FROM THE DIVISION OF ENT DISEASES
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INNATE MECHANISMS IN UPPER AIRWAY INFLAMMATION WITH FOCUS ON EPITHELIUM AND NEUTROPHILS

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Innate Mechanisms in Upper Airway Inflammation with focus on Epithelium and Neutrophils

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my grandfather Rolf Zetterström

ABSTRACT

Mucosal inflammation is a key feature in allergic rhinitis (AR) and chronic rhinosinusitis with nasal polyps (CRSwNP). The traditional idea of the epithelium as a simple barrier and neutrophils as a homogenous cell population, already terminally differentiated, has lately been reconsidered. Recent findings have identified advanced immunological properties of both epithelial cells (ECs) and neutrophil subsets. Toll-like receptors (TLRs) and activin receptor-like kinases (ALKs) constitute important receptors of the ECs in recognizing stimuli leading to inflammatory and biological processes through altered gene expression. The new neutrophil classification, in four various subsets, is based on their expression of FcγRIII (CD16) and L-selectin (CD62L). The various subsets appear to have diverse roles during inflammatory conditions.

The overall aim of this thesis is to investigate the role of the epithelial and neutrophil cells in upper airway innate immunity.

Papers I-III explored the role of nasal epithelial cells (NECs) in antigen presentation as well as TLRs and ALKs on ECs in AR and CRSwNP. Major histocompatibility complex class II (MHC class II) as well as co-stimulatory molecules were found on NECs from mice and humans and were upregulated on OVA-sensitized mice. NECs from sensitized mice could take up, process and present antigens in a class II dependent manner for the activation of antigen-specific $CD4^+$ T cells. NECs from AR patients were able to activate autologous T cells against the major birch allergen protein, Bet v 1, and induce an IL-13 release. Normally, TLR9 can be found in NECs, but was found to be almost absent in NECs from the mucosa close to the polyps of CRSwNP patients. The TLR9 expression could be reconstituted by stimulation with its ligand, CpG, both *in vitro* and *in vivo*. Stimulation also resulted in a downregulation of VEGFR2, a receptor of angiogenesis, and cytokines. Investigation of polyp ECs revealed an upregulation of ALK1-6. Upon ligand stimulation, a downregulation of factors affecting cell proliferation (Ki67) and inflammation (ICAM-1 and IL-8) was seen. This was even more pronounced when microbial infection was mimicked.

In Papers IV and V, neutrophil subsets were described for the first time in the nasal mucosa and a local shift to the activated subset became evident in AR and CRSwNP. In AR, the activated subset $CD16^{high}$ $CD62L^{dim}$ was shown to have T cell priming capacities and an ability to enhance eosinophil migration. Neutrophils from CRSwNP patients displayed an upregulation of CD11b, most clearly emphasized on the cells of the activated subset. The corresponding adhesion molecule ICAM-1 was upregulated on the epithelium of polyps.

In summary, epithelial and neutrophil cells in the nasal mucosa of patients with AR and CRSwNP exhibit an alerted receptor pattern with a deranged immunological response. Functional data including T cell activation, migration, adhesion and cytokine release clearly indicate a role for TLRs, ALKs and activated neutrophils in these upper airway inflammatory diseases. Further, antigen presenting ECs can contribute to mucosal inflammation in AR.

LIST OF SCIENTIFIC PAPERS

I. Arebro J, Tengroth L, Razavi R, Kumlien Georén S, Winqvist O, Cardell LO.
Antigen-presenting epithelial cells can play a pivotal role in airway allergy.
J Allergy Clin Immunol 2016; 137:957-60.e7.

II. Tengroth L, Arebro J, Kumlien Georén S, Winqvist O, Cardell LO.
Deprived TLR9 expression in apparently healthy nasal mucosa might trigger polyp-growth in chronic rhinosinusitis patients.
PLoS One 2014; 9:e105618.

III. Tengroth L, Arebro J, Larsson O, Bachert C, Kumlien Georén S, Cardell LO.
Activation of activin receptor-like kinases curb mucosal inflammation and proliferation in chronic rhinosinusitis with nasal polyps.
Manuscript.

IV. Arebro J, Ekstedt S, Hjalmarsson E, Winqvist O, Kumlien Georén S, Cardell LO.
A possible role for neutrophils in allergic rhinitis revealed after cellular subclassification.
Sci Rep 2017; 7:43568.

V. Arebro J, Drakskog C, Winqvist O, Bachert C, Kumlien Georén S, Cardell LO.
A shift in neutrophil subsets might contribute to inflammation in chronic rhinosinusitis with nasal polyps.
Manuscript.

CONTENTS

ABBREVIATIONS

ANOVA	Analysis of variance
ALK	Activin receptor-like kinase
APC	Antigen presenting cell
AR	Allergic rhinitis
BMP	Bone morphogenetic proteins
CD	Cluster of differentiation
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CpG	Cytosine phosphate-guanine
CT	Computed tomography
DC	Dendritic cell
EC	Epithelial cell
ELISA	Enzyme-linked immunosorbent assay
FESS	Functional endoscopic sinus surgery
FMO	Fluorescence minus one
FSC	Forward scatter
HLA	Human leukocyte antigen
HNEC	Human nasal epithelial cell
ICAM-1	Intercellular adhesion molecule 1
IEC	Intestinal epithelial cell
IFN-γ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharide
MFI	Mean fluorescent intensity
MHC class II	Major histocompatibility complex class II
MNEC	Mouse nasal epithelial cell
NAL	Nasal lavage
NEC	Nasal epithelial cell
NLR	NOD-like receptor

OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PRR	Pattern-recognition receptor
RLR	RIG-like receptor
SAE	Staphylococcus aureus enterotoxin
SSC	Side scatter
Tc	T cytotoxic
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alfa
VEGFR2	Vascular endothelial growth factor receptor 2

AIMS

The overall aim of this thesis is to investigate the role of epithelial and neutrophil cells in upper airway innate immunity. More specifically to:

- Determine if epithelial cells in upper airway inflammation demonstrate an altered receptor pattern in terms of antigen presentation as well as TLR and ALK expression (Papers I, II, III).

- Investigate if stimulation of receptors linked to antigen presentation, TLRs and ALKs affect local nasal inflammation and cell proliferation (Papers I, II, III).

- Explore whether neutrophil subsets are present in the nose and if there is a shift in neutrophil subsets during upper airway inflammation (Papers IV, V).

- Analyze local innate mechanisms in relation to the epithelium and neutrophils in order to characterize their role in AR (Papers I, IV).

- Assess whether local inflammation and disease progression in CRSwNP can be reduced by targeting TLRs, ALKs or activated neutrophils (Papers II, III, V).

INTRODUCTION

The nose and paranasal sinuses

The nose and paranasal sinuses represent the initial upper part if the airways, actively participating in airway dynamics. An adult inhales 10,000 liters of air every day, thus constantly exposing the upper airways to air-borne particles. A pseudo-stratified respiratory epithelium lines the mucosa and contains cells with both innate and acquired immune defense mechanisms. The nasal septum and the three turbinates separate the nasal cavity into different air chambers (Fig. 1).

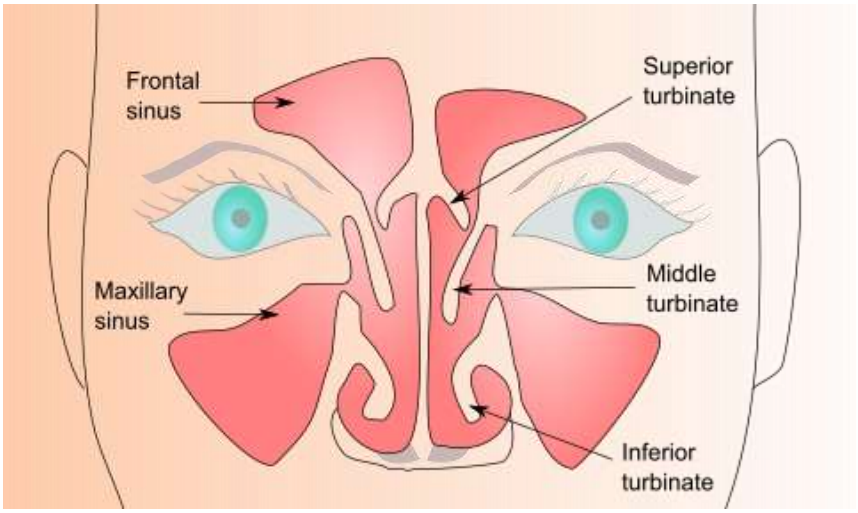

Figure 1. Schematic illustration of the nasal turbinates and the paranasal sinuses. The sphenoid and ethmoid sinuses are not visualized in this coronary projection.

The paranasal sinuses are spaces within the bone that surround the nasal cavity and are normally air-filled. Four sets of paired sinuses are recognized: maxillary, sphenoid, ethmoid and frontal (Fig. 1). The paranasal sinuses maintain a near-sterile environment and lie in close proximity to the nasal cavity, being heavily colonized by myriads of potentially pathological microbes. In addition, particles attach to the nasal mucosa during normal breathing. These particles are removed by mucosal clearance, but the nose also actively participates in the immunological defense of the airways.[1] Given these conditions, it seems remarkable that the mucosa of the nose and paranasal sinuses is in harmony and free from disease in most people.

Epidemiological as well as clinical studies have highlighted the relationship between the upper and lower airways. Up to 60-78 % of patients with asthma also have co-existing allergic rhinitis (AR), leading to the concept of "United Airways" or "Unified Airways".[2, 3] Likewise, 40-75 % of all patients with asthma have concurrent chronic rhinosinusitis (CRS).[4] Interestingly, the association with asthma was even stronger in those patients reporting both CRS and AR.[5] A considerable overlap between asthma and sinonasal comorbidities thus confirm the concept of "United Airways".

UPPER AIRWAY DISEASE

Allergic rhinitis

AR is an inflammatory disease of the sinonasal mucosa, induced by a reaction to a normally harmless antigen. It has troublesome local symptoms such as itching, sneezing, nasal drip and obstruction, resulting in negative impact on quality of life in combination with fatigue.[6] Relatively often, the disease is complicated by asthma and co-existing sinusitis, conjunctivitis and skin manifestations. The diagnosis AR is based on clinical history combined with positive skin prick test and/or radioallergosorbent test (RAST). A positive skin prick test is revealed by a skin reaction within 15 minutes. RAST detects allergen-specific IgE in serum towards the specific airborne allergen tested. The allergen is added to the patient's serum and after washing procedures, polyclonal anti-human IgE is added and if sufficiently bound, the test result turns positive (Fig. 2).

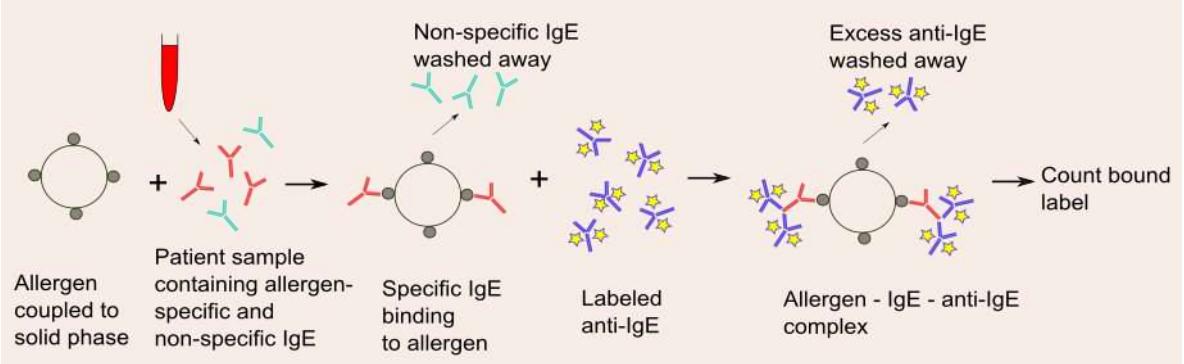

Figure 2. RAST for allergen-specific IgE in serum. Allergen-specific IgE in red, non-specific IgE in green and labeled IgE in blue highlighted with yellow stars.

Previously, AR was considered a relatively rare disease. However, during the last 100 years, the incidence in most industrialized countries has increased. Several hypotheses have been proposed to explain this increase among which the hygiene hypothesis has some strong spokesmen.[7, 8] According to this theory, changes in lifestyle in industrialized countries have led to a lower infectious burden but instead, a rise in both allergic and autoimmune diseases. Today, AR causes major medical and socioeconomic problems, affecting up to 40 % of children and 10-30 % of adults in industrialized countries.[9] In Sweden, rhinitis cost 2.7 billion euro a year due to lost productivity, according to a questionnaire-based study.[10, 11]

Allergic immune responses

The allergic inflammatory response can be divided into two main phases: the initial sensitization phase and the subsequent effector phase. In the sensitization phase, an atopic individual is exposed to an allergen promoting activation of allergen-specific T helper (Th) 2 cells which secrete cytokines (for example interleukin (IL)-4, IL-5 and IL-13) thus inducing IgE-production by B cells. These IgE molecules then bind to the surfaces of mast cells and basophils in a process known as sensitization. Upon re-exposure to the allergen, the effector phase takes place. Cross-linkage of the IgEs leads to degranulation and release of inflammatory

mediators including cytokines and histamine. These mediators affect vascular permeability and may cause smooth muscle contraction. In addition, they recruit eosinophils, neutrophils and Th2 T cells, causing the late phase reactions.

Chronic rhinosinusitis

CRS is a disease defined as chronic inflammation of the nose and paranasal sinuses. It is characterized clinically by symptoms like nasal congestion or discharge, loss of smell, facial blockage and pain causing considerable morbidity lasting for 12 weeks or longer. The diagnosis CRS is verified by endoscopic examination (Fig. 3) and computed tomography (CT) changes (Fig. 4) according to the EPOS guidelines.[1] CRS can be divided into CRS with and without nasal polyps (CRSwNP and CRSsNP). In CRSwNP, nasal polyps are visualized by anterior rhinoscopy or endoscopic examination. Recent data indicates that CRS affects 5-15 % of the population in Europe and in the US. The same increasing trend is seen for allergy, and CRS

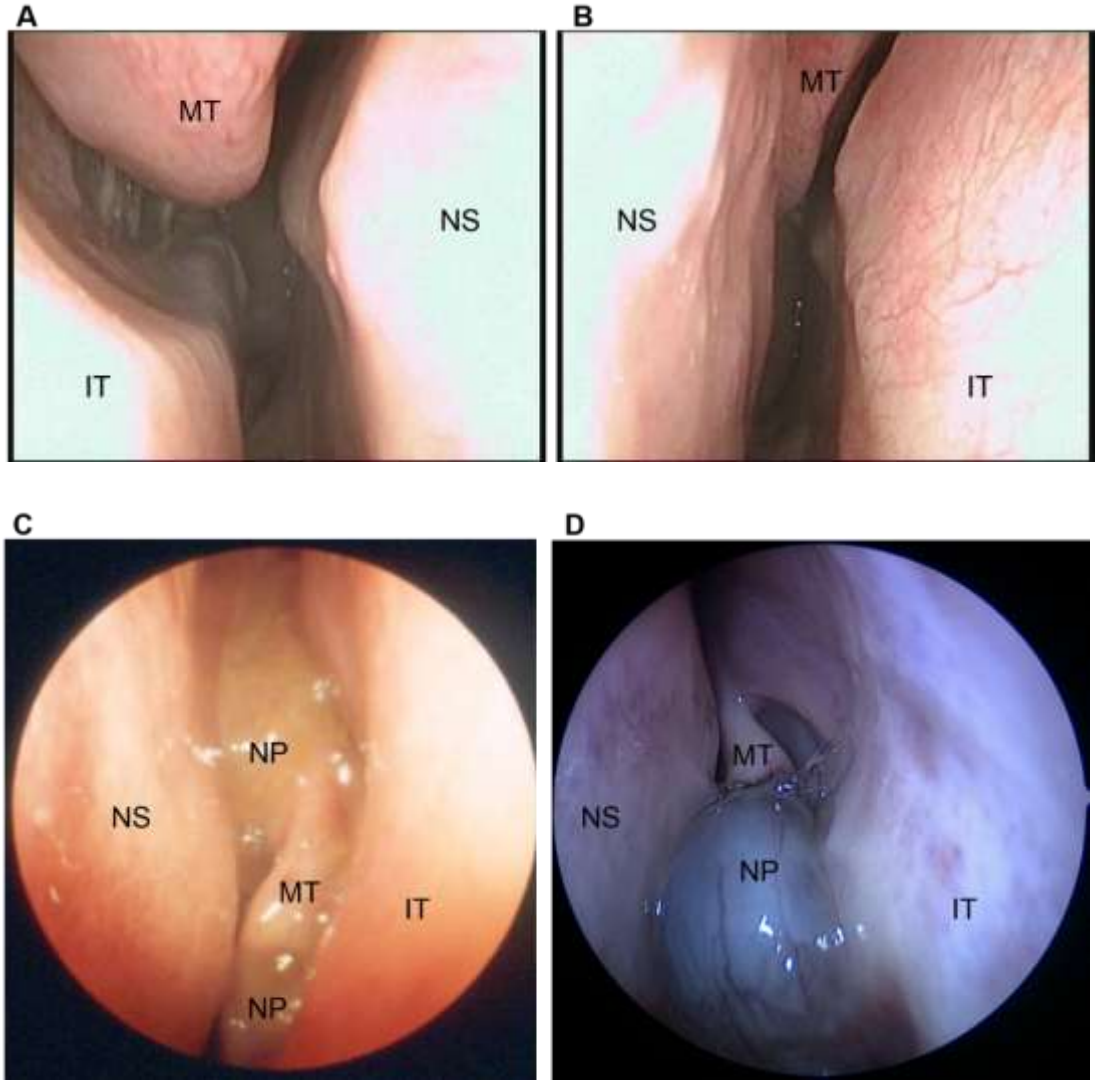

Figure 3. Endoscopic examination of the nose. (**A**) Right and (**B**) left nostril of healthy control without polyps. (**C-D**) Left nostril of two different CRSwNP patients. NS = nasal septum, NP = nasal polyp, IT = inferior turbinate, MT = middle turbinate.

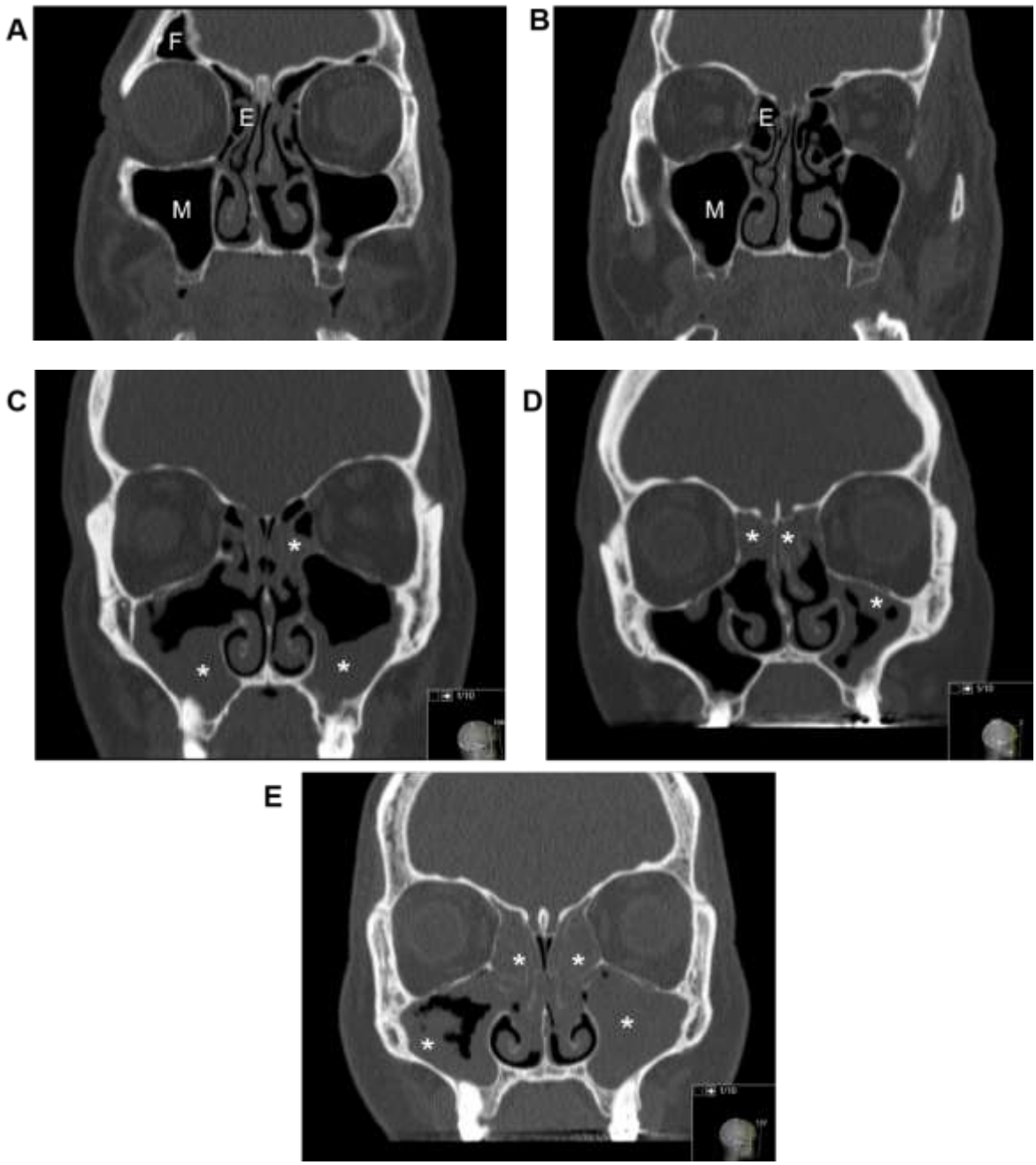

Figure 4. CT scan of the sinuses. (**A**-**B**) Healthy control and (**C**-**E**) three different CRSwNP patients. The patients display different grades of sinus obstruction and opacification, seen as grey instead of black color. F = frontal sinus, E = ethmoid sinus, M = maxillary sinus, * = partial or total opacification of the sinus.

together with allergy are hence frequently associated.[12] CRSwNP and asthma are also associated, albeit their inter-relationship remains poorly understood.[6] Patients suffering from CRSwNP, asthma and aspirin sensitivity at the same time (termed 'Samter's triad') constitute a selected group of patients and often suffers more severe disease.[1] The majority of patients with CRS can be successfully treated with topical corticosteroids, at least during the first years of disease. Occasionally, oral corticosteroids or antibiotics can be required periodically. In addition, a small group of patients require surgery. Functional endoscopic sinus surgery (FESS) involves the removal of polyps and affected polypoid mucosa as well as opening of the sinuses. The clearance of inflammatory tissue, as well as the improvement of sinus ventilation, are the

probable successful mechanisms of FESS. The rate of relapse in CRS symptoms after surgery tends although to be higher for patients with compared to without nasal polyps.[13]

The etiology behind CRSwNP is probably multifactorial, including genetic factors, anatomical circumstances, infections and local inflammatory impairment. It is unknown why some people develop polyps and some do not. A well-documented clinical observation is however that an upper airway infection often precedes the polyp development.[14] The nasal polyps arise from the mucosa in the sinuses or in the nose. The benign masses consist of loose connective tissue, pronounced edema, inflammatory cell infiltration, and capillaries and glands. Eosinophils are the most frequent inflammatory cells in nasal polyps, leading to large concentrations of IL-5.[15] The inflammation seen in Western countries is most often characterized by a robust Th2 response.[16, 17] The development of new monoclonal antibodies like anti-IgE and anti-IL-5 has thus opened up the possibility of a new treatment arsenal in CRSwNP. However, current data yield insufficient evidence to recommend either of them for medical treatment of CRSwNP, especially non Th2 CRSwNP or Asian patients biased towards neutrophilic inflammation.[17] Ongoing investigations may though clarify this matter in the near future.[1, 18] Finally, several publications have established that CRS consists of numerous endotypic clusters highly correlating with phenotypic parameters. This indicates that CRS is a more complex immunological disease than previously thought, warranting more personalized treatment strategies.[16, 19]

THE IMMUNE SYSTEM

The immune system comprises a collection of tissues, cells and molecules that mediate resistance to potentially harmful substances and pathogens. Host defense mechanisms consists of the innate immune system, which mediates the initial rapid protection against pathogens, and the adaptive immune system, which develops more slowly and mediates a more effective and specific defense. These two branches of the immune system have traditionally been described as separate systems. Accumulating evidence has however proven close interactions between the two arms, establishing the view that efficient protections against pathogens is mediated by a tight cooperation between these arms, making strict sectioning an incorrect simplification.[20]

The immune system is required for eliminating foreign structures. However, immune responses are capable of causing tissue damage and disease. Hypersensitivity, for instance, is a reflection of an excessive or aberrant immune response to an antigen instead of a normal, balanced response. Another example of misdirected immune response is autoimmune disease, where the immune system is directed against self (autologous) antigens, as a result of the failure of self-tolerance.

Innate immunity

All individuals are provided with an innate immunity. The skin and the mucosa create a physical barrier that constitutes the first line of the innate immune system. The cellular, second innate line of defense, is primarily facilitated through the actions of monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils) and dendritic cells (DCs) and becomes

activated if the pathogen circumvents this initial defense. The innate immune system is characterized by its rapid action and it does not adapt to the presence of microbial agents like the adaptive immune system.

To be able to respond immediately to invading pathogens, innate immune cells express germline-encoded receptors called pattern-recognition receptors (PRRs). They are found on the cell membrane, in the endosomal compartments and in the cytosol. PRRs recognize and bind to conserved microbial components termed pathogen-associated molecular patterns (PAMPs). The human PRR family consists of at least three receptor families, including toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs). The TLRs are the most well-known group of PRRs and they are essential for innate and adaptive immune responses. Ten different TLRs are known in humans, each recognizing a specific pathogen pattern.[21] TLRs have been demonstrated in various cells and tissues, including eosinophils, neutrophils, DCs, lymphocytes, nasal mucosa and tonsils.[22-25]

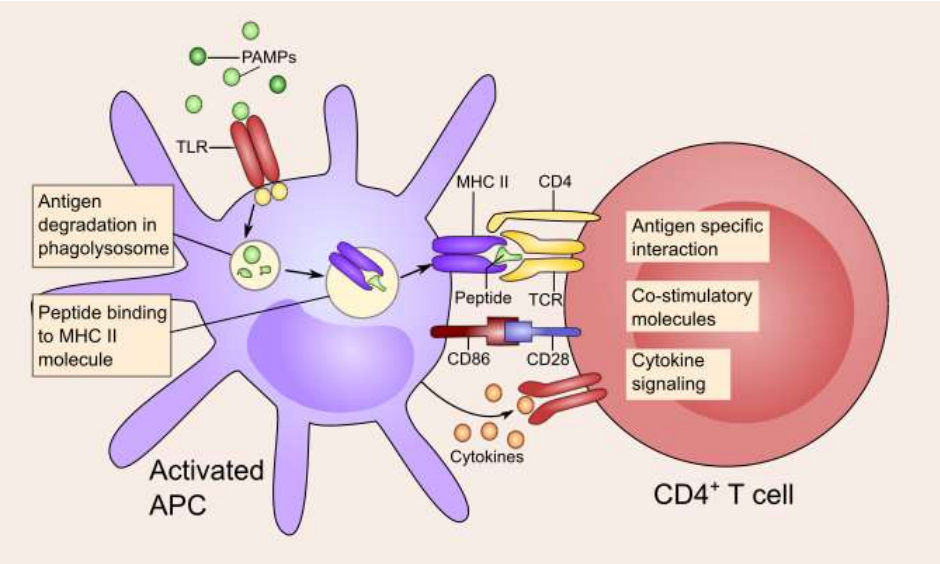

Figure 5. Key events during the interaction between an antigen presenting cell (APC) and a T cell. An antigen (PAMP) binds to a TLR on an APC, whereupon the antigen is endocytosed and processed into peptides. The peptide binds to a MHC class II molecule intracellularly and the complex is subsequently transported to the cell membrane, where the peptide is presented to a T cell. Full T cell activation further requires interaction of co-stimulatory molecules and cytokine signaling.

Adaptive immunity

The adaptive immune system is based on lymphocytes named T and B cells and is initiated when their receptors recognize antigens. Lymphocytes have a vast receptor diversity enabled through somatic recombination during cell development, meaning that lymphocytes can mount specific responses towards infectious agents. Hence, adaptive immunity is more specialized than the innate system and is able to combat infections that have evolved to evade innate responses. This system is also responsible for the generation of our immunological memory. T and B cells circulate in their naïve forms in blood and lymphoid tissues until they are activated by antigen presenting cells (APCs) like DCs. The APCs display peptides through

major histocompatibility complex class II (MHC class II) molecules as well as co-stimulatory molecules (Fig. 5). The MHC molecules in humans are called human leukocyte antigens (HLAs). There are different sets of class II genes, and HLA-DR constitutes one of them. Upon activation, B cells differentiate into plasma cells which secrete antibodies, or memory cells. The T cells can be divided into several categories, for instance into Th cells being $CD4^+$ and T cytotoxic (Tc) cells being $CD8^+$. The naïve Th cells are activated to differentiate into for example Th1, Th2 and Th17 cells, which display different patterns of cytokine secretion. While Th1 cells primary secrete interferon gamma (IFN-γ), the Th2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13. Th17 cells produce IL-17. Allergy and CRSwNP are often characterized by Th2-biased inflammation.

LINKS BETWEEN INNATE IMMUNITY AND UPPER AIRWAY INFLAMMATION

Epithelial cells

Nasal epithelial cells (NECs) constitute a physical barrier but also play an active role in innate and adaptive immune responses.[26] TLRs have been identified on airway epithelial cells (ECs).[27-29] ECs of CRSwNP patients have been shown to secrete more GM-CSF, eotaxins and RANTES than healthy controls, potentially contributing to the recruitment and survival of eosinophils.[30-33] Furthermore, ECs can secrete TSLP, IL-33 and IL-25 affecting Th2 inflammation mainly through DCs and innate lymphoid cells.[34]

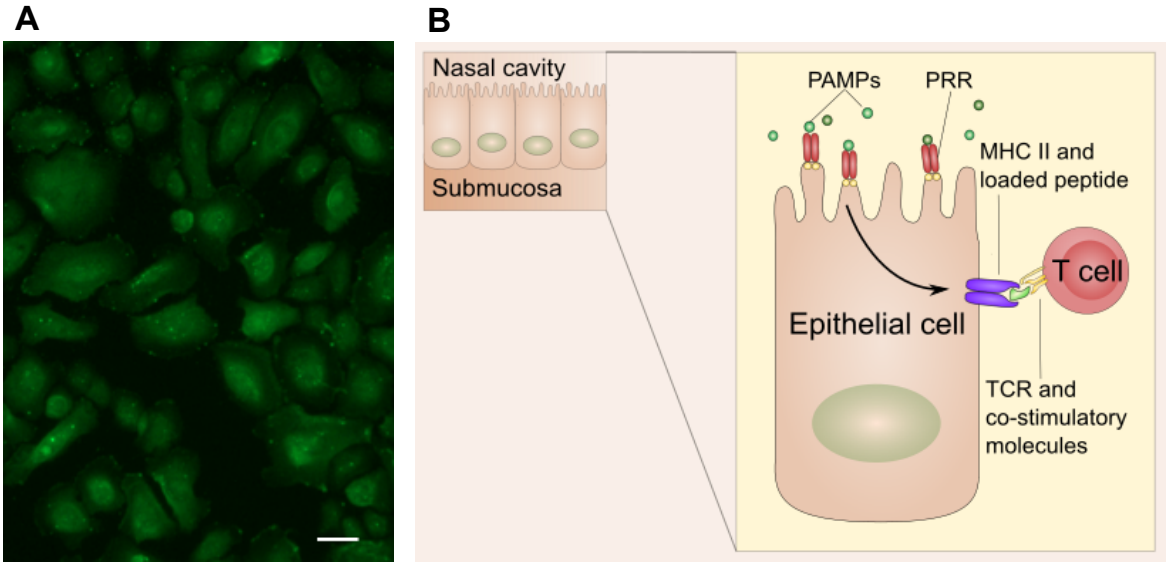

Figure 6. Epithelial cells. (**A**) Immunofluorescent image of epithelial cells from human nasal mucosa expressing MHC class II molecules (green). Scale bar: 50 µm. (**B**) A schematic and simplified figure of antigen presentation by the epithelium. Antigens (PAMPs) bind to PRRs on an epithelial cell and are degraded into peptides intracellularly. Subsequently, the peptides are presented in a class II dependent manner to a T cell.

The ability to recognize and react to foreign antigens and thereafter activate the adaptive immune system has traditionally been assigned to APCs like DCs, macrophages and certain B cells. At homeostasis, MHC class II expression is restricted to these professional APCs, whereas MHC class I molecules are expressed on all nucleated cells. However, during inflammation, ectopic expression of MHC class II can be detected on other cells than professional APCs, and from multiple tissues including esophagus, intestine, kidney, skin and airways (Fig. 6A).[35-40] ECs have thus been suggested to have more complex features than being a barrier, producing mucous, having cilia activity and releasing cytokines. Just like traditional APCs, ECs express MHC class II molecules on their surface, crucial for antigen presentation to $CD4^+$ T cells. [41-43] These findings suggest an antigen-presenting potential of ECs (Fig. 6B), although the molecular mechanisms and immunological importance of this function have not yet been fully examined.

Neutrophils

Neutrophils have a key role in eliminating invading pathogens and in promoting tissue repair, and are thus essential for the innate immune response. Neutrophils are the most abundant leukocyte in human blood and in response to infections, the production of neutrophils from the bone marrow increases rapidly. Upon infection, an upregulation of adhesion molecules on the endothelium of the blood vessel will start a process of neutrophil extravasation. Their half-life in blood is normally relatively short (6-8 hours) but upon migration to inflamed tissue where they phagocytose and kill bacteria, their survival has been shown to be extended.[44] After killing bacteria, the neutrophils undergo apoptosis and are cleared by resident macrophages. Lately, neutrophils have however been demonstrated to have functions beyond the role of killing bacteria. The idea of neutrophils as homogenous, short-lived cells already terminally differentiated has been challenged. Attention has recently been paid to the findings of different neutrophil subsets defined by differences in FcγRIII (CD16) and L-selectin (CD62L) expression.[45, 46] This has resulted in the defining of four different subsets: $CD16^{dim}$ $CD62L^{high}$

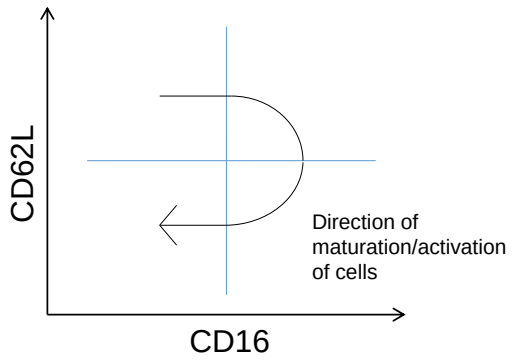

Figure 7. A schematic figure illustrating the maturation or activation process of neutrophils on a flow cytometry dot plot. Immature neutrophils derived from the bone marrow ($CD16^{dim}$ $CD62L^{high}$) express CD62L but hardly CD16 and are displayed in the upper left quadrant. Normal mature neutrophils ($CD16^{high}$ $CD62L^{high}$) express both CD16 and CD62L and are localized in the upper right quadrant. Activated neutrophils ($CD16^{high}$ $CD62L^{dim}$) express CD16 but have lost the CD62L molecules and are found in the lower right quadrant. End state $CD16^{dim}$ $CD62L^{dim}$ neutrophils are displayed in the lower left quadrant.

(with banded nuclear morphology, characteristic of neutrophils derived from the bone marrow and hence considered to be a less mature subset), $CD16^{high}$ $CD62L^{high}$ (phenotypically normal mature neutrophils), $CD16^{high}$ $CD62L^{dim}$ (with a hyper-segmented nucleus, thought to be activated) and $CD16^{dim}$ $CD62L^{dim}$ (not further defined) (Fig. 7).

Eosinophils play an important inflammatory role in airway allergy and CRSwNP. Neutrophils, are at the same time known to increase in allergy.[47, 48] Their relative increase is not as distinct as the one of eosinophils, but their absolute increase deserves certain attention. At the same time, significant numbers of neutrophils accumulate in the nasal mucosa of CRSwNP patients, and the Asian CRSwNP-population is even biased towards neutrophilic inflammation.[17] Their contribution to CRSwNP pathogenesis has so far been unclear. However, the recent reports on different neutrophil subsets, all with diverse roles during inflammatory conditions, raises the question whether these subsets are present in, and affect the course of, upper airway inflammation.

Toll-like receptors

During the last decade, opinion has shifted from considering CRS to have an underlying infectious cause to view it as a disease of constant inflammation.[41] The discovery of TLRs as a part of the innate immune system more than 30 years ago has opened possibilities for exploring the underlying mechanisms of this opinion (Fig. 8). At the same time, increasing evidence indicate that TLRs also are linked to allergy.

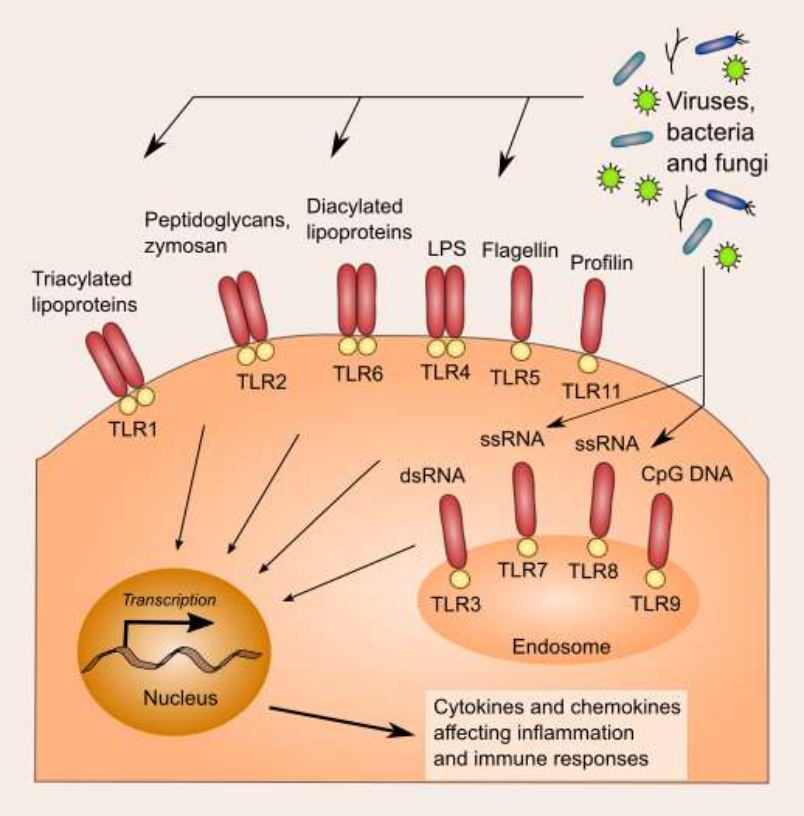

Figure 8. TLRs (red and yellow). TLR1, 2, 4-6 and 11 bind to the cell surface while TLR3 and 7-9 act intracellularly. The TLRs bind ligands derived from viruses, bacteria and fungi. Upon binding, an intracellular signal cascade results in altered transcription of DNA, resulting in immune events.

Nasal TLR expression[25, 49] and the effects of local treatment with TLR8 agonist[50] has recently been studied among patients with AR, suggesting that TLRs have a role in allergic airway inflammation. We have demonstrated the presence of TLR2, 3 and 4 both in mRNA and as proteins in nasal mucosa, and an increase in protein expression has been seen following allergen challenge.[49] A dysregulation of TLR9 genes has been shown among patients with CRSwNP.[51, 52] TLR9 seem to induce a Th1-biased immune response, known to suppress Th2-inflammation often seen in CRSwNP.[53] We have previously detected the expression of TLR9 in healthy nasal mucosa.[54] Cytosine phosphate-guanine oligodeoxynucleotide (CpG-ODN) is a TLR9 ligand found in viral and bacterial DNA. The ability of CpG to induce Th1-related activities has made it an interesting potential target for the treatment of allergy and inflammatory diseases caused by microbes.[55, 56]

Activin receptor-like kinases

The transforming growth factor beta (TGF-β) superfamily consists of more than 30 different ligands in mammals and have been conserved through evolution.[57] They are involved in a range of biological processes, and can be divided into TGF-βs, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs). The TGF-β ligands act through type I transmembrane serine/threonine kinase receptors, also termed activin receptor-like kinases (ALKs), and type II transmembrane serine/threonine kinase receptors.[58] So far, seven ALKs have been described in mammals.[59]

Ligands can bind multiple ALKs but their affinities vary greatly.[60-62] Upon binding, a number of intracellular SMADs are activated through phosphorylation, eventually regulating gene expression (Fig. 9). Disruption in the pathway linked to the TGF-β superfamily has been linked to various diseases, including airway inflammation.[58, 63, 64]

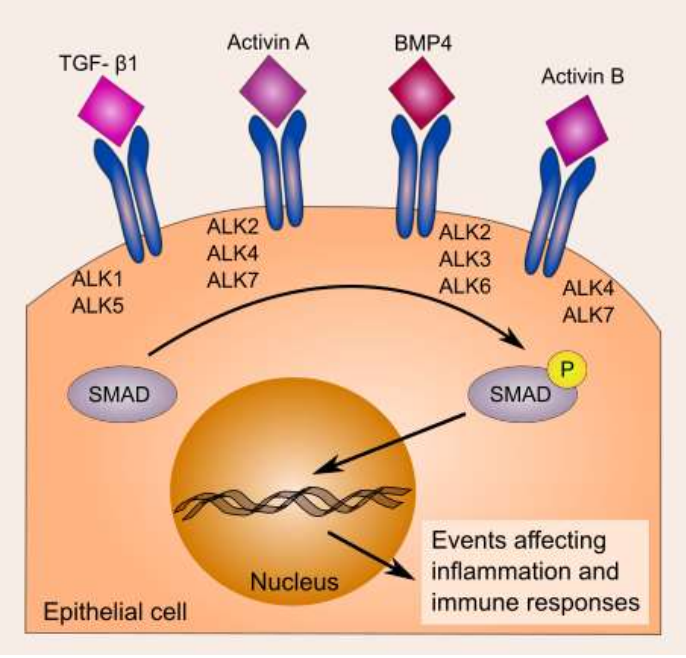

Figure 9. ALKs (blue), illustrated on the cell surface. ALK ligands (purple) can bind multiple ALKs, illustrated in the picture. ALK-ligand interaction causes phosphorylation (yellow P) of intracellular SMADs and regulates gene expression in the nucleus resulting in for example immune events.

MATERIALS AND METHODS

This section contains a brief overview of the materials and methods used in the studies. More details can be found in the individual Papers I-V.

Human study population

CRSwNP patients in Papers II, III and V were defined by EPOS criteria [European Position Paper on Rhinosinusitis and Nasal Polyps] based on symptoms, results of nasal endoscopy and CT of the sinuses.[1]

AR patients in Paper I and IV were diagnosed based on clinical history and positive skin prick test and/or positive RAST for the relevant allergen (phadiatop was used, detecting IgE against birch, timothy, mold, mugwort, mite, cat, horse and dog and in addition Bet v 1 in Paper I).

Healthy controls in Papers I-V had no history of sinus disease, asthma or allergy. None of the healthy controls were pregnant, had a history of steroid use, had a positive skin prick test/RAST upon testing or were smokers.

Human characteristics and samples obtained (defined in brackets) of each Paper:

Paper I: Patients with AR (nasal brushing and peripheral blood) and healthy controls (nasal brushing or nasal biopsies and peripheral blood).

Paper II: Patients with CRSwNP (biopsies of turbinate tissue, polyps and peripheral blood) and healthy controls (nasal biopsies and peripheral blood).

Paper III: Patients with CRSwNP and healthy controls (nasal biopsies and/or nasal brushing and peripheral blood).

Paper IV: Patients with AR and healthy controls (nasal biopsies, nasal lavage (NAL) and peripheral blood).

Paper V: Patients with CRSwNP (biopsies of turbinate tissue, polyps and peripheral blood) and healthy controls (nasal biopsies and peripheral blood).

All studies were performed at the ENT department at Karolinska Institutet or Karolinska University Hospital in Stockholm. Fresh human materials were used in all studies after approval from the Ethical Review Board at Karolinska Institutet or Lund University and an informed consent was obtained from all participants.

Animals

In Paper II, cells from C57BL/6 and OT-II T cell receptor (TCR) transgenic mice were used. The animals were supplied with pelleted food and water *ad libitum* and were housed in a climate-controlled environment with a 12 hours daylight cycle. C57BL/6 mice were sensitized by means of intraperitoneal injection of grade V ovalbumin (OVA) and aluminium hydroxide administered in the morning on days 0 and 7. The study was approved by the Swedish Animal Experimental Review Board.

Human nasal samples

Biopsies from non-polypoid turbinate tissue (inferior or middle) and polyps were taken during FESS or in local anesthesia from patients with CRSwNP. Biopsies from inferior turbinates of healthy controls were taken after topical application of local anesthesia. Tissue used in Papers I, II, IV and V was filtered through a cell strainer into Nutrient Mixture Ham F12 medium (DMEM/F-12) containing serum. Cells were washed and centrifuged and the supernatant was subsequently aspirated and discarded. The cells were stained and analyzed with flow cytometry.

Nasal brushing was performed using a cytology brush to obtain NECs from the middle meatus. Cells were stained and analyzed immediately with flow cytometry or cultured in collagen-coated tissue culture flasks.

NAL fluid was collected by first cleaning excess mucous by forceful exsufflation and 8–10 ml of sterile saline solution was then sprayed into the nostrils and passively collected when dripping out. Cells were stained and analyzed with flow cytometry.

Human nasal challenge

In Paper I, CRSwNP patients were intra-nasally challenged with a spray containing physiological saline solution and 50 mM CpG-ODN or saline solution (placebo). Nasal biopsies and peripheral blood were obtained during FESS the following day and stained and analyzed with flow cytometry. The patients and the person analyzing the samples were blinded regarding which spray that had been used.

Stimulation and co-cultures of tissues and cells

In Paper II, human nasal biopsies were used for *in vitro* experiments to determine whether CpG stimulation had an impact on TLR9 and vascular endothelial growth factor receptor 2 (VEGFR2) expression as well as cytokine release. Tissues were separated into pieces of 0.05 g and incubated on 24-well culture plates in DMEM/F-12 with serum, penicillin, streptomycin and fungizone. The biopsies were subsequently stimulated with modified CpG-ODN for 4 or 24 hours. Cells were stained and analyzed with flow cytometry and supernatants were analyzed with Luminex.

Primary human NECs (HNECs) obtained by nasal brushing were cultured for experiments in Papers I and III. Cells were maintained in collagen-coated tissue culture flasks in Keratinocyte Serum-Free Medium (KSFM) containing epidermal growth factor, bovine pituitary extract, penicillin and streptomycin. Fungizone was in addition added to the cells stimulated with Bet v 1 in Paper I and to all cells in Paper III. Cultured cells were used in passage 2-6 for the experiments. They were positive for the epithelial cell adhesion molecule EpCAM.

In Paper I, the cultured HNECs were subsequently used for co-cultures to determine whether the epithelium could present antigens to T cells and cause cell activation and proliferation. Autologous T cells were isolated from peripheral human blood using negative selecting magnetic antibody cell sorting separation. By adding magnetic labelled antibodies and placing the tube with blood in the magnetic field, the magnetically labeled cells adhered to the wall of the tube. The supernatant, containing the T cells, could then be collected. Residual erythrocytes

were depleted. Cultured HNECs were then stimulated with birch pollen extract or Bet v 1 for 24 hours. Upon washing, T cells were added and re-suspended in TexMacs-medium containing L-glutamine, serum, penicillin, streptomycin and fungizone. Upon 4, 24 or 48 hours of co-culture, T cells and HNECs were collected, stained and analyzed with flow cytometry while supernatants were analyzed with Luminex.

In Paper III, cultured HNECs were used to see whether stimulation with ALK ligands could impact on factors linked to cell proliferation and/or inflammation. Cultured NECs were stimulated for 24 hours with either TGF-β1, Activin A, BMP4 or Activin B. In separate experiments, tumor necrosis factor α (TNF-α) was added the last 4 hours to mimic microbial load. Cells were then stained and analyzed with flow cytometry and supernatants were analyzed with enzyme-linked immunosorbent assay (ELISA).

In Paper IV, human co-cultures with neutrophils and T cells or neutrophils and eosinophils were set up to establish whether activated neutrophils could prime T cells and/or cause eosinophil migration. Peripheral blood was collected in heparin tubes and neutrophils and T cells or eosinophils were isolated using ficoll-paque and additional positive selection using CD15 microbeads further purifying neutrophils, or MACSxpress Isolation Kits for negative selection of neutrophils and eosinophils. Remaining erythrocytes were lysed. Neutrophils were diluted in RPMI-medium containing autologous plasma, penicillin and streptomycin and subsequently activated with 1 μg/ml lipopolysaccharide (LPS), 5 ng/ml TNF-α and 10 ng/ml IL-8 and then washed. For T cell assays, a co-culture with activated neutrophils and lymphocytes was set up in wells for 30 minutes and CD3 was subsequently added for 90 minutes for T cell activation. Transwell plates were used to block cell-cell contact in separate experiments, adding CD3 to the lymphocyte part of the transwell plates. For eosinophil assays, migration set up was conducted with transwell plates for all experiments. Neutrophils were added to the bottom well and eosinophils were added to the insert. After 3 hours of incubation, inserts were removed and cell suspensions were collected. Cell suspensions of both T cell and eosinophil assays were subsequently stained and analyzed with flow cytometry.

In Paper I, mouse nasal epithelial cells (MNECs) were cultured to see if they could act as APCs. The use of mouse cells made it possible to use a transgenic system (OT-II mice) as well as to use naïve T cells as reporter cells, two settings not viable in a human setting. Mice septal mucosa were isolated from nasal septums as previously described [65] and put in DMEM/F-12 and then into dissociation medium for 1 hour containing minimal essential medium, penicillin/streptomycin, pronase and DNase. DMEM/F12 containing FBS was added to stop the reaction. Tissue was filtered through a cell strainer and thereafter cultured in complete culture medium with DMEM/F12 containing penicillin/streptomycin, FBS and ITS Premix. The suspension was incubated for 2 hours on culture dishes to let fibroblasts adhere. Supernatants containing ECs were then collected and cultured in collagen-coated tissue culture dishes containing complete culture medium. To obtain mouse T cells, spleens were taken from mice, filtered through a cell strainer and then washed and centrifuged. The supernatant was discarded and remaining splenic cells were added to ECs stimulated with OVA for 4 or 24 hours. In separate experiments, anti–MHC class II antibodies were added.

Immunohistochemistry

Optical imaging in Papers I and III was carried out using classic immunohistochemistry (IHC) or immunofluorescence (IF). In IHC, a primary and secondary antibody is added and visualization is subsequently accomplished using a chemical reaction to produce a color change. In IF, the target is detected using an antibody and subsequent visualization is made by adding a fluorophore coupled to the secondary antibody.

In Paper I, cultured HNECs from an AR patient were pre-incubated with serum to block non-specific binding and subsequently incubated with an anti–MHC class II primary antibody followed by a fluorescein-coupled secondary antibody to visualize MHC class II expression. Imaging was performed with a Nikon Digital Sight DS-U1 camera coupled to a Nikon Eclipse TE2000-U microscope.

HNECs from another AR patient in Paper I were used to study antigen uptake. Fluorescein coupled to dextran was added to HNECs in complete medium and thereafter washed with PBS to visualize endocytosis. Cells were fixed in formaldehyde, washed with PBS and subsequently incubated with serum to block non-specific binding. MHC class II expression was visualized with an anti–MHC class II primary antibody followed by a fluorescein coupled secondary antibody. Stained cells were maintained in anti-fade reagent with 49-6-diamidino-2-phenylindole (DAPI) to visualize nuclei. For laser confocal microscopy, a Nikon Eclipse TE 300 microscope equipped with a solid-state laser and connected to a Hamamatsu ORCAER camera was used.

In Paper III, nasal biopsies were fixed and embedded in paraffin and subsequently cut into 5 μm sections. Sections were deparaffinized, rehydrated and incubated in Sudan black B (for DAB staining) in 70 % ethanol. Following antigen retrieval in citrate buffer, sections were permeabilized. For 3,3'-diaminobenzidine (DAB) staining, inhibition of the endogenous peroxidase activity was accomplished by treating the tissues with 1 % H_2O_2 followed by incubation with serum and bovine serum albumin (BSA), blocking non-specific binding. For IF, BSA was used to block non-specific binding. Sections were incubated with a primary antibody against IL-8, Ki67, intercellular adhesion molecule 1 (ICAM-1), ALK2, ALK3, ALK5, ALK7 or EpCAM and subsequently incubated with a secondary antibody. Sections for DAB staining were incubated with Avidin-Biotin-Complex followed by DAB and counterstained with Mayer's Haematoxylin. For IF, mouse antibody diluent was used as a negative control. For DAB staining, images were captured with a Nikon Digital Sight DS-U1 camera, coupled to a Nikon Eclipse TE2000-U microscope. For IF, imaging was performed using an Olympus Provis microscope, connected to an Olympus U-PS camera.

Flow cytometry

Flow cytometry was the main method used in Papers I-V. The method analyzes the physical and chemical properties of individual cells in a suspension based on how they scatter light from a laser beam. Using different detectors, the flow cytometer is able to provide information about cell size (displayed as forward scatter, FSC), granularity (displayed by side scatter, SSC) and fluorescence intensity of fluorochrome-conjugated antibodies used for detection of intra- or extracellular antigens. Flow cytometry thus allows simultaneously multi-parametric analyses of both physical and chemical characteristics of up to thousands cells per second. The data can

then be plotted as a single dimension on a histogram (Fig. 10A) or in a two-dimensional dot plot (Fig. 10B-C). The dot plot reveals correlations between two different parameters, characterized on the x- and y-axis. The regions on this plot can be separated based on fluorescence intensity, a process called 'gating' (Fig. 10C). The cells in this gate can be further plotted on a new dot plot, with two new parameters. The cells of a gate (often referred to as a 'population') can be presented in different ways: for example in relation to all cells, in relation to a specific group of cells (leukocytes for instance), in absolute count or as mean fluorescent intensity (MFI).

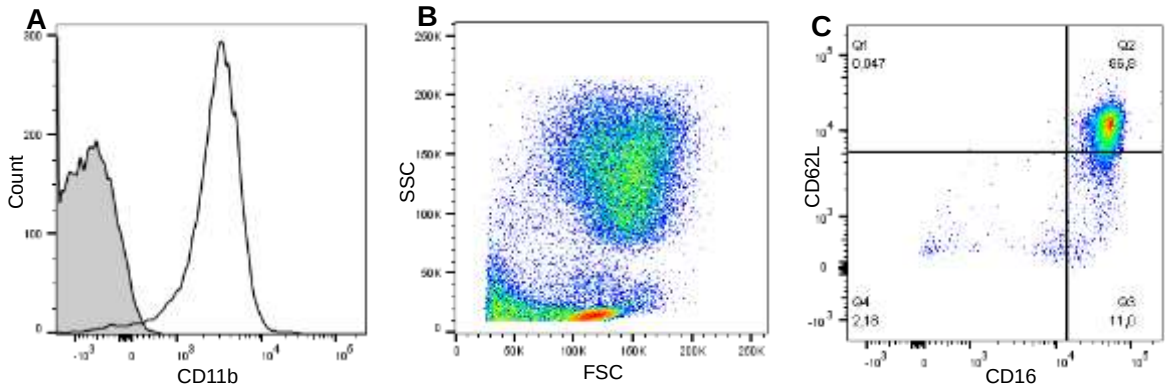

Figure 10. Flow cytometry data of blood from an AR patient. (**A**) A histogram demonstrating CD11b positive cells (open histogram, black line) and negative isotype control (filled histogram, black line). (**B**) A dot plot demonstrating the FSC and SSC of cells in blood. Roughly, the top population (high FSC and SSC) comprises of granulocytes, the bottom right population (high FSC, low SSC) comprises mainly of lymphocytes and monocytes and the bottom left population (low FSC and SSC) comprises of erythrocytes, thrombocytes and dead cells. (**C**) A dot plot displaying neutrophils based on their expression of CD16 and CD62L. The lines constitute gates, separating the cells into four different subsets (fractions displayed in % below 'Q1-Q4').

The intensity read out in flow cytometry is relative and user-adjustable. Hence, unstained or negative controls are needed. Three major issues always have to be taken into account in flow cytometry. Unstained cells emit background fluorescence (called autofluorescence), determined by also analyzing unstained cells. Antibody-antigen interactions can be non-specific: antibodies bind non-specifically to Fc receptors on the surface of many cells and dead cells tend to soak up antibodies. With a correct control, called isotype control, using the same monoclonal antibody but with an irrelevant specificity, this problem can be solved. Alternatively, or in addition, an Fc block can be used. Finally, the emission spectra of different fluorescent dyes overlap and hence, a dual color analysis can result in signals on both fluorescence detectors even for cells which only carry either of the fluorochromes. This is especially a problem in multi-parametric flow cytometry and is solved with compensation. A fluorescence minus one (FMO) control is used, containing all the fluorochromes in a panel except for the one that is being measured. This results in preferences used when defining positive and negative signals. Last but not least, a positive control is desirable to prove that the test antibody is functional and to allow proper settings of the flow cytometer.

Peripheral blood in the Papers was always lysed prior to staining. Staining was performed with antibodies conjugated with fluorochromes. Samples were fixed prior to analysis and an LRSFortessa analyzer and data were processed using FlowJo software.

Enzyme-linked immunosorbent assay

ELISA is a method for detection and quantification of an antibody or an antigen in a sample. The method was used in Paper I to detect secreted IFN-γ in supernatants of mouse co-cultures and in Paper III to detect secreted IL-8 in supernatants of stimulation assays. Sandwich-ELISAs were used based on microplates pre-coated with antibodies against the antigen of interest. When adding a sample to the microplate, potential antigens in the sample will bind to the antibodies. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for the antigen of interest was added and upon adding a substrate solution, a color developed in proportion to the amount of bound substrate.

Luminex

Luminex multiplex immunoassay is a method that quantifies several proteins or peptides at the same time in one sample. The method was used to detect cytokines in the supernatants of the human co-cultures in Paper I and in the human CpG stimulation assays in Paper II. Luminex has generally lower detection levels than ELISA but can be more expensive, depending on how many cytokines that are of interest. Luminex is similar to sandwich-ELISA, with the exception that magnetic beads dyed with fluorescent dyes are covalently coupled to the antibodies directed against the antigen of interest. Each bead thus has a distinct color code permitting discrimination of individual antigens. Human Cytokine Standard 17-plex were used in both Papers I and II and subsequently quantified on the Luminex200 system.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. In all Papers, mean is represented by a horizontal line for data presented as individual values. Remaining data is represented as mean ± standard error of the mean (SEM). A p-value of 0.05 or less was considered statistically significant (*$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$) and *n* equals the number of included subjects.

An unpaired t-test was used to compare two sets of data. For non-repeated measurements, a one-way analysis of variance (ANOVA) with a Bonferroni (Paper I) or a Tukey (Paper IV) post-test was performed. Non-parametric data were analyzed with a Kruskal-Wallis (Papers II and V) or a Friedman test (Papers III and IV) test together with a Dunn's multiple comparisons post-test (for more than two sets of paired data) except for the eosinophil migration experiments in Paper IV where a Tukey's multiple comparisons post-test was used. For more than two sets of matched data, a two-way ANOVA with a Bonferroni (Papers I and IV), a Dunnett's (Paper II) or a Tukey (Paper V) post-test was conducted.

RESULTS AND COMMENTS

EPITHELIAL RECEPTOR PATTERN IN UPPER AIRWAY INFLAMMATION (PAPERS I-III)

Traditionally, the role of ECs in innate immunity has been limited to features like barrier function, mucous production and cilia activity. Today, the role of ECs is believed to be much more complex with a close interaction with the adaptive part of the immune system.

Expression of MHC class II and co-stimulatory molecules

Expression of MHC class II molecules on the cell surface is crucial for antigen presentation to lymphocytes. Hence, we mapped the expression of MHC class II on isolated NECs, as well as co-stimulatory molecules (cluster of differentiation (CD) 86 and CD80) necessary for full T cell activation. We found a marked expression of H2-IAb (a mouse allele of MHC class II), CD86 and CD80 on naïve MNECs (Fig. 11A). We sensitized mice with OVA to mimic allergic patients. NECs from OVA-sensitized mice expressed increased levels of H2-IAb and CD80 (Fig. 11B-C).

Human samples were obtained before the start of the pollen season from healthy controls (Fig. 12A) and from patients with birch pollen-induced AR (Fig. 12B), confirming cell-surface expression of HLA-DR (one of the human MHC class II molecules) and CD86.

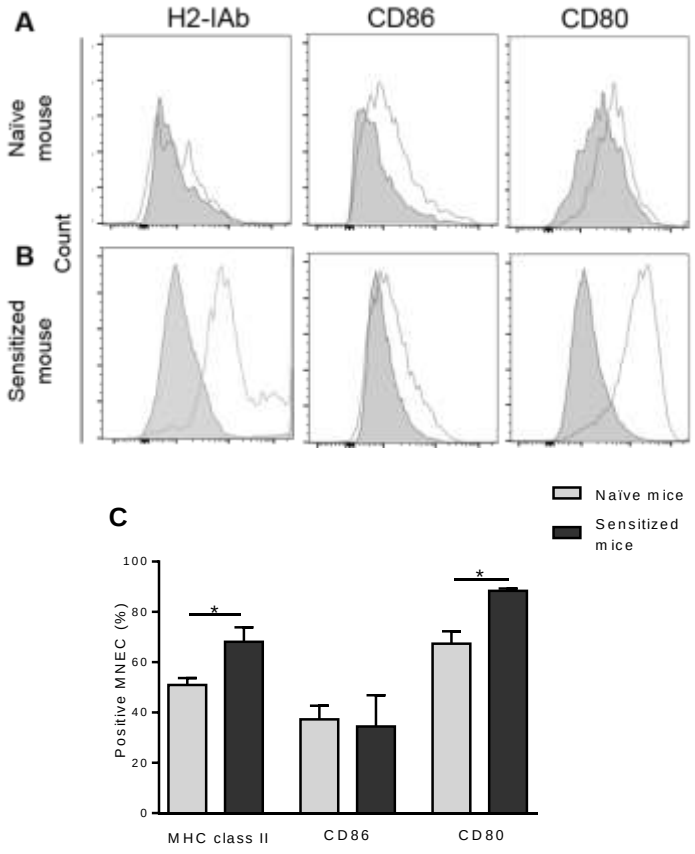

Figure 11. Expression of H2-IAb, CD86 and CD80 on freshly isolated MNECs from (**A**) a naïve mouse and (**B**) from an OVA-sensitized mouse. (**C**) Expression of MHC class II, CD86 and CD80 on cultured MNECs from naïve and OVA-sensitized mice (*n* = 3-4).

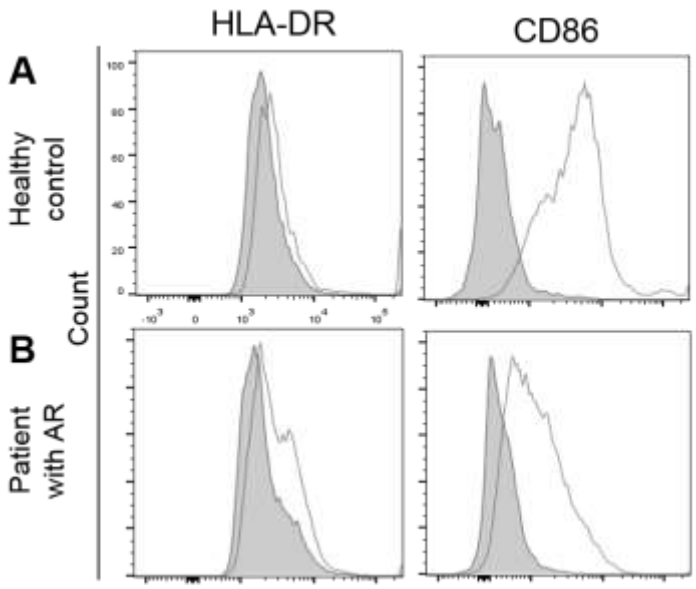

Figure 12. Expression of HLA-DR and CD86 (open histogram) in cultured HNECs from (**A**) a healthy control and (**B**) an AR patient. Filled histogram represents isotype control.

Mucosal TLR expression

We continued studying TLRs, known to initiate an immune response upon binding conserved microbial components. A scarce amount of TLR9 expressing NECs were detected in the turbinate tissue of CRSwNP patients. In contrast, healthy controls had relatively high amounts of TLR9 expressing NECs in turbinate tissue (Fig. 13). Polyp tissue demonstrated an increased percentage of NECs expressing TLR9 compared to surrounding turbinate tissue from corresponding patients. This did however not reach statistical significance.

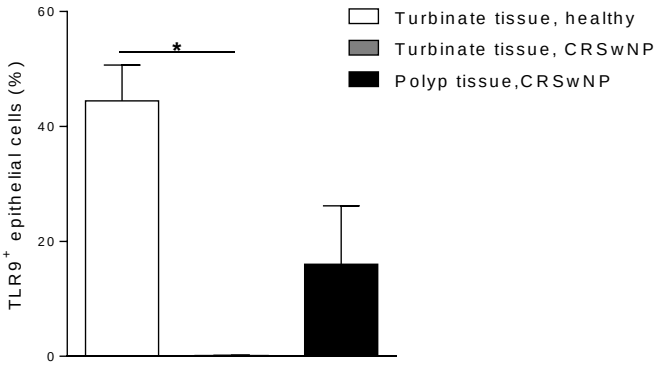

Figure 13. Expression of TLR9 on turbinate epithelial cells from healthy controls compared to turbinate and polyp epithelial cells from CRSwNP patients, $n = 5$.

ALK expression in nasal mucosa

Recent investigations into causes of airway inflammation have revealed a role for the TGF-β superfamily upon binding to ALKs. We continued mapping the expression of ALKs on NECs from CRSwNP patients. IF revealed a strong presence of ALKs in the epithelial layer of the

polyps but merely a low to moderate expression in turbinate tissue from healthy controls (Fig. 14A-D). ALK expression on NECs was further quantified with flow cytometry revealing an increase of ALK1-6 on polyp ECs (Fig. 15). Upon further mapping of local proliferation and inflammation, IHC and flow cytometry revealed an increased expression of Ki67 and ICAM-1 and an increased release of IL-8 from polyp ECs compared to control cells (Fig. 16A-F).

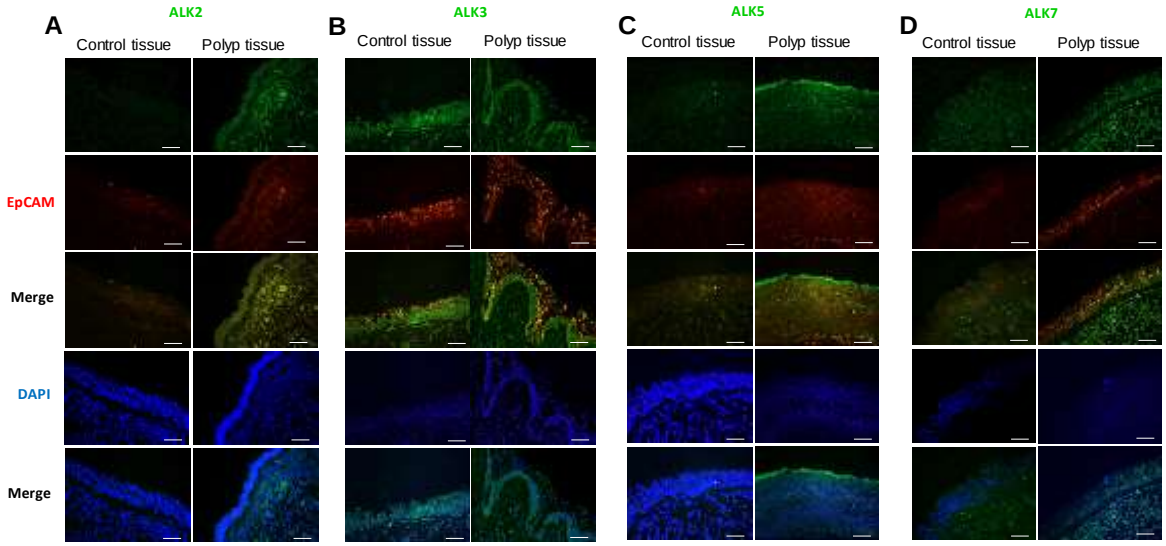

Figure 14. ALK expression in human nasal biopsies from CRSwNP patients and healthy controls (*n* = 3-4). ALK (green), EpCAM (red), co-localized expression of ALK and EpCAM (yellow), nucleus DAPI (blue) and ALK localized in the nucleus (light-blue). (**A**) ALK2, (**B**) ALK3, (**C**) ALK5 and (**D**) ALK7. Scale bar: 50 µm.

COMMENTS

The present studies revealed that NECs have the receptor pattern necessary for antigen presentation and that sensitized mice have upregulated receptor levels. In addition, NECs from CRSwNP patients express less TLR9 on turbinate ECs and more ALK1-6 as well as Ki67, ICAM-1 and IL-8 on polyp ECs compared to healthy controls.

The ability to recognize and react against foreign antigens and activate lymphocytes has been assigned to traditional APCs like DCs, macrophages and certain B cells. At the same time, it has become generally accepted that ECs are far more complex in function than previously thought and that they as well can express MHC class II molecules. MHC class II molecules have previously been reported on cells from multiple tissues during inflammatory conditions.[35-38, 40] We could detect MHC class II and co-stimulatory molecules on NECs from AR patients as well as from mice, thus confirming previous data of the field.[39] The expression of MHC class II molecules and CD86 was upregulated in OVA-sensitized mice, bringing new descriptive knowledge to the subject.

Intestinal ECs (IECs) are just like airway ECs positioned in the interface between the immune system and an environment scattered with antigens. In humans, CD86 was not detected on normal human IECs[66] but upregulated on IECs from patients with inflammatory bowel

disease.[67] Likewise, HLA-DR and CD86 was found to be increased on ECs from patients with eosinophilic esophagitis[35] and on NECs from AR patients in season compared to pre pollen season. However, no comparative data from healthy controls were included in the study.[35] We detected an increased amount of MHC class II molecules and CD80 on MNECs from sensitized mice but no upregulation in AR patients likely because samples were taken outside pollen season.

We have previously demonstrated the expression of TLR9 in nasal mucosa[54] and a reduced expression of TLR9 on ECs from nasal mucosa of rhinitis patients (persistent AR and CRS) has been reported.[68] This downregulation seem transient, since AR patients outside pollen season displayed TLR9 on NECs similar to healthy controls.[25] A decreased TLR9 expression on mRNA level has been found in paranasal sinus mucosa from patients with recurrent CRSwNP, compared to patients experiencing milder disease.[52] We here reveal that the nasal mucosa adjacent to the polyps express almost no TLR9. TLR9 is known to induce Th1 related activities known to suppress Th2-biased immune responses associated with CRSwNP.

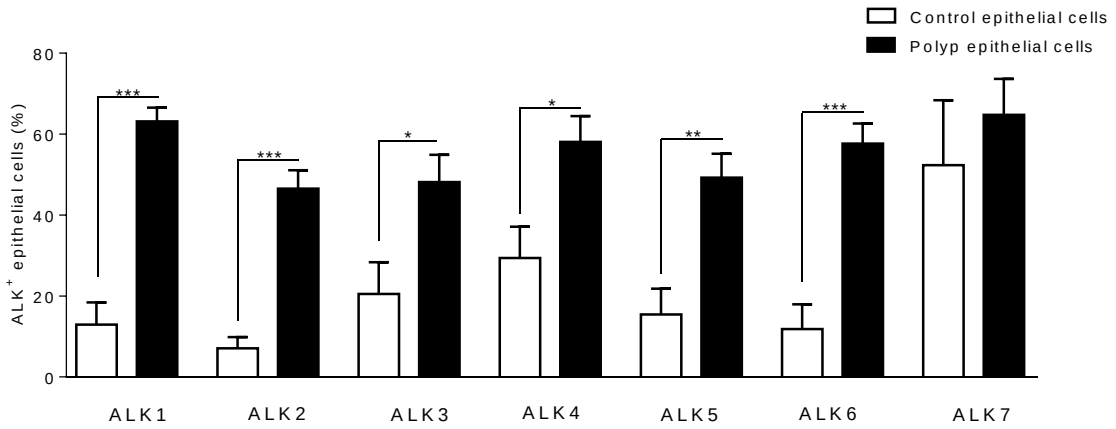

Figure 15. ALK expression on epithelial cells from CRSwNP patients (n = 13) and healthy controls (n = 8) examined with flow cytometry.

Six out of seven ALKs were upregulated on polyp NECs. ALKs are receptors thought to be mainly anti-inflammatory upon ligand stimulation.[58, 69] Limited research has to date been done on the potential distribution of ALKs in humans, and even less in the upper airways. Kariyawasam et al. detected ALKs on bronchial EC from asthma patients.[70] Upon allergen challenge, expression of ALK1 and ALK4 increased while ALK5 expression decreased. Activin A was found to induce proliferation but not chemokine or cytokine release from bronchial ECs. This suggests a role for Activin A signaling through ALK4, in tissue repair and resolution of inflammation in asthma after allergen challenge.

To summarize, this first part of the thesis describes the presence of MHC class II and co-stimulatory molecules on NECs and an upregulated mucosal expression of TLR9 and ALK1-6 in CRSwNP patients. This suggests that the local epithelium can present antigens, initiating an adaptive immune response. Further, the presented data proposes a role for TLR9 and ALK1-6 in CRSwNP.

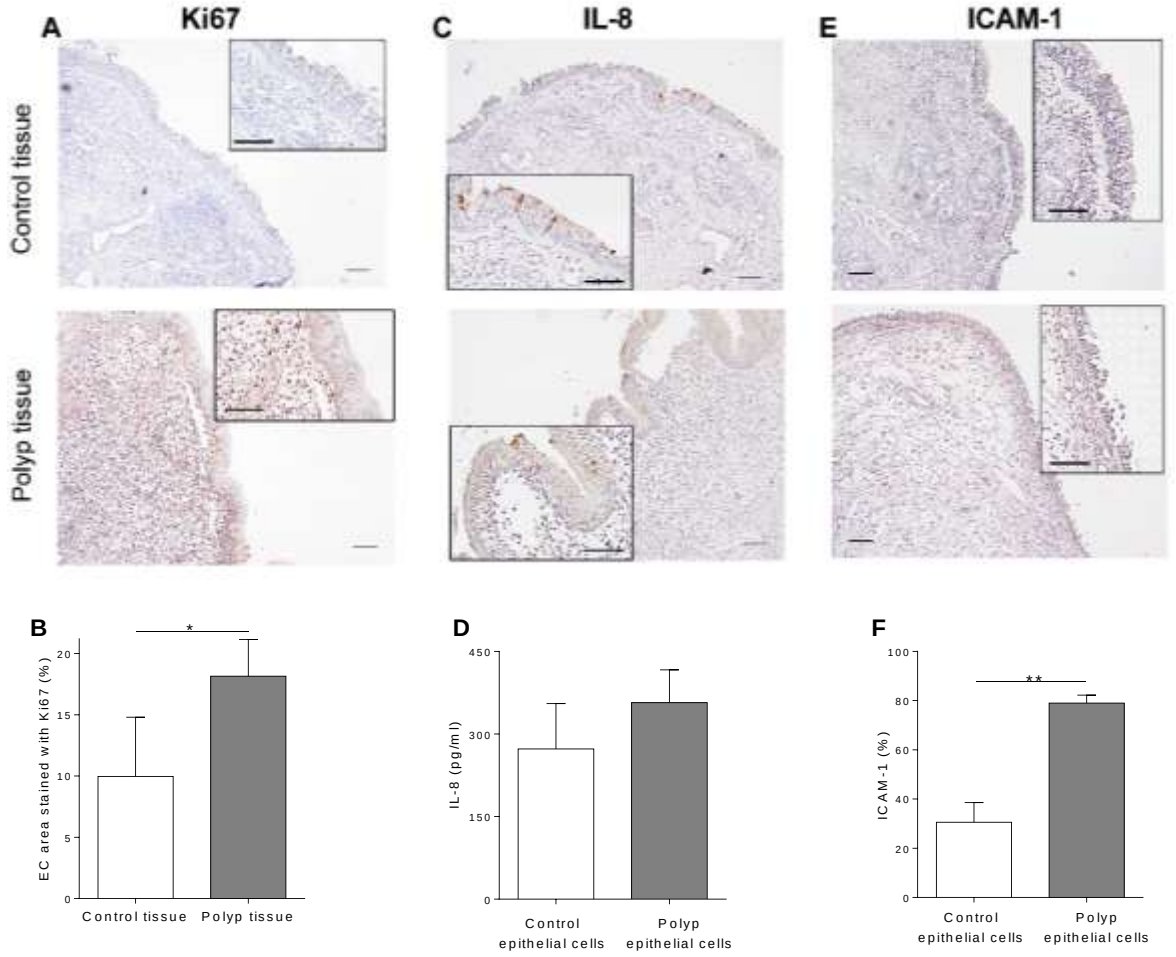

Figure 16. Nasal biopsies from CRSwNP patients and healthy controls. Staining with IHC and quantification with flow cytometry (*n* = 5-8). (**A**) Ki67 staining and (**B**) quantification on ECs. (**C**) IL-8 staining and (**D**) quantified release from cultured ECs. (**E**) ICAM-1 staining and (**F**) quantification on ECs.

FUNCTIONAL ROLES OF NASAL EPITHELIAL RECEPTORS (PAPERS I-III)

In order to further substantiate the role of epithelial receptors, functional experiments were set up using relevant antigens and ligands.

Epithelial cells affect Th2 inflammation

Co-culture experiments were designed to study if NECs could interact with T cells. We chose a well-established transgenic mouse system - OT-II mice carrying a transgenic TCR specific for a H2-IAb presented OVA peptide amino acid 323-339 - as well as OVA-sensitized mice and naïve mice.

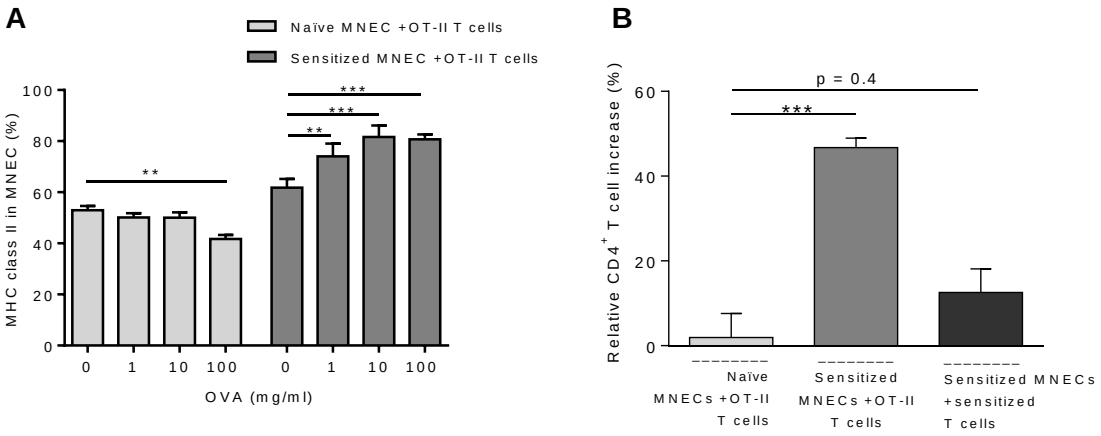

Figure 17. OVA-stimulated MNECs co-cultured with T cells. (**A**) MHC class II expression on MNECs (4 hours). (**B**) $CD4^+$ T cell counts (24 hours).

MNECs were co-cultured with increasing amounts of ovalbumin in the presence of T cells. NECs from sensitized mice exhibited an enhanced expression of MHC class II upon co-culture with OT-II T cells compared to naïve T cells (Fig. 17A). The number of OT-II $CD4^+$ T cells in the same co-cultures was increased and a similar trend was also seen when OVA-sensitized T cells were used as reporter cells (Fig. 17B).

The T cells in the co-cultures were further studied. Activated T cells display activation markers such as CD69 and CD44. CD69 represent the earliest T cell activation marker and is not displayed on resting lymphocytes.[71] CD44 is a late activation marker, mediating adhesion to endothelium upon ligand binding.[72] When NECs from sensitized mice were used as APCs, there was a pronounced increase of activated CD69 expressing OT-II T cells (Fig. 18A-B). The same findings were observed when using T cells from OVA-sensitized mice as reporter cells (Fig. 18A-B). In addition, MNECs from sensitized mice augmented the amount $CD44^+$ OT-II cells, as well as sensitized T cells, seen in a dose-dependent manner (Fig. 18C-D). Sensitized MNECs did not affect the fraction of $CD69^+$ T cells in co-cultures with neutralizing anti-MHC class II antibodies (Fig. 18E). IFN-γ release was measured in the supernatants as a marker of functional T cell activation. Sensitized OVA-stimulated MNECs were able to induce naïve OT-II T cells to release IFN-γ although statistical significance was not reached (Fig. 18F) and a similar tendency was seen from co-cultures with sensitized $CD4^+$ T cells.

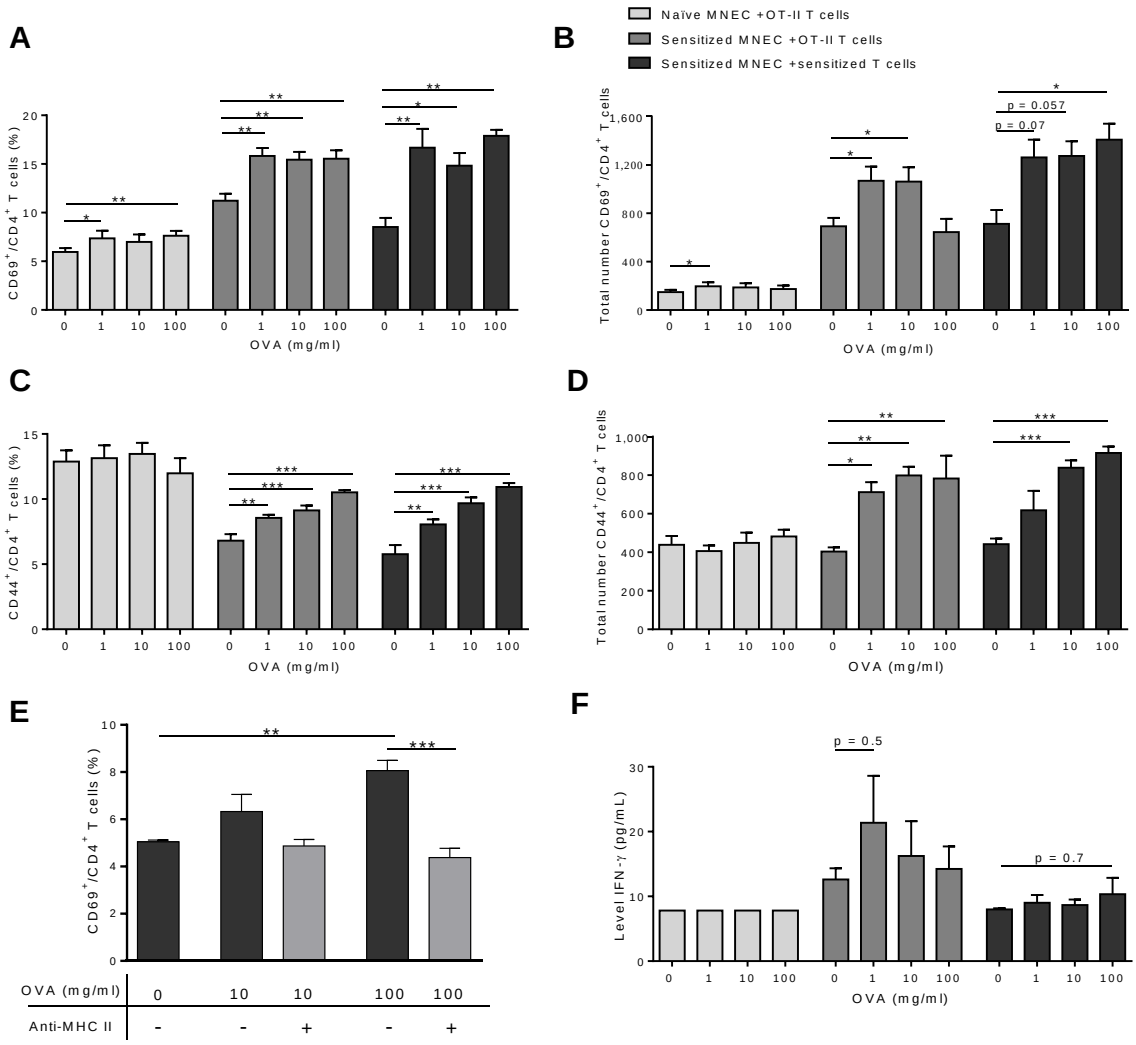

Figure 18. T cells and IFN-γ release upon co-culture with OVA-stimulated MNECs (24 hours). Color box legends valid for A-D and F. (**A**) Fraction and (**B**) total number of $CD69^+/CD4^+$ T cells. (**C**) Fraction and (**D**) total number of $CD44^+/CD4^+$ T cells. (**E**) Co-cultures with MNECs and T cells (both from sensitized mice) with anti–MHC class II antibodies (anti-MHC II). (**F**) INF-γ release in supernatants of co-cultures.

CPG stimulation influences inflammation and angiogenesis

To investigate whether the ligand CpG could affect or even restore the TLR9 expression, NECs were cultured and stimulated with CpG. ECs of turbinate tissue from CRSwNP patients upregulated the TLR9 expression upon CpG stimulation (Fig. 19A). CpG stimulation also decreased the levels of VEGFR2 (Fig. 19B), a receptor of angiogenesis, as well as decreased the levels of secreted G-CSF, IL-6 and MIP-1β (Fig. 20A-C). No differences were seen regarding TLR9, VEGFR2 or cytokines when cultured NECs from patient polyps or healthy controls were stimulated with CpG.

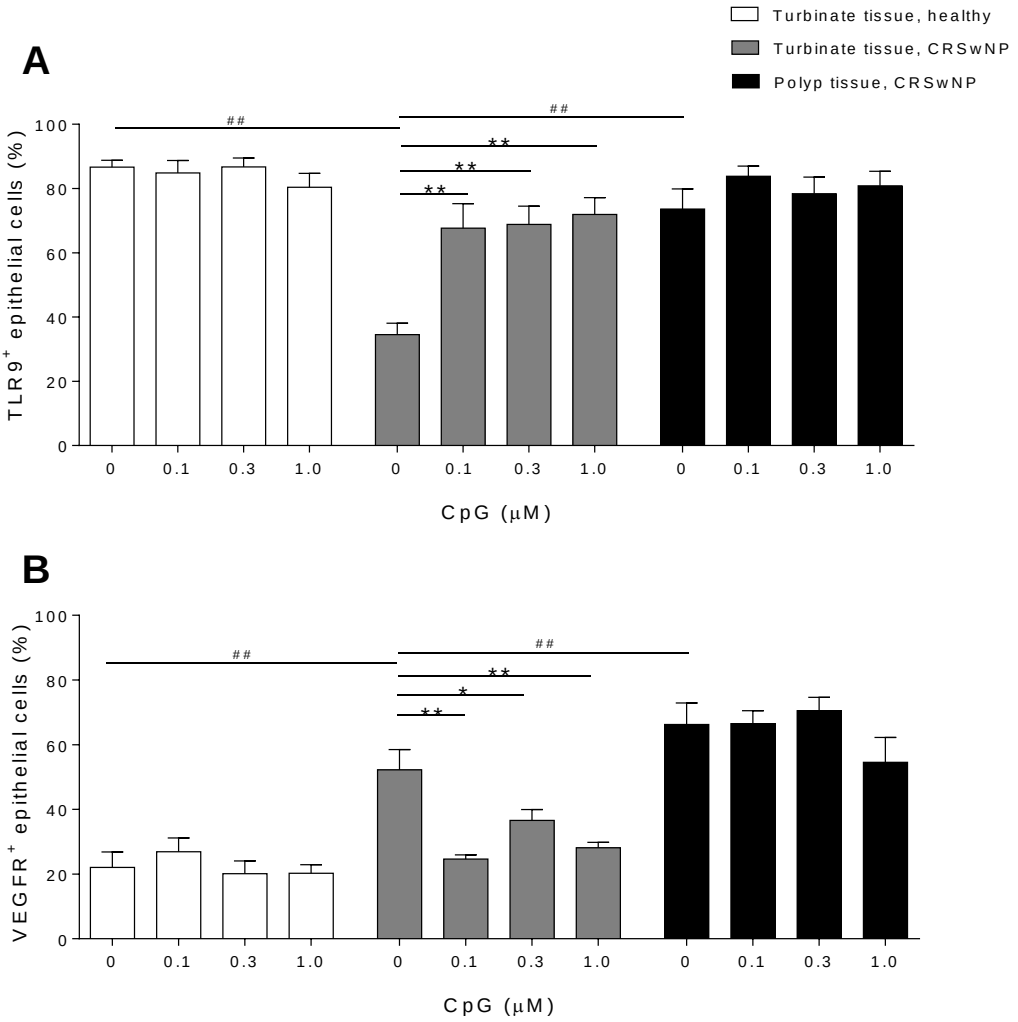

Figure 19. Cultured NECs from patients and healthy controls stimulated with CpG. (**A**) TLR9 expression upon 4 hours and (**B**) VEGFR2 expression upon 24 hours of stimulation. $n = 6$, *$p < 0.05$; **$p < 0.01$ (un-stimulated vs. CpG stimulated) and ##$p < 0.01$ (un-stimulated turbinate tissue from patients vs. un-stimulated turbinate tissue from healthy controls and polyp tissue from patients).

Activation of ALKs affect local proliferation and inflammation

Cultured NECs were stimulated with multiple ALK ligands whereupon markers of proliferation (Ki67) and inflammation (ICAM-1 and IL-8) were measured. Upon stimulating ALK1 and 5 with TGF-β1, a downregulation of Ki67 expression (Fig. 21A) and IL-8 release (Fig. 21B) was detected from polyp ECs. In addition, stimulation of ALK2, 4 and 7 with Activin A or ALK4 and 7 with Activin B decreased ICAM-1 expression and IL-8 release from polyp ECs (Fig. 21C-F). Control ECs showed no change in Ki67 or ICAM-1 expression or IL-8 release upon ALK stimulation (Fig. 21A-F).

COMMENTS

An efficient T cell response is crucial in an inflammatory response. Upon stimulating co-cultures of MNECs and T cells in an antigen-specific manner (OVA), MNECs upregulated their MHC class II expression. No upregulation was seen when MNECs were co-cultured with naïve splenic cells. This demonstrates the essence of antigen presentation and interaction of

antigen-specific T cells. Thus, sensitized MNECs upregulate their antigen presenting capacity upon OVA-stimulation in a TCR-specific manner. In addition, the total number $CD4^+$ T cells and their level of activation, demonstrated by the expression of CD69 and CD44, was increased upon stimulation in the same co-cultures. The OVA peptide used encompasses an allergic antigenic epitope of the OVA protein in mice. Hence, activation of OT-II T cells by OVA requires antigen processing. This event is class II dependent, revealed by the assays with MHC class II-blocking antibodies. Altogether, we claim that sensitized MNECs can take up, process and present antigens and cause an antigen specific $CD4^+$ T cell response in a class II dependent manner.

Kambayashi et al. published a review on the subject 'atypical APCs', questioning what role they could have in immune responses.[73] They concluded that a large number of cells can express MHC class II molecules and present antigens to $CD4^+$ T cells. MHC class II molecules alone are however not sufficient for full APC function, since APCs also need to process antigens and express co-stimulatory molecules for lymphocyte activation. They argue that non-professional APCs are unlikely to replace DCs, since there has hardly been shown that these cell types are able to activate naïve $CD4^+$ T cells. We do not claim that NECs can replace the role of professional APCs. However, the use of a TCR transgenic system (OT-II mice) and murine ECs made it possible for us to use naïve T cells as reporter cells, thus proving that a T cell response against allergens could be initiated by ECs.

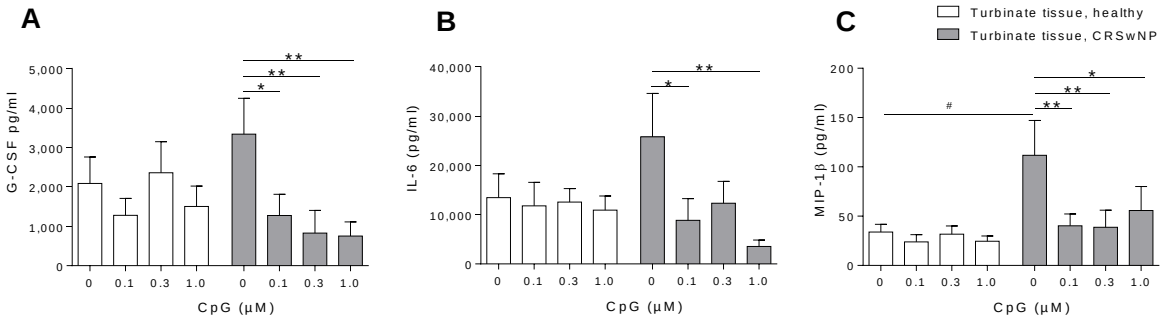

Figure 20. Cytokine secretion from cultured NECs upon 4 or 24 hours of CpG stimulation. (**A**) G-CSF, (**B**) IL-6 and (**C**) MIP-1β. *$p < 0.05$; **$p < 0.01$ (un-stimulated vs. CpG stimulated), # $p < 0.05$ (un-stimulated turbinate tissue from healthy controls vs. from patients).

We continued studying human NECs from CRSwNP patients *in vitro*. Several studies have reported on immunostimulatory properties of CpG *in vitro* in humans[74, 75] and *in vivo* in mice.[76, 77] These findings reveal the ability of CpG to promote a Th1-biased immune response.[56, 75] We here showed that the pronounced decrease in TLR9 expression on patient NECs (Fig. 13) could be restored towards healthy conditions upon CPG stimulation. In addition, CpG stimulation decreased the expression of VEGFR2, a receptor necessary for vascular supply thus facilitating the growth of nasal polyps[78, 79] and often upregulated in CRSwNP.[80, 81] Hence, CpG seem to regulate inflammation and angiogenesis in CRSwNP.

We have previously shown that factors affecting local proliferation (Ki67) and inflammation (ICAM-1 and IL-8) were increased in CRSwNP (Fig. 16A-F). Stimulation with ALK ligands

downregulated all these factors. Since the levels of the two ALK ligands TGF-β1 and Activin A have been shown to be low in CRSwNP, absence of ALK-activation might contribute to inflammation in this disease.[69, 82, 83]

Altogether, these results demonstrate that NECs can activate and proliferate $CD4^+$ T cells in an antigen specific manner. Activation of TLR9 and ALKs resulted in cytokine release and changes in the receptor pattern, linked to inflammation, angiogenesis and cell-proliferation. The presented data suggests a role for these receptors in the local nasal inflammation, something that is further underscored by the changes in the receptor patterns seen during inflammatory conditions.

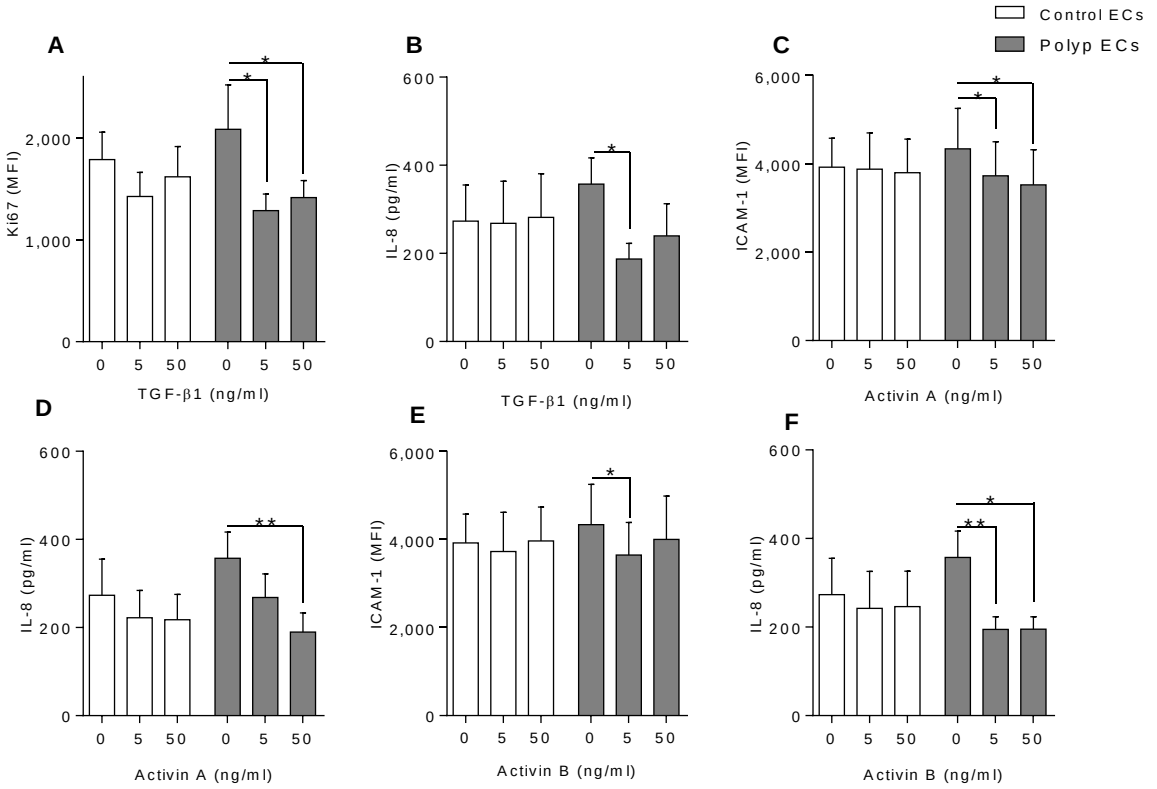

Figure 21. Cultured NECs stimulated with ALK ligands for 24 hours. (**A-B**) TGF-β1, (**C-D**) Activin A and (**E-F**) Activin B. Expression of Ki67 and ICAM-1 as well as secreted IL-8 was measured. Specimen from turbinate tissue from healthy controls and polyp tissue from CRSwNP patients.

NEUTROPHIL SUBSETS IN THE NOSE (PAPERS IV, V)

Neutrophils have long been considered as short-lived, terminally differentiated cells. However, information has recently emerged indicating that neutrophils can be divided into different functional subsets, based on the expression of CD16 and CD62L.

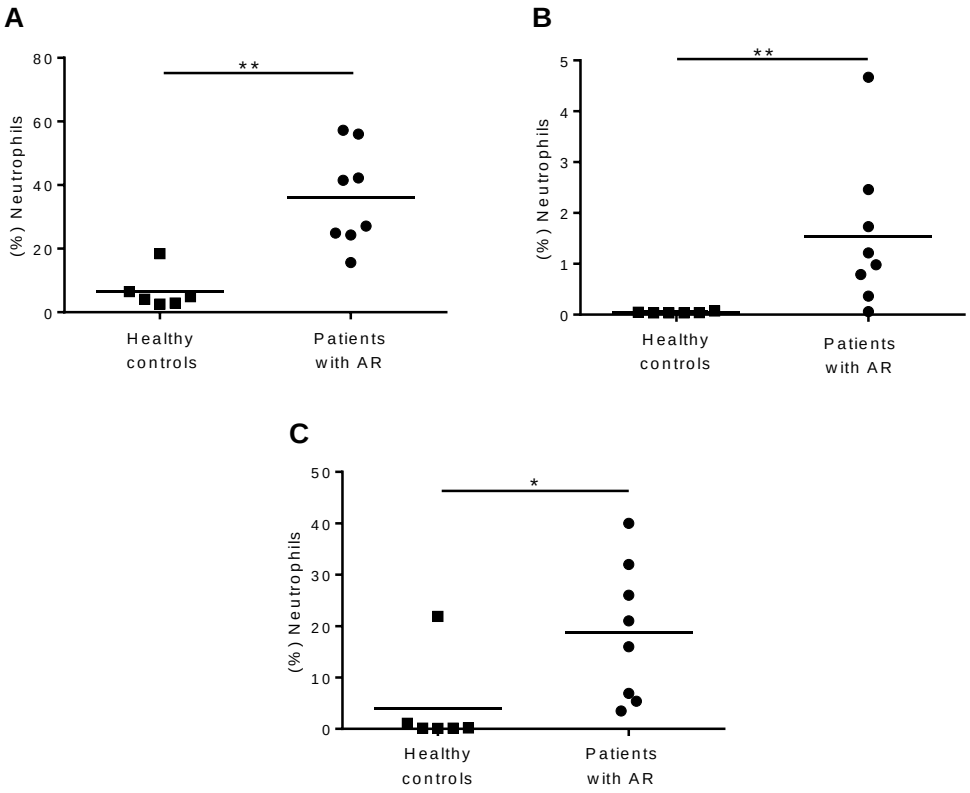

Figure 22. Fractions of neutrophils in (**A**) blood, (**B**) nasal biopsies and (**C**) NAL from patients with AR during pollen season and healthy controls.

The distribution of neutrophil subsets in AR

We started with determining the total neutrophil fractions in AR. Neutrophil fractions were found to be enhanced in blood, nasal biopsies and NAL from AR patients during pollen season, compared to healthy controls (Fig. 22A-C). We classified the neutrophils into four different subsets (Fig. 23A). The less mature subset, $CD16^{dim}$ $CD62L^{high}$, was hardly detectable. The mature subset, $CD16^{high}$ $CD62L^{high}$, was mainly found in blood. In nasal mucosa, both the $CD16^{high}$ $CD62L^{high}$ subset and the activated form, $CD16^{high}$ $CD62L^{dim}$, were elevated. Finally, the subsets $CD16^{high}$ $CD62L^{dim}$ and $CD16^{dim}$ $CD62L^{dim}$, considered the end state neutrophil, were elevated in NAL. Healthy controls exhibited the same general compartmental subset pattern as the AR patients with more differentiated subsets in nasal mucosa and even more in NAL compared to in peripheral blood (data not shown). The activated neutrophil subset, $CD16^{high}$ $CD62L^{dim}$, was elevated compared to the mature neutrophil subset $CD16^{high}$ $CD62L^{high}$ in biopsies from AR patients (Fig. 23B). This was not seen in healthy controls.

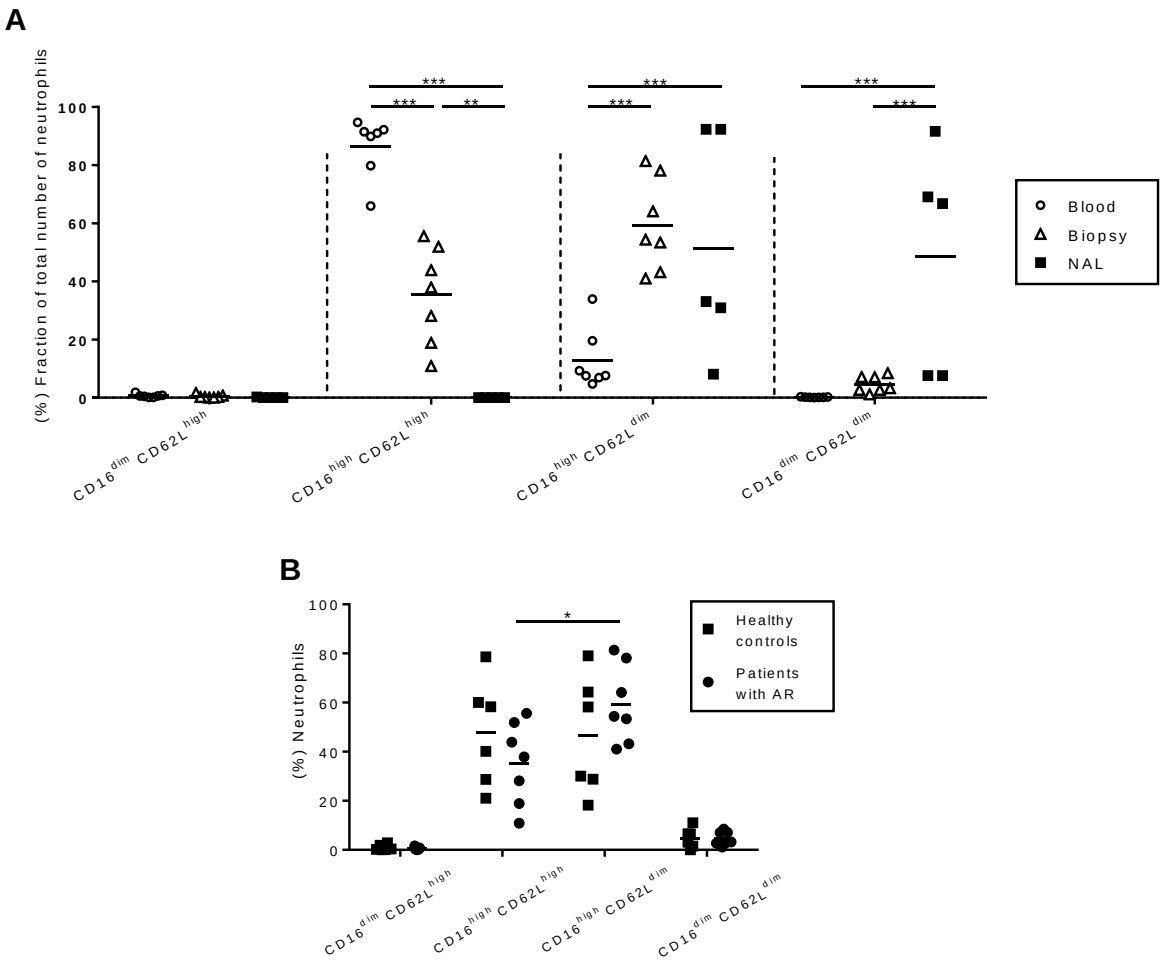

Figure 23. Fractions of different neutrophil subsets. (**A**) Neutrophil subsets in blood, nasal biopsies and NAL from AR patients during pollen season. (**B**) Distribution of neutrophil subsets in nasal biopsies from AR patients and healthy controls during pollen season.

The distribution of neutrophil subsets in CRSwNP

We continued mapping neutrophils in CRSwNP. We saw no differences in the total neutrophil fractions in blood or tissue between patients and controls (Fig. 24A-B). When subtyping the neutrophils as in AR patients, we detected a clear shift. The activated neutrophil subset, $CD16^{high}$ $CD62L^{dim}$, was higher in patient polyp mucosa compared to control mucosa (Fig. 25A). A similar trend, not reaching statistical significance, was also seen for $CD16^{high}$ $CD62L^{dim}$ neutrophils found in the surrounding nasal mucosa of patients ($p = 0.3$). Moreover, the end state subset, $CD16^{dim}$ $CD62L^{dim}$, was also increased in patient polyp mucosa compared to healthy controls. On the other hand, the mature neutrophil subset, $CD16^{high}$ $CD62L^{high}$, dominated in control mucosa (Fig. 25A). Polyps exhibited a significantly higher ratio of activated neutrophils compared to normal neutrophils (Fig. 25B) or end state neutrophils compared to normal neutrophils (Fig. 25C), highlighting the shift towards more activated and differentiated neutrophils in the nasal mucosa from patients.

COMMENTS

Neutrophil subsets are present in the nasal mucosa and activated and differentiated subsets are increased locally in allergy and CRSwNP. The shift towards more activated neutrophils in the mucosa of patients was more pronounced in CRSwNP whereas the increase in total number of neutrophils was more marked in AR, indicating a substantial increase of the activated subsets also in AR.

Eosinophils are known to play a part in both allergy and CRSwNP. Even though the neutrophils far outnumber the number of eosinophils found both locally and systemically during these conditions, the former has been given much less attention as a potential player in the inflammatory reaction. This is probably due to the fact that neutrophils always can be found in the nose, also in the asymptomatic allergic phase, and in healthy controls. The finding of different subsets with different immunological properties has however changed the concept of neutrophil studies.[45] Sauce et al. reported that elderly patients have increased levels of activated $CD16^{high}$ $CD62L^{dim}$ neutrophils in the blood compared to healthy controls.[84] The same subset has been proven to be increased in blood in patients with various cancer diagnoses.[85]

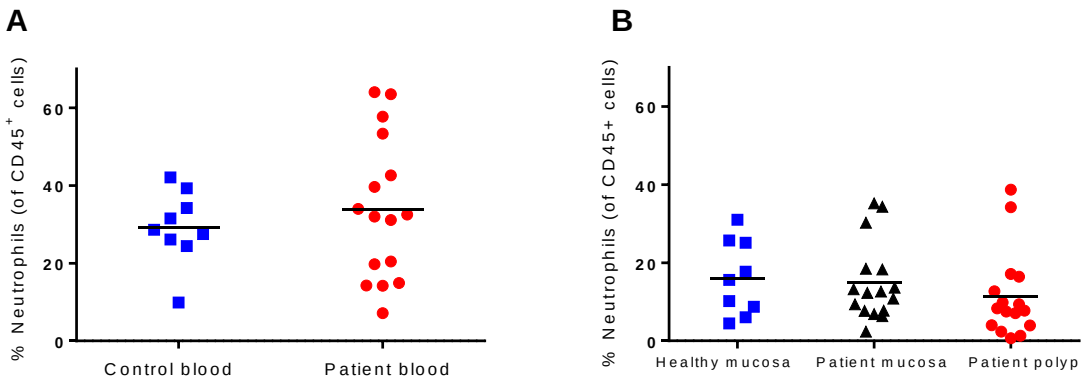

Figure 24. Fractions of neutrophils in (**A**) blood and (**B**) in patient surrounding nasal mucosa and polyps as well as in nasal mucosa from healthy controls.

Neutrophils from allergic patients have at the same time been shown to downregulate the surface expression of CD62L upon allergen stimulation.[86] In line with this, we have previously demonstrated that neutrophils in NAL from allergic patients have a downregulated expression of L-selectin (CD62L) during the peak of the pollen season.[87] The distribution of neutrophil subsets based on their expression of CD16 and CD62L has never been studied in AR. Neither have they been mapped in CRSwNP. In addition, little attention has previously been paid to the end state subset, $CD16^{dim}$ $CD62L^{dim}$ neutrophils. We believe it to still have functional properties as with the $CD16^{high}$ $CD62L^{dim}$ subset, because of the viability experiments. We here report that activated and differentiated neutrophil subsets accumulate locally at the site of

disease, making it tempting to suggest a role for these subsets in nasal allergy as well as CRSwNP.

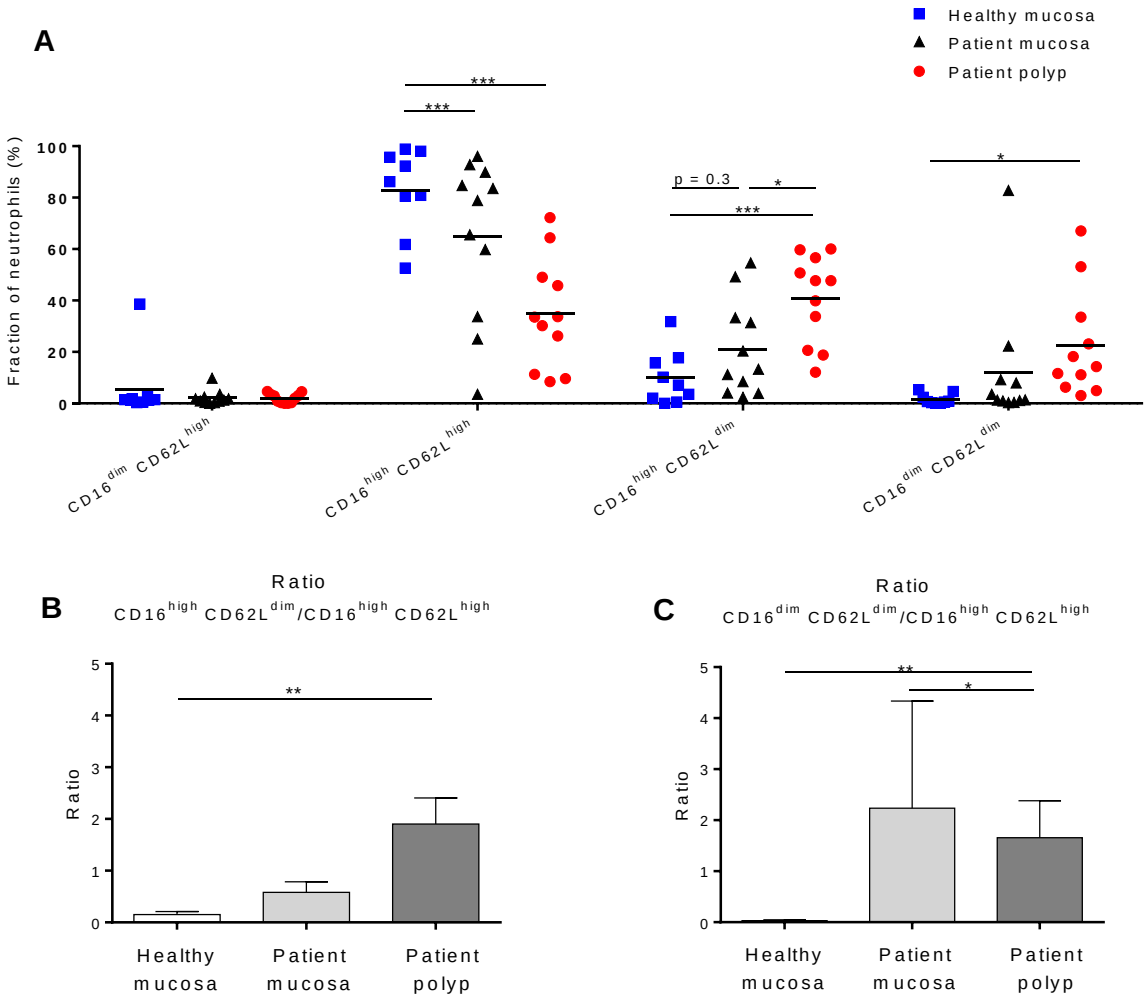

Figure 25. (**A**) The distribution of neutrophil subsets in patient surrounding nasal mucosa and polyps as well as nasal mucosa from healthy controls. Ratio of (**B**) activated neutrophils compared to normal neutrophils and (**C**) end state neutrophils compared to normal neutrophils.

INNATE MECHANISMS OF THE EPITHELIUM AND NEUTROPHILS AFFECT ALLERGIC INFLAMMATION (PAPERS I, IV)

Nasal inflammation, where the innate immune system immediately reacts to invading pathogens, is a major feature of AR. The epithelium and neutrophils play a role in this, but the mechanisms behind this is rather unknown.

Epithelial cells induce autologous CD4$^+$ T cell allergen responses

We wished to further validate the idea of HNECs as APCs, suggested by expression of MHC class II and co-stimulatory molecules (Fig. 12A-B), and to investigate whether this action could be upregulated in allergy. Accordingly, the ability of HNECs to endocytose exogenous antigens was investigated by providing FITC labeled dextran to cultured HNECs derived from an AR patient. IF demonstrated the presence of dextran in intracellular vesicles after 2 hours suggesting that NECs could endocytose exogenous antigens (Fig. 26A-B).

We continued allowing cultured HNECs to endocytose a crude birch pollen extract. Autologous T cells harvested in-season during periods of high pollen counts were subsequently added to the co-cultures. We observed an increase in the number of autologous CD4$^+$ T cells when co-cultured with stimulated HNECs from AR patients (Fig. 26C). In the following experiments, we used the major birch allergen protein, Bet v 1, minimizing endotoxin contamination. Autologous CD69$^+$/CD4$^+$ T cells increased upon co-culture with stimulated HNECs from AR patients (Fig. 27A). IL-13 release was augmented in the same co-cultures (Fig. 27B). We finally analyzed the distribution of different HLA-DR expressing cells in the nasal mucosa, revealing a high number of HLA-DR expressing ECs, compared to HLA-DR expressing professional APCs (Fig. 27C). The expression of HLA-DR was rather high in NECs, demonstrated by the MFI (Fig. 27D).

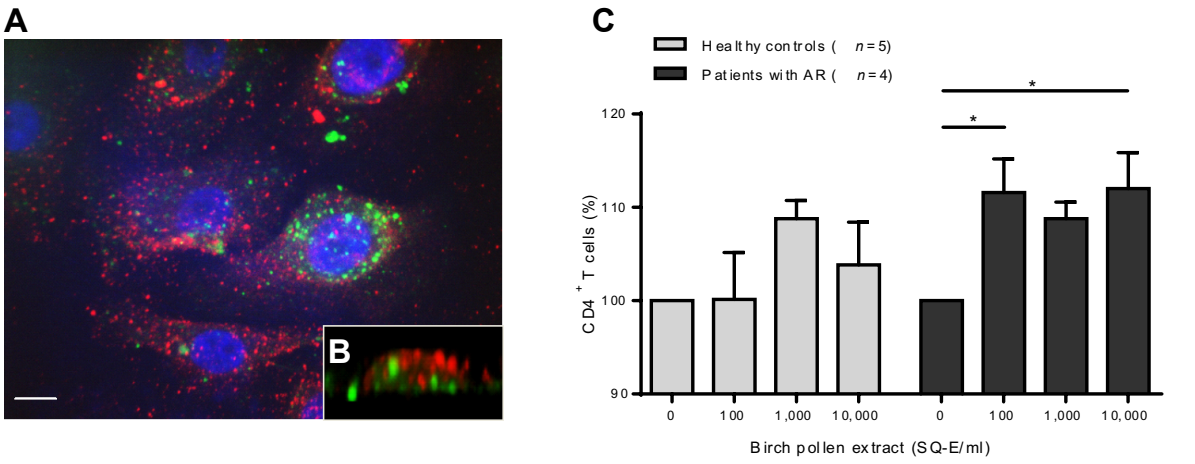

Figure 26. (**A**) Endocytotic uptake of exogenous antigens (dextran, in green) by NECs from a patient with AR, co-localizing with MHC class II molecules (red) intracellularly in vesicles. Nucleus counterstained with DAPI (blue). (**B**) A three-dimensional composite image showing co-localization. Scale bar: 10 µm. (**C**) Levels of CD4$^+$ T cells after co-culture with HNECs stimulated with birch pollen.

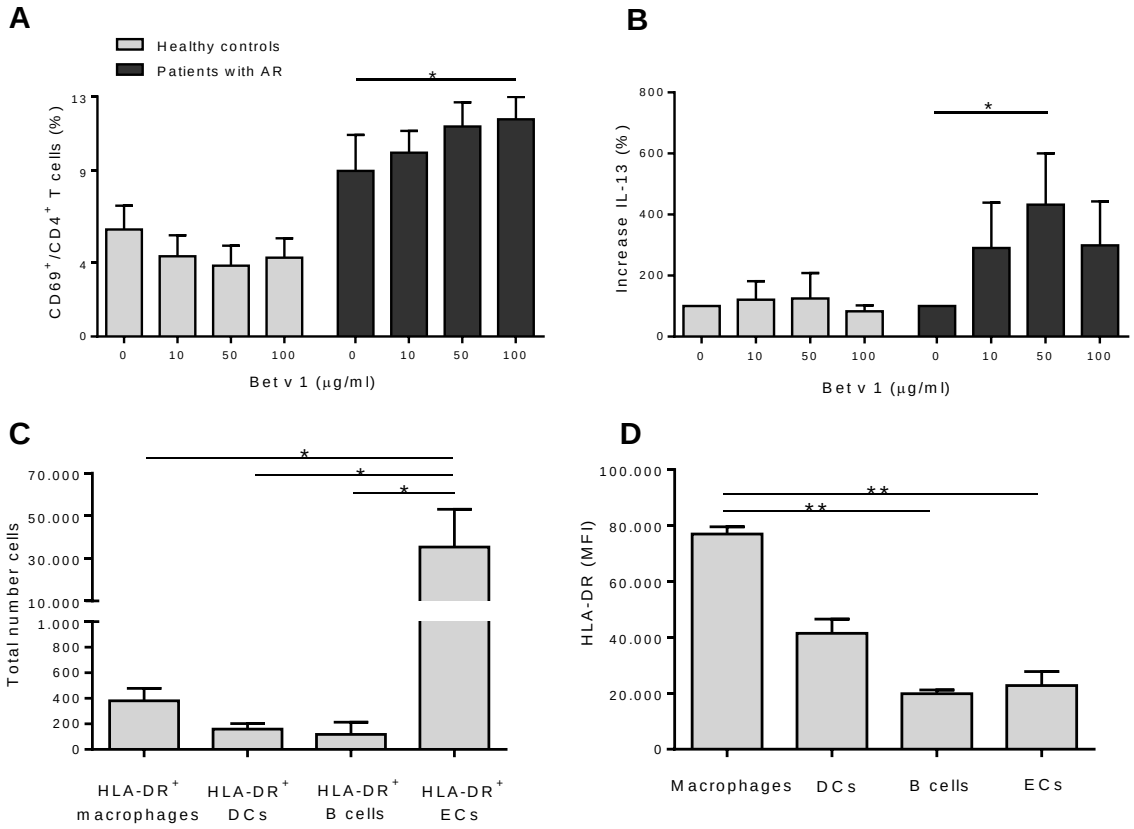

Figure 27. Human NECs. (**A**) The fraction of $CD69^+/CD4^+$ T cells and (**B**) the IL-13 release followed co-culture with HNECs stimulated with Bet v 1. (**C**) Different HLA-DR^+ cell types in nasal mucosa and (**D**) the MFI.

Activated neutrophils can prime T cells and mediate eosinophil migration

Neutrophils were isolated from blood and stimulated with LPS, TNF-α and IL-8 to cause activation. Flow cytometric analysis revealed an increase of the activated neutrophil subset ($CD16^{high}$ $CD62L^{dim}$) (Fig. 28). Autologous T cells from blood were added in co-cultures. T cells primed with activated neutrophils were more likely to get activated by CD3 stimulation, demonstrated by the CD69 expression (Fig. 29A). The same findings were observed when using blood from AR patients (Fig. 29B).

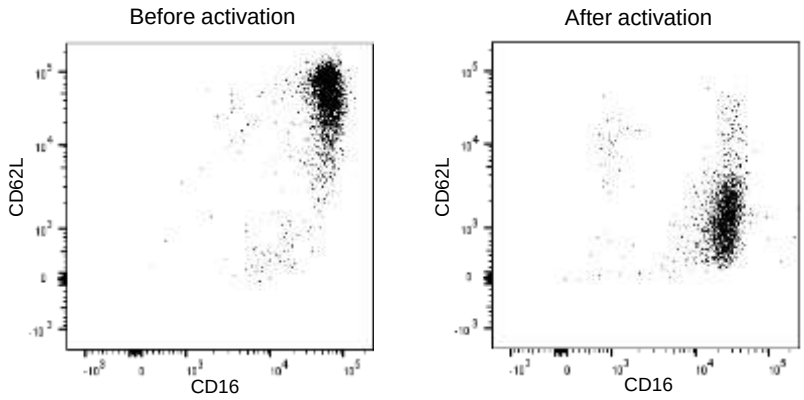

Figure 28. Flow cytometric dot plot of neutrophils from blood before and after activation with LPS, TNF-α and IL-8.

Experiments carried out on transwell plates did not reveal an increase of $CD69^+/CD4^+$ T cells upon priming with activated neutrophils (Fig. 29C), indicating that the enhanced T cell priming of neutrophils was cell-cell contact or close contact dependent. Assays were finally set up to study the impact of activated neutrophils on eosinophils. Isolated neutrophils stimulated with LPS, TNF-α and IL-8 upregulated eosinophilic migration in the transwell system (Fig. 30).

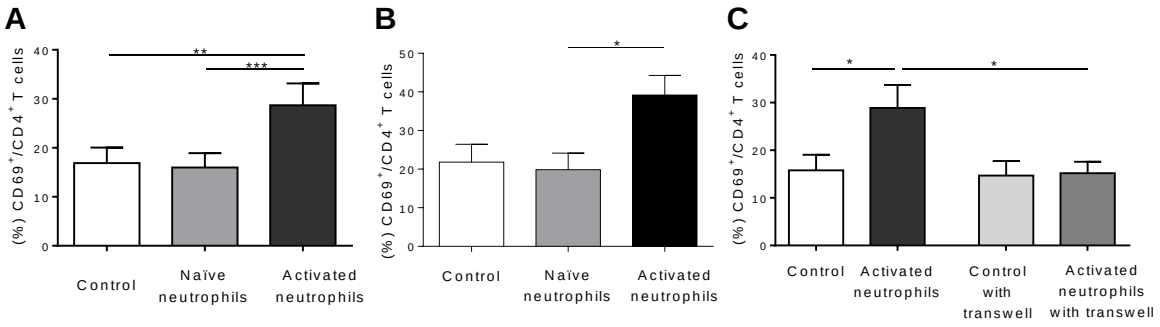

Figure 29. T cells primed by activated neutrophils. The fraction of $CD69^+/CD4^+$ T cells from (**A**) healthy individuals (n = 12) and (**B**) AR patients (n = 5) primed with naïve or activated neutrophils. (**C**) Experiments with and without transwell plates (n = 11). Control = no added neutrophils.

COMMENTS

NECs from AR patients exhibited an increased capacity to activate autologous $CD4^+$ T cells in an allergen-specific manner, further corroborating the mouse data presented (Fig. 17-18). This recall response was likely to result in a Th2-biased response reflected by the secretion of IL-13. In addition, HLA-DR^+ HNECs far outnumbered local HLA-DR^+ DCs, B cells and macrophages. Thus, the ability of NECs to act as APCs is an important event in the first line of defense in the nasal mucosa, being constantly exposed to allergens. In addition, professional APCs are fewer in total numbers[88, 89] and believed to mainly present and subsequently activate T cells in the lymph nodes, not locally in the nose.

The idea of more advanced immunological properties of ECs is gaining acceptance. It enables a rapid and potentially efficient nasal immunological defense considering ECs strategic localization, vast numbers and rapid innate action. Experiments in Paper I was both conducted with mouse and human cells. The different set ups make up for each other since they have different advantages. The mice model allowed the usage of transgenic systems as well as naïve T cells, making it possible to draw more reliable conclusions on the capacities of NECs. Adding human experiments, on the other hand, enabled conclusions on a clinical level. True allergic patients were included, unlike the allergic model used in mice.

How antigen presentation by ECs can contribute to a disease has to date scarcely been investigated. Dotan et al. revealed that IECs from patients with inflammatory bowel disease stimulated $CD4^+$ T cells more efficiently than control IECs.[36] Deleting MHC class II expression exclusively on podocytes, terminally differentiated visceral ECs in kidneys, prevented the induction of anti-GBM nephritis in mice.[37] An upregulated expression of MHC class II molecules on mice type II alveolar ECs has been reported following infection with

Mycobacterium tuberculosis, suggesting that a rapid response with local antigen presenting ECs is important for the initiation of an early adaptive immune response.[90] Several viruses, for example causing severe acute respiratory syndrome (SARS), have developed mechanisms to inhibit MHC class II pathways in ECs, indicating them as important in the defense against viruses.[91] Upregulated levels of HLA-DR and CD86 have been reported on primary NECs from AR patients during the pollen season.[39] Bet v 1 has further been demonstrated to bind to and to enter ECs rapidly (within minutes) after the exposure even in allergic patients during the non-symptomatic season.[92] Despite these findings, the function of airway ECs as significant contributors to the allergic reaction remains to be established.

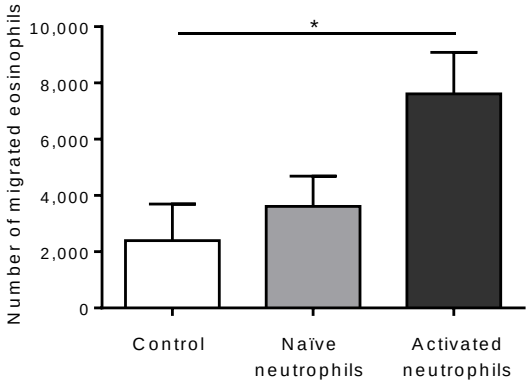

Figure 30. Eosinophil migration upon incubation with naïve or activated neutrophils in transwell systems (n = 4-5). Control = no added neutrophils.

Neutrophils are, just like ECs, an abundant cell type in the nasal mucosa. Different subsets have been suggested to have diverse roles during various inflammatory conditions. The subset $CD16^{high}$ $CD62L^{dim}$ was shown to have a lower phagocytic index as well as a lower oxidative burst compared to the normal mature neutrophil subset.[84] This was suggested to partly explain the susceptibility to microbial infections during aging. On the other hand, we have previously shown activated neutrophils to have protective aspects in head and neck squamous cell carcinoma.[93, 94] We here showed that activated neutrophils could prime T cells, thus facilitating T cell activation. In contrast to this, Pillay et al. reported that activated neutrophils suppressed T cell activation. These experiments were however in model systems using blood from healthy volunteers while our experiments used cells from allergic patients (Fig. 29B). Eosinophils are known to play an important role in Th2-mediated allergic immune responses.[95, 96] The presented data revealed that activated neutrophils could facilitate eosinophil migration. This assigns neutrophils a novel role in allergy. Interestingly, reduced numbers of neutrophils have been recorded following immunotherapy.[97, 98] Thus, we concluded that activated neutrophils are likely to enhance eosinophil migration in allergy, facilitating the local eosinophil infiltration seen in this condition.

To sum up, this is the first time that innate activity within the nasal epithelium has been directly linked to efficient and rapid allergen-induced T cell activation. Activated neutrophils in the mucosa were shown to augment this process. These neutrophils could also enhance eosinophil migration. Allergen exposure is known to result in a high number of activated $CD4^+$ T cells

and eosinophils in the nasal mucosa and an increase of cells expressing Th2 cytokines.[99] Hence, the local epithelium and neutrophils are likely to rapidly contribute to this well-known allergic inflammatory process upon allergen exposure.

TARGETING INFLAMMATION IN CRS THROUGH INNATE MECHANISMS (PAPERS II, III, V)

The theories about the mechanisms involved in CRSwNP progression are becoming increasingly complex, without identifying the pivotal factors behind the growth of polyps.[19] A re-examination of old concepts may thus be necessary to find novel treatment strategies.

CpG challenge *in vivo* has potential anti-inflammatory effects

Patients requiring surgical intervention for CRSwNP were intra-nasally challenged with CpG or placebo. After 24 hours, biopsies from mucosa surrounding the polyps were taken during FESS. Flow cytometry revealed an upregulation of $TLR9^+$ NECs as well as a tendency towards a downregulation of $VEGFR2^+$ NECs in the CpG challenged group (Fig. 31A-B). These data confirmed the *in vitro* data presented (Fig. 19), indicating that CpG augments the TLR9 expression and decreases the VEGFR2 expression. In addition, these data are in line with the *in vitro* data of the epithelium in the mucosa surrounding the polyps and highlights the immunological potential of this mucosa.

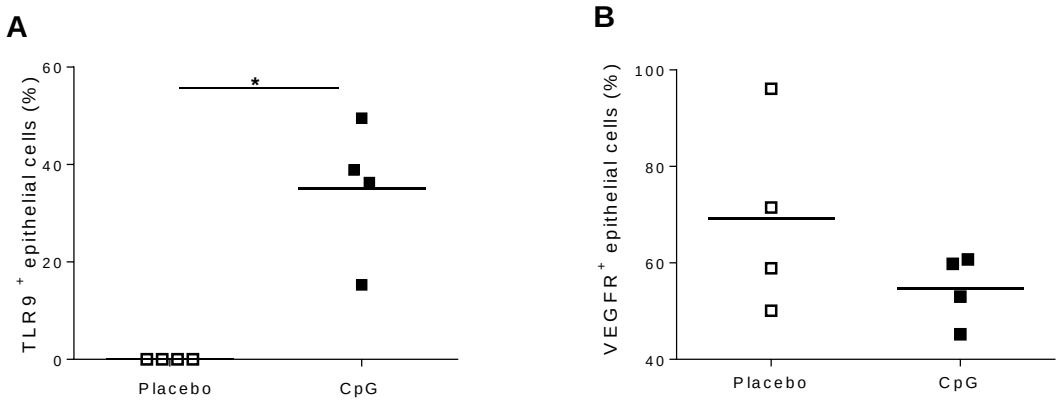

Figure 31. Expression of (**A**) TLR9 and (**B**) VEGFR2 on epithelium surrounding the polyp, 24 hours after *in vivo* nasal challenge with CpG or placebo.

ALK activation reduces mimicked microbial inflammation in CRSwNP

We earlier demonstrated that ALK-stimulation reduced ICAM-1 expression on NECs from CRSwNP patients (Fig. 21C, E). Infections are known to trigger periods of disease progression. Hence, cultured NECs pre-treated with the ALK-ligands TGF-β1, Activin A, BMP4 and Activin B were subsequently exposed to TNF-α, a cytokine known to be produced upon bacterial, fungal and viral infections. Pre-treatment with ALK-ligands significantly inhibited TNF-α induced ICAM-1 expression on polyp ECs (Fig. 32).

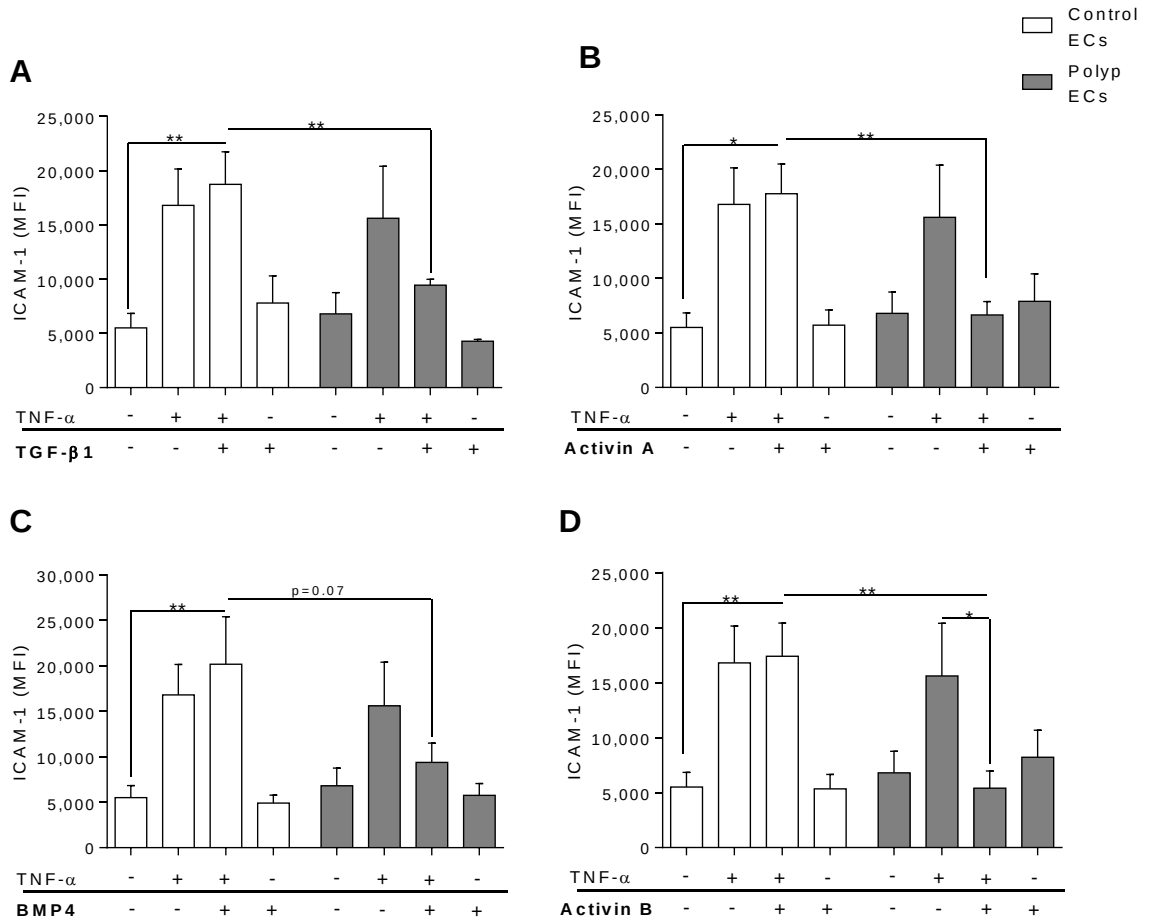

Figure 32. Cultured NECs stimulated with (**A**) TGF-β1, (**B**) Activin A, (**C**) BMP4 and (**D**) Activin B for 20 hours whereupon TNF-α was added and incubated for 4 hours and ICAM-1 expression was determined. Specimen from polyp tissue from CRSwNP patients and turbinate tissue from healthy controls (*n* = 5-7).

Neutrophils from patients have greater possibilities for mucosal adhesion

To further explore the impact of activated neutrophils locally in patients with CRSwNP, we studied the protein subunit CD11b. Together with CD18, it forms a heterodimeric integrin mediating inflammation by regulating leucocyte adhesion and migration, implicating cell activation. This occurs by binding to the cell surface molecule ICAM-1. Neutrophils both in blood (Fig. 33A) and nasal mucosa (Fig. 33B) from CRSwNP patients exhibited higher fractions of $CD11b^{+}$ cells compared to healthy controls. The nasal mucosa surrounding the polyps showed the same pattern regarding CD11b. The expression of CD11b was most prominent on $CD16^{high}$ $CD62L^{high}$ and $CD16^{high}$ $CD62L^{dim}$ neutrophils in patient blood (Fig. 34B). Activated $CD16^{high}$ $CD62L^{dim}$ neutrophils exhibited the highest expression of CD11b both in patient mucosa and polyps (Fig. 34D, E). No such shift was seen in healthy blood or mucosa (Fig. 34 A, C). Correspondingly, ICAM-1 was significantly upregulated on ECs from polyps (Fig. 35).

COMMENTS

Inflammatory exacerbations combined with polyp growth are regularly seen in conjunction with upper airway infections in CRSwNP.[14] Several bacteria have been isolated from the

sinuses in CRS[100], although much focus have been placed on *Staphylococcus aureus* and its enterotoxin products (SAE).[101-103] The relationship between *Staphylococcus aureus* and CRSwNP is still not fully established, even though there is a consensus about the role for a locally disturbed microbial flora in the disorder.[104, 105] It might be that the polyp growth is not directly linked to the invading pathogen, but rather to the trauma that the pathogen causes.[106] Presented ligand stimulation of epithelial ALKs decreased ICAM-1 more prominently in the presence of TNF-α, signaling a tentative anti-inflammatory effect of these receptors.

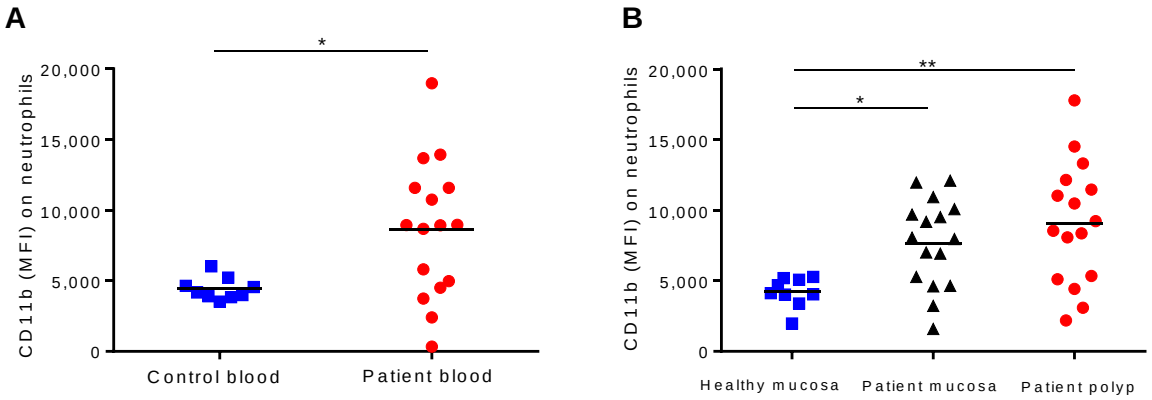

Figure 33. The expression of the surface marker CD11b on neutrophils from (**A**) blood and (**B**) patient surrounding nasal mucosa and polyps as well as nasal mucosa from healthy controls.

CpG is a TLR9 ligand found in viral and bacterial DNA. The ability of CpG to induce Th1 polarization has been suggested to have a potential protective role against Th2 inflammation. A recent study showed no effects on IL-5 or eotaxin-3 upon CpG stimulation.[107] On the other hand, matrix metalloprotease 9 (MMP-9) - an important protein in tissue remodeling and repair - was elevated in stimulated cell cultures from CRS patients. However, the study suffers from some weaknesses. Patients with inverted papilloma were used as controls and patient samples incubated with negative controls also appeared to have increased MMP-9.[107] Hence, we still believe that CpG could have a protective role in CRSwNP patients by lowering inflammation and preventing an angiogenetic milieu of the epithelium.

Kamp et al.[46] reported that the neutrophil subset $CD16^{high}$ $CD62L^{dim}$ had a decreased ability to adhere to endothelium *in vitro*, possibly resulting in less extravasation of activated neutrophils from the blood. The subset was instead suggested to preferentially migrate to peripheral lymphoid organs. The authors reported on upregulated CD11b on activated neutrophils and questioned what consequences this finding could have *in vivo*. We saw no increase in activated neutrophils in blood from patients with CRSwNP, but they were increased in the nasal mucosa. We suggest that the neutrophils are activated in the sinonasal mucosa, probably due to local IL-8 and IL-17. We propose these $CD16^{high}$ $CD62L^{dim}$ neutrophils to have enhanced adhesion to the epithelium in the nasal mucosa by CD11b and ICAM-1 interactions, since these receptors were upregulated on the neutrophils and epithelial cells of patients. Future studies will hopefully answer what role the activated subsets can play in CRSwNP and whether targeting them or CD11b/ICAM-1 can facilitate disease control.

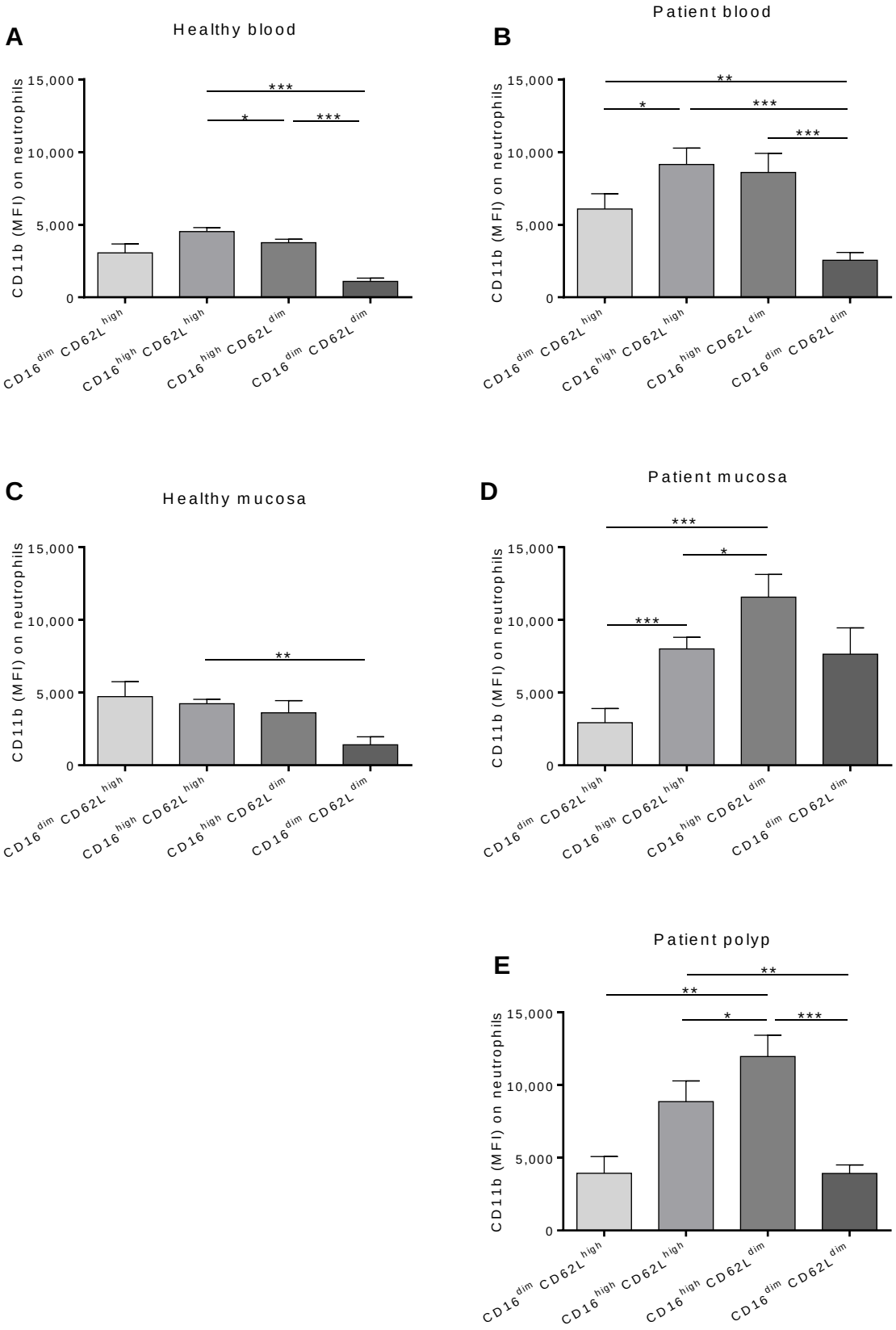

Figure 34. Expression of CD11b on different neutrophil subsets from (**A**) healthy blood, (**B**) patient blood, (**C**) nasal mucosa from healthy controls, (**D**) nasal mucosa from CRSwNP patients and (**E**) polyps from CRSwNP patients (*n* = 11-16).

The nasal mucosa with its epithelial receptors appears to change during inflammatory conditions and targeting these receptors must therefore be considered in future therapeutic efforts. The surrounding nasal mucosa seen not far from the polyps seem to be of special interest for some of these mechanisms. Hence, researchers should consider analyzing not only the diseased tissue of the polyp but also the surrounding non-polypoid mucosa.

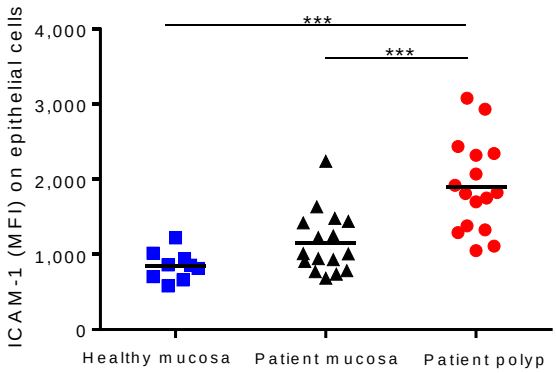

Figure 35. ICAM-1 expression on epithelial cells from patient surrounding nasal mucosa and polyps as well as nasal mucosa from healthy controls.

CONCLUSIONS

- Nasal epithelial cells express MHC class II and co-stimulatory molecules, necessary for antigen presentation, as well as TLR9 and ALKs. The presentation of these signaling molecules was found to differ between inflamed and healthy tissue. The presented data suggest that the local epithelium has the ability to present antigens and to influence the inflammatory process during upper airway disease.

- Mouse nasal epithelial cells could activate and proliferate $CD4^+$ T cells in an antigen specific manner. Stimulation of TLR9 and ALK1-6 resulted in cytokine release and changes in the receptor pattern, linked to inflammation, angiogenesis and cell-proliferation. Hence, activation of these, during inflammatory conditions, modified receptors might have a direct or indirect impact on the ongoing inflammatory process.

- Four neutrophils subsets could be characterized in the human nose and changes in the distribution of these subsets could be linked to the inflammatory process seen in AR and CRSwNP. Generally, the more activated and differentiated subsets were found to accumulate locally in patients, suggesting a role for them in upper airway disease.

- The human nasal epithelium was shown to have the ability to amplify an allergic response thereby contributing to the ongoing $CD4^+$ T cell-mediated inflammation. In addition, activated neutrophils in the nasal mucosa could prime T cells and enhance eosinophil migration. Since both T cells and eosinophils are central in the allergic response, the presented data underscore the role epithelial and neutrophil cells have in the fast response to allergens at the local site of exposure.

- Ligand stimulation of TLR9 and ALK1-7, *in vivo* or in a model of microbial infection, were found to reduce local inflammation, angiogenesis and cell-proliferation. Further, an increased expression of CD11b on activated neutrophils and a corresponding upregulated expression of ICAM-1 on epithelial cells from CRSwNP patients was detected, suggesting a mechanism for a neutrophil-epithelial interaction in nasal polyps. These results indicate that targeting of local innate receptors can reduce inflammation and slow down disease progression in CRSwNP.

SUMMARY AND FUTURE PERSPECTIVES

The traditional idea of the epithelium as a simple barrier and neutrophils as homogenous and terminally differentiated cells have lately been reconsidered. The present thesis details the role of ECs in antigen presentation and for the innate functions of TLRs and ALKs. In addition, it reports on a role for the activated neutrophil subset in nasal allergy and polyposis.

ECs by far exceed the number of professional APCs in the nose. The same is true for HLA-DR^+ cells. The ECs are in place to meet the antigen when it enters the nose, whereas it normally takes several days to recruit professional APCs like DCs to the nose.[108] Further, professional APCs activate T cells preferably in lymphnodes, not locally in the nasal mucosa. These facts emphasize the immunological value of the antigen presenting capacity of local ECs.

New information indicates that neutrophils can be divided into different subsets, each with diverse roles during inflammatory conditions. The present thesis advances this concept further by describing the existence of neutrophil subsets in the nose, that a shift towards more activated subsets is seen in upper airway inflammation and that these subsets seem to have diverse functions in upper airway diseases.

It was revealed that local epithelial and neutrophil cells in the nasal mucosa of patients with AR and CRSwNP, both characterized by a type 2-biased inflammation, exhibited an alerted receptor pattern with a deranged immunological response. Our data on T cell activation, cell migration and adhesion as well as cytokine release, clearly indicated a role for antigen presenting ECs, TLRs, ALKs and various neutrophil subsets in these upper airway inflammatory diseases.

Human experiments (Paper I) disclosed a high percentage of HLA-DR^+ ECs in the nose. It would therefore be of interest to compare the efficacy of these antigen presenting ECs to DCs, macrophages and B cells. An epithelial specific MHC class II knock out mouse would then be useful in order to further understand the role of aberrant class II expression and to explore whether MHC class II lacking selectively on ECs but normally expressed on immune cells would significantly affect the induction of inflammation. In a recent mouse model of inflammatory bowel disease, MHC class II deficient mice were fed *Helicobacter hepaticus* and IL-10 receptor-blocking antibodies. It was shown that transferred $CD4^+$ T cells were unable to induce and reinstate tolerance, demonstrating the need for aberrant MHC class II expression.[109] Thus, it seems to be important for the initial protection against pathogens to have a local rapid response with an induced capability to present antigen and to activate $CD4^+$ T helper cells.

Steroids are commonly used to suppress local inflammation in CRSwNP and AR. These drugs are generally highly un-specific on a molecular level, making them effective in various inflammatory conditions but at the same time, rendering multiple side-effects.[110] Oral steroids have more potent effects than topical steroids. Long-term usage is however not recommended due to the unwanted effects, resulting in recommendations on periodic treatment but with risk of disease recurrence.[111] A key point in the development of new treatment strategies is to find targets to hit, minimizing these side-effects. Monoclonal antibodies, which dampen

Th2 inflammation and IgE related activity, are presently tried out.[18] These biologicals are however costly which limits their use in large patient cohorts.

Stimulation of epithelial TLR and ALK receptors (Papers II and III) affected the inflammatory response as well as factors coupled to angiogenesis and cell proliferation in CRSwNP. It is tempting to speculate on a therapeutic role for the ligands of these receptors in the future treatment of CRSwNP and AR.

Immunotherapy is an effective way to induce allergen tolerance. It requires three to five-years of treatment with daily medications or repeated injections. The tolerance inducing mechanisms are still not fully understood, but areas of interest include Th1/Th2, IL-10 and IgG4. Rather unnoticed is that this therapy also effects neutrophils.[97, 98, 112, 113] Since data in the present thesis showed a role for the activated subset in allergy, it would be valuable to explore the effect of immunotherapy on the different subsets described in Paper IV.

Activated neutrophils might increase airway inflammation through priming of T cells and increasing eosinophil migration. It would be of interest to explore whether blocking the activation procedure of neutrophils, likely taking place in the local mucosa, could have further anti-inflammatory effects. Leukapheresis is another desirable method, however requiring systemic detection of the cell type of interest.[114] Perhaps $CD11b^{+}$ neutrophils could be the target to explore in CRSwNP or AR.

Paper V revealed a shift in neutrophil subsets in CRSwNP, favoring the more activated subset in the mucosa. An upregulation of CD11b and ICAM-1 on neutrophils and ECs respectively was also seen. Experiments evaluating the migrating capacity of $CD16^{high}$ $CD62L^{dim}$ neutrophils might reveal the role for activated neutrophils in the inflammatory drive behind polyp development. Steroid treatment did not affect the findings on either neutrophil subsets nor CD11b or ICAM-1 expression. Thus, possible treatments interfering with neutrophils and the epithelium on this level could open up new perspectives for future management of CRSwNP, especially for the group of patients with weak or absent response to steroid treatment.

The neutrophil subset $CD16^{dim}$ $CD62L^{dim}$ has previously been described, although not further specified.[45] Nor have experiments on potential immunological characteristics of this subset been published. We reported that this subset was increased in NAL of AR patients compared to nasal mucosa and blood, and that the same subset is increased in polyp mucosa from CRSwNP patients. Further experiments, purifying this subset for functional analysis, would answer which implications the presence of this subset could have.

The results of this thesis increase the understanding of upper airway inflammation on a molecular level, hence encouraging future studies of more specific treatment strategies for CRSwNP and AR.

POPULÄRVETENSKAPLIG SVENSK SAMMANFATTNING

Epitelceller i nässlemhinnan utgör en första barriär mot invaderande mikrober och antigener som virus, bakterier och pollen. På senare tid har man insett att detta ytskikt inte bara utgör en fysisk barriär, utan även besitter en immunologisk försvarsförmåga. Medfödda, så kallade innata, receptorer är av speciellt intresse i detta sammanhang. De har förmåga att känna igen och dra igång en immunologisk reaktion som svar på en invaderande mikrob. Denna avhandling har fokuserat på två receptorfamiljer av detta slag; toll-lika receptorer (TLRs) och activin receptor-lika kinaser (ALKs). Hur och i vilken utsträckning aktivering av dessa kan medverka i uppkomst av inflammatoriska tillstånd i näsan är ofullständigt känt. Avhandlingen har även utrett hur epitelceller kan agera som antigenpresenterande celler.

Neutrofiler, den mest frekvent förekommande vita blodkroppen hos människor, finns alltid i blod och vävnad hos såväl friska som sjuka personer. En ökning i antal ses regelmässigt i samband med en infektion men ingen större betydelse har tillmätts neutrofilen i samband med utvecklingen av allergi och näspolypos. Kanske beror detta på att man betraktat neutrofiler som en tillsynes homogen, enhetlig cellpopulation. På senare tid har emellertid flera studier visat att så inte är fallet. Neutrofiler kan delas in i olika subpopulationer med hänsyn till vilka receptorer som vid en viss tidpunkt uttrycks på cellens yta. Variationer i uttrycket av ytreceptorerna FcγRIII (CD16) och L-selectin (CD62L) antas återspegla variationer i neutrofilens immunologiska egenskaper.

Syftet med föreliggande avhandling är att kartlägga på vilket sätt TLR och ALK på epitelceller samt olika neutrofila subgrupper kan påverka uppkomst och utveckling av allergisk rinit och näspolypos. Avhandlingen utreder även hur epitelceller kan agera som antigenpresenterande celler.

Delarbete I-III kartlägger uttrycket av ytreceptorer kopplade till antigenpresentation, TLRs samt ALKs på näsepitelceller. Epitel från såväl människa som mus har receptorer nödvändiga för antigenpresentation. Epitelceller från en allergisk musmodell (så kallade sensitiserade möss) samt från allergiska patienter kan både ta upp och bearbeta antigen och presentera slutprodukten för T celler, som därvid aktiveras. Detta sker inte med friska epitelceller från mus eller människa. Epitelceller verkar således spela en viktig för den allergispecifika inflammationen i näsan. Humana näsepitelceller i närheten av den plats i näsan där polyper vanligen bildas har ett mycket lågt uttryck av TLR9 i jämförelse med motsvarande nässlemhinna hos friska, polypfria individer. Om epitelcellerna stimuleras med ett för receptorn specifikt protein, CpG, återkommer dock det tidigare saknade TLR9-uttrycket. Aktivering av TLR9 hämmar även VEGFR2, en receptor kopplad till lokal kärltillväxt, liksom andra inflammationsdrivande signalsubstanser, så kallade cytokiner. Sålunda förefaller aktivering av TLR9 gynnsamt om man vill förhindra tillväxt av näspolyper. Uttrycket av ALK1-6 är uppreglerat på polypepitelceller från patienter. Om man stimulerade dessa receptorer med deras specifika ligander, vilka ofta är färre i antal hos näspolypospatienter, minskar de faktorer som gynnar celldelning (Ki67) samt inflammation (ICAM-1 och IL-8). ICAM-1 minskningen är speciellt påtaglig om man stimulerar med en tilläggsligand som imiterade effekten av en

attackerande mikrob. Då infektioner är en vanlig orsak till polyptillväxt talar detta för ALK-inblandning i polypsjukdomen.

I delarbete IV och V beskrivs för första gången förekomsten av olika neutrofila subgrupper i näsans slemhinna. En av populationen kan betraktas som aktiverad och deras antal ökar påtagligt hos patienter med allergi och näspolypos. Vi kunde visa att aktiverade neutrofiler i sin tur kan aktivera T celler samt underlätta migration av eosinofiler, två viktiga immunologiska reaktioner av grundläggande betydelse för utveckling av allergiska reaktioner. Även hos patienter med näspolypos förefaller aktiverade neutrofiler vara av betydelse. Dessa uppvisar ett ökat uttryck av CD11b samtidigt som ytepitelet uppvisar fler ICAM-1-molekyler. Dessa två molekyler interagerar och underlättar cell-cell-kontakt och bidrar till att indirekt driva på den inflammatoriska reaktionen.

Sammanfattningsvis visar denna avhandling att receptoruttrycket på epitel och neutrofiler är förändrat vid sjukdomar som allergisk rinit och näspolypos. Detta leder i sin tur till ett förändrat immunologiskt svar och de resultat som presenteras i denna avhandling tyder på att detta kan bidra till dessa sjukdomars uppkomst och utveckling.

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