Der p 1-

specific Treg and Breg cells in allergen immunotherapy and mechanism of immune tolerance

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การศึกษาเม็ดเลือดขาวชนิดทีและบีเซลล์ชนิดควบคุมที่จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่นและกลไกของ
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พ.ท.ธเดช บุญปิยทัศน์

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ธเดช บุญปียทัศน์ : การศึกษาเม็ดเลือดขาวชนิดทีและบีเซลล์ชนิดควบคุมที่จำเพาะต่อสารก่อ
ภูมิแพ้ไรฝุ่นและกลไกของระบบภูมิคุ้มกันที่ทนต่อสารก่อภูมิแพ้ไรฝุ่น. (Der p 1-
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วัคซีนโรคภูมิแพ้คือการฉีดสารก่อภูมิแพ้ซ้ำๆเพื่อให้อาการภูมิแพ้ดีขึ้นและทนต่อสารก่อ
ภูมิแพ้ การสร้างเม็ดเลือดขาวชนิดที-เร็คและบี-เร็คเซลล์ชนิดควบคุมเป็นสิ่งจำเป็นสำหรับภูมิคุ้มกันเพื่อ
ทนต่อสารก่อภูมิแพ้และทำให้การรักษาด้วยวัคซีนโรคภูมิแพ้ประสบความสำเร็จ จุดมุ่งหมายของการ
วิจัยเพื่อศึกษาบทบาทของเม็ดเลือดขาวที-เร็คที่จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่นชนิดที่ทำหน้าที่ควบคุม
ระบบภูมิคุ้มกันที่มีประสิทธิภาพ และที่ไม่มีประสิทธิภาพ และเม็ดเลือดขาวบี-เร็คเซลล์ชนิดควบคุมที่
หลั่งสารอินเตอร์ลิวคิน 10 และ 1RA ว่ามีการเปลี่ยนแปลงระหว่างการได้รับการรักษาด้วยวัคซีนโรค
ภูมิแพ้หรือไม่อย่างไร เป็นการศึกษาในอาสาสมัครโรคภูมิแพ้ที่ได้รับการรักษาด้วยวัคซีนโรคภูมิแพ้ต่อ
สารก่อภูมิแพ้ไรฝุ่นโดยการฉีดเข้าใต้ผิวหนังจำนวน 25 ราย (20 รายตอบสนองดีต่อการรักษา 5 รายการ
รักษาไม่ได้ผล) ทำการตรวจวัดการตอบสนองของเม็ดเลือดขาวชนิดที-เร็คชนิดควบคุมที่จำเพาะต่อสาร
ก่อภูมิแพ้ไรฝุ่นด้วยวิธีการวิเคราะห์ลักษณะพิเศษของเซลล์ที่เป็นบวกต่อ tetramer-allergen-MHC-class
II ในส่วนของเม็ดเลือดขาวบีเซลล์ที่จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่นทำการตรวจด้วยวิธี fluorochrome-
labeled Der p 1 และได้รับการวิเคราะห์โดยวิธี flow cytometry ทำการหาความสัมพันธ์กับการ
ตอบสนองทางคลินิกต่อวัคซีนโรคภูมิแพ้ไรฝุ่น ผลการศึกษาพบว่าวัคซีนโรคภูมิแพ้ไรฝุ่นสามารถทำให้
เกิดการสร้างเม็ดเลือดขาวที-เร็คที่จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่นที่มีประสิทธิภาพเพิ่มขึ้น ใน
ขณะเดียวกันสามารถลดการสร้างเม็ดเลือดขาวที-เร็คที่จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่นที่ไม่มี
ประสิทธิภาพ ในส่วนของเม็ดเลือดขาวบี-เร็คพบว่าวัคซีนโรคภูมิแพ้ไรฝุ่นสามารถทำให้จำนวนของ
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Allergen-specific immunotherapy (AIT) involves the repeated administration of allergen products in order to induce clinical and immunologic tolerance to the offending allergen. The generation and maintenance of functional allergen-specific regulatory T (Treg) cells and regulatory B (Breg) cells are known in bee venom immunotherapy. However, in aeroallergen, house dust mite has not to be studied. The aim of the study is to identify the possible roles of Der p 1-specific active Treg (FOXP3+Helios+CD25+CD127-CD4+), ineffective Treg (ILT3-CD25+CD4+) and Breg (IL-10+IL-1RA+CD73-CD25+CD71+) cells changes during AIT and its correlation to treatment outcomes. We studied 25 allergic patients (20 responders and 5 non-responders) undergoing subcutaneous house dust mite immunotherapy. House dust mite-specific Treg cells responses were investigated by characterization of Der p 1-MHC-class II tetramer-positive cells. House dust mite-specific B cells were detected using fluorochrome-labeled Der p 1 method. Der p 1-specific Treg and Breg cells were analysed by flow cytometry and correlated to clinical response to AIT. We demonstrated that AIT induced activated Der p 1-specific Treg cells and decreased substantially ineffective Der p 1-specific Treg cells. For B cell responses, we found a significant increase of IL-10-and IL-1RA-producing Breg cells after AIT in responding patients. In conclusion, AIT-induced clinical improvement is significantly correlated with the increased of Der p 1-specific Treg and Breg cells.

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Field of Study:	Clinical Sciences	Advisor's Signature	
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TABLE OF CONTENTS

ACRONYMS

AIT: Antigen-specific immunotherapy

AR: Allergic rhinitis

Breg: B regulatory

HDM: House dust mite

IL-1RA: Interleukin-1 receptor antagonist
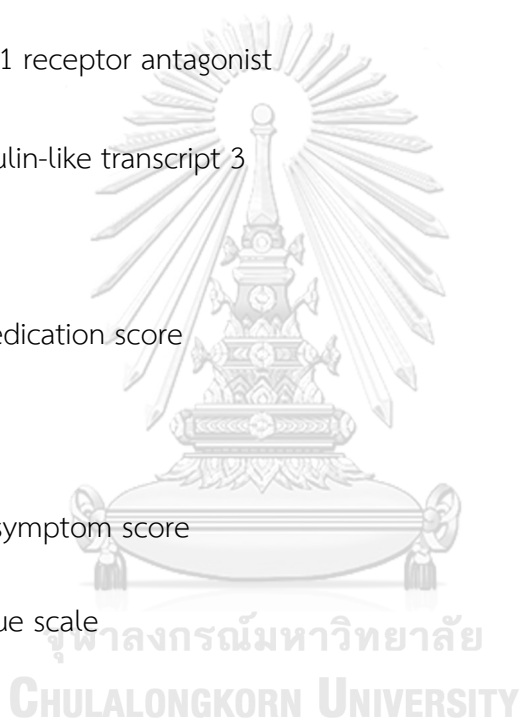
ILT3: Immunoglobulin-like transcript 3

SPT: Skin prick test

SMS: Symptom-medication score

Treg: T regulatory

TNSS: Total nasal symptom score

VAS: Visual analogue scale

1. Introduction

The pathophysiology of the allergic disease is complex and influenced by many factors, including genetic susceptibility, route of exposure, antigen/allergen dose, time of exposure with stimulators of innate immune response, such as infections or commensal bacteria. [1-3] Seasonal and perennial allergens can be inhaled, ingested and can enter the body via the damaged skin, and induce an IgE-mediated local or systemic inflammatory process. Allergen-specific immunotherapy (AIT) is an effective treatment for allergic asthma, allergic rhinitis, and atopic dermatitis as well as venom-induced anaphylaxis. In addition to reducing symptoms of the disease, AIT can provide long-term protection by inducing allergen-specific immune tolerance.[4]

At present, studies assessing allergen-specific T and B cells are a method to monitor immune tolerance in patients during AIT. HLA-peptide tetramer complexes facilitate the identification and characterization of antigen-specific T cells without the need for the cells to express particular functional activities. In allergen-specific B cells, fluorescently labeled antigen has been used to identify B cells with particular B cells receptor specificities by flow cytometry. This project aims to investigate the changing profile of allergen-specific Treg and Breg cells during AIT. IL-10 secreting Treg and Breg cells will be mainly focused. The results of this thesis elaborated a number of novel knowledge that has not been published before which include allergen-specific functional and inactive Treg cells have a role in AIT, allergen-specific B cells, plamablasts and Breg cells changed during house dust mite immunotherapy and Breg produced IL-1RA. These finding will help us better understanding of the complex networks of allergen immune tolerance.

2.   Literature Review

2.1 Allergen-specific immunotherapy (AIT)

Dysregulated immune tolerance plays an essential role in many allergic diseases including asthma, atopic dermatitis, allergic rhinitis, food allergy, venom allergy as well as autoimmune diseases, organ transplantation, tumors and chronic infections. Allergens enter the body via the respiratory tract, gut, conjunctiva, injured skin or insect stings, and most of the time the result is the induction of tolerance as a natural mechanism. AIT is the most efficient procedure to treat allergic patients with asthma, rhinitis, conjunctivitis, atopic dermatitis and stinging insect allergy. AIT has a disease-modifying effect and might also lead to a decrease in requirements for anti-inflammatory and symptomatic medications. AIT can also change the course of allergic disease symptoms and displays a long-term efficacy due to its induction of allergen-specific immune tolerance.[1, 5]

There are two phases to AIT: build up phase and maintenance phase. The build-up phase is initiated by administrations increasing concentrations of allergens. The injections are scheduled once a week for 3 – 6 months. In the maintenance phase, patients are planned to receive the most efficient dose usually every 4 weeks. A decision to stop AIT is made after 3 to 5 years of treatment. Because AIT is very time-consuming for patients and has a success rate of only 60-80%, it is essential to discover biomarkers identifying responder and non-responder patients before the immunotherapy is started.

2.2 Administration route of AIT

Noon and Freeman demonstrated that prophylactic subcutaneous injection (SCIT) with a grass pollen extract was effective in suppressing immediate conjunctival sensitivity to grass pollen.[6] Until recently, SCIT has been the gold standard administration route of AIT.[7] The conventional schedule for SCIT using allergen extracts consists of a dose build-up by injection once weekly, followed by maintenance dose injections at 4- to 8-week intervals.[8] At least 50 injections over 3

years is a significant disadvantage of conventional treatment. To shorten the build-up phase, there is a possibility to use cluster or rush schedules.[9] In cluster protocol, multiple injections are given on non-consecutive days, whereas in rush protocol multiple injections are given on consecutive days, reaching the maintenance phase in few days, but increasing the risk of systemic allergic reaction.[10] The new routes increase its attractiveness by enhancing treatment efficacy, reducing treatment duration, reduce the risk of anaphylactic reaction, minimizing costs of treatment and self-administrable treatment routes.

Currently investigated novel AIT routes include oral (OIT), sublingual (SLIT), intradermal (IDIT), local nasal (LNIT), intralymphatic (ILIT) and epicutaneous (EPIT). OIT involves the regular oral administration of small but increasing amounts of the allergen. OIT provides a long-term solution for food allergy such as milk, egg, peanut and wheat.[11, 12]

SLIT or oral allergy drop involves administering the allergens under the tongue generally on a daily basis. SLIT is safe, effectiveness, short or not need build-up phase.[13] SLIT is indicated in grass pollen and house dust mite allergic rhinitis that may exert a preventative effect on the development of asthma and the onset of new sensitizations.[14, 15] LNIT seems to be effective only on rhinitis symptoms and requires a particular technique of administration.[16] However, LNIT is not documented for clinical use. IDIT was applied into the substance of the skin as same as intradermal test. IDIT is safe and leading to symptoms relief after administration of few doses but is not clinically useful and resulted in worsening of respiratory allergic symptoms.[17] ILIT is a direct intralymphatic injection of the antigen to enhance the efficacy of AIT by a reduced amount of treatment application, reduced treatment duration, fast symptom alleviation and proper safety.[18, 19] However, ILIT requires ultrasound-guided and physician-administrated. EPIT has the potential to emerge as a promising needle-free treatment route and self-administration. EPIT also has a good safety profile, but local eczematous skin reactions may occur.[20]

2.3 Mechanisms of AIT-mediated induction of immune tolerance

The induction of immune tolerance has ideally been a primary objective for the prevention and treatment of allergic diseases. The known events involving the AIT-induced immune tolerance are as follows (Fig 1):[21]

- Rapid desensitization of mast cells and basophils by allergens
- Induction of peripheral T cell tolerance mainly by the generation of allergen-specific Treg cells which is introduced by IL-10 and TGF-β. There is also a suppression allergen-specific Th2 cells.
- Antibody class switching from IgE to IgG4 as indicated by early induction of IgG4 and a late decrease in IgE levels
- Induction of IL-10-producing regulatory B cells (Br1)
- Suppression of mast cells, basophils and eosinophils
- Suppression of inflammatory dendritic cells and induction tolerogenic dendritic cells
- Suppression of mucus production
- Suppression of tissue inflammation
- Suppression of tissue inflammation and T cell homing to tissues
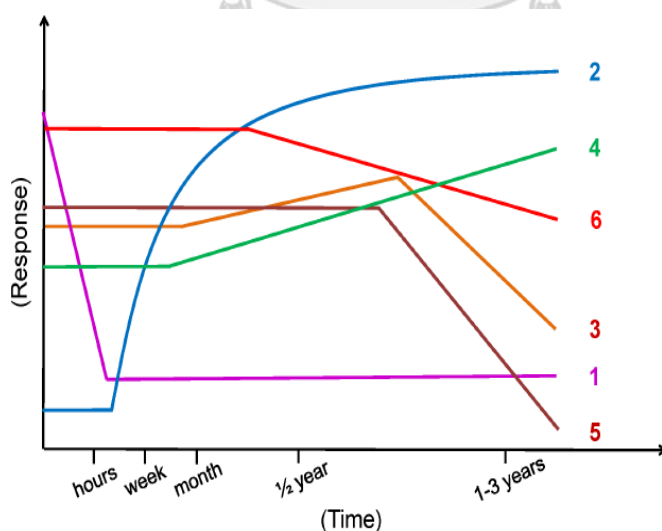
Figure 1. *Specific immune responses are observed during AIT. 1. An early desensitization effect including a decrease in mast cell and basophil degranulation soon after the first administration of allergens. 2. A generation of allergen-specific Treg cells and suppression of effector cells. 3.*

An early increase and a late decrease in specific IgE levels. 4. A relatively early increase in specific IgG4. 5. A late decrease in type I skin test reactivity. 6. A decrease in tissue mast cell and eosinophil numbers and a release of their mediators after a few months.[21]

In T cells tolerance, AIT induces a significant change in allergen-specific T cells subsets. The proportion of IL-4 producing Th2 cells decreases. Meanwhile, IL-10 producing inducible Treg cells increase in number and achieve the function similar to the immune status observed in healthy subjects.[22] The improvement of symptoms had the significant correlation with the increase in Treg cells number during AIT.[23] Two sets characterize the Treg cells: forkhead box protein 3 (Foxp3)-adaptive Treg cells and Foxp3^{-} but IL-10 secreting type 1 regulatory cell (Tr1).[24] Studies investigating the role of the different type of Treg cells during AIT have shown overlapping effects of different Treg cells subsets for the induction of T cell tolerance.[25, 26] It is well understood that there is reciprocal regulation between individual Th cell subsets, such as Th1, Th2, Th9, Th17, and Th22 however; Treg cells play an essential role in the suppression of effector T cell responses (Fig 2).
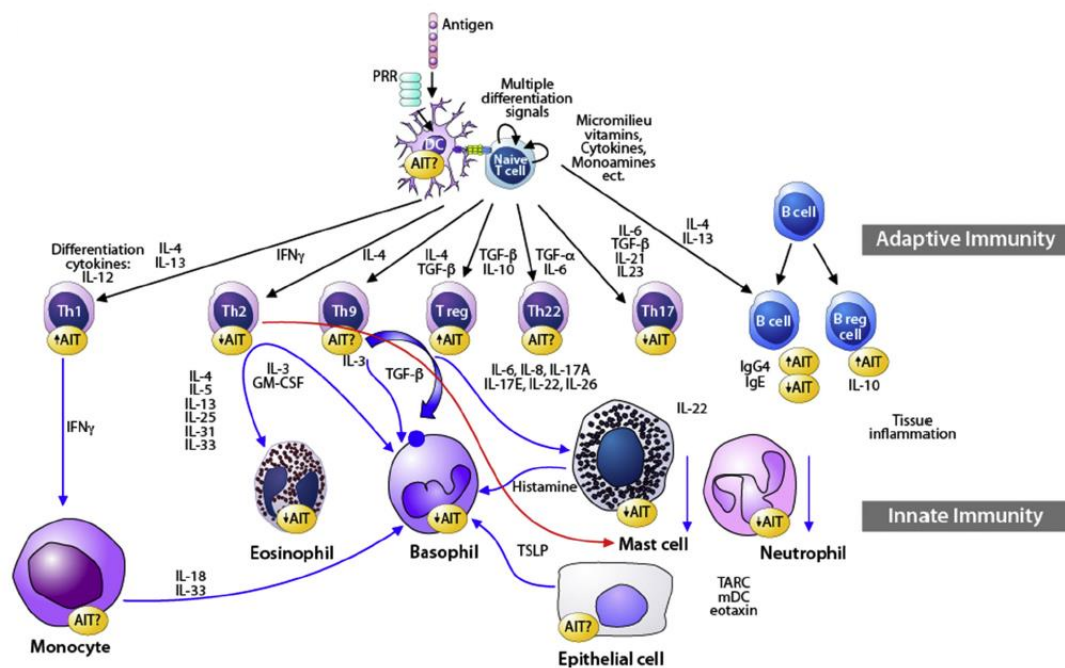
Figure 2. Cellular and molecular changes during AIT. Differentiation of naïve T cells after allergen presentation in the presence of innate immune response substances that trigger

recognition receptors (PRR) and vitamins, monoamines that control cellular differentiation and co-exposed substances with the antigen and status of the cells and cytokines in the microenvironment is shown. Naïve T cells can differentiate into Th1, Th2, Th9, Th17 and Th22 T cells. Based on their respective cytokine profiles, responses to chemokines, and interactions with other cells, these T cells subsets can contribute to general inflammation. The increase in Th1 and Treg cells number play a role in counterbalancing other effector cells. The balance between allergen-specific effector T cells (particularly Th2 cells) and IL-10 producing Treg cells is decisive for the development or suppressive of allergic inflammation. Treg cells and their cytokines suppress Th2-type immune response and contribute to the control of allergic disease in several major ways. Similarly, induction of IL-10 producing Breg cells plays an essential role in suppression of IgE and induction of IgG4.[27]

Helios expression is a marker of T cells activation and expression (Fig 3, A).[28] Also, Helios enhances induced Treg cell function in cooperation with FOXP3.[29] Moreover, Helios is selectively upregulated in $CD4^+$ T cells during Th2 and TFh responses to alum-protein vaccines.[30] Wegrzn et al. reported healthy control and stable asthma had the percentage of $FOXP3^+Helios^+$ Teg cells more than viral and non-viral exacerbation asthma, and correlated with FEV1.[31] The presence of local $FOXP3^+CD25^+CD3^+$ in the nasal mucosa, their increased number after immunotherapy, and their association with clinical efficacy and suppression of seasonal allergic inflammation invigorate the concept of local allergen tolerance depend on Treg cells.[26] The co-expression of $CD4^+CD25^+FOXP3^+$ and $ILT3^+$ represents a distinct Treg subset.[32] Protein kinase CK2 enable Treg cells to suppress excessive Th2 responses. ILT3 is regulated by the protein kinase CK2 and may work as an inactivating the regulatory function of Treg cell (Fig. 3B).[32] $ILT3^+$ Treg cells failed to suppress GATA-3 expression and instead induced the expression of IL-5 and IL-13, which further demonstrated the Th2 cells-promoting ability of $ILT3^+$ Treg cells.[32] Furthermore, the $LT3^+$ Treg cells percentage of healthy donors was a significant lower to compare with allergic patients.[32]
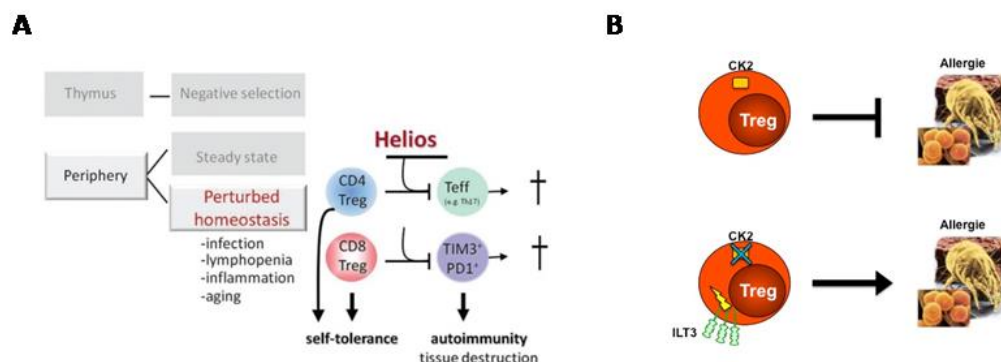
Figure 3. A, *Helios expression in Treg cell enhances function to suppress T effector cells.[14] **B,** Treg cell-specific gene targeting, that the suppression of Th2 cells was dependent on the activity of the protein kinase CK2. The ILT3 expression on Treg cell inhibits the activity of CK2.[18]*

AIT promotes not only IL-10 production by Tr1 cells but also promotes IL-10 secreting B regulatory 1 (Br1), which can regulate immune responses through suppression of antigen-specific CD4+ T cell proliferation and production of non-inflammatory IgG4 antibodies.[33] Distinct from IL-10-secreting DCs, IL-10 secreting allergen-specific Breg cells were shown to exist in bee venom tolerant beekeepers and patients with bee venom allergy who had undergone bee venom AIT.[33] Human Br1 cells are enriched for CD25^{+}CD71^{+}CD73^{-} B cells.[33] IgG4 antibodies have the unique ability to exchange Fab arms, which can lead to the generation of functionally monovalent antibodies that are preventing the formation.[34] Also, IgG4 can play an inhibitory role by interfering with allergen-mediated IgE-crosslinking, thereby preventing mast cell and basophil degranulation.[35, 36] In response to AIT typically a transient increase in serum -specific IgE is observed followed by the gradual decrease over subsequent months.[37, 38] On the other hand, serum specific IgG4 increases during AIT. However, the correlation between serum IgG4 and clinical outcome of AIT remains controversial.[39]

IL-1 is a potential mediator of inflammation and tissue damage in multiple organs. An IL-1 family is a group of 11 cytokines.[40] IL-1α and IL-1β are the most study members. IL-1β is a potent proinflammatory cytokine that is implicated in the

development of chronic inflammatory disease, such as, rheumatoid arthritis, cardiovascular disease and diabetes.[41] IL-1RA binds to the IL-1 receptor and thereby prevents binding of IL-1α and IL-1β that reduced the inflammation-related IL-1 disease.[40] IL-1RA is produced by the IL1RN gene.[42] The deficiency of the IL1RN gene caused very rare autoinflammatory, deficiency of II-1 receptor antagonist (DIRA) which causes the body is unable to regulate inflammation in the body properly.[42] IL-1RA is produced by monocytes, macrophages, neutrophils, epithelial cells, fibroblasts and mesenchymal stem cells, but has not been reported in Breg cells.[43]

IL-10 and IL-1RA-producing B cell changing during AIT are unknown. The effectiveness of IL1RA secreted by mesenchymal stem cells (MSCs) in controlling inflammation was further shown in vivo using the collagen-induced arthritis murine model.[43] MSCs lacking IL1RA expression were unable to protect mice from arthritic progression and even worsened clinical signs, as shown by higher arthritic score and incidence than control arthritic mice. IL1RA(-/-) MSCs were not able to decrease the percentage of Th17 lymphocytes and increase the percentage of Treg cells as well as decreasing the differentiation of B cells toward plasmablasts.[43]

2.4 Innate immune response in AIT

Dendritic cells (DC) is an antigen-presenting cell. DC can either initiate and sustain allergic inflammation or support the induction of tolerance by Treg cell responses.[44] DC subsets, such as regulatory DC (DCreg) cells and type 2 dendritic cells (DC2s), are already implicated in the pathomechanisms of allergic rhinitis.[45] DC2-associated markers, such as CD141, GATA3, OX40L, and receptor-interacting serine/threonine-protein kinase 4 (RIPK4), were downregulated after a 4-month sublingual AIT concomitantly with upregulation of DCreg cell-associated markers, including complement C1q subcomponent subunit A (C1QA), FCγRIIIA, ferritin light chain (FTL), and solute carrier organic anion transporter family member 2B1 (SLCO2B1), in the blood of clinical responders as opposed to nonresponders.[46]

Innate lymphoid cells (ILCs) are a recently identified group of lymphoid cells that lack an antigen receptor and develop from common lymphoid progenitors in fetal liver and bone marrow.[47] ILCs have been classified into 3 groups, and each group produces cytokines and transcription factors resembling each of the T helper cell subsets.[47, 48] Group 1 ILCs, which consists of classical natural killer (cNK) cells and ILC1s, can produce the Th1 cytokine, interferon (IFN)-γ and can express T-box transcription factor (T-bet). They have been implicated in Th1-like immunity against intracellular pathogens, such as viruses and intracellular bacteria.[49, 50] Group 2 ILCs (ILC2s), which consists of natural helper cells and nuocytes (inflammatory ILC2s), they can produce Th2 cytokines: IL-4, IL-5, IL-9, and IL-13 and can express GATA-3.[51-55] They have been implicated in immunity against helminths, allergic diseases, and tissue repair.[47] Group 3 ILCs (ILC3s) consist of 2 major subsets, natural cytotoxicity receptor (NCR), NCR⁺ ILC3s, and NCR⁻ ILC3s.[56] ILC3s can produce the Th17 cytokine, IL-17A - but IL-22 can only be produced by NCR⁺ ILC3s. ILC3s express retinoid-related orphan receptor γt (RORγt) and have a role in immunity against extracellular bacteria and implicated in the pathogenesis of some autoimmune diseases.[56]

Currently, it has been demonstrated that ILCs, especially ILC2s are involved in allergic airway inflammation. ILC2s in peripheral blood and bronchoalveolar lavage fluid (BALF), were increased in asthmatic patients compared with control subjects and were negatively correlated with airway function.[57-60] Many inhalant antigens trigger IL-33 and IL-25 production from epithelial cells and various immune cells and induce eosinophilic asthma-like airway inflammation through activation of lung ILC2s.[61-65] The strong protease activity of house dust mites (HDM) induces IL-33 release from epithelial cells, which induces asthma-like airway inflammation through ILC2 activation.[47] In patients with allergic rhinitis (AR), ILC2s are increased in peripheral blood after a cat allergen challenge and during grass pollen season.[66-68] The levels of ILC2s in HDM AR patients was higher compared with healthy controls and had a positive correlation with plasma IL-13 levels.[69, 70]

An Increase in Treg cells leads to the dose-dependent decrease in cytokine production by ILC2s.[71] Moreover, *in vitro* study shows recombinant TGF-β or IL-10 suppressed both human and murine ILC2s.[72] Also, human inducible Treg cells suppress syngeneic human ILC2s through the ICOSL to control airway inflammation in a humanized ILC2 mouse model.[72]

In Thai HDM AR patients, AIT responders and healthy subjects had a reduced the frequency of circulating ILC2s compared to AIT non-responders and AR patients.[73] Conversely, ILC1s from AIT responders and healthy subjects revaled increased frequency compared to AIT non-responders and AR patients. The frequency of ILC3s NCR[+] and NCR[-] in AIT responders showed significantly lower compared with AR patients and healthy subjects.[73] The ILC1: ILC2 proportion in AIT responder was approximate to that of healthy subjects.[73] Peripheral blood mononuclear cells from patients who were responders to AIT had a significantly lower expression of the activation marker CD69 on ILC2s in response to allergen restimulation compared to AR patients, but no difference compared to AIT non-responders and healthy subjects.[73]

2.5 Biological markers for AIT

Novel biomarkers are much needed for predicting AIT responses. They can be used to identify, before immunotherapy, responders versus non-responders, as well as to follow the therapy responsiveness of patients during the AIT or whether the individual may have a clinical relapse after a remission induced by AIT. The biomarkers are useful in designing appropriate treatment schemes and in deciding to stop AIT with the high degree of confidence. In current clinical practice, the immune monitoring parameter of AIT in patients is commonly restricted to measuring of allergen-specific IgE/IgG4 ratio in sera.[74-76] Recently, there have been newer assays used to determine the success of AIT including simple flow cytometry assay measuring IgE-facilitated allergen binding (FAB), and major histocompatibility complex

class II tetramer staining.[77, 78] Moreover, successful immunotherapy can be predicted by anergic transcriptional phenotypes in single allergen-specific T cells.[79] However, due to its complicated assay procedures, these assays are not routinely used in the clinical setting. Therefore, it is still necessary to identify novel surrogate markers for AIT that can conveniently be measured at the point of care. The assay should detect the biomarkers in easily accessible samples including whole blood, serum, body fluids or nasal scrapings. It should not involve difficult assay procedures and can be performed in a clinical setting that may not have cell culture facilities. Finally, the assay should be cost-effective. In recent years, exploratory technologies, such as proteomic techniques, gene arrays, mRNA arrays, multiplex analysis of secreted proteins in body fluids and multicolor flow cytometry of peripheral blood cells, have been rapidly improved. With the availability of this high throughput screening techniques, novel AIT-specific biomarkers should be more conveniently discovered shortly.

2.6 Der p 1-specific T and B cells

HDM is the most common aeroallergen in Thailand and can be efficiently treated by HDM AIT which also provides a suitable model to investigate allergen/antigen-specific immune tolerance.[80, 81] Der p 1 is the major HDM allergen which leads to augment total and IgE-specific antibody synthesis.[82] Proteolytic activity of Der p1 plays a role in directing the dendritic cell to induce Th2 differentiation and stimulate the epithelial-mesenchymal transition in airway epithelial cells, leading to airway remodeling.[83, 84] Patients allergic to HDM can be successfully treated with HDM AIT [85]. HDM-reactive IL-5$^+$IL-13$^+$CD27$^-$CD161$^+$CD4$^+$ cells and ST2$^+$CD45RO$^+$CD4$^+$ cells were significantly lower in active-responders to HDM sublingual immunotherapy [86].

The investigation of allergen-specific immune response by direct analysis of human peripheral blood T cells without any further culture provides an efficient tool

for the in-depth analyses of changes in allergen-specific T cells in allergen tolerance during AIT.[87] The specific immune responses of CD4^{+} T cells required antigen presentation through APC-displayed MCH-II-peptide complexes and interaction with T cells receptor.[88] Peptide-MHC class-II tetramer staining is one of the methods to characterize antigen-specific CD4^{+} T cells. Such tetramers classically consist of complexes composed of four MHC class II heterodimers loaded with the peptide representing a high-affinity T cells epitope. MHC molecule coupled to biotin can be multimerized subsequently with fluorescently labeled streptavidin, thus providing high-avidity reagents to observe epitope-specific T cells by flow cytometry.[89] However, the limitation of the method is the availability of recombinant MCH class-II molecule, knowledge of epitopes and recruiting participants to match with MHC class-II tetramer.[90]

Currently, several methods have been developed to examine antigen-specific B cells. Because of their low frequency, accurate detection and confirmation of specificity pose a challenge. Fluorescent multimers have been used to detect peanut allergen-specific as well as tetanus toxoid-specific circulating B cells.[91, 92] Moreover, a dual-labeling strategy as we used in this study was applied to investigate Ara h1 and Ara h2-specific B cells in peanut allergy.[93] Peanut-specific immunotherapy has been previously associated with an increased number of allergen-specific class-switched memory B cells and plasmablasts.[92, 93]

In the present study, we investigated the role of allergen-specific B cells in the induction of clinical and immune tolerance to HDM. Allergen-specific B cells are present in circulation at very low frequencies.[94] Small fractions (<0.05%) of B cells express a B cell receptor (BCR) that binds fluorescent dyes such as phycoerythrin (PE).[95] Therefore, accurate detection of allergen-specific B cells requires staining with antigens labeled with two structurally unrelated fluorescent dyes. We present here a reproducible technique to identify Der p 1-specific B cells with the method of dual-color allergen-labeled fluorescence staining.

Accordingly, AIT for the treatment of HDM-allergy will be performed and Der p 1-specific T and B cells will be analyzed in peripheral blood samples during AIT. Currently, a prospective analyzes of Der p 1-specific T and B cell responses during AIT has not been done. This study is for further understanding of the possible roles of Der p 1-specific T and B cell on AIT immune tolerance.

3. Objectives and Hypothesis

Primary objective:

To identify subset of Der p 1-specific functional Treg (FOXP3$^+$Helios$^+$CD25$^+$CD127$^-$CD4$^+$CD3$^+$), inactive Treg (ILT3$^+$CD25$^+$CD4$^+$CD3$^+$) and Breg (IL-10$^+$IL-1RA$^+$CD73$^-$CD25$^+$CD71$^+$CD19$^+$) cells that change during the course of AIT.

Secondary objective:

To identify biological markers for good AIT responsiveness in allergic patients

Hypothesis:

Allergen immunotherapy upregulates functional Treg (FOXP3$^+$Helios$^+$CD25$^+$CD127$^-$CD4$^+$CD3$^+$) and Breg (IL-10$^+$IL-1RA$^+$CD73$^-$CD25$^+$CD71$^+$CD19$^+$), but downregulates non-functioning or inactivation (ILT3$^+$CD25$^+$CD4$^+$CD3$^+$). These specific immunomodulation resulted to AIT-induced immunotolerance and clinical improvement (Fig 4).
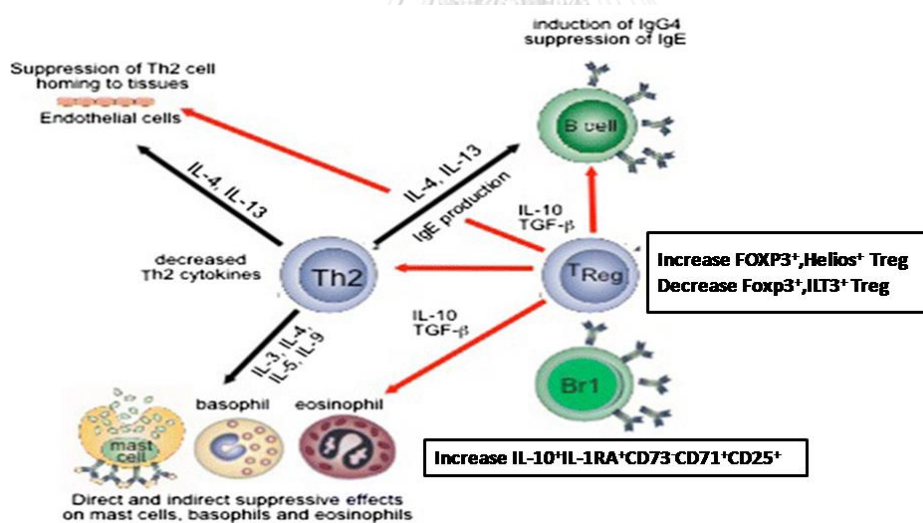

Figure. 4 *Mechanism of AIT includes our hypothesis.*[1]

4. Methods

4.1 Study population

Twenty-five adult allergic patients with allergic rhinitis or allergic asthma who are recommended for AIT by a physician at the Allergy Clinic, Pharmongkutklao Hospital, Bangkok, Thailand will be recruited to participate in this study. All participants met the following enrollment criteria: a history and objective symptoms of HDM-related and positive skin prick test (wheal diameter ≥ 6 mm^2 than negative control) with *D. pteronyssinus* extract (Alk-Abello, Round Rock, TX, USA). Approximately, 80% the allergic patients at the Allergic Clinic, Phramongkutklao hospital displayed positive skin prick test with mites.[96] A 30-week weekly dose escalation protocol was performed with the subcutaneous administration of standardized mite mixes, containing glycerin 50% v/v, phenol 0.4% (preservative), *D. pteronyssinus* 5,000 Allergy Units/ml and *D*. farinae 5,000 Allergy Units/ml (Alk-Abello). The study protocol is shown in Figure 5.
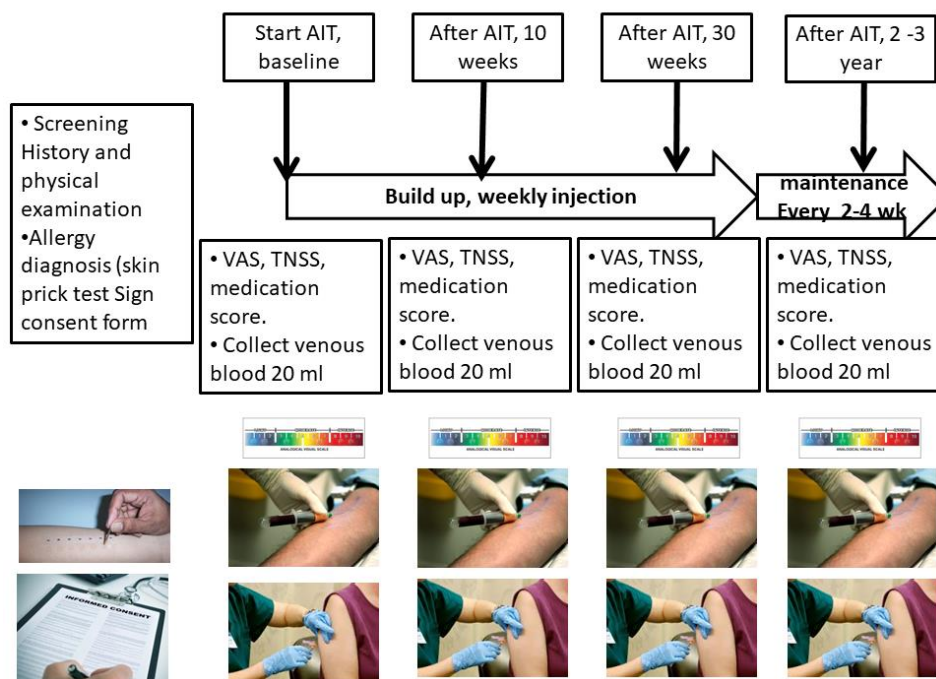
Figure 5. Study protocol. AIT; Allergen-specific immunotherapy, VAS; Visual analogue scale, TNSS; Total nasal symptom score.

Sample size

This study is entirely novel. We used data from the previous pilot study to calculate sample size. Our null hypothesis is that there will be no difference of frequency in Der p 1-specific Treg and Breg cell subsets between different time points. We will study the same subjects at 3 different time points thus. The previous pilot study, we found increased 25% in the frequency of allergen-specific T and B cells during AIT that significantly varies throughout AIT. Then the minimal clinical difference is 1.75 (25% increase from level observed in pilot data). The variance from my pilot data is 3.26. We considered that if the *P*-value is less than 0.05, there is sufficient evidence to reject the null hypothesis, as there is only a small chance of the results occurring if the null hypothesis is true. The formula to calculate sample size presents in figure 6.

$$N = \frac{Z(Z_{1-\alpha/2}+Z_{1-\beta})^2((t-1)p)}{t\left(\frac{MCD}{\sigma}\right)^2}$$

1. $\alpha = 0.05$
2. $\beta = 0.1$ (90% power)
3. $\sigma = 3.26$ (from my pilot study)
4. Minimal clinical difference (MCD) = 1.75
5. $t = 3$ (from 3 time points)
6. Within subject correlation = 0.5

$$N = \frac{21*(3-1)0.5}{3\left(\frac{1.75}{3.26}\right)^2}$$

$$N = 24.30$$

Figure 6. *The calculation of an adequate sample size determined in this study.*

Thus, we will have 25 subjects undergoing AIT for confirmation of changes during therapy.

Sample size calculations were done by Dr. Cameron Paul Hurst with R software.

4.2 Study protocol:

1. Clinical study

The study protocol will be explained to all precipitants before recruiting the study. At baseline, all participants will be interviewed for allergic symptoms and examine allergen by using skin prick test. Then the participants will be started AIT and followed the protocol of injection mite immunotherapy until 3 years of AIT (Table 1). The study protocol was approved by the Institutional Review Board of Royal Thai Army's ethics committee, IRBRTA 67/2556 and the Faculty of Medicine, Chulalongkorn University, IRB No. 193/60. All patients were recruited after obtaining written informed consent.

Table 1. Schedule of injection mite immunotherapy

Visit	Antigenic dilution concentration	Volume (ml)	Antigen (AU)	Accumulate antigen (AU)
W1	1:1000 (5 AU/ml)	0.05	0.25	0.25
W2	1:1000 (5 AU/ml)	0.1	0.5	0.75
W3	1:1000 (5 AU/ml)	0.15	0.75	1.5
W4	1:1000 (5 AU/ml)	0.2	1	2.5
W5	1:1000 (5 AU/ml)	0.25	1.25	3.75
W6	1:1000 (5 AU/ml)	0.3	1.5	5.25
W7	1:1000 (5 AU/ml)	0.35	1.75	7
W8	1:1000 (5 AU/ml)	0.4	2	9
W9	1:1000 (5 AU/ml)	0.45	2.25	11.25
W10	1:1000 (5 AU/ml)	0.5	2.5	13.75

W11	1:100 (50 AU/ml)	0.05	2.5	16.25
W12	1:100 (50 AU/ml)	0.1	5	21.25
W13	1:100 (50 AU/ml)	0.15	7.5	28.75
W14	1:100 (50 AU/ml)	0.2	10	38.75
W15	1:100 (50 AU/ml)	0.25	12.5	51.25
W16	1:100 (50 AU/ml)	0.3	15	66.25
W17	1:100 (50 AU/ml)	0.35	17.5	83.75
W18	1:100 (50 AU/ml)	0.4	20	103.75
W19	1:100 (50 AU/ml)	0.45	22.5	126.25
W20	1:100 (50 AU/ml)	0.5	25	151.25
W21	1:10 (500 AU/ml)	0.05	25	176.25
W22	1:10 (500 AU/ml)	0.1	50	226.25
W23	1:10 (500 AU/ml)	0.15	75	301.25
W24	1:10 (500 AU/ml)	0.2	100	401.25
W25	1:10 (500 AU/ml)	0.25	125	526.25
W26	1:10 (500 AU/ml)	0.3	150	676.25
W27	1:10 (500 AU/ml)	0.35	175	851.25
W28	1:10 (500 AU/ml)	0.4	200	1051.25
W29	1:10 (500 AU/ml)	0.45	225	1276.25
W30	1:10 (500 AU/ml)	0.5	250	1526.25

After 30 weeks, patients undergo 0.5 ml (1:10 concentration) every 2 – 4 weeks until 2 - 3 years.

2. **Clinical assessment**

The participants will assess allergic symptoms by using the visual analogue scale. Total nasal symptoms score (TNSS, possible score 0 -12) is widely used to evaluate the severity of allergic rhinitis.[97] Because we recruited patients with among allergic rhinitis, asthma, and atopic dermatitis, that don't have the specific questionnaire to access various allergic diseases in the same scale. Allergic symptoms are monitored with visual analogue scale (VAS) and validated can be used to follow up symptoms in allergic rhinitis, asthma and atopic dermatitis patients.[98-100] The VAS is a measurement instrument that is determined by measuring the distance (mm) on the 10-cm line. A VAS is a measurement instrument that tries to measure a characteristic or attitude that is believed to range across a continuum of values and cannot easily be directly measured. For example, the amount of allergic symptoms that a patient feels ranges across a continuum from none to an extreme amount of severe. From the patient's perspective, this spectrum appears continuous ± their allergic symptoms do not take discrete jumps, as a categorization of none, mild, moderate and severe would suggest. It was to capture this idea of an underlying continuum that the VAS was devised. The patient marks on the line the point that they feel represents their perception of their current state.[101] The patient had to record on the same questionnaire whenever they used medication (1 point: to use beta-2 agonists, antihistamine, pseudoephedrine and montelukast; 2 points: inhaled/intranasal corticosteroid; 3 points: one tablet of corticosteroid) and were calculated as Total medication score (TMS).[102] Symptom-mediation score (SMS) combines clinical symptom (VAS) and TMS, which represents treatment efficacy and assesses the different outcome measures to discriminate between active treatment and placebo.[103, 104]

3. Laboratory study

The 20 ml venous blood is collected at baseline, 10 weeks, 30 weeks, 2 years and 3 years during AIT. Peripheral blood mononuclear cells (PBMCs) will be isolated by low-density gradient centrifugation (Ficoll-Paque Plus) from peripheral blood.

Plasma will be stored in 2 mL microcentrifuge tube. All samples will be frozen in -80 °C liquid nitrogen until the final analysis at the laboratory at Division of Allergy and Clinical Immunology Department of Medicine Faculty of Medicine Chulalongkorn University.

Der p 1-specific B cells

To define the frequency of Der p 1-specific B cells, 10^7 PBMCs were stained using eF780 viability dye (eBioscience). Then cells were washed in staining buffer (PBS, pH 7.2, 0.5% bovine serum albumin, and 2 mM EDTA) and resuspended in 100 µL staining buffer. FcR blocking reagent (Miltenyi Biotec) was added to cells according to manufacturer's instructions. Afterward, cells were stained with antibodies against surface markers (Table 2) at 4°C for 15 min. Alexa Fluor 488-IgA (Jackson Laboratory, Bar Harbor, ME, USA) and Dy405-IgG1 (Sanquin, Amsterdam, Netherland) were stained to detect IgA and IgG1 respectively. For IgG4 staining, anti-IgG4 Ab (Alta Bioscience, Bermingham, UK) labeled with biotin (Innova Biosciences, Cambridge, UK) and Streptavidin-PE/Dazzle 594 (Biolegend) was used.

Table 2. Antibodies used in flow cytometry for B cells analysis

Antigen	Parameter	Clone	Manufacturer
CD19	BV510	HIB19	Biolegend
CD27	AF700	M-T271	Biolegend
IgM	BV605	MHM-88	Biolegend
IgM	PerCP/Cy5.5	MHM-88,	Biolegend
CD38	BV786	HIT2	BD Biosciences
CD3	eFlour780	UCHT1	eBiosciences
CD14	APC/Cy7	HCD14	Biolegend

CD16	APC/Cy7	3G8	Biolegend
CD73	PE	AD2	Biolegend
CD25	PE/Dazzle594	M-A251	Biolegend
CD71	PE/Cy5	M-A712	BD Biosciences
CD24	BV421	ML5	Biolegend
Streptavidin	PE/Dazzle594		Biolegend
IgA	AF488		Jackson Laboratory, USA
IgG1	Dy405		Sanquin, Netherland
IgG4	Biotin		Alta Bioscience, Bermingham, UK

To identify Der p 1-specific B cells, we selected BV510-CD19 (violet laser, Ex_{max} 415 nm) and labeled Der p 1 with Dy633 (red laser, Ex_{max} 633 nm) and PE-Cy7 (blue laser, Ex_{max} 496 nm) to detect the Der p 1-specific B cells by flow cytometry. After labeling of Der p 1 with Lightning-Link Dy633 Rapid Conjugate System and Lightning-Link PE-Cy7 Tandem Conjugate Kit (both from Innova Biosciences). The conjugated Der p 1 constructs were titrated for optimal concentration for staining. After viability dye staining, FcR blocking reagent (Miltenyi Biotec) was added to cells for blocking unspecific binding. We prepared the mixture of labeled Der p 1 allergens containing Der p 1- Dy633, Der p 1-PE-Cy7 and added all surface staining. We added the mixture into the sample to avoid blocking of the Der p 1-specific BCR that may occur in the case of adding Der p 1 conjugates sequentially. We purified double Der p1-Dy633^{+}, Der p 1-PE-Cy7^{+}, single Der p1- Dy633^{+} and single Der p 1-PE-Cy7^{+} class-switched memory B cells (Fig 7, A). After 7 days of culturing with CD40L and rIL-21, cells were immortalized by transduction with BCL6 and BCL-xl according to method by Kwakkenbos MJ, et al.[105] After in vitro expansion of immortalized B cells, we

found that more than 95% of the double Der p 1- Dy633$^+$Der p 1-PE-Cy7$^+$ B cells retained their double-color positive staining while single positive B cells did not (Fig 7, B). More importantly, we also measured Der p 1-specific-IgG levels in the culture supernatant after 7 days by ELISA. Der p1- Dy633$^+$Der p 1-PE-Cy7$^+$ B cells showed high Der p 1-specific IgG levels while single-color Der p 1$^+$ B cells did not produce Derp 1-specific IgG (Fig 7, C).
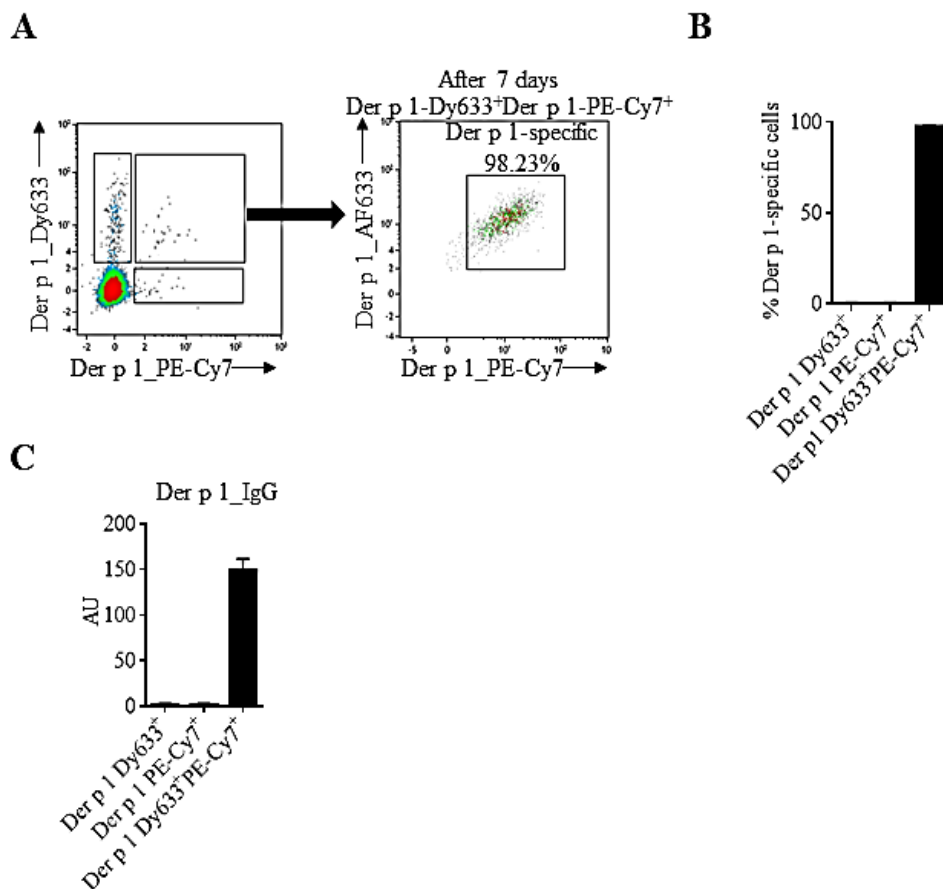
Figure 7. A, We purified double Der p 1-Dy633$^+$, Der p 1-PE-Cy7$^+$, single Der p1- Dy633$^+$and single Der p 1-PE-Cy7$^+$ class-switched memory B cells. The cells were cultured with CD40L and rIL-21cells, and immortalized by transduction with BCL6 and BCL-xl. After 7 days, the cells were stained with Der p 1-Dy633 and Der p 1-PE-Cy7 to identify Der p 1-specific B cells. B, Frequency of Der p 1-specific B cells after 7 days in vitro expansion of immortalized B cells (n = 5). C, Der p 1-specific-IgG levels in the culture supernatant after 7 days by ELISA.

For intracellular cytokine staining, 5x10^6 PBMCs were stimulated for 48 h with 1 µM of the TLR9 ligand CPG2006 (Microsynth, Balgach, Switzerland) in 2 ml

complete RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO USA). After 48 h, PBMCs were stimulated with 20 ng/ml PMA and 1 µg/ml ionomycin for 2 h followed by 2 h in the presence of 10 µg/ml brefeldin A (all from Sigma-Aldrich, St. Louis, MO, USA). After surface staining, cells were washed with staining buffer and fixed and permeabilized using Cytofix/CytopermKit (BD Biosciences, San Jose, CA, USA) and stained with anti-IL-10-PerCP/Cy5.5 (clone JES3-9D7, Biolegend) and anti-IL-1RA-FITC (clone CRM17, eBioscience), incubated for 30 min in the dark at 4°C and washed with staining buffer. All samples were analyzed and sorted with a FACSAria III (Beckton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed by Kaluza Software (Beckman Coulter, Indianapolis, IN, USA).

Purified recombinant Der p 1 (Allergopharma AG, Therwil, Switzerland) was labeled with Lightning-Link Dy633 Rapid Conjugate System and Lightning-Link PE/Cy7 Tandem Conjugate Kit (both from Innova Biosciences). The conjugated Der p 1 constructs were titrated for optimal concentration for staining. Der p 1-specific class-switched B cells were gated as $CD19^+IgM^-$ and double positive for Der p 1-Dy633 and Der p 1-PE/Cy7. As a negative control, cells were incubated with purified non-labeled Der p 1 for 15 minutes in the dark at 4°C to block Der p 1-specific BCRs before adding the conjugated Der p 1.

To measure Der p 1-specific immunoglobulins, 5 µg/ml Der p 1 was coated to a Nunc Maxisorb microtiter plate (Thermo Scientific, Waltham, MA, USA) at 4°C overnight and then blocked with blocking buffer (PBS pH7.4, 1% BSA, 0.05% Tween 20). Diluted plasma samples (in the blocking buffer) were added and incubated for 2 hours at room temperature. For specific IgG4 and IgE detection, mouse anti-human IgG4 and IgE (Bionostics, Devens, MA, USA) was used, followed by incubation with peroxidase-conjugated goat anti-mouse IgG and IgE (Thermo Scientific). For specific IgA detection, goat anti-human IgA-HRP (Bethyl Laboratories, Montgomery, TX, USA) and tetramethylbenzidine (TMB) substrate were used. The reaction was stopped by 2M sulfuric acid, and OD450 nm values were measured by a Mithras LB 940

spectrophotometer (Berthold Technologies, Bad Wildbad, Germany). The results were expressed as arbitrary units (AU) based on the positive in-house pool plasma of HDM allergic patients.

Der p 1-specific T cells

For Ag-specific tetramer, first, we checked HLA-DR typing. Genomic DNA was extracted from PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). HLA-DRB1 low-resolution and high-resolution typing performed using the AllSet+TM Gold DR Low and High Res SSP kits (Invitrogen, Carlsbad, CA, USA), according to the manufacturer protocols. Twelve out of 25 patients who were compatible with Der p 1 peptide (p)-MHCII tetramers HLA-DRB1*0410, HLA-DRB1*1101 and HLA-DRB1* 1501 were enrolled in the study. The patient characteristics are shown in Table 3.

Table 3. Patient characteristics in Der p 1-specific T cells study

Patient no.	Sex	HLA-DRB1	Disease
1	Male	0401	AR, AD
2	Male	1101	AR, AD
3	Female	1501	AR
4	Female	0401	AR
5	Female	1501	AR, AD
6	Male	1501	AR
7	Male	1101	AR
8	Female	1501	AR
9	Male	0401	AR, AA

10	Female	1501	AR, AA
11	Female	1101	AR, AA
12	Female	0401	AR, AA

AA; Allergic asthma, AD; Atopic dermatitis, AR; Allergic rhinitis.

For Der p 1-specific CD4^{+} T cell staining, PBMCs were enriched with anti-CD3 magnetic MicroBead (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated by autoMACS separator (Miltenyi Biotec) according to manufacturer's protocols. After enrichment, cells were stained with eFluor 780 viability dye (eBioscience, San Diego, CA, USA) for 30 minutes at 4 °C and washed in staining buffer (phosphate buffer saline, pH 7.2, 0.5% bovine serum albumin, and 2 mM EDTA). Cells were incubated with 2 μg/ml of phycoerythrin (PE)-Der p 1-tetramers in complete RPMI medium at room temperature for 30 minutes. The PE labeled-empty (without any peptide) HLA-DRB 0401, 1101 and 1501 tetramers, as well as non-MHC matching tetramers, were used as negative control. Epitopes from Der p 1 for HLA-DRB1*0401, DRB1*1101 and DRB1*1501 were did identified by Tetramer Guided Epitope Mapping (Table 4) [106]. Der p 1 specific MHCII-tetramer and empty tetramer-labeled with PE were produced by the Benaroya Research Institute (Seattle, WA, USA). The method to measure pMHCII-tetramer specificity was described in the previous study [87]. Cells were then stained with extracellular antibodies, V500-anti-CD3 (clone SP34-2, BD Biosciences, San Jose, CA, USA), PE/CF594-anti-CD4 (clone RPA-T4, BD Biosciences), APC/Cy7-anti-CD14 (clone HCD14, Biolegend, San Diego, CA, USA), APC/Cy7- anti-CD16 (clone 3G8, Biolegend), APC/Cy7-anti-CD19 (clone HIB19, Biolegend), BV421-anti-CD25 (clone BC6, Biolegend), BV605-anti-CD127 (clone A019D5, Biolegend) and PE/Cy7-anti-ILT3 (clone ZM4.1, Biolegend) in room temperature for 15 minutes. Following this, FOXP3 and Helios intracellular staining buffer (Biolegend) was performed following the manufacturer's protocols. Cells were stained with Alexa Fluor 488-anti-FOXP3 (clone 259D) and Alexa Fluor 647-anti-Helios (clone 22F6, both Biolegend) at room

temperature in the dark for 30 minutes. After washing in staining buffer, cells were resuspended in phosphate buffer saline with 0.1% paraformaldehyde and 2 mM EDTA. Cells were measured by FACSAria III (Beckton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed by Kaluza Software (Beckman Coulter, Indianapolis, IN, USA).

Table 4. Der p 1 T-cell epitopes

Tetramer Reagents	Sequence
DR0401/Der p 1 233-252	HSAIAVIIGIKDLDAFRHYD
DR1101/Der p 1 185-204	GVVQESYYRYVAREQSCRRP
DR1501/Der p 1 265-284	PNYHAVNIVGYSNAQGVDYW

For Intracellular cytokine staining, the anti-CD3 positively enriched cells were treated with phorbol 12-myristate 13 acetates (PMA) and ionomycin 4 hours and then with brefeldin A (all from Sigma-Aldrich, Buchs, Switzerland) 2 hours in cRPMI at 37 °C. Then, viability staining was performed first, followed by PE-Der p 1–tetramers and surface antibodies, V500-anti-CD3 (BD Biosciences), PE/CF594-anti-CD4 (BD Biosciences), APC/Cy7-anti-CD14 (Biolegend), APC/Cy7- anti-CD16 (Biolegend), APC/Cy7-anti-CD19 (Biolegend) and PerCP-anti-CD8 (clone SK1, Biolegend). Fixation/permeabilization staining (BD Bioscience) was performed according to the manufacturer's protocol. Cells were then stained with PE/Cy7-anti-IL-10 (clone JES3-9D7) for 30 minutes at 4 °C. Corresponding isotype-matched antibodies were used as negative controls in all stainings.

For Treg suppression assay, PBMCs from allergic asthma patients from Hochgebirgsklinik, Davos, Switzerland were enriched with CD4$^+$ T cell Isolation Kit (Miltenyi Biotec) and separated by autoMACS separator (Miltenyi Biotec). The enriched CD4$^+$ T cells were selected as the final negative fraction, and the final

positive fraction was irradiated and used as co-cultured cells. Then, CD4$^+$ T cells were stained with viability dye, anti-CD3, anti-CD4, anti-CD25 and anti-ILT3 as described above. ILT3$^+$ and ILT3$^-$ CD4$^+$CD25$^+$ Treg cells and CD4$^+$CD25$^-$ T effector cells were sorted by FACSAria III (Beckton Dickinson). T effector cells were stained with CellTrace violet cell proliferation kit (ThermoFisher, Waltham, MA, USA) according to the manufacturer's protocol. Next, Treg, T effector, and irradiated T cells were cultured with anti-CD3/anti-CD28 coated beads and IL-2 (all Sigma-Aldrich) in complete RPMI medium in 96 U bottom well plate at 37 C, 5% CO2 for 5 days. Treg cells and T effector cells were co-cultured in different Treg: T effector cell ratios. After 5 days, cells were harvested and washed 2 times with phosphate buffer saline. Cells were stained with 0.5 μl viability dye eFluor 780 (eBioscience) for 30 min at 4°C, and then cells were stained with FITC-anti-CD3 (clone 17A2), AF700-anti-CD4 (clone OKT4) and PE-anti-CD25 (clone BC96, all Biolegend) for 15 min at room temperature. Cells were washed 2 times with staining buffer and analysed by flow cytometry.

4. Statistical analysis

Data are expressed as a mean ± standard error of the mean (SEM). The Repeated Measures ANOVA with post-hoc analysis was used to compare related samples between different time points. The Mann-Whitney U test was used to compare differences between responder and non-responder groups. Spearmen's test was performed for correlation analyses. The correlation network was performed with R version 3.4.2 (The R Foundation for Statistical Computing, Vienna, Austria). Statistical

analyses and heat map correlation were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). P value < 0.05 was considered as significant.

5. Results

5.1 Der p 1-specific B cells

5.1.1 Characterisation of responder and non-responder patients after 2 years of AIT

Sixteen patients, who completed 2 years of AIT, were classified as responder or non-responder using SMS measured at baseline and after 2 years of AIT. The baseline patient characteristics are shown in Table 5. Eleven patients in the responder group improved clinically by decreased SMS from 13.23 ± 0.28 to 2.45 ± 0.24, $P = 0.001$ while SMS was 13.1 ± 1.1 to 10 ± 0.9, $P = 0.12$ in five clinically non-responder patients before and after 2 years of AIT, respectively (Fig 8, A). Also, the percentage change of SMS decreased significantly in responders more than non-responders (Fig 8, A). In the responder group, mean wheal diameter of skin prick test reactivity to HDM decreased from 7.0 ± 1.3 mm to 2.7 ± 0.5 mm, $P = 0.001$, whereas it was 7.6 ± 1.1 mm and 5.6 ± 0.5 mm, $P = 0.06$ in non-responder patients (Fig 8, B). Moreover, the relative percentage difference of mean wheal diameter was reduced significantly in responders compared to non-responders (Fig 8, B).

Table 5. *Patient baseline characteristic in Der p 1-specfici B cells study*

| Patient code	Sex	Age	Diagnosis	SPT Der p (mm)		Visual analogue scale				Total medication score	
				Before	2 y	Before	10 w	30 w	2 y	Before	2 y
Responder_AIT1	Male	35	AR	7	3	8	4	1	2	5	1
Responder_AIT2	Female	50	AR	6	3	8.5	5	0	2	6	1
Responder_AIT3	Male	25	AR, AA	8	3	7.5	3	2	1	7	2
Responder_AIT4	Male	41	AR	10	2	8	5	3	2	5	0
Responder_AIT5	Female	26	AR	6	2	8.5	7	2	2	4	1
Responder_AIT6	Male	60	AA	8	3	9	8.5	1	0	5	2
Responder_AIT7	Female	52	AR, AA	6	3	7	4.5	0	0	6	2
Responder_AIT8	Male	24	AR	8	3	7.5	5	2	1	4	0
Responder_AIT9	Female	52	AR, AA	6	2	6	4	1	0	7	2
Responder_AIT10	Female	27	AR, AD	6	2	8.5	7	2	3	4	1
Responder_AIT11	Male	20	AR	6	3	9	7.5	3	2	5	0
Non-responder_AIT1	Male	24	AR	8	6	9.5	9	4	6	5	3
Non-responder_AIT2	Female	64	AR, AA	8	5	9.5	7	2.5	6	6	5
Non-responder_AIT3	Female	18	AR	6	6	6.5	6.5	3	5	4	2
Non-responder_AIT4	Female	15	AR, AD	7	6	9.5	8	4	8	5	3
Non-responder_AIT5	Male	22	AA	9	5	6.5	4	4	6	4	6

AA; allergic asthma, AD; atopic dermatitis, AR; allergic rhinitis, SPT; skin prick test, Der p; Dermatophagoides pteronyssinus,

Total medication score; 1 point: for beta-2 agonists, antihistamine, pseudoehidrine and montelukast, 2 points: inhaled/intranasal steroids, 3 points: one tablet of corticosteroid)
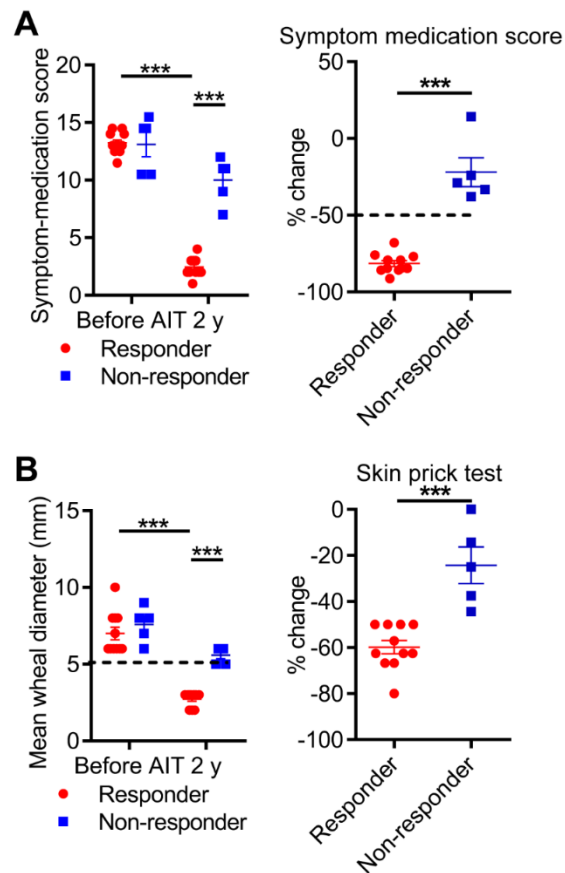
Figure 8. *Decreased clinical symptoms and house dust mite sensitization after 2 years of allergen-specific immunotherapy (AIT) in responder patients.* **A, the** *Clinical response was assessed by symptom-medication score (SMS) in house dust mite allergy undergoing subcutaneous injection AIT (responder patients, n = 11 and non-responder patients, n = 5) and percentage change of SMS between before and 2 years (2 y) AIT.* **B,** *Mean wheal diameter and percentage change of positive Dermatophagoides pteronyssinus skin prick test compared between before and 2 years of AIT. ***p < 0.001.*

In addition, SMS and mean wheal diameter of positive skin prick test showed positive correlation in responders (r = 0.77, *p* = 0.0002) and non-responders (r = 0.18, *p* = 0.43) after 2 years (Fig 9, A). The VAS and TMS also decreased significantly in responders more than non-responders (Fig 9, B and C).
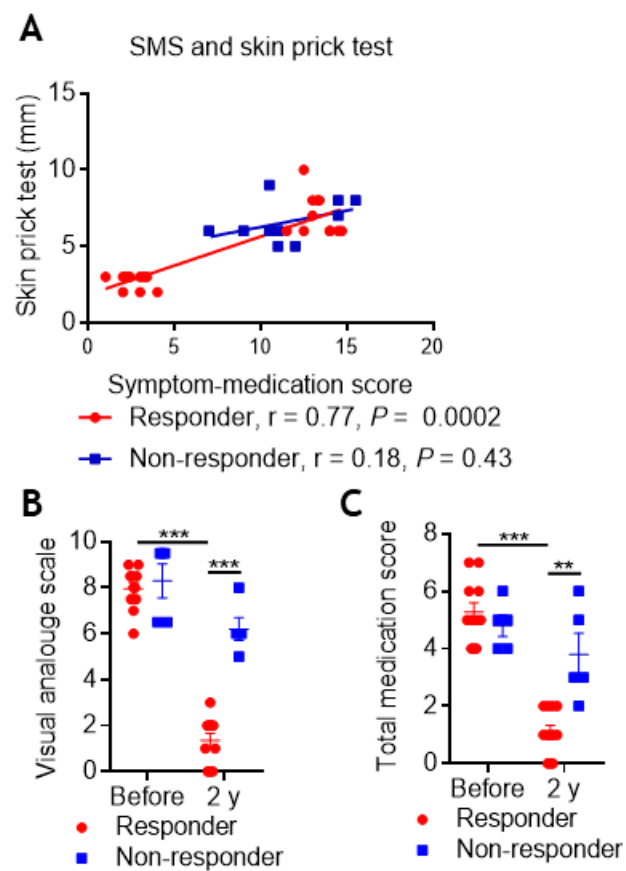
Figure 9. *A, Correlation between symptom-medication score and positive Dermatophagoides pteronyssinus skin prick test in responder and non-responder patients. B and C, Clinical response was assessed by visual analogue scale (VAS) and total medication score (TMS) in house dust mite allergy undergoing subcutaneous injection allergen-specific immunotherapy (AIT) compared between before and 2 years (2 y) of AIT, responder patients, n = 11 and non-responder patients, n = 5. **P < 0.01, ***P < 0.001*

### 5.1.2 Increased frequency of Der p 1-specific B cells during the 2 years of AIT

To study allergen-specific B cell responses during AIT, Der p 1-specific class-switched B cells (CD19$^+$IgM$^-$Der p 1$^+$) were analyzed using flow cytometry. The gating strategy and control are shown in figure 10. The frequency of Der p 1-specific B cells was investigated before the start, at 10 weeks and 30 weeks and after 2 years of AIT.

In responder patients, AIT significantly increased the frequency of Der p 1-specific class-switched B cells after 30 w and 2 years of AIT compared with baseline. Mean changes were from 0.049 ± 0.012 % at the baseline to 0.073 ± 0.019 % at 10 w, 0.097 ± 0.021 % at 30 w and 0.165 ± 0.029 % after 2 years (Fig 11, A and B). Next, we analysed a biomarker for AIT treatment efficacy. Increased Der p 1-specific B cells from baseline to 2 years was observed in responders (3.60 ± 1.20-fold increase after 2 years) compared to non-responder patients (0.59 ± 0.41-fold increase) (Fig 11, C).
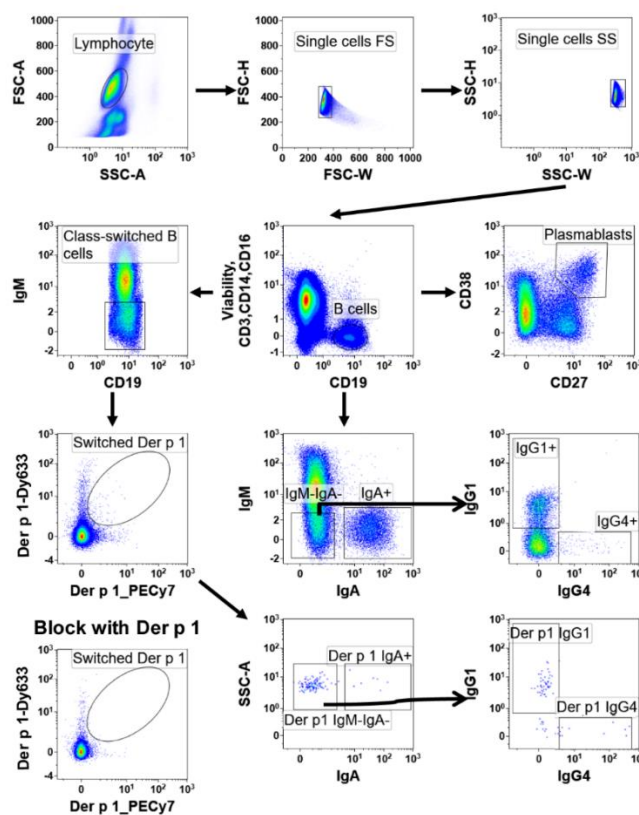
Figure 10. Gating strategy for Der p 1-specific cells in peripheral blood mononuclear cells. Lymphocytes were first gated from the forward and side scatter plot. After doublet discrimination, live CD19⁺CD3⁻CD14⁻CD16⁻ cells were gated. Then IgM⁻ (class-switched B cells) was gated. Der p1-Dy633⁺ and Der p 1-PECy7⁺ were identified as Der p 1-specific B cells. The negative control, cells were incubated with an excess of non-labeled Der p 1 to block subsequent binding of labeled Der p 1 to the B cells receptor. Der p 1-specific IgA⁺ B cells were gated on Der p 1-specific B cells and Der p 1-specific IgG1⁺ and IgG4⁺ B cells were gated on Der p 1-specific IgA⁻IgM⁻ B cells.
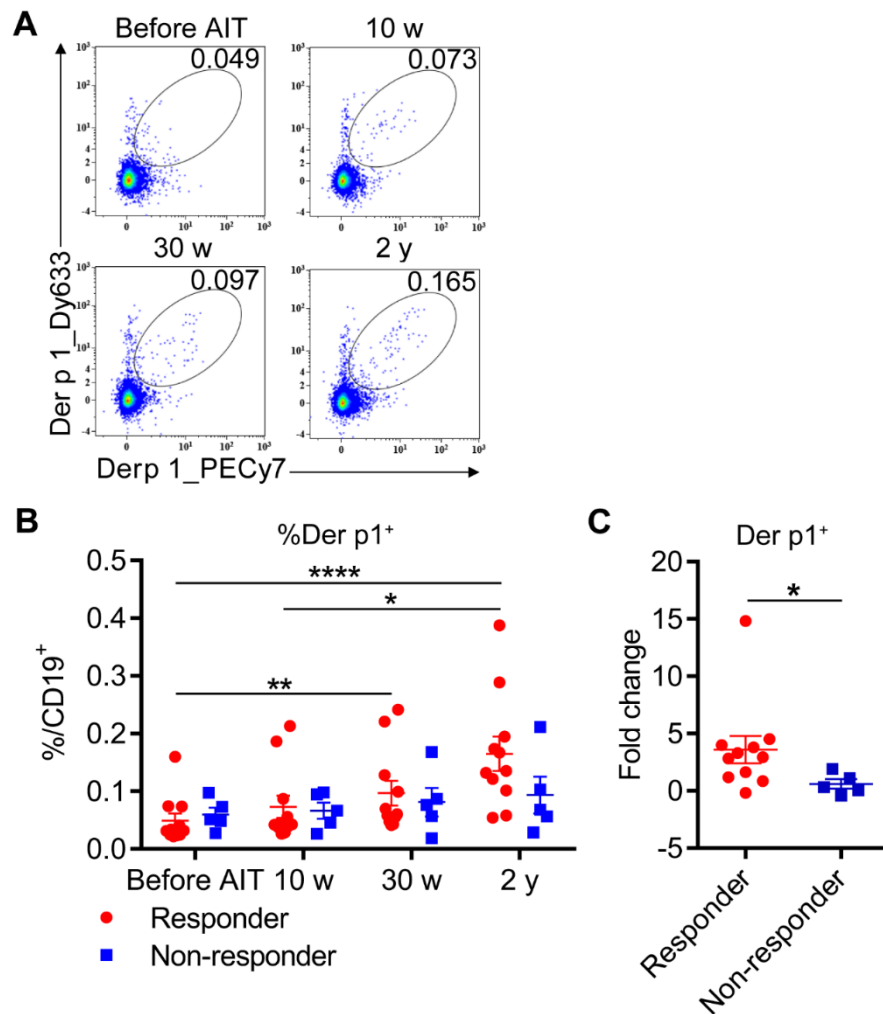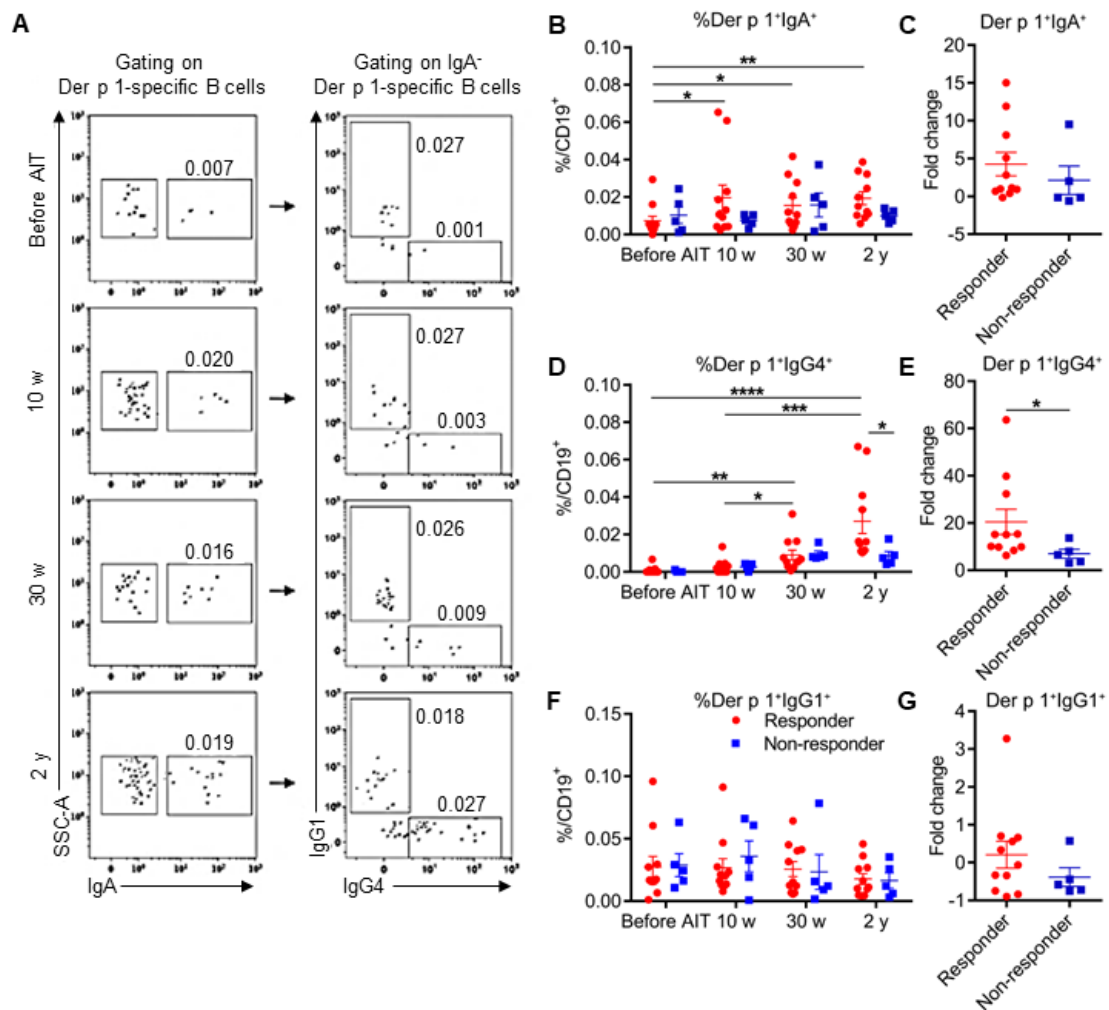
Figure 11. *Increased Der p 1-specific class-switched B cells in responder patients during allergen-specific immunotherapy (AIT).* **A,** *Representative dot plot of a representative responder patient. Cells were gated as CD19+IgM and Der p 1-specific cells were identified as dual stained for Der p 1-Dy633+ and Der p 1-PECy7+.* **B,** *Frequency of Der p 1-specific class-switched B cells before, at 10 weeks (10 w), 30 weeks (30 w) and 2 years (2 y) of AIT.* **C,** *Fold change of Der p 1-specific class-switched B cells between before and after 2 years of AIT among responder patients (n = 11) and non-responder patients (n = 5). *p < 0.05, **p < 0.01, ****p < 0.0001.*

5.1.3 Increased IgA and IgG4 isotypes of Der p 1-specific B cells and antibodies during AIT

We measured the expression of IgA, IgG1 and IgG4 Ig isotypes on Der p 1-specific class-switched B cells. The representative gating strategies used to investigate the expression of IgA, IgG1 and IgG4 of Der p 1-specific (Der p 1+) class-switched B

cells are shown in figure 10. The frequency of IgA$^+$ and IgG4$^+$-secreting-Der p 1$^+$ B cells, as the percentage of total B cells, significantly increased after 2 years (Fig 12, A - E). IgA$^+$ Der p 1-specific B cells showed expansion after 10 weeks and continued to increase during AIT in responder patients (Fig 12, B), without showing any significant difference between responder and non-responder patients (Fig 12, C). IgG4$^+$ Der p 1-specific B cells were detected at a very low frequency in allergic patients before the start of AIT and showed a significant increase after 30 weeks and continued to increase after 2 years of AIT (Fig 12, D). AIT responder patients showed a significantly higher frequency of IgG4$^+$ Der p 1-specific class-switched B cells (0.27 ± 0.006%) compared to non-responder patients (0.008 ± 0.002%) after 2 years of AIT (Fig 12, D). Moreover, the degree of change (between before and 2 years) of IgG4$^+$ Der p 1-specific B cells was significantly higher in responders (20.52 ± 5.34-fold difference) compared to non-responders (6.97 ± 1.91-fold difference) after 2 years (Fig 12, E). The number of IgG1$^+$ Der p 1-specific B cells did not show any difference between responders and non-responders at baseline or during AIT (Fig 12, F and G).

Figure 12. *Increased Der p 1-specific IgA and IgG4-switched B cells in responder patients during allergen-specific immunotherapy (AIT). **A,** Representative dot plots of Der p 1-specific IgA⁺, IgG4⁺ and IgG1⁺ B cells of the responder patients. **B – G,** Frequency of Der p 1-specific IgA⁺, IgG4⁺ and IgG1⁺ B cells before, at 10 weeks (10 w), 30 weeks (30 w) and 2 years (2 y) of AIT and fold change between before and after 2 years of AIT compared between responder patients (n = 11) and non-responder patients (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

Plasma levels of Der p 1-specific IgA, IgG4 and IgE were also determined during AIT. At 2 years, we observed that plasma levels of Der p 1-specific IgA were increased compared to baseline in responders, which was greater compared to non-responders (Fig 13, A). Plasma levels of Der p 1-specific IgG4 continued to increase only in responder patients until 2 years and were significantly higher compared to non-responder patients (Fig 13, B). Meanwhile, plasma levels of Der p 1-specific IgE

decreased significantly after 2 years of AIT in responder patients (Fig 13, C). Collectively, our data demonstrate that plasma levels of Der p 1-specific IgA and IgG4 increase, whereas plasma levels of Der p 1-specific IgE decreae during AIT in responder patients.
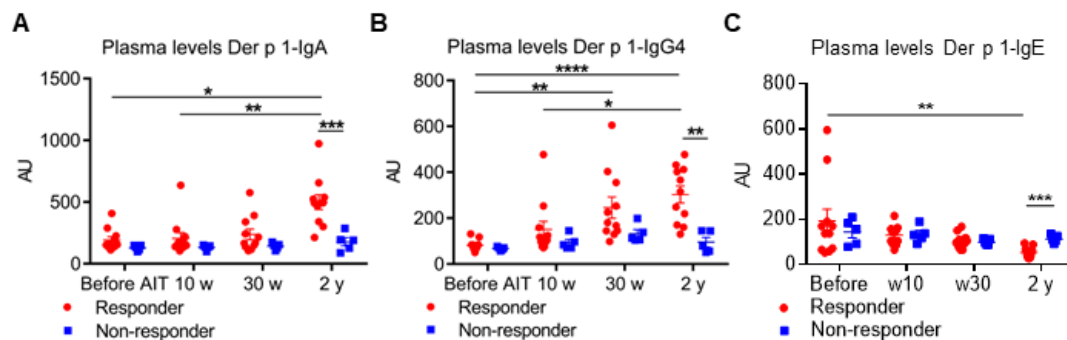
Figure 13. A - C, *Plasma levels of Der p 1-specific IgA, IgG4 and IgE before, at 10 weeks (10 w), 30 weeks (30 w) and 2 years (2 y) allergen-specific immunotherapy (AIT) compared between responder patients (n = 11) and non-responder patients (n = 5). Values are presented in arbitrary unit (AU). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

5.1.4 Increased circulating plasmablasts response during AIT

Plasmablasts were identified as $CD19^+CD27^{hi}CD38^{hi}$ cells in the circulation. Their gating strategy is shown in figure 10. The percentage of circulating plasmablasts increased in both groups, their frequency significantly increased from 0.20 ± 0.01% to 0.30 ± 0.02% after 30 weeks and to 0.43 ± 0.03% after 2 years in responder patients during the course of AIT, whereas the increase in the plasmablasts in non-responders was not significant (Fig 14, *A* and *B*). The mean frequency of plasmablasts in responder patients was also significantly higher compared to non-responder patients after 2 years (Fig 14, *B*). Moreover, plasmablasts increased 1.21 ± 0.2 fold from baseline after 2 years, 0.52 ± 0.16 greater than non-responders (Fig 14, C).
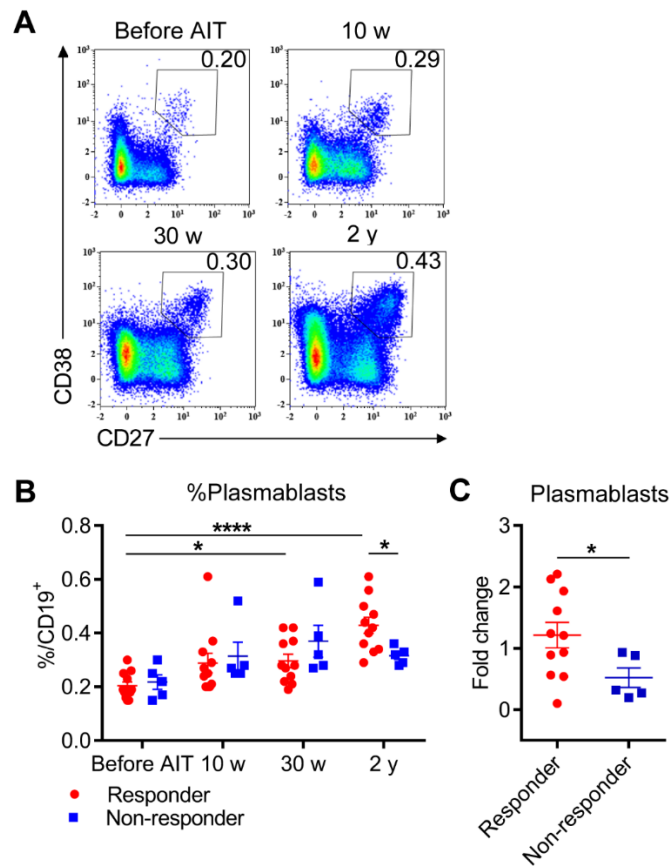
Figure 14. *Increased frequency of plasmablasts in responder patients during allergen-specific immunotherapy (AIT). **A,** Representative dot plot of a representative responder patient. Cells were first gated from CD19⁺ viable cells and plasmablasts were defined as CD27ʰⁱCD38ʰⁱ. **B** and **C,** Frequency of plasmablasts before, at 10 weeks (10 w), 30 weeks (30 w) and 2 years (2 y) of AIT and fold change between before and after 2 years of AIT in responder patients (n = 11) and non-responder patients (n = 5). *p < 0.05, ****p < 0.001.*

5.1.5 IL-10 and IL-1RA single or double positive Breg cells expand in responders during AIT

To identify Breg cells, PBMCs were stimulated with the TLR9 ligand CPG 2006 for 48 hours before the flow cytometry analysis, and we investigated IL-10- and IL-1RA-producing Breg cells.[107] We demonstrate in the present study that 3 subsets of Breg cells are detectable in the peripheral blood. The subsets are defined by staining positive for IL-10, IL-1RA or both. The gating strategy of Breg cells are shown in figure

15, and increased in the frequency of CD73⁻CD25⁺CD71⁺ Breg cells during AIT are shown in figure 16, *A* and *B*.
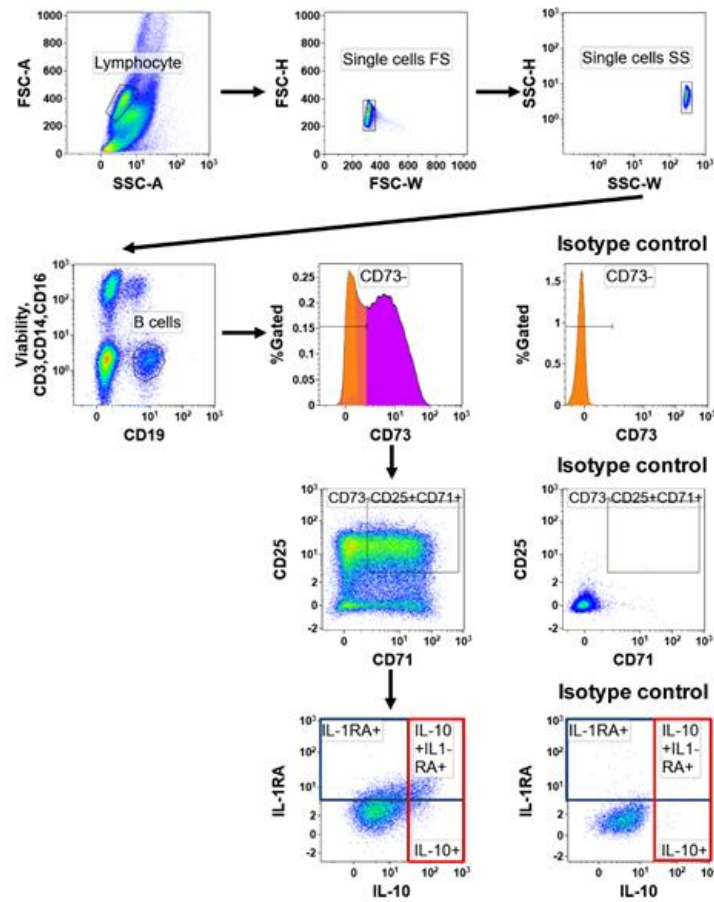

Figure 15. *Gating strategy for Breg cells in peripheral blood mononuclear cells. Lymphocytes were first gated from the forward and side scatter plot. After doublet discrimination, live CD19⁺CD3⁻CD14⁻CD16⁻ cells were gated. Then CD73⁻ and CD71⁺CD25⁺ were gated. Afterward, IL-10 and IL-1RA were gated to show total IL-10 (red box) and total IL-1RA (blue box) and dual IL-10 and IL-1RA-producing Breg cells.*
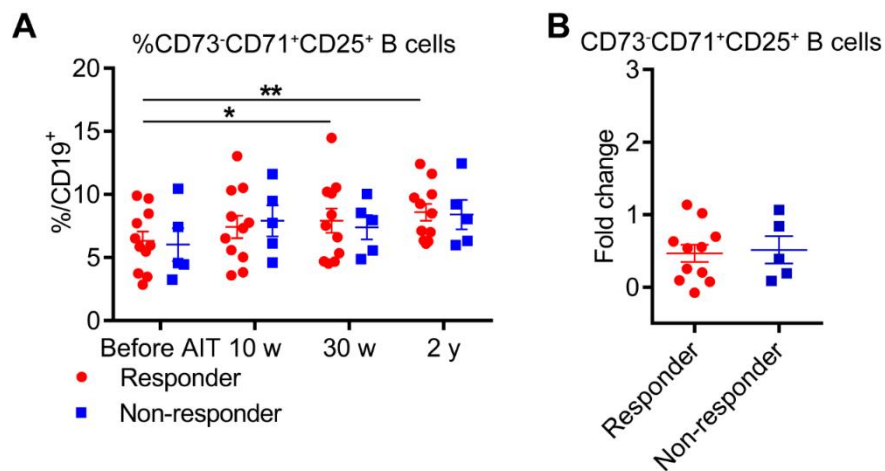
Figure 16. *A* and *B*, Frequency of CD73$^-$CD71$^+$CD25$^+$ B cells before, at 10 weeks (10 w), 30 weeks (30 w) and 2 years (2 y) of AIT and fold change between before and after 2 years of AIT among responder patients (n = 11) and non-responder patients (n = 5). *P < 0.05, **P < 0.01.

Within the CD73$^-$CD25$^+$CD71$^+$ population, the frequency of IL-10$^+$ Breg cells showed a significant difference between responder and non-responder patients. At 30 weeks of AIT, the frequency of total IL-10$^+$ Breg cells was significantly higher in responders (2.27 ± 0.30%) compared to non-responders (1.24 ± 0.13%) (Fig 17, A and B). Furthermore, the frequency of total IL-10$^+$ Breg cells was significantly increased in AIT responders (2.60 ± 0.21%) compared to non-responders (1.75 ± 0.21%) after 2 years (Fig 17, B). The frequency of total IL-1RA$^+$ Breg cells also increased during AIT and showed a significant difference between responders (2.29 ± 0.18%) and non-responders (1.47 ± 0.41%) after 2 years of AIT (Fig 17, C). Also, the frequency of dual positive IL-10$^+$IL-1RA$^+$ Breg cells significantly increased during AIT (Fig 17, D). After 2 years, responders almost doubled the frequency of IL-10$^+$IL-1RA$^+$ Breg cells (1.26 ± 0.09%) compared to non-responder patients (0.65 ± 0.12%) (Fig 17, D). As shown in Fig 17, B and D, either single or double IL-10$^+$, and IL-1RA$^+$ Breg cells showed a tendency to be already higher at the beginning of the study in responders.
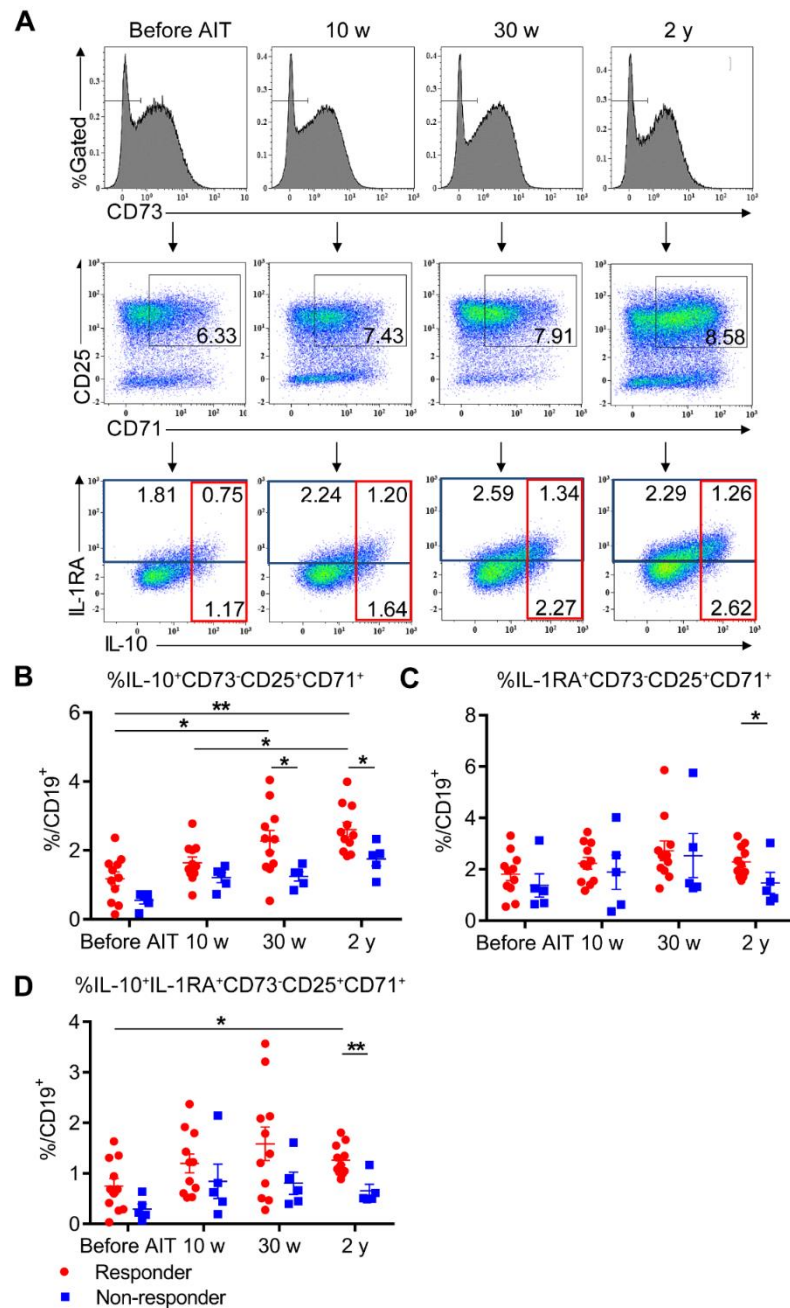
Figure 17. *The frequency of IL-10 and IL-1RA-producing Breg cells increased in responder patients during 2 years (2 y) allergen-specific immunotherapy (AIT).* **A,** *Representative dot plots of responder patient. Cells were first gated from CD19⁺ viable cells and cells were gated as Breg cells. IL-10 and IL-1RA were gated on Breg cells and described as total IL-10⁺ (red), total IL-1RA⁺ (blue) and dual positive IL-10⁺IL-1RA⁺ Breg cells.* **B - D,** *Frequency of total IL-10⁺, total IL-1RA⁺ and dual positive IL-10⁺IL-1RA⁺ Breg cells before, at 10 weeks (10 w), 30 weeks (30 w) and 2 years (2 y) of AIT among responder patients (n = 11) and non-responder patients (n = 5). *p < 0.05, **p < 0.01.*

**5.1.6 Clinical symptoms and B cell responses during AIT**

A correlation analyses of all investigated markers of the study between responder and non-responder patients before and 2 years after AIT are shown in Fig 18, A. It appeared that a strong immune response that includes multiple parameters of antigen-specific B cells and Breg cells during AIT defines those who respond to therapy. An extensive correlation between B cell responses was observed in responders compared to non-responders (Fig 18, A). Also, the correlation between the clinical symptoms observed by SMS and the frequency of B cell subsets were analyzed to investigate the relationship between clinical tolerance and Breg cell-related immune tolerance (Fig 18, B). The IgG4-secreting Der p 1-specific B cells showed a negative correlation with SMS in both among responders (r = -0.75, P < 0.0001) and non-responders (r = -0.89, P = 0.0001). Also, the frequency of plasmablasts negatively correlated with SMS in both responder (r = -0.69, P = 0.0002) and non-responder patients (r = -0.62, P = 0.04). Moreover, increased IL-10$^+$ Breg cells negatively correlated with clinical symptoms during AIT in both responders (r = -0.67, P = 0.0005) and non-responder patients (r = -0.67, P = 0.03). Also, SMS negatively correlated with dual positive IL-10$^+$IL-1RA$^+$ Breg cells, among responder (r = -0.42, P = 0.04) and non-responder patients (r = -0.67, P = 0.03). The frequency of IgG4-secreting Der p 1-specific B cells also positively correlated with the frequency of plasmablasts, and IL-10$^+$ Breg cells in both groups (Fig 18, B). In all these correlation analyses, responders showed a stronger correlation compared to therapy in non-responders. The r-value of correlation and significance of all data of responder and non-responder patients are shown in figure 18, C.
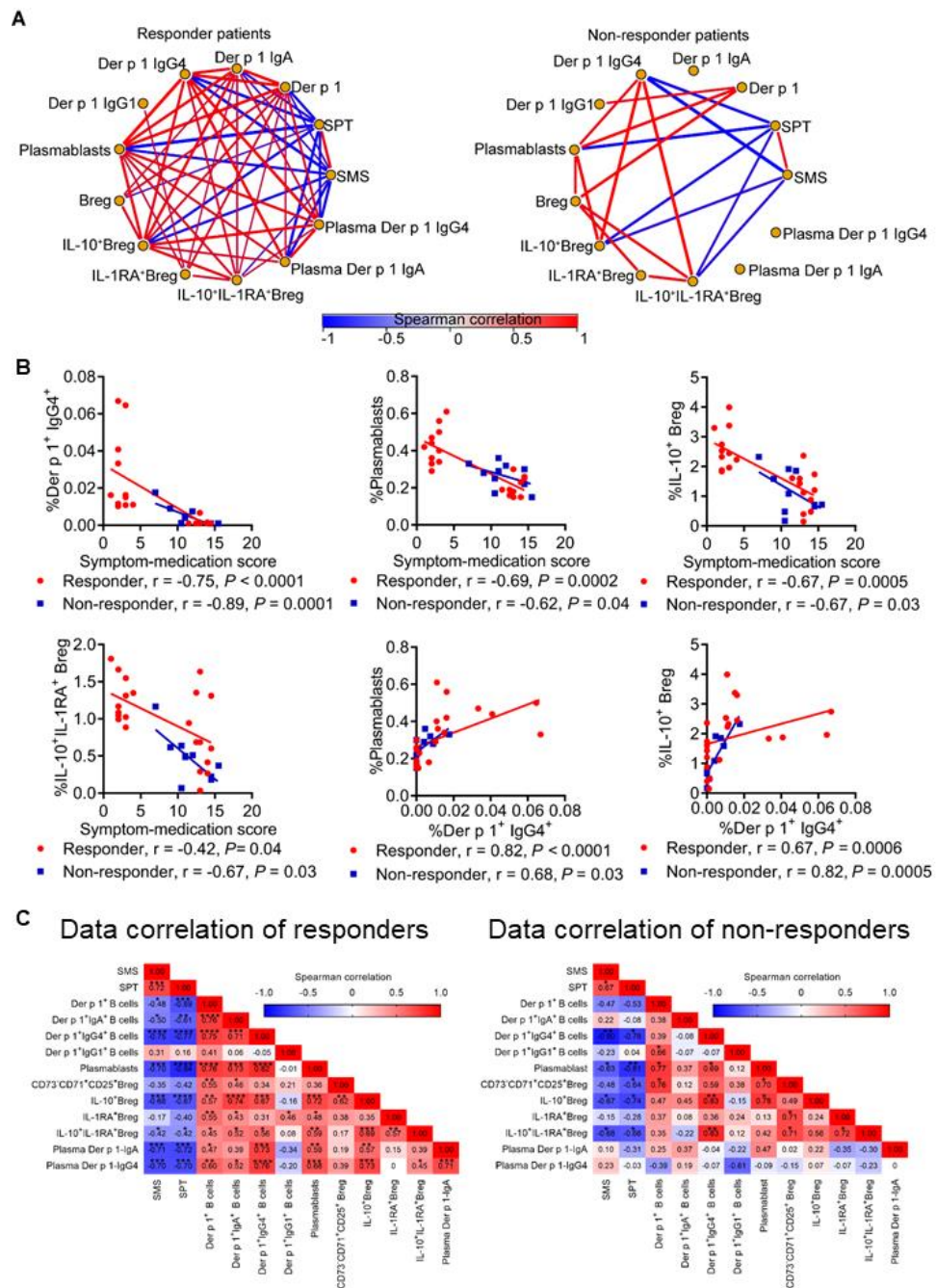
Figure 18. *Correlation data of responder and non-responder patients.* **A,** *Correlation network displaying Spearman correlation between all comparisons in responder and non-responder patients.* **B,** *Correlation between symptom-medication score (SMS) and Der p 1-specific IgG4⁺ B cells, plasmablasts, total IL-10⁺ and dual positive IL-10⁺IL-1RA⁺ Breg cells during allergen-specific immunotherapy (AIT) between before and 2 years after AIT.* **C,** *Heatmap displaying Spearman correlation between all comparisons in responder and non-responder patients. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

5.1.7 Summary results of Der p 1-specific B cells

IgG4[+] and IgA[+] Der p 1-specific B cells showed a significant increase after AIT, with a significantly higher frequency in responders compared to non-responder patients in IgG4^{+} but not in IgA[+] fraction (Fig 19). The frequency of plasmablasts, IL-10 and/or IL-1RA-producing Breg cells was higher among responders compared to non-responders after 2 years (Fig 19). Increased frequency of Der p 1-specific IgG4^{+} B cells, plasmablasts, IL-10^{+} and dual positive IL-10^{+}IL-1RA^{+} Breg cells significantly correlated with improved clinical symptoms throughout AIT.
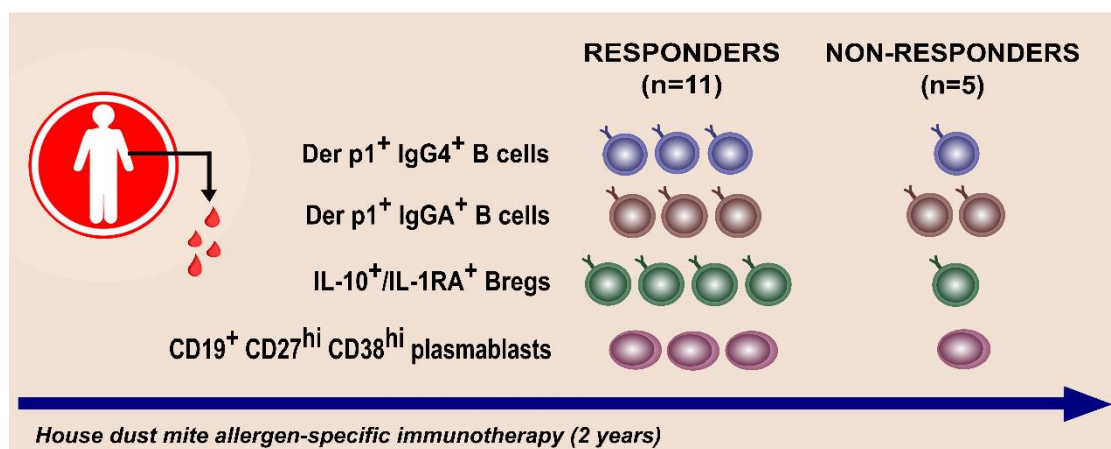

Figure 19. *Increased IgA- and IgG4-expressing Der p 1-specific B cells, plasmablasts, and IL-10^{+} and IL-1RA^{+} Breg cells in house dust mite immunotherapy responder (Graphic abstract made by Anna Goblinska, the graphic editor of Allergy).*

5.2 Der p 1-specific T cells

5.2.1 Der p 1-specific Treg cell responses during AIT

Twelve patients matched with Der p 1-tetramer were investigated Der p 1-specific T cells compared between baseline, 10 weeks, 30 weeks and 3 years after AIT. The gating strategy of Der p 1-specific CD4^{+} Th, CD4^{+}CD25^{+}CD127^{-} Treg and CD4^{+}CD25^{+}ILT3^{+} Treg cells is shown in figure 20, A. Before the AIT, the frequency of FOXP3^{+} and Helios^{+} Der p 1-specific Treg cells constituted only $2.73 \pm 0.53\%$ of Der p 1-specific CD4^{+} T cells (Fig 20, B). We found that the frequency of Der p 1-specific FOXP3^{+}Helios^{+} Treg cells was markedly increased peaking after 30 weeks ($14.73 \pm 1.24\%$) and slightly decreased after 3 years ($6.95 \pm 0.95\%$) of AIT (Fig 20, B). The frequency of Der p 1-specific FOXP3^{+}Helios^{-} Treg cells did not change significantly

during AIT (Fig 20, B). Similarly, non-specific FOXP3⁺Helios⁺ and FOXP3⁺Helios⁻ Treg cells did not change during the therapy (Fig 20, C). Thus, AIT induced allergen-specific Treg cell response, but it did not affect the non-specific Treg cell.
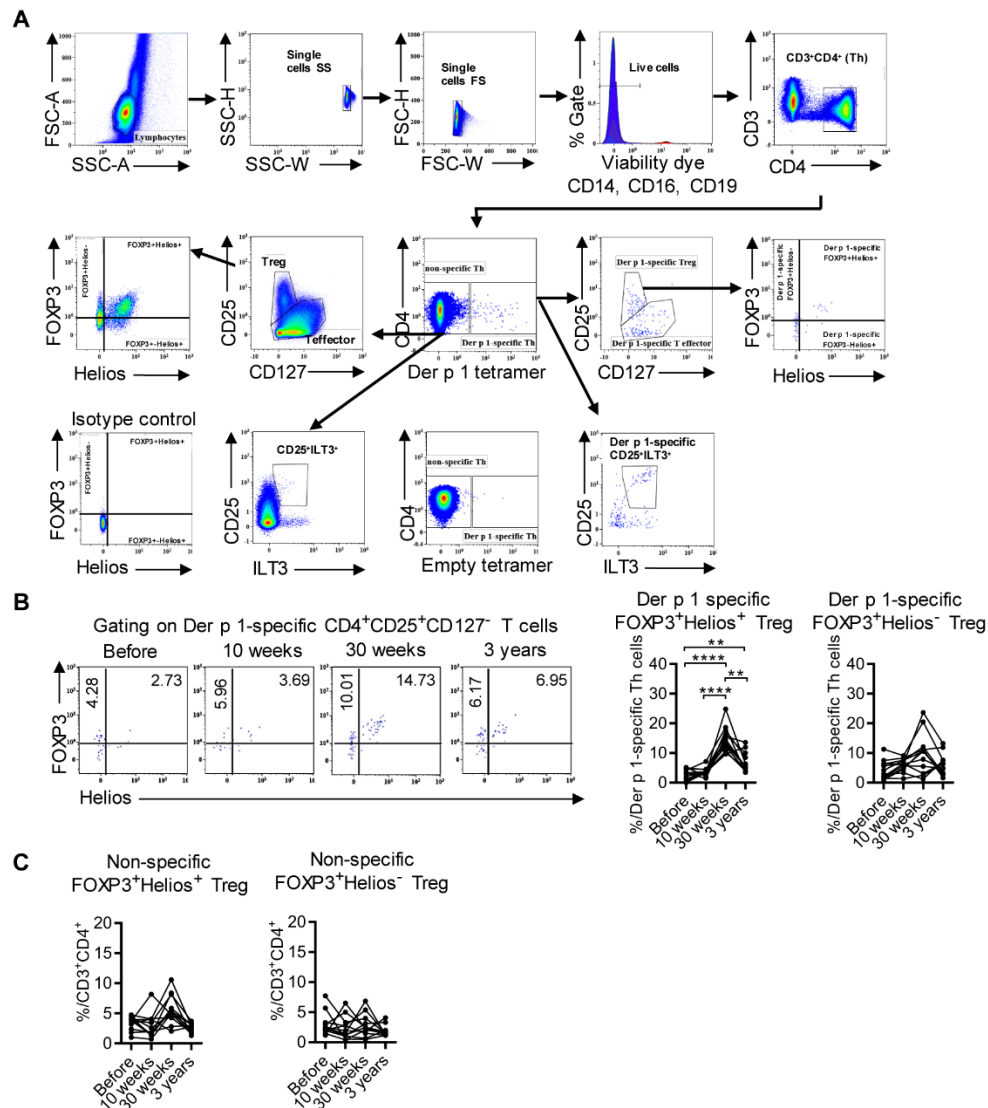
Figure 20. *Increased frequency of Der p 1-specific FOXP3 and Helios positive Treg cells during allergen-specific immunotherapy (AIT).* ***A,*** *Gating strategy of Der p 1-specific Treg cells. Identified cells were first gated on lymphocytes and single cells. Alive CD3⁺ cells were gated after gating out dead cell (viability⁺), CD14⁺, CD16⁺ and CD19⁺. Der p 1-specific CD4⁺ T cells were gated on Der p 1 peptide-MHCII tetramer⁺. Empty peptide-MHCII tetramer was used as negative control. Treg cells were identified by the expression of CD4⁺CD25⁺CD127⁻ and then gated on FOXP3 and Helios. Another Treg population was gated on CD3⁺CD4⁺CD25⁺ILT3⁺ cells. Effector T cells were*

*gated on CD4$^+$CD25$^-$ T cells. **B**, Representative flow cytometry dot plots (left) and the frequency (right) of Der p 1-specific FOXP3$^+$Helios$^+$ and FOXP3$^+$Helios$^-$ Treg cells during AIT (n = 12). **C**, There was no change in the frequency of non-specific FOXP3$^+$Helios$^+$ and FOXP3$^+$Helios$^-$ Treg cells during AIT. The values are presented as the mean value. **P < 0.01, ****P < 0.0001*

Next, we analyzed the frequency of Treg cell subset, which expresses CD25 and ILT3. The frequency of Der p 1-specific CD4$^+$CD25$^+$ILT3$^+$ Treg cells was significantly lower after 3 years of AIT (Fig 21, A). No significant changes in non-specific CD4$^+$CD25$^+$ILT3$^+$ T cells were observed (Fig 21, B). Therefore, we performed *in vitro* suppressive assay to compare functions of ILT3$^+$ and ILT3$^-$ Treg cells. Interestingly, CD4$^+$CD25$^+$ILT3$^+$ Treg cells displayed significantly reduced suppressive function as compared to CD4$^+$CD25$^+$ILT3$^-$ Treg cells which revealed even a higher rate of the proliferation of T effector cells (Fig 21, C). Also, we further examined FOXP3 and Helios expression in ILT3$^+$ and ILT3$^-$ Treg cell. We found that within ILT3$^+$ Treg cells the frequency of FOXP3$^+$Helios$^+$ cells was significantly low compared to ILT3$^-$ Treg cells. Hence, AIT reduced dysfunctional allergen-specific Treg cell response in addition to increasing FOXP3$^+$Helios$^+$ Teg cell frequency.
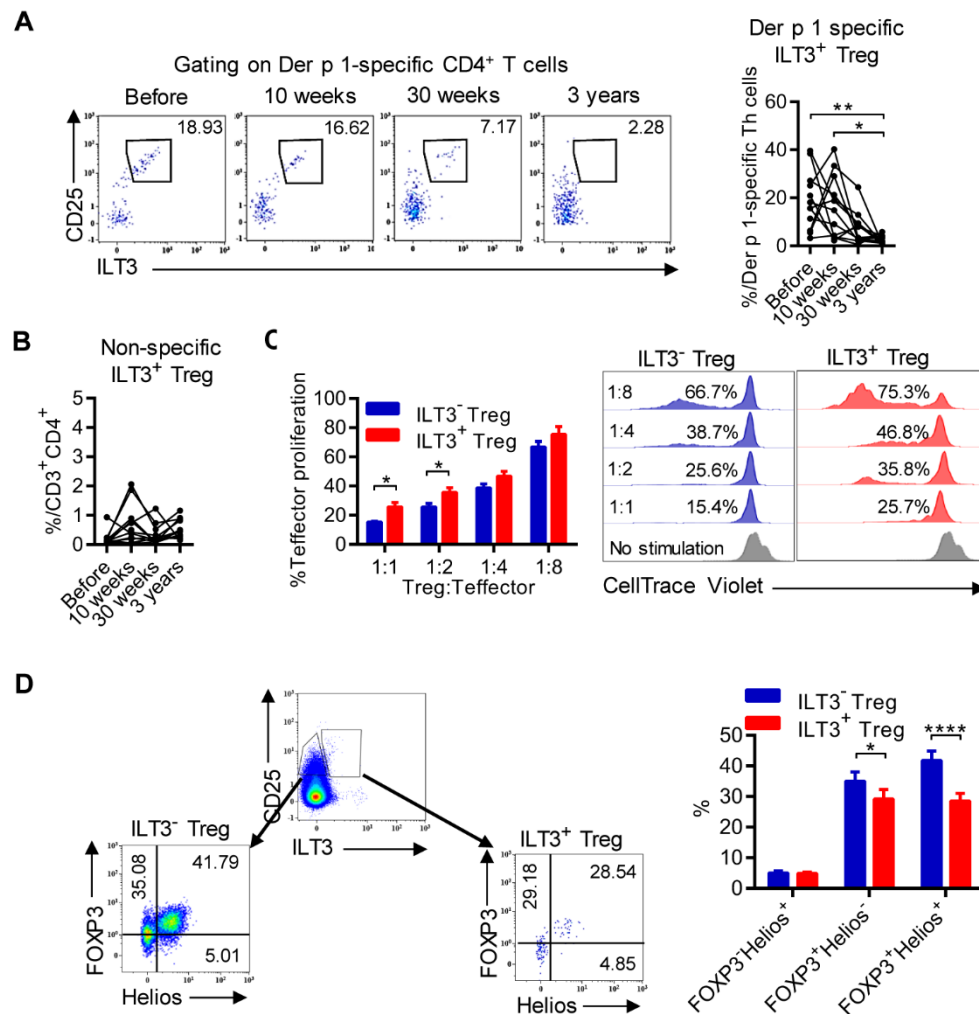
Figure 21. Decreased frequency of Der p 1-specific ILT3⁺ Treg cells during allergen-specific immunotherapy (AIT). **A,** Representative flow cytometry dot plots (left) and the frequency (right) of Der p 1-specific ILT3⁺ Treg cells during AIT (n = 12). **B,** The frequency of non-specific ILT3⁺ Treg cells during AIT. **C,** In vitro Treg suppression assay, comparing the suppressive capacity of ILT3⁻ and ILT3⁺ Treg cells. Sorted T effector cells were labeled with CellTrace Violet, stimulated with anti-CD3 and anti-CD28, and cocultured with sorted ILT3⁻ or ILT3⁺ Treg cells for 5 days. The frequency of T effector cells proliferation (left) and the overlay histogram (right) of ILT3⁻ or ILT3⁺ Treg cells at various ratios (n = 6). **D,** Dot plots (Left) and the frequency (Right) of FOXP3 and Helios expression on ILT3⁻ and ILT3⁺ Treg cells (n = 48). The values are presented as mean value ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001.

5.2.2 Der p 1-specific IL-10 Treg cell responses during AIT

To elucidate the cytokine profile of allergen-specific T cells, we determined Der p 1-tetramer$^+$CD4$^+$ T cells, which are producing IL-10, (Fig 22, A). Der p 1-specific IL-10$^+$ Treg cells displayed low frequency of Der p 1-specific CD4$^+$ T cells before AIT (2.89 ± 0.55%). During AIT, IL-10-producing Der p 1-specific Treg cells expanded already after 10 weeks (5.12 ± 0.67%) and showed the highest frequency after 30 weeks (22.95 ± 3.07%) of AIT (Fig 22, B). At 3 years of AIT, the frequency of IL-10-producing Der p 1-specific Treg cells (17.54 ± 1.71%) was slightly decreased compared to the 30 weeks time point but was still higher compared to baseline (Fig 22, B). For comparison, there was no significant change in IL-10, non-specific CD4$^+$ T cells during AIT (Fig 22, C).
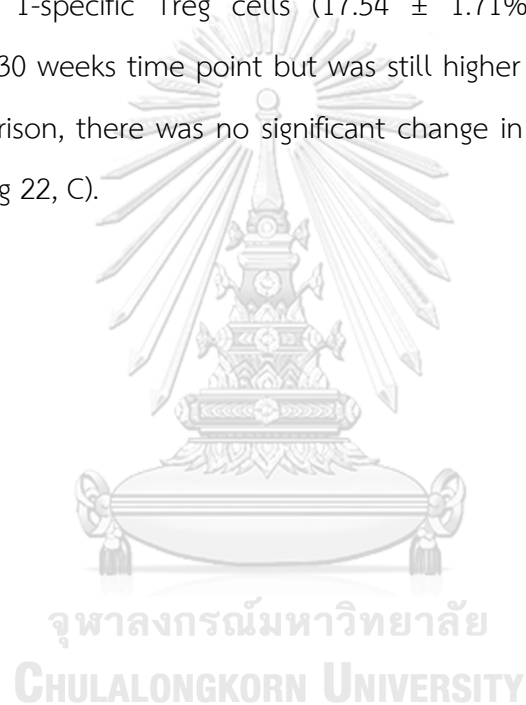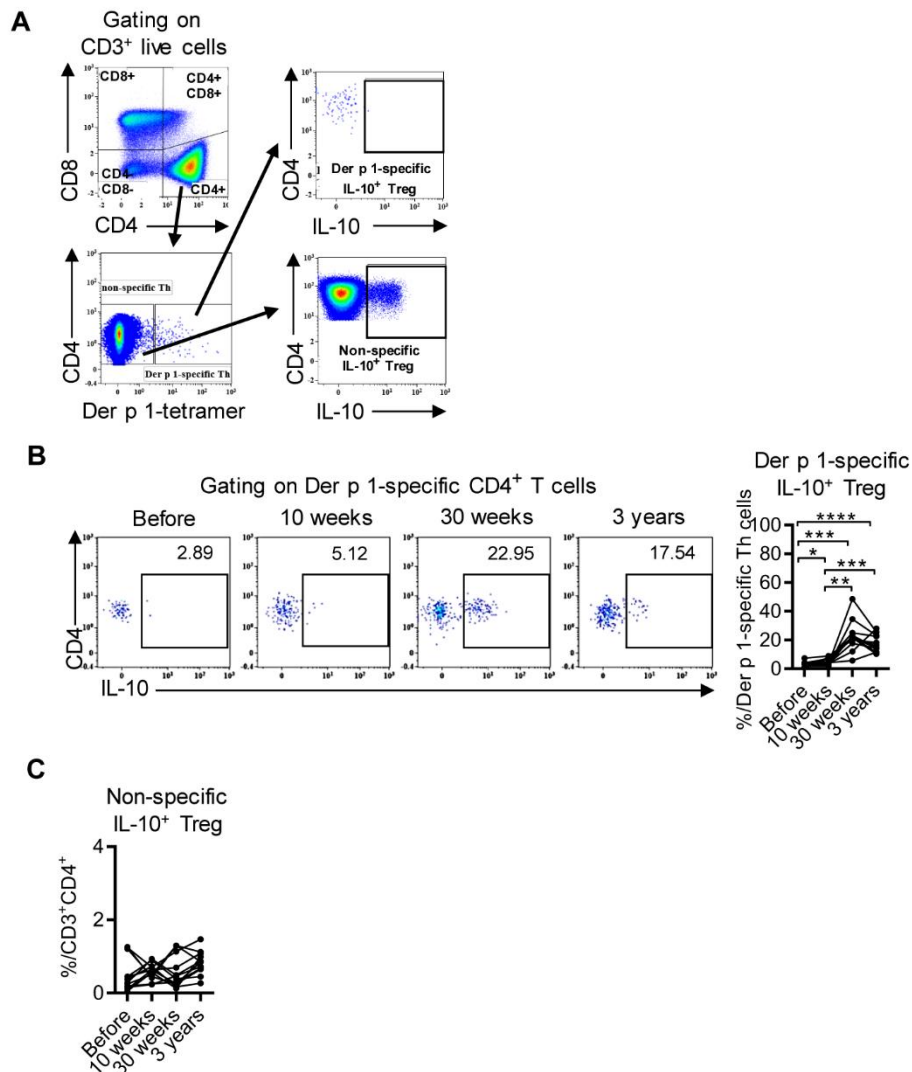
Figure 22. Increased IL-10-producing Der p 1-specific CD4$^+$ T cell responses during allergen-specific immunotherapy (AIT). *A,* Gating strategy of the cytokine profile of non-specific and Der p 1-specific CD4$^+$ T cells. Peripheral blood mononuclear cells were stimulated with phorbol 12-myristate 13 acetates (PMA) and ionomycin for 4 hours followed by brefeldin A for 2 hours before staining and flow cytometry analysis. *B,* Representative dot plots (left) and the frequencies (right) of Der p 1-specific IL-10$^+$ Treg cells during AIT (n = 12). *C,* Frequency of non-specific IL-10-producing Treg cells during AIT. The values are presented as the mean value. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

5.2.3 Der p 1-specific Treg cell response correlates with allergic symptoms

Allergic patients had significantly decreased total nasal symptom score (TNSS) after 30 weeks (-66.08 ± 6.40%) and 3 years (-86.33 ± 3.35%) of AIT (Fig 23, A). The efficacy of AIT and its link to Treg cells was further investigated by its correlation with TNSS. Improved allergic symptoms by reduced TNSS showed a significant correlation with increased frequency of functial Der p 1-specific FOXP3$^+$Helios$^+$ and IL-10$^+$ Treg cells (Fig 23, B). Moreover, reduced allergic symptoms correlated with the decreased frequency of dysfunctional Der p 1-specific ILT3$^+$ Treg cells (Fig 23, B).
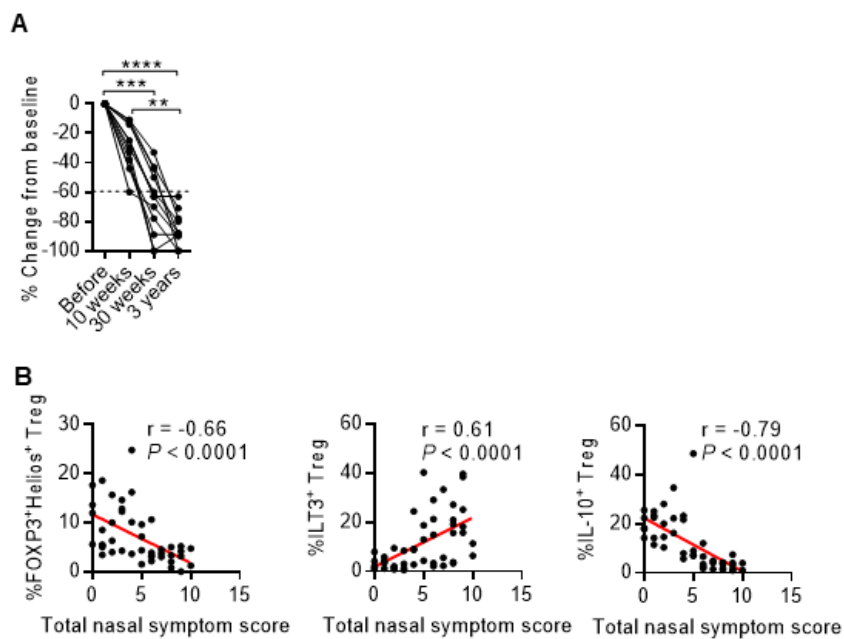
Figure 23. *Reduced total nasal symptom score (TNSS) during AIT was linked to changes in Treg cell responses.* *A,* *Percent change of TNSS from baseline of 12 clinical responder patients during AIT.* *B,* *Scatterplot and Spearman correlation between TNSS and Treg cells responses.* **P < 0.05,* ***P < 0.01, ***P < 0.001, ****P < 0.0001.*

5.2.4 Summary results of Der p 1-specific T cells

Twelve of 25 AIT patients matched with their MHC-class II expression to the Der p 1 peptide-MHC-class II tetramers. A significant increase in the numbers of Der p

1-specific FOXP3^{+}Helios^{+}CD25^{+}CD127^{-} Treg cells after 30 weeks was observed, which slightly decreased after 3 years of AIT (Fig 24). In contrast, Der p 1-specific immunoglobulin-like transcript 3 (ILT3)$^{+}$CD25^{+} Treg cells decreased substantially from baseline after 3 years of AIT (Fig 24). ILT3^{+} Treg cells displayed compromised suppressive function and low FOXP3 expression. Also, Der p 1-specific IL-10 responses have increased after 30 weeks and IL-10^{+} Der p 1-specific Treg cells remained present at high frequency after 3 years of AIT (Fig 24). Increased number of FOXP3^{+}Helios^{+}, IL-10^{+} and decreased ILT3^{+} Treg cell responses correlated with improved allergic symptoms.
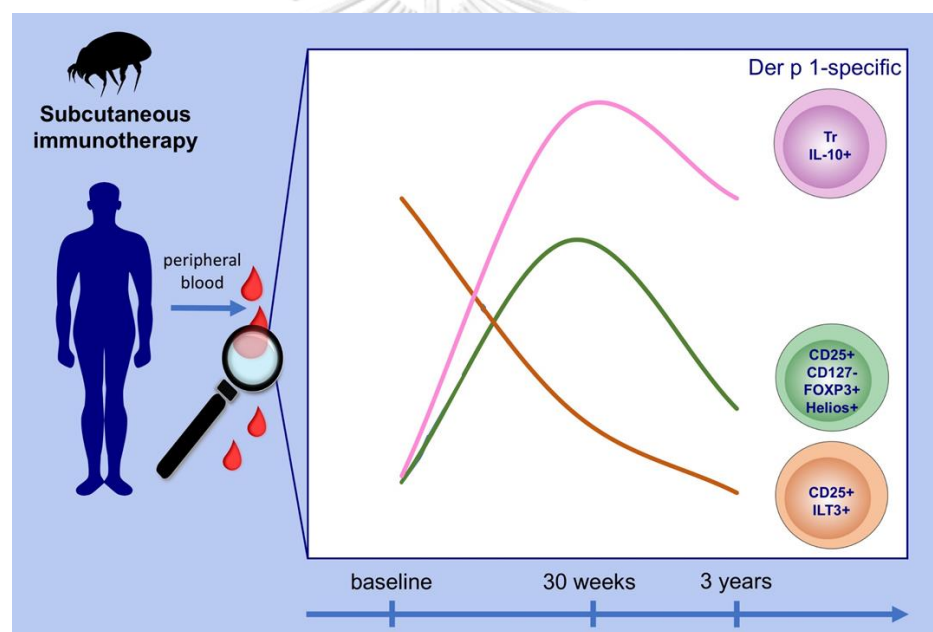
Figure 24. *House dust mite immunotherapy induces an increase in Der p 1-specific activated Treg cells, IL-10-producing Treg cells and reduces Der p 1-specific dysfunctional ILT3^{+}CD25^{+} Treg cells. Increased number of Der p 1-specific FOXP3^{+}Helios^{+}, IL-10^{+} and decreased Der p 1-specific ILT3^{+} Treg cell responses correlated with improved allergic symptoms. House dust miet immunotherapy improves clinical symptoms which relates to the correction of dysregulated T cell responses (Graphic abstract made by Anna Goblinska, the graphic editor of Allergy).*

6. Discussion

6.1 Der p 1-specific B cells

The present study focuses on HDM allergen, Der p 1-specific immune responses during 2 years of AIT in peripheral blood samples taken before, 10 weeks, 30 weeks and 2 years of HDM extract subcutaneous AIT. The data presented here highlight essential aspects of peripheral allergen-specific B cell tolerance and links some of the findings to clinical tolerance. As a critical finding, the frequency of certain circulating allergen-specific B cells, Breg cell subsets and plasma cells changed during AIT and presented differences between therapy responder and non-responder patients.

In this study, a dual-labeling method was used to identify Der p 1-specific B cells. A similar approach has been used for the identification of Ara h 1 and Ara h 2-specific B cells in peanut allergy and PLA-specific B cells in venom allergy.[5,9,20] One of the first findings of the present study is as long as the patients received HDM AIT, their allergen-specific B cell numbers showed a significant increase, during 2 years AIT. Interestingly, this increase in a total number of Der p 1-specific B cells was up to 3 times in 2 years in average responder patients. The findings suggest a steady and continuous but relatively slow increase in responder patients. It shows a difference compared to infectious diseases vaccine-specific B cell responses and booster injections of vaccines, which rather show a stronger and rapid increase after the booster doses.[91]

In this study, we observed an increase in allergen-specific IgA$^+$ and IgG4$^+$ B cells during AIT, which was greater in responder compared to non-responder patients. Due to their very low frequency, IgE$^+$ B cells could not be detected with the same method. We could detect plasma levels Der p 1-specific IgE that decrease during AIT. Increases in serum allergen-specific IgA and IgG4 levels were also found in responder egg-allergic patients during oral immunotherapy.[108, 109] In grass pollen

immunotherapy, serum Phl p 5-specific IgA1 and IgA2 concentrations also increased after 2 years treatment.[110] The increase in Der p 1-specific IgG4$^+$ B cell frequency showed an initial response after low-dose allergen-specific subcutaneous injection. In high dose allergen exposure models, cat owners or beekeepers, as well as bee venom AIT, promotes the generation of functional IL-10-producing Treg cells and high levels of allergen-specific IgG4. [111-114] It is associated with tolerance induction without new sensitizations or asthma development. IgG4 has multiple features that may suggest a role in immune tolerance. IgG4 does not have any role in antibody-dependent cellular cytotoxicity as it does not have a specific Fc receptor.[115] Moreover, IgG4 might restrict inflammation due to an inability to fix complement and control allergy as a result of competing with IgE as a blocking Ab for allergen binding to IgE Fc receptor-expressing cells.[35, 116, 117]

We demonstrate here that circulating plasmablasts are increased during AIT and even highly expressed at 2 years in responder patients compared to non-responders. It was an interesting finding to see a non-allergen-specific population change in an antigen-specific treatment, which can be due to a general vaccine effect and needs further research. The frequency of plasmablasts in HDM-allergic individuals and treated with AIT seems to be much higher than bee venom allergic individuals. In a previous study, we observed an increased frequency of plasmablasts during venom immunotherapy (VIT), but PLA-specific plasmablasts were rarely detected.[107] Ara h 2-specific plasmablasts also increased during oral peanut immunotherapy.[118] Intralymphatic immunotherapy induces grass pollen Ag-specific plasmablasts and increases tolerance to skin prick testing in a pilot study.[119] In the present study, we could not detect Der p 1-specific plasmablasts as they were below the detection limit of flow cytometry. The assessment of immunoglobulin isotypes in allergen-specific plasmablasts by multicolor flow cytometry before immunotherapy is challenging because the number of antigen-specific plasmablasts is deficient.

Antigen-specific plasmablasts expressing specific BCR have been shown to increase intensely after vaccination or natural infection.[27-29]

Here, we demonstrated that B_R1 cells not only produce IL-10 but also produce IL-1RA. IL-10^{+}IL-1RA^{+} Breg cells expanded significantly during AIT and were higher among responder patients. We previously reported IL-10-secreting CD73^{-}CD25^{+}CD71^{+} Breg cells expanded in response to high-dose antigen exposure, both among patients receiving VIT and among beekeepers during beekeeping season.[107] In addition, IL-10^{+} Breg cells suppress T helper cell proliferation and upregulate IgG4 secretion.[33] The present study introduces IL-1RA as a Breg cell cytokine in humans. The link of IL-1RA to IL-10 in B cells was first shown with increased IL-1RA in only IL-10 transfected and overexpressing human B cells, which showed that B cells overexpressing IL-10 gained a suppressive and anti-inflammatory function with increased IL-1RA and decreased proinflammatory cytokines.[120] Various anti-inflammatory functions of IL-1RA has been reported. IL-1 triggers a cascade of inflammatory mediators, chemokines and other cytokines. IL-1RA is an IL-1 inhibitor effective in reducing inflammation by blocking pro-inflammatory signals through the IL-1 receptor.[121] Administration of IL-1RA to mice reduced inflammation in various disease.[122] In humans, IL-1RA reduces the inflammatory effects of IL-1 and preserves cell function in diabetes and subarachnoid hemorrhage.[121, 123] IL-1RA reduces IL-17-producing T cells and induces Treg cell proliferation.[30,31] IL-1RA might support IL-10 to induce immune tolerance by B cells. In a murine model, IL-1RA, produced by mesenchymal stem cells, decreased the differentiation of B cells toward plasmablasts and induced the differentiation of macrophages toward a M2-like phenotype.[19] However, the role of IL-1RA directly upon class-switched B cells in humans remains to be further studied.

In correlation analyses between multiple parameters, we demonstrated here a distinct profile between responders and non-responders. We observed a strongly correlating overall immune response with multiple parameters of B cell responses in

therapy responders. The B cell immune response parameters also showed a negative correlation with SMS and SPT, suggesting an excellent link to clinical improvement. It is not entirely possible to identify which of these changes are fully linked to treatment success. However, increases in IgG4$^+$ allergen-specific B cells, plasmablasts, and IL-10 and/or IL-1RA-producing Breg cells responses during the AIT are particular markers associated with successful treatment. As previously described, increases in allergen-specific IgA and IgG4 and decreases in allergen-specific IgE are the markers of successful immune response during AIT.[118, 124-128] We aimed to demonstrate a biomarker to predict patient responsiveness to AIT. IL-10-producing Der p 1-specific B cells showed a significant difference after 30 weeks of AIT from baseline. Changes in the frequency of IgG4 class-switched B cells differed between responder and non-responder patients. The responder patients also showed expansion of plasmablasts. Also, increased IL-10 and IL-1RA-producing Breg cells in responder patients were observed. However, it appeared that all of these might represent important targets that may predict clinical therapy response and high numbers are needed. It was not possible to demonstrate a single factor that identifies non-responder patients in the present study. Many factors such as gender, age, disease duration, socioeconomic, occupational, medical adherence, house environment, psychology and body mass index may play a role. Besides, additional allergen exposure and sensitization or degree of dust mite exposure might explain the variability in clinical response.

6.2 Der p 1-specific T cells

The present study demonstrates novel immunological mechanisms linked to the induction of peripheral allergen-specific tolerance by AIT. We demonstrated early increases in active Tregs resulting in desensitisation compared to delayed suppression of ineffective Tregs as a potential tolerance mechanism after 3 years. Der p 1-MHC class-II tetramer staining could be used to monitor peripheral allergy tolerance and clinical response of HDM-specific immunotherapy. It has been previously shown that AIT induces an increase in the percentage of allergen-driven Treg cells.[129, 130] In the

present study, we demonstrated that AIT differentially modifies the activation and function of several Treg subsets. AIT *in vivo* increased subsets of allergen-specific Treg cells with distinct phenotypes and mechanism of action include the CD4^{+}CD25^{+}CD127^{-}FOXP3^{+} nTreg and the CD4^{+} IL-10-secreting Tr1 cells.[111, 131] In addition to changes in allergen-specific Treg cells, we observed that in 9 out of 12 patients overall IL-10^{+} Tr1 cells also increased during AIT is concordant with the previous data reported by others. [132]

As the availability of recombinant MCH class-II molecules is limited, knowledge on T cell epitopes and recruitment of participants to match with MHC class-II tetramers.[90] Therefore, our study was not powerful enough to examine the differences between responders or non-responders to the therapy. Interestingly, we found here that TNSS was reduced by 88.5% in matched and by 57.1% in non-matched HLA-DR patients after 3 years of AIT (Fig 25). Hence, allergen-specific Treg cells subsets responses in non-responders to AIT is still a question and requires further studies. Although the present study focuses on Treg cells, their effect on the control of allergen-specific immune responses goes in parallel to allergen-specific memory B cell responses and the importance of allergen-specific effector and regulatory B cell balance should be studied in bigger cohorts.[133]
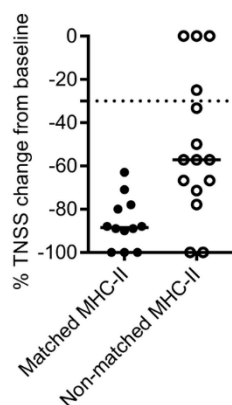
Figure 25. *Change in total nasal symptom score (TNSS) after 3 years allergen-specific immunotherapy from baseline; comparison between matched (n = 12) and non-matched (n = 13) HLA-DR patients. The data present as median.*

Helios is a specific marker for thymus-derived regulatory T cells.[134] Combining FOXP3 and Helios intracellular staining is a useful approach to determine activated Treg cells, because FOXP3+Helios+ Treg cells proliferate *in vitro* significantly less than FOXP3+Helios⁻ Tregs upon TCR stimulation.[135] Unlike FOXP3+Helios⁻ Tregs, FOXP3+Helios+ Tregs secrete IL-10, but not other effector cytokines such as IFN-γ or IL-2.[135] Moreover, Helios is selectively upregulated in CD4+ T cells during Th2 and T follicular helper cell responses to alum containing-protein vaccines.[30] The presence of local FOXP3+CD25+ Treg cells in the nasal mucosa increased number after immunotherapy, and their association with clinical efficacy and suppression of seasonal allergic inflammation revitalize the concept of local allergen tolerance depending on Treg cells.[26]

The dysfunctional allergen-specific ILT3+CD4+CD25+FOXP3+ Treg cells constitute a small subpopulation of allergen-specific Treg cells. We found that the percentage of allergen-specific ILT3+ Treg cells was significantly higher before the treatment, which may suggest the inability of Tregs to efficiently suppress allergen-specific Th2 responses. We showed here that human ILT3+ Treg cells had reduced suppressive activity and low FOXP3 and Helios expression suggesting a negative role for ILT3+ Tregs in allergen tolerance. Supporting our findings, it has been shown in mice that ILT3+ Treg cells fail to suppress GATA-3 expression and instead induce expression of IL-5 and IL-13.[32] FOXP3 plays an essential role in the maintenance of immunological tolerance to self and innocuous foreign antigens.[136] FOXP3 homodimers can mediate transcriptional regulation through direct binding to DNA and FOXP3 has also been shown to interact with several molecular partners forming FOXP3/protein complexes that are required for several regulatory processes.[136]

Recently, new allergen-specific T cells subsets have been identified in allergic patients during AIT. Special AT-rich sequence-binding protein-1 (SATB1) is repressed in FOXP3$^+$ Tregs following grass-specific immunotherapy, and its expression correlates with the clinical response.[137, 138] Allergen-specific CD45RB^{low}CD27$^-$ CRTH2$^+$CD161$^+$CD49d$^+$ T cells (Th2A) are more frequent in allergic patients compared to non-allergic individuals and suggesting a role for the control of clinical allergic reactions by these cells that are induced by immunotherapy.[139] Also in long-term grass-specific immunotherapy, a clinical improvement during 2 years of treatment has been associated with the decrease in numbers of the allergen-specific CRTH2$^+$CCR4$^+$CD27$^-$CD4$^+$ Th2 cells.[140] Indeed, there are now many studies confirming the role of decreased Th2 responses in the control of allergic disease and Treg cells play an essential role in the suppression of Th2 cells.

In the present study, IL-10$^+$ Treg cells had an early response in low-dose AIT and correlated with the reduction of allergic symptoms. Allergen-specific IL-10-secreting Treg cells are essential components of the induction and maintenance of peripheral allergen tolerance enhanced by AIT.[141]

It is also important to emphasize that direct analyses of the *in vivo* situation of circulating allergen-specific T cells is an advantage of the present study compared to cell cultures. Serum specific IgE/total IgE ratio and IgE-FAB, allergen-specific IgG4 have been considered as potential surrogate candidate biomarkers.[44] The area is open for further research to discover biomarkers for point-of-care usage because the early prediction of success and to decide when to stop is essential for the patient compliance, cost-effectiveness as well as avoidance of side effects. These studies require sophisticated techniques. However, a good biomarker should be studied in easily accessible samples with a rapid method. As similar findings have been reported in healthy immune response to allergens, it may suggest that similar mechanisms can be necessary for the prevention of HDM allergy. None the less, a more extensive scale study, in a randomized controlled study, in particular,

comparing HDM-specific antibody-based assays versus Treg-based assays is warranted for identifying proper biomarkers predicting or monitoring AIT responders.

6.3 Limitation of the study

The limitations include the paucity of previous data for the vaccine employed from controlled trials, small numbers, lack of control group, lack of robust efficacy outcomes in the clinical trial. Moreover, extremely low frequencies of allergen-specific T and B cells could detect in peripheral blood. Furthermore, the availability of recombinant MCH class-II molecules is limited, 50% of the patients in this study matched to Der p 1-MHC class-II tetramers. In addition, we did not find the biomarker to predict response to AIT before start allergen immunotherapy. One major handicap as a biomarker is these analyses require sophisticated laboratory approach and cannot be done at the point-of-care.

7. Conclusion

In conclusion, house dust mite immunotherapy involves upregulation of the activated Der p 1-specific Treg cells and downregulation of dysfunctional Der p 1-specific Treg cells subset that are the new knowledge in AIT (Fig 26). Moreover, the present study demonstrates that Der p 1-specific B cells and Breg cell responses over a 2-year period during house dust mite immunothearpy and suggests novel mechanisms of allergen tolerance (Fig 26). In Breg cells, we also find Breg cells produce IL-1RA together with IL-10 increased during the AIT. It is the first discover in the field of immunology that Breg cells can produce IL-1RA. We show house dust mite immunotherapy leads to a reduction of allergic symptoms and medication which correlate to Treg and Breg cells changes. However, we could not find the biomarker to predict responder or non-responder to treatment before start AIT. Extension of the study to a larger number of patients and extending the study period should provide additional information for identifying biomarkers of long-term tolerance and successful AIT response.
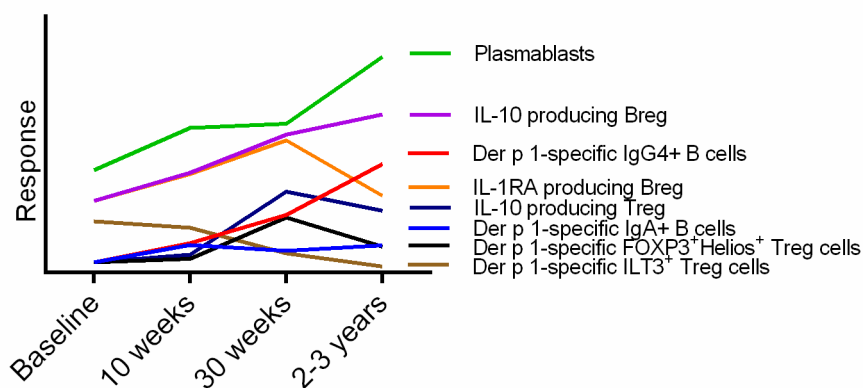
Figure 26. *House dust mite immunotherapy involves upregulation of the activated Der p 1-specific Treg cells and Tr1 cells, meanwhile downregulation of dysfunctional Der p 1-specific Treg cells subset. House dust mite immunotherapy also induces IgG4 and IgA producing allergen-specific B cells, plasmablasts, and IL-10 and/or IL-1RA producing Breg cells. Treg and Breg cells changes correlate to clinical response during the treatment.*

REFERENCES

1. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. J Allergy Clin Immunol 2014; 133:621-31.

2. Sokolowska M, Frei R, Lunjani N, Akdis CA, O'Mahony L. Microbiome and asthma. Asthma Res Pract 2018; 4:1.

3. Sokolowska M, Chen LY, Liu Y, Martinez-Anton A, Logun C, Alsaaty S, et al. Dysregulation of lipidomic profile and antiviral immunity in response to hyaluronan in patients with severe asthma. J Allergy Clin Immunol 2017; 139:1379-83.

4. Globinska A, Boonpiyathad T, Satitsuksanoa P, Kleuskens M, van de Veen W, Sokolowska M, et al. Mechanisms of allergen-specific immunotherapy: Diverse mechanisms of immune tolerance to allergens. Ann Allergy Asthma Immunol 2018; 121:306-12.

5. Jutel M, Akdis CA. Immunological mechanisms of allergen-specific immunotherapy. Allergy 2011; 66:725-32.

6. Noon L. Prophylactic inoculation against hay fever. Historical document. Ann Allergy 1960; 18:287-91.

7. Passalacqua G, Canonica GW. Allergen Immunotherapy: History and Future Developments. Immunol Allergy Clin North Am 2016; 36:1-12.

8. Jutel M, Kosowska A, Smolinska S. Allergen Immunotherapy: Past, Present, and Future. Allergy Asthma Immunol Res 2016; 8:191-7.

9. Sharkey P, Portnoy J. Rush immunotherapy: experience with a one-day schedule. Ann Allergy Asthma Immunol 1996; 76:175-80.

10. Feng S, Xu Y, Ma R, Sun Y, Luo X, Li H. Cluster subcutaneous allergen specific immunotherapy for the treatment of allergic rhinitis: a systematic review and meta-analysis. PLoS One 2014; 9:e86529.

11. Pajno GB, Cox L, Caminiti L, Ramistella V, Crisafulli G. Oral Immunotherapy for Treatment of Immunoglobulin E-Mediated Food Allergy: The Transition to Clinical Practice. Pediatr Allergy Immunol Pulmonol 2014; 27:42-50.

12. Mantyla J, Thomander T, Hakulinen A, Kukkonen K, Palosuo K, Voutilainen H, et al. The effect of oral immunotherapy treatment in severe IgE mediated milk, peanut, and egg allergy in adults. Immun Inflamm Dis 2018; 6:307-11.

13. Passalacqua G, Compalati E, Canonica GW. Sublingual Immunotherapy: Clinical Indications in the WAO-SLIT Position Paper. World Allergy Organ J 2010; 3:216-9.

14. Marogna M, Spadolini I, Massolo A, Canonica GW, Passalacqua G. Randomized controlled open study of sublingual immunotherapy for respiratory allergy in real-life: clinical efficacy and more. Allergy 2004; 59:1205-10.

15. Marogna M, Tomassetti D, Bernasconi A, Colombo F, Massolo A, Businco AD, et al. Preventive effects of sublingual immunotherapy in childhood: an open randomized controlled study. Ann Allergy Asthma Immunol 2008; 101:206-11.

16. Passalacqua G, Canonica GW. Local nasal specific immunotherapy for allergic rhinitis. Allergy Asthma Clin Immunol 2006; 2:117-23.

17. Slovick A, Douiri A, Muir R, Guerra A, Tsioulos K, Hay E, et al. Intradermal grass pollen immunotherapy increases TH2 and IgE responses and worsens respiratory allergic symptoms. J Allergy Clin Immunol 2017; 139:1830-9 e13.

18. Patterson AM, Bonny AE, Shiels WE, 2nd, Erwin EA. Three-injection intralymphatic immunotherapy in adolescents and young adults with grass pollen rhinoconjunctivitis. Ann Allergy Asthma Immunol 2016; 116:168-70.

19. Senti G, Crameri R, Kuster D, Johansen P, Martinez-Gomez JM, Graf N, et al. Intralymphatic immunotherapy for cat allergy induces tolerance after only 3 injections. J Allergy Clin Immunol 2012; 129:1290-6.

20. Senti G, von Moos S, Kundig TM. Epicutaneous Immunotherapy for Aeroallergen and Food Allergy. Curr Treat Options Allergy 2014; 1:68-78.

21. Fujita H, Soyka MB, Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy. Clin Transl Allergy 2012; 2:2.

22. Akdis CA, Akdis M, Blesken T, Wymann D, Alkan SS, Muller U, 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.

23. Suarez-Fueyo A, Ramos T, Galan A, Jimeno L, Wurtzen PA, Marin A, et al. Grass tablet sublingual immunotherapy downregulates the TH2 cytokine response followed by regulatory T-cell generation. J Allergy Clin Immunol 2014; 133:130-8 e1-2.

24. Sugita K, Hanakawa S, Honda T, Kondoh G, Miyachi Y, Kabashima K, et al. Generation of Helios reporter mice and an evaluation of the suppressive capacity of Helios(+) regulatory T cells in vitro. Exp Dermatol 2015; 24:554-6.

25. Mobs C, Ipsen H, Mayer L, Slotosch C, Petersen A, Wurtzen PA, et al. Birch pollen immunotherapy results in long-term loss of Bet v 1-specific TH2 responses, transient TR1 activation, and synthesis of IgE-blocking antibodies. J Allergy Clin Immunol 2012; 130:1108-16 e6.

26. 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, 72 e1.

27. Jutel M, Agache I, Bonini S, Burks AW, Calderon M, Canonica W, et al. International consensus on allergy immunotherapy. J Allergy Clin Immunol 2015; 136:556-68.

28. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. PLoS One 2011; 6:e24226.

29. Takatori H, Kawashima H, Matsuki A, Meguro K, Tanaka S, Iwamoto T, et al. Helios Enhances Treg Cell Function in Cooperation With FoxP3. Arthritis Rheumatol 2015; 67:1491-502.

30. Serre K, Benezech C, Desanti G, Bobat S, Toellner KM, Bird R, et al. Helios is associated with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of Foxp3 expression. PLoS One 2011; 6:e20731.

31. Wegrzyn AS, Jakiela B, Ruckert B, Jutel M, Akdis M, Sanak M, et al. T-cell regulation during viral and nonviral asthma exacerbations. J Allergy Clin Immunol 2015; 136:194-7 e9.

32. Ulges A, Klein M, Reuter S, Gerlitzki B, Hoffmann M, Grebe N, et al. Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. Nat Immunol 2015; 16:267-75.

33.   van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. J Allergy Clin Immunol 2013; 131:1204-12.

34.   van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martinez-Martinez P, Vermeulen E, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 2007; 317:1554-7.

35.   Santos AF, James LK, Bahnson HT, Shamji MH, Couto-Francisco NC, Islam S, et al. IgG4 inhibits peanut-induced basophil and mast cell activation in peanut-tolerant children sensitized to peanut major allergens. J Allergy Clin Immunol 2015; 135:1249-56.

36.   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.

37.   Van Ree R, Van Leeuwen WA, Dieges PH, Van Wijk RG, De Jong N, Brewczyski PZ, et al. Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. Clin Exp Allergy 1997; 27:68-74.

38.   Gleich GJ, Zimmermann EM, Henderson LL, Yuninger 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.

39.   Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. World Allergy Organ J 2015; 8:17.

40.   Herder C, Donath MY. Interleukin-1 receptor antagonist: friend or foe to the heart? Lancet Diabetes Endocrinol 2015; 3:228-9.

41.   Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 2011; 117:3720-32.

42.   Cowen EW, Goldbach-Mansky R. DIRA, DITRA, and new insights into pathways of skin inflammation: what's in a name? Arch Dermatol 2012; 148:381-4.

43. Luz-Crawford P, Djouad F, Toupet K, Bony C, Franquesa M, Hoogduijn MJ, et al. Mesenchymal Stem Cell-Derived Interleukin 1 Receptor Antagonist Promotes Macrophage Polarization and Inhibits B Cell Differentiation. Stem Cells 2016; 34:483-92.

44. Shamji MH, Kappen JH, Akdis M, Jensen-Jarolim E, Knol EF, Kleine-Tebbe J, et al. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper. Allergy 2017; 72:1156-73.

45. O'Mahony L, Akdis CA, Eiwegger T. Innate mechanisms can predict successful allergy immunotherapy. J Allergy Clin Immunol 2016; 137:559-61.

46. Gueguen C, Bouley J, Moussu H, Luce S, Duchateau M, Chamot-Rooke J, et al. Changes in markers associated with dendritic cells driving the differentiation of either TH2 cells or regulatory T cells correlate with clinical benefit during allergen immunotherapy. J Allergy Clin Immunol 2016; 137:545-58.

47. Morita H, Moro K, Koyasu S. Innate lymphoid cells in allergic and nonallergic inflammation. J Allergy Clin Immunol 2016; 138:1253-64.

48. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol 2013; 13:145-9.

49. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat Immunol 2013; 14:221-9.

50. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. Immunity 2013; 38:769-81.

51. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature 2010; 463:540-4.

52. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol 2011; 12:1055-62.

53. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc Natl Acad Sci U S A 2010; 107:11489-94.

54. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. Immunity 2012; 37:649-59.

55. Pelly VS, Kannan Y, Coomes SM, Entwistle LJ, Ruckerl D, Seddon B, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. Mucosal Immunol 2016; 9:1407-17.

56. Melo-Gonzalez F, Hepworth MR. Functional and phenotypic heterogeneity of group 3 innate lymphoid cells. Immunology 2017; 150:265-75.

57. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. J Allergy Clin Immunol 2014; 134:671-8 e4.

58. Liu T, Wu J, Zhao J, Wang J, Zhang Y, Liu L, et al. Type 2 innate lymphoid cells: A novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma. Respir Med 2015; 109:1391-6.

59. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. J Allergy Clin Immunol 2016; 137:75-86 e8.

60. Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani S. Type 2 innate lymphoid cells in induced sputum from children with severe asthma. J Allergy Clin Immunol 2016; 137:624-6 e6.

61. Morita H, Arae K, Unno H, Miyauchi K, Toyama S, Nambu A, et al. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. Immunity 2015; 43:175-86.

62. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. Immunity 2012; 36:451-63.

63. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. J Immunol 2012; 188:1503-13.

64. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. Nat Immunol 2011; 12:631-8.

65. Kim DW, Cho SH. Emerging Endotypes of Chronic Rhinosinusitis and Its Application to Precision Medicine. Allergy Asthma Immunol Res 2017; 9:299-306.

66. Doherty TA, Scott D, Walford HH, Khorram N, Lund S, Baum R, et al. Allergen challenge in allergic rhinitis rapidly induces increased peripheral blood type 2 innate lymphoid cells that express CD84. J Allergy Clin Immunol 2014; 133:1203-5.

67. Lao-Araya M, Steveling E, Scadding GW, Durham SR, Shamji MH. Seasonal increases in peripheral innate lymphoid type 2 cells are inhibited by subcutaneous grass pollen immunotherapy. J Allergy Clin Immunol 2014; 134:1193-5 e4.

68. Lombardi V, Beuraud C, Neukirch C, Moussu H, Morizur L, Horiot S, et al. Circulating innate lymphoid cells are differentially regulated in allergic and nonallergic subjects. J Allergy Clin Immunol 2016; 138:305-8.

69. Zhong H, Fan XL, Yu QN, Qin ZL, Chen D, Xu R, et al. Increased innate type 2 immune response in house dust mite-allergic patients with allergic rhinitis. Clin Immunol 2017; 183:293-9.

70. Fan D, Wang X, Wang M, Wang Y, Zhang L, Li Y, et al. Allergen-Dependent Differences in ILC2s Frequencies in Patients With Allergic Rhinitis. Allergy Asthma Immunol Res 2016; 8:216-22.

71. Krishnamoorthy N, Burkett PR, Dalli J, Abdulnour RE, Colas R, Ramon S, et al. Cutting edge: maresin-1 engages regulatory T cells to limit type 2 innate lymphoid cell activation and promote resolution of lung inflammation. J Immunol 2015; 194:863-7.

72. Rigas D, Lewis G, Aron JL, Wang B, Banie H, Sankaranarayanan I, et al. Type 2 innate lymphoid cell suppression by regulatory T cells attenuates airway hyperreactivity and requires inducible T-cell costimulator-inducible T-cell costimulator ligand interaction. J Allergy Clin Immunol 2017; 139:1468-77 e2.

73. Mitthamsiri W, Pradubpongsa P, Sangasapaviliya A, Boonpiyathad T. Decreased CRTH2 Expression and Response to Allergen Re-stimulation on Innate Lymphoid Cells in Patients With Allergen-Specific Immunotherapy. Allergy Asthma Immunol Res 2018; 10:662-74.

74. Noh G, Ahn HS, Cho NY, Lee S, Oh JW. The clinical significance of food specific IgE/IgG4 in food specific atopic dermatitis. Pediatr Allergy Immunol 2007; 18:63-70.

75. Wang J, Lin J, Bardina L, Goldis M, Nowak-Wegrzyn A, Shreffler WG, et al. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. J Allergy Clin Immunol 2010; 125:695-702, e1- e6.

76. Okamoto S, Taniuchi S, Sudo K, Hatano Y, Nakano K, Shimo T, et al. Correction: Predictive value of IgE/IgG4 antibody ratio in children with egg allergy. Allergy Asthma Clin Immunol 2013; 9:34.

77. Bonvalet M, Wambre E, Moussu H, Horiot S, Kwok WW, Louise A, et al. Comparison between major histocompatibility complex class II tetramer staining and surface expression of activation markers for the detection of allergen-specific CD4(+) T cells. Clin Exp Allergy 2011; 41:821-9.

78. Shamji MH, Wilcock LK, Wachholz PA, Dearman RJ, Kimber I, Wurtzen PA, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. J Immunol Methods 2006; 317:71-9.

79. Ryan JF, Hovde R, Glanville J, Lyu SC, Ji X, Gupta S, et al. Successful immunotherapy induces previously unidentified allergen-specific CD4+ T-cell subsets. Proc Natl Acad Sci U S A 2016.

80. Choovivathanavanich P, Suwanprateep P, Kanthavichitra N. House dust and cockroach sensitivity in allergic Thais. J Med Assoc Thai 1971; 54:476-81.

81.   Pumhirun P, Towiwat P, Mahakit P. Aeroallergen sensitivity of Thai patients with allergic rhinitis. Asian Pac J Allergy Immunol 1997; 15:183-5.

82.   Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen. Clin Exp Allergy 2001; 31:1594-8.

83.   Ghaemmaghami AM, Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. Clin Exp Allergy 2002; 32:1468-75.

84.   Heijink IH, Postma DS, Noordhoek JA, Broekema M, Kapus A. House dust mite-promoted epithelial-to-mesenchymal transition in human bronchial epithelium. Am J Respir Cell Mol Biol 2010; 42:69-79.

85.   Huser C, Dieterich P, Singh J, Shah-Hosseini K, Allekotte S, Lehmacher W, et al. A 12-week DBPC dose-finding study with sublingual monomeric allergoid tablets in house dust mite-allergic patients. Allergy 2017; 72:77-84.

86.   Ihara F, Sakurai D, Yonekura S, Iinuma T, Yagi R, Sakurai T, et al. Identification of specifically reduced Th2 cell subsets in allergic rhinitis patients after sublingual immunotherapy. Allergy 2018; 73:1823-32.

87.   Renand A, Archila LD, McGinty J, Wambre E, Robinson D, Hales BJ, et al. Chronic cat allergen exposure induces a TH2 cell-dependent IgG4 response related to low sensitization. J Allergy Clin Immunol 2015; 136:1627-35 e1-13.

88.   Sadegh-Nasseri S, Natarajan S, Chou CL, Hartman IZ, Narayan K, Kim A. Conformational heterogeneity of MHC class II induced upon binding to different peptides is a key regulator in antigen presentation and epitope selection. Immunol Res 2010; 47:56-64.

89.   Wambre E, Bonvalet M, Bodo VB, Maillere B, Leclert G, Moussu H, et al. Distinct characteristics of seasonal (Bet v 1) vs. perennial (Der p 1/Der p 2) allergen-specific CD4(+) T cell responses. Clin Exp Allergy 2011; 41:192-203.

90.   Wambre E, James EA, Kwok WW. Characterization of CD4+ T cell subsets in allergy. Curr Opin Immunol 2012; 24:700-6.

91. Franz B, May KF, Jr., Dranoff G, Wucherpfennig K. Ex vivo characterization and isolation of rare memory B cells with antigen tetramers. Blood 2011; 118:348-57.

92. Patil SU, Ogunniyi AO, Calatroni A, Tadigotla VR, Ruiter B, Ma A, et al. Peanut oral immunotherapy transiently expands circulating Ara h 2-specific B cells with a homologous repertoire in unrelated subjects. J Allergy Clin Immunol 2015; 136:125-34 e12.

93. Hoh RA, Joshi SA, Liu Y, Wang C, Roskin KM, Lee JY, et al. Single B-cell deconvolution of peanut-specific antibody responses in allergic patients. J Allergy Clin Immunol 2015.

94. Hoh RA, Joshi SA, Liu Y, Wang C, Roskin KM, Lee JY, et al. Single B-cell deconvolution of peanut-specific antibody responses in allergic patients. J Allergy Clin Immunol 2016; 137:157-67.

95. Kadri S, Hinman VF, Benos PV. RNA deep sequencing reveals differential microRNA expression during development of sea urchin and sea star. PLoS One 2011; 6:e29217.

96. Sawad B. RF, Atik S. Clinical data of rhinitis, asthma and eczema patients at the allergy clinic of Pramongkutklao Hospital, Bangkok, Thailand. Allergy 2012; 67:170.

97. Downie SR, Andersson M, Rimmer J, Leuppi JD, Xuan W, Akerlund A, et al. Symptoms of persistent allergic rhinitis during a full calendar year in house dust mite-sensitive subjects. Allergy 2004; 59:406-14.

98. Demoly P, Bousquet PJ, Mesbah K, Bousquet J, Devillier P. Visual analogue scale in patients treated for allergic rhinitis: an observational prospective study in primary care: asthma and rhinitis. Clin Exp Allergy 2013; 43:881-8.

99. Ohta K, Jean Bousquet P, Akiyama K, Adachi M, Ichinose M, Ebisawa M, et al. Visual analog scale as a predictor of GINA-defined asthma control. The SACRA study in Japan. J Asthma 2013; 50:514-21.

100. Reich A, Heisig M, Phan NQ, Taneda K, Takamori K, Takeuchi S, et al. Visual analogue scale: evaluation of the instrument for the assessment of pruritus. Acta Derm Venereol 2012; 92:497-501.

101. Bousquet PJ, Combescure C, Neukirch F, Klossek JM, Mechin H, Daures JP, et al. Visual analog scales can assess the severity of rhinitis graded according to ARIA guidelines. Allergy 2007; 62:367-72.

102. Eifan AO, Akkoc T, Yildiz A, Keles S, Ozdemir C, Bahceciler NN, et al. Clinical efficacy and immunological mechanisms of sublingual and subcutaneous immunotherapy in asthmatic/rhinitis children sensitized to house dust mite: an open randomized controlled trial. Clin Exp Allergy 2010; 40:922-32.

103. Clark J, Schall R. Assessment of combined symptom and medication scores for rhinoconjunctivitis immunotherapy clinical trials. Allergy 2007; 62:1023-8.

104. Calderon MA, Bernstein DI, Blaiss M, Andersen JS, Nolte H. A comparative analysis of symptom and medication scoring methods used in clinical trials of sublingual immunotherapy for seasonal allergic rhinitis. Clin Exp Allergy 2014; 44:1228-39.

105. Kwakkenbos MJ, Diehl SA, Yasuda E, Bakker AQ, van Geelen CM, Lukens MV, et al. Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. Nat Med 2010; 16:123-8.

106. Kwok WW, Roti M, Delong JH, Tan V, Wambre E, James EA, et al. Direct ex vivo analysis of allergen-specific CD4+ T cells. J Allergy Clin Immunol 2010; 125:1407-9 e1.

107. Boonpiyathad T, Meyer N, Moniuszko M, Sokolowska M, Eljaszewicz A, Wirz OF, et al. High-dose bee venom exposure induces similar tolerogenic B-cell responses in allergic patients and healthy beekeepers. Allergy 2017; 72:407-15.

108. Wright BL, Kulis M, Orgel KA, Burks AW, Dawson P, Henning AK, et al. Component-resolved analysis of IgA, IgE, and IgG4 during egg OIT identifies markers associated with sustained unresponsiveness. Allergy 2016; 71:1552-60.

109. Sugimoto M, Kamemura N, Nagao M, Irahara M, Kagami S, Fujisawa T, et al. Differential response in allergen-specific IgE, IgGs, and IgA levels for predicting outcome of oral immunotherapy. Pediatr Allergy Immunol 2016; 27:276-82.

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

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

112. Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet 2001; 357:752-6.

113. Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. J Exp Med 2008; 205:2887-98.

114. Platts-Mills TA, Woodfolk JA. Allergens and their role in the allergic immune response. Immunol Rev 2011; 242:51-68.

115. van de Veen W, Akdis M. Role of IgG4 in IgE-mediated allergic responses. J Allergy Clin Immunol 2016; 138:1434-5.

116. Collins AM, Jackson KJ. A Temporal Model of Human IgE and IgG Antibody Function. Front Immunol 2013; 4:235.

117. James LK, Bowen H, Calvert RA, Dodev TS, Shamji MH, Beavil AJ, et al. Allergen specificity of IgG(4)-expressing B cells in patients with grass pollen allergy undergoing immunotherapy. J Allergy Clin Immunol 2012; 130:663-70 e3.

118. Hoh RA, Joshi SA, Liu Y, Wang C, Roskin KM, Lee JY, et al. Single B-cell deconvolution of peanut-specific antibody responses in allergic patients. J Allergy Clin Immunol 2016; 137:157-67.

119. Schmid JM, Nezam H, Madsen HH, Schmitz A, Hoffmann HJ. Intralymphatic immunotherapy induces allergen specific plasmablasts and increases tolerance to skin prick testing in a pilot study. Clin Transl Allergy 2016; 6:19.

120. Stanic B, van de Veen W, Wirz OF, Ruckert B, Morita H, Sollner S, et al. IL-10-overexpressing B cells regulate innate and adaptive immune responses. J Allergy Clin Immunol 2015; 135:771-80 e8.

121.   Volarevic V, Al-Qahtani A, Arsenijevic N, Pajovic S, Lukic ML. Interleukin-1 receptor antagonist (IL-1Ra) and IL-1Ra producing mesenchymal stem cells as modulators of diabetogenesis. Autoimmunity 2010; 43:255-63.

122.   Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov 2012; 11:633-52.

123.   Galea J, Ogungbenro K, Hulme S, Patel H, Scarth S, Hoadley M, et al. Reduction of inflammation after administration of interleukin-1 receptor antagonist following aneurysmal subarachnoid hemorrhage: results of the Subcutaneous Interleukin-1Ra in SAH (SCIL-SAH) study. J Neurosurg 2017:1-9.

124.   Varshney P, Jones SM, Scurlock AM, Perry TT, Kemper A, Steele P, et al. A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. J Allergy Clin Immunol 2011; 127:654-60.

125.   Kim EH, Bird JA, Kulis M, Laubach S, Pons L, Shreffler W, et al. Sublingual immunotherapy for peanut allergy: clinical and immunologic evidence of desensitization. J Allergy Clin Immunol 2011; 127:640-6 e1.

126.   Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, et al. Clinical efficacy and immune regulation with peanut oral immunotherapy. J Allergy Clin Immunol 2009; 124:292-300, e1-97.

127.   Vickery BP, Lin J, Kulis M, Fu Z, Steele PH, Jones SM, et al. Peanut oral immunotherapy modifies IgE and IgG4 responses to major peanut allergens. J Allergy Clin Immunol 2013; 131:128-34 e1-3.

128.   Burks AW, Jones SM, Wood RA, Fleischer DM, Sicherer SH, Lindblad RW, et al. Oral immunotherapy for treatment of egg allergy in children. N Engl J Med 2012; 367:233-43.

129.   Syed A, Garcia MA, Lyu SC, Bucayu R, Kohli A, Ishida S, et al. Peanut oral immunotherapy results in increased antigen-induced regulatory T-cell function and hypomethylation of forkhead box protein 3 (FOXP3). J Allergy Clin Immunol 2014; 133:500-10.

130. Wambre E, DeLong JH, James EA, LaFond RE, Robinson D, Kwok WW. Differentiation stage determines pathologic and protective allergen-specific CD4+ T-cell outcomes during specific immunotherapy. J Allergy Clin Immunol 2012; 129:544-51, 51 e1-7.

131. Akdis CA, Akdis M. Advances in allergen immunotherapy: aiming for complete tolerance to allergens. Sci Transl Med 2015; 7:280ps6.

132. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, 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.

133. van de Veen W, Stanic B, Wirz OF, Jansen K, Globinska A, Akdis M. Role of regulatory B cells in immune tolerance to allergens and beyond. J Allergy Clin Immunol 2016; 138:654-65.

134. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010; 184:3433-41.

135. Elkord E, Abd Al Samid M, Chaudhary B. Helios, and not FoxP3, is the marker of activated Tregs expressing GARP/LAP. Oncotarget 2015; 6:20026-36.

136. Bin Dhuban K, d'Hennezel E, Nagai Y, Xiao Y, Shao S, Istomine R, et al. Suppression by human FOXP3(+) regulatory T cells requires FOXP3-TIP60 interactions. Sci Immunol 2017; 2.

137. Froese van Dijck A, Kousar L, Layhadi J, Fedina O, Calvosa S, Scadding GW, et al. SATB1 is repressed in FoxP3+Tregs following Grass Pollen Subcutaneous and Sublingual Immunotherapy and Correlates with Clinical efficacy. Journal of Allergy and Clinical Immunology 2017; 139:AB192.

138. Renand A, Shamji MH, Harris KM, Qin T, Wambre E, Scadding GW, et al. Synchronous immune alterations mirror clinical response during allergen immunotherapy. J Allergy Clin Immunol 2018; 141:1750-60 e1.

139. Wambre E, Bajzik V, DeLong JH, O'Brien K, Nguyen QA, Speake C, et al. A phenotypically and functionally distinct human TH2 cell subpopulation is associated with allergic disorders. Sci Transl Med 2017; 9.

140. Scadding GW, Calderon MA, Shamji MH, Eifan AO, Penagos M, Dumitru F, et al. Effect of 2 Years of Treatment With Sublingual Grass Pollen Immunotherapy on Nasal Response to Allergen Challenge at 3 Years Among Patients With Moderate to Severe Seasonal Allergic Rhinitis: The GRASS Randomized Clinical Trial. JAMA 2017; 317:615-25.

141. 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.

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INSTITUTIONS ATTENDED

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- Master degree in medical science; minor in allergy and immunology - Chulalongkorn University, Bangkok, Thailand (2010)

- Bachelor degree in doctor of medicine - Pramongkutklao Military College of Medicine, Mahidol University, Bangkok, Thailand (2001)

Certification:

- EAACI Knowledge Examination in Allergology and Clinical Immunology – European Academy of Allergy and Clinical Immunology (2016)

- Research fellow in allergy - SIAF, Swiss Institute of Allergy and Asthma Research, Switzerland (2013 – 2015)

- International acupuncture - Tianjin University, Tianjin, China (2011)

- Thai board of medicine allergy and clinical immunology - King Chulalongkorn Memorial Hospital, Bangkok, Thailand (2010)

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HOME ADDRESS

PUBLICATION

1. W Mitthamsiri, P Pradubpongsa, A Sangasapaviliya,T Boonpiyathad, Decreased CRTH2 Expression and Response to Allergen Re-Stimulation on Innate Lymphoid Cells in Pateints with Allergen-Specific Immunotherapy. Allergy Asthma Immunol Res. 2018 Nov;10(6):662-674

2. Shan M, Carrillo J, Yeste A, Gutzeit C, Segura-Garzón D, Walland AC, Pybus M, Grasset EK, Yeiser JR, Matthews DB, van de

Veen W, Comerma L, He B, Boonpiyathad T, Lee H, Blanco J, Osborne LC, Siracusa MC, Akdis M, Artis D, Mehandru S, Sampson HA, Berin MC, Chen K, Cerutti A. Secreted IgD Amplifies Humoral T Helper 2 Cell Responses by Binding Basophils via Galectin-9 and CD44. Immunity. 2018 Sep 26:1-16.

3. Głobińska A, Boonpiyathad T, Satitsuksanoa P, Kleuskens M, van de Veen W, Sokołowska M, Akdis M. Mechanisms of allergen-specific immunotherapy: diverse mechanisms of immune tolerance to allergens. Ann Allergy Asthma Immunol. 2018 Sep;121(3):306-312

4. Boonpiyathad T, Mitthamasiri W, Pradubponsa P, Sangasapaviliya A. Urticaria Diagnosis. EMJ. 2018;3(1):98-105

5. Boonpiyathad T, Sangasapaviliya A. Hydroxychloroquine in the treatment of anti-histamine refractory chronic spontaneous urticaria, randomized single-blinded placebo-controlled trial and an open label comparison study. Eur Ann Allergy Clin Immunol. 2017 Sep;49(5):220-224

6. Li S, Morita H, Rückert B, Boonpiyathad T, Neumann A, Akdis C, Type 3 innate lymphoid cells induce proliferation of CD94+ NK cells, J Allergy Clin Immunol. Oct;140(4):1156-1159.e7.

7. Boonpiyathad T, Chantveerawong T, Pradubponsa P, Sangasapaviliya A. Serum Vitamin D Levels and Vitamin D Supplement in Adult Patients with Asthma Exacerbation. J Allergy. 2016, Article ID 4070635, 9 pages

8. Tadech Boonpiyathad, Atik Sangasapaviriya. Autologous serum and plasma skin test to predict 2-year outcome in chronic spontaneous urticaria. Asia Pac Allergy. 2016 Oct;6(4):226-235.

9. Boonpiyathad T, Meyer N, Moniuszko M, Sokolowska M, Eljaszewicz A, Wirz OF, Tomasiak-Lozowska MM, Bodzenta-Lukaszyk A, Ruxrungtham K, van de Veen W. High-dose bee venom exposure induces similar tolerogenic B-cell responses in allergic patients and healthy beekeepers. Allergy. 72(3):407-415, Mar 2017

10. P. Satitsuksanoa, M. Kennedy, D. Gilis, M. Le Mignon, N.

Suratannon, W. T. Soh, J. Wongpiyabovorn, P. Chatchatee, M. Vangveravong, T. Rerkpattanapipat, A. Sangasapaviliya, S. Piboonpocanun, E. Nony, K. Ruxrungtham, A. Jacquet, the Mite Allergy Research Cohort (MARC) study Team (Thantiworasit P, Pulsawat P, Daengsuwan T, Laisuan W, Tongdee M, Dchapaphapeaktak N, Boonpiyathad T).The minor house dust mite allergen Der p 13 is a fatty acid-binding protein and an activator of a TLR2-mediated innate immune response. Allergy. 2016 Oct;71(10):1425-34

11.   Soh W.T.Le Mignon M. Suratannon N. Satitsuksanoa P. Chatchatee P. Wongpiyaboron J. Vangveravong M. Rerkpattanapipat T. Sangasapaviliya A. Nony E. Piboonpocanun S. Ruxrungtham K. Jacquet A. Mite Allergy Research Cohort (MARC) Study Team (Thantiworasit P, Pulsawat P, Daengsuwan T, Laisuan W, Tongdee M, Dchapaphapeaktak N, Boonpiyathad T). The House Dust Mite Major Allergen Der p 23 Displays O-Glycan-Independent IgE Reactivities but No Chitin-Binding Activity. Int Arch Allergy Immunol 2015;168:150-160

12.   Tadech Boonpiyathad, Sittipong Yimsawad, Atik Sangasapaviriya. The Cost of Asthma Treatment in Phramongkutklao Hospital: Population-Based Study in Adults. J Med Assoc Thai. 2016 Jan;99 (1): 51-57

13.   Boonpiyathad T, Pradubpongsa P, Sangasapaviriya A. Vitamin d supplements improve urticaria symptoms and quality of life in chronic spontaneous urticaria patients: a prospective case-control study. Dermatoendocrinol. 2014 Jul 16;6:e29727

14.   Sawad Boonpiyathad and Atik Sangasapaviliya, Refractory Asthma Treatment Is Complicated by Tracheobronchomalacia: Case Reports and Review of the Literature; Case Reports in Medicine, Volume 2013, Article ID 735058, 4 pages

15.   Boonpiyathad S, Pornsuriyasak P, Buranapraditkun S, Klaewsongkram J., Interleukin-2 levels in exhaled breath condensates,

asthma severity, and asthma control in nonallergic asthma. Allergy Asthma Proc. 2013 Sep;34(5):35-41

AWARD RECEIVED

1. Travel grant award of Asia Pacific Association of Allergy, Asthma and Clinical Immunology, 2010

2. Travel grant award of International Severe Asthma Forum, EAACI, 2012

3. EAACI research fellowship award 2013

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