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Glucocorticosteroids administration is associated with increased regulatory T cells in equine asthmatic lungs

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ABSTRACT

Recurrent inflammation in severe equine asthma causes a remodeling of the airways leading to incompletely reversible airway obstruction. Despite the improvement of clinical signs and lung function with glucocorticoids (GC), inflammation, translated by an increased percentage of neutrophils, persists in the airways. Regulatory T cells (Treg) have been shown to have anti-inflammatory properties and play an important role in balancing the immune response by suppressing effector lymphocyte activity. However, interactions between Treg, neutrophils and glucocorticosteroids *in vivo* are unclear, particularly in asthma. Furthermore, the effects of GC on Treg in the airway of asthmatic horses have not been investigated. We hypothesized that horses with severe asthma display a decreased population of pulmonary Treg when compared to heathy controls, and that treatment with GC lead to an increased pulmonary Treg cell population only in affected horses. Using lung function measurements and flow cytometry with surface antigens CD4 and FoxP3, we investigated Treg in airway luminal cells obtained by bronchoalveolar lavage fluid (BALF) from 6 asthmatic horses in exacerbation of the disease and 6 aged-match controls, kept in the same environment, before and following a 2-week treatment with dexamethasone. Results showed that the number of Treg increases only in the lungs of asthmatic horses following GC therapy, despite continued presence of increased numbers of neutrophils. Our results support the complexity of the interaction between Treg, neutrophils and GC.

1. Introduction

Severe equine asthma (heaves, recurrent airway obstruction) is characterized by a recurrent airway obstruction and a chronic neutrophilic airway inflammation (Leclere et al., 2011). The condition is exacerbated by exposures to barn antigens, particularly those present in hay. However, the immune process contributing to the airway inflammation remains ill-defined, and different cell populations, including T cells, appears to play a role in the development and perpetuation of inflammation in equine and human asthma, notably by release of pro-inflammatory cytokines such as IL-1β, IL-4, IL-23 and IL-17 (Robinson et al., 2004; Lloyd and Hawrylowicz, 2009; Voo et al., 2009). Severe asthmatic horses often display Th2 and Th17 responses, which in human is associated with increased airway inflammation (Broide and Firestein, 1991; Robinson et al., 1996; Debrue et al., 2005; Ainsworth et al., 2006; Choy et al., 2015). Th17 cells are of a particular interest, as they promote the recruitment of neutrophils by producing IL-17 to further intensify chronic inflammation (Pelletier et al., 2010). It is known that recurrent inflammation in equine asthma causes a remodeling of the airways, which in turn, leads to severe airway obstruction, and the incurability of the condition (Leclere et al., 2011).

Interestingly, healthy horses also develop airway inflammation when exposed to stable antigens, but without developing lung diseases (Tremblay et al., 1993; Ivester et al., 2014). In these animals, inflammation is generally transient, while it persists in asthmatic horses. These findings suggest that appropriate autoregulation is possibly lacking in the lungs of affected horses and contribute to the persistent inflammation. Regulatory T cells (Treg) have anti-inflammatory properties by suppressing effector lymphocyte activity and therefore play an important role in balancing the immune response in asthma (Kearley et al., 2005; Lewkowich et al., 2005; Kearley et al., 2008). Forkhead box transcription factor FoxP3 is a critical regulator of Treg development and function and its expression is required for T cell immunosupressor activities (Fontenot et al., 2003; Hori et al., 2003). Treg exert these effects by direct contact with the effector cells and also by the production of anti-inflammatory molecules, including IL-10 and TGF-β.

Abbreviations: BALF, bronchoalveolar lavage fluid; FoxP3, forkhead box protein 3 transcription factor; GC, glucocorticosteroid; GILZ, glucocorticoid-induced leucine zipper; P_L, transpulmonary pressure; Treg, regulatory T cell

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Treg and Th17 cells share common differentiation pathways, and microenvironment changes can induce plasticity of these T cell phenotypes (Sehrawat and Rouse, 2017). It has been suggested that the balance between Treg and Th17 cells activities contributes to numerous infectious and non-infectious diseases (Sehrawat and Rouse, 2017).

The production of IL-10 and TGF-β by neutrophils is increased in presence of LPS-activated Treg (Lewkowicz et al., 2013). As the stabling environment of horses is rich in endotoxins (Riihimaki et al., 2008; Elfman et al., 2009; Whittaker et al., 2009), this may represent an additional means by which Treg contribute to homeostasis in healthy horses by dampening inflammation. However, because of its pro-fibrotic properties, Treg could conversely contribute to airway remodeling and to the deposition of collagen observed in equine asthma (Setlakwe et al., 2014, MacDonald et al., 2015). Studies have also shown that neutrophils can modulate recruitment of Th17 and Treg, and that T cells can induce the recruitment, activation and persistence of neutrophils (Pelletier et al., 2010; Lewkowicz et al., 2013). It was recently reported that Treg are increased when asthmatic horses are challenged with moldy hay (Henriquez et al., 2014), suggesting that these cells may be upregulated in equine asthma.

Glucocorticoids (GC) improve the clinical signs and airway obstruction in severe asthmatic horses without resolving the airway neutrophilia when the offending antigen exposure is maintained (Lavoie et al., 2002; Lavoie et al., 2006; Leclere et al., 2011). Treg are among the targets of GC (D'Adamio et al., 1997; Ayroldi et al., 2001; Karagiannidis et al., 2004; Cannarile et al., 2006; Braitch et al., 2009) and it has been suggested that they have a stimulatory effect on these cells (Dao Nguyen and Robinson, 2004; Karagiannidis et al., 2004). However, it is unclear how Treg respond to GC in disease processes and whether they modulate the interactions between Treg and neutrophils (Ayroldi et al., 2001; Karagiannidis et al., 2004; Braitch et al., 2009; Yuksek et al., 2011, Olsen et al., 2015). Thus, in this study, we evaluated the effects of GC on pulmonary Treg of asthmatic horses and in healthy controls kept in the same environment. Based on previous works (Karagiannidis et al., 2004; Lewkowich et al., 2005; Kearley et al., 2008), we hypothesized that 1) horses with severe asthma display a decreased population of pulmonary Treg when compared to controls and 2) that GC would lead to an increased Treg cell population in asthmatic horses.

2. Materials & Methods

2.1. Animals

Horses were part of a larger study evaluating the effects of glucocorticoids in equine asthma, and the animals, experimental protocol, physiological and inflammatory features have been reported previously (Vargas et al., 2017). In brief, 6 asthmatic horses and 6 aged-match controls were antigen-exposed by being stabled and fed hay for 1 month, then treated with dexamethasone (Dominion Veterinary Laboratories ltd, MB, CA) at a dosage of 0.06 mg/kg once daily for 2 weeks. Lung function was measured in unsedated standing horses, using a pneumotachograph attached to a mask and an esophageal balloon catheter as previously described (Jean et al., 1999). Bronchoalveolar lavage fluids (BALF) were collected as previously described (Lavoie et al., 2001) before (Baseline), and after 1 week and 2 weeks of dexamethasone treatment. Cytocentrifuge preparations of BALF were stained with a modified Wright–Giemsa solution (Kwik-Diff, Fisher Scientific, Waltham, MA, USA). Differential cell counts were obtained from 400 cells. BALF were then centrifuged at 1600 rpm at 4 °C for 5 min and cells were resuspended in PBS 1X and further analyzed with flow cytometry.

All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee for the Faculty of Veterinary Medicine of the University of Montreal (deontology Rech-1716).

2.2. Flow cytometry

Total BALF cells were studied by flow cytometry using Treg surface markers CD4 and FoxP3. Cells (3×10^6 cells) were fixed in paraformaldehyde 2% and washed three times in PBS 1X and incubate 10 min with PBS containing 0.1% Triton X-100 (Sigma-Aldrich, St-Louis, MO, USA). Cells were then stained for 30 min with anti-CD4 (Monoclonal Antibody Center, Washington State University, WA, USA; dilution 1/10), and anti-FoxP3-PE (Cedarlane, Burlington, ON, CA; dilution 1/10). All incubation steps were performed at 4 °C. Cells were then washed 3 times in PBS 1X and incubated 30 min in the dark with Alexa488- conjugated anti-IgG antibodies (Thermo Fisher Scientific, Waltham, MA, USA; dilution 1/500). Cells were washed twice and resuspended in 500 µl PBS 1X before flow cytometry acquisition of 10 000 events and analysis using BD Accuri C6 software and instrument (BD Bioscience, San Jose, CA, USA). Isotype-matched control antibodies (rat IgG2a-PE and mouse IgG1; Vector Laboratories, Burlington, ON, CA; Miltenyi Biotec Auburn, CA, USA and AbD Serotec, Raleigh, NC, USA, respectively) were used as negative controls. Pulmonary lymphocytes were gated using forward scatter and side scatter profiles (FSC and SSC parameters, excluding cell debris, neutrophils and macrophages). CD4+ cells were then gated and FoxP3 expression was quantified against CD4+ gated cells regarding its control isotype. Signals greater than those of the isotype-matched controls were considered positive, and degree of staining was evaluated as the mean percentage of positive cells (Fig. 1).

2.3. Statistical analysis

Data were statistically evaluated using a repeated measure 2-way ANOVA, with time as within-subjects factor and treatment as between subject factor. Post-hoc analyses were performed using Dunnett tests and the Fisher's LSD tests, to compare treatment values to baseline within each group, or values between groups at each time point, respectively (GraphPad Prism 7.0c software). A p-value of 0.05 was considered significant and all results were expressed as mean ± standard error of the means (SEM).

3. Results and discussion

In this study, we investigated the Treg response to GC in the airways of horses with severe asthma and healthy controls. As expected, antigen exposure induced an airflow limitation in affected horses with increased transpulmonary pressure values ($p < 0.001$), whereas control horses maintained a normal lung function (Fig. 2A). As the transcription factor Foxp3 was shown to play an essential role in establishing a functional regulatory T cell linage (Fontenot et al., 2003, Hori et al., 2003), we evaluated the proportion of pulmonary $CD4^+$ lymphocytes expressing this marker. We observed that exacerbation of severe equine asthma was not associated with a significantly increased Treg cell population when compared to controls. These results at first sight contrast with the recent report of an increased in Treg in the airways of asthmatic horses after antigen challenge (Henriquez et al., 2014). However, the results of the latter study are difficult to compare to ours, as the Treg response to an acute challenge was previously investigated and it lacked a control group, while our study evaluated the Treg population in the airways after a chronic antigen exposition (> 3 weeks) in healthy and asthmatic horses kept in the same environment. Also, this difference may have been due to our sample size as a power calculation showed that 14 horses in each group would be required to demonstrate such an increase of pulmonary Treg cells at the baseline.

The Treg response to GC was different in the 2 groups of horses, as it was significantly increased with treatments in asthmatic horses ($p = 0.0005$), while the number of these cells were unaffected by therapy in controls ($p = 0.65$; Fig. 2B). GC treatment significantly increased pulmonary Treg cells value only after 2 weeks of treatment in