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Soluble metals in residual oil fly ash alter innate and adaptive pulmonary immune responses to bacterial infection in rats

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Abstract

The soluble metals of the pollutant, residual oil fly ash (ROFA), have been shown to alter pulmonary bacterial clearance in rats. The goal of this study was to determine the potential effects on both the innate and adaptive lung immune responses after bacterial infection in rats pre-exposed to the soluble metals in ROFA. Sprague-Dawley rats were intratracheally dosed (i.t.) at day 0 with ROFA (R-Total) (1.0 mg/100 g body weight), the soluble fraction of ROFA (R-Soluble), the soluble sample subject to a chelator (R-Chelex), or phosphate-buffered saline (Saline). On day 3, rats were administered an i.t. dose of 5×10^4 *Listeria monocytogenes*. On days 6, 8, and 10, bacterial pulmonary clearance was monitored and bronchoalveolar lavage (BAL) was performed on days 3 (pre-infection), 6, 8, and 10. A concentrated first fraction of lavage fluid was retained for analysis of lactate dehydrogenase and albumin to assess lung injury. BAL cell number, phenotype, and production of reactive oxygen (ROS) and nitrogen species (RNS) were assessed, and a variety of cytokines were measured in the BAL fluid. Rats pre-treated with R-Soluble showed elevated lung injury/cytotoxicity and increased cellular influx into the lungs. R-Soluble-treatment also altered ROS, RNS, and cytokine levels, and caused a degree of macrophage and T cell inhibition. These effects of R-Soluble result in increased pulmonary bacterial burden after infection. The results suggest that soluble metals in ROFA increase lung injury and inflammation, and alter both innate and adaptive pulmonary immune responses.
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Keywords: ROFA; *Listeria monocytogenes*; Alveolar macrophage; T cells; Cytokines

Introduction

Residual oil fly ash (ROFA) is an air pollutant by-product of the combustion of fossil fuels and contributes significantly to total primary particulate matter (PM) emissions in the U.S. (EPA, 2004). Increased infectivity and susceptibility to infection have been correlated with high levels of outdoor air pollutants (American Thoracic Society, 1996), and epidemiological and human experimental studies have attributed high metal content of pollutants to increased respiratory inflammation and illness (Leonardi et al., 2000; Schaumann et al., 2004; Soukup et al., 2000). Animal studies have shown that respiratory exposure to ROFA (Antonini et al., 2002; Hatch et al., 1985; Pritchard et al., 1996) or ambient air particulates (Zelikoff et al., 2003) that have a high metal content can increase susceptibility to infection. Roberts et al. (2003) demonstrated that it was the soluble metal constituent of ROFA that appeared to alter pulmonary clearance of *Listeria monocytogenes* instilled in the lungs of rats. The goal of the current study was to characterize the innate and adaptive immune response in the lungs of rats infected with the bacteria after pulmonary exposure to the soluble metal fraction of ROFA.

L. monocytogenes has been used by a number of investigators as a model pathogen to examine respiratory defense mechanisms in response to various environmental exposures (Antonini et al., 2002; Cohen et al., 2001; Van Loveren et al., 1988; Yin et al., 2002, 2003). *L. monocytogenes* is a Gram-positive, facultative, intracellular bacterium that resides in the cytosol of infected cells. It can infect a variety of cell types including epithelial cells and phagocytes (Seder and Gazzinelli,

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1999). Effective clearance of the bacterial infection requires both the innate (non-specific) and adaptive (pathogen-specific) immune responses. The primary adaptive immune response to *L. monocytogenes* is considered to be cell-mediated immunity, consisting of $CD8^+$ T cells and $CD4^+$ T_H1 cells, rather than humoral immunity, where B cells and $CD4^+$ T_H2 cells would be activated (Pamer, 2004).

An effective immune response to a *L. monocytogenes* infection is governed partly by immunomodulatory cytokines which influence both the innate and adaptive immune responses. The innate response of phagocytosis of bacteria by macrophages results in an inflammatory cytokine cascade that includes tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-12. The inflammatory response, in turn, will result in an increase in endothelial barrier permeability, an increase in neutrophil and monocyte recruitment to the infected area, and enhanced activation of phagocytes and natural killer (NK) cells in order to kill the bacteria (Seder and Gazzinelli, 1999). The pro-inflammatory cytokines, such as IL-12, induce the expression of IFN-γ by $CD4^+$ T_H1 cells, $CD8^+$ T cells, and NK cells, and the presence of IL-12 and IFN-γ, in turn, drives the differentiation of naïve $CD4^+$ T cells toward the $CD4^+$ T_H1 subset (Blesson et al., 2002; Hsieh et al., 1993; Mosmann and Sad, 1996). An anti-inflammatory cytokine and negative modulator of this response is IL-10. IL-10 is known to inhibit activation of $CD4^+$ T_H1 and $CD8^+$ T cells, monocytes, macrophages and neutrophils, and has varying effects on NK cells depending on the presence of other cytokines (Moore et al., 2001). These cell functions are necessary in the cell-mediated immune response to *L. monocytogenes*. During the adaptive response to *L. monocytogenes*, T cell subsets will produce cytokines that will effect their own development and proliferation. IL-2 is produced primarily by $CD4^+$ T_H1 cells, by naïve $CD4^+$ and some $CD8^+$ T cells, and promotes growth, proliferation, and clonal expansion of T cells. IL-4 is secreted mainly by the $CD4^+$ T_H2 subset and is involved in the promotion of the humoral immune response (Mosmann and Sad, 1996), which can be inhibitory to the cell-mediated response. The cell-mediated immune response will further promote bacterial killing and clearing of infected cells by macrophages and NK cells.

ROFA and metals associated with it have been shown to alter the function of multiple cell types in the lungs, including those involved in innate immune responses, such as alveolar macrophages (AMs). Antonini et al. (2002) showed that exposure to ROFA prior to infection decreased the ability of AMs to kill bacteria in the lung. Investigators have shown that ROFA can alter the oxidative state of AMs and neutrophils (Antonini et al., 2002; Becker et al., 2002; Goldsmith et al., 1998). The ability of phagocytes to produce reactive oxygen and nitrogen species plays an important role in killing of *L. monocytogenes*; however, excessive oxidative stress and injury may not be beneficial prior to infection. ROFA has also been shown to alter inflammatory cytokine production in the lung by phagocytes and epithelial cells (Gavett et al., 1999; Goldsmith et al., 1998; Lambert et al., 2000), which may affect cell-signaling pathways involved in the immune response. Much of the inflammation and injury observed in response to ROFA has been attributed to the metal content, particularly the soluble metals (Dreher et al., 1997; Dye et al., 1999; Kodavanti et al., 1998).

The direct effects of ROFA on lymphocytes, both B and T cells, in response to infection are not well characterized. Most of the information regarding the function of these cell types in response to ROFA comes from studies that examine the ability of ROFA to enhance sensitization and hyperresponsiveness to allergens in animals (Gavett et al., 1999; Lambert et al., 2000), which demonstrated that ROFA could alter lymphocyte proliferation and cytokine production. It has been shown that PM containing similar metals present in ROFA can also have effects on lymphocytes, including alterations in B and T cell proliferation (Zelikoff et al., 2002) and lymphocyte infiltration into the lung (Dye et al., 2001). Therefore, it is possible that ROFA, particularly the soluble metal constituent, may alter the function of lymphocytes involved in the immune response to bacterial infection in the lung.

The goal of this study was to determine the potential mechanisms by which the soluble metals in ROFA may alter the innate and adaptive immune responses in the lung of rats exposed to the metals prior to pulmonary bacterial infection. We hypothesized that exposure to the soluble metals of ROFA prior to infection would alter the function of a number of cell types involved in the innate and adaptive immune response in the lungs of rats, including the activity of phagocytes and lymphocytes. To investigate this hypothesis, rats were pre-exposed to a single acute dose of ROFA or the soluble fraction of ROFA by intratracheal instillation. Control animals were instilled with phosphate buffered saline as a vehicle control, or the soluble ROFA that had the metal cations removed by a chelator to control for the effects of non-metal and non-cationic soluble constituents. Following exposure to the ROFA samples, rats were intratracheally inoculated with *L. monocytogenes*. Infection was characterized by monitoring animal weight, lung injury, and pulmonary clearance of *L. monocytogenes*. Cellular influx into the lungs of animals pre-exposed to the ROFA samples, the chelated sample, or vehicle control was monitored, and the number and phenotype of cells were determined. The function of cell types involved in the immune response was assessed by measuring production of reactive oxygen (ROS) and nitrogen species (RNS) in the lung and the presence of a variety of cytokines in the bronchoalveolar lavage fluid.

Methods

Animals. Male Sprague-Dawley [Hla:(SD)CVF] rats (Hilltop Laboratories, Scottdale, PA) weighing 250–300 g, approximately 10 weeks old, were used for all experiments in accordance with a protocol approved by the IACUC. They were given the ProLab 3500 diet and tap water *ad libitum*, housed in a clean air and viral- and antigen-free room with restricted access in an AAALAC-approved animal facility, and allowed to acclimate for 1 week before use. The rats were monitored and found to be free of endogenous viral pathogens, parasites, mycoplasms, *Helicobacter*, and CAR *Bacillus*.

Materials. *L. monocytogenes* (strain 10403S, serotype 1) was obtained as a gift from Dr. Rosana Schafer of the Department of Microbiology and Immunology at West Virginia University. Residual oil fly ash (ROFA) was

collected from a precipitator at Boston Edison Co., Mystic Power Plant #4, Everett, MA. The chelating resin, Chelex 100 (iminodiacetic acid), was purchased from Sigma-Aldrich Co., St. Louis, MO.

ROFA characterization. Particle size of the ROFA sample was determined by scanning electron microscopy (JSM-#5600 SEM, JEOL Ltd., Peabody, MA) and previously characterized (Antonini et al., 2002). ROFA particles were of respirable size with a count mean diameter of 2.2 μm. The metal constituents of the ROFA samples were analyzed using inductively coupled argon plasma, atomic emission spectroscopy (NIOSH, 1994).

Experimental design. A timeline for the experiment is presented in Fig. 1. At day 0, animals were pre-exposed to ROFA samples or phosphate buffered saline (vehicle control) by intratracheal instillation. At day 3, the animals were inoculated via intratracheal instillation with 5×10^4 *L. monocytogenes*. Uninfected control animals in each treatment group received saline on day 3. At days 6, 8, and 10, the rats were euthanized, the left lungs of animals were clamped off, removed, homogenized, and the number of colony forming units (CFUs) was counted. The right lungs of animals were lavaged, and the cells and the fluid were retained for analysis including cellular phenotyping, oxidant production, and cytokine production. Bacterial clearance, change in body weight, and lung injury parameters were compared between the whole ROFA sample and the soluble ROFA sample to confirm previous findings (Antonini et al., 2002; Roberts et al., 2003).

ROFA treatment. The ROFA sample (R-Total) was suspended in sterile phosphate-buffered saline (PBS) (6 mg/ml), sonicated for 1 min with a Sonifier 450 Cell Disruptor (Branson Ultrasonics, Danbury, CT), and allowed to shake and incubate for 24 h at 37 °C. The sample was further divided into soluble and insoluble components by centrifugation at 12,000×*g* for 30 min. The supernatant of the sample was recovered and filtered (R-Soluble). According to the manufacturer, Chelex is an insoluble resin that selectively binds divalent cations over monovalent cations with an affinity of 5000:1, and has a strong attraction for transition metals, even in highly concentrated salt solution. Chelex was added to the R-Soluble sample (20 mg Chelex/0.1 mg ROFA) and incubated on a rotary shaker overnight. The sample was centrifuged, and the supernatant of the R-Soluble sample treated with Chelex (R-Chelex) was recovered.

Rats were lightly anesthetized by an intraperitoneal injection (i.p.) of 0.6 ml of a 1% solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN) and intratracheally instilled (i.t.) with 1.0 mg/100 g body weight (bw) of R-Total in 300 μl of PBS, according to the method of Brain et al. (1976). In addition, R-Soluble was administered by i.t. using the soluble portion equivalent to that in the R-Total instillate. Animals in the control groups received 300 μl of sterile PBS (Saline) as a vehicle control or R-Chelex as a control for non-chelatable soluble constituents in that portion of the R-Soluble sample via i.t. The ROFA dose chosen was previously shown to induce inflammation (Antonini et al., 2002), and fell within the range of concentrations consistently used in other animal studies evaluating the pulmonary responses to ROFA (Dreher et al., 1997; Kodavanti et al., 1998; Lambert et al., 2000). There were 8–12 animals per group per time point.

Intratracheal bacteria inoculation. *L. monocytogenes* was cultured overnight in brain–heart infusion broth (Difco Laboratories, Detroit, MI) at 37 °C in a shaking incubator. Following incubation, the bacterial concentration was determined spectrophotometrically at an optical density of 600 nm. The sample was diluted to the concentration of 5×10^4 *L. monocytogenes* in 500 μl of sterile PBS and administered i.t. to the rats in each treatment group 3 days post-ROFA sample instillation ($n=8$ per group per time point). Uninfected control animals from each treatment group received sterile PBS on day 3 ($n=4$ per group per time point).

Morbidity/pulmonary clearance of L. monocytogenes. Morbidity and pulmonary clearance were monitored in animals pre-treated with R-Total, R-Soluble, R-Chelex, or Saline. Animal weights were monitored over the course of the treatment period as an indicator of morbidity. Rats were euthanized with an overdose of sodium pentobarbital, and at days 6, 8, and 10 (corresponding to days 3, 5, and 7 post-infection), and the left lungs were removed from all the rats in each treatment group. The excised tissues were suspended in 10 ml of sterile water, homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA), and cultured quantitatively on brain–heart infusion agar plates (Becton Dickinson and Co., Cockeysville, MD). The number of viable CFUs was counted after an overnight incubation at 37 °C.

Bronchoalveolar lavage (BAL). BAL was performed by washing out the lungs of the rats with aliquots of PBS in order to obtain pulmonary cells for morphological and functional analysis, and the acellular BAL fluid was retained for analysis of indicators of tissue damage and cellular activity. Rats were euthanized with an overdose of sodium pentobarbital, the left lungs were clamped off, and BAL was performed on the right lungs on day 3 prior to infection, and at days 6, 8, and 10. A concentrated first fraction of BAL was obtained as described by Antonini et al. (2002). This concentrated aliquot was withdrawn, retained, kept separately, and was designated as the first fraction of BAL fluid. The following aliquots were 6 ml in volume, instilled once with light massaging, withdrawn, and combined until a 30-ml volume was obtained. For each animal, both fractions of BAL were centrifuged, the cell pellets were combined and resuspended in 1 ml of PBS, and the acellular fluid from the first fraction was retained for further analysis.

Analysis of albumin and lactate dehydrogenase (LDH). The presence of albumin and LDH in the BAL fluid of all treatment groups was measured to evaluate the loss of integrity of the alveolar–capillary barrier and general cytotoxicity, respectively. Measurements of both albumin and LDH in the acellular fluid were obtained using a Cobas Mira analyzer (Roche Diagnostic Systems, Montclair, IN). Albumin was determined by spectrophotometric measurement of the reaction product of albumin with bromcresol green (628 nm) according to a method by Sigma Diagnostics (St. Louis, MO). LDH activity was quantified by detection of the oxidation of lactate coupled to the reduction of NAD^+ at a spectrophotometric setting of 340 nm over time.

Pulmonary cell differentials and phenotyping. Cellular analysis was performed on the BAL samples of rats pre-treated with R-Total, R-Soluble, R-Chelex, or Saline on days 3, 6, 8, and 10. Total BAL cells were counted using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL). Cell differentials were performed to determine the total number of alveolar macrophages (AMs), polymorphonuclear cells (PMNs), and lymphocytes as previously described (Antonini et al., 2002). The percentage of AMs, PMNs, and lymphocytes were multiplied by the total number of cells to calculate the number of each cell type. To determine phenotype of the cell population in the BAL, cells were labeled

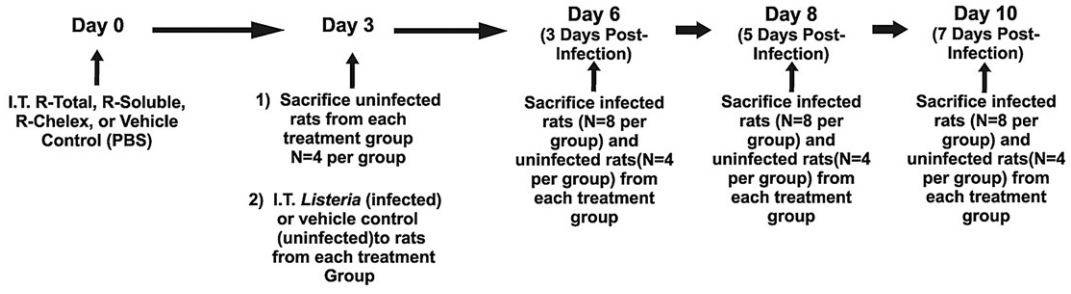

Fig. 1. Timeline of experimental design.

with fluorescently tagged antibodies against specific cell surface markers (BD Biosciences Pharmingen, San Diego, CA). The cell types that were identified were NK cells ($CD161a^+$, $CD3^-$), B lymphocytes ($CD45R^+$), and T lymphocytes ($CD3^+$). The T cells were further differentiated as T-helper (T_H) cells ($CD4^+$) or cytotoxic T cells ($CD8^+$). Using a flow cytometer (FACS Calibur, BD Biosource, San Diego, CA), the lymphocyte population of the BAL cells to be analyzed was determined by size using forward and side scattering, and the viable cells were selected by eliminating the population of dead cells that had stained positive for 7-amino-actinomycin D (7-AAD). Percentages of each cell type in BAL measured with flow cytometry were calculated and multiplied by the total number of lymphocytes determined from the cell differentials.

Measurement of nitric oxide and reactive oxygen species. Nitric oxide (NO) levels and ROS were determined by analysis of BAL from rats pre-treated with R-Total, R-Soluble, R-Chelex, or Saline on days 3, 6, 8, and 10. The presence of NO in acellular BAL was determined as the accumulation of nitrite using a modified microplate assay using the Greiss reagent (Green et al., 1982) and a nitrate reductase to convert any nitrate in the sample to nitrite. The absorbance of the samples was analyzed on a SPECTRAmax TM 250 spectrophotometer (Molecular Devices Co., Sunnydale, CA) at 550 nm. The measurement of total nitrite represents the presence of both nitrate and nitrite (NO_x) in the sample. In addition, total nitrite production by BAL AMs was also measured in the cell-conditioned media after an 18-h incubation as previously described (Antonini et al., 2002; Huffmann et al., 2003).

To estimate total lung oxidant burden, luminol-dependent chemiluminescence (CL) was performed on BAL cells as a measure of the light generated by the production of ROS by AMs and PMNs using a Berthold LB953 luminometer (Wallace Inc., Gaithersburg, MD) as described previously (Antonini et al., 1994). Phorbol 12-myristate 13-acetate (PMA) (10 μM), a soluble stimulant of total BAL phagocytes (AMs and PMNs), or non-opsonized, insoluble zymosan (2 mg/ml), a stimulant of AMs only (Castranova et al., 1990), was added to the assay immediately prior to CL measurement to determine the contribution of both AMs and PMNs to the overall production of ROS in the lungs of the rats. Measurement of CL was recorded for 15 min at 37 °C, and the integral of counts per minute (cpm) per 10^6 cells versus time was calculated. CL was calculated as the cpm of the stimulated cells minus the cpm of the corresponding resting cells, and the value was normalized to total number of BAL cells for PMA-stimulated CL and total number of AMs in the BAL for zymosan-stimulated CL.

Cytokine measurements in BAL. Cytokines present in the BAL fluid of rats pre-treated with R-Total, R-Soluble, R-Chelex, or Saline were analyzed by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits (BioSource International Inc., Camarillo, CA) to determine activity of various cell types involved in the immune response. The following cytokines were quantified: tumor necrosis factor-alpha (TNF-α), interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p70, and interferon-γ (IFN-γ).

Statistical analysis. Results are expressed as means ± standard error of measurement (SE). Statistical analyses were carried out with the SigmaStat 3.1 statistical program (Chicago, IL). The significance of the interaction among different treatment groups for the different parameters at each time point was assessed using analysis of variance (ANOVA). The significance of difference between individual groups was analyzed using the Student–Newman–Keuls method with the criterion of significance set at $p < 0.05$.

Results

ROFA characterization

The ROFA used in this study had been previously characterized (Antonini et al., 2002; Roberts et al., 2003) and metal content is presented in Table 1. Briefly, the R-Total sample consisted primarily of iron, nickel, aluminum, calcium, vanadium, and zinc. The R-Soluble sample was comprised of nickel, iron, aluminum, and zinc, but contained very little vanadium. Both the R-Total and R-Soluble sample were found to be acidic in nature. The chelator, Chelex, successfully removed the majority of metals from the R-Soluble sample leaving only a small amount of iron and less than 1 μg of vanadium.

Table 1
Element mass of ROFA samples (μg/2 mg instillate)

	R-Total (pH=5.00)	R-Insoluble (pH=7.06)	R-Soluble (pH=4.10)	R-Chelex (pH=9.70)
Fe	244	186	37.2	6.76
Al	121	64.1	46.6	N.D.
V	92.0	83.1	1.17	0.574
Ni	76.9	11.0	55.7	N.D.
Ca	61.1	7.16	45.1	N.D.
Zn	10.7	1.13	8.69	N.D.

Trace elements: Ba, Cd, Co, Cr, Cu, Mn, Pb.
N.D.: not detected.

Uninfected treatment groups

Biochemical analysis of BAL fluid: LDH and albumin

Lung injury parameters and BAL cell number for uninfected treatment group data are presented in Tables 2 and 3, respectively. In addition, all data for day 3, which represents the baseline values in the treatment groups prior to infection, are included in the graphs in Figs. 2–8 for reference purposes. The presence of the intracellular enzyme, LDH, in the BAL fluid of rats was used as a marker for general cytotoxicity. LDH was elevated in the uninfected R-Total and R-Soluble group on day 3, with the more severe elevation in the R-Total group at this time point (Fig. 3A, Day 3; Table 2). The increase in LDH persisted in the uninfected rats in the R-Total group and in the R-Soluble group at all time points when compared to Saline controls (Table 2) and on day 6 the elevation was more severe in the R-Soluble group when compared to the R-Total group. Albumin in the BAL fluid, an indicator of the breakdown of the alveolar blood–air barrier, was increased in the R-Soluble group at all time points and in the R-Total group through day 6 when compared to controls (Fig. 3B, Day 3; Table 2). These results indicate that there was a significant lung injury in the R-Soluble and R-Total groups and that the injury was greater and more persistent in the R-Soluble group over time.

BAL cellular profiles

BAL cell numbers and phenotypes were monitored in uninfected animals exposed to R-Total, R-Soluble, R-Chelex, or Saline. Total BAL cell number was counted, and the number of AMs, PMNs, and lymphocytes were calculated from cell differentials (Table 3). On day 3 rats exposed to R-Soluble and R-Total had a significantly greater number of PMNs and lymphocytes when compared to the Saline and R-Chelex groups, indicating an enhanced inflammatory response in this group (Fig. 4, Day 3; Table 3). The increase in PMNs in the uninfected R-Soluble and R-Total groups persisted out to Day 10, and the increase in lymphocytes in the R-Total group continued to day 6. There was a significant increase in AM number in the R-Soluble and R-Total groups on days 6, 8, and

Table 2
BAL fluid LDH and albumin in non-infected rats

Time	LDH (U/L) albumin (mg/ml)	Saline	R-Chelex	R-Soluble	R-Total
Day 3	LDH	147±12.9	165±8.36	461±35.4[a]	627±80.4[b]
	Albumin	0.23±0.03	0.28±0.03	0.58±0.07[a]	1.2±0.18[b]
Day 6	LDH	135±13.9	131±3.85	742±47.1[a]	458±28.1[b]
	Albumin	0.21±0.03	0.22±0.01	0.86±0.07[a]	0.64±0.02[b]
Day 8	LDH	121±15.4	155±23.4	349±47.0[c]	256±68.2[c]
	Albumin	0.22±0.02	0.32±0.07	0.66±0.08[a]	0.39±0.08
Day 10	LDH	79.8±10.6	153±19.7[d]	257±44.0[a]	165±8.40[d]
	Albumin	0.21±0.03	0.33±0.10	0.41±0.08[e]	0.26±0.01

Values are means±SE.
[a] Significantly different from Saline, R-Chelex, and R-Total ($p<0.05$).
[b] Significantly different from Saline, R-Chelex, and R-Soluble ($p<0.05$).
[c] Significantly different from Saline and R-Chelex ($p<0.05$).
[d] Significantly different from Saline and R-Soluble ($p<0.05$).
[e] Significantly different from Saline ($p<0.05$).

10. The increases in cellular infiltration into the lungs of rats treated with R-Total and R-soluble are indicative of an increase in lung inflammation and lung injury. Flow cytometry, in conjunction with cell differential counts, was used to determine the number of NK cells, B cells, total T cells, $CD4^+$ T cells, and $CD8^+$ T cells. There were no significant differences in lymphocyte numbers and NK cell numbers among the uninfected treatment groups (data not shown).

BAL fluid levels and BAL cellular oxidant production

Oxidant production was monitored in all treatment groups. The presence of NO, a known antimicrobial agent, as well as an indicator for increased oxidative stress, was measured as NO_x (nitrate and nitrite). Values for NO_x in the BAL fluid and NO_x production by AMs on day 3 in the uninfected animals are shown in Fig. 5. There were no significant differences among uninfected treatment groups on day 3 or on days 6, 8, and 10 (data not shown). Luminol-dependent CL was performed on BAL cells to determine the level of production of ROS by AMs and PMNs. There were no significant differences among uninfected treatment groups on day 3 after stimulation with PMA (Fig. 6A) or non-opsonized zymosan (Fig. 6B), and there were no significant differences among treatment groups in the absence of infection on days 6, 8 and 10 (data not shown).

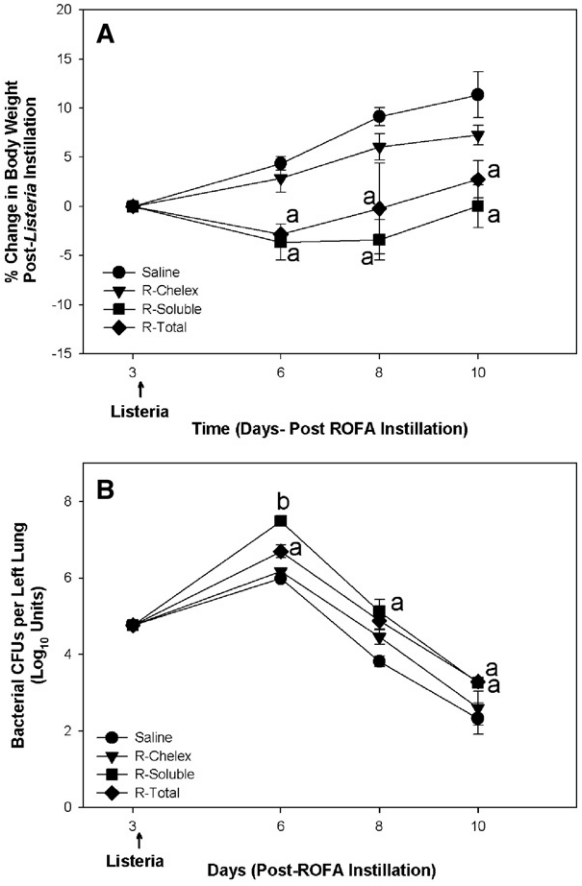

Fig. 2. % Change in body weight post-infection (A) and number of bacterial CFUs in the left lungs (B) of rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or saline 3 days prior to intratracheal inoculation with *L. monocytogenes*. Values are means±SE ($n=8$/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex ($p<0.05$).

Table 3
BAL cell numbers for non-infected rats (10^6 cells)

Time	BAL cell number	Saline	R-Chelex	R-Soluble	R-Total
Day 3	Total cells	7.85±0.47	7.98±0.93	10.2±0.68	10.7±0.80
	AMs	7.80±0.47	7.92±0.93	9.57±0.68	9.16±0.50
	PMNs	0.02±0.01	0.02±0.01	0.41±0.65[a]	1.06±0.24[a]
	Lymphocytes	0.03±0.01	0.04±0.01	0.18±0.05[b]	0.38±0.10[c]
Day 6	Total cells	7.37±0.79	7.17±1.00	19.4±1.95[a]	15.9±1.48[a]
	AMs	7.32±0.77	7.16±1.01	13.6±1.15[a]	14.7±1.58[a]
	PMNs	0.04±0.02	0.01±0.01	5.55±2.06[b]	0.63±0.19[c]
	Lymphocytes	0.01±0.01	0.01±0.01	0.00±0.00	0.30±0.11[c]
Day 8	Total cells	9.69±1.24	11.2±1.81	20.8±4.62[a]	16.3±4.16
	AMs	9.68±1.24	11.1±1.82	20.3±4.52[a]	15.5±3.74
	PMNs	0.01±0.01	0.00±0.00	0.37±0.10[a]	0.65±0.42[a]
	Lymphocytes	0.00±0.00	0.00±0.00	0.03±0.03	0.06±0.02
Day 10	Total cells	9.66±0.37	14.2±0.70[d]	23.9±2.86[b]	16.1±0.58[d]
	AMs	9.66±0.37	14.1±0.74[d]	22.9±2.76[b]	15.4±0.72[d]
	PMNs	0.00±0.00	0.02±0.02	0.74±0.11[a]	0.44±0.19[a]
	Lymphocytes	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.03

Values are means±SE.
[a] Significantly different from Saline and R-Chelex ($p<0.05$).
[b] Significantly different from Saline, R-Chelex, and R-Total ($p<0.05$).
[c] Significantly different from Saline, R-Chelex, and R-Soluble ($p<0.05$).
[d] Significantly different from Saline and R-Soluble ($p<0.05$).

BAL cytokine analysis

Inflammatory cytokines and immunomodulatory cytokines present in the BAL of the R-Total, R-Soluble, R-Chelex, and Saline groups were measured as indicators of cell-specific activity in response to the ROFA samples. There was a slight, but significant increase in IL-6 in the uninfected R-Total group on day 3 when compared to controls (Fig. 7B), indicating a degree of lung injury and inflammation. There was a decrease in IL-2 in the uninfected R-Soluble group on day 3 when compared to controls (Fig. 8A), suggesting that the soluble components of ROFA may have adverse effects on the T cell population. However, this decrease did not persist past this time

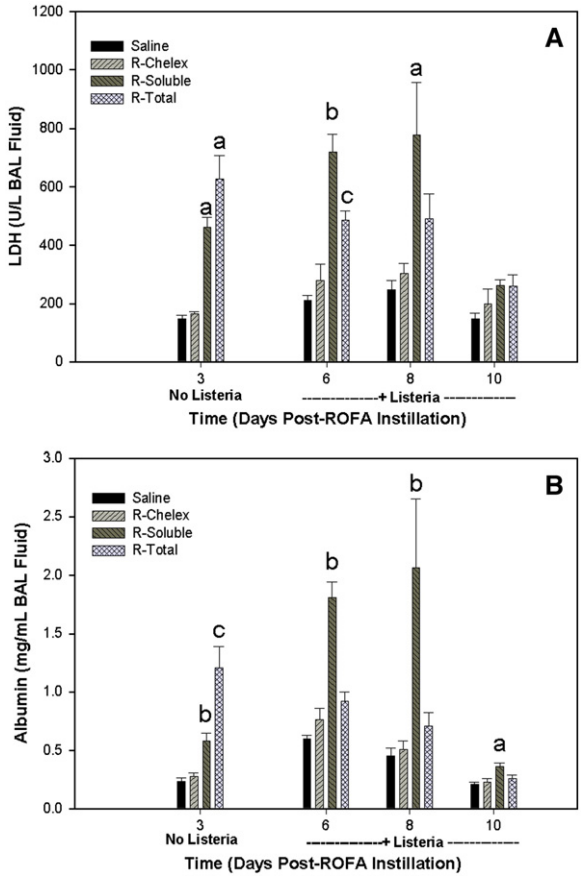

Fig. 3. Lactate dehydrogenase (LDH) (A) and albumin (B) in the BAL fluid of rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or saline 3 days prior to intratracheal inoculation with *L. monocytogenes*. Values are means±SE ($n=8$/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex, [c]significantly different from R-Soluble, R-Chelex, and Saline ($p<0.05$).

point in uninfected rats. On day 3, there were no significant differences in TNF-α (Fig. 7A), IL-12p70 (Fig. 7C), IL-10 (Fig. 7D), IL-4 (Fig. 8B), or IFN-γ (data not shown). There were no significant differences in any of the cytokine levels in uninfected rats on days 6, 8, and 10.

Infected treatment groups

Morbidity and pulmonary bacterial clearance

Morbidity in animals pre-treated with Saline, R-Total, R-Soluble, or R-Chelex was monitored as the % change in body weight after infection with *L. monocytogenes* (Fig. 2A). Rats pretreated with R-Total or R-Soluble lost a significant amount of weight post-infection when compared to controls. Animals in the R-Chelex group did not differ from the saline group. Rats R-Total and R-Soluble treatment groups began to regain body-weight by day 8, although they weighed significantly less at all time points post-infection when compared to the Saline and R-Chelex groups. Uninfected animals from all treatment groups gained weight throughout the course of the study (data not shown). Animals treated with R-Chelex or Saline were able to clear a large portion of bacteria from the lungs by day 10 (Fig. 2B), whereas animals pre-exposed to R-Total or R-Soluble had a significantly greater bacterial lung burden at the early time points post-infection. The significant increase in CFUs in the lungs of the R-Soluble group persisted throughout the time course. These data indicate that the R-Soluble sample resulted in a similar if not greater severity of infection as observed in the R-Total group. A previous study by Roberts et al. (2003) had shown that the acidity of the ROFA samples was not a factor in altered pulmonary bacterial clearance; rather, the clearance of the pathogen depended on the soluble metal content of the ROFA. In addition, the removal of soluble metals from the R-Soluble group resulted in a pattern of infection similar to that of the Saline group, confirming that the alteration in the severity of infection in rats treated with R-Soluble is due entirely to one or more the soluble cations that had been removed.

Biochemical analysis of BAL fluid: LDH and albumin

After infection, animals pre-treated with R-Soluble had significantly higher LDH levels on days 6 and 8 when compared to control animals, which were slightly higher than pre-infection levels in the uninfected R-Soluble rats (Fig. 3A). Also, LDH was increased in the infected R-Total animals on day 6 when compared to controls. The R-Chelex group did not differ from control post-infection. Albumin increased and remained elevated in the R-Soluble group at all time points post-infection when compared to all groups (Fig. 3B). These results indicate that the R-Soluble sample caused a substantial level of lung injury and cytotoxicity that at times exceeded the R-Total sample.

BAL cellular profiles

Total BAL cell number was counted (Fig. 4A), and the number of AMs (Fig. 4B), PMNs (Fig. 4C), and lymphocytes (Fig. 4D) were calculated from cell differentials. In general, in response to the infection, there is a trend for an increase in the number of cells in the airspace in all groups post-inoculation with *L. monocytogenes*. However, the animals treated with R-Soluble had a significantly increased number of total BAL cells when compared to all other groups post-infection, including the R-Total group. The increase can be attributed to the influx of PMNs at the early time points post-infection (Fig. 4C), and to the macrophage and lymphocytes populations at the later time points (Figs. 4B+D).

Flow cytometry, in conjunction with cell differential counts, was used to determine the number of NK cells, B cells, total T cells, $CD4^+$ T cells, and $CD8^+$ T cells. In general, the NK cell and lymphocytes populations gradually increased in all treatment groups post-infection. At the early time points post-infection, the R-Soluble group tended to have lower numbers of NK cells when compared to the saline group, although the difference was not significant, and there were no significant differences in the lymphocytes numbers (data not shown). However, on day 10 there was a significant increase in NK cell number and T cell number in the R-Soluble-treated rats when compared to all other groups (Table 4). This was attributed to an increase in both subsets of the T cell population, $CD4^+$ and $CD8^+$ T cells, at day 10. Over the course of the infection, there is

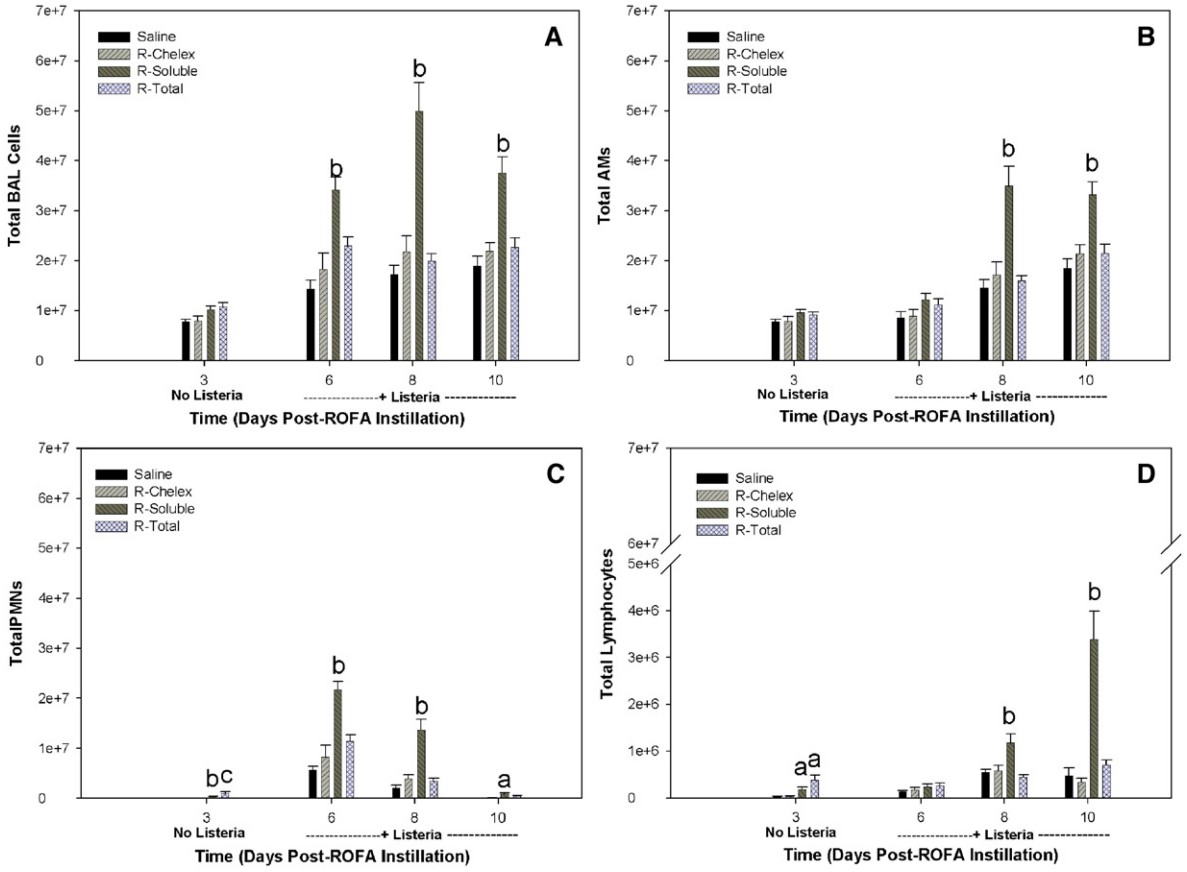

Fig. 4. Total cells (A), AMs (B), PMNs (C), and lymphocytes (D) present in the BAL of rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or Saline 3 days prior to intratracheal inoculation with *L. monocytogenes* as determined by cell differentials. Values are means ± SE ($n = 8$/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex, [c]significantly different from R-Soluble, R-Chelex, and Saline ($p < 0.05$).

a much larger expansion of the T cell population when compared to B cells reflecting the dominance of the cell-mediated immune response to *L. monocytogenes*.

BAL fluid levels and BAL cellular production of nitric oxide

The presence of NO, a known antimicrobial agent, as well as an indicator for increased oxidative stress, was measured as NO_x (nitrate and nitrite) (Fig. 5). NO_x in the BAL fluid was significantly elevated in the R-Soluble group when compared to all other groups at the early time point post-infection (Fig. 5A). Post-infection, NO_x production by cells in all treatment groups increased markedly when compared to levels on day 3; however, AMs from the R-Soluble and R-Total groups produced significantly less NO_x than the control groups on day 6 (Fig. 5B), and on day 8, AMs from the R-Soluble group continued to produce less total nitrite when compared to the rats in the Saline group. At day 10, NO_x production remained elevated for all treatments, and there were no significant differences among groups.

Oxidative potential of phagocytes: PMA- and zymosan-stimulated CL

Luminol-dependent CL was performed on BAL cells to determine the level of production of ROS by AMs and PMNs. After infection, there was an increase in both PMA- and zymosan-stimulated CL (Figs. 6A and B, respectively) in all of the treatment groups; however, the increase in PMA-stimulated and zymosan-stimulated CL was significantly greater in the R-Soluble-treated rats when compared to all other groups on days 6 and 8, and this increased persisted on day 10 after zymosan stimulation. By day 10, PMA-stimulated oxidant production had returned to levels observed prior to infection (Day 3).

BAL cytokine analysis

Cytokines present in the BAL of the R-Total, R-Soluble, R-Chelex, and Saline groups were measured post-infection. TNF-α (Fig. 7A), IL-6 (Fig. 7B), IL-12 (Fig. 7C), and IL-10 (Fig. 7D) were measured in the BAL to evaluate the inflammatory response. On day 6, there was an increase in TNF-α in the BAL in all the groups as a response to the bacterial infection when compared to the pre-infection levels on day 3; however, there were no significant differences among treatments at any of the time points post-infection (Fig. 7A). Post-infection, on days 6 and 8, there was a significant elevation in IL-6 in the animals pre-treated with R-Soluble when compared to all other treatments (Fig. 7B), indicating a more persistent and severe inflammatory response. IL-12 is a pro-inflammatory and immunomodulatory cytokine. The highest levels of IL-12,

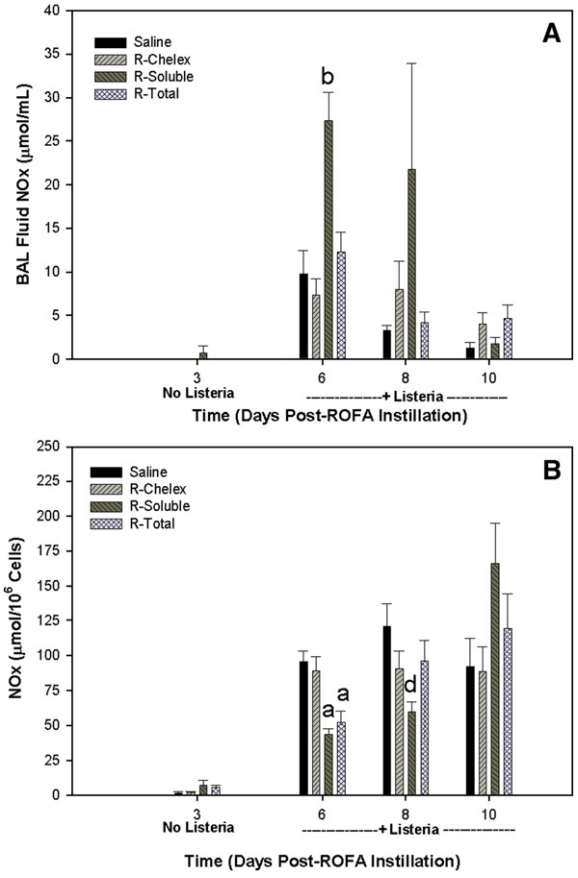

Fig. 5. Nitrate and nitrite (NO_x) in the BAL fluid (A) and cell media from BAL AMs cultured for 18 h (B) from rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or Saline 3 days prior to intratracheal inoculation with *L. monocytogenes*. Values are means±SE ($n=8$/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex, [d]significantly different from Saline ($p<0.05$).

secreted primarily by innate responders, were observed at the early time point post-infection (Fig. 7C). However, there was a significant decrease in the R-Total group in the levels of IL-12 relative to the other groups at this time point, indicating a possible inhibition in the development of the immune response to *L. monocytogenes* in this group. Because IL-12 should have a greater impact on the immune response in the early stages of infection as opposed to the later phase, the slight but significant decrease in IL-12 at day 10 in the R-Soluble group when compared to controls likely did not affect the course of the infection. IL-10 is considered to primarily be inhibitory in nature, and to be an anti-inflammatory cytokine. At day 6, IL-10 levels in the BAL of animals pre-treated with R-Soluble, but not R-Total, remained elevated when compared to the Saline control rats, potentially inhibiting cells involved in the clearance of the bacteria from the lungs (Fig. 7D).

To determine the potential activity of the $CD4^+$ T cell subsets in the lung, levels of IL-2 and IL-4 were measured in the BAL fluid. The decrease in IL-2 that was observed in R-Soluble rats prior to infection on day 3 persisted in infected rats in that treatment group throughout the time course (Fig. 8A). After infection, there was significantly less IL-4 in the BAL of rats pre-treated with R-Soluble when compared to controls on day 6 (Fig. 8B). The trend for the decrease in IL-4 in the R-Soluble group continued on days 8 and 10; however, it was not statistically significant at those time points. Changes in the levels of these cytokines may be indicative of alterations in T cell function. IFN-γ is also produced by $CD4^+$ T_H1 cells, as well as by $CD8^+$ T cells and NK cells. There was a general increase among treatment groups at the first time point post-infection, but there were no significant differences in the cytokine post-infection (data not shown). Levels of this cytokine in the BAL were extremely variable across all time points, as well as within groups at a single time point. Differences in IFN-γ levels may be better detected at an earlier time point post-infection, or by measuring levels in media after culturing of BAL or lymph node cells of infected animals.

Discussion

The goal of this study was to determine the potential effects on both the innate and adaptive immune responses in the lung of rats exposed to the soluble metals in ROFA prior to pulmonary

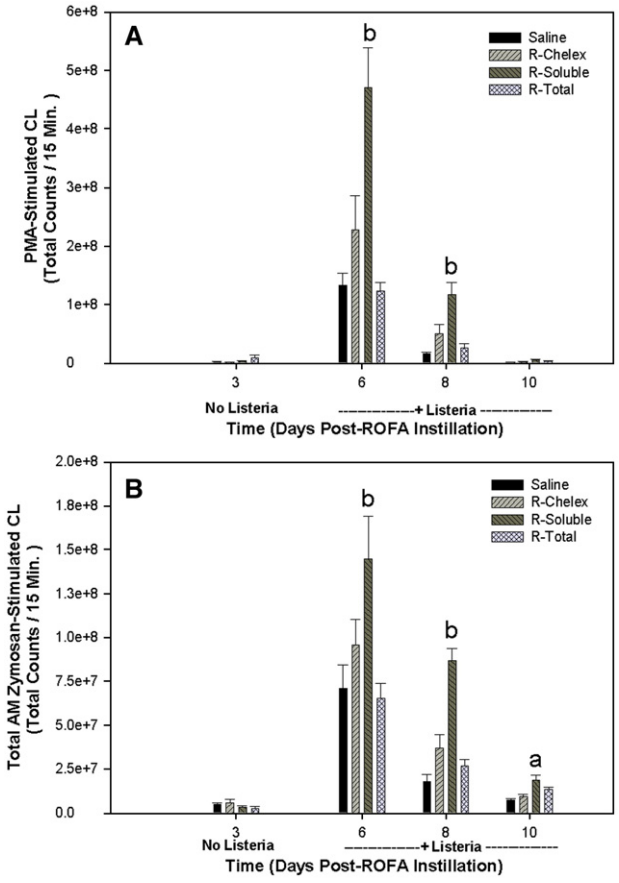

Fig. 6. BAL cellular chemiluminescence (CL) depicted in total counts per 15 min for total BAL cells after stimulation with PMA (A) or for AMs after stimulation with non-opsonized zymosan (B) in rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or Saline 3 days prior to intratracheal inoculation with *L. monocytogenes*. Values are means±SE ($n=8$/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex ($p<0.05$).

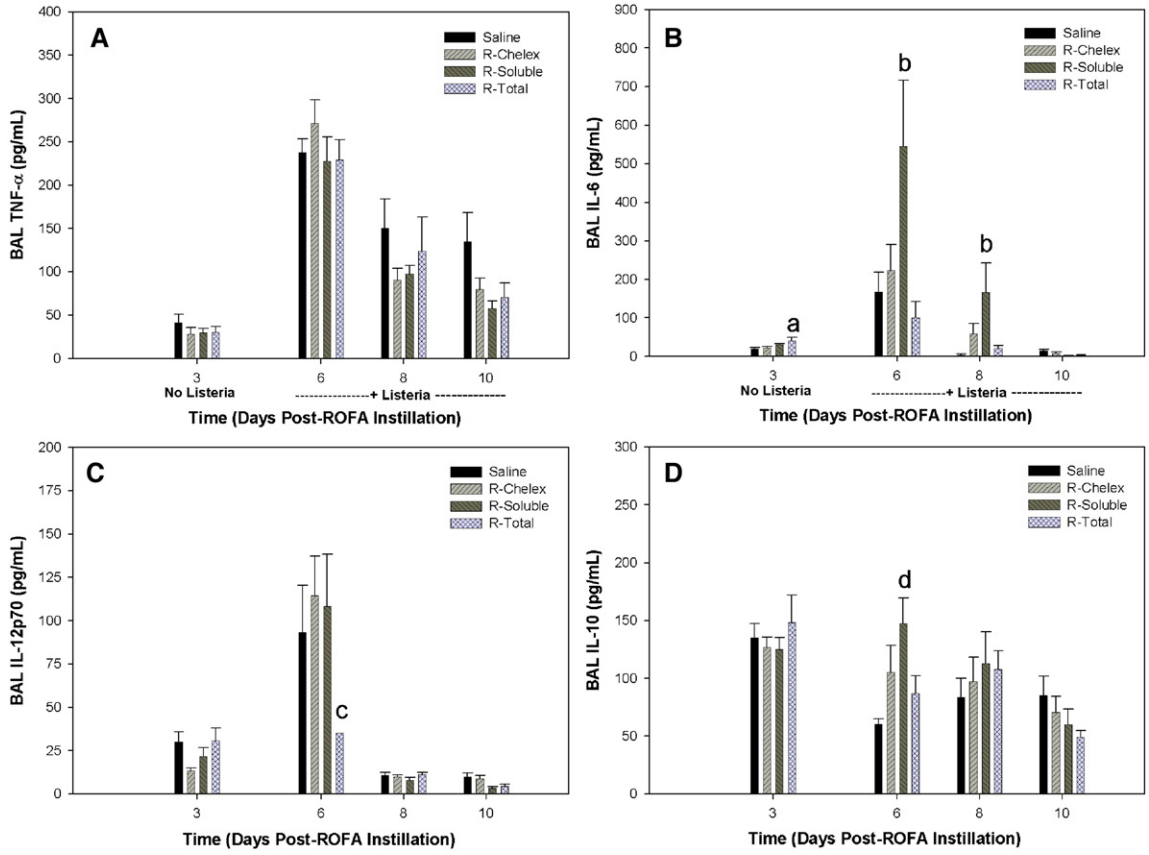

Fig. 7. Concentration of pro-inflammatory cytokines, TNF-α (A), IL-6 (B), and IL-12p70 (C), and anti-inflammatory cytokine, IL-10 (D) in the BAL fluid of rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or Saline 3 days prior to intratracheal inoculation with *L. monocytogenes*. Values are means ± SE ($n = 8$/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex, [c]significantly different from R-Soluble, R-Chelex, and Saline, [d]significantly different from Saline ($p < 0.05$).

bacterial infection. This study found that the soluble metals in ROFA caused increased lung injury and cytotoxicity prior to infection, and that the increased injury and cytotoxicity persisted throughout the time course post-infection. In addition, this study demonstrated that the soluble fraction of ROFA decreased bacterial clearance from the lungs of rats to a comparable, if not greater degree, when compared to that of the whole ROFA sample. The infection in the R-Soluble sample was characterized by a greater influx of neutrophils into the lungs at the early time points post-infection, and macrophages and lymphocytes at the later time points, and appeared to alter the activity of macrophages and lymphocytes ($CD4^+$ T cells, and $CD8^+$ T cells) post-infection. In addition, the soluble sample altered production of ROS and RNS, IL-6, IL-10, IL-2 and IL-4 in the lungs of rats post-infection. This novel finding indicates that the soluble metals may adversely affect phagocyte and lymphocyte function, consequently altering the innate and adaptive immune responses to *L. monocytogenes* infection. These effects were abolished when the metals were removed from the soluble sample by Chelex, confirming that the soluble metals were responsible for the results, rather than the non-metal constituents present in the sample.

At 3 days post-ROFA exposure, and prior to infection, the primary differences that existed among the animals exposed to the soluble metals or the total ROFA when compared with control groups were increases in LDH and albumin, indicators of cytotoxicity and lung injury, and increased neutrophil numbers. The increased injury and inflammation that was observed early after exposure with the soluble fraction of ROFA was consistent with the findings of other investigators (Dreher et al., 1997; Kodavanti et al., 1998). Similar indicators of injury and inflammation are also observed with the soluble metal fractions of Utah Valley Dust (Dye et al., 2001) and welding fumes (Antonini et al., 2004a). Despite the injury and inflammation, 3 days after exposure to the soluble ROFA, reactive oxygen and nitrogen species, as well as pro-inflammatory cytokines do not appear to be altered prior to bacterial challenge. Interestingly, although T cell populations in the lung did not differ significantly among groups prior to infection, there was a decreased level of IL-2 in the soluble group when compared to controls, indicating that the soluble metals may have the ability to directly inhibit T cells. Because the cell-mediated immune response is critical in *L. monocytogenes* infection, it is possible that the direct effects of these metals may be responsible for alterations in the later adaptive cellular responses to the bacteria.

The initial, innate immune response to *L. monocytogenes* infection involves recruitment of neutrophils, macrophages, monocytes, and NK cells to the infected area. It is possible that the injury in the lung induced by the soluble metals in ROFA

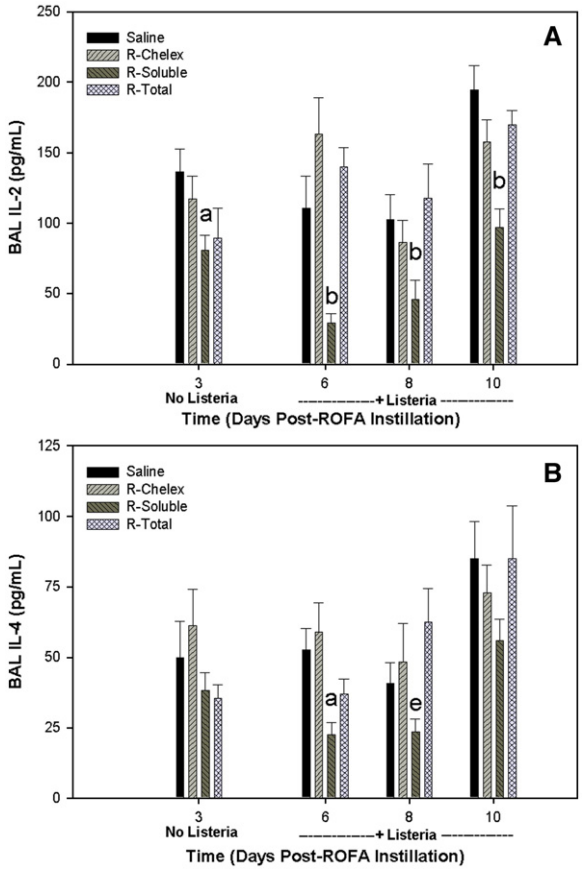

Fig. 8. T cell cytokines, IL-2 (A) and IL-4 (B), in the BAL fluid of rats that were pre-exposed to R-Total, R-Soluble, R-Chelex, or Saline 3 days prior to intratracheal inoculation with *L. monocytogenes.* Values are means±SE (n=8/treatment group/time point); [a]significantly different from Saline and R-Chelex, [b]significantly different from R-Total, Saline, and R-Chelex, [c]significantly different from R-Total ($p<0.05$).

prior to infection may have compromised the host cells' ability to resist infection or that the responsiveness of these cells was altered or inhibited. Antonini et al. (2002) showed that macrophage bactericidal activity in response to *Listeria* challenge was suppressed in animals that were pre-treated with ROFA prior to infection. This study indicates that the soluble metals may be the fraction of the ROFA that is responsible for impaired innate immune response.

Cytokine levels in the BAL and cellular production of reactive species were assessed to try to determine the activity level of cells involved in the innate and adaptive immune response. The pro-inflammatory cytokine cascade, which includes TNF-α, IL-6, and IL-12, is a critical step in the innate immune response to *L. monocytogenes* and leads to a progression that results in the initiation of cell-mediated immunity and further activation of innate responders (Seder and Gazzinelli, 1999). All three of these cytokines were expected to increase post-infection in all groups. IL-6 was found to be significantly elevated in the R-Soluble group post-infection. Although there were no significant differences observed in TNF-α and IL-12 at the time points examined, it is possible that if any differences were to occur it may have taken place earlier after infection, prior to alterations in IL-6.

IL-6 is a pleiotropic cytokine that is produced by many cell types resulting in a variety of effects. IL-6 plays an important role in the acute-phase response, inflammatory response, and, in conjunction with IL-1, IL-6 has been shown to be involved in the initiation of cytotoxic T cell ($CD8^+$) responses (Akira et al., 1990; Ford et al., 1991). In addition, IL-6 may play a critical role in the neutrophil response to *L. monocytogenes* as well. The significant increase in the R-Soluble group on days 6 and 8 corresponded well with the significant influx of neutrophils early on, and cytotoxic T cells ($CD8^+$) later, into the lungs of rats that received the soluble metals prior to infection. The elevation in IL-6 is likely indicative of the increased lung injury and an exacerbated acute-phase response due to a delay in, or the suppression of, the early innate immune response that led to the increased bacterial burden on day 6.

The pro-inflammatory cytokines, including TNF-α, IL-12, and IL-6, are also known to induce production of ROS and RNS in macrophages and neutrophils, either directly or through the induction of IFN-γ production (Billiau, 1996; Borish et al., 1989). ROS and RNS contribute to intracellular bacterial killing (MacMicking et al., 1995; Nacy et al., 1991), and NO has been shown to play a regulatory role in cell-mediated immunity. Alternatively, excessive production of these mediators can lead to oxidative stress which may have adverse effects on the ability of the animal to respond to infection. For example, inducible nitric oxide synthase (iNOS) knock-out mice have an exacerbated T_H1 response to infection (MacLean et al., 1996); and elevated levels of NO can result in inhibition of T cell proliferation (Blesson et al., 2002; Hoffman et al., 2002; Van der Veen et al., 2000) and reduction in IL-2 production (Blesson et al., 2002). These studies indicate that there is a critical level of ROS and RNS that is required to be considered non-disruptive, yet beneficial, in response to an infection.

There was an increase in oxidant production, measured as chemiluminescence, in all groups at the early time point post-infection; however, the increase in the R-Soluble group was significantly greater when compared to all other groups. In addition, NO levels in the BAL were also elevated in the R-Soluble group on day 6. The overall elevation in ROS and RNS burden in the lungs of the R-Soluble rats may contribute to an increased oxidative stress in these animals and further compromise their ability to clear the bacteria. Whether or not the elevations in NO in response to R-Soluble after infection may be altering the course of the immune response to *L. monocytogenes* by directly increasing lung injury and/or altering T cell function requires further investigation.

Table 4
BAL cell phenotype on day 10 determined by flow cytometry (10^4 cells)

Treatment	NK cells	B cells	Total T cells	$CD4^+$ T cells	$CD8^+$ T cells
Saline	6.14±1.47	9.92±2.41	63.9±14.6	17.7±3.90	11.0±2.40
R-Chelex	5.95±0.730	9.10±1.97	51.7±9.85	17.9±3.13	12.3±1.77
R-Soluble	11.8±1.31[a]	17.7±4.63	126±28.8[a]	53.4±11.4[a]	37.0±4.98[a]
R-Total	4.95±1.10	6.39±2.07	34.3±9.05	14.8±4.23	9.31±1.77

Values are means±SE., [a]Significantly different from all groups ($p<0.05$).

Interestingly, when NO_x production was measured on a per cell basis *ex vivo*, there was a significant decrease in the NO_x levels in the R-Soluble- and R-Total-treated animals on day 6, suggesting suppression of AM function. This suggests that the increase in NO_x in the BAL of the soluble group may be a product of the significant influx of neutrophils into the lung or increased NO production by type II epithelial cells, and that the soluble metals in ROFA are suppressing AM function early after infection. Because the metals in the R-Total group have been shown to be redox-reactive, and that this property belongs entirely to the soluble fraction (Antonini et al., 2004b; Lewis et al., 2003), it is also possible that these soluble metals may be altering the NO production or the NO product intracellularly. This reduction in NO production by AMs is in agreement with the findings of Antonini et al. (2002) where at the early time point after infection, animals pre-treated with the total ROFA showed a decrease in AM NO_x production. The authors also showed that AMs from animals exposed to ROFA prior to infection had decreased intracellular killing of bacteria, a mechanism that has been attributed to the ability of AMs to produce nitric oxide (Green et al., 1994; MacMicking et al., 1995).

It is of interest to note that in this study, the changes in inflammatory cytokines and oxidants observed in the soluble sample were not recapitulated in the R-Total sample. One possible explanation for this may involve the relatively low bacterial dose used in this study. The R-Soluble sample was observed to induce a more severe infection early on when compared to the R-Total in this study, and the same pattern was observed in a previous study where a much higher bacterial dose was employed (Roberts et al., 2003). Although the lower dose of bacteria is enough to induce a greater infection in the R-Total sample than compared to controls, it may not be high enough for detection of more subtle cellular changes that would impair the immune responses that were observed in the R-Soluble sample at the same bacterial dose.

AMs are critical in the killing and clearance of *L. monocytogenes*. In fact, suppression of AM activity in the lungs has been shown to be correlated with decreased clearance of *L. monocytogenes* in response to the whole ROFA sample (Antonini et al., 2002) and to other air pollutants, such as diesel (Yang et al., 2001; Yin et al., 2002) and ozone (Van Loveren et al., 1988). Alterations in IL-10 production may be involved in the inhibition of AM killing of *L. monocytogenes*. IL-10 is an anti-inflammatory cytokine produced by a variety of cell types, including activated macrophages, and has numerous effects on a number of different cells types (Moore et al., 2001). IL-10 inhibits activation of macrophages by NK cells and T_H1 cells by blocking IFN-γ and TNF-α signals from these cells, which in turn activate intracellular killing of pathogens by the AMs. It also directly inhibits macrophage production of cytokines that drive the cell-mediated immune response. In addition, *L. monocytogenes*, and other intracellular pathogens that specifically target macrophages for infection, use IL-10 to prolong survival by suppressing the host immune response (Redpath et al., 2001).

The current study found that IL-10 was significantly elevated on day 6 in the rats treated with soluble metals prior to infection when compared to the Saline group, and this increase correlated with the highest bacterial burden in the lungs of these animals. Similar results have also been observed in other studies that utilize the respiratory *L. monocytogenes* model of infectivity (Antonini et al., 2004a; Yin et al., 2004). Antonini et al. (2004a, 2004b) found that IL-10 increased after infection in rats pre-exposed to welding fume, and that bacterial clearance decreased. Interestingly, the factor that appeared to influence bacterial clearance by rats pre-treated with different welding fumes in the study was the metal solubility of the fume, with the most soluble fume having the greatest impact on bacterial clearance.

Activation of the cell-mediated arm of the immune system is critical in long-term elimination of *L. monocytogenes* infection (Emmerling et al., 1975). The adaptive immune response to the infection by all groups included an increase in lymphocyte influx into the lung that included both $CD4^+$ and $CD8^+$ T cells over the time course, as well as an increase in NK cells and AMs. As mentioned previously IL-12 can influence the direction of the adaptive immune response toward a cell-mediated, rather than a humoral, response. IL-12 was observed to be increased on day 6 in all groups except the R-Total group. Although there was no difference in IL-12 in the R-Soluble group when compared to control groups at the early time point post-infection, by day 8 and 10, there was a significant increase in the lymphocyte response in the soluble metals group when compared to all other groups. Increases in active $CD4^+$ T_H1 cells and NK cells in response to *L. monocytogenes* infection further stimulate AMs to kill and clear the bacteria from the lungs. The significant increases in these cell types in the R-Soluble group may have been required to respond to the elevated bacterial burdens in the lungs of these rats. Interestingly, there was significantly less IL-12 in the R-Total group on day 6 when compared to all groups, including the R-Soluble group. This discrepancy between the two groups made it difficult to distinguish how ROFA may be altering antigen presentation and T-cell differentiation. It is possible that the samples may be differentially affecting dendritic cell function. Further investigation in this area is necessary to draw conclusions on dendritic cell responses in this model.

The activity of the T cells that responded to the infection may provide evidence as to why the higher bacterial burdens persisted in the soluble metals group throughout the time course of the infection. Animals treated with R-Soluble prior to infection had significantly reduced levels of IL-4, a $CD4^+$ T_H2 cytokine, and more importantly lower IL-2, a cytokine produce by undifferentiated T cells, as well as $CD4^+$ T_H1 and $CD8^+$ T cells. Although T_H2 cells do not play a major role in the response to *L. monocytogenes*, it has been suggested that an initial burst in IL-4 within the first day of infection aids in recruitment of neutrophils and monocytes to the area of infection by stimulating chemokine production (Kaufmann et al., 1997). IL-2 plays an important role throughout the course of the infection, in an autocrine fashion, acting as a growth and proliferative factor promoting clonal expansion of T_H1 and

cytotoxic T cells (Minami et al., 1993), and in a paracrine fashion, activating NK cells (Henney et al., 1981; Naume et al., 1993) and promoting survival of neutrophils (Djeu et al., 1993) and monocytes (Espinoza-Delgado et al., 1995). Activated T_H1, cytotoxic T, and NK cells would, in turn, secrete cytokines, such as IFN-γ, which would activate and enhance intracellular killing of bacteria by macrophages.

The decrease in both IL-2 and IL-4 prior to and post-infection, and the persistent decrease in IL-2 through day 10, was not likely due to lack of T cell recruitment into the lung, as there was a significant increase in T cells responding to the infection in animals pre-treated with soluble metals on day 10. These data, in conjunction with the apparent alteration in T cell function prior to infection, suggest that the soluble metals may be altering T cell activity directly, resulting in a slowing in the clearance of the bacteria over time. Alternatively, the T cells may be inhibited indirectly. Soluble metals in ROFA may induce production of mediators or second messengers that may inhibit T cell activity, such as NO, which has also been shown to alter T cell cytokine production as well as gene transcription (Blesson et al., 2002).

Although the early inhibition of bacterial clearance in the R-Soluble and R-Total groups may be due to inhibition of macrophage activity, the later responses, which result in a similar pattern of clearance between the two groups, may be due to different mechanisms. Where the soluble metals appear to be altering lymphocyte function directly at the later time points, the total ROFA may be interfering with the necessary signals between the innate and adaptive immune system earlier on, as demonstrated by a low IL-12 production. As mentioned previously, the insoluble metals alone did not alter infectivity (Roberts et al., 2003); therefore, the differences between the soluble ROFA fraction and the total sample may be due to total metal content or to interactions between certain particulate and soluble metals in the total sample, which may alter the bioavailability or distribution of different metals. The presence and quantities of metals in the total sample, such as vanadium, which are absent in the soluble fraction, may act antagonistically with other metals, such as nickel, as suggested by both *in vivo* and *in vitro* studies of metal mixtures (Dreher et al., 1997; Fisher et al., 1986; Geertz et al., 1994; Kodavanti et al., 1998), which may ultimately result in different effects on AMs, NK cells, and lymphocytes.

Despite the selectivity of the chelating agent, Chelex, for divalent transition metals over monovalent cations, there is still a slight possibility that Chelex may have removed other cations from the soluble fraction of ROFA, and this may also be contributing to the responses observed in that sample. To address this, studies are being conducted to determine which soluble metal or group of metals may account for the changes observed in the R-Soluble group, and to address to what degree the metals, and not another unknown soluble component, contribute those responses.

To summarize, the soluble metals in ROFA increased lung injury and appeared to be the primary constituent responsible for the altered immune response to *L. monocytogenes*. Although the effects altering the adaptive response between the soluble metals and the total ROFA may differ, the ultimate result appears to be the same. Inhibition of AM bactericidal activity led to a high bacterial burden early in the infection, and inhibition of, delay in, or alteration of the adaptive immune response resulted in the slowing of the bacterial clearance over time. Experiments that examine lymphocyte production of cytokines harvested from the lung-draining lymph nodes are also currently being conducted to better explain the direction of the early lymphocyte response in these animals.

Acknowledgments

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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