (n=8). Data are expressed as median +/- IQR.
Statistical analysis; Wilcoxon-test

CONCLUSIONS

To conclude, a whole tissue nasal mucosal stimulation model was established which can be used to mimic the early phase of an allergic reaction both in nasal polyps and inferior turbinates.

We observed a significantly higher release of mast cell mediators after equivalent stimulation of nasal polyp tissues compared to inferior turbinates, the mechanism of which remains unclear. It is well recognized that mast cells with distinct functional and histochemical properties are present in human tissues [42, 43]. The functional heterogeneity, the micro-environmental forces that dictate responsiveness and the impact of disease on mast cell response might be important in this process.

As high amounts of nasal polyp tissue are easier to access, and as nasal polyps and inferior turbinate tissue react in the same concentration- dependent manner to IgE- dependent triggers,

nasal polyp tissue could be used to study the effect of inhibitors of the allergic early phase reaction in future settings.

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CHAPTER VI:
STAPHYLOCOCCUS AUREUS ENTEROTOXIN B, PROTEIN A AND LIPOTEICHOIC ACID STIMULATIONS IN NASAL POLYPS

Joke Patou MD, Philippe Gevaert MD, Thibaut Van Zele MD, Gabriele Holtappels, Paul Van Cauwenberge MD, PhD, Claus Bachert MD, PhD

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Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University, Ghent, Belgium

ABSTRACT

Background: Increasing evidence points towards a modifying role of Staphylococcus aureus and its products in the pathogenesis of nasal polyposis.

Objective: The aim of this study was to investigate cytokine and mediator production after stimulation with Staphylococcus aureus derived proteins enterotoxin B, protein A and lipoteichoic acid in nasal polyp and control inferior turbinate tissue.

Methods: Tissue fragments were stimulated with RPMI (negative control), enterotoxin B, protein A and lipoteichoic acid for 30 minutes and 24 hours. Supernatants were measured by Multiplex for pro-inflammatory cytokines (IL-1β, tumor necrosis factor-α) and T cell and subset related cytokines (interferon-γ, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13). Histamine, transforming growth factor-β1, cysteinyl leukotrienes and prostaglandin D_2 were analysed by ELISA.

Results: 30 minutes protein A stimulation resulted in a significant increase of histamine, leukotrienes and prostaglandin D_2. Enterotoxin B stimulation over 24 hours induced a significant increase of IL-1β, tumor necrosis factor-α, interferon-γ, IL-2, IL-4, IL-5, IL-10 and IL-13 in both groups, this increase being significantly higher in nasal polyps compared to controls.

Conclusions: We here show that Staphylococcus aureus products have various effects on mucosal tissues: surface protein A induces mast cell degranulation, whereas enterotoxins induce the release of cytokines, with a Th2-skewed pattern in nasal polyps, supporting the stimulatory role of superantigens in the development of this inflammatory disease.

Clinical Implications: Staphylococcus aureus products affect inflammation in nasal polyps by inducing mast cell degranulation and T-cell activation.

CAPSULE SUMMARY

This study for the first time demonstrates that Staphylococcus aureus derived enterotoxin B can polarize mucosal inflammation in polyp tissue to a Th2-pattern, and protein A induces mast cell degranulation.

INTRODUCTION

Chronic rhinosinusitis with nasal polyposis (NP) is a chronic inflammatory disease of the paranasal sinuses, associated with Th2-biased inflammation[1], an increase of tissue eosinophils[2] and polyclonal IgE production, not related with the allergic status of the patients[3]. The mast cells in the stroma of nasal polyps are often degranulated[4, 5]. In the general population the prevalence of NP ranges from 1 to 4% and the precise mechanism underlying the pathogenesis of NP is unknown and probably multifactorial[2, 6].

The colonization rate with Staphylococcus aureus (*S. aureus*) in the middle meatus is increased in patients with NP versus controls[7]. These bacteria express a number of surface proteins such as lipoteichoic acid (LTA) and protein A (SpA) that have the potential to interfere with host defence mechanisms. LTA has been suggested to be essential for nasal colonization and interaction with human nasal epithelial cells[8] and SpA has been demonstrated to increase histamine release from human basophils[9] and human heart mast cells[10]. SpA appears to activate basophils by interacting through its alternative binding site with immunoglobuline E (IgE) V_H3^+ bound to the high affinity IgE receptor (FcεRI) [9, 10]. In addition, SpA has been proposed to have B-cell superantigenic effects[11, 12].

Moreover, *S. aureus* secretes several toxins with superantigen activity namely the *S. aureus* - derived enterotoxins (SAEs) and the toxic shock syndrome toxin (TSST-1). Superantigens for T-lymphocytes have the ability to crosslink the class II major histocomptability complex of antigen-presenting cells and the T-cell receptor (TCR) β-chain variable regions. This crosslinking takes place outside the conventional antigen-binding grove, and may lead to the stimulation of up to 20-25% of the T-cell population in a non-specific way, compared with stimulation of only about 0.1% via the conventional allergen-specific way[13]. Once activated, T-cells may produce interleukins (IL) including IL-4, IL-5, IL-13, eotaxin and many others, which may lead to an eosinophilic inflammation and local IgE-production.

Specific IgE (sIgE) against SAEs is found more frequently in NP versus controls and correlates with higher levels of IL-5, eotaxin and eosinophil cationic protein (ECP)[2]. Moreover, an increased number of T cells expressing the TCR β-chain variable region, known to be induced by microbial superantigens, was detected in NP and correlated with the presence of sIgE against SAEs[14]. A recent study of our group demonstrated that NP was predominantly characterized by increased Th2- cytokines such as IL-5, eotaxin, IL-2sRalpha and IgE compared to controls[1].

The present study sought to elucidate the modulatory effects of the *S. aureus* surface proteins SpA, LTA and the *S. aureus* enterotoxin B (SEB) in nasal polyp tissue and to determine possible differences from normal nasal (control) tissue. The following cytokines were measured: pro-inflammatory cytokines (IL-1β, tumor necrosis factor (TNF)-α), T cell and subset related cytokines (interferon (IFN)-γ, IL-2, IL-4, IL-5, IL-8, IL-12p70, IL-13,) and immunoregulatory cytokines (IL-10, transforming growth factor (TGF)-β1). Furthermore, mediators such as histamine, cysteinyl leukotrienes and prostaglandin D_2 were analysed to determine the response of local mast cells.

METHODS

Patients

Nasal tissue was obtained from 25 patients at the Department of Otorhinolaryngology of the University Hospital of Ghent. The ethical committee of the Ghent University Hospital approved the study and all patients gave their written informed consent prior to inclusion in the study. None of the subjects received intranasal corticosteroids, anti-histamines or anti-leukotrienes, oral and intranasal decongestants or intranasal anticholinergics within 1 week prior to surgery and none of the subjects received oral and/or intramuscular corticosteroids within 4 weeks prior to surgery. For female subjects pregnancy or lactation was excluded.

Nasal polyp samples were collected during functional endoscopic sinus surgery from 12 patients (median age 43 years, ranging from 24 to 67 years old, 10 male and 2 female patients). Nasal polyposis was diagnosed based on symptoms, clinical examination, nasal endoscopy, and sinus computed tomography (CT) scan according to the EP^3OS guidelines[15].

Furthermore samples were collected from inferior turbinates (controls) from 13 patients undergoing septal surgery and/or turbinotomy because of nasal obstruction (median age 29 years, ranging from 22 to 62 years old, 8 male and 5 female patients).

The atopic status of all patients was evaluated by skin prick tests with a standard panel of 14 inhalant allergens. The reaction to a skin prick test was considered positive if the wheal area caused by the allergen was greater than 7 mm² (diameter >3 mm). Negative and positive controls (10 mg/ml histamine solution) were included with each skin prick test. Five inferior turbinates and five NP were obtained from patients with positive skin prick test for at least one of the most common aeroallergens.

Two NP patients reported mild asthma in history, and all patients were free of aspirin intolerance. Three control patients and one NP patient reported to smoke cigarettes.

The nasal tissue collected during surgery was immediately transported to the laboratory and divided into two parts. One part was immediately snap frozen in liquid nitrogen and stored at –80°C until analysis for immunohistochemistry and until homogenisation. The remaining tissue was used for the *ex-vivo* stimulations.

Mechanical disruption and stimulations of human nasal tissue

The human nasal mucosa and submucosa was cut thoroughly in tissue culture medium consisting of RPMI 1640 (Sigma-Aldrich, Bornem, Belgium), containing 2mM L-Glutamine (Invitrogen, Merelbeke, Belgium), antibiotics (50 IU/ml penicillin and 50µg/ml streptomycin) (Invitrogen) and 0.1% BSA (Bovine Serum Albumin, Sigma). The tissue was passed through a mesh to achieve comparable fragments. The tissue fragments (+/- 0.9 mm^3) were weighed and resuspended as 0.04g tissue/ 1 ml tissue culture medium.

As SpA interacts with immunoglobuline E V_H3^{+} [10], the tissue was preincubated for 1 hour at 37°C 5% CO2 with 1µg/ml human myeloma immunoglobuline E (Calbiochem, VWR International, Leuven, Belgium). After 3 washing steps the tissue fragments were resuspended in the appropriate amount of culture medium and then the fragments were divided into a 48 well plate (BD Falcon, VWR, Leuven) filled with 0.5 ml tissue fragment suspension in each well.

In a following step the tissue fragments (inferior turbinates n=13, NP n=12) were stimulated with culture medium (negative control) and 0.5 µg/ml SEB (Sigma-Aldrich) for 30 minutes and 24 hours. A subgroup of patients (inferior turbinates n=8, NP n=8) also was stimulated with 10µg/ml SpA (Sigma-Aldrich) and 10µg/ml LTA (Sigma-Aldrich), both for 30 minutes and 24 hours.

After that, tissue fragments and supernatants were separated by centrifugation. Aliquots of the supernatants were taken and stored immediately at -20°C until analysis of cytokines and histamine, $LTC_4/D_4/E_4$ and PGD_2.

Measurements of mediators in supernatants of stimulated tissue fragments

Concentrations of IL-1β, TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70 and IL-13 (2.4-10000 pg/ml) were measured on tissue supernatants obtained after the *ex-vivo* stimulations using Multi-spot assays (Meso Scale Discovery, Maryland) following the instructions of the manufacture. The plates were analysed using a Sector Imager 6000 (Meso Scale Discovery).

Concentrations of histamine (2.7-219 ng/ml), $LTC_4/D_4/E_4$ (0.0313-2 ng/ml), PGD_2 (2-250 pg/ml) and TGF-β1 (7.8-1000 pg/ml) were measured using ELISA kits: Histamine (IBL Hamburg, Germany), $LTC_4/D_4/E_4$ (Oxford Biomedical Research, Nuclilab BV, Ede, The Netherlands), PGD_2 (Cayman Chemicals, Ann Arbor, Michigan) and TGF-β1 (R&D Systems Europe Ltd, Abingdon, United Kingdom) following the instructions of the manufacturer.

Measurement of IgE to SAEs mix in tissue homogenates

Snap frozen tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added per every 0.1g tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun Melsungen, Germany) at 1,000 rpm for 5 minutes on ice as described previously. After homogenization, the suspension was centrifuged at 3,000 rpm for 10 minutes at 4°C and the supernatants were separated and stored at -80°C until analysis. All samples were assayed for IgE to SAEs (staphylococcus enterotoxin A, C and TSST-1) (0.35-100 kUA/l) by the UNICAP system (Pharmacia, Uppsala; Sweden).

Immunhistochemistry

Cryostat sections were prepared (6µm) and mounted on SuperFrost Plus glass slides (Menzel Glaeser, Braunschweig, Germany), packed in aluminium paper and stored at -30°C until staining. Sections were immunohistochemically stained with the following mouse monoclonal antibody: CD3 (clone UCHT1, Dako, Glostrup; Denmark) to compare the number of T cells in inferior turbinates and NP.

For immunohistochemical stainings specimens were fixed in acetone. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in TBS containing 0.1 % sodium azide for 20 minutes. Primary antibody or negative control, consisting of the corresponding isotype control, were incubated for 1 hour and detected using the LSAB+ technique conjugated with peroxidase according to the manufacture's instructions. (Labelled streptavidin-biotin, Dako). The peroxidase activity was detected using AEC Substrate chromogen (Dako), which results in a red stained precipitate. Finally sections were counterstained with hematoxylin and

mounted.

The number of positive cells was analysed using a magnification of 400x and scored by two independent observers who did not know the diagnosis and clinical data. A grading scale from 0 to 3 was applied, ranging from absent to numerous stained cells. Score 0 represents no positive cells, score 1 <10 positive cells/field, score 2:10-100 positive cells/field and score 3: >100 positive cells/field. The analysis included all areas of the biopsy and for each sample 10 fields were scored.

Statistical analysis

Statistical analysis was performed using the Wilcoxon test (for paired comparisons). The Mann-Withney U test was used for between-group (unpaired) comparisons. P values of less than .05 were considered as statistically significant. Results are expressed as median +/- IQR (interquartile range).

RESULTS

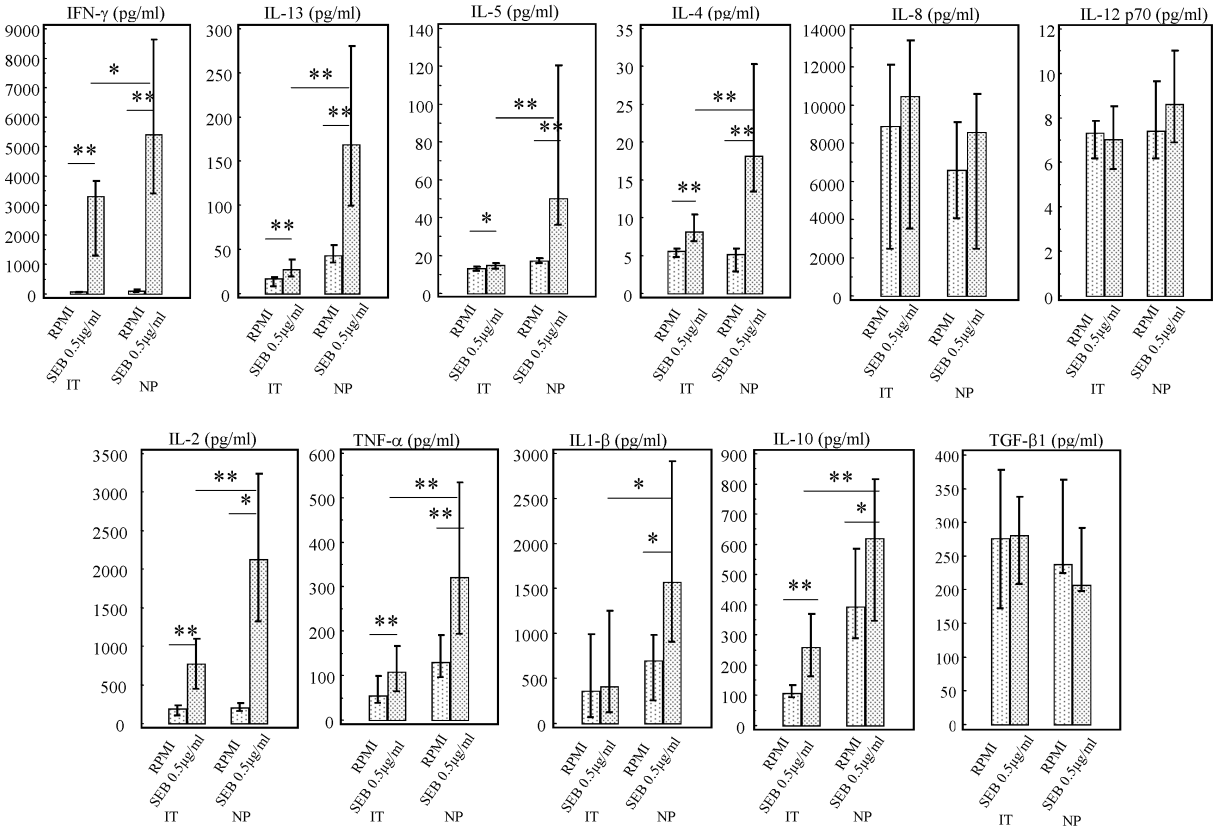

*Fig 1. Effect of 24 hours SEB (0.5 µg/ml) stimulation compared to RPMI on IFN-γ, IL-13, IL-5, IL-4, IL-2, TNF-α, IL-1β, IL-10, IL-8, IL-12p70 and TGF-β1 release. Comparison between nasal polyps (NP) (n=12) and inferior turbinates (IT) (n=13). * p<0.05, ** p<0.01.*

SEB stimulations

The inferior turbinates (n=13) and the nasal polyp explants (n=12) were stimulated for 30 minutes and for 24 hours with culture medium alone (RPMI) and SEB (0.5 µg/ml). SEB stimulation for 30 minutes did not increase the release of cytokines in comparison with culture medium alone in controls or NP (results not shown).

24 hours SEB stimulation demonstrated a significant increase of Th1 and Th2 cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-13) in inferior turbinates and NP compared to RPMI, but not for IL-8, IL-12p70 and TGF-β1. The release of these cytokines in NP was significantly higher compared to inferior turbinates for all measured cytokines, except for IL-8, IL-12p70 and TGF-β1. The pro-inflammatory cytokine IL-1β only demonstrated a significant increase in NP and TNF-α showed a release pattern similar to the above mentioned cytokines (Figure 1).

After 24 hours in RPMI, a significantly higher release was noticed for IL-13, IL-5, TNF-α and IL-10 in NP tissue (Table 1). No difference in release could be found between the allergic patients and the non-allergic patients.

Table 1. Concentrations of cytokines in supernatants after 24 hours in RPMI (spontaneous release).

	Inferior Turbinates	Nasal Polyps	*p-value*
IFN-γ (pg/ml)	57.0 (44.3-68.2)	84.3 (61.5-136.0)	=0.1
IL-13 (pg/ml)	16.5 (8.6-18.2)	42.4 (35.1-54.9)	<0.0001
IL-5 (pg/ml)	13.0 (12.0-14.1)	17.0 (16.1-18.7)	=0.0003
IL-4 (pg/ml)	5.5 (4.8-5.9)	5.1 (2.9-5.9)	=0.36
IL-2 (pg/ml)	186.2 (101.6-235.2)	202.5 (164.2-265.8)	=0.34
TNF-α (pg/ml)	54.2 (38.9-98.9)	129.0 (95.6-191.5)	=0.007
IL-1β (pg/ml)	355.7 (67.4-989.2)	692.0 (253.5-981.5)	=0.29
IL-10 (pg/ml)	104.7 (93.5-134.8)	390.9 (288.6-584.7)	=0.0001
IL-8 (pg/ml)	8860.2 (2458.5-12122.2)	6563.8 (4082.1-9105.4)	=0.27
IL-12p70 (pg/ml)	7.3 (6.2-7.9)	7.4 (6.2-9.7)	=0.41
TGF-β1 (pg/ml)	275.5 (172.6-377.9)	236.7 (224.0-363.2)	=0.67

Inferior turbinates (n=13) versus nasal polyps (n=12). Data are expressed as median +/- IQR (interquartile range).

The cryostat sections were stained for CD3 and semi-quantitatively scored. No difference in the number of T lymphocytes could be found between inferior turbinates and NP (results not shown).

A ratio was calculated between the concentrations of cytokines in culture medium and after SEB stimulation, both for NP and inferior turbinates, and compared to each other (Table 2).

Strikingly, the relative increase in cytokine release in NP was highest (above 2) for IL-5, IL-4 and IL-2, but lowest (0.58) for IL-10 and TGF-β1 (0.73).

SEB did not show an effect on mast cells derived cytokines; after 30 minutes (Fig. 2A) and 24 hours stimulation (Fig. 2B), no increase could be found for histamine, $LTC_4/D_4/E_4$ and PGD_2 in either group. None of the samples were positive for IgE to SAEs (results not shown).

Table 2. Ratio of cytokine concentrations after SEB (0.5 µg/ml) stimulation and RPMI for 24 hours.

	Inferior Turbinates	Nasal Polyps	Ratio (NP/IT)
IFN-γ	54.1	70	1.29
IL-13	3.6	4.5	1.25
IL-5	1.2	4.3	3.58
IL-4	2	5.8	2.9
IL-2	5.6	12.3	2.2
TNF-α	1.8	2.7	1.5
IL-1β	1.5	2.3	1.53
IL-10	2.6	1.5	0.58
IL-8	1.3	1.2	0.92
IL-12p70	1.2	2	1.67
TGF-β1	1.1	0.8	0.73

Inferior Turbinates (IT) (n=13) versus nasal polyps (NP) (n=12).

SpA and LTA stimulations

30 minutes SpA stimulation induced a significant increase of histamine, $LTC_4/D_4/E_4$ and PGD_2 compared to culture medium in inferior turbinates and NP (Fig. 2A). Howewer, no increase of Th1/Th2 cytokines or pro-inflammatory cytokines was measured after SpA and LTA short-time stimulation in comparison with culture medium alone. After 24 hours stimulation with SpA, the production of cysLTs remained significantly increased in inferior turbinates and NP compared to culture medium (Fig. 2B). Furthermore IL-5 was significantly increased in NP, and IL-13 demonstrated an increasing trend, not reaching significance though (Fig 3).

Stimulation with LTA for 30 minutes and 24 hours did not induce any increase in Th1/Th2 or pro-inflammatory cytokines (results not shown), nor in histamine, $LTC_4/D_4/E_4$ and PGD_2 (Fig. 2A and 2B).

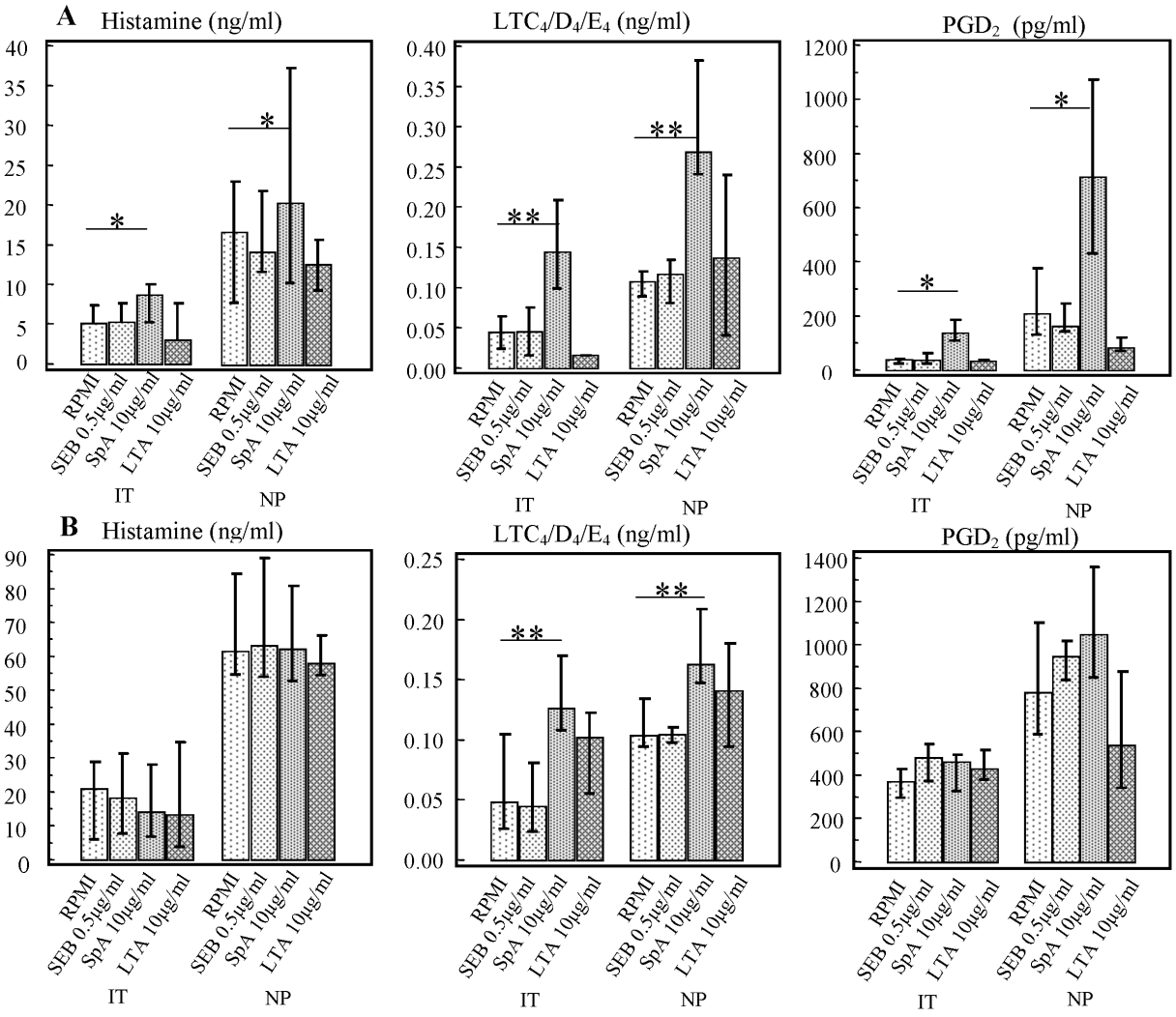

*Fig 2. (A) Effect of 30 minutes stimulation with RPMI (baseline), SEB (0.5 µg/ml), SpA (10 µg/ml) and LTA (10 µg/ml) on histamine, $LTC_4/D_4/E_4$ and PGD_2 release in nasal polyps (NP) (n=8) and inferior turbinates (IT) (n=8). (B) Effect of 24 hours stimulation with RPMI (baseline), SEB (0.5 µg/ml), SpA (10 µg/ml) and LTA (10 µg/ml) on histamine, $LTC_4/D_4/E_4$ and PGD_2 release in nasal polyps (NP) (n=8) and inferior turbinates (IT) (n=8). * $p<0.05$, ** $p<0.01$.*

DISCUSSION

We here shown that staphylococcal products have different effects on nasal mucosal samples: SpA after 30 minutes resulted in the early release of mast cell mediators including histamine, $LTC_4/D_4/E_4$ and PGD_2, whereas SEB after 24 hours induced a late-phase release of numerous immunoregulatory and pro-inflammatory cytokines, favouring Th2-cytokines and disfavouring IL-10 and TGF-β1 in nasal polyps.

There is increasing evidence that the colonization of *S. aureus* and the release of its cell products may be linked to the inflammation in NP[3, 7]. In NP, increased rates of *S. aureus* colonization were found[7] and sIgE against SAEs was more frequently present in NP versus

controls and correlated with higher levels of IL-5, eotaxin and eosinophil cationic protein (ECP)[2]. With the role of *S. aureus* as disease modifier being suggested, a number of diagnostic and therapeutic approaches such as antibiotic treatment or *S. aureus* vaccination may be considered. The potential effect of *S. aureus* eradication in sinus disease has not been studied yet, but large-scale double blind placebo controlled studies are currently ongoing. However, in atopic dermatitis, the role of *S. aureus* and the use of antibiotic treatment has already been established. Antimicrobial treatment leads to a significant, albeit temporary improvement of atopic dermatitis in patients who are colonized with *S. aureus*[16].

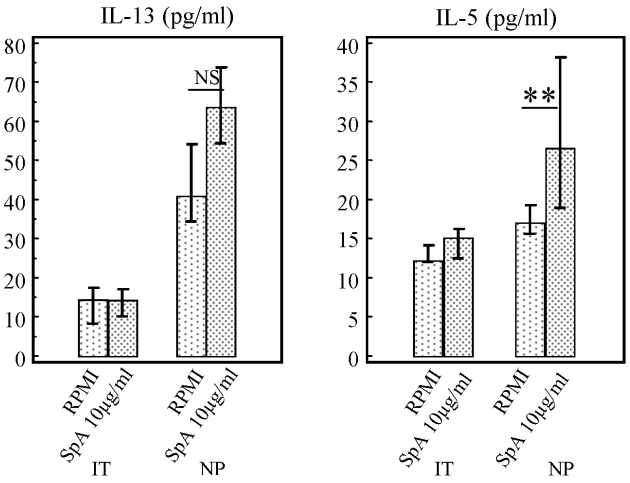

*Fig 3. Effect of 24 hours stimulation with SpA (10 µg/ml) compared to RPMI on IL-13 and IL-5 in inferior turbinates (IT) (n=8) and nasal polyps (NP) (n=8). ** p<0.01, NS= not significant.*

S. aureus secretes several enterotoxins with superantigen activity. Superantigens induce large-scale stimulation of T lymphocytes by a mechanism distinct from conventional antigen presentation, involving direct class II major histocomptability complex binding and stimulation of TCR families based on Vbeta gene usage[17, 18]. In this study, 24 hours stimulation with SEB induced a remarkable mean increase of IFN-γ (54 times and 70 times more for inferior turbinates and NP, respectively, compared to culture medium) and a substantial mean increase of IL-2 (5.6 times for inferior turbinates and 12.3 times for NP). However, this release is not reflected in tissue concentrations of patients with chronic nasal polyposis. A recent study of our group revealed significantly higher IL-5 protein concentrations (used as a Th2-marker) in NP homogenates versus controls, whereas IFN-γ (a Th1-marker) did not demonstrate any difference[1]. In line with these results, we have demonstrated, in the supernatants of tissue fragments cultured for 24 hours with medium alone, a significantly higher expression of IL-5 in NP compared to inferior turbinates (p=0.0003), but no difference in IFN-γ expression (p=0.1). Furthermore, when stimulated with SEB, the cytokine production was further skewed to IL-5, IL-4 and IL-2, but not to IFN-

γ, in NP. Concomitantly, the production of IL-10 and TGF-β1 decreased, indicating a possible lack in T-cell regulation induced by SEB.

In other inflammatory diseases similar effects of SEB were described. Peripheral blood mononuclear cells from patients with active atopic eczema/dermatitis syndrome or asthma and non-atopic controls secreted increased levels of IL-5, IL-4, IL-13 and IFN-γ in response to SEB. Only IL-5 and IL-13 were significantly higher in active atopic eczema/dermatitis syndrome or asthma compared to non-atopic controls[19, 20].

S. aureus enterotoxin B not only has effects on T lymphocytes, but may also affect directly the eosinophil activity by upregulating cell-surface expression of antigens and by inhibiting the eosinophil apoptosis[21]. Furthermore, SEB induced IL-12p40 production in peritoneal mice macrophages[22] and a culture of corneal epithelial cells has been shown to release IL-8 following treatment with SEB[23]. In this study, however, neither IL-12p70 nor IL-8 were upregulated after SEB stimulation in inferior turbinates and NP. However, similar ex vivo studies are required because results of animal studies can't necessarily be projected on humans.

By measuring mediators such as histamine, $LTC_4/D_4/E_4$ and PGD_2 the responses of specific cells, such as mast cells, were analyzed. No increase was measured for these mediators in inferior turbinates and NP after 30 minutes and 24 hours stimulation, which may demonstrate the lack of direct effect of SEB in releasing mast cell mediators. In line with our results, SEB was not shown to release histamine from a human mast-cell line (HMC-1) and led to a dose-dependent inhibition of IL-4 release[24]. Other studies however demonstrated opposite results. Peripheral blood basophils from patients with atopic eczema stimulated with SEB, secreted significantly higher amounts of histamine and leukotriene C_4 than peripheral blood basophils from healthy controls[25] and in rodent mast cells cultures, serotonin was released after SEB stimulation[26]. As none of the patients were positive for sIgE against SAEs, we could not demonstrate here the conventional allergen-mediated reaction in mast cells, basophils and Fcepsilon-receptor bearing cells after SEB stimulation. In a previous report it was demonstrated that isolated basophils released histamine in response to SEB only when patients had sIgE against SEB[27]. The role of IgE and its functionality needs to be further studied.

In contrast, stimulations with SpA, which is a surface protein on *S. aureus*, demonstrated an increase of histamine, $LTC_4/D_4/E_4$ and PGD_2. Marone and colleagues described an increased histamine release after SpA stimulation in basophils[9] and in human heart mast cells[10]. SpA

has a classical site that binds to Fcγ, a constant region of IgG[28], and an alternative site that binds the Fab portion of human polyclonal IgM, IgA, IgG and IgE[29]. Protein A's releasing activity is mediated by interaction with the commonly expressed V_H3 region of IgE, bound to the FcεRI[10]. The concept of the classical superantigens (SAEs and TSST-1) applied to the pathophysiology of allergic disorders, led to the definition of "superallergens" to indicate proteins of various origins able to activate FcεRI$^+$ cells by interacting with membrane-bound IgE[29]. Our results support the "allergenic" effect of SpA, as, in contrast to SEB, inferior turbinate and nasal polyp tissue, stimulated with SpA did give a significant increase of histamine, $LTC_4/D_4/E_4$ and PGD_2, already after 30 minutes. As colonization of *S. aureus* is present in 63.6% of subjects with NP, with rates as high as 66.7% and 87.5% in the subgroups with asthma and aspirin sensitivity compared to rates of 33.3% in controls[7], not only SEB, but also SpA may be relevant in the contribution of the ongoing inflammation in NP.

No increase of Th1/Th2 cytokines or pro-inflammatory cytokines was measured after SpA stimulation in comparison with culture medium alone, except for IL-5 in NP after 24 hours, again emphasising the limited influence of SpA on T cells.

Finally, stimulations with LTA were performed, as LTA is known to be important in the interaction of *S. aureus* with human nasal epithelium cells[8]. LTA stimulation on nasal tissue did not induce any increase of Th1/Th2 cytokines, pro-inflammatory cytokines or mast cell derived mediators, clearly reflecting the limited role of LTA in the inflammatory scene in NP. In future, similar experiments may be done to approach the role of other agents such as viruses, fungi and atypical bacteria in inducing inflammation in nasal mucosal tissue.

In conclusion, these results support the hypothesis that *S. aureus* may be linked to the inflammation in NP. Its enterotoxin SEB is able to induce the release of Th1/Th2-derived and pro-inflammatory cytokines in nasal tissue, with significantly higher release in NP compared to controls. The production is in favour of Th2 cytokines such as IL-5, IL-4 and IL-2 and disfavoured IL-10 and TGF-β1. SEB does not appear to have any allergenic effect in nasal tissue. In contrast, SpA, a surface protein of S. *aureus,* has clearly different properties and is able to increase histamine, $LTC_4/D_4/E_4$ and PGD_2 release in nasal tissue, which demonstrates its allergenic effect and moreover, may therefore, be an additional factor in causing or exacerbating the inflammation in NP.

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PART III: NEW THERAPEUTIC TARGETS

CHAPTER VII:
SYK-KINASE INHIBITION PREVENTS MAST CELL ACTIVATION IN NASAL POLYPS

Joke Patou[1], Gabriele Holtappels[1], Karen Affleck[2], Paul Van Cauwenberge[1], Claus Bachert[1]

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[1]Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University, Ghent, Belgium
[2]GSK, Stevenage, United Kingdom

ABSTRACT

Background: Mast cells are crucial effector cells in the allergic cascade. The cross-linking of the high affinity IgE receptor (FcεRI) activates mast cells and basophils. Spleen tyrosine kinase (Syk) is positioned upstream of the IgE receptor signal transducing pathway and may represent an important target for the treatment of nasal inflammatory diseases.

Objective: We therefore aimed to look at the effect of a specific Syk inhibitor in the release of mast cell mediators in human cord blood-derived mast cells (*in-vitro*) and in human nasal tissue (*ex-vivo*).

Methods: Surgical samples were collected from patients with nasal polyposis who underwent sinus surgery. Tissue cubes of +/- 0.9 mm^3 were primed with myeloma IgE (1µg/ml), preincubated with Syk inhibitor NVP-QAB205 in different concentrations and then stimulated with tissue culture medium, anti-IgE 10µg/ml and anti-IgE 30µg/ml. Supernatants were analysed for concentrations of histamine, $LTC_4/LTD_4/LTE_4$ and PGD_2. Cord blood-derived mast cells were likewise pre-incubated with compound, prior to stimulation with anti-IgE at 10µg/ml.

Results: In cord blood-derived mast cells (CBDMCs) the Syk inhibitor prevented the degranulation assessed by measurement of histamine release and the production of $LTC_4/LTD_4/LTE_4$ and PGD_2. Furthermore, the Syk inhibitor was similarly able to significantly inhibit the release of these granule and newly synthesized mediators by nasal polyp mast cells in a dose dependent manner.

Conclusion: Although the critical role of Syk in the IgE receptor signal transduction pathway has been well documented in vitro, this study supports the importance of Syk in IgE receptor-

mediated degranulation of mast cells ex-vivo within nasal tissue. Thus, inhibition of Syk may represent an important therapeutic strategy for the treatment of upper airway disease with mast cell involvement, such as allergic rhinitis.

INTRODUCTION

Mast cells are multifunctional effector cells of the immune system and play a crucial role in the allergic response. Activation of these cells induces the release of a wide collection of mediators which initiate the cascade of clinical symptoms associated with allergic rhinitis such as itching, sneezing, rhinorrhea and nasal obstruction[1]. First of all there is the release of preformed inflammatory mediators such as histamine and tryptase localized in specialized granules and secondly there is the *de novo* synthesis and secretion of cytokines, chemokines, and eicosanoids [1, 2]. During the acute allergic reaction there is mainly the release of preformed mediators such as histamine but also the release of newly formed leukotrienes (LTC4/D4/E4) and prostaglandin D2 (PGD2)[3].

The IgE/ allergen –mediated stimulation through the cross-linking of the high-affinity receptor for IgE (FcεRI) remains a very important step in the activation of the mast cell. The FcεRI expressed on mast cells and basophils is a tetrameric receptor comprised of the IgE-binding α chain, the signal-amplifying β chain[4] and the two signal transducing γ chains[5]. The transmission of the signals is dependent on the specific sequence of the immunoreceptor tyrosine-based activation motif (ITAM) present on the β and γ chains. After the phosphorylation of the ITAMs by the protein tyrosine kinase Lyn, there is the activation of spleen tyrosine kinase Syk through ITAM binding [6, 7]. Furthermore there is the activation of several adaptor proteins and transcriptional factors. The activation of FcεRI finally leads to degranulation, cytokine and eicosanoid production of mast cells.

Consequently, as Syk is positioned upstream in the cell signalling pathway, therapies targeting Syk should block three mast cell functions: The release of preformed mediators such as histamine, the production of lipid mediators such as leukotrienes and prostaglandins and the secretion of cytokines[8].

We have demonstrated before[9] that nasal polyp tissue can be used for testing the early phase of an allergic reaction. As large amounts of nasal polyp tissue are easier to access than inferior turbinate tissue, and as nasal polyps and inferior turbinate tissue react in the same concentration- dependent manner with the release of histamine, leukotrienes and PGD2 to

IgE- dependent triggers, we here use mast cell stimulation of nasal polyp tissue to study the effect of Syk inhibition of the allergic early phase reaction.

The essential role of Syk has been proven in Syk-deficient murine mast cells, which are defective in receptor-induced degranulation, cytokine synthesis and intracellular signalling-pathway activation[10]. Piceatannol, originally described as a Syk-selective inhibitor has been found to prevent mast cell degranulation in chopped lung fragments in guinea pigs[11] and in human lung mast cells[12]. Nowadays the more selective third-generation Syk inhibitors are used, resulting in more confident interpretation of results.

One study demonstrated inhibition of histamine release in human basophils and cultured CD-34+ mast cells, and showed inhibition of activation of human bronchial mast cells, assessed as less bronchial smooth muscle contraction in human isolated bronchial preparations. In this study a third-generation specific syk inhibitor (NVP-QAB205) was used[13].

Syk may represent an important target for the treatment of allergic rhinitis[14]. Since there is currently no data supporting this hypothesis, the aim of this study was to investigate the ability of NVP-QAB205 to inhibit the release of acute phase mediators in nasal polyp tissue after the stimulation of the high affinity IgE receptor.

MATERIALS AND METHODS

Patients

Nasal polyp samples of 8 patients were collected at the Department of Otorhinolaryngology, Ghent University Hospital. The ethical committee of the Ghent University Hospital approved the study and informed consent was obtained from all subjects prior to inclusion in the study. None of the subjects used any intranasal corticosteroids within 1 week prior to surgery, nor took any oral and/or intramuscular corticosteroids within 4 weeks prior to surgery, nor anti-histamines or anti-leukotrienes within 4 days prior to surgery, nor oral and intranasal decongestants or intranasal anticholinergics within 2 days prior to surgery. For the female subjects there was no current pregnancy or lactation.

Samples were collected during functional endoscopic sinus surgery from 8 patients (median age 38.5 years, ranging from 20 to 54 years old, 4 female and 4 male patients). Nasal polyposis was diagnosed based on symptoms, clinical examination, nasal endoscopy, and sinus computed tomography (CT) scan according to the EP^{3}OS guidelines[15].

The atopic status of all patients was evaluated by using skin prick tests with a standard panel of inhalant allergens. The reaction to a skin prick test was considered positive if the wheal area caused by the allergen was greater than 7 mm² (diameter >3 mm). Negative and positive controls (10 mg/ml histamine solution) were also included with each skin prick test. In the nasal polyp group there were 2 patients with an atopic status. None of the patients had asthma or aspirin intolerance in history.

The nasal tissue collected during surgery was immediately transported to the laboratory for the *ex-vivo* stimulations.

Syk Inhibitor NVP-QAB205

The Syk inhibitor used in these studies (NVP-QAB205) was a kind gift from GlaxoSmithKline (Stevenage, United Kingdom) and its characteristics have been published[16]. In isolated enzyme assays this compound has an IC50 of 0.01µM; hence at the concentrations tested here, it is a selective inhibitory tool.

Mechanical disruption and stimulations of human nasal tissue

Human nasal mucosa and submucosa was cut thoroughly in tissue culture medium consisting of RPMI 1640 (Sigma-Aldrich, Bornem, Belgium), containing 2mM L-Glutamine (Invitrogen, Merelbeke, Belgium), antibiotics (50 IU/ml penicillin and 50µg/ml streptomycin) (Invitrogen) and 0.1% BSA (Bovine Serum Albumin, Sigma). The tissue was passed through a mesh to achieve comparable fragments. The tissue fragments (+/- 0.9 mm^3) were weighed and resuspended as 0.04g tissue/ 1 ml tissue culture medium.

Tissue was preincubated for 1 hour at 37°C, 5% CO_2 with 1µg/ml human myeloma IgE (Calbiochem, VWR International, Leuven, Belgium) and washed, tissue fragments were resuspended in the appropriate amount of culture medium, and 0.5 ml of the fragment suspension was dispensed per well in a 48 well plate (BD Falcon). The fragment suspensions were stimulated with either culture medium, or the Syk inhibitor NVP-QAB205 at concentrations of 10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M for 1 hour. The DMSO concentration was kept constant at 0.1%. Following incubation with the inhibitor the fragments were stimulated with either tissue culture medium (negative control), or ε-chain specific anti-human IgE antibody (Dako Belgium N.V., Heverlee, Belgium) at 10 or 30 µg/ml for 30 minutes. All stimulations were done in duplicate.

Supernatants were separated by centrifugation and stored immediately at -20°C until analysis of histamine, $LTC_4/D_4/E_4$ and PGD_2.

Cord Blood-Derived Mast Cell Culture and Activation Assay

Cord blood-derived mast cells were differentiated from CD34+ progenitor cells (AllCells, Berkeley, California, USA), by culturing with stem cell factor (SCF), IL-6 and IL-10 essentially as described by Ochi et al[17].

The medium used was RPMI 1640 (Sigma) containing 10% heat inactivated CELLect Fetal Bovine Serum (MP Biomedicals), 1% (0.1mM) non-essential amino acids 2 mM L-Glutamine, 100U/ml Penicillin, 100µg/ml Streptamycin , 10µg/ml Gentamicin, 200nM 2-mercaptoethanol (All from InVitrogen), and 100ng/ml SCF, 50ng/ml IL-6, and 10ng/ml IL-10 (all from Peprotech) for 12 weeks.

For inhibitor assays (n=4), cells were plated at $5x10^4$ cells/well in V-bottom 96-well tissue culture plates (Corning), and primed for 6 days 37°C, 5% CO2 in complete medium containing 10ng/ml IL-4 (Peprotech) and 2 µg/ml IgE (1µg/ml kappa, 1µg/ml lambda; Biodesign, AMS Biotechnology). Cells were then washed in assay buffer (RPMI containing additives as above but with only 4% FCS) and resuspended in 90µl of assay buffer per well. The Syk inhibitor was added in 10 µl of medium to give final concentrations ranging between 10^{-9} M and $3x10^{-7}$ M, and incubated for 30 minutes at 37ºC, 5% CO2. Anti-IgE antibody (Sigma) was then added in 10µl to give a final concentration of 10µg/ml and the plate was incubated for a further 30 minutes. The plate was then centrifuged at 1500 rpm in a bench centrifuge for 5 minutes to pellet cells, and supernatants were removed to a new plate, and centrifuged again that the supernatants were cell-free.

Determination of mediator concentrations in supernatants

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure concentrations of histamine (IBL, Germany), LTC4/D4/E4 (Oxford Biomedical Research, Nuclilab BV, Ede, The Netherlands) and PGD2 (Cayman Chemicals, Ann Arbor, Michigan).

Statistical analysis

Statistical analysis was performed using the Wilcoxon test (for paired comparisons). P values of less than .05 were considered as statistically significant.

RESULTS

Effect of Syk Inhibitor on Mediator Release from CBDMCs

Pre-incubation for 30 minutes with inhibitor resulted in significant inhibition of the release of histamine (Fig 1A, IC50 27.5 nM), leukotrienes (Fig 1B, IC50 22.5 nM) and PGD2 (Fig 1C, IC50 26.6 nM) in response to IgE receptor cross-linking induced by anti-IgE antibody. Here, the inhibitory potencies of the Syk inhibitor on the different mediators were similar. These efficacies correlated well with the activity of the inhibitor in *in vitro* kinase assays.

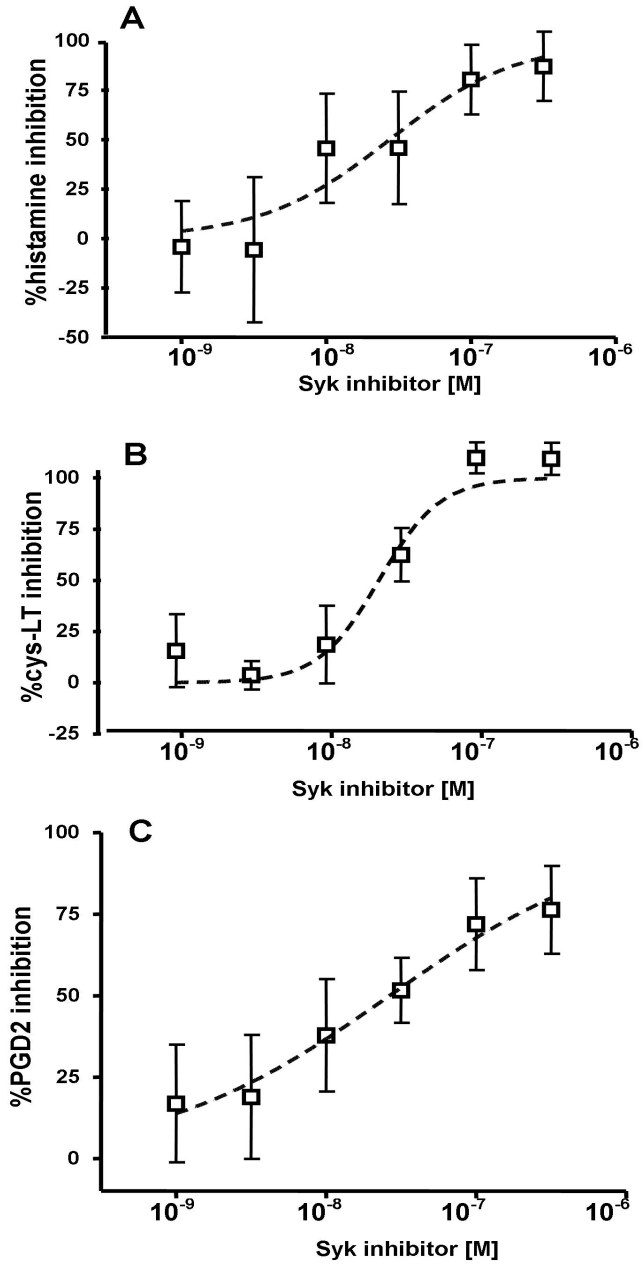

Fig 1. Percent inhibition of mediator release after anti-IgE (10µg/ml) stimulation and increasing NVP-QAB205 concentrations in cord blood derived mast cells. Inhibition of histamine release (A). Inhibition of $LTC_4/D_4/E_4$ release (B). Inhibition of PGD_2 release (C). Results are expressed as mean +/- SEM.

Effect of Syk Inhibitor on Mediator Release from Human Nasal Tissue

Stimulation of nasal polyp tissue (n=8) with anti-IgE (10µg/ml and 30µg/ml) resulted in a dose-dependent release of histamine, cysteïnyl leukotrienes and PGD_2 (Table 1).

Effect of syk inhibitor on histamine release

Increasing concentrations of Syk inhibitor (10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M) significantly inhibited the release of histamine induced by 10µg/ml anti-IgE ($p<0.02$, $p<0.01$, $p<0.02$ and $p<0.02$ respectively) (IC50 13,6 nM). Also the nasal polyp tissue stimulated with 30µg/ml anti-IgE and preincubated with the Syk inhibitor, showed a significant decrease of histamine release, except for 10^{-8} M syk inhibitor ($p<0.01$, $p<0.01$ and $p<0.01$ respectively) (IC50 13,6 nM) (Table 1 and Fig 2A).

Effect of syk inhibitor on $LTC_4/D_4/E_4$ release

In nasal polyps increasing concentrations of Syk inhibitor gave significant increasing inhibition of $LTC_4/D_4/E_4$ release compared to anti-IgE 10µg/ml stimulation alone ($p<0.05$, $p<0.01$, $p<0.01$ and $p<0.01$ respectively) (IC50 5,7 nM) and compared to anti-IgE 30µg/ml stimulation alone ($p<0.05$, $p<0.01$, $p<0.01$ and $p<0.01$ respectively) (IC50 3,9 nM) (Table 1 and Fig 2B).

Effect of syk inhibitor on PGD_2 release

Furthermore, in nasal polyps the release of PGD_2 was almost completely blocked after preincubation with increasing concentrations of Syk inhibitor ($p<0.01$, $p<0.01$, $p<0.01$ and $p<0.01$) in comparison with the release after stimulation with anti-IgE 10µg/ml alone (IC50 80 nM). Also the nasal polyp tissue stimulated with 30µg/ml anti-IgE showed a significant dose-dependent decrease of PGD_2 release ($p<0.01$, $p<0.01$, $p<0.01$ and $p<0.01$ respectively) (IC50 88 nM) (Table 1 and Fig 2C).

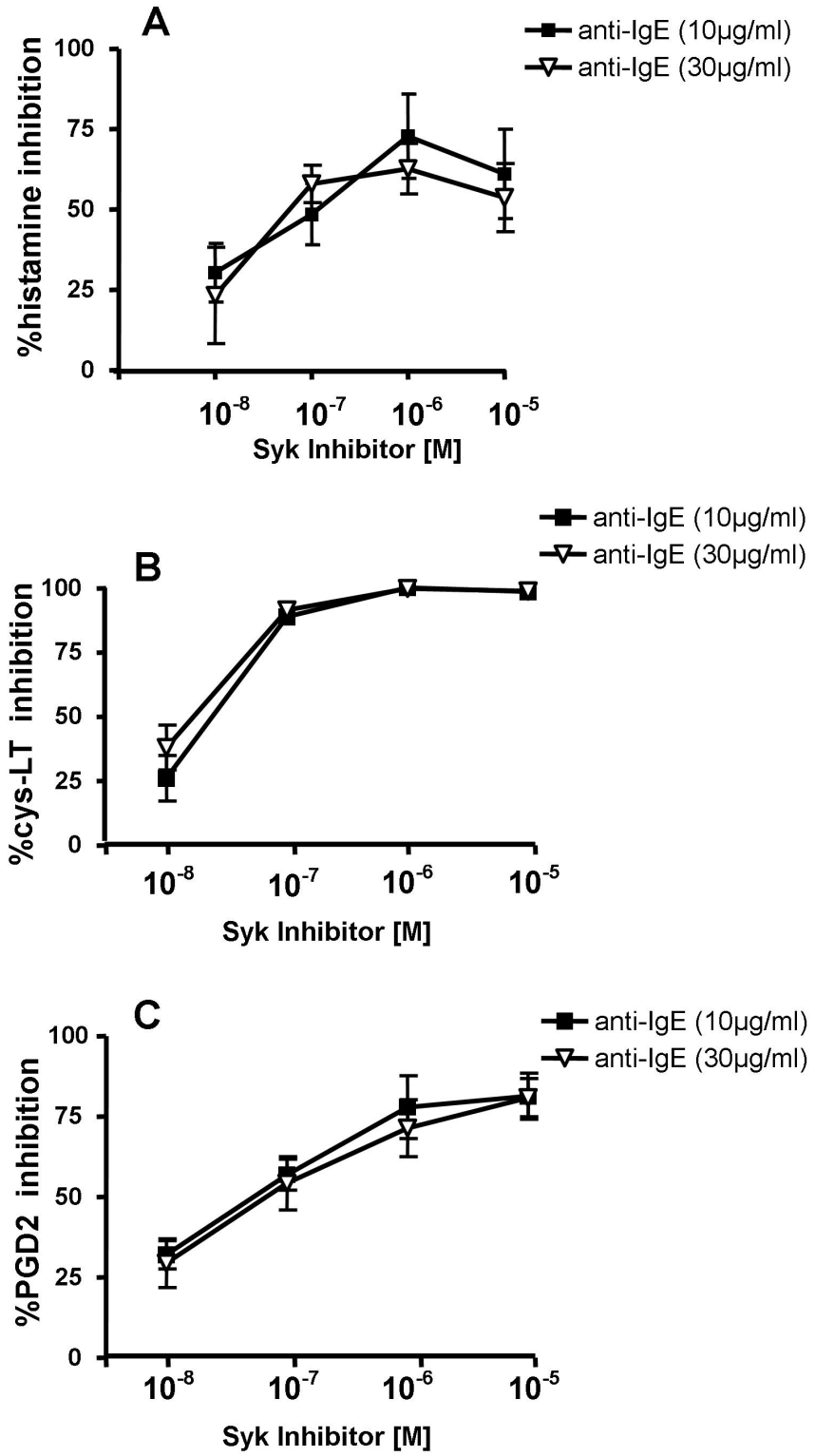

Fig 2. Percent inhibition of mediator release after anti-IgE 10µg/ml (■) and anti-IgE 30 µg/ml (▽) stimulation and increasing NVP-QAB205 concentrations in nasal polyp tissue. Inhibition of histamine release (A). Inhibition of $LTC_4/D_4/E_4$ release (B). Inhibition of PGD_2 release (C). Results are expressed as mean +/- SEM.

DISCUSSION

The involvement of mast cells in upper airway disease such as allergic rhinitis is well established, but the current treatment modalities such as anti-histamines, anti-leukotrienes, and intranasal corticosteroids, are aimed at preventing the activity of single mediators, whereas the mast cell, upon activation by allergen, is able to produce a large number of inflammatory mediators whose activities will be unaffected by such drugs. There is therefore scope to improve on current treatments.

Table 1. Overview of the effect of increasing concentrations of the Syk inhibitor on anti-IgE-induced release of histamine (ng/ml), $LTC_4/LTD_4/LTE_4$ (ng/ml) and PGD_2 (pg/ml) in the nasal polyp group (n=8). Data are expressed as mean +/- SD.

Stimulus	release	$+10^{-8}$ M syk inh	$+10^{-7}$ M syk inh	$+10^{-6}$ M syk inh	$+10^{-5}$ M syk inh
Histamine (ng/ml)					
Baseline	24.4 +/- 11.9	23.8 +/- 11.9	22.2 +/- 12.3	21.1 +/-11.6	22.8 +/- 11.6
Anti-IgE 10µg/ml	42.3 +/- 16.8	37.0 +/- 16.7	32.2 +/- 14	28.1 +/-14.3	29.7 +/- 13.4
*P-value**		*0.0156*	*0.0078*	*0.0156*	*0.0156*
Anti-IgE 30µg/ml	63.0 +/- 20.6	51.6 +/- 24	39.2 +/- 15.3	36.4 +/-12.0	39.5 +/- 16.4
*P-value**		*0.1484*	*0.0078*	*0.0078*	*0.0078*
$LTC_4/LTD_4/LTE_4$ (ng/ml)					
Baseline	0.08 +/- 0.04	0.07 +/- 0.03	0.07 +/- 0.04	0.06 +/- 0.03	0.07 +/- 0.04
Anti-IgE 10µg/ml	0.69 +/- 0.59	0.53 +/- 0.46	0.16 +/- 0.14	0.08 +/- 0.02	0.08 +/- 0.06
*P-value**		*0.0391*	*0.0078*	*0.0078*	*0.0078*
Anti-IgE 30µg/ml	0.92 +/- 0.55	0.66 +/- 0.63	0.17 +/- 0.16	0.07 +/- 0.04	0.08 +/- 0.05
*P-value**		*0.0234*	*0.0078*	*0.0078*	*0.0078*
PGD_2 (pg/ml)					
Baseline	168.0 +/-163.6	169.3 +/- 105.6	150.4 +/- 96.1	91.1 +/- 70.9	113.3 +/- 52.2
Anti-IgE 10µg/ml	2662.3 +/- 1793.3	1805.9 +/- 1170.7	1200.5 +/- 918.7	454.2 +/- 284.2	422.4 +/- 291.6
*P-value**		*0.0078*	*0.0078*	*0.0078*	*0.0078*
Anti-IgE 30µg/ml	5205.5 +/-3575.5	3631.4 +/- 2543.6	2118.5 +/- 1566.9	1022.4 +/- 192.3	775.6 +/- 306.7
*P-value**		*0.0078*	*0.0078*	*0.0078*	*0.0078*

** versus Anti-IgE stimulation (Statistical analysis; Wilcoxon-test)*

One such approach to improve upper airway disease therapies could be the discovery of potent and efficacious mast cell stabilisers, which would effectively block the production of

both early mediators (e.g. histamine, cysteinyl-leukotrienes, PGD2) and cytokines, which should relieve both immediate symptoms experienced on exposure to IgE-dependent triggers, but also halt the subsequent recruitment of inflammatory cells into the nasal mucosa. An understanding of the signalling pathways initiated upon cross-linking of the high affinity IgE receptor has helped identify potential future drug targets, and an example of one such protein is the tyrosine kinase Syk which appears to be critical for mast cell function.

In order to evaluate the mast cell stabilisation potential of novel molecules such as Syk inhibitor NVP-QAB205, inhibition of mediator release of mast cells was here studied in cord blood derived mast cells and in nasal polyps. Our group recently developed a human nasal mucosal stimulation model with anti-IgE stimulations that mimics the allergic acute phase reaction[9]. Stimulations with anti-IgE on IgE-primed nasal tissue fragments lead to a dose-dependent release of histamine, leukotrienes and PGD_2 both in inferior turbinates and nasal polyps. In view of the mediators and the time course after stimulation, those outcome measures are specific for a mast cell response. As inferior nasal turbinate tissue in allergic rhinitis, nasal polyp tissue is characterized by the presence of effector cells such as mast cells and eosinophils, both typical for allergic inflammation[18]. As nasal polyps and inferior turbinate tissue react in the same concentration-dependent manner to IgE-dependent triggers, and nasal polyp tissue is easily provided by surgical procedures performed to remove disease, nasal polyp tissue was used in this pharmacological profiling setting[9].

Syk is not only expressed in mast cells[19, 20] and basophils[21], but also in eosinophils[22], neutrophils[23], macrophages[24], dendritic cells[25] and B-cells[26].

Syk has an essential role in immunoreceptor signalling. Allergen- specific IgE binds to the surface of mast cells and basophils through high affinity Fc receptors for IgE. Following subsequent allergen exposure, activation of mast cells and basophils through crosslinking of the tetrameric FcεRI occurs and causes degranulation and *de novo* synthesis and release of mediators, all important in the allergic inflammation cascade. Receptor crosslinking results in the recruitment and the activation of cytoplasmatic kinase Lyn. Lyn causes tyrosine phosphorylation of ITAM in the cytoplasmatic domains of β- and γ-chains. Phosphorylated ITAM in the γ-chains serve as binding sites for Syk SH2 domains. As a result of its binding to ITAM, syk becomes activated[7, 10, 27]. Activated Syk regulates multiple intracellular signalling pathways. Some important downstream targets of Syk in mast cells include phospholipase Cγ (PLCγ), which may lead to mast cell degranulation, and the mitogen-activated protein kinase (MAPK) pathway which in turn may lead to leukotrienes and prostaglandine production[8].

There is a growing body of literature of the importance of Syk in the development of allergic inflammation in the lower airways. In animal asthma models, aerosolized Syk antisense oligonucleotides (ASO) inhibited many of the central components (f.e. the level of eosinophils, tumour necrosis factor) of allergic asthma[28], and a Syk-selective tyrosine kinase inhibitor prevented mast cell degranulation and airway hyperresponsiveness[11, 29]. In human lung mast cells piceatannol prevented histamine release[12]. Piceatannol was originally described as a syk-specific inhibitor but it inhibits other kinases as well[30] and applied at concentrations inhibiting IgE-induced mediator release from basophils, it doesn't act on syk[31]. Syk inhibitor NVP-QAB205 is considered selective for its respective kinase as it doesn't inhibit other signaling steps thought to be downstream of Syk kinase such as ras-ERK pathway elements. It is known to have an IC 50 of 40 nM in human basophils and inhibition of Syk activity has no influence on either loss from the cell surface or degradation of the IgE receptor. Furthermore, it inhibits histamine release in cultured mast cells and it inhibits bronchial smooth muscle contraction [13, 32].

Supplementary with the limited findings of Syk inhibition in the human bronchial biopsies, this study reports the potency of NVP-QAB205 to inhibit the release of acute phase mediators in human nasal tissue. The inhibitor was able to completely and potently inhibit mediator release from cord blood derived mast cells, thus enabling a comparison of its effect in nasal tissue fragments.

In nasal polyps the syk inhibitor significantly inhibited the release of histamine, $LTC_4/D_4/E_4$ and PGD_2. The IC50 values (13 nM for histamine, about 4.8 nM for leukotrienes and 80 nM for PGD_2) were comparable to IC50 value of histamine in human basophils. In CBDMCs, the potencies of the Syk inhibitor on the different mediators are similar (pLTs=histamine=PGD2) however in nasal polyps they are different (pLTs>histamine>PGD2); probably due to the more complex physiological and disease-relevant system in human tissue. These are all mast cell products that may be detected immediately after exposure to allergens, causing typical symptoms such as rhinorrhea, sneezing, itching and nasal obstruction[1]. Furthermore, through the release of various inflammatory mediators, mast cells may contribute to the induction of eosinophilic inflammation and to the formation and progression of nasal polyps[33]. In nasal fluids from patients with nasal polyps, histamine levels are significantly higher than those observed in patients without nasal polyps[34]. In nasal polyps, preincubation with the syk inhibitor gave a 100% inhibition of the cysLTs production after anti-IgE stimulation and there was an almost total (+/-80%) inhibition of PGD_2 production. There was also a significant

decrease of histamine release; however the highest inhibition here was around 60%. The inhibition of the release of these mediators seemed to be dose-dependent.
A clinical study, where another specific Syk inhibitor (R112) was applied, demonstrated the reduction of symptoms in seasonal allergic rhinitis patients [35]. Our results suggest that this could be due to the inhibition of histamine, cysLTs and PGD_2.

In summary, syk activation is an important upstream event in the pathways activated through FcεRI and may therefore control the synthesis and release of a whole range of mediators of both the early and late allergic responses. This study demonstrates that syk inhibition may prevent the degranulation of and the *de novo* synthesis in mast cells and in human nasal tissue. In future, specific syk inhibitors might provide a new therapeutic possibility in the treatment of upper airway disease with mast cell involvement such as allergic rhinitis.

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CHAPTER VIII :
IKK -2 INHIBITION IN NASAL POLYPS

Joke Patou MD[1], Gabriele Holtappels[1], Karen Affleck[2], Koen Van Crombruggen[1] PhD, Philippe Gevaert MD, PhD[1], Claus Bachert MD, PhD[1]

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[1] Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University, Ghent, Belgium
[2] GSK, Stevenage, United Kingdom

ABSTRACT

Background: Long term therapy with topical glucocorticosteroids is a hallmark therapeutic approach to control airway inflammation, but a complete suppression is seldom achieved. Targeting IKK-2 may circumvent this problem, and represents a growing area of interest, as within the last decade, small molecule inhibitors have become available. Nuclear Factor (NF) -kappaB is held inactive in the cytoplasm bound to I-kappaB. The removal of I-kappaB, via the actions of inhibitor of kappaB kinase-2 (IKK-2), allows NF-kappaB to enter the nucleus and regulate the expression of many inflammatory genes, including cytokines, chemokines, and adhesion molecules. Our aim was to determine the impact of IKK-2 inhibitors in comparison to the topical corticosteroid fluticasone propionate (FP) on the release of inflammatory cytokines, using nasal polyp tissue as a model.

Methods: Surgical samples were collected from patients with nasal polyposis (n=8). Tissue fragments were preincubated for 1 hour with a specific IKK-2 inhibitor and FP in different concentrations and then stimulated with RPMI (negative control) or Staphylococcus aureus enterotoxin B (SEB) for 6 hours. Supernatants were measured by Multiplex for pro-inflammatory cytokines (IL-1β, tumor necrosis factor-α) and T cell and subset related cytokines (interferon-γ, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13).

Results: Stimulation with SEB for 6 hours resulted in a significant increase of IL-2, IL-4, IL-5, IL-13, IFN-γ, IL-1β and TNF-α. The IKK-2 inhibitor and FP were able to almost completely block the release of IL-4, IL-5 and IL-13. For IL-2 and IFN-γ FP had less impact than the IKK-2 inhibitor. Furthermore, the IKK-2 inhibitor could completely block the release of TNF-α and IL1-β, whereas FP had no effect on their release.

Conclusion: To conclude, inhibition of IKK-2 results in a reduction of the release of pro-inflammatory, Th1- and Th2- associated cytokines in nasal polyposis. Our results suggest that the IKK-2 inhibitor has a broader spectrum of inhibitory effects than FP, also including inhibition of pro-inflammatory cytokines.

INTRODUCTION

Nasal polyposis (NP) is a chronic inflammatory disease of the paranasal sinuses, associated with an increase of tissue eosinophils, Th-2 cytokines and IgE, and it is often associated with non-allergic late-onset asthma[1]. In the general population the prevalence of NP ranges from 1 to 4% and the precise mechanism underlying the pathogenesis of NP is unknown and probably multifactorial. Treatment of NP with oral and topical corticosteroids and long-term antibiotics, each with different levels of evidence are described[2].

Topical corticosteroids sprays have a documented effect on the size of bilateral NP and also on symptoms associated with NP such as nasal blockage, secretion and sneezing but the effect on the sense of smell is not high[2]. Several studies underline the effect of fluticasone propionate (FP) to reduce nasal polyp size, reduce complaints of nasal obstruction and increase nasal airflow[3, 4]. Efficacy of treatment of NP with topical glucocorticosteroids is partly associated with their ability to reduce eosinophil viability and activation[5, 6], and to reduce the secretion of chemotactic cytokines[6, 7]. In allergic rhinitis patients, FP reduces the secretion of several cytokines and chemokines after nasal allergen challenge[8, 9].

In NP an induced inflammatory resistance to steroid treatment with higher expression of GRβ isoforms has been described[10, 11], however other data describe no correlation between expression of glucocorticoid receptor isoforms and nasal symptoms[12].

Despite all possible pharmacological treatments, NP remains a poorly controlled disease and when conservative treatment brings little or no lasting improvement, it often needs surgical intervention[13]. Effective treatments such as the development of more specific and potent anti-inflammatory agents that target the inflammation associated with NP are needed.

NF-κB is a major family of transcription factors activated during the inflammatory response. It is a key transcriptional regulator of multiple inflammatory mediators such as TNF-α, interleukins, vascular cell adhesion molecules, etc. In unstimulated cells, NF-κB dimers are inactive, since they are sequestered in the cytoplasm by interaction with inhibitory proteins termed IκBs (inhibitors of NF-κB)[14] . Phosphorylation and degradation of IκBs allows NF-κB

to translocate to the nucleus and to bind DNA to initiate gene expression[15]. The IκB kinase (IKK)-complex, composed of 3 subunits (IKK-1, IKK-2 and NEMO)[16], is responsible for this phosphorylation and degradation.

IKK-2 is an important kinase for the control of inflammatory genes and recently pharmaceutically developed synthetic small molecule inhibitors of IKK-2, have become available[17]. IKK-2 inhibitors might prove useful in inflammatory and autoimmune disorders; however to our knowledge there are no reports on IKK-2 inhibition in NP.

In inferior turbinates and NP, our group demonstrated a significant increase of several Th1, Th2 and pro-inflammatory cytokines after *Staphylococcus aureus* enterotoxin B (SEB) stimulation, with this increase significantly higher in NP compared to controls[18].

The current study aimed at evaluating the therapeutic potential of inhibiting the NF-κB pathway. We wanted to determine the impact of IKK-2 inhibitors in comparison to the topical corticosteroid FP on the release of inflammatory cytokines, using nasal polyp tissue as a model. Their inhibitory properties on T-cell related cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, and IL-13) and pro-inflammatory cytokines (IL-1β, TNF-α) were tested after stimulation with SEB.

MATERIALS AND METHODS

Patients

Nasal polyp samples (n=8) were obtained from patients at the Department of Otorhinolaryngology of the University Hospital of Ghent. The ethical committee of the Ghent University Hospital approved the study and all patients gave their written informed consent prior to inclusion in the study. None of the subjects received intranasal corticosteroids, anti-histamines or anti-leukotrienes, oral and intranasal decongestants or intranasal anticholinergics within 1 week prior to surgery and none of the subjects received oral and/or intramuscular corticosteroids within 4 weeks prior to surgery. For female subjects pregnancy or lactation was excluded.

The nasal polyp samples were collected during functional endoscopic sinus surgery from 8 patients (median age 44 years, ranging from 26 to 68 years old, 7 male and 1 female patient). Nasal polyposis was diagnosed based on symptoms, clinical examination, nasal endoscopy, and sinus computed tomography (CT) scan according to the EP^{3}OS guidelines[19].

The atopic status of all patients was evaluated by skin prick tests with a standard panel of 14 inhalant allergens. The reaction to a skin prick test was considered positive if the wheal area

caused by the allergen was greater than 7 mm² (diameter >3 mm). Negative and positive controls (10 mg/ml histamine solution) were included with each skin prick test. Two NP samples were obtained from patients with positive skin prick test for at least one of the most common aeroallergens. Two NP patients reported mild asthma in history, and one of them suffered of aspirin intolerance. One patient reported to smoke cigarettes.
The nasal tissue collected during surgery was immediately transported to the laboratory and used for the *ex-vivo* stimulations.

Mechanical disruption and stimulations of human nasal tissue

The human nasal polyps were cut thoroughly in tissue culture medium consisting of RPMI 1640 (Sigma-Aldrich, Bornem, Belgium), containing 2mM L-Glutamine (Invitrogen, Merelbeke, Belgium), antibiotics (50 IU/ml penicillin and 50µg/ml streptomycin) (Invitrogen) and 0.1% BSA (Bovine Serum Albumin, Sigma-Aldrich). The tissue was passed through a mesh to achieve comparable fragments. The tissue fragments (+/- 0.9 mm^3) were weighed and resuspended as 0.04g tissue/ 1 ml tissue culture medium. Then 0.5 ml of this fragment suspension was dispensed per well of a 48 well plate (BD Falcon, VWR, Leuven). The fragments were then treated with either culture medium alone, the IKK-2 inhibitor (GSK 657311A) at concentrations of 10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M (based on preliminary experiments) or FP (GSK, Stevenage, UK) at concentrations of 10^{-10} M, 10^{-9} M, 10^{-8} M (based on preliminary experiments) for 1 hour. The DMSO concentration was kept constant at 0.1%. Following incubation with inhibitor and FP, the fragments were stimulated with either culture medium (negative control), or 0.5 µg/ml SEB (Sigma-Aldrich) for 6 hours.
Supernatants were then removed and aliquots were stored immediately at -20°C until analysis of cytokines. The tissue was stored at -80°C.

Measurements of mediators in supernatants of stimulated tissue fragments

Concentrations of IL-1β, TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70 and IL-13 (2.4-10000 pg/ml) were measured on tissue supernatants obtained after the *ex-vivo* stimulations using Multi-spot assays (Meso Scale Discovery, Maryland, Gaithersburg) following the instructions of the manufacture. The plates were analysed using a Sector Imager 6000 (Meso Scale Discovery).

Statistical analysis

Statistical analysis was performed using the Wilcoxon test (for paired comparisons). P values of less than .05 were considered as statistically significant. The results of percentage inhibition were calculated based on the mean values corrected for baseline.

RESULTS

Stimulation with 0.5µg/ml SEB for 6 hours resulted in significant increase of IL-2 ($p<0.01$), IL-4 ($p<0.01$), IL-5 ($p<0.05$), IL-13 ($p<0.01$), IFN-γ ($p<0.01$), IL-1β ($p<0.01$) and TNF-α ($p<0.05$); there was no increase of IL-8, IL-10 and IL-12p70, confirming earlier results[18].

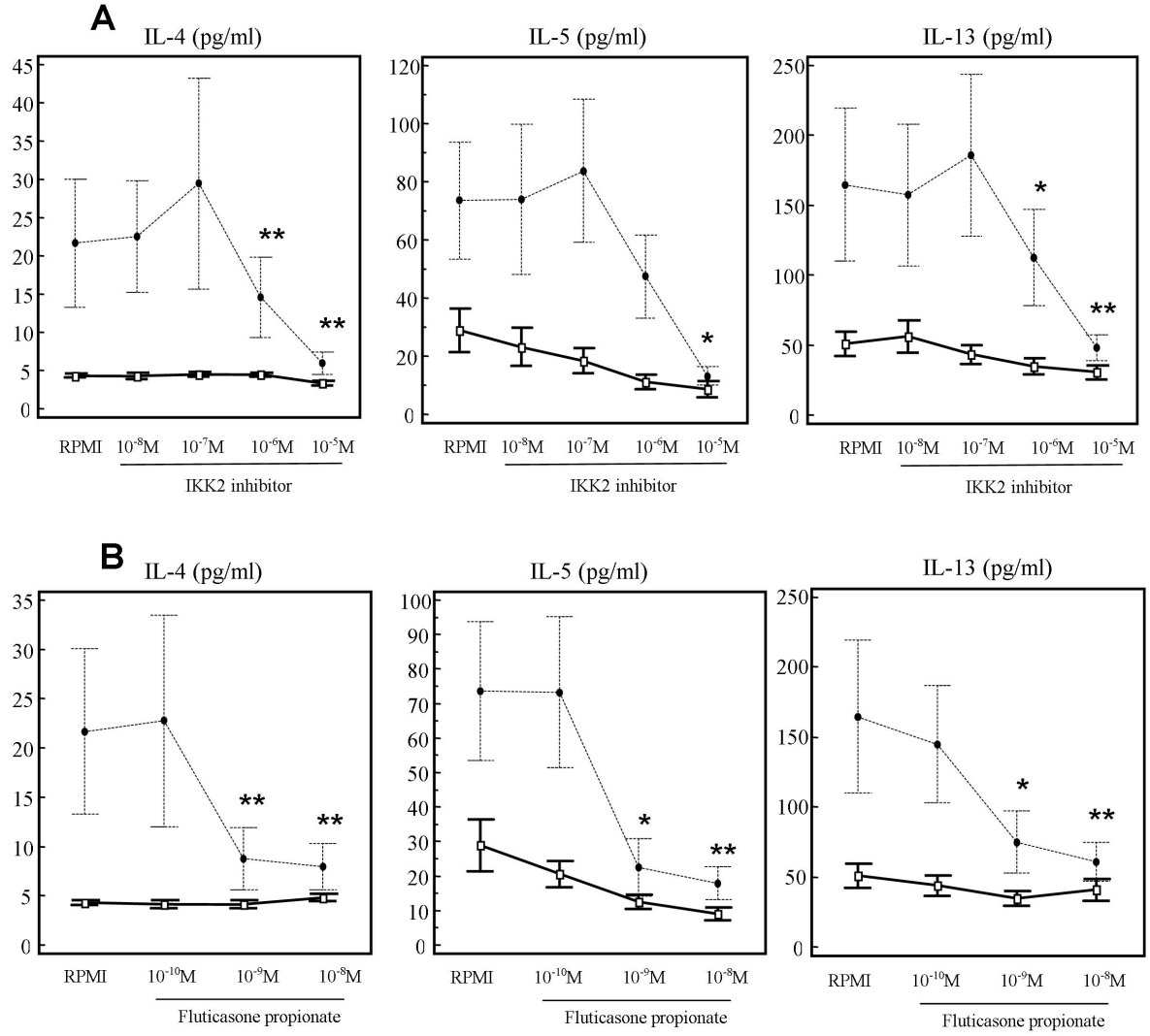

*Fig 1. Inhibition of the release of Th2 cytokines IL-4, IL-5 and IL-13 (pg/ml) after SEB stimulation (0.5 µg/ml) versus control samples. Inhibition with increasing concentrations of the IKK-2 inhibitor (A). Inhibition with increasing concentrations fluticasone propionate (B). * $p<0.05$, ** $p<0.01$. Results are expressed as mean +/- SEM (standard error of the mean).*

Effect on Th2 cytokines

After stimulation with 0.5µg/ml SEB, the IKK-2 inhibitor was able to almost completely block the release of IL-4, IL-5 and IL-13. A decrease of respectively 42% and 85% was observed for IL-4 following incubation with the IKK-2 inhibitor at 10^{-6} M and 10^{-5} M. For IL-5 and IL-13 release, a blockage of respectively 90% and 85% was reached with the highest concentration (10^{-5} M) of the IKK-2 inhibitor (Fig 1A).

Also for FP a significant inhibition of Th2 cytokine release was found, at a concentration of 10^{-9}M. FP 10^{-8} M demonstrated a decrease of IL-4, IL-5 and IL-13 of 82%, 80% and 82% respectively (Fig 1B) (IC50: table 1).

Effect on Th1 cytokines

Not only was the production of Th2 cytokines inhibited, but also that of Th1 cytokines. For IFN-γ, a higher inhibition was achieved with the IKK-2 inhibitor, reaching 80% inhibition at 10^{-5} M (Fig 2A) versus only 47% inhibition with the highest concentration of FP (Fig 2B). For IL-2, an inhibition of 85% was achieved with the IKK-2 inhibitor at 10^{-5} M (Fig 2A) versus 34% with the highest concentration of FP (Fig 2B) (IC50: table 1).

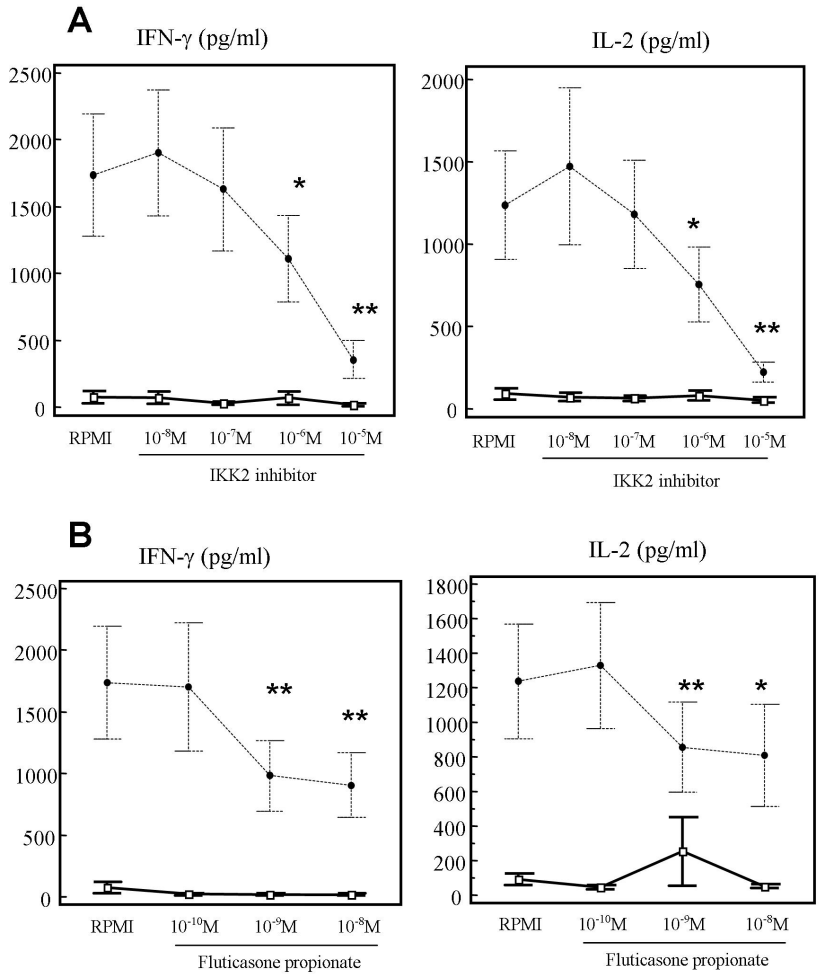

*Fig 2. Inhibition of the release of Th1 cytokines IFN-γ and IL-2 (pg/ml) after SEB stimulation (0.5 µg/ml) versus control samples.. Inhibition with increasing concentrations IKK-2 inhibitor (A). Inhibition with increasing concentrations fluticasone propionate (B). * p<0.05, ** p<0.01. Results are expressed as mean +/- SEM (standard error of the mean).*

Effect on pro-inflammatory cytokines

With regard to the pro-inflammatory cytokines IL1-β and TNF-α, almost a complete inhibition of release was achieved with the IKK-2 inhibitor; 98% and 88%, respectively at 10^{-5} M (Fig 3A). In contrast, no significant inhibition for IL1-β and only a marginal, but significant inhibition of 25% was achieved by FP for TNF-α (Fig 3B) (IC50: table 1).
Furthermore, the spontaneous release of the pro-inflammatory cytokines, but also of IL-5, was decreased (baseline), especially after IKK-2 inhibition.

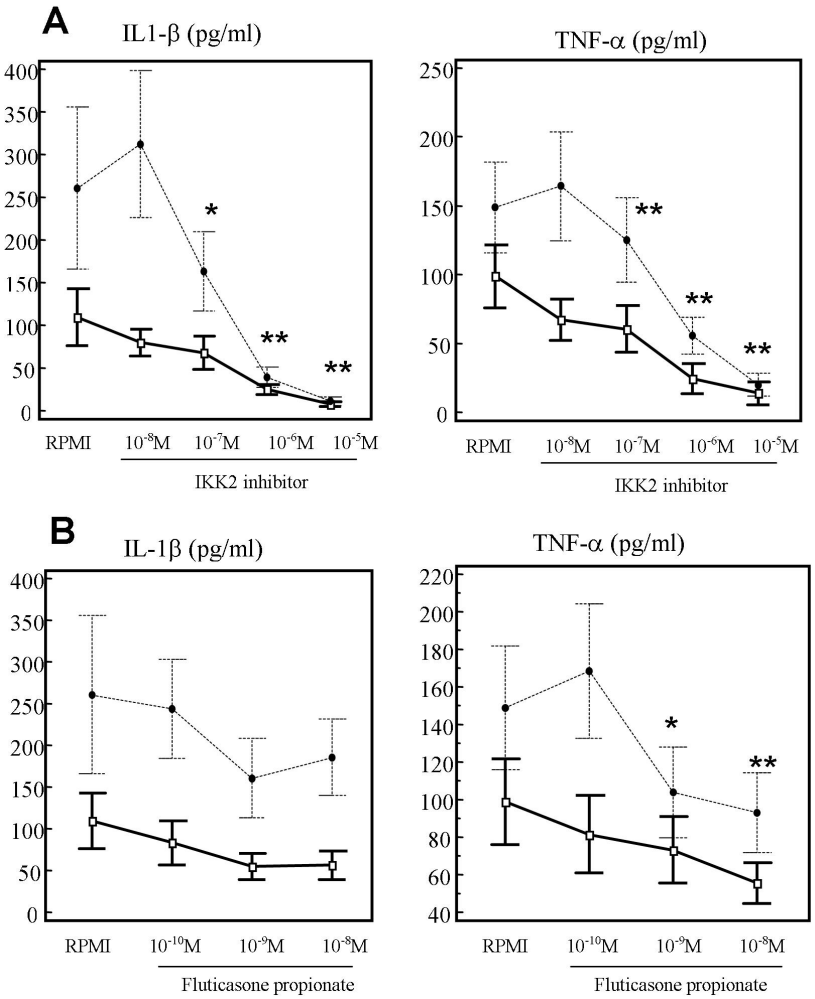

*Fig 3. Inhibition of the release of pro-inflammatory cytokines IL-1β and TNF-α (pg/ml) after SEB stimulation (0.5 µg/ml) versus control samples. Inhibition with increasing concentrations IKK-2 inhibitor (A). Inhibition with increasing concentrations fluticasone propionate (B). * p<0.05, ** p<0.01. Results are expressed as mean +/- SEM (standard error of the mean).*

DISCUSSION

Nasal polyposis is a chronic inflammatory disease of the sinus mucosa and the middle turbinate often associated with non-allergic late onset asthma. Earlier studies demonstrated higher Th2 cytokines present in polyp tissue[1], and stimulation with S. aureus derived

superantigen SEB further increases the Th2 bias and additionally increases Th1 cytokines and pro-inflammatory cytokines[18].

Long term therapy with potent intranasal glucocorticosteroids is frequently performed to control nasal polyp inflammation and growth, but the response to this treatment is often only partially successful and short courses of oral glucocorticosteroids or long-term antibiotics are periodically needed, mainly to quickly improve nasal obstruction. Despite this therapy, nasal polyps may progress and may require surgery.

Table 1. IC50. Results are expressed as mean +/- SEM (standard error of the mean).

	IC50 IKK-2 inhibitor	IC50 FP
Th2 cytokines		
IL-4	$5,4 .10^{-6}$ M (+/- $7,0.10^{-7}$ M)	$7,0.10^{-10}$ M (+/- $1,4. 10^{-10}$ M)
IL-5	$3,0 .10^{-6}$ M (+/- $1,2.10^{-6}$ M)	$4,8 .10^{-10}$ M (+/- $9,3.10^{-11}$ M)
IL-13	$5,9 .10^{-6}$ M (+/- $1,6.10^{-6}$ M)	$1,5 .10^{-9}$ M (+/- $6,6.10^{-10}$ M)
Th1 cytokines		
IFN-γ	$2,6 .10^{-6}$ M (+/- $1,0.10^{-6}$ M)	$3,2 .10^{-9}$ M (+/- $1,6.10^{-9}$ M)
IL-2	$3,8 .10^{-6}$ M (+/- $1,1.10^{-6}$ M)	not possible to calculate
Pro-inflammatory cytokines		
IL-1β	$5,4 .10^{-7}$ M (+/- $9,3.10^{-8}$ M)	not possible to calculate
TNF-α	$1,5 .10^{-6}$ M (+/- $8,2.10^{-7}$ M)	not possible to calculate

For several years, a large number of kinases from different signal transduction pathways have been the target of interest for the treatment of inflammation and autoimmune disorders. One class of such kinases has been the IKK-2 (IκB-kinase 2). The transcription factor Nuclear Factor kappa-B (NF-κB) is known to be a master regulator of the expression of many inflammatory genes, including cytokines, chemokines, and adhesion molecules and upon activation of the cell following the stimulation of receptors such as the TNF receptor, Toll-like receptors or the T-cell receptor (canonical pathway), a signal transduction cascade unravels. NF-κB is held inactive in the cytoplasm, bound to I-κB. The removal of I-κB, via the actions of IKK-2, allows NF-κB to enter the nucleus.

In this regard, stimulation with SEB was used to demonstrate the possible effect of fluticasone propionate and an IKK-2 inhibitor in decreasing the production of Th1/Th2 or pro-inflammatory cytokines[14-16].

Here, stimulation with SEB indeed led to a significant production of IL-2 , IL-4, IL-5, IL-13, IFN-γ, IL-1β and TNF-α, confirming earlier results[18] and inhibition of this production was

realized with an IKK-2 inhibitor. First of all, this is pointing out the importance of the NF-κB pathway in the production of inflammatory cytokines in the superantigen-driven (SEB) stimulation. Moreover, this stimulation might concern specifically the canonical pathway as IKK-2 seems to play an important role and IKK-2 and NEMO are not required for NIK (NF-κB binding kinase) - induced p100 processing (alternative pathway)[20].
Superantigens are able to crosslink the class II major histocompatibility complex of antigen-presenting cells and the T cell receptor β-chain variable regions[21]. SEB stimulate T-cells both in humans and mice through the T cell receptor. Hence, here there is most likely a T-cell intrinsic NF-κB activation after stimulation with SEB through the T cell receptor.

Because of its critical role in canonical NF-κB activation, IKK-2 has been the focus of considerable attention as an anti-inflammatory target. Inhibitors are expected to be efficacious in rheumatoid arthritis by affecting multiple mechanisms and cell types. In the murine collagen induced arthritis model they could inhibit disease severity and NF-κB related cytokines (IL-1β, IL-6, TNF-α and IFN-γ)[22-24]. Furthermore, in inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, characterized by chronic relapsing inflammation, IKK-2 inhibitors ameliorated inflammatory responses in mice[25]. Additionally, IKK-2 inhibition could be beneficial in patients with type 2 diabetes because the activation of NF-κB through IKK-2 induces inflammatory mediators that cause insuline resistance[26]. During insulin resistance tests in mice, IKK-2 inhibition dosed intraperitoneally significantly decreased plasma glucose levels[27]. Furthermore NF-κB plays an essential role in the production of chemokines, cytokines and cell adhesion molecules in allergic asthma[28]. In mice, IKK-2 inhibition suppressed allergen-induced airway inflammation and hyperreactivity, with inhibition of airway eosinophilia, IL- 4, IL-5 and IL-12[29]. Similar results were reported in an antigen-driven model of airway inflammation in rats and in human airway smooth muscle[30, 31]; IKK-2 inhibition decreased inflammatory cell recruitment, TNF-α, IL-1β, IL-4, IL-5, IL-13 and eotaxin.
To our knowledge, there are no reports on IKK-2 inhibition in nasal polyposis. However, the NF-κB pathway may play an important role in this inflammatory disease. There is a higher gene and protein expression of NF-κB in nasal polyps than in control mucosa, whereas transcription factor AP-1 does not seem to play a significant role in this pathological process[32] and the low expression of COX-2 mRNA in nasal polyps from aspirin-sensitive patients is associated with a down-regulation of NF-κB activity[33].

In this study, with the use of human nasal polyp tissue, we achieved similar results with the IKK-2 inhibitor as mentioned in the studies above, for the inhibition of IL-4, IL-5 and IL-13 and here, the inhibitory effect of the IKK-2 inhibitor was comparable to the inhibitory effect of fluticasone propionate for those Th-2 cytokines.

In regard of the inhibition of Th-1 cytokines, the IKK-2 inhibitor was able to block the production of IFN-γ and IL-2 almost completely, whereas fluticasone propionate only reached inhibition of around 40%. Similarly, in nasal lavage of allergic rhinitis patients after allergen challenge, fluticasone propionate caused a decrease of IL-4, IL-5 and IL-13 and levels of IL-2 and IFN-γ were not affected[9].

Furthermore, it could completely block the production of pro-inflammatory cytokines such as IL-1β and TNF-α (98% and 88% respectively), whereas fluticasone propionate was practically unable to block these. Other studies demonstrated more or less similar results, for instance G-CSF (granulocyte-colony stimulating factor) release from primary airway smooth muscle is virtually steroid resistant, whereas it is completely blocked by IKK-2 inhibitors[30] and levels of TNF-α are not affected by fluticasone propionate in allergic rhinitis patients after allergen challenge[9]. Interestingly, the spontaneous release of IL1-β and TNFα was decreased (baseline), especially after IKK-2 inhibition. As both IL1-β and TNF-α may activate NF-κB this can be explained easily.

There are concerns that inhibition of NF-κB may cause side effects such as increased susceptibility to infection, which has been observed in gene disruption studies when components of NF-κB are inhibited[34, 35]. Furthermore, it is suggested that IKK-2 may serve additional functions beyond regulating the NF-κB pathway: the possibility has been raised that the IKK family is involved in control of normal mitosis [36, 37]In addition, IKK-2 knockout mice do not survive due to liver apoptosis. The risks of chronic administration of IKK-2 inhibitors in humans remain to be investigated. More information will become available as structurally distinct IKK-2 inhibitors progress through pre-clinical safety studies.

To conclude, targeting IKK-2 represents a growing area of interest for researchers as small molecule inhibitors have recently become available. However, teratogenicity and susceptibility to infection could be problematic and therefore more research is necessary.

Our results suggest that the IKK-2 inhibitor seemed, in certain respects, to possess a more comprehensive anti-inflammatory profile to that of fluticasone propionate in nasal polyposis. As in nasal polyposis, a subpopulation of patients was reported resistant to glucocorticoid

treatment[11] and as short courses of oral glucocorticosteroids with possible side-effects are often necessary, IKK-2 inhibition could give a more general treatment.

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PART IV: GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

Discovery of novel drugs targeting kinases, an important class of intracellular enzymes that play a critical role in signal transduction pathways controlling a variety of cellular functions, has become the focus of a large number of drug discovery programs in the pharmaceutical and biotech industry. The approved drugs today have been useful for the treatment of a variety of cancers in human; kinase inhibitor discovery programs are now mainly focusing on drugs for the treatment of inflammation and autoimmune disorders, like rheumatoid arthritis, asthma, etc. Less is known about efficacy in nasal inflammatory pathologies. Kinase inhibitors have been tested in a lot of *in vitro* models, mainly animal models. In this work we demonstrate models to test this kind of drugs in *ex vivo* human nasal tissue, which allows testing drugs in human disease relevant tissue in a kind of pre-safety and pre-clinical setting. We also test two kinase inhibitors in these models, namely a Syk inhibitor and an IKK-2 inhibitor.

IgE-dependent early phase model

In chapter V, we established a whole tissue nasal mucosal stimulation model which can be used to mimic the early phase of an allergic reaction both in nasal polyps and inferior turbinates. By using whole tissue preparations, the cells remained in their natural environment, and unchanged surface receptor expression was maintained by not using enzymatic digestion, thus closely mimicking the *in vivo* situation. These, in contrast to using exploration systems of rodent origin, use mast cell lines, or stimulate little amounts of human mast cells after positive isolation and the use of enzymatic digestion. Stimulations were done for 30 minutes with anti-IgE 10 µg/ml and anti-IgE 30 µg/ml in IgE primed nasal tissue fragments in order to study the acute response of the mast cell. Those stimulations resulted in a significantly higher production and release of mediators such as histamine, $LTC_4/D_4/E_4$ and PGD_2, all important in the early phase of an allergic reaction. These mediators were released in a concentration-dependent manner. After correction for spontaneous release, the induced release of histamine, $LTC_4/D_4/E_4$ and PGD_2 was significantly higher in the nasal polyp group compared to the inferior turbinate group**,** although tryptase, FcεRIα positive cells and FcεRIα-chain transcripts were equally present in both groups. No correlation was found

between baseline concentrations of IgE, and the release of histamine, $LTC_4/LTD_4/LTE_4$ and PGD_2 after stimulation. The nasal polyp tissue reacted in the same concentration-dependent manner as the inferior turbinate tissue.

This IgE-dependent early phase model allows us to compare results obtained from nasal polyp tissue to inferior turbinate tissue. This is important in future settings as high amounts of nasal polyp tissue are easier to access.

SEB-dependent late-phase model

In chapter VI, we established a nasal polyp tissue stimulation model with SEB for late-phase release of numerous immunoregulatory and proinflammatory cytokines. SEB stimulation for 30 minutes did not increase the release of cytokines, whereas 24 hours stimulation (late-phase) resulted in a significant increase of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, TNF-α and IL-1β in nasal polyps and inferior turbinates; and the release of these cytokines in nasal polyps was significantly higher compared to inferior turbinates, whereas no difference in the number of T lymphocytes could be found between inferior turbinates and nasal polyps. SEB did not show an effect on mast cell derived cytokines; after 30 minutes and 24 hours stimulation, no increase could be found of histamine, $LTC_4/D_4/E_4$ and PGD_2 in either group, which may prove the lack of direct effect of SEB in releasing mast cell mediators. However, as none of the samples were positive for IgE to SAEs, we could not explore the conventional allergen-mediated reaction in mast cells. Recently, we used this ex-vivo tissue model to test and investigate the anti-inflammatory effect on cytokines of new drugs.

Furthermore, there is an increasing evidence that the colonization of *S. aureus* and the release of its cell products may be linked to the inflammation in nasal polyps[1]. In our study we further elucidated the disease-modifying role of *S. aureus* derived proteins SEB, SpA and LTA in nasal polyps. In the supernatants of tissue fragments cultured for 24 hours with medium alone, a significantly higher spontaneous release of IL-5 in nasal polyps compared to inferior turbinates was demonstrated, but no difference in IFN-γ release. In line with these results, significantly higher IL-5 protein concentrations versus controls, and no difference in IFN-γ concentrations in nasal polyp homogenates were found[2]. The relative increase in cytokine release after SEB stimulation in nasal polyps was the highest for IL-5, IL-4 and IL-2, but not for IFN-γ, demonstrating skewing towards a Th-2 polarisation pattern. The relative increase in cytokine release was the lowest for IL-10 and TGF-β1, indicating a possible lack in T-cell regulation induced by SEB. A more recent study of our group could confirm these results. A decreased expression of FOXP3 (transcription factor for T regulatory cells) was found in

nasal polyps together with low levels of TGF-β1 protein expression, reflecting a deficiency or dysfunction of Treg cells[3].

In contrast to SEB, our results demonstrate an "allergenic" effect of SpA, a surface protein on *S. aureus*. Its activity is mediated by interaction with the commonly expressed V_H3 region of IgE, bound to the FcεRI[4]. After 30 minutes stimulation with SpA, inferior turbinate and nasal polyp tissue did give a significant increase of histamine, $LTC_4/D_4/E_4$ and PGD_2 and no increase of cytokines. After 24 hours, the production of cysLTs remained significantly increased and also IL-5 was significantly increased.

LTA stimulation on nasal tissue did not induce any increase of Th1/Th2 cytokines, pro-inflammatory cytokines or mast cell derived mediators, clearly reflecting the limited role of LTA in the inflammatory scene in nasal polyps.

These data support the hypothesis that *S. aureus* may be linked to the inflammation in nasal polyposis. Not only SEB, but also SpA may prove factors in causing or exacerbating the inflammation in nasal polyposis.

Syk-kinase inhibition

The cross-linking of the high affinity IgE receptor activates mast cells and basophils. As spleen tyrosine kinase is positioned upstream of the IgE receptor signal transducing pathway, therapies targeting Syk should block three mast cell functions: The release of preformed mediators such as histamine, the production of lipid mediators such as leukotrienes and prostaglandins and the secretion of cytokines[5]. Therefore, the inhibition of Syk may represent an important target for the treatment of nasal inflammatory diseases.

In chapter VII we looked at the effect of a specific Syk inhibitor to inhibit the release of early phase mast cell mediators in human cord blood-derived mast cells (*in-vitro*) and in human nasal polyp tissue (*ex-vivo*). The IgE-dependent early phase model was used to test this inhibiting effect.

In cord blood-derived mast cells the Syk inhibitor significantly prevented the degranulation, assessed by measurement of histamine release, and the production of $LTC_4/LTD_4/LTE_4$ and PGD_2. Furthermore, the Syk inhibitor was similarly able to significantly inhibit histamine, $LTC_4/LTD_4/LTE_4$ and PGD_2 in nasal polyp tissue in a dose dependent manner. In cord blood-derived mast cells, the potencies of the Syk inhibitor on the different mediators were similar (pLTs=histamine=PGD_2); however in nasal polyps they were different (pLTs>histamine>PGD_2), probably due to the more complex physiological and disease-relevant system in human tissue.

Supplementary with the limited findings of Syk inhibition described in the human bronchial biopsies[6], our study reports the potency of a specific selective Syk inhibitor (NVP-QAB205) to inhibit the release of acute phase mediators in human nasal tissue. A clinical study, where another specific Syk inhibitor (R112) was applied, demonstrated the reduction of symptoms in seasonal allergic rhinitis patients [7]. Our results suggest that this could be due to the inhibition of histamine, cysLTs and PGD_2.

We suggest that in future, specific Syk inhibitors might provide a new therapeutic possibility in the treatment of upper airway diseases with mast cell involvement, such as allergic rhinitis.

IKK-2- kinase inhibition

Targeting IKK-2 represents a growing area of interest for researchers and pharmaceutical firms as, during the last decade, small molecule inhibitors have become available. IKK-2 is an important kinase in the NF-κB pathway. Inhibition of this kinase could therefore result in the blockage of transcription of multiple inflammatory mediators, such as TNF-α, interleukins, cell adhesion molecules, etc. Long term therapy with potent intranasal or oral corticosteroids is frequently performed to control nasal polyp inflammation and growth, but the response to this treatment is often only partially successful and oral corticosteroids may cause multiple side-effects.

In chapter VIII our goal was to determine, in nasal polyposis, the impact on the release of several Th1, Th2 and pro-inflammatory cytokines when inhibiting IKK-2 in comparison to the impact of the topical corticosteroid fluticasone propionate. We used the SEB-dependent late phase model to test these inhibitory properties and we could demonstrate that the IKK-2 inhibitor and fluticasone propionate were able to almost completely block the release of IL-4, IL-5 and IL-13. In regard of the inhibition of Th-1 cytokines, the IKK-2 inhibitor was able to block the production of IFN-γ and IL-2 almost completely, whether fluticasone propionate only reached an inhibition of around 40%. Furthermore, the IKK-2 inhibitor could completely block the production of pro-inflammatory cytokines such as IL-1β and TNF-α, whereas fluticasone propionate was practically unable to block these. Our results suggest that the IKK-2 inhibitor seemed, in certain respects, to possess a more comprehensive anti-inflammatory profile than that of fluticasone propionate in nasal polyposis. In the future IKK-2 inhibition could be of interest for the treatment of nasal polyposis. However, the risks of chronic administration of IKK-2 inhibitors in humans remain to be investigated as side-effects such as teratogenicity and susceptibility to infection could be problematic. More information will

become available as structurally distinct IKK-2 inhibitors progress through pre-clinical safety studies.

FUTURE PERSPECTIVES

In the future, these kinds of stimulation models make it possible to test drugs in human disease relevant tissue in a kind of pre-safety and pre-clinical setting.

In general, we will be able to test different kinds of promising selective pharmaceutical compounds. Moreover, these kinds of stimulation models give us the opportunity to test different molecules of the same compound on human tissue. In this respect small differences in potency can be detected.

Currently, we are using the early and the late phase model to test Compound A (corticosteroid -like extract from plants), PDE-4 inhibitors (phosphodiesterase-4) and CRAC (Ca^{2+} release-activated Ca^{2+}) channels inhibitors.

Furthermore, we can use these stimulation models on other tissues. Currently there are studies ongoing to stimulate gut tissue and to test relevant inhibitors.

These stimulation models may also be used in more fundamental research settings. We used the supernatants from anti-IgE stimulated polyp tissue to demonstrate that PGD_2 produced by mast cells promotes the migration of Th2 cells through a CRTH2 (chemoattractant receptor-homologous receptor expressed on Th2 cells) dependent mechanism[8].

Development of new nasal tissue models: First of all an *ex-vivo* late phase model should be set up to stimulate mast cells in inferior turbinates and nasal polyps by using IgE/ anti-IgE specific (ε chain specific) stimulations and measure production of several late phase cytokines (i.e. IL-4, IL-5, IL-6, TNF-α, IL-13). This would be very interesting to test new molecules with potential in treating allergic rhinitis. We tried to set up this model and we could achieve a significant release of IL-5 and IL-13 in nasal polyps; however, until now we have not been able to set up a late phase anti-IgE model in inferior turbinates; further studies are necessary.

Recently, we have established a model with specific allergen stimulations[9]. Allergen stimulation with different concentrations of grass pollens, in inferior turbinate and nasal polyp tissue with specific IgE to grass pollen, led to the release of PGD_2 in a Gaussian distribution. Interestingly, in nasal polyp tissue there was a lower release of PGD_2 after grass pollen stimulation compared to the PGD_2 release in inferior turbinate. This in contrast to the significantly higher release of PGD_2 in nasal polyps after anti-IgE stimulation. Moreover, in

this recent study we could demonstrate the direct effect of SEB in releasing mast cell mediators in samples positive for IgE to SAEs.

Finally, the therapeutic purposes for kinase inhibitors will be further explored. On the one hand, inhibition of Syk kinase activity, using small molecules is one approach. On the other hand, although early in the pharmaceutical development stage, suppression of Syk gene expression appears to be a viable approach to reduce allergic responses. Regulation of Syk gene expression can be targeted using siRNA (small interfering RNA) technology. In cooperation with GSK, we will investigate the feasibility of delivering RNAi (RNA interference) to nasal tissue fragments, look at the maintenance of a mast cell response after RNAi delivery and investigate significant functional inhibition of the mast cell response. Further studies will be needed to evaluate these kinds of therapies in terms of efficacy and safety.

GENERAL CONCLUSIONS

This thesis evaluates models to test drugs in human-disease-relevant-nasal tissue in a pre-safety and pre-clinical setting, and demonstrates the effect of two kinase inhibitors namely a Syk inhibitor and an IKK-2 inhibitor. A whole tissue nasal mucosal anti-IgE stimulation model, which can be used to mimic the early phase of an allergic reaction, was set up both in nasal polyps and inferior turbinates, and a SEB tissue stimulation model for late-phase release of numerous immunoregulatory and proinflammatory cytokines was established. Additionally, we have shown that SEB and SpA contribute to the Th-2 polarisation pattern and the mast cell degranulation in nasal polyps, respectively.

The Syk inhibitor we tested significantly inhibited histamine, leukotrienes and PGD_2. Furthermore, our results suggest that the IKK-2 inhibitor has a broader spectrum of inhibitory effects than fluticasone propionate, also including inhibition of pro-inflammatory cytokines. In the future, the Syk - and the IKK-2 inhibitor might therefore be further developed as new therapeutics targets.

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CURRICULUM VITAE

Familienaam:	Patou
Naam:	Joke Agnes Erik
Geboortedatum:	13 april 1979
Geboorteplaats:	Brugge
Nationaliteit:	Belg
Burgelijke staat:	Gehuwd, 2 kinderen
Thuisadres:	Steenakker 11 9000 Gent België
E-mail:	jokepatou@hotmail.com

ONDERWIJS

Secundair onderwijs: St.-Jozef Humaniora, Brugge
1991-1997: Grieks-Latijn / Wiskunde-Wetenschappen

Basisopleiding arts*:* Universiteit Gent, 1997- 2004
* Inleefstage, Kameroen, augustus-september 2000
* Erasmusstage, Amsterdam, VU, mei-juli 2003

Certificaat elektrocardiogram: Universiteit Gent, 2002

Certificaat Radioprotectie: Universiteit Gent, 2004

Arts specialist in opleiding otorhinolaryngologie: Universiteit Gent, 2004 -2011
* Full time wetenschappelijk onderzoek, september 2004 -maart 2007, Upper airways research laboratory
* Dienst neus-, keel-, oorheelkunde en hoofd- en halschirurgie Universitair Ziekenhuis Gent, april 2007-september 2008
* Dienst neus-, keel-, oorheelkunde AZ St.- Lucas Gent, oktober 2008 - september 2009
* Dienst neus-, keel-, oorheelkunde en hoofd-en halschirurgie Universitair Ziekenhuis Gent, oktober 2009 -juli 2011

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Patou J, Holtappels G, Affleck K, Van Cauwenberge P, Bachert C. Syk-kinase inhibition prevents mast cell activation in nasal polyps. Rhinology. 2011 March; 49:100-6.

PRESENTATIES OP CONGRESSEN

Mondelinge Presentaties

1 oktober 2005, Modern research in sinus disease and allergic rhinitis. Presentation Skills Master Class, UZ Gent: “Pathomecanisms of allergic rhinitis” Patou J.

18 november 2005, Koninklijke Belgische Vereniging voor Otorhinolaryngologie, Gelaat- en Halschirurgie, From opinion to advice, Congress Palace, Luik: “Intraepithelial presence of Staphylococcus aureus in nasal polyps” Patou J, Van Zele T, Holtappels G, Claeys S, Gevaert P, Cuvelier C, Van Cauwenberge P, Bachert C.

25 november 2005, Superantigens in airway disease: Immunology and clinics of superantigen-driven inflammation, Novotel Hotel, Gent: “Intraepithelial presence of Staphylococcus aureus in nasal polyps” Patou J, Van Zele T, Holtappels G, Claeys S, Gevaert P, Cuvelier C, Van Cauwenberge P, Bachert C.

9 februari 2006: 6th Symposium on Experimental Rhinology and Immunology of the Nose (SERIN), Barcelona, Spain: “Ex-vivo nasal challenge model to investigate mast cell activation” Patou J, Holtappels G, Gevaert P, Van Cauwenberge P, Bachert C.

20 mei 2006: Koninklijke Belgische Vereniging voor Otorhinolaryngologie, Gelaat- en Halschirurgie, Wetenschappelijke Vergadering, Gasthuisberg, Leuven: “Inhibition of protein tyrosine kinase activity in an ex-vivo nasal challenge model” Patou J, Holtappels G, Thompson N, Van Cauwenberge P, Bachert C.

26 februari 2007: 63rd Annual Meeting of the American Academy of Allergy, Asthma and Immunology (AAAAI), San Diego, California, United States: “Protein A, SEB and LTA stimulations in nasal polyps” Patou J, Van Zele T, Gevaert P, Holtappels G, Williamson R, Van Cauwenberge P, Bachert C.

4 november 2010: European Rhinitis and Asthma Meeting Symposium on Experimental Rhinology and Immunology of the Nose (ERAM/SERIN 2010), Brussels, Belgium: “IKK2 inhibition in nasal polyps” Patou J, Holtappels G, Afleck K, Gevaert P, Bachert C.

Posterpresentaties

juni 2005: XXIV Congres of the European Academy of Allergology and Clinical Immunology (EAACI), München, Germany: “Staphylococcal aureus in epithelial cells and lamina propria of normal mucosa and nasal polyps ” Patou J, Van Zele T, Holtappels G, Claeys S, Gevaert P, Van Cauwenberge P, Bachert C.

februari 2006: 4th EAACI and GA2LEN Davos Meeting, Basic Immunology Research in Allergy and Clinical Immunology, Garmish-Partenkisrchen, Germany: “Ex-vivo nasal challenge model to investigate mast cell activation” Patou J, Holtappels G, Gevaert P, van Cauwenberge P, Bachert C.

juni 2006: XXV Congress of the European Academy of Allergology and Clinical Immunology (EAACI), Vienna, Austria: “Ex-vivo model to challenge and investigate nasal mast cell activation” Patou J, Holtappels G, Gevaert P, Van Cauwenberge P, Bachert C.

29 september-2 oktober 2006, EAACI and GA2LEN Allergy school, Hannover, Germany: “Syk-kinase inhibition prevents mast cell and basophil activation in human nasal tissue” Patou J, Holtappels G, Thompson N, Affleck K, Gevaert P, Van Cauwenberge P, Bachert C.

BEURZEN EN PRIJZEN

EAACI Travel Grant, 29 september-2 oktober 2006, EAACI and GA2LEN Allergy school, Allergy – From diagnosis to treatment, Hannover, Germany

AAAI 2007 Fellows-in-Training International Travel Grant Scholarship, 23-27 februari 2007: 63rd Annual Meeting of the American Academy of Allergy, Asthma and Immunology (AAAAI), San Diego, California, United States

SERIN poster award, 4-6 november 2010: European Rhinitis and Asthma Meeting Symposium on Experimental Rhinology and Immunology of the Nose, Brussels, Belgium

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