ORIGINAL ARTICLE

TNF polymorphisms modify endotoxin exposure-associated longitudinal lung function decline

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Objectives: Endotoxin exposure induces airway inflammation, hyper-responsiveness and higher expression of tumour necrosis factor (TNF). This study was conducted to investigate whether *TNF* polymorphisms modify the effect of endotoxin exposure on chronic declines in lung function.

Methods: Associations between *TNF* and *LTA* polymorphisms, endotoxin exposure and lung function were analysed in 263 cotton workers and 230 silk workers as a reference group, who were prospectively followed for 20 years. Multiple linear regression models were used to assess the association, with adjustment for smoking and other covariates.

Results: Endotoxin exposure was associated with faster lung function decline among genotypes associated with higher TNF expression levels, with estimates of annual FEV_1 change in relation to endotoxin exposure of −2.9 ml and −6.8 ml in the *G/G* and *G/A+AA* genotypes, respectively, for the *TNF* polymorphism; and −2.0 ml, −4.0 ml and −3.6 ml in *A/A*, *A/G* and *G/G* genotypes, respectively, for the *LTA* polymorphism. When joint effects of endotoxin exposure and smoking were considered, the effect modification of *TNF* and *LTA* polymorphisms was prominent in never smokers.

Conclusions: *TNF* and *LTA* polymorphisms may modify the association between occupational endotoxin exposure and longitudinal lung function decline, which was more clearly observed in never smokers.

Long-term occupational exposure to vegetable dust may cause lung function decline and airway disease. Both animal and epidemiological studies suggest that bacterial endotoxin is the major causative agent in organic dust that contributes to acute and chronic airway inflammation and airflow obstruction.[1 2] The incorporation of endotoxin into macrophages and endothelial cells results in local production of inflammatory cytokines, along with subsequent migration of inflammatory cells into the lung and the penetration of cytokines into the blood.[3]

Tumour necrosis factor (TNF), an important regulatory cytokine with strong proinflammatory and immunomodulatory properties, elicits the widest spectrum of biological activities, including defense against bacterial infection.[4] TNF helps mediate the septic shock state induced by bacterial endotoxin and the wasting diathesis that typifies chronic diseases.[5] Natural induction of TNF may have protective effects, but its overproduction may be detrimental and even lethal to the host.[4] TNF may increase airway responsiveness and neutrophil infiltration,[6] promote tracheal smooth muscle proliferation and alter smooth muscle function,[7] suggesting that high TNF expression levels may contribute to the airway inflammation and hyper-responsiveness.

The polymorphic genes of *TNF* and *LTA* (lymphotoxin alpha or *TNF* beta) are arranged in tandem. The *A* allele of the *TNF −308G/A* polymorphism (rs1800629) and the *G* allele of the *LTA 252A/G* polymorphism (rs909253) are associated with higher production and expression of *TNF*[8 9] or *LTA*,[10] respectively, and are the most widely studied polymorphisms for these two genes. Some studies found no associations between *TNF* or *LTA* polymorphisms and lung function decline or the development of chronic obstructive pulmonary disease (COPD),[11–16] while several others suggested that the two *TNF* polymorphisms are associated with the development or prognosis of COPD.[17–19]

In a previous study, we observed that long-term occupational exposure to cotton dust resulted in faster declines in lung function, which were associated most closely with high concentrations of endotoxin.[20 21] It has also been reported that endotoxin exposure may induce increased concentrations of TNF in serum[22] and bronchoalveolar lavage.[23] Polymorphisms relevant to different *TNF* serum levels may affect the association between endotoxin exposure and lung function changes. Based on this evidence, we hypothesised that polymorphisms associated with higher TNF expressions increase the effect of endotoxin exposure on lung function changes. In addition, cigarette smoking, a major risk factor for chronic airway obstruction may, in turn, modify the associations among *TNF* polymorphisms, endotoxin exposure and pulmonary function. The primary aim of this study was to test this hypotheses in a cohort of textile workers, who were followed for 20 years.

METHODS

Study population

The Institutional Review Boards of the Harvard School of Public Health, the Putuo District People's Hospital, and the Human Resources Administration, China approved the study. The study subjects came from a longitudinal cohort study that was undertaken in cotton textile workers in Shanghai, China, whose respiratory health and pulmonary function were followed prospectively for 20 years. Details of this population are described elsewhere.[20 24] In brief, 447 cotton textile workers and 472 silk textile workers were recruited and studied from 1981 onwards. Follow-up surveys were conducted in 1986, 1992, 1996 and 2001. Forced expiratory manoeuvre data were available through 2001. Since cotton dust and endotoxin levels in the silk mill were nearly zero over the period of study, we assigned cotton workers as an endotoxin exposure group and silk workers as a reference group. A total of 501 subjects who returned to the last survey and donated blood samples were

Abbreviations: COPD, chronic obstructive pulmonary disease; PL-EM, Partition Ligation-Expectation Maximization; TNF, tumour necrosis factor

Table 1 Characteristics of subjects in endotoxin exposed and reference groups

	Exposed group n = 263	Reference group n = 230
Gender (%)		
Male	110 (42%)	98 (43%)
Female	153 (58%)	132 (57%)
Age (years)*	57 (10)	56 (10)
Height (cm)*	164 (7)	163 (7)
Smoking status (%)		
Never smoker	181 (69%)	159 (69%)
Ex-smoker	14 (5%)	13 (6%)
Current smoker	68 (26%)	58 (25%)
Pack-years of smoking in smokers	29 (21)	25 (16)
FEV_1 at baseline (ml)	2894 (717)	2883 (647)
Changes in FEV_1 (ml/year)†	−31.50 (17.07)	−27.50 (14.97)
TNF polymorphism		
A/A	1 (1%)	3 (1%)
G/A	35 (13%)	32 (14%)
G/G	227 (86%)	195 (85%)
LTA polymorphism		
G/G	50 (19%)	35 (15%)
A/G	127 (48%)	122 (53%)
A/A	86 (33%)	73 (32%)
TNF haplotype‡		
Haplotype 1 (*TNF_G-LTA_A*)	299 (57%)	268 (58%)
Haplotype 2 (*TNF_G-LTA_G*)	190 (36%)	154 (34%)
Haplotype 3 (*TNF_A-LTA_G*)	37 (7%)	38 (8%)

*Age and height at the last survey.
†Annual changes in FEV_1 over 20 years.
‡Haplotype frequencies, where each subject carries two haplotypes.

included in this analysis, which consisted of 267 (60%) in the exposure group and 234 (50%) in the reference.

Genotyping

DNA was extracted from peripheral blood samples using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). The *TNF -308G/A* and *LTA 252A/G* polymorphisms were genotyped by the 5′ nuclease assay using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers, probes and reaction conditions are available upon request. Genotyping was performed by laboratory personnel blinded to case-control status, and a random 5% of the samples were repeated to validate genotyping procedures.

Table 2 Estimates for annual changes in FEV_1 (ml/year) over 20 years in relation to endotoxin exposure by *TNF* polymorphisms*

Genotypes	n	Estimate (95% CI†)
TNF polymorphism		
G/G	422	−2.87 (−5.31 to −0.43)
GA+AA	71	−6.83 (−13.32 to −0.34)
LTA polymorphism		
A/A	159	−2.03 (−6.39 to 2.34)
A/G	249	−3.97 (−7.09 to −0.85)
G/G	85	−3.85 (−9.13 to 1.97)
A/G+G/G	334	−3.95 (−6.63 to −1.28)
Combined *TNF* and *LTA* polymorphisms‡		
Zero variant allele	159	−2.03 (−6.39 to 2.34)
One variant allele	209	−3.43 (−6.69 to −0.18)
Two or more variant alleles	125	−5.17 (−9.97 to −0.36)

*Adjusting for age, smoking status, smoking amount and baseline FEV_1.
†95% CI for tests that coefficients are different from zero.
‡Combined genotypes based on the number of variant alleles, with zero variant allele in the wild genotype (*G/G* of *TNF* and *A/A* of *LTA*), one variant allele in heterozygous genotype (*G/A* of *TNF* and *A/G* of *LTA*), and two or more variant alleles in homozygous genotype (*A/A* of *TNF* and *G/G* of *LTA*).

Statistical analysis

Differences in the distribution of the principal covariates between the exposure group and reference group were tested using the χ^2, Fisher's exact, and Student's t tests, where appropriate. Annual decline in FEV_1 was calculated based on the differences in pre-shift FEV_1 measurements between the last and baseline survey and divided by 20 (years). The reason that we used the two-point FEV_1 was because the data at these two surveys were available for all of the study subjects. This enabled us to maintain statistical power to detect possible exposure–response relations, given that there were missing data from 14% to 26% at other follow-up surveys. Multiple linear regression models were used to determine the association between *TNF* and *LTA* genotypes, alone or in combination with endotoxin exposure, and the annual decline rate of FEV_1 (treated as a continuous variable). Covariates considered in the models included gender, smoking status (categorical variables), age, height, baseline FEV_1 and smoking amount (continuous variables). Interactions between these variables were also examined.

Detection of linkage disequilibrium between the two polymorphisms was based on Lewontin's D′ in all subjects.[25] Haplotypes of the two *ERCC1* polymorphisms were generated using the Partition Ligation-Expectation Maximization (PL-EM) version 1.0.[26] This software uses an efficient variant of the EM algorithm to reconstruct individual probabilities for individual phasing accuracy, based on unphased genotype data. At the same time, it also provides estimates on the overall haplotype frequencies as well as their standard errors.

In the genotype analyses, the wild *G/G* genotype of *TNF* and *A/A* genotype of *LTA* polymorphisms were treated as reference groups, and the *TNF G/A* and *A/A* genotypes were combined because of sparsity of *A/A* genotype. *TNF* and *LTA* were analysed separately as well as in combination. In addition to determining the overall association between *TNF* genotypes and longitudinal changes in lung function, we assessed possible effect modification of *TNF* polymorphisms on the association between endotoxin exposure and lung function by stratifying *TNF* and *LTA* polymorphisms in the analysis. Finally, we investigated the joint effects of combined *TNF* and *LTA* polymorphisms, endotoxin exposure, and smoking on the longitudinal changes in FEV_1, of which non-exposed, non-smokers with *TNF* ''wild type'' genotype were defined as the reference group. Statistical analyses were carried out using SAS personal computer software (version 9.2, SAS Institute, Cary, NC, USA).

RESULTS

Population characteristics

There was no significant difference in age, gender, height, smoking status and baseline FEV_1 between subjects with and without blood sample in either group ($p>0.05$). A total of 493 out of the 501 subjects were genotyped successfully for both *TNF* (*-308 G/A*) and *LTA* (*252A/G*) polymorphisms, including 263 in the exposed group and 230 in the reference. The characteristics of the exposed and reference groups are shown in table 1. No significant differences were found in the demographic features such as age, gender, height, smoking status, smoking amount and baseline FEV_1 between the two groups ($p>0.05$).

Genotype and haplotype distributions

Both *TNF* and *LTA* polymorphisms were in Hardy–Weinberg equilibrium ($p>0.05$ by χ^2 test). Genotype frequencies in the exposed and reference groups were similar (table 1). The two polymorphisms were in high linkage disequilibrium ($D' = 0.98$), and subjects with wild types of *LTA* (*A/A* genotype) were also carriers of wild types of *TNF* (*G/G* genotype). Applying the PL-EM algorithm, the posterior probabilities of individual haplotypes were all greater than 99%; therefore, we assigned

Table 3 Joint effects of combined *TNF* polymorphisms, endotoxin exposure, and smoking status on annual declines in FEV_1 (ml/year)*

	Endotoxin exposure and smoking joint groups							
	Non-exposed + non-smoking		Non-exposed + smoking		Exposed + non-smoking		Exposed + smoking	
Genotype	Estimate (95% CI)	n	Estimate (95% CI)	n	Estimate (95% CI)	n	Estimate (95% CI)	n
Combined *TNF* and *LTA*†								
Wild genotype	Referent	118	−2.48 (−6.98 to 2.02)	55	−0.65 (−3.89 to 2.59)	131	−11.09 (−14.74 to −1.50)	64
Variant genotype	2.97 (−1.66 to 7.59)	41	−5.64 (−12.59 to 1.31)	16	−2.08 (−6.39 to 2.24)	50	−8.12 (−15.38 to −6.79)	18

*Adjusting for age and baseline FEV_1.
†Combined genotypes of *TNF* or *LTA* based on the number of variant alleles, with zero or one variant allele in ''wild genotype'', two or more variant alleles in ''variant genotype''.

each individual haplotype with the highest posterior probability. Total of 3 haplotypes (*haplotype 1*, *TNF_G-LTA_A*; *haplotype 2*, *TNF_G-LTA_G*; and *haplotype 3*, *TNF_A-LTA_G*) were generated using PL-EM, with the similar haplotype distributions in the exposed and reference groups (table 1). Because of the high linkage disequilibrium between the two polymorphisms, all subjects with the *LTA A/A* genotype had two copies of *haplotype 1*, subjects with the *LTA A/G* genotype had one copy of *haplotype 1*, and subjects with the *LTA G/G* genotype had no copies of the *haplotype 1*. Thus, analysis of gene–gene joint effects was performed subsequently, as the haplotype analysis did not provide additional information.

TNF polymorphisms and the changes in lung function

Overall, the average annual changes in FEV_1 were –31.5 ml in exposed subjects and –27.5 ml in reference (''–'' represents the direction of decline). Regression analysis suggested that endotoxin exposure was associated with –3.2 ml/year change. There was no significant association between *TNF* and *LTA* polymorphisms and lung function decline. For the *TNF* polymorphism, the estimated annual change in FEV_1 was –0.3 ml for *GA+A/A*, relatively to the *G/G* genotype ($p = 0.86$). For the *LTA* polymorphism, the annual changes were –0.4 ml ($p = 0.86$) and 0.3 ml ($p = 0.17$), respectively, for the *A/G* and *G/G* genotypes when compared with the *A/A* genotype.

To determine potential effect modification of *TNF* or *LTA* polymorphisms on the association between endotoxin exposure and annual changes in FEV_1, we fitted multiple liner regression models with *TNF*, *LTA* polymorphism, as well as their combination, respectively. Age, sex, height, baseline FEV_1, smoking status and smoking amount were considered in the initial models as candidate covariates or confounders. Height and sex were removed from the final models because of little contribution to the outcomes of interest, whereas age, smoking amount and baseline FEV_1 were retained in the models. As shown in table 2, exposure to endotoxin was associated with lung function declines in all *TNF* genotype groups. The estimated decline rates, however, were greater in the variant genotypes. Among the group with the *TNF* polymorphism, the average annual change in FEV_1 associated with endotoxin exposure was –6.83 ml for *G/A+A/A* genotypes, in contrast to –2.87 for *G/G*. Among the *LTA* polymorphism groups, the annual changes were –3.97 and –3.58 ml for *A/G*, *G/G*, respectively, in contrast to –2.03 for the *A/A* genotype.

When *TNF* and *LTA* polymorphisms were combined based on the number of variant alleles—that is, heterozygous genotype (*G/A* of *TNF* and *A/G* of *LTA*) had one variant allele, and homozygous genotype (*A/A* of *TNF* and *G/G* of *LTA*) had two variant alleles—the estimated endotoxin exposure associated FEV_1 declines relatively tended to be greater with increasing variant alleles (table 2). The coefficients in variant genotypes were significantly different from zero, whereas this was not the case in variant alleles.

Furthermore, we added the factor of cigarette smoking to the combined *TNF* polymorphisms and endotoxin exposure, and examined their joint effects with multiple linear regression models. Again, other relevant factors were adjusted. As shown in table 3, in subjects with either smoking or exposure to endotoxin, variant *TNF* genotype was associated with a greater FEV_1 decline than corresponding wild *TNF* genotype. Subjects with both endotoxin exposure and tobacco smoking had greater annual declines in FEV_1 than any other subgroups, but the annual decline was not greater in the variant genotype subgroup than in the wild genotype subgroup.

DISCUSSION

In this study, we examined a possible link between *TNF* polymorphisms and chronic lung function changes related to endotoxin exposure in a cohort of textile workers. Overall, the differences in annual FEV_1 declines were greater in exposed subjects with variant genotypes. The magnitude of the effect modification of *TNF* polymorphisms on lung function changes associated with endotoxin exposure ranged, on average, from 2–4 ml per annum, or 40–80 ml for 20 years, indicating that the effect modification was relatively small.

The difference in annual decline of FEV_1 between variant and wild type *TNF* genotypes among those who were both exposed to endotoxin and smoked was barely detectable in the joint effects models (table 3). There are at least two possible explanations for this. Firstly, the coexistence of both endotoxin exposure and cigarette smoking may have exerted a too strong chronic effect on airways that overwhelmed or masked the modest effect modification of *TNF* polymorphisms. Another possibility is that the small number of subjects with the variant genotype in this category may have resulted in inadequate study power to detect the effect modification. The former is more likely, given that the sample sizes in the other cells of the table were similar.

TNF has strong proinflammatory and immunomodulatory properties. However, it has been reported that TNF is associated with increased airway responsiveness in normal subjects, and with airway inflammation with a neutrophil infiltration.[6] Although both of the variant genotypes of *TNF* and *LTA* correspond to higher baseline and inducible TNF expression levels,[9 10] the underlying effects of *TNF* polymorphisms alone on longitudinal changes in lung function may not be strong enough to be detected, without consideration of their interaction with environmental factors. TNF has been found to be central to smoke-induced acute inflammation and resulting connective tissue breakdown, and occurrence of emphysema.[27] Exposure to endotoxin may result in higher expressions of TNF.[22 23] Therefore, the effect of *TNF* polymorphisms can be observed when exposure to endotoxin or cigarette smoking exists, in which *TNF* polymorphisms modify the effect of these environmental exposures on respiratory outcomes.

Among several studies linking *TNF* polymorphisms to the development or prognosis of COPD, an Irish study suggested that the *A/A* genotype of the *TNF* polymorphism was predisposed to more severe airflow obstruction and a worse prognosis in COPD.[17] A Japanese study suggested that the *TNF* polymorphism was significantly associated with smoking-related COPD,[18] and might be partly associated with the extent of emphysematous changes in patients with COPD.[19] Among the studies focusing on occupational populations, a study conducted in China found that the *TNF* polymorphism was associated with coal workers' pneumoconiosis.[28] A French study also reported that interactions of *TNF* and *LTA* with environmental exposure and intermediate response phenotypes were important components in the pathogenesis of coal workers' pneumoconiosis.[29] The current study provides additional information on the connection between *TNF* polymorphisms and chronic changes in lung function in endotoxin-exposed workers.

We acknowledge several limitations in this study. Firstly, this is a study with a moderate sample size. Although we observed effect modification of *TNF* polymorphisms on endotoxin-related lung function decline, gene–environment interaction was not clearly seen in the stratification analysis, quite possibly because of insufficient study power. Secondly, the subjects who donated blood samples accounted for only 55% subjects of the original cohort. To identify whether the subjects being analysed differed from those who were not included in this study, we compared the demographic features and lung function testing data at baseline and follow-up between the participants and non-participants in this study. The results showed no significant differences between the two compared groups, indicating that the loss to follow-up was non-differential. Therefore, the results based on the subjects were unlikely to be substantially biased. Lastly, we evaluated only two polymorphisms of *TNF* that are related to TNF expression levels. It is possible that other polymorphisms of these two genes—for example, *-376G/A, -238G/A* and *489G/A*[30]—or polymorphisms of other cytokine genes may modify the association between environmental exposures and respiratory outcomes. Further studies with a larger sample size are needed to address this issue, in which possible gene–gene interaction or gene–gene joint effects can thus be examined.

In conclusion, *TNF* polymorphisms may modify longitudinal changes in lung function associated with occupational exposure to endotoxin, in which variant *TNF* genotypes (*A* allele of the *TNF* and *G* allele of the *LTA* polymorphism) boost lung function declines. The results highlight the importance of further investigating the roles of host factors in the development of respiratory diseases in occupationally-exposed populations.

Main messages

- Cotton dust and other vegetable dust exposure results in airway inflammation. This inflammation is mediated in part by a protein known as tumour necrosis factor (TNF). The inducibility of this protein is related to variation in the TNF gene.
- Our findings in this prospective follow-up study of textile workers indicate that such genetic variation modifies the association between endotoxin exposure and long-term loss of lung function, but this effect was modest.

Policy implications

- The most effective way to prevent endotoxin-related lung function loss is by reducing organic dust exposures, by setting permissible exposure limits low enough to prevent dysfunction in the most susceptible workers.

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