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Cohabitation with a sick partner increases allergic lung inflammatory response in mice

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ABSTRACT

The bidirectional relationship between the nervous system and the immune system is relevant for homeostatic organism maintenance. Studies from our laboratory showed that 14 days of cohabitation with a sick partner (injected with Ehrlich tumor cells-TAE) produced behavioral, neurochemical, endocrinological and immunological changes. This study analyzes the effects of cohabitation with an Ehrlich tumor-bearing animal on ovalbumin (OVA)-induced lung inflammatory response in mice. Pairs of male mice were divided into three groups: naïve, control and experimental. Animals of the naïve group were kept undisturbed being used for the assessment of basal parameters. One animal of each experimental and control pair of mice was immunized with OVA. On $ED_{(0)}$, these OVA-immunized animals received an OVA booster. At this day ($D_{(0)}$) the experimental mice that were kept undisturbed were inoculated with 5×10^6 Ehrlich tumor cells; their immunized cage-mates were then referred as to CSP ("companion of sick partner"). The undisturbed mice of each control pair were i.p. treated on $D_{(0)}$ with 0.9% NaCl; their sensitized cage-mates were subsequently referred as CHP ("companion of health partner"). The OVA challenge was performed on CSP and CHP mice on $ED_{(12)}$ and $ED_{(13)}$; blood and tissue collection were performed on $ED_{(14)}$. Fourteen days after cohabitation, in comparison to the CHP mice, the CSP mice displayed the following: (1) an increased number of eosinophils and neutrophils in the BAL, (2) a decreased bone marrow cell count, (3) increased levels of IL-4 and IL-5 and decreased levels of IL-10 and IFN-γ in the BAL supernatant, (5) increased levels of IgG1-OVA, decreased levels of IgG2a-OVA and no changes in OVA-specific IgE in the peripheral blood, (6) increased expression of L-selectin in the BAL granulocytes, (7) decreased tracheal reactivity to methacholine measured *in vitro*, (8) no changes in plasma corticosterone levels and (9) increased levels of plasmatic noradrenaline. These results suggest that allergic lung inflammatory response exacerbation in CSP mice is a consequence of the psychological stress induced by forced cohabitation with the sick partner. Strong involvement of the sympathetic nervous system (SNS) through adrenaline and noradrenaline release and a shift of the Th1/Th2 cytokine profile toward a Th2 response were considered to be the mechanisms underlying the cell recruitment to the animal's airways.

1. Introduction

The long-held view that homeostatic mechanisms are integrated by the nervous and endocrine systems has been expanded by information that these systems interact with the immune system (Ader et al., 1990; Dunn and Wang, 1995). Changes in cell-mediated immune function were reported in individuals undergoing distressing life experiences (Gold, 1988; Nagata et al., 1999). Stressors were reported to decrease immune/inflammatory responses (Elenkov et al., 1999; Costa-Pinto and Palermo-Neto, 2010) and to modulate cytokine and peptide production and release (Besedovsky et al., 1986; Theoharides et al., 1995; Elenkov and Chrousos, 1999a) among others, through Hypothalamic–Pituitary–Adrenal (HPA) axis or Sympathetic Nervous System (SNS) activations.

Ehrlich tumor cells were reported to elicit a strong host inflammatory/immune response (Segura et al., 1997; Palermo-Neto et al., 2003, 2008), a fact that, together with other properties, makes this

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tumor an interesting experimental model for the analysis of tumor growth. The data from our laboratory have shown that 14 days of cohabitation of mice with an Ehrlich tumor-bearing conspecific individual increased their locomotor activity within an open-field apparatus (Morgulis et al., 2004), decreased neutrophil and macrophage oxidative burst and phagocytosis in response to induction by myristate acetate or *Staphylococcus aureus* (Alves et al., 2006, 2007), decreased the animal's host resistance to tumor growth (Morgulis et al., 2004), decreased the levels and increased the turnover rate of noradrenaline within the hypothalamus (Alves et al., 2006), increased plasma levels of noradrenaline and adrenaline (Alves and Palermo-Neto, 2014) and induced no changes in corticosterone serum levels (Alves et al., 2006, 2012). Immune impairment has also been observed in companions of B16F10 melanoma-bearing mice (Tomiyoshi et al., 2009). These effects were discussed as being a consequence of the stress imposed by the housing condition. Indeed, it was shown that odor cues released by Ehrlich tumor-bearing mice are aversive to their partners, increasing their plasma levels of noradrenaline and adrenaline, consistent with a SNS activation hypothesis (Alves and Palermo-Neto, 2014).

The existence of interactions between emotional or psychopathological disorders and allergic and/or chronic diseases such as asthma is well established (Nagata et al., 1999; Vamos and Kolbe, 1999; Rietveld et al., 2000). Asthma is a multifunctional disease characterized by pulmonary cellular infiltration, plasma exudation and airway hyper-responsiveness, the latter of which is globally related to the toxic effects of mediators released into the lungs by alveolar macrophages, neutrophils, eosinophils and mast cells (Bochner and Busse, 2005). Studies concerning emotional impacts on immune responses usually employ animal models of aversive stimulation to evaluate an antigen-induced inflammatory response (Persoons et al., 1995; Costa-Pinto and Palermo-Neto, 2010). Airway inflammation induced by ovalbumin (OVA) aerosol challenge in OVA-sensitized animals mimicked some pathological characteristics of asthma, e.g., peribronchial edema and increased number of lymphocytes and eosinophils in the bronchoalveolar lavage fluid (Portela et al., 2001, 2002). These events were augmented by early-life psychological stress (Chida et al., 2007), unpredictable stressor procedures (Datti et al., 2002), foot shock stress (Portela et al., 2001, 2002) and chemically-induced stressors (Ligeiro de Oliveira et al., 2012; Stankevicius et al., 2012; Hamasato et al., 2013).

Stressors are known to shift the Th1/Th2 cytokine balance toward a Th2 cytokine profile, thus suppressing Th1 and upregulating Th2 responses (Elenkov and Chrousos, 1999b), and deregulation of the Th1/Th2 cytokine balance is a key to the pathogenesis of asthma and atopic diseases (Marshall et al., 1998; Ngoc et al., 2005). The over-production of Th2 cytokines results in the recruitment and activation of inflammatory mediators, including mast cells, basophils, and eosinophils, further resulting in airway obstruction (Finkelman et al., 2010). The present study was thus specially designed to analyze the effects of 14 days of cohabitation with an Ehrlich tumor-bearing conspecific on OVA-sensitized and challenged mice, considering the following: cell trafficking into the bronchoalveolar lavage fluid, Th1 and Th2 cytokine production, immunoglobulin plasma levels, adhesion molecule expression, *in vitro* tracheal reactivity, corticosterone serum levels and plasma catecholamine levels.

2. Materials and methods

2.1. Animals

Male Balb/c mice (25–35 g, 8 weeks) from our departmental facilities were used. The animals were maintained under a controlled temperature (22–24 °C) and a 12-h light/dark cycle with free access to food and water. The animals were housed in plastic cages and were handled and used in accordance with the guidelines of the Bioethical Committee for the Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine of the University of São Paulo, Brazil (protocol No.2595/2012); these guidelines are similar to those of the National Institutes of Health (NIH), USA.

2.2. Group formation and induction of allergy

Fig. 1 depicts the protocol used in this study. Experiments were performed according to Good Laboratory Practice (GLP) standards and quality assurance methods. After a habituation period of 8 days, twenty pairs of mice were at random divided into three groups: naïve (4 pairs) control (8 pairs) and experimental (8 pairs). Care was taken to avoid possible stress induced by social hierarchies' establishment. For that, the paired mice were taken from the same cage colony. Fights and/or confrontations were not seen throughout the 8 days of previous cohousing. Animals of the naïve group (N) were kept undisturbed from $ED_{(-8)}$ (Experimental day-8) to $ED_{(13)}$. On $ED_{(-7)}$, one animal of each experimental and control pair of mice was subcutaneously (s.c.) immunized with a suspension of 10 μg OVA (Egg Albumin Grade V, Sigma Chemical Company®, USA) plus 10 mg of aluminum hydroxide. On $ED_{(0)}$, these OVA-immunized animals received a s.c. OVA booster injection (10 μg OVA plus 10 mg $Al(OH)_3$. At this day ($D_{(0)}$) the experimental mice that were kept undisturbed were inoculated with 5×10^6 Ehrlich tumor cells intraperitoneally (i.p.); their immunized cage-mates were then referred as to CSP ("companion of sick partner"). The undisturbed mice of each control pair were i.p. treated on $D_{(0)}$ with 0.9% NaCl; their sensitized cage-mates were subsequently referred as CHP ("companion of health partner"). The pairs of mice were left to cohabitate in the same cage for 14 days; confrontations or fights were not seen during this time period. On $ED_{(12)}$ and $ED_{(13)}$, the CHP and CSP mice, the subjects of this study, were anesthetized with isofluorene and subsequently intranasally challenged with two drops of a 1% OVA solution, as suggested by (Zosky and Sly, 2007). On $ED_{(14)}$, the Ehrlich tumor-injected mice were scored for Ehrlich tumor clinical signs and symptoms as suggested elsewhere (Tomiyoshi et al., 2009; Alves et al., 2010, 2012). Tissues were collected from the N, CSP and CHP mice on $ED_{(14)}$.

2.3. Leukocyte recruitment in the bronchoalveolar lavage fluid (BAL)

On $ED_{(14)}$, the lungs of the N, CSP and CHP mice were lavaged with 1.5 mL phosphate-buffered saline (PBS) solution through a cannula inserted into the trachea. The recovered BAL (approximately 1 mL) was centrifuged (250*g* for 5 min at 20 °C), and the resulting cell pellet was suspended again in 1 mL of PBS. Cell suspensions (90 μl) were stained with 10 μl of 0.2% crystal violet, and the total cell number was determined in Neubauer chambers using a light microscope. Differential cell counts were performed with cytocentrifuge preparations (Cytospin, Fanem, São Paulo, Brazil) that were stained with Rosenfeld's dye using standard morphological criteria.

2.4. Quantification of blood leukocytes and bone marrow cells

Blood aliquots taken from the abdominal aorta of N, CSP and CHP mice on $ED_{(14)}$ were diluted 1:20 in Turk's fluid (3% acetic acid) for total white blood cell counting with an automatic cell counter (ABC Vet®, São Paulo, Brazil). The total number of bone marrow cells taken from the mice was quantified from the femoral marrow lavage fluid. The recovered bone marrow lavage (5 mL) was centrifuged (250*g* for 5 min at 20 °C). The supernatant was

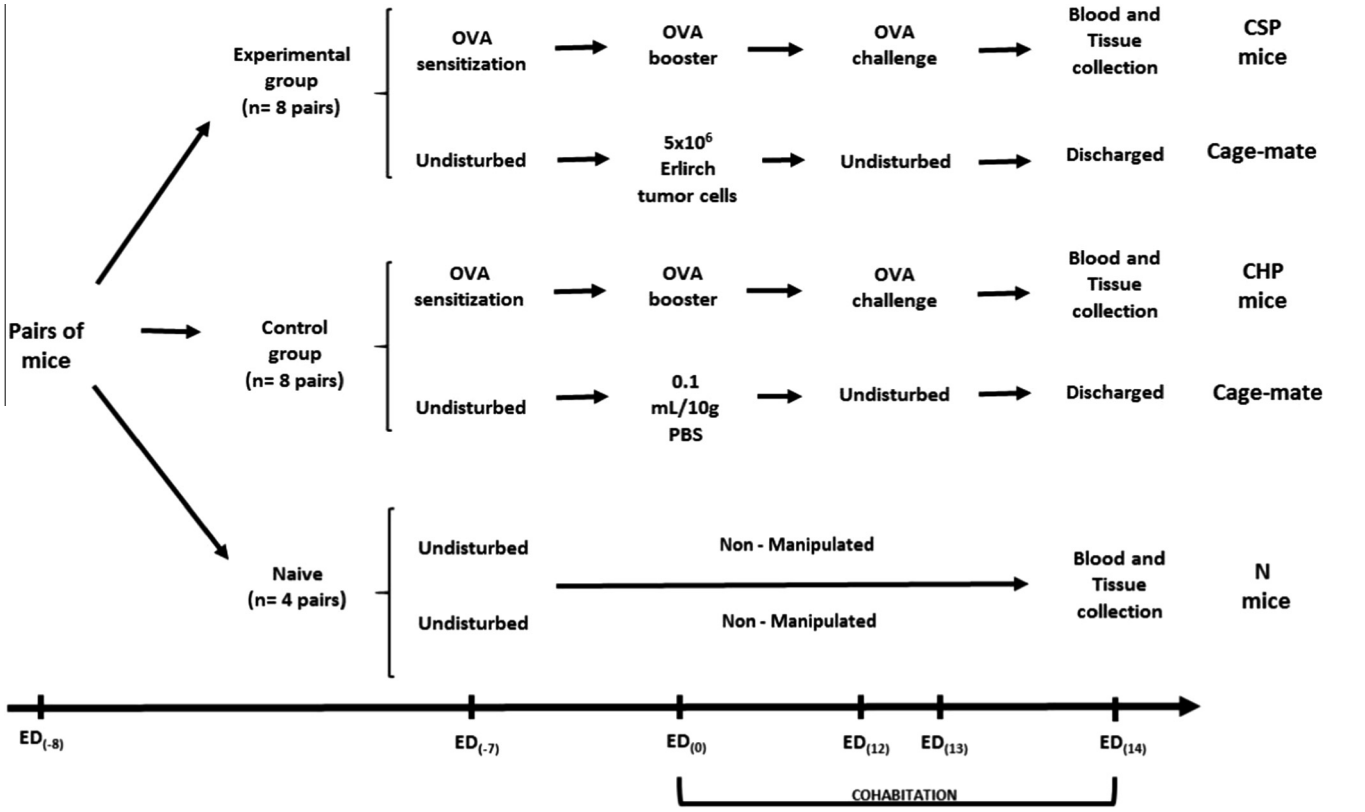

Fig. 1. Schematic representation of the experimental protocol used. Eight days before the beginning of the experiment (Experimental day-8 ($ED_{(-8)}$), pairs of mice were divided into three groups: naïve, control and experimental. Animals of the naïve group (N) were kept undisturbed from $ED_{(-8)}$ (Experimental day-8) to $ED_{(13)}$. On $ED_{(-7)}$, one animal of each experimental and control pair of mice was subcutaneously (s.c.) immunized with a suspension of 10 μg OVA plus 10 mg of aluminum hydroxide. On $ED_{(0)}$, these OVA-immunized animals received a s.c. OVA booster injection. At this day ($D_{(0)}$) the experimental mice that were kept undisturbed were inoculated with 5×10^6 Ehrlich tumor cells intraperitoneally (i.p.); their immunized cage-mates were then referred as to CSP (''companion of sick partner''). The undisturbed mice of each control pair were i.p. treated on $D_{(0)}$ with 0.9% NaCl; their sensitized cage-mates were subsequently referred as CHP (''companion of health partner''). The OVA challenge was performed on CSP and CHP mice on $ED_{(12)}$ and $ED_{(13)}$; blood and tissue collection were performed in N, CHP and CHP mice on $ED_{(14)}$.

discarded and the pellet was resuspended in 1 mL PBS, stained with crystal violet (0.2%) and quantified by optical microscopy, as described above.

2.5. Quantification of cytokines in the BAL supernatant

IL-4, IL-5, IL-10 and IFN-γ levels were determined in samples of BAL supernatant taken from, N, CSP and CHP mice on $ED_{(14)}$. An ELISA kit (R&D Systems, Minneapolis, USA) was used to measure IL-4, IL-5, IL-10 and IFN-γ in the BAL supernatant, and the assays were performed according to the manufactureŕs instructions. The detection limit for IL-4 was 2 pg/mL, IL-5 was 7 pg/mL, IL-10 was 15.6 pg/mL, IL-13 was 7.8 pg/mL, and for IFN-γ it was 2 pg/mL. The results were expressed in pg of interleukin produced per mL.

2.6. Quantification of OVA-specific IgG1and IgG2a levels in peripheral blood

OVA-specific IgG1 and IgG2a antibodies were assayed in CSP and CHP mice by ELISA. Briefly, serum samples were plated on 96 wells previously coated with OVA (2 μg/well). The bound antibodies were revealed with goat anti-mouse IgG1 or IgG2a for 1 h followed by addition of peroxidase-conjugated rabbit antigoat IgG antibodies (Southern Biotechnology, Birmingham, AL) for 1 h. The reaction was developed by the addition of 100 μl/well of *o*-orthophenylenediamine dihydrochloride (Sigma Chemical Company®, USA). The reaction was stopped with 4 M H_2SO_4 and the absorbance of the samples determined at 490 nm. The concentrations of OVA-specific IgG1 and IgG2a antibodies were estimated by comparison with IgG1 and IgG2a standards run in parallel on rabbit anti-mouse (Southern Biotechnology, Birmingham, AL) Ig-coated plates (Faustino et al., 2012).

2.7. Quantification of OVA-specific IgE levels in peripheral blood

Serum OVA-specific IgE was assayed in CSP and CHP mice by sandwich ELISA as previously described (Russo et al., 2001). Briefly, plates were coated with anti-IgE (Southern Biotechnology, Birmingham, USA) and serum was added in 1/10 dilution in PBS. Subsequently, biotin-labeled OVA was added to the wells. The bound OVA-biotin was revealed by extravidin-peroxidase (Sigma Chemical Company, USA). OVA-specific IgE levels of samples were deduced from an internal standard obtained from pooled sera of hyperimmunized mice that was arbitrarily assigned as 100 U/mL (Faustino et al., 2012).

2.8. Analysis of L-selectin expression in BAL granulocytes

BAL cells taken from N, CSP and CHP mice on $ED_{(14)}$ were resuspended in Dulbecco-modified Eagle's medium (DMEM) containing 2% FCS. The cells were blocked with anti-CD16/anti-CD32 at a 1:100 dilution at 4 °C for 20 min to prevent nonspecific binding via the Fc receptor. After Fc blocking, the cells were stained with PE conjugated anti-mouse CD62L (L-selectin), clone MEL-14 (Biolegend, CA, USA) for 30 min at 4 °C according to the manufacturer's instructions. The cells were then washed twice with cold

HBSS, resuspended in paraformaldehyde (1%) and analyzed using a FACS Calibur cytometer (BD Bioscience, San Diego, CA). The data were analyzed using FlowJo software sa.

2.9. In vitro tracheal responsiveness to methacholine (MCh)

Tracheal rings were mounted for the measurement of isometric force quantification by means of two steel hooks in a 30 mL organ bath. Force contraction was registered using a force displacement transducer and a chart recorder (Powerlab®, Labchart, AD Instruments). Briefly, tracheal rings removed from N, CSP and CHP mice on $ED_{(14)}$ were suspended in an organ bath filled with KH at 37 °C. Tissues were continuously aerated (95% O_2 and 5% CO_2), and after the equilibrium period (40 min), the tracheal tension was adjusted to 1 g. Tissue viability was assessed by replacing KH solution with KCl buffer (60 mM) in the bath and comparing the contraction force generated with those obtained previously. Next, cumulative dose–response curves to methacholine (MCh) were constructed.

2.10. Plasma corticosterone determination

An ELISA kit (Life Science, Plymouth Meeting, PA, USA) was used to measure corticosterone in the plasma of CSP and CHP mice on $ED_{(3)}$, $ED_{(6)}$, $ED_{(9)}$, $ED_{(12)}$ and $ED_{(14)}$; blood collection was performed by retro-orbital puncture. The assays were performed according to the manufacturer's instructions. The detection limit for corticosterone was 27.0 pg/mL.

2.11. Plasma catecholamine measurement

Plasma was removed from CSP and CHP mice on $ED_{(9)}$ and $ED_{(14)}$ and immediately flash frozen in liquid nitrogen to halt the degradation of catecholamines. The samples were stored at −80 °C prior to analysis. Plasma concentration of catecholamines was measured by HPLC. The HPLC setup for catecholamine analysis included the following: a 590 Solvent delivery module, U6K injector, 460 eletrochemical detector and M740 data module (Milipore Corp., Waters Chromatography Division, Milford, MA, USA). A Clin-Rep® column and eluent (Pharma Vertriebs GmBH & Co KG, Munich, FRG) were used for the chromatography performed at ambient temperature. Eluent flow was 1.0 mL min^{-1}.

2.12. Statistical analysis

GraphPad Prism 5.0 (GraphPad Software) was used for statistical analysis. The parametric data were analyzed by Student's t test, one-way and two-way ANOVA followed by the Dunnett and Bonferroni post-tests respectively, for multiple comparisons. Since data on catecholamine were non-parametric, they were analyzed by Mann–Whitney's U test. In all experiments, $p \leqslant 0.05$ was considered significant for all comparisons performed. The data are presented as the means and standard derivations.

3. Results

Ehrlich tumor cell injection induced behavioral changes in the sick animals. These alterations arose progressively, being characterized by the presence of lethargy, reduced activity and interest in surroundings, and decreased ability to respond to the companion mice. On $ED_{(14)}$, the sick mice also presented anorexia, dyspnea, rough hair and increased abdominal volume. We performed the experiment using the naïve and the companions of health partner (CHP) and sick partner (CSP) on $ED_{(14)}$.

3.1. Cohabitation with a sick partner exacerbates allergic lung inflammation

As depicted in Fig. 2 (Panel A), an increase ($F_{(2,21)} = 117.0$; $p < 0.05$) in the total number of leukocytes was observed in the BAL of CHP and CSP mice compared to the observed in Naïve (N) mice. Further analysis showed that CSP data were higher ($p < 0.05$) compared to that observed in CHP mice. The differential cell count, presented in Table 1, showed an increased number of eosinophils ($F_{(2,21)} = 80.09$; $p < 0.05$) and neutrophils ($F_{(2,21)} = 127.7$; $p < 0.05$) in the BAL of CSP mice compared to that measured in both N and CHP animals. No differences ($p > 0.05$) were found in mononuclear cells in the BAL taken from N, CSP and CHP mice.

3.2. Effects of cohabitation with a sick partner on the peripheral blood and bone marrow cells of allergic mice

As depicted in Fig. 2 (Panel B), CHP and CSP mice presented increased numbers ($F_{(2,21)} = 21.36$; $p < 0.05$) of total leukocytes in the bone marrow lavage fluid in relation to N animals. However, a decrease ($p < 0.05$) in the total number of these cells was

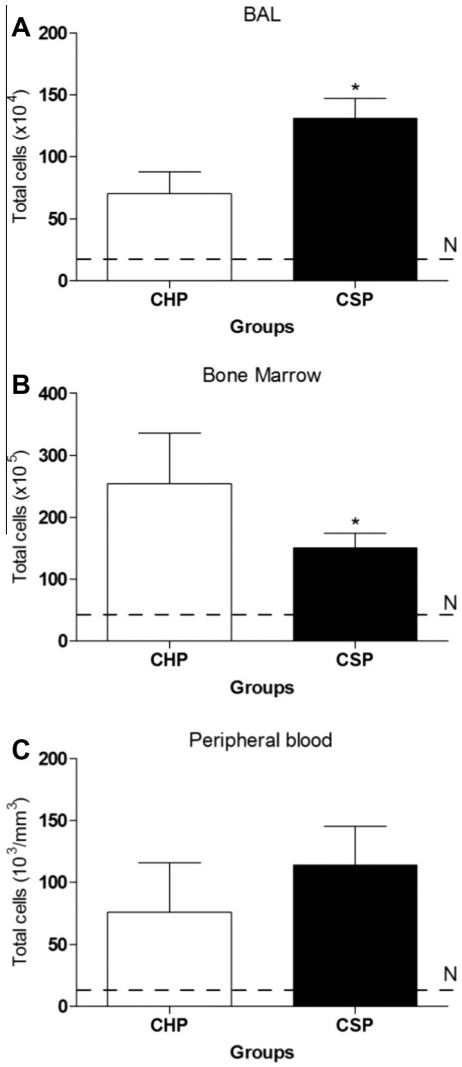

Fig. 2. Effects of cohabitation with sick cage-mate on the total BAL count (A), peripheral blood count (B), and total bone marrow count (C) of CSP and CHP mice. The dotted line depicts (N) naïve mice. $^{(*)}p < 0.05$ compared to the CHP group. Data are expressed as the mean ± SD. (One-way ANOVA followed by Dunnett post-test, $n = 8$ mice/group.)

Table 1
Differential cell count in BAL harvested from mice that lived (CSP) or not lived (CHP) with sick cage mate for 14 days.

Groups	Eosinophils ($\times 10^5$)	Neutrophils ($\times 10^5$)	Mononuclear cells ($\times 10^6$)
Naïve	0.00 ± 0.00	9.50 ± 2.60	4.80 ± 0.60
CHP	2.71 ± 0.88	38.33 ± 5.71	4.50 ± 0.92
CSP	5.10 ± 0.83*	55.97 ± 6.18*	5.11 ± 0.76

CHP: Companion of health partner; CSP: companion of sick partner. Mononuclear cells = macrophages + lymphocytes.
* $p \leqslant 0.05$ (One-way ANOVA followed by Dunnett test); $n = 5$–7 animal/group.

observed in the of CSP mice compared to the N and CHP animals. As presented in Fig. 2 (Panel C), no differences ($p > 0.05$) in the total peripheral blood cell count were found among N, CSP and CHP mice.

3.3. Cohabitation with a sick partner increases IL-4 and IL-5 levels and decreases IL-10 and IFN-γ levels in the BAL supernatant

As show in Fig. 3, cytokines (IL-4, IL-5, IL-10 and INF-γ) were almost not detected in the BAL supernatant of mice of the N group. However, the BAL obtained from CSP mice had increased levels of both IL-4 ($F_{(2,21)} = 54.47$; $p < 0.05$, Panel A) and IL-5 ($F_{(2,21)} = 16.11$; $p < 0.05$, Panel B) and decreased levels of IL-10 ($F_{(2,21)} = 24.33$; $p < 0.05$, Panel C) and μIFN-γ ($F_{(2,21)} = 4.83$; $p < 0.05$, Panel D) in relation to the N and CHP animals.

3.4. Cohabitation with a sick partner increases IgG1-OVA and decreases IgG2a-OVA levels in the peripheral blood

As shown in Fig. 4, CSP mice had increased levels of IgG1-OVA ($F_{(2,21)} = 57.11$; $p < 0.05$, Panel A), decreased levels of IgG2a-OVA ($F_{(2,21)} = 102.3$; $p < 0.05$, Panel B) in relation to the CHP animals. Differences were not found for IgE-OVA between CHP and CSP mice ($p > 0.05$, Panel C). As expected, IgG1-OVA, IgG2a-OVA and IgE-OVA were not detected in mice of the N group.

3.5. Cohabitation with a sick partner affects L-selectin expression in BAL granulocytes

As shown in Fig. 5, CSP mice had increased expression of L-selectin ($F_{(2,21)} = 22.55$; $p < 0.05$) in relation to N and CHP mice. Data from CHP and N groups on L-selectin were not statistically different ($p > 0.05$).

3.6. Cohabitation with a sick partner affects in vitro tracheal reactivity to methacholine

A two-way ANOVA showed significant ($F_{(2,6,147)} = 52.81$; $p < 0.05$) differences among: the three groups, the 5 different doses of MCh used and an interaction between MCh effects and the groups. As depicted in Fig. 6, tracheal responsiveness to MCh was higher in CHP and CSP mice than in Naïve (N) animals ($p < 0.05$). Further analysis showed that the tracheal hyper-responsiveness to MCh was reduced in CSP mice compared to that measured in CHP mice ($p < 0.05$). Consistent with this, the dose–response curve for MCh was shifted to the right, and the maximal contraction achieved was decreased in the mice of the CSP group in relation to CHP group ($p < 0.05$).

3.7. Cohabitation with a sick cage-mate does not affect plasma corticosterone levels

Fig. 7 depicts the plasma corticosterone levels in the CHP and CSP mice. A two-way ANOVA showed significant differences among the days but no differences were found between the groups ($F_{(1,4,70)} = 31.42$; $p < 0.05$). Specifically, no differences were found

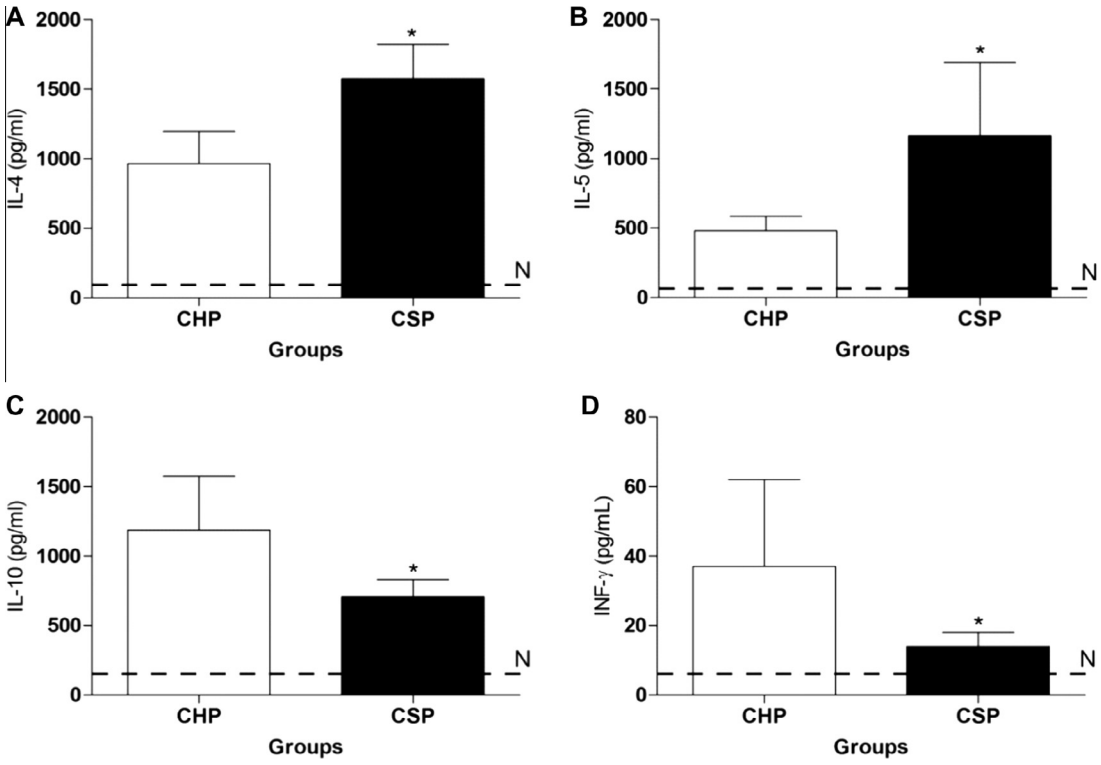

Fig. 3. Effects of cohabitation with sick cage-mate on IL-4 (A), IL-5 (B), IL-10 (C) and IFN-γ (D) levels in the BAL supernatant of CSP and CHP mice. The dotted line depicts (N) naïve mice. (*)$p < 0.05$ compared to the CHP group. Data are expressed as the mean ± SD. (One-way ANOVA followed by Dunnett post-test, $n = 8$ mice/group.)

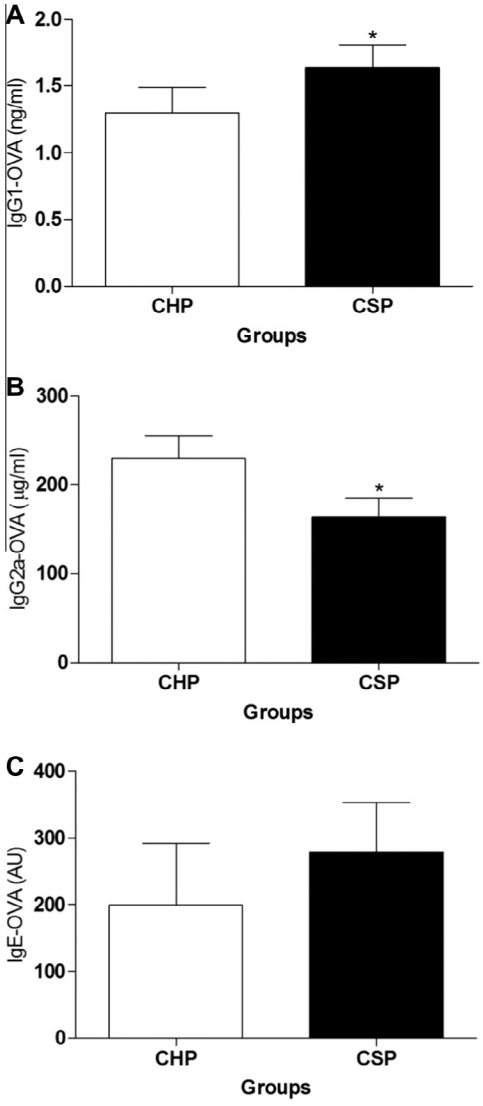

Fig. 4. Effects of cohabitation with sick cage-mate on IgG1-OVA (A) and IgG2a-OVA (B), and IgE-OVA (C) levels in the peripheral blood of CSP and CHP mice. The dotted line depicts (N) naïve mice. $^{(*)}p < 0.05$ compared to the CHP group. Data are expressed as the mean ± SD. (Student's t test, n = 8 mice/group.)

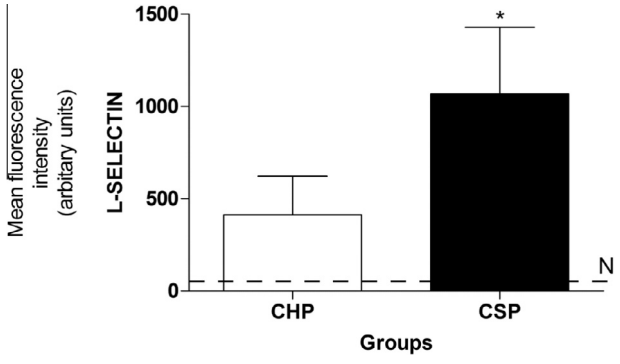

Fig. 5. Effects of cohabitation with sick cage-mate in L-selectin expression in granulocytes taken from the BAL of CSP and CHP mice. The dotted line depicts (N) naïve mice. $^{(*)}p < 0.05$ compared to the CHP group. Data are expressed as the mean ± SD. (One-way ANOVA followed by Dunnett post-test, $n = 8$ mice/group.)

($p > 0.05$) in the plasma corticosterone levels between CHP and CSP mice at any time point, i.e., on $ED_{(3)}$, $ED_{(6)}$, $ED_{(9)}$, $ED_{(12)}$ and $ED_{(14)}$. However, most likely as a consequence of the stress induced by the procedure used for blood collection, the serum corticosterone levels increased ($p < 0.05$) from $ED_{(3)}$ to $ED_{(14)}$ in the mice from both groups. Although no significant differences were observed between the days and groups over time, a tendency toward a smaller increase was observed in CSP mice compared to those of the CHP group.

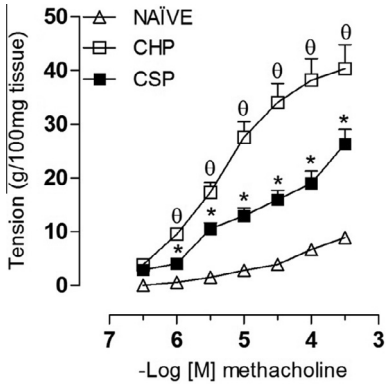

Fig. 6. Effects of cohabitation with sick cage-mate on *in vitro* tracheal reactivity to methacholine in CSP and CHP mice. $^{(*)}p < 0.05$ compared to the CHP group. θ $p < 0.05$ compared to the (N) naïve group. Data are expressed as the mean ± SD. (Two-way ANOVA followed by Bonferroni post-test, $n = 8$ mice/group.)

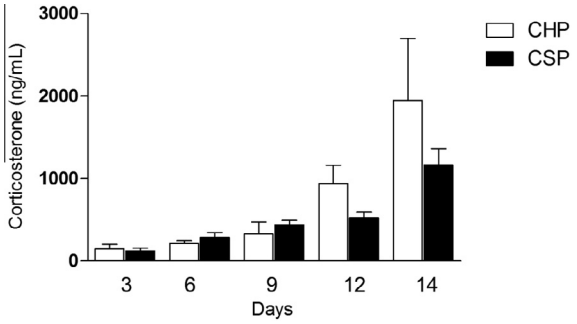

Fig. 7. Effects of cohabitation with sick cage-mate on plasma corticosterone levels of CSP and CHP mice on $ED_{(3)}$, $ED_{(6)}$, $ED_{(9)}$, $ED_{(12)}$ and $ED_{(14)}$. Data are expressed as the mean ± SD. ($p > 0.05$, Two-way ANOVA followed by the Bonferroni post-test, $n = 8$ mice/group.)

Table 2
Plasmatic adrenaline and noradrenaline levels in mice that lived (CSP) or not lived (CHP) with sick cage mate for 9 and 14 days.

Days of cohabitation	Adrenaline (ng/mL)		Noradrenaline (ng/mL)	
	CHP	CSP	CHP	CSP
9	0.29 ± 0.04	0.45 ± 0.11*	1.85 ± 0.19	3.98 ± 0.69*
14	0.22 ± 0.10	0.21 ± 0.06	3.34 ± 0.79	2.29 ± 0.54*

CHP: Companion of health partner; CSP: companion of sick partner.
* $p \leqslant 0.05$ (Mann–Whitney's U test); $n = 5$ animal/group.

3.8. Effects of cohabitation with sick cage-mate on plasma catecholamine levels

Table 2 shows the plasma concentrations of adrenaline and noradrenaline in the CSP and CHP mice, measured on $ED_{(9)}$ and $ED_{(14)}$. Differences between the CSP and CHP mice were found for both adrenaline and noradrenaline. Compared to CHP mice, the concentrations of adrenaline and noradrenaline in CSP mice were higher on $ED_{(9)}$ ($p < 0.05$). Notably, however, the plasma levels of noradrenaline were smaller ($p < 0.05$) in the CSP than in the CHP mice

on $ED_{(14)}$. No differences were found in the plasma adrenaline levels between the CSP and CHP mice on $ED_{(14)}$.

4. Discussion

Cohabitation for 11 days with an Ehrlich tumor-bearing mouse has been reported to produce significant changes in behavior as well as in the immune and endocrine systems (Morgulis et al., 2004; Alves et al., 2006, 2007, 2012). These effects were recently reported to rely on odor clues released by the Ehrlich tumor-bearing mice. Indeed, it was shown that odors released by Ehrlich tumor-injected mice are aversive and induce psychological stress in their cage mates (Alves and Palermo-Neto, 2014). Data from the present study confirm and extend these interesting neuroimmune changes. Indeed, our data demonstrate that mice that were housed for 14 days with an Ehrlich tumor-bearing mouse presented an increased allergic lung inflammatory response.

Our study demonstrates that the CSP mice had decreased cellularity in the bone marrow and an increased number of eosinophils and neutrophils harvested from the BAL compared to CHP animals. Furthermore, a clear tendency toward an increased number of leukocytes in the peripheral blood was also found in CSP mice. The number and proportion of leukocytes present in the bone marrow, peripheral blood and inflammatory sites have been reported to characterize important features of immune system activation and immune cell distribution within the body during an immune/inflammatory response (Ottaway and Husband, 1994; Stefanski, 2000; Engler et al., 2004; Azpiroz et al., 2008). Thus, it seems feasible to state that the stress induced by cohabitation with a sick partner was able to modulate cellular recruitment to the lungs in OVA-sensitized and challenged mice. Indeed, differences were found between CSP and CHP mice in leukocyte redistribution from the bone marrow and peripheral blood to the airway space. Previous studies have also reported that stressors are important factors in both leukocyte trafficking (Dhabhar, 2002, 2003) and the onset and exacerbation of asthma symptoms (Datti et al., 2002; Forsythe et al., 2004; Okuyama et al., 2007; Portela et al., 2007; Quarcoo et al., 2009; Hamasato et al., 2013). Thus, the present data not only agree with the previous studies but also reinforce the suggestion that cohabitation with an Ehrlich tumor-bearing mouse induces psychological stress in their cage-mates (Palermo-Neto and Alves, 2014).

Airway inflammation is one of the main characteristics of bronchial asthma, whereby eosinophils, neutrophils and lymphocytes infiltrate the lung tissue and obstruct the airway space. Stressors have been discussed as being capable of altering the Th1/Th2 cytokine balance (Tausk et al., 2008). The recruitment and migration of eosinophils into the inflamed tissue after antigen challenge is controlled by cytokines, chemokines and inflammatory mediators. Allergic immune responses are linked to Th1/Th2 imbalances with an increase in the Th2 cytokine profile (Marshall et al., 1998; Ngoc et al., 2005). In the course of allergic diseases, Th2 cytokines such as IL-4 and IL-5 increase allergen-specific IgE production and also induce the migration of leukocytes, namely eosinophils, to inflammatory loci through adhesion molecules such as L-selectin (Cotran and Mayadas-Norton, 1998). This work shows that CSP mice had increased levels of IL-4 and IL-5 and decreased levels of IL-10 and IFN-γ in the BAL compared to animals of the CHP group. Increased expression of L-selectin was also reported in the BAL granulocytes of CSP mice. Altogether, the data obtained seem to explain the higher number of leukocytes found in the BAL of the CSP mice and strongly suggest that the psychological stress induced by cohabitation favors the release of Th2 cytokines.

IL-4 is essential for driving the differentiation of naïve Th0 cells into Th2 cells in the course of allergic inflammation (Renauld, 2001). IL-4 was also reported to facilitate isotype switching and the production of immunoglobulin E (IgE), a hallmark of allergy. IL-5 functions to differentiate, activate, and enhance the viability of eosinophils (Barnes, 2008; Finkelman et al., 2010). Activated eosinophils are known to release potent proinflammatory mediators (Holgate, 2008). Th1 cells secrete a specific profile of cytokines, including IFN-γ and TNF-α, that favor the cellular immune response (Nguyen and Casale, 2011). In particular, IFN-γ has been reported to antagonize Th2 cytokine activity (Busse and Rosenwasser, 2003). In contrast, IL-10 is known to regulate the activity of both Th1 and Th2 cells (Hawrylowicz and O'Garra, 2005). Further data from our study showed that CSP mice presented increased levels of IgG1-OVA and decreased levels of IgG2a-OVA compared to CHP mice. No changes in IgE-OVA was observed between CSP and CHP mice, a fact that agree with data previously reported (Chida et al., 2007). According to Nurieva and Chung (2010), IgE and IgG1 are linked to the development of a Th2 response. Therefore, our evaluation of immunoglobulin together with that of the Th1/Th2 cytokine balance are consistent with the idea that OVA-sensitized animals that cohabitated for 14 days with an Ehrlich tumor-bearing mouse experienced a shift in the Th1/Th2 profile toward a Th2 response. The absence of differences in the levels of anti-OVA specific IgE appears to preclude the possibility that the stress of cohabitation may affect antigen sensitization.

Leukocyte migration into inflamed tissue involves complex interactions of the leukocytes with the endothelium through regulated expression of surface adhesion molecules. In the present study, we showed that CSP mice had an increased expression of L-selectin in BAL granulocytes, which might explain the increased number of eosinophils and neutrophils harvested from the BAL of CSP mice that cohabitated with Ehrlich tumor-bearing mice. Indeed, L-selectin acts as a "homing receptor" for leukocytes to enter the secondary lymphoid tissues via high endothelial venules (Scola et al., 2009).

Iwakabe et al. (1998) showed that mice subjected to a restraint stress exhibited increased serum levels of corticosterone, decreased NK cell activity and decreased production of IFN-γ and IL-4, meaning that the stressor induced a shift in the Th1/Th2 balance, favoring Th2 responses. According to Li et al. (2013) OVA-sensitized and challenged Balb/C mice submitted to a psychosocial stress of social confrontation presented behavioral signs indicative of high levels of stress and an exacerbated OVA-induced airway inflammation compared to animals that were OVA-sensitized and not stressed, showing a positive correlation between the behavioral changes observed and the aggravation of the allergic lung inflammatory response. In the present study, no differences in the serum corticosterone levels were found between CSP and CHP mice from $ED_{(3)}$ through $ED_{(14)}$. Corticosterone serum levels of OVA-sensitized and non-sensitized rats (Portela et al., 2007) and mice (Li et al., 2013) were shown to be the same. No differences were found in the expressions of GR protein and GR mRNA in the lung tissue of OVA-sensitized and non-sensitized Balb/C mice (Li et al., 2013). Although unexpected from a stressor, the lack of changes in corticosterone found in CSP mice of the present work agrees with previous data reported elsewhere (Morgulis et al., 2004; Alves et al., 2010). Furthermore, these data do not preclude the presence of changes in HPA activity. Indeed, decreased levels of ACTH in the presence of similar levels of corticosterone have been reported in submissive mice after a psychological stress induced by social confrontation (Lightman et al., 2002; Jahng, 2011). Thus, it is not at all impossible that the lack of significant changes observed in the serum corticosterone levels between CSP and CHP mice might also rely on HPA feedback mechanisms induced by the stress conditions imposed by the 14 days of cohabitation on the CSP animals. Data on serum corticosterone levels between CSP and CHP mice on $ED_{(12)}$ and $ED_{(14)}$ seem to support this notion.

The results of our study also showed that CSP mice had reduced tracheal responsiveness to methacholine. However, as shown in Fig. 6, tracheal responsiveness to methacholine in CSP mice was higher than that measured in naïve animals. This observation is in line with data reported elsewhere because a cholinergic bronchial hyper-responsiveness has been shown in allergic lung inflammation models (Deleuze et al., 2003; Hamid et al., 2003). Notably, the observed hypo-responsiveness to methacholine in CSP mice occurred in parallel with the increase in the allergic lung inflammatory response. Other studies have demonstrated that events underlying inflammation in the lung, such as pulmonary cell influx, can be dissociated from those that govern bronchial smooth muscle tonus, i.e., that induce hypo-responsiveness (Lino dos Santos Franco et al., 2006; Ligeiro de Oliveira et al., 2012). Therefore, the data on airway responsiveness could be viewed as the result of a balance between the release of inflammatory mediators leading to bronchoconstriction (e.g. leuckotrienes, thromboxanes) and those causing bronchodilation (e.g. NO). Such mediators are not only produced by inflammatory cells recruited into the lungs but also by smooth muscle, epithelial and sensorial nervous system cells. Thus, the hypo-responsiveness observed in the CSP mice of the present work might be understood as a reflex of an elevated production of mediators that induce bronchodilation. Vaniotis et al. (2013) showed that activation of G-protein coupled to B-adrenergic receptors induced NO production in cardiac tissue. Although NO release was not quantified in the present study, it seems reasonable to suggest that the tracheal hypo-responsiveness observed in the CSP mice might have been related with the production and release of NO within the airway of CSP mice.

SNS activation in response to stress increases the plasma levels of catecholamines (Elenkov et al., 2000; Kohm and Sanders, 2000; Powell et al., 2013). As shown in Table 2, a huge increase in the plasma levels of catecholamines was observed on $ED_{(9)}$ in CSP mice compared to CHP animals, which is consistent with data reported previously (Alves and Palermo-Neto, 2014). These authors, using an experimental model similar to the one used in this study, reported increased levels of plasma catecholamine in CSP mice from $ED_{(2)}$ till $ED_{(9)}$. Relevant to state that adrenaline and noradrenaline levels of CHP mice were not statistically different from those reported in our laboratory for Naïve animals (data not shown). Catecholamine is known to change immune cell activity through G-protein coupled B-adrenergic receptors.(Kohm and Sanders, 2000). In this respect, catecholamines regulate various aspects of the humoral responses involved in asthma, including: (1) the expression of IL-4, IL-5 and IL-13 following allergen exposure (Torres et al., 2005), (2) the release of histamine by activated mast cells (Elenkov and Chrousos, 1999b) and (3) the recruitment and activation of eosinophils in the airways (Trueba and Ritz, 2013). Thus, activation of the SNS and catecholamine release due to unpleasant housing conditions might be important mediators of the stress-induced lung changes in allergic mice. Notably, however, a significant decrease in plasma noradrenaline levels was found in CSP mice on $ED_{(14)}$, i.e., after 14 days of cohabitation with the sick partners. These data are consistent with previously reported data (Alves and Palermo-Neto, 2014), strongly suggesting that the SNS might also undergo physiological adaptations to the changes imposed by 14 days of cohabitation with the Ehrlich tumor-bearing mice. Notably, a tendency toward decreased levels of corticosterone in CSP mice was also observed after $ED_{(12)}$. Since the levels of noradrenaline were reduced in the plasma of CSP mice on day 14, it is reasonable to infer that this catecholamine has long-lasting effects on immune response, i.e., changes might have occurred during the OVA allergic inflammatory response induction.

In conclusion, the present data provide experimental evidence that the psychological stress induced by 14 days of cohabitation with an Ehrlich tumor-bearing partner exacerbated the allergic lung inflammatory response in CSP mice, as assessed by: (1) the increased migration and presence of eosinophils and neutrophils to the BAL, (2) the increased Th2 cytokine profile, (3) changes in IgG1-OVA and IgG2a-OVA and (4) the increased expression of L-selectin in granulocytes. However, cohabitation with a sick partner reduced tracheal responsiveness to methacholine in OVA-sensitized mice. Strong SNS participation through adrenaline and noradrenaline release seems to underlie these reported effects.

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Competing interests

The authors have declared that no competing interests exist.

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