

Toll-like receptor 4 variants reduce airway response in human subjects at high endotoxin levels in a swine facility

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Background: Toll-like receptor 4 (*TLR4*) variants have been shown to reduce the respiratory responses to inhaled LPS in controlled experiments among healthy volunteers.

Objective: We sought to investigate whether naive subjects with *TLR4* variants showed reduced respiratory response to a complex aerosol including endotoxin as a major constituent.

Methods: Twenty-nine nonsmoking, nonatopic healthy subjects with *TLR4* 299/399 polymorphisms and 29 age- and sex-matched, wild-type *TLR4* control subjects were exposed for 5 hours each in a noncontaminated environment (baseline day) and in a swine confinement facility (exposure day). There were 16 men and 13 women in each of the 2 age- and sex-matched groups.

Results: *TLR4* polymorphic subjects who were exposed to high endotoxin levels (≥ 1550 EU/m³) had less reduction in the percentage across-shift change in FEV₁ from baseline than did wild-type subjects exposed to similar endotoxin levels. Among subjects exposed to higher endotoxin levels, the mean differences in the percentage across-shift changes between baseline and exposure days were significantly less in *TLR4* polymorphic subjects compared with those seen in wild-type subjects in FEV₁ ($-8.48\% \pm 1.52\%$ [mean $\pm$ SE] vs $-11.46\% \pm 1.79\%$, $P = .001$), forced expiratory flow between 25% and

75% of forced vital capacity ($-18.30\% \pm 1.99\%$ vs $-24.14\% \pm 3.28\%$, $P = .009$), and FEV₁/forced vital capacity ratio ($-5.40\% \pm 0.56\%$ vs $-8.53\% \pm 1.51\%$, $P = .04$). These patterns were not observed in IL-6 levels from serum and nasal lavage fluid, IL-8 levels from nasal lavage fluid, white blood cell counts, or blood differential counts.

Conclusion: The association between *TLR4* variants and reduced airway responsiveness to inhaled particulate was observed at high endotoxin concentrations, creating the possibility of certain threshold phenomena for the apparent protective effect of *TLR4* variants. (J Allergy Clin Immunol 2009;123:1-11.)

Key words: Airway hyperresponsiveness, blood counts, cytokines, endotoxin, gene-environment interaction, lung function, swine confinement facility, Toll-like receptor 4 variants

The Toll-like receptor 4 gene (*TLR4*) encodes the principal innate immunity receptor in human subjects for LPS.^{1,2} Recognition of LPS by this receptor leads to release of proinflammatory cytokines, including IL-1 β , IL-6, IL-8, and TNF- α).^{3,4} Two cosegregating single nucleotide polymorphisms at amino acid positions 299 (Asp299Gly) and 399 (Thr399Ile) of the *TLR4* gene have been associated with reduction in the airway and inflammatory responses to LPS in human subjects in the laboratory setting.^{5,6} *TLR4* polymorphisms also are demonstrated to increase the risk of septic shock^{7,8} and infection with respiratory syncytial virus⁹ and decrease the risk of atherosclerosis.¹⁰

However, it is not known whether the airways response to inhaled LPS, which is a purified form of endotoxin, in the laboratory⁵ might relate to the airways response to the occupational setting in which endotoxin comprises an important constituent of the ambient inhaled aerosol. Because the *TLR4* polymorphisms occur in up to 10% of the population and endotoxin is ubiquitous in the indoor environment of swine operations, the effect of these variants on chronic endotoxin exposure represents a relevant concern and might provide important clues to understanding the health of workers in swine operations.

Exposure to bioaerosols, including endotoxin, has been associated with workers' health in swine operations.¹¹ Indoor air quality in swine confinement buildings is characterized by a variety of suspended particulates, including dust, endotoxin, ammonia, and hydrogen sulfide.¹² These exposures have been associated with excess symptoms,¹³ reductions in lung function,¹⁴ increases in airway responsiveness,^{15,16} longitudinal decrease in lung function in exposed swine workers,¹⁷⁻¹⁹ and acute across-shift changes in lung function and inflammatory response in healthy

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Abbreviations used

FEF₂₅₋₇₅: Forced expiratory flow between 25% and 75% of forced vital capacity
 FEV₁: Forced expiratory volume in the first second
 FVC: Forced vital capacity
 PC₂₀: Methacholine dose required for a 20% decrease in FEV₁
 TLR4: Toll-like receptor 4

volunteers.^{3,4,20} Therefore the purpose of this study was to determine whether airways response in this occupational setting with high endotoxin exposures was influenced by the *TLR4* polymorphism in nonsmoking healthy volunteers.

METHODS

Additional details can be found in the **Methods** section of this article's Online Repository at www.jaiconline.org. The study was conducted at the Canadian Centre for Health and Safety in Agriculture and the Prairie Swine Centre, Inc, in the winter of 2006. The study was approved by the Biomedical Research Ethics Board of the University of Saskatchewan. Written consent was obtained from the study participants, who were students from the University of Saskatchewan and were paid an honorarium. In total, 846 students who were never smokers participated in the study, completed a brief screening questionnaire for allergic and respiratory history, and were genotyped for the *TLR4* 299 polymorphism. Of these, 842 were also successfully genotyped for the *TLR4* 399 polymorphism. Hardy-Weinberg equilibrium was met for distributions of both the *TLR4* 299 and 399 variants. Some results from the screening data have been published elsewhere.²¹

For the acute exposure study, 42 subjects with the *TLR4* 299/399 polymorphisms and 68 subjects with wild-type *TLR4* who were without a self-report of hay fever, allergy, wheeze, or asthma were invited to participate in skin prick tests to 25 allergens. Subjects who exhibited 1 or more positive allergy test responses or responded to methacholine tests on the baseline day were excluded. After these exclusions, 29 subjects with the *TLR4* 299/399 polymorphisms and 29 age- and sex-matched, *TLR4* wild-type control subjects were studied. Among the polymorphic group, there were 13 heterozygous subjects each in the male and female groups and 3 subjects homozygous for the variant allele in the male group.

Four subjects, consisting of 2 sets of age- and sex-matched pairs, were usually studied together on the exposure day in the swine facility. The baseline day was always conducted before the exposure day because of the potential residual effect of the exposure day on the baseline day. The research staff and technicians who participated in the study were blinded to *TLR4* genotype. Study participants practiced forced expiratory maneuvers to minimize the learning effect²² and completed a detailed questionnaire on the training day.

On the baseline day, subjects arrived at 30-minute intervals at the laboratory from 7:30 AM until 9 AM for pulmonary function tests, returned to their classes, and came back to the laboratory approximately 5 hours later on the same day for repeat pulmonary function tests, blood sampling, and nasal lavage and methacholine challenge tests.

On the exposure day, which was at least 7 days later than the baseline day, subjects arrived at the swine barn at 7:30 AM to begin a 5-hour exposure in the barn. After reviewing the biosecurity rules of the barn, subjects completed pulmonary function measurements and entered the barn room at 15-minute intervals equipped with an attached personal air sampler, leaving the barn room for approximately 20 minutes at the 2½-hour point for pulmonary function tests. Subjects rode a stationary bicycle for 3 km at 18 km/h for each hour while in the barn to simulate the usual work load in a swine facility. At the end of the 5-hour exposure period, subjects completed pulmonary function tests and provided blood and nasal lavage samples. Methacholine challenge tests were conducted 3.5 hours after exposure to the barn environment.

The percentage across-shift change in lung function was defined as the relative change in lung function at the end of 5-hour exposure in the swine barn in comparison with lung function before entering the barn. It was obtained for

each participant by dividing the difference between the postexposure and pre-exposure lung function by the pre-exposure lung function and then multiplying by 100 to determine the percentage across-shift change in lung function. The statistical analyses were stratified by low and high endotoxin exposures. The cutoff point for the low and high endotoxin exposures was 1550 EU/m³, which was the median value of all personal-sampler measurements taken during the study. This value is very close to the average value of endotoxin concentration observed in swine operations in studies conducted in Sweden and the Netherlands.^{23,24} Wilcoxon signed-rank tests were used to test the differences between baseline and exposure days. Mixed models with random intercepts were used to test the differences in physiologic and immunologic outcomes between the wild-type and polymorphic groups after controlling for matching. Further details of the mixed model are presented in the Methods section of this article's Online Repository.

RESULTS

The distributions of age, sex, height, weight, and baseline lung function measurements were similar in the wild-type and polymorphic groups (Table I). As expected from the study design, mean values of carbon dioxide and ammonia and geometric mean values of dust, endotoxin load, and endotoxin concentration in the indoor environment were similar for the wild-type and polymorphic study groups (Table II). The circadian changes in the log-transformed endotoxin concentrations were stronger than the changes between study days, with circadian changes accounting for 82.5% of the total variation in the log-transformed endotoxin concentration. The log-transformed endotoxin concentration was not significantly correlated with outdoor temperature. The distribution of subjects exposed to low (<1550 EU/m³) and high (≥1550 EU/m³) concentrations of endotoxin was balanced between the study groups for both male and female participants (data not shown).

Mean percentage across-shift changes in forced expiratory volume in the first second (FEV₁), forced expiratory flow between 25% and 75% of forced vital capacity (FEF₂₅₋₇₅), and FEV₁/forced vital capacity (FVC) ratio were significantly greater on exposure days compared with those on baseline days for both low and high endotoxin exposures in the wild-type and polymorphic groups (Table III). This pattern was also observed for FVC, but the difference in the mean percentage across-shift change in FVC between baseline exposure days was significant only in the wild-type group for low and high endotoxin exposures (Table III). After controlling for matching, differences in the percentage across-shift changes in FEV₁, FEF₂₅₋₇₅, and FEV₁/FVC ratio between the baseline and exposure days were statistically different between the wild-type and polymorphic groups for high endotoxin exposure but not for low endotoxin exposure (Fig 1). The reduced response in the percentage across-shift change in FEV₁ for higher endotoxin exposure in the polymorphic group is further shown in Fig 2. The response observed for the polymorphic group, as indicated by the median percentage across-shift change in FEV₁, for high endotoxin exposure was very similar to that observed for the wild-type and polymorphic groups for low endotoxin exposure. The same subject had the lowest percentage across-shift change in FEV₁ on the baseline and exposure days for the higher endotoxin exposure in the polymorphic group.

When adjusted for carbon dioxide and ammonia levels in random intercept models, results for the higher endotoxin exposure were similar to those reported in Fig 1 for the differences in the percentage across-shift changes in FVC ($P = .59$),

TABLE I. Demographic characteristics and baseline lung function values for the *TLR4* 299/399 wild-type and polymorphic groups

	Wild-type group (n = 29), mean (SE)	Polymorphic group (n = 29), mean (SE)
Age (y)	20.5 (0.40)	20.3 (0.42)
Height (cm)	173.6 (1.80)	176.6 (2.00)
Weight (kg)	71.2 (2.62)	74.9 (3.21)
Sex, no. (%)		
Male	16 (55.2)	16 (55.2)
Female	13 (44.8)	13 (44.8)
FVC (L)	4.98 (0.21)	4.98 (0.21)
FEV ₁ (L)	4.10 (0.15)	4.20 (0.16)
FEF ₂₅₋₇₅ (L/s)	4.21 (0.21)	4.48 (0.20)
FEV ₁ /FVC ratio (%)	83.7 (1.38)	85.6 (1.06)

FEV₁ ($P < .001$), FEF₂₅₋₇₅ ($P = .006$), and FEV₁/FVC ratio ($P = .045$) between the baseline and exposure days. In addition, outdoor temperature was not correlated with pre-exposure lung function measurements on the exposure days, and the results were similar to those reported in Fig 1 when adjusted for outdoor temperature in the random intercept model. When stratified by sex, differences in the mean percentage across-shift change in FEV₁ between baseline and exposure days for the polymorphic and wild-type groups for high endotoxin exposure were significantly different for male ($-8.07\% \pm 2.01\%$ [mean $\pm$ SE] vs $-11.51\% \pm 2.45\%$, $P = .06$) and female ($-8.88\% \pm 2.44\%$ vs $-11.41\% \pm 2.82\%$, $P = .002$) subjects. Differences in the mean percentage across-shift change between the baseline and exposure days for the polymorphic and wild-type groups for high endotoxin exposure was significantly different only in male subjects for FEF₂₅₋₇₅ ($-16.19\% \pm 2.44\%$ vs $-25.12\% \pm 4.55\%$, $P < .001$) and FEV₁/FVC ratio ($-5.17\% \pm 0.74\%$ vs $-9.78\% \pm 2.61\%$, $P = .06$).

When both the low and high endotoxin exposure groups were combined, there was an almost significant interaction ($P = .07$) between genotype group and log-transformed endotoxin concentration in the random intercept model for the differences in the percentage across-shift changes in FEV₁ between the baseline and exposure days, indicating a gene-environment interaction (data not shown). A significant interaction was also observed between genotype group and log-transformed endotoxin concentration for the differences in the percentage across-shift changes in FEF₂₅₋₇₅ ($P = .02$).

The methacholine dose required for a 20% decrease in FEV₁ (PC₂₀) was significantly lower after exposure compared with the baseline observation for both low and high endotoxin exposures in the wild-type and polymorphic groups (Table III), but the differences in PC₂₀ between the exposure and baseline days were not statistically significant between the wild-type and polymorphic groups after controlling the matching.

As shown in Table IV, significant changes were observed in the mean white blood cell counts between baseline and exposure days in the wild-type and polymorphic groups for both low and high endotoxin exposure. Neutrophil, lymphocyte, and monocyte counts increased from the baseline day to the exposure day for low and high exposure in both the wild-type and polymorphic groups, but the increase in lymphocyte counts was not statistically significant ($P = .48$) for high exposure in the polymorphic group. No significant changes were observed between the baseline and exposure days in basophil and eosinophil counts for low and

TABLE II. Swine barn indoor environmental characteristics during the 17-day study period by the *TLR4* 299/399 wild-type and polymorphic groups

	Wild-type group (n = 29), mean (SE)	Polymorphic group (n = 29), means (SE)
Area samples		
CO ₂ (ppm)	960.2 (39.0)	960.0 (39.1)
NH ₃ (ppm)	10.8 (0.82)	10.8 (0.82)
Personal samples, geometric mean (95% CI)		
Dust concentration (mg/m ³)	0.89 (0.61-1.29)	0.87 (0.61-1.22)*
Endotoxin load (EU/mg)	1686.3 (1316.3-2160.3)	1700.4 (1397.0-2,070.2)
Endotoxin concentration (EU/m ³)	1500.1 (923.8-2436.7)	1509.7 (942.7-2417.7)*

*Data are missing for 1 observation because of pump malfunction.

high endotoxin exposure in both the wild-type and polymorphic groups.

As seen in Table V, the differences in the mean IL-6 levels from serum and nasal lavage fluid and IL-8 levels from nasal lavage fluid between the baseline and exposure days were statistically significant for low and high endotoxin exposure in the wild-type and polymorphic groups, but there were no significant differences between the 2 study groups after controlling for matching. The mean serum concentration of TNF- α was not significantly different between the baseline and exposure days for low and high endotoxin exposure in the polymorphic and wild-type groups (Table V).

When the 3 homozygous subjects were excluded from the analysis, results for FVC, FEV₁, and FEF₂₅₋₇₅ were very similar to those reported in Fig 1 for the higher endotoxin exposure, but the difference in percentage across-shift change in FEV₁/FVC ratio between the baseline and exposure days was not significant ($P = .09$).

DISCUSSION

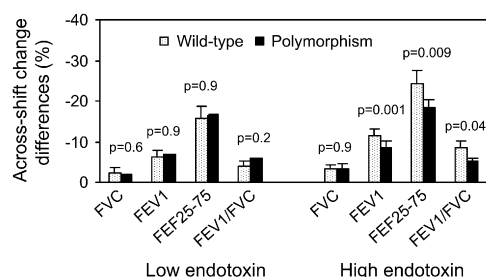
Our results indicate that individuals with the *TLR4* 299/399 polymorphisms showed reduced respiratory responsiveness to inhaled endotoxin when the endotoxin concentration exceeded a certain level (approximately 1550 EU/m³). An endotoxin exposure of 1200 EU/m³ has been reported as the average value in swine operations.^{23,24}

The overall prevalence of homozygous variants is quite low (<1.0%), and the prevalence of heterozygous variants is about 10%. In our study the genotyping on 846 students to obtain the population with and without the *TLR4* variants yielded only 3 homozygous variants. As shown in the work by Arbour et al⁵ and in the current study, heterozygous subjects showed less physiologic response to LPS and bioaerosols containing endotoxin than did wild-type subjects. In our study no significant differences were observed in the blood counts and immunologic outcomes between homozygotes and heterozygotes in the *TLR4* polymorphic group at the exposure levels encountered.

The apparent interaction between *TLR4* gene polymorphisms and endotoxin exposures in a swine operation facility is consistent with what was referred to as "reactive norms" by Martinez²⁵ for establishing a gene-environment interaction. Although our study

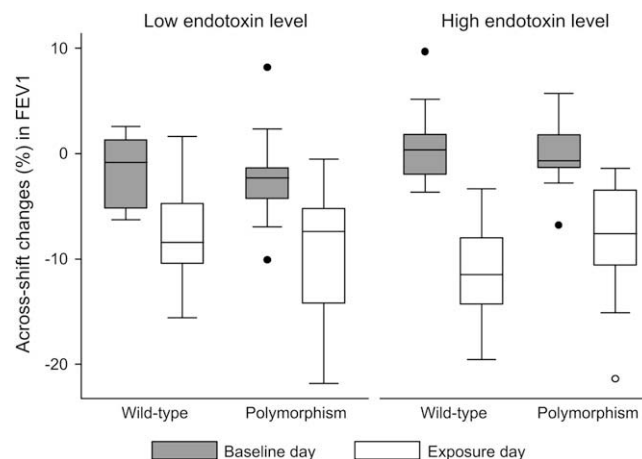
TABLE III. Across-shift changes in lung function and PC₂₀ for the *TLR4* 299/399 wild-type and polymorphic groups on baseline and exposure days for low and high endotoxin exposure

Factors	Wild-type group						Polymorphic group					
	Baseline day, mean (SE)		Exposure day, mean (SE)		Difference, mean (SE)		Baseline day, mean (SE)		Exposure day, mean (SE)		Difference, mean (SE)	
Low endotoxin exposure (<1550 EU/m ³), n = 15 for wild-type and n = 14 for polymorphic groups												
Shift change												
FVC (%)	-1.33	0.63	-3.63	1.06	-2.30†	1.21	-1.91	0.73	-3.56	1.13	-1.65	1.17
FEV ₁ (%)	-1.36	0.82	-7.71	1.20	-6.35†	1.57	-2.28	1.13	-9.04	1.51	-6.76*	1.62
FEF ₂₅₋₇₅ (%)	-1.44	2.27	-17.30	2.27	-15.86†	2.93	-3.32	2.50	-19.85	2.84	-16.52†	2.02
Ratio (%)	-0.35	0.60	-4.23	0.99	-3.88†	1.29	0.14	0.79	-5.74	1.26	-5.88†	1.20
Methacholine PC ₂₀ (mg/mL)	603.0	265.4	54.40	29.7	-548.6†	267.7	437.9	124.5	107.0	67.3	331.0†	75.4
High endotoxin exposure (≥1550 EU/m ³), n = 14 for wild-type and n = 14 for pleomorphic groups												
Shift change												
FVC (%)	-0.77	0.59	-3.91	0.93	-3.14†	1.15	-0.74	0.53	-3.86	1.28	-3.12	1.39
FEV ₁ (%)	0.60	0.96	-10.86	1.27	-11.46†	1.79	-0.12	0.81	-8.59	1.50	-8.48†	1.52
FEF ₂₅₋₇₅ (%)	1.77	1.69	-22.37	2.65	-24.14†	3.28	0.46	1.42	-17.84	2.17	-18.30†	1.99
Ratio (%)	1.21	0.46	-7.32	1.28	-8.53†	1.51	0.40	0.37	-5.00	0.60	-5.40†	0.56
Methacholine PC ₂₀ (mg/mL)	302.4	101.5	47.5	31.2	-254.8†	78.5	523.7	186.7	69.4	34.5	-454.3†	185.4

P* < .05.†*P* < .01 for the significance of the differences in percentage across-shift change in lung function between the baseline and exposure days (Wilcoxon tests).FIG 1.** Mean differences in percentage across-shift lung function changes between baseline and exposure days by low and high endotoxin levels for the *TLR4* 299/399 wild-type and polymorphic groups. Error bars indicate 1 SE. Significance of the differences was determined by using random intercept models after allowing for matching. Low and high endotoxin concentrations are defined as below and above the median (<1550 EU/m³ and ≥1550 EU/m³).

was conducted on young, nonsmoking healthy subjects, the results emphasize the importance of considering gene-environment interaction in studies investigating the health of subjects who are exposed to high endotoxin concentrations.

The association between endotoxin exposure and physiologic outcomes, immunologic outcomes, or both in human subjects has been reported in several studies.^{15,18,26-28} The *TLR4* gene was identified as the principal innate immunity receptor in human subjects for LPS, and 2 polymorphisms in the extracellular domain of this receptor were shown to decrease the response to inhaled LPS.⁵ Comparing our results with those of Arbour et al,⁵ it appears that a critical level of endotoxin or LPS is required to demonstrate a difference in FEV₁ response to endotoxin between the wild-type and polymorphic groups. In the study by Arbour et al, the mean difference in percentage across-shift change in FEV₁ between the polymorphic and wild-type groups was statistically significant when the LPS dose reached 6.5 μg and the percentage reduction in FEV₁ from baseline values reached about 10% in the wild-type group.⁵ In our study the mean difference

**FIG 2.** Distribution of the percentage across-shift changes in FEV₁ on baseline and exposure days for low and high endotoxin levels for the *TLR4* 299/399 wild-type and polymorphic groups.

in percentage across-shift change in FEV₁ between the baseline and exposure days for the polymorphic and wild-type groups was statistically significant when the endotoxin concentration in the ambient environment was greater than the median value (1550 EU/m³). The controlled inhalation of LPS in the study by Arbour et al⁵ is not comparable with that seen in our study, in which subjects were exposed to a complex dust in a swine confinement setting. Assuming that endotoxin levels and ventilation rates in our study were fixed at the median values of 1550 EU/m³ and 0.502 L/s, respectively, during the 5-hour exposure, the total exposure to endotoxin that subjects would have experienced during the 5 hours is approximately 0.19 μg (see the Methods section in this article's Online Repository for a detailed calculation). However, we recognize that these approximated exposures might underestimate the dose of inhaled endotoxin in our study subjects because enhanced ventilation rates during the bicycle exercise

TABLE IV. Blood counts and differentials for the *TLR4* 299/399 wild-type and polymorphic groups on baseline and exposure days for low and high endotoxin exposure

Factors	Wild-type group					Polymorphic group					P value
	Baseline, mean (SE)		Exposure day, mean (SE)		P value*	Baseline day, mean (SE)		Exposure day, mean (SE)		P value*	
Low endotoxin exposure (<1550 EU/m ³), n = 15 for wild-type and n = 14 for pleomorphic groups											
Blood counts											
WBC (10 ⁹ /L)	5.12	(0.29)	8.10	(0.49)	.001	6.13	(0.40)	9.08	(0.50)	.001	.98†
Differentials											
Neutrophils (10 ⁹ /L)	2.51	(0.22)	5.29	(0.42)	.001	3.38	(0.36)	5.64	(0.49)	.001	.37
Lymphocytes (10 ⁹ /L)	1.98	(0.09)	2.15	(0.11)	.03	2.09	(0.15)	2.59	(0.16)	.001	<.001‡
Monocytes (10 ⁹ /L)	0.49	(0.04)	0.55	(0.04)	.09	0.49	(0.04)	0.68	(0.03)	.002	.003‡
Basophils (10 ⁹ /L)	0.01	(0.01)	0.02	(0.02)	.41	0.01	(0.01)	0.02	(0.02)	.41	.95‡
Eosinophils (10 ⁹ /L)	0.13	(0.02)	0.10	(0.02)	.13	0.14	(0.01)	0.14	(0.01)	1.00	.14†
High endotoxin exposure (≥1550 EU/m ³), n = 14 for wild-type and n = 14 for pleomorphic groups											
Blood counts											
WBC (10 ⁹ /L)	5.74	(0.25)	8.84	(0.57)	.001	6.05	(0.35)	9.19	(0.54)	.002	.96‡
Differentials											
Neutrophils (10 ⁹ /L)	2.93	(0.16)	5.55	(0.59)	.001	3.35	(0.26)	6.20	(0.49)	.001	.78‡
Lymphocytes (10 ⁹ /L)	2.23	(0.16)	2.54	(0.14)	.08	2.06	(0.16)	2.18	(0.10)	.48	.39
Monocytes (10 ⁹ /L)	0.42	(0.03)	0.58	(0.05)	.006	0.49	(0.04)	0.69	(0.06)	.01	.39†
Basophils (10 ⁹ /L)	0.01	(0.01)	0.01	(0.01)	1.00	0.03	(0.01)	0.01	(0.01)	.18	.23†
Eosinophils (10 ⁹ /L)	0.13	(0.02)	0.13	(0.02)	1.00	0.14	(0.03)	0.14	(0.03)	.71	.67†

WBC, White blood cell.

*Significant differences between baseline and exposure days from Wilcoxon tests.

†Significance of absolute differences between the wild-type and polymorphic groups after allowing for matching with random intercept models.

‡Maximum likelihood estimation in random intercept models failed because of numeric problems, and significance of absolute differences between the wild-type and polymorphic groups was based on the iterated expectation and maximization method.

TABLE V. Serum and nasal cytokine levels for the *TLR4* 299/399 wild-type and polymorphic groups on baseline and exposure days for low and high endotoxin exposure

Low and high endotoxin exposure											
Factors	Wild-type group					Polymorphic group					<i>P</i> value
	Baseline, mean (SE)		Exposure, mean (SE)		<i>P</i> value*	Baseline, mean (SE)		Exposure, mean (SE)		<i>P</i> value*	
Low endotoxin exposure (<1550 EU/m ³), n = 15 for wild-type and n = 14 for pleomorphic groups											
Serum cytokines											
IL-6 (pg/mL)	0.50	(0.31)	1.55	(0.52)	.001	0.25	(0.06)	0.72	(0.15)	.17	.05‡
TNF-α (pg/mL)	0.83	(0.13)	0.94	(0.14)	.57	0.77	(0.09)	0.85	(0.07)	.01	.36†
Nasal cytokines											
IL-6 (pg/mL)	2.82	(1.13)	144.24	(46.05)	.001	9.95	(5.60)	186.43	(76.13)	.001	.65‡
IL-8, (pg/mL)	256.46	(69.92)	1186.67	(127.96)	.001	225.61	(49.41)	1459.28	(212.13)	.001	.24†
High endotoxin exposure (≥1550 EU/m ³), n = 14 for wild-type and n = 14 for pleomorphic groups											
Serum cytokines											
IL-6 (pg/mL)	0.31	(0.12)	1.03	(0.12)	.001	0.55	(0.33)	1.91	(0.55)	.006	.21‡
TNF-α (pg/mL)	0.86	(0.16)	1.13	(0.16)	.12	1.00	(0.22)	1.36	(0.17)	.14	.27†
Nasal cytokines											
IL-6 (pg/mL)	1.33	(0.41)	123.42	(41.41)	.001	2.89	(0.91)	177.40	(51.22)	.001	.34‡
IL-8 (pg/mL)	179.22	(34.07)	1372.7	(34.07)	.001	428.39	(176.26)	1411.22	(198.03)	.005	.43‡

*Significant differences between baseline and exposure days from Wilcoxon tests.

†Significance of absolute differences between the wild-type and polymorphic groups.

‡Maximum likelihood estimation in random intercept models failed because of numeric problems, and significance of absolute differences between the wild-type and polymorphic groups was based on iterated expectation and maximization method.

periods of 10 minutes every hour might have increased the endotoxin dose.

Although the endotoxin exposure in our study might be lower than the doses reported in the study by Arbour et al,⁵ the biologic potency of the endotoxin might have been quite different because the swine confinement environment results in a much more

complex bioaerosol. Nevertheless, in both studies polymorphic individuals were protected from the airway responses to endotoxin or LPS, and a threshold in the endotoxin/LPS concentration, as well as the respiratory response (approximately 10% reduction in FEV₁ from baseline value), was needed to observe a difference between the wild-type and polymorphic groups.

The relationship between *TLR4* variants and the concentration of endotoxin observed in our study is similar to that reported by Martinez²⁹ for the interaction between *CD14* polymorphisms and the concentration of endotoxin needed to stimulate an allergic response and asthma. Subjects with CC homozygotes for *CD14* –159 were at increased risk of allergic sensitization at low endotoxin exposure but at reduced risk at high endotoxin concentrations. Similar results were replicated for *CD14* polymorphisms in 4 studies conducted in a white population from Tucson, Arizona³⁰; German-speaking children from Western Europe³¹; children from Greater Manchester, United Kingdom³²; and an adult population from Detroit, Michigan.³³ In our study subjects who were AA homozygotes (wild-type) for *TLR4* had increased percentage across-shift change in FEV₁ only for high endotoxin exposure, whereas subjects with GG homozygotes and heterozygotes (AG) for *TLR4* 299 had a modest decrease in percentage across-shift change in FEV₁ for high endotoxin exposure. In aggregate, these findings highlight the importance of environmental exposures in identifying the biologic and clinical significance of the genetic variants.

The results from our study and other studies^{29–32} have shown that some human populations are more susceptible to endotoxin exposure than others and emphasize the importance of considering endotoxin concentration in determining environmental controls in occupations in which workers are exposed to high endotoxin levels. Establishing standards in the occupational setting that protect the most vulnerable individual based on age, sex, genotype, or any other definable feature represents a prudent approach to standard setting that will be increasingly informed by genetic findings.

Inflammatory cytokine levels (IL-6 from serum and nasal lavage fluid and IL-8 from nasal lavage fluid) and blood differential counts increased significantly after exposure in the swine facility for both the low and high endotoxin exposure levels in the *TLR4* wild-type and polymorphic groups. The lack of response in the polymorphic group might be related to the very low prevalence of homozygous variants ($n = 3$) or the possibility that although a threshold level of endotoxin is relevant to the airway response, this same threshold is not relevant to the peripheral blood. Alternatively, inhaled endotoxin might not have resulted in a high enough concentration in the peripheral blood to be relevant for the regulation of the blood cell count and therefore to result in an observed difference between wild-type and polymorphic individuals. For instance, similar increases were also observed for the mean serum concentration of TNF- α for both the wild-type and polymorphic groups, but the differences in TNF- α levels between the baseline and exposure days were not statistically significant for both low and high endotoxin exposure levels in the wild-type and polymorphic groups. The reasons for these nonsignificant increases are not known but will be explored in a future study.

A limitation in our study is that subjects included in the study were nonsmoking college students naive to the swine environment and free of allergy or bronchial hyperresponsiveness. By excluding the atopic subjects, we might have selected the subjects who were more sensitive to endotoxin. The number of positive skin prick test responses has been shown to be associated with sCD14 levels and *CD14* gene polymorphisms,³⁰ which in turn are associated with LPS responsiveness.³⁴ The results from our study cannot be generalized directly to swine workers and workers in other industries who are chronically exposed to bioaerosols and endotoxin. Further

studies on workers with and without *TLR4* polymorphisms are required to extend our results to exposed workers.

Another limitation in our study is that only dusts, endotoxin, ammonia, and carbon dioxide were measured, and other contaminants, including other bioaerosols, hydrogen sulfide, odoriferous fatty acids, glucans, CpG motifs in bacterial DNA, and viruses, were not measured, which might have confounded the exposures and could have induced responses in subjects with and without *TLR4* 299/399 polymorphisms.

In conclusion, our results indicate that individuals with the *TLR4* 299/399 polymorphisms have less reduction in percentage across-shift change in FEV₁ from baseline on exposure to swine confinement operations than do wild-type individuals when exposed to relatively high levels of endotoxin. However, immunologic or systemic effects related to endotoxin exposure were not observed in our study.

Clinical implications: Gene-environment interactions should be considered in studies examining the respiratory health of subjects who are exposed to high endotoxin concentrations.

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METHODS

Animal facility and management

The exposure study was conducted at the Prairie Swine Centre, Inc, as previously described,^{E1,E2} with an all-in-all-out grower-finisher swine production room (5.3 m wide and 14.2 m long). The room had 6 pens; each pen (4.2 × 2.0 m) had a partially slatted floor (one third of the pen area) over a 0.6-m-deep manure pit. The room held 10 pigs per pen for a total of 60 pigs at an average starting weight of about 40 kg. The room had dry feeders with an automatic feeding system and nipple drinkers. Pigs were fed *ad libitum* with standard grow-finish mash diets. The room was mechanically ventilated by using a bank of propeller fans controlled by an automatic electronic controller. Heat was supplied by an unvented natural gas unit heater. Throughout the trials, standard environmental control settings recommended for growing-finishing pigs were used in the room to maintain temperature depending on the age of the pigs and the outdoor weather conditions.

DNA isolation and genotyping

Study participants were instructed to swish 10 mL of mouthwash (Scope; Procter & Gamble, Inc, Toronto, Ontario, Canada) vigorously around their mouths for 30 seconds before spitting into a sterile 50-mL bar-coded tube. Genomic DNA was extracted from oral epithelial cells in the mouthwash sample by using the Genra Autopure robot (Genra Systems, Minneapolis, Minn). Working dilutions of DNA at a concentration of 10 ng/μL in RNase/DNase-free purified water were prepared for subsequent genotyping assays and sequencing. TaqMan allelic discrimination assays for the human *TLR4* A299G and T399I polymorphisms were designed by using Primer Express software (Applied Biosystems, Foster City, Calif), and PCR reactions were run on MJ Tetrad thermal Cyclers (Bio-Rad Laboratories, Hercules, Calif). TaqMan genotypes were determined by means of postamplification plate reading on the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Human subject DNA samples that did not genotype well in 2 TaqMan assays were submitted for direct sequencing of the amplicon by means of fluorescent dye termination sequencing on an ABI 3730 capillary sequencer.

Skin prick testing

Allergen skin prick tests were conducted with a battery of 25 allergens and both positive (histamine) and negative (saline) controls. Allergens used were *Aspergillus* species, *Helminthosporium* species, *Cladosporium* species, *Alternaria* species, egg, milk, peanut, shellfish, house dust mite, house dust, grain dust, wheat dust, feathers, cat dander, dog dander, cattle dander, horse dander, box elder, birch, poplar, willow, mixed tree, mixed grass, mixed weeds, and ragweed (Western Allergy Services Ltd, Burnaby, British Columbia, Canada). Subjects were considered to be atopic if 1 or more allergens produced a raised wheal more than 3 mm in diameter compared with that elicited by the negative control.

Lung function measurements

Lung function measurements (ie, FVC, FEV₁, FEV₁/FVC ratio, and FEF₂₅₋₇₅) were measured with a SensorMedics volume displacement spirometer (SensorMedics, Anaheim, Calif), according to specifications of the American Thoracic Society.^{E3} Calculations were made of the percentage change in pulmonary function test variables from the first measurement to the last measurement on baseline and exposure days. Pulmonary function tests were conducted by certified technicians.

Methacholine challenge

Methacholine challenge was performed by using the method described by Cockcroft et al,^{E4} which involves successive 2-minute exposures. We performed an initial inhalation using 0.9% saline and then successive exposures of doubling concentrations of methacholine, starting at 1 mg/mL and stopping when a decrease of 15% in FEV₁ was produced or when the maximum exposure concentration of 250 mg/mL was reached. The bronchial challenges were performed with a Bennett Twin Jet Nebulizer (Puritan Bennett Corp of California, Carlsbad, Calif) with a mass output of 130 mg/min. The subjects, in the sitting position, inhaled the nebulized solution using a mouthpiece and T-piece while breathing tidally for 2 minutes. FEV₁ was measured with an electronic spirometer (V MAX20; SENSORMEDICS, Yorba Linda, Calif) at both 30 and 90 seconds after the 2-minute inhalation of methacholine. The doses of methacholine were

administered at 5-minute intervals. The methacholine concentration that reduced FEV₁ by 20% (PC₂₀) was interpolated or extrapolated from the last 2 responses. PC₂₀ was used as the indicator of responsiveness of the airways.

Nasal lavage procedure

In the nasal lavage procedure, which was adapted from the method of Naclerio et al,^{E5} the neck was extended to approximately 30°, and 5 mL of normal saline was instilled into each nostril with the subject closing the oropharynx for 10 seconds to 2 minutes before expelling the liquid into a sterile container. The volume was recorded and centrifuged (RT600B Refrigerated Centrifuge; Sorvall, Newton, Conn) at 400 rpm for 10 minutes at 4°C and frozen at -70°C for future analysis.

White blood cell methodology

White blood cells were counted with a Coulter Counter STKS model (Coulter Electronics, Hialeah, Fla) by using individual cell volume, high-frequency conductivity, and laser light scatter. A well-clotted sample was centrifuged at room temperature, and the serum was divided into 1-mL aliquots and frozen at -70° to -80° for future cytokine analysis.

Nasal and serum cytokines

Levels of the proinflammatory cytokines IL-6 and TNF-α were measured in cell-free sera by using high-sensitivity immunoassay kits. In addition, IL-8 and IL-6 were measured in nasal wash supernatant. All kits were obtained from R&D Systems (Minneapolis, Minn).

Measurements of air quality and environment

Assessment of environmental contaminants was similar to those used in our previous studies.^{E1,E2} Concentrations of suspended particulate were measured with personal samplers (Dupont Air Sampler Model P4L; Canada Safety Supply, Saskatoon, Saskatchewan, Canada) by using a 37-mm glass fiber filter of 0.8-μm porosity (Type A-E, no. 61652; Gelman, Montreal, Quebec, Canada). Endotoxin analysis was performed at the Vaccine and Infectious Diseases Organization, University of Saskatchewan, by extracting endotoxin from dust filters individually in 10-mL, sterile, nonpyrogenic water (LAL Reagent Water; BioWhittaker, Walkersville, Md) with the Limulus amoebocyte lysate assay (Kinetic-QCL, BioWhittaker). Endotoxin in dust was converted to endotoxin in air and reported in endotoxin units per cubic meter. Inhaled endotoxin was obtained by multiplying the following factors: median endotoxin concentration (1550 EU/m³), minute volume of ventilation (0.008 m³/min), total exposure time (300 min) and deposition fraction (0.5) which was equal to 1860 EU (= 1550 × 0.008 × 300 × 0.5). Using the formula 10 EU = 1 ng, inhaled endotoxin was equal to 186 ng or 0.19 mg in our study.

An existing manifold system connected to Teflon tubing was used to draw gas streams from the ventilation inlet and exhaust of the swine room every 15 minutes during the exposure day. An ammonia analyzer (Model Chillgard RT; MSA Canada, Edmonton, Alberta, Canada) and a carbon dioxide analyzer (Model Guardian Plus; Topac, Hingham, Mass) were used to monitor levels of ammonia and carbon dioxide, respectively.

Statistical methods

The random effects model used to control for matching between wild-type and polymorphic groups for low and high endotoxin levels was of the following form^{E6}:

$$Y_{ij} = \alpha + \beta X_{ij} + \zeta_j + \epsilon_{ij},$$

where Y_{ij} represent the differences in the outcomes (percentage across-shift lung function, PC₂₀, serum and nasal lavage cytokine levels, or blood counts) between baseline and exposure days for the subject i and matched pair j ; X_{ij} is a dichotomous variable, which is assigned 0 and 1 for the wild-type and polymorphic groups, respectively; α is the fixed intercept; β is the regression coefficient for X_{ij} ; ζ_j is the random effect for the j th match pair; and ϵ_{ij} is the residual. The random effects ζ_j and ϵ_{ij} are assumed to be independent and have normal distributions with mean 0 and variances σ^2 and ψ^2 , respectively.

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