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Journal
American Journal of Respiratory and Critical Care Medicine, 196(4)

ISSN
1073-449X

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Publication Date
2017-08-15

DOI
10.1164/rccm.201611-2346oc

Peer reviewed

ORIGINAL ARTICLE

A Functional Toll-Interacting Protein Variant Is Associated with Bacillus Calmette-Guérin–Specific Immune Responses and Tuberculosis

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Abstract

Rationale: The molecular mechanisms that regulate tuberculosis susceptibility and bacillus Calmette-Guérin (BCG)-induced immunity are mostly unknown. However, induction of the adaptive immune response is a critical step in host control of *Mycobacterium tuberculosis*. Toll-interacting protein (TOLLIP) is a ubiquitin-binding protein that regulates innate immune responses, including Toll-like receptor signaling, which initiate adaptive immunity. TOLLIP variation is associated with susceptibility to tuberculosis, but the mechanism by which it regulates tuberculosis immunity is poorly understood.

Objectives: To identify functional TOLLIP variants and evaluate the role of TOLLIP variation on innate and adaptive immune responses to mycobacteria and susceptibility to tuberculosis.

Methods: We used human cellular immunology approaches to characterize the role of a functional TOLLIP variant on monocyte mRNA expression and *M. tuberculosis*–induced monocyte immune functions. We also examined the association of TOLLIP variation with BCG-induced T-cell responses and susceptibility to latent tuberculosis infection.

Measurements and Main Results: We identified a functional TOLLIP promoter region single-nucleotide polymorphism, rs5743854, which was associated with decreased TOLLIP mRNA expression in infant monocytes. After *M. tuberculosis* infection, TOLLIP-deficient monocytes demonstrated increased IL-6, increased nitrite, and decreased bacterial replication. The TOLLIP-deficiency G/G genotype was associated with decreased BCG-specific IL-2^+ $CD4^+$ T-cell frequency and proliferation. This genotype was also associated with increased susceptibility to latent tuberculosis infection.

Conclusions: TOLLIP deficiency is associated with decreased BCG-specific T-cell responses and increased susceptibility to tuberculosis. We hypothesize that the heightened antibacterial monocyte responses after vaccination of TOLLIP-deficient infants are responsible for decreased BCG-specific T-cell responses. Activating TOLLIP may provide a novel adjuvant strategy for BCG vaccination.

Keywords: bacillus Calmette-Guérin; tuberculosis; Toll-interacting protein; adaptive immunity; genetics

(Received in original form November 22, 2016; accepted in final form May 2, 2017)

Supported by National Institutes of Health (K08 AI102971, J.A.S.; K24 AI089794, T.R.H.; NO1-AI-70022 [Tuberculosis Research Unit], T.R.H. and W.A.H.), the Burroughs Wellcome Foundation (T.R.H.), and the Dana Foundation (T.R.H. and W.A.H.).

Author Contributions: J.A.S., T.R.H., T.J.S., M.H., J.S.C., and W.A.H. conceived of the study design. J.A.S., D.J.H., M.S., W.A.H., M.M., and T.R.H. developed and recruited cohorts that were used in this study. J.A.S., R.D.W., and G.J.P. collected data used in this experiment. J.A.S., T.R.H., and R.D.W. analyzed the data. L.L., R.G., E.G.H., M.D., and D.J.H. contributed to interpretation of results and contributed advanced statistical help. J.A.S. wrote the manuscript with assistance from T.R.H., T.J.S., J.S.C., M.H., and W.A.H. All authors reviewed, revised, and approved the manuscript for submission.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 196, Iss 4, pp 502–511, Aug 15, 2017

Originally Published in Press as DOI: 10.1164/rccm.201611-2346OC on May 2, 2017
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Although tuberculosis is the most common cause of mortality from an infectious disease in the world, current vaccines are imperfect. The genetic factors that regulate the vaccine immune response to bacillus Calmette-Guérin and susceptibility to *Mycobacterium tuberculosis* infection and tuberculosis disease are poorly understood.

What This Study Adds to the Field: We demonstrate that a functional variant of Toll-interacting protein (TOLLIP), an immune regulatory protein, alters TOLLIP mRNA expression in South African infant monocytes. The TOLLIP-deficient genotype was associated with decreased frequency and altered quality of bacillus Calmette-Guérin–specific memory T-cell responses and was also associated with increased susceptibility to multiple tuberculosis clinical phenotypes. Interestingly, TOLLIP-deficient monocytes had increased inflammatory and antimicrobial metabolite production and decreased *M. tuberculosis* replication. These data suggest that increased control of *M. tuberculosis* and bacillus Calmette-Guérin by the innate immune system leads to diminished effective T-cell responses. Our study identifies TOLLIP as a potential target for enhancing vaccine immunity while minimizing potentially harmful innate immune cytokine responses.

Tuberculosis (TB) leads the world as a cause of morbidity and mortality, and so improvement on current therapies and vaccines for TB remains a public health priority (1). The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine was first administered in 1921 and remains the only approved vaccine for TB (2). BCG is effective in infants, but has variable and partial efficacy in adults for preventing pulmonary TB (3). Genetic factors are associated with susceptibility to TB disease, tuberculin skin test sensitivity (4), and tumor necrosis factor (TNF) or IFN-γ responses to BCG or *Mycobacterium tuberculosis* (MTb) (4–9). Although these data suggest that genetic factors regulate susceptibility to TB and BCG vaccine responses, the specific genes and mechanisms are largely unknown, especially for latent and pediatric TB. An improved understanding of the factors that regulate development of effective immune responses to BCG vaccination and susceptibility to TB may uncover novel strategies for vaccine development.

Multiple factors influence the development of effective immune responses to MTb, including autophagy (10–12); endosomal transport (13); Toll-like receptor (TLR) signaling (14, 15); and induction of IL-1β, other cytokines, and antimicrobial molecules (16, 17). Adaptive immunity, particularly initiation and development of effective $CD4^+$ T-cell responses, is also critical for MTb control (18, 19). Toll-interacting protein (TOLLIP) regulates many of these critical immune processes including TLR2, TLR4, and IL-1R signaling, autophagy, and endosomal transport (20–23). Common variation in the TOLLIP gene has been associated with leprosy, leishmaniasis, and idiopathic pulmonary fibrosis (24–27). TOLLIP variation is also strongly associated with susceptibility to adult TB disease (24). We previously identified two TOLLIP variants that were strongly associated with adult TB. One of these variants (haplotype-tagging single-nucleotide polymorphism [SNP] rs5743899) was also associated with decreased TOLLIP mRNA expression and increased IL-6 and TNF secretion in monocytes stimulated with TLR ligands (24). These studies did not identify a causal polymorphism or investigate the effects of TOLLIP variation on live MTb-induced innate and adaptive immune responses.

We hypothesized that a single functional variant in the TOLLIP promoter region alters mRNA expression of TOLLIP in monocytes and critically impacts the innate and adaptive immune response to MTb and BCG. In this study, we identify a common functional variant of the TOLLIP gene and use it to understand how TOLLIP alters human immune function after mycobacterial infection. This framework may lead to improved therapies and vaccines for intracellular infections, including MTb. Some of the results of these studies have been previously reported in the form of an abstract (28).

Methods

Ethics Statement

Approval for all human study protocols was obtained from the human subjects review boards at the University of Washington School of Medicine (Seattle, WA). The South African study included written informed consent from the parent or legal guardian of the participant and approval by the University of Cape Town Research Ethics Committee. Written informed consent was received from all participants before inclusion in the study.

Human Subjects

Subjects with latent TB infection (LTBI) were recruited through the TB Control Program, Public Health–Seattle and King County, and clinical characteristics have been described previously (29) and are summarized below. A close contact was defined as an individual with at least 8 hours' exposure to an AFB-smear positive index case in an enclosed space during the preceding 3 months. The online supplemental METHODS provide complete details of study inclusion and exclusion criteria.

South African study participants were enrolled at the South African Tuberculosis Vaccine Initiative field site in Worchester, South Africa, as part of a larger study on BCG vaccination with 11,680 infants (30). This area has one of the highest rates of TB incidence in the world, with an incidence of 3% among children in the study population (30). A nested genetics case–control study was performed with identification of cases and control subjects during a 2-year prospective observation period after vaccination at birth. The criteria for detection of TB cases and inclusion of control participants have been described previously and is summarized in the online supplemental METHODS (30).

The Seattle study group included 62 healthy volunteers who donated blood samples. The ethnic composition of these subjects was 73% white (45 of 62) and 27% Asian (17 of 62).

All statistical analyses are described in the online supplemental METHODS and were performed using Stata 13.0 (Statacorp LLC, College Station, TX) and Prism 6 (GraphPad Software, La Jolla, CA) software. The remainder of all experimental procedures, including molecular cloning, sample handling and preparation,

microbiologic techniques, and flow cytometry, are described in detail in the online supplemental METHODS.

Results

TOLLIP mRNA Expression Is Genetically Regulated

We previously discovered that SNP rs5743899 was strongly associated with TOLLIP monocyte mRNA expression and susceptibility to adult TB in a Vietnamese cohort (24). To define a functional TOLLIP SNP, we identified common variants in high linkage disequilibrium (LD) with rs5743899 using the 1,000 Genomes database (http://grch37.ensembl.org/index.html) across multiple populations (genomic SNP map) (Figure 1A; *see* Figure E1 in the online supplement). TOLLIP (chromosome 11p15.5) contains six exons and 430 SNPs in the Yoruba population as described in the 1,000 Genomes Project (31). We identified three SNPs (rs5743854, rs7481967, and rs4963035) that were in LD with rs5743899 (R^2 LD > 0.2) in multiple populations (South African cohort LD) (Figure 1B) (1,000 Genomes data, *see* Figure E1). We created transcriptional reporters by fusing 1-kb fragments of TOLLIP promoter containing one of three promoter region variants with the firefly luciferase gene. TOLLIP rs5743854 C allele was associated with 10-fold higher relative luciferase activity compared with the G allele ($P < 0.0001$) (Figure 1C). SNPs rs7481967 and rs4963035 were not associated with differences in promoter-luciferase activity (Figures 1D and 1E).

We then isolated mRNA from monocytes of 147 South African infants and measured transcript levels by reverse transcriptase polymerase chain reaction. TOLLIP rs5743854 genotype was associated with mRNA expression (Figure 1F) ($P = 0.0008$, Kruskal-Wallis test) with the G/G genotype associated with lower mRNA expression ($P = 0.0001$, Mann-Whitney test, recessive model comparing G/G [glyceraldehyde phosphate dehydrogenase–normalized TOLLIP median expression = 1.43; n = 19] vs. GC/CC [median C/C = 3.96, G/C = 3.28; n = 128]). We validated this finding using the GTex Project (www.gtexportal.org/home) and found that rs5743854 is significantly associated with TOLLIP gene expression in several tissue types (data not shown) (32). Together, these data suggest that the G allele of SNP rs5743854 is a promoter region expression quantitative trait locus that is associated with TOLLIP deficiency.

We then reevaluated whether rs5743854 was associated with TB in our original cohort of adult TB disease in Vietnam. Examination of LD between rs57473899 and rs5743854 in the KHV (Kinh in Ho Chi Minh City) (*see* Figure E1) populations using the 1,000 Genomes browser confirms that these two SNPs are in nearly complete LD (R^2 LD = 0.96;

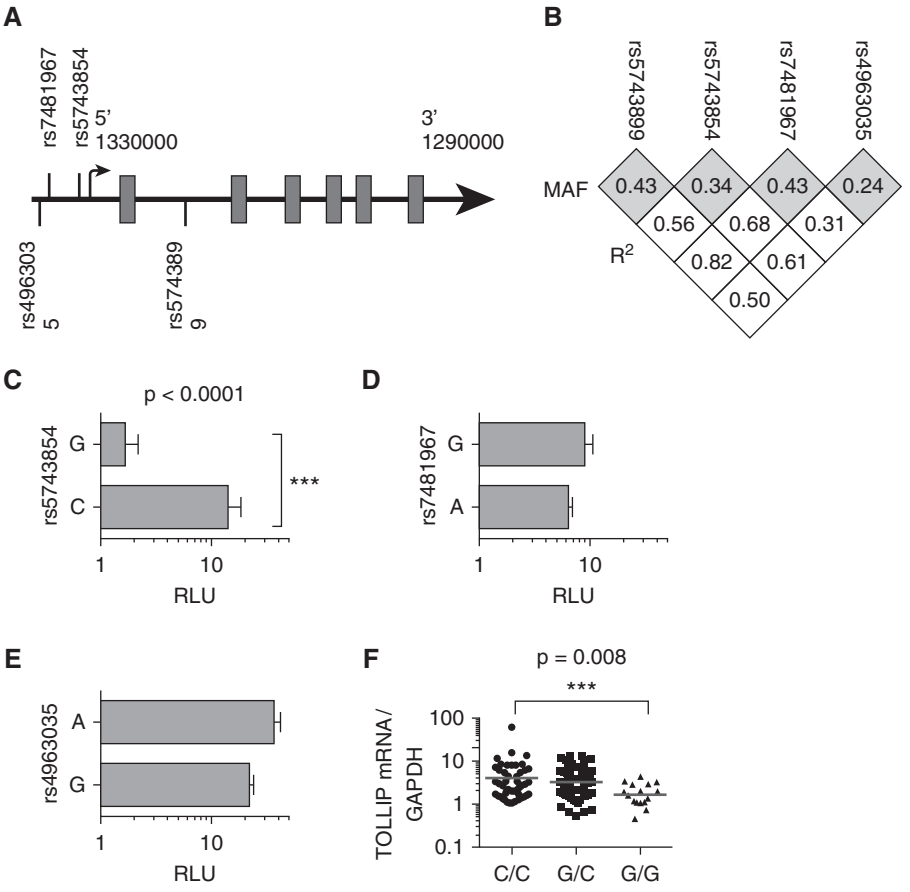

Figure 1. Single-nucleotide polymorphism (SNP) rs5743854 regulates Toll-interacting protein (TOLLIP) mRNA expression in South African infant monocytes. (*A*) Genomic location of TOLLIP SNPs. The TOLLIP coding region ± 10 kb flanking regions among South African case–control genetic study population are shown. TOLLIP spans 36 kb on chromosome 11p15.5 and consists of six exons (*dark gray regions*). Four candidate functional SNPs are shown. *Curved arrow* = transcriptional start site. (*B*) Linkage disequilibrium of four potential regulatory TOLLIP SNPs associated with pediatric tuberculosis in the Cape Mixed Ancestry population. Minor allele frequencies of TOLLIP SNPs of interest (*gray squares*) and R^2 linkage disequilibrium values (*white squares*) are indicated. (*C–E*) Assessment of candidate functional SNPs with luciferase signaling assay. HEK293T cells were transfected with firefly luciferase-expressing plasmids (pGL4) under the control of various promoter fragment sequences. Cells were cotransfected with the *Renilla* luciferase gene under an SV40 promoter; data are reported as relative luciferase units, which are calculated by dividing the firefly luciferase signal by the *Renilla* luciferase signal. Firefly luciferase–expressing pGL4 plasmids under the control of 1-kb promoter sequence fragment containing (*C*) rs5743854 allele G or allele C, (*D*) rs7481967 allele G or allele A, or (*E*) rs4963035 allele A or allele G are shown. All data are representative of at least three independent experiments. ***$P < 0.001$ by Student's two-sided *t* test. (*F*) Correlation between TOLLIP SNP rs5743854 genotype and TOLLIP mRNA expression in peripheral blood monocytes from 156 South African 10-week-old infants. mRNA was isolated from plate adherent monocytes derived from cryopreserved peripheral blood mononuclear cells. The *line* indicates the median value for each genotype. ***$P = 0.008$ by Kruskal-Wallis test. MAF = minor allele frequency; RLU = relative luciferase units.

Table 1. TOLLIP SNP rs5743854 Genotype Frequencies in Case–Control Study of LTBI in Seattle

	Clinical Status	Genotype			HWE *P* Value	Geno *P* Value	Unadjusted Recessive Model	
		C/C	G/C	G/G			*P* Value	OR (95% CI)
rs5743854	No LTBI	65 (0.56)	43 (0.37)	7 (0.07)	0.75	0.007	0.008	3.26 (1.36–7.81)
	LTBI	58 (0.39)	65 (0.44)	26 (0.17)				

Definition of abbreviations: CI = confidence interval; Geno = genotypic genetic model; HWE = Hardy-Weinberg equilibrium; LTBI = latent tuberculosis infection; OR = odds ratio; Recessive = recessive genetic model; SNP = single-nucleotide polymorphism; TOLLIP = Toll-interacting protein.
Data from unadjusted models are presented.

http://grch37.ensembl.org/index.html). Based on these data, we inferred that the G/G genotype of rs5743854 was associated with increased risk for pulmonary and meningeal TB disease in a Vietnamese population based on prior published data (24).

TOLLIP Variation Is Associated with Increased Risk for Latent MTb Infection

We next examined whether this SNP was associated with other TB phenotypes. Because TOLLIP is evolutionarily conserved from yeast and regulates basic processes of cellular stress and inflammation, we hypothesized it may have a role in TB latency, reactivation, and progression (20, 23). We tested the hypothesis that rs5743854 was associated with risk for LTBI in a cohort of MTb-exposed individuals. We genotyped rs5743854 in 143 TB-exposed participants with LTBI and 106 without LTBI (cohort characteristics) (*see* Table E1). rs5743854 was significantly associated with risk for LTBI (Table 1) ($P = 0.007$, genotypic model). This association best fit a recessive model of inheritance (Table 1) (odds ratio [OR], 3.26; 95% confidence interval [CI], 1.36–7.81; $P = 0.008$).

We then evaluated whether the association between rs5743854 and susceptibility to LTBI may have been confounded because of epidemiologic factors. To address ethnic heterogeneity, we genotyped a panel of 95 ancestry informative markers, then performed a principal components analysis and identified the top principal component after comparing models using likelihood ratio tests. The association between rs5743854 remained statistically significant after adjustment of rs5743854 for the top principal component (OR, 2.59; 95% CI, 1.06–6.38; $P = 0.037$) (Table 2) (29, 33). We describe the genetic association between rs5743854 and LTBI susceptibility when stratified by ethnicity (Asian vs. black African) in Table E2 (full details in online supplemental Results). Rs5743854 was associated with LTBI in both populations in a genotypic model, but in the recessive model, was only associated in individuals with black ethnicity. In a multivariate model that included characteristics of the index case (bilateral findings on chest radiography and smear grade) and contacts (age and foreign birth), rs5743854 remained statistically significant (OR, 3.60; 95% CI, 1.34–9.72; $P = 0.011$) (Table 2). Clinical characteristics were determined through stepwise selection, starting with a model with all variables and then removing one variable at a time to find the best performing model. Together, these data suggest that demographic, ethnic, and epidemiologic characteristics do not explain the association of TOLLIP rs5743854 with susceptibility to LTBI.

rs5743854 Genotype G/G Is Associated with Diminished BCG-Specific T-Cell Responses

On discovery of a functional SNP associated with increased risk for TB disease and LTBI, we hypothesized that it may impair critical adaptive immune functions. Multiple lines of evidence have identified T cells, particularly Th1-type $CD4^+$ T cells, as a critical component of the immune response to MTb (17). To test this hypothesis, whole blood was collected from BCG-vaccinated infants at age 10 weeks (n = 174 divided into discovery and validation groups). None of these infants developed TB symptoms during the study period. Samples were stimulated *ex vivo* with media, BCG, or staphylococcal enterotoxin B, and analyzed by flow cytometry to evaluate the quality and magnitude of the BCG-specific T-cell responses (gating strategy) (Figure 2A).

In the discovery cohort (n = 85), the rs5743854 G allele was associated with decreased BCG-specific IL-2 and IL-22 $CD4^+$ T-cell responses (Figure 2B) ($P = 0.014$ and 0.016, respectively;

Table 2. Multivariable Model of TOLLIP rs5743854 Association with Latent *Mycobacterium tuberculosis* Infection in Contacts of Patients with Tuberculosis

Variable	Odds Ratio (95% CI)	*P* Value
Unadjusted additive model		
TOLLIP rs5743854	3.26 (1.36–7.81)	0.008
Additive model adjusted for ethnicity		
TOLLIP rs5743854	2.59 (1.06–6.38)	0.037
Additive model adjusted for clinical variables		
TOLLIP rs5743854	3.60 (1.33–9.72)	0.011
Bilateral radiographic disease	1.82 (0.95–3.46)	0.069
Smear burden	1.11 (0.89–1.37)	0.343
Foreign birth	2.71 (1.14–6.42)	0.024
Age	1.01 (0.99–1.02)	0.395

Definition of abbreviations: CI = confidence interval; TOLLIP = Toll-interacting protein.
Multivariable regression model was created using likelihood ratios in a stepwise fashion.

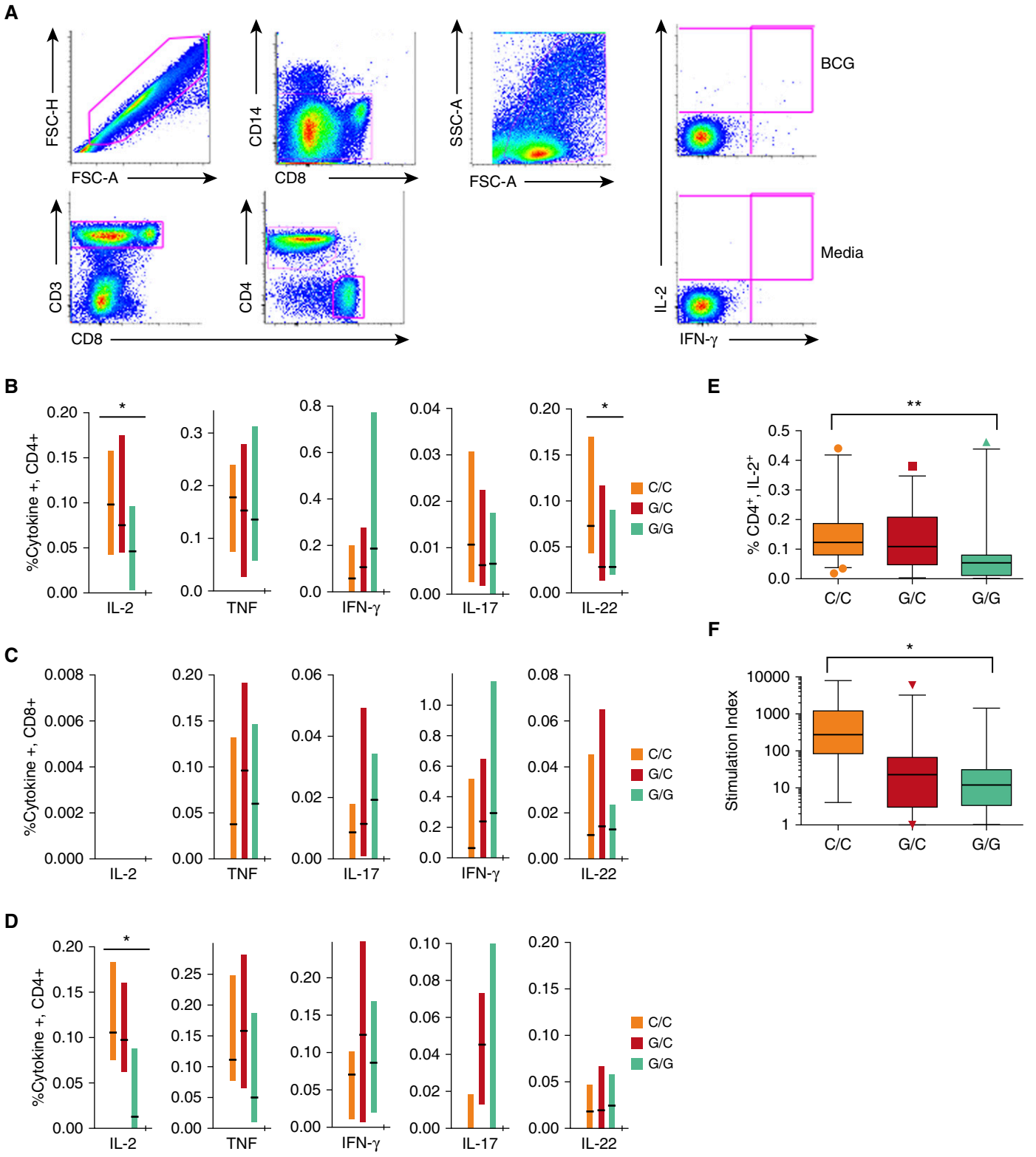

Figure 2. Toll-interacting protein rs5743854 deficiency allele is associated with diminished bacillus Calmette-Guérin (BCG)-specific T-cell cytokine responses and proliferation. Whole blood was incubated with media or 10^5 cfu/ml Danish BCG, combined with anti-CD28/CD49d for 7 hours before adding Brefeldin A for an additional 5 hours, then fixed and permeabilized. (*A*) Gating strategy used for identifying $CD4^+$ and $CD8^+$ T cells. Representative dot plots from a single study participant are shown. From *left* to *right*, leukocytes from whole blood were acquired and cell doublets excluded. $CD14^+$ cells were excluded, then leukocytes were further identified. T cells were selected and then separated into $CD4^+$ and $CD8^+$ T cells. (*B–D*) Frequencies of IL-2,

Mann-Whitney of recessive genetic model). rs5743854 was not associated with BCG-specific TNF, IFN-γ, or IL-17A in $CD4^+$ cells or $CD8^+$ T-cell responses (Figure 2C). In the validation dataset (infant samples, n = 89), we found that rs5743854 G/G was associated with decreased frequency of IL-2^+ $CD4^+$ T cells in a recessive genetic model ($P = 0.011$, Mann-Whitney test) (Figure 2D). The IL-22^+ $CD4^+$ T-cell association did not replicate. We found that rs5743854 G/G genotype was associated with decreased frequency of IL-2^+ $CD4^+$ T cells when all data were combined together in a recessive model ($P = 0.022$, Mann-Whitney test) (Figure 2E). We also evaluated the association between TOLLIP rs5743854 and polyfunctional $CD4^+$ T cells using COMPASS (34), a Bayesian hierarchical framework that models and combines all observed multifunctional cell subset responses into a single polyfunctionality score. We found that the rs5743854 genotype was not significantly associated with polyfunctionality by this method (*see* Figure E3).

Because IL-2 is a proproliferative cytokine that amplifies existing Th1 cytokine responses (35), we evaluated the association between rs5743854 and T-cell proliferation in peripheral blood mononuclear cells from 65 infants vaccinated with BCG and restimulated at 10 weeks of age. rs5743854 was associated with decreased BCG-specific $CD4^+$ T-cell proliferation based on the stimulation index of Oregon Green ($P = 0.048$, linear model) (Figure 2F). Together, these data suggest that individuals with rs5743854 G/G genotype have decreased BCG-specific IL-2^+ $CD4^+$ T-cell responses and T-cell proliferation.

We also evaluated the association between rs5743854 and pediatric TB in 227 cases and 626 control subjects in Cape Town, South Africa (*see* Table E3) (30, 36). rs5743854 was not significantly associated with TB ($P = 0.229$, recessive model) (*see* Table E4), but we observed a trend toward an increased risk (G/G genotype frequency of 0.09 in control subjects and 0.12 in cases). Although the clinical cohorts tested currently and in prior studies (24) are heterogeneous with regards to age of onset of TB and clinical phenotype, there was a consistent association of TOLLIP deficiency with increased risk of LTBI or TB disease ($P = 3.6 \times 10^{-5}$; OR, 1.62 [1.29–2.05], by random effects meta-analysis).

TOLLIP Deficiency Is Associated with Increased Proinflammatory Cytokine Secretion after TLR Stimulation

We next investigated the immune mechanisms underlying the association of TOLLIP deficiency with decreased BCG-induced T-cell responses. We hypothesized that TOLLIP variation altered innate cytokine responses to mycobacteria, influencing inflammation, mycobacterial replication, and priming of the adaptive immune response. In prior studies, TOLLIP negatively regulated inflammation in monocytes (24). We stimulated peripheral blood mononuclear cells with LPS (TLR4 ligand) and *M. tuberculosis* whole-cell lysate (MTb lysate), and measured secreted IL-6. rs5743854 genotype G/G was associated with significantly increased IL-6 after LPS (Figure 3A) ($P < 0.0001$, Mann-Whitney test, recessive model [G/G vs. C/C+G/C]) and MTb lysate stimulation (Figure 3B) ($P < 0.0001$; C/C individuals = 35, C/G = 21, G/G = 8; Mann-Whitney test, recessive model [G/G vs. C/C + G/C]). To confirm this effect was caused by TOLLIP deficiency, we created TOLLIP-deficient THP-1 monocytes through CRISPR/Cas9 gene editing. We confirmed our knockout using sequencing (data not shown) and Western blotting (Figure 3C). TOLLIP-deficient THP-1 cells secreted significantly more TNF (Figure 3D) and IL-6 (Figure 3E) than control cells transfected with an empty vector (control) after stimulation with the TLR2/TLR6 ligand PAM2 (250 ng/ml) or TLR4 ligand LPS (10 ng/ml) for 24 hours ($P < 0.0001$) (Figures 3D and 3E). We validated these results using siRNA knockdown; TOLLIP deficiency was associated with increased IL-6 and TNF responses after LPS and PAM2 (TLR2/TLR6) stimulation ($P < 0.001$) (*see* Figure E4, and full details in online supplemental Results).

TOLLIP Deficiency Decreases MTb Replication in Monocytes

Because TOLLIP deficiency was associated with increased IL-6 and TNF, we hypothesized that TOLLIP deficiency led to diminished intracellular mycobacterial replication, thus influencing priming of adaptive immune responses. We used TOLLIP-deficient THP-1 cells to evaluate the overall effect of TOLLIP on MTb replication in monocytes. TOLLIP-deficient THP-1 (TOLLIP-KO) cells stimulated with live MTb strain H37Rv at multiplicity of infection (MOI) 5 for 24 hours produced significantly higher TNF than control cells ($P < 0.0001$) (Figure 4A). We hypothesized that TOLLIP deficiency activates killing mechanisms within macrophages, including reactive nitrogen production, after MTb infection. TOLLIP-KO cells infected with MTb (MOI 25) for 24 hours produced significantly more nitrite, a stable intermediate of nitric oxide, than control cells, which produced little detectable nitrite after a 24-hour stimulation ($P < 0.01$) (Figure 4B). We evaluated TOLLIP's overall effect on MTb replication in monocytes. After phorbol myristate acetate differentiation, monocytes were infected with an MTb Erdman strain expressing the *luxCDABE* operon from *Vibrio harveyi* (MTb-lux, MOI 5). Luminescence from this organism strongly correlates with MTb colony-forming units *in vitro* ($R^2 = 0.99$) (*see* Figure E5). MTb replication within TOLLIP-KO cells was decreased compared with control after Day 4 ($P < 0.001$) (Figure 4C).

Figure 2. (Continued). tumor necrosis factor, IFN-γ, IL-17, and IL-22 cytokine-expressing T cells (background-subtracted), stratified by rs5743854 genotype. Median with intraquartile range shown. $CD4^+$ T cells (*B*) and $CD8^+$ T cells (*C*) in discovery cohort (n: C/C = 45, G/C = 34, G/G = 10). No $CD8^+$ IL-2 was detected in this cohort. (*D*) Frequency and cytokine expression profile of BCG-specific $CD4^+$ T cells in validation cohort (n: C/C = 40, G/C = 35, G/G = 5). (*E*) Frequency and cytokine expression profile of frequency of all BCG-specific IL-2^+ $CD4^+$ T cells measured in a combined cohort (n: C/C = 85, G/C = 69, G/G = 15). Statistical significance determined by Mann-Whitney test of recessive genetic model (C/C + G/C vs. G/G genotype). (*F*) $CD4^+$ T-cell proliferation after BCG restimulation. Peripheral blood mononuclear cells from 65 infants were stained with Oregon Green overnight and incubated with media or BCG for 6 days. Cells were assessed for Oregon Green and stratified by rs5743854. Tukey plot with the Stimulation Index (percentage of BCG-restimulated, Oregon $Green_{low}$ cells/percentage of media-restimulated, Oregon $Green_{low}$ cells). Statistical significance determined using linear model. $^*P < 0.05$, $^{**}P < 0.01$. FSC = forward scatter; SSC = side scatter; TNF = tumor necrosis factor.

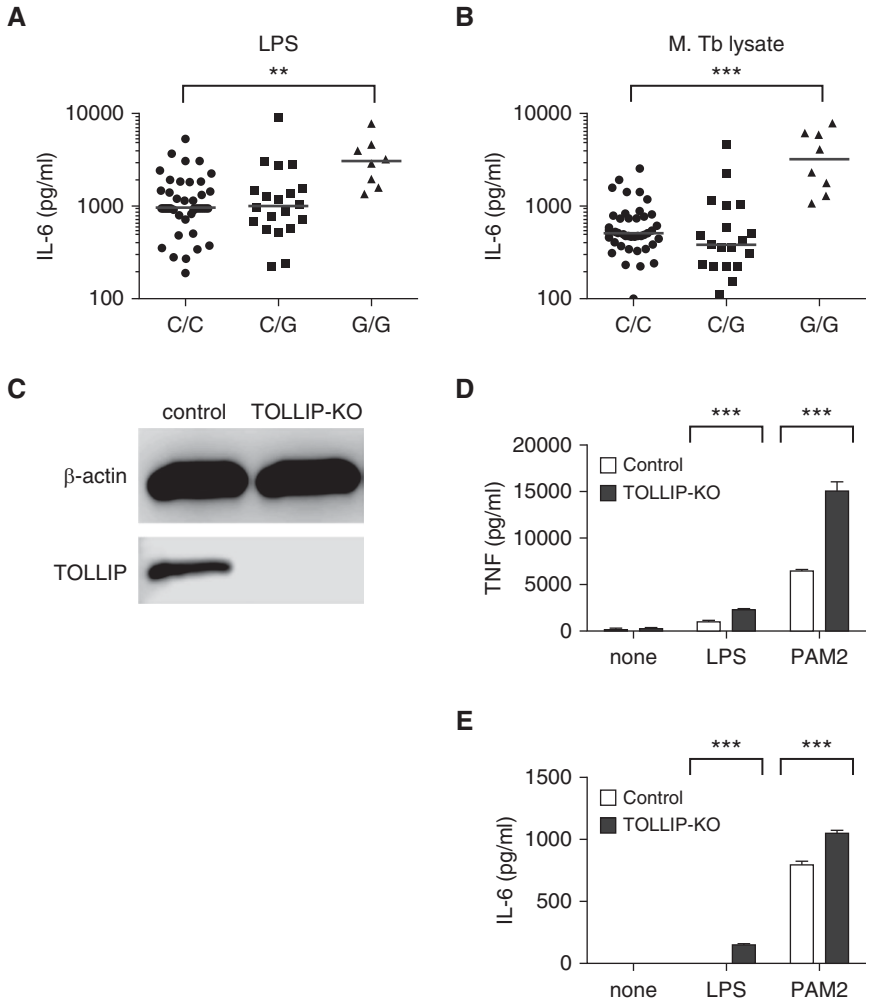

Figure 3. Toll-interacting protein (TOLLIP) deficiency is associated with increased proinflammatory cytokine secretion after Toll-like receptor (TLR) stimulation. Peripheral blood mononuclear cells were collected from 64 healthy volunteers in Seattle and stimulated with LPS (*A*, 10 ng/ml) or *Mycobacterium tuberculosis* whole-cell lysate (*B*, 1 μg/ml) for 24 hours. Supernatants were collected, IL-6 responses were measured by ELISA, and the results were stratified by rs5743854 genotype. C/C individuals = 35, C/G = 21, G/G = 7; Mann-Whitney test of recessive model (G/G vs. G/C + C/C) was used to determine statistical significance. (*C*) Empty vector (control) THP-1 cells and TOLLIP-deficient (TOLLIP-KO) THP-1 cells were generated with CRISPR/Cas9, and TOLLIP protein expression was assessed by Western blot with β-actin comparison as a loading control. (*D* and *E*) Concentrations of tumor necrosis factor (*D*) or IL-6 (*E*) from supernatants of control and TOLLIP-KO cells were compared after 10 ng/ml LPS stimulation (TLR4 agonist) and 250 ng/ml PAM2 stimulation (TLR2/6 agonist) overnight. Student's *t* test was used to determine statistical significance. $^{**}P < 0.01$; $^{***}P < 0.0001$. M. Tb = *Mycobacterium tuberculosis*; TNF = tumor necrosis factor.

Discussion

The principal results of this study are identification of a functional upstream polymorphism of the TOLLIP gene, rs5743854, and its association with decreased TOLLIP mRNA expression in monocytes, decreased BCG-specific IL-2^+, $CD4^+$ T-cell responses, and increased risk of TB disease and LTBI. Furthermore, TOLLIP-deficient monocytes have increased production of proinflammatory cytokines and nitrite as well as diminished intracellular MTb replication. These data suggest that TOLLIP increases risk for TB disease and MTb infection by diminishing the adaptive immune response, possibly through increased control of MTb in macrophages and antigen-presenting cells.

Individuals with rs5743854 G/G genotype, when compared with other genotypes, developed decreased BCG-specific IL-2 production from $CD4^+$ T cells, without significant differences in IFN-γ or TNF, 10 weeks after vaccination. IL-2–producing $CD4^+$ T cells mediate control of chronic MTb infection in murine models (19). This T-cell phenotype may represent a naive or T central memory (T_{CM}) population that is important for effective BCG-induced immunity (19). After BCG vaccination, IL-2 is produced predominantly by T cells with a naive profile ($CD45RA^+CCR7^+$) or a central memory profile (T_{CM}, CD45RA-$CCR7^+$) (37). T_{CM} produce IL-2 and/or TNF (without coexpression of IFN-γ) after BCG vaccination (37). Naive T cells and T_{CM} are characterized by their prominent proliferative capacity and cytokine production, and are important T-cell populations for control of chronic infections (38). Boosting the T_{CM} population in particular is important for development of effective TB vaccine immunity (39) and in the control of TB (40).

We hypothesize that several possible mechanisms may explain how TOLLIP variation leads to diminished BCG-specific IL-2^+ $CD4^+$ T-cell populations. Increased inflammation in the setting of chronic antigen exposure provokes increased T-cell differentiation in experimental models of infection and autoimmmunity (19, 39). Because TOLLIP deficiency leads to a hyperinflammatory phenotype, decreasing TOLLIP may lead to enhanced chronic inflammation that diminishes the proliferative capacity of T cells after BCG vaccination. Furthermore, increased initial inflammation in TOLLIP-deficient individuals after live BCG vaccination may diminish intracellular mycobacterial growth, decreasing the effective dose of vaccine and diminishing antigen presentation by macrophages and dendritic cells. In this paper, we demonstrate several antimicrobial pathways that are influenced by TOLLIP, including TNF induction and nitric oxide activity. These mechanisms may directly decrease the number of BCG bacilli *in vivo*, influencing the duration and intensity of antigen-presenting cell–T-cell interactions. These results may also limit spread of the live vaccine throughout the organism, a critical step in the induction of effective vaccine immunity via BCG (41). Studies in viral vaccines have shown that increased antigen load may increase the

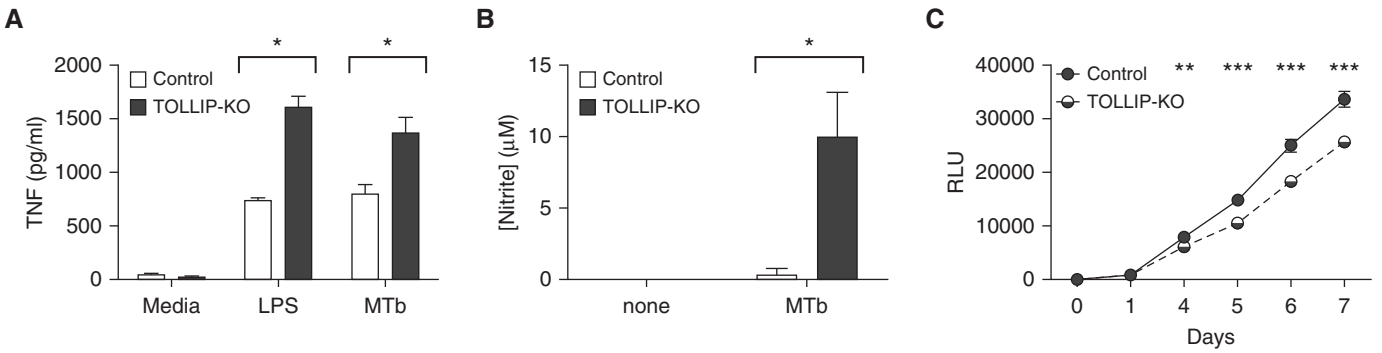

Figure 4. Toll-interacting protein (TOLLIP) regulates immune response and *Mycobacterium tuberculosis* (MTb) replication. Empty vector (control) and TOLLIP-deficient (TOLLIP-KO) THP-1 cells were generated and stimulated in the following manner. (*A*) Concentration of tumor necrosis factor from supernatants of control and TOLLIP-KO cells was compared after 10 ng/ml LPS stimulation or MTb H37Rv infection (multiplicity of infection [MOI] 5) overnight. (*B*) Reactive nitrogen species production was compared in control with TOLLIP-KO cells infected with MTb H37Rv (MOI 25) or media for 24 hours. Nitrite concentrations were determined in supernatants using the Griess reagent system. Limit of detection for reagent system was 1 μM. Cytokine and nitrite results are the mean ± SEM of three replicates per condition and are representative of three independent experiments. Statistical significance was measured by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$. (*C*) MTb replication in control and TOLLIP-KO cells over 7 days. Cells were cultured and then infected with MTb Erdman strain expressing the *lux* gene (MOI 5). Luminescence was measured for each group over the next 7 days. Mycobacterial replication experiments are shown as the mean ± SEM. These experiments were performed independently at least three times to ensure reproducibility. Statistical significance was measured by Student's *t* test for each time point by comparing TOLLIP-KO and control cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$. RLU = relative luciferase units; TNF = tumor necrosis factor.

number of memory T cells producing IL-2 (42). Alternately, the characteristics of macrophages themselves may be influenced by TOLLIP deficiency.

We demonstrate that TOLLIP suppresses nitrite production in human cells, and inducible nitric oxide synthase in mice influences macrophage polarization, inducing a more "M2" phenotype (43). These effects may impact the adaptive immune response in complex ways. Monocyte-specific cytokine production may be altered by TOLLIP deficiency in a way that influences T-cell differentiation after BCG vaccination. For example, after TOLLIP knockdown, monocytes secrete less IL-10 after TLR4 stimulation (24). IL-10 production may provide a basis to control differentiation of T cells and may prolong protection from intracellular infections after vaccination (42).

TOLLIP regulation of TLR1/2/6 -dependent cytokine secretion may lead to altered functional quality of BCG-specific T-cell response. Several studies have shown that TLR2 activation in dendritic cells promotes strong Th2, but not Th1, $CD4^+$ T-cell immune responses (44, 45). Conversely, induction of TLR2 signaling may lead to expansion of antigen-specific T regulatory cell subsets (46). Individuals with hypofunctional TLR1 and TLR6 genetic variants, both necessary for TLR2 signaling, developed increased BCG-specific IL-2 and IFN-γ T-cell responses when compared with those with effective TLR1 and TLR6 (9). Thus, inhibition of TLR2 in the context of a live vaccine, for example via coadministration of a small molecule inhibitor of TLR2, may be a novel method for improving BCG efficacy (47). Because current mechanisms associated with protection from TB disease are unknown, further study is required to elucidate the complex effects of TOLLIP deficiency on the adaptive immune response to TB (48).

Limitations of our study included the possible confounding of the genetic findings by heterogeneity of the population substructure (49). To address this, we performed logistic regression by self-identified ethnicity and also used a set of ancestry informative markers specifically designed to account for the heterogeneity in the South African and Seattle populations (50). Furthermore, we have evaluated TOLLIP rs5743854 in multiple populations with varied ethnicity. Another possible limitation could be misclassification of cases and control subjects. The G/G allele of rs5743854 may be associated with the performance of the diagnostic test rather than truly increasing LTBI risk, because the test itself measures an immune response. However, antigen-specific IFN-γ responses to BCG in this study were no different based on TOLLIP genotype. Furthermore, the frequency of antigen-specific $CD4^+$ $IL\text{-}2^+$ cells was reduced in the TOLLIP-deficient group, suggesting a positive tuberculin skin test or IFN-γ release assay reaction may be less likely in TOLLIP-deficient individuals exposed to MTb. We believe that individuals with the G/G genotype of rs5743854 have increased susceptibility to LTBI rather than an association with an immunologic mechanism underlying the diagnostic test.

Our genetic finding was robust in a variety of clinical phenotypes, including MTb infection and disease, but we did not find a significant association with pediatric TB. The availability of pediatric TB genetic cohorts is currently limited and validation of these genetic findings is an important future step. To our knowledge, this study represents the largest genetic study ever performed on pediatric TB with 227 cases identified (51, 52). Development of larger genetic cohorts of pediatric TB is required to resolve this question. Nevertheless, the immune pathogenesis of pediatric TB is likely different in important ways as compared with LTBI or adult TB (53). Better understanding of the immune responses to TB in both adults and children may address this issue in the future.

Conclusions

We discovered that TOLLIP SNP rs5743854 control subject transcriptional expression of TOLLIP is associated with decreased BCG-specific $CD4^+$ T-cell responses and increased risk for developing adult TB and LTBI. Because TOLLIP deficiency is also

associated with increased cytokine responses to MTb and replication, we hypothesize that this increased inflammation and decreased mycobacterial replication may lead to diminished IL-2–producing BCG-specific T cells. These findings may offer a new target for vaccine adjuvants or host-directed therapeutics for TB or other intracellular infections. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the families who participated in the study. They also thank the immunology and clinical teams at the SATVI research site in Worchester for obtaining informed consent and collecting and processing blood from the study participants. They acknowledge the support of the Cell Analysis Facility Flow Cytometry and Imaging Core in the Department of Immunology at the University of Washington.

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