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IMMUNOMODULATION OF ALLERGIC IMMUNE RESPONSE DURING SPECIFIC IMMUNOTHERAPY

by

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ABSTRACT

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Immunomodulation of allergic immune response during specific immunotherapy

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Atopic, IgE-mediated allergies are one of the major public health problems in Finland and other Western countries. These diseases are characterized by type 2 T helper (Th2) cell predominated immune responses (interleukin-4 (IL-4), IL-5) against ubiquitous environmental allergens. Despite of adequate pharmacological treatment, more than 20% of the patients with allergic rhinitis develop asthma. Allergen specific immunotherapy (SIT) is the only treatment currently available to affect to the natural course of allergic diseases. This treatment involves repeated administration of allergens to the patients either via sublingual route (sublingual immunotherapy, SLIT) or by subcutaneous injections (subcutaneous immunotherapy, SCIT). Successful treatment with SCIT or SLIT has been shown to provide long-term remission in symptoms, and prevent disease progression to asthma, but the immunological mechanisms behind these beneficial effects are not yet completely understood. Increased knowledge of such mechanisms could not only help to improve SIT efficacy, but also provide tools to monitor the development of clinical response to SIT in individual patients, and possibly also, predict the ultimate therapeutic outcome. The aim of this work was to clarify the immunological mechanisms associated with SIT by investigating the specific allergen-induced immune responses in peripheral blood mononuclear cells (PBMC) of allergic rhinitis patients during the course of SLIT and SCIT. The results of this work demonstrate that both therapies induced increases in the protective, Th2-balancing Th1 type immune responses in PBMC, e.g. by up-regulating signaling lymphocytic activation molecule (SLAM) and interferon gamma (IFN-γ) expression, and augmented tolerogenic T regulatory (Treg) cell type responses against the specific allergens, e.g. by increasing IL-10 or Forkhead box P3 (FOXP3) expression. The induction of allergen-specific Th1 and Treg type responses during SLIT were dependent on the treatment dose, favoring high allergen dose SLIT. During SCIT, the early decrease in Th2 type cytokine production - in particular of IL-4 mRNA and IL-4/IFN-γ expression ratio - was associated with the development of good therapeutic outcome. Conversely, increases in both Th2 (IL-5) and Th1 (IFN-γ, SLAM) type responses and IL-10 mRNA production were seen in the patients with less effective outcome. In addition, increase in Th17 type cytokine (IL-17) mRNA production was found in the PBMC of patients with less effective outcome during both SLIT and SCIT. These data strengthen the current hypothesis that immunomodulation of allergen-specific immune responses from the prevailing Th2-biased responses towards a more Th1 type, and induction of tolerogenic Treg cells producing IL-10 represent the two key mechanisms behind the beneficial effects of SIT. The data also give novel insight into the mechanisms why SIT may fail to be effective in some patients by demonstrating a positive correlation between the proinflammatory IL-17 responses, Th2 type IL-5 production and clinical symptoms. Taken together, these data indicate that the analysis of Th1, Th2, Treg ja Th17-associated immune markers such as IL-10, SLAM, IL-4, IL-5 and IL-17 could provide tools to monitor the development of clinical response to SIT, and thereby, predict the ultimate clinical outcome already in the early course of the treatment.

Keywords: immunotherapy, allergy, mechanisms, T-helper cell, cytokine, tolerance

TIIVISTELMÄ

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Allergisen immuunivasteen muokkautuminen siedätyshoidon aikana

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Atooppiset, IgE-välitteiset allergiat ovat yksi keskeisimmistä kansanterveydellisistä ongelmista Suomessa ja muissa länsimaissa. Näitä sairauksia luonnehtii tyypin 2 auttaja T-solujen muodostamat vasteet (IL-4, IL-5) ympäristön tavallisia allergeeneja kohtaan. Oikeasta lääkehoidosta huolimatta noin 20%:lle allergisista nuhapotilaista kehittyy astma jossain elämänvaiheessa. Ainoa tapa vaikuttaa allergisen sairauden kulkuun on siedätyshoito (SIT). Siinä potilasta totutetaan sietämään yhä suurempia allergeenimääriä antamalla allergeenia toistuvina annoksina joko kielenalussiedätyshoitona (SLIT) tai ihonalaissiedätyshoitona (SCIT). Siedätyshoito voi lieventää allergian oireita pitkäkestoisesti ja estää allergian pahenemisen astmaksi, mutta näiden vaikutusten taustalla olevia immunologisia mekanismeja ei vielä täysin tunneta. Tuntemalla siedätyshoidon immunologisia mekanismeja paremmin voitaisiin sen tehoa ehkä entisestään parantaa sekä hoitovasteen kehittymistä potilaissa seurata - mahdollisesti jopa ennustaa lopullista hoitotulosta. Tutkimuksen tarkoituksena oli selvittää siedätyshoidon taustalla olevia immunologisia mekanismeja tutkimalla allergeenispesifisiä immuunivasteita siedätyshoidettujen potilaiden valkosoluviljelmissä (PBMC). Tutkimuksen tulokset osoittavat, että sekä SLIT että SCIT lisäävät suojaavien Th1- (IFN-γ, SLAM) ja Treg- (IL-10, FOXP3) soluvasteiden kehittymistä spesifisiä allergeeneja kohtaan. Th1- ja Treg-soluvasteiden muodostuminen allergeeneja kohtaan SLIT-hoidon aikana riippui käytetystä hoitoannoksesta, suosien suuria allergeenipitoisuuksia. SCIT-hoidossa varhainen lasku allergeenin indusoimissa Th2-tyypin immuunivasteissa, erityisesti IL-4 mRNA ja IL-4/IFN-γ vasteissa, liittyi hyvän hoitotuloksen kehittymiseen. Sen sijaan heikosti siedätyshoidosta hyötyneillä potilailla tapahtui nousua sekä Th2- (IL-5) että Th1- (IFN-γ, SLAM) tyypin immuunivasteissa, mutta myös Treg- (IL-10) vasteissa. Myös tulehdusvastetta edistävän, Th17-soluille tyypillisen IL-17 mRNA:n ekspressio nousi potilailla, joilla siedätyshoidon kliininen teho jäi heikoksi (sekä SLIT että SCIT). Tämän tutkimuksen tulokset vahvistavat nykykäsitystä siitä, että allergeenispesifisten immuunivasteiden tasapainottuminen Th2-vasteista Th1-suuntaan ja tolerogeenisten Treg-soluvasteiden kehittyminen allergeeneja kohtaan ovat kaksi keskeistä mekanismia siedätyshoidon taustalla. Sekä SLIT että SCIT indusoivat pitkäaikaisia muutoksia näissä suojaavissa vasteissa PBMC soluissa. Tutkimuksen tulokset antavat viitettä myös sille, miksi siedätyshoito voi epäonnistua joillain potilailla osoittamalla, että tulehdusvastetta edistävän IL-17 sytokiinin ja Th2-sytokiinivasteiden sekä kliinisten oireiden välillä on positiivinen yhteys. Tämän väitöskirjatutkimuksen tulokset osoittavat, että analysoimalla allergeenien indusoimia Th1, Th2, Treg ja Th17 tyypin immuunivasteita (esim. IL-10, SLAM, IL-4, IL-5, ja IL-17) PBMC-soluissa siedätyshoidon aikana voidaan hoitovasteen kehittymistä seurata sekä hoidon tulosta jo sen alkuvaiheessa mahdollisesti ennustaa.

Avainsanat: siedätyshoito, allergia, mekanismit, auttaja-T-solu, sytokiini, toleranssi

CONTENTS

ABBREVIATIONS

AHR	airway hyperreactivity
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CTLA	cytotoxic T-lymphocyte antigen
CXCL	CXC chemokine ligand
CysLT	cysteinyl leukotriene
ECP	eosinophil cationic protein
FcεRI	high-affinity IgE Fc receptor
FOXP3	forkhead box P3
GATA-3	GATA-binding protein 3
G-CSF	granulocyte colony-stimulating factor
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
MCP-1	monocyte chemotactic protein-1
mRNA	messenger RNA
NKT	natural killer T cell
PBMC	peripheral blood mononuclear cells
PGD_2	prostaglandin D_2
RANTES	regulated upon activation, normal T-cell expressed, and secreted
RORγt	retinoic acid-related orphan nuclear receptor γt (in humans RORC)
SCIT	subcutaneous immunotherapy
SIT	specific immunotherapy
SLAM	signaling lymphocytic activation molecule
SLIT	sublingual immunotherapy
SMS	symptom medication score
TARC	thymus and activation regulated chemokine
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
Th	T helper cell
Treg	T regulatory cell
VIT	venom immunotherapy

LIST OF ORIGINAL PUBLICATIONS

This work is based on the following original communications, which are referred in the text by Roman numerals (I-IV).

I Savolainen J, Nieminen K, Laaksonen K, Laiho T, Jacobsen L, Lahesmaa R, Terho EO, Valovirta E (2007). Allergen-induced *in vitro* expression of IL-18, SLAM and GATA-3 mRNA in PBMC during sublingual immunotherapy. Allergy 62: 949–953.

II Nieminen K, Laaksonen K, Savolainen J (2009) Three-year follow-up study of allergen-induced *in vitro* cytokine and SLAM mRNA responses in PBMC of allergic rhinitis patients undergoing specific immunotherapy. International Archives of Allergy and Immunology. 150(4):370-376.

III Nieminen K, Valovirta E, Savolainen J (2009) Clinical outcome and IL-17, IL-23, IL-27 and FOXP3 expression in peripheral blood mononuclear cells of pollen-allergic children during sublingual immunotherapy. Pediatric Allergy and Immunology. *In press.*

IV Nieminen K, Laaksonen K, Kilpeläinen M, Waris M, Savolainen J. Comparison of Th1-, Th2- and Th17 type responses and therapeutic outcome during specific immunotherapy. *Submitted.*

1 INTRODUCTION

Allergic diseases are among the most common chronic illnesses in humans (Kay 2001). These diseases can manifest in any age group and in many different organs, with typical symptoms including asthma, rhinoconjunctivitis, gastrointestinal symptoms, and characteristic skin lesions (Johansson *et al.* 2001). It has been estimated that over 500 million people worldwide suffer from allergic rhinitis, and over 300 million more from allergic asthma, and in many countries, the prevalence is increasing (Bousquet *et al.* 2008). Allergy as a term (from the Greek words *allos* meaning "other" and *ergon* meaning "work") was introduced in 1906 by von Pirquet to distinguish immune responses that are harmful to host from protective immunity (Kay 2001). In 1967, it was recognized that immunoglobulin E (IgE) was the factor responsible for inducing the allergic reactions (Johansson 1967; Ishizaka & Ishizaka 1967). Atopy (from the Greek word *atopos*, meaning "out of place") means the genetic or personal aptitude to produce IgE specific for common environmental antigens (allergens), and was introduced in 1923 by Coca and Cooke to explain a series of hypersensitivity syndromes with hereditary transmission (Kay 2001; Johansson *et al.* 2001). Nowadays, the role of heredity is small (~30%) in the clinical expression of atopy and in the sensitivity to a particular allergen, with environmental factors appearing to predominate the genetic factors (Bousquet *et al.* 2001). T helper type 2 (Th2) cells, characterized by the production of cytokines, including interleukin-4 (IL-4), IL-5 and IL-13 are critical for the induction of allergen-specific IgE synthesis in B cells and, thus, for the development and maintenance of allergic diseases.

The means to manage allergic diseases are currently based on allergen avoidance, pharmacotherapy, allergen specific immunotherapy (SIT) and patient education (Bousquet *et al.* 2008). Of these, only allergen avoidance and SIT have the potential to affect to the natural course of allergic diseases (Bousquet *et al.* 1998). SIT is indicated for IgE-mediated allergic diseases to clinically relevant allergens (pollens, mites, animal dander, Hymenoptera venoms), and involves administration of allergen extracts or their derivatives in increasing doses until the achievement of a maintenance dose, which is typically continued for 3-5 years to provide long-lasting protective effects against the allergens that are responsible for producing the symptoms (Bousquet *et al.* 2008). SIT was introduced into clinical practice in 1911 and is traditionally given by subcutaneous injections of allergen extracts or their derivates under forearm skin (specifically posterior portion of the middle third of upper arm), and is therefore termed subcutaneous immunotherapy (SCIT) (Cox 2007). Alternative routes for performing SIT have been proposed sporadically since the 1920s (Esch 2008). Increasing body of evidence supports the safety and efficacy of sublingual immunotherapy (SLIT) as a propitious alternative to

SCIT, both in children and in adults (Esch 2008; Wilson *et al.* 2005; Penagos *et al.* 2006; Penagos *et al.* 2008; Larenas-Linnemann 2009). This therapy involves administration of allergens under tongue, where they are kept for few minutes and then swallowed. The hallmarks of SLIT are its improved tolerability and safety profile as compared to SCIT, and these features have also allowed home administration of SIT (Esch 2008).

SIT is recognized as a biological response modifier, capable of influencing or down-modulating the allergen-induced immune responses, and inducing allergen-specific tolerance (Bousquet *et al.* 1998). However, the immunological mechanisms behind this tolerance induction are not yet completely understood. It has been demonstrated that there is immune deviation from the predominated allergen-specific Th2 type cell responses to a more Th1 response, decrease in allergen-specific IgE production in favor of IgG4, and induction of tolerogenic T regulatory (Treg) cells that produce anti-inflammatory cytokines, including IL-10 and/or transforming growth factor beta (TGF-β) (James & Durham 2008; Moingeon *et al.* 2006). So far, however, nothing is known of the effects of this treatment on Th17 type immune responses, a recently characterized T helper cell subclass with pleiotropic functions, and no immunological marker for monitoring of the therapeutic response to SIT has been established. A more complete understanding of the immunological mechanisms that underlie successful SIT could improve the knowledge of the induction of allergen-specific tolerance, and thus, provide means to improve the clinical efficacy of this treatment (James & Durham 2008). It could also provide tools to monitor the development of clinical response to SIT in individual patients, and possibly also, predict the ultimate therapeutic outcome. The aim of this study was to deepen the understanding of the immunological mechanisms associated with SIT by investigating the *in vitro* allergen-induced immune responses, including Th17 type responses, in peripheral blood mononuclear cells (PBMC) of allergic rhinitis patients during the treatment, and by comparing these responses with clinical improvement. The aim of the study was also to investigate the dose-dependency of such responses during SLIT by comparing the responses between patients treated with low and high allergen doses.

2 REVIEW OF THE LITERATURE

2.1 Atopic allergies and allergic inflammation

2.1.1 Atopy, allergy and allergens (definition, manifestations and prevalence)

Allergies are hypersensitivity reactions to innocuous environmental antigens initiated by immunological mechanisms (Johansson *et al.* 2001). Distinct from other hypersensitivity reactions that may occur in the body, allergies are mediated by IgE (termed type I hypersensitivity reaction). The antigens that can induce IgE synthesis in the body, that is sensitization, and subsequent allergic reactions, are known as allergens. Most allergens are soluble proteins or glycoproteins ranging from 2 to 60 kDa in molecular weight, although certain polysaccharides and some low molecular weight chemicals and drugs can also be allergenic (Johansson *et al.* 2001; Donnell & Grammer 2004). Depending on their route of introduction, the allergens can be classified either as airborne allergens (inhaled), food allergens (ingested), occupational allergens, drug allergens or hymenoptera venoms (Bousquet *et al.* 2002). Worldwide, airborne allergens, including pollens, animal dander, mites, and mold cause the most problems for people with allergies (The National Institutes of Health (NIH)). In Finland, the most common allergens causing sensitization are animal dander (cat, dog), and birch and timothy grass pollens (von Hertzen *et al.* 2006). The prevalence of allergic sensitization to at least one of the common environmental allergens in Finland has been shown to range from around 35 to 47% based on recent epidemiological studies (Kilpeläinen 2001; von Hertzen *et al.* 2006; Pallasaho *et al.* 2006).

The genetic or personal aptitude to produce IgE against environmental allergens is known as atopy (Johansson *et al.* 2001). Atopy as such, though, does not indicate a symptomatic inflammation; it has been estimated that only 57% of those with specific IgE to inhalant allergens, have a symptomatic disease, i.e. allergic rhinitis or asthma (Bousquet *et al.* 2008; Kerkhof *et al.* 2000). However, atopy increases the risk of developing symptomatic allergic disease during lifetime. Typically, an atopic person develops a spectrum of atopic diseases with age, sometimes referred as the ‘atopic march’. Gastrointestinal and eczematous skin symptoms, often caused by food allergens, predominate during the first years, whereas asthma and rhinitis to inhalant allergens develop later (Johansson *et al.* 2001).

Allergic diseases are increasing in prevalence at an alarming rate, particularly in countries with a western lifestyle, although in some countries with high prevalence, a plateau seems to be already achieved (Bousquet *et al.* 2008; Ascher 2006). In Europe, it has been estimated that approximately one-quarter of the population is affected by

respiratory allergies (Bousquet *et al.* 2008; Burney *et al*. 1996). The prevalence of allergic disorders, in particular allergic rhinitis and asthma, has steadily increased in Finland since the 1960s (Latvala *et al.* 2005). The reasons for the increased prevalence of allergic diseases in westernized countries remain unresolved, but environmental factors, including the urban lifestyle deprived from microbial contacts, and pollution, have been indicated to play an important role (Bousquet *et al.* 2008; Kilpeläinen 2001; von Herzen *et al*. 2006).

2.1.2 Allergic immune reaction

2.1.2.1 Immunological basis of allergy I - the Th2/Th1 paradigm

The synthesis of specific IgE in B cells is dependent on CD4+ Th2 lymphocytes that secrete cytokines such as IL-4, IL-5, IL-9 and IL-13 (Table 1) (Galli *et al.* 2008). IL-4, in particular, is the key cytokine in inducing antibody isotype switch to IgE in B lymphocytes (Pene *et al.* 1988). In addition, IL-5 contributes to the pathogenesis of allergic inflammation by inducing the synthesis and differentiation of eosinophils from bone marrow precursors, which then recruit to the allergen-exposed tissues and sustain inflammation (Lopez *et al.* 1988; Clutterbuck *et al.* 1989). The biological functions of Th2 cytokines in allergic inflammation are summarized in Table 1. It has been demonstrated that allergen-specific CD4+ T cell clones of allergic patients produce cytokines predominantly of the Th2 type (Wierenga *et al.* 1991; Parronchi *et al.* 1991), and increased synthesis of Th2 type cytokines have also been found in cutaneous biopsies (Kay *et al.* 1991), nasal mucosa (Durham *et al.* 1992) and bronchoalveolar lavage (BAL) fluid (Robinson *et al.* 1993) obtained from allergic individuals after allergen challenge and during natural allergen exposure. Healthy individuals, by contrast, favour the production of Th1 type cytokines in response to allergens (Wierenga *et al.* 1991; Imada *et al.* 1995). Th1 cytokines, in particular interferon gamma (IFN-γ), are known to be important in the suppression of allergic immune responses. IFN-γ inhibits IL-4-induced Th2 cell development from naïve T cells (Maggi *et al.* 1992) and the production of IgE in B lymphocytes (Pene *et al.* 1988). The induction of Th1 cell responses is dependent on IL-12, a heterodimeric cytokine composed of p35 and p40 subunits and produced by activated macrophages and B lymphocytes (Manetti *et al.* 1993). IL-12 also suppresses the synthesis of IgE by IL-4-stimulated human B cells (Kiniwa *et al.* 1992), and has the ability to suppress Th2 cell development (Manetti *et al.* 1993).

Table 1. Biological functions of Th2 cytokines in allergic inflammation (James & Durham 2008).

Cytokine	Function(s)
IL-4	Promotes IgE and (with IL-10) IgG4 production in B cells Induces Th2 cell development and inhibits Th1 differentiation Induces expression of adhesion molecules by endothelial cells Induces mucus production from mucosal glands Up-regulates co-stimulatory molecules on B cells Up-regulates expression of low-affinity IgE receptor (CD23)
IL-5	Induces eosinophil differentiation, activation and survival Enhances IL-4 induced IgE synthesis in B cells
IL-9	Promotes mast cell and basophil growth Promotes eosinophil development (in synergy with IL-5) Up-regulates high-affinity IgE receptor on mast cells (FcεRI)
IL-13	Promotes IgE and (with IL-10) IgG4 production in B cells Induces expression of adhesion molecules by endothelial cells Induces mucus production from mucosal glands Up-regulates expression of low-affinity IgE receptor (CD23)

The development of Th2 cells is influenced by many transcription factors, the key lineage commitment factor involving GATA-binding protein 3 (GATA-3) (Zheng *et al.* 1997; Zhang *et al.* 1997). GATA-3 regulates Th2 cytokine gene expression in naïve CD4+ T cells (Zheng *et al.* 1997; Zhang *et al.* 1997). It concomitantly inhibits the differentiation of Th1 cells by cell-intrinsic mechanism that is not dependent on IL-4 and that may involve repression of IL-12 signaling (Ouyang *et al.* 1998).

2.1.2.2 Immunological basis of allergy II – Treg cell deficit

The imbalance between Th2 and Th1 cell responses formed the basis of understanding allergic immune responses for more than two decades since the discovery of these two subsets in the late 1980s. More recently, T regulatory cells (Treg) have been discovered as another pivotal subset of CD4+ T cells with implications for allergic disease (Holgate & Polosa 2008; Groux *et al.* 1997). These cells are characterized by the expression of transcription factor forkhead box P3 (FOXP3) and IL-2 receptor (CD25) (Fontenot *et al.* 2003; Yagi *et al.* 2004). FOXP3 is required both for the development of Treg cells, as well as maintenance of their regulatory function (Fontenot *et al.* 2003; Yagi *et al.* 2004). In healthy individuals, Treg cells are involved in the active suppression of proliferation and Th2 type cytokine production against allergens, whereas in allergic subjects, deficits in their capacity to suppress allergen-specific CD4+ T effector cell functions have been found (Ling *et al.* 2004; Grindebacke *et al.* 2004; Bellinghausen *et al.* 2005). It has also been demonstrated that in healthy individuals, allergen-specific

Treg cells are present in higher frequencies than their effector counterparts (Th1, Th2), and secrete high amounts of IL-10 and TGF-β when stimulated with allergen, whereas in allergic subjects, the Th2 cell subset and IL-4, IL-5 and IL-13 responses predominate (Jutel *et al.* 2003; Akdis *et al.* 2004). The importance of Treg cells in the immunosuppression of allergic immune responses has been further emerged from studies showing that beekeepers, who have naturally become tolerant to bee venom after being stung several times by bees, have increased numbers of venom-specific IL-10-producing Treg cells and increased production of IL-10 in response to bee venom allergen (Akdis *et al.* 1998). In addition, adoptive transfer of allergen-specific CD4+CD25+ cells in mice has been shown to prevent allergen-induced Th2 responses and IgE production, as well as airway eosinophilia (Cottrez *et al.* 2000; Stock *et al.* 2004).

The mechanisms by which Treg cells exert their immune regulatory activity on allergen-specific CD4+ T effector cells are largely dependent on the secreted cytokines IL-10 and TGF-β (Taylor *et al.* 2006). It has been demonstrated that IL-10 can inhibit the proliferation and cytokine production by both Th1 and Th2 cells, and induce long-term antigen-specific anergy in CD4+ T cells (Del Prete *et al.* 1993; Groux *et al.* 1996; Francis *et al.* 2003). IL-10 also inhibits IL-4-mediated IgE synthesis in B cells, while induces IgG4 production (Punnonen *et al.* 1993; Jeannin *et al.* 1998; Akdis *et al.* 1998). IL-10 also suppresses survival and cytokine production of eosinophils (Takanaski *et al.* 1994) and prevents mediator release from mast cells and basophils (Pierkes *et al.* 1999; Royer *et al.* 2001). Similarly, TGF-β has been shown to suppress both Th1 and Th2 type cytokine responses (Fargeas *et al.* 1992; Jutel *et al.* 2003), and proliferation by allergen-stimulated PBMC (Jutel *et al.* 2003) but, instead of inducing IgG4, it directs the synthesis of IgA in B lymphocytes (Jutel *et al.* 2003; Cazac & Roes 2000). Furthermore, neutralization of either IL-10 or TGF-β in the allergen-stimulated PBMC of healthy individuals has been shown to result in an increased proliferation and in increased synthesis of Th2 type cytokines, including IL-4, IL-5, and IL-13, indicating their active involvement in the suppression of allergic immune responses also *in vivo* (Akdis *et al.* 1998; Jutel *et al.* 2003). The anti-allergic immune effects of IL-10 and TGF-β *in vitro* are summarized in Table 2.

Treg cells are also capable of inducing apoptosis and depletion of allergen-specific T effector cells by direct cell-to-cell contact mechanisms independent of soluble IL-10 or TGF-β (Sun *et al.* 2007; Nagato *et al.* 2007). The cell-contact dependent immunosuppression by Treg cells has been shown to involve surface molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1), as well as cell surface-bound TGF-β (Taylor *et al.* 2006; Nakamura *et al.* 2001; Akdis *et al.* 2004).

Table 2. Anti-allergic immune effects of Treg type cytokines IL-10 and TGF-β (Jutel *et al.* 2006).

Cytokine	Functions
IL-10	Suppresses the synthesis of allergen-specific IgE and induces IgG4 Blocks B7/CD28 costimulatory pathway on T cells Suppresses allergen-specific Th1 and Th2 cells Inhibits dendritic cell maturation, leading to reduced MHC class II and costimulatory ligand expression Upregulates FOXP3 expression Reduces release of proinflammatory cytokines and histamine by mast cells
TGF-β	Suppresses the synthesis of allergen-specific IgE and induces IgA Suppresses allergen-specific Th1 and Th2 cells Down-regulates FcεRI expression on Langerhans cells Associated with CTLA-4 expression on T cells

2.1.2.3 Immediate (type I) hypersensitivity reaction

Allergic hypersensitivity reaction is typically a biphasic reaction, involving an immediate, early-phase reaction, occurring within seconds to minutes of allergen exposure, and a late-phase response, peaking within hours (Galli *et al.* 2008). Chronic allergic inflammation may result if the allergen exposure is frequent or persistent.

The immediate allergic reaction is an IgE-mediated mast cell-driven response. Mast cells are tissue resident inflammatory cells, and increased numbers have been found in mucosa of patients with allergic disease (Bentley *et al.* 1992; Wilson *et al.* 2001b; Nouri-Aria *et al.* 2005). These cells express high-affinity FcεRI receptors and have specific IgE bound on their plasma membrane (Galli et al. 2008; Ying et al. 1998). The cross-linking of IgE and their corresponding receptors on mast cells by binding of bivalent or multivalent allergen molecules results in the release of various proinflammatory mediators to the surrounding milieu. These mediators include histamine, and serine proteases such as tryptase, that are stored preformed in the intracellular granules, and lipid mediators such as cysteinyl leukotrienes (CysLT) and prostaglandins (e.g. prostaglandin D2, PGD_2), that are rapidly formed from membrane phospholipids following mast cell activation (Bousquet *et al.* 2001; Galli *et al.* 2008). These mediators give rise to the acute signs and symptoms of allergic inflammatory reaction, which typically peak at 15-30 min after allergen exposure, and resolve within 1-3 hours (Till *et al.* 2004; Galli *et al.* 2008). The signs and symptoms of this response may vary according to the site of the reaction, but can include 1) vasodilatation, producing erythema (reddening) of the skin and conjunctiva, 2) increased vascular permeability, leading to tissue swelling and, in the eyes, to tear formation, 3) contraction of bronchial smooth muscle, causing airflow obstruction and wheezing, and 4) mucus hypersecretion, exacerbating airflow obstruction in lower

airways and causing a runny nose (Galli *et al.* 2008). Mast cells can also synthesize several cytokines, chemokines and growth factors that increase adhesion molecule expression on vascular endothelial cells and promote the recruitment of other immune effector cells from circulation to the site of allergic inflammation (Kay 2001; Galli *et al.* 2008). Among these mediators are Th2 cytokines such as IL-13, proinflammatory cytokines such as IL-8 (also known as CXC chemokine ligand 8, CXCL8) and tumor necrosis factor-alpha (TNF-α), and chemokines such as CC chemokine ligand 2 (CCL2, formerly known as monocyte chemotactic protein-1, MCP-1) (Bousquet *et al.* 2001). Usually, mast cell mediators are released only locally, but a rapid and systemic release of these mediators from mast cells, or basophils, that also express FcεRI, can lead to a serious, and potentially fatal, allergic hypersensitivity reaction termed anaphylaxis (Galli *et al.* 2008). Anaphylactic reactions are often associated with stinging insect (Hymenopthera venom) hypersensitivity, latex allergy, and some forms of food allergy (seafood, nuts).

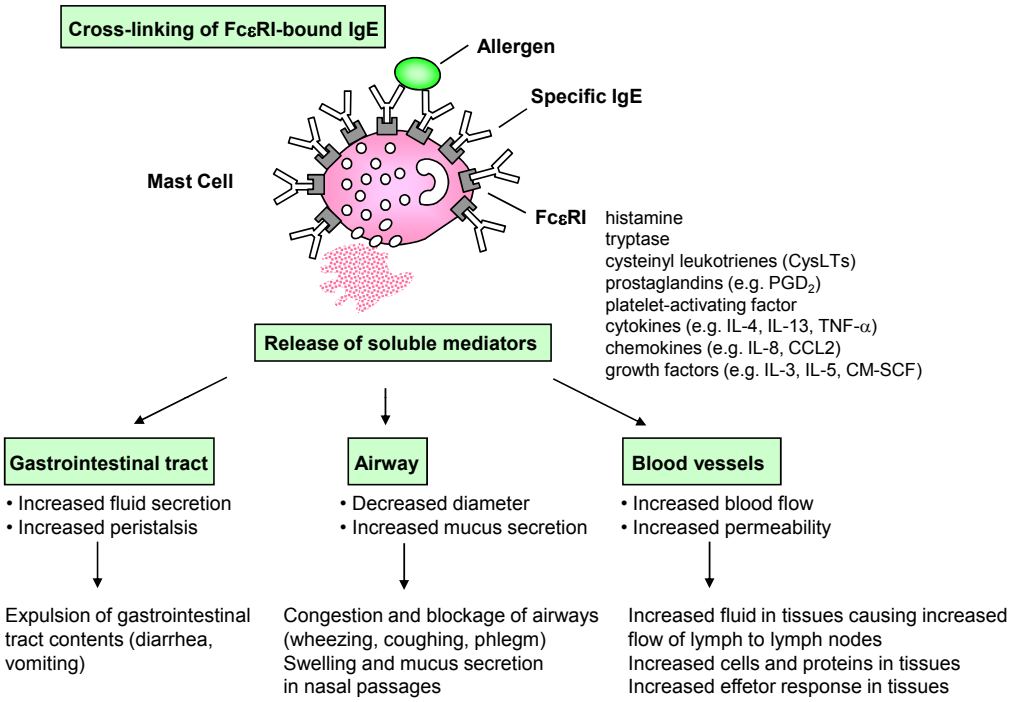

Figure 1. Events associated with immediate IgE-mediated (type I) hypersensitivity reaction. Allergic individuals have specific IgE bound on their mast cells through high-affinity FcεRI receptors. Upon allergen encounter and cross-linking of the FceRI, mast cells release both preformed and newly-synthesized mediators, including histamine, cysteinyl leukotrienes (CysLTs) and cytokines that act on local blood vessels to increase vascular permeability, induce smooth muscle contraction and increase mucus production, which give rise to the acute signs and symptoms of the allergic reaction. Chemokines released by mast cells also induce the recruitment of other inflammatory cells, in particular eosinophils and Th2 cells, to the allergic site, which characterizes the late phase allergic reaction (modified from Janeway *et al*. 2001).

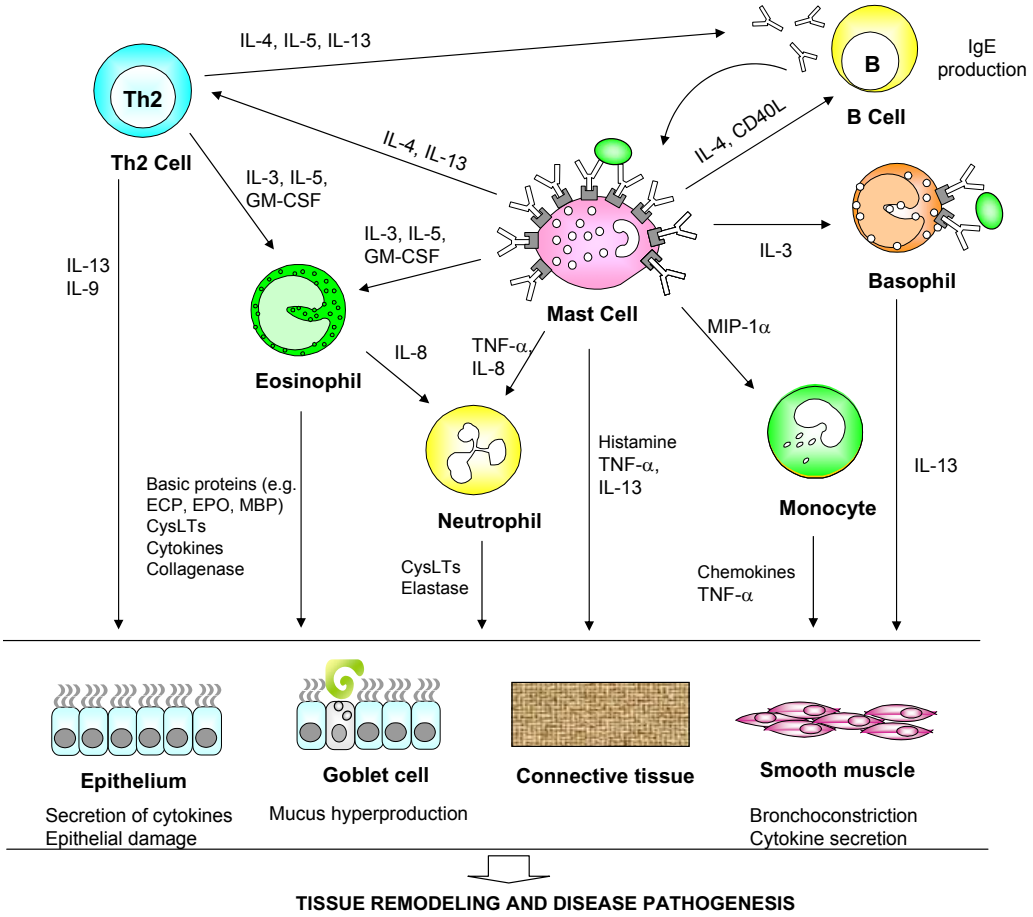

Figure 2. Mediators of late-phase allergic reaction (modified from Galli et al. 2008 and Janeway et al. 2001). Late-phase allergic reactions typically occur within hours of allergen exposure and are mediated by the actions of innate and adaptive immune cells that have been recruited to the site of the allergen exposure from circulation, as well as by inflammatory mediators that are secreted by tissue-resident cells. Eosinophils and neutrophils, for example, are potent sources of proinflammatory mediators, including cysteinyl leukotrienes (CysLTs) and cytokines that induce mucus hypersecretion and smooth muscle cell contraction, enzymes such as elastase and collegenase that activate matrix-degrading metalloproteinases, and basic proteins such as eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and major basic protein (MBP) that can induce tissue damage. Macrophages produce proinflammatory cytokines and activate Th2 cells, that in turn produce cytokines such as IL-4, IL-5, IL-13 and IL-9 and induce IgE synthesis in B cells. Together these mediators and cells sustain inflammation at the affected site, which can lead to tissue remodeling and chronic inflammation, if the allergen exposure is persisting. MIP-1α, macrophage inflammatory protein (also known as CCL4); GM-CSF, granulocyte-macrophage colony-stimulating factor.

2.1.2.4 Late-phase reaction

Depending on the allergen dose and allergen sensitivity of the subject, a second phase of allergic inflammation, the late-phase reaction, may occur (James & Durham 2008). This reaction typically develops 2-6 hours after the allergen challenge, often peaks within 6-9 h, and has fully resolved after 1-2 days, if there is no repeated exposure to the allergen (Galli *et al.* 2008). This response is clinically manifested by an edematous, red and slightly indurated swelling in the skin, sustained blockage in the nose, and by prolonged wheezing in the lung (Kay 2001). It involves infiltration of inflammatory cells from circulation to the allergen-exposed tissue, including neutrophils, that accumulate early, eosinophils, which appear shortly after, and then basophils, which are followed by mononuclear cells, including monocytes, that differentiate into tissue macrophages, and T cells, in particular of those that express Th2 type cytokines (Kay 2001; Charlesworth *et al.* 1989; Zweiman *et al.* 2000; Varney *et al.* 1993; Wilson *et al.* 2001b). The infiltrated inflammatory cells secrete proinflammatory mediators that sustain inflammation and, thereby, contribute to the development of chronic inflammation, which is especially noticeable in allergic asthma (Holgate & Polosa 2008). Persistent allergic inflammation is often associated with changes in the structural cells at the affected tissue and, in many cases, with markedly altered function of the target organs (Galli *et al.* 2008).

2.2 Specific immunotherapy (SIT)

2.2.1 General

Specific immunotherapy (SIT; also known as allergen immunotherapy or allergen vaccination, as well as hyposensitization, or desensitization) is the practice of repeated administration of specific allergens to a patient with an IgE-mediated condition for the purpose of providing protection against the allergic symptoms and inflammatory reactions associated with natural exposure to these allergens (Cox *et al.* 2007). This treatment, originally described by Noon in 1911, was developed to treat ‘hay fever’ or seasonal allergic rhinitis caused by pollen allergens, but is now expanded to include treating allergies caused by other allergen sources too, such as Hymenoptera venoms, house dust mites, animal dander, cockroaches and fungi (Esch 2008; Cox *et al.* 2007; Bousquet *et al.* 2008). SIT is recognized as a biological response modifier, capable of influencing the allergen-driven immunological responses, and is, alongside with allergen avoidance, the only treatment currently available to affect the natural course of allergic diseases (Bousquet *et al.* 2008). The treatment is indicated for patients over 5 years of age who have a demonstrated IgE-mediated allergic disease (i.e. history of allergic symptoms, positive skin prick test and/or *in vitro*-determined specific IgE, which correspond to the clinical symptoms), whose symptoms are inadequately controlled by medication or allergen avoidance, who have adverse effects from medication, or who wish to reduce the long-term use of medication (Cox *et al.* 2007; Bousquet *et al.* 2008).

2.2.2 Regimens (SCIT, SLIT)

The traditional route for administering allergens to the patients during SIT is by subcutaneous injection (SCIT). This treatment is, however, not without a risk of developing serious, and potentially fatal, systemic reactions, in particular during the induction phase (the risk ranges from <1% when using the conventional regimen to even >36% when using rush schedules) (Cox *et al.* 2007; Bousquet *et al.* 2008). Therefore, other routes, including intranasal, oral, bronchial, and sublingual routes, have been explored as an alternative to SCIT (Esch 2008; Bousquet *et al.* 1998). Sublingual immunotherapy (SLIT) was introduced in 1986 and accepted by the World Health Organization (WHO) for routine clinical use in 1998 (Penagos *et al.* 2006; Bousquet *et al.* 1998). SLIT involves administration of allergen extracts in the form of tablets or drops under tongue, where they are kept for 2-3 min and then swallowed. Its hallmarks are improved tolerability and safety profile as compared to SCIT, and these features have also allowed home administration of SIT (Esch 2008). Only few clinical cases on anaphylactic reactions following SLIT have been published: one case was on latex immunotherapy, two other on multi-allergen vaccine, and one on overdose of maintenance dose (Bousquet *et al.* 2008; Dunsky *et al*. 2006; Antico *et al*. 2006; Eifan *et al*. 2007; Blazowski 2008). The increased tolerability of SLIT is likely to relate to the low prevalence of effector cells, including mast cells, basophils and eosinophils, in the oral mucosa (Lima *et al.* 2002; Marcucci *et al.* 2007; Esch 2008).

SCIT typically involves a build-up phase (also known as the up-dosing, introduction, or dose-increase phase) during which the allergens are introduced to the body, by starting with very low quantities, and increasing them until the achievement of maintenance dose, which is typically continued for 3-5 years to induce long-lasting protective effects against the allergens that are responsible for producing the symptoms (Cox *et al.* 2007; Bousquet *et al.* 1998). In SCIT, the starting dose is generally 1000-10,000-fold less than the maintenance dose, the latter being typically in the range of 5-20 μg of major allergen for inhalant allergens, and 100 μg for Hymenoptera venom (Cox *et al.* 2007). Several build-up schedules have been generated for SCIT, the most common one (often termed conventional schedule) involving administration of increasing doses of the allergens at 1 to 2 week intervals, when the maintenance dose is achieved within 4 to 6 months. Accelerated schedules such as cluster and rush SCIT, enable achievement of the maintenance dose in a much shorter period, even within 1-3 days when using the rush schedule, or within 4-8 weeks, when using the cluster schedule, but these methods are associated with a higher risk of generating severe adverse reactions (Cox *et al.* 2007). The risk of generating systemic adverse reactions during the accelerated schedules has been reduced by the usage of depot or modified extracts instead of the aqueous ones (Mellerup *et al.* 2000; Cox 2006). After the maintenance dose is achieved, the dose is typically given in every 4-8 weeks for Hymenoptera venom, and 2-4 weeks for inhalant

allergens (Cox *et al.* 2007). The interval between injections, though, can be progressively increased as tolerated to 4 to 6 weeks according to other practice guidelines (Li *et al*. 2003).

In most trials showing the clinical efficacy of SLIT, maintenance doses in the range of 3 to more than 375 times higher than the maintenance dose of SCIT have been administered, and the current recommendation by the WHO is that doses at least 50-100 times higher than in SCIT should be administered, to reach the same level of efficacy (Moingeon *et al.* 2006; Bousquet *et al.* 2008). To achieve these cumulative doses, allergen preparations must be administered frequently (usually daily) and for long duration (usually year round) (Esch 2008). A possible explanation for this may be an inefficient delivery of allergens to the immune cells, such as oral dendritic cells, and their difficulty to ultimately induce a robust and protective T-cell response (Esch 2008). Also, current SCIT protocols usually rely on the use of an adjuvant (e.g. calcium phosphate or aluminum hydroxide), which are not employed in the sublingual administration (Moingeon *et al.* 2006). The favorable safety profile of SLIT has also unnecessitated the use of a build-up phase, thereby enabling the initiation of SLIT regimens directly with therapeutically effective maintenance doses (Lombardi *et al.* 2009).

2.2.3 Clinical efficacy

Several recent meta-analyses of randomized placebo-controlled trials have concluded that both SCIT and SLIT are clinically effective in the treatment of allergic rhinitis and asthma in adult and pediatric patients (Abramson *et al.* 2003; Calderon *et al.* 2007; Wilson *et al.* 2005; Penagos *et al.* 2006; Penagos *et al.* 2008). Both treatments have been found to significantly reduce allergy symptoms and medication use when compared to placebo group. However, some controversy still exists between the different meta-analyzes in the clinical efficacy of SIT to confer for the reduction of allergic rhinoconjunctivitis symptoms as compared to pharmacological treatment in children (Wilson *et al.* 2003; Röder *et al*. 2008). The discrepancies in the efficacy of SIT in the treatment of IgE-mediated allergies between different clinical trials have been suggested to relate with divergences in the methodological qualities of the trials, differences in the administration form and duration of SIT, and the use of different allergens or different allergen doses (Röder *et al*. 2008). SCIT has been shown to be effective against allergies due to pollens (birch and other *Betulaceae*; grass, *Cupressae*, cypress, olive tree, *Parietaria*, ragweed), animal allergens (cat), house dust mite, fungi (*Alternaria*) and cockroach (Cox *et al.* 2007; Bousquet *et al.* 2008). In SLIT, the efficacy has been demonstrated against birch, cypress, grass, olive and *Parietaria* pollens, and house dust mite (Bousquet *et al.* 2008).

A number of longitudinal clinical studies have shown that the therapeutic effects of SCIT are long-lasting, with clinical efficacy persisting for at least 3-6 years after its discontinuation in adults (Durham *et al.* 1999b; Passalacqua & Canonica 2002), and as recently shown

in children, even for 12 years (Cools *et al.* 2000; Eng *et al.* 2006). SCIT, when given prophylactically to children, may also reduce the onset of new sensitizations to unrelated allergens (Des Roches *et al.* 1997; Pajno *et al.* 2001; Eng *et al.* 2006), and prevent disease progression from allergic rhinitis to asthma (Jacobsen *et al.* 1997; Polosa *et al.* 2004; Jacobsen *et al.* 2007). It has been estimated that nearly 20% of patients with allergic rhinitis will develop asthma in 10 years time despite of an appropriate pharmacological treatment (Linna *et al*. 1992; Bousquet *et al*. 2001). Increasing body of evidence supports that SLIT can also prevent the development of new sensitizations and reduce the onset of asthma when initiated early in childhood (Di Rienzo *et al.* 2003; Novembre *et al.* 2004; Marogna *et al.* 2007a; Inal *et al.* 2007). SLIT has also been shown to confer for long-term remission in symptoms, lasting for at least 4-5 years after its discontinuation in children (Di Rienzo *et al.* 2003). The duration of efficacy of both SLIT and SCIT in the patients has been shown to depend on the length of the treatment course, with 3-4 years treatment period conferring for the best protection (Des Roches *et al.* 1996; Marogna *et al.* 2007a). SIT has also been shown to provide long-lasting protection against Hymenopthera venom anaphylaxis (Golden *et al.* 1996; Ross *et al.* 2000). SIT for Hymenopthera venom allergy is often referred as venom immunotherapy (VIT), and this term is hereafter used to indicate the mechanisms associated with this treatment.

2.3 Immune mechanisms behind the beneficial effects of SIT

2.3.1 Attenuation of immediate hypersensitivity reaction

The clinical efficacy of SIT is largely related to its capacity to inhibit immediate and late-phase allergic responses in the target organs upon subsequent allergen encounters. The changes in these responses can be experimentally tested by local application of allergens on the target mucosa e.g. in nose, lungs or skin by inhalation or intradermal injection, respectively - or in the case of pollen allergy, by investigating the responses during natural allergen exposure. Several studies have shown a significant reduction in the magnitude of immediate allergic reactions to the causative allergens in the allergen-exposed tissues during SCIT, as shown by either diminished wheal-and-flare response in the skin (Iliopoulos *et al.* 1991; Varney *et al.* 1993; Durham *et al.* 1996; Hamid *et al.* 1997; Nish *et al.* 1994; Durham *et al.* 1999a; Keskin *et al.* 2006), reduced sneezing and nasal congestion (Creticos *et al.* 1985; Iliopoulos *et al.* 1991; Otsuka *et al.* 1991; Durham *et al.* 1996; Klimek *et al.* 1999a; Keskin *et al.* 2006; Francis *et al.* 2008), or diminished airway obstruction in the lungs (Arvidsson *et al.* 2004). The dose of allergen needed to induce such responses has also been shown to increase several-fold during SIT (Creticos *et al.* 1985; Pene *et al.* 1998; Klimek *et al.* 1999a; Meissner *et al.* 1999; Durham *et al.* 1999a; Keskin *et al.* 2006; Lent *et al.* 2006).

Significant reduction in the numbers of mast cells in skin (~5x) (Durham *et al*. 1999a) and nasal mucosa (Otsuka *et al*. 1991; Cengizlier *et al*. 1995; Nouri-Aria *et al*. 2005; Plewako *et al*. 2008) has been found after SCIT. In both sites, diminished or even abolished secretion of mast cell-derived mediators, including histamine and PGDB2B or N-α-Tosyl L-arginine methyl ester (TAME)-esterase in response to allergen challenge has been found (Creticos *et al*. 1985; Hedlin *et al*. 1989; Iliopulos *et al*. 1991; Nish *et al*. 1994; Dokic *et al*. 1996). In pollen-allergic patients, the post-seasonally recorded decrease in the numbers of mast cells in skin during SCIT was found to correlate with the clinical improvement during subsequent pollen season, although was not directly associated with the decrease in the size of the late-phase allergic reactions in skin (Durham *et al*. 1999a). SCIT has also been shown to blunt the usual seasonal increases in mast cells in nasal mucosa of pollen allergic patients, although this did not correlate with the clinical improvement during the season (Nouri-Aria *et al*. 2005).

The immediate allergic responses to specific allergens have been shown to decrease already 3 months after the start of SCIT (Hedlin *et al*. 1989; Francis *et al*. 2008), and to remain low up to 3 years after its discontinuation in adult pollen allergic patients (Durham *et al*. 1999b). Recently, in children, there was no return of immediate allergic reactions to specific allergens until 12 years post-SCIT (Eng *et al*. 2006). Both in adults and children, the trend of return in immediate allergic reactions after SCIT to specific allergens was not associated with any relapse in clinical improvement though (Durham *et al*. 1999b; Eng *et al*. 2006).

During SLIT, generally no changes in immediate skin or conjunctival responses to specific allergens have been found during the first 2 years of treatment (Pradalier *et al.* 1999; Lima *et al.* 2002; Bufe *et al.* 2004; Smith *et al.* 2004a; Valovirta *et al.* 2006). After the second treatment year, however, the patients were 6.8 times likely to show reduced nose running and 2.4 more likely to have reduced sneezing as compared to placebo-treated group (Smith *et al.* 2004a). In some studies, earlier reductions in rhinoconjuctivitis symptoms, occurring just 6 months after the initiation of treatment, was found (Marcucci *et al.* 2005a). In addition, reduction in seasonal ocular symptoms in some studies (Pradalier *et al.* 1999) but not all (Clavel *et al.* 1998), and reduction in bronchial asthma symptoms (Pradalier *et al.* 1999) were demonstrated just 4 months after the start of SLIT (Smith *et al.* 2004a). It has been demonstrated that nasal tryptase levels are decreased after allergen challenge after the third year of SLIT (Marcucci *et al.* 2005b). However, only low numbers of mast cells are normally found to be present in the sublingual mucosa, and thereby, no changes in the mucosal tryptase levels have been found during the course of SLIT (Marcucci *et al.* 2001; Marcucci *et al.* 2007).

2.3.2 Attenuation of late-phase allergic reaction

A second key feature of SIT, and the factor most likely to contribute to the capacity of this treatment to attenuate disease progression and pathogenesis, is the suppression of allergen-induced late-phase reactions. This has been demonstrated in skin (Iliopoulos *et al.* 1991; Varney *et al.* 1993; Nish *et al.* 1994; Durham *et al.* 1996; Hamid *et al.* 1997; Durham *et al.* 1999a; Lima *et al.* 2002; Plewako *et al.* 2004; Gardner *et al.* 2004a; Alexander *et al.* 2005), nose (Iliopoulos *et al.* 1991; Durham *et al.* 1996; Wachholz *et al.* 2002) and lungs (Oda *et al.* 1998; Arvidsson *et al.* 2004) during both SCIT and SLIT, as well as in skin after VIT (Jung *et al.* 1997; Nasser *et al.* 2001; Tarzi *et al.* 2006). In skin, the reduction in the late-phase allergic reactions during SCIT and VIT was found to be associated with the diminished infiltration of inflammatory cells, including T cells, eosinophils, basophils, neutrophils and macrophages from circulation to the allergen-challenged site (Varney *et al.* 1993; Nish *et al.* 1994; Nasser *et al.* 2001; Jung *et al.* 1997; Plewako *et al.* 2004). Similarly, in nose, decreased number of infiltrated inflammatory cells, in particular T cells and eosinophils, in response to allergen challenge was found during the course of SCIT (Furin *et al.* 1991; Durham *et al.* 1996). Biopsies taken in and out of pollen season from pollen allergic patients have also revealed significant seasonal decreases in the numbers of infiltrating T cells, as well as eosinophils and basophils, to nasal mucosa during SCIT (Furin *et al.* 1991; Wilson *et al.* 2001b; Wilson *et al.* 2001a; Wachholz *et al.* 2002; Nouri-Aria *et al.* 2005). The changes in the cellular infiltrates in nasal mucosa during SCIT have been shown to relate with the decreased concentration of eosinophil-associated inflammatory mediators such as eosinophil cationic protein (ECP, also known as RNASE 3, from ribonuclease, RNase A family, 3) and tryptase, in nasal secretions during allergen challenge (Klimek *et al*. 1999b; Keskin *et al.* 2006). In pollen allergic patients, the decrease in the numbers of eosinophils in nasal mucosa during pollen season during SCIT was found to correlate with the clinical improvement (Wilson *et al.* 2001a; Wilson *et al.* 2001b; Nouri-Aria *et al.* 2005).

SCIT has also been demonstrated to blunt the usual seasonal increases in eosinophils in BAL fluid of pollen allergic patients with rhinoconjunctivitis and asthma (Rak *et al.* 1991). In addition, SCIT has been shown to blunt allergen-induced increases in eosinophils in peripheral blood and sputum of allergic asthma patients (Oda *et al.* 1998; Arvidsson *et al.* 2004). In some studies, both SCIT and VIT have been demonstrated to result in a diminished number of basophils in peripheral blood and/or their decreased mediator release (Plewako *et al.* 2006a; Jutel *et al.* 1996; Pierkes *et al.* 1999; Shim *et al.* 2003). In asthma patients, this inhibition was found to be directly associated with the decreased asthma symptom scores (Shim *et al.* 2003). SCIT has also been shown to result in a reduced concentration of eosinophil-associated inflammatory mediators, including ECP/RNASE 3 and nitric oxide (NO) in serum (Arvidsson *et al.* 2004; Cevit *et al.* 2007). VIT has been shown to reduce the expression of RANTES (from Regulated upon Activation,

Normal T-cell Expressed, and Secreted, also termed CCL5), IL-8/CXCL8 and MCP-1/CCL2 in PBMC (Akoum *et al.* 1998).

The late-phase allergic reactions to specific allergens in skin have been shown to decrease significantly already 2 weeks after the initiation of SCIT, and thus, to occur earlier than the reduction in immediate allergic responses (Francis *et al.* 2008). The late-phase skin response to specific allergens have been shown to remain virtually absent in the patients treated with SCIT for at least 3 years after the discontinuation, and to relate with the diminished infiltration of T cells and IL-4 mRNA+ cells in the allergen-challenged skin (Durham *et al.* 1999b). The long-term immunomodulatory effects of SCIT on allergen-induced cellular infiltration have been also confirmed in nasal mucosa, wherein a sustained blockage of the usual seasonal increases in eosinophils and cells staining positive for chemokines eotaxin, RANTES/CCL5, CC chemokine receptor 3 (CCR3), and TARC (from thymus and activation regulated chemokine, also known as CCL17) was found 3 to 5 years post treatment (Plewako *et al.* 2008).

The suppression of late-phase allergic responses in skin (Lima *et al.* 2002), nose (Passalacqua *et al.* 1999; Ciprandi 2006b; La Grutta *et al.* 2007) and lungs (Ciprandi *et al.* 2006b; Marogna *et al.* 2005) have also been demonstrated during SLIT, although not in all studies (Valovirta *et al.* 2006). In conjunctiva, the reduction in the late-phase allergic reactivity after SLIT was found to be associated with the diminished infiltration of inflammatory cells, including eosinophils and neutrophils, from circulation, as well as with the decreased expression of intercellular adhesion molecule 1 (ICAM-1) on conjunctival epithelium in response to allergen challenge (Passalacqua *et al.* 1998). Similarly, in nose, decreased infiltration of inflammatory cells, including eosinophils and neutrophils, and decreased expression of ICAM-1 on nasal epithelium were found after the allergen challenge after the treatment with SLIT (Passalacqua *et al.* 1999; Ciprandi 2006b; La Grutta *et al.* 2007). In pollen allergic patients, SLIT has also been shown to significantly reduce the usual seasonal increases in the numbers of infiltrating eosinophils in nasal mucosa (Marogna *et al.* 2005; Marogna *et al.* 2007b; Marogna *et al.* 2009). A trend toward diminished eosinophil count in peripheral blood has also been found during SLIT (Lue *et al.* 2006). After 6 months of treatment, there was also a significant reduction in the sublingual ECP/RNASE 3 concentration, but not of tryptase (Marcucci *et al.* 2005a). However, no changes in the inflammatory cells (CD1a+ dendritic cells, CD68+ macrophages, or total (CD3+) T lymphocytes) in sublingual mucosa have been found during the first 18 months of SLIT (Lima *et al.* 2002). Only low numbers of eosinophils have been normally found in the sublingual mucosa, with no changes in these cells during SLIT (Marcucci *et al.* 2007). However, SLIT has been shown to lead into decreased serum concentration of eosinophil-associated inflammatory mediators, including ECP/RNASE 3, IL-13, and prolactin (Passalacqua *et al.* 1998; Ippoliti *et al.* 2003).

2.3.3 Immunomodulation of allergen-specific T cell responses

2.3.3.1 Induction of tolerogenic Treg cells

The ultimate goal of SIT is to increase tolerance to the allergens that are causing the symptoms. This tolerance can be induced in principle by two immunological mechanisms: either by induction of Treg cells that mediate active suppression of the allergen-induced immune responses or by inducing anergy (functional inactivation) and/or clonal deletion (apotosis) in allergen-specific T effector cells (Faria & Weiner 2005; Moingeon *et al.* 2006). The primary factor that determines which form of peripheral tolerance develops is the dose of antigen administered. Low doses of antigen generally favor the generation of Treg-driven tolerance, whereas high doses of antigen favor anergy-driven tolerance (Friedman & Weiner 1994; Gardner *et al.* 2004b). SIT, initiated with very low allergen doses, and then increasing them up to the maintenance doses, is likely to involve both mechanisms.

It has been demonstrated that there is induction of tolerogenic, allergen-specific CD4+CD25+ Treg cells, in particular of the IL-10-producing type, in peripheral blood during SCIT (Francis *et al.* 2003; Jutel *et al.* 2003; Gardner *et al.* 2004a), VIT (Akdis *et al.* 1998) and SLIT (Bohle *et al.* 2007). These cells are induced early, within 1-3 months after the initiation of these treatments (Akdis *et al.* 1998; Jutel *et al.* 2003; Gardner *et al.* 2004a; Bohle *et al.* 2007). In pollen allergic patients, SCIT has also been shown to result in local increases in IL-10 or TGF-β expressing cells, e.g. in nasal mucosa during pollen season (Nouri-Aria *et al.* 2004; Pillette *et al.* 2007). Several studies have also demonstrated increased peripheral IL-10 production during SIT; the expression has been shown to increase during SCIT for grass or tree pollens (Francis *et al.* 2003; Jutel *et al.* 2003; Savolainen *et al.* 2004; Francis *et al.* 2008), dust mites (Gardner *et al.* 2004a) and cat (Verhoef *et al.* 2005), during SLIT for grass or tree pollens (Savolainen *et al.* 2006; Bohle *et al.* 2007; Burastero *et al.* 2008) or dust mites (Ciprandi 2006a; Ciprandi 2006b) and during VIT for bee or wasp venom (Bellinghausen *et al.* 1997; Akdis *et al.* 1998; Pierkes *et al.* 1999; Nasser *et al.* 2001; Faith *et al.* 2003; Fellrath *et al.* 2003; Tarzi *et al.* 2006). In some studies, increase in peripheral TGF-β production has been shown during SCIT (Jutel *et al.* 2003; Lent *et al.* 2006) and SLIT (Burastero *et al.* 2008).

The IL-10+ cell population found in the nasal mucosa of SCIT-treated patients during pollen season has been shown to represent an immunization-induced phenomenon, as there is no increase in such cells in the nasal mucosa of healthy individuals during pollen season (Nouri-Aria *et al.* 2004). The Treg cells induced during SIT thereby represent an inducible subset of the Treg cells (iTreg) in the body, generated by immunization in the periphery, whereas the naturally occurring Treg cells (nTreg) that maintain tolerance against self antigens, develop in thymus (Bluestone & Abbas 2003; Faria & Weiner 2005). The nTreg cells constitutively express high levels of FOXP3, whereas it is induced in

iTreg cells following activation (Ziegler 2006). Several subtypes of iTreg cells have been identified, including the predominantly IL-10-producing type 1 Treg cells (Tr1), and the predominantly TGF-β-producing Th3 cells (Taylor *et al.* 2006; Chen *et al.* 1994; Groux *et al.* 1997). Oral immunization has been shown to favor the induction of Th3 subtype in mice (Chen *et al.* 1994). It has been demonstrated that FOXP3 mRNA expression increases in PBMC and nasal mucosa during SCIT, and it co-localizes to CD4+ T cells expressing CD25 (Radulovic *et al.* 2008). FOXP3 expression has also been shown to increase in PBMC during SLIT (Bohle *et al.* 2007).

Besides of inducing IL-10 and/or TGF-β production in Treg cells, both VIT and SCIT have also been shown to increase IL-10 production in antigen-presenting cells (APC), including monocytes and B cells (Akdis *et al.* 1998; Nouri-Aria *et al.* 2004). After VIT, nearly 41% of the IL-10-producing cells in peripheral blood were identified as CD14+ monocytes or as CD19+ B cells, and after grass pollens SCIT, 35% of the IL-10 mRNA+ cells in nasal mucosa were found to be CD68+ macrophages (Nouri-Aria *et al.* 2004). Likewise, a large proportion (~35%) of TGF-β mRNA+ cells in the nasal mucosa after grass pollen SCIT was characterized as CD68+ macrophages (Pillette *et al.* 2007). The induction of Treg type immune responses during SIT are summarized in Table 3.

It has been demonstrated that the early induction of IL-10 production by PBMC coincidences with the suppression of late-phase allergic reactions in the skin both during SCIT (Gardner *et al.* 2004a; Francis *et al.* 2008) and VIT (Nasser *et al.* 2001). The blocking of either IL-10 or TGF-β in the PBMC cultures *in vitro* has been shown to result in full restoration of the SIT-induced suppressions in allergen-specific T cell proliferation and Th2 and Th1 cytokine production (Bellinghausen *et al.* 1997; Akdis *et al.* 1998; Jutel *et al.* 2003; Faith *et al.* 2003). IL-10 production induced during SIT has also been shown to contribute to the inhibition of proinflammatory mediator release from allergen-stimulated peripheral blood leukocytes (Pierkes *et al.* 1999) and the reduced expression of CD28-family costimulatory molecules on CD4+ T cells (Bellinghausen *et al.* 2004), since these responses are restored by IL-10 neutralization. In SLIT-treated patients, the increase in IL-10 production during the treatment was found to be associated with the diminished lymphoproliferation of PBMC to specific allergen (Burastero *et al.* 2008), as well as to correlate with TGF-β production (Savolainen *et al.* 2006). The increased IL-10 production during SLIT has also been shown to correlate with the increased forced expiratory flow values (Ciprandi 2006a), as well as with the decreased nasal responses to decongestion testing (Ciprandi *et al.* 2007).

2.3.3.2 Induction of Th2/Th1 shift

In addition to inducing Treg cells, immune deviation from the predominated Th2-biased responses to a more Th1 type in allergen-specific T effector cells is another important phenomenon associated with SIT. During SCIT for grass or tree pollens (Ebner *et al.*

Table 3. Induction of allergen-specific Treg cells and changes in allergen-specific Treg type cytokine production during SIT (↑ increased response, ↓ decreased response, ↔ no change in response).

PERIPHERAL TREG RESPONSES					
SIT regimen	**Observation period**	**Cells**	**Treg cells**	**Treg type cytokine production**	**Reference**
SCIT pollen	3 mo	short-term TCL[b]		IL-10 ↔	Klimek *et al.* 1999a
	up-dosing (2–4 wk)[a], early MP[c] (~4 mo), and 1. season	PBMC		IL-10 ↓ at 2-4 wk, but recovered by MP, and at 1. season ↔ to not-treated	Moverare *et al.* 2000
	1. season (4–5 mo)	PBMC		IL-10+ cells ↔	Gabrielsson *et al.* 2001
	1 yr	PBMC	% IL-10+CD4+CD25+ ↑	IL-10 ↑ (in and out season)	Francis *et al.* 2003
	1 yr	PBMC		IL-10 ↑, TGF-β ↔ (IL-10 also ↑ at MP in good responders)	Savolainen *et al.* 2004
	2 wk – 1yr	PBMC		IL-10 ↑ from wk 4	Francis *et al.* 2008
SCIT dust mite	3 mo	short-term TCL		IL-10 ↔	Oda *et al.* 1998
	7 – 70 d	PBMC	% IL-10+CD4+CD25+ ↑ (d 70)	IL-10 and TGF-β ↑ from d 28	Jutel *et al.* 2003
	3 and 9 mo	PBMC	% IL-10+ CD4+ CD25+ ↑ 9 mo (% IL-10+CD8+ ↔)		Gardner *et al.* 2004a
SCIT animal	30 wk	enriched CD4+	Suppressive activity ↔	IL-10 ↔	Smith *et al.* 2004b
	12-14 wk	PBMC	Suppressive activity induced in CD4+	IL-10 ↑ trend	Verhoef *et al.* 2005
	5 wk	PBMC		TGF-β ↑, IL-10 ↔ high dose	Lent *et al.*2006
SLIT pollen	1 and 2 yr	PBMC		IL-10 ↑ at 2 yr, TGF-β ↔ in high dose and low dose group	Savolainen *et al.* 2006
	4 and 52 wk	PBMC	FOXP3 mRNA ↑ at 4 wk, but ↓ at 52 wk Suppressive activity ↔	IL-10 ↑ at 4 wk, but ↓ at 52 wk TGF-β ↔	Bohle *et al.* 2007
	2 mo	PBMC	% IL-10+CD3+ cells ↑	IL-10 ↑, TGF-β ↑	Burastero 2008
SLIT dust mite	6 mo (& after 22–24 mo)	short-term TCL	% IL-10+CD4+ cells ↑ (after 22–24 mo)	IL-10 ↑, TGF-β ↔ (IL-10 also ↑ after 22–24 mo)	Cosmi *et al.* 2006
	3 yr	PBMC		IL-10 ↑	Ciprandi *et al.* 2006a
SLIT dust mite/ pollen	Up-dosing (2 wk) and early MP (8 wk)	PBMC		IL-10 ↔, TGF-β ↔	Dehlink *et al.* 2006
VIT	1 wk	PBMC		IL-10 ↑ (produced by CD4+ T cells)	Bellinghausen *et al.* 1997
	1 – 28 d	PBMC	% IL-10+CD4+CD25 ↑ from d 7	IL-10 ↑ from d 7	Akdis *et al.* 1998
	8 and 16 wk	short-term TCL		IL-10 ↑ by short-term TCL from 8 wk	Faith *et al.* 2003
	7 - 80 d	short-term TCL		IL-10 ↑ by short-term TCL (peak at d 42)	Fellrath *et al.* 2003
	12 wk	PBMC	FOXP3 mRNA ↑ (trend)	IL-10 ↑	Tarzi *et al.* 2006
LOCAL TREG RESPONSES					
SIT regimen	**Observation period**	**Tissue**	**No. of Treg type cells**		**Reference**
SCIT pollen	2 yr (in and out season)	Nose	IL-10 mRNA+ cells ↑ (in season)		Nouri-Aria *et al.* 2004
	2 yr (in and out season)	Nose	TGF-β mRNA+ ↑ (in season)		Pillette *et al.* 2007
	2. season	Nose	CD4+CD24+ cells expressing FOXP3 ↑		Radulovic *et al.* 2008
SCIT animal	4 wk post SIT (10 wk)	Skin	CD4+CD25+ cells ↑, IL-10+CD4+ cells ↔, IL-10 mRNA+ ↔, TGF-β mRNA ↑ (Eos)		Alexander *et al.* 2005
VIT	3 mo	Skin	IL-10+ cells ↑, TGF-β+ cells ↔		Nasser *et al.* 2001

[a] duration of treatment, [b] TCL, allergen-specific T cell line, [c] MP, maintenance phase

1997), dust mites (Secrist *et al.* 1993; Oda *et al.* 1998; Benjaponpitak *et al.* 1999; Fu *et al.* 2003; Jutel *et al.* 2003; Gardner *et al.* 2004a) and animal allergens (Pene *et al.* 1998; Meissner *et al.* 1999; Smith *et al.* 2004b; Tazaki *et al.* 2004; Verhoef *et al.* 2005; Lent *et al.* 2006), as well as during VIT for Hymenopthera venoms (Jutel *et al.* 1995; McHugh *et al.* 1995; Akdis *et al.* 1996; Akoum *et al.* 1996; Bellinghausen *et al.* 1997; Akdis *et al.* 1998; Faith *et al.* 2003; Fellrath *et al.* 2003; Tarzi *et al.* 2006), a significant reduction in the allergen-induced proliferation by specific T cells, and a shift in the peripheral allergen-specific Th2/Th1 balance, as shown by either decreased Th2 type cytokine (IL-4, IL-5, IL-13) production and/or increased synthesis of Th1 type cytokines (IFN-γ, IL-12), has been demonstrated. Some studies, however, have failed to demonstrate any significant changes in the peripheral allergen-specific T cell responses during SCIT (Till *et al.* 1997; Klimek *et al.* 1999a; Wachholz *et al.* 2002; Francis *et al.* 2003; Francis *et al.* 2008), but rather, have found a shift away from the predominated allergen-induced Th2 type immune responses towards more Th1 type in target organs (Klimek *et al.* 1999a; Wachholz *et al.* 2002).

Local alterations in allergen-specific Th2/Th1 cell balance during SCIT have been demonstrated in numerous studies. Decreases in the numbers of cells expressing mRNAs for Th2 type cytokines (IL-4, IL-5) and/or increases in the numbers of cells expressing mRNAs for Th1 cytokines (IL-2, IFN-γ) or IL-12 have been found after allergen challenge for example in the skin (Varney *et al*. 1993; Nasser *et al*. 2001; Hamid *et al*. 1997) and nose (Durham *et al*. 1996). SCIT has also been shown to blunt the usual seasonal increases in IL-5 or IL-9 mRNA expressing cells in the nasal mucosa of pollen allergic patients (Wilson *et al*. 2001a; Wachholz *et al*. 2002; Nouri-Aria *et al*. 2005). In addition, SCIT has been shown to block the usual seasonal increase in IL-4 and IL-13 production in PBMC (Giannarini & Maggi 1998; Gabrielsson *et al*. 2001). Decreased concentration of IL-5 and increased concentration of IFN-γ have also been found in the nasal secretions of SCIT-treated patients (Klimek *et al*. 1999a). These local changes have been shown to correlate with the clinical improvement. For example, in pollen allergic patients, the increase in the numbers of IFN-γ mRNA+ cells and the decrease in the numbers of IL-5 mRNA+ cells in nasal mucosa during SCIT were found to closely correlate with the clinical improvement during the pollen seasons (Durham *et al*. 1996; Wilson *et al*. 2001a).

In skin, the increase in the numbers of IL-12 mRNA+ cells, most (55-80%) of which were found to co-localize to CD68+ macrophages, has been shown to correlate with the decrease in the numbers of IL-4 mRNA+ cells and the increase in the numbers of IFN-γ mRNA+ cells in dermis after allergen challenge during SCIT (Hamid *et al.* 1997). This finding suggests that the shift in allergen-specific Th2/Th1 balance during SCIT may be driven by IL-12. SCIT has also been shown to result in increase in peripheral IL-12-producing CD14+ monocytes and IFN-γ-producing CD56+ natural killer (NK) cells in patients with allergic asthma (Plewako *et al.* 2006b). However, in sublingual mucosa, no changes in IL-12 mRNA+ cells have been found during SLIT (Lima *et al.* 2002).

Studies on allergen-specific T cell responses during SLIT have demonstrated reduced allergen-induced lymphoproliferation (Fanta *et al.* 1999; Bohle *et al.* 2007), increased synthesis of IFN-γ (Cosmi *et al.* 2006; Ciprandi *et al.* 2008a) and inhibition of IL-5 increase (Savolainen *et al.* 2006) in PBMC. However, some studies have failed to demonstrate any significant changes in peripheral allergen-specific T cell responses during SLIT, due to low dose treatment protocols (Rolinck-Werninghaus *et al.* 2005; Dehlink *et al.* 2006). The capacity of SLIT to induce changes in peripheral allergen-specific T cell responses has been shown to depend on the therapeutic allergen dose, as shown in trials involving both high allergen dose and low dose treated groups (Savolainen *et al.* 2006; Lent *et al.* 2006). The dose-dependency of SLIT in inducing immune deviation in allergen-specific immune responses is thus similar to as earlier demonstrated for SCIT (Pene *et al.* 1998). In allergic rhinitis patients, the increase in IFN-γ production in the PBMC during SLIT was found to correlate with the clinical improvement (Ciprandi *et al.* 2008a). The changes in allergen-specific Th2/Th1 type immune responses in PBMC and target tissues during SIT are summarized in Table 4.

IL-18 and SLAM are two molecules suggested to play a role in the Th2/Th1 shift during SIT (Laaksonen *et al.* 2003; Savolainen *et al.* 2004). SLAM (signalling lymphocytic activation molecule, CD150) is a self-ligand, transmembranic, cell-surface glycoprotein belonging to the CD2 subset of immunoglobulin superfamily, and is preferentially expressed on Th1 cells over Th2 cells (Cocks *et al.* 1995; Castro *et al.* 1999; Hamalainen *et al.* 2000). It has been demonstrated that SLAM induces proliferation and IFN-γ production in T cells, and reverses the cytokine production profile of Th2 clones to a Th0/Th1 type (Aversa *et al.* 1997; Carbadillo *et al.* 1997). In SCIT-treated patients, a significant increase in SLAM mRNA expression was found after 1 year of treatment, and this response tended to increase even earlier, by the time of reaching the maintenance dose, in patients developing a good therapeutic outcome (Laaksonen *et al.* 2003). IL-18, in turn, is a macrophage-derived cytokine originally defined as a factor capable of inducing IFN-γ production in T cells (Okamura *et al.* 1995). Its effects *in vitro*, however, have been shown to depend on co-stimulatory factors, in particular of IL-12. In the presence of IL-12, IL-18 has been shown to enhance IFN-γ production in T cells and B cells, inhibit IgE-production, and prevent antigen-specific Th2-like cell development (Micallef *et al.* 1996; Yoshimoto *et al.* 1997; Hofstra *et al.* 1998). In the absence of IL-12, in contrast, IL-18 has been shown to induce IL-18 receptor alpha (IL-18Rα) expression on mast cells and basophils, and to stimulate IL-4 and IL-13 production, resulting in increased IgE levels (Yoshimoto *et al.* 1999). IL-12 mRNA expression has been shown to increase in macrophages in peripheral blood and target mucosa during SCIT (Hamid *et al.* 1997; Plewako *et al.* 2006b). Accordingly, increased IL-18 mRNA expression has been found in the PBMC of patients treated with SCIT (Savolainen *et al.* 2004).

Table 4. Changes in allergen-specific Th2/Th1 type cytokine responses during SIT (↑ increased response, ↓ decreased response, ↔ no change in response).

PERIPHERAL Th2 and Th1 type RESPONSES						
SIT regimen	**Observation period**	**Proliferation**	**Cells**	**Th2 type cytokine production**	**Th1 type cytokine production**	**Reference**
SCIT pollen	3 mo and 1 yr	↓ from 3 mo	short-term TCL[b]	% IL-4+ cells ↓ from 3 mo	% IFN-γ+ ↑ from 3 mo	Ebner *et al.* 1997
	6-7 yr continuous SIT or 3 yr post SIT (3-4 yr)[a]	↔ both groups	PBMC	IL-5 ↔ both groups	IFN-γ ↔ both groups	Till *et al.* 1997
	1. or 2. season	Blunted (1. & 2.)	short-term TCL	Blunting of seasonal increase in IL-4 (2. season)	Seasonal IFN-γ ↔	Giannarini & Maggi 1998
	3 mo	↔	short-term TCL	IL-4 and IL-5 ↔	IFN-γ ↔	Klimek *et al.* 1999a
	Up-dosing (2–4 wk), early MP[c] (~4 mo), and 1. season	↓ at 2-4 wk, then ↑ by MP to 1. season	PBMC	IL-4 and IL-5 ↓ at 2-4 wk, then recovered by MP, and at 1.season ↑ compared to untreated	IFN-γ ↔	Moverare *et al.* 2000
	1. season (4-5 mo)		PBMC	Blunting of seasonal increase in IL-4+ or IL-13+ cells	IFN-γ+ cells ↔	Gabrielsson *et al.* 2001
	2. season	↔	PBMC	IL-5 ↔	IFN-γ ↔	Wachholz *et al.* 2002
	1 yr	↔	PBMC	IL-4, IL-5, IL-13 ↔	IFN-γ ↔	Francis *et al.* 2003
	MP (<1 yr)		PBMC	IL-5 ↔	IFN-γ ↔	Francis *et al.* 2008
SCIT dust mite	3 and 18 mo	↓ at 3 mo but recovered at 18 mo	short-term TCL	IL-4 and IL-5 ↓ from 3 mo	IFN-γ ↑ from 3 mo	Oda *et al.* 1998
	>1.5 yr		short-term TCL	% IL-4+CD4+ and % IL-4+CD8+ cells ↓	% IFN-γ+ CD4+ or CD8+ cells ↔	Fu *et al.* 2003
	7–70 d	↓ from d 28	PBMC	IL-5 and IL-13 ↓ from d 28	IFN-γ ↓ from d 28	Jutel *et al.* 2003
	3 and 9 mo	↔	PBMC	% IL-4+CD4+ and % IL-4+CD8+ cells ↓ at 9 mo	% IFN-γ+ CD4+ or CD8+ cells ↔	Gardner *et al.* 2004a
	>6 mo (25.7±6.3 mo)		PBMC	IL-4 ↓	IFN-γ and IL-12 ↑	Tazaki *et al.* 2004
SCIT dust mite/ pollen	5 yr (1–17 yr)	↓ trend	CD8-depleted PBMC	IL-4 ↓	IL-2 and IFN-γ ↔	Secrist *et al.* 1993
	Up-dosing and MP (10-53 mo)		CD8-depleted PBMC	IL-4/IFN-γ ↑ during up-dosing and ↓ at MP		Benjaponpitak *et al.* 1999
SCIT animal	6 wk post SIT (6wk)		PBMC	IL-4 ↓ in high dose-treated	IFN-γ ↔	Pene *et al.* 1998
	3 mo (12–14 wk)		PBMC	IL-5 ↓, but IL-4 ↔	IFN-γ ↔	Meissner *et al.* 1999
	30 wk	↓	enriched CD4+	IL-13 ↓, but IL-5 ↔	IFN-γ ↔	Smith *et al.* 2004b
	12-14 wk	↓ (CD4+ and CD8+)	PBMC	IL-5 ↓	IFN-γ ↓ trend	Verhoef *et al.* 2005
	5 wk	↔	PBMC	IL-4 ↓ high dose-treated, IL-5 ↔	IFN-γ ↔	Lent *et al.* 2006
	3 wk and 3 mo		PBMC		IL-12+ monocytes ↑ at 3 mo, IFN-γ+ NK cells ↑ from 3 wk	Plewako *et al.* 2006b
SLIT pollen	Early MP and 1 yr	↓ (MP & 1 yr)	short-term TCL	IL-4 ↔	IFN-γ ↔	Fanta *et al.* 1999
	1 and 2 yr	↔	PBMC	IL-4 ↔	IFN-γ ↔	Rolinck-Weninghaus *et al.* 2005
	1 and 2 yr		PBMC	IL-5 ↑ at 1 and 2 yr, IL-4/IFN-γ ratio ↔ in low dose IL-5 ↔ and IL-4/IFN-γ ratio ↔ at 1 and 2 yr in high dose		Savolainen *et al.* 2006
	4 and 52 wk	↓ (4 & 52 wk)	PBMC	IL-4 ↓ from 4 wk	IFN-γ ↓ at 4 wk, but ↑ at wk 52	Bohle *et al.* 2007
	3 mo		PBMC		IFN-γ+ cells ↑	Ciprandi *et al.* 2008a

Table 4 continues…

PERIPHERAL Th2 and Th1 type RESPONSES						
SIT regimen	**Observation period**	**Proliferation**	**Cells**	**Th2 type cytokine production**	**Th1 type cytokine production**	**Reference**
SLIT dust mite	6 mo (& after 22–24 mo)	↓	PBMC (short-term TCL)	IL-4 ↔	IFN-γ ↑ (IFN-γ also ↑ after 22-24 mo) (% IFN-γ+CD4+ cells ↑ after 22-24 mo)	Cosmi *et al.* 2006
SLIT dust mite/ pollen	Up-dosing (2 wk) and early MP (8 wk)	↔	PBMC	IL-4 ↔ %IL-4+CD4+ cells ↔	IFN-γ ↔ % IFN-γ+CD8+ cells ↔, %IL-2+ CD4+ or CD8+ cells ↔	Dehlink *et al.* 2006
VIT	1–50 d	↓ (d 50)	PBMC	IL-4 ↓ from d 7, IL-5 ↓ from d 21	IFN-γ ↑ from d 7	Jutel *et al.* 1995
	3 wk - >6 mo conventional	↓ at 9 mo	PBMC	IL-4 ↓ from 2-3 mo	IFN-γ ↑ at 2-6 mo, then recover	McHugh *et al.* 1995
	1 d - >3 mo rush	↑ at 1 mo, then ↓ from 3 mo	PBMC	IL-4 ↓ at d 1, then recovered at 1 wk - 2mo, then again ↓ at >3 mo	IFN-γ ↑ from 2 mo	
	2 mo	↓	PBMC	IL-4, IL-5 and IL-13 ↓	IL-2 and IFN-γ ↓	Akdis *et al.* 1996
	15 and 90 d	↓ from d 15	enriched CD3+	No. IL-4+ cells ↓ from d 15	No. IFN-γ+ cells ↑ from d 15	Akoum *et al.* 1996
	1wk	↓	PBMC	IL-4 ↓	IFN-γ ↑	Bellinghausen *et al.* 1997
	14 wk or >3 yr	↔ in 14 wk group ↓ in >3 yr group	short-term TCL	IL-4 ↔ in both groups IL-4/IFN-γ ↔ in 14 wk group, but ↓ in >3 yr group	IFN-γ ↔ in patients treated for 14 wk, but ↑ in patients treated for >3yr	Kämmerer *et al.* 1997
	1 - 28 d	↓ from d 7	PBMC	IL-5 and IL-13 ↓ from d 7	IFN-γ ↓ from d 7	Akdis *et al.* 1998
	8 and 16 wk		short-term TCL	IL-4 and IL-13 ↓ from 8 wk, but IL-5 ↔	IFN-γ ↑ from 8 wk	Faith *et al.* 2003
	7 - 80 d	↑ at d 14, then ↓ by d 80	short-term TCL	IL-4 ↔	IFN-γ ↑ (at d 42)	Fellrath *et al.* 2003
	12 wk	↓	PBMC	IL-13 ↓	IFN-γ ↓	Tarzi *et al.* 2006
LOCAL Th2 and Th1 type RESPONSES						
SIT regimen	**Observation period**	**Tissue**	**No. of Th2 or Th1 type cells**			**Reference**
SCIT pollen	9 mo	Skin	Th2 cytokine (IL-4, IL-5) mRNA+ cells ↔, IL-2 mRNA+ and IFN-γ mRNA+ cells ↑ in 50% of patients			Varney *et al.* 1993
	1 yr	Nose	Th2 cytokine (IL-4, IL-5) mRNA+ cells ↓ trend, IFN-γ mRNA+ cells ↑			Durham *et al.* 1996
	4 yr	Skin	IL-12 mRNA+ cells ↑			Hamid *et al.* 1997
	3 yr post-treatment	Skin	Sustained decrease in IL-4 mRNA+ cells			Durham *et al.* 1999b
	3 mo	Nose	IL-5 ↓ and IFN-γ ↑ in nasal secretions			Klimek *et al.* 1999a
	2 yr (2. season)	Nose	Trend for blunting of seasonal increase in IL-5 mRNA+ cells			Wilson *et al.* 2001a
	2 yr (2. season)	Nose	Blunting of seasonal increase in IL-5 mRNA+ cells, significant increase in IFN-γ mRNA+ cells,			Wachholz *et al.* 2002
	2 yr (2. season)	Nose	Blunting of seasonal increase in IL-9 mRNA+ cells			Nouri-Aria *et al.* 2005
SCIT animal	4 wk	Skin	IL-4 or IL-5 mRNA+ cells ↔, IFN-γ mRNA+ cells ↑			Alexander *et al.* 2005
SLIT pollen	18 mo	Sublingual mucosa	IL-12 mRNA+ cells ↔			Lima *et al.* 2002
VIT	3 mo VIT	Skin	IL-4 mRNA+ cells ↓, IL-13 mRNA+ cells ↔, IFN-γ or IL-12 mRNA+ cells ↔			Nasser *et al.* 2001

[a] duration of treatment, [b] TCL, allergen-specific T cell line, [c] MP, mantenance phase

Another mechanism by which SIT has been suggested to induce immune deviation in allergen-specific Th2/Th1 balance is by inducing selective apoptosis of memory Th2 lymphocytes (CD45RO+ CD4+IL-4+ cells) thereby increasing the proportion of allergen-specific IFN-γ+ cells (Guerra *et al.* 2001). Nearly 40% of the Th2 cells expressing IL-4 obtained from SCIT-treated patients have been shown to undergo apoptosis when exposed to specific allergen - a phenomenon which has not been found in the untreated allergic controls (Guerra *et al.* 2001). This concords with the findings made in mite-sensitive patients with asthma, and in pollen allergic patients suffering from allergic rhinitis, in whom hardly any allergen-specific CD4+ T cell clones could be established after 3 months of SCIT, and the ones that were established after 1 year or 18 months of the treatment, had shifted their cytokine production pattern to a Th1 or Th0 type (Oda *et al.* 1998; Ebner *et al.* 1997). It has been reported that very high allergen concentrations induce T cell deletion (Critchfield *et al.* 1994; Gardner *et al.* 2004b). SCIT has also been shown to induce apoptosis of monocytes in an allergen-specific manner, thereby affecting the capacity of these cells to present allergens to specific T cells (Monteseirin *et al.* 2003). Yet another mechanism by which SCIT has been suggested to influence on allergen-specific T effector cell responses is by modulation of co-stimulatory molecule expression on T cells, rendering them less susceptible to the activation by APCs (Plewako *et al.* 2004; Piconi *et al.* 2007).

2.3.4 Antibody isotype switch and induction of serum-blocking activity

The third main category of immunological changes induced against the allergens during SIT is the modulation of specific antibody responses (Moingeon *et al*. 2006). During SCIT, there is usually an initial rise in serum allergen-specific IgE concentration during the up-dosing and early maintenance phases, which then progressively declines over a period of months to years to the same or lower level compared to baseline (Jutel *et al*. 2006; Lichtenstein *et al*. 1973; Geich *et al*. 1982; Francis *et al*. 2008). In pollen allergic patients, SCIT has also been shown to result in blunting of the usual seasonal increases in serum allergen-specific IgE (Lichtenstein *et al*. 1973; Gleich *et al*. 1982; Nouri-Aria *et al*. 2004; Keskin *et al*. 2006). Accordingly, SLIT for grass pollen allergens has been shown to result in an initial rise in serum specific IgE levels, which thereafter have been shown to decrease over a period of several months back toward the baseline (Lima *et al*. 2006; Dahl *et al*. 2008). SLIT, however, appears not to be able to blunt the usual seasonal increases in serum allergen-specific IgE in pollen allergic patients, but rather, has been shown to result in a diminished expression of allergen-specific IgE in the nasal secretions, and this effect has been shown to depend on the treatment dose, favoring high allergen dose treatment (Marcucci *et al*. 2005a).

Both SCIT and SLIT have been shown to result in a significant and sustained increase in serum allergen-specific IgG concentration, in particular IgG4, and this effect has also been

shown to be treatment dose dependent (Nouri-Aria *et al*. 2004; Keskin *et al*. 2006; Rossi *et al*. 2007; Francis *et al*. 2008; Lue *et al*. 2006; Lima *et al*. 2006; Dahl *et al*. 2008). It has been demonstrated that allergen-specific IgG antibodies begin to increase early, within 7-12 weeks after the initiation of SCIT (Jutel *et al*. 2003; Keskin *et al*. 2006; Francis *et al*. 2008). Some studies have shown that the dominant immunoglobulin type during the early course of SCIT is IgG1, whereas IgG4 begins to appear in significant quantities after prolonged immunization (Peng *et al*. 1992; McHugh *et al*. 1990). However, some studies have found simultaneous increases in IgG1 and IgG4 during the early phase of SCIT (Jutel *et al*. 2003; Keskin *et al*. 2006). Modest increases in serum allergen-specific IgA, in particular IgA2, levels, have also been found during SCIT for pollens (Pilette *et al*. 2007; Francis *et al*. 2008) and dust mite (Jutel *et al*. 2003; Fu *et al*. 2003), as well as after SLIT for dust mite (Bahceciler *et al*. 2005). The increase in allergen-specific IgA response during SCIT has been shown to coincidence with the induction of specific IgG responses (Jutel *et al*. 2003; Francis*et al*. 2008). In addition, increased levels of IgG and IgA have been found in the nasal secretions after pollen SCIT (Platts-Mills *et al*. 1976). The increases in allergen-specific IgG4 and IgA responses during SIT have been shown to relate with the associated increases in IL-10 and TGF-β production (Akdis *et al*. 1998; Jutel *et al*. 2003). Addition of IL-10-producing Treg cells to B cell cultures has been shown to result in the preferential production of IgG4 (Satoguina *et al*. 2005), and the IgA-containing serum fraction of SCIT-treated patients has been shown to correlate with local TGF-β production and induce IL-10 production in monocytes (Pilette *et al*. 2007). A kinetic study has revealed that the allergen-induced IL-10 production by PBMC precedes that of IgG4 increase in serum during SCIT, suggesting that the early IL-10 responses may be required for the induction of IgG4 synthesis in B cells (Francis *et al*. 2008).

During dust mite SCIT, significant increases in allergen-specific IgA, IgG4 and IgG1 were found just 70 days after the start of the treatment, with no change in allergen-specific IgE (Jutel *et al*. 2003). Accordingly, during grass pollen SLIT, increases in specific IgG4 and IgG1 were seen already 90 days after the initiation of the treatment without any consistent change in the allergen-specific IgE throughout the 1.5 years observation period (Pfaar & Klimek 2008). The changes in allergen-specific IgE levels during SIT have thus suggested to be hardly explained by the diminished clinical responsiveness to allergen since the decrease in serum IgE is late, relatively small and does not correlate with clinical improvement (Jutel *et al*. 2006).

SIT has also been shown to induce serum-blocking activity, mediated by allergen-specific IgG, but not either IgA or IgM (van Neerven *et al*. 1999; Pillette *et al*. 2007). This activity has been observed both after grass pollen (van Neerven *et al*. 1999; Wachholz *et al*. 2003; Pillette *et al*. 2007; Francis *et al*. 2008) and house dust mite SCIT (Schubert *et al*. 2009), as well as after grass pollen SLIT (Dahl *et al*. 2008). The IgG-containing serum of SCIT-

treated patients has been shown to inhibit the formation of allergen-IgE complexes, and thereby, to prevent allergen-IgE binding to CD23+ B cells and subsequent allergen-presentation to T cells, resulting in the inhibition of proliferation and cytokine (IL-4, IL-5, IFN-γ and IL-10) secretion by allergen-specific T cells (van Neerven *et al*. 1999; Wachholz *et al*. 2003). The IgG-containing serum of SCIT-treated patients has also been shown to inhibit allergen-induced histamine release from basophils (Francis *et al*. 2008; Schubert *et al*. 2009) and mast cells in an allergen-specific manner (Platts-Mills *et al*. 1976; Lambin *et al*. 1993; Garcia *et al*. 1993; Visco *et al*. 1996). The serum blocking activity has been shown to be induced early, within 6 to 12 weeks after the initiation of SCIT (Francis *et al*. 2008; Schubert *et al*. 2009), and to occur earlier when using the clustered schedule as compared to the usage of conventional up-dosing schedule (Schubert *et al*. 2009). It has been demonstrated that the serum-blocking activity persists for up to 2 years after the end of SCIT, and correlates with the sustained remission in clinical symptoms (Wilcock *et al*. 2005). A summary of the clinical and immunological changes associated with SIT is presented in Figure 3.

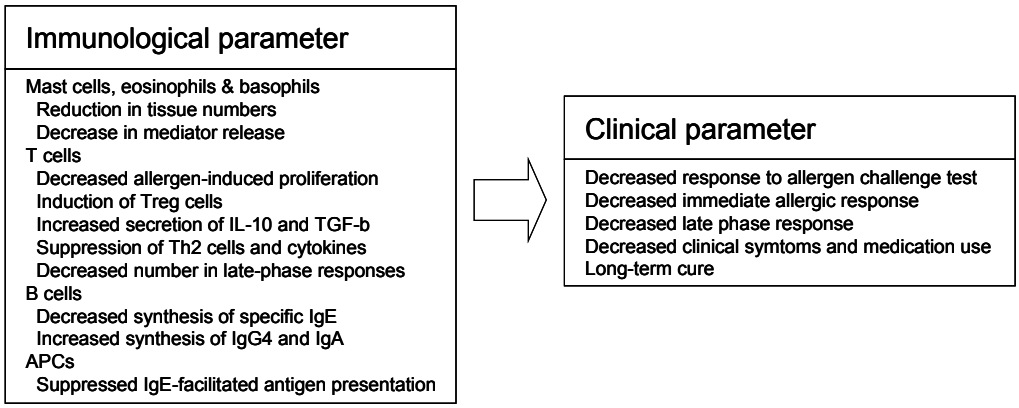

Figure **3**. A summary of clinical and immunological changes associated with SIT (modified from Jutel *et al*. 2006).

2.4 Th17 type immune responses in allergy

2.4.1 The Th17 subset

Th17 cells represent the newest subset of CD4+ T lymphocytes identified in humans. These cells have been named according to their predominant secretion of IL-17 (IL-17A), and they have been further characterized by the expression of transcription factor retinoic acid-related orphan nuclear receptor γt (RORγt), which is termed RORC, variant 2, in humans (Wilson *et al.* 2007; Acosta-Rodriquez *et al.* 2007; Annunziato *et al.* 2007). ROCγt/RORC is required for the lineage commitment of Th17 cells (Ivanov *et al.* 2006). The Th17 cells are contained within the CD45RO+ pool of peripheral CD4+ T

lymphocytes, and typically comprise less than 1% of peripheral CD4+ T lymphocytes (Annunziato *et al.* 2007). However, increased frequencies have been identified in the disease affected tissues of patients with chronic inflammatory diseases, such as reactive arthritis, psoriasis and Crohn's disease (Infante-Duarte *et al.* 2000; Park *et al.* 2005; Annunziato *et al.* 2007).

The development of Th17 cells is dependent on TGF-β in the context of inflammatory cytokines such as IL-1β, IL-6 and IL-23 (Korn *et al.* 2009). These cytokines are produced by activated APCs, such as macrophages and dendritic cells following activation. TGF-β is necessary for the induction of RORC, and the cytokines IL-1β, IL-6 and IL-23 induce IL-17 expression in naïve (CD45RA+) T cells (Manel *et al.* 2008; Volpe *et al.* 2008; Burgler *et al.* 2009). IL-23 has also been suggested to be required for the maintenance of Th17 cells (Korn *et al.* 2009). IL-23 is a heterodimeric cytokine composed of p40 subunit in common with IL-12 and a unique IL-23p19 subunit (Oppmann *et al.* 2000). In humans, IL-23, IL-1β and IL-6 have been shown to enhance IL-17 production by the *in vitro* polyclonally activated CD45RO+ memory Th17 cells, but not to induce their proliferation (Wilson *et al.* 2007; Annunziato *et al.* 2007, Liu & Rohowsky-Kochan 2008).

The development of Th17 cells is inhibited by both Th1 and Th2 type cytokines (Wilson *et al.* 2007), whereas these cytokines appear not to have any immunomodulatory effect on the IL-17 production by polyclonally activated CD45RO+ memory Th17 cells (Liu & Rohowsky-Kochan 2008). In mice, the development of Th17 cells is prevented by IL-27, another novel member of the IL-12 cytokine family derived from activated APCs (Kastelein *et al.* 2007). This cytokine consists of an IL-12p40-related subunit known as Epstein-Barr virus–induced molecule 3 (EBI3) and a p28 subunit, and has been shown to favour Th1 polarization (Pflanz *et al.* 2002, Yoshimura *et al.* 2006).

The requirement of TGF-β for differentiation suggests a reciprocal relationship between the Th17 and Treg cells (Korn *et al.* 2009). Recent data suggest also the capacity of Treg cells to convert into IL-17-producing Th17 cells under proinflammatory conditions (Voo *et al.* 2009; Beriou *et al.* 2009). The Th17 cells have been shown to be able to induce IgM, IgG and IgA, but not IgE synthesis in B cells, but to display low cytotoxic capability as compared to Th1 cells, as well as to resist suppression by autologous Treg cells (Annunziato *et al.* 2007). In addition, Th17 cells have been shown to act on bronchial epithelial cells to induce proinflammatory cytokine expression (Burgler *et al.* 2009). A substantial proportion of the *in vitro* generated or *ex vivo*-derived memory Th17 cells have been shown co-produce IFN-γ or TNF-α (Acosta-Rodriquez *et al.* 2007; Annunziato *et al.* 2007; Wilson *et al.* 2007; Liu & Rohowsky-Kochan 2008).

2.4.2 Interleukin-17 (IL-17)

IL-17 (IL-17A) is a homodimeric, proinflammatory cytokine of 155 amino acids, and the founding member of IL-17 cytokine family, which now includes six members (IL-17A-F) (Fozziez *et al.* 1996; Korn *et al.* 2009). IL-17 was originally described as a cytokine produced by murine cytotoxic T cells and called CTLA-8 (Rouvier *et al.* 1993). In humans, the primary source of IL-17 was found to be the activated peripheral CD4+ CD45RO+ memory T cells, which were later defined as Th17 cells (Yao *et al.* 1995; Fozziez *et al.* 1996). Nowadays, it has been demonstrated that other leukocyte types, including CD8+ T cells (Shin *et al.* 1998), natural killer T (NKT) cells (Rachitskaya *et al.* 2008), eosinophils (Molet *et al.* 2001), and macrophages (Song *et al.* 2008) are also capable of expressing IL-17, but so far no production by stromal cell types has been found.

The receptor for IL-17 (IL-17R or IL-17AR) is ubiquitously expressed in tissues (Yao *et al.* 1997). Accordingly to this, IL-17 has been shown to act on a wide variety of stromal cell types, including epithelial cells, vascular endothelial cells, keratinocytes and fibroblasts to induce the secretion of proinflammatory cytokines such as IL-6 and IL-11, growth factors, including growth-related oncogene α (Groα, also known as CXCL1) and granulocyte colony-stimulating factor (G-CSF), adhesion molecules, including ICAM-1, and chemokines, in particular IL-8/CXCL8 that promote the recruitment and generation of neutrophils both *in vitro* and *in vivo* (Figure 4) (Yao *et al.* 1995; Yao *et al.* 1997; Fozziez *et al.* 1996; Molet *et al.* 2001; Kawaguchi *et al.* 2001; Jones & Chan 2002; Laan *et al.* 1999; Schwarzenberger *et al.* 1998). IL-17 has also been shown to act on leucocytes, including macrophages and eosinophils to induce the secretion of proinflammatory cytokines, as well as to promote the chemotaxis and survival of macrophages *in vitro* (Jovanovic *et al.* 1998; Sergejeva *et al.* 2005; Cheung *et al.* 2008). On the other hand, IL-17 has also been shown to suppress TNF-α- or rhinovirus-induced eosinophil-chemokine RANTES/CCL5 expression on bronchial epithelial cells and fibroblasts, and to inhibit TNF-α-induced Th2-chemokine TARC/CCL17 expression and antigen-uptake by dendritic cells (Andoh *et al.* 2002; Schnyder *et al.* 2005; Wiehler *et al.* 2007), suggesting pleiotropic immunoregulatory functions.

Several microbial stimuli, including bacterial lipopeptides, and pertussis toxin have been shown to induce the production of IL-17 from naïve CD4+ T lymphocytes (Infante-Duarte *et al.* 2000; Chen *et al.* 2007), indicating the primary involvement of Th17 type immune responses in antimicrobial defence. Also, mice deficient in IL-17R have been shown to have impaired host defence due to the reduced G-CSF and macrophage inflammatory protein 2 (MIP-2) production, and consequently diminished neutrophil recruitment (Ye *et al.* 2001).

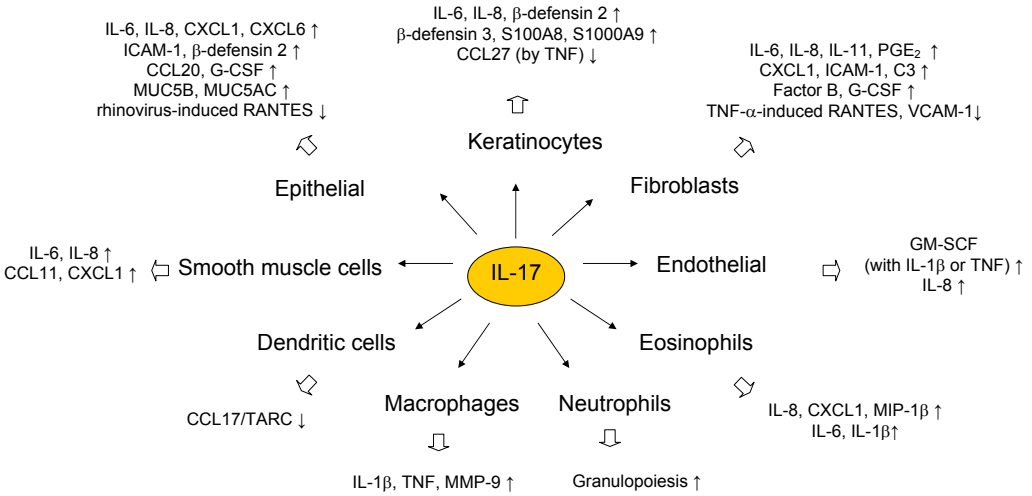

Figure 4. Biological activities of IL-17 (modified from Oboki *et al.* 2008).

2.4.3 Role of IL-17/Th17 cells in allergic inflammation

2.4.3.1 Th17 type immune responses in murine models of allergic asthma

Th17 type immune responses are required for allergen-specific sensitization, as mice deficient in either IL-17 or IL-17R have impaired allergen-specific cellular and humoral responses, including reduced Th2 cytokine production and IgE synthesis (Nakae *et al*. 2001; Schnyder-Candrian *et al*. 2006). Epicutaneous sensitization in mice has been shown to result in cutaneous expression of IL-17, in increased serum IL-17 concentration, and to elicit IL-17-secreting CD4+ T cells in the draining lymph nodes and spleen (He *et al*. 2007). IL-17 and IL-23 expression, and the numbers of IL-17+CD4+ T cells, have been shown to increase in the lungs of sensitized mice after allergen challenge (Hellings *et al*. 2003; Schnyder-Candrian *et al*. 2006; He *et al*. 2007; Wakashin *et al*. 2008). The increased infiltration of IL-17+CD4+ T lymphocytes to the lungs allergen-challenged sensitized mice has been shown to be associated with a significant increase in the BAL fluid neutrophils and with enhanced airway hyperreactivity (AHR) (He *et al*. 2007; Wakashin *et al*. 2008). Neutralization of IL-23 prior to the allergen-challenge has been found to result in a reduced allergen-induced eosinophil and lymphocyte recruitment and in decreased Th2 cytokine production in the airways (Wakashin *et al*. 2008), indicative of the role of Th17 type immune responses in the enhancement of Th2-mediated allergic immune reactions. Furthermore, adoptive co-transfer of allergen-specific Th17 cells with the Th2 lymphocytes to the airways of sensitized mice has been shown to potentiate allergen-induced Th2 type inflammatory responses, resulting, for example, in increased recruitment of eosinophils and augmented AHR (Wakashin *et al*. 2008). On the other hand, in mice with already established asthma, neutralization of IL-17 shortly prior to the allergen provocation has been found to

result in a greater bronchial eosinophilia, and in elevated expression of Th2 cytokines in the lungs (Hellings *et al*. 2003). Accordingly, in another study, administration of IL-17 during the allergen challenge was found to result in a inhibition of eosinophil and lymphocyte recruitment to the mouse airways and to prevent mucus hypersecretion and hyperplasia of goblet cells, and these responses were reversed by the neutralization of IL-17 (Schnyder-Candrian *et al*. 2006). However, administration of neutralizing IL-17 antibodies to the mice 4-7 days prior to the allergen challenge was found to be ineffective in preventing the recruitment of eosinophils to the airways or in inhibiting the Th2 type cytokine production in lungs (He *et al*. 2007), but the administration of neutralizing antibodies repeatedly during the inhalation challenge was found to result in an extravagated lung IL-5 and IL-4 expression, and in increased peripheral blood and bone marrow, but not bronchial, eosinophilia (Hellings *et al*. 2003).

2.4.3.2 Th17 type immune responses in human allergy

Increased numbers of IL-17-expressing T cells and increased levels of either IL-17 protein or mRNA have been found in the airways of patients with asthma (Molet *et al*. 2001; Chakir *et al*. 2003; Bullens *et al*. 2006). In patients with severe allergic asthma, up to 20% of the infiltrating T cells were found to be IL-17+CD4+ T lymphocytes during acute exacerbation (Pene *et al*. 2008). The levels of IL-17 in the sputum of asthma patients have been shown to correlate with AHR (Barczyk *et al*. 2003), and with the disease severity (Chakir *et al*. 2003), although not in all studies significant differences between the mild or moderate-to-severe asthmatics, or between atopic and non-atopic asthmatics with regard of the bronchial IL-17 expression have been found (Bullens *et al*. 2006). Allergic asthma patients have also been reported to have higher plasma levels of IL-17 as compared with healthy (Wong *et al*. 2001), and their PBMC have been shown to produce higher amounts of IL-17 in response to stimulation with the specific allergen (Hashimoto et al. 2005). However, some studies have reported normal serum IL-17 levels in the patients with asthma, and normal spontaneous IL-17 production by their PBMC (Lei *et al*. 2008). In lungs, the expression of IL-17 was found to correlate with CD3γ expression, and with IL-5 and IL-8/CXCL8 mRNAs (Bullens *et al*. 2006). Both IL-17 and IL-8/CXCL8 responses were also found to correlate with sputum neutrophil count, but not with the numbers of eosinophils (Bullens *et al*. 2006).

Increased Th17 responses have also been found in patients with atopic dermatitis and allergic rhinitis (Toda *et al*. 2003; Koga *et al*. 2008; Ciprandi *et al*. 2008b; Ciprandi *et al*. 2009). In patients with atopic dermatitis, increased IL-17 expression and increased numbers of IL-17+CD4+ T cells were found in the disease-affected skin areas, with the responses being more associated with the acute skin lesions than with the chronic lesions, and to correlate with the disease severity (Toda *et al*. 2003; Koga *et al*. 2008). Increased frequencies of IL-17+CD4+ T cells have also been demonstrated in the

PBMC of atopic dermatitis patients but only in those with a severe disease (Koga *et al*. 2008). In allergic rhinitis patients too, increased serum IL-17 responses have been found more in association with the severe disease, as these patients often also have higher serum specific IgE level, lower allergen threshold dose during conjuctival challenge and a tendency toward a higher peripheral blood eosinophil count (Ciprandi *et al*. 2008b). During pollen season, a significant positive correlation was also found between the serum IL-17 levels, symptom severity scores, medication use and the peripheral blood eosinophil count in patients with severe allergic rhinitis (Ciprandi *et al*. 2009). The involvement of Th17 type immune responses in human allergy are summarized in Table 5.

Table 5. Th17 type immune responses associated with allergy in humans.

Human asthma		**Reference**
Sputum & BALF	Increased numbers of IL-17+ cells in sputum and BALF of asthmatic patients (6/11 atopic); eosinophils are IL-17+ in addition to T cells	Molet *et al.* 2001
Plasma	Trend for elevated plasma levels of IL-17 in atopic asthmatics	Wong *et al.* 2001
Bronchial biopsy	Increased levels of IL-17 in bronchial biopsies of atopic asthmatics; IL-17 levels correlate with disease severity	Chakir *et al.* 2003
Sputum	IL-17 levels in sputum correlate with airway hyperreactivity (AHR)	Barczyk *et al.* 2003
PBMC	Increased allergen-specific production of IL-17 by PBMC of atopic asthmatics	Hashimoto *et al.* 2005
Sputum	Increased expression of IL-17 in sputum; IL-17 correlates with CD3γ expression and with sputum IL-5, IL-8 and neutrophil count; no difference in IL-17 expression between mild or moderate-to-severe asthmatics, or between atopic and non-atopic astmatics	Bullens *et al.* 2006
Serum	Normal serum levels of IL-17 in patients with allergic asthma, non-allergic asthma, or COPD	Lei *et al.* 2008
Bronchial biopsy	IL-17+CD4+ T cells constitute ~20% of infiltrating CD4+ T lymphocytes in patients with severe asthma during acute episode (atopy status not stated)	Pene *et al.* 2008
BALF	Increased numbers of IL-17-producing macrophages and increased level of IL-17 in BALF of allergic asthmatics	Song *et al.* 2008
Human allergic rhinitis		
Nasal secretion	Increased levels of IL-17 in viral-triggered rhinitis, but not allergic rhinitis	Klemens *et al.* 2007
Serum	Increased serum levels of IL-17 in birch-monosensitized patients outside season; elevated IL-17 responses associate with higher serum total and specific IgE levels, lower allergen threshold dose during conjunctival challenge and with a trend toward higher eosinophil counts	Ciprandi *et al.* 2008b
Serum	IL-17 levels in serum correlate with seasonal symptom severity scores, medication use and peripheral eosinophil count in patients with persistent moderate-to-severe allergic rhinitis	Ciprandi *et al.* 2009
Human atopic dermatitis		
Skin biopsy	IL-17 expression increased in acute but not chronic skin lesions	Toda *et al.* 2003
Skin biopsy/ peripheral blood	Increased frequencies of Th17 cells in peripheral blood of patients with atopic dermatitis; IL-17+ cells are infiltrated markedly more in acute than chronic lesions	Koga *et al.* 2008

3 AIMS OF THE STUDY

Successful treatment with SCIT and SLIT has been shown to result in long-term remission in allergy symptoms and medication use in allergic rhinitis patients, as well as to prevent disease progression to asthma. The immune deviation from Th2 to Th1 type response in allergen-specific T cells, and induction of tolerogenic Treg cells have been suggested as the two key mechanisms behind these beneficial effects, but little is known of the kinetics of these responses in PBMC during the course of the treatments. Also, no immune marker associated with these immune responses and the clinical efficacy has been established that could be used to monitor the development of clinical response in individual patients during these modalities. In addition, nothing is known of the modulation of Th17 type immune responses, the newly described subset of CD4+ T cells in humans with implications in the pathogenesis of allergic inflammation, during SCIT or SLIT. The aim of this work was to clarify the immunological mechanisms associated with SCIT and SLIT and their clinical efficacy by investigating the *in vitro* specific allergen induced immune responses in peripheral blood mononuclear cells (PBMC) of allergic rhinitis patients during these treatments.

The specific aims of this study were:

1. to investigate allergen-specific Th2/Th1 balance during SLIT by simultaneous analysis of GATA-3, SLAM and IL-18 mRNA expression, and further correlate these parameters with clinical outcome (**I**)

2. to investigate allergen-specific Th2/Th1 balance and Treg responses during SCIT by simultaneous analysis of IL-4, IL-5, IFN-γ, SLAM, IL-18 and IL-10 mRNA expression, and further correlate these parameters with clinical outcome (**II**)

3. to investigate allergen-specific Treg and Th17 responses during SLIT by simultaneous analysis of FOXP3, IL-17, IL-23, and IL-27 mRNA expression, and further correlate these parameters with clinical outcome (**III**).

4. to investigate allergen-specific Th17 responses during SCIT by simultaneous analysis of IL-17 and RORC mRNA expression, and further correlate these parameters with Th2 and Th1 responses, specific antibody (IgE, IgG4) production, and clinical outcome (**IV**).

4 MATERIALS AND METHODS

4.1 Subjects

A total of 80 subjects were included in this work (Figure 5). Seventy of the study subjects had skin-prick test verified pollen allergy, elevated serum allergen-specific IgE, and history of allergic symptoms. Forty of the allergic subjects were adults, aged between 16-44 years, and thirty children, aged between 5-15 years. In addition, ten healthy adults, aged between 25-45 years were included as non-allergic controls in the SCIT studies **II** and **IV**. The study design and patient characteristics are presented in Figure 5. The patients in the SCIT studies **II** and **IV** were enrolled from the outpatient clinic of Department of Pulmonary Diseases and Clinical Allergology, Turku University Central Hospital. The patients in SLIT studies **I** and **III** were randomly selected from a single-centre, randomized, double-blind, placebo-controlled dose response phase II trial as earlier described (Valovirta *et al.* 2006). The studies were approved by local ethics committees (Turku University or Turku Allergy Centre), and performed with the patients' written informed consent.

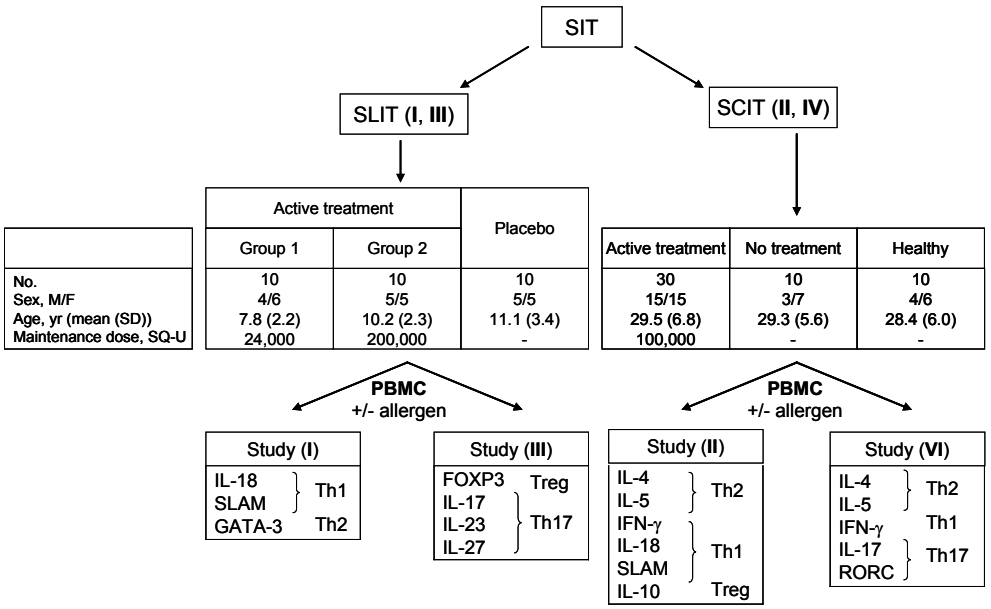

Figure 5. Study design and patient characteristics. A total of 80 subjects were included, seventy of them having clinically documented allergic rhinitis due to tree or grass pollens. Thirty of these allergic subjects were children and randomized to receive sublingual immunotherapy (SLIT) for up to 2 years either with low allergen dose (group 1, weekly cumulative maintenance dose 24,000 SQ-U, n=10), high allergen dose (group 2, weekly cumulative maintenance dose 200,000 SQ-U, n=10), or placebo (n=10). The remaining forty allergic patients were adults and treated either with subcutaneous immunotherapy (SCIT) (monthly maintenance dose 100,000 SQ-U, n=30), or pharmacotherapy only (n=10) for up to 3 years. Ten healthy adults served as non-allergic controls for the baseline analysis in SCIT studies. The cytokines and immune markers investigated in SLIT (I, III) and SCIT (II, IV) studies are displayed in the bottom panel of the figure.

4.2 Specific immunotherapy

4.2.1 Subcutaneous immunotherapy

Specific immunotherapy was conducted either by using the subcutaneous route (SCIT) or according to the sublingual-swallow method (SLIT). SCIT was performed in 30 adult allergic rhinitis patients, 18 of whom were treated according to the conventional up-dosing schedule and 12 by using the clustered rush protocol. The up-dosing schemes are presented in Table 6. The up-dosing extract in the clustered rush SCIT regimen was a water-soluble extract of birch (*Betula verrucosa*) or timothy grass (*Phleum pratense*) pollen (Aquagen SQ; ALK-Abelló, Hørsholm, Denmark). The up-dosing extract in the conventional SCIT regimen was an aluminum hydroxide depot of birch (*B. verrucosa*) and/or timothy grass (*P. pratense*) pollen (Alutard SQ; ALK-Abelló). Total of 18 subjects were treated with birch, 6 with timothy and 6 with a combination of these two allergens. The maintenance extract in both SCIT regimens was Alutard SQ, given at 6-week intervals with dose 100,000 SQ-U for up to 3 years. The maintenance doses corresponded to 20 µg of major allergen of timothy grass pollen (*Phl p*5), and 15 µg of major allergen of birch pollen allergen (*Bet v*1).

Table 6. Up-dosing schedules used in SCIT studies **II** and **IV**.

	Conventional	Clustered rush
Number of subjects	18	12
Allergen extract	Alutard SQ	Aquagen SQ
Birch	9	9
Timothy	3	3
Birch & Timothy	6	-
Dosage per visit (SQ-U)		
Week 1	20	10-50-100-300
Week 2	40	500-800-1000-2000
Week 3	80	4000-6000-8000-10,000
Week 4	200	10,000-20,000-40,000
Week 5	400	50,000-50,000
Week 6	800	100,000*
Week 7	2000	
Week 8	4000	
Week 9	8000	
Week 10	10,000	
Week 11	20,000	
Week 12	40,000	
Week 13	60,000	
Week 14	80,000	
Week 15	100,000*	

* maintenance dose, continued at 6-week intervals for up 3 years (modified from Laaksonen *et al.* 2003)

4.2.2 Sublingual immunotherapy

The allergen preparation in the SLIT studies (**I** and **III**) was a glycerinated mixture of SQ-standardized extracts of birch (*B. verrucosa*), hazel (*Corylus avellana*) and alder (*Alnus glutinosa*) tree pollens (ALK-Abelló). The patients were randomized in three equal-sized groups (n=10 in each): the groups 1 and 2 received active treatment and placebo group a diluent containing 50% glycerol and 50% saline buffer. The up-dosing was conducted within a 5-week period involving five weekly doses (Table 7). The maintenance dose was 4,800 SQ-U for group 1 and 40,000 SQ-U for group 2, given five times a week. The cumulative weekly dose in groups 1 and 2 thus corresponded to 3.6 μg and 30 μg of major allergen *Bet v* 1/*Aln g* 1/*Cor a* 1, respectively. Each dose was kept under tongue for 3 min and then swallowed. SLIT continued for up 18 months.

Table 7. Up-dosing schemes used in SLIT studies **I** and **III**.

	Group 1	Group 2
Number of subjects	10	10
Dosage per day (SQ-U)		
Week 1	60	600
Week 2	160	1600
Week 3	600	4800
Week 4	1600	14,000
Week 5	4800*	40,000*

* maintenance dose, continued with 5 weekly doses for up 18 months (Valovirta *et al.* 2006)

4.3 Clinical evaluation

4.3.1 SCIT

Symptoms and medication scores: Allergy symptoms and the use of medication were scored post-seasonally prior to the commencement of SIT and thereafter after one, two and three years of therapy, and adjusted to pollen counts. The form included visual analogue scale (VAS) of nine different symptoms (conjunctival itching, erythema and discharge; nasal itching, blockage and discharge, nocturnal, daytime and cough induced by exercise). Medication was scored on scale 0-5 [not at all (0), occasionally seldom (1), occasionally often (2), almost daily (3), continuously (4), continuously with the maximal dose (5)] separately for peroral antihistamines, topical nasal steroids and eyedrops (cromones or antihistamine). The mean VAS of the nine different symptoms was calculated as percentage units of the maximal VAS score to represent seasonal symptoms. The natural logarithms of the mean seasonal pollen counts were calculated to represent seasonal pollen exposure (Winther *et al.* 2000). The percentage unit score of VAS symptoms was adjusted to pollen exposure by multiplication with the ratio of the natural logarithms of the longtime and seasonal mean pollen counts. The mean medication scores were calculated as percentage units of maximal medication scores

and adjusted to the pollen counts as described for the VAS scores. A combined symptom medication score (CSMS) was obtained adding together the mean VAS and medication scores.

Pollen counts: Pollen data originates from the monitoring site in Turku, Finland. Sampling and analysis were carried out according to the European standards (Mandrioli *et al.* 1998). Pollen samples were taken with a Burkard spore trap (Burkard Manufacturing Ltd, Rickmansworth, Hertfordshire, UK). The trap was situated on the roof of a university building, 18 meters above the ground. All pollen counts were analyzed daily. The pollen season was 90% of the pollen total. Pollen counts were expressed as number of pollen grains / m3 of air and the mean seasonal count was used as a representative value for the season. High sampling site was representative for pollen types with high source as for instance birch trees, but grass pollen counts were often underestimated, because their source was at a low level and most pollen grains were settled close to the source area (counts 4.4 times higher at the ground level than on the roof) (Rantio-Lehtimäki *et al.* 1991).

Validation of the visual analogue scoring form: For validation of the visual analogue scoring form, 20 allergic rhinitis patients filled a diary card for 7 weeks during birch pollen season. The severity of eye, nose and lung symptoms (0-3 points) and the use of medication were reported daily. The mean VAS of symptoms during one week was evaluated at the end of each week. Post-seasonally, after five to six months, without any earlier notice, the same patients were invited to fill the visual analogue scoring form used in this study to register their seasonal symptoms and medication in retrospect. To compare the results from these two forms, the results were calculated as percentage units of the maximal VAS or medication score.

4.3.2 SLIT

Patient Diary: Symptoms and medications were registered in a patient diary as earlier described (Valovirta *et al.* 2006). The children filled in their diary during the tree pollen season for 12 weeks. The following parameters were registered daily in the patient diary:

Symptom Scores: Allergic, clinical symptoms were categorized as nose symptoms (runny nose, sneezing, blocked nose), eye symptoms (streaming and swelling, redness and itching) and lung symptoms (breathlessness, cough, wheeze and chest tightness). Each symptom was scored by the children: 0 = No symptoms, 1 = Slight symptoms, 2 = Moderate symptoms, and 3 = Severe symptoms.

Medication: The children were supplied with medication for hay fever and asthma symptoms. The daily medication score for each child was calculated as the sum of

medication administered at a particular day: cetirizine tablets (10 mg) - 2 points/tablet; cromolyn eyedrops (40 mg/ml) – 1 point/drop; cromolyn nasal spray (5.2 mg/dose), terbutaline inhalation (0.25 mg/dose) and salbutamol inhalation (0.2 mg/dose) – 1 point/puff; budesonide inhalation (200 µg/dose) and fluticasone propionate inhalation (100 µg/dose) – 4 points/puff and 80 points per course of prednisolone (5 mg/tablet).

Combined symptom medication score: The symptom and medication scores were averaged over the 12-week tree pollen season for each child and a combined symptom medication score (SMS) was obtained by adding together the two scores.

Post-SLIT asthma symptoms: A telephone survey for the assessment of asthmatic symptoms and the use of asthma medication among the study children was conducted five years after the commencement of SLIT as described earlier (Savolainen *et al.* 2006). The survey listed the possible use of any asthma medication during the past 12 months, including the latest prescribed medication and dosage, use of beta-agonists during the last two weeks and the number of months on inhaled corticosteroids. In addition, the daytime, nocturnal and exercise induced cough, dyspnea, and wheezing during the last 12 months were registered. Based on the telephone survey, the 30 study children could be divided into two groups, one group with no signs of bronchial asthma (n=16) and another group being on inhaled steroids and/or having had asthmatic wheezing during the past 12 months. Some patients on inhaled steroids were asymptomatic.

4.4 Blood samples

Blood samples were collected from the SCIT or SLIT treated patients yearly, before the commencement of SIT and after 1, 2 or 3 years of treatment, each out of pollen season. The blood samples from the untreated or placebo-treated allergic control patients were obtained at corresponding time points. The ten healthy subjects who served as non-allergic controls for the baseline analysis in SCIT studies **II** and **IV** donated blood samples only once, that is, when the studies were initiated.

4.5 *In vitro* laboratory methods

4.5.1 Antigens

The *in vitro* stimulation tests were performed with whole allergen extracts of birch (*Betula verrucosa*) or timothy grass (*Phleum pratense*) pollen (kindly provided by ALK-Abelló (Hørsholm, Denmark). Purified protein derivative (PPD) of *M. tuberculosis* (Statens Seruminstitut, Copenhagen, Denmark) served as an unrelated antigen for specificity determination of the SIT-induced immune responses.

4.5.2 Isolation and stimulation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Ficoll (Ficoll-Hypaque, Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. The PBMC were washed twice with Hanks' balanced salt solution, and suspended in RPMI (Gibco, Life Technologies, Paisley, Scottland, UK), supplemented with 5% autologous serum, 2 mM L-glutamine (Gibco, Life Technologies, Paisley, Scottland, UK), 100 U/ml penicillin and 100 μg/ml gentamycine (Nordvacc Media Skärholmen, Sweden). The PBMC were then applied on 48-well culture plates, at a density of $2x10^5$ cells per well, and incubated at +37˚C, in a humidified atmosphere with 5% CO_2 for three days. The stimulation was performed in the presence of 50 μg/ml relevant whole allergen extract or 10 μg/ml PPD. The total culture volume in each duplicate culture was 400 μl. After 72 h stimulation, the PBMC were collected by centrifugation, suspended in 500 μl of Trizol reagent (Gibco Life Technologies, Paisley, Scotland, UK), and stored at -70°C.

4.5.3 Total RNA extraction and cDNA synthesis

Total RNA was isolated from PBMC according to Trizol instructions (Gibco Life Technologies) with the exception that prior to isopropanol precipitation, 1 μl of glycogen was added to enhance RNA precipitation. The extracted RNA was stored in 200 μl of 75% ethanol at -20°C, and prior to RT reaction, suspended to 20 μl of diethylpyrocarbonate (DEPC) treated water. The RT reaction was performed with First Strand cDNA Synthesis Kit (Pharmacia, Sweden) using oligo(dT) primers, and cDNA was stored at -70°C.

4.5.4 Real-time PCR (TaqMan)

The amplification of β-actin, cytokine (IL-4, IL-5, IL-10, IL-17A, IL-18, IL-23p19, IL-27p28, IFN-γ, TGF-β1), SLAM, and transcription factor FOXP3, RORC (variant 2), and GATA-3 cDNAs was performed in MicroAmp® optical 96-well reaction plate (Applied Biosystems, Foster City, CA, USA). Each well contained 1 μl of total cDNA, 300 nM of sequence specific primers and 200 nM of dual-labelled fluorogenic probe in 1x TaqMan® Universal PCR master mix (Applied Biosystems). A negative PCR control without template and a positive PCR control with a template of known amplification were included in each assay. The reaction was performed in ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the standard two-step run protocol (Step 1: 10 min at 95ºC, Step 2: 40 cycles of 15 sec at 95ºC plus 1 min at 60ºC). For analysis of IL-5, IL-10, IL-17A, IL-18, IL-23p19, IL-27p28, TGF-β1, FOXP3, RORC expression cDNA specific assay reagent kits were purchased from Applied Biosystems. The primer and probe sequences for the analysis of β-actin, SLAM, IL-4, IFN-γ and GATA-3 expression were designed using Primer Express software (Applied Biosystems) and labeled with FAM (6-carboxyfluorescein) at the 5` end and with TAMRA

(6-carboxytetramethylrhodamine) at the 3` end as described earlier (Hämäläinen *et al*. 2000; Laaksonen *et al.* 2003). During PCR, the Ct values (the cycle number at which the detected fluorescence exceeds the threshold) for each amplification product were determined using a threshold value of 0.03. The cytokine, SLAM, and transcription factor (RORC, FOXP3 and GATA-3) specific signals were normalized by the constitutively expressed β-actin signals using the formula $2^{-\Delta Ct} = 2^{-(Ct,\beta\text{-actin} - Ct,Cytokine/SLAM/RORC/FOXP3/GATA\text{-}3)}$. The stimulation indexes, indicating the fold change in expression in allergen stimulated versus non-stimulated cultures were thereafter calculated by the formula $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ for stimulated culture} - \Delta Ct \text{ for unstimulated culture})}$ as described in ABI PRISM 7700 SDS Relative Quantitation of Gene Expression-protocol by Applied Biosystems (User Bulletin #2, P/N 4303849). The obtained means of duplicates were used for statistical analysis. All analyses of all genes were performed in the linear range of the amplification, verified by dilution series of each cDNA. β-actin was not regulated during allergen stimulation (unstimulated CT: mean (SD): 18.20 (0.97), stimulated CT 18.19 (1.02), n=180).

4.5.5 Serum specific IgE and IgG determination (IV)

Serum allergen-specific IgE and IgG_4 antibodies were analyzed by Immuno-Cap (Phadia, Uppsala, Sweden).

4.6 Statistical analysis

Mann-Whitney U-test was used for unpaired, comparisons between the groups and Wilcoxon Signed Rank Test for paired comparisons within the groups. Correlation analyses were performed with a non-parametric Spearman Rank-Order Correlation test. A p-value <0.05 was considered to indicate a statistically significant difference.

5 RESULTS

5.1 Clinical efficacy

5.1.1 Changes in symptom and medication scores (I-IV)

Both SCIT and SLIT were clinically effective in terms of reducing seasonal allergy symptoms and rescue medication use. The adjusted end-point symptom and medication scores are presented in Table 9. From a subgroup of SCIT-treated patients (20 of 30) and untreated allergic controls (8 of 10) there were symptom and medication scores available after the complete 3-year treatment course. The patients in SLIT studies **I** and **III** were randomly selected from a single-centre, randomized, double-blind, placebo-controlled dose response phase II trial, and the clinical efficacy data for the total patient population has been previously published (Valovirta *et al.* 2006). The clinical efficacy in SLIT favored the high allergen dose treatment (group 2, cumulative weekly maintenance dose of 200,000 SQ-U compared to group 1, cumulative weekly maintenance dose of 24,000 SQ-U).

Table 9. Symptom and medication scores after SLIT and SCIT.

	SCIT	
	SCIT	No SCIT
Number of subjects	20	8
Treatment dose SQ-U[a]	100,000	-
End-point symptoms[b], mean (SD)	-37.6 (7.0)	-23.4 (6.1)
End-point medication[b], mean (SD)	-35.3 (8.2)	+2.3 (2.7)

	SLIT		
	Group 2	Group 1	Placebo
Number of subjects	10	10	10
Treatment dose SQ-U[c]	200,000	24,000	-
End-point symptoms[d], mean (SD)	1.9 (1.6)	3.2 (2.7)	3.6 (3.4)
End-point medication[d], mean (SD)	3.6 (5.2)	4.9 (6.0)	3.9 (3.8)
Subjects with asthma/no asthma after 5 yr[e]	2/8	6/4	6/4

[a] at the maintenance phase, given at 6-week intervals

[b] after 3 years of treatment, as %-unit reduction of maximal scores and adjusted to pollen counts

[c] at the maintenance phase, weekly cumulative dose

[d] Sum of scores after 2 years of treatment, averaged over the 12-week pollen season

[e] Asthma incidence after 5 years of the commencement of SLIT based on a telephone survey; $p = 0.038$ for astma versus no asthma group according to chi-square test (Savolainen *et al.* 2006).

5.1.2 Asthma symptoms after SLIT (III)

Based on the telephone survey, five years after the start of SLIT and 3 years post-SLIT, the 30 study subjects included were divided into two groups, one group with no signs of bronchial asthma (n=16) and another group being on inhaled steroids and/or having had asthmatic wheezing during the past 12 months. There was more asthma in the low dose (group 1, 24,000 SQ-U/week) and placebo groups (12 of 20) than in the high dose treated group (group 2, 200,000 SQ-U/week, 2 of 10) (Table 9; p=0.038, according to chi-square test, Savolainen *et al*. 2006). Altogether nine children were on inhaled corticosteroids five years later, two from the high dose group and seven from the low dose or placebo groups. These data suggests that high dose SLIT could be effective in preventing disease progression from allergic rhinitis to asthma in pediatric patients.

5.1.3 Specific antibody responses during SCIT (IV)

In study **IV**, serum specific antibody responses during the first year of SCIT were monitored. IgG4 levels significantly increased in the treated group (fold change from baseline 33.8 (27.4), median (MAD)) compared to untreated patients, in whom no change in IgG4 levels were found (fold change from baseline 1.0 (0.2)), (p<0.0001). There was also a slight but significant decrease in the level of specific IgE in serum in the SCIT-treated patients after 1 year of treatment when compared to untreated patients (fold change from baseline 0.8 (0.2) in treated vs. 1.2 (0.3) in untreated group, p=0.026).

5.1.4 Correlation between in retrospect post-seasonally recorded symptom and medication scores and the seasonally recorded scores during SCIT (IV)

The clinical improvement during SIT is generally measured by means of changes in seasonal visual analogue scale of symptoms and medication scoring, usually recorded on a diary card basis during the season. The patients for SIT, however, are typically recruited post-seasonally, making the baseline evaluation of symptoms difficult. According to the results presented in study **IV**, the in retrospect filled post-seasonal visual analogue scale form is reliable and sufficient measure of symptom and medication improvement during SCIT. The retrospectively recorded VAS correlated well with symptoms (r=0.76, p<0.0001) and VAS (r=0.55, p<0.011) input in the diary card (seasonal weekly mean) (**IV**, Figure 1). The medication recorded retrospectively also correlated well with that recorded in the diary card (seasonal weekly mean) (r=0.86, p<0.0001) (**IV**, Figure 1). This allows an assessment of baseline symptoms in situations when patients are recruited post-seasonally.

5.2 IL-18, SLAM and GATA-3 expression in PBMC during SLIT (I)

5.2.1 SLIT up-regulates allergen-specific IL-18 and SLAM mRNA expression in PBMC

In study **I**, peripheral Th1 and Th2 responses during SLIT were investigated. This was done by analyzing the expression of Th1 type immune markers IL-18 and SLAM, and the Th2 cell master transcription factor GATA-3 in *in vitro* allergen-stimulated PBMC after 0, 1 and 2 years of SLIT. Furthermore, the dose dependence of these responses were investigated by collecting the blood samples from the high dose (group 2, dose 40,000 SQ-U per day with cumulative weekly dose of 200,000 SQ-U), low dose (group 1, dose 4,800 SQ-U per day with cumulative weekly dose of 24,000 SQ-U) or placebo-treated children. The expression of IL-18 mRNA was significantly increased in the high-dose treated group (dose group 2) as compared to placebo group after 1 year of treatment (p=0.028) (**I**, Fig. 1). Also SLAM mRNA expression was increased in the dose group 2 as compared to baseline after 1 year of treatment (p=0.028), but this increase was not significant compared to the placebo group (**I**, Fig. 1). No changes were seen in GATA-3 mRNA expression during SLIT (**I**, Fig. 1).

5.2.2 The up-regulation in IL-18 mRNA expression in PBMC during SLIT correlates with decreased late-phase cutaneous responses

After 2 years of treatment with SLIT, there was a significant inverse correlation between allergen-induced IL-18 mRNA responses in PBMC and late-phase reactions in skin (r= -0.41, p=0.041) (**I**, Fig. 3). No correlation was found between the late-phase skin reaction and SLAM or GATA-3 expression (data not shown).

5.2.3 The up-regulation in SLAM mRNA expression in PBMC during SLIT correlates with TGF-β and IL-10 mRNA expression

In dose group 2, the allergen-induced SLAM mRNA expression after 2 years of treatment correlated strongly with TGF-β mRNA (r=0.80, p=0.0037) and IL-10 (r=0.96, p<0.0001) mRNA expression (**I**, Fig. 2). There was no correlation between IL-18 and TGF-β or IL-18 and IL-10 expression. Also, no correlation was found between IL-18 and SLAM mRNAs. Neither IL-18 nor SLAM correlated with any of the Th1 or Th2 cytokines IFN-γ, IL-4 and IL-5 during SLIT (data not shown).

5.2.4 The up-regulation in either IL-18 or SLAM mRNA expression in PBMC during SLIT fails to correlate with the changes in symptom or medication scores

After 1 or 2 years of treatment with SLIT, there was no correlation between the allergen-induced IL-18, SLAM or GATA-3 mRNA expression in PBMC and symptom or medication scores (data not shown).

5.3 Three-year follow-up of immunological changes in allergen-specific responses during SCIT (II)

5.3.1 SCIT induces sustained increases in allergen-specific Treg and Th1 type cytokine responses in PBMC

In study **II**, the expression of Treg type cytokine IL-10, Th2 cytokines IL-4, IL-5, and Th1 cell markers IFN-γ, IL-18 and SLAM in response to specific allergen were investigated in PBMC during the complete 3-year course of SCIT. This was done by collecting blood samples at 0, 1 and 3 years from the start of SCIT. The three-year follow-up samples were available from altogether 20 SCIT-treated patients and from 8 untreated allergic control patients. After the first treatment year, there was a significant decrease in the expression of IL-5 mRNA ($p<0.05$), a tendency towards diminished IL-4 mRNA expression, and a marked reduction in the IL-4/IFN-γ expression ratio ($p<0.05$) in the allergen-stimulated PBMC of SCIT-treated patients when compared to baseline (**II**, Table 3). These changes were accompanied with significant increases in the expressions of IFN-γ ($p<0.02$), IL-18 ($p<0.02$), SLAM ($p<0.01$) and IL-10 ($p<0.01$) mRNAs (**II**, Table 3). IL-18 ($p<0.01$), SLAM ($p<0.01$) and IL-10 ($p<0.05$) expressions remained up-regulated in the allergen-stimulated PBMC of SCIT-treated patients up to 3 years of SCIT, albeit declined from the first year responses (**II**, Table 3). In contrast, the immunomodulatory effects of SCIT on the allergen-induced IFN-γ and Th2 type cytokine responses and IL-4/IFN-γ ratio were no longer evident in the PBMC after 3 years of treatment (**II**, Table 3). No significant changes in either Th2 (IL-4, IL-5), Th1 (IFN-γ, SLAM, IL-18) or Treg (IL-10) type immune responses to specific allergen were found in the PBMC of patients who had not been treated with SCIT during the 3-year follow-up (**II**, Table 3).

5.3.2 The early decrease in Th2 cytokine production is associated with the development of a good therapeutic outcome

Based on the changes in VAS scores after two years of treatment, the SCIT-treated patients were divided in two groups: those who benefited well from SCIT (>40 percentage unit reduction, n=12) and those who had less symptoms improvement (<40 percentage unit reduction, n=8) (**II**, Table 2). Comparison of the immunological markers with clinical improvement revealed that decreases in allergen-specific *in vitro* expression of IL-4 mRNA and IL-4/IFN-γ ratio in the PBMC of SIT-treated patients after 1 year of treatment were associated with the development of a good therapeutic outcome, as set of by more than 40 percentage unit reduction in VAS symptom scores (**II**, Table 3). In patients with less symptoms improvement (<40 percentage unit reduction in VAS), however, there was significant increase in both IL-5 ($p<0.02$) and IFN-γ ($p<0.02$) mRNA expression, and a tendency towards increased IL-4 mRNA expression, during SCIT (**II**, Table 3). The increase in IL-10 mRNA expression after 1 and 3 years of treatment was also associated more with the less beneficial efficacy of SCIT than with the good therapeutic outcome, whereas the increases in the allergen-induced *in vitro* expressions of IL-18 and SLAM mRNAs had no association with the clinical efficacy of SCIT (**II**, Table 3).

5.4 FOXP3 mRNA expression and Th17 type immune responses in PBMC during SLIT (III)

5.4.1 Allergen-specific FOXP3 mRNA expression increases in PBMC during SLIT and correlates with specific IL-10 and TGF-β production

In study **III**, the expression of Treg-associated transcription factor FOXP3, and the Th17 associated cytokines IL-17, IL23, and IL-27 were investigated during SLIT. These transcripts were analyzed from the allergen-stimulated PBMC collected at 0, 1 and 2 years of SLIT from the high dose (group 2, 200,000 SQ-U/week), low dose (group 1, 24,000 SQ-U/week) and placebo-treated children. After two years of SLIT, a significant increase in allergen-stimulated FOXP3 mRNA expression was found in the PBMC of high dose-treated children, both when compared to placebo-treated children (p=0.028) and the baseline expression (p=0.016) (**III**, Table 3). This increase was specific to the allergen, as there was no change in the PPD-induced FOXP3 mRNA expression in the PBMC during SLIT. The changes in the allergen-induced FOXP3 mRNA responses significantly correlated with IL-10 mRNA in the whole study group after one year (r=0.44; p=0.0131) and two years (r=0.48; p=0.0068) of treatment, and with TGF-β1 mRNA expression after one year of treatment (r=0.54; p=0.0017) (**III**, Figure 1), but failed to correlate significantly within the individual dosing groups.

5.4.2 IL-17 mRNA expression remains unchanged in PBMC during SLIT, but correlates with clinical symptoms in the individual level

Allergen-induced IL-17 mRNA expression remained unchanged in the PBMC of all three dosing groups during SLIT when investigated at the group level (**III**, Figure 2). However, in the individual level, there was a significant positive correlation between the allergen-induced IL-17 responses and clinical outcome after 2 years of treatment both in the whole study group (r=0.38, p=0.039), and also, when the three dosing groups were analyzed separately, in the dose group 2 (r =0.74, p=0.027), but not dose group 1 (r= -0.14, p=0.68) or in placebo group (r= 0.44, p = 0.18) (**III**, Figure 3). A clear association of the elevated allergen-induced IL-17 mRNA responses with poor therapeutic outcome, as indicated by high symptom and medication scores, was also evidenced when the study population was divided into two groups based on the clinical outcome: good outcome (SMS<3, n=13) and poor outcome (SMS>3, n=17) (**III**, Table 4). A significantly (p=0.0024) higher allergen-induced IL-17 mRNA expression was seen in the group with poor outcome (SMS>3) (**III**, Table 4). When symptoms and medication were analyzed separately, the high IL-17A expression was associated more with symptoms than medication (**III**, Table 4).

5.4.3 IL-27p28 mRNA expression remains low and unchanged in the PBMC during SLIT, but the expression of IL-23p19 mRNA progressively decreases the high dose treated group

After 1 (p=0.047) and 2 (p=0.013) years from the start of SLIT, a significant progressive decrease was seen in the expression of IL-23p19 mRNA in response to specific allergen in the PBMC of high dose treated children (group 2, 200,000 SQ-U/week) when compared to baseline (**III**, Figure 2). In placebo group, by contrast, there was a significant progressive increase in the non-stimulated expression of IL-23p19 in the PBMC after one (p=0.009) and two (p=0.005) years from the start of SLIT (**III**, Figure 2). IL-27p19 mRNA expression remained low and unchanged in the allergen-stimulated and non-stimulated PBMC of all three different dosing groups during the 2-year course of SLIT (**III**, Figure 2). No significant correlation was found between the allergen- or non-stimulated expressions of IL-17, IL-23p19 and IL-27p28 mRNAs in the PBMC of children treated within the different dosing groups, or the whole patient population (n=30) investigated during the treatment (data not shown).

5.4.4 FOXP3 mRNA expression is transiently increased in the subsequent asthma group, but IL-23p19 expression progressively decreases in the non-asthma group

Based on the telephone survey, five years after the start of SLIT and 3 years post SLIT, the 30 study subjects were divided into two groups, one group with no signs of bronchial asthma (n=16) and another group being on inhaled steroids and/or having had asthmatic wheezing during the past 12 months. There was more asthma in the low dose (24,000 SQ-U/week) and placebo groups than in high dose group (group 2, 200,000 SQ-U/week) (**III**, Table 1). Altogether nine children were on inhaled corticosteroids five years later, two from the high dose group and seven from the low dose or placebo groups. Allergen-induced FOXP3 expression was transiently elevated in the PBMC of asthma group as compared to non-asthma group (p=0.013) after 1 year of SLIT but without change in comparison to baseline expression (**III**, Table 5). However, there was a significant progressive decrease in the allergen-induced IL-23p19 mRNA expression in the PBMC of non-asthma group during SLIT as compared to baseline expression (p=0.034 after 1 year and p=0.044 after 2 years), whereas no change was found in the asthma group (**III**, Table 5). Instead, there was a significant progressive increase in the asthma group the in non-stimulated expression of IL-23p19 mRNA during SLIT as compared to baseline (p=0.002 after 1 year and p=0.004 after 2 years) but no change in the non-asthma group (**III**, Table 5). Both non-stimulated (p=0.034) and allergen-stimulated (p=0.046) IL-23p19 mRNA responses were significantly lower in the PBMC of non-asthma group after 1 year of SLIT as compared to asthma group (**III**, Table 5). There was no significant association between the antigen-induced IL-17A or IL-27p28 mRNA expression in PBMC and subsequent reported asthma (data not shown).

5.5 Th17 type immune responses (IL-17, RORC) in PBMC during SCIT (IV)

5.5.1 Th17 type responses remain unchanged in the PBMC during SCIT, but correlate with clinical improvement in the individual level

In study **IV**, Th17 type immune responses against specific allergen in the PBMC during SCIT were investigated, along with Th1 and Th2 responses. These responses were analyzed from blood samples collected at 0 and 1 year from the start of SCIT from thirty study patients that had been treated with SCIT and from ten other allergic patients who served as untreated controls. After one study year, there were no significant differences in the fold changes from baseline in the mRNA expressions of allergen-induced IL-17 and RORC between the SCIT-treated and untreated allergic control groups (**IV**, Table 3). Also, no significant differences between these groups were found in the fold changes from baseline in the expressions of allergen-induced IFN-γ, IL-4, and IL-5 mRNAs (**IV**, Table 3). However, again in the individual level, there was a significant negative correlation found between the IL-17 mRNA fold changes from baseline and the combined symptom medication score in the SCIT-treated patients (r= -0.45, p=0.015), which was not observed in the untreated control patients (r= -0.094, p=0.80) or the allergic rhinitis group as a whole (r = -0.27, p=0.10) (**IV**, Figure 2).

5.5.2 IL-17 mRNA expression during SCIT positively correlates with IL-5 mRNA, but has a negative association with serum specific IgE

After 1 year of treatment with SCIT, there was a significant negative correlation between the IL-17 mRNA and IgE fold changes from baseline in the treated patients (r= -0.57, p=0.0024) and the whole allergic rhinitis group (r= -0.52, p=0.0013) but not in the untreated controls (r=0.03, p=0.92) (**IV**, Figure 3). In addition, there was a significant positive correlation between the IL-17 and IL-5 mRNA changes in the treated patients (r=0.37, p=0.048) and the whole allergic rhinitis group (r=0.35, p=0.030) but not in the untreated controls (r= -0.35, p=0.30) (**IV**, Figure 3). Significant positive correlations were also found between the IL-4 mRNA and IgE fold changes in the whole allergic rhinitis group (r=0.35, p=0.041) but only marginally in the treated patients (r=0.33, p=0.077) and untreated controls (r=0.58, p=0.099) (**IV**, Figure 3). Significant positive correlations were also found between IL-4 and IL-5 mRNA in treated patients (r=0.56, p=0.0027) and the whole allergic rhinitis group (r=0.50, p=0.0019) but not in the untreated controls (r=0.35, p=0.30) (**IV**, Figure 3). All other correlations remained non-significant.

6 DISCUSSION

6.1 The up-regulation of Th1 and Treg type immune markers in PBMC during SLIT and SCIT indicates a Th2 to a Th1 shift in allergen-specific T cell responses, and induction of tolerogenic Treg cells

The two key mechanisms behind the beneficial effects of SIT have been suggested to include the induction of tolerogenic, allergen-specific CD4+CD25+ Treg cells that produce anti-inflammatory cytokines, such as IL-10 and/or TGF-β, and immune deviation from a Th2 to a Th1 type response in allergen-specific T cells. In pollen allergic patients, the numbers of FOXP3-expressing CD4+CD25+ Treg cells have been shown to increase in nasal mucosa during pollen season, and this increase has been found to correlate with clinical improvement, as well as with the suppression of local allergic inflammation, involving reductions in the numbers of mucosal IL-5 mRNA+ cells and eosinophils (Radulovic *et al.* 2008). Our laboratory and others have previously shown that allergen-specific IL-10 production is up-regulated in the PBMC during SCIT and SLIT (Francis *et al.* 2003; Savolainen *et al.* 2004; Savolainen *et al.* 2006; Cosmi *et al.* 2006; Bohle *et al.* 2007; Francis *et al.* 2008; Burastero *et al.* 2008). In addition, in nasal mucosa and peripheral blood, there is increased expression of Th1 cytokines during the treatment with SCIT or SLIT (Durham *et al.* 1996; Ebner *et al.* 1997; Wachholz *et al.* 2002; Cosmi *et al.* 2006; Bohle *et al.* 2007). In SLIT studies **I** and **III**, and SCIT study **II**, the induction and the kinetics of Th1 and Treg type immune responses during these two treatments were investigated, and increased expression of both Th1 (IL-18 and SLAM) and Treg type (IL-10) cell markers after SCIT and SLIT were found, thus supporting the role of these responses in underlying mechanisms of SIT.

6.2 The induction of tolerogenic Treg response and immune deviation from a Th2- to more Th1 type response in PBMC during SLIT is dependent on the treatment dose, favoring high dose SLIT

It is known that low dose SCIT regimens are clinically ineffective (Bousquet *et al.* 2008; van Metre *et al.* 1980; Hirsch *et al.* 1982). In SLIT studies **I** and **III,** allergen-specific IL-18, SLAM and FOXP3 expressions were found to increase significantly only in the PBMC of children treated with high dose SLIT regimen (group 2, 200,000 SQ-U/week), who also had the greatest reduction in symptoms after the treatment. This demonstrates that the induction of tolerogenic, Treg type immune responses and the immune deviation from Th2 to a Th1 type response is, in fact, dependent on the therapeutic allergen dose, favoring high dose SLIT. This phenomenon has also been previously demonstrated in SCIT (Pene *et al.* 1998).

6.3 The early decrease in Th2 type cytokine production during SCIT is associated with the development of a good therapeutic outcome

A number of recent meta-analyses of double-blind, placebo-controlled trials support the clinical efficacy of SCIT in the treatment of allergic rhinitis and asthma (Abramson *et al.* 2003; Calderon *et al.* 2007). However, a significant heterogeneity in the clinical efficacy of SCIT between different clinical trials and different individuals still exists. When successful, a marked clinical improvement is often achieved already after one preseasonal treatment period, whereas a more pronounced and sustained clinical efficacy usually requires longer treatment courses, typically for at least 3 years. In accordance with the previous studies, we found that SCIT was highly effective in reducing seasonal symptoms and the need for rescue medication already after the first treatment year, and a further improvement in both of these parameters was seen after the second treatment year, which remained similar till the end of SCIT (**II**, Table 2). The decrease in allergen-specific Th2 type cytokine responses after 1 year of treatment - in particular of IL-4 mRNA and IL-4/IFN-γ expression ratio - were associated with the development of a good therapeutic outcome to SCIT. Conversely, increases in both Th2 (IL-5) and Th1 (IFN-γ, SLAM) type responses and IL-10 mRNA production during SCIT were found in patients with less effective treatment.

It has been previously published from our laboratory that allergen-specific IL-10 mRNA responses are increased early, by the time of reaching the maintenance dose, in PBMC of patients who benefited most from SCIT (Savolainen *et al.* 2004). A similar tendency was also found for allergen-induced IL-18 and SLAM mRNAs expression (Laaksonen *et al.* 2003; Savolainen *et al.* 2004), whereas the responses were delayed, peaking not until the end of the first treatment year, in PBMC of patients who had less beneficial outcome. The early induction of allergen-specific IL-10 production in PBMC during SCIT and SLIT has also been reported by other investigators, but often without correlating to symptoms improvement at later stages of SIT (Francis *et al.* 2008; Bohle *et al.* 2007). In study **II**, we extended the investigation of allergen-specific immune responses and symptom improvement to cover the complete 3-year course of SCIT. At years 1 and 3 from the start of SCIT, we could not find any significant association between the increased allergen-induced IL-18 and SLAM mRNA responses in the PBMC and clinical outcome, whereas high IL-10 mRNA expression was found in patients with less beneficial outcome. Thus, early immune deviation in the peripheral allergen-specific immune responses is critical for the development of good therapeutic outcome to SCIT.

6.4 The Th1 and Treg type responses induced in PBMC during SCIT are sustained for years

Previously, the evidence of immunological mechanisms behind SCIT has been collected from short-term studies, maximum of one or two years in duration, and studies extended

to cover the whole treatment period have been lacking. In the 3-year prospective study of SCIT in pollen allergic rhinitis patients (study **II**), it was demonstrated that SCIT induces long-term changes in allergen-specific immune responses in PBMC. Both Th1 (IFN-γ, IL-18, SLAM) and Treg (IL-10) type immune responses that were induced against specific allergen during SCIT remained up-regulated in the PBMC for up to 3 years of treatment, albeit declined from the first year responses. These results concord with the results of study **I** where a peak in peripheral allergen-specific SLAM mRNA expression and a slightly more sustained increase in IL-18 mRNA expression were seen in the PBMC after 1 year of commencement of the high dose SLIT regimen (group 2, cumulative weekly maintenance dose 200,000 SQ-U). In a previous publication from our laboratory (Savolainen *et al.* 2006), a peak also in peripheral allergen-specific IL-10 production was seen after the first treatment year with 200,000 SQ-U/week dose SLIT regimen, but a decline back to baseline after two years of treatment. These findings suggest that there is a shift in allergen-specific immune responses from peripheral blood to target tissues after the first treatment year, where they contributed to clinical improvement. This suggestion is also supported by finding of Wachholz *et al.* (Wachholz *et al.* 2002) who found a shift from Th2 to Th1 responses in nasal mucosa of patients treated for 2 years with SCIT, but was not able to find a similar response in PBMC.

6.5 Allergen-induced IL-17 responses correlate with symptom medication scores during SLIT and SCIT

It has been previously shown that serum IL-17 levels are elevated in patients with pollen allergic rhinitis (Ciprandi *et al.* 2008b; Ciprandi *et al.* 2009). However, until now, little has been known of the allergen-specific Th17 type immune responses in PBMC of pollen allergic rhinitis patients. In study **IV**, we found no differences in the allergen-induced *in vitro* IL-17 mRNA responses between allergic rhinitis patients and healthy subjects at the baseline, when measured outside of pollen season. The increased expression of IL-17 mRNA in both groups compared to non-stimulated cells rather suggests a normal proinflammatory response against a foreign antigen. The cellular source of IL-17 in this experimental setup, though, remains to be further evaluated as several other cell types than Th17 cells, including CD8+ T cells (Shin *et al.* 1998), and NKT cells (Rachitskaya *et al.* 2008) have been shown to be capable of expressing IL-17. Recent data also suggest macrophages as a potential source of IL-17 (Song *et al.* 2008). Interestingly, in a mouse model of allergic asthma, macrophages rather than Th17 cells were found to be the major source of IL-17 in the airways, and these cells were also identified in BAL fluid specimens obtained from allergic asthma patients (Song *et al.* 2008). The enhanced specific allergen-induced IL-17 mRNA responses found in the PBMC of some patients with poor therapeutic outcome after 1 year of SCIT in study **IV** could therefore be macrophage-derived, as there was no change in RORC mRNA expression in the PBMC during this treatment, suggesting that there was no propagation of allergen-specific Th17 cells.

There is evidence of synergistic effects between Th17 and Th2 type immune responses in the enhancement of allergic inflammation in mice (Wakashin *et al.* 2008). In addition, in allergic asthma patients, a significant positive correlation has been found between the bronchial IL-5 mRNA and IL-17 mRNA responses (Bullens *et al.* 2006). Both IL-17 and IL-5 are capable of inducing eosinophil activation, resulting in the release of proinflammatory mediators to the surrounding milieu (Cheung *et al.* 2008). Bronchial IL-17 responses of allergic asthma patients have also been found to correlate with the disease severity (Chakir *et al.* 2003). Recently, in allergic rhinitis patients too, a significant positive correlation was found between the serum IL-17 levels and symptoms severity scores, medication use, and peripheral eosinophil count during pollen season (Ciprandi *et al.* 2009). Taken together, the results presented in paper **IV** with *in vitro* allergen-induced IL-17 mRNA responses of the PBMC of SCIT-treated patients correlating with IL-5 mRNA expression, and also, inversely with the clinical improvement during SCIT, support these prior findings.

During SLIT (study **III**), allergen-induced IL-17 mRNA expression was significantly increased in the study subjects with elevated combined symptom medication score (SMS) after two years of treatment. When the study population was divided in two groups based on the clinical outcome: good outcome (SMS<3, n=13) and poor outcome (SMS>3, n=17), a significantly (p=0.0024) higher allergen-induced IL-17A expression was seen in the group with poor outcome (SMS>3). When symptoms and medication were analyzed separately, the high IL-17A expression was associated more with symptoms than medication.

It has been demonstrated that allergen-induced IL-17 responses of macrophages are down-modulated by IL-10 (Song *et al.* 2008). The early induction of IL-10 production has been shown to be of importance in the induction of allergen-specific T cell anergy during SIT (Bellinghausen *et al.* 1997; Akdis *et al.* 1998), and in the attenuation of late-phase allergic reactions (Francis *et al.* 2008). Furthermore, it has been earlier shown that allergen-induced Th2 responses are decreased early in subjects responding clinically well to SIT, but remain elevated in those failing to respond (Benjaponpitak *et al.* 1999). Therefore, the increased allergen-induced IL-17 mRNA responses along with the enhanced IL-5 production in the PBMC of patients who had poor a therapeutic outcome after 1 year of treatment with SCIT could be a result from the delayed induction of tolerogenic Treg responses against the specific allergen (Paper **IV**).

6.6 IL-17 responses have a dual immunoregulatory role during SCIT

Besides the IL-17 responses were found to play a proinflammatory role in SCIT (Paper **IV**) by correlating positively with IL-5 mRNA expression and inversely with clinical improvement, a significant negative association was also found between the serum

allergen-specific IgE levels and IL-17 mRNA responses after 1 year of treatment. So far, though, little is known of the effects of IL-17 on immunoglobulin synthesis. It has been demonstrated that the deficiency in Th17 type cell responses in humans results hyper-IgE-syndrome (Milner *et al.* 2008), suggesting that Th17 type immune responses could have an immunoregulatory role in the IgE synthesis in B cells. Therefore, IL-17 and the Th17 type responses could have a pleiotropic function during SIT.

6.7 Comparison of immunological mechanisms associated with SCIT and SLIT

Figure 6 schematically summarizes the immunological mechanisms found in this work to be associated with the high dose SLIT (200,000 SQ-U/week), low dose SLIT (24,000 SQ-U/week) and SCIT. It appears that the immunological mechanisms between SLIT and SCIT regimens are comparable, provided that high allergen dose treatment was used in SLIT. The immunological changes induced by high dose SLIT and SCIT regimens also appear to follow similar pattern of kinetics, with a peak in the protective peripheral Th1 and Treg type immune responses being seen after the first treatment year in both regimens, and thereafter a trend toward back the baseline. The subsiding of these responses from peripheral blood after the first treatment year suggests a shift to tissues where they contributed to clinical improvement.

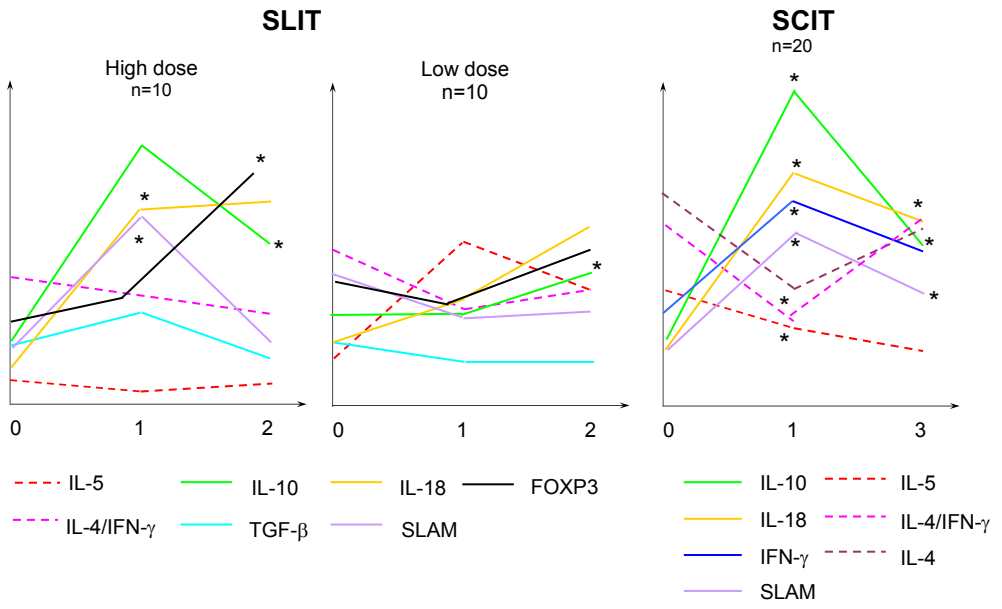

Figure 6. Summary of immunological mechanisms associated with SLIT and SCIT (Papers I-III, and Savolainen *et al.* 2006). Asterisk denotes to a statistically significant difference in expression compared to baseline.

The kinetics of immunological changes associated with SLIT and SCIT were investigated in this work at rather late time points, at years 1, 2 and 3 from start. It has been demonstrated that many immunological changes associated with SIT occur much earlier, within weeks or months from the start, or even days, depending of the administration form or schedule (e.g. conventional SCIT versus rush VIT). These data thus do not exclude the possibility that the dynamics of the immunological changes shown here may differ when investigated at earlier time points, for example at the beginning of the treatment. Not only the administration route and schedule used, but also the type of allergen (e.g. venom versus dust mite versus pollen) and the dose during the treatment are likely to influence on the kinetics of the immunological changes associated with SIT. However, one of the main objectives of this work was to investigate the kinetics of immunological changes associated with SCIT and SLIT in relationship with the clinical outcome and it is well known that the establishment of clinical improvement requires long periods of treatment, usually for at least a year, but often years to provide sustained improvement and long-term cure.

In SCIT studies **II** and **IV**, some of the patients (n=12) were treated with clustered rush regimen and others by conventional schedule (n=18). However, subanalysis of immune responses within these populations revealed no significant differences in the magnitude or the kinetics of the responses at the investigated time points. This is likely to be due to the fact that in both regimens, like in SLIT, at 1 year, which was the earliest time point investigated, the maintenance dose had already been achieved long time ago, and thereby the possible influence of the up-dosing schedule on the responses had subsided. In SLIT, the therapeutic allergen dose though appeared to be a more determining factor in the capacity of this modality to induce changes in the allergen-induced immune responses (Figure 6). Subanalysis of the patients treated with birch and/or timothy grass pollen SCIT unfortunately was not possible due to numerically too unevenly distributed study population.

7 SUMMARY AND CONCLUSIONS

In this work, immunological mechanisms associated with SCIT and SLIT have been investigated. This work demonstrates that both treatments induce an increase in the protective Th1 and Treg type immune responses against the specific allergens in PBMC. The early induction in Th1 and Treg type immune responses, concomitantly with the decreased expression of Th2 type cytokines, was associated with the development of a good therapeutic outcome to SCIT. Furthermore, the data of this work demonstrate that both treatments have long-term immunomodulatory effects on allergen-specific Th1 and Treg type immune responses in PBMC, that endure at least up to 2 to 3 years of therapy, whereas during SCIT, decreased Th2 type cytokine responses were seen only during the early therapy, at one year. The subsiding of these protective immune responses from peripheral blood after the first treatment year, when a peak in expression of both Th1 and Treg type responses was found, suggests a shift to mucosal tissues where they contributed to clinical improvement. Taken together, these data indicate that SIT induces a long-term immune reorientation of the pre-existing, inappropriate Th2-biased immune responses in allergic rhinitis patients. The data of this work also demonstrates that the capacity of SLIT to modulate allergen-specific immune responses is dependent on the treatment dose, favoring high allergen dose (cumulative weekly maintenance dose 200,000 SQ-U), as shown by inadequate induction of the protective Th1 and Treg type immune responses against specific allergens in children treated with low dose regimen (24,000 SQ-U/week).

The specific findings of this work are:

1) SLIT up-regulates SLAM mRNA expression, an activation molecule associated with Th1 cells, and increases the expression of IL-18 mRNA, an APC-derived cytokine, in allergen-simulated PBMC. Both molecules have been earlier suggested to play a role in the Th2 to Th1 shift during SCIT. Both of these immune markers peaked at 1 year of treatment, and then subsided. A significant inverse relationship was found between the peripheral allergen-induced IL-18 mRNA responses and late-phase cutaneous responses after 2 years of treatment. Neither IL-18 nor SLAM, or GATA-3, mRNA expression correlated with the clinical symptoms or medication scores (**I**).

2) SCIT induces a transient decrease in allergen-specific Th2 type cytokine (IL-5) production in PBMC after 1 year of treatment, but increases SLAM, IL-18 and IL-10 expression in a sustained manner. However, a peak in SLAM, IL-18 and IL-10 expression was also found after 1 year of the treatment. IFN-γ expression was transiently up-regulated in the PBMC only after 1 year of treatment. The early decrease in IL-4 mRNA expression and IL-4/IFN-γ ratio were associated with the development of good therapeutic outcome, whereas both Th1 (IFN-γ, SLAM) and Th2 (IL-5) cytokines, as well as IL-10, increased in the patients with less beneficial outcome (**II**)

3) SLIT leads to up-regulation of FOXP3 mRNA expression in allergen-stimulated PBMC, but only in those treated with high allergen dose, indicating dose-dependence of the response. The expression increased after 2 years of treatment. FOXP3 mRNA responses correlated with IL-10 and TGF-β mRNA production in the whole cohort, but not within individual dosing groups. The up-regulation in FOXP3 mRNA expression did not correlate with the changes in symptom or medication scores. IL-17 mRNA expression remained unmodulated between or within the different dosing groups during SLIT, but correlated with symptom medication scores in the level of individual patients. IL-23p19 mRNA expression, a cytokine involved in Th17 development and maintenance, decreased significantly and progressively only in the high dose treated group. The decrease in allergen-specific IL-23p19 mRNA expression during SLIT was also associated with the protection against subsequent asthma symptoms. No change in the originally low IL-27p28 mRNA expression was found in the PBMC during SLIT (**III**).

4) No change in RORC or IL-17 mRNA expression was found in the allergen-stimulated PBMC of SCIT-treated patients 1 year of treatment when compared to untreated controls. However, a negative association was found between the reduced symptom medication scores and IL-17 mRNA fold changes among the treated patients. A significant positive correlation was also found between the IL-17 and IL-5 mRNA fold changes in the treated patients, and a negative association between the IL-17 mRNA and IgE changes after 1 year of treatment, suggesting a pleiotropic role for Th17 type immune responses in SCIT (**IV**).

Taken together, these data strengthen the current concept that immunomodulation of allergen-specific immune responses from the prevailing Th2-biased responses towards a more Th1 type, and induction of tolerogenic Treg cells represent the two key mechanisms behind the beneficial effects of SCIT and SLIT, by demonstrating long-term immune deviation towards such responses in the PBMC of patients undergoing these treatments. The data also give a novel insight into the mechanisms why SCIT and SLIT may fail to be effective in some patients by demonstrating a simultaneous up-regulation in both Th1 and Th2 type immune responses in the patients with less efficient outcome during SCIT, and by a positive correlation between the proinflammatory IL-17 responses and symptom medication scores both during SCIT and SLIT. In addition, this work suggests a mechanism for the established prophylactic efficacy of SIT against asthma development, as shown by a progressive decrease in the allergen-induced IL-23 responses in the PBMC of patients treated with clinically effective doses during SLIT, and in those reporting no asthma symptoms after SLIT, suggesting that successful treatment with SLIT could have the capacity to protect against proinflammatory, Th17-promoting immune responses. These findings suggest that Th17 type immune responses may play a role in the pathogenesis of allergic rhinitis, but further studies, involving larger cohorts and

analysis at protein level, as well as from target tissues are required to confirm these pilot studies. In conclusions, the results of this work indicate that analysis of a combination of Th1, Th2, Treg and Th17-associated immune markers such as SLAM, IL-4, IL-5, IL-10, and IL-17 from allergen-stimulated PBMC of SIT-treated patients during the treatment could provide tools to monitor the development of clinical response to SIT, and thereby, predict the ultimate clinical outcome. PBMC would represent an ideal tissue to investigate such SIT-associated efficacy indicators, as direct analysis from target organs is time-consuming and laborious.

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REFERENCES

Abramson MJ, Puy RM, Weiner JM. Allergen immunotherapy for asthma. Cochrane Database Syst Rev 2003:CD001186.

Acosta-Rodriguez EV, Rivino L, Geginat J, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8:639-46.

Akdis CA, Akdis M, Blesken T, et al. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. J Clin Invest 1996;98:1676-83.

Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102:98-106.

Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;199:1567-75.

Akoum H, Tsicopoulos A, Vorng H, et al. Venom immunotherapy modulates interleukin-4 and interferon-gamma messenger RNA expression of peripheral T lymphocytes. Immunology 1996;87:593-8.

Akoum H, Duez C, Vorng H, et al. Early modifications of chemokine production and mRNA expression during rush venom immunotherapy. Cytokine 1998;10:706-12.

Alexander C, Ying S, A BK, Larche M. Fel d 1-derived T cell peptide therapy induces recruitment of CD4+ CD25+; CD4+ interferon-gamma+ T helper type 1 cells to sites of allergen-induced late-phase skin reactions in cat-allergic subjects. Clin Exp Allergy 2005;35:52-8.

Andoh A, Fujino S, Bamba S, et al. IL-17 selectively down-regulates TNF-alpha-induced RANTES gene expression in human colonic subepithelial myofibroblasts. J Immunol 2002;169:1683-7.

Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. J Exp Med 2007;204:1849-61.

Antico A, Pagani M, Crema A. Anaphylaxis by latex sublingual immunotherapy. Allergy 2006;61:1236-7.

Arvidsson MB, Lowhagen O, Rak S. Allergen specific immunotherapy attenuates early and late phase reactions in lower airways of birch pollen asthmatic patients: a double blind placebo-controlled study. Allergy 2004;59:74-80.

Asher MI, Montefort S, Bjorksten B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006;368:733-43.

Aversa G, Chang CC, Carballido JM, Cocks BG, de Vries JE. Engagement of the signaling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A-sensitive T cell proliferation and IFN-gamma production. J Immunol 1997;158:4036-44.

Bahceciler NN, Arikan C, Taylor A, et al. Impact of sublingual immunotherapy on specific antibody levels in asthmatic children allergic to house dust mites. Int Arch Allergy Immunol 2005;136:287-94.

Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med 2003;97:726-33.

Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J. Insect venom immunotherapy induces interleukin-10 production and a Th2 to-Th1 shift, and changes surface marker expression in venom-allergic subjects. Eur J Immunol 1997;27:1131-9.

Bellinghausen I, Klostermann B, Bottcher I, Knop J, Saloga J. Importance of the inducible costimulator molecule for the induction of allergic immune responses and its decreased expression on T helper cells after venom immunotherapy. Immunology 2004;112:80-6.

Bellinghausen I, Konig B, Bottcher I, Knop J, Saloga J. Regulatory activity of human CD4 CD25 T cells depends on allergen concentration, type of allergen and atopy status of the donor. Immunology 2005;116:103-11.

Benjaponpitak S, Oro A, Maguire P, Marinkovich V, DeKruyff RH, Umetsu DT. The kinetics of change in cytokine production by CD4 T cells during conventional allergen immunotherapy. J Allergy Clin Immunol 1999;103:468-75.

Bentley AM, Jacobson MR, Cumberworth V, et al. Immunohistology of the nasal mucosa in seasonal allergic rhinitis: increases in activated eosinophils and epithelial mast cells. J Allergy Clin Immunol 1992;89:877-83.

Beriou G, Costantino CM, Ashley CW, et al. IL-17 producing human peripheral regulatory T cells retain suppressive function. Blood 2009.

Blazowski L. Anaphylactic shock because of sublingual immunotherapy overdose during third year of maintenance dose. Allergy 2008;63:374.

Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003;3:253-7.

Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J Allergy Clin Immunol 2007;120:707-13.

Bohle B. T cell responses during allergen-specific immunotherapy of Type I allergy. Front Biosci 2008;13:6079-85.

Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;102:558-62.

Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. J Allergy Clin Immunol 2001;108:S147-334.

Bousquet J, Demoly P, Yssel H. Atopy. In: Bousquet J, Dutau G, Grimfeld A, de Prost Y, eds. From atopic dermatitis to asthma. Paris: Expansion Scientifique Francaise, 2002:5-17.

Bousquet J, Khaltaev N, Cruz AA, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy 2008;63 Suppl 86:8-160.

Bufe A, Ziegler-Kirbach E, Stoeckmann E, et al. Efficacy of sublingual swallow immunotherapy in children with severe grass pollen allergic symptoms: a double-blind placebo-controlled study. Allergy 2004;59:498-504.

Bullens DM, Truyen E, Coteur L, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? Respir Res 2006;7:135.

Burastero SE, Mistrello G, Falagiani P, et al. Effect of sublingual immunotherapy with grass monomeric allergoid on allergen-specific T-cell proliferation and interleukin 10 production. Ann Allergy Asthma Immunol 2008;100:343-50.

Burgler S, Ouaked N, Bassin C, et al. Differentiation and functional analysis of human T(H)17 cells. J Allergy Clin Immunol 2009;123:588-95, 595 e1-7.

Burney P, Chinn C, Jarvis D, Luczynska C, Lai E. Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). Eur Respir J 1996;9:687-95.

Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database Syst Rev 2007:CD001936.

Carballido JM, Aversa G, Kaltoft K, et al. Reversal of human allergic T helper 2 responses by engagement of signaling lymphocytic activation molecule. J Immunol 1997;159:4316-21.

Castro AG, Hauser TM, Cocks BG, et al. Molecular and functional characterization of mouse signaling lymphocytic activation molecule (SLAM): differential expression and responsiveness in Th1 and Th2 cells. J Immunol 1999;163:5860-70.

Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity 2000;13:443-51.

Cengizlier R, Saraclar Y, Adalioglu C, Tuncer A. Changes in nasal metachromatic cells during allergen immunotherapy in children. Allergol Immunopathol (Madr) 1995;23:111-6.

Cevit O, Kendirli SG, Yilmaz M, Altintas DU, Karakoc GB. Specific allergen immunotherapy: effect on immunologic markers and clinical parameters in asthmatic children. J Investig Allergol Clin Immunol 2007;17:286-91.

Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. J Allergy Clin Immunol 2003;111:1293-8.

Charlesworth EN, Hood AF, Soter NA, Kagey-Sobotka A, Norman PS, Lichtenstein LM. Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. J Clin Invest 1989;83:1519-26.

Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994;265:1237-40.

Chen X, Howard OM, Oppenheim JJ. Pertussis toxin by inducing IL-6 promotes the generation of IL-17-producing CD4 cells. J Immunol 2007;178:6123-9.

Cheung PF, Wong CK, Lam CW. Molecular mechanisms of cytokine and chemokine release from eosinophils activated by IL-17A, IL-17F, and IL-23: implication for Th17 lymphocytes-mediated allergic inflammation. J Immunol 2008;180:5625-35.

Ciprandi G, Cirillo I, Fenoglio D, Marseglia G, Tosca MA. Sublingual immunotherapy

induces spirometric improvement associated with IL-10 production: preliminary reports. Int Immunopharmacol 2006;6:1370-3. *a*.

Ciprandi G, Fenoglio D, Cirillo I, Milanese M, Minuti P. Sublingual immunotherapy and regulatory T cells. Allergy 2006;61:511-3. *b*.

Ciprandi G, Cirillo I, Tosca MA, Marseglia G, Fenoglio D. Sublingual immunotherapy-induced IL-10 production is associated with changed response to the decongestion test: preliminary results. Allergy Asthma Proc 2007;28:574-7.

Ciprandi G, Fenoglio D, Di Gioacchino M, et al. Sublingual immunotherapy provides an early increase of interferon-gamma production. J Biol Regul Homeost Agents 2008;22:169-73. *a*.

Ciprandi G, Fenoglio D, De Amici M, Quaglini S, Negrini S, Filaci G. Serum IL-17 levels in patients with allergic rhinitis. J Allergy Clin Immunol 2008. *b*.

Ciprandi G, De Amici M, Murdaca G, et al. Serum interleukin-17 levels are related to clinical severity in allergic rhinitis. Allergy 2009.

Clavel R, Bousquet J, Andre C. Clinical efficacy of sublingual-swallow immunotherapy: a double-blind, placebo-controlled trial of a standardized five-grass-pollen extract in rhinitis. Allergy 1998;53:493-8.

Clutterbuck EJ, Hirst EM, Sanderson CJ. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GMCSF. Blood 1989;73:1504-12.

Cocks BG, Chang CC, Carballido JM, Yssel H, de Vries JE, Aversa G. A novel receptor involved in T-cell activation. Nature 1995;376:260-3.

Cools M, Van Bever HP, Weyler JJ, Stevens WJ. Long-term effects of specific immunotherapy, administered during childhood, in asthmatic patients allergic to either house-dust mite or to both house-dust mite and grass pollen. Allergy 2000;55:69-73.

Cosmi L, Santarlasci V, Angeli R, et al. Sublingual immunotherapy with Dermatophagoides monomeric allergoid down-regulates allergen-specific immunoglobulin E and increases both interferon-gamma- and interleukin-10-production. Clin Exp Allergy 2006;36:261-72.

Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response in vivo. J Immunol 2000;165:4848-53.

Cox L. Accelerated immunotherapy schedules: review of efficacy and safety. Ann Allergy Asthma Immunol 2006;97:126-37; quiz 137-40, 202.

Cox D, Li JT, Nelson H, Lockey R (eds.). Allergen immunotherapy: a practice parameter second update. J Allergy Clin Immunol 2007;120:S25-85.

Creticos PS, Adkinson NF, Jr., Kagey-Sobotka A, et al. Nasal challenge with ragweed pollen in hay fever patients. Effect of immunotherapy. J Clin Invest 1985;76:2247-53.

Critchfield JM, Racke MK, Zuniga-Pflucker JC, et al. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. Science 1994;263:1139-43.

Dahl R, Kapp A, Colombo G, et al. Sublingual grass allergen tablet immunotherapy provides sustained clinical benefit with progressive immunologic changes over 2 years. J Allergy Clin Immunol 2008;121:512-518 e2.

Dehlink E, Eiwegger T, Gerstmayr M, et al. Absence of systemic immunologic changes during dose build-up phase and early maintenance period in effective specific sublingual immunotherapy in children. Clin Exp Allergy 2006;36:32-9.

Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 1993;150:353-60.

Des Roches A, Paradis L, Knani J, et al. Immunotherapy with a standardized Dermatophagoides pteronyssinus extract. V. Duration of the efficacy of immunotherapy after its cessation. Allergy 1996;51:430-3.

Des Roches A, Paradis L, Menardo JL, Bouges S, Daures JP, Bousquet J. Immunotherapy with a standardized Dermatophagoides pteronyssinus extract. VI. Specific immunotherapy prevents the onset of new sensitizations in children. J Allergy Clin Immunol 1997;99:450-3.

Di Rienzo V, Marcucci F, Puccinelli P, et al. Long-lasting effect of sublingual immunotherapy in children with asthma due to house dust mite: a 10-year prospective study. Clin Exp Allergy 2003;33:206-10.

Dokic D, Nethe A, Kleine-Tebbe J, Kunkel G, Baumgarten CR. Mediator release is altered in immunotherapy-treated patients: a 4-year study. Allergy 1996;51:796-803.

Donnell AT, Grammer LC. An overview of allergens. Allergy Asthma Proc 2004;25:S2-4.

Dunsky EH, Goldstein MF, Dvorin DJ, Belecanech GA. Anaphylaxis to sublingual immunotherapy. Allergy 2006;61:1235.

Durham SR, Ying S, Varney VA, et al. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. J Immunol 1992;148:2390-4.

Durham SR, Ying S, Varney VA, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. J Allergy Clin Immunol 1996;97:1356-65.

Durham SR, Varney VA, Gaga M, et al. Grass pollen immunotherapy decreases the number of mast cells in the skin. Clin Exp Allergy 1999;29:1490-6. *a*.

Durham SR, Walker SM, Varga EM, et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999;341:468-75. *b*.

Ebner C, Siemann U, Bohle B, et al. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. Clin Exp Allergy 1997;27:1007-15.

Eifan AO, Keles S, Bahceciler NN, Barlan IB. Anaphylaxis to multiple pollen allergen sublingual immunotherapy. Allergy 2007;62:567-8.

Eng PA, Borer-Reinhold M, Heijnen IA, Gnehm HP. Twelve-year follow-up after discontinuation of preseasonal grass pollen immunotherapy in childhood. Allergy 2006;61:198-201.

Esch RE. Sublingual immunotherapy. Curr Opin Otolaryngol Head Neck Surg 2008;16:260-4.

Faith A, Richards DF, Verhoef A, Lamb JR, Lee TH, Hawrylowicz CM. Impaired secretion of interleukin-4 and interleukin-13 by allergen-specific T cells correlates with defective nuclear expression of NF-AT2 and jun B: relevance to immunotherapy. Clin Exp Allergy 2003;33:1209-15.

Fanta C, Bohle B, Hirt W, et al. Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. Int Arch Allergy Immunol 1999;120:218-24.

Fargeas C, Wu CY, Nakajima T, Cox D, Nutman T, Delespesse G. Differential effect of transforming growth factor beta on the synthesis of Th1- and Th2-like lymphokines by human T lymphocytes. Eur J Immunol 1992;22:2173-6.

Faria AM, Weiner HL. Oral tolerance. Immunol Rev 2005;206:232-59.

Fellrath JM, Kettner A, Dufour N, et al. Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. J Allergy Clin Immunol 2003;111:854-61.

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-6.

Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996;183:2593-603.

Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 2003;111:1255-61.

Francis JN, James LK, Paraskevopoulos G, et al. Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. J Allergy Clin Immunol 2008;121:1120-1125 e2.

Friedman A, Weiner HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc Natl Acad Sci U S A 1994;91:6688-92.

Fu CL, Ye YL, Lee YL, Chiang BL. Both allergen-specific CD4 and CD8 Type 2 T cells decreased in asthmatic children with immunotherapy. Pediatr Allergy Immunol 2003;14:284-91.

Furin MJ, Norman PS, Creticos PS, et al. Immunotherapy decreases antigen-induced eosinophil cell migration into the nasal cavity. J Allergy Clin Immunol 1991;88:27-32.

Gabrielsson S, Soderlund A, Paulie S, van der Pouw Kraan TC, Troye-Blomberg M, Rak S. Specific immunotherapy prevents increased levels of allergen-specific IL-4- and IL-13-producing cells during pollen season. Allergy 2001;56:293-300.

Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature 2008;454:445-54.

Garcia BE, Sanz ML, Dieguez I, de las Marinas MD, Oehling A. Modifications in IgG subclasses in the course of immunotherapy with grass pollen. J Investig Allergol Clin Immunol 1993;3:19-25.

Gardner LM, Thien FC, Douglass JA, Rolland JM, O'Hehir RE. Induction of T 'regulatory' cells by standardized house dust mite immunotherapy: an increase in CD4+ CD25+ interleukin-10+ T cells expressing peripheral tissue trafficking markers. Clin Exp Allergy 2004;34:1209-19. *a*.

Gardner LM, O'Hehir RE, Rolland JM. High dose allergen stimulation of T cells from house dust mite-allergic subjects induces expansion of IFN-gamma+ T Cells, apoptosis of CD4+IL-4+ T cells and T cell anergy. Int Arch Allergy Immunol 2004;133:1-13. b.

Giannarini L, Maggi E. Decrease of allergen-specific T-cell response induced by local nasal immunotherapy. Clin Exp Allergy 1998;28:404-12.

Gleich GJ, Zimmermann EM, Henderson LL, Yunginger JW. Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. J Allergy Clin Immunol 1982;70:261-71.

Golden DB, Kwiterovich KA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Discontinuing venom immunotherapy: outcome after five years. J Allergy Clin Immunol 1996;97:579-87.

Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. Clin Exp Allergy 2004;34:1364-72.

Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med 1996;184:19-29.

Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-42.

Guerra F, Carracedo J, Solana-Lara R, Sanchez-Guijo P, Ramirez R. TH2 lymphocytes from atopic patients treated with immunotherapy undergo rapid apoptosis after culture with specific allergens. J Allergy Clin Immunol 2001;107:647-53.

Haahtela T, von Hertzen L, Makela M, Hannuksela M. Finnish Allergy Programme 2008-2018--time to act and change the course. Allergy 2008;63:634-45.

Hamalainen H, Meissner S, Lahesmaa R. Signaling lymphocytic activation molecule (SLAM) is differentially expressed in human Th1 and Th2 cells. J Immunol Methods 2000;242:9-19.

Hamid QA, Schotman E, Jacobson MR, Walker SM, Durham SR. Increases in IL-12 messenger RNA+ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. J Allergy Clin Immunol 1997;99:254-60.

Hashimoto T, Akiyama K, Kobayashi N, Mori A. Comparison of IL-17 production by helper T cells among atopic and nonatopic asthmatics and control subjects. Int Arch Allergy Immunol 2005;137 Suppl 1:51-4.

He R, Oyoshi MK, Jin H, Geha RS. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. Proc Natl Acad Sci U S A 2007;104:15817-22.

Hedlin G, Silber G, Schieken L, et al. Attenuation of allergen sensitivity early in the course of ragweed immunotherapy. J Allergy Clin Immunol 1989;84:390-9.

Hellings PW, Kasran A, Liu Z, et al. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. Am J Respir Cell Mol Biol 2003;28:42-50.

Hirsch SR, Kalbfleisch JH, Cohen SH. Comparison of Rinkel injection therapy with standard immunotherapy. J Allergy Clin Immunol 1982;70:183-90.

Hofstra CL, Van Ark I, Hofman G, Kool M, Nijkamp FP, Van Oosterhout AJ. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsiveness, eosinophilia, and serum IgE levels. J Immunol 1998;161:5054-60.

Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nat Rev Immunol 2008;8:218-30.

Ishizaka K, Ishizaka T. Identification of gamma-E-antibodies as a carrier of reaginic activity. J Immunol 1967;99:1187-98.

Iliopoulos O, Proud D, Adkinson NF, Jr., et al. Effects of immunotherapy on the early, late, and rechallenge nasal reaction to provocation with allergen: changes in inflammatory mediators and cells. J Allergy Clin Immunol 1991;87:855-66.

Imada M, Simons FE, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon-gamma production in primary culture: responses of subjects with allergic rhinitis and normal controls. Immunology 1995;85:373-80.

Inal A, Altintas DU, Yilmaz M, Karakoc GB, Kendirli SG, Sertdemir Y. Prevention of new sensitizations by specific immunotherapy in children with rhinitis and/or asthma monosensitized to house dust mite. J Investig Allergol Clin Immunol 2007;17:85-91.

Infante-Duarte C, Horton HF, Byrne MC, Kamradt T. Microbial lipopeptides induce the production of IL-17 in Th cells. J Immunol 2000;165:6107-15.

Ippoliti F, De Santis W, Volterrani A, et al. Immunomodulation during sublingual therapy in allergic children. Pediatr Allergy Immunol 2003;14:216-21.

Ivanov, II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121-33.

Jacobsen L, Nuchel Petersen B, Wihl JA, Lowenstein H, Ipsen H. Immunotherapy with partially purified and standardized tree pollen extracts. IV. Results from long-term (6-year) follow-up. Allergy 1997;52:914-20.

Jacobsen L, Niggemann B, Dreborg S, et al. Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. Allergy 2007;62:943-8.

James LK, Durham SR. Update on mechanisms of allergen injection immunotherapy. Clin Exp Allergy 2008;38:1074-88.

Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology. New York and London: Garland Science; c2001

Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. J Immunol 1998;160:3555-61.

Johansson SG. Raised levels of a new immunoglobulin class (IgND) in asthma. Lancet 1967;2:951-3

Johansson SG, Hourihane JO, Bousquet J, et al. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. Allergy 2001;56:813-24.

Jones CE, Chan K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. Am J Respir Cell Mol Biol 2002;26:748-53.

Jovanovic DV, Di Battista JA, Martel-Pelletier J, et al. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J Immunol 1998;160:3513-21.

Jung CM, Funk A, Rakoski J, Ring J. Immunohistochemical analysis of late local skin reactions during rush venom immunotherapy. Allergy 1997;52:717-26.

Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. J Immunol 1995;154:4187-94.

Jutel M, Muller UR, Fricker M, Rihs S, Pichler WJ, Dahinden C. Influence of bee venom immunotherapy on degranulation and leukotriene generation in human blood basophils. Clin Exp Allergy 1996;26:1112-8.

Jutel M, Akdis M, Budak F, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33:1205-14.

Jutel M, Akdis M, Blaser K, Akdis CA. Mechanisms of allergen specific immunotherapy--T-cell tolerance and more. Allergy 2006;61:796-807.

Kammerer R, Chvatchko Y, Kettner A, Dufour N, Corradin G, Spertini F. Modulation of T-cell response to phospholipase A2 and phospholipase A2-derived peptides by conventional bee venom immunotherapy. J Allergy Clin Immunol 1997;100:96-103.

Kastelein RA, Hunter CA, Cua DJ. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol 2007;25:221-42.

Kawaguchi M, Kokubu F, Kuga H, et al. Modulation of bronchial epithelial cells by IL-17. J Allergy Clin Immunol 2001;108:804-9.

Kay AB, Ying S, Varney V, et al. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. J Exp Med 1991;173:775-8.

Kay AB. Allergy and allergic diseases. First of two parts. N Engl J Med 2001;344:30-7.

Kerkhof M, Schouten JP, De Monchy JG. The association of sensitization to inhalant allergens with allergy symptoms: the influence of bronchial hyperresponsiveness and blood eosinophil count. Clin Exp Allergy 2000;30:1387-94.

Keskin O, Tuncer A, Adalioglu G, Sekerel BE, Sackesen C, Kalayci O. The effects of grass pollen allergoid immunotherapy on clinical and immunological parameters in children with allergic rhinitis. Pediatr Allergy Immunol 2006;17:396-407.

Kilpeläinen M. Asthma and allergic rhinoconjunctivitis among university students – occurrence and risk factors. Annales Universitas Turkuensis. 2001. Painosalama Oy, Turku, Finland. ISBN-951-29-2056-5.

Kiniwa M, Gately M, Gubler U, Chizzonite R, Fargeas C, Delespesse G. Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. J Clin Invest 1992;90:262-6.

Klimek L, Dormann D, Jarman ER, Cromwell O, Riechelmann H, Reske-Kunz AB. Short-term preseasonal birch pollen allergoid immunotherapy influences symptoms, specific nasal provocation

and cytokine levels in nasal secretions, but not peripheral T-cell responses, in patients with allergic rhinitis. Clin Exp Allergy 1999;29:1326-35. *a*.

Klimek L, Wolf H, Mewes T, et al. The effect of short-term immunotherapy with molecular standardized grass and rye allergens on eosinophil cationic protein and tryptase in nasal secretions. J Allergy Clin Immunol 1999;103:47-53. *b*.

Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible Pathogenic Role of Th17 Cells for Atopic Dermatitis. J Invest Dermatol 2008.

Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol 2009.

La Grutta S, Arena A, D'Anneo WR, et al. Evaluation of the antiinflammatory and clinical effects of sublingual immunotherapy with carbamylated allergoid in allergic asthma with or without rhinitis. A 12-month perspective randomized, controlled, trial. Eur Ann Allergy Clin Immunol 2007;39:40-4.

Laaksonen K, Junikka M, Lahesmaa R, Terho EO, Savolainen J. In vitro allergen-induced mRNA expression of signaling lymphocytic activation molecule by PBMC of patients with allergic rhinitis is increased during specific pollen immunotherapy. J Allergy Clin Immunol 2003;112:1171-7.

Laan M, Cui ZH, Hoshino H, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J Immunol 1999;162:2347-52.

Lambin P, Bouzoumou A, Murrieta M, et al. Purification of human IgG4 subclass with allergen-specific blocking activity. J Immunol Methods 1993;165:99-111.

Larenas-Linnemann D. Sublingual immunotherapy in children: complete and updated review supporting evidence of effect. Curr Opin Allergy Clin Immunol. 2009 Apr;9(2):168-76.

Latvala J, von Hertzen L, Lindholm H, Haahtela T. Trends in prevalence of asthma and allergy in Finnish young men: nationwide study, 1966-2003. Bmj 2005;330:1186-7.

Lei Z, Liu G, Huang Q, et al. SCF and IL-31 rather than IL-17 and BAFF are potential indicators in patients with allergic asthma. Allergy 2008;63:327-32.

Lent AM, Harbeck R, Strand M, et al. Immunologic response to administration of standardized dog allergen extract at differing doses. J Allergy Clin Immunol 2006;118:1249-56.

Li JT, Lockey RF, Bernstein IL, Portnoy JM (eds.). Allergen immunotherapy: a practice parameter. American Academy of Allergy, Asthma and Immunology. American College of Allergy, Asthma and Immunology. Ann Allergy Asthma Immunol 2003;90:1-40.

Lichtenstein LM, Ishizaka K, Norman PS, Sobotka AK, Hill BM. IgE antibody measurements in ragweed hay fever. Relationship to clinical severity and the results of immunotherapy. J Clin Invest 1973;52:472-82.

Lima MT, Wilson D, Pitkin L, et al. Grass pollen sublingual immunotherapy for seasonal rhinoconjunctivitis: a randomized controlled trial. Clin Exp Allergy 2002;32:507-14.

Ling EM, Smith T, Nguyen XD, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 2004;363:608-15.

Linna O, Kokkonen J, Lukin M. A 10-year prognosis for childhood allergic rhinitis. Acta Paediatr 1992;81:100-2.

Liu H, Rohowsky-Kochan C. Regulation of IL-17 in Human CCR6+ Effector Memory T Cells. J Immunol 2008;180:7948-57.

Lombardi C, Incorvaia C, Braga M, Senna G, Canonica GW, Passalacqua G. Administration regimens for sublingual immunotherapy to pollen allergens: what do we know? Allergy 2009;64:849-54.

Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA. Recombinant human interleukin 5 is a selective activator of human eosinophil function. J Exp Med 1988;167:219-24.

Lue KH, Lin YH, Sun HL, Lu KH, Hsieh JC, Chou MC. Clinical and immunologic effects of sublingual immunotherapy in asthmatic children sensitized to mites: a double-blind, randomized, placebo-controlled study. Pediatr Allergy Immunol 2006;17:408-15.

Maggi E, Parronchi P, Manetti R, et al. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. J Immunol 1992;148:2142-7.

Mandrioli P, Comtois P, Dominguez Vilches E, Galan Soldevilla C, Isard S, Syzdek L. Sampling: principles and techniques. In: Mandrioli P, Comtois P, Levizzani V, eds. Methods in aerobiology. Bologna: Pitagora Editrice, 1998:47-112.

Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 2008;9:641-9.

Manetti R, Parronchi P, Giudizi MG, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med 1993;177:1199-204.

Marcucci F, Sensi L, Frati F, et al. Sublingual tryptase and ECP in children treated with grass pollen sublingual immunotherapy (SLIT): safety and immunologic implications. Allergy 2001;56:1091-5.

Marcucci F, Sensi L, Di Cara G, Incorvaia C, Frati F. Dose dependence of immunological response to sublingual immunotherapy. Allergy 2005;60:952-6. *a*.

Marcucci F, Sensi L, Di Cara G, et al. Three-year follow-up of clinical and inflammation parameters in children monosensitized to mites undergoing sub-lingual immunotherapy. Pediatr Allergy Immunol 2005;16:519-26. *b*.

Marcucci F, Sensi L, Incorvaia C, Di Cara G, Moingeon P, Frati F. Oral reactions to sublingual immunotherapy: a bioptic study. Allergy 2007;62:1475-7.

Marogna M, Spadolini I, Massolo A, Canonica GW, Passalacqua G. Clinical, functional, and immunologic effects of sublingual immunotherapy in birch pollinosis: a 3-year randomized controlled study. J Allergy Clin Immunol 2005;115:1184-8.

Marogna M, Bruno M, Massolo A, Falagiani P. Long-lasting effects of sublingual immunotherapy for house dust mites in allergic rhinitis with bronchial hyperreactivity: A long-term (13-year) retrospective study in real life. Int Arch Allergy Immunol 2007;142:70-8. *a*.

Marogna M, Spadolini I, Massolo A, et al. Effects of sublingual immunotherapy for multiple or single allergens in polysensitized patients. Ann Allergy Asthma Immunol 2007;98:274-80. *b*.

Marogna M, Spadolini I, Massolo A, et al. Long-term comparison of sublingual immunotherapy vs inhaled budesonide in patients with mild persistent asthma due to grass pollen. Ann Allergy Asthma Immunol 2009;102:69-75.

McHugh SM, Lavelle B, Kemeny DM, Patel S, Ewan PW. A placebo-controlled trial of immunotherapy with two extracts of Dermatophagoides pteronyssinus in allergic rhinitis, comparing clinical outcome with changes in antigen-specific IgE, IgG, and IgG subclasses. J Allergy Clin Immunol 1990;86:521-31.

McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy. Clin Exp Allergy 1995;25:828-38.

Meissner N, Kochs S, Coutelle J, et al. Modified T-cell activation pattern during specific immunotherapy (SIT) in cat-allergic patients. Clin Exp Allergy 1999;29:618-25.

Mellerup MT, Hahn GW, Poulsen LK, Malling H. Safety of allergen-specific immunotherapy. Relation between dosage regimen, allergen extract, disease and systemic side-effects during induction treatment. Clin Exp Allergy 2000;30:1423-9.

Micallef MJ, Ohtsuki T, Kohno K, et al. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. Eur J Immunol 1996;26:1647-51.

Milner JD, Brenchley JM, Laurence A, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature 2008;452:773-6.

Moingeon P, Batard T, Fadel R, Frati F, Sieber J, Van Overtvelt L. Immune mechanisms of allergen-specific sublingual immunotherapy. Allergy 2006;61:151-65.

Molet S, Hamid Q, Davoine F, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol 2001;108:430-8.

Monteseirin J, Bonilla I, Chacon P, et al. Allergen-dependent CD14 modulation and apoptosis in monocytes from allergic patients. Allergy 2003;58:1027-32.

Moverare R, Elfman L, Bjornsson E, Stalenheim G. Changes in cytokine production in vitro during the early phase of birch-pollen immunotherapy. Scand J Immunol 2000;52:200-6.

Nagato T, Kobayashi H, Yanai M, et al. Functional analysis of birch pollen allergen Bet v 1-specific regulatory T cells. J Immunol 2007;178:1189-98.

Nakae S, Komiyama Y, Nambu A, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity 2002;17:375-87.

Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+) CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001;194:629-44.

Nasser SM, Ying S, Meng Q, Kay AB, Ewan PW. Interleukin-10 levels increase in cutaneous

biopsies of patients undergoing wasp venom immunotherapy. Eur J Immunol 2001;31:3704-13.

Nish WA, Charlesworth EN, Davis TL, et al. The effect of immunotherapy on the cutaneous late phase response to antigen. J Allergy Clin Immunol 1994;93:484-93.

Nouri-Aria KT, Wachholz PA, Francis JN, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. J Immunol 2004;172:3252-9.

Nouri-Aria KT, Pilette C, Jacobson MR, Watanabe H, Durham SR. IL-9 and c-Kit+ mast cells in allergic rhinitis during seasonal allergen exposure: effect of immunotherapy. J Allergy Clin Immunol 2005;116:73-9.

Novembre E, Galli E, Landi F, et al. Coseasonal sublingual immunotherapy reduces the development of asthma in children with allergic rhinoconjunctivitis. J Allergy Clin Immunol 2004;114:851-7.

Oboki K, Ohno T, Saito H, Nakae S. Th17 and allergy. Allergol Int 2008;57:121-34.

Oda N, Yamashita N, Minoguchi K, et al. Long-term analysis of allergen-specific T cell clones from patients with asthma treated with allergen rush immunotherapy. Cell Immunol 1998;190:43-50.

Okamura H, Tsutsi H, Komatsu T, et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 1995;378:88-91.

Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000;13:715-25.

Otsuka H, Mezawa A, Ohnishi M, Okubo K, Seki H, Okuda M. Changes in nasal metachromatic cells during allergen immunotherapy. Clin Exp Allergy 1991;21:115-9.

Ouyang W, Ranganath SH, Weindel K, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 1998;9:745-55.

Pajno GB, Barberio G, De Luca F, Morabito L, Parmiani S. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. Clin Exp Allergy 2001;31:1392-7.

Pallasaho P, Ronmark E, Haahtela T, Sovijarvi AR, Lundback B. Degree and clinical relevance of sensitization to common allergens among adults: a population study in Helsinki, Finland. Clin Exp Allergy 2006;36:503-9.

Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133-41.

Parronchi P, Macchia D, Piccinni MP, et al. Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. Proc Natl Acad Sci U S A 1991;88:4538-42.

Passalacqua G, Albano M, Fregonese L, et al. Randomised controlled trial of local allergoid immunotherapy on allergic inflammation in mite-induced rhinoconjunctivitis. Lancet 1998;351:629-32.

Passalacqua G, Albano M, Riccio A, et al. Clinical and immunologic effects of a rush sublingual immunotherapy to Parietaria species: A double-blind, placebo-controlled trial. J Allergy Clin Immunol 1999;104:964-8.

Passalacqua G, Canonica GW. Long-lasting clinical efficacy of allergen specific immunotherapy. Allergy 2002;57:275-6.

Penagos M, Compalati E, Tarantini F, et al. Efficacy of sublingual immunotherapy in the treatment of allergic rhinitis in pediatric patients 3 to 18 years of age: a meta-analysis of randomized, placebo-controlled, double-blind trials. Ann Allergy Asthma Immunol 2006;97:141-8.

Penagos M, Passalacqua G, Compalati E, et al. Metaanalysis of the efficacy of sublingual immunotherapy in the treatment of allergic asthma in pediatric patients, 3 to 18 years of age. Chest 2008;133:599-609.

Pene J, Rousset F, Briere F, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. Proc Natl Acad Sci U S A 1988;85:6880-4.

Pene J, Desroches A, Paradis L, et al. Immunotherapy with Fel d 1 peptides decreases IL-4 release by peripheral blood T cells of patients allergic to cats. J Allergy Clin Immunol 1998;102:571-8.

Pene J, Chevalier S, Preisser L, et al. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. J Immunol 2008;180:7423-30.

Peng ZK, Naclerio RM, Norman PS, Adkinson NF, Jr. Quantitative IgE- and IgG-subclass responses during and after long-term ragweed immunotherapy. J Allergy Clin Immunol 1992;89:519-29.

Pfaar O, Klimek L. Efficacy and safety of specific immunotherapy with a high-dose sublingual grass pollen preparation: a double-blind, placebo-

controlled trial. Ann Allergy Asthma Immunol 2008;100:256-63.

Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 2002;16:779-90.

Piconi S, Trabattoni D, Saresella M, et al. Effects of specific immunotherapy on the B7 family of costimulatory molecules in allergic inflammation. J Immunol 2007;178:1931-7.

Pierkes M, Bellinghausen I, Hultsch T, Metz G, Knop J, Saloga J. Decreased release of histamine and sulfidoleukotrienes by human peripheral blood leukocytes after wasp venom immunotherapy is partially due to induction of IL-10 and IFN-gamma production of T cells. J Allergy Clin Immunol 1999;103:326-32.

Pilette C, Nouri-Aria KT, Jacobson MR, et al. Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF-beta expression. J Immunol 2007;178:4658-66.

Platts-Mills TA, von Maur RK, Ishizaka K, Norman PS, Lichtenstein LM. IgA and IgG anti-ragweed antibodies in nasal secretions. Quantitative measurements of antibodies and correlation with inhibition of histamine release. J Clin Invest 1976;57:1041-50.

Plewako H, Arvidsson M, Oancea I, Hasseus B, Dahlgren U, Rak S. The effect of specific immunotherapy on the expression of costimulatory molecules in late phase reaction of the skin in allergic patients. Clin Exp Allergy 2004;34:1862-7.

Plewako H, Wosinska K, Arvidsson M, et al. Basophil interleukin 4 and interleukin 13 production is suppressed during the early phase of rush immunotherapy. Int Arch Allergy Immunol 2006;141:346-53. *a*.

Plewako H, Wosinska K, Arvidsson M, Bjorkander J, Hakansson L, Rak S. Production of interleukin-12 by monocytes and interferon-gamma by natural killer cells in allergic patients during rush immunotherapy. Ann Allergy Asthma Immunol 2006;97:464-8. *b*.

Plewako H, Holmberg K, Oancea I, Gotlib T, Samolinski B, Rak S. A follow-up study of immunotherapy-treated birch-allergic patients: effect on the expression of chemokines in the nasal mucosa. Clin Exp Allergy 2008;38:1124-31.

Polosa R, Li Gotti F, Mangano G, et al. Effect of immunotherapy on asthma progression, BHR and sputum eosinophils in allergic rhinitis. Allergy 2004;59:1224-8.

Pradalier A, Basset D, Claudel A, et al. Sublingual-swallow immunotherapy (SLIT) with a standardized five-grass-pollen extract (drops and sublingual tablets) versus placebo in seasonal rhinitis. Allergy 1999;54:819-28.

Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. J Immunol 1993;151:1280-9.

Rachitskaya AV, Hansen AM, Horai R, et al. Cutting Edge: NKT Cells Constitutively Express IL-23 Receptor and ROR{gamma}t and Rapidly Produce IL-17 upon Receptor Ligation in an IL-6-Independent Fashion. J Immunol 2008;180:5167-71.

Radulovic S, Jacobson MR, Durham SR, Nouri-Aria KT. Grass pollen immunotherapy induces Foxp3-expressing CD4+ CD25+ cells in the nasal mucosa. J Allergy Clin Immunol 2008;121:1467-72, 1472 e1.

Rak S, Bjornson A, Hakanson L, Sorenson S, Venge P. The effect of immunotherapy on eosinophil accumulation and production of eosinophil chemotactic activity in the lung of subjects with asthma during natural pollen exposure. J Allergy Clin Immunol 1991;88:878-88.

Rantio-Lehtimaki A, Koivikko A, Kupias R, Makinen Y, Pohjola A. Significance of sampling height of airborne particles for aerobiological information. Allergy 1991;46:68-76.

Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR. Activation of CD4+ T cells, increased Th2 type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin Immunol 1993;92:313-24.

Rolinck-Werninghaus C, Kopp M, Liebke C, Lange J, Wahn U, Niggemann B. Lack of detectable alterations in immune responses during sublingual immunotherapy in children with seasonal allergic rhinoconjunctivitis to grass pollen. Int Arch Allergy Immunol 2005;136:134-41.

Ross RN, Nelson HS, Finegold I. Effectiveness of specific immunotherapy in the treatment of hymenoptera venom hypersensitivity: a meta-analysis. Clin Ther 2000;22:351-8.

Rossi RE, Monasterolo G, Coco G, Silvestro L, Operti D. Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. Vaccine 2007;25:957-64.

Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. J Immunol 1993;150:5445-56.

Royer B, Varadaradjalou S, Saas P, Guillosson JJ, Kantelip JP, Arock M. Inhibition of IgE-induced activation of human mast cells by IL-10. Clin Exp Allergy 2001;31:694-704.

Satoguina JS, Weyand E, Larbi J, Hoerauf A. T regulatory-1 cells induce IgG4 production by B cells: role of IL-10. J Immunol 2005;174:4718-26.

Savolainen J, Laaksonen K, Rantio-Lehtimaki A, Terho EO. Increased expression of allergen-induced in vitro interleukin-10 and interleukin-18 mRNA in peripheral blood mononuclear cells of allergic rhinitis patients after specific immunotherapy. Clin Exp Allergy 2004;34:413-9.

Savolainen J, Jacobsen L, Valovirta E. Sublingual immunotherapy in children modulates allergen-induced in vitro expression of cytokine mRNA in PBMC. Allergy 2006;61:1184-90.

Schnyder B, Schnyder-Candrian S, Pansky A, et al. IL-17 reduces TNF-induced Rantes and VCAM-1 expression. Cytokine 2005;31:191-202.

Schnyder-Candrian S, Togbe D, Couillin I, et al. Interleukin-17 is a negative regulator of established allergic asthma. J Exp Med 2006;203:2715-25.

Schubert R, Eickmeier O, Garn H, et al. Safety and immunogenicity of a cluster specific immunotherapy in children with bronchial asthma and mite allergy. Int Arch Allergy Immunol 2009;148:251-60.

Schwarzenberger P, La Russa V, Miller A, et al. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. J Immunol 1998;161:6383-9.

Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. J Exp Med 1993;178:2123-30.

Sergejeva S, Ivanov S, Lotvall J, Linden A. Interleukin-17 as a recruitment and survival factor for airway macrophages in allergic airway inflammation. Am J Respir Cell Mol Biol 2005;33:248-53.

Shim JY, Kim BS, Cho SH, Min KU, Hong SJ. Allergen-specific conventional immunotherapy decreases immunoglobulin E-mediated basophil histamine releasability. Clin Exp Allergy 2003;33:52-7.

Shin HC, Benbernou N, Esnault S, Guenounou M. Expression of IL-17 in human memory CD45RO+ T lymphocytes and its regulation by protein kinase A pathway. Cytokine 1999;11:257-66.

Smith H, White P, Annila I, Poole J, Andre C, Frew A. Randomized controlled trial of high-dose sublingual immunotherapy to treat seasonal allergic rhinitis. J Allergy Clin Immunol 2004;114:831-7. *a*.

Smith TR, Alexander C, Kay AB, Larche M, Robinson DS. Cat allergen peptide immunotherapy reduces CD4(+) T cell responses to cat allergen but does not alter suppression by CD4(+) CD25(+) T cells: a double-blind placebo-controlled study. Allergy 2004;59:1097-101. *b*.

Stock P, Akbari O, Berry G, Freeman GJ, Dekruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. Nat Immunol 2004;5:1149-56.

Song C, Luo L, Lei Z, et al. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. J Immunol 2008;181:6117-24.

Sun JB, Czerkinsky C, Holmgren J. Sublingual 'oral tolerance' induction with antigen conjugated to cholera toxin B subunit generates regulatory T cells that induce apoptosis and depletion of effector T cells. Scand J Immunol 2007;66:278-86.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100:655-69.

Takanaski S, Nonaka R, Xing Z, O'Byrne P, Dolovich J, Jordana M. Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. J Exp Med 1994;180:711-5.

Tarzi M, Klunker S, Texier C, et al. Induction of interleukin-10 and suppressor of cytokine signalling-3 gene expression following peptide immunotherapy. Clin Exp Allergy 2006;36:465-74.

Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 2006;117:433-42.

Tazaki T, Minoguchi K, Yokoe T, et al. Allergen rush immunotherapy increases interleukin (IL)-12 production and IL-12 receptor beta2 chain expression in patients with allergic asthma. Cell Immunol 2004;228:20-6.

Till S, Walker S, Dickason R, et al. IL-5 production by allergen-stimulated T cells following grass pollen

immunotherapy for seasonal allergic rhinitis. Clin Exp Immunol 1997;110:114-21.

Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. J Allergy Clin Immunol 2004;113:1025-34; quiz 1035.

Toda M, Leung DY, Molet S, et al. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. J Allergy Clin Immunol 2003;111:875-81.

Valovirta E, Jacobsen L, Ljorring C, Koivikko A, Savolainen J. Clinical efficacy and safety of sublingual immunotherapy with tree pollen extract in children. Allergy 2006;61:1177-83.

van Neerven RJ, Wikborg T, Lund G, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. J Immunol 1999;163:2944-52.

Van Metre TE, Adkinson NF, Jr., Amodio FJ, et al. A comparative study of the effectiveness of the Rinkel method and the current standard method of immunotherapy for ragweed pollen hay fever. J Allergy Clin Immunol 1980;66:500-13.

Varney VA, Hamid QA, Gaga M, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. J Clin Invest 1993;92:644-51.

Verhoef A, Alexander C, Kay AB, Larche M. T cell epitope immunotherapy induces a CD4+ T cell population with regulatory activity. PLoS Med 2005;2:e78.

Visco V, Dolecek C, Denepoux S, et al. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. J Immunol 1996;157:956-62.

Volpe E, Servant N, Zollinger R, et al. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. Nat Immunol 2008;9:650-7.

von Hertzen L, Makela MJ, Petays T, et al. Growing disparities in atopy between the Finns and the Russians: a comparison of 2 generations. J Allergy Clin Immunol 2006;117:151-7.

Voo KS, Wang YH, Santori FR, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. Proc Natl Acad Sci U S A 2009;106:4793-8.

Wachholz PA, Nouri-Aria KT, Wilson DR, et al. Grass pollen immunotherapy for hayfever is associated with increases in local nasal but not peripheral Th1:Th2 cytokine ratios. Immunology 2002;105:56-62.

Wachholz PA, Soni NK, Till SJ, Durham SR. Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. J Allergy Clin Immunol 2003;112:915-22.

Wakashin H, Hirose K, Maezawa Y, et al. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. Am J Respir Crit Care Med 2008;178:1023-32.

Wiehler S, Proud D. Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection. Am J Physiol Lung Cell Mol Physiol 2007;293:L505-15.

Wierenga EA, Snoek M, Jansen HM, Bos JD, van Lier RA, Kapsenberg ML. Human atopen-specific types 1 and 2 T helper cell clones. J Immunol 1991;147:2942-9.

Wilcock LK, Wachholz PA, Na DH, et al. Time-course of serum IgG4 levels and IgG inhibitory activity following withdrawal of grass pollen immunotherapy (IT). Journal of Allergy and Clinical Immunology 2005;115:S265.

Wilson DR, Nouri-Aria KT, Walker SM, et al. Grass pollen immunotherapy: symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season. J Allergy Clin Immunol 2001;107:971-6. *a*

Wilson DR, Irani AM, Walker SM, et al. Grass pollen immunotherapy inhibits seasonal increases in basophils and eosinophils in the nasal epithelium. Clin Exp Allergy 2001;31:1705-13. *b*

Wilson DR, Torres LI, Durham SR. Sublingual immunotherapy for allergic rhinitis. Cochrane Database Syst Rev. 2003;(2):CD002893.

Wilson DR, Lima MT, Durham SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. Allergy 2005;60:4-12.

Wilson NJ, Boniface K, Chan JR, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nat Immunol 2007;8:950-7.

Winther L, Malling HJ, Moseholm L, Mosbech H. Allergen-specific immunotherapy in birch- and grass-pollen-allergic rhinitis. I. Efficacy estimated by a model reducing the bias of annual differences in pollen counts. Allergy 2000;55:818-26.

Wong CK, Ho CY, Ko FW, et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin Exp Immunol 2001;125:177-83.

Yagi H, Nomura T, Nakamura K, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 2004;16:1643-56.

Yao Z, Painter SL, Fanslow WC, et al. Human IL-17: a novel cytokine derived from T cells. J Immunol 1995;155:5483-6.

Yao Z, Spriggs MK, Derry JM, et al. Molecular characterization of the human interleukin (IL)-17 receptor. Cytokine 1997;9:794-800.

Ye P, Rodriguez FH, Kanaly S, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 2001;194:519-27.

Ying S, Barata LT, Meng Q, et al. High-affinity immunoglobulin E receptor (Fc epsilon RI)-bearing eosinophils, mast cells, macrophages and Langerhans' cells in allergen-induced late-phase cutaneous reactions in atopic subjects. Immunology 1998;93:281-8.

Yoshimoto T, Okamura H, Tagawa YI, Iwakura Y, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. Proc Natl Acad Sci U S A 1997;94:3948-53.

Yoshimoto T, Tsutsui H, Tominaga K, et al. IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. Proc Natl Acad Sci U S A 1999;96:13962-6.

Yoshimura T, Takeda A, Hamano S, et al. Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism. J Immunol 2006;177:5377-85.

Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 1997;272:21597-603.

Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89:587-96.

Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006;24:209-26.

Zweiman B, Haralabatos IC, Pham NC, David M, von Allmen C. Sequential patterns of inflammatory events during developing and expressed skin late-phase reactions. J Allergy Clin Immunol 2000;105:776-81.