

Polymorphisms in the CD14 Gene Associated with Pulmonary Function in Farmers

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Rationale and Objectives: Farmers experience airway obstruction, which may be attributable in part to endotoxin inhalation. CD14 is a receptor for endotoxin. **Materials and Methods:** Based on our findings of increased circulating CD14 associated with the CD14/–159 T allele, we hypothesized that carriers of this allele would have decreased lung function among endotoxin-exposed individuals. CD14/–159TT farmers ($n = 19$) had significantly lower lung function as measured by FEV₁ ($p = 0.028$) and mean forced expiratory flow during the middle half of the FVC (FEF_{25–75}) ($p = 0.05$) compared with farmers with the C allele ($n = 78$). Also, farmers with the CD14/–1619GG genotype ($n = 11$) were associated with lower lung function (FEV₁, $p = 0.008$; FEF_{25–75}, $p = 0.009$) compared with farmers with the A allele ($n = 86$). **Results:** No association between CD14/–550 and lung function was observed (FEV₁, $p = 0.32$; FEF_{25–75}, $p = 0.11$). Increased prevalence of wheezing was reported in farmers homozygous for CD14/–159T ($p = 0.013$) or CD14/–1619G ($p = 0.019$) compared with farmers with the CC or AA genotype, respectively. No association was found between TLR4/Asp299Gly and lung function or wheeze. **Conclusion:** We conclude that the CD14/–159 or CD14/–1619 loci may play a role in modulating lung function and wheeze among agricultural workers.

Keywords: endotoxin; farming; lipopolysaccharide; occupational exposure; wheeze

Environmental exposure to endotoxin—a cell wall component of gram-negative bacteria (1)—represents a major occupational hazard for the development of lung inflammation and the impairment of pulmonary function in production agriculture (2–5). Several studies have shown that farmers exposed to large amounts of endotoxin by organic dust as they handle grain and engage in swine and poultry production commonly display signs and symptoms of respiratory disease including asthma, rhinitis, chronic bronchitis, and organic dust toxic syndrome (6, 7). Furthermore, an asthma-like syndrome, characterized by cough, wheezing, and airway obstruction, has been described in one-fourth of swine production workers (8). Airway inflammation characterized by the presence of increased number of neutrophils and increased concentration of cytokines has been found in farmers during seasonal grain harvest and in full-time swine

farmers (9, 10). Although these are complex clinical phenotypes, it is apparent that lung inflammation and decreased lung function are common among farmers, and that these findings are linked to the presence of endotoxin in inhaled dust.

Genetic factors are critical determinants of lung inflammation and pulmonary function. At high endotoxin exposure levels, only a portion of farmers experience respiratory symptoms or disease (11). It is hypothesized that genetic differences that affect responsiveness to endotoxin underlie the variability in airway responsiveness. CD14 is a high-affinity receptor for endotoxin (12, 13). CD14 acts by transferring endotoxin (14) and other bacterial ligands from circulating LPS-binding protein to the toll-like receptor 4 (TLR4)/MD-2 signaling complex. Engagement of this complex results in the activation of innate host defense mechanisms such as release of proinflammatory cytokines. Soluble CD14 (sCD14) is found in normal plasma at microgram concentrations (15) and has been shown to be elevated in the lung as a result of inflammation (16), infection (17, 18), or allergen provocation (19). The gene encoding CD14 is localized on chromosome 5q31.1 (20), a region that has been linked to asthma (21, 22) and pulmonary function (23). We and others have reported elevated serum sCD14 levels in homozygous carriers of the T allele at –159 compared with CC individuals (nomenclature relative to the CD14 transcription start site) (24–27). Functional studies have revealed a biologic mechanism for increased expression of sCD14 among CD14/–159TT individuals (28). We found decreased affinity of DNA/protein interactions at the polymorphic Sp binding site with the T allele. The decreased affinity of the inhibitory Sp3 transcription factor resulted in increased transcriptional activity of the CD14 promoter.

Based on these findings of increased circulating CD14 associated with the CD14/–159 T allele, we hypothesized that carriers of this allele would have decreased lung function among endotoxin exposed individuals. We tested this hypothesis using a farming population typically exposed to abnormally high concentrations of endotoxin and with evidence of respiratory disease (4).

Some of these studies have been previously reported in the form of an abstract (29).

METHODS

Study Population

Nonsmoking, adult males (ages 21–80) who were currently farming or had farmed in the past were recruited from the Keokuk County Rural Health Cohort Study (see online supplement) (30). Eligible subjects ($n = 155$) were contacted with the option of participation. Health history and exposure questionnaires were administered by a nurse, followed by spirometry, skin prick test (SPT), and phlebotomy. The University of Iowa, University of Nebraska Medical Center, and the University of Arizona Institutional Review Boards approved the study.

Clinical Tests

All participants were skin prick–tested and considered positive with a wheal diameter greater than 3 mm to one or more allergens (tree pollen

(Received in original form April 22, 2004; accepted in final form December 7, 2004)

Funded in part by NIH grant ES0036 (T.L.), K30 HL004519 (T.L.), HL66447 (F.M.), HL66806 (F.M.), and NIOSH ES 00296–05.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 171, pp 773–779, 2005

Originally Published in Press as DOI: 10.1164/rccm.200404-5300C on December 10, 2004
Internet address: www.atsjournals.org

mix, grass pollen mix, cockroach mix, mold mix, dust mite mix, grain dust mix, soybean dust, insect mix, cat pelt, hog hair, cattle hair, turkey feathers, dog hair, horse hair, chicken feathers and mouse, rat mix). Spirometry was used to assess pulmonary function. Results were adjusted for the mean age, height, and weight of the population (31). Airway obstruction was defined as FEV₁/FVC ratio of less than 70% (n = 22). Subjects with more than 70% FEV₁/FVC ratio were considered to have no airway obstruction (n = 75).

Genetic Analysis

Polymerase chain reaction was performed with DNA isolated from blood, 200 μ M dNTP, MgCl₂, buffer, 0.4 U Taq polymerase, and 0.1 μ M each of the forward and reverse primers (Table 1). Polymerase chain reaction–amplified DNA was digested at 37°C for 16 hours. Allele-specific polymerase chain reaction was used for CD14/–1619 (Table 1). An internal control was used to amplify the granulocyte macrophage-colony stimulating factor (GM-CSF) gene. The forward primer was 5' CTGGAGTCAGAATGAGGCAC and the reverse primer was 5' CTC AGCTGATAAAGGAGCTC. For the TLR4/Asp299Gly polymorphism, the procedure was as described by Michel and colleagues (32).

Statistical Analyses

χ^2 , Fisher's exact test, and analysis of variance (ANOVA) were used to assess statistical significance using SPSS Windows 10.1 software (SPSS, Inc., Chicago, IL). In the ANOVA *post hoc* analysis, the p value for pairwise comparisons is reported using Bonferroni's correction.

Multiple regression was done using a univariate model. Exploratory analysis tested the significance of each independent variable (SPT, asthma, years of farming [tertiles], currently farming, and farm exposures [i.e., corn, oats, soy, hay, poultry, hog, dairy]) on the dependent variables FEV₁ or mean forced expiratory flow during the middle half of the FVC (FEF_{25–75}). Significant independent variables were then added to the model in a step-up approach. Interaction terms were then tested in the model (haplotypes $\times$ years of farming (tertiles), $\times$ harvested hay, and $\times$ raised hogs). The interaction terms, haplotype $\times$ SPT, $\times$ asthma; $\times$ currently farming; $\times$ harvested corn, soybeans, or oats; $\times$ raised dairy; or poultry were not considered in the model because there were less than five subjects per cell in a cross-tab analysis.

Linkage disequilibrium (r^2) was calculated using Linkage Disequilibrium Analyzer software 1.0. Haplotypes were inferred using Phase software (33) with a 95% probability threshold. In the haplotype analysis, pulmonary function results from each subject were associated with each of the two haplotypes carried by the same subject; consequently, the analyses were performed by chromosomes rather than by subject (34).

RESULTS

Ninety-seven nonsmoking, male, white farmers with a mean age of 59 years (range 25–89) were studied. The participation rate was 62.5% of all eligible subjects. Subjects with airway obstruction were significantly older ($p < 0.0001$) and reported a higher prevalence of wheeze ($p = 0.077$) and cough/phlegm ($p < 0.0001$) than nonobstructed subjects (Table 2). A similar proportion of obstructed and nonobstructed subjects harvested corn, soybeans, hay, and oats (data not shown), or raised hogs, dairy, or poultry. The lack of association between airway obstruction and asthma,

TABLE 2. STUDY POPULATION DEMOGRAPHICS—COMPARISON BETWEEN OBSTRUCTED AND NONOBSTRUCTED FARMERS

Phenotype	Obstructed [§]	Nonobstructed	p Value
N	22	75	
Age, mean yr ($\pm$ SE)	70.0 (2.8)	55.6 (1.6)	< 0.0001
Ethnicity, white, n (%)	22 (100%)	75 (100%)	1.0
Respiratory variables			
FEV ₁ , L, mean ($\pm$ SE)*	2.9 (0.2)	3.4 (0.1)	0.01
FEF _{25–75} , L/s, mean ($\pm$ SE)*	1.7 (0.1)	2.9 (0.1)	< 0.0001
Cough or phlegm, n (%)†	13 (59%)	10 (13%)	< 0.0001
Doctor-diagnosed asthma, n (%)	2 (10%)	4 (6%)	0.53
Wheeze, n (%)‡	8 (36%)	13 (17%)	0.077 [§]
Positive SPT, n (%)	4 (18.2%)	11 (14.7%)	0.69
Farming variables			
Currently farming, n (%)	16 (73%)	59 (80%)	0.49
Farming yr ($\pm$ SE)	39.3 (4.9)	36.0 (2.0)	0.51
Hog farming, n (%)	13 (59%)	42 (56%)	0.80
Dairy farming, n (%)	8 (36%)	13 (17%)	0.077 [§]
Poultry farming, (%)	5 (23%)	6 (8%)	0.118 [§]

* FEV₁ and FEF_{25–75} values adjusted for mean age, height, and weight of population.

† Questionnaire: Do you usually have a cough? or Do you usually bring up phlegm from your chest?

‡ Questionnaire: Do you ever wheeze?

§ Obstructed subjects: FEV₁/FVC < 70%; nonobstructed subjects: FEV₁/FVC $\geq$ 70%.

¶ Fishers exact test.

corn, poultry, and dairy may be the result of too few subjects and the lack of statistical power.

TLR4/Asp299Gly Gene Polymorphism and Susceptibility to Airway Obstruction

The allele frequencies for the TLR4/Asp299Gly polymorphism were A = 94.4% and G = 5.6% and were found to be in Hardy-Weinberg equilibrium. No association was found between the TLR4/Asp299Gly polymorphism and either lung function or wheezing (data not shown). The TLR4/Thr399Ile variant was not analyzed in this study because of the report by Arbour and colleagues indicating complete linkage disequilibrium between these two loci (35).

CD14 Proximal Promoter Single Nucleotide Polymorphisms

Ninety-seven adult farmers were genotyped for three CD14 promoter polymorphisms at locations –1619, –550, and –159 relative to the transcription start site (Figure 1A). The allele frequencies were CD14/–1619 (A = 64%, G = 36%), CD14/–550 (C = 76%, T = 24%) and CD14/–159 (C = 55%, T = 45%) and were found to be in Hardy-Weinberg equilibrium. The strength of pairwise linkage disequilibrium between the three single nucleotide polymorphisms (SNPs) is as follows: –1619 and –550, $r^2 = 0.18$; –1619 and –159, $r^2 = 0.68$; –550 and –159, $r^2 = 0.27$.

TABLE 1. POLYMERASE CHAIN REACTION–BASED RESTRICTION FRAGMENT ASSAYS FOR CD14 POLYMORPHISMS

	Forward Primer	Reverse Primer	MgCl ₂ (mM)	Annealing (°C)	Enzyme	Products
CD14/–159	5'GTGCCAACAGATGAGGTTTAC	5'GCCTCTGACAGTTTATGTAATC	1.5	56	5 U Ava II	C allele: 497 bp T allele: 353, 144 bp
CD14/–550	5'GGAAGGGGGAATTTTCTTTAGGC	5'GGCAGTGTCTCTGATGACTCA	2.4	60	5 U Hae III	C allele: 236 bp T allele: 258 bp
CD14/–1619 A allele	5'GAACAAAACCTCCGTCTCAAATAA TAAATAAATA	5'CTAGACCTCTGGGGAAAGAA CTATATGAAA	2.0	58	None	A allele: 376 bp GMCSF: 960 bp
CD14/–1619 G allele	5'GAACAAAACCTCCGTCTCAAATAA TAAATAAATG	5'CTAGACCTCTGGGGAAAGAA CTATATGAAA	2.0	58	None	G allele: 376 bp GMCSF: 960 bp

Cycling conditions were 95°C for 2 min, 35 cycles of 95°C for 30 s, annealing temperature for 30 s, 72°C for 40 s, and a final extension of 72°C for 10 min.

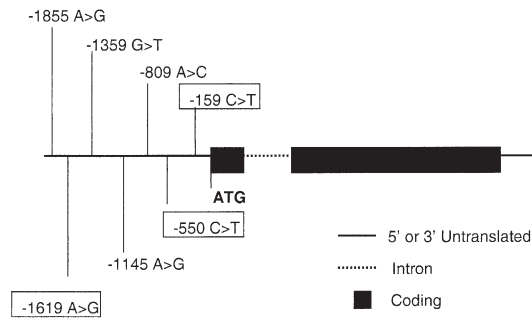


Figure 1. Gene structure and location of CD14 promoter polymorphisms (> 10% frequency). ATG represents translational start codon. Boxed polymorphisms are those selected for genotyping. Polymorphism nomenclature is relative to transcription start site (GenBank Accession No. U00699).

CD14 Gene Polymorphisms and Susceptibility to Airway Obstruction and Wheeze

When using lung function as a dichotomous variable (< 70% or $\geq$ 70% FEV₁/FVC ratio), the CD14/–1619, CD14/–550, and CD14/–159 loci showed no significant association with airway obstruction (Table 3), although the odds of having airway obstruction was 2–3 times greater in carriers of the G allele for CD14/–1619 and of the T allele for CD14/–159. There was a significant association between CD14 polymorphisms and self-reported wheeze (Table 4). Individuals homozygous for –1619G or –159T had a higher prevalence of wheeze than individuals with the A or C allele, respectively ($p = 0.019$ and $p = 0.013$). There was no association between the CD14/–550 loci and wheeze.

Pulmonary function data were used as a continuous variable to investigate the association between FEV₁, FEF_{25–75}, and CD14 polymorphisms. There was a strong association between CD14/–1619 genotypes and lung function (ANOVA; FEV₁, $p = 0.008$; FEF_{25–75}, $p = 0.009$, respectively) (Figure 2). As indicated from the pairwise comparisons, FEV₁ levels for AG subjects were significantly lower than those for subjects with the AA genotype ($p = 0.006$). Similarly, FEF_{25–75} levels associated with the GG genotype were lower than the AA homozygotes ($p = 0.017$).

A significant association was observed between the CD14/

–159 polymorphism and FEV₁ (ANOVA; $p = 0.028$) or FEF_{25–75} (ANOVA; $p = 0.05$) (Figure 2). This significant association was due to lower FEV₁ (pairwise comparison, $p = 0.06$) and FEF_{25–75} (pairwise comparison, $p = 0.05$) values for the TT subjects compared with individuals with the CC genotype. There was no association between the CD14/–550 loci and lung function (Figure 2).

To incorporate the relative contribution of both CD14/–1619 and CD14/–159 polymorphisms on decreased lung function, haplotypes were constructed using the two SNPs: –1619 and –159. Three haplotypes were identified in the population. Their estimated frequencies were 54.6% for A|C (–1619, –159), 36.1% for G|T, and 9.3% for A|T. There was a significant association between the three haplotypes and FEV₁ (ANOVA; $p = 0.045$) or FEF_{25–75} (ANOVA; $p = 0.014$) values (Figure 3). The A|C haplotype showed consistently higher lung function than the G|T haplotype as measured by FEV₁ (pairwise comparison, $p = 0.04$) and FEF_{25–75} (pairwise comparison, $p = 0.014$). Some variability in the A|T haplotype was observed depending on the lung function test. When the CD14/–550 locus was included in the haplotype analysis, similar results were obtained (data not shown).

Multiple Regression Analysis

To rule out potential confounding interactions and evaluate other main effects, we reevaluated the effect of haplotypes on FEV₁ and FEF_{25–75} when testing for the following variables in a step-up approach: SPT, asthma, years of farming (tertiles), currently farming, and farm exposures (i.e., corn, oats, soy, hay, poultry, hog, and dairy). Table 5 shows the results of the multiple regression analysis for factors related to FEV₁ and FEF_{25–75}. The only independent variables that were significant predictors of lung function, and therefore were included in the final model, were CD14/haplotypes and exposure to corn. For example, the G|T haplotype was associated with lower FEV₁ and FEF_{25–75} levels (1.39 and 1.10 units, respectively) than was the A|C haplotype. Subjects who grew corn as their main crop were more likely to have lower FEV₁ and FEF_{25–75} levels (1.19 and 0.70 units, respectively) than were those who did not grow corn. However, because of the low number of subjects not exposed to corn ($n = 7$), the importance of exposure to corn as a risk factor for decreased lung function in this population must be

TABLE 3. FREQUENCY OF CD14 POLYMORPHISMS IN OBSTRUCTED AND NONOBSTRUCTED FARMERS

Polymorphism	Nonobstructed [§] (<i>n</i> = 75)	Obstructed [§] (<i>n</i> = 22)	OR (95% CI)	<i>p</i> Value [¶]
CD14/–1619*				
AA	44%	23%	1.0	
AG	47%	59%	2.45 (0.79, 7.63)	0.18
GG	9%	18%	3.77 (0.80, 17.72)	0.18
CD14/–550†				
CC	53%	59%	1.0	
CT	43%	41%	0.87 (0.33, 2.28)	0.81
TT	4%	0%	ND	ND
CD14/–159‡				
CC	33%	14%	1.0	
CT	49%	59%	2.93 (0.76, 11.3)	0.15
TT	17%	27%	3.85 (0.83, 17.9)	0.13

ND = not determined.

* p Value (χ^2) = 0.16.

† p Value (χ^2) = 0.61.

‡ p Value (χ^2) = 0.18.

[§] Obstructed subjects: FEV₁/FVC < 70%; nonobstructed subjects: FEV₁/FVC $\geq$ 70%.

[¶] Fisher's exact test.

TABLE 4. FREQUENCY OF CD14 POLYMORPHISMS IN EVER-WHEEZE AND NEVER-WHEEZE FARMERS

Polymorphism	Never-wheeze (n = 76)	Ever-wheeze [§] (n = 21)	OR (95% CI)	p Value [¶]
CD14/-1619*				
AA	45%	19%	1.0	
AG	47%	57%	2.8 (0.83, 9.62)	0.102
GG	8%	24%	7.1 (1.46, 34.5)	0.019
CD14/-550 [†]				
CC	51%	67%	1.0	
CT	45%	33%	0.6 (0.21, 1.59)	0.326
TT	4%	0%	ND	ND
CD14/-159 [‡]				
CC	36%	5%	1.0	
CT	47%	67%	10.5 (1.30, 83.3)	0.008
TT	17%	28%	12.5 (1.36, 111.1)	0.013

ND = not determined.

* p value (χ^2) = 0.034.[†] p value (χ^2) = 0.36.[‡] p value (χ^2) = 0.021.[§] Questionnaire: Do you ever wheeze?[¶] Fisher's exact test.

considered cautiously. Interestingly, there was a significant interaction between CD14 haplotypes and years of farming (Table 5). The relationship between CD14 haplotypes and lung function described was observed only in subjects who farmed, on average, 37.9 years or less. There was no relationship between CD14 haplotypes and lung function among subjects who farmed, on average, 57.2 years. Note that of the subjects with the G/T haplotype, the number of subjects with many years of farming (mean 57.2 years) tended to be less than those with fewer years of farming (mean 15.4 years).

The gene by gene interaction for CD14 and TLR4 polymorphisms was not investigated because of the lack of association between airway obstruction and TLR4 polymorphisms.

DISCUSSION

This study provides data on the association between three SNPs in the 5' flanking region of the CD14 gene and lung function using

genotype and haplotype analysis. In this population sample, we demonstrate that the genotypes CD14/-1619GG and CD14/-159TT are associated with decreased FEV₁ and FEF₂₅₋₇₅ among nonsmoking, male farmers. In contrast, no association with lung function was found at the CD14/-550 locus. The association between self-reported wheeze and CD14/-1619 and CD14/-159 polymorphisms substantiates these results and suggests that CD14 gene polymorphisms are important determinants of airway obstruction among farmers.

As part of the Program for Genomic Applications of the NHLBI, 37 SNPs in the CD14 gene were identified by sequencing the complete coding region of CD14, intron I, and approximately 6 kb of 5' genomic DNA for 90 individuals (36, 37). Only nine SNPs had a minimal allele frequency greater than 10%, and none of the SNPs resulted in a putative amino acid change. Of the nine frequent SNPs in the CD14 gene, seven were identified in the proximal promoter region (2 kb) (36, 37). Of these seven SNPs, almost complete linkage disequilibrium was reported

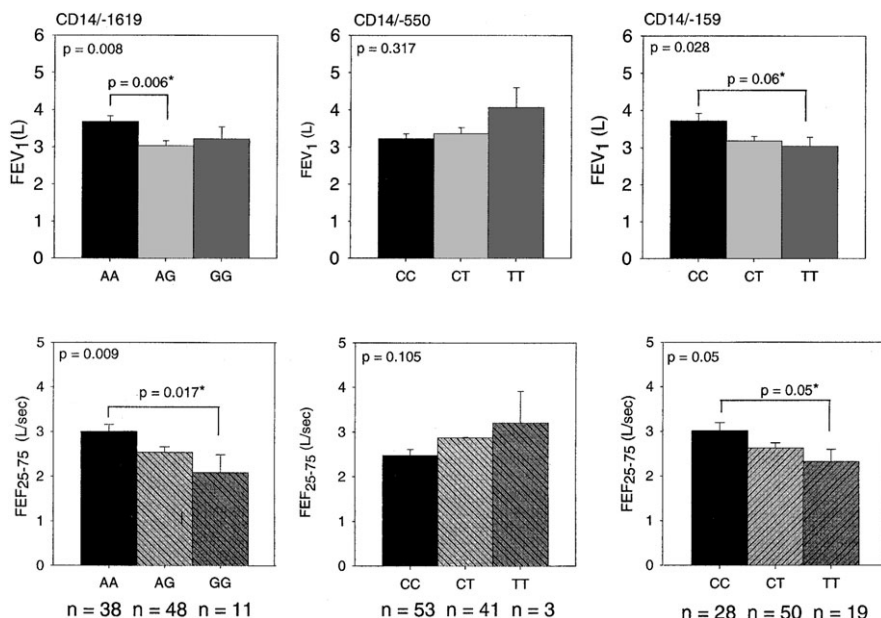


Figure 2. Association of FEV₁ (L) and FEF₂₅₋₇₅ (L/second) with CD14/-1619 and CD14/-159 genotypes. (Left) Lung function values versus CD14/-1619 genotypes. (Middle) Lung function values versus CD14/-550 genotypes. (Right) Lung function values versus CD14/-159 genotypes. Overall p value was determined by analysis of variance. *Pairwise comparisons (n = 3) were done using Bonferroni correction. n = number of subjects for each genotype.

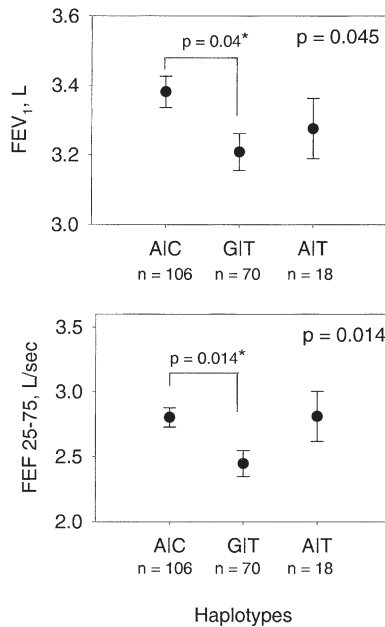


Figure 3. Association of FEV₁ and FEF₂₅₋₇₅ with CD14 haplotypes. Haplotypes (AC, GT, AT) are defined as CD14/–1619 and CD14/–159. Overall p value was determined by analysis of variance. *Pairwise comparisons (n = 3) were done using Bonferroni correction. n = number of chromosomes.

among CD14/–1855, CD14/–1359, CD14/–809, and CD14/–550, and between CD14/–1145 and CD14/–159 (36). Consequently, allelic association analyses performed with only three SNPs at positions –1619, –550, and –159 represent all SNPs in the proximal promoter region of the CD14 gene.

Farmers who were homozygous for the G allele at the –1619 loci were more likely to have decreased pulmonary function as measured by FEV₁ and FEF₂₅₋₇₅ and a higher prevalence of wheeze compared with those with the A allele. In addition, we observed a strong association between the CD14/–159 T allele and decreased lung function and increased wheeze compared with farmers with the C allele. Haplotype analysis duplicated

the pulmonary function and wheeze results at the individual –1619 and –159 loci, suggesting that either the –1619 or –159 locus is a strong predictor of lung function and wheeze. It is difficult to ascertain which polymorphism is functionally associated with lung function in farmers because these SNPs are highly redundant as a result of the high linkage disequilibrium between these two loci.

Our group has previously described the gene polymorphism at CD14/–159 (26). In a longitudinal cohort, we found that children carrying the CD14/–159TT genotype have higher serum sCD14 levels than carriers of the CC or CT genotypes. Similarly, sCD14 was found to be elevated in carriers of the CD14/–159T allele among healthy adults (LeVan and colleagues, unpublished) and in adult patients with inflammatory diseases such as ulcerative colitis (27), *Helicobacter pylori* infection (25), and stable coronary artery disease (24). Functional studies have revealed a potential biologic mechanism for increased expression of sCD14 among CD14/–159TT individuals (28). We found that CD14/–159 had enhanced (32% increase) transcriptional activity in luciferase reporter assays compared with CD14/–159C. By electromobility shift assays, the sequence of the CD14 promoter encompassing the SNP was found to be a Sp1, 2, and 3 protein binding motif. Furthermore, the binding affinity of Sp1, 2, and 3 proteins were found to be enhanced for an oligo containing the CD14/–159C allele compared with the T allele. Sp1 has been shown to function as a transcriptional activator, whereas Sp3 as a repressor. Therefore, we hypothesized that enhanced binding affinity of Sp3, as well as Sp1 and Sp2, at CD14/–159C could result in transcriptional repression of CD14/–159C relative to CD14/–159T. Functionality of the –1619 SNP has not yet been demonstrated; however, its location in the 5' untranslated region of the CD14 gene corresponds to a hepatocyte nuclear factor-3 consensus site, which is juxtaposed to two Alu sequences (38). Interestingly, this transcription factor is expressed in the liver, which is a major site of sCD14 synthesis (39). Further studies are necessary to understand the role of the –1619 locus and its interaction with the –159 locus on CD14 gene regulation.

The mechanism by which CD14 polymorphisms affect pulmonary function is uncertain; however, we hypothesize that farmers homozygous for the –159 T allele have decreased pulmonary function and increased wheeze because of increased levels of sCD14 and inflammation in the lung. As described previously, we have defined the molecular mechanism by which CD14/–159 T affects sCD14 levels; therefore, there is strong evidence to suggest that CD14/–159TT farmers may have increased levels of serum sCD14. Genetically determined higher levels of sCD14 in the lung may result in increased responsiveness to environmental endotoxin and subsequent increased levels of inflammatory cells and cytokines in the lung. There is increasing evidence showing a strong relationship between bronchoalveolar lavage (BAL) sCD14 levels and lung inflammation. Increased sCD14 levels were shown in BAL fluids from subjects with acute respiratory distress syndrome and were correlated with lung inflammatory markers such as polymorphonuclear neutrophils and total protein (16). In mice, blockade of CD14 in the lungs via anti-CD14 monoclonal antibodies was found to attenuate lung inflammation (40). Virchow and colleagues found increased levels of sCD14 in BAL fluids from asthmatic subjects after segmental allergen provocation (41). Many allergen extracts are contaminated with endotoxin, which may account for some of the effects of segmental allergen challenge. Also, increased levels of sCD14 in BAL fluid were correlated with impaired pulmonary function in subjects with tuberculosis and subjects with sarcoidosis (42). Altogether, these results suggest that CD14/–159 may play a role in modulating sCD14 levels and inflammation in the lung and subsequent pulmonary function and wheeze.

TABLE 5. ASSOCIATION OF FEV₁ AND FEF₂₅₋₇₅ WITH CD14 HAPLOTYPES (–1619 | –159): MULTIPLE REGRESSION MODELS

Variable*	FEV ₁ (L) β (SE)	FEF ₂₅₋₇₅ (L/s) β (SE)
Intercept	4.75 (0.34)	3.25 (0.35)
Haplotype [†]		
A/C	—	—
A/T	–0.86 (0.57)	0.28 (0.58)
G/T	–1.39 (0.38)	–1.10 (0.38) ^{**}
Corn as main crop [‡]	–1.19 (0.26)	–0.70 (0.26) ^{**}
Years of farming (tertiles) [§]	0.10 (0.11)	0.09 (0.11)
Haplotype × years farming [†]		
A/C	—	—
A/T	0.30 (0.29)	–0.15 (0.30)
G/T	0.55 (0.17) ^{**}	0.39 (0.18) ^{††}
Adjusted r ² [¶]	0.147	0.102

* Other variables tested in the model were SPT, doctor-diagnosed asthma, currently farming, and farm exposures (i.e., oats, soy, hay, poultry, hog, poultry, and dairy).

[†] The reference category was A/C. Haplotype defined by –1619 | –159.

[‡] “Did not have corn as main crop” was the reference category.

[§] Few years: 15.4 ± 1.0 (SE); moderate years: 37.9 (0.6); and many years: 57.2 (0.9). The “many” years of farming was the reference category.

[¶] Adjusted r² is a measure of the variability in FEV₁ or FEF₂₅₋₇₅ accounted for by the model.

^{||} p < 0.0001.

^{**} p < 0.01.

^{††} p < 0.05.

Few studies have explored the possible genetic risk factors for lung disease (43–47), specifically endotoxin-induced lung disease. Genetic studies performed on mice have shown that the TLR4 gene, which plays a key role in endotoxin signal transduction, regulates endotoxin responsiveness (48). Mutations of the TLR4 gene were shown to abolish responses to endotoxin, causing these animals to be hypersensitive to infection by gram-negative bacteria. Arbour and colleagues found that the missense mutations Asp299Gly and Thr399Ile were associated with hyporesponsiveness to inhaled endotoxin in normal human subjects (35). A blunted response to endotoxin was defined as a failure to decrease FEV₁ by less than 20% after inhalation challenge. In addition, LeVan and colleagues found these missense mutations were also associated with a decreased inflammatory response to inhaled endotoxin as measured by plasma levels of C-reactive protein, LPS-binding protein, and white blood cells (32). Thus it seems likely that the mutations of the TLR4 gene play an important role in regulating endotoxin responsiveness. However, we found no association between the TLR4/Asp299Gly polymorphism, lung function, and wheeze in this population. This result may have been due to the low frequency of the TLR4/Asp299Gly polymorphism (5.6%), thus limiting the power of our analysis. Also, acute exposure to endotoxin may have different effects on pulmonary function compared with intermittent or chronic exposure such as that found in the farming occupation. Last, endotoxin is but one causative agent modulating lung function in farmers. Despite numerous studies indicating endotoxin as the primary agent affecting lung function in farmers, the farming environment is complex and composed of gram-negative and gram-positive bacterial ligands, aeroallergens, and fungi, as well as many undefined inhalants that may very well modulate lung function (4, 49).

For reasons of both statistical significance and biological plausibility, the final model for FEV₁ or FEF_{25–75} in farmers included exposure to corn, CD14 haplotypes, and the interaction between CD14 haplotypes and years of farming. We hypothesize that decreased lung function associated with exposure to corn is potentially a result of the high endotoxin content of dust from stored corn (50). Farmers are potentially exposed to high levels of corn dust while performing various tasks that include cleaning the bin where the corn is stored. Studies by Schwartz and colleagues (51, 52) have demonstrated that inhaled grain dust and endotoxin produce similar physiologic and biological effects in humans, which include airflow obstruction chronic cough and wheeze (53–55). Farmers with the CD14 haplotype, G/T, were at higher risk for impaired lung function than farmers with the A/C haplotype; however, this relationship was only observed in farmers with on average of 37.5 years or less of active farm work. There was no relationship between CD14 haplotypes and lung function among farmers with an average of 57.2 years of farming. We propose this interaction between CD14 haplotypes and lung function is due to a healthy worker effect. Specifically, farmers with the G/T haplotype and at risk for airway obstruction may quit farming earlier than those farmers with the low-risk haplotype A/T. This notion is supported by the fact that among subjects with the G/T haplotype, there were fewer subjects that had farmed on average 57.2 years compared with subjects who had farmed 15.4 years. From a public health perspective, farmers with the susceptible genotype may develop chronic airway symptoms on farm exposures and hence be forced to terminate or change employment. Future investigations will evaluate the length of exposure to corn to validate our hypothesis.

Subjects included in this study were all male, nonsmoking farmers. These selection criteria can be viewed as strengths or limitations of the study. With restricted selection, confounding of the association between CD14 polymorphisms and lung function by gender and smoking status was controlled. The study

was limited to male farmers because men perform the majority of the labor in production agriculture. Also, previous studies have shown that smoking (56) is associated with airway obstruction. Therefore, we considered that it was important to control for both of these factors in the process of subject selection. However, because of the selection criteria used, the results of our study could not be generalized to farmers who do not match the selection criteria used herein.

Another potential confounding factor that affects genetic association studies is population stratification. Fifty percent of the population was of German descent; however, there was no significant difference in the prevalence of airway obstruction among Germans and non-Germans. This would suggest that association from population heterogeneity is unlikely; however, it cannot be completely excluded.

In summary, we have demonstrated that variations in the CD14 gene may play an important role in the development of airway obstruction among adult, male farmers.

Conflict of Interest Statement: T.D.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.V.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.J.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; G.P.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; F.D.M. received \$13,000 in 2002 and \$3,000 in 2003 and \$2,500 from Merck for serving as a member on the Merck Scientific Advisory Board and received money from GlaxoSmithKline for presenting at a sponsored event in 2003 (\$1,500) and as a member of the AstraZeneca Speakers Bureau received \$10,000 in 2002 and \$2,500 in 2003 for lectures; M.M.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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