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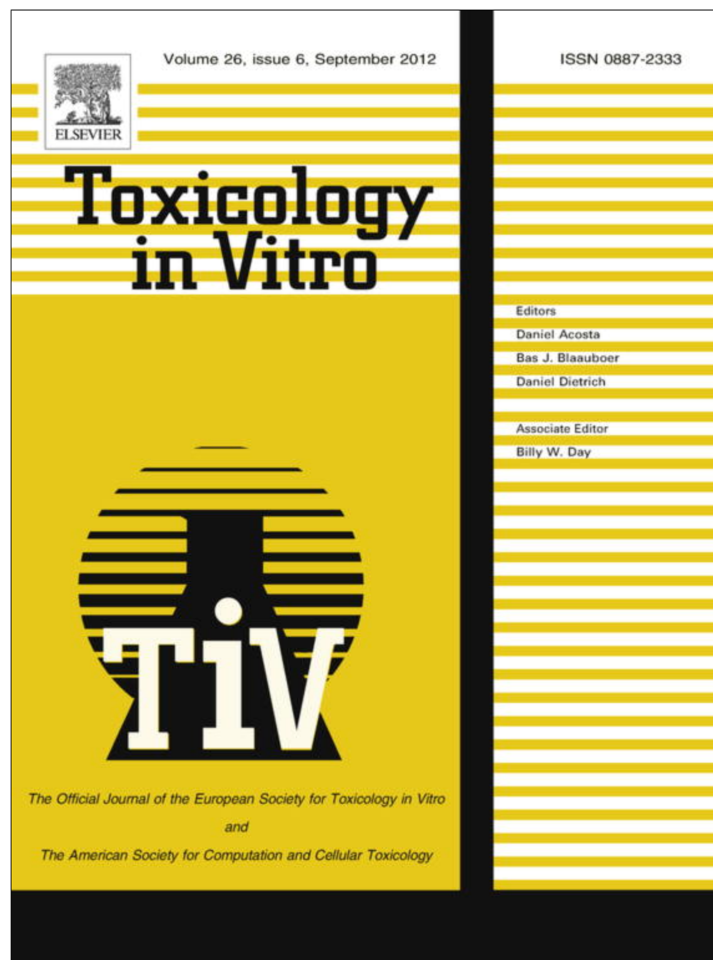

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Low levels of residual oil fly ash (ROFA) impair innate immune response against environmental mycobacteria infection *in vitro*

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Epidemiological studies have shown that pollution derived from industrial and vehicular transportation provokes adverse health effects causing broad spectrum of ambient respiratory diseases. Therefore, air pollution should be taken into account when microbial diseases are evaluated. Environmental mycobacteria (EM) are opportunist pathogens in a variety of immunocompromised patients eliciting significant impact on human morbidity and mortality. The aim of this study was to evaluate the *in vitro* effects of residual oil fly ash (ROFA) on the alveolar macrophages (AMs) response to opportunistic bacteria.

AMs from young Wistar rats were obtained by bronchoalveolar lavage and co-cultured with *Mycobacterium phlei* (MOI 10). We exposed AM cultures to ROFA to characterize the effect of low ROFA concentrations (0, 2.5, and 5 μg/ml) and evaluated the response of pre-exposed AM against the bacilli. Low ROFA concentrations induced superoxide anion and nitrites production ($p < 0.001$). Pre-exposure to ROFA (2.5 and 5 μg/ml) caused a significant reduction on TNFα ($p < 0.001$) and superoxide anion ($p < 0.001$) production but, did not modify the nitrite production when AM were co-cultured with *M. phlei*. In addition, ROFA significantly diminished AM killing ability in culture ($p < 0.001$). Hence, our results indicate that pre-exposure to low levels of ROFA modifies the innate pulmonary defence mechanisms against environmental mycobacteria.

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1. Introduction

The environmental mycobacteria (EM), also known as atypical nontuberculous mycobacteria, or mycobacteria other than tuberculosis, are a group of human and animal opportunistic pathogens which have relevant impact on health (Castronovo, 2008; Grange et al., 1995; Iseman and Marras, 2008; Khatter et al., 2008; Primm et al., 2004). Environmental opportunistic mycobacteria are distinguished from the members of the *Mycobacterium tuberculosis* complex (and *Mycobacterium leprae*) by the fact that they are not obligate pathogens but are true inhabitants of the environment. Furthermore, the knowledge of EM as etiological agents of pulmonary diseases (like cavitary disease, opportunistic infection, and nodular disease associated with bronchiectasis) and disseminated infection is increasing (Castronovo, 2008; Iseman and Marras, 2008; Khatter et al., 2008; Khoor et al., 2001). Human activities impact on mycobacterial ecology and promote human exposure to these organisms. Water and particulate matter suspended in the air are the main source of contact between man and mycobacteria (Goslee and Wolinsky, 1976). Most commonly, humans are exposed to mycobacteria through aerosols generated from different activities such as drinking, swimming, and bathing. Dust and cigarette smoke can also be sources of environmental mycobacterial infection (Le Dantec et al., 2002; Primm et al., 2004). Furthermore, tobacco smoking, passive smoking, indoor air pollution from biomass and fossil fuels have been implicated as risk factors for mycobacterial infection as tuberculosis (Lin et al., 2007; Smith, 2002; Tremblay, 2007). Nowadays, exposure to air pollution can be considered an inescapable part of modern life in urban areas throughout the world. Many major cities in the world are overwhelmed by environmental problems, with air pollution being one of the most relevant public health concerns (Valavanidis et al., 2008; Yang and Omaye, 2009).

Air pollution, including air particulate matter (PM) and gases like CO, NO_x, SO_x and O_3 certainly interfere with nonspecific and specific lung defenses, thus facilitating the development of

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pulmonary diseases, such as exacerbation of chronic obstructive pulmonary disease (COPD), allergies and asthma (D'Amato et al., 2005; Halonen et al., 2008; Ponka, 1991; Rusznak et al., 1994; Schwartz et al., 1991). Airborne PM, a major component of urban air pollution, consists of a mixture of particle components. Residual oil fly ash (ROFA), a combustion product of fuel oil, is chemically complex. In its composition are present sulfates, silicates, and metallic traces (iron, vanadium, nickel), which are able to exert potent adverse biological effects (Ghio et al., 2002; Ostachuk et al., 2008). For its characteristics, ROFA 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).

Alveolar macrophages (AM) are the main responsible cell type for the uptake and clearance of inhaled microorganisms and environmental particles. However, macrophages are also the primary host cells for mycobacteria (Leemans et al., 2005). The initial contact between mycobacteria and the host proceeds through the interaction of mycobacteria with alveolar macrophages permitting the intracellular growth of mycobacteria (Beltan et al., 2000). It is known that PM alters the redox metabolism and the production of pro-inflammatory cytokines and mediators of innate immune response in AM (Li et al., 1997; Martin et al., 2007; Olivieri and Scoditti, 2005). In addition, it has been shown that exposure to air pollution particles can increase susceptibility to lung infection. Antonini et al. (2002) and Roberts et al. (2004), demonstrated that ROFA exposure reduces microbicide activity of alveolar macrophages. It also increases pulmonary damage and susceptibility to *Listeria monocytogenes* infection. In addition, Zhou and Kobzik (2007) observed that *in vitro* pre-exposure of AM to concentrate ambient particles (CAPs) reduce *Streptococcus pneumoniae* internalization. Urban pollution and mycobacteriosis produced by EM are emerging problems. However, to the best of our knowledge, no studies describing either *in vivo* or *in vitro* effects of air pollutants upon the innate immune response against EM are available. Taking into account that the respiratory tract is the main target organ for air pollutants, and that alveolar macrophages are the key lung cell type involved in the response against bacteria, we hypothesize that ROFA could negatively impact on the host innate immune response against EM-infection. Hence, the aim of this work was to study the biological effects of ROFA on the response to EM-infection in cultured rat AMs.

2. Material and methods

2.1. Animals

Male Wistar rats (3 months old) were bred at the School of Dentistry, University of Buenos Aires breeding facility and housed in a controlled environment at the School of Science and Technology, University of San Martín. The animals received a normal protein diet and water *ad libitum*. The experiments reported were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (NIH, 1996), the EC Directive 86/609/EEC and the committee *ad hoc* of the School of Science and Technology, National University of General San Martín.

2.2. Bacterial strain and culture condition

An isolate of *Mycobacterium phlei* was selected from the collection of the Biotechnology Institute-INTA. The identity of the isolate was confirmed as *M. phlei* type I, by PCR restriction fragment length polymorphism analysis technique (Telenti et al., 1993) and by partial sequencing of the *hsp65* gene. The strain was grown in Middlebrook 7H9 liquid medium (BD Difco) supplemented with 0.5% glycerol, and when necessary kanamycin (25 $\mu g\ ml^{-1}$) was added. Briefly, cells were grown at 37 °C with shaking to an optical density at 600 nm of 0.5–0.8, then centrifuged and washed three times with PBS (phosphate saline buffer, Sigma–Aldrich). 25–21 G-needle passages (four times each) were performed to avoid clamps, cells were let stand for 10 min and suspensions were aliquoted and conserved at 4 °C until use. Colony forming units were determined in each experiment.

2.3. M. phlei transformation

Electrocompetent mycobacterium cells were prepared according to Parish and Stoker (2009). Mycobacterial strains were transformed with the replicative vector pFPV27-GFP by electroporation using a Bio-Rad Gene Pulser (Parish and Stoker, 2009). Following electroporation, *M. phlei* was plated onto Middlebrook 7H10 medium supplemented with kanamycin.

2.4. Residual oil fly ash

Residual oil fly ash (ROFA) was employed as a recognized, standard, harmful ambient PM and was generously provided by J. Godleski (Harvard School of Public Health, Boston, MA, USA). It was collected in Boston Edison Co., Mystic Power, plant number 4, CT, USA.

2.5. Isolation of alveolar macrophages and co-culture with EM

Rat alveolar macrophages (AM) were obtained by bronchoalveolar lavage as described elsewhere (Tasat and de Rey, 1987). Briefly, the thoracic cavity was partly dissected; the trachea was cannulated with an 18-gauge needle and infused twelve times with 1 ml of cold PBS. The bronchoalveolar lavage fluid (BALF) was immediately centrifuged at 800×g for 10 min at 4 °C and the BALF cells were resuspended in PBS. Total cell number was determined using a hemocytometer. BALF was employed when, based on morphological criteria, the proportion of AM was >95%. AMs were cultured in RPMI-1640 (Sigma–Aldrich) with penicillin (100 UI/ml), streptomycin (10 μg/ml), and 10% fetal calf serum (FCS). In all cases, except when otherwise stated, 1.25×10^5 macrophages were seeded per dish (24-well plate, diameter 15.6 mm). After 20 min incubation, cells were carefully washed to remove any non-adherent cells and were incubated overnight at 37 °C in a 5% CO_2 atmosphere. The AMs were then exposed to different ROFA concentrations (0, 2.5, 5, 10, 25, 50 and 100 μg/ml, depending on the experiment) in RPMI-1640 with antibiotics and 10% FCS for 24 h at 37 °C in a 5% CO_2 atmosphere (Mitschik et al., 2008). After incubation with ROFA, AM were co-cultured with *M. phlei* (MOI: 10), suspended in RPMI-1640 with 10% FCS for 24 h.

2.6. Phagocytosis of M. phlei

Macrophages adhered to glass coverslips (Marienfeld) were co-cultured with *M. phlei* (MOI: 10) for 15 min, 30 min, 2 h, 24 h and 48 h at 37 °C in 5% CO_2. Extracellular bacteria were removed by washing with warm PBS three consecutive times. The coverslips were removed and cells were fixed in 4% paraformaldehyde for 15 min. Then, coverslips were mounted on glass slides with the addition of DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, Sigma–Aldrich). Stained coverslips were observed by fluorescence microscopy (BX-60 Olympus IX-71 microscope, Olympus) with the appropriate filters for GFP and DAPI. Duplicate coverslips were observed for each experimental condition and at least 100 cells were counted per coverslip. Any macrophage containing at least one bacillus was scored as infected.

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2.7. Macrophage metabolic activity: MTT Assay

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma–Aldrich) assay was performed as previously described for macrophages (Molinari et al., 2000, 2003; Morgan, 1998). Briefly, AMs were seeded at a density of 1.25×10^5 cells/well on a 24-well plate. Medium from control and ROFA-treated cultures was removed and 0.5 ml fresh complete growth medium supplemented with 50 μl MTT (4 mg/ml in PBS) was added for 3 h. Then 10% SDS was added to stop the MTT reaction and to solubilize the formazan precipitate. The optical density (OD) of the final solution was measured at 570 nm in a spectrophotometer (Shimadzu UV-1201V).

2.8. Superoxide anion generation: NBT Assay

Superoxide anion (O_2^-) was evaluated on control and treated AM cultures by the Nitro Blue Tetrazolium (NBT) assay (Segal, 1974). This assay was performed by adding 1 ml 0.1% NBT (Sigma–Aldrich) in PBS to each well containing AM. All wells were incubated with NBT for 45 min at 37 °C. After incubation time, 0.4% paraformaldehyde was added to stop the reaction and fix the cells. The presence of a blue formazan precipitate was indicative of reactive cells. Cells were counted by light microscopy as described elsewhere (Molinari et al., 2000). At least 200 cells per well and three wells for each experimental condition were counted under a Nikon Alphaphot-2 V52 microscope. Results are expressed as the percentage of reactive cells.

2.9. Nitrite production: Griess reaction

Nitrite production was assessed by Griess reaction using the modified Griess reagent (Sigma–Aldrich). The culture supernatant was evaluated according to the manufacturer instructions. Nitrite Standard curve was carried out with $NaNO_2$ (Cicarelli) and nitrite-free water. Absorbance was measured at 595 nm on a microplate reader (Bio-Rad, Benchmark).

2.10. Tumor necrosis factor alfa (TNFα) production: ELISA

The production of the cytokine tumor necrosis factor alpha (TNFα) was detected in cell-free culture supernatant using a commercial specific enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. After each treatment, culture supernatant was collected, centrifuged to remove residual debris and frozen at −80 °C until use. 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% FCS for 1 h at RT. Cytokine standards and AM supernatants were added to wells in triplicate and incubated for 2 h. Following three washes, biotinylated cytokine-specific detection antibody 1:250 was added for 1 h. After washing, the detection agent streptavidin-peroxidase was used with the substrate TMB for 30 min. Absorbance was measured at 655 nm on a micro plate reader (Bio-Rad, Benchmark).

2.11. Bacterial killing assay

As mentioned under Section 2.5, after incubation with ROFA (0, 2.5, 5 μg/ml) for 24 h, AMs were incubated with *M. phlei* (MOI: 10) for an additional 24 h. Unphagocytozed mycobacteria were washed away with PBS three times. To measure total bacteria uptake, the cells were lysed with 0.1% Triton X-100 (Sigma–Aldrich). The lysates were diluted in PBS and plated onto 7H10 (BD Difco, NJ) agar plates and incubated at 37 °C. Colony-forming units were counted after 4–5 days incubation.

2.12. Statistical analysis

Three independent experiments and in quadruplicates were conducted for all assays with the exception of the *Phagocytosis* of *M. phlei* (Section 2.6) in which two independent assays were done. All 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.

3. Results

In order to evaluate the effect of ROFA in rat alveolar macrophages (AMs), we exposed AM cultures to different ROFA concentrations (0, 5, 10, 25, 50 and 100 μg/ml). ROFA concentrations higher than 10 μg/ml significantly reduced the AMs metabolic activity (Fig. 1). 10 μg/ml produced a 20% reduction while doses greater than 25 μg/ml reduced this parameter by 50%. Based on these results, in this work we selected ROFA doses lower than 10 μg/ml (2.5 and 5 μg/ml).

The production of nitrite, superoxide anion and TNFα were evaluated on AMs after 24 h of exposure to ROFA. As is shown in Fig. 2A and B when AMs were exposed to 2.5 μg/ml of ROFA, both nitrite production and the intracellular generation of superoxide anion significantly increased ($p < 0001$). However, TNFα production was not modified by the exposure to ROFA (Fig. 2C).

Since *M. phlei* has not been extensively studied *in vitro* and parameters as infection time are unknown, we evaluated the optimal co-culture time counting the number of macrophages infected at each time by fluorescence microscopy (Fig. 3). Based on these results, the infection time selected was 24 h.

To evaluate the effect of ROFA on the response of AMs to the infection of *M. phlei*, we incubated the ROFA pre-exposed cell culture with the bacilli for 24 h. Then, we evaluated nitrite, superoxide anion and TNFα production in the AMs culture supernatants (Fig. 4). No differences were found on nitrite production in response to *M. phlei* infection neither in control AMs nor when AMs were pre-exposed to ROFA (Fig. 4A). On the other hand, whereas *M. phlei* infection incremented the TNFα and ROS production, doses of 2.5 and 5 μg/ml ROFA decreased this response significantly (Fig. 4B and C). From these results, it is clear that AMs response to *M. phlei in vitro* is altered by ROFA exposure.

In addition, we studied intracellular survival of *M. phlei* after 24 h of co-culture. We observed that 5 μg/ml ROFA significantly diminished the killing of the mycobacteria, while 2.5 μg/ml ROFA did not show any effect ($p < 0.001$) (Fig. 5). Again, our results indicate that ROFA affects AM response by altering the killing activity.

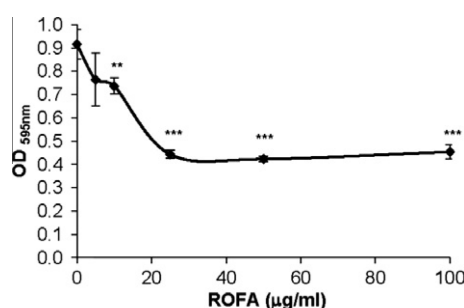

Fig. 1. Macrophage metabolic activity. Effect of different ROFA concentrations on the metabolic activity of cultured AM determined by MTT assay. Results are expressed as OD_{595} mean ± SD; ($n = 4$). $^{***}p < 0.001$, $^{**}p < 0.01$ are statistically different from controls (0 μg/ml). Results are representative of three independent experiments.

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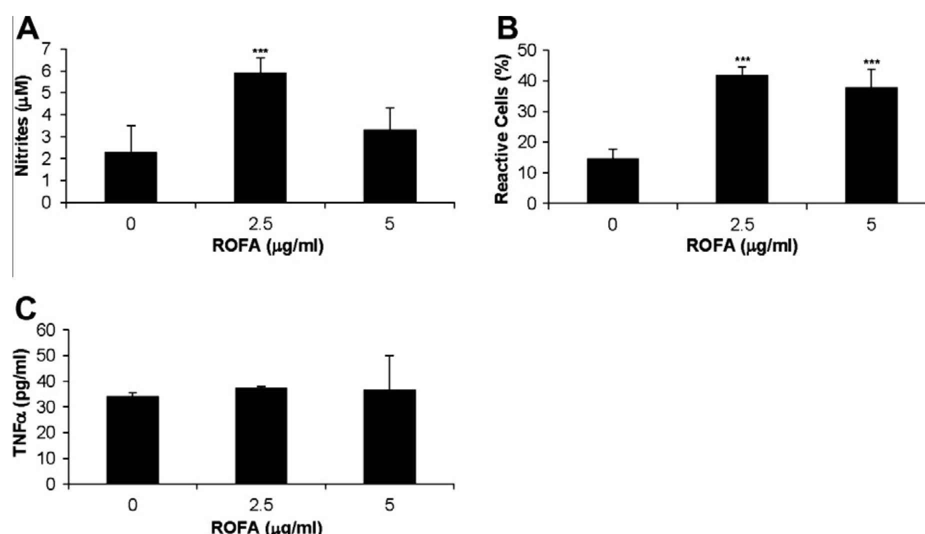

Fig. 2. Evaluation of nitrites, superoxide anion and TNFα production on AM exposed for 24 h to ROFA. (A) Nitrites production was analyzed in AM culture supernatant by Griess reaction. (B) Superoxide Anion production was analyzed by NBT assay. (C) TNFα production was analyzed in AM culture supernatant by ELISA. All data are presented as mean ± SD; (n = 4–7). ***p < 0.001, **p < 0.01 and *p < 0.05 are statistically different from the control (0 µg/ml). Results are representative of three independent experiments.

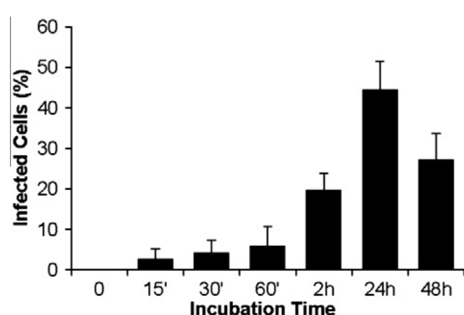

Fig. 3. Evaluation of *M. phlei* infection at different co-culture times. The number of macrophages infected by *M. phlei* was analyzed at different time points by fluorescence microscopy. All data are presented as mean ± SD; duplicate coverslips for each experimental condition and at least 100 cells were counted per coverslip. Results are representative of two independent experiments.

4. Discussion

It is well known that exposure to suspended air particulate matter and gases cause broad spectrum of ambient respiratory diseases. Our results suggest that ROFA concentrations higher than 10 µg/ml reduce the metabolic activity of alveolar macrophages (AMs). ROFA cytotoxicity could explain this observation (Jiang et al., 2000; Dye et al., 1997). This is consistent with that observed by Holian et al. (1998), who analyzed cellular viability in human AMs. Although several previous works evaluated the effects of particulate matter on AMs *in vitro*, they did not consider ROFA concentrations lower than 10 µg/ml (Mitschik et al., 2008). Accordingly, we first characterized the effects of low ROFA concentrations and then we focused our study on its biological impact on the susceptibility to EM infection. It is well known that phagocytosis of microorganism induce the activation of enzymes like nitric oxide sintase (iNOS) and NADPH oxidase complex. Therefore, reactive

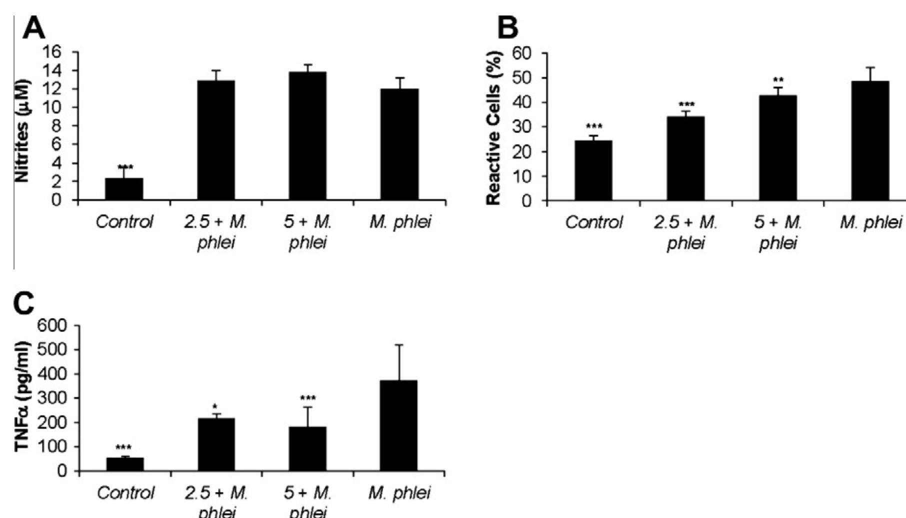

Fig. 4. Evaluation of nitrites, superoxide anion and TNFα production on AM exposed for 24 h to ROFA and co-cultured with *M. phlei*. (A) Nitrites production was analyzed in AM culture supernatants by Griess reaction. (B) Superoxide Anion production was analyzed by NBT assay. (C) TNFα production was analyzed in AM culture supernatants by ELISA. All data are presented as mean ± SD; (n = 3–6). ***p < 0.001, **p < 0.01 and *p < 0.05 are statistically different from the control (*M. phlei*). Results are representative of three independent experiments.

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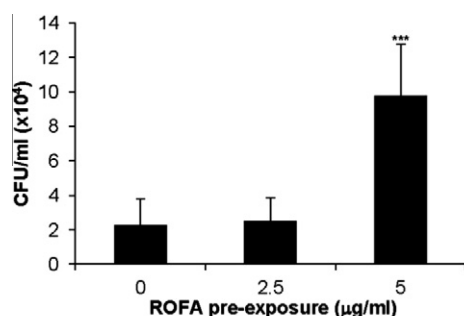

Fig. 5. Killing assay. Intracellular survival of *M. phlei* from AM cultures exposed to different concentrations of ROFA. All data are presented as mean ± SD; ($n = 4$). $^{***}p < 0.001$ is statistically different from the control (0 μg/ml). Results are representative of three independent experiments.

nitrogen species (RNS) and reactive oxygen species (ROS) are produced because of their microbicide activity (Sharma et al., 2007). Phagocytes are also able to produce proinflammatory cytokines which are essential for resistance to infections (Roberts et al., 2007). Evidence on the induction of nitric oxide (NO) production in response to particulate matter in AM is scarce. Huang et al. (2003) have demonstrated that NO production is associated with human AM apoptosis for high ROFA concentrations (100–200 μg/ml) (Huang et al., 2003). Nevertheless, we were able to observe that nitrite production in AMs *in vitro* was induced with ROFA concentrations as low as 2.5 μg/ml, reinforcing the harmful effect of this pollutant. In addition, in agreement with several authors, we found that ROFA was able to induce the production of another oxidative metabolite, the superoxide anion (Becker et al., 2002; Goldsmith et al., 1997; Huang et al., 2003). Although in these studies ROFA played as a potent cell response modulator, it should be taken into account that these authors did not assay low concentrations of ROFA. TNFα is a proinflammatory cytokine and a very important macrophage activation mediator. Our data in agreement with other studies (Mitschik et al., 2008; Tao and Kobzik, 2002) suggest that exposure to low ROFA concentrations for 24 h did not modify TNFα production.

Different previous studies showed that pre-exposure to particulate matter increase AM susceptibly to different infections (Roberts et al., 2004; Saito et al., 2002; Zhou and Kobzik, 2007). However, as the surviving strategies inside target cells differs between pathogens, it is important to characterize the scenario for each one. *M. phlei* has been isolated from patients with a wide range of clinical diagnosis (peritonitis, conjunctivitis and urethritis) and from patients affected by cystic fibrosis or infected with HIV (Oriani and Sagardoy, 2007; Shojaei et al., 2011; Singh et al., 2007). Furthermore, *M. phlei* was the most frequently isolated EM species in sputum specimens from chest symptomatic patients living in Bangalore, India (Chauhan, 1993). In spite of these reports, *M. phlei* is considered essentially non-pathogenic mycobacteria.

Our results show that pre-exposure to ROFA alters the defense mechanisms of AMs against *M. phlei* infection by decreasing the production of important cell mediators involved in cellular response such as superoxide anion and TNFα and finally affecting the killing capacity of phagocytes. 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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