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3,4-Methylenedioxymethamphetamine (Ecstasy) Decreases Inflammation and Airway Reactivity in a Murine Model of Asthma

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Key Words

3,4-Methylenedioxymethamphetamine · Ecstasy · Allergic lung inflammation · Corticosterone · Leukocyte migration · Airway reactivity

Abstract

Objective: 3,4-Methylenedioxymethamphetamine (MDMA), or ecstasy, is a synthetic drug used recreationally, mainly by young people. It has been suggested that MDMA has a Th cell skewing effect, in which Th1 cell activity is suppressed and Th2 cell activity is increased. Experimental allergic airway inflammation in ovalbumin (OVA)-sensitized rodents is a useful model to study Th2 response; therefore, based on the Th2 skewing effect of MDMA, we studied MDMA in a model of allergic lung inflammation in OVA-sensitized mice. **Methods:** We evaluated cell trafficking in the bronchoalveolar lavage fluid, blood and bone marrow; cytokine production; L-selectin expression and lung histology. We also investigated the effects of MDMA on tracheal reactivity in vitro and mast cell degranulation. **Results:** We found that MDMA

given prior to OVA challenge in OVA-sensitized mice decreased leukocyte migration into the lung, as revealed by a lower cell count in the bronchoalveolar lavage fluid and lung histologic analysis. We also showed that MDMA decreased expression of both Th2-like cytokines (IL-4, IL-5 and IL-10) and adhesion molecules (L-selectin). Moreover, we showed that the hypothalamus-pituitary-adrenal axis is partially involved in the MDMA-induced reduction in leukocyte migration into the lung. Finally, we showed that MDMA decreased tracheal reactivity to methacholine as well as mast cell degranulation in situ. **Conclusions:** Thus, we report here that MDMA given prior to OVA challenge in OVA-sensitized allergic mice is able to decrease lung inflammation and airway reactivity and that hypothalamus-pituitary-adrenal axis activation is partially involved. Together, the data strongly suggest an involvement of a neuroimmune mechanism in the effects of MDMA on lung inflammatory response and cell recruitment to the lungs of allergic animals.

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA), or ecstasy, is a synthetic drug used recreationally, mainly by young people. MDMA is a potent releaser of neurotransmitters in the central nervous system and has psychostimulant properties in animals and humans [1, 2]. MDMA is also known for its empathogenic euphoria and its ability to increase energy and sexual arousal [3], which leads to an enhanced response to sensory stimulation [4–6]. Moreover, it has been reported that MDMA induces hyperthermia, increases heart rate, anxiety [7], neurodegeneration of serotonergic [8] and catecholaminergic pathways [9, 10], and induces hypothalamo-pituitary-adrenal (HPA) axis activation [11] and immunological alterations [11, 12].

Recently, we showed that MDMA decreases neutrophil activity and alters leukocyte distribution [11]. MDMA decreased the neutrophil oxidative burst and phagocytosis, increased the number of circulating neutrophils while decreasing the number of lymphocytes. MDMA also decreased bone marrow cellularity, increased spleen cellularity, and decreased spleen relative weight. Moreover, we showed that MDMA increases HPA axis activity through the noradrenergic pathway in the hypothalamus. Therefore, we hypothesized that the observed effects induced by MDMA could be mediated by indirect mechanisms involving glucocorticoids and/or catecholamines, as we did not observe any effect of MDMA on neutrophil activity *in vitro*.

MDMA has been described to induce immunosuppressive actions. It has been reported that MDMA increases susceptibility to infectious diseases in humans [13–15] and in laboratory animals [16, 17]. Moreover, MDMA was shown to suppress the generation of lipopolysaccharide-induced proinflammatory cytokines such as tumor necrosis factor- α and interleukin (IL)-1 β [18, 19] and to increase the lipopolysaccharide-induced production of the anti-inflammatory cytokine IL-10 [20]. Boyle and Connor [21] reported that MDMA suppresses interferon- γ secretion and signaling in mice. In humans, it was shown that MDMA increases the number of circulating natural killer cells and transforming growth factor- β and IL-10 levels [13, 22]. It was found that MDMA decreases the number of CD4⁺ T-helper cells [23] and reduces phytohemagglutinin-stimulated T-cell proliferation [24]. Finally, a Th cell skewing effect following MDMA administration has been suggested in rodents, in which Th1 cell activity is suppressed and Th2 cell activity is increased [12].

Asthma is a disease involving a high Th2 immune response and is characterized by chronic airway inflammation and poor respiratory function [25]. Experimental allergic airway inflammation in ovalbumin (OVA)-sensitized rodents is a useful model to study this condition. Therefore, based on the Th2 skew effect of MDMA, we studied MDMA in a model of allergic lung inflammation in OVA-sensitized mice. We evaluated cell trafficking in the bronchoalveolar lavage fluid, blood and bone marrow; cytokine production; L-selectin expression and lung histology. We also investigated the effects of MDMA on tracheal reactivity *in vitro* and mast cell degranulation.

Material and Methods

Animals

Male Balb/c mice from our own colony weighing 25–35 g were used. Animals were housed in controlled temperature (22–24°C) and in artificially lit rooms on a 12-hour light/12-hour dark cycle (lights on at 7.00 a.m.) with free access to rodent chow and water. The experiments were performed in a different room with the same temperature as the animal colony from which the animals were transferred. Animals were maintained in their home cages 7 days before the beginning of the experiments. Animals were housed and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil (Protocol No. 772/2005).

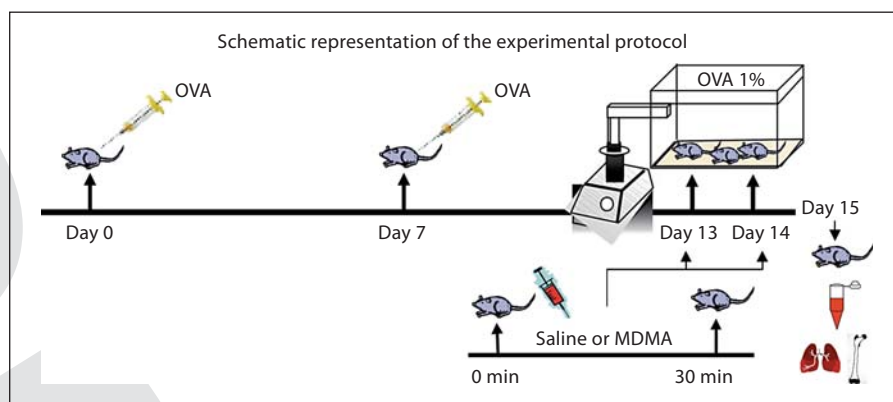
Drugs

MDMA was extracted from tablets kindly provided by the Department of Investigation on Narcotics, a police institution responsible for the investigation of illicit drugs in the state of São Paulo (São Paulo, Brazil). Upon delivery to the laboratory, the drug was extracted and purified from tablets as previously reported [26]. MDMA (99% pure) was dissolved in a 0.9% NaCl solution to a concentration of 10 mg/kg. MDMA or 0.9% saline was administered by intraperitoneal injection at a volume of 0.1 ml/10 g. The MDMA dose was chosen based on a previous experiment conducted in our laboratory [11]. Mifepristone (RU 486; Tocris Bioscience, Ellisville, Mo., USA), a glucocorticoid receptor GR11 antagonist, was dissolved in PEG-400 to a concentration of 5 mg/ml and administered by subcutaneous injection of 100 μ l/mouse. Methysergide (Tocris Bioscience), a serotonin receptor antagonist, was dissolved in 0.9% saline to a concentration of 5 mg/kg for intraperitoneal injection at a volume of 0.1 ml/10 g. Methacholine (a non-specific muscarinic agonist) was used to induce tracheal constriction at concentrations ranging from 10^{-3} to 10^{-9} M.

Model of Allergic Lung Inflammation

Mice were sensitized through intraperitoneal administration of 10 μ g OVA (Egg Albumin Grade V, Sigma Chemical Company®, USA) adsorbed with 10 mg aluminum hydroxide. Seven days later, animals were boosted with the same solution by subcutaneous injection. Lung inflammation was induced by exposing mice to a 1% OVA aerosol challenge on days 13 and 14 after primary

Fig. 1. Schematic representation of the experimental protocol used to analyze the effects of MDMA given prior to OVA challenge in OVA-sensitized mice.



sensitization. Aerosol challenge was achieved by placing 5 mice into an inhalation chamber ($18.5 \times 18.5 \times 13.5$ cm) connected to an ultrasonic nebulizer (Inalamax NS model S3®, Brazil), which generates a mist aerosol (mean size: $0.5\text{--}1.0\ \mu\text{m}$) for 20 min twice a day. Figure 1 shows a schematic representation of the experimental protocol used.

Leukocyte Distribution

Bronchoalveolar Lavage Fluid. Bronchoalveolar lavage fluid (BAL) was collected as previously described by Ligeiro-Oliveira et al. [27]. Briefly, 24 h after the last OVA aerosol challenge, mice were sacrificed by exsanguination through the abdominal aorta after deep xylazine ($20.0\ \text{mg/kg}$) and ketamine ($75.0\ \text{mg/kg}$) intraperitoneal anesthesia. Lungs were flushed with 3-ml aliquots of phosphate-buffered saline (PBS) solution through a cannula inserted into the trachea. BAL fluid was harvested and centrifuged at $250\ \text{g}$ for 5 min. The remaining cell pellet was resuspended in 1 ml of PBS. Total leukocyte counts were performed after adding $10\ \mu\text{l}$ of crystal violet to $90\ \mu\text{l}$ of the cell suspension. Neubauer chambers were used for cell counting under a light microscope.

Blood. White blood cells were counted with a C530 automatic cell counter (CELM®, São Paulo, Brazil).

Bone Marrow. The total number of bone marrow cells was quantified from femoral marrow lavage. Epiphyses of the femurs were cut off on both sides, and the bone marrow was flushed with 5 ml ice-cold PBS. The cell suspension was centrifuged at $250\ \text{g}$ for 5 min, and the remaining cell pellet was resuspended for total leukocyte counts in Neubauer chambers.

Cytokine Production

IL-10, IL-5 and IL-4 levels were determined in supernatants of lung explant cultures [28]. Briefly, lungs were flushed with 20 ml PBS by inserting a cannula into the right atrium to remove the intravascular blood. Four small pieces of lung were chopped randomly, placed into 24-well plates and cultured with 1 ml Dulbecco's modified Eagle's medium containing 0.5% penicillin-streptomycin ($10,000\ \text{IU}$ to $10\ \text{mg/ml}$) for 24 h at 37°C with 5% CO_2 . Results were expressed as picogram of cytokine per milligram of dry lung tissue. IL-10, IL-5 and IL-4 were quantified using ELISA kits purchased from R&D Systems (Minneapolis, Minn., USA). Samples were analyzed in duplicate using standard curves according to the manufacturer's instructions.

L-Selectin Expression

Briefly, $100\ \mu\text{l}$ whole blood and BAL cells (1×10^6 cells/ $100\ \mu\text{l}$) were used. Erythrocytes were lysed with 2 ml sterile 0.2% NaCl for 20 s, and the isotonicity was immediately restored by adding 2 ml sterile 1.6% NaCl. Samples were then centrifuged at $250\ \text{g}$ for 5 min and cell pellets resuspended in $100\ \mu\text{l}$ cold PBS with the anti-CD62-L monoclonal antibody conjugated with phycoerythrin for 60 min according to the manufacturer's specifications. A flow cytometer (FACSCalibur®, Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) interfaced with a Macintosh G4 computer was used to sort cells. L-selectin expression was determined by gating cell types according to their properties on forward scatter and side scatter plots, as described elsewhere [29]. Fluorescence data were collected on a log scale, and red fluorescence was measured at $585 \pm 42\ \text{nm}$ (FL2 detector); data were analyzed using FlowJo software.

In vitro Tracheal Responsiveness to Methacholine

Tracheal rings were mounted for the measurement of isometric force by means of two steel hooks in a 30-ml organ bath [30]. Force contraction was registered using a force displacement transducer and a chart recorder (Powerlab®, Labchart, AD Instruments). Briefly, tracheal rings were suspended in an organ bath filled with KH solution at 37°C . Tissues were continuously aerated with 95% O_2 and 5% CO_2 . After a 40-min equilibrium period, tracheal tension was adjusted to 1 g. Tissue viabilities were assessed by replacing the KH solution with a 60 mM KCl buffer in the bath and comparing the contraction force generated with those obtained previously. Next, cumulative dose-response curves to methacholine were constructed [31]. Methacholine, a non-specific muscarinic receptor agonist, was used to induce tracheal constriction at concentrations ranging from 10^{-9} to $10^{-2}\ \text{M}$.

Mast Cell Degranulation

Lungs were excised, fixed in 2% paraformaldehyde and 2% glutaraldehyde in PBS, pH 7.4, for 24 h at 4°C . Lungs were then fragmented, washed, dehydrated in ethanol, clarified in xylene and embedded in Histosec™ (Merck, USA). Sections were cut ($3\ \mu\text{m}$; HYRAX M60, Zeiss, GR), mounted on slides and stained with a 0.25% toluidine blue and 0.25% borate sodium solution. Quantification of mast cell number and activation in the lung tissue was performed using a high-power objective ($\times 40$) through the measurement of the area of analysis with Axiovision software.

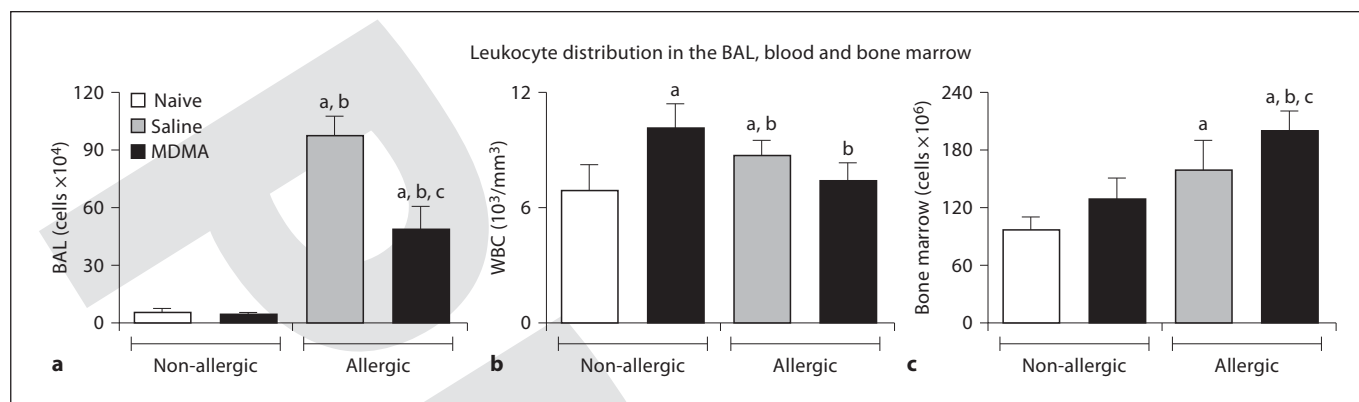


Fig. 2. Effects of MDMA on leukocyte distribution in OVA-sensitized mice. BAL count (a), white blood cell (WBC) count (b), and bone marrow count (c). Data are presented as the mean $\pm$ SD (n = 10 mice/group). ^a p < 0.05 compared to naive non-allergic mice; ^b p < 0.05 compared to MDMA non-allergic mice; ^c p < 0.05

compared to saline-treated allergic mice. Statistics were calculated by one-way ANOVA followed by Tukey-Kramer or KW followed by Dunn's multiple comparison tests (for parametric and non-parametric data, respectively).

(Carl Zeiss Canada Ltd., Toronto, Ont., Canada). Data were reported as cells/mm², and at least 10 distinct sections per mouse were analyzed.

Statistical Analysis

GraphPad Prism 5.0 (GraphPad Softwares, Inc.) was used for statistical analysis. Parametric data were analyzed by one-way ANOVA followed by the Tukey-Kramer multiple comparison test. Kruskal-Wallis (KW) followed by Dunn's multiple comparison tests were used to analyze non-parametric data. In all experiments, p < 0.05 was considered significant. Data are presented as the mean $\pm$ SD.

Results

MDMA Alters Leukocyte Distribution in BAL, Bone Marrow and Blood and Reduces Inflammation in OVA-Sensitized Mice

First, we tested if MDMA treatment would change leukocyte distribution and, as a consequence, modify lung inflammation. Importantly, OVA sensitization was effective in inducing lung inflammation, as can be observed by the number of leukocytes found in the BAL (KW = 33.132; p < 0.05). We also observed that MDMA treatment alone did not alter leukocyte migration into the lung (KW = 33.132; p < 0.05) (fig. 2a). However, in OVA-sensitized mice, MDMA treatment decreased leukocyte migration into the lung (KW = 33.132; p < 0.05) (fig. 2a).

In blood, we observed that MDMA treatment increased circulating leukocytes ($F_{3,36} = 17.643$; p < 0.001)

(fig. 2b) compared to non-allergic mice. In OVA-sensitized mice, an increase in the number of circulating leukocytes was observed; however, there were fewer cells than what was observed in non-allergic MDMA-treated mice ($F_{3,36} = 17.643$; p < 0.001) (fig. 2b). MDMA treatment of OVA-sensitized mice precluded the increase in leukocyte number ($F_{3,36} = 17.643$; p < 0.001) (fig. 2b).

In bone marrow, we observed that OVA sensitization increased leukocyte counts (KW = 26.677; p < 0.01) compared to non-allergic mice. Also, MDMA treatment in OVA-sensitized mice induced an increase in bone marrow cellularity, as the number of cells was higher than that measured in the OVA-sensitized group (KW = 26.677; p < 0.001) (fig. 2c).

Finally, we analyzed lung morphology in order to confirm the occurrence of lung inflammation. Microscopic analysis revealed that MDMA-treated mice had reduced leukocyte infiltration into the pulmonary parenchyma (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000334098).

MDMA Modifies Th2 Cytokine Production in the Lung of OVA-Sensitized Mice

We showed that MDMA was able to alter leukocyte distribution and to reduce inflammation. We next investigated whether MDMA could change Th2 cytokine production in lungs of OVA-sensitized mice. We observed that lung explants from OVA-sensitized mice had increased production of both IL-4 ($F_{2,21} = 23.91$; p < 0.0001) and IL-5 ($F_{2,21} = 28.18$; p < 0.0001) (fig. 3a, b). MDMA-

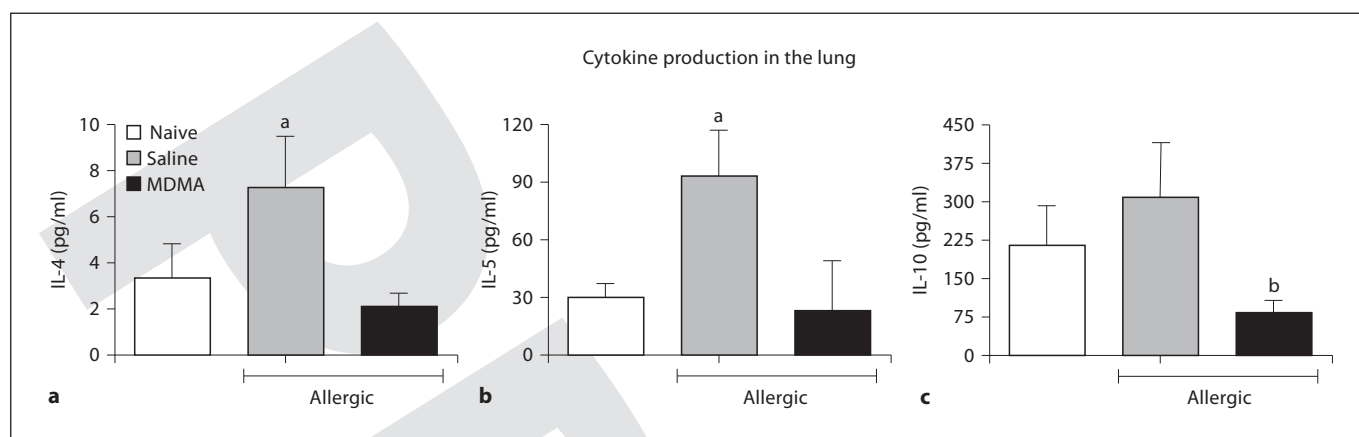


Fig. 3. Effects of MDMA on Th2-like cytokines of lung explants. IL-4 (a), IL-5 (b) and IL-10 (c) production in lung explant supernatants. Data are presented as the mean $\pm$ SD (n = 8 mice/group). ^a p < 0.05 compared to naive non-allergic mice; ^b p < 0.05 compared to saline-treated allergic mice.

pared to saline-treated allergic mice. Statistics were calculated by one-way ANOVA followed by Tukey-Kramer's multiple comparison test (parametric data).

treated mice had a decrease in both cytokines at the same level in non-allergic mice (fig. 3a, b). OVA-sensitized mice did not have an increase in the level of IL-10 compared to non-allergic mice, although a tendency was observed. MDMA treatment of OVA-sensitized mice decreased IL-10 production in the lung explant compared to both non-allergic and OVA-sensitized mice ($F_{2,21} = 16.55$; $p < 0.0001$) (fig. 3c).

MDMA Decreases L-Selectin of Leukocytes in the Lung and Blood of OVA-Sensitized Mice

After analyzing Th2 cytokines, we analyzed the expression of L-selectin (CD62-L) in leukocytes in both BAL and blood in order to clarify the effects of MDMA on leukocyte distribution. Figure 4 shows a schematic representation of the data analyzed by flow cytometry.

We observed that CD62-L expression in lymphocytes and monocytes in blood was not altered by OVA sensitization (table 1). Importantly, we observed that MDMA treatment decreased monocyte expression of CD62-L ($F_{2,27} = 8.665$; $p < 0.05$) (table 1). However, OVA sensitization in both 0.9% saline- and MDMA-treated mice reduced CD62-L expression in a subset of neutrophils compared to non-allergic mice (KW = 9.974; $p < 0.01$ and 0.05, respectively). In BAL lymphocytes, we observed that CD62-L expression was not altered; however, we observed that OVA sensitization increased CD62-L expression in a subset of neutrophils (KW = 14.720; $p < 0.01$) and that MDMA treatment reduced such an increase (KW = 14.720; $p < 0.05$) (table 1).

Table 1. Effects of MDMA on L-selectin (CD62-L) in OVA-sensitized mice

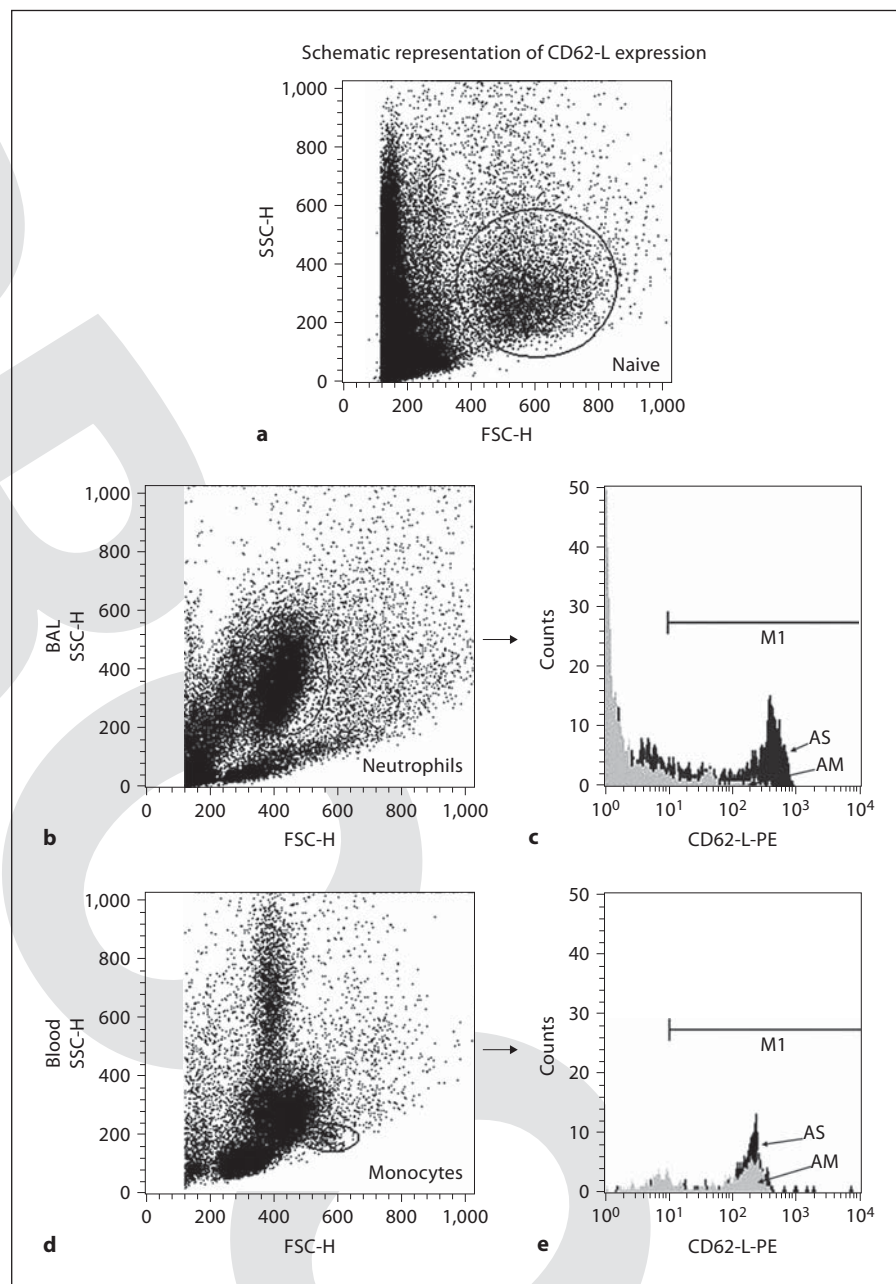
	Naive	Allergic saline	MDMA
<i>Blood</i>			
Lymphocytes (MFI)	68.7 $\pm$ 9.6	65.3 $\pm$ 6.5	63.6 $\pm$ 3.8
Monocytes (MFI)	170.8 $\pm$ 14.8	163.9 $\pm$ 11.4	145.5 $\pm$ 16.8 ^{a, b}
Neutrophils (MFI)	70.4 $\pm$ 15.5	38.9 $\pm$ 4.6 ^a	43.1 $\pm$ 12.7 ^a
<i>BAL</i>			
Lymphocytes (MFI)	21.0 $\pm$ 1.6	19.3 $\pm$ 4.0	21.1 $\pm$ 1.6
Neutrophils (MFI)	13.4 $\pm$ 12.6	124.6 $\pm$ 77.8 ^a	54.6 $\pm$ 33.6

Data are expressed as the mean $\pm$ SD (n = 10 mice/group). MFI = Mean fluorescent intensity. ^a p < 0.05 compared to naive non-allergic mice; ^b p < 0.05 compared to saline-treated allergic mice. Statistics were calculated by one-way ANOVA followed by Tukey-Kramer or KW followed by Dunn's multiple comparison tests (for parametric and non-parametric data, respectively).

The Glucocorticoid Receptor Antagonist RU-486 Partially Prevents MDMA Effects on BAL Leukocyte Migration

Due to prior experience with amphetamine in a rodent model of asthma [27], we decided to analyze the role of glucocorticoid hormones on leukocyte migration into the lung. As previously reported, we observed that MDMA treatment in OVA-sensitized mice decreased the number of leukocytes in BAL (KW = 29.832; $p < 0.001$).

Fig. 4. Representative dot plots and histograms used in the analyses of CD62-L expression. Dot plot of BAL harvested from naïve non-allergic mice (**a**); dot plot of BAL harvested from OVA-sensitized allergic mice (**b**); comparative histogram of BAL from both saline-allergic mice and MDMA-allergic mice (**c**); dot plot of whole blood harvested from OVA-sensitized allergic mice (**d**); comparative histogram of blood from saline-treated allergic mice (AS) and MDMA-treated allergic mice (AM) (**e**).



Interestingly, pretreatment with RU-486 partially reversed this decrease in MDMA-treated mice ($KW = 29.832$; $p < 0.001$) (table 2).

MDMA Decreases Airway Reactivity to Methacholine in vitro

Based on all the results obtained, we analyzed airway reactivity to methacholine in vitro. We observed that OVA-sensitized mice had an increase in tracheal reactivity to methacholine ($F_{3,12} = 11,751$; $p < 0.05$) (fig. 5). We also ob-

served that MDMA treatment in OVA-sensitized mice decreased tracheal reactivity ($F_{3,12} = 11,751$; $p < 0.001$) (fig. 5). Interestingly, pretreatment with methysergide did not prevent this decrease ($F_{3,12} = 11,751$; $p < 0.05$) (fig. 5).

MDMA Decreases Pulmonary Mast Cell Degranulation

Finally, we analyzed the effects of MDMA on mast cell degranulation in order to better understand the results of tracheal reactivity. Analysis of the non-allergic group in-

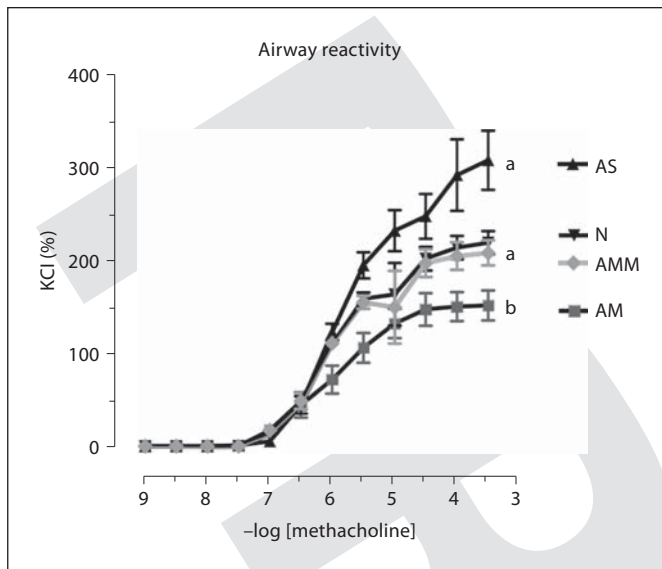


Fig. 5. Effects of MDMA on tracheal hyperresponsiveness in OVA-sensitized mice. A methacholine concentration-effect curve was constructed using an organ bath system. AS = Saline-treated allergic mice; N = naive non-allergic mice; AMM = methysergide + MDMA-treated allergic mice; AM = MDMA-treated allergic mice. Data are expressed as the mean $\pm$ SD ($n = 6$ mice/group). ^a $p < 0.05$ compared to naive non-allergic mice; ^b $p < 0.05$ compared to saline-treated allergic mice. Statistics were calculated by one-way ANOVA followed by Tukey-Kramer's multiple comparison test (parametric data).

indicated that animals did not have mast cell degranulation in the lung parenchyma (0% degranulation) (table 3; fig. 6). However, mast cell degranulation was observed in all cells analyzed (100% degranulation) in OVA-sensitized mice ($F_{2,20} = 28.29$; $p < 0.001$) (table 3; fig. 6). On the other hand, MDMA treatment in OVA-sensitized mice reduced this degranulation by 80% (20% degranulation; $F_{2,20} = 28.29$; $p < 0.001$) (table 3; fig. 6). A representative microscopic photo is shown in the supplementary material (online suppl. fig. 2).

Discussion

MDMA studies on humans and laboratory animals have become a topic of great interest because the use of MDMA has increased exponentially in the past decades. MDMA has been described to induce immunosuppressive effects. We report here that MDMA given prior to OVA challenge in OVA-sensitized allergic mice is able to reduce lung inflammation and airway reactivity.

Table 2. Effects of HPA axis activation on leukocyte migration into the lung

	Allergic		Allergic + RU-486	
	saline	MDMA	saline	MDMA

Total, cells $\times 10^5$ 84.0 ± 8.8 40.5 ± 19.9^a 85.7 ± 16.9^b 63.0 ± 7.4

Data are expressed as the mean $\pm$ SD ($n = 10$ mice/group). ^a $p < 0.05$ compared to saline-treated allergic mice; ^b $p < 0.05$ compared to MDMA-treated allergic mice. Statistics were calculated by KW followed by Dunn's multiple comparison test (non-parametric data).

Table 3. Effects of MDMA on mast cell degranulation in OVA-sensitized mice

	Naive	Allergic	
		saline	MDMA

Total, cells/mm ²	1.1 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
Intact, cells/mm ²	1.1 ± 0.1	0.0 ± 0.0^a	1.2 ± 0.1^b
Degranulated, cells/mm ²	0.0 ± 0.0	1.2 ± 0.1^a	0.3 ± 0.1^b
Degranulation, %	0	100	20

Data are expressed as the mean $\pm$ SD ($n = 7-8$ mice/group). ^a $p < 0.05$ compared to naive non-allergic mice; ^b $p < 0.05$ compared to saline-allergic mice. Statistics were calculated by one-way ANOVA followed by Tukey-Kramer's multiple comparison test (parametric data).

Initially, we found that MDMA decreases leukocyte migration into the lungs in OVA-sensitized mice, as shown by the BAL count. We have previously shown that amphetamine decreases leukocyte migration into lungs of OVA-sensitized rats; specifically, it was shown that amphetamine decreased BAL eosinophil, macrophage and neutrophil counts [27]. These data indicate a possible MDMA-induced anti-inflammatory effect, a fact corroborated by the lung histological analysis, which clearly showed a reduced number of leukocytes in lungs of MDMA-treated mice. Together, these data suggest that MDMA has an anti-inflammatory role in a model of allergic lung inflammation. Although the literature postulates a possible increase in the Th2 response after MDMA treatment [12], we showed that MDMA treatment previous to OVA challenge in OVA-sensitized mice is able to decrease inflammatory parameters. It is noteworthy that these effects on lung inflam-

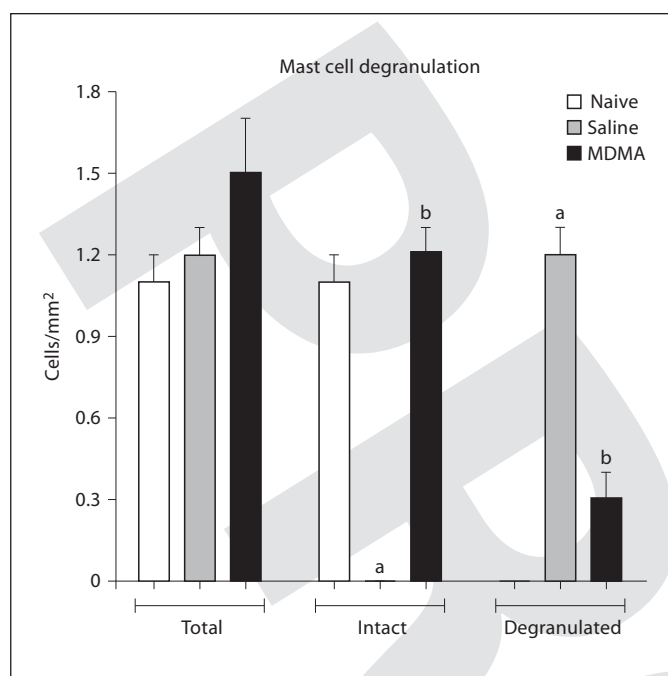


Fig. 6. Effects of MDMA on mast cell degranulation in OVA-sensitized mice. Total, intact and degranulated mast cells were counted after each of the indicated treatments. Data are expressed as the mean $\pm$ SD ($n = 6$ mice/group). ^a $p < 0.05$ compared to naive non-allergic mice; ^b $p < 0.05$ compared to saline-treated allergic mice. Statistics were calculated by one-way ANOVA followed by Tukey-Kramer's multiple comparison test (parametric data).

mation rely on the phase of the immune response taking place. MDMA was administered in the effector phase of the allergic response, i.e. prior to OVA challenge, and this should be taken into account when interpreting the findings.

Based on results obtained, we tested if MDMA would change Th2-like cytokine levels, which are extremely important not only for the development of allergic responses, but also for the effector response to the antigenic allergen. We found that MDMA in OVA-sensitized mice decreases the concentration of IL-4, IL-5 and IL-10 in lung explants. These data indicate that MDMA was able to decrease two of the most important cytokines in the establishment of the inflammation in allergic responses (IL-4 and IL-5) and one of the most important cytokines responsible for regulating immune responses (IL-10). IL-4 is known to induce the commitment of Th0 cells towards the Th2 lineage [32] and to induce B-cell class switching to produce IgE [33]. Moreover, IL-4 induces the release of proinflammatory cytokines and the

expression of adhesion molecules, which facilitates leukocyte migration into the lung [34]. Notably, MDMA-treated mice had reduced levels of IL-4, which seems to be involved in the reduction of CD62-L expression, as both cytokines were diminished in this experiment. Leukocyte migration into inflamed tissues involves complex interactions of leukocytes with the endothelium through regulated expression of surface adhesion molecules. L-selectin (CD62-L) acts as a 'homing receptor' for leukocytes to enter secondary lymphoid tissues via high endothelial venules. Ligands present on endothelial cells bind to leukocytes expressing CD62-L, slowing leukocyte trafficking through the blood and facilitating entry into a secondary lymphoid organ at that point [35]. Indeed, we observed that CD62-L expression is reduced not only in blood monocytes, but also in BAL neutrophils. Consistent with our data, it has recently been shown that MDMA treatment decreased ICAM production [12]. Moreover, it is possible that in our experimental protocol, other adhesion molecules such as ICAM and MAC-1 might also be diminished. These data suggest that MDMA might lead to reduced expression of adhesion molecules by decreasing IL-4 production, which in turn would reduce leukocyte migration into the lung. IL-5 is known to induce a selective differentiation of eosinophil lineage [36]. We showed that MDMA decreases the BAL eosinophil count, which is likely to be a consequence of decreased IL-5 production, which in turn would lead to reduced eosinophil production and decreased chemoattractant molecules released by mast cells and T cells. These responses would decrease eosinophil/neutrophil recruitment to the site of inflammation [37, 38]. These hypotheses were investigated by showing that MDMA-treated mice have decreased IL-5 production in lung explant and degranulation of mast cells.

IL-10 is a powerful anti-inflammatory cytokine [39]. We showed that MDMA decreases IL-10 production in lung explants. Therefore, we hypothesize that MDMA decreases all other proinflammatory parameters reported here, thereby leading the organism to a state where IL-10 production would be unnecessary to regulate the immune response. It is also possible that MDMA decreases IL-10 production due to its immunosuppressive actions, regardless of the character of the cytokines, i.e. proinflammatory, anti-inflammatory and/or regulatory. It has been reported that MDMA treatment increases production of IL-4 and IL-10 [20–22, 24]. Differences in methodologies, experimental protocols, or the phase of the immune response in the animals receiving MDMA might account for these variations. The data from stud-

ies using the lung explant technique are interesting because they allow the analysis of the continued generation of mediators released by the lung, i.e. analysis of the functional state not only of the lung structural cells, but also of cells recruited to the lung. More importantly, events were analyzed in the absence of influences of the regulating inflammatory mechanisms observed *in vivo*, which allow us to study the local control of inflammation.

In our work, MDMA was able to reduce mast cell degranulation in OVA-sensitized mice by 80%. Upon activation, mast cells release preformed granules containing histamine, serotonin, eicosanoids, free radicals and Th2-like cytokines [33]. These data are relevant because they might explain the MDMA anti-inflammatory response involving leukocyte migration into the lung, decreased Th2-cytokine production and the effects on airway hypersensitivity. We showed that MDMA decreases tracheal reactivity to methacholine *in vitro*. It is known that histamine and serotonin induce the contraction of airway smooth muscle, mucus secretion and vasodilatation [40]; therefore, we believe that by decreasing histamine and serotonin release, MDMA would also decrease airway reactivity. Although we have not tested it, MDMA may also decrease IgE serum levels, which could provide an alternative explanation for the observed decrease in mast cell degranulation and, as a consequence, the reduced airway reactivity. However, we used a serotonin receptor antagonist (methysergide) in MDMA-treated mice, and we were unable to see a reversion of methacholine-induced airway reactivity; therefore, serotonin seems not to be involved in this process.

Stressors are known to reduce immune system activity. We have shown that MDMA (10 mg/kg) increased HPA axis activity, as shown by an increase in serum levels of corticosterone, probably through the activation of the noradrenergic pathway in the hypothalamus [11]. The increase in corticosterone serum levels persisted up to 6 h after MDMA treatment (online suppl. fig. 3). Therefore, it is not surprising that the effects observed after MDMA treatment may involve HPA axis activation. We have shown that chemical stressors, such as cyhalothrin [41–44] and amphetamine [27, 45, 46], were able to decrease several parameters of the immune system. In fact, amphetamine was able to change leukocyte distribution in lungs in OVA-sensitized rats [27]. Similarly, acute amphetamine treatment, given prior to OVA challenge, decreased BAL leukocyte count. Importantly, metyrapone, an inhibitor of corticosterone synthesis, given prior to amphetamine treatment partially reversed

this effect [27]. Furthermore, we also showed in this work that RU-486, an antagonist of the glucocorticoid receptor, given prior to MDMA treatment partially abrogated the inhibition of leukocyte migration into the lung in OVA-sensitized mice. Therefore, we suggest that both MDMA- and amphetamine-induced HPA axis activation are at least partially responsible for the inhibition of leukocyte migration into the lung. In support of the present data, we recently showed that OVA-sensitized rats treated with amphetamine had reduced cell recruitment into the lung, vascular permeability and cellular expression of adhesion molecules (ICAM-1 and Mac-1). Also, amphetamine treatment decreased lung explant levels of IL-4 while increasing IL-10 in OVA-sensitized rats [46]. Moreover, tracheal responsiveness to methacholine was reduced by amphetamine treatment. Together, these data reinforce our hypothesis that MDMA acts as a chemical stressor and releases corticosterone through HPA axis activation, thereby decreasing cellular migration into lungs of OVA-sensitized rats and mice. Consistent with our data, corticosterone is known to exert potent anti-inflammatory and immunosuppressive effects that include a reduction in both expression of adhesion molecules [47] and histamine release from rat peritoneal mast cells [48].

Together, the previous and present data strongly suggest a neuroimmune mechanism as part of the effects of MDMA on lung inflammatory response and cell recruitment to the lung in allergic animals. It is likely that such effects rely at least in part on HPA axis activation and corticosterone serum levels. Nonetheless, care should be taken when extrapolating the data of these studies to humans. Some discrepancies have already been reported between the effects of drugs in animal models of asthma and human asthmatics [49].

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