

A Single Exposure to Organic Dust of Non-Naïve Non-Exposed Volunteers Induces Long-Lasting Symptoms of Endotoxin Tolerance

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Key Words

Endotoxin · Occupational exposure · Tumor necrosis factor- α · Interleukin-6 · CD14 · HLA-DR

Abstract

Background: Work with occupational levels of organic dust is associated with a chronic inflammatory response that must somehow be controlled. Endotoxin tolerance has previously been described in vitro and animal studies as a mechanism that modifies the threshold at which response occurs. **Objective:** We investigated the response of non-naïve, currently non-exposed persons to a single exposure of organic dust in a swine confinement building. **Methods:** We exposed 16 non-naïve persons in a swine confinement building with low-to-moderate representative levels of organic dust and characterized their acute immune response. **Results:** Under work-like 3-hour exposure conditions, non-naïve volunteers developed an inflammatory response documented by an increase in interleukin-6 (IL-6) in bronchoalveolar lavage (BAL) from 3.1 to 6.1 pg/ml and visual indices of bronchial inflammation. Similarly, serum IL-6 increased with a peak 3 h after exposure. Tumor necrosis factor (TNF)- α was not detected in BAL, and serum TNF- α was reduced

from 3.7 pg/ml at baseline to less than 2 pg/ml within 3 h after exposure, and remained decreased until 2 weeks after exposure. This is a cardinal marker for immune suppression which was confirmed by other markers: reduction in HLA-DR expression on alveolar macrophages and CD14 expression on blood monocytes. **Conclusion:** We report findings that suggest that long-lasting endotoxin tolerance and immune suppression may be induced by a brief exposure to organic dust concentrations in the medium-low range of occupational levels.

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Introduction

Endotoxin in organic dust is a constituent of the human environment that derives from gram-negative bacteria around and on us. As the concentration of organic dust and endotoxin can vary by orders of magnitude from setting to setting (for example at home [1] and in agricultural industry [2]), the immune system must be able to determine the environmental levels to set thresholds for an inflammatory reaction that may protect against infection. Concentrations of the lipopolysaccharides (LPS) are sensed through the CD14-TLR4 system, and can be mea-

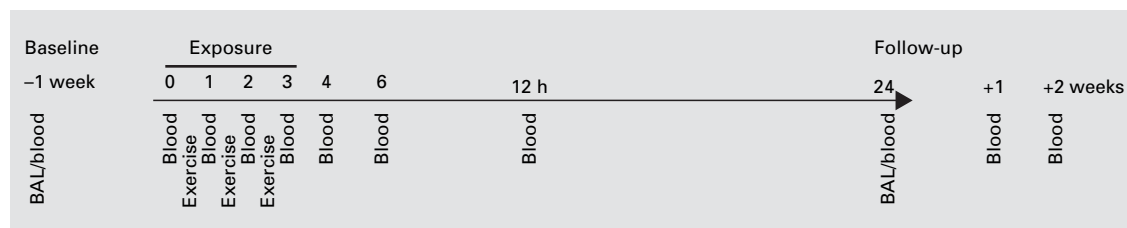


Fig. 1. Exposure plan.

sured with the limulus amebocyte lysate test [3]. IL-6 in bronchoalveolar lavage (BAL) or serum is a reliable marker for an inflammatory response to LPS [4]. The major route of exposure is in the lung through breathing. Most investigations into the consequences of inhaled endotoxin focus on events in naïve subjects following high levels of exposure that do not necessarily reflect the occupational exposure [2]. In occupational exposure studies, elevated levels of endotoxin can be associated with symptoms that are worst on Monday morning (after having recovered during the weekend), and are characterized by an adaptive process that occurs during the working week [5].

Endotoxin tolerance has been investigated in numerous animal studies (reviewed by West and Heagy [6]). Typically, animals were challenged with a low dose of endotoxin, which enabled them to survive rechallenge with a much higher dose the next day. This adaptation is transient and is associated with characteristic changes in the monocytes: (1) a reduction in secreted and serum tumor necrosis factor (TNF)- α [6] and (2) a reduction in the expression of HLA-DR and CD14 on monocytes and macrophages. Although these experiments were done with high levels of endotoxin, similar effects have been observed with levels of endotoxin similar to the ones observed in agriculture [7, 8]. In vitro studies have shown that endotoxin tolerance can also be induced on blood cells from normal human volunteers [9] where secreted TNF- α and monocyte HLA-DR were reduced during adaptation. Peri- and postoperatively, serum TNF- α , monocyte HLA-DR and monocyte CD14 were significantly reduced but recovered after 1 week [10].

We have exposed non-naïve, but currently non-exposed volunteers in an experimental exposure to work-like conditions and monitored the pulmonary and systemic inflammatory response. Here we present data suggesting that an acute inflammatory response in the lung is associated with systemic desensitization that persists for at least 2 weeks.

Subjects and Methods

Characteristics of the study participants have been described previously [11]. Former farming apprentices ($n = 16$) who had previously worked with swine were recruited to participate in an experimental exposure study. Their mean age was 26 years at the time of the study (range 25–29 years). They had worked in swine confinement buildings for a mean of 1.6 years (range 0.2–4.3 years) but had not worked with pigs during the last 3.5–9.0 years (mean 5.6 years). Eight persons smoked. The observations reported here were similar for smokers and nonsmokers. They were examined in detail 1 week before, during and 1 day after the exposure, and they were followed until 2 weeks after exposure (fig. 1). The exposure of all participants, either in pairs or singly, lasted 3 h in the same building. Each person cycled for the first 10 min of each hour of exposure on an exercise bicycle set at 50 W. Backpack samplers were used to collect ambient air samples for analysis of dust and LPS [11]. IOM dust samplers [12] were used to collect inhalable dust samples, and cyclone dust samplers from SKC (50% cutoff effectiveness value of 5 μm) for collecting the respirable fraction. The sampling rates were adjusted to 2.0 l/min for the inhalable dust and 1.9 l/min for the respirable dust. The concentration of airborne dust was determined gravimetrically from the dust mass collected on a filter through which a known volume of air had passed. To determine LPS concentrations, dust-loaded filter discs were extracted and vortexed in endotoxin-free borosilicate glass vessels with 10 ml endotoxin-free water for 1 h at 20°C. Afterwards the suspensions were centrifuged for 10 min at 1,000 g. The resulting supernatants were serially diluted and then used in a limulus amebocyte lysate (LAL) test, which was applied as chromogenic kinetic method. For calibration, a reference endotoxin standard from *Escherichia coli* (EC6) was used.

During bronchoscopy [13], 1 week before and 1 day after exposure, an inflammatory index (score 0–3) was assigned to five subcategories (erythema, edema, secretions, friability and cough) by the attending physician, who was not blinded to the study. The scores were summed up and the values of bronchoscopy obtained at baseline were compared to those after exposure.

Blood for serum harvesting was drawn repeatedly during the exposure into vacutainers (fig. 1) and was frozen immediately. BAL was obtained both at baseline and 1 day after exposure. Prewarmed saline was instilled into the right middle lobe in three 60-ml aliquots, withdrawn immediately and stored on ice. The pooled lavage was filtered through a 40-nm nylon mesh and centrifuged, and the supernatant was stored at -80°C until analysis. Serum and BAL IL-6 (detection limit 0.7 pg/ml) and TNF- α (detection limit 0.12 pg/ml) were measured in duplicate with Quantikine ELISA assays from R&D Systems.

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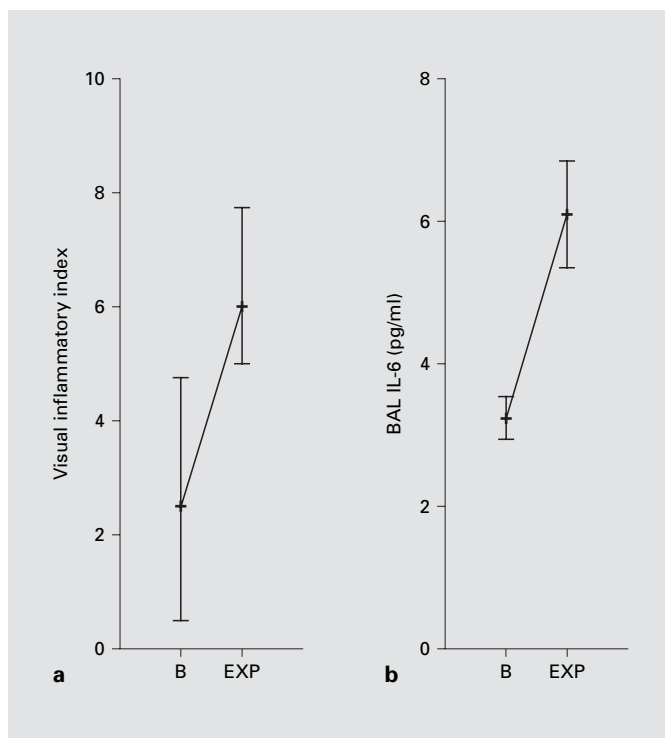
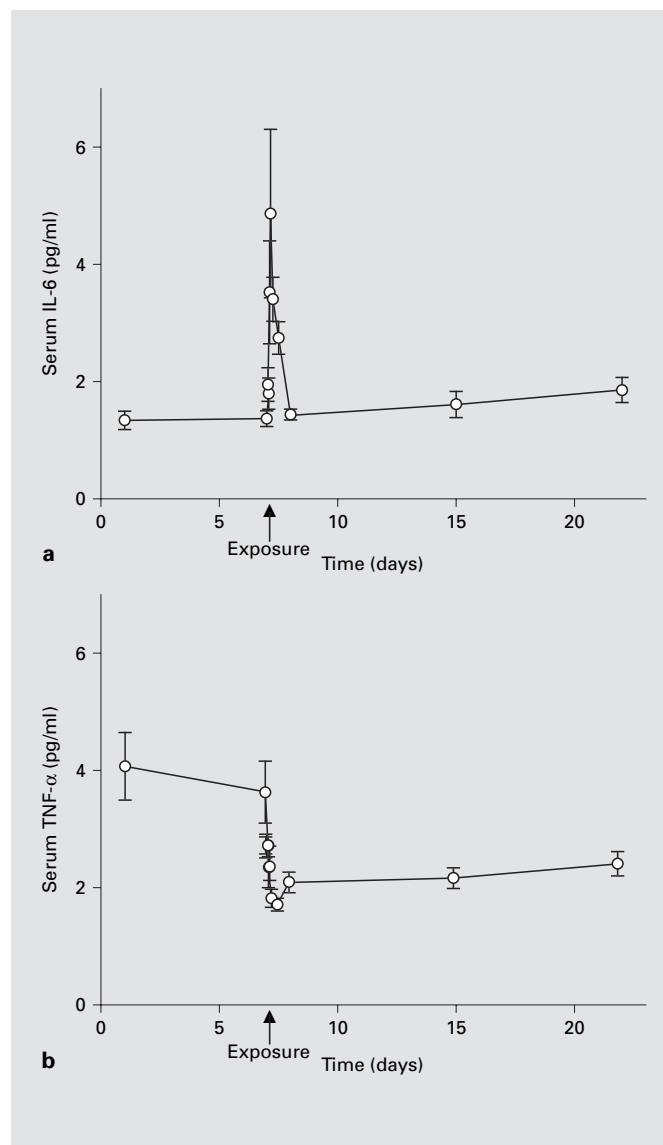


Fig. 2. Markers of lung inflammation. B = Baseline; EXP = exposure. **a** Inflammatory index change during bronchoscopy from baseline to 1 day after exposure. Median, and 25th and 75th percentiles are shown. $p = 0.003$, Wilcoxon's matched-pair signed-rank test. **b** BAL IL-6 concentration change from baseline to 1 day after exposure (mean $\pm$ SEM, $p < 0.001$, paired t test).

Fig. 3. Serum cytokine response. **a** Serum IL-6 increased from exposure start, and remained significantly elevated 3–12 h after exposure ($p < 0.001$, Wilcoxon's matched-pair signed-rank test). **b** Serum TNF- α was reduced from 2 h after exposure ($p < 0.05$, paired t test), and remained decreased from 3 h after exposure to 2 weeks after exposure ($p < 0.006$, paired t test).

One week before and 1 day after exposure, BAL cells and heparinized, hemolyzed blood were stained with directly conjugated antibodies to CD14 APC (Serotec) and HLA-DR PerCP (Becton-Dickinson), with isotype controls (Serotec). Data (50,000 events) collected on a FACSCalibur flow cytometer were blindly analyzed in batches. Monocytes were identified on a forward scatter versus side scatter plot. Alveolar macrophages were identified as HLA-DR bright cells with high forward and side scatter. Mean fluorescence intensity (MFI) was compared between baseline and exposure readings.

Statistical significance was evaluated with SPSS version 10. Normal distribution of data was determined with the Kolmogorov-Smirnov and Shapiro-Wilks tests. Data not normally distributed were log transformed and retested. Non-normally distributed data were evaluated with the Wilcoxon test, and normally distributed data with a paired t test.



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Results

Exposure Measures: LPS and Dust

The median exposure was 710 EU/m³ (25th and 75th percentiles 471 and 1,868 EU/m³) inhalable and 12.05 EU/m³ (25th and 75th percentiles 0.73 and 29.44 EU/m³) respirable endotoxin, and 1.23 mg (25th and 75th percentiles 0.97 and 1.42 mg) inhalable and 0.04 mg (25th and 75th percentiles 0.02 and 0.05 mg) respirable dust. Endotoxin in the inhalable fraction correlated with the respirable fraction (Pearson's correlation coefficient $r = 0.926$, $p < 0.001$).

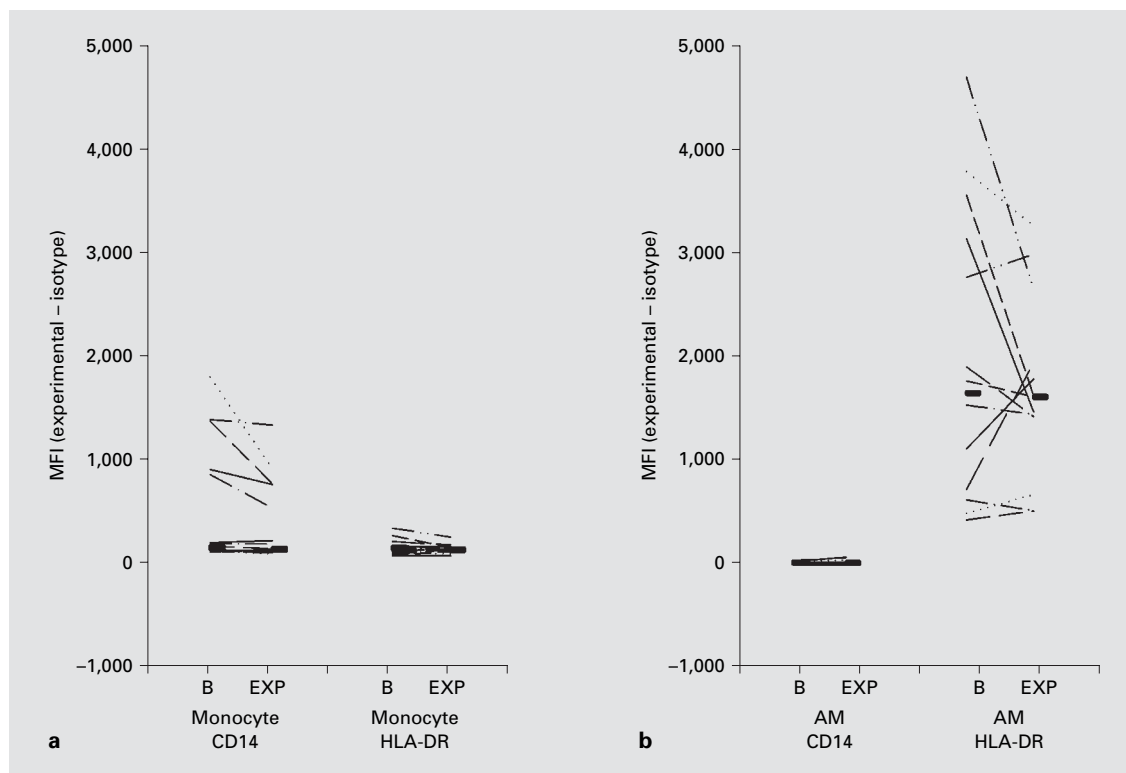


Fig. 4. Expression of CD14 and HLA-DR on alveolar macrophages (AM) and blood monocytes. All data were acquired with the same settings and are hence comparable. B = Baseline; EXP = exposure. **a** Decrease in monocyte surface CD14 ($p = 0.008$, Wilcoxon's matched-pair signed-rank test) and HLA-DR (nonsignificant). **b** Decrease in alveolar macrophage surface CD14 (nonsignificant) and HLA-DR ($p = 0.03$, paired t test).

The Inflammatory Response in the Lung

When participants were bronchoscoped approximately 20 h after exposure, clear signs of bronchial inflammation were seen compared with bronchoscopy at baseline. The total indices for erythema, edema, secretions, and friability increased significantly from baseline to exposure ($p < 0.006$, Wilcoxon, fig. 2a). The total BAL cell number was high at baseline ($48.3 \pm 38.9 \times 10^6/\text{ml}$) as well as after exposure ($53.3 \pm 39.8 \times 10^6/\text{ml}$, not shown). BAL IL-6 increased from $3.08 \pm 1.1 \text{ pg/ml}$ at baseline to $6.11 \pm 3.2 \text{ pg/ml}$ the day after exposure ($p = 0.001$, fig. 2b). TNF- α was not detectable in BAL.

Systemic Inflammatory Responses

Serum IL-6 and TNF- α . Serum IL-6 (fig. 3a) was significantly elevated from 3 to 12 h ($p \leq 0.001$), with a maximum 6 h after exposure start (median 2.95 pg/ml), which returned to baseline (median 1.1 pg/ml) values after 24 h (median 1.45 pg/ml). Serum TNF- α (fig. 3b) decreased from baseline values (median 3.7 pg/ml) to a min-

imum 6 h after exposure (median 1.7 pg/ml), and remained significantly reduced until the end of the study period (2 weeks, median 2.3 pg/ml , $p \leq 0.006$, paired t test, from 3 h to 2 weeks). There was no correlation to exposure measures.

CD14 and HLA-DR Expression on Blood Monocytes and Alveolar Macrophages. To investigate whether other markers of immune suppression are associated with the exposure investigated here, the CD14 and HLA DR expression was evaluated on blood monocytes (fig. 4a). On exposure to dust and endotoxin in swine confinement buildings, monocyte CD14 MFI was reduced $14 \pm 22\%$ compared to baseline ($p = 0.008$), whereas the reduction in HLA-DR MFI was not significant. On alveolar macrophages, after exposure HLA-DR MFI was reduced by $17 \pm 20\%$ compared to baseline ($p = 0.03$), whereas CD14 MFI was unchanged (fig. 4b). The change in HLA-DR expression on alveolar macrophages correlated with the amount of inhalable LPS ($r = -0.579$, $p = 0.048$, $n = 12$) and nearly with respirable LPS ($p = 0.052$).

Discussion

Sixteen former farming apprentices who had previously worked with swine were exposed to a 3-hour work session in a swine confinement building. Exposure to dust and LPS in this study was representative for Danish swine confinement buildings [2], but approximately tenfold lower than in other studies of swine confinement workers. An inflammatory response in the lung has been suggested before [11], and was documented by both visual indices of bronchial inflammation and BAL IL-6 levels. BAL cell number was unchanged, and CD14 expression on alveolar macrophages was not detectable. The BAL cell number was high as half of the participants smoked. As an index for systemic inflammation, serum IL-6 was elevated 3–12 h after exposure start and returned to pre-exposure values after 24 h.

Serum TNF- α decreased within 3 h after the start of exposure, reaching a minimum level after 6 h and remaining decreased for 2 weeks. A similar decrease in TNF- α had been found in two other studies. In a study characterizing bronchial and systemic inflammatory responses following bronchial endotoxin instillation, serum IL-6 increased from 0.4 to 12.8 pg/ml 6 h after exposure, whereas TNF decreased from 1.6 to 1.37 pg/ml at the same time [14]. The exposure to 4 ng/kg endotoxin resulted in a 32-fold increase in serum IL-6, and a 0.85-fold reduction in serum TNF. In the present study, the median exposure was 0.012 ng/kg endotoxin (25th and 75th percentiles were 0.001 and 0.048 ng/kg) – approximately 300-fold lower than in a previous study [14]. It resulted in a 2.5-fold increase in serum IL-6 and a 0.46-fold reduction in serum TNF. Whereas the IL-6 response wanes after 12 h, it appears as though the TNF depression is long lasting. In studies where induced sputum was used to monitor the inflammatory response during experimental exposure to increasing doses of LPS [15], the TNF- α concentration at the lowest dose of LPS was lower than the TNF concentration during the response to saline [16]. This has not been noted because the focus of that study was on the inflammatory response at higher concentrations of LPS. In several studies on the induction of endotoxin tolerance in animals, normal responsiveness returns after 8 days of tolerance [6]. In our study, one aspect of tolerance, the reduced serum TNF- α level, persisted for at least 2 weeks suggesting that tolerance in man may last significantly longer. Our findings are thus corroborated by observations in previous studies with LPS challenge and measurement of TNF. The TNF response may be beyond a linear response, or may not be related to endotoxin alone, as there was no

correlation between LPS exposure levels and reduction in serum TNF. The effects of other Toll-like receptor ligands (e.g. β -glucans [17]), that also signal an elevated pollution level, may be required to adjust the threshold at which an immune response to LPS is mounted.

We investigated another aspect that may be related to endotoxin tolerance: the monocyte/macrophage response associated with immune suppression. In a study of surgical patients, those with depressed levels of CD14 and HLA-DR on blood monocytes developed endotoxin tolerance [10]. In *in vitro* experiments with purified monocytes from healthy volunteers incubated with varying doses of LPS, HLA-DR and CD86 were decreased [9]. CD14 had not been investigated in this study. On alveolar macrophages, which do not express appreciable levels of CD14, a significant reduction in the marker HLA-DR after exposure, correlating with the inhalable endotoxin exposure, further supports an adaptive response. Expression of monocyte CD14 was significantly reduced after exposure in swine confinement buildings, whereas HLA-DR expression was unaltered. Adaptation effects to endotoxin that involve macrophages have been suggested previously [7, 8], and a reduction in CD14 on rabbit alveolar macrophages treated with LPS has recently been demonstrated [18], supporting our observation that exposure to low levels of LPS reduces the innate immune response.

Reduction of serum TNF- α , monocyte CD14 and HLA-DR on alveolar macrophages suggested induction of endotoxin tolerance in the non-naïve, non-exposed subjects during a 3-hour exposure in swine confinement buildings. This endotoxin tolerance may persist for more than 2 weeks. This is consistent with and supports the hypothesis that environmental exposure to endotoxin is a central factor in the development of tolerance, regarding both sensitization to allergens [19] and the severity of asthma [1]. Studies in *Drosophila* (reviewed in [20]) suggest that TNF may be a key link between the innate and adaptive immune systems. We are planning to repeat these measurements before and after controlled exposure to nebulized LPS, and to characterize the role of TNF- α in response to low concentrations of LPS and organic dust.

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Note Added in Proof

In a recent letter, tolerance induced in healthy persons to inhaled endotoxin was documented by a significantly reduced number of sputum neutrophils after a second challenge with inhaled endotoxin separated from the first challenge by 4 weeks.

Loh LC: Tolerance to repeat exposure of inhaled endotoxin: An observation in healthy humans. *Eur Respir J* 2005;26: 363–364.