Published in final edited form as:
J Toxicol Environ Health A. 2012 ; 75(24): 1456–1470. doi:10.1080/15287394.2012.722186.

Chronic obstructive pulmonary disease patients have greater systemic responsiveness to *ex vivo* stimulation with swine dust extract and its components versus healthy volunteers

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Abstract

Chronic obstructive pulmonary disease (COPD) is characterized by an airway and systemic inflammatory response. Bioaerosols/organic dusts are important agricultural pollutants that may lead to COPD. These environments are complex containing a rich source of various microbial components. The objective of this study was to determine whether individuals with COPD have enhanced systemic responsiveness to settled swine facility organic dust extract (ODE) or its main pathogenic components (peptidoglycan [PGN], lipopolysaccharide [LPS]) versus healthy volunteers. A modified whole blood assay (WBA) that included occupational levels of ODE and concentrations of LPS and PGN found in ODE was used to determine systemic responsiveness (mediator release), and sputum inflammatory markers were measured to explore for systemic and airway associations. Sputum samples were evaluated for cell counts, and TNF-α, IL-8/CXCL8, IL-6, and IL-10. *Ex vivo* whole blood stimulation with ODE, LPS, and PGN each resulted in significant mediator release in all subjects, the highest occurring with ODE; PGN resulted in significantly enhanced TNF-α and IL-8 as compared to LPS. COPD subjects demonstrated greater systemic responsiveness using the modified WBA versus healthy controls. Within COPD subjects, blood baseline TNF-α, IL-8, and IL-10 and ODE, PGN, and LPS-stimulated IL-8 levels significantly correlated with lung function. In conclusion, dust-induced mediator release was robust, and PGN, in part, resembled dust-induced mediator release. Subjects with COPD demonstrated increased mediator release following *ex vivo* whole blood stimulation with bioaerosol components suggesting that circulating blood cells in COPD subjects may be primed to respond greater to microbial/inflammatory insult.

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Keywords

Lipopolysaccharide; Peptidoglycan; COPD; Farm; Dust; Cytokine; Whole blood assay; sputum; swine; systemic inflammation

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation and airway inflammation leading to accelerated lung function decline (Donaldson et al. 2005). Exposures to inhaled substances such as cigarette smoke and air pollutants play an important putative role in the lung inflammatory process leading to COPD and exacerbation of COPD (Bhalla et al. 2009). Bioaerosols in many occupational environments, especially agricultural settings, are important air pollutants that are recognized to be an independent risk factor for COPD development (Eduard et al. 2009; Spurzem et al. 2002). These bioaerosols or organic dusts are complex and diverse mixtures containing rich sources of microbial cell wall components from Gram-positive and Gram-negative bacteria, which are also capable of activating innate immune inflammatory pathways postulated to be important in the development of airways disease (Kline et al. 2004; Sarir et al. 2008). Whereas lipopolysaccharide (LPS) is one common inflammatory toxin present in the bioaerosols (Cleave et al. 2010; Cormier et al. 2000; Poole et al. 2007, 2008, 2009; Rask-Andersen et al. 1989; Romberger et al. 2002; Vogelzang et al. 2000), it may not be the prime inflammatory exposure agent in all occupational settings, particularly large animal farming environments where peptidoglycans (PGN) as derived from Gram-positive bacteria (i.e. 85% of total cell wall) and to lesser degree Gram-negative bacteria (i.e. 5% of cell wall), are also highly prevalent (Burch et al. 2010; Nehme et al. 2008; Poole et al. 2008, 2010). In addition, fungal fragments and strict anaerobic microorganisms such as arachaebacteria, have also recently been reported in these environmental settings (Nehme et al. 2008, 2009). Exposure to these complex mixtures of organic dusts and/or specific individual microbial cell wall components may be important in the susceptibility of an individual to develop airway disease.

In individuals with COPD, there is increasing evidence for systemic inflammatory consequences such as skeletal muscle dysfunction (Anonymous 1999), cardiovascular disease (Man et al. 2012), osteoporosis (Graat-Verboom et al. 2012), and diabetes (Rana et al. 2004). Moreover, subjects with COPD demonstrate raised markers of systemic oxidative stress, activation of circulating blood cells, and increased plasma levels of proinflammatory mediators (Aldonyte et al. 2003; Papaioannou et al. 2010; Schols et al. 1996; Sinden and Stockley 2010). In addition, circulating monocytes from individuals with COPD produce more TNF-α when stimulated with lipopolysaccharide (LPS) than those from healthy controls (de Godoy et al. 1996). Interestingly, Lambert et al. also demonstrated that LPS-induced tumor necrosis factor (TNF)-α release in whole blood has been associated with farmers reporting chronic bronchitis, but was not associated with lung function. Other studies demonstrated that *ex vivo* LPS-induced release of interleukin (IL)-10 and TNF-α in whole blood reflects high occupational endotoxin exposure in exposed workers (Cleave et al. 2010; Smit et al. 2009). However, there are few studies assessing the role of other key bioaerosols in an *ex vivo* assays such as the whole blood assay (WBA), which might be

important in order to gain a more complete understanding of the systemic response in individuals with COPD.

The aim of this study was to determine whether individuals with COPD display enhanced WBA responsiveness to large animal (swine) facility organic dust extract (ODE) or its main components (PGN, LPS) versus healthy volunteers. A modified WBA that included ODE, LPS and PGN was used to assess WBA responsiveness *ex vivo*, and sputum inflammatory markers were measured to explore for associations between systemic and airway responses. Because Poole's 2008 work demonstrated that PGN appears to be a critical factor in ODE-induced cell responses, it was hypothesized that PGN stimulation would closely resemble ODE-stimulated responses. It was also postulated that WBA responsiveness would differ between healthy volunteer and subjects with COPD, which would favor use of the WBA as a systemic biomarker. In this study, healthy control adult subjects were compared to subjects with COPD, and the relationships between lung function, sputum inflammatory markers and WBA responsiveness were assessed.

METHODS

Study Design

Two groups of adult subjects, healthy control and COPD, were recruited and informed consent was obtained prior to enrollment after approval from the institutional review boards at both enrollment sites (Omaha Veterans Administration and University of Nebraska Medical Center). The study was registered on clinicaltrials.gov: #NCT00871637. The inclusion criteria for all subjects were age between 50 and 75 years to represent the patient population typically affected with COPD, and study was designed such that control subjects would be similar in age range to subjects with COPD to account for potential differences associated with aging. All subjects were included if deemed medically stable to participate in an induced sputum protocol by medical history and physical examination. Healthy subjects were non-smokers (defined as having smoked less than 100 cigarettes in their life-time), and demonstrated a pre-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) [FEV_1/FVC ratio] ≥ 70% and FEV_1 ≥ 80%. COPD subjects were enrolled if demonstrated a post-bronchodilator FEV_1/FVC of less than 70%. Subjects were excluded if they had a diagnosis of lung cancer, autoimmune disease, or other respiratory disease such as asthma or interstitial lung disease. Subjects were also excluded if they reported a respiratory illness in the prior month or were on systemic corticosteroid or immunosuppressive therapy.

All subjects completed a health, symptom, medication, and work exposure questionnaire. Allergy phenotype of subjects was determined by skin prick testing to a panel of 8 allergens (Timothy grass, cottonwood tree, ragweed, cat dander, dog dander, *Aspergillus*, *Alternaria*, and dust mite). Blood was drawn for C-reactive protein (non-specific marker of systemic inflammation), complete blood count, and for use in the WBA.

Spirometry and Sputum Induction

Prior to sputum induction, a base line spirometry by the Puritan Bennett Renaissance II Spirometry System was obtained. Subjects were treated with 2.5 ml of nebulized albuterol using a Sidestream® disposable nebulizer prior to sputum induction with spirometry repeated and recorded. The sputum induction protocol followed a standard published protocol with safety measures (Alexis et al. 2001). Briefly, three 7-min inhalation periods of nebulized hypertonic saline (3%, 4%, 5%) were followed by expectoration of sputum into a sterile specimen cup in subjects with an FEV_1 greater than one L. Subjects with an FEV_1 less than one L received only 3% hypertonic saline for all treatment periods. After each inhalation period, spirometry was repeated and subjects were monitored for 10 and 20% drops in post-bronchodilator FEV_1. Subjects whose FEV_1 dropped greater than 20% after a hypertonic saline treatment were removed from the protocol and allowed to recover. Subjects whose FEV_1 dropped between 10 - 19% received their next treatment with the previous dose of hypertonic saline.

Sputum Processing

Within 2 hr of collection, sputum plugs were separated from whole sputum and processed according to previously published methods (Alexis et al. 2001). In brief, sputum plugs were macroscopically identified and manually selected from their surrounding fluid and treated with 0.1% dithiothrietol (sputolysin, Calbiochem, Gibbstown, NJ), a mucolytic dispersing agent. Samples were subsequently filtered (48-52 μm pore) and centrifuged (500 × *g*) to separate sputum cells from sputum supernatant. Cell-free supernatant was frozen at −80 °C and stored for later evaluation. Total cell count and viability by trypan blue exclusion method were determined with a hemacytometer. Sputum cells were spun onto slides with a Cytopro Cytocentrifuge (Wescor Inc, Logan, UT) and stained with DiffQuik (Dade Behring, Newark, DE). Differential cell counts were performed by counting 400 leukocytes per slide per subject.

Whole Blood Assay

Approximately 4-5 ml of peripheral blood per subject was collected by venipuncture. Heparinized blood was diluted in a ratio of 1:4 with complete L-glutamine-RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2-mercaptoethanol (5×10^{-5} M), 50 μg/ml streptomycin (Invitrogen). Cells (1 ml/well) were stimulated with LPS (100 ng/ml and 10 ng/ml), PGN (10 μg/ml and 1 μg/ml), organic dust extract (1% and 0.1%) and saline (phosphate buffered saline, control) at 37 °C, 5% CO_2 incubator. After 24 hr, cell-free supernatants were collected and stored at −80°C for later cytokine/chemokine analysis. All blood samples for the WBA were processed within 2 hr of collection as this has been shown to be a critical variable (van der Linden et al. 1998; Wouters et al. 2002).

Organic dust extract (ODE) from settled surface dust from swine confinement animal feeding operation facilities was prepared as previously described (Poole et al. 2007; Romberger et al. 2002). Briefly, batches of ODE were prepared by placing dust into solution and sterile filtered (0.2 μM pore) and aliquots were frozen at −80 °C. Analysis of the dust and extract was previously published (Poole et al. 2008). In brief, analysis of the organic

dust prior to placing into extract form revealed trace metals and predominance of Gram-positive bacteria (98%; *Staphylococcus*, *Bacillus*, *Streptomycetes*, and *Enterococcus* species) as identified by means of colony morphology (Poole et al. 2008). Moreover, chemical marker analysis by means of gas chromatography-tandem mass spectrometry demonstrated high muramic acid (mean 203.5 ng/mg; marker of PGN/bacterial biomass) and 3-hydroxy fatty acids (0.0723nmol/mg; marker of endotoxin). The endotoxin equivalent concentration in ODE 1% is 48 ng/ml (range: 26-70 ng/ml) as determined by the Limulus amebocyte lysate gel clot assay (Cambrex, Walkersville, MD). The total protein concentration in ODE 1% is approximately 10-40 μg/ml, by Bradford protein assay (Bio-Rad, Hercules, CA). These ranges were utilized to approximate comparable concentrations of LPS and PGN with ODE in the side-by-side *ex vivo* WBA studies. Thus, 100 ng/ml of LPS is approximately two-fold higher than LPS detected in a 1% ODE concentration, and PGN 10 μg/ml is approximate to protein concentration in a 1% ODE concentration. However, it is recognized that the Bradford assay is not a direct measurement of PGN, but there is no commercially available assay to directly PGN in the dust extract. LPS from *Escherichia coli* (O55:B5)-, and *Staphylococcus aureus* PGNs were purchased from Sigma (St. Louis, MO). A 1 and 0.1% concentration of ODE was utilized to arbitrarily represent a relative high and low swine barn exposure. Although direct comparisons with cell culture system are not feasible, it is noted that others have defined low endotoxin and dust exposure in a swine barn as a mean LPS of approximately 45.2 ± 6.6 ng/m^3 (Dosman et al. 2006). Concentration of LPS and PGN were adjusted to be comparable to these relative high and low swine barn exposure of ODE designations. These concentrations also do not elicit any cellular toxicity (Poole et al. 2008).

Mediator Analysis

Ex vivo stimulated whole blood cell-free supernatant was evaluated for TNF-α, IL-6, IL-8/CXCL8, and IL-10 by means of sandwich ELISA, as previously published (Poole et al. 2007). To determine concentration of cell-free sputum supernatant of same mediators, sandwich ELISA was modified such that standard controls were diluted in 0.05% dithiothrietol to remain consistent with the sputum processing and compared to ELISA with standard controls not diluted in dithiothrietol. Although trends were similar between methods, data presented are from utilizing 0.05% dithiothreitol in the ELISA. TNF-α, IL-6, IL-8/CXCL8, and IL-10 were chosen as these cytokines/chemokines have either been previously implicated in mediating bioaerosol/organic dust-induced airway disease in farmers (Deetz et al. 1997; Wang et al. 1997) or associated with airway symptoms in a WBA (Smit et al. 2009).

Statistical Analysis

Comparisons in subject characteristics, sputum cell and mediator characteristics between control and COPD group were made using t-test or Wilcoxon rank-sum tests for continuous variables, and chi-square tests or Fisher's exact tests for categorical variables. Wilcoxon matched-pair signed-rank tests were used to determine blood cytokine differences before and after ODE, LPS and PGN stimulation. Friedman tests, nonparametric version of repeated measures ANOVA were used to determine blood cytokine differences among ODE, LPS, and PGN treated groups. Pair wise comparisons with a Bonferroni adjustment

were made for each pair treatment groups. One-tailed Wilcoxon rank sum tests were used to determine if blood cytokine differences between the COPD and healthy control group occurred. Spearman correlation was used to determine the relationship between blood baseline cytokine levels and stimulated cytokine levels with sputum inflammatory markers, lung function, and dyspnea score within the COPD group. Significant difference accepted at $p<0.05$.

RESULTS

Population Characteristics

The characteristics of two subject populations are shown in Table 1. The healthy control population was younger. This recruitment effect was secondary to the majority of the COPD group being enrolled at the Veterans Hospital while the healthy controls were recruited from the university setting. Body mass index (BMI), gender, race and ethnicity were not significantly different between groups. Total white blood cell count did not differ between groups, but circulating monocytes were numerically higher in subjects with COPD as compared to healthy controls, but this was not statistically significant. There was no significant difference in circulating neutrophils and lymphocytes between groups. C-reactive protein levels did not differ between groups, but it was noted that 80% of the subjects with COPD were on a cholesterol-lowering agent (HMG-CoA reductase inhibitor) as compared to 10% of the healthy control group. Exposure to farming, dusts, solvents, and asbestos was reported in 80% of the subjects with COPD as compared to 60% of the healthy controls. To further phenotype the subjects, allergic status was determined. Four of the healthy controls demonstrated allergic sensitization (positive skin prick test to ≥ 1 allergen) as compared to two subjects with COPD.

Lung function and symptoms were consistent with enrollment criteria expectations (Table 1). The average FEV_1 for the healthy controls was 3.37 L or 96% of predicted as compared to subjects with COPD, which had a significant FEV_1 group average of 1.65 L or 51% of predicted. In addition, the Medical Research Council Dyspnea Scale (Fletcher et al., 1959) demonstrated that subjects with COPD experienced more dyspnea as compared to controls.

Sputum Cell and Mediator Characteristics

To identify the current airway inflammatory status of the subjects at the time of the WBA, an induced sputum protocol was conducted as described in the methods section. All subjects tolerated the procedure well without adverse reactions. Subjects with COPD demonstrated a significant increased % of sputum neutrophils as compared to healthy control subjects (Table 2). In addition, healthy control subjects had an increased % of sputum macrophages as compared to COPD subjects, reflecting a near absent inflammatory sputum profile. Cell-free sputum supernatant was evaluated for pro-inflammatory mediators (Table 2). Sputum IL-8/CXCL8 levels were significantly elevated in subjects with COPD as compared to healthy controls. There was no significant difference in sputum IL-6 between groups. Sputum TNF-α and IL-10 were not detected.

Whole blood assay (WBA) results

Ex vivo whole blood stimulation with high and low ODE and comparable high and low LPS and PGN concentrations resulted in significant release of TNF-α, IL-6, IL-8, and IL-10 compared to baseline (diluent) in all subjects (N=20, Figure 1(A-D)). There were significant variations in the stimulatory ability of the respective environmental agent to release specific mediators from whole blood. Overall, ODE (1%) resulted in significant increases in all cytokine/chemokines measured as compared to LPS and PGN. PGN (10 μg/ml) resulted in significantly elevated TNF-α and IL-8 as compared to LPS, and the increases were most similar in magnitude to ODE (1%) versus all other stimuli.

As specifically shown in Figure 1, both concentrations of ODE (1 and 0.1%) induced a significant TNF-α release as compared to both concentrations of LPS (100 and 10 μg/ml) and low concentration PGN (1 μg/ml). Stimulation with high concentration PGN (10 μg/ml) resulted in significantly elevated TNF-α release as compared to both concentration of LPS and low concentration PGN (1 μg/ml). Stimulation with high concentration ODE (1%) induced significantly increased levels of IL-8 as compared to high concentration LPS (100 ng/ml), but there was no marked difference in ODE-induced IL-8 release as compared to PGN-induced IL-8 release. ODE (1 and 0.1%)-induced significantly elevated levels of IL-10 as compared to LPS and PGN (Figure 1C). High concentration ODE (1%)-induced IL-6 release was significantly increased as compared to both concentrations of LPS (100 and 10 ng/ml) and low concentration PGN (1 μg/ml).

Next, it was of interest to determine if whole blood cytokine release assay would differ between healthy control and COPD subjects. Utilizing the same data set as presented in Figure 1 (all subjects combined), but presenting the data from the healthy controls versus COPD subjects, significant differences were demonstrated for TNF-α, IL-8, IL-10, and IL-6 (Figure 2A-D). Figure 2 illustrates TNF-α and IL-8 release after stimulation with organic dust, LPS, and PGN. Subjects with COPD demonstrated enhanced whole blood TNF-α release following simulation with 1% ODE, and trends were observed for PGN and LPS. Figure 2 also demonstrates that ODE- (0.1%), LPS- (10 and 100 ng/ml), and PGN-induced IL-8 release was significantly elevated in subjects with COPD as compared to healthy controls with trends observed for all other concentrations. Only 1% ODE-induced IL-10 and IL-6 release was significantly increased in subjects with COPD as compared to healthy controls. These studies demonstrate that subjects with COPD have enhanced cytokine/ chemokine release as compared to healthy volunteers and that differences are greatest with complex organic dust extract of the materials tested.

Next, within the COPD group, it was sought to determine if relationships existed between whole blood cytokine levels and sputum inflammatory cells and cytokines, lung function and dyspnea scores because there is mounting evidence that systemic inflammation is associated with COPD (Sinden and Stockley 2010). Within COPD subjects, there were significant correlations with lung function and baseline blood IL-10, IL-8, and TNF-α, but not IL-6, as outlined in Table 3. Sputum macrophages (%) significantly correlated inversely with baseline blood IL-10 (Spearman correlation coefficient: −0.71) and TNF-α (−0.68). There was also an inverse correlation of sputum IL-8 levels with blood baseline IL-6 levels

within the COPD group (−0.857). Significant correlations with baseline blood cytokine levels and dyspnea scores were not observed.

There were also significant correlations with lung function and *ex vivo* stimulated blood IL-8 levels, but not TNF-α, IL-6, and IL-10, within the COPD group as shown in Table 3. There were also significant correlations with stimulated blood cytokine levels and sputum endpoints. There was a significant inverse correlation with sputum IL-6 levels and 0.1% ODE stimulated IL-10 (Spearman correlation coefficient: −0.79) and 10 μg/ml PGN stimulated IL-10 (−0.90). There was also an inverse correlation with sputum macrophages and 0.1% ODE stimulated IL-8 (−0.72) and 10 μg/ml and 1 μg/ml PGN (−0.90 and −0.83, respectively). ODE (0.1%) and PGN (10 μg/ml) stimulated blood IL-10 inversely correlated with sputum IL-6 (−0.79 and −0.90, respectively). PGN (1 μg/ml) stimulated TNF-α significantly correlated with sputum IL-6 (0.76) and trends were observed for ODE (0.1%) stimulated TNF-α and sputum IL-8 levels (0.69). Dyspnea scores only correlated significantly with LPS (100 ng/ml) stimulated blood IL-6 (0.62, p).

DISCUSSION

COPD is characterized as an airway inflammatory disease, but increasingly, it is recognized that individuals with COPD have increased evidence of systemic inflammation (Agusti and Soriano 2008; Sinden and Stockley 2010). This current study supports a relationship between airways disease and systemic inflammation in subjects with COPD. Data showed that whole blood from subjects with COPD demonstrated increased cytokine/chemokine release following stimulation with occupational levels of ODE and its pathogenic components (PGN, LPS) as compared to healthy volunteers. In addition, unstimulated, baseline levels of IL-10, TNF-α, and IL-8 in the whole blood supernatants significantly correlated with lung function parameters within the COPD group. *Ex vivo* stimulated IL-8 levels also significantly correlated with lung function parameters within the COPD group. These data suggest that circulating blood cells in COPD subjects may be primed to respond to microbial/inflammatory insult. Furthermore, in all subjects, ODE-induced mediator release in the WBA was superior to PGN and LPS, but PGN and not LPS-, induced pro-inflammatory mediator release was most comparable to ODE-induced responses. This may have important consequences in assessing various occupational exposure environments with respiratory health outcomes.

In support for the concept that COPD is characterized as a systemic inflammatory disease is that isolated peripheral blood monocytes in subjects with COPD respond greater to *ex vivo* stimulation with LPS as compared to healthy controls (Aldonyte et al. 2003; de Godoy et al. 1996; Wehlin et al. 2005). While our study findings are in agreement with these previous reports, the observation was extended to a modified *ex vivo* whole blood stimulation assay. Namely, it was demonstrated that subjects with COPD have enhanced propensity to release higher levels of certain pro-inflammatory mediators from whole blood following *ex vivo* stimulation with occupational concentrations of swine facility ODE and its pathogenic microbial components, LPS and PGN. However, the findings were not global in that not all agents investigated in the WBA showed the ability to discriminate between subjects with and without COPD. The ability of the ODE to stimulate cytokine/chemokine release was

greater than LPS or PGN with ODE showing marked differences for all mediators investigated, TNF-α, IL-8, IL-6, and IL-10. Differences in the ability of PGN and LPS to induce whole blood mediator release between subjects with COPD and healthy volunteers was only significant for TNF-α and IL-8. A potential biologic explanation for this observation is that the ODE utilized should be viewed as a potent inflammatory agent because it is a diverse and complex mixture, composed of a variety of cell wall components of many types of bacteria as well as trace metals and particles. It is also recognized that due to the biologic complexity of the samples, variability in the samples among different animal confinement facilities will be inevitable. Nonetheless, in all subjects, the ODE induced increased levels of cytokine/chemokine release compared to LPS or PGN alone. Thus, our study supports the notion that combinations of microbial cell wall components versus that of individual components induce greater activation of host defense cells. Organic dust is an example of an environmental agent that contains a combination of microbial cell wall components from Gram positive and negative bacteria, information that will be useful for future preventative and therapeutic options.

Given the inherent complexity with the ODE samples, the effects of LPS and PGN were also investigated individually at concentrations comparable to that found in ODE. Whereas LPS is commonly utilized by others to study *ex vivo* responsiveness, it is also highlighted that there is a critical role for PGN in eliciting pro-inflammatory molecule release from whole blood. These differences are likely explained by the differing pattern recognition receptors and signaling pathways utilized by the host defense system to recognize specific inflammatory molecules. LPS is recognized by the Toll-like receptor (TLR)4/CD14 complex, and PGN can be recognized by TLR2 and mCD14, nucleotide oligomerization domain (NOD)1, NOD2, and a family of PGN recognition proteins (Dziarski 2004; Sarir et al. 2008; Xiang and Fan 2010). Data showed that PGN-induced pro-inflammatory cytokine/chemokine levels were to some extent comparable to ODE-induced levels. This finding suggests that PGN needs to be considered when studying real-life agriculture environmental-induced lung disease, particularly large animal faming environments. This concept is also supported by our previous study as well as others demonstrating that the endotoxin component in swine barn dust does not completely explain the innate immune inflammatory response *in vitro* (Larsson et al. 1999; Muller-Suur et al. 2002; Poole et al. 2007; Poole et al. 2009; Romberger et al. 2002). However, it is recognized that PGN concentrations have not been directly measured in organic dust extracts due to absence of commercial available assays, but instead, muramic acid, a backbone of PGN, has been routinely detected by mass spectrometry methods (Poole et al. 2010). Thus, the role of PGN in the dust extract is inferred, but future confirmatory studies are warranted.

Results from this study showed that TNF-α and IL-8 release from whole blood was most consistently enhanced in subjects with COPD following stimulatory challenges. TNF-α (a potent activator of endothelial cells, epithelial cells, and neutrophils) and IL-8 (a chemoattractant for neutrophils) are well-recognized mediators thought to play a role in the inflammatory process of COPD. These mediators may be derived from a number of circulating blood cells including monocytes, lymphocytes, neutrophils, and eosinophils (Borish and Steinke 2003). Differences between study groups for IL-10 and IL-6 release

were only observed following ODE challenge. IL-6, produced mainly by mononuclear phagocytic cells, but also lymphocytes, exerts several pro-inflammatory effects, but may also mediate anti-inflammatory effects. In comparison, IL-10, produced by monocytes and lymphocytes, is regarded as an anti-inflammatory agent. Interestingly, Smit et al. showed that among agricultural workers, high IL-10 responders had higher prevalence of airway symptoms. Although total leukocytes count and lymphocyte count did not differ between groups, there was a numerical increase, but not statistically significant, in monocytes between groups, which may, in part, contribute to these observations.

The observation reported here that baseline blood cytokine/chemokine levels correlated with lung function parameters within the COPD group is important. Although serum levels of pro-inflammatory levels have been associated with COPD (Schols et al. 1996), to the best of our knowledge, this is a first report that collection of mediators from unstimulated whole blood (collected over 24 hr) within COPD subjects correlates to lung function parameters and severity. Of the mediators, blood baseline TNF-α was most associated with lung function within the COPD group. Correlations with sputum endpoints were less clear. In addition, a correlation in lung function and sputum endpoints within the COPD group was also observed after stimulation with environmental agents. Interestingly, as opposed to *ex vivo* stimulated TNF-α, IL-6, IL-10, significant correlations were only observed with *ex vivo* stimulated IL-8 and lung function. This apparent systemic predisposition towards IL-8 production in subjects with COPD may be important for the airway neutrophil/inflammatory response. Others reported associations with whole blood LPS-induced TNF-α and IL-10 release and respiratory symptoms in occupationally exposed workers (Lambert et al. 2005; Smit et al. 2009). Collectively, our findings support the view that circulating blood cells from COPD subjects are primed.

Results from this study shown here were also evident despite no difference in C-reactive protein levels. C-reactive protein is an acute phase protein that is a non-specific marker of systemic inflammation and has been associated with subjects with COPD (Kony et al. 2004). It is possible that the levels of C-reactive protein were similar between groups due to the effect of high use of lipid-lowering agents in the COPD group as compared to healthy control subjects because lipid-lowering agents are well-recognized to affect C-reactive protein levels (Albert et al. 2001). However, it was still possible to detect inter-individual differences in subjects with COPD on lipid-lowering agents, which suggest that although these lipid-lowering agents are important, these agents may not completely abrogate systemic inflammatory responses in subjects with COPD.

A potential weakness of this study is that all subjects with COPD had a significant history of smoking, but cigarette smoke exposure is a potential inherent confounder when studying COPD populations. However, despite that all subjects with COPD admitted to tobacco use, they were clinically stable, not on systemic anti-inflammatory medications, and were age-group matched to the healthy controls (but were generally older). Future studies could explore the role of smoking alone on WBA responsiveness. Another potential confounder is farm exposure as 60% of the subjects with COPD had significant farm exposure reported in their lifetime, and repeated exposure to organic dusts in farming environments is associated with an adaptation resulting attenuation of inflammatory responses (Von Essen and

Romberger 2003). Thus, future studies should focus on current agricultural workers to determine if there are important differences in cytokine responsiveness. In addition, this is a relatively small sample size, which also lacked representation from the African American population. Nonetheless, this current study was able to find important significant inter-individual differences in a well-phenotyped group of COPD subjects suggesting that this relatively easy, modified WBA may be useful in studies of individuals exposed to various agricultural environments to assess risk of lung development and/or severity of disease.

In conclusion, this study found significant differences in whole blood mediators with and without stimulation of occupational levels of large animal (swine) facility ODE and its components (LPS, PGN) in subjects with COPD as compared to healthy volunteers. This study provides support that markers of systemic inflammation may be determined by utilization of a modified WBA tool in subjects with COPD and that circulating whole blood cells of subjects with COPD show heightened propensity to respond to microbial/inflammatory stimuli. This study can next be applied to larger clinical studies with focus on occupational, agriculture exposed workers with COPD or at risk of developing COPD to understand the profile of mediator release and potential agents driving the complex mechanisms involved with chronic inflammation in COPD.

Acknowledgments

We wish to thank the UNMC and VA clinical research personnel, particularly, LuAnn Larsson and Tamar Chadwell, for their assistance in scheduling and arranging for these studies.

Study supported by grants from the Center for Clinical and Translational Research, University of Nebraska Medical Center, Omaha, NE and the National Institute of Environmental Health Sciences (K08: ES015522-01; JAP). None of the authors have conflicts of interest or a financial relationship with a biotechnology and/or pharmaceutical manufacturer.

Abbreviations

COPD	Chronic obstructive pulmonary disease
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
IL	Interleukin
LPS	Lipopolysaccharide
ODE	Organic Dust Extract
PGN	Peptidoglycan
PBS	Phosphate buffered saline
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
WBA	Whole blood assay

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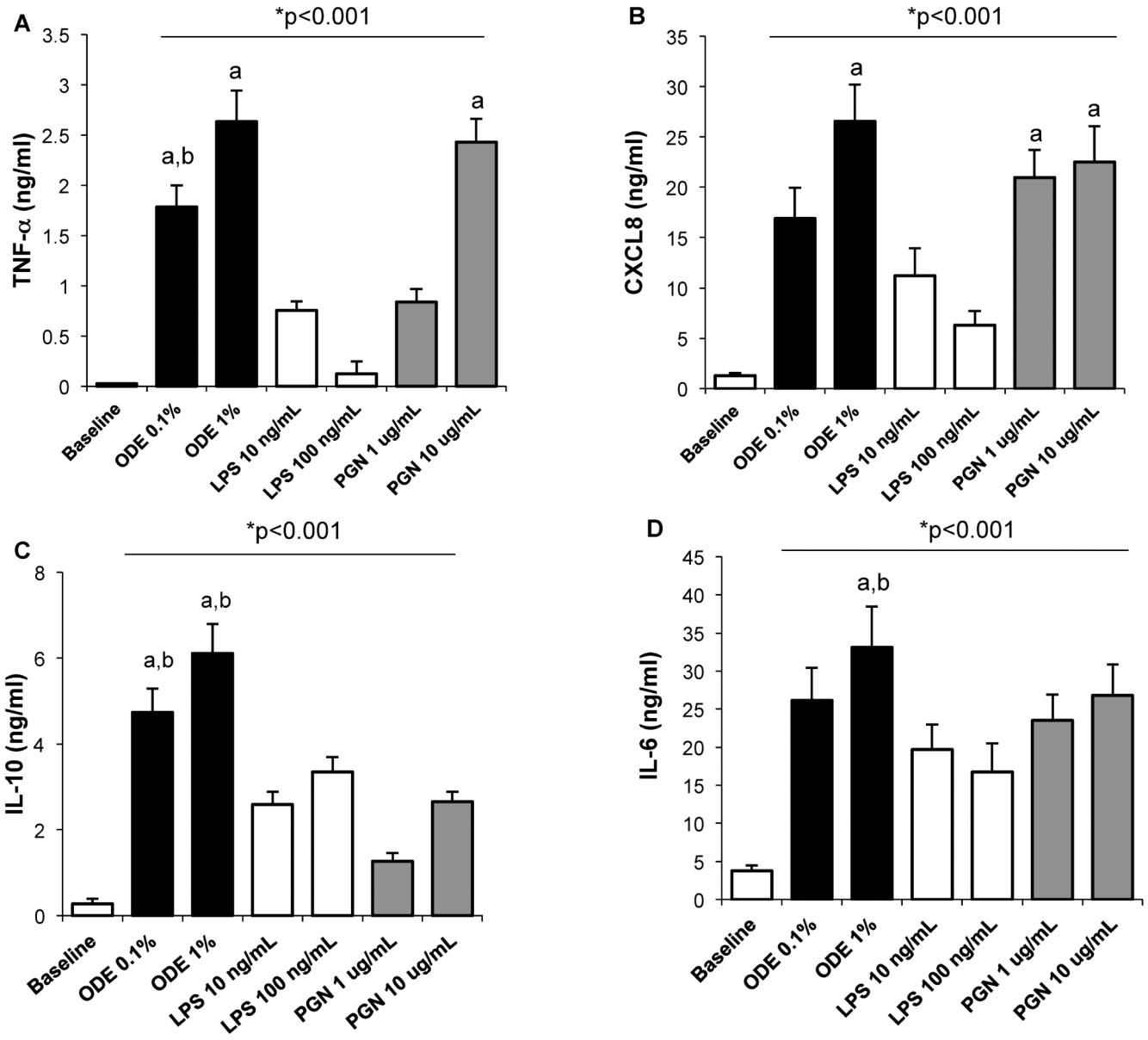

Figure 1.

(A-D). *Ex vivo* whole blood stimulation with high and low organic dust extract (ODE), lipopolysaccharide (LPS), and peptidoglycan (PGN) resulted in significant cytokine/chemokine release in all subjects as compared to baseline (*p<0.001). Results of cell-free supernatant release of TNF-α (**A**) IL-8/CXCL8 (**B**), IL-10 (C), and IL-6 (D) release following 24hr incubation with high and low concentrations of ODE (1%, 0.1%) and comparable concentrations of LPS (100 and 10 ng/ml) and PGN (10 and 1 μg/ml) by ELISA. Unstimulated (baseline) levels are also shown. Data are presented as mean concentration (ng/ml) with standard error bars shown (N=20 subjects). By Bonferroni adjustment, a) denotes significant difference as compared to LPS stimulation, and b) denotes significant difference as compared to PGN stimulation.

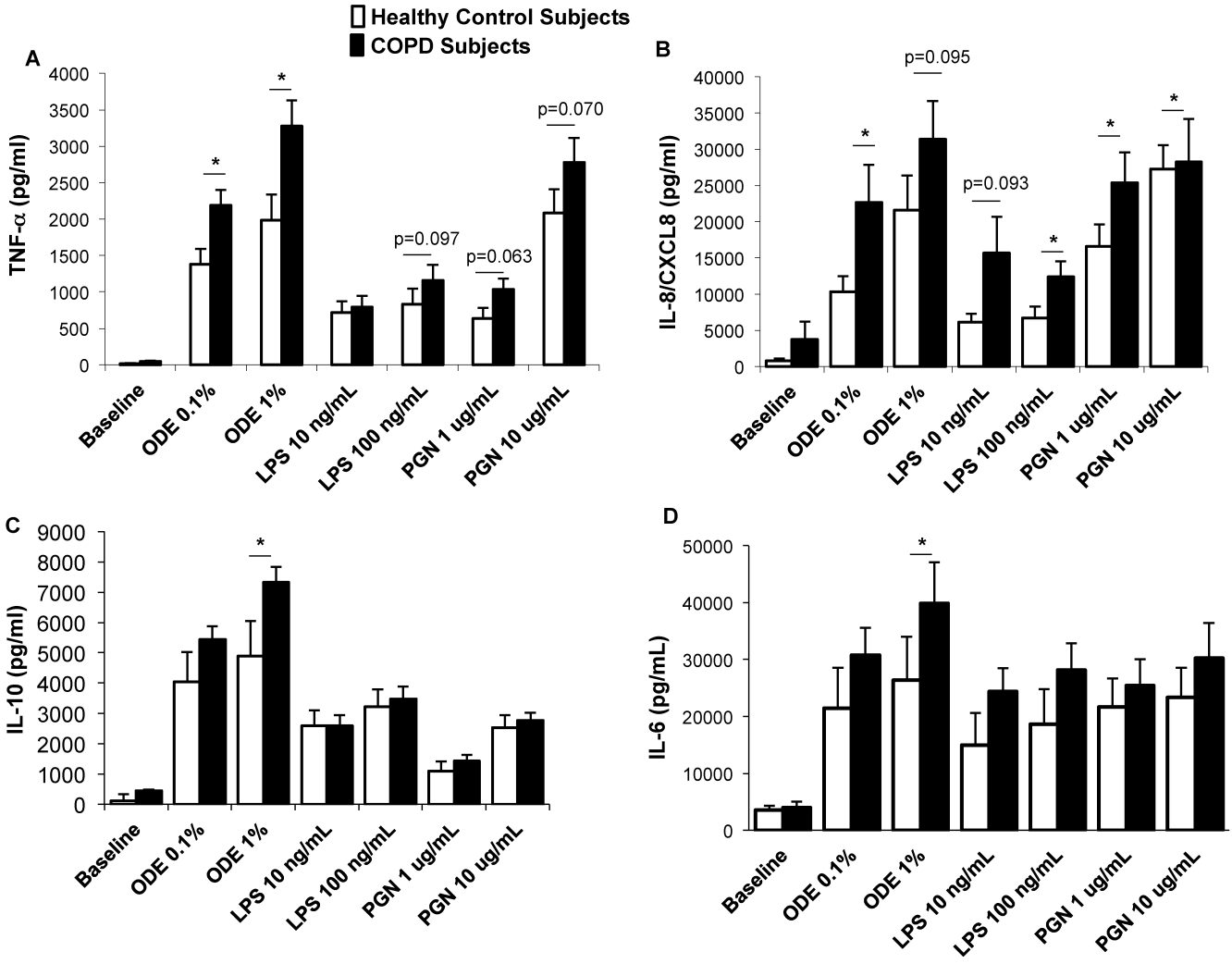

Figure 2.

(A-D). Differences in TNF-α (**A**), IL-8/CXCL8 (**B**), IL-10 (**C**), and IL-6 (**D**)-hyper-responsiveness to organic dust extract (ODE), lipopolysaccharide (LPS), and peptidoglycan (PGN) stimulated whole blood from subjects with COPD as compared to healthy controls are depicted. Unstimulated (baseline) levels are also shown. Data are presented as mean concentration (pg/ml) with standard error bars shown (N=10/group). P values <0.10 are given, and * denotes statistically significant ($p<0.05$).

Table 1

Group Demographics, Lung Function, and Symptoms

	Healthy Control Subjects (n=10)	COPD Subjects (n=10)
Age, mean	53.2	62.2
Gender, % Male	60%	100%
History of Smoking, %	0%	100%
Average Pack Years (range)	0	70 (34-120)
BMI, kg/m^2 (±SE)	30.8 (2)	30.7 (2.3)
Race, %		
White, Non-Hispanic	90%	70%
White, Hispanic	10%	0%
Black	0%	30%
C-reactive protein, unit (±SE)	0.72 (0.17)	0.94 (0.32)
Total White Blood Cell Count × $10^3/\mu L$, unit (±SE)	7.19 (0.54)	6.93 (1.1)
Monocytes	0.53 (0.7)	0.70 (1.0)
Neutrophils	4.27 (0.36)	4.53 (0.43)
Lymphocytes	2.25 (0.24)	2.67 (0.39)
FEV_1 % (L)	96% (3.37)	51% (1.65)
FVC % (L)	93% (4.13)	68% (2.97)
FEV_{25-75} (L)	113% (3.49)	25% (0.76)
FEV_1/FVC, % ratio	82%	54%
Daily Cough	0%	60%
Daily Sputum Production	0%	50%
Daily Wheezing	0%	90%
Daily Dyspnea	20%	100%
Dyspnea Index[a]	1.9	4.3

1 = I am not troubled by shortness of breath

2 = I am not troubled by shortness of breath except with strenuous exercise

3 = I am troubled by shortness of breath when hurrying on level ground or up a slight hill

4 = I walk slower than people of my own age on level ground because of shortness of breath

5 = I stop for breath after walking 100 yards or after a few minutes on level ground

6 = I am too short of breath to leave the house and/or I am short of breath when dressing or undressing

[a]Medical Research Council Dyspnea Index Scale(28)

Table 2
Sputum Cell Differential and Mediators in Health Control and COPD Subjects

	Healthy Control Subjects (n=10)	COPD Subjects (n=10)	p-value
Cell Differential, % (±SE)			
Epithelial cells	27.4% (4.7)	35.1% (9.9)	0.48
Eosinophils	0.05% (0.05)	0.1% (0.07)	0.52
Lymphocytes	0.04% (0.03)	0% (0)	0.19
Neutrophils	12.9% (2.8)	42.8% (9.1)	0.02
Macrophages	59.6% (4)	22% (1.8)	<0.001
Sputum Cytokines, pg/ml (±SE)[a]			
IL-6	852 (330)	447 (136)	0.28
IL-8/CXCL8	340 (62)	716 (144)	0.03
TNF-α	Not detected	Not detected	-
IL-10	Not detected	Not detected	-

[a] Missing data due to inadequate sample; N=1 for control subjects (inadequate sample to determine cytokines) and N=2 for COPD subjects (1 inadequate sample for cell differential and cytokines, and 1 additional sample inadequate to determine TNF-α).

Table 3

Baseline and stimulated blood cytokine levels correlate with lung function within COPD subjects (N=10)*

	TNF-α		CXCL8/IL-8		IL-10	
	Spearman correlation coefficient	**p-value**	**Spearman correlation coefficient**	**p-value**	**Spearman correlation coefficient**	**p-value**
Baseline (unstimulated)						
FVC % predicted	0.63	0.05	0.73	0.02	0.71	0.02
FEV_1 % predicted	0.75	0.01	0.71	0.02	0.72	0.02
FEV_1/FVC ratio	0.70	0.02				
FEF_{25-75} % predicted	0.74	0.01	0.48	0.16	0.56	0.09
1% ODE stimulated						
FEV_1/FVC ratio			0.81	0.004		
FEF_{25-75} % predicted			0.71	0.02		
PGN (10 μg/ml) stimulated						
FEV_1/FVC ratio			0.79	0.007		
FEF_{25-75} % predicted			0.65	0.043		
PGN (1 μg/ml) stimulated						
FEV_1 % predicted			0.85	0.0016		
FEV_1/FVC ratio			0.94	<0.0001		
FEF_{25-75} % predicted			0.88	0.0008		
LPS (100ng/ml) stimulated						
FEV_1 % predicted			0.73	0.016		
FEV_1/FVC ratio			0.75	0.01		
FEF_{25-75} % predicted			0.65	0.04		

*Unfilled areas represent statistically non-significant data.