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**Mycobacterium bovis Bacillus Calmette-Guérin Infection Induces TLR2-Dependent Peroxisome Proliferator-Activated Receptor γ Expression and Activation: Functions in Inflammation, Lipid Metabolism, and Pathogenesis**


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Macrophages have important roles in both lipid metabolism and inflammation and are central to immunity to intracellular pathogens. Foam-like, lipid-laden macrophages are present during the course of mycobacterial infection and have recently been implicated in mycobacterial pathogenesis. In this study, we analyzed the molecular mechanisms underlying the formation of macrophage lipid bodies (lipid droplets) during Mycobacterium bovis bacillus Calmette-Guérin (BCG) infection, focusing on the role of the lipid-activated nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). We found that BCG infection induced increased expression of PPARγ that paralleled the augmented lipid body formation and PGE2 synthesis in mouse peritoneal macrophages. BCG-induced PPARγ expression and lipid body formation were diminished in macrophages from TLR2-deficient mice, suggesting a key role for TLR2. The function of PPARγ in modulating BCG infection was demonstrated by the capacity of the PPARγ agonist BRL49653 to potentiate lipid body formation and PGE2 production; furthermore, pretreatment with the PPARγ antagonist GW9662 inhibited BCG-induced lipid body formation and PGE2 production. BCG-induced MIP-1α, IL12p70, TNF-α, and IL6 production was not inhibited by GW9662 treatment. Nonpathogenic Mycobacterium smegmatis failed to induce PPARγ expression or lipid body formation. Moreover, inhibition of PPARγ by GW9662 enhanced the mycobacterial killing capacity of macrophages. Our findings show that PPARγ is involved in lipid body biogenesis, unravels a cross-talk between the innate immune receptor TLR2 and the lipid-activated nuclear receptor PPARγ that coordinates lipid metabolism and inflammation in BCG-infected macrophages, thereby potentially affecting mycobacterial pathogenesis.

The resurgence of tuberculosis worldwide has intensified research efforts to investigate host defense and elucidate the cellular and molecular mechanisms involved in Mycobacterium tuberculosis infection. M. tuberculosis is an intracellular pathogen that survives and replicates within cells of the host immune system, primarily macrophages. Differentiation of macrophages into foamy cells is a common pathological observation in tuberculous granulomas and pleuritis in both experimental and clinical settings (1–4). The foamy aspect of macrophages was shown to occur due to cytoplasmic lipid accumulation into lipid bodies (lipid droplets) (4–6). Recent studies have demonstrated that newly formed lipid bodies are structurally distinct cytoplasmic organelles involved in lipid mediator synthesis with immunomodulatory functions during bacillus Calmette-Guérin (BCG) infection (4, 6). Moreover, mycobacteria-induced lipid bodies often exhibiting intimate contact with bacteria-containing phagosomes. Significantly, mycobacteria-induced lipid body biogenesis and targeting may provide an escape mechanism during infection due to down-modulation of the macrophage response and/or acquisition of nutrients, leading to enhanced survival and replication in host cells (4, 6–11). However, the molecular mechanisms that regulate lipid body biogenesis during mycobacterial infection and their contribution to the pathophysiology of tuberculosis are not well understood.

Peroxisome proliferator-activated receptor (PPAR) γ is a member of the lipid-activated nuclear receptor family and has been demonstrated to function as a key transcriptional regulator of cell differentiation, inflammation, and lipid metabolism in macrophages and dendritic cells (for review, see Ref. 12). The PPAR γ transcription factor directly regulates the expression of several genes that coordinate lipid metabolism and inflammation (1, 6). The molecular mechanisms underlying the formation of macrophage lipid bodies (lipid droplets) during Mycobacterium bovis bacillus Calmette-Guérin (BCG) infection, focusing on the role of the lipid-activated nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). We found that BCG infection induced increased expression of PPARγ that paralleled the augmented lipid body formation and PGE2 synthesis in mouse peritoneal macrophages. BCG-induced PPARγ expression and lipid body formation were diminished in macrophages from TLR2-deficient mice, suggesting a key role for TLR2. The function of PPARγ in modulating BCG infection was demonstrated by the capacity of the PPARγ agonist BRL49653 to potentiate lipid body formation and PGE2 production; furthermore, pretreatment with the PPARγ antagonist GW9662 inhibited BCG-induced lipid body formation and PGE2 production. BCG-induced MIP-1α, IL12p70, TNF-α, and IL6 production was not inhibited by GW9662 treatment. Nonpathogenic Mycobacterium smegmatis failed to induce PPARγ expression or lipid body formation. Moreover, inhibition of PPARγ by GW9662 enhanced the mycobacterial killing capacity of macrophages. Our findings show that PPARγ is involved in lipid body biogenesis, unravels a cross-talk between the innate immune receptor TLR2 and the lipid-activated nuclear receptor PPARγ that coordinates lipid metabolism and inflammation in BCG-infected macrophages, thereby potentially affecting mycobacterial pathogenesis.
genes participating in fatty acid uptake, lipid storage, and inflammatory response by binding to specific DNA response elements in target genes as heterodimers with the retinoid X receptors (13–15). Indeed, PPARγ is highly expressed in macrophage-derived foam cells within atherosclerotic lesions where it plays an important role in lipid homeostasis and metabolism (16–19). PPARs are expressed by leukocytes including macrophages, dendritic cells, T cells, and B cells, where a role for these receptors in inflammation and immunoregulation has been proposed (20, 21).

Of major interest regarding the roles of PPARγ during pathogen infection, it has been demonstrated that PPARγ may repress target inflammatory genes, including proinflammatory cytokines and inducible NO synthase, through ligand-dependent transrepression of NF-κB target genes (22, 23). Although the role for PPARγ as a master regulator of lipid metabolism and inflammation has been described in different conditions, the involvement and relevance of PPARγ activation in the immune response and ability of macrophages to respond to intracellular pathogen infection has not yet been elucidated.

Considering the role of PPARγ in lipid metabolism, adipocyte and myeloid cell differentiation, and inflammatory control, we hypothesize that PPARγ is regulated and active in lipid body-enriched cells and PPARγ may regulate processes associated with lipid body formation in leukocytes during intracellular pathogen infection. In this study, we demonstrate that mycobacterial infection induces PPARγ expression and activation. Mycobacteria-induced PPARγ expression and activation is demonstrated to be centrally involved in regulating lipid metabolism in macrophages through the modulation of lipid body biogenesis and PGE2 production and to have effects on the host response to infection. Moreover, our results reveal novel interactions between the innate immune receptor TLR2 and the lipid-activated nuclear receptor PPARγ.

Materials and Methods

Animals

C57BL/6 mice were obtained from the Fundação Oswaldo Cruz breeding unit. TLR2 knockout (TLR2−/−) and TLR6 knockout (TLR6−/−) mice in a homozygous C57BL/6 background (24) were donated by Dr. S. Akira (Osaka University, Osaka, Japan). Animals were bred and maintained under standard conditions at the breeding unit of the Oswaldo Cruz Foundation, Brazil. Animals were caged with free access to food and water in a room at 22–24°C and a 12-h light/dark cycle in the Department of Physiology and Pharmacodynamics animal facility until they were used. Animals weighing between 20 and 25 g from both sexes were used. All protocols were approved by the Fundação Oswaldo Cruz Animal Welfare Committee.

Bacterial strains

Mycobacterium bovis BCG (Moreau strain) vaccine was obtained from the Fundação A'haadpho da Paiva (25). The freeze-dried vaccine was stored at 4°C and resuspended in RPMI 1640 medium just before use. Mycobacterium smegmatis (mc155) and zymosan were stored at −70°C and resuspended in RPMI 1640 just before use. GFP-M. bovis BCG was provided by M. A. O’Donnell (Department of Urology, University of Iowa, Iowa City, IA).

Macrophage culture and in vitro infection

Peritoneal cells from naive C57BL/6, TLR2−/−, or TLR6−/− mice were harvested with sterile RPMI 1640 cell culture medium. Peritoneal cells were allowed to adhere for 2 h at 37°C in a 5% CO2 atmosphere and were vigorously washed twice with PBS to remove nonadherent cells. Macrophages (1 × 106 cells/ml) were adhered to cover slides within culture plates (24 wells) overnight with RPMI 1640 cell culture medium containing 2% FCS. Macrophages were infected with BCG (multiplicity of infection [MOI], 1:1) or M. smegmatis (MOI, 1:1) or stimulated with TLR2 ligands zymosan (1:1) or Pam3Cys (10 μM) for 24 h at 37°C in a CO2 atmosphere. Alternatively, macrophages were treated for 30 min before infection with BRL49653 (5 μM) or GW9662 (1 μM) at 37°C and then infected with BCG (MOI, 1:1) for 1 h, followed by three PBS washes to remove noninternalized BCG. The vehicle (DMSO 0.01%) was used as the control. The cell-free supernatants were recovered and stored at −20°C. Cell viability was assessed by trypan blue exclusion at the end of each experiment and was always >90%.

Human macrophages were derived from monocytes obtained from platelet-freeuffy coats isolated from healthy donors by Ficoll-Hypaque (Pharmacia) gradient centrifugation and immunomagnetic cell separation using anti-CD14-conjugated microbeads (VarilMACS; Miltenyi Biotech) according to the manufacturer’s protocols. Purified monocytes were resuspended (1.5 × 106/ml) and differentiated in RPMI 1640 supplemented with 10% FCS, 500 U/ml penicillin-streptomycin (Life Technologies), and 2 mM 1-glutamine (Life Technologies). Human macrophages were infected with GFP-BCG (MOI, 1:1).

Lipid body staining and enumeration

Macrophages were fixed in 3.7% formaldehyde in Ca2+/Mg2+-free HBSS (pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained in 1.5% osmium tetroxide (30 min), rinsed in water, immersed in 1.0% thioracil-hydrazide (5 min), rinsed in water, rinsed in 0.1 M cacodylate buffer, re-incubated in 1.5% osmium tetroxide (3 min), rinsed in distilled water, dried, and mounted for further analysis. The morphology of fixed cells was observed and lipid bodies were enumerated by light microscopy with a ×100 objective lens for 50 consecutive macrophages in each slide.

PGE2 measurement

PGE2 levels were measured directly in the supernatant from culture cells obtained 2, 6, or 24 h after BCG injection. PGE2 was assayed in the cell-free supernatant by enzyme-linked immunosorbent assay according to the manufacturer’s instructions (Cayman Chemical).

Cytokine analysis

Supernatants from in vitro BCG-infected macrophages after 24 h of infection were collected and stored at −20°C until the day of analysis. Cytokines were analyzed simultaneously using LumineX technology on the Bio-Plex system (Bio-Rad). Fifty microliters of sample was analyzed using a mouse multiplex cytokine kit obtained and assayed according to the manufacturer’s instructions (Upstate Biotechnology). Data analyses were performed with the Bio-Plex Manager software.

Western blot analysis

Cell lysates were prepared in reducing and denaturing conditions and subjected to SDS-PAGE. Samples were submitted to electrophoresis in 10% acrylamide gradient SD-G-PAGE gels. After transfer onto nitrocellulose membranes, nonspecific binding sites were blocked with 5% nonfat milk in TBST (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20). Membranes were probed with the polyclonal Ab anti-PPARγ (H100; Santa Cruz Biotechnology) or anti-β-actin mAb (BD Transduction Laboratories) in TBST with 1% nonfat dry milk. Proteins of interest were then identified by incubating the membrane with HRP-conjugated secondary Abs in TBST, followed by the detection of Ag-Ab complexes by Supersignal Chemiluminescence (Pierce). For the densitometry analysis, the images from developed films were analyzed in the software Image 2D (GE Healthcare). The spotting and the analysis parameters were performed by a colleague blind to the identity of the sample.

Immunodetection of PPARγ in human and murine macrophages

Human macrophages obtained from monocytes, noninfected or infected with GFP-BCG (6 × 106 cells/group), were pelleted and fixed in 4% paraformaldehyde (pH 7.3) for 24 h at 4°C. Each cell block was then embedded in paraffin followed by sectioning and mounting on the same glass slide. After deparaffinization, rehydration, and Ag unmasking, immunofluorescence staining was performed by using a mAb to PPARγ (clone E8, 1/75 dilution; Santa Cruz Biotechnology). Briefly, PPARγ was detected by incubating sections for 1 h at room temperature with the primary Ab followed by HRP-labeled anti-mouse secondary IgG-Fab” treatment. The visualization was made with a tyramide-conjugated red fluorescent amplification kit using tetramethylrhodamine (TSA-TRM System; PerkinElmer Life Science). The nuclear counterstain was made with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). To ensure the staining specificities, negative controls were also included by using isotype-matched control IgG (DakoCytemation) in place of the primary Ab. Positive controls for PPARγ staining were made on normal human adult adipose tissue sections that exhibited nuclear staining in the majority of adipocytes. Fluorescence images were obtained using an Olympus BX51 microscope.
equipped with a narrowband tricolor excitation filter and DP71 digital camera. Fluorescent photomicrographs were captured with a single exposure, which simultaneously visualized both the green (the presence of GFP mycobacteria), the red (PPARγ/H9253 protein), and the blue (DAPI) fluorescent lights. For transferring and editing images for documentation, Viewfinder and Studio Lite software version 1.0.136 of 2001 Pixera (Digital Imaging Systems) and Adobe Photoshop version 8.0 were used.

For the immunolocalization of PPARγ in murine macrophages, cells were stimulated with lipoarabinomannan (LAM; 300 ng/ml). PPARγ was detected by incubating formalin (3.7%)-fixed macrophage-containing coverslips for 1 h at room temperature with the primary pAb to PPARγ (clone H100; Santa Cruz Biotechnology). After a vigorous wash, cells were incubated with anti-rabbit Alexa Fluor 546-labeled secondary Ab (Molecular Probes). Nonimmune rabbit serum was used as negative control (The Jackson Laboratory). The slides were analyzed by confocal laser-scanning microscopy on a Zeiss LSM 510-META. The nuclear counterstain was made with DAPI (Sigma-Aldrich).

Mycobacterial viability determined by flow cytometry
A live/dead staining protocol based on the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) was applied to study the viable vs nonviable BCG obtained from GW9662-treated or vehicle-treated macrophages. In brief, peritoneal macrophages (1 × 10^6/well) in a 24-well plate were pretreated with either GW9662 (1 μM) or vehicle for 30 min at 37°C, then infected with BCG (MOI, 1:1) for 1 h, followed by three PBS washes to remove any noninternalized BCG. Macrophages were then incubated for 12 h after infection in RPMI 1640 cell culture medium containing 2% FCS and reconstituted with GW9662 (1 μM) or vehicle. Macrophages were lysed with 0.1% saponin and bacterial-containing suspensions were incubated with a LIVE/DEAD BacLight Bacterial Viability Kit according to the manufacturer’s instructions. The percentages of live and dead bacteria were determined by flow cytometry as previously described (26). Flow cytometric measurements were performed on a FACSCalibur (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

Statistical analysis
The results are expressed as mean ± SEM and were analyzed statistically by means of variance followed by the Newman-Keuls-Student test or Student’s t test with the level of significance set at p < 0.05.

Results
M. bovis BCG infection induces PPARγ expression
Lipid-laden (foamy) macrophages are present in mycobacteria infection, but the molecular mechanisms underlying their formation are currently unknown. Considering the role of lipid-activated nuclear receptors in lipid metabolism, macrophage differentiation,
and inflammation control, we investigated the role of PPARγ in lipid body formation induced by BCG. Using an experimental model of mouse peritoneal macrophages infected by *M. bovis*, BCG, we investigated the effect of *Mycobacterium* infection on PPARγ expression by Western blot analysis. As shown in Fig. 1A, BCG infection induced a time-dependent increase of PPARγ protein expression in macrophages. Increased PPARγ protein content was observed within 2 h and was at its maximum within 24 h after

**FIGURE 2.** Effect of PPARγ agonist BRL49653 and PPARγ antagonist GW9662 on BCG-induced lipid body biogenesis and PGE2 production. Peritoneal macrophage were treated with vehicle, BRL49653 (5 μM), or GW9662 (1 μM) for 30 min before infection with BCG. Lipid body counting (A) and PGE2 production (B) in peritoneal macrophages infected with BCG (MOI, 0.05:1 bacterium:macrophage) treated with vehicle or BRL49653. Lipid body counting (C) and PGE2 production (D) in peritoneal macrophages infected with BCG (MOI, 1:1) treated with vehicle or GW9662. Each bar represents the mean ± SEM from three independent pools of 10 animals each. Differences between control and infected with treatment groups are indicated by asterisks (p < 0.05). +, Differences between BCG and BCG in the presence of BRL49653 or GW9662. E, Representative images of macrophages treated with vehicle or GW9662 followed by infection with BCG after osmium staining, as observed by light microscopy (original magnification, ×100).

**FIGURE 3.** Cytokine production during BCG infection is independent of PPARγ activation. Analysis of cytokine synthesis by macrophages infected by BCG (MOI, 1:1) pretreated with GW9662 (1 μM) or vehicle. Cytokine production was analyzed using Luminex technology at 24 h of infection. Each bar represents the mean ± SEM from three independent pools of 10 animals each. Differences between control and infected with treatment GW9662 groups are indicated by asterisks (p < 0.05).
BCG infection (Fig. 1A). The increased PPARγ protein expression and nuclear localization upon BCG infection was confirmed in human monocytes infected with fluorescent-labeled BCG (GFP-BCG; Fig. 1B).

Foam cells within atherosclerotic lesions contain high expression levels of PPARγ (17). The foamy-like phenotype is also observed in Mycobacterium-infected macrophages in vivo (1–3). Next, we evaluated whether the in vitro macrophage infection by BCG directly caused lipid body biogenesis. As seen in Fig. 1C, there were markedly increased numbers of lipid bodies in BCG-stimulated macrophages when compared with control. BCG-induced lipid body biogenesis paralleled the induction of PPARγ expression (Fig. 1C). Macrophage lipid bodies have been characterized as key compartmentalization environments for inflammatory mediator production (4, 27–29). We analyzed whether the BCG-induced increase in lipid body numbers is associated with PGE₂ production by macrophages in vitro. We observed that M. bovis BCG-induced PPARγ expression and lipid body biogenesis were accompanied by enhanced PGE₂ generation after the infection (Fig. 1D).

Ligands of PPARγ modulate BCG-induced lipid body biogenesis and PGE₂ generation

PPARγ is a lipid-activated transcription factor that has been intimately linked to lipid metabolism and storage in fat cells and foam cells. Since macrophage infection by BCG induced expression of PPARγ and its nuclear localization, we investigated whether PPARγ is involved in lipid body biogenesis within mycobacteria-infected macrophages. To investigate the functional role of PPARγ activation during M. bovis BCG infection, we used a specific agonist (BRL49653) or antagonist (GW9662) for the receptor. As shown in Fig. 2, BRL49653 (5 μM) potentiated lipid body formation (Fig. 2A) and PGE₂ production (Fig. 2B) induced by suboptimal concentrations of BCG (0.05:1, bacterium:macrophage) at 24 h in vitro. Conversely, pretreatment with the selective PPARγ antagonist GW9662 significantly inhibited lipid body biogenesis (Fig. 2, C and E) and PGE₂ production (Fig. 2D) induced by BCG (1:1, bacterium:macrophage) infection at 24 h in vitro, thus indicating a required role for PPARγ signaling activation in lipid body biogenesis and further prostanoid production during BCG infection.

Cytokine production during BCG infection is independent of PPARγ activation

Recent studies have shown that PPARγ activation can inhibit the NF-κB and MAPK pathways, two of the most important signaling pathways regulating proinflammatory responses triggered by TLR activation (23, 30). The effect of pretreatment with PPARγ antagonist GW9662 on BCG-induced MIP-1α, IL12p70, IL-6, and TNF-α production by macrophages was investigated during BCG infection. As shown in Fig. 3, BCG infection significantly

**FIGURE 4.** TLR2-dependent PPARγ expression in response to infection with BCG in peritoneal macrophages in vitro. A. Analysis of PPARγ expression by Western blot in peritoneal macrophages obtained from TLR2+/+ and TLR2−/− mice 24 h after infection with BCG (MOI, 1:1). Total macrophage cell lysates (4×10⁶ cells/lane) were separated by SDS-PAGE (10%) and subjected to Western blotting for PPARγ. B. Total macrophage cell lysates (4×10⁶ cells/lane) were separated by SDS-PAGE (10%) and subjected to Western blotting for PPARγ. The image is representative of at least two different blots. C. TLR2-dependent PPARγ expression and nuclear localization 24 h after LAM (300 ng/ml) stimulation assessed by confocal laser microscopy analysis. As opposed to nonstimulated cells predominantly showing the blue nuclear counterstain, there is an increase in the amount of PPARγ-specific red nuclear fluorescence after LAM stimulation. PPARγ-specific red nuclear fluorescence after LAM stimulation was diminished in TLR2−/−. D. Lipid body formation (C), PGE₂ synthesis (D), and TNF-α protein expression (E) were evaluated in macrophages from TLR2−/− and TLR2+/+ mice 24 h after infection in vitro with BCG (MOI, 1:1). Each bar represents the mean ± SEM from n = 3 pools of 10 animals in three independent experiments. Differences between control and infected groups are indicated by asterisks (p < 0.05). +, Differences between wild-type and deficient mice.
increased the synthesis of MIP-1α, IL12p70, IL-6, and TNF-α within 24 h. Pretreatment with GW9662 failed to modify BCG-induced cytokine production by macrophages (Fig. 3).

**Induction of PPARγ expression by BCG infection depends on TLR2 but not TLR6 activation**

It has been demonstrated that lipid body formation in leukocytes is a highly regulated event that depends on the interaction of cellular receptors with their ligands, and lipid bodies were shown to be involved in the production of inflammatory mediators and as markers of leukocyte activation (31). The role of TLR-mediated pattern recognition and activation in the mechanism of lipid body formation has been documented (4, 5, 28, 32). The role of TLR2 in regulating PPARγ expression during BCG infection in vitro was investigated. As shown in Fig. 4A, BCG infection failed to induce PPARγ expression in macrophages from TLR2−/− mice while it induced PPARγ expression in TLR2+/+ mice after 24 h of in vitro infection. The role of TLR2 in increased PPARγ protein expression and nuclear localization was confirmed in mouse macrophages stimulated with the BCG cell wall component LAM (Fig. 4B). As shown in Fig. 4B, LAM stimulation induced increased expression and nuclear localization of PPARγ. This effect was drastically reduced in TLR2−/− mice, demonstrating a key role for TLR2 signaling in PPARγ expression and activation. Confirming results obtained in vivo with BCG infection (4, 5), lipid body formation, PGE₂, and TNF-α generation in TLR2−/− mice macrophages were drastically inhibited when compared with macrophages from TLR2+/+ mice (Fig. 4, C–E). Stimulation of macrophages in vitro with *M. smegmatis* (1:1), zymosan (1:1), or Pam3Cys (10 μM) or vehicle. A. Total macrophage cell lysates (4 × 10⁶ cells/lanes) were separated by SDS-PAGE (10%) and subjected to Western blotting for PPARγ. The image is representative of at least two different blots. B–D. Each bar represents the mean ± SEM from at least three pools of 10 animals. Differences between control (Ctr) and infected groups are indicated by asterisks (p < 0.05).

**Inhibition of PPARγ by GW9662 leads to enhanced mycobacterial killing by macrophages**

Accumulating evidence has suggested that lipid body formation may favor intracellular mycobacterial survival and/or replication (4, 34–36). Since PPARγ activation was shown to be important in lipid body biogenesis in the course of BCG-induced infection, we asked whether PPARγ has a role in BCG pathogenesis. To gain insights into the role of PPARγ in macrophages on mycobacterial survival, we evaluated the effect of PPARγ inhibition by GW9662 on mycobacterial killing. As shown in Fig. 6, pretreatment with GW9662 (1 μM) significantly enhanced the capacity of macrophages to kill *M. bovis* BCG as assessed by live/dead bacterial staining by flow cytometry. This suggests that *M. bovis* BCG might utilize PPARγ signaling for survival.

### Table I. Lipid body formation and PGE₂ and TNF-α synthesis is independent of TLR6 signaling during BCG infection

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<th>Parameters</th>
<th>TLR6+/+ Vehicle</th>
<th>BCG</th>
<th>TLR6−/− Vehicle</th>
<th>BCG</th>
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<td>Lipid bodies (no./cell)</td>
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<td>3.5 ± 0.6</td>
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<td>PGE₂ (ng/ml)</td>
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<td>4.6 ± 0.1</td>
<td>107.8 ± 0.4²</td>
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<tr>
<td>TNF-α (ng/ml)</td>
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<td>1.1 ± 0.09</td>
<td>4.6 ± 0.2²</td>
<td></td>
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</tbody>
</table>

*Macrophages isolated from TLR6+/+ and TLR6−/− mice were stimulated in vitro with BCG or vehicle for 24 h. Results were expressed as means ± SEM from three pools of macrophages from five animals each.

*Value of p < 0.05 when vehicle and BCG-infected cells were compared.

*Value of p > (nonsignificant) when BCG-infected cells isolated from TLR6+/+ and TLR6−/− were compared.
we investigated the effect of a selective PPARγ antagonist, GW9662. We demonstrated that pretreatment with GW9662 significantly inhibited BCG-induced lipid body formation and PGE2 production, but not the production of TNF-α, IL-6, IL-12, and MIP-1, demonstrating the ability of PPARγ to differentially modulate the response of macrophages to infection.

The mechanisms involved in BCG-induced PPARγ expression were analyzed. Engagement of TLR proteins activates the expression of proinflammatory mediators by macrophages and has been shown to regulate host susceptibility to pathogens. Recent studies have demonstrated that bacterial components may regulate PPARγ expression and function. For instance, LPS, a component of the Gram-negative bacterial cell wall that acts through TLR4-dependent signaling as well as experimental sepsis, down-regulates PPARγ expression in macrophages and hepatic cells (43, 44). In contrast, TLR4 activation was shown to positively regulate the expression of PPARγ in epithelial cells (45). Different members of the TLR family, including TLR2, TLR4, and TLR9, as well as TLR6 and TLR1 when dimerized with TLR2, have been implicated in the host response to mycobacterial infection to mediate intracellular signaling in mycobacterial Ag recognition, cytokine production, and lipid metabolism (46–49). In particular, TLR2 appears to be critical for sensing mycobacteria and is classically recognized as a principal inducer of signals in mycobacterial infection (46). Lipid body biogenesis induced by Mycobacterium BCG was shown to be highly dependent on TLR2-dependent signaling (4–6). Thus, we asked whether TLR2 activation was involved in the regulation of PPARγ expression and/or activation during experimental infection by M. bovis, BCG. BCG-induced PPARγ expression, lipid body formation, and PGE2 and TNF-α generation were drastically inhibited in TLR2 deficient mice, demonstrating a requisite role for TLR2 in BCG-mediated macrophage up-regulation of PPARγ protein content. Interestingly, activation of macrophages in vitro with Mycobacterium smegmatis failed to induce PPARγ expression, lipid body formation, or PGE2 production, while still inducing TLR2-dependent TNF-α production. This finding suggests that TLR2 activation, although essential for mycobacteria-induced lipid body formation, is not sufficient to trigger pathways of lipid body formation and other cofactors may be involved. Indeed, cofactors for TLR activation to form lipid bodies have been described in LPS stimulation and Histoplasma capsulatum infection (27, 50). Accordingly, TLR2-dependent signaling may be modulated by the concomitant interaction with coreceptors and, depending on the coreceptor used, distinct downstream signaling pathways may be recruited, leading to differential cellular compartmentalization and responses (51–54). One such cofactor for TLR2 activation is TLR6. However, our findings demonstrate that TLR6 deficiency does not modify the ability of BCG to induce lipid body formation and inflammatory mediator production, indicating that TLR6 is not required for lipid body biogenesis. Additional studies will be necessary to characterize the accessory pathways for TLR2 signaling involved in lipid body biogenesis.

Our observation that nonpathogenic Mycobacterium smegmatis failed to trigger PPARγ expression in macrophages suggests that PPARγ may participate in the pathogenesis of infection. Strikingly, PPARγ inhibition in macrophages not only leads to decreased lipid body biogenesis, but also enhances the ability of macrophages to kill mycobacteria, supporting the hypothesis that PPARγ expression and activation may have implications in the pathogenesis of mycobacterial infection. Future studies in animal models as well as in Mycobacterium tuberculosis infection will be necessary to further characterize the role of PPARγ in the pathogenesis of tuberculosis and as targets for therapeutic intervention.

FIGURE 6. Pretreatment of macrophage with GW9662 enhances mycobacterial killing. Viable vs nonviable BCG obtained from GW9662-treated or vehicle-treated macrophages were evaluated by a LIVE/DEAD BacLight Bacterial Viability Kit. The percentages of live and dead bacteria were determined by flow cytometry 12 h after infection. Differences between treated and untreated groups are indicated by asterisks (p < 0.05), n = 8.

Discussion

Lipid bodies are still enigmatic cytoplasmic organelles, which is believed to play major roles in the physiological and pathological process (31, 37). It has been increasingly recognized that lipid bodies are involved in different aspects of innate immunity to infection (6, 38). Lipid bodies were demonstrated to act as platforms for enhanced PGE2 synthesis during BCG infection and to constitute nutrient-rich sources for mycobacterial growth, thus suggesting that lipid bodies and lipid body-derived PGE2 might have implications for the pathogenesis of mycobacterial infection (4–6). However, the molecular mechanism by which mycobacterial infection is associated with lipid synthesis and lipid accumulation in lipid bodies is unknown. Our study offers the first evidence that Mycobacterium bovis BCG is able to increase macrophage lipid accumulation and PGE2 formation through the increased expression and activity of PPARγ. Moreover, we uncovered novel connections between TLR signaling activation and PPARγ expression and activation, which adds support to the growing body of evidence that places PPARγ as a key component in inflammation and innate immunity.

We demonstrated that BCG infection in macrophages led to increased expression of PPARγ in mouse macrophages over a 24-h period. PPARγ expression is apparent within 2 h and reaches maximal levels within 24 h after the infection. Induction of PPARγ expression by BCG was also confirmed in human monocytes via an immunofluorescent analysis, which found increased nuclear PPARγ immunoreactivity. BCG-induced macrophage PPARγ expression was accompanied by enhanced lipid body biogenesis and increased PGE2 production by the infected cells, prompting us to investigate whether PPARγ activation was involved in the mechanisms of lipid body biogenesis.

To this end, we analyzed the effect of PPARγ activation during BCG infection. We observed that the PPARγ agonist BRL49653 potentiated lipid body formation and PGE2 production induced by a suboptimal dose of BCG. Accordingly, PPARγ activation regulates the accumulation of lipids and the expression of several genes involved with lipid metabolism and accumulation in macrophages (16, 17, 19), including ADRP, which is a protein associated with the surface of lipid bodies in numerous cells including macrophages and is believed to play a major role in the maintenance of lipid stores (39–41). Indeed, de Assis et al. (42) have demonstrated that the PPARγ agonists BRL49653 and hexadecil azeloyl phosphatidylcholine potentiate lipid body biogenesis in peritoneal macrophages after oxidized phospholipid stimulation. To confirm the involvement of PPARγ in BCG-induced lipid body formation,
which confers to PPARγ the ability to polarize monocyte macrophage differentiation toward an alternative anti-inflammatory phenotype with implications for the ability of macrophages to kill Leishmania parasites (55, 56).

In conclusion, our findings demonstrate that mycobacterial infection induces PPAR expression in a highly regulated manner that is dependent on TLR2 signaling. Moreover, PPARγ acts in a TLR2-dependent signaling pathway as a key modulator of lipid metabolism and inflammation in BCG-infected macrophages. These findings suggest a role for PPAR in mycobacteria-induced lipid body formation and PGE2 production, thereby potentially affecting mycobacterial pathogenesis.

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Disclosures
The authors have no financial conflict of interest.

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