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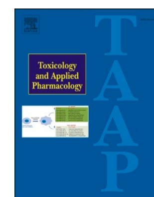

Biological effects of inhaled crude oil. VI. Immunotoxicity

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ABSTRACT

Crude oil is an unrefined petroleum product that is a mixture of hydrocarbons and other organic material. Studies on the individual components of crude oil and crude oil exposure itself suggest it has immunomodulatory potential. As investigations of the immunotoxicity of crude oil focus mainly on ingestion and dermal exposure, the effects of whole-body inhalation of 300 ppm crude oil vapor [COV; acute inhalation exposure: (6 h × 1 d); or a 28 d sub-chronic exposure (6 h/d × 4 d/wk. × 4 wks)] was investigated 1, 28, and 90 d post-exposure in Sprague-Dawley rats. Acute exposure increased bronchoalveolar lavage (BAL) fluid cellularity, CD4+ and CD8+ cells, and absolute and percent CDllb+ cells only at 1 d post-exposure; additionally, NK cell activity was suppressed. Sub-chronic exposure resulted in a decreased frequency of CD4+ T-cells at 1 d post-exposure and an increased number and frequency of B-cells at 28 d post-exposure in the lung-associated lymph nodes. A significant increase in the number and frequency of B-cells was observed in the spleen at 1 d post-exposure; however, NK cell activity was suppressed at this time point. No effect on cellularity was identified in the BALF. No change in the IgM response to sheep red blood cells was observed. The findings indicate that crude oil inhalation exposure resulted in alterations in cellularity of phenotypic subsets that may impair immune function in rats.

1. Introduction

This is the sixth manuscript in a series of seven tandem papers in which the potential toxicity of crude oil vapor (COV) has been comprehensively investigated. This manuscript focuses on immunomodulation after inhalation exposure of rats to COV. The reader is directed to the first paper in the series (Fedan, 2022), which reviews the existing information about crude oil's biological effects and describes the approach to the overall investigation. The other studies in this series have described the inhalation exposure system designed for this investigation (McKinney et al., 2022) and the effects of COV inhalation on pulmonary, cardiovascular, kidney and neurological function (Sager et al., 2022; Fedan et al., 2022; Krajnak et al., 2022; Sriram et al., 2022). The studies' overall findings are summarized in Investigative Team (2022).

Crude oil is the unprocessed oil obtained *via* drilling that is subsequently refined in a production facility. Petroleum is produced after the processing of crude oil and is a general grouping including both crude oil and petroleum products; the terms crude oil and petroleum are usually used interchangeably. Crude oil is mostly made up of hydrocarbons, varying in length and structure and is categorized as heavy or light based on its American Petroleum Institute (API) gravity with heavy oil having a higher density, viscosity, and lower solubility due to longer, more complex hydrocarbon chains. Occupational exposure to crude oil can occur during the initial phases of drilling, production, and maintenance of the machines along with downstream refinery operations and distribution (Kirkeleit et al., 2006a, 2006b; Kirkhus et al., 2015). In addition to production, occupational exposure to those involved in containment and abatement can also occur as a result of accidental oil spills (Moore and Burns, 2011). Dermal and inhalation are the most common occupational routes of exposure.

Crude oil contains a complex mixture of organic compounds (Gough and Rowland, 1990), including branched alkyl aromatic hydrocarbons (benzenes, tetralins, naphthalenes, polycylic aromatic hydrocarbons) (Booth et al., 2007). The polar organic compounds found in crude oil can also contain nitrogen, sulfur, and oxygen atoms, giving them increased dissolution. Lighter hydrocarbons (including benzene) are considered volatile organic compounds (VOCs) and can evaporate quickly due to a high vapor pressure (Hanna and Drivas, 1993).

Some oil components have been found to affect immune function,

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with benzene being the most widely studied and known to have toxic effects on the blood and bone marrow. Occupational benzene exposure in humans has been associated with hematological changes, including significant decreases of red blood cells (RBC), white blood cells (WBC), and neutrophils that were related to exposure concentration (Qu et al., 2002). Another group of workers exposed to levels of benzene below the U.S. occupational standard of 1 part per million (ppm) were identified to have decreases in WBC and platelets (Lan et al., 2004). Additionally, occupational exposure to petroleum fumes was found to cause a reduction in hematological indices, including RBC count, hemoglobin concentration, and hematocrit, which worsened with prolonged exposure (Okoro et al., 2006). In mice, inhalation of 100 ppm benzene decreased total numbers of B- and T-lymphocytes in the spleen, providing evidence for potential immunological consequences (Farris et al., 1997).

Although limited, studies have investigated the effects of crude oil exposure on the immune response. Immune suppression was observed in workers maintaining tanks containing crude oil residues, as characterized by decreases in CD4+ T-cells in the blood and serum IgM and IgA concentrations. This immune suppression correlated with benzene concentrations in the blood and urine of the workers (Kirkeleit et al., 2006a, 2006b). Additionally, in a cohort study, responders to the 2010 Deepwater Horizon oil spill who reported any exposure to crude oil had an elevated risk of asthma (Rusiecki et al., 2018). In reference to epidemiology studies, it is difficult to determine if alterations in immune function were due to dermal or inhalation exposure or a combination of both. Numerous *in vivo* studies show changes in some aspects of immune function due to crude oil exposure. However, these studies have mainly investigated exposure *via* ingestion or dermal exposure. In one study, oral exposure to crude oil led to increased thymus and spleen atrophy in mice (Leighton, 1990). Oil-contaminated mussels fed to rats caused an increase in DNA damage in the liver and bone marrow; the magnitude of the damage correlated with increased levels of chemical contamination in the mussels (Lemiere et al., 2005). Additionally, after minks were fed a diet that included fuel oil, increases in peripheral blood CD3+ T-cells and monocytes, along with increases in WBC, were observed (Schwartz et al., 2004). Dermal exposure of crude oil and jet fuel has been shown to be immunosuppressive in mice (Burnham and Bey, 1991; Ullrich and Lyons, 2000). However, no studies investigating alterations on immune function due to crude oil exposure *via* inhalation were identified.

The present study was undertaken to evaluate immunotoxicity in rats following whole-body inhalation exposure to COV in which a surrogate for the crude oil that leaked from the Deepwater Horizon oil spill was utilized. To evaluate immunotoxicity, endpoints investigated were immune cell phenotyping, natural killer (NK) cell activity, and antibody response to sheep red blood cells (SRBC). The results indicate a slight change in immune response following COV inhalation, with alterations occurring in CD4+ T cells and B-cells in the spleen and lung lymph nodes (LLN) and suppression of NK cell function.

2. Methods

2.1. Animals

All studies were conducted in facilities accredited by AAALAC International, were approved by the CDC-Morgantown Institutional Animal Care and Use Committee (protocols 13-JF-R-014, 14-JF-R-011 and 16-JF-R-020 v. 3 and v. 4) and were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats [H1a: (SD) CVF], approximate body weight of 200–275 g at arrival, were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All animals were free of viral pathogens, parasites, mycoplasm, *Heliobacter* and cilia-associated respiratory bacillus. Animals were acclimated for one week and housed in ventilated micro-isolator units supplied with HEPA-filtered laminar flow air (Lab Products OneCage; Seaford, DE), with Teklad Sanichip and Teklad Diamond Dry cellulose bedding or Shepherd Specialty Paper's Alpha-Dri cellulose (Shepherd Specialty Papers; Watertown, TN) bedding instead of Diamond Dry. They were provided tap water and autoclaved HaCXan Teklad Global 18% protein rodent diet (Harlan Teklad; Madison, WI) *ad libitum*. Rats were housed in pairs under controlled light cycle (12 h light/12 h dark) and temperature (22–25 °C) conditions.

2.2. Crude oil exposures

Rats (8 per group) were exposed in a whole-body inhalation chamber to filtered air or 300 ppm COV for 6 h × 1 d (acute exposure) or 6 h/d 4 d/wk. × 4 wks (sub-chronic exposure) (McKinney et al., 2022). Experimental endpoints were examined at 1 and 28 days post exposure (dpe) for the acute exposure study, and at 1, 28, and 90 days post exposure for the sub-chronic exposure study.

2.3. Tissue processing

On days 1, 28, and 90, following the last crude oil or filtered air exposure, rats were given an i.p. injection of sodium pentobarbital (100–300 mg/kg; Fatal Plus; Vortech Pharmaceuticals; Dearborn, MI). Following exsanguination, the trachea was cannulated, the chest cavity was opened, the right bronchus was clamped off, and bronchoalveolar lavage (BAL) fluid was collected from the left lung. The first lavage sample was obtained by filling the left lung with 3 ml of phosphate-buffered saline (PBS), massaging for 30 s, withdrawing, and repeating the process one more time. This concentrated aliquot was withdrawn, retained, kept separately, and was designated as the first fraction of BAL fluid. Subsequent aliquots of PBS in 5 ml volumes were instilled once with light massaging, withdrawn, and combined until a 30 ml total volume was obtained. For each animal, both lavage fractions were centrifuged (10 min, 598 *g*), and the cell pellets were combined and resuspended in 1 ml of PBS.

The lung lymph nodes (LLNs) and spleen were removed from each animal, cleaned of connective tissue, and placed in sterile PBS or complete medium (CM)[RPMI-1640, 10% fetal bovine serum (FBS; HyClone, Waltham, MA) and 100 IU penicillin/100 μg streptomycin (Sigma-Aldrich; St. Louis, MO) per ml of medium], respectively. LLN single cell suspensions were prepared by mechanical disruption of tissues between frosted microscope slides in PBS. Spleens were prepared using a 30 ml syringe plunger and subsequently passing the homogenate through a cell strainer to obtain a single cell suspension. Cells were washed with PBS and resuspended in CM. To enumerate the total number of cells, 20 μl of cells was added to 10 ml of Isoton II diluent (1:500; Beckman Coulter; Brea, CA) and two drops of ZAP-OGLOBIN (Beckman Coulter) were added to lyse red blood cells. Cells were then counted using a Coulter counter.

2.4. Flow cytometry

For phenotypic analysis, single cell suspensions obtained from the BAL, spleen, and LLN (1–2 × 10^6) were dispensed into a 96-well, round-bottom plate and washed in staining buffer (PBS + 1% bovine serum albumin +0.1% sodium azide). Erythrocytes were lysed *via* RBC lysis buffer before staining of the spleen. For blocking of Fc receptors, cells were resuspended in staining buffer containing α-rat CD32 antibody. Cells were then incubated with a staining cocktail of fluorochrome-conjugated antibodies specific for rat cell surface epitopes: CD3-FITC (clone G4.18), CD4-APC-Cy7 (OX-35), CD8-PerCP (OX-8), CD11b-V450 (WT.5), CD45RA-PE (OX-33), CD45-PE-Cy7 (OX-1), CD161a-AF647 (10/78). Following an incubation, cells were washed twice in staining buffer and fixed in Cytofix buffer according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Within 24 h, cells were resuspended in staining buffer and analyzed on an LSR II flow cytometer (BD Biosciences). Data analysis was performed with FlowJo

7.6.5 software (TreeStar Inc.; Fenton, MI). Cells were first gated on single cells using SSC-A × SSC-H doublet discrimination, followed by cellular discrimination using FSC-A and SSC-A parameters, then cells were identified as CD4+ T cells (CD4+ CD3+), CD8+ T cells (CD8+ CD3+), B cells (CD45RA+ CD3-), NK cells (CD161a^{hi} CD3-), and CD11b + myeloid cells.

2.5. Natural killer (NK) cell assay

NK cell activity was evaluated using Yac-1, a murine T-cell lymphoma cell line (ATCC; Manassas, VA) as the target cell. Target cells were cultured in complete media (CM), maintained at 37 °C in a humidified, 5% CO_2 atmosphere, and monitored daily. Cells were harvested in the log phase of growth, washed with Dulbecco's PBS, and counted using 0.4% trypan blue solution. Only the cultures with >95% cell viability were selected for use in the assay. Target cells were labeled with carboxyfluorescein succinmidyl ester (CellTrace™ CFSE Proliferation Kit; Life Technologies; Waltham, MA) according to the manufacturer's directions. Cells were pelleted, washed twice in CM to wash off the unincorporated dye, counted in 0.4% trypan blue solution, and diluted to the desired concentration in CM. Splenocytes collected and processed as described above were used as the effector cells in this assay. The cytotoxicity assay was performed in 96-well, round bottom tissue culture-treated microtiter plates. Effector cells were seeded in a 100 μl volume/well with a fixed number of CFSE-stained target cells (20,000/well) with effector to target ratios ranging from 50:1 to 150:1. Control wells for spontaneous death of target contained CFSE-stained target with no effectors (0:1, E:T ratio). Recombinant rat interleukin (IL)-2 (final concentration 0.05 ng/μl; R&D Systems; Minneapolis, MN) was added to all wells for enhancement of baseline cytotoxic activity. The contents of each well were mixed gently by pipetting, centrifuged briefly at 526 *g*, and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. Killing was stopped by placing the plate on ice for 5 min. Appropriate wells were subsequently stained with Live/Dead Fixable Violet Dead Cell Stain and fixed in Cytofix fixation buffer (BD Bioscience) according to the manufacturer's directions. Effectors only, unstained target, and Live/Dead Fixable Violet Dead Cell (Invitrogen; Waltham, MA)-stained targets were also included as assay controls. Stained samples were analyzed on LSR II flow cytometer (BD Biosciences). The results were expressed as percentage of dead targets on a cell-to-cell basis.

2.6. Hematology and serum chemistries

Following administration of an overdose of sodium pentobarbital and exsanguination, whole blood was collected from the abdominal aorta of the rats. A 150 μl aliquot was used for hematological evaluations, and 0.5 ml serum (collected from 5 ml of centrifuged blood) was used for serum chemistry analysis. Selected serum chemistries were evaluated using a Catalyst DX Chemistry Analyzer (IDEXX Laboratories, Inc.; Westbrook, ME). Endpoints analyzed included: albumin (ALB), globulin (GLOB), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), urea nitrogen (BUN), creatinine (CREA), glucose (GLU), total protein (TP), creatine kinase (CK), triglycerides (TRICL), cholesterol (CHOL), ALB/GLOB ratio, and BUN/CREA ratio. Selected hematological parameters were evaluated using a ProCyte DX Automatic Hematology Analyzer (IDEXX). Endpoints analyzed included peripheral erythrocyte and leukocyte counts, leukocyte differentials (lymphocytes, neutrophils, monocytes, basophils, and eosinophils), reticulocytes, platelet counts, hematocrit, hemoglobin levels, mean corpuscular hemoglobin (MCH) and hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean platelet volume (MPV), platelet hematocrit (PCT), and platelet distribution width (PDW).

2.7. Spleen IgM response to SRBC

The primary IgM response to SRBC was enumerated using a modified hemolytic plaque assay (Jerne and Nordin, 1963; Marrocco et al., 2015). Four days prior to euthanasia, the rats were immunized with 2×10^8 SRBC (in 1 ml volume) by i.v. injection. All SRBC for these studies were drawn from a single donor animal (Lampire Laboratories; Pipersville, PA). On days 1, 28, and 90 following the last exposure to COV or air, rats were euthanized by CO_2 asphyxiation, body and spleen weights were recorded, and spleens were collected in 10 ml of Hank's balanced salt solution (HBSS). Blood was also retrieved in serum collection tubes following transection of the abdominal aorta and serum was stored at −20 °C for subsequent analysis of serum anti-SRBC IgM levels (see below). Single cell suspensions of the spleens from individual animals were prepared in HBSS by disruption using a 30 ml syringe plunger and subsequent passage through a cell strainer to filter cellular debris. To quantify the total number of spleen cells, 20 μl of cells was added to 10 ml of Isoton II diluent (1:500; Beckman Coulter) and two drops of ZAP-OGLOBIN (Beckman Coulter) were added to lyse red blood cells. Cells were then counted using a Coulter counter.

Dilutions (1:60 and 1:120) of spleen cells were then prepared and 100 μl of each dilution were added to test tubes containing a 0.5 ml warm agar/dextran mixture (0.5% Bacto-Agar; Thermo Scientific; Waltham, MA) and 0.05% DEAE dextran (Sigma-Aldrich), 25 μl of 1:1 ratio of SRBC suspension, and 25 μl of 1:4 dilution (1 ml lyophilized) guinea pig complement (Cedarlane Laboratories; Burlington, Ontario). Each sample was vortexed, poured into a petri dish, covered with a microscope coverslip, and incubated for 3 h at 37 °C. The plaques (representing antibody-forming B-cells) were then counted. Results were expressed in terms of both specific activity (IgM plaque forming cells (PFC) per 10^6 spleen cells) and total activity (IgM PFC per spleen).

2.8. Statistical analyses

To determine statistically significant differences in immunological endpoints, a 2-tailed unpaired *t*-test was used to compare rats exposed to filtered air or COV at the specified time point. Presented data are based on the means ± SEM of 8 rats for each treatment group at each time point, except for the NK cell assay, in which 4 rats per exposure group were used. Differences between control and experimental groups were accepted as significant when $P < 0.05$ or $P < 0.01$. Statistical analysis was performed using Graph Pad Prism version 5.0 (San Diego, CA).

3. Results

3.1. Acute exposure study

An acute exposure study was conducted to determine COV toxicity. A significant reduction in NK cell activity was observed, but only at the 1 d time point (Supplemental Fig. 1). However, no change in the IgM response to SRBC was demonstrated (Supplemental Fig. 2). No changes in LLN immune cell populations were identified (Supplemental Table 1). A significant increase in the frequency of NK cells was observed in the spleen but only at the 1 d time point (Supplemental Table 2). Lymphocytes were measured in the BAL as an increase in lymphocyte population would signify inflammation and an airway disease. In the BAL, significant increases were identified in total cellularity, CD4+ T cells, CD8+ T cells and in the number and frequency of CD11b + cells, but only at 1 d post-exposure (Supplemental Table 3). Significant decreases in ALT and CK were identified in the serum, but only at the 28-d time point (Supplemental Table 5). Hematological analyses demonstrated significant increases in the number of erythrocytes and levels of hemoglobin and hematocrit, but only at 1 d post-exposure (Supplemental Table 4).

3.2. Sub-chronic exposure study

3.2.1. Immunophenotyping

Flow cytometry was used to conduct phenotypic analysis of immune cells isolated from the BAL, LLN, and spleen. In the BAL, no effect of COV

was seen with respect to total cellularity or any of the evaluated immune cell subsets at any timepoint (Table 1). While exposure to 300 ppm COV had no effect on total cellularity, a significant decrease in frequency of CD4+ T-cells in the LLN was observed at 1 d post-exposure (Table 2). At 28 d post-exposure, COV induced a significant increase in number and frequency of B-cells in the LLN; however, this was not observed at the other time points (Table 2). Although there was no increase in total cellularity in the spleen a significant increase in the numbers and frequency of B-cells was observed but only at 1 d post-exposure (Table 3).

3.2.2. Natural killer cell assay

Sub-chronic exposure to 300 ppm COV suppressed NK cell function at 1 d post-exposure (Fig. 1A). A significant decrease in the percent killing was observed at two effector to target ratios, with 1:25 inducing a decrease of 10% and 1:50 causing a decrease of 8% at 1 d post-exposure (Fig. 1A). No changes were observed at 28 d post-exposure at any effector to target ratios (Fig. 1B).

3.2.3. Hematology and serum chemistries

No consistent hematological changes were observed at the time points evaluated. However, at 90 d post-exposure, the percent of circulating neutrophils increased (Supplemental Table 6) along with levels of urea nitrogen (BUN), alkaline phosphatase (ALKP), and alanine aminotransferase (ALT) in the crude oil exposed rats (Supplemental Table 7). Additionally, at 1 d post-exposure there was a decrease in ALT (Supplemental Table 7) and at 28 d post-exposure an increase in percent monocytes (Supplemental Table 6). No hematological changes were observed at 1 d post-exposure. It is important to note that while these changes were identified to be statistically significant, the values still fell within what is considered to be the “normal” range.

3.2.4. IgM response to SRBC

To evaluate if exposure to crude oil was immunosuppressive, the IgM response to SRBC was examined. After exposure to COV, no statistically significant reduction in the PFC/spleen or specific (PFC/10^6 cells) IgM antibody activity against SRBC was observed at any of the post-exposure time points (Fig. 2).

Table 1
BAL phenotyping of rats after sub-chronic exposure to COV.

	1 Day		28 Days[b]		90 Days	
BAL parameter[a]	Air	COV	Air	COV)	Air	COV
Cellularity ($\times 10^6$)	3.92 ± 0.56	4.08 ± 0.15	3.79 ± 0.12	3.95 ± 0.22	4.53 ± 0.26	4.52 ± 0.20
CD4+	24,300 ± 4082	29,610 ± 2402	25,660 ± 7026	21,700 ± 836.6	33,160 ± 3727	40,160 ± 2840
CD4+ (%)	0.69 ± 0.10	0.73 ± 0.06	0.69 ± 0.21	0.56 ± 0.04	0.76 ± 0.11	0.90 ± 0.07
CD8+	2290 ± 352.9	2846 ± 353.7	4548 ± 909.9	2738 ± 353.1	3580 ± 268.8	4121 ± 811.0
CD8+ (%)	0.06 ± 0.01	0.07 ± 0.01	0.12 ± 0.02	0.07 ± 0.01	0.081 ± 0.01	0.091 ± 0.02
B-cells	30,770 ± 6130	28,300 ± 2091	18,020 ± 3145	16,450 ± 2949	9442 ± 3301	9294 ± 2700
B-cells (%)	0.75 ± 0.09	0.70 ± 0.06	0.48 ± 0.10	0.43 ± 0.09	0.22 ± 0.08	0.21 ± 0.07
NK	2287 ± 622.5	1738 ± 182.2	2262 ± 238.3	1919 ± 174	1656 ± 239.4	1908 ± 309.8
NK (%)	0.06 ± 0.02	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.039 ± 0.01	0.042 ± 0.01
CD11b+	68,610 ± 15,850	56,350 ± 6982	90,310 ± 23,800	66,330 ± 11,190	40,660 ± 4074	59,660 ± 10,390
CD11b + (%)	1.76 ± 0.31	1.40 ± 0.19	2.42 ± 0.67	1.72 ± 0.35	0.89 ± 0.06	1.33 ± 0.25

Note that 1 Day, 28 Days, and 90 Days refers to days post exposure.
[a] Values are expressed as the means ± SEM for each group (n = 8 rats/group).
[b] 4 air controls and 7 COV-treated animals for this timepoint.

Table 2
LLN phenotyping of rats after sub-chronic exposure to COV.

	1 Day		28 Days		90 Days	
LLN parameter[a]	Air	COV	Air	COV	Air	COV
Cellularity ($\times 10^7$)	1.62 ± 0.14	1.73 ± 0.21	1.46 ± 0.17	1.74 ± 0.13	1.65 ± 0.21	1.74 ± 0.10
CD4+ ($\times 10^6$)	5.98 ± 0.46	4.72 ± 0.26	4.61 ± 0.61	5.34 ± 0.47	4.18 ± 0.54	4.10 ± 0.44
CD4+ (%)	38.34 ± 0.78	31.33 ± 2.87*	31.21 ± 1.39	30.65 ± 0.88	25.66 ± 1.17	23.53 ± 1.94
CD8+ ($\times 10^6$)	2.67 ± 0.22	2.10 ± 0.16	1.79 ± 0.25	2.30 ± 0.21	2.23 ± 0.14	2.10 ± 0.018
CD8+ (%)	17.26 ± 1.16	13.91 ± 1.42	12.27 ± 0.87	13.15 ± 0.49	14.11 ± 0.73	12.11 ± 0.84
B-cells ($\times 10^6$)	2.60 ± 0.33	3.55 ± 0.56	3.24 ± 0.44	4.69 ± 0.40*	4.07 ± 0.76	4.70 ± 0.50
B-cells (%)	16.59 ± 1.36	21.46 ± 2.37	22.24 ± 1.17	26.85 ± 1.04*	23.61 ± 1.33	26.78 ± 1.96
NK	52,000 ± 9396	48,060 ± 5687	44,880 ± 9894	48,970 ± 7279	52,190 ± 5517	60,400 ± 12,500
NK (%)	0.34 ± 0.06	0.31 ± 0.04	0.30 ± 0.04	0.28 ± 0.03	0.32 ± 0.02	0.34 ± 0.06
CD11b+	60,850 ± 11,410	59,970 ± 10,160	45,700 ± 8873	35,210 ± 9570	53,140 ± 5615	48,000 ± 9484
CD11b + (%)	0.39 ± 0.05	0.38 ± 0.06	0.34 ± 0.09	0.21 ± 0.06	0.34 ± 0.04	0.28 ± 0.05

Note that 1 Day, 28 Days, and 90 Days refers to days post exposure.
[a] Values are expressed as the means ± SEM for each group (n = 8 rats/group).
* $P < 0.05$.

Table 3
Spleen phenotyping of rats after sub-chronic exposure to COV.

	1 Day		28 Days		90 Days	
Spleen parameter[a]	Air	COV	Air	COV	Air	COV
Cellularity ($\times 10^8$)	8.59 ± 0.53	10.04 ± 0.75	11.55 ± 0.63	10.16 ± 0.49	10.53 ± 0.47	10.77 ± 0.83
CD4+ ($\times 10^8$)	1.21 ± 0.07	1.48 ± 0.12	1.97 ± 0.12	1.87 ± 0.12	1.29 ± 0.58	1.41 ± 0.90
CD4+ (%)	16.38 ± 0.51	17.36 ± 0.84	17.23 ± 1.18	18.31 ± 0.50	12.33 ± 0.54	13.50 ± 1.00
CD8+ ($\times 10^7$)	6.61 ± 0.44	7.80 ± 0.81	8.62 ± 0.82	8.81 ± 0.97	8.02 ± 0.47	8.27 ± 0.44
CD8+ (%)	8.94 ± 0.37	9.07 ± 0.56	7.48 ± 0.67	8.56 ± 0.57	7.65 ± 0.38	7.89 ± 0.55
B-cells ($\times 10^8$)	2.09 ± 0.25	2.77 ± 0.16*	3.01 ± 0.21	2.79 ± 0.13	2.73 ± 0.15	3.16 ± 0.35
B-cells (%)	27.81 ± 1.79	32.74 ± 1.14*	26.06 ± 1.26	27.55 ± 0.71	25.85 ± 0.79	28.79 ± 1.35
NK ($\times 10^7$)	2.28 ± 0.08	2.40 ± 0.27	3.24 ± 0.20	3.21 ± 0.22	2.73 ± 0.25	3.55 ± 0.50
NK (%)	3.14 ± 0.19	2.81 ± 0.23	2.89 ± 0.26	3.17 ± 0.17	2.51 ± 0.19	2.90 ± 0.22
CD11b + ($\times 10^7$)	2.88 ± 0.27	3.13 ± 0.18	3.94 ± 0.21	3.23 ± 0.12	4.07 ± 0.32	4.75 ± 0.70
CD11b + (%)	3.88 ± 0.27	3.74 ± 0.26	3.41 ± 0.09	3.20 ± 0.11	3.84 ± 0.16	4.30 ± 0.45

Note that 1 Day, 28 Days, and 90 Days refers to days post exposure.
[a] Values are expressed as the means ± SEM for each group (n = 8 rats/group).
* $P < 0.05$.

4. Discussion

These studies were undertaken to investigate the immunotoxicological effects of inhaled COV due to the large number of exposed workers and the lack of existing toxicity data. The studies in this section focused on immunomodulation resulting from inhaled COV in rats. Following extended whole-body inhalation exposure to 300 ppm crude

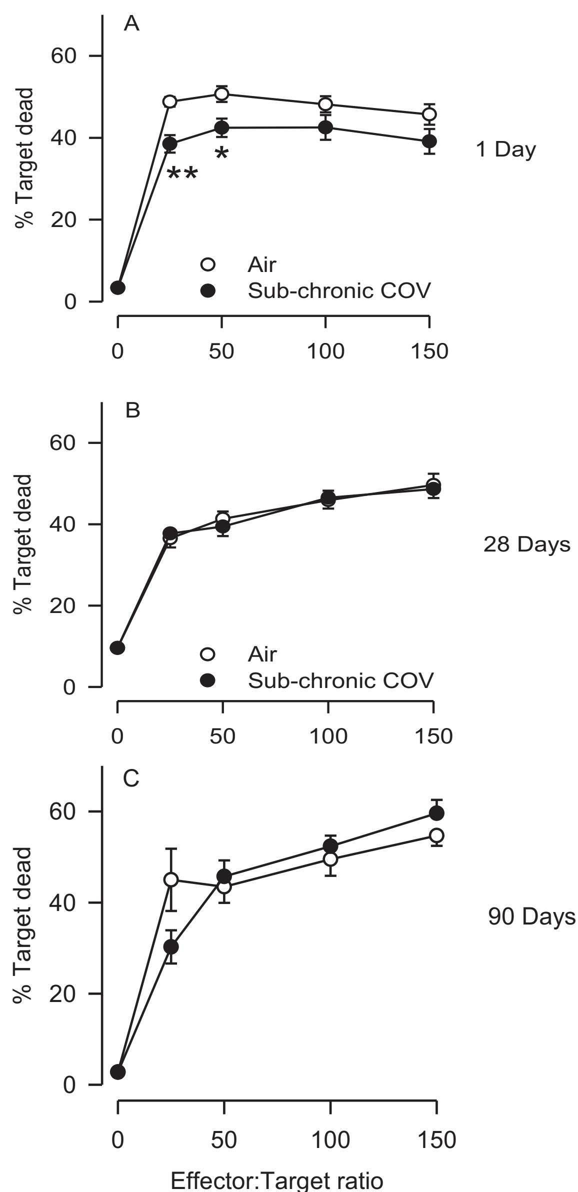

Fig. 1. Inhalation sub-chronic exposure to COV suppresses spleen NK cell activity. NK cell activity in splenocytes was evaluated after *in vivo* sub-chronic exposure to 300 ppm COV. NK cell percent killing of target cells is illustrated at 1 d (A), 28 d (B) and 90 d (C) post-exposure. Data shown as the means ± SEM of 4 rats per group. *P < 0.05, **P < 0.01 *vs.* air controls at indicated effector: target ratios.

oil, quantitative changes in B-cells in both the spleen and the LLN and changes in CD4+ T-cells in the LLN were observed in the present study. While none of the phenotypic changes in the LLN or spleen were significant over multiple time points, the CD4+ T-cell and B-cell changes in the LLN suggest trending decreases and increases, respectively, at all post-exposure time points investigated. Additionally, the increases in B-cell number and frequency suggest the induction of adaptive immune responses, although as these changes were relatively minor and were not observed consistently between time points and tissues, which raises questions about the biological relevance. While the acute exposure study did identify changes in the BAL (cellularity, CD4+, CD8+, CDllb+), these effects were only observed 1 d post-exposure, a finding which is not consistent with those from the sub-chronic exposure study, suggesting an acute inflammatory response. However, as these changes were relatively minor and not observed over multiple measurements, therefore, the biological relevance is debatable. Previous studies on benzene exposure also found effects on B-cells in the spleen; however, the B-cell numbers were decreased in these cases (Farris et al., 1997; Aoyama, 1986). This could be explained by benzene exposure causing B-cell cytotoxicity, which does not occur with the entire cocktail of chemicals in crude oil exposure (Aoyama, 1986). Although occupational exposure to benzene has been shown to reduce IgM levels in the blood (Kirkeleit et al., 2006a, 2006b), no changes in immune suppression following inhalation exposure to COV were identified, as evaluated through measurements of the IgM antibody production. The PFC assay measures the humoral immune response *via* production of antigen-specific antibodies (IgM isotype), which involves the cooperation and interaction of macrophages, T-lymphocytes, and B-lymphocytes. This assay is considered one of the most sensitive endpoints to assess immunomodulation. Supporting the data presented in the current study, rats exposed to gasoline *via* inhalation showed no changes in IgM response to SRBC evaluated by PFC/spleen or specific PFC/10^6 cells (White Jr. et al., 2014). However, the same study did find a decrease in IgM response when the gasoline was mixed with ethanol and diisopropyl ether. Based on the results presented in the current study, lack of effect on humoral immune response in addition to observed changes only at early time points suggests that the immune changes are inflammatory in nature.

However, a suppression of NK cell function after COV inhalation exposure was observed, but only at the early timepoint after the acute and sub-chronic exposures. NK cells can act as a bridge between innate and adaptive immune responses. Evaluation of the functional capacity of NK cells to induce cytotoxicity has been determined to be important in assessing immunotoxicity. Jet fuel is processed from crude oil and contains similar aromatic hydrocarbon species (*e.g.*, benzene, toluene) (Agency for Toxic Substances and Disease Registry, 2017). Like the present study, inhalation of jet fuel was shown to suppress NK cell function in mice (Harris et al., 2000). Jet fuel-exposed mice exhibited decreased NK cell-mediated cytotoxicity against target cells compared to control mice. Additionally, a population exposed to an oil spill was investigated seven years after the incident, and no significant changes in most lymphocyte subsets or circulating cytokines were found. However, there was a decrease in the frequency of NK cells in exposed individuals compared to reference individuals (Laffon et al., 2013). Although a change in NK cell frequency or number was not observed in our study, the change in NK cell function suggests a possible functional immune defect. Again, this change occurred only at 1 d post-exposure, suggesting that NK cell function had recovered and returned to air exposure control levels by 28 d post-exposure.

A limited number of epidemiological studies have evaluated functional immune outcomes in occupational and residential populations. A large cohort study of over 50,000 members of the U.S Coast Guard was conducted on responders (exposed/not exposed) and non-responders to the Deepwater Horizon oil spill. Oil-exposed responders acquired an elevated relative risk for asthma compared to non-responders. Also, a positive association between crude oil exposure and numerous acute symptoms such as wheezing, shortness of breath, and skin rash was observed in the responders (Rusiecki et al., 2018). Additionally, long-term studies were conducted following the Hebei Spirit oil spill, which occurred near a residential area in South Korea, wherein the effects of the accident on children were examined one, three, and five years after the exposure. Estimates of oil exposure were based on the distance from the oil spill and modeled estimates of crude oil compounds (including benzene and toluene). Oil exposure level estimates were significantly associated with asthma in children at all time points evaluated following the oil spill (Noh et al., 2019). Additionally, in a population living near an oil spill, increases in lymphocytes and eosinophils in blood samples were identified (Khurshid et al., 2008). In human airway epithelial cells exposed to crude oil, *in vitro* alterations in expression of genes associated with asthma including C3, C1R, and MIF were found (Liu et al., 2016). Also altered were genes involved in cell junctions and angiogenesis, both

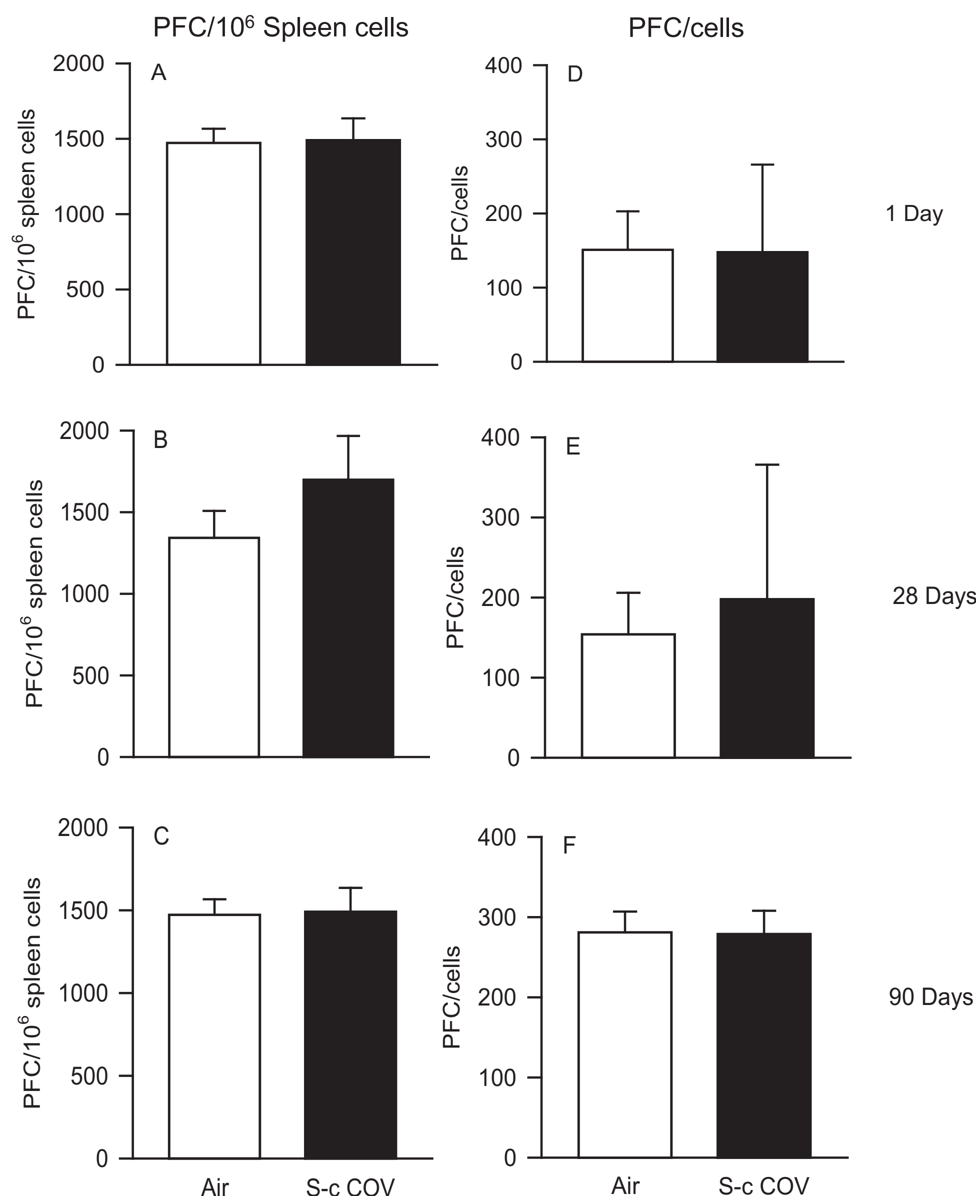

Fig. 2. Inhalation sub-chronic exposure to COV does not suppress the spleen IgM response to SRBC. The spleen IgM response to sheep red blood cells (SRBC) was evaluated after sub-chronic exposure to 300 ppm COV. The IgM response to SRBC/10^6 splenocytes and IgM response to SRBC/spleen is illustrated at 1 d (A and B), 28 d (C and D) and 90 d (E and F) post-exposure. Data shown as the means ± SEM of 8 rats per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of which have been found to be associated with asthma (Ribatti et al., 2009; Georas and Rezaee, 2014). The increase in B-cells observed both in the LLN and spleen in the studies described in this paper emphasizes the potential impact of COV on allergic responses, suggesting a possible mechanism for the increased risk of asthma seen in the epidemiological studies following oil exposure. However, more research is needed to investigate whether the increase in these immune cell populations is also leading to increased production of IgE antibodies.

While numerous studies have focused on dermal and ingestion as routes of exposure, fewer studies have investigated the toxicity of inhaled COV. In one study, investigators used particulate matter that was collected from *in situ* burn plumes of the Deepwater Horizon oil spill for the exposure (Jaligama et al., 2015). Results from this investigation revealed changes in immune function with increases in macrophages and eosinophils in the BAL fluid of mice. An increase in the T_H2 cell response in a mouse model of asthma also was observed. In that study, it was suggested that it is the *particulate matter* produced from the burning oil that led to the observed biological effects. Also, in that study three exposures were conducted over a 5-day period, compared to the study presented in this paper in which *vapor* exposures lasted for four weeks, suggesting that early inflammation might occur with shorter exposures and could be a possible explanation for the differences in the findings. Another study investigated inhalation exposure to crude oil in rats and found increases in DNA damage in peripheral white blood cells; however, this study was focused on genotoxicity and other immune responses were not investigated (Valdiglesias et al., 2012).

While numerous investigations have suggested the occurrence of immune effects in response to oil exposure, most of these have been largely influenced by the mixed composition of the test materials. Interestingly, in factory workers exposed to benzene, toluene co-exposure reduced benzene hematological toxicity (Robert Schnatter et al., 2010). Conversely, a study of a crude oil-exposed population in Scotland found no difference in hematological or biological markers compared to a control, non-exposed population (Campbell et al., 1993). Additionally, in an occupational setting where males were exposed to mixed organic solvents containing toluene (but not benzene), toluene levels were associated with an increase in B-lymphocytes and a decrease in CD4+ T-lymphocytes in blood (Tanigawa et al., 2001). This is also supported by findings that co-exposure of toluene with benzene (both components of crude oil) altered adverse effects of benzene alone (Ikeda

et al., 1972; Robert Schnatter et al., 2010). Exposures occurring with mixtures that contain multiple immunomodulating substances can be difficult to interpret. The crude oil utilized in our study contains a mixture of both toluene and benzene and the results presented in this manuscript are more similar to those described in the literature for toluene exposure. While the majority of studies investigating petroleum exposure determine that the greatest alterations in immune function occur following single chemical exposures, it is important to keep in mind that these experiments may not fully represent what is occurring in occupational settings where the entire petrochemical "cocktail" is involved in the exposure. In general, the single exposure studies do suggest that benzene alone is more hazardous to the immune system than what was observed in the present study with COV exposure and collectively suggest that immunomodulation *via* petroleum exposure is influenced by co-exposure to additional organic compounds.

As discussed above, there is a wide range of results in epidemiological studies investigating immune function following crude oil exposure (McLoone et al., 2019). Interpretation of these findings needs to be done with caution as these exposures can occur *via* multiple routes and with co-exposure to other environmental or occupational toxicants. The type of crude also needs to be considered, as heavy crude oil has longer hydrocarbon chains with a higher density, viscosity, and lower solubility, and these chemical properties of the hydrocarbon could alter the potential for disease (Curtis and Sergent, 2020).

In summary, in the present study, inhalation exposure to COV resulted in functional impairment of the innate immune system, inflammation, and limited quantitative changes in cellularity in rats at early post-exposure time points. The suspected health effects of crude oil due to the mixture of multiple chemicals (including benzene) along with the risk of occupational exposure, demonstrates the need for additional studies on human health to evaluate the potential adverse effects due to COV exposure.

5. Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of brand name does not constitute product endorsement.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest in relation to this publication.

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