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RESEARCH ARTICLE

Systemic immune cell response in rats after pulmonary exposure to manganese-containing particles collected from welding aerosols

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Abstract
Welding fume inhalation affects the immune system of exposed workers. Manganese (Mn) in welding fume may induce immunosuppressive effects. The goal was to determine if Mn in welding fume alters immunity by reducing the number of circulating total leukocytes and specific leukocyte sub-populations. Sprague-Dawley rats were treated by intratracheal instillation (ITI) with either a single dose (2.00 mg/rat) or repeated doses (0.125 or 2.00 mg/rat for 7 weeks) with welding fumes that contained different levels of Mn. Additional rats were treated by ITI once a week for 7 weeks with the two doses of manganese chloride ($MnCl_2$). Bronchoalveolar lavage was performed to assess lung inflammation. Also, whole blood was recovered, and the number of circulating total leukocytes, as well as specific lymphocyte subsets, was determined by flow cytometry. The welding fume highest in Mn content significantly increased lung inflammation, injury, and production of inflammatory cytokines and chemokines compared to all other treatment groups. In addition, the same group expressed significant decreases in the number of circulating $CD4^+$ and $CD8^+$ T-lymphocytes after a single exposure, and significant reductions in the number of circulating total lymphocytes, primarily $CD4^+$ and $CD8^+$ T-lymphocytes, after repeated exposures (compared to control values). Repeated $MnCl_2$ exposure led to a trend of a reduction (but not statistically significant) in circulating total lymphocytes, attributable to the changes in the $CD4^+$ T-lymphocyte population levels. The welding fume with the lower concentration of Mn had no significant effect on the numbers of blood lymphocytes and lymphocyte subsets compared to control values. Evidence from this study indicates that pulmonary exposure to certain welding fumes cause decrements in systemic immune cell populations, specifically circulating T-lymphocytes, and these alterations in immune cell number are not dependent exclusively on Mn, but likely a combination of other metals present in welding fume.

Keywords: Welding fume, immunotoxicity, manganese, blood analysis, particulate matter

Introduction

Electric arc welding is a common industrial process used to join metals. Arc welding processes generate complex aerosols that are composed of potentially hazardous metals, such as iron (Fe), manganese (Mn), and chromium (Cr). Greater than 400,000 workers are classified as full-time welders in the US (Bureau of Labor Statistics, 2007), and several million more workers are regularly exposed to welding aerosols worldwide. The health effects of welders are oftentimes difficult to determine because welders are a heterogeneous workforce employed in a variety of workplace settings that may include confined, poorly ventilated spaces, or well-ventilated indoor and open-air sites. In addition, their exposure may vary due to the vast selection of different materials/processes commonly used in welding. Most long-time welders have experienced some type of pulmonary disorder during their career (as reviewed by Sferlazza and Beckett, 1991; Antonini, 2003). Respiratory effects have included bronchitis, metal fume fever, reversible lung function decrements, and a possible increase in the incidence of lung cancer. For decades, an area of concern within the occupational health community has been the observation that welding fume inhalation may cause decrements in immune function (as reviewed by Zeidler-Erdely et al., submitted). Worker studies assessing the adverse effects

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of welding fume exposure on immune function are limited.

Limited information is available about the systemic changes in leukocyte number after welding fume exposure. Kim et al. (2005) observed a significant increase in peripheral leukocyte and neutrophil numbers in non-smoking welders immediately following an acute exposure. The oxidative stress ratio of peripheral blood monocytes (du Plessis et al., 2010) and biomarkers of systemic inflammation (Nuernberg et al., 2008) have been shown to be significantly elevated in workers exposed to welding fume compared to non-exposed controls. Hanovcova et al. (1998) indicated that exposure to stainless steel welding fume increased the number of peripheral lymphocyte and lymphocyte subsets. However, Tuschl et al. (1997) observed no change in circulating lymphocyte numbers in welders, but did report a significant decrement in the cytotoxic activity of natural killer (NK) cells.

Animal studies provide additional evidence of systemic immune responses. In a study by Rim et al. (2004), gene expression profiling of peripheral blood mononuclear cells collected from rats after 30 days of inhalation to a relatively high concentration of stainless steel welding fume indicated that ~3-times more genes, many of which are associated with immune function and response, were down-regulated as opposed to being increased. Moreover, microarray technology and subsequent pathway analysis indicated a strong immunologic transcriptional response to welding fume in a study comparing lung tumor susceptible and resistant mouse strains (Zeidler-Erdely et al., 2010). Another study specifically evaluated the systemic humoral immune response after pulmonary exposure to a highly water-soluble stainless steel welding fume in rats (Anderson et al., 2007). *In vitro* exposure of splenocytes to stainless steel fume reduced IgM activity in response to sheep red blood cells (SRBC). In addition, splenocytes harvested after an *in vivo* exposure to 20 mg/kg of the stainless steel fume showed a significant decrease in the IgM response to SRBC.

Welding fume is a complex mixture of different metals, many of which may alter the host's immune system after exposure. Nakata et al. (2006) and Yuan et al. (2006) hypothesized that Mn in welding fume may induce systemic immunosuppressive effects in exposed workers. Smialowicz et al. (1988) indicated that manganese chloride ($MnCl_2$) significantly enhanced systemic natural killer NK cell activity in young mice that was mediated by production of interferon (IFN)-α and -β. Mn is an essential element in most welding electrodes. Mn increases hardness and strength, prevents steel from cracking during manufacture, and improves metallurgical properties (Harris, 2002). Nakata et al. (2006) showed that welders who had a high concentration of Mn in their blood displayed significant reductions in total lymphocytes, B-lymphocytes, total T-lymphocytes, and CD8^{+} T-lymphocytes compared to levels in workers

with low blood Mn levels. In contrast, Yuan et al. (2006) observed no differences in number of circulating total lymphocytes and lymphocyte subsets in welders who had elevated blood Mn levels compared to unexposed controls. It was the goal of the current study to develop an animal model to determine if pulmonary exposure to Mn in welding fume alters systemic immunity by causing changes in the number of circulating total lymphocytes and specific lymphocyte sub-populations.

Materials and methods

Experimental design

Male Sprague-Dawley rats were exposed (by intratracheal instillation [ITI]) to multiple doses of welding fumes that contained different levels of Mn. Short- and long-term dosing regimens included an acute, single ITI, or a repeated ITI regimen that lasted 7 weeks (once a week × 7 weeks). Additional sets of rats were treated by ITI once a week for 7 weeks with pure $MnCl_2$. Whole blood was recovered from all treated animals, and the number of circulating total leukocytes, monocytes, polymorphonuclear leukocytes (PMN), and lymphocytes, as well as specific lymphocyte subsets, was determined by flow cytometry. Bronchoalveolar lavage (BAL) was performed only in the repeated dosing regimen study to assess onset/persistence of any lung injury and inflammation.

Welding fume characterization

Bulk samples of different welding fumes were collected by Lincoln Electric Co. (Cleveland, OH). The fumes were generated in a cubical open front fume chamber (volume = 1 m³) by a skilled welder using a manual or semi-automatic technique appropriate to the electrode and collected on 0.2 μm Nuclepore filters (Nuclepore Co., Pleasanton, CA). The fume samples were generated using two different processes: (1) gas metal arc welding using a mild steel E70S-3 electrode (GMA-MS; L-50 carbon steel electrode, Lincoln Electric Co., Cleveland, OH), and (2) shielded manual metal arc welding using a flux-covered stainless steel hard-surfacing electrode (MMA-HS; Wearshield 15CrMn, Lincoln Electric Co., Cleveland, OH). Particle sizes of bulk samples of the two collected fumes were determined using scanning electron microscopy and found to be in the respirable size range with count mean diameters of <1.0 μm.

A portion of the collected fume samples was digested, and the metals were analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) by the Division of Applied Research and Technology (DART, Cincinnati, OH), according to NIOSH method 7300 modified for microwave digestion (National Institute for Occupational Safety and Health, 1994). The metals that were measured included Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Sr, Te, Ti, Tl, V, Zn, and Zr. Metal content of blank filters were analyzed for control purposes.

Animals

Male Sprague-Dawley [Hla:(SD) CVF] rats from Hilltop Lab Animals (Scottsdale, PA), weighing 250–300 g and free of viral pathogens, parasites, mycoplasma, *Helicobacter*, and CAR *Bacillus*, were used for all exposures. The rats were acclimated for at least 6 days after arrival, and were housed in ventilated polycarbonate cages on Alpha-Dri cellulose chips and hardwood Beta-chips as bedding. All rats were provided HEPA-filtered air, and had *ad libitum* access to irradiated Harlan Teklad 2918 diet (Frederick, MD) and tap water. The animal facilities were specific pathogen-free, environmentally controlled (20–25°C, 30–40% relative humidity, and 12-h light/dark cycles), and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal procedures used during the study were reviewed and approved by the National Institute for Occupational Safety and Health (NIOSH) Animal Care and Use Committee.

Welding fume treatment

Two dosing regimens were used for the study: (1) single treatment—one intratracheal instillation (ITI) of 2.0 mg/rat of GMA-MS or MMA-HS welding fume; (2) repeated treatment—one ITI a week for 7 weeks, with 0.125 or 2.00 mg/rat of GMA-MS or MMA-HS welding fume. Additional sets of rats were treated by ITI once a week for 7 weeks with 0.125 or 2.00 mg/rat of manganese (II) chloride tetrahydrate (MnCl$_2$; Sigma-Aldrich Chemical Co., St. Louis, MO). For the ITI procedure, rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% [w/v] solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN) and treated by ITI with the welding fume sample suspended in 300 μl of sterile phosphate-buffered saline (PBS, pH 7.4). Vehicle control animals were treated by ITI with 300 μl of sterile PBS.

Whole blood and bronchoalveolar lavage collection

At 1 day after the final ITI for each dosing regimen, animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (>100 mg/kg body weight [BW]; Sleepaway. Ft. Dodge Animal Health, Wyeth, Madison, NJ). Whole blood was collected via the inferior vena cava and each rat was exsanguinated (by severing abdominal aorta). BAL was performed on the animals used in the repeated dosing regimen at 1 day after the last of the seven weekly ITI treatments to assess lung injury and inflammation.

The lungs were first lavaged with a 1 ml/100 g BW aliquot of calcium-, magnesium-free PBS (pH 7.4). The first fraction of recovered bronchoalveolar lavage fluid (BALF) was centrifuged (500 ×g, 10 min), and the resultant cell-free supernatant analyzed for various biochemical parameters. The lungs were further lavaged with 6 ml aliquots of PBS until 30 ml were collected. These samples also were centrifuged (10 min, 500 ×g), and the cell-free BALF discarded. The cell pellets from all washes for each rat were combined, washed, and re-suspended in 1 ml of PBS buffer and evaluated as described below.

Whole blood cell differentiation and quantification

Whole blood cells were differentiated and quantified as previously described (Erdely et al., 2011). Briefly, 100 μl of the blood cell suspension was added to a flow tube with 100 μl of 300 μg/ml mouse IgG (Sigma) in FACS buffer. After 10 min incubation, 50 μl of pre-mixed antibodies in FACS buffer were added to the tube and stained for 30 min at room temperature on a shaker. The antibody mixture contained 5 μg/ml of the following antibodies: CD45R-FITC (clone HIS24), CD4-FITC (clone OX35), CD8-PerCP (clone OX-8), and CD3-APC (clone 1F4). All antibodies were purchased from PharMingen (Becton Dickinson, San Diego, CA). Red blood cells were then lysed with 100 μl Caltag Cal-lyse lysing solution (GAS-010, Invitrogen, Carlsbad, CA) for 10 min in the dark. Caltag counting beads (PCB-100, Invitrogen) were added for cell enumeration prior to analysis using a FACSCalibur flow cytometer (Becton Dickinson Biosystems, San Jose, CA). Samples were acquired through a live gate without compensation.

After collecting 3500 counting beads, the data of all cells were exported to the analysis software FlowJo (Treestar, Costa Mesa, CA). Leukocytes were separated by side scattering (SSC) and forward scattering (FSC) into three gates: lymphocytes, monocytes, and eosinophils + neutrophils. The lymphocytes were further classified by FSC/SSC and specific surface markers. CD4 cells were identified by expression of CD3$^+$ and CD4$^+$; CD8 cells were identified as double positive in CD3$^+$ and CD8$^+$ channels. B-Lymphocytes were distinguished from T-lymphocytes by CD45R expression in the lymphocyte gate. Natural killer (NK) cells were identified by expression of NKR-PIA, but not CD3, marker. NK-T cells were identified as double positives in CD3 vs NKR-PIA plots. The NK antibody staining was needed for the calculation of the total CD8$^+$ population.

Lung injury and inflammation
Lung cell differentiation

Total cell numbers recovered by BAL were determined using Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cells were prepared on cytospin slides (5 × 10^4 cells) using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, UK). Cells (200/rat) were identified after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA) as alveolar macrophages, polymorphonuclear leukocytes (PMN), and lymphocytes.

Biochemical parameters of lung injury

Using the acellular first fraction of BALF, albumin content (index to quantify increased permeability of bronchoalveolar-capillary barrier) and lactate dehydrogenase (LDH) activity (indicator of general cytotoxicity) were measured. Albumin content was determined

colorimetrically at 628 nm (based on albumin binding to bromcresol green) using an albumin BCG diagnostic kit (Sigma). LDH activity was determined by measuring oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurements were performed with a COBAS MIRA auto-analyzer (Roche Diagnostic Systems, Montclair, NJ).

BALF cytokine and chemokine determination

Levels of cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, -2, -6, and -10, and the chemokines monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) were assayed in the first fraction of BALF. The selection of the cytokines and/or chemokines was based on their role in lung inflammatory and immune responses after particulate exposure. Cyto-/chemokine protein concentrations were determined using enzyme-linked immunoabsorbent assay (ELISA) kits (Biosource International, Inc., Camarillo, CA). Results of the colorimetric assay were obtained with a Spectramax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Devices Corp., Sunnyvale, CA).

Serum and BALF antibody quantification

The concentrations of rat immunoglobulins (IgA, IgG, and IgM) and complement factor 3 (C3) were determined in the serum and/or first fraction BALF by ELISA (GenWay Biotech, Inc., San Diego, CA) according to manufacturer's instructions. The Spectramax 250 plate reader was used to measure absorbance values for each immunoasssay.

Statistical analysis

All data are presented as mean [± SE]. Analyses were performed using JMP® 9 Statistical Discovery Software (SAS, Cary, NC). Treatments were compared by a one-way Analysis of Variance (ANOVA) followed by a *post-hoc* Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Fume characterization

Metal composition of the two welding fume samples (Table 1) was determined in a previous study (Antonini et al., 2010). Briefly, the GMA-MS fume was relatively insoluble in water and composed of Fe and Mn. The MMA-HS fume was significantly more water-soluble, and composed of predominately Mn, Fe, and Cr and alkali metals, potassium (K), and sodium (Na). The percentage of total Mn (50.90%) was more than double in the MMA-HS fume compared to the Mn (21.70%) in GMA-MS.

Systemic immune cell response; single welding fume treatment

Total leukocytes, monocytes, PMN, and lymphocytes, as well as lymphocyte subsets were determined in

Table 1. Metal composition and solubility properties of welding fume samples.

Fume samples	Weight % total metals[a]	Soluble/insoluble ratio[b]
GMA-MS	Fe 72.2%	0.0139
	Mn 21.7%	
MMA-HS	Fe 19.3%	0.2182
	Mn 50.9%	
	Cr 8.46%	
	K 12.1%	
	Na 6.73%	
	Ni 0.09%	

Note: Metal composition was determined in particles suspended in saline by ICP-AES.
[a] Relative to all metals analyzed.
[b] Soluble metal composition was determined in filtered soluble fraction (supernatant) of particle suspension after overnight incubation at 37°C and centrifugation (Antonini et al., 2010).

whole blood collected 1 day after a single 2.00 mg dose of the two different welding fumes (Table 2). A single intratracheal treatment with the two fumes had no significant effect on the number of total circulating leukocytes, monocytes, and lymphocytes. Blood PMN levels were significantly elevated 1 day after a single treatment with both the GMA-MS and MMA-HS welding fumes, compared to control values. Among the lymphocyte subsets, CD4+ and CD8+ T-lymphocyte levels were significantly reduced below control values 1 day after a single treatment with the MMA-HS fume, but not the GMA-MS welding fume. B-Lymphocyte numbers in the blood were unchanged after a single treatment with either of the two fumes.

Local lung response; repeated welding fume treatment

Multiple parameters of lung injury and inflammation were measured in the recovered BALF 1 day after 7 weeks of treatment with two doses of the welding fumes and $MnCl_2$ groups (Table 3). In terms of lung injury, repeated lung treatment with each welding fume and the $MnCl_2$ groups at the two doses significantly increased both LDH and albumin, compared to control host values. Treatment with the 2.00 mg dose of the MMA-HS and $MnCl_2$ groups caused significant increases in both LDH and albumin compared to values seen in the other welding fume groups.

In the assessment of lung inflammation, repeated treatment with all of the welding fume and $MnCl_2$ groups caused significant elevations in the number of PMN and lymphocytes recovered from the lungs by BAL. Both BALF PMN and lymphocyte numbers were significantly increased after repeated treatment with 2.00 mg of the MMA-HS and $MnCl_2$ groups compared to values seen in the other welding fume groups (Table 3). Both $MnCl_2$ groups and only the 2.00 mg dose for the two fume groups caused a significant increase (compared to control) in BALF alveolar macrophage number and MIP-2 levels. BALF MIP-2, MCP-1, TNFα, and IL-6 levels were

Table 2. Immune cell differential of whole blood after a single welding fume treatment.

Whole blood leukocytes (10^6/ml)	Saline control	GMA-MS (2.0 mg)	MMA-HS (2.0 mg)
Total Leukocytes	8.37 ± 0.68	8.69 ± 1.10	7.50 ± 0.69
Monocytes	0.36 ± 0.05	0.34 ± 0.04	0.35 ± 0.03
PMN	0.91 ± 0.09	2.01 ± 0.29*	1.47 ± 0.15*
Total Lymphocytes	7.10 ± 0.54	6.33 ± 0.80	5.68 ± 0.58
B-Lymphocytes	2.60 ± 0.24	2.44 ± 0.44	2.55 ± 0.33
T-Lymphocytes	4.30 ± 0.32	3.68 ± 0.34	2.92 ± 0.38*
CD4^{+} Lymphocytes	2.98 ± 0.21	2.61 ± 0.24	2.08 ± 0.26*
CD8^{+} Lymphocytes	1.41 ± 0.12	1.15 ± 0.11	0.91 ± 0.13*

Note: Immune cell differential in whole blood at 1 day after a single intratracheal instillation of 2.0 mg gas metal arc-mild steel (GMA-MS) or manual metal arc-hard-surfacing (MMA-HS) welding fume. Control animals were treated with sterile saline. PMN = polymorphonuclear leukocytes. Values are means ± SE ($n = 6$).
* Value significantly different from saline control group for particular cell type ($p < 0.05$).

Table 3. Lung injury and inflammation in BALF after repeated welding fume treatments.

Lung parameters	Saline control	GMA-MS (0.125 mg)	GMA-MS (2.0 mg)	MMA-HS (0.125 mg)	MMA-HS (2.0 mg)	MnCl$_2$ (0.125 mg)	MnCl$_2$ (2.0 mg)
Lung injury							
LDH (U/L)	43 ± 6	89 ± 8[a]	163 ± 13[a]	106 ± 11[a]	807 ± 99[a,b]	127 ± 13[a]	452 ± 82[a,c]
Albumin (mg/ml)	0.10 ± 0.01	0.16 ± 0.01[a]	0.25 ± 0.02[a]	0.24 ± 0.02[a]	0.85 ± 0.10[a,b]	0.24 ± 0.02[a]	1.14 ± 0.25[a,b]
Cellular (10^6)							
AM	7.34 ± 0.51	8.48 ± 0.26	17.40 ± 1.40[a]	9.23 ± 0.67	21.60 ± 4.20[a]	11.06 ± 1.04[a]	13.02 ± 0.92[a]
PMN	0.04 ± 0.02	0.37 ± 0.03[a]	3.78 ± 0.72[a]	0.88 ± 0.09[a]	41.40 ± 5.61[a,b]	5.79 ± 1.44[a]	13.98 ± 3.0[a,c]
Lymph	0.02 ± 0.01	0.12 ± 0.01[a]	0.94 ± 0.40[a]	0.26 ± 0.02[a]	3.28 ± 0.27[a,b]	0.39 ± 0.05[a]	0.73 ± 0.32[a]
Chemokines (pg/ml)							
MCP-1	37 ± 19	36 ± 13	51 ± 8	16 ± 7	751 ± 116[a,b]	46 ± 7	567 ± 189[a,b]
MIP-2	460 ± 39	431 ± 23	604 ± 14[a]	553 ± 24	1035 ± 35[a,b]	666 ± 69[a]	1047 ± 32[a,b]
TNFα	21 ± 4	23 ± 3	18 ± 1	19 ± 3	48 ± 2[a,b]	32 ± 3	61 ± 14[a,b]
IL-6	267 ± 71	171 ± 12	127 ± 30	124 ± 18	639 ± 102[a,b]	301 ± 79	1011 ± 239[a,b]
Antibody							
IgG (pg/ml)	880 ± 102	1070 ± 195	1908 ± 429	3004 ± 790[a]	9663 ± 6307[a]	3908 ± 1108[a]	4078 ± 1979[a]

Note: Biochemical and cellular parameters measured in bronchoalveolar lavage fluid (BALF) at 1 day after repeated intratracheal instillations of 0.125 or 2.0 mg of gas metal arc-mild steel (GMA-MS) and manual metal arc-hard-surfacing (MMA-HS) welding fume or MnCl$_2$ once a week for 7 weeks. Control animals were treated with sterile saline.
LDH, lactate dehydrogenase; AM, alveolar macrophages; PMN, polymorphonuclear leukocytes; Lymph, lymphocytes. Values are means ± SE ($n = 5$–6).
[a] Value significantly different from saline control when comparing groups within a parameter; [b] value significantly greater than that noted in all other groups for the given parameter; [c] value significantly greater than that noted in all other groups except the MMA-HS 2.0 mg group for the given parameter ($p < 0.05$).

significantly elevated in the 2.00 mg MMA-HS and MnCl$_2$ groups compared to values noted with rats in all the other groups. Treatment with the welding fumes and MnCl$_2$ had no significant effect on BALF IL-1β, IL-2, or IL-10 levels. Both doses of the MMA-HS and MnCl$_2$ groups caused a significant increase in BALF IgG levels after repeated treatment compared to control.

Systemic immune cell response; repeated welding fume and MnCl$_2$ treatment

Total leukocytes, monocytes, PMN, and lymphocytes were determined in whole blood collected 1 day after 7 weeks of treatment with two doses of the different welding fumes and MnCl$_2$ groups (Figure 1). Repeated welding fume treatment had no statistically significant effect on the number of circulating total leukocytes and monocytes (Figures 1A and 1B). Both doses of MMA-HS and the 2.00 mg dose of MnCl$_2$ resulted in significantly increased circulating PMN compared to saline control (Figure 1C), indicating the capacity of these groups to induce systemic inflammation. Intratracheal treatment for 7 weeks with 2.00 mg of the MMA-HS fume caused a significant decrease in circulating total lymphocytes compared to saline control values (Figure 1D). No effect on total lymphocyte number was found in the other fume or MnCl$_2$ groups (compared to control).

Subsets of lymphocytes were also measured in whole blood after repeated treatment with the two different welding fumes and MnCl$_2$ groups (Figure 2). The number of circulating B-lymphocytes was unaffected by welding fume and MnCl$_2$ treatment (Figure 2A). T-Lymphocyte, CD4^{+}, and CD8^{+} cell levels were significantly decreased after repeated treatment with 2.00 mg of MMA-HS fume as compared to values noted in the saline control hosts (Figures 2B–2D). None of the other welding fume or MnCl$_2$ groups demonstrated a significant effect from
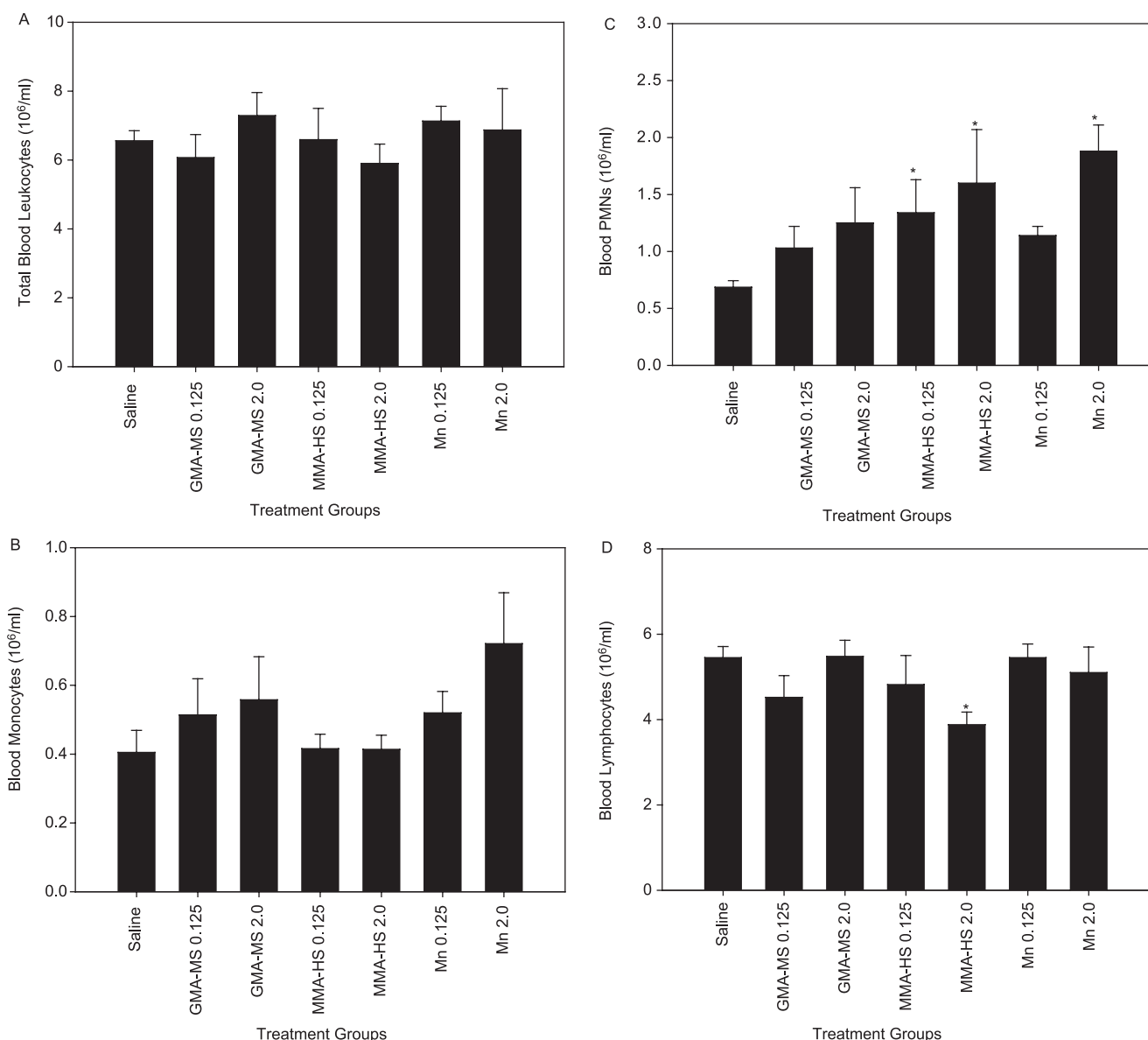
Figure 1. Systemic response; repeated treatment. Total (a) leukocytes, (b) monocytes, (c) PMN, and (d) lymphocytes in whole blood at 1 day after repeated intratracheal instillations of 0.125 or 2.0 mg of gas metal arc–mild steel (GMA-MS) and manual metal arc–hard-surfacing (MMA-HS) welding fume or $MnCl_2$ once a week for 7 weeks. Control animals were treated with sterile saline. Values are means ± SE (n = 5–6). * Value significantly different from saline control group for a particular cell type ($p < 0.05$).

treatments on T lymphocytes, CD4+, and CD8+ cell levels relative to those associated with the saline controls.

Different immunoglobulins were measured in the serum after repeated welding fume treatment. None of the welding fume treatments had a statistically significant effect on serum concentrations of IgG, IgA, IgM, or C3 compared to control (data not shown).

Discussion

In agreement with several worker studies, systemic PMN numbers were increased in most cases after welding fume treatment in the current animal study. In terms of circulating lymphocyte number, the high Mn, MMA-HS fume had the greatest effect on the systemic immune

T-lymphocyte differential, whereas neither dose of the low Mn, GMA-MS fume caused a significant change in the number of circulating total T-lymphocytes or T-lymphocyte sub-populations. Significant reductions in circulating total T-lymphocytes and lymphocyte subsets were observed after treatment with 2.00 mg of MMA-HS fume. The response was similar, regardless of whether the animals were treated with a single or multiple treatments with 2.00 mg of MMA-HS fume, indicating that the effect was acute and persisted over time. This reduction in the number of circulating lymphocytes may be attributed partly to increased translocation of the cells to the alveolar space (Table 3) or lung-associated lymph nodes. Previously, Anderson et al. (2007) observed a significant increase in the total number of both B- and T-lymphocytes
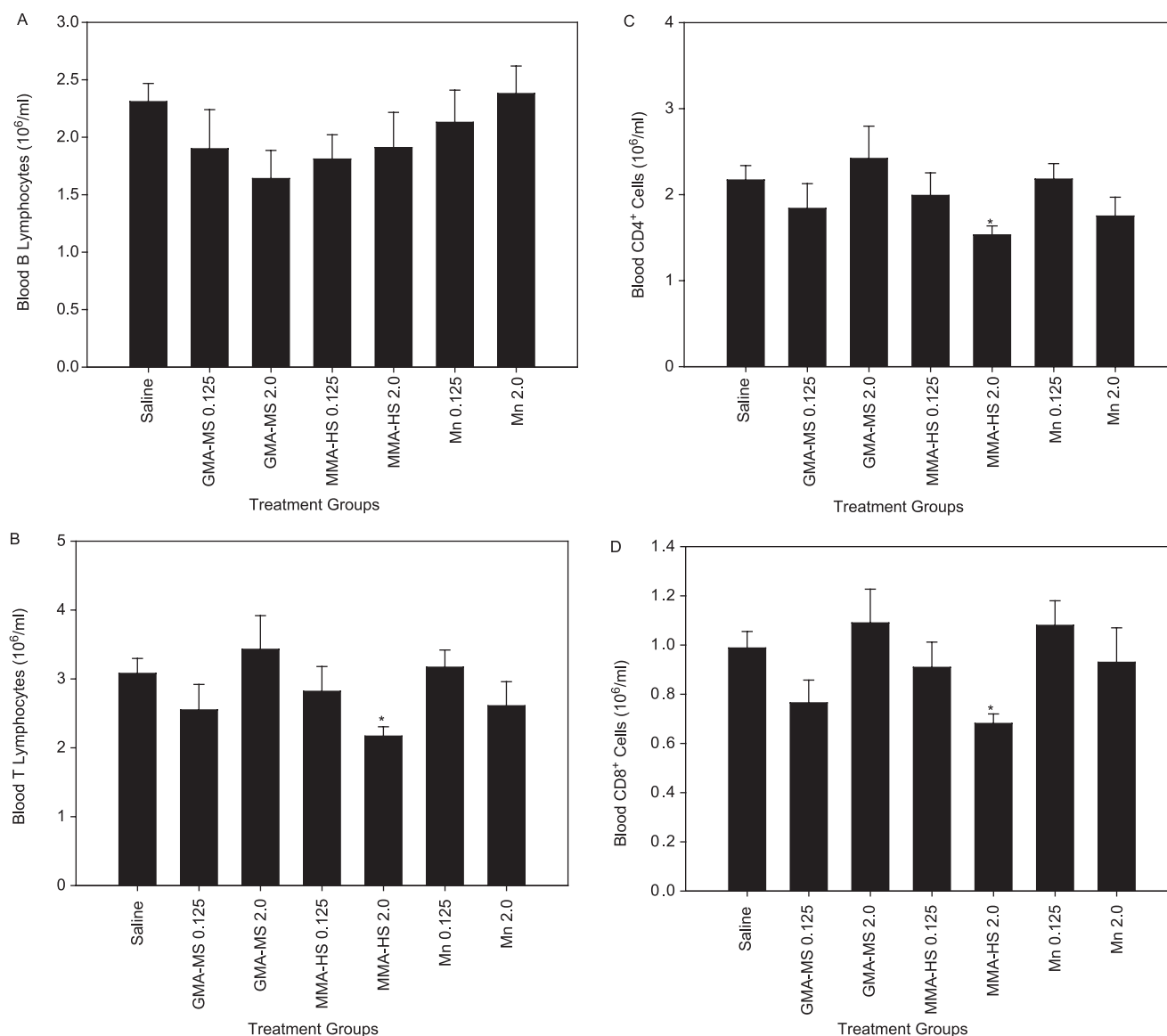
Figure 2. Systemic response; repeated treatment. (a) B-lymphocytes, (b) T-lymphocytes, (c) CD4+ lymphocytes, and (d) CD8+ lymphocytes in whole blood at 1 day after repeated intratracheal instillations of 0.125 or 2.0 mg of gas metal arc-mild steel (GMA-MS) and manual metal arc-hard-surfacing (MMA-HS) welding fume or MnCl2 once a week for 7 weeks. Control animals were treated with sterile saline. Values are means ± SE (n = 5–6). * Value significantly different from saline control group for a particular cell type ($p < 0.05$).

in the lung-associated lymph nodes after lung treatment with welding fume. Interestingly, no changes in the number of whole blood B-lymphocytes were observed after treatment with the MMA-HS fume.

The effect on blood T-lymphocytes observed in the current study was mostly supported by Nakata et al. (2006), who showed that the number of total lymphocytes, total T-lymphocytes, and CD8+ T-lymphocytes were significantly lower in welders that had higher blood Mn concentrations compared to workers with lower blood Mn levels. In contrast to the Nakata et al. (2006) study, Yuan et al. (2006) observed no significant difference in the number of peripheral T-lymphocytes and T-lymphocyte subsets when comparing Mn-exposed welders with non-exposed controls. Even though mean blood Mn levels for the exposed welder group in both studies were comparable,

i.e., 1.7 mg/dl (Nakata et al., 2006) vs 4.8 mg/dl (Yuan et al., 2006), the average workplace Mn exposure concentration for the Nakata et al. (35.7 mg/m³ in one factory and 4.80 mg/m³ in the second factory) study was substantially higher compared to the Mn levels measured in the Yuan et al. study (i.e., 0.138 mg/m³) study. The difference in airborne Mn exposure concentration, and ultimately the pulmonary exposure of the worker, and not blood Mn level, may better explain the varied systemic cell response observed in the two studies. This observation provides additional evidence that blood Mn concentrations may be a poor correlate for workplace exposure to Mn (Ellingsen et al, 2003; Lu et al., 2005; Dorman et al., 2008). Another possible explanation of the observed difference from the Nakata et al. (2006) and Yuan et al. (2006) studies was that Yuan et al. (2006) used the percentage of lymphocytes

for each subset, whereas Nakata et al. (2006) utilized the absolute counts of the lymphocyte subset.

The effect of welding fume exposure on circulating immunoglobulin levels has been demonstrated to be variable in worker studies. Borska et al. (2003) observed significant reductions in serum IgG, IgA, and IgM and a significant increase in serum C3 in welders compared to a non-exposed control group, whereas Boshnakova et al. (1989) observed only a significant decrease in serum IgG in welders, but no changes in serum IgA and IgM. Tuschl et al. (1997) observed no differences in circulating IgG, IgA, and IgM when comparing welders and non-welders. In the current animal study, none of the welding fume treatments caused significant changes in the serum concentrations of IgG, IgA, IgM, or C3. In agreement, Nakata et al. (2006) also observed no change in circulating levels of IgG, IgA, and IgM when comparing groups of workers exposed to varying concentrations of Mn in welding fume. On the other hand, IgG was significantly increased in the BALF after treatment with both doses of the MMA-HS fume in the current study. This increase in IgG in the BALF was likely the result of translocation of IgG from the circulation to the airspaces as another serum protein, albumin, was also significantly elevated in the BALF after treatment with the MMA-HS fume.

Even at relatively high repeated doses (~2-fold higher than Mn amount in the MMA-HS fume), pure $MnCl_2$ did not cause a significant decrease in circulating T-lymphocytes in the current study, although there was a trend for reduced numbers of total T-lymphocytes, primarily $CD4^+$ T-lymphocytes. Thus, the influence of other metals present in welding fume on the immune system cannot be ignored, and these other metals may account for the observation that $MnCl_2$ only showed a trend for decreased T-lymphocytes as compared to the total MMA-HS fume. It has been hypothesized that Fe, the primary component of most welding fume, may pre-dispose welders and other metal workers to infectious pneumonia (Palmer et al., 2003, 2006). It has been proposed that free Fe may serve as a nutrient for infectious microorganisms and promote their growth and viability (Ghio and Cohen, 2005; Weinberg, 2009). However, a previous animal study has shown that ITI of iron oxide (Fe_2O_3) had no effect on local immune defense responses, and did not alter the clearance of a bacterial pathogen (Antonini and Roberts, 2007).

The immunosuppressive effects of Cr have been well-documented (Cohen et al., 1998; Cohen, 2004). Measureable levels of soluble Cr were observed in the MMA-HS fume used in the current study. Previous studies have shown that lung treatment with pure soluble Cr at concentrations found in welding fume or stainless steel welding fume that contain soluble Cr before pulmonary infection with a bacterial pathogen, *Listeria monocytogenes*, significantly increased bacterial lung burden when compared to control (Antonini et al., 2004, 2007; Antonini and Roberts, 2007). Importantly, the amount of Cr (8.5%) in the MMA-HS fume used in the current study was

substantially less compared to the amount of Cr in the fumes, i.e., 29% and 20%, used in, respectively, Antonini et al. (2004) and Antonini et al. (2007). Even though Cr levels were much lower and Mn levels were substantially higher in the MMA-HS fume used in the current study, the systemic T-lymphocyte and T-lymphocyte subset response induced by the MMA-HS fume likely was not based solely on the influence of one metal, but a combination of metals. It could be hypothesized that the effect on systemic T-lymphocyte populations may be the result of an additive or synergistic interaction between the Cr and Mn in the welding fume. A similar observation was made in an earlier study that showed oxidant-induced lung injury caused by welding fume was not dependent exclusively on a single metal or the solubility of the metal (Taylor et al., 2003).

In summary, an animal model was established that examined the effect of pulmonary exposure to welding fumes that contained different levels of Mn on the systemic immune cell profile. The welding fume with the highest concentration of Mn caused significant decreases in the number of circulating T-lymphocytes and specific T-lymphocyte subsets after acute and repeated lung treatment. None of the welding fume treatments used in the study caused significant changes in serum concentrations of IgG, IgA, IgM, or C3. Importantly, a high repeated pulmonary dose of $MnCl_2$ did not cause a significant decrease in circulating T-lymphocytes and lymphocyte subsets, indicating that the immunomodulatory effects observed in welders are likely due to pulmonary exposure to a combination of metals.

Disclaimer

The findings and conclusions of this paper have not been formally disseminated by NIOSH and should not be construed to represent any agency determination or policy.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Notice of correction:

This paper published online on 28 Februrary 2012 was incorrectly titled as a REVIEW ARTICLE. It has been corrected to a RESEARCH ARTICLE.