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Suppression in lung defense responses after bacterial infection in rats pretreated with different welding fumes

James M. Antonini*, Michael D. Taylor, Lyndell Millecchia, Alicia R. Bebout, Jenny R. Roberts

Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505 USA

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Abstract

Epidemiology suggests that inhalation of welding fumes increases the susceptibility to lung infection. The effects of chemically distinct welding fumes on lung defense responses after bacterial infection were compared. Fume was collected during gas metal arc (GMA) or flux-covered manual metal arc (MMA) welding using two consumable electrodes: stainless steel (SS) or mild steel (MS). The fumes were separated into water-soluble and -insoluble fractions. The GMA-SS and GMA-MS fumes were found to be relatively insoluble, whereas the MMA-SS was highly water soluble, with the soluble fraction comprised of 87% Cr and 11% Mn. On day 0, male Sprague–Dawley rats were intratracheally instilled with saline (vehicle control) or the different welding fumes (0.1 or 2 mg/rat). At day 3, the rats were intratracheally inoculated with 5×10^3 *Listeria monocytogenes*. On days 6, 8, and 10, left lungs were removed, homogenized, cultured overnight, and colony-forming units were counted to assess pulmonary bacterial clearance. Bronchoalveolar lavage (BAL) was performed on right lungs to recover phagocytes and BAL fluid to measure the production of nitric oxide (NO) and immunomodulatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-2, IL-6, and IL-10. In contrast to the GMA-SS, GMA-MS, and saline groups, pretreatment with the highly water soluble MMA-SS fume caused significant body weight loss, extensive lung damage, and a dramatic reduction in pulmonary clearance of *L. monocytogenes* after infection. NO concentrations in BAL fluid and lung immunostaining of inducible NO synthase were dramatically increased in rats pretreated with MMA-SS before and after infection. MMA-SS treatment caused a significant decrease in IL-2 and significant increases in TNF-α, IL-6, and IL-10 after infection. In conclusion, pretreatment with MMA-SS increased production of NO and proinflammatory cytokines (TNF-α and IL-6) after infection, which are likely responsible for the elevation in lung inflammation and injury. In addition, MMA-SS treatment reduced IL-2 (involved in T cell proliferation) and enhanced IL-10 (involved in inhibiting macrophage function) after bacterial infection, which might result in a possible suppression in immune response and an increase in susceptibility to infection.

Keywords: Welding fume; *Listeria monocytogenes*; Bacterial clearance; Nitric oxide; Macrophage; Cytokines

Introduction

Acute upper and lower respiratory tract infections have been reported to be increased in terms of severity, duration, and frequency among welders (Howden et al., 1988). Doig and Challen (1964) found that the excess in mortality observed among welders was due to pneumonia. Wergeland and Iversen (2001) announced that the Norwegian Labor Inspection Authority has warned all Norwegian physicians about the possible lethal risk of an association between pneumonia and the inhalation of metal fumes. Coggon et al. (1994) analyzed three sets of occupational mortality data that spanned the years 1959–1990 and observed a significant increase in mortality from pneumonia among welders. Importantly, retired welders did not demonstrate an increase in pneumonia deaths, leading the authors to rule out nonoccupational confounding factors. Recent evidence has

* Corresponding author. Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505. Fax: +1 304 285 5938.
E-mail address: jga6@cdc.gov (J.M. Antonini).

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suggested that ferrous and other metal fumes inhaled in the workplace may increase the susceptibility to infection (Palmer et al., 2003).

The chemical properties of welding fumes can be quite complex (Antonini, 2003). Most welding materials are alloy mixtures of metals characterized by different steels that may contain Fe, Mn, Cr, Ni, and others. Fumes generated from stainless-steel (SS) electrodes usually contain significant levels of Cr and Ni, whereas fumes from mild steel (MS) welding processes are predominantly Fe without the presence of Cr or Ni. In flux-covered manual metal arc welding (MMA), fluxes are incorporated into the consumable electrode and used to protect the weld from oxidation. The molten fluxes, which commonly contain high levels of fluorides and silicates, help carry away impurities from the weld in a liquid stream. The fluxes then are commonly a source for inhalation exposures.

Differences also exist in the water solubility of the metals found in different types of welding fumes. Fumes generated during MMA welding are generally more water-soluble than fumes collected from gas metal arc welding (GMA) processes, in which inert gases are used to protect the weld (Antonini et al., 1999). The presence of soluble metals (which are likely more bioavailable) have been shown to be important in the potential toxic responses observed after in vivo (Taylor et al., 2003; White et al., 1982) and in vitro (Antonini et al., 1999) welding fume exposure.

It is possible that pulmonary exposure to welding fumes may increase the susceptibility to lung infection in welders. Animal studies investigating the effects of welding fumes on immune responses are lacking in this regard. It was the objective of the current study to determine whether welding fumes of different chemical and physical characteristics suppress lung defense responses. Rats were pretreated with different welding fumes of vastly different metal compositions and solubility properties before pulmonary inoculation with a bacterial pathogen. It was hypothesized that soluble metals present in welding fumes may affect lung defenses and increase the susceptibility to infection in rats.

Materials and methods

Animals. Male Sprague–Dawley [Hla:(SD) CVF] rats from Hilltop Lab Animals (Scottdale, PA), weighing 250–300 g and free of viral pathogens, parasites, mycoplasmas, *Helicobacter*, and CAR Bacillus, were used for all experiments. The rats were acclimated for at least 6 days after arrival and were housed in ventilated polycarbonate cages on Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, and provided HEPA-filtered air, autoclaved Prolab 3500 diet, and tap water ad libitum. The animal facilities are specific pathogen-free, environmentally controlled, and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Welding sample collection and characterization. Bulk samples of different welding fume samples were collected by Kenneth Brown of Lincoln Electric Co. (Cleveland, OH) and have been used in a previous study (Antonini et al., 1999). The fumes were generated in a cubical open front fume chamber (volume = 1 m^3) by a skilled welder using a manual or semiautomatic technique appropriate to the electrode and collected on 0.2 μm Nuclepore filters (Nuclepore Co., Pleasanton, CA). The fume samples were generated in three different ways: (1) gas metal arc welding using a mild steel E70S-3 electrode (GMA-MS); (2) gas metal arc welding using a stainless-steel ER308L Si electrode (GMA-SS) with argon and CO_2 shielding gases to protect the weld from oxidation; and (3) manual metal arc welding using a flux-covered stainless steel E308-16 electrode (MMA-SS). Particle sizes of the welding samples were previously characterized by scanning electron microscopy and were of respirable size with count mean diameters for the GMA-MS, GMA-SS, and MMA-SS fumes measuring 1.22, 1.38, and 0.92 μm, respectively (Antonini et al., 1999).

Welding fume samples (GMA-MS, GMA-SS, and MMA-SS) were suspended in sterile phosphate-buffered saline (PBS), pH 7.4, and sonicated for 1 min with a Sonifier 450 Cell Disruptor (Branson Ultrasonics, Danbury, CT). The GMA-MS and GMA-SS samples were found to contain negligible soluble metals. Because the MMASS sample was found to be highly water-soluble in a previous study (Antonini et al., 1999), it was further divided into soluble and insoluble components. The particle suspension (MMA-SS-Total) was incubated for 24 h at 37 °C, and the sample was centrifuged at 12,000 × *g* for 30 min. The supernatant of the sample (MMA-SS-Sol) was recovered and filtered with a 0.22 :m filter (Millipore Corp., Bedford, MA). The pellet (MMA-SS-Insol) was resuspended in PBS. Analysis of the metal constituents of the welding fume, as well as the soluble and insoluble fractions of the MMA-SS fume, was previously reported (see Table 1) and determined by inductively coupled argon plasma atomic emission spectroscopy (Antonini et al., 1999).

Table 1
Welding fume characterization

Sample	Metal analysis (wt.%)	Soluble/insoluble ratio	pH
MMA-SS	Fe 41	0.345	Total 6.92
	Mn 17	Soluble metals:	Sol 7.05
	Cr 29	Cr 87%	Insol 7.09
	Ni 3	Mn 11%	
GMA-SS	Fe 53	0.006	Total 6.94
	Mn 23		Sol 6.97
	Cr 19		Insol 7.01
	Ni 5		
GMA-MS	Fe 85	0.020	Total 7.02
	Mn 14		Sol 7.44
			Insol 7.03

The data presented were modified from Antonini et al. (1999).

Experimental design. On day 0, rats were pretreated by intratracheal instillation of the welding fume samples or saline (vehicle control). On day 3, animals from each group were intratracheally inoculated with either saline or 5×10^3 *Listeria monocytogenes*. At days 3 (preinfection), 6, and 10, the left lungs were tied off, and bronchoalveolar lavage (BAL) was performed on the right lungs of some animals from each group. The recovered cells were differentiated and chemiluminescence (CL) and nitric oxide (NO) production were determined as indices of macrophage function. Albumin and lactate dehydrogenase (LDH), two indices of lung injury, and several immunomodulatory cytokines were measured in the acellular bronchoalveolar lavage fluid (BALF). From the same animals from days 6, 8, and 10, the nonlavaged left lungs were removed, homogenized, and the number of bacterial colony-forming units (CFUs) was determined to assess bacterial load. In a separate set of animals from each of the treatment groups, histopathological analysis was performed on nonlavaged lungs. Animal weights and morbidity were monitored over the course of the treatment period to evaluate the general health status of the infected animals.

Welding fume treatment. Rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN) and intratracheally instilled with 0.1 or 2 mg/rat of the welding fume samples (MMA-SS, GMA-SS, and GMA-MS) in 300 μl of saline, according to the method of Reasor and Antonini (2001). The welding fume doses of 0.1 and 2.0 mg/rat were chosen based on results from a previous study (Antonini et al., 1996). In addition, the MMA-SS-Total sample was divided into soluble (supernatant) and insoluble (the pellet resuspended in initial particle suspension volume) fractions as described in the Welding Sample Collection and Characterization section. The animals in the MMA-SS-Sol group received intratracheal instillations of the microgram equivalent of the soluble fraction of the Total sample, whereas the animals in the MMA-SS-Insol group received intratracheal instillations of the microgram equivalent of the insoluble fraction of the Total sample. Animals in the vehicle control group were intratracheally sham exposed with 300 μl of sterile saline.

Intratracheal bacterial inoculation. L. monocytogenes (strain 10403S, serotype 1) 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 at an optical density of 600 nm using a spectrometric method and diluted with sterile saline to a concentration of 5×10^3 of *L. monocytogenes*/500 μl of sterile saline. At day 3, the rats treated with welding fume and saline were further divided and inoculated intratracheally with 5×10^3 of *L. monocytogenes* or sterile saline. In a previous pilot study, this bacterial dose did not elicit an inflammatory response in the lungs, gave a uniform infection, and did not kill untreated naive Sprague–Dawley rats (Antonini et al., 2001a).

Pulmonary clearance of L. monocytogenes. At days 6, 8, and 10, the left lungs were removed from rats in each treatment group. The excised tissues were suspended in 10 ml of sterile water, homogenized using a Polytron 2100 homogenizer (Brinkmann Instruments, Westbury, NY), and cultured on brain heart infusion agar plates (Becton Dickinson and Co., Cockeysville, MD). The number of viable CFUs were counted after an overnight incubation at 37 °C.

Histopathology. Histopathological analysis was performed on the nonlavaged lungs of selected rats from each group. Rats were euthanized with sodium pentobarbital (>100 mg/kg body weight, Butler Co., Columbus, OH), and the lungs were preserved with 10% neutral buffered formalin by airway fixation at total lung capacity. The entire lungs were removed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Bronchoalveolar lavage. The rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (>100 mg/kg body weight, Butler) and then exsanguinated by severing the abdominal aorta. At days 3 (preinfection), 6, and 10, the left bronchus was clamped off, and BAL was performed on the right lungs of selected rats from each group. The right lungs were first lavaged with a 1 ml/100 g body weight aliquot of calcium- and magnesium-free PBS, pH 7.4. The first fraction of recovered BALF was centrifuged at $500 \times g$ for 10 min, and the resultant cell-free supernatant was analyzed for various biochemical parameters and cytokine levels. The lungs were further lavaged with 6-ml aliquots of PBS until 30 ml were collected. These samples also were centrifuged for 10 min at $500 \times g$ and the cell-free BALF discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 ml of PBS buffer and evaluated as described below.

Cellular evaluation. Total cell numbers were determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cells were differentiated using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England). Cell suspensions (5×10^4 cells) were spun for 5 min at 800 rpm and pelleted onto a slide. Cells (200/rat) were identified after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA).

Biochemical parameters of injury. Using the acellular first fraction of BALF, albumin content, an index to quantify increased permeability of the bronchoalveolar–capillary barrier, and LDH activity, an indicator of general cytotoxicity, were measured. Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromocresol green using an albumin BCG diagnostic kit (Sigma Chemical Co., St. Louis, MO). LDH activity was determined

by measuring the oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurements were performed with a COBAS MIRA autoanalyzer (Roche Diagnostic Systems, Montclair, NJ).

Chemiluminescence. Luminol-dependent CL measures the light generated as reactive oxygen/nitrogen species are produced by activated cells. CL was performed with an automated Berthold Autolumat LB 953 luminometer (Wallace, Inc., Gaithersburg, MD) as previously described (Antonini et al., 1994). Luminol was used as an amplifier to enhance detection of the light, and 2 mg/ml of unopsonized zymosan (Sigma) was added immediately before the measurement of CL to activate the AMs. Rat neutrophils (PMNs) have not been observed to respond to unopsonized zymosan in our system, therefore, the zymosan-stimulated CL produced is generated by AMs. Measurement of CL was recorded for 15 min at 37 °C using 5×10^5 AMs, and the integral of counts vs. time was calculated. Zymosan-stimulated CL was calculated as the total counts of stimulated cells minus the total counts of the corresponding resting cells.

Nitric oxide production/immunohistochemistry. The reactive nitrogen intermediate, NO, has been shown to play an important role in antibacterial defenses as well as lung cell toxicity. Acellular BALF was frozen immediately after collection for later determination of in vivo production of NO. The NO oxidation products nitrate (NO_3) and nitrite (NO_2), collectively called NOx, were assayed. BALF samples were first incubated with *E. coli* nitrate reductase to convert NO_3 to NO_2. Then, NO_2 was measured using a colorimetric method with the Greiss reaction (Green et al., 1982). NOx levels were determined by comparing values to sodium nitrite standards. Conversion of NO_3 to NO_2 was confirmed in every assay by measuring the formation of NO_2 from NO_3 standards.

Immunohistochemistry was performed to measure the expression of inducible nitric oxide synthase (iNOS) by staining excised lung tissue from treated animals (Porter et al., 2002). iNOS is the enzyme responsible for the formation of NO. Paraffin sections were cut at 5 μm, deparaffinized in xylene, and rehydrated. Slides were placed in citrate buffer (pH 6.0) and microwaved. After endogenous peroxidase was blocked in a 1:1 mixture of 3% H_2O_2 and methanol, slides were placed in 1% bovine serum albumin for 30 min at room temperature and then incubated overnight at 4° C with a monoclonal antibody, anti-iNOS Type II, #610329, dilution 1:100 (BD Biosciences, San Diego, CA). A LSAB-2 kit for rat specimens, K0609 (DAKO, Carpinteria, CA) was used to label the antibody, with AEC (Zymed, South San Francisco, CA) as the chromogen. Sections were counterstained with Mayer's hematoxylin.

Lung lavage fluid cytokine analyses. Levels of cytokines, tumor necrosis factor-α (TNF-α), IL-2, IL-6, and IL-10, were assayed in the first fraction of BALF at days 3 (preinfection), 6, and 10. The selection of the cytokines to be assayed was based on their potential role in lung inflammatory and immune responses to intracellular bacterial infection. Cytokine protein concentrations were determined using enzyme-linked immunoabsorbent assay (ELISA) kits (Biosource International, Inc., Camarillo, CA). The results of the colorimetric assay were obtained with a Spectramax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Devices Corp., Sunnyvale, CA).

Statistical analysis. Results are expressed as means ± standard error of measurement (SE). Statistical analyses were carried out with the JMP IN statistical program (SAS, Inc., Belmont, CA). The significance of difference among the treatment groups was determined using an analysis of variance (ANOVA) and Tukey–Kramer post hoc test. For all analyses, the criterion of significance was set at $P < 0.05$.

Results

Elemental analysis of fume samples

The welding fume samples were suspended in saline and the relative amounts of different metals were determined as weight percentages. The elemental composition of the three fumes was found to be quite different (Table 1). The GMA-MS sample was comprised of Fe (85%) and Mn (14%), whereas the two SS samples (MMA-SS and GMA-SS) contained much less Fe with the addition of Cr and Ni. The GMA-SS and GMA-MS samples were found to be relatively water insoluble with soluble-to-insoluble ratios of 0.006 and 0.020, respectively. The flux-covered MMA-SS sample was much more water soluble than the other two samples with a soluble-to-insoluble ratio of 0.345. The majority of the soluble fraction of the MMA-SS fume was comprised of mostly Cr (87%) with some Mn (11%). The pH of the welding fume samples and their soluble and insoluble fractions was mostly neutral.

Morbidity/body weight

Pretreatment with MMA-SS fume (2 mg/rat) had a significant effect on animal survival after infection (data not shown). By day 10, 30% of the rats from the MMA-SS groups had died. Pretreatment with the MMA-SS-Insol and MMA-SS-Sol fractions before infection did not have an effect on survival (data not shown). All rats from the saline, GMA-SS, and GMA-MS groups survived during the 10-day period.

In the assessment of body weight change, rats from the MMA-SS group lost significantly more weight at each time point after infection compared to the other groups (Fig. 1A). Of the animals from the MMA-SS group that had survived, body weights appeared to be returning to control values at

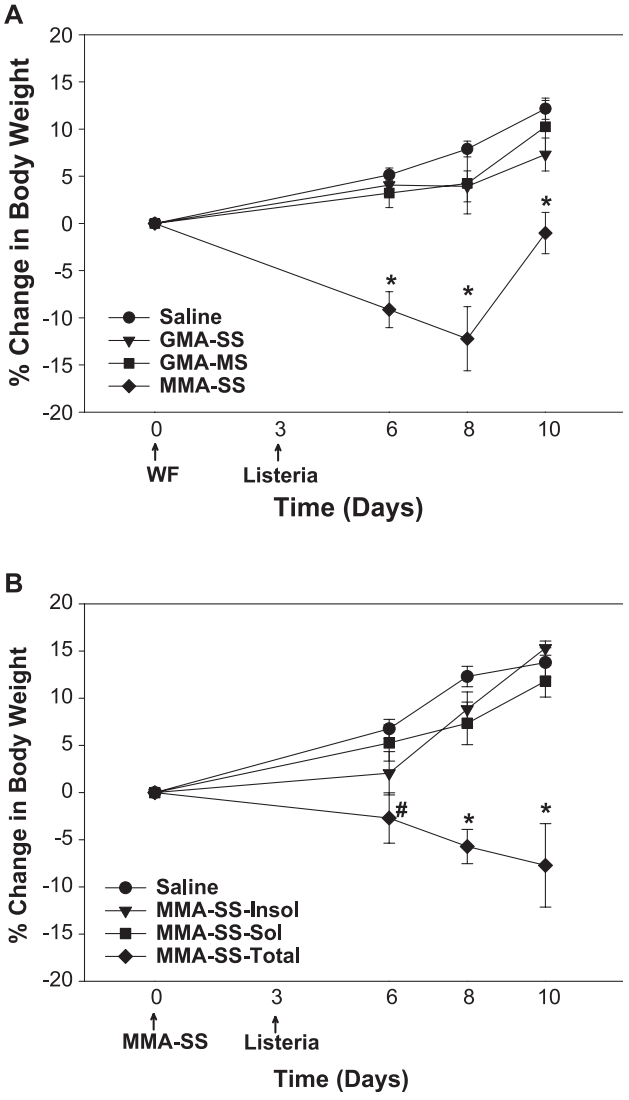

Fig. 1. Percent change in body weights of rats preexposed to (A) gas metal arc-stainless steel (GMA-SS), gas metal arc-mild steel (GMA-MS), and manual metal arc-stainless steel (MMA-SS) welding fumes or (B) the insoluble (MMA-SS-Insol) and soluble (MMA-SS-Sol) fractions of MMA-SS welding fume. The welding samples (2 mg/rat) were intratracheally instilled 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE (n = 8–12); *, significantly different from all other groups at each time point; #, significantly different from MMA-SS-sol and saline groups ($P < 0.05$).

day 10. Pretreatment with the MMA-SS-Insol and MMA-SS-Sol fractions before infection did not cause a loss in body weight (Fig. 1B). None of the welding fume samples had an effect on survival or body weight after treatment with the lower 0.1 mg/rat dose before infection (data not shown).

Bacterial clearance

Pretreatment with the MMA-SS fume (2 mg/rat) before infection caused a dramatic delay in the pulmonary clearance of *L. monocytogenes* (Fig. 2A). Significant increases of 407-, 1511-, and 24-fold were observed in lung CFUs for the MMA-SS group compared with the saline group at days 6, 8, and 10, respectively. Significantly more bacteria were present in the lungs of the MMA-SS group compared with the GMA-SS and GMA-MS groups at days 6 and 8. Pretreatment with the GMA-SS and GMA-MS fumes before infection caused slight, but not significant, increases in lung CFUs at days 8 and 10 compared with the saline group. A significant reduction in pulmonary bacterial clearance was observed at each time point after treatment with the lower 0.1 mg/rat dose of the MMA-SS fume (but not for the other fume samples) when compared with the saline control (data not shown).

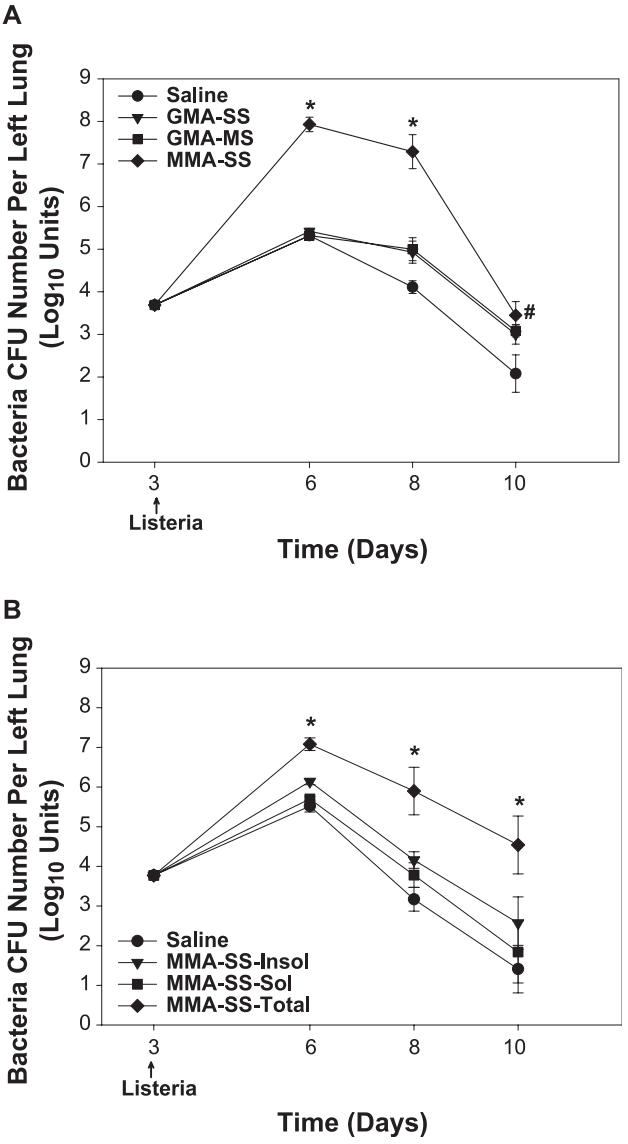

Fig. 2. Pulmonary clearance of bacteria by rats preexposed to (A) gas metal arc-stainless steel (GMA-SS), gas metal arc-mild steel (GMA-MS), and manual metal arc-stainless steel (MMA-SS) welding fumes or (B) the insoluble (MMA-SS-Insol) and soluble (MMA-SS-Sol) fractions of MMA-SS welding fume. The welding samples (2 mg/rat) were intratracheally instilled 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means in log 10 base units ± SE (n = 8–12); *, significantly greater than all other groups at each time point; #, significantly greater than saline group ($P < 0.05$).

In examining the effect of the different fractions of the MMA-SS fume on bacterial clearance, significant increases in lung CFUs were observed for the MMA-SS-Total sample (2 mg/rat) compared with the saline group at all three time points (Fig. 2B). Bacterial clearance of rats preexposed to the MMA-SS-Sol and MMA-SS-Insol fractions before infection was not significantly different from saline controls.

Histopathology

Histopathological analysis was performed on lungs from the treatment groups, and representative micrographs from each group on day 8 are depicted in Figs. 3A–F. Lungs from the saline group appeared mostly normal at each time point after infection with *L. monocytogenes*. A few localized areas of mild inflammation were observed for the saline group at the earlier time points after infection. Mild to moderate areas of inflammation were observed in the lungs of the groups treated with GMA-SS, GMA-MS, MMA-SS-Insol, and MMA-SS-Sol groups after infection on days 6 and 8. By day 10, most of pulmonary inflammation observed for these groups had cleared. Severe pneumonitis, characterized by a peribronchiolar accumulation of neutrophils, consolidation, edema, and the appearance of multiple granulomatous lesions, was observed throughout the lungs at each time point for the groups treated with MMA-SS-Total before infection.

Lung inflammation and injury

Lung cells recovered from each group were counted and identified. MMA-SS pretreatment caused a significant increase in BALF macrophages, PMNs, eosinophils, and lymphocytes before and after infection compared with the saline control group (Figs. 4A–D). Pulmonary inoculation with *L. monocytogenes* of saline-pretreated animals caused a significant increase in PMNs at day 6 and in lymphocytes at days 6 and 10 compared with noninfected controls (Figs. 4B,D).

Different markers of pulmonary injury were measured in the acellular BALF after treatment. MMA-SS pretreatment

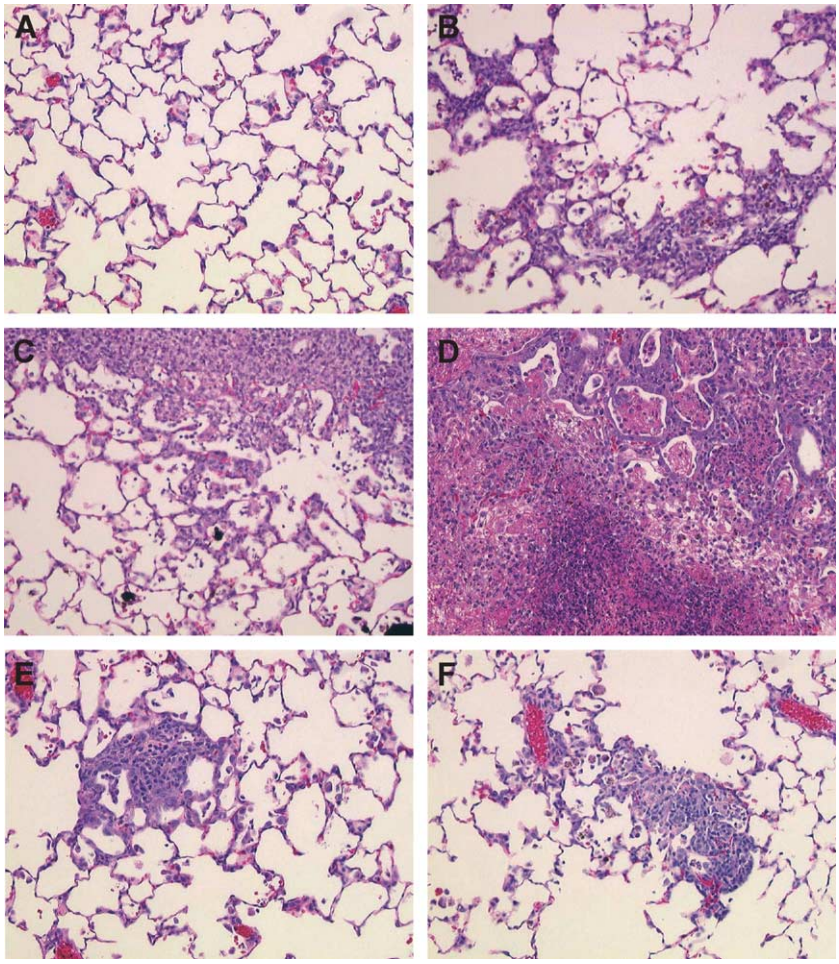

Fig. 3. Light micrographs of rat lungs stained with hematoxylin and eosin at day 8 after exposure to (A) saline + *L. monocytogenes*; (B) GMA-SS-Total + *L. monocytogenes*; (C) GMA-MS-Total + *L. monocytogenes*; (D) MMA-SS-Total + *L. monocytogenes*; (E) MMA-SS-Sol + *L. monocytogenes*; and (F) MMA-SS-Insol + *L. monocytogenes*. The welding samples (2 mg/rat) were intratracheally instilled 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Original magnification: 100×.

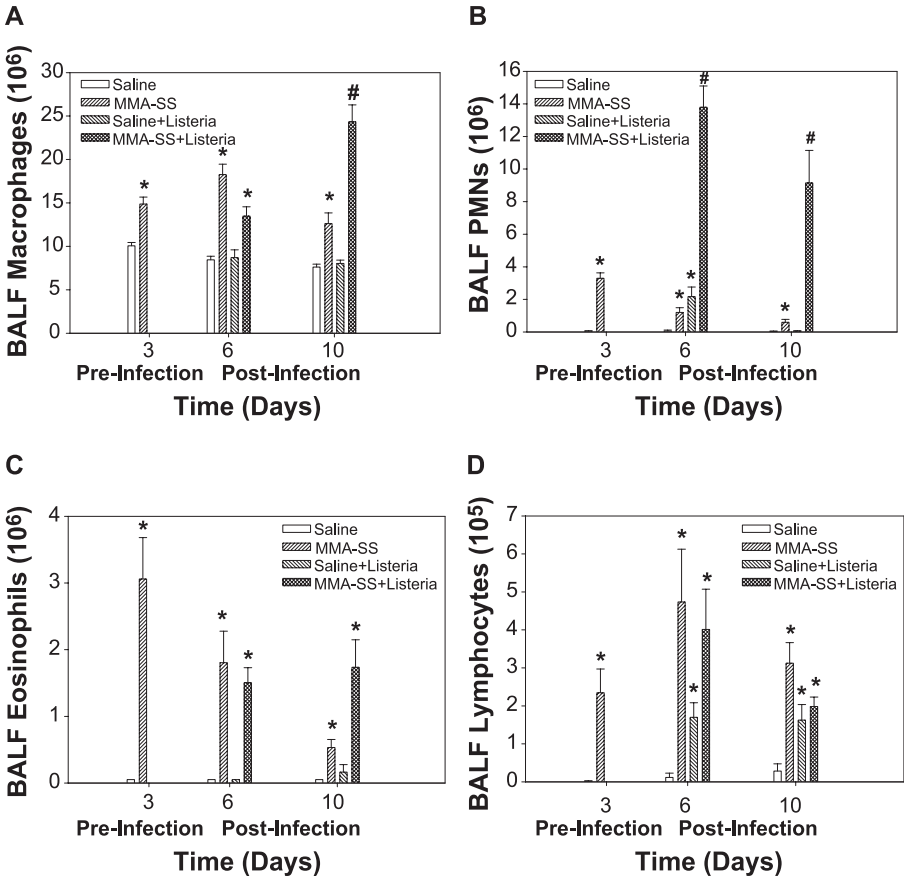

Fig. 4. (A) Macrophages, (B) PMNs; (C) eosinophils, and (D) lymphocytes recovered from the BALF. Rats were preexposed to saline or MMA-SS welding fume (2 mg/rat) by intratracheal instillation 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE (n = 8–12); *, significantly greater than saline control (noninfected) within a time point; #, significantly greater than all other groups within a time point; $P < 0.05$.

caused significant increases in LDH activity (Fig. 5A) and albumin (Fig. 5B) before and after pulmonary inoculation with *L. monocytogenes* compared to the noninfected saline control group.

Lung defense responses

To assess the response of reactive oxygen/nitrogen species to infection, chemiluminescence and NOx were measured in recovered lung phagocytes and acellular BALF, respectively. Before infection (day 3), MMA-SS pretreatment caused a significant increase in CL and BALF NOx (Figs. 6A,B). After infection, CL was significantly increased in both the saline and MMA-SS groups inoculated with *L. monocytogenes* at day 6, but only in the MMA-SS + *L. monocytogenes* group at day 10 (Fig. 6A). *L. monocytogenes* infection of saline-treated rats had no effect on BALF NOx levels at days 6 and 10, whereas MMA-SS pretreatment significantly increased NOx at each time point after infection (Fig. 6B).

Minimal areas of positive iNOS staining (red) were observed in the saline control group before infection (Fig. 7A). At day 6, an increase iNOS staining (which appeared to be associated with macrophages) was observed in the saline + *L. monocytogenes* group (Fig. 7B), whereas iNOS levels returned to noninfected control levels by day 8 (data not shown). Before and after infection at all time points, MMA-SS pretreatment caused a substantial increase in iNOS staining (Figs. 7C–F). iNOS staining for the MMA-SS groups was associated with localized areas of significant inflammatory cell accumulation.

A number of pulmonary cytokines important in inflammatory and immune responses were measured in the acellular BALF. Significant elevations in TNF-α and IL-6 were observed for the saline and MMA-SS groups inoculated with *L. monocytogenes* at day 6 compared with the noninfected control (Figs. 8A,B). TNF-α and IL-6 were significantly elevated for the MMA-SS + *L. monocytogenes* groups compared to all other groups at day 6 and days 6 and 10, respectively. Conversely, MMA-SS pretreatment caused significant reductions in IL-2 before and at day 6 infection compared with the noninfected control (Fig. 9A). A significant reduction in IL-10 was observed for the MMA-SS group before infection compared with the noninfected saline control (Fig. 9B). However, MMA-SS pretreatment

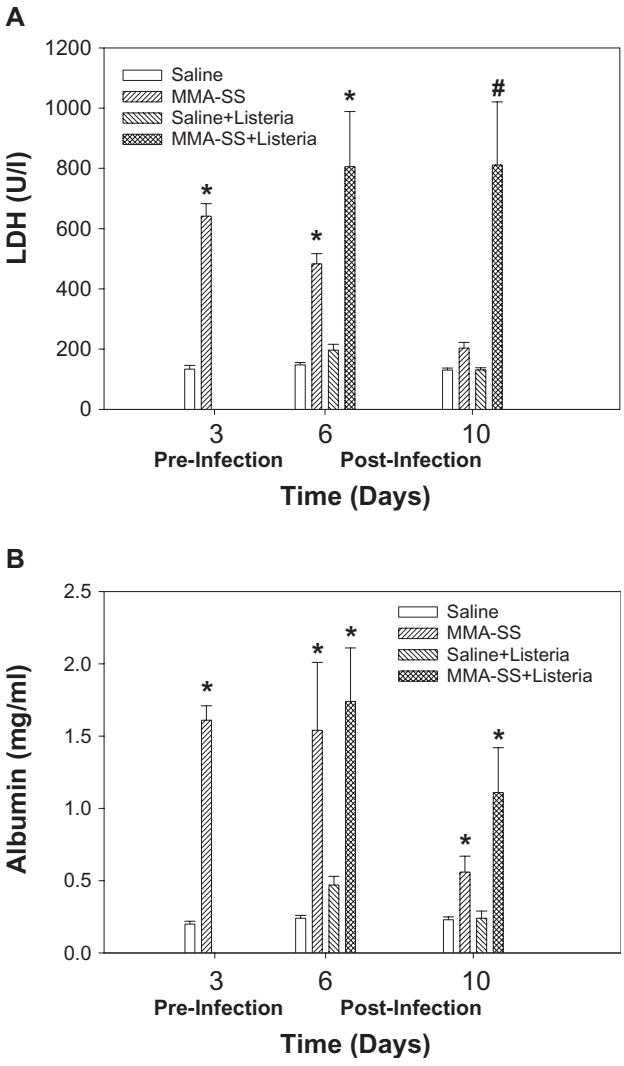

Fig. 5. (A) Lactate dehydrogenase (LDH); (B) albumin measured in the acellular BALF. Rats were preexposed to saline or MMA-SS welding fume (2 mg/rat) by intratracheal instillation 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE (n = 8–12); *, significantly greater than saline control (noninfected) within a time point; #, significantly greater than all other groups within a time point; $P < 0.05$.

caused a significant increase in IL-10 at day 6 after infection compared with the noninfected controls.

Discussion

It was the goal of this study to elucidate the potential mechanisms by which different welding fumes may increase the susceptibility to bacterial lung infection. Welding fumes with different elemental compositions and solubility properties were used to determine which chemical component may be responsible for possible alterations in lung defense responses. An animal bacterial infectivity model developed by our group (Antonini et al., 2000, 2001a, 2001b) and others (Cohen et al., 2001; Jakab, 1993; Van Loveren et al., 1988) was used. Rats were intratracheally instilled with different welding fumes before pulmonary inoculation with a gram-positive, intracellular, facultative bacterial pathogen, *L. monocytogenes*. Both innate (nonspecific) and adaptive (cell-mediated) lung immune responses are required to resolve infection with *L. monocytogenes* (Portnoy et al., 2002; Shen et al., 1998; Unanue, 1997).

Of the three welding fumes examined, only the MMA-SS sample had a significant effect on lung infectivity. Pretreatment with MMA-SS caused a significant delay in the pulmonary clearance of the bacteria and led to a measurable loss in animal body weight compared to the two insoluble welding fumes. In addition, the histopathological changes after infection were more extensive, significantly more pronounced, and persisted for a longer period of time in the

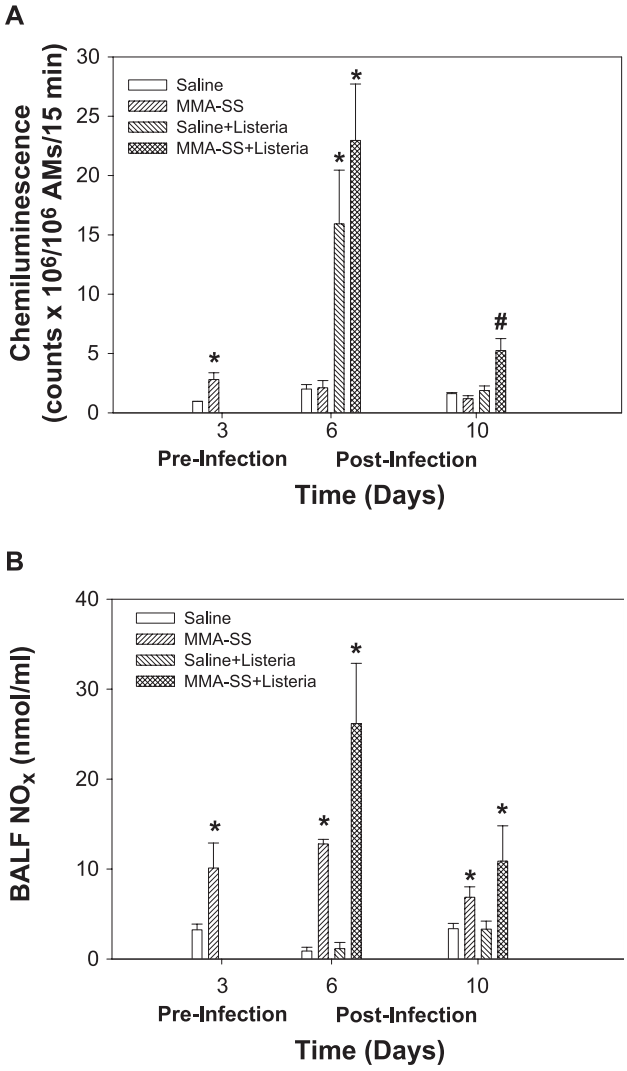

Fig. 6. (A) Macrophage chemiluminescence (CL); (B) BALF nitric oxide. Rats were preexposed to saline or MMA-SS welding fume (2 mg/rat) by intratracheal instillation 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE (n = 8–12); *, significantly greater than saline control (noninfected) within a time point; #, significantly greater than all other groups within a time point; $P < 0.05$.

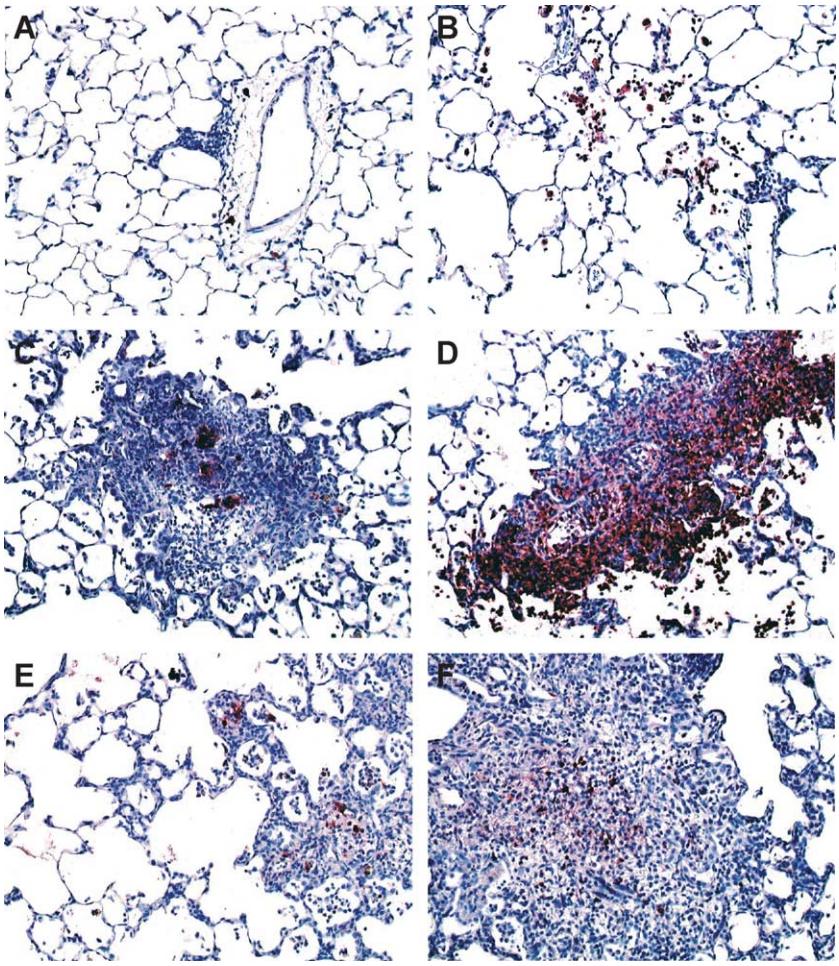

Fig. 7. Immunohistochemical localization of inducible nitric oxide synthase (iNOS) in rat lungs exposed to: (A) Saline (preinfection), 3 days, (B) saline + *Listeria*, 6 days; (C) MMA-SS (preinfection), 3 days; (D) MMA-SS + *Listeria*, 6 days; (E) MMASS + *Listeria*, 8 days; (F) MMA-SS + *Listeria*, 10 days. Red staining indicates positive labeling of iNOS.

MMA-SS group. Because of the differences in water solubility of the welding fumes, it was hypothesized that the soluble component of the MMA-SS fume (comprised of 87% Cr and 11% Mn) was responsible for the difference observed in the infectivity when comparing the different fumes.

Previously, our group and others have shown that residual oil fly ash (ROFA) slows the pulmonary clearance of bacteria and alters lung defenses (Antonini et al., 2002; Hatch et al., 1985; Pritchard et al., 1996). The immunosuppressive effect in the lung was observed to be exclusively dependent on the soluble metals associated with ROFA (Roberts et al., 2004). However, when the MMA-SS welding fume was separated into soluble and insoluble components, a different pattern in the infectivity response was observed compared to ROFA. Neither the soluble or insoluble fraction had a significant effect on the pulmonary clearance of the bacteria or caused a change in body weight compared with the saline control group. It appears that the effect of the MMA-SS sample on lung defense against infection is dependent on the combination of both the soluble (Cr, Mn) and insoluble (Fe, Mn, Ni, Cr) metals of the fume, thus disproving earlier our hypothesis. The question still remains as to which metal or metals are responsible for the effects observed. Ongoing studies are currently examining the role that individual or combinations of welding fume metals play on local lung immune responses.

Because the MMA-SS fume caused significant decrements in lung defense and increased the susceptibility to infection, several additional assays were performed using this fume in an attempt to determine the mechanisms involved. Pulmonary treatment with the MMA-SS fume increased lung injury as assessed by BALF analysis. LDH and albumin levels were significantly elevated in the MMA-SS fume group compared with the control group before and after infection.

In the assessment of the cellular response for the current study, it was observed that the number of macrophages, PMNs, eosinophils, and lymphocytes recovered from the lungs of the MMA-SS group were significantly increased before and after infection. Earlier studies have shown that treatment with the soluble fraction of the MMA-SS fume was responsible for the elevated eosinophil response,

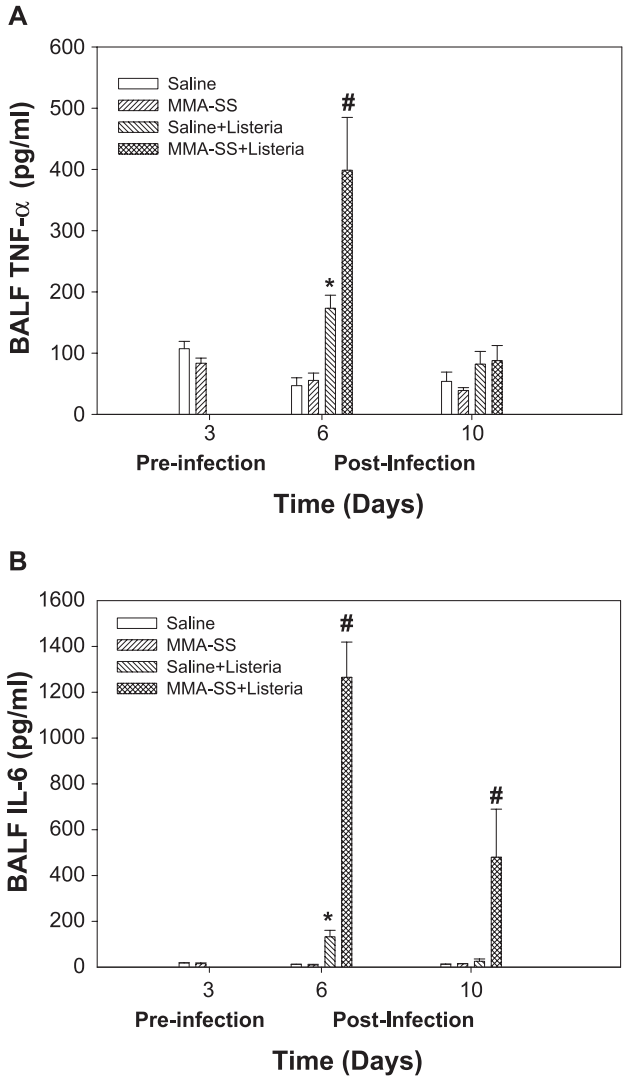

Fig. 8. (A) TNF-α and (B) IL-6 measured within the bronchoalveolar lavage fluid (BALF). Rats were preexposed to saline or MMA-SS welding fume (2 mg/rat) by intratracheal instillation 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE (n = 8–12); *, significantly greater than saline (noninfected) and MMA-SS (noninfected) groups within a time point; #, significantly greater than all other groups within a time point; $P < 0.05$.

whereas the insoluble component led to the recruitment of the PMNs (Taylor et al., 2003). This further supports the idea that both the soluble and insoluble components of the MMA-SS sample are required for lung defense responses observed in the total MMA-SS group. These observed elevations in lung cell influx may be indicative of altered immune and inflammatory responses after MMA-SS treatment. It would be expected that increases in the presence of these immune cells in the lungs would enhance the response to *L. monocytogenes* infection. However, this does not appear to be the case.

Previously, we have demonstrated that extensive lung injury may have a minimal effect on defense against infection. Despite the presence of lung fibrosis and massive inflammation caused by short-term treatment with high doses of crystalline silica, lung defense responses were more than sufficient to control the spread of *L. monocytogenes* infection (Antonini et al., 2000). It was observed that alterations in local immune responses caused by silica were most likely due to an up-regulation of phagocytic cell infiltration and function.

One mechanism by which lung phagocytes kill microbes and control the spread of infection is to produce biologically reactive oxygen (e.g., superoxide anion, hydroxyl radical, hydrogen peroxide) and nitrogen (e.g., NO) species. Both in vivo and in vitro studies have shown that oxygen/nitrogen intermediates are important in the killing of *L. monocytogenes* (Kradin et al., 1999; MacFarlane et al., 1998; Ogawa et al., 2001; Ohya et

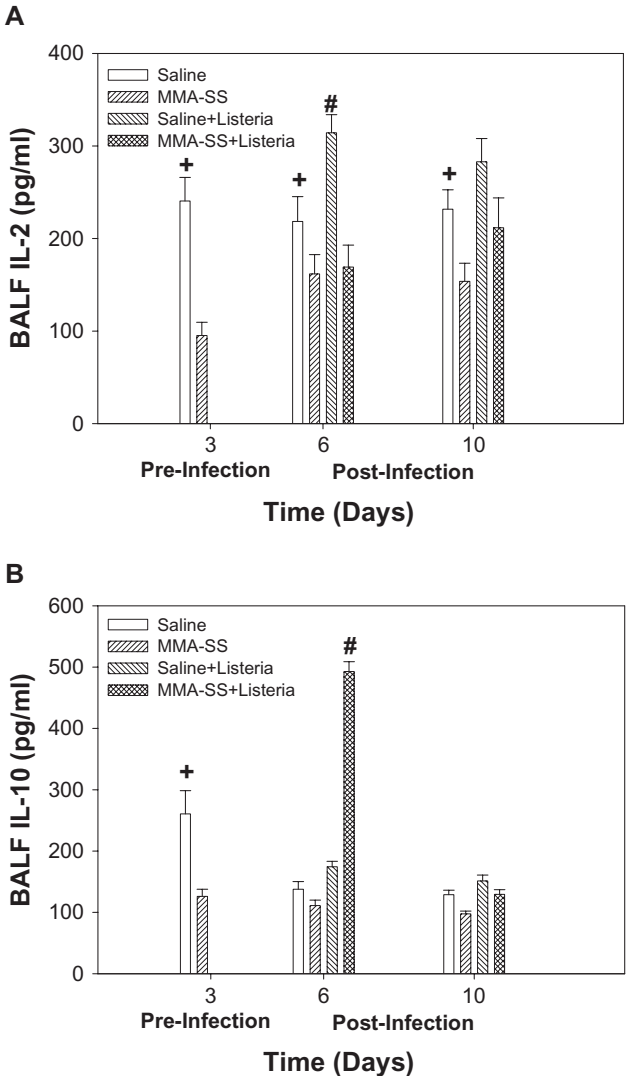

Fig. 9. (A) IL-2 and (B) IL-10 measured within the bronchoalveolar lavage fluid (BALF). Rats were preexposed to saline or MMA-SS welding fume (2 mg/rat) by intratracheal instillation 3 days before intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE (n = 8–12); #, significantly greater than all other groups within a time point; +, significantly greater than MMA-SS group (noninfected); $P < 0.05$.

al., 1998). NO can promote the cytotoxic activities of phagocytes and modulate cell-mediated immunity (Lyons, 1995; Nathan and Xie, 1994). NO has been observed to combine with superoxide anion to form the highly reactive substance, peroxynitrite (Pryor and Squadrito, 1995). Moreover, Hickman-Davis et al. (1999) have demonstrated that peroxynitrite generation is an important mechanism in the killing of bacterial pathogens.

In previous studies, it has been observed that pretreatment with diesel exhaust particles (Yang et al., 2001) and ozone (Van Loveren et al., 1988) slows the clearance of *L. monocytogenes* from the lungs. A suppression in both macrophage activation and the production of reactive oxidant intermediates appeared to be involved in the immunomodulatory effects associated with exposure to diesel exhaust (Yin et al., 2002) and ozone (Cohen et al., 2001). However, treatment with MMA-SS welding fume before infection activated lung phagocytes as measured by an increase in chemiluminescence and NO production. In addition, BALF NO levels were significantly elevated at all time points after infection. Thus, the observed increase in oxidant intermediate production caused by MMA-SS treatment likely plays only a minor role in the killing of the bacteria. Instead, the increase in oxygen/nitrogen intermediate generation may contribute to the elevations observed in lung injury caused by the MMA-SS fume. It is well established that excessive production of peroxynitrite can cause significant lung cell and tissue damage (Beckman and Koppenol, 1996; Pryor and Squadrito, 1995). Importantly, it was observed in the current study that staining for iNOS, the enzyme responsible for generating NO, was increased and associated with localized areas of inflammation and injury after treatment with MMA-SS.

To determine if MMA-SS treatment altered immune cell signaling, the production of several cytokines important in lung defense was measured. TNF-α and IL-6, two cytokines with prominent pro-inflammatory properties, were significantly elevated after infection in the MMA-SS group compared to the other groups. This result was not surprising because IL-6 is an important mediator of acute phase reactions and is involved in the development of oxidative stress and tissue damage (Borish et al., 1989; Taga and Kishimoto, 1997). Elevations in TNF-α and IL-6 may be the cause of the oxidative lung injury and inflammation that were seen in the MMA-SS group before and after pulmonary infection.

In addition, IL-10 was measured in the acellular BALF of the different treatment groups. IL-10 is an anti-inflammatory cytokine that inhibits production of other cytokines (e.g., IL-2 and interferon-γ) by both T cells and natural killer cells indirectly via inhibition of innate immune responses, such as macrophage function and activation (de Waal Malefyt et al., 1993; Moore et al., 2001; Taga et al., 1993). Before infection, MMA-SS treatment caused a significant suppression in IL-10 that was most likely due to an activation of lung phagocytes in response to the presence of the particles in the alveolar spaces. However, after infection, IL-10 levels were increased in the lungs of the welding fume group, indicating that *L. monocytogenes* inoculation after MMA-SS treatment may be indirectly affecting cell-mediated immune responses.

To assess whether adaptive, cell-mediated immune processes were altered, IL-2 was measured in the BALF of the MMA-SS and saline control groups before and after infection. IL-2 is a lymphokine secreted by CD4+ T helper cells and is involved in T cell growth and differentiation (Mosmann, 1992). In addition, IL-2 is a driving force in the differentiation of CD4+ T cells toward the TH1 rather than a TH2 cell subset that is associated with cell-mediated immune responses as opposed to humoral responses (Mosmann and Coffman, 1989). MMA-SS treatment before and after infection with *L. monocytogenes* led to a significant reduction in lung IL-2 levels, which is likely indicative of either a suppression in the T cell response or an improper T cell response to the bacterial challenge.

In summary, the effect of three chemically different welding fumes on lung defense against infection was examined. Treatment with MMA-SS, a highly water soluble welding fume, altered lung defenses, whereas no effect was observed after pulmonary exposure to the other two more insoluble welding fumes. The MMA-SS fume is a complex mixture of both soluble and insoluble metals. The significant slowing of the pulmonary clearance of the bacterial pathogen and the associated elevation in lung morbidity caused by MMA-SS treatment were dependent on both the soluble and insoluble metals. The observed MMA-SS-induced lung damage was likely the result of an excessive influx of inflammatory cells into the lungs and an overproduction of reactive oxygen/nitrogen intermediates. Alterations in defense against infection could be attributed to the effect of MMA-SS exposure on immune cell signaling in lungs. Significant changes were observed in the lung levels of cytokines important in both innate and adaptive immune responses before and after infection. Additional studies are ongoing in the laboratory, which are assessing the role of other cytokines, such as IL 12, IL 4, and interferon-γ, on the effect of welding fume exposure on lung defense responses to infection. Also, studies are needed to determine which metal or combination of metals may be responsible for the decrements in lung defense caused by welding fume exposure.

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