Toxoplasma gondii Virulence Factor ROP18 Inhibits the Host NF-κB Pathway by Promoting p65 Degradation*

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Background: ROP18 is a Toxoplasma secreted Ser/Thr protein kinase important for acute virulence. Results: ROP18 phosphorylates host p65 at Ser-468 and targets this protein to the ubiquitin-dependent degradation. Conclusion: ROP18 inhibits the host NF-κB pathway by promoting p65 degradation. Significance: These findings reveal a novel molecular mechanism by which type I strain manipulates the host immune system to facilitate infection.

The obligate intracellular parasite Toxoplasma gondii secretes effector molecules into the host cell to modulate host immunity. Previous studies have shown that T. gondii could interfere with host NF-κB signaling to promote their survival, but the effectors of type I strains remain unclear. The polymorphic rhoptry protein ROP18 is a key serine/threonine kinase that phosphorylates host proteins to modulate acute virulence. Our data demonstrated that the N-terminal portion of ROP18 is associated with the dimerization domain of p65. ROP18 phosphorylates p65 at Ser-468 and targets this protein to the ubiquitin-dependent degradation pathway. The kinase activity of ROP18 is required for p65 degradation and suppresses NF-κB activation. Consistently, compared with wild-type ROP18 strain, ROP18 kinase-deficient type I parasites displayed a severe inability to inhibit NF-κB, culminating in the enhanced production of IL-6, IL-12, and TNF-α in infected macrophages. In addition, studies have shown that transgenic parasites carrying kinase-deficient ROP18 induce M1-biased activation. These results demonstrate for the first time that the virulence factor ROP18 in T. gondii type I strains is responsible for inhibiting the host NF-κB pathway and for suppressing proinflammatory cytokine expression, thus providing a survival advantage to the infectious agent.

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4 The abbreviations used are: iNκB, NF-κB; HFF, human foreskin fibroblast; m.o.i., multiplicity of infection; IRG, IFN-γ-induced immunity-related GTPase.
degradation of IκB, an event involved in the nuclear translocation of p65/RelA to activate NF-κB. However, despite the initiation of NF-κB signaling, infection with *T. gondii* did not lead to the activation of NF-κB but to its termination. The reason for disabling NF-κB is associated with blocking of p65 translocation to the nucleus (10, 12, 13). Other studies have demonstrated that *T. gondii* activates NF-κB, which up-regulates the expression of anti-apoptotic genes to facilitate the replication of the pathogen (15–17). These results suggested that type I strains promoted the phosphorylation of IκB and induced nuclear translocation of p65 (14, 16, 18). Therefore, studies of the nuclear translocation of p65 in type I strain infection have yielded conflicting results. Importantly, none of these studies demonstrated which effectors of the type I strain manipulate the host NF-κB signaling to elicit a survival response during infection. The ROP18 kinase has been identified as a key virulence determinant conferring a high mortality phenotype of type I strains. Accordingly, we screened the ROP18 interacting host proteins using the yeast two-hybrid method. To our surprise, p65 was found as a target protein of ROP18. Then we sought to elucidate the relationship between the kinase activity of ROP18 and p65 degradation. Furthermore, we investigated ROP18-mediated host NF-κB suppression and the phenotype of infected macrophages.

Our results presented here showed that ROP18 phosphorylates p65 at Ser-468 to promote its ubiquitin-dependent degradation; thus, the nuclear localization of p65 cannot be observed in infected cells with type I strains, consistent with previous studies (9, 12, 13, 19, 20). Our data demonstrated that infection with *T. gondii* type I results in p65 ubiquitin-dependent degradation, which blocks the nuclear translocation of p65 and induces the consequent termination of the NF-κB pathway. Therefore, the study presented here gave a reasonable explanation for the initiation and termination of NF-κB pathway by *T. gondii* type I infection (10). Consistently, compared with wild-type ROP18 strain, kinase-deficient ROP18 type I parasites displayed a severe inability to inhibit the NF-κB pathway, culminating in the enhanced production of IL-6, IL-12, and TNF-α. In addition, transgenic parasites carrying kinase-deficient ROP18 parasites induced M1-biased activation. The data indicated for the first time that the *T. gondii* type I virulence factor ROP18 is responsible for inhibiting the host NF-κB pathway and for suppressing proinflammatory cytokine expression, thereby providing a survival advantage to this infectious agent.

**EXPERIMENTAL PROCEDURES**

*Ethics Statement*—Ethical permission was obtained from the Institutional Review Board of the Institute of Biomedicine at Anhui Medical University (permit number AMU 26 – 093628), which records and regulates all research activities in the school. The Institutional Review Board of the Anhui Medical University approved both animals and humans protocols. The approval from the Institutional Review Board includes the permission of using mice under euthanasia, and all the experimental procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Cells, Mice, and Parasites**—The 3–4-week-old female KunMing mice or BALB/c mice used in this study were obtained from the Experimental Animal Center of Anhui Province. All animal experiments were conducted with the approval of the Animal Care and Use Committee of Anhui Medical University. The wild-type ROP18-Ty1 RH strain (overexpressing wild-type ROP18 RH strain) and the kinase-deficient ROP18-Ty1 RH strain (overexpressing kinase-deficient ROP18 RH strain) were kindly provided by Professor J. F. Dubremetz (Universite de Montpellier, Montpellier, France). The Δku80Δhxgprt RH strain and ROP18 knock-out Δku80 RH strain were kindly provided by Professor John C. Boothroyd (Stanford University School of Medicine). The HFF (human foreskin fibroblast cell line), RAW264.7 (mouse macrophage cell line), U937 (human macrophage cell line), and HEK293T cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 5% CO₂ atmosphere. All parasite strains and cell lines were routinely assessed for mycoplasma contamination, and no contamination was detected.

**Reagents and Plasmids**—Amplification of the open reading frame encoding *T. gondii* ROP18 (GenBank™ ID AM075204.1) was achieved through RT-PCR of the whole *T. gondii* tachyzoite RNA (21, 22). Point mutations were introduced using the QuikChange method (Stratagene). The plasmid vectors pGBK7 (Clontech), pGADT7 (Clontech), 3×FLAG (Sigma), and pEGFP-C2 (BD Biosciences) were used to generate mammalian and yeast expression constructs carrying full-length ROP18, the N-terminal portion of ROP18 (amino acids 1–252), ROP18Δ27-WT (lacking signal peptide), ROP18Δ27-MUT (D394A, lacking signal peptide), ROP18Δ240 (lacking amino acids 1–240), p65-N1 (amino acids 1–285), p65-N2 (amino acids 1–190), p65-M (amino acids 190–285), and p65-C (amino acids 285–550). Site-directed mutagenesis was performed using a standard molecular biology protocol. All constructs were confirmed through DNA sequencing. The full-length and deletion mutants for ROP18 or p65 were cloned into the bacterial expression plasmids pGEX-6P-1 (GE Healthcare) and pET-28a (Novagen). Lipopolysaccharides (LPS) were from Sigma, rhTNF-α and rmTNF-α were from R&D Systems (Minneapolis, MN), TRIZol reagent and PrimeScript™ RT reagent kit were from Invitrogen, MG132 was from Millipore, three kinds of p65 antibodies were purchased from Abcam (ab177895) and Upstate (06-418), anti-FLAG M2 gel, monoclonal anti-Ty1, monoclonal anti-GFP, and monoclonal anti-FLAG antibody M2 were from Sigma. Anti-SAG1 was from ViroStat, anti-CD86, anti-PD-L1, anti-PD-L2, and anti-MHC2 were from BD Biosciences, and anti-ubiquitin (#3936) and phospho-p65 Ser-468 (#3039) were from Cell Signaling. The IL-6, IL-12p40, and TNF-α levels were measured using an ELISA kit (R&D Systems). The reporter genes (3×κB-Luc and pFR-Luc) were kindly provided by Prof. Wancheng Li (University of Nebraska Medical Center).

**Generation of Over-expressing ROP18-WT and MUT Type I T. gondii in ROP18 Knock-out Background**—The 5′-UTR region of ROP18 from type I *Toxoplasma* genomic DNA was amplified by PCR (forward, 5′-GAACATCTGGTCCTCAGCAG-TTGCACAGGGACGACGATCT-3′; the BglII site was under-
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lined; reverse, 5′-CATGCCATGGCAACTTTCACACAAA-TCGGACTGCGGTG-3′; the NcoI site was underlined). The 3′-UTR region of ROP18 was amplified with primers (forward, 5′-TCCCCCGGGGAAGCTCAAATGAAAGGGGAACG- TGGCC-3′; the Saml site is underlined; reverse, 5′-GCTCTAGA-GACTGCTACGCGCCCTTGTAGTTGT-3′; the XbaI site is underlined). Genomic DNA containing the ROP18 wild-type or mutant genes were amplified from the wild-type ROP18-Ty1 or the kinase-deficient ROP18-Ty1 RH strains (kindly provided by Professor J. F. Dubremetz, Université de Montpellier, France) by PCR (forward, 5′-GAAACATCTGCTACGAGTTCGAC-AGGGAGACGAGCT-3′; the BglII site was underlined; reverse, 5′-GCTCTAGACTGCTACGCGCCCTTGTAGTTGT-3′; the XbaI site was underlined) and subcloned as the BglII/XbaI fragment to generate a 6.95-kb-long fragment and digested as NcoI/SamI fragment. Then the PCR product of 5′-UTR digested with NcoI was connected with the NcoI/SamI fragment and was connected with the 3′-UTR digested with SamI. The 5′-UTR-TUB promoter-ROP18-Ty1-HXGPR-3′-UTR was amplified by PCR with the primers (forward, 5′-GAACATCTGCTACGAGTTCGAC-AGGGAGACGAGCT-3′; the BglII site was underlined; reverse, 5′-GCTCTAGACTGCTACGCGCCCTTGTAGTTGT-3′; the XbaI site was underlined) and subcloned as the BglII/XbaI fragment. The 100-μg fragment was transfected into the Δku80Δhxgprt RH strain parasites (kindly provided by Professor John C. Boothroyd, Stanford University) by electroporation.

Electroporation was done in a 2-mm cuvette (Bio-Rad) with 2 mM ATP (MP Biomedicals) and 5 mM GSH (EMD) in a Gene Electroporation was done in a 2-mm cuvette (Bio-Rad) with 2 mM ATP (MP Biomedicals) and 5 mM GSH (EMD) in a Gene

In Vitro Phosphorylation Assay—The His-tagged full-length p65 and its truncates (p65-N1 and p65-C) were expressed in *Escherichia coli* and subjected to 10% SDS-PAGE followed by immunoblot analysis. 

Yeast Two-hybrid Analysis—All p65 variants constructed in pGADT7 (BD Bioscience) were mated with the AH109 strain transformed with pGBK7-T7-ROP1825−251. Briefly, the yeast cells were transformed with the bait construct using the lithium acetate method followed by the selection of bait-containing, auxotrophic yeast cells via the appropriate nutritional marker in the selection medium. The bait-containing cells were subsequently transformed with the library constructs, and the resulting transformants were grown on medium for the selection of either the expression of both AD and BD vectors or an interaction between the expressed fusion proteins via nutritional reporter gene expression. Clones expressing all three reporter genes, His3, Ade2, and x-gal, were further analyzed. Interactions between bait and prey were selected via colony growth on plates lacking tryptophan, leucine, and histidine.

**GST Pulldown Assay**—GST-ROP18 or GST was purified and conjugated to glutathione-Sepharose 4B beads. Purified His-p65-M was incubated with GST-ROP18-conjugated or GST-conjugated beads at 4 °C for 2 h. Subsequently, the beads were washed 3 times with pre-cooled PBS containing 1% Triton X-100 followed by 3 washes with PBS. The bound proteins were fixed in Laemmli loading buffer, incubated at 100 °C for 10 min, and subjected to 10% SDS-PAGE followed by immunoblot analysis.

**Luciferase Assay**—The cells were seeded onto 24-well plates and transfected with reporter plasmids and other plasmids as indicated. After transfection, the cells were treated with the indicated reagents or left untreated. Luciferase activity was evaluated using a Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. The assays were performed in triplicate.

**Immunofluorescence**—The cells were harvested and plated onto glass coverslips. Subsequently, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked using a solution containing 10% bovine serum albumin. The coverslips were incubated with anti-Ty1, anti-p65, and anti-GFP at 4°C overnight. FITC-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-rabbit IgG, and DAPI dye were used for antigen and DNA visualization. The images were captured using an Olympus BX60 Upright Fluorescence microscope with the appropriate filters and objectives and with identical acquisition parameters for each experiment.

**Immunoprecipitation and Immunoblot Analysis**—The 293T or HFF cells and parasites were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, and 10 g/ml pepstatin A) containing a protease inhibitor mixture (Sigma). The cell lysates were separated through SDS-PAGE, transferred to nitrocellulose membranes, probed with the corresponding antibodies, and developed using an ECL kit. For immunoprecipitation, the cell lysates were preclared using anti-FLAG M2 affinity gel for 4 h with rotation at 4 °C. The immunoprecipitants were washed three times with lysis buffer and three times with PBS and then eluted through boiling with Laemmli loading sample buffer. The eluates were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

**Flow Cytometric Analysis**—A total of 2×10^6 U937 cells were stained with phosphatidylethanolamine-conjugated anti-CD86, anti-CD273 (PD-L2), or anti-CD274 (PD-L1) antibodies or phosphatidylethanolamine-Cy5-conjugated anti-MHC Class II antibodies. The stained cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences) using FCS Express 4 Plus Research Edition software.

**ELISA Analysis**—RAW264.7 cells were seeded (10^6 per well) onto 12-well plates and incubated overnight at 37 °C in 5% CO₂. The cells were infected with freshly lysed *T. gondii*
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ROP18 Interacts with p65 and Mediates Its Proteasome-ubiquitin-dependent Degradation—To further assess the specificity of this interaction, we expressed GFP-tagged ROP18 lacking the signal peptide (ROP18Δ27-WT-GFP) and the N-terminal deletion of ROP18 (ROP18Δ240-GFP) together with FLAG-tagged p65 in 293T cells (Fig. 2A). Interestingly, we observed that the overexpression of ROP18Δ27-WT-GFP rather than ROP18Δ240-GFP dramatically reduced the level of FLAG-p65 (Fig. 2B). In addition, ROP18Δ27-WT-GFP, but not ROP18Δ240-GFP, reduced the levels of p65 in a dose-dependent fashion in dually transfected 293T cells (Fig. 2, C and D). To determine whether the ROP18-dependent decrease in p65 protein level is mediated through proteasomal degradation, FLAG-p65 was coexpressed with ROP18Δ27-WT-GFP in the presence of the proteasome inhibitor MG132. Under this condition, the level of p65 was stabilized, and the interaction between ROP18Δ27-WT-GFP and p65 was confirmed through coimmunoprecipitation (Fig. 2E). Additionally, the cells were co-transfected with the various ROP18 plasmids and myc-tagged ubiquitin for charactering the effect of ROP18-mediated p65 proteasomal degradation. As the result, ubiquitination of p65 was significantly increased by ROP18Δ27-WT-GFP, but not ROP18Δ240-GFP, in the presence of MG132 (Fig. 2F). Taken together, these results support that the N-terminal portion of ROP18 associates with p65 and targets it to a proteasome-ubiquitin-dependent degradation pathway.

ROP18 Phosphorylates p65 at Ser-468 to Promotes Its Degradation—Although the kinase activity of ROP18 is essential for the virulence of type I parasites (22, 26–28), the biological significance of the kinase activity of ROP18 and NF-κB remains unknown. To determine if ROP18 phosphorylates p65, eukaryotic expression ROP18 was incubated with recombinant p65 and its truncates in a kinase assay in vitro (Fig. 3A). The observed autophosphorylation band confirmed that the eukaryotic expression of ROP18 has kinase activity (Fig. 3A, black arrow). Incubation of the recombinant p65 and its truncate proteins with [32P]ATP and GFP-tagged ROP18 resulted in the incorporation of [32P] into the full-length and C-terminal portion of recombinant of p65 (Fig. 3A, white arrow). Thus, the results showed that ROP18 primarily phosphorylates the C-terminal portion of p65. To define the residues of p65 were phosphorylated by ROP18, we co-transfected the cells with GFP-tagged-ROP18-WT or MUT (kinase- deficient) and p65-FLAG plasmids and treated the cells with or without TNF-α. Calyculin A was used as the phosphatase inhibitor to block dephosphorylation. The results demonstrated that wild-type but not kinase-deficient ROP18 phosphorylated p65 at Ser-468, whereas other phosphor-p65 antibodies did not give rise to any signal (Fig. 3B), indicating that ROP18 may phosphorylate p65 at Ser-468. To determine whether ROP18 phosphorylates p65 at Ser-468 in vivo, HFF cells were infected at an m.o.i. of ~1 with ROP18-WT RH, ROP18-MUT RH, or RH strain. After immunoprecipitation of p65, Western blotting revealed the Ser-468 phosphorylation of p65 from the ROP18-WT RH strain and RH strain compared with the ROP18-MUT RH strain (Fig. 3C). To confirm if Ser-468 is ROP18 substrate, we generated non-phosphorylate-able p65 by mutating serine 468 to alanine and repeated the in vitro phosphorylation experi-
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A

1. BD-ROP18[25-291]+AD-p65-full length
2. BD-ROP18[25-291]+AD-p65-C
3. BD-ROP18[25-291]+AD-p65-M
4. BD-ROP18[25-291]+AD-p65-N2
5. BD-ROP18[25-291]+AD-p65-N1
6. BD-lam+AD-T (negative control)
7. BD-53+AD-T (positive control)

B

Rel homology domain (RHD)

| Domain          | DNA binding domain | Dimerizing domain | NLS | TAD | ROP18 binding |
|-----------------|--------------------|-------------------|-----|-----|---------------|
| 11              | 11                 | 190               | 285 | 312 | 550           |
| p65-N1          | -                  | 190               | 285 |     | +             |
| p65-N2          | 1                  | 190               | 285 |     | -             |
| p65-M           | 1                  | 190               | 285 |     | +             |
| p65-C           | 1                  | 190               | 285 |     | -             |

C

|         | Input | Pull-down |
|---------|-------|-----------|
| p65-M-His | +     | -         |
| ROP18-GST | -     | +         |
| GST      | -     | -         |

D

|         | Input | IP |
|---------|-------|----|
| ROP18-△27-GFP | +     | -  |
| GFP vector    | -     | +  |

E

ROP18-WT RH | p65 | DAPI | Merge | BF

Ty1 \ p65 \ DAPI \ BF
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The Kinase Activity of ROP18 Suppresses NF-κB Activation—To investigate the effect of ROP18 on NF-κB-mediated target gene activation, luciferase reporter assays were performed. The cells were transiently transfected with a luciferase reporter construct containing three copies of the NF-κB binding site (3xκB-Luc) together with ROP18-WT or ROP18-MUT vectors and treated with TNF-α for 6 h. The reporter assays showed that the overexpression of ROP18-WT significantly inhibited TNF-α-induced NF-κB activation (Fig. 6A) and considerably reduced NF-κB transcriptional activity induced by LPS (Fig. 6B). Additionally, transfection with ROP18-Δ27-WT-GFP or ROP18Δ240-GFP plasmids at different doses revealed that ROP18-Δ27-WT-GFP inhibited the activity of κB-luciferase in a dose-dependent manner. However, in cells expressing ROP18Δ240-GFP (without p65 interacting domain), the activity of κB-luciferase was largely unaffected (Fig. 6C). Additional experiments employing ROP18-WT or ROP18-MUT or ROP18-KO RH strain consistently showed that ROP18 WT-RH strain induced a dramatic inhibition of NF-κB activity compared with ROP18-MUT and ROP18-KO parasites (Fig. 6D). Taken together, the results suggested that the kinase activity of ROP18 is required for the inhibition of NF-κB activation.

The Kinase Activity of ROP18 Suppresses the Expressions of NF-κB Target Genes in Vitro—Previous results have shown that infection with type I RH tachyzoites inhibits macrophage responses to LPS and other TLR ligands, and this inhibition phenotype is associated with the NF-κB (19, 29–31). Therefore, we examined whether ROP18 kinase is the effector to manipu-
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RAW264.7 cells were transfected with ROP18-WT, ROP18-MUT, or empty vectors and stimulated with LPS for 6 h. The macrophages stimulated by LPS produced robust amounts of IL-6, IL-12p40, and TNF-α, whereas cells expressing ROP18-WT induced low levels of IL-6, IL-12p40, and TNF-α compared with ROP18-MUT (Fig. 7, A–C). Further-
FIGURE 3. ROP18 phosphorylates p65 at serine 468 to promote its degradation. A, bacterially expressed His-full-length p65 and its truncates (p65-N1 and p65-C) as substrates. Both His-p65 full-length and truncate proteins were purified on Ni²⁺-Sepharose beads and phosphorylated in vitro using [³²P]ATP and eukaryotic express ROP18 as described. Samples were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue-stained (right) and subsequently incubated with x-ray film. The autoradiogram was also shown (left). Casein was used as a positive control. Note that in the presence of ROP18, the dramatic autophosphorylation of ROP18 (black arrow) and the incorporation of ³²P into full-length and the C-terminal portion of p65 protein were observed (white arrow). B, ROP18 phosphorylates p65 at serine 468. 293T cells were co-transfected with ROP18-WT-GFP or ROP18-MUT-GFP and p65-FLAG. At 24 h after transfection, the cells were treated with or without TNF-α (10 ng/ml) for 6 h and immunoprecipitated (IP) with FLAG antibodies. Calyculin A was used as the phosphatase inhibitor to block dephosphorylation. The p65 phosphorylation was detected by the indicated phospho-p65 antibodies. IB, immunoblot. C, using wild-type and kinase-deficient ROP18 expressing transgenic strains to determine that ROP18 phosphorylates p65 at Ser-468. The HFF cells were infected with the indicated parasites at an m.o.i. of 1. At 12 h after infection, immunoprecipitation (IP) of p65 from infected cell lysates was detected with phospho-p65 Ser-468 antibody. D, ROP18 phosphorylates p65 serine 468 in vitro. Bacterially expressed GST-p65-C fragments (amino acids 285–550) as substrates, both wild-type and S468A mutant GST-p65-C proteins, were purified on glutathione-agarose beads and phosphorylated in vitro using [³²P]ATP and eukaryotic express ROP18 as described. Samples were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue (lower) and subsequently incubated with x-ray film. The autoradiogram was also shown (upper). Note that in the presence of ROP18 there was dramatic incorporation of ³²P into wild-type, but not S468A mutant, GST-p65-C protein. E, verifying that ROP18 phosphorylation Ser-468 of p65 regulates its degradation. 293T cells were co-transfected with FLAG-tagged p65-wild-type or S468A mutant and ROP18-WT-GFP. At 24 h after transfection, the cells were immunoprecipitated with anti-FLAG and immunoblotted with the indicated antibodies, respectively. F, verifying that ROP18 kinase selectively phosphorylates p65 but not IκBα. 293T cells were transfected with ROP18-WT-GFP plasmid and immunoprecipitated with p65 or IκBα antibody. IgG antibody was used as the negative control. After immunoprecipitation, p65 and IκBα phosphophorylations were detected by phospho-Ser/Thr antibody immunoblotting. G, ROP18 did not influence THF-α-induced IκBα degradation. Cells were transfected with ROP18 wild-type, kinase-deficient expression plasmid, and control vector, then treated with THF-α for the indicated times. Western blotting was performed on the cell extracts to check the degradation of IκBα.
more, U937 cells infected with different strains were stimulated with LPS for 6 h. Although cells infected with ROP18-WT RH displayed an effective ability to suppress LPS-induced cytokine production, cells infected with ROP18-MUT or ROP18-KORH strains strongly enhanced LPS-induced IL-6, IL-12p40, and TNF-α production (Fig. 7, D–F). Thus, we conclude that the interaction of ROP18 with p65 mediates p65 degradation, thereby down-regulating the expression of NF-κB target genes.

**M1 Polarization Infected with ROP18 Kinase-deficient Strain**—T. gondii primarily infects nucleated cells, including macrophages, and associated mononuclear phagocytes in vivo. Recent literature has stated that type I and type III strains elicit a gene expression profile similar to that of alternatively activated macrophages (termed M2), whereas type II strains induce a classically activated phenotype (termed M1) (29). M1 macrophages typically express chemokines and cytokines that activate antimicrobial activity in cells, whereas M2 macrophages secrete anti-inflammatory molecules that down-regulate TH1 type responses (2, 32). To determine the association of ROP18 with a M1/M2-polarized phenotype, macrophages were infected with ROP18-WT, ROP18-MUT, or RH strains. FACS assays showed that ROP18-MUT tachyzoites, unlike ROP18-WT RH and RH strains, elicited the expression of the M1-associated markers (CD86 and PD-L1), which is consistent with the inability of this strain to inhibit the NF-κB pathway.
(Fig. 8, A and B). The M2 signature (MHC2 and PD-L2) was clearly noted in macrophages infected with ROP18-WT RH and RH strains (Fig. 8, C and D). Thus, the kinase activity of ROP18 contributes to the M1/M2-biased phenotype of the host macrophages.

DISCUSSION

Transcription factors control many innate immune effectors that enhance or regulate the overall immune response to invading microorganisms. In turn, successful intracellular pathogens have developed strategies to undermine important host cell...
immune pathways to promote their survival. Recent studies have revealed that, like other pathogens, *T. gondii* injects virulence factors into host cells using the so-called “kiss and spit” model to provide a survival advantage to parasites (2, 29).

ROP16 of type I and type III strains phosphorylates host signal transducers and activators of STAT3 and STAT6, resulting in the prolonged activation of these two transcription factors and the subsequent up-regulation of IL-4 and antagonizing induction of IL-12 (33–35). A dense-granule protein of type II parasite strains, GRA15, integrated to parasitophorous vacuole membrane, activates TRAF6, which activates IκB kinase, leading to the phosphorylation and proteasomal degradation of IκB to the activation of NF-κB pathway. Recent research shows that IRGs (IFN-γ-induced immunity-related GTPases) are crucial for the control of toxoplasmosis in mice. The finding suggests that ROP18 and ROP5 are involved to avoid IRG recruitment, which is unlikely to happen in species that do not have the IRG system such as humans (36–38). Our result here demonstrated that ROP18 in type I strains is involved in the host NF-κB pathway present in murine as well as the human immune system.

We confirmed for the first time that the virulence factor ROP18 in type I strains is responsible for inhibiting host NF-κB and for suppressing proinflammatory cytokine, and the disruption of ROP18 kinase activity in type I strain compromised the ability of the parasite to inhibit NF-κB for the degradation of p65 and increased expression of IL-12, IL-6, and TNF-α (Fig. 7). Corresponding to the key role of ROP18 in driving M2 polarization, kinase-deficient ROP18 parasites induced M1-biased activation compared with the wild-type ROP18 strain (Fig. 8).

In addition, we elucidated the molecular mechanisms of the relationship between ROP18 and the host NF-κB pathway. Evidence for the interaction between p65 and ROP18 was obtained through communoprecipitation, GST pulldown, and yeast two-hybrid assays. We further demonstrated that the N-terminal portion of ROP18 is associated with the dimerization domain of p65. ROP18 phosphorylates p65 at Ser-468 and degrades this protein via ubiquitin-dependent degradation pathway and subsequently suppresses NF-κB activation. Kinase-deficient ROP18 forms a more stable interaction with

![Figure 6. Kinase activity of ROP18 suppresses NF-κB activation.](image-url)
p65 in infected cells (Fig. 5B), suggesting that kinase-deficient ROP18 disabled to induce the phosphorylation and degradation of p65. Previous studies have reported that Ser-468 phosphorylation of p65 allows for binding of the COMMD1 complex and subsequent ubiquitination (39–42). COMMD1 has been identified in a complex with an E3 ubiquitin ligase complex composed of TCEB1/elongin C, CUL2, SOCS1, and RBX1 (40, 41). COMMD1 promotes ubiquitination of p65 and its subsequent proteasomal degradation (39). Therefore, our results implicate that ROP18 phosphorylates p65 at Ser-468 to promote its binding of COMMD1 and subsequent ubiquitination.

The modulation of the NF-κB pathway through *T. gondii* infection has long been an area of debate, with some studies showing that *T. gondii* activates NF-κB and other studies showing that *T. gondii* inhibits NF-κB activation (9). Previous investigations showed that infection with type I strains activates NF-κB in host cells, whereas infection with type I strains results in the activation of IkB kinase and induces the phosphorylation and proteasomal degradation of the inhibitor of NF-κB (IκB) (10, 16, 18). Other studies have shown that infection with type I strains inhibits NF-κB activation, as type I strains do not activate NF-κB compared with LPS and TNF stimulation (10, 12–13). Our immunofluorescence experiments showed that
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Type I strains slightly activate p65 translocation, consistent with the results of previous studies. Interestingly, we also observed that the kinase activity of ROP18 induces p65 ubiquitin-dependent degradation and the subsequent suppression of NF-κB activation. Functionally, p65 ubiquitination is a potential mechanism regulating the termination of NF-κB by the intracellular T. gondii and may also contribute to the oscillation of nuclear p65. The wild-type ROP18 RH strain suppresses NF-κB-regulated proinflammatory cytokines compared with kinase-deficient ROP18 tachyzoites. This result is consistent with previous observations that infections with type I strains inhibit the NF-κB pathway and down-regulate the induction of IL-12, thus limiting protective T helper 1 (Th1)-type cytokine responses (2, 9, 12, 13, 19, 29). Thus, the present results provide a reasonable explanation for this debate. In addition, we further demonstrated that kinase-deficient ROP18 parasites induce M1-biased activation compared with wild-type ROP18 parasites, indicating a ROP18-induced inhibition of

FIGURE 8. Inducing the M2 phenotype with type I strains expressing wild-type ROP18. A, U937 cells were infected with ROP18-WT RH strain, ROP18-MUT RH strain, or RH strain. At 24 h post-infection, macrophages were stained for CD86 or PD-L1, and fluorescence-activated cell sorting assays were performed (dotted lines, uninfected macrophages; solid lines, macrophages infected with RH strain; heavy lines, macrophages infected with ROP18-MUT RH strain; shaded, macrophages infected ROP18-WT RH strains). B, histograms depict the percentage of positively stained CD86 or PD-L1 cells analyzed in A. Indicated values are the means ± S.D. of triplicates. ***, p < 0.001, compared with the RH strain control. C, U937 cells were infected with ROP18-WT RH strain, ROP18-MUT RH strain, or RH strain. At 24 h post-infection, macrophages were stained for MHC2 or PD-L2, and fluorescence-activated cell sorting assays were performed (dotted lines, uninfected macrophages; solid lines, macrophages infected with RH strain; heavy lines, macrophages infected with ROP18-MUT RH strain; shaded, macrophages infected ROP18-WT RH strain). D, the histograms depict the percentage of positively stained MHC2 or PD-L2 cells analyzed in C. Indicated values are the means ± S.D. of triplicates. ***, p < 0.001, compared with the RH strain control.
host NF-κB pathway and of IL-12 production, thereby causing M2-biased phenotypes. Taken together, our data indicate that ROP18 in type I RH strain is responsible for inhibiting host NF-κB pathways, facilitating the M2-biased response and consequently promoting the survival and proliferation of invading pathogens.

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