NOVEL POTENTIAL TARGETS FOR TREATMENT OF AIRWAY INFLAMMATION

TERESE HYLANDER

Karolinska Institutet

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"JU MER MAN TÄNKER,
DESTO MER INSER MAN ATT DET INTE
FINNS NÅGOT ENKELT SVAR"

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ABSTRACT

Allergy is a complex biological response mediated by several different cell types including B lymphocytes, T lymphocytes and eosinophils. From clinical observations it is well-known that microbial infections, in particular viruses, can cause exacerbation of allergic rhinitis and asthma. The underlying mechanisms are still far from understood but recent data indicate that an activation of the immune system via pattern-recognition receptors (PRRs) including NOD-like receptors (NLRs), RIG-like receptors (RLRs) and Toll-like receptors (TLRs) might play a role. These receptors recognize invading microorganisms and enable them to interact directly with an ongoing inflammatory response. In addition, the receptors have been linked to atopic disorders such as allergic rhinitis. Today, allergen-specific immunotherapy is the only treatment that in addition to relieving symptoms also changes the progress of the underlying allergic airway disease. This therapy is traditionally administered through subcutaneous injections during three to four years but recently, intralymphatic allergen-specific immunotherapy (ILIT) emerged as an effective and less time-consuming alternative.

The aim of this thesis is to investigate novel targets of potential use for treatment of airway inflammation with special focus on PRRs and ILIT.

The first part of the thesis (PAPERS I-III) is focused on NLRs and RLRs in human leukocytes. A range of NLRs and RLRs were detected at mRNA and protein levels. Their expression in B lymphocytes was generally higher in cells derived from peripheral blood than in cells from tonsils. In T cells, a differentiated expression was seen among $CD4^+$ and $CD8^+$ tonsillar cells. Stimulation with cognate ligands in combination with triggering of the B cell receptor (via IgM or IgD) or the T cell receptor (CD3/CD28) promoted lymphocyte activation as shown by enhanced proliferation, up-regulated expression of cell-surface markers, prolonged survival and secretion of cytokines. Concomitant stimulation via the NLR and TLR systems synergistically enhanced the proliferative responses of B cells. Altogether this indicates that lymphocytes have the ability to recognize pathogens via the PRR system and it supports the idea of a role for innate receptors also in the adaptive branch of the immune system.

Eosinophils expressed both NLRs and RLRs and stimulation with the NOD1 and NOD2 ligands promoted activation as manifested by the release of cytokines, enhanced survival, regulated expression of cell-surface markers and induced chemotactic migration. These events appeared to be related to the NF-κB pathway. Stimulation with the Th2-like cytokines IL-5 and GM-CSF augmented NLR-mediated activation. These findings suggest the NLRs to be a new activation pathway for eosinophils and possibly a link between respiratory infections and allergic exacerbations.

In the second part of the thesis (**PAPERS IV-V**), the clinical and cellular effects of ILIT are evaluated. Actively treated patients exhibited a clear improvement of their seasonal allergic symptoms as well as their nasal symptoms upon allergen challenge. The treatment increased the levels of allergen-specific IgE and decreased nasal inflammatory responses. On a cellular level, activation of $CD4^+$ T cells and granulocytes was induced along with reduced Th2 and regulatory T lymphocyte activity. These findings appear to confirm ILIT as a safe and effective therapy for allergic rhinitis and reveal new insights on the cellular mechanisms underlying its beneficial effects.

In summary, this thesis demonstrates a role for PRRs in lymphocytes and eosinophils and that ILIT is a safe and effective route that can be used for treating patients with allergic airway disorders. In the future, we hope that these findings can be used in the development of new and more effective treatment strategies for airway inflammation.

2.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Människans immunförsvar brukar vanligtvis indelas i ett medfött (ospecifikt) och ett förvärvat (specifikt) försvar. Det medfödda immunförsvaret känner omedelbart igen strukturer som är främmande för kroppen och detta försvar medieras bl.a. av eosinofiler som har en viktig roll i bekämpandet av parasiter men som även förknippas med astma och allergi. Det förvärvade immunförsvaret utvecklas till följd av infektion och utgörs av B- och T-celler som producerar antikroppar och inflammatoriska ämnen som kan bekämpa inträngande mikroorganismer. I avhandlingens första del har vi studerat kopplingen mellan medfödd och förvärvad immunitet vid igenkänningen av bakterier och virus, medan den andra halvan fokuserar på immunförsvarets betydelse vid allergivaccination.

Det är välkänt att infektioner, framförallt av virus, kan försämra såväl allergisk rinit (hösnuva) som astma, men hur detta i detalj går till är fortfarande okänt. Mycket talar dock för att s.k. patogen-igenkännande receptorer kan ha en betydande roll i denna process genom sin förmåga att aktivera det medfödda försvaret som i sin tur drar igång det förvärvade svaret. Dessa receptorer kan delas in i olika familjer och däribland finns de NOD-lika och RIG-lika receptorerna. När dessa receptorer känner igen bakterier respektive virus startar olika processer i cellen vilket leder till frisättning av ämnen som orsakar inflammation. De NOD-lika och RIG-lika receptorerna har nyligen blivit upptäckta och ganska lite känt om vart de finns och vad deras funktion är. Målet med avhandlingens första del (ARBETE I-III) var att kartlägga förekomst och funktion av NOD-lika och RIG-lika receptorer i B-celler, T-celler och eosinofiler.

För att undersöka detta isolerades celler från blod och halsmandlar. Förekomst av de NOD-lika och RIG-lika receptorerna analyserades i de olika celltyperna och cellerna odlades med eller utan olika bakterie- och virusstrukturer med känd förmåga att aktivera NOD-lika och RIG-lika receptorer. Hur cellerna reagerade (dvs. deras funktion) registrerades. Vi fann att NOD-lika och RIG-lika receptorer var vanligt förekommande i B-celler, T-celler och eosinofiler och att stimulering med mikrobiella strukturer aktiverade cellerna vilket resulterade i inflammatoriska reaktioner. I eosinofiler kunde vi också visa att inflammatoriska ämnen som är karaktäristiska för allergiker gör att cellerna reagerar mer uttalat på bakteriella strukturer.

Detta är första gången som närvaro av NOD-lika och RIG-lika receptorer visas i B-celler, T-celler och eosinofiler vilket innebär att receptorer som tillhör det medfödda immunförsvaret finns på celler som tillhör såväl det medfödda (eosinofiler) som det förvärvade immunförsvaret (B-celler och T-celler). Detta fynd suddar ut gränsen mellan immunförsvarets två grenarna då det har ansetts att det medfödda immunförsvarets igenkänning av mikroorganismer är en förutsättning för att det förvärvade immunsvaret ska engageras. Vidare verkar det som om individens allergiska status påverkar hur eosinofiler reagerar vid bakterie- och virusinfektion,

vilket skulle kunna vara en bidragande orsak till att allergiska besvär kan förvärras vid t.ex. förkylning.

Det har visat sig att fel i de NOD-lika och RIG-lika receptorerna kan leda till utveckling av allergi och astma. Vid allergi reagerar immunförsvaret mot ofarliga ämnen i omgivningen (t.ex. pollen, födoämnen och husdjur) och utsöndrar då olika inflammatoriska substanser som kan ge upphov till symptom som nästäppa, rinnande ögon och hosta. Det finns flera varianter av symptomlindrande behandling men endast allergivaccination påverkar sjukdomens vidare utveckling. Förenklat kan man säga att allergivaccination manipulerar immunförsvaret så att kroppens reaktioner mot allergenet minskar. Allergivaccination ges vanligtvis med sprutor i armen, men då det normalt krävs injektioner var sjätte vecka under 3-5 år, avstår många patienter från behandling. Nyligen har det framkommit data som tyder på att tre sprutor, givna direkt i en lymfkörtel i ljumsken med en månads mellanrum, kan vara lika effektivt som 3-5 års traditionell behandling. Dock vet man inte idag om denna behandlingsmetod är helt säker för patienten eller vad som händer i kroppen när man behandlar allergi på detta sätt. Därför behöver den nya behandlingen utvärderas innan den kan erbjudas som alternativ till traditionell allergivaccination.

I avhandlingens andra del (ARBETE IV-V) har vi utvärderat om det är säkert att behandla pollenallergiska patienter med allergen-injektioner direkt in i en lymfkörtel. Vi fann att denna behandlingsmetod är säker för patienten och att den inte orsakar fler biverkningar än traditionell allergivaccination. Efter genomförd behandling rapporterade patienterna färre allergiska symtom när de sprayades med allergen i näsan och de upplevde en klar förbättring av sina allergisymtom under följande pollensäsong. Därefter undersökte vi om denna behandlingsmetod kan förändra immunförsvaret på liknande sätt som när vaccinationen ges via sprutor i armen. Resultaten tyder på att behandlingen är associerad med förändringar i cellernas reaktivitet, inflammatoriska svar och immunförsvarets sammansättning av celler, vilket stämmer väl överens med vad som sker vid traditionell allergivaccination. Detta tyder på att sprutor som ges direkt in en lymfkörtel i ljumsken kan vara ett tidssparande och effektivt sätt för behandling av allergiska besvär.

Sammantaget så hoppas vi att det som framkommit i denna avhandling i framtiden ska kunna användas som underlag vid utveckling av nya och mer effektiva behandlingsstrategier för sjukdomar i luftvägarna.

4.

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My *friends*, for joyful gatherings and fun times. I look forward spending more time with you.

My *husband Björn and my Family*, for always being there for me, your endless encouragement and your love. What would I have done without you?!

6.

ABBREVIATIONS

ANX-V	Annexin V
BCR	B cell receptor
Ct	Cycle threshold
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern
EDN	Eosinophil-derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
FSc	Forward scatter
GM-CSF	Granulocyte macrophage-colony stimulating factor
iE-DAP	γ-D-glutamyl-*meso* diaminopimelic acid
Ig	Immunoglobulin
ILIT	Intralymphatic allergen-specific immunotherapy
IPAF	ICE protease activating factor
LGP-2	Laboratory of genetics and physiology 2
MDA-5	Melanoma differentiation associated gene 5
MDP	Muramyl dipeptide
NAIP	Neuronal apoptosis inhibitor proteins
NALP	NACHT domain-, LLR- and pyrine domains-containing proteins
NF-κB	Nuclear factor- κB
NLR	NOD-like receptor
NOD	Nucleotide-bindling oligomerization domain
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PI	Propidium iodide

PRR	Pattern-recognition receptor
RIG	Retinoic acid inducible gene
RLR	RIG-like receptor
RT	Reverse transcription
SCIT	Subcutaneous allergen-specific immunotherapy
SEM	Standard error of the mean
SLIT	Sublingual allergen-specific immunotherapy
SSc	Side scatter
SQ-U	Standard quality units
TCR	T cell receptor
Th	T helper
Th1	Th type 1
Th2	Th type 2
TLR	Toll-like receptor

8.

ARTICLES INCLUDED IN THE THESIS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals (**I-V**). The papers are appended at the end of the thesis.

➢ *Petterson T.,* Jendholm J., Månsson A., Bjartell A., Riesbeck K. and Cardell LO.
Effects of NOD-like receptors in human B lymphocytes and crosstalk between NOD1/NOD2 and Toll-like receptors.
J Leukoc Biol. 2011 Feb;89(2):177-87.

➢ *Petterson T.,* Månsson A., Riesbeck K. and Cardell LO.
Nucleotide-binding and oligomerization domain-like receptors and retinoic acid inducible gene-like receptors in human tonsillar T lymphocytes.
Immunology. 2011 May;133(1):84-93.

➢ Månsson A., *Petterson T.* and Cardell LO.
Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs) in human eosinophils: activation by NOD1 and NOD2 agonists.
Immunology. 2011 Nov;134(3):314-25.

➢ *Hylander T*.,* Latif L., Petersson-Westin U. and Cardell LO.
Intralymphatic allergen-specific immunotherapy: an effective and safe alternative treatment route for pollen-induced allergic rhinitis.
J Allergy Clin Immun. 2013;131:412-20

➢ *Hylander T*.,* Petersson-Westin U., Eriksson M., Winqvist O. and Cardell LO.
Mechanisms and cellular effects of intralymphatic allergen-specific immunotherapy.
(Manuscript)

*Change of family name from Petterson to Hylander (2012)

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AIMS

The aim of this thesis project is to investigate novel targets of potential use for treatment of airway inflammation with special focus on pattern-recognition receptors and intralymphatic allergen-specific immunotherapy. More specifically, the aims are:

➢ To characterize expression and functional activities of NOD-like receptors in human tonsillar and blood-derived B cells (**PAPER I**).

➢ To create an expression profile for NOD-like receptors and RIG-like receptors in human tonsillar T cells and to analyze their functional relevance (**PAPER II**).

➢ To characterize the expression of NOD-like receptors and RIG-like receptors in human eosinophils and to investigate their potential role in microbe-induced exacerbations of allergic airway inflammation (**PAPER III**).

➢ To evaluate the safety and clinical effects of intralymphatic allergen-specific immunotherapy for treatment of pollen-induced allergic rhinitis (**PAPER IV**).

➢ To explore cellular changes induced by intralymphatic allergen-specific immunotherapy and to assess the mechanisms involved (**PAPER V**).

INTRODUCTION

THE IMMUNE SYSTEM

The immune system consists of numerous cells, tissues and mediators that defend the host from potentially harmful substances and pathogens. However, the ability to eliminate foreign structures comes with a risk of misdirected immune responses towards innocuous antigens, which might lead to the development of allergic and autoimmune diseases [1, 2].

The vertebrate immune system is often described as two separate branches; the innate and the adaptive immunity. However, accumulating evidence point to a close interaction between the two arms and it is now apparent that efficient protection against pathogens is mediated via cooperation of the blunt but rapid innate immune response and the highly specific adaptive immune response. In this thesis, the two branches will be presented separately and focus will be on cells and receptors of importance to the present work (as highlighted in **FIGURE 1**).
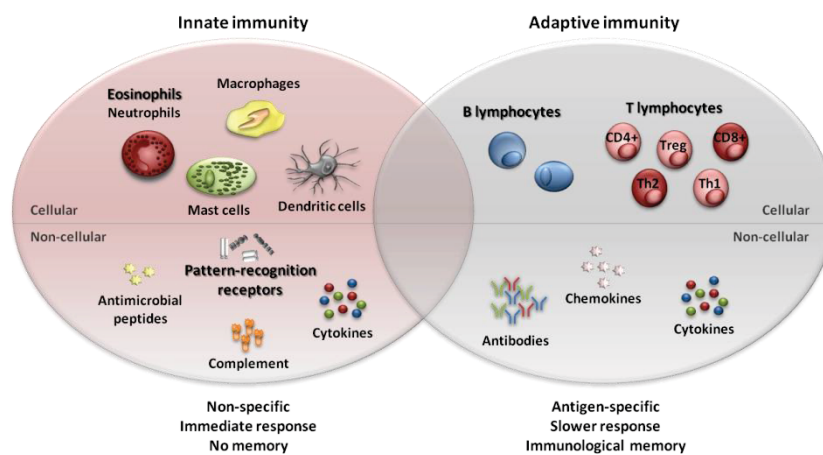
FIGURE 1. Schematic overview of the innate and adaptive immunity with cells and receptors of importance to the thesis highlighted.

INNATE IMMUNITY

The innate immune system provides a non-specific defense that is ready to mobilize within minutes after the first signs of danger. The first line of defense is provided by physical and chemical barriers that include epithelial surfaces, antimicrobial peptides and the complement system. If the pathogen is able to circumvent this initial defense, then the innate immune cells, including granulocytes, monocytes, macrophages, natural killer cells and dendritic cells become activated [2, 3]. These cells have the ability to destroy the invading pathogen, to call the attention of phagocytes to the microbe and to activate the adaptive branch of the immune system.

To be able to respond immediately to invading pathogens, the cells of the innate immune system express pattern-recognition receptors (PRRs). They recognize microbial motifs termed pathogen-associated molecular pattern (PAMPs), which are highly conserved components of the microorganism not present on mammalian cells, or endogenous molecules produced by injured or dying cells called danger-associated molecular patterns (DAMPs) [1, 2, 4].

PATTERN-RECOGNITION RECEPTORS

Germline-encoded PRRs constitute an essential part of the innate immune system allowing recognition of invading microorganisms. To date, the human PRR family consists of at least three receptor families, including Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs), which recognize different classes of pathogens, including bacteria, viruses and fungi. To ensure effective detection and clearance of infections, the PRRs are positioned in different cellular compartments. The TLRs are located at the cell surfaces and in the endosomes, whereas the NLRs and RLRs are found in the cytosol [5-8]. A schematic overview of the innate immune receptors and their cognate ligands is presented in **FIGURE 2.**
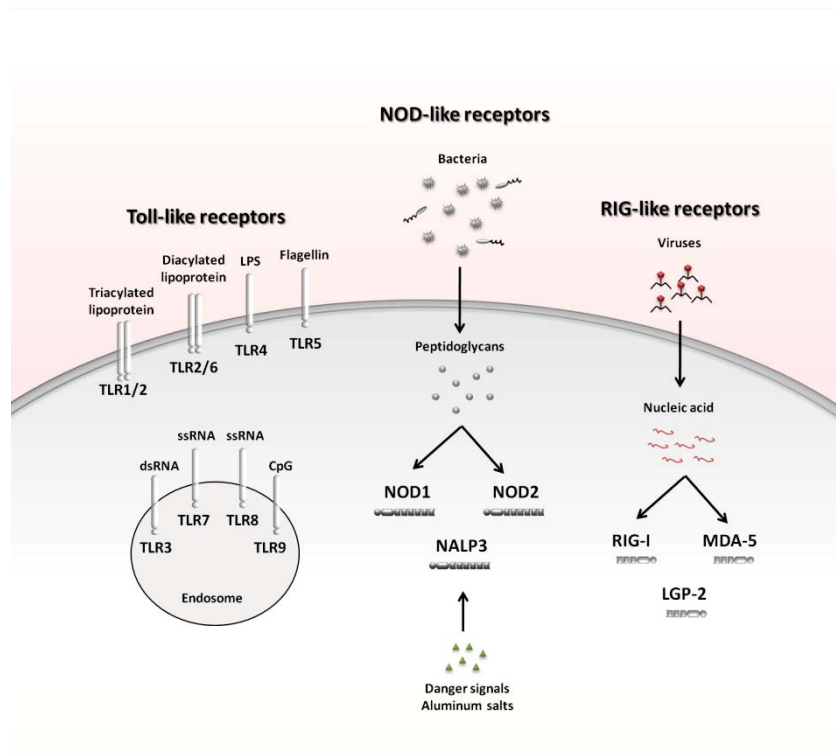
FIGURE 2. Schematic overview of Toll-like receptors, NOD-like receptors and RIG-like receptors and their cognate ligands.

TOLL-LIKE RECEPTORS

The first PRRs to be discovered were the TLRs and therefore these receptors have been most extensively studied among the PRRs. Today, the human TLRs consist of 10 members and they are found both extracellularly (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) and intracellularly (TLR3, TLR7, TLR8 and TLR9).

In general, the receptors positioned at the cell surface are involved in the recognition of bacterial cell wall components such as lipoproteins (TLR1, TLR2 and TLR6), lipopolysaccharide (LPS, TLR4) and flagellin (TLR5). In contrast, the intracellular TLRs primarily respond to viral components including dsRNA (TLR3), ssRNA (TLR7 and TLR8) and DNA-containing unmethylated CpG motifs (TLR9) [5, 8-11].

TLRs are found in a range of cells and tissues, including eosinophils, neutrophils, monocytes, epithelial cells, lymphocytes, tonsils and nasal mucosa [12-18].

NOD-LIKE RECEPTORS

The human NLR family consists of more than 20 members but the physiological functions of most members are poorly understood. Based on their N-terminal domains, the NLRs can be divided into three major subfamilies; caspase-activating and recruitment domain (CARD)-containing nucleotide-binding oligomerization domains (NODs), pyrine domain (PYD)-containing NACHT-, LRR- and PYRIN-domain containing proteins (NALPs) and baculovirus inhibitor repeat (BIR)-containing neuronal apoptosis inhibitor proteins (NAIPs). The NLRs are located within the cytosol where they primarily respond to bacterial infections and danger signals [5, 8, 19]. So far, cognate ligands have only been described for the best characterized members; NOD1, NOD2 and NALP3.

NOD1 and NOD2 recognize peptidoglycan that is a major component of bacterial cell walls. More specifically, NOD1 senses the substructure γ-D-glutamyl-*meso* diaminopimelic acid (iE-DAP) found in Gram-negative bacteria, whereas NOD2 detects muramyl dipeptide (MDP) common to Gram-negative and Gram-positive bacteria.

Both NOD1 and NOD2 recruit the kinase RIP2 upon activation, which subsequently activates NF-κB-dependent transcription of proinflammatory cytokines [10, 20]. NALP3 (also known as NLRP3) responds to danger signals released from injured or dying cells by forming caspase-1-activating protein complexes called inflammasomes, which eventually drives release of active IL-1β and IL-18. Additionally, recent studies have demonstrated a role for NALP3 in the recognition of aluminum salts (alum), commonly used as adjuvants in vaccines [21, 22].

Expression of functional NLRs has been demonstrated in an emerging number of studies, but so far most of them have focused on innate immune cells including monocytes, dendritic cells and neutrophils [23-25]. Information regarding these receptors in human eosinophils and lymphocytes has until now been lacking [26-28].

RIG-LIKE RECEPTORS

The RLRs represent the least characterized family of the innate immune receptors. To date, only three receptors have been identified; retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP-2). In contrast to the antiviral TLRs located in the endosomes, the RLRs screen the cytoplasm and mediate responses against viruses replicating inside the cell. Recognition of viral components activates signaling mechanisms that eventually result in production of interferons (IFNs) [5, 8, 9, 19].

Both RIG-I and MDA-5 are capable of recognizing many different viruses. However, RIG-I preferably detects short dsDNA and ssRNA with a 5'-triphosphate moiety, whereas MDA-5 favors recognition of long dsRNA and poly(I:C) [29-31]. The role of LGP-2 is still under investigation as no ligand has yet been identified, but it is thought to function as a regulator of RIG-I and MDA-5 responses [32].

IMPLICATIONS OF PRRS IN HUMAN DISEASES

Although PRRs function to protect the host from infection, an increasing number of studies have demonstrated that they are implicated in various immunological disorders, such as airway inflammation and allergic rhinitis [33, 34]. In this context, especially TLR4 has gained interest through its involvement in the hygiene hypothesis. This hypothesis suggests that the reduced microbial burden during childhood in westernized countries causes a skewing of the immune system towards a Th2 profile that in turn can explain the increasing prevalence of allergic diseases seen during the last decades [35, 36]. For the newly discovered cytosolic PRRs, such a connection has not been fully established but there is reason to suspect that these receptors could play a similar role. Although the field of NLR research is relatively new, polymorphisms in the genes encoding NOD1 (*CARD4*) and/or NOD2 (*CARD15*) have already been linked to allergic disorders [37-40].

EOSINOPHILS

Eosinophils are multifunctional leukocytes that mature in the bone marrow under the influence of IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) and they are thereafter released into the blood stream. Traditionally eosinophils are thought to be involved in innate immune defenses against parasitic helminthes, but they have been shown to play important roles in Th2-mediated immune responses and in the defense against viral and bacterial infections [41, 42]. During allergic airway inflammation, the eosinophils are attracted to the airways by IL-8 and eotaxin and by regulating their expression of adhesion molecules such as CD11b and CD62L, they migrate from the circulation to the airways where their effector functions are exerted.

Eosinophils are capable of releasing granule proteins including eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO) and major basic protein (MBP). In addition, they can synthesize and secrete numerous cytokines, lipid mediators, leukotrienes and prostaglandins. This has attributed a role for eosinophils in allergic airway inflammation, airway hyperresponsiveness and airway remodeling [41, 43-45]. An overview of the mechanisms by which eosinophils might affect the allergic airways is provided in FIGURE 3.

The eosinophil

T cell activation and polarization

Smooth muscle cell proliferation
Extracellular matrix production

Smooth muscle cell contraction

Mast cell activation

Epithelial activation and damage

Extracellular matrix production
Mucus secretion
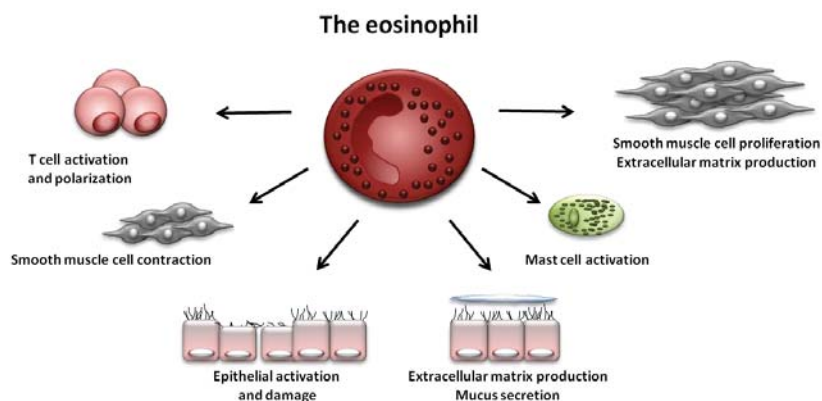
FIGURE 3. Schematic overview of the possible involvement of eosinophils in allergic responses of the airways.

ADAPTIVE IMMUNITY

Most pathogenic infestations can be handled by the innate immune system, but if it fails to clear the infection, B and T lymphocytes of the adaptive immunity kick in. In contrast to the innate immunity recognizing generic patterns using a fixed set of PRRs, the adaptive immune system provides custom-made responses towards each specific pathogen. This highly sophisticated defense is created as a consequence of recombination of receptor genes during lymphocyte development, which generates receptors specific for virtually any antigen [46].

LYMPHOCYTE ACTIVATION

Both B and T cells originate from stem cells in the bone marrow. The former stays in the bone marrow to mature, whereas the latter migrates to the thymus for further development. After maturation, lymphocytes are released to the circulation where they migrate and concentrate in secondary lymphoid organs such as tonsils and lymph nodes. Within these secondary lymphoid organs, the rare antigen-specific lymphocytes are brought together with antigen-presenting cells and this interaction with the innate immunity promotes the onset of the adaptive immunity [2, 47].

The activation of lymphocytes is traditionally thought to depend on two distinct signals; a theory called the "two-model signal of lymphocyte activation". The first signal is provided when lymphocytes recognize their corresponding antigen via their cell-bound B or T cell receptors (BCR or TCR, respectively). The BCR is made up by cell-bound antibodies that sense complex 3D structures, whereas the TCR responds to short linear peptides bound to MHC class I or II on antigen-presenting cells.

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The antigen-binding to the BCR/TCR is stabilized by co-receptors such as CD19 on B cells and CD4 or CD8 on T cells.

For B cells, the second activation signal can be provided by interaction between CD40 on the B lymphocytes and CD40L on T lymphocytes and in return, the B lymphocyte up-regulates CD80/CD86 that interacts with CD28 on the T cell, thereby providing its second activation signal [2, 3, 48, 49]. The lymphocytes thereafter start to expand and participate in pathogen killing by producing antibodies and various mediators that in most cases promote clearance of the infection. The generation of sufficient amounts of antigen-specific cells takes approximately a week, which explains why the adaptive responses are slower than those of the innate immunity. However, upon a second pathogen-encounter, memory B and T lymphocytes will mount antigen-specific responses within a couple of days [46].

B LYMPHOCYTES

The major role of B cells is to produce antibodies that are crucial in the defense against pathogens as they recognize, neutralize and target microbes for elimination, but the antibodies also function as BCRs. In addition to producing antibodies, B cells function as antigen-presenting cells and have the ability to secrete immunomodulatory cytokines that influence the polarization and activation of T lymphocytes and dendritic cells [50, 51]. After antigen-encounter and contact with T lymphocytes in the lymph nodes, the B cells become activated and differentiate into long-lived memory cells or antibody-secreting plasma cells [51, 52]. Depending on the cytokines released in the lymph nodes, the B cells produce antibodies of a specific subclass, either Immunoglobulin A (IgA), IgD, IgE, IgG or IgM that all have different functions [1]. For example, IgE is involved in the defense against parasites but also plays a pivotal role in initiation of allergic diseases, whereas IgG is implicated in immune responses against bacteria, viruses and fungi. An overview of the roles of B cells in immune responses is provided in FIGURE 4.
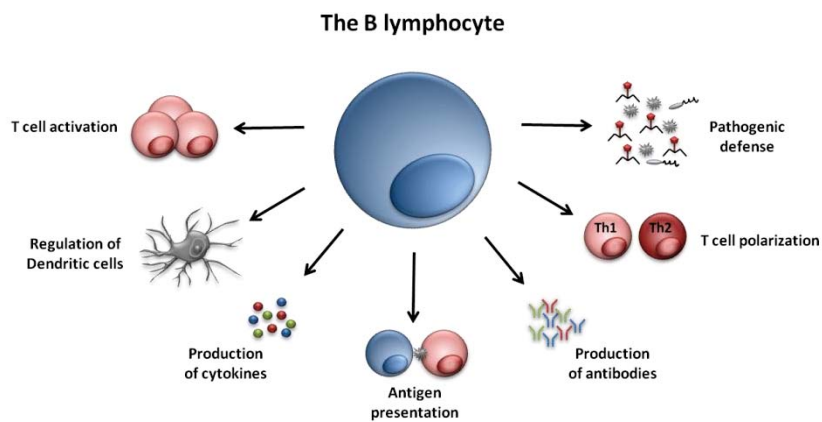
The B lymphocyte

FIGURE 4. Schematic overview of the roles of B cells in various immune responses.

T LYMPHOCYTES

During maturation in the thymus, the T cells undergo rearrangement of TCRs and start to express either CD4 or CD8 co-receptors. Thereafter, they undergo positive and negative selection based on their affinity to MHC:self-peptide complexes. Cells that bind to MHC class I complexes become cytotoxic T lymphocytes (CTLs) expressing the CD8 co-receptor, whereas cells that recognize peptides on MHC class II differentiate to T helper cells (Th cells) expressing the CD4 co-receptor [2]. An overview of the roles of T cells in immune responses is provided in FIGURE 5.

The T lymphocyte
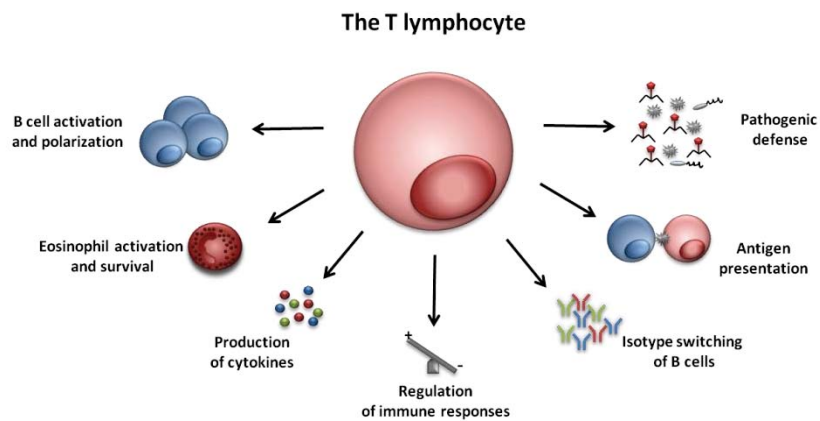
FIGURE 5. Schematic overview of the roles of T cells in various immune responses.

18.

T HELPER CELLS

Depending on the surrounding cytokine milieu and means of infection, CD4$^+$ Th cells can differentiate into several different subsets, including Th1, Th2 and regulatory T cells [53].

The Th1 and Th2 subsets were the first to be identified. Th1 cells produce for instance IFN-γ, which stimulates CTLs and promotes immunity against intracellular bacteria and viruses. Th2 cells secrete cytokines, including IL-4, IL-5 and IL-13 that are involved in clearance of extracellular pathogens but also mediate antibody production and isotype-switching of B cells [50, 53]. The Th1 and Th2 subsets also have the ability to suppress each other. In various immunological diseases, an imbalance can be seen between Th1 and Th2 cells. Allergic disorders are associated with a predominance of Th2 cells and cytokines which can promote production of IgE in B cells and survival of eosinophils [50].

Another Th subset is the regulatory T cells. This is a diverse population that can be characterized in many different ways depending on their expression of cell-surface markers and transcription factors. However, they all have the ability to balance immune responses by inducing antigenic tolerance in other immune cells. For example, this can be achieved by secretion of IL-10 that suppresses IgE production and promotes IgG4 secretion [50].

CYTOTOXIC T CELLS

When a cell becomes infected by intracellular pathogens, the infested cell will present virus-derived peptides on MHC class I in order to call the attention of the immune system. These peptides will be discovered by CD8$^+$ CTLs that upon recognition release cytotoxic granules that promote apoptosis of the infected cell. In this way, spreading of the infection to adjacent cells can be prevented [3].

ALLERGIC AIRWAY INFLAMMATION

Allergic diseases, such as allergic rhinitis, are mediated via both the innate and the adaptive immune system, which initiate complex inflammatory responses against otherwise harmless environmental antigens like allergens. The clinical manifestations of allergic disorders can vary depending on how the allergen is introduced to the body, i.e. aeroallergens can cause nasal and ocular symptoms and they are often associated with asthma.

ALLERGIC IMMUNE RESPONSES

Many inflammatory cells and mediators are involved in the allergic process but the exact mechanisms behind this are still under investigation. However, the allergic inflammatory response can be divided in two main stages; the sensitization phase and the elicitation phase. In atopic individuals, initial exposure to allergen promotes activation of allergen-specific Th2 cells that secrete cytokines (e.g. IL-4 and IL-13), which in turn induce IgE production by B lymphocytes. Secreted IgE then binds to the surfaces of mast cells and basophils, in a process known as sensitization. The elicitation phase takes place upon re-encounter of the allergen. Then the IgE receptors on basophils and mast cells become cross-linked, which leads to degranulation and release of inflammatory mediators including cytokines and histamine. These mediators are responsible for early and late allergic responses. The former promotes the characteristic features of allergic rhinitis including nasal blockage (induced by vasodilatation and increased vascular permeability), rhinorrhea (by hypersecretion of water and mucus) and sneezing and itching (promoted by stimulation of afferent nerves).

The latter is mediated via chemokines secreted from mast cells that recruit inflammatory cells, including eosinophils and Th2 cells, which contribute to tissue damage, sustained nasal blockage and wheezing in asthmatics [54-58]. Some characteristic features of allergic inflammation are presented in **FIGURE 6.**
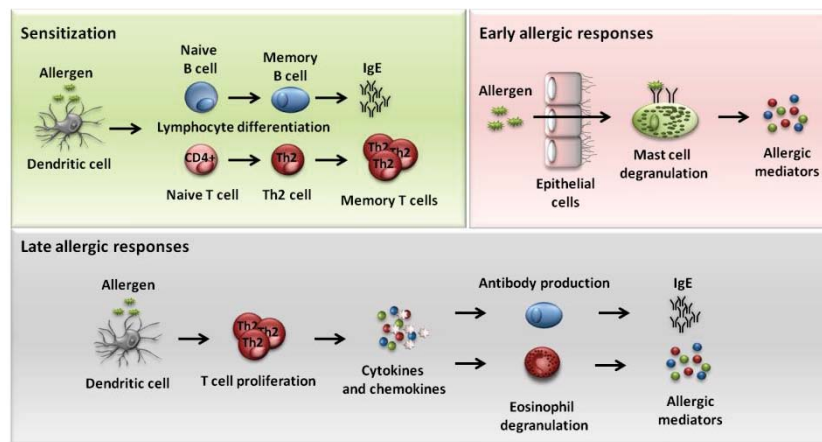
FIGURE 6. Some characteristic features of allergic inflammation.

ALLERGIC RHINITIS

The incidence of allergic rhinitis has increased in most industrialized countries during the last 20 years. The reasons are still unclear but several different theories (including the hygiene hypothesis) have been put forward. However, the etiology of allergic rhinitis appears to be influenced by both genetic and environmental factors where tobacco smoke and respiratory pathogens have been suggested to dominate the latter [56]. Clinically, allergic rhinitis is defined as a systematic disorder with nasal and ocular symptoms that include itching, sneezing, secretion and blockage upon allergen contact. It affects social life, sleep, school, work and quality of life [56, 59]. Most patients with allergic rhinitis can be treated with pharmacotherapy, including antihistamines, intranasal corticosteroids and eye drops. However, for patients who have symptoms that are inadequately controlled by conventional pharmacotherapy, who cannot avoid the allergen, who suffer from adverse events of the allergy medications, or who wish to reduce the long-term use of medication, allergen-specific immunotherapy is recommended as the first-line therapy [59-61].

ALLERGEN-SPECIFIC IMMUNOTHERAPY

During allergen-specific immunotherapy, the dose of a specific allergen is gradually increased and administered to an allergic individual to induce tolerance. The treatment is indicated for patients with IgE-mediated allergic diseases that have been verified by i.e. history of allergic symptoms, positive skin prick test and presence of allergen-specific IgE in serum, and clinical symptoms [59, 62].

Allergen-specific immunotherapy is the only treatment that affects the cause of the allergic disease and that has been shown to diminish allergic symptom, improve quality of life, prevent new sensitization and reduce development of asthma in allergic rhinitis patients [63-65]. Currently, there are in principle four different routes for administration of allergen-specific immunotherapy; subcutaneous, sublingual, intralymphatic and epicutaneous.

SUBCUTANEOUS ALLERGEN-SPECIFIC IMMUNOTHERAPY

The traditional route for administration of allergen-specific immunotherapy is subcutaneous injections and the efficacy of subcutaneous allergen-specific immunotherapy (SCIT) for treatment of respiratory disorders has been confirmed in numerous clinical studies [61, 62]. However, only 5 % of allergic patients undergo SCIT and one reason for this is the long duration of the therapy [66, 67]. The treatment protocol typically consists of an initiation phase where increasing amounts of allergen is injected weekly to the upper arm until a maintenance dose is reached, which thereafter is administered every 6-8 week for 3-5 years [62]. Another disadvantage of SCIT is the risk of adverse events. For aeroallergens, up to 30% of the patients suffer from systemic reactions after allergen administration and redness, itch and edema in proximity to the injection site are often observed [62, 68].

Although SCIT is considered the golden standard for allergen-specific immunotherapy, much effort has been put into finding more patient-friendly, non-invasive and less time-consuming alternatives.

SUBLINGUAL ALLERGEN-SPECIFIC IMMUNOTHERAPY

A more recent, non-invasive administration route is sublingual allergen-specific immunotherapy (SLIT), where the allergen extract is self-administrated in the form of tablets that are placed under the tongue once daily for approximately three years [69, 70]. Similar to SCIT, SLIT reduces allergic symptoms and provides long-term protective effects after discontinuation. In addition, the sublingual route has been associated with a better safety profile than SCIT since most adverse events are local and transient [69, 71-73]. However, the duration of the treatment is the same as for SCIT and leaving the patient to self-medicate during such a long time can cause problems with compliance.

EPICUTANEOUS ALLERGEN-SPECIFIC IMMUNOTHERAPY

In search of a needle-free, potentially self-administrable route for treatment of allergic disorders, the skin has gained increasing amount of interest. The skin has a high density of antigen-presenting cells and by applying allergen-containing patches to the epidermis, efficient antigen-delivery is ensured [74]. Promising data, including reduced allergic symptoms during pollen season, have recently been presented for grass pollen allergy [75, 76]. This treatment route has been described as convenient for the patients and although local eczematous skin reactions have be observed, EPIT appears to be safe and efficient [77].

INTRALYMPHATIC ALLERGEN-SPECIFIC IMMUNOTHERAPY

As immune responses are initiated within secondary lymphoid organs, direct intralymphatic injections of antigen emerged as a promising alternative when exploring novel potential administration routes for vaccines.

During intralymphatic allergen-specific immunotherapy (ILIT), the allergen is injected into the inguinal lymph nodes under ultrasound guidance. Clinical trials have demonstrated that three intralymphatic injections with considerably lower allergen doses are able to induce tolerance and the treatment appears to be associated with fewer adverse events than SCIT. Clinically, ILIT with grass pollen extract has been demonstrated to ameliorate hay fever symptoms, to decrease skin prick test reactivity and to increase allergen tolerability. In addition, ILIT appears to reduce levels of allergen-specific IgE and induce IgG4 and IL-10 production [78].

MECHANISMS OF ALLERGEN-SPECIFIC IMMUNOTHERAPY

The clinical effects and immunological mechanisms of allergen-specific immunotherapy are evaluated continuously [62]. The treatment does not only affect clinical parameters (such as allergic symptoms during pollen season and upon allergen challenge, early and late allergic responses, quality of life and usage of allergy medications), but also several immunological parameters [79]. The impact on cellular responses is particularly well-documented for SCIT and SLIT [80, 81].

In the adaptive part of the immune system, allergen-specific immunotherapy has been shown to reduce activation of Th2 cells in favor for Th1 cells resulting in decreased IL-4 and IL-5 production and increased secretion of IFN-γ. It also promotes generation of IL-10-secreting regulatory T cells and decreases allergen-specific proliferation. In addition, allergen-specific immunotherapy affects the production of antibodies from B cells. Initially, an increase in allergen-specific IgE levels can be observed, which is followed by a long-term decrease in favor for allergen-specific IgG4.

Allergen-specific immunotherapy also affects the innate branch of the immune system. For example, successful treatment is associated with reduced recruitment of inflammatory cells (including eosinophils and mast cells) and prevented release of proinflammatory mediators [61, 67, 79, 81]. An overview of some of the clinical and immunological effects of allergen-specific immunotherapy is presented in FIGURE 7. Although SCIT, SLIT, ILIT and EPIT all appear to induce similar clinical effects, at least in a short perspective, the mechanisms of tolerance induction remains to be settled.

Clinical effects:
↓ allergic symptoms
↓ usage of allergy medications
↓ early and late allergic responses
↑ quality of life
• prevention of progression into asthma
• prevention of new sensitizations

Allergen-specific immunotherapy

B lymphocytes:
↓ levels of allergen-specific IgE
↓ seasonal increase in IgE
↑ levels of allergen-specific IgG4
↑ production of IL-10

Eosinophils and Mast cells:
↓ tissue numbers
↓ release of mediators

T lymphocytes:
↓ allergen-specific proliferation
↓ levels of Th2 cytokines
↑ levels of Th1 cytokines
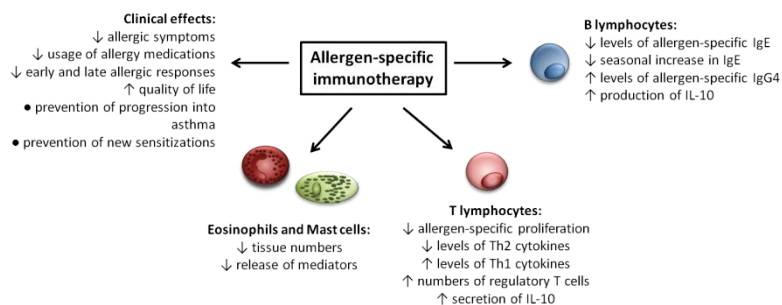↑ numbers of regulatory T cells
↑ secretion of IL-10

FIGURE 7. Overview of clinical and immunological changes induced by allergen-specific immunotherapy.

24.

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 (PAPERS I-V).

HUMAN STUDY POPULATIONS

In all studies, fresh human materials were utilized.

➢ In PAPER I, tonsils were acquired from 28 pediatric patients undergoing tonsillectomy due to tonsillar hyperplasia. Buffy coats were obtained from 21 healthy donors.

➢ PAPER II is based on tonsils from 28 pediatric patients undergoing tonsillectomy due to recurrent infections or tonsillar hyperplasia.

➢ In PAPER III, fresh peripheral blood and buffy coats were collected from allergics and healthy individuals.

➢ In PAPER IV, peripheral blood was drawn and nasal lavage fluids were collected from 28 patients with allergic rhinitis before and after treatment with active or placebo ILIT.

➢ In PAPER V, blood samples were acquired from 35 subjects with allergic rhinitis before and after treatment with active or placebo ILIT.

METHODS

This thesis can be divided into two partly integrated parts, the first (PAPERS I-III) focusing on the expression and function of PRRs in subsets of leukocytes (B lymphocytes, T lymphocytes and eosinophils, respectively), and the second (PAPERS IV-V) evaluating the clinical and cellular effects of ILIT.

All studies were performed at Skåne University Hospital Malmö after approval from the Ethics committee of Lund University and/or Karolinska Institutet. Written informed consent was obtained from all participants or the parents of pediatric subjects.

PART I.

In **PAPERS I-III**, mononuclear cells or granulocytes were isolated from tonsils and/or peripheral blood by density gradient centrifugation. Occasionally, the separation was followed by negative selection using antibody-conjugated magnetic beads. Isolated cells were analyzed for mRNA and protein expression using real-time reverse transcription (RT)-polymerase chain reaction (PCR), immunohistochemistry and flow cytometry. Cells were also used in various *in vitro* studies after culture in absence or presence of specific PRR ligands. Expression of cell surface and activation markers (flow cytometry), cytokine release (enzyme-linked immunosorbent assay; ELISA), viability and apoptosis (flow cytometry), and proliferation ([*methyl-^{3}H*]Thymidine incorporation) were assessed as depictured in **FIGURE 8**.
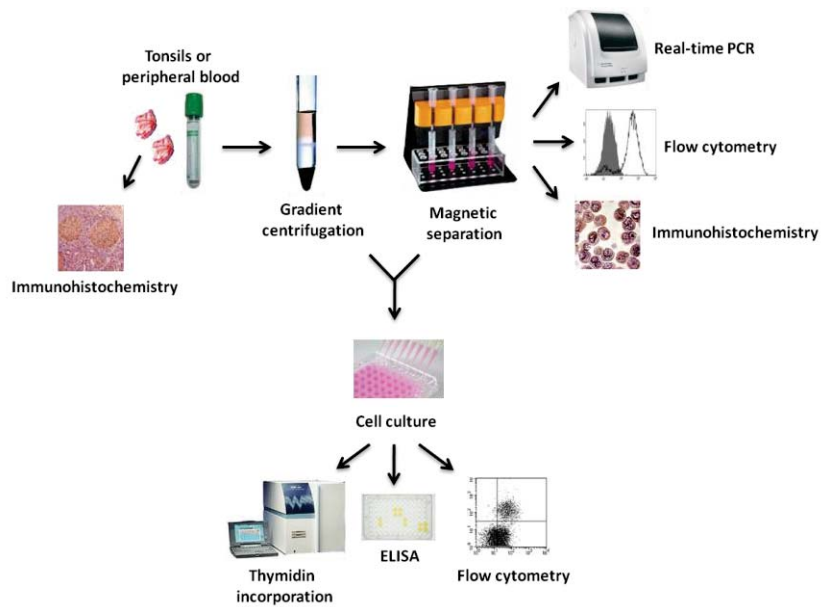

FIGURE 8. Schematic overview of the methods used in papers I-III.

CELL ISOLATION

Ficoll-Paque™, can be used to separate cells of different density. Granulocytes and erythrocytes with high density quickly sediment to the bottom of the tube, whereas mononuclear cells (including lymphocytes and monocytes) with light density are found in the interface of plasma (above) and Ficoll-Paque™ (below).

The gradient centrifugation was occasionally followed by magnetic separation to obtain highly purified cell populations (such as B lymphocytes, T lymphocytes and eosinophils). Then, freshly isolated cells were incubated with a cocktail of primary antibodies directed towards unwanted cells (such as NK cells, macrophages, monocytes) followed by antibody-conjugated antibodies against the primary antibodies. Finally, the cell suspension was passed through a magnetic column and stained cells were retained in the column, whereas unlabelled cells (CD19$^+$ B cells in **PAPER I**, CD3$^+$ T cells in **PAPER II** and CD16$^-$ eosinophils in **PAPER III**) passed through.

REAL-TIME RT-PCR

Real-time PCR enables quantification of gene expression. The procedure begins with extraction of total RNA from isolated cells followed by reverse transcription of RNA into complementary DNA (cDNA). During the quantification process, cyclic heating and cooling denatures the double-stranded cDNA, enables attachment of sequence-specific DNA probes and promotes annealing/extension of new DNA strands. During amplification, the quencher fluorophore of the probe is separated from the reporter fluorophore, giving rise to measureable fluorescence. When the emitted fluorescence reaches a predetermined value, a cycle threshold (Ct) value can be determined. Using the comparative Ct method, the obtained Ct value of the investigated gene is subtracted with the value for the housekeeping gene (β-actin) and in this thesis, the amount of mRNA is presented in relation to 100,000 molecules of β-actin as 100,000 x $2^{-\Delta Ct}$ [12, 13].

In all three papers, RNA was extracted from isolated cells using the RNeasy mini kit (Qiagen) and the RNA quality and concentration was estimated based on the wavelength absorption ratio (260/280 nm) determined by spectrophotometry. During the second step, RNA was reversibly transcribed into cDNA using Omniscript reverse transcriptase kit (Qiagen) with oligo-dT primer.

Quantitative PCR was performed either on a Smart cycler II (Cepheid, **PAPER I**) or a Stratagene Mx3000 (Agilent Technology, **PAPERS II-III**) using FAM-labelled probes from Applied Biosystems and Taqman® Universal PCR Mastermix, No AmpErase UNG and Assay on-Demand gene expression products (Applied Biosystems) or Stratagene Brilliant® QPCR Mastermix (Agilent technology), respectively.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry can be used for detection of various proteins in tissues and cells. The method is based on visualization of specific antigens using primary antibodies that subsequently are detected with enzyme-labelled polymers conjugated to secondary antibodies. After incubation with a substrate (for example 3,3-diaminobenzidine (DAB) or Fast Red), positive immunoreactivity can be identified as brown or red precipitates, respectively. To give contrast to the sections and to visualize nuclei, counterstaining with haematoxylin was performed and slides were examined using bright field microscopy. To rule out unspecific background staining, universal negative control for mouse or rabbit primary antibodies and antibody diluents were used.

Immunohistochemistry was applied for detection of NLR and RLR proteins. In **PAPERS I-II**, paraffin-embedded tonsillar tissues were used. To identify the locations of B and T lymphocytes within the tissues, primary antibodies against CD3 and CD20 were utilized. CD20$^+$ B cells were primarily detected within the germinal centers, whereas CD3$^+$ T cells were found in the adjacent areas. In **PAPERS I-III**, highly purified B cells, T cells and eosinophils were assessed for NLR and RLR protein expression.

FLOW CYTOMETRY

Flow cytometry can simultaneously measure multiple physical and chemical properties of individual cells based on how they scatter incident light from a laser beam. Using different detectors, the flow cytometer provides information about cell size (displayed by forward scatter; FSc), granularity (displayed by side scatter; SSc) and fluorescence intensity of fluorochrome-conjugated antibodies used for detection of intra- or extracellular antigens. By gating on FSc and SSc, lymphocytes, monocytes and granulocytes can easily be distinguished. In the present thesis, analyses were performed on Epics XL or FC500 flow cytometer from Beckman Coulter and data were analyzed using Expo32 ADC software or CxP Analysis software (Beckman coulter).

In **PAPERS I-II AND IV,** B and T lymphocytes were identified as CD19$^+$ and CD3/CD4/CD8$^+$ lymphocytes and monocytes as CD14$^+$ cells, and in **PAPERS III AND V,** eosinophils and neutrophils were discriminated as CD16$^-$ or CD16$^+$ granulocytes, respectively (**FIGURE 9**).

Flow cytometry was used for determination of intracellular protein expression of various members of the NLR and RLR families (**PAPERS I-III**) and regulation of extracellular activation markers (**PAPERS I-V**). In **PAPERS I AND III,** the viability of stimulated cells was determined using staining with Annexin V (ANX-V) and propidium iodide (PI). The former binds to phosphatidylserine that is translocated to the plasma membrane during apoptosis, whereas the latter is a nucleic acid dye that can be used to discriminate between dead and apoptotic cells, respectively [82].
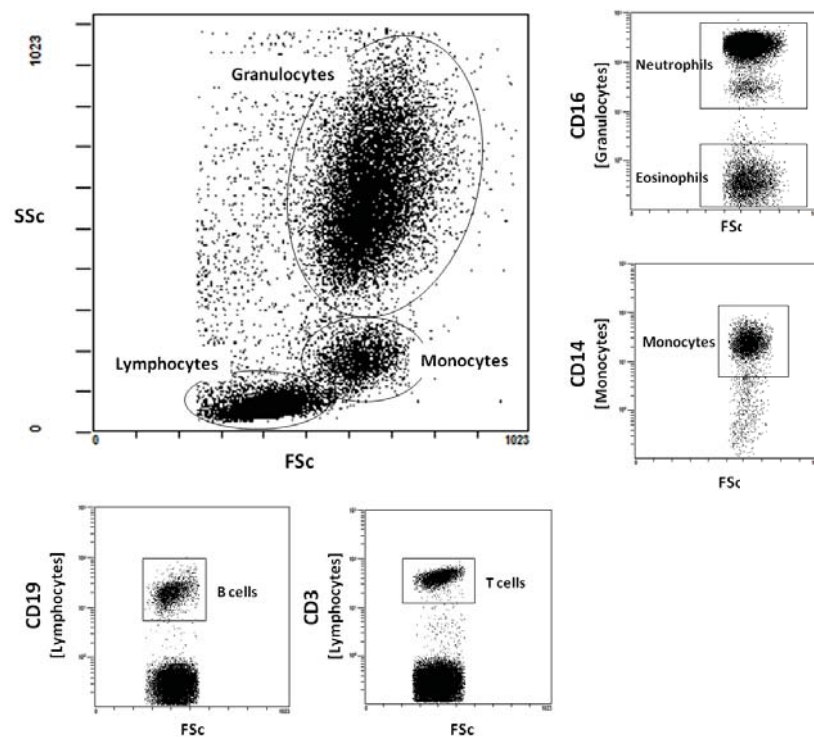
FIGURE 9. Flow cytometric identification of cell population based on FSc and SSc properties.

ELISA

ELISA is a sensitive method for detection and quantification of antigens and/or antibodies present in for instance serum, cell culture supernatants and nasal lavage fluids. In this thesis, the ELISAs used are of so-called "sandwich" type. In short, a capture antibody directed towards the antigen of interest is coated to a microplate. When standards (with known concentration) or samples are applied, the antigen binds to the immobilized antibody and thereafter an antigen-specific enzyme-linked polyclonal antibody is added for detection. Lastly, a substrate initiates color development that is proportional to the amount of antigen in the sample.

In **PAPER II,** commercial ELISA kits from RnD systems were used to determine levels of IL-2, IL-4, IL-10 and IFN-γ in supernatants. In **PAPER III**, cell culture supernatants were assessed for IL-8 and RANTES using plates from RnD systems and levels of EDN and IFN-α using kits from MBL and PBL Biomedical laboratories, respectively. In **PAPER IV**, levels of IL-8 in nasal lavage fluids and IL-10 secretion were measured with ELISA plates from RnD systems.

[*METHYL-³H*]THYMIDINE INCORPORATION

The [*methyl-³H*]Thymidine incorporation assay is a method used for quantification of proliferation. It is based on measurements of incorporated levels of radio labelled Thymidine during mitotic cell division. After cell culture in the presence of tritiated Thymidine, cells are harvested onto glass fiber filter discs and the extent of cell divisions is determined by a scintillation beta-counter based on the levels of radioactivity in the DNA recovered from the cells.

In **PAPER I**, proliferative responses to NLR and TLR ligands in purified tonsillar and peripheral B cells were determined. In **PAPER II,** Thymidine incorporation was measured in cultured CD3$^+$ tonsillar T cells and tonsillar mononuclear cells, and in **PAPER IV** allergen-specific proliferation of peripheral blood mononuclear cells was assessed.

PART II.

PAPERS IV AND V are based on a pilot study followed by a double-blind placebo-controlled clinical trial of intralymphatic administration of allergen-specific immunotherapy as treatment for pollen-induced allergic rhinitis (**FIGURE 10**).

Leukocyte numbers and distribution, levels of total and specific serum-Igs as well as various cell surface markers were analyzed in whole blood. Nasal allergen provocations with symptom scoring were performed and nasal lavage fluids were collected and analyzed for inflammatory cells and mediators.

All study subjects were treated with three intralymphatic 0.1 ml injections of either placebo (allergen diluent, ALK Abéllo) or 10,000 SQ-U/ml of a standardized, aluminum hydroxide-adsorbed, depot birch- or grass-pollen vaccine (Alutard®, ALK Abéllo). The injections were given with an interval of approximately four weeks.
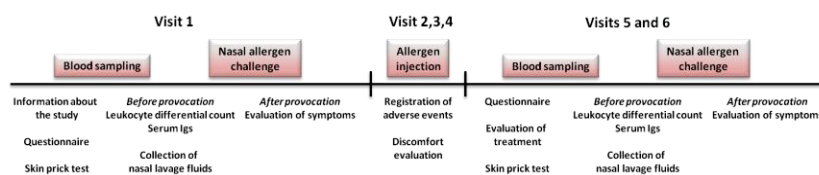

FIGURE 10. Study outline of papers IV and V.

SUBJECT CHARACTERIZATION

In PAPERS IV AND V, recruited patients exhibited moderate to severe birch/grass-pollen induced rhinoconjunctivitis with symptoms including itchy nose and eyes, sneezing, nasal congestion and secretion. Allergy was verified by a positive skin prick test, presence of allergen-specific IgE in serum and a positive nasal provocation test [62]. General contraindications were pregnancy or nursing, wish for pregnancy, autoimmune and collagen disease, cardiovascular disease, current persistent asthma, upper airway disease (non-allergic sinusitis, nasal polyposis), chronic obstructive and restrictive lung disease, hepatic and renal disease, cancer, previous immuno- or chemotherapy, major metabolic disease, alcohol or drug abuse, mental incapability of coping with the study or medication with a possible side-effect of interfering with the immune response.

SKIN PRICK TEST

Skin prick tests were performed using a standard panel of 11 common airborne allergens (ALK Abéllo) including pollen (birch, timothy, mug worth and ragweed), house dust mite (*D. pteronyssinus* and *D. farinae*), molds (*Cladosporium* and *Alternaria*) and animal allergens (cat, dog, horse). Testing was performed on the volar side of the forearm with saline buffer as negative control and histamine chloride (10 mg/ml) as positive control. All included patients presented a wheal reaction diameter of more than 3 mm towards birch or grass after 15 min.

BLOOD TESTING

Blood was obtained from participants at baseline, after completion of treatment, and at the end of the pollen season following end of treatment. Levels of serum allergen-specific IgE and IgG4 were determined using the Phadia CAP system and total and differential leukocyte counts were determined in a Counter LH750/GenS cell counter (Beckman Coulter).

Nasal allergen challenge with symptom scoring

Purified birch or grass allergen extracts (10,000 SQ-U) were delivered in each nasal cavity by a mechanical pump [83, 84]. Subjects were asked to record the occurrence and severity of allergic symptoms (including nasal itch, secretion and congestion) using a scale from 0 to 3 (0= no symptoms, 1= mild symptoms, 2= moderate symptoms and 3= severe symptoms) before and after (5, 15 and 30 min after) the provocation [85].

In **PAPER IV,** study subjects were intranasally challenged before and 4 weeks after ILIT to confirm seasonal allergic rhinitis and to monitor the treatment outcome. In **PAPER V,** nasal provocation tests were performed before and 4 weeks after the treatment as well as at the end of the following pollen season.

Nasal lavage and cell counts

Collection of nasal lavage fluids is a non-invasive method used for studying the inflammatory responses of the nose. In **PAPER IV,** a sterile saline solution was alternately aerosolized into the nostrils after clearing of excess mucus. The nasal fluids were passively collected in a test tube until 7 ml was recovered [83, 84, 86]. The derived fluids mainly contain inflammatory mediators and cells (leukocytes and epithelial cells) [87]. The numbers of cells were counted in a Bürker chamber and the fluids were thereafter centrifuged, separated from the cells and stored at -70°C until analysis. The IL-8 levels in the collected nasal lavage fluids were analyzed with ELISA.

Questionnaires

In **PAPERS IV AND V**, the trial staff recorded the occurrence of local and systemic reactions during the first hour after every intralymphatic injection. Late reactions during the next 24 hours were self-registered by the patients. The study subjects were also asked to estimate the discomfort associated with the injection using an arbitrary scale ranging from 0-10 (0- completely painless and 10- worst experienced pain) and relate it to the discomfort of a venous puncture (more painful, the same pain, less painful, no pain at all).

At the end of the first allergy season after the finished treatment, study subjects were asked to compare their allergic symptoms during the recent pollen season with the symptoms they experienced the season just before treatment. For this, a visual analog scale (VAS) ranging from 0 (unchanged symptoms, no improvement) to 10 (total symptom relief, complete recovery) was used. In addition, patients recorded if their total use of allergy medication (including oral histamines, intranasal corticosteroids, eye drops, inhaled β2-agonists and systemic corticosteroids) changed during the pollen season as a result of the treatment.

STATISTICAL ANALYSES

Statistical analyses were performed using Graphpad Prism. In **PAPERS I-III,** data are presented as mean ± standard error of the mean (SEM) and in **PAPERS IV-V** as mean ± SEM or individual values in combination with a horizontal line representing the mean value. A *p*-value of 0.05 or less was considered statistically significant and *n* equals the number of performed experiments or included subjects.

Distribution of data was assessed using D'Agostino and Pearson omnibus normality test. Normally distributed data were analyzed 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 signed rank tests or Mann-Whitney tests were used for non-parametric data. For more than two paired data sets, one-way repeated measures ANOVA along with Dunnett's or Bonferroni post-test were utilized.

Nonrandom associations between two categorical variables were determined by Fisher exact test.

NOD-LIKE AND RIG-LIKE RECEPTORS IN HUMAN LEUKOCYTES

RESULTS PAPERS I-III

NLRs and RLRs are recently discovered cytosolic PRRs that detect conserved microbial structures and trigger innate immunity. Their expression has been reported in several different cell types, but studies regarding their presence in the human system are limited. Information about their functional activities is even more restricted as synthetic ligands only are available for NOD1 (iE-DAP), NOD2 (MDP), NALP3 (aluminum hydroxide) and RIG-I/MDA-5 (poly(I:C)/LyoVec). In the first part of the thesis, the aim was to characterize expression and functional activities of NLRs and RLRs in subsets of human leukocytes.

At mRNA level, a range of NLRs and RLRs, including NOD1, NOD2, NALP3, RIG-I and MDA-5, was found in B lymphocytes, T lymphocytes and eosinophils (**FIGURE 11**). When tonsillar and peripheral cells were compared, a differential expression was found. In general, blood-derived B cells exhibited higher levels of the receptors than lymphoid cells. However, no differences were seen among naïve, germinal center or memory B cells and neither between Th1 nor Th2 tonsillar cells. Unpublished data from our department show that patients suffering from allergic rhinitis exhibit the same expression levels of NOD-like and RIG-like receptors as healthy individuals without allergy.
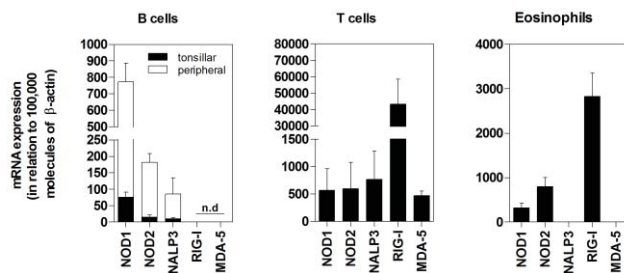

FIGURE 11. mRNA expression of selected NOD-like and RIG-like receptors in human B cells, T cells and eosinophils was determined using real-time RT-PCR. Data are presented in relation to the internal control gene β-actin as $2^{-\Delta Ct} \times 10^5$ and depicted as mean ± SEM.

Protein expression of the receptors was confirmed using immunohistochemistry (**Figure 12**) and flow cytometry. Staining of tonsillar tissue sections revealed presence of NLRs and RLRs both in the germinal centers were B cells reside, and in the adjacent T cell areas. Corresponding proteins were found in purified B and T lymphocytes. Immunostaining of eosinophils showed expression of NOD1 and NOD2, low levels of RIG-I and MDA-5, whereas NALP3 protein was completely absent.
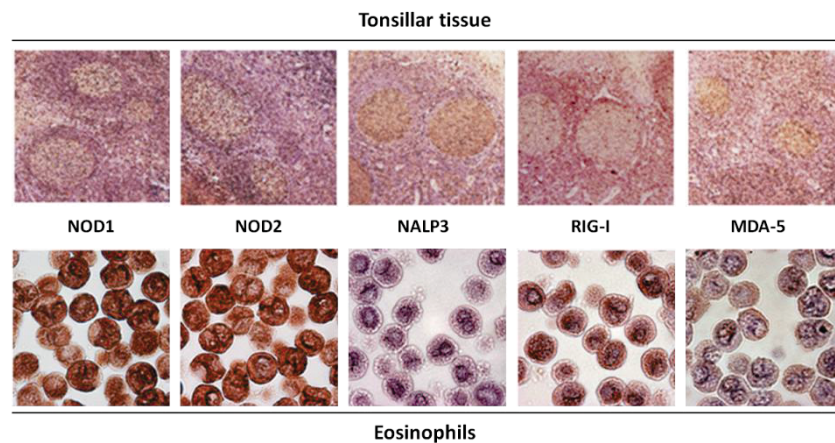

Figure 12. Protein expression of NOD1, NOD2, NALP3, RIG-I and MDA-5 in tonsillar tissue and purified eosinophils was confirmed using immunohistochemistry. Slides were counterstained with haematoxylin and analyzed by microscopy.

To study the functional relevance of NLRs and RLRs, purified cells were cultured in the absence or presence of agonists specific for the receptors. However, due to the ongoing debate regarding the specificity of Alum and poly(I:C)/LyoVec along with the low protein levels of NALP3 and RIG-I/MDA-5 observed, only the functional responses of NOD1 and NOD2 will be presented.

In lymphocytes, none of the ligands were able to induce activation without concomitant signals via the BCR or the TCR, respectively. However, upon stimulation via IgM/IgD or CD3/CD28, an activated B and T cell phenotype was observed as manifested by induced proliferation (**Figure 13**), enhanced viability, up-regulated expression of cell surface markers and increased cytokine secretion. Co-stimulation via CD40L (to mimic the interaction with T cells) failed to induce further B cell activation. The functional responses were generally higher in B cells obtained from blood than tonsillar tissues, which is well in line with the expression profile.
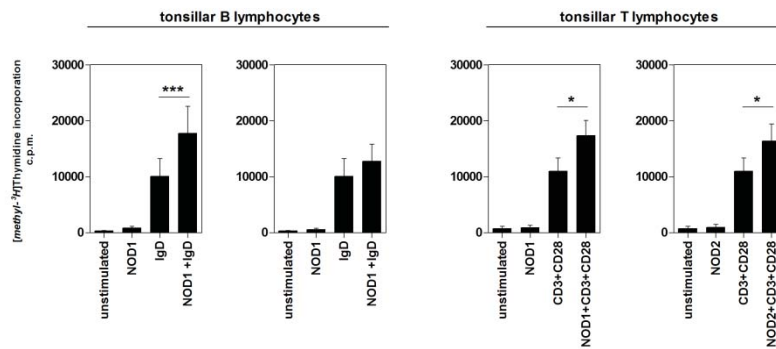
FIGURE 13. Proliferation of tonsillar B and T lymphocytes was determined using [*methyl-*^{3}*H*]Thymidine incorporation. Data are presented as mean ± SEM. * p<0.05, *** p<0.001.

Although all PRRs are involved in the defense against invading pathogens, their cellular locations vary. The NLRs and the RLRs are located in the cytosol, whereas the TLRs are found on the cell surface or within the endosomes. Emerging evidence suggest that the receptor families cooperate to ensure effective detection and clearance of infections. To investigate if such an interaction applies also in B lymphocytes, purified cells were stimulated with iE-DAP or MDP in combination with agonists for TLR1/2 (Pam$_3$CSK$_4$), TLR7 (R-837) or TLR9 (CpG) in the absence or presence of BCR triggering. Results revealed that the NLR and the TLR systems could cooperate to potentiate proliferation (**FIGURE 14**).

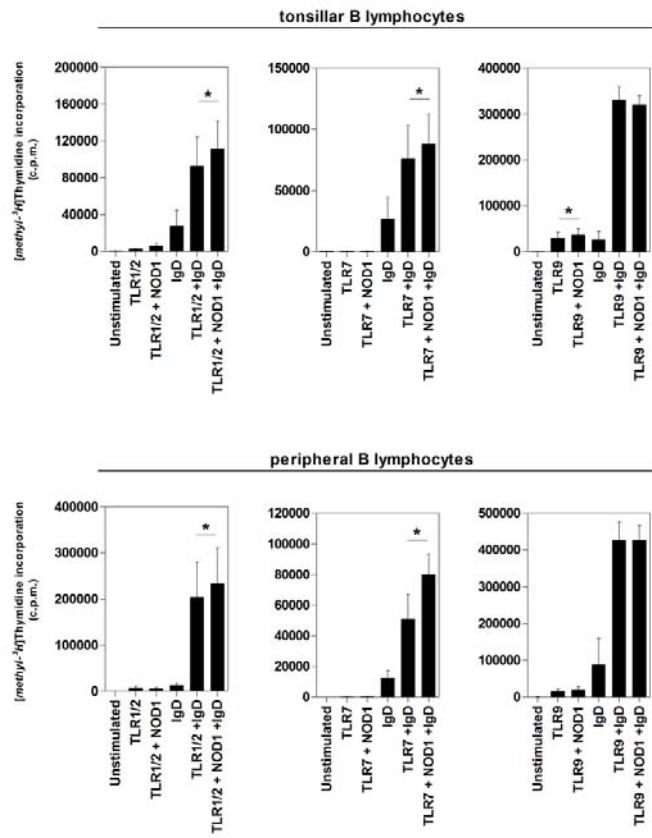**FIGURE 14.** Cooperative effects of the NOD1 ligand and TLR agonists on proliferative responses of tonsillar and peripheral B cells was determined using [*methyl-³H*]Thymidine incorporation. Data are presented as mean ± SEM. * p<0.05.

Unlike lymphocytes that constitute the cellular elements of the acquired immunity, eosinophils are part of the innate immune system. They are involved in the elimination of parasitic infections, but a growing body of evidence suggests that they also have a function in the recognition and killing of bacteria and viruses. Respiratory infections induced by these pathogens are known to promote hyperreactive responses, but the mechanisms behind such microbe-induced exacerbations are still far from understood. One possible explanation could be an activation via the PRR system.

Both the NOD1 and NOD2 ligands were able to activate purified eosinophils, as displayed by changed levels of the activation markers CD11b, CD62L and CD69, induced secretion of IL-8 and enhanced chemotactic migration (**FIGURE 15**). This is in agreement with the observed mRNA and protein expression.
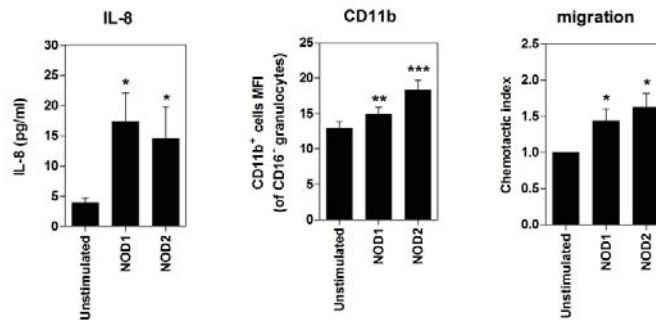
FIGURE 15. IL-8 secretion, regulation of CD11b and chemotactic migration of eosinophils was assessed using ELISA, flow cytometry and a migration assay, respectively. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To evaluate possible involvement of these receptors in microbe-induced exacerbations of allergic airway inflammation, eosinophils were also stimulated with NOD agonists in the absence and presence of Th1- and Th2-like cytokines. In the presence of cytokines regulating Th2 immunity (IL-5 and GM-CSF), the NLR induced activation was augmented, which was evidenced by increased levels of inflammatory mediators (**FIGURE 16**).
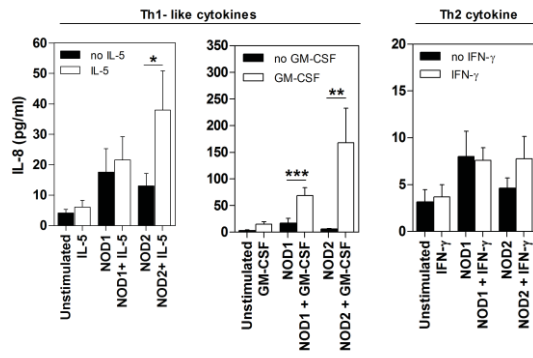
FIGURE 16. After priming of NOD1 and NOD2 responses using IL-5, GM-CSF and IFN-γ, secretion of IL-8 by eosinophils was determined using ELISA. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

COMMENTS PAPERS I-III

In the first part of the thesis, the presence of a range of NLRs and RLRs is demonstrated in human primary leukocytes. We also provide evidence for their importance in the recognition and clearance of bacterial infections and suggest a role for these receptors as a link between respiratory infections and exacerbations of allergic diseases.

Although PRRs belong to the innate branch of the immune system, we amongst others have previously demonstrated an active role of Toll-like receptors in lymphocytes [12, 13]. Encouraged by these observations, presence of the related NOD-like and RIG-like receptors was assessed in B and T lymphocytes. Lymphocytes were found to exhibit a range of NLRs and RLRs and differences in expression profiles and functional activities were observed between blood-derived and tonsillar cells. However, the evaluation of functionalities was limited since ligands are only available for NOD1 (iE-DAP), NOD2 (MDP), NALP3 (aluminum hydroxide) and RIG-I/MDA-5 (poly(I:C)/LyoVec). Also, it is worth mentioning that there is an ongoing discussion regarding the specificities of some of these agonists.

The NOD proteins are involved in the recognition of peptidoglycans where NOD1, specifically respond to iE-DAP and NOD2 to the L-D isomer of MDP. In agreement, both lymphocytes and eosinophils were activated by these agonists while being completely unresponsive to the control compounds D-glutamyl-lysine (iE-Lys) and the D isomer of MDP. In contrast, NALP3 is known to respond to microbial products and host-derived danger signals. There are recent studies demonstrating a role for the NALP3 inflammasome in the recognition of the adjuvant Alum, but it seems to be unclear whether this interaction is NALP3-specific.

RIG-I and MDA-5 take part in the recognition of intracellular viruses and these receptors can, according to the manufacturer, be activated by the synthetic ligand poly(I:C)/LyoVec. However, this ligand consists of poly(I:C)s complexed to the transfection agent LyoVec[TM] allowing intracellular delivery. Therefore, its effects via endosomal TLR3 cannot be completely ruled out.

The different responses of tonsillar and peripheral B cells might be related to divergent cellular compositions of the two compartments and also to variations in the antigenic load. There is a larger proportion of resting B cells in the periphery than in lymphoid tissues and obviously blood lacks germinal center B cells [88]. Also, tonsillar lymphocytes are constantly exposed to pathogens, which might affect the receptor expression. In contrast to studies on TLRs showing that the differentiation process and microbial infections influence the expression profiles [13, 89-92], the NLR levels were unaffected by both the differentiation stage of the B cell and ongoing tonsillar infections.

The onset of adaptive immunity is traditionally believed to be dependent on the recognition of pathogens by innate immune cells expressing PRRs. Over the last decades, this view has been modified as an increasing number of studies have demonstrated that various PAMPs (such the TLR9 ligand CpG) can directly activate B cells even in the absence of co-stimulatory signals [13]. In contrast to some TLR ligands, stimulation with the NOD1 and NOD2 agonists alone is not sufficient to induce lymphocyte activation. However, when the lymphocytes are provided with the first activation signal (via the BCR/TCR), the cells are able to respond unspecifically to certain microbial components via their PRRs. Therefore, it seems reasonable to update the traditional "two-signal model" of lymphocyte activation to include signals provided via the PRR system.

Emerging numbers of studies have demonstrated a close interaction between different members of the PRR families. In agreement, we demonstrate cooperative effects of NLRs and TLRs in B lymphocytes. During bacterial infections, invading pathogens most probably engage several different PRRs simultaneously. It has been proposed that the cytosolic PRRs function as a backup system for the recognition of bacteria and viruses having escaped the initial detection via cell surface TLRs or released from the endosomal compartments. The cooperation between these receptor families might provide the immune system with a valuable defense mechanism ensuring effective detection and clearance of invading pathogens.

Although the PRRs are supposed to protect the host from infections, these receptors have been increasingly implicated in various human disorders [33, 34]. During the last decades, the prevalence of allergic disorders has increased [59]. This might be related to the so-called hygiene hypothesis that suggests that a reduced microbial exposure during childhood is linked to increased susceptibility to develop allergic diseases. A well-known example of this is the exposure to LPS (recognized via TLR4) in farmer's children, who appear to be protected against allergy development [35, 36]. For the related NLRs, such a link is under investigation but polymorphisms in the *NOD1* and *NOD2* genes have already been associated with allergic disorders, asthma and atopy-related traits [37-40]. In addition, our group has shown that patients suffering from symptomatic allergic rhinitis exhibit lower levels of NOD1 and NALP3 than healthy controls and allergics outside pollen season [93]. However, in lymphocytes (unpublished observations) and eosinophils obtained from healthy individuals and allergic subjects, no differences in NLR expression or activation profiles have been observed.

Eosinophils are important in early innate immune responses, but they also have a role in allergic disorders affecting inflammation, hyperresponsiveness and remodeling of the airways [41, 43, 94]. In the allergic airways, bacteria and viruses can be as potent as allergens in promoting hyperreactive responses and aggravating allergic symptoms [95-97]. The mechanisms behind these microbe-induced exacerbations are still far from understood, but one explanation could be an activation of eosinophils through their PRRs. We demonstrate that eosinophils can

be activated via the NLR system and that the responses are augmented in the presence of Th2-promoting cytokines. This is in agreement with a previous study from our laboratory that shows TLR-induced responses of eosinophils to be enhanced upon pre-treatment with IL-5 and that patients with allergic rhinitis respond more strongly to TLR-stimulation than healthy individuals [98, 99]. Together, these results make it tempting to speculate that these receptors constitute a pathway linking allergic exacerbations with respiratory infections. However, the responses induced by the NLR agonists alone are of a fairly low magnitude, especially as compared to stimulation with whole bacteria. Nonetheless, these receptors may function as fine-tuners in the mounting of appropriate immune responses. In a biological system, it is most likely the combined actions of several receptors and mediators that are of importance and not the effects exerted by the individual receptors alone.

In conclusion, the first part of this thesis demonstrates functional NLRs and RLRs in both lymphocytes and eosinophils. The active role of PRRs in cells traditionally belonging to the adaptive immune response emphasizes the important cross-talk between the two branches of the immune system. In addition, we show that PRR-induced eosinophil responses can be enhanced in the presence of cytokines associated with an allergic phenotype. This together with the knowledge that eosinophils are accumulated in hyperreactive airways further highlight the possible role of PRRs not only in virus-induced but also in bacteria-induced exacerbations of allergic respiratory diseases.

INTRALYMPHATIC ALLERGEN-SPECIFIC IMMUNOTHERAPY

RESULTS PAPERS IV-V

Allergen-specific immunotherapy can be used to treat patients with allergic rhinitis and asthma. The clinical improvement following treatment is extensively documented but despite its proven efficacy, only a minority of all patients eligible for treatment undergoes therapy. One reason for this is the long treatment duration of conventional SCIT. However, as immune responses are initiated within secondary lymphoid organs, intralymphatic allergen administration has recently emerged as a less time-consuming alternative to conventional treatment. In the second part of the thesis, the aim was to evaluate ILIT for treatment of pollen-induced allergic rhinitis.

Using ultrasound guidance, the superficial inguinal lymph nodes were identified as hypoechoic nodules (**FIGURE 17**), and 1,000 SQ-U of allergen extract was slowly injected. The patients reported that the injections were virtually painless and not associated with greater discomfort than a venous puncture. Treatment-induced reactions were in most cases restricted to minor swelling, redness and itch in conjunction to the injection site. No severe adverse events were reported. However, it is worth mentioning that one patient who was not included in the initial studies (a placebo patient from the first study that later was given active treatment), reported local redness and swelling in a somewhat greater area around the injection site as a result of the first allergen injection. This reaction was recorded during the first 30 min period after the injection and it was resolved within an hour.
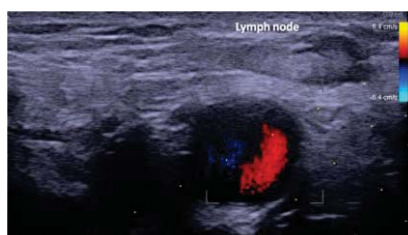
FIGURE 17. Ultrasound image of an inguinal lymph node.

Actively treated patients showed improved symptoms upon allergen challenge along with a decreased inflammatory response in the nose as manifested by reduced numbers of inflammatory cells and IL-8 levels in nasal lavage fluids (**FIGURE 18**). Additionally, the clinical improvements were accompanied with increased levels of allergen-specific IgE, whereas no significant effects were seen in allergen-specific IgG4 levels.
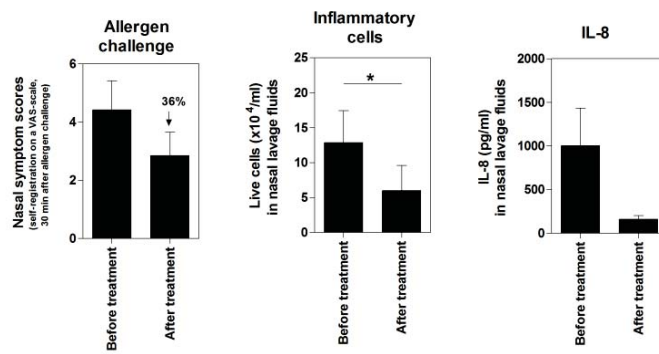
FIGURE 18. Changes of clinical parameters after treatment with intralymphatic allergen-specific immunotherapy. Data are presented as mean ± SEM. * $p < 0.05$.

Patients receiving active treatment also reported an improvement of their seasonal symptoms (**FIGURE 19**) and a reduction in their use of allergy medication.
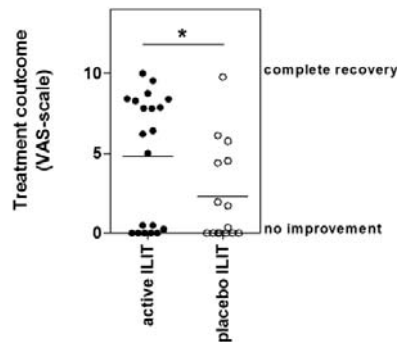
FIGURE 19. Patient-recorded treatment outcome. The study subjects were asked to rank the change in their allergic symptoms as compared to the pollen season preceding intralymphatic treatment. * $p < 0.05$.

44.

After the evaluation of the safety and the clinical effects of ILIT, the next step was to identify cellular and molecular changes mediated by the treatment. Among the actively treated patients, ILIT promoted both short-term and long-term activation of neutrophils and eosinophils as shown by an up-regulated expression of cell surface markers (**FIGURE 20**).
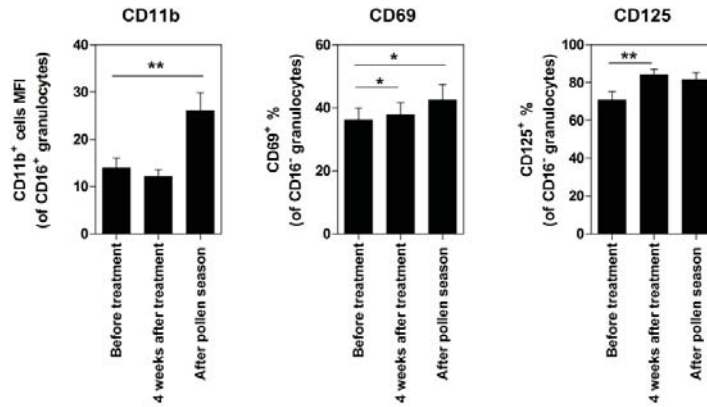
FIGURE 20. Activation of granulocytes after intralymphatic allergen-specific immunotherapy. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$.

In contrast to the enhanced activation seen among granulocytes, a reduced percentage and activation of Th2 cells were observed along with fewer regulatory T cells (**FIGURE 21**).
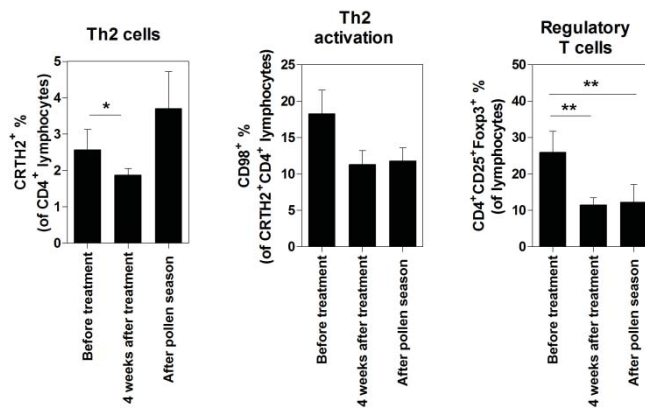
FIGURE 21. Activation of lymphocytes after intralymphatic allergen-specific immunotherapy. Data are presented as mean ± SEM. * p<0.05, ** p<0.01.

As can be seen in **FIGURE 19**, the actively treated patients can be divided in two subgroups, one consisting of 12 patients reporting a clear improvement of their seasonal symptoms and another subgroup of 8 patients that did not experience any relief. The former group also exhibited a marked reduction of reported symptoms in response to the nasal allergen challenge, whereas the latter described no such change (**FIGURE 22**). The patients in the improved actively treated subgroup reported that they were able to reduce their intake of allergy medication during pollen season.
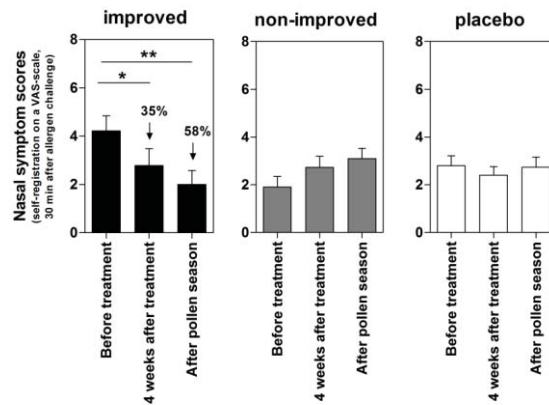
FIGURE 22. Allergic symptoms upon allergen provocation among improved subjects, non-improved patients and placebo controls. Data are presented as mean ± SEM. * p<0.05, ** p<0.01.

46.

COMMENTS PAPERS IV-V

The potency of intralymphatic administration of vaccines has been studied in various models [78, 100-103] and the first reported use of ILIT for treatment of allergic rhinitis in humans by Senti and colleagues [78] attracted the attention of many allergologists as the intralymphatic route appeared to be an efficient alternative to conventional SCIT. In the second part of the thesis, we have evaluated the effects of ILIT for treatment of birch- or grass-pollen induced allergic rhinitis. We demonstrate that ILIT is safe for the patient, reduces allergic symptoms upon nasal allergen challenge and during pollen season, decreases the inflammatory response of the nose and affects the activation profiles of peripheral lymphocytes and granulocytes.

We have used the same approach as Senti and colleagues and administered three intralymphatic injections of 1,000 SQ-U of allergen with 4 weeks intervals [78] as this time interval has been proven to be sufficient to allow formation of antigen-specific B cell responses and for affinity maturation of memory B cells [104, 105]. The allergen injected in the present investigation is the same as we use for regular SCIT treatment, but the administered dose is about 100 times lower. For SCIT the maintenance dose is 100,000 SQ-U and it has been reported that up to 30% of patients treated with conventional SCIT suffer from systemic reactions after allergen administration [68]. The ILIT dose of 1,000 SQ-U might be one explanation for the good safety profile seen with this administration route. Only one of our patients (who was not part of the initial study) suffered from a moderate local reaction and otherwise the treatment-induced adverse events were restricted to redness, itch and swelling in close proximity to the injection site.

In the inguinal regions, the lymph nodes are often found just a few millimeters under the skin surface and they can easily be detected using ultrasound. The sparse sensory innervation of these nodes can explain the lack of discomfort during and after the injections [106]. Although intralymphatic injections can be administered within minutes by skilled physicians, there are some technical aspects that need to be considered. Once the lymph nodes have been identified, 0.1 ml allergen should be injected into a lymph node with an average size of 0.5-1.0 cm. In addition to the requirement of ultrasound equipment, the procedure necessitates simultaneous work with both hands, holding the probe in one and injecting the allergen with the other. Even when using a fine needle for administration, it can be difficult to penetrate the capsule surrounding the lymph node. This could lead to inadequate administration of the allergen near instead of in the lymph node. There is also a risk for allergen "leak out" when the needle is removed. These factors might limit the broad use of this treatment route at the allergy clinics.

Reported clinical effects of SCIT include e.g. improved quality of life, diminished seasonal allergic symptoms and medication use as well as reduced skin prick test reactivity [63-65]. In our studies, we demonstrate that patients receiving active ILIT experience improvement of their seasonal allergic symptoms in combination with decreased nasal symptoms upon allergen challenge. In addition, there seems to be a reduction in the nasal inflammatory response which might reflect a skewing towards a more non-allergic, non-inflammatory cytokine profile.

It is tempting to speculate that ILIT and SCIT might share some humoral and cellular hallmarks. It is well-known that SCIT can promote an initial increase of allergen-specific IgE followed by a long-term decline in favor for IgG4 [60, 107, 108]. For ILIT, an induction of allergen-specific serum IgE was observed, whereas no effects on IgG4 production were found. On a cellular level, SCIT reduces the activation of Th2 cells in favor of Th1 responses and induces activation of regulatory T cells [61, 64, 65, 109]. Regarding the effects on peripheral innate immune responses, little information is available. Among actively treated patients, ILIT was shown to promote continuous activation of granulocytes, which most likely is due to the retention of alum at the injection site. Although this continuous stimulation might cause a local chronic inflammation, it appears as if this innate immune activation is required for tolerance induction.

In the adaptive part of the immune system, ILIT promoted an initial activation of Th cells followed by reduced activation of Th2 lymphocytes and regulatory T cells. Although ILIT appears to share some markers of SCIT, some defining features seem to differ. However, it is possible that the altered administration route induces at least partly different mechanisms without interfering with the clinical outcome. Further studies will be needed to identify which treatment-induced changes that induce beneficial effects and which events that are merely a marker for such effects.

In summary, the second part of this thesis demonstrates that ILIT constitutes a safe and effective treatment strategy for pollen-induced allergic rhinitis. From an economical, societal and health economic perspective, ILIT might constitute a new paradigm in allergen-specific immunotherapy as the treatment requires fewer treatment visits, fewer injections and considerably lower allergen doses than conventional SCIT.

SUMMARY AND CONCLUSIONS

➢ A differentiated expression of NOD1, NOD2, NALP1, NALP3 and NAIP was found in tonsillar and blood-derived B lymphocytes. The expression was generally higher in peripheral B cells. Stimulation with the NOD1 and NOD2 agonists alone failed to induce B cell activation but concomitant triggering of the BCR increased proliferation, induced expression of cell surface markers and prolonged survival. Peripheral B cells were activated by the NOD1 and NOD2 ligands, whereas tonsil-derived B lymphocytes responded solely to NOD1. Additionally, NLR ligands were found to enhance TLR-induced proliferation of B cells, whereas co-stimulation via CD40L failed to induce activation.

These results indicate that during infection, B lymphocytes can recognize pathogens via NLRs, which point to a mechanism ensuring effective clearance of microbes by bridging innate and adaptive immunity.

➢ Tonsillar T lymphocytes expressed a broad range of NLRs and RLRs, comprising NOD1, NOD2, NALP1, NALP3, NAIP, IPAF and RIG-I, MDA-5 and LGP-2, respectively. Stimulation with the NOD1, NOD2 and RIG-I/MDA-5 ligands in combination with TCR triggering induced proliferation, enhanced secretion of IL-2 and IL-10 and up-regulated expression of CD69 of tonsillar mononuclear cells. In purified tonsillar CD3$^+$ T cells, stimulation of the TCR promoted proliferative responses but they were not further enhanced by the cognate ligands.

These data support the idea of an important role of innate immune receptors in the adaptive branch of the immune system. Also, T cells appear to play a role in the response towards bacterial and viral infections.

➢ Prominent expression of NOD1 and NOD2 along with low levels of RIG-I and MDA-5 was found in eosinophils. Stimulation with the NOD1 and NOD2 ligands induced activation mediated via the NF-κB pathway. This was evidenced by secretion of IL-8, prolonged survival, regulated expression of cell surface markers and chemotactic migration. The NOD2 ligand also increased secretion of EDN and NOD1- and NOD2-induced activation was augmented by IL-5 and GM-CSF, but not by IFN-γ.

These findings suggest the NLR system as a new pathway for eosinophil activation and as the responses are enhanced in the presence of cytokines regulating Th2 immunity, the NLRs might have a role in linking respiratory infections with allergic diseases.

> ILIT was found to be safe for the patients and none of the injections elicited any severe adverse events. Among patients receiving active treatment, increased levels of allergen-specific IgE and induced peripheral T cell activation were seen. A clinical improvement of nasal allergic symptoms upon challenge was recorded along with a decreased inflammatory response in the nose as characterized by lowered levels of IL-8 and reduced levels of inflammatory cells in nasal lavage fluids. Additionally, the patients reported an improvement of their seasonal allergic disease. No such changes were seen in the placebo group.

> Our data highlight ILIT as a safe and effective administration route for treatment of pollen-induced allergic rhinitis and this treatment strategy might constitute a less time-consuming and more cost-effective alternative to conventional SCIT.

> On a cellular level, active ILIT enhanced activation of granulocytes and reduced activation of Th2 lymphocytes and regulatory T lymphocytes. Among actively treated patients reporting successful treatment and improvement of their allergies, reduced symptoms upon challenge were demonstrated in combination with reduced use of pharmaceutical treatment products.

> These results indicate that the clinical effects of ILIT are accompanied by alterations in the activation profiles of peripheral leukocytes and reveal new insights on mechanisms underlying the beneficial effects of the treatment.

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FUTURE PERSPECTIVES

One of the key findings in immunology research was the essential role of PAMP recognition by PRRs in the induction of immune responses made by Charles Janeway [110]. Since then, these receptors have been extensively studied and new insights about their diverse roles in health and disease are constantly being provided. It is now apparent that the PRRs are important in the induction of both innate and the adaptive immunity and we amongst others have demonstrated roles in eosinophils, neutrophils and epithelial cells as well as in B and T lymphocytes [12-17, 23, 111]. Therefore, it seems reasonable that the two branches of the immune system should be viewed as complementary and cooperating.

In most studies, one PRR in a specific cell type in a certain environment has been investigated, but in order to fully understand the complex roles of PRRs, the interaction between various PRR members, cells and mediators needs to be assessed. Single-cell systems in which an isolated cell type is stimulated via a single receptor by a specific agonist poorly represent the natural biological environment. Therefore, advances in the area of cooperation between different PRRs are likely of importance. Interestingly, synergistic effects between NLRs and TLRs were observed in B lymphocytes, which strengthen the idea that the combined actions of the receptor families are needed to ensure effective clearance of infections. Failure in this defense can, during certain circumstances, lead to the development of allergic diseases and also promote exacerbations of allergic diseases. The presented relation between PRRs and Th2-promoting cytokines in eosinophils seems to corroborate such a notion. However, the link between microbes, PRRs and allergy will need to be further explored.

Upon infection or an allergic reaction, microbial components or allergens are effectively drained to secondary lymphoid organs where adaptive responses are initiated. When this process is mimicked in terms of delivery of allergen directly to the lymph node during the course of ILIT, protective immune responses are quickly induced. Already after three injections tolerance against alum-adjuvanted allergens can be observed but the mechanisms behind these beneficial effects need to be further dissected. PRRs might play a role in this process as the immunostimulatory properties of Alum recently have been attributed to the interaction with the NALP3 inflammasome [21, 22]. Indeed, the PRR system is being extensively utilized in the development of new types of vaccines based on its ability to skew the immune system in the desired direction.

As compared to SCIT, ILIT has a better safety profile, requires considerably lower allergen doses but also fewer treatment visits and allergen injections. This opens up for greater flexibility for the patients but also creates new opportunities for dissection of the protective mechanisms behind allergen-specific immunotherapy. From a socioeconomic perspective, the benefits of ILIT stand clear. In Sweden, the entire ILIT protocol with three injections is estimated to cost approximately 800

EUR/patient and allergen. This could be compared to approximately 9000 EUR/patient and allergen for SCIT which requires more than 50 injections over a 4 year period [112]. To this, the indirect costs associated with the long SCIT treatment need to be added. In addition to providing a safe and effective treatment route for allergic diseases, ILIT might therefore represent a new paradigm in allergen-specific immunotherapy.

Taken together, we hope that the results presented in this thesis can constitute a foundation for using PRRs and ILIT in the development of new and more effective treatment strategies for inflammatory airway diseases and for prevention of allergic exacerbations.

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APPENDICES