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Research paper

CD4⁺CD25⁺ T cells expressing FoxP3 in Icelandic horses affected with insect bite hypersensitivity

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ABSTRACT

Insect bite hypersensitivity (IBH) is an IgE-mediated dermatitis caused by bites of midges from the genus *Culicoides*. We have shown previously that peripheral blood mononuclear cells (PBMC) from IBH-affected horses produce higher levels of IL-4 and lower levels of IL-10 and TGF- β 1 than those from healthy horses, suggesting that IBH is associated with a reduced regulatory immune response. FoxP3 is a crucial marker of regulatory T cells (Tregs). Here we have determined the proportion of CD4⁺CD25⁺FoxP3⁺ T cells by flow cytometry in PBMC directly after isolation or after stimulation with *Culicoides* extract or a control antigen (Tetanus Toxoid). There were no differences between healthy and IBH horses either in the proportion of FoxP3⁺CD4⁺CD25⁺ cells in freshly isolated PBMC or in the following stimulation with Tetanus Toxoid. However, upon stimulation of PBMC with the allergen, expression of FoxP3 by CD4⁺CD25^{high} and CD4⁺CD25^{dim} cells was significantly higher in healthy than in IBH horses. Addition of recombinant IL-4 to PBMC from healthy horses stimulated with the allergen significantly decreased the proportion of FoxP3 expressing cells within CD4⁺CD25^{high}. These results suggest that IBH is associated with a decreased number of allergen-induced Tregs. This could be a consequence of the increased IL-4 production by PBMC of IBH-affected horses.

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1. Introduction

Insect bite hypersensitivity (IBH) or sweet itch is a recurrent, seasonal, IgE-mediated allergic dermatitis affecting horses. IBH is caused by bites of *Culicoides* species (Halldorsdottir et al., 1989; Hellberg et al., 2006) that are prevalent in mainland Europe and on other continents but not in Iceland. Thus, horses born and living in Iceland do not develop IBH. However, the incidence of IBH is >50% when these horses are imported to mainland Europe (1st gener-

ation) where they are extensively exposed to the allergens (Bjornsdottir et al., 2006). Conversely, Icelandic horses born in mainland Europe (2nd generation) have a considerably reduced incidence of IBH (<10%) (Bjornsdottir et al., 2006; Marti et al., 2008). We have shown previously that upon *Culicoides*-specific stimulation, the proportion of IL-4 producing T cells was highest in PBMC from IBH-affected 1st generation horses compared to those from IBH 2nd generation horses as well as healthy 2nd and 1st generation Icelandic horses (Hamza et al., 2007). IL-4 production was usually not detected in healthy 2nd generation horses. This was due to a suppression by the regulatory cytokines IL-10 and TGF- β 1 (Hamza et al., 2008), as neutralization of these cytokines resulted in an increased number of IL-4 producing T cells.

Regulatory T cells (Tregs) provide self-tolerance and negative control (suppression) of immune responses

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(Sakaguchi, 2004). Most regulatory T cells are CD4⁺ and characterized by expression of CD25 and the transcription factor FoxP3, which is required for both the development and the function of Treg cells (Fontenot et al., 2003). Two general subsets of CD4⁺ Treg cells were identified: natural Treg which are produced in the thymus and migrate into the periphery (Raimondi et al., 2007) and adaptive Treg which are induced in the periphery upon encounter with an antigen (Akbar et al., 2003).

There are multiple mechanisms through which Treg cells operate, none of which has been identified exclusively (Shevach, 2009). In humans and mice, subsets of regulatory T cells (Tregs) are important sources of IL-10 and TGF- β 1 (Akdis et al., 2006; Jutel et al., 2003). Studies in humans have shown that Treg cells in healthy individuals may control Th2 responses through their ability to produce high levels of IL-10 and TGF- β 1 (Jutel et al., 2003). On the other hand, IL-4 promotes Th2 cell differentiation (Ansel et al., 2006) and may block the induction of Treg cells (Hadjur et al., 2009; Dardalhon et al., 2008; Prochazkova et al., 2009). Therefore, the balance between allergen-specific Treg cells and Th2 cells could be decisive in the development of allergy (Akdis et al., 2004).

CD4⁺ Treg cells are characterized by expression of CD25 and FoxP3 (Yamazaki et al., 2006; Ziegler and Buckner, 2006). A previous study originally presented at the 8th IVIS, had demonstrated cross-reacting antibodies against CD25 and FoxP3 in horses (Steinbach et al., 2009) and the characterization of equine CD4⁺CD25⁺ T cells and their regulatory function has been presented at the 9th IVIS (Hamza et al., 2010a). So far only one other most recently published study has also shown the phenotype of Treg in equine PBMC (Robbin et al., 2011). The fact that Tregs might be involved in IBH is underlined by the significantly lower expression of FoxP3 mRNA in the skin of IBH-affected horses (Heimann et al., 2011).

The aim of this study was to investigate whether there is a difference between IBH-affected 1st generation and healthy 2nd generation Icelandic horses in the proportion of FoxP3-expressing CD4⁺ T cells using both freshly isolated PBMC and following culture with allergen extract. In addition, we intended to determine the effect of IL-4 on the proportion of FoxP3-expressing CD4⁺ T cells.

2. Materials and methods

2.1. Study group

Twenty-seven adult horses of the Icelandic breed living in mainland Europe for several years were included in the study. They fell into two groups: IBH-affected Icelandic horses imported from Iceland as adults (1st generation IBH; $n=10$), and healthy Icelandic horses born in mainland Europe (2nd generation healthy horses; $n=17$). The IBH-affected horses had been imported from Iceland to Switzerland at least five years prior to the study. All horses were vaccinated regularly against tetanus and equine influenza and were de-wormed. IBH and healthy horses were from the same stables, i.e. were both exposed to insect bites with similar intensity. The diagnosis of IBH was based

on clinical signs and history, as described (Hamza et al., 2007).

2.2. Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from healthy horses and IBH-affected horses by Ficoll-Hypaque as described (Hamza et al., 2007). 4×10^6 freshly isolated PBMC were examined for the presence of circulating FoxP3⁺ CD25⁺CD4⁺ T cells using flow-cytometry as described below. Additionally, 4×10^6 PBMC from the same horses were cultured for 4 days with *Culicoides nubeculosus* extract (10 μ g/ml; Marti et al., 1999) or with Tetanus Toxoid, (10 μ g/ml; Schweizerisches Serum and Impfinstitut Bern, Switzerland) as control antigen, or left unstimulated (Mock). In a pilot study, we found that stimulation of PBMC induced expression of FoxP3 at day 3 which peaked at day 4 compared to day 1 and 2 (data not shown).

In another experiment, PBMC from healthy horses were cultured for 4 days with *Culicoides* extract in the presence or absence of recombinant equine IL-4 (rIL-4, R&D systems, 50 ng/ml). A dose response experiment (10–1000 ng/ml) performed first showed that a concentration of 50 ng/ml rIL-4 was optimal for our study (data not shown). Cells were harvested and FoxP3 expression by different CD4⁺ T cells subsets was measured by flow-cytometry.

All cell cultures were performed in RPMI 1640 containing FCS serum (10%), penicillin (100 IU/ml), streptomycin (100 μ g/ml), non-essential amino acids (1%), MEM vitamins (100 μ M), sodium pyruvate (1 mM) and 2-mercaptoethanol (50 μ M).

2.3. Measurement of FoxP3-expressing CD4⁺CD25⁺ T cells by flow-cytometry

Freshly isolated or 4 days cultured PBMC were labelled with 5 μ l mouse-anti-equine CD4 (CVS4, Serotec, Düsseldorf, Germany) at 4 °C for 30 min, followed by donkey-anti-mouse IgG-FITC (Jackson ImmunoResearch Europe Ltd, UK). This was followed by staining with goat-anti-human CD25 (R&D systems, London, UK, (Steinbach et al., 2009) at 4 °C for 30 min. A secondary antibody, donkey-anti-goat IgG-APC (Jackson ImmunoResearch) was used. The buffer used for labelling and washing steps for cell surface staining was PBS containing EDTA (13.4 mM), gelatine (1%) and sodium azide (0.02%). Thereafter, cells were fixed and permeabilized using Fix-perm buffer (ebioscience, Hatfield, UK) at 4 °C for 15 min, followed by washing twice with PBS containing 0.5% Saponin (Sigma–Aldrich, St. Louis, MO, USA) and 0.5% BSA (PPA laboratories GmbH, Pasching, Austria). Whole mouse-IgG molecules (10 μ g/ml) were added for 15 min to block remaining antibody binding sites of secondary antibody from CD4 to avoid cross reaction of anti-mouse FoxP3 with second antibody of CD4. This was followed by staining with rat-anti-mouse FoxP3 PE (FJK-16s, ebioscience) for 30 min on ice, followed by 2 further washes in PBS containing 0.5% Saponin and 0.5% BSA. Isotype control used was rat anti mouse IgG-PE (ebioscience). Washed cells were then resuspended in PBS and measured by flow-cytometry using LSRII FACS machine (Becton-Dickinson, Mountain View, CA, USA). Analysis was

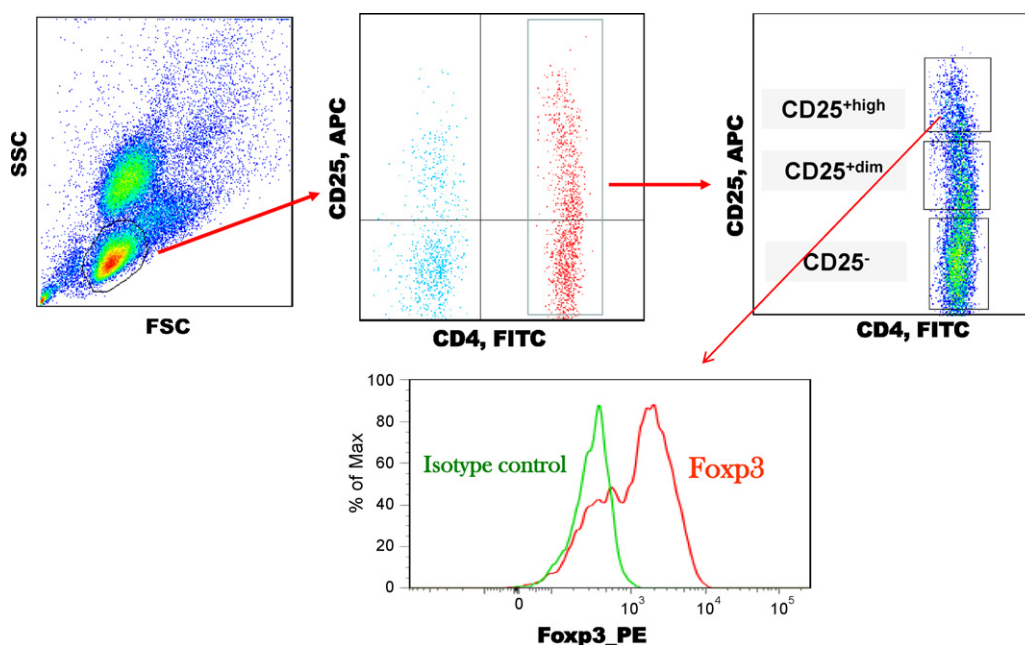


Fig. 1. Gating strategy used to determine FoxP3-expressing CD4⁺CD25⁺ T cells in PBMC from Icelandic horses. A gate was positioned around lymphocytes. Cells within this gate were used for gating CD4 positive cells. Cells within the CD4 gate were then analysed for expression of CD25. Three gates were placed in a way to clearly distinguish the CD25⁻, CD25^{dim} or CD25^{high} populations. FoxP3 histogram plots for each of the three gates were overlaid with their respective isotype control antibodies to discriminate negative from positive populations.

performed using Flowjo software (TreeStar Inc-Ashland). A gate was set around lymphocytes and gated cells were analysed for CD4 expression where another gate was positioned. CD4⁺ cells were analysed for the expression of CD25. Three gates were then placed, one for CD4⁺CD25⁻, one for CD4⁺CD25^{dim} and a third one for CD4⁺CD25^{high} cells (Fig. 1). Percentage of FoxP3-expressing cells was determined within each of the three gates.

2.4. Statistical analyses

NCSS 2007 software program (NCSS, Kaysville, Utah 84037, USA) was used for statistical analyses. Descriptive statistics by group were run on the data and kolmogrov–Smirnov test showed that the data are normally distributed. ANOVA was performed to compare the differences in the expression of FoxP3 by the 3 CD4⁺ T cell subsets (CD4⁺CD25⁻, CD4⁺CD25^{dim}, CD4⁺CD25^{high}). The Bonferroni test was used to correct for multiple comparisons.

Two sample unpaired *t*-test was used to compare the frequency of FoxP3 between healthy and IBH-affected horses. Paired *t*-test was used to compare stimulation with *Culicoides* extract or Tetanus Toxoid to Mock cells, likewise to compare the frequency of FoxP3⁺ T cells following culture with or without rIL-4. Overall, *p* values ≤ 0.05 were regarded as significant.

3. Results and discussion

In a previous study, we have reported a lower frequency of IL-4 producing cells in healthy horses compared to IBH-

affected Icelandic horses (Hamza et al., 2007). This was paralleled by higher levels of the regulatory cytokines IL-10 and TGF-β1 (Hamza et al., 2007, 2010b), and neutralization of these cytokines had increased the proportion of IL-4-producing cells (Hamza et al., 2008). Therefore, it was of interest to examine whether healthy horses have a higher proportion of FoxP3⁺ T cells compared to IBH horses using freshly isolated PBMC and following culture with allergen extract.

Freshly isolated PBMC from healthy and IBH Icelandic horses were examined for the existence of circulating CD4⁺CD25⁺ T cells expressing FoxP3. Using flow-cytometry, PBMC from 2nd generation healthy (H) or 1st generation IBH-affected (IBH) horses showed that a minority of circulating CD4⁺ T cells were indeed CD25⁺. The frequency of CD4⁺CD25⁻ however, was significantly higher compared to CD4⁺CD25^{dim} and ^{high} T cell subpopulations (Fig. 2a). There were no differences between healthy and IBH-affected horses in the proportion of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells. Interestingly, the significantly highest percentage of FoxP3⁺ T cells was detected within the CD4⁺CD25^{high} cells as compared to CD4⁺CD25^{dim} and CD4⁺CD25⁻ T cells (Fig. 2b). Importantly, no significant differences were found in the proportion of CD4⁺CD25⁺FoxP3⁺ T cells between healthy and IBH-affected horses. This indicates that there is no general defect in the frequency of circulating CD4⁺CD25⁺FoxP3⁺ T cell population in IBH-affected horses.

Subsequently, we addressed the ability of IBH-affected horses to induce FoxP3 expression upon *Culicoides* allergen stimulation compared to healthy horses. PBMC from healthy and IBH horses were cultured for 4 days with *Culi-*

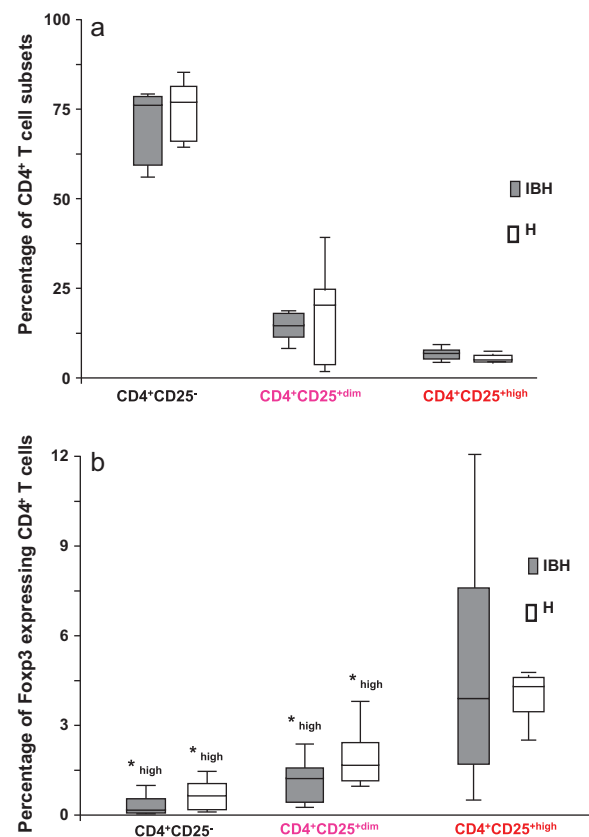


Fig. 2. Proportion of circulating CD4⁺CD25⁻, CD4⁺CD25^{dim} and CD4⁺CD25^{high} T cells (a) and expression of FoxP3 within these 3 subpopulations (b) as measured by flow-cytometry. Results are represented as box plots. The centre horizontal line of the box plot marks the median of the sample. The edges of the box mark the first and third quartiles. The whiskers define the upper adjacent value which is the largest observation that is less than or equal to the 75th percentile plus 1.5 times the interquartile range (IQR) and the lower adjacent value which is the smallest observation that is greater than or equal to the 25th percentile minus 1.5 times IQR. (a) Freshly isolated PBMC from both healthy 2nd generation (H, *n*=9) and IBH 1st generation (IBH, *n*=10) Icelandic horses contain small populations of either CD4⁺CD25^{dim} or CD4⁺CD25^{high} T cells. There is no significant difference between the 2 horse groups in the expression of FoxP3 (b) as measured by flow-cytometry. Results are represented as box plots. The centre horizontal line of the box plot marks the median of the sample. The edges of the box mark the first and third quartiles. The whiskers define the upper adjacent value which is the largest observation that is less than or equal to the 75th percentile plus 1.5 times the interquartile range (IQR) and the lower adjacent value which is the smallest observation that is greater than or equal to the 25th percentile minus 1.5 times IQR. (*high) indicates statistical significance difference to CD4⁺CD25^{high}. There were no significant differences in the frequency of CD25 subpopulations (a) nor in the percentage of FoxP3⁺ cells within the 3 subpopulations (b) between IBH-affected and healthy control horses.

coides extract or a control recall antigen (Tetanus Toxoid) or left unstimulated (Mock). There was a significant increase in the percentage of FoxP3⁺ cells within the CD4⁺CD25^{dim} and ^{high} subpopulations following stimulation with *Culicoides* or Tetanus Toxoid in the healthy horses compared to mock (Fig. 3). While the CD4⁺CD25^{dim} and ^{high} subpopulations from IBH-affected horses also showed a significant increase in the expression of FoxP3 upon Tetanus Toxoid stimulation, culture with *Culicoides* extract did not result in any increase of FoxP3 expression (Fig. 3). Consequently, IBH-affected horses had a significantly lower frequency of FoxP3⁺ CD4⁺CD25^{dim} and CD25^{high} T cells induced by the

Culicoides allergen compared to healthy horses, while there were no significant differences between the two horse groups following stimulation with Tetanus Toxoid or in the unstimulated cells (Mock). These findings reflect a normal ability of CD4⁺CD25⁺ T cells from IBH-affected horses similar to healthy horses to express FoxP3 after antigenic stimulation. However, this capability was impaired in IBH horses but not healthy horses when the cells were stimulated with *Culicoides* allergen. Other studies (Chen et al., 2004; Schmidt-Weber and Blaser, 2005) have suggested that the expression of FoxP3 represents a default immune response of T cells after antigenic stimulation which is influenced by the local environment such as the presence of certain cytokines. Our hypothesis is that either PBMC cultures from IBH horses stimulated with the *Culicoides* allergen contain factors which inhibit the expression of FoxP3 or PBMC cultures from healthy horses contain factors enhancing the expression of FoxP3. For example TGF-β1 (Andersson et al., 2008) and recently IFN-γ have been shown to amplify FoxP3 expression (Ouaked et al., 2009), while IL-4 a prototype of Th2 cytokine can inhibit the induction of FoxP3 (Dardalhon et al., 2008; Hadjur et al., 2009; Prochazkova et al., 2009). We have previously reported that stimulation with *Culicoides* resulted in lower IL-10 and TGF-β1 but higher IL-4 production in IBH horses compared to healthy horses (Hamza et al., 2010b). Since no IFN-γ production could be detected upon *Culicoides* stimulation (Hamza et al., 2007), the question arises whether a low FoxP3 expression following *Culicoides* stimulation in IBH-affected horses is caused by a lack of IL-10 and TGF-β1 or whether the high IL-4 could suppress FoxP3 expression.

In the present study we have investigated whether the addition of rIL-4 to *Culicoides* stimulated cells from healthy horses reduces FoxP3 expression (Fig. 4). PBMC from healthy horses were cultured with *Culicoides* or left unstimulated in the presence or absence of rIL-4 and then FoxP3 expression was measured using flow-cytometry (Fig. 4). Our results showed that addition of rIL-4 to the culture significantly decreased the frequency of FoxP3-expressing CD25⁺CD4⁺ T cells. This demonstrates that IL-4 can modulate the FoxP3 expression and could be responsible for the lower FoxP3 expression observed following *Culicoides* stimulation of PBMC from IBH horses. Further studies using neutralising antibodies against IL-4 need to be performed.

Taken together, our results confirm the existence of circulating FoxP3⁺CD4⁺CD25⁺ T cells in horses and for the first time show the induction of FoxP3 expressing T cells upon antigenic stimulation. Furthermore, the proportion of circulating FoxP3⁺CD4⁺CD25⁺ T cells does not allow for a distinction between healthy and allergic horses. However, *Culicoides* allergen stimulation induced more FoxP3 in CD4⁺CD25⁺ T cells from healthy horses compared to IBH-affected horses. This suggests that the allergen-induced FoxP3⁺ T cells might play a role in the regulation of an allergic-specific immune response in healthy horses. Moreover, the presence of IL-4 upon encounter IBH allergen can inhibit the induction of FoxP3. This can explain the lack of a regulatory immune response in IBH horses.

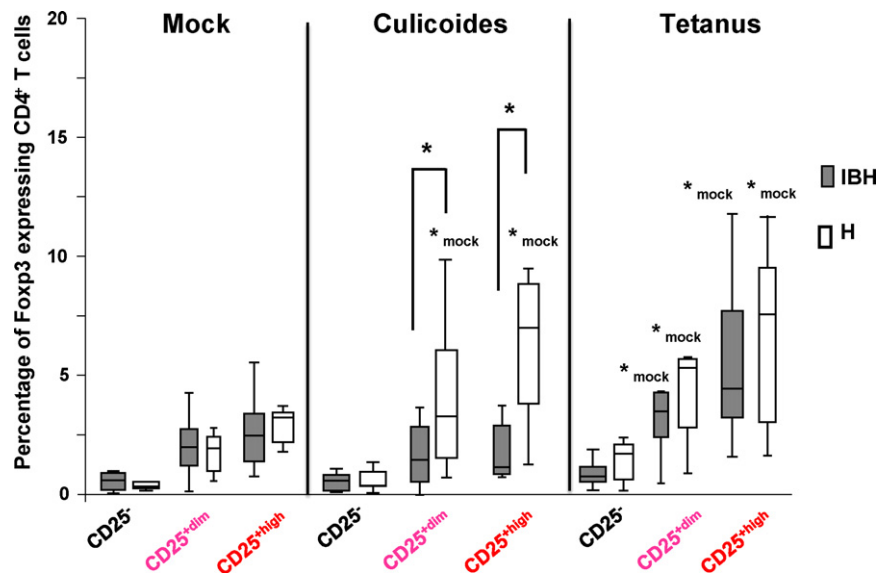


Fig. 3. Percentage of FoxP3 expressing T cells following antigenic stimulation. Freshly isolated PBMC from healthy 2nd generation and IBH 1st generation horses (same horses as in Fig. 2) were cultured with *Culicoides* extract, Tetanus Toxoid (Tetanus) or left unstimulated (Mock). Four days later, cells were harvested and percentages of FoxP3-expressing T cells were determined using flow-cytometry. Results are represented as box plots. Compared to mock, there is a significant increase in the proportion of FoxP3 expressing cells within CD4⁺CD25^{dim} and CD4⁺CD25^{high} in healthy horses after stimulation with *Culicoides* (*mock). Compared to mock, stimulation with Tetanus significantly increased FoxP3 expressing cells in both healthy and IBH horses (*mock). * and lines denotes a statistically significant difference between healthy and IBH horses. *p* value ≤ 0.05.

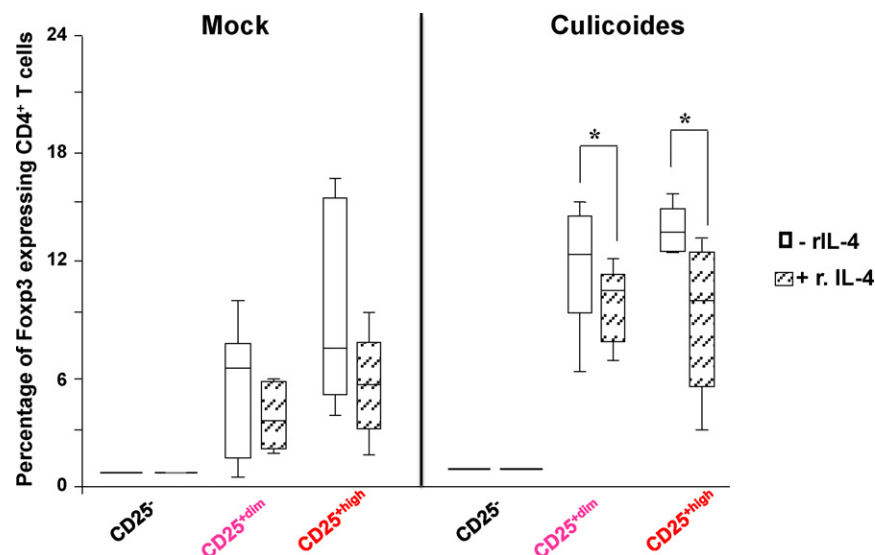


Fig. 4. Influence of IL-4 on the percentage of FoxP3-expressing T cells. PBMC from healthy 2nd generation horses (*n*=8) were cultured with *Culicoides* extract or left unstimulated (Mock) with or without addition of recombinant equine IL-4. Four days later, cells were harvested and percentages of FoxP3-expressing T cells were determined within CD4⁺CD25⁻, CD4⁺CD25^{dim} and CD4⁺CD25^{high} cells using flow-cytometry. Results are represented as box plots. rIL-4 significantly reduced the number of FoxP3 expressing cells within CD4⁺CD25^{dim} and CD4⁺CD25^{high} populations (*). *p* value ≤ 0.05.

In the future, we will investigate whether the circulating FoxP3⁺CD4⁺CD25⁺ T cells exhibit a suppressive function and thus fully represent Treg cells and whether this function is impaired in IBH-affected horses.

Conflict of interest

The Authors certify that they have no affiliation with or financial involvement in any organization or entity with a

direct financial interest in the subject matter or materials discussed in the manuscript. Any research or project support is identified in the “Acknowledgment” section of the manuscript.

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