Original Article

Epistatic effect of TLR-1, -6 and -10 polymorphisms on organic dust-mediated cytokine response

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Exposure to organic dust from agricultural environments is associated with inflammatory respiratory conditions. The putative causal agents in organic dust include viral, microbial and fungal components, which are recognized by the family of Toll-like receptors (TLRs) and drive host innate and adaptive responses. Our aim in this study was to determine whether responsiveness to organic dust among agricultural workers was dependent on polymorphisms in the TLR10-TLR1-TLR6 gene cluster. We stimulated whole blood from 509 agricultural workers with organic dust, triacyl lipopeptide N-palmitoyl-S-dipalmitoylglyceryl Cys–Ser–(Lys)₄ (Pam3CSK4) and the diacyl-lipopeptide peptidoglycan. Several of the tagging polymorphisms and haplotypes conferred hyper-responsiveness to organic dust with an increase in interleukin-6 (IL-6; P < 0.005), but not tumor necrosis factor-α (TNF-α), secretion. We conclude that genetic variation in the TLR10-TLR1-TLR6 gene cluster mediates responsiveness to organic dust, but indicates different signaling pathways for IL-6 and TNF-α. These studies provide new insight into the role of the TLR10-TLR1-TLR6 gene cluster and the innate immune response to organic dust.

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Introduction

Inhalation of components from airborne microorganisms, as found in organic dust from agricultural environments, may lead to several inflammatory respiratory conditions including rhinosinusitis, asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD) and hypersensitivity pneumonitis.¹–³ The putative inflammatory agents in organic dust include viral, microbial and fungal components.⁴,⁵ A challenge in defining mechanisms of organic dust-induced inflammatory responses is the complex nature of the dust. We and others have found a strong predominance of Gram-positive bacteria in organic dust from swine confinement facilities.⁶,⁷ Specifically, the anaerobes from the genera Clostridium, Lactobacillus, Ruminococcus, Eubacterium and Prevotella constituted the majority of bacteria in the swine dust.⁸ In a mass spectrometry analysis, swine dust was found to have high concentrations of muramic acid, a component of peptidoglycan (PGN), which originates from Gram-positive bacteria (that is, 85% of total cell wall) and to a lesser extent Gram-negative bacteria (5% of cell wall).⁹ The family of Toll-like receptors (TLRs) recognizes these environmental components and drives the acute production of proinflammatory mediators, including cytokines, chemokines and cell-adhesion molecules, which are critical for an effective host defense and adaptive immune response.⁸

Ten human TLRs have been identified to date and they each recognize restricted ligands.⁹,¹⁰ In humans, the TLR10, TLR1 and TLR6 genes are tandemly arranged on chromosome 4p14 and are believed to have arisen from duplication events.¹¹ Phylogeny supports the theory that TLR10 existed before the gene duplication event that generated TLR1 and TLR6.¹²,¹³ Although most TLRs signal as homodimers, TLR10, TLR1 and TLR6 require ligation with TLR2. The TLR1/2 and TLR6/2 heterodimers can discriminate between the acylation state of bacterial lipopeptides from microorganisms recognizing triacyl- and diacyl-lipopeptides, respectively.¹⁴–¹⁶ The synthetic triacyl lipopeptide N-palmitoyl-S-dipalmitoylglyceryl Cys–Ser–(Lys)₄ (Pam3CSK4) has been shown to stimulate specifically via TLR1/2,¹⁴,¹⁷ whereas, TLR6/2 heterodimers recognize diacyl-lipopeptides, for example, PGN.¹⁸ Despite extensive research on TLRs, the ligand(s) and function for human TLR10 has been uncertain. Recently, Guan et al.¹⁷ showed that TLR10/2 senses triacylated lipopeptides (Pam3CSK4) and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6. However, TLR10 alone or in cooperation with TLR2 fails to activate typical TLR downstream signaling pathways.¹⁹

The ability of the host to respond to organic dust found in the environment is highly variable. Differences between individuals have been reported in the release and synthesis of cytokines from host cells stimulated with bacterial components and this variability has been attributed, in part, to genetic variation in the TLR genes.²⁰–²⁵ Much of the variation in response to TLR2 agonists is thought to be driven by polymorphisms in the TLR10-TLR1-TLR6 gene cluster.²⁰ Others have found that among individuals working in agriculture, some develop respiratory symptoms and disease, whereas others remain healthy. Thus, the aim of this study was to determine whether individual differences among agricultural workers, in response to organic dust, are dependent upon single-nucleotide polymorphisms (SNPs) in the TLR10-TLR1-TLR6 gene cluster using a whole-blood assay.

Results

TLR10-TLR1-TLR6 gene cluster

The location of tagging SNPs, linkage disequilibrium (LD) and minor allele frequencies (MAFs) in the TLR10-TLR1-TLR6 gene cluster.
cluster are shown in Figure 1 and Supplementary Table S1. The tagging SNPs in each gene had considerable LD within the TLR10-TLR1-TLR6 gene cluster (r² > 0.8). Four of the chosen tagging SNPs had putative amino-acid substitutions, one in the TLR10 gene, two in the TLR1 gene and one in the TLR6 gene. The MAFs for the tagging SNPs were consistent with frequencies reported from the HapMap Project in the Centre d’Etude du Polymorphisme Humain pedigrees.26

Study population
There were 681 veterans enrolled in the AgLUNG study.27 However, for this study, only participants with genotyping data and organic dust- and Pam3CSK4-stimulated interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels were included in the analysis (n = 509). Study population characteristics stratified by organic dust-stimulated IL-6 and TNF-α level are presented in Table 1. Reflecting demographic trends of the VA population in the urban Midwest,28 the AgLUNG population was composed primarily of white men (97%) with a median age of 65 (interquartile range = 60–71 years). The length of time participants worked on a farm was substantial, with a median of 22 years (interquartile range = 13–43 years). The prevalence of ever smokers and COPD was 79% and 37%, respectively. Lower organic dust-stimulated IL-6 and TNF-α levels were associated with individuals that were younger and never smokers.

Organic dust-stimulated IL-6 and TNF-α levels and TLR10, TLR1 and TLR6 gene polymorphisms
Organic dust-stimulated IL-6 was associated with several TLR10, TLR1 and TLR6 polymorphisms assuming a dominant model (Table 2). TLR10 SNPs rs11466645, rs11466617 and rs11725309 showed increased organic dust-stimulated IL-6 levels in individuals carrying the minor allele compared with those homozygous for the dominant allele. Similarly, the minor allele at TLR1 rs4833095, rs5743595 and rs5743580, as well as TLR6 rs5743795 and rs5743788, was associated with increased IL-6 levels compared with individuals homozygous for the major allele. In contrast, one TLR6 SNP rs5743815 was associated with decreased levels of organic dust-stimulated IL-6 when comparing individuals carrying the minor allele to those homozygous for the major allele. All associations were strengthened after adjustment for covariates in a multivariable regression model (including age, body mass index, education, sex, COPD/smoke status, race and years worked on a farm), and passed adjustment for a false discovery rate (FDR) of either 1 or 5%. Interestingly, marginal associations were observed between TLR10, TLR1 and TLR6 polymorphisms and organic dust-stimulated TNF-α levels; however, statistical significance was lost after applying a FDR adjustment.

Organic dust-stimulated IL-6 and TNF-α levels, and TLR10, TLR1 and TLR6 haplotypes
Given the high degree of LD within the TLR10-TLR1-TLR6 gene cluster, haplotypes were determined using Haploview Tagger.29 Ten of the 15 tagging SNPs were included in the haplotypes defined as rs11466657, rs7660429, rs3923647, rs4833095, rs5743594, rs5743582, rs5743580, rs5743827, rs5743815 and rs5743788. One haplotype showed significant increased organic dust-stimulated IL-6 levels when compared with the most frequent haplotype in both univariate and multivariable analysis. None of the haplotypes were associated with organic dust-stimulated TNF-α (Table 3).
lower levels of IL-6 in those carrying the minor allele. Three of the four TLR6 tagging SNPs were significantly associated with Pam3CSK4-stimulated IL-6 levels in the univariate analysis and all remained significant after adjustment for age, body mass index, education, sex, race, COPD/smoke and a FDR adjustment at the 1% level.

Pam3CSK4-stimulated IL-6 and TNF-α levels, and TLR10, TLR1 and TLR6 gene polymorphisms

Similar associations were observed between Pam3CSK4-stimulated TNF-α levels and each of the tagging SNPs in the TLR10, TLR1 and TLR6 genes (Table 4), except for TLR1/rs5743582, TLR6/rs5743827 and TLR6/rs5743815. The SNPs rs5743582 and rs5743827 were associated with stimulated levels of TNF-α, yet were not associated with IL-6. In contrast, the SNP rs5743815 was not associated with Pam3CSK4-stimulated TNF-α, yet associated with stimulated IL-6 levels. Most associations remained significant after adjustment for covariates and a FDR at the 1 or 5% level.

Pam3CSK4-stimulated IL-6 and TNF-α levels, and TLR10, TLR1 and TLR6 haplotypes

Two of the haplotypes from the TLR10-TLR1-TLR6 gene cluster showed significant increases in Pam3CSK4-stimulated IL-6 levels when compared with the most frequent haplotype (Table 5). These associations were retained after adjusting for age, body mass index, education, sex, COPD/smoke status, race and years worked on a farm in a multivariate model. Both of the haplotypes that were associated with increased Pam3CSK4-stimulated IL-6 were also associated with increased Pam3CSK4-stimulated TNF-α.

PGN-stimulated cytokines, TLR10, TLR1 and TLR6 gene polymorphisms and haplotypes

To assess the ligand specificity of our results, we performed a whole-blood assay using PGN, the ligand for the TLR6/2 heterodimer. Only two of the TLR6 tagging polymorphisms were associated with PGN-stimulated IL-6 levels after adjustment for the FDR (Table 6). In contrast, two SNPs in the TLR1 gene and one in the TLR6 gene were associated with PGN-stimulated TNF-α. One of the haplotypes showed significant increases in PGN-stimulated IL-6 and TNF-α levels when compared with the most frequent haplotype (Table 7). These associations were retained after adjusting for age, body mass index, education, sex, COPD/smoke status, race and years worked on a farm in a multivariate model.

DISCUSSION

Due to the diverse population of Gram-positive bacteria in dust from agricultural environments and because the TLR1 and TLR6 genes recognize cell wall components of Gram-positive bacteria, we focused on polymorphisms in the TLR10-TLR1-TLR6 gene cluster as a likely locus in modifying dust-induced inflammation. We demonstrate that responsiveness to organic dust, in an assay using whole blood from agricultural workers, was highly dependent on nine polymorphisms in the TLR10, TLR1 and TLR6 genes. This dependence was demonstrated for all SNPs by an increase in IL-6 production in carriers of the minor allele compared with those homozygous for the major allele. These associations remained significant even after correction for a FDR. Five out of six of the TLR10 tagging SNPs were associated with Pam3CSK4-stimulated IL-6 levels in both univariate and multivariate models. Of the significant TLR1 SNPs, rs3923647 and rs4833095 were missense mutations. All of these TLR1 SNPs passed adjustment for the FDR. For four of the TLR1 SNPs, veterans with the minor allele showed increased responsiveness and higher levels of Pam3CSK4-stimulated IL-6 levels compared with individuals homozygous for the major allele. The opposite was observed for rs5743594, where we found decreased responsiveness to Pam3CSK4 and

| Table 1. Characteristics of study population |
|---------------------------------------------|
|                                            |
| N  | ln IL-6 (pg ml⁻¹ per lymphocytes) n = 509 | ln TNF-α (pg ml⁻¹ per lymphocytes) n = 509 |
|---------------------------------------------|
| Mean | s.d. | Mean | s.d. |
|---------------------------------------------|
| Age (years) |
| 39–50 | 31 | 8.854b | 0.441 | 6.545b | 0.604 |
| 51–60 | 104 | 8.981 | 0.716 | 6.788 | 0.764 |
| 61–70 | 238 | 9.152 | 0.647 | 7.058 | 0.740 |
| 71–80 | 136 | 9.244 | 0.593 | 7.122 | 0.855 |
| Sex |
| Male | 496 | 9.132 | 0.647 | 6.994 | 0.788 |
| Female | 13 | 8.801 | 0.501 | 6.789 | 0.710 |
| BMI (kg m⁻²) |
| < 25 | 70 | 9.181 | 0.701 | 6.997 | 0.909 |
| 25–29.9 | 149 | 9.152 | 0.606 | 7.014 | 0.941 |
| ≥ 30 | 290 | 9.095 | 0.653 | 6.974 | 0.659 |
| Race |
| White | 481 | 9.131 | 0.632 | 7.002 | 0.777 |
| Other | 22 | 8.973 | 0.901 | 6.721 | 0.924 |
| Education |
| ≤ High school | 221 | 9.167 | 0.628 | 7.037 | 0.745 |
| > High school | 271 | 9.107 | 0.613 | 6.959 | 0.812 |
| Smoking status |
| Never | 108 | 8.898b | 0.569 | 6.657b | 0.750 |
| Former | 292 | 9.198 | 0.669 | 7.122 | 0.794 |
| Current | 104 | 9.159 | 0.589 | 6.972 | 0.677 |
| COPD |
| No | 319 | 9.143 | 0.660 | 6.999 | 0.785 |
| Yes | 188 | 9.084 | 0.623 | 6.965 | 0.790 |
| Worked on a farm (years) |
| < 10 | 75 | 9.092 | 0.796 | 6.918 | 0.765 |
| 10–19.9 | 145 | 9.099 | 0.607 | 6.901 | 0.739 |
| 20+ | 281 | 9.153 | 0.608 | 7.057 | 0.812 |

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume 1; FVC, forced vital capacity. *Whole-blood assay; organic dust stimulated. **Significant difference between groups, overall test, P < 0.05. †Numbers do not add up to 100% due to missing data. FEV1/FVC < 0.70.
TLR10 is unknown; however, an intracellular portion of the protein and this TIR domain is important for intracellular signaling in other TLR receptors. The ligand for the extracellular portion of the TLR10 receptor is unknown, but it is known to cause an amino-acid change in the TIR domain of the protein (rs4129009, Ile775Val). The rs4129009 SNP is associated with increased TLR10 messenger RNA levels and active TLR10 receptor. Previous studies have shown that rs4129009 is involved in the intronic region of TLR10 and modulates organic dust-stimulated IL-6 production.

Pam3CSK4-stimulated IL-6 levels. These results suggest that TLR10 may have a role in the production of organic dust-stimulated IL-6. The fact that we see very similar results with Pam3CSK4 stimulation suggests a role for triacylated lipopeptides in organic dust-induced IL-6. In contrast, TLR10 SNPs did not alter PGN-stimulated IL-6 production, which implies that PGN does not signal through the TLR10 receptor. These three TLR10 SNPs share a high degree of LD with other SNPs in the gene region.

Table 2. Association of TLR10, TLR1 and TLR6 polymorphisms with organic dust-stimulated IL-6 and TNF-α

| SNP         | Gene | Ln IL-6 (pg ml⁻¹ per lymphocytes) | Ln TNF-α (pg ml⁻¹ per lymphocytes) |
|-------------|------|-----------------------------------|-------------------------------------|
| rs11466657  | TLR10| 0.143                             | 0.284                               |
| rs11466645  | TLR10| 0.127                             | 0.194                               |
| rs11466617  | TLR10| 0.132                             | 0.143                               |
| rs7660429   | TLR10| 0.006                             | 0.001                               |
| rs11725309  | TLR1  | 0.156                             | 0.157                               |
| rs3923642   | TLR1  | 0.062                             | 0.060                               |
| rs4833095   | TLR1  | 0.105                             | 0.113                               |
| rs5743595   | TLR6  | 0.204                             | 0.177                               |
| rs5743594   | TLR6  | 0.011                             | 0.053                               |
| rs5743582   | TLR6  | 0.007                             | 0.077                               |
| rs5743580   | TLR6  | 0.022                             | 0.181                               |
| rs5743582   | TLR6  | 0.076                             | 0.063                               |
| rs5743815   | TLR6  | -0.660                            | -0.319                              |
| rs5743795   | TLR6  | 0.162                             | 0.104                               |
| rs5743788   | TLR6  | 0.134                             | 0.031                               |

Abbreviations: COPD, chronic obstructive pulmonary disease; FDR, false discovery rate; IL-6, interleukin-6; SNP, single-nucleotide polymorphism; TNF-α, tumor necrosis factor-α. SNPs estimates are based on a dominant model. β represents a natural log increase or decrease in stimulated Ln IL-6 and Ln TNF-α (pg ml⁻¹ per lymphocytes), with the dominant genotype as the reference. Univariate P-value results are from t-tests. Multivariable results (P_adj) are adjusted for age, body mass index, education, sex, race, COPD/smoke and years worked on a farm.

Table 3. Association of TLR10, TLR1 and TLR6 haplotypes with organic dust-stimulated IL-6 and TNF-α levels

| TLR10-TLR1-TLR6 haplotypes | Ln IL-6 (pg ml⁻¹ per lymphocytes) | Ln TNF-α (pg ml⁻¹ per lymphocytes) |
|-----------------------------|-----------------------------------|-------------------------------------|
| TCATCCGGTG                  | 0.143                             | 0.284                               |
| CCACCCAGTC                  | 0.127                             | 0.194                               |
| TCACCCAGTC                  | 0.132                             | 0.143                               |
| TCATCTGAC                  | 0.005                             | 0.001                               |
| TGATCTGAC                  | 0.013                             | 0.014                               |
| TGATATGAC                  | 0.053                             | 0.077                               |
| TGATGATGAC                 | 0.134                             | 0.031                               |

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; IL-6, interleukin-6; SNP, single-nucleotide polymorphism; TNF-α, tumor necrosis factor-α. Haplotypes defined as rs11466657, rs7660429, rs3923642, rs4833095. Sample sizes estimated from haplotype frequency. β represents the change compared with the reference haplotype. Univariate P-value results are from t-tests.

consistent with our findings. Our data do not show that TLR10 SNPs modulate organic dust-stimulated TNF-α production; yet several of the TLR10 SNPs were significantly associated with increased production of Pam3CSK4-stimulated TNF-α, suggesting a signaling pathway independent of TLR10 for the stimulation of TNF-α by organic dust. Future work will need to identify and dissect the functionality of TLR10 SNPs and its signaling pathways.

Next we found that TLR1 polymorphisms modulate innate immune responses to organic dust and Pam3CSK4 (a synthetic triacylated lipopeptide). Triacylated lipopeptides are found in Gram-positive bacteria and are ligands for TLR1/2 heterodimers. Three SNPs in the TLR1 gene (rs4833095, rs5743595 and rs5743580) were associated with both organic dust- and Pam3CSK4-stimulated IL-6 production. This is consistent with the known composition of Gram-positive bacteria in organic dust from swine confinement. In contrast to Pam3CSK4, PGN signals through TLR6/2 heterodimers, thus, as expected; we did not observe an association between TLR1 SNPs and PGN-stimulated IL-6 production. We found moderate LD (r² > 0.6) among the TLR1 SNPs (rs4833095, rs5743595 and rs5743580), indicating that their association with organic dust-stimulated IL-6 production may be due to only one of these SNPs or possibly another SNP in LD at another location in the gene region.

TLR1 rs4833095 (Asn248Ser) is a known composition of Gram-positive bacteria in organic dust from swine confinement. In contrast to Pam3CSK4, PGN signals through TLR6/2 heterodimers, thus, as expected; we did not observe an association between TLR1 SNPs and PGN-stimulated IL-6 production. We found moderate LD (r² > 0.6) among the TLR1 SNPs (rs4833095, rs5743595 and rs5743580), indicating that their association with organic dust-stimulated IL-6 production may be due to only one of these SNPs or possibly another SNP in LD at another location in the gene region. TLR1 rs4833095 (Asn248Ser) is a known composition of Gram-positive bacteria in organic dust from swine confinement. In contrast to Pam3CSK4, PGN signals through TLR6/2 heterodimers, thus, as expected; we did not observe an association between TLR1 SNPs and PGN-stimulated IL-6 production. We found moderate LD (r² > 0.6) among the TLR1 SNPs (rs4833095, rs5743595 and rs5743580), indicating that their association with organic dust-stimulated IL-6 production may be due to only one of these SNPs or possibly another SNP in LD at another location in the gene region. TLR1 rs4833095 (Asn248Ser) is a known composition of Gram-positive bacteria in organic dust from swine confinement. In contrast to Pam3CSK4, PGN signals through TLR6/2 heterodimers, thus, as expected; we did not observe an association between TLR1 SNPs and PGN-stimulated IL-6 production. We found moderate LD (r² > 0.6) among the TLR1 SNPs (rs4833095, rs5743595 and rs5743580), indicating that their association with organic dust-stimulated IL-6 production may be due to only one of these SNPs or possibly another SNP in LD at another location in the gene region.
Additional studies confirmed hyper-responsiveness to Pam3CSK4 with rs5743618 and also found that carriers of the isoleucine allele demonstrated higher surface expression of the TLR1 on peripheral monocytes and higher NF-κB activity in response to Pam3CSK4 relative to the serine allele. Taken together, rs5743618, found in the transmembrane domain of TLR1, may be the functional variant and that surface trafficking of TLR1 contributes to its function to regulate the innate immune inflammatory response.

We identified three SNPs in the TLR6 gene that modified innate immune responses to organic dust. Two of these SNPs (rs5743795 and rs5743788) were associated with hyper responsiveness to organic dust and increased IL-6 production. The SNP rs5743795 is located in the intronic region of the TLR6 gene and was in strong LD with several TLR1 and TLR6 SNPs, namely, the TLR1 missense SNP rs4833095. Differential IL-6 secretion through TLR1, and not TLR6, is supported by our data showing that rs5743795 was associated with altered IL-6 production in response to stimulation with the TLR1 ligand Pam3CSK4, but not the TLR6 ligand PGN (Table 6). Furthermore, this SNP was shown recently to be associated with differential responses to Pam3CSK4. Wurfel and coworkers found a missense SNP located in the extracellular domain of the receptor and was previously found to increase innate immune responses to Pam3CSK4. However, studies investigating the functionality of this SNP have been inconsistent. Additional studies confirmed hyper-responsiveness to Pam3CSK4 with rs5743618 and also found that carriers of the isoleucine allele demonstrated higher surface expression of the TLR1 on peripheral monocytes and higher NF-κB activation in response to Pam3CSK4 relative to the serine allele. Taken together, rs5743618, found in the transmembrane domain of TLR1, may be the functional variant and that surface trafficking of TLR1 contributes to its function to regulate the innate immune inflammatory response.

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**Table 4.** Association of TLR10, TLR1 and TLR6 polymorphisms with Pam3CSK4-stimulated IL-6 and TNF-α

| SNP            | Gene | Ln IL-6 (pg ml⁻¹ per lymphocytes) | Ln TNF-α (pg ml⁻¹ per lymphocytes) |
|----------------|------|-----------------------------------|-------------------------------------|
| rs11466657     | TLR10| 0.537 ± 0.186                     | 0.0039d                            |
| rs11466645     | TLR10| 0.610 ± 0.089                     | < 0.0001d                          |
| rs11466617     | TLR10| 0.614 ± 0.086                     | < 0.0001d                          |
| rs7660429      | TLR10| 0.150 ± 0.100                     | 0.14                               |
| rs11723509     | TLR10| 0.614 ± 0.088                     | < 0.0001d                          |
| rs39323647     | TLR10| 0.412 ± 0.208                     | 0.049                              |
| rs4833095      | TLR10| 0.666 ± 0.081                     | < 0.0001d                          |
| rs5743595      | TLR10| 0.658 ± 0.087                     | < 0.0001d                          |
| rs5743594      | TLR10| −0.174 ± 0.087                    | 0.047                              |
| rs5743582      | TLR10| 0.023 ± 0.107                     | 0.83                               |
| rs5743580      | TLR10| 0.657 ± 0.088                     | < 0.0001d                          |
| rs5743827      | TLR6 | −0.027 ± 0.083                    | 0.74                               |
| rs5743815      | TLR6 | −0.728 ± 0.234                    | 0.0019d                            |
| rs5743795      | TLR6 | 0.571 ± 0.086                     | < 0.0001d                          |
| rs5743788      | TLR6 | 0.416 ± 0.094                     | < 0.0001d                          |

**Table 5.** Association of TLR10, TLR1 and TLR6 haplotypes with Pam3CSK4-stimulated IL-6 and TNF-α levels

| TLR10-TLR1-TLR6 haplotypes | N° | Ln IL-6 (pg ml⁻¹ per lymphocytes) | P-value | P_adj-value | N° | Ln TNF-α (pg ml⁻¹ per lymphocytes) | P-value | P_adj-value |
|-----------------------------|----|-----------------------------------|---------|-------------|----|-----------------------------------|---------|-------------|
| TCATCCGGTG                  | 232| 0.703 (0.175)                     | < 0.0001 | < 0.0001    | 232| 0.674 (0.205)                     | < 0.0001 | < 0.0001    |
| CCACCCAGTC                  | 13 | 0.609 (0.087)                     | < 0.0001 | < 0.0001    | 13 | 0.669 (0.103)                     | < 0.0001 | < 0.0001    |
| TCACCCAGTC                  | 67 | 0.028 (0.080)                     | 0.73     | 0.74        | 84 | −0.029 (0.089)                    | 0.74     | 0.78        |
| TCATTGATC                   | 36 | 0.158 (0.115)                     | 0.17     | 0.13        | 36 | −0.128 (0.132)                    | 0.33     | 0.23        |
| TGATCTGATC                  | 77 | 0.257 (0.079)                     | 0.001    | 0.001       | 77 | 0.117 (0.093)                     | 0.21     | 0.23        |

Abbreviations: COPD, chronic obstructive pulmonary disease; FDR, false discovery rate; IL-6, interleukin-6; SNP, single-nucleotide polymorphism; TNF-α, tumor necrosis factor-α. SNPs estimates are based on a dominant model. β represents a natural log increase or decrease in stimulated Ln IL6 and TNF-α (pg ml⁻¹ per lymphocytes), with the dominant genotype as the reference. P_adj-value results are from t-tests. Multivariable results (P_adj) are adjusted for age, body mass index, education, sex, COPD/smoke and years worked on a farm. Passed the FDR adjustment at 1% level. Passed the FDR adjustment at 5% level.
rs5743580 (TLR1) and rs5743788 (TLR6), relative to the reference haplotype. These results are in concordance with our individual SNP analysis. It must be emphasized that these three polymorphisms studied in the haplotype analysis do not act in isolation because of their close proximity on chromosome 4p14 and the high LD among these genes.

In addition to these tagging SNPs, we must consider the possibility that other SNPs not genotyped in our study, but are in LD with the rs5743580, rs5743594, rs5743788, and rs5743795 SNPs of TLR10-TLR1-TLR6 gene cluster to account for variability of IL-6 production in response to organic dust exposure in humans. Furthermore, the presence of genetic variation leads to upregulation of the innate immune response upon organic dust challenge of whole blood from agricultural workers. Polymorphisms within the TLR10-TLR1-TLR6 locus have been associated with altered susceptibility to diseases such as mycobacterial infections of leprosy (TLR1 Serr602ile in high LD with TLR1 N248S), sepsis (TLR1 rs5743551 in LD with TLR1 N248S), prostate cancer (TLR1 N248S; TLR10 N241H in LD with TLR10 rs11466645), non-Hodgkin’s lymphoma (TLR1 N2448S), Crohn’s disease (TLR1 N2448S), asthma (TLR10 ile775Val in LD with TLR10 rs11466645; TLR1 N2448S) and chronic sarcoidosis (TLR10 N241H in LD with TLR10 rs114666445),2,23,33,37-40 Possible future directions for study include the relevance of SNPs in this locus with acute and chronic disease in agricultural workers.

**MATERIALS AND METHODS**

**Study population and clinical assessments**

The Agricultural Lung (AgLUNG) study is a cross-sectional study of veterans that have worked on a farm for >2 years as an adult. The study was designed to assess the relationship between agricultural exposures and chronic respiratory disease in persons seeking care at the General Medicine clinics in the VA Nebraska Western Iowa Health Care System. There were 681 veterans recruited from 2008 to 2013 that were between the ages of 40 and 80 years; however, 509 participants are included in this analysis due to availability of genotyping data and organic dust- and Pam3CSK4-stimulated IL-6 and TNF-α levels. Veterans were excluded if they had a history of lung cancer, metastatic cancer to the lungs or interstitial lung disease such pulmonary fibrosis, asthma, sarcoidosis, hypersensitivity pneumonitis or if they had a history of an infection in the 3 weeks before study enrollment. Eligibility information was obtained by self-report and medical chart confirmation. Subject demographics, respiratory symptoms, smoking habits and agricultural exposures were obtained by in-person and
telephone interviews. A participant was considered to be a smoker if they had smoked >100 cigarettes in their lifetime. All veterans underwent spirometry and if they had a FEV/FVC < 0.70, then post-bronchodilator spirometry with 0.083% albuterol was performed. COPD status was ascertained for each participant using the Global Initiative for Chronic Obstructive Lung Disease definition of post-bronchodilator FEV/FVC < 0.70.43 FEV, and FVC were adjusted for height, weight, age, gender and ethnicity based on NHANESIII reference equations.42 All participants signed a written informed consent document at study enrollment. This study was approved by the VA Nebraska Western Iowa Healthcare Systems Institutional Review Board.

Whole-blood assay
Heparinized blood was diluted with l-glutamine-RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) at a 1:1 ratio and stimulated with organic dust extract (1%), triacyl lipopeptide N-palmitoyl-S-palmitoylglycercyl Cys–Ser–Lys (Pam3CSK4, 1 ng ml\(^{-1}\)), PGN (10 μg ml\(^{-1}\)) or phosphate-buffered saline. Blood was incubated for 24 h at 37 °C with 5% CO\(_2\) and then centrifuged at 500 g for 5 min. Cell-free supernates were stored at −80 °C for later cytokine analysis. Blood samples were processed within 2 h of collection.

Organic dust extract
Swine confinement animal feeding operation facility organic dust was collected and prepared as previously described.44 In brief, settled surface dust samples from local swine confinement feeding operations were extracted in Hank’s balanced salt solution, centrifuged filter sterilized and stored at −20 °C (100% dust extract). The extract prepared in this manner contains no particulate matter larger than 0.2 μm in diameter. The extract was diluted to a final concentration of 1% (vol/vol) for all experiments.

TNF-α and IL-6 enzyme-linked immunosorbent assays
For IL-6 and TNF-α measurement, a sandwich enzyme-linked immunosorbent assay was employed.44 In brief, flat-bottomed polystyrene microtiter plates were coated with 200 μl per well of purified (goat) anti-human IL-6 or (mouse) anti-human TNF-α antibody (2 μg ml\(^{-1}\); both from R&D Systems, Minneapolis, MN, USA) in carbonate buffer (pH 9.6) overnight at 4 °C. After washing the plates three times in phosphate-buffered saline/Tween 20 (PBS-T), cell-free whole-blood assay supernates were dispensed in duplicate wells and incubated at room temperature for 2 h. Plates were again washed three times with PBS-T and incubated with (rabbit) anti-human IL-6 antibody (Sigma-Aldrich, St Louis, MO, USA) diluted 1:1000 or biotinylated (goat) anti-human TNF-α (1:250; R&D Systems) in PBS-T/BLOTO (0.2% instant nonfat milk, PBS-T/B) for 1 h. After three plate washes, human serum-absorbed peroxidase conjugated (goat) anti-rabbit IgG (Rockland Immunochemicals, Limerick, PA, USA) was added at 1:2000 (IL-6) or streptavidin-HRP (1:200, for TNF-α) in PBS-T/B for 1 h. The plates were again washed three times and 200 μl per well of peroxidase substrate (10 ng ml\(^{-1}\) orthophenylenediamine containing 0.003% H\(_2\)O\(_2\); Sigma-Aldrich) was added to IL-6 plates, and 100 μl per well TMB substrate (R&D Systems) for the TNF-α plates. The reaction was terminated with 27.5 μl per well of 8 M sulfuric acid, and plates were read at 490 or 450 nm using the VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cytokine concentrations were interpolated from an integrated 8-point standard curve created using purified recombinant human proteins. The limits of detectability for the human cytokine assays were: IL-6, 60 pg ml\(^{-1}\) and TNF-α, 15 pg ml\(^{-1}\).

Genetic analysis
As part of the HapMap Project and the Innate Immunity Program in Genomic Applications, the TLR10, TLR1 and TLR6 genes were resequenced from DNA obtained from 30 trios from Utah residents with Northern or Western European ancestry (the Centre d’Etude du Polymorphisme Humain population). A haplotype tagging strategy using publicly available software44 and SNPs identified in the intronic sequence, ~ 6 kb of 5‘-genomic DNA and 2 kb of 3‘-genomic DNA was implemented to reduce the number of SNPs analyzed and to capture the polymorphic structure of the gene. The algorithm was based on polymorphic sites that exceeded a 10% MAF and a within-bin LD exceeding an r\(^2\) value of 0.7. Additional missense SNPs were included based on their MAF and functional significance.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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