

## LARGE-SCALE CANDIDATE GENE STUDY OF TUBERCULOSIS SUSCEPTIBILITY IN THE KARONGA DISTRICT OF NORTHERN MALAWI

JODENE FITNESS, SIAN FLOYD, DAVID K. WARNDORFF, LIFTED SICHALI, SIMON MALEMA,  
AMELIA C. CRAMPIN, PAUL E. M. FINE, AND ADRIAN V. S. HILL

Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom; Department of Tropical Hygiene,  
London School of Hygiene and Tropical Medicine, London, United Kingdom; Karonga Prevention Study,  
Chilumba, Karonga District, Malawi

**Abstract.** Twenty-seven polymorphisms from 12 genes have been investigated for association with tuberculosis (TB) in up to 514 cases and 913 controls from Karonga district, northern Malawi. Homozygosity for the complement receptor 1 (CR1) Q1022H polymorphism was associated with susceptibility to TB in this population (odds ratio [OR] = 3.12, 95% Confidence interval [CI] = 1.13–8.60,  $P = 0.028$ ). This association was not observed among human immunodeficiency virus (HIV)-positive TB cases, suggesting either chance association or that HIV status may influence genetic associations with TB susceptibility. Heterozygosity for a newly studied CAAA insertion/deletion polymorphism in the 3'-untranslated region of solute carrier family 11, member 1 (SLC11A1, formerly NRAMPI) was associated with protection against TB in both HIV-positive (OR = 0.70, 95% CI = 0.49–0.99,  $P = 0.046$ ) and HIV-negative (OR = 0.65, 95% CI = 0.46–0.92,  $P = 0.014$ ) TB cases, suggesting that the SLC11A1 protein may have a role in innate TB immune responses that influence susceptibility even in immunocompromised individuals. However, associations of other variants of SCLA11A with TB reported from other populations were not replicated in Malawi. Furthermore, associations with vitamin D receptor, interferon- $\gamma$ , and mannose-binding lectin observed elsewhere were not observed in this Karonga study. Genetic susceptibility to TB in Africans appears polygenic. The relevant genes and variants may vary significantly between populations, and may be affected by HIV infection status.

### INTRODUCTION

Approximately eight million new cases and almost 1.8 million deaths attributable to tuberculosis (TB) occur each year.<sup>1</sup> In many populations, this situation is exacerbated by an increasing prevalence of infection with human immunodeficiency virus (HIV). Tuberculosis is caused by *Mycobacterium tuberculosis*, an intracellular pathogen that survives within macrophages. It is primarily a pulmonary disease, but it may affect organs other than the lungs, and it has been estimated that only 10% of infected individuals develop clinical disease.<sup>2</sup> Evidence from family, twin, and case-control studies indicates that host genetic factors play a role in determining the outcome of exposure and infection.<sup>3</sup>

The first convincing evidence related to HLA genes: the HLA-DR2 molecule is associated with TB susceptibility in some populations, while DQ1 alleles are associated in others.<sup>4</sup> Non-HLA genes have also been implicated in TB susceptibility. Variants of solute carrier family 11, member 1 (SLC11A1, formerly NRAMPI) have been associated with TB susceptibility in populations from The Gambia, Guinea-Conakry, Korea, Brazil and Japan.<sup>5–9</sup> Also, evidence for linkage of a TB susceptibility locus to a 2q35 region that contains SLC11A1 has been reported in a Canadian Aboriginal pedigree.<sup>10</sup> A variant in the vitamin D receptor (VDR) has been associated with TB susceptibility in The Gambia.<sup>11</sup> There is limited and conflicting evidence suggesting that variants in mannose-binding lectin (MBL2) may be associated with either protection or susceptibility to TB.<sup>12,13</sup> Recently, an interferon- $\gamma$  (IFNG) polymorphism (+874 T/A) that may cause increased IFNG transcription in response to NF- $\kappa$ B has been found to be associated with resistance to TB in Sicilians and South Africans.<sup>14–16</sup>

The Karonga Prevention Study (KPS), a long-term epidemiologic study of mycobacterial disease in the Karonga District of northern Malawi, provides an ideal opportunity to evaluate genetic associations with TB that have been reported from other populations, as well as to generate and investigate

new hypotheses. In recognition of this, both case control and multicase family studies have been set up in this population to investigate genetic risk factors for both TB and leprosy. This paper reports on the TB case-control study. Given that HIV is a major risk factor for TB in this population, as elsewhere in southern Africa, and the possibility that the influence of some genetic factors may depend upon the level of immune competence of the host, both HIV-negative and HIV-positive TB patients were studied.

Twenty-seven non-HLA polymorphisms from 12 genes were investigated for association with TB susceptibility in this population. Variants in SCL11A1, VDR, MBL2, and IFNG were included because these genes have been associated with TB susceptibility in other populations. Interleukin-10 (IL10) is a key anti-inflammatory cytokine that has recently been implicated in leprosy susceptibility in Brazil.<sup>17</sup> Polymorphisms in tumor necrosis factor (TNF, formerly TNF $\alpha$ ), and lymphotoxin alpha (LTA, formerly TNF $\beta$ ) have been associated with leprosy susceptibility. The toll-like receptors 2 and 4 (TLR2 and TLR4) are pattern recognition molecules involved in both innate and adaptive immune responses. Variants of toll receptors have recently been associated with leprosy, malaria and pneumococcal disease (Cooke G, Aucan C, Walley AJ, Segal S and others, unpublished data).<sup>18</sup> The chemokine (C-C motif) ligand 3 (CCL3, formerly MIP1 $\alpha$ ) was selected as a functional candidate due to its involvement in T helper 1 cell (TH1) differentiation. A variant in intracellular adhesion molecule 1 (ICAM1) may be associated with susceptibility to severe malaria.<sup>17</sup> Finally, since complement receptor 1 (CR1) can be used by *M. tuberculosis* to gain entry into macrophages, it was hypothesized that some recently defined variants in CR1 may influence susceptibility to mycobacterial disease.<sup>17</sup>

### MATERIALS AND METHODS

The study protocol was reviewed and approved by the National Health Sciences Research Committee of Malawi and by

the Ethics Committee of the London School of Hygiene and Tropical Medicine.

**Recruitment and sample collection.** The population, basic field methods, and TB case finding and diagnostic procedures of the KPS have been described in detail elsewhere.<sup>19,20</sup> Tuberculosis cases recruited into the study were ascertained largely by passive self-reporting at health centers, although a small proportion were identified in the context of other studies. Sputum samples and lymph node aspirates were examined (fluorescence and Ziehl-Neelsen stain) and cultured at project headquarters in Chilumba, with species confirmation by the United Kingdom Public Health Laboratory Service Mycobacterial Reference Unit (Dulwich, United Kingdom). Biopsy specimens were sent to Professor S. Lucas (St. Thomas' Hospital, London, United Kingdom). Inclusion in this study required confirmation of TB by culture, smear, or histology (excluding those whose only evidence of TB was a single scanty smear with fewer than 10 bacilli).

Recruitment for this study began in 1996, with targets of 250 HIV-positive and 250 HIV-negative cases. Cases diagnosed from November 1996, together with cases diagnosed before this and known to be HIV-negative at the time of diagnosis (as all TB cases in Karonga have been offered HIV counseling and testing since 1988), were included. For cases diagnosed before 1998, up to four individually matched controls were selected using project databases. Individuals were eligible for selection as controls if they had no history or evidence of either TB or leprosy and they were not a first- or second-degree relative of the case to which they were being matched. Matching criteria were age (within age groups 15–19, 20–24, 25–29, 30–34, 35–44, 45–54, 55–64, and  $\geq 65$  years old), sex, and area of residence (within 1 km of the case). From 1998 a field-based method was used to identify controls frequency-matched to cases for age (using date of birth in 10-year calendar periods), sex, and area of residence.<sup>21</sup> Approximately two controls were recruited for each case. Analyses reported here include cases and controls recruited up to the end of September 2001.

Cases and controls were interviewed by project paramedical staff who explained the study, provided counseling, and invited the patients to provide a blood sample for HIV testing and genetic analyses. Blood (7.5 mL) for HIV and genetics testing was collected into EDTA, and placed in vaccine carriers with wet ice until it reached the project laboratory within three days of collection, where cells were separated and stored at  $-70^{\circ}\text{C}$ . The DNA was separated with Nucleon kits (Scotlab, Coatbridge, United Kingdom) following the manufacturer's instructions, and shipped at  $4^{\circ}\text{C}$  to the Wellcome Trust Centre for Human Genetics (Oxford, United Kingdom) for analysis. Those who wished to know their HIV status were given post-test counseling in their homes. The HIV testing was done in duplicate using a particle agglutination test (Edware modification of Serodia; Mast Diagnostics, Ltd., Bootle, Merseyside, United Kingdom) and an ELISA (Vironostika HIV Uni-form II plus 0; Organon Teknika, Ltd., Cambridge, United Kingdom), with retesting of discordant samples.<sup>22</sup>

**Genotyping.** The methods used for genotyping the 27 variants investigated have been described elsewhere.<sup>17</sup> The ligation detection reaction was used to type polymorphisms in VDR, MBL, IL10, TNF, ICAM1, CR1, and TLR4.<sup>17</sup> The SLC11A1, CCL3, TLR2, and LTA polymorphisms were typed by fluorescence polymerase chain reaction (PCR) and

an amplification refractory mutation system PCR<sup>17</sup> was used to type the IFNG variant.

**Statistical analysis.** Analysis of the individually-matched and frequency-matched studies was done separately. For the individually-matched studies, conditional logistic regression was used to quantify the association between disease and genotype, with and without controlling for ethnic group. For the frequency-matched study, logistic regression was used. All analyses were stratified on HIV status of the case.

In general, HIV-negative controls were used as the comparison group for both HIV-negative and HIV-positive cases. For HIV-negative cases, HIV-negative individuals are the most valid control group. For HIV-positive cases, HIV-positive individuals are the ideal control group. However, relatively few HIV-positive controls were identified, and restricting the control group to them would considerably limit study power. If the genes studied are not related to the risk of HIV infection, then it is acceptable to use HIV-negative individuals, rather than HIV-positive individuals, as the control group for HIV-positive cases. The association between genotype and HIV status was thus assessed among the controls. For the polymorphisms where there was preliminary evidence of an association, HIV-positive controls were used as the comparison group for HIV-positive cases.

Two estimates of effect (odds ratios [OR]) were obtained for each polymorphism, one from the individually matched study and one from the frequency-matched study. A summary estimate was obtained by combining the two estimates in a meta-analysis.

Genotyping was not always successful, and for some polymorphisms genotyping was attempted on only a proportion of individuals. Thus, the number of individuals genotyped was less than the total number of individuals who gave blood for genetics and HIV testing.

## RESULTS

Four hundred and thirty-one individuals were recruited into the individually matched study (133 cases and 298 controls). The HIV status was known for all individuals except for one control, and DNA was obtained from 418 individuals. A total of 1,589 individuals were recruited into the frequency-matched case-control study (546 cases and 1,043 controls). Of these individuals, 1,132 provided blood for testing (412 cases and 720 controls), and 174 controls and 1 case provided a buccal swab. Genetic data were analyzed for slightly different numbers of individuals for each gene up to a total of 1,188 individuals. The HIV data were available for 85% of these individuals; thus, 1,010 individuals (386 cases and 624 controls) were included in the analyses.

Preliminary comparisons of genotype frequencies between HIV-positive controls ( $n = 82$ ) and HIV-negative controls ( $n = 542$ ) indicated that the polymorphisms TNF-238, VDR-*Bsm* I, and CR1-4795 may be associated with HIV in Malawians (compare the controls in Tables 1 and 2). In each case the rarest genotype showed a weak association ( $P = 0.01$ ,  $0.02$ , and  $0.04$ , respectively). Thus, for these polymorphisms, HIV-positive TB cases were compared with HIV-positive controls to assess the association between TB and genotype (Table 1). Homozygosity for the *Bsm* I polymorphism in VDR appeared to provide protection against TB among HIV-

TABLE 1  
Genotype frequencies and odds ratios (ORs) for HIV-positive cases and HIV-positive controls\*

Gene	Polymorphism	Genotype	HIV+ cases		HIV+ controls		HIV+ cases compared to HIV+ controls OR (95% confidence limits) <i>P</i> values adjusted for age, sex, zone, ethnic group
			%	n	%	n	
CR1	A→G at 4795 K1590E (McC <sup>a</sup> →McC <sup>b</sup> )	a/a	64	161	68	52	1.06 (0.59-1.92) 0.837
		a/g	30	75	32	24	
		g/g	6	16	0	0	
TNF	-238 (G→A)			252	76		
		g/g	85	122	88	43	
		g/a	15	21	8	4	2.01 (0.65-6.28) 0.228
		a/a	0.7	1	4	2	0.11 (0.005-2.58) 0.171
VDR	C→T ( <i>Bsm</i> I) (b→B)			144	49		
		c/c	62	123	60	36	
		c/t	35	69	23	14	1.65 (0.78-3.52) 0.194
		t/t	3	6	17	10	0.12 (0.04-0.40) 0.001
				198	60		

\* Only those polymorphisms for which there was evidence that genotype was associated with HIV status are shown. HIV = human immunodeficiency virus; CR1 = complement receptor 1; TNF = tumor necrosis factor; VDR = vitamin D receptor.

infected individuals (OR = 0.12, 95% CI = 0.04-0.4,  $P = 0.001$ ). However, this association results from a high frequency of the rarer homozygote (17%) in the small ( $n = 60$ ) HIV-positive control group and could be a chance finding. There was no evidence that the TNF-238 and CR1-4795 variants were associated with TB in HIV-infected individuals.

The 24 polymorphisms that did not show evidence for association with HIV were tested for association with TB by comparing genotype frequencies of both HIV-negative and HIV-positive cases against those of HIV-negative controls. Results are shown in Table 2. Among HIV-negative individuals, homozygosity for the CR1 variant Q1022H was associated with TB (OR = 3.12, 95% CI = 1.13-8.60,  $P = 0.028$ ). Evidence for association with this variant was not found among HIV-positive TB cases.

On comparing HIV-positive TB cases with HIV-negative controls, associations were observed with MBL2 G57E heterozygotes (OR = 1.69, 95% CI = 1.04-2.75,  $P = 0.034$ ), and IL10-1082 G homozygotes (OR = 0.37, 95% CI = 0.18-0.76,  $P = 0.007$ ). While there was no association between either of these variants and HIV-negative TB, the same genotype trends were observed, suggesting that these variants may have a subtle influence on TB susceptibility, the impact of which is more pronounced in individuals already immunocompromised by HIV infection.

There was borderline evidence that the SLC11A1 exon 2 deletion may be associated with TB susceptibility in HIV-positive cases (OR = 2.60, 95% CI = 0.97-6.99,  $P = 0.059$ ). Heterozygosity for the SLC11A1 CAAA polymorphism was associated with protection in both HIV-positive (OR = 0.70, 95% CI = 0.49-0.99,  $P = 0.046$ ) and HIV-negative (OR = 0.65, 95% CI = 0.46-0.92,  $P = 0.014$ ) TB cases.

## DISCUSSION

In the Karonga population, heterozygosity for the 3'-untranslated (UTR) region CAAA insertion/deletion in SCL11A1 is associated with protection against TB, regardless of HIV status. This variant has apparently not been previously investigated for association with TB susceptibility. Furthermore, an association between any SCL11A1 variant and TB susceptibility in HIV-positive individuals has not been

described previously. That this association is observed in both HIV-negative and HIV-positive cases suggests that the SCL11A1 gene product acts to protect against TB even during the immunosuppressed state of HIV infection, which may provide insight into how SCL11A1 influences TB susceptibility. The gene product is thought to act as a divalent ion pump that can influence acidity and/or iron content of the phagosome, and thus be involved in the direct killing of mycobacteria. However, it is also involved in regulation of the development of the adaptive immune response. As the adaptive immune response becomes impaired by infection and destruction of T cells in the course of HIV infection, these findings suggest SCL11A1 may be more important in the innate immune response to TB. It is interesting to contrast this with the growing evidence that suggests that SCL11A1 may influence development of the acquired immune response to leprosy.<sup>23</sup>

It is not known whether the CAAA variant has any effect on the function or expression of the SCL11A1 gene product. Other SCL11A1 variants that have been associated with TB susceptibility elsewhere (including the promoter microsatellite, which does have a putative effect on the levels of SCL11A1 expression) were not associated in this Malawi series. The association of several variants of this gene with mycobacterial disease is unlikely to be due to chance, but the failure to identify a consistently associated variant may indicate that other, as yet undescribed, polymorphisms in this gene are responsible for the observed associations. A preliminary sequencing project of a 244-basepair segment of the 3'-UTR of the SCL11A1 gene in Malawians has not shown any novel polymorphisms. An alternative possibility is that the associations have arisen due to linkage disequilibrium with a variant in some neighboring gene that influences TB susceptibility.

There is some evidence that SCL11A1 genotype may influence susceptibility to HIV infection in Colombians.<sup>24</sup> While preliminary results suggest that this is not the case in Karonga district, the possibility that the association observed in HIV-positive cases is due to susceptibility to HIV infection rather than TB infection cannot be ruled out.

On phagocytic cells complement receptor 1 mediates the adherence and phagocytosis of complement-opsonized pathogens. Since mycobacteria use this mechanism to gain entry

TABLE 2  
Genotype frequencies for cases and controls and odds ratios (ORs)\*

Gene	Polymorphism	Genotype	HIV+ cases		HIV- cases		HIV- controls		HIV- cases compared to HIV-controls: OR (95% confidence limits) P values		HIV+ cases compared to HIV-controls: OR (95% confidence limits) P values		
			%	n	%	n	%	n					
CCL3 (MIPI- $\alpha$ )	Promoter microsatellite (TA) <sub>n</sub> at -906	331/331	48	99	48	70	39	226					
		331/327	24	50	27	39	28	162	0.69 (0.40-1.16)	0.156	0.74 (0.46-1.18)	0.204	
		331/333	18	37	15	22	19	113	<b>0.55 (0.28-1.03)</b>	<b>0.062</b>	0.65 (0.38-1.09)	0.104	
		Other	10	20	11	16	14	79	0.65 (0.32-1.33)	0.242	0.67 (0.35-1.28)	0.224	
			206		147		580						
CR1	A→G at 4795 K1590E (McC <sup>a</sup> →McC <sup>b</sup> )	a/a	64	161	61	119	62	459					
		a/g	30	75	32	63	33	239	0.96 (0.65-1.42)	0.844	0.81 (0.56-1.17)	0.255	
		g/g	6	16	7	13	5	36	1.75 (0.82-3.74)	0.147	1.23 (0.59-2.56)	0.588	
				252		195		734					
	G→A at 4828 R1601G (S11→S12)	g/g	46	115	50	97	48	350					
		g/a	40	101	38	73	42	306	0.91 (0.62-1.32)	0.609	0.96 (0.67-1.38)	0.829	
		a/a	14	35	12	24	10	72	1.48 (0.82-2.66)	0.192	1.33 (0.77-2.32)	0.309	
				251		194		728					
	A→G at 4870 I1615V	g/g	80	201	78	151	79	578					
		g/a	18	46	21	41	19	138	1.30 (0.84-2.01)	0.232	0.90 (0.58-1.41)	0.655	
		a/a	1	3	1	2	2	11	1.04 (0.20-5.23)	0.965	0.53 (0.10-2.96)	0.470	
				250		194		727					
	A→G at 3650 H1208R	a/a	63	32	59	48	60	107					
		a/g	33	17	37	30	33	58	1.12 (0.55-2.28)	0.759	0.55 (0.18-1.65)	0.287	
g/g		4	2	5	4	7	12	1.20 (0.27-5.44)	0.809	0.86 (0.08-9.30)	0.904		
			51		82		177						
T→C at 2078 I684T	t/t	77	95	79	96	76	262						
	t/c	13	16	13	16	15	53	0.53 (0.22-1.27)	0.155	0.88 (0.36-2.15)	0.778		
	c/c	10	13	7	9	9	30	0.79 (0.29-2.15)	0.644	0.86 (0.35-2.13)	0.747		
			124		121		345						
G→T at 3093 Q1022H	g/g	71	164	68	133	74	496						
	g/t	27	62	28	55	24	160	<b>1.45 (0.97-2.16)</b>	<b>0.073</b>	1.25 (0.83-1.87)	0.290		
	t/t	2	4	4	8	2	14	<b>3.12 (1.13-8.60)</b>	<b>0.028</b>	0.99 (0.26-3.76)	0.987		
			230		196		670						
C→G at 5507 P1827R	c/c	73	186	72	151	74	541						
	c/g	24	62	25	53	24	177	1.14 (0.76-1.68)	0.529	1.14 (0.77-1.69)	0.515		
	g/g	2	6	3	7	2	14	2.32 (0.84-6.37)	0.104	1.29 (0.42-3.94)	0.652		
			254		211		732						
ICAM1	A→T at 179 K29M (ICAM-1 <sup>ref</sup> →ICAM-1 <sup>Kilifi</sup> )	a/a	41	89	45	94	45	268					
		a/t	40	86	37	78	37	222	0.96 (0.65-1.42)	0.852	0.97 (0.62-1.51)	0.890	
		t/t	19	42	18	37	18	106	0.97 (0.60-1.57)	0.915	0.94 (0.55-1.62)	0.827	
			217		209		596						
IFNG	Intron 1 + 874 T→A	a/a	65	154	71	151	66	467					
		a/t	31	74	28	60	31	219	0.92 (0.63-1.32)	0.617	0.96 (0.66-1.43)	0.885	
		t/t	4	10	1	2	2	17	0.40 (0.08-1.84)	0.233	0.67 (0.58-4.06)	0.386	
			238		213		703						
IL10	-592	c/c	38	94	40	85	42	297					
		c/a	49	120	47	98	43	301	1.02 (0.70-1.49)	0.905	1.23 (0.85-1.80)	0.274	
		a/a	13	32	13	27	15	107	0.91 (0.53-1.57)	0.745	0.92 (0.54-1.57)	0.758	
				246		210		705					
	-819	c/c	38	93	40	85	41	287					
		c/t	49	122	47	98	43	303	0.98 (0.68-1.43)	0.922	1.13 (0.78-1.65)	0.513	
t/t		13	32	13	27	15	108	0.87 (0.51-1.51)	0.628	0.86 (0.51-1.48)	0.593		
			247		210		698						
-1082	a/a	47	73	41	69	38	203						
	a/g	42	65	46	78	46	251	1.16 (0.76-1.76)	0.497	<b>0.55 (0.34-0.88)</b>	<b>0.013</b>		
	g/g	11	17	13	23	16	87	0.83 (0.46-1.50)	0.538	<b>0.37 (0.18-0.76)</b>	<b>0.007</b>		
			155		170		541						
LTA (TNF $\beta$ )	5'UTR microsatellite (AC/GT) <sub>n</sub> at -3.5 kb†	101 absent	89	211	87	172	85	599					
		101 1 copy	10	24	13	26	15	105	0.89 (0.52-1.52)	0.658	<b>0.64 (0.38-1.07)</b>	<b>0.090</b>	
		101 2 copies	1	2	0	0	0.4	3					
					237		198		707				
		103 absent	66	156	62	122	62	436					
		103 1 copy	30	71	34	67	34	241	1.04 (0.74-1.47)	0.827	0.78 (0.55-1.11)	0.163	
		103 2 copies	4	10	4	9	4	30					
					237		198		707				
		105 absent	47	111	52	102	54	380					
		105 1 copy	43	101	40	80	39	274	1.06 (0.77-1.45)	0.728	1.12 (0.83-1.51)	0.444	
			10	25	8	16	7	53					
			237		198		707						

TABLE 2  
Continued

Gene	Polymorphism	Genotype	HIV+ cases		HIV- cases		HIV- controls		HIV- cases compared to HIV-controls: OR (95% confidence limits) P values	HIV+ cases compared to HIV-controls: OR (95% confidence limits) P values	
			%	n	%	n	%	n			
MBL2 (MBP, MBL)	G→A at 239 G57E (C variant)	111 absent	94	223	95	188	94	668	1.26 (0.55-2.85) 0.583	0.86 (0.41-1.78) 0.677	
		111 1 copy	6	14	5	10	6	39			
		111 2 copies	0	0	0	0	0	0			
				237		198		707			
		g/g	62	95	65	110	66	362			
		g/a	34	53	31	52	29	160			
		a/a	4	6	4	6	5	24			
				154		168		546			
				62	163	62	145	60			469
				30	79	31	71	34			266
SLC11A1 (NRAMP1)	Promoter microsatellite (GT) <sub>n</sub> (199 = allele 3, 201 = allele 2)	199/199	62	163	62	145	60	469	0.88 (0.62-1.25) 0.481	0.75 (0.52-1.08) 0.123	
		199/201	30	79	31	71	34	266			
		201/201	6	15	3	7	4	32			
		Other	2	4	4	9	1	11			
				261		232		778			
				58	126	51	112	50			353
				34	74	42	91	40			287
				8	17	7	15	10			69
					217		218				709
				50	129	49	117	42			317
TGTG ins/del	ins/del del/del	ins/ins	58	126	51	112	50	353	1.01 (0.71-1.42) 0.970	0.72 (0.49-1.07) 0.108	
		ins/del	34	74	42	91	40	287			
		del/del	8	17	7	15	10	69			
				217		218		709			
				50	129	49	117	42			317
				40	103	38	90	48			367
				10	27	13	32	10			78
					259		239				762
				93	152	93	155	94			493
				7	11	7	11	6			32
TLR2	Intron 2 microsat†	Deletion present	7	11	7	11	6	32	0.88 (0.38-2.02) 0.757	<b>2.60 (0.97-6.99) 0.059</b>	
				163		166		525			
		216 absent	86	215	89	192	89	658			
		216 1 copy	14	34	10	21	11	83			
		216 2 copies	0	0	1	2	0.1	1			
				249		215		742			
				77	191	75	162	74			550
				21	52	23	49	25			182
				2	6	2	4	1			10
					249		215				742
TLR4	Exon 4 A→G at 896 D299G	222 absent	77	191	75	162	74	550	0.94 (0.63-1.40) 0.763	1.04 (0.70-1.53) 0.863	
		222 1 copy	21	52	23	49	25	182			
		222 2 copies	2	6	2	4	1	10			
				249		215		742			
				43	106	42	90	41			304
				43	107	43	93	48			358
				14	36	15	32	11			80
					249		215				742
				79	196	76	164	77			569
				18	46	23	49	22			166
TNF	-238	226 absent	79	196	76	164	77	569	0.96 (0.64-1.44) 0.841	1.15 (0.76-1.72) 0.507	
		226 1 copy	18	46	23	49	22	166			
		226 2 copies	3	7	1	2	1	7			
				249		215		742			
				93	112	90	146	91			389
				7	8	10	16	9			38
				0	0	0	0	0			0
					120		162				427
				85	122	90	162	87			464
				15	21	10	19	13			69
VDR	T→C 352 silent ( <i>Taq I</i> , T→t)	a/a	93	112	90	146	91	389	0.82 (0.45-1.49) 0.515	1.37 (0.68-2.73) 0.378	
		g/a	7	8	10	16	9	38			
		g/g	0	0	0	0	0	0			
				120		162		427			
				85	122	90	162	87			464
				15	21	10	19	13			69
				0.7	1	0	0	0			0
					144		181				533
				82	97	82	132	83			344
				18	21	17	27	17			71
TNF	-308	g/g	82	97	82	132	83	344	1.15 (0.67-1.99) 0.610	1.31 (0.59-2.90) 0.511	
		g/a	18	21	17	27	17	71			
		a/a	0	0	1	2	0.2	1			
				118		161		416			
				82	98	78	129	72			301
				17	20	20	33	26			109
				1	1	2	3	2			7
					119		165				417
				82	98	78	129	72			301
				17	20	20	33	26			109
TNF	-863	a/c	17	20	20	33	26	109	0.73 (0.45-1.19) 0.204	0.59 (0.29-1.20) 0.145	
		a/a	1	1	2	3	2	7			
				119		165		417			
				59	132	61	129	57			384
				36	82	34	72	36			241
				5	11	5	11	7			47
					225		212				672
				49	97	48	70	53			287
				41	82	43	63	39			210
				10	20	9	13	8			46
TNF	-238	g/g	85	122	90	162	87	464	0.82 (0.45-1.49) 0.515	1.37 (0.68-2.73) 0.378	
		g/a	15	21	10	19	13	69			
		a/a	0.7	1	0	0	0	0			
				144		181		533			
				82	97	82	132	83			344
				18	21	17	27	17			71
				0	0	1	2	0.2			1
					118		161				416
				82	98	78	129	72			301
				17	20	20	33	26			109
TNF	-308	a/c	17	20	20	33	26	109	0.73 (0.45-1.19) 0.204	0.59 (0.29-1.20) 0.145	
		a/a	1	1	2	3	2	7			
				119		165		417			
				59	132	61	129	57			384
				36	82	34	72	36			241
				5	11	5	11	7			47
					225		212				672
				49	97	48	70	53			287
				41	82	43	63	39			210
				10	20	9	13	8			46
VDR	G→T ( <i>Apa I</i> , a→A)	t/t	49	97	48	70	53	287	1.36 (0.88-2.11) 0.169	1.23 (0.81-1.87) 0.322	
		t/g	41	82	43	63	39	210			
		g/g	10	20	9	13	8	46			
				199		146		543			
				59	132	61	129	57			384
				36	82	34	72	36			241
				5	11	5	11	7			47
					225		212				672
				49	97	48	70	53			287
				41	82	43	63	39			210
		10	20	9	13	8	46				

TABLE 2  
Continued

Gene	Polymorphism	Genotype	HIV+ cases		HIV- cases		HIV- controls		HIV- cases compared to HIV-controls: OR (95% confidence limits) P values	HIV+ cases compared to HIV-controls: OR (95% confidence limits) P values
			%	n	%	n	%	n		
	C→T	c/c	62	123	61	89	58	314		
	( <i>Bsm</i> I, b→B)	c/t	35	69	37	54	35	192	1.05 (0.67-1.65)	0.824
		t/t	3	6	3	4	7	39	0.70 (0.22-2.25)	0.546
				198		147		545		0.85 (0.56-1.28) 0.432
										0.64 (0.23-1.77) 0.386

\* Statistically significant or marginally significant associations are shown in **bold**. HIV = human immunodeficiency virus; CCL3 = chemokine (C-C motif) ligand 3; CR1 = complement receptor 1; ICAM1 = intercellular adhesion molecule 1; IFNG = interferon- $\gamma$ ; IL10 = interleukin 10; LTA = lymphotoxin alpha; TNF $\beta$  = tumor necrosis factor  $\beta$ ; UTR = untranslated region; MBL = mannose-binding lectin; SLC11A1 = solute carrier family 11, member 1; ins = insertion; del = deletion; TLR2 = toll-like receptor 2; TNF = tumor necrosis factor; VDR = vitamin D receptor.

† Baseline alleles other than 101, 103, 105, and 111.

‡ Baseline alleles other than 216, 222, 224, 226.

into macrophages, it was hypothesized that variants in CR1 may influence susceptibility to TB.<sup>17,25,26</sup> Five polymorphisms that encode amino acid substitutions in the extra-membrane portion of the protein and which may affect either ligand binding or CR1 stability were investigated.<sup>17</sup> Homozygosity for Q1022H was associated with TB susceptibility (OR = 3.12, 95% CI = 1.13-8.60,  $P = 0.028$ ). This variant occurs in a ligand binding domain, close to an aspartic acid residue that is important in C3b binding. It has been suggested that substitution of glutamine to a weakly basic and more hydrophobic histidine residue may alter ligand binding by CR1.<sup>27</sup> If this variant does reduce CR1 binding of C3b, it appears that rather than providing protection by limiting *M. tuberculosis* uptake, it increases TB susceptibility, indicating that uptake via CR1 may invoke protective mechanisms. Lack of an association between Q1022H and TB in HIV-positive individuals suggests that this protective mechanism is inhibited or ablated in immunosuppressed individuals.

Interferon- $\gamma$  is an essential and central regulator of the human response to infection. Homozygosity for the T allele of an intronic single nucleotide polymorphism (SNP) at +874 that may influence IFN- $\gamma$  production is associated with susceptibility to TB in Sicilians and in South Africans.<sup>14-16</sup> This SNP was not associated with TB susceptibility in Malawi. This could be because the lower allele frequency in Malawi (20% compared with 50%) reduces the power to detect such an association, or because the TB association is due to another polymorphism that is in linkage disequilibrium (LD) with the +874 SNP in Sicily but not in Malawi, or because this allele does not affect TB susceptibility in this population. In some populations it appears that the +874 SNP is in complete LD with a microsatellite allele that is associated with increased IFN- $\gamma$  production. Genotyping of this microsatellite indicates that while there is evidence of strong LD between it and the +874 SNP in Malawi, this is not absolute.

The gene product of MBL2, mannose-binding lectin, binds to mannose groups on a variety of bacteria, resulting in complement activation and opsonophagocytosis. Three polymorphisms in exon 1 influence serum MBL levels. Low serum concentrations of MBL may be associated with recurrent infections in young children, and yet low producing alleles are maintained in all populations, leading to the hypothesis that these variants are maintained by balancing selection.<sup>17</sup> Protection from mycobacterial infection by limiting phagocytotic entry of mycobacteria into host cells has been proposed as a mechanism by which such alleles may be maintained.<sup>17</sup> There is some evidence that such variants may be protective against

meningeal TB in Cape Coloureds but no association with protection against pulmonary TB in The Gambia was found.<sup>13,28</sup> In Malawi, we found no convincing evidence of association with TB.

Interleukin-10 is a key anti-inflammatory cytokine. Three promoter polymorphisms (-1082, -819, -592) that may influence IL10 levels were investigated for susceptibility to TB.<sup>17</sup> The HIV-positive, but not HIV-negative, Malawians homozygous for the -1082 G allele appeared to be protected against TB. The -1082 SNP lies in a putative transcription factor binding site, but reports on the function of the IL10 promoter variants are contradictory.<sup>29</sup> The -1082 G allele has been reported to be associated with reduced IL10 transcription, but haplotypes bearing the G variant are usually associated with increased IL10 levels.<sup>30,31</sup> It is unclear how increased IL10 production would provide protection against TB. However, there is some evidence that other, more distal IL10 promoter polymorphisms may be more important in controlling IL10 levels than the proximal polymorphisms investigated here.<sup>32</sup> Furthermore, one haplotype of these distal polymorphisms is reported to be associated with increased IL10 production and is linked to each of the three common proximal haplotypes, perhaps explaining the variable results of studies of the proximal polymorphisms. In light of the observed -1082 G association, an investigation of these distal polymorphisms and the haplotypes that are present in the Malawi population is warranted.

Intracellular adhesion molecule 1 (ICAM1/CD54) mediates both leukocyte-leukocyte and leukocyte-endothelial interactions during an immune response.<sup>33</sup> An apparently deleterious polymorphism, K29M, which reduces binding to leukocyte function associated antigen-1 and fibrinogen, two of the key ligands for ICAM1, has been postulated to be maintained by balancing selection. However, there was no evidence to suggest that this polymorphism influences TB susceptibility in Malawi.<sup>33</sup>

Vitamin D receptor is the receptor for the active form of vitamin D (1 $\alpha$ 25(OH)<sub>2</sub>D<sub>3</sub>), which in addition to influencing bone mass, has important immuno-modulatory effects including suppression of cytokine synthesis, immunoglobulin production and lymphocyte proliferation. Epidemiologic evidence suggests vitamin D deficiency is associated with susceptibility to TB. There is evidence that vitamin D impedes growth of *M. tuberculosis* in human macrophage and monocytic cell lines, and it has been suggested that vitamin D-containing medications may be useful in treating some forms of leprosy.<sup>17,34</sup> Homozygosity for a *Taq* 1 polymorphism (tt)

in the ligand binding domain of VDR was associated with resistance to TB in The Gambia.<sup>11</sup> Although this genotype was associated with susceptibility to leprosy, there was no evidence that it influences TB susceptibility in Malawians.<sup>17</sup> Failure to replicate the association observed in The Gambia may indicate that VDR does not influence TB susceptibility in Malawi, but it is also possible that the Gambian association was due to linkage disequilibrium between the silent *Taq* 1 SNP in codon 352 and another polymorphism with a functional effect, which does not exhibit the same LD in the Malawi population. Among HIV infected Malawians (Table 1) homozygosity for the intronic VDR polymorphism *Bsm* I (BB) appeared to be protective against TB, but this mainly reflects a high incidence of this genotype in the small sample of HIV-positive controls. Interestingly, the same BB genotype has been associated with rapid HIV disease progression in Spaniards, but association between VDR and HIV susceptibility was not assessed in that study.<sup>35</sup>

On recognition of pathogens, mammalian toll-like receptors send signals to activate innate and adaptive immunity. Both TLR2 and TLR4 can mediate cellular activation in response to components of *M. tuberculosis*.<sup>36</sup> An intronic microsatellite that may influence TLR2 expression levels has been identified in intron 2 of the TLR2 gene but no alleles were associated with TB susceptibility in Malawi (Segal S and others, unpublished). A TLR4 polymorphism that results in an amino acid substitution (Arg299Gly) in the receptor's extra-cellular domain, and thus impairs lipopolysaccharide-induced signaling, was found not to associate with TB susceptibility in Malawi.<sup>17</sup>

Tumor necrosis factor is a proinflammatory cytokine with pleiotropic effects. Its critical role in protection against TB is illustrated by the observation that some patients with inflammatory conditions that have been treated with the anti-TNF drug infliximab develop active TB.<sup>37</sup> Several promoter polymorphisms exist that may influence TNF expression and some have been associated with both autoimmune and infectious diseases, although none have been associated with TB susceptibility.<sup>17,38–40</sup> Consistent with these findings, no TNF promoter polymorphisms were associated with TB susceptibility in Malawi.

Some limited evidence that an LTA microsatellite allele may be protective against TB in HIV-positive Malawians was found. Like TNF, LTA is located in the HLA class III region, and encodes a cytokine with pleiotropic immunomodulatory effects. Lymphotoxin alpha appears to be crucial in organizing the granulomatous response that is required for controlling *M. tuberculosis* infection in mice.<sup>41</sup> Recently, two LTA SNPs were shown to be associated with susceptibility to myocardial infarction, and, these SNPs appear to influence the expression and/or function of LTA.<sup>42</sup> It would be of interest to investigate the relationship between the upstream microsatellite and these putative functional polymorphisms. Any investigation of association with TNF and LTA is complicated by the fact that these genes lie in the HLA region and there may be linkage disequilibrium between HLA alleles that influence TB susceptibility. There was some evidence that the TNF-238a allele may be associated with HIV infection in Malawi. Variants in TNF have been associated with HIV progression in some populations, but not with susceptibility.<sup>43</sup>

The chemokine (C-C motif) ligand 3, formerly known as the macrophage inflammatory protein-1, induces activation of

macrophages and proliferation and TH1 differentiation of T cells.<sup>44</sup> It belongs to the C-C chemokine family and binds receptors CCR-1, CCR-4, and CCR-5. Expression of CCL3 is induced by *M. tuberculosis* infection of human alveolar macrophages, with avirulent strains inducing greater expression than virulent strains.<sup>45</sup> There was borderline evidence that the promoter microsatellite 333 allele might be associated with protection against TB in HIV-negative Malawians. However, the functional effect, if any, of this promoter microsatellite is unknown.

This large-scale investigation of candidate genes has shown that genetic variants in *SCLA11A1* appear to influence TB susceptibility in Malawi, as has been found in other populations. Furthermore, this influence may extend to HIV-infected individuals. However, the association in Malawi is with a different variant of *SCLA11A1* from that reported in other populations, and the molecular basis of this interpopulation heterogeneity requires further analysis. Previously reported associations between variants of VDR, MBL, and IFNG and TB susceptibility in HIV-negative individuals were not replicated in this Malawian population. An association with a novel candidate gene, CR1, was shown. This is the first study to report analysis of genetic variants that may influence TB susceptibility in individuals immunocompromised by HIV infection, and we found preliminary evidence that both an IL10 promoter variant and the *Bsm* I polymorphism in VDR may influence TB susceptibility in such individuals.

Overall, this extensive analysis highlights two important features of TB genetic susceptibility: the likely existence of important interpopulation differences in relevant susceptibility loci and the potential for identification of false-positive associations by analysis of variants in multiple genes. To address both issues, future studies of polygenic susceptibility will require larger sample sizes than generally studied to date, as well as replication studies, ideally in both the original and in different populations.

Received August 28, 2003. Accepted for publication March 11, 2004.

**Acknowledgments:** We thank the many field, laboratory, and data management staff of the KPS who have carried out the work in Karonga District since 1979, the people of Karonga District, and the Ministry of Health and Population and the National Health Sciences Research Committee of Malawi for their encouragement of the KPS over many years. We also thank past and present Wellcome Trust Centre for Human Genetics (WTCHG) researchers and collaborators including Peter Zimmerman, Patricia Ramaley, and Graham Cooke for their role in developing the genotyping methods used here, as well as WTCHG core facilities staff, and Christophe Aucan, Kerrie Tosh, and Branwen Hennig for various assistance.

**Financial support:** This study was supported by the Wellcome Trust. Adrian V. S. Hill is a Wellcome Trust Principal Research Fellow.

**Authors' addresses:** Jodene Fitness, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand, E-mail: jodene.fitness@vuw.ac.nz. Sian Floyd and Paul E. M. Fine, Department of Tropical Hygiene, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom, E-mails: sian.floyd@lshtm.ac.uk and paul.fine@lshtm.ac.uk. David K. Warndorff, Lifted Sichali, Simon Malema, and Amelia C. Crampin, Karonga Prevention Study, PO Box 46, Chilumba, Karonga District, Malawi. Adrian V. S. Hill, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, United Kingdom, E-mail: adrian.hill@molecular-medicine.oxford.ac.uk.

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