นิพนธ์ต้นฉบับ • Original Article

การตอบสนองของเซลล์ทีเอชพีวันที่ถูกกระตุ้นต่อเชื้อวัณโรค (*Mycobacterium tuberculosis*) ในระหว่างการรักษาด้วยไอโซไนอะซิดและไรแฟมพิซินในหลอดทดลอง

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Responses of Activated THP-1 Cells to *Mycobacterium Tuberculosis* Infection During Isoniazid and Rifampicin *in vitro* Treatment

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หลักการและวัตถุประสงค์: แมคโครฟาจมีบทบาทสำคัญในภูมิคุ้มกันแบบจำเพาะและไม่จำเพาะต่อวัณโรค ยาต้านวัณโรคอาจมีผลต่อปฏิสัมพันธ์ระหว่างแมคโครฟาจและเชื้อวัณโรค ปฏิสัมพันธ์ระหว่างเชื้อวัณโรคและแมคโครฟาจระหว่างการรักษาด้วยยาต้านวัณโรคยังไม่เคยมีการศึกษามาก่อน การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาปฏิสัมพันธ์ระหว่างทีเอชพีวันเซลล์ที่ถูกกระตุ้นแล้วและเชื้อวัณโรคในระหว่างการรักษาด้วยยาต้านวัณโรค

วิธีการศึกษา: เซลล์ทีเอชพีวันที่ถูกกระตุ้นแล้ว นำมาติดเชื้อมัยโคแบคทีเรียมทูเบอคูโลซีสแล้วเติมยาต้านวัณโรค เปรียบเทียบกับเซลล์ไม่ติดเชื้อที่เลี้ยงในสภาวะเดียวกัน โปรตีนถูกนำมาวิเคราะห์ด้วย GeLC MS/MS และวิเคราะห์ความสัมพันธ์ของโปรตีนตามวิถี KEGG โดยใช้ซอฟแวร์ Ingenuity

ผลการศึกษา: โปรตีนที่พบทั้งหมด 4,173 โปรตีน เป็นโปรตีนที่พบเฉพาะในสภาวะติดเชื้อ 98 โปรตีน เกี่ยวข้องกับการกระตุ้นลิมโฟไซค์ชนิดที (IL2/ CD28) การตายของเซลล์แบบอะพอพโทซิส (CASP3 และ TGFB1) การอยู่รอดของเซลล์ (NFkB Akt) และการควบคุมการถอดรหัสดีเอ็นเอ (Myc BCL6) และพบเฉพาะสภาวะไม่ติดเชื้อ 74 โปรตีน เกี่ยวข้องกับภูมิคุ้มกันต่อต้านเชื้อ (IL17A) การแบ่งตัวและการอยู่รอดของเซลล์ (ERK และ Myc) และการเจริญเติบโตและการ

Background and **Objective**: Macrophages are cells that play a key role in both innate and adaptive immune responses to TB. Anti-TB drugs might affect the interaction between macrophages and *Mycobacterium tuberculosis* (*Mtb)*. However, as of yet there have been no studies that reporting on the interaction between *Mtb* and macrophages during anti-TB treatment. This study aims to study the response of activated THP-1 cells to *Mtb* infections during *isoniazid* and *rifampicin* treatments *in vitro*.

Methods: The activated THP-1 cell line was cultured and infected with *Mtb* and was subsequently treated with isoniazid and rifampicin. A culture of uninfected cells as also treated using the same method. Comparative proteomic analysis using GeLC MS/MS was performed. The protein networks were analyzed based on KEGG pathway using Ingenuity software.

Results: Overall, this study found 4,173 proteins, 98 of which were found exclusively in the infected cells and 74 of which were found only in the uninfected cells. The proteins that were expressed in the infected cells were associated with T cell activation (IL2 and CD28),

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เคลื่อนที่ของเซลล์ (TP53/CTNNB1/Axin1)
สรุป: การศึกษานี้ให้ข้อมูลเกี่ยวกับปฏิสัมพันธ์ระหว่างเซลล์ที่เอชพีวันที่ถูกกระตุ้นและเชื้อวัณโรคในระหว่างการรักษาด้วยยาต้านวัณโรค

apoptosis (CASP3 and TGFB1), cell survival (NFkB and Akt) and transcriptional regulation (Myc and BCL6). The proteins that were suppressed in the infected cells associated with immune response (IL17A), cell proliferation and survival (ERK and Myc), cell growth and migration (TP53, CTNNB1 and Axin1).
Conclusion: Our study provides information regarding the activated THP-1 cell response to *Mtb* infection during anti-TB drug treatment *in vitro*.
Keywords: THP-1 cells, *Mycobacterium tuberculosis*, isoniazid, rifampicin, comparative proteomic, GeLC MS/MS.

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Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is a leading cause of morbidity and mortality worldwide. Alveolar macrophages (AM) are the first line of defense and play an important role in both innate and adaptive immune responses to *Mtb* infection,[1] as well as other types of bacterial infection.

The infection of macrophages activate several immune responses against the pathogen, such as those of IL12[2] and IL17A[3] and activate cell survival via ERK and Myc[4]. In the case of TB, the interaction between *Mtb* and macrophages has been investigated. *Mtb* can be phagocytosed by macrophages but it can survive inside the cells through the inhibition of phagosome-lysosome fusion[1, 5, 6]. The interaction between *Mtb* and phagocytes leads to cell activation and the production of various cytokines and chemokines, which help to control the infection[7]. The infected macrophage can activate naive T-cells via the activation of the CD28 receptor leading to an immune response against the pathogen[8]. The supernatant of *Mtb* infected macrophages also induced T cell apoptosis[9]. The putative clearance marker of *Mtb* infected macrophage and treated with isoniazid and rifampicin has been studied[10]. However, the interaction between macrophages and *Mtb* infection during anti-TB drug treatment has yet to be investigated.

The standard regimen for TB treatment is 6 months of combined drug therapy in which isoniazid (INH) and rifampicin (RIF) are the core drugs used throughout the treatment course. Although the pathway of host-mycobacterial interaction was elaborately depicted by KEGG,[11] the interaction of the tubercle bacilli and the host during anti-TB treatment might be different. Study of the mechanisms of host-pathogen interaction during anti-TB treatment might contribute increasing the efficacy of current TB treatments, as well as the discovery of new treatments.

In previous studies, the microparticles containing INH and RIF activated TNF-α secretion from infected cells[12] and induced the apoptosis[13]. This study addressed the enhancement of the macrophages in order to destroy *Mtb* through the use of drugs containing microparticles and focused only on particular pathway of host responses. Previous studies have investigated the transcriptional expression of *Mtb* lipid and macrophages[14] and the proteins expressed from macrophage-*Mtb* interaction[15]. However, there has yet to be research conducted that elucidates the high throughout analysis that can investigate the whole response of the host and the tubercle bacilli during anti-TB treatment.

In this study, we aim to investigate the activated THP-1 cells response to *Mtb* infection during combined of INH and RIF treatment *in vitro* using Gel electrophore-

sis-liquid chromatography tandem mass spectrometry (GeLC-MS/MS). The network analyses were analyzed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), which details well-identified pathways among protein interactions in humans using Ingenuity software. The identified proteins from the host-mycobacterial interaction during anti-TB drug treatment might provide more insight into the mechanisms of adaptation for drug resistance that might facilitate intervention in the case of *Mtb* infection.

Materials and Methods

Cell culture and infection

THP-1 cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS (Hyclone™, GE Healthcare Life Science, USA). THP-1 cells were activated using 50 nM/μl PMA followed by incubation at 37°C, 5% CO_2 for 24 hr. The *Mtb* H37Rv was grown in Middlebrook 7H9 with OADC for 14 days. The *Mtb* H37Rv suspension dispersion was achieved by repeated passage through a 26-gauge needle set. The *Mtb* was adjusted to 0.5 McFarland standards, and diluted in RPMI medium. Activated THP-1 cells were infected with *Mtb* H37Rv (MOI = 1) for 4 hr. After incubation, 3 μg/ml INH and 9 μg/ml RIF in fresh RPMI media were used for treatment of infected cells for 24 hr. The un-infected cells cultured in the same conditions (adding the same anti-TB drugs) were used as controls. Three independent experiments were performed on different occasions as biological replicates.

Proteome collection

The cell supernatant and cell lysate proteins were collected after 24 hr of incubation. The 3 ml of cell supernatant samples were filtered using the syringe filter (Whatman™, GE Healthcare Life Science, USA) and were then transferred into 10 ml conical tube. The protein collection tubes were kept at -80°C for extraction and precipitation. The cell lysate proteins were washed twice using PBS the infected cells were scraped off and transferred into conical tubes. Then, the remaining solution was transferred into a 15 ml conical tube and the remaining protein was collected by adding with 500 ul PBS and pipetting all supernatant into the tube. The infected cells were lysed by adding 0.5% (w/v) SDS. The tube was wrapped using paraffin and sonicated at 53 KHz 37°C for 30 min. Tubes of protein suspension were kept at -80°C for further extraction and precipitation.

Protein extraction and preparation

Protein extraction and preparation were followed the protocol described previously[10]. Two volumes of acetone were added to the culture supernatants and cell lysate samples. Protein suspensions were incubated at -20°C for 8 hr and then centrifuged at 8,000xg for 30 min at 4°C. The protein pellets were dried and re-suspended in 30 μl of a sample storage buffer (50 mM NaH_2PO_4, 5 mM DTT, 0.25 M NaCl solution and proteinase inhibitor). The quantification of the protein samples was performed using the Lowry method. Protein samples and protein standards (0, 2, 4, 6, 8, 10 μg/ml BSA) were transferred into 96-well plates. Then, 200 μl of solution A (2.5% SDS, 2.5% Na_2CO_3, 0.2 N NaOH, 0.025% $CuSO_4$ and 0.05% tartaric acid) was added, and incubated at room temperature for 30 min. Then, 50 μl of solution B (20% Folin-Ciocalteu phenol reagent) was added, and incubated at room temperature for 30 min. The protein samples were measured at OD750 and compared with the standards to estimate the concentrations.

SDS PAGE and in-gel digestion

The 50 μg proteins were run by SDS-PAGE and stained with Coomassie blue. The gel was sliced and cut into 1-mm^3 cubes. The gel pieces were transferred into low binding 96-well plates and incubated with 25 mM NH_4HCO_3 at room temperature for 10 min. Then, 200 μl *of acetonitrile* (ACN) was added and incubated for 10 min with shaking. After ACN removal, the gel pieces were incubated at 56°C for 1 hr with 50 μl of 10 mM DTT in 10 mM NH_4HCO_3. Next, 50 μl of 100 mM iodoacetamide in 10 mM NH_4HCO_3 was added, and incubated for 1 hr in the dark. After that, the samples underwent two cycles of adding of 200 μl of ACN, shaking for 5 min and removing of all liquid. Then, 10 μl of

enzyme solution (10 ng/μl trypsin in 10 mM NH_4HCO_3) was added to the gel pieces and incubated at 37°C for 3 hr. The peptide from the gel pieces was extracted by 3 cycles of adding 50 μl of 50% ACN and shaking for 10 min. The peptide solutions were dried at 40°C and kept at -20°C until analysis.

LC MS/MS analysis

The peptide samples were resuspended in 0.1% formic acid then mixed with a pipette 100 times and transferred into low-binding tubes. The samples were centrifuged at 8,000 xg for 10 min and the peptide solution were transferred into vial tubes. Then, 4.5 μl of peptide sample was injected into a LC MS/MS analyzer (*SYNAPT*™ HDMS mass spectrometer, Waters, UK).The quadrupole mass analyzer was adjusted such that ions from m/z 300 to 1,800 were efficiently transmitted. The MS/MS survey was performed with the range 50 to 1,990 Da and 0.5 sec scan time. The values were normalized using a BSA external intensity control.

Bioinformatics and data analyses

The data from LC MS/MS was analyzed using DeCyderMS 2.0 differential analysis software (DeCyderMS, GE Healthcare, UK). The signal intensities of the peptides was detected using the PepDetect module. The peptides were matched across different signal intensity maps among the tested conditions using the PepMatch module. The $\log_2$ intensities of the sample were used for normalization of ion intensity distribution of BSA. An average abundance ratio > 2-fold higher than BSA external intensity control was determined to be an identified and expressed protein with a significant standard t-test p-value < 0.05. The analyzed data was run through the NCBI database using Mascot software (Matrix Science, London, UK). Database interrogation parameters were as follows: taxonomy (human or eukaryote), enzyme (trypsin), variable modifications (carbamidomethyl, oxidation of methionine residues), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (± 1.2 Da), fragment mass tolerance (± 0.6 Da), peptide charge state (1+, 2+, and 3+), and max missed cleavages. Group-to-group comparisons were performed by linear regression with p-values adjusted by the Benjamini-Hochberg false discovery rate procedure. The network analyses were analyzed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), which details the well identified pathways among protein interaction in humans, using Ingenuity software (Qiagen, Germany). The protein networks were defined based on the network score according to the fit of that network to the user-defined set of focus genes. The score is the –log10(p-value) of a Fishers-exact test and, hence, indicates the likelihood of the focus genes in a network not being found together due to random chance.

Results

To identify the proteins that responded to the infection of *Mtb* infected macrophage during INH and RIF treatment, the *Mtb* H37Rv were infected with activated THP-1 cell and treated with INH and RIF. The PMA-activated THP-1 cells without *Mtb* H37Rv infection and treated with INH and RIF were used as a control. Overall, there were 4,173 unique peptides from intracellular and extracellular proteins of *Mtb* infected and uninfected THP-1 cells treated with INH and RIF detected by LC-MS/MS (Figure 1).

Patterns of protein expression of *Mtb* infected macrophage during anti-TB treatment

The proteomic analysis of uninfected macrophages protein treated with INH and RIF (uninfected) were compared with *Mtb* infected macrophages treated with INH and RIF (infected). The whole proteomic analysis revealed that there were 4,001 proteins found in both infected and uninfected samples. There were 74 proteins found in the uninfected samples and 98 proteins in the infected samples.

The intracellular (cell lysate) and extracellular (culture supernatant) compartments of proteomes were separately analyzed. There were 2,736 intracellular proteins found in both infected and uninfected samples, of which 50 were found only in the uninfected samples and 66 found only in the uninfected samples. There were 1,447 extracellular proteins found in both infected and uninfected samples, of which 24 and 32 were found only in uninfected and infected samples, respectively (Figure 1).

Network analysis of proteomes responded to *Mtb* infection during anti-TB treatment

The network analysis of proteomes was performed to elucidate possible protein-protein interaction and possible mechanisms related to host-pathogen interaction during anti-TB treatment. The proteins expressed only in the infected samples during treatment were found in four important networks. Network A is centered on IL2 and CD28 and consists of proteins involved T cell activation. Network B is centered on CASP3 and TGFB1 and consists of proteins involved in apoptosis. Network C is centered on NFkB and Akt and consists of proteins involved in B-cell activation. Network D is centered on Myc and BCL6 and consists of proteins involved in transcription regulation (Figure 2).

The pathway analysis of proteins that were expressed in the *Mtb* infected samples belonged to three networks. Network A is centered on Akt, NFκB and IL17A and consists of proteins involved in immune response. Network B is centered on TP53, ERK, Myc and consists of proteins involved in cell proliferation and survival. Network C is centered on TP53, CTNNB1 and consists of proteins involved in cell growth and migration (Figure 3).

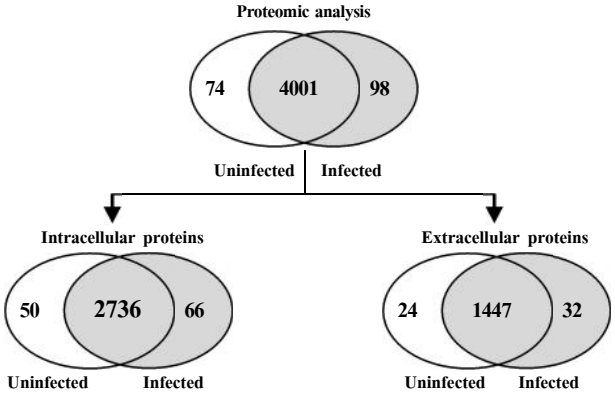

Figure 1 A Venn diagram of the whole proteome analysis. There were 4,001 proteins found in both infected and uninfected samples and there were 74 proteins found only in the uninfected samples and 98 proteins found only in infected samples. There were 2,736 intracellular proteins found in both infected and uninfected samples, of which 50 were found only in uninfected samples and 66 were found only in infected samples. There were 1,447 extracellular proteins found in both infected and uninfected samples, of which 24 and 32 proteins were found only in uninfected and infected samples, respectively.

Discussion

Mtb is an intracellular pathogen that can survive inside the macrophages. INH and RIF are the main drugs in the standard regimen for treatment of TB. Multi-drug resistant (MDR) TB is the label given to *Mtb* that is resistant to these drugs. Although, the mechanism of host-mycobacterial interaction has previously been described, interaction during drug treatment may differ. The proteins from the infected cells that responded during anti-TB treatment might lead to the discovery of better treatments for TB. In this study, we used the PMA activated THP-1 cells infected with *Mtb* and treated with INH and RIF followed by GeLC MS/MS to identify and quantify peptides that were expressed or suppressed in *Mtb* infected cells during INH and RIF treatment. Our research demonstrated the proteins of activated THP-1 cells responses to *Mtb* infection during anti-TB drug treatment.

The activated THP-1 cells proteins expressed after *Mtb* infection and not found in the uninfected cells were associated with the T-cell activation, apoptosis, cell survival and transcription regulation of host cells. The network of T-cell activation centered on CD28 and IL-2. CD28 is the receptor that plays roles in antigen presentation and activation of naive T-cells and plays a central role in immune responses against pathogens[8]. IL-2 is the cytokine that functions as a factor in the growth and death of antigen-activated T lymphocytes and promotes T-cell development[16]. The IL-2 protein is correlated with *Mtb* antigen load after drug treatment and can be used as an immunological marker of mycobacterial load and clinical status of TB[17]. Another pathway centered on CASP3 and TGFB1 also supports a response of infected macrophages in the apoptosis pathway. Both CASP3 and TGFB1 induced the apoptosis[18]. The network of proteins involved in cell

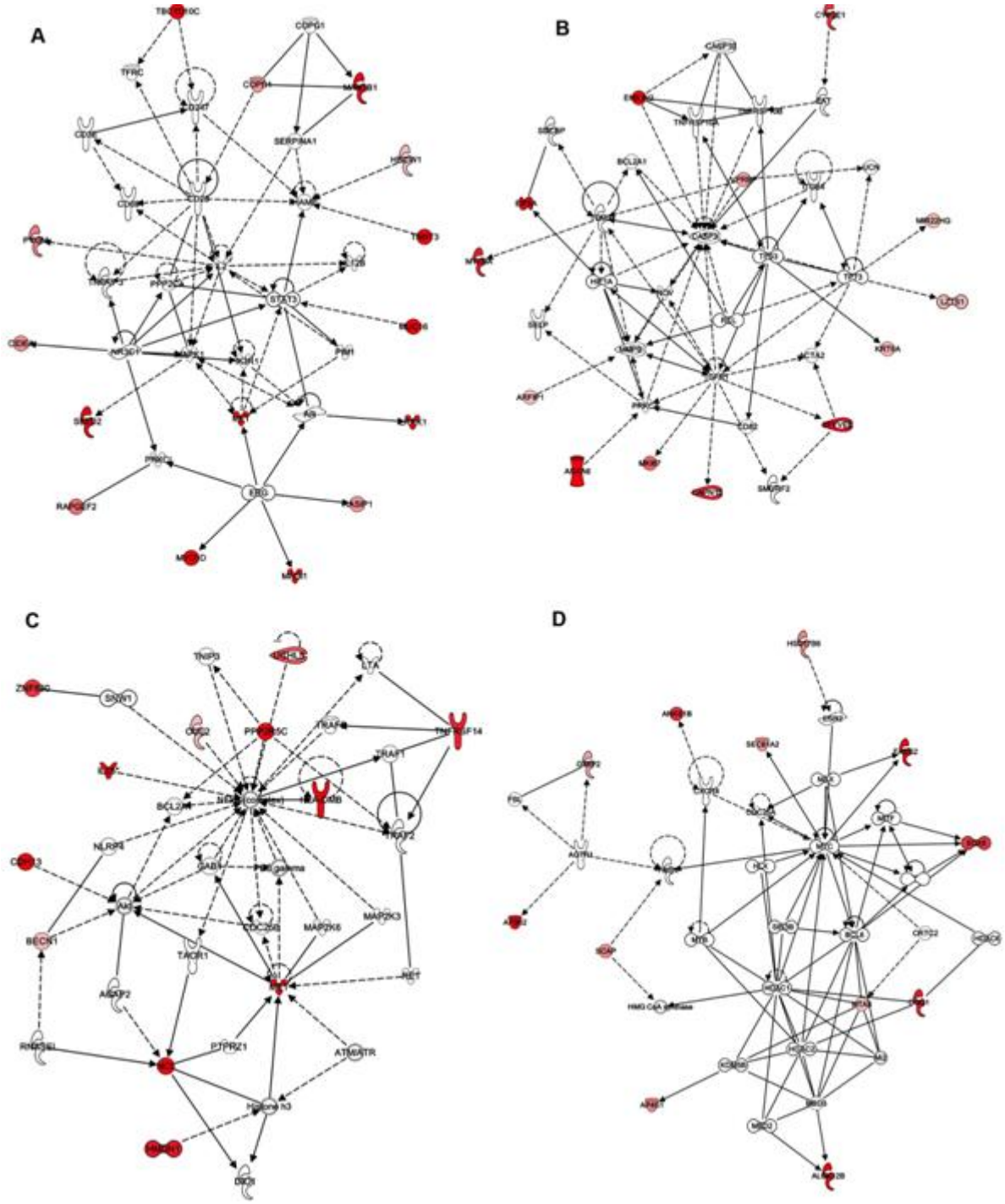

Figure 2 Network analysis of proteins expressed in *Mtb* infected cells during anti-TB treatment. Network A is centered on IL2 and CD28 and consisted of proteins involved in T cell activation. Network B is centered on CASP3 and TGFB1 and consisted of proteins involved in apoptosis. Network C is centered on NFκB and Akt and consisted of proteins involved in cell survival. Network D is centered on Myc and BCL6 and consisted of proteins involved in cell differentiation, antimicrobial response and phagocytosis of *Mtb*. Solid lines denote a direct protein-protein interaction, such as binding; dotted lines denote other relationships, such as co-expression, regulation and activation. The intensity of protein expression is denoted in shades of color proportionate to the level of expression.

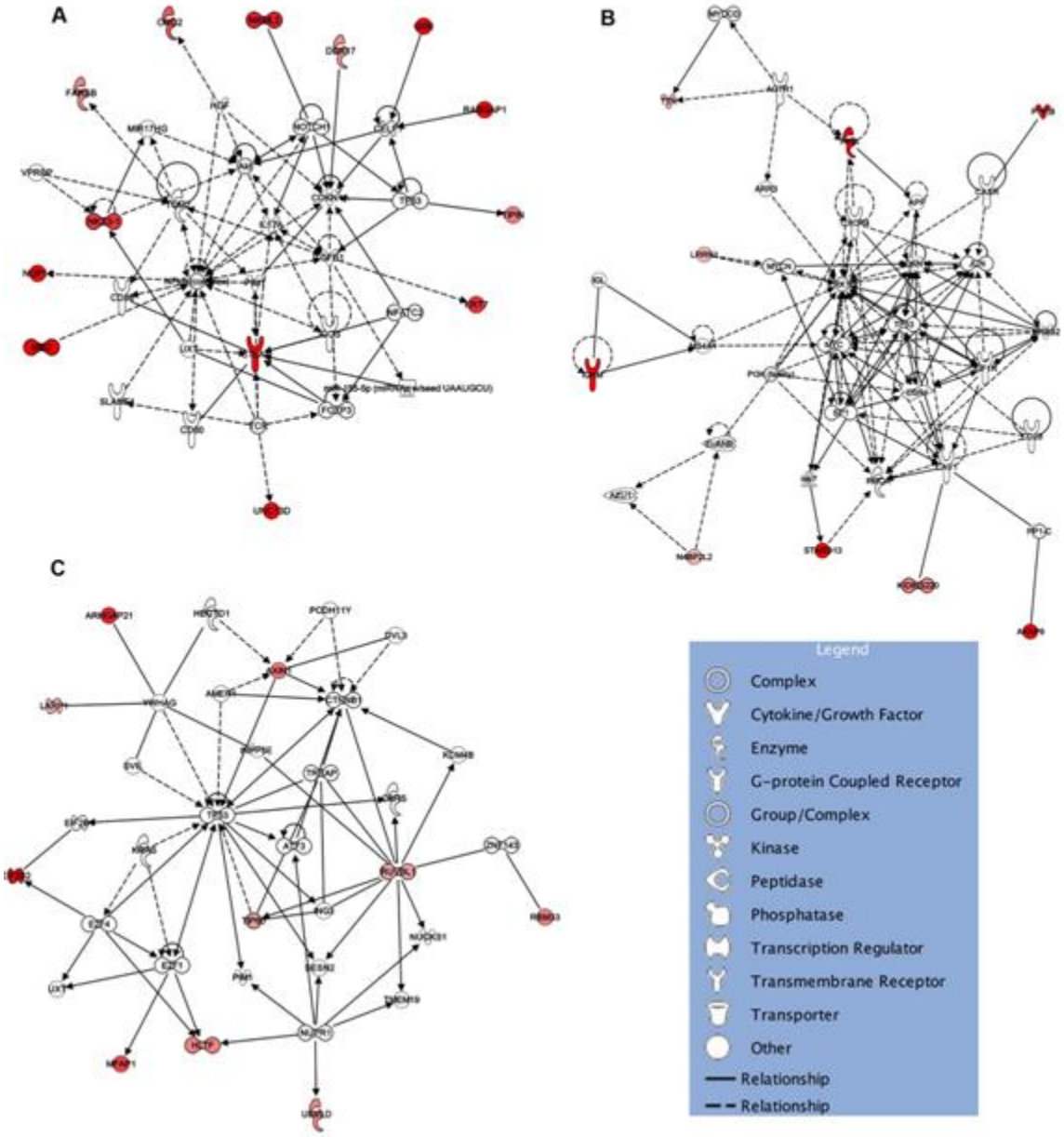

Figure 3 Network analysis of proteins that were suppressed in *Mtb* infected cells during anti-TB treatment. Network A is centered on IL17A and consisted of proteins involved in immune response through induction of granuloma formation and inflamation. Network B is centered on ERK, TP53, Myc and consisteds of proteins involved in regulation of cell proliferation and survival. Network C is centered on TP53, CTNNB1, Axin1 and consisted of proteins involved in cell growth and migration regulation. Solid lines denote a direct protein-protein interaction, such as binding; dotted lines denote other relationships, such as co-expression, regulation and activation. The intensity of protein expression is denoted in shades of color proportionate to the level of expression.

survival was centered on NFKB and Akt. The signaling of Akt-mediated activation of NFKB plays a key role in the survival of macrophages[19]. The Akt contributed to host cell survival and enhanced the *Mtb* survival in *Mtb*-infected samples[20]. The cell differentiation network was centered on Myc and BCL6 and was associated with HDAC1/HDAC2. BLC6 was the transcription repressor controlling the cell differentiation[21]. Myc is the universal amplifier of expressed genes in lymphocytes[22] and also involved the anti-mycobacterial response[23] and phagocytosis of *Mtb*[24]. BCL6 involved in translocation in lymphoma, B-cell differentiation and inflammation[25]. The Myc and BCL6 centered network also involved the HDAC1/HDAC2 that was involved in cell differentiation[26,27]. Activated THP-1 cells during combined INH and RIF drug treatment showed the activation of immune responses against *Mtb* infection via the activation of CD28 and IL-2 expression, which activated the T-cell responses. The activation of the apoptosis pathway also limited *Mtb* survival. However, the induction of host cell survival and control of cell differentiation might be beneficial to *Mtb* pathogens as a host for intracellular growth.

The proteins of the activated THP-1 cells present in uninfected cells but absent after *Mtb* infection, i.e. proteins that were repressed during infection, were associated with the immune response, cell proliferation and survival and cell growth and migration. The network of immune response centered on IL17A plays a critical role in the prevention of *Mtb* infection through the induction of mature granuloma formation and inflammation[3]. The repression of IL17A might be explained by the interference granuloma formation and inflammation by *Mtb,* allowing it to survive. The cell proliferation and survival network centered on ERK and Myc, which functioned as regulators of cell proliferation. Moreover, ERK can activate nitric oxide synthase[28], IL-12 and TNF-α during *Mtb* infection[29]. The repression of ERK and Myc during infection might indicate immune interference with *Mtb* via the reduction of nitric oxide synthase, cytokine and proinflammatory cytokine production aiding in the *Mtb*'s survival[4]. The cell growth regulation network was centered on TP53 and consisted of proteins involved in apoptosis activation such as CTNNB1and Axin1, which also functions as a tumor suppressor[30]. This network protein suppression might indicate the interference of apoptosis with the intracellular survival of *Mtb*. Taken together, the protein networks suppressed during infection might be the target of *Mtb*, aiding in the disease's survival by interfering with host immune responses, such as granuloma formation (IL17A), cell differentiation and nitric oxide synthase (ERK) and cell growth control (TP53, CTNNB1, Axin1). This enhances the ability of *Mtb* to survive inside macrophages.

First, we have compared the results from our study with the protein expression profile of *Mtb*-infected macrophages from a previous study[15]. It was found that there were no proteins in common between the two studies. Second, comparison of the results from our experiment with the transcriptomic profile of human macrophage responded after 4 hr of *Mtb* infection[31] found only the spermidine N-acetyltransferase (SSAT) in common between two studies. This might be explained by the macrophages responses against *Mtb* infection differing based on whether or not anti-TB treatment was administered.

Alternatively, other confounding factors such as the type of antigen (lipid antigen vs. antigen from lived *Mtb* cells), differing cell types (activated THP-1 cells vs. human macrophages) and varying methodological approaches (transcriptomics vs proteomics) might lead to the differences in the results. Nonetheless, the study of protein that is the final product of gene expression better represents the actual biology of the host-mycobacterial response. Moreover, in our study, we used activated THP-1 cells, the medium of which was daily replenished and which were cultured longer after PMA activation and, thus, possessed characteristics close to those of the primary human macrophages[32]. We used the PMA-activated macrophages with anti-TB drugs in the comparison between the infected and non-infected conditions. This means that the differential proteins between the two conditions were not affected by the PMA or anti-TB drugs due to the background subtraction during data analysis. Notably, we sampled the protein from the proteomic result to verify our findings using a western blot, which gave us concordant

results. This is described in another study conducted by our group (data not shown). Furthermore, the proteomic results from our study were derived from three independent experiments, ensuring the reproducibility of the results. Additional studies investigating the host as well as the pathogen responses using primary human cells from a sample of people that represents the host heterogeneity might provide a clearer finding.

In conclusion, we showed that the protein expression of activated THP-1 cell responded to *Mtb* infection during anti-TB drug treatment was associated with the protein networks in the network of IL2 and CD28, network of CASP3 and TGFB1, network of NFΚB and Akt and network of Myc and BCL6. The suppressed protein of activated THP-1 cell response to *Mtb* infection during anti-TB drug treatment was associated with the protein in network of IL17A, network of ERK, TP53, Myc and network of TP53, CTNNB1, Axin. These proteins might indicate the mechanism associated with intracellular infection of *Mtb* during anti-TB drug treatment.

In summary, we showed several proteins and possible mechanisms of interaction between macrophages and *Mtb* during INH and RIF treatment. The analysis of proteins from *Mtb*-infected macrophages revealed several processes of host defense against *Mtb* infection. Alternatively, there were several host proteins with which *Mtb* interfered, contributing to its survival. The host and *Mtb* interaction during anti-TB drug treatment showed the different expressed proteins when compared to the condition in which no anti-TB drugs were used from a previous study.

Acknowledgements

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