

Human *IL10* Gene Repression by Rev-erb α Ameliorates *Mycobacterium tuberculosis* Clearance^{*[5]}

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Background: Transcriptional modulation of *IL10*, a cytokine that blocks phagolysosome maturation, is not well understood.

Results: This study demonstrates human *IL10* gene repression by direct binding of Rev-erb α on Rev-DR2 in the proximal promoter.

Conclusion: Rev-erb α binds to *IL10* proximal promoter, represses expression, and impedes *Mycobacterium tuberculosis* in human macrophages.

Significance: This study provides rationale to target Rev-erb α as a therapeutic intervention that might support host defense in tuberculosis.

Nuclear receptors modulate macrophage effector functions, which are imperative for clearance or survival of mycobacterial infection. The adopted orphan nuclear receptor Rev-erb α is a constitutive transcriptional repressor as it lacks AF2 domain and was earlier shown to be present in macrophages. In the present study, we highlight the differences in the relative subcellular localization of Rev-erb α in monocytes and macrophages. The nuclear localization of Rev-erb α in macrophages is subsequent to monocyte differentiation. Expression analysis of Rev-erb α elucidated it to be considerably more expressed in M1 phenotype in comparison with M2. Rev-erb α overexpression augments antimycobacterial properties of macrophage by keeping *IL10* in a basal repressed state. Further, promoter analysis revealed that *IL10* promoter harbors a Rev-erb α binding site exclusive to humans and higher order primates and not mouse, demonstrating a species barrier in its functionality. This direct gene repression is mediated by recruitment of co-repressors NCoR and HDAC3. In addition, our data elucidate that its overexpression reduced the survival of intracellular pathogen *Mycobacterium tuberculosis* by enhancing phagosome lysosome maturation, an event resulting from *IL10* repression. Thus, these findings suggest that Rev-erb α bestows protection against mycobacterial infection by direct gene repression of *IL10* and thus provide a novel target in modulating macrophage microbicidal properties.

Macrophages are immune system sentinels with a major role to play in both innate and adaptive immunity. They are the key effectors in antimicrobial defense, atherogenesis, autoimmunity, and many other inflammatory diseases (1). Although the

activation, function, classification, and plasticity of these cells have been studied extensively with regard to cellular signaling, the cytokine environment, and surface, cellular, or secretory markers, studies of the underlying molecular mechanism have mostly addressed NF- κ B (2). Similarly, modulation of macrophage function and alteration of disease pathology by small molecules such as heme, lipids, or drugs such as rifampicin are well understood at the translational level of the effectors (3, 4), but the transcriptional mechanism involving interactions of these ligands with transcriptional molecules and the resulting expression patterns have not been investigated.

This study focuses on Rev-erb α , an adopted orphan nuclear receptor that belongs to the steroid/thyroid hormone receptor superfamily and is a known heme sensor (5). It is encoded on the opposite strand of the thyroid receptor α (*c-erbA α*) gene (6). Rev-erb α modulates proinflammatory cytokines through NF- κ B (7, 8) and in that regard is similar to heme, its physiological ligand (5). Rev-erb α is structurally unusual in that it lacks the C-terminal tail (helix 12) of the ligand binding domain, which is required for co-activator recognition, hence functions as a transcriptional repressor (9). Rev-erb α may bind to its response element either as a monomer or as a homodimer. As a monomer, it binds to the consensus nuclear receptor half-site motif flanked by a 6-bp AT-rich sequence (A/T)₆ PuGGTCA; as a homodimer, it binds to a direct repeat of the core motif separated by 2 bp (DR2) (10). Rev-erb α competes with ROR α ,² liver X receptor α (LXR α), and peroxisome proliferator-activated receptor α (PPAR α) for these response elements and has opposing effects on transcription (11–13). In several instances, Rev-erb α DR2 overlaps the TATA box (14, 15), which suggests the possibility that Rev-erb α is a component of a basal repressive transcription complex.

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[5] This article contains supplemental Figs. 1–6.

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² The abbreviations used are: ROR α , RAR-related orphan receptor α ; RAR, retinoic acid receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; NCoR, nuclear receptor co-repressor; HDAC3, histone deacetylase 3; PMA, phorbol 12-myristate 13-acetate; MDM, monocyte-derived macrophage.

Mycobacterium tuberculosis latently infects one-third of the human race with incidences of active cases, which are rising alarmingly in immunocompromised individuals, especially people infected with HIV. Now with an increase in the cases of multidrug-resistant tuberculosis and with the emergence of extremely drug resistant and total drug resistant strains of *M. tuberculosis*, tuberculosis has become a major global public health threat. The last decade has seen a hunt for new cellular targets to control and eliminate tuberculosis infection. Nuclear receptors have emerged as decisive cellular targets with the potential of regulating host immune responses to pathogens. Nuclear receptors including PPAR γ , testicular receptor 4 (TR4), and LXRs have been shown to effect *M. tuberculosis* survival or clearance via modulating macrophage function (16–18); the role of Rev-erb α in *M. tuberculosis* infection has not been addressed, although it has been reported to modulate macrophage function, and its ligand heme has been shown to exhibit antimicrobial properties. Among cytokines, IL10 is a master regulator of macrophage plasticity and function; it antagonizes the expression of co-stimulatory molecules, blocks the release of proinflammatory cytokines, and inhibits phagolysosome maturation and key events in apoptosis (19–21). IL10 ameliorates immunopathology and prevents host injury, but also has been reported to impede clearance of several pathogens such as *Mycobacterium* spp., *Plasmodium* spp., *Listeria monocytogenes*, and *Leishmania major* (22). Despite the pleiotropic effects of IL10, its regulation at the level of signal transduction, epigenetics, and transcription factor binding has been addressed in a limited fashion, mostly in regard to IL10 gene activation (23). Understanding the molecular events and associated transcription factors that constitute basal repression of IL10 is a requirement for design of newer strategies for infectious disease intervention.

In this study, we demonstrate that Rev-erb α binds *in vitro* and *in vivo* to the human IL10 putative Rev-erb α DR2 response element, which is preceded by an A/T-rich sequence. Rev-erb α forms a repressive complex by associating with NCoR-HDAC3 upon heme binding and keeps IL10 in a basal repressed state. This repression of human IL10 provides microbicidal phenotype characterized by increased phagolysosome maturation and creation of a macrophage niche that reduces survival of the intracellular parasite *M. tuberculosis*. Further, the proficiency of Rev-erb α to regulate human IL10 selectively and precisely makes it a valuable target for pharmacological exploitation in infection and tumor regression. Thus, this study will allow us to understand a hitherto unknown mechanism for direct gene regulation of human IL10 by Rev-erb α and utilize the ligand binding domain of Rev-erb α to design small molecules with microbicidal properties.

EXPERIMENTAL PROCEDURES

Cells and Reagents—THP-1 (National Centre for Cell Science (NCCS), India) cells were maintained in RPMI 1640 medium (Gibco) with 10% FBS (Gibco), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). MG132 (carbobenzoxycarboxy-Leu-Leu-leucinal, Sigma). Peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by Ficoll-Hypaque density centrifugation. Recombinant human

GM-CSF and M-CSF and cytokines (eBioscience) were used for differentiation of monocytes into macrophages.

Plasmids and Bacterial Strains—pCMV-XL5-Rev-erb α construct was supplied by OriGene. Full-length Rev-erb α was PCR-amplified using a forward primer with a ClaI restriction site and a reverse primer with a KpnI restriction site and then cloned into pCMV2-FLAG vector. pAdeno-X cloning of Rev-erb α was performed according to the manufacturer's instructions (Clontech). Adenoviral particles were produced, titrated, and stored at -70°C . pSC301 GFP vector capable of expressing in *Mycobacterium* sp. and in *Escherichia coli* was kindly provided by Dr. Yossef Av-Gay. GFP-H37Rv and H37Ra were made by electroporation and selection as described previously (24).

Cell Differentiation and Polarization—THP-1 cells obtained from the NCCS and maintained in RPMI 1640 with 10% FBS and penicillin/streptomycin were plated at a density of 1×10^6 /well in 6-well plates and stimulated with phorbol 12-myristate 13-acetate (PMA) (30 ng/ml) for 6 h. After 6 h, the medium was replaced by fresh complete RPMI 1640 with PMA plus either IFN γ (20 ng/ml) and LPS (100 ng/ml) or IL4 (20 ng/ml) for another 18 h (supplemental Fig. 1). Cells treated with only PMA were taken as controls. Peripheral blood was drawn from healthy volunteers. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation. The isolated mononuclear cells were then resuspended in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS and plated at 5×10^6 cells/well for 2 h ($37^{\circ}\text{C}/5\% \text{CO}_2$) to allow monocyte adherence. After 2 h, nonadherent lymphocytes were removed by PBS washes, and fresh complete medium was then added to the wells. After 24 h, adherent cells were washed with PBS, detached from the wells by scraping with rubber scraper, and counted after trypan blue dye staining. The cells ($>85\%$ monocytes as determined by flow cytometric analysis after staining with anti-CD14 mAbs) were plated either in 96-well plates at the density of 2×10^5 cells/well or in 12-well plates at the concentration of 1×10^6 cells/well and were cultured for in complete RPMI 1640 along with either GM-CSF (50 ng/ml) for M1-type cells or M-CSF (50 ng/ml) for M2-type cells for 5–6 days at 37°C in $5\% \text{CO}_2$ to promote their full differentiation into monocyte-derived macrophages (MDMs). After 5–6 days, these MDMs ($>95\%$ CD14) were stimulated by IFN γ (50 ng/ml) and LPS (10 ng/ml) or IL4 (50 ng/ml) for another 24 h (supplemental Fig. 2) (25, 26).

Transfection and Transduction—siRNA and/or plasmids were transfected into the cells by using Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen). Overexpression of Rev-erb α was performed by infecting primary human monocyte-derived macrophages with adenoviral particles (500–1000 plaque-forming units/cell) in Opti-MEM for 2–4 h followed by the addition of fresh medium and additional incubation of 24 h.

Electrophoretic Mobility Shift Assay—Oligonucleotides were annealed and then end-labeled using T4-poly nucleotide kinase and [γ - ^{32}P]ATP. pCMV-XL5-Rev-erb α was transcribed *in vitro* by T7-polymerase and subsequently translated using the TNT-coupled transcription and translation system (Promega) according to the manufacturer's protocol. A DNA protein bind-

ing assay was performed (27). For all competition experiments, cold oligonucleotides were added after labeled probe.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed according to standard protocol (28). ChIP and re-ChIP-purified DNA fragments along with input DNA were PCR-amplified using primers specific to putative Rev-erb α response elements on the IL10 promoter (+1 to -350). The following primers were used for the detection of ChIP products: ChFwd IL10, 5'-AAT CAA CTT TTT TTA ATT GAG AAG CT-3' and ChRev IL10, 5'-GCC TTC TTT TGC AAG TCT GTC T-3'; ChRevDR2(+), forward, 5'-GGT GGA CTA CAA ATC CCG ACA GTC TT-3' and ChRevDR2(+) reverse, 5'-TGG GAC AGA GGG CTC TGC GC-3'; β -actin forward, 5'-ACT GTT ACC CTC AAA AGC AG-3' and β -actin reverse, 5'-GTG GGT CAC TAG GGA GAG ACC-3'.

Preparation of *M. tuberculosis* and Infection—*M. tuberculosis* H37Ra and H37Rv cultures were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma) and 10% Middlebrook oleic acid albumin dextrose catalase (BD Biosciences) to log phase. Cultures of log phase were used for infection. In brief, cultures were centrifuged for 5 min and resuspended in RPMI 1640 medium containing 10%FBS. For single bacterial cell preparation, culture was syringe-passed carefully for 20–30 times for 3–5 min and then used for infection. To ascertain the multiplicity of infection, 1×10^6 PMA-treated THP-1 macrophages were infected with different dilutions of bacteria for 4 h and then washed three times with medium to remove any unphagocytosed bacteria. Cells were again incubated for 24 h in complete RPMI medium, and then acid-fast staining or confocal imaging was performed to count the cell-associated mycobacteria (29). On the basis of this screening, multiplicity of infection of 1:5 was used for H37Ra and H37Rv infection. At this multiplicity of infection, most macrophages confirmed the presence of 5–10 bacilli per cell.

Mycobacterial Viability Determination by Flow Cytometry and CFU Assay—Infected M1-MDMs with knockdown of Rev-erb α or ectopically overexpressed Rev-erb α were lysed with 0.06% SDS after 24 h of incubation, and bacterial suspensions were used with the LIVE/DEAD BacLight bacterial viability and counting kit as per the manufacturer's instructions (Invitrogen). The percentage of live and dead bacteria was determined by flow cytometry (BD FACSCalibur, BD Biosciences) after staining with SYTO9 and propidium iodide. For CFU determination, after macrophage solubilization, the bacterial suspensions were serially diluted, 50 μ l of each sample was plated, and CFU were counted. Final calculations included the dilution factor and the volume of diluted sample for plating. Anti-IL10 neutralizing antibody (purified Rat anti-human IL10, BD Biosciences) or isotype control was used 30 min prior to infection with H37Ra or H37Rv.

Cell Cytometry—Analysis of cell surface marker expression was performed by surface staining of cells at a density of 10^5 for 30 min at 4 °C by using the appropriate isotype controls with phycoerythrin-conjugated anti-CD86, anti-CD80, anti-CD40, and anti-CD-206 and FITC-conjugated anti-CD-68 (eBioscience/BD Biosciences). Samples were analyzed on a FACSCalibur using Cell-Quest software (BD Biosciences).

ELISA—Cell culture supernatants were harvested after 36 h of siRNA transfection or 24 h of viral transduction and analyzed for IL10 using a commercial kit (BD Biosciences) in accordance with the manufacturer's instructions.

Real-time Quantitative RT-PCR and Western Blotting—Total RNA was isolated by the TRIzol method from macrophages and monocytes, and 1 μ g was reverse-transcribed (Fermentas) according to the manufacturer's protocol and subsequently amplified by PCR using specific primers. GAPDH mRNA was used as a loading control. The relative abundance of gene was calculated using the formula $2^{-\Delta C_T}$, where ΔC_T is calculated as the difference between target gene and GAPDH C_T values. For immunoblotting, 40 μ g of total protein extract was resolved by SDS-PAGE on a 10% acrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. β -Actin, tubulin, and lamin-B (Santa Cruz Biotechnology, Santa Cruz, CA) were used as loading controls for the whole cell extract, cytoplasmic fraction, and nuclear fraction.

Confocal Microscopy—THP-1 cells were treated with PMA (30 ng/ml) and grown on poly-L-lysine-coated coverslips. THP-1, PMA-treated THP-1 cells, and transfected PMA-treated THP-1 cells were then fixed with 4% paraformaldehyde and processed for immunostaining. Rev-erb α (Santa Cruz Biotechnology) was stained using mouse-anti-Rev-erb α or mouse anti-FLAG tag followed by Texas Red conjugated with goat-anti-mouse antibody. Tubulin was stained with Tubulin Tracker, and DAPI (Sigma) was used as a nuclear stain. For immunostaining of Rev-erb α in M1-MDM and M2-MDM, goat-anti-mouse FITC was used as secondary antibody. For co-localization of mycobacteria with lysosomes, GFP-H37Ra and GFP-H37Rv were used to infect macrophages. Cells were then incubated for another 24 h followed by the addition of 100 nM LysoTracker for 30 min at 37 °C and fixation with 4% paraformaldehyde. The coverslips were washed thoroughly with PBS and mounted on slides with antifade reagent (Invitrogen, Molecular Probes). The stained cells were observed with an LSM 510 Meta Carl Zeiss confocal microscope and Nikon A1R confocal microscope. The co-localization of LysoTracker and GFP-H37Ra and H37Rv was quantified by selecting a region of interest and determining the overlap coefficient (30).

DHR123 Assay—For total reactive oxygen species measurement, the cells were incubated with 10 μ M DHR123 probe for 30 min at 37 °C. The cells were harvested and subjected to flow cytometry to determine the levels of reactive oxygen species.

Ethics Statement (Human Peripheral Blood Mononuclear Cell 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 Ethics and Biosafety Committee of the Institute of Microbial Technology (IMTECH), Sector 39A, Chandigarh, India (01/2011/IMT/IEC-Blood; 12/2010IMT/IBSC). 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

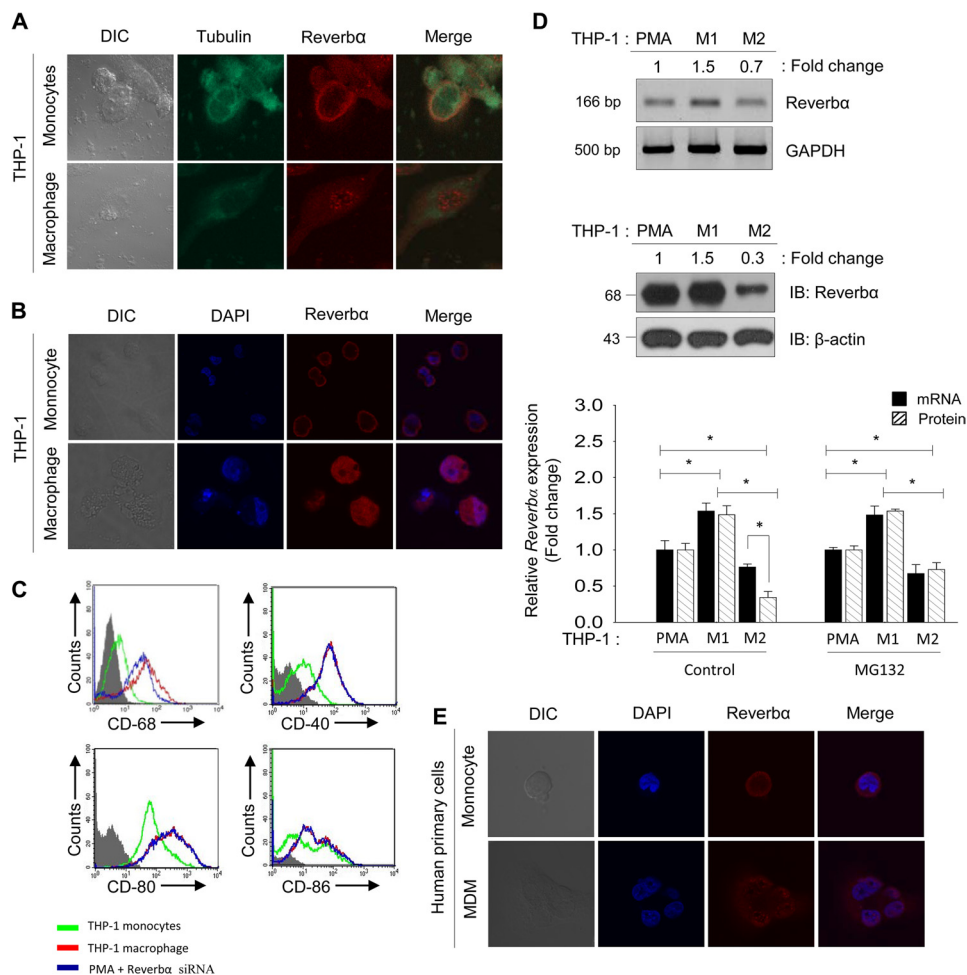


FIGURE 1. Expression and subcellular localization of Rev-erb α in THP1 monocyte-macrophage cells. *A*, THP-1 monocytes and PMA differentiated THP-1 macrophage cells were stained with antibody against Rev-erb α using mouse monoclonal anti-Rev-erb α followed by Texas Red conjugated with goat anti-mouse antibody. Tubulin was stained with tubulin tracker. *DIC*, differential interference contrast. *B*, subcellular localization of ectopically expressed Rev-erb α (FLAG tag) in THP1 monocytes/macrophages using mouse anti-FLAG followed by Texas Red conjugated with goat-anti-mouse antibody. Nucleus was stained with DAPI. *C*, FACS analysis of differentiation and activation markers of macrophage in the Rev-erb α knockdown background. Rev-erb α nuclear localization is consequent to and without any bearing on PMA-induced THP1 monocyte-macrophage differentiation and activation; in its knockdown backgrounds, surface CD68, CD86, CD80, and CD40 were similar to scrambled knockdown. The *green line* indicates THP-1 monocyte cells, the *red line* indicates PMA-treated THP-1 with scrambled RNA, and the *blue line* indicates PMA-treated THP-1 cells with Rev-erb α knockdown. *D*, expression of Rev-erb α was analyzed by RT-PCR and immunoblotting (upper panel and middle panel). To ascertain the relative fold change in expression of Rev-erb α , PMA-stimulated and M1 and M2-programmed THP-1 cells were treated with MG132 (10 μ M) and then subjected to quantitative RT-PCR and immunoblotting (lower panel). Vehicle-treated cells were taken as control. Relative gene and protein expressions were significantly higher in M1 cells in comparison with M2 cells. *E*, human monocytes and MDMs obtained from buffy coat were also stained for endogenous Rev-erb α , as described above. All images were acquired at 60 \times . Asterisks denote significant differences (*, $p < 0.05$). Data are representative of three independent experiments with similar results and are shown as mean \pm S.D. of the indicated number of experiments.

prior to his/her induction in the study. Information to healthy volunteers and consent forms were in languages (English, Hindi, and Punjabi) familiar to the volunteers.

RESULTS

Rev-erb α Expression and Cellular Localization in Monocyte/Macrophage Cell Lines—Rev-erb α has been shown to express in various cell types. It can modulate adipocyte and myocyte differentiation, which led us to question of whether Rev-erb α affects monocyte differentiation to macrophages. THP-1 (THP-1 cells treated with PMA) macrophages are a very well recognized and widely used model for a differentiated tissue macrophage that closely resembles native monocyte-derived macrophage differentiation (supplemental Fig. 1). This model system was utilized to first investigate the localization of Rev-erb α in both monocytes and macrophages. Interestingly, sub-

cellular localization patterns were found to be distinct. In monocytes, Rev-erb α was exclusively cytoplasmic, whereas in macrophages, it was predominantly nuclear; this discrete subcellular localization of Rev-erb α in monocytes and macrophages was confirmed by confocal microscopy and immunoblotting (Fig. 1*A* and supplemental Fig. 3*A*). Ectopic expression of Rev-erb α in monocytes and differentiated macrophages led to a similar localization pattern (Fig. 1*B*). Noise-to-signal ratio was ascertained for anti-Rev-erb α antibody by staining the cells with Rev-erb α knockdown background. Using FACS analysis, we determined surface expression of macrophage differentiation marker CD68, co-stimulatory molecules CD80 and CD86, and CD40 in THP-1 monocytes and THP-1 derived macrophages in Rev-erb α knockdown backgrounds and compared them with a THP-1 macrophage control (Fig. 1*C*). Upon silencing Rev-erb α in THP-1-derived macrophages, no change in

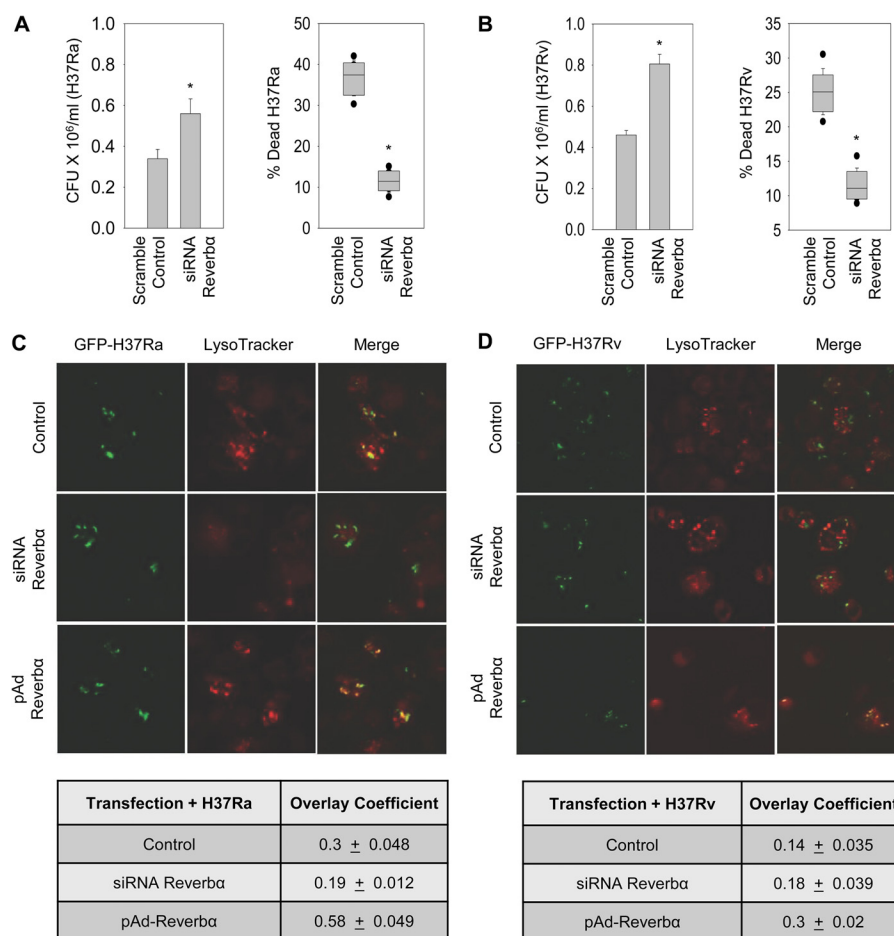


FIGURE 2. Rev-erb α promotes phagosome maturation leading to *M. tuberculosis* clearance. *A* and *B*, loss of function (RNAi; 60 nM; knockdown efficiency ~80%) of Rev-erb α augments intracellular *M. tuberculosis* load, as monitored by CFUs and by monitoring the percentage of dead bacteria by flow cytometry for both H37Ra and H37Rv. *C* and *D*, the extent of co-localization of GFP-H37Ra and H37Rv with acidified lysosomes (stained with LysoTracker) was determined in M1-programmed MDMs (control, Rev-erb α knockdown, and ectopically expressed pAd-Rev-erb α). Images shown for each group are those obtained for mycobacteria (GFP-H37Ra and H37Rv), acidified lysosomes (LysoTracker), or a merge of the two (*Merge*) at 60 \times . The overlap coefficient (at a scale of 1) for both GFP-H37Ra and H37Rv with LysoTracker for 40 consecutive infected macrophages was determined, negating the outliers. The results were verified by six repetitions of the experiments, each of which was conducted in triplicate. A significant increase in intracellular *M. tuberculosis* clearance was observed in macrophages overexpressing pAd-Rev-erb α in contrast to Rev-erb α silenced or control macrophages. Data are representative of three independent experiments with similar results. CFU counts are plotted as the mean \pm S.D., and flow cytometry results are plotted as the median.

expression of differentiation marker or co-stimulatory molecule was observed, suggesting that Rev-erb α had no effect on PMA-induced THP-1 monocyte-to-macrophage differentiation or activation (Fig. 1C). There was a striking difference in the abundance of Rev-erb α in M1- and M2-polarized THP-1 macrophages, with less Rev-erb α (mRNA and protein) present in the alternative M2 macrophages (Fig. 1D, *upper* and *middle* panel). Further, to determine whether proteasomal activity is involved in this difference at the level of both transcription and protein stability, PMA-induced THP-1 cells and M1- and M2-polarized THP-1 macrophages were treated with MG132, a specific inhibitor of 26 S proteasome. Noticeably, MG132 promoted Rev-erb α accumulation in M2-polarized THP-1 macrophages as evident by ubiquitination of Rev-erb α in M2- but not M1-polarized THP-1 macrophages (Fig. 1D, *lower* panel and *supplemental Fig. 3B*). To corroborate the findings in the primary cells, human MDMs were utilized, and an endogenous Rev-erb α staining was performed as mentioned above (Fig. 1E and *supplemental Fig. 3C*). A similar observation of nuclear localization of Rev-erb α was also found in MDMs. Also, real-

time PCR analysis of Rev-erb α mRNA expression was performed on human MDMs programmed into M1 and M2 macrophages (*supplemental Fig. 3D*). Thus, cytoplasmic localization of Rev-erb α , a nuclear receptor, explains its inability to modulate monocyte-macrophage differentiation, but its nuclear localization after differentiation suggests that it may regulate some of the genes and modulate macrophage function.

Rev-erb α Knockdown Cells Are More Susceptible to *M. tuberculosis*—Our previous study has illustrated the role of nuclear receptors in modulating macrophage effector functions in mycobacterial clearance and survival (16). Given that Rev-erb α finds enhanced expression in bactericidal M1 macrophages, we investigated its antimycobacterial role. We employed an RNAi approach to knock down Rev-erb α in M1 macrophages followed by infection with avirulent *M. tuberculosis* H37Ra or virulent *M. tuberculosis* H37Rv and looked for the intracellular *M. tuberculosis* load in the cell by performing a CFU assay. Silencing of Rev-erb α enhanced survival and multiplication of both the bacterial strains. To ascertain the *M. tuberculosis* viability in the cells with Rev-erb α knockdown

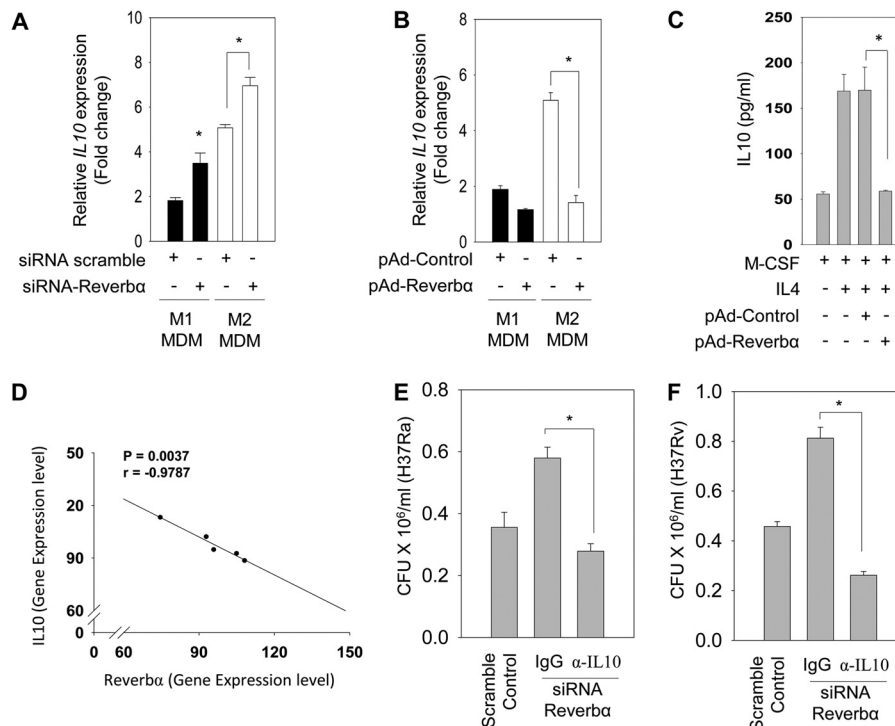


FIGURE 3. Rev-erb α and IL10 display correlation in expression and consequent function. A and B, real-time PCR analysis of IL10 mRNA in M1- and M2-MDMs in Rev-erb α knockdown background and M1 and M2-MDMs infected with pAd-Rev-erb α or pAd-control. C, levels of IL10 secretion were assessed in M2-MDMs infected with pAd-Rev-erb α or pAd-control. D, M2-programmed MDMs infected with pAd-Rev-erb α evaluated for time course expression kinetics of exogenous Rev-erb α and endogenous IL10 mRNA by real-time PCR (at 0, 2, 4, 6, 8, and 12 h) showed negative correlation, with Pearson coefficient, $r = -0.9787$, and p value = 0.0037. Unprogrammed MDMs were taken as control. E and F, M1 macrophages with Rev-erb α knockdown background were treated with anti-IL10 antibody (50 μ g/ml) or isotype control before infection with H37Ra or H37Rv. Cells were later lysed and assessed for mycobacterial growth by CFU assay. Asterisks denote significant differences (*, $p < 0.05$). Data are representative of three independent experiments with similar results. Error bars indicate mean \pm S.D.

background, we used the LIVE/DEAD BacLight viability assay. SYTO9 stains both live and dead bacteria; however, propidium iodide is taken up efficiently by bacteria with damaged membrane, and we found that the percentage of dead bacteria was reduced significantly for both H37Ra and H37Rv (Fig. 2, A and B). Phagolysosome biogenesis is a prime requisite for the elimination of the pervading mycobacterium (31, 32); therefore, we investigated survival of the intracellular pathogen *M. tuberculosis* in these macrophages. *M. tuberculosis* GFP-H37Ra and GFP-H37Rv co-localized better with lysosomes (stained with LysoTracker dye) in control cells and cells that overexpressed Rev-erb α than in Rev-erb α knockdown cells (Fig. 2, C and D). The results from the phagolysosome and bacteria co-localizations correlate and compliment CFU and BacLight assays for both H37Ra and H37Rv.

Rev-erb α Modulation of Antimycobacterial Response of Macrophages Is through IL10—IL10 is secreted in response to mycobacterial infection and is associated with decreased resistance to infection (19, 33). There is clear evidence that the IL10-mediated resistance is associated with blocking of endogenous TNF α production, which inhibits antimicrobial effects of IFN γ ; and also by impeding phagolysosome maturation (20, 34). Therefore, we measured the relative mRNA expression of IL10 in M1- and M2-MDMs with Rev-erb α knockdown background. We found that upon knockdown, the relative expression of IL10 was increased in both M1-MDM and M2-MDM cells as compared with the scramble control (Fig. 3A). To corroborate the

expression analysis, M1- and M2-MDMs were infected with pAd-Rev-erb α adenoviral particles to ectopically express Rev-erb α . We observed that relative IL10 expression was reduced in both the cell types with significant decrease in M2-MDMs (Fig. 3B). Confirmation of ectopic expression by pAd-Rev-erb α in M1- and M2-polarized human MDMs was performed by immunoblot (supplemental Fig. 4). To explore the effect of Rev-erb α on IL10 protein, M2-MDMs were infected with pAd-Rev-erb α or pAd-control, and we determined the IL10 protein expression by ELISA. We found a stark reduction in IL10 expression upon ectopic expression of Rev-erb α in M2-MDMs (Fig. 3C). Earlier studies have mentioned Rev-erb α in the bidirectional regulation of cytokine IL6 as well as in modulation of IL10, which implies that its role is as an equilibrant (35, 36). Also, due to observed antimicrobial effects in Rev-erb α -overexpressing cells, oxidative stress in MDMs overexpressing Rev-erb α was monitored by a DHR123 assay, which indicated significant change in reactive oxygen species (supplemental Fig. 5). The significant reduction in IL10 expression upon Rev-erb α overexpression intrigued us enough to examine time-course gene expression analysis between ectopic Rev-erb α IL10 in M2 cells (Fig. 3D). IL10 expression was negatively modulated by Rev-erb α , and a good correlation was obtained in their expression kinetics. These results suggest that the IL10 gene could be a direct target of Rev-erb α . To further verify whether the decrease in mycobacterial survival upon ectopic expression of Rev-erb α functions through down-regulation of IL10, a CFU

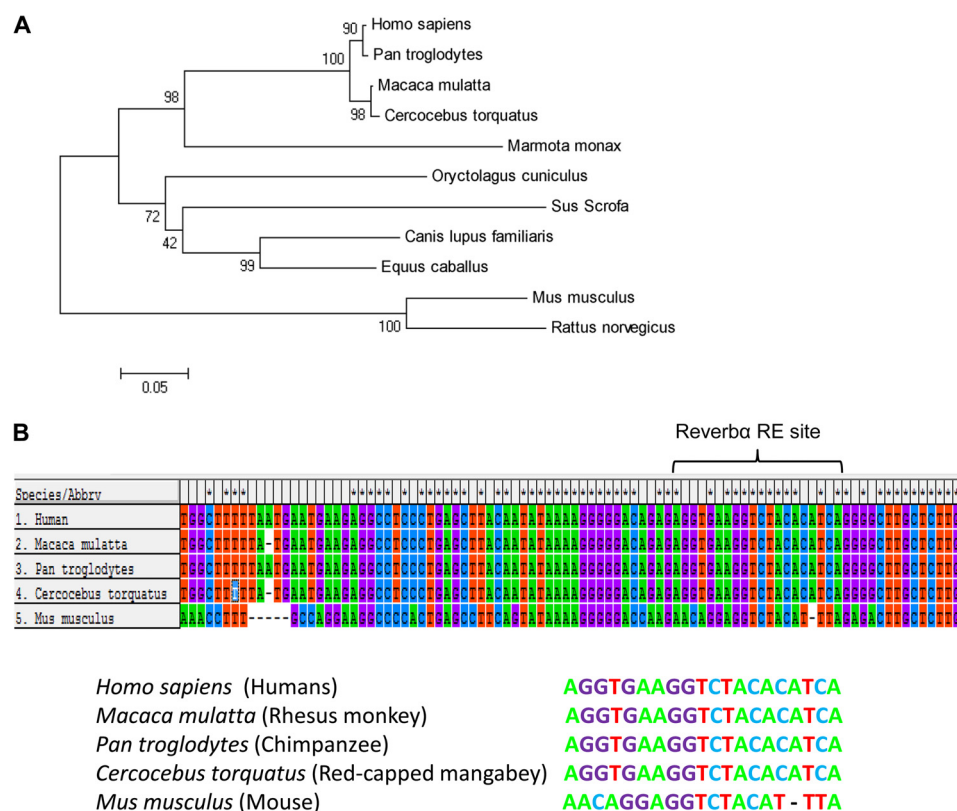


FIGURE 4. **Comparative analysis of IL10 promoter containing Rev-erb α response element.** A, phylogenetic tree of IL10 promoters containing the conserved response element of Rev-erb α constructed by MEGA5 (Molecular Evolutionary Genetics Analysis) software using the neighbor joining method, with 1000 bootstrap replicates. B, multiple pairwise sequence alignment of IL10 promoters encompassing Rev-erb α response elements among various mammalian species, exhibiting heterogeneity in the murine IL10 promoter. Asterisks denote significant differences (*, $p < 0.05$). Data are representative of three independent experiments with similar results.

assay was performed. Cells with knockdown background of Rev-erb α were treated with anti-IL10 antibody for 30 min before infection with H37Ra or H37Rv. Rev-erb α knockdown cells treated with anti-IL10 antibody exhibited lower CFU titers as compared with cells treated with isotype control (Fig. 3, E and F).

Comparative Phylogenetic Analysis of the IL10 Promoter with the Rev-erb α Response Element—On the basis of the above obtained results, we also looked into the correlation between Rev-erb α and IL10 in murine macrophages; however, only subtle changes were observed (data not shown). This observation intrigued us enough to examine the promoter of IL10 gene for the putative response element for Rev-erb α in the IL10 promoter, and to our surprise, response element for Rev-erb α binding was found in the human IL10 promoter. The evolutionary relationship between the promoter sequences of 10 other species comprising the putative response element was also studied by phylogenetic reconstruction (Fig. 4A). Multiple pairwise alignment of IL10 gene promoters containing Rev-erb α response elements of different mammalian species exhibited homology in humans, monkeys, and higher primates (Fig. 4B). In the mouse IL10 gene promoter, however, the half-site and the second core motif of Rev-erb α DR2 were disrupted. This indicates that in mammals, IL10 direct gene regulation by Rev-erb α may be limited to humans and higher order mammalian species and explains the species barrier observed in mouse.

IL10 Is a Direct Target Gene of Rev-erb α —We next determined whether Rev-erb α could bind to the IL10 promoter. We performed ChIP, using anti-Rev-erb α antibodies in human macrophages (Fig. 5A and supplemental Fig. 6). PCR analysis with primers that amplify the proximal IL10 promoter revealed direct and equivalent Rev-erb α binding. Primer specific for Rev-erb α DR2 on its own promoter was used as a positive control for the successful immunoprecipitation by Rev-erb α specific antibody; β -actin served as a negative control. Controls showed that PCR amplification was specific. A bioinformatic search revealed a putative half-site motif flanked by 6 bp of AT-rich sequence, (A/T)₆ PuGGTCA, overlapping with the direct repeat (DR2) of the core motif separated by 2 bp in the proximal region: (−49)-ACAGAGAGGTGAAGGTCTACATCA(−22). Bold nucleotides indicate the purine rich half site prior to the Rev-DR2 sequence; underlined nucleotides indicate the Rev-DR2 sequence. Therefore, to explore whether this DNA region in human IL10 is capable of conferring the repressible action of Rev-erb α , we cloned the proximal region (−70 to +30) in pGL3-Luci, a firefly luciferase reporter plasmid. The basal activity of this IL10 promoter reporter was significantly reduced in Cos-1 cells when Rev-erb α plasmid was co-transfected as compared with control plasmid (Fig. 5B, left panel). Further, a rescue in promoter reporter activity was observed in human MDM programmed M1 and M2 macrophages when the transfection of pGL3-IL10 promoter was performed in the Rev-erb α knockdown background as compared

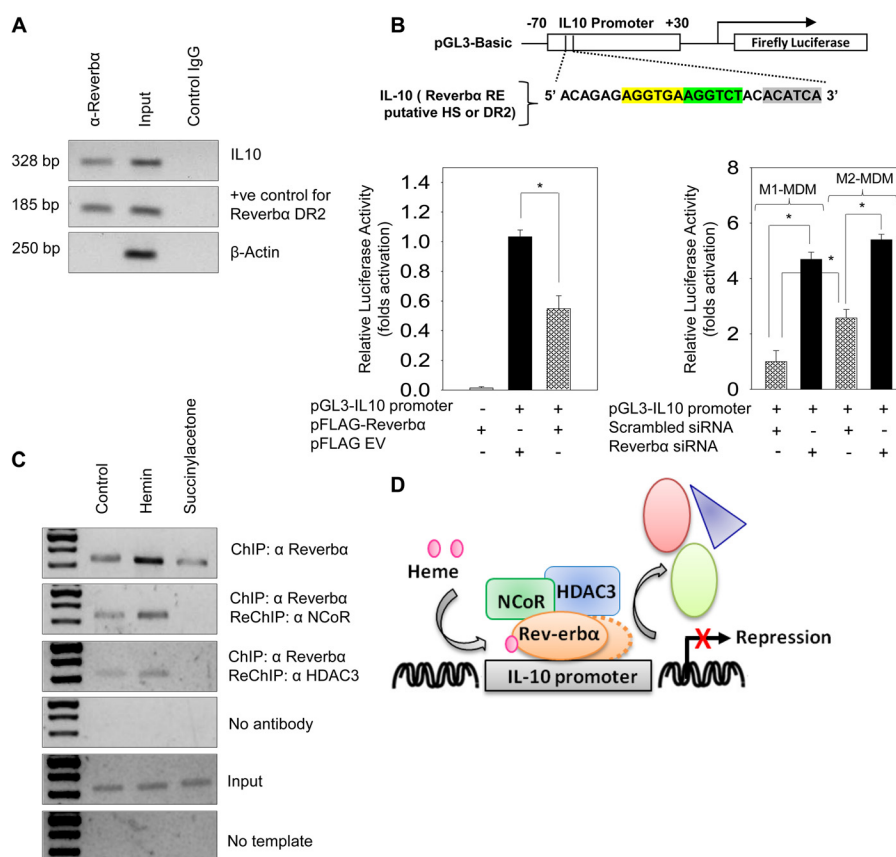


FIGURE 5. The human IL10 proximal promoter contains a functional Rev-erb α response element. *A*, ChIP analysis of Rev-erb α binding at the human IL10 proximal promoter region was performed using chromatin isolated from M1-programmed human MDMs. Cross-linked lysates were immunoprecipitated with isotype control antibody or ChIP-grade Rev-erb α antibody. DNA isolates were subjected to PCR with a primer pair covering the -1 to -350 region of the IL10 gene proximal promoter (top) or the β -actin gene promoter (bottom). PCRs without DNA (Control IgG) or with nonimmunoprecipitated genomic DNA (Input) were also performed. +ve control, positive control. *B*, sequence of IL10 promoter with the putative response element for Rev-erb α (top). Left panel, activity of a pGL3 luciferase reporter driven by the human Rev-erb α response element containing the IL10 gene promoter (-70 to $+30$) upon co-transfection with Rev-erb α in Cos-1 cells. Right panel, activity of the promoter reporter transfected in M1- and M2-programmed MDM cells with control and Rev-erb α knockdown background. Luciferase activity was measured 24 h after transfection. EV, empty vector. Asterisks denote significant differences (*, $p < 0.05$). Data are representative of three independent experiments with similar results. Error bars indicate mean \pm S.D. *C*, identification of the Rev-erb α repressive complex on the IL10 proximal promoter in control cells (vehicle: dimethyl sulfoxide (DMSO)-treated), in the presence of exogenous hemin (6 μ M), or after heme depletion (serum-free medium supplemented with 5 mM succinylacetone). Cells were analyzed for coimmunoprecipitation of NCoR-HDAC3 with Rev-erb α by sequential ChIP assay. *D*, schematic representation of mechanism underlying modulation of protective immune response. Rev-erb α binds to its response element on human IL10 gene promoter by replacing the co-activators followed by recruitment of co-repressors NCoR and HDAC3, resulting in down-regulation of IL10 gene expression.

with scrambled knockdown (Fig. 5*B*, right panel). This demonstrates that IL10 is a target gene of Rev-erb α . Because Rev-erb α negatively regulates IL10, and its repressive function has been shown to be dependent on recruitment of the NCoR-HDAC3 complex, which is dependent on heme, the only known endogenous ligand of Rev-erb α , we looked at the associated transcriptional complex in control, heme-depleted, and exogenous heme (hemin)-treated macrophages. Although hemin treatment slightly augmented Rev-erb α interaction with NCoR-HDAC3 over controls, heme depletion abrogated this interaction and slightly reduced Rev-erb α binding as well (Fig. 5*C* and supplemental Fig. 6). On the whole, the ChIP assay and the reporter assay data indicate that Rev-erb α regulates IL10 promoter negatively. On the basis of the chromatin immunoprecipitation experiments, a schematic was drawn that depicts the underlying mechanism of Rev-erb α -mediated repression of human IL10 (Fig. 5*D*). Binding of nuclear receptor Rev-erb α to the IL10 promoter and heme regulated recruitment and stabilization of the transcriptional co-repressors NCoR and HDAC3 to form a repressive complex that allows down-regulation of IL10 by Rev-erb α .

Rev-erb α Binding as Dimer on Human IL10 Promoter Is Necessary for Its Repressive Activity—To further verify that IL10 is a direct target gene of Rev-erb α ; we performed an electrophoretic mobility shift assay (Fig. 6*A*). The nuclear extract of macrophages was used as an endogenous source for Rev-erb α protein and incubated with labeled IL10 probe. The following complex induced a clear shift in the probe containing the IL10 promoter, but a decreased intensity in the shifted bands was observed upon the addition of anti-Rev-erb α antibody, possibly caused by interference of the antibody with protein-DNA binding. The specificity of sequence recognition of Rev-erb α was analyzed by competitive electrophoretic mobility shift assay (EMSA). The same shifts were produced with *in vitro*-translated Rev-erb α , and the binding was specific as it could be competed with unlabeled (cold) wild-type probe (Fig. 6*B*) and unlabeled, consensus Rev-erb α -DR2 probe, demonstrated as dose-dependent reduction in band intensity as compared with control (Fig. 6*C*). Furthermore, to verify the functional relevance of the nucleotides, mutants of the wild type IL10 probe were prepared. Although mutation in the first and second core

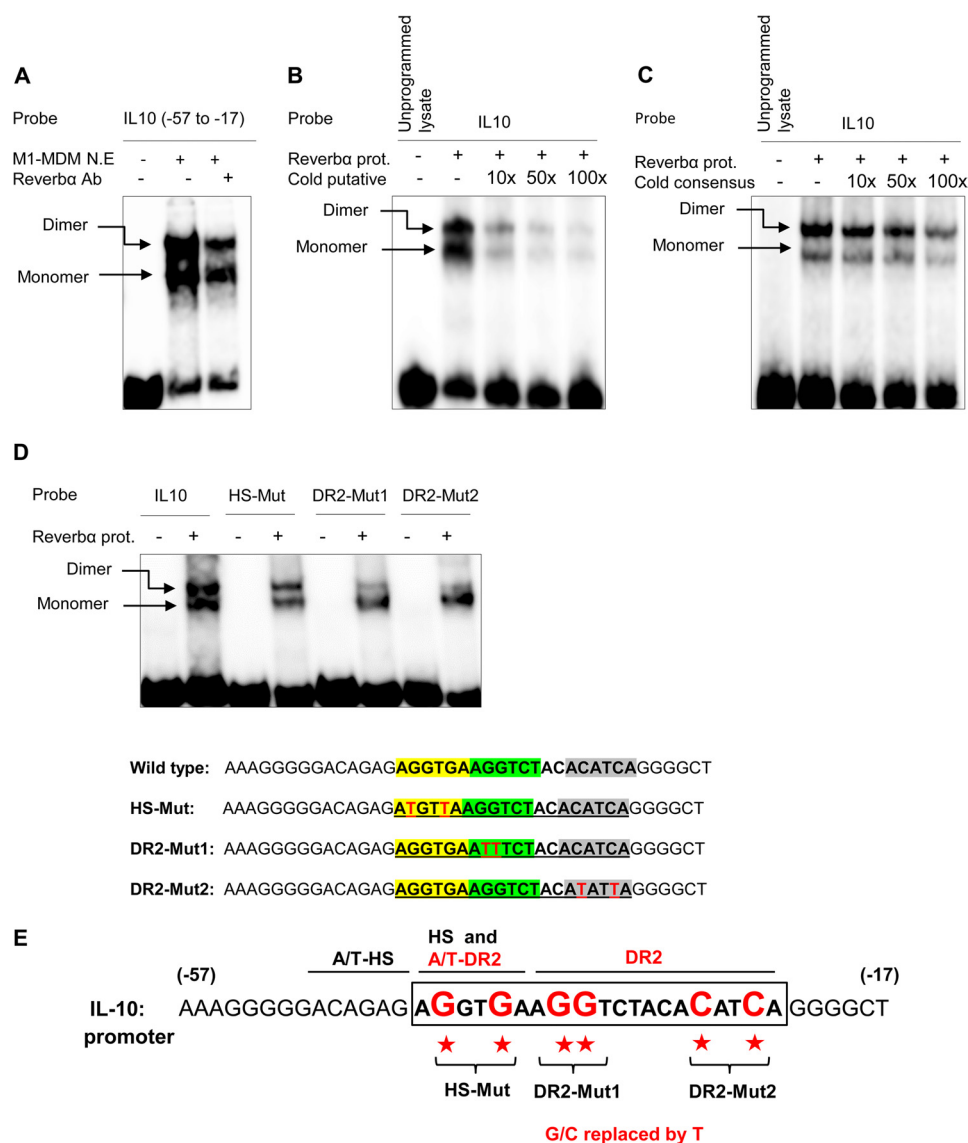


FIGURE 6. Rev-erb α binds to the human-IL10 promoter Rev-erb α DR2 as a homodimer. A, EMSA of putative Rev-erb α response element on IL10 gene promoter with nuclear extract (N.E.) from M1-programmed MDMs. Ab, antibody. B and C, EMSA performed using the end-labeled oligonucleotide bearing the half-site and DR2 (-57 to -17) in the presence of *in vitro* translated Rev-erb α or unprogrammed reticulocyte lysate. Competition experiments were performed by adding 10-, 50-, and 100-fold excess of cold putative IL10 Rev-erb α response element (B) and Rev-erb α DR2 consensus (C). prot., protein. D, EMSA performed using end-labeled oligonucleotide bearing the wild type, half-site mutant (HS-Mut), DR2-Mut1 (DR2 first core motif mutant), and DR2-Mut2 (DR2 second core motif mutant). E, sequences for the wild-type IL10 probe and its subsequent designed mutants.

motif of DR2 abolished the abundance of Rev-erb α binding as a dimer, mutating the half-site preceded by the DR2 sequence such that it retained the preceding A/T-rich region of DR2 had no effect on Rev-erb α binding, indicating that Rev-erb α binds to DR2 and not the overlapping half-site (Fig. 6, D and E).

DISCUSSION

A number of genes that are known to modulate microbicidal properties have been identified in host macrophages. However, identifying new target genes and acquiring an improved understanding of the hierarchy of host functions that lead to mycobacterial clearance remain the focus in designing relevant treatment strategies.

In this study, we found that Rev-erb α augments macrophage microbicidal properties by acting as a transcriptional repressor of IL10 gene. Rev-erb α overexpression in macrophages resulted

in increased phagolysosome maturation and subsequently enhanced bactericidal activity. Mechanistically, Rev-erb α was found to be a repressor of IL10 gene, where along with NCoR and HDAC3, it is recruited at IL10 gene promoter at a Rev-DR2 motif well conserved in higher primates but missing from mice. Rev-erb α was earlier shown to be involved in the induction of adipocyte and myocyte differentiation (37, 38); however, we did not find any bearing of Rev-erb α on monocyte differentiation.

Many nuclear receptors have been implicated in *M. tuberculosis* survival or clearance and inflammation. PPAR γ and TR4 provoke anti-inflammatory and pro-mycobacterium properties of macrophages (16, 17, 25), whereas vitamin D receptor and LXRs on the other hand have anti-inflammatory but anti-infective or bactericidal properties (39, 40). Similarly, we observe that Rev-erb α facilitates *M. tuberculosis* clearance as an effect of its role in reducing IL10 production (Fig. 3, A–C). Rev-erb α

overexpression contributes to induction of certain pro-inflammatory genes such as IL6 and COX-2 through NF- κ B pathway by increasing p65 nuclear translocation (8). Similar findings have also been mentioned in astrocytes, where Rev-erb α competes with ROR α for the up-regulation of IL6 in NF- κ B-dependent pathway (35). Rev-erb α competition with ROR α on IL6 gene promoter, however, has been shown to repress IL6 (35, 36). Temporal differences between Rev-erb α and cytokine expression to different stimuli, cellular milieu, and the circadian clock are suggestive of its role as an equilibrist in inflammation.

IL10 production was first described in Th2 cells; later it was found that other cells of the immune system, including macrophages, dendritic cells, CD8⁺ T cells, B cells, mast cells, and neutrophils also express IL10 (41). Various transcriptional activators that control IL10 expression in T cells and antigen-presenting cells have been identified. JUN proteins and GATA3 regulate IL10 production in Th2 cells (42, 43), whereas transcription factor FOS regulates IL10 production in macrophages and dendritic cells (23, 44). The list of transcription factors that activate IL10 production is increasing; however, only a handful of transcription factors are known to repress its expression (23). Transcription factors ETS1 and T-bet repress *IL10* expression in Th1 cells, BCL-6 inhibits IL10 production in Th2 cells, MHC class II transactivator (CIITA) negatively regulates *IL10* expression in mouse dendritic cells, and BCL-3 represses *IL10* production in macrophages (23, 45–48). However, the quest for master regulators with a direct binding site on the *IL10* promoter still remains. Promoter analysis of *IL10* revealed that it harbors a response element for the binding of the Rev-erb α . *In silico* investigation proved this response element to be exclusive to humans and higher order primates and not mouse, thereby making Rev-erb α a plausible target for therapeutic intervention. Because Rev-erb α is a transcriptional repressor, we analyzed the promoter reporter activity of *IL10* gene and observed that *IL10* gene is being repressed. This repression is mediated by the recruitment of co-repressor complex molecules NCoR and HDAC3 on the promoter of the target gene. Thus, to our knowledge, this is the first study demonstrating *IL10* transrepression by direct binding of a transcription factor and nuclear receptors to its promoter. Further, enhanced Rev-erb α -mediated *IL10* repression generated macrophages that have improved bactericidal competence and better ability to clear the intracellular pathogen *M. tuberculosis*. We observed that there is an increased co-localization of both *M. tuberculosis* avirulent strain H37Ra and virulent strain H37Rv with lysosomes in Rev-erb α knockdown macrophages than in control macrophages. Our results blended perfectly with a previous study indicating that *IL10* expression impeded phagolysosomal maturation (19). Moreover, experiments have shown that an excess of free heme stimulates the generation of free radicals and increases cellular susceptibility to oxidant-mediated killing (49). In our system, heme repressed IL10 production by augmenting Rev-erb α interaction with co-repressor machinery (Fig. 5C). Further, assessment of oxidative stress indicated significant change in reactive oxygen species in MDMs overexpressing Rev-erb α (supplemental Fig. 5). This result combines with the previous studies advocating antimicrobial properties of heme. Taken together, the findings presented here reveal what we

believe is a novel role of Rev-erb α in modulating the antimycobacterial response. Our finding that Rev-erb α is a transcriptional repressor of *IL10* may have ramifications in other macrophage intracellular microparasites as well as in tumor regression because there is growing evidence that IL10 induction is an important component of tumor regression. The antitumor property of IL10 is attributed to its ability to activate natural killer cells, to reduce the surface expression of MHC molecules on target cells, thereby making it more susceptible to natural killer cell lysis, and to block tumor angiogenesis and invasiveness through the induction of metalloproteinase inhibitors (50).

In summary, we have demonstrated the mechanism by which Rev-erb α down-regulates human *IL10* by recruiting NCoR and HDAC3 in macrophages, which alleviates the antimycobacterial host response. Agonist and antagonists that can fit into the ligand binding pocket of Rev-erb α and modulate *IL10* might provide potential therapeutic application.

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