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Mycobacterium tuberculosis Modulates Macrophage Lipid-Sensing Nuclear Receptors PPARγ and TR4 for Survival

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Mycobacterium tuberculosis–macrophage interactions are key to pathogenesis and clearance of these bacteria. Although interactions between M. tuberculosis–associated lipids and TLRs, non-TLRs, and opsonic receptors have been investigated, interactions of these lipids and infected macrophage lipid repertoire with lipid-sensing nuclear receptors expressed in macrophages have not been addressed. In this study, we report that M. tuberculosis–macrophage lipids can interact with host peroxisome proliferator-activated receptor γ and testicular receptor 4 to ensure survival of the pathogen by modulating macrophage function. These two lipid-sensing nuclear receptors create a foamy niche within macrophage by modulating oxidized low-density lipoprotein receptor CD36, phagolysosomal maturation block by induction of IL-10, and a blunted innate response by alternative polarization of the host and pathogen. Relative mRNA expression levels of these receptors in PBMCs derived from clinical samples convincingly implicate them in tuberculosis susceptibility. These observations expose a novel paradigm in the pathogenesis of M. tuberculosis amenable for pharmacological modulation. The Journal of Immunology, 2012, 188: 5593–5603.

M. tuberculosis is the etiologic agent of tuberculosis in humans and is responsible for more morbidity than any other bacterial disease (Global Tuberculosis Control, World Health Organization report 2010). The resurrection of tuberculosis globally, despite the use of bacillus Calmette-Guérin (BCG) vaccine and the introduction of directly observed therapy in many high-burden regions, has led to increased efforts to understand the cellular mechanisms of M. tuberculosis–macrophage interactions. In this study, we investigate host–pathogen interactions, particularly those between host lipid-sensing nuclear receptors (LSNRs) and pathogen lipids, that contribute to M. tuberculosis pathogenesis or clearance. Our approach is to look beyond NF-κB to other nuclear receptors that have recently been reported to be expressed and function in macrophages and other immune cells (1, 2). We also look beyond the interactions of M. tuberculosis–associated lipids with membrane receptors, such as TLRs, non-TLRs, and opsonic receptors (3, 4), to the lipids’ ability to modulate host LSNRs for macrophage survival (via apoptosis or autophagy) (17, 30).

M. tuberculosis, a facultative intracellular pathogen, primarily targets macrophages, cells of the innate immune response system. The successful survival of M. tuberculosis inside macrophages rests upon its ability to usurp its host’s innate defense pathways, including production of reactive oxygen species (ROS) and/or reactive nitrogen species, phagolysosome biogenesis, and modulation of macrophage survival (via apoptosis or autophagy) (17, 30). This work was supported by Department of Biotechnology-India project BT/01/IYBA/2009.

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18). Moreover, *M. tuberculosis* stimulates the differentiation of macrophages into foam cells, which are characterized by lipid body biogenesis (19, 20). These lipids provide nutrients to *M. tuberculosis*, leading to an enhanced ability to survive and replicate in host cells. This pathogen-induced manipulation of the host machinery is responsible for the survival of *Mycobacterium* inside the macrophage. However, the underlying molecular mechanisms are not well understood.

We investigated *M. tuberculosis* survival within macrophages by looking at LSNR loss of function in a bacterial survival assay. Several of the LSNRs tested showed significant differences in *M. tuberculosis* survival. Although knockdown of PPARγ and TR4 seems to support *M. tuberculosis* clearance, LXRx knockdown leads to increased *M. tuberculosis* burden. The mechanism of these responding transcription factors in orchestrating pathogen survival and clearance was investigated. PPARγ and TR4 synergistically contribute to *M. tuberculosis*-induced lipid biogenesis by increased expression of CD36 and modulation of foam cell formation by priming macrophages to a less microbicidal alternative phenotype. This study is the first report, to our knowledge, of cross talk between host LSNRs (PPARγ and TR4) and *M. tuberculosis* lipids and the total lipid homeostasis repertoire generated during *M. tuberculosis* infection of macrophages.

**Materials and Methods**

**Cells, small interfering RNA-mediated silencing, stable cell lines, and GFP-H37Ra**

Human macrophage cell line THP1 was grown in RPMI 1640 (Life Technologies Laboratories) supplemented with 10% FBS (HyClone). For transient silencing, THP1 cells are transfected with 60 nM scrambled control small interfering RNA or a pool of two gene-specific small interfering RNA (30 nM each) targeting two different regions of an mRNA. For stable cell lines, cells were infected with short hairpin RNA (shRNA) lentiviral particles containing three to five expression constructs encoding target-specific nucleotide shRNA as per the manufacturer’s protocol (Santa Cruz Biotechnology). The stable clones expressing the shRNA underwent puromycin dihydrochloride selection. Before experiments, THP1 and the selected clones were differentiated with 30 ng/ml in the presence of 30 nM each targeting two different regions of an mRNA. For stable cell clones were differentiated with 30 ng/ml PMA for 10 h followed by a 24-h resting period. pSC301 GFP vector capable of expressing in *Mycobacterium* sp. and *Escherichia coli* was kindly provided by Dr. Yossef Av-Gay (University of British Columbia, Vancouver, BC, Canada). GFP-H37Ra was made by electroporation and selection as described previously (21).

**Mycobacterial viability determination by flow cytometry and CFU assay**

THP1 or the knockdown cell lines for PPARγ, TR4, and LXRα were infected with H37Ra or H37Rv (multiplicity of infection 1:1) for 4 h, washed three times with medium to remove any unphagocytosed bacteria, and incubated with repletion medium for 24 h. Macrophages were then solubilized with 0.06% SDS, and bacterial suspensions were used with the Live/Dead BacLight Bacterial Viability and Counting kit as per the manufacturer’s instructions (Invitrogen). The percentages of live and dead bacteria were determined by flow cytometry (BD FACSCalibur; BD Biosciences) after staining with SYTO 9 and propidium iodide (PI). For CFU determination, after macrophage solubilization, the bacterial suspensions were serially diluted, 50 μl each sample was plated, and CFUs were counted. Final calculations included the dilution factor and the volume of diluted sample used for plating.

**Confocal microscopy**

THP1 macrophages were seeded onto 16-mm diameter glass coverslips precoated with poly-l-lysine in 12-well tissue culture plates at a density of 0.4 × 10^5 cells per coverslip and infected with GFP-H37Ra and GFP-H37Rv as detailed above. Cells were then incubated with 100 nM LysoTracker (Invitrogen/Molecular Probes) for 30 min at 37°C and fixed with 4% paraformaldehyde. The coverslips were washed thoroughly with PBS and mounted on slides with antifade. The stained cells were observed with an LSM 510 Meta Carl Zeiss confocal microscope (Carl Zeiss) and A1R-A1 Nikon microscope (Nikon). The colocalization of LysoTracker and GFP-H37Ra was quantified by selecting a region of interest and then determining the overlap coefficient as described previously by Manders et al. (22).

**Cell cytometry**

Analysis of cell-surface marker expression was performed by surface staining of cells at a density of 10^6 for 30 min at 4°C by using appropriate isotype controls and PE-conjugated anti-CD-206 (BD Biosciences). Samples were analyzed on an FACSCalibur using CellQuest software (BD Biosciences).

**Lipid body staining and enumeration**

Cells and infection were performed as above. The cells were fixed with 4% paraformaldehyde for 5 min, stained with 0.5% Oil red O for 30 min at room temperature, and counterstained with hematoxylin to stain nuclei. Cells were rinsed with PBS, mounted on glass slides, and imaged. Lipid bodies were enumerated by light microscopy with a ×100 objective lens for 50 consecutive macrophages on each slide.

**Free radicals and biochemical assays**

**Griess assay.** For NO measurement, postinfecion of macrophages with *M. tuberculosis*, the supernatant was collected, centrifuged, mixed in a 1:1 ratio with Griess reagent (100 μl; 0.5% sulphanilamide and 0.05% naphthylethlenediamine-HCl in 1.25% H3PO4), and incubated for 15 min at room temperature. The absorbance was recorded at 540 nm. Nitrite concentrations in the supernatant were calculated relative to standard concentrations of NaNO2 dissolved in medium or PBS.

**Arginine assay.** A 0.1 M glycine buffer with 10 mM MnCl2 was added to cell lysate and incubated at 37°C for 10 min. An additional 0.25 M arginine was added, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding a mixture of sulfuric acid (96%), phosphoric acid (85%), and water (at a 1:3:7 ratio) and mixed well. A 4% isonitrosopropiophenone solution was then added, and the mixture was incubated at 95°C for 1 h. The solution was cooled to room temperature (23), and the absorbance at 540 nm was recorded.

**DHR123 assay.** For total reactive ROS measurement, the cells were incubated with 10 μM DHR123 probe for 30 min at 37°C. The cells were harvested and subjected to flow cytometry to determine the levels of ROS.

**Cytokine analysis**

Supernatants were harvested at 24 h postinfection. The levels of immunoreactive inflammatory cytokines (IL-6, IL-12p70, IL-10, and TNF-α) were determined by ELISA using a commercial kit (BD Biosciences) in accordance with the manufacturer’s instructions.

**Cell viability and apoptosis/necrosis assay**

**MTT assay.** Macrophages were cultured in a 24-well dish. A 20-μl aliquot of MTT (5 mg/ml in PBS) was added to the wells and incubated for 4 h at 37°C. The medium was removed carefully, 200 μl DMSO was added and mixed, and the absorbance was read at 540 nm.

**Staining with Annexin V and PI**

Cells were stained with the Annexin V-FITC Apoptosis Detection Kit (Calbiochem) in Annexin binding buffer according to the manufacturer’s instructions. Cells were analyzed with a BD FACSCalibur flow cytometer (BD Biosciences).

**Quantitative RT-PCR**

RNA was extracted using a commercially available RNeasy kit according to the manufacturer’s instructions after harvesting the cells in 1 ml TRIzol reagent (Invitrogen). The quantitative real time PCR was performed in 96-multitwell plates with a quantitative real time PCR kit (Invitrogen). PCR was performed in an iCycler iQ real-time detection system (Bio-Rad), and the PCR baseline-subtracted data were computer generated as described by the manufacturer (Bio-Rad). β-actin mRNA was used as a reference housekeeping gene for normalization.

**Lipid extraction**

Mycobacterial cell suspensions were autoclaved and harvested by centrifugation. The centrifuged cells were washed and freeze dried. The freeze-dried cell mass was extracted using chloroform/methanol (2,1, v/v) at 50°C. Dried extracts were dissolved in a mixture of chloroform/methanol/water (8:4:2, v/v/v); the contents of the lower, organic phase were recovered and dried to yield the washed total lipid extract (23). THP1 macrophages alone or with infection (multiplicity of infection 1:1) were centrifuged. They were serially diluted, 50 μl aliquots were collected, centrifuged, mixed in a 1:2 ratio with Griess reagent (100 μl; 0.5% sulphanilamide and 0.05% naphthylethlenediamine-HCl in 1.25% H3PO4), and incubated for 15 min at room temperature. The absorbance was recorded at 540 nm. Nitrite concentrations in the supernatant were calculated relative to standard concentrations of NaNO2 dissolved in medium or PBS.
were lysed and extracted using methanol/chloroform (2:1). A homogenous single phase was obtained; the pellets from this phase consist mostly of proteins and were discarded, and the supernatant was dissolved in citric acid (50 mM)/water/chloroform (1:2:1). The lower, organic phase was recovered and dried to yield the total lipids. The total lipids were solubilized in DMSO at 1 μg/ml or in chloroform-methanol for further analysis. Total lipids were further verified by TLC. Polar and apolar lipids were extracted by solvent partitioning.

**Cells, transfections, and reporter assays**

COS-1/THP1 macrophages were plated in 24-well dishes and allowed to grow to 70% confluency in appropriate medium. Cells were transiently transfected with a total of 500 ng each expression plasmid using Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer’s instructions. A dual luciferase expression system (Promega) PG5 GAL4 reporter and CD36 promoter reporter was used. Firefly luciferase was used as the reporter, and Renilla luciferase expression was used to monitor transfection efficiency and for normalization. Normalized luciferase activities (relative light units) were plotted as the average (± SD) of triplicate samples from typical experiments (SigmaPlot).

**Plasmids and expression**

The cDNA clone hTR4 (a generous gift from Prof. James Douglas Engel, University of Michigan, Ann Arbor, MI) was subcloned by PCR into pFLAG (Sigma-Aldrich) with EcoRI and XbaI and into pBIND with KpnI and XbaI. hPPARγ (Addgene) was subcloned into pFLAG with Clal and KpnI and into pBIND with Sall and XbaI. hLXRα was subcloned into pFLAG with HindIII and XbaI and into pBIND with BamHI and XbaI. All constructs were verified by sequencing, transfected into COS1 cells, and analyzed by Western blot with anti-FLAG (Sigma-Aldrich) and nuclear receptor-specific Abs. The CD36 promoter containing PPARγ and TR4 response element, with the sequence 5′-AAAGTCAGGGCCA-3′, was cloned into the pGL3 basic vector (Promega) by PCR-based cloning using a forward primer with an MluI restriction site and a reverse primer with a BglII site. This CD36 promoter (300 bp), containing PPARγ and TR4 response element linked to a luciferase reporter in pGL3, was tested for reporter activity assay. The pBIND and pG5 vectors were kindly provided by Dr. Shailesh Surapureddi (National Institute of Environmental Health Sciences, Research Triangle Park, NC).

**Generation of human macrophages**

Human PBMCs were derived from fresh blood isolated from healthy donors by Ficoll-Hypaque (Sigma-Aldrich) density centrifugation. Cells were rested for 3 h at 37°C, followed by vigorous washing with PBS to remove the nonadherent cells. Surface staining was performed on adherent cells, and they were found to be 95% monocytes. Monocyte-derived macrophages were generated by culturing monocytes in the presence of M-CSF (50 ng/ml) for 7 d. These human macrophages were used for M. tuberculosis survival assay.

**Ethics statement (human PBMC isolation)**

The project was approved by the ethics committee of the Government Medical College and Hospital (GMCH), Sector 32, Chandigarh, India (GMCH/TA-1-19/2011/Agenda No. 2) and ethics and biosafety committee of the Institute of Microbial Technology (IMTECH), Sector 39A, Chandigarh, India (01/2011/IMT/IEC-Blood; 12/2010/IMT/IBSC). It was approved to Dr. Pawan Gupta (IMTECH) with collaborators Dr. Javed N. Agrewala (IMTECH) and Dr. Ashok Kumar Janmeja (Pulmonary Medicine, GMCH). The study was conducted strictly in accordance with the ethical guidelines for biomedical research on human subjects by Central Ethics Committee on Human Research, Indian Council of Medical Research-2000 and those as contained in the Declaration of Helsinki. Each subject was provided with written information about the study under the title Informed consent to patient and written consent on the patient consent form was obtained from each patient prior to his/her induction in the study. Information to patient and consent forms were in languages (English, Hindi, and Panjabi) familiar to the patients. Human PBMCs were derived from fresh blood drawn from donors (healthy individual, close contact, and tuberculosis patients) by Ficoll-Hypaque gradient centrifugation (Sigma-Aldrich).

**Statistics**

The results are expressed as mean ± SD unless otherwise mentioned. SigmaPlot and SPSS were used for statistical analysis. The efficiency of PCR amplification for each gene was calculated with the standard curve method \(E = 10^{-\frac{1}{N(2)} \text{cycle slope}}\). Relative mRNA abundance among all genes considered in the study was calculated as reported earlier (24) using the inverse of PCR efficiency raised to the power Δ cycle threshold (Ct) (gene abundance = \(1/E^{\Delta Ct}\)), where \(\Delta C = C_t - C_r\) reference and calculated as: \(\{[1/E^{\Delta C}]\text{gene of interest/sum (1/E^{\Delta C}) of all genes measured}\}\). The relative expression of each target mRNA in PBMCs derived from close contact and tuberculosis patients was calculated by the comparative 2^(-ΔΔCt) relative to healthy individual.

**Results**

**M. tuberculosis survival assay**

To test for involvement of LSNRs in the possible modulation of survival and clearance of M. tuberculosis in macrophages, we performed loss-of-function studies (Fig. 1A). A set of LSNRs, including PPARγ, TR4, vitamin D receptor, and LXRs have been shown to be expressed in human as well as murine macrophages (1, 2); we used RNA interference (RNAi) to knock down expression of these proteins in human THP1 monocyte/macrophage cells (Supplemental Fig. 1). We then infected the cells with M. tuberculosis strain H37Ra and looked at the intracellular M. tuberculosis load from cell lysates, by performing CFU assay. The uptake of bacteria remains nearly the same in all knockdowns as assessed by determining the CFU immediately postinfection.

There was a 6-fold decrease in phagocytosed bacteria within 24 h of infection and as such was found to be adequate time point for the study. Although RNAi knockdown of PPARγ significantly reduced intracellular H37Ra load compared with the control (2.5-fold), knockdown of TR4 marginally reduced bacterial load (1.4-fold). Conversely, knockdown of vitamin D receptor and LXRs enhanced M. tuberculosis load by 2-fold and 5-fold, respectively. Knockdown of other tested LSNRs did not significantly change M. tuberculosis survival.

Though transient knockdown with RNAi was efficient (~95%), stable knockdown cell lines were generated to further rule out any artifacts arising from incomplete silencing. The loss of expression of PPARγ, TR4, and LXRs in these stable cell lines was determined (Supplemental Fig. 2). To assay M. tuberculosis viability in the stable cell lines, PPARγ-, TR4-, or LXRs-knockdown THP1 macrophages were infected with H37Ra. The intracellular M. tuberculosis was later isolated and stained with SYTO 9, a green fluorescent nucleic acid stain that stains both live and dead cells, and PI, a red fluorescent nucleic acid stain that penetrates only bacteria with damaged membranes. The bacteria were evaluated by flow cytometry, and the percentages of dead bacteria were calculated (Fig. 1B). The results from this assay complemented the CFU data but were short of perfect corroboration, perhaps due to experimental sensitivities. The percentage of dead H37Ra was increased in the stable PPARγ- and TR4-knockdown cell lines, whereas the percentage of dead bacteria was significantly reduced in LXRs-knockdown cells.

To ensure survival within its niche macrophage, M. tuberculosis is known to manipulate phagolysosomal maturation, an important host innate immune response to intracellular infection. We therefore quantified M. tuberculosis survival at the level of phagolysosomal maturation in stable knockdown THP1 macrophages and quantified the observation. Clearly, PPARγ- and TR4-silenced macrophages showed more efficient phagolysosomal fusion compared with control macrophages (Fig. 1C, Supplemental Fig. 3). LXRs knockdown, in contrast, significantly reduced phagolysosomal maturation.

Together, the CFU data, cell viability data, and imaging data in transient and stable knockdown backgrounds suggest that M. tuberculosis might use PPARγ and TR4 for its survival, whereas its host might use LXRs to control the M. tuberculosis infection.
PPARγ and TR4 contribute to H37Ra-induced foamy macrophages

Lipid-laden (foamy) macrophages are present in M. tuberculosis infections, but the mechanistic details underlying their formation have not been adequately investigated. Because many lipid-activated nuclear receptors have been shown to modulate lipid metabolism, we investigated their functional role in foam cell formation during infection with H37Ra (Fig. 2A). An increased number of lipid bodies characteristic to the foamy-like phenotype was observed upon macrophage infection with H37Ra. The number of lipid bodies was significantly reduced in PPARγ- and TR4-silenced macrophages. Furthermore, H37Ra was able to induce significantly higher lipidogenesis in LXRα-silenced macrophages.

We also investigated the expression of CD36 and scavenger receptor A (SRA), which account for most of the oxidized low-density lipoprotein (oxLDL) uptake leading to foamy macrophage formation. In addition, we looked at the expression of cholesterol efflux transporters like ATP-binding cassette transporters ABCA1 and ABCG1, which abrogate the foamy phenotype (Fig. 2B). H37Ra infection significantly induced CD36, which was abrogated in PPARγ- or TR4-silenced macrophages but augmented in LXRα knockdown. H37Ra also, to a lesser extent, induced SRA expression in the control cell line, which was abrogated only in TR4-silenced macrophages. Interestingly, H37Ra infection reduced the basal expression of ABCA1 and ABCG1 in the control cell line; ABCG1 expression was further reduced in the LXRα knockdown, but not in the PPARγ and TR4 knockdowns. The relative mRNA abundance of these genes confirms that H37Ra leads to foamy biogenesis by upregulating oxLDL receptors (CD36 and SRA) and downregulating cholesterol efflux transporters (ABCA1 and ABCG1) (Fig. 2C). Although PPARγ and TR4 largely mediate H37Ra upregulation of CD36, LXRα antagonizes this effect along with H37Ra downregulation of ABCG1, so these LSNRs differentially contribute to H37Ra modulation of foamy macrophages.

We also investigated H37Ra modulation of a CD36 promoter reporter in control, PPARγ-, and TR4-silenced macrophages (Fig. 2D). Although H37Ra infection caused significant activation of the CD36 promoter reporter as monitored by luciferase activity, CD36 promoter activity was significantly reduced in the PPARγ- or TR4-silenced background. A complete loss of activity was observed in the double knockdown of PPARγ and TR4, confirming synergism between these two adopted orphan receptors.

Heterogeneous macrophage polarization and cytokine repertoire during mycobacterial infection

Because heterogeneous and alternative activation of macrophages deprives them of a coordinated defense program to M. tuberculosis (25, 26) and as PPARγ, one of the LSNRs that affected M. tuberculosis survival, is known to trigger alternative programs (27), we investigated PPARγ, TR4, and LXRα as possible mediators or attenuators of H37Ra modulation of macrophage polarity. As expected, H37Ra-induced expression of inducible NO synthase (iNOS) was less than that induced by LPS; however, expression of arginase I and II was significantly high (Fig. 3A). An increase in the expression of dectin-1 and mannose receptor (MR) was also observed. Although silencing of PPARγ significantly enhanced H37Ra induction of iNOS, silencing of TR4 and LXRα had marginal effects. Furthermore, whereas H37Ra induction of arginase I and, to a lesser extent, arginase II was abrogated in PPARγ- and TR4-silenced backgrounds, only arginase II induction was abrogated in LXRα-silenced macrophages. H37Ra-induced expression of dectin-1 and MR was abrogated in PPARγ- and TR4-silenced backgrounds. It seems that PPARγ and TR4 mediate H37Ra reprogramming of macrophages toward a less microbicidal alternative phenotype.
Further, we investigated arginine derivitization to NO or polyamine with Griess and arginase assays (Fig. 3B). As evident in Fig. 3B, H37Ra shifts arginine metabolism toward polyamine synthesis at the expense of NO production. Whereas the levels of NO generated were identical to the reported levels for human macrophages (28), they were much less when compared with murine macrophages (25). Interestingly, PPARγ knockdown and, to a lesser extent, TR4 knockdown abrogated the H37Ra-induced shift in arginine metabolism.

Levels of proinflammatory cytokines such as IL-6, IL-12p70, and TNF-α and an anti-inflammatory cytokine, IL-10, were checked to assess the inflammatory response during H37Ra infection in the context of loss of function of the LSNRs that affected M. tuberculosis survival (Fig. 3C). H37Ra infection, much like LPS treatment, showed a characteristic burst of proinflammatory cytokines including IL-6, IL-12, and TNF-α. H37Ra infection also showed a significant surge in IL-10. Although silencing of PPARγ and TR4 slightly enhanced H37Ra induction of IL-6 and TNF-α, it completely abrogated H37Ra induction of IL-10. LXRα knockdown did not show any appreciable effect on H37Ra induction of IL-6 and IL-10. H37Ra induction of PGs (PGE2) was abrogated in knockdowns of PPARγ and TR4, but not of LXRα (Fig. 3D).

Macrophages orchestrate the activation of innate immunity to directly attack an invading pathogen by secreting a battery of ROS (29), including hydroxyl radical (•OH), superoxide anions (O2•−), singlet oxygen (1O2), hydrogen peroxide (H2O2), and peroxynitrite (ONOO−). This mechanism is vital for the clearance of mycobacteria. We therefore investigated the total ROS status of H37Ra-infected macrophages and the possible role of LSNRs. LPS treatment or H37Ra infection both led to generation of ROS as measured in a DHR123 assay (Fig. 3E). Although knockdown of PPARγ or TR4 only slightly increased induction of ROS, LXRα knockdown completely abrogated this induction. CD206, a macrophage, receptor is upregulated following IL-4 stimulation or LPS treatment or H37Ra infection both led to generation of ROS. LPS treatment or H37Ra infection both led to generation of ROS. LPS treatment or H37Ra infection both led to generation of ROS.
bility after H37Ra infection (Fig. 4A). Furthermore, in PPARγ- and TR4-silenced macrophages, a marginal rescue of macrophage viability after H37Ra infection was observed. Intriguingly, in LXRα-silenced macrophages, a significant rescue and increase in viable macrophages was evident. Because *M. tuberculosis* induces apoptosis in infected macrophages (30, 31), and apoptosis creates an intracellular environment that reduces the viability of intracellular bacteria (32, 33), we further monitored apoptosis in the PPARγ-, TR4-, or LXRα-knockdown cell lines postinfection with H37Ra (Fig. 4B). PPARγ and TR4-silenced macrophages did not show any significant change in H37Ra-induced apoptosis, but intriguingly, in LXRα-deficient macrophages, apoptosis was significantly reduced. Although this observed effect could be seen as a way to reduce toxicity in the cell by abrogation of a ROS generator, it can also be addressed from the point of view of a species barrier to LXR function (see Discussion). No significant changes in cell viability were observed for uninfected control knockdowns of PPARγ, TR4, or LXRα for the time frames tested.

**H37Ra modulates expression kinetics of LSNRs**

Because the LSNRs in this study are either aiding *M. tuberculosis* survival or contributing to host defenses, we investigated if H37Ra can modulate changes in their gene expression (Fig. 5A, 5B). Interestingly, whereas gene expression of PPARγ fluctuated and showed up as an immediate early and late marker, TR4 gene expression remained unchanged and seems constitutive (Fig. 5A). LXRα seems to be an early and intermediate marker. The relative mRNA expression levels of these three LSNRs were quantitated by real-time PCR and normalized to GAPDH. Whereas the basal expression levels of PPARγ and LXRα were similar to each other, expression of TR4 was slightly lower (Fig. 5B).

**Cross talk of host LSNRs with H37Ra lipids and with total lipids associated with H37Ra-infected macrophages**

To test if host LSNRs are modulated by the *M. tuberculosis* lipid repertoire and to total macrophage–*M. tuberculosis* lipids associated with homeostasis upon host–pathogen interaction, we performed a standard GAL4-based transactivation assay (Promega) (Fig. 5C). GAL4 fusion constructs of PPARγ, TR4, or LXRα were transfected into COS1 cells, and transactivation by total lipid extracts derived from H37Ra, H37Ra-infected macrophages, and control macrophages was evaluated. A 3-fold transactivation in H37Ra lipid extract was observed for GAL4 fusions with PPARγ and TR4; however, no significant activation was observed for GAL4–LXRα. Transactivation was significantly higher with total lipid extract from H37Ra-infected macrophages for GAL4 fusions with PPARγ, TR4, or LXRα. The control lipid extract did not show significant activation with any of the GAL4 fusion proteins. We further investigated this cross talk on a CD36 promoter reporter (Fig. 5D) as a direct measure of activation, as CD36 is a target gene for both PPARγ and TR4 (16, 34). Inductions similar to those in the transactivation assay were observed. The observed transactivation and induction were comparable to those observed for the positive controls, rosiglitazone, γ-linoleic acid, and TO-901317.
transactivation of PPAR

Total lipid extract from H37Rv-infected macrophages produced a characteristic trait of mycobacterial infection (39). However, the underlying M. tuberculosis-triggered mechanism that regulates foamy biogenesis and eventually contributes to the pathophysiology of tuberculosis is not well understood. Although oxLDL uptake promotes foamy biogenesis, cholesterol efflux antagonizes it. M. tuberculosis seems to modulate both of these key events (Fig. 2). This study implicates, for the first time to our knowledge, that PPARγ synergizes with TR4 in augmenting bug survival.

Formation of foamy macrophages rich in cholesterol, fatty acids, and their derivatives has been convincingly implicated in mycobacterial persistence. The lipids are a carbon source for pathogen in the host (36), and the foam cells inhibit phagosome maturation while enhancing phagocytosis (37, 38). The presence of foamy macrophages, safe havens harboring multiple bacilli, constitutes a characteristic trait of mycobacterial infection (39). However, the underlying M. tuberculosis-triggered mechanism that regulates foamy biogenesis and eventually contributes to the pathophysiology of tuberculosis is not well understood. Although oxLDL uptake promotes foamy biogenesis, cholesterol efflux antagonizes it. M. tuberculosis seems to modulate both of these key events (Fig. 2). This study implicates, for the first time to our knowledge, that PPARγ synergizes with TR4 in augmenting bug survival.

In order to confirm our in vitro cell-culture findings obtained in a THP1 human representative cell line and as these LSNRs have an associated species barrier, we further extended our study to clinical samples for in vivo evidence. mRNA expression of PPARγ, TR4, and LXRα in PBMCs derived from tuberculosis patients, close contacts, and healthy individuals was determined and normalized to that of β-actin, a housekeeping gene for which expression is relatively constant (Fig. 7). Although expression $[2^{-\Delta\Delta Ct}]$ of PPARγ was significantly higher for tuberculosis patients when compared with healthy controls, that of LXRα was lower, and no significant change in expression was observed for TR4 (Fig. 7A). Although no striking relatedness was observed in PPARγ, TR4, and LXRα expression in close contacts, interestingly, an elevated expression of PPARγ and/or TR4 was observed in few close contacts (Fig. 7B). A health follow-up of these and other close contacts is being planned. Further, given the elevated expression of PPARγ/TR4 in diseased individuals and close contacts, we investigated modulation of H37Rv survival by rosiglitazone, a PPARγ/TR4 agonist/activator in human monocyte-derived macrophages (16). Expectedly, rosiglitazone increased H37Rv survival by 2-fold (Fig. 7C).

Discussion

Recent studies have shown evidence that M. tuberculosis hijacks host cellular factors for its survival (11, 25, 35). Interactions of M. tuberculosis lipids with macrophage membrane receptors for sensing and phagocytosis of M. tuberculosis have also been reported (3, 4). This study reports, for the first time to our knowledge, evidence of an intricate mechanism by which M. tuberculosis lipids and changes in lipid homeostasis in M. tuberculosis-infected macrophages can act as a heterologous and derivitized ligand source to which the host LSNRs PPARγ and TR4 can respond. These two LSNRs create a foamy niche with a blunted innate response by polarization of the macrophage to an alternative phenotype. Previous work has established that LXRα decreases the intracellular M. tuberculosis burden (6, 7, 10), whereas PPARγ of all isoforms augments M. tuberculosis survival (11, 25, 35). The role of TR4 in those reports was not clear. This study shows, for the first time to our knowledge, that PPARγ synergizes with TR4 in augmenting bug survival.

Although oxLDL uptake promotes foamy biogenesis, cholesterol efflux antagonizes it. M. tuberculosis seems to modulate both of these key events (Fig. 2). This study implicates, for the first time to our knowledge, H37Rv-triggered expression of the macrophage scavenger receptor CD36, and to some extent, SRA, which are known to account for a large portion of oxLDL uptake by macrophages (40) via...
activation of PPARγ and/or TR4 (Fig. 2). CD36 is a known target gene of PPARγ and TR4 (16, 34) that has recently been reported to be crucial for mycobacterial infection (41). Although TR4 has been unequivocally implicated in foam cell formation (16), reports on PPARγ are less clear. PPARγ can boost lipoprotein uptake, but at the same time, it can also enhance cholesterol efflux via LXR-dependent and -independent pathways by upregulating ABCA1 and ABCG1 (13). It is interesting to note that H37Ra downregulation of the cholesterol efflux transporters ABCA1 and ABCG1 is antagonized by LXRα, but not PPARγ, and seems to be an H37Ra-specific effect. Intriguingly, H37Ra, although promoting PPARγ pathways leading to oxLDL uptake, somehow restricts PPARγ alternative pathways that are known to lead to cholesterol efflux. It is interesting to note that the PPARγ response in infectious diseases is in variance with other known complex diseases. A similar role of PPARγ in lipid body biogenesis has been reported for M. bovis BCG, but not for M. smegmatis (11). LXRα is known to antagonize foam cell formation (42), which could explain M. tuberculosis-augmented foam cell formation in LXRα-silenced macrophages.

Several of the bacterial pathogens, including M. tuberculosis, are known to induce heterogeneous macrophage programs to ensure survival (25, 26, 43). Classical (M1) macrophages are extremely microbicidal and inflammatory, whereas alternative (M2) macrophages are poorly microbicidal and are rather immunomodulatory (43). PPARγ and TR4 contribute to M. tuberculosis induction of macrophage polarization into a heterogeneous but alternative phenotype by upregulating expression of surface markers (CD36 and to some extent dectin-1 and CD206), cellular markers (arginase I), and secretory markers (IL-10), while downregulating ROS/NO generation. PPARγ and TR4 contributing to M. tuberculosis downregulation of NOS/ROS could be ascribed to their role in PGE2 synthesis, which is known to abrogate NOS/ROS (35). PPARγ has previously been shown to regulate BCG-induced PGE2 (11). The ability of PPARγ and TR4 to modulate M. tuberculosis-induced IL-10 secretion could explain their role in phagolysosome maturation block (Fig. 1C, Supplemental Fig. 3). The role of IL-10 in modulating phagosome maturation block and pathogen survival is well understood (44), though alternative pathways also exist (45). Whereas the role of PPARγ in modulation of IL-10 expression has been well studied (27, 46), the role of TR4 was unclear. CD206 and CD36 are described as the hallmark markers for M2 macrophages (47). The ability of TR4 to modulate the expression of CD206, a M2 macrophage marker, was earlier unknown. We found in this work that TR4 regulates CD206 expression and hence might be crucial in regulating alternative activation of macrophages. Further, the involvement of PPARγ in controlling the expression of CD206 and CD36 during alternative activation of macrophages has already been reported (47). Furthermore, CD36 has been shown to be a direct target gene for both TR4 and PPARγ (16, 34). So a double knockdown of PPARγ or TR4 brings out a change in plasticity gear against M2 phenotype, which leads to a consequential and additive effect of phagolysosome maturation, ROS/NO production, and decrease in foam phenotype, which overall might be responsible for the decreased bug burden in PPARγ- and TR4-silenced macrophages. It further remains to be seen whether these macrophage polarizations regulated by PPARγ or TR4 are plastic and reversible.

LXRα does not seem to have a significant effect on macrophage polarization and only partly skews arginine metabolism to polyamines by increased expression of arginase II (48). The observed role of LXRα in mycobacterial killing may because of its role in ROS generation (Fig. 3E), together with its ability to inhibit foam cell formation (Fig. 2). This is in agreement with previous reports that, although suggesting a species barrier in the LXRα response to intracellular pathogen, implicate TLR4 and NADPH oxidase-induced production of antibacterial ROS selectively in human, but not murine, macrophages (5). Conversely, the microbicidal effect

FIGURE 5. PPARγ, TR4, and LXRα cross talk with H37Ra lipids and total infected macrophage lipid repertoire. (A) H37Ra exposure results in a rapid, sequential, and transient expression cascade of PPARγ (early and late marker) and LXRα (early and intermediate marker), with no significant changes detected for TR4. Expression of nuclear receptors at every time point of infection was normalized to uninfected control. The maximum expression detected over the entire experimental time course for each indicated receptor was assigned an expression value of 100. (B) Relative mRNA abundance among all genes considered in the study was calculated as: (1/EΔCt) gene of interest/sum (1/EΔCt) of all genes measured. Transactivation of PPARγ, TR4, and LXRα by H37Ra lipids, total infected macrophage lipid repertoire, and macrophage lipids was monitored on pG5 GAL4 reporter (416.6 μg) (C) or on CD36 promoter reporter (416.6 μg) (D) transfected in COS1 cells along with GAL4 fusion and CMV constructs of PPARγ, TR4, or LXRα (83.3 μg). A total of 1 μL of 1 μg/mL of total lipids was used for the transactivation assay. Positive controls rosiglitazone (PPARγ ligand), α-linoleic acid (TR4 ligand), and TO-901317 (LXRα ligand) were used at 1 μM. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. Error bars show SD. *p < 0.01, **p < 0.05, significant differences with vehicle control or otherwise depicted.
of LXRα in murine macrophages has been ascribed to expression of antiapoptotic factors not detectable in human macrophages. It seems murine and human macrophages have evolved divergent ways to combat intracellular bacteria via LXRs as the common player. It is, therefore, not out of place to observe LXRα silencing leading to increased macrophage survival in our experiments (Fig. 4); increased survival would be a consequence of reduced toxicity to the cells caused by abrogation of a ROS generator (5, 49). This could also be an H37Rω-specific effect in ensuring macrophage survival, as both apoptosis and necrosis have been implicated equivocally and also have been debated for bacterial clearance (30–33).

*M. tuberculosis* cell wall possesses a repertoire of complex lipids (50). Mycolic acids (varying chain length unsaturated fatty...
acid), the major constituent of the M. tuberculosis cell wall, have been highlighted as potent inducers of foamy macrophages (39, 51) in which another cell wall lipid, trehalose dimycolate, has been shown as a potent inducer of granuloma formation (52). Although a plethora of literature is available that describes M. tuberculosis lipid interactions with macrophage surface sensing receptors, their cross talk with host LSNRs over the course of infection has not been addressed. This study confirms for the first time, to our knowledge, cross talk of M. tuberculosis lipids with host LSNRs (Fig. 5C, 5D). PPARγ and TR4 have been shown as a potent inducer of granuloma formation (52). Further fatty acids can get oxidized and nitrated. Hydroxyoctadecadienoic acid and hydroxyeicosatetraenoic acid, which can account for >60% of all lipid peroxidation (53, 54). Further fatty acids can also get oxidized and nitrated. Hydroxyoctadecadienoic acid, hydroxyeicosatetraenoic acid, and nitrated linoleic acid are natural ligands for PPARγ and TR4 (16, 53, 55).

It seems that although M. tuberculosis lipids can act as a ligand source for LSNRs, they may also get oxidized and derivitized in response to oxidative stress in the infected host (17) or may induce lipid homeostasis in the infected macrophage. This extensive lipid repertoire may act as potential ligands to PPARγ and TR4. M. tuberculosis-induced oxLDL uptake seems to be a source of LXRs ligand, as evidenced by increased transactivation of LXRs in the context of the infected macrophage lipid repertoire. Our ongoing efforts involve an extensive and exhaustive mass spectrometry-based lipidomics study to identify the heterologous and derivitized ligands for these LSNRs.

A significant increase in PPARγ and TR4 transactivation by H37Rv versus H37Ra lipids can explain the observed higher survival index of the virulent strain. However, both the avirulent H37Ra and virulent H37Rv strains have the ability to modulate plasticity and induce foamy biogenesis, and hence, both the strains have been used in the experiments to verify that PPARγ and TR4 seem to act synergistically for M. tuberculosis survival. Even though H37Ra and H37Rv differ in trafficking within macrophages, and the number of H37Ra bacilli colocalized with lysosomes was significantly more than that of H37Rv bacilli, the overall pattern of colocalization of these two strains in different knockdown cell lines remained the same. Further, H37Ra and H37Rv has similar growth rates in human monocyte-derived macrophages (56). Moreover, the relative mRNA expression pattern of responding LSNRs in PBMCs from diseased and close contacts relative to healthy individuals complements experimental findings (Fig. 7). However, unlike in the THP-1 macrophage infection experiments, in which TR4 expression remained constant throughout, it is interesting to note that few of the close contacts had elevated expression of TR4. M. tuberculosis infection may not have any bearing on these expressions, as they may be mere signatures of susceptibility or resistance. Strikingly, rosiglitazone widely prescribed for inflammatory disorders and a PPARγ/TR4 agonist significantly increased H37Rv survival in human monocyte-derived macrophages.

This study, for the first time to our knowledge, reports and confirms the idea of cross talk between an array of complex, derivitized M. tuberculosis-macrophage lipids with PPARγ and TR4, which are known lipid sensors that contribute to M. tuberculosis survival. The existence of the heterologous ligand–receptor pair could simply be a case of coevolution or adaptive evolution. LSNR cross talk with the M. tuberculosis lipid repertoire suggests these receptors as new therapeutic targets for pharmacological modulation. Further studies are needed to decipher M. tuberculosis-induced selective induction of the PPARγ lipid biogenesis pathway and downregulation of the alternative pathway that leads to reduced cholesterol efflux. In addition, in light of the results presented in this study, caution is advised when pursuing PPARγ ligand therapy for inflammatory disorders, particularly on the Indian subcontinent and in other geographic hot spots for tuberculosis, because the ligands may cause reactivation of tuberculosis and compounded atherogenesis in patients with a history of tuberculosis.

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Disclosures

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