

The role of IL-33/ST2, IL-4, and eosinophils on the airway hyperresponsiveness induced by *Strongyloides venezuelensis* in BALB/c mice

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Abstract Strongyloidiasis is a neglected chronic nematode infection, in which the control of autoinfection rate and severity of disease is dependent on type 2 immune responses. *Strongyloides* also causes Th2 responses in the lung of infected animals and changes in airway function, including airway hyperresponsiveness (AHR). Mechanisms of AHR during *Strongyloides venezuelensis* infection are not entirely known, and we investigate here the role of IL-4, eosinophils, and IL-33/ST2. AHR was evaluated in infected mice by determining changes in lung function after increasing doses of methacholine. Balb/C, but no C57Bl/6, mice developed AHR, tissue eosinophilia, and increased local IL-4 and IL-5 production. Functional changes peaked at day 4 and 7, after the larva had left the lungs. AHR was clearly dependent on IL-4 but not on eosinophils, as evaluated by experiments in IL-4 and Gata-1-deficient mice. Experiments in ST2-deficient mice showed that this pathway was not needed for induction of AHR but was necessary for the maintenance of AHR and for Th2 responses in the lung. These studies clearly show a crucial role for IL-4 in the induction of AHR following *S. venezuelensis* infection and for IL-33/ST2 in maintaining AHR and lung Th2 responses.

Keywords *Strongyloides venezuelensis* · Airway hyperresponsiveness · IL-33/ST2 pathway · IL-4 and eosinophils

Introduction

Nematode parasites infect over one billion people worldwide, causing great morbidity, increased susceptibility to other infections, and, in some cases, death (Hotez et al. 2008; McCarty et al. 2014). Among these nematodes, *Strongyloides stercoralis* afflicts 30 to 100 million people in 70 countries, primarily in tropical and subtropical regions of the world (Siddiqui and Berk 2001; Puthiyakunnon et al. 2014). Nematode infections induce a predominantly type 2 immune response that results in eosinophilic inflammation, differentiation and activation of goblet cells, alternatively activated macrophage and mast cells, and increased production of IgE and IgG₁. These changes are associated with host protection in many experimental models (Urban et al. 1992; Allen and Maizels 1996; Finkelman et al. 1997; Onah and Nawa 2000; Patel et al. 2009), including *Strongyloides venezuelensis* infection in a mouse model (Negrão-Corrêa et al. 2006). However, the immune response induced by nematodes has also been associated with functional and physiological changes, including airway hyperresponsiveness (Hall et al. 1998; Palmer et al. 2002; Silveira et al. 2002), increased mucus production and secretion (Tomita et al. 2000; Khan and Collins 2004), increased fluid secretion and mucosal permeability (Cliffe et al. 2005), and enhanced intestinal propulsive activity (Khan and Collins 2004; Zhao et al. 2003). These physiological changes contribute to nematode control but also to morbidity by intensifying symptoms, such as diarrhea,

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weight loss (Kobayashi and Horii 2008), wheezing, or lung functional disorders (Alcântara-Neves et al. 2010; Walsh 2011). The mechanisms responsible for the physiological changes induced during nematode infections are not well established; however, in some experimental models, these changes are also associated with the induction of the type 2 immune response (Zhao et al. 2003; Khan and Collins 2004).

Experimental infection with *S. venezuelensis*, a nematode that naturally infects wild rats, is frequently used in experimental studies because its life cycle is well characterized and easily maintained in laboratory rodents. Similar to *S. stercoralis* infection in humans, the infective larvae of *S. venezuelensis* penetrate the skin and undergo an obligatory migration through the rodent lungs prior to establishment in the duodenal mucosa (Takamure 1995). In previous studies, we demonstrated that in infected rats, the majority of the parasite larvae pass through the lungs after 48 h and induce airway hyperresponsiveness (AHR) after 5 days. This AHR is coincident with the peak of lung eosinophilic inflammation, IgE production, and the production of mucus and proinflammatory cytokines (Silveira et al. 2002; Ferreira et al. 2007). In this experimental model, neutralization of IL-13 during nematode infection did not alter the induction of AHR, suggesting that this mechanism was IL-13 independent (Ferreira et al. 2010), which differs from the IL-13-dependent murine model of allergic rhinitis and asthma (Wynn 2003; Wang and McCusker 2005) and from other nematode infections, such as *Nippostrongylus brasiliensis* (Marsland et al. 2008). However, the rat model is not as efficient as the mouse model for the identification of the possible mechanisms associated with the physiological changes induced by *Strongyloides* infection because of the limited availability of adequate experimental tools. In light of these considerations, the objective of the present study was to evaluate whether lung functional alterations occurred in mice infected with *S. venezuelensis* and directly address the role of IL-33/ST2 activation pathway, IL-4, and eosinophils in the process.

Materials and methods

Animals

The experimental procedures received prior approval from the local animal ethics committee (protocols 104/2007 and 159/2012) and were in accordance with the ethical principles in animal research adopted by the Brazilian National Council on Animal Experimentation (CONCEA).

For the experiments described herein, 10–12-week-old female BALB/c and female C57BL/6 mice, acquired from an established colony at the university's mouse facility (Centro de Bioterismo (CEBIO), ICB UFMG, Brazil), were used. For some experiments, IL-4-deficient mice (IL-4^{-/-}), ST2

receptor-deficient mice (ST2^{-/-}), and Δ dblGATA mice, all on a BALB/c background, were also used to evaluate the role of IL-4 production, IL-33/ST2, and eosinophil activation in the functional alteration induced by *S. venezuelensis* infection, respectively. IL-4^{-/-} mice (Noben-Trauth et al. 1996) were obtained from the Oswaldo Cruz Foundation animal facility. ST2^{-/-} mice, animals deficient in the receptor that is activated by IL-33 binding (Townsend et al. 2000), were originally purchased from Jackson Immuno-Research Laboratories and kindly provided by Dr. João Santana (Faculdade de Medicina de Ribeirão Preto, USP, Brazil). The Δ dblGATA mice, animals that harbor a target deletion of the high-affinity GATA-binding site in the promoter for the transcription factor GATA-1 that resulted in selective loss of eosinophil lineage (Yu et al. 2002), were kindly donated by Dr. A. Humbles (Harvard, Boston, MA) to Dr. Mauro Martins Teixeira. During the experimental procedure, the animals were housed at the animal facility for infected animals of the Parasitology Department (UFMG, Brazil), fed with laboratory chow (Nuvilab®, Colombo, Parana, Brazil), and provided tap water ad libitum.

Parasite infection model

S. venezuelensis, a nematode that has an obligatory migration through the host lungs before establishment in the duodenal mucosa, was used in the experiments. The nematode was isolated from *Rattus norvegicus* and has been maintained in the Department of Parasitology of the UFMG by serial passage in Wistar rats.

For the experiments described herein, *S. venezuelensis* infective filiform larvae (L3) were obtained from vermiculite culture of infected-rat feces. The cultures were kept for 48–72 h at 28 °C, and the infective larvae were collected and concentrated using a Baermann apparatus. Subsequently, the recovered larvae were filtered, washed several times in phosphate-buffered saline (PBS), and counted, and their concentration was adjusted to 7000 L3/mL. For infection, each mouse was subcutaneously inoculated with 100 μ L of PBS containing the infective larvae (700 L3/mouse) in the abdominal region. Airway hyperresponsiveness was evaluated at 0, 2, 4, 7, and 10 days after infection, as described below. The lung, small intestine, and the feces were analyzed to determine parasite burden, as previously described by Negrão-Corrêa et al. (2004). For some infected animals, lung samples were collected for histopathological analyses and for cytokine and eosinophil peroxidase (EPO) measurements.

Evaluation of lung function

Changes in lung function were evaluated by the variation of intratracheal pressure after injection of increasing doses of a bronchial constriction agent as an indirect measurement of

lung resistance, which was based on the protocol established in rats (Silveira et al. 2002; Ferreira et al. 2007), with modifications detailed below. At different time points after parasite inoculation, 8–10 female mice were kept anesthetized by i.p. inoculation with a mixture of ketamine (80 mg/kg; Dopalen, Ceva Santé Animale—Paulinia/SP-Brazil) and xylazine (15 mg/kg; Kensol, König Brazil) solution, and the jugular vein and trachea were cannulated. A three-way connector was attached to the tracheotomy tube with two ports connected to a ventilator (Model 686, Harvard Apparatus Inc., South Natick, Massachusetts, USA; 10 mL of air/kg animal at a rate of 150 breaths/min) and one port connected to a pressure transducer (Physiological Pressure Transducer—Ohmeda). The smooth muscles of the mice were paralyzed with i.p. injection of 120 µg/mouse pancuronium bromide (Pancuron—Cristália-Itapira/SP, Brazil). Increasing doses (0.3, 0.9, 3, 6, 9, and 30 µg/mouse) of acetyl-β-methacholine chloride (Sigma Chemical) were injected through the jugular cannula, and the variation in the intratracheal pressure was used as an indirect measurement of lung resistance, as described by Silveira et al. (2002). The data are expressed as the percent increase in the intratracheal pressure compared to the baseline.

Histopathology

Lung (right lobe) samples were collected, fixed in 10 % buffered formalin, and embedded in paraffin, and 5-µm sections were prepared for histology. Sections of each tissue were stained with hematoxylin and eosin (H&E) to assess the overall inflammatory response.

Measurement of type 2 cytokines and eosinophil infiltration in pulmonary homogenates

Cytokines were quantified in homogenates from the lungs obtained by homogenizing 100 mg of the left lobe of the lung from BALB/c, C57BL/6, or ST2^{-/-}-deficient mice at 0, 4, and 7 days p.i. The tissue samples were prepared in a tissue homogenizer (Power General 125; Fisher Scientific, Pittsburgh, PA) in 1 mL of PBS containing 0.5 % of bovine serum albumin and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM of benzethonium chloride, 10 mM ethylene diamine tetra acetate, 20KI aprotinin A, and 0.05 % Tween 20). Each sample was then centrifuged for 10 min at 10,000×g, 4 °C. Cytokine production was measured in the supernatants, and the pellet was stored at -20 °C for EPO assays.

Concentrations of IL-4 and IL-5 were measured by the ELISA technique using commercially available kits (R&D Systems, Minneapolis, MN, EUA) according to manufacturer's instructions. Known concentrations of the recombinant

proteins were used to generate a standard curve to convert OD readings of samples to pg/mL.

The eosinophil peroxidase activity in lung homogenates of the experimental mice was estimated according to the protocol previously described (Strath et al. 1985) and modified in the laboratory (Silveira et al. 2002). Briefly, the pellet obtained from lung homogenized tissue was re-suspended in phosphate buffer (PBS, pH 7.4) containing 0.5 % hexadecyltrimethylammonium bromide, and the solution was submitted to three freeze-thaw cycles in liquid nitrogen. The homogenate was centrifuged (20 min at 3000×g, 4 °C), and the supernatant samples were added in equal volume to the substrate solution (1.5 mM of *o*-phenylenediamine (OPD) in 0.075 mM Tris-HCl buffer pH 8.0 containing 6.6 mM of hydrogen peroxide). After 30 min at room temperature, the reaction was stopped with 50 µL of 1 M of H₂SO₄. Reaction intensity was read at 492 nm on a microplate reader (Status-Labsystems Multiskan RC, Helsinki, Finland) and results shown as absorbance units.

Statistical analysis

The data are reported as the means and the standard error of the means (SEM). The means of the normally distributed data were compared using either Student's *t* test (two groups) or one-way analysis of variance (ANOVA). In the latter analysis, *P* values were assigned using the Student–Newman–Keuls test. Differences of *P* < 0.05 were considered significant.

Results

The migration of *S. venezuelensis* larvae through the lungs of BALB/c mice induced a transient inflammatory process that intensified at 4 days after infection (Fig. 1b), a time point in which parasite larvae had already left the lung and established in the intestinal mucosae. Cellular infiltration in lung was greatly reduced after 10 days after infection (Fig. 1c). Transient cellular infiltration after parasite migration was also observed in C57BL/6-infected mice (data not shown). In both, C57BL/6 and BALB/c mouse strains, subcutaneous infection with 700 L3 of *S. venezuelensis* leads to larvae migration through the lungs and development of adult worms in the small intestine. The greatest number of larvae was recovered from the host lungs at day 2 of infection in BALB/c- and C57BL/6-infected mice (Fig. 1d). All mice that were infected with 700 L3 of *S. venezuelensis* exhibited worms in the small intestine and eggs in the feces between 7 and 10 days after infection (Fig. 1e, f). However, worms from BALB/c mice produced a lower number of eggs that were eliminated earlier from the intestine than from C57BL/6 mice (Fig. 1e, f).

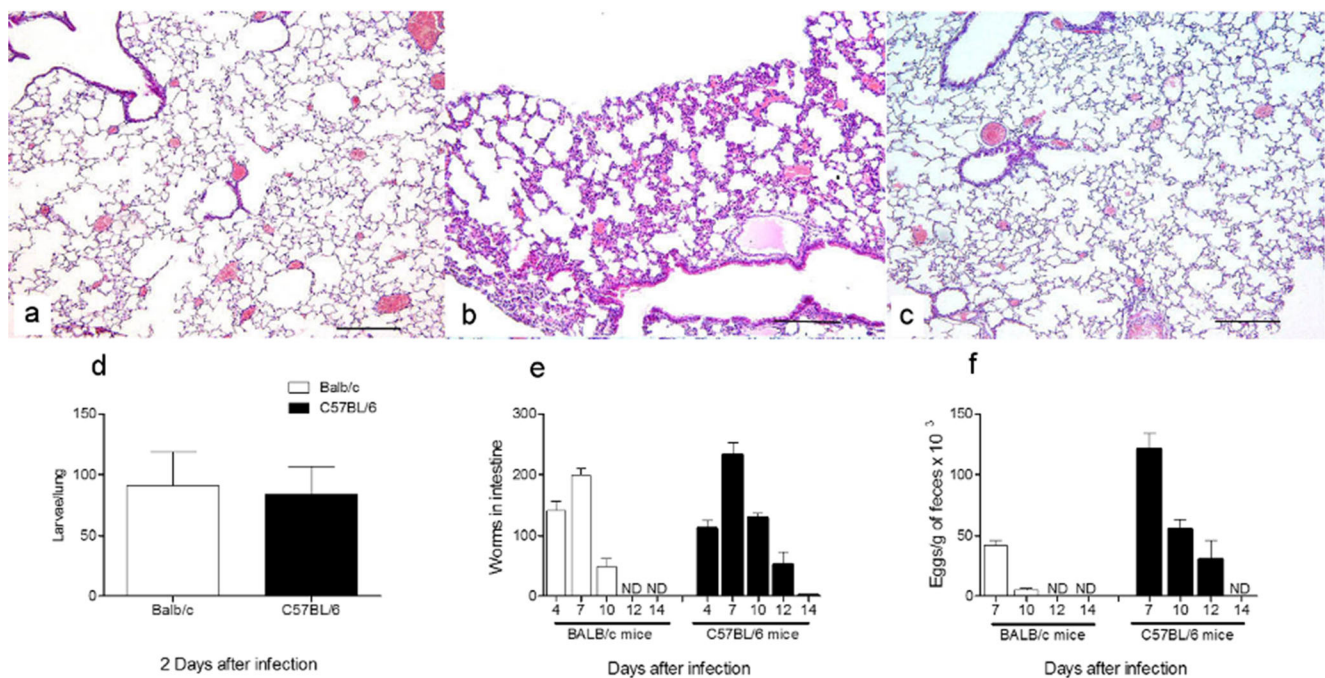


Fig. 1 Kinetic of *Strongyloides venezuelensis* infection and histopathological appearance of the lungs in experimentally infected mice. BALB/c or C57BL/6 mice were subcutaneously infected with 700 L3 of *S. venezuelensis*/mouse, and the lungs, small intestine, and feces of infected animals were collected to estimate parasite burden at different time after infection. At upper panel, lung sample of BALB/c mice at 0, 4, and 10 days after nematode infection were fixed with buffered formalin, embedded in paraffin, and 5- μ m sections were stained with H&E. Sections of lungs obtained from uninfected (a), 4-day infected BALB/c mice (b), and 10-day infected BALB/c mice (c).

Note the cellular infiltration and hemorrhage in lung of 4-day infected mice (b). Bars = 100 μ m. The lower panel showed the parasite burden and infection kinetic in BALB/c or C57BL/6 mice. In d, the average number of parasite larvae recovered in lungs of 2-day infected BALB/c and C57BL/6 mice. Adult worms from the small intestine and eggs eliminated in feces of BALB/c and C57BL/6 mice during the experimental infection are shown in e and f, respectively. ND no detected parasite in the sample. Each value represents the mean $\pm$ SEM of six mice per group, and the results are representative of three independent experiments

Hyperresponsiveness and lung inflammation in C57BL/6- and BALB/c-infected mice

To verify the functional alterations associated with parasite migration through the lungs of different strains of mice, we measured the variation of the intratracheal pressure in response to intravenous injection of increasing doses of methacholine. The results indicated that the response to methacholine injection in 2-day infected BALB/c mice was similar to that of uninfected mice. However, methacholine led to a leftward shift of the dose–response curve in 4–10-day infected BALB/c mice (Fig. 2a). The variation of the intratracheal pressure in response to the injection of 30 μ g of methacholine, which is the dose that induced the maximum response, clearly demonstrated a significant increase in the lung response at 4 days after infection (Fig. 2b). In contrast, airway responses to methacholine in infected C57BL/6 mice were low and not different from uninfected animals (Fig. 2c).

The airway hyperresponsiveness detected in *S. venezuelensis*-infected BALB/c mice was accompanied by increased EPO activity and increased concentration of IL-4 and IL-5 in lung homogenate during the infection (Fig. 3 (a1–a3)). In contrast, the concentration of IL-5 in lung

homogenate of C57BL/6 mice did not change during *S. venezuelensis* infection (Fig. 3 (b3)), and there was a significant reduction of EPO activity and IL-4 concentration at 4 and 7 days of infection, respectively (Fig. 3 (b1 and b2)). These results suggest that the mouse strain is determinant for the type of pulmonary inflammation induced by *S. venezuelensis* and consequently for airway hyperresponsiveness.

Participation of type 2 immune response in the hyperresponsiveness and lung inflammation induced by *S. venezuelensis* infection in mice

Since airway hyperresponsiveness was induced only in BALB/c mice and the functional alteration was accompanied by induction of a local type 2 response, we used IL-4 deficient and Δ dblGATA mice with the BALB/c background to directly evaluate the participation of IL-4 and eosinophils on lung inflammation and functional changes induced by *S. venezuelensis*. The lungs from 4-day infected BALB/c mice had several foci of inflammation at the pulmonary parenchyma and exhibited intense leukocyte infiltration, hemorrhage, pulmonary edema, and destruction of

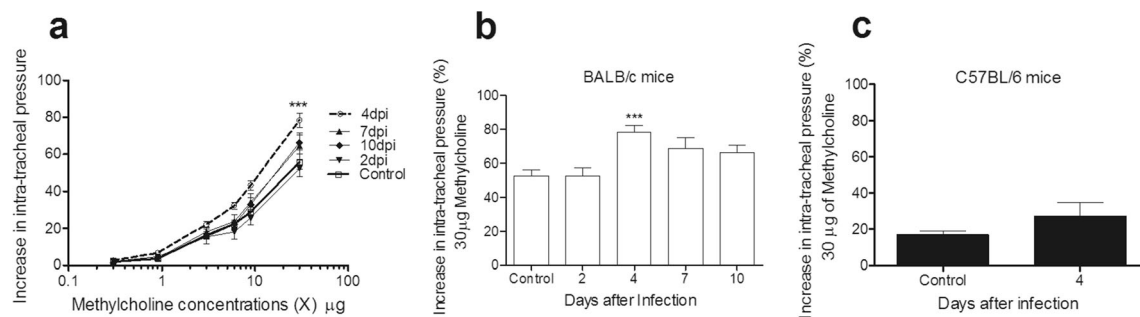


Fig. 2 Airway functional response during *Strongyloides venezuelensis* infection in different mouse strains. **a** Percent increase in the intratracheal pressure induced by intravenous injection of increasing doses (0.3–30 μg) of methylcholine in BALB/c mice during parasite infection. **b** Percent increase in the intratracheal pressure produced by the injection of 30 μg of methacholine in uninfected and 2-, 4-, 7-, and 10-day infected BALB/c

mice. **c** Percent increase in the intratracheal pressure produced by the injection of 30 μg of methacholine in uninfected and 4-day infected C57BL/6 mice. *** $P < 0.001$ compared with the airway response of uninfected mice of the same strain. Each value represents the mean $\pm$ SEM of 8–10 mice per group, and the results are representative of two independent experiments

the alveolar wall (Fig. 4a). *S. venezuelensis* migration also induced cellular infiltration and destruction of the alveolar wall in $\Delta\text{dblGATA}$ and $\text{IL-4}^{-/-}$ -infected mice (Fig. 4b, c). However, cellular infiltration in lung tissue of 4-day infected $\text{IL-4}^{-/-}$ or $\Delta\text{dblGATA}$ mice was basically composed by monocytes, and no eosinophils were found (Fig. 4 inserts).

As shown in Fig. 5, the increase of intratracheal pressure in response to methacholine detected in 4-day infected BALB/c mice was not detected in IL-4-deficient mice (Fig. 5a, b). However, the absence of eosinophils in $\Delta\text{dblGATA}$ mice did not alter the increase in intratracheal pressure induced by the parasite (Fig. 5c, d), indicating that lung functional

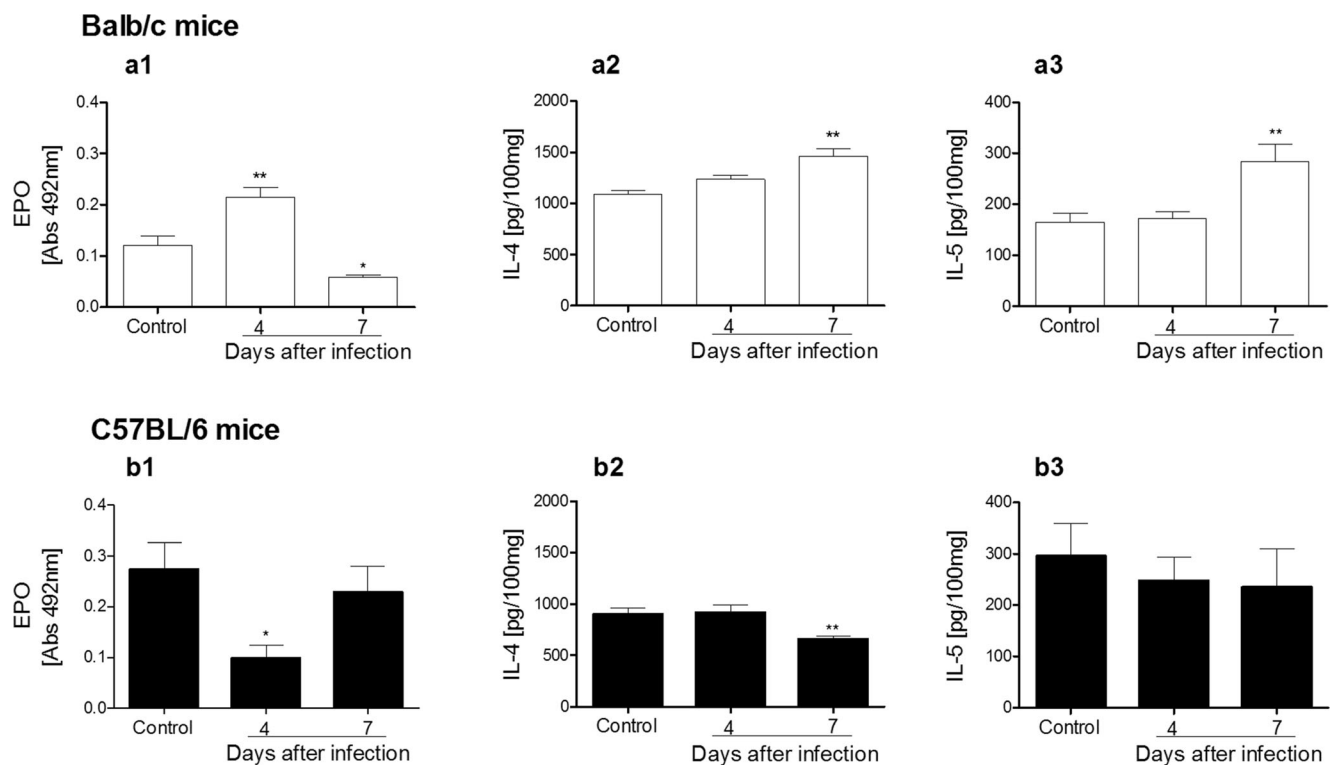


Fig. 3 Type 2 cytokines and eosinophil infiltration in lungs of BALB/c and C57BL/6 mice during *S. venezuelensis* infection. BALB/c or C57BL/6 mice were subcutaneously infected with 700 L3 of *S. venezuelensis*/mouse, and 100 mg sample of lung were collected, homogenated, and used to estimate cytokine concentration and EPO activity at 0, 4, and 7 days after the infection. Data from BALB/c mice are shown in panel a. Peroxidase of eosinophil activity (a1), IL-4 concentration (a2), and IL-

5 concentration in lung homogenate. C57BL/6 mice data are shown in panel b. Peroxidase of eosinophil activity (b1), IL-4 (b2), and IL-5 concentration (b3). ** $P < 0.01$ and * $P < 0.1$ compared with uninfected mice of the same strain. Each value represents the mean $\pm$ SEM of 8–10 mice per group, and the results are representative of two independent experiments

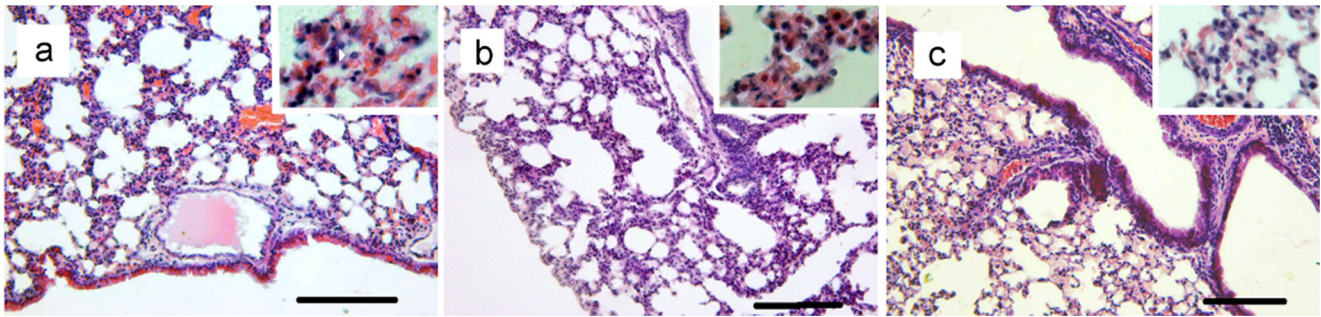


Fig. 4 Cellular infiltration in lungs of BALB/c, IL-4^{-/-} and ΔdblGATA at 4 days of *Strongyloides venezuelensis* infection. Mice were subcutaneous infected with 700 L3/mouse, and lung sample of 4-day infected mice was fixed with buffered formalin, embedded in paraffin,

and 5-μm sections were stained with H&E. Sections of lungs obtained from wild-type BALB/c mice (a), ΔdblGATA BALB/c mice (b), and IL-4^{-/-} BALB/c mice c. Note focal cellular infiltration and hemorrhage in all the lung samples. Bars = 100 μm. Arrow in insert a indicates eosinophil

alteration induced by *S. venezuelensis* is dependent on early IL-4 production but not related to the eosinophilia.

Experimental data have shown that IL-33/ST2 activation is an important mechanism of the innate immune response that has been associated with induction of type 2 immunity

(Townsend et al. 2000; Kondo et al. 2008; Kim et al. 2012). Therefore, we evaluated the relevance of the IL-33/ST2 pathway for the airway hyperresponsiveness induced by the nematode infection. Infection of ST2^{-/-} mice induced a significant increase in airway response to methacholine that was

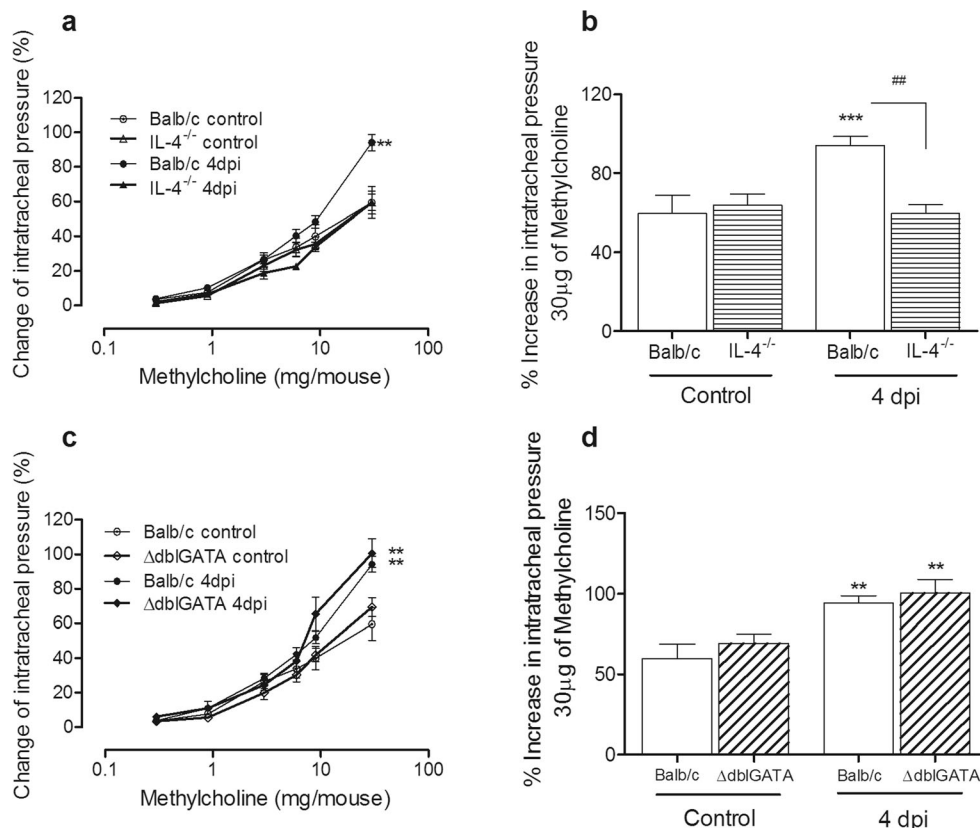


Fig. 5 Effect of IL-4 and eosinophil in airway functional response induced by *Strongyloides venezuelensis* infection in BALB/c mice. **a** Percent increase in the intratracheal pressure induced by intravenous injection of increasing doses (0.3–30 μg) of methacholine in uninfected and 4-day infected BALB/c mice or IL-4-deficient BALB/c mice. **b** Percent increase in the intratracheal pressure produced by the injection of 30 μg of methacholine in uninfected and 4-day infected BALB/c mice or IL-4-deficient BALB/c mice. **c** Percent increase in the intratracheal pressure induced by intravenous injection of increasing doses (0.3–30 μg) of methacholine in uninfected and 4-day infected BALB/c mice

or eosinophil-deficient BALB/c mice (ΔdblGATA). **d** Percent increase in the intratracheal pressure produced by the injection of 30 μg of methacholine in uninfected and 4-day infected BALB/c mice or ΔdblGATA BALB/c mice. ****P* < 0.001 and ***P* < 0.01 compared with the airway response of uninfected mice of the same strain. ##*P* < 0.01, comparing data from BALB/c mice and IL-4^{-/-} BALB/c mice at the same time point. Each value represents the mean ± SEM of 8–10 mice per group, and the results are representative of two independent experiments

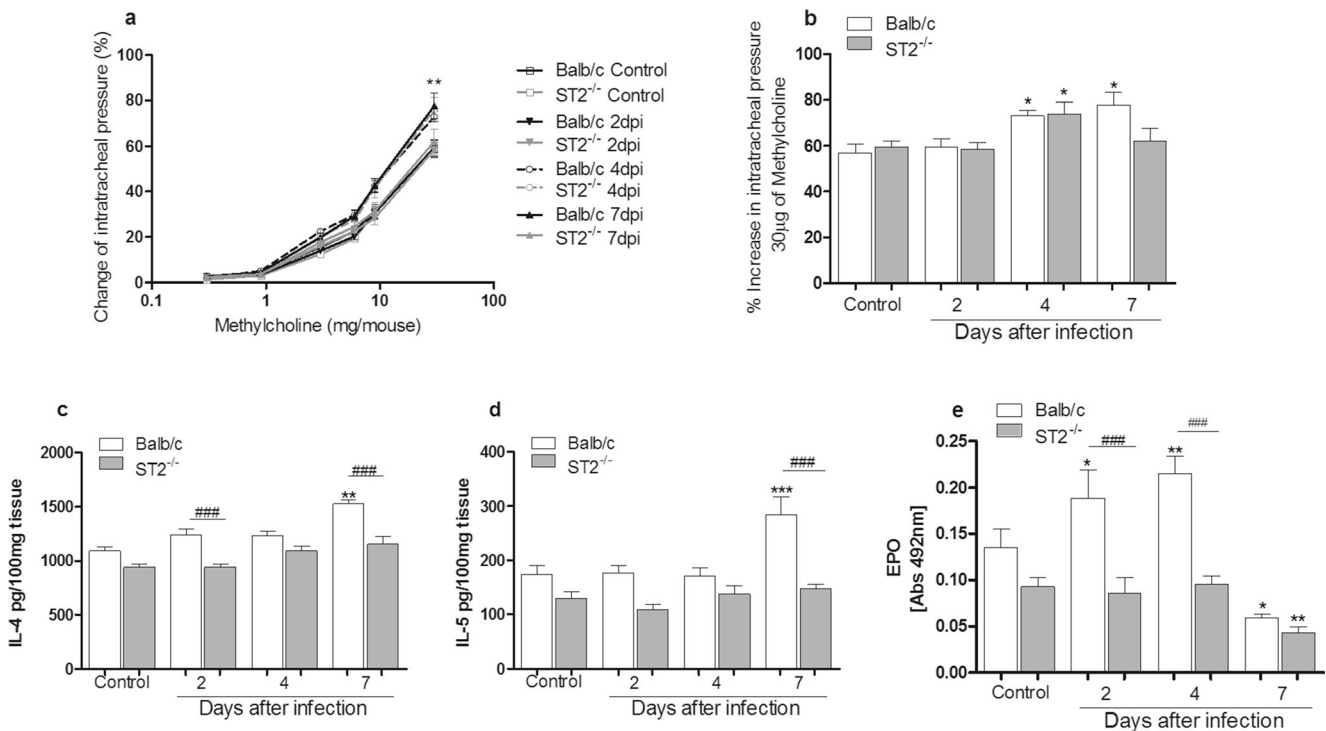


Fig. 6 Effect of IL-33/ST2 pathway in airway functional response and pulmonary inflammation induced by *Strongyloides venezuelensis* infection. **a** Percent increase in the intratracheal pressure induced by intravenous injection of increasing doses (0.3–30 µg) of methacholine in uninfected and 4-day infected BALB/c mice or ST2^{-/-} BALB/c mice. **b** Percent increase in the intratracheal pressure produced by the injection of 30 µg of methacholine in uninfected and 4-day infected BALB/c mice or ST2^{-/-} BALB/c mice. Concentration of IL-4 (c), IL-5

(d), and eosinophil peroxidase activity (e) in lung homogenate of BALB/c mice or ST2^{-/-} BALB/c mice during *S. venezuelensis* infection. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.1$ compared with uninfected mice of the same strain. ### $P < 0.001$, comparing data from BALB/c mice and ST2^{-/-} BALB/c mice at the same time point. Each value represents the mean $\pm$ SEM of 8–10 mice per group, and the results are representative of two independent experiments

comparable to that observed in WT-infected mice. However, the lung functional change induced by the infection was reversed at 7 days of infection in ST2^{-/-} but not WT mice (Fig. 6a, b). The absence of IL-33/ST2 activation also affected the local increase in IL-4 and IL-5 concentration after *S. venezuelensis* migration through the host lung and blocked the eosinophil infiltration and/or activation to this tissue (Fig. 6c–e).

Discussion

This experimental work demonstrates that primary infection with *S. venezuelensis* in BALB/c mice induced AHR between 4 and 7 days after infection, a time point at which the parasite larvae have already left the host lung and the eosinophilic inflammatory response was more intense. These data are similar to our previous studies in rats (Silveira et al. 2002; Ferreira et al. 2007). In contrast to BALB/c mice, C57BL/6 mice exhibited a weak lung response to methacholine, and *S. venezuelensis* did not significantly alter the lung functional response at day 4 in C57BL/6 mice. As seen in Fig. 2a, this is the peak of AHR in BALB/c mice. Moreover, C57BL/6 mice were more susceptible to *S. venezuelensis*, showing a higher

number of adult worms in the intestine and parasite eggs in the feces of the mice. In a previous study (Ferreira et al. 2007), we demonstrated that *S. venezuelensis*-induced AHR did participate in early host control of the parasite. Indeed, the previous data showed that decreased pulmonary functional responses produced by salbutamol treatment during parasite migration throughout the rat lungs appeared to favor parasite survival and increased host susceptibility to infection (Ferreira et al. 2007). In addition to the absence of airway reactivity, lung homogenates of C57BL/6 mice showed no increase in the concentration of type 2 cytokines and eosinophils after the nematode infection. Lower lung response detected in C57BL/6 mice would also favor parasite migration throughout the host lungs allowing that a larger number of worms reach the intestine.

Our experimental data demonstrated for the first time differences in the lung inflammatory and functional responses between BALB/c and C57BL/6 during nematode infection. Experimental studies reporting nematode-induced AHR in C57BL/6 mice were done only in immunized and challenged animals (Hall et al. 1998; Mehlotra et al. 2001). However, models of asthma have previously demonstrated that the genetic background of mice and duration of allergen exposure

have profound impact on lung responses (Gueders et al. 2009; Hirota et al. 2009; Van Hove et al. 2009). For example, Gueders et al. (2009) showed that ovalbumin (OVA)-induced asthma in BALB/c mice resulted in a stronger production of type 2 cytokines in the lungs, tissue infiltration of mast cells, and greater levels of airway reactivity to methacholine than in C57BL/6 mice. Van Hove et al. (2009) and Hirota et al. (2009) demonstrated that OVA sensitization and brief intranasal challenge led to Th2-driven inflammation in both C57BL/6 and BALB/c mice. However, changes in airway structure and hyperresponsiveness after allergen challenge were restricted to BALB/c mice. Moreover, the authors reported that even PBS-treated C57BL/6 mice were less responsive to methacholine than BALB/c mice (Van Hove et al. 2009). A more detailed evaluation showed that in BALB/c mice, but not in C57BL/6, IL-13 production led to phosphorylation of STAT6 briefly after allergen exposure and later phosphorylation of SMAD2, a downstream element of TGF- β 1 pathway, that would be responsible for differences in lung responsiveness observed in the mouse strains (Hirota et al. 2009). Webb et al. (2004) identified a polymorphism in IL-4R α that alters the avidity of the receptor to IL-4 among different mouse strain that may also contribute to the differences in lung response of C57BL/6 and BALB/c. Therefore, this is the first report of changes in airway responses between strains in a model of nematode infection, and these changes are in overall agreement with those of the literature in asthma, i.e., BALB/c mice tend to have more changes in airway function than C57BL/6 mice.

Airway hyperresponsiveness has also been reported after primary infection by *N. brasiliensis* (Coyle et al. 1998; Marsland et al. 2008) and *Toxocara canis* in mice (Pinelli et al. 2007). Mice that were primary infected with *Brugia malayi* (Cadman et al. 2014) or immunized with soluble antigen and challenged with live microfilaria also developed AHR (Hall et al. 1998; Mehlotra et al. 2001). Nematode-induced airway hyperresponsiveness was accompanied by pulmonary inflammation with eosinophil accumulation and mucus secretion, similar to the lung inflammation reported in allergic asthma models. Investigations in animal models and in asthmatic patients indicate a crucial role of type 2 cytokines in allergic airway inflammation and hyperresponsiveness. Studies have shown the direct effect of IL-4 production on muscle cells (Moore et al. 2002) or the indirect effect of IL-4 and/or IL-13 production on mucus production (Zhu et al. 1999; Wills-Karp et al. 1998), mast cells (Brightling et al. 2002; Carroll et al. 2002), and eosinophil stimulation (Teixeira et al. 1995; Humbles et al. 2004; Lee 2004; Wills-Karp and Karp 2004). The crucial role of IL-13 in airway hyperresponsiveness has been experimentally confirmed in protocols of murine asthma (Walter et al. 2001; Shore 2004; Kasaian and Miller 2008). However, the role of the immune response on airway hyperresponsiveness induced by nematode infection is not

well established. In a previous study, we demonstrated that neutralization of IL-13 with antibodies did not interfere with the induction of hyperresponsiveness in *S. venezuelensis*-infected rats (Ferreira et al. 2010). The establishment of a mouse model capable of detecting airway responses during *S. venezuelensis* infection was an important step for mechanistic studies as far more tools are available to investigate the role of particular cytokines or pathways in mice. The data of the present work clearly show that the increase in airway response observed in infected BALB/c mice is dependent on IL-4 production but independent of eosinophils. Coyle et al. (1998) also reported a non-essential role of eosinophils for *N. brasiliensis*-induced AHR in primary-infected mice. The authors demonstrated that the absence of IL-5 production in mice experimentally infected by *N. brasiliensis* resulted in abrogation of eosinophil infiltration in the mice lung, but the AHR was unimpaired (Coyle et al. 1998). It is important to notice that, differently from the transient AHR induced by *S. venezuelensis*, *N. brasiliensis*-infected mice also developed chronic pulmonary damage that persists longer after worm elimination and the reduction of inflammatory cell infiltration. The chronic lung lesion induced by *N. brasiliensis* infection was accompanied by airway hypersensitivity that persisted up to 300 days after infection, and the physiological change was dependent on IL-13 production and modulated by IFN- γ (Marsland et al. 2008). In contrast to our studies or in *N. brasiliensis*-induced AHR, PHIL and EPO^{-/-} mice showed reduction in airway response after *B. malayi* infection, indicating that eosinophils participate in lung dysfunction induced by the *Brugia* infection (Cadman et al. 2014). Filarial-induced AHR in immunized mice was also dependent on IL-4, IL-5, and modulated by IFN- γ (Hall et al. 1998; Mehlotra et al. 2001). These data suggest that mechanisms responsible for nematode-induced AHR would vary with the parasite species and time of infection.

In *S. venezuelensis*-infected BALB/c mice, AHR was dependent on IL-4 production, occurred early in infection, and it was transient, suggesting that *S. venezuelensis*-induced AHR might occur in the absence of Th2 cells and adaptive immunity; therefore, innate immune cells and their cytokines would have a role in the process. Recent data has shown that innate lymphoid cells and IL-33 and/or IL-25 production would be involved in some forms of asthma that occurs independently of Th-2 establishment, such as airway hyperresponsiveness triggered by air pollution, stress, and infection (Matangkasombut et al. 2009; Kim et al. 2012). IL-33 is a member of the IL-1 family of cytokines that is constitutively expressed in the nuclei of airway epithelial and endothelial cells that can be activated and secreted after tissue injury. The bioactive form of IL-33 binds to the IL-33 receptor (also called ST2) and promotes differentiation and expansion of local Th2 cell responses, influencing DC maturation and activity and recruiting Th2 cells to lymph nodes and tissue

(Coyle et al. 1999; Nakae et al. 2013). The role of IL-33/ST2 activating pathway in the induction of AHR was directly demonstrated after intranasal inoculation of IL-33 in WT or RAG2^{-/-} mice. Treatment with IL-33, in both WT and RAG2^{-/-} mice, resulted in lung accumulation of IL-5 and IL-13-producing innate cells, eosinophil recruitment, and AHR (Kondo et al. 2008; Ikutani et al. 2012). Moreover, IL-33 was essential for manifestation of OVA-induced allergic airway inflammation, including cellular infiltration in lungs, especially eosinophils, and airway hyperresponsiveness (Oboki et al. 2010). The participation of IL-33/ST2 pathway in the pathogenesis of asthma was also demonstrated in humans. Recent studies demonstrated increased expression of IL-33 in bronchial epithelium cells and airway smooth muscle cells of asthmatic patients (Préfontaine et al. 2009), a large concentration of this cytokine in bronchoalveolar lavage fluid (BALF) of asthmatic patients following an allergen challenge (Fux et al. 2013), and a strong association between polymorphisms in genes for IL-33 or its receptor (ST2) and asthma susceptibility and severity (Savenije et al. 2014; Bønnelykke et al. 2014). In contrast to the asthma models, our data clearly showed that IL-33/ST2 activation was not necessary for the early (day 4) AHR response induced by *S. venezuelensis* in mice. However, activation of ST2 receptor was essential for eosinophil recruitment and increased concentration of IL-4 and IL-5 in lung homogenates during *S. venezuelensis* infection. The participation of IL-33 production in lung eosinophilic inflammation observed in *S. venezuelensis*-infected mice was also demonstrated by Yasuda et al. (2012), using IL-33-deficient mice. Even though the IL-33/ST2 signal pathway was not necessary to induce AHR in 4-day infected mice, the airway response returned to basal level at 7 days of infection in ST2^{-/-} but not in WT BALB/c-infected mice. The results indicate that IL-33/ST2 activation is essential for induction of lung TH-2 response and to prolong the AHR induced by *S. venezuelensis* infection.

The early cell source of IL-4 and the mechanism induced by this cytokine that result in *S. venezuelensis*-induced AHR was not determined. However, recent experimental work (Wehrmann et al. 2016) demonstrated that $\gamma\delta$ T cells isolated from the alveolar space are an important source of IL-4 in LPS-induced acute lung injury. This cell population produces IL-4 as early as 1 day after LPS stimulation, and the early production of IL-4 modulates TNF- α production by alveolar macrophages, decreasing activation of proinflammatory M1 macrophages in the lung and the tissue injury. The early production of IL-4 in the lung would alter muscarinic receptor expression and/or function on neuron and smooth muscle cells, affecting muscle contractility (Akiho et al. 2002; Zhao et al. 2003; Horsnell et al. 2007; Darby et al. 2015). Moreover, experimental data demonstrated that IL-4/IL-13 induces AHR in mice that express IL-4R α only on smooth muscle cells,

suggesting that these cytokines would act directly on muscle to induce hyperresponsiveness (Perkins et al. 2011).

In summary, the strain of mice used was determinant for the airway hyperresponsiveness induced by *S. venezuelensis* infection. Moreover, nematode-induced AHR depends on innate production of IL-4, and IL-33/ST2 activation was essential to stimulate type 2 inflammatory response in lungs of BALB/c-infected mice and to maintain parasite-induced AHR.

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Authors' contribution Conceived of or designed the study: ESA, CAJP, MMT, and DNC

Performed parasitological and histopathological experiments: ESA, CAJP, JRL, MCR, JMPM, and DNC.

Optimized the methodology and performed AHR in infected animals: ESA, CAJP, ATMP, and MMT

Analyzed data: ESA and DNC

Wrote the manuscript: ESA, MMT, and DNC

The authors read and approved the final manuscript.

Compliance with ethical standards

Competing interest The authors declare no conflicts of interest.

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