Human TOLLIP Regulates TLR2 and TLR4 Signaling and Its Polymorphisms Are Associated with Susceptibility to Tuberculosis


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Human TOLLIP Regulates TLR2 and TLR4 Signaling and Its Polymorphisms Are Associated with Susceptibility to Tuberculosis


Tuberculosis, one of the leading causes of death worldwide, stimulates inflammatory responses with beneficial and pathologic consequences. The regulation and nature of an optimal inflammatory response to *Mycobacterium tuberculosis* remains poorly understood in humans. Insight into mechanisms of negative regulation of the TLR-mediated innate immune response to *M. tuberculosis* could provide significant breakthroughs in the design of new vaccines and drugs. We hypothesized that TOLLIP and its common variants negatively regulate TLR signaling in human monocytes and are associated with susceptibility to tuberculosis. Using short hairpin RNA knockdown of TOLLIP in peripheral blood human monocytes, we found that TOLLIP suppresses TNF and IL-6 production after stimulation with TLR2 and TLR4 ligands. In contrast, secretion of the anti-inflammatory cytokine IL-10 was induced by TOLLIP. We also discovered two common polymorphisms that are associated with either decreased levels of mRNA expression (rs3750920) or increased IL-6 production (rs5743899) in a sample of 56 healthy volunteers. Furthermore, in a case-population study in Vietnam with 760 cord blood samples and 671 TB case patients, we found that SNPs rs3750920 and rs5743899 were associated with susceptibility to tuberculosis \( (p = 7.03 \times 10^{-16} \text{ and } 6.97 \times 10^{-7}) \), respectively. These data demonstrate that TOLLIP has an anti-inflammatory effect on TLR signaling in humans and that TOLLIP deficiency is associated with an increased risk of tuberculosis. To our knowledge, these data also show the first associations of TOLLIP polymorphisms with any infectious disease. These data also implicate an unexpected mechanism of negative regulation of TLR signaling in human tuberculosis pathogenesis.


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conclusive (34–36). Together, these studies suggest that the role of IL-10 in the pathogenesis of TB remains unclear and further study is required.

Negative regulators have been discovered at nearly every step of the TLR signaling cascade (37–39) and include Toll-interacting proteins (TOLLIP, also called IL-1RacPip), A20, Single Ig IL-1R (SIGIRR, also called TIR8) (41), and IL-1R–associated kinase 3 (IRAK-3, also called IRAK-M) (42). TOLLIP is a 274-aa protein with highly conserved C2 (amino acids 54–186, similar to that found in phosphoinositol-specific phospholipase C-dll) and C-terminal ubiquitin-associated domains. It was initially discovered as an IL-1R–interacting protein that linked IRAK to the IL-1R pathway (43), and later was implicated in suppression of the TLR2 (44) and TLR4 pathways (45). However, in vivo murine knockout models demonstrated that TOLLIP induced proinflammatory pathways, in contrast to in vitro experiments (46). To our knowledge, there are no studies examining the effects of TOLLIP on TLR signaling pathways in humans.

Several lines of evidence suggest that host genetics regulates susceptibility to TB, including twin-based, linkage, candidate gene association, and genome-wide association studies. We, along with other investigators, have identified associations between common polymorphisms in innate immunity genes and susceptibility to TB and clinical phenotypes (22, 47, 48). Much of this work has focused on pattern recognition receptors and their associated adaptor proteins, including TLR 1, 2, 4, 6, and 9. Several candidate gene association studies have shown associations between single nucleotide polymorphisms (SNPs) in TLR2 and TLR4, including 597CC within a Vietnamese population (8, 49). A polymorphism in TLR1 (T1805G, I602S) regulates lipopeptide-induced cytokine secretion and TLR1 surface expression, and is associated with susceptibility to TB as well as leprosy (48, 50–52). Genetic studies have also found associations of TLR4 polymorphisms with TB susceptibility (10, 24, 25). These studies suggest that genetic control of TLR1/2/4/6 signaling by negative regulators may alter susceptibility to TB. In contrast to TLRs, the functional and clinical significance of genetic variation of TOLLIP and other negative regulators of TLR signaling is poorly studied and only partially understood. In this study, we examined the function of TOLLIP in human monocytes with knockdown studies and examination of common TOLLIP variants. We also examined whether these polymorphisms were associated with susceptibility to TB in Vietnam. These studies suggest that TOLLIP is a critical regulator of the TLR pathway in humans and that this immune regulation has a critical role in the susceptibility to TB.

Materials and Methods

Materials

RPMI 1640 medium, t-glutamine, penicillin, and streptomycin were obtained from Life Technologies (Carlsbad, CA). Ultrapure LPS (TLR4 ligand) isolated from *Salmonella minnesota* R595 was obtained from List Biological Labs (Campbell, CA). The synthetic lipopeptides PAM2 (PAM2CSKKKK, S-[2,3-bis-(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/4 ligand) and PAM3 (Pam3CSKKKK, N-palmitoyl-S-[2,3-bis-(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/6 ligand) and PAM3 (Pam3CSKKKK, N-palmitoyl-S-[2,3-bis-(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine, TLR2/6 ligand) were obtained from List Biological Labs (Campbell, CA). The synthetic lipopeptides PAM2 and PAM3 were used to stimulate human peripheral blood monocytes with 10% FCS. Cells were incubated with TLR ligands LPS, or with *M. tuberculosis* whole cell lysates for 24 h. Subsequently, supernatants were evaluated for levels of cytokine (IL-6, IL-10, TNF) via ELISA Duoset (R&D Systems, Minneapolis, MN). Each sample was assayed in duplicate or triplicate, and experiments shown were performed at least twice to ensure reproducibility.

Genotyping and linkage disequilibrium

Genotyping was performed with Sequenom’s MassARRAY technique as described previously (54). This technique uses allele-specific primer extension reactions to discriminate genotypes. We also confirmed the SNPs of interest using Taqman genotyping technology (Applied Biosystems, Carlsbad, CA). We identified haplotype-tagging SNPs from the CHB (Han Chinese in Beijing) and CEU (Utah residents with European ancestry) populations from the International HapMap Project (http://www.hapmap.org) and other public databases with the Genome Variation Server (http://www.ncbi.nlm.nih.gov/SNP/ and www.innateimmunity.net). We searched a region on chromosome 11p15.5, 10 kb upstream and downstream of TOLLIP for tagged SNPs using an R2 cutoff of 0.8 for linkage disequilibrium and a minor allele frequency cut-off of 5%. Stata/Intercooled version 11.0 software program PWLD (StataCorp, College Station, TX) was used to calculate R2 and D’ as measurements of linkage disequilibrium between the polymorphisms.

Molecular biology

TOLLIP mRNA levels were measured in monocytes from peripheral blood of healthy donors in the Seattle area. RNA was obtained after cell lysis and RNA purification (Qiagen, Valencia, CA). After synthesis of cDNA, real-time PCR was performed. TOLLIP mRNA levels were normalized with a GAPDH control using the following primer-probe sets (5′-J56-FAM/CTCCGGGAT7ZEN/GTG-3′, 5′-CTAGGCTGTACACCCTTG-3′, 5′-GGCGTTGACCTTTTATC-3′; Integrated DNA Technologies, Coralville, IA). Quantitative PCR was performed on a Taqman machine.

Gene silencing

Knockdown studies were performed using plasmids encoding three TOLLIP-specific short hairpin RNA (shRNA; Santa Cruz Biotechnology, Santa Cruz, CA) that were then packaged into a nonreplicating lentiviral particle to improve transfection efficiency using a standard protocol (UCSF Viracore Facility, San Francisco, CA). These lentiviral particles were then incubated with monocytes at a multiplicity of infection of 2 for 24 h before cells were stimulated and harvested for cytokine analysis. Knockdown was confirmed using quantitative PCR for TOLLIP mRNA transcript.

Human subjects

Approval for human study protocols was obtained from the human subjects review boards at the University of Washington School of Medicine (Seattle, WA), the Hospital for Tropical Diseases (Ho Chi Minh City, Vietnam), Pham Ngoc Thach Hospital for Tuberculosis and Lung Diseases (Ho Chi Minh City, Vietnam), Health Services of Ho Chi Minh City, Hung Vuong Hospital (Ho Chi Minh City, Vietnam), and the Oxford Tropical Research Ethics Committee (Oxford, U.K.).

Genomic DNA was purified from saliva and blood samples. The Seattle study group included 56 healthy volunteers who donated blood and saliva samples. The ethnic composition of these subjects was 70% Caucasian (39/56), 27% Asian (15/56), and 4% African American (2/56). Study subjects with tuberculous meningitis (TBM) were recruited from two centers in Ho Chi Minh City, Vietnam: Pham Ngoc Thach (PNT) Hospital for Tuberculosis and the Hospital for Tropical Diseases (HTD). These 500-bed hospitals serve the local community and act as tertiary referral centers for TB (PNT) and infectious diseases (HTD) for southern Vietnam. Individuals at least 15 y old, admitted to these centers with clinical meningitis (defined as nuchal rigidity and abnormal cerebrospinal fluid parameters), a negative HIV test result, and a positive Ziehl–Neelsen stain for acid-fast bacilli or *M. tuberculosis* culture, or both, from cerebrospinal fluid were recruited (‘*definite TBM*’). In addition to define TBM, the cohort included subjects with “probable TBM,” defined as clinical meningitis plus at least one of the following: chest radiograph consistent with active TB, acid-fast bacilli found in any specimen other than cerebrospinal fluid, and clinical evidence of other extrapulmonary TB (55). From 2003 through 2004, subjects with pulmonary TB were recruited from a network of district TB control units within Ho Chi Minh City that
provide directly observed therapy to TB patients. These subjects were 15 y old, had no previous history of treatment for TB, no evidence of miliary or extrapulmonary TB, chest radiograph results consistent with nonmiliary pulmonary TB, negative HIV test results, and sputum smear positive for acid-fast bacilli or M. tuberculosis cultured from sputum. In addition, pulmonary TB subjects enrolled were recruited from PNT hospital from 2006 through 2008.

Control subjects were enrolled at Hung Vuong Hospital in Ho Chi Minh City from blood collected from the umbilical cord of babies after birth. All case and control participants were unrelated, and greater than 95% were of the Vietnamese Kinh ethnicity. Written, informed consent was obtained from patients or their relatives if the patient could not provide consent (i.e., was unconscious). Parents provided consent for cord-blood controls.

Out of the study subjects that self-identified their ancestry as Vietnamese Kinh, we formally tested for levels of background genetic differences between cases and controls by genotyping the cohort for a panel of 24 independent SNP markers. The mean $\chi^2$ statistic for the 24 SNPs comparing allelic frequencies between cases and control was 1.6 ($p = 0.20$), suggesting that no significant population stratification was present in our study population.

**Statistical methods**

All analyzed SNPs were tested for Hardy-Weinberg equilibrium in control subjects using a $\chi^2$ goodness-of-fit test. In our primary analysis, we examined whether polymorphism genotype frequencies were associated with any type of TB in a case-population study design using Stata MP11 and the user written package “genassoc” (58). For secondary analyses, SNPs were investigated for associations under additional genetic models (dominant, recessive, and additive) and for association with the clinical subtypes of TB (pulmonary or meningeal). In the recessive model, carriers of allele 1 (00 genotype) were compared with homozygous subjects for allele 2 (11 genotype). In the dominant model, carriers of allele 2 (01 and 11 genotypes) were compared with homozygous subjects for allele 1 (00 genotype). Measures of linkage disequilibrium were assessed in controls using “pwld” in Stata.

Nine TOLLIP polymorphisms were genotyped in the Vietnam and Seattle cohorts. Two (rs4744015 and rs5744016) were monogenic and one (rs5743890) was not in Hardy-Weinberg equilibrium and thus not analyzed further. Six are shown in all subsequent analyses. If we correct for multiple comparisons with a Bonferroni test ($p$ value/6), only $p$ values less than 0.008 remain significant. However, as we had two single nucleotide polymorphisms of interest based upon prior functional data, we show uncorrected $p$ values in these tables.

The Pearson-$\chi^2$ test and Student $t$ test were used to assess categorical and continuous clinical variables, respectively. For analysis of associations between TOLLIP polymorphisms and mRNA levels or cytokines, we used an ANOVA analysis with Mann–Whitney tests for significance.

**Results**

**TOLLIP regulates the TLR signaling pathway in humans**

In order to determine the functional role of TOLLIP in the human innate immune system, we performed shRNA knockdown experiments on peripheral blood monocytes from healthy volunteers (Fig. 1). Using a lentiviral delivery system in primary monocytes, we knocked down TOLLIP mRNA expression by more than 50% compared with monocytes alone or those treated with control lentiviral particles (Fig. 1A; $p < 0.001$, Student $t$ test). We also tested and confirmed that TLR2 and TLR4 mRNA expression was not altered by TOLLIP shRNA (Fig. 1A; $p = 0.78$ for TLR2, $p = 0.14$ for TLR4; Student $t$ test comparing control to TOLLIP shRNA viral infection). After stimulation with PAM2 (TLR2/6 ligand) and PAM3 (TLR2/1 ligand), TOLLIP-deficient monocytes produced elevated levels of IL-6 compared with either untransfected monocytes or control-lentiviral particle-transfected monocytes ($p < 0.01$; Fig 1B–F). Transfection with control shRNA led to a mild increase in IL-6 that we attributed to the

![FIGURE 1. Lentiviral shRNA knockdown of TOLLIP in primary human monocytes. Peripheral blood monocytes were isolated and incubated with media, lentiviral particles encoding scrambled shRNA, or lentiviral particles encoding shRNA for three areas within the TOLLIP gene for 24 h. These cells were then incubated with TLR ligands for 24 h, and secreted cytokine responses were measured in the supernatant. (A) mRNA levels of expression after lentiviral knockdown of peripheral blood monocytes. This data shows one representative experiment of two. IL-6 (B–F), TNF (G–K), and IL-10 (L–P) cytokine responses after media, PAM2, PAM3, or LPS stimulation in primary monocytes incubated with nothing, control shRNA, or TOLLIP shRNA-encoding lentiviral particles. M. tuberculosis stimulation is from an independent experiment. *$p < 0.01$, ANOVA with Mann–Whitney $U$ test.](http://www.jimmunol.org/doi/pdf/1739)
innate inflammatory response to lentivirus infection and cytosolic DNA. In contrast, LPS (TLR4 ligand)-induced IL-6 levels were similar under all conditions (Fig. 1E). TOLLIP knockdown led to increased TNF production after stimulation with PAM2, PAM3, LPS, or M. tuberculosis whole cell lysate ($p < 0.01$; Fig. 1G–K). In cells stimulated with PAM2, PAM3, or M. tuberculosis whole cell lysate, there were no significant differences in IL-10 production between cells stimulated with nonspecific lentiviral particles or TOLLIP-specific viral particles ($p < 0.01$; Fig. 1I). Intriguingly, TOLLIP knockdown in LPS-stimulated cells led to significant decreases in secreted IL-10 from monocytes ($p < 0.01$; Fig 1O).

In a separate experiment with stimulation with M. tuberculosis H37Rv whole cell lysate, we found that TOLLIP knockdown led to increased IL-6 and TNF secretion, but not IL-10 compared with controls ($p < 0.01$; Fig. 1F, 1K, 1P).

These data suggest that TOLLIP regulates the innate immune response in humans via two mechanisms—by suppressing proinflammatory cytokines via TLR2 and TLR4 and by inducing IL-10 through a TLR4-specific mechanism.

Association of TOLLIP SNPs with mRNA Expression

Next, we examined whether common variants of TOLLIP regulated its function. We genotyped nine haplotype-tagging SNPs in 84 healthy volunteers and examined their association with TOLLIP mRNA expression in monocytes. (Fig. 2, polymorphisms listed in genomic order) For SNP rs3750920, the minor homozygote was significantly associated with increased mRNA expression compared with either heterozygotes or major homozygotes ($p < 0.01$ by genotypic model; CC = 26, CT = 32, TT = 16; Fig. 2D). This association was also significant in a recessive model compared TT homozygotes with CT/CC genotypes ($p < 0.01$). SNP rs5743899 genotype GG was associated with decreased mRNA transcript in a recessive model ($p < 0.01$; AA = 49, AG = 31, GG = 4; Fig. 2A).

The other 4 TOLLIP SNPs were not significantly associated with mRNA expression (Fig. 2B, 2C, 2E, 2F). Three SNPs (rs3793964, CC = 36, CT = 24, TT = 16; rs3829223, CC = 19, CC = 32, TT = 27; and rs3168046, CC = 15, CT = 22, TT = 14) showed a trend toward an association with mRNA expression (Fig. 2C, 2E, 2F; $p = 0.26$, 0.22, and 0.14, respectively). These SNPs were in linkage disequilibrium with SNP rs3750920 (Supplemental Fig. 1A; $R^2 = 0.43, 0.55, 0.73$ for pairwise comparisons of three SNPs with rs3750920). These results suggest that at least one TOLLIP polymorphism is associated with mRNA expression and is a genetic marker of human TOLLIP deficiency.

Association of TOLLIP SNP rs5743899 with cytokine responses to TLR ligands

We next examined whether polymorphisms rs5743899 and rs3750920 were associated with cytokine responses in monocytes. We stimulated PBMCs with TLR ligands (PAM2, PAM3, and LPS) as well as M. tuberculosis whole cell lysate, and measured secreted IL-6 and IL-10 in the culture supernatants (Fig. 3A, 3B). Minor homozygotes (GG) of polymorphism rs5743899 were associated with significantly higher levels of IL-6 compared with heterozygotes or major homozygotes (Fig 3C, 3D, 3E; AA individuals = 35, AG = 21, GG = 7; $p < 0.01$, ANOVA with Mann–Whitney U test) after stimulation with PAM2 (TLR2/TLR6) or PAM3 (TLR2/TLR1), but not LPS (TLR4). Furthermore, IL-6 levels were significantly increased after stimulation with M. tuberculosis whole cell lysate (Fig. 3F). In addition, IL-10 levels were significantly decreased in a recessive pattern for GG individuals after LPS stimulation ($p = 0.03$; Fig 3G).

There were not any significant changes in IL-10 production after PAM2 or PAM3 stimulation. By way of contrast, minor homo-
zygotes from rs3750920 were not associated with IL-6 levels when compared with heterozygotes or major homozygotes (Fig. 4A–D; CC = 18 individuals, CT = 24, TT = 12). Furthermore, no associations were noted in IL-10 production (Fig 4E–H). These data demonstrate that the minor homozygote of rs5743899 is associated with increased levels of IL-6 after PAM2, PAM3, or *M. tuberculosis* whole cell lysate stimulation, as well as decreased IL-10 after LPS stimulation.

**Association of TOLLIP SNPs with susceptibility to TB**

To assess the role of TOLLIP deficiency in human disease, we performed a candidate gene case-population association study to

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**FIGURE 3.** TOLLIP SNP rs5743899 and cytokine responses after TLR and *M. tuberculosis* stimulation of PBMCs. PBMCs were isolated from 64 healthy volunteers in Seattle and stimulated with media or TLR ligands (LPS at 10 ng/ml, PAM2 at 250 ng/ml, PAM3 at 250 ng/ml, *M. tuberculosis* whole cell lysate at 1 μg/ml) for 24 h. Secreted IL-6 (A) and IL-10 (B) levels were measured in supernatants via ELISA. (C–F) IL-6 responses after TLR stimulation, stratified by rs5743899 genotype. AA individuals = 35, AG = 21, GG = 7. (G–J) IL-10 responses after TLR stimulation, stratified by rs5743899 genotype. *p < 0.01; **p = 0.03 by Mann–Whitney U test in a recessive model.

**FIGURE 4.** TOLLIP polymorphism rs3750920 and cytokine responses after TLR and MTb stimulation of PBMCs. Identical experimental details as Figure 3, except that data were stratified by genotype rs3750920; CC = 18 individuals, CT = 24, TT = 12. IL-6 (A–D) responses and IL-10 (E–H) responses after stimulation with media or TLR ligands (LPS at 10 ng/ml, PAM2 at 250 ng/ml, PAM3 at 250 ng/ml, *M. tuberculosis* whole cell lysate at 1 μg/ml) for 24 h.
examine associations between TOLLIP polymorphisms and susceptibility to TB. We examined 671 cases (394 pulmonary TB [PTB] and 277 TBM) and 760 cord blood controls in our cohort. We first analyzed polymorphisms rs3750920 and rs5743899 because of their association with functional phenotypes. TOLLIP polymorphisms had similar linkage disequilibrium patterns in Seattle and Vietnam, as well as the Northern European (CEU) and Han Chinese (CHB) HapMap populations (Fig. 5, Supplemental Figs. 1 and 2). Polymorphisms rs3750920 and rs5743899 were associated with susceptibility to TB using a genotypic model (Table I; rs3750920, $p = 7.03 \times 10^{-16}$; and rs5743899, $p = 6.97 \times 10^{-12}$). Three other SNPs (rs3793964, rs3829223, and rs3168046) were associated with TB, and these SNPs were in a high degree of linkage disequilibrium with SNP rs3750920 in the Vietnamese population (Supplemental Table I). All these SNPs were associated with TB after a conservative Bonferroni correction for multiple comparisons. One SNP (rs5743942) was associated with TB in an unadjusted analysis, but not after a Bonferroni correction.

Secondary analysis of rs3750920 and rs5743899

For the two significant polymorphisms, we examined these associations further under different genetic models and clinical phenotypes. Testing the association with TB under different genetic models for rs3750920, we found that the strongest association was consistent with a dominant model (Table II; odds ratio [OR], 0.453; $p = 6.28 \times 10^{-12}$). In contrast, SNP rs5743899 was most strongly statistically associated with all forms of TB in a recessive model (OR, 1.641; $p = 0.0004$). We also examined the associations with different clinical forms of TB (e.g., PTB) and TBM. Both SNPs were associated with PTB and TBM to a similar degree (Table II). Finally, we evaluated whether these two SNPs were associated with death or disability from TB, as well as with CSF and serum cytokine responses at the time of presentation (for subjects with TBM). We did not find any significant associations with these outcomes (data not shown). These data suggest that two TOLLIP polymorphisms are associated with susceptibility to TB as well as TOLLIP expression levels or regulation of TLR-mediated cytokine secretion.

Discussion

Our study shows that TOLLIP regulates human TLR signaling pathways in monocytes by suppressing proinflammatory cytokines (IL-6 and TNF) and inducing anti-inflammatory cytokine (IL-10) secretion in peripheral blood monocytes. In addition, we found two common genetic variants of TOLLIP that are associated with TOLLIP mRNA expression or TLR-mediated cytokine release, or both. Finally, these genetic markers of TOLLIP deficiency were associated with an increased risk for developing TB in a case-population study in Vietnam.

Our data from knockdown of TOLLIP in peripheral blood monocytes indicates that TOLLIP is a negative regulator of TNF and IL-6 after PAM2, PAM3, and LPS stimulation. Although these data demonstrate that TOLLIP regulates human TLR2 and TLR4 function, the findings appear to be in contrast to murine models, where Tollip−/− bone marrow-derived macrophages and dendritic cells had decreased LPS-induced IL-6 and TNF in comparison with wild type cells (46). The reasons for this discrepancy are uncertain. It is possible that the unique gene splicing profile of murine TOLLIP led to the proinflammatory profile in the knockout (59) or that cell type or assay conditions were different be-

![FIGURE 5. Chromosomal map and linkage disequilibrium plots of TOLLIP polymorphisms in a Vietnamese cohort. (A) Chromosomal map shows genomic location of polymorphisms. Boxes show exons within gene on chromosome 11. rs3168046 was located on the 3' UTR for the TOLLIP gene. (B, C) Linkage disequilibrium plots with R² and D' values for controls in Vietnam. White boxes on top of plot show frequency of polymorphisms. Degree of shading is proportionate to D' or R² value.](http://www.jimmunol.org/DownloadedFrom)
polymorphisms that are associated with a loss of function as they mimic the phenotype elicited by shRNA knockdown. The mechanism that leads to its loss of function is unclear. We found that minor homozygotes of rs5743899 were associated with a trend of less TOLLIP mRNA in comparison with heterozygotes and major homozygotes, suggesting that differences in TOLLIP expression levels regulate cytokine responses. Our analysis of the case-population data supports this theory, because there is an association between rs5743899 and risk for developing TB in a recessive model. The causative polymorphisms are not known, and this complicates interpretation of the data. In addition to regulation of transcription, the possible mechanisms include linkage disequilibrium of the associated SNPs with a nonsynonymous coding region functional SNP that changes protein localization or conformation and leads to abnormal TOLLIP function. The C2 domain of TOLLIP contains a phosphorylation domain that is hypothesized to be important for IRAK-4 binding (66). This may affect TLR signaling transduction and lead to the cytokine differences noted in this study. A mutation in this domain is a potential location for a loss-of-function mutation, and in vitro cell culture models have shown that abolishing the phosphorylation domain reduces the regulatory capacity of TOLLIP (45). Further study is needed to determine the molecular mechanism of action that leads to this phenotype.

In contrast to rs5743899, polymorphism rs3750920 was associated with protection from TB and increased levels of TOLLIP mRNA. The functional change that leads to the association of rs3750920 with TB is a subject of ongoing study. There are several possible mechanisms that could lead to rs3750920 affecting risk of developing TB, but not IL-6 or TNF cytokine responses in monocytes. First, polymorphisms at rs3750920 might affect cytokines that were not examined during this study, such as IL-1β or IL-18, which could affect inflammatory activity. Second, rs3750920 may affect cytokines that are not expressed in monocytes, such as IL-12p70 or IL-23, but that are expressed in much between the human and murine experiments. Possibly, another set of regulatory molecules may exist apart from TOLLIP and distinguish TLR2 and TLR4 signaling pathways in mice and humans. These species-specific findings underscore the well-recognized importance of TOLLIP and its role in regulating IL-12, which is a critical cytokine for developing TB, but not IL-6 or TNF cytokine responses in monocytes, presumably by activation of similar transcription factors such as NF-kB (60). However, there are several examples of the IL-10 induction pathway diverting from the IL-6 or TNF induction pathway. For example, IL-10 is induced through the TLR2/TLR4 pathway (61) and modified by CARD9 as well as IFN-β. (62) In addition, Card9⁻/⁻ mice are unable to control *M. tuberculosis* infection and do not produce significant IL-10, despite IL-6 and TNF production. (63) Furthermore, differential regulation of proinflammatory and anti-inflammatory signaling has been observed in HIV-infected macrophages that upregulate IL-10 and downregulate IL-12. (64) Several possible mechanisms could explain how TOLLIP could differentially regulate proinflammatory and anti-inflammatory cytokine production. For example, the C-terminal domain of TOLLIP contains a Tom-1 binding domain and likely plays a role in cellular trafficking and ubiquitination of proteins that could affect TLR signal transduction. In addition, TOLLIP can traffic from the cytosol to the cell nucleus via sumoylation, suggesting a role for TOLLIP regulation of transcription factors (65). Although the mechanism by which TOLLIP differentially regulates cytokines is not clear, this finding may help to elucidate the signaling pathway of IL-10.

To our knowledge, this report is the first description of TOLLIP polymorphisms that are associated with a loss of function as they

### Table I. TOLLIP gene region single nucleotide polymorphisms and genotype frequencies in the control and tuberculosis groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group</th>
<th>00</th>
<th>01</th>
<th>11</th>
<th>χ²</th>
<th>p Value</th>
<th>H-W</th>
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<tbody>
<tr>
<td>rs3750920</td>
<td>Control</td>
<td>308 (0.415)</td>
<td>355 (0.479)</td>
<td>78 (0.105)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>328 (0.610)</td>
<td>134 (0.253)</td>
<td>75 (0.129)</td>
<td>69.8</td>
<td>7.03 × 10⁻¹⁶</td>
<td>0.101</td>
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<tr>
<td>rs5743899</td>
<td>Control</td>
<td>252 (0.334)</td>
<td>391 (0.520)</td>
<td>110 (0.145)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>261 (0.397)</td>
<td>252 (0.383)</td>
<td>144 (0.220)</td>
<td>28.4</td>
<td>6.97 × 10⁻⁷</td>
<td>0.163</td>
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</table>

*SNPs are arranged in the order that they are located on the chromosome. SNPs are listed by reference SNP ID.*

### Table II. Associations of rs3750920 and rs5743899 with TB meningitis and pulmonary tuberculosis

<table>
<thead>
<tr>
<th>SNP</th>
<th>Group</th>
<th>00</th>
<th>01</th>
<th>11</th>
<th>OR CI (95%)</th>
<th>p Value</th>
<th>OR CI (95%)</th>
<th>p Value</th>
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<tbody>
<tr>
<td>rs3750920</td>
<td>Control</td>
<td>308 (0.415)</td>
<td>355 (0.479)</td>
<td>78 (0.105)</td>
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<td></td>
<td>PTB</td>
<td>144 (0.415)</td>
<td>60 (0.255)</td>
<td>35 (0.134)</td>
<td>0.445</td>
<td>0.21–0.78</td>
<td>6.34E–09</td>
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<tr>
<td></td>
<td>PTB</td>
<td>184 (0.617)</td>
<td>74 (0.248)</td>
<td>40 (0.134)</td>
<td>0.453</td>
<td>0.36–0.78</td>
<td>6.28E–12</td>
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<tr>
<td></td>
<td>TB</td>
<td>328 (0.610)</td>
<td>134 (0.253)</td>
<td>75 (0.129)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>262 (0.397)</td>
<td>252 (0.383)</td>
<td>144 (0.220)</td>
<td>0.763</td>
<td>0.61–0.95</td>
<td>0.015</td>
<td>1.641</td>
<td>1.23–2.2</td>
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</tbody>
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*Values for calculations of ORs, each group was compared with the control group. 0, common allele; 1, allele with minor frequency. Hardy-Weinberg (H-W) equilibrium was calculated from control subjects only.

A dominant and recessive allelic analysis was performed.

SNPs are listed by reference SNP ID.

“All TB” includes both PTB and TB.

For calculations of ORs, each group was compared with the control group. 0, common allele; 1, allele with minor frequency. CI, Confidence interval.
higher quantities in macrophages (67) and dendritic cells. Third, SNP rs3750920 may regulate protein trafficking and functions such as endocytosis and phagocytosis. TOLLIP is known to bind to ubiquitinated proteins and localize with the protein Tömöl to the early endosome, and it may be important in bacterial entry (68–70). Finally, TOLLIP polymorphisms may regulate effector mechanisms that lead to bacterial killing (such as NO production). Studies are ongoing to examine these possibilities and to determine the causative SNP in linkage disequilibrium with rs3750920. The genetic and mRNA data from these two SNPs likely represent two independent effects with distinct mechanisms.

Interestingly, our genetic data suggests that a hypofunctional TOLLIP genotype (rs7543899 GG) is associated with an increased risk of TB, as well as increased levels of proinflammatory cytokines. There is a long history of observations in the TB field of the potentially deleterious effects of too much inflammation. Steroid treatment of patients with TB and pericarditis is used to dampen the immunopathologic consequences of inflammation (55, 71). In this regard, TNF has been shown to have protective and deleterious effects in different models of TB. For example, although knockout of TNF in murine models is deleterious with increased M. tuberculosis replication, evidence also suggests that a hyperinflammatory state can lead to increased TB replication and worsened disease (72–74). Furthermore, studies of leukotriene A4 hydrolyase, an enzyme that regulates eicosanoid and TNF production in zebra fish and humans, suggest that optimal control of M. tuberculosis requires balanced signaling with deleterious outcomes associated with insufficient or excessive TNF secretion (73). TOLLIP regulation of the anti-inflammatory cytokine IL-10 provides an additional mechanism to inhibit inflammatory pathways (61). Card9-deficient mice do not produce any IL-10 and are unable to control M. tuberculosis infection (63). In addition, IL-10–producing T cells promote anergy to PPD in TB patients and may alter the T cell response and overall control of latent tuberculosis infection (33). These data suggest that inhibition of inflammation is partially beneficial for clinical TB outcomes. However, the nuances of how and when to inhibit inflammation during clinical treatment of TB remain poorly understood.

Our study has several potential limitations. The association findings from the case-control study might not be due to polymorphisms within TOLLIP, but this seems unlikely. TOLLIP is fairly isolated on chromosome 11 in humans and is flanked by the mucin 2 precursor and BRSK2, both ∼100 kbp upstream and downstream, respectively. Neither protein has been associated with immune responses. A second limitation of any genetic study relates to multiple comparisons that require adjustment of significance thresholds for p values depending on the number of analyses performed. Even with the most conservative Bonferroni correction of our genetic association data (multiplying the p value by 6), our findings remain highly significant. Furthermore, our experimental data suggests that two polymorphisms are genetic markers of TOLLIP function. A third limitation of this study and case-population studies is the misclassification of controls, as some of the cord-blood subjects may develop TB in the future. Although the use of cord blood samples can lead to a modest loss of power, the misclassification of controls underestimates the genetic risk of polymorphisms. Finally, candidate gene association studies are subject to confounding because of population substructure. However, our study population was the Vietnamese Khinh, a relatively homogenous population in South East Asia. We previously found no evidence of population stratification in this population using control genomic SNPs (57).

These data suggest that TOLLIP has an important role in the pathogenesis of TB by regulating both proinflammatory and anti-inflammatory pathways. Although further study is required, modulating TOLLIP activity and function may lead to important breakthroughs in TB vaccine design and immune drug development.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Table I.** TOLLIP gene region single nucleotide polymorphisms and genotype frequencies in the control and tuberculosis groups.

<table>
<thead>
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<th>SNP</th>
<th>Group</th>
<th>Genotype</th>
<th>Genotype Analysis</th>
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<td></td>
<td>00</td>
<td>01</td>
<td>11</td>
<td>P</td>
<td>H-W</td>
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<tr>
<td>rs5743899</td>
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<td>252 (.335)</td>
<td>391 (.519)</td>
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<tr>
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<td>rs3750920</td>
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<td>296 (.460)</td>
<td>242 (.376)</td>
<td>106 (.165)</td>
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</table>
HapMap LD Matrix (CEU)

A

B

HapMap LD Matrix (CHB)

C

D
**Supplemental Figure 1.** Linkage disequilibrium plots of TOLLIP polymorphisms in Seattle cohort. Linkage disequilibrium plots with $R^2$ (A) and $D'$ (B) values for subjects in Seattle. White boxes on top of plot show frequency of polymorphism. Degree of shading proportionate to $D'$ or $R^2$ value.

**Supplemental Figure 2.** Linkage disequilibrium plots of TOLLIP polymorphisms from HapMap populations of European descent in Utah (CEU) (A, B) and Han Chinese in Beijing (CHB) (C, D). Disequilibrium plots with $R^2$ (A, C) and $D'$ (B, D) values. White boxes show frequency of haplotype. Degree of shading proportionate to $D'$ or $R^2$ value.