Autophagy Gene Variant IRGM —261T Contributes to Protection from Tuberculosis Caused by Mycobacterium tuberculosis but Not by M. africanum Strains

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Abstract

The human immunity-related GTPase M (IRGM) has been shown to be critically involved in regulating autophagy as a means of disposing cytosolic cellular structures and of reducing the growth of intracellular pathogens in vitro. This includes Mycobacterium tuberculosis, which is in agreement with findings indicating that M. tuberculosis translocates from the phagolysosome into the cytosol of infected cells, where it becomes exposed to autophagy. To test whether IRGM plays a role in human infection, we studied IRGM gene variants in 2010 patients with pulmonary tuberculosis (TB) and 2346 unaffected controls. Mycobacterial clades were classified by spoligotyping, IS6110 fingerprinting and genotyping of the pks1/15 deletion. The IRGM genotype —261TT was negatively associated with TB caused by M. tuberculosis (OR 0.66, CI 0.52–0.84, Pnominal 0.0009, Pcorrected 0.0045) and not with TB caused by M. africanum or M. bovis (OR 0.95, CI 0.70–1.30, P 0.8). Further stratification for mycobacterial clades revealed that the protective effect applied only to M. tuberculosis strains with a damaged pks1/15 gene which is characteristic for the Euro-American (EUAM) subgroup of M. tuberculosis (OR 0.63, CI 0.49–0.81, Pnominal 0.0004, Pcorrected 0.0019). Our results, including those of luciferase reporter gene assays with the IRGM variants —261CC and —261TT, suggest a role for IRGM and autophagy in protection of humans against natural infection with M. tuberculosis EUAM clades. Moreover, they support in vitro findings indicating that TB lineages capable of producing a distinct mycobacterial phenolic glycolipid that occurs exclusively in strains with an intact pks1/15 gene inhibit innate immune responses in which IRGM contributes to the control of autophagy. Finally, they raise the possibility that the increased frequency of the IRGM —261TT genotype may have contributed to the establishment of M. africanum as a pathogen in the West African population.

Introduction

Autophagy is induced by the formation of intracellular double-membrane structures which form autophagosomes to sequester and, after further maturation to autolysosomes, degrade cytosolic protein aggregates and corrupted cellular organelles. Thereby, it allows a re-cycling of amino acids, which is of particular importance during periods of cell starvation. Autophagy is also an efficient innate defense mechanism in that it may lead to deposition of intracellular pathogens into an autophagosome for subsequent autolysosomal acidification and peptidase-mediated degradation. The net effect of well functioning autophagy is protein degradation, optimized loading of Human Leukocyte Antigen (HLA) class II molecules and intensified antigen presentation [1].

The activation pathways of autophagy and phagocytosis are closely related in that they share phosphatidylinositol-3-phosphate (PI3P) and other factors in facilitating the fusion step and closure of the phagophore at the final stage of phagosome and autophagosome formation [2]. Among other factors, immunity-related GTPases exert significant effects on both autophagy and phagosome maturation [3]. It was recently shown in human U937 cells that inhibition of the immunity-related GTPase IRGM by siRNA caused impaired conversion of light chain 3 (LC3), an exclusive marker of the autophagy cascade, into its active form which is required for the elongation of the double membranes and
eventual completion of the autophagosome [4]. In these experiments, impaired LC3 conversion resulted also in an extended survival of BCG in phagosomes. These experiments were based on earlier experiments in mice where Irgm1 (syn.: LRG-47; encoded by Irgm1), the murine homologue of IRGM, was critically involved through induction of autophagy in the control of several intracellular pathogens, including *M. tuberculosis*. IRGM (syn: LRG47, IFI1) is encoded by the immunity-related GTPase protein family, M gene (*IRGM*; 5q33.1; OMIM *608212).* IRGM consists of a long first exon encoding 181 amino acids, and four shorter putative exons that span more than 50 kilobases (kb) downstream of the first exon [7]. Most recent evidence indicates that the *IRGM* gene was deactivated during evolution but has regained its function after the insertion of a retroviral ERV9 segment and an Alu repeat sequence (Figure 1; [8]). Genetic variants of *IRGM*, including a 20.1 kilo-base (kb) upstream deletion polymorphism, have been found to confer in Caucasians an increased risk of developing Crohn’s disease, whereby coding-sequence variation has been excluded to be the liable source of the association [9–11].

The phylogenetic tree of the *M. tuberculosis* complex contains two independent clades. Clade 1 represents all *M. tuberculosis sensu stricta* lineages which are exclusively pathogenic for humans, while clade 2 comprises lineages that are pathogenic for both humans and animals (*M. africanum, M. bovis*) [12]. A major branch of *M. tuberculosis sensu stricta* is the *M. tuberculosis* Euro-American (EUAM) lineage that recently was shown to be prevalent and cause significant infections in West Africa as well [13]. While *M. tuberculosis* lineages occur throughout the world, *M. africanum* strains are almost exclusively restricted to West Africa [12,14], suggesting the existence of factors favoring spread and preservation of *M. africanum* in West Africa.

Based on the *in vitro* evidence on the role of IRGM and autophagy in experimental *M. tuberculosis* infections of murine and human cells [3,4] we hypothesized that naturally occurring *IRGM* variants, including the large upstream 20.1 kb deletion, might also be relevant to the phenotype of *in vivo* human pulmonary tuberculosis (TB). We have, in a case-control design, re-sequenced fragments of the *IRGM* gene and genotyped distinct genetic variants. An influence exerted by these variants on susceptibility or resistance to TB should be reflected in a large sample of active sputum-positive cases with pulmonary TB that we collected in Ghana, West Africa, and compared it to a control group of notable size.

**Results**

Power of the association study, Hardy-Weinberg equilibrium

A power of >90% of detection was achieved for multiplicative and additive models, assuming an approximative TB prevalence of 0.004 in West Africa, a frequency of 0.1 for high risk alleles, and a genotype relative risk of 1.3 (*p* = 0.03) with our sample size (case-control ratio = 1.18).

All variants tested were in HWE in cases and controls except IRGM alleles IRGM −261T and IRGM −71, where the distribution of alleles was in HWE among cases, but deviated in controls. The deviation could be traced to the subgroup of controls of Ewe ethnic background. As IRGM −261T and IRGM −71 are in LD but were determined by independent genotyping assays, a genotyping failure appears to be highly unlikely. The most plausible explanation is that the deviation results from the low number of Ewe controls (5%). Calculation exclusion of this subgroup from statistical analyses did not affect the significance of our results.

As data on the frequency of the *IRGM* −261T variant in other ethnic groups are not available so far, we have typed this variant also in a small panel of 47 healthy Caucasian subjects. The genotypes CC, CT and TT were observed at frequencies of 0.81, 0.15 and 0.04, respectively, versus frequencies of 0.44, 0.43 and 0.13 in the Ghanaian study population. Although not representative due to the low number of Caucasian individuals and to the bias imposed by a non-randomly selected African study group, the data suggests that the *IRGM* −261T allele occurs by far more frequently among West Africans.

**Novel IRGM variants and genotypes identified by re-sequencing**

Re-sequencing revealed, in addition to previously recognized polymorphisms, ten yet unidentified *IRGM* variants which were submitted to the NCBI dbSNP database (Table 1). The positions and preliminary NCBI rs numbers of the novel variants are as follows: IRGM −908A>C (rs105106760), −797C>T (rs105106761), −420C>T (rs105106763), −284G>A (rs105106764), 281C>A (rs105106765), 370A>G (rs105106766), *IRGM* intron +2T>C (rs105106767), intron +106T>C (rs105106768) and a deletion of two bases at positions −386/−387delAG (rs105106769). A tetrancleotide polymorphism (rs68000371; repeats starting at nucleotide position −308) was newly recognized to appear as triple repeat. This variant is located at the 3′-end of the Alu segment [Figure 1; [13]]. Potential accumulation of transcription factor binding sites (FOXP3, GR, PR A/PR B) may occur, depending on the number of tetrancleotide repeats. All novel variants were confirmed by forward and reverse DNA sequencing and were observed in at least two different DNA samples. The *IRGM* genotypes identified by re-sequencing of 69 DNA samples are given in Table 1.

**Mycobacterial genotypes**

Out of the total of 1567 isolates that were obtained and characterized, 1029 (65.7%) were *M. tuberculosis* Euro-American

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**Author Summary**

Autophagy is a process in which cell components are degraded by the lysosomal machinery. It has recently been described that activation of autophagy reduces the viability of *M. tuberculosis* in phagosomes due to an intimate autophagy-phagocytosis interaction. *M. tuberculosis* may also be directly accessible to autophagy, as *M. tuberculosis* was found to translocate into the cytoplasm. The immunity-related GTPase protein family, M gene (*IRGM*; 5q33.1; OMIM *608212).* IRGM consists of a long first exon encoding 181 amino acids, and four shorter putative exons that span more than 50 kilobases (kb) downstream of the first exon [7]. Most recent evidence indicates that the *IRGM* gene was deactivated during evolution but has regained its function after the insertion of a retroviral ERV9 segment and an Alu repeat sequence (Figure 1; [8]). Genetic variants of *IRGM*, including a 20.1 kilo-base (kb) upstream deletion polymorphism, have been found to confer in Caucasians an increased risk of developing Crohn’s disease, whereby coding-sequence variation has been excluded to be the liable source of the association [9–11].

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Based on the *in vitro* evidence on the role of IRGM and autophagy in experimental *M. tuberculosis* infections of murine and human cells [3,4] we hypothesized that naturally occurring *IRGM* variants, including the large upstream 20.1 kb deletion, might also be relevant to the phenotype of *in vivo* human pulmonary tuberculosis (TB). We have, in a case-control design, re-sequenced fragments of the *IRGM* gene and genotyped distinct genetic variants. An influence exerted by these variants on susceptibility or resistance to TB should be reflected in a large sample of active sputum-positive cases with pulmonary TB that we collected in Ghana, West Africa, and compared it to a control group of notable size.
(EUAM) strains (pks1/15 7 base-pair [bp] deletion), 472 (30.1%) were M. africanum, and 10 (0.6%) were M. bovis (the latter two lineages exhibiting the RD9 deletion). Fifty-six (3.6%) isolates belonged to the M. tuberculosis East-African-Indian (EAI), Beijing or Delhi lineages.

Allele, genotype and haplotype associations

Genotyping of eight IRGM variants was performed in 2010 HIV-negative TB cases and 2346 controls. The genotyping detection rate was >94% for all variants.

In the entire study group, no allelic or genotypic association with disease or resistance was observed. A trend for an association with resistance to TB was, however, seen for the distribution of the IRGM −261TT genotype (OR 0.79, CI 0.65–0.96, uncorrected nominal P value [Pnom] 0.017; Table 2). Stratification for the two major phylogenetic mycobacterial clades, M. tuberculosis sensu strictu and M. africanum/M. bovis, revealed a significant difference in the distribution of the IRGM −261 genotype among cases and controls. IRGM −261TT was significantly associated with protection from TB caused by M. tuberculosis, but not by M. africanum/M. bovis (OR 0.66, CI 0.52–0.84, Pnom 0.0009, Pcorr 0.0045 vs. OR 0.95, CI 0.70–1.30, Pnom 0.8). The association was confirmed in additive and recessive statistical models (ORadd 0.86, CI 0.77–0.95, Pcorr 0.023 and ORrec 0.68, CI 0.53–0.83, Pcorr 0.005, respectively; Table 3). Further stratification with respect to mycobacterial genotypes revealed that the association of the −261TT genotype applied exclusively to carriers of the M. tuberculosis Euro-American (EUAM) genotype, but not to individuals infected with M. tuberculosis East-African-Indian (EAI), Beijing or Delhi genotypes (OR 0.63, CI 0.49–0.81, Pnom 0.0004, Pcorr 0.0019 vs. OR 1.20, CI 0.57–2.52, P nom 0.6). Again, the association was substantiated in the additive and recessive models (ORadd 0.85, CI 0.76–0.95, Pcorr 0.0017, respectively; Table 4). No association was observed for carriers of the heterozygous IRGM −261CT genotype (OR 0.96, CI 0.82–1.13, P 0.6).

For the low numbers of patients infected with mycobacteria of the EAI/Beijing/Delhi group, the statistical power might not allow to obtain significant results and a possible association could be easily overlooked. To rule out this possibility statistically, a supplementary test of interaction was performed [16]. This test permitted to verify complete independence of ORs and validation of the exclusive liability of the M. tuberculosis EUAM genotype for the observed association. Cluster analyses of cases were done as previously described [17]. The distribution of the two variants at IRGM position −261 among and within clusters of cases did not differ significantly. This applied also when stratifications for ethnicity were performed.

Haplotypes and linkage disequilibria (LD) were reconstructed with the UNPHASED and Haploview softwares, respectively (Table 5, Figure 1). The corrected global P value of 0.017 for haplotypes after 1000 permutations comprising all variants that

Figure 1. Schematic structure of the IRGM gene. A) Gene segments with lengths and all SNPs that are recognized so far are given. SNPs associated with Crohn’s Disease, SNPs genotyped in the present study and the SNP associated with protection from TB caused by the EUAM-lineage of M. tuberculosis are indicated. The nucleotide positions of SNPs indicated by rs/ss numbers are listed in Table 1. B) Estimates of pairwise linkage disequilibria (LD) between IRGM variants with the LD measure of r² assuming Hardy-Weinberg equilibrium. C) Potential loss of transcription factor binding sites (ARNRT, PAX5, AHR) in sequences carrying the IRGM −261T allele. D) Potential accumulation of transcription factor binding sites (FOXP3, GR, PR A/PR B), depending on the number of tetranucleotide repeats (rs60800371; [15]).

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Table 1. IRGM variants and minor allele frequencies identified by re-sequencing.

| Localization | rs/-ss number | Substitution | MAF  

| S'-UTR  | -964 | rs10059911 | A>C | 0.46 (A) |
| S'-UTR  | -908* | rs105106760 | A>C | 0.01 (C) |
| intron  | -844 | rs10052068 | T>C | 0.23 (C) |
| intron  | -797* | rs105106761 | C>T | 0.06 (T) |
| intron  | -787 | rs17111376 | T>C | 0.5 |
| intron  | -566 | rs17111339 | G>C | 0.21 (C) |
| intron  | -420* | rs105106763 | C>T | 0.06 (T) |
| S'-UTR  | -386/387* | rs105106769 | AG>del | 0.04 (del) |
| Alu sequence  | -3087* | rs60800371 | 1, 3 or 4 'GGTT' |
| Alu sequence  | -284* | rs105106764 | G>A | 0.04 (A) |
| Alu sequence  | -261 | rs9637876 | C>T | 0.31 (T) |
| Alu sequence  | -71 | rs9637870 | C>T | 0.31 (A) |
| exon 1  | 281* | rs105106765 | C>A | 0.01 (C) |
| exon 1  | 313 | rs10056172 | C>T | 0.49 (T) |
| exon 1  | 370* | rs105106766 | A>G | 0.01 (G) |
| Intron  | +2* | rs105106767 | C>T | 0.01 (C) |
| Intron  | +87 | rs7705542 | G>A | 0.49 (G) |
| Intron  | +106* | rs105106768 | T>C | 0.01 (C) |

Positions refer to the reference sequence and are given relative to the ATG start codon (reference sequence: chromosome 5 contig, GenBank accession number NW_922784.1, region 23935681…23938058; contains at position –308 one ‘GGTT’ motif). MAF, minor allele frequency; * novel variant identified in the present study population; , microsatellite repeat of 1, 3 or 4 ‘GGTT’ tandem motifs.

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were tested indicated that the observed association might apply also to a distinct haplotypic combination. Detailed analyses including 1000 permutations for each combination showed that the IRGM haplotype 20kdel_DEL/−4299G/−566G/−420C/ rep4/−261T/−71A/313T was associated with resistance to TB caused by EUAM strains (OR 0.83, CI 0.73–0.93, P 0.001; Table 5), albeit the haplotype was not found to exert a stronger effect than the −261TT variant alone when occurring homozgyously (Tables 4 and 5). This underscores the significance of the IRGM −261TT genotype in protection from human pulmonary tuberculosis caused by the species M. tuberculosis.

Reporter gene assay

A two-tailed student’s t-test revealed a significant difference in the normally distributed ratio of firefly:Renilla luciferase activity of five independent transfections per IRGM variant in the luciferase reporter gene assay, indicating increased gene expression in cells transfected with pGL3-Control Vector carrying the mutant variant IRGM-261T than in cells transfected with the pGL3-Control Vector with the IRGM wild-type variant (−261C) (P = 0.013) (Figure 2).

Discussion

We found in a large Ghanaian study group of HIV-negative patients with pulmonary TB and healthy control individuals that a distinct IRGM genotype, IRGM −261TT, was as a trend associated with decreased susceptibility to TB. After stratification for the two major mycobacterial clades and molecular subtypes the trend observed in the entire study group could be traced back to an association of IRGM −261TT with tuberculosis exclusively when caused by the M. tuberculosis EUAM lineage. No association was observed in comparisons of cases caused by M. tuberculosis EAI, Beijing, Delhi and M. africanum/M. bovis with controls. The frequencies of the synonymous variant 313T>C, the variant at position −4299 and of the 20.1 kb deletion, all found to be associated with Crohn’s disease in other studies [9–11], did not differ significantly between TB patients and controls.

Upon infection of macrophages, M. tuberculosis initially resides in phagosomes. During their normal maturation, phagosomes fuse with lysosomes and establish a hostile environment for the pathogen, characterized by lysosomal enzymes, acid pH, reactive oxygen and nitrogen intermediates, and toxic peptides. Most macrophage-mediated killing of intracellular pathogens occurs within the phagolysosome and pathogens have developed several mechanisms to avoid this vacuolar attack by escape and multiplication in the cytoplasm, inhibition of phagosome-lysosome fusion or circumventing the common endocytic pathway (reviewed in [18]). With regard to M. tuberculosis H37Rv, translocation of the bacteria from phagosomes to cytosolic compartments has been observed to occur in nonapoptotic cells [19]. Notably, escape into the cytosol has also been observed of the M. tuberculosis strain CDC1551 in laboratory infections of the amoeba Dictyostelium discoideum [20] and loss of phagosomal membranes with release of mycobacteria has been observed in an in vitro system five days post infection [21].

Autophagy induced by IRGM can efficiently interfere with cytosolic replication of pathogens by trapping and recapture and subsequent degradation of bacteria [22,23]. In the LC3 dependent activation pathway, induction of autophagy contributes not only to the degradation of cytosolic components, but also to the maturation of mycobacterial phagosomes [3,4]. While M. tuberculosis can impair phagosome maturation by blocking the normal PI3P dependent pathway, this effect is restored and outbalanced by the alternate activation mode triggered by LC3. This is consistent with the finding in mice and in human U937 cells that murine Irgm1 and human IRGM, respectively, play an important role in the containment of M. tuberculosis through efficient induction of autophagy [4].

The polymorphism that is associated with relative resistance to TB, IRGM −261T, in particular when occurring homozygously as TT genotype, might enhance expression of the mature IRGM protein which triggers autophagic degradation of translocated bacteria. The transcription factor AHR is expressed in the monocytic cell line THP1 that we used in our transcription experiments and inhibits differentiation of monocytes in vitro [24,25]. This suggests that an unaffected AHR/ARNT transcription factor complex is likely to occur in the IRGM wild-type gene promoter, may decrease innate immune responses mediated by macrophages. Inversely, and based on our luciferase reporter gene assays, the loss of the potential PAX5, AHR and ARNT transcription factor binding sites predicted for the IRGM variant −261T upstream of the coding region is likely to contribute to increased IRGM gene expression and, thus, enhanced innate responses. The fact that IRGM is not inducible by IFN-γ as are Irg genes in mice [4,7,26] but initiates successfully autophagy as does IFN-γ, argues for additional resistance mechanisms in individuals carrying the IRGM −261T variant homozygously, independent of other Th1-mediated immune responses.

The association that we observed was restricted to infections caused by the M. tuberculosis EUAM lineage. The major characteristic of that lineage is the pks11/15 7b deletion which does not occur in M. africanum, M. bovis and in the other M. tuberculosis lineages identified in our study, EAI, Beijing, and Delhi.
### Table 2. *IRGM* genotype associations.

| GT       | Cases (%) | Controls (%) | OR   | CI    | P nom | P corr | ORadd CI | P nom | P corr | ORrec CI | P nom | P corr |
|----------|-----------|--------------|------|-------|-------|--------|----------|-------|--------|----------|-------|--------|
| All genotypes |           |              |      |       |       |        |          |       |        |          |       |        |
| deletion  |           |              |      |       |       |        |          |       |        |          |       |        |
| WT/WT    | 484 (24.7)| 577 (25.2)   | 1    |       | 0.97  | [0.89–1.05] | 0.4    | 0.90  | [0.78–1.04] | 0.2 |
| WT/DEL   | 1009 (51.5)| 1131 (49.4) | 1.05 | [0.90–1.22] | 0.524 |       |          |       |        |          |       |        |
| DEL/DEL  | 466 (23.8)| 584 (25.5)   | 0.93 | [0.78–1.11] | 0.430 |       |          |       |        |          |       |        |
| −4299    |           |              |      |       |       |        |          |       |        |          |       |        |
| CC       | 545 (27.8)| 677 (29.9)   | 1    |       | 1.05  | [0.97–1.15] | 0.2    | 1.03  | [0.89–1.19] | 0.7 |
| CT       | 989 (50.4)| 1104 (48.8) | 1.11 | [0.97–1.28] | 0.14 |       |          |       |        |          |       |        |
| TT       | 429 (21.9)| 484 (21.4)   | 1.10 | [0.93–1.31] | 0.3  |       |          |       |        |          |       |        |
| −566     |           |              |      |       |       |        |          |       |        |          |       |        |
| CC       | 117 (6.0)| 118 (5.2)    | 1    |       | 0.99  | [0.89–1.09] | 0.8    | 1.01  | [0.89–1.14] | 0.8 |
| CG       | 703 (36.1)| 855 (37.3)   | 0.84 | [0.64–1.11] | 0.2  |       |          |       |        |          |       |        |
| GG       | 1125 (57.8)| 1318 (57.5) | 0.87 | [0.66–1.14] | 0.3  |       |          |       |        |          |       |        |
| −261     |           |              |      |       |       |        |          |       |        |          |       |        |
| CC       | 901 (45.2)| 1011 (43.5) | 1    |       | 0.91  | [0.84–0.99] | 0.04   | 0.80  | [0.67–0.96] | 0.015 |
| CT       | 862 (43.3)| 988 (42.5)   | 0.98 | [0.86–1.11] | 0.7  |       |          |       |        |          |       |        |
| TT       | 229 (11.5)| 324 (14.0)   | 0.79 | [0.65–0.96] | 0.017 |       |          |       |        |          |       |        |
| −71      |           |              |      |       |       |        |          |       |        |          |       |        |
| GG       | 372 (39.0)| 856 (38.9)   | 1    |       | 0.97  | [0.89–1.06] | 0.5    | 0.85  | [0.71–1.01] | 0.07 |
| AG       | 465 (48.7)| 999 (45.4)   | 1.08 | [0.94–1.23] | 0.290 |       |          |       |        |          |       |        |
| AA       | 118 (12.4)| 347 (15.8)   | 0.89 | [0.73–1.07] | 0.215 |       |          |       |        |          |       |        |
| 513      |           |              |      |       |       |        |          |       |        |          |       |        |
| TT       | 280 (27.9)| 675 (29.2)   | 1    |       | 1.04  | [0.95–1.13] | 0.4    | 1.01  | [0.87–1.17] | 0.9 |
| TC       | 506 (50.4)| 1144 (49.5) | 1.09 | [0.94–1.25] | 0.2  |       |          |       |        |          |       |        |
| CC       | 218 (21.7)| 491 (21.3)   | 1.07 | [0.90–1.27] | 0.5  |       |          |       |        |          |       |        |

GT, genotype; OR, odds ratio; CI, 95% confidence interval; ORadd, estimates of an additive genetic model; ORrec, estimates of a recessive genetic model; Pnom, nominal P value; Pcorr, P value after Bonferroni correction (correction factor = 5); WT, wild-type, no deletion; DEL, 20.1 kb deletion; nc, not calculable.

*Number of tandem repeats given in digits.

**homozygous 4/4 genotype compared to all other combinations.

P values are adjusted for age, gender and ethnicity.

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### Table 3. *IRGM* −261 genotype associations after stratification for the two major groups of *M. tuberculosis* and *M. africanum/M. bovis*.

| GT       | Cases (%) | Controls (%) | OR   | CI    | P nom | P corr | ORadd CI | P nom | P corr | ORrec CI | P nom | P corr |
|----------|-----------|--------------|------|-------|-------|--------|----------|-------|--------|----------|-------|--------|
| *M. tuberculosis* |           |              |      |       |       |        |          |       |        |          |       |        |
| −261     |           |              |      |       |       |        |          |       |        |          |       |        |
| CC       | 502 (46.8)| 1011 (43.5) | 1    |       | 0.86  | [0.77–0.95] | 0.005 | 0.025 | 0.68 [0.53–0.85] | 0.001 | 0.005 |
| CT       | 464 (43.3)| 988 (42.5)   | 0.95 | [0.82–1.11] | 0.6  |       |          |       |        |          |       |        |
| TT       | 106 (9.9)| 324 (14.0)   | 0.66 | [0.52–0.84] | 0.0009 | 0.0045 |          |       |        |          |       |        |
| *M. africanum/M. bovis* |           |              |      |       |       |        |          |       |        |          |       |        |
| −261     |           |              |      |       |       |        |          |       |        |          |       |        |
| CC       | 204 (42.5)| 1011 (43.5) | 1    |       | 1.00  | [0.87–1.15] | 0.99  | 0.99  | [0.89–1.10] | 0.9 |
| CT       | 214 (44.6)| 988 (42.5)   | 1.07 | [0.86–1.32] | 0.5  |       |          |       |        |          |       |        |
| TT       | 62 (12.9)| 324 (14.0)   | 0.95 | [0.70–1.30] | 0.8  |       |          |       |        |          |       |        |

GT, genotype; OR, odds ratio; CI, 95% confidence interval; ORadd, estimates of an additive genetic model; ORrec, estimates of a recessive genetic model; Pnom, nominal P value; Pcorr, P value after Bonferroni correction (correction factor = 5). P values are adjusted for age, gender and ethnicity.

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Why relative protection by IRGM−261TT is exclusively provided against disease caused by the lineage that exhibits the pks1/15 deletion remains to be explained further. One may hypothesize that absence or presence of the pks1/15 deletion may contribute to a modulation of the pathogenic potential of infecting strains in different ethnicities, leading to an adaption of mycobacterial lineages to their sympatric host population. Inhibition of innate immune responses and a tendency of increased spread of bacteria by phenolic glycolipid TB (PGL-tb), the product of undamaged pks1/15 gene, has been demonstrated [27,28]. It is, therefore, reasonable to assume that lineages which do not produce PGL-tb as a suppressor of the innate immune response are more susceptible to IRGM-triggered innate immune mechanisms, namely phagosome maturation and autophagy. However, a more recent report indicates that PGL-tb itself does not confer hypervirulence, but rather differentially may modulate early cytokine response of the host [29]. As such, variable PGL-tb levels in conjunction with other yet inadequately defined mycobacterial virulence factors might result in a lineage-specific immunogenicity and/or degree of virulence that is related to the occurrence of human genetic variants in a particular population (14,29). Since translocation of bacteria from the phagosome has only been observed for M. tuberculosis H37rv and M. leprae, but not for M. bovis BCG [19], and was not tested for other lineages it is also conceivable that distinct pathogenic strains are subjected to mechanisms preventing translocation to cytosolic compartments and allowing escape from autophagy, a hypothesis that awaits further substantiation. This would support the view of increased virulence of lineages carrying intact pks1/15 genes such as M. tuberculosis Bejing and other lineages, but also underline the equivalent pathogenic potential of M. africanum compared to M. tuberculosis as has been demonstrated [6].

| Table 4. IRGM −261 genotype associations after stratification for strains positive and negative for the mycobacterial pks1/15 deletion. |
|GT| Cases (%)| Controls (%)| OR| CI| Pnom| Pcorr| ORadd| CI| Pnom| Pcorr| ORrec| CI| Pnom| Pcorr|
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|M. tuberculosis EUAM|−261|CC|476 (46.9)|1011 (43.5)|1|0.85 [0.76–0.95]|0.003|0.016|0.64 [0.50–0.82]|0.0003|0.0017|
|CT|444 (43.7)|988 (42.5)|0.96 [0.82–1.13]|0.6|
|TT|96 (9.5)|324 (14.0)|0.63 [0.49–0.81]|0.0004|0.0019|
|M. tuberculosis EAI/Beijing/Delhi|−261|CC|26 (46.4)|1011 (43.5)|1|1.02 [0.70–1.49]|0.93|0.056|1.35 [0.67–2.70]|0.4|
|CT|20 (35.7)|988 (42.5)|0.78 [0.43–1.41]|0.4|
|TT|10 (17.9)|324 (14.0)|1.20 [0.57–2.52]|0.6|

GT, genotype; OR, odds ratio; CI, 95% confidence interval; ORadd, estimates of an additive genetic model; ORrec, estimates of a recessive genetic model; Pnom nominal P value; Pcorr, P value after Bonferroni correction (correction factor = 5). EUAM, Euro-American lineages of Mycobacterium tuberculosis; EAI, East-African-Indian lineages of Mycobacterium tuberculosis. P values are adjusted for age, gender and ethnicity. doi:10.1371/journal.ppat.1000577.t004

| Table 5. IRGM haplotype associations (8 polymorphisms). |
|---|---|---|---|---|---|---|---|---|---|
|Haplotype| GT| n (%)| OR| CI| Pnom|
|---|---|---|---|---|---|
|Del−4299|65 (1.5)|1.06 [0.66–1.70]|0.8|
|Del−566|97 (21.9)|1.16 [0.80–1.69]|0.4|
|Del−420|50 (1.1)|0.98 [0.54–1.78]|0.95|
|Del−261|1009 (22.8)|1.05 [0.92–1.20]|0.4|
|Del−71|39 (0.9)|1.93 [1.14–3.27]|0.01|
|Del−313|784 (17.7)|1.00 [0.87–1.16]|0.96|
|Del−340|102 (2.3)|0.84 [0.57–1.23]|0.3|
|Del−37|52 (1.2)|1.25 [0.78–2.01]|0.4|
|Del−25|37 (0.8)|1.38 [0.75–2.53]|0.3|
|Del−264|553 (12.5)|1.10 [0.93–1.29]|0.3|
|Del−554|1459 (32.9)|0.83 [0.73–0.93]|0.001|
|Del−94|176 (3.9)|1.26 [0.97–1.63]|0.09|

EUAM, Euro-American lineages of Mycobacterium tuberculosis; del, deletion; rep, repeat (number of tetranucleotide repeats given in digits; see also Figure 1); OR, odds ratio; CI, 95% confidence interval; Pnom, nominal P value; WT, wild-type; DEL, 20.1 kb deletion. P values refer to comparisons of one haplotype to all other combinations and are adjusted for age, gender and ethnicity. Corrected global P value in cases caused by EUAM infections after 10000 permutations = 0.02. Missing genotypes are compensated for by simulations of all possible completions of the data. doi:10.1371/journal.ppat.1000577.t005
observed in our study group [17]. It is intriguing to speculate that the high prevalence of IRGM −261TT in our Ghanaian study population and the relative protection that it confers from TB caused by M. tuberculosis EUAM might reflect one of multiple exclusive propagation of M. africanum in West Africa, an assumption to be verified in other data sets of mycobacterial and human genotypes which are not yet available. The phylogeography of mycobacteria implies that lineages have become differentially adapted to different ethnicities with allelic variations conferring traits associated with certain infection phenotypes [14].

While the association of the IRGM polymorphism that we found cannot unquestionably confirm the role of IRGM in tuberculosis, it adds evidence to the in vivo experiments in mice and human cells [3,4] and supports the relevance of autophagy in the control of tuberculosis. Unfortunately, genetic replication data on the role of human IRGM polymorphism in infections caused by M. tuberculosis subtypes that have unambiguously been determined by mycobacterial genotyping are not available so far, neither for Caucasian nor for African or Asian study groups. Data of functional experiments on the effect of IRGM in tuberculosis caused by strains that produce PGL-tb are not available. The inhibition of the innate immune mediators TNF-alpha and other interleukins by PGL-tb has been described earlier [26]. A corresponding inhibition of IRGM would explain our findings and contribute to understanding the role of PGL-tb as a factor of virulence and modulator of innate immune responses.

Materials and Methods

Ethics statement

The study protocol was approved by the Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University, Kumasi, and the Ethics Committee of the Ghana Health Service, Accra. Patients were treated according to the “Directly Observed Treatment, Short-course” (DOTS) strategy organized by the Ghanaian National Tuberculosis Programme. Blood samples for genetic analyses and HIV testing were taken only after a detailed explanation of the study aims and written or thumb-printed consent for participation provided, including HIV testing.

Patients and controls

Study participants were recruited in Ghana, West Africa, between September 2001 and July 2004. The recruitment area and the enrollment procedure have previously been described [17,30]. Patients were enrolled at the two Teaching Hospitals in Accra and Kumasi and at additional hospitals or policlinics in Accra, Tema, Kumasi, Obuasi, Agona, Mampang, Agogo, Konongo and Nkwie (Ashanti Region), Kwakwaw and Anbie (Eastern Region), and Asin Fosu and Dunkwa (Central Region). Characterization of patients included i) the documentation of the medical history on standardized structured questionnaires, including self-reported duration of cough and symptoms of TB (dyspnea, chest pain, night sweats, fever, hemoptysis, weight loss), ii) two independent examinations of non-induced sputum specimens for acid-fast bacilli, iii) serological determination of the HIV status and confirmation of positive results by an alternative test system, iv) cultivating and molecular differentiation of phylogenetic mycobacterial lineages, and v) a posterior-anterior chest radiography. Inclusion criteria were two sputum smears positive for acid-fast bacilli, no history of previous TB or anti-mycobacterial treatment and an age between 6 and 60 years. Two sputum smears were examined in order to corroborate and confirm the phenotype. Patients were also included if only one smear and the culture for mycobacterial growth was positive. Exclusion criteria were incomplete information provided on the questionnaire, HIV positivity, evidence of alcoholism, drug addiction and other apparent generalized disease. A total of 2010 patients fulfilling the criteria for participation were enrolled.

Unrelated personal contacts of cases and community members from neighboring houses of cases and public assemblies were recruited as controls. The leading criterion for enrollment as a control was no history of TB or previous anti-mycobacterial treatment. Characterization of controls included a medical history, posterior-anterior chest X-ray radiography and a tuberculin skin test (Tuberculin Test PPD Mérieux, bioMérieux, Nürtingen, Germany). 1211 personal contacts and 1135 community members fulfilled the criteria for participation and were available as controls.

Study participants belonged to the following ethnic groups (cases/controls): Akan including Ashanti, Fante, Akuapem (63.6%/59.1%), Ga-Adangbe (14.5%/19.8%), Ewe (7.1%/9.3%) and ethnic groups of northern Ghana including Dagomba, Sissala, Gonja, and Kusasi (12.9%/10.4%). The proportions of ethnicities among patients and controls did not differ significantly.

Disclosure of HIV test results was dependent on the documented willingness of participants to be informed and included for HIV-positive patients their prompt referral to counseling and treatment provided by the Ghanaian AIDS Control Programme.

Typing of mycobacterial isolates

The firm diagnosis of pulmonary TB was made as described previously [13,17,30]. M. tuberculosis complex isolates were cultured on Lowenstein-Jensen (LJ) media and shipped to the German National Reference Centre for Mycobacteria (Borstel, Germany) for minute analyses of biochemical, growth and molecular
characteristics. Molecular differentiation of 1567 mycobacterial isolates included spoligotyping, IS6110 fingerprinting and typing of the pks1/15 deletion as described previously [31–33]. Mycobacterial strains were further stratification grouped according to the major phylogenetic lineages [12].

The stepwise procedure of typing of mycobacteria included an initial cluster analysis of IS6110 fingerprinting data and lineage identification according to specific spoligotype signatures. Assignment of lineages was based on the MIRU-VNTRplus webpage (www.miru-vntrplus.org) [34] and a reference strain collection using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Classification was confirmed by random selection of 20 strains of each group and testing for the presence of lineage-specific deletions as follows: pks1/15 for M. tuberculosis Euro-American, region of difference (RD) 239 for M. tuberculosis East African Indian (EAI), RD9 and RD711 for M. africanum West African 1, RD9 and RD702 for M. africanum West African 2, and RD9 and RD4 for M. bovis. All M. tuberculosis strains with mycobacterial strains exhibiting the same IS6110 fingerprinting data and lineage identification according to specific spoligotype signatures. Assignment of lineages was based on the MIRU-VNTRplus webpage (www.miru-vntrplus.org) [34] and a reference strain collection using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Classification was confirmed by random selection of 20 strains of each group and testing for the presence of lineage-specific deletions as follows: pks1/15 for M. tuberculosis Euro-American, region of difference (RD) 239 for M. tuberculosis East African Indian (EAI), RD9 and RD711 for M. africanum West African 1, RD9 and RD702 for M. africanum West African 2, and RD9 and RD4 for M. bovis. All M. tuberculosis strains were for further stratification grouped according to the major phylogenetic lineages [12].

Mycobacterial strains were for further stratification grouped according to specific spoligotype signatures. Assign-
ethnicity of Yoruba (Caucasians: G 1.0; Yoruba, originating from Nigeria: G 0.74, C 0.26; www.hapmap.org/cgi-perl/snp_details?name/rs17111379&source=hapmap_B35). The variant at position −420C/T (rs105106765) was included as it was identified as a novel variant in our study population and was not in strong linkage with other polymorphisms. The IRGM variant −261 (rs9637876) was selected for genotyping, because, according to an in silico prediction of transcription factor binding properties, the allele causes loss of several binding sites (PAX3, ARNT, AHR; http://alleggen.bsi.ups.es/recerca/menu_recreca.html). IRGM −71 (rs9637870) and the microsatellite repeat (rs60800371; repeats starting at position −308) are in LD with IRGM −261 and were included in genotyping to more reliably identify a potentially causative variant. The synonymous exonic variant 313C/T was not subjected to genotyping, as they were either in perfect LD with IRGM −261 or away from the region of deletion. The assay enabled the determination of IRGM −71, −261 and −308. Enzymatic digestions and ligations were performed according to the instructions of the manufacturer. Small-scale preparations were done applying the NucleoSpin Plasmid Kit (Macherey and Nagel, Düren, Germany). Plasmid DNAs were propagated in E. coli XL1-Blue cells (Stratagene, La Jolla, CA, USA) and prepared using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). DNA sequences of the final constructs were confirmed by sequencing.

1 × 10^6 cells of the human monocytic cell line THP1 (German Resource Centre for Biological Material, DMSZ, Braunschweig, Germany) were transfected with either 0.5 μg of the two plasmid constructs, with the addition of 0.5 μg of the plasmid phRL-CMV which contains the gene encoding Renilla luciferase (Promega, Mannheim, Germany) in order to normalize results. Transfection with 0.5 μg of the unmodified pGL3-Control Vector and 0.5 μg phRL-CMV, as well as a mock transfection, were performed for control and to minimize effects of reagents on the cells. All transfections were performed with the Amma Cell Line Nucleofector Kit V (Lonza, Cologne, Germany). Four hours after transfection, cells were harvested and luciferase activities were measured (Dual-Luciferase Reporter Assay System; Promega, Mannheim, Germany). Firefly luminescence was measured using a single tube Junior LB9509 luminometer (Berthold Technologies, Bad Wildbad, Germany). After a 10 second measurement period, 100 μl 1 x Stop & Glo Reagent were added for the detection of Renilla luminescence. Measurements of luminescence are expressed as relative light units (RLU). For each variant, five independent transfections were performed.

Databases, statistics

Demographic data, self-reported signs and symptoms as documented on structured questionnaires as well as laboratory results were double-entered into a Fourth Dimension database (San Jose, CA, USA). Bacteriological data were provided as Excel datasheets. Data were locked before using them in a pseudonymized form for statistical analyses. Power calculations were performed with the public CATS software (http://www.sph.umich.edu/csg/abecasis/CaTS/).

Multivariate logistic regression analyses were calculated for different models to determine odds ratios (OR) for allele and genotype distributions (STATA 10.0MP software; Stata Corporation, College Station, TX, USA). As age, sex and ethnicity were significant confounders, they were appropriately adjusted for. Analyses of allele distributions and Hardy-Weinberg equilibria (HWE) were calculated with a public STATA module (www-genome.cimr.cam.ac.uk/clayton/software/stata/; David Clayton, Cambridge, UK). Haplotypes were estimated with the UNPHASED software (version 3.0.13; Frank Dudbridge, http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/), whereby incomplete haplotypes were subjected to simulations comprising all possible haplotypic completions of available data. In order to verify the independence of ORs that were obtained for associations of different mycobacterial clades/genotypes with phenotypes, ORs were subjected to tests of interaction according to the method described in [16].

Corrections of nominal P values (Pnom) for multiple testing applied to the number of variants tested and when stratifications by mycobacterial lineages were made. Bonferroni corrected P values (Pcorr) < 0.05 were considered significant and correction values are indicated where applicable.

The tetranucleotide repeat rs96080371 and SNPs at positions −261 (rs9637876) and −71 (rs9637870) are in strong LD (r^2 = 0.8) and were, therefore, combined as a single correction entity. Global P values of haplotype associations and of distinct haplotypes were subjected to 1000 permutations (UNPHASED software).
Shapiro-Wilk tests and a two-tailed student’s t-test were calculated to confirm a normal distribution and differences in the ratio of firefly:Renilla luciferase activity in the reporter gene assay.

Accession numbers

**Genes and genomic contig.** Immunity-related GTPase family; IRGM (NCBI BC120163.1); Homo sapiens chromosome 5 genomic contig (NCBI NW_922784.1). PGR; PR A/PR B (HGNC HGNC:8910); receptor; GR (HGNC HGNC:7978); Progesterone receptor/receptor subfamily 3, group C, member 1/glucocorticoid receptor; GR (HGNC HGNC:7978); Nuclear Transcription factor I; FOXP3 (HGNC HGNC:6106); Nuclear Transcription factor II; PAX5 (HGNC HGNC:8619); Aryl hydrocarbon receptor; AHR (HGNC HGNC:700); 17. Meyer CG, Scarisbrick G, Niemann S, Browne EN, Chinbuah MA, et al. (2008) Genotyping of IRGM tetranucleotide promoter oligorepeats by allele-specific PCR amplification and the reporter gene assays, respectively. Volker Heussler was helpful with the reporter gene assays and provided laboratory reagents.

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**Author Contributions**

Conceived and designed the experiments: TT ENLB MAC AE JOG IO EOD SRG RDH CGM. Performed the experiments: CDI TT SN SH. Analyzed the data: CDI TT SN SH. Contributed reagents/materials/analysis tools: CDI TT SN SH. Performed the experiments: MAC AE JOG IO EOD. Sample collection supervision Ghana: ENLB. Study design and phenotyping of patients and controls: ENLB MAC JOG IO EOD. Sample collection: MAC AE IO EOD. Sample collection supervision: JOG.

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