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RP105 Engages Phosphatidylinositol 3-Kinase p110δ To Facilitate the Trafficking and Secretion of Cytokines in Macrophages during Mycobacterial Infection

Chien-Hsiung Yu,* Massimo Micaroni,† Andreas Puyskens,* 1 Thomas E. Schultz,* Jeremy Changyu Yeo,† Amanda C. Stanley,† Megan Lucas,‡ Jade Kurihara,‡ Karen M. Dobos,‡ Jennifer L. Stow,† and Antje Blumenthal* 8,§

Cytokines are key regulators of adequate immune responses to infection with Mycobacterium tuberculosis. We demonstrate that the p110δ catalytic subunit of PI3K acts as a downstream effector of the TLR family member RP105 (CD180) in promoting mycobacteria-induced cytokine production by macrophages. Our data show that the significantly reduced release of TNF and IL-6 by RP105−/− macrophages during mycobacterial infection was not accompanied by diminished mRNA or protein expression. Mycobacteria induced comparable activation of NF-κB and p38 MAPK signaling in wild-type (WT) and RP105−/− macrophages. In contrast, mycobacteria-induced phosphorylation of Akt was abrogated in RP105−/− Cal-101, and small interfering RNA–mediated knockdown of p110δ by RP105−/− mycobacteria-induced cytokine production by macrophages. Our data show that the significantly reduced release of TNF and IL-6 formation (3). Macrophages are the major host cells for pathogenic lymphocyte activation and recruitment, as well as granuloma infections such as antimicrobial defense of infected macrophages. Such interference with p110δ diminished mycobacteria-induced TNF secretion by WT but not RP105−/− macrophages. Such interference with p110δ activity led to reduced surface-expressed TNF in WT but not RP105−/− macrophages, while leaving TNF mRNA and protein expression unaffected. Activity of Bruton’s tyrosine kinase was required for RP105-mediated activation of Akt phosphorylation and TNF release by mycobacteria-infected macrophages. These data unveil a novel innate immune signaling axis that orchestrates key cytokine responses of macrophages and provide molecular insight into the functions of RP105 as an innate immune receptor for mycobacteria. The Journal of Immunology, 2015, 195: 000–000.

Effective cytokine responses are central to the host control of infections with pathogenic mycobacteria. This is exemplified by the enhanced susceptibility of individuals with genetic defects in the IL-12/IFN-γ signaling axis (1) as well as the increased risk for reactivation of latent tuberculosis in patients receiving TNF-neutralizing biologicals in the treatment of inflammatory disorders (2). The concerted actions of cytokines orchestrate key elements of host responses to mycobacterial infections such as antimicrobial defense of infected macrophages, lymphocyte activation and recruitment, as well as granuloma formation (3). Macrophages are the major host cells for pathogenic mycobacteria. In innate immune responses expressed by macrophages, including TLRs, c-type lectin receptors, and NOD-like receptors, play central roles in the uptake of mycobacteria and the activation and regulation of host responses to mycobacterial infection (4). Ligation of innate immune receptors triggers stereotypical cellular responses such as activation of NF-κB and MAPK signaling culminating in the expression of genes that shape the host response to infection, including inflammatory cytokines (5). Besides mRNA expression, key regulatory checkpoints of macrophage cytokine responses include posttranscriptional mRNA stabilization by p38 MAP-mediated MK2 activation and microRNAs (6). Posttranscriptional regulation of Mycobacterium tuberculosis–induced TNF production downstream of TLRs has been suggested (7) and microRNAs 125b and 155 have been implicated in the fine-tuning of macrophage TNF production induced by lipomannans of different mycobacterial species (8). An additional key checkpoint of cytokine responses is the directed cytokine trafficking and release via the trans-Golgi network and delivery to the cell surface for release (9). Current understanding of the molecular events that orchestrate trafficking and release of cytokines is almost exclusively derived from studies utilizing soluble TLR ligands. Whether cytokine responses to mycobacterial infection are regulated through protein trafficking and targeted release is unknown.

Radioprotective 105 kDa (RP105; CD180) is a member of the TLR family that is expressed by B cells, macrophages, and myeloid dendritic cells (10–13). RP105 is phylogenetically most closely related to TLR4 (11). Similar to all TLR family members, RP105 contains extracellular leucine-rich repeat domains (13). Similar to TLR4, which forms a functional complex with MD-2, the soluble MD-2 homolog, MD-1, is critical for cell surface expression and functions of RP105 (12, 14–16). Owing to the lack of an intracellular Toll/IL-1R (TIR) domain, which is essential for TLR

*The University of Queensland Diamantina Institute, University of Queensland, Translational Research Institute, Brisbane, Queensland 4102, Australia; †Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia; ‡Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523; and §Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, Queensland 4072, Australia.

Address correspondence and reprint requests to Dr. Antje Blumenthal, The University of Queensland Diamantina Institute, 37 Kent Street, Brisbane, QLD 4102, Australia. E-mail address: a.blumenthal@uq.edu.au

The online version of this article contains supplemental material.

Abbreviations used in this article: BCG, bacillus Calmette-Guérin; Bir, Bruton’s tyrosine kinase; HPRT, hypoxanthine phosphoribosyltransferase; RP105, radioprotective 105 kDa; siRNA, small interfering RNA; TAPI, N-(3,4-di(hydroxymethyloxamoyl) methyl)-4-methylpentanoyl-L-naphthylalanoyl-L-alanine amide; TIR, Toll/IL-1R; WT, wild-type.

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signaling, it has been postulated that RP105 might require a signaling-competent partner to mediate cellular activation (17). B cells proliferate in response to an anti-RP105 Ab. This response has been reported to be mediated by a CD19/Lyn/Vav complex and involve signaling via protein kinase Cζ/II, MEK, PI3K, and Bruton’s tyrosine kinase (Btk) (18, 19). In macrophages and dendritic cells, RP105 has been reported to curb LPS-induced cytokine production (11). Proposed molecular mechanisms for this regulatory role include sequestration of LPS by the RP105/M-1 complex (11, 20) as well as spatial separation of the TIR domains of TLR4 homodimers (20, 21). In contrast to the negative regulation of TLR4 functions, we identified RP105 as a coreceptor for TLR2 in the recognition of mature mycobacterial lipoproteins. RP105 expression by macrophages was required for optimal cytokine production upon infection with M. tuberculosis and stimulation with the M. tuberculosis 19 kDa lipoprotein. RP105 physically and functionally interacted with TLR2. However, canonical TLR signaling events such as phosphorylation of JNK and p38 MAPKs and the NF-xB regulator, IkBα, remained intact in RP105-deficient macrophages (10). This suggested that RP105 contributed to mycobacteria-induced cytokine production by macrophages not merely by facilitating prototypical TLR2 signaling. However, the molecular mechanisms of RP105-dependent macrophage cytokine production remained elusive. PI3Ks are signaling enzymes that orchestrate a multitude of cellular functions, including apoptosis, differentiation, metabolism, cytoskeletal remodeling, and trafficking of intracellular organelles (22). Within this large family, the heterodimeric class I PI3K kinases are comprised of a regulatory subunit (class IA, p85 or p55 subunits; class IB, p84/87 or p101 subunits) and one of four p110 catalytic subunits (class IA, α, β, δ; class IB, γ) (23). The p85 regulatory subunit of class IA PI3Ks is recruited to membranes via phosphorylated tyrosine residues in conserved YXXM motifs contained in receptor tyrosine kinases or adaptor proteins. Activated PI3Ks convert the lipid substrate phosphatidylinositol 4,5-bisphosphate into the second messenger phosphatidylinositol 3,4,5-trisphosphate, which exerts regulation of key cellular functions via various downstream adaptors, protein kinases (e.g., Akt/PKB1), and regulatory factors for GTPases (24). In contrast to the ubiquitous expression of PI3K p110α and p110β, the p110δ subunit is predominantly found in hematopoietic cells. Besides its fundamental roles in B cell development and functions (25), p110δ is implicated as a central regulator of intracellular vesicle trafficking associated with inflammatory cytokine responses in different immune cells such as mast cells (26), NK cells (27), B cells (28, 29), and T cells (30). In macrophages, p110δ was required for fission of TNF-containing vesicles from the Golgi apparatus and transport to the cell surface upon stimulation with the TLR4 agonist LPS (31). A PI3K p110δ inhibitor also impaired TNF secretion by glucose-deprived primary microglia and ameliorated inflammation and injury in a murine stroke model (17).

In this study, we demonstrate that PI3K p110δ acts as a downstream effector of RP105 in promoting mycobacteria-induced cytokine production by macrophages. Our data show that RP105-mediated PI3K p110δ activity regulates the trafficking and surface delivery of TNF, revealing this as a new molecular checkpoint for the regulation of mycobacteria-induced host responses imposed by RP105.

Materials and Methods

**Bacteria, reagents, Abs**

*Mycobacterium bovis* bacillus Calmette–Güerin (BCG) strain Pasteur (American Type Culture Collection) was grown to midlog phase in 7H9 Middlebrook medium (BD Biosciences) with 5% BSA, 2% dextrose, 0.85% NaCl, and 0.05% Tween 80. *Listeria monocytogenes* 10403s (gift from Prof. Eric Pamer, Memorial Sloan-Kettering Cancer Center, New York, NY) was grown to early log phase in brain heart infusion medium (BD Biosciences) and diluted in DMEM for infections. Gamma-irradiated *M. tuberculosis* H37Rv (catalog no. NR-14819) was obtained from BEI Resources, Colorado State University, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Pam3CSK4 was from InvivoGen. N-(R)-(2-(hydroxymyicosanoic acid)ethyl)-4-methylpentanoyl-n-hexadecyl-t-alanine amide (TAPl, TACE inhibitor) was from Enzo Life Sciences. Cell signaling molecule inhibitors were LY294002 (Selleck Chemicals), wortmannin (Alomone Labs), YM204 (ICOS), AS252424 (Cayman Chemical), TGX-221, Cal-101 (Symansis), PP2 (Cayman Chemical), piceatannol, and ibrutinib (Selleck Chemicals). Abs against p-p38, p-p65, p-Akt (Ser473), Akt (Cell Signaling Technology), PI3K p110α, α-tubulin (Abcam), and HRP-conjugated goat anti-rabbit IgG (Invitrogen) were used for Western blot. Abs against mouse TNF (allophycocyanin, MP6-XT22), IL-6 (PE, MP5-20F3) (BioLegend), and F4/80 (FITC, MB8; E Bioscience) were used for flow cytometry.

**Purification of the M. tuberculosis 19 kDa lipoprotein**

*M. tuberculosis* cell wall was obtained and extracted with Triton X-114 as described previously (33). The *M. tuberculosis* 19 kDa lipoprotein was purified from the detergent extract similar to methods described previously (34) with modifications to ensure low endotoxin contamination. First, the whole gel eluates was pretreated and washed with LPS-binding buffer (10 mM Tris-free, 0.2 M NaCl, 0.25% deoxycholic acid sodium salt, 0.5 M EDTA [pH 8] in endotoxin-free water) prior to elution of the 19 kDa lipoprotein. Second, the electroelution was performed using 0.1 M ammonium bicarbonate (pH 8) in endotoxin-free water, and fractions containing purified 19 kDa lipoproteins were tested for endotoxin (*Limulus amebocyte lysate assay*; Lonza, Basel, Switzerland) and immediately freeze-dried. *Limulus amebocyte lysate assays* demonstrated <4 ng endotoxin units/mg 19 kDa lipoprotein protein in all samples tested.

**Mice**

RP105−/− (35) and TLR2−/−/RP105−/− (10) mice were obtained from Christopher L. Karp (Cincinnati Children’s Research Center, Cincinnati, OH). C57BL/6 mice (Charles River Laboratories) were used as controls. All mice were bred locally under specific pathogen-free conditions and were age and sex matched for all experiments. All animal procedures adhered to the guidelines of the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes and were approved by the The University of Queensland Animal Ethics Committee (DI/56709; DJ/571/12).

**Primary macrophage culture**

Bone marrow of 8- to 10-wk-old mice was differentiated into macrophages for 6–7 d in DMEM with 20% L cell–conditioned medium, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (pH 7.4) (Invitrogen). Mice were sacrificed by CO2 asphyxiation, and bone marrow was removed from the femur and tibia. Bone marrow was flushed with DMEM, and cells were counted by trypan blue exclusion. Cells were cultured with Fc Block and anti-Abrin Ab followed by Alexa Fluor 647–conjugated secondary Ab (Life Technologies), BCG and irradiated *M. tuberculosis* were prepared as single-cell suspensions (36). PI3K inhibitors were added 30 min after stimulation. Cytokine concentrations were measured by ELISA (TNF, R&D Systems; IL-6, BD Biosciences). For mRNA expression, cells were lysed in RNAzol (Sigma-Aldrich) and total RNA was isolated. RNA was reverse transcribed using oligo(dT) nucleotides. Primers for mouse Tnf and Rpl4 were as described (37); others were designed using the Integrated DNA Technologies online tool (Table I). Quantitative real-time PCR was performed using SYBR Green real-time master mix (Applied Biosystems) and a 7900HT (Applied Biosystems, PerkinElmer).

**Flow cytometry**

For detection of intracellular cytokines, macrophages were treated with monensin (2.4 μM; BD Biosciences) 30 min postinfection or stimulated with anti-Tnf Ab followed by Alexa Fluor 647–conjugated secondary Ab (Life Technologies). BCG and irradiated *M. tuberculosis* were prepared as single-cell suspensions (36). PI3K inhibitors were added 30 min after stimulation. Cytokine concentrations were measured by ELISA (TNF, R&D Systems; IL-6, BD Biosciences). For mRNA expression, cells were lysed in RNAzol (Sigma-Aldrich) and total RNA was isolated. RNA was reverse transcribed using oligo(dT) nucleotides. Primers for mouse Tnf and Rpl4 as described (37); others were designed using the Integrated DNA Technologies online tool (Table I). Quantitative real-time PCR was performed using SYBR Green real-time master mix (Applied Biosystems) and a 7900HT (Applied Biosystems, PerkinElmer).

**Western blotting**

Macrophages were stimulated and lysed (120 mM Tris–HCl [pH 6.8], 4% SDS, 20% glycerol, 10% DTT, bromophenol blue). Samples were separated
on 10% SDS-PAGE gels and subsequently blotted onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% nonfat milk in TBS/T0.1% Tween 20 before incubation with primary Abs overnight at 4°C, followed by HRP-conjugated secondary Ab. Proteins were visualized using SuperSignal West Dura extended duration substrate (Thermo Scientific), imaged (Fusion SL, Vilber Lourmat), and analyzed (ImageJ).

**Fluorescence microscopy**

Macrophages were seeded onto glass coverslips and infected with BCG, stimulated with M. tuberculosis 19 kDa lipoprotein or Pam3CSK4 in the presence of TAPI (5 μM), TNF staining was performed as described (38). Cells were fixed and incubated with rabbit polyclonal anti-mouse TNF Ab (Calbiochem), followed by an Alexa Fluor 555–conjugated anti-rabbit Ab (Invitrogen). Subsequently, macrophages were permeabilized and incubated with rat anti-mouse TNF Ab (MP6-XT22; BD Biosciences) followed by Alexa Fluor 488–conjugated anti-rat Ab (Invitrogen). DAPI was used for visualizing nuclei. Epifluorescence microscopy used a ×63 oil objective (BX51; Olympus). Image analysis occurred blinded with identified sample specifications: 1) percentages of cells displaying nuclear strain of nuclei. A 647–conjugated anti-rabbit Ab (Invitrogen). DAPI was used for visualizing nuclei. Epifluorescence microscopy used a ×63 oil objective (BX51; Olympus). Image analysis occurred blinded with identified sample specifications: 1) percentages of cells displaying nuclear strain of nuclei. A 647–conjugated anti-rabbit Ab (Invitrogen). DAPI was used for visualizing nuclei. Epifluorescence microscopy used a ×63 oil objective (BX51; Olympus). Image analysis occurred blinded with identified sample specifications: 1) percentages of cells displaying nuclear strain of nuclei. A 647–conjugated anti-rabbit Ab (Invitrogen). DAPI was used for visualizing nuclei. Epifluorescence microscopy used a ×63 oil objective (BX51; Olympus).

**Small interfering RNA knockdown**

PI3K p110δ expression was silenced using predesigned stealth small interfering RNAs (siRNAs). A nontargeting scrambled siRNA was used as control (all Life Technologies). Macrophages were differentiated for 6 d and transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen). Subsequent experiments were carried out 48 h after transfection.

**Data representation and statistical analyses**

mRNA expression is represented as gene expression relative to hypoxanthine phosphoribosyltransferase 1 (Hprt) expression (2(Ct Hprt ~ Ct gene)). Where indicated, relative gene expression in infected/stimulated cells was normalized to expression in untreated cells of the same genotype in the same experiment and is represented as fold change. Two groups were compared by a two-tailed Student t test (95% confidence interval); multiple conditions were analyzed by one- or two-way ANOVA followed by a Sidak or Dunnett multiple comparison test as appropriate. GraphPad Prism 6 was used for all charts and statistical analyses.

**Results**

**RP105 mediates cytokine production by macrophages in response to mycobacteria**

RP105 was previously demonstrated to facilitate cytokine production in response to infection with M. tuberculosis. Mature mycobacterial lipoproteins, such as the 19 kDa lipoprotein, were identified as RP105 agonists (10). M. bovis BCG is an attenuated strain of M. bovis lacking the RD-1 region, which contains important virulence determinants (39). However, it still shares many features with M. tuberculosis and M. bovis, including the expression of lipoproteins (40). Similar to infection with M. tuberculosis, RP105−/− bone marrow–derived macrophages infected with BCG displayed reduced release of cytokines, including TNF and IL-6, compared with wild-type (WT) cells (Fig. 1A), confirming the presence of RP105 agonists in mycobacterial species other than M. tuberculosis. Reduced cytokine release by RP105−/− macrophages was also observed in response to gamma-irradiated M. tuberculosis (Fig. 1B), confirming RP105-dependent macrophage activation by mycobacteria independent of active infection. In contrast to mycobacteria, RP105-deficient macrophages did not display reduced cytokine production in response to infection with Listeria monocytogenes (Fig. 1C), an intracellular pathogen that infects macrophages, contains lipoproteins, and activates macrophage cytokine responses in a TLR2-dependent manner (41). This is in concordance with our previous observations that not all TLR2 agonists require RP105

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**Table I. Real-time PCR primers**

<table>
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<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
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<td>TGAAGATGTTAATCCGAGTG</td>
</tr>
<tr>
<td>Pik3ca</td>
<td>TCAAGGAGAACAAAGAAGGCG</td>
<td>AGCTTCTGCACATTCTACC</td>
</tr>
<tr>
<td>Pik3cb</td>
<td>GTGTAGGCTCTTATGTCCTCG</td>
<td>AGGTACTCGCTCCCTTTT</td>
</tr>
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<td>Pik3cd</td>
<td>TTTTCTTCACAATCTGTCG</td>
<td>AGCCTGTTGAACTTCAGCC</td>
</tr>
<tr>
<td>Pik3cg</td>
<td>GCAGCTGGAAGGCTGTCTTAC</td>
<td>TCCAGAAGATGTTGACAGAG</td>
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<tr>
<td>Hprt</td>
<td>CCCCAGAAATGTTGAGATTGCC</td>
<td>AAGAAAATGGCTGATATC</td>
</tr>
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**FIGURE 1.** RP105-dependent cytokine production in response to mycobacteria, but not L. monocytogenes. (A) WT and RP105−/− bone marrow–derived macrophages were infected with BCG. (B) stimulated with gamma-irradiated M. tuberculosis, (C) infected with L. monocytogenes (all 5 and 10 bacteria/macroage), or left uninfected (0). TNF and IL-6 concentrations in cell culture supernatants were determined 8 h postinfection per stimulation. Data are means ± SD of triplicates from one representative of three independent experiments. See also Supplemental Fig. 1. LM, L. monocytogenes; Mtb (irr.), irradiated M. tuberculosis.
for macrophage activation (10). Thus, RP105 promotes macrophage cytokine responses in mycobacterial infections.

Canonical TLR signaling events and protein expression of cytokines remain unaltered in RP105-deficient macrophages

We next sought to elucidate the nature of the contribution of RP105 to macrophage cytokine production during mycobacterial infection. To this end, we interrogated the activation of key TLR signaling events and regulatory checkpoints of cytokine expression in WT and RP105−/− macrophages. Canonical TLR signaling events, such as activation of MAPKs and the NF-κB pathway, were previously demonstrated to occur with similar kinetics and strength in WT and RP105−/− macrophages in response to stimulation with the M. tuberculosis 19 kDa lipoprotein (10). This was also observed in the context of infection with whole mycobacteria with no changes in MAPK and NF-κB signaling (Supplemental Fig. 1A, 1C). Similarly, infection with L. monocytogenes, which induced macrophage cytokine production independent of RP105, did not show differences between WT and RP105−/− macrophages in the activation of MAPK and NF-κB signaling (Supplemental Fig. 1B). We next examined whether RP105 contributed to early macrophage cytokine responses at the level of mRNA expression. Upon infection with BCG and stimulation with gamma-irradiated M. tuberculosis, Tnf and Il6 mRNA expression was comparable between WT and RP105−/− macrophages between 2 and 8 h postinfection (Fig. 2A, Supplemental Fig. 2A). This contrasts with the significantly reduced cytokine concentrations in supernatants of RP105−/− macrophage cultures at 8 h postinfection (Fig. 1A, 1B). These observations indicate that RP105 orchestrates mycobacteria-induced cytokine responses downstream of gene transcription.

Therefore, we next examined mycobacteria-induced intracellular TNF and IL-6 protein expression by fluorescence staining. We observed a similar percentage of WT and RP105−/− macrophages expressing TNF and IL-6 protein at comparable levels in response to stimulation with BCG, gamma-irradiated M. tuberculosis, and the M. tuberculosis 19 kDa lipoprotein, as well as the RP105-independent TLR2/1 agonist Pam3CSK4 (Fig. 2B–D, Supplemental Fig. 2B–D). However, mycobacteria-induced TNF and IL-6 protein expression was abrogated in macrophages deficient for both TLR2 and RP105 (Fig. 2B–D, Supplemental Fig. 2B–D), suggesting divergence in the cellular pathways mediating cytokine production downstream of RP105 and TLR2. These observations led us to hypothesize that RP105 contributed to the trafficking and release of cytokines rather than their expression and synthesis.

RP105 promotes TNF trafficking in macrophages

TNF is expressed as a membrane-bound precursor that is transported via the trans-Golgi network and recycling endosome to the cell surface, requiring proteolytic activity of the matrix metalloprotease TACE for ultimate release (42). These characteristics of TNF allow for tracking its trafficking from the Golgi apparatus to the cell surface. We analyzed TNF distribution in WT and

![FIGURE 2. Mycobacteria-induced TNF mRNA and protein expression are comparable in WT and RP105−/− macrophages. WT, RP105−/−, and TLR2/RP105−/− bone marrow–derived macrophages were infected with BCG or stimulated with gamma-irradiated M. tuberculosis (each five bacteria/macrophone), Mycobacterium 19 kDa lipoprotein (LP) (1 μg/ml) and Pam3CSK4 (10 ng/ml) for 2, 4, and 8 h. (A) Tnf mRNA expression was normalized to Hprt as relative gene expression and represented as fold change compared with unstimulated cells. Data are means ± SEM from three independent experiments compared between WT and RP105−/− cells per time point by two-way ANOVA and a Sidak multiple comparison test. (B) Representative flow cytometry plots of intracellular TNF staining in WT, RP105−/−, and TLR2/RP105−/− bone marrow–derived macrophages upon mycobacterial infection or stimulation. (C) Percentages of F4/80+TNF+ macrophages and (D) median fluorescence intensity (MFI) of TNF staining in F4/80+TNF+ macrophages 4 h after infection or stimulation. Data are means ± SEM from three independent experiments, analyzed by two-way ANOVA and a Dunnett multiple comparison test. ***p < 0.001, ****p < 0.0001. See also Supplemental Fig. 2. Mtb (irr.), irradiated M. tuberculosis.](http://www.jimmunol.org/DownloadedFrom)
RP105−/− macrophages by utilizing immunolabeling of surface TNF in cells treated with the TACE inhibitor, TAPI, followed by permeabilization and staining of intracellular TNF with a second round of immunolabeling using a different fluorophore (38) (Fig. 3A). Upon infection with BCG or stimulation with the M. tuberculosis 19 kDa lipoprotein, the percentages of WT and RP105−/− macrophages that stained positive for Golgi-associated TNF were comparable, corroborating our finding that RP105 deficiency did not impair TNF protein expression (Fig. 3). In contrast, there was a significant reduction in the percentage of RP105-deficient macrophages that displayed surface TNF compared with WT cells (Fig. 3B). Furthermore, in RP105−/− macrophages significantly lower amounts of surface TNF were detected at filopodia and lamellipodia, sites of active TNF release (43) (Fig. 3). In contrast to the findings with mycobacteria, WT and RP105−/− macrophages stimulated with the RP105-independent TLR2/1 agonist, Pam3CSK4, showed no differences in the percentages of cells displaying surface well as Golgi-associated TNF or the amounts of TNF detectable at filopodia and lamellipodia (Fig. 3), confirming that there is no general deficiency in TNF trafficking in RP105−/− macrophages upon TLR stimulation. These findings strongly suggest that RP105 contributes to the regulation of post-Golgi intracellular cytokine trafficking and surface delivery in macrophages during mycobacterial infection.

RP105-dependent activation of PI3K signaling

We next determined which cellular signaling cascades implicated in cytokine trafficking might be activated in an RP105-dependent manner. PI3K signaling has been shown to be involved in regulating TNF delivery from the Golgi apparatus to the cell surface in LPS-stimulated macrophages (31). A previous study showed that PI3K signaling was activated in B cells upon stimulation with an anti-RP105 Ab (44). We thus investigated whether mycobacteria-induced activation of PI3K signaling required RP105. Phosphorylation of Akt (Ser473) was used as readout for the activation of

**FIGURE 3.** RP105−/− macrophages display reduced surface TNF in response to mycobacteria. WT and RP105−/− bone marrow-derived macrophages were analyzed for surface and intracellular TNF after infection with BCG (10 bacteria/macrophage), stimulation with M. tuberculosis 19 kDa lipoprotein (1 μg/ml) or Pam3CSK4 (10 ng/ml). (A) Representative fluorescence microscopy images at 4 h of stimulation (Scale bar, 20 μm; blue shows DAPI). (B) Cells displaying surface as well as Golgi-associated TNF were quantified and compared with cells displaying only Golgi-associated TNF in response to BCG, M. tuberculosis 19 kDa lipoprotein, and Pam3CSK4 at 2, 4, and 8 h. Data are means ± SEM of 300–500 cells cumulative from three independent experiments. Fluorescence intensity of surface TNF was determined at lamellipodia and filopodia at 2, 4, and 8 h after infection/stimulation. Data are means ± SEM of 100–250 cells cumulative from three independent experiments. Comparisons between genotypes by two-way ANOVA and a Sidak multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
PI3K. Phosphorylation of Akt was induced in WT macrophages in response to mycobacterial infection (Fig. 4A, 4C). This was inhibited by the pan-PI3K inhibitors wortmannin and LY294002 (Supplemental Fig. 3A). In contrast to WT macrophages, mycobacteria-induced phosphorylation of Akt was abrogated in RP105−/− macrophages (Fig. 4A, 4C, and 4D).

We noted that Pik3r1, the gene encoding the class I PI3K regulatory subunits p85α, p55γ, and p50δ (45), is proximal to Rpi105/Cd180 on chromosome 13. However, we confirmed that Pik3r1 mRNA expression was not affected in RP105−/− macrophages (Supplemental Fig. 3B). Furthermore, Akt phosphorylation was comparable between WT and RP105−/− macrophages upon infection with L. monocytogenes (Fig. 4B) and stimulation with Pam3CSK4 (Supplemental Fig. 3C), demonstrating that there is no general defect in activating PI3K signaling in RP105−/− macrophages. Taken together, these findings identify PI3K signaling as a downstream effector of RP105-dependent cellular activation in macrophages in response to mycobacterial infection.

**PI3Kδ promotes mycobacteria-induced TNF secretion dependent on RP105**

It was recently described that p110δ mediates the delivery of TNF-containing vesicles from the Golgi apparatus to the cell surface in LPS-stimulated macrophages (31). In light of a previous hypothesis that PI3K p110δ promotes the delivery of TNF-containing vesicles from the Golgi apparatus to the cell surface in LPS-stimulated macrophages (31), we investigated whether activity of p110δ was dependent on RP105. Comparable p110δ protein expression was observed in WT and RP105−/− macrophages (Supplemental Fig. 4B). In WT macrophages, p110δ knockdown reduced 1) mycobacteria-induced Akt phosphorylation (Fig. 3D), suggesting that the p110δ reservoir was not affected by RP105 deficiency.

Cal-101, a selective inhibitor for PI3K p110δ (46), reduced the BCG-induced Akt phosphorylation in WT macrophages in a dose-dependent manner (Supplemental Fig. 4A) and, at higher concentration, also diminished the basal Akt phosphorylation in RP105−/− macrophages (Supplemental Fig. 4A). Cal-101 reduced mycobacteria-induced TNF secretion by WT macrophages in a dose-dependent manner (Fig. 5A). This was not associated with reduced TNF protein expression (Fig. 5B), but with a reduced percentage of macrophages displaying TNF at the cell surface as well as lower amounts of surface-associated TNF located at filopodia and lamellipodia (Fig. 5C). In contrast, the BCG-induced residual TNF secretion by RP105−/− macrophages was not impaired by Cal-101, and neither the percentage of RP105−/− macrophages positive for cell surface TNF nor the fluorescence intensity of surface TNF in membrane regions of cytokine release was altered by Cal-101 treatment (Fig. 5C). Moreover, treatment of macrophages with inhibitors for the p110α, β, and γ subunits diminished BCG-induced TNF release by both WT and RP105−/− macrophages (data not shown).

To further assess the requirement of p110δ activity for RP105-mediated TNF trafficking, siRNAs were used to silence p110δ protein expression in macrophages. Two of the three siRNAs tested, in particular siRNA2, were found to significantly diminish endogenous p110δ protein expression in macrophages by >90% (Fig. 5D), without significant effects on expression of other PI3K p110 isoforms (Supplemental Fig. 4B). In WT macrophages, p110δ knockdown reduced 1) mycobacteria-induced Akt phosphorylation (Fig. 3D), suggesting that the p110δ reservoir was not affected by RP105 deficiency.
FIGURE 5. PI3K p110δ is a downstream effector of RP105 and mediates TNF trafficking in mycobacteria-infected macrophages. (A) TNF production by WT and RP105\(^{−/−}\) bone marrow–derived macrophages infected with BCG (five bacteria/macrophage) for 8 h compared with
phosphorylation (Supplemental Fig. 4C), 2) mycobacteria-induced TNF secretion (Fig. 5E), and 3) the percentage of macrophages expressing TNF at the cell surface and the amounts of surface TNF in these cells (Fig. 5F). In contrast, p110δ knockdown in RP105−/− macrophages did not affect mycobacteria-induced TNF secretion or surface expression (Fig. 5E, 5F). Taken together, these data identify PI3K p110δ as a downstream effector of RP105 that promotes mycobacteria-induced trafficking and release of TNF by macrophages.

Note that whereas mycobacteria-induced IL-6 release by macrophages was RP105-dependent (Fig. 1, Supplemental Fig. 2A–D), this was independent of PI3K p110δ activity (Supplemental Fig. 2E, 2F). This suggests p110δ-independent cellular mechanisms downstream of RP105 that promote IL-6 trafficking and release by mycobacteria-infected macrophages.

Btk activity links RP105 and PI3K signaling

Previous studies have implicated tyrosine kinases, including Btk, Syk, and the Src family member, Lyn, in connecting TLR signaling to cytokine trafficking and release through PI3K activation (47–49). We assessed whether Btk, Src, or Syk activity was required for RP105-dependent cytokine production as well as Akt phosphorylation in macrophages during mycobacterial infection. Ibrutinib, a selective inhibitor for Btk, was shown to block both mycobacteria-induced TNF secretion and Akt phosphorylation in WT but not in RP105−/− macrophages. Ibrutinib, a selective inhibitor for Btk, or Syk (piceatannol) diminished mycobacteria-induced TNF secretion and Akt phosphorylation in WT but not in RP105−/− macrophages (Fig. 6A, 6D). As this was not associated with diminished intracellular cytokine expression (Fig. 6B), these observations mirrored the pattern observed with the p110δ inhibitor, Cal-101, and the siRNA-mediated knockdown of p110δ expression (Fig. 5, Supplemental Fig. 4A, 4C). In contrast, inhibitors targeting Src (PP2) and Syk (piceatannol) diminished mycobacteria-induced TNF production by both WT and RP105−/− macrophages (Fig. 6C). These data identify Btk as a downstream effector of RP105 whose activity is required for mycobacteria-induced PI3K activation and TNF release.

**Discussion**

TNF plays a central role in controlling infections with pathogenic mycobacteria. In conjunction with IFN-γ, TNF enhances the production of reactive radicals and the antimycobacterial activity of macrophages (51). Data from patients receiving TNF-neutralizing therapies as well as mouse studies demonstrate that effective TNF blockade is achieved. TNF expression is required for granuloma formation and maintenance during active as well as latent tuberculosis (52). TNF expression by macrophages in response to mycobacteria is known to occur downstream of TLRs and c-type lectin receptors via the activation of NF-κB and MAPKs signaling, and is regulated by microRNAs and SHIP (4, 8, 53).

In this study, we reveal that mycobacteria-induced TNF responses by macrophages are regulated at the level of intracellular cytokine trafficking and release through RP105-dependent activation of PI3K signaling. First, mycobacteria-induced TNF mRNA and protein expression were comparable between WT and RP105−/− macrophages. In contrast, fewer RP105−/− macrophages displayed surface-bound TNF and at lower abundance, and TNF was present at significantly lower concentrations in culture supernatants of RP105−/− macrophages. Second, the mycobacteria-induced phosphorylation of the PI3K downstream effector, Akt, required RP105. Third, inhibition of PI3K p110δ activity and p110δ knockdown impaired mycobacteria-induced TNF responses at the level of cytokine trafficking and release in WT but not RP105−/− macrophages.

TLR2 and TLR3 have been implicated in the mycobacteria-induced activation of PI3K signaling in macrophages (54–56). In the present study, we show that in mouse bone marrow–derived macrophages, RP105 deficiency abrogated mycobacteria-induced Akt phosphorylation. We cannot exclude that this RP105-dependent activation of PI3K signaling also required TLR2. However, most investigations on PI3K activity in response to TLR2 agonists have reported crosstalk with MAPK and NF-κB activation (57, 58). In contrast, RP105 deficiency in macrophages did not affect mycobacteria-induced phosphorylation of p38 and JNK or phosphorylation of p65 or IkBα (Ref. 10 and Supplemental Fig. 1). Furthermore, the differential effects of RP105 deficiency and RP105/TLR2 deficiency on TNF and IL-6 protein expression in mycobacteria-stimulated macrophages suggest a distinct RP105-dependent signal that occurs independent of canonical TLR signaling.

In contrast to the signaling cascades that activate NF-κB, MAPKs, and IFN regulatory factors downstream of TLRs, the molecular events connecting TLR engagement and PI3K activation are less well understood. The regulatory PI3K p85 subunits contain SH2 domains, which enable recruitment of catalytic p110 subunits to phosphorylated tyrosine residues contained within YXXM motifs (22). Several modes of p85 recruitment to TLRs have been proposed. These include physical interaction with TLRs and c-type lectin receptors via the activation of PI3K downstream of TLRs and c-type lectin receptors via the activation of PI3K, and RP105/TLR2 deficiency on TNF and IL-6 protein expression compared with scrambled control RNA. A representative Western blot is depicted. p110δ knockdown impaired mycobacteria-induced TNF responses at the level of cytokine trafficking and release in WT but not RP105−/− macrophages.
TIRAP/MAL (61), and BCAP (47, 62) have been described, and tyrosine kinases, including Lyn, Syk, and Btk, have been implicated in linking TLR signaling and PI3K activation (48, 49, 63). Our data place Btk upstream of PI3K activation and as a molecular link between RP105 and TNF trafficking in mycobacteria-infected macrophages.

Whereas RP105 lacks the TIR domain that is required for the recruitment of TLR signaling adaptors (17), it contains at least one tyrosine residue in its cytoplasmic tail, which, however, is not embedded into a classical YXXM motif. It remains to be determined whether this tyrosine is phosphorylated in response to activating engagement and might provide a recruitment platform for Btk, PI3K regulatory subunits, or other signaling-competent membrane-associated proteins that engage Btk and PI3K. In B cells, a stimulatory anti-RP105 Ab activated PI3K signaling involving CD19, Btk, and Lyn (18, 19). Moreover, the YXXM motifs in BCAP and CD19 were required for BCR-mediated PI3K/Akt activation (64). These observations suggest that RP105-mediated PI3K activation can occur independently of other TLRs, but might require interactions with a YXXM-containing partner. Although it is currently unknown whether RP105 is phosphorylated in macrophages in response to mycobacteria, it is tempting to speculate that this might provide a molecular basis for signal diversion between RP105 and TLR2 when sensing mycobacteria.

Mycobacteria-induced PI3K signaling in macrophages has been reported to contribute to the production of cytokines (TNF, IL-6, G-CSF) and chemokines (CCL5, CCL8) (55, 65), as well as cell polarization (54), bacterial uptake (66), and inhibition of phagosome maturation (67). The positive effects on mycobacteria-induced inflammatory cytokine responses seem to contrast the reported inhibition of proinflammatory cytokine production by PI3K signaling in the context of TLR stimulation, in particular LPS. However, note that there are conflicting results describing TLR-induced PI3K activity as either a positive or negative regulator of macrophage cytokine production. Varying protocols utilizing the pan-PI3K inhibitors, LY294002 and wortmannin, as well as yet to be identified functions of regulators of the PI3K signaling cascade might account for some of these differences (68).

The different PI3K p110 catalytic subunits have distinct, non-redundant functions. However, pan-PI3K inhibitors or genetic
ablation of regulatory PI3K subunits does not allow attribution of specific contributions by individual p110 subunits expressed by macrophages. Thus, p110 subunit-specific inhibitors, siRNA knockdown, and genetic deletion in mice are valuable tools to gain refined insight into the multifaceted roles of PI3K signaling in macrophages. This is well exemplified by observations that PI3K p110 promotes LPS-induced TNF release by macrophages and microglia (31, 32). Together with our findings, this identifies PI3K p110 as a critical regulator of TNF trafficking and release in macrophages. PI3K p110 acted downstream of RP105 upon mycobacterial stimulation. Although physical interaction between RP105/MD-1 and TLR4/MD-2 have been shown, it is currently unknown whether LPS binding to RP105/MD-1 mediates PI3K p110 activation to facilitate cytokine trafficking. In light of the reported net inhibition of LPS-induced macrophage proinflammatory cytokine production by RP105 (11), however, such contributions would be masked. M. tuberculosis-derived TLR4 agonists have been reported (69, 70), and it seems possible that these may engage RP105 together with TLR4 when activating macrophages. However, in the context of infection with whole viable M. tuberculosis, TLR4+/−/− macrophages did not differ in their TNF and IL-12p40 release from WT controls, and RP105/TLR4 double-deficient macrophages phenocopied RP105 single-deficient cells (10), suggesting that any contribution of an RP105/TLR4 interaction would be minor.

Neither p110 inhibition or knockdown nor RP105 deficiency fully abolished the mycobacteria-induced cytokine release by macrophages, which is consistent with previous observations (10, 31). Differential involvement of p110 in the secretion of TNF and IL-6 from macrophages is in concordance with previous findings in the context of LPS stimulation, which indicated engagement of p110 with p230-positive tubules that facilitate TNF exit from the trans-Golgi network (31). In contrast to TGF-β, IL-6 secretion occurs via multiple types of trans-Golgi network carriers (71). Differences in the molecular machinery that facilitate sorting and transport of cytokines may account for the lack of impact displayed by the p110-specific siRNA and small molecule inhibitor on mycobacteria-induced IL-6 release by macrophages in the present study. The molecular nature of p110-mediated cytokine trafficking remains to be clearly defined. In addition to direct modulation of vesicle fission (31), indirect action by facilitating the expression or subcellular positioning of essential effectors involved in cytokine trafficking, such as Rab GTPases and SNARE proteins (9), might occur. A role for RP105 in the secretion of both TNF and IL-6 in response to mycobacterial infection suggests that in addition to engagement of p110, this TLR family member facilitates cytokine trafficking at multiple molecular levels.

The present study unveils a novel molecular link between innate immune recognition of mycobacteria by RP105 and a key regulator of cytokine trafficking in macrophages, PI3K p110. It elucidates molecular mechanisms of RP105-dependent macrophage activation and reveals a thus far unknown regulation checkpoint of TNF release by mycobacteria-infected macrophages. The multilayered control mechanisms orchestrating TNF expression and release underpin the central role this cytokine plays in the host defense but also its potential to induce pathology in chronically inflamed tissues.

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**Disclosures**

The authors have no financial interests of conflict.

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Supplemental Figure 1. Comparable activation of p38 MAPK and NF-κB signalling in WT and RP105−/− macrophages in response to *M. bovis*, BCG and *L. monocytogenes*. Western blot analysis for phosphorylation of p38, p65, IκB-α, and α-Tubulin in cell lysates from WT and RP105−/− bone marrow-derived macrophages (BMMs) infected with (A) *M. bovis*, BCG (MOI 10) or (B) *L. monocytogenes* (MOI 5) for the indicated times. Normalised signal intensities are shown as means ± SEM from 3-4 independent experiments compared between WT and RP105−/− cells per individual time point by two-way ANOVA and Sidak’s multiple comparison test; *n.s.* not significant. (C) Microscopy analyses of P-p65 (indicated by white arrows) co-localized with nuclei (DAPI) in WT and RP105−/− BMMs infected with BCG. Percentages of cells displaying nuclear P-p65 were determined. Data are means ± SEM from 3 independent experiments compared by two-way ANOVA and Sidak’s multiple comparison test between WT and RP105−/− cells at each time point; *n.s.* not significant.
Supplemental Figure 2. RP105-independent expression of mycobacteria-induced IL-6.

WT, RP105−/− and TLR2/RP105−/− BMMs infected/stimulated with *M. bovis*, BCG, γ-irradiated Mtb (each 5 bacteria/cell), Pam3CSK4 [10 ng/ml] or unstimulated (Unst). (A) *Il6* expression normalized to *Hprt* and represented as fold change compared to unstimulated cells. Means ± SEM from 3-4 independent experiments compared by two-way ANOVA and Sidak’s multiple comparison test. (B) Representative FACS plots of intracellular IL-6 in WT, RP105−/− and TLR2/RP105−/− BMMs. (C) Percentages of F4/80+ IL-6+ BMMs and (D) IL-6 median fluorescence intensity (MFI) in F4/80+ BMMs 8h post infection/stimulation. Means ± SEM of 3 independent experiments compared by two-way ANOVA and Dunnett’s multiple comparison test. *P<0.05, **P<0.01, ****P<0.0001. IL-6 secretion by BMMs treated with (E) Cal-101 or DMSO [0 µM]; (F) p110δ siRNA2 or scrambled control. Means ± SEM from 3 independent experiments analyzed by two-way ANOVA and Sidak’s multiple comparison test. *n.s. not significant.
Supplemental Figure 3. RP105 deficiency does not result in defective PI3K signaling.

(A) Western blot for P-Akt and α-Tubulin in WT BMMs infected with *M. bovis*, BCG (10 bacteria/macrophage) for 2h in the presence of Wortmannin [0.03, 0.1 µM] and LY294002 [3, 10 µM]. One representative of 2 independent experiments. (B) Basal mRNA expression of *Pi3kr1* in WT and RP105−/− BMMs. Each dot represents cells from an individual mouse. Means ± SEM from 6 mice analyzed by paired t-test. (C) Western blot for P-Akt, total Akt and α-Tubulin in lysates of WT and RP105−/− BMMs stimulated with Pam3CSK4 [10 ng/ml] for the times indicated. P-Akt normalized to α-Tubulin is represented as fold change compared to unstimulated cells. Means ± SEM of normalized P-Akt from 6 independent experiments compared per time point by two-way ANOVA and Sidak’s multiple comparison test. (D) Western blot for PI3K p110δ in WT and RP105−/− BMMs, normalized to α-Tubulin. Means ± SEM from 3 independent experiments analyzed by paired t-test. n.s. not significant.
Supplemental Figure 4. Inhibition of PI3K p110δ activity. Western blot for P-Akt and Akt in WT and RP105^{-/-} BMMs treated with (A) Cal-101 [0.01-10 μM] or DMSO [0 μM]; (C) siRNA2 targeting p110δ, and infected with M. bovis, BCG (10 bacteria/cell) for 2h. P-Akt normalized to Akt represented as fold-change compared to unstimulated cells. Means ± SEM from 3 independent experiments analyzed by two-way ANOVA and Sidak’s multiple comparison test (A) or paired t-test (C) comparing treatment to control per genotype; *P<0.05, ***P<0.001, ****P<0.0001. (B) mRNA expression of PI3K p110 isoforms upon p110δ knockdown by siRNAs or scrambled control. Gene expression normalized to Hprt is depicted as fold-change compared to non-transfected cells. Means ± SEM from 3 independent experiments analyzed by one-way ANOVA and Sidak’s multiple comparison test; *P<0.05, **P<0.01, ***P<0.001. n.s. not significant.