

MECHANISMS

Genetic Variation in the *TNFA* Promoter Region and *TNFA* Gene Expression in Subjects with Asthma

BRIDGETTE L. JONES,* BELINDA K. GRAHAM, AMANDA K. RIFFEL, HONGYING DAI, LANNY J. ROSENWASSER, AND CARRIE A. VYHLIDAL

Children's Mercy Hospital, Kansas City, MO, USA.

Objective. Asthma is a chronic disease that affects millions of people. Messenger RNA (mRNA) expression of specific inflammatory markers has been associated with asthma and corticosteroid response. Tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine, has been shown to have increased expression in airways of asthmatics and may be related to corticosteroid sensitivity. The purpose of this study was to determine how genetic variants within the promoter region of the *TNFA* gene differ between subjects with asthma and controls. We also investigated how genetic variation affects gene expression. **Methods.** We enrolled 94 subjects between 5 to 54 years of age who met the inclusion and exclusion criteria. *TNFA* mRNA expression was determined by qRT-PCR on total RNA isolated from the buccal mucosa. Genotyping was performed for *TNFA*-1031T/C, -857C/T, and -308G/A on genomic DNA isolated from blood with commercially available assays. Gene expression was log-2 transformed and corrected with 2 normalization genes. General linear model, Chi-square test, Fisher's exact test, and Cochran–Mantel–Haenszel test were performed with $p < .05$. **Results.** The *TNFA*-857C/T polymorphism is associated with asthma in this cohort. The *TNFA*-857T allele is underrepresented in pediatric subjects with asthma relative to those without asthma (3% and 29% of individuals, respectively, $p = .01$). Furthermore, a *TNFA* haplotype combination containing -1031T/-857C/-308G and -1031T/-857T/-308G is associated with lower expression of TNF- α mRNA ($p = .01$) in pediatric subjects. **Conclusions.** Presence of the *TNFA*-857T allele may be protective in the development of asthma and a haplotype combination that contains the *TNFA*-857T allele is associated with *TNFA* expression.

Keywords biomarker, cytokine, gene expression, inflammatory markers, polymorphisms

INTRODUCTION

Asthma is a chronic disease that affects millions of people worldwide. According to the American Lung Association and the National Health Interview Survey, approximately 38.4 million Americans are diagnosed with asthma within their lifetime (1). Various mediators are known to be involved in the pathophysiology of asthma. Tumor Necrosis Factor- α (TNF- α), a pro-inflammatory cytokine, is one such mediator. TNF- α plays a key role in the host response to microorganisms and is produced by numerous cells including macrophages, monocytes, dendritic cells, eosinophils, and even epithelial and smooth muscle cells. The cytokine plays multiple roles in the pathogenesis of asthma which include: T cell activation and cytokine release, upregulation of adhesion molecules in the airway and vascular endothelium, increased airway smooth muscle hyperresponsiveness, recruitment of eosinophils, neutrophils, macrophages and monocytes, and increased histamine release from airway mast cells (2). The importance of TNF- α in the pathogenesis of asthma has also been demonstrated by the findings of Howarth et al. who reported that the bronchoalveolar lavage fluid levels of TNF- α protein and messenger RNA (mRNA) expression in the airway was higher in those with severe asthma compared to mild disease (3). Others have also found evidence that the mediator is particularly important in severe disease. Berry et al. found that membrane-bound

TNF- α , TNF- α converting enzyme, and TNF- α receptor expression in peripheral monocytes was increased in subjects with refractory asthma compared to subjects with mild-to-moderate asthma (4). Recent data regarding anti-TNF therapy in patients with asthma have provided mixed conclusions. Earlier studies have suggested that anti-TNF therapy may be beneficial in certain sub-populations of asthma patients (3, 4). However, a recent study of anti-TNF therapy in patients with asthma was terminated early due to severe adverse effects (5).

Asthma is a complex disease which is likely to be a result of synergistic environmental, genetic, and developmental factors (6). The gene that expresses TNF- α , *TNFA*, is located in the major histocompatibility complex region on chromosome 6p21.3, a highly variable region. Genetic polymorphisms within the *TNFA* promoter are suggested to be associated with the disease. Various investigators have found associations between the *TNFA*-308 G/A polymorphism and asthma. However, conflicting reports exist regarding the effect of this variation (7–12). For example, the A allele at *TNFA*-308 was associated with increased susceptibility to asthma and elevated *TNFA* transcription (7–10). Other investigators found that the G allele was associated with asthma (10). A study in Japanese children identified the -857C allele and the -1031T-863C-857C haplotype as being associated with asthma. The study also suggested that the -857 allele and -1031T-863C-857C haplotype may be associated with low promoter activity (13). However, a mechanistic cause that explains these associations has not been identified.

*Corresponding author: Bridgette Jones, Children's Mercy Hospital, Kansas City, MO, USA; E-mail: bljones@cmh.edu

We aimed to determine if there were genetic differences in the promoter region of *TNFA* between subjects with and without asthma in children and in adults. We also evaluated whether genetic variation in the *TNFA* promoter region affected mRNA expression of TNF- α in the buccal mucosa, a potential surrogate marker for epithelial-based changes in the lungs of patients with asthma.

METHODS

Study Population

Children were recruited from the Children's Mercy Hospitals and Clinics and adults from the Truman Medical Center for this study, which was approved by both the respective Institutional Review Boards. Three cohorts of children were recruited: (1) without asthma, (2) asthma subjects on step 1 therapy, and (3) asthma subjects on step 2 or 3 therapy. Two cohorts of adults were recruited: (1) without asthma and (2) asthma on step 2 or 3 therapy. Step therapy was defined by the 2007 National Heart, Lung, and Blood Guidelines for the Diagnosis and Management of Asthma. Participants were enrolled after written informed consent or parental permission and child assent. Asthma was defined by spirometry according to the American Thoracic Society guidelines (14) or by clinical history in children unable to perform spirometry. Participants without asthma were also recruited from clinics at the Children's Mercy Hospitals and Clinics and the Truman Medical Center, and confirmation of non-asthma was reconciled with the treating physician. Exclusion criteria included: tobacco smokers, subjects with chronic lung disease, immunodeficiency, uncontrolled gastroesophageal reflux, pneumonia in the past 6 months, use of intranasal steroids within the past 2 weeks (due to concerns for topical effect on the buccal mucosa), use of systemic steroids within the past 6 weeks, and current immunomodulator treatment.

Genotyping

Genomic DNA was extracted from blood using the Illustra Blood Genomic Prep Mini Spin Kit (GE Healthcare, Piscataway, NJ). Genotyping assays were performed on genomic DNA (12–16 ng) using commercially available TaqMan assays to detect rs1799964 (-1031), rs1799724 (-857), and rs1800629 (-308) SNPs (assay id: C_7514871_10, C_11918223_10, and C_7514879_10, respectively, (Applied Biosystems, Foster City, CA)), and KAPA Probe Fast qPCR master mix (Kapa Biosystems, Boston, MA). Haplotype information was inferred by using PHASE (15).

RNA Extraction

Buccal cells were obtained with a sterile soft-bristled toothbrush after rinsing the mouth. The toothbrush was immediately stored in Saliva Protect RNA stabilization solution (Qiagen, Valencia CA). The samples were immersed in the solution for 24–72 hours, at 4°C prior to RNA extraction. RNA was extracted from 9–30 mg of

buccal cells using the Illustra RNAspin Mini RNA isolation kit (GE Healthcare, Piscataway, NJ).

Gene Expression

RNA (20 ng) was reverse transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems). Pre-amplification was performed using the TaqMan Pre-Amplification Kit (Applied Biosystems) according to the manufacturer's protocol and reactions were diluted 1:40. Quantitative PCR was performed on 3.2 μ l diluted, pre-amplified cDNA using PerfeCTa qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD) and TaqMan Gene Expression assays (HS00174128_ml for *TNFA*, 4333764F for *GAPDH*, and 4333763F for *PPIA* (Applied Biosystems)). Gene expression values were log₂ transformed and corrected by the geometric mean *GAPDH* and *PPIA*.

Statistical Analysis

Descriptive statistics, Chi-square test, and *t*-test were used to compare the demographics between subjects with and without asthma. Chi-square and Fisher's exact test were used to detect the association between asthma and *TNFA* genotype and haplotype. The impact of race on the association between asthma and genotype was evaluated by Cochran–Mantel–Haenszel test. General linear model was applied to compare gene expression by gender, race, age, and genotypes. Non-parametric analysis (Wilcoxon rank sum test or Kruskal–Wallis test) was used to compare the rank of *TNFA* gene expression among the different *TNFA* genotypes and haplotypes and results were consistent with the General linear model analysis. At last, logistic regression was also performed to evaluate the association between genotype and asthma susceptibility by adjusting for age and race. Statistical significance was claimed at 95% confidence level ($p < .05$). Analyses were performed using SAS 9.2 (Cary, NC) and SPSS 19.

RESULTS

Study Population

Evaluable data were available for 88 subjects between 5 and 54 years of age (mean $\pm$ standard deviation: 19.3 $\pm$ 15.0 years of age). Data from 6 subjects were removed from analysis due to low or no expression for all genes in the study, suggesting poor sample quality. Fifty-six percent of the subjects were male. The cohort largely included subjects who classified themselves as African American (52%) and the remaining subjects were classified as White non-hispanic (37%), or other (11%). There was no significant difference in gender related to genotype, haplotype, or gene expression ($p > .05$) for *TNFA*. See demographics in Table 1.

Genotyping Results

Figure 1 A shows the minor allele frequencies for *TNFA*-1031 T $\rightarrow$ C (rs1799964), -857 C $\rightarrow$ T (rs1799724), and -308 G $\rightarrow$ A (rs1800629) in all control subjects and study participants with asthma. The minor allele frequency for *TNFA*-1031T/C,

TABLE 1.—Subject demographics^a.

	Non-asthma	Asthma Step 1 level therapy	Asthma Step 2 or 3 level therapy
Adults			
<i>n</i>	10		17
Males (%)	3 (33%)		5 (29%)
Caucasian (%)	5 (50%)		4 (24%)
African American (%)	2 (20%)		12 (71%)
Median age (range)	38 (27–54)		44 (21–54)
Children, ages 5–11			
<i>n</i>	11	13	9
Males (%)	7 (64%)	9 (69%)	7 (78%)
Caucasian (%)	2 (18%)	5 (38%)	3 (33%)
African American (%)	6 (55%)	7 (54%)	5 (55%)
Median age (range)**	9 (5–11)	9 (6–11)	8 (6–10)
Children, ages 12–18			
<i>n</i>	10	10	8
Males (%)	5 (50%)	7 (70%)	6 (75%)
Caucasian (%)	5 (50%)	5 (50%)	4 (50%)
African American (%)	4 (40%)	5 (50%)	4 (50%)
Median age (range)**	14 (12–18)	14 (12–17)	13 (12–18)

^aAdult and pediatric cohort participants: number of adults and children recruited for each asthma severity group and age group.

**The distribution of healthy subjects and asthmatic subjects at two severity levels were not significantly different by gender or race ($p > .05$); **within each age group, age was comparable among control, step 1, and step 2/3 subjects ($p > .05$).

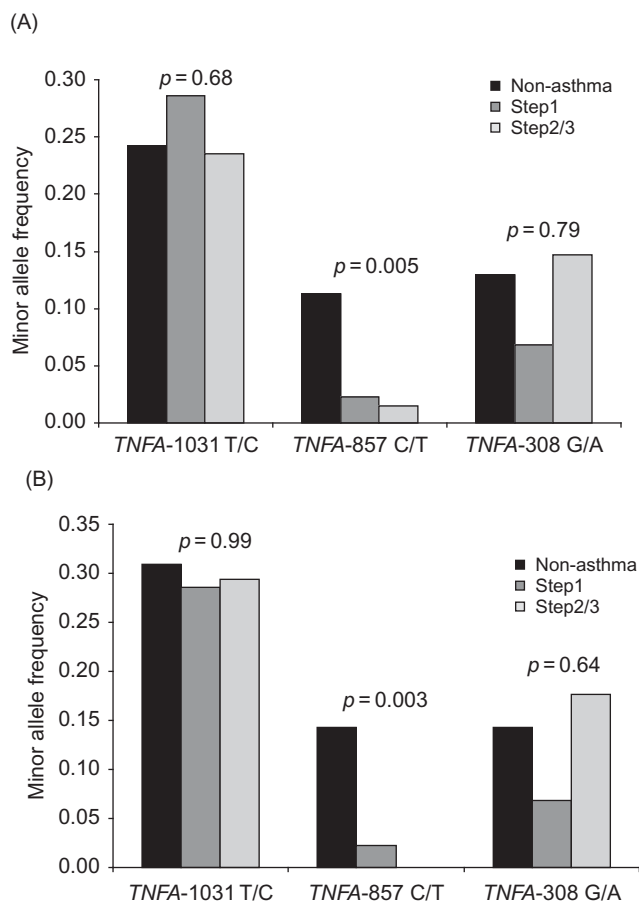


FIGURE 1. Minor allele frequencies of *TNFA*-857, -1031, -308 in participants without asthma and those with asthma that are on Step 1 therapy and Step 2 therapy. (A) Differences in minor allele frequencies in all subjects. (B) Differences in minor allele frequencies in the pediatric cohort. * p -values for differences in minor allele frequency between non-asthma, Step 1, Step 2 & 3 asthma are shown.

-857C/T, and -308G/A in the entire cohort were 0.25, 0.05, and 0.12, respectively. Genotype frequencies did not deviate from the expected Hardy–Weinberg equilibrium in control participants and participants with asthma ($p > .05$). The *TNFA*-857C/T polymorphism was associated with the diagnosis of asthma. The T allele was observed in 3.6% of individuals with asthma compared to 22.6% of those without the diagnosis of asthma, $p < .01$. The minor allele was also associated with less severe disease. The T allele was present in only 1 subject on Step 2/3 asthma therapy (3%) compared to 5% of subjects on step 1 therapy and 23% of control subjects ($p < .05$) (Figure 1A), which suggests that this allele may serve a protective role in the development of severe asthma. When analyzing the pediatric and adult cohorts separately, differences in allele frequency for *TNF*-857 were observed between the two cohorts. In the adult cohort, there was no difference in the T allele frequency between participants with and without asthma. However, in the pediatric population, a significant difference in allele frequency was observed, whereby the variant allele was present in 2.6% of the children with asthma as compared to 28.6% of the children without asthma ($p = .01$ adjusted by race and age; Bonferroni corrected $p = .03$). The T allele was not present at all in the children on Step 2/3 asthma therapy (Figure 1 Panel B). There were no participants in either cohort (pediatric or adult) who were homozygous for the T allele at the -857 position. The *TNFA*-857T allele was also underrepresented in subjects who identified as African American (95% homozygous C at *TNFA*-857, $p = .03$).

There was no difference in the minor allele frequency for *TNFA*-1031 or -308 between subjects with asthma vs. those without asthma in the combined cohort or stratified by children and adults (Figure 1A, B). We also did not observe a difference in ethnicity for these SNPs.

Haplotypes present in this cohort are represented in Figure 2. The *TNFA* haplotype, 1031T/-857C/-308G, occurred most frequently (59%) in the entire cohort. The

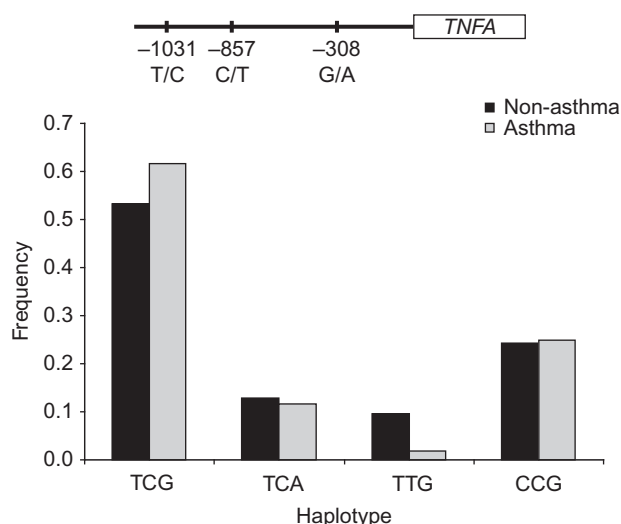


FIGURE 2. Haplotype frequencies of *TNFA*-1031T/-857T/-308G in participants without asthma and those with asthma that are on Step 1 therapy and Step 2 therapy. * p -values $>.05$ for all comparisons of haplotype frequency by asthma vs. non-asthma.

haplotype -1031T/-857T/-308G was underrepresented in subjects with asthma (1.8%) compared with subjects without asthma (9.7%), although this difference did not meet the statistical significance. This finding supports our previous findings which suggest a protective effect for *TNFA*-857T. There were no significant differences observed in the relationships between *TNFA* haplotype and disease when stratified by children and adults.

Gene Expression Results

No statistical differences were observed in the mRNA expression of *TNFA* in the buccal mucosa in relation to the -857 genotype or -1031 or -308 genotype (Figure 3) for the entire cohort or in the separate pediatric and adult cohorts. Gene expression levels for *TNFA* differed by diplo-type, whereby *TNFA*-1031T/-857T/-308G when also paired with *TNFA*-1031T/-857C/-308G appeared to be associated with lower expression of mRNA relative to subjects with other haplotype combinations ($p = .01$) in the pediatric cohort (Figure 4A). However, this association was not statistically significant in the adult group (Figure 4B).

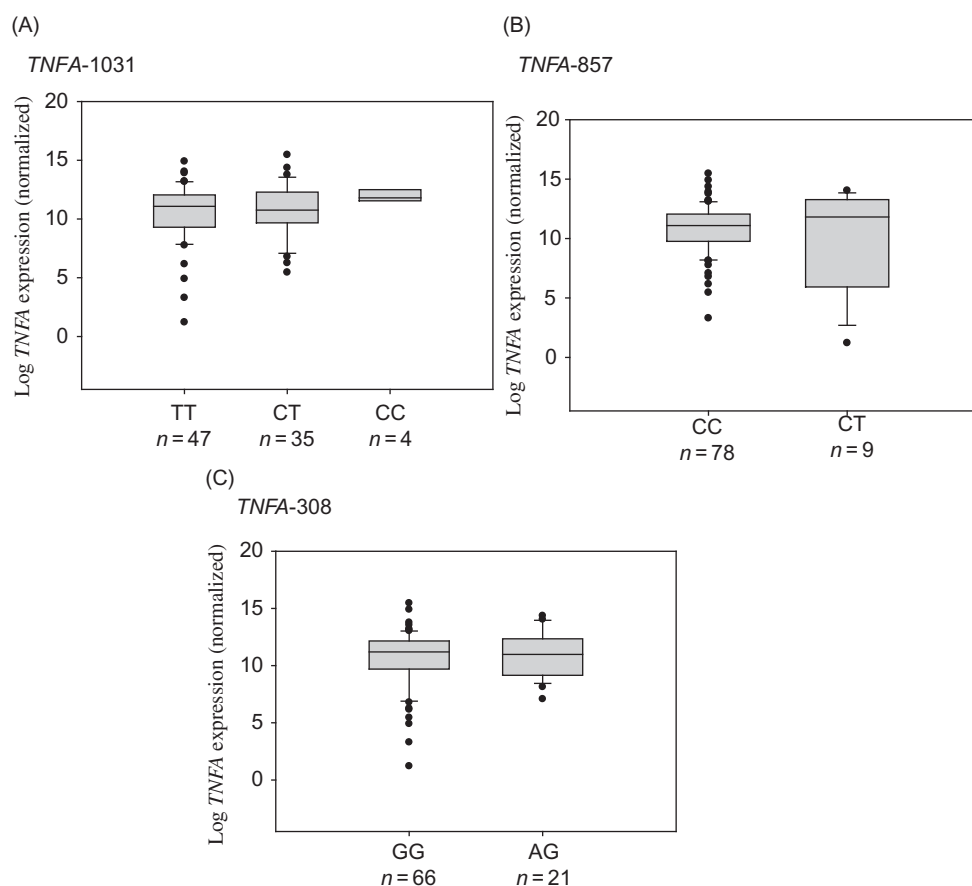


FIGURE 3. Effect of *TNFA* genotypes on mRNA expression. Differences in the log mRNA *TNFA* expression relative to observed genotype. (A) effect of *TNFA*-1031 on mRNA expression, (B) effect of *TNFA*-857 on mRNA expression, (C) effect of *TNFA*-308 on mRNA expression. * p -values $>.05$ for all comparisons of genotype and mRNA expression.

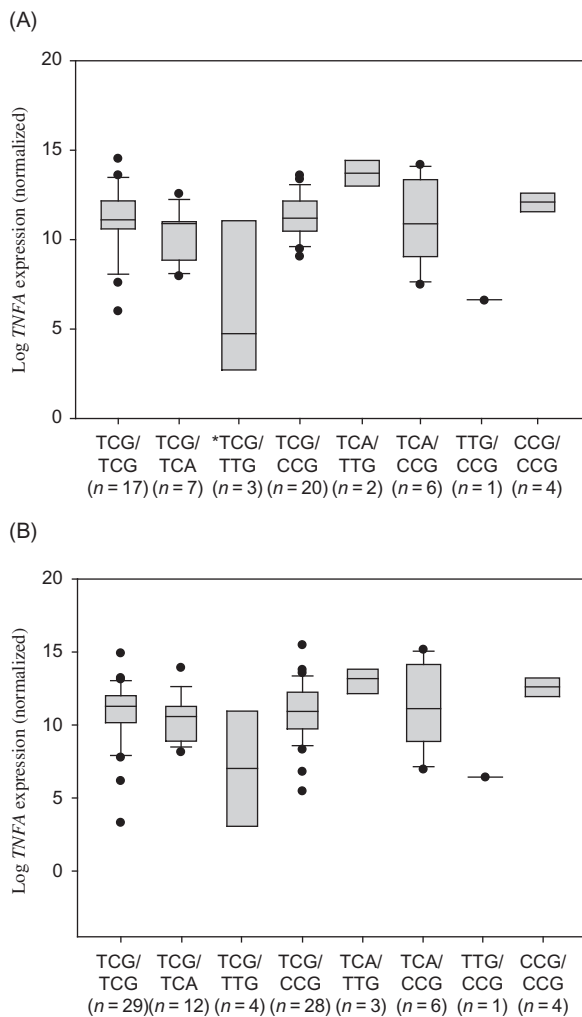


FIGURE 4. Effect of haplotype on mRNA expression. (A) Differences in the log mRNA *TNFA* expression relative to observed haplotypes in the pediatric cohort. *TCG/TTG haplotype is associated with decreased mRNA *TNFA* expression relative to other represented haplotype combination, $p = .01$. (B) Differences in the log mRNA *TNFA* expression relative to observed haplotypes in the combined cohort.

DISCUSSION

The *TNFA*-857T allele appears to be protective in the development of asthma; however, the results presented here suggest that this variant may be only protective in the pediatric populations. In children, the *TNFA*-857 T allele was underrepresented in subjects with asthma compared to subjects without asthma. Our findings are consistent with a previous study by Noguchi et al., conducted in a relatively large pediatric cohort, which demonstrated that *TNFA*-857C is associated with asthma (13). Other studies have not identified an association between the *TNFA*-857 polymorphism and asthma (11, 15, 16); however, these studies were primarily conducted in adult cohorts (11, 16). Perhaps the *TNFA*-857 SNP is most important in the pathogenesis of the disease during childhood. Our findings along with those of Noguchi et al., confirm that the *TNFA*-857 SNP is an important determinant in asthma and that further work should be done in evaluating the role of this SNP in children with the disease. We also found that the

TNFA-857 T allele was significantly underrepresented in the African American subjects. Previous studies, which evaluated polymorphisms in the *TNFA* promoter region, have been conducted in other ethnic populations (Caucasian, Korean, Indian, and Hispanic) and did not find an association between the SNP and asthma (11, 12, 15, 16). A majority of our study participants identified as African American and 95% of them did not possess a T allele at -857. To our knowledge, this study is the first evaluation of the *TNFA* promoter region in a largely African American cohort with asthma suggesting that the -857C/T SNP may be most important within certain ethnic populations (e.g. African Americans). We also observed a “dose-response” effect in relation to the protective effects of the *TNFA*-857 T allele and the severity of the disease in that the T allele was underrepresented in subjects with more severe disease compared to subjects with mild disease, which provides internal validity to our finding of a protective effect of the allele.

While others have reported higher *TNF- α* levels in subjects with severe asthma relative to mild disease (3, 4), we were not able to determine a biological mechanism underlying the apparent protective effects of the *TNFA*-857 T allele. We did not observe a statistically significant association between mRNA expression and *TNFA* genotype in either the pediatric or the adult populations. However, there was a trend toward lower expression of *TNFA* mRNA in subjects with genotype -857TC relative to individuals with the -857CC genotype. We also did not observe a statistical difference in gene expression for the *TNFA*-1031T/C or -308G/A SNPs. None of the participants possessed the *TNFA*-857TT or *TNFA*-308AA genotype and only 4 subjects had the *TNFA*-1031CC genotype which may have prevented us from observing significant differences in gene expression between the variant groups. In addition, subjects who were homozygous for the T allele at *TNFA*-1031, possessed a T allele at *TNFA*-857, or were homozygous for the G allele at *TNFA*-308 appeared to have a lower range of expression of the gene when compared to other represented haplotypes.

The *TNFA*-1031T/-857T/-308G haplotype was underrepresented in subjects with asthma relative to those without the disease, but this difference did not meet the statistical significance. Noguchi et al. also found that the *TNFA*-1031T/-863C/-857C haplotype was transmitted from parents to offspring with asthma more often than expected ($p = .00002$) and the haplotype -1031T/-863C/-857T was undertransmitted to children with asthma ($p = .0027$), suggesting a protective effect of this haplotype. A study conducted by Sharma et al. in an adult population found that an extended haplotype that contained -1031T/-857C/-308G was more common in subjects with asthma when compared to control subjects (16). These data support our results in which the *TNFA*-857T allele occurs at a lower frequency in children with asthma, suggesting that the -857T allele may provide a protective effect in the pathogenesis of asthma.

Although we did not find an association between the *TNFA* haplotypes and asthma, the *TNFA*-1031T/-857T/-308G

haplotype was associated with lower expression of *TNFA* when inherited along with a haplotype that contains a G allele at *TNFA*-308 and T allele at *TNFA*-1031 (homozygous for T allele at *TNFA*-1031, and homozygous for G allele at *TNFA*-308) in the pediatric group. We are unable to fully elucidate the possible additive effects of this haplotype combination. However, our previous observation regarding subjects who are homozygous GG at *TNFA*-308, homozygous TT at *TNFA*-1031, or possess a T allele at *TNFA*-857 with a tendency to have a lower range of gene expression may be related to this finding. Thereby, possessing a combination of these gene variants may lead to a more significantly reduced level of expression than observed if inherited alone, which may be especially true in specific ethnic populations and/or age groups. Alternatively, the observed association between the *TNFA* genotype and asthma may be due to other factors related to the effect of age, environmental factors, and/or genes that may be in linkage with *TNFA* which may actually be causative.

The *TNFA*-308G/A SNP has been the most investigated SNP in the *TNFA* promoter region in relation to asthma. Various studies have found an association between *TNFA*-308G/A and asthma (7, 8, 10, 17–20). We did not observe a statistically significant association between the *TNFA*-308G/A polymorphism and asthma among our cohort. However, the A allele at *TNFA*-308 was more commonly observed in subjects with more severe disease relative to those without asthma or with less severe disease. One study conducted in children found that *TNFA*-308GG genotype reduced the risk of asthma; however, this effect seemed to be associated with the environmental factor of living in a low-ozone area (17). Another study, conducted in Mexico City (a high-ozone area), found that the *TNFA*-308 A allele was associated with an increased risk of asthma and this effect was much stronger in children of non-smoking parents (15). Ozone and tobacco smoke exposure was not assessed as a part of our study; however, the Kansas City Metropolitan Area is generally considered a high-ozone area (21). Conflicting reports exist regarding the importance of the *TNFA*-308G/A polymorphism and *TNFA* mRNA expression (22, 23). Our overall findings are complimentary and biologically plausible in that the haplotype and genotype combinations associated with decreased disease are in turn associated with decreased expression of *TNFA*, one of the major mediators of the disease.

In our study, we focused on SNPs that have been previously evaluated in relation to asthma, although there are several additional SNPs within the *TNFA* promoter region (-863 C/A, -851 C/T, -419G/C, -376G/A, -238G/A, -162G/A, and -49G/A) that have yet to be fully explored. Perhaps other SNPs within *TNFA* and/or more complex haplotype structures are more strongly related to asthma and/or gene expression in this cohort. Another limitation is that our study was conducted in a relatively small sample size and some genotypes were not represented in our cohort. The limited sample size may have precluded us from observing previously identified associations and may have limited our ability to discern significant differences

between the variant groups relative to disease and/or gene expression. Our study cohort also only included subjects with relatively mild to moderate disease as we excluded those who were steroid-dependent and who were on immunomodulators (e.g. Omalizumab). The -857 T allele was significantly underrepresented ($n = 1$) in subjects with more severe asthma, however, the protective effect of this polymorphism in those with mild disease and the potential dose response may be more readily observed in a cohort that includes those with truly severe disease (e.g. those on Step 4 therapy). Furthermore, asthma severity was inferred based on the level of asthma therapy at the time of enrollment and additional assessments of severity and asthma control were not obtained (e.g. asthma symptom scores, methacholine challenge). Lastly, our study was conducted as a part of another investigation designed to assess the feasibility of epithelial cells of the buccal mucosa as a suitable tissue to evaluate the mRNA expression of genes that are associated with asthma (24). Although we have found that *TNFA* was expressed in the buccal mucosa in adequate quantities perhaps this tissue may not be the most suitable for evaluation of changes in *TNFA* expression in relation to activity in the lungs of patients with asthma. However, previous studies have observed similar gene expression patterns between buccal mucosal tissue and respiratory tissue (25). Other tissues, such as blood and respiratory tissue, should also be investigated to fully explore the possible associations between genetic variation and gene expression.

The *TNFA*-857T SNP and -1031T/-863C/-857T haplotype along with inheritance of a G allele at *TNFA*-308, and T allele at *TNFA*-1031 may be important in the pathogenesis of asthma and may be associated with a specific asthma phenotype. We have identified that specific inheritance patterns may be especially important in the pathogenesis of asthma in the African American population and may even be more important in children, especially those with increased asthma severity. As African American children suffer disproportionately from asthma, as it relates to morbidity and mortality, this type of data may allow better targeted treatments in these groups (1). This study suggests that genetic data may be useful in defining patient populations that may benefit most significantly from targeting the TNF pathway. Future studies should focus on the relationship between genetic variation of *TNFA* in larger but more targeted cohorts which have not been previously explored (e.g. African American children with severe disease) and should also take into account other potential modifiers such as environmental factors (e.g. cigarette smoke and ozone levels).

CONCLUSIONS/KEY FINDINGS

The *TNFA*-857T SNP and -1031T/-863C/-857T haplotype along with inheritance of a G allele at *TNFA*-308, and T allele at *TNFA*-1031 is associated with a diagnosis of asthma particularly among children and African American subjects. This finding identifies a potential

genotype/phenotype relationship that may be important in predicting the therapeutic response to treatments which target TNF- α .

DECLARATION OF INTEREST

All authors declare no known conflicts of interest.

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