International Immunopharmacology 74 (2019) 105674

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Effects of allergen-specific immunotherapy on peripheral blood regulatory T cells and serum concentrations of cytokines and immunoglobulins in horses with allergic dermatitis

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ARTICLE INFO

Keywords:
Allergen-specific immunotherapy
Treg cells
Cytokines
Immunoglobulins
Allergic dermatitis
Horses

ABSTRACT

The aim of this study was to assess the effect of allergen-specific immunotherapy (ASIT) on the immunological responses of horses. Blood samples were taken from thirty-two horses with allergic dermatitis 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. Clinical improvement was appreciated by the majority of the horses' owners (56.6%). No effect of ASIT on $CD4^+CD25^{High}$ Treg cells was found during the one year treatment period. No differences in the percentage of $CD4^+$ T cells were observed between the groups, and no effects of ASIT over time were observed. 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%, $p < 0.001$). 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. A reduction in the serum levels of total IgE in the horses with allergic dermatitis was observed at the 6th month ($p < 0.05$), but increased again at the end of the study. The results 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. More studies are needed with new vaccine compositions and administration protocols to improve the immunological responses of the horses with allergic dermatitis.

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 [1,2]. These diseases are atopic dermatitis (AD) and recurrent airway obstruction (RAO) generated by aeroallergens as well as insect bite hypersensitivity (IBH) provoked by insects, mainly *Culicoides* genus [1,2]. 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 [2,3].

An alternative treatment for atopic dermatitis (aeroallergens) 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 [4–9]. ASIT has been used in human and small animal dermatology and represents specific approach to the treatment of IgE-mediated hypersensitivity [5,7,10]. ASIT has been used in horses with allergic dermatitis, and in a retrospective study by Stepnik et al. [11], owners reported an 84% clinical improvement in the disease.

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 [5,8,9,12,13]. Successful ASIT is associated with the shift 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 [7–9,14]. 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 [6–8,12,13]. IL-10 has a potent immunosuppressive capacity and has an essential role in the establishment of peripheral tolerance,

Abbreviations: ASIT, allergen-specific immunotherapy

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https://doi.org/10.1016/j.intimp.2019.105674
Received 4 February 2019; Received in revised form 20 May 2019; Accepted 29 May 2019
Available online 10 June 2019
1567-5769/

inhibiting the production of proinflammatory cytokines [4,7,8,15]. 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 [8,10]. The expression of IL-10 and TGF-β contribute to Treg function and immunoglobulin switching to IgG4 and IgA, respectively, which have inhibitory activities [6,8,13,16].

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 [5,6,9,12]. It has been suggested that upregulation of $CD4^+CD25^+$ Treg cells plays a role in ASIT treatment [12]. 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 [17–19]. The influence 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 [20]. 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 [21] and increasing IFN-γ expression to produce a shift to the Th1 dominant state [22]. 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 [11], and there was also a randomized study of the efficacy of ASIT in controlling IBH in horses [23]. However, these articles did not study how the T cells or other immunological components are affected by ASIT treatment in horses with allergic dermatitis.

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 dermatitis 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 serum levels of IgA, total IgE and IgG4. These cytokines and immunoglobulins appear to be keys in the pathogenesis and resolution by immunotherapy of the allergic diseases showed in dogs [21,22,24] and in human medicine [6,14,25,26].

2. Materials and methods

2.1. Animals

A total of 42 horses were included in this study. Thirty-two horses showed clinical signs of atopic 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. Horses were diagnosed with a dermatological protocol procedure including scraping of the lesions to detect mites and lice, trichogram and fungal culture for the isolation of dermatophytes. 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. More details of the diagnosis and inclusion criteria of allergic dermatitis of the horses used in this study have been recently published [27]. 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 Allercept© ELISA test (Heska Corp., Fribourg, Switzerland), designed to detect the presence of allergen-specific IgE. Only horses with clinical signs compatible with allergic dermatitis 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. Before enrolling a horse into the study an informed consent was obtained from both horse owners and veterinarians. All horses were patients of the Spanish veterinarians who wanted take part in the study.

Table 1
Composition of the inoculums injected in 32 horses. Allergens were identified with Allercept© ELISA test. The outcome was the assessment made by the owners of the horses after one year of ASIT.

Horse	Composition of the inoculum	Outcome[a]
1	*D. farinae*, *D. pteronyssinus*	Positive
2	*Grama*, *A. siro*, *T. putrescentiae*, *D. farinae*	Positive
3	*D. glomerata*, *Grama*, *S. cereale*, *T. putrescentiae*	Positive
4	*C. tarsalis*, *Tabanus*, *Culicoides*, *B. germanica*	NR
5	*D. glomerata*, *R. crispus*, *D. farinae*, *D. pteronyssinus*	Negative
6	*D. glomerata*, *R. crispus*, *D. farinae*, *D. pteronyssinus*, *T. putrescentiae*	Negative
7	*S. cereale*, *R. crispus*, *P. lanceolata*, *P. pratense*, *T. putrescentiae*	Negative
8	*T. putrescentiae*, *Tabanus*, *C. tarsalis*	NR
9	*D. glomerata*, *S. cereale*	Negative
10	*D. farinae*, *D. pteronyssinus*, *C. tarsalis*, *Tabanus*	Positive
11	*T. putrescentiae*, *C. tarsalis*, *Tabanus*, *B. germanica*	Positive
12	*D. glomerata*, *R. crispus*, *T. putrescentiae*	NR
13	*D. farinae*, *D. pteronyssinus*, *T. putrescentiae*, *C. tarsalis*, *Tabanus*	Negative
14	*R. crispus*, *A. siro*, *D. farinae*, *D. pteronyssinus*	Negative
15	*R. crispus*, *T. putrescentiae*, *C. tarsalis*, *Tabanus*, *Culicoides*	Positive
16	*C. tarsalis*, *Tabanus*, *Culicoides*	Positive
17	*Chenopodium*, *P. lanceolate*, *D. farinae*, *D. pteronyssinus*	NR
18	*S. cereal*, *R. crispus*, *F. sylvatica*, *D. glomerata*, *T. putrescentiae*, *Tabanus*, *Culicoides*	NR
19	*B. germanica*	Negative
20	*R. crispus*, *T. putrescentiae*, *Culicoides*	Positive
21	*S. cereale*, *A. siro*, *D. glomerata*, *T. putrescentiae*, *Culicoide*, *B. germanica*	Positive
22	*Chenopodium*, *D. glomerata*, *P. lanceolata*, *R. crispus*, *T. putrescentiae*	Positive
23	*C. tarsalis*, *Tabanus*, *Culicoides*, *B. germanica*	Positive
24	*R. crispus*, *S. caprea*	Negative
25	*C. tarsalis*, *Culicoides*	Negative
26	*A. siro*, *D. farinae*, *D. pteronyssinus*, *T. putrescentiae*, *C. tarsalis*, *Tabanus*, *Culicoides*	Negative
27	*D. farinae*, *D. pteronyssinus*, *T. putrescentiae*	Positive
28	*D. glomerata*, *R. crispus*, *S. cereale*, *D. farinae*, *D. pteronyssinus*, *Culicoides*	Negative
29	*A. siro*, *D. farinae*, *D. pteronyssinus*, *T. putrescentiae*	Positive
30	*R. crispus*, *B. alba*, *T. putrescentiae*, *C. tarsalis*	Positive
31	*C. tarsalis*	Positive
32	*R. crispus*, *A. siro*, *D. farinae*, *T. putrescentiae*	Negative

NR = no response.
[a] A positive response was considered if the horses improved in their symptoms by 50% or more and negative response if it was < 50%. The questioner was response by 27 owners; there were 15 positive response (56.6%) and 12 negative response (44.4%). A positive response was considered if the horses improved in their symptoms by 50% or more and negative response if it was < 50%.

2.2. ASIT treatment

Horses in the ASIT group received specific-allergen immunotherapy (ASIT) formulated with aluminium hydroxide-adsorbed allergens produced by Diavet Laboratorios. The inoculums had a mixture of allergens expressly prepared to each horse according to the results of the serum analysis for allergen-specific IgEs. Table 1 shows the allergen composition of the inoculums injected into horses and clinical outcome according to the assessment of the owners. The injection protocol was started following the manufacturer's instructions, and it entailed increasing subcutaneous doses over the course of one year. The horses did not receive any other treatment during the study, 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. All inoculums were started between May and October and the treatment was extended for one year.

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 min 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. [17] 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 min. The lysed blood was centrifuged at 300*g* for 5 min 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 min. A vial with 1×10^5 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 min. 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 min 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 60× magnification using a combination of transmitted light, scattered light, polarized light, and fluorescence. The cell sample acquisition (10,000 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 $CD4^+CD25^{High}$ T cells following the descriptions made by Hamza et al. [18] and Henríquez et al. [20]. The $CD4^+CD25^{High}$ cells were considered Treg cells because the FoxP3 marker is expressed primarily in this subpopulation in equine PBMC [17–20]. The percentage of $CD4^+CD25^{High}$ (Treg) cells was quantified within the population of lymphocytes labelled with the antibody anti-CD4 (lymphocytes $CD4^+$). Fig. 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, total IgE and IgA immunoglobulins were determined by ELISA. For the serum concentrations of equine IL-10, TGF-β and total 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 total 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 over time. For parametric data, ANOVA with *post hoc* Duncan's test was performed. Comparison between groups at each time point was tested with the non-parametric unpaired Mann-Whitney *U*test and unpaired Student's *t*-test when the data was normally distributed. General Linear Models (GLM) were analysed to assess the influence of “Group” (ASIT, control) and “Time” (T0, T03, T06, T09, T12) and their paired interactions on the T cell subset, cytokines and immunoglobulin concentrations. 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 2); 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 ($p < 0.01$). The GLM analysis showed that the interaction effect between “Group” and “Time” was significant for $CD4^+CD25^-$ T cells ($p = 0.038$, Table 3).

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

Table 4 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 ($p < 0.05$). No effects of the immunotherapy 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 dermatitis or in the healthy horses (Table 4). Total IgE serum levels were higher in the ASIT group than in the control horses at the beginning of the treatment ($p < 0.05$). Immunotherapy

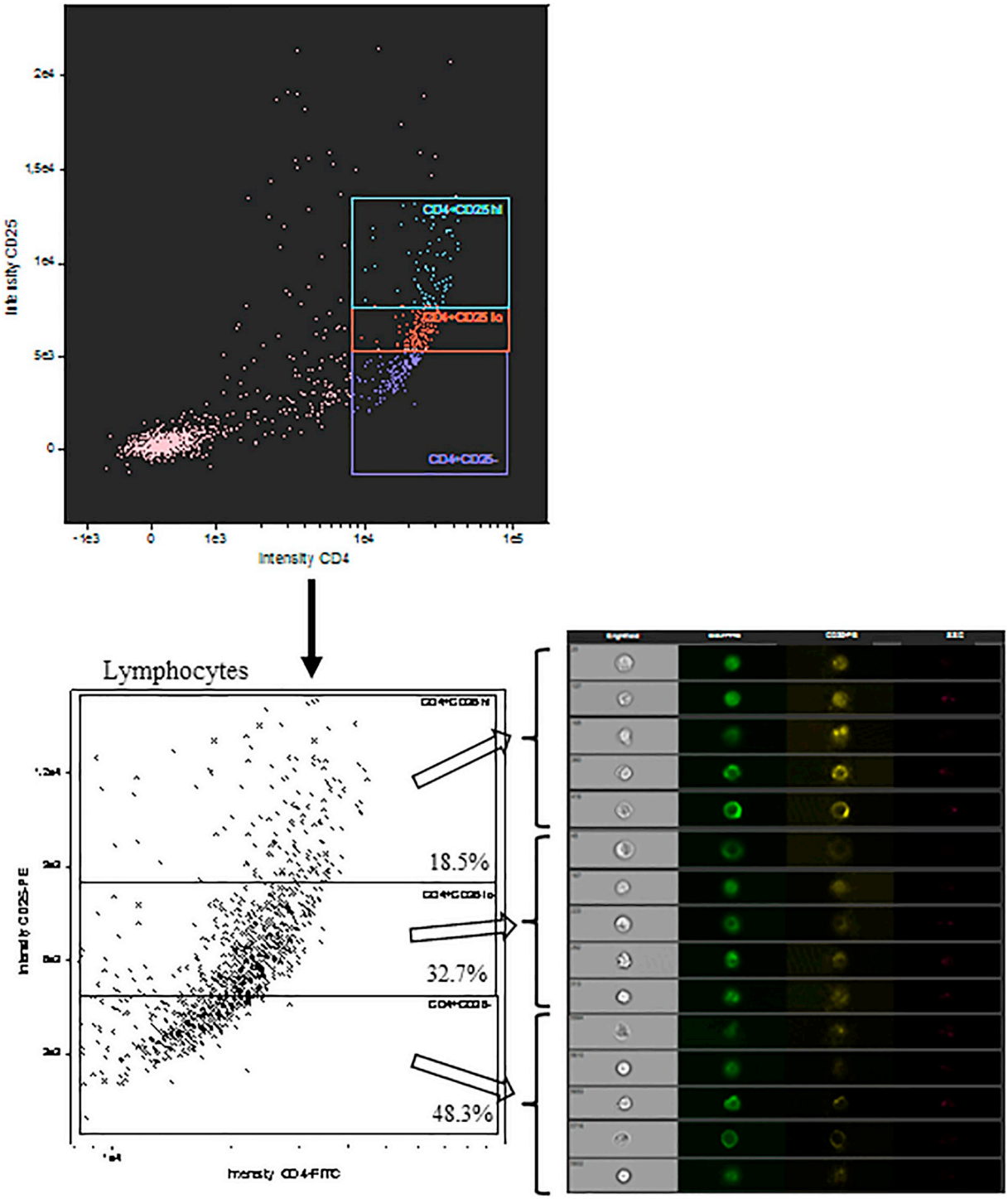

Fig. 1. Gating strategy to distinguish among $CD4^+CD25^-$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{High}$ subpopulations in peripheral PBMC of a horse treated with specific IgE immunotherapy. A gate was positioned around the lymphocytes to identify the $CD4^+$ T cells. The $CD4^+CD25^-$, $CD4^+CD25^{dim}$ 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.

decreased the total IgE serum levels at the 6th month of treatment ($p < 0.05$), but not at the 12th month. 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 ($p > 0.05$). No interaction effect in the GLM analysis between "Group" and "Time" was observed for any cytokine and immunoglobulin concentrations ($p > 0.05$, Table 5).

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 dermatitis over the course of one year of treatment in comparison with healthy horses during the same time. This study included horses vaccinated with different numbers of allergens (one to seven) including aeroallergens and from insects in the vaccine

Table 2
Percentages of subsets of T cells analysed by imaging flow cytometry in the peripheral blood from horses with allergic dermatitis treated by immunotherapy and healthy horses over time.

		T0	T03	T06	T09	T12	*p* value[a]
Lymphocytes	ASIT	29.7 ± 15.6	36.7 ± 12	28.3 ± 13	37.9 ± 10.3	33.2 ± 11	0.116[KW]
	Control	22.1 ± 14.3	19.3 ± 6.1	22 ± 6.1	ND	25.5 ± 3.5	0.663[KW]
	p value[mw][b]	0.070	0.001	0.151		0.002	
$CD4^+$	ASIT	50.1 ± 18.1	53.5 ± 11.8	50.2 ± 11.6	44.7 ± 16.3	48.9 ± 13.6	0.595[A]
	Control	52.1 ± 10.6	45.3 ± 15.8	57.5 ± 8.3	ND	60.1 ± 18.8	0.085[A]
	p value[t]	0.745	0.108	0.07		0.031	
$CD25^+$	ASIT	20.8 ± 15.7	18.7 ± 9.6	15 ± 7.2	16.1 ± 10.8	17.5 ± 10	0.755[KW]
	Control	6.6 ± 4.8	7.3 ± 4.7	8.7 ± 4.2	ND	9.7 ± 3.9	0.254[KW]
	p value[mw]	0.001	0.025	0.009		0.001	
$CD4^+CD25^-$	ASIT	66.5 ± 11.9	61.3 ± 9.6	61 ± 9.9	64.2 ± 10.1	62.3 ± 11.2	0.349[KW]
	Control	54 ± 11.7	60.2 ± 13.8	52.7 ± 8	ND	52.2 ± 17.4	0.541[KW]
	p value[mw]	0.082	0.408	0.038		0.057	
$CD4^+CD25^{dim}$	ASIT	16 ± 8.7	19.3 ± 7	19.5 ± 7.2	18.6 ± 6.8	16.2 ± 7.6	0.232[A]
	Control	22.6 ± 7.1	23.4 ± 7.9	28.1 ± 11.2	ND	27.5 ± 13	0.641[A]
	p value[t]	0.038	0.175	0.026		0.004	
$CD4^+CD25^{High}$	ASIT	17.6 ± 9.6	19.3 ± 6.8	19.6 ± 10	17.3 ± 6.3	21.5 ± 6.4	0.518[A]
	Control	23.3 ± 8.5	16.5 ± 6.7	20.2 ± 7.1	ND	20.2 ± 7.4	0.277[A]
	p value[t]	0.104	0.311	0.877		0.762	

mw: Mann-Whitney test; t: Student's t-test; KW: Kruskal Wallis; A: ANOVA.
ND = not determined. Data are means ± SD.
[a] Comparison over time is shown in the rows.
[b] Comparison between groups (ASIT, Control) for each time shown is in the columns.

composition, and in field conditions. Horses were kept in their original housing during the entire study with regular visits by their primary veterinarian. In this study, according to the owners, half of the horses improved the clinical outcomes as consequence of ASIT treatment. The efficacy of ASIT to control clinical signs of allergic dermatitis has been variable. In this sense, ASIT in horses failed to provide a significant improvement in the clinical signs of dermatitis [23] in horses with ASIT formulated to *Culicoides*, and in another study, clinical signs of 59% of mostly aeroallergen reactive horses were able to be controlled with ASIT alone [11] mainly affected by aeroallergens. To accurately assess the clinical efficacy of ASIT, more studies would be needed based on the evaluation of the clinical score performed by veterinarians.

It is essential to understand the immunological response of Treg cells, cytokines and immunoglobulins to understand the pathogenesis and control of allergic diseases [4,8,9,13]. Currently, Treg cells are defined by the expression of CD4CD25FoxP3-positive cells in horses [17–20,28] and in dogs [29,30]. 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 [31,32]. Additionally, among nonhuman species, FoxP3 is highly expressed in the $CD4^+CD25^{High}$ subpopulation in ovine PBMC [33], and in porcine (94.1%) [34] and canine activated PBMCs [35]. Only a minority of the equine $CD4^+CD25^{High}$ T cells express FoxP3 but they have strong suppressive activity [17] with the major proportion found in foals or in yearlings rather than in adult horses [18]. 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 our 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. [28]. In our study, as in the study of Hamza et al. [28], 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, contrary that has been reported in dogs with atopic dermatitis, which had more circulating Treg cells than the healthy controls [29,30]. 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. [20]. CD25 (IL-2Rα) is the principal cell surface marker of Treg cells [32], and CD25 expression increases upon *in vitro* stimulation of equine PBMC with pokeweed mitogen [19]. 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 dermatitis. 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 [21]. Additionally, individuals receiving immunotherapy against grass pollen have higher levels of Treg cells than people with untreated atopy and healthy people [25]. However, other reports showed that the Treg proportions remained unchanged during ASIT treatment against birch pollen, although patients displayed reduced skin prick test reactivity [36]. 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 [4]. The

Table 3
General Linear Model (GLM) for T cell subsets. Statistical significance (*p*) of the factors evaluated in each T cell subsets and their paired interactions are shown.

	Lymphocytes	$CD4^+$	$CD25^+$	$CD4^+CD25^-$	$CD4^+CD25^{dim}$	$CD4^+CD25^{High}$
Group	0.000	0.242	0.000	0.474	0.000	0.724
Time	0.453	0.437	0.877	0.492	0.505	0.612
Group × Time	0.725	0.067	0.504	0.038	0.484	0.231

Table 4
Serum changes in the concentrations of cytokines and immunoglobulins analysed by ELISA tests in horses with allergic dermatitis treated by immunotherapy and in healthy horses over time.

		T0	T06	T12	*p* value[§]
TGF-β (pg/ml)	ASIT	1138 ± 350	972 ± 350	936 ± 438	0.103[A]
	Control	845 ± 288	ND	546 ± 257	0.033[t]
	p value[t,*]	0.028		0.019	
IL-10 (pg/ml)	ASIT	411 ± 214	400 ± 256	414 ± 271	0.935[KW]
	Control	596 ± 188	ND	514 ± 319	0.273[mw]
	p value[mw]	0.069		0.394	
IFN-γ (pg/ml)	ASIT	1319 ± 1463	1360 ± 135	1043 ± 882	0.818[KW]
	Control	661 ± 794	ND	456 ± 666	0.499[mw]
	p value[mw]	0.314		0.045	
IgG4 (μg/ml)	ASIT	231 ± 164	216 ± 142	217 ± 137	0.977[KW]
	Control	272 ± 83	ND	297 ± 99	0.463[mw]
	p value[mw]	0.403		0.118	
IgE (ng/ml)	ASIT	5.53 ± 4.71[a]	2.35 ± 1.69[b]	4.49 ± 2.64[ab]	0.048[KW]
	Control	0.84 ± 0.32	ND	1.51 ± 1.26	0.488[W]
	p value[mw]	0.035		0.012	
IgA (ng/ml)	ASIT	690 ± 503	686 ± 447	471 ± 285	0.147[KW]
	Control	658 ± 500	ND	895 ± 696	0.892[mw]
	p value[mw]	0.904		0.306	
Ratio IgE/IgG4	ASIT	0.029 ± 0.02	0.021 ± 0.023	0.029 ± 0.02	0.473[KW]
	Control	0.006 ± 0.004	ND	0.007 ± 0.006	0.999[mw]
	p value[mw]	0.041		0.023	

mw: Mann-Whitney test; t: Student's t-test; KW: Kruskal Wallis; A: ANOVA. Different superscripts ([a,b]) are statistically significant differences over time in each group in the same row.
ND = not determined. Data are means ± SD.
[§] Comparison over time is shown in the rows.
[*] Comparison between groups (ASIT, Control) for each time is shown in the columns.

Table 5
General Linear Model (GLM) for cytokine and immunoglobulin concentrations. Statistical significance (*p*) of the factors evaluated in each parameter and their paired interactions are shown.

	TGF-β	IL-10	IFN-γ	IgG4	IgE	IgA	Ratio IgE/IgG4
Group	0.003	0.467	0.126	0.160	0.026	0.719	0.091
Time	0.011	0.862	0.542	0.880	0.149	0.876	0.353
Group × Time	0.659	0.460	0.696	0.648	0.496	0.426	0.516

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 [7,14,37]. The effect of ASIT on peripheral Treg cells could also be influenced by these factors. Nevertheless, some horses had a positive clinical response even though the results obtained for the whole data did not show of allergen-tolerant state. Perhaps, other immunological parameters were not studied in this study, such as suppression of eosinophils, mast cells, or the effect of ASIT on B cells in the synthesis of antibodies may explain the clinical improvement of some horses. New perspectives in vaccine development with recombinant allergens or pure salivary gland proteins of *Culicoides* have been investigated [38] 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 [10,39,40] mainly with IBH and asthma affected horses. Perhaps the improvement in the formulation of the vaccine could increase the peripheral blood Treg cell percentage in horses undergoing immunotherapy. To respond to this hypothesis, more studies with a different formulation of the inoculums and to test other immunological analysis to confirm allergen-tolerance in experimental conditions are needed.

Cytokines have an important role in the pathogenesis of allergic diseases characterized by a Th2 cytokine response [10]. Changes observed during ASIT include the increased expression of IL-10 and TGF-β secreted by Treg cells, resulting in the suppression of allergen-induced specific T cells [6,7,10]. The effect of ASIT on the serum levels of cytokines was variable throughout the study. 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 in horses. The effects of the allergic diseases and immunotherapy on the levels of cytokines are variable depending on the experimental protocol and animal species studied. Niedźwiedź et al. [41] showed that horses affected by RAO, a disease of allergic aetiology, had higher IFN-γ serum levels than healthy controls, as was seen during the length of our study. However, in a recent study, Klier et al. [39] found a significant decrease in serum IFN-γ concentration of horses nebulized with gelatin nanoparticles CpG indicating a reduction of the inflammatory response. 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 [22]. 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 [26], contrary to our results. The results from the scientific literature indicate that effects of IFN-γ are complex and can contribute to both anti-inflammatory and proinflammatory processes [42].

The immunotherapy 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 [6,8,10,15]. In veterinary medicine, increases in IL-10 levels have been shown in canine immunotherapy against atopic dermatitis together with increases in $FoxP3^+CD4^+$ T cells [21] and have been detected in the supernatant of canine PBMC stimulated with alimentary allergens [24]. Additionally, increases in the serum levels of IL-10 and TGF-β1 have been detected in human patients with atopic disease after two years of ASIT treatment [26]. In our study, the statistically significant differences in TGF-β serum levels that were observed between the two groups appear to be 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 [5]. 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. [26] in human patients. Furthermore, the dose of allergens can influence the clinical efficacy of ASIT [7] 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 [27]. This heterogeneous variety of allergens could influence the cytokine expression [5,13] and the use of these vaccines at the same time should be possible in multisensitized patients [10]. An improvement of the clinical outcomes in horses due to ASIT with vaccines composed of multiple allergens has been recorded [11], but no reduction in clinical score could be demonstrated by Ginel et al. [23] in another ASIT immunization with *Culicoides* extract. These papers only studied the clinical outcomes of the horses after ASIT, not the cellular and humoral responses in the animals.

In our study, the increase in total serum concentration of IgE in affected horses is characteristic of allergic diseases with signs of skin hypersensitivity [2,3,43] and confirms the allergic nature of the disease when matched with the history of seasonal flares. During ASIT, the concentration of allergen-specific IgE initially increases and then decreased to pretreatment levels during the maintenance phase [5,10,13]. The total concentration of IgE was higher in the horses with allergic dermatitis than in the control horses, and the total IgE levels in ASIT group remained high and not reached the basal level at the end of the study. The effects of ASIT treatment on IgE levels are 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 [44] nor were significant differences found even after two years of ASIT treatment [26]. However, Keppel et al. [21] 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 in horses. In our study, 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 [15].

ASIT treatment is associated with elevated concentrations of IgG4, which has inhibitory activity, and with high concentrations of IgA [5,9,15]. 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 [9,15] 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, 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 [9,15]. Additionally, the allergen concentration or type of allergen has been demonstrated to influence the regulatory activity of human $CD4^+CD25^+$ T cells [37]. The influence exerted by these factors in veterinary immunology has not been completely investigated and is likely important to the immunotherapy response.

The paper is the first report regarding the immunological mechanisms in horses with allergic dermatitis 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 were also not significant, with the exception of decreases in the serum concentration of total IgE after the six month treatment interval. The results could indicate that there are no similarities between ASIT for horses with allergic dermatitis 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, heterogeneous horse population, 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 dermatitis treated with immunotherapy.

Funding

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

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank the cooperating local veterinarians who sent us blood samples from their patients with allergic diseases to conduct this study.

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