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Epistatic effect of TLR −1, −6 and −10 Polymorphisms on Organic Dust-Mediated Cytokine Response

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Abstract

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 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 IL-6 ($p < 0.005$), but not 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.

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 (1, 4, 5). A challenge in defining

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None of the authors have any competing financial interests in relation to the work described.

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 (4). In a mass spectrometry analysis, swine dust was found to have high concentrations of muramic acid, a component of PGN, which originates from gram-positive bacteria (i.e. 85% of total cell wall) and to a lesser extent gram-negative bacteria (5% of cell wall) (5). 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 differentially 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, peptidoglycan (PGN) (18). Despite extensive research on TLRs, the ligand(s) and function for human TLR10 has been uncertain. Recently, Guan et al showed that TLR10/2 senses triacylated lipopeptides (Pam3CSK4) and a wide variety of other microbial- derived agonists shared by TLR1, but not TLR6 (16). 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 while 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 are shown in Figure 1 and Table S1. The tagging SNPs in each gene had considerable LD within the *TLR10-TLR1-TLR6* gene

cluster ($r^2 > 0.8$). 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 (CEPH) 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- stimulated and Pam3CSK4- stimulated IL-6 and 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 [IQR] = 60 – 71 years). The length of time participants worked on a farm was substantial, with a median of 22 years (IQR = 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 to 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 to 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, BMI, education, sex, COPD/smoke status, race and years worked on a farm), and passed adjustment for a false discovery rate 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 false discovery rate 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 to the most frequent haplotype in both univariate and multivariable analysis. None of the haplotypes were associated with organic dust- stimulated TNF-α (Table 3).

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

Associations between Pam3CSK4- stimulated IL-6 levels and *TLR10*, *TLR1* and *TLR6* gene polymorphisms were examined using a dominant model (Table 4). Four out of five of the *TLR10* tagging SNPs were significantly associated with stimulated IL-6 levels in both the univariate and multivariate models, including the missense mutation rs11466657. All four of the *TLR10* SNPs showed increased responsiveness to Pam3CSK4 with higher levels of IL-6 among individuals carrying the minor allele compared to those homozygous for the major allele. These associations remained significant even after correction for a false discovery rate. Five out of six of the *TLR1* 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 false discovery rate. For four of the *TLR1* SNPs, veterans with the minor allele showed increased responsiveness and higher levels of Pam3CSK4- stimulated IL-6 levels compared to individuals homozygous for the major allele. The opposite was observed for rs5743594, where we found decreased responsiveness to Pam3CSK4 and 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 false discovery rate adjustment at the 1% level.

Pam3CSK4- stimulated 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 false discovery rate 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 to the most frequent haplotype (Table 5). These associations were retained after adjusting for age, BMI, 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.

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

In order to assess the ligand specificity of our results, we performed a whole blood assay using peptidoglycan, the ligand for the TLR6/2 heterodimer. Only two of the *TLR6* tagging polymorphisms were associated with peptidoglycan- stimulated IL-6 levels after adjustment for the false discovery rate (Table 6). In contrast, two SNPs in the *TLR1* gene and one in the *TLR6* gene were associated with peptidoglycan- stimulated TNF-α. One of the haplotypes

showed significant increases in PGN- stimulated IL-6 and TNF-α levels when compared to the most frequent haplotype (Table 7). These associations were retained after adjusting for age, BMI, 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 to those homozygous for the major allele. However, organic dust-stimulated production of TNF-α was not affected by these polymorphisms, suggesting different inflammatory signaling pathways between these two cytokines. To our knowledge, this is the first report to demonstrate an *ex vivo* functional role for the *TLR10-TLR1-TLR6* gene cluster in mediating inflammatory responses to complex organic dust from agricultural environments.

Three *TLR10* polymorphisms (rs11466645, rs11466617 and rs11725309) were strongly associated with both organic dust- and Pam3CSK4- stimulated IL-6 levels. These results suggest that *TLR10* may play 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 ($r^2 > 0.8$) and appear to represent a single association. Though the function of these SNPs in the intronic region of *TLR10* is unknown, they are in high LD (based on a reference population) with the missense SNP rs4129009 (*TLR10* Ile775Val). The rs4129009 SNP is known to cause an amino acid change in the TIR domain of the intracellular portion of the protein and this TIR domain is important for intracellular signaling in other TLR receptors (30). The ligand for TLR10 is unknown; however, an *in vitro* study by Guan et al showed that the extracellular portion of the TLR10 receptor recognizes Pam3CSK4 when coupled with TLR1, yet intracellular signaling does not occur (16). However, our data shows increased IL-6 response to organic dust and Pam3CSK4 for *TLR10* SNPs, which may suggest that a SNP in *TLR10* (rs4129009), results in a functionally active TLR10 receptor. Previous studies have shown that rs4129009 was associated with increased TLR10 mRNA levels and conferred hyper responsiveness to Pam3CSK4 (21, 31), an observation 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 confinements (4–7). 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 missense SNP located in the extracellular domain of the receptor and was previously found to increase innate immune responses to Pam3CSK4 (21, 31, 32). However, studies investigating the functionality of this SNP have been inconsistent (33, 34). Wurfel and coworkers found that this SNP did not alter responsiveness to Pam3CSK4 in cloning and transfection experiments. However, the SNP rs5743618 (Ser602Ile) in high LD with rs4833095, did increase responsiveness (22, 35, 36). 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 (35, 36). 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 *TLR10* 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 (22). Interestingly, rs5743815 had the opposite effect compared to rs5743795, where those with the minor allele had decreased organic dust-stimulated IL-6 production compared to individuals homozygous for the major allele. The SNP rs5743815 was not in LD with the other tagging SNPs studied in the *TLR10-TLR1-TLR6* gene cluster. It is a missense SNP (Val427Ala) with unknown function located in the extracellular leucine-rich domain of TLR6. To further resolve the complex causal relationships, functional analyses should be undertaken. Only then will we be able to conclusively identify the contributions of single SNPs in this TLR cluster on the overall effects of organic dust- stimulated cytokine production.

Haplotypes were constructed using 10 of the 15 SNPs investigated in this study. Association analysis showed that one defined haplotype was associated with organic-dust stimulated IL-6 release. The same haplotype was also associated with Pam3CSK4- and PGN-stimulated IL-6. In the haplotype analysis, increased IL-6 was driven by the minor allele in

three SNPs, rs4833095 (*TLR1*), 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 these variants, are the functional polymorphisms responsible for increased IL-6 stimulation. Proving this will require *in vitro* and animal experiments using engineered TLR receptors. Furthermore, rare variants were not investigated in this study and future studies would require a larger population than the current for sufficient statistical power.

In conclusion, the present study demonstrates an important role of the *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* Ser602Ile 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* N248S), Crohn's disease (*TLR1* N248S), asthma (*TLR10* Ile775Val in LD with *TLR10* rs11466645; *TLR1* N248S) and chronic sarcoidosis (*TLR10* N241H in LD with *TLR10* rs11466645) (22, 31, 35, 37–40). Possible future directions for study include the relevance of SNPs in this locus with acute and chronic disease in agricultural workers.

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 Heath 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- stimulated 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 three weeks prior to 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 more than 100 cigarettes in their lifetime. All veterans underwent spirometry and if they had a $FEV_1/FVC < 0.70$, 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 (GOLD) definition of post-bronchodilator $FEV_1/FVC < 0.70$ (41). FEV_1 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) at a 1:1 ratio and stimulated with organic dust extract (1%), triacyl lipopeptide N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-$(Lys)_4$ (Pam3CSK4, 1 ng/ml), peptidoglycan (PGN, 10 μg/ml) or phosphate buffered saline. Blood was incubated for 24 hr 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 hr of collection.

Organic Dust Extract

Swine confinement animal feeding operation facility organic dust was collected and prepared as previously described (43). Briefly, 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 dust extract was diluted to a final concentration of 1% (vol/vol) for all experiments.

TNF-α and IL-6 ELISAs

For IL-6 and TNF-α measurement, a sandwich ELISA was employed (43). In brief, flat-bottomed polystyrene microtiter plates were coated with 200 μl/well of purified (goat) anti-human IL-6 or (mouse) anti-human TNF-α antibody (2 μg/mL) (both from R & D Systems, Minneapolis, MN) 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 hr. Plates were again washed three times with PBS-T and incubated with (rabbit) anti-human IL-6 antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:1000 or biotinylated (goat) anti-human TNF-α (1:250) (R & D Systems) in PBS-T/BLOTTO (0.2% instant nonfat milk, PBS-T/B) for 1 hr. After three plate washes, human serum-absorbed peroxidase conjugated (goat) anti-rabbit IgG (Rockland Immunochemicals, Limerick, PA) was added at 1:2000 (IL-6) or streptavidin-HRP (1:200, for TNF-α) (R & D Systems) in PBS-T/B for 1 hr. The plates were again washed three times and 200 μl/well of peroxidase substrate (10 ng/ml orthophenylenediamine containing 0.003% H_2O_2 (Sigma-Aldrich) was added to IL-6 plates, and 100 μL/well TMB substrate (R & D Systems) for the TNF-α plates. The reaction was terminated with 27.5 μl/well of 8M sulfuric acid, and plates were read at 490nm or 450nm using the VERSAmax microplate reader. 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 and TNF-α, 15 pg/mL.

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 CEPH population). A haplotype tagging strategy using publicly available software (44) 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% minor allele frequency (MAF) and a within-bin linkage disequilibrium (LD) exceeding an r^2 value of 0.7. Additional missense SNPs were included based on their MAF and functional significance.

QiaAMP DNA Blood and Tissue Mini Kit was used to isolate genomic DNA from whole blood (Qiagen, Valencia, CA, USA). Genotype information was obtained using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Agena Bioscience, San Diego, CA, USA). SpectroDesigner software (Agena Bioscience) was used to design the multiplex polymerase chain reaction assays and associated extension reactions. Primer extension products were loaded onto a 384-element chip with a nanoliter pipetting system (Agena Bioscience) and analyzed with a MassArray mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Mass spectra peak identification was obtained using SpectroTyperRT 4.0 software. Hardy-Weinberg calculations were done to determine genotyping quality control and whether each marker was in the expected allelic population equilibrium. The following SNPs were analyzed for this study, *TLR10*: rs11466657, rs11466645, rs11466617, rs7660429, rs11725309; *TLR1*: rs3923647, rs4833095, rs5743595, rs5743594, rs5743582, rs5743580; and *TLR6*: rs5743827, rs5743815, rs5743795, rs5743788. The polymorphism rs5743810 was excluded from the analysis, because it was not in Hardy-Weinberg equilibrium.

Statistical Analyses

In a post-hoc power calculation, a sample size of 509 achieves 90% power to detect a change in slope (β) from 0 to 0.25 when the standard deviation (SD) of the independent variable is 0.40, the SD of dependent variable is 0.60, and the two-sided α-level is 0.01, which is Bonferroni adjusted for multiple comparisons.

Due to the skewed nature of IL-6 and TNF-α level, all analyses were conducted on the natural logarithm scale, to meet the normality assumption. Stimulated IL-6 and TNF-α levels were compared by patient characteristics using t-test and ANOVA models. Associations between stimulated IL-6 and TNF-α levels and *TLR10*, *TLR1* and *TLR6* polymorphisms were examined utilizing t-tests, assuming a dominant model for the polymorphisms, variances were found to be similar between groups. A combination variable (COPD/smoke) was created to account for both COPD and smoking status in the multivariable models and contained three categories: 1) COPD, 2) no COPD, ever smoker, and 3) no COPD, never smoker. Multivariable linear regression models were used to examine *TLR* polymorphisms as predictors of IL-6 and TNF-α level, while adjusting for age, gender, body mass index (BMI), education level, race, COPD/smoke and years worked

on a farm. Adjustments for multiple comparisons were made using Benjamini Hochberg false discovery rate (FDR) methodology (45).

TLR haplotypes were constructed using Haploview software version 4.2 (29) and haplotype blocks were estimated using the confidence interval for r^2 values. To select SNPs in the haplotype, we used Haploview Tagger analysis to reduce the number of SNPs included from 15 to 10 using an r^2 threshold of 0.8. Considering an r^2 threshold of 0.7 gave the same resulting SNPs included in the haplotype as that found for $r^2 = 0.8$. SNPs rs5743580, rs5743595, rs5743580, rs11725309, rs5743795, rs11466617, rs11466645 are all highly correlated and the Tagger analysis indicated that rs5743580 could capture the information contained from the other six SNPs.

The association between IL-6 and TNF-α levels and *TLR* haplotypes was tested using the R function haplo.glm contained in the R package haplo.stats. Haplo.glm fit univariate and multivariable linear regression of stimulated IL-6 and TNF-α level on haplotype, which allowed for ambiguous haplotypes, interactions and covariates. The model is fit using an iterative two-step expectation-maximization (EM) algorithm, with the posterior probabilities as weights to update the regression coefficients, and the regression coefficients are used to update the posterior probabilities (46). Both multivariable adjusted models and univariate models were considered. Multivariable models were adjusted for age, gender, body mass index, education, race, COPD/smoke and years worked on a farm. Multivariable linear regression models considered an interaction between *TLR* haplotypes and the COPD/smoke variable; however, none were significant and thus excluded from the final model. All tests were two-sided. Models that do not include haplotype information were fit using SAS 9.3 (SAS Institute Inc., Cary, NC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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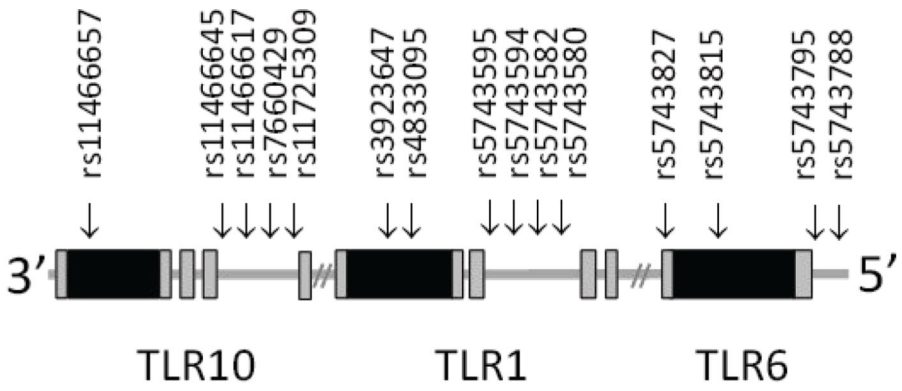

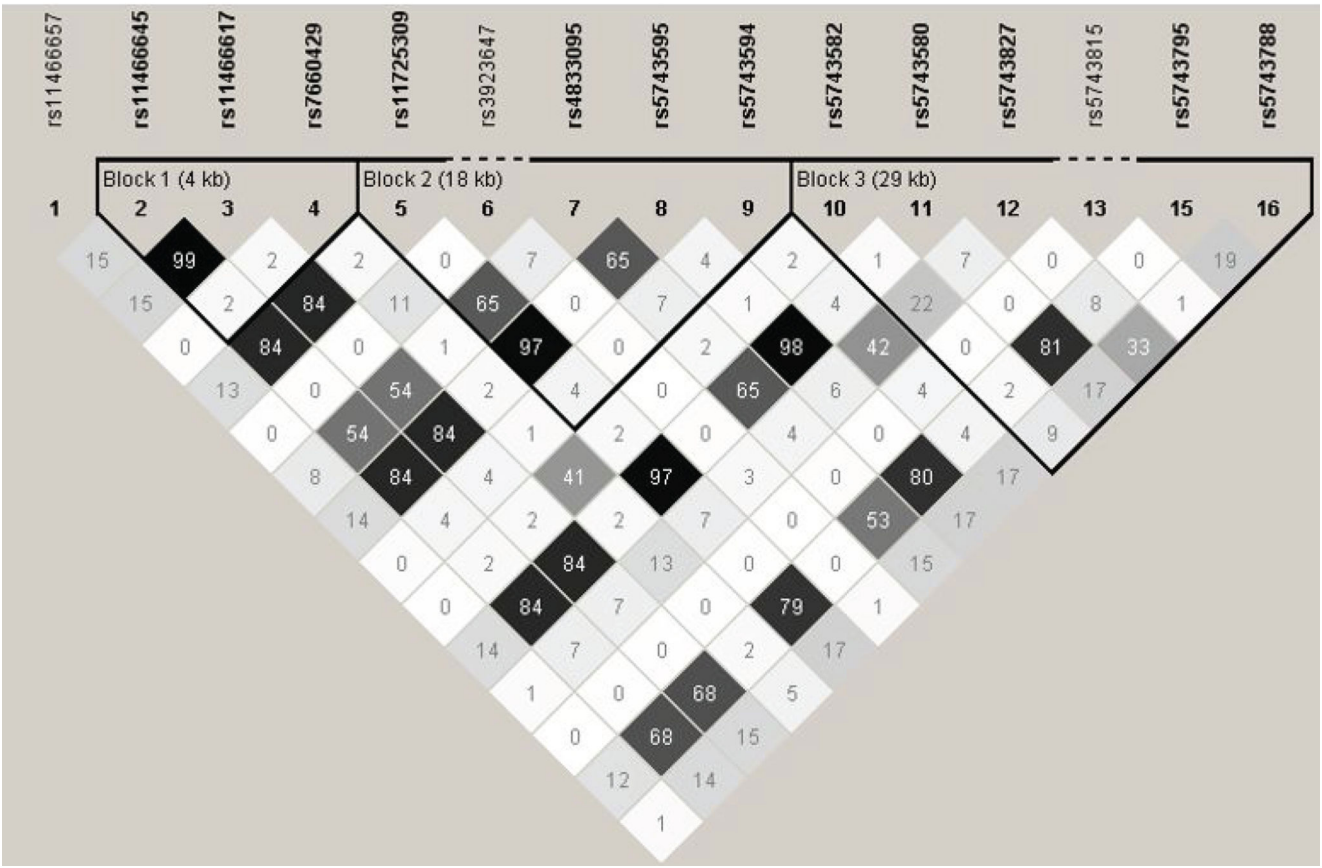

Figure 1.

Schematic diagram of the *TLR10-TLR1-TLR6* gene cluster on chromosome 4. Top: Each tagging polymorphism is indicated by an arrow. Black boxes represent coding region; grey boxes represent non-coding untranslated exonic regions; grey line represents intronic regions and parallel vertical lines represent intragenic regions. TLR, Toll-like receptor. Bottom: Linkage disequilibrium (LD) between tagging polymorphisms. LD values presented as $r^2 \times 100$.

Table 1

Characteristics of Study Population

		ln IL-6[1] (pg/mL/lymphocytes) n=509		Ln TNF-α[1] (pg/mL/lymphocytes) n=509	
	N	**Mean**	**(SD)**	**Mean**	**(SD)**
Age, years					
39–50	31	8.854^	(0.441)	6.545^	(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[2]					
White	481	9.131	(0.632)	7.002	(0.777)
Other	22	8.973	(0.901)	6.721	(0.924)
Education[2]					
≤ High School	221	9.167	(0.628)	7.037	(0.745)
> High School	271	9.107	(0.613)	6.959	(0.812)
Smoking Status[2]					
Never	108	8.898^	(0.569)	6.657^	(0.750)
Former	292	9.198	(0.669)	7.122	(0.794)
Current	104	9.159	(0.589)	6.972	(0.677)
COPD[2]					
No	319	9.143	(0.660)	6.999	(0.785)
Yes	188	9.084	(0.623)	6.965	(0.790)
Worked on a Farm, yrs[2]					
<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 and Definitions: BMI, body mass index; COPD, $FEV_1/FVC < 0.70$.

[1]Whole blood assay; organic dust- stimulated

[2]Numbers don't add up to 100% due to missing data

^Significant difference between groups, overall test, $p<0.05$.

Table 2

Association of *TLR10*, *TLR1*, and *TLR6* Polymorphisms with Organic Dust- Stimulated IL-6 and TNF-α

		Ln IL-6 (pg/mL/lymphocytes)				Ln TNF-α (pg/mL/lymphocytes)			
SNP	**Gene**	**[1]β_{SNP}**	**[1]SE_{SNP}**	**P-value[2]**	**P_{adj}-value[3]**	**[1]β_{SNP}**	**[1]SE_{SNP}**	**P-value[2]**	**P_{adj}-value[3]**
rs11466657	*TLR10*	0.143	0.129	0.27	0.37	0.284	0.159	0.08	0.07
rs11466645		0.127	0.064	0.046	0.008^	0.149	0.078	0.058	0.03
rs11466617		0.132	0.062	0.04	0.006^	0.143	0.076	0.059	0.03
rs7660429		−0.006	0.070	0.93	0.64	0.001	0.085	0.99	0.51
rs11725309		0.156	0.063	0.014^	0.0017*	0.157	0.078	0.045	0.03
rs3923647	*TLR1*	0.062	0.141	0.66	0.16	−0.040	0.176	0.82	0.71
rs4833095		0.105	0.058	0.07	0.026^	0.113	0.073	0.12	0.04
rs5743595		0.204	0.062	0.001*	0.0005*	0.177	0.077	0.02	0.02
rs5743594		0.011	0.061	0.85	0.66	−0.053	0.075	0.48	0.20
rs5743582		0.007	0.074	0.93	0.78	−0.077	0.090	0.39	0.56
rs5743580		0.202	0.062	0.0012*	0.0006*	0.181	0.077	0.02	0.02
rs5743827	*TLR6*	0.076	0.057	0.18	0.13	−0.063	0.070	0.37	0.40
rs5743815		−0.660	0.160	<0.0001*	<0.0001*	−0.319	0.201	0.11	0.13
rs5743795		0.162	0.061	0.0084^	0.0006*	0.104	0.076	0.17	0.11
rs5743788		0.134	0.064	0.04	0.019^	0.031	0.081	0.70	0.49

[1] SNP-estimates are based on a dominant model. β estimates represent a natural log increase or decrease in stimulated Ln IL-6 and Ln TNF-α (pg/mL/lymphocytes), with the dominant genotype as the reference

[2] Univariate p-value results are from t-tests

[3] Multivariable results (p_{adj}) are adjusted for age, body mass index, education, sex, race, COPD/smoke and years worked on a farm

* Passed the FDR adjustment at 1% level;

^ passed the FDR adjustment at 5% level

Table 3

Association of *TLR10*, *TLR1*, and *TLR6* Haplotypes with Organic Dust-Stimulated IL-6 and TNF-α Levels

***TLR10-TLR1-TLR6* Haplotypes**[1]	**n**[2]	**Ln IL-6 (pg/mL/lymphocytes) β (SE)**[3]	**P-value**[4]	**P_{adj}-value**[5]	**n**[2]	**Ln TNF-α (pg/mL/lymphocytes) β (SE)**[3]	**P-value**[4]	**P_{adj}-value**[5]
TCATCCGGTG	232	0	Ref[6]	Ref[6]	232	0	Ref[6]	Ref[6]
CCACCCAGTC	13	0.209 (0.128)	0.10	0.13	13	0.231 (0.155)	0.14	0.12
TCACCCAGTC	67	0.205 (0.065)	0.002	<0.001	67	0.109 (0.078)	0.16	0.12
TCATTCGATC	84	0.088 (0.058)	0.13	0.20	84	−0.019 (0.071)	0.78	0.61
TGATCTGATC	36	0.034 (0.084)	0.68	0.31	36	−0.053 (0.102)	0.61	0.98
Other	77	−0.029 (0.059)	0.62	0.98	77	−0.116 (0.071)	0.10	0.38

[1] Haplotypes defined as rs11466657, rs7660429, rs3923647, rs4833095, rs5743594, rs5743582, rs5743580, rs5743827, rs5743815, and rs5743788.

[2] Sample sizes estimated from haplotype frequency

[3] β represents the change compared to the reference haplotype

[4] Univariate p-value results

[5] Multivariable results (p_{adj}) are adjusted for age, BMI, education, sex, COPD/smoke status, race and years worked on a farm

[6] Pairwise comparison to the reference haplotype

Table 4

Association of *TLR10*, *TLR1*, and *TLR6* Polymorphisms with Pam3CSK4- Stimulated IL-6 and TNF-α

		Ln IL-6 (pg/mL/lymphocytes)				Ln TNF-α (pg/mL/lymphocytes)			
SNP	**Gene**	**[1]β_{SNP}**	**[1]SE_{SNP}**	**P-value[2]**	**P_{adj}-value[3]**	**[1]β_{SNP}**	**[1]SE_{SNP}**	**P-value[2]**	**P_{adj}-value[3]**
rs11466657	*TLR10*	0.537	0.186	0.0039*	0.0077^	0.803	0.224	0.0004*	0.0008*
rs11466645		0.610	0.089	<0.0001*	<0.0001*	0.822	0.104	<0.0001*	<0.0001*
rs11466617		0.614	0.086	<0.0001*	<0.0001*	0.823	0.103	<0.0001*	<0.0001*
rs7660429		0.150	0.100	0.14	0.14	−0.066	0.121	0.58	0.46
rs11725309		0.614	0.088	<0.0001*	<0.0001*	0.848	0.103	<0.0001*	<0.0001*
rs3923647	*TLR1*	0.412	0.208	0.049	0.022^	0.467	0.250	0.063	0.034^
rs4833095		0.666	0.081	<0.0001*	<0.0001*	0.880	0.096	<0.0001*	<0.0001*
rs5743595		0.658	0.087	<0.0001*	<0.0001*	0.912	0.103	<0.0001*	<0.0001*
rs5743594		−0.174	0.087	0.047	0.037^	−0.260	0.106	0.015^	0.016^
rs5743582		0.023	0.107	0.83	0.96	−0.302	0.138	0.018^	0.0094^
rs5743580		0.657	0.088	<0.0001*	<0.0001*	0.912	0.103	<0.0001*	<0.0001*
rs5743827	*TLR6*	−0.027	0.083	0.74	0.83	−0.265	0.099	0.0076^	0.0078^
rs5743815		−0.728	0.234	0.0019*	0.0010*	0.080	0.287	0.78	0.94
rs5743795		0.571	0.086	<0.0001*	<0.0001*	0.756	0.103	<0.0001*	<0.0001*
rs5743788		0.416	0.094	<0.0001*	<0.0001*	0.294	0.114	0.010^	0.012^

[1]SNP-estimates are based on a dominant model. β estimates represent a natural log increase or decrease in stimulated Ln IL-6 and TNF-α (pg/mL/lymphocytes), with the dominant genotype as the reference

[2]Univariate p-value results are from t-tests

[3]Multivariable results (p_{adj}) are adjusted for age, body mass index, education, sex, race, COPD/smoke and years worked on a farm

*Passed the FDR adjustment at 1% level;

^passed the FDR adjustment at 5% level

Table 5

Association of *TLR10*, *TLR1*, and *TLR6* Haplotypes with Pam3CSK4-Stimulated IL-6 and TNF-α Levels

***TLR10-TLR1-TLR6* Haplotypes**[1]	**n**[2]	**Ln IL-6 (pg/mL/lymphocytes) β (SE)**[3]	**P-value**[4]	**P_{adj}-value**[5]	**n**[2]	**Ln TNF-α (pg/mL/lymphocytes) β (SE)**[3]	**P-value**[4]	**P_{adj}-value**[5]
TCATCCGGTG	232	0	Ref[6]	Ref[6]	232	0	Ref[6]	Ref[6]
CCACCCAGTC	13	0.730 (0.175)	<0.0001	<0.0001	13	0.764 (0.205)	<0.0001	0.001
TCACCCAGTC	67	0.609 (0.087)	<0.0001	<0.0001	67	0.669 (0.103)	<0.0001	<0.0001
TCATTCGATC	84	0.028 (0.080	0.73	0.74	84	−0.029 (0.089)	0.74	0.78
TGATCTGATC	36	0.158 (0.115)	0.17	0.13	36	−0.128 (0.132)	0.33	0.23
Other	77	0.257 (0.079)	0.001	0.001	77	0.117 (0.093)	0.21	0.23

[1] Haplotypes defined as rs11466657, rs7660429, rs3923647, rs4833095, rs5743594, rs5743582, rs5743580, rs5743827, rs5743815, and rs5743788.

[2] Sample sizes estimated from haplotype frequency

[3] β represents the change compared to the reference haplotype

[4] Univariate p-value results

[5] Multivariable results (p_{adj}) are adjusted for age, BMI, education, sex, COPD/smoke status, race and years worked on a farm

[6] Pairwise comparison to the reference haplotype

Table 6

Association of *TLR10*, *TLR1*, and *TLR6* Polymorphisms with Peptidoglycan-Stimulated IL-6 and TNF-α

		Ln IL-6 (pg/mL/lymphocytes)				Ln TNF-α (pg/mL/lymphocytes)			
SNP	**Gene**	**[1]β_{SNP}**	**[1]SE_{SNP}**	**P-value[2]**	**P_{adj}-value[3]**	**[1]β_{SNP}**	**[1]SE_{SNP}**	**P-value[2]**	**P_{adj}-value[3]**
rs11466657	*TLR10*	0.083	0.118	0.48	0.61	0.180	0.158	0.25	0.30
rs11466645		0.059	0.055	0.28	0.24	0.157	0.075	0.037	0.052
rs11466617		0.074	0.055	0.18	0.19	0.168	0.074	0.024	0.037
rs7660429		0.070	0.062	0.25	0.095	0.005	0.083	0.95	0.53
rs11725309		0.084	0.088	0.13	0.093	0.169	0.075	0.026	0.034
rs3923647	*TLR1*	0.240	0.129	0.064	0.039	0.106	0.176	0.55	0.43
rs4833095		0.088	0.052	0.091	0.041	0.149	0.071	0.036	0.021
rs5743595		0.123	0.055	0.026	0.022	0.215	0.075	0.0042^	0.0058^
rs5743594		0.039	0.054	0.47	0.75	0.042	0.072	0.56	0.86
rs5743582		0.033	0.065	0.61	0.39	−0.041	0.087	0.64	0.97
rs5743580		0.121	0.055	0.029	0.024	0.215	0.075	0.0043^	0.0062^
rs5743827	*TLR6*	0.094	0.051	0.064	0.054	0.043	0.068	0.53	0.47
rs5743815		−0.472	0.142	0.0009^	0.0006*	−0.278	0.191	0.15	0.085
rs5743795		0.118	0.055	0.032	0.021	0.129	0.074	0.082	0.11
rs5743788		0.141	0.057	0.014	0.0065^	0.178	0.078	0.023	0.010^

[1] SNP-estimates are based on a dominant model. β estimates represent a natural log increase or decrease in Ln IL-6 or Ln TNF-α, with the dominant genotype as the reference

[2] Univariate p-value results are from t-tests

[3] Multivariable results (p_{adj}) are adjusted for age, body mass index, education, sex, race, COPD/smoke and years worked on a farm

* Passed the FDR adjustment at 1% level;

^ passed the FDR adjustment at 5% level.

Table 7

Association of *TLR10*, *TLR1*, and *TLR6* Haplotypes with Peptidoglycan-Stimulated IL-6 and TNF-α

***TLR10-TLR1-TLR6* Haplotypes**[1]	**n**[2]	**Ln IL-6 (pg/mL/lymphocytes) β (SE)**[3]	**P-value**[4]	**P_{adj}-value**[5]	**n**[2]	**Ln TNF-α (pg/mL/lymphocytes) β (SE)**[3]	**P-value**[4]	**P_{adj}-value**[5]
TCATCCGGTG	232	0	Ref[6]	Ref[6]	232	0	Ref[6]	Ref[6]
CCACCCAGTC	13	0.186 (0.116)	0.11	0.18	13	0.245 (0.152)	0.11	0.17
TCACCCAGTC	67	0.152 (0.059)	0.010	0.003	67	0.187 (0.077)	0.015	0.014
TCATTCGATC	84	0.079 (0.053)	0.14	0.18	84	0.069 (0.070)	0.32	0.46
TGATCTGATC	36	0.096 (0.076)	0.21	0.095	36	0.041 (0.100)	0.68	0.48
Other	77	0.042 (0.053)	0.42	0.12	77	−0.035 (0.069)	0.61	0.97

[1] Haplotypes defined as rs11466657, rs7660429, rs3923647, rs4833095, rs5743594, rs5743582, rs5743580, rs5743827, rs5743815, and rs5743788.

[2] Sample sizes estimated from haplotype frequency

[3] β represents the change compared to the reference haplotype

[4] Univariate p-value results

[5] Multivariable results (p_{adj}) are adjusted for age, BMI, education, sex, COPD/smoke status, race and years worked on a farm

[6] Pairwise comparison to the reference haplotype