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Genetic and Shared Environmental Influences on Interferon-γ Production in Response to *Mycobacterium tuberculosis* Antigens in a Ugandan Population

Li Tao, Sarah Zalwango, Keith Chervenak, Bonnie Thiel, LaShaunda L. Malone, Feiyou Qiu, Harriet Mayanja-Kizza, W. Henry Boom, and Catherine M. Stein* for the Tuberculosis Research Unit (TBRU)

Department of Medicine, and Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio; Uganda – CWRU Research Collaboration; College of Health Sciences, Makerere University and Mulago Hospital, Kampala, Uganda

Abstract. Interferon-γ (IFN-γ) is a key cytokine in the immune response to *Mycobacterium tuberculosis* (Mtb). Many studies established IFN-γ responses are influenced by host genetics, however differed widely by the study design and heritability estimation method. We estimated heritability of IFN-γ responses to Mtb culture filtrate (CF), ESAT-6, and Antigen 85B (Ag85B) in 1,104 Ugandans from a household contact study. Our method separately evaluates shared environmental and genetic variance, therefore heritability estimates were not upwardly biased, ranging from 11.6% for Ag85B to 22.9% for CF. Subset analyses of individuals with latent Mtb infection or without human immunodeficiency virus infection yielded higher heritability estimates, suggesting 10–30% of variation in IFN-γ is caused by a shared environment. Immunosuppression does not negate the role of genetics on IFN-γ response. These estimates are remarkably close to those reported for components of the innate immune response. These findings have implications for the interpretation of IFN-γ response assays and vaccine studies.

INTRODUCTION

Tuberculosis (TB) is a growing public health problem globally. According to the World Health Organization (WHO), one-third of the world is infected by *Mycobacterium tuberculosis* (Mtb), and almost 9.4 million new cases of active TB occur annually, resulting in 1.7 million TB-attributable deaths (http://www.who.int/tb/country/data/profiles/en/index.html). The immune response to Mtb involves the interplay between antigen presenting cells, T cells, and cytokines. The coordinated "cross talk" between these essential components of the immune response is necessary for control of Mtb and preventing progression to TB.[1]

Several studies have shown an association between risk of TB disease and human genetic factors.[2,3] Fewer studies have focused on host genetic influences on cytokines essential for the immune response to Mtb.[4–6] Because of the relevance to interferon-γ response assays (IGRAs) and immune responses to T-cell subsets in vaccine development, it is important to understand the genetic influences on interferon-γ (IFN-γ) production in response to Mtb antigens. Our previous study found an estimated heritability of IFN-γ in response to Mtb culture filtrate (CF) of 17%.[7] A twin study of IFN-γ responses to the proteins PPD-RT48, 70 kDa, and 85 kDa estimated heritability to be between 0% and 40%.[8] Another twin study of responses to PPD-RT49, short-term Mtb culture filtrate, killed *Mtb*, 65 kDa, and 85 kDa estimated heritability to be between 3% and 41%.[9] Cobat and others[10] estimated heritability of IFN-γ response to be 43–58% in response to Bacillus Calmette Guerin (BCG), purified protein derivative (PPD), and ESAT-6 in sibling pairs.

However, these studies are not directly comparable because of the differing strategies used to estimate heritability. In twin studies, dominance genetic and shared environmental effects are confounded and cannot be separately estimated.[11] Study designs using only sibling pairs estimate broad-sense heritability, which is the sum of additive and dominance genetic variance, and may be confounded by shared environmental effects.[12] Methods that include all pair types, including spousal pairs, can parse out the effect of shared environment, and estimate narrow-sense heritability, which is caused by additive genetic effects only.[7] The impact of shared environment is not negligible in studies of genetic susceptibility to TB[13] or the immune response to Mtb.[14] In addition, previous studies were hampered by small sample sizes, which could result in imprecise estimates of these variance components. They also excluded human immunodeficiency virus (HIV)-infected individuals, and therefore could not assess the joint impact of HIV and host genetics. Therefore, it is unclear if previously mentioned differences in heritability estimates are dependent on antigens assayed or differences in study design. A large study of multiple relative pair types may allow for more accurate estimates of IFN-γ heritability.

It is well established that antigen-induced IFN-γ has a protective role in immune response to Mtb.[15] The IFN-γ is a pro-inflammatory cytokine produced by T cells necessary for the containment of Mtb by macrophages.[16] The IGRAs measuring T-cell responses to specific Mtb antigens have been developed to diagnose latent Mtb infection (LTBI).[17] The Mtb-specific antigens elicit clinically relevant IFN-γ responses.[18] The ESAT-6 is a component of IGRAs,[19,20] and Antigen 85B (Ag85B) is a vaccine candidate.[21,22] The IFN-γ responses are also used to assess candidate TB vaccines.

The purpose of this study was to estimate heritability of IFN-γ production in response to CF, ESAT-6, and Ag85B. Cytokine responses were assayed as part of a large ongoing epidemiological study of Mtb transmission, providing a large sample size for analysis. Furthermore, because this study used a household contact study design, we have data on various relative pair types, allowing us to use a robust heritability estimation method.[7]

MATERIALS AND METHODS

Study design. Study design and enrollment procedures have been described in detail elsewhere.[5,7,23] Study participants were enrolled between 2002 and 2006 as part of a household

* Address correspondence to Catherine M. Stein, Department of Epidemiology and Biostatistics, Case Western Reserve University, 2103 Cornell Road, Wolstein Research Bldg. 1316, Cleveland, OH 44106. E-mail: catherine.stein@case.edu

contact study of *Mtb* infection and TB disease conducted in Kampala, Uganda. Households were ascertained through an individual with culture-positive TB (index case). All individuals were HIV tested except for very young children when neither parent was HIV infected. All individuals received tuberculin skin tests (TST) using purified protein derivative (PPD). The TST positivity was defined as an induration of 5 mm or greater for children < 5 years of age or HIV-positive individuals; a cutoff of 10 mm was used otherwise. Based on these tests, individuals were classified into three clinical groups: active TB cases, latent TB infected individuals (LTBI) with positive TST, and TST negative. The institutional review boards at University Hospitals of Cleveland and the Uganda Council for Science and Technology approved the study. All individuals within the households provided written informed consent (or in the case of children, assent was given by the child and the parent/guardian provided written informed consent).

Immunological assay. Blood samples were obtained from each study participant. Whole blood was stimulated with Mtb antigens, and supernatants collected for storage at −70°C after 7 days of stimulation as described elsewhere.[18] Whole blood stimulations were done as one, 1 mL culture. Antigens included CF, ESAT-6, and Ag85B. The IFN-γ response in supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (Thermo Scientific, Rockford, IL). Whole blood cultured without antigen stimulation served as a negative control. Though CF assays were conducted for all individuals, assays for specific antigens were not conducted for all individuals, resulting in missing values in the analysis ($N = 215$ for ESAT-6 and $N = 224$ for Ag85B).

Data analysis. To measure antigen-specific IFN-γ responses relative to no antigen, IFN-γ response to medium alone was subtracted from antigen-stimulated readings for each individual. Negative differences were truncated to 0. These differences were then log-transformed, because of the extreme skewness of values. Log-transformed values were used in the subsequent segregation analyses.

Heritability estimation. Heritability is defined as the proportion of trait variation caused by genetic factors, and can be estimated using relative pair correlations. In our previous work,[7] we developed a method for heritability estimation that accounts for non-zero spousal correlations and unequal parent–offspring and sibling pair correlations. This flexibility accounts for effects of shared environment, so that heritability estimates are not upwardly biased. Because TB and its immune response are influenced by shared environment, it is important to properly account for it. We estimated relative pair correlations using the SEGREG programs in S.A.G.E. (v.6.1) (Case Western Reserve University, Cleveland, OH). Using a one mean model under Bonney's class D regressive model,[24] this program simultaneously transforms the data, adjusts for covariates, and estimates parent–offspring, sibling, and marital correlations. We used a George-Elston transformation[25] to reduce skewness and kurtosis, so not to artificially inflate estimates of relative pair correlations. Covariates included HIV status, age, and clinical group (BCG vaccination status and sex were not significant). The number of relative pairs used in this analysis is provided in Table 1.

Because of the strong effect of HIV seropositivity on IFN-γ responses, we repeated the heritability estimation on the subset of HIV negative individuals; this analysis used 304 parent–offspring pairs, 254 sibling pairs, and 34 spousal pairs. To assess the impact of including the entire range of belonging to different clinical groups (TST negative, LTBI, and active TB) in the overall analysis, we also repeated the estimation of relative pair correlations in LTBI individuals only as a sub-analysis. Many relative pairs were lost in this sub-analysis (only 134 parent–offspring pairs and 114 sibling pairs available), because if one individual in a pair was not in the LTBI clinical group, that pair could not be included in the segregation analysis. Thus, based on the low number of spousal pairs ($N = 2$–4), we could not repeat heritability estimation using our method, but do present parent–offspring and sibling correlations. A similar analysis could not be done in the subset of individuals who had active TB or were TST negative because there were too few relative pairs that were concordant for these clinical outcomes.

TABLE 1
Descriptive statistics of analysis sample*

	N (%)
Clinical/demographic characteristics	
Total number of individuals	1,104
Clinical group	
TST negative at baseline	280 (25.4%)
TST positive at baseline	522 (47.3%)
TB cases	296 (26.8%)
Males/females	508 (46.0%)/596 (54.0%)
HIV-positive	200 (18.1%)
Presence of BCG scar	693 (62.8%)
Age distribution	
0–2	76 (6.9%)
3–5	111 (10.1%)
6–14	263 (23.8%)
15+	654 (59.2%)
Pedigree characteristics	
Number of pedigrees	277
Number of parent–offspring pairs	511
Number of sibling pairs	263
Number of marital pairs	64

*TST = tuberculin skin test; TB = tuberculosis; HIV = human immunodeficiency virus; BCG = Bacillus Calmette Guerin.

RESULTS

This analysis included 1,104 individuals from 277 households. Of these individuals, 26.8% had active TB disease and another 47.3% had LTBI based on the TST (Table 1). Furthermore, 18.1% were HIV seropositive, and 62.8% had scars characteristic of BCG vaccination. Of the individuals that were TST negative at baseline, 255 were HIV negative (91.1%), 21 were HIV positive (7.5%), and 4 did not have an HIV result. Of the TST positive individuals, 459 (87.9%) were HIV negative, 52 (10.0%) were HIV positive, and 11 had unknown HIV status. Finally, of the TB cases, 169 (57.1%) were HIV negative, 126 (42.6%) were HIV positive, and 1 had unknown HIV status. As expected, differences were observed in IFN-γ responses to each of the antigens across the clinical groups (Figure 1A–C and Supplemental Material).

Relative pair correlations for IFN-γ responses were estimated using SEGREG (S.A.G.E. version 6.1) (Table 2). Spousal correlations were greater than parent–offspring correlations, showing an influence of shared environment. In addition, sibling correlations were greater than parent–offspring, showing a considerable influence of dominance genetic effects.

The heritability values estimated from these relative pair correlations are also shown in Table 2. Heritability estimates for IFN-γ response variables ranged from 11% (Ag85B) to 22%

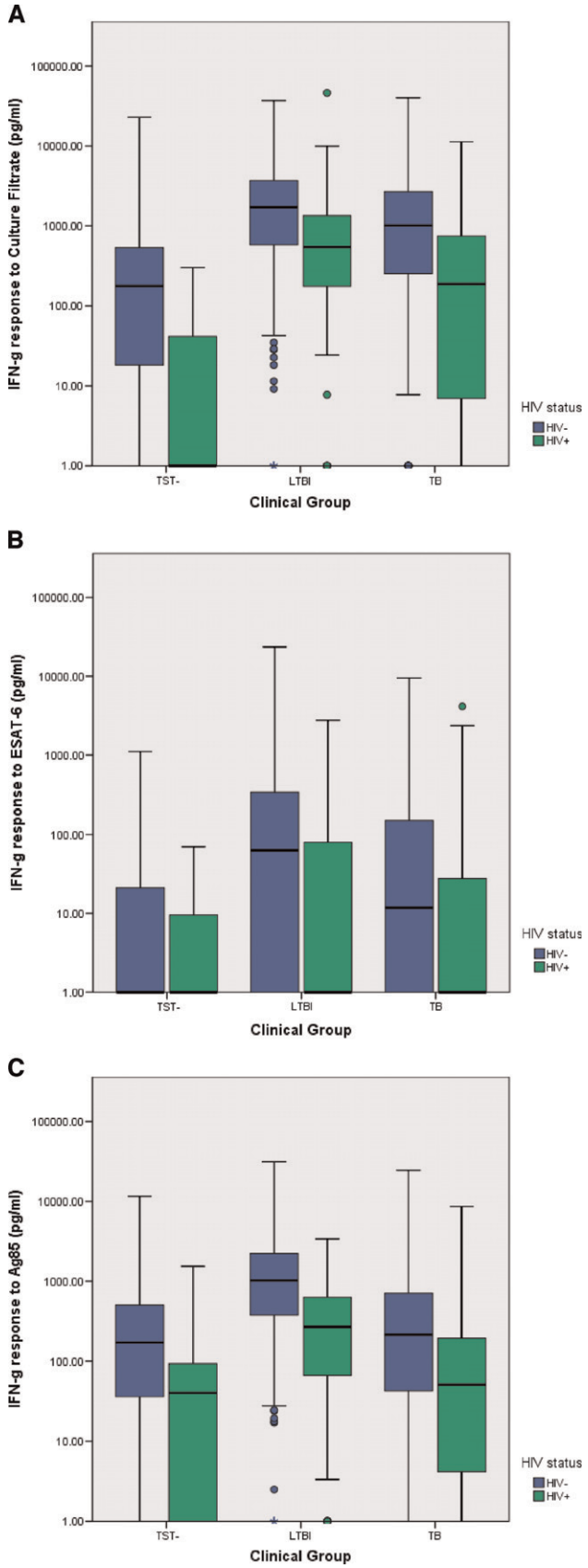

FIGURE 1. (**A**) Interferon-γ (IFNγ) response to culture filtrate (CF) by clinical group; (**B**) IFNγ responses to ESAT6 by clinical group; (**C**) IFNγ responses to Ag85B by clinical group.

TABLE 2

Relative pair correlations and heritability estimates obtained from all individuals*

	Relative pair correlations			
IFN-γ response	Spousal	Parent–offspring	Sibling	Heritability (95% CI)
CF	0.1813	0.1073	0.3517	22.86 (5.0–40.7)
ESAT-6	0.0703	0.0854	0.0842	15.53 (0.0–34.6)
Ag85B	0.1479	0.0535	0.2588	11.62 (0.0–30.2)

*CI = confidence interval; IFN-γ = Interferon-γ; CF = culture filtrate.

(CF). Given its standard error, the heritability estimate of CF was significantly different than zero at the $\alpha = 0.05$ level.

Next, we estimated heritability in the HIV-negative subset of individuals (Table 3). These values are not directly comparable to those in Table 2, because the sample size is smaller. Nevertheless, all of the heritability estimates are higher than in Table 2, and were significantly different than 0 except for Ag85B. Even in the setting of immune suppression, IFN-γ responses retain a heritable component.

Finally, we conducted segregation analysis, and estimation of relative pair correlations, in the subset of LTBI individuals. Parent–offspring and sibling correlations are given in Table 4. All of these correlations are higher than those for the entire dataset except for two. One can estimate narrow-sense heritability using two times the parent–offspring correlation,[12] though this method does not fully account for shared environment. With these data, we obtained the following estimates for heritability within the LTBI subset: 34% for CF, 48% for ESAT-6, and 34% for Ag85B (data not shown).

DISCUSSION

Our primary objective was to estimate the heritability of IFN-γ in response to both CF- and Mtb-specific antigens relevant for IGRAs and vaccine responses, and we found some of these heritability estimates to be significantly different from zero. The heritability of responses to CF was found to be most significant, whereas responses to ESAT-6 and Ag85B were less heritable. This is not surprising given that CF includes multiple antigens, with ESAT-6 and Ag85B among them. Furthermore, these heritability estimates are depressed when HIV-positive individuals are included in the analysis. Genetic studies of TB susceptibility have overlooked the importance of shared environment in terms of epidemiological risk and exposure to infectious cases[14] and Mtb strain,[13] therefore it is essential to account for shared environment in heritability estimation.

One noteworthy comparison is that of heritability of innate versus adaptive immune responses. Here, we analyzed IFN-γ, part of the adaptive immune response. Previously, we

TABLE 3

Relative pair correlations and heritability estimates obtained from HIV negative individuals*

	Relative pair correlations			
IFN-γ response	Spousal	Parent–offspring	Sibling	Heritability (95% CI)
CF	0.1654	0.1739	0.3527	35.06 (15.2–54.9)
ESAT-6	–0.0177	0.1364	0.0988	26.92 (5.9–48.0)
Ag85B	0.1140	0.0705	0.2497	15.04 (–6.6–36.7)

*CI = confidence interval; IFN-γ = Interferon-γ; CF = culture filtrate.

TABLE 4
Relative pair correlations obtained from only LTBI (TST+) individuals*

IFN-γ response	Parent–offspring	Sibling
CF	0.1692	0.5072
ESAT-6	0.2401	0.0702
Ag85B	0.1714	0.1994

*LTBI = latent Mtb infection; IFN-γ = Interferon-γ; CF = culture filtrate.

estimated the heritability of early tumor necrosis factor-α responses (TNF-α), a key component of the innate immune response to TB, and found an estimate of 34% in response to CF in this same study population.[14] The analysis included both HIV-positive and HIV-negative individuals, as well as individuals from all stages of TB pathogenesis, therefore was clinically comparable to the individuals examined in Table 2. Though the heritability of TNF-α is certainly greater than IFN-γ, it is not as great a difference as one might expect when contrasting innate and adaptive immune responses.

Another important contribution of this analysis is the assessment of the role of HIV infection. As expected, HIV infection showed a significant influence on IFN-γ responses, and that the heritability obtained by including both HIV-positive and HIV-negative individuals are less than the heritability from the HIV-negative subset. This shows that HIV negates some of the genetic influence on IFN-γ response; yet importantly, host genetics are still influential in HIV-infected individuals. This also suggests a potential host gene by HIV interaction, which has been seen in a previous candidate gene study.[5]

Our previous study estimated the heritability of IFN-γ response to CF at 17%[7] compared with 22.8% in this analysis. There are a number of possible reasons for this small difference in estimates. First, the source of CF differed,[14] as did the ELISA kit used. Second, we had more complete data in this phase of the study, therefore this heritability estimate may be more precise. An analysis of twin pairs in Gambia estimated the heritability of IFN-γ in response to Ag85B to be 40%.[8] The study did not find a genetic component for IFN-γ response to CF, and found that environmental components accounted for a larger part of variance in IFN-γ secretion than genetic components for all measures. Another study in Gambia involving infant twin pairs produced a similarly low heritability of 12% for CF (confidence interval [CI] 0–46%) and 3% for Ag85B (CI 0–35%), further illustrating the importance of environment in explaining variance in IFN-γ response.[9] In contrast, a study of South African subjects calculating heritability using sibling pairs estimated IFN-γ response heritability to be 43% for BCG bacilli as antigen and 58% for ESAT-6.[10]

Several methodological issues explain these differences in heritability values. Our study used all types of relative pairs (parent–offspring, sibling, and spousal), whereas others used only twin pairs[8,9] or sibling pairs.[10] Twin studies are limited in that the components of variance caused by shared environment versus dominance genetic effects cannot be estimated separately, and heritability estimates based on sibling pairs alone cannot partition out the effect of shared environment or dominance genetic variance.[7,11] Our method estimates narrow-sense heritability (additive component of genetic variance only) and partitions out the effect of shared environment, the latter of which is of particular relevance for an infectious disease like TB. Second, our study was quite large (1,104 individuals), thus resulting in very robust estimates of heritability. Other studies excluded or did not separate HIV-positive individuals and BCG-vaccinated individuals. Our study encompasses the entire range of TB pathogenesis (uninfected, infected, and diseased), and we were able to adjust for such clinical factors by including them as covariates in analysis. Finally, the Mtb strains endemic to Uganda, the Gambia, and South Africa differ,[26] which may impact the proportion of IFN-γ response that is caused by shared environment.

We also conducted separate analyses for the entire sample and then restricted our analysis to the LTBI subset to examine how stable our heritability estimates are when including various clinical groups. The heritability estimates for LTBI are considerably higher, and suggest that there is heterogeneity in the TST negative group and/or the active TB group (most likely the latter) that results in weaker correlations when these clinical groups are included in the analysis. However, it is important to point out that the heritability estimates in the LTBI subset were obtained without data from spousal pairs, which means they likely include variance caused by shared environment and genetics. This suggests that the influence of shared environment on IFN-γ responses may be anywhere between 10% and 30%. Thus, the estimates obtained from the full data may be considered lower limits of the heritability, but may also be more realistic, because this is what the study population looks like clinically.

These results may have implications for the global use of IGRAs and vaccine studies. As we and others have found, variation in IFN-γ response to Mtb *in vitro* has a moderate genetic influence. Because the distribution of genetic polymorphisms also differs globally, these findings further suggest that IGRA responses may differ across world populations as a result of host genetic factors and TB prevalence. Discrepancies between studies of IGRA sensitivity/specificity[20] could be explained by such genetic variation between study sites. Our study cannot answer that question, however it does warrant further investigation. Finally, vaccine studies use IFN-γ response as a measure of response to the vaccine. As more TB vaccines are developed, it will be important to account for global variability in IFN-γ responses.

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Authors' addresses: Li Tao, Department of Medicine, Case Western Reserve University, Cleveland, OH, E-mail: li.tao@case.edu. Sarah Zalwango, Uganda – CWRU Research Collaboration, Kampala, Uganda, E-mail: szalwango@mucwru.or.ug. Keith Chervenak, Bonnie Thiel, LaShaunda L. Malone, and W. Henry Boom, Department of Medicine and Uganda – CWRU Research Collaboration, Case Western Reserve University, Cleveland, OH, E-mails: kac2@case.edu, bat2@case.edu, llm19@case.edu, and whb@case.edu. Feiyou Qiu, Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, OH, E-mail: fxq10@case.edu. Harriet Mayanja-Kizza, Uganda – CWRU Research Collaboration and College of Health Sciences, Makerere University and Mulago Hospital, Kampala, Uganda, E-mail: hmk@mucwru.or.ug. Catherine M. Stein, Department of Epidemiology and Biostatistics, and Uganda – CWRU Research Collaboration, Case Western Reserve University, Cleveland, OH, E-mail: catherine.stein@case.edu.

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