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Human ULK1 Variation and Susceptibility to *Mycobacterium tuberculosis* Infection

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Background. Unlike tuberculosis, few studies have evaluated a host genetic basis for variability in susceptibility to latent *Mycobacterium tuberculosis* infection (LTBI). We performed a candidate gene association study of autophagy-related genes and LTBI.

Methods. We enrolled close contacts of individuals with pulmonary tuberculosis, assessed LTBI status, and determined clinical and sociodemographic risk factors for LTBI. In participants who self-identified as Asian or black, we compared haplotype-tagging single-nucleotide polymorphisms (SNPs) in *ULK1* and *GABARAP* between cases (n = 143) and controls (n = 106). Using CRISPR/Cas9 in U937 monocytes, we investigated the effect of ULK1 deficiency on cytokine expression, autophagy, and *M. tuberculosis* replication.

Results. In Asian participants, we identified 2 *ULK1* SNPs (rs12297124 and rs7300908) associated with LTBI. After adjustment for population admixture and clinical risk for LTBI, each rs12297124 minor allele conferred 80% reduction in LTBI risk (odds ratio, 0.18; 95% confidence interval, .07–.46). Compared with controls, ULK1-deficient cells exhibited decreased tumor necrosis factor secretion after stimulation with Toll-like receptor ligands and *M. tuberculosis* whole-cell lysate, increased *M. tuberculosis* replication, and decreased selective autophagy.

Conclusions. These results demonstrate a strong association of rs12297124, a noncoding *ULK1* SNP, with LTBI and a role for ULK1 regulation of TNF secretion, nonspecific and *M. tuberculosis*–induced autophagy, and *M. tuberculosis* replication in monocytes.

Keywords. autophagy; candidate gene analysis; single nucleotide polymorphism; genetic susceptibility; tuberculosi.

Mycobacterium tuberculosis infects one third of the world's population and is a leading cause of infectious disease–related death [1]. Infection with *M. tuberculosis* may result in several outcomes, including immediate bacillary multiplication resulting in primary tuberculosis, latent *M. tuberculosis* infection (LTBI), or reactivation tuberculosis. The mechanisms responsible for different tuberculosis outcomes are poorly understood. Several lines of evidence support the influence of host genetics on susceptibility to tuberculosis, including twin-based, candidate gene association, linkage, and genome-wide association studies [2–4]. The vast majority of studies that have evaluated a host genetic basis for variability in tuberculosis outcomes have assessed associations with active tuberculosis. However, the direct study of tuberculosis susceptibility in humans allows for unique insights not possible in animal models, including investigations into LTBI [5].

Despite a massive global burden, the host genetic basis for LTBI susceptibility is understudied [6]. The heritability of responses to LTBI tests—the tuberculin skin test (TST) and the interferon γ (IFN-γ)–release assay—is estimated to be high [7, 8]. Genome-wide linkage studies identified several loci associated with LTBI [9–11], and candidate gene association studies identified associations between polymorphisms in the genes encoding interleukin 10 and interleukin 4 and TST responses [12, 13]. Although these studies suggest genetic regulation of LTBI susceptibility, the mechanisms remain unknown. A better understanding of the impact of host genetics on LTBI is key to understanding tuberculosis pathogenesis.

The innate immune system, in particular alveolar macrophages, likely plays an important role in determining tuberculosis outcomes following inhalation of *M. tuberculosis* into the lungs and prior to T-cell priming. We and others have identified associations between common polymorphisms in innate immunity genes and susceptibility to tuberculosis [14–16]. Much of this work has focused on pattern-recognition receptors and their associated adaptor proteins [17–19]. In recent years there has been a growth in interest and understanding of autophagy, which protects the host by targeting invading pathogens for elimination (in addition to other functions) and has a major role in host tuberculosis defenses [20–23]. Autophagy is initiated by a ULK1-containing complex under regulation

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by mTOR and AMP kinase. GABARAP regulates later stages of autophagosome maturation. A number of candidate gene studies have demonstrated associations between polymorphisms in IRGM, an autophagy gene, and tuberculosis in various populations [24–26]. However, IRGM polymorphisms were not associated with the outcome of LTBI in one study [27]. To our knowledge, associations of genetic variants in autophagy genes with LTBI have not been identified.

We hypothesized that common variation in autophagy-related genes is associated with susceptibility to LTBI following tuberculosis exposure. With few exceptions [9], studies of LTBI susceptibility and genetic variation occurred in tuberculosis-endemic regions (geographic differences may affect responses to tuberculosis treatments) [28, 29] and did not adjust for epidemiologic risk factors, potentially leading to loss of study power. We enrolled close contacts of tuberculosis cases through a US tuberculosis program to examine whether variation in autophagy genes is associated with susceptibility to *M. tuberculosis* infection. To our knowledge, this is the first reported association of a *ULK1* single-nucleotide polymorphism (SNP) with a tuberculosis-related outcome.

METHODS

Human Subjects

We recruited subjects through the Tuberculosis Control Program (TBCP), Public Health–Seattle and King County (Seattle, WA). Eligible study subjects were close contacts of patients with culture-confirmed pulmonary tuberculosis. Generally, close contact was defined as >8 hours of exposure to an index case in an enclosed space during the preceding 3 months. All participants were ≥18 years of age and without a prior history of tuberculosis. We excluded individuals who were immunocompromised, including known those with known human immunodeficiency virus (HIV) infection. HIV testing was not routinely offered to close contacts unless they received a diagnosis of active tuberculosis.

LTBI status was evaluated with tuberculin skin tests (TSTs) and/or QuantiFERON Gold-in-Tube (QFT) assays at the discretion of the TBCP. TSTs were performed using 5 tuberculin units (0.1 mL) of purified protein derivative (RT 23 solution, Sanofi-Pasteur) and read at 48–72 hours. A TST yielding an induration diameter of ≥5 mm was considered positive for LTBI [30]. The QFT assay was performed and interpreted according to manufacturer guidelines. Participants who were tested with both the TST and the QFT assay had blood collected before TST placement, and any positive result was considered evidence of LTBI. Per TBCP protocol, close contacts with a negative result of the initial test for LTBI were retested 8–10 weeks after the last tuberculosis exposure. We defined converters as participants who initially tested negative for LTBI but had a positive result on retesting.

SNP Selection

We selected 2 candidate autophagy genes, *GABARAP* and *ULK1*, and ancestry-informative markers [31]. We chose ULK1 on the basis of its central role in the initiation of autophagy and GABARAP because of its role in later stages of autophagosome maturation. Neither gene was examined in a previous study of tuberculosis genetic susceptibility [32]. Haplotype tagging SNPs were chosen using the 1000 Genomes Browser in Asian (CHB, CHS, and JPT) and African (ASW, LWK, and YRI) populations [33]. Selection criteria included a minor allele frequency of 2.5% (for *GABARAP)* or 5% (for *ULK1*), an R^2 cutoff of 0.8 for linkage disequilibrium, and a region that included the gene ± 1 kb.

Genomic Techniques

We limited genotyping to participants who self-reported as Asian or black, to maximize study efficiency, as these ethnicities are the most frequent among individuals with tuberculosis in King County, Washington, and limit confounding due to population stratification. Included cases and controls were unrelated. Genomic DNA was prepared from saliva (Oragene Discover, DNA Genotek). By use of Illumina's GoldenGate Assay and a custom multiplex oligo pool, genomic DNA samples (250 ng) underwent allele-specific primer extension and amplification. Amplified products were then hybridized to Universal BeadChips and subsequently imaged on the Illumina iScan+. Cluster plots were visually inspected to ensure accurate genotyping calls. The call rate exceeded 95% for 92% of all genotyped SNPs. Genotypes were confirmed in a subset of individuals through genotyping with the Fluidigm BioMark microfluidic system. All *GABARAP* and *ULK1* SNPs were in Hardy-Weinberg equilibrium ($P > .001$ by the exact test) based on the χ^2 goodness-of-fit test [34].

CRISPR/Cas9 Gene Editing

Guide RNA targeting *ULK1* (sequence GGACGCCTC-CATGCTCAGCG[TGG]) was selected using ChopChop (available at: https://chopchop.rc.fas.harvard.edu/) [35]. The guide RNA and scaffold were cloned into a lentiviral plasmid, gRNA-Cas9-t2a-puromycin pRRL (a gift from Elizabeth Gray, University of Washington), using Clontech InFusion Cloning Kit (Takara Bio). Nontargeting control gRNA-CAs9-t2a-puromycin pRRL plasmids (a gift from Elizabeth Gray) were used as negative controls (sequences were obtained from the GeCKO v2 library, Addgene) [36]. Lentivirus was produced in HEK 293 T Lenti-X cells (Clontech) by transfecting ULK1-pRRL plasmid with packaging plasmids pRSV-Rev, pMD2.g, and pMDLg/pRRE (Addgene numbers 12253, 12259, and 12251, respectively), in Opti-MEM and TransiT-LT1 (Mirius Bio). Supernatants were filtered and incubated with U937 cells and 5 µg/mL polybrene (EMD Millipore). Transduced U937 cells were allowed to rest for 24 hours in fresh medium after incubation with lentiviral particles. After puromycin selection, Crispr-Cas9 targeting

was evaluated by restriction fragment–length polymorphism (RFLP) screening, using restriction sites located within the Cas9 cut site. Genomic DNA was isolated and sequenced to confirm the presence of an insertion/deletion mutation (AGCCCCTCGCCCCCTGCCCACGCT[:/T]GAGCATGGA GGCGTCCT). To evaluate cell viability, cells were plated at 1×10^5 cells/well, removed daily, stained with trypan blue, and counted in a hemocytometer. Both viable and nonviable cells were recorded, and percentage viability calculated. Viability experiments were performed on 2 separate days, and data were pooled.

Cytokine Expression

U937 cells were plated at 5×10^4 cells/well in Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum, 2 mM L-glutamine, and HEPES, differentiated with 50 ng/mL phorbol myristate acetate (PMA; Invivogen, San Diego, CA) for 48 hours, washed with Hank's balanced salt solution (HBSS), and rested for 24 hours in complete medium. Cells were incubated with lipopolysaccharide (LPS; List Biological Labs, Campbell, CA), PAM2/PAM3 (Invivogen), or whole-cell lysate from *M. tuberculosis* strain H37Rv (Colorado State University, Fort Collins) for 24 hours. In addition, we used an NLRC4 ligand to activate the inflammasome with a chimeric protein of the type 3 secretion system needle protein of *Burkholderia thailandensis* fused to the binding domain of *Bacillus anthracis* lethal factor, which is used for cytoplasmic delivery when administered with *B. anthracis* protective antigen (a gift from Russell Vance, University of California, Berkeley). We evaluated supernatants for interleukin 1β (IL-1β) and tumor necrosis factor (TNF) levels, using the DuoSet enzyme-linked immunoassay (R&D Systems, Minneapolis), and for interferon β (IFN-β), using the TaqMan assay (Thermo Fisher Scientific, Waltham, MA) and primer Hs.PT.56a.39481063.g (IDT, Coralville, IA).

Autophagy Studies

U937 cells were differentiated using PMA for 24 hours, then exposed to medium, starvation, or *M. tuberculosis* expressing mCherry at a multiplicity of infection (MOI) of 5 for 4 hours. Cells were washed (3 times) in phosphate-buffered saline and fixed in 100% methanol at −20°C for 5 minutes. Cover slips were incubated in Alexa488 anti-LC3 (clone D3U4C, Cell Signaling) at a dilution of 1:50 overnight at 4°C. After washing, the cover slips were mounted onto glass slides, using Prolong Gold anti-fade reagent (Molecular Probes). Images were acquired on a Nikon Eclipse Ti microscope (60× objective), corrected for background, and the number of LC3 puncta >1 µm in diameter were measured (Volocity 6.3, PerkinElmer, Waltham, MA) for at least 100 cells per condition. Each experiment was performed in triplicate. Overlaid fluorescent images were analyzed to determine the number of LC3-positive autophagosomes containing mycobacteria. A minimum of 100 phagosomes were analyzed for each cellular condition, with at least 3 independent experiments performed.

Table 1. Characteristics of 273 Participants With and 296 Without Latent *Mycobacterium tuberculosis* Infection (LTBI)

Characteristic	LTBI	No LTBI	*P* Value
Age, y, mean ± SD	41.3 ± 15.9	41.0 ± 15.2	.80
Male sex	153/273	153/296	.30
Ethnicity			<.001
Asian	113/273	82/296	
Black	58/273	45/296	
Latino	31/273	25/296	
Pacific Islander	35/273	21/296	
White	21/273	36/296	
Other	14/273	17/296	
Foreign birth (n = 558)	211/266	145/292	<.001
BCG vaccination (n = 563)	165/270	118/293	<.001
BMI,[a] median	25.0	25.8	.17
Homeless	33/273	56/296	.03
Nocturnal exposure (n = 568)	53/272	42/296	.09
Smoking (n = 562)	63/272	70/290	.79
Alcohol use (n = 566)	89/272	111/294	.21
Employed >20 h/wk	121/273	137/296	.61
Highest education level (n = 559)			.04
Less than grade school	10/268	8/291	
Grade school	50/268	30/291	
High school	134/268	148/291	
College	55/268	77/291	
>College	19/268	28/291	
Tuberculosis history in first-degree relative	61/273	48/296	.06
Index characteristic			
Bilateral disease detected by chest radiography (n = 461)	120/237	78/224	.001
Cavitation(s) detected by chest radiography (n = 466)	146/240	110/226	.008
AFB smear finding			<.001
Negative	11/236	36/232	
1+ or 2+	53/236	67/232	
3+ or 4+	172/236	129/232	
LTBI diagnosis, participants, no.			
TST (n = 256)	105	151	
IGRA (n = 358)	171	187	
Dual testing (n = 86)	49	36	

Data are percentage of participants, unless otherwise indicated.

Abbreviations: AFB, acid-fast bacilli; IGRA, interferon γ release assay; TST, tuberculin skin test.

[a] Body mass index (BMI) is calculated as the weight in kilograms divided by the height in meters squared.

M. tuberculosis Infection

U937 cells with the ULK1 knockout, empty vector CRISPR control, or wild type were plated in sextuplet at a density of 75 000 cells/well and differentiated with PMA (50 ng/mL) overnight, washed twice with HBSS, and rested for 24 hours. A frozen aliquot of *M. tuberculosis* Erdman strain expressing lux (MTB-lux; a gift from Jeffrey Cox, University of California, San Francisco) was thawed, centrifuged, and resuspended in

Table 2. 2 ULK1 Single-Nucleotide Polymorphisms (SNPs) Are Associated With Latent *Mycobacterium tuberculosis* Infection (LTBI) in Asian Contacts

	Genotype Frequency in Controls				Genotype Frequency in Cases With LTBI				*P* Value	
SNP	00	01	11	Total	00	01	11	Total	Genotypic	Trend test
ULK1 rs12297124	0.657	0.286	0.057	70	0.837	0.163	0.000	92	.005	.003
ULK1 rs7300908	0.657	0.329	0.014	70	0.837	0.163	0.000	92	.01	.006

Abbreviations: 00, homozygous common allele; 01, heterozygous allele; 11, homozygous rare allele.

RPMI with 10% fetal calf serum. Luminescence from this organism strongly correlates with the number of *M. tuberculosis* colony-forming units on an agar plate ($R^2 = 0.99$; data not shown). A separate set of wells was included with mLux in the absence of U937 cells as a control for background luciferase expression. After incubation for 4 hours (MOI, 10), wells were washed once with medium and then incubated with fresh medium for 7 days. Relative light units were measured at days 0, 1, and 4–7 (Synergy H4 reader, BioTek Instruments). The experiment was performed 3 separate times.

Statistical Methods

Differences were tested using the χ^2 test, the Student *t* test, or the Wilcoxon rank sum test as appropriate and indicated in figure legends. We assessed associations between candidate SNPs and LTBI in genotypic exact models stratified by ethnicity, using Stata 11 (Stata, College Station, TX). SNPs with a significant association ($P < .05$) were evaluated using dominant, recessive, and additive models. To assess for population stratification, we performed principal components analysis using ancestry-informative markers and examined scree and score plots. Before candidate gene association analyses, we removed genetic outliers from each ethnicity on the basis of the first 2 principal components (n = 4). We evaluated significant SNP associations with LTBI, using multivariable logistic regression that adjusted for principal components and demographic and exposure-related variables that were associated with LTBI in the entire cohort.

Table 3. Multivariable Model of ULK1 rs12297124 Association With Latent *Mycobacterium tuberculosis* Infection in Asian Contacts

Variable	Odds Ratio (95% CI)	*P* Value
Unadjusted additive model		
ULK1 rs12297124	0.36 (.19–.72)	.004
Additive model adjusted for first principal component		
ULK1 rs12297124	0.30 (.15–.61)	.001
Additive model adjusted for clinical variables[a]		
ULK1 rs12297124	0.18 (.07–.46)	.0004
Bilateral radiographic disease	1.89 (.78–4.54)	.16
Smear burden (high, low, negative)	2.09 (1.09–4.00)	.03
Foreign birth	0.82 (.16–4.14)	.81
Age	1.04 (1.01–1.06)	.01

Abbreviation: CI, confidence interval.

[a] Adjusted for PC1.

We chose demographic and exposure variables for evaluation, based on published and hypothesized associations with LTBI, including index case characteristics (acid-fast bacilli [AFB] smear burden, categorized as smear negative, low (1+/2+), or high (3+/4+); and radiographic evidence of bilateral disease or cavitations), exposure setting, and contact characteristics (sex, ethnicity, homelessness, education level, employment status, alcohol use, body mass index, smoking, foreign birth, BCG status, and tuberculosis history in first-degree relative). We included age in multivariable models regardless of bivariate significance. We compared logistic regression models, using partial likelihood ratio tests (nested models) or the Akaike information criterion (nonnested models). We report uncorrected *P* values throughout the article.

Expression Quantitative Trait Loci (eQTL) Analysis

We used publicly available data from the Genotype-Tissue Expression project (GTEx) to examine associations between SNPs of interest and messenger RNA levels in associated genes in a variety of cells and tissues [37]. GTEx generates eQTL *P* values based on linear regression analysis assuming an additive model, using a 2-tailed *t* test.

Ethics

This study was approved by the University of Washington Institutional Review Board, and informed consent was obtained from all participants.

RESULTS

Study Population

From 2010 to 2014, we enrolled 569 close contacts of tuberculosis index cases. Although we limited our candidate gene association study to individuals who self-identified as black or Asian, any close contact was eligible for enrollment to determine epidemiological and clinical characteristics associated with LTBI. In our cohort, 273 participants (48%) had LTBI (Table 1). Agreement between TST and QFT was moderate ($\kappa = 0.54$) [38]. LTBI test conversions were observed in 44 participants. In multivariable analysis of demographic, clinical, and exposure-related variables, radiographic evidence of bilateral disease (odds ratio [OR], 2.3; 95% confidence interval [CI], 1.5–3.5) and AFB smear grade (OR, 1.3; 95% CI, 1.1–1.5) in the index case and black ethnicity (compared with white; OR,

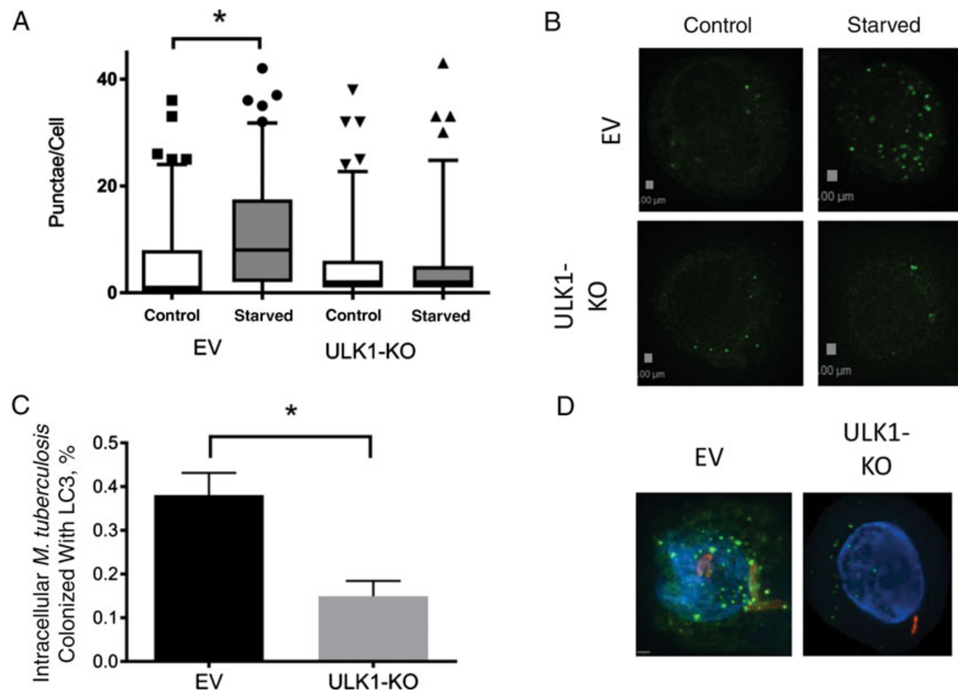

Figure 1. ULK1 deficiency inhibits nonselective autophagy and *Mycobacterium tuberculosis*–selective autophagy in U937-derived monocytes and macrophages. *A* and *B*, Nonselective autophagy is inhibited without ULK1. ULK1-deficient (ULK1-knockout [KO]) or empty-vector (EV) U937 cells were incubated in nutrient-rich medium or starvation medium for 4 hours and then fixed, permeabilized, and stained for LC3. Images were obtained via fluorescent microscopy, and the number of LC3-positive puncta were measured per cell for each condition. *A*, Tukey plot of the number of puncta/cell in each condition. *B*, Deconvolved images of LC3 staining from individual cells in each condition, representative of each. Green images indicate LC3, and blue images indicate DAPI nuclear staining. Each experiment was performed with at least 100 individual observations and performed 3 times to ensure reproducibility. *C* and *D*, Selective autophagy of *M. tuberculosis* is inhibited in cells lacking ULK1. *C*, Bar graph of the proportion of intracellular bacteria colocalized with LC3-positive puncta. Bars indicate mean values, with error bars indicating standards error of the mean. *D*, Deconvolved images of intracellular *M. tuberculosis* colocalized with autophagosomes in an EV cell and intracellular *M. tuberculosis* not colocalized with LC3 in an ULK1-KO cell. Green images indicate LC3, red images indicate mycobacteria, and blue images indicate DAPI nuclear staining. To calculate statistics, at least 100 cells were measured for each condition, and each experiment was performed 3 times to ensure reproducibility. $^*P < .05$ by the Wilcoxon rank sum test.

3.7; 95% CI, 1.7–8.0) and foreign birth (OR, 2.7; 95% CI, 1.5–5.0) in close contacts were independently associated with LTBI.

Genotyping Results by Ethnicity

To determine whether candidate polymorphisms in *ULK1* and *GABARAP* were associated with susceptibility to *M. tuberculosis* infection, we performed genotyping in 143 close contacts with LTBI and 106 without LTBI. We evaluated 15 SNPs in *ULK1* and 5 SNPs in *GABARAP* (Supplementary Table 1). Using genotypic models, we identified 2 SNPs in *ULK1* in Asian participants that were significantly associated with LTBI (unadjusted $P < .05$): rs12297124 and rs7300908 (Table 2). No associations between *GABRAP* polymorphisms and LTBI were identified (Supplementary Table 1). We found that rs12297124 (OR, 0.36; 95% CI, .19–.72; $P = .004$) and rs7300908 (OR, 0.37; 95% CI, .18–.77; $P = .007$) had the strongest associations with LTBI in additive models (Table 3). As rs12297124 was in high linkage disequilibrium with rs7300908 ($r^2 = 0.88$; Supplementary Figure 1), we focus the remainder of this article on understanding LTBI susceptibility with rs12297124, an intronic SNP that involves a G → T transversion.

ULK1 Genetic Associations

We genotyped ancestry-informative markers and performed a principal components analysis, which indicated that the cases and controls were genetically well matched and that clusters by principal components agreed with self-identified ethnicity (Supplementary Figure 2). After comparing models by using likelihood ratio tests, we included only the first principal component (PC1) in multivariate models. Among Asian participants, adjustment of rs12297124 for PC1 strengthened the association with LTBI (OR, 0.30; 95% CI, .15–.61; Table 3). In a multivariable model that included index case characteristics (bilateral disease on radiographs and smear grade), contact characteristics (foreign birth and age), and PC1, rs12297124 remained independently associated with LTBI (OR, 0.18; 95% CI, .07–.46; Table 3). Among black participants (50 cases and 36 controls), ULK1 rs12297124 was not significantly associated with LTBI in a model that included exposure-related variables and PC1 (OR, 0.67; 95% CI, .14–3.22). When we evaluated Asian and black participants together in a multivariable model, ULK1 rs12297124 remained independently associated with LTBI (OR, 0.32; 95% CI, .16–.66; $P = .002$). We also

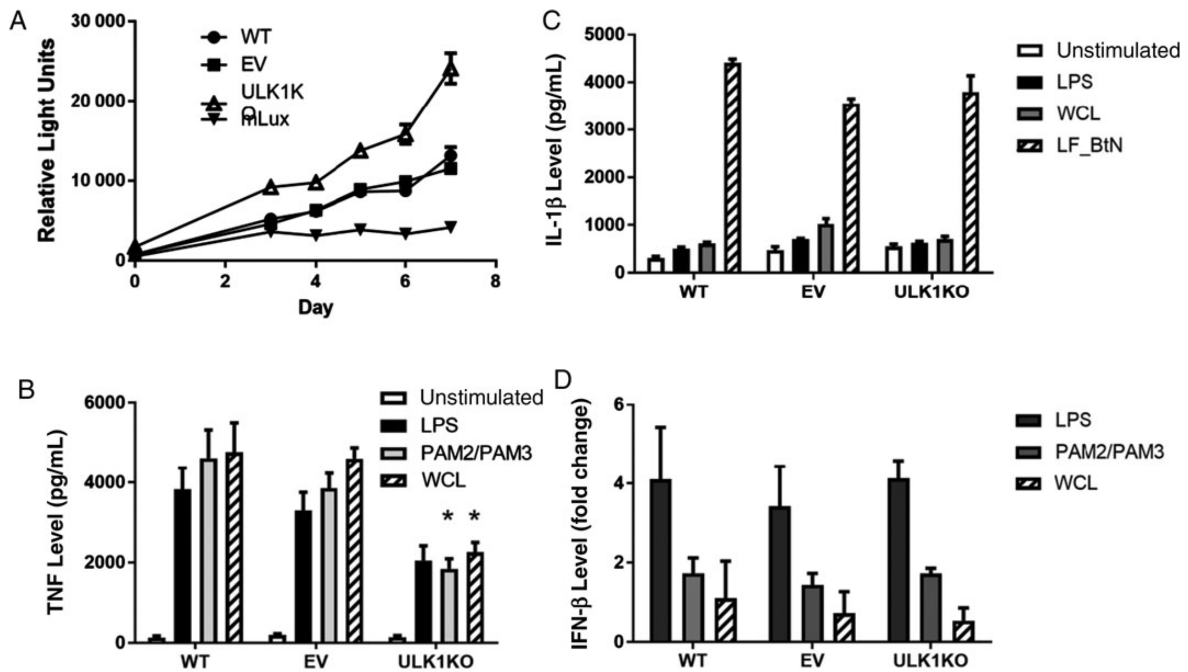

Figure 2. Differential responses in ULK1-deficient monocyte derived macrophages. U937 cells with either the ULK1 knockout (KO), the empty-vector CRISPR control (EV), or wild type (WT). *A*, *Mycobacterium tuberculosis* replication in ULK1-deficient monocyte-derived macrophages. KO, EV, and WT cells infected with luciferase-expressing *M. tuberculosis* at a multiplicity of infection (MOI) of 10. A separate set of wells was included with luciferase-expressing *M. tuberculosis* (mLux) in the absence of U937 cells as a control for background luciferase expression. Relative light units were measured at the indicated time points. Each point represents the mean of 6 separate wells. $P = .004$ by the Wilcoxon rank-sum test on days 3–7 for ULK1-deficient cells as compared to cells with EV or WT. *B*, After stimulation with PAM2/PAM3 (250 ng/mL each), lipopolysaccharide (LPS; 100 ng/mL), and *M. tuberculosis* whole-cell lysate (WCL; 25 µg/mL) for 24 hours, tumor necrosis factor (TNF) was measured by enzyme-linked immunosorbent assay (ELISA). ULK1-deficient cells produced significantly less TNF in response to PAM2/PAM3 (*$P = .004$ by the Student *t* test) and WCL ($P < .001$) as compared to cells with WT or EV. *C*, After stimulating the inflammasome with *Bacillus thailandensis* needle protein fused to lethal factor for cytoplasmic delivery (LF_Btn), interleukin 1β (IL-1β) secretion was measured by ELISA . *D*, After stimulation with PAM2/PAM3 (250 ng/mL, each), LPS (100 ng/mL), and WCL (25 µg/mL) for 24 hours, interferon β (IFN-β) was measured by reverse transcription–polymerase chain reaction. Linearized transcription values are expressed as fold change as compared to unstimulated cells. Graph includes merged data from 2 independent experiments.

evaluated rs12297124 associations with LTBI as defined separately by QFT assay positivity and TST positivity in Asian participants: after adjustment for principal components, rs12297124 was associated with QFT assay–defined LTBI (OR, 0.31; 95% CI, .13–.73; $P = .007$) and TST-defined LTBI (OR, 0.08; 95% CI, .01–.64; $P = .02$).

eQTL Findings

Using the GTEx browser (available at: http://www.gtexportal.org/home/), we evaluated associations between *ULK1* expression and haplotype-tagging SNPs. Of the 15 *ULK1* SNPs that we genotyped, only rs12297124 was significantly associated with ULK1 expression in tissue from esophageal muscularis mucosae ($P = .005$; Supplementary Figure 3).

ULK1-Deficient Cells Are Associated With Increased *M. tuberculosis* Replication, Decreased TNF Secretion, and Decreased Autophagy

To evaluate potential mechanisms through which ULK1 affects LTBI susceptibility, we created an ULK1-deficient U937 cell line, using the CRISPR/Cas9 gene editing technique. We inserted a single base pair into ULK1 that generated a premature stop codon, which we verified by a RFLP assay (Supplementary Figure 4). We assessed nonselective autophagy to confirm that ULK1 function was impaired and found that starvation conditions did not induce autophagy in ULK1-deficient cells, as measured by the number of LC3-positive puncta per cell. This was in contrast to cells created with empty vector during CRISPR/Cas9 processing, in which starvation led to a significant increase in the number of LC3-positive puncta per cell (Figure 1*A* and 1*B*). Percentage cell viability did not differ between ULK1-deficient U937, empty vector, and wild-type cells. We infected ULK1-deficient, empty vector, and wild-type U937 cells with *M. tuberculosis* expressing the lux operon to assess differences in control of infection. Using a bioluminescent assay, we observed increased *M. tuberculosis* replication in ULK1-deficient cells compared to empty vector control and wild-type cells ($P = .004$ for days 3–7; Figure 2*A*).

To determine a mechanism for ULK1-dependent replication, we assessed whether ULK1-deficient U937 cells displayed different cytokine responses to stimulation conditions as compared to empty vector and wild-type control cells. ULK1-deficient cells produced significantly less TNF in response to PAM2 and PAM3 ($P = .004$ and $P = .001$) and *M. tuberculosis*

whole-cell lysate ($P < .001$ and $P < .02$), compared with wild-type and empty vector cells, respectively (Figure 2*B*). However, TNF responses to LPS stimulation were not significantly different between ULK1-deficient cells and control cells. When we stimulated the inflammasome with an NLRC4 ligand, IL-1β production was not different when comparing ULK1-deficient cells to empty vector or wild-type cells (Figure 2*C*). As ULK1 negatively regulates IFN-β [39], we assessed IFN-β responses in our cell lines to various stimulation conditions. Compared with empty vector and wild-type cells, ULK1-deficient cells had similar changes in IFN-β responses as compared to unstimulated states (Figure 2*D*).

We next investigated the impact of ULK1-deficiency on selective autophagy and infected cells with mCherry-expressing *M. tuberculosis* (Figure 1). In comparison to control cells, ULK1-deficient cells had significantly fewer intracellular bacteria colocalized with LC3-positive puncta (Figure 1*C* and 1*D*).

DISCUSSION

The primary finding of our study is that *ULK1* polymorphisms were associated with LTBI in Asian individuals, with a reduction of >80% in the LTBI risk for each copy of the minor allele. ULK1-deficient monocytes had increased *M. tuberculosis* replication, decreased TNF response to stimulation with TLR ligands and *M. tuberculosis* whole-cell lysate, and impaired autophagy. Furthermore, variation in rs12297124 was associated with ULK1 expression in esophageal muscularis mucosa cells. Previous murine studies demonstrated that autophagy pathway genes regulate *M. tuberculosis*–specific pathways in monocytes and influence tuberculosis susceptibility during in vivo infection studies [21, 40–42]. Autophagy is initiated by an ULK1-containing complex under regulation by mTOR and AMP kinase. ULK1 activation leads to phosphorylation of beclin-1, which ultimately results in autophagosome formation [43]. Although polymorphisms in *IRGM*, an autophagy gene, have been associated with tuberculosis in diverse populations [24–26], a study of 22 autophagy genes in an Indonesian population did not identify any associations with tuberculosis [32]. To our knowledge, *ULK1* is only the second autophagy gene associated with tuberculosis in genetic studies and the first to be associated with LTBI [24, 25, 27].

The mechanism of how ULK1 variants regulate susceptibility to human *M. tuberculosis* infection is not known. Based on its well-described role in autophagy initiation, we speculated that human ULK1 deficiency leads to decreased autophagy and increased *M. tuberculosis* replication in monocytes. Our studies in ULK1-deficient monocytes support this model. However, additional mechanisms may be important, including ULK1 regulation of *M. tuberculosis* replication through effects on cell signaling. We showed decreased TNF responses, a key cytokine in control of *M. tuberculosis* infection, after stimulation of ULK1-deficient monocytes with *M. tuberculosis* whole-cell lysate, which is similar to previously reported TNF decreases in *M. tuberculosis*–exposed cells after 3MA-induced inhibition of autophagy [44, 45]. ULK1 phosphorylates STING [39] with negative regulation of IRF3-dependent IFN-β, which negatively regulates IL-1β, a cytokine that is protective in murine tuberculosis [46]. Autophagy has been shown to limit inflammasome activity, and inhibition of autophagy led to increased IL-1β secretion after infection with *M. tuberculosis* [44, 47]. In addition, ULK1 has been demonstrated to negatively regulate NLRP3 and caspase-1 activity with decreases in interleukin 18 expression [48]. Our data suggest that ULK1 mediates TLR and *M. tuberculosis*–induced TNF secretion, but not NLRC4-mediated IL-1β production. We currently do not know which ULK1 variants regulate gene function. Further insight into human ULK1 function will require fine-mapping and functional studies to discover which SNPs regulate ULK1 function and their effects on *M. tuberculosis* immunoregulation.

Our study has several limitations. Our small sample size for black participants may have limited our ability to detect small-to-moderate genetic relative risks in candidate genes. However, our use of controls who were exposed to infectious tuberculosis likely improved study power by reducing misclassification bias [49]. An additional limitation is that LTBI tests are imperfect and may lead to misclassification of study subjects. As with any candidate gene study, our findings should be replicated in a new population.

Infection with *M. tuberculosis* leads to a spectrum of outcomes that include sterilizing immunity, LTBI, subclinical disease, and symptomatic tuberculosis. Host genetic variation may impact tuberculosis pathogenesis at any stage in the spectrum. The majority of genetic studies of tuberculosis susceptibility has focused on the outcome of active tuberculosis and were unable to determine which steps in the tuberculosis spectrum were impacted. Given the orders of magnitude of difference in LTBI versus active tuberculosis prevalence, the specific genetic variations associated with susceptibility would be expected to differ between LTBI and active tuberculosis.

In summary, we found that a common ULK1 genetic variant was associated with protection against LTBI. To our knowledge, this is the first study to suggest a role for ULK1 in immunoregulation of *M. tuberculosis*. Our findings prompt further evaluation of this gene to validate our results and to further probe mechanistic explanations.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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D. J. H. and T. R. H. conceived and designed the study. D. J. H., A. D. G., J. A. S., M. N., and T. R. H. contributed to analyses and interpretation of analyses. All authors contributed to data acquisition and preparation and critical revision of the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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