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NASAL EPITHELIAL CELLS: INNATE IMMUNITY AND INFLAMMATION

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Nasal Epithelial Cells: Innate Immunity and Inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Det hade varit bra att veta lite tidigare…

ABSTRACT

The surface epithelium that lines the nasal passages is often the first tissue in the airway to encounter inhaled pathogens. It collaborates closely with the innate immune system, a subsystem of the immune system that defends the host from infection by organisms, mainly by initiating a local inflammatory reaction. Pattern-recognition receptors (PRRs) are important in pathogen recognition, cell activation and regulation of immune responses and include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs). The transforming growth-factor beta (TGF-β) superfamily and their type I receptors, the activin receptor-like kinases (ALKs), are important mediators that promote remodelling and have recently also been shown to regulate airway inflammation. Even though PRRs and ALKs are essential in preventing disease, disruption of these systems is generally believed to be involved in the pathogenesis of airway inflammatory diseases such as asthma and chronic rhinosinusitis with nasal polyp (CRSwNP). Hence, the overall aim with this thesis was to investigate the role of PRRs and ALKs in airway inflammation.

Human airway smooth muscle cells (HASMCs) are essential for the regulation of airflow; importantly, they are also involved in the shortness of breath that characterises microbial-induced exacerbations of asthma. The present thesis showed that stimulation of TLR2, TLR3, TLR4, TLR7 and NOD1 on HASMCs resulted in cytokine release, upregulation of inflammatory cell surface markers and downregulation of receptors involved in smooth muscle cell contraction.

The nasal epithelium was found to express TLR3, TLR7, TLR9, RIG-I and MDA-5 and stimulation resulted in an increased inflammatory response characterised by the release of chemokines and cytokines. In addition, a specific role for TLR9 was found in patients with CRSwNP that might be linked to polyp growth via downregulation of VEGFR expression and lowered release of inflammatory cytokines.

Virus-related ligand stimulation of TLR7 induced a rapid release of the neuropeptide, substance P (SP), from human nasal epithelial cells (HNECs) and sensory neurons. The released SP promptly upregulated the epithelial TLR expression. This suggests a role for SP in rapid priming of the innate immune system during viral infections.

Polyp epithelial cells from patients with CRSwNP expressed high levels of ALK1-6. Polyp epithelial cells stimulated with ALK-ligands demonstrated a potential anti-inflammatory role for ALKs in polyps. Previous reports have demonstrated low levels of ALK-ligands in patients with CRSwNP, suggesting that ALKs could contribute to uncontrolled inflammation promoting the progression of CRSwNP. BMP4, an ALK-ligand, suppressed inflammation and hyperplasia in the turbinate tissue of patients with CRSwNP. This effect was absent in the corresponding polyp, suggesting that BMP4-ALK3 interaction might be involved in polyp growth in patients.

In summary, this thesis demonstrates a role for specific epithelial PRRs and ALKs in CRSwNP and for smooth muscle PRRs in asthma. In addition, it proposes a novel role for substance P in

kick starting the innate immune system by upregulating PRRs in response to microbial stimulation. These findings could generate new potential targets for the treatment of inflammatory airway diseases.

LIST OF SCIENTIFIC PAPERS

I. Anne Månsson Kvarnhammar, Lotta Tengroth, Mikael Adner and Lars Olaf Cardell
Innate immune receptors in human airway smooth muscle cells: activation by TLR1/2, TLR3, TLR4, TLR7 and NOD1 agonists
PLoS One, 2013, Jul 4;8(7):e68701

II. Lotta Tengroth, Camilla Rydberg Millrud, Anne Månsson Kvarnhammar, Susanna Kumlien Georén, Leith Latif and Lars Olaf Cardell
Functional effects of toll-like receptor (TLR)3, 7, 9, RIG-I and MDA-5 stimulation in nasal epithelial cells
PLoS One, 2014, Jun 2;9(6):e98239

III. Lotta Tengroth, Julia Arebro, Susanna Kumlien Georén, Ola Winqvist and Lars Olaf Cardell
Deprived TLR9 expression in apparently healthy nasal mucosa might trigger polyp-growth in chronic rhinosinusitis patients
PLoS One, 2014, Aug 18;9(8):e105618

IV. Olivia Larsson*, Lotta Tengroth*, Yuan Xu, Rolf Uddman, Susanna Kumlien Georén and Lars Olaf Cardell
*These authors contributed equally to this work.
Substance P represents a novel first-line defense mechanism in the nose
J Allergy Clin Immunol, 2017, Feb 20. pii: S0091-6749(17)30241-5.

V. Lotta Tengroth, Julia Arebro, Olivia Larsson, Claus Bachert, Susanna Kumlien Georén and Lars Olaf Cardell
Activation of activin receptor-like kinases (ALKs) curb mucosal inflammation and proliferation in chronic rhinosinusitis with nasal polyps
Manuscript

VI. Lotta Tengroth, Olivia Larsson, Julia Arebro, Claus Bachert, Susanna Kumlien Georén and Lars Olaf Cardell
Impaired effects of BMP4 release in CRSwNP; a potential mechanism for polyp development
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AHR	Airway hyperresponsiveness
ALK	Activin receptor-like kinase
BMP	Bone morphogenetic protein
CRS	Chronic rhinosinusitis
CRSwNP	Chronic rhinosinusitis with nasal polyps
CRSsNP	Chronic rhinosinusitis without nasal polyps
DAMP	Danger-associated molecular pattern
ER	Endoplasmic reticulum
EMT	Epithelial–mesenchymal transition
FESS	Functional endoscopic sinus surgery
HASMC	Human airway smooth muscle cell
HNEC	Human nasal epithelial cell
IFN	Type I interferon
MDA-5	Melanoma differentiation-associated gene-5
MFI	Medium fluorescence intensity
MNEC	Murine nasal epithelial cell
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerisation domain
PAMP	Pathogen-associated molecular pattern
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene-1
RLR	RIG-I-like receptor
SP	Substance P
TGF-β	Transforming growth-factor-beta
TGN	Trigeminal sensory neuron
Th2	T helper cell type 2
TLR	Toll-like receptor

1. BACKGROUND

1.1 CHRONIC RHINOSINUSITIS

Chronic rhinosinusitis (CRS) is a disabling disease that affects more than 10% of the European population[1]. CRS was initially seen as one homogenous disease, but recently several phenotypes and endotypes have been established in a step towards developing personalised medicine for these patients[2, 3]. CRS is clinically associated with nasal obstruction, nasal discharge, facial pain or pressure and/or reduction/loss of smell persisting for longer than 12 weeks[1]. The cause of CRS is not entirely known but defects in the sinonasal epithelial barrier, malfunctioning mucociliary clearance and tissue remodelling are all processes that contribute to the chronic inflammatory condition, characteristic of CRS[1]. CRS can be divided in chronic rhinosinusitis with and without nasal polyps (CRSwNP and CRSsNP)[1]. CRS, allergic rhinitis and asthma are often co-morbid and share the same trend of increasing prevalence[4]. Recent studies also demonstrate that innate immune responses are involved in the pathogenesis[5].

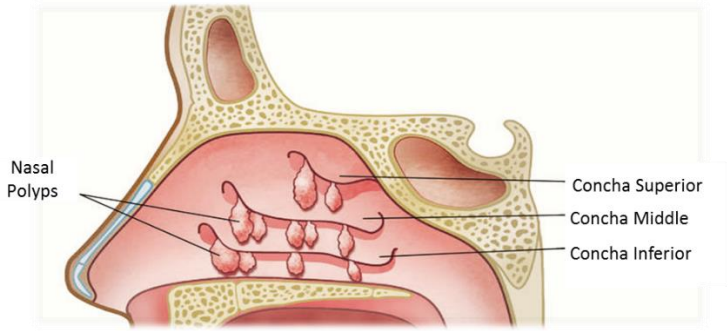

Figure 1. Overview of polyps in the nose. From Medical Dictionary, © 2009 Farlex and Partners.

1.1.1 Chronic rhinosinusitis with nasal polyps

CRSwNP was reported to have a prevalence of 2.7% in the total Swedish population[6]. CRSwNP is regarded as a type 2 (Th2) inflammatory disease in Europe[1] that is driven by cytokines like IL-4, IL-5 and IL-13, and enhanced infiltration of eosinophils, basophils and mast cells[7, 8]. In Asian patients, CRSwNP is instead characterised by a non-eosinophilic inflammation and a mixed T cell immune response[9, 10]. Nasal polyps are characterised by eosinophils, oedema formation, lack of collagen in the extracellular matrix and pseudocysts consisting of albumin accumulation[11-13]. Nasal polyps arise from the mucosa of the nasal sinuses (commonly at the outflow tract of one or more of the sinuses) or from the mucosa of the nasal cavity (Figure 1). The origin and progression of nasal polyposis is still unknown. One hypothesis is that an inaccurate or excessive immune response to foreign agents results in extended mucosal inflammation and an increased cellular infiltration[14, 15]. Virus, bacteria, fungi and allergens are the agents most associated with the pathogenesis of CRSwNP and could be one explanation for the progression of polyposis seen in these patients[1].

1.1.2 Chronic rhinosinusitis without nasal polyps

Chronic rhinosinusitis without nasal polyps (CRSsNP) is, in contrast to CRSwNP, characterised by increased neutrophilic inflammation and fibrosis formation within the extracellular matrix of the nasal mucosa[16]. A thickening of collagen fibres can be detected, but pseudocysts are not present[12].

1.2 ASTHMA

Asthma is a chronic inflammatory airway disease affecting more than 300 million people worldwide, with prevalence still rising[17]. The pathophysiological manifestations of asthma are reversible airflow obstruction, airway inflammation and acute airway hyperresponsiveness (AHR)[18]. Airflow obstruction is a consequence of airway smooth muscle cell contraction, airway wall thickening, airway oedema or a combination of all of these factors. Asthma may be either allergic or non-allergic, depending on the inflammatory cascade. Allergic asthma is often eosinophilic, whereas non-allergic asthma, such as aspirin-, exercise- and infection-induced asthma, is often neutrophilic. Non-allergic asthma is often severe and associated with steroid-resistance[19].

1.3 SMOOTH MUSCLE CELLS

Human airway smooth muscle cells (HASMCs) line the lower airways and are involved in AHR, remodelling and inflammation in asthma[20]. Contraction of HASMCs narrows the airway. Phenotypically, HASMCs can be either contractile or proliferative; the latter phenotype induce airway thickening and release multiple cytokines and chemokines, like IL-6, IL-8 and eotaxin, initiating airway inflammation[20]. HASMCs can respond to various stimuli, including inflammatory mediators, neurotransmitters or exogenous substances and constrict or relax depending on the stimulus[21].

1.4 EPITHELIAL CELLS

The airway epithelium constitutes a first line of defence against pathogens and consists of the basement membrane, basal cells, goblet mucous cells, epithelium with pseudostratified columnar epithelial cells and the mucous layer[22]. Epithelial cells are important as a physical barrier, as well as in the production of cytokines and chemokines and the control of innate and acquired immune responses[22, 23]. The epithelium also retains and activates innate lymphoid cells (ILCs), which is important for the defence against viruses[24]. Epithelial cells have protective functions but are also involved in the pathogenesis of various inflammatory airway diseases. Decreased tight junctions with increased permeability, impaired mucociliary clearance and a reduced production of antimicrobial peptides are all epithelial-related deficiencies described in CRSwNP and allergic rhinitis[25-27]. In addition, epithelial cells

demonstrate impaired or dysregulated function of innate immune receptors such as pattern-recognition receptors (PRRs), contributing to inflammatory airway diseases[28].

1.5 PATTERN-RECOGNITION RECEPTORS

PRRs recognise conserved molecular motifs of microbial origin termed pathogen-associated molecular patterns (PAMPs) or endogenous molecules produced by injured or dying cells called danger-associated molecular patterns (DAMPs)[29, 30]. PRRs consists of three receptor families: Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Figure 2).

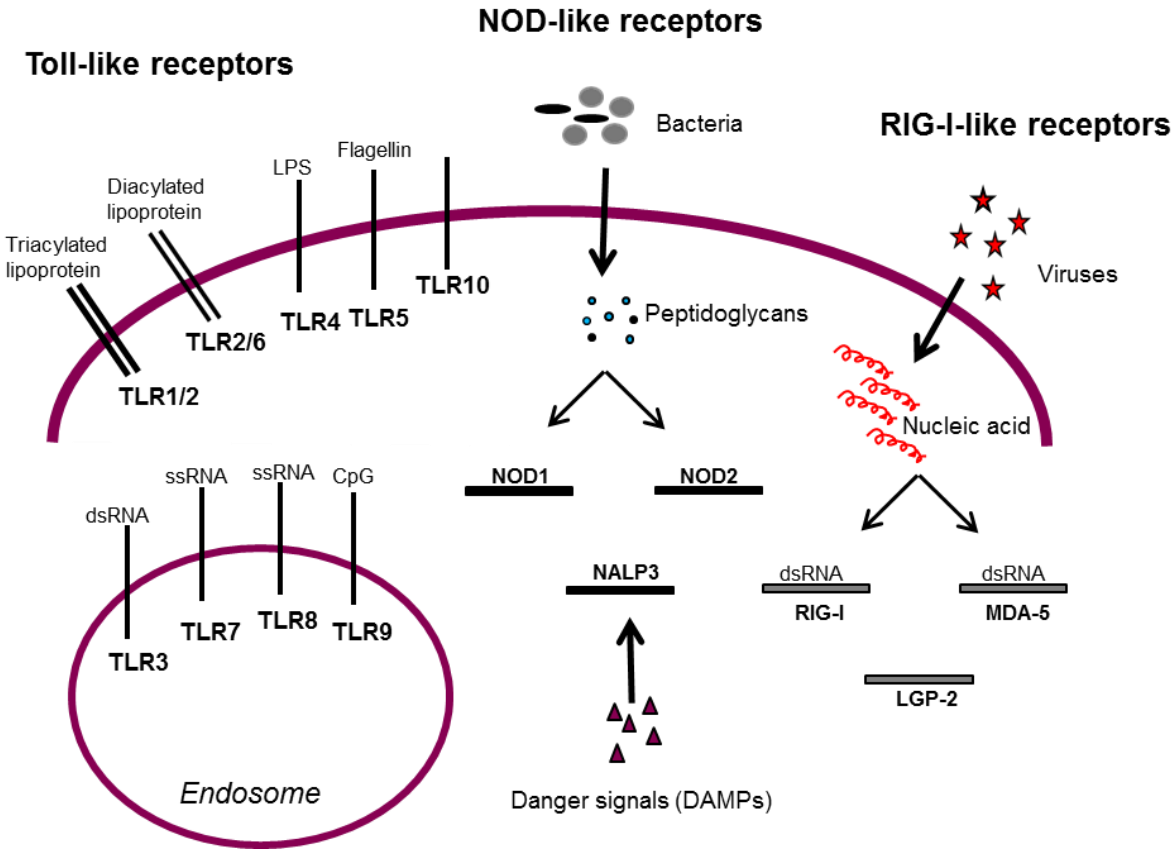

Figure 2. Schematic picture of TLRs, NLRs and RLRs and their respective ligands.

1.5.1 Toll-like receptors

Ten different TLRs have been discovered in humans and include both intracellular and extracellular receptors[31] (Figure 2). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are mainly expressed on the cell surface and recognise extracellular microorganisms like viruses, bacteria and fungi. The ligand for TLR10 is currently not known but TLR10 is thought to heterodimerise with TLR2[32]. TLR3, TLR7, TLR8 and TLR9 are intracellular and recognise microorganisms and ligands that have already entered the cell. TLR11-TLR13 have been shown in mice, these TLRs recognise parasites, but are still believed to be absent in humans[33, 34]. TLRs are involved in the protection against pathogens, but also contribute to pathogenesis of airway diseases[35].

1.5.2 TLR3, TLR7, TLR8 and TLR9

In resting cells, TLR3, TLR7, TLR8 and TLR9 are synthesised and stored in the endoplasmic reticulum (ER). From there, the intracellular TLRs are folded and translocated to cytosolic endosomes via numerous chaperone proteins, one of which is named UNC-93B[36, 37]. TLR3, TLR7/TLR8 and TLR9 recognise different molecular motifs (e.g., TLR3: double-stranded RNA (dsRNA); TLR7/8: single-stranded RNA (ssRNA) and TLR9: unmethylated CpG motifs in viral and bacterial DNA), common to many respiratory viruses[38, 39]. In addition, TLR7 can recognise miRNA and anti-viral compounds of the imidazoquinolines family[40, 41]. TLR3, TLR7 and TLR8 sense viral RNA from, for example rhinoviruses, influenza viruses and respiratory syncytial viruses (RSV), which are all common upper respiratory viruses, and induce a robust immune response[42-45]. Upon recognition, TLR7, TLR8 and TLR9 use the MyD88-dependent pathway to initiate signalling. TLR3 instead signals via the TRIF-dependent pathway to induce the production of both pro-inflammatory cytokines and interferons[46].

1.5.3 TLR4

Resting respiratory epithelial cells express intracellular TLR4, located in pools in the Golgi complex[47]. Upon cell activation, TLR4 is rapidly transferred to the cell surface for pathogen recognition[48]. TLR4 in complex with myeloid differentiation factor 2 (MD-2) recognise LPS from gram-negative bacteria[49]. The response to LPS is also dependent on CD14[50]. TLR4 signalling uses a MyD88-dependent and MyD88-independent pathway. The MyD88-dependent pathway activates NFκB and mainly takes place at the plasma membrane[51], whereas the MyD88-indenpendent pathway signals through TRIF, leading to interferon regulatory factor-3 (IRF3) activation in early endosomes[52].

1.5.4 Nod-like receptors

To date, 22 Nod-like receptors in humans have been characterised. The NLR family has been divided in four subgroups based on the variation in their N-terminal domain: acidic domain containing (NLRA), BIR domain containing (NLRB), CARD domain containing (NLRC) and pyrin domain containing (NLRP)[53]. It has been shown that the NLRC members, nucleotide-binding oligomerisation domain 1 (NOD1) and NOD2, are important bacterial sensors that recognise muropeptides, peptides released from the peptidoglycan layer of the bacterial cell membrane that stimulate innate immunity[54, 55]. Other NLRs like NLRP1, NLRP3, NLRP6 and NAIP form inflammasomes that activate inflammatory processes[56]. All NLRs have been associated with human airway diseases[56, 57].

1.5.5 Rig-I-like receptors

The RLR family includes three receptors located in the cytoplasm: retinoic acid-inducible gene-1 (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP-2). RIG-I is activated by dsRNA or 5′-triphosphate moiety from viral RNA, whereas MDA-5 is activated only by dsRNA[58]. This viral activation results in production of cytokines such as type I interferons (IFNs)[59]. LGP-2 remains poorly described but is believed to regulate RIG-I and MDA-5 signalling[60].

1.6 NEURONS AND NEUROPEPTIDES

The upper and lower airways are innervated by sensory nerves[61, 62]. Sensory nerves release neuropeptides including tachykinins, such as substance P (SP), neurokinin A (NKA) and neurokinin B (NKB)[63]. Tachykinins bind and act through their NK receptors[64]. The release of SP induces vasodilation, increased vascular permeability and leucocyte recruitment, phenomena that are collectively referred to as neurogenic inflammation[65, 66]. Sensory nerve fibres are hypersensitive in airway inflammatory diseases and play a role in airway disease pathology[67-70]. Elevated levels of SP have been reported during viral infection, stimulating both eosinophil accumulation in the nasal mucosa as well as bronchoconstriction in the lower airways[71, 72]. SP has also been shown to be upregulated during exacerbations in asthmatic patients[73].

1.7 TISSUE REMODELLING

Chronic inflammation in tissues is often accompanied by structural changes, referred to as remodelling[74]. Remodelling is thought to originate from persistent inflammation and aberrant repair mechanisms. The remodelling pattern differs in all airway diseases. In CRSwNP, remodelling is characterised by oedematous stroma with albumin deposition, pseudocyst formation, hyperplasia and subepithelial and perivascular infiltration of inflammatory cells[12, 75, 76]. Plasma proteins are enriched due to vascular leakage and transportation through the dysfunctional epithelial layer, enabling an increased oedema formation[77]. In CRSsNP, remodelling is characterised by fibrosis, basement membrane thickening and goblet cell hyperplasia.

1.8 TRANSFORMING GROWTH-FACTOR BETA SUPERFAMILY

The transforming growth factor beta (TGF-β) superfamily consists of 33 members, which can be divided in subgroups that include the TGF-βs, activins/inhibins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs)[78]. The TGF-β ligands bind to type I transmembrane serine/threonine kinase receptors, also named activin receptor-like kinases (ALKs), and type II transmembrane serine/threonine kinase receptors[78]. Upon binding, activation of downstream signalling mediators occurs by phosphorylation of multiple Smads. Smads act as signal integrators and translocate to the nucleus to interact with other signalling

pathways that regulate gene transcription[79, 80]. Additionally, TGF-β can activate a Smad-independent pathway via activation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun-N-terminal kinase (JNK)[80]. A role for the TGF-β superfamily in airway inflammation has been demonstrated[81, 82].

TGF-β is an important mediator promoting remodelling and fibrosis and is elevated in CRSsNP[83]. However, in CRSwNP the reported levels of TGF-β have been contradictory. Some studies demonstrate low levels of TGF-β in CRSwNP compared to control tissue[83, 84], while others demonstrate elevated levels of TGF-β[85, 86]. Activin A, another important member of the TGF-β superfamily, shows a similar release pattern to TGF-β, with low levels in CRSwNP and high levels in CRSsNP[84].

1.8.1 Bone morphogenetic protein

BMPs, a group of growth factors, regulate tissue architecture throughout the body. BMPs, like other members of the TGF-β superfamily, binds to type I and II transmembrane serine/threonine kinase receptors, the former receptor also termed ALKs. Unlike other TGF-β members, BMPs are able to bind and signal via ALKs in the absence of type II receptors[87]. Activated BMP receptors phosphorylate Smads, which regulate gene transcription[80]. BMP4 binds primarily to ALK3 and ALK6 on airway epithelial cells where it inhibits proliferation and epithelial–mesenchymal transitions (EMT)[88]. BMP4 release is upregulated in murine ovalbumin-induced lung inflammation[89] and has anti-inflammatory properties, inhibiting epithelial pro-inflammatory cytokines[90]. Mutations in the genes encoding BMP4 cause juvenile polyposis, polyp formation and growth in the colon[91].

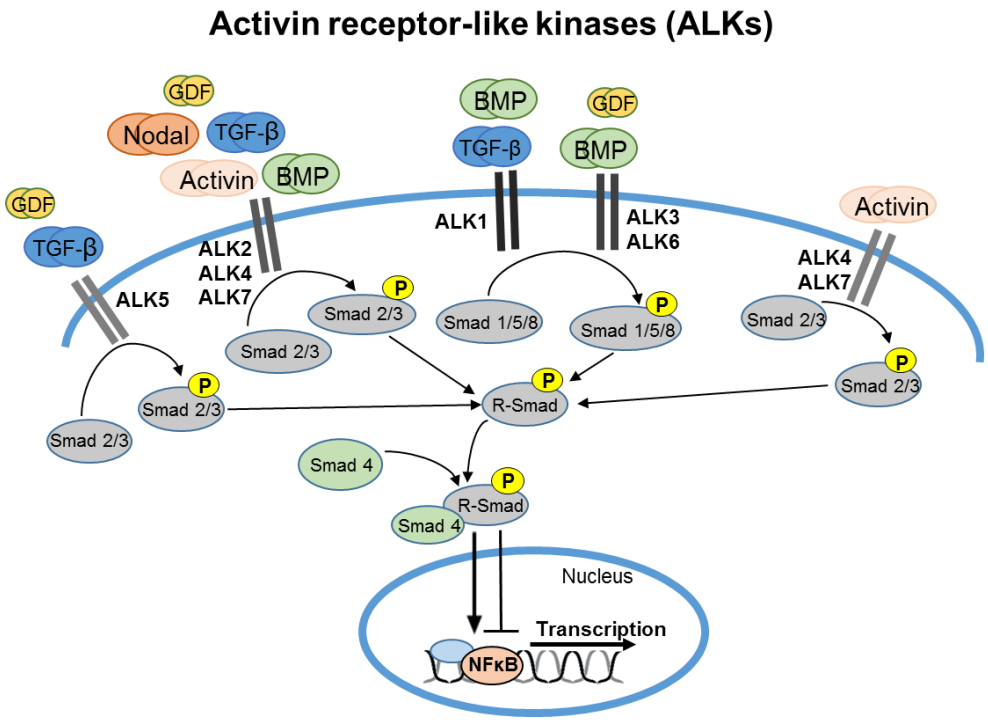

Figure 3. Schematic representation of the TGF-β-superfamily pathway in epithelial cells, including ligands, ALKs and Smads. Formation of the ligand-receptor complex leads to phosphorylation and activation of Smad. This subsequently activates or blocks NFκB and gene transcription. P indicates phosphorylation.

1.9 ACTIVIN RECEPTOR-LIKE KINASES

To date, seven type I transmembrane serine/threonine kinase receptors have been identified in mammals, termed activin receptor-like kinases 1- 7. ALK 1-7 are utilised by numerous ligands in the TGF-β superfamily[92] (Figure 3). The ALKs form a hetero-tetrameric complex pairing with type II receptors resulting in downstream signalling[93]. Ligands have the ability to bind multiple ALKs, but the affinities between different ALKs vary. ALK1 (ACVL1) acts as a main receptor for TGF-βs and BMPs[94]. ALK2 (ACVR1) is activated by various TGF-βs, activins and BMPs[95]. ALK3 (BMPRIA) binds BMP2 and BMP4 with high affinity[80]. ALK4 (ACVR1B) is activated by activins, binding Activin A with high affinity[96]. ALK5 (TGFBR1) is characterised as the primary receptor for TGFβ1-3[97]. ALK6 (BMPRIB) binds BMP2 and BMP4[98]. Little is known about ALK7 (ACVR1C), but it is known to bind Activin B with high affinity[99] (Figure 3). Activation of ALKs has both pro-inflammatory and anti-inflammatory effects, and has additionally been shown to be profibrogenic in human airways[100].

1.10 INFLAMMATORY CYTOKINES AND RECEPTORS

Studies have demonstrated that pro-inflammatory cytokines, such as IL-6, IL-8, CSF and MIP-1β, can promote survival and/or proliferation, activation and differentiation of multiple cells. In addition, they play a crucial role in microbial-induced exacerbations of inflammatory airway diseases[101]. IL-6 induces B-cell antibody production and T-cell activation and differentiation[102]. The major role for IL-8 is to recruit and activate neutrophils[103]. CSF stimulates eosinophil and neutrophil infiltration[104, 105], whereas MIP-1β attracts lymphocytes, eosinophils, neutrophils and monocytes[106]. IFN-β has antiviral properties, upregulating TLR expression and inducing protection against subsequent viral infections by inhibiting viral replication[107, 108].

In addition to pro-inflammatory cytokines, multiple receptors also have pro-inflammatory functions, as they are involved in microbial intrusion and increase inflammation. ICAM1 is the main receptor for rhinoviruses[109]. HLA-DR on epithelial cells from allergic patients drives the inflammatory process by increasing eosinophilic inflammation and activation of other inflammatory cells[110], while VEGFR participates in angiogenesis by enhancing proliferation, migration and vascular permeability[111].

2. AIMS OF THE THESIS

The overall aim of this thesis is to investigate the interaction between the epithelium, airway inflammation and innate immunity in upper airway diseases, including infectious rhinitis and chronic rhinosinusitis with nasal polyps (CRSwNP).

The specific aims of the thesis are to:

- Characterise pattern-recognition receptors (PRRs) on human airway smooth muscle cells.
- Examine the expression and function of viral recognising PRRs in human nasal epithelial cells.
- Analyse the role of Toll-like receptor 9 (TLR9) on polyp and turbinate tissues from patients with CRSwNP.
- Reevaluate the role of neuropeptides in airway disease by investigating links between substance P (SP) release and epithelial TLR activity.
- Describe the expression and function of activin receptor-like kinases (ALKs) in the polyp epithelium of patients with CRSwNP, focusing on proliferation and local mucosal inflammation.
- Investigate the function of bone morphogenetic protein 4 (BMP4) in the epithelium of patients with CRSwNP by exploring its effects on angiogenesis, proliferation and inflammation.

3. MATERIALS AND METHODS

This section contains a brief overview of the materials and methods used in the thesis. For more detailed descriptions, the reader is referred to the individual articles, paper I-VI.

3.1 SUBJECTS AND STUDY DESIGN

The local ethics committee approved all studies. All participants gave their written informed consent. All procedures were conducted according to the principles expressed in the Declaration of Helsinki.

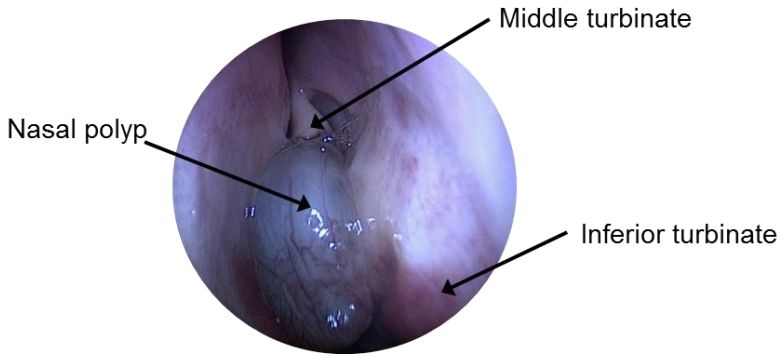

Figure 4. Picture visualising a nasal polyp, the middle turbinate and the inferior turbinate in a patient with CRSwNP.

Patients with chronic rhinosinusitis with nasal polyp (CRSwNP) were defined by historic and endoscopic criteria and computed tomography (CT) changes[1]. Biopsies were taken during functional endoscopic sinus surgery (FESS) or local anaesthesia and collected from polyps and turbinate tissue. Turbinate tissue was defined as the area where the mucosa had a nonpolypoid appearance and bordered the polyp or the tissue showing polypoidal changes. The location of the turbinate tissue was close to the middle nasal meatus, from the middle turbinate or the inferior turbinate (Figure 4). Biopsies from healthy controls were taken from the inferior turbinate after topical application of local anaesthesia or obtained in conjunction with nasal surgery (resection of the lower turbinate).

In all patients, steroids were withheld for at least 4-6 weeks (topically) and 8-12 weeks (systemically) prior to participation. Patients on daily inhaled steroid medication were excluded from the study. Steroids have anti-inflammatory, immunosuppressive and anti-proliferative effects and have been demonstrated to increase innate immune receptors[112]. A "washout" period minimises the appearance of steroid-related artefacts. In paper III, patients with more than four episodes of FESS were excluded from the study. Further inclusion criteria are specified in paper I-VI.

3.2 NASAL ADMINISTRATION OF CPG

In paper III, the *in vivo* effects of CpG were studied in patients with CRSwNP. These patients were randomised to receive physiological saline solution with or without CpG, applied by intranasal spray to both nostrils. Biopsies from polyps and turbinate tissue were taken 24 h after the CpG/placebo administration, during FESS.

3.3 TISSUE CULTURE

Biopsies from polyps or turbinate tissue were taken from patients with CRSwNP. Biopsies from healthy controls were taken from the inferior turbinate. Biopsies were analysed immediately with flow cytometry or used for stimulation experiments. The tissue pieces used for stimulation were separated into equally sized pieces, stimulated and incubated on 24-well culture plates in DMEM/F-12 supplemented with foetal bovine serum (FBS), penicillin, streptomycin and amphotericin B.

3.4 ISOLATION OF HUMAN CELLS

Primary cells are a biologically relevant tool when studying human biology and lack the genetic changes that allow indefinite cultivation *in vitro*. To study primary human nasal epithelial cells (HNECs), nasal brushings of polyps and turbinate tissue were performed on patients with CRSwNP, as well as of turbinate tissue on healthy controls. Nasal brushing is a gentle and efficient way to collect epithelial cells without harming the cells or the patient. Epithelial cells derived from nasal brushings were either analysed immediately with flow cytometry or cultured for *in vitro* experiments. Flow cytometry was performed to verify protein expression on cultured cells compared to fresh cells.

3.5 CELL CULTURE

3.5.1 Culture of human cells

Human pharyngeal epithelial cell lines Detroit-562 (CCL-138) and FaDu (HTB-43) were obtained from ATCC. Detroit-562 and FaDu were cultured in MEM medium with Earl's salts and L-glutamine, FBS, penicillin and streptomycin. The medium for Detroit-562 also contained sodium pyruvate, non-essential amino acids, gentamicin and amphotericin B.

To improve our studies on cell lines, we developed cultures of primary human nasal epithelial cells (HNECs). To study multiple functions of these cells, HNECs were cultured in collagen-coated flasks in keratinocyte serum-free medium (KSFM) supplemented with bovine pituitary extract, epidermal growth factor, penicillin, streptomycin and amphotericin B (complete KSFM), specific for epithelial cell growth. In all experiments, cells from passages 2–7 were

used, and cells were all positive for EpCAM. EpCAM is an epithelial specific marker[113], enabling separation of epithelial cells from other cells in the nose.

To study airway smooth muscle cells, tracheal and bronchial human airway smooth muscle cells (HASMCs) from non-asthmatic, healthy subjects were obtained from Promocell or Lonza in passage 2. HASMCs were cultured in smooth muscle cell growth medium (SMCGM) supplemented with FBS, epidermal growth factor, basic fibroblast growth factor, insulin, penicillin, streptomycin and amphotericin B. Cells were cultured up to passage 7.

All cells were cultured at 37°C in a humidified 5% CO_2 air atmosphere. Cells were passaged and plated on multiwell culture plates at a density of 100,000- 250,000 cells/ml medium and incubated to reach confluence. For HASMC culture, cells were growth-arrested in serum-free medium for 24 h, and then incubated in SMCGM containing 2% FBS during cell stimulation.

3.5.2 Isolation and culture of murine trigeminal sensory neurons

To study the specific function of ganglia, murine trigeminal ganglia were quickly dissected from six-to-eight-week female C57BL/6N mice and digested. Following mechanical dissociation, cells were separated from debris and enriched for neurons by two-step centrifugation. Cells were seeded at a concentration of 500,000 cells/well, onto 24-well cell culture plates or on glass coverslips. Cells were cultured in F12 medium, supplemented with FBS, penicillin, streptomycin, Amphotericin B and recombinant β-Nerve Growth Factor.

3.5.3 Isolation and culture of murine nasal epithelial cells

To compare the findings in human nasal epithelial cells with murine trigeminal ganglia (TGN), murine nasal epithelial cells (MNECs) were used to compare epithelial cell function over the species barrier. C57BL/6N mice were sacrificed and the septal mucosa was dissected. Nasal septa were pooled and transferred to MEM supplemented with Pronase and DNase for 1 h, after which DMEM/F12 containing FBS was added to stop the reaction. Tissue was passed through a cell strainer twice and resuspended in DMEM/F12 with penicillin, streptomycin, amphotericin B, FBS, epidermal growth factor and ITS liquid media supplement. The suspension was incubated on Primaria culture dishes to eliminate non-epithelial cells. Remaining cells were seeded onto collagen-coated 24-well plates at 250,000 cells/ml.

3.6 IMMUNOSTAINING AND MICROSCOPY

Immunohistochemistry can be used for the detection and localisation of multiple proteins in tissues. Immunohistochemistry can also be used for quantification of protein expression in tissues.

3.6.1 Immunohistochemistry of biopsies

Biopsies were fixed, embedded in paraffin and subsequently cut into 5μm sections on a microtome. Following deparaffinisation, rehydration and heat-mediated antigen retrieval, sections were permeabilised and blocked for non-specific binding. Biopsies from patients with CRSwNP and controls were incubated in a Sudan black B solution to minimise autofluorescence. The sections were incubated at room temperature (RT) (1 h) or at 4°C (overnight) with antibodies targeting ALK2, 3, 5, 7, TLR2, 3, 4, 7, 9, NOD1, RIG-I, MDA-5, EpCAM, substance P or β III tubulin and subsequently incubated with secondary antibodies.

To visualise or analyse expression of a single protein in tissue, DAB staining was performed. To analyse multiple proteins or specify location or movement in a cell, immunofluorescence staining was performed.

In paper I-II, the labelled streptavidin biotin ($LSAB^+$) System-horseradish peroxidase (HRP) or the Dako Cytomation Envision$^+$ System HRP kit was used. Sections were incubated with HRP-labelled polymer followed by 3,3'-diaminobenzidine (DAB) substrate-chromogen and then rehydrated. In paper V, sections were incubated with Avidin-Biotin-Complex followed by 3,3'-DAB. To visualise nuclei, counterstaining with Mayer's haematoxylin was performed. The sections were examined using light microscopy. Image analysis was carried out using ImageJ. Images were initially deconvoluted to separate the DAB and haematoxylin channels; subsequently the total DAB stained epithelial area was measured.

In paper IV-VI, all sections were incubated with a fluorescent-labelled secondary antibody. Sections were mounted in ProLong Diamond Antifade Mountant with DAPI. Negative control sections were stained with secondary antibody only. Imaging was performed on a Zeiss LSM800 confocal microscope or on an Olympus Provis microscope, connected to an Olympus U-PS camera.

3.6.2 Immunocytochemistry of cultured cells

In paper I-II, cells were seeded (50,000-300,000 cells/chamber) in 4-well chamber slides, cultured, fixed, permeabilised and treated with hydrogen peroxidase. The cells were incubated at RT for 1 h with antibodies targeting TLR2, 3, 4, 7, 9, NOD1, RIG-I and MDA-5. Cells were later treated with HRP-labelled polymer, followed by 3,3′-DAB substrate-chromogen. To visualise nuclei, counterstaining with Mayer's haematoxylin was performed. The sections were examined using light microscopy.
In paper IV, cultured cells were stimulated and subsequently fixed, permeabilised and blocked for non-specific binding. Cells were incubated with antibodies against TLR4, 7 and Neurofilament H followed by fluorescent-labelled secondary antibodies. Coverslips were mounted onto slides with ProLong Diamond Antifade Mountant with DAPI. All imaging was performed on a Zeiss LSM800 confocal microscope or Olympus Provis microscope, connected to an Olympus U-PS camera.

3.7 FLOW CYTOMETRY

Flow cytometry can measure multiple physical and chemical properties on individual cells based on how they scatter light from laser beams. Multiple filters then enable the detection of information about the cell, regarding cells size (Forward scatter, FSC), granularity (Side scatter, SSC) and the intensity of fluorochrome-conjugated antibodies. The number of positively labelled cells and the medium fluorescence intensity (MFI) can be calculated. By gating cells on EpCAM on the X-axes, epithelial cells can be distinguished (Figure 5A). Doublets can be excluded by gating on Forward-Height (FSC-H) and Forward-Area (FSC-A) (Figure 5B). The Vybrant Apoptosis Assay Kit was used to assess the percentage of viable cells (Figure 5B).

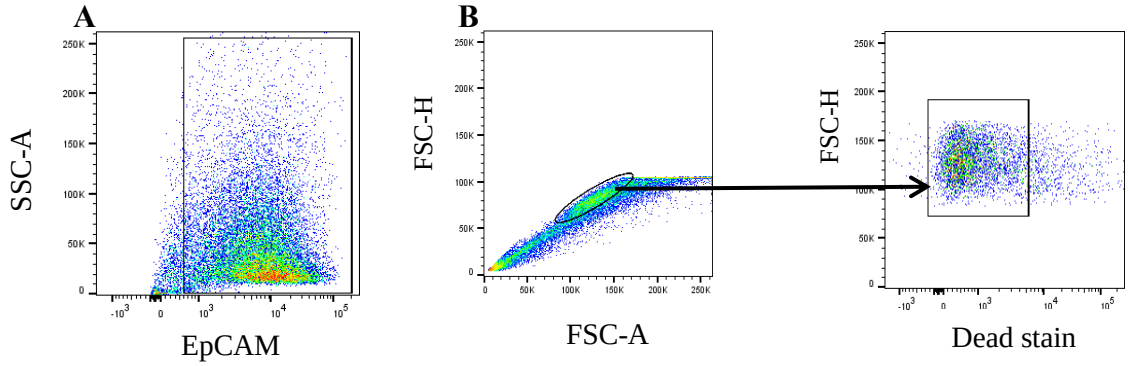

Figure 5. Epithelial cells gated based on expression of EpCAM (**A**).
Single cells gated based on Forward Height (FSC-H) and Forward Area (FSC-A) and viability (**B**).

Biopsies used for flow cytometry were first placed through a 100-µm cell strainer, into DMEM/F-12 containing FBS and incubated for 5 min. The cells were washed and centrifuged, after which the supernatant was aspirated and discarded. All cells were gated based on forward and side scatter and events in the range of 10,000–50,000 were collected.

In papers II-VI, the epithelial-specific marker EpCAM was used to identify epithelial cells. In papers I-IV, cells were analysed for their expression of TLR1-9, NOD1, RIG1, MDA-5, CysLT1R, β_2-AR, ICAM1, HLA-DR, VEGFR2 and NK1R. The IntraPrep Permeabilisation Reagent kit was used to detect intracellular proteins. Isotype controls relevant for each antibody were used for the detection of background staining. In papers V-VI, cells were analysed for their expression of ALK1-7, ICAM1, VEGFR, BMI-1, HLA-DR, and Ki67. A transcription factor buffer set was used to detect the intracytoplasmic and intranuclear proteins. For analysis of Smad phosphorylation, cells were incubated with warm Phosflow, washed and subsequently incubated with cold Phosflow Perm Buff III to minimise auto-phosphorylation.

For all antibody stainings, cells were incubated with antibody or isotype control for 15-20 min at RT, thereafter washed and fixed in formaldehyde.

In paper I-II, cells were analysed on a Coulter Epics XL flow cytometer (Beckman Coulter). Data was analysed with Expo32 ADC (Beckman Coulter). In paper III-VI, cells were

analysed on an LRSFortessa analyser (BD). Data was analysed with FlowJo Analysis Software (©Tree Star).

3.8 CELL PROLIFERATION

To examine proliferation and viability of smooth muscle cells, alamarBlue® Cell Viability Reagent was used. The number of cells was proportional to the colour change. AlamarBlue reagent was added to plates containing HASMCs. After 2 h of incubation, the absorbance was measured on a spectrophotometer. The proliferation was depicted as percent difference in reduction between control and treated cells according to the equation in paper I.

3.9 ELISA

ELISA is a specific method for detection and quantification of proteins present in, for example, cell culture supernatant. In a sandwich ELISA, a capture antibody detecting the antigen of interest is coated onto a microplate. Standard and samples are added and the specific antigen binds to the capture antibody. Thereafter, an antigen specific enzyme-linked antibody is added for detection. Lastly, a substrate initiate's colour development proportional to the amount of antigen in the sample. In paper I-II and V-VI, ELISA kits from R&D Systems were used to determine levels of IL-6, IL-8, GM-CSF, TGF-β1, eotaxin, RANTES and BMP4. In paper I, ELISA kits from PBL Interferon Source were used to measure IFN-β and ELISA kit from PBL Biomedical Laboratories was used to detect IFN-α. In paper IV, substance P was measured using an EIA Kit allowing detection of human and mouse substance P. All samples were analysed in duplicates to control the stability of the method.

3.10 MULTIPLEX CYTOKINE MEASUREMENT

Multiplex cytokine measurement is a method that quantifies a large number of proteins or peptides simultaneously in one sample of cell culture supernatants. The assay principle is similar to a sandwich ELISA, but the antibodies directed against an antigen are covalently coupled to magnetic beads dyed with fluorescent dyes. As the Multiplex assays can measure multiple proteins at the same time, less sample is required. In addition, multiplex assays have a broader range and a lower minimal detection limit of all measured proteins as compared to an ELISA assay. In paper III, a Human Cytokine Standard 17-plex (Bio-Rad Laboratories) was used and quantified on the Luminex200 system.

3.11 RNA EXTRACTION AND REAL-TIME PCR

Real-time PCR enables quantification of gene expression. Investigating mRNA in combination with protein levels provides a total picture of the receptor expression. The

procedure starts with extraction of total RNA followed by reverse transcription of RNA into complementary DNA (cDNA). Cyclic heating and cooling denatures the double stranded cDNA, where attachment of DNA probes binds and enables annealing/extension of new DNA strands. During amplification, light is emitted and a threshold cycle (Ct) value is determined.

To investigate the gene expression of various receptors, biopsies and cells were lysed and RNA was extracted using an RNeasy Mini Kit. The quality and quantity of the obtained RNA was determined by spectrophotometry using the wavelength absorption ratio (260/280 nm).

Reverse transcription of total RNA into cDNA was performed using the Omniscript reverse transcriptase kit (Qiagen) with oligo(dT)16 primer in a Mastercycler personal PCR machine. The RNA samples were denatured (65° for 5 min), chilled (4° for 5 min) and amplified (37°C for 1 h) in a final volume of 20 µl using a Mastercycler PCR machine (Eppendorf, Hamburg, Germany).

Real-time reverse transcription PCR was performed using Stratagene Brilliant QPCR with FAM™ labelled probes for TLR3, 7, 9, RIG-I, MDA-5 and β-actin. Stratagene Mx3000P was also used with FAM™ labelled probes for TLR1-10, NOD1-2, NLRP3, RIG-I, MDA-5, LGP-2 and GAPDH together with Brilliant® QPCR Master Mix. FAM™ labelled probes were used with the TaqMan-based detection, a specific and sensitive method, allowing detection of low number of copies and two different sequences in one tube. The thermal cycler was set to perform an initial set-up (95°, 10 min) and 45 cycles of denaturation (95°, 15 or 30 sec) followed by annealing/extension (60°, 1 min). In paper I, primers for MLCK and GAPDH were designed and synthesised by DNA Technology A/S. PCR reactions were performed using the Brilliant® II SYBR® Green QPCR Master Mix. SYBR® Green-based detection can be used to detect any double stranded DNA sequence and no probe is required. The thermal cycler was set to perform 95°C for 15 min, followed by 46 cycles of 94°C for 30 s and 55°C for 60 s. For SYBR® Green-based detection, melting curve analysis was performed to ensure specificity of the amplified PCR products.

The relative amount of mRNA for the specific genes was determined by subtracting the Ct values for the gene of interest from the Ct value for the control genes (GAPDH or β-actin) (ΔCt). The amount of mRNA was expressed in relation to 10^5 β-actinmolecules or GAPDH ($2^{-\Delta Ct} \times 10^5$).

3.12 STATISTICS

Data was analysed using GraphPad Prism Software (San Diego, CA, USA). Results are expressed as individual dots with mean or mean ± standard error of mean (SEM). In paper I-II, n equals the number of independent experiments (passages) or donors. In paper III-VI, n equals the number of human donors. In paper IV, experiments involving cultured murine epithelial cells or neurons, n is equal to the number of individual replicate measurements.

Normally distributed data was analysed using parametric tests and data not normally distributed by non-parametric tests. For comparison of two data sets, paired or unpaired t-tests were employed for parametric data, whereas Wilcoxon's matched-pairs signed rank tests were used for non-parametric data. For more than two paired data sets, one-way repeated measures ANOVA followed by Dunnett's or Bonferroni's multiple comparison post-test was used for parametric data. A Kruskal-Wallis test or a Friedman's test followed by a Dunn's multiple comparison post-test was used for non-parametric data. In paper I, a Grubbs' outlier test was carried out on the replicate analyses to identify possible outliers. A p-value of 0.05 or less was considered statistically significant.

4. RESULTS AND COMMENTS

4.1 PATTERN-RECOGNITION RECEPTORS IN HUMAN AIRWAY SMOOTH MUSCLE CELLS (PAPER I)

Human airway smooth muscle cells (HASMCs) are central to airway hyperresponsiveness (AHR), remodelling and inflammation in asthma patients[114]. The first study was designed to characterise the expression and function of pattern-recognition receptors (PRRs), involved in microbe-induced exacerbation[115], on HASMCs. HASMCs were cultured with PRR-ligands to study release of cytokines and chemokines and expression of cell surface molecules. Poly(I:C) (TLR3) induced a significant release of IL-6, IL-8, GM-CSF, eotaxin and RANTES. Pam_3CSK_4 (TLR1/2), FSL-1 (TLR2/6), LPS (TLR4), R-837 (TLR7) and iE-DAP (NOD1) stimulation induced release of IL-6, IL-8 and GM-CSF (Figure 6A-E). To verify this effect, cells were incubated with Pam_3CSK_4, poly(I:C), LPS, R-837 and iE-DAP in multiple concentrations. This revealed a concentration-dependent increase in the release of IL-6 and IL-8 with an exception of LPS stimulation where the release of IL-6 and IL-8 plateaued after the lowest LPS concentration. TGF-β1 release was not altered by PRR stimulation (Figure 6F). In addition, Pam_3CSK_4, poly(I:C), LPS, R-837 and iE-DAP stimulation increased the expression of ICAM-1 and HLA-DR. As the specificity of R-837 for TLR7 has been questioned, HASMCs were pre-treated with the TLR7 antagonist IRS661. Results showed that IRS661 eliminated the R-837-induced IL-6 release.

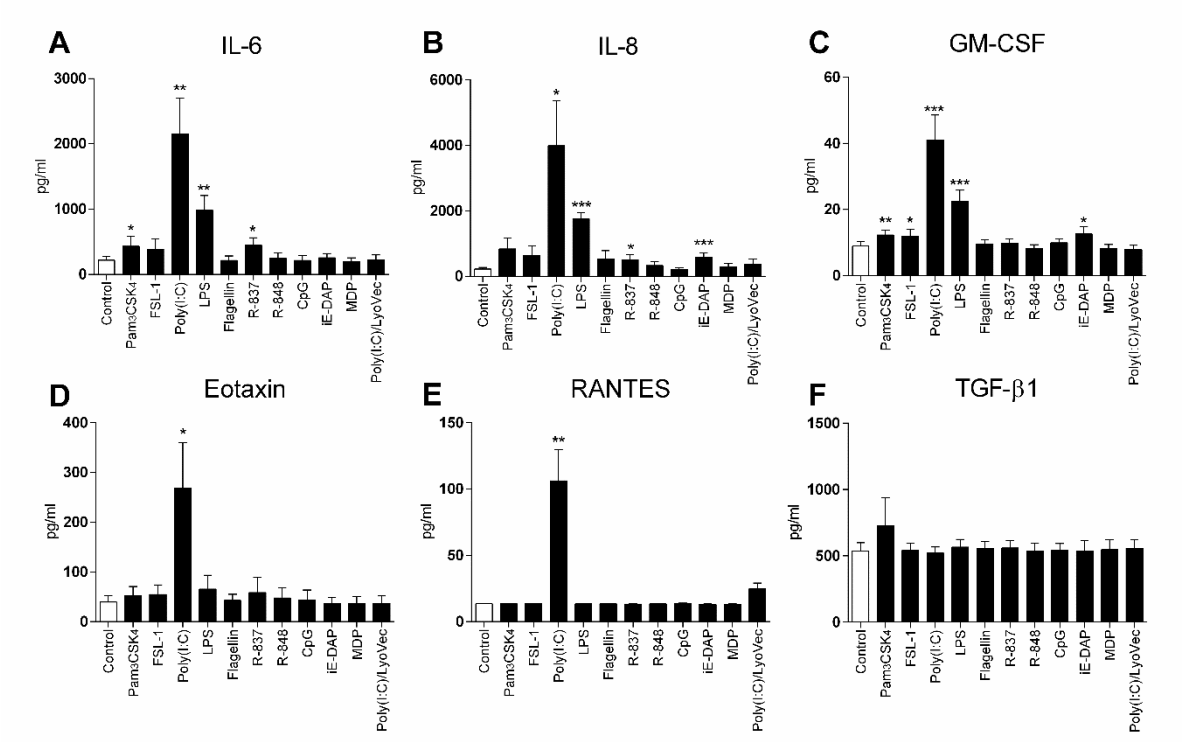

Figure 6. HASMCs were cultured (24h) with/ without Pam_3CSK_4 (TLR1/2), FSL-1 (TLR2/6), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), R-837 (TLR7), R-848 (TLR7/8), CpG (TLR9), iE-DAP (NOD1), MDP (NOD2) and poly(I:C)/LyoVec (RIG-I/MDA-5). (**A**) IL-6 (n=14), (**B**) IL-8 (n=14), (**C**) GM-CSF (n=14), (**D**) eotaxin (n=7), (**E**) RANTES (n=7) and (**F**) TGF-β1 (n=6) was analysed using ELISA. All values: mean ±SEM.

TLR1/2, TLR3, TLR4, TLR7 and NOD1 were expressed on both mRNA and protein levels on cultured HASMCs. Pam_3CSK_4, poly(I:C), LPS, R-837 and iE-DAP stimulation also downregulated expression of myosin light-chain kinase (MLCK) and cysteinyl leukotriene 1 receptor (CysLT1R) (Figure 7A-B). In addition, poly(I:C) stimulation increased expression of β2-adrenoceptor (β_2AR) (Figure 7C).

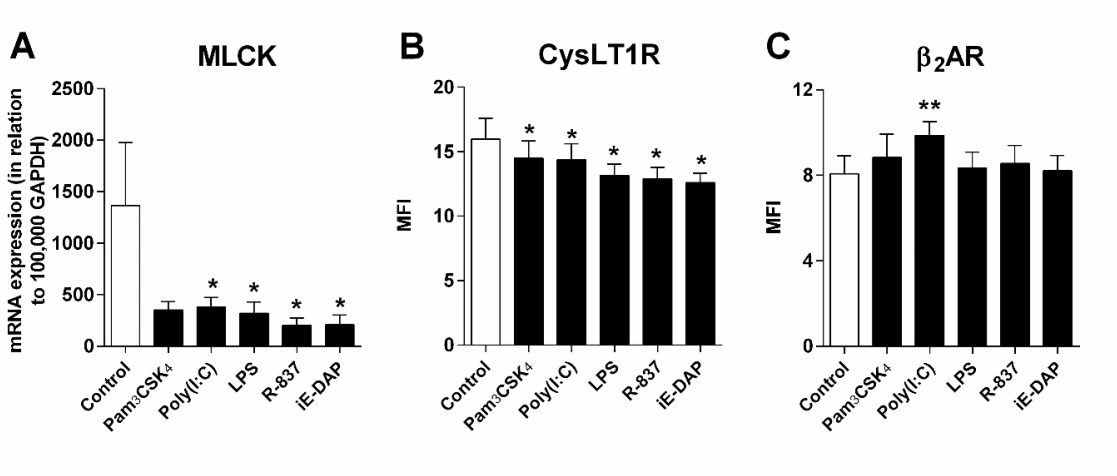

Figure 7. HASMCs were cultured (24 h) in the absence or presence of Pam_3CSK_4, poly(I:C), LPS, R-837 and iE-DAP. (**A**) MLCK mRNA expression was analysed using real-time RT-PCR. (**B)** CysLT1R and (**C**) β_2AR expression was analysed using flow cytometry (n=7). All values: mean ±SEM.

4.2 COMMENTS (PAPER I)

HASMCs are involved in asthma pathogenesis, as they play a role in airway contraction, airway wall thickening and generation of cytokines and chemokines that leads to airflow obstruction, remodelling and local inflammation[18].

Cultured HASMCs can have a contractile and a synthetic/proliferative phenotype, the former being characterised by high expression of contractile proteins and the latter by low expression[116]. The switch between these phenotypes is referred to as phenotypic plasticity. HASMCs grown in 5-10 % serum develop a synthetic phenotype[117]. A long starvation period can induce a hyper-contractile phenotype in HASMCs[118]. However, HASMCs need serum to proliferate and grow *in vitro*[119]. In addition, plasticity is modulated by confluency of the cell culture[118]. The phenotypic plasticity occurs *in vitro*, but whether it also occur *in vivo* is not known. However, optimising *in vitro* culture models/conditions is important as phenotypic plasticity of HASMCs is associated with changes in contractile protein and ion channel expression, which can be functionally significant when conducting *in vitro* experiments[120].

A high TLR3 expression was evident on the HASMCs and the strongest responses were seen upon stimulation with the TLR3 ligand poly(I:C), demonstrated by the release of pro-inflammatory cytokines, eotaxin and RANTES. HASMCs stimulated with poly(I:C) have previously been demonstrated to increase the chemotactic activity on eosinophils[121], which may be related to the release of these mediators. Poly(I:C) additionally downregulated MLCK and CysLT1R, and induced β_2AR expression. The function of MLCK is to phosphorylate the myosin light chain, leading to contraction, and elevated levels of MLCK have been found in

asthmatic HASMC[114]. β_2AR binds β_2-agonists and mediate smooth muscle relaxation via the release of cAMP. The β2AR-cAMP axis is abnormally regulated in asthma and is a common target in asthma treatment[122]. These findings strengthen the function of TLR3 as both limiting contractile receptors and promoting relaxant receptors. Unlike our data, Morishima *et al*. have demonstrated that poly(I:C) does not affect the expression of CysLT1R[123]. These discrepancies may be explained by different culture models/conditions discussed above, or by donor and origins differences of the HASMCs (tracheal vs. bronchial). However, our results emphasise that TLR3 is an important receptor on HASMCs, regulating both local inflammation and relaxation.

Results from studies on the expression of TLR7 on HASMCs have been contradictory. By immunohistochemistry, functional TLR7 expression has been demonstrated on guinea pig airway smooth muscle cells[124]. However, other human studies demonstrate TLR7 expression on nerve fibres innervating the airways, and not on airway smooth muscle cells[125]. Comparatively, our study clearly demonstrates functional TLR7 expression on cultured HASMCs. In line with this, TLR7 expression on cultured foetal and adult HASMCs has been demonstrated[126]. R-837 induced release of IL-6 and IL-8 and downregulated expression of MLCK and CysLT1R, indicating that R-837 induces pro-inflammatory cytokine release and relaxation of HASMCs.

It is important to note that the TLR7 agonist R-837 has a multitude of different effects. Binding of R-837 to TLR7 can induce pro-inflammatory cytokines or rapidly initiate a calcium release[127, 128]. Previous studies have shown that R-837 administration induced a rapid airway relaxation, both *in vivo* and *in vitro* via disruption of calcium homeostasis in HASMCs or through nitric oxide release from nerves[125, 129]. Studies have also showed that certain effects of R-837 are independent of TLR7 expression[130]. Synthetic TLR7 and TLR8 agonists are structurally related to imidazoquinolines and quinolines and have been demonstrated to relax pre-contracted guinea pig and human airways[131]. Topical R-837 induces strong responses in skin of Tlr7−/− mice, independently of TLR7 and adaptive immune responses[132]. The receptor involved in this response remains unknown[133]. Therefore, R-837 studies have to be carefully designed to not include/exclude cells based purely on their expression of TLR7. In addition, it is vital to conduct experiments that include TLR7 agonists, that do not belong to the quinolone family, or to block TLR7, to demonstrate whether the effects are TLR7-dependent. In our study, a TLR7 specific antagonist termed IRS661 was used and demonstrated that our cytokine release was TLR7-dependent.

To summarise, expression of TLR2, TLR3, TLR4, TLR7 and NOD1 was found on HASMCs and activation of these receptors promotes the development of a synthetic phenotype of HASMCs. This was characterised by a release of various cytokines, an upregulation of several inflammatory cell surface markers and downregulation of receptors involved in smooth muscle cell contraction.

4.3 PATTERN-RECOGNITION RECEPTORS IN THE NASAL EPITHELIUM (PAPER II-III)

The airway epithelium provides protection against pathogens through barrier functions, as well as the release of epithelial-derived chemokines and cytokines[22]. The first study aimed to evaluate the presence of virus recognising PRRs on primary human nasal epithelial cells (HNEC) and their role in inflammation in healthy subjects. First, mRNA expression of TLR3, TLR7, TLR9, RIG-I and MDA-5 was demonstrated in nasal biopsies. Immunohistochemistry (IHC) was used to determine the location of the receptors and revealed that the expression of all five receptors was most abundant on the surface epithelium (Figure 8).

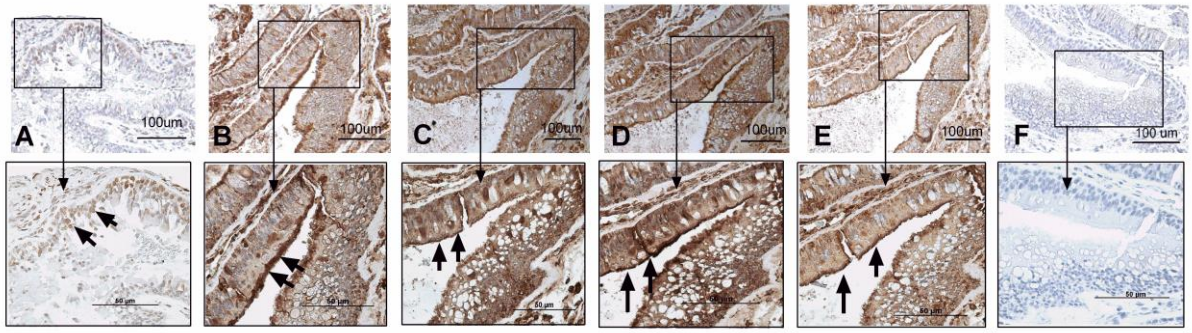

Figure 8. Sections of nasal biopsies stained for TLR3 (**A**), TLR7 (**B**), TLR9 (**C**), RIG-I (**D**), MDA-5 (**E**) and control slides (**F**), visualised by 3, 3′-DAB (brown). All slides were counterstained with haematoxylin (blue). The figure shows one representative biopsy out of four. The arrows indicate positive stained cells.

Since the highest expression of PRRs was evident in the nasal epithelium, receptor expression was evaluated in HNEC and in the nasopharyngeal epithelial cell lines Detroit-562 and FaDu. mRNA expression for TLR3, TLR7, TLR9, RIG-I and MDA-5 was detected in HNEC. These findings were verified in Detroit-562 and FaDu with the exception of TLR7 and TLR9, which were barely detectable. In contrast, protein expression of TLR3, TLR7, TLR9, RIG-I and MDA-5 could be demonstrated in all cells using IHC and flow cytometry.

The release of IL-6, IL-8, GM-CSF and IFN-β was evaluated after PRR-ligand stimulation of nasal biopsies. Poly(I:C) (TLR3) induced release of IL-6 whereas R-837 (TLR7) induced release of IL-6 and GM-CSF. CpG (TLR9) stimulation resulted in a significant upregulation of IL-8 whereas poly(I:C)/LyoVec (RIG-I/MDA-5) stimulation resulted in a release of IFN-β (Figure 9A). In HNECs, Poly(I:C) induced release of IL-6, IL-8 and GM-CSF whereas R-837 induced release of IL-6. Poly(I:C)/LyoVec stimulation resulted in a release of IFN-β (Figure 9B). In addition, ICAM1 expression on HNEC was upregulated after poly(I:C) stimulation. Stimulating Detroit-562 and FaDu with PRR-ligands demonstrated similar release patterns; however, certain differences could be detected.

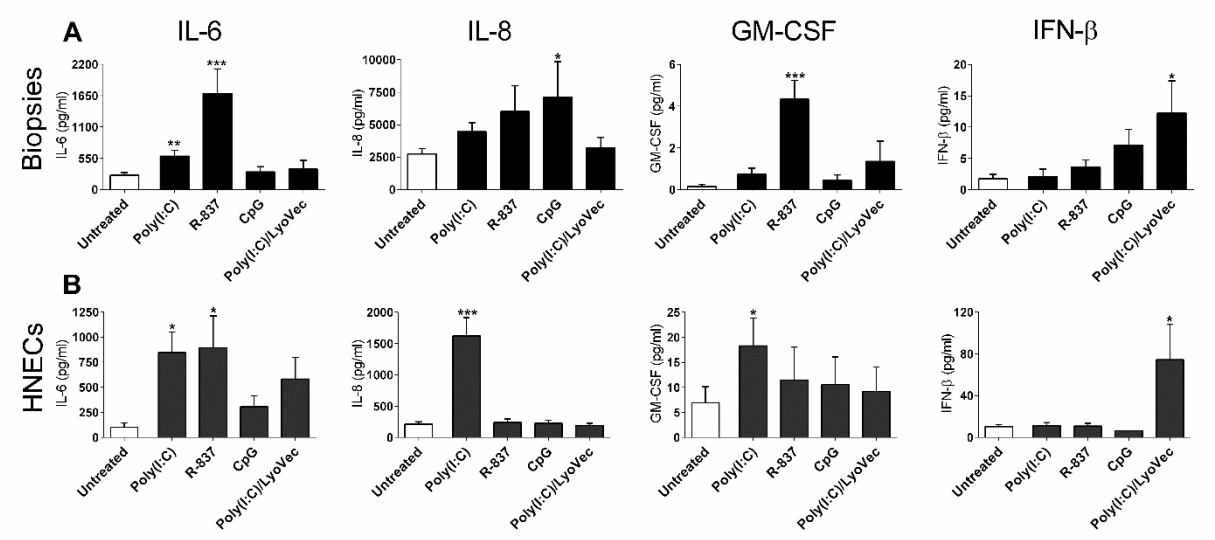

Figure 9. Nasal biopsies (**A**) and HNECs (**B**) were cultured in the absence (Untreated) or presence of poly(I:C) (TLR3), R-837 (TLR7), CpG (TLR9) and poly(I:C)/LyoVec (RIG-I/MDA-5). After 24 h, supernatants were collected and analysed for levels of IL-6, IL-8, GM-CSF and IFN-β using ELISA (n=5-9). All values: mean ± SEM.

Disruption in the PRR immune response contributes to pathogenesis of airway diseases[5, 35]. One hypothesis in the progression of chronic rhinosinusitis with nasal polyps (CRSwNP) is that an inaccurate immune response to foreign agents results in an extended mucosal inflammation[14, 15]. Virus recognising TLRs were evaluated in the nasal mucosa of patients with CRSwNP. TLR9 expression was not evident in epithelial cells derived from turbinate tissue of patients whereas it was present in epithelial cells derived from polyps of the same patients, and epithelial cells derived from healthy controls (Figure 10A). However, following *in vitro* CpG stimulations, and *in vivo* CpG-treatment, the TLR9 expression on epithelial cells derived from turbinate tissue was restored (Figure 10B). To investigate if CpG stimulations altered cytokine and chemokine release, supernatants were analysed. The release of G-CSF, IL-6 and MIP-1β from turbinate tissue was reduced towards levels demonstrated in healthy controls. In addition, epithelial VEGFR2 expression was downregulated after CpG stimulations on turbinate tissue (Figure 10C), in vitro, and a small decrease could be detected after CpG-treatment *in vivo*.

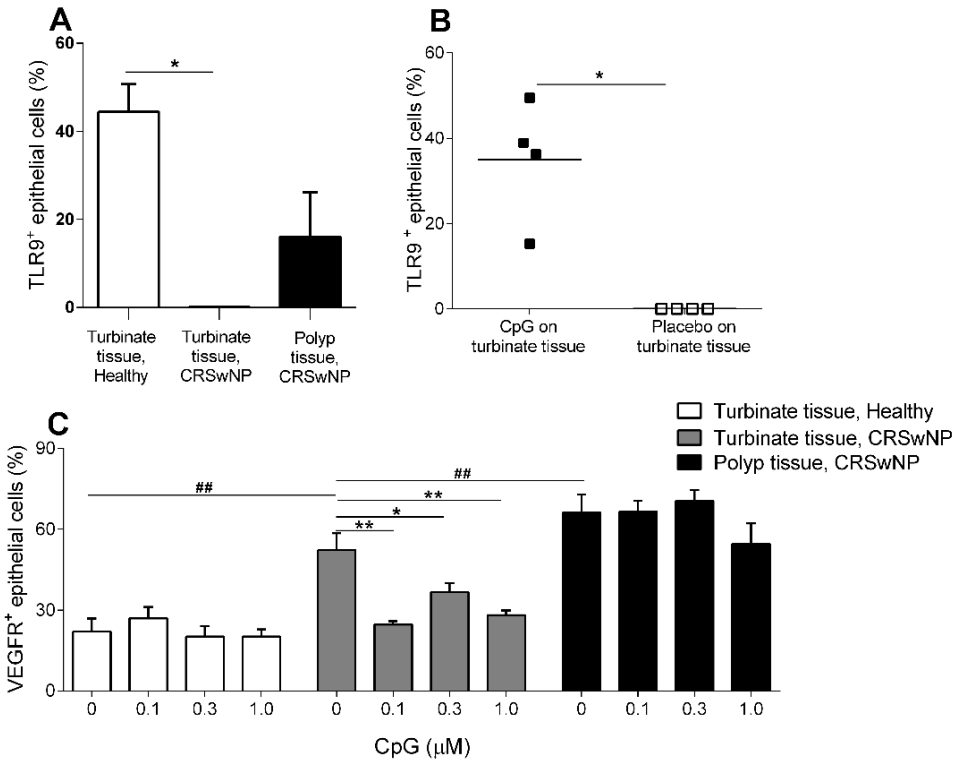

Figure 10. TLR9 expression on epithelial cells (*n*=5) (**A**). CRSwNP-patients nasally challenged with CpG (*n*=4) or placebo (*n*=4). Epithelial TLR9 expression was analysed in biopsies from turbinate tissue obtained after 24 h (**B**). Epithelial VEGFR2 expression after 24 h of culture with vehicle/CpG analysed using flow cytometry (*n*=5) (**C**). All values: mean ±SEM.

Comparisons of cytokine and chemokine release between polyp and turbinate tissue demonstrated that IL-5 and IL-10 release from polyps was significantly higher compared to turbinate tissue from patient and healthy control.

4.4 COMMENTS (PAPER II-III)

TLR3 activation via poly(I:C) stimulation induced the release of multiple cytokines from both nasal biopsies and HNECs, demonstrating that HNECs are important players in the release of pro-inflammatory cytokines seen following infections in the nose. Among viruses that may infect nasal epithelial cells, rhinovirus is the most common and is recognised by TLR3[134]. TLR3 activation on HNECs is important for the antiviral responses upon rhinovirus infections in the nasal mucosa. However, TLR3 is also involved in unwanted effects in airway diseases[135]. CRS is generally characterised by Th2 inflammation driven by cytokines like IL-4 and IL-13[136]. Th2 cytokines greatly enhance TLR3 signalling on epithelial cells[137], resulting in excessive release of TLR3-induced pro-inflammatory cytokines, driving inflammation during viral-induced exacerbations. In addition, activation of TLR3 resulted in an upregulation of ICAM1 on HNECs. ICAM1 is the main receptor to which dsRNA viruses such as rhinoviruses binds[109], and an upregulation could enhance the susceptibility to more severe or prolonged airway infections. Since TLR3 activation on HNECs increases pro-inflammatory cytokines and

enhances ICAM1-binding of airway viruses, this PRR could be important with regards to virus-induced exacerbations of airway diseases.

The finding that RIG-I/MDA-5 activation on HNECs is responsible for the majority of IFN-β release in the nasal epithelium is central. IFN-β is involved in the protection of epithelial cells against further viral infections. Impairment of virus-induced IFN-β release is associated with increased viral replication in epithelial cells[107, 108]. Prolonged infections may lead to chronic inflammatory conditions such as allergic airway inflammation or CRS. Indeed, in CRS, HNECs demonstrate a delayed clearance of virus, less released IFN-β and lower MDA-5 expression upon rhinovirus infections[138]. Altogether, therapeutics towards RIG-I/MDA-5 activation could be of use to inhibit prolonged inflammation and progression of CRS and other airway inflammatory conditions.

To summarise, this study demonstrates that TLR3, TLR7, TLR9, RIG-I and MDA-5 are expressed on HNECs and recognise virus-related products causing an increased inflammatory response. The induction of IFN-β underscores that RIG-I/MDA-5 on HNECs are important PRRs involved in viral clearance in the nose. These receptors may have the ability to affect an ongoing inflammatory process in the nasal mucosa.

TLR9 activation is known to induce a Th1 immune response[139], reversing and preventing a Th2 inflammation that is associated with CRSwNP. Studies have shown that the TLR9 agonist CpG has immunomodulatory properties *in vitro*[140] and *in vivo* in humans[141, 142], and in mice[143]. These findings show that CpG activates a Th1-biased immune response in multiple cells. The deficient TLR9 expression on turbinate tissue in CRSwNP could be of relevance to the malfunctioning immune response upon viral intrusions, known to cause aggravated inflammation and progression of polyps[144]. Restoration of TLR9 with CpG stimulation may curtail the predominant Th2 inflammation, as well as more quickly deplete viral infections, lowering the risk for polyp growth. In line with this, studies in mice have shown that activation of innate immune defences by CpG can protect against a wide range of pathogens, including respiratory syncytial virus, *Mycobacterium tuberculosis* and herpes simplex virus[145].

Recent studies have also demonstrated various factors important in the expression of TLR9. DNases are required for processing of viral DNA into shorter products, enabling TLR9 recognition[146]. As DNases control TLR9-ligands, they consequently control the regulation and expression of TLR9. In addition, cleavage of TLR9 seems to be required for correct TLR9 activation[147]. Whether TLR9 deficiency is a consequence of a Th2 inflammation or that other factors, like those mentioned above, alter the TLR9 expression and recognition, lowers the Th1 inflammation and therefor enables a stronger Th2 inflammation, remains to be established.

Upon CpG stimulation, the release of G-CSF, IL-6 and MIP-1β of turbinate tissue from patients with CRSwNP was reduced to levels demonstrated in healthy controls. IL-6 induces B-cell antibody production, T-cell activation and differentiation[102]. G-CSF stimulates eosinophil and neutrophil infiltration[104, 105], whereas MIP-1β attracts eosinophils and neutrophils[106]. An inhibition of these cytokines could hinder a further progression of CRSwNP and possibly delay polyp recurrence, even after the polyp has been surgically removed.

In summary, defects in the TLR9-mediated microbial defence were evident in turbinate tissues from patients with CRSwNP. CpG stimulation upregulated expression of TLR9, downregulated the expression of VEGFR and the release of IL-6, G-CSF and MIP-1β. Activation of TLR9 may therefore have a role in restricting nasal polyp growth or recurrence.

4.5 SUBSTANCE P REPRESENTS A NOVEL FIRST-LINE DEFENSE MECHANISM IN THE NOSE (PAPER IV)

Substance P (SP) has previously been shown to play a critical role in animal models of continuous airway inflammation[148, 149], but this has been hard to validate clinically[150], suggesting an alternative role for SP in humans. Our experiments were designed to study if TLR activation could contribute to early SP release during viral infection and if SP, subsequently, affected the innate immune response.

Our study demonstrated a widespread expression of TLR3, TLR4 and TLR9 in human nasal mucosa, whereas TLR7 was almost exclusively found on epithelial cells and nerve fibres. To further examine the level of neuronal-TLR expression, human trigeminal ganglia were evaluated. This revealed high TLR7 and TLR9 expression and low TLR3 and TLR4 expression in the ganglia. TLR7 was also demonstrated to co-localise with SP on innervating sensory nerve fibres.

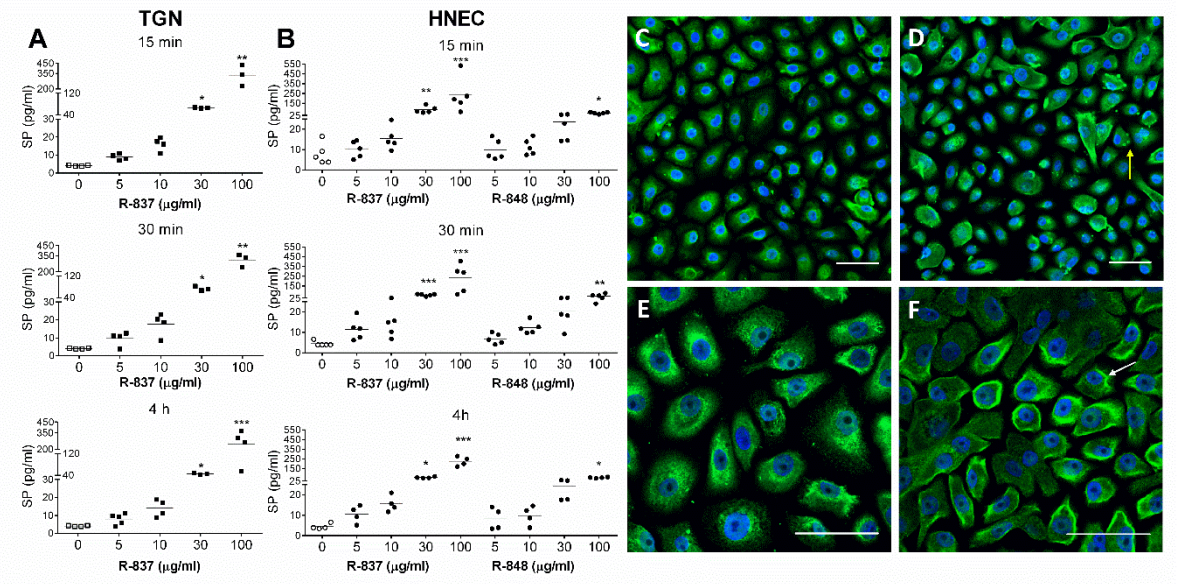

Figure 11. Substance P (SP) production by TGN (**A**) and HNEC (n=4-5) (**B**), comparing unstimulated vs. stimulated cells. Black line: Mean. Epithelial TLR4 expression in unstimulated HNEC (**C, E**) and SP-stimulated HNEC (100nM SP, 30 min) (**D, F**). Representative images from one healthy subject demonstrating expression of TLR4 (green) and nucleus (blue) (n=5). Scale bar: 50 µm.

Stimulation with the TLR7 agonist R-837 and the TLR7/8 agonist R-848 resulted in a rapid (15 min) concentration-dependent release of SP from cultured murine sensory neurons (TGN) and HNEC (Figure 11A-B). Levels remained significantly high after 30 min and 4 h (Figure 11A-B). R-848 stimulation on HNECs resulted in a similar SP release, but the effect was less prominent (Figure 11B).

Stimulation with SP on HNECs induced upregulation of TLR1, TLR3, TLR4, TLR7 and TLR9 expression of HNECs within 30 min. TLR2, TLR5, TLR6 and TLR8 were unresponsive to SP stimulation. The TLR4 upregulation on HNECs was shown to be the result of TLR-movement from intracellular compartments close to the nucleus (Figure 11C, E), to the cell surface (Figure 11D, F) after 30 min of SP stimulation. Some cells demonstrated punctate (yellow arrow) or polarised (white arrow) TLR expression (Figure 11D, F). The SP receptor, neurokinin 1 receptor (NK1R), was found to be expressed on HNECs (Figure 12A). Upregulation of TLR1, TLR4, TLR7 and TLR9 expression was blocked when HNECs were pre-treated with the NK1R antagonist, aprepitant, prior to SP stimulation (Figure 12B, D, E, F). TLR3 upregulation was not blocked by aprepitant (Figure 12C).

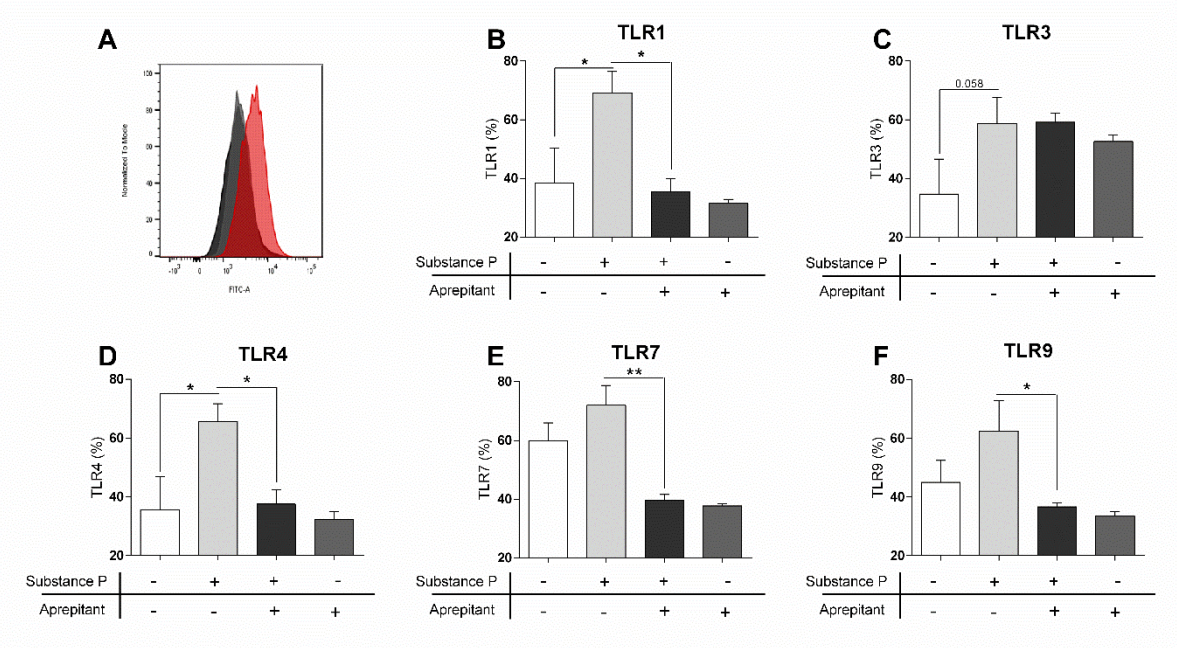

Figure 12. HNEC unstained (black histogram), isotype (grey histogram) and NK1R stained (red histogram) (**A**). HNEC expression of TLR1 (**B**), TLR3 (**C**), TLR4 (**D**), TLR7 (**E**) and TLR9 (**F**) 30 min after SP stim (100 nM), with/without prior incubation (3 min) with Aprepitant (10 nM) (n=5). All values: mean ±SEM.

4.6 COMMENTS (PAPER IV)

SP production in response to airway viral infection is a well-established phenomenon[151, 152], and results suggest that SP increases inflammation and symptoms upon infection[153]. This is the first study to show that both sensory neurons and HNECs release SP in a single burst within minutes of TLR7 stimulation. The release of SP from sensory neurons was found to be 500 times higher than the release from HNEC, when the SP release was expressed in relation to the number of cells involved. Although this demonstrates a profound capability for neurons to produce SP, HNECs are local and they far outnumber neurons in the nose, indicating that they have a powerful role in releasing SP in the nasal mucosa. Upon recognition, TLR7 traditionally uses the MyD88-dependent pathway to initiate signalling[46]. Recently, activation of TLRs has been demonstrated to induce calcium-signalling[154], possibly by sensitising receptors responsible for calcium release[155]. TLR7 can bind and activate the transient receptor potential ankyrin 1 (TRPA1), initiating SP release via an influx of extracellular Ca^{2+} [156]. Activated TLR7

interacts with TRPA1 on sensory neurons[157] and on airway epithelial cells[158]. Since our results show that the release of SP is rapid, one could speculate that this release is linked to calcium fluxes. The ability for both sensory neurons and epithelial cells to rapidly secrete SP in response to TLR activation represents a novel first-line defense mechanism for these cells. Neuropeptides may be designed for kick starting the immune system, locally regulating or priming acute inflammation in the airways. More importantly, this regulation is also present in resident non-neuronal cells.

Since the TLR upregulation after SP stimulation was rapid, it is likely due to alterations in intracellular trafficking, rather than *de novo* synthesis[159]. Recently, the intracellular trafficking of different viral-recognising TLRs has emerged as an important factor in TLR recognition. TLRs are often sequestered into intracellular stores. However, TLR-recognition only occurs in acidified endosomes or on the cell membrane, since other compartments contain chloroquine or ammonium chloride, preventing acidification[160]. Resting respiratory epithelial cells express intracellular TLR4, located in pools in the Golgi complex[47]. Upon cell activation, TLR4 is folded and transported to the cell surface for pathogen recognition, within minutes[48]. TLR3, 7 and 9 reside both in the endoplasmic reticulum (ER) and in the Golgi complex in resting cells and traffic from these stores to the endosomes upon stimulation[37, 161-163]. Proteins involved in folding and intracellular trafficking of TLRs are gp96, CNPY3, CNPY4 and UNC93B1[37, 164, 165]. The recycling endosome resident GTPase, Rab11a, has also been showed to be involved in redistribution of TLR4 and TLR9[166, 167]. In addition to this, SP-induced recycling of NK1R is also dependent on Rab11a[168]. One possible explanation for SP-induced TLR-transportation, demonstrated in our paper, could be due to the activation of Rab11a.

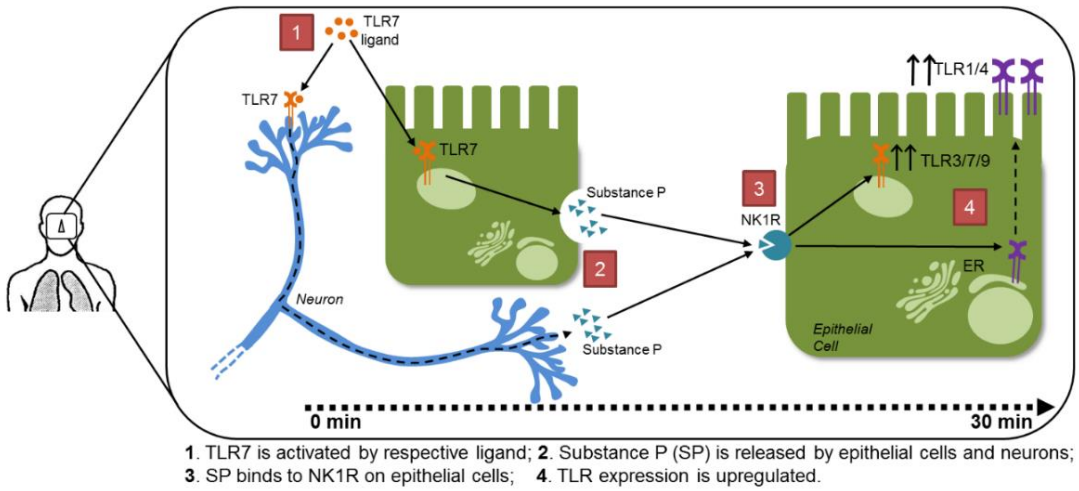

Figure 13. Summary of the main findings in Paper IV.

The conclusions from this study are that TLR7 ligands stimulate a rapid release of neuronal and epithelial SP. The released SP acts as an initial defensive response by upregulating epithelial TLR expression, via relocation of TLR within the epithelial cell (Figure 13). In line with this, pre-treating cells with SP has been shown to enhance the response to TLR-ligands[169].

Altogether, this study demonstrates that SP has the ability to kick-start the immune system and thereby prepare the epithelium against incoming microbial antigens (Figure 13).

4.7 ACTIVATION OF ACTIVIN RECEPTOR-LIKE KINASES CURB MUCOSAL INFLAMMATION AND PROLIFERATION IN CHRONIC RHINOSINUSITIS WITH NASAL POLYPS (PAPER V)

The transforming growth-factor beta (TGF-β) superfamily and their type I receptors, named activin receptor-like kinases (ALKs) have recently been proposed to be involved in local airway inflammation in CRSwNP[83]. The study was designed to examine the presence and potential function of ALKs in CRSwNP.

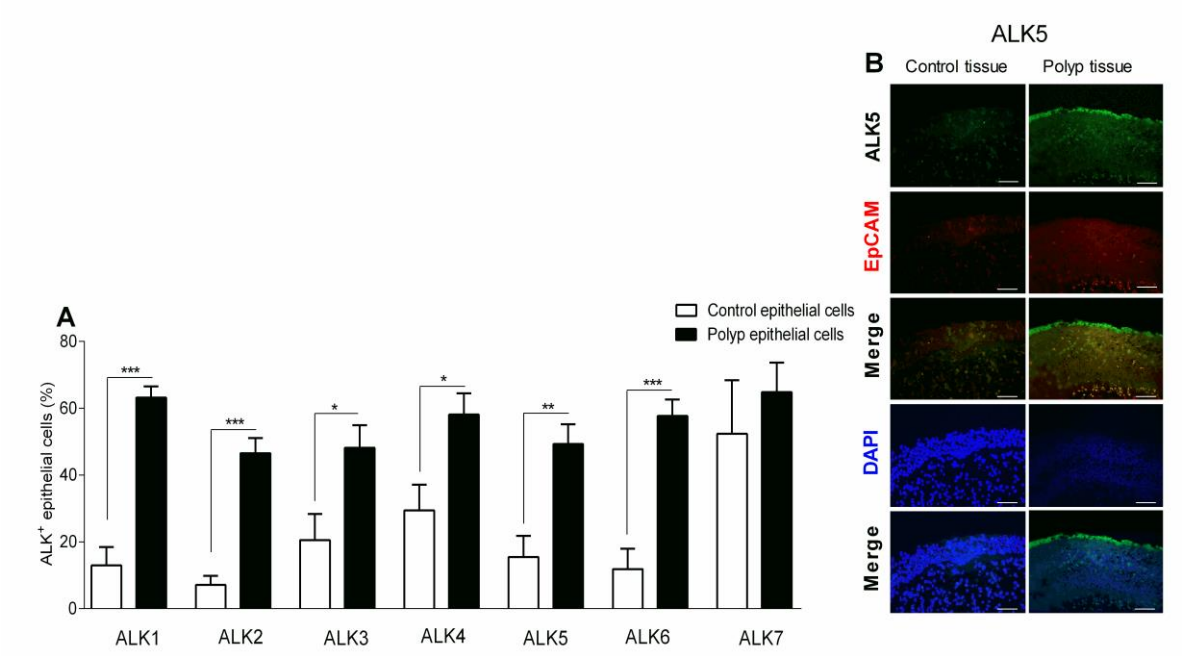

Figure 14. ALK on epithelial cells from controls (n=8) and CRSwNP-patients (n=13) (**A**). All values: mean±SEM. Representative pictures from one control and one patient demonstrating ALK5 (green), EpCAM (red), nucleus (DAPI) (**B**), scale bar: 50 µm. 3-4 controls and patients were stained.

Expression of ALK1-6 was significantly elevated on epithelial cells from polyps compared to controls (Figure 14A). The high expression of ALKs was confirmed by immunohistochemistry (IHC). IHC revealed an abundance of ALK5 in the epithelial layer of the polyps compared to control tissue (Figure 14B). In addition, strong expression of ALK2, ALK3 and ALK7 was demonstrated in the epithelial layer of the polyps. ALK2 and ALK7 expression was also seen in submucosal compartments. Low to moderate expression levels of ALK2, ALK3, ALK5 and ALK7 was seen in the nasal mucosa of healthy controls. Ki67 immunostaining was increased in polyp epithelium compared to control epithelium, with expression being confined to basal cells. Expression of ICAM1 was significantly upregulated and the release of IL-8 was slightly increased in polyps compared to controls, both present in the epithelium.

Stimulation with TGF-β1 resulted in a downregulation of Ki67 expression on cultured polyp epithelial cells, whereas stimulation with Activin A, BMP4 and Activin B did not affect Ki67 expression. Control epithelial cells exhibited no change in Ki67 upon ligand stimulation.

Stimulation with TGF-β1, Activin A and Activin B downregulated IL-8 release and ICAM1 expression in polyp epithelial cells, whereas no corresponding changes were found in control epithelial cells.

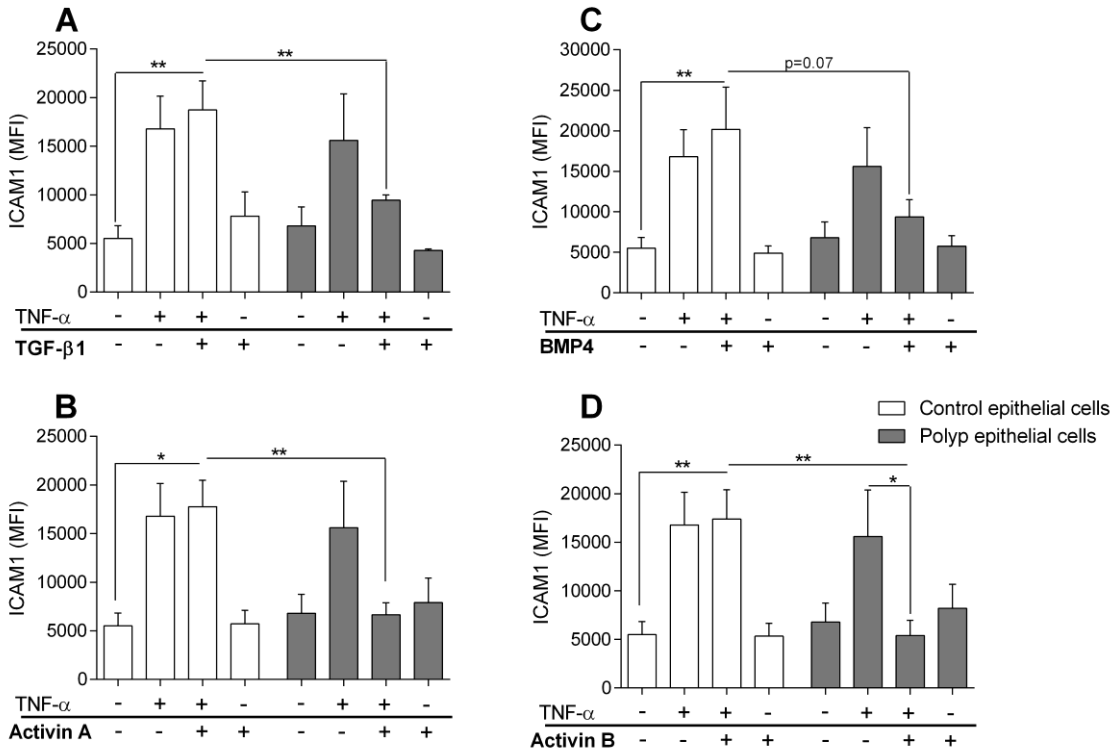

Figure 15. Epithelial cells stimulated with TGF-β1 (**A**), Activin A (**B**), BMP4 (**C**) or Activin B (**D**), for 20h after which TNF-α was added (4h) and ICAM1 expression was analysed (n=5-7). All values: mean±SEM.

To investigate if ALK-ligands had the ability to inhibit the local inflammatory response in epithelial cells, cells were exposed to TNF-α with and without the presence of TGF-β1, Activin A, BMP4 and Activin B. TNF-α caused an upregulation of ICAM1 expression compared to unstimulated cells in both polyp and control epithelial cells (Figure 15A-D). Pre-treatment for 20 h with TGF-β1, Activin A or Activin B, respectively, markedly inhibited TNF-α-induced ICAM1 expression on polyp epithelial cells compared to controls (Figure 15A, B, D). Further, pre-treatment with BMP4 inhibited TNF-α-induced ICAM1 expression on polyp epithelial cells, although this did not reach statistical significance (Figure 15C).

4.8 COMMENTS (PAPER V)

ALK signalling was previously believed to be restricted to endothelial cells[170]. The present study demonstrates that polyp epithelial cells express high levels of 6 out of 7 ALKs and that the expression of ALK5 is the one most prominent on the surface epithelium of polyps. This strengthen the impression that ALK signalling together with cytokines produced in the tissue micromilieu is important in disease remodelling and inflammation[171, 172].

Ki67 is a marker for proliferating cells and a positive correlation between Ki67 expression and the severity of epithelial remodelling has been described[173-175]. Studies have also demonstrated

a correlation between high Ki67 expression and polyp eosinophilia[176]. In addition, steroid treatment, the most effective pharmacologic therapy for nasal polyps, has been reported to downregulate the expression of Ki67[176]. Our study demonstrated that the polyp epithelium expresses increased Ki67 and that TGF-β1 stimulation downregulates this expression. These results suggest that TGF-β1 may inhibit polyp epithelial cells with abnormal or defective proliferation. This effect may be even more pronounced in patients with high eosinophilia, a group considered more difficult to treat and in frequent need of repeated surgery[177].

IL-8 release and ICAM1 expression are events necessary for leukocytes recruitment[178] and are important in the pathogenesis of nasal polyps[179]. The presented results show that stimulation with TGF-β1, Activin A and Activin B downregulated ICAM1 expression and decreased IL-8 release. Interestingly, studies have shown a low release of both TGF-β1 and Activin A from nasal polyps[180]. Activin A was specifically decreased at sites in the polyp exhibiting a massive B-cell infiltration[181]. Activin B has been shown to stimulate wound closure and cause reepithelialisation in mice[182]. This implies that a reduction of TGF-β1, Activin A and B decrease the anti-inflammatory effects they normally convey, increasing the local inflammation in CRSwNP.

Patients with CRSwNP often experience periods of disease worsening, characterised by increased polyp growth, triggered by local infections in the nose. TNF-α is produced upon bacterial, fungal and/or viral infections and is known to play a role in this process[183, 184]. In this study, TNF-α stimulation induced an upregulation of ICAM1 expression on both polyp and control cells. Pre-treatment with TGF-β1, Activin A or Activin B, respectively, inhibited TNF-α-induced ICAM1 expression on polyp epithelial cells. TGF-βs, Activins and BMPs bind to their corresponding receptor and phosphorylate Smads, which trigger a nuclear translocation of the Smad complexes[185]. In epithelial cells, the phosphorylated Smad complex brings myocardin-related transcription factors (MRTFs) into the nucleus through direct interactions[186, 187]. Upon translocation of MRTFs into the nucleus, MRTFs form a complex with NFκB, blocking NFκB activity. NFκB plays a key role in ICAM1, IL-8 and HLA-DR gene transcription. Blocking NFκB thereby inhibits inflammatory responses such as ICAM1 gene expression[188-190]. Altogether, this could be one explanation to the now reported anti-inflammatory effects of TGF-β1, Activin A and Activin B on polyp epithelial cells.

In conclusion, the present study demonstrates that polyp epithelial cells express high levels of six ALKs. It also presents data indicating an anti-inflammatory role for TGF-β1, Activin A and Activin B in polyps. Previous studies have demonstrated low levels of TGF-β1 and Activin A in nasal polyps[83, 180], and a further downregulation of these mediators by virus infections[191, 192]. This generally reduced ALK activation could possibly contribute to uncontrolled inflammation promoting the progression of CRSwNP.

4.9 IMPAIRED EFFECTS OF BMP4 RELEASE IN CRSWNP; A POTENTIAL MECHANISM FOR POLYP DEVELOPMENT (PAPER VI)

The expression of ALK3 and release of BMP4 has been reported to be upregulated in airway inflammation[89]. They demonstrate important anti-inflammatory effects by inhibiting epithelial pro-inflammatory cytokine release[90]. This study was designed to evaluate the role of BMP4 in nasal polyposis.

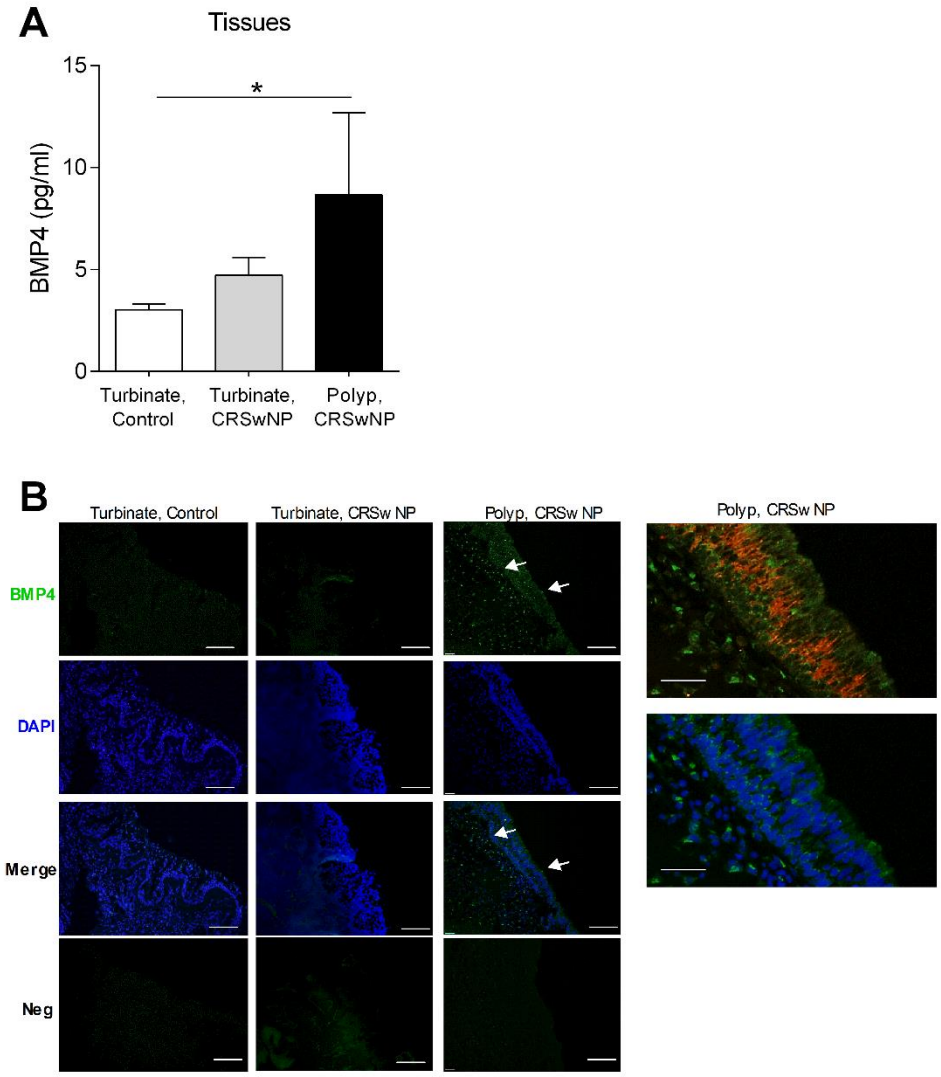

Figure 16. BMP4 release from biopsies after 24 h of culture, measured using ELISA (**A**) (n=6-9). All values: mean± SEM. Representative images demonstrating BMP4 in control, turbinate and polyp tissue with magnifications of images demonstrating BMP4 in polyp. (n=3-5). BMP4 (green); DAPI (blue); EpCAM (red), negative controls (Neg) (**B**). Scale bar: 50µm.

A higher release of BMP4 from polyps was noted, as compared to turbinate tissue from patients and controls (Figure 16A). Using IHC, BMP4 expression was detected in the surface epithelial layer as well as in the submucosal layer of polyps (Figure 16B, right panels). In contrast, no BMP4 expression could be detected in control tissue or in turbinate tissue (Figure 16B, left and middle panels).

A high expression of ALK3 was found in turbinate and polyp tissue from patients with CRSwNP, most abundant in the epithelial layer. In contrast, low expression of ALK3 was found in turbinate tissue from controls. The high expression of ALK3 was subsequently

evaluated on HNECs. The expression of ALK3 on turbinate epithelial cells from patients was significantly elevated compared to control epithelial cells. To test the function of ALK3, epithelial cells were stimulated with the ALK3-ligand BMP4. This revealed a strong intracellular phosphorylation of Smad in turbinate epithelial cells from patients(Figure 17A). This phosphorylation did not occur in epithelial cells from polyps or control tissue (Figure 17A).

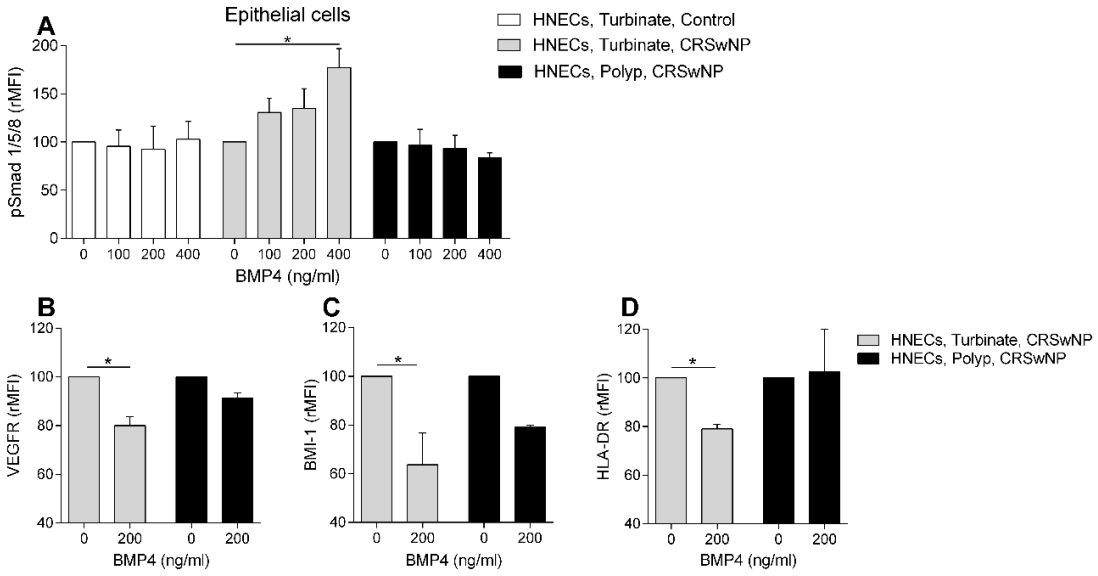

Figure 17. Phosphorylated Smad1/5/8 (pSmad1/5/8) in epithelial cells after BMP4 stimulation (2 h) (**A**) (n=3-4). Epithelial cells cultured without (0) or with BMP4 (200ng/ml) (48h) and expression of BMI-1 (**B**), VEGFR (**C**) and HLA-DR (**D**) was analysed using flow cytometry (n=3-5). All values: mean± SEM.

To further investigate the effects of BMP4, markers for angiogenesis, proliferation and inflammation were analysed on epithelial cells. Expressions of VEGFR, BMI-1 and HLA-DR was downregulated on turbinate epithelial cells upon BMP4 stimulation (Figure 17B-D). No changes could be detected on polyp epithelial cells (Figure 17B-D).

4.10 COMMENTS (PAPER VI)

In contrast to the other ALK-ligands, the effects of BMP on polyp epithelial cells was limited. However, the effects on the turbinate mucosa close to the polyp was more prominent and mediated Smad phosphorylation. Many patients demonstrate high eosinophil infiltration in their polyps[193]. Eosinophilic nasal polyposis is a disease that is difficult to control and with a high risk of polyp recurrence[194]. Eosinophils release toxic mediators like eosinophilic peroxidase (EPO) and major basic protein (MBP) that damage the upper airway mucosa[195, 196]. EPO, MBP and eosinophil-derived neurotoxin (EDN) have been demonstrated to relocate ALK3 from the membrane to the nucleus, preventing ALK3 to activate Smad signalling[197]. This relocation could explain the loss of functional ALK3 activation in polyp epithelial cells. The disrupted BMP4-ALK3 signalling in polyps, due to the eosinophilic infiltration could be

a factor contributing to the high recurrence of polyps. Eosinophilic depletion might therefor be a way to reduce the disease thereby restoring the BMP4 signalling.

Expression of ALK3 is also regulated by other factors, one of them being the TGF-β type III receptor[198]. The TGF-β type III receptor co-localises with ALK3 and retains the receptor on the cell surface[198], thereby enabling signalling. Downregulated expression of TGF-β type III receptor has been reported in nasal polyps[12]. This could be an additional factor contributing to the non-functional BMP4 signalling and the malfunctioning epithelium in polyps.

VEGF receptors can be expressed on polyp epithelial cells and it is known to promote cell hyperplasia in polyposis [199, 200]. BMI-1 is involved in epithelial cell proliferation and contributes to polyp growth[201]. BMP4 reduced the expression of VEGFR and BMI-1 on turbinate epithelial cells from patients with CRSwNP suggesting an ability for BMP4 to lower hyperplasia in the turbinate tissue. As no such reduction was seen in the polyps from the same patients, this could be an additional factor behind disease progression in patients with CRSwNP.

To summarise, this study demonstrates that BMP4 initiate a phosphorylation of Smad in turbinate epithelial cells and this activation suppresses hyperplasia and inflammation in the turbinate tissue of patients with CRSwNP. This effect was absent in corresponding polyp epithelial cells, which may explain the polyp development and growth seen in these patients. Smad signalling in nasal polyps could become a target in attempts to treat CRSwNP.

5. CONCLUSIONS

- Human airway smooth muscle cells were found to express functional TLR2, TLR3, TLR4, TLR7 and NOD1. Stimulation with the corresponding agonists Pam_3CSK_4, poly(I:C), LPS, R-837 and iE-DAP resulted in a release of various cytokines, an upregulation of several inflammatory cell surface markers and downregulation of receptors involved in smooth muscle cell contraction. These results indicate a role for PRRs in the development of a synthetic phenotype of HASMCs in respiratory diseases.

- It was established that human nasal epithelial cells express functional TLR3, TLR7, TLR9, RIG-I and MDA-5. Activation of these receptors via recognition of virus-related products resulted in epithelial cell responses through the release of inflammatory cytokines. PRRs on nasal epithelial cells could affect an ongoing inflammatory process in the nasal mucosa.

- Defects in the TLR9-mediated microbial defence were evident in turbinate tissues from patients with CRSwNP. This was not seen in the polyp tissue or in the healthy turbinate tissue. CpG stimulation upregulated TLR9 expression and downregulated VEGFR expression in turbinate tissues from patients with CRSwNP. Activation of TLR9 may therefore restrict nasal polyp growth or recurrence.

- Virus-related ligand stimulation of TLR7 induced a rapid release of SP from nasal epithelial cells and sensory neurons. The released SP promptly upregulated the epithelial TLR expression, via SP-induced redistribution. This suggests a role for SP in rapid priming of the innate immune system during viral infections.

- Activin receptor-like kinases (ALKs) were demonstrated in the nasal polyp epithelium. Concurrently, these receptors was found on epithelial cells from the nasal polyps with increased levels of ALK1-6 in comparison to nasal epithelial cells from healthy controls. Activation of these receptors reduced the aberrant proliferation and inflammation that characterise polyp epithelial cells, proposing a role for ALKs in restricting progression of CRSwNP.

- BMP4 suppressed hyperplasia and inflammation in the turbinate tissue derived from patients with CRSwNP via phosphorylation of Smad. This effect was absent in the corresponding polyps. The lack of suppression in the polyp might contribute to the progression of the disease. Hence, targeting Smad signalling might be a future therapeutic target in CRSwNP.

6. GENERAL DISCUSSION

6.1 *IN VITRO* CULTURES

In most papers in the present thesis, cells have been isolated from patients and healthy control individuals, cultured *in vitro*, and subsequently analysed using various methods. Single cell cultures do not mirror the function of cells *in vivo*, where they interact with surrounding cells and the unique tissue milieu, but they may still reveal important information. In paper II, both nasal biopsies and HNECs were stimulated with the same PRR-ligands, enabling a comparison of functions of a single cell with that of the whole tissue. In paper III, the cell function of CpG *in vitro* was confirmed by the study of cell function *in vivo*. Importantly, in all our studies, primary cells were taken directly from their normal environment in patients or controls for immediately analyse or culture. The signalling pattern observed in these cells is therefore, at least to some extent, still affected by the intrinsic components of the host, like the imprint of genetic and epigenetic factors. Epithelial cell lines are easy to grow, but they are either immortalised or cancerous and are therefore less ideal representations of the nasal airway epithelium[202]. To minimise the off-target effects seen using serum, our HNEC cultures are serum-free. This is especially important when investigating PRRs.

There are few animal models of relevance in polyp research. Previously, rabbits have been used to study polyp formation[203], but recently a new murine model was presented[204]. The morphology of the rabbit and mouse polyp is clearly different from that of the human polyp, especially evaluating the epithelium[204, 205]. This increases the need for better *in vitro* cultures. An attempt in this direction is the use of air-liquid interface (ALI) system of polyp epithelial cells[206]. In this model, human cells maintain roughly the same transcriptomes as *in vivo* epithelial cells[207], enabling improved conditions hopefully resulting in outcomes with improved clinical relevance. These cultures contain ciliated columnar epithelial cells, goblet cells and mucous cells[206], and can be used for analysis of inhalationtoxicity, ciliary coordination, hyperplasia, infection and colonisation, as well as for pharmaceutical prevention or treatment studies. Additional methodological development, using three-dimensional (3D) culture system, might further improve the relevance of the outcome, especially when it comes to the role of tight junctions and inflammatory responses[208].

6.2 TURBINATE TISSUE FROM PATIENTS WITH CRSWNP

Studies on patients with CRSwNP have long been focused on evaluating the role of the polyp itself. Our articles have broadened that perspective, by investigating two different locations in the nose of patients with CRSwNP, the polyp itself and the seemingly healthy turbinate mucosa close to the polyp. Previous studies have shown that polyp formation is only one of several signs of a generally inflamed nasal mucosa[209]. In line with this, it is well established that asthma and allergy both are signs of a more generally systemic Th2 type inflammatory conditions, further underscored by the frequent coexistence of these diseases, not seldom also in combination with CRSwNP[193, 210]. Altogether, this made us to believe that in certain phenotypes of CRSwNP, the cause of the disease is to find in the turbinate mucosa rather than in the polyp itself. This might be especially relevant when seeking a cause for the 40% risk of

polyp recurrence seen after surgery, among certain patients with CRSwNP[177, 211]. Future CRSwNP research should therefore include the mucosa surrounding the polyp. It is also tempting to speculate that, in these cases, a more complete removal of sinus mucosa during the polyp surgery may reduce the risk of symptoms and disease recurrence.

6.3 FUTURE PERSPECTIVES

In these studies, PRRs have proven to be important receptors in mediating a robust immune response via the release of inflammatory cytokines. PRRs are also involved in initiating a Th1 immune response and reducing smooth muscle cell contraction. Different PRRs have multiple functions and are known to cooperate with each other. PRRs clearly play vital roles, but there are risks in manipulating these receptors, as they are expresses on almost all cells in the body. More promising treatment options need to minimise the systemic effects of PRR stimulation. It is equally important to consider factors like dosing and timing of the PRR-ligands. However, their beneficial effects as bronchodilators, anti-inflammatory mediators and as anti-virals in inflammatory airway diseases, are important. To clarify if there are any use of PRRs therapy one first needs to specify which patients would best benefit from such manipulations, both for therapy and surgery. As research is starting to focus on more specific and personalised medicine, this could open up new ways to carefully use PRRs in driving inflammation in a more appropriate direction.

Despite great functional effects in animal models, treatment targeting substance P and its receptors have had no great clinical effects in humans until recently[150]. Our presented results could open up new ways of using neuropeptides to inhibit or monitor exacerbations of viral-induced airway diseases. To do this one must evaluate the functions of neuropeptides on PRR-regulation in asthmatics, allergics and CRSwNP patients. As a subgroup of patients with CRSwNP still suffers from high recurrence of nasal polyps, one must first characterise these patients and evaluate their SP levels. Neuropeptides may in the future be used as biomarkers and markers for uncontrolled viral-induced exacerbations.

ALKs on epithelial cells demonstrate a possible role in restoring a malfunctioning epithelium in polyps. Understanding the complete role of defects in the mucosal defence and barrier function in polyps would enable further ways of inhibiting progression of polyposis.

ALKs are also expressed on other cells such as regulatory T-cells (Tregs). Nasal polyps are characterised by impaired Treg presence and function[10]. TGF-β and ALK5 are essential components for the generation of Tregs. In mice, depletion of ALK5 depletes generation of Treg[212]. Activin A induces generation of Tregs that suppress Th2 inflammation and provide protection against allergic airway disease[213]. These reports could indicate that a loss of ALKs is important for multiple cells in nasal polyps and therefore needs to be further assessed.

7. POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvaret kan förenklat delas in i en medfödd och en förvärvad, adaptiv del. Det medfödda immunförsvaret är snabbt, relativt ospecifikt och utan minne. Det utgörs bland annat av patogen-igenkännande receptorer (PRRs). PRRs finns på många olika celler och dess uppgift är att känna igen främmande mikrober som bakterier, virus och svampar. När en PRR stöter på en främmande mikrob svarar cellen den sitter på med ett inflammatoriskt svar. På så vis kan mikrober bekämpas tidigt och om det inflammatoriska svaret är framgångsrikt förhindras en infektion. Det har dock visats att mutationer eller felaktiga och långdragna aktiveringar av dessa PRRs kan leda till sjukdomar i luftvägarna som t.ex. astma och näspolyper. En annan grupp av receptorer utgörs av så kallade ALKs. Dessa proteiner finns, likt PRRs, på flertalet celler och aktivering av dessa bidrar till strukturförändringar i vävnader och ändringar av kroppens inflammatoriska försvar. Felaktig aktivering av ALKs finns beskrivet för en rad olika sjukdomar där några är i luftvägarna.

Astma är en inflammatorisk sjukdom som ger förträngning av luftvägarna och svårighet att andas och detta styrs till stor del av celler i den glatta muskulaturen. När dessa celler drar ihop sig krymper luftvägarna och man kan få andnöd. I avhandlingen undersöktes funktionen av PRRs på luftvägarnas glattmuskelceller. Det visade sig att aktivering av PRRs orsakade en relaxation av den glatta muskulaturen samtidigt som den startar ett inflammatoriskt svar. Detta betyder att aktivering av PRRs kan ge en direkt luftvägsdilatation, något som skulle kunna användas vid luftvägssjukdomar som astma.

I näsan finns, likt luftvägarna i övrigt, ett yttre skikt bestående av epitelceller. Flera av avhandlingens delarbeten undersökte PRRs funktion på dessa epitelceller. Resultaten visade att aktivering av PRRs bidrar till kroppen snabba försvar genom att epitelcellerna släppte ut signalämnen som kan locka till sig andra celler och starta ett inflammatoriskt svar. I ett av delarbetena undersöktes epitelceller från patienter med näspolyper. Vi kunde konstatera att PRR uttrycket på den till synes friska slemhinnan bredvid polypen var avvikande. Denna slemhinna uppvisade ett felaktigt uttryck av PRR och kan inte ge ett bra försvar mot inträngande mikrober. Detta ger en ökad infektionsrisk och en ökad inflammation som kan bidra till en polyptillväxt. Genom att förbättra PRRs funktioner på slemhinnan hos patienter med näspolyper kan nya framtida terapimöjligheter utvecklas.

Neuropeptider är speciella signalsubstanser i kroppen som frisätts av nerver. I ett av delarbetena i avhandlingen har en neuropeptid, substans P, studerats och hur denna påverkar PRR uttrycket i näsan. Eftersom forskning nyligen visat att substans P också kan frisättas från epitelceller så studerades även dessa celler. Virus-liknande molekyler kunde bidra till snabb frisättning av substans P både från nerver och epitelceller. Substans P aktiverade i sin tur PRRs på näsans epitelceller. Detta delarbete visar att neuropeptider påverkar vårt medfödda immunförsvar på ett helt nytt sätt och öppnar upp för ett nytt sätt att se på funktionen av neuropeptider som del av vårt snabba immunförsvar.

I avhandlingens sista arbeten kartlades uttrycket av ALK hos patienter med näspolyper. Epitelceller från patienter med näspolyper uttryckte höga nivåer av ALK. Aktivering av dessa

visade sig ge ett skydd mot inflammation vid infektion. Näspolyper innehåller dock låga nivåer av molekyler som aktiverar ALK, i jämförelse med frisk nässlemhinna. Man kan därför tänka sig att denna brist på ALK aktivering i polypen kan bidra till ett lokalt försämrat infektionsförsvar och därmed indirekt även bidra till tillväxten av polyper i samband med infektion. Resultaten visade även att BMP4 som aktiverar en speciell ALK minskar den inflammatoriska reaktionen i slemhinnan runt polypen. Detta skulle kunna användas i terapeutiskt syfte för att förhindra att polyper växer tillbaka i slemhinnan efter en operation.

Sammanfattningsvis visar denna avhandling att både PRRs och ALKs på epitelceller utgör en viktig del av vårt immunförsvar. Förändringar av dessa receptorers uttryck och funktion påverkar flera luftvägssjukdomar som näspolyper. I ett av delarbetena visas det att substans P snabbt frisätts av virus-liknande molekyler och kan aktivera PRRs på epitelceller. Avhandlingen stärker betydelsen av PRRs, ALKs och neuropeptider vid ett immunologiskt skydd mot invaderande mikroorganismer.

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