

# Role of Toll-like receptors in airway inflammation

Anne Månsson

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Laboratory of Clinical and Experimental Allergy Research  
Department of Otorhinolaryngology, Malmö University Hospital  
Lund University, Sweden

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## List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals (I-VI):

- I.** Månsson A, Adner M and Cardell LO. Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis. *Respir Res.* 2006, 7:36
- II.** Månsson A, Adner M, Höckerfelt U and Cardell LO. A distinct Toll-like receptor repertoire in human tonsillar B cells, directly activated by Pam<sub>3</sub>CSK<sub>4</sub>, R-837 and CpG-2006 stimulation. *Immunology.* 2006, 118:539
- III.** Månsson A and Cardell LO. Role of atopic status in Toll-like receptor (TLR)7 and TLR9-mediated activation of human eosinophils. *J Leukoc Biol.* *in press*
- IV.** Månsson A, Fransson M, Adner M, Benson M, Uddman R, Björnsson S and Cardell LO. TLR3 in human eosinophils: functional effects and decreased expression during allergic rhinitis. *Int Arch Allergy Immunol; cond. accepted*
- V.** Månsson A, Bachar O, Adner M and Cardell LO. Nasal CpG oligodeoxynucleotide administration induces a local inflammation response in non-allergic individuals. *Allergy, in press*
- VI.** Månsson A, Bachar O, Adner M, Björnsson S and Cardell LO. Rush immunotherapy against birch pollen-induced allergic rhinitis induces leukocyte phenotype alterations. *submitted*

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## **Abstract**

Toll-like receptors (TLRs) are germline-encoded pathogen-recognition receptors that detect different microbial structures and activate the immune system. The human TLR family presently comprises ten members (TLR1-TLR10), each with distinct properties. Although the TLRs protect the host from infection, they have become increasingly implicated in the pathogenesis of various inflammatory diseases, including asthma and allergy. The aim of this thesis was to characterize the presence and function of TLRs in different cell types involved in upper airway inflammation, with special emphasis on recurrent tonsillar infection and allergic rhinitis. We also sought to map cellular and molecular changes occurring as a result of allergen-specific immunotherapy.

In the first two studies, a distinct expression of TLRs was demonstrated in subsets of B and T lymphocytes isolated from human tonsils. The expression levels seemed to be affected by ongoing tonsillar infection. This was most evident in T cells. By studying the functional activity of the expressed receptors, it was found that the corresponding ligands promoted B cell activation. These data indicate that TLRs have a direct role not only in activation of the innate part of our immune system, but also of the adaptive branch.

In the next two studies, presence of the virus-sensing receptors TLR3, TLR7 and TLR9 was shown in eosinophils. Stimulation with the cognate ligands gave rise to an activation manifested by an increased eosinophil survival, release of damaging mediators and ability to migrate to inflamed areas. It was also found that the response was higher in atopic patients with allergic rhinitis than in healthy subjects, and in the presence of a Th2-like cytokine milieu. Thus, activation of eosinophils via these TLRs might engender a link between viral infection and allergic exacerbations.

In the following work, the effect of nasal administration with the TLR9 agonist CpG was described in healthy subjects and in patients with allergic rhinitis. CpG exposure resulted in an increased nasal resistance, production of nasal nitric oxide, infiltration of inflammatory cells and release of pro-inflammatory Th1-type cytokines. These responses were generally more marked among the healthy subjects, most likely due to the ongoing persistent inflammation seen in the allergic group. Taken together, these results show that CpG induces a local inflammation that skews the immune response towards a Th1-like phenotype.

Lastly, cellular and molecular effects induced by rush immunotherapy were analyzed in patients with birch pollen-induced allergic rhinitis. The clinical improvements seen as a result of three years of treatment were mirrored by several changes in antibody and receptor levels. Of these, a reduction in serum allergen-specific IgE antibodies, correlating with cell-bound IgE and IgG expression, along with a general activation of T cells were most prominent. The novel findings in this report included a shift in monocyte populations and decrease in levels of TLR2 and TLR4.

To summarize, the results presented in this thesis demonstrate a role for TLRs in lymphocyte and eosinophil biology, as well as an involvement in tonsillar and allergic inflammation. Although their exact role in airway inflammation is unclear, the TLR system holds great promise in the development of new therapeutic alternatives for allergic and inflammatory diseases.

## Abbreviations

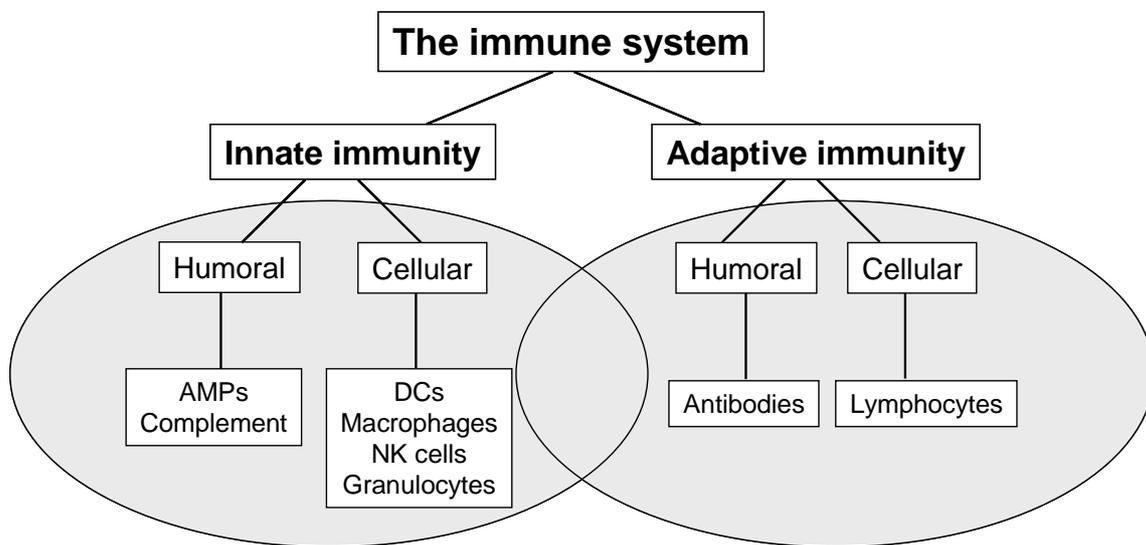
AHR	airway hyperresponsiveness
AMP	antimicrobial peptide
ANXV	annexin V
APC	antigen-presenting cell
BCR	B cell receptor
Ct	cycle threshold
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ds	double-stranded
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EPO	eosinophil peroxidase
FSc	forward scatter
GC	germinal center
GM-CSF	granulocyte macrophage-colony stimulating factor
HEV	high-endothelial venule
HI	<i>Haemophilus influenzae</i>
HRP	horse-radish peroxidase
IFN	interferon
Ig	immunoglobulin
IDC	interdigitating DC
IL	interleukin
IRF	IFN regulatory factor
LRR	leucine-rich repeat
LPS	lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MFI	Mean fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NAL	nasal lavage

NAR	nasal airway resistance
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NK	natural killer
NLR	Nod-like receptor
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PMA	phorbol myristate acetate
poly(I:C)	polyinosinic-polycytidylic acid
PRR	pathogen-recognition receptor
RIT	rush immunotherapy
RLR	Rig-like receptor
SEM	standard error of the mean
ss	single-stranded
SSc	side scatter
SIT	specific allergen immunotherapy
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
Th1	Th type 1
Th2	Th type 2
TIR	Toll IL-1 receptor
TLR	Toll-like receptor
Treg	T regulatory cell

## Introduction

### 1. The immune system

Protection against invading pathogens requires a range of different cells and molecules, all of which are crucial for mounting an immune response. Traditionally, the vertebrate immune system can be divided into two separate branches; the innate and the adaptive (Fig. 1). The innate immune system is present from birth and is responsible for the most basic forms of protection, such as physical and chemical barriers. Cellular and molecular actions are also included in this defense, which comprises dendritic cells (DCs), macrophages, natural killer (NK) cells and granulocytes along with antimicrobial peptides (AMPs) and components of the complement system (1, 2). The adaptive immune system is more sophisticated, mounting a highly specific response combined with the formation of an immunological memory. Antibody-producing B cells, and T cells with various effector functions constitute the backbone of this system (3). There is a clear and necessary crosstalk between the two systems, and it is the combined actions of the rapid innate and the slower but specific adaptive immunity that enable the host to effectively clear infections.



**Figure 1.** Conceptual outline of the two mutually supportive branches of the vertebrate immune system.

#### 1.1 The innate immune system

The innate immunity protects the host immediately upon infection, and it exercises its full effect even before the onset of adaptive immunity. It encompasses a range of host defense

mechanisms. The non-cellular part includes simple barrier functions that prevent the entry of pathogens, and the complement cascade that destroys the pathogen directly or call them to attention of phagocytes (4). AMPs like lysozyme, defensins and psoriasin are additional components of this system (5-7). The cellular elements include DCs, monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils) and NK cells, along with epithelial surfaces that form the interface between the microbe and the host (4, 8). Cells of the innate immunity are able to recognize conserved microbial structures, termed pathogen-associated molecular patterns (PAMPs) by using evolutionary conserved proteins called pathogen-recognition receptors (PRRs). The human PRRs include the Toll-like receptors (TLRs) recognizing bacteria, viruses, fungi and protozoa; the Nod-like receptors (NLRs) that sense bacteria; and the retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) identifying viruses (9, 10).

The activation of the innate immune response is a prerequisite for the onset of adaptive immunity. This stems from the need for antigen-presentation mediated by antigen-presenting cells (APCs), predominantly DCs. Uptake and phagocytosis of microbes is facilitated by receptor-mediated recognition of microbial molecules (11). In this communication, the PRR-PAMP interaction plays a central role. Upon recognition of pathogens, the DC undergo a differentiation programme called DC maturation, characterized by up-regulation of major histocompatibility complex (MHC) molecules containing pathogen-derived peptide fragments, up-regulation of co-stimulatory molecules such as CD40, CD80 (B7.1) and CD86 (B7.2), and increased production of cytokines. Together, these events allow an optimal interaction with naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells (2, 3, 11-14).

## **1.2 The adaptive immune system**

B and T lymphocytes are the effector cells of the adaptive immunity, responsible for cell-mediated and humoral immune responses as well as generation of immunological memory that provides protection against re-infections. Humoral immunity is provided by antibodies produced by B cells, which recognize, neutralize and target pathogens for elimination. The cellular component is provided by T cells that kill infected cells and eliminate reservoirs of infection. Unlike cells of the innate immune system that utilize a fixed set of receptors, recognition of pathogens by B and T cells is flexible, as they undergo a recombination of their antigen receptors in order to create novel and unique receptors for each antigen (4).

### **1.2.1 B lymphocytes**

The main function of B cells is to produce antibodies against invading pathogens. Antibodies, encoded by heavy and light immunoglobulin (Ig) genes, constitute the B cell receptor (BCR). They can be either secreted or cell surface-bound, and are divided into different isotypes; IgM, IgD, IgA, IgE and IgG. They recognize tertiary (three-dimensional) structures of the antigen. At first, B cells produce pentameric IgM, but then undergo recombination, isotype switching and affinity maturation, leading to production of IgG, IgE or IgA subtypes along with selective survival of B cells with the highest affinity for the specific antigen (4).

### **1.2.2 T lymphocytes**

The T cell receptor (TCR) recognizes processed antigens presented as peptides bound to class I or class II MHC proteins. T cells can distinguish between the two MHC classes, and are classified accordingly as CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) recognizing peptides presented on MHC class I, or CD4<sup>+</sup> T helper (Th) cells responding to MHC class II peptides (8). CD4<sup>+</sup> cells can be further phenotypically and functionally divided into Th type 1 (Th1), Th type 2 (Th2) and T regulatory (Treg) cells. Very simplified, Th1 cells induce cell-mediated immunity by secreting interferon (IFN)- $\gamma$ , activating macrophages and stimulating CTLs. In contrast, Th2 cells produce interleukin (IL)-4, IL-5 and IL-13, and stimulate B cells to produce antibodies, in particular IgE, thereby inducing humoral immunity (4, 15, 16). Tregs act to suppress the responses of other T cells either by cell-cell contact or by the production of IL-10 or transforming growth factor (TGF)- $\beta$  (17, 18).

## **2. The upper respiratory tract**

The human respiratory tract can be divided into the upper and the lower airways. The former comprises the nose, the paranasal sinuses, pharynx and larynx. The latter is composed of the trachea, bronchi and lung alveoli (19). This chapter will focus on the defense mechanisms of the upper respiratory tract.

### **2.1 The nose**

The nasal mucosa constitutes the first line of defense against airborne particles as most breathing occurs through the nose. The nose is constantly exposed to both pathogenic and non-pathogenic antigens. These antigens are removed non-immunologically by the first

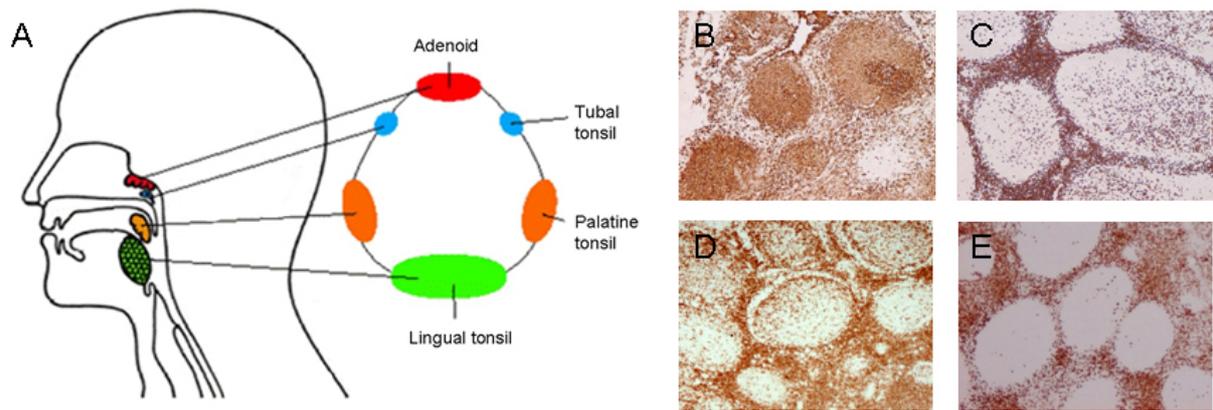
defense layer of the mucosa, comprising mucus, ciliated epithelial cells, AMPs and glycoproteins/lysozymes. If the antigens pass this layer, both non-specific and specific immunological mechanisms set in. The former consists of phagocytic cells like neutrophils and macrophages, as well as activation of the complement system. The latter is composed of antibodies, mainly of IgA and IgG isotypes, and immunocompetent cells present in the nasal mucosa. Failure of these defense mechanisms might result in upper respiratory tract infections and/or allergy (20, 21).

## **2.2 Lymphoid organs in the respiratory tract**

The so called Waldeyer's ring is located at the opening of the respiratory and digestive tract. It consists of the adenoids, the paired tubal tonsils, the lingual tonsils and the paired palatine tonsils (Fig. 2A). They are all secondary lymphoid organs belonging to the mucosa-associated lymphoid tissue (MALT). Due to the anatomical location, they are continuously exposed to foreign airborne and ingested antigens (22, 23).

### **2.2.1 The palatine tonsils**

The palatine tonsils, from now on referred to simply as tonsils, are sites where innate immunity leads to the onset of adaptive immunity (24). They are covered by a non-keratinized stratified squamous epithelium supported by an underlying capsule of connective tissue containing blood vessels, nerves and lymphatics. Also, high-endothelial venules (HEV) are present, which are necessary for the migration of B and T cells from the blood stream into the tonsils. The tonsils contain four specialized lymphoid compartments; the reticular crypt, which increases the surface area for antigen stimulation, the extrafollicular area, the mantle zones of lymphoid follicles, and the follicular germinal centers (GCs) (22, 25). Leukocytes, predominantly lymphocytes are found in all the tonsillar compartments. Seven tonsillar B cell subsets can be identified, including naïve B cells, GC founder cells, GC B cells and memory cells (26, 27), along with T cells ( $CD4^+ > CD8^+$ ), macrophages, follicular DCs and interdigitating DCs (IDCs) (23). The localization of B and T cells in the GCs and the extrafollicular T cell zones, respectively, is shown in Fig. 2B-E.



**Figure 2.** (A) Waldeyer's ring with the adenoids, the paired tubal tonsils, the paired palatine tonsils and the lingual tonsil. Figure adapted from (23). (B-E) Immunohistochemistry of human palatine tonsils demonstrating the typical distribution of (B) B cells (CD20<sup>+</sup>), (C) T cells (CD3<sup>+</sup>), (D) T helper cells (CD4<sup>+</sup>), and (E) cytotoxic T cells (CD8<sup>+</sup>) (magnification  $\times 40$ ).

Naïve B and T lymphocytes circulate between the blood and the secondary lymphoid organs in search of antigens. Once an antigen is encountered in the tonsil, the passing lymphocytes are retained. The antigen-specific B cells undergo clonal expansion. Some cells differentiate into low-affinity IgM antibody-secreting plasma cells. Others undergo somatic hypermutation, isotype switching and affinity maturation, leading to the generation of memory B cells and plasma cells secreting high-affinity IgA, IgG or IgE antibodies (22, 27-30). This process takes 3-5 days (21), and is T cell-dependent. B cells that have recognized an antigen by the BCR are stimulated in the extrafollicular zones by activated CD4<sup>+</sup> T cells to which IDCs have presented processed antigen. They express the necessary co-stimulatory molecule CD40L that binds to CD40 on the B cells. In return, B cells deliver specific antigenic peptides to the TCR and co-stimulatory CD80/CD86 that interact with CD28 on the T cells (22, 31). This model is known as the two-signal theory of lymphocyte activation (32, 33).

### Recurrent tonsillitis and tonsillar hypertrophy

Tonsillar diseases are common health-related problems in the general population (34). Hyperplasia of the tonsils is most frequent among children, whereas recurrent tonsillitis becomes more widespread in teens (35). The indications for tonsillectomy are usually multiple episodes ( $\geq 4$  times a year) of tonsillitis for more than two years, and/or hyperplastic obstructing tonsils which means that they are close to the midline (34, 35). Tonsillitis has generally been considered as an infection caused by *Streptococcus pyogenes*, especially group

A  $\beta$ -hemolytic streptococci. During recent years, *Haemophilus influenzae* (HI), which previously has been regarded as a normal habitant, has been associated with the occurrence of tonsillitis (35, 36). However, high isolates of HI has also been found in hyperplastic tonsils (37). Determining the microbial flora in completely normal tonsils is a difficult task as the bacteria reside in the deep crypts that are not accessible unless the tonsils are surgically removed. Thus, tonsils can be used as a good model for studying lymphoid organs, or cells thereof, and enable comparisons between inflamed/infected and healthy non-infected tissues.

### **3. Allergic airway inflammation**

#### **3.1 The allergic immune response**

The allergic immune response is an IgE-mediated hypersensitivity reaction towards innocuous antigens, referred to as allergens. The development of an allergic response can be divided into a sensitization and an elicitation phase. During the former, the allergen is recognized by the immune system as harmful, which results in an IgE production by B cells. The IgE molecules in turn bind to high-affinity Fc receptors on mast cells and basophils. Upon second exposure (elicitation phase), crosslinking of allergen and cell-bound IgE occurs, leading to mast cell and basophil degranulation and subsequent release of histamine, leukotrienes and prostaglandins. These soluble mediators are responsible for the early phase response, characterized by vasodilatation and increased vascular permeability (leading to edema and nasal blockage), mucus secretion (causing rhinorrhoea) and stimulation of afferent nerves (promoting itching and sneezing). The late phase response, occurring 4-24 hours later, is manifested by Th2 cell activation, tissue eosinophilia and infiltration of effector cells (38, 39).

IgE production and eosinophilia are highly T cell-dependent. Upon allergen recognition, the so called DC type 2 (named after their ability to induce Th2-type T cell responses) is critically important (40). Th2 cells secrete a range of cytokines that affect the allergic response at several levels. IL-4 and IL-13 stimulate the production of IgE by B cells, IL-5 promotes growth, differentiation and activation of eosinophils, IL-9 induces growth and activation of mast cells, and granulocyte macrophage-colony stimulating factor (GM-CSF) affects growth of eosinophils and activation of APCs (38, 41).

### **3.1.1 Eosinophils**

Classically, eosinophils are involved in the immune response against helminth infections. However, they also have a central role in allergic diseases, affecting allergic airway inflammation, airway hyperresponsiveness (AHR) and airway remodeling by the release of tissue-damaging mediators (42-44). Elevated numbers is known to correlate with the disease severity (45). Eosinophils mature in the bone marrow, are released into the circulation where they make up 1-3% of the circulating leukocytes, and are eventually recruited to various tissues (46). Their development is governed by IL-3, IL-5 and GM-CSF. IL-5 is the most specific eosinophil cytokine, responsible for growth, differentiation and activation. Under homeostatic conditions, eosinophils are predominantly found in the gastrointestinal tract, but upon allergic inflammation, they are attracted to the airways by the actions of Th2 cytokines (43, 47, 48). Eosinophils are bi-lobed granulocytes that possess secondary granules containing four toxic basic granule proteins; major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). In addition, they release leukotrienes, prostaglandins and a range of cytokines and chemokines (43). These molecules have pro-inflammatory effects, including up-regulation of the adhesion system, and modulation of cellular trafficking, vascular permeability and smooth muscle constriction (45, 49). The biological role of eosinophils remains a matter of controversy. Although peripheral blood and tissue eosinophilia are hallmarks of parasitic infection, the way this is of benefit for the host is unclear. Similarly, it should be stressed that in the allergic setting, the eosinophils are not beneficial for the host in any way (47). It all boils down to whether accumulation of eosinophils is a cause of allergic disease, or whether they are just bystander cells that can be used as a clinical marker (48, 50).

### **3.2 Allergic rhinitis**

Allergic rhinitis is a common global disease, affecting 10-25% of the population. The prevalence has increased dramatically during the last decades, particularly in countries with a western lifestyle (51-53). According to the 2008 ARIA (Allergic Rhinitis and its Impact on Asthma) document, rhinitis is defined as an inflammation of the lining of the nose, characterized by nasal symptoms including anterior or posterior rhinorrhea, sneezing, blockage and/or itching. It is the most common form of non-infectious rhinitis and it is associated with an IgE-mediated immune response against allergens (54). Allergic rhinitis can further be subdivided into persistent and intermittent, previously termed perennial and

seasonal. The persistent form is characterized by symptoms present for  $\geq 4$  days a week and during  $\geq 4$  weeks consecutively, mostly induced by indoor allergens like house dust mites and animal dander. Otherwise, it is intermittent, usually caused by pollen (53-55).

The etiology of allergic rhinitis is influenced by several factors, including genetic susceptibility and environmental factors such as route of exposure, dose and characteristics of the allergen (52). There is also a clear relationship between allergic rhinitis and asthma. Epidemiological studies have established that about 19-38% of patients with allergic rhinitis have coexistent asthma, whereas more than 80% of patients with allergic asthma have concomitant rhinitis symptoms. This link suggests that these two airway disorders are manifestations of the same disease and is called the "integrated airway hypothesis" (56, 57).

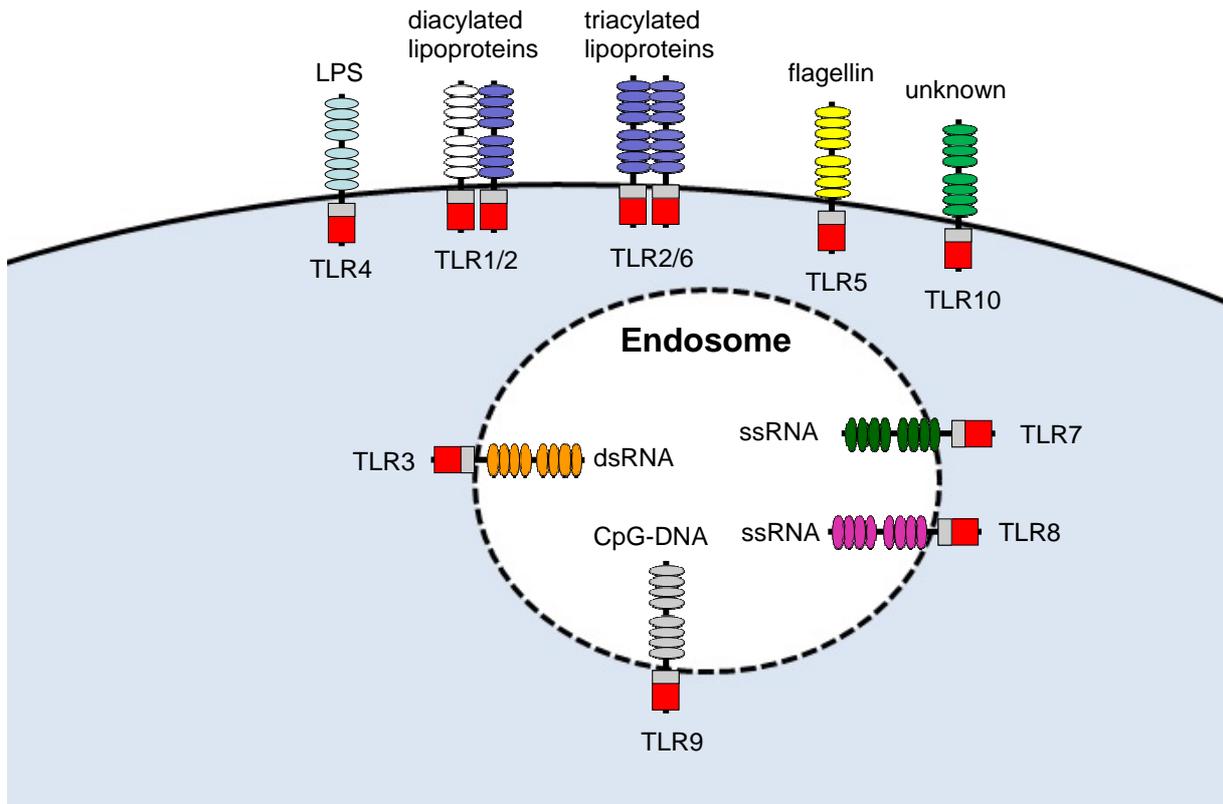
### **3.2.2 Specific allergen immunotherapy (SIT)**

Most patients suffering from rhinitis can be treated with conventional pharmacotherapy, including oral antihistamines, intranasal glucocorticosteroids, often in combination with eye drops containing antihistamines or mast cell stabilizers. Antileukotrienes can be recommended as single therapy, but more often as "add on" when the patient is suffering from concomitant allergic symptoms from the lungs and/or skin (54). In addition to this, specific allergen immunotherapy (SIT) can be used for treatment of carefully selected, highly motivated patients with a moderate to severe form of IgE-mediated disease. It is especially preferred when specific allergens cannot be avoided and/or conventional therapy is not tolerated (51). While other treatments only take care of the symptoms of allergic disease, SIT is the only available treatment that can modify the natural course of the disease by reducing the sensitivity to allergens. A three-to-four-year individually tailored regimen induces long-term remission following discontinuation, and prevents onset of new sensitization and reduces disease progression of rhinitis to asthma in children (51, 58-60). A recently approved sublingual tablet (Grazax<sup>®</sup>), containing a grass pollen extract, appears to be similarly effective, with few side effects, and can be self-administered at home (61). Different protocols for injection immunotherapy can be used, all starting with a low dose of allergen, followed by a gradually increase in dose until the maintenance dose is reached (58). The up-dosage phase can be achieved within weeks to months (conventional regime), over a few consecutive days (rush), or in a single day (ultra-rush). However, the primary concern with the faster protocols is the higher rate of systemic reactions (58, 62, 63).

The protective effects of SIT are being attributed to changes in antibodies, cellular events and in cytokines (64). To name a few, a reduction in serum allergen-specific IgE in favor of IgG<sub>1</sub> and IgG<sub>4</sub> antibodies have been reported, along with a suppressed infiltration of effector cells, a shift from a Th2- to a Th1-biased immune response and an induction of Tregs (51, 52, 60, 65).

#### **4. Toll-like receptors (TLRs)**

The discovery of the TLR family began with the identification of the Toll protein in *Drosophila*. In 1997, a human Toll homologue was described by Medzhitov and colleagues, later designated TLR4 (66). Since then, additional proteins have been added to the human TLR family, now comprising ten members (TLR1-TLR10). TLRs are type I integral membrane glycoproteins, characterized by leucine-rich repeat (LRR) motifs in the recognition domain and a Toll IL-1 receptor (TIR) domain that make up the cytoplasmic signaling domain (67-70). TLR1, -2, -4, -5, -6 and -10 are positioned at the cellular surface where they predominantly recognize proteins, lipoproteins and polysaccharides from bacteria. In contrast, TLR3, -7, -8 and -9 are located in intracellular endosomal compartments where they sense different forms of nucleic acids from viruses (13, 71-73). Each TLR recognizes specific PAMPs. TLR2 acts as a heterodimer in concert with TLR1 or TLR6, and mediate responses to e.g. lipoproteins, lipoteichoic acids, peptidoglycan and zymosan. Depending on the heterodimer, TLR2 can discriminate between diacyl (TLR1/2) and triacyl (TLR2/6) lipopeptides. There are indications that TLR10 also has the ability to form heterodimers with TLR1 and TLR2, but the specific ligands have not yet been identified. TLR3 is involved in the recognition of double-stranded (ds) RNA from viruses and the synthetic dsRNA polyinosinic-polycytidylic acid (poly(I:C)). TLR4 is the main lipopolysaccharide (LPS) receptor. However, it is dependent on the cooperation with LPS binding protein, CD14 and MD-2. TLR5 recognizes flagellin, an essential component of flagella found in both Gram-positive and Gram-negative bacteria. TLR7 and TLR8 mediate responses to single-stranded (ss) viral RNA, but they also respond to immunomodulatory drugs such as loxoribine and imidazoquinolones, including imiquimod (R-837) and resiquimod (R-848). TLR9 respond to bacterial and viral DNA containing unmethylated CpG motifs (3, 13, 68, 74-76). An overview of the cellular location of the TLRs and their ligands is presented in Figure 3.



**Figure 3.** Outline of the human TLR family. The surface-exposed TLRs detect protein structures mainly from bacteria, whereas the endosomal members sense nucleic acids primarily from viruses.

#### 4.1 Presence of TLRs in the airways

Expression of TLRs has been found in various tissues and cells of the airways, including tonsils and adenoids (77, 78), the nasal mucosa (79), epithelial cells (80, 81), and smooth muscle cells (82, 83). In addition, presence of TLRs have been demonstrated in different cell types such as mast cells, NK cells, DCs, macrophages, monocytes, granulocytes and lymphocytes (67). In Table 1, the TLR expression in different immunocompetent cells is summarized.

**Table 1.** TLRs in different human immune cells. Summarized from references (84-95).

	pDC	mDC	Monocytes	Neutrophils	Eosinophils	B cells	T cells
<b>TLR1</b>	+	++	++	+	-/+	++	+
<b>TLR2</b>	-	++	+++	+++	+++	+	+
<b>TLR3</b>	-	++	-	-	-/+	-	+
<b>TLR4</b>	-	-	+++	++	-/+	-	-/+
<b>TLR5</b>	-	+	++	+	+++	-	++
<b>TLR6</b>	++	++	+	+	-	-/+	-
<b>TLR7</b>	+++	-/+	-/+	-/+	+++	+++	+
<b>TLR8</b>	-	+++	++	-/+	-	-	-
<b>TLR9</b>	+++	-	-	+++	++	+++	+
<b>TLR10</b>	+	+	-	+	+	+++	+

+, ++, +++ and - indicated the intensity of the expression of each TLR. -/+ indicate that there are contrasting data. pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells.

## 4.2 TLR signaling

The activation of TLR signaling pathways starts from the cytoplasmic TIR domain (96). Downstreams of TIR, adaptor molecules are required, including MyD88, TIRAP (TIR-domain-containing adaptor molecule), TRIF (Toll-receptor-associated activator of interferon) and TRAM (TRIF-related adaptor molecule). The most commonly used pathway is the MyD88-dependent pathway, utilized by all TLRs except TLR3. A complex series of events culminate in the activation of nuclear factor (NF)- $\kappa$ B, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, which regulate transcription of pro-inflammatory genes. In contrast, the MyD88-independent pathway, selectively used by TLR3 (and to some extent TLR4), uses TRIF as an adaptor and activates IFN regulatory factor (IRF)-3. However, as for MyD88-dependent signaling, NF- $\kappa$ B and MAPK are ultimately activated (13, 69, 97, 98).

## 4.3 TLRs in allergic disease

In spite of the protective effects of TLRs upon infection, faulty TLR signaling and polymorphisms in the TLR genes are increasingly implicated in the pathogenesis of allergic diseases. One explanation has been related to the so called hygiene hypothesis. This hypothesis states that a lack of early childhood exposure to infectious agents, symbiotic microorganisms (e.g. gut flora) and parasites increases the susceptibility to allergic diseases by modulating the development of the immune system. During normal circumstances,

infectious stimuli (via TLRs) lead to Th1-mediated responses. A reduction in TLR activation reduces the Th1 responses, resulting in unrestrained Th2-mediated immunity. This might explain the increase in allergic diseases seen in the western world during the last decades (99-101). The most well-studied example of this is exposure to LPS (via TLR4), though the effects are highly dependent on the time, dose and route of exposure (102). LPS during the early years can decrease the incidence of atopic asthma later in life (103), whereas the disease condition is aggravated in patients suffering from asthma following LPS exposure (104). Supporting this hypothesis, LPS exposure and atopy rate is inversely correlated in farmers' children (105). Farmers' children also have higher levels of TLR2 and CD14 (an LPS co-receptor) than non-farmers' children, suggesting a higher microbial burden in the former group (106). In line with this, polymorphisms in the *TLR2* and *CD14* genes have been associated with increased susceptibility to develop allergic diseases and/or its severity (104, 107). Also, some groups have found genetic variations in the *TLR4* gene, whereas others have not (108). Another TLR ligand that has gained a lot of interest in this field is CpG (via TLR9), because of its ability to skew the immune system towards a Th1 profile that counteracts the allergic Th2 response (70, 109-111). CpG is currently being used in both pre-clinical and early clinical studies as a novel therapeutic agent to treat allergic asthma and related diseases (112-114).

## The present investigation

### ***Aims***

The aim of this thesis was to investigate how TLR stimulation affects different cell types involved in upper airway inflammation, with special emphasis on allergic rhinitis. We also sought to identify cellular and molecular changes occurring as a result of specific allergen immunotherapy.

The specific aims were to:

- Analyze the expression of TLR1-TLR10 in purified human CD4<sup>+</sup> and CD8<sup>+</sup> tonsillar T cells, and compare the TLR expression in cells obtained from patients with recurrent tonsillitis and tonsillar hyperplasia (*paper I*).
- Study the expression and functional relevance of TLR1-TLR10 in purified human tonsillar B cells, and determine if the expression is altered during the B cell development or by ongoing tonsillar infection (*paper II*).
- Explore the role of virus-recognizing TLRs in purified human eosinophils, focusing on the role of atopic predisposition and presence of Th2-type cytokines (*paper III-IV*).
- Investigate the effects of nasal CpG administration, as a mimic of upper airway infection, in healthy subjects and in patient with allergic rhinitis (*paper V*).
- Monitor the leukocyte phenotypes of patients with birch pollen-induced allergic rhinitis undergoing rush immunotherapy (RIT) treatment (*paper VI*).

## **Methods**

A general theme for the first four papers included in this thesis is the use of antibody-coupled magnetic beads to isolate different human leukocyte subsets from peripheral blood and tonsils, including B cells, T cells and eosinophils, for various *in vitro* studies. The cells were subsequently analyzed by flow cytometry, real-time PCR, ELISA and Luminex Multiplex Immunoassay. The last two papers are more clinically angled, focusing on differences between either healthy individuals and patients with allergic rhinitis or allergic patients before and after treatment with RIT. The main methods used during the clinical investigations were flow cytometry to study cell surface markers on leukocytes from whole blood, rhinomanometry to measure nasal resistance, ELISA to determine cytokine secretion in nasal lavage (NAL) fluid, along with measurements of nasal and pulmonary nitric oxide (NO) levels. A more detailed description of the study groups and the specific techniques used is given in brief below.

## **Human study populations**

All studies included in this thesis are conducted on human material. The different study populations included are as follows:

- In paper I, tonsils were obtained from 39 children undergoing tonsillectomy due to recurrent infection or tonsillar hyperplasia.
- In paper II, patient material included tonsils from 33 patients and peripheral blood from 6 healthy volunteers.
- In paper III, peripheral blood was obtained from healthy subjects and patients with seasonal allergic rhinitis outside pollen season.
- Paper IV is based on bone marrow samples and peripheral blood acquired from 9 healthy volunteers and 21 patients with seasonal allergic rhinitis of whom 10 were sampled outside, and 11 during pollen season.
- In paper V, 10 healthy controls and 10 patients with allergic rhinitis examined out of season were included.
- Paper VI analyzed blood samples from 10 patients with birch pollen-induced allergic rhinitis before and after RIT treatment.

All studies were performed at Malmö University Hospital after approval from the Ethics Committee of Lund University. A written informed consent was obtained from all participants.

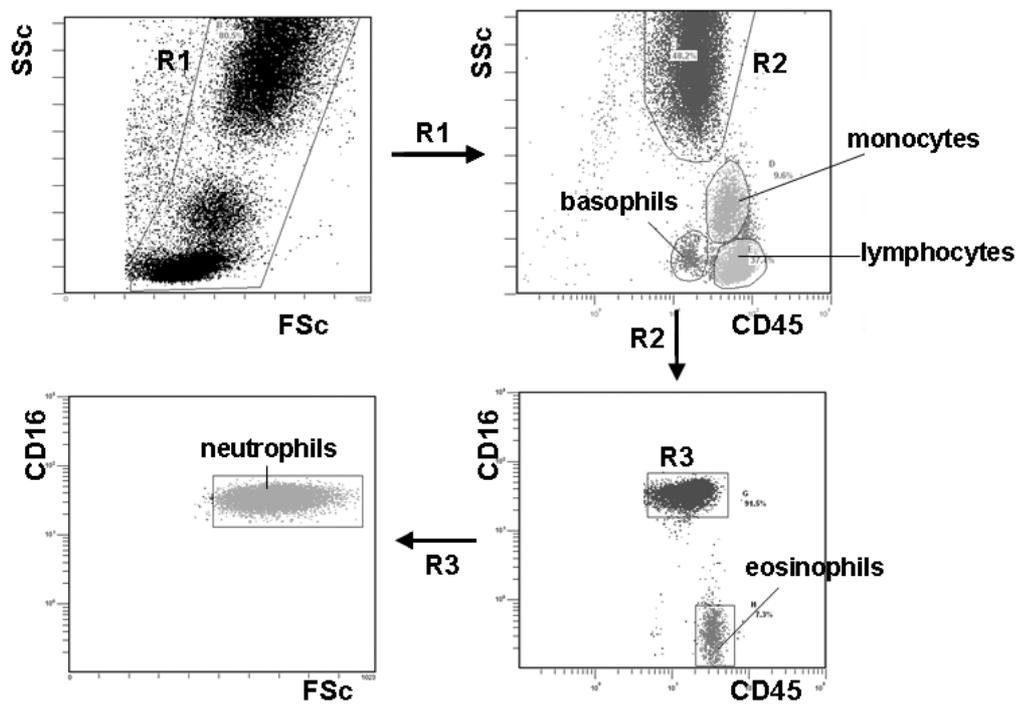
### **Real-time PCR**

Real-time PCR was used to quantitatively determine the mRNA expression of TLR1-TLR10 in isolated cells from peripheral blood and tonsils (paper I, II, IV). In a first step, cells are lysed in RLT buffer supplemented with 2-merkaptoethanol (1%). RNA is extracted using RNeasy Mini Kit (Qiagen), and the concentration and quality is determined by spectrophotometry based on the wavelength absorption ratio (260/280 nm). Thereafter, the mRNA is reversely transcribed into cDNA by use of Omniscript reverse transcriptase kit (Qiagen) with oligo-dT. In the present thesis, the PCR assays were performed on a Smart Cycler (Cepheid) using TaqMan Universal PCR Master Mix, No AmpErase UNG and Assay-on-Demand Gene Expression products (Applied Biosystems) containing unlabeled primers and MGB probe (FAM™ dye-labeled) (115). An advantage of using probe-based systems of this sort is that non-specific amplification is prevented. The gene expression of TLR1-TLR10 was assayed using the comparative cycle threshold (Ct) method where the relative amount of TLR mRNA is determined by subtracting the Ct value for the gene of interest with the Ct value for the housekeeping gene  $\beta$ -actin ( $\Delta$ Ct). Data are expressed in relation to 100,000 molecules of  $\beta$ -actin as  $100,000 \times 2^{-\Delta Ct}$ . Real-time PCR is a good alternative to easily quantify and compare expression of genes in different samples. However, there are clear limitations associated with this method. As the gene expression not always correlates with the actual proteins expressed, results should be interpreted with caution. Additional analyses, using alternative methods, are usually required to get the whole picture.

### **Flow cytometry**

Flow cytometry is a method that distinguishes individual cells based on their physical and chemical properties. It enables multi-parametric and quantitative analyses in real-time. Briefly, a beam of light is directed towards a stream of fluid containing the cells of interest, labeled with fluorescent antibodies. The flow cytometer is equipped with different detectors, including forward scatter (FSc), side scatter (SSc) and one or more fluorescence detectors, that subsequently detect the light passing through the cells. The combination of scattered and

fluorescent light picked up by the detectors provides information about the cellular size, granularity and phenotype. Different cell types from tonsil and peripheral blood can easily be distinguished based on their FSc, SSc, CD45 and CD16 properties (Fig. 4) (79). In this thesis, flow cytometry by means of fluorescently labeled antibodies was used to study extra- and intracellular expression of TLRs in tonsillar, peripheral or bone marrow-derived cells (paper I-V), as well as regulation of cell surface activation markers on various lymphoid and circulating immune cells (paper II-IV, VI). In addition, staining with Annexin V (ANXV) and Propidium iodide (PI) was utilized for detection of viable and apoptotic cells (paper III). ANXV binds to phosphatidylserine that is translocated from the inner to the outer leaflet of the plasma membrane during apoptosis, and PI is a nucleic acid binding dye used to discriminate apoptotic from dead cells (116). All analyses were performed using a Coulter Epics XL flow cytometer together with Expo32 ADC software (Beckman Coulter) for data acquisition and analysis.



**Figure 4.** Separation of different leukocyte subsets in peripheral blood. Gate R1 represents live cells. By plotting SSc versus CD45 from R1, granulocytes (R2), monocytes, lymphocytes and basophils can be distinguished. By further plotting CD16 versus CD45 from R2, neutrophils (R3) and eosinophils can be separated. Neutrophils from R3 can be further enriched based on CD16 and FSc expression.

## **Immunohistochemistry**

In immunohistochemistry, antibodies are used to detect proteins in tissue or cell preparations. In the present thesis, enzymatic staining was used to investigate the morphological localization of TLRs in paraffin-embedded tonsillar sections (paper I and II). The B and T cell markers CD20 and CD3 were also used in order to determine the cellular location. CD20<sup>+</sup> cells are located mainly within the GCs, whereas CD3<sup>+</sup> cells predominantly reside in the T cell zones of the tonsils. The currently used protocol is based on detection of an antigen by a primary mouse or rabbit antibody, followed by addition of a secondary goat anti-mouse or goat anti-rabbit antibody conjugated with a horse-radish peroxidase (HRP)-labeled polymer. After incubation with a substrate, 3,3'-diaminobenzidine or 3-amino-9-ethylcarbazole, a color reaction occurs that stains the tissue brown or red, respectively. Positive immunoreactivity can be identified by bright field microscopy. Immunohistochemistry using enzymatic staining is a good qualitative technique to visualize a protein within a tissue or cell preparation. However, the reliability of performing quantitative measurements of protein expression in different samples is limited. Also, this method usually requires low antibody dilutions (range 1:10-1:100), which increases the risk of cross-reactivity with other peptides.

## **ELISA**

ELISA is a sensitive and specific method for quantification of antibodies and antigens present in e.g. cell culture supernatants, serum, plasma and NAL fluids. The presently used ELISA:s are of so called "sandwich" type. In simple terms, a microplate is pre-coated with a capture antibody against the antigen of interest. Samples and standards (of known concentrations) are added, which bind to the capture antibody. Thereafter, a detection antibody that in turn binds to the antigen is applied, followed by an enzyme-linked secondary antibody. In the last step, a substrate is added that converts the enzyme into a detectable color that can be measured using a microplate reader. The amount of antigen present in the sample is proportional to the intensity of the color. In this thesis, commercial ELISA plates to detect IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IFN- $\alpha$ , IFN- $\gamma$ , macrophage inflammatory protein (MIP)-1 $\alpha$  and EDN have been utilized (paper II-V).

### **Luminex Multiplex Immunoassay**

The Luminex Technology can be used for measurements of up to 100 different antigens in a single sample. It enables studies of extracellular antigens, including cytokines and chemokines, along with phosphorylated and total levels of intracellular analytes and activation of transcription factors. In the present thesis, the presence of total and phosphorylated MAPKs (Akt, p38 and JNK) was investigated (paper IV). Briefly, beads with defined spectral properties conjugated to analyte-specific capture antibodies are incubated together with samples and standards. After washing, analyte-specific biotinylated detector antibodies are added that bind to epitopes on the analyte. After another washing, Streptavidin conjugated to a fluorescent protein is added, which in turn binds to the biotinylated detector antibody. After a last washing step to remove unbound fluorescent Streptavidin, the beads are analyzed using the Luminex instrument. By monitoring the spectral properties of the beads and the amount of fluorescent protein, the concentration of each analyte can be determined from the standard curve (117). The Luminex technology is a cost-effective and sample-saving method as compared to e.g. ELISA. However, these advantages are at the expense of the sensitivity.

### **The nose model**

The upper airways are relatively easy to access and offer the opportunity to perform provocations with allergens and/or different inflammatory mediators in conjunction with repeated sampling in the nose and lungs with a minimum risk and discomfort for the patient. In this way, the nose can be used as a tool to study the effects *in vivo* both in the upper and lower airways. This has prompted us to use allergic rhinitis as an experimental model for allergic inflammation. For this purpose, we are using techniques such as rhinomanometry, measurements of nasal and pulmonary NO levels and cellular infiltration and cytokines in NAL fluid. In the present thesis, this model was used to study the effects of CpG administration on airway inflammation (paper V).

### **Rhinomanometry**

Rhinomanometry is a technique for evaluating the degree of nasal obstruction by measuring the airflow and pressure within the nose during respiration. Increased nasal airway resistance (NAR) reflects an increased thickness of the nasal mucosa. In the present study, active

anterior rhinomanometry was used. A pressure catheter is connected to one nostril. The flow is measured by a pneumotachograph via an air-tight face mask connected to a rhinomanometer (GM Instruments). An X-Y recording of a pressure-flow curve is presented on a computer, and total NAR is presented as  $V_2$  according to the polar coordinate of Broms (118). Each nostril was measured separately, and the combined values for both nostrils are referred to as  $V_{2tot}$ .

### ***Nitric oxide measurements***

NO plays a key role in the non-specific host defense by its antimicrobial activity and in the pathophysiology of airway disease. NO is detectable in exhaled air of normal individuals, but is increased during asthma, periods of allergic rhinitis and respiratory tract infections (119). The levels of nasal and pulmonary NO levels were determined using a NIOX<sup>®</sup> NO analyzer (Aerocrine AB). During the measurements, air is continuously drawn into the analyzer at a constant sampling rate. Fractional exhaled NO is sampled in exhaled air from the lungs via the mouth using a tube, and from the nose using a nasal olive. The background NO value is automatically subtracted from the NO measurements (120, 121).

### ***Nasal lavage***

Collection of NAL fluid is a non-invasive method to study mechanisms involved in airway inflammation. In the design used in the present investigation (paper V), the nose is first cleared from excess mucus. A room temperatured sterile saline solution (0.9% NaCl) is then sprayed into both nostrils alternately. The fluid is allowed to return passively into a test tube until 7 ml is collected (122). The obtained fluid contains mainly epithelial cells and neutrophils, inflammatory mediators from the mucosal surface exudation and secretion (123). Advantages with the current protocol for NAL fluid collection is a high cellular exchange and quality as well as a good reproducibility. In contrast, a drawback is that the fluid is diluted and may have to be concentrated depending on the application. Despite great efforts, we have not been able to find a good reference protein.

### **Statistics**

In all papers, data are presented as mean  $\pm$  standard error of the mean (SEM), and  $n$  equals the number of subjects or experiments performed. Normally distributed data were analyzed using

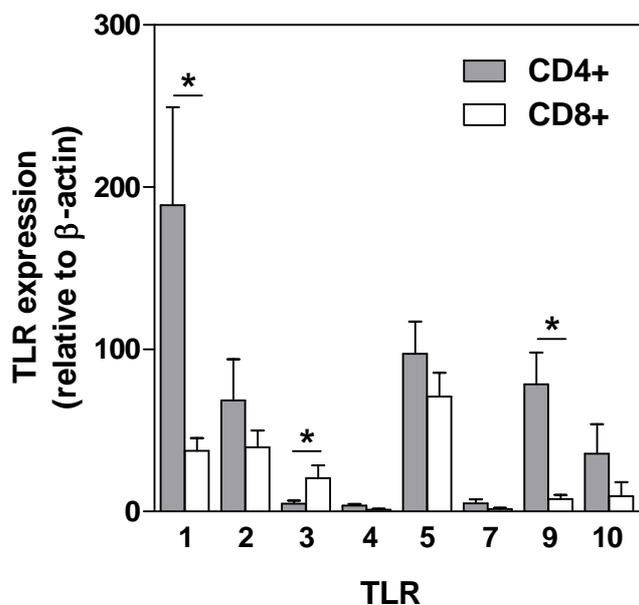
parametric tests. For comparisons of two sets of data, unpaired or paired *t*-test was used. For comparison of more than two data points, one-way Repeated Measures ANOVA with Dunnett's post test was employed. Data not normally distributed were analyzed with non-parametric Kruskal-Wallis test in combination with Dunn's Multiple comparison test. In all cases, p values < 0.05 were considered statistically significant.

## Results and comments

### TLR expression in tonsillar T cells (paper I)

The tonsils are sites where innate immunity closely can interact with the adaptive immunity; the latter a process mediated by B and T lymphocytes (24). Expression of TLRs has been demonstrated in the tonsils and in different immune cells, including monocytes, DCs and B cells (77, 89). However, expression of TLRs in human T cells has been debated. The present study aimed to investigate the expression profile of TLR1-TLR10 in different subsets of purified tonsillar T cells. Also, the ability of TLRs to respond to different PAMPs might be of importance for mounting immune reactions in the tonsils. Therefore, TLR expression was compared *ex vivo* in T cells from recurrently infected and hyperplastic tonsils.

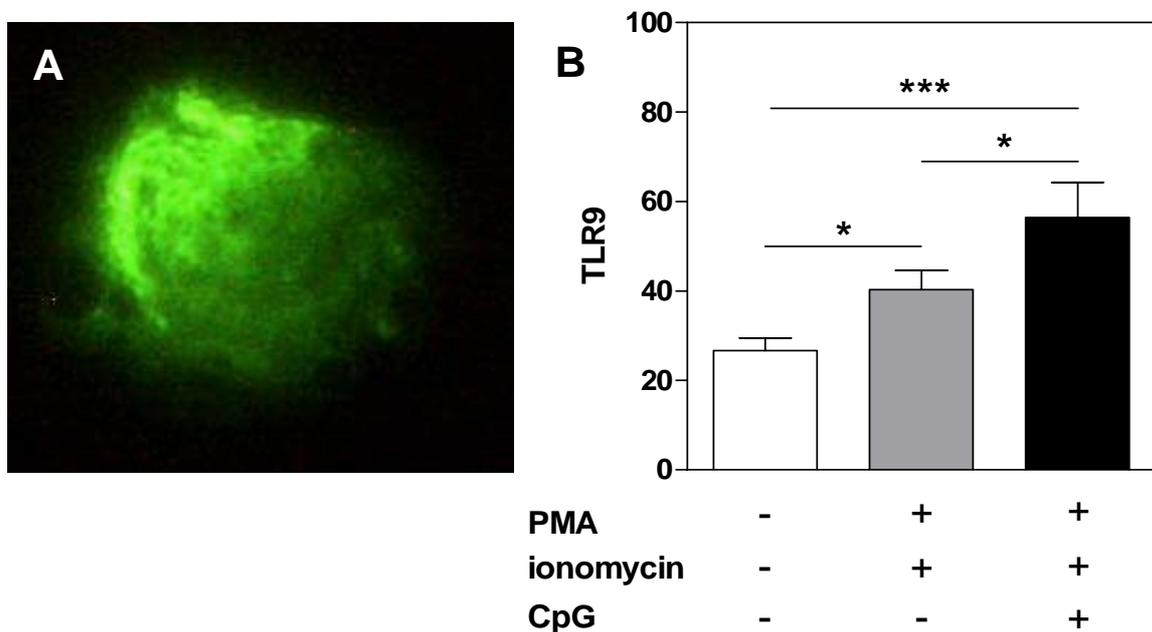
T cells were found to express mRNA encoding 8 out of the 10 known TLRs, and among them TLR1, -2, -5, -9 and -10 were most prominent. TLR6 and -8 were completely absent. Comparing the expression levels of single TLRs in the different cellular subsets revealed a differential expression in CD4<sup>+</sup> Th cells and CD8<sup>+</sup> CTLs (Fig. 5). No further differences were found in CD4<sup>+</sup>/CRTH2<sup>-</sup> Th1 cells and CD4<sup>+</sup>/CRTH2<sup>+</sup> Th2 cells. Expression of the receptors was confirmed by immunohistochemistry and FACS analysis.



**Figure 5.** TLR expression in human tonsillar T cells. Freshly isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were analyzed for expression of TLR1-TLR10 using real-time PCR. Data depicted in relation to β-actin as  $2^{-\Delta Ct} \times 10^5$  (CD4<sup>+</sup>, n=22; CD8<sup>+</sup>, n=21; \* p<0.05).

To study whether microbial exposure affects the TLR expression, T cells were isolated from recurrently infected tonsils, harboring  $\beta$ -hemolytic streptococci (group A, C and G) or anaerobes, and hyperplastic culture-negative tonsils serving as controls. Results revealed that TLR2, -3 and -5 mRNA and proteins were up-regulated in CD8<sup>+</sup> cells from infected tonsils, whereas in CD4<sup>+</sup> cells, TLR9 mRNA was down-regulated.

The present study shows that human tonsillar T lymphocytes express a broad range of TLRs. Presence of innate immune receptors on T cells is an interesting observation as T cells traditionally are not thought to play a role in the initial phase of an immune response. Functional studies on TLRs in T cells are scarce, but mostly evolving around TLR9 and the ligand CpG. However, if the reported effects are direct or mediated via APCs is still a matter of controversy (124-127). Our unpublished studies show that purified tonsillar CD4<sup>+</sup> T cells express TLR9, visualized using immunofluorescence (Fig. 6A), and that CpG up-regulates its own recognition receptor (Fig. 6B), induces expression of CD69 and increases the production of IFN- $\gamma$  and IL-4 in T cells activated by phorbol myristate acetate (PMA) and ionomycin.



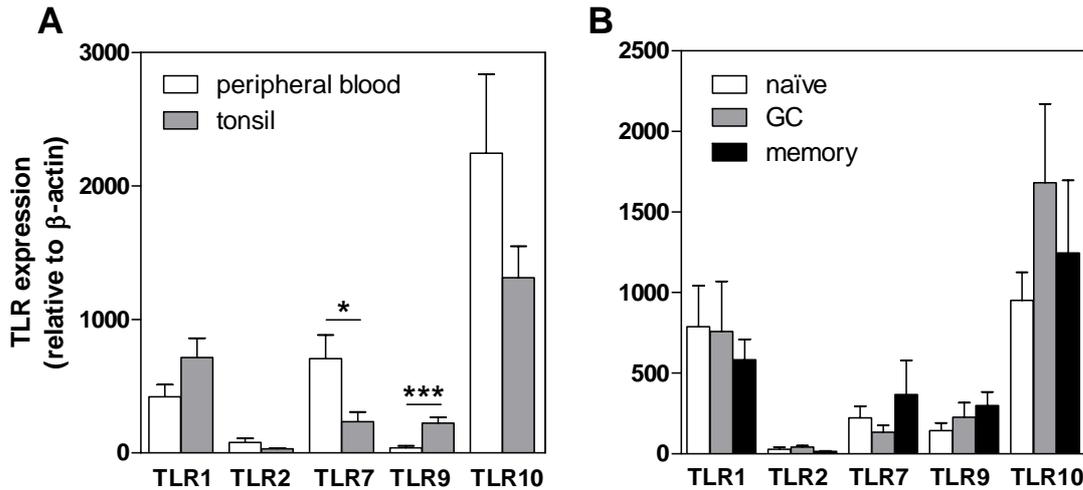
**Figure 6.** CpG induces expression of TLR9. (A) Purified CD4<sup>+</sup> T cells were stained with FITC-conjugated anti-TLR9 mAb. (B) Purified cells were stimulated for 24 h in the absence or presence of PMA (0.1 ng/ml), ionomycin (0.5  $\mu$ g/ml) and CpG (0.3  $\mu$ M). Thereafter, expression of TLR9 was determined by use of flow cytometry. Mean fluorescence intensity of TLR9 in cells from eight different donors are summarized (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001).

The differential expression of TLRs in CD4<sup>+</sup> and CD8<sup>+</sup> cells is intriguing. Most interesting is that CTLs have higher levels of TLR3, which is involved in recognition of viral dsRNA. This indicates that TLR3 is yet another way for CTLs to recognize viruses, and thus play a role in cytotoxic T cell responses. Lastly, we have shown an altered TLR profile upon tonsillar infection. The reason for why only certain TLRs are altered might be related to the presence of the cognate natural ligand. Further, if the expression is positively or negatively regulated seems to be dependent on the cell type. Taken together, presence of TLRs in T cells highlights the biological importance of TLRs in the adaptive immunity, and raises questions regarding the role of TLRs in T cell activation both in the absence and presence of APCs. Infection-dependent alterations in TLR expression further support the well-founded notion of TLRs as important players in the combat against infections.

### **Tonsillar B cells are directly activated by a distinct set of TLR ligands (paper II)**

Recognition of PAMPs by innate immune cells expressing TLRs is traditionally thought to induce antigen presentation, up-regulation of co-stimulatory molecules and release of cytokines that in turn activates the adaptive immune system comprising B and T lymphocytes (128). Over the years, the picture has been slightly revised as there are numerous studies showing that PAMPs, in particular CpG, can directly induce an adaptive immune response in B cells (84, 129-131). The purpose of the present study was to investigate if the TLR profile is regulated during the differentiation process that occur in the tonsils, explore possible tissue-specific differences in TLR expression between B cells from tonsils and blood, and determine if the TLR expression is altered in response to infection. Also, we aimed to analyze the ability of a range of TLR ligands to directly induce B cell activation.

Purified tonsillar CD19<sup>+</sup> B cells expressed a distinct set of TLRs, comprising TLR1, -2, -7, -9 and -10. The remaining TLRs were at the lower detection limit or undetectable. A similar TLR profile was seen in peripheral B cells, although there were some compartmental differences (Fig. 7A). The presence of TLRs was confirmed using immunohistochemistry and flow cytometry analysis. To study whether the expression is regulated during the B cell differentiation, TLR mRNA levels were determined in naïve, GC and memory B cells. However, no differences were found (Fig. 7B). Moreover, the TLR expression levels did not appear to be affected by ongoing microbial infection.



**Figure 7.** Expression of TLRs in human B cells. **(A)** CD19<sup>+</sup> tonsillar (n=20) and peripheral B cells (n=6). **(B)** Tonsillar B cells divided into naïve, GC and memory subsets (n=19-23). Expression was determined using real-time PCR, and is depicted in relation to  $\beta$ -actin as  $2^{-\Delta Ct} \times 10^5$  (\* p<0.05, \*\*\* p<0.001).

To study the ability of TLR ligands to induce B cell activation, purified tonsillar CD19<sup>+</sup> cells were stimulated for 48 h with or without Pam<sub>3</sub>CSK<sub>4</sub> (TLR1/2), LPS (TLR4), flagellin (TLR5), R-837 (TLR7) and CpG (TLR9). It was found that Pam<sub>3</sub>CSK<sub>4</sub>, R-837 and CpG induced IL-6 secretion and up-regulated expression of MHC class II. In accordance with the lack of TLR4 and TLR5, no effects were seen with LPS or flagellin.

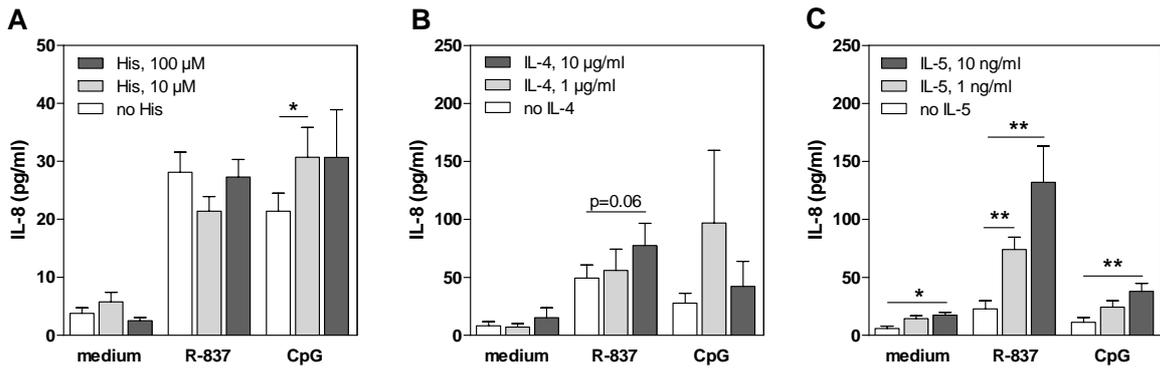
This study is first to show expression of TLRs in purified subsets of human tonsillar and peripheral B cells at both mRNA and protein level. B cells exhibit a distinct TLR repertoire with high levels of TLR1, -2, -7, -9 and -10. No differences were found among naïve, GC or memory B cells, indicating that the differentiation process does not affect the TLR repertoire. On the other hand, the expression appeared to be regulated by tissue-specific factors. This suggests that the differential expression is not a result of the differentiation process, but instead a consequence of cells progressing from a resting to an activated state. In contrast to T cells (paper I), ongoing microbial infection did not markedly affect the TLR levels. Lastly, all TLRs expressed in tonsillar B cells, for which specific ligands are available, were able to induce B cell activation. No B cell activating ability was seen with LPS or flagellin, although LPS is a powerful T cell-independent agonist in mice. However, TLR4 is a good example of species-related differences between mouse and man. It is well known that murine B cells respond vigorously to LPS via TLR4, whereas human B cells do not (132). Collectively, these findings extend the view of an active role of TLRs in B cell activation in the absence of T cell

help. Also, identification of the ligand for TLR10 will open new avenues in the field of TLR-induced B cell activation.

### **Role of atopic status in TLR-mediated activation of eosinophils (paper III)**

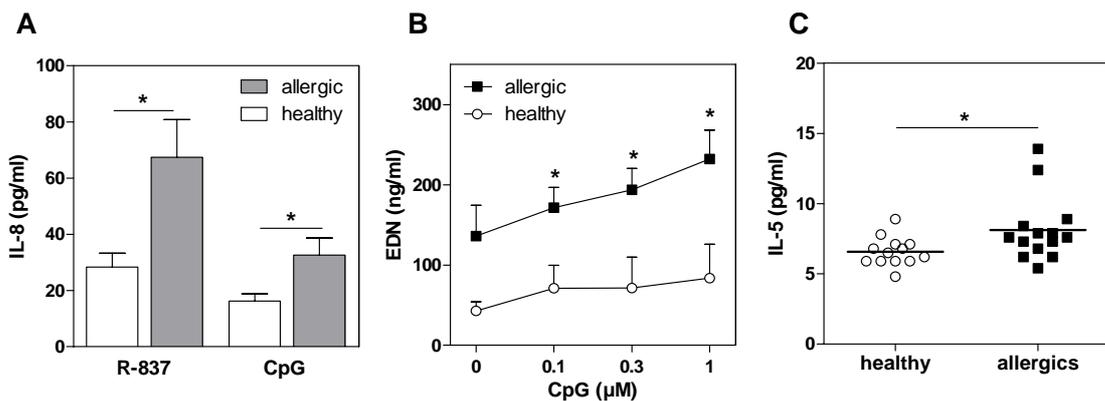
It is well known that viral respiratory infections can aggravate the disease conditions for patients suffering from allergic rhinitis and asthma (133-135). In the present paper, we investigated whether this could be due to a virus-induced activation of eosinophils through their TLRs. The virus-recognizing TLRs are TLR3, TLR7/8 and TLR9, responding to viral dsRNA, viral ssRNA and unmethylated CpG-containing DNA from bacteria and viruses, respectively (74, 76, 85). When this study was designed, presence of TLR7 and TLR9 had been demonstrated in human eosinophils (91, 92). Naïve hypereosinophilic mice had been shown to express TLR3 (136), but there was no support for this finding in the human system. Therefore, we decided to focus on the expression and function of TLR7 and TLR9.

Firstly, expression of TLR7 and TLR9 was verified in purified human eosinophils. R-837 and CpG, ligands for TLR7 and TLR9, respectively, were found to activate eosinophils at several levels, i.e. prolong survival, regulate adhesion molecule expression and activation markers, enhance migration and induce cytokine secretion. Also, CpG promoted degranulation, while this was not seen with R-837. After having found that several basic eosinophil functions are affected by TLR7- and TLR9-induced activation, we next wanted to investigate whether the responses could be regulated by pre-treatment with histamine, IL-4 and IL-5. A trend towards an enhanced response was seen with histamine and IL-4, whereas a clear increase in both TLR7- and TLR9-mediated activation was observed with IL-5 (Fig. 8A-C). This suggests that allergic mediators, in particular IL-5, may sensitize eosinophils for TLR activation.



**Figure 8.** IL-5 priming augments TLR-induced activation. Purified eosinophils ( $1 \times 10^6$  cells/ml) were pre-treated with histamine (His), IL-4 or IL-5 for 1 h before addition of R-837 or CpG. Levels of IL-8 were measured in the cell culture supernatants using ELISA. (A) IL-8 secretion after stimulation for 24 h with R-837 (10  $\mu$ g/ml) and CpG (1  $\mu$ M) in combination with histamine (10 and 100  $\mu$ M, n=8), (B) IL-4 (1 and 10  $\mu$ g/ml, n=5) or (C) IL-5 (1 and 10 ng/ml, n=5) (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Next, the TLR responses in atopic patients with allergic rhinitis were compared with responses seen among healthy individuals. Eosinophils from allergic subjects responded more strongly to R-837 and CpG than those from healthy controls, manifested by an increased secretion of IL-8 and the granule protein EDN (Fig. 9A, B). Increased levels of IL-5 in serum were found in the allergic group (Fig. 9C), suggesting the higher responsiveness to be the result of enhanced IL-5-mediated priming.



**Figure 9.** Allergic patients are more responsive to TLR stimulation than healthy subjects. Purified eosinophils ( $1 \times 10^6$  cells/ml) obtained from healthy subjects and patients with allergic rhinitis were stimulated with either R-837 and CpG. Cell culture supernatants were recovered and levels of IL-8 and EDN were measured by ELISA. (A) IL-8 release by allergic (n=6) and healthy (n=6) individuals after 24 h stimulation with R-837 (10  $\mu$ g/ml) and CpG (1  $\mu$ M). (B) Release of EDN by allergic (n=5) and healthy (n=4) subjects after 3 h culture with CpG. (C) Levels of IL-5 in serum of allergic (n=14) and healthy (n=13) subjects measured by ELISA (\*  $p < 0.05$ ).

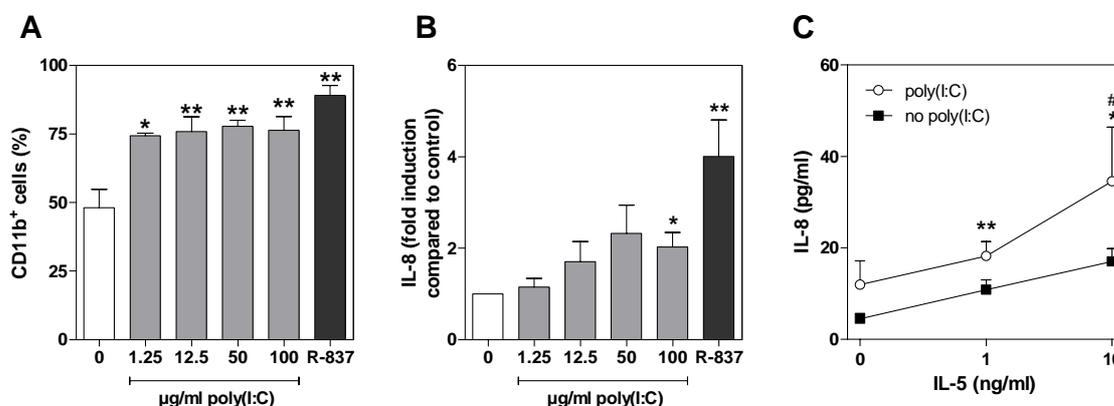
Having established a role for R-837 and CpG in the induction of eosinophilic inflammation, we aimed to investigate whether the two ligands also have the ability to affect the development of AHR, another hallmark of asthma. Results showed that CpG-mediated activation of eosinophils promoted airway epithelial damage by inducing epithelial cell death and cytokine release. However, this was not seen with R-837, suggesting the involvement of cytotoxic granule proteins such as EDN.

The present study shows that human eosinophils can be activated via TLR7 and TLR9, leading to an inflammatory response characterized by prolonged eosinophil survival, regulated expression of adhesion molecules leading to enhanced migration and increased cytokine production. The mechanisms behind TLR7 and TLR9 activation appears to be slightly different as CpG, but not R-837, induces release of cytotoxic granule proteins that in turn might affect AHR. Further, it is demonstrated that the presence of a Th2-like cytokine milieu as well as the atopic status of the donor may affect the outcome of the response. It is therefore tempting to conclude that eosinophils activated via the TLR system might engender a link between viral infections and allergic exacerbations.

#### **Link between TLR3 in eosinophils and allergic rhinitis (paper IV)**

The involvement of TLR7 and TLR9 in eosinophilic inflammation and possibly AHR, together with signs of TLR3 in eosinophils in a previous flow cytometric screening project, encouraged us to look more specifically at TLR3 in eosinophils. Hence, a study was designed to in detail examine the expression of TLR3 in bone marrow and peripheral blood eosinophils obtained from healthy subjects and patients with allergic rhinitis both during and outside pollen season. Presence of TLR3 was found in both bone marrow and peripheral blood eosinophils. The expression was higher in bone marrow-derived than in circulating cells, and it was down-regulated in both compartments during symptomatic allergic rhinitis. To find out if this receptor is functionally active, purified eosinophils from peripheral blood were stimulated with poly(I:C). It was found that poly(I:C) induced up-regulation of the adhesion molecule CD11b and secretion of IL-8 (Fig. 10A, B). As for the experiments with TLR7 and TLR9 (paper III), cells were also pre-treated with Th2 cytokines. No effect was seen with histamine or IL-4, but IL-5 augmented the poly(I:C)-induced activation (Fig. 10C). Lastly, the signaling pathways induced by poly(I:C) stimulation were investigated using specific pathway

inhibitors in combination with Luminex Multiplex Immunoassays. An involvement of p38 MAPK and NF- $\kappa$ B signaling pathways could be demonstrated.



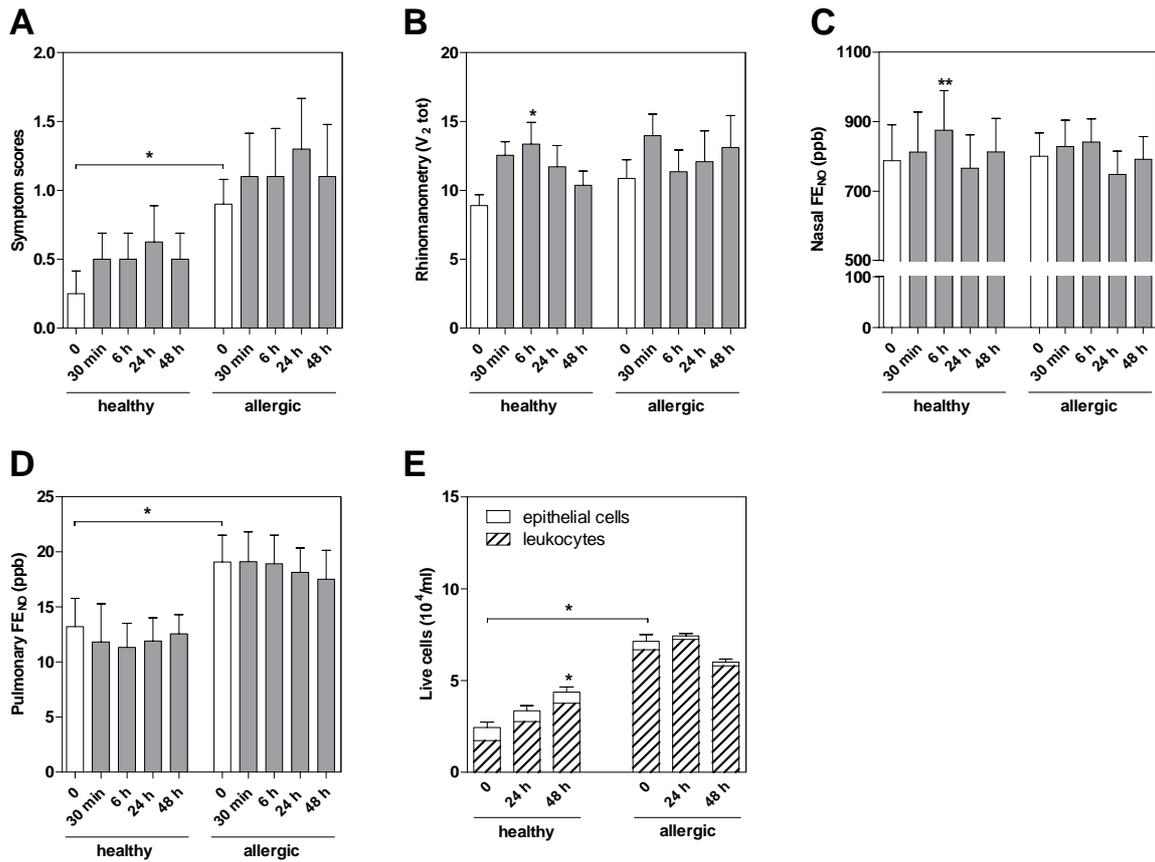
**Figure 10.** Poly(I:C) induces eosinophil activation. Purified cells ( $1 \times 10^6$  cells/ml) were incubated for 24 h with or without poly(I:C) or IL-5 and analyzed for expression of CD11b using flow cytometry and IL-8 using ELISA. R-837 (10  $\mu$ g/ml) was used as a positive control. (A) Percentage of CD11b positive cells, and (B) IL-8 production (n=4). (C) Eosinophils pre-treated with IL-5 for 1 h before addition of 12.5  $\mu$ g/ml poly(I:C). Levels of IL-8 were measured by use of ELISA (n=5) (\* p<0.05, \*\* p<0.01 compared to untreated control; # p<0.05 white circle compared to black square).

The present study demonstrates expression and a functional role of TLR3 in human eosinophils. The increase in CD11b expression and in IL-8 levels in response to poly(I:C) stimulation suggests that eosinophils activated via TLR3 might be destined to home to inflamed tissues, which in the context of allergic rhinitis correspond to the nose, and to direct leukocyte traffic into inflamed areas. The decreased level of TLR3 during pollen season indicates a link between TLR3 regulation and allergic rhinitis. Although the effects exerted by poly(I:C) are not as evident as the effects induced by R-837 and CpG, these findings altogether make the picture complete in that virus-recognizing TLRs in eosinophils have an important role in allergic airway disease.

### **Nasal administration with CpG induces a local inflammation (paper V)**

Allergens are not the sole trigger of allergic rhinitis, as respiratory infections, organic compounds and environmental pollutants are known to cause disease exacerbations (137, 138). We have in a previous study demonstrated that nasal administration with LPS (via TLR4) in conjunction with allergen causes a more marked inflammatory response than both LPS and allergen alone (121). The purpose of this study was to investigate the effects of CpG via TLR9, another TLR member, in the human nose. TLR9 has gained a lot of interest in the field of allergy research because of its ability to induce a Th1-biased immune response that counteracts the Th2 response associated with allergic reactions.

The effect of nasal administration with CpG was investigated in healthy subjects and in patients with allergic rhinitis outside pollen season. An increase in nasal resistance, nasal NO levels and in the number of live cells in NAL fluid were seen as a result of CpG exposure. This inflammatory response was more prominent in healthy subjects than among patients with a history of allergic rhinitis, although a higher basal inflammatory response was recorded in the allergic group (Fig. 11A-E). Further, the NAL fluids were analyzed for a range of different cytokines. The secretion of IL-1 $\beta$ , IL-6 and IL-8 was increased in the healthy group 24 h post CpG challenge, whereas no effect was seen among the allergics. However, a higher basal cytokine release was seen in the allergic group, along with a trend towards an increased level of IFN- $\gamma$  and IL-12 p70. Lastly, to determine the cellular sources behind the effects, neutrophils isolated from peripheral blood together with the airway epithelial cell line Detroit-562 were used. Both cell types expressed TLR9. CpG gave rise to a secretion of IL-1 $\beta$ , IL-6 and IL-8 in neutrophils, whereas only IL-8 release was induced by the cultured epithelial cells.



**Figure 11.** Nasal administration with CpG induces a local inflammatory response. Nasal challenge with CpG (10  $\mu$ M) in healthy subjects (n=10) and in patients with a history of allergic rhinitis (n=10). **(A)** Symptom scores, **(B)** nasal resistance, **(C)** nasal NO levels, **(D)** pulmonary NO levels, and **(E)** number of live cells in NAL fluid (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

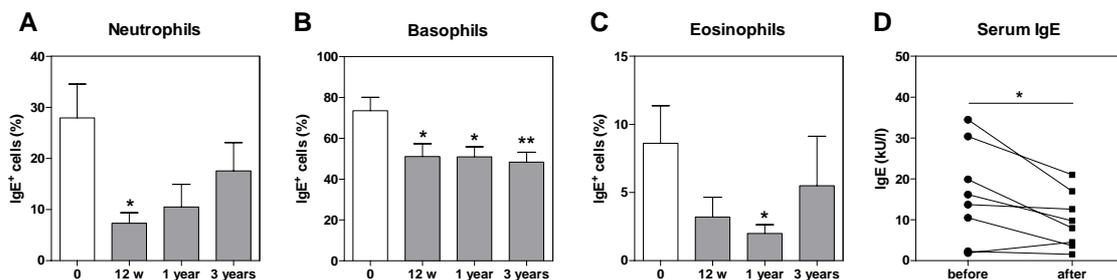
The present study is the first to investigate the effects of CpG administration in the human nose. We show that CpG induces a local inflammatory response in non-allergic individuals, manifested by an increase in nasal resistance, nasal NO levels, cellular infiltration and in cytokine levels. It appears as these effects are mediated primarily by nasal epithelial cells and neutrophils. The pulmonary NO levels remained unchanged throughout the study, indicating that the inflammatory process is restricted to the upper airways. The allergic subjects overall displayed a higher basal inflammatory response but were almost unresponsive to CpG. This ongoing, so called minimal persistent inflammation, might have resulted in a higher threshold for activation, making them less responsive. It should also be noted that the study was conducted out of season. The allergic Th2 response is likely to be minimal during this period, and possible effects of CpG could consequently be masked by this fact. Therefore, the possibility that effects are seen during pollen season or upon co-administration with allergen

should not be ruled out. The cytokine profile seen upon CpG administration suggests the induction of pro-inflammatory and Th1-type (IL-1 $\beta$ , IL-6, IL-8 and IL-12 p70), rather than a Th2-type (IL-4, IL-5 and IL-10), immune response. Nasal administration with CpG in conjunction with allergen would enable us to further validate the idea of CpG as a modifier of allergic immune responses.

### Rush immunotherapy induces leukocyte phenotype alterations (paper VI)

The clinical improvements following SIT are well documented, but the exact mechanisms behind these effects are not fully known (139). The present study followed a group of 10 patients undergoing RIT, an accelerated protocol for SIT, against birch pollen-induced allergic rhinitis during three years. The short- and long-term effects on peripheral blood leukocyte phenotypes were monitored using a flow cytometric approach.

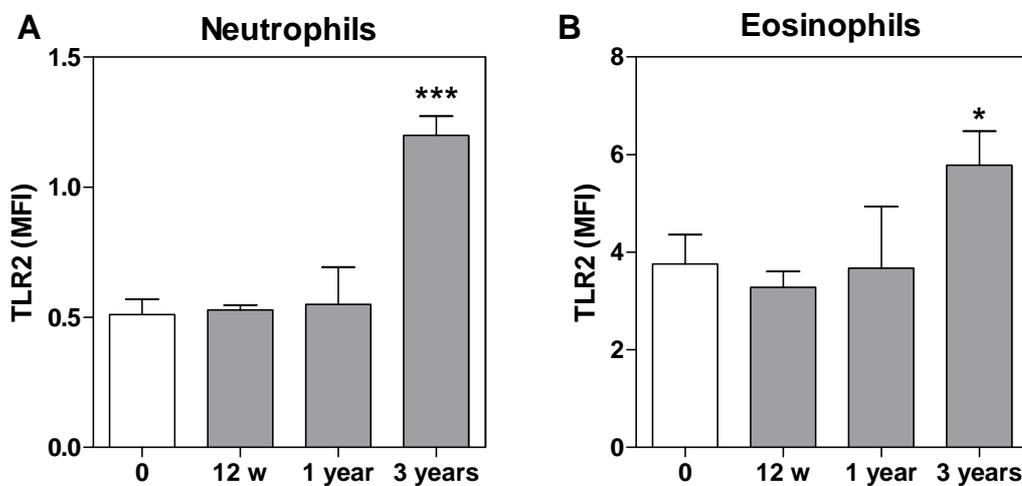
One out of ten patients suffered from a systemic reaction during the up-dosage phase and was therefore excluded. Remaining patients tolerated the RIT protocol well and continued throughout the 3-year period. They all experienced considerable improvements in symptoms and in medication requirements during the upcoming pollen seasons. RIT induced an initial increase in eosinophil numbers, and generated an onset of neutrophils, lymphocytes and total leukocytes upon allergen provocation. RIT also caused a decrease in cell-bound IgE on granulocytes (Fig. 12A-C), along with a lower level of allergen-specific IgE in serum (Fig. 12D). A corresponding increase in the high-affinity IgG receptor was seen.



**Figure 12.** IgE decreased during RIT. Expression of membrane-bound IgE on (A) neutrophils, (B) basophils, and (C) eosinophils. Blood samples obtained before, 12 weeks, 1 year and 3 years after RIT were stained with antibodies against IgE, and analyzed by use of flow cytometry (D) Levels of allergen-specific IgE before and 3 years after RIT (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Further, the treatment induced a decrease in neutrophil CD11b levels, a shift in monocyte subsets in favor of CD14<sup>high</sup> CD16<sup>+</sup> cells, and an activation of T lymphocytes. The latter was manifested by an up-regulation of the activation markers CD69 and CD98, as well as an expansion of the CD4<sup>+</sup> CD25<sup>+</sup> T cell pool during the initial phase of RIT.

After having looked at basic phenotypical changes, we wished to assess the influence of RIT on the TLR expression. Four TLRs were selected; TLR2, -3, -4 and -9. No significant changes were seen in TLR3 and TLR9 expression. However, an up-regulation in TLR2 (Fig. 13A, B) and TLR4 was found in neutrophils and eosinophils.



**Figure 13.** Up-regulation of TLR2 in (A) neutrophils and (B) eosinophils following RIT treatment. Blood samples obtained before, 12 weeks, 1 year and 3 years after RIT were stained with antibodies against TLR2, and analyzed by use of flow cytometry (\* p<0.05, \*\*\* p<0.001).

This 3-year follow-up study shows that the included patients experienced lasting reductions in symptoms and medication requirements, effects that were mirrored by changes in antibodies and in cellular events. Although this study is based on a relatively small group of patients, there are several findings that both confirm and complement previous studies. In particular, the reduction of IgE, in favor of IgG, is corroborated at both the serum and the receptor level. A role for CD4<sup>+</sup> CD25<sup>+</sup> cells that might be part of the Treg pool is also supported by the present findings. We also extend the current knowledge about the cellular mechanisms linked to the protective effects of immunotherapy by proposing a role for TLR2 and TLR4, as well as a shift in monocyte subsets, as novel events involved. With this study, new targets for immunomodulation are put to attention.

## **Summary and conclusions**

- A broad range of TLRs was discovered in human tonsillar T cells, with a clear predominance for TLR1, -2, -5, -9 and -10. A differential expression of TLRs in CD4<sup>+</sup> and CD8<sup>+</sup> cells was found, along with alterations in TLR expression as a consequence of recurrent tonsillar infection. These regulations in expression suggest that TLRs are of importance to adaptive immune cells and that TLRs have a direct role in immune reactions against infections.
- Human tonsillar B cells expressed a distinct TLR repertoire, comprising TLR1, -2, -7, -9 and -10. The expression was similar in naïve, GC and memory B cells, and was not affected by ongoing infection. Purified B cells responded directly to TLR1/TLR2, TLR7 and TLR9 stimulation with increased cytokine secretion and enhanced antigen presentation. These findings suggest that B cells, via TLRs, have the ability to directly respond to microbial infections, and that TLRs may function as regulators of B cell activation.
- Purified eosinophils from peripheral blood expressed TLR7 and TLR9. Stimulation with R-837 and CpG, ligands for TLR7 and TLR9, respectively, affected several eosinophil functions, including adhesion molecule expression, migration, cytokine secretion, degranulation and survival. Priming with the allergic mediators histamine, IL-4, and most prominently IL-5, augmented the TLR-induced responses, thereby sensitizing eosinophils for TLR7 and TLR9 activation. The TLR responses were more marked in patients with allergic rhinitis than in healthy subjects. In line with this, serum levels of IL-5 were higher in the allergic group, indicating increased IL-5-mediated priming. Altogether, these findings suggest that TLR-mediated eosinophil activation might engender a link between viral infection and allergic exacerbations.
- TLR3 was found in eosinophils from both peripheral blood and bone marrow, but was higher in the latter. A reduced expression was observed in eosinophils from both compartments during symptomatic allergic rhinitis. Stimulation with poly(I:C) increased the adhesion molecule expression and the IL-8 secretion, suggesting a role for TLR3 in eosinophil aggregation and leukocyte recruitment. These effects were mediated via p38 MAPK- and NF-κB-related signaling pathways. As for TLR7 and TLR9, the Th2 cytokine IL-5 augmented the TLR3-induced activation. All together, TLR3 appears to be a

functionally active receptor in eosinophils, with a possible role in allergic airway inflammation.

- Nasal administration with CpG increased the nasal resistance, the local NO production, as well as the leukocyte numbers and cytokine levels in NAL fluid. *In vitro* experiments suggested these effects to emanate from neutrophils and epithelial cells. Moreover, the CpG-induced response was more prominent in healthy subjects than in patients with allergic rhinitis. These findings suggest that CpG induces a local airway inflammation, and cytokine analyses reflect the ability of CpG to induce a pro-inflammatory Th1-like immune response.
- RIT caused a decrease in cell-bound IgE on granulocytes along with a corresponding increase in the high-affinity IgG receptor on eosinophils. In line with this, a lower level of allergen-specific IgE was found in serum after RIT. Further, the treatment induced a decrease in neutrophil CD11b levels, a shift in monocyte subsets in favor of CD14<sup>high</sup> CD16<sup>+</sup> cells, and an increase in activated T lymphocytes, manifested by an up-regulation of CD69 and CD98, as well as an expansion of the CD4<sup>+</sup> CD25<sup>+</sup> T cell pool. Also, TLR2 and TLR4 levels were affected by RIT. The presented data extends the current knowledge of cellular events that might be linked to the protective effects of allergen immunotherapy, thereby offering new targets for immunomodulation.

## Concluding remarks

The TLRs are key regulators of innate and adaptive immunity, and they are involved in both health and disease. In the present thesis we have studied the role of TLRs in lymphocyte and eosinophil biology, and documented their implication in tonsillar and allergic inflammation. However, a few findings are of particular interest.

One intriguing observation, made by ourselves and other groups, is that both B and T cells as part of the adaptive immunity express TLRs and respond to their ligands, both in the absence and presence of co-stimulatory signals (95, 130, 140, 141). In line with these observations, unpublished studies from our lab show presence of NLRs, another family of innate immune receptors, in human lymphocytes. We have discovered a range of NLRs in B cells, and that stimulation with the Nod1 and Nod2 ligands induces B cell activation upon concomitant BCR triggering. Even more interesting, a synergistic effect between TLR and NLR stimulation upon BCR engagement has been shown in the absence of T cell help, leading to a vigorous proliferation (142). Also, we have found that T cells express NLRs, and preliminary data indicate an induction of proliferation upon simultaneous stimulation via CD3 and CD28 (143). All these findings point in the same direction, that PRRs are highly active in the human lymphocyte setting. Thus, the “two-signaling model” of lymphocyte activation needs to be revised to include signals via PRRs.

Rhinovirus, influenza virus and respiratory syncytial virus (RSV) are common respiratory tract pathogens. As mentioned earlier, viral respiratory infections like these often precede asthma exacerbations, and during certain circumstances may lead to the development of allergic disease. This phenomenon is likely to comprise several different cells, mediators and events, but an activation of TLRs in eosinophils appears to be one such mechanism. The role of the eosinophil in atopic diseases has been widely disputed during the last decades. From being considered the principal cause of asthma, primarily by the initiation of AHR, the eosinophil was degraded to being a bystander cell and was almost forgotten in the allergic arena. This can partly be explained by the failure of eosinophil-targeted therapies (such as anti-IL-5 antibodies) to abolish eosinophilia and AHR. However, during the last couple of years, the eosinophil has been resurrected as an important player in allergic diseases due to its role in the perpetuation of airway inflammation, its potential to induce AHR and airway remodeling. The present findings suggest that eosinophils engender a link between viral

infection and allergic exacerbations. This idea is in line with the old concept that eosinophils, as part of the innate immunity, have a role in helminth infections. Thus, it might be that viruses cause eosinophil activation via similar TLR-related mechanisms as helminths, suggesting a role for eosinophils not primarily in the induction or propagation of airway inflammation but in mediating acute asthma exacerbations.

The hygiene hypothesis suggests that a reduced microbial burden during childhood can result in allergic diseases later on in life. In explaining this, TLR2 and TLR4 have gained a lot of attention because of their ability to recognize endotoxins. We have previously demonstrated an up-regulation of TLR2 and TLR4 in the nasal mucosa of patients suffering from symptomatic allergic rhinitis, suggesting these TLRs to be involved in the allergic inflammation (115). In line with this study, we have in the present thesis demonstrated a down-regulated expression of TLR2 and TLR4 upon immunotherapy treatment, suggesting that the treated patients acquire TLR levels similar to those of healthy individuals. In contrast to our data, farmers' children have been reported to have higher levels of TLR2 and CD14 (co-receptor for TLR4) than non-farmers' children, a finding attributed to the higher endotoxin exposure (106). However, in this context it is important to distinguish the effects seen during early life (before the onset of allergic disease) from the effects seen during adulthood. Once the allergic airway inflammation has been established, bacterial and viral infections may be as important as allergens in inducing hyperreactive responses.

Another issue that needs clarification is the involvement of TLR9 in allergic rhinitis. On one hand, we have demonstrated a higher responsiveness to CpG in eosinophils obtained from allergic individuals, whereas we on the other hand have shown that allergic patients are almost anergic to nasal administration with CpG. First of all, the cell types involved were different. In the former study we used eosinophils isolated from peripheral blood, whereas nasal epithelial cells and neutrophils were the primary target cells in the latter. Secondly, the origin of the cells differed, using circulating cells versus tissue-based cells. Cells circulating in the blood stream generally are in a quiescent state, but once recruited to the tissues they differentiate, become active and exert their effector functions. Thirdly, the first study was performed *in vitro* using a defined medium and additives, whereas the second study was conducted *in vivo* where the natural environment was preserved. Also, the type and concentration of CpG differed in the two studies. Thus, it appears as the effect of a single TLR is dependent on the cell type, route, dose and time of exposure.

Several TLR agonists or antagonists are currently being used by the pharmaceutical industry in an attempt to find novel treatment alternatives for asthma and allergy. CpG has so far been considered a promising candidate based on animal models and a few clinical trials, using an inhalation or injection approach (113, 114). However, in our study the effects of CpG applied in the nose were examined, which is the natural route of exposure for allergens. We now plan to extend this study by a simultaneous co-administration of CpG and allergen in the nose, with the hope of inducing Th1 cytokines and reducing the allergic response. With this study, the general picture of CpG as a regulator of allergic disease will be more complete, by showing both systemic and local effects.

One last remark that could be made is the use of different TLR and NLR ligands as adjuvants in vaccine development. CpG is, as mentioned above, currently being used as an adjuvant in allergy vaccines in various clinical trials (144). Another common adjuvant is alum, which has been used in vaccines for more than 70 years. Its immunostimulatory properties were just recently attributed to the interaction with Nalp3, a member of the NLR family (145, 146). In view of this, it is tempting to speculate that several other ligands of the growing family of PRRs might be exploited in the future design of new adjuvants for allergy vaccination.

## **Future perspectives**

The discovery of the TLRs in the late 1990's and later their ability to link innate and adaptive immunity has changed our view of the immune system. Since then, other innate immune receptors have been identified, so far including the NLR and RLR families. The NLRs presently comprise more than 20 members (147), and the RLR family encompasses three different proteins (148), but new members are constantly being added. The ligands for many of the receptors are still unidentified, and consequently, many of their functions are unknown. Accumulating evidence suggest that there is a crosstalk between the three receptor families (10). This together with the future discovery of their ligands will increase our understanding of the complex role of PRRs in host defense in both health and disease.

## Populärvetenskaplig sammanfattning på svenska

Den här avhandlingen består av sex olika, men delvis integrerade, arbeten som handlar om immunförsvarets s.k. Toll-likareceptorer (TLRs) och deras betydelse för luftvägsinflammation. TLRs är en receptorfamilj med för närvarande tio kända medlemmar, vilka ingår i den medfödda/ospecifika delen av vårt immunförsvar. Var och en av dessa receptorer känner igen olika strukturer på bakterier och virus. Den primära rollen av TLRs är att skydda oss från infektioner. Det har dock visat sig att felaktig aktivering eller mutationer i TLR-generna kan ge upphov till ett flertal inflammatoriska sjukdomstillstånd såsom cancer, autoimmuna sjukdomar och astma/allergi.

Avhandlingens första del kännetecknas av att olika vita blodkroppar, s.k. leukocyter, har isolerats från blod och tonsiller (halsmandlar) med hjälp av antikropps-konjugerade magnetiska kulor och därefter använts för olika *in vitro* studier. Vi har härvid valt att fokusera på tre celltyper; B-celler, T-celler och eosinofiler, vilka anses vara av central betydelse för inflammation i allmänhet, och allergisk inflammation i synnerhet. I **delarbete I och II** tittade vi på uttryck och funktion av TLRs på tonsillära B- och T-lymfocyter, vilka tillhör den förvärvade/anpassningsbara delen av vårt immunförsvar. Traditionellt sett anses dessa celler aktiveras enligt en ”två-signal-modell” som förenklat innebär att de behöver två signaler vardera för aktivering. Den första via B- respektive T-cellsreceptorn och den andra från co-stimulatoriska molekyler som de förser varandra med. Resultaten från dessa båda studier visade att såväl B- som T-celler uttrycker en väldefinierad TLR-profil. Kronisk inflammation i tonsillerna (p.g.a. återkommande halsfluss) verkade dessutom förändra TLR-uttrycket, i synnerhet hos T-celler. Genom att studera den funktionella aktiviteten av de uttryckta receptorerna såg vi även att deras motsvarande liganderna ger upphov till en direkt aktivering av B-celler, och i viss mån även T-celler. Dessa data tyder på att även celler i det förvärvade immunförsvaret kan nyttja ”medfödda” receptorer, något som gör att ”två-signal-modellen” för lymfocytaktivering bör omvärderas.

Att virala luftvägsinfektioner kan förvärra sjukdomstillståndet för allergiker och astmatiker är sedan länge välkänt. Hur detta sker är dock höljt i dunkel. I **delarbete III och IV** utgick vi från hypotesen att detta kunde ske genom en aktivering av TLRs på eosinofiler. Våra resultat visade att eosinofiler uttrycker TLRs som känner igen virala byggstenar, samt att stimulering av dessa ger upphov till en eosinofil aktivering kännetecknad av en förlängd överlevnad, ökad

frisättning av skadliga mediatorer och ökad benägenhet att migrera ut till inflammierade områden. Vi kunde också konstatera att patienter med hösnuva svarar starkare på stimulering än friska individer, samt att närvaron av s.k. Th2-cytokiner (allergiska mediatorer) förstärker svaret. Sammantaget tyder resultaten från dessa båda studier på att eosinofiler har TLRs som kan ge upphov till en antiviral immunrespons, samt att det svar som följer tycks påverkas av individens allergiska status. Mycket talar därför för att vår idé om en roll för eosinofiler i utvecklingen av hösnuva är riktig.

**Delarbete V** är centrerat kring TLR9 och dess ligand CpG. CpG har tidigare visat sig kunna styra immunförsvaret mot en icke-allergisk fenotyp vilket gör att den anses vara en lovande kandidat vid utvecklingen av nya terapialternativ mot allergiska sjukdomar. Dess direkta effekter på human luftväg har dock tidigare ej undersökts. I arbetet sprayade vi CpG i näsan på friska frivilliga och på patienter med hösnuva. Resultaten visade att CpG ger upphov till en lokal inflammation, kännetecknad av ökad nästäppa, produktion av kväveoxid, tillströmning av leukocyter samt frisättning av pro-inflammatoriska Th1-baserade (icke-allergiska) cytokiner. Svaret på CpG-tillförelsen föreföll också mer uttalad hos de friska deltagarna än hos de med hösnuva. Vi avser att fullfölja denna kartläggning av effekterna av CpG i näsan med en ny studie där CpG tillförs nasalt i kombination med allergen.

Avslutningsvis i **delarbete VI** undersöktes vad som sker på cellnivå under och efter behandling med allergen-specifik immunterapi. De uppmätta cellulära förändringarna återspeglade väl de uppnådda terapeutiska resultaten. Vi såg en minskning i allergen-specifika IgE-antikroppar och en ökning av s.k. regulatoriska T-celler, något som stämmer bra överens med tidigare publicerade data. Dessutom visade vi att behandlingen förändrar balansen mellan olika sorters monocyter samt att uttrycket av TLR2 och TLR4 nedregleras. Kunskapen om det senare skulle kunna användas i framtida utveckling av nya former av terapeutisk immunomodulering.

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## **Appendices**