Tuberculosis resistance protein TOLLIP controls immune pathology by resolving the integrated stress response in alveolar macrophages.

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Abstract

A functional polymorphism responsible for TOLLIP deficiency is associated with increased tuberculosis risk. However, TOLLIP's role in TB pathogenesis is unknown. We found that *Tollip*^{-/-} mice develop severe *Mycobacterium tuberculosis* (Mtb) disease, characterized by lipid-laden foamy macrophages. This phenotype was intrinsic to alveolar macrophages (AM), as *Tollip*^{-/-} AM from mixed bone marrow chimeras selectively accumulated lipid and harbored Mtb. Global gene expression analysis of Mtb-infected, *Tollip*^{-/-} AM demonstrated increased EIF2 signaling, a key regulator of the integrated stress response (ISR) to variable environmental conditions. The ISR and foam cell formation were linked, as Mtb lipid mycolic acid incubation with *Tollip*^{-/-} macrophages led to lipid accumulation, ISR responses, and increased Mtb replication. Correspondingly, ISRIB, a small-molecule ISR inhibitor, restored Mtb control in *Tollip*^{-/-} mice and substantially improved Mtb outcomes in resistant C57BL/6 mice. Targeting the ISR broadly improves Mtb control and immunopathology, offering a promising target for host-directed tuberculosis therapy.

Main

A common, functional promoter variant in Toll-Interacting Protein (TOLLIP) is associated with *TOLLIP* deficiency and increased risk for *Mycobacterium tuberculosis* (Mtb) disease^{1,2,3}. TOLLIP is a ubiquitin-binding protein, expressed in all tissues, that organizes the transport and delivery of endosomal cargo^{4,5,6}. Its cargo include intracellular lipids⁷, insoluble protein aggregates^{8,9}, cell surface and endosomal signaling receptors¹⁰, and mitochondria^{11,12}, but its complex and varied role in endosomal transport remains incompletely defined. Via this activity, TOLLIP diminishes TLR2, TLR4, and IL1-R signaling, stabilizes Type I interferon secretion via the endosomal immune receptor STING, and significantly reduces immune pathology during prolonged LPS stimulation^{10,13,14,15,16}. TOLLIP influences pathogenesis of several chronic conditions, including neurodegenerative disease^{8,9}, pulmonary fibrosis¹⁷, stroke¹⁸, and myocardial infarction¹⁹. Thus, TOLLIP regulates a plethora of diverse cellular functions and pathological conditions, but how it influences Mtb pathogenesis is unknown.

Macrophages are good candidate mediators for TB susceptibility in TOLLIP-deficient individuals. Macrophages are the primary intracellular niche for Mtb *in vivo*, and the relative activation state of the macrophage determines whether this niche is permissive or restrictive to Mtb replication. TOLLIP's diverse functions significantly overlap with critical host defense pathways responsible for Mtb control. TLR2, TLR4, IL-1R, and STING participate in Mtb recognition by innate immune cells and contribute to Mtb host defense^{20,21,22}. Complex ER endosomal sorting restricts intracellular Mtb growth²³, and oxidative damage from senescent mitochondria induces Mtb spread in macrophages^{24,25}. Accumulation of lipids and insoluble protein aggregates within macrophages leads to foamy macrophage formation and Mtb persistence *in vivo*^{26,27,28}. Therefore, understanding how TOLLIP restricts Mtb growth within macrophages may provide insight into essential Mtb host response pathways, especially during chronic infection. To better understand how TOLLIP influences Mtb-macrophage interactions *in vivo*, we

conducted a series of experiments in genetically modified mice to identify the lung-resident macrophage subsets most dependent on TOLLIP for Mtb host defense and the specific cellular mechanisms responsible for this phenotype.

Results

TOLLIP is required to control Mtb infection

Human TOLLIP-deficient macrophages developed hyperinflammatory cytokine responses after TLR stimulation or live Mtb infection^{1, 2, 3}. We hypothesized that murine TOLLIP deficiency might be linked to similarly dysregulated cytokine responses. To test this, we evaluated cytokine responses in *Tollip*^{-/-} macrophages after TLR stimulation or Mtb infection. *Tollip*^{-/-} peritoneal macrophages (PEM) secreted more TNF but less IL-10 after LPS (TLR4 ligand), PAM3 (TLR2/1 ligand), or Mtb whole cell lysate stimulation (**Figure S1A-B**), and more TNF and IL-1b, but less IL-10 after overnight Mtb infection (**Figure S1C-E**; H37Rv strain, MOI 2.5). These findings are consistent with our prior studies and suggest that Tollip-mediated regulation of macrophage function is similar in humans and mice³.

TOLLIP maintains immune balance in macrophages after prolonged stimulus, and hyperinflammatory immune activation is associated with worsened Mtb outcomes²⁹. Therefore, we hypothesized that *Tollip*^{-/-} mice would develop worsened Mtb disease. We infected *Tollip*^{-/-} mice and littermate controls (B6) with ~ 50 colony forming units (CFU) of Mtb H37Rv via aerosol and monitored weight, bacterial burden, and survival over time (Fig. 1A). Lung bacterial burdens were diminished in *Tollip*^{-/-} mice 7, 14 (Fig. 1B) and 28 days (Fig. 1C) post-infection but developed increased bacillary burden 56 days post-infection and onward. By 180 days post-infection, lung bacterial burdens were increased ten-fold in *Tollip*^{-/-} mice (Fig. 1C). In the spleen, Mtb dissemination was delayed in *Tollip*^{-/-} mice, as Mtb was not detected in 2/5 spleens 14 days post-infection (Fig. 1D). We detected fewer Mtb CFU in the spleens of *Tollip*^{-/-} mice 28 days post-infection but increased Mtb CFU 56 days post-infection and onward (Fig. 1E). *Tollip*^{-/-} mice exhibited greater weight loss (Fig. 1F) and met criteria for euthanasia before B6 controls (Fig. 1G). Histopathologic analysis of lung sections revealed distinct patterns in *Tollip*^{-/-} mice 56 days after infection, displaying increased numbers of inflammatory infiltrating cells with finely fibrillary cytoplasmic appearance, consistent with lipid-laden “foamy” macrophages (Fig. 1H). Taken together, *Tollip*^{-/-} mice developed worsened Mtb disease and large numbers of foamy macrophages during prolonged infection.

TOLLIP mRNA and protein expression is ubiquitous in both hematopoietic and non-hematopoietic cells^{5, 30}. To assess the relative contribution of each cell type to Mtb immunity, we generated bone-marrow chimeric mice and infected them with aerosolized Mtb. Bacterial burdens were determined 60 days after infection (Fig. 1I). *Tollip*^{-/-} bone marrow -> *Tollip*^{-/-} mice exhibited higher lung bacterial burdens than B6 -> B6 controls. Importantly, *Tollip*^{-/-} -> B6 chimeras developed elevated bacterial burden,

while bacillary load in B6 → *Tollip*^{-/-} was not significantly different than B6 → B6 controls (Fig. 1J). Therefore, TOLLIP influences Mtb protection via expression in radiosensitive hematopoietic cells.

We also measured the contribution of TOLLIP to Mtb host defense in the absence of mature B and T cells. We compared bacterial burden of *Tollip*^{+/+}, *Tollip*^{-/-}, *Rag1*^{-/-}, and *Rag1*^{-/-}*Tollip*^{-/-} mice 28 days after aerosol infection with Mtb. *Rag1*^{-/-}*Tollip*^{-/-} mice demonstrated significantly increased Mtb CFU in their lungs (2.2x higher CFU) compared to *Rag1*^{-/-} mice (Fig. 1K). These data demonstrate that TOLLIP is required for an optimal innate immune response against Mtb, in the absence of T and B cell responses.

***Tollip*^{-/-} alveolar macrophages selectively harbor increased Mtb**

Lung-resident macrophage subset populations demonstrate distinct, genetically regulated functional characteristics that influence responses to Mtb^{31,32}. Only some *Tollip*^{-/-} macrophages developed a foamy phenotype, and TOLLIP was required for myeloid cell Mtb control. Therefore, we considered whether TOLLIP's effects were selective to specific macrophage subpopulations. *Tollip*^{-/-} and B6 mice had similar numbers of alveolar macrophages (AM; CD11c + SiglecF+), monocyte-derived macrophages (MDM; SiglecF-CD11b + CD11c + MHCII+), interstitial macrophages (IM; SiglecF-CD11b + CD11c-MHCII+), neutrophils (PMN; SiglecF-CD11b + Ly6G+), and dendritic cells (DC; SiglecF-CD11b-CD11c + MHCII+) after 28 or 56 days of infection with Mtb expressing an mCherry fluorescent reporter (Mtb-mCherry; Fig. 2A-B, gating in **Figure S2**)^{33,34}. In *Tollip*^{-/-} mice, fewer AM and PMN were infected with Mtb-mCherry 28 days postinfection (Fig. 2C), but after 56 days, a significantly greater proportion of AM and MDM were Mtb-infected (Fig. 2D). The frequency of Mtb-infected AM, but not MDM or PMN, was significantly increased in *Rag1*^{-/-}*Tollip*^{-/-} mice, compared with *Rag1*^{-/-} control mice, 28 days after infection (Fig. 2E). These data suggest that *Tollip*^{-/-} AM may be selectively susceptible to Mtb, and that T and B cells delay, but do not eliminate, this effect.

We hypothesized that intrinsic TOLLIP activity led to selective effects on AM. To test this, we generated mixed bone marrow chimeric mice, which permit evaluation of TOLLIP function at the single cell level. B6.SJL-*Ptprca* *Pepcb*/BoyJ (CD45.1) mice were lethally irradiated and bone marrow was reconstituted with a 1:1 mix of bone marrow from *Tollip*^{-/-} (CD45.2+) and B6 (CD45.1 + CD45.2+) mice, followed by 10 weeks of rest (Fig. 2F). After confirming equal proportions of immune cells from each lineage (Fig. 2G), we infected chimeras with Mtb-mCherry and compared the proportion of Mtb-infected myeloid cell subsets by genotype (gating strategy depicted in **Figure S3A-D**). AM were the primary cell infected 14 days post-infection, with increasing proportions of PMN and MDM becoming infected over time, consistent with prior findings (Fig. 2H)³⁴. 14 days post infection, proportionally fewer *Tollip*^{-/-} AM, but not other subpopulations, were Mtb-infected (Fig. 2I). By contrast, proportionally more *Tollip*^{-/-} AM were Mtb infected and intracellular Mtb MFI was increased, suggesting greater intracellular Mtb burden, but other *Tollip*^{-/-} myeloid subsets did not demonstrate relative Mtb accumulation (Fig. 2J-K). *Tollip*^{-/-} AM demonstrated increased NOS2 + frequency (**Figure S3E**) with increased MFI (**Figure S3F**), confirming that

Mtb persistence occurred in the setting of hyperinflammation. Therefore, absence of *Tollip* in AM leads to selectively increased Mtb burden during prolonged infection.

Tollip^{-/-} AM develop increased EIF2 signaling after prolonged Mtb infection

We next sought to identify the gene expression networks in AM that were associated with prolonged Mtb infection. We compared the global transcriptional networks of sorted, Mtb-infected and bystander B6 and *Tollip^{-/-}* AM from mixed bone marrow chimeric mice 28 days after Mtb infection (sorting strategy is shown in **Figure S4**). We identified 194 differentially expressed genes (DEG) between B6 and *Tollip^{-/-}* Mtb-infected AM (Fig. 3A; FDR < 0.05; gene names in **Dataset S1**) and 157 DEGs between B6 and *Tollip^{-/-}* Mtb-uninfected “bystander” AM, and with a more liberal FDR < 0.3, we identified 2599 DEGs between Mtb-infected AM and 2042 DEGs from bystander AM (Fig. 3B; **Dataset S1**). We performed Ingenuity Causal Network Analysis comparing DEGs from Mtb-infected B6 and *Tollip^{-/-}* AM to identify functional nodes that were associated with their increased susceptibility to Mtb infection³⁵. The top node discovered in *Tollip^{-/-}* AM was “Increased EIF2 Signaling” (Fig. 3C).

EIF2 signaling is activated via phosphorylation at Ser51 of EIF2A, which triggers the integrated stress response (ISR), a set of cellular adaptations to harsh environmental or cellular conditions³⁶. We validated our transcriptional observations by measuring phosphorylated EIF2A (pEIF2) in Mtb-infected lungs by immunohistochemistry. We found increased pEIF2 in the lungs of *Tollip^{-/-}* mice 56 days after aerosol Mtb infection (Fig. 3D-E)³⁷ with no difference in total EIF2A expression (Fig. 3F-G). To summarize, *Tollip^{-/-}* AM in mixed chimeric mice demonstrated increased EIF2 signaling 28 days after infection via transcriptional and protein analysis, which correlated with increased bacterial burden.

TOLLIP and EIF2 kinase expression is increased in caseous human TB granulomas

A defining characteristic of human TB granulomas is the presence of “caseum,” a lipid-rich center of necrosis that is surrounded by foam cells and other inflammatory cells induced by Mtb infection^{38,39}. Because we noted that TOLLIP acts selectively in AM to influence EIF2 activation, we hypothesized that TOLLIP and EIF2 kinase expression are increased in human caseous granulomas. EIF2 is phosphorylated by one of four upstream kinases – *EIF2AK1* (HRI), *EIF2AK2* (PKR), *EIF2AK3* (PERK), and *EIF2AK4* (GCN2)⁴⁰. We evaluated the gene expression of TOLLIP, EIF2A (*EIF2S1*), and EIF2 kinases in human caseous granulomas compared with samples from healthy areas of the same lung, using a publicly available dataset⁴¹. *EIF2AK1*, *EIF2AK2*, and *EIF2AK3* demonstrated significantly increased expression in caseous granulomas, but not *EIF2S1* (Fig. 4A-D). *EIF2AK4* and *TOLLIP* expression were increased (Fig. 4E-F; FDR = 0.25 and 0.08, respectively) but less strongly than other EIF2 kinases. In contrast to our observations in granulomas, *TOLLIP* and EIF2 kinase expression was not changed in the peripheral blood of individuals with active or latent TB (**Figure S5A-D**)⁴². Together, EIF2 kinases and TOLLIP are selectively enriched in the TB granuloma, providing a tissue-specific role for these genes during human TB disease.

Mtb cell wall lipid mycolic acid induces lipid accumulation and increases *Mtb* replication in *Tollip*^{-/-} macrophages

Next, we sought to assess how TOLLIP deficiency shapes ISR activation in *Mtb*-infected AM. TOLLIP prevents lipid droplet delivery to lysosomes⁷, excess lipid induces the ISR⁴³, and lipid accumulation may contribute to myeloid cell dysfunction via cellular stress pathways^{44, 45}. Therefore, we considered the possibility that lipid accumulation in *Tollip*^{-/-} AM contributes to *Mtb* spread by inducing the ISR via pEIF2⁴⁶. To understand this pathway better, we evaluated two known mechanisms for inducing lipid accumulation and foam cell formation in *Mtb*-infected macrophages – Type I interferon and the *Mtb* cell wall lipid mycolic acid (MA)^{47, 48}. In *Tollip*^{-/-} mice, *Ifnb* gene expression was diminished in *Mtb*-infected lung tissue 60 days after infection (Fig. 5A)¹⁵. Therefore, we evaluated how TOLLIP influenced MA-induced lipid accumulation in macrophages. We confirmed the capacity of *Tollip*^{-/-} bone marrow-derived macrophages to perform bulk macroautophagy, a major lipid clearance pathway^{43, 49, 50}. In these cells, we measured protein levels of LC3II and p62 after treatment with Bafilomycin A (BafA) to prevent autophagosome/lysosome fusion⁵¹. *Tollip* did not significantly affect levels of LC3II, nor the induction of LC3II by BafA treatment (**Figures S6A-B**). Levels of the adaptor protein p62, which is cleared during autophagy, were also similar (**Figures S6C**). Therefore, TOLLIP is dispensable for bulk autophagic flux in bone marrow-derived macrophages.

Next, we evaluated lipid accumulation after MA treatment. *Tollip*^{-/-} PEM selectively accumulated lipid droplets after treatment with a mix of three MA isoforms found in *Mtb* (a-MA, methoxy-MA, and keto-MA) for 72 hours (Fig. 5B-D)^{27, 52, 53}. MA-treated *Tollip*^{-/-} PEM had increased *Mtb* replication, measured by luminescence, after infection with *Mtb* H37Rv expressing the *luxCDABE* luminescent reporter from *Vibrio harveyi* (*Mtb-lux*; MOI 1), which closely correlates with bacterial CFU (Fig. 5E)². We observed similar phenotypes *in vivo*. In mixed bone marrow chimeric mice, *Tollip*^{-/-} AM, but not MDM, accumulated lipid 28 days after *Mtb* infection (Fig. 5F-G, gating in **Figure S4**). Taken together, MA is sufficient to induce lipid accumulation and promote *Mtb* replication in *Tollip*^{-/-} macrophages, and we observed selective lipid accumulation in *Tollip*^{-/-} AM that corresponded with increased *Mtb* burden in mixed bone marrow chimeras.

EIF2 inhibition with ISRIB controls *Mtb* replication and improves *Mtb* outcomes

After determining that lipid accumulation was associated with *Mtb* replication in *Tollip*^{-/-} macrophages, we evaluated the effect of MA on ISR activation. MA treatment of *Mtb*-infected, *Tollip*^{-/-} PEM led to significant increases in *Ern1* (**Figure S7A**), *Eif2ak3* (**Figure S7B**), and *Atf6* (**Figure S7C**), which maintain immune function during lipid excess and activate the ISR via *Eif2ak3*^{44, 46, 54}. Because MA is linked to ISR activation, we evaluated the influence of the ISR on *Mtb* replication directly, by treating PEM with the EIF2 activator raphin-1 or the EIF2 inhibitor ISRIB^{55, 56}. Neither raphin-1 (10μM) or ISRIB (5nM) treatment induced cell death in macrophages or altered *Mtb* replication in broth culture (data not shown). Raphin-1

treatment increased Mtb replication in PEM (Fig. 6A) while ISRIB decreased Mtb replication in MA-treated, *Tollip*^{-/-} PEM (Fig. 6B). Overall, these data confirm that ISR activation permits Mtb replication independently of lipid accumulation, and that MA-induced Mtb replication is mediated through ISR activation.

Next, we tested the effectiveness of ISRIB on Mtb control *in vivo*. We infected mice with Mtb via aerosol, and after allowing 15 days for Mtb growth, treated daily with vehicle control, ISRIB (1mg/kg intraperitoneally), or raphin-1 (1mg/kg intraperitoneally). 56 days post infection, Mtb CFU were measured from lungs and spleen, and one lung lobe was preserved for pathology analysis (Fig. 6C). ISRIB treatment restored Mtb control in the lungs and spleens of *Tollip*^{-/-} mice (Fig. 6D-E). Moreover, ISRIB significantly reduced overall bacterial burden in resistant B6 control mice (Fig. 6D-E). Treatment of B6 mice with raphin-1 increased bacterial burden in both lung and spleen (Fig. 6F-6G). In pathology analysis, ISRIB significantly reduced lung cellularity in the hyperinflammatory *Tollip*^{-/-} mouse model, but neither ISRIB or raphin-1 significantly altered lung cellularity in B6 mice (Fig. 6H-6I)^{9, 57}. We detected fewer cells and increased hematoxylin staining in raphin-1 treated, *Tollip*^{-/-} mice, consistent with cellular necrosis, which is a risk factor for Mtb spread²⁹. In summary, ISRIB treatment rescued the Mtb susceptibility phenotype in *Tollip*^{-/-} mice and significantly improved Mtb control in B6 mice.

Discussion

TOLLIP deficiency in mice was associated with enhanced Mtb control in the early stages of Mtb infection, but ultimately resulted in exacerbated disease. The worsened immunopathology in *Tollip*^{-/-} mice was associated with an abundance of lipid-laden foamy macrophages, and a failure to control bacterial burdens during chronic infection. AM were shown to be the primary cellular drivers of this phenotype, as AM TOLLIP-deficiency resulted in intracellular lipid accumulation, which triggered an EIF2-mediated stress response. EIF2 inhibition resulted in improved pathology and Mtb control, providing a mechanistic link between TOLLIP deficiency and Mtb disease, and identifying this pathway as a potential target for host-directed therapy.

A schematic of our proposed model is shown in Fig. 6J. *Tollip*^{-/-} AM accumulate lipids intracellularly during Mtb infection due to selectively impaired homeostatic lipid clearance of Mtb lipids. Mtb cell wall-derived lipids, such as MA, induce ISR activation, which contributes to increased intracellular Mtb replication, as EIF2 inhibition via ISRIB restores macrophage host defense in *Tollip*^{-/-} mice. However, in TOLLIP-deficiency, AM provide a reserve niche for Mtb persistence during chronic infection.

It is interesting that AM-intrinsic TOLLIP-deficiency only contributes to exacerbated disease during prolonged infection, but not in the first few weeks after aerosol infection. In previous *in vitro* experiments, we observed improved Mtb control in *Tollip*^{-/-} macrophages after 48 hours, and improved control of *L. pneumophila* infection, an acute intracellular bacterial infection, in *Tollip*^{-/-} mice. These data are consistent with an enhanced and beneficial innate immune response during acute infections^{2, 9}. However,

Tollip^{-/-} AM developed lipid accumulation that corresponded with impaired Mtb control that corresponds with lipid accumulation during the chronic phase of Mtb infection. Therefore, TOLLIP may have two functions that are related but independent. First, the beneficial innate immune activation improves Mtb control transiently but incompletely, then impaired lipid clearance induces ISR and cellular dysfunction later in the course of infection. There is precedent for a dual role for TOLLIP, as it balances clearance of insoluble endoplasmic reticulum proteins with stabilizing the cytoplasmic DNA sensor STING¹⁵. These findings may provide an explanation for the biphasic response to Mtb seen in *Tollip*^{-/-} mice.

AM are the primary cell infected by Mtb during early infection, but their role during chronic infection is less well understood^{34, 58}. Chronically activated AM disproportionately contribute to immunopathology in influenza and RSV infection^{32, 59} and exacerbate fibrotic responses in autoimmune diseases and idiopathic pulmonary fibrosis⁶⁰. Our data suggest that TOLLIP prevents AM dysregulation during Mtb infection, and its absence leads to Mtb-induced immunopathology. These data identify a novel, genetically regulated role for AM during chronic Mtb disease. Specifically targeting therapeutics to AM during chronic Mtb infection may be effective for controlling persisting Mtb bacilli.

Tollip^{-/-} mice demonstrated increased EIF2 phosphorylation in AM and *in vivo*, leading to immune pathology and Mtb progression. In prior studies, EIF2 was induced in Mtb-infected bone marrow-derived macrophages from *sst1.B6* mice that produce excess Type I interferon⁴⁷. *Tollip*^{-/-} cells demonstrated impaired Type I interferon secretion compared to B6 mice due to decreased STING stability¹⁵, suggesting an alternate mechanism for ISR activation. MA induced the ISR in Mtb-infected, *Tollip*^{-/-} macrophages⁴⁸. In models of hepatic steatosis and atherosclerosis, lipid accumulation in the ER induces EIF2 phosphorylation^{61, 62, 63}. Lipid-induced EIF2 activation may interact with inflammatory stress. Excess TNF induces programmed necrosis and Mtb spread by sphingosine-induced calcium flux and oxidative stress^{24, 25, 64}. Notably, we found strong enrichment of lipid and sphingolipid metabolic transcriptional pathways in Mtb-infected *Tollip*^{-/-} AM, and MA metabolizes into sphingosine in macrophages⁶⁵. In summary, TOLLIP contributes to the degradation of Mtb-derived lipids to protect from Mtb immunopathology.

EIF2 activation and the downstream ISR may play an important role in the transition from asymptomatic Mtb infection to symptomatic TB disease. Studies from the preantibiotic era regularly describe “post-primary” tuberculosis as an acute, paucibacillary, caseating lipoid pneumonia obstructing bronchioles in a “tree-in-bud” pattern^{66, 67}. Within the lipoid pneumonia, obstructed alveoli attract macrophages with a foamy appearance, leading to subsequent necrosis and granuloma formation. Pathologically, this obstructive lipoid pneumonia precedes the transition from asymptomatic to symptomatic TB disease³⁹. Our model mimics important aspects of this observation, whereby *Tollip*^{-/-} AM accumulate lipid, which permits Mtb growth via the EIF2 pathway. A combination of host and bacterial factors that obstruct clearance of lipid debris may contribute to Mtb progression by inducing EIF2 in AM.

This study revealed a critical role for TOLLIP within AM that governs immunopathology and Mtb infection outcomes. However, TOLLIP is ubiquitously expressed and may influence the function of other immune cell subsets relevant for Mtb host responses, particularly T cells and dendritic cells. EIF2 signaling influences dendritic cell function, particularly in tumors and during chronic viral infections^{68, 69, 70}. TOLLIP may also impact T cell activation and differentiation. The selective autophagy receptor TAX1BP1, which is structurally and functionally similar to TOLLIP, is essential for the biosynthetic demands of T cells during the metabolically and synthetically demanding initial proliferative period⁷¹. Thus, future studies are needed to investigate how TOLLIP and ISR signaling pathways shape immune function across tissues and immune lineages.

We showed that ISRIB treatment improved Mtb control even after infection was established, a critical requirement for translation of any host-directed therapy to human disease. Often host-directed interventions are tested in mice by administration either prior to or very early after infection and may show lesser efficacy when used during chronic infection^{72, 73, 74}. However, our studies show that the EIF2 pathway is required to clear lipids that would otherwise impair immunity during prolonged infection, providing a promising host-directed target for established disease. Importantly, we showed that EIF2-inhibition enhanced immunity in both TOLLIP-deficient and B6 hosts. Thus, ISRIB is candidate host-directed therapeutic agent that should be explored further in future studies.

Online Methods

Reagents

A complete list of reagents can be found in **Table S1**.

Mice

All mice were housed and maintained in specific pathogen-free conditions at the University of Washington and Seattle Children's Research Institute, and all experiments were performed in compliance with the Institutional Animal Care and Use Committee from each institution. Mice used in the experiments were 6–12 weeks of age. B6.Cg-Tollip^{tm1Kbns/Cnrm} (*Tollip*^{-/-}) mice were obtained from the European Mutant Mouse Archive (www.infrafrontier.eu)⁷⁵. Mice were backcrossed 11 times on C57BL/6J background and were confirmed to be > 99% C57BL/6J genetically by screening 150 SNP ancestry informative markers (Jax, Inc). Genotyping was performed using DNA primers for neomycin (Forward sequence: AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG; Reverse sequence AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG) and the first exon of TOLLIP (Forward sequence: AGC TAC TGG GAG GCC ATA CA; Reverse sequence: CGT GTA CGG GAG ACC CAT TT). Protein expression was confirmed in both knockout and backcrossed alleles by qPCR and Western blot. All wild type control mice were age-matched littermates to ensure a common genetic background.

Model of Mtb aerosol infection

Aerosol infections were performed with Mtb, H37Rv strain transfected with a mCherry reporter plasmid, described previously³⁴. Mice were enclosed in a Glas-Col aerosol infection chamber and ~50–100 CFU were deposited into mouse lungs. Doses were confirmed using control mice by plating lung homogenates on 7H10 agar immediately after aerosol infection. Mice were sacrificed at indicated timepoint, and lungs were gently homogenized in PBS-containing 0.05% Tween using a gentleMacs dissociator (Miltenyi Biotec). Tissue homogenates were serially diluted on 7H10 agar and lung CFU was enumerated.

Lung Cell Flow Cytometry

Lung single cell suspensions were washed and stained for viability with Zombie Aqua viability dye (BioLegend) for 10 min at room temperature in the dark. After incubation, 100 µl of a surface antibody cocktail diluted in 50% FACS buffer/50% 24G2 Fc block buffer was added and surface staining performed for 30 min at 4°C. Antibody lists can be found in **Table S1**. The cells were washed once with FACS buffer and fixed with 2% paraformaldehyde for 1 h prior to analyzing on an LSRII flow cytometer (BD Biosciences). In some experiments, intracellular staining was performed after surface staining. Permeabilization was performed with Fix-Perm buffer (eBiosciences) for minimum of 60 min before the addition of intracellular antibodies. Then cells were fixed with 2% paraformaldehyde for 1 h prior to analyzing on an LSRII flow cytometer (BD Biosciences). 28 days after infection, mice were sacrificed, and AM were sorted on a BD FACS Aria in a BSL-3 facility for infected and uninfected populations. The samples were spun and stored – 80° C in Trizol.

Chimera Generation

: *Tollip*^{-/-} mixed bone marrow chimeras were generated in the following manner: B6.SJL-Ptprca Pepcb/BoyJ (CD45.1+; Jax, Inc.) F1 mice were lethally irradiated (1000 cGy). 5-10x10⁶ bone marrow cells comprised of a 1:1 mixture of CD3-depleted (Miltenyi Biotec) *Tollip*^{-/-} (CD45.2+) and F1 generation of C57BL/6J (CD45.1 + 45.2+) bone marrow was provided intravenously. For full bone marrow chimera generation, and *Tollip*^{-/-} mice were irradiated, followed by hemopoietic reconstitution by adoptive transfer of 5-10x10⁶ bone marrow cells via intravenous injection.

Tissue Preparation and Evaluation

Mice were euthanized and lungs were gently homogenized in HEPES buffer containing Liberase Blendzyme 3 (70 µg/ml; Roche) and DNaseI (30 µg/ml; Sigma-Aldrich) using a gentleMacs dissociator (Miltenyi Biotec). The lungs were then incubated for 30 min at 37°C and then further homogenized a second time with the gentleMacs. The homogenates were filtered through a 70 µm cell strainer, pelleted for RBC lysis with RBC lysing buffer (Thermo), and resuspended in FACS buffer (PBS containing 2.5% FBS and 0.1% NaN₃). To prepare organs for histology, lung sections were inflated to 15cm water pressure with 4% paraformaldehyde, fixed in the same solution, embedded in paraffin, and sliced into 4µm sections. Sections stained with hematoxylin and eosin were examined by a pathologist blinded to mouse genotype.

Intracellular Mtb replication assay.

Frozen Mtb was thawed and cultured on a shaking incubator for two doubling cycles. One day prior to infection, cultures were back-diluted into an optical density (OD) of 0.2–0.4 in 7H9 media supplemented with glycerol (4%), Middlebrook ADC Growth Supplement (100 mL/L), and Tween 80 (0.05%). At the time of infection, Mtb was filtered through a 5 µm syringe filter to remove bacterial clumps, and cells were inoculated at indicated MOI. The inoculum was prepared in RPMI-10 medium and applied to cells, which were centrifuged at 1200rpm for 5 minutes and incubated for 4 hours at 37°C. Supernatants were removed and washed twice with prewarmed PBS to remove unbound bacteria, before adding per warmed RPMI supplemented with 10% FBS. Intracellular growth was determined using luminescence on a Synergy H4 multimode microplate reader (Biotek Instruments) daily from Day 0 to Day 3. In some experiments, 24hrs post infection cell culture supernatants were collected and filtered twice using 0.22µm filter and frozen at -80°C.

Macrophage Preparation

Resident peritoneal cells, mainly consisting of *in vivo* differentiated macrophages, were isolated using standard methods⁷⁶. Briefly, 10ml of cold PBS was injected using 27g needle, and peritoneum was gently massaged. Cells were removed with PBS, centrifuged at 4° C and placed into warm RPMI media. PEMs (1×10^5 /well) were plated in 96-well plates in 200µL of antibiotic-free RPMI 1640 containing with 10% Fetal Bovine Serum (Atlas Biologics). Cell were rested for minimum 24hrs before further experimentation. Bone marrow was harvested from mice and grown in RPMI supplemented with 10% heat inactivated FBS and M-CSF (40ng/ml) in tissue culture treated plates. Cells were then incubated at 37° C for 6 to 7 days. Bone marrow-derived macrophages (BMDM) were used after 7 days of culture. BMDMs were detached by gentle scraping and were then plated in RPMI 1640 supplemented with 10% heat inactivated FBS.

Mycolic acid preparation

α-mycolic acid (C80) was obtained from Avanti Polar Lipids. Powdered lipids were solubilized in chloroform, which was evaporated in the plate and the lipids recovered in RPMI with 10% FCS. The suspension was sonicated at 37°C for 20 min, followed by addition of cells. Control suspensions were prepared similarly but without the addition of MA and sonicated before administration. 50µg of mycolic acid were administered to cells of interest for 72 hours prior to measurement, as previously described²⁷. MA was free from endotoxin as determined by a chromogenic Limulus amoebocyte lysate assay (Genscript, Piscataway, NJ, USA).

Cellular Studies

Cell-culture supernatants were collected at indicated time and frozen at -20° C until analysis. The cytokine concentrations in the culture supernatant were determined using quantitative ELISA (Mouse TNF and IL-10 DuoSet; R&D Systems) as recommended by the manufacturer. For detection of lipid bodies, PEMs from B6 and *Tollip*^{-/-} mice were plated (1×10^5 /well) in a glass bottom chamber slide. Cells were washed twice and resuspended in PBS solution of HCS LipidTOX Deep Red Neutral Lipid stain (ThermoFisher Scientific) according to manufacturer instruction. After incubation, cells were washed twice with PBS and

fixed in 2% PFA for 30min. Cells were mounted in medium containing DAPI stain (ProLong Gold, Thermo, Inc.). Lipid droplets were counted from 100 cells identified randomly selected high-powered fields.

Western blotting

Immunoblots were performed as described previously ⁷⁷. Briefly, 5–20µg of cell or tissue protein extract was separated by SDS-PAGE, transferred onto PVDF membranes and immunoblotted with primary antibodies, listed in **Table S1**. Secondary antibodies conjugated to horseradish peroxidase were added and luminescence was quantitated.

Preparation of total RNA and sequencing

Total RNA from sorted cells was isolated using the manufacturer's instructions (TRIzol, Invitrogen). The RNA purity and quantified was assessed by RNA-Tapestation (Agilent 4200). cDNA was prepared using SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio USA, Inc). RNA sequencing libraries were prepared using the Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Samples were sequenced as 50 bp paired-end reads on a Illumina HiSeq 2500 and assessed using FastQC v0.11.8 to visualize sequence quality ⁷⁸. Sequences were filtered using AdapterRemoval v2.3.1 to remove adapters, remove sequences with > 1 ambiguous base, and trim ends to a 30 + Phred score ⁷⁹. Sequences were aligned to the mouse genome mm10 using STAR v2.7.4a ⁸⁰, and alignments were assessed with Picard v2.18.7 ⁸¹ and samtools v1.10 ⁸². Total reads in gene exons were quantified using featureCounts v2.0.1 ⁸³.

Differential expression analysis

Gene expression analyses were performed in R v3.6.1 with the tidyverse v1.3.0 ⁸⁴. Overall, samples were very high-quality with > 32 million aligned sequences per sample and low variability in gene coverage (median coefficient of variation coverage < 0.6). Genes were filtered to protein coding genes with biomaRt (N = 21850) with > 0.1 counts per million (CPM) in at least three samples (N = 14215) ⁸⁵. Counts were normalized for RNA composition using edgeR and log2 CPM voom normalized with quality weights using limma ^{86,87}. Differentially expressed genes were determined using limma using a contrasts model comparing B6 and *Tollip*^{-/-} cells in uninfected and infected groups (**Table S2**). Genes with FDR < 0.3 for at least one contrast (N = 3899) were included in Ingenuity Pathway analysis. The Benjamini–Hochberg correction for multiple comparisons was applied to P-values and significance was assessed at FDR < 0.05 for all tests.

Statistics

For mouse experiments, groups of five mice were compared with one another unless otherwise indicated. Cellular measurements were compared using a two-sided *t*-test unless otherwise specified. A value of *p* < 0.05 was considered a statistically significant result unless otherwise specified. Statistics were calculated using Prism version 8.0 (GraphPad, Inc.).

Declarations

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Figures

Figure 1

TOLLIP is required for *Mtb* control in mice and its absence induces foam cell formation. Mice were infected with *Mtb* H37Rv (50-100 CFU) via aerosol and monitored over time.

A) Experimental timeline.

B-C) Lung bacterial burden in B6 (clear circle) and *Tollip*^{-/-} (black square) mice B) 1 and 2 weeks after infection and C) 4 weeks and onward. * p < 0.05, ** p < 0.01, *** p < 0.001 at each time point; two-sided t-

test at each time point; n = 50. Overall association between genotypes – p = 0.0123; mixed effects model.

D-E) Spleen bacterial burden D) 1 and 2 weeks after infection and E) 4 weeks and later after infection. * p < 0.05; ** p < 0.01, ***p < 0.001, two-sided t-test at each time point; n = 30. Overall association between genotypes – p = 0.0092; mixed effects model.

F) Percentage of initial body weight after Mtb infection *Circle* – B6 mice; *square* - *Tollip*^{-/-} mice. N = 20 *** p < 0.001, two-way ANOVA accounting for time and genotype.

G) Survival curve analysis of B6 (*clear circle*) and *Tollip*^{-/-} (*black square*) mice after aerosol infection with 50-100 CFU Mtb H37Rv. N = 20. *** p < 0.0001, Mantel-Cox test.

H) Hematoxylin and eosin staining of Mtb-infected lung tissue from B6 and *Tollip*^{-/-} mice 28 and 56 days after aerosol infection.

I) Experimental outline for bone marrow chimera experiments.

J) Lung bacterial burden of bone marrow chimeric mice, 56 days after aerosol Mtb infection. N = 5 mice/group.

K) B6, *Tollip*^{-/-}, *Rag1*^{-/-}, and *Rag1*^{-/-}*Tollip*^{-/-} mice were infected with Mtb and bacterial burden was measured 28 days after infection (n = 5-10 mice/group). * p < 0.05, ** p < 0.01, *** p < 0.001, two-sided t-test.

Figure 2

Tollip^{-/-} AM are preferentially infected with Mtb due to intrinsic activity.

A-B) Lungs from Mtb-infected full knockout mice were gently dissociated, and flow cytometry was performed. Gating strategy is described in **Figure S2**. The proportion of AM, MDM, IM, and PMN from B6 (clear circle) and *Tollip*^{-/-} (black square) mice were compared **A)** 4 and **B)** 8 weeks after aerosol infection with 50-100 CFU mCherry-expressing Mtb; n = 5; * p < 0.05, two-sided t-test.

C-D) The proportion of Mtb-infected (mCherry+) AM, MDM, IM, and PMN from B6 (*clear circle*) and *Tollip*^{-/-} (*black square*) mice were compared **C)** 28 and **D)** 56 days after infection. n = 5/group; * p < 0.05, two-sided t-test.

E) The proportion of Mtb-infected AM, MDM, and PMN from *Rag1*^{-/-} (clear circle) and *Rag1*^{-/-}*Tollip*^{-/-} mice 28 days post infection. N = 7 in *Rag1*^{-/-}; N = 9 in *Rag1*^{-/-}*Tollip*^{-/-}; **** p < 0.0001 two-sided t-test.

F) Mixed bone marrow chimera experimental strategy and timeline. Mixed bone marrow chimeras were generated by transferring a 1:1 mix of B6 (CD45.1+/CD45.2+) and *Tollip*^{-/-} (CD45.2+) bone marrow to a CD45.1+ murine host. Immune cell reconstitution was permitted for 10 weeks. The gating strategy is described in **Figure S4**.

G) Distribution of CD45 expression in naïve mixed bone marrow chimeric mice. N = 8.

H) Composition of Mtb-infected lung myeloid cell types at multiple time points after aerosol infection with Mtb.

I-J) Distribution of Mtb-infected (mCherry+) lung-specific myeloid cells at **H)** 14 and **I)** 28 days post-infection in mixed bone marrow chimeric mice stratified by B6 (*white circle*) and *Tollip*^{-/-} (*black square*) genotype. Lines connect genotypes from paired samples. * p < 0.05, ** p < 0.01, paired two-sided t-test. N = 5/group.

K) Median fluorescence intensity of mCherry in Mtb-infected (mCherry+) AM 8 days post-infection in mixed bone marrow chimeric mice stratified by B6 (*white circle*) and *Tollip*^{-/-} (*black square*) genotype. Paired samples are connected by lines. * p < 0.05, paired two-sided t-test N = 5.

AM – alveolar macrophage, MDM – monocyte-derived macrophage, IM – interstitial macrophage, PMN – neutrophil, DC – dendritic cell. Experiments were performed at least three times for reproducibility.

Figure 3

Figure 3

***Tollip*^{-/-} AM develop increased EIF2 signaling after prolonged Mtb infection.** Mixed bone marrow chimeric mice were infected with mCherry-expressing Mtb. 28 days after infection, Mtb-infected and Mtb-uninfected B6 and *Tollip*^{-/-} AM were sorted and RNA-seq was performed. Sorting strategy can be found in Figure S3.

A-B) Volcano plot of gene expression ($\log_2$ fold change) and significance ($-\log_{10}(\text{FDR})$) for genes between *Tollip*^{-/-} (CD45.2) and B6 (CD45.1+CD45.2+) in **A)** Mtb-uninfected and **B)** Mtb-infected AMs. Horizontal lines indicate significant genes ($\text{FDR} < 0.05$) and genes of interest ($\text{FDR} < 0.3$).

C) Ingenuity Causal Network Analysis on differentially expressed genes between B6 and *Tollip*^{-/-} Mtb-infected AM subgroup ($\text{FDR } q < 0.3$). P value cutoff $< 10^{-4}$.

D) Semiquantitative analysis of EIF2 phosphorylated at Ser51 (pEIF2) expression in the lungs of B6 and *Tollip*^{-/-} mice eight weeks after Mtb infection.

E) Representative 20x imaging sections of lung tissue sections stained for pEIF2 by immunohistochemistry.

F) Semiquantitative analysis of EIF2S1 expression in the lungs of B6 and *Tollip*^{-/-} mice two months after Mtb infection.

G) Representative 20x imaging sections of lung tissue sections stained for pEIF2 by immunohistochemistry. * $p < 0.05$, ** $p < 0.01$, two-sided t-test. N = 5 mice/group.

Figure 4

Figure 4

TOLLIP and EIF2 kinase transcripts are enriched in caseous TB granulomas.

A-F) Gene transcript expression of A) *EIF2AK1*, B) *EIF2AK2*, C) *EIF2AK3*, D) *EIF2AK4*, E) *EIF2S1*, and F) *TOLLIP* in human caseous granuloma tissue compared with healthy-appearing lung tissue from different lobes in the same individuals. Data are derived from the analysis of the publicly available GEO dataset GSE20050 using the indicated probe sets. FDR – false discovery rate. FDR is indicated above each data point.

Figure 5

Figure 5

Tollip^{-/-} macrophages accumulate excess lipid and increased Mtb replication after mycolic acid incubation.

A-C) B6 and *Tollip*^{-/-} peritoneal exudate macrophages (PEM) were isolated and incubated with Mtb cell wall lipid mycolic acid (MA) for 72hrs. PEM were stained with LipidTOX Red dye (red) and DAPI (blue), fixed, and visualized with fluorescent microscopy.

A) Representative image of lipid droplet (LD) accumulation in B6 and *Tollip*^{-/-} PEM.

B) Percentage of cells with visible LD. At least 100 cells imaged per experiment. Bar represents mean $\pm$ SD values.

C) Total number of LD per cell of 100 cells with detectable LD. Bar – error bars (mean $\pm$ SD).

D) Median fluorescence intensity of LipidTox neutral lipid stain from mixed bone marrow chimeric mice in alveolar macrophages (AM) and monocyte-derived macrophages (MDM) 28 days after Mtb infection, stratified by B6 (white circle) and *Tollip*^{-/-} (black square) genotype. Infection and gating were performed as in **Figure S4**. Lines connect B6 and *Tollip*^{-/-} AM from the same mouse. * $p < 0.05$, paired two-sided t-test; $N = 5$. Experiment was performed five times.

E) Representative histogram of AM and MDM lipid staining (B6 – blue; *Tollip*^{-/-} – red).

F) Luminescence of B6 and *Tollip*^{-/-} PEM incubated with mycolic acid (MA; 10 μ g/ml) and infected with Mtb H37Rv expressing *luxCDABE* luminescence gene from *Vibrio harleyi* (MOI 1). PEMs were isolated and

incubated with MA for 72 hr before infection. Luminescence was measured daily over 48-72 hours; N = 6. Experiments were repeated three times; all data is shown. * $p < 0.05$; 3-way ANOVA for an effect of MA in *Tollip*^{-/-} PEM when controlling for time.

Figure 6

ISRIB treatment restores immune control in *Tollip*^{-/-} mice and improves host defense in B6 mice.

A) B6 PEM (*black circles*) were treated with vehicle control or the EIF2 activator raphin-1 (*black and white circles*; 10 μ M), infected with Mtb H37Rv expressing *luxCDABE* luminescence gene from *Vibrio harleyi* (Mtb-lux; MOI 1) and luminescence was measured over time. Error bars – SD. * $p < 0.05$; 2-way ANOVA for an effect of raphin-1 controlling for time. N = 6, experiment was performed three times.

B) *Tollip*^{-/-} PEM were treated with vehicle control, MA, EIF2 inhibitor ISRIB (200nM), or both, then infected with Mtb-lux (MOI 1). Luminescence was measured 24 and 48 hours after infection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, 2-way ANOVA accounting for time and drug treatment. N = 6, experiment was performed three times.

C) Experimental design. B6 and *Tollip*^{-/-} mice were infected with 50-100 CFU Mtb and 15 days after infection, vehicle control, ISRIB (1mg/kg/day), or raphin-1 (1 mg/kg/day) were instilled intraperitoneally. Eight weeks after infection, mice were euthanized, bacterial CFU was measured, and tissue was collected for pathology analysis.

D-E) Mtb CFU in D) lungs and E) spleen from mice treated with ISRIB.

F-G) Mtb CFU in F) lungs and G) spleens from mice treated with raphin-1.

H) Representative images of lung (5x magnification) stained with H&E from B6 and *Tollip*^{-/-} mice, with and without ISRIB treatment.

I) Number of nucleated cells per 20x high-powered field in Mtb-infected mice after ISRIB and raphin-1 treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, 2-way ANOVA accounting for genotype and drug treatment. N = 11, experiment was performed twice.

J) Model of TOLLIP function and EIF2 activation during Mtb infection. During chronic Mtb infection, lipid products are released in Mtb-infected AM. TOLLIP prevents lipid accumulation, which contributes to ongoing host control of Mtb and maintains EIF2 signaling at a basal level. The resulting homeostasis of host-Mtb interaction results in ongoing host control. In chronically Mtb-infected *Tollip*^{-/-} AM, the absence of TOLLIP permits ongoing lipid accumulation, which activates EIF2. EIF2 signals create conditions acceptable for increased bacterial burden. As a result, foam cell formation prevents Mtb killing and permits ongoing Mtb expansion. ISRIB inhibition restores Mtb control overall, including in the resistant B6 mouse, making it an effective host-directed therapeutic across genetic backgrounds.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS2.csv](#)
- [Supplemental021422.docx](#)