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Biodiesel versus diesel exposure: Enhanced pulmonary inflammation, oxidative stress, and differential morphological changes in the mouse lung

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Abstract

The use of biodiesel (BD) or its blends with petroleum diesel (D) is considered to be a viable approach to reduce occupational and environmental exposures to particulate matter (PM). Due to its lower particulate mass emissions compared to D, use of BD is thought to alleviate adverse health effects. Considering BD fuel is mainly composed of unsaturated fatty acids, we hypothesize that BD exhaust particles could induce pronounced adverse outcomes, due to their ability to readily oxidize. The main objective of this study was to compare the effects of particles generated by engine fueled with neat BD and neat petroleum-based D. Biomarkers of tissue damage and inflammation were significantly elevated in lungs of mice exposed to BD particulates. Additionally, BD particulates caused a significant accumulation of oxidatively modified proteins and an increase in 4-hydroxynonenal. The up-regulation of inflammatory cytokines/chemokines/growth factors was higher in lungs upon BD particulate exposure. Histological evaluation of lung sections indicated presence of lymphocytic infiltrate and impaired clearance with prolonged

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Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.07.006.

retention of BD particulate in pigment laden macrophages. Taken together, these results clearly indicate that BD exhaust particles could exert more toxic effects compared to D.

Keywords

Aspiration exposure; Cytokine panel; Pulmonary toxicity; Biodiesel particle retention; Inflammation; Lipid droplets

Introduction

Despite the widespread use of petroleum-based diesel (D) fuels, interest in vegetable oils as an alternative fuel source was reported in several countries as early as the 1920s and 1930s. The potential interest in alternative fuels was not evidenced until the fuel-energy crisis in the late 1970s and early 1980s, after which vegetable oil derived fuels gained their prominence as a potential alternative energy source (Hill et al., 2006; Ragauskas et al., 2006). One of the key issues of biodiesel (BD) use is to reduce the emissions of particulate matter (PM) and greenhouse gasses (GHG). The combustion of vegetable oil-derived biodiesel fuels was proven effective in producing similar or less emissions compared to petroleum-based D (Koonin, 2006). Regardless of its broad use in different operational areas, including transportation (on- and off-road vehicles), and other manufacturing/production (mining, oil and gas industry) sectors, inadequate attention has been paid to the possible health hazards of BD (Bunger et al., 2007; Krahl et al., 2001; Swanson et al., 2007).

Exposure to diesel exhaust in humans has been shown to cause a number of adverse health outcomes. For instance, acute exposure to diesel particulate matter (DPM) was shown to facilitate pulmonary inflammation with influx of phagocytic cells (Holgate et al., 2003a, 2003b), while long-term exposure was strongly associated with a greater incidence of cough, phlegm, and chronic bronchitis (Pronk et al., 2009). Additionally, exposure to DPM has been associated with a number of long-term adverse effects, such as exacerbation of pre-existing lung disease, respiratory infections, bronchoconstriction and cancer (Sawyer et al., 2010; Silverman et al., 2012). High-molecular weight polycyclic aromatic hydrocarbons (PAHs), and PAH-derivatives, such as nitro-PAHs from combustion exhaust are considered to be potent mutagens and carcinogens (Garshick et al., 2004; Tokiwa and Ohnishi, 1986). Additional adverse effects of DPM may also include vascular damage, cardiac malfunctions and pro-thrombotic effects (Mills et al., 2005, 2007; Nemmar et al., 2007; Rivero et al., 2005; Tornqvist et al., 2007). Clinical studies involving humans have shown impairment of vasodilation, and increased incidence of arrhythmias and myocardial infractions along with changes in heart rate (Hazari et al., 2011; Mills et al., 2005, 2007; Nemmar et al., 2009; Peretz et al., 2008; Watkinson et al., 1998).

BD derived from vegetable oils of various sources (rapeseed, soybean, corn, sunflower, etc.), is gaining popularity as an alternative fuel for its use in diesel engines to meet the requirements of the Clean Air Act. Currently the majority of BD fuels are produced by the trans-esterification of vegetable oils with methanol or another alcohol, generating fatty acid alkyl esters (Knothe et al., 2010). The major components of these oils are mono-unsaturated oleic acid (C18:1) and polyunsaturated linoleic acid (C18:2) and linolenic acid (C18:3) that

are susceptible to oxidation upon combustion leading to formation of peroxides and a variety of secondary oxidation products (Knothe, 2005; Song et al., 2000). A detailed list of fatty acids composition of BD from various sources can be found in Supplemental Materials (Table S1). Combustion of BD was shown to reduce mass emissions of PM, unburned hydrocarbons (HC), PAHs, nitrogen oxides levels, carbon monoxide, and aldehyde like compounds (Garshick et al., 2004; Tokiwa and Ohnishi, 1986). Previous published studies showed the potential of neat BD and BD blends (Bugarski, 2006; Bugarski et al., 2010; McDonald et al., 1997) to lessen occupational exposures to elemental carbon (EC) and non-volatile fractions of DPM (Purcell et al., 1996). However, BD was found to increase particle-bound volatile organic fraction of PM and carbonyl emissions (Liu et al., 2009; Purcell et al., 1996). The semi-volatile fractions of BD exhaust demonstrated increased cellular toxicity compared to that of PM (Liu et al., 2009). Additional studies have compared the acute toxicity exerted by BD and D emissions using mutagenicity assays (Bunger et al., 1998). While the emissions of BD are less mutagenic than D with high sulfur content fuel (Kado and Kuzmicky, 2003), recent studies, including our own, demonstrated that BD is more mutagenic when compared to ultralow sulfur diesel fuel (ULSD) (Bunger et al., 2007; Kisin et al., 2013; Krahl et al., 2009). The non-methylated esters of BD were shown to be more mutagenic than both methylated BD and petroleum D (Bunger et al., 2000). Further, Ackland et al. has shown that exposure to 80% and 20% BD blends increased formation of multinucleated cells by 16% and 52%, respectively (Ackland et al., 2007). Studies by Brito et al. (2010) suggest that BD displays equal and/or more toxic effects compared with D fuel. While exposure to D, neat BD and a 50% BD blend induced lung inflammation in rats 1 h post exposure, no disparity between groups exposed to different fuels was found (Brito et al., 2010). These results were similar to those reported for the chronic BD exposure studies (Finch et al., 2002), where no difference between exposure of animals to the combustion of these different fuels was found.

As reported previously, differences in the toxicity induced by BD could be due to several factors including the chemical composition of BD, different additives used in engines, age and operating conditions of the engine (Obert, 1973; Ullman, 1989). These parameters could in turn facilitate a number of varied health outcomes found in occupational settings (Groves and Cain, 2000; Pronk et al., 2009). Considering BD fuel consists of unsaturated fatty acids, which are easily oxidized during combustion, we hypothesize that BD exhaust particles could induce pronounced adverse outcomes compared to D.

Materials and methods

Animals

Specific pathogen-free adult female C57BL/6 mice (8–10 weeks) were supplied by Jackson Laboratories (Bar Harbor, ME) and weighed 20.0 ± 1.9 g when used. Animals were housed one mouse per cage receiving filtered high efficiency particulate air (HEPA) in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-accredited National Institute of Occupational and Safety Health (NIOSH) animal facility. All animals were acclimated in the animal facility under controlled temperature and humidity for one week prior to use. Beta Chips (Northeastern Products

Corp., Warrensburg, NY) were used for bedding which was changed weekly. Animals were supplied with water and certified chow 7913 (Harlan Teklad, Indianapolis, IN) ad libitum, in accordance with the guidelines and policy set forth by the Institute of Laboratory Animal Resources, National Research Council. All experimental procedures were conducted in accordance with a protocol approved by the NIOSH Institutional Animal Care and Use Committee (IACUC).

Details of exhaust/emission generation system and fuels

The DPM samples were collected at the diesel laboratory at NIOSH Office of Mine Safety and Health research (OMSHR). The exhaust samples were collected using a single batch of neat corn-based fatty acid methyl ester (FAME) BD, acquired from Peter Cremer (Cincinnati, OH, NEXSOL BD-100) and single batch of petroleum-based ultralow sulfur diesel (ULSD) fuel, acquired from a local supplier. The BD fuel met ASTM D 6751 standard. The samples of BD and D a particulates were collected from the exhaust of a mechanically controlled, naturally-aspirated directly injected Isuzu C240 (Isuzu Motors Limited) diesel engine equipped with diesel oxidation catalytic converter (DOC, Lubrizol, New Market, ON). The specifications for the engine are given in Table S2. The engine was coupled to a water-cooled eddy-current dynamometer from SAJ (Pune, India, Model SE150). The engine was exercised over four steady-state operating conditions (Table S3). These four loads are part of the International Standards Organization for Standardization (ISO) 8-mode test cycle ISO 8178 C1 (ISO, 1996). The exhaust particles originating from the four different loads were collected and combined for performing the toxicity studies as described in this study.

DPM sample collection system

A high volume sampling system was developed to advance the methods of collecting representative samples of diesel particulates for toxicity analysis. This system allows for collecting nano-sized and ultrafine DPM aerosols in liquid media, therefore preserving to the highest possible level physical and chemical characteristics of sampled aerosols. Collecting and assaying particulates in water minimizes non-physiologic agglomeration, dissolution, and surface conditioning of particulates> and destruction of the particulate properties that can occur in filter collection, solvent extraction, or the preparation of collected material. The diesel particulate samples were collected from partial-flow dilution system (dekati, Model FPS-4000) using custom designed sampling system made with a version of a versatile aerosol concentrator enrichment system (VACES) (Khlystov et al., 2005; Kim et al., 2000; Sioutas et al., 1999) (see Fig. S1) and BioSampler® (SKC, Eighty Four, PA) (see Fig. S1). The VACES system was used to grow diesel aerosols by condensing deionized water on surface of those aerosols. The VACES system was operated with one condenser column, one virtual impactor, and one BioSampler®. The diluted exhaust with temperatures ranging between 295 K and 305 K (function of the engine operating condition) was flown above body of deionized water heated at ~315 K. The water was condensed on the DPM in a single chilled condenser column. The temperature of the coolant in the chiller was maintained at 268 K. As a result of the condensation process, the median diameter of the newly generated aerosol was ~5 μm. The enlarged aerosols were separated from the rest of flow using a virtual impactor (VI). The major VI flow of 55 l/min was maintained using mass flow

controller (Sierra Instruments, Model 850) and vacuum pump (Oerlikon Leybold, Model SOGEVAC SV25B). The minor VI flow of 5 l/min was maintained using critical orifice (BGI, Model SO1) and vacuum pump (Oerlikon Leybold, Model SOGEVAC SV25). The minor flow was directed through BioSampler®. Three three-hour samples were collected for each of four engine operating conditions (Table S3). The samples collected for each of the fuels were combined and further concentrated using a rotating evaporator, Eppendorf vacufuge (Hamburg, Germany). Such concentrated samples were either used directly or further diluted using sterile water for toxicity studies in mice. The particle suspensions were sonicated briefly using vibra cell (Sonics & Materials, CT, USA) before administering them to animals.

Total carbon content analyses

The aqueous mixtures solutions of BD and D exhaust particulates were applied directly to a pre-cleaned high purity, quartz filters (Pallflex 2500QAT-UP, Pallflex Inc., Putnam, CN) and analyzed by NIOSH Method 5040 (Birch, 1998, 2002, 2004; Birch and Cary, 1996; NMAM, 2003). The method is based on a thermal–optical analysis technique for organic and elemental carbon (OC and EC). The thermal–optical analyzer (Sunset Laboratories, Inc., Forest Grove, OR) has been described in detail previously (Birch and Cary, 1996; NMAM, 2003). In the typical application, air samples collected on quartz-fiber filters are analyzed. A 1.5 cm2 filter portion is removed for the analysis and OC–EC results (in $\mu g/cm^2$) for the portion are multiplied by the deposit area to calculate the OC–EC filter mass. In this study, entire sample volume (about 200 μL) of the PM mixture was analyzed in multiple aliquots. Aliquots of the aqueous mixtures of BD and D exhaust particles were applied directly to 1.5 cm^2 portions of pre-cleaned, high purity, quartz fiber filters (Pallflex 2500QAT-UP, Pallflex Inc., Putnam, CN) and the portions were analyzed. In addition, the inner vial wall was wiped with quartz media (1.5 cm^2 portion) after all the liquid was analyzed to recover any material clinging to wall. Results for the wiped sample and multiple aliquots were summed to give the total carbon (TC = OC + EC) in the sample.

Particulate aspiration

The bolus administration of BD or D particulates to C57BL/6 mice was performed via pharyngeal aspiration. Briefly, after anesthetization with a mixture of ketamine and xylazine (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), the mouse was placed on a board in a near vertical position and the animal's tongue extended with lined forceps. A suspension (approximately 60 μl) of BD or D particles (0, 9, and 18 μg/mouse of total carbon) prepared in United States Pharmacopeia (USP) grade sterile water was placed posterior on the tongue, which was held until the suspension was aspirated into the lungs. Control mice were administered water as a vehicle. The mice revived unassisted after approximately 30–40 min. All mice from the control (water), BD, and D groups survived this exposure procedure and exhibited no negative behavioral or health outcomes. The animals were weighed and sacrificed with intra-peritoneal injection of sodium pentobarbital (> 100 mg/kg) and exsanguinated, after 24 h, 7 days and 28 days following the pharyngeal aspiration. Five animals per study group were utilized for all in vivo assays. The dose of BD and D particulates employed in this study reflects concentrations within the exposure limits in coal mines and other occupations associated with use of diesel equipment (EPA, 2002). A

permissible exposure limit (PEL) of 160 μg/m^3 of TC was established by Mine Safety and Health Administration (MSHA) in 2008. More detailed explanation of human equivalent exposure at the dose employed can be found in Supplemental material.

Obtaining bronchoalveolar lavage (BAL)

Mice were weighed and sacrificed with intraperitoneal injection of sodium pentobarbital (> 100 mg/kg) and exsanguinated. The trachea was cannulated with a blunted 22 gauge needle, and BAL was performed using cold sterile USP grade Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) at a volume of 0.9 ml for first lavage (kept separate) and 1.0 ml for subsequent lavages. Approximately 5 ml of BAL fluid per mouse was collected in sterile centrifuge tubes. Pooled BAL cells for each individual mouse were washed in PBS by centrifugation (800 × g for 10 min at 4 °C). Cell-free first fractions of BAL aliquots were used immediately or stored at 4 °C for LDH assays, while the remainder of samples were frozen at −80 °C for protein and cytokine evaluations.

BAL cell counting and differentials

The degree of inflammatory response induced by pharyngeal aspiration of BD, and D particulates was estimated by quantitating total cells, alveolar macrophages (AMs), and polymorphonuclear leukocytes (PMNs) recovered by BAL. Cell counts were performed using an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer II with a 256C channelizer, Coulter Electronics, Hialeah, FL). AMs and PMNs were identified by their characteristic cell shape in cytospin preparations stained with Diffquick (Fisher Scientific, Pittsburgh, PA), and differential counts of BAL cells was carried out. At least 300 cells per slide were considered for each sample for this analysis.

Lung homogenate preparation

The whole mouse lung was separated from other tissues and weighed before being homogenized with a tissue tearer (model 985-370, Biospec Products Inc., Racine, WI) in PBS (pH 7.4) for 2 min. The homogenate suspension was aliquoted and frozen at −80 °C until processed.

Total protein and lactate dehydrogenase (LDH) activity

Measurement of total protein in the BAL and tissue homogenates was performed by a modified Bradford assay according to the manufacturer's instructions (BioRad, Hercules, CA) with bovine serum albumin as a standard. The activity of LDH was assayed spectrophotometrically by monitoring the reduction of nicotinamide adenine dinucleotide at 340 nm in the presence of lactate using Lactate Dehydrogenase Reagent Set (Pointe Scientific, Inc., Lincoln Park, MI).

Myeloperoxidase (MPO) activity

Inflammatory response in the lung of mice was assessed by measurement of myeloperoxidase (MPO) activity by Enzyme Linked Immunosorbent Assay (ELISA). The concentration of MPO in tissue homogenates was measured using a commercially available ELISA immunoassay kit (Cell Sciences, Canton, MA) with detection limit ranging from

1.02 to 250 ng/ml. Each measurement of MPO activity in tissue homogenates was assayed in at least triplicate and normalized to total protein content in tissue samples.

Levels of oxidative stress markers

Oxidative damage to the lung following administration of BD or D was evaluated by the presence of 4-hydroxynonenol (4-HNE) and protein carbonyls in tissue homogenates. 4-HNE, a byproduct of lipid peroxidation, was measured in lung homogenates by ELISA using the OxiSelect HNE-His adduct kit (Cell Biolabs, Inc, San Diego, CA). The quantity of oxidatively modified proteins as assessed by measurement of protein carbonyls in lung homogenates was determined using the Biocell PC ELISA kit (Northwest Life Science Specialties). Sensitivity of the assay is <0.1 nmol/mg protein.

Measurement of cytokines and chemokines using Bio-Plex

Cytokines and chemokines in the BALF and lung homogenates from mice exposed to BD and D particulates were analyzed using a Bio-Plex system (Bio-Rad, CA, USA). Using mouse cytokine group I panel 23-Plex assay kit, both BALF and lung homogenates were assayed for the following 23 cytokines and chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, INF-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. In addition, the lung homogenates were also assayed for: IL-15, IL-18, Basic-FGF, LIF, M-CSF, MIG, MIP-2, PDGF-BB, and VEGF, using Bio-Plex Pro mouse cytokine Th17 panel B group II panel 9-Plex assay kit. The 40-fold diluted aliquots of lung homogenates and BALF (50 μl taken as is) in each case were used for analyzing and estimating the concentrations of cytokines, chemokines and growth factors. The concentrations were calculated using Bio-Plex Manager 6.1 software (Bio-Rad, Tokyo) based on standard curves.

Dynamic light scattering studies

Size of BD and D particulates was determined by dynamic light scattering using Nanotrac 252 (Microtrac, Montgomeryville, PA). The particle sizes of BD and D combustion exhaust particulates before and after sonication with a probe sonicator (Branson Sonifier 450, 10 W continuous outputs) were determined.

Imaging using transmission electron microscopy (TEM)

For AM ultra-structure analysis by TEM, cell pellets of BAL cells were fixed in Karnovsky's fixative (2.5% gluteraldehyde and 3.5% paraformaldehyde in 0.1 M Sodium Cacodylate Buffer) then post-fixed in 2% Osmium Tetroxide for 1 h. The samples were then dehydrated and embedded in epon. The blocks were thin sectioned and stained with uranyl acetate and lead citrate. The images were photographed on a JEOL 1220 transmission electron microscope.

Lung histopathology

Lung tissues were harvested at 1, 7, and 28 days post exposure to vehicle or BD and D particulates and inflation fixed in situ with 4% paraformaldehyde at constant pressure of 10 cm H_2O for 10 min with the chest cavity open. Coronal sections were cut from the lungs.

The lungs were embedded in paraffin and sectioned at a thickness of 5 μm with an HM 320 rotary microtome (Carl Zeiss, Thornwood, NY). Thus prepared sections were stained with hematoxylin and eosin (H&E), and histological evaluation was performed to examine BD or D induced pathological severity. Sample identification was coded to ensure unbiased evaluation.

Statistical analysis

Statistical analysis was performed using SigmaPlot 11.0 (San Jose, CA). Data are presented as mean ± standard error of the mean (SEM). Statistical significance of the observed outcomes i.e., treatment related differences was analyzed by Student's t-test, analysis of variance (ANOVA), Dunnett's and Bonferroni tests as appropriate. A p value less than 0.05 was considered statistically significant.

Results

Analysis of BD and D particulates

Total carbon (TC) content is often used as a surrogate for DPM, since it can be measured accurately at low concentrations. Samples from BD and D particulates were analyzed for OC and EC, with TC (representing over 80% of DPM) as a measure of PM. The OC content in the BD exhaust was higher compared to D samples. While BD particulates were composed of 47% and 53% OC and EC, respectively, the D particles had 32% OC and 68% EC.

Further, dynamic light scattering (DLS) measurements were employed to determine the hydrodynamic diameter of BD and D particulates (Supplementary Fig. S2). Prior to sonication, both concentrated BD and D exhaust particles exhibited sizes of ~720 nm and larger (data not shown). However, after sonication BD and D particulates had a diameter of ~216 nm and ~312 nm, respectively, as estimated by DLS, indicating further dispersion by sonication.

Enhanced recruitment of immune cells in mice exposed to BD particulates

In order to understand the recruitment of phagocytic immune cells upon neat BD and D particulate exposure in the lungs, the cell profiles in BAL fluid were determined 1, 7, and 28 days following pharyngeal aspiration. A dose dependent increase in total cells was observed on days 1 and 7 post exposure with BD (265.0 ± 60.0 and 532.0 ± 82.0% after 24 h at 9 μg and 18 μg TC vs control mice) and D (230.0 ± 12.0 and 322.0 ± 32.0% after 24 h at 9 μg and 18 μg TC vs control mice). However, in both cases on day 28 post exposure, the levels of total cells returned to control levels. An accumulation of PMNs/leukocytes on day 1 post exposure was observed after pharyngeal aspiration with BD and D particulates (Fig. 1c), followed by an influx of AMs (311.0 ± 54.0 and 167.0 ± 22.0% compared to control, respectively, Fig. 1b) peaking on day 7. In comparison, exposure to BD particulates induced a significant inflammatory response with a maximum PMN influx (1500.8 ± 232.8 folds vs control) occurring on day 1 post exposure. While the PMN influx on day 7 post BD particulate exposure was still significantly elevated (307.16 ± 67.89 folds vs control), a substantial decrease in PMNs (~17.2 ± 4.0 folds vs 573.7 ± 67.5 folds on day 1) was

observed after D exposure (Fig. 1c). By day 28 post BD and D exposure PMNs in BAL fluid decreased and AMs returned to control levels; however, the total number of PMNs still remained elevated to a similar extent (~4 fold) as compared to control. These results indicate that the magnitude of phagocytic cell responses is significantly higher in animals exposed to BD particulates.

Increased permeability of lung upon BD particle exposure

Exposure to BD and D particulates caused increased lung permeability, as evidenced by elevated total protein in the BAL fluid (Fig. 2a). BD exposure (18 μg of TC/mouse) induced a percent increase of 204.7 ± 6.9, 125.5 ± 6.3, and 100.4 ± 3.0 in BAL protein on days 1, 7, and 28 post exposure, respectively (Fig. 2a). In comparison, D particulates exposure at the same concentration only resulted in elevated protein levels (129.9 ± 2.5% increase vs control) after day 1 and they returned to control levels on days 7 and 28 post exposure. A maximal increase found after 24 h post exposure with BD particulates clearly indicates increased lung permeability as compared to D (Fig. 2a).

Pulmonary damage and inflammatory responses following BD or D particulate exposures

The degree of pulmonary cytotoxicity and inflammatory responses elicited by BD or D exhaust particle exposures were assessed by LDH and MPO activity in the lung homogenates (Figs. 2b, c). LDH levels were significantly elevated upon BD particulate exposure (18 μg/mouse; 129.3 ± 4.5% change vs control mice) on days 1 and 7 post exposure (Fig. 2b). On day 28 post BD exposure, LDH levels still remained significantly (149.0 ± 4.2%) elevated as compared to control mice. The release of LDH in response to D particulates was significantly lower than with BD. For example, the LDH levels peaked on day 28 post exposure in the case of BD particulates (149.0 ± 4.2%), a maximum increase (114.6 ± 3.9% increase vs control mice) in LDH activity was found at 7 days post D exposure.

An increase of upto 115.5 ± 3.7% and 135.9 ± 2.9% in MPO activity compared to control was found in lungs after pharyngeal aspiration with 9 μg and 18 μg of TC/mouse of BD particulates, respectively. In contrast, D particulate exposure either resulted in a slight decrease or no change in MPO activity compared to control mice (Fig. 2c, MPO – red vs blue bars). Overall, these results clearly indicate that BD exhaust is more potent in inducing acute pulmonary cell damage as well as inflammatory response compared to D.

Oxidative stress in the lungs is increased upon exposure to BD particulates

Oxidative damage, assessed by the levels of oxidatively modified proteins (protein carbonyls) and 4-hydroxynonenal (4-HNE), marker of lipid-peroxidation, in the lungs of mice exposed to BD and D particulates is presented in Fig. 3. Most significant and sustained increase in the accumulation levels of protein carbonyls (1.26, 1.32, 1.44 fold vs control) was found on 1, 7, and 28 days post exposure at the highest concentration (18 μg of TC) of BD particulates tested, respectively. In contrast, the levels of protein carbonyls in the lungs of mice exposed to D particulates (9 and 18 μg of TC/mouse) either decreased or remained similar to controls (Fig. 3a). In addition, an increase in the amount of 4-HNE was observed on 28 days (1.43 fold vs control) post exposure to BD particulate exposure (Fig. 3b).

However, the levels of 4-HNE in the lungs of D particulate exposed mice either slightly decreased or remained similar to control levels. Overall, the magnitude of oxidative damage in the lungs is more pronounced in mice treated with BD exhaust.

Cytokine, chemokine and growth factors following BD or D exhaust exposures in mice

The release of inflammatory cytokines, chemokines and growth factors was used as a marker of the pro- or anti-inflammatory responses in the mouse lungs exposed to BD and D particulates. The data (represented as the mean ± SEM) obtained at a dose (18 μg TC/mouse) investigated for the two exposure groups (BD and D) after 24 h is shown in Fig. 4, and a detailed list of changes at each time point is presented in Supplementary Tables S4 and S5.

Responses in cytokines

The cytokine levels were found to be elevated in BAL fluid and in lung tissue homogenates of mice after BD and D 24 h post exposure (Fig. 4a). A total of seven cytokines out of 16 tested in the tissue homogenates and six out of 14 cytokines tested in BAL fluid exhibited significant increase (> 1.5 folds vs control) upon exposure to BD. The changes found in the cytokine levels from tissue homogenates were in the order: IL-1α > IL-1β > IL-6 > IL-12p40 > IL-12p70 > IL-4 NIFNγ and from BAL fluid were: IL-6 > IL-1α > IL-12p40 > IL-5 NIL-13 > IL-4 (Fig. 4a). However, the changes in IL-1β, IL-12p70, and IFNγ levels in lungs and IL-5 and IL-13 levels in BAL were only found in mice exposed to BD particulates (Fig. 7). At the later time points of BD particulate exposure (7 and 28 days), only IL-1α, and IL-12p40 were elevated (Supplementary Fig. S3a).

Upon 24 h post D particulate exposure, only IL-1α was increased in lungs. In contrast, the changes found in BAL fluid were different. Four out of 14 cytokines measured, IL-1α, IL-5, IL-6, and IL-12p40, were significantly up-regulated in BAL fluid compared to controls (Fig. 4a). However, after 7 and 28 days post D particulate exposure, no detectable increase in cytokines was found either in lungs or BAL fluid (Supplementary Table S4). Overall the cytokine levels upon BD particulate exposure were more prominent both in BAL fluid and in lungs as compared to D.

Responses in chemokines/growth factors

Similar to cytokines, the changes in the levels of 16 different chemokines and growth factors were monitored following BD and D particulate exposures in mice at 24 h (Fig. 4b), and at 7 and 28 days (Supplementary Fig. S3).

After 24 h post exposure, a total of 11 chemokines/growth factors in lungs and 7 in BAL fluid were significantly up-regulated in mice after pharyngeal aspiration with BD particulates. During acute phase of BD particulate exposure the changes in lungs were as follows: MIP-2 > G-CSF > KC > MIP-1α > MCP-1 > MIP-1β > MIG > RANTES > M-CSF > LIF > Eotaxin, and in BAL fluid were: G-CSF > RANTES > MIP-1α > KC > MCP-1 > TNF-α > MIP-1β. Similarly upon D exposure, an upregulation of 7 chemokines/growth factors in lungs: MIP-2 > KC > RANTES > G-CSF > MIP-1α > M-CSF > LIF, and increase in 6 in the BAL fluid: G-CSF > RANTES > KC > MIP-1β > MCP-1 > MIP-1α.

The accumulation of chemokines/growth factors, in particular MIP-2, KC, RANTES, MIP-1α and MIG in lungs was still higher (> 1.2 folds vs control) after 7 and 28 days post BD particulate exposure (Fig. S3, Supplementary Table S5). The changes found in the lungs on day 7 post BD exposure were as follows: MIP-2 > KC ≥ RANTES > MIP-1α > MIG. On day 28 post BD particulate exposure, a significant increase (> 1.5 folds vs control) in only PGDF-BB was observed. In addition to PGDF-BB, a slight increase (~1.2 folds vs control) in MIG, MIP-1α and MIP-2 was also detected at 28 days post BD particulate exposure (Supplementary Table S5). However, upon D exposure, inflammatory chemokines and growth factors (except for MIP-2 and RANTES) either remained unchanged or slightly decreased compared to control (Supplementary Fig. S3 and Table S5). On day 28 post BD and D particulate exposures, no significant changes in any of the chemokines/growth factors was found in BAL fluid.

Transmission electron microscopy of alveolar macrophages

The TEM images of AMs exposed to BD and D particulates collected at different end points, along with their control, are shown in Fig. 5. BD and D particulates are clearly seen as electronic dense inclusions in cytoplasm, inside small vesicular lysosomes of AMs after 24 h post exposure (Fig. 5a). In contrast, after 7 and 28 days post BD particulate exposure, PM was localized to specialized cytoplasmic inclusions inside AMs (Figs. 5b, c). These spherical structures were 1–2 μm in size, resembling lipid droplets or foam organized cells (Reue, 2011). However, such structures were not seen in either the control group or in mice exposed to D particulates.

Pathohistological evaluation of lung sections

Hematoxylin and eosin (H&E) stained sections of the lungs in the control mice revealed normal histology of conductive and respiratory airways (data not shown). Severe endobronchial, peribronchial, and perivascular inflammatory infiltrate, composed mainly of neutrophils, was observed in the lung sections of mice 24 h post BD and D particulate exposure. However, the intensity of acute inflammation was higher in BD group, where focal destruction of bronchiolar epithelium was seen (Fig. 6, 24 h — red arrow). Pigment laden macrophages, containing brown-black material were also present in high numbers in both BD and D exposed mice. A similar pattern of histological alterations was also found after 7 days post exposure with BD and D particulates albeit to a moderate level. The inflammatory infiltrate at this end point was predominantly composed of lymphocytes (Fig. 6, 7 d). At 28 days post exposure, a mild chronic inflammation in the form of peribronchial lymphocytic infiltrate was only observed in lung sections of mice exposed to BD particulates. Numerous pigment laden macrophages were also focally present in BD group. In contrast, no significant inflammation was seen in mice after 28 days post exposure to D particulates (Fig. 6, 28 d). Overall the intensity of pulmonary inflammation was higher in BD group compared to D group.

Discussion

Biodiesel is an oxygenated fuel that is derived from animal fats and vegetable oils. It is considered as the sole alternative fuel that is cheaper and can be efficiently used in any

original diesel engines, releasing the same amount of power as petroleum diesel (Balat and Balat, 2010). It is assumed that health effects of biodiesel to humans are certainly better as it provides substantial reduction to exhaust emissions by reducing greenhouse effects, black smoke, air toxins similar to carbon monoxide, unburned hydrocarbons and any PM. As the use of BD replaces fossil fuels, it becomes important to establish the biological responses and health effects that stem from BD particulates.

The pulmonary airways form the first line of defense against airborne irritants, pollutants, and other infectious agents. Ineffective clearance and longer retention times of impacted particles at the site of exposure is an important factor leading to adverse health effects. In addition to providing a mechanical barrier, airway epithelium also produces chemokines and cytokines that recruit and activate phagocytic cells to clear inhaled particles/agents and damaged cells. An increase in phagocytic inflammatory cells and elevated protein levels in BAL fluid upon exposure to BD and D PM was reported previously (Brito et al., 2010; Finch et al., 2002; Hemmingsen et al., 2011; Tzamkiozis et al., 2010). This is also evident in our studies, where an enhanced recruitment of phagocytic cells in BAL fluid was observed after 24 h, and 7 d post exposure following BD and D (Fig. 1). Bolus administration of BD and D particulates via pharyngeal aspiration in mice induced accumulation of PMNs/leukocytes on day 1 post exposure, followed by an influx of AMs peaking on day 7 (Fig. 1). This enhanced recruitment of AMs at 7 days post exposure, required to clear injured/dying neutrophils, is further validated by the presence/incidence of particle-containing AMs in BAL (Fig. 5) as well as pigment-laden macrophages in pulmonary tissue (Fig. 6). This is also in agreement with previous studies reporting increased number of macrophages and presence of particle-containing AMs in lungs upon subchronic inhalation exposures to soybean based BD (Finch et al., 2002). While no significant inflammation was observed after 28 d post exposure to D particulates, our study indicates presence of numerous pigment laden macrophages with non-resolved mild chronic inflammation upon BD exposure. These data demonstrate an impaired clearance and/or prolonged retention of PM, which can further lead to respiratory maladies. In fact, a number of long-term adverse effects including exacerbation of pre-existing lung disease, respiratory infections, and cancer (Sawyer et al., 2010; Silverman et al., 2012) were shown to be associated with PM exposures.

Exposure to DPM has been shown to elicit inflammatory pulmonary responses, activation of cellular signaling pathways and release of pro-inflammatory mediators (Bonvallot et al., 2001; Holder et al., 2007; Totlandsdal et al., 2012). To date, limited studies examining release of inflammatory mediators in response to BD particulate exposures are available. To the best of our knowledge, this is the first study reporting broad assessment of inflammatory mediators in mouse lungs after BD and D exposure. The majority of inflammatory mediators up-regulated in BAL fluid, including IL-6, KC, G-CSF, and RANTES (Fig. 4) upon BD and D exposures are consistent with the recruitment of phagocytic cells such as neutrophils, and macrophages (Fig. 1), during acute phase of inflammation. Most importantly, overexpression of IL-4 and IL-13, seen only upon BD particulate exposure, is associated with allergic inflammation and induction of type 2 T helper cell (T_h2) responses (Venkayya et al., 2002; Wills-Karp et al., 1998). The elevated levels of T-cell cytokines IL-4, IL-5, IL-6, and IL-13 seen in this study upon BD exposure are also paralleled by the accelerated accumulation of lymphocytic infiltrates observed in lungs of mice up to 28 days post BD

exposure (Fig. 6). Both IL-4 and IL-13 have been shown to enhance IL-12p70 production by dendritic cells and monocytes/macrophages (Hochrein et al., 2000; Kalinski et al., 2000; Ma and Trinchieri, 2001). This is consistent in our studies demonstrating an elevated level of IL-12p70 in lungs of mice exposed to BD particulates and not D particulates (Fig. 7). The up-regulation in IFN-γ seen upon BD particulate exposure (Supplementary Table S4) further validates the accumulation of IL-12p70, an active form of IL-12, that stimulates production of IFN-γ thus promoting the differentiation of T_h0 into T_h1 cells (Hamza et al., 2010). Additionally, accumulation of TNF-α and IL-1β, pro-inflammatory cytokines mainly produced by activated macrophages, was found only in mice exposed to BD particulates (Fig. 7, Supplementary Table S4). These two cytokines, acting synergistically (Dinarello, 2000), are implicated in the pathogenesis of many acute and chronic non-infectious/infectious inflammatory respiratory diseases. Further, an incremental change in the release of cytokines, notably in the levels IL-8, IL-6, G-CSF, RANTES, and MCP-1, was reported upon stimulation of epithelial cells (ECs) with IL-1β/TNF-α (McDougall et al., 2008). This is strongly supported by our studies where KC and MIP-2 (homologues of human IL-8), IL-6, G-CSF, RANTES and MCP-1 in addition to IL-1β/TNF-α are also up-regulated to a higher extent in BD particulates exposed mice (Fig. 7, Supplementary Tables S4 & S5). BD particulate exposure also resulted in accumulation of MIG (at 1, 7 and 28 days), a T-cell chemoattractant induced by IFN-γ. Especially the prolonged accumulations observed in IL-1α, MIP-1α, MIP-2 and MIG, all produced by macrophages, are further supported by persistent presence of particle containing macrophages in the BAL fluid and pigment laden macrophages in lungs upon BD particulate exposure compared to D particulates (Figs. 5–6). These findings are consistent with the hypothesis that BD particulates, composed of (non-)oxidized unsaturated fatty acids and its combustion products, are capable of triggering long-term adverse effects compared to D particulates. These studies clearly indicate that BD particulates can potentiate distinct and prolonged inflammatory responses, as evidenced by significant increase in the level of peribronchial and perivascular lymphocytic infiltrate – a hallmark of inflammatory response – compared to D particulates.

Results from our study indicate increased organic carbon in BD particulates compared to D. These results are consistent with previous published studies showing that combustion of BD increases soluble organic fraction (SOF) of the exhaust (Graboski et al., 2003). The increase in the organic fraction was correlated with its oxidative potential (Biswas et al., 2009). The accelerated oxidative stress detected in this study, assessed by accumulation of 4-HNE and protein carbonyls in the lungs of mice exposed to BD particulates, is probably due to high level of organic matter (Fig. 3). While it becomes difficult to attribute these changes to a distinct chemical species in BD particulates, increased soluble organic content, including aldehyde-like compounds as well as fatty acid esters, and the presence of transition metals in BD exhaust emissions have been shown previously to induce oxidative stress (Bonvallot et al., 2001; Liu et al., 2009; Swanson et al., 2007). The combustion of BD is shown to release unique chemical compounds, such as fragments of methylated fatty acids and FAMEs themselves due to their incomplete combustion (Ratcliff et al., 2010; Tsai et al., 2010). Considering that BD fuel is mainly composed of poly-unsaturated fatty acids (PUFA), its emissions are more prone to peroxidation. Based on this, we speculate that the accelerated oxidative stress and lipid-peroxidation upon BD exposure could further trigger accumulation

of oxidized lipids leading to formation of foam cells similar to those seen in early events of atherosclerosis (Kruth, 2001; Webb and Moore, 2007). It is well established that excessive amounts of unsaturated and saturated free fatty acids can trigger formation of lipid droplets in macrophages/monocytes whereby their esterification into triacylglycerols or cholesterol esters prevents lipotoxicity (Blouin et al., 2010; den Hartigh et al., 2010; Melo et al., 2011; Robinson et al., 2009; Scifres et al., 2011). In addition to acting as storage lipid reservoirs, lipid droplets – due to their hydrophobic nature – can also accumulate different lipophilic xenobiotics and drugs, including aliphatic and polyaromatic hydrocarbons from the combustion exhaust (Murphy et al., 2008). In fact, our TEM studies suggested sequestration and localization of BD PM to spherical lipid organelles ranging between 1 and 2 μm in diameter in macrophages, mimicking lipid droplets or foam cells. Based on this, we hypothesize that the formation of lipid droplets, specific to BD particulate exposures, is facilitated by unsaturated fatty acids present in BD (Melo et al., 2011). Assuming that products of BD combustion, e.g., PAHs, can exert toxic effects in cells, their sequestration by lipid droplets might enhance their adverse effects via slow release into intracellular compartments (Fujimoto et al., 2008). Further, slow release of the accumulated oxidized lipids – in their free or esterified form – from their storage reservoirs and their interactions with critical molecular targets in cells may contribute to the overall toxicity of BD. Previous studies have shown that accumulation of oxidized lipids in macrophages induces marked changes of their phenotype (toward pro-inflammatory M1 phenotype and/or a newly identified Nrf2-dependent Mox phenotype) (Adamson and Leitinger, 2011), associated with their modified recognition and clearance (Arroyo et al., 2002; Kagan et al., 2002).

In conclusion, our studies indicate pronounced adverse effects induced by combustion emissions from neat BD in relation to petroleum D fuel, as characterized by enhanced recruitment of BAL inflammatory cells, increase in tissue damage and oxidative stress, and enhanced release of inflammatory mediators. Prolonged retention and impaired clearance of particulates was found in mice exposed to BD particulates. Moreover, the presence of pigment laden macrophages in lung tissue and prolonged retention of PM in BAL macrophages with in lipid droplets or foam cells at 28 days post BD particulate exposure clearly warrants further investigation. Future studies focusing on the detailed analysis of combustion products from BD as well as specific mechanism of interactions of these emissions in relation to the observed inflammatory responses and impaired clearance is needed to better explain adverse outcomes of BD use on human health. The consequences of lower particulate mass emissions and indicated higher toxicity associated with BD were not investigated as part of this study. However, studies evaluating the adverse outcomes upon inhalation exposure to BD and D combustion exhaust are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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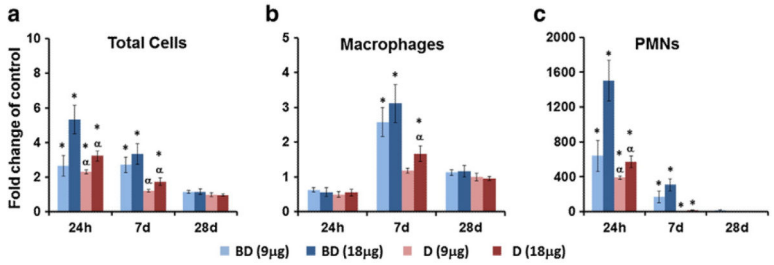

Fig. 1.

Cell profile in BAL fluids of C57BL/6 mice after exposure to neat BD and D particulates via pharyngeal aspiration. (a) Total cells; (b) alveolar macrophages (AMs); and (c) polymorphonuclear leukocytes (PMNs). Light and dark blue columns — exposure with 9 μg and 18 μg of total carbon/mouse of BD particulates; Light and dark red columns — exposure with 9 μg and 18 μg of total carbon/mouse of D particulates. Mice were exposed via pharyngeal aspiration to doses indicated. Average control values for total cells (cells, $\times 10^3$) on day 1, 7 or 28 post exposure were 430.28 ± 31.85, 468.75 ± 61.93 or 705.41 ± 69.59, respectively. Average control values for AMs (cells, $\times 10^3$) on day 1, 7 or 28 post exposure were 427.66 ± 31.95, 466.10 ± 60.43 or 699.56 ± 66.69, respectively. Animals were sacrificed 1, 7, and 28 days post exposure. Average control (water-treated mice) values for PMNs (cells, $\times 10^3$) on day 1, 7 or 28 post exposure were 1.67 ± 0.87, 0.97 ± 0.61 or 1.79 ± 1.46, respectively. Means ± SEM (n = 5 mice per group). *$p < 0.05$ vs. control mice, $^{\alpha}p < 0.05$ vs. mice exposed to BD particulates.

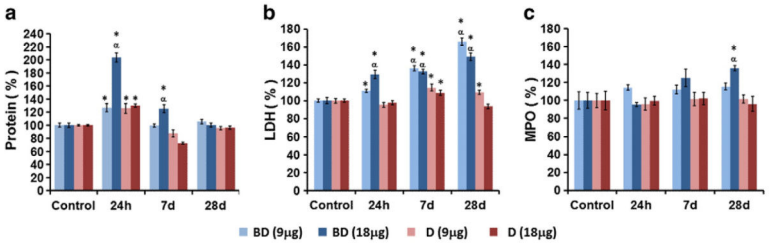

Fig. 2.

Tissue damage and inflammation as evaluated by changes in total protein, the activity of LDH, and MPO. Changes in (a) total protein content, (b) LDH and (c) MPO levels in the lung homogenates of mice sacrificed on days 1, 7 and 28 after pharyngeal aspiration with BD or D particulates (9 μg and 18 μg deposited dose of total carbon). The data is represented as percent compared to control in all cases. *$p < 0.05$ vs. control (water treated) mice. $^{\alpha}p < 0.05$ vs. D particulates exposed mice. Means ± SEM (n = 5 mice per group).

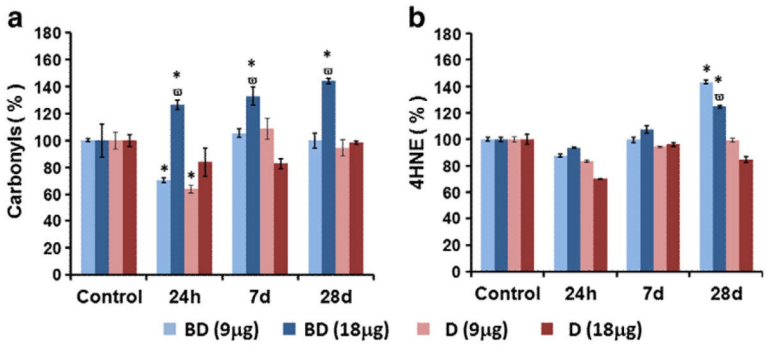

Fig. 3.

Biomarkers of oxidative stress in the lungs after days 1, 7, and 28 post exposures of C57BL/6 mice following pharyngeal aspiration of BD and D particulates. The levels of (a) 4-hydroxynonenal (4-HNE), and (b) protein carbonyls assessed in the lungs without and at various concentrations (9 μg and 18 μg of TC/mouse) of BD or D particulates. Blue columns correspond to the exposure with BD exhaust; and red columns correspond to exposure with D particulates. Means ± SEM (n = 5 mice per group). *$p < 0.05$ vs. control (water treated) mice. $^{\varpi}p < 0.05$ vs. mice exposed to D particulates.

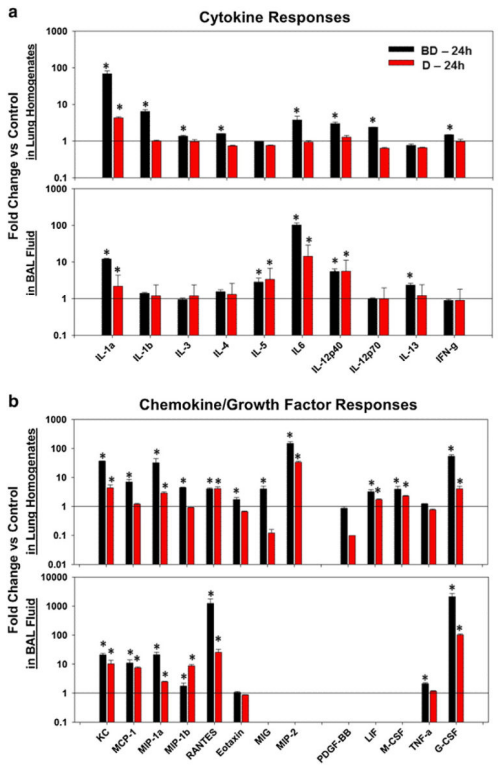

Fig. 4.

Differential responses in cytokines, chemokines and growth factors upon exposure to BD and D particulates. Semi-logarithmic plot of the levels of inflammatory cytokines (a), chemokines and growth factors (b) in the lung homogenates and BAL fluid of C57BL/6 mice (n = 5) following aspiration of 18 μg/mouse of total carbon of BD or D particulates. These measurements were performed using Bio-rad 23-plex and 9-plex mouse assay kits, composed of a combination of pro- and anti-inflammatory cytokine along with a sub set of chemokine's and growth factors. The data are presented as logarithm of means ± SEM of fold increase compared to controls in each case. *$p < 0.05$ increase compared to control (water treated) mice.

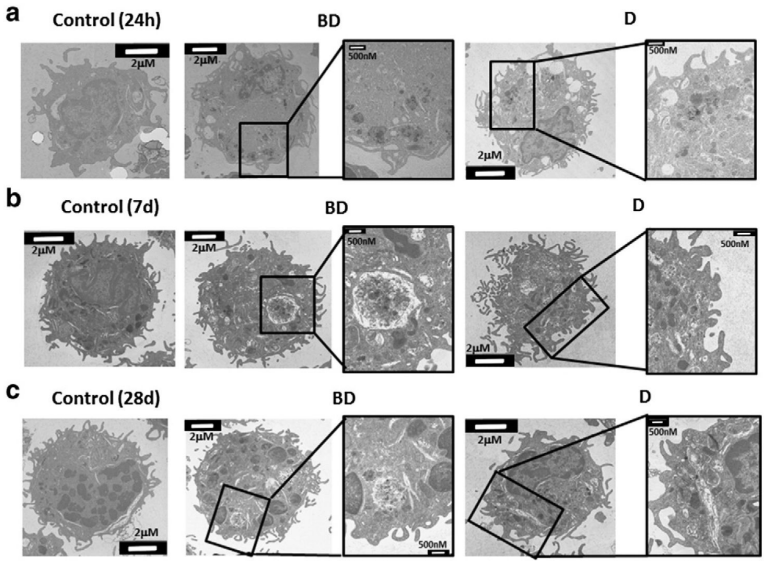

Fig. 5.

TEM micrographs of alveolar macrophages from BAL fluid of mice exposed to BD and D particulates. Alveolar macrophages in neat BD and D particulates exposed lungs using TEM after (a) day 1, (b) day 7, and (c) day 28 post exposure via pharyngeal aspiration.

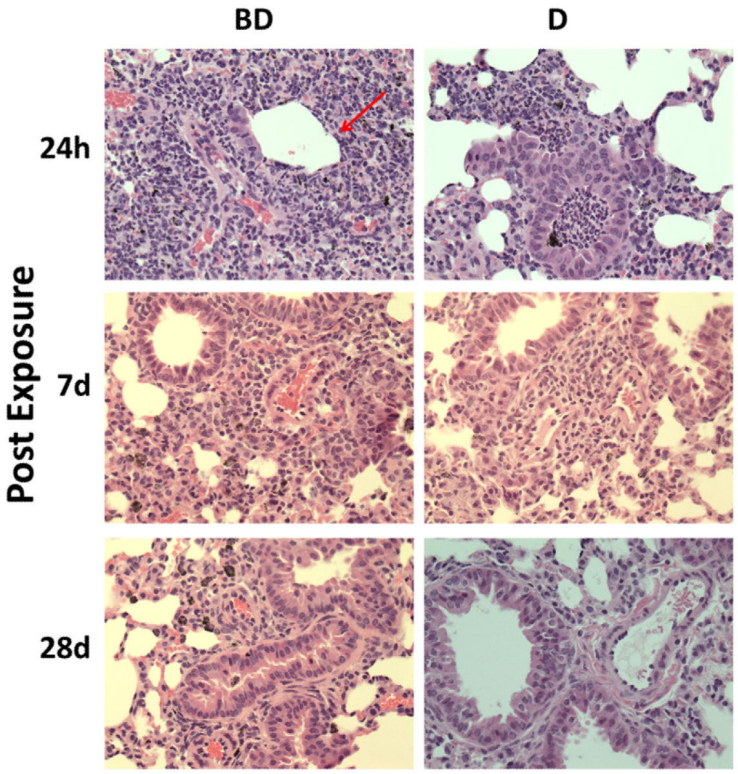

Fig. 6.

Lung histology 24 h, 7 and 28 days post exposure with BD and D particulates. All sections were stained with H&E and the images were taken at 400× magnification. At 24 h post exposure, a severe acute endobronchial, peribronchial and perivascular inflammation was more prominent in BD group, with focal destruction of bronchial epithelium (arrow). At 7 and 28 days post exposure, a moderate and mild chronic peribronchial and perivascular inflammation was more prominent in lung sections, respectively of mice treated with BD particulates. Numerous pigment-laden histiocytes are also present.

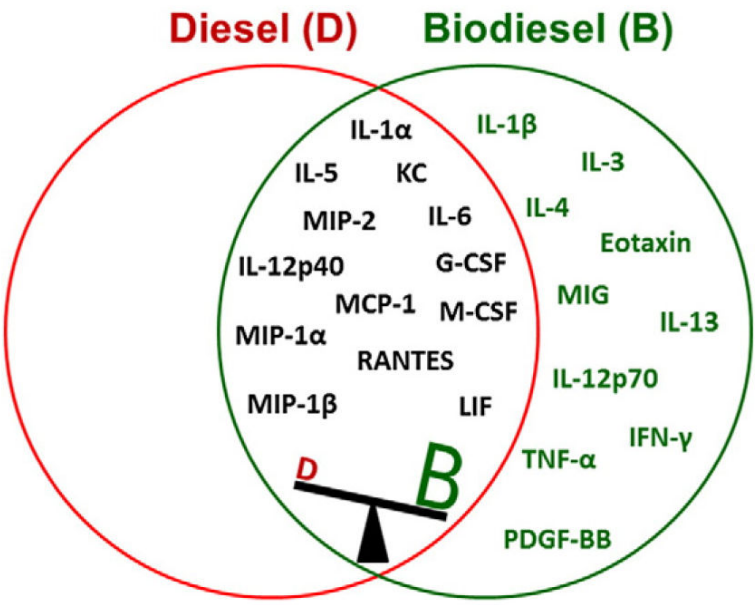

Fig. 7.

A Venn diagram depicting the differential responses in inflammatory mediators upon neat Biodiesel (BD) and Diesel (D) particulate exposures. A set of 32 inflammatory mediators, including cytokines, chemokines and growth factors was assessed in the BAL and pulmonary tissue of mice exposed to BD and D particulates. The responses common to both groups (BD and D) are colored in black and those only seen after BD particulate exposure are colored in green.