The role of viruses and *Staphylococcus aureus* in propagating a Th2 response in human nasal polyps

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This thesis consists of 6 chapters. The first two chapters are reviews which are meant to introduce into the airways. Then, questions of my experimental works and hypothesis are followed. Finally, I mentioned three papers which have been published as original papers.

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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.

Feng Lan, Nan Zhang, Jie Zhang, Olga Krysko, Quanbo Zhang, Junming Xian, Lara Derycke, Yanyu Qi, Ka Li, Sixi Liu, Pin Lin, Claus Bachert. Forkhead box protein 3 in human nasal polyp regulatory T cells is regulated by the protein suppressor of cytokine signaling 3. Journal of Allergy and Clinical Immunology 2013 Dec; 132(6):1314-21.

Feng Lan, Nan Zhang, Gabriele Holtappels, Natalie De Ruyck, Olga Krysko, Koen Van Crombruggen, Sebastian L Johnston, Nikolaos G Papadopoulos, Claus Bachert. *Staphyloccoccus aureus* induces type 2 cytokines via TSLP and IL-33 release in human airway mucosa. To be submitted.

Feng Lan[*], Xiangdong Wang[*], Hans J Nauwynck, Gabriele Holtappels, Luo Zhang, Sebastian L Johnston, Nikolaos G Papadopoulos, Claus Bachert, Nan Zhang. Th2 biased upper airway inflammation is associated with an impaired response to viral infection with Herpes simplex virus 1. Rhinology (Accepted on 09/12/15).

Feng Lan, Nan Zhang, Elien Gevaert, Luo Zhang, Claus Bachert. Regulatory T cell in allergic airway diseases. Clinical & Experimental Allergy (under revision).

Feng Lan, Nan Zhang, Elien Gevaert, Luo Zhang, Claus Bachert. Viruses and bacteria in Th2 biased allergic airway disease. Allergy (under revision).

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LIST OF ABBREVIATIONS

AERD: Aspirin-exacerbated respiratory disease;

AHR: Hyper-responsiveness;

APCs: Antigen presenting cell;

AR: Allergic rhinitis;

ARI: Acute respiratory infection;

BAL: Broncho-alveolar lavage fluid;

CCR: Chemokine receptor;

CRS: Chronic rhinosinusitis;

CRSwNP: Chronic rhinosinusitis with nasal polyps;

CRSsNP: Chronic rhinosinusitis without nasal polyps;

CRTH2: Chemoattractant receptor homologous molecule expressed on Th2 cells;

CTLA-4: Cytotoxic T lymphocyte-associated antigen-4;

DCs: Dendritic cells;

DNAzyme: DNAzyme deoxyribozymes;

dsRNA: Double stranded RNA;

ECP: Eosinophil-cationic protein;

EPOS: European Position Paper on Rhinosinusitis and Nasal Polyps;

Foxp3: Fork head box p3;

FcεRI: High affinity IgE receptor;

GITR: Glucocorticoid-induced tumor necrosis factor receptor;

HDM: House dust mite;

HMT: Healthy mucosal tissue;

HRV: Human rhinovirus;

HRSV: Human respiratory syncytial virus;

HSV: Herpes simplex virus;

ICOS: Inducible T cell co-stimulator;

IFN-α: Interferon alpha;

IFV: Influenza virus;

IgE: Immunoglobulin E;

IL-6: Interleukin 6;

ILCs: Innate lymphoid cells;

ICL2 : Type 2 innate lymphoid cells;

IL-1ra: IL-1 receptor antagonist;

IL13Rα1: IL-13 receptor α1 chain;

iNKT: Invariant Natural Killer T;

IT: Inferior turbinate;

iTreg: Induced regulatory T cell;

JAK: Janus kinase;

LAG-3: Lymphocyte activation gene3;

lfTSLP: Long form TSLP;

MASA: Methicillin-resistant *Staphylococcus aureus*;

mTECs: Medullary thymic epithelial cells;

NF-κB: Nuleare factor-κappaB;

NP: Nasopharynx;

Nrp1: Neuropilin-1;

ODN: CpG oligodeoxynucleotides;

OVA: Ovalbumin;

PANC-1: Pancreatic carcinoma epithelial-like cell;

PD-1: Programmed cell death-1;

PIFV: Parainfluenza viruses;

pSTAT: Phosphorylated signal transducer and activator of transcription;

RORγt: RAR-related orphan receptor gamma;

RSV: Respiratory syncytial virus;

SEB: Staphylococcal enterotoxin B;

S. aureus: *Staphylococcus aureus*;

S. epidermidis: *Staphylococcus epidermidis*;

sfTSLP: Short form TSLP;

SIT: Specific immunotherapy;

SOCS3: Suppressor of cytokine signaling 3;

ST2L: ST2 receptors;

STAT: Signal transducer and activator of transcription;

TCR: T cell receptor;

TGF-β: Transforming growth factor beta;

TNF-αlpah: Tumor necrosis factor;

Tr1: Type 1 regulatory T cell;

Treg: Regulatory T cell;

nTreg: Naturally occurring regulatory T cell;

Th: T helper cells;

TIM4: T cell immunoglobulin mucin domain molecule 4;

TLR-2: Toll-like receptor 2;

TSLP: Thymic stromal lymphopoietin;

TSLPR: TSLP receptor;

SUMMARY

Multiple distinct viruses and bacteria have been detected in the airways. Recently, it has been confirmed that the microbiome of allergic individuals differ from those of healthy subjects, showing a close relationship with the type 2 response in airway disease. Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory upper airway disease. A smaller population of Chinese CRSwNP patients, but 85% of European CRSwNP patients show a Th2-biased and eosinophilic inflammation. An increase in colonization with *Staphylococcus aureus* (*S. aureus*) and the presence of *S. aureus* enterotoxin-specific IgE antibodies have been demonstrated in the mucosa of CRSwNP subjects in comparison with control or chronic rhinosinusitis without nasal polyp (CRSsNP) patients. Moreover, a high prevalence of viruses such as human rhinovirus (HRV), human respiratory syncytial virus (HRSV), influenza virus (IFV), and herpes simplex virus is also observed in CRSwNP mucosa. However, it is unknown to which extent viruses and bacteria directly impact on the Th2 response in CRSwNP.

CRSwNP is also characterized by a deficit in fork head box P3 $(Foxp3)^+$ T regulatory (Treg) cells in European and Chinese patients. Therefore, restoring Treg cell efficiency may be a useful strategy to inhibit Th2 responses in CRSwNP. In this thesis, we established human nasal *ex vivo* infection models to study the inflammatory response after infection, and we aimed to confirm the hypothesis that microbes affect the Th2 response in CRSwNP. Furthermore, we investigated ways to inhibit Th2 responses in CRSwNP tissue by reinforcing the suppressive function of Treg cells.

In the first model, we have demonstrated that *S. aureus* infection increased IL-33, Thymic stromal lymphopoietin (TSLP), IL-5, and IL-13 expressions in CRSwNP tissue accompanied by elevated expressions of the TSLP and IL-33 receptors, which were predominantly expressed on $CD3^+$ T cells. Healthy inferior turbinate (IT) tissue did release TSLP after *S. aureus* infection, but not type 2 cytokines. Compared to *S. aureus*, *Staphylococcus epidermidis* did not induce any IL-33, TSLP and type 2 cytokine release in neither CRSwNP nor healthy IT tissues. Increased levels of IL-33 and TSLP were also induced by *S. aureus* in BEAS-2B bronchial epithelial cells, associated with an activation of nuclear factor-κappaB (NF-κB) pathways. Blocking toll-like receptor (TLR) 2 using a specific antagonist CU-CPT22 reduced the effect of *S. aureus* infection on release of TSLP and IL-

33 and the activity of NF-κB signaling in BEAS-2B cells, pointing to the role of TLR2 in this activation.

In the thesis we also have demonstrated that CRSwNP tissue showed a significant deficit in IFN-γ and IL-17 release within 24 to 72h after herpes simplex virus 1(HSV1) infection, while releasing significantly more pro-inflammatory cytokines including IL-1β and TNF-α during the same time frame. These findings were associated with significantly higher viral invasion scores at 48 and 72h in CRSwNP mucosa compared to those for IT. There was a significantly higher spontaneous release of IL-5 at 24h and 48h in CRSwNP vs. IT, but this was independent from HSV1 infection. These observations support the hypothesis that CRSwNP tissue provides an inadequate defense against virus infection when compared to healthy tissue, which may contribute to more and longer symptoms upon acute infection, but also to the persistence of inflammation in CRSwNP tissue. In CRSwNP tissue, we have observed that suppressor of cytokine signaling 3 (SOCS3) gene and protein expression was up-regulated in inflammatory cells, whereas Foxp3 gene and protein expression was down-regulated. For the first time we showed that nasal mucosal Treg cells co-express both proteins. Switching off the expression of SOCS3 in human airway mucosa resulted in Foxp3 up-regulation, whereas inducing it in a cancer cell line PANC-1 led to Foxp3 down-regulation. We also found that phosphorylation of signal transducer and activator of transcription 3 (STAT3) was decreased in inflamed mucosa, and we hypothesized that SOCS3 may be responsible; indeed, phosphorylation of STAT3 increased upon silencing SOCS3 expression in inflamed mucosa and decreased upon SOCS3 plasmid transfection in PANC-1 cells. Finally, the expression of the Th2 cytokine IL-5 decreased in CRSwNP tissue upon silencing SOCS3 expression using siRNA transfection, indicating that this intervention is functional; increased Foxp3 expression in CRSwNP tissue inhibited the Th2 response.

In summary, we have demonstrated that *S. aureus* contributes to the Th2 response via the epithelial cell derived cytokines TSLP and IL-33 in CRSwNP tissue. CRSwNP tissue shows a significant deficit in the defense against viruses such as HSV1 and HRV, and against *S. aureus* infection when compared to healthy tissue, which may contribute to the persistence of inflammation in CRSwNP tissue. Silencing SOCS3 expression inhibits IL-5 cytokine expression in CRSwNP

tissue via an up-regulation of Foxp3 expression, which may represent an innovative therapeutic strategy in Th2-biased CRSwNP disease.

SAMENVATTING

Verschillende virussen en bacteriën werden gedetecteerd in de luchtwegen. Recent onderzoek heeft bevestigd dat het microbioom van allergische individuen verschilt met dat van gezonde individuen, wat aantoont dat er een nauwe relatie bestaat met een type 2 (Th2) immuunrespons in luchtwegaandoeningen. Chronische rhinosinusitis met neuspoliepen (CRSwNP) is een chronische ontstekingsziekte van de bovenste luchtwegen. Een kleiner deel van de Chinese CRSwNP patiënten, maar 85% van de Europese CRSwNP patiënten hebben een Th2 gemedieerde en eosinofiele inflammatie. In CRSwNP patiënten werd een verhoogde kolonisatie met *Staphylococcus aureus* (*S. aureus*) en een verhoogde aanwezigheid van specifieke antibodies tegen *S. aureus* enterotoxine waargenomen, vergeleken met gezonde individuen en patiënten met chronische rhinosinusitis zonder poliepen. Daarenboven werd in het mucosa van CRSwNP patiënten een hoge prevalentie van virussen zoals het humane rhinovirus (HRV), humane respiratory syncytial virus (HRSV), influenza virus (IFV) en herpes simplex virus waargenomen. Nochtans is het onbekend in welke mate virussen en bacteriën een directe impact hebben op de Th2 respons in CRSwNP.

CRSwNP wordt ook gekarakteriseerd door een defect in fork head box P3 $(Foxp3)^+$ T regulatory (Treg) cells in Europese en Chinese patiënten. Daarom zou het herstel van de efficiëntie van deze Treg cellen een bruikbare strategie kunnen zijn om de Th2 respons in CRSwNP patiënten te inhiberen. In deze thesis werd een humaan nasaal *ex vivo* infectie model ontwikkeld om de inflammatoire respons na infectie te bestuderen en met het doel de hypothese te bevestigen dat micro-organismen de Th2 respons kunnen beïnvloeden in CRSwNP. Daarenboven werden er manieren gezocht om de Th2 respons in CRSwNP weefsel te inhiberen via het versterken van de suppressieve functie van de Treg cellen.

In het eerste model, hebben we aangetoond dat *S. aureus* infectie zorgt voor een verhoging van IL-33, thymic stromal lymphopoeitin (TSLP), IL-5 en IL-13 expressie in CRSwNP weefsel. Dit effect ging gepaard met een verhoogde expressie van de TSLP en IL-33 receptoren die voornamelijk op $CD3^+$ T cellen tot expressie kwamen. Inferiour turbinate weefsel van gezonde individuen (IT) toonde een vrijstelling van TSLP na *S. aureus* infectie, maar niet van type 2 cytokines. Vergeleken met *S. aureus*, zorgde *Staphylococcus epidermidis* niet voor de inductie van IL-33, TSLP en type 2

cytokines in CRSwNP of gezond IT weefsel. In BEAS-2B werden eveneens verhoogde levels van IL-33 en TSLP ge ïnduceerd na infectie met S. aureus. Dit was geassocieerd met een activatie van de nuclear factor-kappaB (Nf-kB) pathway. Het blokkeren van toll-like receptor (TLR) 2 met de specifieke antagonist CU-CPT22, verminderde het effect van *S. aureus* infectie op de vrijstelling van TSLP en IL-33 en de activiteit van NFkB signaling in BEAS-2B cellen. Dit wijst op een rol van TLR2 in deze activatie.

In de thesis werd ook aangetoond dat CRSwNP weefsel een significant defect in de vrijstelling van IFN-γ en IL-17 vertoont 24 tot 72 uur na herpes simplex virus infectie, terwijl significant meer pro-inflammatoire cytokines waaronder IL-1β en TNF-α werden vrijgesteld in ditzelfde tijdsframe. Deze bevindingen waren geassocieerd met significant hogere virale invasie scores na 48 en 72 uur in CRSwNP mucosa, vergeleken met deze van de IT. Er was een significant hogere spontane vrijgave van IL-5 na 24 en 48 in CRSwNP versus IT, maar dit was onafhankelijk van HSV1 infectie. Deze observaties ondersteunen de hypothese dat CRSwNP weefsel voor een inadequate verdediging zorgt tegen virus infectie wanneer met die vergelijkt met gezond weefsel. Dit kan bijdragen tot meer een langere symptomen bij acute infectie maar ook tot de persistentie van de inflammatie in CRSwNP weefsel. Een op regulatie van suppressor of cytokine signalling 3 (SOCS3) genexpressie en prote ïne expressie in inflammatoire cellen van CRSwNP weefsel werd geobserveerd, terwijl Foxp3 gen- en eiwit expressie een down regulatie vertoonden. Wij toonden voor de eerste keer aan dat nasale mucosale Treg cellen beide prote ïnen tot expressie brengen. Het uitschakelen van de expressie van SOCS3 in humane luchtwegmucosa resulteerde in een op regulatie van Foxp3, terwijl het induceren in de kanker cel lijn PANC-1 leidt tot Foxp3 down regulatie. De fosforylatie van signal transducer and activator of transcription 3 (STAT3) was verminderd in ontstoken mucosa en we stelden als hypothese dat SOCS3 hiervoor verantwoordelijk zou kunnen zijn. Inderdaad, de fosforylatie van STAT3 was verhoogd na silencing van SOCS3 expressie in ontstoken mucosa en verminderd na SOCS3 plasmide transfectie in PANC-1 cellen. De expressie van het Th2 cytokine IL-5 was verminderd in CRSwNP weefsel na silencing van SOCS3 expressie door midden van siRNA transfectie. Dit toont aan dat deze interventie functioneel was: verhoogde Foxp3 expressie in CRSwNP weefsel inhibeerde de Th2 respons.

Samengevat, hebben we aangetoond dat *S. aureus* bijdraagt aan de Th2 respons door middel van de epitheliale cel geproduceerde cytokines TSLP en IL33 in CRSwNP weefsel. CRSWNP weefsel toont een significant gebrek in de defensie tegen virussen zoals HSV1 en HRV, en tegen *S. aureus* infectie vergeleken met gezond weefsel, wat kan bijdragen aan de persistentie van de inflammatie in CRSwNP weefsel. Het silencen van SOCS3 expressie inhibeert de expressie van het IL-5 cytokine in CRSwNP weefsel via een op regulatie van Foxp3 expressie, wat een mogelijk innovatieve therapeutische strategie voorstelt voor de behandeling van Th2 gemedieerde CRSwNP.

CHAPTER 1

VIRUSES AND BACTERIA

IN TH2 BIASED ALLERGIC AIRWAY DISEASES

Viruses and bacteria in Th2 biased allergic airway disease

Feng Lan, Nan Zhang, Elien Gevaert, Luo Zhang, Claus Bachert

Abstract:

Allergic airway diseases are typically characterized by a type 2-biased inflammation. Multiple distinct viruses and bacteria have been detected in the airways. Recently, it has been confirmed that the microbiome of allergic individuals differs from those of healthy subjects, showing a close relationship with the type 2 response in allergic airway disease. In this paper, we summarize recent findings on the prevalence of viruses and bacteria in type 2-biased airway diseases and on mechanisms employed by viruses and bacteria in propagating type 2 responses. The understanding of the microbial composition and post-infectious immune programming is critical for the reconstruction of the normal microflora and immune status in allergic airway diseases.

Introduction

The role of environmental and gastrointestinal pathogens in allergic airway inflammatory pathology (whether protective or provocative) has been widely studied. The hygiene hypothesis proposes that a low prevalence of childhood asthma is associated with exposure of infants to a microbe-rich environment[1,2]. A low diversity of the gut microbiome in early infancy precedes asthma at school age[3]. However, the Copenhagen Prospective Studies on Asthma in Childhood recently suggested that individual susceptibility and inflammatory consequences after infection, rather than the specific microbial trigger, are associated with school-age asthma development[4]. Therefore, understanding the microbial composition and post-infectious immune programming in type 2-biased airway diseases are important for therapeutic interventions in the future. Allergic asthma, allergic rhinitis and chronic rhinosinusitis with nasal polyps (CRSwNP) are typical airway disorders that are characterized by a type 2-biased inflammation. In this review, we summarize the prevalence of viruses and bacteria in type 2-biased airway diseases, on the mechanisms employed by viruses and bacteria in propagating type 2 responses and the impact of T helper 2 (Th2) cytokines on the defense against infections.

The prevalence of viruses in the airways

Viral infections starting from the nasopharynx in early life are reportedly associated with an increased risk of developing asthma later in life (**Table1**). Viral infections that affect allergic upper airway diseases can also lead to acute asthma exacerbations in the lower airways[5]. To clarify the prevalence of viral communities in the development of asthma, nasopharyngeal samples were obtained from infants at scheduled visits at 2, 6, and 12 months of age, or within 48h after onset of an acute respiratory infection (ARI) during the first year of life in the extension of the Childhood Asthma Study[6]. The most common viruses found in the nasopharynx of healthy children during ARI were human rhinovirus (HRV) (40%) and human respiratory syncytial virus (HRSV) (11%)[6]. Among those viruses, HRV-C rather than the other HRV subtypes or HRSV, which was detected in nasopharynx of healthy children during ARI accompanied by wheezing symptoms, was positively associated with later chronic wheezing among all children and particularly for those who were atopic by 2 years[6]. In a recent study, HRSV and HRV induced lower respiratory tract infections in

412 pediatric patients up to the age of 3 years were associated with the development of wheezing and exacerbations[7]. In addition, pediatric patients with HRV induced wheezing were more likely to develop subsequent recurrent wheezing and asthma during the following 3 years[7]. Both HRV and HRSV are well known risk factors for subsequent wheezing or asthma development in infancy, but they also have specific susceptibilities according to their age. HRV infection carries a markedly higher risk for persistent wheezing in younger infancy (under 2 years of age)[8], while HRSV infections are related to the development of asthma in children at school age[9].

Table 1. The prevalence of viruses in airways.

Characteristic of subjects	Sample source	Identification techniques	The common viruses	Ref.
Healthy infants (<12m) during acute respiratory infection	nasopharynx secretion	RT-PCR;	HRV (40%); HRSV (11%);	6-7
Asthmatic children (3-16y) during asthma exacerbation	nasopharynx secretion	RT-PCR; VNT;	HRV (30.3%); HRSV (40.9%); IFV (15.9%); HSV-1 (56.8%);	10-13
Asthmatic adults during asthma exacerbation	nasopharynx secretion	RT-PCR;	HRV-A,B,C (10.4%) HRSV-A (10.4%); Metapneumovirus (6.3%);	14
Chronic rhinosinusitis patients	nasal tissue or epithelial cell scraping;	RT-PCR;	Picornavirus (29%); HRSV (15%); IFV (20%); Parainfluenza viruses-3(18 %);	15-16
Allergic rhinitis patients	nasal swab	RT-PCR;	HRV (14.1%);	23

HRV: human rhinovirus; HRSV: human respiratory syncytial virus; IFV: influenza viruses; VNT: virus neutralization test; RT-PCR: reverse transcription polymerase chain reaction; 16S rRNA: 16S ribosomal RNA, m: month; y: year.

In hospitalized children aged between 3 to 16 years, HRV and HRSV were the most frequent viruses in nasopharynx secretion during asthma exacerbations[10]. Similarly, a Japanese group also demonstrated that HRSV was related to wheezing episodes and was more frequent in patients with

a history of asthma[11]. Besides HRSV and HRV, also influenza viruses (IFV) were involved in asthma exacerbations in children particularly with atopic sensitizations, but not in non-atopic children[12]. In atopic Turkish children with asthma and allergic rhinitis, significantly higher herpes simplex type 1 (HSV-1) IgG seropositivity was detected in the serum than in the matched non-atopic group[13], suggesting a possible relationship between pediatric allergic airway diseases and HSV-1 infections. In adults, higher incidences of HRV A/B/C, HRSV A, and metapneumovirus infections were observed in nasopharyngeal swabs during asthma exacerbations *Corynebacterium pneumonia*[14].

The rate of picornavirus, HRSV, IFV, and parainfluenza viruses was significantly increased in CRS patients compared with that seen in controls (64% vs. 30%)[15]. Particularly, HRV was the only virus that shared a significant increase in infection rate in both nasal lavage and epithelial cells scrapped from nasal inferior turbinate of CRS patients vs. controls. On the contrary, a high rate of this virus was also found in epithelial cells scrapped from the nasal middle meatus of Chinese CRSwNP patients, but with no significant difference when compared with controls[16]. Thus, sampling from different places of the nose contributes to the different results. Concordantly, a high prevalence of HRV was also present in sinus tissues in Brazilian CRS patients, and there was a peak of detection during the autumn and winter seasons, when respiratory viruses are far more prevalent[17]. Unfortunately, CRS was not further differentiated in that study. In contrast to Th2-biased inflammatory characteristics of CRSwNP, CRSsNP often exhibits a Th1-cell inflammation pattern[18]. Due to their different inflammatory patterns, it is necessary to characterize the prevalence of viruses in each subgroup of CRS in the future.

Allergic rhinitis is an allergic inflammation of the upper airways, and it is also a known risk factor for wheeze and asthma. In general, asthma occurs in 10–40% of patients with allergic rhinitis, whereas allergic rhinitis occurs in 20–80% of patients with asthma[19,20]. Interestingly, both the cross-sectional survey of Singaporean and the Pollution and Asthma Risk: an Infant Study birth cohort studies demonstrated that allergic rhinitis may start as early as in the first 2 years of life[21,22]. In early childhood, HRV was the most frequent virus detected in nasal swab samples of prolonged/recurrent

rhinitis[23]. Interestingly, HRV-positive infants with recurrent rhinitis had a higher rate of wheeze compared to HRV-negative infants[23].

Table 2. The prevalence of bacteria in airways.

Subjects	Sample	Identification technique	Bacterial species	Ref.
Healthy infants (2m) without acute respiratory infection	nasopharynx secretion	16S rRNA pyrosequencing; bacterial culture;	*Staphylococcus* (41%); *Corynebacterium*(22%); (main bacteria) *Alloiococcus* (14%); *Moraxella* (9%); *Haemophilus* (9%);	6, 29
Healthy infants (12m) without acute respiratory infection	nasopharynx secretion	16S rRNA pyrosequencing;	*Moraxella*(41%); *Alloiococcus*(26%); (main bacteria) *Staphylococcus* (11%); *Corynebacterium* (12%);	6,
Healthy children (24m)	nasopharynx secretion	bacterial culture;	*Streptococcus pneumoniae (72.8%); Moraxella catarrhali (78.6%); Haemophilus influenzae (57.3%); Staphylococcus aureus (3.9%)*	25
Healthy adults (18-66y)	middle meatus swab	16S rRNA pyrosequencing; RT-PCR;	*Staphylococcus epidermidis* (96.4%); *Staphylococcus aureus* (67.9%); *Propionibacterium acnes* (92.9%);	34
Asthmatic patients (>18y) during asthma exacerbation	nasopharynx swab	RT-PCR	*Streptococcus pneumoniae* (18.8%); *Haemophilus influenzae (12.5%);*	14
Chronic rhinosinusitis without asthma patients	sinus swab (ethmoid region)	RT-PCR	*Staphylococcus (19.3%)*; *Fusobacterium* (9.0%);	35,36
Chronic rhinosinusitis with asthma patients	sinus swab (ethmoid region)	RT-PCR	*Staphylococcus* (28.4%); *Ralstonia* (8.2%); *Acinetobacter* (2.3%);	35,36

RT-PCR: Reverse transcription polymerase chain reaction; 16S rRNA: 16S ribosomal RNA, m: month; y: year.

Similar to childhood allergic rhinitis, an abundance of respiratory viruses, especially HRV, was detected in nasal lavage samples of adult perennial allergic rhinitis patients, but respiratory viral infection was independently correlated with symptom scores[24].

The prevalence of bacteria in the airways

Bacterial community composition is a strong determinant of general health and disease, as well as a potential modulator of inflammatory processes contributing to airway diseases. Here bacteria which affect airway diseases are listed in **Table 2**. In infants, the nasopharynx microbiome is qualitatively dominated by six common genera: *Haemophilus*, *Streptococcus*, *Moraxella* (those three genera are common in ARI), *Staphylococcus*, *Alloiococcus* and *Corynebacterium* (more common in healthy samples)[6], which is consistent with previous studies in children[25,26] and in adults[27]. Similarly, *Streptococcus pneumoniae*, *Haemophilus influenzae, Moraxella catarrhalis*, *Streptococcus pyogenes* and *Staphylococcus aureus* (*S. aureus*) were also observed in nasopharynx swabs of Asian infants[28]. However, the Asian infants had a higher colonization of *S. aureus* (88.9–100.0%) than in other studies at the first year of life, and 50% of *S. aureus* was methicillin-resistant *S. aureus*[28]. Nevertheless, the composition of microbial communities seems unstable over time. Early colonization typically involved *Staphylococcus* or *Corynebacterium*, which later was replaced by *Moraxella* or *Alloiococcus* in healthy infants, indicating that the composition of microbiome undergoes dynamic changes[6]. Whether the early microbiome dynamic change is related with later allergic airway diseases, requires more research. Clearly neonatal colonization with *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* had a strong correlation with subsequent recurrent wheeze and childhood asthma[6,29,30]. Evidence was also available that *S. aureus* acted as a risk factor for asthma development in children and young adults, but not in older adults[31-33]. It is an evident that bacterial organisms act as important contributors to asthma exacerbations. A study of the bacterial composition of the nasopharynx in adult asthmatic patients indicated that *Streptococcus pneumoniae* infection was also associated with asthma exacerbations in clinical practice[14].

In CRS, inflammation development takes place in the middle meatus. A high prevalence of *Staphylococcus epidermidis* (96.4%), *S. aureus* (67.9%), and *Propionibacterium acnes* (92.9%) was

found in middle meatus specimens of healthy adults[34]. Similarly to healthy adults, bacteria such as *Pseudomonas*, *Staphylococcus*, and *Streptococcus* were numerically dominant in sinus tissues of CRS patients[35]. Interestingly, a higher abundance of *Staphylococcus*, *Acinetobacter*, *Pseudomonas aeruginosa*, and *Ralstonia* species, and a lower level of *Prevotella*, *Fusobacterium* and *Campylobacter* species were detected in CRS patients with concomitant asthma than in CRS patients without asthma[35,36]. Additionally, patients with abundant expression of *Corynebacterium* at the time of endoscopic surgery had an optimal outcome, while patients with high *S. aureus* had a suboptimal outcome[35]. Coherently, an increase of nasal *S. aureus* carriage and the presence of *S. aureus* enterotoxin-specific IgE antibodies have been described in the mucosa of CRSwNP subjects and perennial allergic rhinitis patients[33,37], and this is even higher in CRSwNP patients with asthma comorbidity and aspirin sensitivity. The further analysis on antibiotics resistance bacteria in allergic rhinitis highlighted that an increase of methicillin assistant *staphylococcus aureus* burden provides an insight into the difficulty of eradicating the bacterium[38].

Viruses and type 2 responses in allergic airway diseases

HRV, as one of the most common viruses in human airways, is associated with the development and exacerbation of allergic asthma. Epithelial cell derived cytokines such as IL-33, thymic stromal lymphopoietin (TSLP) and IL-25 are critical in propagating a type 2 immune response by affecting Th2 cells, type 2 innate lymphoid (ILC2) cells and dendritic cells[39]. HRV infections induce mucosal IL-33 production *in vivo*, which can further initiate type 2 cytokine release from Th2 cells or ILC2 cells[40]. HRV-induced IL-25 amplified type 2 responses via activation of IL-25 receptors on murine Th2 cell, ILC2s and basophils[41]. Elevated levels of TSLP in allergic asthma subjects positively correlated with the severity of asthma[42], but so far there is no direct evidence showing the effect of HRV infection on TSLP production. In a recent study, $CD11b^+$ exudative macrophages bearing M2 macrophage markers have been identified as a new source for IL-13 production in a OVA asthma mouse model after HRV infection[43].

Respiratory syncytial virus (RSV), a member of the paramyxoviridae family, is also associated with type 2 responses in allergic airway diseases. An increased level of IL-13 was observed in the lungs of BALB/c mice, mediated by IL-33 and TSLP production after intranasal RSV incubation[44,

[45]. Furthermore, the interactions between a glycosyltransferase lunatic fringe and Notch ligands 4-Notch were reportedly responsible for the augmented Th2 inflammation in mouse during RSV asthma exacerbations[46]. Thus, IL-33, TSLP and Notch signaling might be the possible therapeutic target in RSV-induced Th2 responses. In addition to the classical Th2 cells and $ST2L^+$ cells, a novel human subset of IL-6Rα^{high} effector memory $CD8^+$ T cells was identified to exclusively release IL-5 and IL-13 after HRSV infection[47]. IL-6Rα^{high} effector memory $CD8^+$ T cells were detected in high numbers in the peripheral blood of asthma subjects[47], suggesting a role of the cells in promoting Th2 responses in asthma **(Figure 1)**.

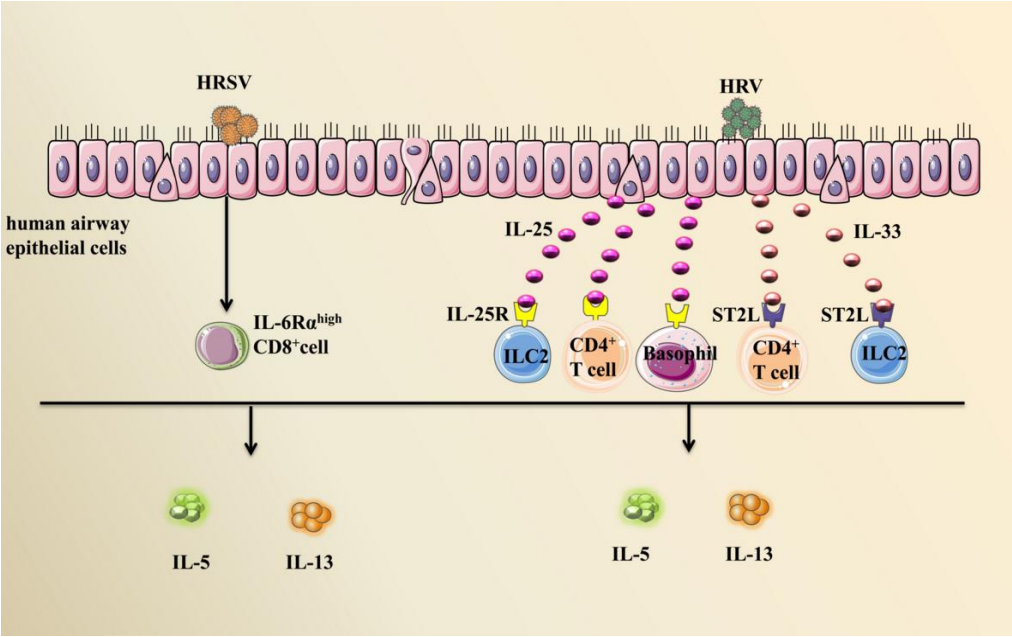

Figure 1: The role of viruses induces type-2 immune response in airways. Human airway epithelial cells produce IL-33, IL-25 in response to human rhinovirus (HRV)[41-42]. IL-33 and IL-25 subsequently drive IL-13 (yellow balls) and IL-5 (green balls) productions via binding to their receptors ST2L and IL-25R respectively on Th2 cells, type 2 innate immune cells (ILC2s) and basophils[42]. Human respiratory syncytial virus (HRSV) also can induce type-2 cytokine release in airways from IL-6Rα^{high} effector memory $CD8^+$T cells[48].

Similarly, IFV may induce the release of IL-33 from Natural killer T cells and alveolar macrophages in mice, leading to a robust IL-5 release from ILC2 cells[48]. Not only viruses but also viral nucleotide analogues can contribute to IL-13 expression by triggering mast cells in mice[49]. Taken together, the effect of viruses including HRV, RSV and IFV on type 2 responses are well established in the lower airways, but their role in upper airways awaits further investigation.

Bacteria and type 2 responses in allergic airway diseases

Airway bacteria such as *Moraxella*, *Corynebacterium pneumonia*, *Streptococcus pneumoniea* and *S. aureus* are associated with the development and exacerbations of asthma, but the underlying molecular mechanisms have not yet been unveiled. Endonasal instillation of ovalbumin (OVA) plus staphylococcal enterotoxin B (SEB) induced a murine pulmonary inflammation, which is characterized by an increase in the number of eosinophils and the production of Th2 cytokines[50]. It has been reported that exposure of mouse splenocytes and peritoneal macrophages to CpG oligodeoxynucleotides from *Streptococcus thermophilus* enhanced IL-33 expression[51], which may further initiate Th2 responses. However, not all bacteria exhibit the ability to enhance Th2 response; *Haemophilus influenzae* infection synergized with OVA mouse model of asthma elevates Th17 responses, not Th2 responses[52].

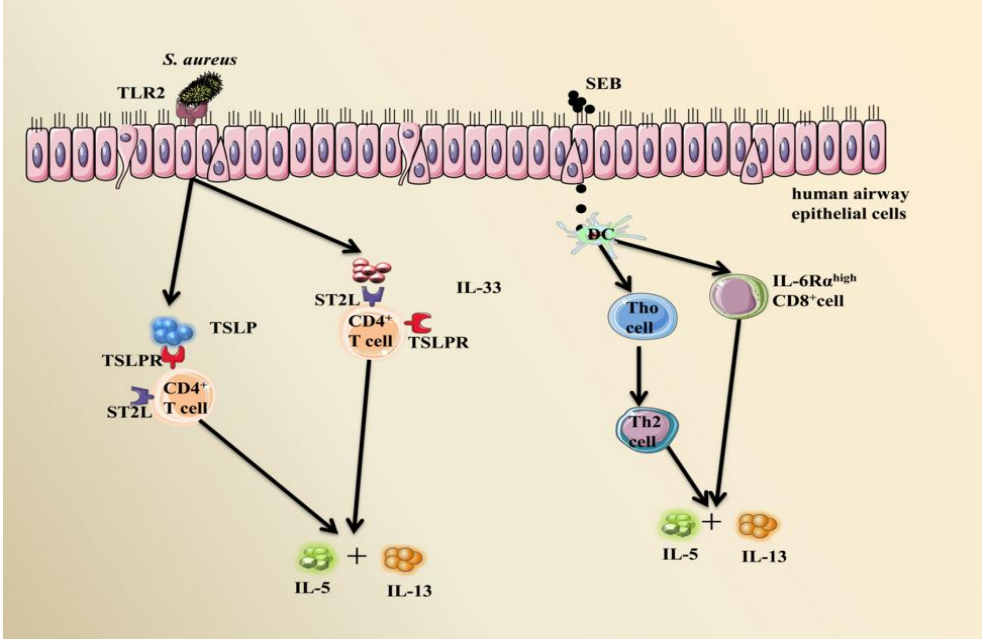

Figure 2: The role of bacteria induces type-2 immune respones in airways. After binding to toll-like receptor 2 (TLR2), *Staphylococcus aureus (S. aureus*) induces type 2 cytokines productions via thymic stromal lymphopoietin (TSLP) and IL-33 release from human airway epithelial cells (data not published). The staphylococcal enterotoxin B (SEB) induces type 2 cytokine releases by affecting the differentiation of T helper (Th) 2 cells and IL-6Rα^{high} CD8^{+}cells[48].

In upper airways, our group has proved that SEB induces considerable release of Th2-associated cytokines including IL-4, IL-5 and IL-13 and also enhances eosinophilic inflammation in human nasal tissues[53-56] (**Figure 2**). Without the effect of the enterotoxin, CRSwNP tissue exposure to *S. aureus* alone led to IL-5 expression via TLSP and IL-33 productions (data not

published). Even though IL-6Rα^{high} effector memory CD8^{+}T cells are involved in virus-induced Th2 response, they also trigger IL-5 and IL-13 expressions in response to the bacterial superantigens SEB and TSST-1[47]. In mouse, an adhesion molecule of *S. aureus* "iron-regulated surface determinant A'' mediated IL-4 secretion from splenocytes[57]. In summary, the effect of bacteria on Th2 response especially in upper airways implicates an important role of bacteria in allergic airway diseases.

Type 2 cytokines upon viral and bacterial infection

In allergic airway diseases, IL-4 is critical for Th2 cell differentiation and causes a switch to IgE production by differentiating B cells[58]. IL-5 is associated with eosinophil development and activation[59]. IL-13 regulates IgE isotype switching in B cells, as well as activating mast cells, eosinophils, and neutrophils[60,61]. As mentioned above, microbial infection can induce type 2 cytokine releases. Simultaneously, type 2 cytokines also can exaggerate inflammation in response to respiratory infection. IL-4 and IL-13 pre-treated human airway epithelial cells promoted higher expression of pro-inflammatory chemokines such as IL-8, CXCL9, CXCL10, CXCL11 in response to polyriboinosinic: polyribocytidylic acid stimulation[62]. IL-13 enhanced goblet cell differentiation [63], and the induction of mucous metaplasia inhibited ciliogenesis and ciliary beat frequency, thereby changing the apical membrane structure which increased susceptibility of human airway epithelium to HRV16 infection[63, 64]. HRV16 infection in turn resulted in further dysfunction of ciliary clearance and hyper-secretion of mucus, aggravating the inflammation in airways[65]. Additionally, IL-4 and IL-13 increased HRV16 replication by reducing the interferon producing capacity of human bronchial epithelial cell line[66]. Thus, type 2 cytokines increase susceptibility to viral infection in airways via changing the epithelial structure and the production of interferons.

In the upper airway, a disruption of the tight junctions such as occludin and zona occludens-1, and an irregular expression pattern were observed in the IL-4–stimulated primary nasal epithelial cells[67]. Zona occludens-1 expression became discontinuous in human nasal epithelial cells exposed to the *S. aureus* conditioned media[68], however, there is no direct evidence showing a correlation between the reduced tight junctions and higher susceptibility to bacterial infection. Indeed, IL-13, not IL-4, markedly down-regulated antimicrobial protein psoriasin expressions in human nasal

epithelial cells[69], which resulted in an increase in susceptibility to bacterial infection. In contrast, IL-4 and IL-13 exposure to human bronchial epithelial cells during mucociliary differentiation contributed to the antimicrobial defense against *Pseudomonas aeruginosa* infection via induction of antimicrobial peptide human beta defensin-2[70]. Therefore, the multifaceted role of Th2 cytokines has been shown in anti-microbial protein production.

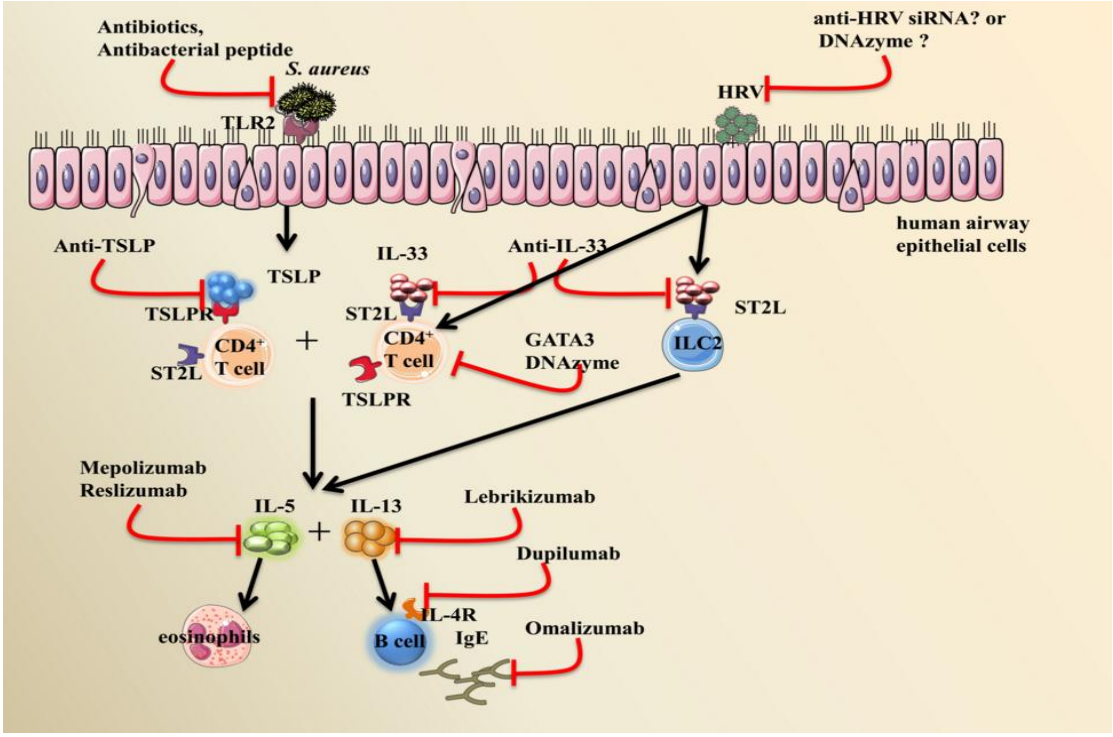

Figure 3: Targets to block type 2 immune responses in airways with viral and bacterial infections. Microorganisms, epithelial cell derived cytokines such as TSLP and IL-33, type 2 cytokines such as IL-4, IL-5, IL-13 and their receptors are targets to inhibit type 2 biased immune response in airways [79-82]. TLR2: toll-like receptor, siRNA: small interfering RNA, TSLP: thymic stromal lymphopoietin, TSLPR: TSLP receptor; ST2L: IL-33 receptor; ILC2: type 2 innate immune cells; IgE: immunoglobulin E; GATA3 DNAzyme: GATA binding protein 3 deoxyribozymes.

Ways to block type 2 responses in allergic airway disease

As mentioned above, there is a link between infection and type 2 response. Type 2 cytokines and their binding receptors, mechanisms of inducing type 2 cytokines during infection and microorganisms become potential targets to block human type 2 responses allergic airway diseases (**Figure 3**). Intra-nasally pretreated anti-IL-4 and anti-RSV siRNAs reduced eosinophils in broncho-alveolar lavage fluid of a mouse model of asthma during asthma exacerbations[71]. It provides

promising “silencing” techniques such as siRNA and DNAzyme against viruses or bacteria to be used in human model to diminish type 2 allergic immune responses. Likewise, non-cytotoxic anthraquinone derivatives ameliorated IL-4 and IL-13 expressions through de-phosphorylation AKT in an OVA-asthmatic mouse model with RV exacerbations[72]. Anti-IL-25 antibody had a similar inhibitory effect on Th2 inflammation induced by murine pneumonia viruses, but it also promoted a Th17 response[73]. Based on the findings in a mouse model, *monoclonal* blocking *antibodies* against type 2 cytokines are used in asthmatic patients in clinic trails. Targeting IL-13 (lebrikizumab) or both IL-4 and IL-13 signaling have demonstrated therapeutic benefits for treating moderate-to-severe asthma patients with increased eosinophils or periostin[74,75]. Additionally, neutralization of IL-5 by mepolizumab is a promising intervention for patients with severe eosinophilic asthma[76, 77]. Furthermore, blocking a subunit of IL-4 receptor by dupilumab can also efficiently inhibit both IL-4 and IL-13 signaling in patients with persistent, moderate-severe asthma [78]. Omalizumab, a humanized monoclonal antibody to block interaction between IgE and high affinity IgE receptor (FcεRI), can significantly improve the lung functions and markedly advance asthma control of severe asthma patients[79]. FcεRI activation is also associated with reciprocal down-regulation of type‖interferon production during viral asthma exacerbations[80]. Thus, omalizumab blocks the type 2 response in allergic asthma and consequently enhances viral clearance by restoring type I interferon production. The expression and the production of Th2 cytokines is also controlled by the transcription factor human GATA binding protein 3 (GATA3) which is relevant for the differentiation of Th2 lymphocytes. Applied in a human asthma model, GATA3 specific-deoxyribozymes (DNAzyme), a DNA molecule docks the GATA3 RNA expression, led to the reduction of Th2 response[81]. In the upper airways, the monoclonal antibodies mepolizumab, reslizumab, omalizumab and dupilumab have been subjected to proof-of-concept studies. Those therapeutic approaches efficiently suppress Th2 immune responses and also may counteract the consequences related to it in terms of deficits in mucosal defense mechanisms[82]. MiR-143, with a direct inhibitory role on IL-13 receptor α1 chain expression, suppresses IL-13-induced inflammatory cytokine and mucus production in nasal epithelial cell from allergic rhinitis patients[83]. Besides the use of monoclonal antibodies against Th2 cytokines and their receptors, interferon-

β produced a strong and consistent abrogation of Th2 cytokine production from human periphery blood mononuclear cells in the presence of HRV infection[84]. MiR-143 with a direct inhibitory role on IL-13 receptor α1 chain (IL13Rα1) expression, suppressed IL-13-induced inflammatory cytokine and mucus production in nasal epithelial cell from AR patients[83]. Specific immunotherapy for 3 months also can inhibit Th2 response in allergic rhinitis patients by modulating T cell immunoglobulin mucin domain molecule 4 (TIM4)/TIM1 interaction on dendritic cells[85].

The interaction between virus and bacteria

Most of the investigations focused on the effect of virus alone or bacterium alone in allergic airway diseases, whereas little is known about viral and bacterial co-infection. In the clinic, virus and bacteria co-infections increased the readmission risk of asthmatic patients[86], and a high frequency of co-infections was observed in asthma exacerbation patients[14]. In mouse model, preceding virus infection may enhance the bacterial adherence such as galectins for *pneumococcal* adhesion[87], and depress the recruitment of neutrophils and natural killer cells which play an important role in the bacterial clearance[88,89]. Conversely, exposure of *influenzae* to primary human epithelial cells significantly enhanced the binding of HRV16, probably through up-regulation of intercellular adhesion molecule and toll-like receptor -3 expression[90]. Our recent study also showed that herpes simplex virus type 1 infection facilitates invasion of *S. aureus* into the nasal mucosa and CRSwNP tissue[91]. In humans with acute respiratory illnesses, however, children with HRSV and *Mycoplasma pneumonia* co-infection had more severe airway inflammation than those with HRSV infection alone[92], bacteria (*influenzae*, *catarrhalis* and *pneumoniae*) are more likely to be detected among virus-negative specimens compared to virus-positive burden[93]. Similarly, mice with pre-symptomatic influenza infection are less susceptible to secondary methicillin-resistant *S. aureus* infection[94], due to the persistence of IL-13 signaling that was advantageous for resolving methicillin-resistant *S. aureus* infection. These observations suggest a profound deference of host defense upon different pathogen species infection. Further investigations addressing potential interactions between specific viruses and bacteria in allergic airway diseases are therefore warranted.

Perspectives

In this paper, we have summarized the evidence for viruses and bacteria to initiate or maintain type 2 responses in the airways. Those infectious agents are commonly associated with Th1 and Th17 responses. We here stress that apart from allergens, specific germs and viruses also may play a critical role in the induction of type 2 immune responses. We assume that not one single event will be enough to initiate and certainly not to maintain Th2 inflammation, but rather that multiple hits are necessary over time, involving microbes, allergens and environmental factors (**Figure 4**).

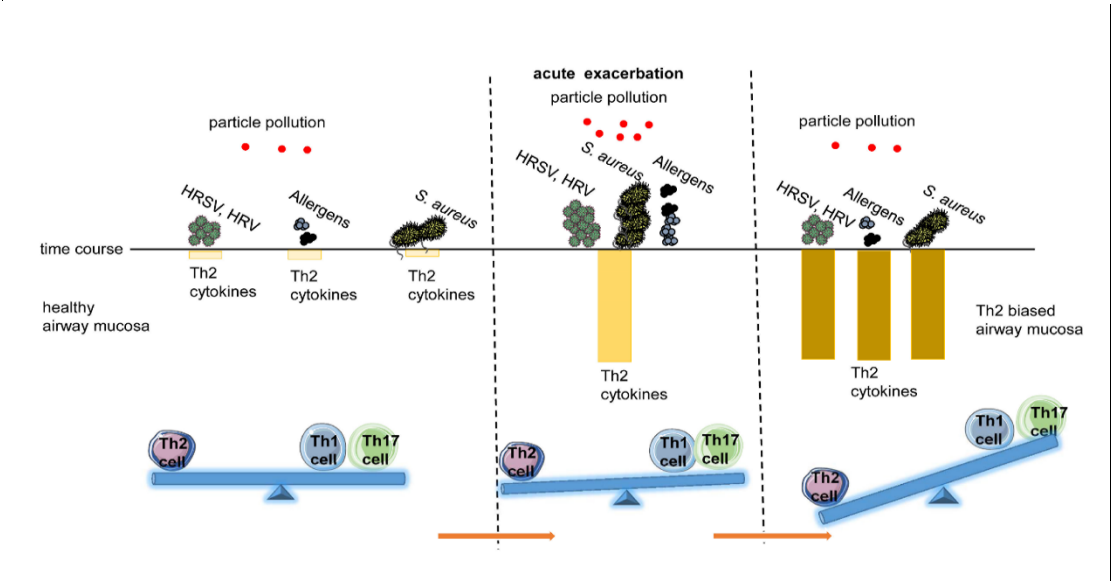

Figure 4 Multiple hits may initiate or maintain Th2 biased immune response in airways. Low numbers of viruses and /or bacteria may persistently exert pressure as colonizers, biofilm formers or intra-mucosal intruders. And high number of microbes may hit the airway mucosa during an acute infection exacerbation and result in plenty of Th2 cytokine release. The balance among Th1, Th2, and Th17 responses, the status of airway mucosa and the some environment are also involved in Th2 biased immune response initiation or maintenance in airways. HRV: human rhinovirus, HRSV: human respiratory syncytial virus, *S. aureus*: *staphylococcus aureus*, Th: T helper.

The impact of such events probably depends on the quantity of viruses and/or bacteria and the time course, e.g. high numbers of microbes may hit the airway mucosa during an infection, and low numbers may persistently exert pressure as colonizers, biofilm formers or intra-mucosal intruders. Furthermore, the immune status of the airway mucosa will make a difference, with a Th1/Th17 biased mucosa being more resistant to Th2 pressure than an already Th2-biased inflammatory status. As we have discussed, these different Th milieus may co-exist, and thus the balance among Th1, Th2, and Th17 responses may determine the course and speed of disease development. Finally, a Th2 response may establish itself within the mucosa, which leads to further negative effects; a Th2

bias indices an insufficiency to defend the mucosa against viral and bacterial infections. For example, our group has demonstrated that *S. aureus* can induce type 2 cytokine release in CRSwNP tissue, but not in healthy control mucosa (publication submitted).

Some literatures indicated that there is no difference of the prevalence rate of viruses and bacteria between allergic airway diseases and controls. The following limitations have to be taken into account: Although culture-independent techniques have the ability to detect more microbes than culture techniques, culture-dependent methods so far remain a better approach for microbiome isolation. In this review, only a cited paper from Michael Inouye's[6] group has carried out additional culture dependent approach in parallel to reduce the possibility of false findings. Some another factors such as samples from different part of airways, the influence of medication especially the use of antibiotics, an acute respiratory infection occurred in the intervening period between sampling, and sampling from different seasons also affect results. Therefore, standardized microbiome identifications are very necessary to gain useful data.

In children, the most common viruses found in respiratory tracts are HRV and HRSV during acute infections, which are associated with a high risk for subsequent wheezing and asthma exacerbation. Thus, HRSV or HRV vaccination has been recommended in high-risk children for prevention of childhood asthma. However, bacteria such as *Haemophilus* and *Staphylococcus* were abundant after applying the seven-valent pneumococcal conjugate vaccine, targeting *Streptococcus pneumoniae* serotypes[25]. Thus, the subsequent bacterial composition when implementing viral vaccines needs careful monitoring.

In the absence of effective anti-viral therapies, targeting pathogenic bacteria for manipulating the microbiome by antibiotics may be an alternative approach. In contrast, several epidemiological studies reported that antibiotic consumption in the first year of life was associated with a risk of asthma in later life[6, 95]. Due to the "common mucosal response" concept which means alterations in immune function of the respiratory tract are linked to the immunomodulatory activity of the gut microbiome, oral administration of probiotics was used for treating allergic airway diseases by changing the gut microbiome[3]. Some investigations have shown that oral administration of probiotics and prebiotics may benefit allergic rhinitis patients[96-98]. Moreover, the local nasal

administration of Lactococcus lactis NZ9000 can affect local and systemic immune responses against *Streptococcus pneumoniea*[99]. Probiotics seem a potentially therapeutic approach for allergic airway diseases, but there are still many controversial issues to be solved. The meta-analysis by Elazab *et al*. has demonstrated that early probiotic administration does decrease atopic sensitization, but it does not reduce the risk of developing asthma[100]. The differences in study design and the usage of different probiotic strains or combinations can lead to completely different results. Adequately controlled experiments with standardized criteria using specific strains, dosages and timing are anticipated to provide more insight.

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CHAPTER 2

REGULATORY T CELLS IN ALLERGIC AIRWAY DISEASES

Regulatory T cell in allergic airway diseases

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Abstract:

Allergic airway diseases are characterized by a skewed T helper 2 (Th2) response and decreased numbers of regulatory T (Treg) cells. Treg cells potentially suppress deleterious activities of effector T cells and maintain a state of tolerance against antigens in airway mucosa. Therefore, understanding the role of Treg cells in allergic airway diseases is crucial for therapeutic interventions. This paper briefly reviews the naturally occurring Treg (nTreg) cells and different phenotypes of induced Treg (iTreg) cells in allergic airway diseases. Recent findings on their identification, generation, migration, and suppressive function as well as their modulation in airway diseases are also discussed.

Introduction

The airway mucosa is constantly exposed to airborne allergens and exogenous particles. The immune system analyzes and appropriately responds to these exposures, forming a state of immune tolerance. Regulatory T (Treg) cells play a crucial role in tolerance induction in airway mucosa. A deficiency or dysfunction of Treg cells may cause allergic airway inflammatory diseases with exacerbated effector T cell proliferation and cytokine production against foreign pathogens.

Allergic airway diseases discussed in this study are chronic rhinosinusitis (CRS), allergic rhinitis (AR) and allergic asthma. CRS, representing an inflammation of the nose and paranasal cavities, is further divided into two subgroups: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). European CRSwNP, AR and allergic asthma are characterized by a skewed T helper 2 (Th2) response, elevated IgE concentrations and decreased number of Treg cells[1-3]. The reinforcement suppressive function of Treg cells is therefore regarded as a plausible strategy to modulate Th2 immune response. However, adoptive transfer of naturally occurring Treg (nTreg) cells in a mouse model of asthma did not provide satisfactory results[4]. The two main reasons could be: i) difficulty in obtaining sufficient numbers of Treg cells for therapeutic interventions since Treg cells constitute 1%–2% of $CD4^+$T cells, ii) alteration in the phenotypes and functional characteristics of nTreg cells caused by repetitive T cell receptor (TCR) stimulation [5]. In addition, the inflammatory environment with pro-inflammatory cytokines such as IL-6 and TNF-α could also convert nTreg cells into Th17 cells[6,7]. Although nTreg cells and induced Treg (iTreg) cells share a similar function in both prevention and treating allergic response in an ovalbumin (OVA) induced mouse model of asthma[8], iTreg cells have superior functional characteristics including resistance to Th17 conversion and anti-apoptosis[9]. Therefore, adoptive transfer of iTreg cells may be a promising therapeutic approach in allergic airway diseases. The hypothesis, however, awaits more investigations to be confirmed. In this review, we will focus on recent findings on identification, generation, migration, and suppressive function modulation of nTregs and different types of iTregs such as $CD4^+CD25^+Foxp3^+$iTreg cells, Type 1 regulatory T (Tr1) cells, $Foxp3^+$ invariant Natural Killer T (iNKT) cells, and $Foxp3^+$ gammadelta (γδ) T cells, which are closely associated with airway

inflammatory diseases. The biomarkers for different Treg cells identification are listed in the **Table 1.**

Table 1. The phenotypes of regulatory T cells in airway disease.

Cell type	Cell markers	Origins	Ref.
nTreg cell	$CD4^+$, $CD25^+$, $CD127^{low}$, $Foxp3^{high}$, $CD6^-$ GITR, CTLA-4 (activated nTreg)	Thymus; $Foxp3^-CD25^+$nTreg cell precursor	18-20, 23-24
$CD4^+CD25^+Foxp3^+$ iTreg cell	$CD4^+$, $CD25^+$, $CD127^{low}$, $Foxp3^{high}$,	Periphery; $CD4^+CD25^-$T cells	18-22
Tr1 cell	$IL\text{-}10^+$, $Foxp3^-$, $ICOS^+$, $PD\text{-}1^+$, $CD49b^+$, $LAG\text{-}3^+$, $CD226^+$	Periphery; conventional or naïve or $CD4^+$T cell (human); $CD4^+CD62L^-Foxp3^-$ cell; (human and mouse)	59-63
$Foxp3^+$ iNKT cell	$CD25^+$, $Foxp3^+$, $CD1d^+$, invariable TCR, $CTLA4^-$ or $GITR^-$	Periphery; iNKT cells include in: $CD1d^+V\alpha14J\alpha18$ with Vβ8, or Vβ7 or Vβ2 cell (mouse); $CD1d^+V\alpha24J\alpha18V\beta11$ cell (human);	74-75, 81
$Foxp3^+$ γδ T cells	$TCR\gamma\delta^+$, $CD25^+$, $Foxp3^+$	Periphery; human central memory γδ T cells($CD27^+CD45RA^-$); mouse splenocytes;	93-94

nTreg, natural occurring regulatory T cell; iTreg, induced regulatory T cell; γδ, gammadelta; Tr 1, Type 1 regulatory T cell; Foxp3, fork head box p3, CTLA, cytotoxic T lymphocyte-associated antigen-4; GITR, glucocorticoid-induced tumor necrosis factor receptor; TCR, T cell receptor LAG-3, lymphocyte activation gene3; ICOS, Inducible T cell co-stimulator; PD1, programmed cell death-1.

Naturally occurring regulatory T cells

Regulatory T cells are generally divided into nTreg cells and iTreg cells. nTreg cells mature in the thymus under the influence of interactions between TCR and autoantigens; while iTreg cells are generated from their precursors in the periphery of secondary lymphoid organs or mucosa tissues. The generation of nTreg cells in thymus requires two steps: TCR/CD28 and RelB-dependent medullary thymic epithelial cells control $Foxp3^{-}CD25^{+}$nTreg cell precursors generation; IL-2 signaling subsequently induces the generation of $Foxp3^{+}CD25^{+}$nTreg cells from the $Foxp3^{-}CD25^{+}$nTreg cells[10-13]. IL-2 is dispensable for nTreg cells development in thymus[14], whereas it is indispensable for nTreg cell peripheral survival [11]. The proliferation of nTreg cells in periphery is associated with an increased TCR-mediated signaling or CD28 co-stimulation[15-17].

To identify nTreg cells, biomarkers $CD4^{+}$, $CD25^{+}$, $CD127^{low}$ and $Foxp3^{high}$ are required. A high expression of Helios, neuropilin-1(Nrp1), programmed cell death-1(PD-1) and Swap70 on nTreg cells is also used to distinguish nTreg cells from iTreg cells[18-20]. However, conflicting data shed doubt on their specific roles[21,22]. Recently, human nTreg cells were reportedly characterized by low CD6 expression [23]. Moreover, the active form of nTreg cells selectively expresses markers such as glucocorticoid-induced tumor necrosis factor receptor (GITR) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)[24].

nTreg cells are generated in the thymus, mediating their suppressive function in lymphoid organs and peripheral inflammatory sites[25]. Therefore, knowing its migration from thymus to secondary lymphoid organs and further to periphery *in vivo* helps us understand the underlying mechanisms of nTreg-mediated suppression. nTreg cells are primed by interacting with antigens in draining lymph nodes, and then the active nTreg cells concomitantly control the proliferation of responder T cells[26]. Although mouse nTreg cells express both chemokine receptor (CCR)4 and CCR7[27], the migration of nTreg cells to lymph nodes to interact with antigens largely depends on CCR7 not CCR4[28]. Thus, without priming in lymph nodes, an increase of $CCR7^{-/-}$ nTreg cells in lung tissue of mouse inefficiently suppresses Th2 and Th17 responses induced by house dust mite (HDM)[29]. In periphery, CCR4 and CCR7 were involved in the migration of mouse nTreg cells at different stages of airway allergic response. CCR7 mediated Treg cells migration to the lymph nodes is essential during an allergen sensitization phase, whereas CCR4-dependent Treg cell homing to lungs or even

to broncho-alveolar lavage fluid is required to suppress a recall response to an inhaled allergen and consecutive allergic pulmonary inflammation[30, 31]. Consistent with mouse studies, human subjects with asthma had an increase in CCR4 expression in nTreg cell of lungs after the segmental allergen challenge[30]. In nasal tissue, the number of nTreg cells trafficking towards nasal airway mucosa decreased in CRSwNP subjects in comparison with controls due to the reduced chemotactic response of nTreg cells to CCL1[32]. Hereby, modulation of chemokine receptor expression on nTreg cells or chemotactic response of nTreg cells may restore the deficiency or dysfunction of nTreg cells in airway allergic diseases.

$CD4^+CD25^+Foxp3^+$ induced regulatory cells

Surface markers of $CD4^+CD25^+Foxp3^+$iTreg cells are similar to nTreg cells. Distinguishing $CD4^+CD25^+Foxp3^+$iTreg cell from nTreg cell or other T cell subtypes has been widely hampered due to lack of specific markers. However, Helios, Nrp1, PD-1 and Swap70 described above are still useful for $CD4^+CD25^+Foxp3^+$iTreg cell identification.

In a TCR-transgenic mouse model, $CD4^+CD25^+Foxp3^+$iTreg cells were generated in the absence of nTreg cells, implicating that the generation of iTreg cells may be independent of nTreg cells *in vivo*[33]. Both mouse and human $CD4^+CD25^-$T cells were converted into $CD4^+CD25^+Foxp3^+$iTreg cells by triggering TCR in the presence of transforming growth factor beta (TGF-β) or in combination with IL-2[34, 35]. 1, 25-Dihyroxyvitamin D3 binding to the vitamin D response elements in the Foxp3 conserved non-coding sequence region, also can induce Foxp3 expression in human $CD4^+CD25^-$T cells[36]. Additionally, mouse lung tissue resident macrophages contributed to the conversion of $CD4^+$T cells into $CD4^+CD25^+Foxp3^+$iTreg cells in a TGF-β and retinoic acid dependent manner[37]. The induction of Foxp3 is critical for $CD4^+CD25^+Foxp3^+$iTreg cell generations. Therefore, a better understanding of Foxp3 regulation is useful for obtaining more iTreg cells in periphery. Foxp3 hypermethylation was associated with pediatric asthma exacerbations or asthma development in response to air pollutant exposures[38,39]. Reportedly, DNA hypermethylation inhibitors boosted Foxp3 expression and increased the expansion of $CD4^+CD25^+Foxp3^+$iTreg cells to alleviate airway inflammation in the OVA-sensitized mice[40]. Recently, our group demonstrated that suppressor of cytokine signaling 3 (SOCS3) indirectly

regulates Foxp3 expression in human CRSwNP tissues[41], which may also contribute to the induction of $Foxp3^+$iTreg cells in allergic upper airway diseases. In comparison to nTreg cells, $CD4^+CD25^+Foxp3^+$iTreg cells efficiently decreased Th2 asthmatic inflammation and reduced specific IgE levels in the airways[42, 43], which confirms that $CD4^+CD25^+Foxp3^+$iTreg cells could be a possible therapeutic approach for airway allergic inflammatory diseases.

iTreg cells are induced in periphery or *ex vivo*, however understanding their migration to peripheral inflammatory sites is also important for immune homeostasis. Defective trafficking properties of $CD4^+CD25^+Foxp3^+$iTreg cells have been found in mucosal of the OVA-induced persistent hyper-responsiveness (AHR) model. Intranasal transfer of aeroallergen loaded airway mucosal dendritic cells from naïve rats into AHR susceptible rats during prolonged aerosol challenge markedly boosted subsequent iTreg cells accumulation in the airway mucosa[44]. In other words, changing the properties of antigen presenting cells will attract more $CD4^+CD25^+Foxp3^+$iTreg cells to peripheral inflammatory sites. This may be relevant to immunotherapy for allergic disease by up-regulating the expansion of specific Treg populations via repeated mucosal exposure to allergens.

Different from nTreg cell, $CD4^+CD25^+Foxp3^+$iTreg cells exhibit non-Ag-specific bystander activity *in vitro*. Both nTreg cells and iTreg cells suppress responder T cells and antigen presenting cells (APCs) via two ways: cell-cell contact or soluble inhibitory cytokines. Active nTreg cells kill $CD4^+$ and $CD8^+$ T cells and other cell types in a perforin-dependent, Fas-FasL-independent manner[45,46]. After activation, nTreg cells are less dependent on co-stimulatory signals in comparison to responder T cells. Thus, the blockage of B7/CD28 and CD40/CD40L signals alters the balance between responder T cells and nTreg cells, which is in favor of nTreg cells activities in the OVA induced mouse model of asthma[47,48]. Both the responder T cells and APCs can be the targets of Treg cells, but the mechanisms of Treg cells on the responder T cells are different from those on dendritic cells (DCs). The interactions of CTLA-4 on nTreg cells with CD80/CD86 on DCs are an important pathway by which nTreg cells could mediate their suppressive function on DCs *in vitro*[49]. IL-10 produced from Ag-specific iTreg cells, not CTLA-4, plays a critical role in down-regulation of CD80/CD86 on DCs in CTLA-4 deficient mice[50]. More recently, the adhesion molecule lymphocyte

activation gene-3(LAG-3) was also reportedly involved in the suppressive activity on nTreg cells by binding to MHC class II on DCs and interfering with the maturation and immune stimulatory capacity of DCs[51]. Surprisingly, no appreciable difference in ability to suppress T cell proliferation *in vivo* between wild type nTreg cells and LAG-3$^{-/-}$ nTreg cells has been described[52]. LAG-3$^{-/-}$T cells show a relatively persistent ability to be suppressed, which means effector T cells with LAG-3 expression are more susceptible to suppression[52]. Apart from cell-cell contact, inhibitory cytokines IL-10, TGF-β, adenosine and IL-35 are associated with the suppressive function of nTreg cells and iTreg cells[53-55]. IL-10 and adenosine showed their suppressive properties by influencing the maturation and co-stimulatory molecule expression of DCs[53, 54]; TGF-β maintains Foxp3 expression in Treg cells [56]. In allergic airways of mice, nTreg cells exhibit a more effective suppressive function towards Th1 than Th2 responses via cAMP[57]. IL-35 produced by mouse nTreg cells was responsible for suppressing IL-17 dependent AHR[58] .

Type 1 regulatory T cells

Tr1 cells are an inducible regulatory T cell population in human CRSwNP tissue and mouse lung tissue, characterized by the abundant production of IL-10 and absence of Foxp3 expression[59, 60]. Inducible T cell co-stimulator (ICOS), PD-1, CD49b, LAG-3 and CD226 are expressed on Tr1 cells, although, none of them can be regarded as a specific marker[61,62]. At present, the co-expression of CD49b, CD226 and LAG-3 are used for human and mouse Tr1 cell purification and isolation[63].

Human Tr1 cells are induced from naive CD4^{+}T cells or conventional CD4^{+}T cell precursors triggered by IL-27 and IL-10 respectively[64, 65]. CD3 and CD46 stimulation in the presence of IL-2 contributes to human Tr1 cells conversions from CD4^{+}T cells[66]. However, poor proliferation of Tr1 cells was observed owing to a high rate of cell death[67]. Nasal administration of CD3 antibodies induced Tr1 cells from CD4^{+}CD25^{-} T cells depending on IL-27 secreted by upper airway resident DCs[68]. In addition, a component of extracellular matrix, hyaluronan, promoted Tr1 cell generations from human or mouse conventional CD4^{+}CD62L^{-}Foxp3^{-} T cell precursors[69]. nTreg cells are also a candidate for Tr1 cell inductions upon the stimulation of CD46 and TNF-α[70], implicating that the local environment is important for the plasticity of nTreg cells. In AR patients, the number of Tr1

cells and not nTreg cells were decreased in peripheral blood[71]. Thus, Tr1 cell induction in local nasal tissue could be an attractive target in inhibiting the allergic inflammatory response in AR.

The suppressive function of Tr1 cells is mainly mediated by inhibitory cytokines, specifically IL-10 and TGF-β[72]. However, neutralizing antibodies against IL-10R and TGF-β cannot completely diminish Tr1 cell suppressive functions[72]. Reportedly, human Tr1 cells specifically lyse APCs via granzyme B and perforin, which confirms that cell-cell contact is also involved in the suppressive activity of Tr1 cells[73].

Foxp3$^+$ invariant Natural Killer T cells

iNKT cells are unique CD1d-restricted innate-like T cells, which shares the properties of both T cells and natural killer cells. Type 1 iNKT cells have been better described than type 2 iNKT cells owing to their invariable TCRs (Vα14Jα18 α-chains paired with a limited number of β-chains Vβ8, Vβ7, Vβ2 in mice, Vα24Jα18 α-chains paired with Vβ11 chain in humans)[74, 75]. Activated iNKT cells in the mice models of asthma or peripheral blood from asthmatic patients are capable of producing Th1, Th2, or Th17 cytokines[76-80]. When Foxp3 is induced, Foxp3$^+$iNKT cells obtain immunosuppressive properties, with the expression of CD25 but not necessarily CTLA4 or GITR[81]. Thus, combined markers (CD25$^+$, Foxp3$^+$, CD1d$^+$ and invariable TCRs) can be used for human and mouse Foxp3$^+$iNKT cell identification.

Human Foxp3$^+$iNKT cells were induced from CD25$^+$CD1d$^+$iNKT cells in the presence of TGF-β plus rapamycin[81]. In contrast to an earlier study, it was found that IL-10 plus rapamycin rather than TGF-b induced Foxp3 expression in freshly isolated peripheral blood Vα24$^+$Vβ11$^+$iNKT cells [82]. However, Foxp3$^+$iNKT cells which are generated from different ways show the same suppressive activity. Recently, it was also reported that the selected human iNKT cell clones (CD4$^+$, double negative) acquired a dramatic increase of Foxp3 expression upon anti-CD3 stimulation; the induction of Foxp3 was also observed in a CD4$^+$ iNKT cell clone upon PMA-ionomycin treatments[83].

Although, Foxp3$^+$iNKT cells inhibit the proliferation of conventional CD4$^+$ cells in a cell-cell contact manner, the mechanisms mediating the suppressive function is still unclear[81]. Foxp3$^+$iNKT

cells are excluded from lymph nodes and preferentially migrate to peripheral tissues in association with the expression of CD103 cells[84]. However, further investigations are still required to detail the migration of Foxp3$^+$iNKT cells.

Foxp3$^+$ gammadelta T cells

γδT cells, with γδ TCR expression, directly recognize conserved non-peptide antigens and lipids independent of MHC/peptide complexes, which is different from classical αβ T cells. According to the usage of Vδ-chain, γδT cells can be mainly divided into 2 subsets Vδ1 and Vδ2; the different TCR arrangement directly affects their eventual principle tissue of residence. In humans, Vδ1 cells with Vγ1 or Vγ4 are mainly located in lung and nasal mucosa[85, 86]; Vδ2 cells with Vγ9 account for 50–95% of circulating γδ T cells in the peripheral blood[87]. In mice, Vγ1 and Vγ4 are present in the lymphoid tissues and Vγ1, Vγ4, and Vγ6 are present in the lungs[88]. The surface markers CD45RA and CD27 further classify Vδ2$^+$ or Vδ1$^+$ cells into naive (CD45RA$^+$CD27$^+$), central memory (CD45RA$^-$CD27$^+$), effector memory (CD45RA$^-$CD27$^-$), and effector (CD45RA$^+$CD27$^-$) statuses[89]. Untill now, the role of γδT cells in allergic airway diseases remains ambiguous. Mouse Vγ1$^+$δ1 T cells promoted airway inflammation or AHR by enhancing the systemic IgE response. In contrast, Vγ4$^+$δ1 T cells alleviated AHR, the Th2 airway allergic response and eosinophilic infiltrations in OVA sensitized mice[90,91]. Increased γδT cells were found in the peripheral blood of moderate/severe house dust mite positive AR patients, which was positively correlated with IL-17 secretions[92]. Surprisingly, inflammatory γδT cells obtain a suppressive function when Foxp3 is induced, and different phenotypes of Foxp3$^+$γδT cell have different surface markers. Human Foxp3$^+$Vδ2 T cells express CD25$^+$ CD27$^+$ CD45RA$^+$[93], while human Foxp3$^+$Vδ1 T cells exhibit CD25$^+$ CD27$^+$ CD45RA$^-$ expression[94]. Similar to human Foxp3$^+$γδT cell, TCRγδ and CD25 are also used for mouse Foxp3$^+$γδT cell isolation[95]. Recently, a novel mouse regulatory γδ T cell CD39$^+$γδ T cell, with the expression of CD25, CD27, CD39 and CD122 but not Foxp3, has been identified both *in vitro* and *in vivo*[96]. Taken together, TCRγδ, CD25 and Foxp3 are used for human and mouse Foxp3$^+$γδ T cell identification.

Casetti *et al*. [93] reported that the induction of Foxp3$^+$Vγ9Vδ2 T cells from human central memory γδ T cells (CD27$^+$CD45RA$^-$) in the presence of Ag stimulation and cytokines (TGF-β1, IL-15) had

an anti-proliferative effect on $CD4^+$ T cells. Quite surprisingly, freshly isolated Vδ2γδ T cells without Foxp3 expression also can suppress the activity of responder $CD4^+CD25^-$αβ T cell via the interactions between CD86 on Vδ2 T cells and CTLA-4 on αβ T cells[97]. Therefore, it seems that Vγ9Vδ2 T cells with or without Foxp3 expression have suppressive functions. However, whether they have their suppressive function *in vivo* or allergic airway diseases awaits further investigations. In mice, γδTCR and TGF-β stimulation induced $Foxp3^+$γδT cell from mouse splenocytes[95].

The inducible $Foxp3^+$Vδ1 Tregs showed a potent anti-proliferative effect on $CD4^+$ T cells via cell-cell contact, although, cytokine-dependent mechanism might also play a role in suppressive functions [93]. The question whether $Foxp3^+$Vδ1 γδ regulatory T cells are capable of mediating suppressive functions to $CD4^+$γδ T cells is yet to be answered.

The effect of allergic airways treatments on Treg cells

Treg cells are very important for Th2 response alleviation in allergic airway diseases. At the same time, clinical therapy may also affect the role and the number of Treg cells in allergic disease. In the treatment of allergic airway disease, corticosteroids provide temporary suppression of inflammatory cytokines and immune cells. Pre-incubation of human nTreg cells with fluticasone propionate increased the subsequent suppressive actions of nTreg cells in allergen-stimulated $CD4^+CD25^-$T cells by an IL-10-dependent mechanism[98]. Allergen-SIT has been used as a desensitizing therapy for allergic diseases. HDM-specific immunotherapy (SIT) can induce specific Tr1 cells that abolish HDM-induced proliferation of Th1 and Th2 cells, as well as their cytokine production[99]. Beside Tr1 cells, $CD4^+CD25^+Foxp3^+$iTreg cells generated by Allergen-SIT also contribute to the suppression of airway eosinophilia in OVA-mouse model of asthma upon OVA challenge[100].

Conclusions

Most of the current information relating to the role of Treg cells in allergic airway diseases focuses on asthma. Allergic asthma is mainly characterized by a Th2-biased response, thus the findings of Treg cells in asthma may also have important implications for understanding their role in other Th2 biased diseases, such as AR and CRSwNP. However, with an increasing emphasis on the

distribution and contribution of Treg cell subtypes in allergic diseases, extensive research from asthma to upper airway allergic disease or from animal models to human samples is required in the near future.

Innate T cells such as iNKT cells and γδ T cells are located in the airway mucosa[101]. T Activated iNKT cells in peripheral blood from asthmatic patients are capable of producing Th1, Th2, or Th17 cytokines[76-80]. Increased γδT cells were found in the peripheral blood of moderate/severe house dust mite positive allergic rhinitis patients, which was positively correlated with IL-17 secretions[92]. Therefore, attempts to restrict the activities of iNKT cells or γδT cells in allergic airway disease by converting them into Foxp3$^+$iNKT cells or Foxp3$^+$γδT cells will be beneficial for treating allergic airway diseases.

Over the last 5–10 years, the role of nTreg cells and CD4$^+$CD25$^+$Foxp3$^+$iTreg cells in airways has been studied extensively. Certainly, understanding the appropriate markers to identify nTreg cells and iTreg cells is essential to study their frequency and function. However, research demonstrated the role of Treg cells in the airways without distinguishing their subtypes, especially of nTreg cells and CD4$^+$CD25$^+$Foxp3$^+$iTreg cells. Thus, it is important to define the different subtypes of Treg cells and further investigate their ability to modulate inflammation in allergic airway diseases. As mentioned above, Tr1 cells, Foxp3$^+$iNKT cells and Foxp3$^+$γδT cells do suppress allergic responses **(Figure1)**. Generation of human Treg cells and enhancing their functions in the

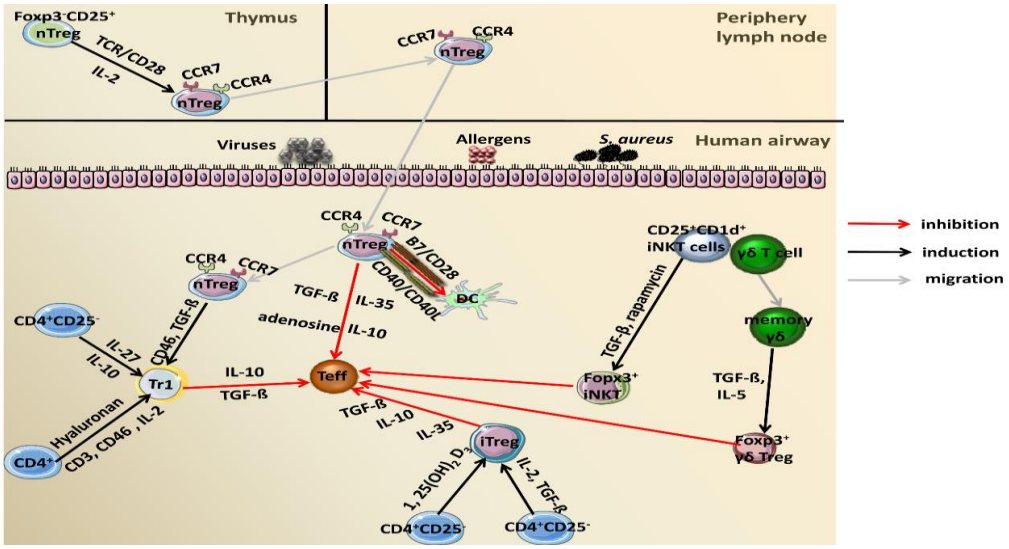

Figure 1. Different subtypes of regulatory T cell (Treg) in allergic airway disease. Allergic airway disease is caused by inappropriate Th2-driven immune responses to viruses, bacteria, and allergen in the

environment. nTregs are generated in thymus thereafter , they migrate from thymus to periphery lymph node or from periphery lymph node to human airway tissue which is mediated by different chemokine receptors (CCRs). The interactions between nTreg cells and DCs could mediate their suppressive function on dendritic cells (DCs), at the same time nTreg cells can be activated after interactions. In turn, active nTreg cells suppress T effector (Teff) cells via two ways cell-cell contact and soluble inhibitory cytokine release such as TGF-β, IL-10, IL-35. Induced regulatory T (iTreg) cells, generated from $CD4^{+}CD25^{-}$ T cell in periphery, obtain suppressive function similar as nTreg cells. Another three iTreg cells such as type 1 regulatory T (Tr1) cell, $Foxp3^{+}$ invariant Natural Killer T (iNKT) cells, $Foxp3^{+}$gammadelta(γδ)T cells can also be induced in human airway tissues. They do show a potent anti-proliferative effect on $CD4^{+}$ T cells, however, the mechanisms are partially unveiled.

airways may be an attractive concept. In parallel, a defective migration also leads to the deficiency and dysfunction of nTreg cells; thus, enhancing the migration of nTregs or iTregs by increasing the chemotactic response may be a possible strategy for allergic airway disease treatment. Of importance, the environment of Treg cells has influence on peripheral Treg cells maintenance. Thus, comprehensive treatment, considering iTreg cell generation and their suppressive function and enhancement of migration to restore Treg cell efficiency in allergic airway disease should be useful to modulate allergic responses in the future.

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AIMS OF THE STUDY

The definition, diagnosis of CRS. Chronic rhinosinusitis (CRS) is a heterogeneous group of diseases of the nose and the paranasal sinuses that lasts for three months or longer. According to the European position paper on rhinosinusitis and nasal polyps[1], symptoms of CRS may include the following: nasal obstruction, nasal secretion and/or post-nasal drip, headaches and/or facial pains, a reduction of smelling for more than 12 weeks during the last year while at least one of the first two mentioned symptoms should be observed. The clinical diagnosis of CRS is currently based on symptoms and duration of symptoms, clinical examinations, nasal endoscopy and CT-scan. With reference to the endoscopic findings, the difference is made between the clinical phenotypes of CRS without nasal polyps (CRSsNP) and CRS with nasal polyp (CRSwNP)[1]. Histologically, CRSsNP is characterized by fibrosis of the mucosa and the basal membrane, while CRSwNP is characterized by an important edema with deposition of albumin and the development of pseudocysts. The clinical pattern of symptoms is overlapping in patients with CRSsNP and CRSwNP. The differentiation of the inflammatory subtypes based on T helper (Th) cells allows a more differentiated classification according to pathomechanical principles into so-called endotypes within the clinical phenotypes which can finally be used to define innovative therapeutic approaches.

Epidemiology of CRS. Recently, the Global Allergy and Asthma Network of Excellence[2] published a first multicenter prevalence study on chronic rhinosinusitis based on 56,000 questionnaires by 19 centers in 12 European countries. A random sample was also submitted to clinical examination by a specialist in order to confirm the diagnosis. The investigation came to the conclusion that the prevalence of CRS in Europe amounts to 10.9%, between 6.9 and 27.1% in different European cities [3]. In the USA, the prevalence of CRS is slightly higher [4]. Among all CRS patients in the USA, about 20–33% suffer from CRSwNP, 60–65% suffer from CRSsNP, and 8–12% from allergic fungal sinusitis[4]. CRS was more common in smokers than non-smokers[3]. Co-morbidities such as asthma and aspirin hypersensitivity are frequent in nasal polyposis.

Therapeutic options. Medical treatment consisting of nasal corticosteroids and antibiotics is the first step in treatment. Topical intranasal corticosteroids have shown to be safe and effective in reducing polyp size[5]. There anti-inflammatory effect is localized and their systemic absorption has been shown to be negligible, however, efficacy is limited. The use of systemic corticosteroids has

been widely used in treatment of CRSwNP, however systemic side effects limit its usefulness. Adverse effects of corticosteroid use include diabetes, peptic ulcer disease, hypertension, and osteoporosis. Nowadays, functional endoscopic sinus surgery has become the standard procedure to restore sinus ventilation and drainage by opening the key areas while preserving sinus mucosa, according to the European position paper on rhinosinusitis and nasal polyps[1]. However, recurrence after surgery is frequent, and other solutions are urgently need. Immunoglobulin E (IgE) antibodies directed against staphylococcal superantigens and other staphylococcal proteins as well as against inhalant allergens have been found in the nasal tissues of CRSwNP patients; Th2 cytokines are also highly expressed in this subgroup of patients. In addition, IgE antibodies directed against staphylococcal superantigens have been found in the nasal tissues of CRSwNP patients[6]. Recently, humanized antibodies such as omalizumab (anti-IgE), reslizumab and mepolizumab (anti-IL-5), as well as dupilumab (anti-IL-4/13) open new perspectives, and these are currently evaluated in clinical studies[7-9]. Th2 associated mediators and IgE antibodies are targets for these humanized antibodies in CRSwNP.

Innate lymphoid cells in CRS. A progress in the field of immunology is that a novel family of CD45 expressing haematopoietic effector cells has been identified. These cells have important effector and regulation functions in the innate immune defense and remodeling of tissue but lack rearranged antigen specific surface receptors of adaptive immune cells and are therefore called innate lymphoid cells (ILCs)[10]. ILCs are classified into three categories based on the characteristic patterns of the cytokines they produce and the transcription factors that are necessary for their development and function analogous to the T helper cells: Group 1 ILCs (ILC1) produce interferon γ and are dependent on the transcription factor T-box expressed in T cells (T-bet), while group 2 ILCs (ILC2) can produce Th2 cytokines such as IL-5 and IL-13 and need the transcription factor GATA binding protein 3 (GATA3). Group 3 ILCs (ILC3) also comprise lymphatic tissue inducing cells. ILC3s can produce IL-17 and/or IL-22 and require RAR-related orphan receptor gamma (RORγt) as transcription factor[10]. In this regard, ILCs are very similar to T helper cells, however, they are part of the innate immune defense and according to the current knowledge, they likely form the link between epithelium and T cell compartment[11]. Chemoattractant receptor homologous

molecule expressed on Th2 cells (CRTH2) and ST2L (IL-33 receptor) positive ILC2s have been detected in healthy human lungs; however, it is currently unclear which functions ILC2s have in humans in comparison to Th2 effector cells (that both produce IL-5 and IL-13) in the context of pulmonary hemostasis. Also, $CRTH2^{+}$ILC2s have been identified in CRSwNP tissue[12]. TSLP, an ILC2 activators produced by epithelial cells, was increased in epithelial cells in patients with severe asthma[13] or CRSwNP[14]. Only little is known about the function of other ILC subgroups in the airways.

T helper cell pattern of CRS. CRSsNP appears as an only moderate, mainly neutrophil Th1 or Th17 polarized inflammation; CRSwNP is characterized by a moderate to high eosinophilic Th2 polarized inflammation besides a neutrophil component, at least in Caucasians[15]. Presumably, the reduced TGF-β expression in the CRSwNP tissue contributes to a deficit of Tregs[16]. The relative deficit of Tregs could be the reason for the inability to suppress the eosinophilic inflammation in CRSwNP patients. In Caucasians, more than 80% of the polyps express a Th2 profile with clear expression of interleukin-5 protein and consecutive tissue eosinophilia whereas this profile is found in less than 20% of all central Chinese CRSwNP patients. Instead, there are Th17 cells that induce a mainly neutrophil inflammation reaction[17].

Microbiome and CRS. The nasopharynx is regarded as a considerable reservoir for bacteria in our body. With the advent of the field of molecular biology, culture independent methods have been developed in order to examine microorganisms based on their genetic patterns. As a consequence of this, a more complex flora was discovered in the upper airways than had previously been suspected. The rate of picornavirus, human respiratory syncytial virus, influenza virus, and parainfluenza viruses was significantly increased in CRS patients compared with that seen in the controls[18]. Frequently occurring germs of bacterial infections of CRS patients with asthma are germs like *Staphylococcus aureus*, *Hemophilus influenza*, *Pseudomonas aeruginosa, and Moraxella catharralis*[19]. Today it is well known that the nasal mucosa is always, at any time in life, colonized with hundreds of different bacteria that – as far as their presence is balanced – guarantee a healthy condition. However, the disturbed balance of the microbiome in Th1, Th2, and Th17 biased responses may determine the course and speed of disease development. Of interest, knowing the

role of microbes present in nasal tissue on the balance may help us understand the pathomechanical principles of CRS.

The aims of the studies described in this thesis are to investigate the role of *S. aureus* and herpes simplex virus 1(HSV1) in Th2 biased CRSwNP disease. For this purpose, we took advantage of our nasal *ex vivo* infection model. We exposed the CRSwNP tissue with *S. aureus* and HSV1, and then evaluated the post-infection immune response. Furthermore, the ways to restore the balance and inhibit the Th2 response in CRSwNP tissue was investigated.

Specific aims of this thesis are:

1) To investigate the role of *S. aureus* in the induction of the Th2 response in CRSwNP tissue. We exposed human nasal tissue cubes to *S. aureus* for 2h, and then placed tissue cube at air-liquid conditions. We aimed to unravel whether *S. aureus* without enterotoxin secretion can induce Th2 cytokines and the mechanisms involved in this process. Therefore, we measured Th2 cytokines and epithelial cell derived cytokines at mRNA level and protein level (**Chapter 3**).

2) To study whether HSV1 also affect Th2 cytokine release in CRSwNP disease. Human nasal tissue cubes were infected by viruses HSV1 for 1h, and then placed at air-liquid conditions. We measured Th1, Th2, and Th17 associated cytokines in supernatants of human nasal the *ex vivo* HSV1 infection model. We also used virus titers and immunofluorescence staining to compare the difference of HSV1 invasion in CRSwNP tissue and health tissue (**Chapter 4**)

3) To explore the ways to inhibit the Th2 response in CRSwNP tissue. We switched off the expression of SOCS3 in human airway mucosa, and over-expressed SOCS3 in PANC-1 cell to monitor the increased expression of Foxp3. In addition, we also evaluated Th2 cytokine expressions after Foxp3 was up-regulated in CRSwNP tissue (**Chapter 5**).

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CHAPTER 3

STAPHYLOCOCCUS AUREUS INDUCES TYPE 2 CYTOKINES VIA TSLP AND IL-33 RELEASE IN HUMAN AIRWAY MUCOSA

Staphyloccoccus aureus induces type 2 cytokines via TSLP and IL-33 release in human airway mucosa

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Abstract：

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a T helper cell (Th)2-skewed upper airway inflammation. Mucosal *Staphylococcus aureus* (*S. aureus*) colonization is found in the majority of CRSwNP patients. *S. aureus* may induce type 2 cytokine release via superantigens. Additionally, we speculated that *S. aureus* without the ability to secrete enterotoxins may release epithelial cell-derived cytokines critical in propagating a type 2 immune response.

Methods: Thymic stromal lymphopoietin (TSLP) and IL-33 and consecutive type 2 cytokines were assessed in a human mucosal tissue model upon *S. aureus* infection.

Results: *S. aureus* infection upregulated active forms of IL-33 and TSLP, and IL-5 and IL-13 expression in CRSwNP tissue, accompanied by elevated expressions of TSLP and IL-33 receptors, which were predominantly expressed on $CD3^+$ T cells. Healthy inferior turbinate (IT) tissue after *S. aureus* infection released TSLP, but not IL-5, and *Staphylococcus epidermidis* did not induce any epithelial cell-derived cytokine release in CRSwNP or healthy IT tissue. Increased levels of IL-33 and TSLP were also induced by *S. aureus* in BEAS-2B epithelial cells, accompanied by an activation of nuclear factor-κappaB (NF-κB) pathways. Blocking toll like receptor 2 (TLR2) using a specific antagonist CU-CPT22 significantly reduced the release of TSLP and IL-33 and the activity of NF-κB signal in BEAS-2B cells with *S. aureus* infection.

Conclusions: We here demonstrate for the first time that *S. aureus* can directly induce epithelial cell-derived cytokine release via binding to TLR2, consecutively propagating type 2 cytokine expression in CRSwNP tissue.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory condition in the upper airways which is often accompanied by co-morbid asthma. A smaller population of Chinese CRSwNP patients, but 85% of European CRSwNP patients show T helper cell (Th) 2-biased and eosinophilic inflammation[1-5]. An increase in colonization with *Staphylococcus aureus* (*S. aureus*) and the presence of *S. aureus* enterotoxin-specific Immunoglobulin E (IgE) antibodies have been demonstrated in the mucosa of CRSwNP subjects in comparison with control or chronic rhinosinusitis without nasal polyps (CRSsNP) patients[6, 7], being even higher in CRSwNP patients with asthma comorbidity and aspirin sensitivity. Critically, staphylococcal enterotoxin B (SEB) of *S. aureus* induces considerable release of pro-inflammatory and Th2-associated cytokines including IL-4, IL-5 and IL-13 and also enhances eosinophilic inflammation in human nasal and in mouse bronchial tissue[8-11]. However, a link between *S. aureus* biofilms and skewing of the Th2 response has also been demonstrated independent from enterotoxin activities[12]. Thus *S. aureus* may impact on Th2 responses not only via enterotoxins; those pathomechanisms await discovery.

Epithelial cells act as the first physical defense barrier. Cytokines derived from epithelial cells such as IL-33 and thymic stromal lymphopoietin (TSLP), potentially released by various viral and bacterial stimuli, are critical in orchestrating innate immune responses, specifically a Th2 immune response. IL-33 has been implicated in asthma, and pretreatment of a mouse model of asthma with anti-IL-33 antibodies result in a decrease of serum IgE, eosinophils and concentrations of IL-4, IL-5 and IL-13 in broncho-alveolar lavage fluid. Similar to IL-33, TSLP also can affect Th2-type airway inflammation by trigging dendritic cells[13]. Recently, Nagarkar *et al*. have successfully shown that truncated TSLP with biological activity is increased in CRSwNP, enhancing IL-5 production from mast cells[14]. Besides the classical Th2 cells, a new group of innate lymphoid cells (ILC2s) also can produce type 2 cytokines including IL-5, IL-9 and IL-13 in response to IL-25 and IL-33[15]. These evidences identify a close relationship between epithelial cell derived cytokines and type 2 skewed airway diseases.

Importantly, TSLP can be induced by rhinovirus or double stranded RNA as a surrogate for viral RNA in human airway epithelial cells[16]. IL-33 was related to virus-induced asthma exacerbation

severity and rhinovirus-induced IL-33 was able to strongly induce type 2 cytokines in human T cells and ILC2s[17]. Even though increased levels of IL-33 are shown in an atopic dermatitis mouse model after SEB exposure[18], and SEB reportedly disrupts epithelial integrity through cytotoxic effects and reduces epithelial tight junction expression, increasing cellular permeability in rat intestinal mucosa[19]. However, the role of *S. aureus* inducing type 2 immune responses independent of enterotoxins in human chronic airway disease via epithelial cell derived cytokines has not been elucidated.

S. aureus colonization is frequently found in Th2-biased inflammation in CRSwNP patients with asthma comorbidity[4]. Making use of formerly described human nasal tissue models[20], we here developed a human mucosal tissue *S. aureus* infection model to investigate the possible role of *S. aureus* in a Th2 response in chronic upper airway disease. We specifically analyzed epithelial cell derived cytokine release in CRSwNP tissue induced by a *S. aureus* strain unable to secrete classical enterotoxins such as SEB, and studied the subsequent type 2 cytokines expression.

Methods

Patients Nasal tissues were obtained from 20 CRSwNP patients and 21 control patients at the department of Oto-Rhino-Laryngology, Ghent University Hospital. None of the patients had taken oral or nasal corticosteroids for four weeks and antibiotics for two weeks before surgery. The diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal polyps (EPOS) 2007 guidelines[21]. The inferior turbinates of patients with septal deviation

were used as controls. The clinical data of all the patients are shown in **Table 1**. Informed consent was obtained from all patients before sample collection. The ethics committees of the Ghent University Hospital approved the study.

Table 1 Clinical data of included patients.

	Chronic rhinosinusitis with nasal polyps (CRSwNP) subjects	Control subjects
No. of subjects	20	21
Age, median (range)	47.8(30-65)	31.2 (15-58)
Gender female/male	9/11	10/11
Atopy	7/20	3/21
Asthma	13/20	0/21
AERD	5/20	0/21

AERD, Aspirin-exacerbated respiratory disease;

***S. aureus* and *S. epidermidis* stocks** GFP labeled *S. aureus* RN6390, containing the plasmid pALC1743, a pSK236-derived shuttle plasmid carrying an active *S. aureus* promoter (RNAIII promoter), was chosen as infection strain in this study. In particular, this strain does not produce *S. aureus* enterotoxins which had been confirmed by a lack of enterotoxin detection. Enterotoxins A, B, C, D were tested using SET-RPLA kit in Trypticase Soy medium (BD Belgium) after *S. aureus* overnight culture at 37 ℃, while toxic shock syndrome toxin 1 was assayed using (TST-RPLA KT) (both from Oxoid, Hampshire, UK). To investigate the specific role of *S. aureus*, *Staphylococcus epidermidis* (*S. epidermidis*) (ATCC 14990) was also used in infection model. A concentration of bacteria 20×10^6 CFU/well was prepared and used according to previous experiments[20].

Human airway infection model CRSwNP tissue or control tissue were cut into small cubes approximately 25 mm^2 in size and washed three times by TCM with antibiotics (50 IU/mL penicillin) (Invitrogen, Belgium) and 50 μg/mL streptomycin (Invitrogen). Then tissue cubes were placed on

a triangle metal mesh in a 6-well plate at air liquid interface conditions at 37 ℃ in humidified air with 5% CO_2 over-night. After 2h of *S. aureus* or *S. epidermidis* infection, all tissue cubes were transferred on triangle metal mesh again in a 6-well plate for another 24h or 48h incubation. The tissue cubes in tissue culture medium alone were taken as control group. Human bronchial epithelial cell line BEAS-2B cells ($5x10^4$ cells/well) were placed in a 24-well plate at 37 ℃ in 5% CO_2 overnight. When reaching 70%-80% confluence, BEAS-2B cells were stimulated by *S. aureus* or *S. epidermidis* for 15min at different concentrations from 10^6 to 10^2cfu/ml, and were washed 2 times by PBS for another 10h or 24h incubation. A concentration of bacteria of 10^6 cfu/ml and the 10h incubation time were suitable for further epithelial derived cytokine pathway investigations in terms of induction of cell death, TSLP and IL-33 cytokine release. To block TSLP and IL-33 releases from BEAS-2B cell after *S. aureus* infection, cells were pretreated with the novel Toll like receptor (TLR)2 antagonist CUCPT22 (Tocris Bioscience, *Ellisville*, *MO*) for 1h, followed by *S. aureus* infection. Experiments were repeated four times. The supernatant and the protein of cells were collected for further study.

Stimulation of nasal single cell suspension Single cell suspensions of human CRSwNP mucosa were prepared as described[22]. $5x10^6$ human nasal single cells per well were stimulated with recombinant human (rh) TSLP, rhIL-33 and both cytokines together (R&D, Minneapolis) with different doses for 24h. Finally, recombinant TSLP and IL-33 were used at a concentration of 100ng/ml for the induction of IL-5 release. After 24h incubation, cytokines were measured in the supernatants of single cell suspensions.

qRT-PCR After extraction of total RNA by using the RNeasy kit (QIAGEN GmbH, Hilden, Germany), 5 ng cDNA was synthesized by using the iScript Advanced cDNA-synthesis Kit (BioRad laboratories, CA, USA). The target product was detected by Sso Advanced SYBR Green Super mix (BioRad) on the LightCycler 480 Instrument II (Roche Applied Science, Penzberg, Germany) using the primer sequences as shown in **Table 2**. To normalize for transcription and amplification variations among the samples, two housekeeping genes were used: Succinate Dehydrogenase Complex and elongation factor 1. The relative quantities expressed were calculated with the commercially available qBasePlus software (BioRad)[23].

Table 2 The primers sequence used for qRT-PCR.

Targets	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)	Accession number
IL-33	TGAATCAGGTGAC GGTGTTGATGG	TGAAGGACAAAGAAGGCC TGGTC	150	NM_033439.3
TSLP	ACTTGCCAACAGG TAAGAGGA AGC	AGGCTTGTTCCACAGTAAG TGCTC	77	NM_033035.4
IL-25	AGTCCTGTAGGGC CAGTGAAGATG	CCGGTTCAAGTCTCTGTCC AACTC	86	NM_022789.3
IL-5	AACTCTGCTGATA GCCAATGAGAC	ACTCTCCAGTGTGCCTATT CCC	106	NM_000879.2
IL-4	ACAGCCTCACAGA GCAGAAGAC	TCTCATGGTGGCTGTAGAA CTGC	138	NM_000589.3
IL-13	CAACATCACCCAG AACCAGAAGGC	AGGTTGATGCTCCATACCA TGCTG	63	NM_002188.2

Cytokine measurements Cytokine measurements were performed in BEAS-2B cells as well as in supernatants or tissue homogenates of tissue from human airway infection model. Tissue homogenates were prepared as described earlier[24]. IL-4, IL-5, and IL-13 measurements were assayed by using Luminex xMAP suspension array technology in a Bio-Plex 200 system (BioRad, MI). Kits for total TSLP and IL-33 measurement were purchased from R&D Company. All cytokine measurements were expressed as pg/ml. Cytokine measurement in supernatants of tissue from human mucosal tissue *S. aureus* infection model was calibrated by tissue cube weight.

Immunofluorescence staining The CRSwNP tissue from infection model was embedded using frozen tissue medium (Klinipath, Duiven, the Netherlands). Sections were incubated with IL-33 antibody from R&D (7.5μg/ml) to evaluate the expression of IL-33 in CRSwNP tissue with infection. Additionally, to determine the location of the TSLP receptor (TSLPR) and IL-33 receptor

long form ST2 receptor (ST2L), double staining was performed on cryosections by incubation with 7.5 μg/ml TSLPR antibody (R&D) and CD3 monoclonal antibody (clone F7.2.38; 1:50; DAKO, Trappes, France), or 7.5 μg/ml ST2L (R&D) antibody and CD3 for 2h at room temperature. At the end of this incubation, the sections were mounted with glycerin-DABCO (Janssen Chemica, Beerse, Belgium). Images were obtained using a Leica TCS SPE laser scanning spectral confocal system linked to a DM B fluorescence microscope (Leica Microsystems). Leica confocal software was used for image acquisition.

Western blotting

Total protein was extracted from CRSwNP tissue and BEAS-2B cells after *S. aureus* infection using the RAPI buffer (Invitrogen) with cocktail proteinase inhibitor (Roche). The following antibodies were used for western blotting: anti-P50 (1:700), anti-phospho-P50 (Tyr705) (1:700), anti-P65 (1:800), anti-phospho-P65 (Tyr694) (1:600), anti-IL-33 (0.2 μg/ml), anti-TSLP (0.1 μg/ml), anti-TSLPR (1 μg/ml) and anti-ST2L (0.1 μg/ml). All antibodies were from Cell Signaling Technology (Cambridge, UK) except anti-P50, anti-phospho-P50, anti-TSLPR, anti-ST2L antibody, and HRP-conjugated secondary antibody (1:6000). Anti-P50, anti-phospho-P50, HRP-conjugated secondary antibodies were from Santa Cruz (Santa Cruz, CA); anti-IL-33, anti-TSLP, anti-TSLPR and anti-ST2L antibodies were from R&D. IL-33 and TSLP were detected using Tris-Tricine gel, while the other proteins described in the manuscript were measured using Tris-glycine gel. The relative band densities of the target protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Invitrogen, Belgium) were estimated by Lab image Analysis Software (Bio-Rad, CA).

Flow cytometry CRSwNP tissue cubes with *S. aureus* infection 24h (n=3 in *quadruplicate*) were collected for single cell suspensions as described above. To measure the expression of TSLPR, the single cells were stained with anti-CD3-Vioblue anti-TSLPR-APC antibodies for 30 min. The IL-33 receptor ST2L is internalized after binding to IL-33, reducing IL-33-induced cytokine release[25]. Thus, following 30min anti-CD3-Vioblue incubation, single cells from *S. aureus* infection model were intracellularly stained by anti-ST2L-PE (R&D) after fixation and permeabilization. Data was acquired on a BD FACS Canto II using FACS Diva software (BD Bioscience) and FlowJo software (Tree Star Inc., Ashland, OR) for analysis.

Statistical analyses The data were analyzed using graphpad prism software (GraphPad Software, san Diego, CA). Statistical analysis was performed by using the Wilcoxon test for paired comparisons. Data comparison within different groups was performed using the Kruskal-Wallis test. A p value < .05 was considered statistically significant.

Results

Active forms of TSLP and IL-33 were induced in CRSwNP tissue in the presence of *S. aureus*, but not *S. epidermidis*. Two human transcript variants of TSLP were described; RT-PCR analysis demonstrated that the mRNA level of lfTSLP, an active form of TSLP, increased in CRSwNP tissue and healthy control tissue 24h after *S. aureus* infection; in contrast, the expression of sfTSLP, possessing antibacterial property, decreased in CRSwNP and healthy control tissue 24h and 48h after *S. aureus* infection (**Figure 1A**). The level of TSLP protein was significantly higher in the supernatants of CRSwNP and healthy control tissue after 48h exposure to *S. aureus* than that in the supernatants of unexposed tissue (**Figure 1B**). IL-33 mRNA in CRSwNP and healthy control tissue did not significantly increase due to *S. aureus* incubation (**Figure 1A**). However, IL-33 protein was increased in CRSwNP tissue homogenates, but not in control tissue, 24h after *S. aureus* infection (**Figure 1B**). The ELISA measurements of TSLP and IL-33 did not distinguish different variants. Therefore, full length IL-33, processed IL-33 (active form) and long-form TSLP were studied after separation in Tris-Tricine gel in the CRSwNP tissue after *S. aureus* exposure (**Figure1C**). Recombinant, N terminally truncated human IL-33 proteins, human lfTSLP and sfTSLP were used as controls. Understanding the location of IL-33 in *S. aureus* infected tissue also helps to evaluate its functional status; therefore IL-33 immunofluorescence staining was performed. A high amount of IL-33 was observed in the extracellular space between epithelial cells of CRSwNP after *S. aureus* stimulation for 24h (**Figure 1D**).

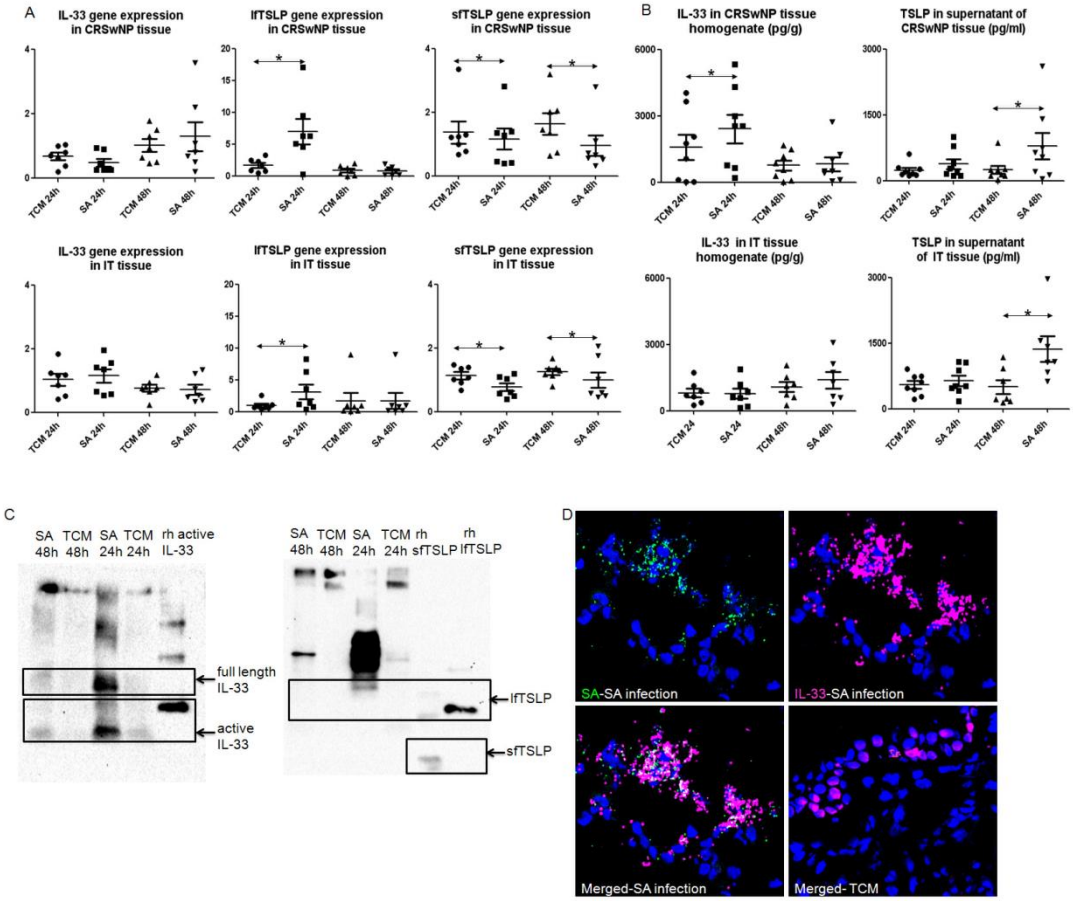

Figure 1. Epithelial cell derived cytokines TSLP and IL-33 were induced in CRSwNP tissue in the presence of *S. aureus*. (A)Gene expressions of long form TSLP (lfTSLP), short form TSLP (sfTSLP) and IL-33 in CRSwNP (n=7) and healthy inferior turbinate (IT) tissues (n=8) with staphylococcus aureus (*S. aureus*) infection for 24h or 48h. Tissue cubes in tissue culture medium (TCM) were taken as controls (**P*<.05). (B) The protein level of TSLP and IL-33 CRSwNP and IT tissues with *S. aureus* infection for 24h or 48h (**P*<.05). (C) Full-length IL-33, active IL-33, long form TSLP and short form TSLP were identify by Tris-Tricine gel. (D) IL-33 was observed in the extracellular space of epithelial cells in CRSwNP tissue with *S. aureus* infection for 24h. Low expression of IL-33 was found in control conditions without any infection. (All slides viewed at 630x magnifications; pink fluorescence demonstrates the presence of IL-33 and green fluorescence the presence of *S. aureus* (SA) in the nasal mucosa; isotype control staining for IL-33 was completely negative (not presented here)).

S. epidermidis, the same staphylococcus genus of *S. aureus*, was used in the human mucosal tissue infection model as a control to demonstrate specificity of the effect of *S. aureus* on epithelial cell-derived cytokine release. *S. epidermidis* did not induce TSLP nor IL-33 proteins in both the CRSwNP tissues (**Figure 2A**). A low basal epithelial expression of IL-33 without any release could be found in CRSwNP tissue after 24h *S. epidermidis* infection (**Figure 2B**), suggesting that specifically *S. aureus* infection, but not *S. epidermidis* induced IL-33 release in CRSwNP tissue.

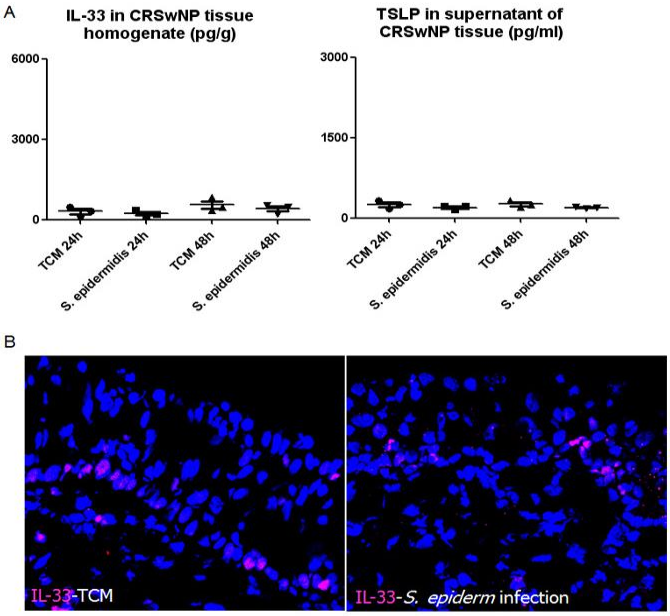

Figure 2. The expression of epithelial cell derived cytokines in CRSwNP tissue after *Staphylococcus epidermidis* (*S. epidermidis*) infection. (A) Protein levels of TSLP and IL-33 were measured in CRSwNP tissue with *S. epidermidis* infection 24 or 48h (n=3). (B) The immunofluorsence staining of IL-33 expression in CRSwNP tissue with *S. epidermidis* infection for 24h. Tissue cubes with tissue culture medium (TCM) were taken as controls. (All pictures viewed at 630x magnifications. Blue fluorescence indicates the nuclear of cell, and pink fluorescence stands for the location of IL-33.)

$CD3^+TSLPR^+$ and $CD3^+ST2L^+$ cells increased in CRSwNP tissue after *S. aureus* infection. To further unveil TSLP and IL-33 signaling pathways in the human airway infection model, expression and localization of TSLPR and ST2L upon *S. aureus* exposure were evaluated by immunofluorescence staining, western blot and flow cytometry. Increased levels of TSLPR and ST2L were observed in the epithelial layer of CRSwNP mucosa after 24h (**Figure 3A-B**). The majority of TSLPR and ST2L was expressed on $CD3^+$ T cells in the epithelial and sub-epithelial layers. In line with the staining results, the expression of TSLPR and ST2L proteins were significantly up-regulated 48h after *S. aureus* infection in comparison with non-infected mucosal tissue using western blot assay (**Figure 3C-D**). Additionally, the number of $CD3^+ST2L^+$ and $CD3^+TSLPR^+$ cells in tissue increased in response to *S. aureus* exposure (**Figure 3E**). ST2L and

TSLPR were expressed on $CD3^-$ T cells, whereas the number of $CD3^-ST2L^+$and $CD3^-TSLPR^+$ cells increased marginally only after *S. aureus* infection (**Figure 3F**).

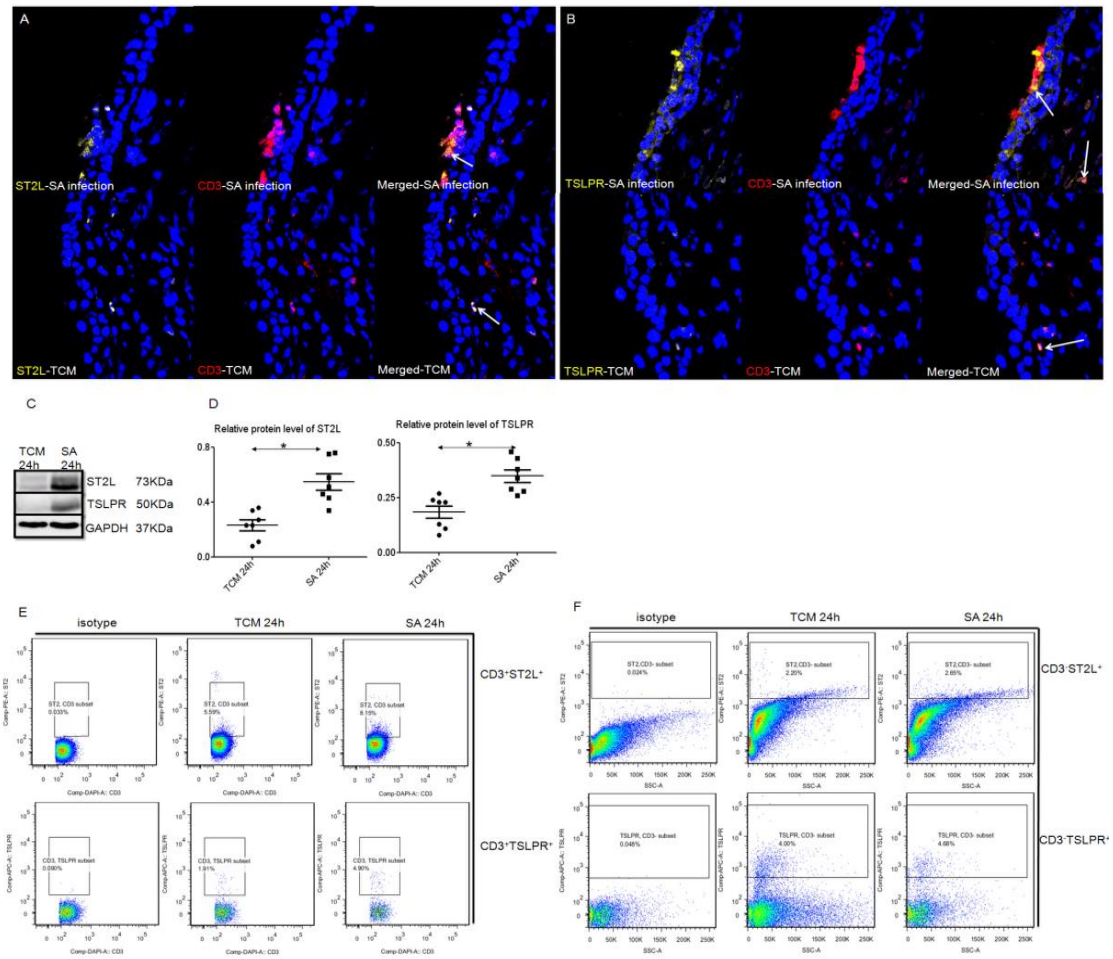

Figure 3. TSLPR and ST2L positive T cells increased in CRSwNP tissue after *S. aureus* infection. (A-B) Immunofluorescence staining pictures of TSLP receptor $(TSLPR)^+CD3^+$cells (white arrow) and IL-33 receptor $(ST2L)^+CD3^+$ cells (white arrow) in CRSwNP tissue with S*taphylococcus aureus* (*S. aureus*) infection for 24h. CRSwNP tissue cubes with tissue culture medium (TCM) were taken as controls (All slides viewed at 630x magnification; yellow fluorescence demonstrated the presence of receptors and red fluorescence stood for the presence of $CD3^+$ cell in the nasal mucosa; isotype control staining for TSLPR and ST2L was completely negative (not presented here)). (C) Western blot pictures of TSLPR and ST2L in CRSwNP tissue with *S. aureus* infection for 24h. (D) Quantification for TSLPR and ST2L proteins in CRSwNP tissues with *S. aureus* (SA) infection (**P*<.05). (E) The expressions of $TSLPR^+CD3^+$ and $ST2L^+CD3^+$ cells were identified in CRSwNP tissue with *S. aureus* infection (n=3). (F) The expressions of $TSLPR^+CD3^-$ and $ST2L^+CD3^-$ cells were identified in CRSwNP tissue with *S. aureus* infection (n=3).

A Th2 response is induced in CRSwNP tissue, but not in healthy control tissue, in the presence of *S. aureus*. To determine the contribution of TSLP and IL-33 to type 2 cytokine release in the

human airway infection model, IL-4, IL-5, IL-13 were measured. IL-5 and IL-13 mRNA expression and IL-5 protein release were up-regulated in CRSwNP tissue 48h after *S. aureus* infection, but not in control tissue (**Figure 4A**). Human nasal single cell suspensions of CRSwNP patients were stimulated with rhTSLP and rhIL-33, both individually and together. IL-5 can be induced from single cell suspensions in the presence of rhTSLP or rhIL-33 alone (**Figure 4B**). Analysis of IL-5 expression in single cell suspensions after combined rhTSLP and rhIL-33 stimulation demonstrated a further significant increase over rhTSLP alone and rhIL-33 alone (**Figure 4B**).

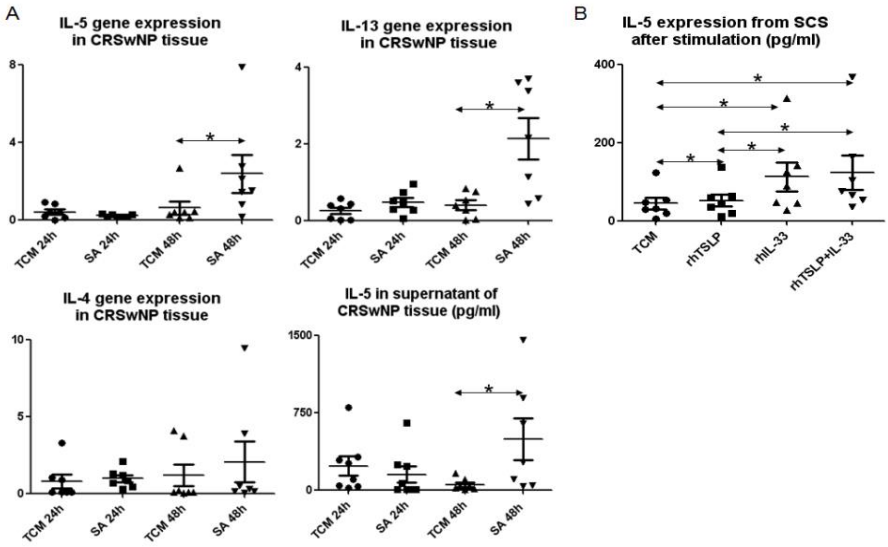

Figure 4. Th2 response is induced in CRSwNP tissue in the presence of *S. aureus*. (A) The expression of IL-4, IL-5 and IL-13 in CRSwNP and healthy inferior turbinate (IT) tissues with S*taphylococcus aureus* (*S. aureus*) infection for 24h or 48h. Tissue cubes with tissue culture medium (TCM) were taken as controls (**P*<.05). (B) IL-5 cytokine increased in single cell suspensions with rhTSLP and rhIL-33 stimulation for 24h (**P* <.05).

***S. aureus* induced release of IL-33 and TSLP partially via Toll-like receptor 2.** To further confirm what had been observed in the human mucosal tissue infection model, epithelial BEAS-2B cells were infected with *S. aureus*. In BEAS-2B cells, we confirmed that *S. aureus* induced TSLP and IL-33 release in a dose-dependent fashion (**Figure 5A**). The relevant NF-κB signaling pathways were investigated to analyze epithelial cell derived cytokine production mechanisms in BEAS-2B cells. Activation of the NF-κB pathway in epithelial cells was evident from an increase in phosphorylation of P50 and P65 at early time points in BEAS-2B cell after *S. aureus* exposure (**Figure 5A**). In contrast, no induction of TSLP or IL-33 expression (data not shown) or up-

regulation of the NF-κB in BEAS-2B cells was observed after *S. epidermidis* infection (data not shown). The result demonstrated that the novel TLR2 antagonist CUCPT22 inhibited the phosphorylation of P50 and P65 in BEAS-2B cell 10h after *S. aureus* infection. Consequently, the downstream IL-33 and TSLP expressions decreased in BEAS-2B cells in a dose-dependent manner (**Figure 5B**).

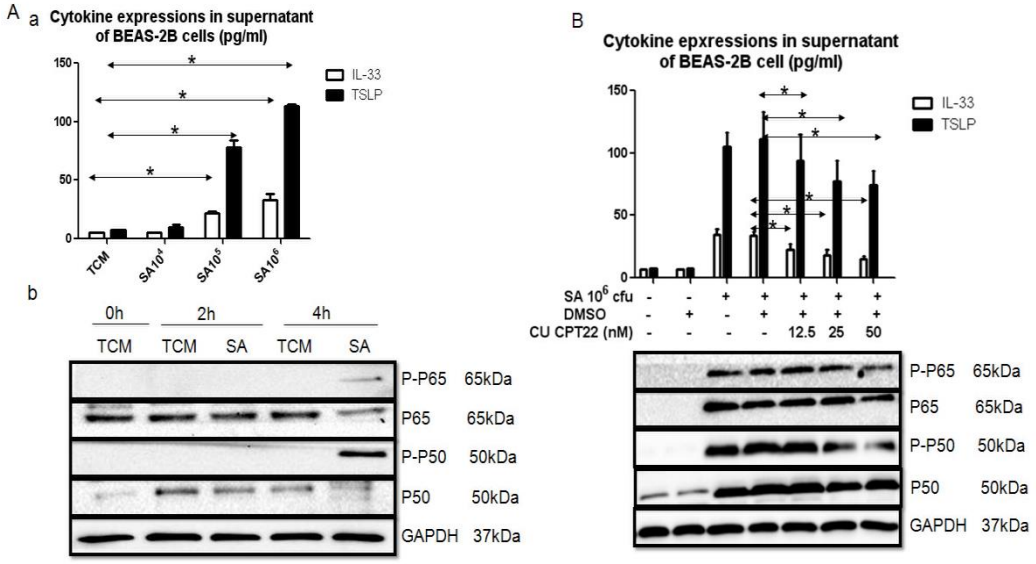

Figure 5. Expression of TSLP and IL-33 in supernatants of the bronchial epithelial cell line BEAS-2B after *S. aureus* infection; possible induction pathway. (A) a, TSLP and IL-33 expression in supernatants of bronchial epithelial cell line BEAS-2B cells after exposure to different concentrations of S*taphylococcus aureus* (*S. aureus*) for 10h. b, Correlation of time-dependent increase in P50 and P65 phosphorylation in BEAS-2B cells with *S. aureus* infection. SA means *S. aureus* (**P* <.05) (B) Novel toll like receptor 2 (TLR2) antagonist CU-CPT22 partially inhibited the phosphorylation of P50 and P65 and their downstream of IL-33 and TSLP expression in BEAS-2B cells 10h after *S. aureus* infection. (**P* <.05)

Discussion

We demonstrate for the first time that *S. aureus* can directly induce the release of the active forms of epithelial cell-derived cytokines TSLP and IL-33 from human nasal tissue, accompanied by the up-regulation of $CD3^+TSLPR^+$ and $CD3^+ST2L^+$ cells, IL-5 and IL-13 mRNA expression and IL-5 protein release in CRSwNP, but not in control tissue. NF-κB signaling is involved in the production of TSLP and IL-33 after binding of *S. aureus* to TLR2; *S. epidermidis* infection did not induce any cytokines.

The tissue explants here serve as a bridge between cell culture and *in vivo* environment. In this study, we infected nasal tissue explants, instead of cell lines or primary cells, with *S. aureus* to efficiently reflect the *in-vivo* situation in the nose after infection. We also focused on the role of *S. aureus* itself, not the extractions from *S. aureus* or toxins produced by *S. aureus*, in Th2 biased upper airways disease. As demonstrated earlier, enterotoxins are known to work as superantigens and induce high amounts of Th2 cytokines and induce IgE formation in CRSwNP tissue[26]; $CD4^+$ T cells from BALB/c mouse with subcutaneous footpad *S. aureus* infection produce a high amount of Th2 cytokines like IL-4 and IL-13[27]. In this study, we successfully demonstrated that a *S. aureus* strain without enterotoxin secretion can directly induce IL-5 and IL-13 in CRSwNP tissue, but not IL-4, via the epithelial cytokines IL-33 and TSLP. Our data also unveiled that type 2 cytokines can only be induced by *S. aureus*, not by *S. epidermidis* in CRSwNP tissue. The downstream activities of epithelial cell derived cytokines are well studied, whereas the factors that affect epithelial cell-derived cytokine release are only partially known. Rhinovirus exposure appears to trigger TSLP expression in the lung of mice with allergic airway inflammation[28]. Mouse epithelial cells MLE-15 and human alveolar epithelial cells A549 infected with influenza virus result in IL-33 release[29]. TSLP is produced by murine dendritic cells in response to certain microbial pathogen products such as lipopolysaccharide, flagellin, FLS-1 and pam3csk4[30]. *S. aureus* membrane and diacylated lipopeptides can induce TSLP release in human keratinocytes[31]. Heat inactivated *S. aureus* infection increases IL-33 in human and murine epidermal keratinocytes and murine dermal macrophages[32]. However, there was no understanding of the relationship between *S. aureus* germs and epithelial cell-derived cytokines or Th2 responses in human. We formerly demonstrated that staphylococcal enterotoxins can induce Th2 cytokines, but also Th1 and Th17 related cytokines, in nasal healthy and CRSwNP tissue[26]. Here we show that enterotoxin non-producing *S. aureus* can induce both TSLP and IL-33 release in human CRSwNP tissue and human bronchial epithelial cells. Even though the predominant form of TSLP remains controversial in humans, two different TSLP forms have been described: lfTSLP is understood as the functional human TSLP[33]; whereas human sfTSLP exhibits a marked antibacterial activity in skin and salivary glands[34]. We demonstrate that lfTSLP increased at mRNA and protein level in CRSwNP tissue with *S. aureus* infection with the potential to propagate Th2 immune responses; while sfTSLP decreased in CRSwNP with *S. aureus* infection,

further decreasing the mucosal defense. IL-33 is a chromatin-associated nuclear cytokine from the IL-1 family that functions as an ‘‘alarmin’’. It has been shown to be constitutively expressed in the nuclei of epithelial cells[35] and to be released into the extracellular space after cell necrosis[36] to elicit innate immune responses. Recently, it has been reported that the biological activity of full-length IL-33 is increased by neutrophil elastase and cathepsin G[37,38]. Based on our immunofluorescence staining, a high amount of IL-33 was observed in the extracellular space between epithelial cells, which was in accordance with increased CRSwNP tissue homogenate protein levels of IL-33 after *S. aureus* infection for 24h. In contrast, IL-33 preferentially was located in the nuclei of nasal basal cells in CRSwNP tissue without stimulation, or after *S. epidermidis* exposure, not impacting on the mucosal immune response. Additionally, full-length IL-33 and processed IL-33 were observed in CRSwNP tissue with *S. aureus* infection, indicating that induced IL-33 in the nasal infection model is functional.

Besides the release of TSLP and IL-33, *S. aureus* infection also can increase the expression of their receptors. The increased TSLPR and ST2L receptors were mainly expressed on $CD3^+$ T cells in CRSwNP tissue, reinforcing the signals. Thus, $CD3^+$T cells here are likely to contribute to the IL-5 production after infection. This does not exclude the contribution of ILC2 cell and mast cell, even though the number of $CD3^-TSLPR^+$ and $CD3^-ST2L^+$ increased only marginally after infection.

Interestingly, a consequent IL-5 protein release was propagated in CRSwNP tissue, but not in control tissue, upon infection; this could be related to the typical Th2 bias in CRSwNP with increased numbers of activated Th2 cells present. Furthermore, defense mechanisms against *S. aureus* may be impaired in CRSwNP, with a high rate of alternatively activated macrophages in CRSwNP vs. healthy mucosa, not suited to phagocytize and kill *S. aureus* efficiently[39]. This consecutively may lead to a continuous stimulation of the mucosal tissue in CRSwNP patients by *S. aureus* contributing to the persistence of inflammation.

It remains to be studied whether S. aureus can amplify preexisting type 2 airway inflammation only, or initiate it. Furthermore, the role of IL-25 in this situation needs further focus; *S. aureus* exposure induced IL-25 protein in BEAS-2B cells (data not shown), but not in the mucosal tissue models.

In summary, we here highlight for the first time that *S. aureus*, but not *S. epidermidis*, induces the release of type 2 cytokines in CRSwNP tissue via epithelial derived cytokines TSLP and IL-33. TLR2 and NF-κB signal are involved in the production of TSLP and IL-33 after *S. aureus* exposure. With the known high colonization of CRSwNP tissue by *S. aureus*, this likely contributes to the initiation or persistence of the Th2-biased inflammation in this disease.

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CHAPTER 4

TH2 BIASED UPPER AIRWAY INFLAMMTION IS ASSOCIATED WITH AN IMPARIED RESPONSE TO VIRUAL INFECTION WITH HERPES SIMPLEX VIRUS 1

Th2 biased upper airway inflammation is associated with an impaired response to viral infection with Herpes simplex virus 1

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Rhinology (accepted)

Abstract

Background: We aimed to elucidate possible differences in antiviral defense in chronic rhinosinusitis with nasal polyps (CRSwNP) mucosal tissue compared to healthy mucosal tissue (HMT) upon herpes simplex virus 1 (HSV1) exposure.

Material and methods: HMT and CRSwNP samples were infected with HSV1. We visualized the virus location by immunofluorescence and monitored invasion by a score. The mediators Interferon (IFN)-α, IFN-β, IFN-λ, IFN-γ, Interleukin (IL)-6, IL-1β, Tumor necrosis factor (TNF)-α, IL-17, IL-5, IL-10 were measured in culture supernatants at baseline and at 24h, 48h and 72h after virus incubation.

Results: CRSwNP mucosal tissue showed a significant deficit in IFN-γ and IL-17 release within 24 to 72h after infection in comparison to HMT, at the same time releasing significantly more pro-inflammatory cytokines including IL-1β and TNFα. These findings were associated with significantly higher viral invasion scores at 48 and 72h in CRSwNP mucosa compared to those for the HMT.

Conclusions: We demonstrate for the first time in a human *ex-vivo* mucosal model that the inadequate response of CRSwNP may be associated with a deeper intrusion of viruses into the mucosal tissue, and may contribute to more and longer symptoms upon acute infection, but also to the persistence of inflammation in CRSwNP tissue.

Introduction:

Chronic rhinosinusitis with nasal polyps (CRSwNP) represents a preferentially eosinophilic type of inflammation of the upper airways, characterized by the overproduction of IL-5, eosinophil-cationic protein (ECP) and immunoglobulin (Ig)E; CRSwNP also is frequently associated with comorbid asthma[1]. Viral infections are known as the most frequent cause of acute rhinitis and may cause asthma exacerbations in children and adults, with human rhinoviruses being identified as the most prominent, but not exclusive agent[2-4]. Although natural and experimental rhinovirus infections have been studied extensively with respect to clinical manifestation and pathophysiological responses in healthy subjects[5, 6], little is known about viral infections in patients with chronic inflammatory disease of the nose and sinuses such as CRSwNP.

Herpes simplex virus 1 (HSV1) infections are highly prevalent in humans. HSV1 is characterized by a comparatively high global sero-prevalence with rates of infection between 65% and 90% worldwide[7-9]. Although people infected with HSV often develop labial or genital lesions, the majority are either undiagnosed or display no physical symptoms; individuals with no symptoms are described as asymptomatic or as having subclinical herpes. Findings from a recent study have shown that 8% (2/23) of human nasal polyps may be infected with HSV1 at a given moment in time; although this seems a low incidence, it underlines the possible role of HSV1 in upper airway disease[10]. Furthermore, HSV infection is a risk factor for nasal carriage of *Staphylococcus aureus* (S. *aureus*) in human immunodeficiency virus -infected patients[11]. For herpes virus infection in animals, the nasal mucosa is considered the primary site of replication[12].

It was recently shown that the response to viral infections (rhinovirus) is also impaired in asthmatics vs. healthy controls, with a deficit in the mucosal production and release of interferons in response to the viral challenge, which may impair the mucosal defense and allow the virus to replicate at a high rate[13]. The mucosa in asthmatics is characterized by a Th2 bias, similar to the situation in CRSwNP. In the current study we therefore aimed to study the cytokine response related to a viral agent, HSV1, in our CRSwNP whole mucosal tissue model compared to control samples to elucidate possible differences in antiviral defense.

Materials and methods

Nasal mucosa tissue

Inferior turbinate healthy mucosal tissue (HMT) was obtained from 7 non-allergic patients (average age 36 years (range: 23-52)) scheduled for turbinate surgery due to septal deviations or turbinate hypertrophy without asthma and other diseases. Nasal polyp tissues were obtained from 7 patients with CRSwNP (average age 48.5 years (range: 17.4-68.8)) by endoscopic sinus surgery at the department of Oto-Rhino-Laryngology, Ghent University Hospital. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and CT scanning of the sinuses according to the European Position Paper on Rhinosinusitis and Nasal Polyps[14]. The atopic status of patients was evaluated by skin prick tests with the European standard panel of 14 inhalant allergens. Negative and positive controls (10mg/mL histamine solution) were included with each skin prick tests. One CRSwNP patient had a positive skin prick test, two patients reported mild asthma, and one patient reported aspirin intolerance.

The ethics committee of the Ghent University Hospital approved the study, Belgium registration number B670201317380; all patients were asked to refrain from oral corticosteroids or antibiotics 4 weeks and topical corticosteroids 2 weeks preoperatively and gave their written informed consent.

Measurement of cytokine and IgE levels in tissue homogenates

Freshly obtained tissue specimens were homogenized, as previously described[15], and assayed for IL-5, IFN-γ and IL-17, by using commercially available Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) following the instructions of the manufacture. IgE and ECP were measured by using the UNICAP system (Pharmacia, Uppsala, Sweden).

HSV1 stocks

HSV1 (ATCC, VR-733, strain F) was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and propagated to large quantities by infection of African green monkey kidney (Vero) cells (ATCC CCL-81; Rockville, MD, USA). The virus strains were passaged twice and diluted in serum-free medium (50% RPMI medium 1640 (Invitrogen, Merelbeke, East Flanders, Belgium) and Dulbecco's Modified Eagle Medium (Invitrogen, Belgium), 50IU/mL penicillin (Invitrogen, Belgium) and 50 µg/mL streptomycin (Invitrogen, Belgium) to a final concentration of 10^7 $TCID_{50}$ (50% tissue culture infectious dose of a virus)/ml. Tissue culture

medium (TCM, contains equal amounts of RPMI medium 1640 (Invitrogen, Merelbeke, East Flanders, Belgium) and Dulbecco's Modified Eagle Medium (Invitrogen, Belgium)) were used for all subsequent experiments involving infection of nasal turbinate tissue *ex-vivo*.

Culture and infection of healthy nasal turbinate and nasal polyp explants with HSV1 (Figure 1)

Nasal turbinate and nasal polyp tissue obtained from each patient following surgery was immediately washed three times with serum-free medium supplemented with antibiotics (50IU/mL penicillin (Invitrogen, Belgium) and 50μg/mL streptomycin (Invitrogen, Belgium) and cultured according to the following protocol. The washed tissue explant was cut into smaller cubes approximately 25mm^2 in size. Nasal tissue cubes of each turbinate or nasal polyp explants were used for further investigation, divided into two equal groups of two cubes each (Group 1 (A,B wells)= HSV1 infection group; Group 2 (A, B wells)= control, non-infection group, Group 1B and Group 2B cubes were divided into 2 parts before any treatment). Each cube was placed with the epithelial surface upwards on sterile fine-meshed gauze in a 6-well tissue-culture plate (Falcon, BD Biosciences, Erembodegem, East Flanders, Belgium) and 3 ml serum-free medium supplemented with antibiotics was added to each well to create an air-liquid interface. All tissue cubes were conditioned as explant cultures by incubation for 24h at 37 ℃ in 5% CO_2 in air atmosphere, and then transferred to a 24-well tissue-culture plate (Falcon, BD Biosciences, Belgium). Groups 1 tissue cubes were inoculated with 1.0 mL inoculum containing 10^7 $TCID_{50}$ of HSV1, and 1.0mL of serum-free tissue culture medium (TCM) was added to the tissue cubes in Groups 2 as mock-condition, all tissue cubes were incubated for 1h at 37 ℃ in 5% CO_2 in air atmosphere. All tissue cubes were washed three times, transferred onto sterile fine-meshed gauze and incubated in a 6-well tissue-culture plate for either 24h or 48h under air-liquid interface culture conditions as before. At the end of each incubation period, the culture supernatants from Group 1A and Group 2A were collected, and tissue cube parts from Group 1B and Group 2B were collected, weighted and snap-frozen respectively at 24h or 48h time point, store at -80 ℃ for further evaluation. In order to investigate the spontaneous release, the tissue cubes which underwent 48h culture were transferred to a 6-well tissue-culture plate with fresh tissue culture medium for a final 24h incubation period (in total 72h tissue incubation) on fine-meshed gauze at an air-liquid interface. At the end of the

culture, the culture supernatant were collected and the tissue cubes were weighed and snap-frozen in liquid nitrogen and stored at -80 ℃ until further assessment. In the process of developing this model, we used uninfected vero cell lysates, heat inactivated HSV1 and tissue culture medium (TCM) as controls vs HSV1; as the controls showed the same morphology and cytokine response patterns, here we only used TCM as control.

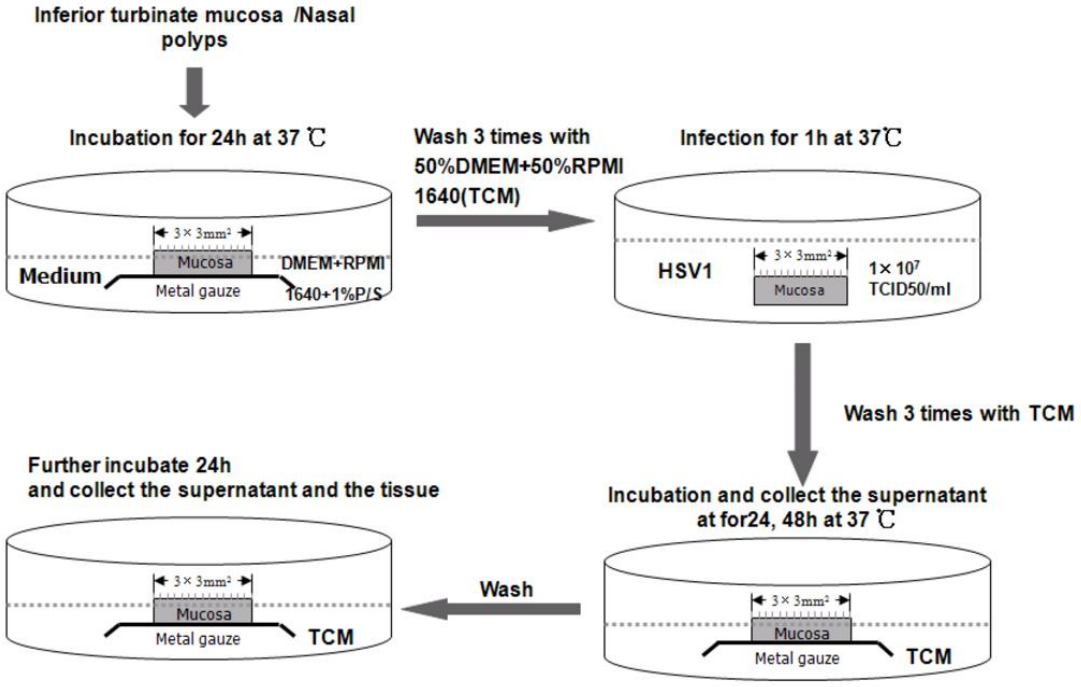

Figure 1: Study flow. Culture and infection of nasal turbinate healthy mucosa tissue and nasal polyp explants with HSV1: Nasal tissue cubes were divided into two equal groups of two cubes each (Group 1 = HSV1 infection group; Group 2 = control, non-infection group). Group 1 tissue cubes were inoculated with HSV1, and serum-free medium was added to the tissue cubes in Group 2 as mock-condition.

Immunofluorescence staining for evaluation of HSV1 invasion

As previously reported[16, 17], 10 serial cryosections (5um per section) of each tissue cube were incubated for 1h at 37 ℃ in the presence of mouse anti-HSV1-gD antibodies (Santa Cruz, Heidelberg, Baden-Wurttemberg, Germany) (100mg/mL, 1:100 in 10% NGS) or mouse IgG2 as an isotype specific negative control antibody (Dako, Glostrup, Region Hovedstaden, Denmark). Following three washings with PBS, the sections were incubated for a further 1h at 37 ℃ in the presence of goat anti-mouse-Texas Red antibodies (Molecular Probes, Invitrogen, Belgium) (2 mg/mL, 1:50 in 10% NGS).

Immunofluorescence-stained slides were evaluated for viral invasion by viewing at 630

magnifications using a fluorescence microscope (Axioplan 2, Carl Zeiss, Gottingen, Lower Saxony, Germany). All stained slides were evaluated by two independent observers, who were blinded to the tissue-treatment protocol and assessed the entire epithelium in each section by viewing up to 8–10 adjacent fields. HSV1 invasion in each field was graded on a 5-point scale (0= epithelium not infected, 1 = epithelium superficially infected, 2= basal cells infected, 3 = basement membrane and HSV1 co-localization, HSV1 do not penetrate the basement membrane, 4 = HSV1 penetrated the basement membrane into the lamina propria). The mean of total scores in the ten sections on each slide was used as the final invasion score for each explant.

Inflammatory cytokines were measured by ELISA in tissue culture supernatants

Inflammatory mediators IFN-γ, IL-6, IL-1β, TNF-α, IL-17, IL-5, IL-10 were measured in culture supernatants at baseline and at 24h, 48h and 72h after virus incubation by means of commercially available Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA). IFN-β (Invitrogen), IFN-α (Invitrogen), and IFN-λ (R&D systems, USA) release was measured in culture supernatants at baseline and at 24h, 48h and 72h after virus incubation, by using ELISA, according to the manufacturer's instructions.

Statistical analysis

Data were expressed as median and interquartile ranges (IQR). The Kruskal–Wallis test was used to assess the significance of intergroup variability; the Wilcoxon test was used for paired comparisons, and the Mann–Whitney U 2-tailed test was used to assess significance for between-group comparisons. *P* values $\leq$ 0.05 were considered to be statistically significant.

RESULTS

Patient characteristics, cytokines and IgE levels in tissue homogenates

The two groups of patients were comparable in terms of clinical characteristics, allergic status and comorbid disease. At baseline, tissues from CRSwNPs showed significantly higher total IgE, ECP and IL-5 concentrations vs inferior turbinate tissues from control samples (p<0.05). IL-17 was

below detection limit in both groups, whereas IFN-γ could be detected in 3 control tissues (**Table 1**).

Table 1. Cytokines and IgE levels in tissue homogenates.

Tissue	Detection limit after sample handling dilution	Inferior turbinate healthy mucosal tissue (HMT) n=7, median (IQR)	*P* value	Chronic Rhinosinusitis with nasal polyps (CRSwNP) n=7, median (IQR)
Total IgE (kUA/L)	1.9	16.6 (9,24-42.3)	0.003	205.3 (56.4-244.2)
ECP (ug/ml)	11.0	357.5 (98-504.3)	0.015	13609.7(5236.3-20570)
IL-5 (pg/ml)	7.0	BDL	0.007	263.7 (39.4-510.4)
IFNγ (pg/ml)	42.9	42.9 (42.9-77.2)	n.s	BDL
IL-17(pg/ml)	7.0	BDL	n.s	7 (7-17.6)

BDL: below detection limit

HSV1 replicates and invades in the nasal mucosal tissue after 24, 48 and 72 hours cultivation

Infection of the nasal inferior turbinate mucosal tissue explants with HSV1 led to focal infection of outer epithelial cells within 24h (**Figure 2A**) with distribution up to the basement membrane and damage of epithelial structural integrity after 48 h (**Figure 2B**). Incubation of the tissue for 72h (**Figure 2C**) following inoculation with HSV1 led to infection of basal epithelial cells, followed by the loss of epithelium and subsequent invasion of HSV1 into the lamina propria. In contrast, HSV1 infected the whole epithelium of nasal polyp tissue within 24h (**Figure 2D**), already causing epithelial damage after 48h (**Figure 2E**), and causing significant damage to the epithelium and invasion into the lamina propria through the basement membrane after 72h (**Figure 2F**).

The depth of mucosal invasion for HSV1 in nasal polyp tissue was similar to turbinate mucosa at 24h, but the invasion scores at 48 and 72h were significantly higher than those for the turbinate mucosa (p<0.05) (**Figure 2G**), indicating that more viruses infected deeper mucosal layers. Immunofluorescence staining for untreated cryosections was also performed by using anti-HSV1-gD monoclonal antibodies. Neither in those cryosections nor in TCM treated HMT and CRSwNP tissues, HSV1 was detectable.

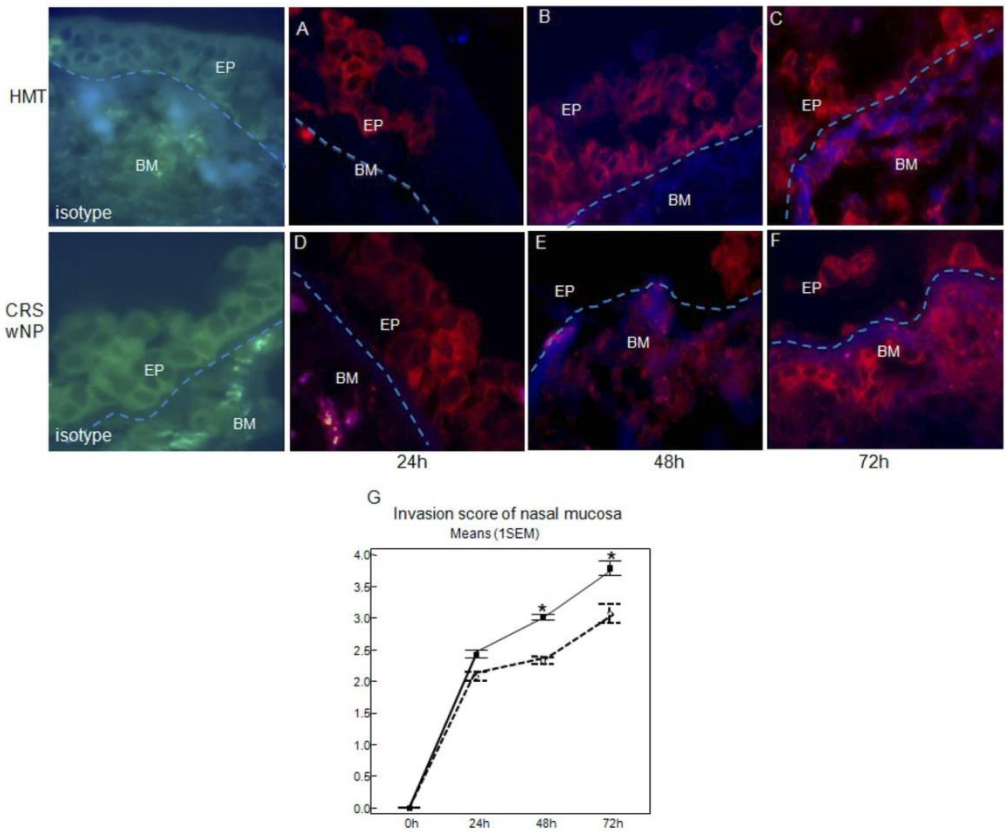

Figure 2. Immunofluorescence-stained sections of inferior turbinate healthy mucosa tissue (HMT) and nasal polyp tissue samples (CRSwNP) after HSV1 infection. (A-C) HMT tissue was infected by HSV1 and incubated for 24h, 48h, 72h. (D-F) CRSwNP tissue was infected by HSV1 and incubated for 24h, 48h, 72h. Isotype: mouse IgG2 antibody on the sections of the tissue samples after 24h HSV1 infection. Red is the positive HSV1 signal. Invasion score of HMT and CRSwNP tissue samples by HSV1. Results are presented as mean scores ± SEM for HMT from 7 patients and CRSwNP from 7 patients. *P<0.01.

Inflammatory mediator release after HSV-1 infection HSV1 induced a significant release of IL-6 after 48h and 72h in both HMT and CRSwNP tissues, without significant difference between groups. However, in CRSwNP samples, we could demonstrate a significant release of IL-1β at 48h (p=0.031), whereas no IL-1β was released in HMT before 72h post inoculation (**Figure 3A**); the

concentration of IL-1β in the supernatants was significantly higher in CRSwNP compared to HMT at 72h (p=0.01). In line with these findings, the release of TNFα was significantly increased after 48h (p=0.031) and 72h (p=0.031) in CRSwNP only.

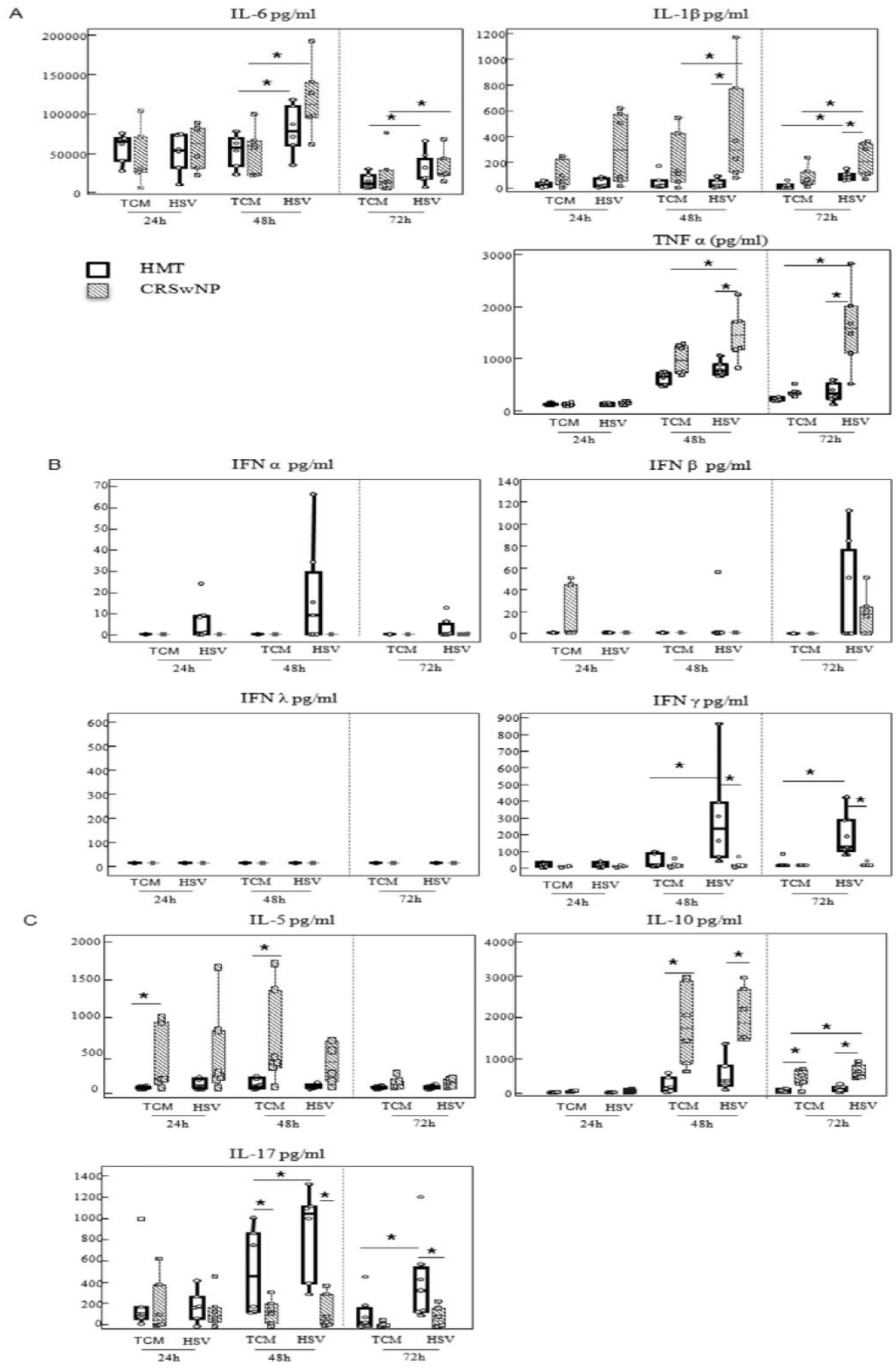

Figure 3 Inflammatory mediator release after HSV-1 infection. Inflammatory mediators IL-6, IL-1β, TNF-α, IFN-α, IFN-β, IFN-γ, IFN-λ, IL-5 and IL-10 were measured in infection model. Data were expressed as median and interquartile ranges (IQR) for HMT from 7 patients and CRSwNP from 7 patients. * P value<0.05. TCM: tissue culture medium, indicating the uninfected culture.

IFN-α could be detected in 4 out of 7 HMT samples after HSV1 incubation at 24 and 48h, but not

in any CRSwNP samples; however, this difference did not reach significance. IFN-β was found in the supernatants of 3 HMT and 4 CRSwNP samples at 72h incubation. IFN-λ was not detectable at any of the time points in any of the samples. However, there was a significant increase of IFN-γ release in HMT samples at 48 (p=0.031) and 72h (p=0.016), but no induction of IFN-γ in CRSwNP tissue upon HSV1 infection (**Figure 3B**).

There were also significant differences in terms of T cell cytokine release in the response of CRSwNP and HMT to HSV1 infection (**Figure 3C**). The expression of IL-17 was only up-regulated in HMT tissues after 48 and 72h, but not in CRSwNP tissues. There was a significantly higher spontaneous release of IL-5 at 24 (p=0.037) and 48h (p=0.025) in CRSwNP vs. HMT, but this was independent from the impact of HSV infection. A significantly increased release of IL-10 was observed in CRSwNP vs. HMT at 48 (p=0.004) and 72h (p=0.004), which was further increased significantly by HSV1 infection at 72h (p=0.031). No increase in IL-10 was observed in HMT samples.

Discussion

This study shows for the first time a major difference in the response of healthy mucosa vs. mucosal tissue derived from chronic rhinosinusitis with nasal polyps (CRSwNP) in response to viral exposure, in this case HSV1. CRSwNP mucosal tissue shows a significant deficit in IFN-γ and IL-17 release within 24 to 72h after infection in comparison to healthy mucosa, while at the same time releasing significantly higher levels of the pro-inflammatory cytokines IL-1β and TNF-α. These findings were associated with significantly higher viral invasion scores at 48 and 72h in CRSwNP mucosa compared to those for the healthy mucosa, indicating that CRSwNP nasal tissue provides less antiviral activity and at the same time releases disease progressing factors, allowing HSV1 to easier penetrate and spread through CRSwNP tissues, although the viral replication was similar between tissues.

Mucosal environments in disease are characterized by a distinct set of cytokine profiles, which impact on the extracellular matrix including the basement membrane, the type of inflammation (neutrophil/eosinophil), the innate and adaptive immune responses to microorganisms, and finally the potential to resolve ongoing inflammation[18]. Mucosal inflammation in nasal polyps mainly is orchestrated by Th2 cytokines, characterized by an increased eosinophilic inflammation and

formation of IgE antibodies. This endotype is associated with co-morbid asthma[15] as well as recurrence of disease in Caucasian patients[19]. All 7 CRSwNP samples in this study were classified as IL-5 positive, with high IgE and ECP concentrations, and 2 out of 7 nasal polyp patients had mild asthma. This biochemical and clinical presentation contrasts to the healthy mucosa and asthma status of the control patients. The specific immune profile in Th2-biased CRSwNP has also been associated with defects in innate or adaptive immunity, such as the alternative activation of macrophages, the suppression of T regulatory activity[20,21], or the epithelial barrier tight junction molecule expression[22, 23].

IFNs are critical for innate and adaptive immunity against viral, some bacterial and protozoal infections[24]. Type I IFNs (IFN-α and IFN-β) can be produced by all nucleated cells as the first line of host antiviral defense[25]. Type II IFN (IFN-γ),–affects activities of macrophages, NK cells, dendritic cells (DC), and T cells by enhancing antigen presentation, cell trafficking, and cell differentiation and expression profiles, conveying antiviral signals from the innate to the adaptive immune response in order to fully activate host antiviral immunity[26]. Recently identified type III IFNs (IFN-λ1, IFN-λ2 and IFN-λ3) can induce antiviral activity in a variety of target cells that express the IFN-λ receptor[27]. Aberrant IFN expressions are associated with a number of inflammatory and autoimmune diseases. An inadequate response to HRV infection has been recently described in severe asthma, suggesting that the innate anti-viral response to viruses, here HRVs, may be impaired in those patients[28].

The significant release of IFN-γ in healthy mucosal tissue (HMT) samples at 48 and 72h is considered a normal response to a viral infection, whereas the lack of induction of IFN-γ release upon HSV1 infection in CRSwNP tissues may result in a deficit to limit viral replication. Our previous findings demonstrated that HSV1 has an increased invasive ability into nasal polyp tissue accompanied by more serious damage of epithelium compared with nasal turbinate mucosa[16, 17]. Also Type I and III IFNs have been shown to play an important role in combating HSV-1 infection, and studies have shown that the magnitude and swiftness of IFN-α/β induction correlates with the relative resistance of C57BL/6 mice to HSV-1 infection[29]. In this present study, IFN-α could be detected in 4 out of 7 HMT samples after HSV1 incubation at 24 and 48h, but not in any CRSwNP samples. IFN-β was found in the supernatants of 3 HMT and 4 CRSwNP samples at 72h incubation.

These findings were rather inconsistent and did not reach significance, but may point to a broader defect in IFN response in CRSwNP tissue.

IL-17 and related components of Th17 immune function are increasingly identified as contributors to the pathogenesis of many infections, including respiratory infections caused by viruses. IL-17 facilitates the recruitment of neutrophils to the airways, and contributes to the clearance of the virus. In our human nasal mucosa model, normal healthy controls responded with a significant increase in IL-17 release upon HSV1 infection, whereas CRSwNP tissue also lacked this adequate immune response with possible consequences; unfortunately, the recruitment of neutrophils cannot further be investigated in this model.

Structural cells such as epithelial cells form part of the first line of defense and play a key role in the initiation of the immune responses including the release of IL-1 family members early on during the development of the inflammatory cascade. Secretion of IL-1β is an important outcome of the inflammasome activation, involving NF-κB activation and the expression of pro-inflammatory cytokines[29]. In the present study, we could demonstrate a significant release of IL-1β and TNF-α in CRSwNP tissue, whereas no IL-1β was released in HMT before 72h post inoculation. This suggests that viral infection induces a more vivid inflammatory response in CRSwNP than in healthy control HMT. This observation is supported by a study reporting increased levels of the pro-inflammatory mediator IL-1β and one of its antagonists, IL-1 receptor antagonist (IL-1ra), in nasal lavage of asthmatic, but not non-asthmatic patients during experimental rhinovirus infection[30]. Wang et al showed no difference in IL-6 and IL-8 release in healthy mucosa vs. nasal polyp tissue after HRV infection in-vitro[31]; in agreement with their findings, we also found no difference for IL-6 (IL-8 was not measured here), but well for other cytokines, prominently for type 2 IFN, IL-17, IL-1β and TNFα.

Herpes simplex virus (HSV)-specific T cells are essential for viral clearance. However, T cells do not prevent HSV during latent infection or reactivation. A recent study using PBMCs has shown that HSV-infected T cells stimulated through the TCR selectively synthesized IL-10, a cytokine that suppresses cellular immunity and favors viral replication[32]. This finding further was supported by studies in mice showing that the immunoregulatory cytokine IL-10 is a key host factor in inducing and maintaining T cell exhaustion, facilitating viral persistence[33,34]. In fact, 9% of human nasal

polyps were infected with HSV1[10], and persistent infections may contribute to this number. This hypothesis requires further research, focusing on IL-10 is a key player in the establishment and perpetuation of viral persistence.

As it is unethical to remove healthy ethmoidal mucosa from a non-diseased person, and it is seldom indicated to remove parts of the middle turbinate from healthy subjects; inferior turbinates have been used as controls for decades. We have shown in the past that inferior turbinates do show the same changes in terms of adaptive and innate immune reactions as the sinus mucosa in CRSsNP and CRSwNP[35]. In that sense we may expect that healthy inferior turbinates do represent healthy sinus mucosa. We assume that the structural changes from nasal polyps to normal sinus mucosa are much greater than the differences from ethmoidal to turbinate mucosa. However, we have to admit the fact that the tissue is isolated and the model therefore is not suitable for studies on cell migration.

In conclusion, we demonstrate here for the first time in a human *ex-vivo* mucosal model that the response upon viral infection by HSV1 of tissues derived from nasal polyps of chronic rhinosinusitis (CRSwNP) patients vs. inferior turbinate of healthy subjects differs considerably. CRSwNP shows a significant deficit in IFN-γ and IL-17 response, but in contrast releases significantly higher amounts of the pro-inflammatory cytokines IL-1β and TNF-α, and IL-10. This inadequate response is associated with a deeper intrusion of the virus upon acute infection, but also may lead to a different acute inflammatory response in CRSwNP tissue.

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CHAPTER 5

ENHANCEMENT of FOXP3 IN HUMAN AIRWAY REGULATORY T CELLS INHIBITS TH2 RESPONSE BY REGULATING THE SUPPRESSOR PROTEIN SOCS3 IN CRSwNP.

Foxp3 in human nasal polyp regulatory T cells is regulated by suppressor protein SOCS3

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Abstract:

Background: In persistent upper airway inflammation, the number of fork head box P3 (Foxp3)$^+$ T regulatory (Treg) cells is reduced but the regulation of Foxp3 expression in Tregs is poorly understood.

Objective: We investigated the interaction between SOCS3 and Foxp3 expression in the airway mucosa.

Methods: Expression of SOCS3 and Foxp3 was measured in CRSwNP tissue and control tissue. Co-expression of SOCS3 and Foxp3 was evaluated in PBMC and in CRSwNP tissue. We also switched off and overexpressed SOCS3 in CRSwNP tissues and in PNAC-1 cell lines and examined the effect on Foxp3 expression.

Results: SOCS3 gene and protein expression was up-regulated in inflammatory cells in airway mucosa, whereas Foxp3 gene and protein expression was down-regulated. Mucosal Treg cells co-expressed both proteins. Switching off the expression of SOCS3 in human airway mucosa resulted in Foxp3 up-regulation, whereas inducing it in PANC-1 cells led to Foxp3 down-regulation. We also found that phosphorylation of STAT3 was decreased in inflamed mucosa, and we hypothesized that SOCS3 was responsible. Phosphorylation of STAT3 increased upon silencing SOCS3 expression in inflamed mucosa and decreased upon SOCS3 plasmid transfection in PANC-1 cells.

Conclusions: We demonstrate for the first time that SOCS3 and Foxp3 are co-expressed in Treg cells in human nasal mucosa, and that SOCS3 negatively regulates Foxp3 expression in human airway mucosa, possibly by phosphorylation of STAT3. Hence, SOCS3 could be a potential target for restoring Foxp3 expression in Treg cells in persistent mucosal inflammation.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory condition in the upper airways that is often accompanied by co-morbid asthma. Two different inflammatory patterns

have been described in CRSwNP: either a T helper cell (Th)2 bias or a mixed Th1 and Th17 cell preponderance. Both patterns are accompanied by a deficit in fork head box P3 ($Foxp3)^+$ T regulatory (Treg) cells in European and Chinese patients[1-4].

$Foxp3^+$ Tregs play a critical role in immune regulation by maintaining peripheral self-tolerance, thereby preventing chronic inflammatory and autoimmune disease [5]. A loss-of-function mutation in the Foxp3 gene in mouse and human models is associated with immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome[5]. Overexpression of Foxp3 in Th cells imparts on them a suppressive phenotype[6]. However, regulation of Foxp3 expression in Tregs is still poorly understood. The role of suppressor of cytokine signaling3 (SOCS3) protein in the development and function of Tregs remains controversial. SOCS3 expression in antigen presenting cells (APCs) indirectly suppresses the development of Tregs[7]. On the other hand, Foxp3 might interact with SOCS3 by binding to the promoter or to other regulatory sites[8]. SOCS3 mediates the suppression of Tregs proliferation and the suppression of Foxp3 and CTLA-4 by reducing IL-2 levels [9]. The inactivation of SOCS3 in $CD4^+$ cells prevents development of the symptoms associated with asthma, such as airway eosinophilia in mouse models[10].

The SOCS family of proteins is at present composed of eight members characterized by the presence of a Src homology 2 domain and a C-terminal conserved domain called the SOCS box[11]. SOCS proteins negatively modulate the activation of signal transducer and activator of transcription (STAT) in the Janus Kinase (JAK)-STAT signaling pathway, which mediates the cytokine negative feedback plural loops[11]. The binding of cytokines such as IL-6, IL-2, IL-23, IL-4, INF-γ and GH to their specific receptors stimulates the cross-phosphorylation of STATs to induce SOCS genes. The SOCS proteins in turn bind to a cytokine-receptor complex, degrade the complex by SOCS-box, and interact with JAK enzyme through their SH2 region. This results in decrease of phosphorylated STATs (pSTATs)[11,12]. SOCS3 protein acts mainly on the phosphorylation of STAT3 and STAT5[13].

In mouse models, SOCS3 protein is a major regulator of the IL-23–mediated Th17 response[14]. SOCS3 has an important role in balancing Th1/Th2 towards Th2-type not Th1 differentiation[15]. High levels of SOCS3 in mouse T cells were also shown to cause Th2 skewing and allergic responses[15].

We analyzed whether SOCS3 is involved in the regulation of Foxp3 expression in human nasal mucosal tissue of CRSwNP patients and studied the mechanisms of this interaction. We used human nasal mucosa as a model to investigate the regulatory role of SOCS3 in the deficit of $Foxp3^+$ Treg cells.

Methods:

Patients. Nasal tissue was obtained from 71 CRSwNP patients and 12 control patients at the Department of Oto-Rhino-Laryngology of the West China Hospital of Sichuan University, Chengdu, China. None of the patients had taken oral corticosteroids for at least two months and topical medication for at least four weeks before surgery. The diagnosis of nasal polyps was made according to European Position Paper on Rhinosinusitis and Nasal Polyps **(**EPOS) 2007 guidelines[16]. The inferior turbinates of patients with septal deviation were used as controls. Prior to surgery, the rhinosinusitis symptoms were evaluated by a physician on a 4-points scale of 0-3 (0=no symptom, 1=mild symptom, 2=moderate symptom, 3=severe symptom) and polyps were graded by size and extent in both left and right nasal fossa, according to the Davos classification (0 to 4 per side). The clinic data of all the patients were shown in **Table E1** in the Online Repository. The study was approved by the local Ethics Committee on Humans.

Table E1. Patients characteristics and symptom scores

	Controls	CRSwNP
Number of patients	12	71
Age(years, range)	29.75(17-60)	38.2(17-66)
Female/male	3f/9m	35f/36m
Asthma in history	0/12	7/71
Aspirin intolerance (history)	0/12	0/71
Duration of symptoms (years, range)	n.a.	8.0(0.5-28)
Bilateral CT score (Lund-Mackay)	n.a.	16.08(11-24)
Bilateral polyp score (Davos)	0	4.16(2-6)
Total symptom score	0.9(0-4)	4.7(2-10)
Nasal congestion	0.8(0-3)	2.1(0-3)
Sneezing	0	0.6(0-3)
Rhinorrhea	0.2(0-1)	1.2(0-3)
Loss of smell	0	0.8(0-3)

Experimentation of the West China Hospital, Sichuan University. Informed consent was obtained from all patients and healthy donors before sample collection.

Immunohistochemistry and immunocytochemistry. The nasal tissues were fixed in 10% neutral buffered formalin and processed to paraffin. Sections of 4–5 μm were incubated overnight at 4℃ with primary antibody against SOCS3 (1:100) (Cell Signaling Technology, Beverly, MA). The secondary antibody was anti-goat (Zhongshanjingqiao, Beijing, China). Positive cells were visualized with the diaminobenzidine chromogenic reaction. Quantitative analysis of the histological sections was performed by two independent observers who counted a total of 300 cells in 10 fields per tissue section from 12 controls and 12 CRSwNP patients. Additionally, the sorted Treg cells from PBMC of 3 healthy donors were fixed and incubated with anti-SOCS3 antibody (1:100). As control, the specimens were incubated with isotype antibody IgG.

qRT-PCR. This was used to estimate the mRNA levels of SOCS3, Foxp3, STAT3 and STAT5A/B in 71 CRSwNP patients and 12 control patients. After extraction of total RNA by using RNeasy kit following the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany), cDNA was synthesized starting with 40 ng total RNA by using the Prime Script RT Reagent Kit (Takara, Dalian, China). Total RNA was quantified with RT-PCR by the iCycler iQ Real-Time PCR Detection System (BioRad, CA) using the following primer sequences: SOCS3 (5'-ATG GTC ACC CAC AGC AAG TT-3'; 5'-ACT GAG CAG CAG GTT CG C-3'), Foxp3 (5'-GAA ACA GCA CAT TCC CAG AGT TC-3'; 5'-ATG GCC CAG CGG ATG AG-3'), STAT3 (5'-GCA CAT TCA TGC TAA GAT TCA G-3'; 5'-TGA TGC TTT GTG TAT GGT TCC-3'), STAT5A (5'-CCA CAG ATG AAG CAA GTG GTC-3'; 5'-ATC GAG TAC ATG CTC ATG GTT C-3'), STAT5B (5'-GAT GCC TTT TAC CAC CAG AGA C-3'; 5'-AGT AGC AGA CTC GCA GGG AAC T-3'). PCR amplification consisted of 40 cycles of denaturation at 95℃ for 15 sec and annealing and extension cycles at 60℃ for 45 sec each. The target product was detected by SYBR Premix Ex Taq II kit (Takara, Dalian, China). To normalize for transcription and amplification variations among the samples, three housekeeping genes were used: β-actin, hydroxymethylbilane synthase, and elongation factor 1. The relative quantities expressed were assessed by the Gene Expression Macro Version 1.1 software (BioRad Laboratories Inc).

Western blotting. Protein was extracted with the Protein Extraction Reagent (KaiJi, Shanghai, China) from the nasal tissue of 35 CRSwNP patients and 12 control patients. Protein concentration was measured by Bicinchoninic Acid Assay kit (KaiJi, Shanghai, China). The following antibodies were used for western blotting: anti-SOCS3 (1:700), anti-STAT3 (1:800), anti-p-Tyr-STAT3 (Tyr705) (1:700), anti-STAT5 (1:600), and anti-p-Tyr-STAT5 (Tyr694) (1:600). All the above-mentioned antibodies were purchased from Cell Signaling Technology. Anti-Foxp3 antibody (1 μg/ml) was purchased from Abcam (Cambridge, United Kingdom). The HRP-conjugated secondary antibody (1:10000) was from Santa Cruz (Santa Cruz, CA). The relative band densities of the target protein to β-actin (Boaosen, Beijin, China) were estimated by Bio-Rad Quantity One Analysis Software (Bio-Rad, CA).

Cell isolation. Primary mononuclear cells were isolated from the blood of 3 healthy donors by Ficoll-Hypaque density gradient centrifugation according to the standard protocol. Cells were plated at $2x10^6$ cells/well in a 24-well plate and cultured at 37 ℃ in 5% CO_2 for 5 days in 1ml RPMI1640 culture medium (Sigma, St. Louis, MO) alone or with recombination human IL-2 (10 ng/ml) and recombinant human IL-15 (10ng/ml) (Miltenyi Biotec, Bergisch Gladbach, Germany).

Single cell suspension and flow cytometry. Single cell suspensions of human nasal mucosa were prepared as described [17]. To analyze the co-expression of Foxp3 and SOCS3 proteins in Tregs, the cultured cells and single cells of CRSwNP tissue (n=3) were collected and incubated with anti-CD3-PE-Cy, anti-CD4-PerCP-Cy5.5 and anti-CD25-FITC antibodies for 30 min. After fixation and permeabilization, the cells were stained intracellularly with anti-Foxp3-PE and a 1:100 rabbit anti human SOCS3 (Abcam, Cambridge, UK) for 30 min followed by goat anti-rabbit APC secondary antibody (Invitrogen, Merelbeke, Belgium) for 20 min. PBMCs from healthy donors were stained with anti-CD4-FITC, anti-CD25-PerCP-Cy5-5 (BD Biosciences, Erembodegem, Belgium) and anti-Foxp3-PE antibody (BD Biosciences) and Treg cells were sorted using standard protocols and a FACS Aria instrument (BD, Biosciences).

RNAi experiments. Three SOCS3-specific siRNAs (siG0933161433, siG0933161455 and siG0933161508) and a negative control siRNA (siN058151221487) were synthesized (RiBo Bio, Shanghai, China) and the target sequence was validated (5'-GAC CCA GTC TGG GAC CAA G-3'). The following sequences were used: SOCS3 sense, GAC CCA GUC UGG GAC CAA G dTdT;

SOCS3 anti-sense CUU GGU CCC AGA CUG GGU C TdTd. The transfection efficacy of siRNA in nasal polyp tissue was estimated by using siR-Ribo™ Transfection Control (siN05815122148) (RiBo Bio, Shanghai, China). The result is shown in **Figure E1**. More detailed information about transfection efficiency has been provided in the **Methods E1** of the Online Repository. Nasal polyp tissue (n=5) was washed with PBS containing 100 IU/ml penicillin and 100 μg/ml streptomycin three times and sliced into cubes. The 80 mg of tissue cubes were placed into each well of a six-well plate containing 1.3 ml of RPMI 1640 medium and transfected with SOCS3-specific siRNA (40nM) or negative control siRNA (40nM) using 5 μl of the lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the standard protocol. After 4h of incubation, fetal serum (10% final concentration) (Hyclone, Logan, UT) was added to each well to terminate transfection and tissues were incubated at 37 ℃ in humidified air with 5% CO_2 for 48h. The tissue cubes were collected for RT-PCR and western blot.

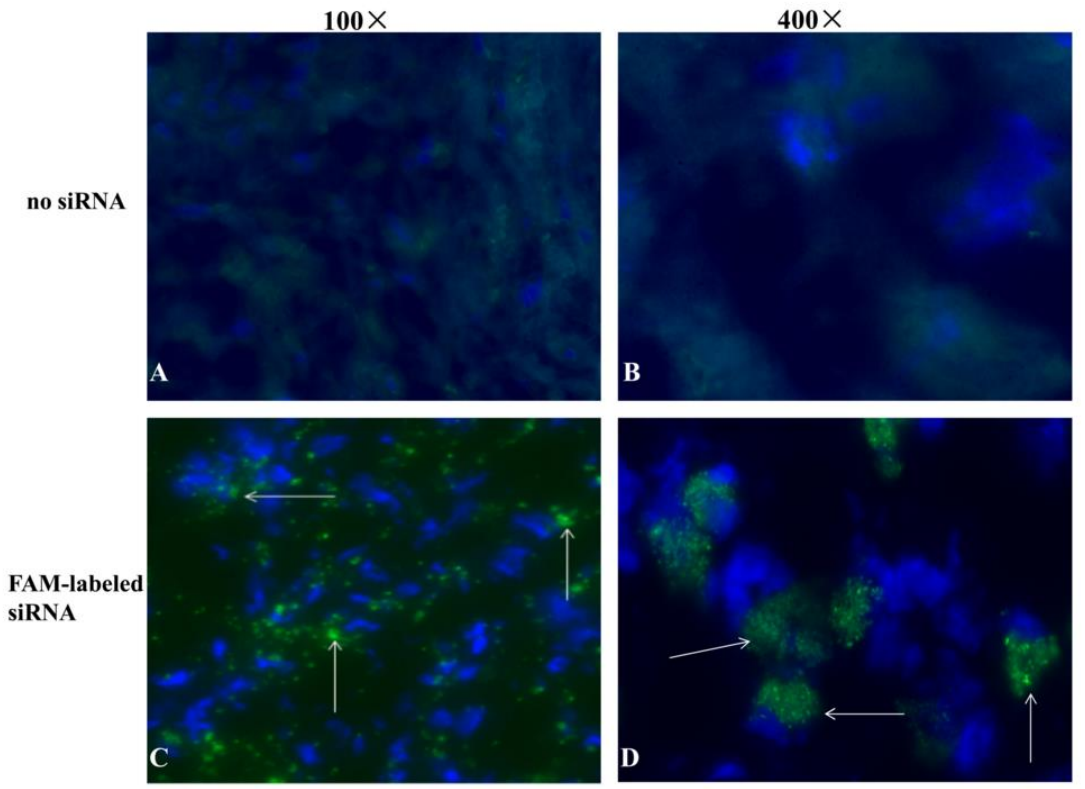

Figure E1: Transfection efficiency of siRNA in nasal polyp tissue fragments. The blue fluorescence represents the nuclei of cells, the green dots fluorescein-labeled siRNA transfection control. (A, B) Fluorescence images of nasal polyp tissue fragments without fluorescein-labeled siRNA. (C, D) Fluorescence images of CRSwNP tissue fragments with fluorescein-labeled siRNA. The merged version of green and blue fluorescence indicates the siRNA intracellular location (white arrows). Magnification: 100 × (A, C) and 400 × (B, D).

Transient expression of SOCS3 in PANC-1 cells. Human pancreatic carcinoma epithelial-like cells (PANC-1), a cancer cell line with strong gene expression of Foxp3[18], was used for transient expression of SOCS3. pEF-FLAG-I/m vector and pEF-FLAG-I/m SOCS3 plasmid (2μg) were separately transfected into 5×10^6 PANC-1 cells by using lipofectamine 2000 transfection reagent. Total RNA and proteins were extracted from transfected PANC-1 cells after 48 h of incubation. These experiments were repeated three times.

Statistics. A one-way ANOVA test, Mann-Whitney test and T test were performed using SPSS 12.0 (SPSS, Inc, Chicago). A p value < .05 was considered statistically significant.

Results:

SOCS3 expression is increased in patients with CRSwNP. Immunohistochemical analysis showed that CRSwNP patients had significantly stronger expression of SOCS3 than controls (**Figure 1**). In CRSwNP tissue, SOCS3 protein was preferentially expressed in inflammatory cells, epithelium and submucosal glandular epithelium. Quantitative analysis of the histological sections performed by two independent observers showed a significant increase in $SOCS3^+$ cells in nasal

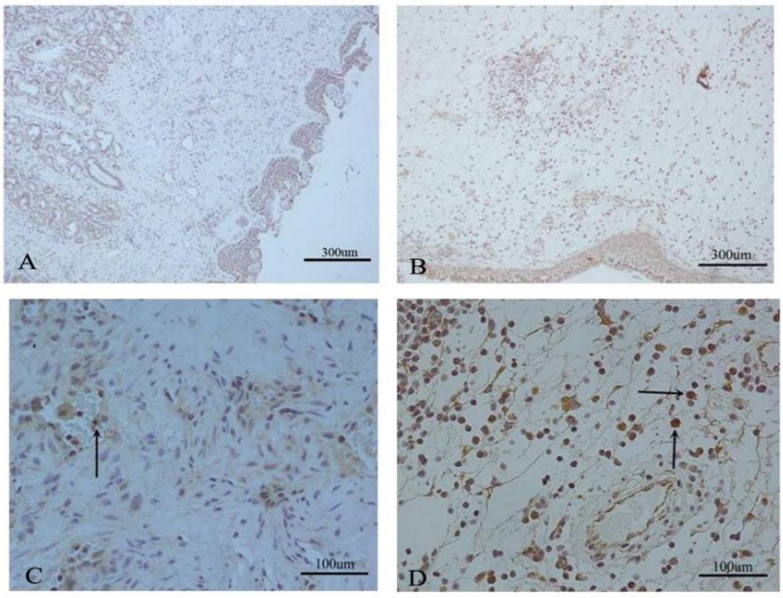

Figure 1 SOCS3 expression in CRSwNP tissues. Representative images of SOCS3 immunohistochemistry in CRSwNP tissues (n=12) and controls (n=12). (A, C) SOCS3-positive cells (arrows) in controls. (B, D) SOCS3-positive stained cells (arrows) in CRSwNP. Magnification: 100× (A, B) and 400× (C, D).

polyp tissue, in which 33.3 ±9.06% of the cells were positively stained, as compared to only 6.67 ±3.58% controls (*P*<.0001). The levels of SOCS3 gene expression were higher in CRSwNP, and this was confirmed by western blot (**Figure 2A, C**). Importantly, the expression of Foxp3 protein in CRSwNP was significantly increased (**Figure 2 B, D**).

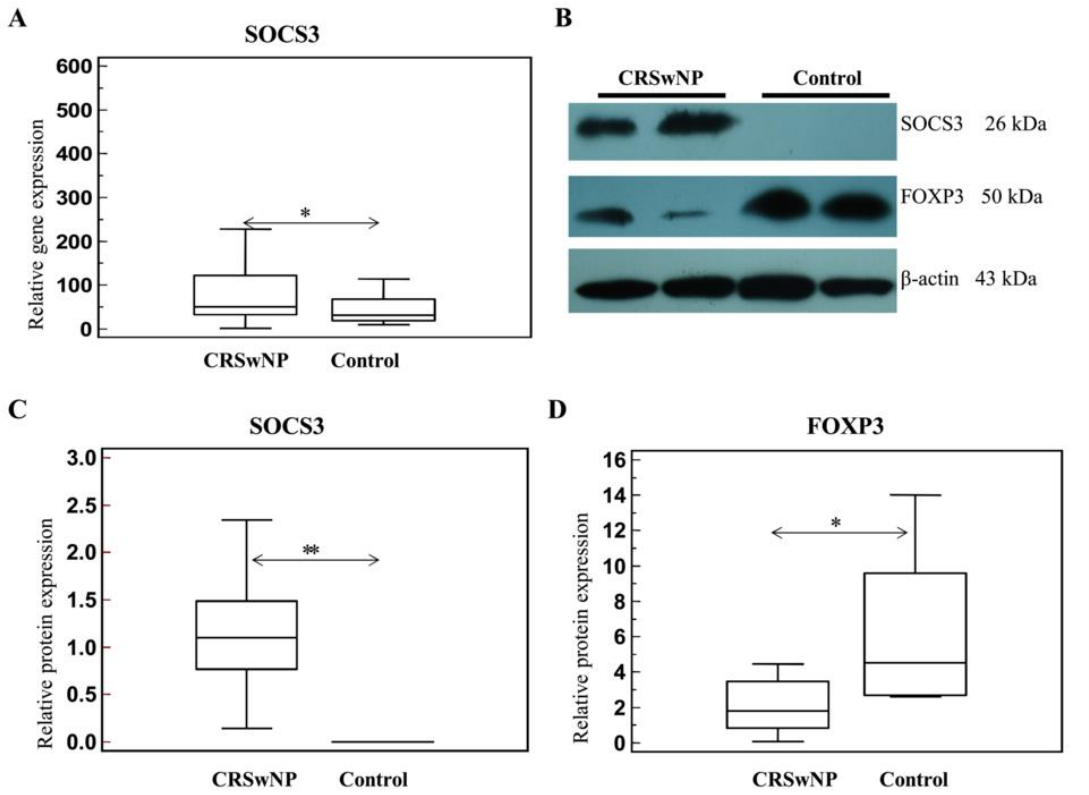

Figure 2 The expressions of SOCS3 and FOXP3 in CRSwNP. (A) RT-PCR showing SOCS3 expression in CRSwNP (n=71) and controls (n=12). (B) Western blotting pictures for SOCS3 and FOXP3 proteins in CRSwNP (n=35) and controls (n=12). (C) Quantification for SOCS3 protein in CRSwNP tissues. (D) Quantification for Foxp3 protein in CRSwNP tissues (**P*<.05, ***p*<.01).

Foxp3-positive Tregs express SOCS3 protein. In non-stimulated conditions, 86% of $CD4^+CD25^+Foxp3^+$ Tregs in human peripheral blood expressed SOCS3 (**Figure 3A-b**). In PBMCs treated with 10 ng/ml of IL-2 and IL-15 for 5 days (**Figure 3A-c**), 96% of $CD4^+CD25^+Foxp3^+$ Tregs co-expressed SOCS3. Therefore, most $CD4^+CD25^+Foxp3^+$ cells co-expressed SOCS3 in both stimulated and non-stimulated conditions. In addition, $CD4^+CD25^+Foxp3^+$ cells were sorted from PBMCs of three healthy donors and stained with anti-SOCS3 antibody. The result confirmed the

co-expression of these proteins in 96% of Tregs (**Figure3B**). In human nasal mucosal tissue of CRSwNP patients, 45.1% of $CD3^+CD4^+CD25^+$cells were $SOCS3^+Foxp3^+$ (**Figure 3C**).

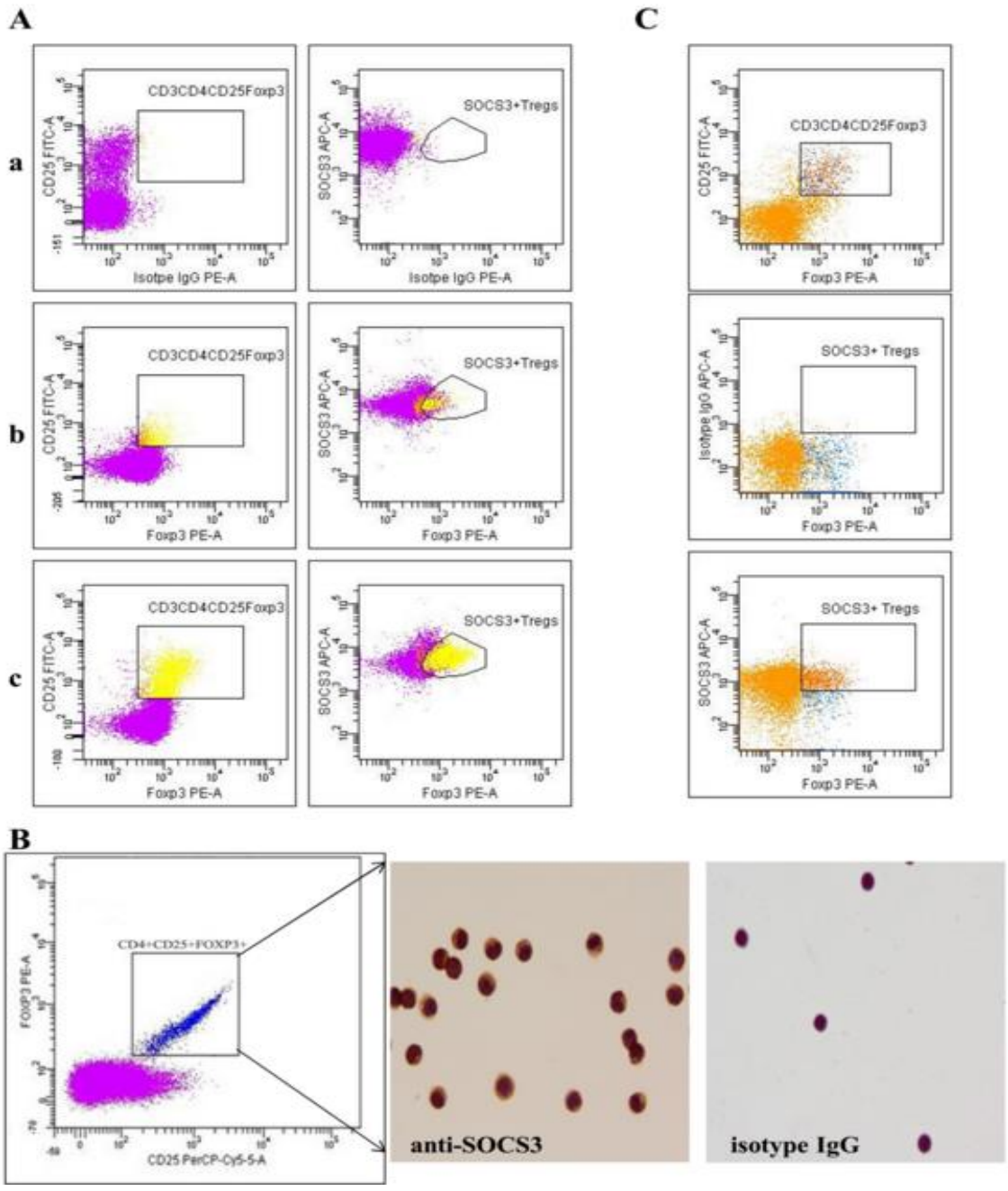

Figure 3 The expression of SOCS3 and FOXP3 proteins in Treg cells. PBMCs of healthy donors gated on living $CD3^+CD4^+CD25^+Foxp3^+$ cells cultured in RPMI: (A-a) is the isotype control. (A-b) Non-stimulated. (A-c) Stimulated with IL-2 and IL-15. (n=3) (B) $CD4^+CD25^+Foxp3^+$cells sorted from PBMCs of healthy donors and stained with anti-SOCS3 antibodies. (n=3) (C) The single cells of CRSwNP tissue gated on living $CD3^+CD4^+CD25^+Foxp3^+SOCS3^+$cells. (n=3)

Modulation of SOCS3 levels and Foxp3 expression. As immunohistochemical analysis showed high levels of SOCS3 in epithelium, glandular epithelium, and inflammatory cells in CRSwNP

tissue, human nasal mucosal tissues were used in silencing experiments. Three different SOCS3-specific siRNAs were designed to block the function of SOCS3 protein. The siRNA construct that knocked down SOCS3 protein most efficiently was selected. Blocking SOCS3 gene expression by siRNA decreased SOCS3 gene expression by 60% (**Figure 4A**). Analysis of Foxp3 gene expression after blocking SOCS3 expression demonstrated a three-fold increase in the same samples of CRSwNP tissue (**Figure 4A**). The RNA expression results were confirmed by western blot analysis: Foxp3 protein expression increased significantly upon silencing SOCS3 in CRSwNP tissue (**Figure 4 B, C**).

The levels of SOCS3 mRNA and protein were increased in PANC-1 cells upon transfection with the SOCS3-encoding plasmid (**Figure 4D-F**), while Foxp3 protein expression was attenuated by up to 80%, as evident from measuring the relative expression levels by western blotting (**Figure 4E**). Taken together, our data demonstrate that SOCS3 negatively modulates Foxp3 expression.

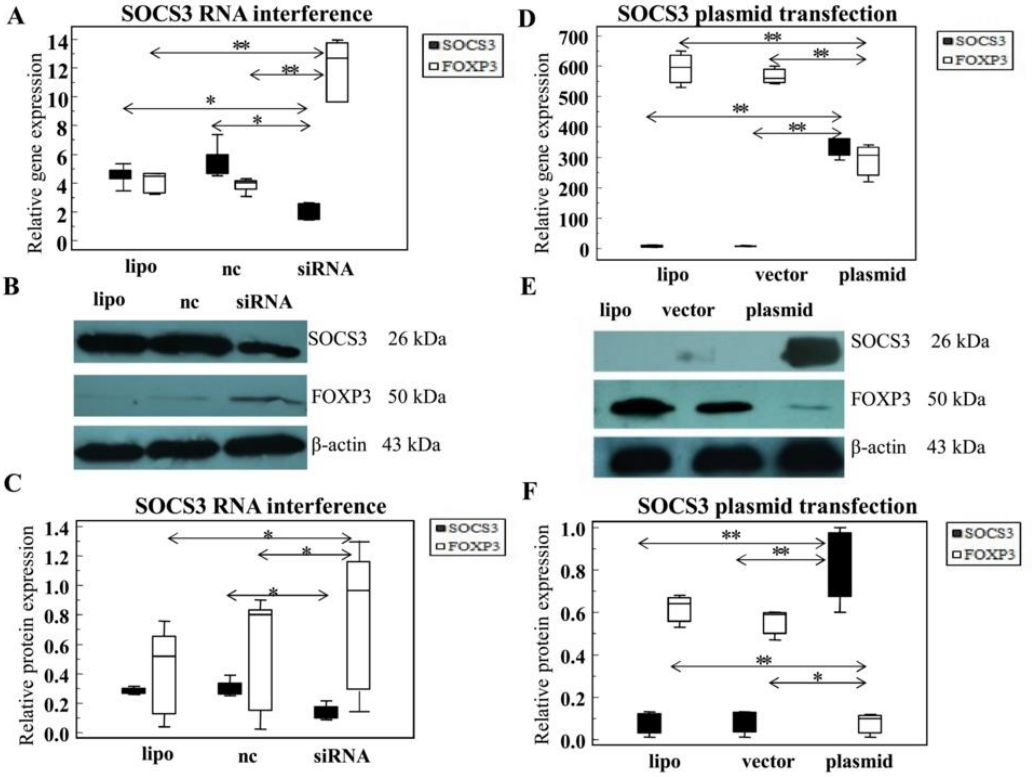

Figure 4 SOCS3 regulates FOXP3 expression in CRSwNP tissues. (A-C) SOCS3 and Foxp3 expression in CRSwNP (n=5) upon addition of lipofectamine 2000 (lipo), transfection with negative control of siRNA (nc), or with SOCS3-specific siRNA (siRNA). (D-F) SOCS3 and Foxp3 expressions in PANC-1 cells upon addition of lipo, transfection with pEF-FLAG-I/m vector (vector) or pEF-FLAG-I/m SOCS3 plasmid (plasmid) (n=3) (**P*<.05, ***p*<.01).

SOCS3 regulation of Foxp3 might depend on the presence of pSTAT3. To further study the regulation of Foxp3 expression by SOCS3, the expression of STAT3 and STAT5A/B genes were investigated in the nasal tissue. STAT3, STAT5B and STAT5A gene expression were not different between CRSwNP and control tissue (data not shown). However, SOCS3 might attenuate the phosphorylation of STATs (pSTATs), which are the activated forms of STATs in the JAK-STAT pathway. We demonstrate that pSTAT3 was significantly down-regulated in nasal mucosa of CRSwNP patients in comparison with controls (**Figure 5 A, B**). The levels of STAT5 and pSTAT5 proteins were undetectable in western blots. As shown in **Figures 5 C** and **D**, the relative expression of pSTAT3 was up-regulated by 65% after silencing SOCS3 protein expression in nasal mucosal tissue. In contrast, the relative expression of pSTAT3 was decreased by 75% when SOCS3 was overexpressed from a plasmid (**Figure 5 E, F**).

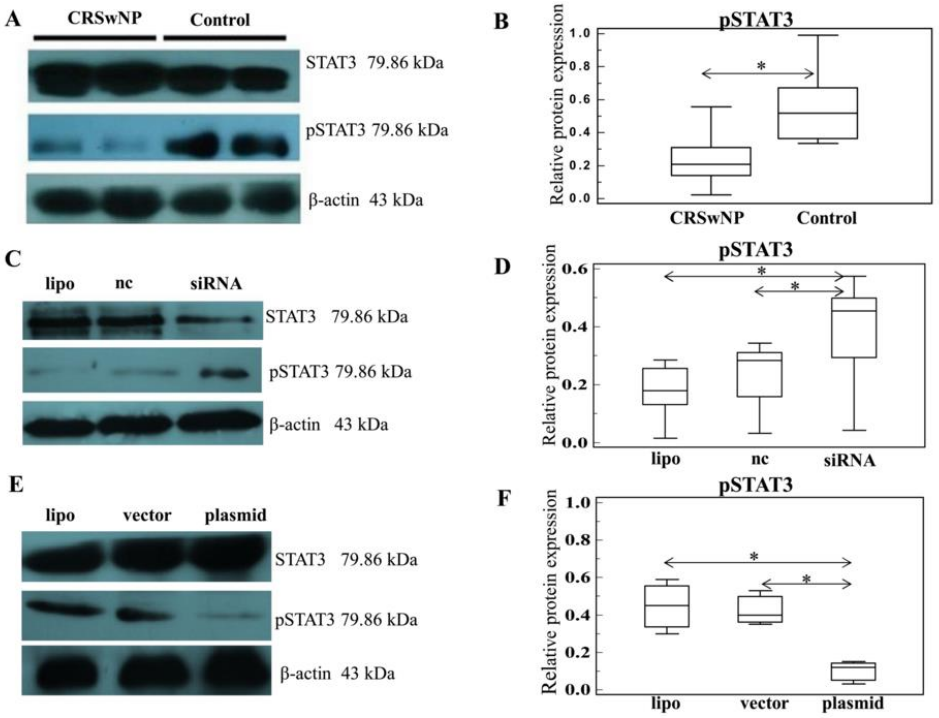

Figure 5 The role of pSTAT3 in regulation of FOXP3. (A-B) pSTAT3 expression in nasal tissue. (C-D) pSTAT3 expression in CRSwNP (n=5) upon addition of lipofectamine 2000 (lipo), transfection with negative control of siRNA (nc), or SOCS3-specific siRNA (siRNA). (E-F) pSTAT3 expression in PANC-1 cells upon addition of lipo, transfection with pEF-FLAG-I/m vector (vector), or pEF-FLAG-I/m SOCS3 plasmid (plasmid) (n=3) (**P*<.05, ***p*<.01).

Discussion:

Chronic rhinosinusitis with nasal polyp is characterized by a Th2-skewed eosinophilic inflammation in European patients and by a Th17-biased inflammatory profile in Asian patients. However, regardless of the inflammatory profile, significantly weaker Foxp3 mRNA expression and significantly stronger T-bet and GATA-3 mRNA expression was observed in both groups of patients[3]. The mechanisms of altered T cell plasticity in CRSwNP are not yet clear. We propose that SOCS3 protein is an important regulator of Foxp3 in CRSwNP. We have demonstrated increased levels of SOCS3 protein in CRSwNP compared to controls. The expression of SOCS3 protein was mostly observed in inflammatory cells, epithelium, and glandular epithelium in the submucosa.

Importantly, SOCS3 expression is not limited to T effector cells[14,15]. Also $CD4^+CD25^+Foxp3^+$ T regulatory cells stained intensely for SOCS3. To our knowledge, this is the first study to demonstrate the co-expression of SOCS3 and Foxp3 proteins in Tregs from human nasal mucosal tissue. In contrast, Pillemer *et al*.[9] did not find SOCS3 protein expression in mouse Tregs. However, in mice lacking SOCS3 expression in T cells, a preferential Th3 and Treg differentiation was observed[19,20]. This T cell polarization bias could be explained by increased production of IL-10 and TGF-β in conditions of SOCS3 deficiency. In addition, the impaired expression of SOCS3 in APCs indirectly induces the promotion of $CD4^+CD25^+Foxp3^+$ cells[7]. In recent experiments, Nguyen *et al*. showed that induction of SOCS3 expression in Tregs by pharmacologic means abrogated a Treg proliferative response induced by serum amyloid A and monocytes[21]. However, the mechanism of SOCS3–Foxp3 interaction and its role in Treg development are not completely understood.

The role of SOCS3 protein in T cell differentiation and function is well established[22], but its role in Treg function is just being elucidated. The differentiation and survival of Tregs are regulated by Foxp3. The NF-κB family members, CREB/ATF, ETS-1 and FOXO1/3, were shown to facilitate Foxp3 transcription and to bind to its promoter and other regulatory elements, as reviewed[23]. Additionally, STAT3 and STAT5 phosphorylation is critical for Foxp3 induction in precursor cells[24]. Phosphorylated STAT3 and STAT5 were also shown to interact with the Foxp3 promoter and to modulate Foxp3 gene expression[25]. SOCS3 protein acts mainly *via* phosphorylated STAT3 and STAT5[26].

In our RNAi experiments, abolishing SOCS3 expression in the nasal polyp tissue increased the levels of Foxp3. In contrast, when overexpression of SOCS3 was achieved by transient transfection, Foxp3 levels were down-regulated. These data confirm that in human nasal polyp tissue, SOCS3 serves as a negative regulator of Foxp3 expression. Intranasal administration of Foxp3 strongly prevents the ovalbumin induced allergic airway inflammation in mice[27]. Moreover, up-regulation of Foxp3 by corticosteroids in asthmatic patients restores T regulatory cell function to suppress the Th2 response[28]. We therefore suggest that up-regulation of Foxp3 expression by SOCS3 down-regulation might present a therapeutic approach in nasal polyps. In addition, we found that the levels of phosphorylated STAT3 protein are reduced in CRSwNP. We also confirm that phosphorylation of STAT3 increased upon silencing SOCS3 expression in inflamed mucosa and decreased upon SOCS3 overexpression from a plasmid transfected in PANC-1 cells. The regulatory role of pSTAT3 on Foxp3, unlike pSTAT5, is still controversial. pSTAT3 is reported to play a negative role on Foxp3 in allergic or graft-versus-host mice models[29,30], however, pSTAT3 signaling also can promote Foxp3 expression in the same graft-versus-host mice model[25]. Furthermore, induced Foxp3 in human Treg cells is pSTAT3 dependent[31], which is in accordance with our results in human nasal polyp tissue. Consequently, we suggest that phosphorylation of STAT3 might be involved in the SOCS3-induced regulation of Foxp3.

The following limitations have to be taken into account: In this study we used CRSwNP patients from China, and there is evidence that Chinese nasal polyps are different from Caucasian polyps, so that our findings might not be readily transferable to all patients; however, there is evidence that the same down-regulation of Foxp3 applies to both the Chinese and the Caucasian polyps, and we also demonstrated that up-regulation of SOCS3 can be demonstrated in Caucasian polyp tissue (data not shown). We therefore believe that our data is valid for all nasal polyp patients. Our patients were asked to stop taking oral corticosteroids for at least two months and topical medication for at least four weeks before surgery; however, we had no possibility of controlling compliance. Also, we speculated on a possible therapeutic effect of silencing SOCS3 to increase the activity of Foxp3 regulatory T cells, which might then lead to a suppression of the inflammatory process, but have not yet proven this here; further studies are ongoing. We also noticed that there is a difference in percentage of Foxp3 expression in SOCS3 positive cells in nasal polyp tissue vs. PBMC from

healthy donors. It is currently unclear whether this difference is due to the fact that we compare peripheral blood to tissue or healthy to diseased patients; further studies are needed to investigate this question.

In summary, we demonstrate that SOCS3 negatively regulates Foxp3 expression in CRSwNP, and this regulation may involve pSTAT3. As down-regulation of SOCS3 leads to an increase in Foxp3 expression, this might improve the suppressive capacities of Tregs[32]. Therefore, SOCS3 could be a potential target for restoring Foxp3 expression in Treg cells to consecutively suppress persistent mucosal inflammation. Thus, SOCS3 siRNA or other promising therapeutic technology such as SOCS3 specific DNAzyme, a new class of antisense molecules that combines the specificity of DNA base pairing with an inherent RNA-cleaving enzymatic activity[33], could possibly be used to inhibit SOCS3 expression for therapeutic intervention in nasal polyp disease.

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Methods E1:

Nasal polyp tissue was cut thoroughly in tissue culture medium consisting of RPMI 1640 and antibiotics (50IU/ml penicillin and 50μg/ml streptomycin). The tissue was passed through a mesh to achieve comparable fragment. Tissue fragments ($\pm 0.9\ mm^3$) were re-suspended and pre-incubated in tissue culture medium for 1 h. 80 mg of tissue fragments were placed into each well of a six-well plate containing 1.3 ml of RPMI 1640 medium without antibiotics. Fluorescein -labelled siRNA, siR-Ribo™ Transfection Control (40 nM), was transfected into the tissue by using 5 μl of lipofectamine 2000 transfection reagent. After 4h of incubation, fetal serum (10% final concentration) was added to each well to terminate transfection and tissues were incubated at 37℃ in humidified air with 5% CO_2 for 48h. Tissue fragments with or without fluorescein-labeled siRNA were washed for three times by PBS before collection, frozen and prepared for cyrosections. 5μm thickness cryosections were fixed in 70% ethanol and dried at room temperature, prior to being mounted with DAPI (1:50 dilution). The transfection efficiency was evaluated by the formula: transfection efficiency=cells expressing green fluorescence/total accounted cells *100%. Images were obtained with two magnifications (100×, 400×) by a fluorescence microscopy (Axioplan 2, Carl Zeiss, Gottingen, lower Saxony, Germany).

Supplementary experiment which has been done based on the previous findings:

To further study the inflammatory response changes in CRSwNP tissue after SOCS3 silencing, inflammatory cytokines such as TNF-α, IL-5, IL-17, IFN-γ and IL-10 were measured in supernatants of CRSwNP tissue with SOCS3 siRNA transfection. All cytokines were assayed using Luminex xMAP suspension array technology in a Bio-Plex 200 system (BioRad, MI, USA), and the final data was expressed as pg/ml. The protein level of IL-5 decreased in the CRSwNP tissue 48h after silencing SOCS3 expression. To our Surprised, IL-10 cytokine also decreased after silencing SOCS3 expression in CRSwNP tissue. Although SOCS3 is very important for Th17 cell generation and IL-23–mediated Th17 response, no significant changes of IL-17 expression were observed in CRSwNP tissue with SOCS3 silencing (**Figure 6**). Thus, we confirm that blocking SOCS3 expression restores Foxp3 expression in Treg cells and inhibits Th2 response in persistent mucosal inflammation.

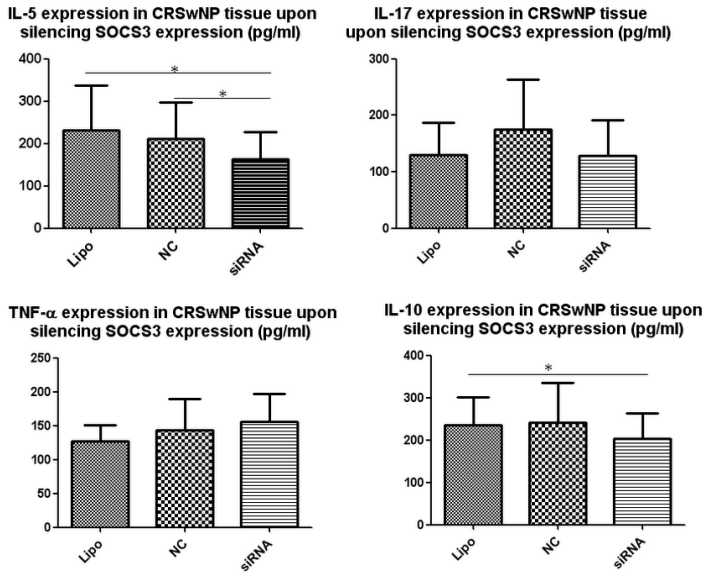

Figure 6: Blocking SOCS3 expression inhibits Th2 response in CRSwNP tissue. Cytokine expressions in supernatant of CRSwNP tissue with SOCS3siRNA transfection. (**P*<.05, ***p*<.01)

CHAPTER 6

DISCUSSION AND PERSPECTIVES

Chronic rhinosinusitis (CRS) is an ill-defined umbrella term for sinus diseases. CRS is defined as disease of the nose and the paranasal sinuses that lasts for three months or longer before the doctor's interview. The disease affects 15% of the total population in the United States; a recent European survey reports a prevalence of 10.8%[1]. However, CRS is not one disease, but distinct pathophysiologies have been described in different CRS subgroups. CRS without nasal polyposis (CRSsNP) often displays a Th1 based neutrophilic inflammation, while CRS with nasal polyposis (CRSwNP) in Europe is in approximately 80% of all cases dominated by Th2 driven eosinophilic inflammatory mechanisms, and may be accompanied by aspirin sensitivity and asthma [2, 3]. Recently, our group has confirmed that the colonization with *Staphylococcus aureus* (*S. aureus*) and the presence of *S. aureus* enterotoxin-specific IgE antibodies significantly increase in the mucosa of CRSwNP subjects in comparison with control or CRSsNP patients. This suggests a link between *S. aureus* colonization or infection and a Th2 response.

S. aureus induces a type 2 response in CRSwNP via epithelial cell derived cytokines

In Chapter 3, we have focused on the association between *S. aureus* and the Th2 response in CRSwNP tissue. Critically, staphylococcal enterotoxin B (SEB) induces considerable release of pro-inflammatory and Th2-associated cytokines including IL-4, IL-5 and IL-13 in human nasal and in mouse bronchial tissue[4, 5]. However, the role of *S. aureus* independent of enterotoxins in inducing type 2 immune responses in human chronic airway disease has not been elucidated. Thus, we used a *S. aureus* strain, which does not produce *S. aureus* enterotoxins in our infection model. Nasal epithelial cell lines or primary epithelial cells and certain microbial pathogen surrogates are frequently used to understand the post-infection immune response. However, as a bridge between simple cell lines and complex *in vivo* environments, nasal tissue explants are better suitable than primary epithelial cells or airway cell lines to investigate the role of microbes in airway diseases. These models can efficiently reflect the *in vivo* situation in the nose during infection.

In recent years, it has been shown that cytokines derived from epithelial cells such as IL-33 and thymic stromal lymphopoietin (TSLP) released by various stimuli, are critical in propagating Th2 immune responses[6, 7]. Our human nasal *ex vivo* infection model suggests that IL-33 and TSLP can be induced in CRSwNP tissue in the presence of *S. aureus*. Although TSLP is also induced in healthy

inferior turbinate (IT) tissue after *S. aureus* infection, a consequent IL-5 protein release is propagated in CRSwNP tissue only, not in healthy IT tissue**.** Therefore, CRSwNP tissue and IT tissue show different reactions upon *S. aureus* infection. Besides the increased IL-33 and TSLP expression, the receptors of epithelial cell derived cytokines TSLPR and ST2L were significantly up-regulated 48h after *S. aureu*s infection, reinforcing the signal of epithelial cell derived cytokines. To analyze the following Th2 response, we measured the mRNA and protein level of IL-4, IL-5 and IL-13 cytokines in human nasal *ex vivo* infection model. We successfully demonstrated that the *S. aureus* strain without enterotoxin secretion can directly induce IL-5 and IL-13 in CRSwNP tissue, but did not find IL-4. To evaluate the specific role of *S. aureus* in IL-5 release, *S. epidermidis* was also included in the models. Our data unveiled that type 2 cytokines can only be induced by *S. aureus*, not by *S. epidermidis* in CRSwNP tissue.

As *S. aureus* could induce TSLP and IL-33 in our human nasal *ex vivo* infection model, we also explored the pathway of cytokine productions induced by *S. aureus*. We could show that activation of the NF-κB pathway was evident from an increase in phosphorylation of P50 and P65 at early time points in BEAS-2B cells after *S. aureus* exposure, accompanied by an increase of IL-33 and TSLP release. The novel TLR2 antagonist CU-CPT22 inhibited the phosphorylation of P50 and P65. Consequently, the expression of the downstream IL-33 and TSLP decreased in a dose-dependent manner. Thus, *S. aureus* can directly induce epithelial cell-derived cytokine release via binding to TLR2 and consecutively propagates type 2 cytokine expressions in CRSwNP tissue **(Figure 1)**.

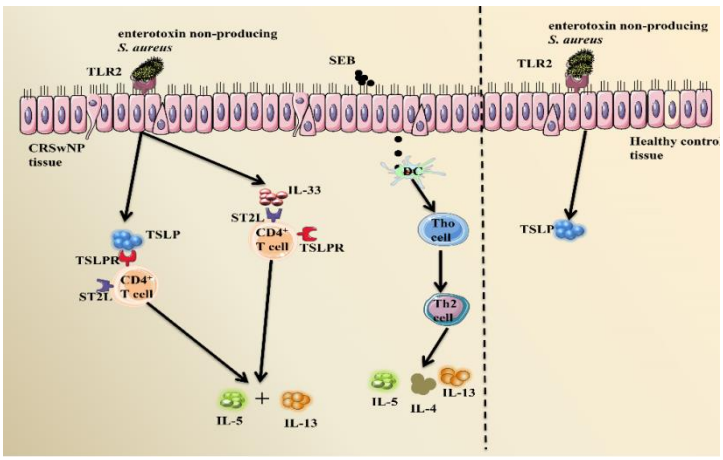

Figure 1: *Staphylococcus aureus* induces Th2 responses via epithelial cell derived cytokines TSLP and IL-33 in a nasal *ex vivo* infection model.

C. Bachert first described IgE to *S. aureus* enterotoxins in CRSwNP and confirmed its role as a marker of severe inflammation in the upper and lower airways, supporting the role of *S. aureus* in CRSwNP tissue[8]. However, the current work for the first time studies direct effects of *S. aureus* on Th2 cytokine induction independent of enterotoxin activity. The defense mechanisms against *S. aureus* may be impaired in CRSwNP, with a high rate of alternatively activated macrophages in CRSwNP vs. healthy mucosa, not suited to phagocytize and kill *S. aureus* efficiently[9]. Products of *S. aureus*, specifically superantigens, induce Th2 cytokines in CRSwNP and healthy mucosal tissue; however, these products need to be in direct contact with the immune cells. Thus, the presence of *S. aureus* within the mucosal tissue of CRSwNP patients may lead to persistent inflammation, but it remains unclear whether *S. aureus* - extra- or intramucosally - actually can initiate type 2 disease. In addition, we here elaborate that *S. aureus* induces Th2 cytokines via epithelial cell derived cytokines after binding to the TLR2 receptor. IL-5 release was not totally blocked by TLR2 blockage, indicating that another production pathway via which *S. aureus* induces the epithelial cell derived cytokines to propagate Th2 cytokines could be involved and needs to be unraveled. From our findings listed above, more efficient therapeutic interventions like specific eradication of *S. aureus*, blocking TLR2 receptor expression, TSLPR and ST2L expression and using specific antibody neutralization of TSLP and IL-33 in CRSwNP are perspectives for the further investigations.

Th2 biased upper airway inflammation is associated with an impaired response to viral infection with Herpes simplex virus 1

Viral infections are known as the most frequent cause of acute rhinitis and asthma exacerbations in children and adults. A high frequency of human rhinovirus (HRV) was found in epithelial cells scraped from the middle nasal meatus of CRSwNP patients; natural and experimental rhinovirus infections have been well studied in the lower airways. However, little is known about the effect of viral infections in patients with chronic inflammatory disease such as CRSwNP. It is also reported that 8% (2/23) of human CRSwNP tissues may be infected with herpes simplex virus 1 (HSV1) at a given moment in time[10]. Moreover, the nasal mucosa is considered the primary site of herpes virus

replication. In Chapter 4, nasal tissues were infected with HSV1 for 24, 48 and 72h to study the post-infection immune response in CRSwNP tissue. We here demonstrate for the first time that the depth of mucosal invasion for HSV1 in CRSwNP tissue at 48 and 72h was significantly higher than that in IT mucosa. Simultaneously, CRSwNP mucosal tissue showed a significant deficit in IFN-γ and IL-17 release within 24 to 72h after HSV infection compared to healthy mucosa, at the same time releasing significantly higher levels of the pro-inflammatory cytokines IL-1β and TNF-α. These findings indicate that CRSwNP nasal tissue provides less antiviral activity and at the same time releases disease progressing factors, allowing HSV1 to penetrate and spread easier through CRSwNP tissues. With the viral infection, also *S. aureus* has been shown to intrude into the nasal mucosa; the virus here works as a "door opener" for a germ which can further stimulate and maintain type 2 inflammation[11]. This might be a crucial mechanism which allows *S. aureus* to reside intramucosally and grants an enormous advantage to this germ. We could confirm that CRSwNP mucosal tissue exerts a defective defense ability against HSV 1 in comparison with controls. Although a high prevalence of HSV1 is observed in upper airways, HRV is one of the most common viruses in human airways and is associated with the development and exacerbation of asthma[12]. HRV infections induce IL-33 production *in vivo*, which can further initiate type 2 cytokine release from Th2 cells or innate lymphoid type 2 (ILC2) cells[13]. Although HSV1 infections could not induce Th2 responses in CRSwNP tissue upon infection, the effect of HRV infections on CRSwNP disease and the relationship between HRV infection and Th2 responses clearly merits further investigations.

Enhancement of Foxp3 in human airway regulatory T cells inhibits IL-5 release by regulating suppressor of cytokine signalling 3 in CRSwNP

Two different inflammatory patterns have been described in CRSwNP: a Th2 bias or a mixed Th1 and Th17 cell preponderance[14]. Regardless of the inflammatory profile in CRSwNP, a deficit in fork head box P3 $(Foxp3)^+$ T regulatory (Treg) cells is observed in European and Chinese patients[15]. However, Foxp3 regulation in Tregs is poorly understood. In Chapter 6, we focused on the enhancement of Foxp3 expression in CRSwNP to alter T cell plasticity in CRSwNP.

We started from an observation in nasal tissue showing that the levels of SOCS3 were

significantly higher in CRSwNP compared to controls. Moreover, the increased SOCS3 protein was predominantly located in inflammatory cells, epithelium, and glandular epithelium in the submucosa. Further immunofluorescence staining demonstrated the co-expression of SOCS3 and Foxp3 proteins in Tregs from human nasal mucosal tissue. Although the role of SOCS3 protein in T cell differentiation and function is well established[16], its role in Treg function is just being elucidated. In our SOCS3 RNAi experiments, abolishing SOCS3 expression in the nasal polyp tissue increased the levels of Foxp3. In addition, when overexpression of SOCS3 was achieved by transient transfection, Foxp3 levels were down-regulated. This SOCS3-based regulation may involve pSTAT3. The findings confirm our hypothesis that SOCS3 is able to regulate Foxp3 expression in CRSwNP, and we speculate on a possible therapeutic effect of silencing SOCS3 to increase the activity of Foxp3 regulatory T cells, which might then lead to a suppression of the inflammatory process. Thus, we measured Th1, Th2 and Th17 associated cytokines after silencing of SOCS3 in a RNAi experiment, and demonstrated that TNF-α, IL-5 and IL-17 decreased in CRSwNP tissue upon silencing SOCS3 expression.

A deficit in $Foxp3^+$ Treg cells is observed in European and Chinese patients[15], thus restoration of the deficiency of Treg cells is regarded as a plausible strategy to modulate Th2 biased allergic airway disease. However, adoptive transfer of Treg cells does not provide satisfactory results as observed in an asthmatic mouse model when compared to induced Treg cells. In addition, a better understanding of Foxp3 regulation is useful for obtaining more iTreg cells in the periphery. In Chapter 6, we have elaborated that silencing SOCS3 increased the activity of Foxp3 regulatory T cells which then inhibited Th2 cytokine release in CRSwNP. This provides further perspective in the role of SOCS3 siRNA or other promising therapeutic technology targeting at SOCS3 expression in therapeutic interventions on the Th2 response in type 2 airway disease.

General discussion:

In this thesis, we have demonstrated that the presence of *S. aureus* in CRSwNP nasal mucosa provides the possibility to induce Th2 cytokines via epithelial the cell derived cytokines TSLP and IL-33, which may aggravate and maintain type 2 immune responses in CRSwNP tissue. CRSwNP furthermore shows a significant deficit in IFN-γ and IL-17 response, but in contrast releases

significantly higher amounts of pro-inflammatory cytokines IL-1β and TNF-α compared to healthy controls, and IL-10 upon virus HSV1 infection; CRSwNP tissue thus displays a deficit against viral infections, which may serve as a door opener for *S. aureus* and may allow this germ to invade into the nasal mucosa. In consequence, products of *S. aureus* can directly interact with local immune cells, with the potential to create a type 2 inflammatory environment with IgE production. The presence of IgE antibodies to *S. aureus* enterotoxins is a hallmark of disease severity not only in upper, but also lower airway disease. We here stress that apart from allergens, specific germs and viruses also may play a critical role in the induction of type 2 immune responses; those infectious agents are commonly associated with Th1 and Th17 responses. We assume that not one single event will be sufficient to initiate and certainly not to maintain Th2 inflammation, but rather that multiple hits are necessary over time, involving microbes, allergens and environmental factors (**Figure 2**), to create a persistent type 2 bias within the tissue. The impact of such events probably depends on the quantity of viruses and/or bacteria and the time course, e.g. high numbers of microbes may hit the airway mucosa during an acute infection, and low numbers may persistently exert pressure as colonizers, biofilm formers or intramucosal intruders. Furthermore, the immune status of the airway mucosa will make a difference, with a Th1/Th17 biased mucosa being more resistant to Th2 response than an already Th2-biased inflammatory status. As we have discussed, these different T helper cell milieus may co-exist, and thus the balance among Th1, Th2, and Th17 responses may determine the course and speed of disease development. Finally, a Th2 response may establish itself within the mucosa, which leads to further negative effects; a Th2 bias induces an insufficiency to defend the mucosa against viral and bacterial infections, which further amplifies the inflammatory process.

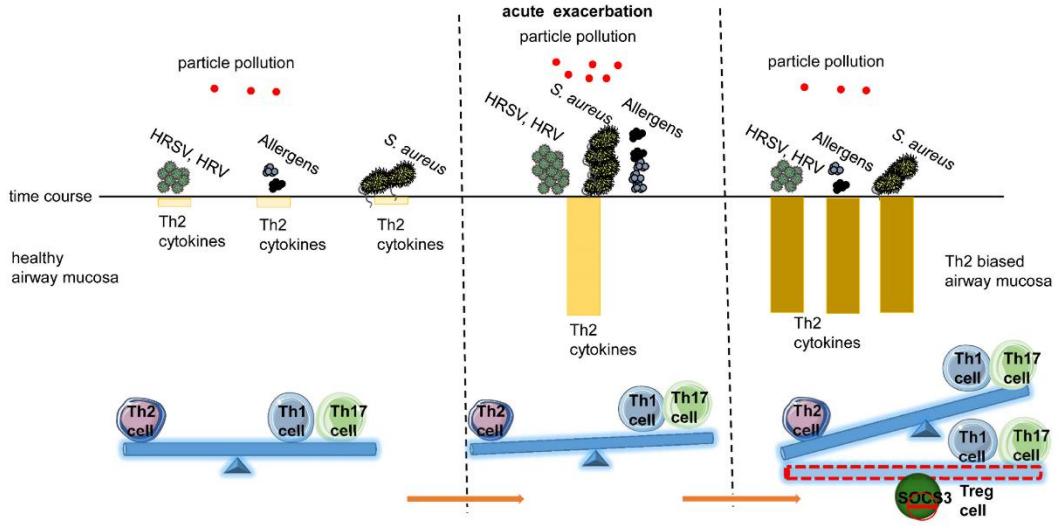

Figure 2. Multiple hits may initiate or maintain type 2 biased immune responses; SOCS3 offers a way to inhibit type 2 immune responses in airways. Low numbers of viruses and /or bacteria may persistently exert pressure as colonizers, biofilm formers or intra-mucosal intruders. High number of microbes may hit the airway mucosa during an acute infection and result in plenty of type 2 cytokine release. The balance among Th1, Th2, and Th17 responses, the status of the airway mucosa and the environment are also involved in Th2 biased immune response initiation or maintenance in the airways. The disturbed balance among Th1, Th2, and Th17 induced by microbial infections may be restored by Treg cells induced by SOCS3 silencing. HRV: human rhinovirus, HRSV: human respiratory syncytial virus, *S. aureus*: S*taphylococcus aureus*, Th: T help. Treg: regulatory T cell. SOCS3: suppressor of cytokine signaling 3.

Finally, we have elaborated that silencing SOCS3 increased the activity of Foxp3 regulatory T cells which then inhibit Th2 cytokine release in CRSwNP tissue. In addition, the further investigations to identify the suppressive function of Treg with SOCS3 silencing are also of interest. Regulatory T cells potentially suppress deleterious activities of effector T cells and maintain a state of tolerance against various stimuli such as bacteria and viruses in airway mucosa. The presence of *S. aureus* can induce Th2 cytokines in CRSwNP tissue. Therefore, we assume that enhancing the functions of Treg cell in CRSwNP tissue may be a potential approach to inhibit Th2 immune response induced by *S. aureus.*

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1. **Feng Lan**[*], XiangDong Wang[*], Hans J Nauwynck, Gabriele Holtappels, Luo Zhang, Sebastian L Johnston, Nikolaos G, Papadopoulos, Claus Bachert, Nan Zhang. Th2 biased upper airway inflammation is associated with an impaired response to viral infection with Herpes simplex virus 1. Rhinology (2015 Dec 23).
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Oral presentations:

23/6/13: Oral presentation at the EAACI-WAO congress 2013 in Milan, Italy: "Forkhead box P3 in human airway regulatory T cells is regulated by suppressor of cytokine signaling 3".

11/4/14: Oral presentation at the 1st SERIN-ASIA in Beijing, China: "Th17 response is partially suppressed in human rhinovirus and *staphyloccoccus aureus* co-infection in nasal polyp tissue".

13/6/14: Oral presentation at the AIReWAY II 2nd annual scientific meeting in Liège, Belgium: "*Staphyloccoccus aureus* induces epithelial cell-derived cytokine production in human nasal polyp tissue".

21/2/15: Oral presentation at the AAAAI congress in Houston, USA: "Staphylococcus Aureus Induces a Th2 Response via TSLP and IL-33 Release in Human Airway Mucosa".

Poster presentations:

30/1/14: Poster presentation at the 12th EAACI Immunology Winter School Brasov, Romania: "Th17 response is partially suppressed in human nasal polyp tissue after human rhinovirus and staphylococcus aureus co-infection".

10/6/14: Poster presentation at the EAACI-WAO congress 2014 in Copenhagen, Denmark: "Staphylococcus aureus elicit epithelial cell-derived cytokine productions in human nasal tissue".

11/12/14: Poster presentation at the Cell Symposia: The Multifaceted Roles of Type 2 Immunity Bruges, Belgium: "*Staphylococcus aureus* induces a Th2 response via TSLP and IL-33 release in human nasal polyps mucosa".

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