



Research paper

Participation of T regulatory cells in equine recurrent airway obstruction



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ABSTRACT

Recurrent airway obstruction (RAO) is an equine immune-mediated disease with a high incidence worldwide. The aim of this work was to contribute to the understanding of RAO pathogenesis by studying T cells bearing regulatory markers in peripheral blood (PB) and in bronchoalveolar lavage fluid (BALF) recovered from the same group of susceptible horses before and after exposure to moldy hay, which has been shown to induce RAO signology in our horse herd. With this purpose, mononuclear cells were obtained from the BALF and PB from horses before and after antigenic challenge and were stained with fluorochrome-conjugated antibodies against CD4, CD25 and Foxp3 and subsequently analyzed by flow cytometry. The results indicated that the percentage of CD4⁺, Foxp3⁺ cells clearly increased in PB and BALF obtained from horses with RAO. In addition, the percentage of CD4⁺, CD25^{high} cells was greatly augmented in BALF of RAO positive horses compared with a baseline. No changes were observed in the PB compartment. The percentage of CD4⁺, CD25^{high}, Foxp3⁺ cells in BALF increased in horses with active disease compared to horses in remission; this cell population also does not show changes in the PB compartment when RAO positive and RAO negative horses were compared. On the other hand, when the percentage of CD4, Foxp3 positive cells were compared with the percentage of CD4⁺, CD25^{high} cells, the numbers were very similar. This observation was true for PB and BALF cells from non exposed horses as well as horses exposed to antigen. In all the experimental situations studied, the population expressing all of the markers CD4⁺, CD25^{high}, Foxp3⁺ represent only a minor percentage of CD4⁺, CD25^{high} or CD4⁺, Foxp3 subpopulations; therefore, an significant number of CD4⁺, CD25^{high}, Foxp3⁻ and CD4⁺, CD25^{null}, Foxp3⁺ cells must exist. Finally, we conclude that horses with RAO show an airway accumulation of T cells bearing regulatory markers that probably are modulating the course of this disease, and that these T cells may be involved in the resolution of immune-mediated bronchial inflammation.

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1. Introduction

The negative modulation of the immune response by T cells received renewed interest after the description of CD4⁺, CD25⁺ regulatory cells by Sakaguchi et al. (1995). However, it is now evident that these regulatory T cells (Tregs) are only a part of the cell-mediated suppression

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machinery used by the immune system to regulate the response to pathogens, tumors and self-antigens. In fact, today it has been recognized that a plethora of cell subpopulations play an active role in immune regulation, including Tr1 (Groux et al., 1997), Th3 (Chen et al., 1994), CD8 positive T cells, other lymphocytes bearing the Foxp3 transcription factor (Kapp et al., 2006), B regulatory cells (Bregs) (Mizoguchi et al., 2002), subpopulations of dendritic cells (DC) (Lambrecht and Hammad, 2012), natural killers cells (NK) (Shi et al., 2011) and innate lymphoid cells (Spits and Cupedo, 2012). The best studied of all of these cell subpopulations are the CD4 T cells that express the transcription factor Foxp3 and the membrane-molecule CD25, which corresponds to the α chain of the receptor for IL-2. Unfortunately, neither Foxp3 nor CD25 can be considered an absolute marker for Tregs. Although CD4 positive T cells bearing one of these markers have been considered previously to have regulatory functions, today the usual way to identify Tregs is the simultaneous identification of CD4, CD25, Foxp3 markers. Currently, this population includes thymus-derived Tregs also called natural Tregs (nTregs) and the peripherally induced Tregs (iTregs) derived from CD4+, CD25^{null}, Foxp3⁻ cells. The CD4+, CD25+, Foxp3+ cell population, consisting of nTregs and iTregs, has been demonstrated to control the response to parasitic, viral, fungal and bacterial infections in mice and humans (Belkaid and Tarbell, 2009). Related to this function, exposing mice to the conidia of *Aspergillus fumigatus* by the intranasal route induced an important increase of CD4+, CD25+, Foxp3+ cells in the bronchiolar space (Montagnoli et al., 2006). These authors also demonstrated that the early arrival of Tregs to the inflamed lung acts mainly by inhibiting polymorphonuclear neutrophils (PMN) via both a contact-dependent mechanism (CTLA-4/IDO) and a contact-independent mechanism (IL-10 production). In contrast, Tregs that arrive late produce IL-10 and express the CTLA-4 molecule, yet fail to inhibit PMN. These late-arriving Tregs are particularly active in regulating Th2 cells that are responsible for the induction of the adaptive humoral response, which may include IgE production and the subsequent allergic reaction. Therefore, it is hypothesized that different CD4+, CD25+, Foxp3+ Treg subpopulations with diverse physiological characteristics play different roles in allergic airway inflammation; however, the extent to which nTregs and iTregs differentially regulate the allergic response is not yet known.

Despite recent advances toward understanding the types and function of Tregs in mice and humans, little is known of the existence, physiology and subsets of Tregs in domestic animals. In horses, Robbin et al. (2011) described the existence of Foxp3 positive lymphocytes in PB, as well as in the CD4 positive subpopulation, and demonstrated that cells expressing this regulatory marker can be stimulated to produce high amounts of IL-10 and IFN- γ (Robbin et al., 2011). Hamza et al. (2011) describe that, similar to mice and humans, a high percentage of the CD4+, CD25^{high} lymphoid population in horses expresses the transcription factor Foxp3 and that when this cells were sorted out they were able to suppress lymphocyte-induced proliferation when were added to the culture of CD4+CD25⁻ cells. The modulatory capacity of this population appears to be

mediated by both a close contact-dependent manner and through secretion of soluble factors, such as IL-10 and TGF- β . A more recent study of Tregs in Icelandic horses revealed that the proportion of CD4+, CD25^{high}, Foxp3+ cells in PB of horses with severe allergic dermatitis due to insect bite hypersensitivity (IBH) was similar to levels found in non-sensitized animals of the same breed. However, after in vitro stimulation of lymphocytes with the insect antigen, the expression of Foxp3 by CD4+, CD25^{high} and CD4+, CD25^{dim} cells was significantly higher in cells isolated from healthy horses compared to the IBH group, suggesting that horses with allergic dermatitis have a decreased capacity to generate Tregs (Hamza et al., 2012).

Recurrent airway obstruction (RAO), or heaves, is highly prevalent among stabled animals and is characterized by airway bronchoconstriction, neutrophilic inflammation, excessive mucous production and airway remodeling. This disease results in reduced dynamic lung compliance, increased pulmonary resistance and pleural pressure excursions (Derksen et al., 1985; Jackson et al., 2000). In susceptible animals exposed to allergens, RAO induces episodes of acute airway obstruction (crisis), followed by periods of disease remission in the absence of the inducing antigens (Robinson et al., 1996).

The pathogenesis of RAO is complex; environmental antigens induce airway inflammation, migration of PMN cells to the bronchioalveolar space and a Th2 adaptive immune response with production of IgE and IgG antibodies. Whereas IgE is known to be responsible for triggering type I hypersensitivity in affected airways (Eder et al., 2000; Halliwell et al., 1993; Horohov et al., 2005; Moran et al., 2012), IgG triggers a type III hypersensitivity response that activates the production of proinflammatory factors such as C-X-C chemokines that stimulate PMN migration into the affected area (Lavoie-Lamoureux et al., 2010; Lavoie et al., 2001). One of the most common allergens responsible for RAO in horses is *A. fumigatus*, which is known to damage epithelial structures via the production of elastase, protease, catalase, dismutase, and cytotoxic ribonuclease (Purkayastha et al., 2000). In addition, PAMPs from *A. fumigatus* are known to stimulate pattern recognition receptors PRRs on DC, inducing the secretion of cytokines that drive Th1, Th2 and Th17 responses (Murdock et al., 2011). Similarly, the activation of toll-like receptor-2 signaling by the phospholipomannan components of *A. fumigatus* results in the production of cytokines TGF- β and IL-10, which are iTreg inducers, from local dendritic cells (Hughes et al., 2011; Wuthrich et al., 2012). Therefore, *A. fumigatus* has the capacity to induce physical damage, stimulate various innate and adaptive pathways of immunity and may activate Tregs as a counterbalance mechanism against inflammation. In this work, we demonstrate that there is an important and significant enhancement in the percentage of Tregs in the airways and PB of horses displaying acute RAO symptoms following exposure to *A. fumigatus*.

2. Materials and methods

2.1. Animals

A RAO-susceptible herd of 9 Chilean Criollo mestizo horses ranging in age from 12 to 20 years weighing

420 to 450 kg were used. They were housed in pasture facilities near the Veterinary Hospital and were regularly de-wormed and clinically evaluated twice daily during the study protocol to maintain optimal health conditions. Samples of PB and BALF were collected to determine basal conditions of parameters in the remission status before challenge, and then the horses were transported to a conditioned stable for airway challenge. At the onset of RAO symptoms, a second set of PB and BALF samples were obtained to evaluate parameters of acute inflammation. Finally, the horses were medically treated and returned to pasture for prompt resolution of RAO symptoms. This study was designed to avoid animal distress and was approved Bioethical Committee of the University.

2.2. Hay/straw challenge

For the hay/straw challenge, horses were housed in a stable with controlled ventilation and fed with hay which was previously contaminated with *A. fumigatus* and incubated at 37 °C in order to induce the mold growth. This environment has previously been shown to induce airway inflammation and dysfunction in horses with RAO (McGorum et al., 1993). After 4 days, the response to the challenge environment was evaluated using the clinical score method described by Rush et al. (1998), endoscopic examination of the upper respiratory tract and BALF cytology as outlined below.

2.3. Physical and endoscopic examination and monitoring

Clinical scoring and physical examination for body temperature, heart rate and respiratory frequency were performed on each horse twice daily. Before, during and after antigenic challenge, the extent of the airway allergic reaction was graded according the same clinical score table mentioned above. Under basal conditions, the average reaction score was 2, but was elevated to an average of 4.5 during the antigenic challenge.

For endoscopic examination, horses were restrained in stocks and sedated by intravenous administration of butorphanol (Fort Dodge, Fort Dodge, IA) (0.01 mg/kg) and detomidine (Pfizer Exton, PA) (0.01 mg/kg). After sedation, the endoscope was passed through the nasal passages into the trachea for upper respiratory tract evaluation and mucus grading. The amount of visible mucus in the trachea was graded on a scale from 0 to 5, as previously described by Gerber et al. (2004).

2.4. Lymphocyte collection from peripheral blood and bronchoalveolar lavage fluid

Blood samples (10 ml) were obtained by jugular venipuncture and stored in sterile tubes containing EDTA as the anticoagulant. For collection of BALF, the endoscope was advanced until wedged, air was aspirated from the area with a syringe, and three 100 ml aliquots of sterile saline pre-warmed to 37 °C, infused and re-aspirated. The aspirated fluid was pooled, mixed in a sterile specimen cup and placed on ice; the recovered fluid from the respiratory

tract and the blood were processed within 20 min after collection to determinate the relative percentage of neutrophils. Thereafter, mononuclear leukocytes isolated from PB and BALF were subjected to density gradient centrifugation using Ficoll–Hypaque (1.077 g/ml). The recovered cells were washed, counted and adjusted to a concentration of 1×10^6 cells/ml. Under basal conditions, the percentage of neutrophils was lower than 5%, but increased to $57.3 \pm 15.7\%$ (mean $\pm$ SD) after 4 days of antigenic challenge.

2.5. Cell staining and flow cytometry

To label membrane markers, 1×10^5 cells were first suspended in 100 μ l of Flow Cytometry Staining Buffer (eBioscience, Hatfield, UK) containing 0.5 μ l mouse anti-equine CD4 (HB61A, VMRD, Inc.) and incubated at 4 °C for 30 min. After washing, the cells were resuspended and treated with the secondary antibody donkey anti-mouse IgG–Alexa 488 (IgG–FITC) (Jackson ImmunoResearch, Europe Ltd., Suffolk, UK); excess secondary antibody was removed by proper washing. Next, the cells were stained with goat anti-human CD25 (R&D Systems, London, UK) as described previously (Hamza et al., 2011; Steinbach et al., 2009); and donkey anti-goat IgG–APC (Jackson ImmunoResearch) was used as the secondary antibody. Controls for this step were performed by staining with secondary antibody only. Thereafter, cells were fixed and permeabilized using Fix/Perm Buffer (eBioscience) at 4 °C for 30 min, followed by washing twice with cold permeabilization buffer. For Foxp3 staining, cell preparations were incubated with rat anti-mouse Foxp3–PE (FJK–16s; eBioscience) or isotype control (eBioscience) antibodies at 4 °C for 30 min followed by two washes with permeabilization buffer. Finally, cells were resuspended in PBS and analyzed by flow cytometry as shown in Fig. 1 and as described previously (Hamza et al., 2011; Robbin et al., 2011).

2.6. Statistical analysis

SigmaPlot 11.0 was used for statistical analyses. Using the Kolmogorov–Smirnov test, it was determined that the data were normally distributed. ANOVA was performed to compare the differences in the expression of FoxP3 by the CD4+ T cell subsets. The two sample paired *t*-test was used to compare the frequency of Tregs between basal conditions and RAO affected horses. Overall, *p* values <0.05 were regarded as significant.

3. Results

3.1. Foxp3 positive cells in peripheral PB and BALF of in basal conditions and RAO-affected horses

Based on previous findings that the transcription factor Foxp3 plays an essential role in establishing a functional regulatory T cell lineage (Fontenot et al., 2003; Hori et al., 2003), we investigated the proportion of CD4 positive lymphocytes obtained from the PB and airways of horses before challenge in comparison to RAO-affected animals. Data presented in Fig. 2 demonstrate that $3.75 \pm 1.48\%$ of CD4+ T

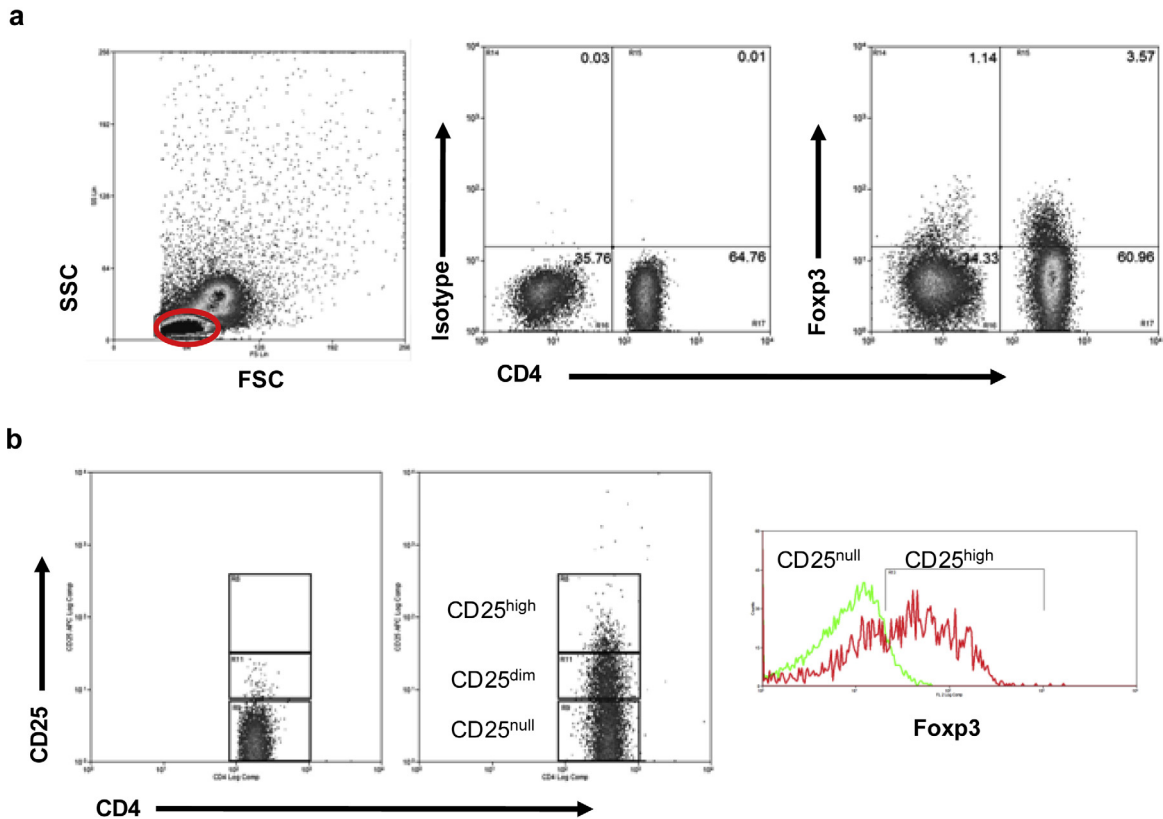


Fig. 1. (a) A gate was positioned around lymphocytes. Cells within the CD4 gate were then analyzed for Foxp3 distribution in CD4+ T cells from PBMC and BALF. (b) Gating strategy used to determine CD25 expressing CD4+ T cells in PBMC and BALF from horses with and without exposure to a primary antibody. Three gates were placed to clearly distinguish the CD25^{null}, CD25^{dim} or CD25^{high} populations. The histogram shows the difference of Foxp3 expression between CD4+ CD25⁻ and CD4+ CD25^{high} subpopulations.

cells in PB of horses in basal conditions express Foxp3, whereas in RAO-affected horses, the percentage of Foxp3+ cells increased to $6.35 \pm 1.12\%$. Similarly, $2.49 \pm 0.84\%$ of lymphocytes recovered from BALF of horses before challenge expressed Foxp3, which was substantially lower than the results obtained in PB. Induction of RAO greatly increased the proportion of Foxp3 positive lymphocytes in BALF to $6.97 \pm 0.74\%$. Therefore, a general conclusion of this first set of determinations is that RAO dramatically augmented the proportion of Foxp3+ cells (Fig. 2).

3.2. Distribution of CD25+ cells within the CD4+ population in blood and BALF in basal condition and RAO-affected animals

The expression of CD25 in CD4 positive T cells has been used to index the suppressive capacity of lymphocytes (Takahashi et al., 1998; Thornton and Shevach, 1998) and correlates with the degree of suppression in humans (Baecher-Allan et al., 2001). Therefore, we next evaluated the percentage of CD25^{null}, CD25^{dim} and CD25^{high} lymphocytes in the CD4 positive population of PB and BALF. No significant differences in the proportions of cells expressing CD25^{null} or CD25^{dim} were detected among animals before challenge and RAO-affected horses. In contrast, the fraction of the CD4, CD25^{high} subset obtained

from BALF at baseline was $3.25 \pm 0.62\%$, but notably higher in RAO Affected horses ($7.39 \pm 1.60\%$) (Table 1). To further emphasize these findings, individual data obtained from PB and BALF of each experimental group is presented in Fig. 3.

3.3. Foxp3 expression in CD4 positive cells separated in CD25^{null}, CD25^{dim} and CD25^{high}

Co-expression of Foxp3 with CD25 is considered a hallmark feature of Tregs in humans and mice. Therefore, we investigated the percentage of cells expressing Foxp3 in CD25^{null}, CD25^{dim} and CD25^{high}. Due to the degree of variability in the data, there were no statistical differences

Table 1

Percentage of CD25^{null}, CD25^{dim} and CD25^{high} subsets within CD4 positive population, from PB and BALF obtained from animals in basal and RAO conditions ($n=9$).

		CD25 ^{null}	CD25 ^{dim}	CD25 ^{high}
PB	Basal	84.79 ± 4.66^a	11.51 ± 2.46	3.69 ± 2.42
	RAO	85.55 ± 4.64	10.05 ± 2.73	3.40 ± 1.94
BALF	Basal	80.63 ± 6.81	16.18 ± 6.86	3.25 ± 0.62
	RAO	76.00 ± 5.67	16.84 ± 3.64	7.39 ± 1.60^b

^a The data in the table show means and standard deviations.

^b Indicate statistical differences between healthy and RAO horses ($p < 0.05$).

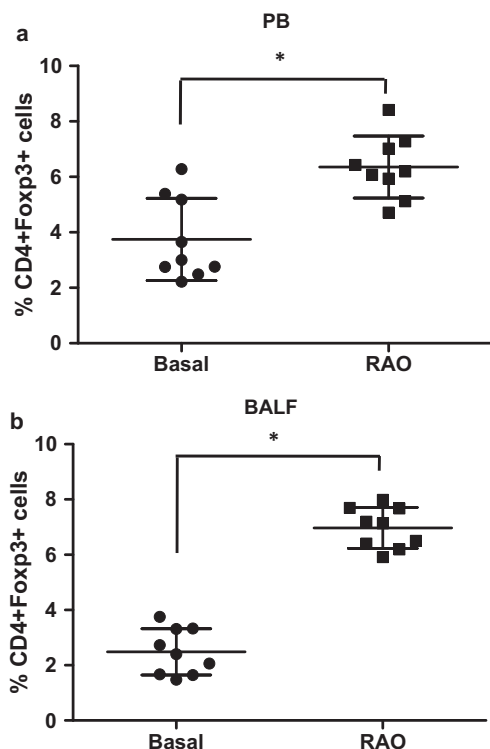


Fig. 2. Effect of antigenic challenge on percentage of CD4+, Foxp3+ cells. The percentage of CD4+ cells that express Foxp3+ obtained from PB (a) and BALF (b) was compared between horses in basal conditions and after antigenic challenge. Bars indicate mean and standard deviation ($n=9$) (* $p<0.05$).

in Foxp3 expression in the three CD25 subsets between basal and RAO condition. However, there is a constant increase in Foxp3 expression when non exposed animals were compared with horses with allergic airway inflammation (Table 2).

3.4. Presence of CD4+, CD25^{high} and Foxp3+ regulatory cells in PB and BALF obtained from animals in basal conditions or RAO-affected horses

After exposure to *A. fumigatus*, the fraction of CD4+, CD25^{high} and Foxp3+ lymphocytes in the PB compartment was $1.02 \pm 0.14\%$, which was comparable to non-exposed animals ($1.16 \pm 0.36\%$). In BALF of animals before challenge this cell population comprised $1.53 \pm 0.46\%$ CD4+ lymphocytes. As expected, induction of RAO increased the percentage of CD4+, CD25^{high}, and Foxp3+ to $3.97 \pm 0.97\%$ (Fig. 4).

Table 2

Expression of Foxp3 within the CD25^{null}, CD25^{dim} and CD25^{high} subsets in PB and BALF obtained from animals in basal and RAO conditions ($n=9$).

		CD25 ^{null}	CD25 ^{dim}	CD25 ^{high}
PB	Basal	4.12 ± 0.85^a	24.19 ± 2.91	45.50 ± 7.76
	RAO	5.04 ± 2.02	35.23 ± 11.55	49.75 ± 7.39
BALF	Basal	6.64 ± 3.11	29.16 ± 4.55	46.69 ± 9.06
	RAO	9.04 ± 3.46	36.43 ± 15.68	53.81 ± 6.79

^a The data in the table show means and standard deviations.

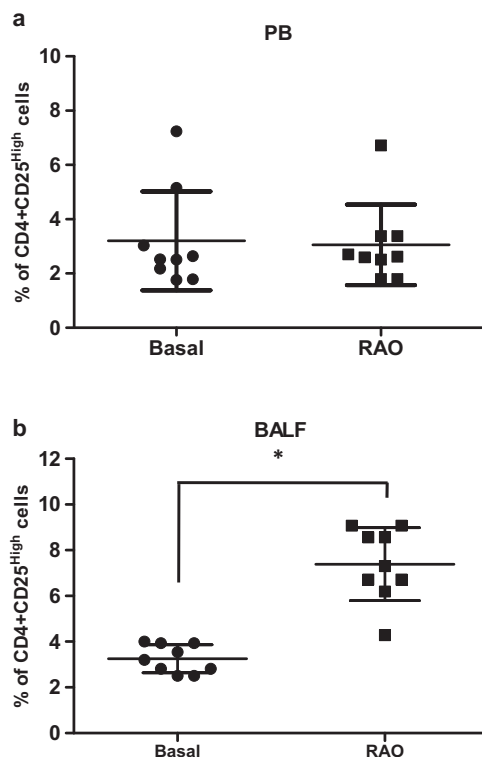


Fig. 3. Effect of antigenic challenge on percentage of CD4+, CD25^{high} cells. The percentage of CD4+ cells that express CD25^{high} obtained from PB (a) and BALF (b) was compared between horses in basal conditions and after antigenic challenge. Bars indicate mean and standard deviation ($n=9$) (* $p<0.05$).

4. Discussion

Regulatory T lymphocytes play an important, yet somewhat controversial, role in conditioning the immune system to antigenic stimulation. For example, humans with a low percentage of Tregs prior to the onset of asthma are more prone to develop an exaggerated immune response. Although there are no studies connecting airway inflammation with Tregs in horses, however Hamza et al. (2011) evaluating horse dermatitis triggered by insect bite arrive to similar conclusion: Foxp3 expression levels in CD4+, CD25+ cells population after challenge with specific antigen determine the susceptibility to develop an allergic reaction. In the work presented here, we demonstrated an increased percentage of lymphoid cells with regulatory markers in the airway space of horses after antigenic challenge with mouldy hay. A previous study in mice demonstrated a similar phenomenon (Montagnoli et al., 2006). This increase in Tregs may occur by two mechanisms: by active recruitment of cells dependent on expression of chemokine receptors, such as CCR4 and CCR8 by Tregs, as well as the presence of the ligands CCL17, CCL22 and CCL1 at the site of inflammation (Faustino et al., 2013); and by the generation of iTregs from CD4+, CD25^{null} naive T cells.

We examined various subpopulations of Tregs, e.g., CD4+, Foxp3+; CD4+, CD25^{high}; and CD4+, CD25^{high}, Foxp3+

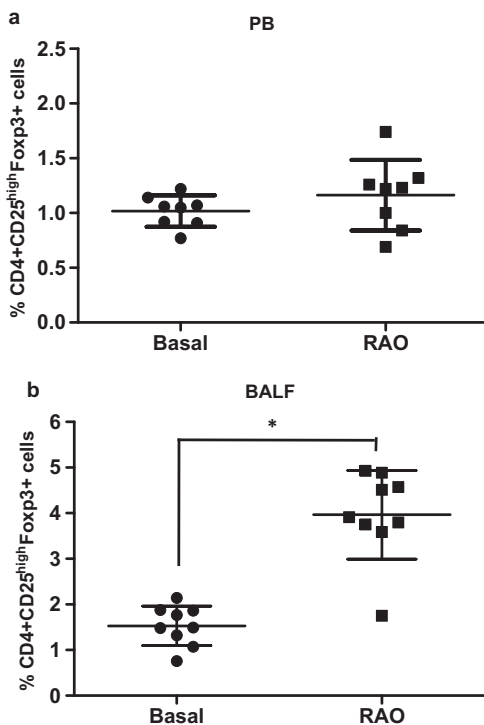


Fig. 4. Effect of antigenic challenge on percentage of CD4+, CD25^{high}, Foxp3+ cells. The percentage of CD4+ cells that co-express the regulatory markers CD25^{high} and Foxp3+ obtained PB (a) and BALF (b) was compared between horses in basal conditions and after antigenic challenge. Bars indicate mean and standard deviation ($n=8$) (* $p<0.05$).

in PB and BALF of animals before and after challenge. The results indicated that these populations were increased in BALF obtained from RAO-affected animals, but only the CD4+, Foxp3+ cells were increased in PB. This generalized increase of Foxp3 positive cells within the CD4+ population may be due to transient expression of Foxp3 in naive T cells (Wang et al., 2007) or the generation of iTregs by the inflammatory milieu in the lung (Xu et al., 2012). In our study, probably both of these mechanisms were operating.

With regard to the CD4+, CD25^{high} subpopulation, we did not find changes in PB when animals cursing with RAO and non exposed animals were compared, but an increase of CD4+, CD25^{high} cells were observed in BALF obtained from RAO-affected animals. This increase may also be due to selective recruitment of nTregs, the local generation of iTregs or expression of IL-2R in locally activated conventional effector T cells. The fraction of CD4, CD25^{high} positive cells in BALF of RAO-affected animals is similar to the levels of CD4+, Foxp3+ cells detected under comparable experimental conditions. It is unlikely that CD4+, Foxp3+ cells detected in BALF of RAO-affected animals are the same as CD4+, CD25^{high} cells because the CD4+ subpopulation that expressed CD25^{high} and Foxp3 together was less than 25% of the individual populations (CD4+, CD25^{high} or CD4+, Foxp3+). In other words, CD4+, CD25^{high} and CD4+, Foxp3+ populations contain a minimal number of cells bearing both markers simultaneously.

As shown in Table 1, RAO uniquely enhanced the proportion of the CD25^{high} lymphoid subset derived from BALF; no significant changes in the proportion of CD25^{null} or CD25^{dim} cells were detected in PB or BALF, irrespective of whether the cells were derived from PB or BALF collected at baseline or in RAO-affected horses. When the three CD25 subpopulations were studied for Foxp3 expression, no statistically significant differences were observed between the different experimental groups, which are similar to the observations of Robbin et al. (2011) that compared female and male horses of different age groups. However, as shown in the data in Table 2, although insignificant, a trend towards an increase regulatory markers was observed in CD25^{null}, CD25^{dim} and CD25^{high} populations in BALF of RAO-affected animals, which appears to be the in vivo equivalent of the results reported by Hamza et al. (2011) in which equine lymphoid cells cultivated in vitro under proper conditions increase the expression of Foxp3 marker cells that were originally CD25^{null}, CD25^{dim} and CD25^{high}.

5. Conclusion

We may conclude that in RAO-affected horses Tregs cells show a percentage increase in the airways as consequence of allergic inflammation, that these cells are heterogeneous population, probably with different physiologic attributes and roles in the regulation and final resolution of the airway allergic inflammation.

Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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