A Functional Haplotype in the 3′Untranslated Region of *TNFRSF1B* Is Associated with Tuberculosis in Two African Populations

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***Rationale*: Susceptibility to tuberculosis is not only determined by *Mycobacterium tuberculosis* infection, but also by the genetic component of the host. The pleiotropic cytokine tumor necrosis factor-α is essential to control tuberculosis infection, and various tumor necrosis factor family members and their respective receptors may contribute to tuberculosis risk.**

***Objectives*: To investigate four functionally relevant polymorphisms in the tumor necrosis factor receptor 2–encoding gene, tumor necrosis factor receptor superfamily member 1B, for association with tuberculosis susceptibility.**

***Methods*: Genotyping of four polymorphisms was performed in independent populations from South Africa (429 cases and 482 control subjects) and Ghana (640 cases and 1,158 control subjects), and the association of the variants with tuberculosis was tested using two case-control association studies.**

***Measurements and Main Results*: Single-point and haplotype analysis in South Africans revealed an association in the 3′untranslated region of the investigated gene. The T allele of rs3397 alone and/or the 3′ untranslated region haplotype GTT may confer protection against tuberculosis insofar as both allele and haplotype frequencies were significantly lower in case subjects than in controls. The GTT genotype had previously been shown to increase the decay of tumor necrosis factor receptor 2 messenger ribonucleic acid, and messenger ribonucleic acid destabilization may represent a key molecular mechanism for disease susceptibility. Interestingly, the association signal appeared to be restricted to women. The genetic finding was validated in female participants from Ghana. The combined *P* value in the haplotype analysis was $P = 0.00011$.**

***Conclusions*: Our finding emphasizes the importance of tumor necrosis factor/tumor necrosis factor receptor–mediated immune responses in the pathogenesis of tuberculosis.**

Keywords: *Mycobacterium tuberculosis*; genetic susceptibility; association study

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Tuberculosis, an infectious disease caused by *Mycobacterium tuberculosis*, is estimated to result in more than 2 million deaths annually, mostly in developing countries. There is growing evidence that genetic variation in the human host also plays an important role in resistance or susceptibility to the disease.

What This Study Adds to the Field

This study provides evidence that variation in the 3′untranslated region of the tumor necrosis factor receptor superfamily member 1B gene, which has been shown to be implicated in messenger ribonucleic acid destabilization, contributes to tuberculosis susceptibility.

The world is significantly burdened by the infectious disease, tuberculosis (TB), with developing countries being most affected (1). Latent infection with *Mycobacterium tuberculosis* is detectable in one-third of the world's population, but only 10% of infected individuals will ever develop active TB. TB susceptibility is not only determined by infection status and environmental factors but also by the host genetic component, as documented by numerous clinical, epidemiological, twin (2–4), and adoption (5) studies. Previous association analyses have identified several susceptibility genes (6).

Evidence exists that the pleiotropic cytokine tumor necrosis factor (TNF)-α is essential to control TB infection. Patients with inflammatory bowel diseases exhibit increased serum levels of TNF-α and are successfully treated with TNF-α-neutralizing agents such as infliximab and etanercept (7, 8). In patients with latent TB, infliximab treatment led to disease reactivation (9), indicating that TNF-α–mediated immune responses are necessary to control infection. Moreover, the functional single nucleotide polymorphism (SNP) in the promoter of *TNF* (-308 G > A, rs1800629) has been associated with TB susceptibility (10, 11). A recent meta-analysis of ten studies that investigated this SNP indicated that it is not involved in TB susceptibility (12), a result that is similar to our previous study (unpublished data) where we examined the well-known promoter SNPs -308 G > A and -1211 C > T (rs1799964) of *TNF* in a South African population. Given the pivotal role of TNF family members and their receptors in the regulation of immune responses, it is anticipated that other members of the TNF/TNF receptor superfamilies may also contribute to TB risk.

TNF-α exerts its pleiotropic function by activating intracellular signaling cascades via binding to two types of receptors

(*Received in original form May 6, 2009; accepted in final form December 9, 2009*)

Supported by the DFG Excellence Cluster "Inflammation at Interfaces" and the German Federal Ministry of Science and Education (BMBF) through the National Genome Research Network (NGFN) and the BMBF Tuberculosis Research Network. SAC sample collection was funded by a grant from the Wellcome Trust to Paul van Helden and Eileen Hoal.

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

Am J Respir Crit Care Med Vol 181. pp 388–393, 2010
Originally Published in Press as DOI: 10.1164/rccm.200905-0678OC on December 10, 2009
Internet address: www.atsjournals.org

with distinct expression profiles and signaling capacities. Although the high-affinity receptor TNF receptor 1 (TNFR1) is expressed on most human cell types, TNF receptor 2 (TNFR2) expression is restricted to cells of the myelomonocytic lineage and to endothelial cells (13). TNFR2, encoded by the tumor necrosis factor receptor superfamily member 1B gene (*TNFRSF1B*), can influence the biological activity of TNF-α both in a membrane-bound and a soluble form (14). Whereas membrane-bound TNFR2 facilitates activation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling cascades upon binding of TNF-α, soluble TNFR2 (sTNFR2) is capable of binding and inactivating circulating TNF-α. Thus, TNFR2 can act both as an agonist and antagonist of its ligand (15). Certain mycobacterial strains induce the release of sTNFR2 by infected alveolar macrophages. This leads to the inactivation of circulating TNF-α and decreases TNF-α-mediated apoptosis of the infected macrophages, which is necessary to prevent mycobacterial growth (16). A knockout mice study showed that TNFR2 plays a role during *Mycobacterium bovis* bacillus Calmette-Guérin infection (17). A genetic study of Japanese individuals found an upstream *TNFRSF1B* polymorphism (rs496888) to be associated with TB (18), but no association was detected using *TNFRSF1B* microsatellites in Ugandans (19).

Several genetic variants in *TNFRSF1B* are functionally important. We previously showed that a nonsynonymous coding SNP, rs1061622 (Met196Arg), significantly alters TNFR2-mediated NF-kB activation and target gene expression (20). Moreover, haplotypes in the 3′untranslated region (UTR) have been reported to affect TNFR2 expression by influencing messenger ribonucleic acid (mRNA) decay rate (21). Given the pivotal role of TNFR2 in antimycobacterial immune responses, we investigated functional *TNFRSF1B* polymorphisms for association with TB susceptibility.

METHODS

Study Populations

Study participants were collected from three populations, namely, South African "Coloureds" (South Africans of mixed racial origin) (SAC, 429 TB case subjects and 482 control subjects), Ghanaians (640 female TB case subjects and 1,158 female control subjects), and Germans (736 unrelated healthy individuals). *See* the online supplement for a detailed description of the study populations. Known HIV-positive individuals were excluded from the study.

Genotyping and Statistical Analysis

The four functionally relevant SNPs (the exonic SNP rs1061622 and the three neighboring 3′UTR markers rs1061624, rs5030792, and rs3397) in the *TNFRSF1B* gene were typed using TaqMan SNP Assays (*see* Table E1, in the online supplement) or the SNPlex Genotyping System (Applied Biosystems, Foster City, CA) as previously described (22, 23). Samples from positive and negative control subjects were included on each genotyping plate and checked for consistency. Cluster plots were visually inspected to ensure accurate genotyping calls. The call rate for each marked exceeded 92%. In addition, all analyzed SNPs were tested for Hardy-Weinberg equilibrium in control subjects before inclusion in the analyses ($P > 0.01$). Further genotyping validation was done by sequencing of the exonic rs1061622 SNP in a subset of samples (n = 15) from the SAC population using previously published primers (24). The sequencing results were concordant with the calls obtained from the TaqMan SNP Assay.

Single marker case-control analyses on allele frequency data were performed with chi-square statistics using two-tailed tests in the open-source software PLINK, version 1.03 (http://pngu.mgh.harvard.edu/purcell/plink) (25). An allelic *P* value of less than 0.05 was considered nominally significant and Bonferroni correction for multiple testing of four SNPs was applied to the single-point results of the replication sample. Haplotype frequencies were inferred using the expectation maximization algorithm as implemented in the program COCAPHASE, version 2.403, which is part of the UNPHASED suite (http://portal.litbio.org) (26). COCAPHASE was also used to evaluate the global statistical significance of haplotype frequency differences between cases and controls after 10,000 permutations. Power and sample size calculations were performed using the PS Power and Sample Size Program (http://medipe.psu.ac.th/episoft/pssamplesize) (27).

RESULTS

Analysis of *TNFRSF1B* SNPs in SAC

The functionally relevant *TNFRSF1B* polymorphisms rs1061622, rs1061624, rs5030792, and rs3397 (20, 21, 28) were genotyped in the SAC population. All markers were in Hardy-Weinberg equilibrium in the control group. Of the four tested SNPs, only rs3397, which is located in the 3′UTR of *TNFRSF1B*, showed a nominal allelic association with TB in the single-marker analysis ($P = 0.049$; Table 1), with the minor T allele being over-represented in control subjects compared with patients. Because allelic variants may potentially influence TB susceptibility in men and women differently (29, 30), we also performed a sex-stratified analysis that was not decided *a priori*. The association was found to involve only the females in the sample set ($P = 0.048$; Table 2), because no significant association was detected in men (data not shown), and the association signal in females reflected a similar *P* value and estimated odds ratio (OR; Table 2) as that in the entire sample set.

Haplotype analysis was performed for the three neighboring 3′UTR SNPs, rs1061624-A/G, rs5030792-G/T, and rs3397-C/T. Of the eight possible haplotypes, four were common with

TABLE 1. ASSOCIATION STATISTICS FOR FOUR *TNFRSF1B* SNPS IN MALE AND FEMALE SAMPLES FROM THE SOUTH AFRICAN COLOURED POPULATION

		Min AF				
dbSNP ID	Alleles	Case Subjects (*n = 429*)	Control Subjects (*n = 482*)	*P* Value*	OR†	95% CI‡
rs1061622	(G/T)	0.386 (G)	0.348	0.101	1.175	0.969–1.426
rs1061624	(A/G)	0.261 (G)	0.301	0.070	1.219§	0.984–1.512
rs5030792	(G/T)	0.008 (G)	0.016	0.101	2.174§	0.840–5.631
rs3397	(C/T)	0.306 (T)	0.350	**0.049**	1.220§	1.001–1.489

Definition of abbreviations: CI = confidence interval; dbSNP ID = single nucleotide polymorphism identification; min AF = minor allele frequency; OR = odds ratio.

* *P* value was obtained from an allele-based case-control comparison using a two-tailed chi-square test with one degree of freedom. Value in boldface type is statistically significant ($P<0.05$).

† OR for developing TB with the minor allele in controls as reference allele.

‡ 95% CI for OR.

§ Inverted OR.

TABLE 2. SUBGROUP ANALYSIS (FEMALE SUBJECTS ONLY) OF *TNFRSF1B* SINGLE NUCLEOTIDE POLYMORHISMS IN SOUTH AFRICAN COLOURED SUBJECTS

		Min AF				
dbSNP ID	Alleles	Case Subjects (*n = 203*)	Control Subjects (*n = 371*)	*P* Value*	OR†	95% CI‡
rs1061622	(G/T)	0.382 (G)	0.342	0.183	1.188	0.922–1.532
rs1061624	(A/G)	0.249 (G)	0.304	0.058	1.320§	0.990–1.761
rs5030792	(G/T)	0.011 (G)	0.016	0.545	1.425§	0.450–4.507
rs3397	(C/T)	0.293 (T)	0.350	**0.048**	1.304§	1.002–1.698

For definition of abbreviations, *see* Table 1.

* *P* value was obtained from an allele-based case-control comparison using a two-tailed chi-square test with one degree of freedom. Values in boldface type are statistically significant ($P<0.05$).

† OR for developing TB with the minor allele in controls as reference allele.

‡ 95% CI for OR.

§ Inverted OR.

a frequency greater than 1% each (Table 3). For the global testing, we joined haplotypes with frequencies less than 1% each into a single group of "rare" haplotypes that accounted for approximately 1.0% of the variation. Potential phenotypic association for one of these very rare haplotypes would not be detectable given the sample size investigated here. For the entire population, there was no global association between the haplotype frequency distribution and the case-control status, although one of the major individual haplotypes (rs1061624-G, rs5030792-T, rs3397-T [GTT]) was nominally associated ($P = 0.030$; Table 3).

Haplotype analysis for females revealed a global significance of $P = 0.021$ (after 10,000 permutations; Table 4). Subsequent explorative testing of each haplotype against all other combined haplotypes showed that the two haplotypes GTT and ATC contributed to the difference in frequency distribution, with GTT having the larger effect ($P = 0.0049$; Table 4). One of its marker alleles, rs3397-T, was also associated in single-point analysis in female subjects (Table 2). Haplotype analysis for the male subgroup showed no significant association (data not shown).

Replication of TB Association for rs3397 and the GTT Haplotype in Ghanaians

To confirm the significant association results observed in SAC female subjects, rs3397 alone and the 3′UTR-haplotype (defined by the three SNPs rs1061624, rs5030792, and rs3397) were analyzed in an independent population of female Ghanaians (640 TB case subjects and 1,158 control subjects), selected on the basis of *a priori* knowledge gained from the SAC analysis. The minor T allele of rs3397 ($P = 0.007$; corrected $P = 0.028$) and the GTT haplotype ($P = 0.0091$; global significance $P = 0.034$) were both found to be statistically significantly enriched in control subjects relative to patients (Tables 5 and 6), thus supporting the findings in the SAC. Although the frequency of rs3397-T was considerably lower in the Ghanaians than in the SAC (Table 7), the OR of approximately 1.3 was very similar in both groups (Table 2 and 5). When female SACs and Ghanaians were examined together, evidence for association was obtained with $P = 0.00093$ (corrected $P = 0.00372$) for rs3397 and $P = 0.00011$ for the GTT haplotype (global significance $P = 0.0012$).

TABLE 3. RESULTS OF THE *TNFRSF1B* HAPLOTYPE ANALYSIS IN MALE AND FEMALE SAMPLES FROM THE SOUTH AFRICAN COLOURED POPULATION

Common Haplotypes*	Case Subjects, freq	Control Subjects, freq	*P* Value†
ATC	0.553 (392)‡	0.509 (419)‡	0.057
ATT	0.187 (133)	0.181 (149)	0.902
GTC	0.147 (105)	0.152 (125)	0.614
GTT	0.108 (77)	0.146 (120)	**0.030**
Global significance			0.136

Definition of abbreviation: freq = frequency.

* The TNFR2 haplotype is shown in the variant order rs1061624, rs5030792, and rs3397.

† *P* value obtained from haplotype analysis. Value in boldface type is statistically significant ($P<0.05$).

‡ *TNFR2* haplotype frequencies (absolute numbers are in parentheses).

Allele Frequencies of Investigated Haplotype SNPs in Different Populations

The SAC have a background of mixed ancestry that includes several European and sub-Saharan African groups. For a comparative analysis, we used the Ghanaians and a population-representative sample of Germans as approximation for the SAC parental populations. The allele frequencies of the investigated haplotype SNPs differed markedly among the three groups (Table 7). For this marker set, the SAC were between Africans and Germans, which is consistent with their mixed origin.

DISCUSSION

We have identified genetic variation in the *TNFRSF1B* gene as an influencing factor for susceptibility to TB infection. Significant association results were observed for a particular 3-SNP haplotype (GTT based on rs1061624, rs5030792, and rs3397) in the 3′UTR and for one of its markers, rs3397, by single point analysis. Interestingly, the signal appeared to be restricted to female subjects. Genetic replication of both the GTT-haplotype and the SNP rs3397 in an independent sample of female Ghanaians confirmed the initial observation (*see* Figure E1).

TABLE 4. RESULTS OF THE *TNFRSF1B* HAPLOTYPE ANALYSIS IN SOUTH AFRICAN COLOURED FEMALE SUBJECTS

Common Haplotypes	Case Subjects, freq	Control Subjects, freq	*P* Value
ATC	0.563 (181)	0.501 (319)	**0.034**
ATT	0.186 (60)	0.184 (117)	0.821
GTC	0.157 (51)	0.153 (97)	0.890
GTT	0.085 (27)	0.150 (96)	**0.0049**
Global significance			**0.021**

For definition of abbreviations, *see* Table 3.

Values in boldface type are statistically significant ($P<0.05$).

TABLE 5. REPLICATION OF THE TB ASSOCIATION OF *TNFRSF1B* SNP RS3397 IN FEMALE GHANAIANS

		Min AF				
dbSNP ID	Alleles	Case Subjects (*n = 640*)	Control Subjects (*n = 1,158*)	*P* Value*	OR†	95% CI
rs3397	(C/T)	0.128 (T)	0.162	**0.007**	1.315	1.079–1.602

For definition of abbreviations, *see* Table 1.
* Corrected $P = 0.028$. Value in boldface type is statistically significant ($P<0.05$).
† Inverted OR.

The GTT haplotype is the rarest (among the four major ones) in both African populations analyzed (~15% in SAC and ~7% in Ghanaian controls). In Germans, this haplotype is the most common, with an estimated frequency of 45% (data not shown); a similar figure was also reported by Puga and colleagues (21) for their European sample. It would be interesting to determine whether the association of TB with the rs3397 T allele and the GTT haplotype can also be observed in Europeans or whether it is restricted to populations with an African background. Given that GTT appears to be overrepresented in controls, and is the most frequent haplotype in a European population, it is possible that its rarity in African populations may contribute to their perceived increased susceptibility to TB.

Genetic variants in the 3′UTR of *TNFRSF1B* are important for a number of pathologies including obesity and insulin resistance (31) and Crohn's disease (32), a chronic inflammatory disorder of the bowels. Interestingly, a recent study showed that this region affects *TNFR2* expression by influencing mRNA stability (21). According to this report, a U-rich region within the 3′UTR increases the decay rate of the *TNFR2* mRNA following T cell activation, and thus is predicted to protect cells from exaggerated TNFR2 effects. This destabilizing effect is mediated through GUUUG repeats within the 3′UTR that are believed to represent important *cis*-regulatory elements to control mRNA decay. Interestingly, these regulatory elements are located in close proximity to the polymorphic region in the *TNFR2* mRNA and thus might easily be affected by allelic variations. The regulatory capacity of this region is underscored by the finding that 3′UTR-haplotype GTC results in significantly decreased mRNA decay rates compared with the other haplotypes, as shown by luciferase-based–reporter gene studies in Jurkat T cell line (21). Nevertheless, the GTC haplotype itself has never been shown to contribute to disease susceptibility in any study including the one presented here. This, and the inability to identify a single *TNFR2* 3′UTR haplotype as a major genetic risk factor for several diseases, argues for another level of complexity in the regulation of TNFR2 that has not been completely unraveled and is a limitation of this study.

Our data indicate that the T allele of rs3397 alone and/or the 3′UTR haplotype GTT may confer protection against TB insofar as both allele and haplotype frequencies are lower in TB case subjects than in control subjects. With regard to the above-mentioned effect on mRNA decay, these protective genotypes are considered to confer unaltered decay rates of TNFR2 mRNA. Consequently, individuals carrying these genotypes might show lower cell-surface expression of TNFR2 and reduced sTNFR2 levels in the serum. Given the well-characterized role of sTNFR2 as an antagonist of TNF-α activity, it is tempting to speculate that reduced sTNFR2 levels might accentuate protective effects initiated by TNF-α and thus improve host resistance to TB infection. The fact that the protective GTT genotype represents one of four different UTR haplotypes exhibiting the same kind of mRNA destabilization, whereas only a single haplotype (GTC) results in impaired mRNA decay rates, argues for a further, and to date undetermined, mode of regulation.

High expression levels of murine *tnfr2* characterize a subset of mouse regulatory T cells (cluster of differentiation [CD]4^+ $CD25^+$) that play a major role in immune response during active TB (33–35). TNFR2-mediated signaling directly affects expansion and function of these regulatory T cells and thus facilitates the exertion of their maximal suppressive activity on activated T lymphocytes (36). With respect to the crucial role of T cell–mediated immune responses for progression of TB, genotype-dependent decay of *TNFR2* mRNA might affect regulatory T cell activity and hereby contribute to disease susceptibility.

Despite its strengths as discussed above, this research had certain limitations. The screening panel from the SAC population had 80% power to detect a variant with an OR of 1.5 or higher at the 5% significance level, assuming a frequency of the disease-associated allele of at least 20% in control subjects (*see* Figure E2A). It is therefore possible that our study did not have the power to detect effect sizes smaller than this (e.g., for SNP rs5030792 with a frequency of 2% in control subjects). But for the replication of SNP rs3397 (assuming a minor allele frequency of 0.35 in the control subjects and an allelic OR of 1.3 accordingly to the SAC discovery sample) the Ghanaian replication sample was expected to have approximately 80% power (*see* Figure E2B). In addition, we selected polymorphisms based only on their functional relevance and have therefore not included all the common genetic variants of *TNFRSF1B*. This study did not use a tagging SNP approach, which has been successful in other studies done by our group (37). The reason for this is that the tagging approach is only as

TABLE 6. REPLICATION OF THE TB ASSOCIATION OF *TNFRSF1B* HAPLOTYPE IN FEMALE GHANAIANS

Common Haplotypes	Case Subjects, freq	Control Subjects, freq	*P* Value
ATC	0.667 (842)	0.655 (1,492)	0.326
ATT	0.076 (96)	0.090 (205)	0.050
GTC	0.209 (263)	0.184 (418)	0.113
GTT	0.048 (61)	0.071 (163)	**0.0091**
Global significance			**0.034**

For definition of abbreviations, *see* Table 3.
Values in boldface type are statistically significant ($P<0.05$).

TABLE 7. COMPARISON OF *TNFRSF1B* SNP ALLELE FREQUENCIES IN DIFFERENT POPULATIONS

dbSNP ID	AF G	AF SAC	AF GH
rs1061624-G	0.574	0.301	0.256
rs5030792-G	0.038	0.016	0.000
rs3397-T	0.625	0.350	0.154

Definition of abbreviations: AF = allele frequency; dbSNP ID = single nucleotide polymorphism identification; G = Germans; GH = Ghanaians; SAC = South African Coloured.

good as the reference from which the tagging SNPs are selected. Normally, haplotype mapped populations (38, 39) can be used to deduce a minimal set of tagging SNPs to be typed. However, it is not clear which of the currently available haplotype mapped populations is a good proxy for the SAC study population.

Our analyses suggest a sex-specific effect of the protective GTT haplotype favoring female individuals. Genetic variation of TNFR2 has previously been shown to contribute to susceptibility for metabolic disorders that are restricted to females, namely polycystic ovary syndrome and hyperandrogenism (40). One possible explanation for this linkage might be that the TNF signaling cascade is estimated to influence serum levels of androgens (41) and thus modulates hormone-mediated processes that also affect immune responses to bacterial infection. Interestingly, it has recently been shown in *tnfr2*-deficient mice that sex-specificity of Tnfr2-mediated cardioprotection during ischemia/reperfusion is caused by differential expression and activation of signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signaling 3 (SOCS3), and c-Jun N-terminal kinase (JNK) pathways between male and female individuals (42). Because components of these pathways are also involved in the immune regulatory program initiated during TB (43, 44), it is possible that similar mechanisms contribute to the sex-specific effect of TNFR2 observed here. Further studies are needed to analyze the extent to which TNFR2 regulation and function represent a novel target mechanism for successful treatment of tuberculosis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank the patients and volunteers who participated as control subjects, and the field workers, nurses, and physicians involved in the recruitment of participants. In particular, we thank the staff of the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) in Ghana and the excellent assistance of Emmanuel Abbeyquaye, Lincoln Gankpala, Sonja Burmester, Birgit Muntau, Gerd Ruge, and Jürgen Sievertsen.

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