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Biomonitoring for assessment of organic dust-induced lung inflammation

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ABSTRACT: Inhalation exposure to particulate matter containing endotoxin (or lipopolysaccharide (LPS)) occurs in a variety of occupations. Nasal lavage and induced sputum have been used to evaluate lung inflammation resulting from such exposures. Whole blood assay (WBA) measures cytokine production of leukocytes after *ex vivo* stimulation with LPS. The present study examined the effectiveness of WBA for evaluating inflammatory responses and susceptibility.

C3HeB/FEJ mice were tolerised by LPS injection or sham tolerised with saline. Animals then inhaled either swine barn dust extract containing endotoxin or saline. Bronchoalveolar lavage (BAL) fluid was assayed for leukocyte counts and pro-inflammatory cytokines (interleukin-6, tumour necrosis factor-α). Whole blood was stimulated with 10 or 100 ng·mL^{-1} of LPS, incubated for 5 or 18 h and assayed for cytokines.

Barn dust-exposed groups revealed significantly higher total cells, neutrophils and cytokines in BAL compared with saline-exposed groups. Animals tolerised to LPS and exposed to barn dust demonstrated lower cellular and cytokine BAL responses. Similarly, WBA yielded significantly elevated cytokines with barn dust exposure and reduced responses with tolerisation.

This study demonstrates the efficacy of whole blood assay as a biomarker of inhalation exposure to inflammatory agents and its use for assessing susceptibility to organic dust-induced lung inflammation.

KEYWORDS: Biomonitoring, endotoxin, exposure assessment, organic dust, whole blood assay

Organic dust is a complex bio-aerosol consisting of bacterial and fungal products and their metabolites, animal, insect, and plant components. Inhalation of organic dust may cause severe acute pulmonary and systemic inflammation and lead to occupational asthma and other obstructive pulmonary diseases [1, 2]. Studies have shown that a nonallergic, neutrophil-mediated response accounts for these pulmonary health outcomes and that pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, play a major role in the inflammatory process [2–4]. A significant component of organic dust responsible for lung inflammation is endotoxin, also referred to as lipopolysaccharide (LPS). Endotoxin exposure is associated with adverse respiratory effects in the indoor environment [5] and is a well-recognised occupational hazard in: 1) swine, poultry and dairy barns; 2) grain handling facilities; 3) vegetable and cotton processing; 4) sawmills, metal machining, fibreglass production operations; and 5) composting and waste handling [6]. Endotoxin forms the exterior layer of the outer cell membrane of nearly all Gram-negative bacteria and is known to have potent pro-inflammatory effects attributed to the lipid-A region of the molecule [7–9]. Since this region is imbedded in the bacterial cell membrane, endotoxin is most potent when released by intact cells or upon disruption of the membrane [10].

Studies have demonstrated that a decline in forced expiratory volume in one second following endotoxin or organic dust exposure is associated with increased production of inflammatory mediators by alveolar macrophages and peripheral blood monocytes [11]. It may therefore be possible to evaluate immune responses to organic dust-induced airway inflammation through analysis of the *ex vivo* release of cytokines by blood monocytes or alveolar macrophages. However, isolating and culturing these cells is time consuming and not conducive to studies of multiple subjects. In addition, isolated cells may not respond in the same manner as when maintained in their natural matrix. Although not a perfect substitute, an *ex vivo* whole blood assay (WBA) attempts to preserve various blood cell interactions necessary for cytokine response and cell viability and may be a suitable alternative [12, 13]. Unlike nasal lavage or induced sputum production, the volume of blood used for the assay is invariable, which allows for the determination of accurate total cell

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counts. Several implementations of whole blood analysis have been used to examine immune responses to various agents and conditions, including pyrogens [14–16], sepsis [17, 18], infectious disease [17, 19, 20], allergens [21, 22] and other environmental agents [23–25]. Using methods similar to this study, WOUTERS *et al.* [26] assessed within- and between-subject variation of the WBA in normal, unexposed volunteers and found that there was relatively low within-subject variance compared with between-subject variance, particularly for IL-1β and IL-6. Intra-individual variances for these two cytokines at LPS stimuli of 12.5 and 100 $ng \cdot mL^{-1}$ were 0.15 and 0.18 for IL-1β and 0.14 and 0.12 for IL-6, respectively. As with other studies, the whole blood responded in a dose–response relationship to the inflammatory agents LPS and curdlan. Of the four cytokines analysed, the greatest consistency of response was observed with IL-1β and IL-6 [26].

Previous research in the present authors' laboratory demonstrated that mice tolerised to endotoxin prior to organic dust exposure had a reduced pulmonary inflammatory response upon inhalation exposure to grain dust as compared to their naïve counterparts [27]. This phenomenon has been previously reported in the literature [28] and has been referred to as endotoxin or LPS ''tolerance'' or ''adaptation'' [2, 27, 29, 30]. The present study evaluates WBA response as a biomarker of exposure to organic dust and uses the endotoxin-tolerised mouse as a model for workers tolerised to endotoxin through daily occupational exposure. These experiments sought to test whether mice exposed to organic dust from swine confinement facilities have a more vigorous whole blood cytokine response to endotoxin than sham-exposed animals and if the cytokine response of mouse whole blood stimulated *ex vivo* increases with higher concentrations of endotoxin stimulant [31]. A second objective was to determine whether LPS tolerance led to reduced responsiveness as assessed using WBA.

MATERIALS AND METHODS

Animals

Male, 6-week-old C3HeB/FeJ mice (Jackson Labs, Bar Harbor, ME, USA) were housed in an American Association for Accreditation of Laboratory Animal Care-accredited rodent vivarium. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Iowa (Iowa City, IA, USA). Mice were quarantined for 12 days before the experiments were initiated. Four mice were reserved as sentinels and necropsied at the beginning and end of the experiment to verify animal health. Thirty-two mice were divided into two exposure groups: a sham exposure group, which received nebulised saline; and an organic dust exposure group, which was exposed to a nebulised extract of dust collected from a concentrated animal feeding operation (CAFO) housing swine (CAFO dust). The strain of mouse chosen for the current study has been previously characterised as ''endotoxin sensitive'' in an organic dust exposure model [27].

Induced lipopolysaccharide tolerance

Endotoxin tolerance was induced in half of the mice from each exposure group, as previously described [27, 32]. Briefly, mice received daily intraperitoneal (*i.p.*) injections of increasing doses of *Escherichia coli* O111:B4 endotoxin (Sigma, St Louis, MO, USA) as follows. Day 1: 100 $\mu g \cdot kg^{-1}$; day 2: 500 $\mu g \cdot kg^{-1}$; day 3: 1,000 $\mu g \cdot kg^{-1}$; day 4: 5,000 $\mu g \cdot kg^{-1}$. Endotoxin concentration of the stock solution was confirmed by kinetic chromogenic Limulus amebocyte lysate (LAL) assay (BioWhittaker Inc., Walkersville, MD, USA). The remaining 16 control mice were administered pyrogen-free saline in a similar fashion.

Swine CAFO dust extract

Dust samples were obtained from an Eastern Iowa swine CAFO by vacuuming vertical surfaces with a sampling vacuum. An extract of the dust was prepared at a concentration of 100 $mg \cdot mL^{-1}$ in sterile, physiological saline. The dust was eluted by first vortexing for 2 min and then shaking on a laboratory rotator for 1 h at ambient temperature. Insoluble particles were removed by centrifuging and decanting twice at $3,350 \times g$ for 15 min each. The final supernatant was adjusted to pH 7.2.

Exposure

Mice were exposed in a whole-body exposure chamber [33] for 4 h to either nebulised saline (n=16) or nebulised CAFO dust extract (n=16). In each exposure group, half of the mice were from the induced tolerance (LPS *i.p.*) group and the remaining were controls (saline *i.p.*). Aerosols were generated with a six-jet Collison nebuliser (BGI Inc., Waltham, MA, USA) with a 138-kPa air supply [7]. Airflow through the chamber was regulated by an exhaust flow of 22.5 $L \cdot min^{-1}$. Passive airflow into the exposure chamber was filtered through an organic vapour cartridge and P100 particulate filter (North Safety Products, Cranston, RI, USA). Endotoxin concentration within the exposure chamber was measured by hourly collection of 15-min air samples collected on 47-mm glass fibre filters.

Bronchoalveolar lavage fluid and whole blood collection

At 1 h post-exposure (5 h after the initiation of the inhalation exposure), animals were anaesthetised, euthanised and exsanguinated by cardiac puncture to obtain heparinised whole blood. Blood from all animals of an exposure group was pooled and held at 4°C. Pooling of blood from individual mice was necessary to obtain sufficient blood for the WBA. Immediately following blood collection, bronchoalveolar lavage fluid (BALF) was obtained for total and differential cell counts and cytokine analyses. The lungs were lavaged with sterile, pyrogen-free saline at a pressure of 25 cmH_2O in 1-mL increments for a total volume of 4 mL. BALF was stored at 4°C and processed as soon as possible after collection. The BALF was centrifuged for 5 min at $200 \times g$ and the resulting supernatant was decanted, divided into equal volume aliquots and frozen at -80°C for later cytokine analysis. The remaining cell pellet was re-suspended in RPMI media and prepared for total and differential cell counts. Total counts were performed using an improved Neubauer hemocytometer (Reichert, Buffalo, NY, USA), and the Diff Quick Stain Set (Harleco, Gibbstown, NY, USA) was used for staining for differential enumeration by microscopy.

Whole blood assay

Whole blood samples were pooled by endotoxin tolerance and exposure groups, and diluted with an equal volume of saline. The diluted whole blood was pipetted into tissue culture tubes

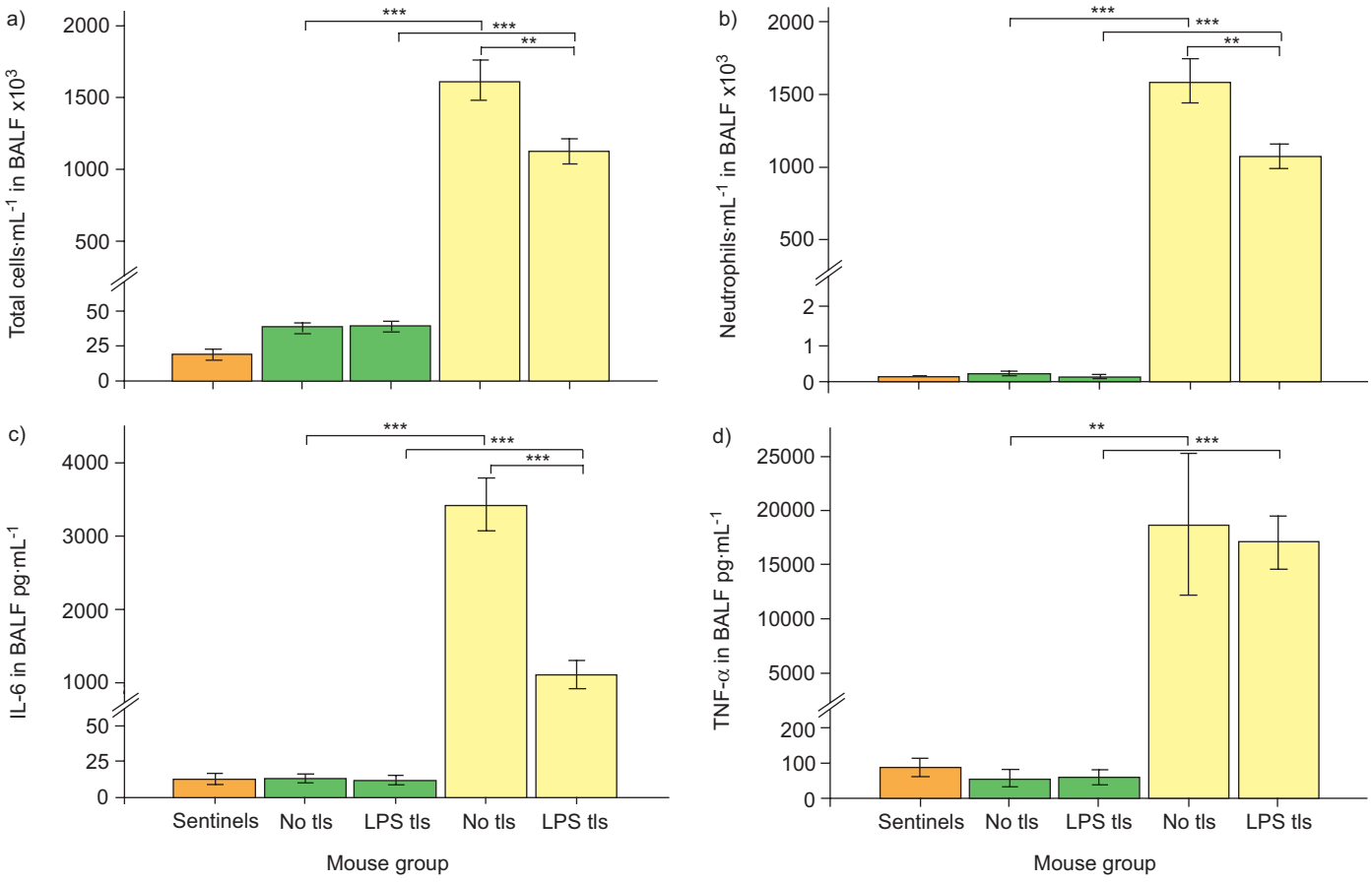

FIGURE 1. a) Total cells, b) neutrophils, c) interleukin (IL)-6 and tumour necrosis factor (TNF)-α in bronchoalveolar lavage fluid (BALF) from sham-tolerised (No tls) and tolerised (lipopolysaccharide (LPS) tls) mice exposed to saline (bars shaded green) and concentrated animal feeding operation dust (bars shaded yellow); sentinels are represented by orange shading. Error bars indicate mean±SEM. **: $p<0.01$; ***: $p<0.001$.

in duplicate aliquots of 900 μL. To each of these tubes, 100 μL of LPS was added to stimulate cytokine release as follows: high LPS stimulant (100 ng·mL^{-1}), low LPS stimulant (10 ng·mL^{-1}), and saline control. LPS was purchased from BioWhittaker Inc. as a lyophilised extract of *E. coli* serotype O55:B5. Tubes with blood and stimulant were incubated at 37°C at 95% humidity and 5% carbon dioxide in duplicate. Following either a 5- or 18-h incubation, 500 μL saline was added to each tube before centrifugation at 1,000×*g* for 15 min. The resulting plasma supernatants were decanted and stored at -80°C until cytokine analysis.

ELISA and Limulus amebocyte lysate assays

Murine IL-6 and TNF-α cytokine concentrations in bronchoalveolar lavage (BAL) supernatant and WBA plasma samples were determined using commercially available ELISA kits (BioSource International, Camarillo, CA, USA). Endotoxin concentrations of air filter samples were determined using kinetic chromogenic LAL assay, performed as previously described [7].

Statistical methods

Primary dependent variables included BAL total and differential cell counts, BALF cytokine concentrations, and WBA cytokine production with and without LPS stimulation. For the WBA, net induced cytokine production was calculated by subtracting the nonstimulated control from the corresponding LPS stimulated result. Unpaired t-tests were used to analyse BALF data. Comparisons were considered significant at $p \leqslant 0.01$.

RESULTS

Exposure

The concentration of the endotoxin delivered in the CAFO dust extract exposure averaged 28.4±4.1 μg·m^{-3}. This concentration corresponds to a body burden of 490 ng·kg^{-1} (4,900 EU·kg^{-1}), which is ~10 times the lung burden a CAFO worker would amass over an 8-h working day in a CAFO operated without any exposure controls [34].

Results of bronchoalveolar lavage fluid analysis

Significantly higher total and neutrophil cell counts as well as cytokine concentrations were observed for both CAFO dust-exposed groups as compared with the sham-exposed groups ($p<0.001$; fig. 1). Mice exposed to CAFO dust had a far higher concentration of total BAL cells than the sham-exposed group mean ($p<0.001$; fig. 1a). This response was marked by an influx of neutrophils, resulting in an increase from 1% neutrophils in controls to 97% in CAFO dust-exposed mice. Figure 1b illustrates that neutrophils increased in concentration from 230

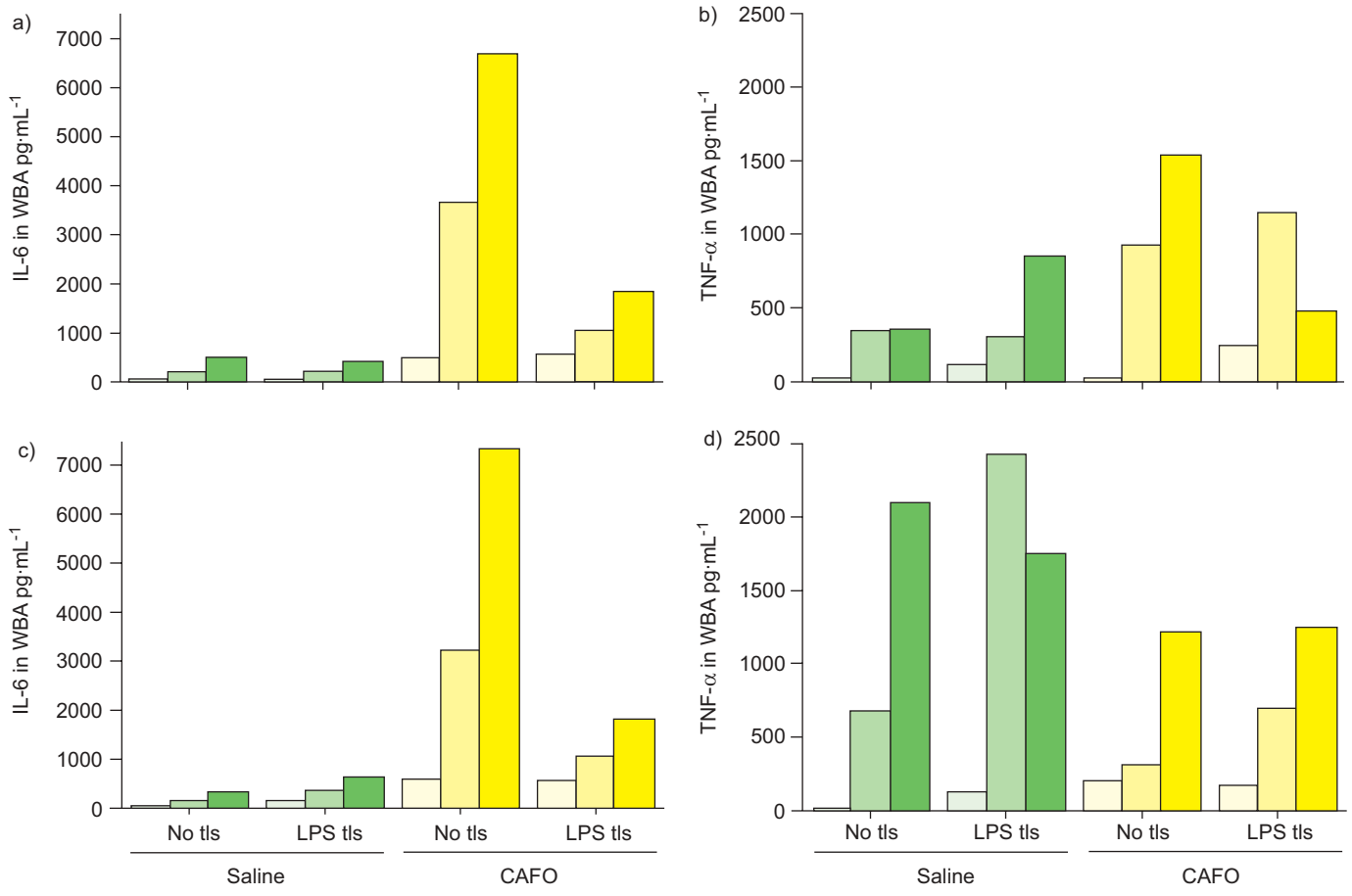

FIGURE 2. Whole blood analysis (WBA) cytokine results for a) interleukin (IL)-6 and b) tumour necrosis factor (TNF)-α following 5-h incubation of the whole blood after lipopolysaccharide (LPS) stimulation and c) IL-6 and d) TNF-α following 18-h incubation. WBA from the saline (green bars) and concentrated animal feeding operation (CAFO; yellow) groups were either unstimulated (lightest bars), stimulated with low LPS (mid-shaded bars) or stimulated with high LPS (darkest bars). No tls: nontolerised; LPS tls: tolerised.

cells·mL^{-1} in controls to 1,600,000 cells·mL^{-1} in the CAFO dust-exposed mice.

Animals tolerised to LPS and exposed to CAFO dust had significantly lower BALF total cells (1.13×106 *versus* 1.61×106 cells·mL^{-1}; $p<0.001$) and neutrophils (1.08×106 *versus* 1.59×106 cells·mL^{-1}; $p<0.001$) compared with animals that received the same exposure but without tolerisation (fig. 1a and b). Furthermore, as shown in figure 1c, tolerised animals in the CAFO dust-exposed group did not have as vigorous a pulmonary inflammatory response as the nontolerised animals, as measured by IL-6 release (3,400 *versus* 1,100 pg·mL^{-1}; $p<0.001$). There was no difference in TNF-α between these two groups (fig. 1d). However, the TNF-α response was extremely high (>17,000 pg·mL^{-1}) for both the tolerised and nontolerised groups exposed to CAFO dust. There were no significant differences in pulmonary inflammatory responses between the control (sham-exposed) tolerised and nontolerised groups.

Whole blood assay

Results of the WBA on pooled blood from the animals described previously are shown in figure 2. The whole blood was incubated for either 5 or 18 h with no *in vitro* stimulation or stimulation with low or high amounts of LPS. In most cases, there was an increased release of cytokines *in vitro* with increasing endotoxin stimulation. Cytokine release was higher in non-tolerised CAFO dust-exposed mice than sham-exposed mice at the 5- and 18-h time points for IL-6 but not TNF-α. Tolerising animals to LPS prior to CAFO dust-extract exposure dramatically reduced their IL-6 response compared with the exposed, nontolerised animals for both the 5- and 18-h time points. As with BALF, this difference was not generally reflected in the sham-exposed groups. The pattern of responsiveness between exposure (sham *versus* CAFO dust) and tolerance (saline *versus* LPS) groups for TNF-α suggested a stronger response to LPS stimulation in the animals that were not exposed to CAFO dust. This may be due to the fact that TNF-α response among mice exposed to CAFO dust peaked before the 5-h time point.

DISCUSSION

Mice exposed to inhaled swine CAFO dust developed a profound lung inflammatory response as demonstrated by a 40-fold increase in BALF cells (30-fold for the tolerised group), a 6,500-fold increase in neutrophils (8,500 for tolerised group) and a ~250-fold increase in two cytokines that serve as markers of inflammation: TNF-α and IL-6. Sham-exposed mice

who breathed nebulised, pyrogen-free saline did not demonstrate any changes in these outcome variables as compared with sentinel animals.

The present study demonstrated that mice tolerised to endotoxin prior to inhalation exposure to swine CAFO dust have significantly fewer total cells and neutrophils in BALF. These mice also responded less vigorously in WBA than nontolerised mice receiving the same exposure. Differences in susceptibility between exposure groups were evident, as demonstrated by the greater WBA IL-6 cytokine responses in both CAFO dust-exposed groups as compared with the sham-exposed groups. The same effect was found to a lesser degree for TNF-α after 5-h WBA incubation but not 18-h incubation. It is possible this was due to the timing of the assay, since TNF-α production peaks much earlier than IL-6 [13]. Furthermore, in human WBA studies, TNF-α production has been found to be much more variable both within and between individuals [12, 26]. These data indicate that WBA functions as a biomarker assay of exposure effect and of reduced susceptibility due to induced tolerance, and suggest that IL-6 might be a more reliable marker of this effect.

Endotoxin exposures were ~10-fold higher than would be experienced by CAFO workers [6, 34]. Despite this difference in exposure used to elicit a response, WBA might also be applicable to human inhalation exposure studies. It is known that individuals vary in their response to inhalation of organic dusts and LPS in both occupational and experimental settings [11, 35]. The nature of this difference may relate to factors in the pathway of signal transduction, from endotoxin to toll-like receptor (TLR)4 *via* LPS-binding protein, CD14 and MD2 [36]. Another significant factor may be genetic determinants of immune responses to LPS. TLR4 polymorphisms may account for some of this variability, but other genes also seem to play an important role [37–39]. ARBOUR *et al.* [40] found that two common TLR4 mutations (Asp299Gly and Thr399Ile) were associated with a decreased response to inhaled LPS in humans. Insertion of the wild-type allele into primary airway epithelial cell or alveolar macrophage cell cultures from individuals with the aforementioned TLR4 mutations could reverse this effect *in vitro*. Some of this variability may also depend on the type of endotoxin (cell-bound *versus* purified) present in the exposure [41] and pre-existing conditions such as asthma [42]. A whole blood *in vitro* stimulation assay attempts to account for this variability by maintaining an ''intact'' cellular milieu; it may therefore be a good predictor of an individual's immune response to various environmental and microbial agents [43].

Workers exposed to high endotoxin in CAFOs, in grain-dust or composting facilities, or in vegetable-washing operations may become tolerised to endotoxin. One way in which this is apparent is in ''Monday morning fever'' in cotton and textile workers, where those exposed to endotoxin early in the week (*i.e.* after a weekend off from work) have more significant respiratory symptoms than they do later in the week [44]. Data from this study suggest that cytokine production in the WBA for cotton would diminish over the course of the working week.

Several previous studies have sought to validate WBA [15, 16, 26, 45]. WOUTERS *et al.* [26] determined that between-individual variability in WBA with LPS and β-glucan stimulation was more significant than within-individual variability. WBA was a reliable, reproducible measure of an individual's responsiveness. However, that study used unexposed normal subjects so no inference could be made regarding the utility of WBA for biomonitoring exposures to inflammatory agents. Other studies have characterised LPS-induced cytokine responses of cultured cells, including monocytes and lymphocytes, but did not investigate WBA [13, 39, 46]. The role of occupationally induced LPS tolerance in WBA has not been adequately explored.

In conclusion, the present study in a murine inhalation exposure model suggests that whole blood analysis may serve as a biomarker of exposure and an alternative to various methods that are either nonspecific or more invasive and not applicable to large, population-based occupational exposure studies. Further research is needed in human exposure models to confirm these findings. One such study is currently underway in the present authors' laboratory.

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