

Mycobacterium tuberculosis 19-kDa lipoprotein induces Toll-like receptor 2-dependent peroxisome proliferator-activated receptor γ expression and promotes inflammatory responses in human macrophages

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Abstract. *Mycobacterium tuberculosis* (M.tb) enhances its survival in macrophages by suppressing immune responses, in part through its complex cell wall structures. M.tb 19-kDa lipoprotein (P19), a component of the complex cell wall structures of M.tb, is a Toll-like receptor (TLR) agonist, and may induce immune responses through TLR2. Furthermore, the activation of peroxisome proliferator-activated receptor γ (PPAR γ) is also involved in M.tb-induced immune responses in macrophages. In the present study, specific agonists/antagonists and siRNA were used to investigate the role of PPAR γ in P19-induced immune responses in human macrophages, including TLR2 activation, p38 phosphorylation and cytokine production. In the present study, PPAR γ expression, p38 phosphorylation and cytokine production were upregulated following M.tb H37Rv infection or P19 treatment. By pretreating macrophages with a specific PPAR γ agonist or antagonist, it was demonstrated that phosphorylation and IL-6 production are modulated in macrophages by PPAR γ activity. Following TLR2 knockdown in macrophages, the expression of PPAR γ was significantly decreased in the presence or absence of P19 treatment. Furthermore, p38 phosphorylation and cytokine production were significantly reduced in TLR2 knockdown macrophages following P19 treatment. It was demonstrated in the current study that PPAR γ was induced and activated by M.tb infection and that P19-induced PPAR γ expression, p38 phosphorylation and cytokine production in macrophages are dependent on TLR2. These findings suggest a role for PPAR γ and TLR2 in P19-induced p38 phosphorylation and cytokine production, thereby potentially influencing M.tb pathogenesis.

Introduction

Tuberculosis is a common infectious disease usually caused by *Mycobacterium tuberculosis* (M.tb) (1). Tuberculosis most regularly affects the lungs, but can potentially affect almost any other body organ. M.tb is spread when individuals with active M.tb infection cough, sneeze, or otherwise transmit respiratory fluids through the air (2). The majority of M.tb infections are asymptomatic and latent, but approximately one in ten latent infections eventually progresses to active tuberculosis which, if left untreated, is fatal in >50% of cases.

Peroxisome proliferator-activated receptor (PPAR), a member of the lipid-activated nuclear receptor family, is a key transcriptional regulator of cell differentiation, inflammation, and lipid metabolism in macrophages and dendritic cells (3). PPARs are expressed in leukocytes, including macrophages, dendritic cells, T cells and B cells, and a role for these receptors in inflammation and immunoregulation has previously been proposed (4,5). There are three types of human PPARs: α , γ , and β/δ , and each type is the product of a different gene (6). PPAR γ , a nuclear receptor superfamily member, is a transcriptional factor that regulates inflammation and is highly expressed in alternatively activated alveolar macrophages and macrophage-derived foam cells, both of which are closely associated with the pathogenesis of tuberculosis (7). A previous study demonstrated that PPAR γ is involved in lipid body biogenesis, revealing a cross-talk between the innate immune receptor Toll-like receptor 2 (TLR2) and the lipid-activated nuclear receptor PPAR γ that coordinates lipid metabolism and inflammation in the *Bacillus Calmette-Guérin* (BCG)-infected macrophages, thereby potentially altering mycobacterial pathogenesis (8). Although it is well established that PPAR γ acts as a master regulator in lipid metabolism and inflammation, the involvement of PPAR γ in the immune response of macrophages to intracellular pathogen infection remains to be elucidated.

Tumor necrosis factor α (TNF- α) is crucial in establishing and maintaining the inflammatory response against infections (9). The blockage of TNF- α has marked effects on the progression of tuberculosis in experimental models. For example, neutralization of TNF- α in a murine model has been demonstrated to lead to tuberculosis aggravation or

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reactivation (10). Furthermore, increased levels of TNF- α are frequently detected in the culture supernatants of peripheral blood mononucleated cells from patients with pulmonary tuberculosis stimulated with mycobacterial antigens (11,12). Similar to TNF- α , IL-6 is involved in chronic inflammatory diseases (13). IL-6-deficient (IL-6^{-/-}) mice are resistant to the induction of various experimental inflammatory diseases (14). The mitogen-activated protein kinase (MAPK) pathway is important for immune response and mycobacterial pathogenesis (15-17), and MAPK family members include extracellular signal-regulated kinase, p38 MAPK (p38), and stress-activated protein kinase/c-Jun N-terminal kinase (18). It has been reported that p38 was activated in monocytes following M.tb infection (19). The involvement of TLR2 in the M.tb infection is well-defined, as patients with TLR2 polymorphisms exhibit increased susceptibility to M.tb infection, whereas TLR2^{-/-} mice are unable to mount optimal immune responses against mycobacteria (20). However, potential additional signaling pathways involved in M.tb-induced molecular regulation are unknown.

The aim of the current study was to investigate the role of PPAR γ in P19-induced immune responses, including TLR2 activation, p38 phosphorylation and cytokine production.

Materials and methods

Reagents and antibodies. PPAR γ agonist BRL49653, antagonist GW-9662 and fetal bovine serum (FBS) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Cell culture reagents, medium, L-glutamine and antibiotics were obtained from Gibco-BRL (Rockville, MD, USA). Monoclonal antibodies (all produced from immunized rabbits) against PPAR γ , phospho-p38, MAPK (Thr180/Tyr182), total p38, TLR2 and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). TLR2 small interfering (si)RNA and control siRNA (fluorescein isothiocyanate-conjugated) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Bacterial strains and P19 isolation. Lyophilized M.tb H37Rv (ATCC 25618) and *Mycobacterium smegmatis* (*M. smegmatis*; ATCC 700084) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), reconstituted and used as described previously (21). The concentration of bacteria was determined by counting in a Petroff-Hausser chamber (Hede Biotechnology, Beijing, China). Bacteria prepared in this manner are $\geq 90\%$ viable as assessed by colony forming unit assays.

Purified M.tb 19-kDa lipoprotein (P19) was obtained as described previously (22). In brief, cell-wall fractions were obtained by sonication of suspended M.tb H37Rv at 20 kHz in iced water (5 cycles for 5 min each). Protein (40 μ g) were mixed with a reducing sample buffer (0.05 mM EDTA, 0.1% SDS, 1% glycerol, 10% 2-mercaptoethanol, and 0.5 mM/ml Tris-HCl pH 6.8), heated for 5 min at 95°C and loaded onto 12% SDS-PAGE gels. Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and stained with Ponceau S red (Energy Chemicals, Shanghai, China) to identify the 19 kDa band; the identity of this band was confirmed in parallel blots with the IT-19 monoclonal antibody. The band was then excised, solubilized in dimethylsulfoxide

(DMSO) and precipitated with carbonate/bicarbonate sodium buffer (0.05 M, pH 9.6). The pellet was rinsed three times with phosphate-buffered saline (PBS; pH 7.4) and stored at -20°C. The concentration of the protein was measured with the Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Cell culture and mycobacterial infection. The WBC 264-9C macrophage cell line (HB-8902; ATCC) was cultivated in cultured in RPMI-1640 medium supplemented with 15% FBS, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2 mM L-glutamine, and 50 μ g/ml gentamicin at 37°C in a humidified incubator containing 5% CO₂. For P19 stimulation or infection with M.tb H37Rv or *M. smegmatis*, WBC 264-9C cells were placed in 12-well culture plates with glass coverslips at a density of 5x10⁵ cells/ml for 24 h. Cells were then washed and incubated for an additional 18 h in the medium with 0.1% FBS. The macrophage monolayers in the tissue culture plates were washed with prewarmed RPMI-1640 medium and replaced with 1 ml RPMI-1640 medium containing 10 mM HEPES and 0.4% human serum albumin, and viable M.tb H37Rv or *M. smegmatis* at a multiplicity of infection of 1:10 macrophage/bacteria, or treated with 5 μ g/ml P19, and incubated at 37°C for various time periods prior to analysis. Alternatively, cells were pretreated with BRL49653 (5 μ M), GW-9662 (1 μ M) or 0.01% DMSO (as vehicle-control) at 37°C for 30 min prior to bacterial infection. The cells and cell-free supernatants were collected and stored at -20°C for subsequent assays. Cell viability was assessed by a trypan blue (Energy Chemicals) exclusion method at the end of each experiment (viability of $\geq 90\%$ was required).

TLR2 siRNA transfection. To silence the expression of TLR2, macrophages were transfected with the TLR2 siRNA. The siRNA transfection was performed according to the manufacturer's instructions. In brief, WBC 264-9C macrophages were incubated in the siRNA Transfection medium (Santa Cruz Biotechnology, Inc.) at a density of 2x10⁵ cells/well in 12-well cell culture plates, followed by the addition of the TLR2 siRNA or negative control siRNA, and incubated at room temperature for 30 min. A transfection efficiency of $>95\%$ was demonstrated by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). Following transfection, the TLR2 expression was analyzed by reverse transcription (RT)-quantitative polymerase chain reaction (qPCR) and western blot analysis. The cytokine expression levels in the supernatant of the transfected macrophages were measured by ELISA.

Western blot analysis. The macrophages were rinsed with prewarmed PBS, and lysed in an ice-cold extraction buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 10% glycerol; 1 mM EDTA; 1 mM EGTA; 1% NP-40; 1 mM dithiothreitol; and protease inhibitor cocktail (Roche, Basel, Switzerland). The homogenate was incubated on ice for 20 min, then centrifuged at 13,000 x g for 20 min at 4°C. The supernatant was collected, and the concentration of the protein in the supernatant was determined using the Bradford Protein Assay kit. The whole cell lysates from the macrophages were subjected to 12% SDS-PAGE, and subsequently blotted onto a PVDF membrane. The membrane was incubated with the antibodies of interest.

GAPDH was used as an internal control. The quantitation of protein bands was performed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

RT-qPCR. Total RNA from the macrophages was extracted using an RNAiso Plus kit (Takara, Dalian, China) according to the manufacturer's instructions. The extracted total RNA was quantified by absorbance at 280 nm using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA in the total RNA was reverse transcribed to complementary DNA using a PrimeScript RT Reagents kit (Takara). qPCR was performed in the iCycler iQ5 (Bio-Rad) using SYBR Premix Ex Taq II (Takara) with the following conditions: 30 sec at 95°C, 40 cycles of 5 sec at 95°C, and 30 sec at 60°C. The mRNA expression levels, which were normalized against GAPDH, were calculated and expressed as $2^{-\Delta\Delta CT}$. The primers used for qPCR were as follows: PPAR γ : F 5'-CATTCTGGCCCACTTTGG-3', and R 5'-TGG AGATGCAGGCTCCACTTTG-3' (229 bp); TLR2: F 5'-AAG AGGAAGCCCAAGAAAGC-3', and R 5'-CAATGG GAATCCTGCTCACT-3' (80 bp); GAPDH: F 5'-ATGGGG AAGGTGAAGGTCG-3', and R 5'-GGGGTCATT GATGGCAACAA-3' (156 bp).

Cytokine assays. Following infection with M.tb H37Rv or *M. smegmatis*, or treatment with P19, the levels of the cytokines (IL-6 and TNF- α) from the supernatant of the macrophages were measured with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. All data in the present study were analyzed by the statistics package SPSS, version 13.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean \pm standard error. A direct comparison between two groups was conducted with the Student's non-paired t-test, and analysis of variance (ANOVA) with Tukey's post-hoc tests was used to compare the means of three or more groups. $P < 0.05$ was considered to represent a statistically significant difference.

Results

PPAR γ expression, p38 phosphorylation and cytokine production were upregulated following M.tb H37Rv infection or P19 treatment. To investigate the effect of P19 treatment on PPAR γ expression, p38 phosphorylation and cytokine production, human macrophages were incubated with 5 μ g/ml purified P19 for various time periods (from 0 to 48 h). M.tb H37Rv or *M. smegmatis* infection were used as controls. The expression of PPAR γ was significantly upregulated by M.tb H37Rv infection ($F_{(4,10)}=35.34$, $P < 0.0001$) and P19 stimulation ($F_{(4,10)}=19.24$, $P < 0.01$) (Fig. 1A). Post hoc analysis revealed that the expression levels of PPAR γ were upregulated at 48 h compared with the levels at 0 h, following M.tb infection (5.40 ± 0.40 -fold, $P < 0.0001$) or P19 treatment (4.10 ± 0.41 -fold, $P < 0.0001$). *M. smegmatis* infection did not significantly affect PPAR γ levels ($F_{(4,10)}=0.11$, $P=0.87$), as expected. The phosphorylation level of p38 was expressed as the ratio of phospho-p38 to total p38 (Fig. 1B). ANOVA revealed significant differences in levels of p38 phosphorylation following M.tb H37Rv infection ($F_{(4,10)}=35.34$, $P < 0.0001$) and following P19 treatment ($F_{(4,10)}=19.24$, $P < 0.01$).

Phospho-p38 levels were 3.11 ± 0.20 -fold ($P < 0.01$, M.tb H37Rv infection) and 2.50 ± 0.34 -fold ($P < 0.01$, P19 treatment) greater at 48 h than those at 0 h, whereas p38 phosphorylation was not significantly affected by *M. smegmatis* infection ($F_{(4,10)}=0.11$, $P=0.87$). The levels of IL-6 (Fig. 1C) and TNF- α (Fig. 1D) were also analyzed. Two-way ANOVA revealed significant effects of time ($F_{\text{time}(4,30)}=16.29$, $P < 0.0001$) and infection/treatment ($F_{\text{infection}(2,30)}=57.64$, $P < 0.0001$) on IL-6 production, as well as an interaction between them ($F_{\text{interaction}(8,30)}=4.46$, $P < 0.01$). Similarly, the levels of TNF- α were also significantly affected by time ($F_{\text{time}(4,30)}=25.92$, $P < 0.0001$) and infection/treatment ($F_{\text{infection}(2,30)}=3.94$, $P < 0.05$); however, no significant effect of time-infection interaction was detected ($F_{\text{interaction}(8,30)}=2.95$, $P=0.07$).

P19-induced p38 phosphorylation and IL-6 production is modulated by PPAR γ . Since M.tb H37Rv infection and P19 treatment upregulate the expression of PPAR γ , the involvement of PPAR γ in p38 phosphorylation and cytokine production induced by P19 in macrophages was investigated. As expected, the levels of p38 phosphorylation were significantly elevated following 24-h P19 treatment compared with those in the vehicle control ($P < 0.05$; Fig. 2A). Pretreatment with a specific PPAR γ agonist (BRL49653) or antagonist (GW-9662) did not significantly affect p38 phosphorylation compared with that in the vehicle control. P19 treatment combined with GW-9662 pretreatment significantly upregulated p38 phosphorylation ($P < 0.01$ vs. vehicle control). Additionally, the activation of PPAR γ by BRL49653 attenuated P19-induced upregulation of p38 phosphorylation ($P=0.21$).

Similarly, 24 h after P19 treatment, the expression levels of IL-6 were significantly elevated ($P < 0.0001$ vs. control; Fig. 2B). The expression levels of IL-6 were not significantly affected by treatment with the PPAR agonist or antagonist alone. However, when compared with P19 alone, treatment with the PPAR γ antagonist significantly upregulated the levels of IL-6 production ($P < 0.05$), while the PPAR γ agonist attenuated them ($P=0.72$).

P19-induced PPAR γ expression, p38 phosphorylation and cytokine production are dependent on TLR2. To confirm the involvement of the TLR2 in P19-induced PPAR γ expression and p38 phosphorylation, the expression of PPAR γ , p38, IL-6 and TNF- α were measured in TLR2 knockdown macrophages (Fig. 3A). Two-way ANOVA revealed a significant effect of TLR2 knockdown ($F_{\text{TLR2-siRNA}(1,8)}=70.4$, $P < 0.0001$) and P19 treatment ($F_{\text{P19}(1,8)}=23.3$, $P < 0.01$) on PPAR γ mRNA expression, as well as an interaction between them ($F_{\text{interaction}(1,8)}=21.8$, $P < 0.01$) (Fig. 3B). Tukey's post hoc tests indicated that the levels of PPAR γ mRNA were significantly reduced following TLR2 knockdown, regardless of whether P19 treatment is used or not ($P < 0.0001$ vs. control).

With regards to p38 phosphorylation, significant effects of TLR2 knockdown ($F_{\text{TLR2-siRNA}(1,8)}=7.81$, $P < 0.05$), P19 treatment ($F_{\text{P19}(1,8)}=14.8$, $P < 0.01$) and interaction factor ($F_{\text{interaction}(1,8)}=5.97$, $P < 0.05$) were also detected (Fig. 3C). As expected, the levels of phospho-p38 were increased following P19 treatment ($P < 0.01$ vs. control). In the absence of P19 treatment, no significant difference in the levels of p38 phosphorylation between the TLR2 knockdown

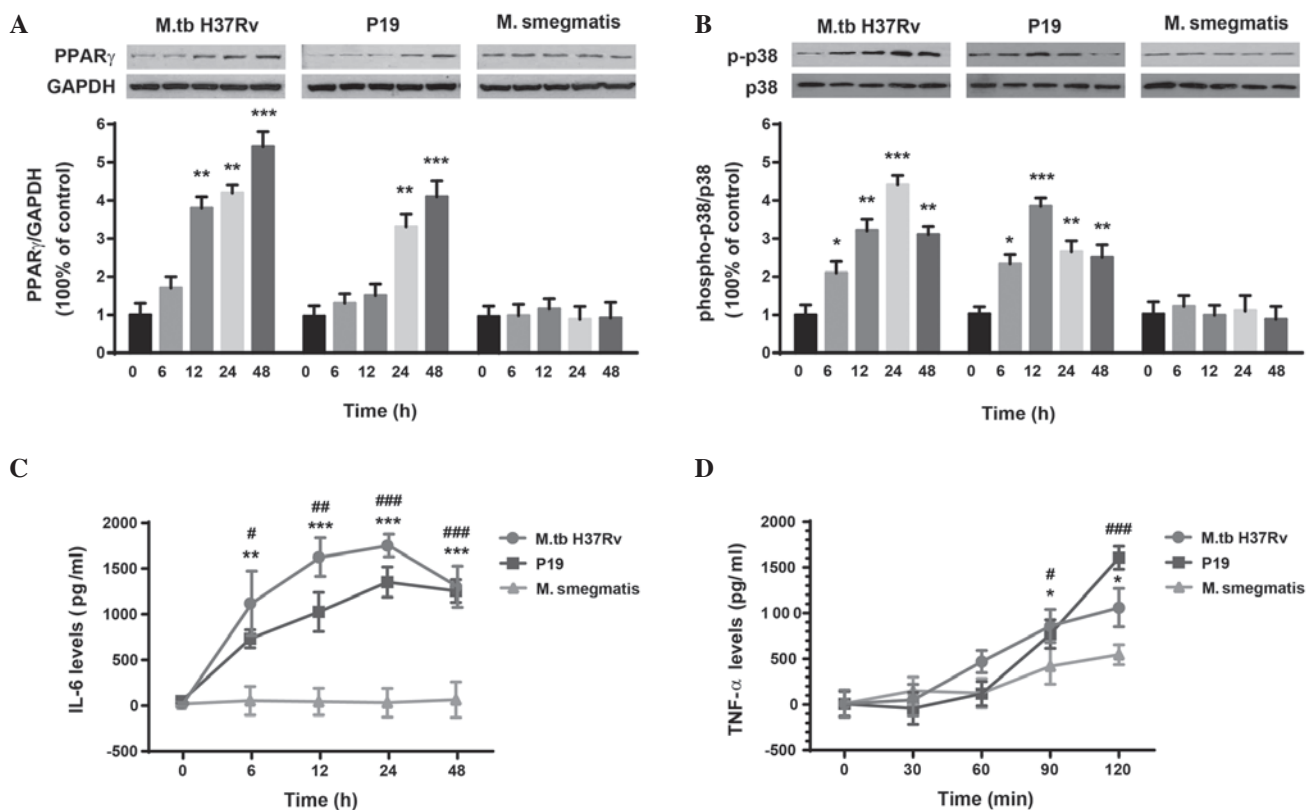


Figure 1 *M.tb* H37Rv and P19 upregulate the expression of (A) PPAR γ , (B) p38 phosphorylation, (C) IL-6 and (D) TNF- α in human macrophages. As a control, macrophages were also incubated with *M. smegmatis*. The phosphorylation state of p38 was expressed as the ratio of phospho-p38 to total p38. Data are expressed as the mean \pm standard error (n=3). For (A) and (B): *P<0.05, **P<0.01, ***P<0.0001 vs. 0 h within each group. For (C) and (D): *P<0.05, **P<0.01, ***P<0.0001, *M.tb* H37Rv infection vs. *M. smegmatis* infection at the same time point; #P<0.05, ##P<0.01, ###P<0.0001, P19 treatment vs. *M. smegmatis* infection at the same time point. PPAR, peroxisome proliferator-activated receptor; *M.tb*, *Mycobacterium tuberculosis*; P19, *M.tb* 19 kDa lipoprotein; *M. smegmatis*, *Mycobacterium smegmatis*; TNF, tumor necrosis factor.

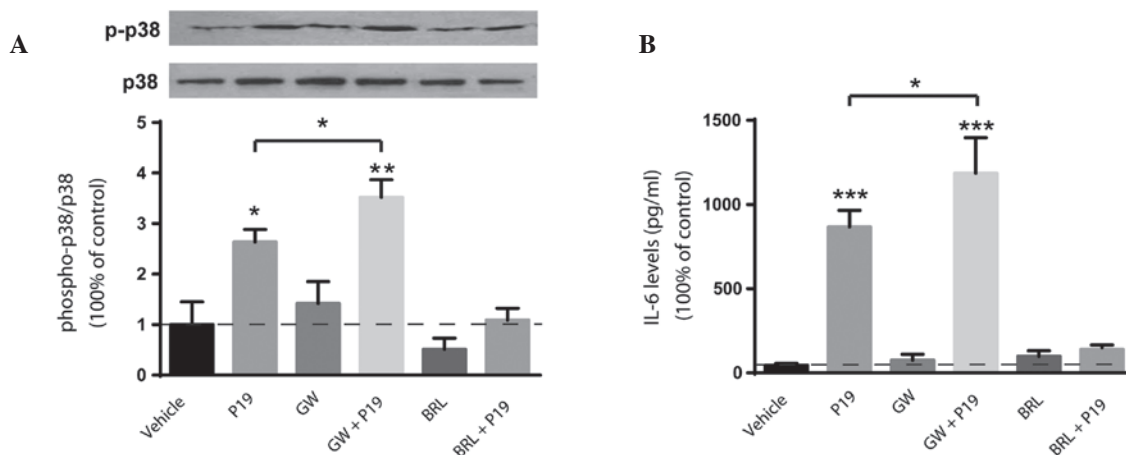


Figure 2 P19-induced p38 phosphorylation is modulated by PPAR γ . Macrophages were pretreated with the specific PPAR γ antagonist GW or agonist BRL alone, or in combination with P19 treatment. (A) The levels of P38 phosphorylation, expressed as the ratio of phospho-p38 to total p38. (B) The levels of IL-6. Data are expressed as the mean \pm standard error (n=3). *P<0.05, **P<0.01, ***P<0.0001 vs. vehicle control, unless otherwise indicated. P19, *M.tb* 19kDa lipoprotein; PPAR, peroxisome proliferator-activated receptor.

macrophages and negative siRNA-treated macrophages was observed (P=0.94). However, TLR2 knockdown combined with P19 treatment significantly decreased the levels of p38 phosphorylation (P<0.01), compared with levels following P19 treatment without TLR2 knockdown. The levels of IL-6

and TNF- α were also analyzed (Fig. 3D and E, Table I). The results demonstrated that TLR2 knockdown combined with P19 treatment significantly reduced the expression levels of both IL-6 and TNF- α compared with the levels following P19 treatment without TLR2 knockdown.

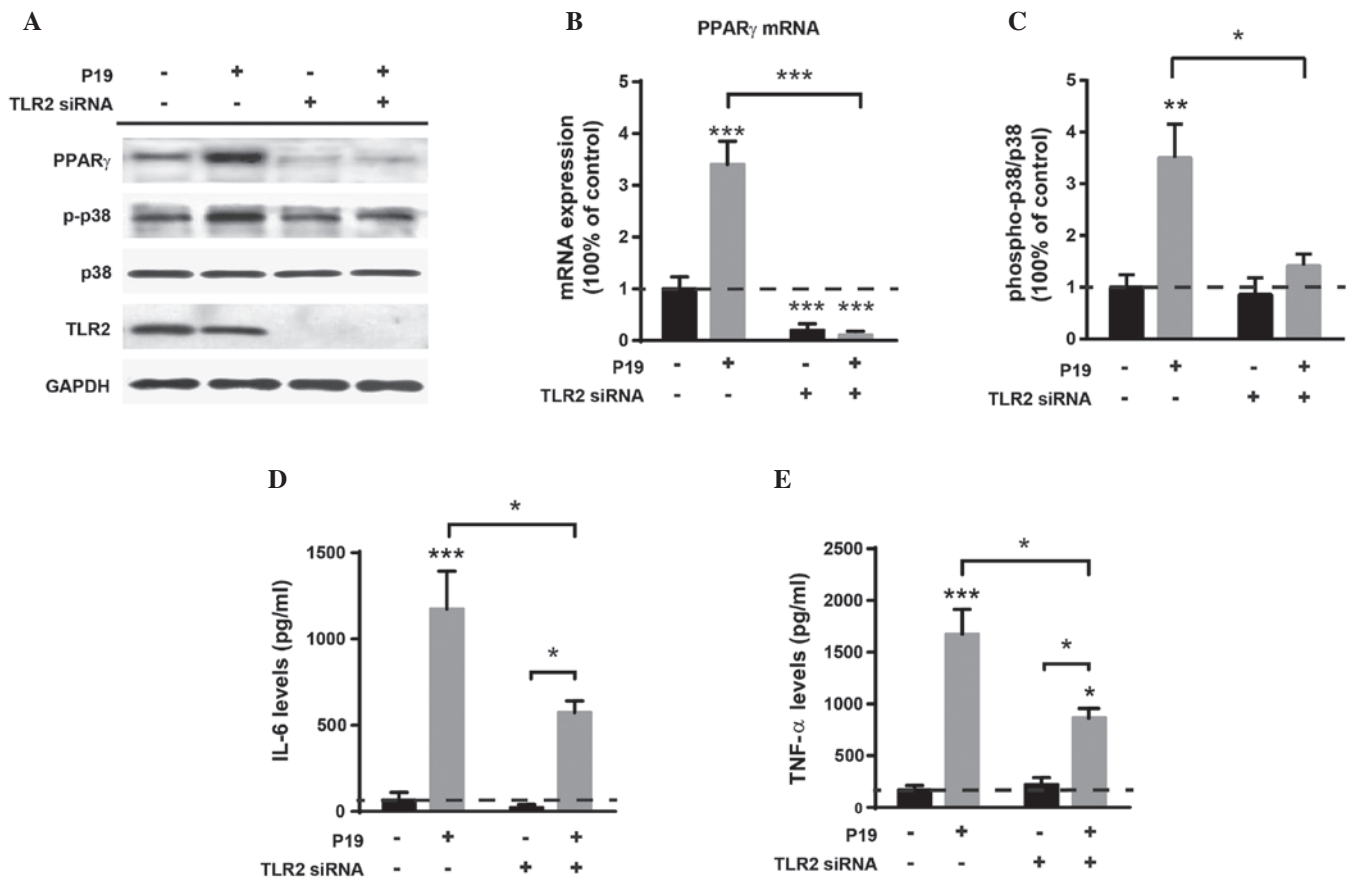


Figure 3. P19-induced PPAR γ expression, p38 phosphorylation and cytokine production in macrophages are dependent on TLR2. (A) A representative western blot of PPAR γ , phospho-p38 and total p38 expression. The expression levels of TLR2 following TLR2 knockdown by TLR2 siRNA were also determined. GAPDH was used as a loading control. (B) PPAR γ mRNA expression. (C) The levels of p38 phosphorylation, expressed as the ratio of phospho-p38 to total p38. (D) The levels of IL-6. (E) The levels of TNF- α . Data are expressed as the mean \pm standard error (n=3). *P<0.05, **P<0.01, ***P<0.0001 vs. the control unless otherwise indicated. TLR, Toll-like receptor; P19, M.tb 19kDa lipoprotein; PPAR, peroxisome proliferator-activated receptor; si, small interfering; TNF, tumor necrosis factor.

Table I. Statistical results for IL-6 and TNF- α production.

Factor	F _(1,8) value	P-value
IL-6 levels		
P19	49.80	<0.0001 ^c
TLR2-siRNA	7.48	0.0256 ^a
Interaction	5.62	0.0453 ^a
TNF- α levels		
P19	62.80	<0.0001 ^c
TLR2-siRNA	7.74	0.0239 ^a
Interaction	10.10	0.0131 ^a

Levels of IL-6 and TNF- α were analyzed using two-way ANOVA. ^aP<0.05; ^bP<0.01; ^cP<0.0001. TNF, tumor necrosis factor; TLR, Toll-like receptor; si, small interfering; ANOVA, analysis of variance.

Discussion

Modulation of the host immune response is essential in mycobacterial pathogenesis. M.tb enhances its survival in

macrophages by suppressing immune responses, in part through its complex cell wall structures. The purified mycobacterial cell wall lipoprotein P19 is well-defined as a TLR2 agonist (23). PPAR γ is a prime candidate for an intracellular molecular switch based on its central role in controlling the inflammatory response in macrophages (24), and although PPAR γ has been extensively investigated in other diseases (25), its immunoregulatory role in infectious diseases (particularly tuberculosis) is just beginning to be recognized (8,26,27). In the present study, the expression of PPAR γ in human macrophages was enhanced by M.tb H37Rv and P19, but not *M. smegmatis*. It was also observed that P19 strongly induces p38 phosphorylation and cytokine (IL-6 and TNF- α) production.

A previous study established that the activation of PPAR γ may repress target inflammatory genes, including proinflammatory cytokines and inducible nitric oxide synthase, through ligand-dependent transrepression of NF- κ B target genes (28). de Assis *et al* (29) demonstrated that the PPAR γ agonist BRL49653 potentiates lipid body biogenesis in peritoneal macrophages following oxidized phospholipid stimulation. A similar role for PPAR γ in lipid body biogenesis was reported in *M. bovis* BCG, but not *M. smegmatis* (8). In the present study, the effect of PPAR γ activation during P19 infection was analyzed. It was demonstrated that pretreatment with

the PPAR γ antagonist GW-9662 significantly upregulated P19-induced p38 phosphorylation and IL-6 production. However, pretreatment with the PPAR γ agonist BRL49653 or antagonist GW-9662 alone did not affect p38 phosphorylation compared with that of the vehicle control. These results indicate that PPAR γ may modulate P19-induced immune response through p38 phosphorylation.

Since TLRs and PPAR γ contribute to *M.tb*-induced immune responses and have been indicated to regulate host susceptibility to pathogens, TLR2 activation involvement in the regulation of PPAR γ expression, p38 phosphorylation and cytokine production in the presence of P19 treatment was investigated in the current study. Following TLR2 knockdown in macrophages, the expression of PPAR γ was significantly decreased in the presence or absence of P19 treatment. Furthermore, the levels of p38 phosphorylation and cytokine production were significantly reduced in TLR2 knockdown macrophages in the presence of P19 treatment compared with those in non-knockdown macrophages treated with P19. These results demonstrated that PPAR γ expression, p38 phosphorylation and cytokine production in human macrophages are associated with TLR2. Results of the present study are consistent with those of previous studies that observed *Mycobacterium bovis* BCG-induced PPAR γ expression, lipid body formation, and PGE $_2$ generation inhibition in TLR2-deficient mice (8).

In conclusion, the findings of the current study demonstrate that the *M.tb* cell wall component P19 induces PPAR γ expression in a TLR2-dependent manner. In the TLR2-dependent signaling pathway, PPAR γ acts as a key modulator of inflammation in P19-stimulated macrophages. These findings suggest a role for PPAR γ and TLR2 in P19-induced p38 phosphorylation and cytokine production, thereby potentially affecting the *M.tb* pathogenesis. Future studies in animal models are required to further characterize the role of PPAR γ and TLR2 in the pathogenesis of tuberculosis.

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