Specific immunoglobulin G4 inhibits Th2 cytokine production in allergic asthmatics with *Dermatophagoides pteronyssinus* subcutaneous immunotherapy

Qiujuan Su, MD[1*], Nina Ren, MD[1, 2*], Xueni Zeng, MD[1], Yan Dong, MD[1], Mo Xian, PhD[1], Xu Shi, PhD[1], Tian Luo, MD[1], Gang Liu, MD[3], Mulin Feng, MD[1#], Jing Li, MD[1, 4#]

[1] Department of Allergy and Clinical Immunology, Guangzhou Institute of Respiratory Health, State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China
[2] Department of Respiratory Medicine, Affiliated Hospital of Guangdong Medical university, Zhanjiang, China
[3] Clinical Research Center, Affiliated Hospital of Guangdong Medical University, Zhanjiang, China
[4] Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology, Guangzhou Medical University, Guangzhou, China

* These authors contributed equally to the work described in this paper.

 # Corresponding authors
Jing Li, MD
Department of Allergy and Clinical Immunology
State Key Laboratory of Respiratory Disease
The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China
Tel: +86-20-34296151; Fax: +86-20-34298996; Email: lijing@gird.cn

Mulin Feng, MD
Department of Allergy and Clinical Immunology
State Key Laboratory of Respiratory Disease
The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China
Tel: +86-20-34296151; Fax: +86-20-34298996; Email: pinsium@163.com

Abstract

Background: The modulations of subcutaneous allergen immunotherapy (SCIT) on lymphocyte subsets and cytokine productions are not fully clarified.

Objective: We investigated the changes of T-lymphocyte subsets and serum *Dermatophagoides pteronyssinus* specific immunoglobulin G4 (Der-p sIgG4), and cytokine productions in allergic asthmatics during Der-p SCIT.

Methods: This study involved 20 allergic asthmatics receiving 156-week Der-p SCIT, 20 patients without SCIT (non-SCIT). Symptom and medication scores (SMS), serum Der-p sIgG4 levels, $CD4^+CD25^+Foxp3^+$ T regulatory (Treg), $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ T-helper (Th) 1, $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ Th2 lymphocyte percentage in peripheral blood mononuclear cells (PBMCs) with/without Der-p extract stimulation at weeks 0, 4, 12, 16, 52, 104, and 156 were measured. Serum from SCIT and non-SCIT patients were incubated with Der-p allergen and Der-p sensitized PBMCs. Levels of interleukin (IL) -4, IL-5, IL-10, IL-13, IL-17, interferon (IFN) -γ, tumor necrosis factor (TNF) -α and transfer growth factor (TGF) -β1 in supernatant were detected.

Results: SCIT patients had significantly lower SMS after week 52. Der-p sIgG4 levels in SCIT patients significantly increased at week 16 compared to non-SCIT subjects. $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ Th2 percentage in SCIT patients showed a significant decrease from week 104 to 156 comparing to week 0, while no change was observed in $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ Th1 percentage. IL-5, IL-13, IL-4, IL-17, and TNF-α levels in supernatant of Der-p sensitized PBMCs, cultured with serum of SCIT patients after 16 weeks decreased significantly compared with non-SCIT patients, and showed significant reverse associations with Der-p sIgG4 levels.

Conclusion: SCIT down-regulated Th2 cytokine productions associated reversely with Dep-p sIgG4 levels in Der-p allergic asthma patients.

Keywords: asthma; rhinitis; allergen specific immunotherapy; regulatory T cells; T helper cells

ABBREVIATIONS

Der-p: *Dermatophagoides pteronyssinus*

Der-f: *Dermatophagoides farinae*

FEV_1: forced expiratory volume in one second

FVC: forced vital capacity

SMS: combined symptom medication score

AIT: allergen immunotherapy

SCIT: subcutaneous allergen immunotherapy

sIgE: specific immunoglobulin E

sIgG4: specific immunoglobulin G4

Treg: regulatory T cell

Foxp3: forkhead box protein 3

Th: T helper cell

SPT: skin prick test

PBMC: peripheral blood mononuclear cell

TNF: tumor necrosis factor

TGF-β: transforming growth factor-β

CRIA: cytokine release inhibition assay

INTRODUCTION

Allergen immunotherapy (AIT) is an effective treatment for allergic rhinitis and allergic asthma in terms of reducing symptom score and medication requirements, improving quality of life, and change the course of allergic disease and induce allergen-specific immune tolerance (1). The mechanisms that are associated with immunotherapy involve changes in the humoral immune response and cellular reactions (2). Cellular changes include generation of allergen specific T regulatory (reg) and Breg cells and suppression of allergen specific T-helper (Th) 2 cells (3). Tregs have been shown to governing peripheral self-tolerance in experimental animals and humans, suppress allergic responses to aeroallergens such as house dust mite and grass pollen, and shift Th2 to Th1, thereby reduce Th2 type cytokines such as interleukin (IL) -4, IL-5, and IL-13 release. The humoral immune response involved switch allergen-specific B-cells produce IgG4 instead of produce allergen-specific immunoglobulin E (sIgE) by Treg cells secreted IL-10 and transforming growth factor-β (TGF-β) (4). Our previous and other studies have found that AIT could induce substantial production in allergen specific immunoglobulin G4 (sIgG4) antibodies (5, 6). Specific IgG4 antibodies are responsible for competing with sIgE to form allergen-IgE complex that inhibit the binding of sIgE to receptor–expressing effector cells (7, 8), and further reduce the cytokine release. Although these observations helped to clarify mechanisms of how AIT promotes the induction of allergen tolerance, the detail immunosuppression factors on reduction in cytokine release through modulating the *reactivity* of effector cells when exposed to allergen stimulation and whether blocking antibody to reduce the *sensitivity* or the threshold of effector cell activation to submaximal allergen concentrations stimulation during the long-term AIT are not clear. In this study, we investigated the changes of Treg, T helper cells, and IgG4 antibodies and their modulations on cytokine release during 3 years of house dust mite subcutaneous allergen immunotherapy (SCIT) in patients with allergic rhinitis and/or asthma.

METHODS

Study design and population

The study included 60 subjects (37 males and 23 females, 5–58 years of age), of which 20 cases received *Dermatophagoides pteronyssinus* (Der-p) SCIT, and 20 patients received standardized asthma and rhinitis medications (non-SCIT), and 20 health subjects were as baseline control. All patients came from the allergy and clinical immunology department of Guangzhou Institute of Respiratory Diseases, fulfilled the ARIA guideline for allergic rhinitis and/or GINA guideline for mild-to-moderate asthma (9, 10), and had positive skin prick test (SPT) and sIgE to Der-p. The health subjects were recruited from Guangzhou Medical University. Patients visited the hospitals for treatments and clinical evaluations. Blood samples were collected before treatment and at specific time points during treatment (week 0, 4, 12, 16, 52, 104, and 156) (Figure 1). This study was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University and registered in the Chinese Clinical Trial Registry (ChiCTR-OOC-15006207). Written informed consent was obtained from all adult patients, or parents of the children.

Skin prick tests

Sensitization to house dust mite aeroallergens including Der-p, *Dermatophagoides farinae* (Der-f) and other common allergens (Soluprick SQ, ALKAbello A/S, Horsholm, Denmark) was assessed. A positive skin reaction was defined as a wheal size ≥ 3 mm after subtraction of the negative control.

Detection of serum IgE and IgG4

The levels of total IgE and sIgE against Der-p, Der-f and other common allergens in all serum samples were measured by Pharmacia CAP fluorescence enzyme immunoassay system (ThermoFisher, Sweden) at week 0. The sIgE results are reported as kU/l, with a cut-off value of 0.35 kU/l and upper sIgE detection limit of 100 kU/l. Any sample with sIgE level > 100 kU/l was diluted and tested again. Serum Der-p sIgG4 levels were measured by a four-layer sandwich ELISA using methods we reported previously (11).

Spirometry and histamine bronchial provocation test

Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were

measured (week 0, 4, 12, 16, 52, 104, and 156) using a MicroQuark Spirometer (Cosmed), which met the standards of the American Thoracic Society and the European Respiratory Society (12); the findings were presented as percent predicted value (FEV_1%).

SCIT protocol

The patients were treated with subcutaneous injections of standardized aluminum-formulated Der-p Alutard-SQ vaccine (ALK-Abello A/S, Horsholm, Denmark). The treatment protocol followed the recommended updosing schedule of 16 weeks before reaching a maintenance dose of 100,000 Alutard-SQ, which was maintained to complete 3 years of SCIT.

Clinical evaluations

The patients were requested to complete a symptom and medication diary during the whole course of treatment. Patients were asked to rate the symptoms of asthma (daytime: 0–5; night time: 0–4) and rhinitis (day or night time: 0–2) according to the severity and frequency of the symptoms in disturbing daily activities and sleep (13). The medication score is calculated by assigning a score of 1 to 160 μg of budesonide or the equivalent dose of inhaled corticosteroid, or 130 μg of budesonide or the equivalent dose of nasal corticosteroid; each puff of salbutamol/terbutaline or the equivalent dose of other inhaled β2-agnoist; 10 mg of oral loratidine or the equivalent dose of other oral anti-histamine. Combined symptom medication score (SMS) were defined as the sum of symptom scores and medication scores (9).

Assessment of T-lymphocyte subsets

For analysis of $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ Th2 cells, peripheral blood mononuclear cell (PBMC) were isolated from peripheral blood using Ficoll-Paque and incubated with 25 μg/ml Der-p extract (ALK-Abello, Denmark) over a period of 72 h at 37°C, during which 25 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μg/ml ionomycin, and 1.7 μg/ml monensine (all from Sigma, USA) were added for the final 4 h of the incubation period. At the end of incubation, the cells were stained with anti-CD4-PC5, anti-IL-4-PE, anti-IFN-γ-FITC, or isotype antibodies as control, and then assessed by flow cytometry (all antibodies and reagent from eBioscience, USA). For analysis of $CD4^+CD25^+Foxp3^+$ Treg cells, PBMCs were

incubated with 25 μg/ml Der-p extract over a period of 72 h at 37℃. At the end of incubation, the supernatant from each culture was collected and stored at –80°C until analysis of cytokines. The cells were then stained with anti-CD4-FITC, anti-CD25-PC5, and anti-Foxp3-PE followed the instruction of antibody. And then detected by flow cytometry (Beckman Coulter Epics XL-MCL, USA) and analyzed using FCS Express software (version 4).

Effector cell cytokine release inhibition assay

Serum inhibitory activity against Der-p stimulate effector cell release cytokines was performed by cytokine release inhibition assay (CRIA). Briefly, 10 μl of serum from SCIT and non-SCIT patients were incubated with 30 μl of Der-p allergen at a 96 well plate (the final concentration of Der-p was 0.15 μg/ml or 15 μg/ml, the allergen concentration was reference to the basophil activation test as we described previously (5)) at 37°C for 1 h. And then added 200 μl of PBMCs (from three severe HDM sensitized donors, 2×10^6 cells/ml) and incubated at 37°C for 2 h, after centrifugation, the supernatants were collected for cytokine determination. The supernatant concentration of IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ, TNF-α and TGF-β1 were tested by Bio-Plex Cytokine Assay (Bio-rad, USA) according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0. An independent-samples *t*- test for unpaired data was used to analyze between group differences in FEV_1%, SMS, Der-p sIgG4, Treg, Th1, Th2, cytokines, and CRIA. A paired-samples *t*-test was used to analyze within-group differences. Values are shown as mean ± SE. Linear regression was employed to analyze the relationship between log Der-p sIgG4, Treg, Th2, Th1, and cytokines. Differences were considered significant at values of $P < 0.05$.

RESULTS

Patient characteristics and the baseline levels of T-cell subsets

The baseline demographic data, SMS, antibody levels and T-cell subsets levels of all subjects are shown in Table 1. There were no differences between the SCIT and non-SCIT groups in gender, SMS, FEV_1%, SPT-diameters, peripheral eosinophil counts, serum IgE levels and percentage of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ Th1 cells ($P > 0.05$), while $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ Th2 levels in SCIT and non-SCIT subjects were higher than health controls in the baseline ($P < 0.05$).

Changes of clinical outcomes

SMS of asthma and rhinitis decreased significantly after 12 weeks of treatment in both SCIT and non-SCIT patients ($P < 0.05$), with significantly more decline in SCIT subjects at week 52 to 156 ($P < 0.05$). FEV_1% did not change significantly during treatment in either group ($P > 0.05$, Supplement figure 1A and 1B).

Time course of Treg, Th1, Th2, and serum sIgG4

Percentage of $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ Th2 cells in SCIT group decreased significantly at week 104 to 156 compared with week 0 with and without Der-p stimulation ($P < 0.05$, Figure 2E). No significant changes were observed for percentage of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ Th1 cells during the time course of treatment comparing with week 0 in SCIT and non-SCIT patients ($P > 0.05$, Figure 2C, 2D). The levels of Der-p sIgG4 in SCIT patients significantly increased starting from week 12 compared to week 0 ($P < 0.05$), and significantly increased from week 16 to week 156 when compared with non-SCIT subjects ($P < 0.01$) (Figure 2F).

Inhibition of effector cell cytokine release

Compared to week 0, levels of cytokines in supernatant of HDM sensitized PBMCs stimulated with 0.15 μg/ml of Der-p allergen and serum from SCIT patients decreased significantly for IL-5, IL-13, IL-17, and TNF-α after 16 weeks ($P < 0.05$), for IL-4 after 52 weeks ($P < 0.05$), but not for IL-10, IFN-γ, and TGF-β1, and not for serum from non-SCIT patients ($P > 0.05$, Figure 3). Levels of supernatant cytokines were not change when stimulated with 15 μg/ml of Der-p allergen and serum from SCIT and non-SCIT patients (Supplement Figure 2).

The relationship between levels of cytokines and Der-p sIgG4 and T-cell subsets

Levels of Der-p sIgG4 had significant negative linear associations with supernatant IL-5, IL-13, IL-17, TNF-α, and IL-4 in sensitized PBMCs stimulated with 0.15 μg/mL of Der-p allergen and serum of SCIT subjects at all time points after the treatment (IL-5, $R^2 = 0.64$, $P < 0.001$; IL-13, $R^2 = 0.66$, $P < 0.001$; IL-17, $R^2 = 0.60$, $P < 0.001$; TNF-α, $R^2 = 0.53$, $P < 0.001$; IL-4, $R^2 = 0.29$, $P < 0.001$; IFN-γ, $R^2 = 0.02$, $P > 0.05$; IL-10, $R^2 = 0.02$, $P > 0.05$; and TGF-β1, $R^2 = 0.00$, $P > 0.05$; Figure 4). The above correlations were not found in non-SCIT subjects (Supplement figure 3). There was no correlation between percentage of Treg, Th1, and Th2 and supernatant cytokine with/without Der-p allergen stimulation in the SCIT and non-SCIT patients at all time points (data not show).

DISCUSSION

In this study, we found that Der-p SCIT results in significant improvement in asthma symptoms and reduction in medication requirement starting after 12 weeks of SCIT. The percentage of Th2 cell in SCIT patients was significantly decreased from week 104 to 156 comparing with baseline. Serum obtained from SCIT patients significantly inhibited cytokine release from effector cells, and a significant inverse relationship was demonstrated between levels of Der-p sIgG4 and effector cell cytokine release.

Due to ethical and practical considerations, it was impracticable to perform a double-blind, placebo-controlled study in a real world setting as we demonstrated previously (13) in this 3-year clinical observation. The current investigation confirmed AIT is an effective treatment for allergic disease as consistency with our previous studies (11, 13) and other studies (14, 15). The mechanism of AIT involved the deviation from a Th2 to a Th1 cell-dominated immune response. Studies have been demonstrated that the proliferative response of peripheral blood T cells to allergen reduced after venom or pollen immunotherapy (16, 17), and shift from a Th2 to a Th1 milieu in the birch pollen immunotherapy (18). In this study, we found that AIT could down-regulate the frequency of Th2 cells, but not accompanied with an increase of Th1 cells. In contrast, similar changes of Th2 cells was not observed in other studies after AIT (3, 19, 20). Wei et al. demonstrated that the frequency of allergen specific Th2 cells was no significant decrease after immunotherapy (21). The controversy of the change of Th2 cells during the AIT may relate to the duration and administration type of AIT. Overall, the variable of Th1/Th2 immune deviation in the peripheral circulations after immunotherapy may not sufficiently reflect the clinical features of immunotherapy (22).

AIT-induced allergen tolerance also associated with generation of natural Treg (nTreg) which characterized by expression of Foxp3 at the early stage of AIT (23). Radulovic et al. found that the numbers of $CD4^+Foxp3^+$ Treg cells in the nasal mucosa increased after grass immunotherapy and correlated with clinical efficacy which supported the induction of immunosuppression of Treg cells in AIT immune tolerance. Similarly, Lin et al. (20) demonstrated that AIT could induce the expression of Foxp3 and up-regulate the functional activity of nTreg cells. In contrast, other studies (19, 21) found the percentage of peripheral $Foxp3^+$ Treg cells did not change after 1 year of house dust mite immunotherapy. In the current study, we found that the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells did not significantly change in SCIT group during the

treatment. In general, AIT may preferentially induce Treg cells in the local tissue rather than peripheral blood, the different markers such as $CD4^{+}Foxp3^{+}$ Treg or CD4+CD25+Foxp3+ Treg used in the studies may also have yielded conflicting data. Moreover, some studies suggest that the inducible IL-10–producing type 1 regulatory (Tr1) cells are more critical than nTreg on the regulation of humoral immune response (4). Those studies found IL-10 and TGF-β secret by Tr1 may switch allergen-specific B-cells induce IgG4 instead of induce sIgE (4).

This study showed that baseline level of Th2 cytokines (IL-4, IL-5, IL-13, IL-17, and TNF-α) in SCIT and non-SCIT subjects increased significantly after Der-p stimulation, confirmed that cross-linking of IgE receptors on the surface of effector cells by IgE-allergen complexes could trigger an immediate-type immune reaction and release of Th2 cell cytokines in allergic patients (24). Relatively, IL-10 may also have other functions besides induces IgG antibodies including inhibits T cell proliferation and cytokine production (25), induction of T cell anergy (26), and induces mast cell apoptosis (27). TGF-β1 is an important immunosuppressive cytokine produced by many cell types including Treg cells (28). The change of IL-10 and TGF-β1 in patients receiving AIT is controversial. Studies have shown that AIT could induce allergen specific IL-10-secreting cells at the early stage of immunotherapy (18, 29). Wei et al. (22, 30) showed that the level of IL-10 in the supernatant of PBMCs were significantly increased after AIT. In contrast, other study (31) found that no significant change of IL-10 secreted by allergen-specific PBMCs after 8 week of AIT. As for TGF-β, Jutel et al. (29) found that the TGF-β was increased after AIT, while other study (32) reported that the level of TGF-β in the supernatants of PBMCs was not changed. Those inconsistent results may be due to the production of cytokines in the PBMCs supernatant involve a complex network of immune effector cells especially mast cells and eosinophils, and may also relate to the allergens used in AIT, duration and administration type of AIT, and the status of atopic disease.

We found that AIT could induce a substantial increase of Der-p sIgG4 in the current study, which is consistent with our previous findings and those of other studies, conformed sIgG4 was a prominent immunological change induced by AIT (6). The magnitude of increase in sIgG4 concentrations may related to the concentration of the allergen (33) and the duration of AIT treatment (11). Specific IgG4 has been proposed to responsible for compete with sIgE to form allergen-IgE complex, thereby inhibiting

complex binding to B cells (32, 34) and prevents the allergen dependent T cells activation (35), also associated with the inhibition of allergen-induced effector cell activation (36, 37), and reduced allergen sensitivity (38). Our previous basophil activation test inhibition experiments showed that AIT-induced serum inhibiting antibodies reduced basophil allergen threshold sensitivity but had no effect on basophil reactivity (5). In our current cytokine release inhibition experiments, we found that serum obtained from SCIT patients inhibited cytokine release from effector cells beginning at week 16 at submaximal allergen concentrations stimulation, and continued to decrease during the 3-year treatment period. We also found a significant inverse correlation between levels of Der-p sIgG4 and cytokine release. However, the serum obtained from AIT patients could not inhibited effector cell cytokine release at maximal allergen concentrations stimulation, which may suggest SCIT could not reduce the reactivity of anaphylactic cell in PBMCs. Thus, IgG4 antibodies may play an important role in reducing the sensitivity of effector cell cytokine production during AIT, and the function of inhibition ability enhanced along with the increasing of sIgG4, but the precise mechanism needs further investigation.

In conclusion, AIT is an effective treatment for allergic diseases. The mechanisms of AIT involve both the cellular and humoral immune responses during allergen immunotherapy. AIT could reduce percentage of Th2 cells and massively induce allergen specific IgG4 antibodies production. SCIT reduced cytokine release from effector cells in Der-p allergic rhinitis and/or asthma patients may through induction of sIgG4.

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Table list

Table 1. The baseline (week 0) information, percentage of Treg, Th1 and Th2 and levels of supernatant cytokines in PBMCs in SCIT, non-SCIT and health subjects.

Figure legends

Figure 1. Flowchart of the study design. (A), and dosing schedule of SCIT injection and visits for clinical evaluation and blood sampling in SCIT group and non-SCIT group (B). SCIT, subcutaneous allergen immunotherapy; SPT, skin prick test; IgE, immunoglobulin E; EOS, peripheral eosinophil counts; SMS, symptom and medication score; FEV1, forced expiratory volume at 1s; PBMC, peripheral blood mononuclear cells; Th, T helper cell; Treg, regulatory T cell; CRIA, cytokine release inhibition assay; sIgG4, specific immunoglobulin G4.

Figure 2. Time course of mean percentage of $CD4^+CD25^+Foxp3^+$ Treg, $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ Th2 in SCIT group and non-SCIT group. Lymphocytes are identified by their scatter properties, the Treg, Th1 and Th2 cells gated as $CD4^+CD25^+Foxp3^+$ (A), $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ (B) and $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ (B) respectively. The mean percentage of Treg (C), Th1 (D) and Th2 (E) cells in $CD4^+$ T cells from the SCIT group and non-SCIT group. Time course of Der-p sIgG4 (F) in SCIT group and non-SCIT group, the y-axis is a log scale. * $P < 0.05$, *' $P < 0.01$, *" $P < 0.001$ when compared with week 0; #' < 0.01, #" $P < 0.001$ when compared with non-SCIT group. Foxp3, forkhead box protein 3; Th, T helper cell; Treg, regulatory T cell; IL, interleukin; IFN-γ, interferon-γ; Der-p, *Dermatophagoides pteronyssinus*; sIgG4, specific immunoglobulin G4.

Figure 3. Time course of inhibition of cytokine release at submaximal allergen concentration. The effector cell cytokine release inhibition assay was performed with serum from SCIT group and non-SCIT group incubated with 0.15 μg/ml Der-p allergen. * $P < 0.05$, *' $P < 0.01$, *" $P < 0.001$ when compared with week 0; # $P < 0.05$, #" $P < 0.001$ when compared with non-SCIT group respectively. Der-p, *Dermatophagoides pteronyssinus*; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; TGF-β1, transforming growth factor-β1.

Figure 4. Linear regression between Der-p sIgG4 and submaximal allergen concentration stimulated cytokine release inhibition assay in the SCIT group at all time points during the treatment. IL-5 (A), IL-13 (B), IL-17 (C), TNF-α (D), IL-4 (E), IFN-γ (F), IL-10 (G), TGF-β1 (H). Der-p, *Dermatophagoides pteronyssinus*; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; TGF-β1, transforming growth factor-β1.

Supplementary figures

Supplement figure 1. SMS and FEV_1% over time. Time course of mean SMS (A) and FEV_1 % predicted (B) in SCIT group and non-SCIT group. * $P < 0.05$, *" $P < 0.001$ when compared with week 0; # $P < 0.05$, #' $P < 0.01$ when compared with control. SMS, combined symptom medication score; FEV_1 = forced expiratory volume in one second; SCIT, subcutaneous allergen immunotherapy.

Supplement figure 2. Time course of inhibition of cytokine release at maximal allergen concentration. The effector cell cytokine release inhibition assay was performed with serum from SCIT group and non-SCIT group incubated with 15 μg/ml Der-p allergen. Der-p, *Dermatophagoides pteronyssinus*; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; TGF-β1, transforming growth factor-β1.

Supplement figure 3. Linear regression between Der-p sIgG4 and maximal allergen concentration stimulated cytokine release inhibition assay in the SCIT group at all time points during the treatment. IL-5 (A), IL-13 (B), IL-17 (C), TNF-α (D), IL-4 (E), IFN-γ (F), IL-10 (G), TGF-β1 (H). Der-p, *Dermatophagoides pteronyssinus*; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; TGF-β1, transforming growth factor-β1.

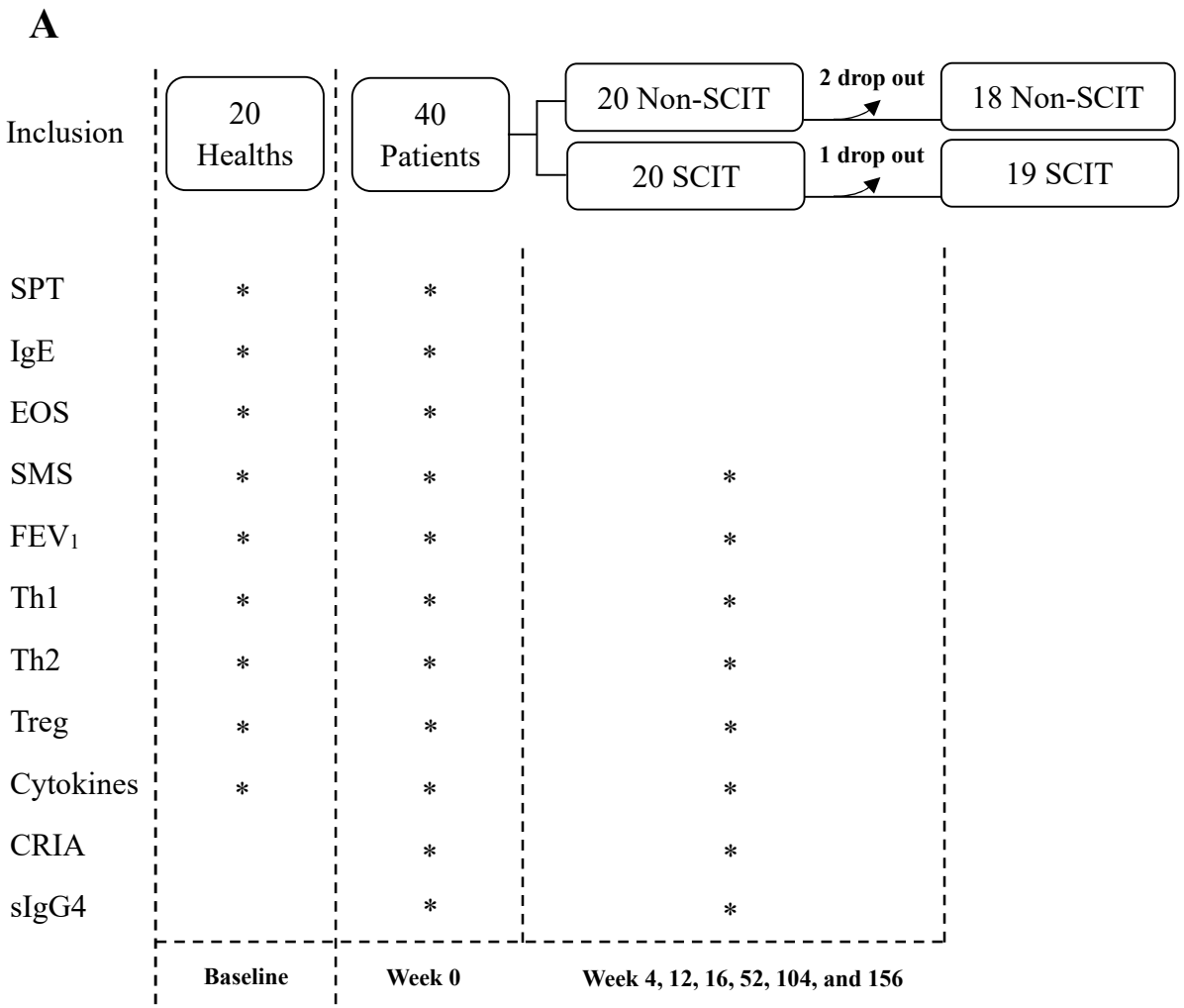
Table 1. The baseline (week 0) information, percentage of Treg, Th1 and Th2 and levels of supernatant cytokines in PBMC in SCIT, non-SCIT and health subjects.

Variable	**SCIT group**		**Non-SCIT group**		**Healthy group**	
Patients, *n* §	20		20		20	
Gender (male/female), *n* §	13/7		14/6		10/10	
Age distribution (years) §	24.4 ± 1.5		19.5 ± 1.3		22.7 ± 1.4	
SPT-diameter						
Der-p (mm) §	9.9 ± 0.7		9.1 ± 0.6		0.9 ± 0.0 †††, ###	
Der-f (mm) §	8.2 ± 0.6		8.3 ± 0.5		0.8 ± 0.0 †††, ###	
sIgE to Der-p (kU/l) §	73.2 ± 8.8		76.9 ± 8.4		0.2 ± 0.0 †††, ###	
sIgE to Der-f (kU/l) §	84.1 ± 9.5		89.4 ± 9.6		0.2 ± 0.0 †††, ###	
Total IgE (kU/l) §	665.7 ± 42.3		539.7 ± 40.8		49.6 ± 8.9 †††, ###	
SMS §	3.6 ± 0.1		4.0 ± 0.1		0.1 ± 0.0 †††, ###	
FEV_1 (%predicted) §	90.7 ± 1.8		92.5 ± 1.7		95.1 ± 1.9	
EOS ($\times 10^9$/L) §	0.5 ± 0.0		0.5 ± 0.0		0.1 ± 0.0 †††, ###	
Der p treated	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
T cells (%)						
Treg §	5.5 ± 1.9	6.3 ± 1.8	5.9 ± 2.2	5.3 ± 2.0	5.2 ± 1.7	6.1 ± 1.9
Th1 §	13.4 ± 4.6	11.9 ± 5.0	10.8 ± 6.0	12.1 ± 6.4	14.3 ± 6.1	12.6 ± 6.8
Th2 §	2.9 ± 1.6	2.4 ± 1.3	2.7 ± 1.7	2.5 ± 1.1	1.7 ± 1.3 †, #	1.8 ± 1.4 †, #
Cytokines (pg/ml)						
IL-4 §	2.1 ± 0.4	3.2 ± 0.7 *	1.4 ± 0.3	2.3 ± 0.6 *	0.7 ± 0.1 †, #	0.8 ± 0.1 ††, ##
IL-5 §	12.2 ± 5.4	1083.4 ± 236.3 ***	11.4 ± 6.8	908.0 ± 360.6 ***	9.8 ± 4.4	13.6 ± 5.7 †††, ###
IL-10 §	18.2 ± 6.4	25.2 ± 29.4	15.5 ± 22.7	21.9 ± 26.2	27.9 ± 6.9	31.8 ± 5.0
IL-13 §	38.7 ± 12.7	494.5 ± 87.5 ***	28.1 ± 15.0	373.2 ± 90.4 ***	32.7 ± 11.2	42.7 ± 15.3 †††, ###
IL-17 §	17.0 ± 5.3	69.0 ± 19.6 ***	12.7 ± 4.0	46.1 ± 8.2 ***	9.1 ± 3.2	13.1 ± 5.7 †††, ###
IFN-γ §	42.2 ± 10.6	48.0 ± 14.0	25.4 ± 6.4	41.5 ± 8.4	21.5 ± 5.7	35.9 ± 7.2
TNF-α §	62.1 ± 26.4	316.3 ± 66.7 ***	102.5 ± 53.7	360.1 ± 106.8 ***	30.5 ± 8.1 †, #	44.6 ± 17.0 †††, ###
TGF-β1 §	2042.1 ± 241.4	2250.4 ± 246.1	1688.0 ± 235.0	1797.7 ± 224.3	1821.5 ± 307.2	2023.3 ± 315.6

Data are presented as mean ± SE. § $P > 0.05$ when compared the variables between SCIT group and non-SCIT group respectively; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ when compared with SCIT group respectively; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ when compared with non-SCIT group respectively; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with unstimulated respectively. PBMC, peripheral blood mononuclear cell; SCIT, subcutaneous allergen

immunotherapy; SPT, skin prick test; IgE, immunoglobulin E; sIgE, specific IgE; EOS, peripheral eosinophil counts; SMS, combined symptom medication score; FEV_1, forced expiratory volume in one second; PBMC, peripheral blood mononuclear cells; Th, T helper cell; Treg, regulatory T cell; Der-p, *Dermatophagoides pteronyssinus*; Der-f, *Dermatophagoides farina*; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; TGF-β, transforming growth factor-β.

Figure 1


A
Inclusion
20 Healths
40 Patients
20 Non-SCIT
2 drop out
18 Non-SCIT
20 SCIT
1 drop out
19 SCIT
SPT
IgE
EOS
SMS
FEV1
Th1
Th2
Treg
Cytokines
CRIA
sIgG4
Baseline
Week 0
Week 4, 12, 16, 52, 104, and 156

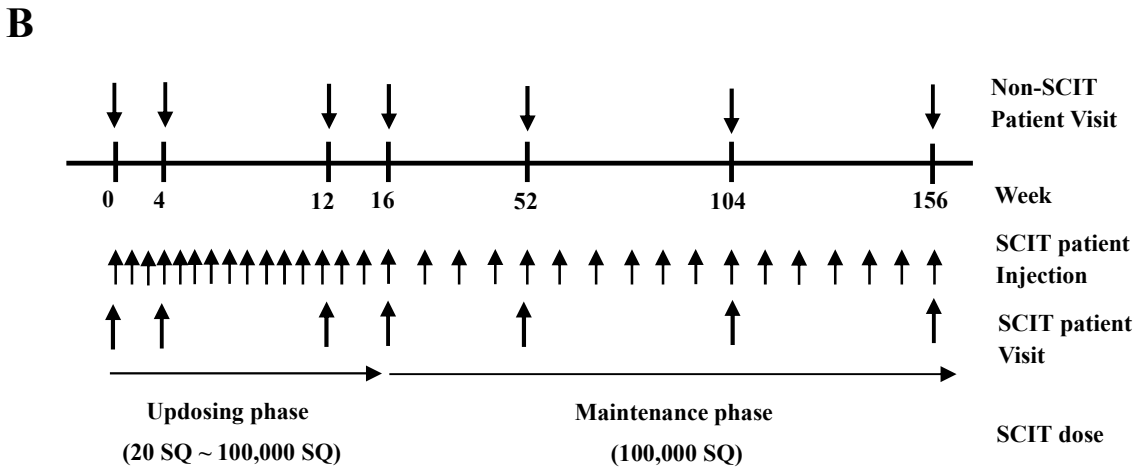

B
Non-SCIT Patient Visit
0
4
12
16
52
104
156
Week
SCIT patient Injection
SCIT patient Visit
Updosing phase
(20 SQ ~ 100,000 SQ)
Maintenance phase
(100,000 SQ)
SCIT dose

Figure 2

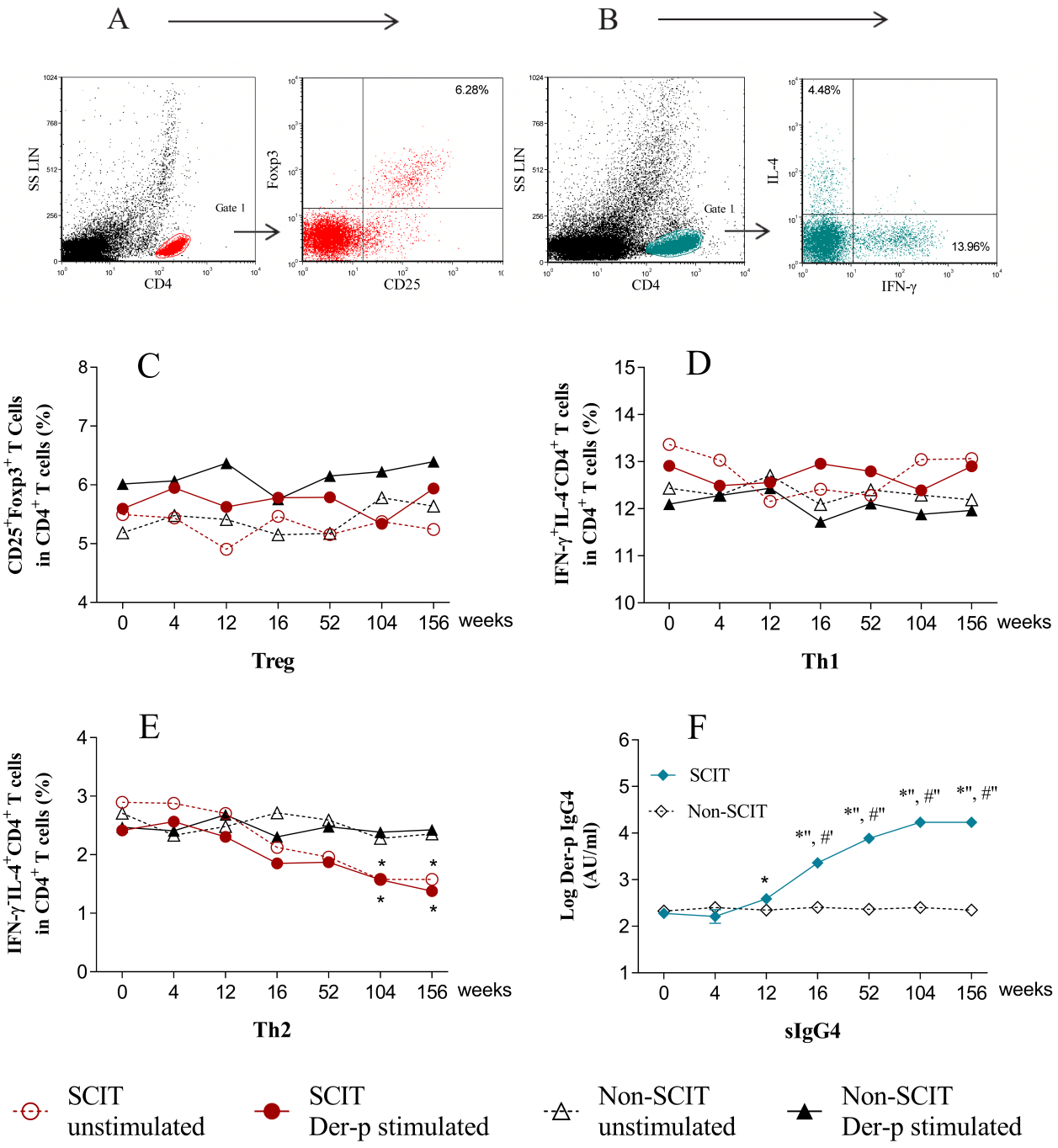

A
B
SS LIN
CD4
Gate 1
Foxp3
CD25
6.28%
IL-4
IFN-γ
4.48%
13.96%
C
CD25+Foxp3+ T Cells in CD4+ T cells (%)
Treg
D
IFN-γ+IL-4-CD4+ T cells in CD4+ T cells (%)
Th1
E
IFN-γ-IL-4+CD4+ T cells in CD4+ T cells (%)
Th2
F
Log Der-p IgG4 (AU/ml)
SCIT
Non-SCIT
sIgG4
weeks
SCIT unstimulated
SCIT Der-p stimulated
Non-SCIT unstimulated
Non-SCIT Der-p stimulated

Figure 3

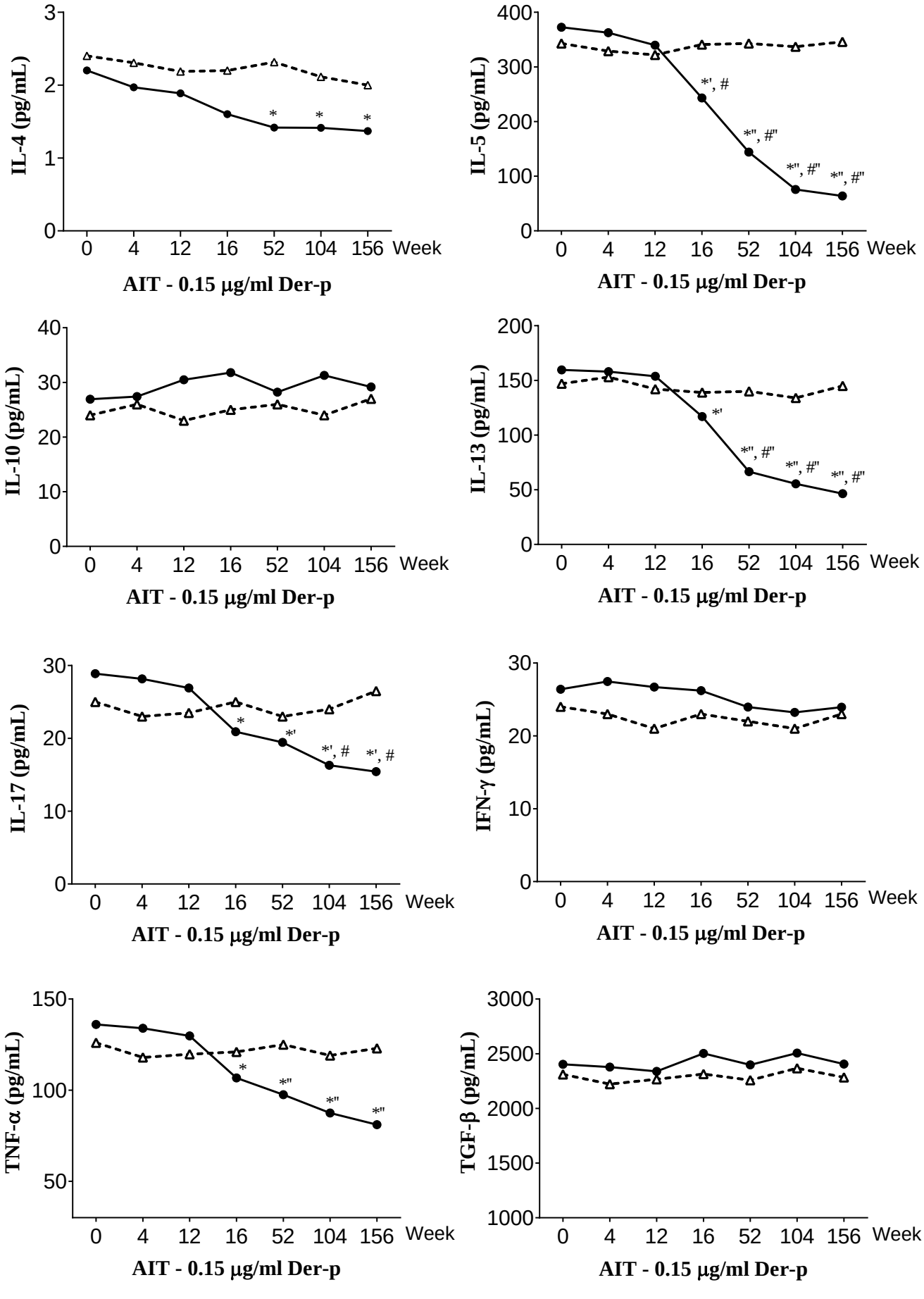
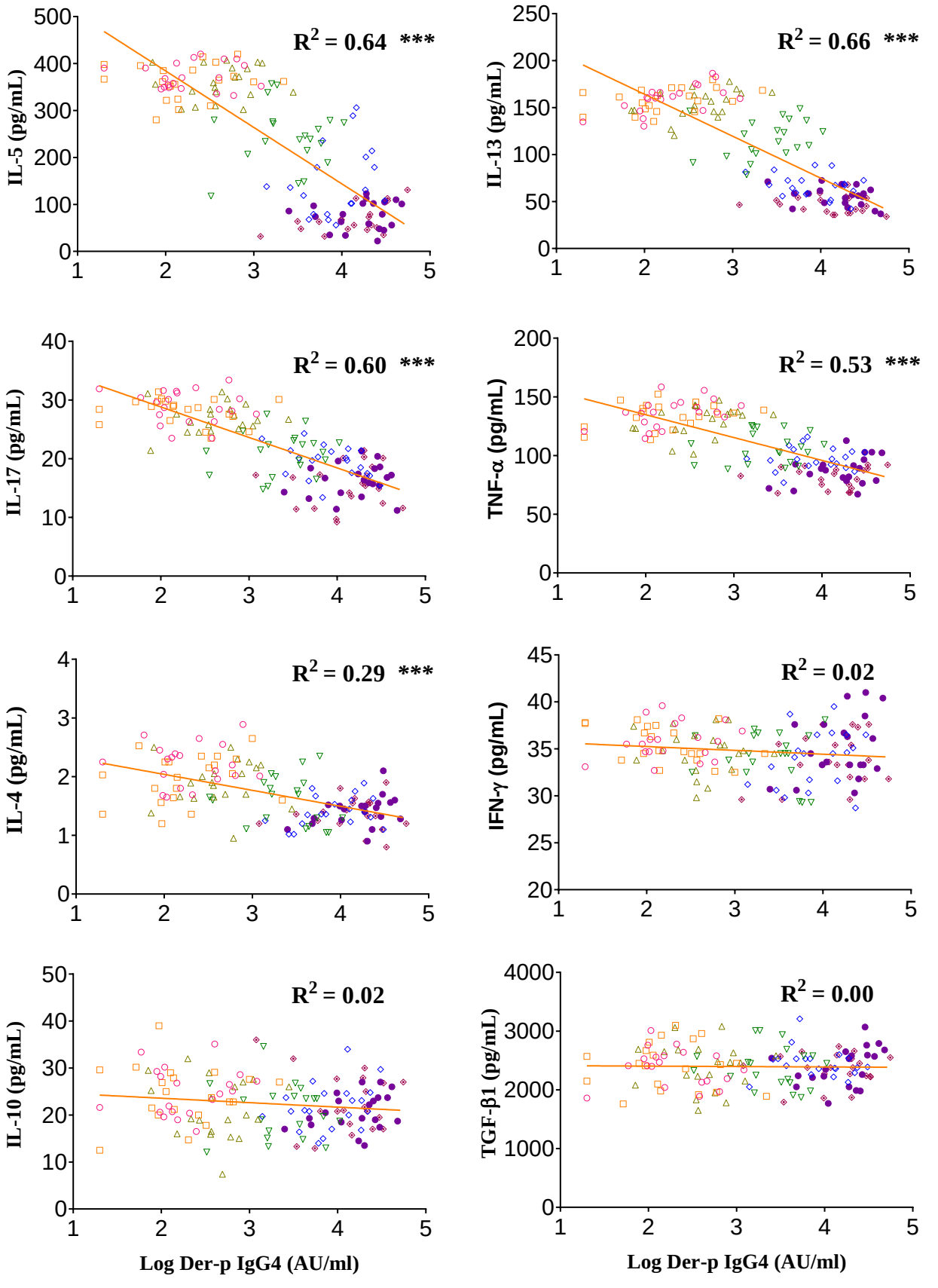
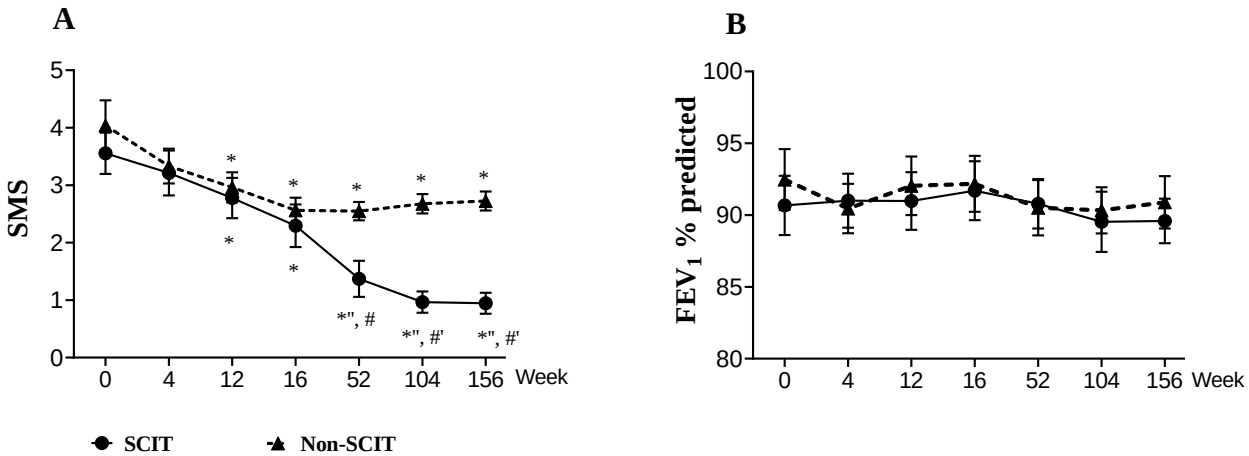

IL-4 (pg/mL)
IL-5 (pg/mL)
IL-10 (pg/mL)
IL-13 (pg/mL)
IL-17 (pg/mL)
IFN-γ (pg/mL)
TNF-α (pg/mL)
TGF-β (pg/mL)
0 4 12 16 52 104 156 Week
AIT - 0.15 μg/ml Der-p
SCIT
Non-SCIT

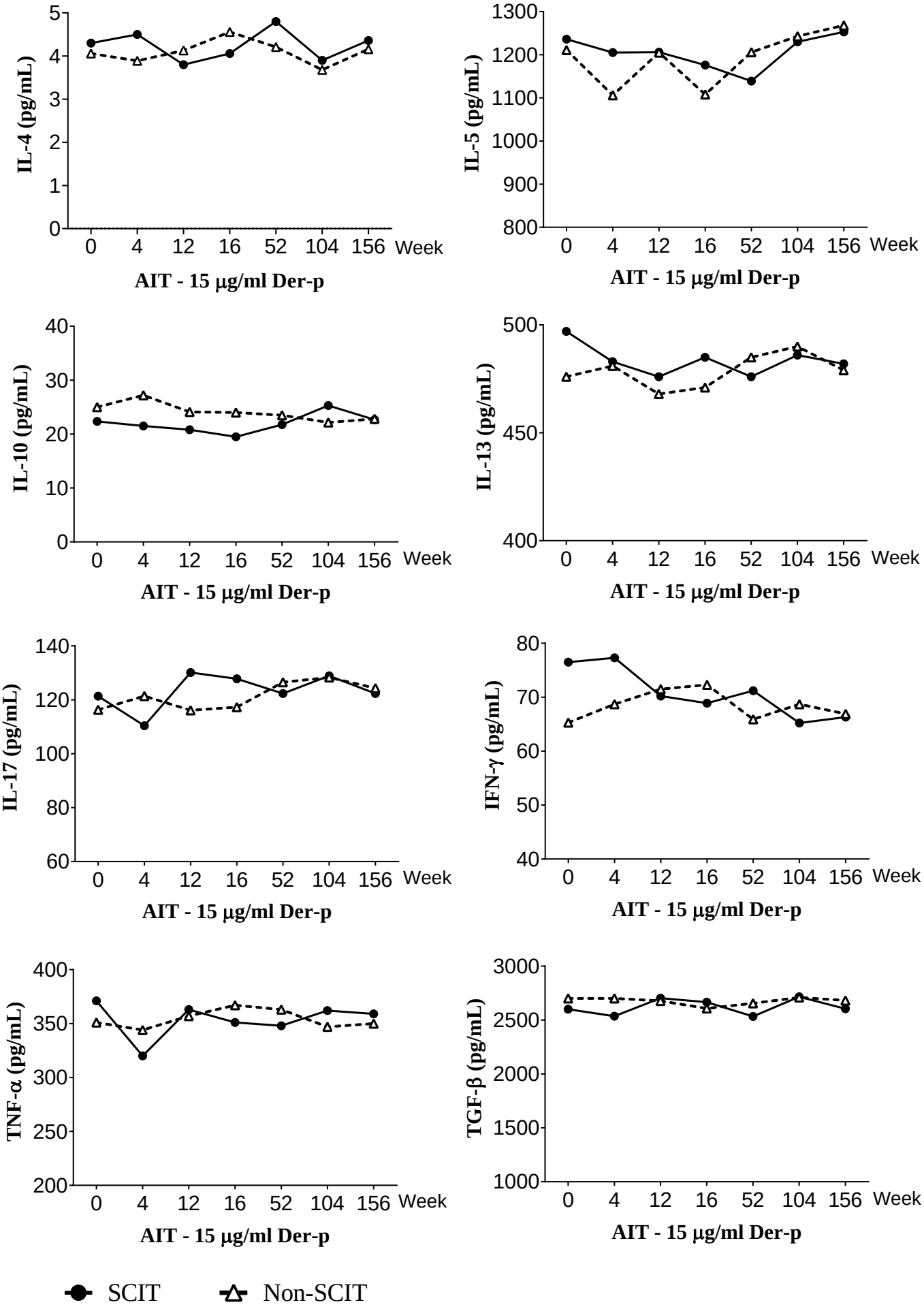
Figure 4


IL-5 (pg/mL)
R2 = 0.64 ***
IL-13 (pg/mL)
R2 = 0.66 ***
IL-17 (pg/mL)
R2 = 0.60 ***
TNF-α (pg/mL)
R2 = 0.53 ***
IL-4 (pg/mL)
R2 = 0.29 ***
IFN-γ (pg/mL)
R2 = 0.02
IL-10 (pg/mL)
R2 = 0.02
TGF-β1 (pg/mL)
R2 = 0.00
Log Der-p IgG4 (AU/ml)
Log Der-p IgG4 (AU/ml)
0 wk
4 wk
12 wk
16 wk
52 wk
104 wk
156 wk

Supplement figure 1



Supplement figure 2



Supplement figure 3