1. Introduction

Asthma is a chronic lung inflammatory disease driven by a Th2 response to inhaled allergens in genetically pre-disposed individuals. Lung inflammation in asthma is characterized by infiltration of eosinophils, CD4 lymphocytes and neutrophils, as well as secretion of pro-inflammatory mediators such as Th2 cytokines (Busse and Rosenwasser, 2003). An important imbalance between pro-inflammatory cells and cells that regulate inflammation seams to occur in asthmatic patients, since they present quantitative and functional impairment of pulmonary regulatory T cells, termed Tregs (Hartl et al., 2007; Ling et al., 2004; Nguyen et al., 2009). Besides being constantly stimulated by aeroallergens, the reduced amount of Tregs in asthmatics might also limit the resolution of lung inflammation. Therefore therapeutic measures are of crucial importance in asthma.

Conventional therapy for asthma is generally based on the anti-inflammatory treatment with inhaled glucocorticoid combined preparations with a long-acting β2-agonist bronchodilator (Barnes, 2004). Asthmatic patients in general respond well to this therapy, although about 5% need additional and prolonged oral treatment with glucocorticoids, which leads to higher risk of adverse effects (Barnes, 2011a, 2011b; Montuschi and Barnes, 2011). The main mechanisms of action of glucocorticoids are thought to be the direct inhibition of expression of pro-inflammatory mediators and induction of expression of anti-inflammatory mediators, such as IL-10 (Barnes, 2011a).

Pieces of evidence in the literature state that glucocorticoids increase the number and activity of Tregs in asthmatic patients and experimental models of lung allergic disease (Hartl et al., 2007; John et al., 1998; Karagiannidis et al., 2004; Peek et al., 2005). However, most of these studies evaluated Treg numbers in peripheral blood, but not in the lungs. Moreover, detection of Tregs has been done for a long time by using markers that are also present in other lymphocytes such as CD4 and CD25 molecules. Most recently, other groups have shown that glucocorticoids can induce a reduction of Treg numbers in normal mice as well as in different inflammatory models (Sbiera et al., 2011; Stock et al., 2005;
2. Material and methods

2.1. Animals

Male A/J and BALB/c mice (18–20 g) were obtained from Oswaldo Cruz Foundation breeding facility (Rio de Janeiro, Brazil), and kept at a controlled room temperature (22–25 °C) with a 12 h light/dark cycle. Experimental procedures were approved by the Animal Ethics Committee of the Oswaldo Cruz Foundation (LW-23/10; CEUA-FIOCRUZ, Rio de Janeiro, Brazil). Animal sensitization, challenge and treatment protocol, as well as cell recovery from lungs and IL-10 detection are detailed in the online Supporting information.

2.2. Sensitization, challenge and treatment protocol

A/J or BALB/c mice were exposed to 25 μl of HDM extract (Greer laboratories, Lenoir, NC, USA; Dermatophagoides pteronyssinus) intranasally (25 μg of protein), for 3 days/week during 3 weeks. Control mice were challenged only with 25 μl of saline. A group of A/J mice were immunized and challenged with ovalbumin as described previously (Serra et al., 2012).

Challenged mice were treated with dexamethasone or budesonide (Fig. 1). Distinct protocols of dexamethasone treatment were used. Protocol 1: dexamethasone oral administration (1 mg/kg) on the same days of HDM challenge. Protocol 2: dexamethasone oral administration (1 mg/kg) was performed once a day on the last week of challenge for 6 consecutive days. Treatment with nebulized budesonide (7.5 mg/ml) was performed on the same days as protocol 2 of dexamethasone using an aerosol nebulizer (Aeroneb® Lab Nebulizer System; Aerogen Ltd., Galway, Ireland) and Buxco-Nebulizer Control-5 (Buxco, Wilmington, USA) which performed 3 inhalation cycles of 10 min each. Dexamethasone was dissolved in sterile saline solution immediately before use. Budesonide was dissolved in a solution of Tween 80 (0.5%) and methanol (0.5%). A group of allergenic-challenged mice was treated with the budesonide vehicle (Tween 80 and methanol). Since this vehicle treated group did not show any difference when compared to the group of challenged mice, it was omitted from the graphs.

2.3. Respiratory mechanics

Airways responsiveness was determined as a change in airway function after the last allergen challenge with aerosolized methacholine in a FinePointe R/C Buxco Platform (Buxco Electronics, Sharon, CT, USA). Lung resistance and dynamic lung compliance were assessed in anesthetized (nembutal 60 mg/kg, intraperitoneal), tracheostomized, and mechanically ventilated mice, as previously described (Olsen et al., 2011).

2.4. Cell recovery

BALF was retrieved by gentle aspiration and centrifuged (250 g, 10 min at 4 °C). Cell pellets were resuspended in PBS.

Lungs were perfused with saline containing 10 mM EDTA. The right lung was chopped and digested for 1 h with 0.15 mg/ml collagenase (Type D, Roche Diagnostics, Lewes, UK) and 0.025 mg/ml DNAse (Type 1, Roche Diagnostics). Cells were retrieved, washed and resuspended in 1 ml RPMI media (10% FBS, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin).

Cervical, inguinal and axillary lymph nodes were extracted, pooled in 1 ml PBS and mechanically homogenized. Thymus was excised and cells suspensions were obtained.

Total cells were counted in Neubauer chamber and differential cell count values were achieved after cytocentrifugation and staining with May-Grunwald-Giemsa. Eosinophils, neutrophils, macrophages and lymphocytes were determined by means of standard morphologic criteria under 1000 × magnification.

2.5. Flow cytometry analyses

CD4CD25Foxp3 characterization was performed with an eBiosciences kit (San Diego, CA, USA).

For characterizing CD4IL10 cells intracellular IL-10 was stained as described previously (Gregory et al., 2009). Samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinsonbiosciences, San Jose, CA, USA). Analyses were performed on FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

2.6. Detection of IL-10

Lung tissue was homogenized as previously described (Serra et al., 2012). Commercial ELISA kits were used for the measurement of IL-10 (Duoset R&D Systems, Minneapolis, MN, USA) in lung homogenates following instructions of the manufacturer.

2.7. Statistical analyses

Statistical analyses were done using one-way analysis of variance (ANOVA) followed by Newman–Keuls–Student test. Lung mechanics data were analyzed using two-way ANOVA with post-hoc Bonferroni correction. Data were expressed as mean ± Standard Error Median (S.E.M.). P < 0.05 (two-tailed test) was considered significant.

3. Results

3.1. Effects of dexamethasone on HDM induced lung inflammation and airway hyperreactivity

HDM challenges induced an increase in total leukocyte numbers detected in the BALF collected 1 day after the last challenge (Fig. 2A). The elevation in leukocyte counts was accounted for by an increase in the numbers of eosinophils, lymphocytes, macrophages and neutrophils. Eosinophil and lymphocyte changes were sensitive to treatment with dexamethasone (Fig. 2A). The leukocyte levels, including eosinophils and lymphocytes, naturally decreased in BALF samples during the resolution of lung inflammation, as assessed 7 and 14 days after the last HDM-challenge (Fig. 2C and E). Dexamethasone accelerates resolution, as evidenced by the significant reduction in the levels of these cells recovered in the BALF of treated animals (Fig. 2C and E).
Antigen challenge with HDM exacerbated airway resistance lung response to inhaled methacholine (3–27 mg/ml) as compared with control mice challenged with PBS, at 24 h after the last challenge (Fig. 2B). During the resolution phase there was also an increase in airway resistance response to methacholine in HDM-challenged mice, compared to PBS (Fig. 2D). By day 14 after the last challenge, there was no difference seen in airway resistance between groups challenged with HDM or PBS (Fig. 2F). Dexamethasone treatment reduced airway resistance in A/J mice HDM-challenged on the peak of inflammation (Fig. 2B), and during resolution (Fig. 2D and F). These results demonstrate that dexamethasone inhibits lung allergic inflammation in A/J mice challenged with HDM.

3.2. Dexamethasone inhibited Treg airway influx during the peak of inflammation as well as during the resolution phase

As shown in other lung inflammation models (Gregory et al., 2009), allergic challenge increased the numbers of Tregs in the airways of A/J mice, when compared to PBS-challenged mice. Increase in Treg numbers in BALF from HDM-challenged A/J mice peaked at 1 day and decreased during resolution days (Fig. 3). Unexpectedly, dexamethasone treatment inhibited Treg numbers in BALF from HDM-challenged A/J mice during the peak of inflammation (Fig. 3A), as well as during the resolution phase (Fig. 3B and C).

3.3. Glucocorticoids inhibit lung infiltration of Tregs on HDM challenged A/J mice

As glucocorticoids are mostly thought to increase Treg cell numbers (John et al., 1998; Karagiannidis et al., 2004; Peek et al., 2005), we next sought to test a different glucocorticoid drug and a different treatment approach (protocol 1). HDM-challenged A/J mice showed increased numbers of CD4+CD25+Foxp3+ and CD4+IL10+ both in the airways (Fig. 4A and C) and lung tissue homogenates (Fig. 4B, D and Fig. S1). Dexamethasone pre-treatment (protocol 1) as well as dexamethasone (protocol 2) or budesonide treatment performed on the last week of challenge inhibited Treg numbers both in BALF and digested lung (Fig. 4). Budesonide vehicle had no effect on HDM challenged mice (data not shown).

3.4. Glucocorticoids fail to increase lung secretion of IL-10 on HDM challenged A/J mice

Studies have shown that glucocorticoid treatment increases Treg activity, by enhancing IL-10 secretion (John et al., 1998; Peek et al., 2005). On A/J mice challenged with HDM, neither dexamethasone nor budesonide treatments increased IL-10 production in lungs (Fig. 5). In fact, dexamethasone pre-treatment (protocol 1) actually...
reduced secretion of IL-10 in lungs of HDM-challenged mice (Fig. 5), suggesting that glucocorticoids might reduce Treg cell activity.

3.5. Glucocorticoids decrease production and circulation of Treg on HDM-challenged A/J mice

Because glucocorticoids have been described to inhibit lymphocyte migration (Baschant and Tuckermann, 2010; Schweingruber et al., 2014), we sought to study if Treg numbers were decreased in the lungs because they were accumulated in lymphoid organs such as the thymus and lymph nodes. Allergic challenge did not change Treg counts in the thymus (Fig. 6A) or lymph nodes (Fig. 6B), compared to PBS, 1 day after the last challenge. Oral dexamethasone, as well as nebulized budesonide, led to a reduction in CD4⁺CD25⁺Foxp3⁺ Treg numbers in thymus (Fig. 6A) and lymph nodes (Fig. 6B). These results suggest that glucocorticoids can inhibit Treg cell production and circulation in peripheral lymphoid organs.

HDM challenge did not change size or cell count of thymuses, compared to PBS (Fig. 7). Both dexamethasone protocols, as well as the inhaled budesonide treatment, reduced thymuses weight (Fig. 7A) and cell counts (Fig. 7B), suggesting that different glucocorticoid treatment strategies might affect thymus structure in lung allergic inflammatory models.

3.7. Glucocorticoids inhibit lung infiltration of Treg cells post-HDM challenge on BALB/c mice

As expected, HDM challenge of BALB/c mice induced an increase in Treg cell numbers in the airways (Fig. 8A) and lungs (Fig. 8B), 1 day after the last challenge. Corroborating the results obtained with A/J mice, nebulized budesonide and oral dexamethasone treatment (protocol 2) also decreased Treg cell numbers in the airways (Fig. 8A) and in lung digests (Fig. 8B) of HDM-challenged BALB/c mice. These results suggest that glucocorticoids inhibit lung infiltration of Treg cells post-HDM challenge independent of the mouse strain studied.

3.8. Glucocorticoids inhibit lung infiltration of Treg on A/J mice challenged with OVA

OVA sensitization and challenge also induced an increase in Treg cell numbers in the airways (Fig. 8C) and lungs (Fig. 8D) of A/J mice, compared to PBS-challenged mice at 1 day after the last challenge. As seen with HDM-challenge, dexamethasone oral treatment (protocol 2) and nebulized budesonide decreased Treg cell numbers in the airways (Fig. 8C) and lung digests (Fig. 8D) of OVA-challenged A/J mice. These results suggest that glucocorticoid treatment inhibits lung infiltration of Treg on A/J mice independent of the allergen used to induce the lung inflammation.
4. Discussion

Tregs have been arousing interest as target cells for the improvement of asthma symptoms; therefore it is crucial to understand the effect of glucocorticoid treatment on these cells in mouse models of allergic lung disease. This study investigated the effect of oral dexamethasone or nebulized budesonide on lung Tregs from allergen-challenged mice. We reported that glucocorticoids inhibit...
lungs and airways hyperresponsiveness of A/J mice challenged with HDM at the peak of inflammation, 1 day after the last challenge, and during the resolution phase, especially 7 days after the last challenge. Glucocorticoid treatment, administered systemically or topically, decreases the levels of Tregs in the lungs of allergenic-challenged mice, independent of mouse strain or allergen type used. In the experimental models tested here, reduction on numbers of pulmonary Tregs induced by glucocorticoid might be due to a decrease in the production of lymphocytes in the thymus. These findings support the interpretation that glucocorticoid treatment may not favor the increase of Treg cell numbers or activity in asthma.

Different mice strains have genetic variations that influence respiratory mechanics and airway hyperresponsiveness (Ewart et al., 2000; Held and Uhlig, 2000; Reinhard et al., 2002; Tankersley et al., 1999). Genetic and functional studies have shown that A/J mice are prone to allergic lung in 1999). Genetic and functional studies have shown that A/J mice are prone to allergic lung in 1999). Genetic and functional studies have shown that A/J mice are prone to allergic lung inflammation, since they can regulate immune responses through colinergic stimulation (Antunes et al., 2009; De Sanctis et al., 1995; Ewart et al., 1996; Wills-Karp and Ewart, 1997). We studied a lung allergic inflammatory model in A/J mice by challenging these animals with one of the most relevant allergens in the clinical setting, house dust mite (Nelson et al., 1996). HDM A/J-challenged mice presented several features of severe asthma, such as lung inflammation and AHR, as well as mucus production and collagen deposition (data not shown), reinforcing the importance of this model for pre-clinical studies. Dexamethasone oral administration two weeks after the first HDM challenge, when inflammation was already present, inhibited lung inflammatory cell infiltration as well as the AHR at the peak of inflammation, as previously reported (Olsen et al., 2011; Ulrich et al., 2008).

GC had been known to accelerate the resolution of inflammation through diverse mechanisms (Brannan, 2010; Hutchinson et al., 2011). In our asthma mouse model, allergen induced inflammation and AHR decrease during the resolution phase, 7 and 14 days post-challenge. As expected, dexamethasone oral treatment administered during the last week of HDM challenge accelerated the natural resolution of inflammation, noted by a reduction of the eosinophil anti-inflammatory influx and AHR. Others found that GCs may improve resolution of inflammation by increasing Treg numbers and their anti-inflammatory activity (Chen et al., 2006; Karagiannidis et al., 2004). Leech et al. showed that Tregs migrate to lungs and draining lymph nodes after the allergenic challenge and reach their peak in number just before the resolution starting point (Leech et al., 2007). Tregs might play a pivotal role in resolution of lung allergic inflammation, since they can regulate immune responses through cell-to-cell contact or secretion of immunosuppressive cytokines, such as IL-10 (Kearley et al., 2005; Lewkowich et al., 2005; Zhang et al., 2014). Surprisingly, we have seen that dexamethasone oral treatment, although accelerating the resolution of inflammation, reduced Treg numbers in the lungs and lymph nodes of HDM-challenged mice, contradicting other studies (Hartl et al., 2007; Karagiannidis et al., 2004). Interestingly, there is a large heterogeneity in the characterization of Tregs, since most groups characterize these cells only by the expression of CD4 and higher expression of CD25, while other groups search only for the indirect presence of Tregs by testing IL-10 production or Foxp3 expression (Hartl et al., 2007; Stelmach et al., 2002). Although there are several additional surface markers used to identify Tregs, the best way to define natural Tregs is probably to analyze the expression of Foxp3 in CD4+CD25+ cells (Sakaguchi et al., 2010; Wieckiewicz et al., 2010). Furthermore, Treg cell populations have been analyzed on blood samples instead of relevant inflammatory sites for asthma, such as the lungs (Karagiannidis et al., 2004). The present study demonstrated that GCs decrease the numbers of Treg cells, characterized by expression of CD4, CD25 and Foxp3, in the BALF and lung digests of allergen-challenged mice.

Aside from the thymus-derived CD4+CD25+Foxp3+ Treg cells, other T regulatory cells also play a role in maintaining peripheral tolerance to airway allergens. These cells are known as adaptive or induced Tregs (iTreg) because they are induced in the periphery. iTregs are capable of secreting high levels of anti-inflammatory IL-10 and TGF-β which might be important in the protection against asthma (Hawrylowicz and O’Garra, 2005). Some studies have shown that GCs increase IL-10 production (John et al., 1998; Stelmach et al., 2002), we observed that lung secretion of IL-10 was reduced by the dexamethasone pre-treatment and unaltered by dexamethasone and nebulized budesonide treatment that started two weeks after the first HDM challenge. These results suggest that dexamethasone treatment can impair IL-10 secreting Treg quantity and activity in murine models of asthma.

Although we have used two different steroid drugs through distinct administration routes, budesonide and dexamethasone were both able to decrease Treg levels in lungs of allergen-challenged mice. In addition, dexamethasone was tested in two distinct protocols, one beginning at the same time as the first HDM challenge and the other starting on the third week of antigen challenge, when lung inflammation was already established. Notably, both dexamethasone protocols were able to decrease Treg lung infiltration. These results are in line with a prior study, which reported reduced Treg cell numbers in spleen and blood of C57BL6 normal mice after dexamethasone intraperitoneal treatment (Sbiera et al., 2011). Sbiera et al. demonstrated that the decrease of Treg cell population induced by GC therapy is not dose dependent. It is noteworthy that the inhaled budesonide used in our study, which is used clinically for the treatment of asthma, had the same effects as oral dexamethasone. Nonetheless, the daily dose of budesonide or dexamethasone administered to mice in our study is lower than the amount generally used by asthmatic patients (Powell and Gibson, 2003; Szefler et al., 2002). Therefore, it seems possible that continuous GC treatment in humans also reduce lung Treg cell numbers.

It is known that inhaled GC in asthma causes fewer side effects compared to oral GCs (Hagan et al., 2014). We showed that nebulized budesonide treatment, as well as oral dexamethasone treatment, reduced thymic size, thymic cell counts and amount of Tregs in the thymus and lymph nodes. These results indicate that GCs inhibit Treg thymic production regardless of the route of administration. It is likely that the decrease in Treg cell numbers observed in the lungs of allergenic-challenged mice was due to the immunosuppressive effects of GCs in the thymus.

Our observations demonstrate that systemic or localized GC treatment can impair Treg quantity and activity in lungs of allergenic-challenged mice, independent of mouse strain or type of allergen tested. As Tregs are important target cells for the treatment of asthma, it is relevant to carefully analyze the effect of in vivo treatment with GCs on these cell populations in lungs of asthmatic patients.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2014.11.034.