Differential Host Gene Signatures in Response to *Mycobacterium tuberculosis* Infection

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Short title: Differential host response to tuberculosis

ABSTRACT

Tuberculosis (TB) represents a global public health threat and is the leading cause of morbidity and mortality worldwide. Effective control of TB is complicated with the emergence of multidrug resistance. Yet, there is still a fundamental gap in understanding the complex and dynamic interactions between different *Mycobacterium tuberculosis* strains and the host. In this project, we investigated the host immune response to different *M. tuberculosis* strains, including avirulent or virulent and rifampin-resistant or isoniazid-resistant strains in THP-1 cells. We identified major differences in the gene response profiles in response to infection with these strains. The expression of IDO1 and IL-1β in the infected cells was stronger in all virulent *M. tuberculosis* strains. The most striking result was the overexpression of many interferon-stimulated genes (ISGs) in cells infected with the isoniazid-resistant strain, compared to the rifampin-drug resistant strain and the drug-sensitive strain. A transcription regulation analysis of the differentially expressed genes in infected THP-1 cells implicated two major transcription factors, NF-κB and STAT1. The differentially expressed ISGs in response to the isoniazid-resistant *M. tuberculosis* strain were associated with STAT1 signaling, while the expression of many cytokines, such IL-1β, was associated with NF-κB signaling. Our data suggest that the isoniazid-resistant *M. tuberculosis* strain preferentially activates STAT1 in response to cGAS-STING activation and induces a host immune response signature that is characteristic of isoniazid resistance. This study has a potential to provide important new insights into TB pathogenesis and to characterize host gene signatures specifically involved in isoniazid-resistant TB.

INTRODUCTION

Tuberculosis (TB) represents a disease of global public health importance and a leading cause of morbidity and mortality worldwide, surpassing HIV/AIDS (1, 2). One third of the world's population is currently infected with *Mycobacterium tuberculosis*, with more than 10 million new cases of active TB reported worldwide resulting in more than 1.6 million deaths annually. The emergence of drug-resistant TB and the increasing incidence of multidrug-resistant (MDR) and, more recently, of extensively drug-resistant (XDR) and totally drug-resistant (TDR) *M. tuberculosis* strains are a highly significant public health threat and jeopardize current efforts to TB control and prevention (3-5).

Besides the ability to acquire mutations in target genes conferring resistance to antimicrobial drugs, *M. tuberculosis* strains also show a high degree of intrinsic resistance to most common antibiotics (6). This allows the mycobacteria to efficiently resist anti-tuberculous treatment. As a consequence, the molecular characterization of drug-resistant *M. tuberculosis* strains remains technically challenging (7-9). There is an urgent need to develop alternative and more sensitive approaches to identify *M. tuberculosis* strains in order to implement better measures to minimize the acquisition of further drug resistance, prevent TB transmission, and ultimately improve TB control and prevention strategies.

The *M. tuberculosis* cell wall has a complex composition and structure. It is considered to be a major virulence factor and to promote the natural resistance of *M. tuberculosis* to antibiotics (10). Mycolic acids represent the hallmark component of the *M. tuberculosis* cell wall and their biosynthesis and regulation are the targets of isoniazid (INH), one of the cornerstone drug of TB treatment (11, 12). Interestingly, the cell wall is significantly thicker in drug-resistant *M.*

tuberculosis strains compared to drug-sensitive strains, further underscoring the importance of mycolic acids biosynthesis in INH-resistant *M. tuberculosis* strains (13).

Cell wall lipids in *M. tuberculosis* play a significant role in modulating the host immune response, as well as its pathogenic processes and virulence (14-17). However, our understanding of the host-*M. tuberculosis* interaction is still limited, and many fundamental gaps remain in how this interaction could be altered in response to different *M. tuberculosis* strains. Infections with different *M. tuberculosis* genotypes have major impacts on host-pathogen interaction, which can lead to substantial differences in the host immune responses (18-20). Some mycobacterial lineages (e.g., Beijing) even showed polymorphic properties in immune response genes, suggesting a possible human-pathogen co-evolution (21). The immune response of infected macrophages also showed a wide variation in the response to different *M. tuberculosis* lineages (22). However, further work is needed to explore the host immune response to different *M. tuberculosis* strains. In this study, we tackled this question and investigated the host immune response to different *M. tuberculosis* strains, including avirulent or virulent and rifampin-resistant or isoniazid-resistant strains in THP-1 cells. Shifting the focus on the host-pathogen interaction in *M. tuberculosis* infection and the identification of specific host responses to *M. tuberculosis* strains have the potential to develop biomarkers for novel and more sensitive diagnostic tools for TB. The identification of TB patients at an early stage, particularly those carrying drug-resistant strains, is crucial in order to begin an appropriate therapy as quickly as possible and improve our efforts for TB control.

MATERIALS AND METHODS

Cell line treatment and *M. tuberculosis* infection

For this study, we used human monocytic THP-1 cells (American Type Culture Collection; Catalog no. TIB-202). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C and 5% CO_2 in a humidified incubator. THP-1 cells were subsequently differentiated for 48 hours with 10 ng/ml phorbol 12-myristate 13-acetate (PMA). The cells were then infected with four different reference mycobacterial strains (H37Ra, H37Rv, H37Rv-INH-R, H37Rv-RIF-R), kindly provided by Dr. Lisa Y. Armitige (Heartland National TB Center). Both H37Ra (American Type Culture Collection; Product no. 25177) and H37Rv (American Type Culture Collection; Product no. 27294) are drug-sensitive, whereas H37Rv-INH-R (American Type Culture Collection; Product no. 35822) and H37Rv-RIF-R (American Type Culture Collection; Product no. 35838), both of which are derivatives of H37Rv, are resistant to isoniazid and rifampin, respectively. We used two time points for infection (4 and 24 hours) in order to monitor differences in gene expression patterns for differentially expressed genes. In addition to the infected THP-1 samples, we also used uninfected control cells (mock-infected with PBS) as negative controls.

RNA isolation and processing

Total RNA was extracted from controls and infected cells (10 samples in total, with 5 samples for each time point), using Trizol according to the manufacturer's protocol. RNA was then suspended in RNase-free water and stored at -80°C until further use. RNA quality was assessed to verify its integrity using Agilent 2100 Bioanalyzer (Agilent Technologies) and RNA quantity was evaluated by spectrophotometry using NanoDrop 2000 (NanoDrop Technologies). All RNA samples showed good RNA yield and no RNA degradation. Total RNA was then reverse transcribed to cDNA, amplified, labeled and hybridized to separate arrays using the GeneChip Human Exon 1.0 ST

Array (Affymetrix, USA), according to the manufacturer's instructions. For transcriptional profiling, we used triplicate hybridization assays in our microarray experiments in order to assess variability among independent labeling reactions and hybridizations. Quality control of the hybridized arrays was also performed for each sample. A visual inspection of the scanned images was conducted looking for any defects, areas of high background, or areas of low signal. The spike-in controls were checked as well to examine for hybridization uniformity.

Microarray data analysis

Data from all 10 samples were preprocessed, summarized at the transcript-cluster (gene) level, and RMA normalized using Affymetrix Power Tools. Prior to differential expression analysis, low-variability genes were filtered out, leaving 13,460 genes. Differential expression analyses were conducted using the R/Bioconductor package *limma* (23). Significant differential expression was defined by an adjusted p-value of less than 0.05. The visualization of the identified differentially expressed genes in the volcano plots was performed using base R graphics.

RESULTS

In this study, we generated gene expression profiles of THP-1 cell lines infected with different reference *M. tuberculosis* strains (H37Ra, H37Rv, H37Rv-INH-R, H37Rv-RIF-R). The choice for this study design allowed us to focus on *M. tuberculosis* strain-specific alterations in the transcriptional response and to avoid putative confounding differences that might be related to the genetic variability of clinical *M. tuberculosis* strains and macrophages isolated from different subjects. The multidimensional scaling (MDS) plot for the microarray expression data clearly

showed well-separated grouping according to infections vs mock infections and time points of infections, and also that replicate samples clustered together (Fig. 1).

Among the total number of differentially expressed genes between infected THP-1 cells and mock-infected cells, we found major differences in those genes, depending on the *M. tuberculosis* strain used for infection. A higher number of differentially expressed genes was detected in infected cells with all virulent *M. tuberculosis* strains (H37Rv, H37Rv-INH-R, H37Rv-RIF-R), compared to the infection with the non-virulent strain (H37Ra) (Fig. 2). This difference was even more pronounced at 24 h post-infection when we performed pairwise comparisons in infected cells (Fig. 3). Based on this finding, the subsequent analyses were therefore based on that time of infection (24 h), instead of 4 h post-infection. A total of 3,622 differentially expressed genes ($p<0.05$) was identified in response to H37Rv-INH-R infection.

At 24 h post-infection, we identified differences in the gene response profiles between the strains. The expression of IDO1 and IL-1β was enhanced in all infections with the virulent reference strains, except H37Ra (Fig. 4). However, the most striking and unexpected result was the overexpression of many interferon-stimulated genes (ISGs) in the THP-1 cells infected with H37Rv-INH-R, in comparison to cells infected with the other *M. tuberculosis* strains (Fig. 5). Our data also showed that protein kinase R (PKR/eIF2aK2) is most strongly induced in THP-1 cells infected with H37Rv-INH-R. A subset of the differentially expressed host genes is shown in figure 5. In this figure, we can see a panel of differences in the host expression profile. The gene expression changes between infections with H37Rv-INH-R vs the other strains are also shown in the volcano plots (Fig. 6).

To determine the key transcription factors that potentially regulate the differentially expressed genes in infected THP-1 cells, we performed a transcription regulation analysis using

pathway relation network (pathway-net: http://pathwaynet.princeton.edu/) (24). Two major transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 1 (STAT1), were identified in this analysis. Our analysis showed that the differentially expressed ISGs in H37Rv-INH-R were associated with STAT1 signaling, while the expression of many cytokines, such IL-1β, was associated with NF-κB signaling.

DISCUSSION

Our study showed major differences in the host response to different *M. tuberculosis* strains. We also showed that the infection with H37Rv-INH-R induced an unexpected overexpression of ISGs in THP-1 cells. Our data indicated that STAT1 is the major transcription factor associated with the differentially expressed genes (e.g., ISGs) in response to H37Rv-INH-R infection.

Multiple recognition and signaling pathways for the host innate immune responses to *M. tuberculosis* have been documented and characterized (25). The main signaling pathways in *M. tuberculosis* are TLR2-MyD88 and cGAS-STING. In this study, the overexpression of ISGs in the cells infected with H37Rv-INH-R suggests a stronger induction of STAT1 as a result of cGAS-STING activation, rather than TLR2-MyD88 activation in the differential host response to INH-resistant TB. In addition to the overexpression of ISGs in the cells infected with the isoniazid-resistant *M. tuberculosis* strain, we also found a higher induction of PKR/eIF2aK2 in those cells. This result is even more exciting, since there has been recently an increasing interest in PKR/eIF2aK2, a key player in the innate immune response to viral infections, for its potential ability to improve TB control (26-29). Recent studies have also shown an important role of NF-

182 κB dynamics in TB pathogenesis (30, 31). NF-κB was the other major transcription factor
183 associated with the differentially expressed genes, which was identified in this study.

184 Resistance to INH is linked to all multidrug-resistant forms of TB (MDR, XDR, TDR).
185 Several studies have shown that INH resistance is acquired first, followed by resistance to rifampin
186 and the other anti-TB drugs (32-36), which highlights the significant impact of INH in the success
187 of treatment in active and latent TB and warrants further efforts to understand the global burden
188 of INH-resistant TB (37). Fig. 7 shows our proposed model for the host signaling response to the
189 different reference *M. tuberculosis* strains (H37Ra, H37Rv, H37Rv-INH-R, H37Rv-RIF-R).

190 The mechanism of INH resistance is complex and has been thoroughly studied in an
191 attempt to improve early diagnosis of INH-resistant *M. tubeculosis* strains (36, 38-44). It is mainly
192 mediated by mutations in the *katG* gene or in the *inhA* regulatory regions (45). INH is activated
193 by the catalase-peroxidase encoded by *katG*. This process interferes with the biosynthesis of
194 mycolic acids by inhibiting NADH-dependent enoyl-ACP reductase encoded by *inhA*. Mutations
195 in other genes have been also associated with INH resistance, however they are not as common as
196 the ones in *katG* (42 to 95%) and *inhA* (6 to 43%) in *M. tuberculosis* clinical strains and their mode
197 of action in INH resistance has not been fully elucidated (46). Nevertheless, not all INH-resistant
198 strains harbor defined genetic mutations associated with resistance to this drug, which complicates
199 efforts to identify those strains. There are also INH resistance-conferring mutations that cannot be
200 detected by current molecular diagnostics approaches (33). In addition, some studies indicated that
201 some *M. tuberculosis* strains acquire drug resistance at higher rates, suggesting a higher mutation
202 rate in these strains and a higher probability that these strains will develop multidrug resistance
203 (47, 48).

Given our results, the differential host immune response to different *M. tuberculosis* strains (e.g. overexpression of ISGs in the cells infected with H37Rv-INH-R) provides opportunities to develop host biomarkers for INH resistance and defines a new facet of host-pathogen interactions that differentially regulate signaling pathways in response to INH-resistant and other *M. tuberculosis* strains. The characterization of such host response signatures has the potential to develop novel diagnostic tools and improve our understanding of the complex and dynamic interactions between *M. tuberculosis* strains and the host.

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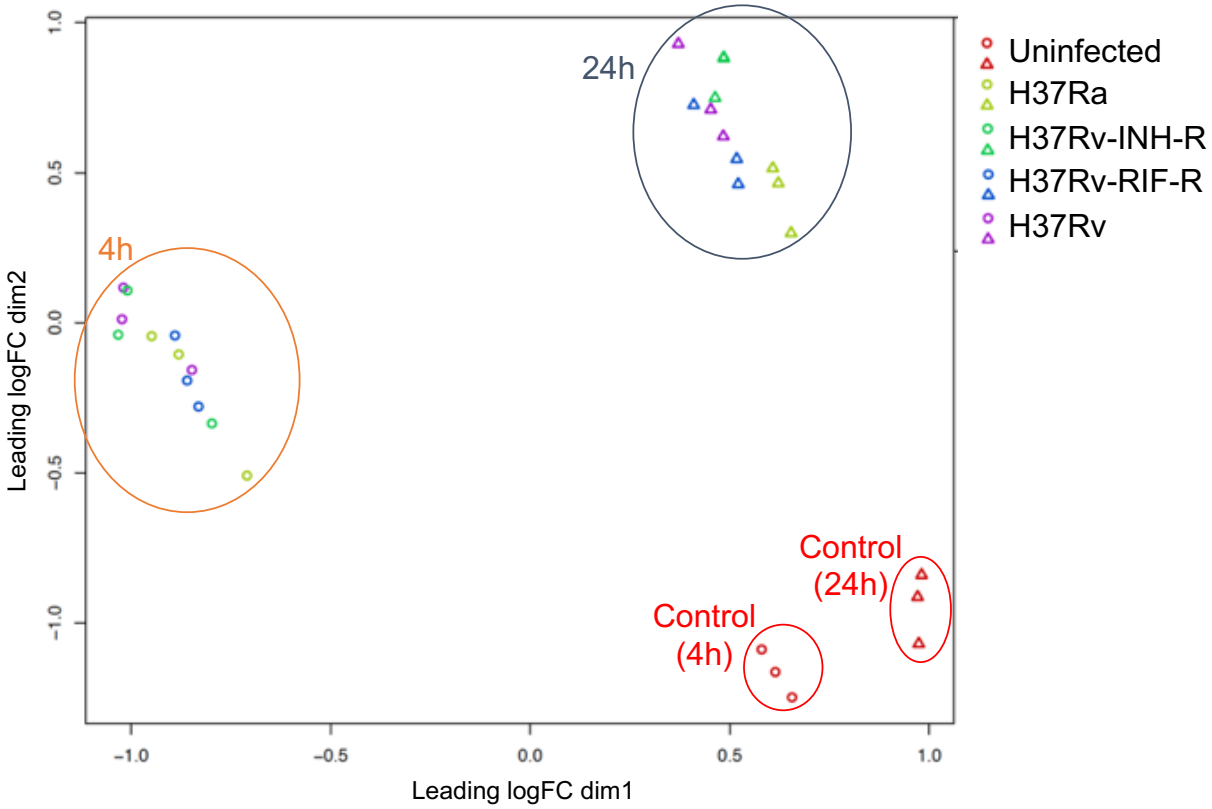
363 **FIGURES**

364 **Figure 1:**

365 MDS Plot for differential gene expression of *M. tuberculosis*-infected vs mock-infected THP-1

366 cell lines at 4 h and 24 h post-infection.



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376 **Figure 2:**

377 Number of differentially expressed genes in *M. tuberculosis*-infected THP-1 cell lines, relative to

378 mock-infected cells, at 4 h and 24 h post-infection ($p<0.05$).

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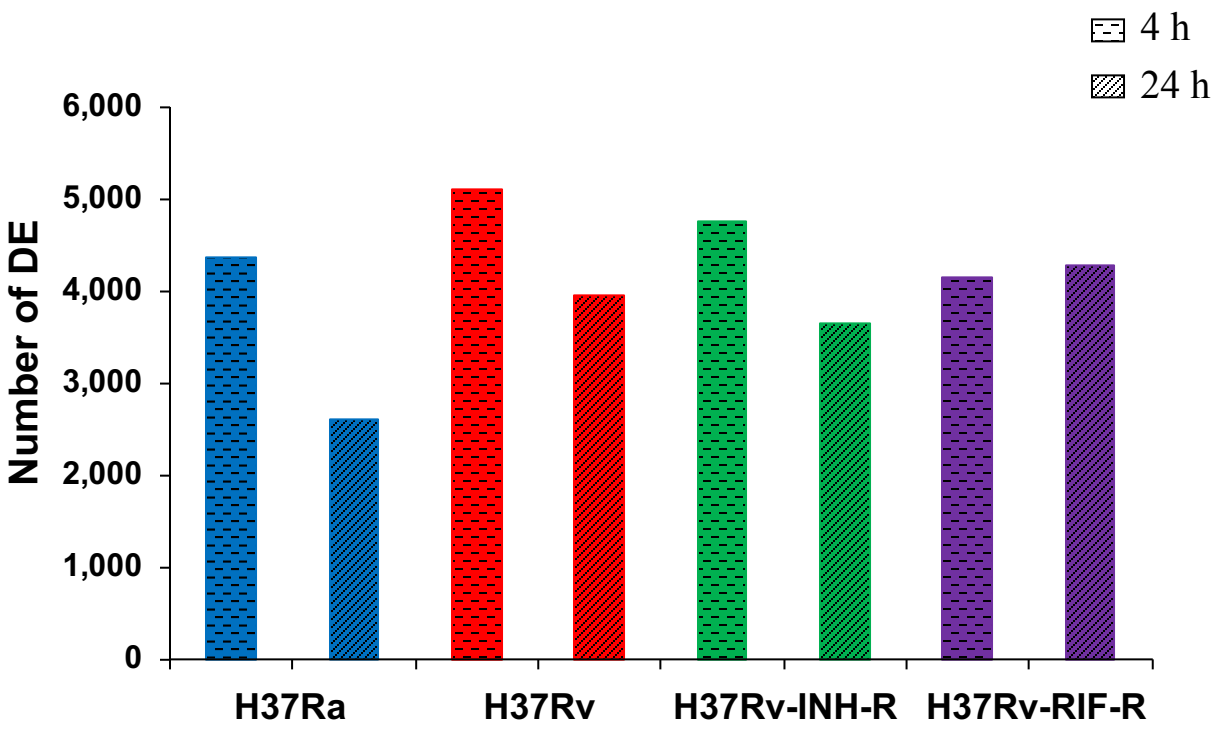

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388 **Figure 3:**

389 Number of differentially expressed genes in the pairwise comparisons of *M. tuberculosis*-infected

390 THP-1 cell lines at 4 h and 24 h post-infection ($p<0.05$).

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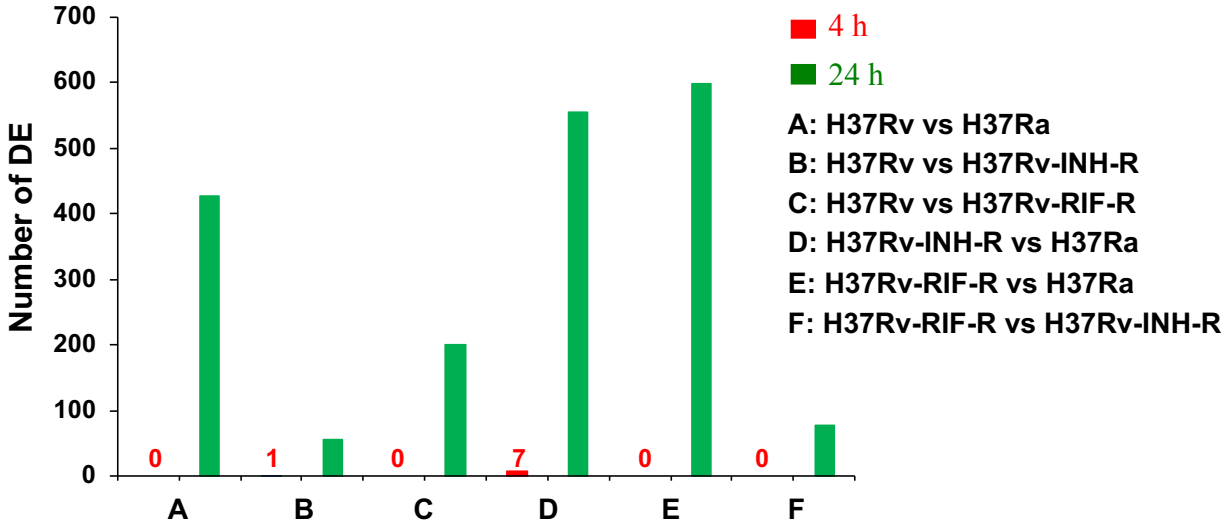

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405 **Figure 4:**

406 Fold change in the expression of IDO1 and IL-1β in THP-1 cell lines infected with reference *M.*

407 *tuberculosis* strains (24 h post-infection; $p<0.05$).

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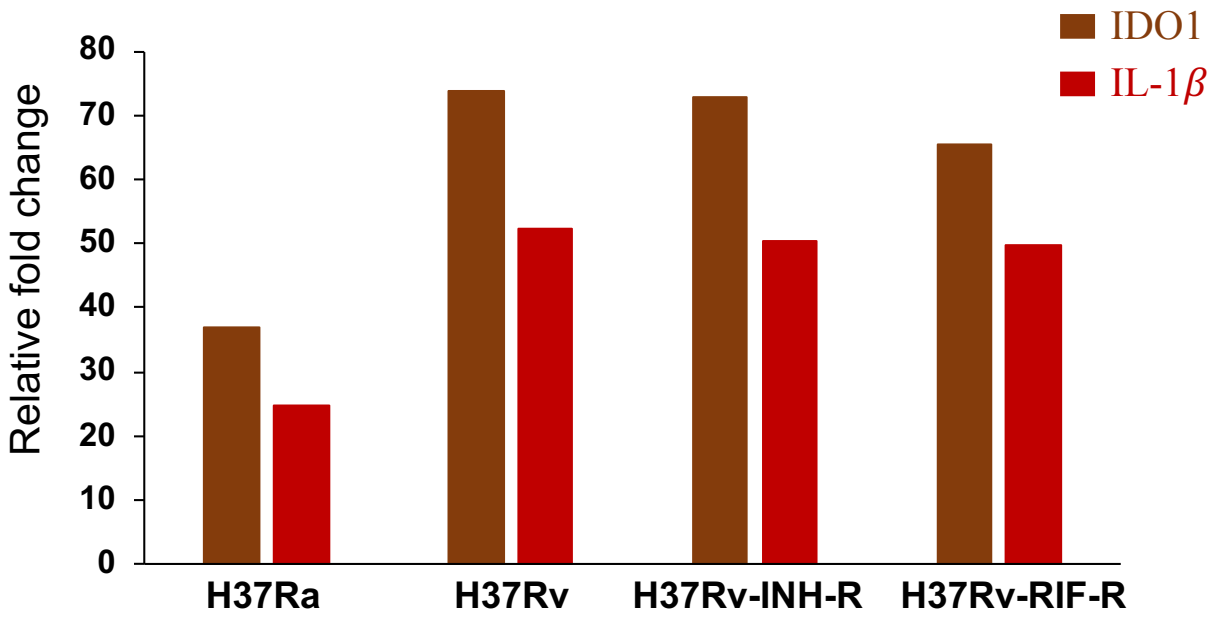

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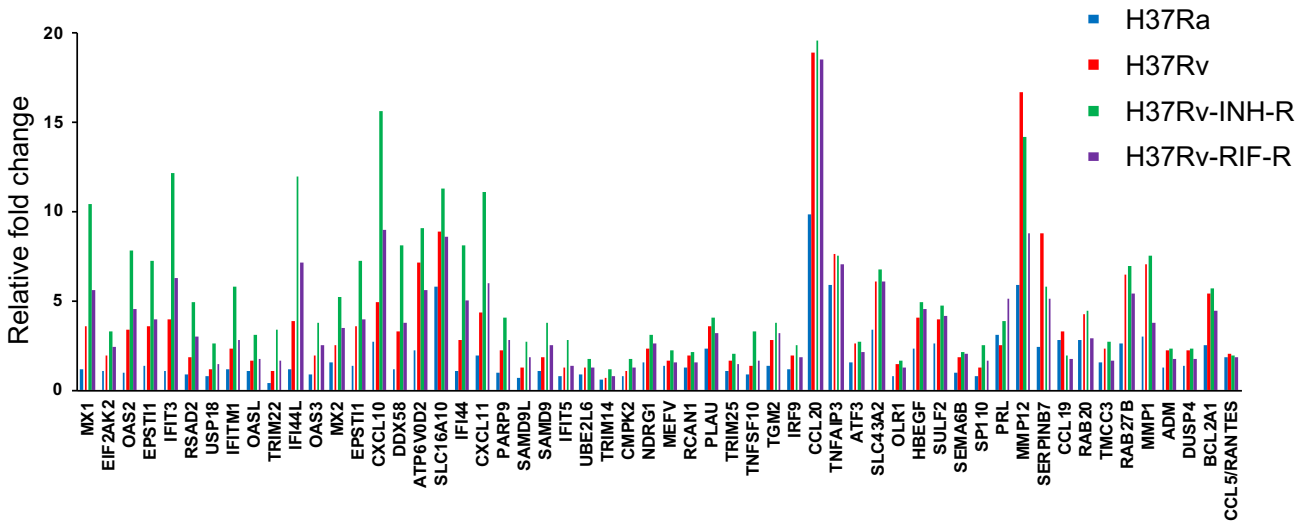
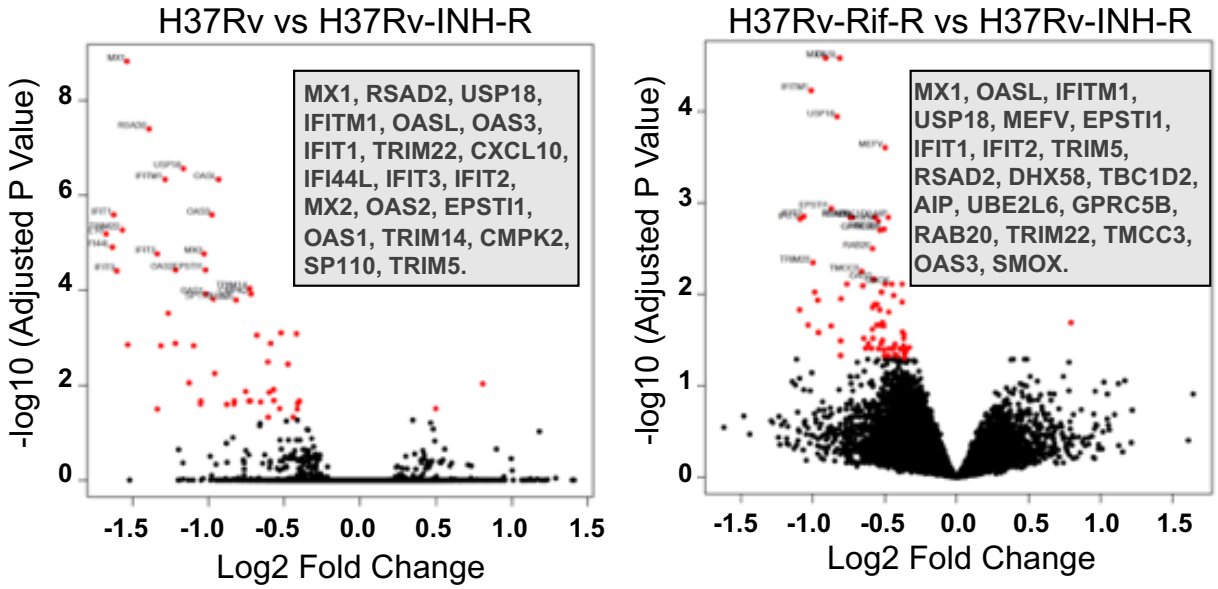
Figure 5:

Fold change in gene expression in THP-1 cell lines infected with reference *M. tuberculosis* strains (24 h post-infection; $p<0.05$).



437 **Figure 6:**

438 Volcano plots displaying differential expression genes in THP-1 cells in three different comparison
439 groups (24 h post-infection; $p<0.05$). The 20 highly significant differentially expressed genes in
440 each plot are indicated in the insets.



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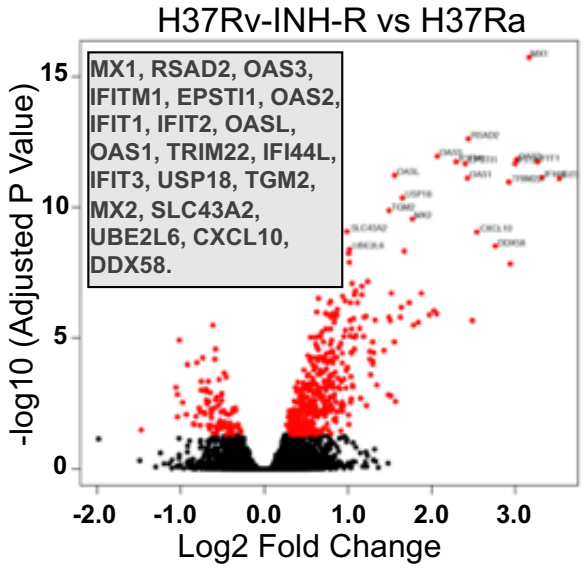

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Figure 7:

Proposed model for host signaling response to reference *M. tuberculosis* strains. H37Rv-INH-R induces the highest expression of ISGs and PKR, compared to the other strains, as indicated by the circles and the big font size.