

***Ex vivo* cytokine release reflects sensitivity to occupational endotoxin exposure**

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ABSTRACT

Not everyone exposed to endotoxin develops respiratory symptoms, even at very high exposure levels. The aim was to investigate whether *ex vivo* LPS-induced cytokine release may be predictive of individual sensitivity to occupational endotoxin exposure.

In 412 agricultural workers, TNF- α , IL-1 β , and IL-10 release was measured in supernatants from LPS-stimulated whole blood, lung function was measured, and respiratory symptoms were assessed by questionnaire. For each cytokine, the population was dichotomized into low and high responders according to median cytokine concentrations. Endotoxin exposure levels were determined based on 249 personal exposure measurements.

High IL-10 responders had a higher prevalence of airway symptoms than low IL-10 responders (ORs between 2.03 and 5.10, $p < 0.05$). TNF- α response was positively, but not significantly, associated with symptoms, whereas no relationship was found between IL-1 β response and symptoms. For all three cytokines, subjects with above-median responses showed significant positive dose-response relationships between endotoxin exposure and asthma symptoms, and significant associations between endotoxin exposure and a lower FEV₁ ($p < 0.05$). In contrast, exposure-response relationships were weak and statistically non-significant for low responders.

The *ex vivo* inflammatory response to LPS reflects to a large extent whether individuals are susceptible to adverse respiratory effects induced by high occupational endotoxin exposure.

Abstract: 199 words

KEYWORDS: asthma; epidemiology; farming; lung function; occupational exposure; whole blood assay

INTRODUCTION

Occupational exposure to airborne endotoxins, cell wall components of Gram-negative bacteria, is associated with respiratory health effects in a dose-dependent manner. Epidemiological studies in occupationally exposed populations like farmers and cotton textile workers have shown exposure-response relationships between endotoxin exposure and acute lung function changes, accelerated chronic lung function decline, and non-allergic asthma [1-8]. Airway inflammatory responses to inhaled endotoxin are characterized by increased levels of infiltrating neutrophils and production of proinflammatory cytokines as shown by nasal lavage, induced sputum and bronchoalveolar lavage studies [9, 10].

We recently reported significant exposure-response relationships between occupational endotoxin exposure and asthma-like symptoms in a large adult population of farmers and agricultural processing workers [1]. The majority of exposed subjects did, however, not report any respiratory symptoms, even at very high endotoxin exposure levels. Individual differences in sensitivity to inhaled endotoxin are likely to play a role and have already been described more than two decades ago [11-13]. Castellan et al. screened naïve, healthy volunteers with a normal baseline FEV₁ for inclusion in short-term endotoxin inhalation experiments. After precautionary exclusion of those with a decrease in FEV₁ >30% on their first exposure, the authors found that endotoxin exposure elicited a measurable response in approximately one third of the remaining subjects, whereas others did not respond at all [11]. The precise biological mechanism which explains that some individuals appear to be more sensitive to inhaled endotoxin than others is not known, although multiple genetic factors arguably play an important role. For instance, polymorphisms in genes of the innate immunity pathway such as toll-like receptor 4 (*TLR4*) and *CD14* have been associated with endotoxin responsiveness in experimental, occupational, and domestic settings [14-17]. It has also been shown that responsiveness to inhaled LPS is correlated with the ability of inflammatory cells to produce

cytokines in response to LPS stimulation [13]. We therefore tested the hypothesis that heterogeneity in respiratory responses to occupational endotoxin exposure may be explained by inter-individual variation in cytokine responsiveness of LPS-stimulated inflammatory cells. We measured cytokine responses to purified endotoxin (lipopolysaccharide, LPS) in an *ex vivo* whole blood assay (WBA). Production of inflammatory cytokines tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) were measured as these cytokines are known to play a key role in endotoxin-induced inflammatory responses. In addition, the regulatory cytokine IL-10 was measured. The specific objectives of the present study were to investigate associations between LPS-induced cytokine release and respiratory health outcomes in a population with medium and high exposure to airborne endotoxin, and more specifically, to study whether the thus defined cytokine responsiveness modifies associations between endotoxin exposure and respiratory health parameters.

METHODS

Study population

The study population consisted of 434 Dutch agricultural processing workers and farmers sampled from a previously described survey [1, 18]. An overview of the recruitment is presented in Figure 1. A total of 342 agricultural processing workers (91% of 376 eligible) consented to blood sampling. A total of 340 farmers (65% of 525 eligible) indicated that they would not object being contacted for a medical examination. For logistical reasons, however, only approximately 100 farmers could be included. A random sample of 122 farmers was approached by telephone, and 92 (75%) participated.

During a visit to the worksite at the start of the workday, venous blood samples were taken for *ex vivo* cytokine induction in a whole blood assay (WBA), monocyte counts which were determined using an automated hematology analyzer (Cell-Dyn Sapphire), and serology. Lung function was assessed according to standard guidelines [19]. A standardized questionnaire with items of the European Community Respiratory Health Survey (ECRHS) questionnaire was used to assess asthma-like symptoms [1, 20]. Atopy was defined as serum IgE to one or more common allergens [18].

In total, data from 412 of 434 participating subjects were used (323 agricultural processing workers, 89 farmers), because WBA could not be performed with blood from 15 subjects, and we excluded four subjects aged <18 or >65 yrs and three subjects with incomplete data. Farmers who participated in the medical examination did not differ significantly from farmers in the original sample with respect to endotoxin exposure, personal characteristics and the prevalence of reported wheeze ($p > 0.05$; t-test or χ^2). Nevertheless, selection bias in farmers was a potential concern and, therefore, sensitivity analyses were performed by repeating all statistical analyses in the subpopulation of agricultural processing workers only. The study

protocol was approved by the institutional ethics committee and all participants gave written informed consent.

Endotoxin exposure

Endotoxin sampling, quantification, and modelling has been described before [1]. Briefly, 249 personal full-shift inhalable dust samples were collected on glass fiber filters using portable pumps and GSP sampling heads. Filters were extracted and supernatants were analysed by the quantitative kinetic chromogenic Limulus Amebocyte Lysate (LAL) assay. Airborne endotoxin exposure levels were assigned to each participant, by using a model which predicted endotoxin exposure by work environment and job title. Endotoxin exposure levels ranged over three orders of magnitude (~ 10 -10,000 EU/m³), and the geometric mean (GM) in the present study (319 EU/m³) was similar to the GM of the original sample (265 EU/m³).

Whole blood assay

The procedure for LPS stimulation in a WBA was based on preliminary optimization studies, in which heparin- and EDTA-anticoagulated blood of 2-12 healthy donors was incubated with or without LPS or other stimuli in various concentrations, and cell supernatants were harvested after one or two days of culture [21].

In the present study, heparinised blood samples were kept on ice until use in the WBA. Average time between blood collection and start of the cell culture was 4.1 ± 1.4 h. Whole blood samples (80 μ l aliquots) were transferred into sterile round bottom 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) and diluted with an equal volume of RPMI 1640 supplemented with 100U penicillin/100 μ g streptomycin per ml (Gibco, Breda, The Netherlands). LPS (Fluka, Buchs, Switzerland) was added to the culture medium to a final concentration of 1 ng/ml (~ 91 EU/ml in the LAL assay). For each subject, negative controls

were included by using medium without LPS. After culture for 18h at 37°C, 5% CO₂, and 96% relative humidity, the plates were centrifuged (15 min, 100xg) and supernatants were stored at -20°C. TNF- α , IL-1 β , and IL-10 were measured in supernatants using a Bio-plex assay [22]. The lower detection limits were 2.2 pg/ml, 20.8 pg/ml, and 2.3 pg/ml for TNF- α , IL-1 β , and IL-10 respectively. On the basis of the preliminary results in 12 subjects [21] it was decided not to measure IL-6 and IL-8, as intra-individual variation exceeded inter-individual variation for these cytokines.

LPS-induced cytokine production was calculated by subtracting cytokine concentrations in the negative control wells from corresponding LPS-stimulated samples. Cytokine concentrations were inversely related to the time that elapsed between venapuncture and start of the cell culture: TNF- α , IL-1 β , and IL-10 production decreased with 32%, 12%, and 8% per hour, respectively. Therefore, cytokine concentrations were adjusted to t=0h by applying a correction factor. For each cytokine, the population was dichotomized into low and high responders according to median cytokine concentrations for each cytokine separately. Distribution of high and low cytokine responses over individuals was however hardly influenced by the correction for time-until-incubation. Categorizations on the basis of adjusted or unadjusted levels were ~90% concordant.

Data analysis

Results from 412 individuals with complete data were available. Associations between cytokine production and respiratory symptoms were assessed by logistic regression analysis. Odds ratios (ORs) for associations between endotoxin exposure and respiratory symptoms were estimated in strata of low and high cytokine responders. Associations between ln-transformed endotoxin exposure levels and respiratory symptoms were presented as ORs and 95%CI for an interquartile range (IQR) increase of endotoxin exposure [1]. The shape of the

exposure-response relationships was further analyzed by means of generalized additive modelling (smoothing; SAS Proc GAM), using Akaike's information criterion to select the degree of smoothing [23]. Associations between endotoxin exposure and FEV₁ in low and high cytokine responders were analyzed by linear regression analysis in the 363 male subjects of the study population. In the main data analysis, cytokine levels were *a priori* dichotomized by the median to keep all 412 subjects in the analyses. Additional sensitivity analyses were performed by using the upper and lower tertiles, by treating cytokine production as a continuous variable, and by comparing subjects with a low or high response for all three cytokines.

RESULTS

Determinants of cytokine response

Figure 2 shows LPS-induced cytokine levels. High variability in cytokine release between participants was observed (2-3 orders of magnitude), in particular for TNF- α . Production of the three cytokines was significantly correlated, with pairwise correlations between ln-transformed cytokine levels of 0.57 for IL-1 β and IL-10, 0.63 for IL-1 β and TNF- α , and 0.61 for IL-10 and TNF- α (Pearson correlation coefficients, $p < 0.001$). Women ($n=49$) had a significantly lower LPS-induced cytokine production than men, in particular for IL-1 β (factor 0.78; $p=0.04$) and TNF- α (factor 0.56; $p=0.001$). The sex difference in cytokine production was independent of age or endotoxin exposure. Dichotomization into low and high responders was therefore performed separately for men and women. Furthermore, we calculated cytokine levels adjusted for the number of blood monocytes, and for all three cytokines the concentrations “per 10^6 monocytes” correlated strongly with the non-adjusted cytokine levels “per ml” (Pearson’s r 0.93 to 0.96; ln-transformed levels). When we dichotomized the population into low and high responders on the basis of monocyte-adjusted cytokine levels, we found excellent concordance with classifications on the basis of non-adjusted levels (85-90%).

Table 1 shows the distribution of potential determinants over high and low TNF- α , IL-1 β , and IL-10 responders. There was no association with smoking habits or atopy for any of the cytokines. Blood monocyte counts were higher in high IL-1 β and IL-10 responders than in low responders (0.57 and 0.56 vs. 0.51 and $0.52 \times 10^9 \text{ L}^{-1}$, respectively; $p < 0.05$). High IL-10 responders were on average 3.5 y younger and had less often grown up on a farm than low IL-10 responders. These relations could also be confirmed among non-farmers ($p < 0.05$). Higher age and a farm childhood were independent determinants of a lower IL-10 response in a multiple logistic regression model with ORs of 0.97 (0.95-0.99) and 0.57 (0.38-0.85),

respectively. Monocyte levels were also independently associated with IL-1 β and IL-10. Analyses with cytokine concentrations as continuous variables or upper and lower tertiles yielded very similar results.

A weak but statistically significant positive association between occupational endotoxin exposure and high IL-1 β and IL-10 response was found. Although endotoxin exposure was associated with cytokine levels, the difference in average exposure levels between high and low responders was small and a strongly overlapping range of exposure levels was found in each subgroup. ORs for an interquartile range increase in endotoxin exposure were 1.25 (1.05-1.49) for IL-1 β and 1.20 (1.01-1.42) for IL-10. When cytokine production was treated as a continuous variable, a significant but low correlation was also found between endotoxin exposure and IL-1 β (ln-transformed values, Pearson's $r=0.11$, $p=0.02$), but not between exposure and IL-10 or TNF- α ($p>0.2$). Endotoxin exposure was also weakly correlated with blood monocyte levels ($r=0.12$, $p=0.02$), and adjustment for monocytes slightly attenuated the associations between endotoxin exposure and IL-1 β and IL-10.

Associations between cytokine response and respiratory symptoms

High IL-10 responders had wheeze symptoms more often than low IL-10 responders (Table 2). TNF- α response was also positively, but non-significantly, associated with symptoms (ORs between 1.32 and 1.86), whereas no relationship between IL-1 β response and symptoms was found. Cough symptoms (daily cough, cough up phlegm, nocturnal cough) were not associated with cytokine production (data not shown). No evidence of confounding or effect modification by variables such as age, farm childhood, smoking or atopy was found, and exclusion of farmers or women did not influence results. Endotoxin exposure was not a confounding factor for the association between cytokine response and respiratory outcomes, as was expected on the basis of the weak associations between endotoxin exposure and

cytokine responses. Monocytes were not associated with symptoms ($p>0.3$ for all symptoms), and adjustment for monocytes did not change the results. Similar patterns were observed when cytokine production was treated as a continuous variable, or when the lowest and highest tertiles of cytokine production were compared.

Cytokine responses as effect modifiers

We earlier found a clear dose-response relationship between endotoxin exposure and the prevalence of respiratory symptoms [1], and we found similar results in the present study. In stratified analyses these dose-response relationships appeared to be largely restricted to the subjects with high cytokine responses (Table 3). For all three cytokines, high responders showed significant positive relationships between occupational endotoxin exposure and wheeze, wheeze with shortness of breath, and wheeze without a cold (ORs between 1.54 and 2.13). In contrast, weak and non-significant associations were found in low responders (ORs between 0.98 and 1.26). ORs for wheeze and wheeze with shortness of breath were significantly different between low and high TNF- α responders (formal test for interaction, $p<0.05$). Exposure-response relationships between endotoxin exposure and cough symptoms were not modified by cytokine response (data not shown).

The shape of the relationships between endotoxin and wheeze seemed log-linear for both high and low TNF- α responders (Figure 3A). A non-linear relationship was suggested for IL-1 β and IL-10 high responders. A steep exposure-response relationship was observed, but only at very high endotoxin exposure levels (>1000 EU/m³). Curves for low responders flattened at high exposures (Figure 3B and 3C; spline $p<0.1$).

Results changed only marginally when female workers were excluded, or when farmers were excluded. In agricultural processing workers, ORs between 1.55 and 2.05 were found for

wheeze-related symptoms in high cytokine responders, and between 0.78 and 1.29 for low responders.

FEV₁ did not differ between low and high responders ($p>0.2$, data not shown). However, in high TNF- α , IL-1 β and IL-10 responders, a significantly lower FEV₁ was found at increasing levels of endotoxin (80 to 92 ml lower for an IQR increase in endotoxin exposure, $p<0.05$), whereas in low responders a much smaller and non-significant difference in FEV₁ (18 to 30 ml lower) was found at the same increase in exposure (Table 4). Formal tests for interaction were, however, not significant for any of the cytokines. Similar results were obtained when analyses shown in Tables 2, 3, and 4 were repeated with low and high responders classified on the basis of monocyte-adjusted cytokine levels.

Sensitivity analyses with combined cytokine response

Because LPS-induced production of the three cytokines was highly correlated, we also combined responses into a single entity. A comparison of subjects with a low or high response to LPS for all three cytokines (low: $n=124$; 30.1% and high: $n=123$; 29.9%), and ignoring subjects with discordant responses ($n=165$; 40.1%), showed that high cytokine production was positively associated with symptom prevalence, with ORs between 1.69 and 3.43 ($p<0.05$ for wheeze without a cold). Effect modification of the exposure-response relationships between endotoxin exposure and symptoms or FEV₁ was very similar to results shown in Table 3 and 4. High responders had ORs between 1.68 and 2.07 for respiratory symptoms ($p<0.05$ for wheeze, wheeze with SOB, wheeze without a cold) and a 172 ml lower FEV₁ ($p=0.002$) for an IQR increase in endotoxin exposure. In contrast, corresponding ORs for low responders were between 0.80 and 1.17 for symptoms ($p>0.05$) and their FEV₁ was on average only 27 ml lower when endotoxin exposure increased over an IQR. The interaction

between cytokine response and endotoxin exposure was significant for the association with wheeze (p-value interaction=0.03).

DISCUSSION

In the present study we found that susceptibility to adverse respiratory effects induced by high occupational endotoxin exposure is reflected by the inflammatory response to LPS, measured as *ex vivo* TNF- α , IL-1 β , and IL-10 responses in a WBA. For high cytokine responders, exposure-response relationships between occupational endotoxin exposure and FEV₁ or respiratory symptoms were relatively steep and statistically significant. In low cytokine responders, associations were weak and non-significant.

On theoretical grounds, dichotomization of subjects into high and low responders according to median cytokine production would be in agreement with the finding that more than one third of individuals appeared responsive to acute, experimental endotoxin inhalation [11]. Sensitivity analyses showed that results were robust when tertiles were used or when cytokine production was treated as a continuous variable. Moreover, the number of blood monocytes did not account for the differences in susceptibility between low and high responders. Heterogeneous results were observed for the three cytokines under study, in particular for associations between cytokine production and symptom prevalence. A possible biological meaning of this difference is, however, not directly available, and should be interpreted with caution. Alternatively, categorization based on all three cytokine responses together is expected to be less sensitive for measurement error of individual cytokines, and thus strengthened the finding that *ex vivo* LPS-induced cytokine response is associated with susceptibility to endotoxin-induced respiratory disorders. On the basis of our findings it cannot be concluded whether the three different cytokine responses should be interpreted as three separate biologically meaningful responses. *In vivo*, the immunologic role of TNF- α and IL-1 β as inflammatory mediators and IL-10 as an inhibitor of cytokines are quite distinct. However, we stimulated whole blood with LPS during 18h, resulting in stimulation of

monocytes and not of IL-10 producing T-cells. Therefore, the cytokine responses could also be interpreted as a single correlated response of monocytes to LPS.

It has been shown that time between blood collection and start of incubation in the WBA is a critical variable for cytokine release [24, 25]. Although we made every effort to minimize this time period by optimizing fieldwork and laboratory procedures, we could not start the *in vitro* incubation of all blood samples within two hours after collection, and we therefore adjusted all measured cytokine levels for the time-until-incubation. In our study, variation in time-until-incubation was small, and correction had only a minor impact on the categorization of high and low responders.

We found lower TNF- α and IL-1 β production from whole blood samples of women compared with men. A similar sex difference has been observed previously in a study among volunteers, and was attributed to the influence of sex hormones [26]. We therefore categorized men and women separately into high and low responders, and we also showed that results were very similar when we excluded women from analyses.

Prolonged exposure to high levels of endotoxin has been shown to lead to persistent airway symptoms and obstruction, probably as a result of chronic mucosal airway inflammation. Apparently, high occupational exposure may also have a systemic effect by increasing the number of circulating monocytes and enhancing the ability of blood monocytes to produce cytokines, as suggested by the weak but positive association between endotoxin exposure and IL-1 β and IL-10 (Table 1). Also when cytokine responses were combined (all low vs. all high), we found a positive association between endotoxin exposure and the cytokine response (OR 1.31 (1.04-1.66)). In a previous study among grain workers, TNF- α release from unstimulated and stimulated peripheral blood monocytes (3 ng/ml LPS) increased significantly across the workweek in exposed workers, and the increase in TNF- α levels was associated with grain dust exposure levels [27]. In our study, blood was collected from Monday to

Wednesday at the start of the workday, and there was no association between day of the week and cytokine production ($P>0.3$). A lower response in the WBA has been described in mice who had developed tolerance to LPS [28], but in human studies including the present one, there is no evidence of development of tolerance at adult age to occupational endotoxin exposure, at least not at the level of cytokine responses [27].

Childhood exposure to microbial agents in a farm environment has been associated with lower LPS-induced IL-10 responses by Braun-Fahrlander et al. [29]. They found an inverse association between endotoxin load in children's mattress dust and LPS-induced IL-10. Our finding that workers who had grown up on a farm had a lower IL-10 response (Table 1) appears to be in accordance with that study. Whether our observation relates to a long-lasting down regulation of IL-10 responses as a result of childhood exposures to endotoxin and other farm-associated microbial agents, or to other factors correlated to a farm childhood, however, is not clear.

LPS-induced release of IL-6 and IL-8 by alveolar macrophages and peripheral blood monocytes correlates with airflow obstruction in response to experimental LPS inhalation [13]. This suggests that cytokine release from inflammatory cells is predictive of *in vivo* susceptibility. A WBA is a simple alternative to stimulating isolated monocytes or alveolar macrophages, especially in large-scale population studies with complicated logistics. The WBA reproducibly measures an individual's cytokine responsiveness, with considerably larger differences in cytokine release between individuals than within individuals when repeated during a period between one and six weeks [24, 25, 30]. It is as yet unknown whether individual responses are reproducible over a longer period of time. Moreover, longitudinal studies are needed to assess the predictive value of cytokine responsiveness.

In conclusion, we have shown that TNF- α , IL-1 β , and IL-10 production by *ex vivo* LPS-stimulated blood monocytes is associated with *in vivo* susceptibility to occupational endotoxin

exposure-associated respiratory effects. Exposure-response relationships between endotoxin exposure and respiratory symptoms and FEV₁ were relatively steep and statistically significant for high cytokine responders, whereas weak, non-significant associations were found for low cytokine responders.

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TABLE 1 Determinants of cytokine response

| | TNF- α response | | IL-1 β response | | IL-10 response | |
|--|------------------------|-------------|-----------------------|-------------|----------------|--------------|
| | Low | High | Low | High | Low | High |
| Age (yrs), mean (SD) | 42.6 (10.2) | 41.6 (10.6) | 42.9 (9.7) | 41.2 (11.0) | 43.9 (9.5) | 40.3 (10.9)* |
| Smoking habits (%) | | | | | | |
| Never | 42.0 | 38.8 | 41.0 | 39.8 | 43.4 | 37.4 |
| Former | 29.3 | 33.5 | 29.8 | 33.0 | 28.3 | 34.5 |
| Current | 28.8 | 27.7 | 29.3 | 27.2 | 28.3 | 28.2 |
| Farm childhood (%) | 47.8 | 44.2 | 47.8 | 44.2 | 54.2 | 37.9* |
| Atopy (%) | 20.0 | 18.5 | 21.5 | 17.0 | 16.1 | 22.3 |
| Blood monocytes (x 10 ⁹ L ⁻¹), GM (GSD) | 0.53 (1.4) | 0.55 (1.4) | 0.51 (1.3) | 0.57 (1.4)* | 0.52 (1.4) | 0.56 (1.3)* |
| Endotoxin exposure (EU/m ³), GM (GSD) | 276 (4.9) | 307 (5.5) | 238 (5.1) | 356 (5.2)* | 247 (5.2) | 343 (5.2)* |

The population was dichotomized into low (n=206) and high (n=206) responders according to median cytokine concentrations for each cytokine separately.

* p<0.05, t-test or χ^2 -test

EU: endotoxin unit; GM: geometric mean; GSD: geometric standard deviation

TABLE 2 Prevalence of respiratory symptoms in 206 low cytokine responders and in 206 high cytokine responders and association with cytokine responses above the median

| | TNF- α response | | IL-1 β response | | IL-10 response | |
|-------------------------|------------------------|------|-----------------------|------|------------------|------------------|
| | Low | High | Low | High | Low | High |
| | % | % | OR (95% CI) | % | OR (95% CI) | % |
| Wheezing | 10.7 | 15.1 | 1.45 (0.80-2.63) | 13.2 | 0.91 (0.50-1.65) | 8.8 |
| Wheezing with SOB | 7.3 | 9.7 | 1.32 (0.65-2.67) | 9.3 | 0.77 (0.38-1.57) | 4.9 |
| Wheezing without a cold | 4.9 | 8.8 | 1.86 (0.83-4.17) | 6.3 | 1.13 (0.52-2.46) | 2.4 |
| Awakened due to SOB | 2.4 | 4.4 | 1.77 (0.58-5.41) | 3.4 | 0.95 (0.32-2.79) | 1.5 |
| | | | | | | 5.3 |
| | | | | | | 3.51 (0.93-13.2) |

SOB: shortness of breath. ORs are adjusted for sex, age, smoking habits, and farm childhood. Bold ORs represent significant associations between cytokine response and symptoms ($p < 0.05$).

TABLE 3 Association between occupational endotoxin exposure and respiratory symptoms in 206 low cytokine responders and in 206 high cytokine responders

| | TNF- α response | | IL-1 β response | | IL-10 response | |
|--------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| | Low | High | Low | High | Low | High |
| | Endotoxin exposure OR (95% CI) | Endotoxin exposure OR (95% CI) | Endotoxin exposure OR (95% CI) | Endotoxin exposure OR (95% CI) | Endotoxin exposure OR (95% CI) | Endotoxin exposure OR (95% CI) |
| Wheezing | 1.02 (0.66-1.56) | 1.82 (1.24-2.67)** | 1.14 (0.76-1.72) | 1.66 (1.13-2.45) | 1.12 (0.70-1.78) | 1.54 (1.08-2.20) |
| Wheezing with SOB | 1.02 (0.63-1.66) | 2.13 (1.33-3.41)** | 1.26 (0.79-2.00) | 1.74 (1.08-2.81) | 1.24 (0.68-2.28) | 1.56 (1.04-2.34) |
| Wheezing without a cold | 0.98 (0.54-1.77) | 1.92 (1.21-3.04)* | 1.20 (0.70-2.06) | 1.69 (1.05-2.72) | 1.05 (0.45-2.49) | 1.63 (1.07-2.47) |
| Awakened due to SOB | 1.79 (0.73-4.35) | 1.86 (0.99-3.49) | 2.16 (1.01-4.61) | 1.55 (0.82-2.95) | 0.88 (0.29-2.68) | 2.28 (1.22-4.28)* |
| SOB: shortness of breath | | | | | | |

* p interaction <0.10; ** p interaction <0.05; Bold ORs represent significant associations between endotoxin exposure and symptoms (p<0.05).

Data are presented as the odds ratio (OR) for an interquartile range increase in endotoxin exposure with 95% confidence interval (95% CI). ORs are adjusted for sex, age, smoking habits, and farm childhood.

TABLE 4 Association between occupational endotoxin exposure and FEV₁ (ml) in 363 male subjects according to cytokine response

| | Low responders (n=181) | | High responders (n=182) | |
|---------------|------------------------|------|-------------------------|------|
| | β (95%CI) | p | β (95%CI) | p |
| TNF- α | -30 (-111 to 51) | 0.47 | -80 (-154 to -6) | 0.03 |
| IL-1 β | -18 (-97 to 62) | 0.66 | -80 (-157 to -3) | 0.04 |
| IL-10 | -28 (-103 to 47) | 0.46 | -92 (-170 to -14) | 0.02 |

Data are presented as the reduction in FEV₁ at an interquartile range increase in endotoxin exposure. Regression coefficients (β) and 95%CI are adjusted for standing height, age, atopy, smoking habits and farm childhood.

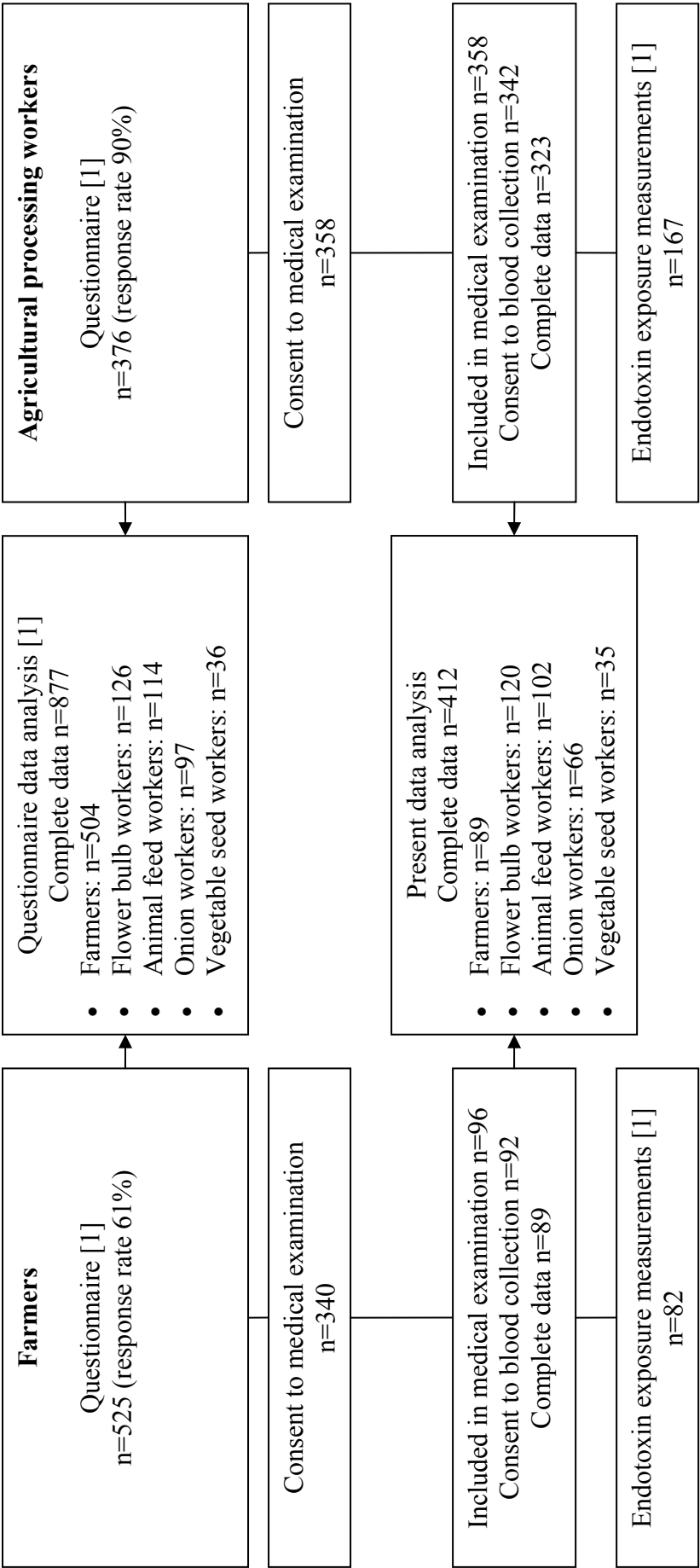


FIGURE 1 Flow diagram representing study population recruitment.

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FIGURE 1 Flow diagram representing study population recruitment.

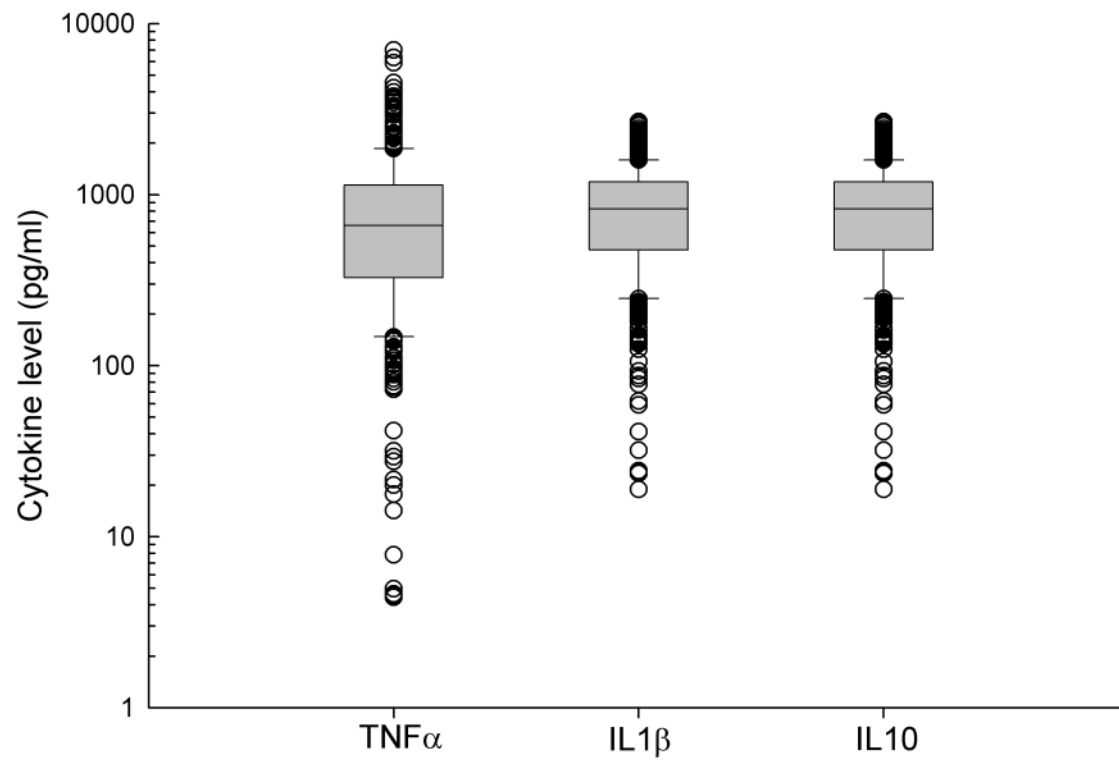


FIGURE 2 Box plots representing cytokine production in response to 1 ng/ml LPS in all study participants (n=412). Boxes delineate the 25th and 75th percentiles with a horizontal line at the median and whiskers depict the 10th and 90th percentiles.

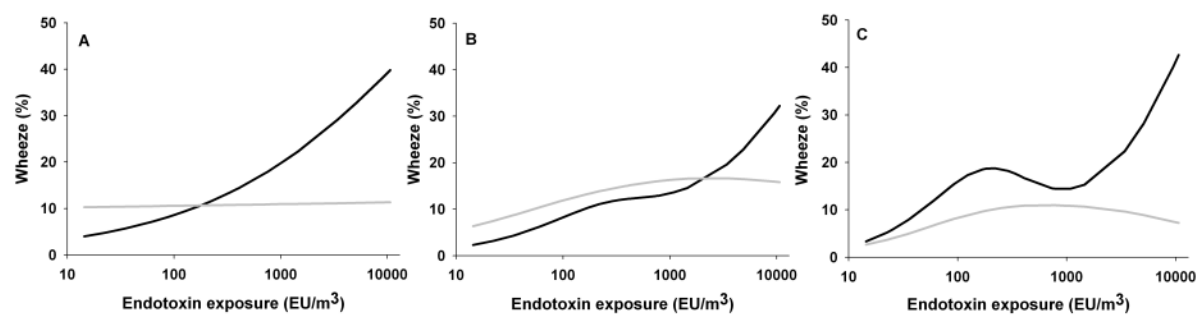


FIGURE 3 Smoothed plots representing dose-response relationships of endotoxin exposure and current wheeze in 206 high responders (— black curve) and 206 low responders (— grey curve) for TNF- α (A), IL-1 β (B), and IL-10 (C).