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Conflict of Interest

The authors have no financial conflicts of interest.

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Respiratory syncytial virus increases eosinophil extracellular traps in a murine model of asthma

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ABSTRACT

Background: Respiratory viral infections are the leading cause of asthma exacerbations. Eosinophil activation results in the formation of eosinophil extracellular traps (EETs), which release web-like structures of DNA and proteins that bind, disarm and extracellularly kill pathogens.

Objective: We investigated whether the respiratory syncytial virus (RSV) *in vitro* could induce EETs in bronchoalveolar lavage fluid eosinophils in a murine model of asthma.

Methods: BALB/cJ mice (6–8 weeks old) were sensitized with 2 subcutaneous injections of ovalbumin (20 µg) on days 0 and 7, followed by three intranasal challenges with ovalbumin (100 µg) on days 14, 15, and 16 of the protocol. The control group received Dulbecco's phosphate-buffered saline. Bronchoalveolar lavage fluid eosinophils of ovalbumin group or control group were stimulated with RSV (10³ PFU/mL) *in vitro* for 3 hours. After that, culture supernatant was collected to perform the analyses proposed in this study.

Results: We verified an increase in extracellular DNA concentration in bronchoalveolar lavage fluid eosinophils from ovalbumin group stimulated with RSV (10³ PFU/mL) *in vitro*, which was confirmed by confocal microscopy. We demonstrated that most cells are negative for annexin V and propidium iodide in all groups evaluated. Also, RSV *in vitro* decreased interferon-γ in culture supernatant when compared to the ovalbumin group.

Conclusion: In this study, we demonstrated for the first time that RSV *in vitro* induces EETs formation in eosinophils from asthmatic mice.

Keywords: Asthma; Inflammation; Eosinophil peroxidase; Extracellular traps; Respiratory syncytial viruses

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INTRODUCTION

During episodes of asthma exacerbation, respiratory viral infection can be detected in nearly 80%–85% of school-age children [1]. Respiratory syncytial virus (RSV) is a main cause of bronchiolitis in children and may increase susceptibility to the development of asthma [2]. Eosinophil activation results in the eosinophil extracellular traps (EETs) formation which is capable of killing bacteria in the extracellular space [3]. In this context, our group described that EETs are released in bronchoalveolar lavage fluid (BALF) and lung tissue of asthmatic mice [4-7]. Currently, there are no available data in the literature regarding the production of EETs induced by RSV. Therefore, we investigated whether RSV *in vitro* induces EETs in BALF cells in a murine model of asthma.

MATERIALS AND METHODS

Sensibilization and challenge

Six- to eight-week-old specific pathogens-free female BALB/cJ mice weighing approximately 20 g from the Center for Experimental Biological Models (CeMBE, PUCRS) were used in the experiments. Mice were sensitized with 2 subcutaneous injections of ovalbumin (20 µg) (OVA - Grade V, Sigma, St. Louis, MO, USA) on days 0 and 7, followed by intranasal challenges with OVA (100 µg) on days 14, 15, and 16 of the protocol [5]. The control group received only Dulbecco's phosphate-buffered saline.

Bronchoalveolar lavage fluid

On day 17 of the protocol, the animals were anesthetized with ketamine (0.4 mg/g) and xylazine (0.2 mg/g) and the trachea was cannulated with a blunt needle. BALF was performed with phosphate-buffered saline (PBS) 2% fetal bovine serum (FBS) (1 mL). BALF was centrifuged, and the pellet was resuspended in PBS 2% FBS. For the confirmation of the experimental model of asthma induction, total cell counts were determined in a Neubauer chamber (BOECO, Hamburg, Germany) and differential cell counts of BALF was determined by cytospin preparations stained with H&E (Panótico Rápido - Laborclin, Brazil).

Virus culture and eosinophils stimulation

The production of RSV A2 strain (kindly donated by Dr. Fernando Polack, Vanderbilt University School of Medicine, Nashville, TN, USA) was obtained in VERO cells (ATCC CCL-81, ATCC, Manassas, VA, USA) with 2% FBS at 37°C under 5% CO₂. BALF cells (2×10^5 /mL) were stimulated with RSV (10^3 PFU/mL) for 3 hours at 37°C under 5% CO₂ while others remained unstimulated. The mentioned RSV concentration was used because higher concentrations (10^4 – 10^6 PFU/mL) did not increase extracellular DNA traps. Thus, we showed that RSV induces EETs release in a concentration-dependent manner. We thought that higher concentrations are cytotoxic to eosinophils.

Quantification of extracellular DNA traps

Extracellular DNA traps were quantified in BALF cells supernatants stimulated with RSV (10^3 PFU/mL) or unstimulated using Quant-iT dsDNA HS (Invitrogen, Carlsbad, CA, USA) and measured in the Quibit 2.0 fluorimeter (Invitrogen), according to the manufacturer's recommendations. Visualization of EETs by fluorescence microscopy BALF cells (2×10^5 /mL) were stimulated with phorbol-12-myristate-13-acetate (50 nM). After that, cells stimulated with RSV (10^3 PFU/mL) and unstimulated ones were incubated for 3 hours, fixed with 4%

paraformaldehyde and stained with anti-eosinophil peroxidase (EPO) and anti-histone H2B (1:250; Santa Cruz Biotechnology, Dallas, TX, USA) for 45 minutes. Later, cells were incubated with fluorescein isothiocyanate (1:100; Santa Cruz Biotechnology) and alexa fluor 633 (1:100; Invitrogen) for 30 minutes. Next, the cells were stained with Hoechst 33342 DNA dye (1:2,000; Invitrogen) for 4 minutes. Confocal images were taken on a Leica TCS-SP8 exciter microscope (Leica Microsystem, Wetzlar, Germany).

Determination of cell death

Cell death in BALF was analyzed by annexin V and propidium iodide (PI) (BD Pharmingen, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cells were incubated with annexin V and PI and analyzed by flow cytometry (FACS Canto II, BD Bioscience). Data were analyzed using FlowJo software version X.0.7 (TreeStar, Woodburn, Oregon, USA).

Cytokine levels

Interleukin-4 (IL-4) and interferon- γ (IFN- γ) levels in culture supernatant were measured by the multiplex technique (MAGPIX TECHNOLOGY, MILLIPIX MAP, Merck Millipore, Burlington, MA, USA) and analyzes were performed on the Magpix equipment (Millipore, Darmstadt, Germany). Results were analyzed using the xPONENT Solutions software (Luminex Corp., Austin, TX, USA).

EPO activity

EPO activity was measured in culture supernatant and was determined through colorimetric assay based on the oxidation of O-phenylenediamine (Sigma) in the presence of hydrogen peroxide [8].

Statistical analysis

Data were expressed as mean \pm standard deviation. Results were analyzed using GraphPad Prism ver. 5 (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance followed by the Tukey *post hoc* test, or Student *t* test was used. A *p* value less than 0.05 was considered statistically significant.

Ethics

This study was approved by the Animal Ethics Committee (CEUA) of our Institution (Protocol number: 14/00387).

RESULTS

Firstly, we evaluate BALF cells in order to experimental asthma model establishment. We observed that OVA group had a significant increase in the total cell counts, as well as an increase in eosinophils, neutrophils, macrophages, and lymphocytes cell counts compared to the control group (**Fig. 1A-E**). There are more than 70% of eosinophils in BALF in OVA group.

Later, we investigated whether RSV in a concentration dependent manner (10^3 – 10^6 PFU/mL) would induce the release of EETs in BALF cells of asthmatic mice. RSV was able to stimulate EETs only in RSV 10^3 PFU/mL concentration (**Fig. 2A**). Thus, we decided to stimulate BALF cells from asthmatic mice with RSV 10^3 PFU/mL.

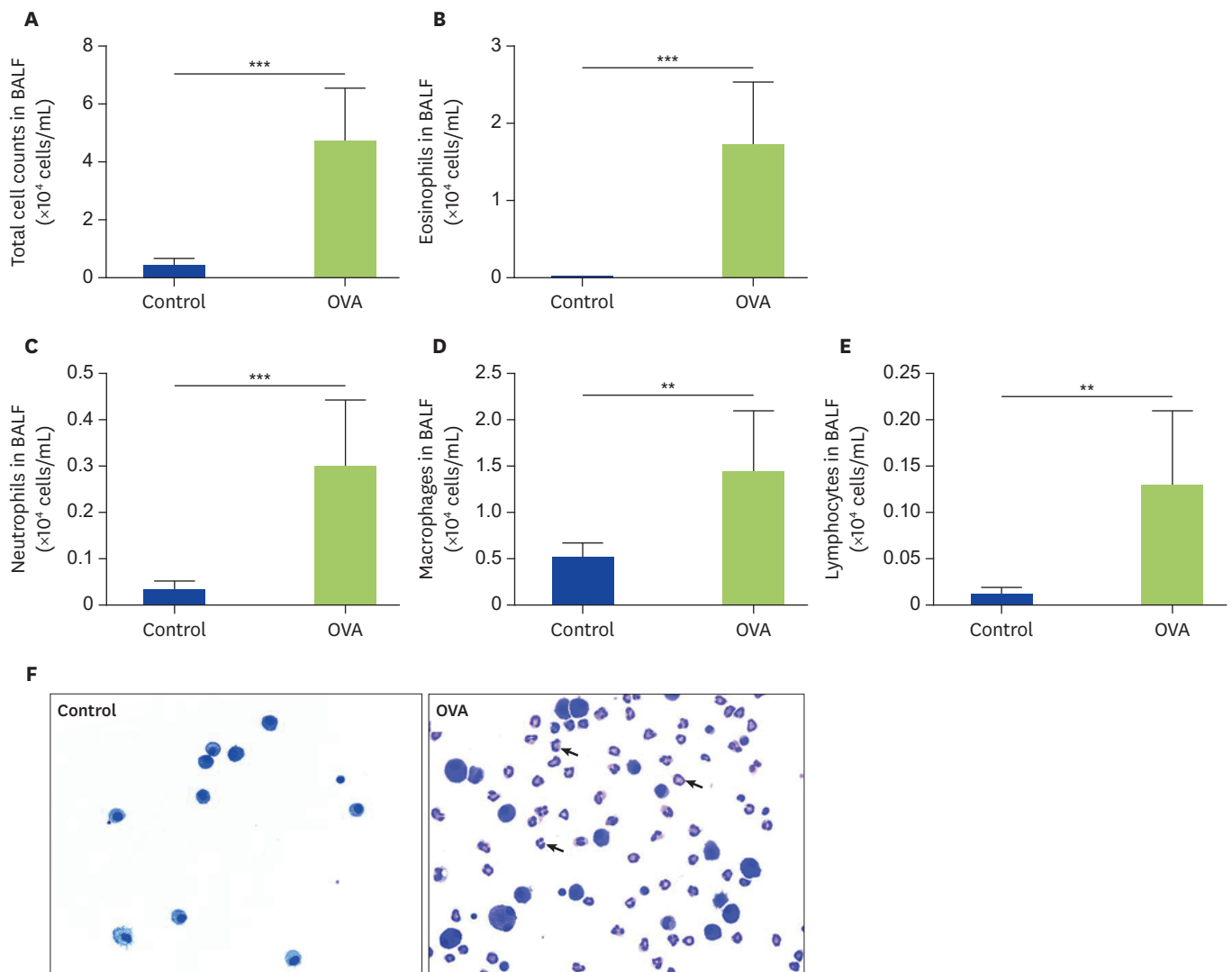


Fig. 1. Ovalbumin-induced airway eosinophilic inflammation. (A) Total cell counts in BALF. (B-E) Differential cell counts in BALF (eosinophils, macrophages, neutrophils, and lymphocytes). (F) Representative illustration of BALF cells stained with H&E ($\times 400$, arrows indicate eosinophils). Results are expressed as mean \pm standard deviation, for 7–10 animals in each group, of 3 independent experiments, ** $p < 0.01$, *** $p < 0.001$. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin.

Eosinophils from BALF-asthmatic mice stimulated with RSV increased extracellular DNA in the culture supernatant when compared to the OVA group (Fig. 2B). In confocal microscopy, we observed multiple extracellular DNA extrusions in eosinophils colocalized with EPO but not colocalized with histone H2B in BALF cells in the OVA group stimulated with RSV (Fig. 2C). EETs release with RSV stimulation was not due to apoptosis showing that most cells were negative for annexin V and PI (Fig. 2D).

Finally, we showed that in culture supernatant from BALF cells stimulated with RSV from asthmatic mice there was no alteration in IL-4 levels (Fig. 3A). On the other hand, we showed a decrease in IFN- γ levels when compared to the OVA group (Fig. 3B). In BALF cells from the OVA group there was an increase in EPO activity but we could not see any alteration in this parameter when stimulated with RSV *in vitro* (Fig. 3C).

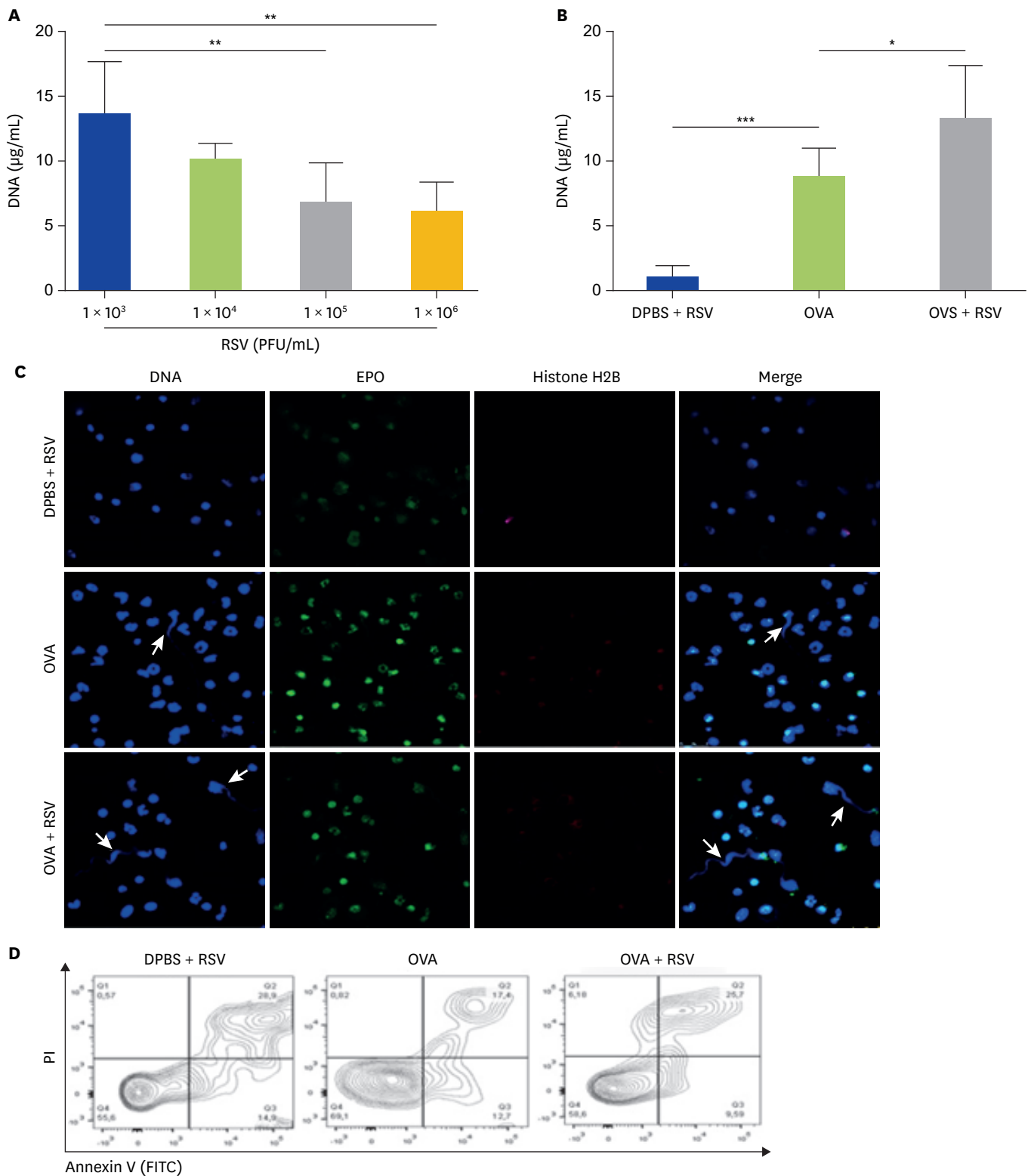


Fig. 2. RSV *in vitro* induces EETs colocalized with EPO in BALF cells from asthmatic mice. (A) Effect of different concentrations of RSV (10^3 – 10^6 PFU/mL) *in vitro* in extracellular DNA concentration. (B) Extracellular DNA concentration in BALF cells from asthmatic and control mice stimulated with RSV (10^3 PFU/mL) *in vitro* or unstimulated. (C) EETs release in BALF cells from asthmatic and control mice stimulated with RSV (10^3 PFU/mL) *in vitro* or unstimulated ($\times 630$, arrows indicate EETs formation). (D) Analysis of annexin V binding and PI uptake in BALF cells from all groups. Results are expressed as mean \pm standard deviation, for 7–10 animals in each group, of 3 independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. BALF, bronchoalveolar lavage fluid; DPBS, Dulbecco's phosphate-buffered saline; EPO, eosinophil peroxidase; OVA, ovalbumin; PFU, plaque-forming unit; PI, propidium iodide; RSV, respiratory syncytial virus; FITC, fluorescein isothiocyanate.

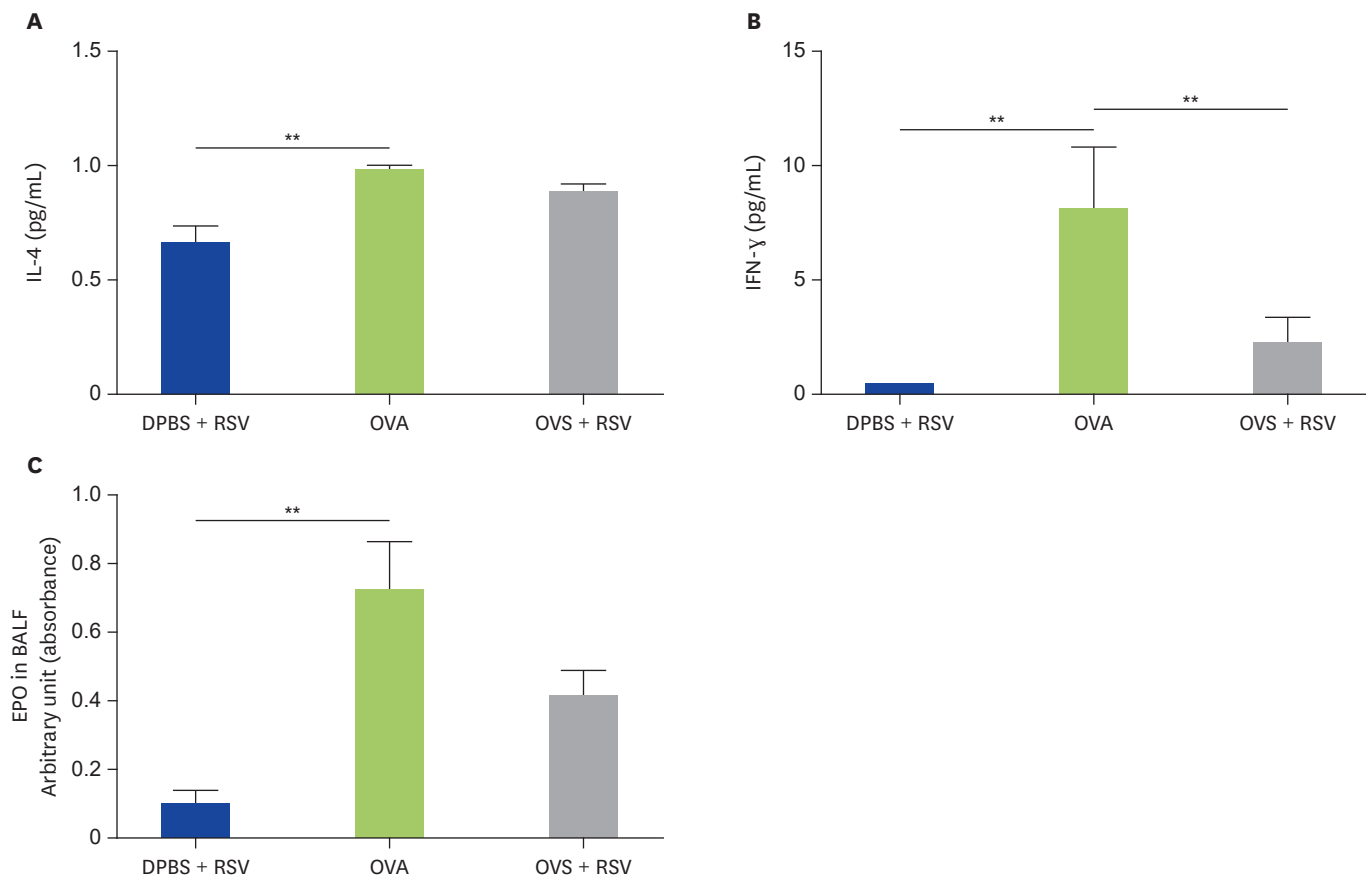


Fig. 3. Effect of RSV in BALF cells from asthmatic mice in cytokines levels and EPO activity. (A) IL-4, (B) IFN- γ , and (C) EPO activity in from BALF cells (2×10^5 /mL) from asthmatic mice stimulated with RSV (10^3 PFU/mL) *in vitro* or unstimulated. Results are expressed as mean \pm standard deviation, for 5 animals in each group, of 3 independent experiments, ** $p < 0.01$. BALF, bronchoalveolar lavage fluid; EPO, eosinophil peroxidase; DPBS, Dulbecco's phosphate-buffered saline; IL-4, interleukin-4; IFN- γ , interferon- γ ; OVA, ovalbumin; RSV, respiratory syncytial virus.

DISCUSSION

EETs are web-like networks of expelled DNA covered with microbicide and cytotoxic proteins [3] which are able to efficiently kill bacteria through DNA traps and may also contribute to lung dysfunction. The first evidence of EETs formation in asthma was in endobronchial biopsies from human atopic asthmatic patients [9]. Moreover, studies have demonstrated that viruses such as the RSV are capable of inducing neutrophil extracellular traps formation [10]. Therefore, we demonstrated for the first time that RSV *in vitro* can induce EETs colocalized with EPO in BALF cells from asthmatic mice. Previous studies of our group have shown that BALF cells produced EETs without showing sign of apoptosis [4-7]. We showed that RSV stimulation *in vitro* did not induce cell death. Furthermore, we demonstrated that EETs were not colocalized with histone H2B, which suggests that DNA released by EETs is of mitochondrial origin. RSV infection enhances T helper type 2 (Th2) cytokine in lung tissue [11]. IL-4 levels were elevated in the OVA group when compared to the control group while that RSV stimulation *in vitro* did not alter IL-4 levels in BALF cells in asthmatic mice. Moreover, RSV *in vitro* reduces IFN- γ levels, a T helper type 1 (Th1) cytokine when compared to the OVA group. Aberle et al. [12] showed that severe RSV bronchiolitis is associated with decreased mRNA IFN- γ expression in peripheral blood. Sanz et al. [13] showed that EPO serum levels are significantly higher in asthmatic patients than in healthy controls. In addition, EPO activity in BALF cells was elevated in the OVA

group when compared to the control group, but the RSV stimulation *in vitro* did not alter this parameter. Taken together our results showed, for the first time, that RSV *in vitro* increases EETs release in BALF cells from asthmatic mice, which probably contributes to airway obstruction and tissue damage in asthma.

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