Mycobacterium tuberculosis Keto-Mycolic Acid and Macrophage Nuclear Receptor TR4 Modulate Foamy Biogenesis in Granulomas: A Case of a Heterologous and Noncanonical Ligand-Receptor Pair

Hedwin Kitdorlang Dkhar,* Ravikanth Nanduri,* Sahil Mahajan,* Sandeep Dave,* Ankita Saini,* Arun Kumar Somavarapu,* Ashish Arora,† Raman Parkesh,* Krishan Gopal Thakur,* Shanmugam Mayilraj,* and Pawan Gupta*

The cell wall of Mycobacterium tuberculosis is configured of bioactive lipid classes that are essential for virulence and potentially involved in the formation of foamy macrophages (FMs) and granulomas. Our recent work established crosstalk between M. tuberculosis cell wall lipids and the host lipid-sensing nuclear receptor TR4. In this study, we have characterized, identified, and adopted a heterologous ligand keto-mycolic acid from among M. tuberculosis lipid repertoire for the host orphan NR TR4. Crosstalk between cell wall lipids and TR4 was analyzed by transactivation and promoter reporter assays. Mycolic acid (MA) was found to transactivate TR4 significantly compared with other cell wall lipids. Among the MA, the oxygenated form, keto-MA, was responsible for transactivation, and the identity was validated by TR4 binding assays followed by TLC and nuclear magnetic resonance. Isothermal titration calorimetry revealed that keto-MA binding to TR4 is energetically favorable. This keto-MA–TR4 axis seems to be essential to this oxygenated MA induction of FMs and granuloma formation as evaluated by in vitro and in vivo model of granuloma formation. TR4 binding with keto-MA features a unique association of host nuclear receptor with a bacterial lipid and adds to the presently known ligand repertoire beyond dietary lipids. Pharmacologic modulation of this heterologous axis may hold promise as an adjunct therapy to frontline tuberculosis drugs. The Journal of Immunology, 2014, 193: 000–000.

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as an inducer of foamy macrophages during granuloma formation (16). In \textit{M. tuberculosis}, MAs are classified as \(\alpha\)-MA, methoxy-MA, and keto-MA (17). These different classes of MAs are not only used as signatures in the identification of different mycobacterial species and strains but also seem to define the virulence property of the strain (18). Although it has been known for some time that MA and the arabinogalactan layer of the \textit{M. tuberculosis} cell wall are covalently associated (19–21), recently the occurrence of free MA in the oxygenated MA class, which is absent in the nonpathogenic strains, survival and pathogenesis of \textit{M. tuberculosis} (22, 23) has been reported. Pathogenic strains such as \textit{M. tuberculosis} (24) and \textit{M. avium} (25) produce the oxygenated MA class, which is absent in the nonpathogenic strains, such as \textit{M. smegmatis} (26). Therefore, it is tempting to hypothesize about and investigate the role of the oxygenated MA class in the survival and pathogenesis of \textit{M. tuberculosis}.

Within the host macrophage, in addition to membrane receptors involved in the phagocytosis of the pathogen, TLRs and some nuclear receptors are considered to be important lipid sensors as well as integrators of lipid signaling, and they have been shown to be integral elements of foamy biogenesis (27–31). Testicular receptor 4 (TR4), peroxisome proliferator-activated receptor (PPAR), and liver X receptor (LXR) function as fatty acid sensors and can sense an array of complex lipids (28, 31–33). TR4 is widely accepted as having a role in macrophage foamy biogenesis (28, 31), and it has been suggested that PPAR-\(\gamma\) is involved in modulating lipid biogenesis (34). On the other hand, LXR-\(\delta\) has the opposite role of inhibiting foam cell formation (35). We recently reported the crosstalk of \textit{M. tuberculosis} lipids with TR4, which leads to bacterial survival, although the identity of the heterologous ligand was unknown.

To determine the exact identity of this heterologous ligand for the lipid-sensing nuclear receptor (LSNR) TR4, we first screened for crossstalk between TR4 and various \textit{M. tuberculosis} cell wall components. Transactivation and promoter reporter assays revealed a ligand-like behavior of MA with TR4. MA extraction and isolation were performed to distinguish which of the distinct classes of MA was involved in the transactivation. Transactivation and promoter reporter assays confirmed the response of TR4 to keto-MA. A TR4 binding assay followed by one-dimensional (1D) and two-dimensional (2D) TLC corroborated the identity of keto-MA. Isothermal titration calorimetry (ITC), circular dichroism and nuclear magnetic resonance (NMR) analysis further confirmed a stable and high-affinity binding between keto-MA and TR4. We have shown that the heterologous ligand-receptor pair, keto-MA–TR4 crossstalk modulates foamy biogenesis and causes granuloma induction. We have reported keto-MA as the heterologous ligand for the orphan nuclear receptor, TR4.

### Materials and Methods

#### Bacterial strain and culture conditions

Mycobacteria strains used were \textit{M. tuberculosis} (H37Ra and H37Rv), \textit{M. smegmatis}, and \textit{M. phlei} and were obtained from Institute of Medical Technology (IMTECH)-Microbial Type Culture Collection Chandigarh or National Institute for Research in Tuberculosis, Chennai, India. They were cultured in 7H9 broth medium containing 5% glycerol and 0.015% Tween 80. OADC (10%) was added as a supplement, and the culture was incubated at 37°C on a shaker. Log phase cultures were spun down at 6000 rpm at room temperature. Cell pellets were stored at \(-80\)°C and lyophilized into powder form. MA was freshly extracted by two different methods, the first of which was acid methanolysis (24). Dried cell mass (150 mg) was added to methanol (2.5 ml), toluene (2.5 ml), and concentrated sulfuric acid (0.1 ml) in a 10-ml polytetrafluoroethylene screw-capped tube. The contents were mixed thoroughly and kept at 75°C for 12–16 h. After methanolysis, the reaction mixture was cooled to room temperature. MA was extracted by adding hexane (2 ml) to the mixture with vigorous shaking and centrifuging to separate the solvent. The upper phase containing the MA was transferred to a fresh tube, and the volume was reduced to 100 \(\mu\)l on a rotatory evaporator. Samples were stored at 4°C until further use.

The second method for MA extraction was based on saponification (18) followed by methyl ester derivatization with trimethylsilyldiazomethane. Dried cell mass was dissolved in 15% aqueous tetrabutylammonium hydroxide and heated at 70°C overnight. The acidified hydrolysis was washed twice with diethyl ether by centrifugation. The washed residue was further hydrolyzed by the addition of tetrabutylammonium hydroxide until no spot of MA was observed on a TLC plate. The extracted MA was run through a silica gel column that was eluted first with hexane followed by a series of diethyl ether:hexane mixtures (6:100, 1:9, and 3:7, v/v) TLC on each mixture was run to check for MA. The crude MA fraction obtained was added to trimethylsilyldiazomethane and incubated for 48–72 h on a rotary shaker. The mixture was loaded onto a silica gel column and eluted first with hexane followed by diethyl ether:hexane (3:7, v/v) to obtain the methyl mycolate fraction.

#### Thin layer chromatography

Extracted crude MA was loaded onto a thin layer plate (Merck), and the chromatogram was analyzed with 1D TLC with the solvent system petroleum ether:dichloromethane (85:15, v/v) (24). MA spots were visualized by placing the plate under UV light after spraying with phosphomolybdic acid (PMA) and visualized on a TLC plate at 100°C for 10–15 min. For 2D TLC, a triple development with petroleum ether:acetone (95:5, v/v) in one direction was followed by a single development at 95°C to the first direction with toluene:acetone (97:3, v/v), as described previously (24, 36). The different spots of MA were designated \(\alpha\)-MA, methoxy-MA, and keto-MA based on the functional groups, as reported previously (37). For purification of each class of MA, the crude MA was run on a preparative TLC plate, and spots were scraped off the plates and extracted with diethyl ether.

#### Cell culture, stimulation, and infection

PBMCs were isolated from fresh-drawn blood by Ficoll-Hypaque gradient centrifugation (Sigma-Aldrich). PBMCs were cultured and obtained as described previously (28). Cells were incubated for 3 h at 37°C and then washed with PBS to remove nonadherent cells. Human monocyte–derived macrophages (hMDMs) were obtained by culturing the cells with M-CSF (50 ng/ml) for 7 d. AHTP-1 stable knockdown cell line (shTR4) was generated and cultured as described in our previous study (28). For the foamy macrophage study or in vitro \textit{M. tuberculosis} survival assay, hMDMs, THP-1 macrophage cells, or both were stimulated with different classes of MA along with \textit{M. tuberculosis} infection at a multiplicity of infection 1:5. Lipid droplets were enumerated by Nile Red or Oil Red O staining. Mycobacterial viability determination was done by CFU assay and flow cytometry. Briefly, hMDMs were infected with \textit{M. tuberculosis} H37Rv or \textit{M. smegmatis} (multiplicity of infection 1:5) along with treatment of the cells with MA or solvent control. After infection, cells were allowed to phagocytose for 1–4 h, and any nonphagocytosed bacteria were cleared by washing with PBS or incomplete medium and incubating with replession medium for 24–48 h. Macrophages were then solubilized and phospholipid suspensions were used with the Live/Dead BacLight Bacterial Viability and Counting kit per the manufacturer’s instructions (Invitrogen). The percentage of live and dead bacteria were determined by flow cytometry (BD FACSCalibur) after staining with SYTO 9 and propidium iodide. For CFU determinations, the bacterial suspensions were serially diluted after macrophage solubilization, 50 \(\mu\)l of each sample were plated, and CFUs were counted. Final calculations included the dilution factor and the volume of diluted sample used for plating.

#### Luciferase reporter assay

The luciferase reporter assay was performed as described (28). COS-1 cells were plated in 24-well plates, and at 70% confluency the media was changed to Opti-MEM, and the cells were transfected with pBIND human TR4, pc5 for the transactivation assay and with pFLAG human TR4, pG5 for the promoter reporter assay. In another experiment, pGL3-CD36 and pBIND plasmids were transfected to control and TR4 and PPAR\(\gamma\) stably knocked down THP-1 cell lines. Firefly luciferase was used as reporter, and \textit{Renilla} luciferase was used to check transfection efficiency.
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and for normalization. Normalized luciferase activities (relative light units) were plotted as the average (± SD) of triplicate samples from typical experiments (SigmaPlot).

**Lipid body enumeration by microscopy**

hMDCMs or THP-1 macrophages were cultured on a coverslip and then stimulated with MA or infected with *M. tuberculosis* as detailed above, or both. The hMDCMs were fixed with 4% paraformaldehyde, and intracellular lipids were stained with Nile Red (3.3 μg/ml) for 15 min. DAPI was used to stain the nuclei. Images were acquired with a Nikon AIR confocal microscope using a 60× objective. The THP-1 macrophages were fixed and stained with 0.5% Oil Red O for 30 min at room temperature, and counterstained with hematoxylin for 5 min to stain nuclei. Cells were imaged with PBS, mounted on glass slides, and imaged. Lipid bodies were enumerated from 10–15 fields containing 20 consecutive macrophages on each slide by light microscopy using a 40× objective lens.

**Determination of intracellular lipid bodies by flow cytometry**

To check for intracellular lipid content after MA stimulation or infection with *M. tuberculosis* as described above, cells were stained with Nile Red, a lipophilic dye that labels fat accumulation within the cytosol. After 48 h of stimulation or infection, adhered cells were washed twice with PBS and trypsinized for detachment from the plate. Following centrifugation at 1500 rpm for 5 min, the cell pellets were resuspended in 1 ml PBS with 1 μg/ml Nile Red and incubated for 15–20 min at room temperature. Nile Red fluorescence was measured by flow cytometry on a Becton Dickinson FACSCalibur system vide FL2 emission channel. Data were acquired and analyzed with CellQuest Pro software from BD Biosciences.

**Virus production**

Replication-deficient adenovirus was produced by transfection into the 293A cell line of either adenoaviral construct pAd/BLOCK-iT-DEST carrying short hairpin RNA (shRNA) for LacZ or shRNA specific for murine or human TR4, according to the manufacturer’s instructions (Invitrogen). Supernatants were collected when 80% cytopathic effect was seen and processed according to the manufacturer’s protocol (Invitrogen). Viral particles were filtered through a 0.2 μm filter, and thawed at the time of transduction. The silencing efficiency of these constructs was monitored by RT-PCR analysis. Mouse cell line RAW or human cell line THP-1 was used to check for knockdown efficiency.

**In vitro granuloma formation**

One hundred micrograms TDM (Sigma), total MA, or keto-MA were mixed with Polybead Polystyrene Microspheres inert beads suitable for microscopy applications and with capability of passive adsorption from Poly-science Ltd. The mixture was rotated overnight at room temperature to allow uniform and homogeneous coating of the beads. The efficiency of lipid coating on the beads was evaluated. Approximately 100 coated beads were incubated with 106 PBMCs in a 12-well plate in a total volume of 1 ml for up to 8 h at 37°C in a 5% CO2 atmosphere. Alternatively, 24 h before incubation with beads, PBMCs were transduced with adenoaviral supernatant having control shRNA or with shRNA TR4. The cellular aggregates were observed on an Olympus IX51 (Olympus) using cellSens imaging software.

**Animal study and ethics**

C57BL/6 mice (male, 6–8 wk old) were procured from the animal facility at the IMTECH and were housed at the Biosafety Level 3 facility of the Institute. The animals were maintained with proper food and water and monitored regularly. All experiments were approved (IAEC/11/18: Study of Nuclear Receptors in Tuberculosis Granuloma) by the Institutional Animal Ethics Committee of the Institute of Microbial Technology and performed according to the National Regulatory Guideline issued by the Committee for the Purpose of Supervision of Experiments on Animals (No. 55/1999/CPCSEA), Ministry of Environment and Forest, Government of India.

**Aerosol infection and determination of Mycobacterium burden**

Experimental mice were injected in the tail vein and aerosol challenged (with a nebulization system Glas-Col; Terre Haute, IN) with control adenovirus (shRNA LacZ) or carrying the shRNA TR4 in the BSL3 (IMTECH). The lungs were dissected, and RT-PCR was performed to check the expression of TR4 in alveolar macrophages at different time points. After adenovirus treatment, the mice were exposed to aerosol inhalation of *M. tuberculosis* H37Rv (∼100 CFU per lung, as per the standardized dose observed after day 1 of infection) using the inhalation exposure system. The bug burden in the experimental animals was determined at different time point after aerosol challenge (5, 30, and 60 d). Animals were kept in separate chambers, and the exposure setting was set for 45 min (nebulization setup) with a standardized CFU count. *Mycobacterium* burden per lung was determined by serial diluting the total lung homogenates and plating them on Middlebrook 7H11 plates supplemented with OADC enrichment. Plates were monitored weekly, CFU count was observed within 3–4 wk of incubation at 37°C, and the total bug load of the lung tissue was recorded.

**Histopathology and body weight analysis**

Control mice and TR4-knockdown mice infected with *M. tuberculosis* H37Rv or *M. avium* were sacrificed after 60 d postinfection, and lung tissue was dissected and fixed in buffered 10% formalin. Histologic sections of the lungs were stained with H&E to check for in vivo granuloma formation. Images were captured on Olympus CH 20L and ocular micrometer disc was used for counting the area. In addition, the body weights of other groups of control and TR4-knockdown mice were monitored weekly for 20 wk after aerosol exposure.

**Isothermal titration calorimetry**

Isothermal titration calorimetry experiments were performed with a Microcal VP-ITC microcalorimeter. Full-length human TR4 protein (hTR4) was purified and extensively dialyzed against the buffer used in ITC, 20 mM TrisHCl (pH 7.4), 250 mM NaCl, and 10% glycerol. The same buffer was used for titration. The stock of ligand was prepared in diethyl ether, and the ligand concentrations used were in 15–20 M excess of TR4. The ligand was dissolved in titration buffer, and an equivalent amount of buffer was added to the protein. Titrations consisted of 45 injections of 6 μl separated by 120 s. The titrations were performed with 3–6-μM concentrations of protein and 45–60-μM concentrations of ligand. Data was analyzed with Origin 7 software to determine the binding constant (Kd), apparent stoichiometry (N), and change in enthalpy (DH) and entropy (ΔS).

**Structural modeling and docking studies**

The human TR4 ligand binding domain (LBD) active-form homology model was generated with a Swiss model server by using human RXRα LBD (Brookhaven Protein Data Bank ID: 1FM6) as a template (38). The 3D structural coordinates of γ-linoleic acid and keto-MA were generated by a Pro-DRG2 server. Docking was performed with AutoDock 4 (39). Figures were created using Pymol.

**Circular dichroism**

Full-length hTR4 was purified and extensively dialyzed against buffer 20 mM Tris HCl (pH 7.4), 250 mM NaCl, and 10% glycerol. Measurements were recorded with a JASCO circular dichroism instrument. For temperature melting experiments, a gradient of 20–90°C was used with increments of 1°C. The change in circular dichroism signal was measured at 223 nm. Protein concentration was 0.2 mg/ml. Experiments were performed with hTR4 in the presence and absence of keto-MA.

**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 Number 2) and the Ethics and Biosafety Committee of the Institute of Microbial Technology (IMTECH), Sector 39A, Chandigarh, India (01/2011/MT/IEC-Blood; 12/2010/MT/GBC). The study was conducted strictly in accordance with the Ethical Guidelines for Biomedical Research on Human Subjects by the 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, and written consent on the consent form was obtained from each healthy volunteer before his or her induction in the study in the language (English, Hindi, and Punjabi) familiar to them.

**Statistics**

The results are expressed as mean ± SD, and SigmaPlot and SPSS were used for statistical analysis unless otherwise mentioned.

**Results**

M. tuberculosis keto-MA is the heterologous ligand for macrophage nuclear receptor TR4

To determine whether *M. tuberculosis* cell wall components are a possible source of heterologous ligands for TR4, a GAL4-based
**FIGURE 1.** *M. tuberculosis* keto-mycolic acid binds to macrophage nuclear receptor TR4. (A) Gal4-based dual-reporter assay was performed in COS-1 cells. When *M. tuberculosis* H37Rv cell wall components (TDM, sulfolipids-1, PIM, PIM 6, LAM, LM, AG, PG, and m-AGP) were evaluated for their ability to transactivate Gal4-TR4, MA was found to cause significant transactivation. Linoleic acid was used as a positive control. (B) Total MA extracted from *M. tuberculosis* H37Rv and H37Ra, *M. smegmatis*, and *M. phlei* was confirmed with 1D TLC. *M. smegmatis* contains only α and α’-MA, *M. phlei* contains α-MA and keto-MA, whereas H37Rv and H37Ra contains all three forms, α-MA, methoxy-MA, and keto-MA. (C) Total MA from each of these species was evaluated for their ability to transactivate Gal4-TR4. Total MA from *M. tuberculosis* H37Rv and H37Ra and *M. phlei* showed an increased transactivation of TR4 as compared with that of *M. smegmatis*, which lacks the oxygenated form of MA. (D) Individual MA from *M. tuberculosis* H37Rv was separated by 1D and 2D TLC (Supplemental Fig. 1A), and spots corresponding to α-MA, methoxy-MA, and keto-MA were scraped and purified. (E) α-MA, methoxy-MA, and keto-MA from *M. tuberculosis* H37Rv were evaluated for their ability to transactivate TR4 by Gal4-based dual reporter and (F) CD36 (a target gene of TR4) gene promoter reporter assays. For transactivation or promoter reporter assay, 1 mg/ml of individual lipids was used. Positive control linoleic acid was used at 1 mM. (G) TR4-coated beads were incubated along with total MA followed by repeated washing. Precipitated beads were solvent extracted to release the bound MA, which was run on analytical TLC plates for 1D and (H) 2D TLC. The spot of the bound MA corresponds to that of the keto-MA in the standard total MA from H37Rv. (I) hMDMs with control or TR4 knockdown (shRNA) background were either stimulated with keto-MA or left unstimulated and then infected with either *M. smegmatis* (for 24 h) or *M. tuberculosis* H37Rv for 48 h. Intracellular bacilli load in the infected macrophages was monitored by counting the number of colonies (i.e., CFU) in 7H11 plates. Interestingly, keto-MA was able to rescue *M. smegmatis* for survival in control but not TR4 knockdown background. Data are representative of three independent experiments with similar results and are shown as mean ± SD of the indicated number of experiments. *p < 0.05, with linoleic acid control in reporter assay or with vehicle control and as otherwise depicted for CFU assay.
transactivation reporter assay was performed in COS1 cells (Fig. 1A). Interestingly, the crude MA fraction significantly transactivated TR4 compared with TDM, sulfolipids-1, PIM, PIM 6, LAM, LM, AG, PG, and m-AGP etc of M. tuberculosis H37Rv. To gain insight into the fraction or type of MA responsible for the transactivation of TR4, total MA was extracted from M. tuberculosis H37Rv and nonpathogenic strains, including M. tuberculosis H37Ra, M. smegmatis, and M. phlei, and examined with 1D and 2D TLC (Fig.1B, Supplemental Fig. 1A). The total MA of both M. tuberculosis H37Rv and H37Ra contained α-MA, methoxy-MA, and keto-MA, whereas the total MA of M. smegmatis lacked keto-MA and methoxy-MA and that of M. phlei lacked methoxy-MA (18, 37). Interestingly, a 10–14-fold transactivation of GAL4–TR4 was observed with the total MA from M. tuberculosis H37Rv/H37Ra and M. phlei; however, no significant transactivation was observed with the total MA from M. smegmatis (Fig. 1C). These reporter assay observations suggest the possible involvement of keto-MA in the modulation of TR4.

The separation and characterization of total MA from M. tuberculosis H37Rv was performed with 1D and 2D TLC as described (24, 37), and the spots corresponding to α-MA, methoxy-MA, and keto-MA, were scraped and further extracted for analysis in the GAL4-based transactivation assay. The results of these assays confirm the involvement of keto-MA in the modulation of TR4 (Fig. 1D, 1E). A gene promoter reporter assay was also performed on TR4 target gene C3D6 to have a direct measure of the MA–TR4 crosstalk in modulating promoter activity (Fig. 1F (31)). Keto-MA was observed to have the highest induction of TR4, which is similar to the results of the transactivation assay. Linoleic acid was used as a positive control in all the assays.

To clarify further the class of MA that can function as a ligand for TR4, we used a conventional bead-based pull-down approach in which bead-bound TR4 was allowed to bind in solution to the total MA extract from M. tuberculosis H37Rv containing all three classes of MA. Precipitated beads were solvent extracted to separate the bound MA from the TR4. This MA fraction was evaluated and analyzed with 1D and 2D TLC, which confirmed that the identity of the bound MA was keto-MA (Fig. 1G, 1H). We also confirmed the pull-down fraction to be keto-MA by analyzing the TLC run on hexane:diethyl ether with similar elution profile as that of petroleum ether:diethyl ether or dichloromethane elution system in which spot III (methoxy-MA) and IV (keto-MA) merge because of similar mobility (37) (Supplemental Fig. 1B). We were also able to rule out methoxy-MA in the pull down fraction by NMR characterization (Supplemental Fig. 1C).

To assess the role of keto-MA as a virulence factor in Mycobacterium survival, we examined the fate of M. smegmatis and M. tuberculosis H37Rv/H37Ra in hMDMs in control and TR4 knockdown background (95% efficiency). Macrophages were infected in the presence and absence of keto-MA. At 48 h after infection, intracellular bacteria was isolated and their viability was determined by CFU assay (Fig. 1I). Using an alternative approach, bacteria were stained with green fluorescent SYTO9, which stains both live and dead cells, and red fluorescent propidium iodide, which penetrates only bacteria with ruptured membranes. Flow cytometry was performed and the percentage of dead bacteria was calculated (Supplemental Fig.1D). Interestingly, the addition of keto-MA rescued M. smegmatis from clearance by the host compared with the M. smegmatis-infected sample without added keto-MA. The keto-MA–mediated increase in bug survival was abrogated in TR4 knockdown background.

The above evidence firmly support the hypothesis that M. tuberculosis H37Rv keto-MA crosstalks with host NR, TR4. We were then set to unravel the mechanism by which the keto-MA crosstalk with host TR4 augments the survival of M. tuberculosis.

M. tuberculosis keto-MA induces foamy macrophages via host nuclear receptor TR4

Formation of foamy macrophages (FMs) has been convincingly implicated in mycobacterial persistence, because the lipids within the granulomas in the host serve as a carbon source for the pathogen during starvation (15). It has been shown that oxygenated MA from M. tuberculosis plays a vital role in FM generation from macrophages (16), although the underlying mechanism has not been elucidated. To our knowledge, the present study is the first to establish a link between keto-MA and the host nuclear receptor TR4 that leads to FMs, as observed with Nile Red and Oil Red O staining (Fig.2, Supplemental Fig. 2). Because TR4 has recently been reported as a lipid sensor and has a role in foamy biogenesis (28, 31), we hypothesized that the underlying mechanism of the keto-MA induction of FMs could largely be attributed to its ability to crosstalk with the host NR TR4. hMDMs were stained with Nile Red after incubation with MA, M. tuberculosis H37Rv, or M. smegmatis and evaluated by confocal microscopy and flow cytometry (Fig. 2). As expected, cells infected with M. tuberculosis H37Rv but not M. smegmatis differentiated into FMs. Strikingly however, M. tuberculosis H37Rv induction of FMs in TR4 knockdown cells (95% efficiency) was significantly reduced. The addition of keto-MA was effective in inducing FMs in hMDMs, which was significantly reduced in a TR4 knockdown background. We also evaluated the induction of FMs in THP-1 macrophages by Oil Red O staining (Supplemental Fig. 2A, 2B). A similar pattern was observed. FM induction was maximum in control THP-1 cells infected or induced with M. tuberculosis H37Rv (∼70% of the infected cells) or keto-MA (∼70% of the total cells compared with control), followed by methoxy-MA (∼10%), and α-MA (∼5%). THP-1 macrophages from cells with a stable knockdown of TR4 (95% efficiency) had less induction of FMs after stimulation with M. tuberculosis H37Rv (∼25% of the infected cells) or keto-MA (∼25% of total cells compared with control), whereas methoxy-MA (∼11%) and α-MA (∼4%) addition showed no significant change. Our data suggest a definite mechanism by which M. tuberculosis H37Rv keto-MA interacts with the host NR TR4 to ensure pathogen survival by inducing FMs, which behave as a nutritional niche for M. tuberculosis and contribute to granuloma formation.

Keto-MA crosstalk with TR4 functions in M. tuberculosis survival and induction of tuberculous granuloma in vitro and in vivo

Granuloma formation, which is a hallmark of tuberculous infection, involves recruitment and formation of FMs that subsequently are surrounded by lymphocytes (40). Our observations thus far indicate a strong role for keto-MA in FM formation via crosstalk with TR4. To further define the role of MA, and in particular keto-MA, in granuloma induction, keto-MA–coated beads and total MA-coated beads were incubated with human PBMCs, and the formation of in vitro granulomas was monitored for 8 d as described earlier (41). Uncoated beads were used as a negative control as they have earlier been shown to have minimal recruitment of the cells. TDM-coated beads, which are known to induce granulomas, were used as a positive control. Total MA- and keto-MA–coated beads induced the recruitment of cells and the formation of a cellular monolayer starting from day 1. The monolayer continued growing, and granuloma-like structures were seen at day 5 (Fig. 3A). We further evaluated the role of the crosstalk between keto-MA and the host NR TR4 in modulating granuloma
formation. The knockdown of TR4 in human PBMCs was found to decrease migration of the cells around keto-MA–coated beads (Fig. 3B, Supplemental Fig. 3A). In keeping with its reported role, TDM was a potent inducer of granulomatous response in all backgrounds, which is understandable because alone it fails to transactivate TR4 (Fig. 1A, Supplemental Fig. 3B).

To assess specifically the role of the host NR TR4 in M. tuberculosis H37Rv survival in vivo, an adeno-based knockdown of TR4 in mice was attempted through delivery by tail vein injection and aerosols. The observed TR4 knockdown of 50–60% (Supplemental Fig. 3C) in alveolar macrophages corresponds with the reduced survival of M. tuberculosis H37Rv determined at different times 15, 30, and 60 d after infection. (Fig. 3C). Histopathology of the lung dissected 60 d after infection from TR4 knockdown mice compared with lung from control mice showed a reduction in the size of the developing follicular granulomas as visualized by H&E staining (Fig. 3D). This reduction in the size of granulomas is seemingly a consequence of increased clearance of M. tuberculosis H37Rv (Fig. 3C, 3D). In addition, when monitored over a period of 20 wk, the control mice had prevailing disease symptoms of laziness and slow movement, particularly at later time points, with a slight change in the body weight as compared with the TR4 knockdown

FIGURE 2. Keto-MA–TR4 axis induces FM phenotype in hMDMs. (A) Control or (B) TR4 knockdown hMDMs were infected with either M. tuberculosis H37Rv or M. smegmatis or stimulated with keto-MA for 48 h and stained with Nile Red. The extent of FMs was assessed by fluorescence microscopy (original magnification ×60) and flow cytometry. Data are representative of three independent experiments with similar results.

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mice (Supplemental Fig. 3D). Lung histopathology of the mice infected with *M. smegmatis* appears normal without any sign of granuloma formation (Supplemental Fig. 3E, 3F). However, when keto-MA from *M. tuberculosis* H37Rv was administered along with *M. smegmatis* infection, there was multiple well-formed granuloma structure. Interestingly, the coadministration in the TR4 knockdown mice, had a reduced number of granuloma formation, which clearly explains the importance of TR4 for the keto-MA mediated granuloma formation.

Noncanonical binding of keto-MA to host NR TR4

Docking and ITC studies were performed to demonstrate the observed binding of keto-MA to TR4. We performed in silico rigid docking studies by making a grid around the predicted and modeled TR4 ligand binding domain (LBD: 349–615) and docking keto-MA. Interestingly, whereas linoleic acid, a known ligand of TR4, showed a negative binding energy that falls in the range observed with other TR4 known ligands (−26.54 to −3.92; data not shown) and is suggestive of a stable complex formation, keto-MA showed a positive binding energy (Fig. 4A). ITC titration experiments in which the sample cell contained full-length human TR4 (1–615) and the syringe contained keto-MA revealed a sigmoidal titration with an analyzed N value of ∼1.59, which indicates that keto-MA is probably binding to TR4 at two binding sites (Fig. 4B). Complete saturation was not obtained, which has also been reported for heme-Reverb binding (42). The process was energetically favorable. ITC titration experiments performed with α-MA and methoxy-MA did not show any binding pattern (Fig. 4B, Supplemental Fig. 4A). Docking studies and ITC experiments suggest that keto-MA acts as a heterologous ligand with presumably a noncanonical binding to a TR4 full-length dimer. Circular dichroism experiments were performed to investigate the effect of keto-MA binding on the thermal stability of hTR4. Interestingly, keto-MA increased the melting temperature (Tm) of hTR4 full-length from 62˚C to 65˚C (Fig. 4C). Finally, the dose-dependent activation of hTR4 by keto-MA was observed by transactivation reporter assay (Fig. 4D), which further supports our claim on the specificity of the binding of keto-MA to hTR4. Our data lead to the conclusion that keto-MA functions as a virulent factor for *M. tuberculosis* by hijacking the host NR TR4 function. This report proposes the noncanonical binding of a host orphan receptor, TR4, with a heterologous ligand, keto-MA (Fig. 5).

**Discussion**

*M. tuberculosis* lipids are known to be efficiently shared and secreted within the infected macrophage and into the extracellular
Among the secreted lipids, the cell-wall lipid TDM and LM have already been characterized as bioactive components during granuloma formation and differentiation of macrophages into multinucleated giant cells, respectively (44–47). Recently, the role of oxygenated forms of MA during foam-cell formation was highlighted (16). To our knowledge, the present study is the first to provide mechanistic insight into keto-MA–induced FM, which involves crosstalk with the host LSNR TR4. TR4 reporter assays with cell wall components and MA from different Mycobacterium species as well as biochemical (TLC) and biophysical (ITC, NMR) characterization of the TR4-bound MA fraction identified keto-MA as a heterologous ligand that shows a noncanonical binding to the host NR TR4. This crosstalk provides insight into the previously observed role of oxygenated MA in the differentiation of macrophages to FMs and in granuloma formation (16).

The relevance of keto-MA in FM formation is emphasized by the fact that M. smegmatis, which lacks keto-MA, induces only ∼5% of the FM formation seen with M. tuberculosis (16). In comparison, transformed M. smegmatis that carries the hma gene coding for keto-MA induced up to ∼60% FM (16).

TR4 has been shown unequivocally to induce FMs partly by regulating oxidized LDL receptor CD36 (28, 31). Our previous study also suggested that crosstalk between host TR4 and members of the M. tuberculosis lipid repertoire is a prerequisite for the induction of FM in M. tuberculosis, although the exact identity of the heterologous ligand was lacking (28). The present study establishes the host–pathogen axis as keto-MA–TR4, in which the host factor TR4 is indispensable for keto-MA–induced FM. This was evident in the experiment with hMDMs with a TR4 knockdown background that were treated with keto-MA, stained with Nile red and Oil Red O, and monitored with imaging and flow cytometry (Fig. 2, Supplemental Fig. 2). FMs are the sites that sustain the pathogen within the granuloma core (16, 45). Given the involvement of TR4 in the modulation of FM, it also possibly plays a role during granuloma formation. As FMs appear among M. tuberculosis/PPD/TDM granuloma, there might be a resemblance in tuberculous granuloma structure arising from these determinants and keto-MA. Pathogen-induced FMs have been reported in cases of Chlamydia (49) and toxoplasma infections (50) and in diseases such as atherosclerosis (51). It is tempting to speculate that the lipid-metabolite-host LSNR cellular deregulation observed in TB and atherosclerosis (28, 52) could be generic to other infections and diseases.

The dynamics of aggregation of immune cells during TB infection leads to granuloma formation (53). This involves changes in macrophage morphology, differentiation, and polarization. Mature macrophages appear epithelial (54); however, they may form multinucleated giant cells (55) or FMs with increased lipid bodies (40). Although oxygenated MA from M. tuberculosis H37Rv has been shown to modulate granuloma formation, the interacting...
partners from the host were unknown. Experiments in the current study involving an in vitro model of granuloma formation (16, 41, 47) validate the presence of the keto-MA–TR4 axis in the granuloma, as seen from the increased number of cells bound to the beads coated with keto-MA, similar to the number with the positive control TDM (Fig. 3A). Furthermore, it is clear that TR4 is indispensable to keto-MA–induced granuloma formation (Fig. 3B). In vivo infection studies in mice showed significant abatement in bacterial burden and consequent reduction in size of developing follicular granuloma in lungs of mice with a TR4 knockdown background. Taken together, these observations confirm that LSNR TR4 functions as a host factor augmenting the survival of *M. tuberculosis* by impeding its clearance and inducing granuloma. Our previous report (28) showed that TR4 polarizes macrophages toward the less microbicidal and immunomodulatory M2 phenotype with a consequential increase in FMs, blocks phagolysosome maturation, and reduces levels of ROS/NO, and as result aids in the survival of *M. tuberculosis*. These observations, as well as the effects of other unknown interactions by keto-MA–TR4, expose the vulnerability of the host. Greater comprehensive insight into the downstream physiology and crosstalk is required for a more concerted intervention.

The roles and relevance of several host molecules that are manipulated by the pathogen are underestimated and thus far inadequately addressed. Because of emergent threats of multidrug-resistant and extensively drug-resistant strains, it is exigent to identify host factors playing combatants and cohorts and to assess their amenability to pharmacologic modulation. A novel design would be a combinatorial therapy that, while targeting the pathogen with frontline drugs, also targets the host cohorts or empowers the host combatants. Several host factors have recently been implicated in *M. tuberculosis* survival and clearance. These pathogen-mediated factors include prostanoid receptor EP2 (56), protein kinase R (57), genes involved in the calcium and cysteine protease pathways (58), the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflamasome (59), and several host-dependent survival factors screened by siRNA (60). Our recent work along with other reports has shown that PPARγ and TR4 provoke the anti-inflammatory and pro-*M. tuberculosis* properties of macrophages (28, 32, 34). In addition, different groups have also reported other nuclear receptors such as vitamin D receptors, Rev-erbo, and LXRα anti-infectives functions against *M. tuberculosis* (27, 28, 61–65).

NRs in particular have been shown to modulate host effectors that are imperative for clearance or survival of intracellular microparasites *Listeria monocytogenes*, *Salmonella typhimurium*, *Leishmania donovani*, and *M. tuberculosis* (27, 32, 34, 66–69). Compared with members of a signaling network, NRs are functionally less pleiotropic, less redundant, and more amenable to pharmacologic modulation and, as such, better therapeutic targets. Given their physiologic importance in human disease, not surprisingly NRs have been reported to be modulated by various agonists and antagonists composed of both biologically and pharmacologically synthesized drugs, a few of which have already been approved by the U.S. Food and Drug Administration (70–74). However, the use of such drugs has not been evaluated in other heterologous contexts, such as infection, and the present TB drug regimens primarily target pathogens (75, 76). We have extensively studied and identified keto-MA as a heterologous specific ligand for TR4, which was also confirmed by gene promoter reporter assay in the PPARγ and TR4 knockdown backgrounds and the failure of PPARγ to pull down keto-MA (Fig. 4D, Supplemental Fig. 4C). Design of inhibitory analogs to this heterologous ligand-receptor binding and evaluation of their efficacy together with frontline TB drugs holds promise as a combinatorial adjunct therapy.

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**Disclosures**

The authors have no financial conflicts of interest.
Supplemental Fig 1. Characterisation of \textit{M. tuberculosis} mycolic acid by 1D/2D TLC; $^1$H-NMR and Percentage of dead baccilli monitored by FACS analysis.

(A) Total-MA from different strains of Mycobacteria (\textit{M. tuberculosis} H37Rv & H37Ra, \textit{M. smegmatis} and \textit{M. phlei}) was separated by 2D TLC using the specified solvent. (B) The identification of the TR4 pulled down MA as keto-MA was confirmed by analyzing the 1D-TLC ran on a different solvent (hexane:diethyl ether) with similar spotting profile (~ Rf value) as that of petroleum ether:diethyl ether or alternate solvent dichloromethane, where in spots III (methoxy-MA) and IV (keto-MA) merge as a single spot because of similar mobility. (C) $^1$H-NMR spectra of the TR4 pulled down MA was compared with total \textit{Mtb}-MA (control) which shows absence of the peak at 3.34 ppm (in pull down MA) that corresponds to methoxy-MA. (D) hMDM's were either stimulated with keto-MA or left unstimulated and then infected with either \textit{M. tuberculosis} H37Ra or \textit{M. smegmatis} for 48 hr. Intracellular baccilli load in the infected macrophages was monitored by the percentage of dead versus live baccilli as stained with SYTO (live and dead baccilli) and PI (dead baccilli only) performed by FACS analysis.
Supplemental Fig 2. Keto-MA-TR4 axis induces foamy macrophage phenotype in THP-1 macrophages

(A) Control and TR4 stable knockdown THP-1 differentiated macrophages were stimulated with α-MA, methoxy-MA or keto-MA. The formation of foamy macrophages was analyzed by Oil Red O staining for the lipid bodies inside the cells. *M. tuberculosis* H37Rv infected macrophages were used as a positive control. Percentages of foamy cells were calculated from 5 fields containing 50 cells each, and imaging was done under bright field microscopy with 40x magnification. (B) Quantification of FMs to each of these treatments was keeping in mind that control cells have Oil Red O droplets ranging between four and eight per cell and so cells containing Oil Red O droplets (>10) were considered FMs. Data are representative of three independent experiments with similar results. Error bars indicate mean ± S.D. Asterisks show significant differences (p < 0.05), with control and as otherwise depicted.
Supplemental Fig 3. *M. tuberculosis* lipids in modulation of granuloma

(A) PBMCs were infected *ex vivo* with control shRNA (LacZ) or TR4 specific shRNA adenovirus for 24 hr and incubated with uncoated or beads coated with keto-MA and the recruitment of leukocytes was counted. The cellular aggregation around the bead was scored; 0: no cell binds to bead; 1: ≤5 cell binds to the bead; 2: > than 5 but ≤25 cell bind to the beads; 3: >25 but ≤50 cell bind to the beads; 4: >50 cell bind to the beads; 5: Blast transformation.

(B) PBMCs were infected *ex vivo* with control shRNA (LacZ) or TR4 specific shRNA adenovirus for 24 hr and incubated with uncoated or beads coated with TDM and granuloma generation was monitored. (C) Adenovirus based TR4 knockdown was checked in the lung alveolar macrophages (~4 mice each of control and shTR4 injected mice) which showed a 50-60% reduction at the RNA level as normalised using beta-actin. (D) Control and TR4 knockdown mice were infected by aerosols of *M. tuberculosis* H37Rv. Body weights were monitored weekly in order to determine the condition of the mice. (E-F) Granulomas were monitored in controls and TR4 knockdown background mice infected with *M. smegmatis* and keto-MA from *M. tuberculosis* H37Rv (20X and 200X). Statistics as mentioned in Supplement Fig 2.
Supplemental Fig 4. Keto-MA specific interaction with TR4

ITC experiment representing the titration of (A) alpha-MA or (B) methoxy-MA with hTR4 full length protein. The titration contained 0.5-1 μM of TR4 in the sample cell and 7.5-15 μM of MA in the ligand syringe. Background (titration of MA into buffer) was subtracted from the data points from titrating MA with TR4 full length protein. (C) CD36 promoter reporter assay was performed in control and PPARγ, TR4 stable knockdown backgrounds. TR4 knockdown completely abolish the activation of CD36 promoter in presence of keto-MA, while PPARγ knockdown doesn’t show any effect. (D) 2D-TLC of PPARγ pull down of keto-MA shows no spot.