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ALLERGIC RHINITIS AND INTRALYMPHATIC VACCINATION; IMMUNE RESPONSE AND TOLERANCE

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ALLERGIC RHINITIS AND INTRALYMPHATIC VACCINATION; IMMUNE RESPONSE AND TOLERANCE

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By

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”Let the goal loom on the horizon but be sure to enjoy the journey there.”

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vi har studerat patienter med allergi mot björk och gräs. För patienter med lindriga besvär finns det idag många mediciner, men för de med mer uttalade symptom räcker inte alltid dessa preparat till. Ett väl beprövat alternativ kan då vara allergen-specifik immunterapi (AIT), också kallat allergivaccinering. Här tillförs det allergen som patienten inte tolererar under strikt kontrollerade former, antingen som subkutana injektioner på sjukhus var sjätte vecka eller som en daglig tablett under tungan i hemmet. För effekt krävs att behandlingen pågår kontinuerligt under minst tre år. En framgångsrik behandling ger symptomreduktion med kvarstående tolerans som sträcker sig många år efter avslutad terapi. Problemet med dagens AIT ligger i den långa behandlingstiden. Därtill vid subkutana injektioner kommer behovet av tät sjukvårdskontakt samt en ringa men icke försumbar risk för svåra biverkningar. Vid AIT med tablettbehandling är det stora problemet att många patienter inte har den uthållighet som krävs för att dagligen ta tabletter under lång tid.

Avhandlingens två första projekt kartlägger nya immunologiska mekanismer som kan påverka uppkomsten och utvecklingen av pollenallergi. Resultaten visar på nya, alternativa vägar för terapiutveckling inom området, vilka skulle kunna leda till mediciner som kompletterar dagens behandling.

I de följande tre arbetena studeras en tredje och fortfarande experimentell form av AIT kallad intralymfatisk immunterapi (ILIT). Här injiceras allergenet, men hjälp av ultraljudsguidning, direkt in i en lymfkörtel i ljumsken. Tidigare studier har visat att tre injektioner med fyra veckors mellanrum ger en symptomlindring som förefaller vara densamma som vid de två etablerade treårsbehandlingarna. Antalet biverkningar är få och milda. I det tredje delarbetet visar vi att det är möjligt att injicera två allergen samtidigt utan risk för ökat antal biverkningar. Biverkningarna vid traditionell AIT är direkt relaterade till den dos allergen som används. En högre dos förväntas ge bättre effekt på bekostnad av fler och svårare biverkningar. I det fjärde delarbetet undersökte vi om en högre dos vid ILIT skulle kunna ge förbättrad symtomlindring. Vi fann, något oväntat, att en högre dos inte resulterade i någon förbättrad tolerans. Vid en påtaglig dosökning sågs också tydlig risk för svåra biverkningar. I det avslutande delarbetet följde vi efter 5 år upp de patienter vi tidigare vaccinerat med två allergen. Även om den initialt goda symptomreduktionen avtagit så kvarstod tydliga tecken på immunologisk toleransetablering i blodet.

ABSTRACT

The overall goal of this thesis was to study novel immunological mechanisms for the development of pollen-induced allergic rhinitis (AR) and to evaluate the clinical response in combination with immunological changes in AR patients treated with intralymphatic immunotherapy (ILIT).

In paper I, an increased fraction of neutrophils were detected in the nasal mucosa of AR patients compared with healthy controls. This accumulation was mainly due to a rise in a specific neutrophil subtype, $CD16^{high}CD62L^{dim}$. Studies of the biological functions revealed that $CD16^{high}CD62L^{dim}$ neutrophils increased T-cell activation and induced eosinophil migration.

Paper II investigated the expression of Notch receptors on $CD4^+$ T-cells and the presence of their corresponding ligands on epithelial cells and neutrophils. The fraction of $CD4^+Notch1^+$ and $CD4^+Notch4^+$ T-cells was higher in AR patients than in healthy controls. The expression levels of Notch ligand Jagged-1 (JAG-1) and Delta-like ligand-1 (DLL-1) were increased in nasal epithelial cells among AR patients. Likewise, neutrophils in nasal mucosa and blood displayed increased expression of JAG-1. Together this signals an increased activity in the Notch1/4 - JAG-1/DLL-1 pathways among allergic individuals suggesting that Notch signaling may participate in the regulation of T-cells in AR.

In paper III the safety and efficacy of intralymphatic immunotherapy (ILIT) with two allergens given concomitantly were assessed. ILIT with two allergens appears to be a safe procedure with limited side effects. Allergen challenge, quality of life scores, and consumption of rescue medication indicated that ILIT reduced rhinitis symptoms. In patients treated with active ILIT timothy-specific IgG4, effector memory Tregs, Th1 central memory $CD4^+$ T-cells, and effector memory $CD4^+$ T-cells in the lymph nodes were increased after treatment, further supporting the rationale for this alternative administration route.

Paper IV describes the outcome of two randomized, double-blinded, placebo-controlled trials. The first included patients that had recently ended three years of subcutaneous immunotherapy (SCIT), and the second contained patients without prior allergen-specific immunotherapy treatment. The dosage of 1000-3000-10000 SQ-U with one month in between was evaluated. This protocol was safe for patients previously treated with SCIT. The combined symptom and medication score (CSMS) was improved compared to the placebo group, and the timothy-specific IgG4 levels in the blood were doubled. In ILIT de novo, the first two patients that received active treatment developed severe adverse reactions at 5000 SQ-U. A modified up-dosing schedule, 1000-3000-3000 SQ-U, appeared safe but failed to improve the CSMS, quality of life, and nasal provocation response. Flow cytometry analyses could not detect T-cell changes, while lymph node-derived dendritic cells showed increased activation.

In Paper V, patients treated with ILIT 5-6 years earlier returned for a follow-up visit to study the remaining clinical effects and persisting immunological changes. To gain statistical power, AR patients without previous AIT were included in the control group. The nasal provocation

test displayed no difference between active ILIT and the control group. Still, the combined symptom and medication score were reduced in active ILIT compared to the control group. Timothy-specific IgE was decreased compared to pretreatment levels. Timothy-specific IgG4 and memory T-cells in lymph nodes were increased. Basophils displayed characteristics of reduced allergen sensitivity.

In summary: $CD16^{high}CD62L^{dim}$ neutrophils may play a role in AR pathology by priming $CD4^{+}$ T-cells and enhancing eosinophil migration. Notch signaling appears to be another novel pathway for the development of pollen allergy involving T-cell regulation. These results suggest novel targets for the development of future AR therapy. In ILIT, two allergens can be concomitantly injected without risk of tangible side effects. In contrast, an increase in the dose, from 1000SQ-U to 5000SQ-U, is associated with a severe risk for anaphylactic reactions and should be avoided. A moderate dose increase to 3000SQ-U does not seem to improve the therapeutic outcome further. It is evident that the favorable effects of ILIT remain long after the last injection, but a booster might be needed after three to five years. Altogether the presented ILIT data further support the future use of ILIT in clinical praxis.

LIST OF SCIENTIFIC PAPERS

I. Arebro J, Ekstedt S, **Hjalmarsson E**, Winqvist O, Kumlien Georén S, Cardell LO. A possible role for neutrophils in allergic rhinitis revealed after cellular subclassification. Sci Rep. 2017 Mar 8;7:43568.

II. **Eric Hjalmarsson**, Marianne Petro, Susanna Kumlien Georén, Ola Winqvist, Lars Olaf Cardell. Upregulated expression of Notch1/4 - JAG-1/DLL-1 in allergic rhinitis. Submitted manuscript.

III. Hellkvist L, **Hjalmarsson E**, Kumlien Georén S, Karlsson A, Lundkvist K, Winqvist O, Westin U, Cardell LO. Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol. 2018 Oct;142(4):1338-1341.e9.

IV. Hellkvist L, **Hjalmarsson E**, Weinfeld D, Dahl Å, Karlsson A, Westman M, Lundkvist K, Winqvist O, Georén SK, Westin U, Cardell LO. High-dose pollen intralymphatic immunotherapy: Two RDBPC trials question the benefit of dose increase. Allergy. 2022 Mar;77(3):883-896.

V. **Hjalmarsson E***, Hellkvist L*, Karlsson A, Winquist O, Kumlien Georén S, Westin U, Olaf Cardell L. A five-year open follow up of a randomized, double-blind placebo-controlled trial of intralymphatic immunotherapy for birch and grass reveals remaining beneficial effects. J Investig Allergol Clin Immunol. 2022 Jun 2:0. doi: 10.18176/jiaci.0832.
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Häyry V, Kågedal Å, **Hjalmarsson E**, Neves da Silva PF, Drakskog C, Margolin G, Georén SK, Munck-Wikland E, Winqvist O, Cardell LO. Rapid nodal staging of head and neck cancer surgical specimens with flow cytometric analysis. Br J Cancer. 2018 Feb 6;118(3):421-427.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AIT	Allergen-specific immunotherapy
APC	Antigen-presenting cell
AR	Allergic Rhinitis
ARIA	Allergic Rhinitis and its Impact on Asthma
BCL	B-cell lymphoma
BMI	Body mass index
Breg	B-regulatory
CCR	C-C Motif Chemokine Receptor
CD	Cluster of differentiation
CSMS	Combine symptoms and medication score
CXCR	C-X-C Motif Chemokine Receptor
DC	Dendritic cell
DLL	Delta like ligand
ECP	Eosinophil cationic protein
EPIT	Epicutaneous Immunotherapy
Fc	Fragment crystallizable
FDC	Follicular dendritic cell
GC	Germinal center
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILIT	Intralymphatic Immunotherapy
iTreg	Inducible T regulatory cell
JAG	Jagged
MBP	Major basic protein
MFI	Mean fluorescence intensity

MHC	Major histocompatibility complex
MS	Medication score
NAL	Nasal lavage
NPT	Nasal allergen provocation
PBMC	Peripheral blood mononuclear cells
QoL	Quality of life
ROR-γt	Retinoic-acid-receptor-related orphan nuclear receptor gamma
RQLQ	Rhino conjunctivitis Quality of Life Questionnaire
SQ-U	Standardized quality units
SCIT	Subcutaneous Immunotherapy
SLIT	Sublingual Immunotherapy
SLO	Secondary lymphoid organs
SPT	Skin prick test
SS	Symptoms score
T-bet	Tbx21
TcR	T-cell receptor
Tfh	T-follicular helper cell
Tfr	Regulatory follicular helper T-cell
TGF	Transforming growth factor
Th	T-helper
TH2	TH2 inflammation
TNF	Tumor necrosis factor
Treg	T regulatory cell
VAS	Visual analog scale

1 INTRODUCTION

1.1 ALLERGIC RHINITIS

Allergic rhinitis (AR) is an IgE-mediated (Type I) allergic disease broadly categorized as an inflammation of the nasal mucosa. Clinically AR is defined as a condition with four primary symptoms: rhinorrhea, sneezing, nasal itching, and nasal congestion[1]. AR-related symptoms can be felt in many areas of daily living, including performance at work and school, poor quality of sleep, and a sense of reduced quality of life[2]. AR patients are also at a higher risk of developing asthma[3] and sensitization to other allergens[4]. Today, AR is a common disease, and the latest estimate in European countries is that 20 to 30% of the adult population and up to 40% of children are affected[5]. The high prevalence of AR also induces high costs socioeconomically[6].

The development of an IgE-mediated allergic disease requires a sensitization phase[5]. During the sensitization phase, allergen-specific immunoglobulin E (IgE) binds to FcεR1 expressed by mast cells, sensitizing them to that specific allergen. Both genetics and environmental components are believed to play a role in allergen sensitization[7]. Individuals that are genetically more prone to develop IgE-mediated disease are referred to as atopic individuals[1].

When a sensitized patient is re-exposed to the same allergen, the allergen binds to mast cells and basophils crosslinking IgE receptors on their cell surface[4]. The IgE crosslinking leads to degranulation and release of mediators. In the early phase of the response, which starts minutes after degranulation, released mediators like histamine cause the nasal symptoms associated with AR[2]. Some patients also develop ocular symptoms with itching, watering, and redness[2].

The late phase of the allergic response typically develops 2-9 hours after mast cell and basophil degranulation and resolves after 1-2 days. Immunologically this phase is characterized by the cellular recruitment of eosinophils, neutrophils, basophils, macrophages, T- and B-cells. (Fig. 1) [4]. Prolonged repetitive exposure to allergens induces chronic tissue inflammation characterized by the presence of a large number of infiltrated leukocytes but also changes in the number, phenotype, and function of structural cells[2, 4]. Together this sustains and aggravates the allergic inflammation.

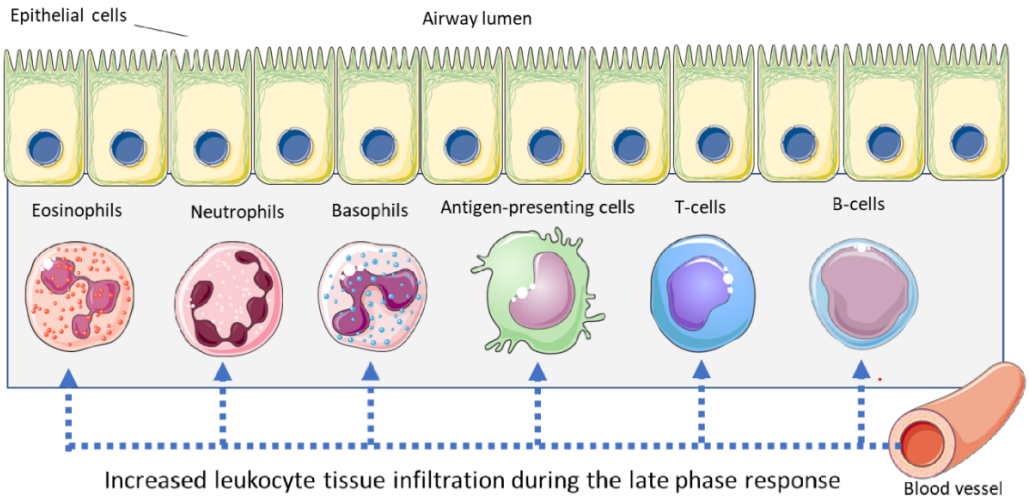

Figure 1. Leukocyte tissue infiltration during the late phase response. The late-phase reactions typically occur hours after basophil and mast cell degranulation. The released mediators directly or indirectly induce tissue infiltration with eosinophils, neutrophils, basophils, antigen-presenting cells, T-cells, and B-cells. Leukocytes in the tissue promote TH2 inflammation which aggravates and sustain the allergic immune response.

AR has historically been categorized according to two symptom patterns, seasonal (occurs during a specific season) or perennial (occurs throughout the year) [8]. Seasonal allergic rhinitis symptoms are usually easily identifiable and are directly associated with seasonal allergen exposure such as tree, grass, and weed pollens. Seasonal AR was used to classify patients in the clinical trials included in this thesis. This categorization of AR symptoms has recently been changed, and intermittent or persistent AR is now used to classify AR symptoms[8]. Depending on disease severity, AR is also classified as mild, moderate, or severe[8]. The Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines have classified "intermittent" AR as symptoms duration less than four days per week and for less than four consecutive weeks and "persistent" AR as symptoms duration for more than four days per week or lasting more than four straight weeks[9]. The AR severity is classified as mild when patients have no impairment in sleep and performance in everyday activities. AR is categorized as moderate to severe if it significantly affects sleep or activities of daily living or if they are considered bothersome by the patient[9]. This categorization enables proper diagnostics and treatment planning and can be used as inclusion criteria for allergen specific-immunotherapy.

2 LITERATURE REVIEW

2.1 PHARMACOTHERAPY

AR treatment aims to reduce or eliminate current symptoms while preventing long-term complications[10]. The management today includes allergen avoidance, pharmacotherapy, and immunotherapy. Immunotherapy should be considered from moderate-intermittent and mild-persistent to severe-persistent symptoms[9, 11, 12].

The use of pharmacologic treatments for AR depends on disease severity. For symptoms progressing from mild-intermittent to severe-persistent, the standard of care are oral antihistamines and intranasal corticosteroids. Short-term systemic corticosteroids are often prescribed when the standard of care fails, like in the middle of a severe pollen season. Even though systemic steroids are commonly used, they are not recommended in current guidelines due to the risk of side effects and lack of documented efficacy[9, 11, 12]. It is essential to recognize that despite widespread availability and frequent use of the standard of care medication, most AR patients are unsatisfied and report a marked impairment in their quality of life[13].

2.2 ALLERGEN-SPECIFIC IMMUNOTHERAPY

Allergen-specific immunotherapy (AIT) has been used for more than 100 years to treat AR[14]. By exposing patients to specific allergens using a strict protocol, the immune system changes the response in a way that suppresses inflammation and promotes the development of long-standing tolerance. Despite positive results, less than 5% of eligible patients are offered AIT as a treatment alternative[15]. This is mainly due to the long treatment protocol and the risk for severe side effects, and the treatment is today labor-intensive and costly. In the current concept of how AIT induces tolerance, tolerogenic antigen-presenting cells have a significant role[16]. It is also believed that an increased T regulatory (Treg) cell response and deviation from a T helper 2 (Th2) to a T helper 1 (Th1) cell response is essential[17]. Also, B-cells have a vital role in allergen tolerance by increasing the expression of IL-10, IgG4 and IgA[18]. Despite well-documented effects on symptoms and inflammation, a complete understanding of the mechanisms leading to tolerance remains to be discovered.

2.2.1 SCIT

Subcutaneous immunotherapy (SCIT) was for decades the standard administration route for AIT[19]. SCIT is administered subcutaneously, most often in the upper arm. At the injection site, the allergen is taken up by dendritic cells that migrate to the draining lymph nodes[20]. To achieve tolerance in response to SCIT, a high dose of the allergen is given[20]. To assure safe administration of the allergen, SCIT involves an initiation phase of weekly injections (7-15 injections), followed by a maintenance phase with injections given every 6-8 weeks for three years or more. SCIT has proven to reduce allergen-induced symptoms and the need for medication during the pollen season[21]. The rate of reduction of symptoms and the medication score is reported to be as high as 80% in many randomized, placebo-controlled trials[5]. The effect of SCIT is in some studies reported to last for more than 8 years, and in some cases lifelong[15].

SCIT is known to be associated with numerous adverse reactions. Up to 30% of treated patients suffer from systemic reactions and a far greater number of patients experience redness, itching and edema at the site of injection[22]. In Sweden, this therapy is therefore mainly performed at hospitals. Taken all together this limits its widespread use.

2.2.2 SLIT

During the last decade, sublingual immunotherapy (SLIT), comprising daily placement of allergen extract tablets under the tongue has become more commonly used. The tablets can be taken at home after an initial start dose at the hospital, and the treatment duration of SLIT is three years or more[5]. In SLIT antigen-presenting cells within the oral mucosa internalize allergens and migrate to nearby lymph nodes. The oral mucosa has high permeability for allergens, enabling a tolerogenic immune response with a lower dose compared to SCIT[23]. At present, SLIT is widely used to treat grass and tree-pollen allergies. Studies comparing SCIT and SLIT revealed both to be effective for seasonal AR[24]. The long duration of the treatment together with frequently occurring local side effects have resulted in a significant problem with compliance. It has been reported in Sweden that 30-40% of patients treated with SLIT terminate their medication prematurely[25].

2.2.3 EPIT

Epicutaneous immunotherapy (EPIT) is an experimental administration route for AIT that delivers allergen by repeated application to the skin[26]. By targeting antigen-presenting cells and avoiding activating mast cells or entering the circulation, EPIT appears to offer a satisfying safety profile. Other advantages of EPIT are that no adjuvant is needed[5]. More data on the clinical effect is required to evaluate the clinical usefulness of EPIT.

2.2.4 ILIT

Intralymphatic allergen-specific immunotherapy (ILIT) is an emerging form of AIT that uses a novel route of delivery with a shorter duration (3 injections over 8 weeks), good compliance and only mild side effects[27] (Fig. 2). In ILIT the allergen dose used is 100-fold lower compared to SCIT. Still, the allergen dose in the lymph nodes is at least 100-fold higher compared with any other AIT route[5, 27]. This high allergen dose locally in the lymph node is believed to trigger the immune system more effectively, compared to other AIT, and induce tolerance within a much shorter time frame[27]. As a result of the low treatment dose used, the safety profile is much more favorable for ILIT compared to SCIT. Only a handful of studies have compared the efficacy of SCIT and SLIT and there are no studies comparing ILIT with other AIT. So far, the general impression is that the efficacy of SCIT, SLIT, and ILIT is similar[28]. ILIT is a treatment method still under development and there is yet to determined what the optimal protocol is in the respect to adjuvant, dose, time between injections, number of injections and if an allergen dose escalation is needed for improved clinical effect.

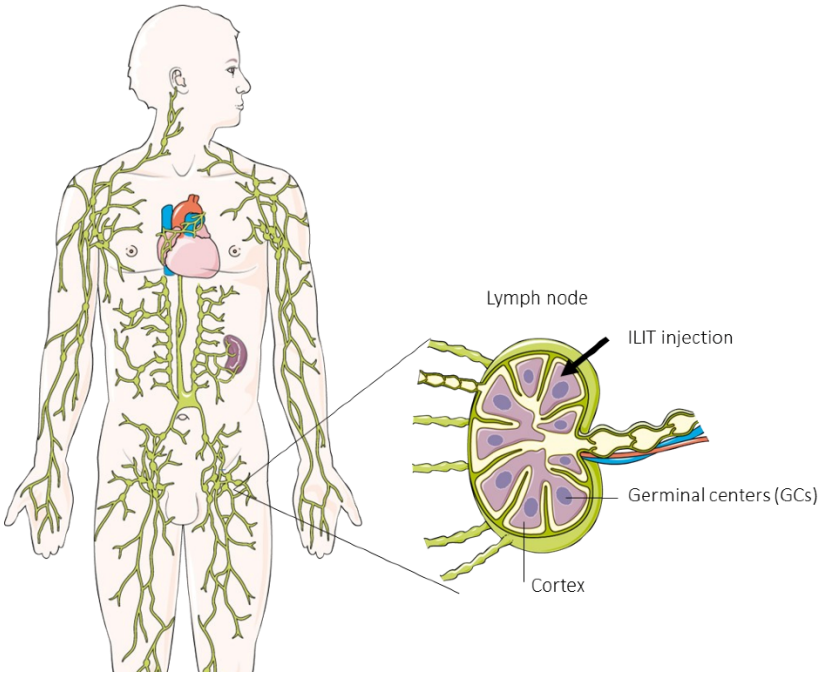

Figure 2. Illustration of placement of ILIT injections. In ILIT the lymph nodes in the groin are most often targeted. The injections are performed with an aseptic technique and ultrasound guidance, the outer cortex of the lymph nodes was targeted. The same lymph node was targeted for all three injections

2.3 THE IMMUNE RESPONSE TOWARDS ALLERGENS

The immune system is a complex network that protects the host from a number of pathogens or malignant cells while keeping a state of tolerance to self and innocuous non-self-antigens like allergens[23]. Normally, immune activation in response to extracellular helminth infections renders a TH2 type response. In allergic patients, the same immune response is activated against allergens[4]. The TH2 response and the allergic immune reaction involve antigen processing and presentation by antigen-presenting cells, Th2 cell differentiation, B-cell class switching to IgE, IgE coating of mast cells and basophils, and other changes in leukocytes not specified[4]. Some of the mechanisms previously described will be further reviewed below.

2.3.1 Allergen sensitization

In the presented project in this thesis, AR patients sensitized to birch and grass allergen have been studied. A schematic illustration allergen sensitization is presented in Figure 3. Sensitization is initiated by the uptake of allergens by DCs in peripheral tissue. DC sample allergens in the airway lumen or encounter them through a leaking epithelial layer[4]. The epithelial layer may further promote allergen sensitization by exposing DCs to an inflammatory milieu that favors a Th2 cell differentiation[29].

In reaction to the uptake of allergens, DCs migrate to regional lymph nodes or sites locally in the mucosa to activate $CD4^+$ T-cells. During the migration, the allergens are processed in antigen-processing compartments into antigenic peptides. These peptides are then loaded on MHC II molecules, which are transported to the cell surface, enabling activation of allergen-specific $CD4^+$ T-cells[30]. During the migration, the expression of co-stimulatory molecules CD80 and CD86 also increases; this is essential to fully activate T-cells and to initiate proliferation and differentiation[31]. Other co-stimulatory molecules may further enhance Th2 cell differentiation, like the expression of Notch ligand JAG-1[32]. Basophils locally in the lymph node may also promote the differentiation of naïve $CD4^+$ T-cells to Th2 cells by the release of IL-4[33].

Th2 cells produce IL-4 and IL13. In the presence of these cytokines, and under the ligation of CD40 with CD40L and CD80 or CD86 with CD28, B-cells undergo immunoglobulin class switching. During this process, gene segments that encode for immunoglobulin heavy gene segments are rearranged, and antibodies such as of IgE class are produced[4]. Secreted IgE then enters the lymphatic vessels and blood and is distributed systemically. IgE in circulation binds to the high-affinity IgE receptor (FcεR1) on tissue-resident mast cells thereby sensitizing them to respond upon a second encounter.

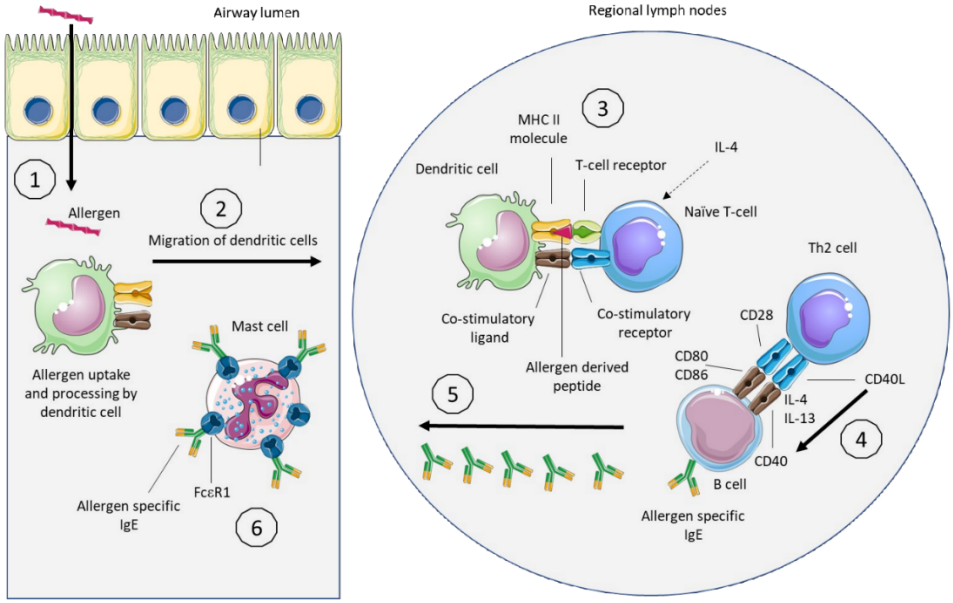

Figure 3. Sensitization to allergens in the airway. 1) Allergens pass through the nasal epithelial barrier and are internalized by DCs in the nasal mucosa. 2) DCs migrate to regional lymph nodes. During the migration DCs process the allergen and present peptides in the MHC II pocket. DCs also mature and increase the expression of co-stimulatory ligands and cytokines. 3) In the lymph node DCs activate allergen-specific $CD4^+$ T-cells by TcR, and CD28 interaction. IL-4 promotes the differentiation of naïve $CD4^+$ T-cells to Th2 cells. 4) Th2 cells interact with allergen-specific B-cells and promote class switching to IgE by interacting with CD80/CD86 and CD40, and the production of IL-4 and IL-13. 5) IgE^+ B-cells then produce and secrete allergen-specific IgE antibodies. 6) The secreted antibodies bind to FcεR1 receptors expressed by mast cells. This process leads to mast cells in the tissue sensitized to a specific allergen. (Figure adapted from Galli et.al. [4])

2.3.2 Antigen-presentation

Dendritic cells (DCs), macrophages, and B-cells are considered professional antigen-presenting cells (APCs) and have a constant capacity to present antigens and activate T-cells [34]. These cells are critical for the activation and differentiation of naïve $CD4^+$ T-cells. Eosinophils, neutrophils, and basophils can behave as antigen-presenting cells. However, the contribution of these cells in the activation of $CD4^+$ T-cells and the development and progression of AR is not known[34]. For T-cells to become activated three signals are needed. For $CD4^+$ T-cells the first signal is the binding of the T cell receptor (TcR) to a specific peptide presented in the major histocompatibility complex II (MHC II) pocket[31]. MHC II presents peptides derived from extracellular antigens. For $CD8^+$ T-cells the first signal is the binding of the T cell receptor (TcR) to a specific peptide presented in the major histocompatibility complex I (MHC I) pocket[31]. MHC I present peptides derived from intracellular antigens. Signal two is the activation of co-stimulatory receptors by the antigen-presenting cell. An array of co-stimulatory receptors including CD28, CTLA-4, PD-1, and Notch have evolved to properly regulate T-cell responses[31, 35, 36]. Activation of CD28 by CD80/CD86 expressed by antigen-presenting cells is a crucial signal for T-cells to become activated and differentiate into a specific Th subtype[31, 36]. Antigen presentation with little co-stimulation has been shown to result in T-cell anergy or induction of Treg cells[37]. The third signal is the secretion of cytokines. Some of the cytokines that influence Th cell differentiation are presented in Figure 4.

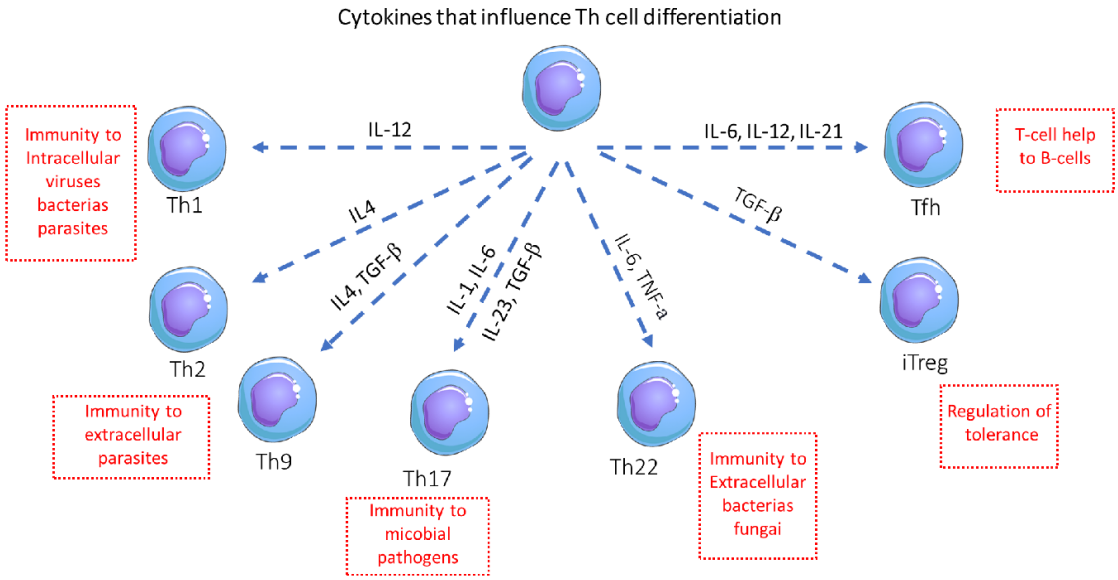

Figure 4. Cytokines influencing CD4⁺ T-cell differentiation.

2.3.3 Notch signaling

Notch signaling is a co-stimulatory pathway that has been shown to influence T-cell activation and differentiation[32]. In mammals, the Notch signaling pathway consists of five ligands (Jagged (JAG) 1 and 2; delta-like ligand (DLL) 1,3 and 4 and four receptors (Notch 1-4) [38]. There are conflicting models regarding how Notch ligands regulate T-cell functions in humans. There is evidence for an instructive model where JAG-1, JAG-2, Notch1 and Notch2 interaction initiates Th2 cell differentiation, and DLL-1, DLL-4, and Notch3 promote Th1 cell differentiation. There is also evidence for a model where Notch acts as an unbiased amplifier, regulating T-cell activation. In this model, Notch receptor-ligand interaction lowers the threshold for activation and optimizes rather than initiates immune responses [32]. In animal studies, Notch signaling seems crucial for the generation of Th2 cells and the development of allergic rhinitis [39, 40]. However, the importance of Notch signaling in IgE-mediated inflammation in a human setting is currently unknown. It is reported that soluble JAG-1 is increased in the blood of allergic patients and that the levels positively correlate with symptom severity[41].

2.3.4 T-cells

Mature CD4⁺ and CD8⁺ T cells express T-cell antigen receptors (TcR) that bind to peptides presented into MHC class II or class I molecules expressed by antigen-presenting cells[31]. CD4⁺ T cells differentiate into various subtypes of helper cells (Th1, Th2, Th9, Th17, Tfh, and Treg) to fight intra- and extracellular infections, to regulate immune activation of T cells, B cells, and antigen-presenting cells[31]. The function of CD8⁺ T-cells is to defend against intracellular pathogens, including viruses and bacteria, and for tumor surveillance[42].

The Th1 cells are induced by IL-12; they are defined by the expression of the transcription factor T-bet and the production of IFN-γ[31]. It has recently been suggested that in directing T-

cell polarization, the collective magnitude and duration of TcR and co-stimulatory signaling heavily affect the polarization[35]. This seems especially important for Th1 cell differentiation. It has been reported that T cells receiving strong antigenic signals selectively upregulate the IL-12Rβ2 subunit, priming them to receive IL-12 signaling and to undergo Th1 cell differentiation[35]. Th1 cells are induced in response to infections by intracellular bacteria and viruses[43]. In the context of AR, allergen-specific Th1 cells producing IFN-γ are detected in allergic and non-allergic patients at the same levels[44]. However, in non-allergic patients, a subtype of Th1 cells expressing type I and II interferon response genes IFI6, MX1, ISG20, OAS1, IFIT1, IFI44L have been detected at high levels[44]. Those T-cells have not been detected in allergic individuals.

Th2 cells are induced by IL-4 and defined by expressing the transcription factor GATA-3 and the production of IL-4 and IL-13[31]. For induction of Th2 cells, there is reported that Th2 differentiation may be the default outcome, occurring in the absence of alternative stimuli[35]. There are also studies reporting that JAG-1 expressing $CD11b^{+}CD301b^{+}PDL2^{+}$ DCs are a subset specialized in inducing Th2 cells by activation of co-stimulatory receptors[35]. Th2 cell differentiation is usually initiated in response to helminth infections[4]. In allergic patients, allergen-specific Th2 cells are detected at elevated levels; these cells are not detected in non-allergic individuals[44]. In the total Th2 cell population, a subtype of cells with the following phenotype $CD4^{+}CD27^{-}CD45RB^{-}CRTH2^{+}CD49d^{+}CD161^{+}$ have been identified. These cells are classified as Th2A and are detected at an elevated level in blood in allergic patients and low levels in non-allergic patients[45]. A subtype of T-cells closely related to Th2 cells are Th9 cells. These cells are induced by IL-4 and TGF-β and defined by the expression of the transcription factor PU.1 and IL-9[46]. Th9 cells have been shown to promote AR by enhancing tissue infiltration of eosinophils and mast cells and enhancing B-cell differentiation[46].

Th17 cells are induced by IL-1, IL-6, IL-23, and TGF-β. They are defined by expressing the transcription factor ROR-γt and the production of IL-17 and IL-22[31]. Th17 cells are induced in response to extracellular bacterial infections and fungal pathogens. To respond to infections, Th17 cells express CCR6 to facilitate migration to the inflammatory site[47]. The role of Th17 cells in AR inflammation is currently uncertain. Th22 cells are a Th subtype closely associated with Th17 cells. Th22 cells have been defined by their production of IL-22 and the absence in the production of IFN-γ, IL-4, and IL-17[48]. Th22 differentiation is induced by IL-6 and TNF-a [49]. The primary function of Th22 cells is to protect epithelial barriers such as in the nose and lung and modulate inflamed and injured tissue[48]. The role of Th22 cells in IgE-mediated inflammation is unclear today[50].

T regulatory cells (Treg) are necessary for restraining excessive or improper T-cell activation. T-cell activation against self-antigens, fetal antigens, and environmental antigens, can have catastrophic effects[51]. To control immune activation against food and environmental antigens, Treg cells can be induced in the periphery (iTreg) [51]. The milieu in the periphery that promotes iTreg cells, is characterized by elevated levels of TGF-β, retinoic acid, and short fatty acids. Treg cells are defined by the expression of FoxP3 or the increased expression of CD25[31]. Treg

cells suppresses T-cell activation by cell-cell contact, local secretion of inhibitory cytokines, and local competition for growth factors[5]. By these mechanisms, Tregs potently inhibit T-cell activation and proliferation.

T follicular helper cells (Tfh) are a specialized subset of $CD4^+$ Th cells that help B cells produce antibodies against foreign pathogens[52]. Tfh cells are defined by the expression of CXCR5, PD-1, Bcl-6, and IL-21[31]. Tfh cells are induced by IL-21, IL-6, IL-12, and typically both DC and B-cell interaction is needed for T-cells to differentiate to Tfh[53]. Tfh resides in secondary lymphoid organs (SLOs), including the tonsil, spleen, and lymph nodes. SLOs contain numerous B- and T-cells, and they are separated into specific zones[52]. Uniquely, mature Tfh cells are found in the B-cell zone interacting with B-cells. Tfh cells are essential for forming germinal centers (GCs), a distinct structure within the B cell zones. B cells within germinal centers undergo rapid proliferation and antibody diversification, allowing the production of many types of antibodies with greater affinity for their targets[54]. Tfh directs this process by providing co-stimulation and producing the cytokine IL-21, which drives B cell proliferation[55]. Additional cytokine production by Tfh determines the type of antibody produced. Most of the IL-4-induced class switching to IgE is produced by Tfh cells. IL-4-producing Th2 cells are more likely to be found in the peripheral tissue.

2.3.5 B-cells

Mature terminally differentiated B-cells, plasma cells, are known as secretors of immunoglobulins (Igs) [56]. The produced Igs are integral to humoral immunity and essential for neutralizing infections before they spread uncontrollably[57]. B-cells can also function as professional antigen-presenting cells activating naïve and memory $CD4^+$ T-cells [58]. The precise role of B-cell antigen presentation in AR is not entirely understood[58]. Further understanding of how B-cells initiate the allergic immune response is essential for developing future treatments against AR. It has been shown that IgE-facilitated antigen presentation and activation of antigen-specific T-cells sustain allergic inflammation[59]. B-cell activation of naïve $CD4^+$ T-cells has also been shown to promote the differentiation of Tfh cells[60].

The humoral immune response begins with the recognition and binding of the cognate antigen by a cell surface B-cell receptor, leading to activation and internalization of the antigen. In lymph nodes, activated B-cells migrate to the border between the B-cell follicles and the paracortex containing mainly T-cells[57]. At this step, B-cells which do not receive help from Tfh cells primarily differentiate into IgM antibody-secreting plasma blasts. B-cells can also migrate deep into the B-cell follicles and generate a germinal center (GC) response[57]. In the GC, B-cells go through multiple rounds of proliferation and sequential interaction with follicular dendritic cells (FDC) and Tfh. The interaction between B-cells, FDC, and Tfh cells in the GC leads to antigen affinity maturation and Ig class switch recombination. Depending on the cytokines in the local environment, B-cell can class switch into IgG1, IgG2, IgG3, IgG4, IgE, IgA1, or IgA2 [61, 62]. IgG is mainly involved in opsonizing pathogens for engulfment by phagocytes and activation of the complement system[63]. Structural differences in the four IgG subclasses translate into different biological effector functions. IgG1 and IgG3 activate

complement efficiently, whereas IgG2 is less efficient, and IgG4 does not appear to activate complement[62]. IgG4 is particularly interesting in allergen tolerance development for its capacity to block IgE-mediated cell activation[5]. IgE antibodies are mainly involved in the clearance of extracellular helminth infections. The binding of infectious agents to specific IgE antibodies bound to mast cells and basophils triggers activation of these cells and the releases of potent chemical mediators that induce reactions, such as coughing, sneezing, and vomiting, that can expel the infectious agents [4]. IgA is believed to have a primary role in protecting from infections by binding to infectious agents at the epithelial surfaces[61].

2.3.6 Basophils and Mast cells

Basophils and mast cells are TH2 inflammatory effector cells[4]. Mast cells are tissue-resident and barely detected in the blood[64]. Basophils can be detected both in the blood and in the tissue. In sensitized patients mast cells and basophils have allergen-specific IgE bound to FcεR1 expressed on the cell surface[4]. Basophils and mast cells exert the effector function when allergen binds to IgE and crosslink FcεR [4]. Crosslinking leads to degranulation and release of mediators like; histamine, leukotrienes, prostaglandins, and kinins[2]. These mediators induce some of the symptoms associated with AR. Beyond the release of cytokines that induce the symptomatic reactions, activated basophils also migrate to lymph nodes directing immune activation towards a Th2 cell activation by the secretion of IL-4[33].

2.3.7 Neutrophilic Granulocytes

Neutrophils are produced in the bone marrow and are the most abundant leukocyte detected in human blood[65]. During homeostatic conditions, neutrophils circulate in the blood and migrate into the tissue to execute their functions. Neutrophils are classically considered to only play a role in the first line defense against invading pathogens by responding to infections by phagocytosis, degranulation of stored mediators, and release of nuclear material in the form of neutrophil extracellular traps (NETs) [65]. Recent data suggest that neutrophils are more complex and may be involved in regulating adaptive immune responses as well. By cell-to-cell contact or the release of mediators, neutrophils have been reported to cross-talk with lymphocytes to regulate their function[66]. It is proposed that neutrophils is comprised of different subtypes with different roles in inflammation[67]. Neutrophils has been linked to increased autoimmunity and possibly IgE-mediated allergic disease by increasing the number of T-cells that respond during an immune response[66].

2.3.8 Other Immune cells

Eosinophils are known to be elevated in blood and tissue in AR patients[68]. Eosinophils are detected by the expression of Siglec-8 and the low expression of FcεR1. Eosinophils express FcγR1 receptors, a high affinity receptor for IgG[69]. In response to activation by an antigen, eosinophils release high amounts of Major basic protein (MBP), and eosinophil cationic protein (ECP) leading to activation of mast cells and other inflammatory cell[68]. Besides activating inflammatory cells MBP and ECP are cytotoxic and important for clearing infections against

parasites, including helminth infections. MBP and ECP ate also cytotoxic for human cells causing inflammation[70].

Eosinophils are also capable of producing TH2 inflammatory cytokines and chemokines, including IL-4, IL-5, IL-8, IL-10, and IL-13 promoting TH2 inflammation and allergic disease. Innate lymphoid cells (ILCs) are a lymphocyte subtype that resembles T-cells in functions but lacks the expression of T-cell receptors. ILCs reside mainly in mucosal tissues[71], where they respond quickly to environmental pathogens and allergens through receptors for cytokines, as well as receptors for nutrient components, microbial products, lipid mediators, and neuronal transmitters[72]. ILCs are divided into three groups: ILC1s, ILC2s, and ILC3s. ILC1s resemble Th1 cells and secrete IFN-γ, ILC2s resemble Th2 cells and secrete cytokines such as IL-5, IL-9, and IL-13, and ILC3s resemble Th17 cells and secrete IL17 and IL-22[72]. In multiple studies, ILC2s promote AR and asthma by rapidly responding to allergens and releasing TH2 cytokines[5]. ILCs will not be further addressed in this thesis.

2.4 INDUCTION OF ALLERGEN TOLERANCE BY AIT

Multiple immunological changes are detected in patients receiving AIT, particularly in patients who respond to the therapy. The most prominent findings are changes in DCs, T-cells, B-cells, humoral immunity, and changes in basophils and mast cells[16-18, 73-75]. A summary of different immunological mechanisms involved in allergen tolerance is presented in Figure 5.

Dendritic cells play a crucial role in the development of allergen tolerance. As a professional antigen-presenting cell, dendritic cells can either initiate or hamper allergenic inflammation. The markers C1Q and FcγRIIIa reflect changes in regulatory DCs (DCreg), and CD141, GATA3, and RIPK4 reflect changes in pro-allergic DCs[18]. Changes in this set of markers in favor of regulatory DCs markers can be used to monitor the effectiveness of AIT at an early stage[16]. DCreg primarily promote tolerance by their reduced expression of co-stimulatory receptors and expression of IL-10 and other anti-inflammatory cytokines[76].

For T-cells, multiple changes are associated with the induction of allergen tolerance. Th2A cells are increased in AR patients compared to non-allergic patients. The reduction of these cells in peripheral blood positively correlates with clinical response[45]. Also, immune deviation towards Th1 cell polarization is one of the mechanisms related to allergen tolerance[73, 77]. A recent study revealed that a subtype of Th1 cells expressing type I and II interferon response genes are unique for non-allergic patients[44]. It is possible that the induction of these cells in AR patients promotes allergen tolerance. Moreover, increasing the levels and function of regulatory follicular cells (Tfr) has been shown to positively correlate with clinical response[78]. Also, the induction of iTreg cells is another critical mechanism favoring allergen tolerance. iTreg cells exert their immunosuppressive abilities by secretion of anti-inflammatory cytokines IL-10 and TGF-β[5]. Additionally, iTregs cells producing IL-35 have been identified in patients treated with AIT[79]. IL-35 has been shown to suppress IgE-mediated inflammation[80].

B-cells ability to inhibit IgE-mediated allergic inflammation mainly resides in the production of allergen binding competing IgA and IgG antibodies. IgG4, in particular, binds to allergen

epitopes otherwise used by IgE, thereby competing and dampening IgE-mediated allergic inflammation. Recent studies also indicate that allergen-specific IgG2 increases in response to AIT, high concentrations of IgG2 were especially detected in patients benefiting the most [20, 81]. The evidence for the importance of Breg cells in the induction of allergen tolerance is accumulating[5]. In response to AIT, this B-cell subset is reported to be the exclusive producer of IgG4. This demonstrates the importance of B regs in allergen tolerance by the dual capacity of both IL-10 and IgG4-induced immune suppression[5].

AIT also reduces basophil allergen sensitivity, thereby reducing allergen-induced basophil activation and subsequently the release of mediators that induce AR symptoms. Two mechanisms are reported to be involved in reduced basophil activation, early desensitization, and the blocking effect of IgG4[5, 73]. In early desensitization, repeated activation of basophils below the threshold for activation is reported to make basophils less responsive to allergen induce degranulation[73]. The importance of basophil desensitization for AIT's long-term effect is unknown. The mechanism of IgG4 in reducing allergen-induced basophil activation depends on the capacity to block IgE-mediated activation[82].

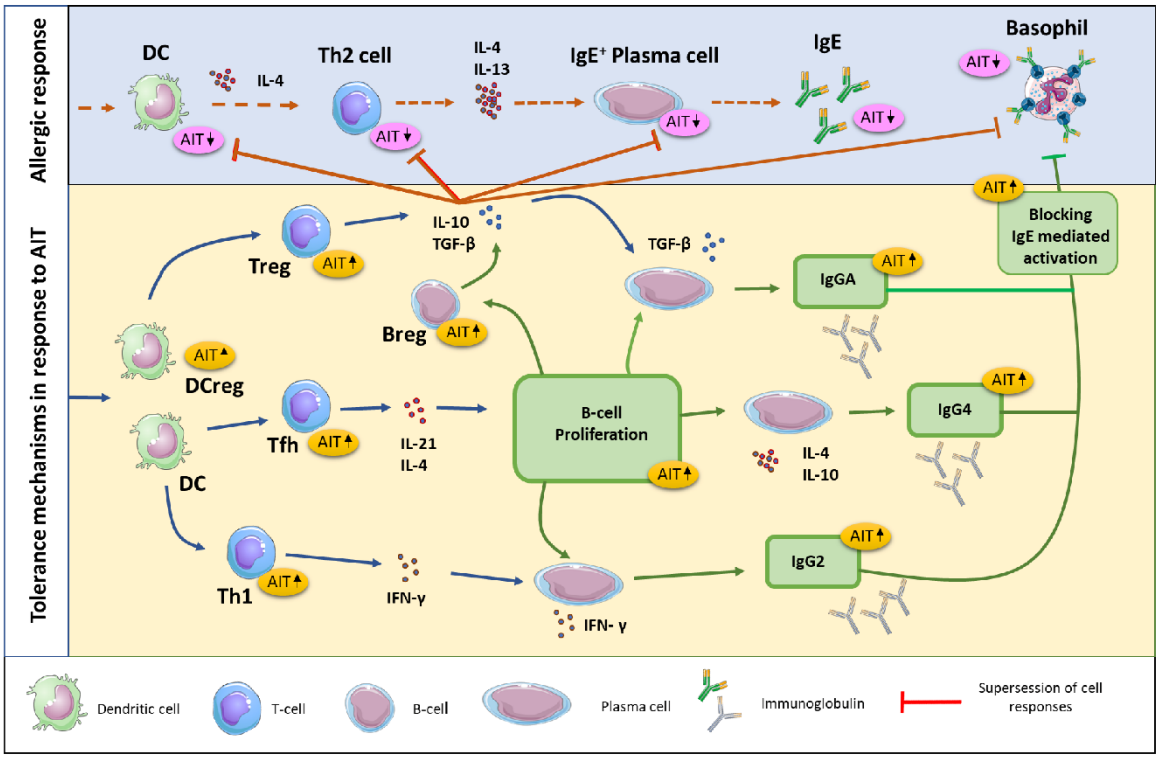

Figure 5. Mechanism of allergen tolerance in response to AIT. In response to AIT, DCreg promotes naïve T-cells to differentiate into Treg cells while conventional DCs promote differentiation into Tfh or Th1 cells.Tfh cells produce IL-4 and IL-21, promoting B-cell maturation, proliferation, and class switching. B-cell, under the influence of IFN-γ class switches to IgG2; in response to IL-4 and IL-10, the B-cells class switches to IgG4, and under the influence of TGF-β, the B-cell class switches to IgA. The secreted IgG2, IgG4, and IgA compete with IgE and reduce IgE-mediated cell activation. Additionally, in response to allergen-specific immunotherapy, B-cells differentiate into Breg cells. Secreted IL-10 and TGF-β from Breg and Treg suppress DCs, Th2, IgE⁺ B-cells, and Basophils, thereby inducing allergen tolerance. Additionally, Th1 cell secretion of IFN-γ suppresses Th2 cell differentiation, further reducing IL-4 and IL-13 induced class switching to IgE. (Figure adapted from Pavon-Romero et.al[20]).

3 RESEARCH AIMS

The overall goal of this thesis was to study novel immunological mechanisms behind the development of allergic rhinitis (AR) and to evaluate the clinical and immunological responses in allergic rhinitis patients treated with intralymphatic immunotherapy (ILIT).

The specific aims were to:

- Identify neutrophil subsets in the blood and nasal mucosa and characterize their role in AR

- Characterize the expression of Notch receptors on T-cells and the appearance of their corresponding Notch ligands on nasal epithelial cells and neutrophils in patients with AR

- Study the clinical outcome and immunological responses in ILIT with two concomitant allergens

- Investigate allergen doses to optimize the clinical outcome of ILIT

- Evaluate the longterm clinical improvement and immunological outcomes 5-6 years after ILIT

4 MATERIALS AND METHODS

This section contains a brief overview of the methods used in the papers I to V.

4.1 STUDY DESIGN

4.1.1 Paper I

Biopsies, Nasal lavage (NAL), and peripheral blood were acquired during the pollen season to analyze neutrophil subtypes. New blood samples were collected outside the pollen season for functional assay. 8 AR patients and 6 non-allergic patients were included.

4.1.2 Paper II

Nasal brush and blood samples were acquired outside the pollen season to analyze Notch pathway proteins on epithelial cells, neutrophils, and T-cells. 16 AR patients and 18 non-allergic patients were included in the study.

4.1.3 Paper III

This study was a randomized placebo-controlled clinical trial with 60 patients recruited between 2012-2015 at the Karolinska Hospital in Stockholm, and Skåne University Hospital in Lund. The patients were randomized 1:1 to either active treatment with ALK Alutard® birch and grass 1000 SQ-U or placebo treatment with ALK diluent. The patients were given three intralymphatic injections at 3-4 weeks intervals. The grass allergen injection was given in a lymph node in the left groin, while the birch allergen injection was given in a lymph node in the right groin. The study's primary outcome was a Nasal provocation test (NPT) with grass allergen. The secondary outcome was the safety of the treatment, allergen-specific IgE, allergen-specific IgG4, SPT, Rhinitis Quality of life Questionnaire (RQLQ), use of pharmacological treatment during the pollen season, and T-cell changes in blood and lymph nodes. Follow-up 1 was performed 2-4 weeks after the last injection, and follow-up two was performed 6-9 months after the last injection. The study outline is presented in Figure 6.

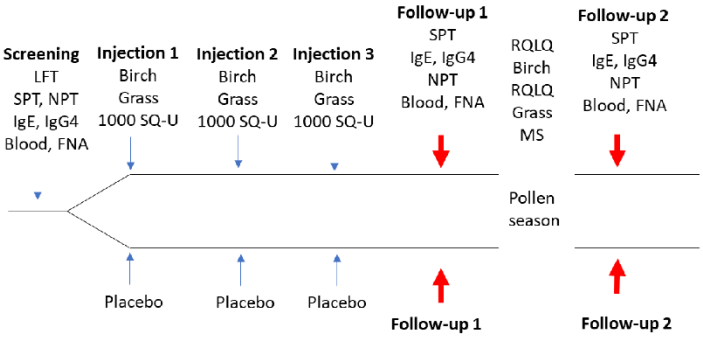

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Figure 6 Study outline of Paper III. LFT= Lung function test, SPT = skin prick test, NPT= Nasal provocation test, FNA= Fine needle aspiration, RQLQ= Rhinitis Quality of life Questionnaire. MS= Medication Score.

4.1.4 Paper IV

This study included two clinical trials: ILIT after SCIT-10000 and ILIT de novo-3000.

ILIT after SCIT-10000

This study was a clinical trial with 29 patients recruited between 2015-2016 at the Karolinska Hospital Stockholm and Skåne University Hospital Lund. The patients included in the trial had recently (<20 months) completed a 3-year SCIT treatment for grass allergen without reaching total symptom relief. The patients were randomized 1:1 to either active treatment with ALK Alutard® Grass or placebo treatment with ALK diluent. Patients treated with active ILIT received; Treatment 1: 1000 SQ-U, Treatment 2: 3000 SQ-U, and Treatment 3: 5000 SQ-U + 5000 SQ-U with 60 minutes of observation between injections. The primary outcome was CSMS during the pollen season. The secondary outcome was safety, allergen-specific IgE, allergen-specific IgG4, SPT, NPT, and RQLQ. The study outline is presented in Figure 7.

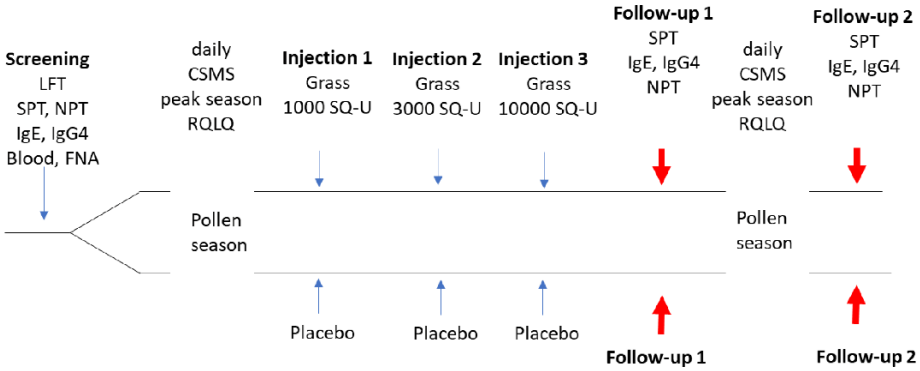

Figure 7. Study outline of ILIT after SCIT. LFT= Lung function test, SPT = skin prick test, NPT= Nasal provocation test, FNA= Fine needle aspiration, CSMS= Combined symptom and medication score, RQLQ= Rhinitis Quality of life Questionnaire.

ILIT de novo-3000

This study was a clinical trial with 39 patients recruited in 2016 at the Karolinska Hospital in Stockholm and Skåne University Hospital in Lund. The patients were randomized 1:1 to either active treatment with ALK Alutard® Grass or placebo treatment with ALK diluent. In the initial treatment, patients were treated with the same protocol as previously described for ILIT after SCIT-10000. However, due to non-acceptable adverse reactions, the treatment protocol was changed to; Treatment 1: 1000 SQ-U, Treatment 2: 3000 SQ-U, and Treatment 3: 3000 SQ-U. The same outcome of the study was used as previously described for ILIT after SCIT-10000, with the addition of an analysis of DCs, and T-cells in lymph nodes and blood. Follow-up one was performed four weeks after the last injection, and follow-up two was performed eight months after the last injection. The study outline is presented in Figure 8.

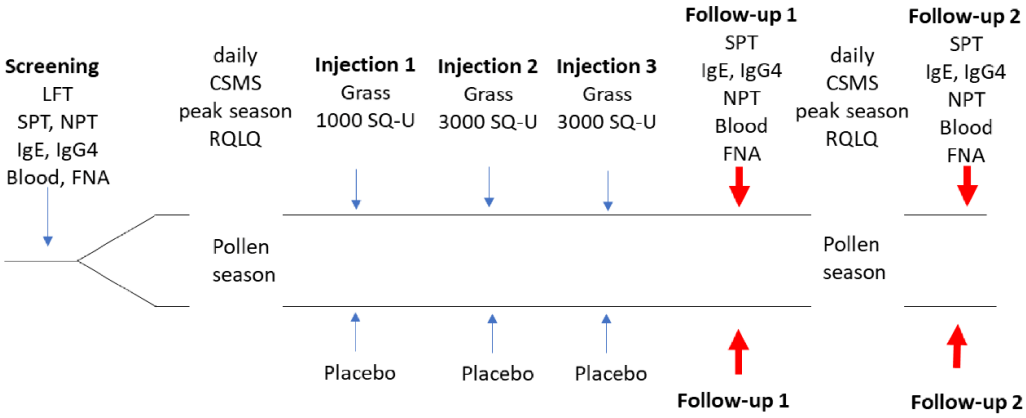

Figure 8. Study outline of ILIT de novo-3000. The first two patients were treated with 5000 SQ-U at injection 3. This induced severe adverse events and the protocol was changed to 3000 SQ-U for the remaining study patients. LFT= Lung function test, SPT = skin prick test, NPT= Nasal provocation test, FNA= Fine needle aspiration, CSMS= Combined symptom and medication score, RQLQ= Rhinitis Quality of life Questionnaire.

4.1.5 Paper V

This study was performed during 2018-2019 and was an open follow-up study of patients included in paper III. In study V, 20 patients treated with active ILIT 5-6 years earlier were compared to 14 control patients: 8 placebo-treated patients and six newly recruited non-AIT treated AR patients. The primary outcome parameter was NPT with grass and birch. The secondary outcome measures were CSMS during the pollen season, allergen-specific IgE, IgG4, RQLQ, basophil function, and analysis of T and B-cells in lymph nodes and blood. The study outline is presented in Figure 9.

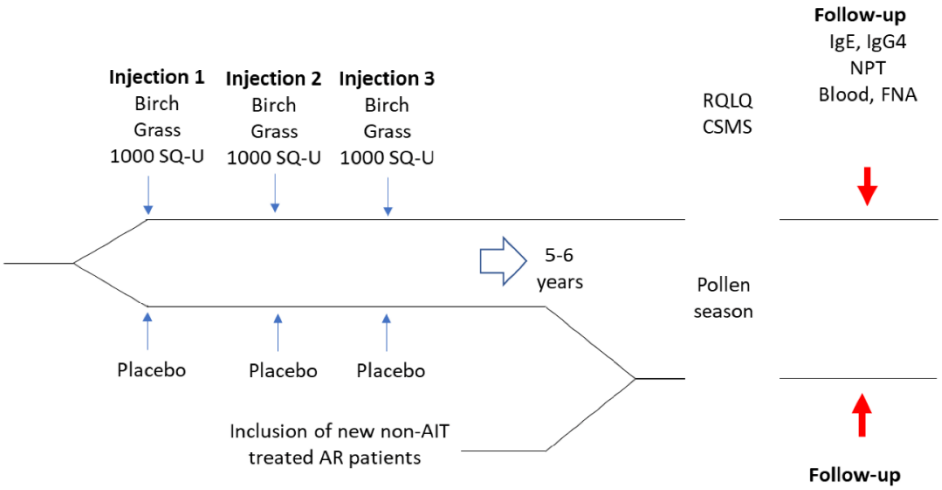

Figure 9. Study outline of Paper V. NPT= Nasal provocation test, FNA= Fine needle aspiration, CSMS= Combined symptom and medication score, RQLQ= Rhinitis Quality of life Questionnaire.

4.1.6 Intralymphatic injections

In study III-V the ILIT injections targeted lymph nodes in the subcutaneous tissue in the groin. The injections were performed with an aseptic technique and ultrasound guidance. To target the same lymph node for all three injections, a picture of the ultrasound was saved. If the allergen were visibly injected into the lymph node, the injection was scored as successfully performed.

4.2 PATIENT SELECTION

4.2.1 Paper I

The diagnosis of AR was based on clinical history, a positive skin prick test (SPT), and a positive test for birch or grass-specific IgE. Healthy controls had no history of sinus disease, asthma, or allergy. None of the healthy controls had a history of steroid use; all had a negative SPT and a negative IgE for birch or grass allergen or other allergens detected with ImmunoCAP Rapid.

4.2.2 Paper II

In study II the diagnosis of AR was based on the clinical history and a positive test for birch or grass-specific IgE. Healthy controls had no history of AR disease and a negative test IgE for birch or grass allergen or other allergens detected with ImmunoCAP Rapid.

4.2.3 Paper III-V

For the inclusion of patients in the clinical trials, the general indications for conventional AIT were used.

Patients with a history of moderate to severe AR during the pollen season according to ARIA guidelines, positive SPT, and allergen-specific IgE >0.3kU/L. Exclusion criteria were as follows: severe atopic dermatitis, uncontrolled perennial asthma, symptomatic sensitization to house dust mites or furry animals with daily exposure, use of beta blockers, ACE inhibitors, pregnancy, nursing, or planning for a pregnancy. Autoimmune or collagen diseases, obesity with BMI>30, or other significant diseases.

All studies I-V were approved by the Ethical review board in Stockholm and/or Lund. The clinical trials were also approved by the Swedish Medical product agency and conducted according to good clinical practice guidelines. The studies are registered at ClinicalTrials.gov.

4.3 EVALUATION OF CLINICAL IMPROVEMENT

4.3.1 Visual analog scale

The visual analog scale (VAS) is a fast and easy way to assess the overall symptomatic effect of AR in a patient[83]. In collecting VAS data, patients usually grade their symptoms on a continuous scale, ranging from 0-10, where 0 means ”no symptoms”, and 10 represents the “highest level” of symptoms. VAS can also be used comparatively[84]. In this case, 0 means “no relief and 10 means “complete relief”. The data refer to the experienced changes in symptoms before and after treatment. The comparative fashion of VAS was used in studies III – V.

4.3.2 Nasal provocation test

A nasal provocation test (NPT) can be used to evaluate rhinitis symptoms in response to allergen provocation. NPT can be done by increasing the allergen concentration in steps to determine the allergen threshold that induces symptoms[85-87]. NPT can also be done by evaluating symptoms after only one allergen dose[88, 89]. In study III-V, the patients were challenged with one allergen dose of 1000 SQ-U of the appropriate allergen in each nostril. The patients scored rhinitis and conjunctivitis symptoms from 0-3 at 0, 5, 15, and 30 minutes after the allergen challenge.

4.3.3 Quality of life

Quality of life (QoL) can be an essential parameter when assessing the disease burden of AR patients. For high sensitivity, a disease-specific questionnaire is preferred[90]. The Juniper Rhino conjunctivitis Quality of life Questionnaire (RQLQ) is recommended for AR and was the questionnaire used in study III-V[90-92]. The score was calculated as the average of 28 questions, ranging from 0-6. The maximum calculated RQLQ score was 6 points, and the minimal clinically significant improvement was 0.5 points.

4.3.4 Daily combined symptoms and medication score

Following the combined symptoms and medication score (CSMS) daily during the pollen season is the preferred method to evaluate treatment response to AIT[93]. The symptom score (SS) includes symptoms of the eyes and nose. The symptoms of the eyes include ocular itching, grittiness, redness, and tearing. The nose symptoms include nasal itching, sneezing, rhinorrhea, and nasal obstruction. These symptoms are scored 0-3 every day during the pollen season. The medication score (MS) includes the use of AR medication during the pollen season. In scoring medications, a common approach is to give the use of antihistamines 1 point and the use of steroids 2 points[93]. The SS and MS can be analyzed separately or in combination. In paper III, we used CSMS as the primary outcome measurement. For correct estimation of MS, the patients were instructed to use their medication stepwise if needed according to the ARIA guidelines[94]. The registration of symptoms and medication use was performed during the pollen season before the treatment as a baseline and during the pollen season after the treatment.

4.3.5 Modifications to symptom and medication score

In Paper III, the use of antihistamine tablets, ocular antihistamines drops, intranasal steroid spray, corticosteroid spray, corticosteroid tablets, β2 inhalant spray, and corticosteroid inhalation spray were assessed as reduced or unchanged use. In paper V, the same CSMS score was used as in paper IV but repeated six times during the birch and grass pollen season.

4.4 IMMUNOLOGICAL METHODS

4.4.1 Allergen-specific immunoglobulins

For the detection of allergen sensitization, ImmunoCAP™ Rapid was used in study I and II. In this test, blood was pipetted to the ImmunoCAP™ plate; this allows for allergen-specific IgE in the blood to bind to various allergens pre-coated in the plate. In the next step, a visualization fluid was pipetted to the ImmunoCAP™ plates to enable a yes or no detection of allergen-specific IgE. ImmunoCAP Rapid can detect the presence of IgE in blood to ten common airborne allergens, including pollen (birch, timothy, mugwort, olive, wall pellitory), house dust mites, mold, and common animal allergens (cat, dog, cockroach). In study III-V, the concentrations of birch, respectively grass specific IgE and IgG4 in serum were analyzed at Karolinska University Laboratory.

4.4.2 Flow cytometry

Flow cytometry was used in all studies to perform a single-cell analysis measuring the expression of specific proteins on the cell surface. The samples were analyzed on an LSRFortessa. The flow cytometry data were processed using FlowJo software© Flow cytometry is widely used to analyze cells and particles in a suspension. Both physical and chemical properties can be measured. Flow cytometry uses the scattering of the light from a laser to measure the size of a cell (forward scattering, FSC) and the granularity or internal complexity of a cell (side scattering, SSC). This can be used to differentiate leukocyte cell types (Fig. 10A).

To detect the expression of proteins or other molecules expressed by cells, specific monoclonal antibodies linked to a fluorochrome are most often used. Various fluorochromes emit light at different wavelengths. By building a panel with different fluorochromes conjugated antibodies, where each fluorochrome emits light at a specific wavelength, multiple targets can be detected on the same cell. Fluorochromes used in flow cytometry emit light in a relatively broad spectrum. The detectors used to detect light from a specific fluorochrome also receive light from other fluorochromes. This distortion of the data is corrected through a process called compensation. During compensation, the emitted light from every antibody fluorochrome conjugate is detected individually. This process allows the overlap of fluorescent light between fluorochromes to be measured and accounted for.

To analyze the data, a gate is often used to determine the fraction of cells expressing a specific antigen. Most often, internal control can be used (Fig. 10B). This means that a cell known not to express the target antigen can be used as a reference for gating. When this is not applicable,

fluorescence minus one control can be used. In this situation, all antigens are stained except for the one antigen the gating is applied to. To account for unspecific binding of antibodies, immunoglobulin isotype controls may be used to interpret the data accurately. Mean fluorescence intensity (MFI) can also be used to analyze the data. The measured intensity of the staining positively correlates with the expression levels of the antigen.

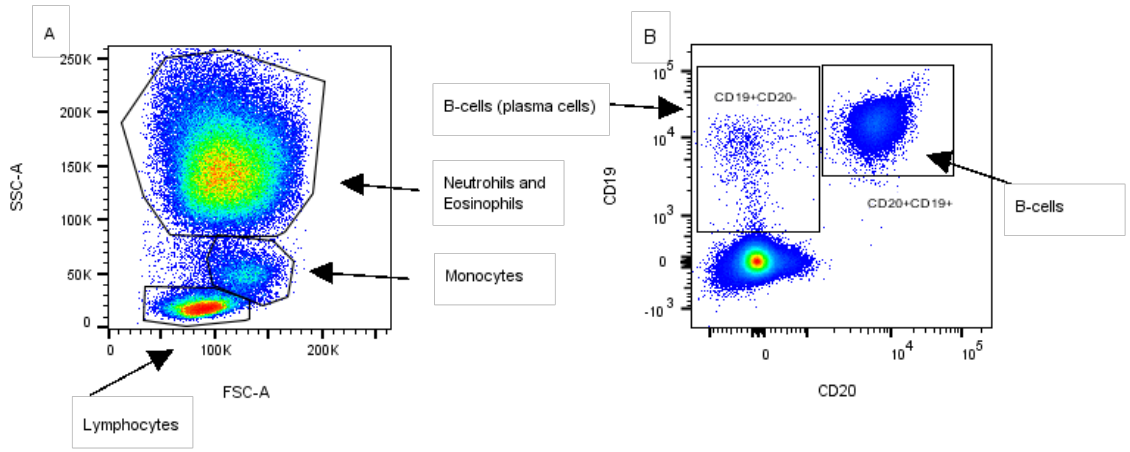

Figure 10. Representative flow cytometry plots. A) Dot plot displaying leukocyte population in peripheral blood using SSC and FSC on the y-axis respectively x-axis. B) Dot plot displaying staining of PBMC with CD19 and CD20 fluorochrome conjugated antibodies, which is shown on the y-axis and x-axis.

4.4.3 Co-culture of Neutrophils with PBMCs and Eosinophils

Blood was collected in heparin-coated blood tubes. Density gradient isolation of peripheral blood mononuclear cells (PBMC) and neutrophils was performed using Ficoll-Paque according to the manufacturer's instructions. The PBMC interface fraction from the Ficoll-Paque isolation was collected and washed with PBS. The bottom fraction from the Ficoll-Paque isolation was treated with ammonium chloride solution to lyse all erythrocytes. To further enrich the neutrophil population, magnetic beads targeting CD15 were used. To purify eosinophils, MACSxpress® kit was used according to the manufacturer's instructions.

To activate the $CD16^{high}CD62L^{high}$ neutrophils to $CD16^{high}CD62L^{dim}$, purified neutrophils were stimulated with 1µg/ml LPS, 5ng/ml TNFα, and 10ng/ml IL-8 for 15 minutes at degrees Celsius. Different neutrophil subsets were cocultured with PBMC for 30 minutes before T-cell activation with anti-CD3. The stimulation was stopped after 90 minutes, and the T-cell activation marker CD69 was measured with flow cytometry. To block cell-cell contact a transwell system was used.

Eosinophil migration was analyzed in a transwell system. The neutrophil subtypes and eosinophils were isolated as described above. The neutrophils were added to the bottom of the plate, and the eosinophils were added on top of the membrane. The eosinophil migration was stopped after 180 minutes. The cell count in the lower compartment was analyzed with flow cytometry.

4.4.4 Analysis of Basophil function

In project IV, we assessed the expression of FcεR1 and bound IgE on basophil in combination with analyzing allergen-induced basophil activation. Blood was collected into sodium heparin-coated tubes. Before allergen stimulation, the blood was washed with PBS. The stimulation was performed with either birch or grass allergen (ALK Aquagen) at 37 degrees Celsius and stopped with ice after 30 minutes. The samples were stained with appropriate fluorochrome-conjugated antibodies to detect basophils and the surface expression of FcεR1, the level of bound IgE, and the expression of activation marker CD63 and analyzed with flow cytometry.

4.4.5 Activation of allergen-specific T-cells

In project IV, the amount of allergen-specific $CD4^+$ T-cells was analyzed in peripheral blood. In short: Density gradient isolation of PBMC was performed using Ficoll-Paque according to the manufacturer's instructions. The PBMC interface from the Ficoll-Paque isolation was collected and washed with PBS. The PBMC were incubated for 16 hours at 37 degrees Celsius with 20000 SQ-U of grass allergen (Aquagen ALK). To accommodate the detection of CD154, the PBMC were pre-incubated with anti-CD40. Allergen-specific cells were quantified based on the expression of CD4, CD69, CD154, and CD137 analyzed by flow cytometry.

4.4.6 Immune cell phenotypes used in paper I-V

	Cell type	Phenotype used for identification
Paper 1	Neutrophils	$CD15^+CCR3^-CDw125^-$
Paper 1	$CD4^+$T-cells	$CD5^+CD4^+CD8^-$
Paper 1	Eosinophils	$Siglec8^+CD11b^+$
Paper 2	$CD4^+$T-cells	$CD5^+CD4^+$CD8-
Paper 2	Neutrophils	$CD15^+CD16^+$
Paper 2	Epithelial cells	$Epcam^+CD45^-$
Paper 3	$CD4^+$ Effector memory T-cells	$CD5^+CD4^+CCR7^-CD45RA^{+/-}$
Paper 3	$CD4^+$ Central memory T-cells	$CD5^+CD4^+CCR7^+CD45RA^+$
Paper 3	$CD8^+$ Effector memory T-cells	$CD5^+CD4^-CCR7^-CD45RA^{+/-}$
Paper 3	$CD8^+$ Central memory T-cells	$CD5^+CD4^-CCR7^+CD45RA^+$
Paper 3	$CD4^+$ Effector memory T-reg cells	$CD5^+CD4^+CD45RA^-CD25^{++}$
Paper 3	$CD4^+$ Central memory Th1 T-cells	$CD4^+CD5^+CCR7^+CD45RA^+CCR5^+$
Paper 4	$CD4^+$ Effector memory T-reg cells	$CD5^+CD4^+CD45RA^-CD25^{++}CD127^-$
Paper 4	$CD4^+$ Allergen specific T-cells	$CD4^+CD5^+CD69^+CD154^+$
Paper 4	Dendritic cells (tissue)	$SSC^{++}FSC^{++}CD45^+CD11c^+$
Paper 4	Dendritic cells (blood)	$SSC^+FSC^{++}CD45^+CD11c^+CD14^-$
Paper 5	Basophils	HLA $DR^-IgE^+Fc\varepsilon R1^+$

Figure 11. Description of immune phenotypes used in Paper I-V.

4.4.7 Statistical methods

Statistical analyses was performed using GraphPad Prism software or R Version R.3.3.3 GUI 1.69.

Statistical significance was set at $p< 0.05$. When comparing two unpaired groups, an unpaired t-test was used if the data was parametric. If the data was nonparametric, the Mann-Whitney test was used. The paired t-test was used to compare two matched groups if the data was parametric. If the data was non-parametric, the Wilcoxon matched pairs signed rank test was used. For more than two sets of data, a two-way ANOVA with Bonferroni post-tests was used.

For repeated measurements, a non-parametric Friedman test with Dunn's post-test, Tukey's multiple comparisons test, and a 2-way ANOVA with Fisher's LSD test were used.

In study III the proportion of patients that changed medication was analyzed with the Fisher exact tests or Chi-square test. Power calculations were performed in all clinical trials with a 2-sample t-test for the different primary outcomes. In paper IV, a generalized additive model was used in a time series analysis to evaluate the relative risk for allergy symptoms in relation to the detected pollen levels.

5 RESULTS AND COMMENTS

5.1 PAPER I - A POSSIBLE ROLE FOR NEUTROPHILS IN ALLERGIC RHINITIS REVEALED AFTER CELLULAR SUBCLASSIFICATION

The contribution of neutrophils to allergic inflammation is not well characterized compared to other granulocytes. It may well be that neutrophils are overlooked and have a significant role in allergic inflammation. Recent findings have revealed that neutrophils can be divided into different subsets with diverse roles in inflammation[95]. The purpose of the present study was to analyze the impact of different neutrophil subpopulations on allergic airway inflammation.

AR patients displayed increased levels of neutrophils in the blood, nasal mucosa biopsies, and nasal lavage compared to healthy controls (Fig. 12A-C). The distribution of neutrophil subtypes in peripheral blood, biopsies and NAL were similar in AR and healthy controls (Fig. 12D). $CD16^{dim}CD62L^{high}$ neutrophils are classified as immature neutrophils and were detected only at low levels in the blood, nasal tissue, and nasal lavage. The $CD16^{high}CD62L^{high}$ neutrophils are classified as "mature non-activated" and were the dominant neutrophil subtype in the blood. $CD16^{high}CD62L^{dim}$ neutrophils are classified as "mature activated" and were detected in nasal tissue and nasal lavage. The $CD16^{dim}CD62L^{dim}$ neutrophils are believed to be terminally differentiated just before apoptosis and were detected only in nasal lavage.

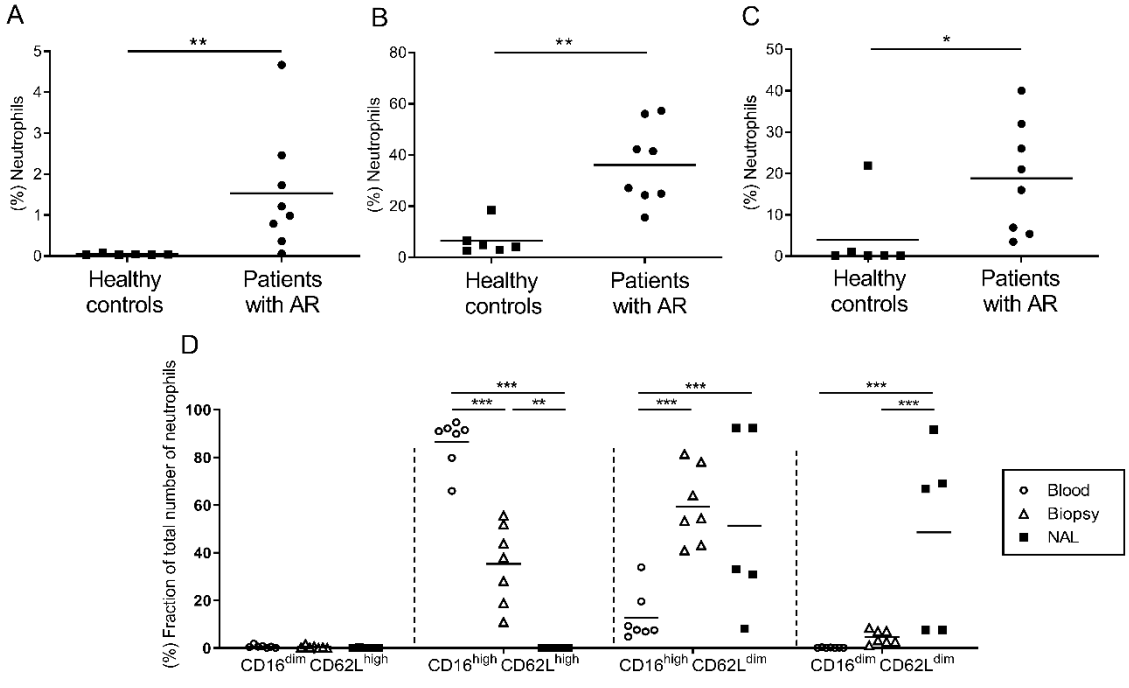

Figure 12. Neutrophils are increased in AR patients. A) Peripheral blood, B) Nasal mucosa biopsies, and C) Nasal lavage. D) The distribution of neutrophil subtypes in AR; blood, biopsies, and nasal lavage (NAL).

When the distribution of neutrophil subpopulations in nasal mucosa biopsies was compared between allergic patients and healthy control subjects, a skewed distribution in AR patients was detected. In AR patients, the level of $CD16^{high}CD62L^{dim}$ was higher than $CD16^{high}CD62L^{high}$ (Fig. 13). This difference in neutrophil subpopulations was not seen in the healthy control subjects.

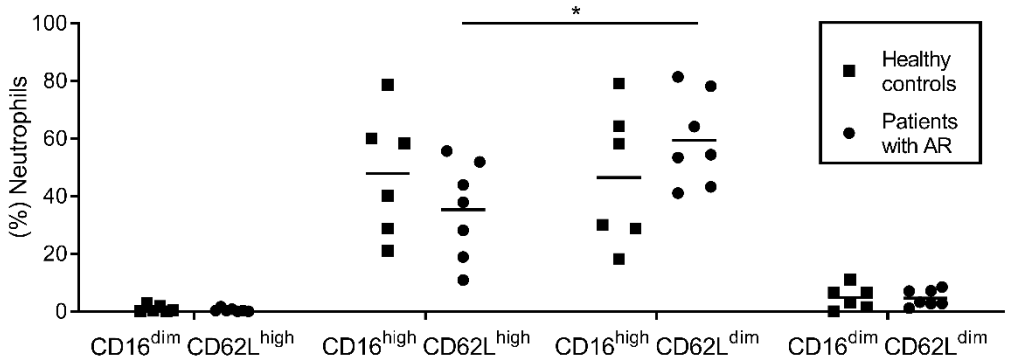

Figure 13. The distribution of neutrophils subtypes in the nasal mucosa.

Subsequently, we performed experiments to address the immunological importance of neutrophil subpopulations on T-cell activation. Blood-derived neutrophils and neutrophils activated *in-vitro* with LPS, TNF-α and IL-8 were incubated with autologous $CD4^+$ T-cells. Anti-CD3 was used to mimic antigen presentation and induce TcR mediated activation. The activation was detected by upregulation of CD69 expression. In a co-culture system with neutrophils and autologous $CD4^+$ T-cells, $CD16^{high}CD62L^{dim}$ were shown to prime $CD4^+$ T-cells and increase their response to anti-CD3 activation. The priming effect of $CD16^{high}CD62L^{dim}$ neutrophils on $CD4^+$ T-cells was detected both in allergic patients and in the healthy control subjects (Fig. 14A, B). By blocking the cell-cell contact between $CD4^+$ T-cells and neutrophils, the priming effect of $CD16^{high}CD62L^{dim}$ neutrophils was inhibited (Fig. 14C). This indicates that the T-cell priming induced by $CD16^{high}CD62L^{dim}$ neutrophils was dependent on the expression of cell surface molecules.

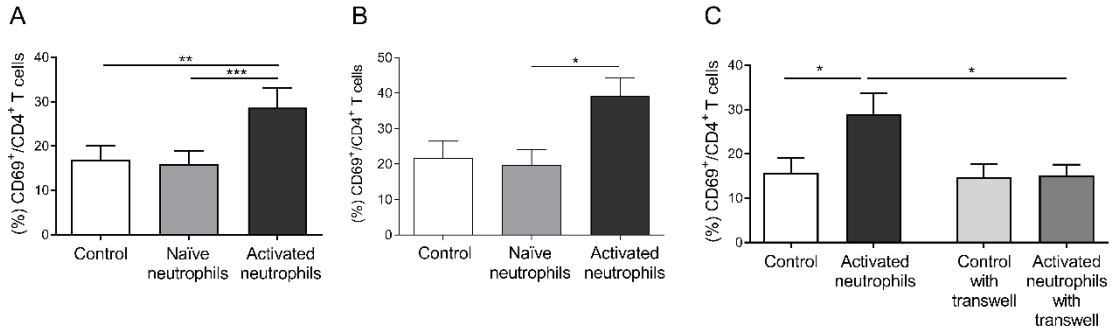

Figure 14. $CD16^{high}CD62^{dim}$ neutrophils increase T-cell activation. A) Healthy control. B) Allergic rhinitis. C) Experiment with and without a transwell. Control= neutrophils not added.

Experiments were also performed to investigate the impact of mature activated neutrophils on eosinophil migration. Blood-derived neutrophils and neutrophils activated *in-vitro* with LPS, TNF-α and IL-8 were incubated with autologous eosinophils. In a transwell system, activated $CD16^{high}CD62L^{dim}$ neutrophils increase eosinophil migration after 3 hours of incubation (Fig. 15). Blood-derived $CD16^{high}CD62L^{high}$ neutrophils did not affect eosinophil migration.

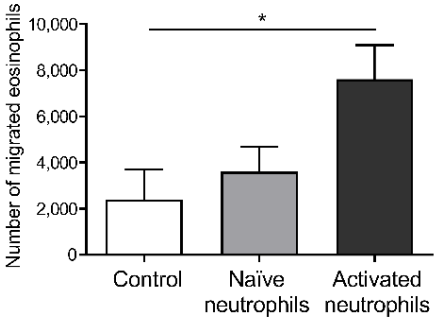

Figure 15. $CD16^{high}CD62^{dim}$ neutrophils increase eosinophil migration. Control= neutrophils not added.

5.1.1 Comments

In the nose, inflammatory signals induce neutrophils to migrate from bone-marrow to blood and into mucosa to remove pathogens and heal damaged tissue[96]. In the mucosa, neutrophils also respond to infections by secreting chemokines attracting eosinophils, mast cells, and basophils to the inflammatory site. The present study demonstrated that AR patients had an increased fraction of neutrophils in the blood, nasal mucosa, and nasal lavage during the pollen season compared to healthy control. Particularly in the nasal mucosa where AR patients displayed a 30 times higher fraction of neutrophils than the control patients. It is not inconceivable to think that inflammation in AR mucosa promote the infiltration of neutrophils. Neutrophils has been reported to increase in the tissue during the allergic late-phase reaction, and their presence is associated with the progression of allergic disease by enhancing allergic inflammation and tissue remodeling[97].

By analyzing cell surface expression of CD16 (FcβRIIIB) and CD62L (L-selectin), recent findings reveal that neutrophils can be divided into different subsets with diverse roles in inflammation[95]. In the present study, AR patients had an increased fraction of $CD16^{high}CD62L^{dim}$ neutrophils in the nasal mucosa compared to non-allergic control patients. Other researchers have shown that $CD16^{high}CD62L^{high}$ can differentiate into $CD16^{high}CD62L^{dim}$ by viral, bacterial, and microbial activation[65, 98, 99]. Our study demonstrated that allergens have the same ability to activate neutrophils by direct or indirect mechanisms. Recent data propose that $CD16^{high}CD62L^{dim}$ neutrophils can be detected in the bone marrow and may be recruited to the blood and peripheral tissue in response to inflammatory stress[100]. If inflammatory stress in AR patients increases the fraction of $CD16^{high}CD62L^{dim}$ neutrophils in the bone marrow is not known.

The immunological function of $CD16^{high}CD62L^{dim}$ neutrophils on the adaptive immune response is inconclusive. The present study showed that $CD16^{high}CD62L^{dim}$ neutrophils

generated *in-vitro* from blood-derived $CD16^{high}CD62L^{high}$ with IL-8, LPS, and TNF-α increased T-cell activation. We also demonstrate that the increased activation of $CD4^+$ T-cells most likely involves cell surface receptors expressed on $CD16^{high}CD62L^{dim}$ neutrophils. Our results are in contrast with those of Pillay et.al. where the $CD16^{high}CD62L^{dim}$ neutrophils suppressed T-cell activation[67]. One discrepancy in the experiments can be addressed by the time difference between the two experiments. Our study used a short incubation time of 90 minutes and focused on what effect living $CD16^{high}CD62L^{dim}$ neutrophils have on T-cell activation. Pillay et.al. used an incubation time of 96 hours and their setup may have focused on a more long-term impact of neutrophil and T-cell interaction. In a study by Hampton et.al. they reported that $Ly6G^{high}CD62L^{dim}$ mouse neutrophils promote T-cell proliferation, these results are in accordance with our data[101].

In another experiment, $CD16^{high}CD62L^{dim}$ neutrophils were shown to induce eosinophil migration. Kikuchi et.al. demonstrated that neutrophils activated with IL-8 increased eosinophil migration[102]. These results concur with our findings where we show that; IL-8, LPS, and TNF-α differentiate $CD16^{high}CD62L^{high}$ neutrophils into $CD16^{high}CD62L^{dim}$ neutrophils and they enhance eosinophil migration.

The limitation of our study was that the mechanism responsible for the T-cell priming was not elucidated. Nor was it analyzed if any T-cell subtype was more prone to be primed by $CD16^{high}CD62L^{dim}$ neutrophils.

Our finding with increased levels of neutrophils and especially activated $CD16^{high}CD16^{dim}$ neutrophils in AR nasal mucosa could open new therapeutic possibilities. To evaluate the effect of activated neutrophils in AR inflammation, an interesting intervention against allergic disease would be to selectively block neutrophil activation or deplete activated neutrophils. This may potentially affect the severity of the inflammation by influencing $CD4^+$ T-cells and eosinophils and other TH2 inflammatory cells.

5.2 PAPER II - UPREGULATED EXPRESSION OF NOTCH 1/4 – JAG-1/DLL-1 IN ALLERGIC RHINITIS

Co-stimulation of TcR is crucial for T-cell differentiation into distinct Th subtypes, proliferation, and effector functions[4, 31]. Notch signaling pathways have emerged as an additive co-stimulatory factor during T-cell activation in airways[103]. Epithelial cells and neutrophils have been shown to influence T-cell activation[104, 105]. If their expression of Notch ligands contributes to T-cell activation, and the allergic inflammation is currently unknown. This study aimed to analyze the expression of Notch receptors on $CD4^+$ T-cells and the expression of their corresponding Notch ligands on epithelial cells and neutrophils in AR patients and non-allergic control patients.

In the present study, AR patients displayed a significantly increased fraction of $CD4^+Notch1^+$ and $CD4^+Notch4^+$ cells in the nasal mucosa compared to the healthy control subjects (Fig. 16). No difference in the fraction of $CD4^+Notch2^+$ or $CD4^+Notch3^+$ cells could be detected between AR patients and healthy control subjects.

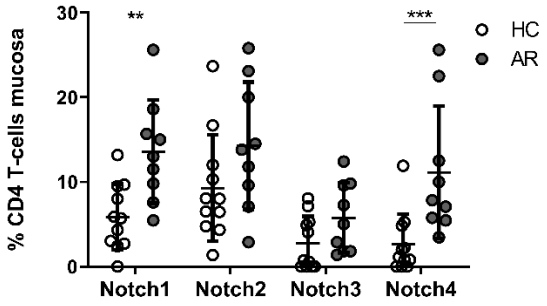

Figure 16. Nasal mucosa derived T-cells expressing Notch1 and Notch4 are increased in AR patients. HC= Healthy control, AR= Allergic rhinitis.

Next, we addressed the activation of Notch receptors by analyzing the expression of Notch ligands on nasal epithelial cells and neutrophils. Epithelial cells in the nasal mucosa of AR patients displayed significantly increased expression of JAG-1 and DLL-1, compared to the control subjects (Fig. 17A). Only a low expression of JAG-2 and DLL-4 was detected on nasal epithelial cells in AR patients and in the healthy control subjects. The fraction of nasal epithelial cells expressing JAG-1 and DLL-1 was also significantly increased in AR patients compared to the control subjects (Fig. 17B). Even though only a small difference was detected, it corroborates the finding of an increased expression of JAG-1 and DLL-1 on nasal epithelial cells in AR patients.

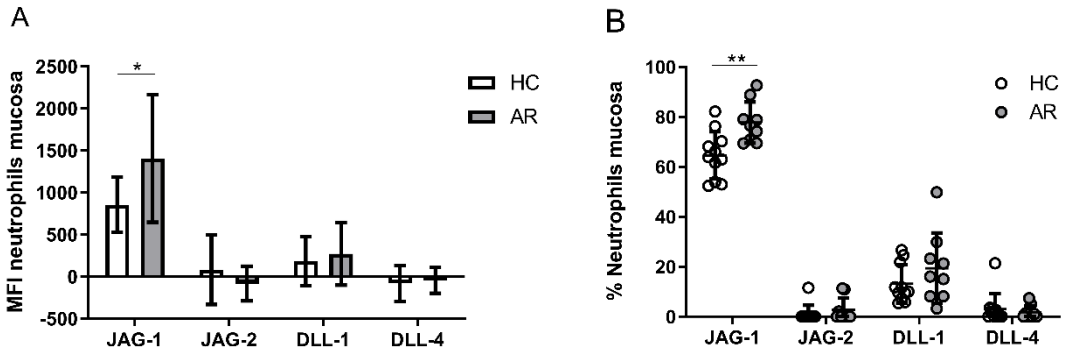

Figure 17. Nasal epithelial cells express increased levels of JAG-1 and DLL-1 in AR patients. A) The expression level of Notch ligands. B) The fraction of cells expressing Notch ligands. HC= Healthy control, AR= Allergic rhinitis.

In line with the finding on nasal epithelial cells, neutrophils also displayed increased expression of JAG-1. This was seen both in neutrophils derived from nasal mucosa and peripheral blood (Fig. 18A), (data from blood not shown.). Only a weak expression of JAG-2, DLL-1, and DLL-4 was seen in AR patients and in the healthy control subjects. The fraction of neutrophils expressing JAG-1 was significantly increased in AR patients compared to the non-allergic control patients (Fig. 18B).

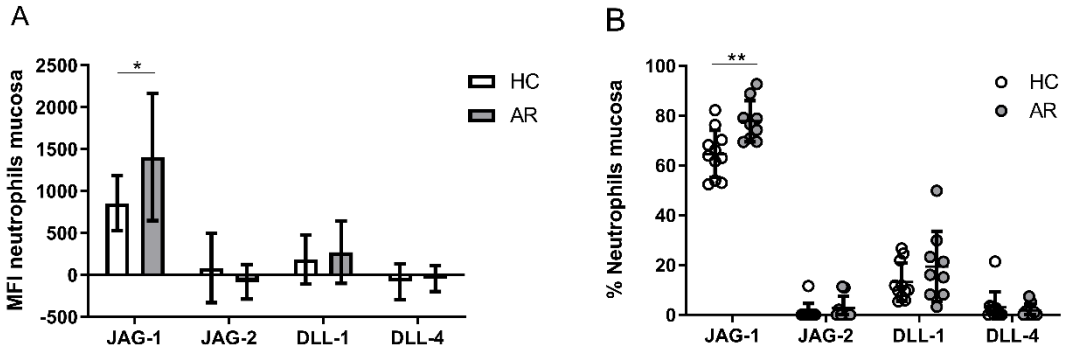

Figure 18. Nasal mucosa derived Neutrophils express increased levels of JAG-1. A) The level of Notch ligand expression. B) The fraction of cells expressing Notch ligands. HC= Healthy control, AR= Allergic rhinitis.

5.2.1 Comments

We demonstrated that the fraction of $CD4^+Notch1^+$ and $CD4^+Notch4^+$ T-cells was increased in the nasal mucosa of AR patients. We also revealed that AR patients had increased expression of Notch ligand JAG-1 and DLL-1 in the nasal mucosa, suggesting that the Notch signaling pathway is active in AR.

In animal studies, it has been shown that Notch signaling is crucial for the development of AR [106, 107]. On the other hand, how the involved cell types and the specific Notch signaling are involved in the development and progression of AR in animal models and humans are still unknown largely unknown[106]. Jiao et.al. have shown that JAG-1 and Notch1 are increased in AR compared to control [41]. Our results are in-line with these findings indicating that JAG-1 expression on antigen-presenting cells and Notch1 expression on T-cells may promote allergic inflammation in humans.

Xu et.al. also identified increased expression of Notch1 on $CD4^+$ T-cells in AR, and that blocking Notch1 may be a novel approach in treating AR by promoting the development of $CD4^+$ Treg, which induces allergen tolerance and reduces allergic inflammation[108]. In an animal model by Xia et.al. JAG-1 expression on antigen-presenting cells and Notch4 expression on $CD4^+$ T-cells was reported to promote the differentiation of Th2 cells and the development of allergic inflammation[40]. Further, Moya et.al. showed that Treg in peripheral tissue expressing Notch4 had a reduced capacity to maintain allergen tolerance[109]. Our findings are in accordance with these results indicating that Notch4 expression on $CD4^+$ T-cells may be a new target to regulate allergic inflammation. In contrast to our findings, Jiao et.al. showed that Notch2 expression was reduced in AR compared to the control group[110]. There are methodological differences between the two studies that could explain the discrepancy. In our study, Notch was analyzed on a protein level on $CD4^+$ T-cells, while Jiao et.al. used gene expression of Notch on the total cell count in the nasal mucosa.

A limitation in our study is that additional evidence is needed to prove that the detected differences in Notch ligand expression between AR and healthy control subjects have biological relevance. Still, this study presents novel data suggesting that Notch signaling may participate in the regulation of T-cells in AR.

5.3 CLINICAL RESPONSE TO ILIT

Allergen-specific immunotherapy aims to reduce the allergic symptoms of the patients. There are several methods to detect the clinical response. NPT are a frequently used and assess symptoms derived from allergen provocation[93]. Other methods commonly used to measure clinical response are RQLQ and CSMS which measure symptom and medication use during the pollen season[93]. In study III, ILIT-1000, the primary outcome measurement was NPT score after grass allergen challenge. In patients treated with active ILIT, clinical improvement was detected 6-9 months after treatment (Fig. 19B). No reduction in symptoms were seen in patients treated with a placebo (Fig. 19A).

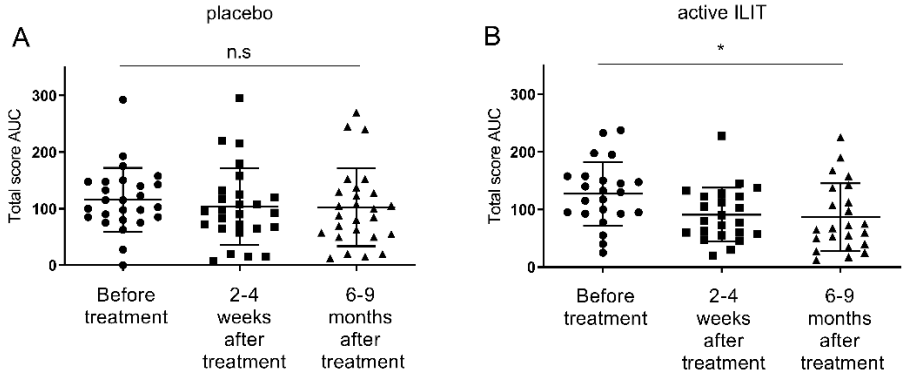

Figure 19. Reduced NPT total score in active ILIT patients. Timothy allergen was used for Nasal allergen provocation (NPT). A) Placebo. B) active ILIT.

At the follow-up of ILIT-1000, 5-6 years after the initial treatment NPT was still the primary outcome measurement. No clinical improvement could be detected when comparing the symptoms scored before treatment with those scored 5-6 years after treatment (Fig. 20A). When comparing the scores between active ILIT and the control group in the follow-up study 5-6 years after, the active ILIT patients scored significantly lower for grass allergen provocation compared to non-AIT controls (Fig. 20B). This clinical improvement was not detected for birch allergen (Fig. 20C).

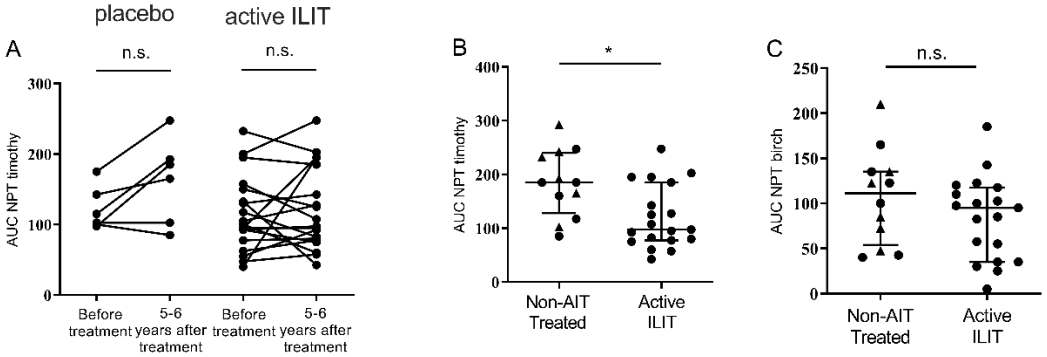

Figure 20. NPT is reduced in active ILIT patient s5-6 years after the initial ILIT treatment. A) Paired comparison. B-C) Un-paired comparison 5-6 years after ILIT. Timothy and birch allergens were used for Nasal allergen provocation (NPT).

In study III, Juniper RQLQ scores were used as a secondary outcome of clinical improvement. We could detect a trend for clinical improvement during the birch allergen season (Fig. 21). No clinical improvement could be seen during the grass allergen season. At the 5–6-year follow-up, a trend with lower scores for both birch and grass allergens was detectable in patients treated with active ILIT compared to the non-AIT treated group, although not significant (data not shown).

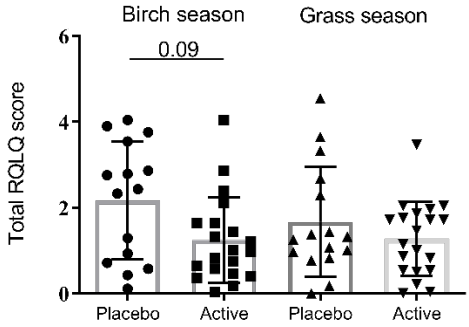

Figure 21. A trend for reduced RQLQ in active ILIT patients.

The follow-up study 5-6 years after the initial treatment used CSMS as a secondary outcome. CSMS is currently recommended as the optimal method to evaluate allergic symptoms and clinical response to allergen-specific immunotherapy. At the follow-up, we detected significantly lower CSMS and MS during the birch and grass allergen seasons in active ILIT compared to non-AIT treated controls (Fig. 22A, C).

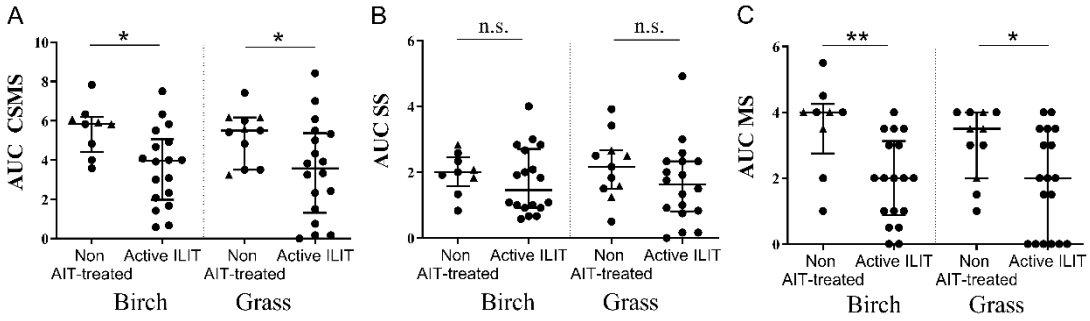

Figure 22. CSMS is reduced in active ILIT patients. A) Combined symptoms and medication score (CSMS). B) Symptom score (SS). C) Medication score (MS). Measurements were acquired during the birch and grass pollen season. AUC= Area under the curve.

In ILIT after SCIT-10000, we could detect a significant reduction in CSMS and MS in patients treated with active ILIT (Fig. 23A, C). No difference was seen in the placebo group. In ILIT-de novo-3000, CSMS was significantly reduced in the active ILIT group and in the non-AIT-treated group respectively (Fig. 23D).

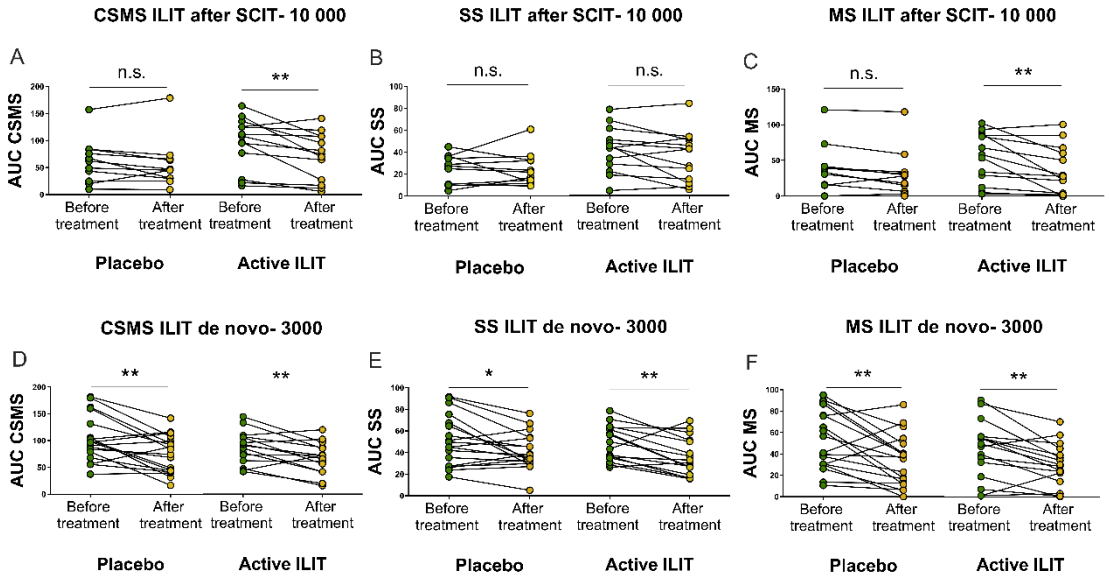

Figure 23. CSMS is reduced in ILIT after SCIT-10000 CSMS patients. A, D) Combined symptoms and medication score (CSMS). B, E) Symptom score (SS). C, F) Medication score (MS). Measurements were acquired during the grass pollen season. AUC= Area under the curve.

5.3.1 Comments

In study III, ILIT-1000, we identified a reduced reaction to nasal provocation with grass allergen. The reduction in symptom scores for active ILIT patients was 32%, and for placebo-treated patients, 12%. The overall decrease in symptoms scores for active ILIT treated patients was around 20% over placebo, which is a level that is believed to be clinically significant [111]. At the follow-up, 5-6 years after the initial treatment, the reduction in symptoms score for NPT could no longer be detected. The low power of the study is a weakness and prevented us from seeing any clinical improvement below 28%. Few studies have investigated the long-term effect of ILIT. In a small open-label study, Ahlbeck et.al. demonstrated that clinical improvement could be sustained for at least three years, supporting a long-term response for ILIT[112]. A double-blinded placebo-controlled study by Skaarup et.al. convincingly demonstrated a substantial clinical improvement in year one after ILIT[113]. However, only a clinical improvement trend was detected in years two and three.

Comparing the NPT scores between active ILIT and the non-AIT treated group revealed a significant clinical improvement for active ILIT at the 5–6-year follow-up. A weakness with this methodology is the possibility of bias in the comparison since not all patients were randomized and included simultaneously. Baseline characteristics with VAS show higher scores for the non-AIT treated group than for the active ILIT group. However, CSMS and NPT values lie in the same range for placebo-ILIT patients and the new control patients. With this in mind, we believe a comparison between active ILIT, and the non-AIT treated group is valid. Still, it is impossible to rule out that the disease severity before the treatment is a factor in the difference detected between active ILIT and the control.

In study III, ILIT-1000, we included RQLQ questionnaires as a secondary outcome measurement. A trend for reduced symptom scores during the birch allergen season was detected in patients treated with active ILIT, while no difference could be detected during the grass allergen season. A shortcoming with the RQLQ questionnaires is that medication use is not addressed. However, during the study, medication scores were analyzed separately. We could detect a higher medication use in the placebo group compared to the patients treated with active ILIT. It might be that the increased use of medication explains why the study failed to detect significant treatment effect for birch and grass allergen. The medical questionnaires did not discriminate at what time point during the pollen seasons patients used their medications. It is possible that the patient started to use the medicine after sensing symptoms during the birch pollen season and continued their use during the grass allergen season. The reduced symptom burden then prevented us from detecting any symptomatic difference between active ILIT and placebo.

At the follow-up, 5-6 years after the initial treatment a trend for reduced RQLQ scores was detected for both birch and grass allergen in patients treated with active ILIT. We also detected a reduced use of medication in the active ILIT group. It is possible that the difference in medication use prevents a significant read out in RQLQ scores between active ILIT and the non-AIT treated patients. To improve and standardize clinical AIT trials the European Academy of Allergy and Clinical Immunology now recommends the primary outcome measurement to be the combined symptom and medication score CSMS[93].

In the 5–6-year follow-up study, CSMS was used as a secondary outcome to detect a remaining clinical effect of ILIT. We could see significantly lower scores for CSMS in patients treated with active ILIT compared to the placebo group for both grass and birch allergens. A weakness in our CSMS data is that the study was an open-label study, which can bias the CSMS scores. The CSMS score combines the allergic symptoms and the medication used during the pollen season. The major difference detected between active ILIT, and placebo was medication use. Since medication use is less prone to bias, this strengthens the validity of the CSMS results.

In ILIT after SCIT-10000 and ILIT de novo-3000, CSMS was used as the primary outcome to detect the clinical response. We could see a reduction in CSMS for ILIT after SCIT-10000 in patients treated with active ILIT. However, only a 3% improvement over placebo was detected, questioning the biological relevance of the results. It is possible that differences in pollen count between seasons reduced the symptoms burden and prevented us from detecting a more biologically relevant difference between active ILIT and placebo patients.

In ILIT de novo-3000, both active ILIT and placebo-treated patients had reduced CSMS. The pollen count was 25%-46% lower the year after treatment and is a likely factor in the reduced symptom burden detected both for active ILIT and placebo. No trend for clinical improvement could be seen when comparing active ILIT with placebo-treated patients. We conclude that the allergen doses used in ILIT de novo-3000 do not add any beneficial clinical effect compared to ILIT-1000.

5.4 T-CELL CHANGES IN RESPONSE TO ILIT

In study III, ILIT-1000, 1000SQ-U/ml were used for the three lymph node injections of birch and grass allergen. FNA and peripheral blood were sampled before the treatment and 2-4 weeks after the last injection. In study IVa, ILIT after SCIT-10000, patients previously treated with SCIT were treated with three grass allergen lymph node injections according to protocol presented in Material and Methods Figure 7. Peripheral blood was sampled before treatment and four weeks after and eight months after the treatment. In study IVb, ILIT de novo-3000, allergic patients were treated with three grass allergen lymph node injections according to protocol presented in Material and Methods Figure 8. FNA and peripheral blood were analyzed before the treatment, and 2-4 weeks after the last injection. Blood was also analyzed at the 8-month follow-up. In study V, a follow-up of study III, lymph node and blood samples were sampled again 5-6 years after the initial treatment.

In allergic patients, it is well established that allergen-specific Th2 cells promote the initiation and progression of the allergic immune response. During allergen-specific immunotherapy, it is also clear that regulatory T-cells increase and play a crucial role in inducing allergen tolerance. This part of the study aimed to analyze the effect of intralymphatic immunotherapy on T-cells in lymph nodes and in peripheral blood.

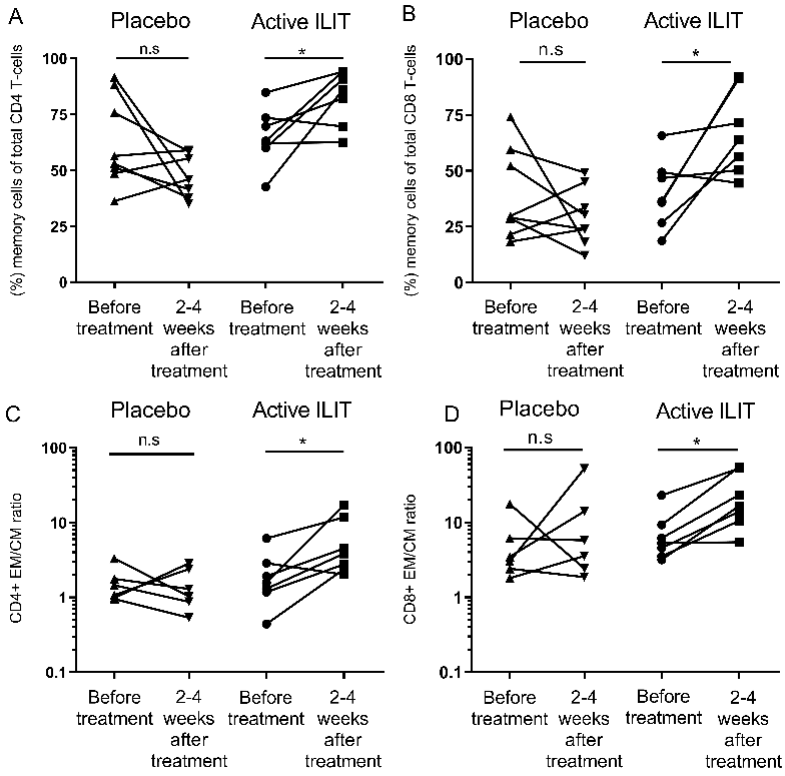

Figure 24. EM/CM ration is increased in lymph node derived T-cells. A, B) The fraction of memory T-cells. C, D) The ratio of effector memory (EM) T-cells and central memory (CM) T-cells.

In Study III, ILIT-1000, a double-blinded placebo-controlled trial, patients were treated with active ILIT or with a placebo. Patients treated with active ILIT displayed an increase of $CD4^+$ and $CD8^+$ memory T-cells in lymph nodes 2-4 weeks after treatment (Fig. 24A, B). A trend for

increased lymph node infiltration of naïve T-cells was detected in placebo-treated patients (Fig. 24A, B). In patients treated with active ILIT the $CD4^+$ and $CD8^+$ T-cells displayed an increased proportion of effector memory cells in lymph nodes 2-4 weeks after the treatment (Fig. 24 C, D). This increased proportion of effector memory T-cells was not detected in the patients treated with a placebo (Fig. 24C, D).

At the long-term follow-up, 5-6 years after the initial treatment, a significantly higher fraction of $CD4^+$ memory T-cells was detected in lymph node material from patients treated with active ILIT than in the control group (Fig. 25 A). No difference was seen for memory $CD8^+$ T-cells between patients treated with active ILIT and the control group (Fig. 25B). Nor was any difference detected in the ratio of effector memory T-cells and central memory T-cells between patients treated with active ILIT and the control group (Fig. 25 C, D).

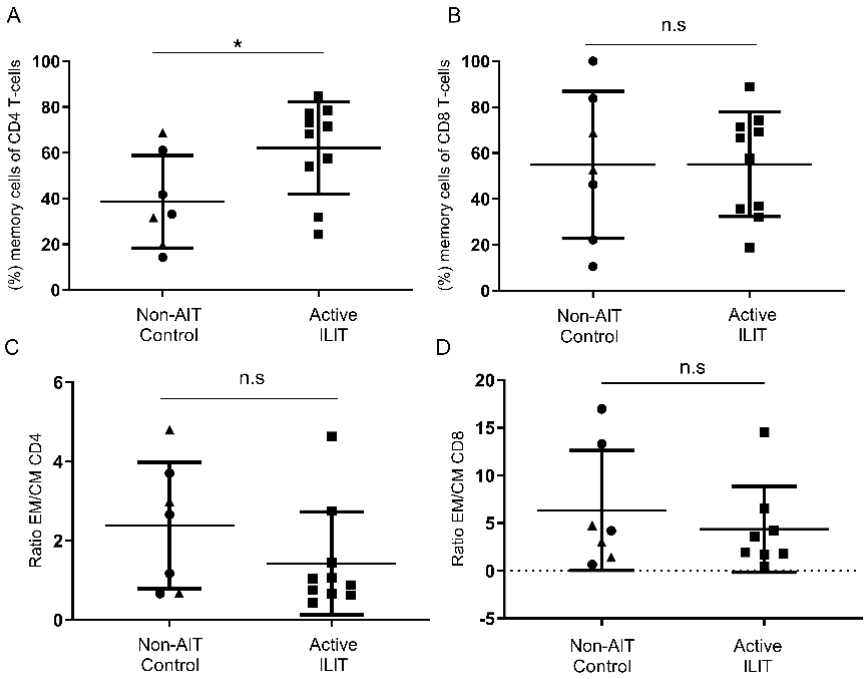

Figure 25. Memory $CD4^+$ T-cells was increased in lymph node derived T-cells 5-6 years after ILIT-1000. A, B) The fraction of memory T-cells. C, D) The ratio of effector memory (EM) T-cells and central memory (CM) T-cells.

In blood-derived T-cells, there was an increase in $CD4^+$ central memory T-cells ($CCR7^+CD45RA^-$) expressing CCR5 in patients treated with active ILIT at 2-4 weeks after treatment (Fig. 26A). Further, patients treated with active ILIT displayed increased fraction of regulatory effector memory T-cells ($CCR7^-CD4^+CD25^{++}$) in blood 2-4 weeks after treatment (Fig. 26B). None of these changes was detected in the placebo-treated patients.

In study IVb, ILIT de novo-3000, a double-blinded placebo-controlled trial, patients were treated with active ILIT or with three injections with a placebo. In blood-derived T-cells from patients treated with active ILIT or placebo, no changes in the levels of $CD4^+$ central memory T-cells ($CCR7^+CD45RA^+$) expressing CCR5 or effector memory Treg ($CD4^+CD127^{dim}CD25^{++}$) could be detected 4 weeks after treatment (Fig. 26C, D).

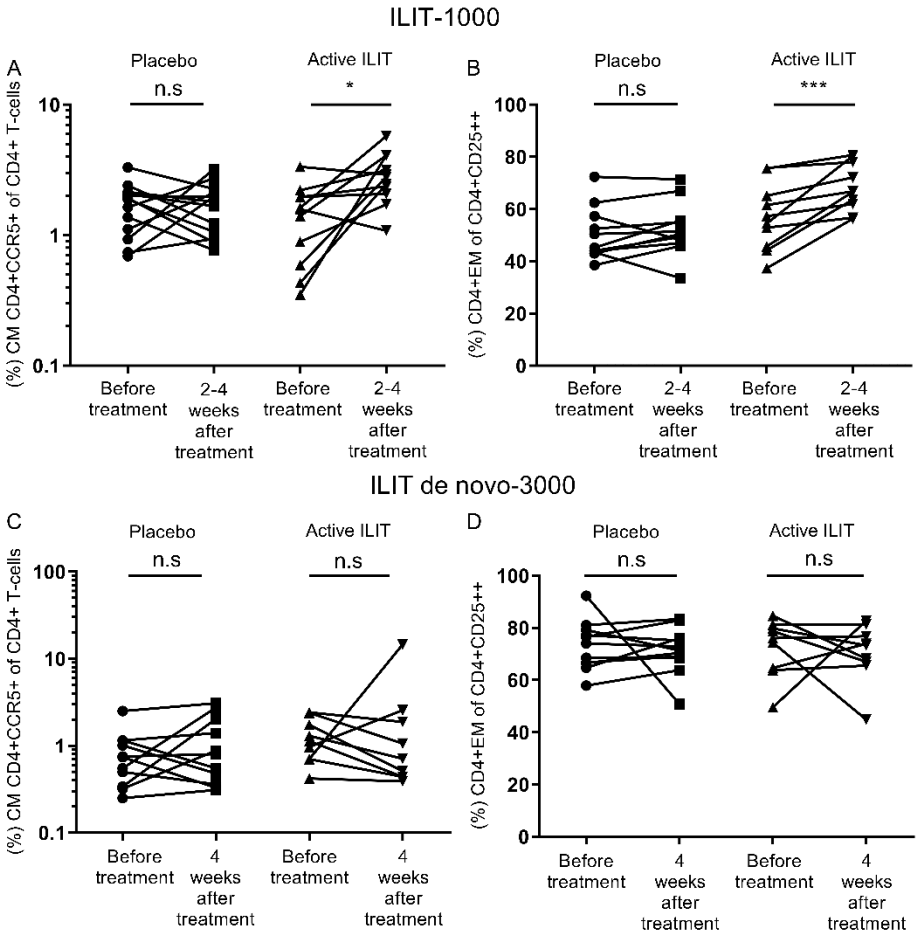

Figure 26. ILIT-1000 increase the fraction of $CD4^+$ CM $CCR5^+$ and $CD4^+$ EM $CD25^{++}$ in blood. A, B) ILIT-1000. C, D) ILIT de novo-3000. EM= Effector memory T-cells. CM= Central memory T-cells.

In ILIT de novo-3000 the levels of allergen-specific T-cells were measured 4 weeks after treatment in blood. To detect allergen-specific $CD4^+$ T-cells, PBMC were purified from whole blood. The cells were then incubated with a CD40 antibody to block CD154 down-regulation and 5000 SQ-U/ml of timothy allergen or control medium. The detection of allergen-specific $CD4^+$ T-cells expressing CD69, and CD154 was successful in both active ILIT and placebo-treated patients (Fig. 27A, B). However, no difference in the levels of allergen-specific $CD4^+$ T-cells could be detected between patients treated with active ILIT or placebo (Fig. 27C).

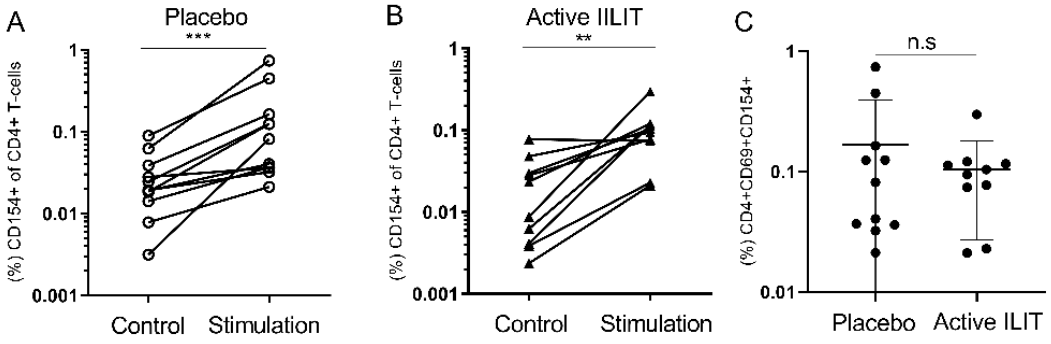

Figure 27. Placebo and active ILIT display similar levels of allergen specific T-cells. Control= no allergen added. Stimulation= Timothy allergen added.

5.4.1 Comments

In response to ILIT with 1000 SQ-U/ml, memory $CD4^+$ and $CD8^+$ T-cells were increased in lymph nodes after active treatment, and a trend for increased levels of naïve $CD4^+$ T-cells was seen in patients treated with placebo. The findings suggest that lymph node injections increase the infiltration of naïve T-cells into the lymph nodes. This infiltration of immune cells may be caused by tissue destruction during the needle injections and the release of alarmins from injured cells[114]. The results also indicate that antigen-presenting cells in the patients treated with active ILIT present antigens to both naïve $CD4^+$ and $CD8^+$ T-cells and activate them to become memory T-cells.

ILIT-1000 is the first human ILIT study to analyze the immune activation in lymph nodes. We found an increased fraction of effector memory T-cells among the memory T-cells in the lymph nodes of patients treated with active ILIT. Effector memory T-cells have a reduced or absent expression of CCR7, enabling their migration from lymph nodes to peripheral blood and into peripheral tissue[115]. This finding supports the immunological idea of ILIT, with the activation of tolerance-inducing T-cells in the lymph nodes that migrates to blood and into peripheral tissue to promote allergen tolerance and inhibit allergic inflammation.

Patients treated with active ILIT displayed an increased fraction of memory $CD4^+$ and $CD8^+$ in lymph nodes-derived T-cells. Activation and differentiation of $CD4^+$ Treg, and $CD4^+$Th1 in lymph nodes are suggested to have a significant role in the induction of allergen tolerance [18]. Far less is known about the role of memory $CD8^+$ T-cells in allergen tolerance. There is evidence that allergen-specific $CD8^+$ T-cells have suppressive functions and protect from allergic inflammation in peripheral tissue[116, 117]. A weakness in our study is that no detailed analysis of the memory T-cell properties in lymph nodes was performed. Unfortunately, the limited sample size from lymph nodes fine needle aspirations only allowed for the performed flow cytometry analysis.

In the follow-up study 5-6 years after ILIT-1000, an increased fraction of $CD4^+$ memory T-cells was detected in patients treated with active ILIT. The clinical significance of this finding is unclear. There is evidence that the adjuvant-allergen complex persists in tissues and is suggested to be involved in the maintenance of immunological memory[118]. In a study by McDougall et.al. they showed that aluminum hydroxide may persist in tissue for up to 40 years[119]. It may be that the adjuvant-allergen complex injected into the cortex of the lymph nodes, persists for a long time, continuously triggering immune activation.

In peripheral blood, we could detect an increased fraction of effector memory Treg cells in patients treated with active ILIT. These results are in line with other allergen-specific immunotherapy studies, implying that Tregs increase following successful treatment [120, 121]. In our study, we used a high expression of the IL-2 receptor (CD25) to identify Treg. The designed flow cytometry panel allowed for a clear separation of the $CD4^+CD25^{++}$ population. Still, it is a weakness that we did not include FOXP3 to define the Treg population.

We also detected increased levels of central memory T-cells expressing CCR5, a receptor associated with Th1 cells. Induction of Th1 cells in combination with selective removal of Th2 cells is widely accepted as a mechanism that induces tolerance[75, 122]. A weakness in our identification of Th1 cells is the use of only CCR5 expression. Inclusion of transcription factor, T-bet, or membrane receptor CXCR3 would have strengthened our finding[123]. Our study would additionally been improved by an extended analysis of the $CD4^+CCR5^+$ T-cell population. Seumois et.al. demonstrated that non-allergic individuals have a subpopulation of Th1 cells expressing type I and II interferon response genes I[44]. It is possible that a subtype of $CD4^+CCR5^+$ T-cells might be a future biomarker for the induction of allergen tolerance.

Analysis of T-cells was also performed in study IVb, ILIT de novo-3000. No clinical improvement could be detected in this study. In line with the absence of clinical improvement, no changes in $CD4^+CD25^+CD127^{dim}$ (Treg) or $CD4^+CCR5^+$ (Th1) could be detected in the blood. The increased allergen dose may induce a different T-cell response not detectable by our analysis. However, the fact that allergen specific-T-cells in patients treated with active ILIT and placebo were at a similar level four weeks after the treatment, activation, and proliferation of allergen-specific memory $CD4^+$ T-cells in patients treated with active ILIT-den novo-3000 is unlikely to have happened.

5.5 B-CELL CHANGES AND HUMORAL CHANGES IN RESPONSE TO ILIT

The development of an IgE-mediated allergic disease depends on class switching of B-cells to IgE. IgE binds to FcεR1 on basophils and mast cells; allergen cross-linking then induces degranulation and histamine release, which causes symptomatic allergic disease[68]. B-cell class switching to IgG4 increases in patients treated with active ILIT and positively correlates with clinical response to AIT treatment[73, 74].

In patients treated with active ILIT, grass-specific IgE was increased 2-4 weeks after treatment (Fig. 28A). At the follow-up 6-9 months after treatment, the increase in IgE was no longer detectable. In the follow-up, 5-6 years after treatment, grass-specific IgE was decreased in patients treated with active ILIT compared to the levels detected before the treatment (Fig. 28B). For the placebo-treated patients, a reduction in grass-specific IgE was seen 2-4 weeks after treatment (Fig. 28A). At the follow-up, 6-9 months after treatment, and in the follow-up 5-6 years after treatment, no changes in grass-specific IgE were detected compared to the levels before the treatment. Moreover, in patients treated with active ILIT an increased level of grass-specific IgG4 was detected at the 2–4-week follow-up, at the follow-up after 6-9 months, and in the long-term follow-up 5-6 years after treatment (Fig. 28C, D). In patients treated with placebo, no changes in the level of grass-specific IgG4 were detected in any follow-up (Fig. 28C).

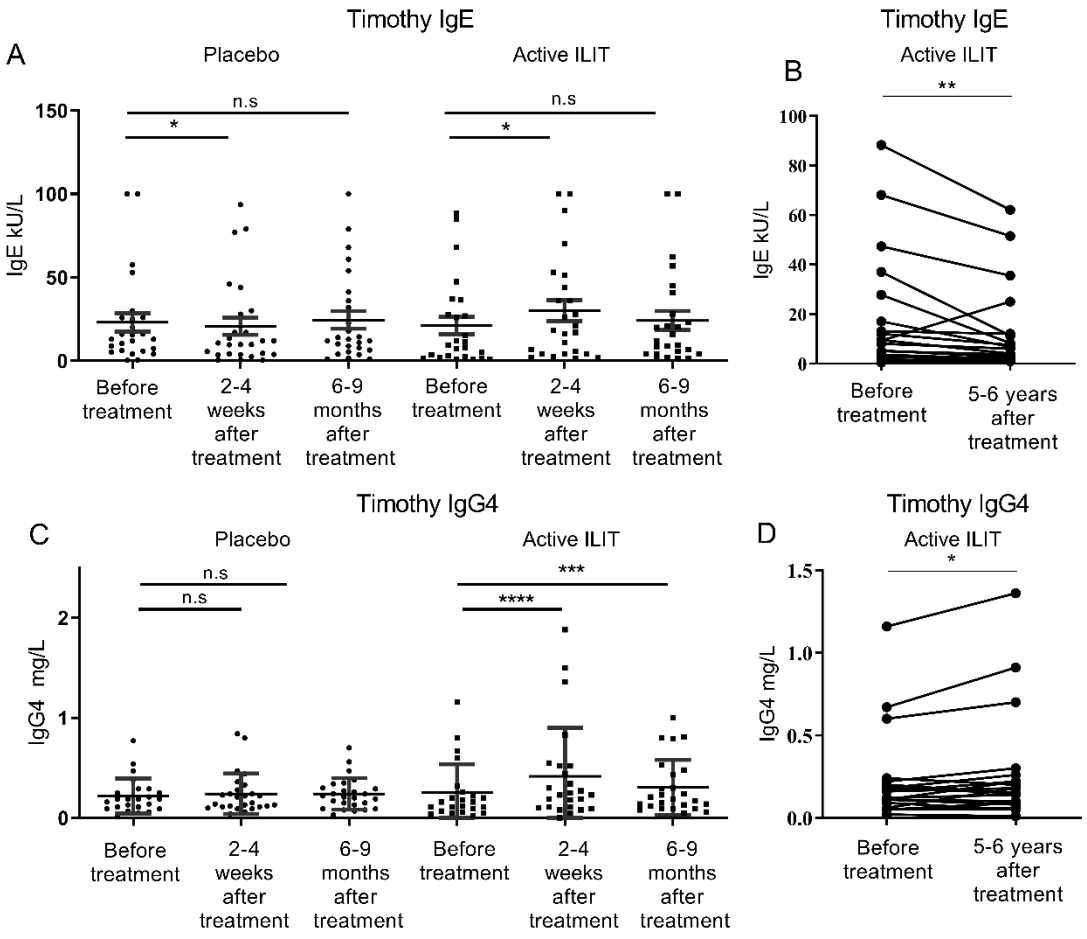

Figure 28. A, B) The levels of Timothy specific IgE. C, D) The levels of Timothy specific IgG4.

In the study IVa, ILIT after SCIT-10000, patients, previously treated with subcutaneous allergen-specific immunotherapy with an unsatisfactory clinical response were treated with ILIT. In the patients treated with active ILIT, the levels of grass-specific IgE were increased four weeks after the treatment (Fig. 29A). No change in grass-specific IgE could be detected eight months after treatment (Fig. 29A). In the patients treated with a placebo, no changes in the levels of grass-specific IgE were detected (Fig. 29A). In the patients treated with active ILIT, an increase in grass-specific IgG4 was detected four weeks after ILIT (Fig. 29C). At the follow-up visit, eight months after ILIT, the levels of grass-specific IgG4 had returned to the pretreatment levels (Fig. 29C). In the placebo group, a steady decline in the levels of grass-specific IgG4 was detected at the follow-up visit four weeks and eight months after ILIT (Fig. 29C).

In the study IVb, ILIT de novo-3000, patients without earlier allergen-specific vaccination were also included. In the patients treated with active ILIT increased levels of grass-specific IgE could be detected at the follow-up visit 4 weeks after treatment, the increased levels of grass-specific IgE were still present at the follow-up visit 8 months after ILIT (Fig. 29B). No changes in the levels of grass-specific IgE could be detected in the placebo group compared to before treatment. For grass-specific IgG4 increased levels could be detected four weeks after and eight months after treatment (Fig. 29D). No changes in grass-specific IgG4 could be detected in the placebo group.

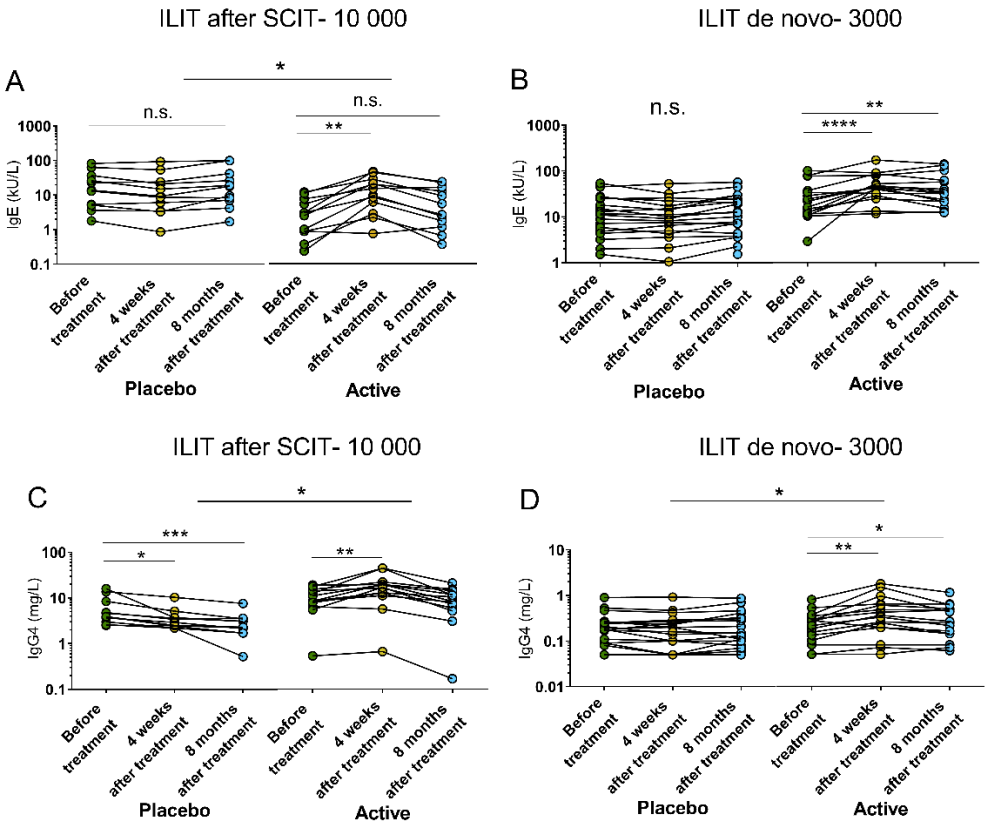

Figure 29. A, B) The levels of Timothy specific IgE. C, D) The levels of Timothy specific IgG4.

5.5.1 Comments

In the study ILIT-1000, we identified an initial increase of grass-specific IgE followed by a steady decline reaching a reduction of around 40% after 5-6 years. At the same time, we could detect an increase in grass-specific IgG4 of about 20% compared to the levels before the start of the treatment. A rise in IgE followed by a steady decline is expected in AIT studies[73, 74]. It is likely that the initial increase in grass-specific IgE is dependent on the activation of plasma cells in the bone marrow and nasal tissue, and that the produced grass-specific IgE spill out into the circulation[124]. The long-term decrease in grass-specific IgE could be a result of IgE memory plasma cells apoptosis. The reduction of IgE could also be dependent on the ability of IgG4 to block the activation of IgE-producing B-cells[125].

IgG4 been shown to increase in response to prolonged allergen exposure[126]. The function of IgG4 is to slow down or stop an inflammatory process guarding against excessive IgG and IgE mediated immune activation. In the present study, we detected increased levels of IgG4 in response to active ILIT. In accordance with our data, increased levels of IgG4 have repeatedly been shown in patients that respond to allergen-specific immunotherapy against inhaled allergens[73, 74]. The level of allergen-specific IgG4 has been shown to decrease with time, and it is suggested that a booster dose could increase the levels of allergen-specific IgG4 and enhance the effect of ILIT[127]. However, the relevance of the level of allergen-specific IgG4 is questioned in a study by Shamji et.al., where it was shown that the blocking capacity more accurately detected clinical response to AIT than the immunoreactive levels of IgG[82].

The mechanism and location for B-cell induced class switching to IgG4 in response to AIT are not fully elucidated. It is known that IL-4 or IL-13 control B-cell class switching to both IgG4 and IgE. The amount of class switching to IgG4 in relation to IgE has been shown to increase in the presence of IL-10, VEGF, IL-12, and IL-21[125]. It is generally considered that class switch recombination occurs in lymphoid tissue, but it has also been shown that class switch recombination can occur in the nasal mucosa[128]. A weakness in our study design was that we did not include an analysis of B-cell Ig subtypes and cytokine production in the nasal mucosa and lymph nodes. This would have increased the current knowledge of IgG4 class switching

In the study ILIT after SCIT-1000, patients treated with active ILIT displayed increased levels of allergen-specific IgE, IgG4, and clinical improvement compared to placebo. Interestingly, the patients previously treated with SCIT had increased levels of allergen-specific IgG4 induced by the SCIT treatment but still an unsatisfactory clinical improvement. In previously non-AIT treated AR patients, the ILIT injection with 100µl of 5000 SQ-U/ml induced anaphylactic reactions. This treatment was tolerated in AR patients previously treated with SCIT. It could be that the increased level of allergen-specific IgG4 in blood in the patients previously treated with SCIT induced systemic tolerance but not peripheral tolerance. This idea is supported by the fact that beekeepers have increased levels of specific IgG4 in their blood, which protects them from anaphylactic reactions[129].

In ILIT de novo-3000 patients treated with active ILIT displayed a significant increase in grass-specific IgG4. However, the expected long-term decrease in IgE was not detected. It is possible that the allergen doses used in ILIT de novo-3000 direct the immune response towards TH2 inflammation and promote IgE class switching over IgG4 promoting an allergic immune response over allergen tolerance.

5.6 DENDRITIC CELL CHANGES IN RESPONSE TO ILIT

A critical step for the induction of allergen tolerance is the reaction of the immune system to the injected allergen. In allergen-specific immunotherapy, reactions that favor Treg and Th1 over Th2 differentiation are believed to be favorable for induction of tolerance. There are no current data on the response of dendritic cells in lymph nodes to the injected allergen during intralymphatic immunotherapy.

In ILIT de novo-3000, the expressions of CD80 and CD141 in lymph node-derived dendritic cells were increased in patients treated with active ILIT (Fig. 30A, C). No changes could be detected on dendritic cells in peripheral blood in patients treated with active ILIT or placebo (data not shown).

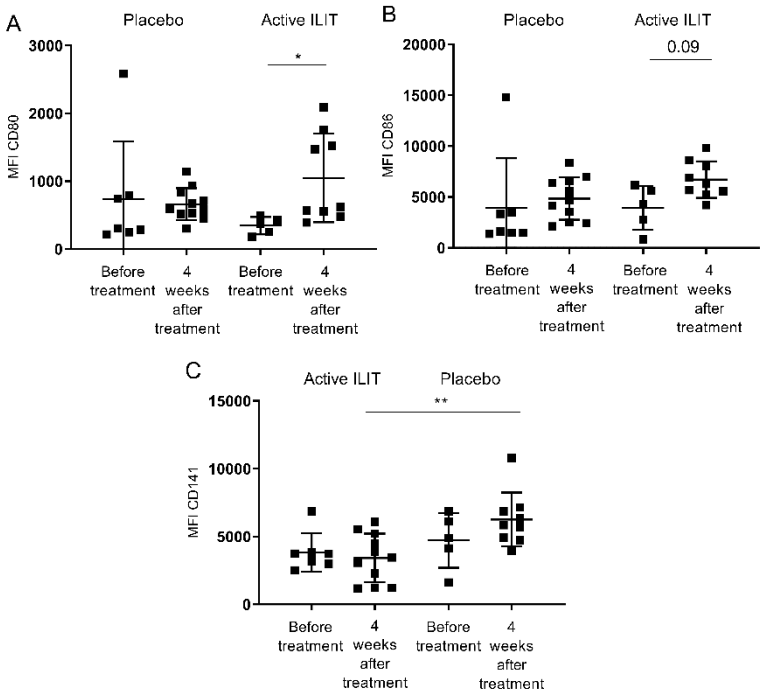

Figure 30. Increased expression of CD80 and CD141 on lymph node derived dendritic cells.

5.6.1 Comments

In ILIT de novo-3000, patients treated with active ILIT showed increased expression of the co-stimulatory molecules CD80, and increased expression of CD141 on dendritic cells. No clinical improvement could be seen in this study.

Wilson et.al. demonstrated that most of the DCs in lymph nodes exhibit an immature phenotype with low expression of CD80 and CD86 in the absence of inflammatory signals[130]. The immature DCs are tolerogenic since the low expression of co-stimulatory signals promotes differentiation of $CD4^+$ Treg and tolerance[131]. In patients treated with active ILIT, we revealed increased expression of co-stimulatory factors in lymph node derived DCs. It may be that the increased concentration of allergen and aluminum hydroxide adjuvants is not optimal for ILIT since it seems to activate DCs and increase their expression of co-stimulatory molecules, promoting T-cell effector cell differentiation. A study by Klimec et.al. supports this, reporting

that aluminum hydroxide induces inflammation at the injection site[132]. The inflammation which recruits and activates DCs is beneficial for SCIT but may be detrimental for ILIT-induced allergen tolerance.

We also detected increased expression of CD141 on DCs in patients treated with active ILIT. It has been reported that CD141 expressing DCs are pro-allergic[133]. Furthermore, it has also been reported that CD141 expression in blood is reduced in patients that respond to allergen-specific immunotherapy[16]. Yu et.al. reported that CD141 expressing pro-allergic DCs induces Th2 cell differentiation[134]. This supports that the DCs expressing CD141 rather than inducing tolerance promote an allergic immune response. The aluminum hydroxide adjuvant may cause a TH2 inflammatory environment. Depending on the injection site and concentration, aluminum hydroxide has been shown to promote TH2 inflammation. The increased concentration in ILIT de novo-3000 compared to ILIT-1000 may favor activation and differentiation of pro-allergic DCs [135].

The limitation of our study is that we cannot compare the level of expression to DCs that promote allergen tolerance. Another weakness is that we used the expression level on the total DC population and did not divide them into subpopulations. Still, we hypothesize that ILIT-3000 induces an excessive immune reaction in lymph nodes, activating DCs and possibly promote a TH2 inflammatory response. Increasing allergen concentration may also cause activation of mast cells locally, further promoting a TH2 inflammatory response by the release of mediators that attract eosinophils, basophils, Th2 cells, and neutrophils. Findings by McKee et.al. supports this hypothesis. They reported that aluminum adjuvant attracts mast cells to the injection site[136]. In ILIT-3000, 84% of the patients treated with active ILIT had adverse events with redness or swelling after injection three, for ILIT-1000, this was only seen in 3.5 % of the patients. It might be that the local allergic reaction is disadvantageous for the induction of allergen tolerance. Pretreating with anti-IgE, up dosing allergen for ILIT-1000, or using a more extended period between injections to clear the allergic reaction may all be alternatives to improve the clinical outcome of ILIT.

5.7 BASOPHIL CHANGES IN RESPONSE TO ILIT

Measuring basophil allergen sensitivity is a promising biomarker to detect clinical response to AIT[137]. In the follow-up study performed 5-6 years after treatment with ILIT-1000, basophil activation test along with markers associated with allergen sensitivity was analyzed.

In blood-derived basophils the level of bound IgE and surface expression of FcεR1 was reduced in Active ILIT compared to controls (Fig. 31A, B). A positive correlation between IgE and FcεR1 could also be detected (Fig. 31C).

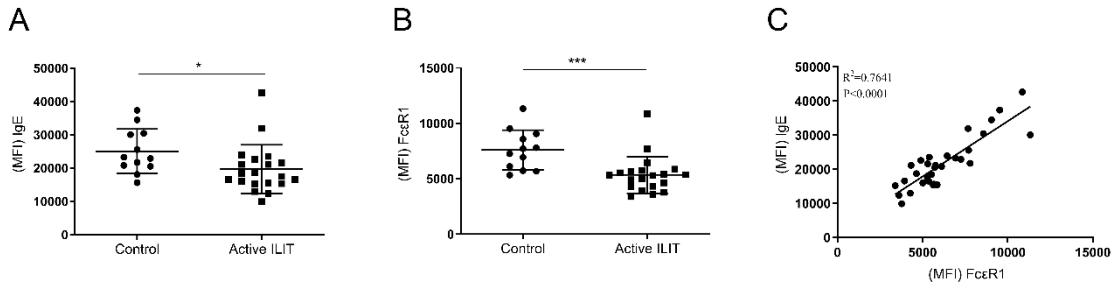

Figure 31. Blood derived basophils express reduced levels of FcεR1 in active ILIT patients. A) Level of bound IgE. B) Expression level of FcεR1. C) Linear regression of FcεR1 and IgE.

Allergen-induced basophil activation displayed a trend for reduced activation to grass allergen (Fig. 32A). For birch allergen, no difference could be detected (Fig. 32B).

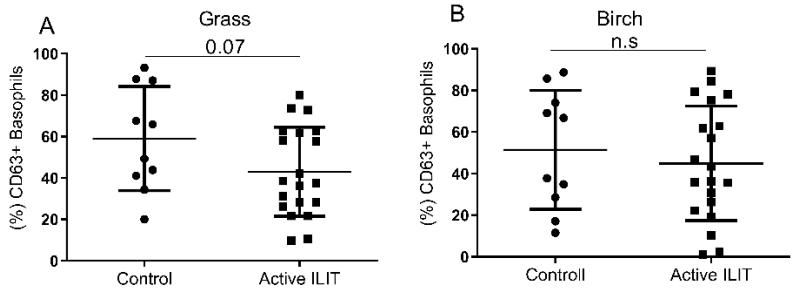

Figure 32. Fraction of basophils activated by allergen stimulation.

5.7.1 Comments

In the 5–6-year ILIT follow-up study, we could detect a reduced expression of FcεR1 on basophils in patients treated with active ILIT. The reduction of FcεR1 expression in basophils is likely due to an overall decrease of IgE in blood. This is supported by Berings et.al. who reported that the total IgE level in blood regulates the expression of FcεR on basophils[138]. Reducing free IgE in blood by anti-IgE treatment have also been shown to reduce FcεR1 expression on basophils. In the present study, reduced expression of FcεR1 was concurrent with reduced levels of bound IgE. This positive correlation between FcεR1 and IgE have been shown earlier by MacGlashan et.al. [139]. Activation of basophils is dependent on the surface density of FcεR1 and bound allergen-specific IgE. Both these factors are reduced in patients treated with active ILIT making them in theory less susceptible to allergen cross-linking and activation.

We also analyzed allergen-crosslinking by allergen-induced basophil activation with grass and birch allergen in the present study. We could demonstrate a trend for reduced basophil activation with grass allergen in patients treated with active ILIT. The mechanism responsible for the reduced reaction to grass-allergen is most likely the reduced levels of grass-specific IgE and the increased levels of grass-specific IgG4 detected in blood in patients treated with active ILIT. No changes in basophil activation were seen for birch allergen.

A weakness in our methodology is that the basophil activation was not performed in the initial study. Analyzing the individual response in each patient had improved the sensitivity of the assay. Also, only using one allergen concentration is a weakness. If multiple concentrations had been used, ranging from 0.1 SQ-U/ml to 1000 SQ-U/ml it would have been possible to determine basophil allergen sensitivity, which is a better estimate of clinical response to AIT [140]. Still, the novel finding of reduced expression of FcεR1 on basophils in patients treated with active ILIT display an immune system transforming away from a TH2 inflammatory response supporting our clinical findings.

6 DISCUSSION

AR is a chronic condition with a 20-30% prevalence in European countries[141]. Typical AR symptoms include rhinorrhea, nasal obstruction or blockage, nasal itching, and sneezing. AR is also associated with increased tiredness. Altogether the combination of symptoms is known to negatively affect work, school performance and a reduction in perceived quality of life[12]. The high prevalence and shortcoming in treatment lead to a massive loss in productivity (presenteeism), resulting in high AR-related costs for society[142]. There are different options to treat AR-induced symptoms, however, despite the frequent use of standard care medication, most AR patients are still unsatisfactorily treated and commonly report impairment in their quality of life[13].

The immunological mechanisms in AR are highly complex, involving multiple immune cells and mediators. Thus, the treatment of AR has proven to be a challenge[2]. Improved techniques have enabled the detection of different cell subpopulations within a population of immune cells depending on their various roles in the immune system. $CD4^+$ T-cells, for example, have been divided into Th1, Th2, Th9, Th17, and Treg, depending on their different effector functions. Accumulating data are indicating that neutrophils also contain different subpopulations with varying roles in homeostasis, cancer, and inflammation. Fridlender et.al. reported that neutrophils can have both pro and anti-tumor properties which significantly impact cancer progression[143]. Pillay et.al. describe that during an inflammatory response, different neutrophil subtypes emerge in peripheral blood[67]. Based on the expression of CD16 and CD62L, they identified an increase of $CD16^{high}CD62L^{dim}$ neutrophils in the blood. This subset was shown to regulate adaptive immunity by inhibiting T-cell activation through the local release of ROS. Further, Polak et.al. has reported that neutrophils may have a novel role in IgE-mediated inflammation by achieving antigen-presenting capacities under certain inflammatory conditions[97].

In our studies, we detected that AR patients had increased fraction of neutrophils in the blood and nasal mucosa. Using the same cell surface markers as Pillay et.al., we could further report that AR patients had an increased fraction of $CD16^{high}CD62L^{dim}$ neutrophils in the nasal mucosa. The $CD16^{high}CD62L^{dim}$ neutrophil subpopulation detected in AR patients may promote allergic inflammation by increasing the T-cell response and inducing eosinophil migration. We could also report that neutrophils in AR expressed increased levels of JAG-1, a T-cell costimulatory factor known to promote Th2 cell differentiation[39, 40]. Besides regulating adaptive immunity, the high levels of neutrophils detected in nasal mucosa could also lead to tissue damage and excessive inflammation that may further promote allergic inflammation[144]. Altogether this makes neutrophils a novel target for future development of AR therapies.

Why neutrophils accumulate in AR tissue is not known. One hypothesis is defective neutrophil clearance. This is supported by Ekstedt et.al., who reported that activated neutrophils had increased expression of CD47 and increased tissue survival[145]. The CD47 pathway is currently

under clinical investigation for antibody-based blocking in cancer[146]. It would be interesting to investigate if anti-CD47 blocking also has a clinical function in AR.

In combination with new pharmacotherapy, further development of AIT could be a game-changer for AR patients. Currently, AIT is the only treatment that changes the course of the disease and reduces AR symptoms long-term. The two current forms of AIT available in standard health care today are SCIT and SLIT. Unfortunately, due to the long treatment protocol (3-5 years), the risk of severe side effects, compliance, and high cost, AIT is today offered to less than 5% of eligible AR patients[15].

We have investigated ILIT as a new treatment route for AIT for a couple of years. One advantage of ILIT is bypassing the skin, which reduces the risk of local adverse events. Also, using a low treatment dose reduces the risk of systemic reactions and severe adverse events. The injection of allergen into the lymph node is also believed to increase the amount of DCs that internalize allergens and present them to T-cells. In SCIT and SLIT, DCs internalize antigens in the periphery and migrate to nearby lymph nodes. At the injection site or locally in the mucosa, only a small number of DCs are present to detect antigens. In lymph nodes, a much higher number of DCs are present. In theory, this enables more interaction between DCs, allergen-specific T-cells, and B-cell, inducing tolerance within a superior time frame compared to SCIT and SLIT.

The protocol used in most ILIT studies consists of three injections four weeks apart. The allergen treatment dose primarily used is 1000 SQ-U/ml. The effect of ILIT was assessed in three systematic reviews published in 2021. Aini et.al. included 11 studies in their analysis and concluded ILIT to be safe but ineffective in treating AR patients[147]. Hoang et.al. included 13 studies and reported that ILIT was effective in treating seasonal AR but not perennial AR[148]. Werner et.al. reported ILIT effective for seasonal and perennial allergens; their conclusions were based on 17 studies[149]. The differences in the outcome of their analyses could be because most of the ILIT studies performed are small-scale studies, and the measured primary outcome differed between studies. To harmonize clinical trials the European Academy of Allergy and Clinical Immunology (EACI) currently recommends the use of CSMS as the primary outcome measurement of clinical effect[93]. For ILIT to induce clinical effects, the number of successfully performed injections seems to have a profound effect on the treatment outcome[150]. It is worth noting that one recent placebo-controlled ILIT trial using grass allergen, reported a high level of successful injection, and also as high as a 50% reduction in CSMS compared to placebo[113].

Our placebo-controlled ILIT-1000 study with concomitant administration of grass and birch allergen further supports the current conception that ILIT is safe. We could also detect a 20% reduced reaction to NPT with grass allergen one year after treatment. Studies measuring the long-term effect of ILIT so far are rare. In a 3-year small open-label study by Ahlbeck et.al., they reported that the reduction in symptoms detected one year after treatment was sustained for at least three years[112]. There is also a study reporting that the detected clinical effect during the first year was not sustained in year three[113]. Our 5-6 years follow-up study supports the long-term effect of ILIT.

To further improve the clinical outcome of ILIT, we performed two placebo-controlled studies with increased allergen doses. Unfortunately, the take-home message from these studies is that an increased allergen dose does not give any beneficial clinical effect compared to 1000 SQ-U/ml.

Our data support that ILIT induces a clinically relevant reduction in symptoms for seasonal allergens that may persist for up to 5 years. Still, for ILIT to become an available treatment, more extensive studies must be performed. However, the small-scale studies completed may give enough evidence that ILIT can be a future treatment for AR to support funding for more extensive, more costly clinical phase II and III trials.

One major hurdle in performing AIT trials is the confounding effect of differences in pollen levels between different seasons. Low levels of allergens in the air the years after treatment may prevent the detection of differences between active treatment and placebo. Therefore, developing a consensus statistical approach adjusting for the differences in pollen level is important. To fully address the usefulness of ILIT in treating AR, it may be of value to divide the patients receiving active ILIT into non-responders and responders. Calculating the reduced CSMS in the patients that respond to therapy is essential to estimate the beneficial effect of ILIT correctly. Also, if a biomarker can detect the patients responding to ILIT and other AIT treatments, that might increase the therapeutic use of AIT by reducing the overall cost and increasing the measured treatment effect.

In combination with assessing the clinical results, immunological analysis was also performed in our ILIT studies to detect biomarkers and improve our understanding of how ILIT induces allergen tolerance. The current understanding of immunological changes that induce allergen tolerance involves DCs, B-, and T-cell changes. Our results indicate that effector memory T-cells are increased in the allergen-injected lymph nodes and that these cells migrate to blood and peripheral tissue. Another possible explanation for our finding of $CD4^+$ T-cells with low expression of CCR7 in lymph nodes is that these cells are Tfh cells[151]. It has been reported that Tfr cells increases in response to AIT and positively correlate with clinical response[78]. Unfortunately, CXCR5, Bcl-6, or FOXP3 were not included in our experiment to confirm Tfh or Tfr differentiation

We could detect signs of a T-cell response deviating from Th2 to Th1 and induction of Treg in peripheral blood. These findings comply with the T-cells changes seen during other AIT treatments[152]. Wambre et.al. also describe the reduction of a Th2 cell phenotype consistent with immune deviation from Th2[45]. It is probable that with further development, the reported changes in T-cells could be a biomarker for early detection of clinical response to AIT.

Changes in B-cells are crucial for the induction of allergen tolerance. Typical findings are the increase of allergen-specific IgG4 and a long-term reduction in allergen-specific IgE. Why the level of IgG4 increases in response to AIT is currently unknown. One hypothesis presented by Aalberse et.al. suggests that long-term exposure to allergen induces subsequent B-cell class switching that foster an IgG4 response[126]. The mechanism for the reduction in IgE involves

IgG4 binding to allergens and thereby blocking B-cell activation[153]. IgG4 also promotes tolerance by inhibiting allergen-induced basophil activation and blocking IgE-facilitated allergen presentation (IgE-FAP) [125]. IgG4 activation of FcγRIIb expressed on B-cells has also been shown to suppress B-cell activation and mediate apoptosis[154]. In our study, we could detect an increase in grass-specific IgG4 in patients treated with active ILIT both short and long-term. However, the level of specific IgG4 correlated poorly with the clinical response.

In the ILIT studies with a higher treatment dose, we could not detect a clinical response, despite an increase in IgG4. This result reveals that the induction of IgG4 is not the causal mechanism for developing allergen tolerance. The study also reveals that induction of IgG4 is not linked to Th2 cell deviation and development of Tregs, since no changes in T-cells were detected despite the increase in specific IgG4.

In the accepted mechanisms for AIT-induced allergen tolerance, DCreg, $CD4^+$ Treg, and Breg cells all have significant roles [16-18, 73-75]. Interestingly, what causes these changes is currently unknown. A possible early anti-inflammatory signal could be dependent on IgG4. It has been shown by Bianchini et.al. that antigen-presenting cells induce recruitment of Treg cells and start to produce and secrete IL-10 in an environment promoting IgG4-mediated FcγRIIb signaling[125]. If this mechanism is relevant to AIT induction of tolerance is currently not known, affirming the need for more immunological studies regarding the mechanism behind the induction of allergen tolerance.

7 CONCLUSIONS AND POINTS OF PERSPECTIVE

- Patients with AR exhibited a higher proportion of $CD16^{high}CD62L^{dim}$ neutrophils in the nasal mucosa than controls. This neutrophil subset could lower the T-cell activation threshold and facilitate eosinophil migration, it is not inconceivable that $CD16^{high}CD62L^{dim}$ neutrophils play a role in AR pathology.

- AR patients displayed an increased proportion of $CD4^+$ T-cells with an expression of Notch1,4 compared to controls. They also exhibited an increased expression of JAG-1 on nasal epithelial cells and neutrophils than the controls. Nasal epithelial cells and neutrophils in AR mucosa may promote $CD4^+$ Th2 cell development and the progression of AR by their increased expression of JAG-1.

- ILIT with birch and grass allergen, when given concomitantly in three doses of 1000 SQ-U one month apart, appeared safe. It reduced the need for symptom-controlling medication during the pollen season and the allergic response to provocation with grass pollen. An increase in memory T-cells in lymph nodes and an increase in memory $CD4^+CCR5^+$ (Th1) and $CD4^+CD25^{++}$ (Treg) in blood were associated with clinical response. The same was true for the increase in allergen-specific IgG4 and the reduction of allergen-specific IgE in serum, indicating the development of allergen tolerance.

- An increase of the allergen doses used in the three-step ILIT protocol did not improve the clinical outcome, and expected changes among T-cells did not occur. Doses up to 3000 SQ-U appeared to be safe, but a further increase of the allergen concentration caused anaphylactic reactions in two patients and should be avoided. In patients previously treated with SCIT. ILIT “re-vaccination“ with 1000-5000-10000 SQ-U reduced the combined symptom and medication score (CSMS) without compromising safety.

- A 5-6-year follow-up of ILIT in patients treated with birch and grass allergen given concomitantly in three doses of 1000 SQ-U revealed a remaining clinical effect for grass allergen assessed with CSMS. Increased activation of $CD4^+$ T-cells in lymph nodes suggests that the effects of a successful ILIT treatment remain for serval years. This assumption was further supported by the finding of increased levels of allergen-specific IgG4 and reduced levels of grass-specific IgE in blood, along with a reduced expression of FcεR1 on basophils.

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