EFFECTS OF ALLERGEN-SPECIFIC IMMUNOTHERAPY TREATMENT ON PERIPHERAL BLOOD REGULATORY T CELLS AND SERUM CONCENTRATIONS OF CYTOKINES AND IMMUNOGLOBULINS IN HORSES WITH ALLERGIC DISEASES

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Abstract

Allergic diseases are important conditions in horses, with a high prevalence worldwide. The treatment of these diseases is a constant challenge for veterinarians and is based on the control of clinical symptoms with medicines. An alternative is the use of allergen-specific immunotherapy (ASIT) to induce a state of immune tolerance. The aim of this study was to assess the effect of ASIT on the immunological responses of horses. Blood samples were taken from thirty-two horses with allergic diseases treated with ASIT and 10 healthy control horses at 0, 3, 6, 9 and 12 months to investigate the evolution of the percentage of regulatory T cells (Treg) in the peripheral blood and the serum levels of cytokines and immunoglobulins. No effect of ASIT on $CD4^{+}CD25^{high}$ Treg cells was found after one year of treatment, and the percentage of Treg cells (approximately 20%) was similar to that at the beginning of the treatment. No differences in the percentage of $CD4^{+}$ T cells (50%) were observed between the groups, and no effects of ASIT over time were observed. The main difference between the two groups was that the percentage of $CD25^{+}$ T cells was always higher in the ASIT group (17.9 ± 11.3%) than in the control group (7.3 ± 4.4%, $P < 0.001$), which could indicate the stimulation of this T cell subset by natural allergens. We did not detect any effect of ASIT on the serum levels of TGF-β, IL-10 and IFN-γ or on the serum concentrations of IgA and IgG4 after one year of immunotherapy. Throughout the entire study, the horses with allergic diseases maintained higher levels of TGF-β, IFN-γ and IgE than the control group ($P < 0.001$) and had lower levels of IL-10 and IgG4 ($P < 0.05$) than the healthy controls. A reduction in the serum levels of IgE in the horses with allergic diseases was observed at the 6th month ($P < 0.05$), but at the end of the study, the IgE levels in the treated horses (4.49 ± 2.64 ng/ml) were higher than those in the controls (1.51 ± 1.26 ng/ml, $P < 0.01$). The results of this study indicate that immunotherapy was insufficient to induce significant changes that could indicate T cell tolerance, a shift in cytokine production to more protective Th1 cells and an increase in

the IgG4 levels. The results obtained could be the consequence of an inappropriate composition of the inoculum or the duration of the ASIT treatment. To confirm this hypothesis, more studies are needed with new vaccine compositions and administration protocols to improve the clinical and immunological responses of the horses with allergic diseases.

Keywords: allergen-specific immunotherapy, Treg, cytokines, immunoglobulins, allergic horses

1. Introduction

Allergic diseases in horses have a wide distribution and prevalence worldwide and represent a challenge for veterinary clinicians in terms of both diagnosis and treatment (Scott and Miller, 2011). These diseases are allergic dermatitis (AD) and recurrent airway obstruction (RAO) generated by aeroallergens as well as insect bite hypersensitivity (IBH) provoked by insects, mainly *Culicoides* (Schaffarzik et al., 2012; Scott and Miller, 2011). The treatment of these diseases is based on preventing or reducing allergen exposure, rigorous insect control and the administration of glucocorticosteroids used for symptomatic treatment (Marsella, 2013; Scott and Miller, 2011).

An alternative treatment for allergic diseases in both human and veterinary medicine is allergen-specific immunotherapy (ASIT), consisting of the administration of increasing doses of allergens and thereby inducing a state of immune tolerance (Bohle, 2008; Jutel et al., 2011, 2016; Larché et al., 2006; Loewestein and Mueller, 2009; Palomares et al., 2014, 2017). ASIT has been used in human and small animal dermatology and represents the only curative and specific approach to the treatment of IgE-mediated hypersensitivity (Jutel and Akdis, 2009; Jutel et al., 2016; Loewenstein and Mueller, 2009). ASIT is an effective treatment, reducing symptom scores and medication use and improving the quality of life of the animals, sometimes for a long time (Keppel et al., 2008; Rees, 2001; Stepnik et al., 2011). ASIT has been used in horses with allergic dermatitis, and in a retrospective study by Stepnik et al., owners reported an 84% clinical improvement in the disease (2011).

The aim of ASIT is the induction of a tolerant state against allergens that is characterized mainly by the generation of allergen-specific regulatory T (Treg) cells (Akdis and Akdis, 2009; Jutel and Akdis, 2011; Jutel et al., 2006, 2016; Palomares et al., 2014, 2017). Successful ASIT is associated with the immunodeviation of a Th2 response to a more protective Th1 phenotype and with the presence of IL-10- and TGF-β-producing Treg cells in

the blood (Loewenstein and Mueller, 2008; Maggi et al., 2012; Palomares et al., 2014, 2017). The increase in the activity of adaptative Treg (aTreg) cells has been emphasized as the main or even unique explanatory mechanism for the clinical efficacy of subcutaneous immunotherapy in human patients (Maggi et al., 2012). Many molecular and cellular mechanisms have been attributed to ASIT such as the regulatory cytokines IL-10 and TGF-β produced by Tr1 cells contributing to the control of allergen-induced immune responses (Akdis and Akdis, 2009; Jutel et al., 2006; Larché et al., 2006; Loewenstein and Mueller, 2009; Palomares et al., 2014). IL-10 has a potent immunosuppressive capacity and has an essential role in the establishment of peripheral tolerance. This cytokine inhibits the production of proinflammatory cytokines (Bohle, 2008; Loewenstein and Mueller, 2009; Palomares et al., 2014; Taylor et al., 2006). TGF-β is a pleiotropic cytokine with a potent regulatory capacity that has a very important role in the suppression of the immune response, and Treg cells, particularly Th3 cells, are the major source of TGF-β (Jutel et al., 2016; Palomares et al., 2014). The expression of IL-10 and TGF-β contribute to Treg function and immunoglobulin switching to IgG4 and IgA, respectively, which have inhibitory activities (Jutel et al., 2006; Larché et al., 2006; Palomares et al., 2014 Shamji et al., 2011). During ASIT, it has been shown that the humoral response is characterized by a transient increase in allergen-specific IgE followed by a decrease in IgE and a gradual increase in allergen-specific IgGs, mainly IgG4 (Jutel et al., 2006, 2016; Palomares et al., 2017).

The generation and maintenance of allergen-specific regulatory T cells and the involvement of their suppressive cytokines and surface molecules are essential for the induction of a state of tolerance against allergens (Palomares et al., 2017; Zhang et al., 2017). ASIT can modify the response of T cells to allergens by generating allergen-specific Treg cells that can suppress the response of effector T cells, memory $CD4^+$ and $CD8^+$ T cells and increase the production of cytokines with regulatory activity (Akdis and Akdis, 2009; Jutel

and Adkis, 2011; Larché et al., 2006; Palomares et al., 2017). It has been suggested that upregulation of $CD4^+CD25^+$ Treg cells plays a role in ASIT treatment (Akdis and Akdis, 2009). In healthy horses, as in human beings, it has been demonstrated that the circulating $CD4^+CD25^{high}$ lymphocytes subpopulation contains Treg cells and that it has a strong suppressive activity (Hamza et al., 2011, 2015; Robbin et al., 2011). The participation of Treg cells has been demonstrated in equine allergic diseases such as RAO, in which $CD4^+FoxP3^+$ T cells are clearly increased in the peripheral blood and in the bronchoalveolar lavage fluid (Henríquez et al., 2014). In a recent paper, these authors showed that Treg cells may play a major role in the regulation and resolution of equine asthma, based on a murine model of severe equine asthma (Henríquez et al., 2017). In veterinary medicine, ASIT has mainly been used to treat canine atopic dermatitis, inducing a significant increase in both Treg cells and IL-10 serum concentrations during 12 months of treatment (Keppel et al., 2008) and increasing IFN-γ expression to produce a shift to the Th1 dominant state (Shida et al., 2004). In equine medicine, there was a retrospective study of the response to ASIT treatment in horses based on the improvement of clinical signs of equine atopic disease (Stepnik et al., 2011), and there was also a randomized study of the efficacy of ASIT in controlling IBH in horses (Ginel et al., 2014). However, these articles did not study how the T cells or other immunological components are affected by ASIT treatment in horses with allergic diseases.

At present, there is not a study describing how Treg cells are modified by ASIT in horses with allergic diseases such as AD or RAO. The aim of this study was to investigate the evolution of different subsets of lymphocytes in the peripheral blood of horses diagnosed with allergic diseases and treated with ASIT for one year. The results were compared with a group of healthy horses (control) that received saline placebo injections for one year. Because the molecular response to ASIT is still unknown in vivo, we also studied the changes in the serum concentrations of IL-10, TGF-β and IFN-γ induced by the treatment as well as the effects on

the levels of IgA, IgE and IgG4. These cytokines and immunoglobulins were shown to be key components in the pathogenesis and resolution by immunotherapy of the allergic diseases in dogs (Keppel et al., 2008; Maina et al., 2017; Shida et al., 2004) and in human medicine (Francis et al., 2003; Larché et al., 2006; Maggi et al., 2012; Zhong et al., 2015).

2. Material and Methods

2.1. Animals

A total of 42 horses were included in this study. Thirty-two horses showed clinical signs of allergic dermatitis such as seasonal pruritus and skin lesions due to scratches in the mane and tail or on the body produced by aeroallergens or insect bites. These horses were diagnosed with allergic dermatitis. This clinical diagnosis was supported by an ELISA test that identified specific IgE serum levels higher than 150 EU. These horses composed the allergic group and were treated by ASIT. Ten horses were clinically healthy with normal blood counts, no alterations in the clinical examination, and no history of any allergic diseases; these horses composed the control group. Serum samples from the horses were analysed by Diavet Laboratorios (Leganés, Madrid, Spain), and environmental and insect allergens were identified with a noncompetitive Allercept© ELISA test (Heska Corp., Fribourg, Switzerland), designed to detect the presence of allergen-specific IgE. Only horses with clinical signs compatible with allergic disease and positive ELISA tests were included in the allergic group in this study. There were seven stallions, 20 mares and 15 geldings, with ages between 2 and 10 years. The most common breed was the Spanish Pura Raza Española (PRE, 15 horses), and the rest were other breeds and crossbreeds. All horses had been regularly vaccinated against tetanus and equine influenza and had been dewormed.

2.2. ASIT treatment

Horses in the ASIT group received ASIT formulated with aluminium hydroxide-adsorbed allergens produced by Diavet Laboratorios. The inoculums had a mixture of

allergens specific to each horse according to the results of the serum analysis for allergen-specific IgEs. The injection protocol was administered following the manufacturer's instructions, and it entailed increasing subcutaneous doses over the course of one year. The horses did not receive any treatment during the assay, except dermatologic shampoo with fatty acids or insect repellent for some of them. For the control group, a placebo with saline was subcutaneously injected following the same inoculation protocol used in the ASIT group.

2.3. Blood samples

Ten ml of blood was collected from the jugular vein into a Vacutainer® tube with EDTA as an anticoagulant and another tube without anticoagulant to obtain serum. The tubes were immediately cooled at 4°C in preparation for the analysis of the T cells by imaging flow cytometry. Blood samples were obtained before ASIT treatment (T0) and at 3, 6, 9 and 12 months (T03, T06, T09, T12) after ASIT treatment was started to assess the evolution of the percentages of T lymphocytes and the serum levels of cytokines and immunoglobulins. In the control group, no blood samples were obtained at T09. The serum samples were centrifuged in the laboratory at 3000 rpm at 4°C for 15 minutes and then kept at -20°C until analysis for the determination of the serum levels of cytokines and immunoglobulins by ELISA.

2.4. Measurement of T cells by imaging flow cytometry

Peripheral blood mononuclear cells (PBMC) were obtained from fresh blood and T cells were labelled following the method proposed by Hamza et al. (2011) with modifications. Briefly, 1 ml of blood with EDTA was lysed with 5 ml of Tris-buffered ammonium chloride (10 ml TRIS 0.17 M pH 7.65 and 90 ml of NH_4Cl 0.16 M, final pH 7.2) for 2 minutes. The lysed blood was centrifuged at 300 g for 5 minutes at 4°C to separate the debris. The supernatant was removed, and the pellet was washed three times with PBS at 300 g at 4°C for 10 minutes. A vial with $1x10^5$ cells in PBS was labelled with 5 µl of mouse anti-horse CD4-FITC (clone MCA 1078F, Bio-Rad-Serotec, Hercules, CA, USA) and 5 µl of monoclonal

anti-human IL-2Rα (CD25)-phycoerythrin (clone 24212, R&D Systems, MN, USA) at 4°C for 45 minutes. The cells were washed with PBS to remove the excess antibodies. The stained cells were resuspended in 200 µl of PBS and fixed with 20 µl of paraformaldehyde for 10 minutes at room temperature before the cytometric analysis. Imaging flow cytometry was conducted in an ImageStream®X cytometer (Amnis®, Seattle, WA, USA) that captures up to twelve images of each cell in different spectral bands at 60x magnification using a combination of transmitted light, scattered light, polarized light, and fluorescence. The cell sample acquisition (10000 events) was performed with INSPIRE® software and analysed with the manufacturer's software (IDEAS v6.1).

The results were defined as $CD4^+$, $CD25^+$, $CD4^+CD25^-$, $CD4^+CD25^{dim}$, and $CD4^+CD25^{high}$ following the descriptions made by Hamza et al. (2015) and Henríquez et al. (2014). The $CD4^+CD25^{high}$ cells were considered Treg cells because the FoxP3 marker is expressed primarily in this subpopulation in equine PBMC (Hamza et al., 2011, 2015; Robbin et al., 2011; Henríquez et al., 2014). The percentage of $CD4^+CD25^{high}$ (Treg) cells was quantified within the population of lymphocytes labelled with the antibody anti-CD4 (lymphocytes $CD4^+$). Figure 1 shows the gating strategy used to identify each $CD4^+$ subpopulation.

2.5. Serum concentrations of cytokines and immunoglobulins

The serum levels of the cytokines IL-10, TGF-β and IFN-γ as well as the concentrations of the IgG4, IgE and IgA immunoglobulins were determined by ELISA. For the serum concentrations of equine IL-10, TGF-β and IgE, commercial Nori® Equine ELISA kits (Genorise Scientific, Glen Mills, PA, USA) were used. The detection limits were 25 pg/ml for IL-10, 6 pg/ml for TGF-β and 0.15 ng/ml for IgE. The serum concentration of IFN-γ was analysed by the Equine IFN-γ VetSet™ ELISA Development kit (Kingfisher Biotech, Saint Paul, MN, USA) with a detection limit of 57.8 pg/ml. The IgG4 serum level was

determined by the Horse IgG4 ELISA kit (MyBiosource, CA, USA), with a detection limit of 5 μg/ml. The Horse IgA ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA), with a detection limit of 15.6 ng/ml, was used for the quantification of IgA. All serum samples were analysed in duplicate according to the manufacturer's instructions. The levels of the cytokines and immunoglobulins were measured colorimetrically using a microplate reader (Labsystem Multiskan RC, Vantaa, Finland) and their concentrations were determined by interpolation from a standard curve obtained with controls provided in the kits.

2.6. Statistical analysis

IBM SPSS 22 for Windows (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. A Shapiro-Wilk test was performed to determine if the data were normally distributed. A non-parametric unpaired Kruskal-Wallis test and *post hoc* Dunn's test were used for three-way comparisons to compare differences between the groups over time. For parametric data, ANOVA with *post hoc* Duncan's test was performed. Paired analyses for the control group for the levels of cytokines and immunoglobulins at T0 and T12 were performed utilizing the Wilcoxon signed-rank test. Differences between the control and ASIT groups at each time point were tested with the non-parametric unpaired Mann-Whitney U-test and unpaired Student's t-test when the data was normally distributed. The adjusted value for significance was $P < 0.05$.

3. Results

3.1. Effect of ASIT treatment on the percentages of lymphocyte subpopulations in horses over the course of one year

No effect of the immunotherapy was observed over the course of one year on any lymphocyte subpopulation of T cells when the data were analysed by a Kruskal-Wallis test or ANOVA ($P > 0.05$) (Table 1); no differences were observed in the control group during the same period. However, differences were observed between the two groups at each analytical

time point. The percentage of lymphocytes was higher in the ASIT group than in the control group throughout the study ($P < 0.001$). The percentage of the $CD4^+$ subset was close to 50% in both groups, and only at T12 was the percentage of the $CD4^+$ subset lower in the ASIT group than in the control group ($P < 0.05$). The percentage of $CD25^+$ cells was always higher in the ASIT group than in the control group throughout the study ($P < 0.01$). When the population of CD4CD25 was gated in three subpopulations, the percentage of $CD4^+CD25^-$ cells was higher in the ASIT group than in the control group ($P < 0.05$). Additionally, there were statistically significant differences in the population of $CD4^+CD25^{dim}$, which was lower in the ASIT group than in the control group ($P < 0.01$).

Significant differences were found between the ASIT and control group in terms of the percentage of T cells as determined by imaging flow cytometry (Fig. 2) when data were grouped over the course of the year of the study. The percentage of lymphocytes was higher in the ASIT group (32.3 ± 13.3%) than in the control group (21 ± 5.1%, $P < 0.001$). The percentage of $CD25^+$ cells was higher in the ASIT group (17.9 ± 11,3%) than in the control group (7.3 ± 4.4%, $P < 0.001$). There were statistically significant differences between the control group (54.7 ± 12.9%) and the ASIT group (63.1 ± 10.7%) in terms of the proportion of $CD4^+CD25^-$ T cells ($P < 0.001$). A decrease in the percentage of $CD4^+CD25^{dim}$ cells was observed in the ASIT group (17.8 ± 7.7%) when compared with the control group (25.2 ± 10.5%, $P < 0.001$). No effects of the immunotherapy were observed on the percentages of the $CD4^+$ and $CD4^+CD25^{high}$ subsets throughout the year of treatment ($P > 0.05$).

3.2. Effects of the ASIT treatment on the levels of cytokines and immunoglobulins in horses over the course of one year

Table 2 shows the evolution of the serum concentrations of three cytokines in the ASIT and control groups. The concentrations of TGF-β decreased over time in the control group ($P = 0.033$), and TGF-β serum levels were higher in the ASIT group than in the control

group at the beginning of the study and after 12 months of treatment ($P < 0.01$). No effects of the immunotherapy treatment over time were observed on the serum levels of IL-10 and IFN-γ ($P > 0.05$) nor was there any effect over time in the control group. After 12 months, the serum levels of IFN-γ were higher in the ASIT group than in the control group ($P < 0.05$).

No variations were observed in IgG4 and IgA levels over time or between groups ($P > 0.05$) after one year of ASIT treatment in either the horses with allergic diseases or in the healthy horses (Table 2). IgE serum levels were higher in the ASIT group than in the control horses at the beginning of the treatment ($P < 0.05$). Immunotherapy treatment decreased the IgE serum levels at the 6th month of treatment ($P < 0.05$). The IgE/IgG4 ratio was higher in the ASIT group than in the healthy horses ($P < 0.05$) at each time point analysed, but no effect of the ASIT was identified ($P > 0.05$).

The data of the serum levels of cytokines and immunoglobulins were grouped and analysed throughout the year of study (Fig. 3). The ASIT group had a higher concentration of TGF-β (1018 ± 383 pg/ml) than the control group (696 ± 306 pg/ml, $P < 0.001$). The ASIT group had a higher concentration of IFN-γ (1252 ± 1201 pg/ml) than the control group (565 ± 719 pg/ml, $P < 0.05$). In contrast, the ASIT group had lower serum levels of IL-10 (415 ± 245 pg/ml) than the control group (555 ± 255 pg/ml, $P < 0.05$).

The serum levels of IgG4 and IgE changed significantly during the study (Fig. 4). The ASIT group had a lower concentration of IgG4 (220 ± 145 µg/ml) than the control group (285 ± 89 µg/ml, $P < 0.05$). Serum levels of IgE were higher in the ASIT group (4.44 ± 3.6 ng/ml) than in the control group (1.29 ± 1.04 ng/ml, $P < 0.01$). However, no effect of the treatment was found ($P > 0.05$) on IgA serum levels over the course of one year. Data from the end of the experiment were similar to the values found at the beginning of the treatment (T0), indicating no effect of ASIT.

4. Discussion

The present study investigated the effect of ASIT on Treg cells in the peripheral blood and on cytokines and immunoglobulins in the serum of horses affected by allergic diseases over the course of one year of treatment in comparison with healthy horses during the same time. It is essential to understand the immunological response of Treg cells, cytokines and immunoglobulins to understand of the pathogenesis and resolution of allergic diseases (Akdis and Akdis, 2009; Bohle, 2008; Akdis et al., 2005; Jutel et al., 2006; Palomares et al., 2014, 2017; Zhang et al., 2014). Currently, Treg cells are defined by the expression of CD4CD25FoxP3-positive cells in horses (Hamza et al., 2011, 2012, 2015; Henríquez et al., 2014; Robbin et al., 2011) and in dogs (Hauck et al., 2016; Majewska et al., 2016). For a long time, $CD4^+CD25^+$ T cells were the most accepted definition for Treg cells in mice and humans, and the majority of FoxP3 expression is found in the $CD4^+CD25^{high}$ subset (Garden et al., 2011; Lastovicka, 2013). Additionally, among nonhuman species, FoxP3 is highly expressed in the $CD4^+CD25^{high}$ subpopulation in ovine PBMC (Rocchi et al., 2011), and in porcine (94.1%) (Käser et al., 2008) and canine activated PBMCs (Mizuno et al., 2009). Only a minority of the equine $CD4^+CD25^{high}$ T cells express FoxP3 but they have strong suppressive activity (Hamza et al., 2011), with the major proportion found in foals or in yearlings rather than in adult horses (Hamza et al., 2015). Due to the evidence in the scientific literature, in this paper, the $CD4^+CD25^{high}$ T cells were considered Treg cells.

The effect of ASIT treatment on Treg cells has varied in different studies conducted with humans and animals. In this study, there was no influence of the ASIT treatment on the percentages of different lymphocyte subsets over the courses of one year of study. The results obtained indicated that the percentages of the different subpopulations of T cells did not change from their levels before the beginning of the immunotherapy (T0), and no effect of the ASIT could be demonstrated. In this sense, there were no differences between healthy and IBH-affected horses in terms of their proportions of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells just

as there was no difference in $CD4^+CD25^+FoxP3^+$ T cells reported by Hamza et al. (2012). In this study, as in that study, allergy-affected horses had a lower percentage of $CD4^+CD25^{dim}$ T cells than healthy horses. Our results suggest that allergic conditions in horses do not induce an increase in Treg cells, such as has been reported in dogs with atopic dermatitis, which had more circulating Treg cells than the healthy controls (Hauck et al., 2016; Majewska et al., 2016). No changes were found in the Treg subsets in the peripheral blood in equines with RAO between the baseline and after four days of *A. fumigatus* challenge in a study by Henríquez et al. (2014). CD25 (IL-2Rα) is the principal cell surface marker of Treg cells (Lastovicka, 2013), and CD25 expression increases upon stimulation of equine PBMC with PWM (Robbin et al., 2011). Throughout this study, the proportion of $CD25^+$ T cells was higher in the ASIT group than in the control group, and this result could indicate the stimulation of this T cell subset by natural allergens, causing the proportion to remain high in the horses with allergic diseases. There have been studies that showed that ASIT can affect Treg cells, increasing their proportion in the peripheral blood of dogs over the course of one year of treatment, which coincided with the improvement of the clinical signs of atopy (Keppel et al., 2008). Additionally, individuals receiving immunotherapy treatment against grass pollen have higher levels of Treg cells than people with untreated atopy and healthy people (Francis et al., 2003). However, other reports showed that the Treg proportions remained unchanged during ASIT treatment against birch pollen, although patients displayed reduced skin prick test reactivity and diminished clinical symptoms (Möbs et al., 2010). Nevertheless, there have been no studies of the effect of ASIT on peripheral Treg cells in equine medicine with which to compare our results. Another possibility that has been suggested is the rapid turnover of Treg cells in the peripheral blood over time during ASIT (Bohle, 2008). The clinical efficacy of ASIT depends on many factors such as allergen and patient selection, the dose of the allergens, the interval between administrations and the time

required for effects to appear (Bellinghausen et al., 2005; Loewenstein and Mueller, 2009; Maggi et al., 2012). The effect of ASIT on peripheral Treg cells could also be influenced by these factors. New perspectives in vaccine development with recombinant allergens or pure salivary gland proteins of *Culicoides* have been investigated (Jonsdottir et al., 2015) to induce an immune response without increasing the production of IgE. Additionally, the introduction of new injection routes and adjuvants could improve immunoprophylaxis for patients with allergic diseases (Jutel et al., 2016). Perhaps the improvement in the formulation of the vaccine could increase the peripheral blood Treg cell percentage in horses undergoing immunotherapy.

Cytokines have an important role in the pathogenesis of allergic diseases characterized by a Th2 cytokine response (Jutel et al., 2016). IL-10 and TGF-β have potent immunosuppressive capacities and essential roles in the establishment of peripheral tolerance to allergens (Palomares et al., 2014; Taylor et al., 2006; Zhang et al., 2014). Changes observed during allergen-specific immunotherapy include the increased expression of IL-10 and TGF-β secreted by Treg cells, resulting in the suppression of allergen-induced specific T cells (Jutel et al., 2016; Larché et al., 2006; Loewenstein and Mueller, 2009). The effect of ASIT on the serum levels of cytokines was variable throughout the study. The horses receiving ASIT treatment had higher concentrations of TGF-β and IFN-γ and a lower concentration of IL-10 than the controls, but the differences were not statistically significant. There have not been any studies that investigated the serum concentrations of cytokines in horses treated with immunotherapy, and little is known about the systemic alterations provoked by allergic diseases. The effects of the allergic diseases and immunotherapy on the levels of cytokines are variable depending on the experimental protocol and animal species studied. Niedzwiedz et al. (2016) showed that horses affected by RAO, a disease of allergic aetiology, had higher IFN-γ serum levels than healthy controls, as was seen in our study at

T0. IFN-γ expression in PBMC stimulated with house dust mite antigens tended to be lower in atopic dogs than in nonatopic dogs, and this expression increased after immunotherapy (Shida et al., 2004). Additionally, IFN-γ concentrations were enhanced without affecting IL-4 levels in dogs with adverse food reactions (Maina et al., 2017). In human patients, IFN-γ concentrations after two years of ASIT were higher than the baseline levels, as were the levels of IL-10 and TGF-β1 (Zhong et al., 2015). The results from the scientific literature indicate that effects of IFN-γ are complex and can contribute to both anti-inflammatory and proinflammatory processes (Schroder et al., 2004).

TGF-β serum levels were higher in the ASIT group than in the control group (Fig. 3), but no effect of the immunotherapy over the course of the year of the study was observed. No response to ASIT was found in terms of IL-10 concentrations over time, and horses treated with the immunotherapy had lower levels of IL-10 than the healthy horses. The immunotherapy treatment is able to induce specific Tr1 cells that abolish the allergen-induced proliferation of Th1 and Th2 cells by increasing IL-10 and TGF-β levels (Jutel et al., 2016; Larché et al., 2006; Palomares et al., 2014; Taylor et al., 2006). In veterinary medicine, increases in IL-10 levels have been shown in canine immunotherapy against atopic disease together with increases in $FoxP3^{+}CD4^{+}$ T cells (Keppler et al., 2008) and have been detected in the supernatant of canine PBMC stimulated with alimentary allergens (Maina et al., 2017). Additionally, increases in the serum levels ofIL-10 and TGF-β1 have been detected in human patients with atopic disease after two years of ASIT treatment (Zhong et al., 2014). Statistically significant differences in TGF-β serum levels were observed between the two groups in this study was due to a decrease in the control group rather than the influence of the immunotherapy. The results obtained by analysing the cytokines confirm that there was no effect of the immunotherapy on the state of immune homeostasis in the horses. Cytokine production by Treg cells can vary depending on the type of organ and allergens, and Tr1 cells

can produce either IFN-γ or IL-10 (Jutel and Akdis, 2011). Perhaps a longer duration of treatment is needed to detect significant increases in the serum concentrations of IL-10 and TGF-β; two years were needed to see results in the study by Zhong et al. (2015) in human patients. Furthermore, the dose of allergens can influence the clinical efficacy of ASIT (Loewenstein and Mueller, 2009) and could be influenced by the cellular and molecular response to the immunotherapy.

Horses in our study were vaccinated against a wide variety of allergens that were identified by allergen-specific IgE serum analysis including mites, aeroallergens and allergens from insects. This heterogeneous variety of allergens could influence the cytokine expression (Jutel and Akdis, 2011; Jutel et al., 2006) and the use of these vaccines at the same time should be possible in multisensitized patients (Jutel et al., 2016). An improvement of the clinical outcomes in horses due to ASIT with vaccines composed of multiple allergens has been recorded (Stepnik et al., 2011), but no reduction in clinical score could be demonstrated by Ginel et al. (2014) in another multiple allergen ASIT study in horses. These papers only studied the clinical outcomes of the horses after ASIT, not the cellular and humoral responses in the animals.

Treg cells induced by ASIT treatment produce high levels of IL-10 and TGF-β that induce the production of IgG4 and IgA, respectively, by B cells and suppress the effect of IgE (Jutel and Akdis, 2011; Jutel et al., 2006, 2016; Shamji et al., 2011). Before the beginning of the treatment, the serum levels of IgE were higher in allergic horses than in the control horses; the same trend was not observed for IgG4 and IgA values. The increase in the total serum concentration of IgE that was detected is characteristic of allergic diseases with signs of skin hypersensitivity (Marsella, 2013; Scott and Miller, 2011; Wagner, 2009) and confirmed the allergic nature of the disease. During ASIT, the concentration of allergen-specific IgE initially increases and then decreased to pretreatment levels during the maintenance phase (Jutel et al.,

2006, 2016; Jutel and Akdis, 2011). The total concentration of IgE was higher in the horses with allergic diseases than in the control horses, and a significant decrease in the serum level of IgE was observed in the 6th month of treatment. However, the IgE levels in ASIT group remained high and had not reached the control level at the end of the study. The effects of ASIT treatment on IgE levels is variable. One study did not find significant differences in specific IgE levels after 70 days of treatment against house dust mites in people with allergies (Jutel et al., 2003) nor were significant differences found even after two years of ASIT treatment (Zhong et al., 2015). However, Keppel et al. (2008) demonstrated that dogs responsive to ASIT had significant decreases in allergen-specific IgE levels over the course of one year. Our study analysed total IgE serum levels, not allergen-specific IgE, and we do not know if ASIT could decrease the levels of some specific IgEs. The high levels of IgE could be the result of a new sensitization to allergens during the allergic season in addition to those produced by long-lived memory B cells that have been exposed to repeated seasons of allergens (Shamji et al., 2011).

ASIT treatment is associated with elevated concentrations of IgG4, which has inhibitory activity, and with high concentrations of IgA (Jutel and Akdis, 2011; Palomares et al., 2017; Shamji et al., 2011). In our study, no changes in serum IgA and IgG4 levels over time were recorded. To the best of our knowledge, studies have not been performed to investigate the serum levels of IgG4 and IgA in horses undergoing ASIT treatment. ASIT treatment did not induce a change in the IgE/IgG4 ratio, which is characteristic of immunotherapy treatment (Palomares et al., 2017; Shamji et al., 2011) due to the increase in IgG4 levels, indicating the predominance of IgE in the allergic horses in our study. The results of the immunoglobulin levels indicated that immunotherapy treatment, given the conditions analysed in this investigation in horses, was insufficient to induce the significant changes that could be expected in the serological biomarkers that indicate T cell tolerance

during immunotherapy (Palomares et al., 2017; Shamji et al., 2011). Additionally, the allergen concentration or type of allergen have been demonstrated to influence the regulatory activity of human $CD4^+CD25^+$ T cells (Bellinghausen et al., 2005). The influence exerted by these factors in veterinary immunology has not been completely investigated and is likely important to the immunotherapy response.

This paper describes the first results regarding the immunological mechanisms in horses with allergic diseases treated by immunotherapy over the course of one year. Differences in the percentages of lymphocytes, $CD25^+$, $CD4^+CD25^-$ and $CD4^+CD25^{dim}$ T cells were observed between horses with allergic diseases and control horses. Only small changes were detected in different T cell subpopulations over the course of the year of treatment, and their effects on the cytokine and immunoglobulin response was also not significant, with the exception of decreases in the serum concentrations of IgE and TGF-β. The results could indicate that there are no similarities between ASIT for horses with allergic diseases and human immunotherapy for allergic diseases or with immunotherapy for canine atopy. Another possibility is that the duration of the treatment and other features such as the composition of allergen extracts, the number of allergens or immunological status of the animals resulted in the lack of a significant stimulus to the immune system of the horse to induce an allergen- tolerant state. All these considerations can influence the success of ASIT and need to be further considered. Further studies are needed to provide data to resolve this issue and improve both the immunological and clinical responses of the horses with allergic diseases treated with immunotherapy.

Funding

Funding was obtained through the Laboratorios Diavet (Leganés, Madrid, Spain).

Acknowledgements

We thank the cooperating local veterinarians who sent us blood samples from their patients with allergic diseases to conduct this study. Additionally, we appreciate the invaluable work performed by Álvaro Casanova on the ImageStream®X Imaging flow cytometer.

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Highlights

- Differences in T cell subsets between horses with allergic diseases and control horses were observed
- No effect of the immunotherapy on peripheral T cells was detected during the study
- IgE and TFG-β serum levels decreased as a consequence of the immunotherapy

Figure captions

Fig 1: Gating strategy to distinguish among $CD4^+CD25^-$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{high}$ subpopulations in peripheral PBMC of a horse treated with specific IgE immunotherapy. A gate was positioned around the lymphocytes to identify the $CD4^+$ T cells. The $CD4^+CD25^-$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{high}$ cells were distinguished based on the fluorescence signal of the $CD25^+$ cells. The percentages of the different subpopulations are expressed. The flow cytometry was conducted with an ImageStream®X cytometer.

Fig. 2: Percentage of cells in each subset as analysed by imaging flow cytometry in the peripheral blood of horses treated with immunotherapy (ASIT n = 111) and healthy horses (control n = 38) over the course of one year. The results are shown as box and whisker plots. Each box represents the 25th to 75th percentiles, the horizontal line represents the median, the whiskers represent the 10th and 90th percentiles, and the plotted points represent the range. Statistically significant differences between groups were analysed by the non-parametric Mann-Whitney test for lymphocytes, $CD25^+$ and $CD4^+CD25^-$ and unpaired t-tests were used for $CD4^+$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{high}$ T cells.

Fig. 3: Serum concentrations of cytokines in horses treated by immunotherapy (ASIT) and healthy horses (control) over the course of one year of treatment. For TGF-β, ASIT n = 85 and control n = 18; for IL-10, ASIT n = 81 and control n = 14; and for IFN-γ, ASIT n = 62 and control n = 15. The results are shown as box and whisker plots. Each box represents the 25th to 75th percentiles, the horizontal line represents the median, the whiskers represent the 10th and 90th percentiles, and the plotted points represent the range. Statistically significant differences between groups were analysed by the Mann-Whitney test, except for TGF-β for which an unpaired t-test was performed.

Fig. 4: Serum concentrations of immunoglobulins of of horses treated by immunotherapy and healthy horses over the course of one year (IgG4 (µg/ml) (ASIT n = 67, Control n = 16), IgA

(ng/ml) (ASIT n = 83, Control n = 15) and IgE (ng/ml), (ASIT n = 36, Control n = 6)). The results are shown as box and whisker plots. Each box represents the 25th to 75th percentiles, the horizontal line represents the median, the whiskers represent the 10th and 90th percentiles, and the plotted points represent the range. Statistically significant differences between groups were analysed by the non-parametric Mann-Whitney test.

Table 1

Percentages of subsets of T cells analysed by imaging flow cytometry in the peripheral blood from horses with allergic diseases treated by immunotherapy (ASIT) and healthy horses (control) over time.

		T0	T03	T06	T09	T12	*P* value
Lymphocytes	ASIT	29.7 ± 15.6	36.7 ± 12	28.3 ± 13	37.9 ± 10.3	33.2 ± 11	0.116
	Control	22.1 ± 14.3*	19.3 ± 6.1***	22 ± 6.1	ND	25.5 ± 3.5***	0.663
CD4^{+}	ASIT	50.1 ± 18.1	53.5 ± 11.8	50.2 ± 11.6	44.7 ± 16.3	48.9 ± 13.6	0.595
	Control	52.1 ± 10.6	45.3 ± 15.8	57.5 ± 8.3	ND	60.1 ± 18.8*	0.085
CD25^{+}	ASIT	20.8 ± 15.7	18.7 ± 9.6	15 ± 7.2	16.1 ± 10.8	17.5 ± 10	0.755
	Control	6.6 ± 4.8**	7.3 ± 4.7**	8.7 ± 4.2**	ND	9.7 ± 3.9**	0.254
CD4^{+}CD25^{-}	ASIT	66.5 ± 11.9	61.3 ± 9.6	61 ± 9.9	64.2 ± 10.1	62.3 ± 11.2	0.349
	Control	54 ± 11.7**	60.2 ± 13.8	52.7 ± 8*	ND	52.2 ± 17.4*	0.541
CD4^{+}CD25^{dim}	ASIT	16 ± 8.7	19.3 ± 7	19.5 ± 7.2	18.6 ± 6.8	16.2 ± 7.6	0.232
	Control	22.6 ± 7.1*	23.4 ± 7.9	28.1 ± 11.2**	ND	27.5 ± 13**	0.641
CD4^{+}CD25^{High}	ASIT	17.6 ± 9.6	19.3 ± 6.8	19.6 ± 10	17.3 ± 6.3	21.5 ± 6.4	0.518
	Control	23.3 ± 8.5	16.5 ± 6.7	20.2 ± 7.1	ND	20.2 ± 7.4	0.277

ANOVA and an unpaired t-test for $CD4^{+}$, $CD4^{+}CD25^{dim}$ and $CD4^{+}CD25^{High}$ T cell percentages were performed to find statistically significant differences over time and between groups; for the rest of parameters, a non-parametric Kruskal-Wallis test and an unpaired Mann-Whitney test were used.

No statistically significant differences over time in each group (ASIT or control) for each T cell subset were found ($P > 0.05$).

Differences between groups (ASIT and control) (*,**,***) for each time in the same column and for each T cell subset are shown.

ND = not determined. Data are means ± SD.

Table 2

Serum changes in the concentrations of cytokines and immunoglobulins analysed by ELISA tests in horses with allergic diseases treated by immunotherapy (ASIT) and in healthy horses (control) over time.

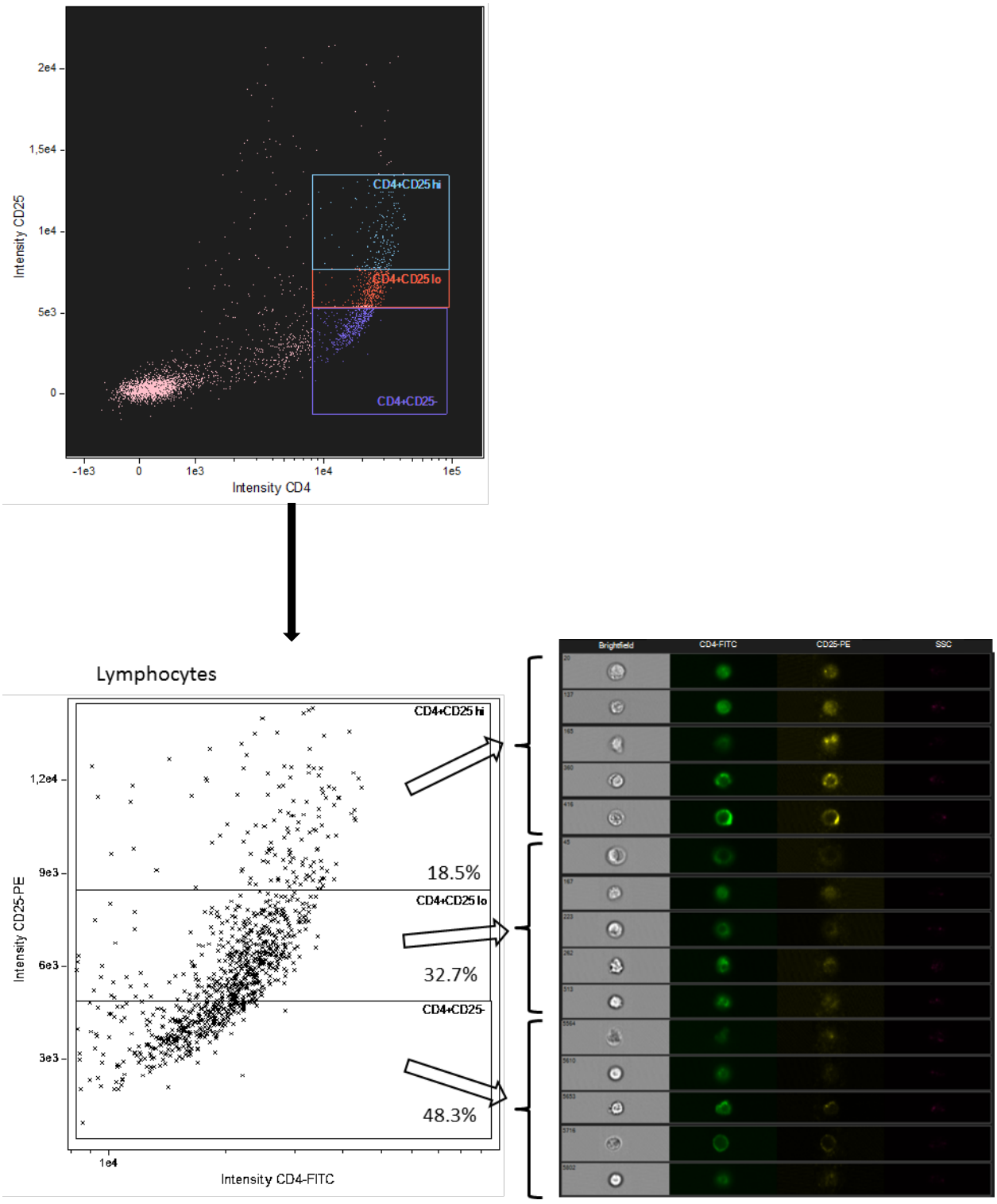
		T0	T06	T12	*P* value
TGF-β (pg/ml)	ASIT	1138 ± 350	972 ± 350	936 ± 438	0.103
	Control	845 ± 288[a*]	ND	546 ± 257[b**]	0.033
IL-10 (pg/ml)	ASIT	411 ± 214	400 ± 256	414 ± 271	0.935
	Control	596 ± 188	ND	514 ± 319	0.273
IFN-γ (pg/ml)	ASIT	1319 ± 1463	1360 ± 135	1043 ± 882	0.818
	Control	661 ± 794	ND	456 ± 666[*]	0.499
IgG4 (µg/ml)	ASIT	231 ± 164	216 ± 142	217 ± 137	0.977
	Control	272 ± 83	ND	297 ± 99	0.463
IgE (ng/ml)	ASIT	5.53 ± 4.71[a]	2.35 ± 1.69[b]	4.49 ± 2.64[ab]	0.048
	Control	0.84 ± 0.32[*]	ND	1.51 ± 1.26[**]	0.488
IgA (ng/ml)	ASIT	690 ± 503	686 ± 447	471 ± 285	0.147
	Control	658 ± 500	ND	895 ± 696	0.892
Ratio IgE/IgG4	ASIT	0.029 ± 0.02	0.021 ± 0.023	0.029 ± 0.02	0.473
	Control	0.006 ± 0.004[*]	ND	0.007 ± 0.006[*]	0.999

ANOVA and an unpaired t-test for TGF-β serum levels were performed to find statistically significant differences over time and between groups; for the rest of parameters, a non-parametric Kruskal-Wallis test and an unpaired Mann-Whitney test were used.

Different superscripts ([a,b]) are statistically significant differences over time in each group (ASIT or control) in the same row, and the *P* values are shown.

Differences between groups (*,**) (ASIT and control) for each time in the same column and for each parameter are shown .

ND = not determined. Data are means ± SD.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Lymphocytes
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Intensity CD25-PE
CD4+CD25 hi
18.5%
CD4+CD25 lo
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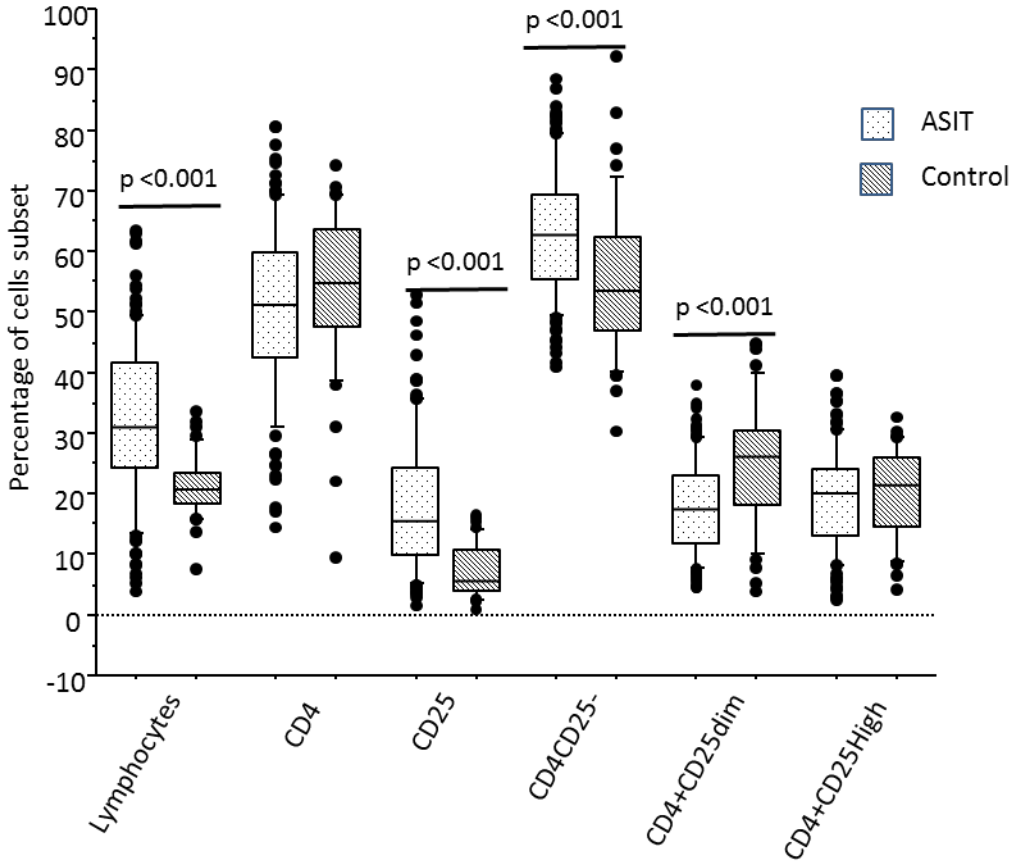
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Percentage of cells subset
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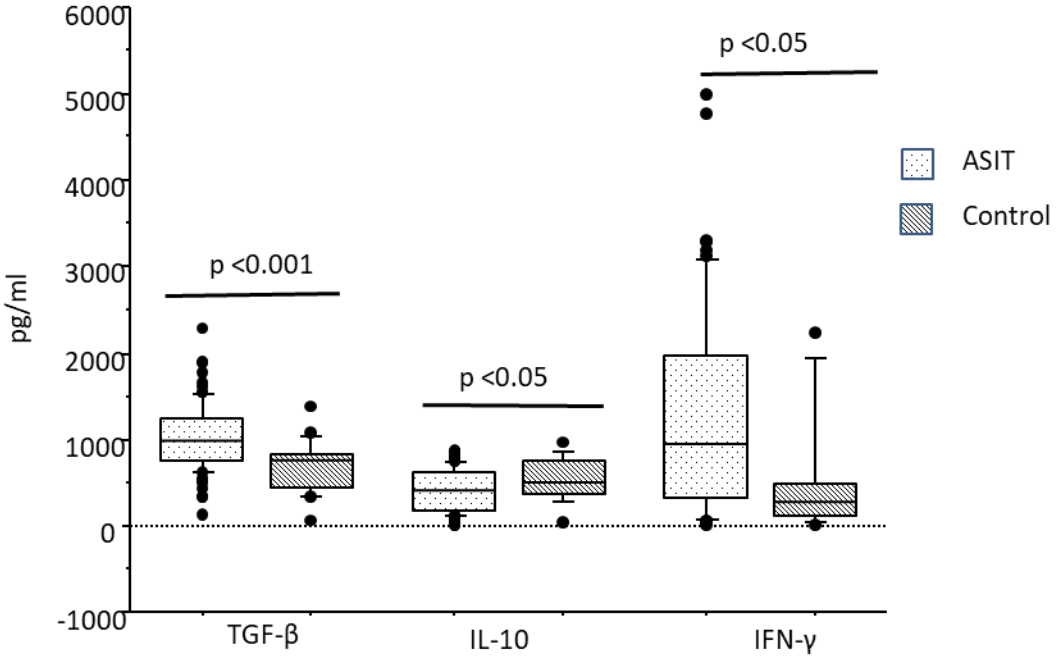
ASIT
Control
Lymphocytes
CD4
CD25
CD4CD25-
CD4+CD25dim
CD4+CD25High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ASIT
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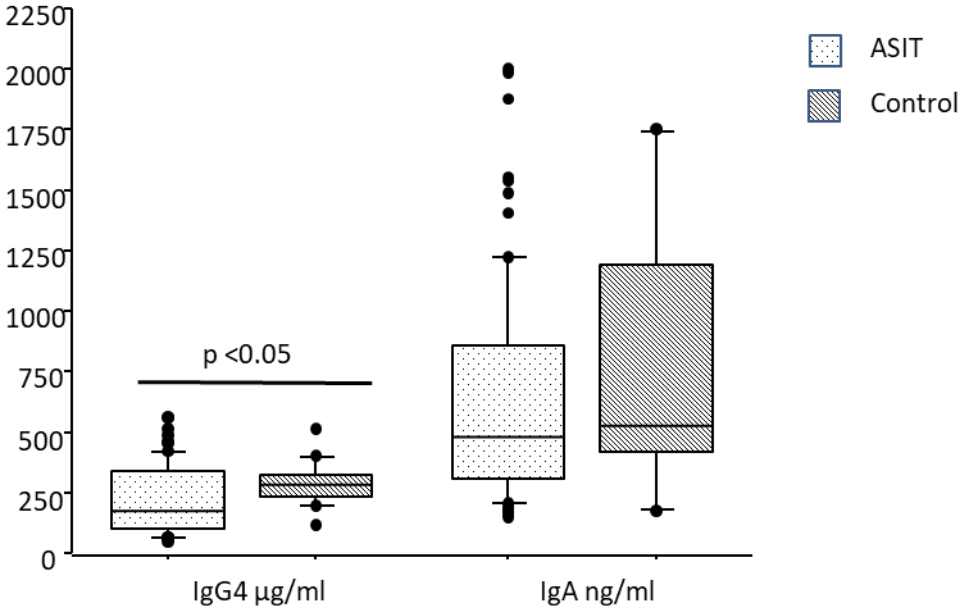
IL-10
IFN-γ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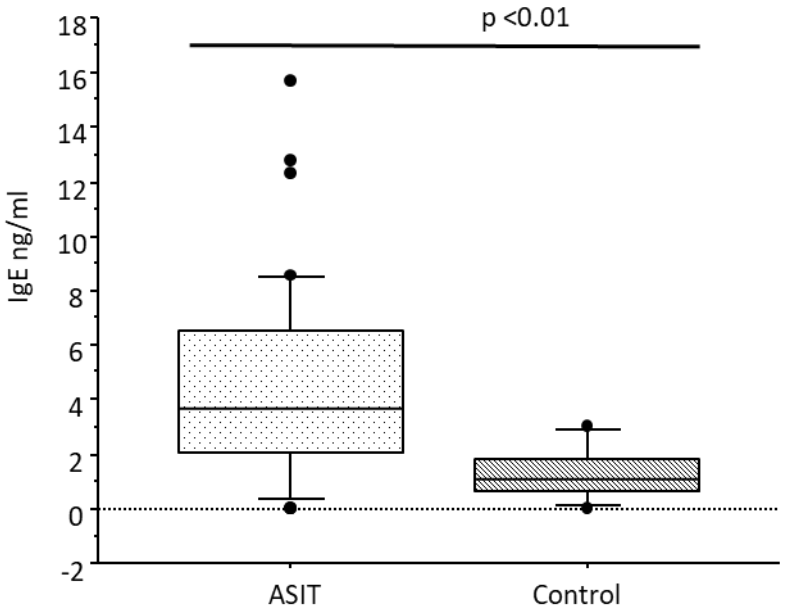
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