Interferon regulatory factor 1 eliminates mycobacteria by suppressing p70 S6 kinase via mechanistic target of rapamycin signaling

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SUMMARY

Objectives: Although it has been reported that Interferon regulatory factor 1 (IRF1) inhibits *Mycobacterium tuberculosis* (Mtb) infection via inducible nitric oxide synthase (iNOS) in mice, how it counteracts with mycobacterial infection in human remains largely obscure. This study was conducted to investigated the effect of IRF1 on Mtb infection in human macrophages (Mϕs).
Methods: We thus investigated the IRF1 expression by using PBMC and monocytes of pulmonary tuberculosis (TB) patients and human monocyte-derived macrophages (hMDMs) and THP-1-derived macrophages (THP-1-Mϕ). We used gain-of-function and loss-of-function approaches to explore the role of IRF1 on Mtb infection.
Results: IRF1 was significantly induced in PBMC and monocytes of pulmonary TB patients *in vivo* and in human Mϕs *in vitro*. We demonstrated that IRF1 protects Mϕs from Mtb infection. Concurrently, IRF1 promotes the expression of several pro-inflammatory cytokines including IL-6, TNF-α and IL-8, indicating IRF1-mediated activation of innate immunity upon Mtb infection. Gain-of-function and loss-of-function approaches have demonstrated that IRF1 suppresses the mechanistic target of rapamycin (mTOR)/p70 S6 kinase (p70 S6K) cascade to exert its anti-Mtb effect.
Conclusions: The discovery of a novel function of IRF1 in facilitating anti-mycobacterial effect through suppressing mTOR/p70 S6K signaling in Mϕs may provide a promoting therapeutic target for tuberculosis.

Introduction

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* (Mtb) infection, represents the most prevalent infectious disease and spreads to approximately one-third of the global population.[1] The host defense system becomes crucial in TB patients particularly those with drug resistance TB who have failed to be cured with first-line anti-Mtb drugs including isoniazid (INH).[2,3] Interferons (IFNs) production which can be induced by Mtb infection, serves as potent immune regulator and evokes multiple immune responses to various pathogens including bacteria infection.[4,5] Hence, IFNs either alone or in combination with the first-line antibiotics have been considered an effective therapy for TB, especially the drug-resistant patients in clinics.[6] Upon binding to their specific receptors, IFNs result in phosphorylation of signal transducer and activator of transcription (STAT) molecules and production of a subset of interferon-stimulated genes (ISGs) to establish the host defense state.[7] As one of the most important ISGs,

transcriptional activator interferon regulatory factor 1 (IRF1) can effectively regulate immune responses to pathogen infections.[8]

Previous studies have shown that IRF1 targets inducible NO synthase (iNOS) to eliminate mycobacterial infection in mice.[9,10] During viral infection, IRF1 effectively constrains virus replication through IFN production and/or activating the JAK-STAT cascade in cell culture models.[11,12] Moreover, IRF1 exerts antitumor function by regulating M1 polarization of macrophages (Mϕs)[13] and affects tumor metastasis and epithelial-mesenchymal-transition (EMT) by manipulating mechanistic target of rapamycin signaling (mTOR) signaling.[14]

Recent studies have suggested that mTOR, which plays a key role in cell growth and metabolism controls, such as regulating translation, transcription, nutrient uptake, ribosome biogenesis and autophagy in response to environmental cues,[15,16] should be harnessed to promote host cell defense mechanisms against invading pathogens including Mtb.[17,18] IRF1 plays important roles in both activation and inhibition of mTOR signaling in different settings.[14,19] Ribosomal p70 S6 kinases (p70 S6K) and autophagy are the key mTOR downstream effectors and suggested as host-targeted therapies for Mtb infection.[20,21]

Given the importance of IRF1 in innate immune response and in light of insufficient mechanistic investigation in respect to Mtb, we studied the effect and mechanism-of-action of IRF1 on Mtb infection. We found that IRF1 was evoked by Mtb infection in pulmonary TB patients and *in vitro*. Furthermore, we identified a novel host defense function of IRF1 in facilitating anti-mycobacterial effect through suppressing mTOR/p70 S6K signaling in Mϕs. We further demonstrated that first-line TB drug INH treatment could lead to the reduction of mTOR/p70 S6K signaling activity and the antimycobacterial effect in IRF1 overexpression cells.

Methods

Ethics approval

This study was approved by the recommendations of the International Committee of Medical Journal Editors and the Ethics Committee of the Southern Medical University with written informed consent from all subjects. Written informed consent was obtained from all TB patients and healthy control subjects enrolled in this study. The protocol was approved by the ethics committee of the Southern Medical University.

Patients and control subjects

Primary active pulmonary TB patients were diagnosed in and recruited from the Guangzhou Chest Hospital (Guangzhou, Guangdong, China). 70 patients with active pulmonary TB (40 males and 30 females) ageing from 15 to 88 years (median 48) were recruited between May 2016 to January 2018. Healthy control (HC) subjects were recruited from Nanfang Hospital and Southern Medical University (Guangzhou, Guangdong, China) and were chosen from consenting adults drawn from the same background and locality as the TB patients. 71 HC (48 males and 23 females) ranged in age from 18 to 60 years (median 37).

Reagents

Dimethylsulfoxide (DMSO), trypan blue were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Texas Red were from Invitrogen (Invivogen, Life Technologies, Carlsbad, CA, USA). 20 µg/ml cGAS-STING agonist cGAMP (Invivogen, Life Technologies, Carlsbad, CA, USA), 66.2 nM TLR2/TLR1 agonist Pam3CSK4 (Invivogen, Life Technologies, Carlsbad, CA, USA), 100 ng/ml TLR4 agonist lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA), and

5 µM TLR9 agonist ODN 2395 (Invivogen, Life Technologies, Carlsbad, CA, USA) were applied. cGAMP and ODN 2395 were transfected to cells with 5 µl/ml medium Lipofectamine 2000 (Invitrogen, MA, USA). 2 µM TBK1 inhibitor BX795 (Selleckchem, Houston, TX, USA), 100 µM NF-κB inhibitor JSH-23 (Selleckchem, Houston, TX, USA), 10 µM p38 MAPK inhibitor SB203580 (Selleckchem, Houston, TX, USA), 20 µM MyD88 inhibitor ST 2825 (MCE, Monmouth Junction, NJ, USA), 10 µM S6 kinase inhibitor PF-4708671 (MCE, Monmouth Junction, NJ, USA) and 10 µM STAT1 inhibitor fludarabine (MCE, Monmouth Junction, NJ, USA) of were applied respectively. 100 ng/ml Human IFN-γ (Pepro Tech, Rocky Hill, USA), 10 µM Rapamycin (MCE, Monmouth Junction, NJ, USA), 5 mM 3-MA (Selleckchem, Houston, TX, USA), 10 µM wortmanin (Selleckchem, Houston, TX, USA), 0.5, 1, 2 µM MHY1485 (MCE, Monmouth Junction, NJ, USA), IFN-γ antibody (25718) (Novus Biologicals, CO, USA), 1 µM Bafilomycin A1 (Baf.A1) Cell Signaling Technology (Danvers, MA, USA) and 0.1 µM isoniazid (INH) from Taiji southwest pharmaceutical co. LTD (Guanghzou, China) were used. Anti-GAPDH (17AF0412, ZSGB-BIO, Beijing, China) primary antibody and other primary antibodies including IRF1 (8478S), phospho-Stat1 (Tyr701) (9167S), Stat1 (14994S), STAT2 (72604), IRF9 (76684S), MX1 (37849S), Viperin (13996S), JAK1 (3344S), LC3 A/B (12741), AKT (4691S), phosphor-AKT (Ser473) (4060S), mTOR (2972S), phospho-mTOR (Ser2448) (5536S), p70 S6 Kinase (9202S),phospho-p70 S6 Kinase (Thr389) (9206S), S6 ribosomal protein (2217S), phospho-S6 ribosomal protein (Ser235/236) (4858S), phospho-p38 MAPK (Thr180/Tyr182) (4511S), Atg5 (12994S), Atg7 (8558S), Atg12 (4180S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Caspase-1 (sc-56036) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). LysoTracker ^{TM} Blue DND-22 (Invitrogen, MA, USA) was also used.

PBMCs isolation

120 ml of peripheral blood were collected, 7 ml of them were collected into vacutainer tuber containing EDTA-K2, mixed with $1 \times$ phosphate buffered saline (PBS), layered onto a Lymphoprep^{TM} (Alere Technologies AS, Oslo, Norway) and isolated by using centrifugation on a Ficoll-Histopaque density gradient at 800 g for 20 min at 25 °C. The PBMC layer was separated and washed three times with 10 ml $1 \times$ PBS, each time centrifuged at 1500 rpm for 10 min at 4 °C to discard the supernatant. The cells were harvested and resuspended with 5 ml PBS and cell numbers were adjusted to 1×10^6/ml.

Cell culture of hMDM and THP-1-Mϕ

Peripheral blood were collected from healthy volunteers and TB patients. Human monocyte-derived macrophages (hMDMs) derived from PBMCs. Briefly, isolated monocytes were cultured overnight in 6-well culture plates in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning, New York, NY, USA) containing 10% fetal bovine serum (FBS) (Corning, New York, NY, USA) at 37 °C, 5% CO$_2$. The suspension cells were discarded and other cells were continued to culture in the presence of 100 ng/ml human granulocyte macrophage colony-stimulating factor (GM-CSF) (Pepro Tech, NJ, USA) in RPMI medium with 10% FBS. Non-adherent cells were repeatedly washed and the medium was changed every two days. An acute monocytic leukemia-derived human cell line of THP-1 cells (CELLCOOK, Guangzhou, China) were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS and penicillin (100 U/ml), streptomycin (100 µg/ml) at 37 °C, 5% CO$_2$. Treating the cells with 100 ng/ml phorbol-12-myristate-13-acetate (PMA) (Pepro Tech, NJ, USA) for 48 h incubation, and they matured macrophage-like phenotype and the adherent cells were THP-1-derived macrophage (THP-1-Mϕ). Non-adherent cells were

removed and washed with RPMI without FBS. THP-1-Mϕ were maintained in complete RPMI media without PMA for 24 h for further experimental purpose.

Short interfering RNA (siRNA) and transfection

IRF1 siRNAs targeting NM_002198.2 and siNC (negative control) with scrambled targeting sequence were designed by Ribo-Bio (Guangzhou, China). 5×10^5/ml THP-1-Mϕ or hMDMs were seeded in 12-well plate. Fresh siRNA transfection reagent of Lipofectamine 2000 (Invitrogen, MA, USA) containing siRNA (final concentration 100 nM) and optiMEM (Gibco, Life Technologies, NY, USA) were performed according to the manufacturer's instruction. All the reagents of 400 µl were added to each well and inoculated for 4–6 h. Then the supernatant was discarded and fresh complete growth medium was added for further 48 h inoculation. Confirmation of siRNA-mediated down-regulation of target gene comparing siNC was detected by qRT-PCR and Western blot analysis.

Lentiviral shRNA production and transduction

X-tremeGENE HP DNA transfection reagent (Roche, Berlin, Germany) was performed according to the manufacturer's instruction. For IRF1 overexpression, pTRIP.CMV.IVSb.ISG.ires.TagRFP based IRF1 overexpression vector (kind gift from Prof. Charles M. Rice, the Rockefeller University was used to package lentiviral pseudoparticles.[4] Meanwhile, vector expressing reporter genes Photinus pyralis luciferase (Fluc) has been used as a control (also a kind gift from Prof. Charles M. Rice). Lentiviral pseudoparticles were generated in HEK293T cells following the method described previously.[11] Virus-containing medium was collected after 72 h post-transfection and concentrated by Lenti-Concentin Virus Precipitation Solution 5× (exCELL EMB810A-1). Lentiviral pseudoparticles were stored at −80 °C. Spinoculation method was used as described for IRF1 transduction assay, THP-1 cell lines were seeded into 6-well plates at density of 5×10^5 cells per well and transduced with lentiviral pseudoparticles at 37 °C for 72 h. Extended the passaging cells for seven days and collected all the cells by flow cytometry (MoFlo XDP) (Beckman Coulter, CA, USA) to select the red fluorescent protein (RFP)-marked positive ones. RFP-marked THP-1 cells transfected with Photinus pyralis luciferase (Fluc) lentivirus has been used as control cells.

Mycobacterial infection and colony-forming units (CFU) assays

Mycobacterium tuberculosis H37Rv (American Type Culture Collection) and *Mycobacterium bovis* BCG (SINOPHARM, Beijing, BJ, China) were cultured in Middlebrook 7H9 broth with 10% BBL Middlebrook oleic acid-albumin-dextrose-catalase (OADC) at 37 °C with 5% CO_2. Mycobacteria were grinded to generate a single bacterium suspension in RPMI. Then both PBMCs and THP-1-Mϕ cells were placed on a 12-well cell culture plates (CELLTER, Guangzhou, China) and were continuously infected with H37Rv and BCG at MOI of 2 to the corresponding time points. Colony-forming units (CFU) assays were utilized to ensure the reliable quantification of intracellular mycobacterial infection. The cells were infected for 1 h with H37Rv and BCG at MOI of 2 and washed for three times with PBS, and RPMI-1640 medium containing 10% FBS was added. After various periods of incubation, cells were lysed with 0.01% TritonX-200 (Solarbio, Beijing, China) to release intracellular bacteria. The aliquots of the lysates of infected cells were then diluted 10-fold in 0.01% TritonX-200. Direct effects of various drugs on bacterial survival were detected by CFU assay without cells at 48 h infection. Each sample with four dilutions were plated individually on 7H10 agar plates. Count the mycobacterial colony-forming units after 14–21 days incubation at 37 °C with 5% CO_2.

Assay of cell viability

Cell viability was assessed with a TransDetect Cell Counting Kit-8 (CCK-8) (TransGene Biotech, Beijing, China). hMDMs and THP-1-Mϕ were incubated with the DMSO or with the pharmaceutical agent for appropriate time. Upon infection with Mtb for 48 h, CCK-8 solution was diluted for 100 times with 10% FBS-RPMI 1640 and added into each well. After 4 h inoculation at 37 °C. The absorbance was measured at 450 nm and 630 nm with a Varioskan Flash (Thermo Fisher Scientific, Carlsbad, CA, USA).

Nitrite (NO) measurement

Griess Reagent Kit (Promega, Madison, USA) was used to detected the NO production. NO standard reference curve was prepared according to the instruction. 50 µl of the supernatant of cells was added to 96 well-plate and 50 µl of the sulfanilamide solution was added. All the samples were incubated at room temperature for 5–10 min with protecting from light. Added 50 µl of the NED solution to all wells and incubated them for 5–10 min with protecting from light. A purple/magenta color will begin to form immediately. Measure absorbance within 30 min at 450 nm and 630 nm with a Varioskan Flash (Thermo Fisher Scientific, Carlsbad, CA, USA).

Assay of intracellular reactive oxygen species (ROS)

ROS was measured by ROS Activity Assay Kit (KeyGEN BioTECH, Jiangsu, China) with the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2′, 7′-dichlorofluorescein (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. Macrophages seeded at a density of 5×10^5/well in 12-plate were transfected with siRNA-IRF1 and infected with BCG in 6 h. To obtain dissociated microglia for the ROS assay, culture medium was removed and the cells were washed three times with PBS. DCFH-DA, diluted to a final concentration of 10 µM with RPMI 1640, was added to cultures and incubated for 20 min at 37 °C. The fluorescence was read at 488 nm for excitation and 525 nm for emission with a Varioskan Flash (Thermo Fisher Scientific, Carlsbad, CA, USA).

RNA extraction and quantitative RT/real time PCR

RNA was extracted from macrophages (hMDMs and THP-1-Mϕ) using Total RNA Kit I (200) (OMEGA, Norcross, GA, USA) according to the operating instructions. RNA concentration was measured by NanoDrop 2000 (Thermo Fisher Scientific, Carlsbad, CA, USA) and 20 µg of total RNA was synthesized to cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the operating instructions. The mRNA expression of GAPDH, *IRF1, Il-6, Il-8, Tnf, Ifn-a, Ifn-b, Ifn-g, Il-10,* other interferon stimulated genes (ISGs), autophagy-related genes (Atgs) and genes related to reactive nitrogen species (RNS) and reactive oxidative species (ROS) were assessed by SYBR® Premix Ex Taq^{TM} II (Tli RNaseH Plus) (TaKaRa, Beijing, China) on Mastercycler ep realplex4 (Eppendorf, Hamburg, Germany). After initial denaturation at 95 °C for 120 s, targeted genes were amplified and quantitated (95 °C for 15 s, 60 °C for 15 s) for 45 cycles followed by a final extension at 68 °C for 20 s. All the PCR products were normalized with respect to GAPDH transcript, then relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold change. Full primers were shown in Supplementary Table 1.

Protein preparation and Western blot analysis

Cells were inoculated in 12-well plate and harvested with lysis buffer containing 455 mM Tris (pH 6.8) (Sangon Biotech, China), 41.6 mM SDS (Zhuosheng Biotech, Shanghai, SHH, China), 26.9 µM, 30% (v/v) glycerol (SIGMA, St. Louis, MO, USA) and 10 µM DL-Dithiothreitol (DTT) (SIGMA, St. Louis, MO, USA). After denaturation at 96 °C heating for 5 min, proteins were separated by electrophoresis on 10% or 15% SDS-polyacrylamide gel and transferred onto polyvinylidene (PVDF) membranes (Merck KGaA, Darmstadt, DA, Germany) by wet transfer method. Membranes were blocked with 5% (w/v) BSA (SIGMA, St. Louis, MO, USA) in PBS containing 0.1% (v/v) Tween-20 (PBS-T) for 1 h before incubation with primary antibodies at a dilution of 1:1000 at 4 °C overnight. Followed by incubation with secondary antibodies goat anti-rabbit at a dilution of 1:3000 or anti-mouse IgG-horseradish peroxidase conjugates at a dilution of 1:3000 (ZSGB-BIO, Beijing, China) for 1 h at room temperature. Washed the membranes with PBS-T three times, each time for 10 min. The immunoblots were visualized by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Carlsbad, CA, USA) on FluorChem Systems (ProteinSimple, CA, USA). Each measurement was performed in triplicated and one of the representative gel image was shown. Band integrated density data of each immunoblot has been quantified by Image J Software.

Multiplex Luminex analysis

Supernatants were analyzed for cytokines (IL-6, IL-8, TNF-α, IFN-α, IFN-β, IFN-γ, IL-10) using the ProcartaPlex® Multiplex Immunoassay (eBioscience, Carlsbad, CA, USA) according to the manufacturers' recommendations and acquired using the MAGPIX-xPONENT system (Luminex, Texas, USA).

Immunofluorescence and confocal fluorescence microscopy analysis

For protein immunofluorescence staining, 1×10^4 cells were seeded on glass coverslips in a 12 well-plate. After fixation with 4% paraformaldehyde for 15 min, permeabilization with 0.2% Triton X-200 for 10 min, and blocking for 1 h with 10% BSA-PBS, the cells were inoculated with primary antibody of IRF1 (1:100, Rabbit mAb, 8478) and LC3 A/B (1:200, Rabbit mAb, 12741) 4 °C overnight as previously described.[11] Removed excess primary antibodies and washed the cells for three times, inoculate the cells with Alexa Fluor®488 donkey anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) Alexa Fluor™ Plus 647 secondary antibody (Invitrogen, MA, USA). Nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Coverslips were mounted by ProLong Gold Antifade reagent (Invitrogen, MA, USA) and detected with confocal fluorescence microscopy with the Zeiss Axiovert LSM 880 instrument (Zeiss, Gottingen, Germany).

Statistical analysis

Results were shown as means values ± SEM of at least 3 independent experiments. Statistical analysis were performed using Student *t*-test or unpaired Mann–Whitney U test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered as statistically significant. All statistical analyses were performed with Prism 7.0 (GraphPad software).

Results

Mycobacterial infection promotes IRF1 expression in patients and in vitro

To investigate how Mtb infection regulates IRF1 expression in patients, we examined mRNA expression of IRF1 in PBMCs, mono-

cytes and the ratio of monocyte: PBMC of HC and active TB patients. The level of IRF1 expression was significantly higher in both PBMCs and monocytes from TB patients, but not in the ratio of monocyte: PBMC (Fig. 1A). Next, we found that *M. bovis* BCG and *M. tuberculosis* H37Rv infection robustly enhanced IRF1 expression at mRNA and protein levels in a time-dependent manner in hMDMs (Fig. 1B) and THP-1-Mϕs (Fig. 1C).

Mtb infection activates innate immune response through pattern recognition receptors (PRRs) including cGAS-STING and toll-like receptor (TLR)-2, -4 and -9 signaling.[22,23] We observed that IRF1 expression was significantly induced by cGAMP (cGAS-STING ligands), Pam3CSK4 (TLR2 ligands) and lipopolysaccharide (LPS) (TLR4 ligands), but not ODN2395 in hMDMs, suggesting cGAS-STING, TLR-2 and -4 as critical PRRs for Mtb-induced IRF1 expression (Supplementary Fig. 1A). Remarkably, H37Rv-induced IRF1 mRNA and protein expression were potently impaired by treating with STAT1 inhibitor fludarabine, but not with other inhibitors of downstream elements including BX795 (TBK1 inhibitor), ST2825 (MyD88 inhibitor), JSH-23 (p65 NF-κB inhibitor) and SB203580 (p38 MAPK inhibitor) (Supplementary Fig. 1B). All the inhibitors have been detected with efficient function. Direct activation of TLR-2 or -4 significantly promoted *Ifn-g, Tnf, Il-1b, Il-6, Il-8* mRNA levels (Supplementary Fig. 1C). However, only IFN-γ treatment significantly induced *IRF1* gene expression in hMDMs and THP-1-Mϕs (Supplementary Fig. 1D). Next, we applied IFN-γ antibody to investigate whether the Mtb-induced IFN-γ production is responsible for IRF1 expression. Although IFN-γ antibody treatment significantly reduced exogenous IFN-γ-induced IFN-g and IRF1 gene expression, it did not influenced the H37Rv-induced IRF1 expression (Supplementary Fig. 1E). These results indicated that Mtb infection might promote IRF1 expression through STAT1 signaling activation independent of IFN-γ production in Mϕs.

IRF1 restricts intracellular growth of mycobacterial infection in Mϕs

To directly assess the role of IRF1 in Mtb infection, we first tested the effect of forced IRF1 expression on mycobacterial survival by colony-forming unit (CFU) assay in THP-1-Mϕs. Successful over-expression of IRF1 was detected by the reporter of red fluorescent protein (TagRFP) expression under the confocal fluorescence microscopy assay when the bicistronic lentiviral vector co-expressing IRF1 and a TagRFP reporter were stably transfected into THP-1 (Supplementary Fig. 2). Furthermore, IRF1 mRNA and protein levels were increased in IRF1-THP-1-Mϕs while the intracellular BCG survival rate was significantly attenuated at 6, 24 and 48 h post-infection (h.p.i.) by CFU assay (Fig. 2A). Consistently, H37Rv infected IRF1-THP-1-Mϕs exerted a significant increase of IRF1 expression and decrease of intracellular mycobacterial survival at 48 h.p.i (Fig. 2B). Next, we knocked down IRF1 with transfecting specific siRNA in THP-1-Mϕs and hMDMs. IRF1 expression levels of mRNA and protein were attenuated upon BCG infection with IRF1 silencing. Correspondingly, CFU assay showed that the knockdown of IRF1 significantly promoted BCG survival in both cells at 48 and 72 h.p.i (Fig. 2C). Consistent results were observed in hMDMs with H37Rv infection (Fig. 2D). Hence, these data provide a direct and strong evidence that IRF1 plays an important role in restricting intracellular Mtb infection in Mϕs.

IRF1 is essential for antimicrobial inflammatory responses to mycobacterial infection in Mϕs

Although IRF1-induced iNOS was shown to be a powerful innate anti-mycobacterial defense mechanism in mice,[9] we did not find that IRF1 silencing affected iNOS as well as dual oxidases 2 (DUOX2), NADPH oxidase 1, 2, 5 (NOX1, 2, 5) mRNA expression and NO, ROS levels in BCG-infected hMDMs (Supplementary Fig. 3A
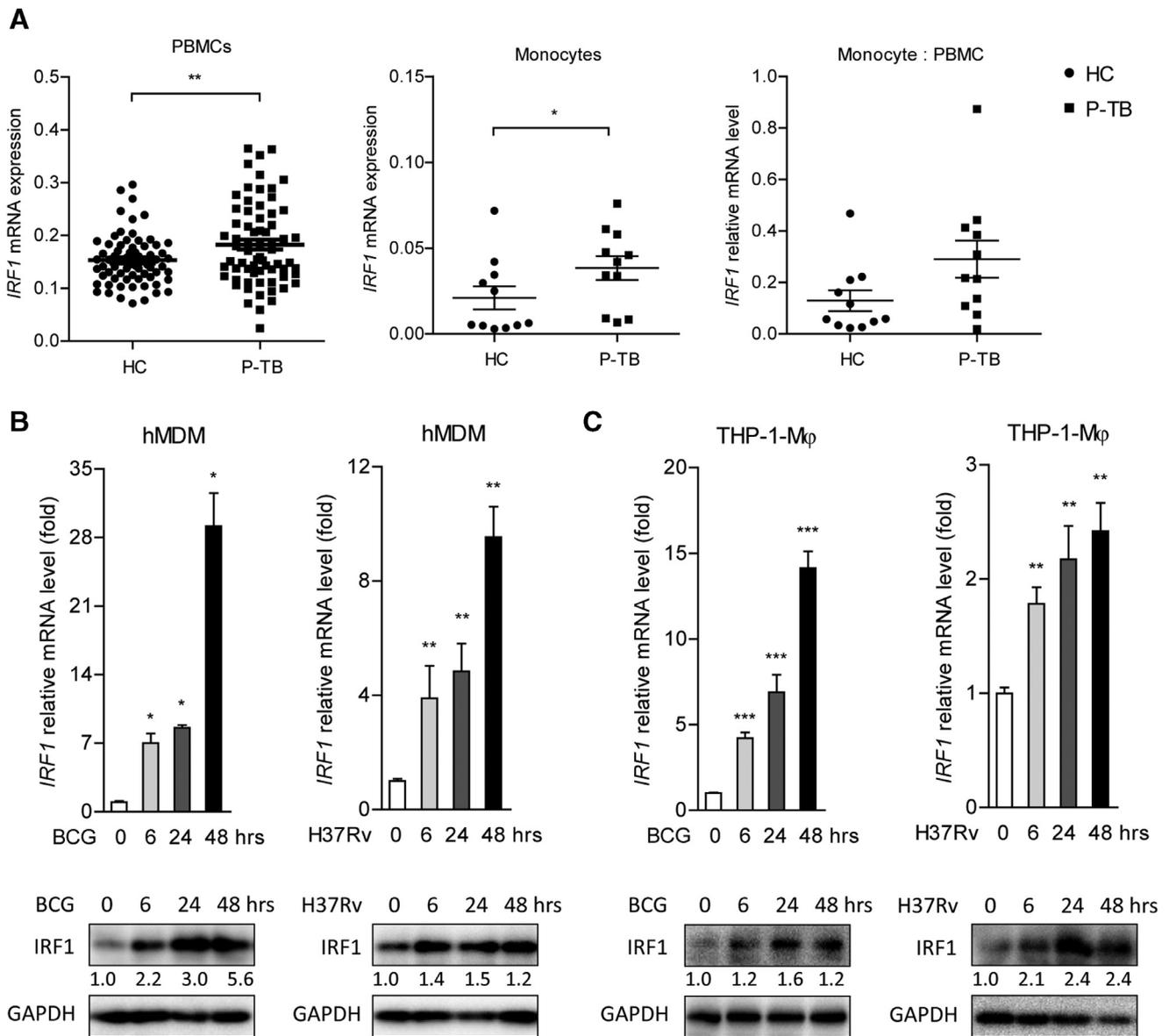
Fig. 1. IRF1 is induced by mycobacterial infection *in vivo* and *in vitro*. (A) Expression of IRF1 mRNA in PBMC of TB patients (Mean ± SEM, $n = 70$) and healthy controls (Mean ± SEM, $n = 71$) and in monocytes and the ratio of monocyte: PBMC of TB patients (Mean ± SEM, $n = 11$) and healthy controls (Mean ± SEM, $n = 11$) were detected by qRT-PCR. The statistical calculations were performed with the Student *t*-test. (**B, C**) In cell culture system, both BCG (MOI = 2) and H37rV (MOI = 2) stimulated IRF1 mRNA expression (Data presented as mean ± SEM, $n = 3$ independent experiments with each 2 replicates) and protein levels in hMDMs (**B**) and THP-1-Mϕs (**C**) during 6, 24, 48 h.p.i. Data are expressed as mRNA fold change relative to uninfected cells. GAPDH served as an internal reference. The statistical calculations were performed with the unpaired Mann–Whitney U test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered as statistically significant. The numbers below immunoblot correspond to band integrated density ratio of IRF1/GAPDH. HC (solid circle), healthy controls; P-TB (solid square), TB patients.

and B). Recently, antimicrobial peptide was recognized as an important component of host defense factor against mycobacterial infection.[24] Here in our study, the expression of two well-studied anti-Mtb peptide hBD1 and LL37 were not influenced by IRF1 (Supplementary Fig. 3C). Subsequently, hMDMs transfected with siIRF1 did not influenced the inflammatory cytokines of *Il-6, Il-8, Tnf, Ifn-a, Ifn-b, Ifn-g* and *Il-10* mRNA expression upon BCG infection (Fig. 3A). Surprisingly, multiplex Luminex assay showed that IRF1 silencing significantly decreased IL-6, TNF-α, IL-8 and IL-10 (to a slight extent), but not IFN-α, β and γ production (Fig. 3B). Matrix metalloproteinases (MMPs) are involved in IRF1 induced inflammation,[25] but IRF1 silencing did not affect MMP-2, 3 and 9 mRNA expression during Mtb infection (Supplementary Fig. 3D). These data indicated that IRF1 promotes pro-inflammatory IL-6, TNF-α and IL-8 secretion without affecting their mRNA expression levels, indicating IRF1-mediated activation of innate immunity upon Mtb infection.

Antimicrobial effect of IRF1 does not rely on STAT1 activation mediated induction of ISG

The genome-wide IRF1 chromatin immunoprecipitation-sequencing (ChIP-seq) data retrieved from the ENCODE ChIP-seq Experiment Matrix database showed that IRF1 directly bound to the promoter region of STAT1, the key element of IFN signaling (Supplementary Fig. 4A). Although silence of IRF1 did not affect Mtb-induced interferons production in Mϕs (Fig. 3B), it inhibited the total protein level and phosphorylation level of STAT1 and decreased the transcription levels of a subset of BCG and H37rV-induced ISGs in hMDMs (Fig. 4A). Consistently, the activation of STAT1 and ISG production were facilitated by IRF1 overexpression upon BCG infection (Fig. 4B). Moreover, pharmacological STAT1 inhibitor of fludarabine almost totally blocked STAT1 activation and ISG protein expression including STAT2, IRF9, MX1, Viperin and JAK1 (Fig. 4C), without influencing their mRNA levels
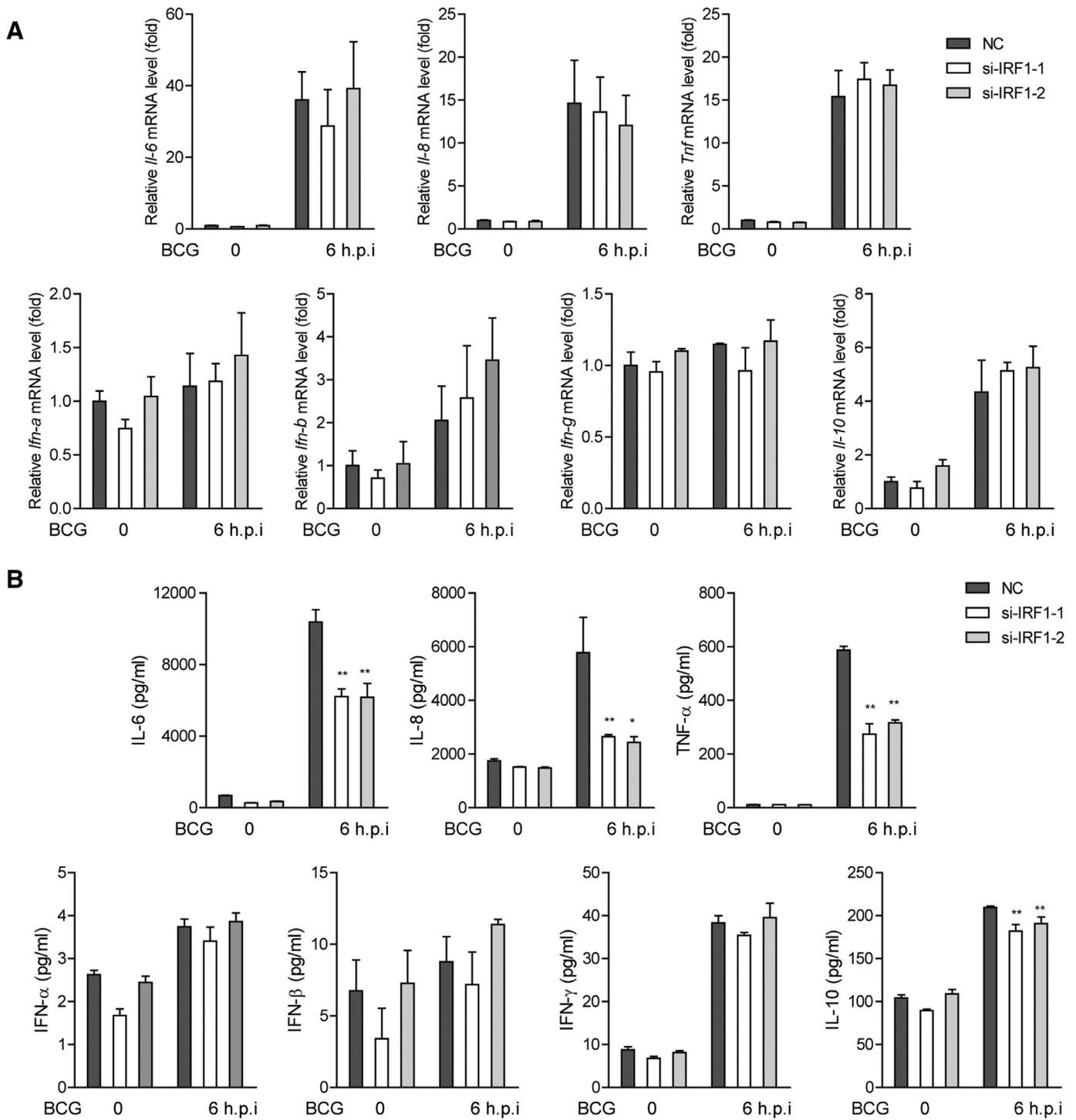
Fig. 2. IRF1 suppresses intracellular mycobacterial infection in Mφs. (A) IRF1 mRNA levels and protein levels have been detected in IRF1 overexpressing THP-1-Mφs upon BCG infection for 24 h (MOI = 2) (Data presented as mean ± SEM, $n = 3$ independent experiments with each 2 replicates). The survival of BCG in Fluc (Control) and IRF1 (IRF1 overexpression) THP-1-Mφs have been determined by CFU assay at 6, 24, 48 and 72 h.p.i. (Data presented as mean ± SEM, at least two independent experiments with each 4 replicates). **(B)** IRF1 mRNA and protein levels were detected in H37Rv (MOI = 2) infected THP-1-Mφs (Data presented as mean ± SEM, $n = 3$ independent experiments with each 2 replicates) upon H37Rv infection for 24 h (MOI = 2). CFU assay has been performed to analyze H37Rv survival at 48 h.p.i. **(C)** IRF1 mRNA levels and protein levels have been detected by qPCR and Western blot assay in si-IRF1-1 and si-IRF1-2 silencing THP-1-Mφs (up) and hMDMs (down) upon 24 h infection of BCG (MOI = 2). CFU was determined for BCG survival at 6, 24, 48 and 72 h.p.i. (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). **(D)** IRF1 silencing of si-IRF1 has been detected for mRNA and protein expression in hMDMs after H37Rv infection (MOI = 2) for 24 h (Data presented as mean ± SEM, $n = 3$ independent experiments with each 2 replicates). CFU assay has been performed to analyze H37Rv survival at 48 h.p.i. (Data presented as mean ± SEM, at least two independent experiments with each 4 replicates). Data are expressed as mRNA fold change relative to uninfected cells. Vector expressing reporter genes Photinus pyralis luciferase (Fluc) has been used as a control. GAPDH served as an internal reference. The survival of BCG and H37Rv at 0 h post infection have been recognized as 100%. The statistical calculations were performed with the unpaired Mann–Whitney U test. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered as statistically significant. The numbers below immunoblot correspond to band integrated density ratio of IRF1/GAPDH.

A

B

Fig. 3. IRF1 is crucial for inflammatory cytokine production during mycobacterial infection in Mφs. (A) Inflammatory cytokines of *Il-6, Il-8, Tnf, Ifn-a, Ifn-b, Ifn-g* and *Il-10* have been detected in BCG infected (for 6 h) IRF1 silencing hMDMs for mRNA levels by qRT-PCR (Data presented as mean ± SEM, $n = 3$ independent experiments with each 2 replicates. Data was presented with 3 replicates of one donor). **(B)** hMDMs transfected with si-IRF1-1 and −2 RNA upon BCG infection for 6 h have been analyzed for cell secreted cytokines including IL-6, TNF-α, IL-8, IL-10 and IFN-α, β and γ by Luminex assay (Data presented as mean ± SEM, $n = 2$ independent experiments. Data was presented with 3 replicates of one donor). Data are expressed as mRNA fold change relative to uninfected NC cells. GAPDH served as an internal reference. The statistical calculations were performed with the unpaired Mann–Whitney U test. $*p < 0.05$ and $**p < 0.01$ were considered as statistically significant.

(Supplementary Fig. 4B). However, the anti-mycobacterial capability of IRF1 was not blocked by STAT1 inhibition (Fig. 4D). Next, we found that fludarabine did not affect cell viability and mycobacteria growth *in vitro* without cells (Supplementary Fig. 4C and D). These data indicated that activation of STAT1 and subsequent ISG production are not required for IRF1 to restrict intracellular mycobacteria in Mφs.

IRF1 inhibits intracellular mycobacteria through suppression of mTOR signaling pathway

It has been reported that mitogen-activated protein kinases (MAPKs) p38 isoforms, caspase-1 activation and mTOR activation are involved in IRF1 expression.[14,26,27] In our study, no difference of phosphorylation level of ERK1/2, p38, pro-caspase-1 or
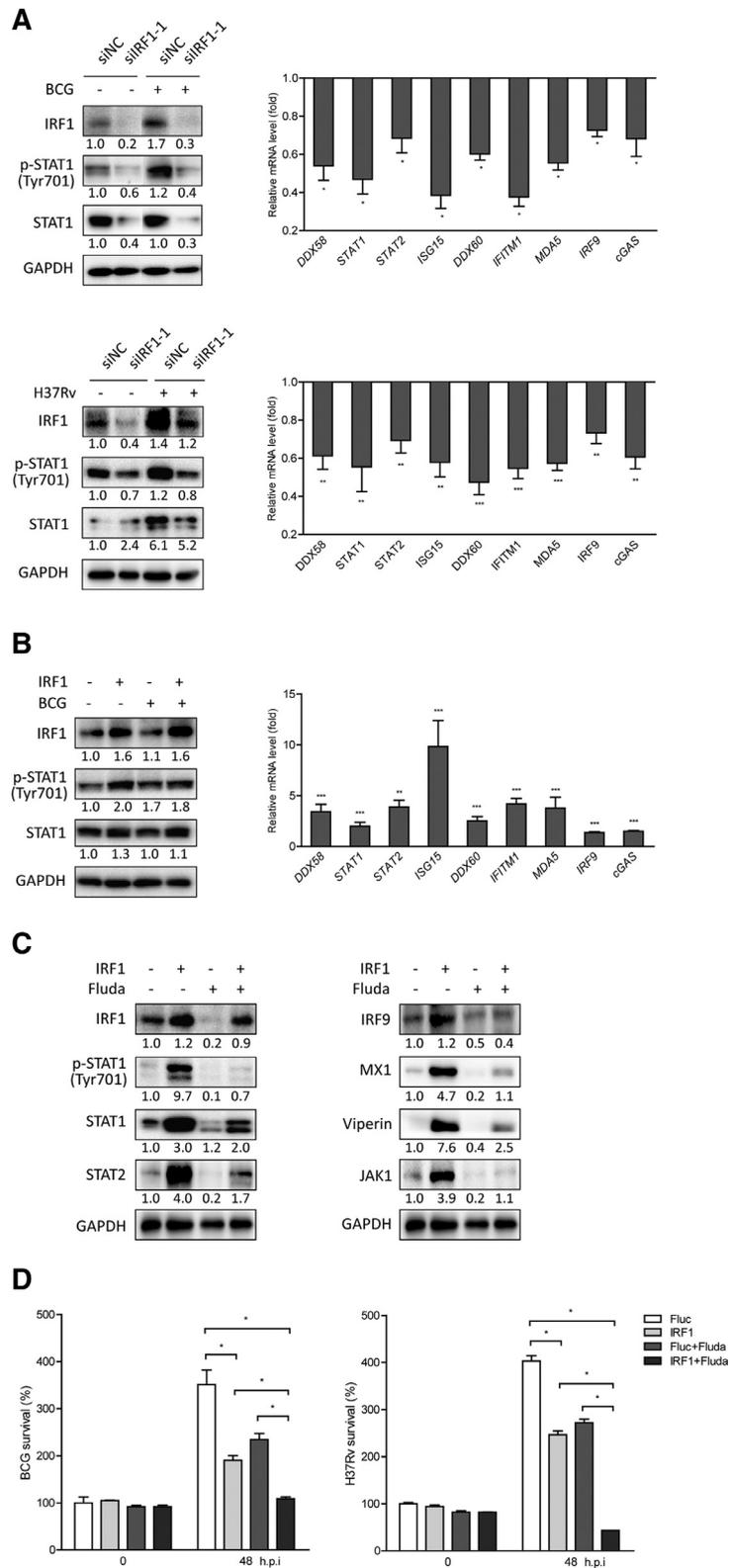
Fig. 4. IRF1 diminishes Mtb infection independent of STAT1 activation and ISG induction. (A) IRF1 silencing attenuated the total STAT1 protein level and phosphorylation level in BCG (MOI = 2) and H37Rv (MOI = 2) infected hMDMs for 24 h. A subset of mRNA expression of ISGs induced by BCG and H37Rv infection showed significant decreases in IRF1 knock downed hMDMs for 24 h (Data presented as mean ± SEM, $n = 3$ independent experiments with each 1–2 replicates). (B) IRF1 overexpression showed potent increase of total STAT1 protein and phosphorylation level and ISGs mRNA levels upon BCG (MOI = 2) infection for 24 h in THP-1-Mφs (Data presented as mean ± SEM, $n = 3$ independent experiments with each 2 replicates). (C) IRF1 overexpressing THP-1-Mφs were pretreated with 10 μM fludarabine for 24 h and the ISGs protein levels have been detected by Western blot analysis upon BCG (MOI = 2) infection for 24 h. (D) CFU have been determined for BCG and H37Rv survival in IRF1 overexpressing THP-1-Mφs under the treatment of 10 μM fludarabine at 48 h.p.i. (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). Data are expressed as mRNA fold change relative to uninfected NC cells. The survival of BCG and H37Rv at 0 h post infection have been recognized as 100%. Vector expressing reporter genes Photinus pyralis luciferase (Fluc) has been used as a control. GAPDH served as an internal reference. The numbers below immunoblot correspond to band integrated density ratio of target band/GAPDH. The statistical calculations were performed with the unpaired Mann–Whitney U test. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered as statistically significant. Fluda, Fludarabine.

cleaved caspase-1 p10 subunit expression was found between IRF1 silencing and control cells during Mtb infection (Supplementary Fig. 5A, B). Surprisingly, IRF1 silencing potently increased mTOR phosphorylation levels in hMDMs (Fig. 5A). Consistently, IRF1 overexpression potently inhibited both phosphor-mTOR and phosphor-AKT levels. However, PI3K inhibition by wortmannin did not restore the antimicrobial effect of IRF1 overexpression (Fig. 5B). Next, rapamycin showed profound inhibition of phosphor-mTOR levels in both IRF1 silencing and overexpressing cells. Furthermore, rapamycin alone remarkably eliminated the mycobacteria infection in both hMDMs and THP-1-Mϕs, but it did not has further effect on Mtb infection in IRF1 silencing or overexpressing Mϕs at 48 h.p.i. (Fig. 5C, D). Consistently, MHY1485 which specifically led to a constitutive activation of mTOR signaling as detected by phosphorylation level of S6K, partly restored IRF1 overexpression-induced S6K activation and the anti-mycobacterial effect of IRF1 overexpression at 48 h.p.i. (Fig. 5E). MHY1485 did not affect cell viability and mycobacteria growth without cells (Supplementary Fig. 4C and D). Hence, these results demonstrated that IRF1 prevents Mtb infection by attenuating mTOR activity.

IRF1 constrains intracellular mycobacterial infection partially depending on autophagy machinery

As one of the most important effector mechanism downstream of mTOR signaling, autophagy has been recognized as a powerful host defense machinery to eliminate mycobacteria in Mϕs.[21] IRF1 could directly regulate autophagy in cancer.[28] Our results also confirmed that IRF1 silencing dramatically attenuated LC3-II (the lipidated form of LC3 found in autophagosomes membranes) expression by Western blot assay and LC3 puncta formation and lysosome-associated membrane protein 1 (LAMP1) expression by confocal fluorescence microscopy analysis (Fig. 6A and Supplementary Fig. 5C). In line with this, IRF1 overexpression exerted a potent increase of LC3-II expression and LC3 puncta formation and lysosomes formation during Mtb infection (Fig. 6B and Supplementary Fig. 5D). IRF1 induced LC3-II protein level was not influenced by STAT1 inhibition (Supplementary Fig. 5E). However, IRF1 did not affect the expression levels of Atgs including Atg5, 7 and 12, et al. (Supplementary Fig. 6). Wortmannin is an inhibitor of autophagy flux to form autolysosomes and makes LC3-II accumulating. Although wortmannin led to LC3-II accumulation during Mtb infection (Fig. 6C), it did not influence the anti-mycobacterial activity of IRF1 (Fig. 5B). Next, in IRF1-THP-1 cells we found that 3-methyladenine (3-MA) of the direct inhibitor of autophagy machinery potently inhibited LC3-II expression, but it only partially restored IRF1 mediated suppression of H37Rv survival (Fig. 6D). These results indicated that IRF1 constrains mycobacterial infection via mTOR pathway partially depending on autophagy machinery.

IRF1 eliminates mycobacteria by suppressing ribosomal protein S6 kinase via mTOR

It is well known that p70 S6K represents the key downstream element of mTOR signaling, thus we investigated whether the anti-mycobacterial effect of IRF1 is mediated by ribosomal protein p70 S6K. We observed that IRF1 silencing facilitated phosphorylation levels of mTOR substrates p70 S6K in THP-1-Mϕs and hMDMs upon BCG or H37Rv infection (Fig. 7A). PF 4708671 as p70 S6K inhibitor has been used to treat with IRF1 silencing hMDMs upon H37Rv infection and the result showed that IRF1 silencing promoted the mTOR, p70 S6K and S6 phosphorylation and they were inhibited by PF 4708671 treatment MHY1485 did not affect cell viability and mycobacteria growth without cells. (Fig. 7B). Moreover, PF 4708671 significantly decreased the survival of intracellular mycobacteria and blocked the pro-mycobacterial

effect of IRF1 silencing (Fig. 7B). However, it did not affect the cell viability and mycobacteria growth without cells (Supplementary Fig. 4C and D). Correspondingly, IRF1 overexpression led to down-regulation of the phosphorylation levels of mTOR and p70 S6K (Fig. 7C). Furthermore, we found that the treatment of PF 4708671 and IRF1 overexpression alone could inhibit the p70 S6K phosphorylation and H37Rv growth in THP-1-Mϕs (Fig. 7C). These results demonstrated that IRF1 eliminates Mtb infection through diminishing ribosomal protein S6 kinase phosphorylation via mTOR signaling pathway inhibition. However, treatment of PF 4708671 did not restore IRF1 silencing-reduced cytokine production, indicating that IL-6, IL-8 and TNF-α production were independent of p70 S6K induction (Supplementary Fig. 7). IFNs have been used as combination with the first-line drug such as INH for treating TB.[6] Thus, we investigated the combinatory effect of INH and IRF1 over-expression on phosphorylation of mTOR and p70 S6K. Interestingly, INH could further promote IRF1-reduced phosphorylation levels of both mTOR and p70 S6K. Consistently, INH and IRF1 overexpression alone could suppressed the mycobacterial infection and INH could still exert an anti-mycobacterial effect in IRF1 overexpression THP-1-Mϕs (Fig. 7D). These results demonstrated that INH and IRF1 could both led to reduction of mTOR/p70 S6K activity and the anti-Mtb effect independently.

Discussion

Although IFNs or in combination with first line anti-tuberculosis therapy have been effectively used in clinics,[6] the paradoxical effects of IFNs on the immune response and pathogenesis of tuberculosis have been strongly supported by studies in diverse models.[5,7] These paradoxical effects might have resulted from unknown divergent function of ISGs whose transcription is activated by the downstream signaling of IFN in mycobacteria infection. Certain ISGs have been reported as positive host defense factors in restricting Mtb infection.[29,30] However, how most of ISGs impacting the host immune response to manipulate Mtb infection remain poorly understood. IRF1 is one of the most important ISGs which is indispensable for anti-viral and anti-tumor activities.[31,32] More than two decades ago, Kamijo et al. suggested that IRF1 plays a putative protective role in combating against Mtb infection via the production of iNOS and nitric oxide (NO) in mice.[9] Here we found that iNOS was not induced by IRF1 in human Mϕs, partly because this critical anti-mycobacterial mechanism differs markedly between mice and humans. In conclusion, our results suggested that IRF1 plays a role in host protection against Mtb infection by restraining p70 S6K activation through mTOR inhibition in human Mϕs.

Various ISGs including IRF1 have been demonstrated with up regulation in purified blood neutrophils and monocytes of active TB patients compared with healthy people, or in Mtb infected alveolar macrophages and monocytes in cell culture models.[10,33–35] In our study, IRF1 expression is significantly enhanced in PBMCs and monocytes of TB patients *in vivo* and in Mtb-infected Mϕs *in vitro*. Although it has been reported that the level of hundreds of genes was significantly associated with the monocyte: lymphocyte (ML ratio) *in vivo* between healthy individuals and Mtb patients, we did not observed significant difference of IRF1 expression in the ratio of monocyte: PBMC between HCs and TB patients.[36] These results indicated that IRF1 could be generally raised in PBMCs in TB patients *in vivo*. However, more samples and further study are needed to define whether the ratio of monocyte in PBMCs is also induced in PBMCs. Some studies have shown that IRF1 can be induced by various pathogens and agents, including double-stranded RNA (dsRNA), IFNs, TNF-α, IL-6, and several other cytokines in different cell types.[27,35] cGAS-STING, TLR-2 and -4 signaling are
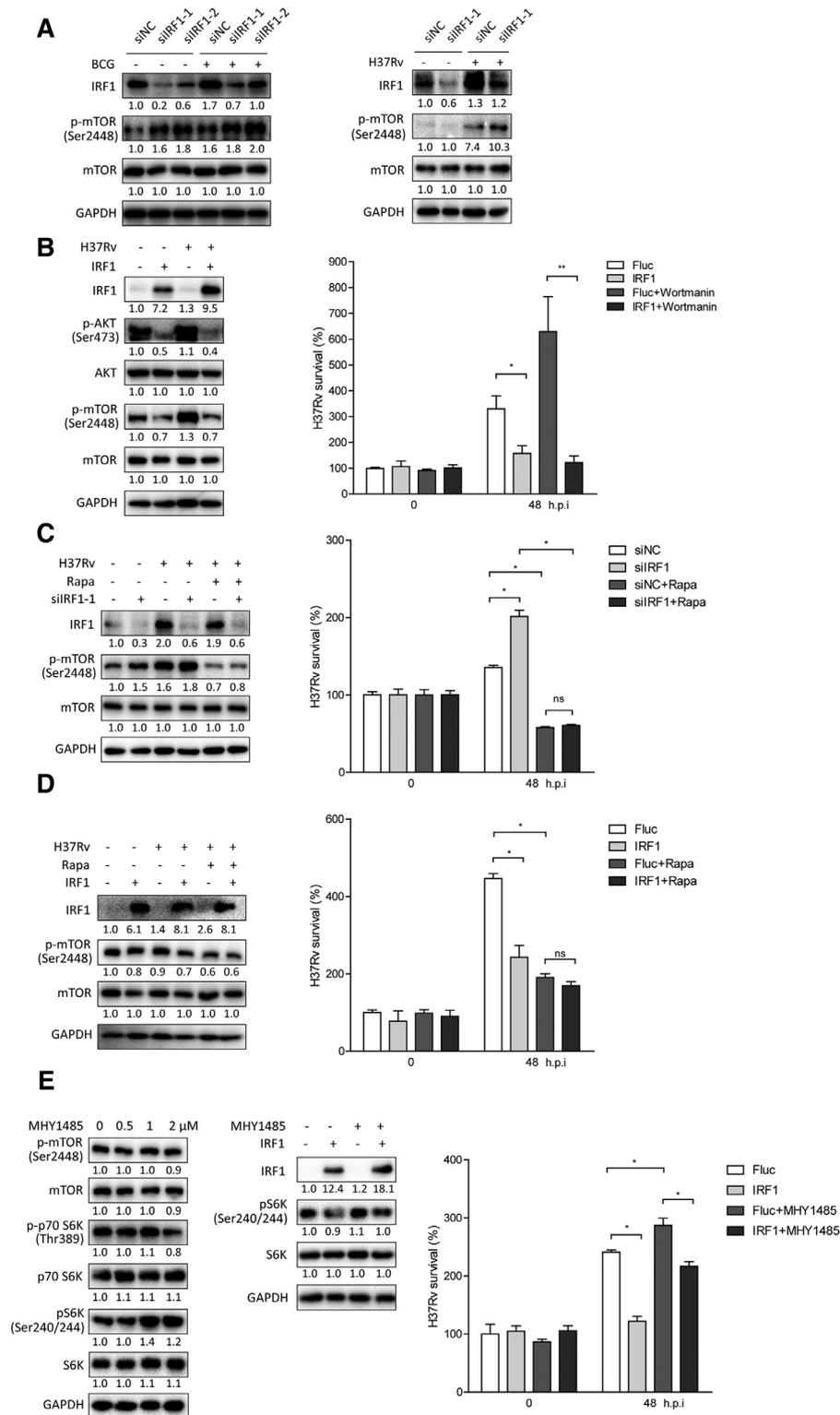
Fig. 5. IRF1 reduces intracellular Mtb infection by inhibiting mTOR signaling pathway. (A) The phosphorylation levels of mTOR protein have been detected by Western blot assay in IRF1 silenced hMDMs upon BCG (MOI = 2) and H37Rv infection (MOI = 2) for 24 h. **(B)** The phosphorylation levels of AKT and mTOR proteins have been analyzed in IRF1 overexpressing THP-1-Mϕs with H37Rv inoculation for 24 h. CFU assay has been performed to determine H37Rv survival in IRF1 overexpressing THP-1-Mϕs with treatment of 10 μM wortmannin at 48 h.p.i. (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). **(C)** hMDMs were transfected with IRF1 siRNA-1 for 48 h and infected with H37Rv for 24 h, 10 μM rapamycin was pretreated for 24 h. Expression levels of phosphor-mTOR has been detected by Western blot assay. The intracellular mycobacterial viability was determined by CFU assay at 48 h.p.i. (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). **(D)** IRF1 overexpressing THP-1-Mϕs were treated with 10 μM rapamycin and infected H37Rv for 24 h. The phosphorylation and total of mTOR protein level was detected. CFU assay at 48 h.p.i. has been performed (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). **(E)** THP-1-Mϕs were treated with 0.5, 1 and 2 μM MHY1485 and infected H37Rv for 24 h. The phosphorylation and total of mTOR, p70 S6K, S6K protein levels were detected. IRF1 overexpressing THP-1-Mϕs were treated with 2 μM MHY1485 and infected H37Rv for 24 h. The phosphorylation and total of p70 S6K protein level was detected. CFU assay at 48 h.p.i. has been performed (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). The survival of BCG and H37Rv at 0 h post infection have been recognized as 100%. The statistical calculations were performed with the unpaired Mann–Whitney U test. $^*p < 0.05$ and $^{**}p < 0.01$ were considered as statistically significant. The numbers below immunoblot correspond to band integrated density ratio of target band/GAPDH. Ns, not significant. Rapa, Rapamycin.
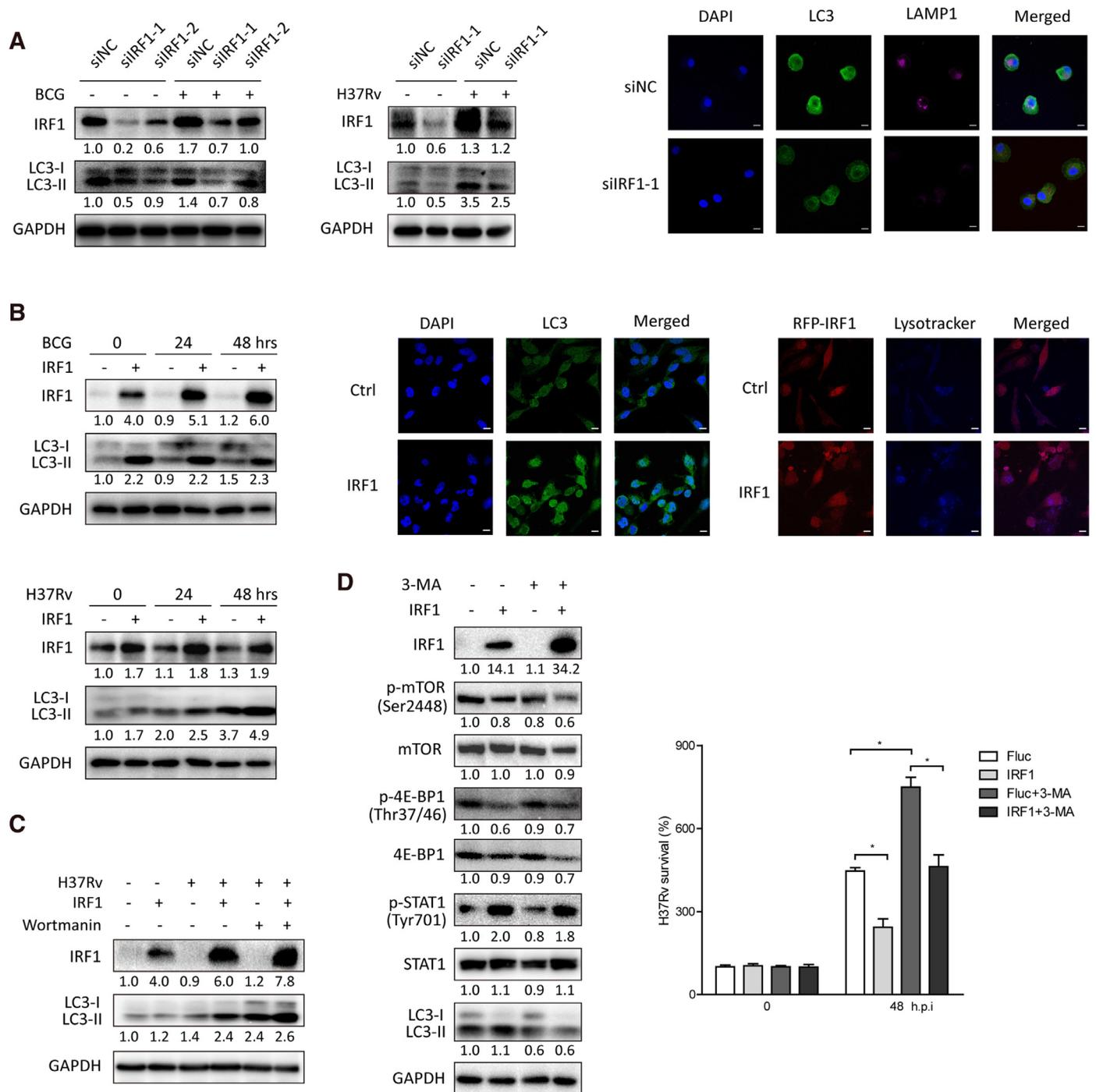
Fig. 6. Antimicrobial effect of IRF1 partially relies on the activation of autophagy machinery. (A) IRF1 was silenced by siRNA for 48 h with subsequent BCG and H37Rv infection for 24 h in THP-1-Mϕs. The LC3 level was detected by Western blot analysis. LC3 puncta and LAMP1 protein levels were monitored with fluorescence microscopy in IRF1 silencing THP-1-Mϕs. (B) In IRF1 overexpressing THP-1-Mϕs, LC3 protein levels were analyzed upon BCG and H37Rv infection for 24 and 48 h. LC3 puncta and autophagosomes determined by lysotracker staining have been detected with fluorescence microscopy. (C) IRF1 overexpressing THP-1-Mϕs were pretreated with 10 μM wortmannin for 24 h and infected with H37Rv for another 24 h, LC3 protein level was detected by Western blot assay. (D) With pretreatment of 5 mM 3-MA for 24 h, H37Rv infected IRF1 overexpressing THP-1-Mϕs were detected for LC3 protein level and phosphorylation level of mTOR, 4E-BP1, STAT1 proteins. Nucleus was stained with DAPI (Blue). LC3 (Green), LAMP1 (Purple), IRF1 (Red) and Lysotracker (Blue) were detected. The survival of H37Rv was determined by CFU assay at 48 h.p.i. IRF1 overexpressing THP-1-Mϕs with 5 mM 3-MA treatment for 24 h (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). The survival of H37Rv at 0 h post infection have been recognized as 100%. The statistical calculations were performed with the unpaired Mann–Whitney U test. *$p < 0.05$ was considered as statistically significant. The numbers below immunoblot correspond to band integrated density ratio of target band/GAPDH. (Scale bar: 20 μm).

critical PRRs that play key roles in sensing and recognizing Mtb.[22,23] In this study, we demonstrated that IRF1 could be induced by activation of cGAS-STING, TLR-2 and -4 signaling. In addition, although a previous study showed that IRF1 is an additional member involved in MyD88 signaling,[37] our data demonstrated that Mtb-induced IRF1 expression was independent of MyD88 expression during Mtb infection. We confirmed that their downstream molecules of TBK1, MyD88, p65 NF-κB, p38 MAPK as well as cytokines of TNF-α, IL-1β, IL-6 and IL-8 were not responsible for IRF1 production. Finally, IRF1 expression might be potentiated
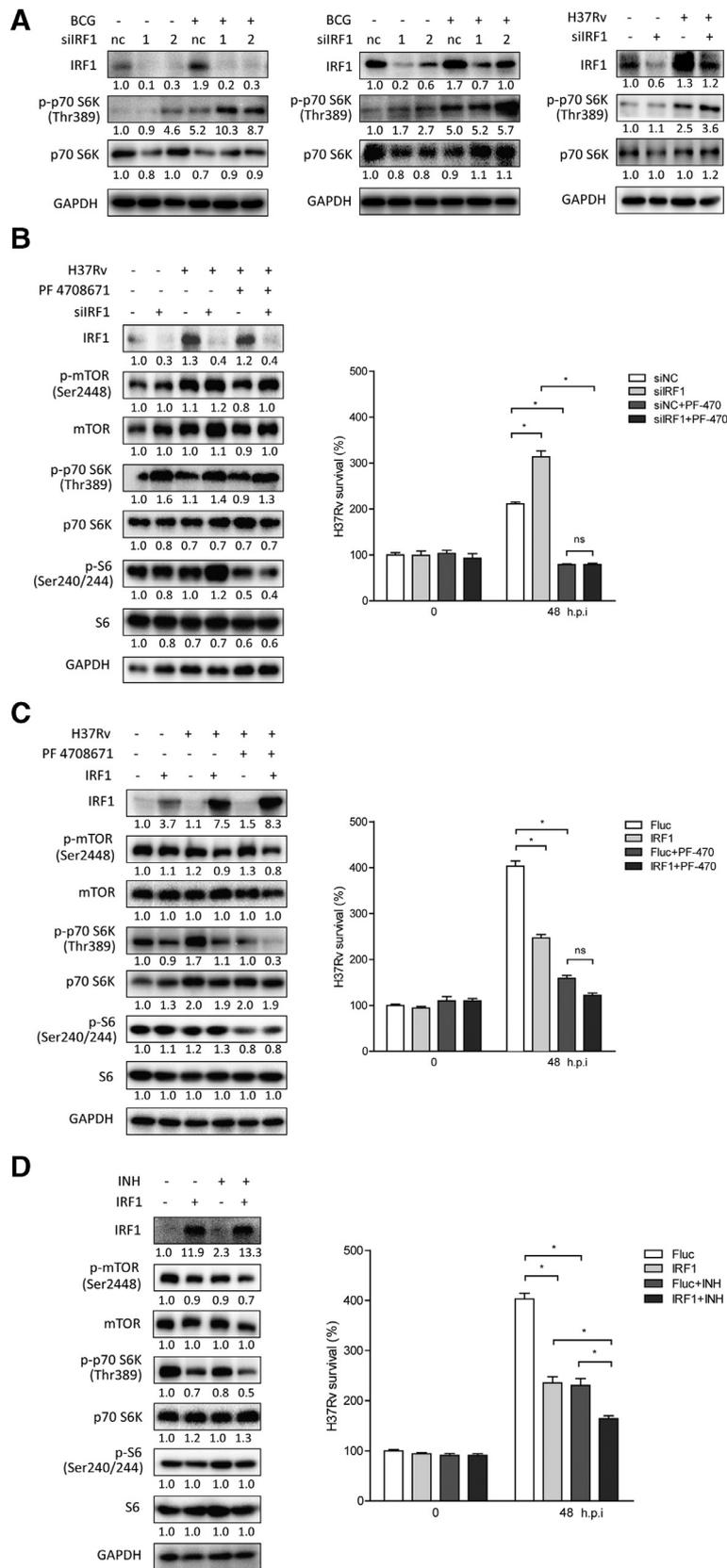
Fig. 7. IRF1 reduces intracellular mycobacteria survival by reducing ribosomal protein S6 kinase expression via mTOR inactivation. **(A)** Phosphorylation of p70 S6K as the downstream element of mTOR pathway has been detected by Western blot assay in IRF1 silencing THP-1-Mφs and hMDMs upon BCG or H37Rv infection for 24 h. **(B, C)** S6, p70 S6 kinase, mTOR and their phosphorylation levels analyzed by Western blot assay and CFU assay to determine H37Rv survival levels at 48 h.p.i. have been performed in **(B)** IRF1 silencing hMDMs and **(C)** IRF1 overexpressing THP-1-Mφs with 10 μM pretreatment of PF 4708671 for 24 h. **(D)** Western blot assay and CFU assay were performed in IRF1 overexpressing THP-1-Mφs with 0.1 μM INH for 24 h (Data presented as mean ± SEM, at least two independent experiments with each 3–4 replicates). The survival of H37Rv at 0 h post infection has been recognized as 100%. The statistical calculations were performed with the unpaired Mann–Whitney U test. $*p < 0.05$ and $**p < 0.01$ were considered as statistically significant. Ns, not significant. The numbers below immunoblot correspond to band integrated density ratio of target band/GAPDH.
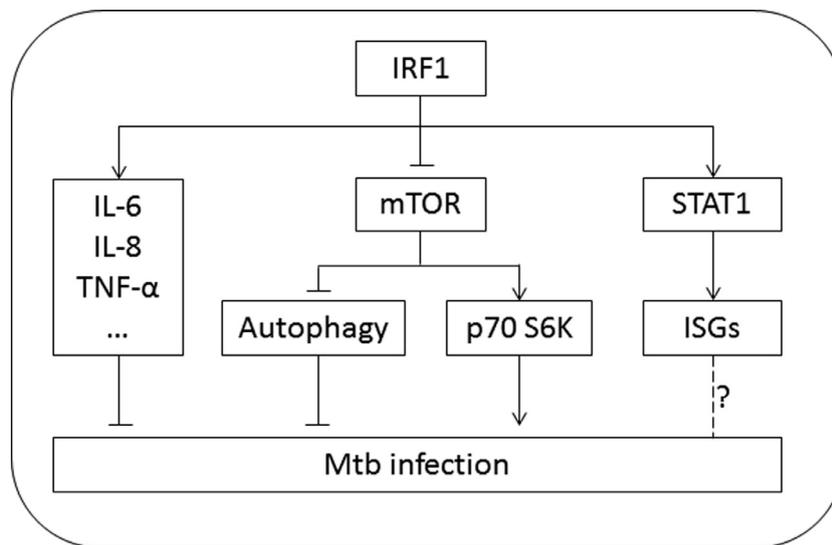
Fig. 8. Illustration of the effects on Mtb infection by IRF1 and downstream pathways.

by STAT1 activation independent of IFN-γ production. These data implicate a potential role of IRF1 in Mtb infection.

Mϕs not only provide residence for Mtb, but also represent one of the most important host defense cells against Mtb infection.[38] As one of the most important ISGs, IRF1 plays a critical role in the host defense mechanism of immune and inflammatory responses against pathogen infection.[13,39–41] Thus, we investigated the direct effect of IRF1 on Mtb infection in human Mϕs. The loss-of-function and gain-of-function of IRF1 have been exploited to confirm that IRF1 serves as a host protective factor to eliminate intracellular mycobacterial infection. Although in mice IRF1 protected the host against Mtb infection by iNOS production, which is a powerful anti-mycobacterial approach in Mϕs,[9] iNOS is not necessary for IRF1 to resist *Toxoplasma gondii* infection in mice.[42] In this study, we observed that IRF1 did not mediated the induction of iNOS in Mtb-infected human Mϕs at all. This means that there should be another recondite mechanism for IRF1 to exert the anti-Mtb potential. The cells uptake the mycobacteria and subsequently activate the innate host defense, such as the production of pro-inflammatory cytokines.[38] Despite IRF1 being considered as a transcription factor and, in some cases, responsible for IFN expression,[43,44] we did not find that IFNs, including IFN-α, β and γ production, were regulated by IRF1 upon Mtb infection. We further detected the production of IL-6, IL-8 and TNF-α, which are the main host protective inflammatory cytokines against Mtb infection, and found that their secretion levels, but not mRNA expression levels, were significantly reduced by IRF1 interfering in hMDMs. The anti-inflammatory cytokine of IL-10 was significantly inhibited by IRF1 silencing only to a slight extent. Therefore, these studies implicate a potent role of IRF1 in pro-inflammatory response production, which is the major arm of anti-mycobacterial defense.[45,46]

Host defence peptides of either hBD1 or LL37, phosphorylation of p38 and caspase-1 production have been suggested as effector molecules of IRF1.[24,26,27,47] However, we did not find any apparent difference of any one of them between control and IRF1 silenced hMDMs upon Mtb infection. Moreover, IRF1 could induce DNA binding of STAT1, leading to STAT1 phosphorylation and activation of its downstream ISG expression.[11,12] Our retrieved IRF1-ChIP-seq data implied that IRF1 could promote the binding of the promoter region of STAT1 gene. Indeed, we found that IRF1 could positively regulate STAT1 activation and ISG production upon Mtb infection. Despite the pharmacological inhibition of STAT1 resulted in abrogation protein levels of STAT1 and ISG expression, it did

not affect the mRNA levels of some genes. We speculated that Mtb infection could downregulate postinitiation mRNA biogenesis pathways to maintain excessive response to STAT1 inhibition. As previous study demonstrated that manipulating postinitiation mRNA biogenesis could restore an unconstrained Mϕs response to IFN-γ during Mtb infection.[48] Moreover, STAT1 inhibition did not block IRF1-mediated anti-mycobacterial effect. This might be because various ISGs gain different functions or exert opposite effects on host defense against Mtb infection, however, more detailed study is needed.

Furthermore, it has been suggested that IRF1 activates mTOR activity in suppressing tumor growth with an unknown mechanism.[14] To the contrary, as shown in our study, mTOR signaling is negatively regulated by IRF1 expression during Mtb infection. Constitutive activation of mTOR signaling could partly restore the anti-mycobacterial effect of IRF1 overexpression, indicating that IRF1 reduced mTOR activation to exert the anti-Mtb effect in Mϕs. Importantly, our previous study suggested that autophagy as a key downstream effector of mTOR signaling plays an important role in attenuating Mtb infection.[21] IRF1 could positively regulate autophagy machinery in certain circumstance.[28] Conversely, others have found that IRF1 knockdown induced autophagy by increasing BECN1 and blunting mTOR survival signaling.[19] Here, we demonstrated that IRF1 overexpression leads to suppression of mTOR activation and autophagy activation without affecting important Atgs expression. Furthermore, 3-MA could inhibit LC3-II formation and partially restore the IRF1-mediated anti-Mtb effect in Mϕs. These data suggested that IRF1 does need mTOR repression in combating intracellular Mtb infection and partially depending on activated autophagy machinery, but this is not the major anti-mycobacterial mechanism. Hence, we speculate that another mechanism downstream of mTOR signaling is exploited by IRF1 to eliminate mycobacterial survival.

In addition, as the key element downstream of mTOR signaling pathway, p70 S6K, is responsible for protein synthesis and cell growth and could regulate the innate immune response to Mtb infection.[20] In our research, over-expression and knock-down of IRF1 mRNA was inversely correlated with the level of p70 S6K phosphorylation in Mtb-infected Mϕs. Inhibition of p70 S6K by specific inhibitor could reduce Mtb infection and blocks the pro-mycobacterial infection of IRF1 silencing in Mϕs. MMPs have been reported as critical factors involved in AKT/p70 S6K signaling induced regulation in monocytes infected with Mtb[20]; however, we

did not observe that IRF1 affects MMPs expression during Mtb infection. Therefore, further studies are required to determine how IRF1 regulate mTOR/p70 S6K axis to display protective function upon Mtb infection.

In conclusion, we characterized Mtb-induced IRF1 as an important host defense factor and revealed a novel mechanism by which IRF1 deficiency-mediated mTOR induced p70 S6K activation, resulting in an anti-mycobacterial activity and serving as the host defense program (Fig. 8). Furthermore, the reduction of mTOR/p70 S6K activation and the anti-Mtb effect of INH is independent of IRF1 and can work together to combat Mtb infection. This study has shed new light on the development of new anti-mycobacterial strategies.

Author contributions

XZ contributed to the study concept and design, obtained funding, XZ and JY acquisition of data, analysis and interpretation of data, drafting of the manuscript, and statistical analysis; ZZ, LZ, LL, BZ, LX, YG, XD, CZ, YH, RW, HL, and YL contributed to acquisition of data and critical revision of the manuscript. QW, SH and CZ contributed to study concept and critical revision of the manuscript; QP contributed to the critical revision of the manuscript; LM contributed to study concept and design, obtained funding, study supervision, and critical revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.06.007.

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