Innate and adaptive host responses and their genetic control in tuberculosis : studies in Indonesia, a highly TB endemic setting

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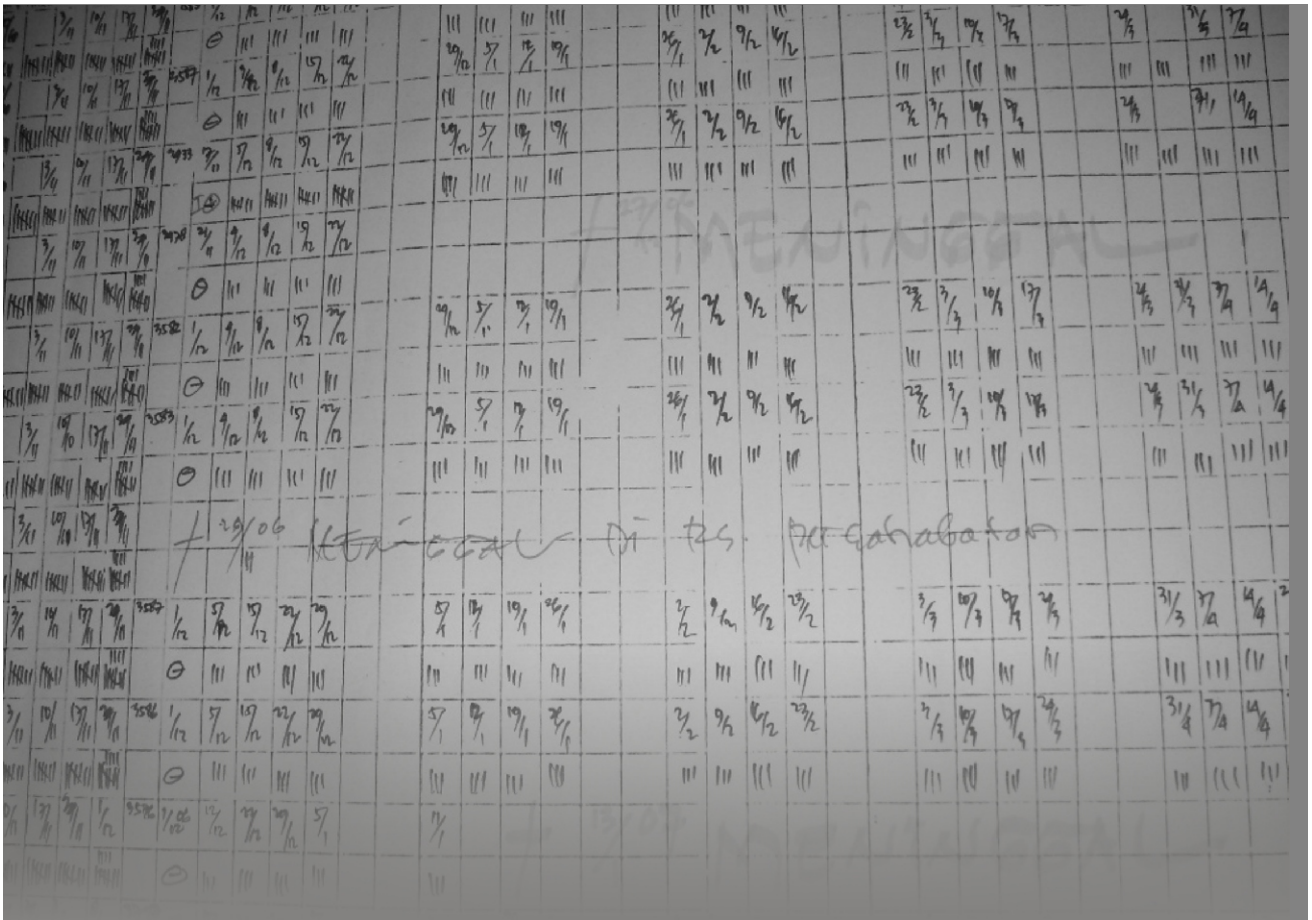

Zooming in on the drugstaking scheme list:
everybody will notice when patients do not come to complete the therapy or die in the middle of therapy (see: † MENINGGAL)

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Association of polymorphisms in IL-12/IFN-γ pathway genes with susceptibility to pulmonary tuberculosis in Indonesia

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SUMMARY

Upon infection with mycobacteria the IL-12/IFN-γ axis plays an essential role in the activation of cell-mediated immunity required for the elimination of pathogens. Mutations in genes of the IL-12/ IFN-γ axis are known to cause extreme susceptibility to infection with environmental mycobacteria, and subtle variations in these genes may influence susceptibility to more virulent mycobacteria.

We analyzed the distribution of polymorphisms in four essential genes from the IL-12/ IFN-γ axis, *IL12B*, *IL12RB1*, *IFNG* and *IFNGR1*, in 382 pulmonary tuberculosis patients and 437 healthy controls from an endemic region in Jakarta, Indonesia. The *IL12RB1* gene was sequenced in a subset of individuals. Nine known single nucleotide polymorphisms (SNPs) and two new silent variations, 135G>A and 1056C>T, were detected in *IL12RB1*. Six functional SNPs (-2C>T, 467G>A, 641A>G, 1312C>T, 1573G>A, 1781G>A) in *IL12RB1*, an *IL12B* promoter insertion/deletion polymorphism and CA repeats in *IFNG* and *IFNGR1* were analyzed in the cohort. The *IFNGR1* allele CA12 ($p = 0.004$) and genotype CA12/CA12 ($p = 0.01$; OR = 0.5) were associated with protection from pulmonary tuberculosis. Interestingly, *IL12B* promoter heterozygosity was associated with protection from tuberculosis in BCG-vaccinated individuals ($p = 0.03$; OR = 0.6). This new finding supports the role that IL-23 -of which *IL12B* encodes a subunit- plays in generation of memory T cells.

INTRODUCTION

Tuberculosis (TB) causes nearly 2 million deaths a year, which makes it the second most common cause of death due to an infectious disease (after AIDS).[1] Although one-third of the world population is infected with *Mycobacterium tuberculosis* only 5-10 % of infected people develop clinical TB. One of the factors influencing susceptibility to TB is host genetics, with evidence of this first shown in twin studies.[2,3] Many candidate genes have since been investigated for their role in TB, and several have been shown to be associated with TB susceptibility, for instance *HLA*,[4] *VDR*,[5] *NRAMP1*,[6] and *MBL*[7] (reviewed in [8]). These associations are, however, not sufficient to account for the genetic contribution identified in the twin studies.

In the past decade, patients with Mendelian Susceptibility to Mycobacterial Disease (MSMD) were found to have defects in type-1 cytokine pathway leading to severe infections with mainly environmental mycobacteria and salmonellae species. MSMD patients are unable to produce or respond to IFN-γ upon encountering intracellular bacterial pathogens, resulting in the inability to mount an adequate cell-mediated immunity response. These defects resulted from mutations in five genes coding for proteins in the IL-12/IFN-γ axis: *IL12B* (encoding IL-12p40), *IL12RB1* (encoding the *ß*1 chain of the IL-12 receptor), *IFNGR1*, *IFNGR2* (encoding the two chains of the IFN-γ receptor) and *STAT1* (reviewed in [9]). More subtle variations in these genes may account for the variation in susceptibility to more virulent mycobacteria and salmonellae species such as *M. Tuberculosis* or *Salmonella typhii.*

Indeed, associations with susceptibility to TB have thus far been detected with four genes from the IL-12/IFN-γ axis: *IL12RB1*, *IL12B*, *IFNG* and *IFNGR1*. One of the two major haplotypes of *IL12RB1*, R214-T365-R378 (RTR) that has been shown to be a lower IL-12 responder,[10,11] was reported to be associated with susceptibility to pulmonary TB in the Japanese population.[10] In Moroccan and Korean patients the major *IL12RB1* haplotypes were not found to be associated with susceptibility to TB.[12,13] In the Moroccan TB patients an association was however found with two polymorphisms in the *IL12RB1* promoter.[12] An insertion/deletion polymorphism in *IL12B* was shown to influence *IL12B* mRNA expression and IL-12p70 production[14,15] and was found to contribute to TB susceptibility in Hong Kong Chinese.[15] A CA repeat in intron 1 of *IFNG* that is linked to IFN-γ production *in vitro* [16] was also found to be associated with TB in Hong

Kong Chinese.[17] In the *IFNGR1* gene a particular allele of the CA repeat in intron 5 has been found to be associated with pulmonary TB in Croatia,[18] although this same polymorphism was not found to be associated with TB in The Gambia.[19]

We investigated whether polymorphisms in the IL-12/IFN-γ axis are associated with susceptibility to pulmonary TB in a case-control study in a highly TB endemic area in Jakarta, Indonesia, a country harboring >10% of all TB cases worldwide. We first identified new variations in *IL12RB1* by sequencing a subset of patients and controls. We analyzed various SNPs in *IL12RB1* as well as the above-described polymorphisms in *IL12B*, *IFNG*, and *IFNGR1* in order to determine whether any of the alleles or genotypes in these polymorphisms influence susceptibility to pulmonary TB in the Indonesian population.

MATERIALS AND METHODS

Patients and control subjects

In a case-control study design, carried out from June 2001 to December 2004, newly diagnosed pulmonary TB-patients (n = 382) aged 15 or older were recruited from an outpatient TB clinic in Central Jakarta, Indonesia. Diagnosis of TB was based on the WHO definition [27] which includes the presence of clinical symptoms, chest X-ray (CXR) examination, and microscopic detection of acid-fast bacilli in Ziehl-Nielsen stained sputum smear or positive culture of *M. tuberculosis*. All patients were tested for HIV serology. Patients with known immunosuppression were excluded (HIV positive, pregnancy or immunosuppressive therapy). HIV infected individuals (<2 %) were referred for post-test counseling and appropriate care, according to the national guidelines.

In the same period, community controls (n = 437) were recruited, matched by age (±10%), sex, ethnic background if possible, socio-economic class, and area of residence. Controls were interviewed using the same standard questionnaire and underwent the same physical, blood, and CXR examination as cases. Controls were excluded if they had a history of prior anti-TB therapy, signs and symptoms suggestive of active TB or infiltrates in the CXR. Controls were not tested for HIV.

There were 231 males (60.5%) and 151 females (39.5%) in the patient group with a mean age ±standard deviation (s.d.) of 33.2±11.9 years and there were 245 males (56.1%) and 192 females (43.9%) in the control group with a mean age ±s.d. of 33.8±11.5 years. Sex and age were not significantly different between the groups. A BCG scar was found in 39.5% of TB patients and in 48.1% of controls (1% of controls unknown). Self and parental ethnicities were recorded upon recruitment. Of both the patient and control group more than 80% were of Javanese origin, the non-Javanese were born on other Indonesian islands. Written informed consent was obtained from all subjects, and the study was approved by the ethical committees of the Medical Faculty of the University of Indonesia and of the Eijkman Institute.

Identification of new *IL12RB1* variations

Peripheral blood mononuclear cells (PBMCs) from 36 patients and 28 controls were isolated from heparinized blood using density centrifugation over a Ficoll-Hypaque gradient (Pharmacia). PBMCs (2×10^6/ml) were incubated in a 24-well plate (Costar) with 2 µl/ml phytohaemagglutinin-16 (PHA, Remel) in Iscove's Modified Dulbecco Medium (IMDM, Bio-Whittaker) supplemented with 2 mM GlutaMAX (Gibco), 10 % Fetal Calf Serum (Gibco), 100 U/ml IL-2 (Chiron), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco). PHA blasts were cultured for 14 days in supplemented IMDM without PHA before RNA isolation using Trizol (GibcoBRL) according to the manufacturer's protocol. For reverse transcription 1 µg of RNA was preincubated with 0.5 µg oligo(dT) $_{12\text{--}18}$ primer (Invitrogen) for 10 minutes at 70°C followed by a 1h incubation at 37°C in the presence of 200 U M-MLV Reverse Transcriptase (Invitrogen), 24 U RNAsin (Promega), 500 µM dNTPs (Invitrogen), 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$ and 8 mM DTT (Invitrogen) in a total volume of 20 µl. RT enzyme was inactivated at 70°C for 10 minutes. Standard protocols [28] were used to amplify full-length *IL12RB1* isoform 1, followed by either nine or three subsequent nested PCRs to obtain the appropriate size products for DGGE analysis or direct sequencing, respectively (primer sequences available on request). DGGE analysis was performed essentially as described.[29] PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequence reactions were performed as described [30] using the ABI PRISM® Big Dye Terminators v 3.1 Cycle Sequencing Kit (Applied Biosystems). Reaction products were analyzed on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems).

Genotyping *IL12RB1* single nucleotide polymorphisms

Genomic DNA was isolated from whole blood essentially as described.[28] PCRs were performed using 100 ng of genomic DNA, 200 μM of each dNTP, 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Promega) in a total volume of 25 μl (primer sequences available upon request). PCR products were digested using the following restriction enzymes: *Bam*HI (-2C>T), *Nde*I (467G>A), *Pvu*II (641A>G), *Bse*LI (1312C>T), *Mwo*I (1573G>A), *Bst*NI (1781G>A) according to the manufacturer's recommendations. Digested products were analyzed on Spreadex gels in a SEA2000 submarine electrophoresis unit (Elchrom Scientific). Interpretation of alleles was done independently by two individuals.

Genotyping using fragment length analyses

PCRs for *IL12B* ins/del, *IFNG* CA, and *IFNGR1* CA polymorphisms were performed using 100 ng of genomic DNA, 200 μM of each dNTP, 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Promega) in a total volume of 25 μl. Forward primers were 5'-labeled with HEX (*IL12B*) or FAM (*IFNG* and *IFNGR1*), primer sequences and cycle conditions are available on request. PCR products were diluted in H_2O, 1 μl of diluted product was added to 8.8 μl HiDi Formamide, 0.2 μl 400 HD-ROX size standard (Applied Biosystems) and heated to 95°C for 5 min. Products were run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems), results were analyzed using GeneScan Analysis and Genotyper software (Applied Biosystems). Several homozygous alleles were sequenced to verify allele lengths.

Statistical analysis

Data from the questionnaires, physical exams, laboratory analyses and genotypings were imported into SPSS version 11.5 (SPSS Inc., Chicago, IL) for statistical analysis. The Hardy-Weinberg equilibrium of each polymorphism was checked using the program HWE.[31] The program CONTING was used to calculate χ^2 and associated *p*-values for contingency tables.[31] All *p*-values are 2-sided, *p*-values < 0.05 were considered significant.

RESULTS

Identification of variations in *IL12RB1*

In order to identify *IL12RB1* polymorphisms present in the Indonesian population we analyzed the *IL12RB1* gene in mRNA from a subset of patients and controls. It should be noted that this approach excludes screening of introns and detection of potential regulatory polymorphisms within the gene. *IL12RB1* isoform 1 was analyzed as this encodes the IL-12Rß1, whereas isoform 2 encodes a putative protein in which the two fibronectin type III repeat domains, the transmembrane domain, as well as the complete intracellular domain required for signal transduction have been replaced by a domain with 80% identity to an Alu-encoded domain. Although it can not be ruled out that isoform 2 encodes a functional protein no evidence to this effect has been reported.

The previously described variations -2C>T, 387G>C, 467G>A, 641A>G, 684C>T, 1094T>C, 1132G>C, 1573G>A, and 1781G>A were all detected in these samples (Table 1). In addition, two new silent variations were identified, 135G>A (S45S in exon 3) and 1056C>T (N352N in exon 10) (Table 1). These were confirmed by sequencing genomic DNA. The 135G>A variation was found in 1 out of 40 chromosomes of TB patients and in 0 out of 36 chromosomes of the controls. The 1056C>T variation was found in 1 out of 36 chromosomes of controls and in 0 out of 32 chromosomes of the cases.

In order to determine the frequencies of *IL12RB1* SNPs in the Indonesian population and whether they contribute to TB susceptibility we typed 314 patients and 316 controls for the SNPs that were expected to have a functional effect, e.g. the coding variations and the -2C>T SNP in the Kozak sequence. The SNPs 641A>G, 684C>T, 1094T>C and 1132G>C have been shown to be in one haplotype block in the Japanese population.[10] Based on the distribution of these SNPs in the sequenced samples, as well as similar findings in a Moroccan study [12] and the Dutch population (van de Vosse, unpublished results) we concluded that the same was likely to be true for the Indonesian population. For practical purposes only one SNP of this block was retained for further analysis; 641A>G. The SNP 1312C>T, causing the amino acid change H438Y, was not identified in the sequenced samples but was nonetheless analyzed in the full cohort since it had been identified together with 1573G>A and 1781G>A in a small group of TB patients from Japan.[20]

Table 1
IL12RB1 variations identified in Indonesians.

Polymorphism	Official designation*	Alleles	Effect on protein	First reported
-2C>T	rs436857	C/T	----	Saito *et al.* 2004[32]
135G>A	----	G/A	Silent (S45S)	This report
387G>C	rs11086087	G/C	Silent (V129V)	Remus *et al.* 2004[12]
467G>A	rs11575926	G/A	R156H	van de Vosse *et al.* 2003[33]
641A>G	rs11575934	A/G	Q214R	Sakai *et al.* 2001 † [20]
684C>T	rs17852635	C/T	Silent (P228P)	Akahoshi *et al.* 2003[10]
1056C>T	—-	C/T	Silent (N352N)	This report
1094T>C	rs375947	T/C	M365T	Sakai *et al.* 2001[20]
1132G>C	rs401502	G/C	G378R	Sakai *et al.* 2001[20]
1573G>A	rs11575935	G/A	A525T	Sakai *et al.* 2001[20]
1781G>A	----	G/A	G594	Sakai *et al.* 2001[20]

Polymorphisms identified by DGGE and/or sequence analysis of *IL12RB1* mRNA from 36 patients and 28 controls.
* In dbSNP build 126, released May 2006 (www.ncbi.nlm.nih.gov).
† First reported by Altare *et al.*[34] as a mutation, later found to be a SNP by Sakai *et al.*[20]

Distribution of *IL12RB1* single nucleotide polymorphism alleles and genotypes

The distribution of the alleles and genotypes of the *IL12RB1* SNPs is presented in Table 2. The polymorphisms 467G>A, 1312C>T and 1781G>A were extremely rare in the Indonesian population and were not analyzed further for association with TB. The Hardy-Weinberg equilibrium was calculated for the remaining *IL12RB1* polymorphisms. The genotypes of -2C>T and 641A>G were in equilibrium in the total group of individuals as well as in the healthy controls and patients. The genotypes of the 1573G>A SNP were in equilibrium in the controls but not in the patients ($p = 0.01$). This can be accounted for by 2 patients with the genotype AA instead of 1 expected AA individual which is not a major deviation from the equilibrium.

Allele and genotype distributions of the *IL12RB1* SNPs were analyzed to determine whether an association with susceptibility to TB was present (Table 2). No significant differences were observed between the TB patients and controls.

Distribution of alleles and genotypes of *IL12B*, *IFNG* **and** *IFNGR1*

The distribution of the alleles and genotypes of the fragment length polymorphisms in *IL12B*, *IFNG* and *IFNGR1* are presented in Table 3-5. The Hardy-Weinberg equilibrium was calculated for these polymorphisms. The *IL12B* ins/del polymorphism as well as the *IFNG* and *IFNGR1* CA repeats were in equilibrium in the total group of individuals, in the healthy controls and in the patients.

The allele and genotype distributions of the *IL12B* promoter polymorphism were analyzed (Table 3). We did not detect a significant association between any allele or genotype and susceptibility to TB. Heterozygotes appear to be protected from TB but this finding is not significant ($p = 0.07$). Because IL-23, for which *IL12B* encodes a subunit, is important for the generation of memory T cells we also analyzed the distribution of *IL12B* genotypes in individuals with or without a BCG scar separately. In the individuals with a BCG scar, a significant association ($p = 0.03$) is detected between *IL12B* heterozygosity and protection from TB (OR = 0.6, 95% CI: 0.4-0.95).

The allele and genotype distributions of the *IFNG* CA repeat were also analyzed (Table 4) without detecting a significant association between any allele or genotype and susceptibility to TB. Finally the allele and genotype distribution of the *IFNGR1* CA repeat were analyzed (Table 5). The allele distribution was significantly different between TB patients and controls ($p = 0.003$). The most common allele, CA12 appeared to be associated with protection from developing TB. Individuals with this protective allele CA_{12} had an OR for developing TB of 0.7 ($p = 0.004$, 95% CI: 0.6-0.9). The genotype distribution was also significantly different between TB patients and healthy controls ($p = 0.01$). Homozygosity for the protective CA_{12} allele was associated with protection from developing TB (OR = 0.5, 95% CI: 0.3-0.8).

DISCUSSION

The IL-12/IFN-γ axis plays an essential role in the activation of cell-mediated immunity required for elimination of mycobacteria. Mutations in IL-12/IFN-γ axis genes cause extreme susceptibility to infection with environmental mycobacteria. Subtle variations in these genes are expected to influence susceptibility to more virulent mycobacteria. We set out to determine the

Table 2
Distribution of *IL12RB1* SNP alleles and genotypes.

Polymorphism	Allele or genotype	Frequency in cases (%)	Frequency in controls (%)	*p*-value
-2C>T	C	590 (94.9)	587 (94.4)	0.71
	T	32 (5.1)	35 (5.6)	
	CC	279 (89.7)	277 (89.1)	0.79*
	CT	32 (10.3)	33 (10.6)	
	TT	0 (0)	1 (0.3)	
467G>A	G	530 (99.6)	513 (99.8)	n.a.
	A	2 (0.4)	1 (0.2)	
641A>G	A	377 (66.1)	400 (66.4)	0.91
	G	193 (33.9)	202 (33.6)	
	AA	127 (44.6)	137 (45.5)	0.95
	AG	123 (43.2)	126 (41.9)	
	GG	35 (12.3)	38 (12.6)	
1312C>T	C	601 (99.8)	604 (100)	n.a.
	T	1 (0.2)	0 (0)	
1573G>A	G	590 (96.4)	578 (96.0)	0.72
	A	22 (3.6)	24 (4.0)	
	GG	286 (93.5)	278 (92.4)	0.60 †
	GA	18 (5.9)	22 (7.3)	
	AA	2 (0.7)	1 (0.3)	
1781G>A	G	621 (99.2)	625 (98.9)	n.a.
	A	5 (0.8)	7 (1.1)	

At the time of these analyses only 314 patients and 316 controls were available.
No differences were observed in the distribution of *IL12RB1* SNP alleles or genotypes between TB patients and healthy controls (using χ^2 tests).
* genotypes CT and TT combined for analysis.
† Genotypes GA and AA combined for analysis. n.a. = not analyzed.

contribution of polymorphisms in IL-12/IFN-γ axis genes to susceptibility to or protection from TB in an Indonesian population. We have analyzed various SNPs in *IL12RB1*, as well as fragment length polymorphisms in *IL12B*, *IFNG* and *IFNGR1* of which the CA repeat in *IFNGR1* and the insertion/deletion polymorphism in *IL12B* were both associated with protection from pulmonary TB in Indonesia.

Table 3
Distribution of alleles and genotypes of the *IL12B* ins/del polymorphism.

Alleles or genotypes	Allele lengths (bp)*	Frequency in cases (%)	Frequency in controls (%)	*p*-value	OR (95% CI)
Alleles	337	419 (56.0)	485 (56.4)	0.88	
	341	329 (44.0)	375 (43.6)		
Genotypes	337 / 337	125 (33.4)	133 (30.9)	0.24	
	337 / 341	169 (45.2)	219 (50.9)		
	341 / 341	80 (21.4)	78 (18.1)		
Genotypes of vaccinated †	Homozygotes	90 (58.4)	95 (46.6)	0.03	Reference
	Heterozygotes	64 (41.6)	109 (53.4)		0.6 (0.4-0.95)

A significant difference in genotype distribution between TB patients and controls was detected among vaccinated individuals (using χ^2 tests).
* The short allele contains GC that in the long allele is replaced by CTCTAA, resulting in a 4 nt difference in length.
† As evidenced by presence of BCG scar.

The cause of TB in Indonesia, a high TB-endemic country, can be either re-activation or, more likely, re-infection. The common practice of BCG vaccination in combination with exposure to environmental mycobacteria renders Mantoux testing to determine exposure of controls impossible. Based on the IFN-γ production of healthy controls in response to *M. tuberculosis*-specific antigens it was concluded that the vast majority of adult healthy controls in Indonesia have been exposed to *M. tuberculosis* (R. van Crevel, unpublished data). We therefore assume that the patients and controls in this cohort have been similarly exposed.

IL12RB1

The two major haplotypes of *IL12RB1*, QMG and RTR, have been shown to differ in IL-12 responsiveness.[10,11] In a Japanese population the RTR haplotype was found to be associated with TB, whereas in Moroccan and Korean populations it was not. In the Moroccan population an association with the -2C>T SNP in *IL12RB1* was found instead. Somewhat unexpectedly, we did not find an association of either of the major haplotypes nor of any of the other SNPs tested with TB susceptibility in the Indonesian population. A large difference in allele frequencies of the QMG/RTR haplotypes appears

Table 4
Distribution of alleles and genotypes of the *IFNG* CA repeat.

Alleles or genotypes	Allele lengths (no. of CA repeats)	Frequency in cases (%)	Frequency in control (%)	*p*-value
Alleles	CA_{11}	0 (0)	1 (0.1)	0.79 *
	CA_{12}	269 (35.6)	317 (36.4)	
	CA_{13}	168 (22.2)	178 (20.5)	
	CA_{14}	4 (0.5)	3 (0.3)	
	CA_{15}	226 (29.9)	278 (32.0)	
	CA_{16}	5 (0.7)	6 (0.7)	
	CA_{17}	1 (0.1)	0 (0)	
	CA_{18}	83 (11.0)	86 (9.9)	
	CA_{20}	0 (0)	1 (0.1)	
Genotypes	CA_{12} / CA_{12}	52 (13.8)	60 (13.8)	0.97 †
	CA_{12} / CA_{13}	59 (15.6)	66 (15.2)	
	CA_{12} / CA_{15}	76 (20.1)	101 (23.2)	
	CA_{12} / CA_{18}	27 (7.1)	27 (6.2)	
	CA_{12} / CA_{minor}	3 (0.8)	3 (0.7)	
	CA_{13} / CA_{13}	19 (5.0)	20 (4.6)	
	CA_{13} / CA_{15}	47 (12.4)	47 (10.8)	
	CA_{13} / CA_{18}	20 (5.3)	23 (5.3)	
	CA_{13} / CA_{minor}	4 (1.1)	2 (0.5)	
	CA_{15} / CA_{15}	37 (9.8)	46 (10.6)	
	CA_{15} / CA_{18}	26 (6.9)	33 (7.6)	
	CA_{15} / CA_{minor}	3 (0.8)	5 (1.1)	
	CA_{18} / CA_{18}	5 (1.3)	1 (0.2)	
	CA_{18} / CA_{minor}	0 (0)	1 (0.2)	
	CA_{minor} / CA_{minor}	0 (0)	0 (0)	

No differences were observed in the distribution of alleles or genotypes between TB patients and healthy controls (using χ^2 tests).
* Minor alleles with frequencies < 1% (CA_{11}, CA_{14}, CA_{16}, CA_{17}, CA_{20}) were pooled for this analysis.
† Minor genotypes with frequencies < 1.5% were pooled for this analysis.

not to be responsible for the inability to detect an association in the Moroccan, Korean and Indonesian populations. The frequency of the RTR haplotype was 36% in Japanese healthy controls,[10] 36% in Korean controls [13] and 34% in our

Indonesian controls. In the Moroccan study the RTR haplotype frequency of 25% may not reflect the frequency in the population as only TB families (*n* = 101) were studied.[13] Due to the larger cohort sizes both the Korean (230 cases, 302 controls) and the Indonesian study (285 cases, 301 controls typed for these alleles) have a higher power (>0.80) to detect potential associations than the Japanese study in which only 98 cases and 197 controls were analyzed. This suggests the association between the *IL12RB1* RTR haplotype and TB is either specific for the Japanese population or this association is a false positive result. To investigate this, replication of the study in a larger, independent Japanese cohort would have to be performed.

The *IL12RB1* -2C>T SNP that was present in 10% of the Moroccan TB families,[12] was unfortunately not analyzed in either the Japanese or Korean studies. In the Indonesian cohort the frequency of the -2C>T minor allele was 5.6% in controls resulting in a power of 0.95 if a similar effect size of 2.7 would be present. If the effect size in the Indonesian population would however be smaller (≤2) the power would drop below 0.75, making it less likely to detect such an association if present.

IL12B

IL-12p40 is an essential component of both IL-12 and IL-23. Defects in IL-12/IL-23 signal transduction affect not only IFN-γ mediated immunity but also IFN-γ independent pathways. These include, amongst others, TNF-α and GM-CSF production by T and NK-cells, which activate macrophages, IL-23/IL-17 mediated immunity, as well as IL-23 dependent generation of memory cells (reviewed in [21]). Heterozygotes for the *IL12B* promoter polymorphism were found by Morahan *et al.*[14] to have both a significantly lower *IL12B* mRNA expression and IL12p70 production than homozygotes. In Hong Kong Chinese a similar trend of lower IL-12p70 production in heterozygotes was found, and heterozygosity was found to be associated with susceptibility to TB.[15] In the Indonesian cohort in contrast, heterozygosity for the *IL12B* promoter polymorphism was weakly associated with protection from TB. Interestingly, when we compared only individuals who were BCG vaccinated (defined by presence of a BCG scar), a significant association was detected between *IL12B* heterozygosity and protection from TB. Moreover, in a parallel project we observed that in accordance with the opposed TB-

Table 5
Distribution of alleles and genotypes of the *IFNGR1* CA repeat.

Alleles or genotypes	Allele lengths (no. of CA repeats)	Frequency in cases (%)	Frequency in controls (%)	*p*-value	OR (95% CI)
Alleles	CA_{12}	219 (29.0)	311 (35.7)	0.003 *	
	CA_{13}	6 (0.8)	3 (0.3)		
	CA_{16}	19 (2.5)	17 (1.9)		
	CA_{17}	25 (3.3)	12 (1.4)		
	CA_{18}	104 (13.8)	112 (12.8)		
	CA_{19}	87 (11.5)	121 (13.9)		
	CA_{20}	23 (3.0)	28 (3.2)		
	CA_{21}	13 (1.7)	13 (1.5)		
	CA_{22}	117 (15.5)	95 (10.9)		
	CA_{23}	77 (10.2)	82 (9.4)		
	CA_{24}	57 (7.5)	74 (8.5)		
	CA_{25}	7 (0.9)	3 (0.3)		
	CA_{26}	2 (0.3)	1 (0.1)		
Alleles	CA_{12}	219 (29.0)	311 (35.7)	0.004	0.74(0.6-0.9)
	CA_{other}	537 (71.0)	561 (64.3)		reference
Genotypes	CA_{12} / CA_{12}	29 (7.7)	58 (13.3)	0.01	0.5(0.3-0.8)
	CA_{12} / CA_{other}	161 (42.6)	195 (44.7)		0.8(0.6-1.1)
	CA_{other} / CA_{other}	188 (49.7)	183 (42.0)		reference

A significant difference was detected in allele and genotype distribution between TB patients and controls (using χ^2 tests).
* Minor alleles with frequencies <1% (CA_{13}, CA_{25}, CA_{26}) were pooled for this analysis.

protective genotypes, heterozygote individuals produced *higher* levels of IL-12p40 than homozygotes in response to stimulation with IFN-γ, LPS and *M. tuberculosis* this difference was however only significant in response to LPS (Table 6). These findings are compatible with the notion that developing adequate memory in response to BCG vaccination is IL-12p40-dependent. This is not surprising given that IL-23, of which IL-12p40 is a subunit, is particularly important for the generation of memory T cells. In both mice and humans the major target cells of IL-23 appear to be $CD4^+$ memory T cells [22,23] and experiments in IL-23p19$^{-/-}$ mice have shown that lack of IL-23 leads to

inefficient responses by memory $CD4^+$ T cells.[24] In addition, a defect in memory T-cell formation was observed in an IL-12Rß1-deficient individual. The patient lacked most memory $CD4^+$ T cells of the CCR7 neg/dull subset of $CD4^+$ T cells and had an impaired response to tetanus toxoid.[25]

The opposing TB-associated and IL-12p40 production-associated genotypes between the populations suggest that this *IL12B* promoter polymorphism does not influence *IL12B* mRNA expression directly, but more likely is linked to a functional polymorphism elsewhere (possibly in the promoter). Extensive analysis of *IL12B* regulatory and promoter regions may aid in identification of the actual functional variant(s) that causes variation in *IL12B* expression and thereby in TB susceptibility. Tso et al. were able to detect an association with the *IL12B* promoter polymorphism[15] without stratifying for BCG status. This may be due to the fact that in the Hong Kong Chinese the BCG coverage rate is much higher (80% since 1962, 100% since 1972[17]) than in the Indonesian population (48% in our controls), thus obscuring the interesting finding that this association is only present in vaccinated individuals.

IFNG

We did not find associations between *IFNG* intron 1 CA repeat alleles or genotypes and susceptibility to TB in the Indonesian population. The frequencies in the cases and controls are very close to the expected frequencies. PBMCs from Caucasians with the CA_{12}/CA_{12} genotype were found to produce significantly higher IFN-γ levels than PBMCs with other genotypes.[16] The genotype CA_{12}/CA_{12} was specifically found to be associated with TB in Hong Kong Chinese,[17] rendering it unlikely that in Hong Kong Chinese the CA_{12}/CA_{12} genotype is also associated with higher IFN-γ production as this would be expected to confer protection from TB rather than susceptibility. We observed higher IFN-γ production (in response to *M. tuberculosis*) in CA_{12}/CA_{12} genotype TB patients as well, but this was not significant since it was only analyzed in small groups (data not shown). Sahiratmadja *et al.* [26] showed in Indonesian pulmonary TB patients that, although *M. tuberculosis* induced IFN-γ production was strongly depressed during active TB, this IFN-γ production is normalized at the end of anti-TB therapy, suggesting *M. tuberculosis* induced IFN-γ production is not inherently different between patients and controls when analyzed in groups. PHA-induced IFN-γ

Table 6
IL-12p40 production in relation to *IL12B* genotypes.

Stimulus	Genotype	IL-12p40 median	*p*-value
50 U/ml IFN-γ	Heterozygotes, *n* = 59	102 pg/ml	0.98
	Homozygotes, *n* = 62	60 pg/ml	
100 ng/ml LPS	Heterozygotes, *n* = 59	435 pg/ml	0.01
	Homozygotes, *n* = 62	299 pg/ml	
10 µg/ml MTB	Heterozygotes, *n* = 59	109 pg/ml	0.94
	Homozygotes, *n* = 62	91 pg/ml	

IL-12p40 production data were generated in a parallel project.[26] In brief, whole blood from controls was stimulated with various stimuli and incubated for 24 hrs at 37ºC. IL-12p40 production was measured in the supernatants using ELISA.

production, however, remained significantly lower in patients after completion of 6 months of anti-tuberculous therapy, suggesting a prolonged or perhaps intrinsic defect in IFN-γ production may be present in these patients.

IFNGR1

Importantly, we found significant associations between *IFNGR1* CA repeat alleles and genotypes in intron 5, and protection from TB that could be attributed to the CA_{12} allele. In a small Croatian study *IFNGR1* CA repeat alleles were also found to be associated with protection from TB, however, in that population it was attributed to another allele, CA_{21}.[18] In a larger study in The Gambia no associations were detected between *IFNGR1* CA repeat alleles or genotypes and TB. Together these three studies suggest that certain alleles may be in linkage disequilibrium with a functional polymorphism in *IFNGR1*, but that these CA repeat alleles are themselves not functional polymorphisms. Further analyses of the *IFNGR1* gene may reveal such variations.

Conclusions between genetic association studies are often conflicting. There are several explanations for this. It is likely that many genes and pathways contribute to the pathogenesis of TB. The different genetic backgrounds, and therefore variations in specific genes, of the various populations in which these studies are conducted may contribute in different

ways to the observed phenotypes. The size of the cohort studied and the frequency of (minor) alleles therein may also greatly influence the outcome, with large cohorts in general producing more robust results. Finally, when the polymorphisms studied are themselves not functional they may be in linkage disequilibrium with a functional variant in one population but not in the next. The *IL12RB1* polymorphisms studied here are all functional polymorphisms, either causing a change in the amino acid sequence or affecting the translation start site. The fragment length polymorphisms studied here are unlikely to represent functional polymorphisms and further research is needed to identify the actual functional variants.

Further research, focusing on identifying the actual functional variants in IL-12/IFN-γ axis genes and, subsequently, on analyzing potential associations of such functional variants with TB will be needed to better understand the contribution of (variants in) the IL-12/IFN-γ axis to the development of TB.

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