In Vivo Short-Term Exposure to Residual Oil Fly Ash Impairs Pulmonary Innate Immune Response Against Environmental Mycobacterium Infection

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ABSTRACT: Epidemiological studies have shown that pollution derived from industrial and vehicular transportation induces adverse health effects causing broad ambient respiratory diseases. Therefore, air pollution should be taken into account when microbial diseases are evaluated. Environmental mycobacteria (EM) are opportunist pathogens that can affect a variety of immune compromised patients, which impacts significantly on human morbidity and mortality. The aim of this study was to evaluate the effect of residual oil fly ash (ROFA) pre-exposure on the pulmonary response after challenge with opportunistic mycobacteria by means of an acute short-term *in vivo* experimental animal model. We exposed BALB/c mice to ROFA and observed a significant reduction on bacterial clearance at 24 h post infection. To study the basis of this impaired response four groups of animals were instilled with (a) saline solution (Control), (b) ROFA (1 mg kg^{-1} BW), (c) ROFA and EM-infected (*Mycobacterium phlei*, 8×10^6 CFU), and (d) EM-infected. Animals were sacrificed 24 h postinfection and biomarkers of lung injury and proinflammatory madiators were examined in the bronchoalveolar lavage. Our results indicate that ROFA was able to produce an acute pulmonary injury characterized by an increase in bronchoalveolar polymorphonuclear (PMN) cells influx and a rise in O_2^- generation. Exposure to ROFA before *M. phlei* infection reduced total cell number and caused a significant decline in PMN cells recruitment ($p < 0.05$), O_2^- generation, TNFα ($p < 0.001$), and IL-6 ($p < 0.001$) levels. Hence, our results suggest that, in this animal model, the acute short-term pre-exposure to ROFA reduces early lung response to EM infection. Environ Toxicol 00: 000–000, 2013.

Keywords: environmental mycobacteria; *Mycobacterium phlei*; ROFA; innate immune response; *in vivo* acute mice model

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INTRODUCTION

Nowadays almost any major city in the world is overwhelmed by environmental problems including air pollution, which represents one of the most relevant public health

concerns (Valavanidis et al., 2008; Yang and Omaye, 2009; Olmo et al., 2011). Air pollution, including air particulate matter (PM) and gases certainly interfere with nonspecific and specific lung defenses, thus facilitating the development of pulmonary diseases, such as exacerbation of chronic obstructive pulmonary disease, allergies, and asthma (Ponka, 1991; Schwartz et al., 1991; Rusznak et al., 1994; D'Amato et al., 2005; Halonen et al., 2008; D'Amato, 2011; Patel et al., 2011). The respiratory system is the most important target for air pollution since it is constantly exposed to the external environment. Therefore, exposure to airborne PM can be considered an inescapable part of modern life in urban areas throughout the world.

Among air PM, dust, irritants, and pathogenic and nonpathogenic microorganisms contact the respiratory system every day leading to different outcomes depending on the identity of the substance and the host response. Effective elimination of PM deposited in the respiratory tract is an important defense function to protect the organism from potentially adverse effects of inhaled particles. In addition, environmental mycobacteria (EM), also known as atypical nontuberculous mycobacteria, are a group of human and animal opportunistic pathogens, which have relevant impact on health (Grange et al., 1995; Primm et al., 2004; Castronovo, 2008; Iseman and Marras, 2008; Khatter et al., 2008; Simons et al., 2011). EM infections acquired from diverse environmental reservoirs (water, air, and soil) are not transmitted between humans and its progression to clinical disease requires one or more predisposing host conditions (Grange et al., 1995; Primm et al., 2004; Castronovo, 2008; Iseman and Marras, 2008; Khatter et al., 2008). EM has been isolated from household water, hot tubs, swimming pools, and workplaces, including hospitals (Toma, 1998; Falkinham, 2002; Tichenor et al., 2012). Consequently, humans are most commonly exposed to mycobacteria through aerosols generated from different activities such as drinking, swimming, and bathing. In the early 1900s, tuberculosis (TB): EM disease ratio was probably 100:1 (TB:EM). Back then, EM disease seemed to be masked by TB. On the contrary, nowadays, in suburban areas where the incidence of TB is low, this disease ratio may be nearly reversed (Cook, 2010). Currently, disease caused by EM is not considered a public health problem in countries where its prevalence is unknown or presumably low. However, in different countries, EM is the cause of important clinical problems (Imperiale et al., 2012). Even more, since the first decade of 21st century, indoor and outdoor air pollution has been implicated as an additional risk factor for mycobacterial infections such as TB (Smith, 2002; Lin et al., 2007; Tremblay, 2007).

Hence, in this context, and taking into account that the respiratory tract is the main target in the organism for both, air pollutants and EM infection, air pollution may be considered as an external factor, which in turn can bias individuals to EM infection.

Regarding air pollutants, it is known that, depending on its physicochemical characteristics, PM can alter lung redox metabolism as well as the production of proinflammatory cytokines and mediators of the innate immune response increasing its susceptibility to infection (Li et al., 1997; Olivieri and Scoditti, 2005; Martin et al., 2007). residual oil fly ash (ROFA), derived from fuel oil combustion, presents in its chemical composition sulfates, silicates, and metallic traces (iron, vanadium, and nickel), and has been used as a surrogate for ambient particles to study the biological effect of air pollution (Saldiva et al., 2002; Schroeder et al., 1987). Due to its morphochemical properties, ROFA is able to exert potent adverse health effects (Ghio et al., 2002; Ostachuk et al., 2008).

Because of the limitations on conducting human exposure experiments, the deposition and fate of airborne particles are often studied in animals. The results of such studies can be extrapolated to humans to estimate equivalent doses and subsequent responses. In fact, Antonini et al. (2002) and Roberts et al. (2004) have demonstrated that ROFA exposure reduces microbicide activity of rat alveolar macrophages and increases pulmonary damage and susceptibility to *Listeria monocytogenes* infection. Moreover, we have previously showed that pre-exposure to ROFA alters alveolar macrophage defense mechanisms against EM-infection *in vitro*. We demonstrated that incubation with ROFA before *Mycobacterium phlei* (*M. phlei*) infection lead to a decrease in superoxide anion and TNFα production, two important mediators involved in cell response, and affects phagocyte killing capacity (Delfosse et al., 2012).

Even though, urban pollution and mycobacteriosis produced by EM are emerging problems, to the best of our knowledge, there are no reports of *in vivo* studies describing the effect of air pollutants upon the innate immune response against EM. We hypothesize that ROFA could negatively impact on the host innate immune response against EM-infection *in vivo*. In this context, the aim of this study was to evaluate the biological effect of ROFA pre-exposure on the response to EM-infection in the respiratory system in a mouse experimental model.

MATERIAL AND METHODS

Animals

BALB/c mice (2–3 months of age) were bred at the Faculty of Exact and Natural Sciences, University of Buenos Aires breeding facility and housed in a controlled environment at the School of Science and Technology, National University of General San Martín. The animals received a normal protein diet (15–20%) and water *ad libitum*. The experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (NIH, 1996), the European Commission Directive 86/609/EEC (The Council of European Communities), and the committee

ad hoc of the School of Science and Technology, National University of General San Martín.

Bacterial Strain and Culture Condition

A field isolate of *M. phlei* was selected from the collection of the Biotechnology Institute-INTA. This isolate was previously typed by PCR restriction fragment length polymorphism analysis technique (Delfosse et al., 2012). The strain was grown in Middlebrook 7H9 liquid medium (BD Difco) supplemented with 0.5% (v/v) glycerol. Briefly, cells were grown at 37°C under continuous shaking (200 rpm) to an optical density of 0.5–0.8 at 600 nm (5 days). Then, cells were harvested from the bacterial culture (100 mL), washed three times with phosphate saline buffer (PBS, Sigma-Aldrich) and resuspended in 40 mL of PBS. Passages through syringes (25 and 21 gauge, four times each) were performed to avoid bacterial aggregates. All these steps were performed in polypropylene tubes (Corning). Then, cells were let stand for 10 min and suspensions were aliquoted and conserved at 4°C until use (generally, suspensions were conserved no longer than 72 h). For each experiment, colony forming units (CFU) were determined by serial dilution. *M. phlei* was spread onto Middlebrook 7H10 (BD Difco) agar plates and incubated at 37°C for 4–5 days. After incubation, the colonies were counted to determine CFU.

Animal Exposure to ROFA and *M. phlei* Infection

BALB/c mice were exposed to ROFA particles by intranasal instillation (Southam et al., 2002). Previously, ROFA was suspended in PBS (pH = 7.2–7.4) and sonicated during 10 min (Klein-Patel et al., 2006; Martin et al., 2010). Mice were anesthetized intraperitoneally with 1 mL kg^{-1} body weight (BW) of xylazine (2%) and ketamine (50 mg mL^{-1}) and then were intranasally instilled with 50 μL of ROFA suspension (1 mg kg^{-1} BW) according to Magnani et al. (2013) and Marchini et al. (2012). EM infection was also performed by intranasal instillation (Logan et al., 2008) 24 h post-ROFA exposure. *M. phlei* suspension (50 μL) containing 8×10^6 CFU was delivered. Animals were sacrificed with an overdose of xylazine-ketamine 24 h postinfection (Table I). To evaluate the clearance of *M. phlei*, mice lungs were excised, homogenized in 1 mL sterile PBS and plated onto Middlebrook 7H10 (BD Difco) agar plates. CFU were determined after 4–5 days of incubation at 37°C. In all experiments, 4–6 animals were employed per group unless otherwise stated.

TABLE I. *In vivo* experimental protocol

	Days		
Experimental Groups	1	2	3
Control	–	–	Sacrifice
ROFA	ROFA-exposure	–	Sacrifice
Inf	–	EM-Infection	Sacrifice
ROFA + Inf	ROFA-exposure	EM-Infection	Sacrifice

Animals were divided into four groups: nonexposed/noninfected (Control); exposed to ROFA (ROFA); EM-infected (Inf); and exposed to ROFA and EM-infected (ROFA + Inf).

ROFA was employed as a recognized standard surrogate ambient PM. ROFA, collected at the Boston Edison, Mystic Power, plant number 4, CT, was generously provided by Dr. J. Godleski (Harvard School of Public Health, Boston, MA).

Pulmonary Cells Obtained by Bronchoalveolar Lavage (BAL): Total Cell Number (TCN) and Cell Differential (CD) Quantification

BAL was performed as described elsewhere (Tasat and de Rey, 1987; Delfosse et al., 2012). Briefly, the thoracic cavity was partly dissected; the trachea was cannulated with an 18 G-needle and infused 10 times with 1 mL of cold PBS. The BAL fluid containing the pulmonary cells was immediately centrifuged at 800 × *g* for 10 min at 4°C. TCN and CD were evaluated on the BAL from all animal groups. TCN was determined using a Neubauer chamber. BAL-CD was performed by fixing the cells in methanol and staining them with modified Wright-Giemsa (TINCION-15, Biopur SRL). At least 200 cells were scored in each sample by light microscopy (Nikon Alphaphot-2 V52 microscope).

Evaluation of Superoxide Anion Generation

Intracellular release of superoxide anion (O_2^-) produced by phagocytes was evaluated through quantification of the blue formazan precipitate found in the cells following nitro blue tetrazolium (NBT) reduction, as previously described (Segal, 1974). Pulmonary cells obtained from BAL were incubated with 1% NBT in PBS for 45 min at 37°C under continuous agitation. The number of reactive and nonreactive cells was scored using light microscopy (Nikon Alphaphot-2 V52 microscope). The staining intensity was analyzed semiquantitatively by light microscopy as described elsewhere (Molinari et al., 2000). Three to four animals per treatment and at least 200 cells per individual were evaluated. The percentage of positive cells was calculated.

Protein Content and Proinflammatory Cytokines Determination

Total protein concentration and proinflammatory cytokine production were evaluated on the centrifuged first milliliter of cell-free BAL (800 × *g*, 10 min). Protein concentration was evaluated at 280 nm in a spectrophotometer (Nanodrop ND1000) using bovine serum albumin as a standard (Jussila et al., 2002; Dougan et al., 2011).

For cytokine determinations, the first BAL milliliter was collected, centrifuged to remove residual debris and frozen at −80°C until use. Tumor necrosis factor alpha (TNFα)

was detected by using a commercial specific enzyme linked immunosorbent assay (ELISA) kit (BD OptEIA™ Mouse TNF ELISA Kit). IL-6 was evaluated by ELISA using commercial antibodies (BD Pharmingen). All procedures were carried out according to the manufacturer's instructions. Briefly, ELISA plates (Nunc) were coated with 1:125 cytokine-specific capture antibody diluted in coating buffer (0.1 M Na_2CO_3, pH 9.5) at 4°C overnight. Wells were blocked with PBS containing 10% fetal calf serum for 1 h at room temperature. Cytokine standards and BAL samples were added to wells in triplicate and incubated for 2 h. Following three washes, biotinylated cytokine-specific detection antibody (1:250) was added and incubation was carried out for 1 h. After washing, the detection agent streptavidin-peroxidase was incubated with the tetramethylbenzidine substrate solution for 30 min. Absorbance was measured at 655 nm using a microplate reader (Bio-Rad, Benchmark).

Statistical Analysis

Three independent experiments were conducted. Four to six animals were employed per group in each assay. Data are presented as mean ± SD. Control and experimental conditions were compared by one-way ANOVA followed by Newman-Keuls test. For all analyses, a value of $p < 0.05$ was considered statistically significant.

RESULTS

The *in vivo* ROFA effect on the innate immune response against *M. phlei* infection was evaluated employing an acute short-term-exposure animal model. Nonexposed (Control) and ROFA-exposed (ROFA) mice were infected intranasally with *M. phlei* (Inf and ROFA + Inf, respectively; Table I).

First, we evaluated *M. phlei* clearance in mice lungs at different time points post infection (Fig. 1). The bacterial load in ROFA pre-exposed mice was statistically higher than in animals treated only with *M. phlei* (Inf, without ROFA exposure). Clearly, bacterial killing capacity was diminished, suggesting that ROFA impairs lung defense mechanisms. Clearance capacity at 7 and 14 days post infection showed no differences between pre-exposed ROFA (ROFA + inf) and EM-infected (Inf) animals, indicating that short-term exposure to ROFA was only capable to affect the initial response to infection.

In this context, we studied which events from the innate immune response were altered. For this purpose, we evaluated an array of biological parameters to test ROFA effect on pulmonary innate immune response against environmental mycobacterium infection. We first assayed TCN, CD, and protein concentration for all treatments performed in this study. According to our acute model, ROFA did not alter TCN while EM-infection, by contrast, induced an increase in this parameter, as expected. When the animals were exposed to ROFA prior to EM-infection, TCN diminished with respect to the infected control animals (Table II). Regarding CD, the number of polymorphonuclear (PMN) cells increased in all treated groups with respect to the controls group ($p < 0.05$; Table II), being EM-infected mice the group with the highest recruitment of PMNs into the alveolar space. However, this response was significantly reduced when animals were pre-exposed to ROFA ($p < 0.001$). The relation between AM: PMN was reduced by both ROFA and the EM-infection independently.

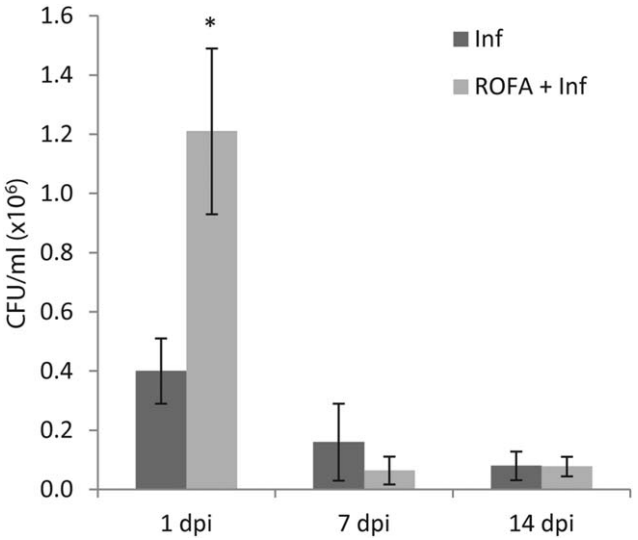

Fig. 1. Clearance of *M. phlei* from mouse lungs. ROFA exposed and nonexposed mice were inoculated intranasally with 8.10^6 CFU. Recovery of CFU from lungs was performed at 1, 7, and 14 days post infection (dpi). All data represent mean ± SD; ($n = 4 - 5$). *$p < 0.05$ is statistically different respect to infected group.

To indirectly assess vascular leakage, we evaluated total protein concentration on BAL. ROFA alone was unable to induce vascular damage. By contrast, EM-infection *per se* caused a significant increase in total protein concentration with respect to the control group ($p < 0.05$; Table II). Accordingly, in the ROFA pre-exposed group, the vascular leakage was similar to that of the infected control group.

To elucidate the effect of ROFA on the pulmonary cell response to *M. phlei* infection, several endogenous inflammatory mediators, such as: superoxide anion, IL-6, and TNFα production were evaluated (Fig. 2). Superoxide anion produced by BAL cells increased in all treated animal groups with respect to the control group [$p < 0.001$; Fig. 2(A)]. The percentage of reactive cells in the infected group showed the maximum value. However, although ROFA alone was able to increase the percentage of reactive cells, the ROFA pre-exposure unexpectedly restricted the response to EM-infection [Fig. 2(A)].

The production in the BAL of both proinflammatory cytokines, IL-6, and TNFα was unaltered by ROFA, whereas it was significantly increased in the EM-infected group (Inf) in comparison with the control group [Fig. 2(B,C)].

TABLE II. BAL composition

		CD		
	TCN (0.10^6)	AM (%)	PMN (%)	Total protein (μg/mL)
Control	1.34 ± 0.35	94 ± 2	6 ± 2	397 ± 21
ROFA	1.32 ± 0.26	83 ± 4^{a}	17 ± 4^{a}	377 ± 15
Inf	2.36 ± 0.38^{b}	42 ± 4^{a}	58 ± 4^{a}	618 ± 43^{b}
ROFA + Inf	1.22 ± 0.18^{e}	$53 \pm 10^{a,d}$	$47 \pm 10^{a,d}$	660 ± 98^{c}

Total Cell Number (TCN) and Cell Differential (CD). TCN was counted with a Neubauer chamber, while for CD, alveolar macrophages (AM) and PMN cells were identified by staining with modified Wright-Giemsa. Total protein concentration was determined in the first BAL milliliter. All data are shown as mean ± SD; ($n = 4 - 6$). Results are representative of three independent experiments.

[a] $p < 0.001$ are statistically different respect to Control
[b] $p < 0.05$ are statistically different respect to Control
[c] $p < 0.01$ are statistically different respect to Control
[d] $p < 0.001$ are statistically different respect to Inf
[e] $p < 0.01$ are statistically different respect to Inf

Interestingly, as seen in Figure 2, the pre-exposure to ROFA induced again a significant decrease in the cytokine secretion in response to *M. phlei* infection. This clearly shows that ROFA exposure impaired the proinflammatory response induced by mycobacterial infection.

DISCUSSION

Diverse stressors of chemical and microbial source can produce lung inflammation and adverse respiratory health effects. In spite of this, the interactions between microbial and chemical stress have received little attention. Several studies have shown that PM increases pulmonary susceptibility to *Listeria monocytogenes*, (Roberts et al., 2004); *Streptococcus pneumoniae* (Sigaud et al., 2007; Zhou and Kobzik, 2007); and Bacillus Calmette-Guerin-BCG-infections (Saito et al., 2002; Saxena et al., 2003). However, to the best of our knowledge no studies have been carried out to test the combined impact of EM and air pollution on health. Considering that strategies to survive inside target cells differ among different pathogens and hosts, it is relevant to characterize the possible scenario for each one. *M. phlei* has been isolated from patients with a wide range of clinical diagnosis (e.g., peritonitis, conjunctivitis, and urethritis) and a history of immunosuppressive syndromes, including cystic fibrosis and HIV infection (Oriani and Sagardoy, 2007; Singh et al., 2007; Shojaei et al., 2011). Moreover, in an epidemiological survey conducted in Bangalore, India, *M. phlei* was the most frequently EM species isolated from the sputum of chest symptomatic patients (Chauhan, 1993). In spite of these evidences, *M. phlei* is still considered essentially nonpathogenic mycobacterium. It is worth to note that as aerosols are the main human exposure source to EM; hence, in our study we employed intranasal instillation, an effective and noninvasive technique either for exposure to ROFA or mycobacteria inoculation (Southam et al., 2002) and considered a good experimental approach to mimic the primary contact with the host. The selected dose of ROFA corresponds to an acute and high air PM exposure. Particularly, this dose mimics the exposure in big cities, being above the standardized limits in large cities of the developing world (150 $\mu g/m^3$). Under this acute ROFA-exposure protocol (Marchini et al., 2012; Magnani et al., 2013), we observed a significantly altered response against EM-infection when animals were pre-exposed to ROFA. We could observe the negative effect of ROFA on lung innate defense through the different parameters evaluated. Noteworthy, the negative effect on lungs clearance lasted for a short period of time, since seven days post infection, the bacterial killing capacity from ROFA exposed and non-exposed animals reached the same level. To evaluate the effect of ROFA in the progression of the infection through time, it would be necessary to develop a chronic model of exposure where infected animals would be exposed to ROFA during the time course of *M. phlei* infection.

All biological parameters evaluated in BAL such as AM:PMN ratio, O_2^- generation and IL-6 and TNF-α levels, from ROFA pre-exposed animals showed that the pollutant bias the mice response to EM-infection toward a more attenuated inflammatory phenotype. Several studies have demonstrated that metallic traces and organic compounds such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls present in air PM are the main cause of PMN influx to the alveolar space. In accordance with previous results from our laboratory and other research groups, ROFA comprises several metallic traces including sulfur, vanadium, nickel, ferric, sodium, silica, and magnesium (Dreher et al., 1997; Martin et al., 2007). In this sense, we found an increase in PMN recruitment into the alveolar space after instillation of ROFA, as previously reported (Walters et al., 2001; Huang et al., 2003; Roberts et al., 2004) and a significantly rise on PMN subpopulation after EM-infection, consistent with the observations reported by

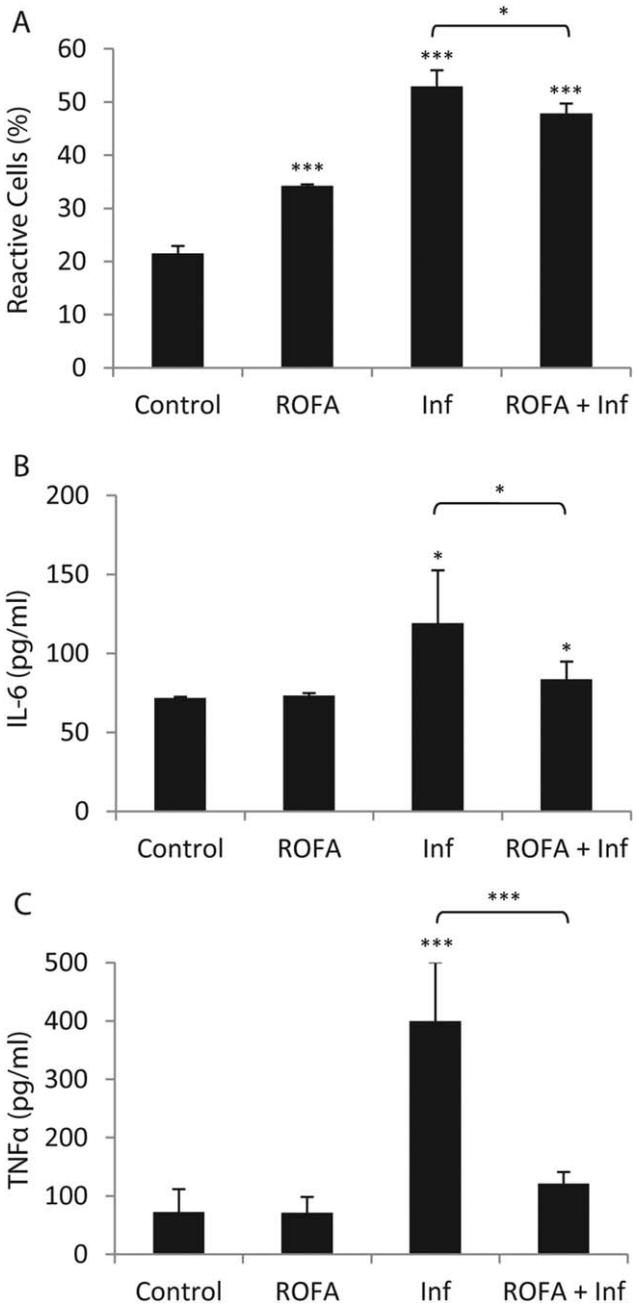

Fig. 2. Superoxide anion (O_2^-), interleukin 6 (IL-6) and tumor necrosis factor alfa (TNFα) evaluated in BAL. A: O_2^- was analyzed by the NBT test on BAL cells. B and C: IL-6 and TNFα were analyzed by ELISA on BAL fluid. All data represent mean ± SD; ($n = 3 - 4$). ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ are statistically different respect to Control otherwise stated. Results are representative of three independent experiments.

Jussila et al. (2002). However, although the pre-exposure to ROFA prior to the infection with *M. phlei* caused a slight, but significant, increase in AM: PMN ratio compared with the nonexposed mice, this increase was lower compared with that of the infected group without such pre-exposure. This observation was independent of endothelial permeability.

Although host defense against mycobacteria is poorly understood, cytokines have been established to have a major role in determining the outcome of infection. Cytokines have an important role in the adaptive immune response as both effectors and regulators of mycobacterial immunity (Cooper et al., 2011). We found that pre-exposure to ROFA reduces the production of TNFα against EM-infection. This result is in agreement with those of Saito et al. (2002) who worked with pre-exposed macrophages to diesel exhaust particles and BCG-infection. Regarding IL-6, we found that *M. phlei* infection increased its production in lungs consistently with Jussila et al. (2002) and Huttunen et al. (2001) who described an increase on the production of this cytokine both, *in vivo* and *in vitro* when mice were infected with *M. terrae*, another known EM. Activated phagocytes (macrophages and neutrophils) respond to xenobiotics through changes in their physiological metabolism, mainly through the respiratory burst or the abrupt consumption of oxygen, which in turns generates superoxide anion as a by-product (Robinson, 2009). Our results indicated that the pre-exposure to ROFA diminishes AM and PMN capacity to generate superoxide anion in response to *M. phlei*, which could be responsible, at least in part, of the dramatic reduced killing ability observed in lung. This result is in agreement with previous AM *in vitro* experiments where we observed that ROFA diminishes AM killing ability in culture (Delfosse et al., 2012). Taking together, TCN and PMN proportion along with TNF-α, IL-6, and superoxide anion assays showed a bimodal effect of ROFA. Where, ROFA alone was able to alter superoxide anion generation by clearly increasing reactive cell percentage. Whereas, when ROFA was administered prior to EM-infection, a marked and significantly reduction in all parameters studied was evidenced. This response could suggest that a defective or impaired phagocytosis could lead to a lower infection rate and a subsequent reduced immune-modulators production. As was demonstrated by Zhou and Kobzik (2007), concentrate ambient particles cause a functional impairment of the antibacterial capacities of murine macrophages inhibiting the internalization of *Streptococcus pneumoniae* due to PM-mediated oxidative stress in the cells. Other possible hypothesis could be a down-regulation of cell surface receptors necessary for mycobacteria to attach and invade macrophages or a physical interference at the cell surface. These issues remain to be evaluated.

Evidence is emerging to support the concept that the pollutants can exert profound effects on human health and disease. Our results showed that pre-exposure to ROFA alter pulmonary defense mechanisms against *M. phlei* infection decreasing the production of important inflammatory cell mediators (superoxide anion and proinflammatory cytokines) involved in the host response to pathogens affecting the killing capacity. This study underlines the potential impact of

pollution on opportunistic mycobacterial infections and highlights the importance of air pollution as a contributing factor in pulmonary infection.

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