A human xenobiotic nuclear receptor contributes to nonresponsiveness of *Mycobacterium tuberculosis* to the antituberculosis drug rifampicin

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**ABSTRACT**

*Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). It acquires phenotypic drug resistance inside macrophages, and this resistance mainly arises from host-induced stress. However, whether cellular drug efflux mechanisms in macrophages contribute to nonresponsiveness of *M. tuberculosis* to anti-TB drugs is unclear. Here, we report that xenobiotic nuclear receptors mediate TB drug nonresponsiveness by modulating drug efflux transporters in macrophages. This was evident from expression analysis of drug efflux transporters in macrophages isolated from TB patients. Among patients harboring rifampicin-susceptible *M. tuberculosis*, we observed increased intracellular survival of *M. tuberculosis* upon rifampicin treatment of macrophages isolated from patients not responding to anti-TB drugs compared with macrophages from patients who did respond. Of note, *M. tuberculosis* infection and rifampicin exposure synergistically modulated macrophage drug-efflux transporters *in vitro*. We also found that the xenobiotic nuclear receptor pregnane X receptor (PXR) modulates macrophage drug efflux transporter expression and activity, which compromised the anti-TB efficacy of rifampicin. We further validated this finding in a TB mouse model in which use of the PXR antagonist ketoconazole rescued rifampicin anti-TB activity. We conclude that PXR activation in macrophages compromises the efficacy of the anti-TB drug rifampicin. Alternative therapeutic strategies, such as use of the rifampicin derivatives rifapentine and rifabutin, which do not activate PXR, or of a PXR antagonist, may be effective for tackling drug nonresponsiveness of *M. tuberculosis* that arises from drug efflux systems of the host.

*M. tuberculosis* is the primary causative agent of human TB and is responsible for maximum deaths than any other single
bacterial pathogen today. The current choice for TB treatment is the use of chemotherapeutic drugs against various molecular targets of the pathogen. The greatest challenge in the treatment of TB is the rapid emergence of drug resistant *M. tuberculosis*. Therefore, there is an urgent need to study the dynamics of drug resistance and to develop therapeutic strategies that can reduce the chances of development of drug resistant pathogen (1).

The present treatment for TB comprises of combination of chemotherapeutic drugs like isoniazid, rifampicin, pyrazinamide, and ethambutol for at least six months to achieve acceptable cure and relapse rates (2). However, a substantial variability in response to TB therapy is observed, even in patients harboring drug-susceptible strains (3). In some patients, *M. tuberculosis* is rapidly eradicated and patients are cured within 2-3 months of chemotherapy. Yet, in others, viable bacilli persist in sputum for considerably more time, in spite of being drug susceptible *in vitro*. The observed differential response to anti-TB drugs among individuals is poorly understood and could be emanating from host factors such as xenobiotic sensing nuclear receptors, drug metabolizing enzymes and drug efflux transporters (4-7). The majority of drug metabolic processes take place in enterohepatic tissues (liver and intestine) as the drug metabolizing enzymes which facilitate these processes are highly expressed in these tissues. The expression and activity of such enzymes in non enterohepatic tissues is very less and here the drug response is majorly regulated by drug efflux transporters (8-11). In addition to this, microRNAs have been implicated in drug response and chemotherapeutic resistance by modulating the expression of drug metabolizing enzymes and transporters through post transcriptional regulation (12).

Previously it has been reported that *M. tuberculosis* acquires drug nonresponsiveness inside macrophages through induction of its intrinsic drug efflux transporters (13). However, macrophage innate drug efflux mechanisms have not been addressed so far. In this study, we explored the host-pathogen and drug interactions in macrophages to identify the host cell factors contributing to drug nonresponsiveness. Our results highlight the role of host cell xenobiotic nuclear receptor PXR and macrophage drug efflux transporters in the differential drug responsiveness observed in patients infected with drug susceptible *M. tuberculosis*. In addition to this, we have also demonstrated that infection of macrophages with *M. tuberculosis* and treatment with anti-TB drug rifampicin modulates the expression of macrophage drug efflux transporters through PXR. Our *in vitro* observations have been further validated in an *in vivo* mouse model of TB infection.

**RESULTS**

Anti-tuberculosis efficacy of rifampicin is compromised in drug nonresponders harboring the drug susceptible *M. tuberculosis*—To understand the contribution of host cell determinants in the therapeutic outcome of TB, patients were divided into two categories, responders and nonresponders based on the sputum conversion from Acid- Fast Bacilli positive...
(AFB+) to Acid- Fast Bacilli negative (AFB-) after two months (Intensive phase) of DOTS therapy. AFB+ and AFB- patients were considered as nonresponders and responders respectively. The patients who were harboring drug resistant bacteria were excluded from the study. The drug susceptibility of the *M. tuberculosis* was evaluated by culturing as well as by multidrug resistant-TB (MDR-TB) rapid genotypic test of sputum as per Revised National Tuberculosis Control Programme (RNTCP) guidelines. We evaluated the intracellular survival of *M. tuberculosis* in human monocyte derived macrophages (hMDMs) isolated from responders and nonresponders in presence or absence of rifampicin, isoniazid or ethambutol by colony forming unit (CFU) assay. *M. tuberculosis* survival was significantly higher in macrophages treated with rifampicin, isolated from nonresponders as compared to responders (Fig. 1A). However, we did not find any difference in *M. tuberculosis* survival in macrophages treated with the other two frontline drugs isoniazid and ethambutol. The uptake of *M. tuberculosis* was assessed after 4 h of infection in the absence of drug and was found similar in both the study groups (Fig. 1B). The above data suggests that host cell mechanisms influence the anti-mycobacterial efficacy of rifampicin and the intracellular *M. tuberculosis* survival, despite the intrinsic susceptibility of the bacteria to rifampicin.

Expression and activity of macrophage drug efflux transporters are modulated by *M. tuberculosis* infection and exposure to rifampicin—It has been reported that macrophage drug efflux transporters are likely to play a crucial role in drug responsiveness in intracellular infections (14-16). We monitored the expression of P-glycoproteins/ ATP-binding cassette transporter subfamily B member 1 (P-gp/ABCB1); multidrug-resistance associated proteins (MRP1/ABCC1 and MRP2/ABCC2) and breast cancer resistant protein (BCRP/ABCG2), the prototype drug efflux transporters by quantitative real-time PCR (qRT-PCR) and immunoblot analysis in hMDMs (isolated from healthy donors) infected with rifampicin sensitive or resistant *M. tuberculosis* and treated with or without rifampicin (Fig. 2A and B). A dynamic host–pathogen and host–drug interaction was discovered.

We observed an increased expression of ABCC2 and ABCG2 but not of ABCB1 and ABCC1 in macrophages infected with rifampicin sensitive or resistant *M. tuberculosis* (Fig. 2A and B). However, rifampicin treatment upregulates the expression of all the four transporters. Further, the expression of ABCC2 and ABCG2 was significantly increased in macrophages infected with rifampicin resistant strain and cotreated with rifampicin as compared to either of them. (Fig. 2A and B). This synergistic effect was not observed in macrophages infected with rifampicin sensitive strain. This could be due to the anti-microbial activity of rifampicin in macrophages infected with rifampicin sensitive strain. Furthermore, we also looked for the expression of primary xenobiotic sensing nuclear receptors PXR and constitutive androstane receptor (CAR) which are reported to transcriptionally regulate drug efflux transporters in various tissues (17,18). However, we did not observe any significant change in the expression of PXR and CAR in *M. tuberculosis* (rifampicin sensitive or resistant) infection or rifampicin treatment (Fig. 2A and B). We further monitored the functional activity of these transporters by
their ability to efflux drug like fluorescent molecules: rhodamine 123 (substrate for P-gp), 5,6-carboxyfluorescein diacetate (CFDA; specific for MRP mediated efflux) and mitoxantrone (substrate for ABCG2) in hMDMs infected with rifampicin sensitive or resistant *M. tuberculosis* and treated with or without rifampicin (Fig. 2C). We observed that *M. tuberculosis* (rifampicin sensitive or resistant) infected hMDMs were more efficient in the efflux of mitoxantrone and CFDA but not rhodamine 123 when compared to uninfected control. Moreover, efflux of mitoxantrone and CFDA was further increased in rifampicin resistant *M. tuberculosis* infected hMDMs treated with rifampicin (Fig. 2C). This clearly suggests that *M. tuberculosis* infection and rifampicin treatment synergistically modulates the expression and activity of some of the macrophage prototype drug efflux transporters.

*M. tuberculosis* infection and rifampicin treatment modulates the macrophage drug efflux potential by modulating the ABC transporters expression through xenobiotic nuclear receptor—As stated above PXR and CAR are known to regulate the expression of drug efflux transporters and rifampicin is known to activate both PXR and CAR (19). Also, activated PXR and CAR leads to induction of a set of overlapping target genes (20). Therefore, we investigated the role of PXR and CAR in modulating the macrophage efflux transporter expression induced by *M. tuberculosis* infection and by rifampicin treatment. We monitored the expression of *ABCB1, ABCC1, ABCC2 and ABCG2* in hMDMs with control, PXR or CAR knockdown background which were infected with rifampicin resistant *M. tuberculosis*, in the presence or absence of rifampicin (Fig. 3).

As observed earlier (Fig. 2), the expression of *ABCB1* and *ABCC1* was not modulated by *M. tuberculosis* infection but increased only upon rifampicin treatment. Interestingly, this increase was abrogated in PXR but not in CAR knockdown background (Fig. 3A and B). On the other hand the expression of *ABCC2 and ABCG2* was increased in both infected or rifampicin treated control cells, with a synergistic increase in control infected macrophages cotreated with rifampicin. This increase was abrogated in PXR but not in CAR knockdown background (Fig. 3C and D). This suggests that PXR but not CAR plays a crucial role in altered expression of the macrophage efflux transporters induced by *M. tuberculosis* infection as well as rifampicin. Further, to validate the role of PXR in the modulation of expression and activity of drug efflux transporters in hMDMs, PXR gain and loss of function studies were done. In PXR knockdown cells, the expression and activity of drug efflux transporters was very less in comparison to control and PXR overexpressed cells (Fig. 4A). Upon comparing uninfected and infected hMDMs treated with or without rifampicin, we found that the expression of *ABCC2 and ABCG2* as well as efflux of CFDA and mitoxantrone was comparatively high in infected cells than in uninfected cells which were further increased upon rifampicin treatment both in control and PXR overexpressed hMDMs but not in PXR knockdown hMDMs. This clearly suggests that the expression of PXR plays a key role in the modulation of macrophage drug efflux transporters mediated by *M. tuberculosis* infection as well as rifampicin.
PXR contributes to nonresponsiveness to rifampicin—Given that PXR activation modulates macrophage drug efflux potential, we investigated the function of PXR in drug-mediated intracellular \textit{M. tuberculosis} clearance. We assessed the survival of rifampicin sensitive \textit{M. tuberculosis} in PXR overexpressing macrophages exposed to an increasing concentration of rifampicin in presence or absence of ketoconazole (an antagonist of PXR) (Fig. 5A). After 48 h of infection, the intracellular survival of \textit{M. tuberculosis} was evaluated by using the CFU assay. We observed an increase in the intracellular survival of \textit{M. tuberculosis} as well as an increase in the minimal inhibitory concentration (MIC) value of rifampicin in PXR overexpressed hMDMs as compared to LacZ control hMDMs. This increase was abrogated with ketoconazole treatment. We also verified the activation of PXR with CYP3A4-XREM gene promoter assay and found that PXR was activated at all the used concentrations of rifampicin and this activation of PXR was abrogated when we used ketoconazole (Fig. 5B). We then selectively investigated the ability of rifampicin to cross talk with PXR and modulate \textit{M. tuberculosis} survival by employing a rifampicin resistant \textit{M. tuberculosis} strain in an intracellular bacterial survival CFU assay (Fig. 5C and D). We compared the survival of rifampicin sensitive and resistant strains of \textit{M. tuberculosis} in control, PXR knockdown or PXR overexpressing hMDMs in the presence or absence of rifampicin. A significant increase in the intracellular survival of both rifampicin resistant and rifampicin sensitive \textit{M. tuberculosis} strains was observed in PXR overexpressed hMDMs as compared to LacZ control or PXR knockdown hMDMs. Interestingly, survival of rifampicin resistant \textit{M. tuberculosis} was significantly increased upon rifampicin treatment of PXR overexpressing hMDMs. However, rifampicin sensitive \textit{M. tuberculosis} was effectively cleared in control and PXR knockdown hMDMs and to a lesser extent in PXR overexpressing hMDMs upon treatment with rifampicin. This clearly suggests that PXR overexpression leads to nonresponsiveness to rifampicin treatment in human macrophages.

Relative expression of xenobiotic nuclear receptors and drug efflux transporters in macrophages derived from patients responding differentially to anti-TB drugs—To expand our findings that xenobiotic nuclear receptors are responsible for drug nonresponsiveness to frontline anti-TB drug rifampicin, we extended our study to clinical samples. We compared the relative expression of PXR and CAR in macrophages isolated from patients who were responding differentially to anti-TB drugs by using qRT-PCR (Fig. 6A-F). Interestingly, we observed a significant higher expression of PXR in drug nonresponsive patients when compared to drug responders (Fig. 6A and B). In addition to PXR and CAR, we also monitored the expression of drug efflux transporters (\textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2} and \textit{ABCG2}). Expression of \textit{ABCC2} and \textit{ABCG2} was significantly higher in drug nonresponsive patients when compared to drug responders (Fig. 6E and F).

Human PXR modulates drug nonresponsiveness in mice model of TB—In order to validate our \textit{in vitro} findings in an \textit{in vivo} setting we used the C57BL/6 mice model of TB. We overexpressed hPXR in C57BL/6 mouse lungs using an adenoviral overexpression system. Control LacZ and
hPXR overexpressing mice were infected with rifampicin sensitive *M. tuberculosis*. After 15 days of infection, mice were divided into six groups as shown in Figure 7 and treated with respective single drug or combination of drugs. After 1 month of treatment the total number of bacilli in lungs was determined by CFU assay (Fig. 7). We observed a significant increase in number of bacilli in the lungs of hPXR overexpressing mice as compared to LacZ control mice and this increase was abrogated upon ketoconazole treatment which is an antagonist of activated hPXR. Further, on treatment with the frontline TB drug rifampicin or its derivative rifabutin, there was a significant decrease in the number of bacilli in the lungs as compared to untreated mice both in control LacZ and hPXR overexpressed mice. However, when we compared the anti-mycobacterial efficacy of rifampicin and rifabutin in LacZ and hPXR overexpressed mice, we observed that the efficacy of rifampicin and not rifabutin is compromised in PXR overexpressed mice as compared to LacZ mice and this effect was abrogated when mice were cotreated with ketoconazole. This could be due to the fact that rifabutin activates PXR to a lesser extent as compared to rifampicin (21). Above described observation suggests that increased expression and activity of hPXR plays a crucial role in drug nonresponsiveness to rifampicin.

**DISCUSSION**

The prevalence of drug-resistant TB poses a major risk to public health (22). The mechanisms by which *M. tuberculosis* is able to withstand the action of antibiotic therapy are highly diverse (23) and are largely defined by the genetic and epigenetic determinants of *M. tuberculosis* (24). Our study expands the drug resistance mechanisms exploited by *M. tuberculosis* and reveals a novel interaction at the interface of infection and consequent antimycobacterial drug exposure. Here, we have explored the role of macrophage xenobiotic nuclear receptors in drug nonresponsiveness exhibited by individuals infected with *M. tuberculosis*. We found that the intracellular survival of drug susceptible *M. tuberculosis* was higher in macrophages treated with rifampicin, isolated from drug nonresponders as compared to the responders (Fig. 1A). This result validates the contribution of host cell factors in anti-TB efficacy of rifampicin in TB patients who are not responding to anti-TB treatment inspite of harboring drug susceptible *M. tuberculosis*.

Drug efflux transporters play a crucial role in modulating the efficacy of antimicrobial compounds which target intracellular pathogens (14-16). We found that rifampicin treatment modulates the expression and activity of macrophage drug efflux transporters ABCB1, ABCC1, ABCC2 and ABCG2 and *M. tuberculosis* infection modulates ABCC2 and ABCG2 only. Further, infected macrophages cotreated with rifampicin showed a synergistic increase in the expression and activity of ABCC2 and ABCG2 (Fig. 2). PXR and CAR are known to play a key role in modulating the host cell drug efflux transporters and it has also been reported that rifampicin modulates efflux transporters by modulating the activity of PXR (17,18,25). Moreover, our previous study demonstrated that *M. tuberculosis* modulates PXR activity through its cell wall lipids (26). These reports instigated us to monitor the expression and activity of drug efflux transporters in *M. tuberculosis* infected macrophages with PXR and CAR knockdown background in presence or
absence of rifampicin. We observed a decrease in the expression of \textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2} and \textit{ABCG2} in rifampicin treated hMDMs with PXR knockdown as compared to control (Fig. 3). However, we observed a partial decrease in \textit{ABCC2}, \textit{ABCG2} (Fig. 3C and D) in \textit{M. tuberculosis} infected hMDMs with PXR knockdown as compared to control. This effect could be due to some other host pathways being modulated by \textit{M. tuberculosis} in addition to PXR. Further, no change was observed in CAR knockdown hMDMs.

Proof of concept experiment using PXR gain of function, revealed that hMDMs overexpressing PXR were more efficient in the efflux of drug like fluorescent molecules CFDA, rhodamine 123 and mitoxantrone as compared to the hMDMs with control and PXR knockdown background (Fig. 4B). This enhanced efflux was observed due to the increased expression of \textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2} and \textit{ABCG2} (Figure 4). In addition to this, a significant increase in the expression of \textit{ABCC2} and \textit{ABCG2} was observed in \textit{M. tuberculosis} infected macrophages as compared to the uninfected control which resulted in efflux of CFDA and mitoxantrone but not rhodamine 123. Our observations also support the previous finding that bone marrow mesenchymal stem cells expressing ABCG2 provide an ideal niche for non-replicating \textit{M. tuberculosis} (27). Similarly, PXR overexpressing macrophages seem to be an ideal protective niche for \textit{M. tuberculosis} to evade from drug therapy.

Rifampicin, a frontline antibiotic for TB treatment also activates hPXR, therefore we investigated how hPXR expression and activity modulates the effective dosage of rifampicin treatment and intracellular survival of \textit{M. tuberculosis}. Interestingly, an increase in the MIC value of rifampicin was observed in hPXR overexpressing hMDMs which was abrogated in presence of ketoconazole (Fig. 5A). This suggests that expression and activity of hPXR modulates the efficacy of rifampicin. This was further confirmed by using rifampicin sensitive and resistant strains of \textit{M. tuberculosis} (Fig. 5C and D). To the best of our knowledge, we are first to report the role of PXR in conferring TB drug nonresponsiveness, and to show that the expression of PXR correlates with TB drug nonresponsiveness. Furthermore, we profiled the expression levels of human PXR, CAR and drug efflux transporters in drug responding and nonresponding TB patients (Fig. 6). We discovered that expression of PXR and \textit{ABCC2} and \textit{ABCG2} were relatively high in drug nonresponders in comparison to the drug responders harboring the drug susceptible strain of \textit{M. tuberculosis} (Fig. 6A, E and F). Our data suggests that the expression of PXR and its target genes may affect the tissue specific pharmacokinetics of anti-TB drugs at the site of infection. Previously it has been reported that intracellular drug tolerance in \textit{M. tuberculosis} infection is acquired through induction of its own drug efflux transporter inside macrophages (13). Here in this study, we have shown that \textit{M. tuberculosis} and host transcription factor PXR together modulate the expression of macrophage drug efflux transporters, which in turn leads to drug nonresponsiveness. It seems that intracellular drug tolerance or drug nonresponsiveness exhibited by \textit{M. tuberculosis} to frontline drugs such as rifampicin could be due to this mutual cross-regulation of drug efflux transporters of macrophage and \textit{M. tuberculosis}.

To validate our \textit{in vitro} observations, we generated a humanized PXR mouse.
model by transducing hPXR in mouse. Comparing the efficacy of rifampicin and rifabutin in control and hPXR overexpressed mice, we observed that the efficacy of rifampicin but not rifabutin is compromised, which was restored when mice were cotreated with ketoconazole (Fig. 7). These results suggested that PXR expression and activity may affect the outcome of TB infection and TB therapy. Consequently, inclusion of a PXR antagonist in combinatorial drug treatment is likely to be a promising strategy to treat TB.

EXPERIMENTAL PROCEDURES

Human Ethics Statement— The project was approved by the Ethics Committee of the Government Medical College and Hospital, Sector 32 (Chandigarh, India), and the Ethics and Biosafety Committee of IMTECH, Sector 39A (Chandigarh, India). 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 (26).

Animal Study and Ethics Statement— C57BL/6 mice (male, 6–8 week old) were procured from the animal facility at IMTECH and were housed at the Biosafety Level 3 (BSL3) facility of the Institute. All experiments were approved by the Institutional Animal Ethics Committee of IMTECH as stated earlier (26).

Study plan and study subjects—To assess the role of host cell determinants in the differential drug responsiveness in TB, adult patients (18–55 years) diagnosed with active TB were enrolled into the study based on certain decisive factors: (i) Therapeutic response to anti-TB drugs after initial two months (Intensive phase) of DOTS therapy (ii) Harboring drug susceptible M. tuberculosis (iii) HIV negative. These approaches reduce the possibility of potential confounding effects of treatment failure as a result of bacterial drug resistance and disparity in therapeutic response due to altered immune functions. Patients were divided into two categories - responders and nonresponders based on the sputum conversion from AFB+ to AFB- after initial two months of DOTS therapy. The drug susceptibility of the M. tuberculosis in these patients was evaluated as per Revised National Tuberculosis Control Program (RNTCP) guidelines. Gene expression profiling of xenobiotic nuclear receptors and drug transporters was performed to identify host cell genes with putative roles in differential drug responsiveness in TB patients. Macrophages derived from peripheral blood derived monocytes (PBMCs) were used for functional validation assays.

Aerosol infection and determination of M. tuberculosis burden—Experimental 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 (Glas-Col; Terre Haute, IN) in the BSL3 facility, at IMTECH as described previously (28). Following infection, mice were divided into different treatment group (five mice per group) and were allowed to establish the infection for 15 d. After 15 d of infection mice were treated with drugs 5 d per week for a month and after one month of treatment mice were
sacrificed and lungs were homogenized in 2 ml sterile PBS. Serial dilutions of lung homogenate were plated on Middlebrook 7H11 agar plates. Colonies were counted after 20 d of incubation at 37°C, and CFUs per lung were determined. Drugs were used in the experiments at the following concentrations: Ketoconazole-75 mg/kg; Rifampicin-10 mg/kg; and Rifabutin-150 mg/kg. Control group mice were treated with vehicle.

**Bacterial strains and culture conditions**—*M. tuberculosis* (H37Rv) was obtained from IMTECH-Microbial Type Culture Collection Chandigarh or the National Institute for Research in Tuberculosis, Chennai, India. Rifampicin resistant *M. tuberculosis* (Zopf) Lehmann and Neumann (ATCC-35838) was obtained from the American Type Culture Collection (ATCC). The two strains were cultured in 7H9 broth medium (BD Difco Laboratories, 271310) containing 0.2% glycerol and 0.05% tween 80. 10% Middlebrook oleic acid albumin dextrose catalase (OADC) (BD Difco Laboratories, 211886) was added as a supplement, and the culture was incubated with shaking at 37°C. Frozen stocks were made by resuspending the log phase cultures in sterile 7H9 broth containing 15% glycerol and were stored at −80°C until use.

**Bacterial viability assays**—Macrophages were washed with incomplete RPMI medium after 4 h of infection with *M. tuberculosis*, and incubated in complete RPMI media for 48 h. After 48 h the cells were then solubilized in 100 µL of 0.06% SDS in PBS, and the bacterial suspension was serially diluted and 100 µL of each diluted sample was plated on 7H11 agar plates. The plates were then incubated at 37°C for 15 to 20 d and after that CFUs were counted.

**Luciferase reporter assays**—hMDMs were transiently transfected with CYP3A4-XREM, pSG5-hPXR and pBind plasmids for promoter reporter assay using turbofect transfection reagent (Thermo Scientific, R0533). pBind vector having Renilla luciferase was used as transfection control. Dual luciferase activity was monitored as described previously (29). Firefly luciferase activity was normalized against Renilla luciferase and the normalized luciferase activities (relative light units) were plotted as an average of triplicate samples.

**Gene silencing and overexpression**—For gene silencing, we used siRNA and shRNA approach. For overexpression studies, we used recombinant adenovirus expressing LacZ (Ad-LacZ) and hPXR (Ad-PXR), which were produced by using Adeno-X Expression System 1 (Clontech, catalog number 631513), according to the manufacturer’s instructions. For transduction in vitro, hMDMs were incubated on day 7 of culture with adenovirus in RPMI 1640 medium supplemented with 10% FBS and penicillin streptomycin (100 U/ml). The cells were incubated in a humidified, CO₂ (5%) incubator at 37°C for 24 h, followed by aspiration of supernatant and replacement of media. Cells were further incubated for an additional 24 h before experimental assays. For in vivo studies, mice were injected in the tail vein and aerosol challenged with Ad-LacZ or Ad-PXR by using the nebulization system as described previously (26).

**Efflux assay**—hMDMs were seeded on 12 well plate at a density of 5x10⁵ cells/well and allowed to adhere overnight. Cells were then transduced with adenovirus either for knockdown or overexpression of hPXR. After 48 h of transduction, cells were infected with *M. tuberculosis* H37Rv as...
described in infection section and incubated for 48 h in presence or absence of rifampicin. Cells were then washed with PBS and incubated in 2 μM rhodamine123; 5 μM of CFDA or 3 μM mitoxantrone for 15 min. at 37°C. Cells were then washed with cold PBS and incubated for 30 min. in 500 μL HBSS at room temperature to allow the efflux. This conditioned HBSS was then filtered and fluorescence was measured by using Biotech synergy fluorimeter (30).

Cell culture and differentiation—PBMCs were isolated from fresh blood using a Ficoll-paque gradient centrifugation. Cells were incubated for 2-3 h at 37°C and then washed with phosphate buffered saline (PBS) to remove non-adherent cells, followed by 7 d of incubation in RPMI supplemented with 10% fetal bovine serum (FBS) and macrophage colony-stimulating factor (M-CSF; 50 ng/ml) at 37°C and 5% CO₂ (26).

Immunoblot analysis—Whole cell lysates were prepared and standard procedures were followed for immunoblotting. Briefly, equal amounts of total proteins were separated by SDS-PAGE on a 10% acrylamide gel and then transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% BSA for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies against ABCB1, ABCC1, ABCC2, ABCG2, PXR, CAR and β-actin. Membranes were then washed three times with 1X PBST (pH 7.4) and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for an hour at room temperature and developed with chemiluminescent HRP substrate.

Infection—hMDMs were infected either with M. tuberculosis H37Rv or rifampicin resistant strains at a multiplicity of infection (MOI) 1:5 (5 bacteria/cell). After 4 h, infected cells were washed thrice with RPMI to remove unphagocytosed bacteria and incubated with repletion medium.

RNA isolation and quantitative real-time PCR (qRT-PCR)—Total RNA from hMDMs was isolated by the TRIzol reagent. 1 μg RNA was reverse-transcribed for cDNA synthesis and subsequently subjected to qRT-PCR using gene specific primers. 18s rRNA was used as control. The relative abundance of the gene was calculated by using the formula $2^{-\Delta\Delta Ct}$ as described previously (31).

Statistical analysis—Results are expressed as the mean ± S.D unless otherwise mentioned. Sigma Plot was used for statistical analysis. Two-tailed Student’s $t$ tests and Mann-Whitney test were performed to obtain $p$ values. Statistical significance was established at $p < 0.05$ (*).
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Conflict of interest
The authors have no financial conflicts of interest.

Author’s Contribution
EB, PG designed the experiments, EB, DT, NA, RN, AS, RK and SK, performed the experiments, EB, PG and AKJ analyzed the data, EB and PG wrote the manuscript, PG supervised the project.
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FOOTNOTES
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The abbreviations used are: Acid-Fast Bacilli (AFB), Breast cancer resistant protein (BCRP/ABCG2), Colony forming unit (CFU), Constitutive androstane receptor (CAR), 5,6-carboxyfluorescein diacetate (CFDA), Human monocyte derived macrophages (hMDMs), multidrug-resistance associated proteins (MRP1/ABCC1 and MRP2/ABCC2); P-glycoproteins/ATP-binding cassette transporter subfamily B member 1 (P-gp/ABCB1); Pregnane X receptor (PXR), Tuberculosis (TB).

FIGURE LEGENDS
FIGURE 1: Anti-tuberculosis efficacy of rifampicin, isoniazid and ethambutol against the intracellular survival of \textit{M. tuberculosis} in macrophages isolated from drug responders and nonresponders. (A) Intracellular survival of drug susceptible \textit{M. tuberculosis} in hMDMs of TB drug responding (n=8) or nonresponding (n=8) patients in absence or presence of frontline anti-TB drugs (rifampicin, isoniazid or ethambutol) for 48 h. (B) Intracellular bacterial load in hMDMs of responders and nonresponders following 4 h of infection in the absence of drug. Bacterial survival was measured by CFU assay. Horizontal lines represent the mean.*,, \( p < 0.05 \) by the Mann-Whitney test.

FIGURE 2: Expression and activity of macrophage drug efflux transporters are modulated by \textit{M. tuberculosis} infection and exposure to rifampicin. (A) qRT-PCR; (B) Immunoblot analysis of ABC transporters (\textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2}, and \textit{ABCG2}) and xenobiotic nuclear receptors (PXR and CAR) in hMDMs isolated from healthy volunteers infected with rifampicin sensitive or resistant \textit{M. tuberculosis} (\textit{r-M.tb}) and treated with or without rifampicin. (C) rhodamine 123, CFDA and mitoxantrone efflux potential of hMDMs isolated from healthy volunteers infected with rifampicin sensitive or resistant \textit{M. tuberculosis} (\textit{r-M.tb}) and treated with or without rifampicin. Data are mean ± SD from three independent experiments each performed in triplicate. *, \( p < 0.05 \) by two-tailed student’s \( t \) test.

FIGURE 3: \textit{M. tuberculosis} infection and rifampicin treatment modulates the macrophage drug efflux potential by modulating the ABC transporters expression through xenobiotic nuclear receptor. (A-D) qRT-PCR analysis of ABC transporters (\textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2}, and \textit{ABCG2}) in control (Scrambled RNA), PXR knockdown (PXR si-RNA) and CAR (CAR si-RNA) knockdown hMDMs isolated from healthy volunteers infected with rifampicin resistant \textit{M. tuberculosis} (\textit{r-M.tb}) and treated with or without rifampicin. Data are mean ± SD from three independent experiments each performed in triplicate. *, \( p < 0.05 \) compared to control or as indicated.

FIGURE 4: PXR expression and activation are crucial for the macrophage drug efflux potential modulated by \textit{M. tuberculosis} infection and rifampicin treatment. (A) qRT-PCR analysis of ABC transporters (\textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2}, and \textit{ABCG2}) in control, PXR knockdown and PXR overexpressing hMDMs isolated from healthy volunteers infected with rifampicin resistant \textit{M. tuberculosis} (\textit{r-M.tb}) and treated with or without rifampicin. (B) rhodamine 123, CFDA and mitoxantrone efflux potential of hMDMs isolated from healthy volunteers infected with rifampicin resistant \textit{M. tuberculosis} (\textit{r-M.tb}) and treated with or without rifampicin. Data are mean ± SD from three independent experiments each performed in triplicate. *, \( p < 0.05 \) compared to control or as indicated.
FIGURE 5: Human PXR activation in hMDMs leads to drug nonresponsiveness. (A) Intracellular survival assay of *M. tuberculosis* H37Rv, exposed to an incremental concentration of rifampicin (0.025 to 1 μg/ml) in presence and absence of ketoconazole in control and PXR overexpressing hMDMs. (B) CYP3A4-XREM promoter reporter assay in COS1 cells overexpressing hPXR treated with different concentrations of rifampicin (0.025 to 1 μg/ml) in the presence or absence of ketoconazole. (C and D) Intracellular survival of rifampicin sensitive (C) and rifampicin resistant (D) *M. tuberculosis* as monitored by CFU assay in control, PXR knockdown, and PXR overexpressing hMDMs in the presence or absence of 0.25 μg/ml of rifampicin for 48 h. Data are mean ± SD from three independent experiments each performed in triplicate. *, *p* < 0.05 compared to control or as indicated.

FIGURE 6: Relative mRNA abundance of xenobiotic nuclear receptors and drug transporters in hMDMs derived from TB patients. (A-F) Relative mRNA abundance of PXR, CAR and drug transporters (ABCB1, ABCC1, ABCC2 and ABCG2) in hMDMs derived from drug responding and nonresponding TB patients. Relative mRNA abundance was calculated as 2^-(ΔΔCt) relative to healthy individuals. Horizontal lines represent the mean.*, *p* < 0.05 by the Mann-Whitney test.

FIGURE 7: Expression and activity of hPXR plays a crucial role in drug nonresponsiveness to rifampicin in mice model of TB. Humanized PXR mice model was generated by overexpression of hPXR in C57BL/6 mouse lungs using an adenoviral overexpression system and LacZ was used as a control. These control LacZ and hPXR overexpressing mice were infected with *M. tuberculosis* H37Rv via an aerosol challenge. After 15 days of infection the mice were divided into six treatment groups as shown in figure and treated with respective single or combination of drugs. After 30 days of treatment the total number of bacilli in lungs was determined by CFU assays. Each group contained five mice. Horizontal lines represent the mean.*, *p* < 0.05 by the Mann-Whitney test.
Figure 1

A

Responders
Non responders

untreated
Rifampicin
Isoniazid
Ethambutol

Percentage survival

B

Responders
Non responders

x 10^6 CFU/ml

NS

*NS
Figure 2

A

ABCB1 expression

ABCC1 expression

ABCC2 expression

ABCG2 expression

Efflux of CFDA (% of Control)

Efflux of Rhodamine 123 (% of Control)

Efflux of mitoxantrone (% of Control)

Specific for ABCB1 and ABCC1 and ABCC2 mediated efflux

Specific for ABCB1 mediated efflux

Specific for ABCG2 mediated efflux

Control
M.tb
r-M.tb
Rifampicin
Rifampicin + M.tb
Rifampicin + r-M.tb

B

PXR expression

CAR expression

X-ray film

180
180
180
75

ABCB1

ABCC1

ABCC2

ABCG2

β -actin

PXR

CAR

Control
M.tb
r-M.tb
Rifampicin
Rifampicin + M.tb
Rifampicin + r-M.tb
Figure 4

(A) ABCB1 expression

(B) ABCB2 expression

Vehicle control
Rifampicin resistant *M. tuberculosis*
Rifampicin
Rifampicin resistant *M. tuberculosis* + Rifampicin
Figure 5

**A**

Graph showing the effect of Rifampicin and Ad PXR + Ketoconazole on relative luciferase activity.

**B**

Bar chart comparing Rifampicin and Rifampicin + Ketoconazole (10 µg/ml) on relative luciferase activity.

**C**

Graph showing the effect of Rifampicin on rifampicin-sensitive M. tuberculosis.

**D**

Graph showing the effect of Rifampicin on rifampicin-resistant M. tuberculosis.
Figure 6

A

PXRI mRNA (relative)

Responders

Non responders

B

CAR mRNA (relative)

Responders

Non responders

C

ABCB1 mRNA (relative)

Responders

Non responders

D

ABCC1 mRNA (relative)

Responders

Non responders

E

ABCC2 mRNA (relative)

Responders

Non responders

F

ABCG2 mRNA (relative)

Responders

Non responders
Figure 7

![Graph showing bacterial counts in LacZ and Adeno PXR mice. The x-axis represents different treatments, and the y-axis represents bacterial counts (x10^6 CFU/lung). The graph includes control, ketoconazole, rifampicin, rifampicin + ketoconazole, rifabutin, and rifabutin + ketoconazole. There are significant differences indicated by asterisks (*) and non-significant differences indicated by 'NS'.](http://www.jbc.org/Downloaded from)
A human xenobiotic nuclear receptor contributes to nonresponsiveness of Mycobacterium tuberculosis to the antituberculosis drug rifampicin
Ella Bhagyaraj, Drishti Tiwari, Nancy Ahuja, Ravikanth Nanduri, Ankita Saini, Rashi Kalra, Sumit Kumar, Ashok Kumar Janmeja and Pawan Gupta

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