รายงานวิจัยฉบับสมบูรณ์

โครงการ

การศึกษาโปรติโอมของปฏิกิริยาระหว่างเชื้อวัณโรคและเซลล์แมคโครฟาจของคนระหว่างการรักษาด้วยยาต้านวัณโรค
(Proteomic study of host-Mycobacterium tuberculosis interaction during anti-tuberculous drugs treatment in human macrophage)

โดย

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สัญญาเลขที่ MRG5580024

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มีการประมาณการว่าหนึ่งในสามของประชากรโลกติดเชื้อวัณโรคแบบแอบแฝง หรือเรียกว่า Latent Tuberculosis infection (LTBI) ปัจจุบันยังไม่มีตัวบ่งชี้ทางชีวภาพที่สามารถบอกถึงสภาวะปราศจากเชื้อของผู้ป่วยภายหลังการรักษาได้ คณะผู้วิจัยจึงมีวัตถุประสงค์ในการศึกษาและวิเคราะห์หาตัวชี้วัดสภาวะปราศจากเชื้อ (clearance stage) จากเซลล์ภายหลังการใช้ยาต้านเชื้อก่อวัณโรค โดยใช้แมคโครฟาจที่ติดเชื้อ *Mycobacterium tuberculosis* (MTB) เป็นต้นแบบ แล้วเปรียบเทียบโปรตีนที่ถูกสร้างขึ้นในระยะติดเชื้อกับสภาวะปราศจากเชื้อ ด้วยวิธีตัดโปรตีนด้วยเอ็นไซม์ trypsin ในเจล (in-gel tryptic digestion) ต่อด้วยการทำ liquid chromatography-tandem mass spectrometry (LC MS/MS) โดยนำโปรตีนรวมนอกเซลล์ (จากน้ำเลี้ยงเซลล์) และ โปรตีนรวมในเซลล์ (องค์ประกอบในเซลล์จากการทำให้แตกแล้ว) โดยใช้เซลล์แมคโครฟาจชนิด THP-1 (จำนวน 3 x 10^6 เซลล์) ที่ติดเชื้อวัณโรคสายพันธุ์ H37Rv ด้วยอัตราส่วนระหว่างเชื้อและเซลล์ 1:1 ซึ่งเชื้อในเซลล์ถูกฆ่าด้วยยาสัดส่วน isoniazid 3 ไมโครกรัม และ ยา rifampicin 9 ไมโครกรัม จากนั้นโปรตีนรวมจึงถูกเก็บมาวิเคราะห์ในวันที่ 1 (สภาวะติดเชื้อ) และวันที่ 5 หลังจากเติมยาแล้ว (สภาวะปราศจากเชื้อ) โดยสภาวะปราศจากเชื้อวัดจากการไม่พบ MTB ในเซลล์แมคโครฟาจ จากนั้นจึงเตรียมโปรตีนที่สกัดได้เพื่อวิเคราะห์ด้วย LC MS/MS ผลการศึกษาพบว่ามีชนิดของโปรตีนภายในเซลล์จากการทดลอง 3 ซ้ำ และอีก 1 ตัวอย่างโปรตีนรวมจากสามซ้ำของการทดลองรวมกันที่แตกต่างกันระหว่างสภาวะติดเชื้อและปราศจากเชื้อ ในส่วนภายในเซลล์ จำนวน 5,142 ชนิด และภายนอกเซลล์จำนวน 2,680 ชนิด โดยมีโปรตีนที่พบเฉพาะสภาวะติดเชื้อ (ไม่พบในสภาวะปราศจากเชื้อ) อย่างน้อย 2 ใน 4 ซ้ำ จากภายนอกเซลล์ 4 ชนิด และภายในเซลล์จำนวน 18 ชนิด และมีโปรตีนที่พบเฉพาะสภาวะปราศจากเชื้อ (ไม่พบในสภาวะติดเชื้อ) อย่างน้อย 2 ใน 4 ซ้ำ จากภายนอกเซลล์จำนวน 9 ชนิด และภายในเซลล์ 12 ชนิด โดย CAECAM18 และ ras GTPase เป็นโปรตีนที่มีแนวโน้มที่ดีในการใช้เป็นตัวบ่งชี้สภาวะปราศจากเชื้อวัณโรค เนื่องจากพบซ้ำๆ ในการทดลองพบปริมาณสูง และเป็นโปรตีนที่มีมีหน้าที่สัมพันธ์กับการติดเชื้อวัณโรค โปรตีนเหล่านี้อาจจะสามารถนำมาเป็นตัวชี้วัดสภาวะปราศจากเชื้อวัณโรคภายหลังจากการรักษาได้

คำสำคัญ *Mycobacterium tuberculosis*, ติดเชื้อวัณโรคแอบแฝง, comparative proteomic, LC MS/MS, clearance marker

Abstract

Project Code: MRG5580024

Project Title: Proteomic study of host-Mycobacterium tuberculosis interaction during anti-tuberculous drugs treatment in human macrophage

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One-third of the world population was infected with *Mycobacterium tuberculosis* (MTB) and was defined as latent tuberculosis infection (LTBI). So far, no biomarker for determining the clearance stage of MTB infection after treatment. We aim to analyze the potential biomarkers indicating the clearance stage of MTB infection by using macrophage infection model. Comparative proteomic analysis between MTB infection stage and clearance stage were performed by using in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry (GeLC MS/MS). Extracellular (culture supernatant) and intracellular (cell lysate) proteomes from activated THP-1 macrophages (3 x 10^6 cells) infected with MTB H37Rv strain (MOI = 1) and treated with 3 μg of isoniazid and 9 μg of rifampicin for 1 day (infection stage) and 5 days (clearance stage) post infection were extracted, processed for GeLC MS/MS. Clearance stage was defined as the condition with no viable MTB from infected macrophages. Three different proteomes from 3 independent experiments and one pooled sample from those were analyzed. There were overall 5,142 and 2,680 protein elements were found in intracellular and extracellular proteome, respectively, in at least one experiment from all independent experiments. There were 4 extracellular and 18 intracellular that uniquely found in the infection stage (at least 2 in 4 replica experiments) and were not found in non-infection control conditions. There were 9 extracellular and 12 intracellular proteins in the clearance stage. CAECAM18 and ras GTPase were promising to use as MTB clearance biomarker due to their consistent results from independent experiments, presence with high quantity and the associated function to MTB infection. These proteins were the potential biomarkers to indicate the clearance stage of MTB infection after anti-tuberculous treatment.

Keywords: Mycobacterium tuberculosis, latent Mycobacterium tuberculosis infection, comparative proteomic, LC MS/MS, clearance marker

Executive summary

เนื่องจากปัจจุบันยังไม่มี biomarker ที่มีประสิทธิภาพที่บ่งชี้ถึงสภาวะปราศจากเชื้อจากร่างกายของผู้ป่วยหลังจากการรักษาด้วยยาต้านวัณโรค วัตถุประสงค์ของโครงการนี้ คือ การศึกษาถึง proteomic profile จาก host macrophages และ *M. tuberculosis* interaction ในขณะที่มียาต้านวัณโรค และเป็นการตรวจหา biomarker เมื่อเชื้อวัณโรคถูกทำลายออกไปจากเซลล์ ซึ่งสามารถนำไปใช้เป็นตัวบ่งชี้ในการตรวจติดตามการรักษาผู้ป่วยวัณโรคต่อไป

แนวความคิด คือ หากกำจัดเชื้อ *M. tuberculosis* ที่ infect ใน macrophages ด้วยยา anti -TB drugs แล้ว จะมี proteomic profile เปลี่ยนไป (เพิ่ม/ลด) อย่างไร เมื่อเปรียบเทียบกับ proteomic profile ที่มาจากมาจาก macrophages อย่างเดียวที่เติมยา (ตัวควบคุม) โดยจะดูทั้ง whole cell protein (cell pellets) และ secreted protein (supernatants) การใช้ macrophages model ทำให้แน่ใจถึงสภาวะปราศจากเชื้อจากเซลล์ ซึ่งต่างจากการศึกษาในมนุษย์ที่ไม่สามารถแน่ใจได้ว่าเชื้อได้หมดไปจากเนื้อเยื่อแล้วจริงๆ

การดำเนินการวิจัยนั้น เริ่มจากการที่เลี้ยงเชื้อวัณโรคโดยใช้ *M. tuberculosis* H37Rv (laboratory virulent strain) ในอาหารเลี้ยงเชื้อเหลว จากนั้นนำมาทำการติดเชื้อให้กับ human macrophage cell line (THP-1 cells) ซึ่งถูกกระตุ้นด้วยสาร PMA เพื่อให้เป็น activated macrophages ที่สัดส่วน MOI = 1 (สัดส่วนที่ macrophages เซลล์ส่วนใหญ่จะถูกติดเชื้อ แต่ก็ไม่มากจนเกินกว่าจะถูกทำลายได้ด้วย anti-TB drug ภายในเวลา 5 วัน) จากนั้นเติม anti TB drugs เพื่อฆ่าเชื้อวัณโรค (ทั้งภายนอกเซลล์และในเซลล์) จากนั้นจึงเก็บ protein จาก whole cell protein (cell pellets) และ secreted protein (supernatants) เพื่อนำมาทำ GeLC MS/MS เพื่อวิเคราะห์หา การเพิ่มขึ้นหรือลดลง ของ protein (ทางผู้วิจัยคาดว่า protein จาก macrophages คือ protein เป้าหมาย) ซึ่งทำการทดลอง 3 ครั้งต่างช่วงเวลากัน และจากตัวอย่างผสมรวมจากทั้ง 3 การทดลอง (pooled sample) เพื่อยืนยันผลการทดลอง

ผลการทดลองพบ macrophage protein ที่มีความแตกต่างอย่างมีนัยสำคัญระหว่างสภาวะที่มีเชื้อ (infection stage) และสภาวะที่ปราศจากเชื้อ (clearance stage) และไม่พบในกลุ่มควบคุม (เซลล์ไม่ติดเชื้อที่เติมยา) อย่างน้อยหนึ่งในสามการทดลอง จำนวน 5,142 ชนิด ซึ่งมาจาก intracellular proteins จำนวน 2,969 ชนิด และจาก extracellular protein จำนวน 2,680 ชนิด โดยพบว่ามี extracellular protein จำนวน 4 ชนิด (protein ที่ให้ mass intensity สูงสุด ที่ 9.65±1.14 คือ APCDD1-like protein) และ intracellular protein จำนวน 18 ชนิด (protein ที่ให้ mass intensity สูงสุด ที่ 9.07±0.68 คือ TLN2) ที่พบเฉพาะ infection stage แต่ไม่พบใน clearance stage จากสองในสี่ตัวอย่างต่างการทดลอง และพบ extracellular protein จำนวน 9 ชนิด (protein ที่ให้ mass intensity สูงสุดที่ 9. 7.31±0.69 คือ drebrin) และ intracellular protein จำนวน 18 ชนิด (protein ที่ให้ mass intensity สูงสุดที่ 8.16±0.13 คือ IL-11) ที่พบเฉพาะ clearance stage แต่ไม่พบใน infection stage จากสองในสี่ตัวอย่างต่างการทดลอง โดย CAECAM18 และ ras GTPase เป็นโปรตีนที่มีแนวโน้มที่ดีในการใช้เป็นตัวบ่งชี้สภาวะปราศจากเชื้อวัณโรค เนื่องจากพบซ้ำๆ ในการทดลองพบปริมาณสูง และเป็นโปรตีนที่มีมีหน้าที่สัมพันธ์กับการติดเชื้อวัณโรค โปรตีนเหล่านี้อาจเป็น biomarker ที่สามารถใช้ในการบ่งชี้สภาวะปราศจากเชื้อของผู้ป่วยวัณโรคที่ได้รับยาต้านเชื้อวัณโรค และอาจช่วยในการศึกษาถึงยาต้านหรือวัคซีนวัณโรคได้ ทั้งนี้อาจมีการศึกษาเพิ่มเติมเพื่อยืนยันผล biomarker เหล่านี้ต่อไป

<u>เนื้อหางานวิจัย</u>

1. บทนำ

Tuberculosis (TB) is one of the greatest public health problems. One-third of the global population is infected with *Mycobacterium tuberculosis* (MTB), the causative agent of TB (1). MTB contain ability to enter dormant stage in host tissue, enabling long term survival in humans. Despite tremendous efforts have been directed toward for controlling TB, the TB epidemic is not under control and number of new case increase every year. Annually, two million people will be dead due to TB. The rate of drug resistant especially multi-drug and extensively-drug resistant TB is increasing. The emerging of virulence strain of MTB and increasing trend of human immunodeficiency virus (HIV) are also the enhancing factors for TB problem (2). The understanding of host-pathogen interaction during the treatment course may provide more information on drug resistant mechanism and drug-drug interaction and is essentially needed for proper interventions.

For TB eradication, TB vaccine is one of the tools that are inevitably needed. The effective TB vaccine is lacking. BCG vaccine may protect only severe form of TB (3, 4). Other strategies that can contribute to the eradication of TB are the identification and treatment of latent TB infection (LTBI) cases. At present, the interferon gamma releasing assays (IGRAs), compare to tuberculin skin test, is a more reliable tool to identify LTBI cases (5). Six to nine months of isoniazid (INH) mono-therapy was suggested to be a standard regimen for LTBI treatment in Europe and USA. The shorter course, four month rifampicin (RIF), was also suggested for LTBI treatment (6). However, the effective and reliable tool or marker that can be used to predict the success and monitoring the treatment of both active and LTBI is lacking. Furthermore, the evaluation of novel anti-tuberculous agents and vaccine is hampered by the lack of biological tools to help predict efficacy, from early drug and vaccine development to clinical trials.

In nature, MTB infected cases may develop active disease, progress into the latent stage that contain the bacilli in tissue or limit by the host immune system that may eliminate the bacilli as in the sterilized condition. Ten percent of infected cases contain the bacilli can develop the disease within the first two years whereas the other 90 percent contain dormant pathogen in tissue can develop into the active disease in lifelong. Presently, there is no tool or marker to differentiate between the people who used to be infected with MTB but the pathogen was cleared versus the LTBI patients (contain the timing bomb bugs). The differentiation between the people who still has latent MTB versus the people who used to be infected with MTB (cleared case) could contribute to the LTBI treatment. In the global scale, such tool or marker can contribute the possibility of the mass treatment of LTBI by narrowing down the target group of the LTBI patients who really require the treatment.

2. วัตถุประสงค์ของโครงการ

To analyze the potential biomarkers indicating the infection and clearance stage of MTB infection by using THP-1 macrophage infection model based on comparative intracellular and extracellular proteomic study using GeLC MS/MS.

3. วิธีการทดลอง

The conceptual framework and experimental scheme was described (figure 1 and 2).

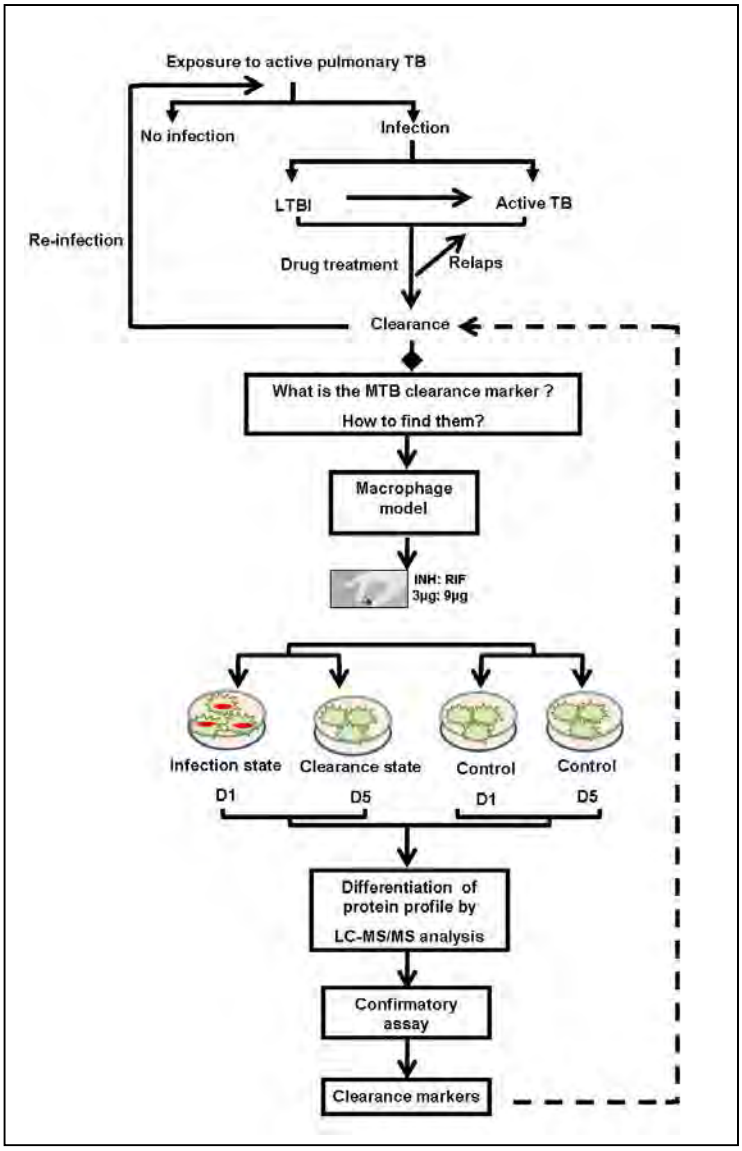


Figure 1. Conceptual framework of the study.

After expose to a source of pulmonary TB, the exposed individual can become infected or non-infected with *M. tuberculosis* (MTB). The infected people can develop into latent TB (LTBI) or active TB. After anti-TB treatment, MTB can be sterilized, non-sterilized or become ill (infected) again. Nonetheless, there is no biomarker indicate the clearance stage of MTB infection after treatment. So in this study we aim to find the biomarkers indicating the clearance stage of MTB by using macrophage model (THP-1) infected with MTB H37Rv and treated with anti-tuberculous drugs. Intracellular and extracellular proteins were collected and analyzed by LC-MS/MS. The data from 3 independent experiments and pooled sample were analyzed to confirm the validity of the result.

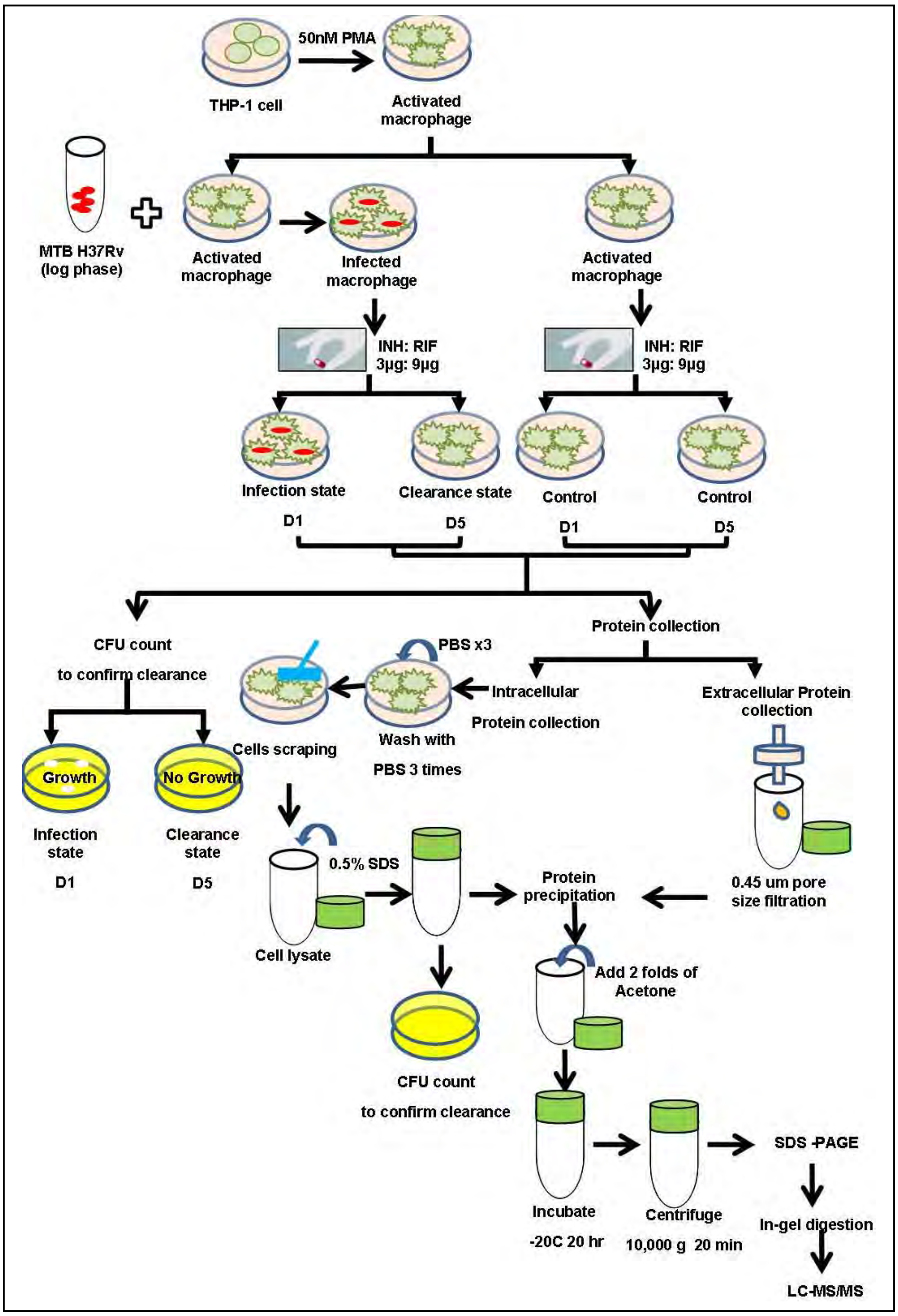


Figure 2. Schematic diagram of experiment.

THP-1 cell was activated by 50 nM PMA and infected with MTB H37Rv that was grown at late log phase. The infected macrophages were treated with 3µg INH and 9µg RIF for 1 day (MTB were remaining in cells, i.e. infection stage) and 5 days (No MTB in cells, i.e. clearance stage) and drug treated THP-1 cell (without MTB) for 1 day and 5 day were used as controls. The culture supernatant and cell lysates were collected. The extracellular proteome (supernatants) were filtrated with 0.45 µm pore size filter. To collect intracellular proteome, infected cells were washed 3 times with PBS, scrapped and lysed by using o.5% SDS. CFU count was performed to confirm the clearance stage of MTB from intracellular and extracellular compartments. The protein solutions were precipitated by adding 2 fold of acetone and centrifuge at 10,000 g for 20 min and re-suspended by protease inhibitor containing solution. The proteins were fractionated by SDS-PAGE and digested by in-gel digestion using trypsin. The proteomic profiles were analyzed by LC-MS/MS.

3.1 THP-1 cell culture and activation

THP-1 cells were cultured in RPMI1640 supplemented with 2 mM L-glutamine and 10% FBS. THP-1 cells were activated using PMA by centrifugation at 800 rpm for 5 min then remove the supernatant and re-suspend with 2 ml cRPMIl, count the cell by hema-cytometer (by dilute 2 µl of cell suspension with 10 µl trypan blue). THP-1 cell were diluted into 7.5 x 10^{5} cell/ml for 48 ml of THP-1 cell (monocyte stage), activated by adding of 150 µl of 16 µg/ml PMA (final concentration = 50 nM PMA) for 48 ml into 2 ml of cell suspension, transferred to 6 well plate (1.5 x10^{6} cells/well), follow by incubation at 37°C, 5% CO_2 for 24 hr. To reduce the effect of PMA from THP-1 cell, the old media was discarded and refill by fresh 3 ml cRPMI and incubate at 37°C and 5%CO_2 for 24 hour.

3.2 MTB culture and preparation of inoculums

MTB H37Rv was grown in Middlebrook 7H9 with OADC for 14 days. The 14 day-H37Rv were measured and adjusted by 0.5 MCFarland standards. Then the cells were diluted into 7.5 x 10^{5} cells/ml for 48 ml in cRPMI. The clumping of MTB cell suspensions were removed by passing through a 26-gauge needle set.

3.3 Infection experiments

After discard the media of THP-1 cells from 6 well plates, MTB H37Rv (1.5 x10^{6} cell/well, MOI = 1) in cRPMI suspension was immediately refilled and then incubated at 37°C and 5% CO_2 for 4 hour. After 4 hour of incubation, the infected cells were treated with 3 ml of 3 µg/ml INH and 9 µg/ml RIF. The drug solutions were changed every 24 hour. The cell lysate and culture supernatant of the Infected cell and control (uninfected cells with the same anti-tuberculous drugs) were collected for 1 day (infection stage) and 5 days (clearance stage) post infection.

3.4 Proteomes collection

Extracellular (culture supernatant) and intracellular (cell lysate) proteomes were collected. After 24 hr incubation, for extracellular protein collection, the culture plate was gently tapped at 4 sides of the plates for 5 times each side and then 3 ml supernatants from each well were collected. The supernatants from each well (approx. 3 ml) were pipetted by syringe, then filtered and transferred into 10 ml conical tubes. All 4 tubes (2 tests and 2 controls from 2 time points) were kept at -80 °C for extraction and precipitation with other supernatants from additional 2 independent experiments. For the intracellular proteins, infected cells were washed 2 times using PBS and scrapped off using chilled Phosphate buffered saline (PBS) solution 500 µl/well and then completely transferred into 15 ml conical tubes. Then, 400 µl of 6% SDS were added into the pooled PBS pellets from replicate wells (final concentration of SDS at 0.5% (w/v)). The tubes were incubated for 5 min and followed by sonication at 53 KHz 37°C for 30min. Tubes of protein lysate suspensions were kept at -80°C for extraction and precipitation with additional 2 independent experiments.

3.5 Protein extraction and preparation

For protein extraction and precipitation, protein suspensions were thawed at room temperature. Two fold volumes (6 ml and 4.4 ml) of acetone were added into culture supernatant and cell lysate suspension, respectively. Protein suspensions were incubated at -20 °C for 8 hours and then centrifugation at 10,000 g for 30 minutes at 4 °C and supernatant was discarded and left to air dry for 30 minutes. The protein pellets were re-suspended by using 30 µl sample storage buffer and left to dissolve at room temperature for 1 hour. The protein suspensions were then transferred to 1.5 ml Eppendorf tube. The quantities of the extracted proteins were measured in triplicate by Lawry method Assay using bovine serum albumin (BSA) as a standard. The BSA standard was diluted into 2 mg/ml and serially diluted into 2, 4, 6, 8, 10 and 0 µg. Intra- and extra-cellular protein samples were diluted 10 and 20 times, respectively, for the appropriate concentration of the MS analysis .Five µl of protein standard and samples were transferred into 96 well plate (triplicate) and 200µl of solution A (2.5% SDS, 2.5% Na_2CO_3, 0.2N NaOH, 0.025% $CuSO_4$ and 0.05% Tatalic acd) was added and incubated 30 min at room temperature. Then, 50 µl of solution B (20% Folin and Ciocalteu's phenol reagent) was added and incubated for 30 min at room temperature. The protein concentrations were measured by densitometer at OD 750 and the concentrations were calculated by their absorbance values compared to the standards.

3.6 SDS PAGE and in-gel digestion

The protein samples were diluted into 50 µg for 10 µl (50µg/well). Then 10 µl of protein samples and 10 µl of 2x loading buffer were added into micro-centrifuge tubes and mixed. The protein samples were incubated at 60°C for 10 minutes. The proteins samples were then run by SDS-PAGE gel at a constant current of 15 mA for 80 min. After SDS PAGE completion, the gels were stained with coomassie blue and incubate for 1 hour and de-stain with de-staining solution (10% glacial acutic acid, 40% methanol). The gel were washed with DW (incubate for 10 min for 2 times) and cut into 1 mm^3 cubes and transferred into 96 well-plates. The gel pieces were washed with 200 µL of DW and shake for 10 minutes, and then all liquids were removed. The coomassie blue dye was removed by 3 cycles of adding 200 µL of 25 mM NH_4HCO_3 and shake for 10 min, and then all liquid was removed. The remaining chemical was removed by 3 cycles of adding 200 µL of DW and shaking for 10 min and removing of all liquid. Then, 2 cycles of adding 200 µL acetonitrile (ACN), shaking for 10 min and removing of all liquid. All gel pieces were dried at room temperature for 10 minutes. Then, 50 µL of 10 mM DTT and 10 mM NH_4HCO_3 was added and incubated at 56°C for 1 hour. Then, 50 µL of 100 mM IAA and 10 mM NH_4HCO_3 was added and incubated at room temperature for 1 hour (with light protection), and then all liquid was removed. Then, 2 cycles of adding 200 µL of ACN was added, shaking for 5 min and removing of all liquid were performed. Enzymatic protein digestion was performed by adding of 10 µL of enzyme solution (10 ng/µl trypsin in 10mM NH_4HCO_3) covering the gel pieces and incubated at 37°C for 3 hours. The peptide was extracted by 3 cycles of adding of 50 µl 50% ACN and shaking at room temperature for 10 minutes/cycle. Then the peptde solutions were transferred into new low binding 96 well-plates and dry the solution at at 40 °C until the solution dried. The dry peptides were kept at -20°C until analyzed.

3.7 LC MS/MS

The adjustment of background intensity was performed by adding 12 µl/well of 0.1% formic acid in LC/MS-grade water. The peptides were re-suspense with 0.1% formic acid and mixed by auto-pipette for 100 times. Then the samples were transferred into the low-binding tubes. Samples were centrifuged at 10,000 rpm for 10 minutes and then transferred into the vial tubes for injection. The volume of injection to LC- MS/MS analysis (SYNAPT G1 HDMS mass spectrometer, WATERS) was 4.5 µl.

Nano scale LC separation of tryptic peptides was performed with a NanoAcquity system equipped with a Symmetry C18 5 µm, 180-µm x 20-mm Trap column and a BEH130 C18 1.7 µm, 100-µm x 100-mm analytical reversed phase column. The samples were initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 15 µl/min for 1 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were separated with a gradient of 15–50% mobile phase B over 15 minutes at a flow rate of 600 nl/minute followed by a 3-minutes rinse with 80% of mobile phase B. The column temperature was maintained at 35 °C. The lock mass was delivered from the auxiliary pump of the Nano Acquity pump with a constant flow rate of 500 nl/min at a concentration of 200 fmol/µl of fibrino peptide B to the reference sprayer of the Nano Lock Spray source of the mass spectrometer. All samples were analyzed at once. Analysis of tryptic peptides was performed using a SYNAPTTM HDMS mass spectrometer (Waters Corp., Manchester, UK). For all measurements, the mass spectrometer was operated in the V-mode of analysis with a resolution of at least 10,000 full-width half-maximum. All analyses were performed using positive nanoelectrospray ion mode. The time-of-flight analyzer of the mass spectrometer was externally calibrated with fibrinopeptide B from 50 to 1600 m/z with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu1] fibrinopeptide B. The reference sprayer was sampled with a frequency of 20 sec. Accurate mass LC-MS data were acquired with data direct acquisition mode. The energy of trap was set at collision energy of 6 V. In transfer collision energy control, low energy was set at 4 V. The quadrupole mass analyzer was adjusted such that ions from m/z 300 to 1800 were efficiently transmitted. The MS\MS survey was over the range 50 to 1990 Da and scan time was 0.5 sec.

3.8 Data analysis

The data from LC MS/MS analysis was analyzed by Decyder MS 2.0 differential analysis software (Amersham biosciences, UK) and searched by mascot database (www.ncbi.nlm.nih.gov, National Center for Biotechnology Information, U.S. National Library of Medicine, USA). The parameters for analysis were 0.1 unit m/z shift tolerance and 5.0% m/z shape tolerance. Group-to-group comparisons were performed by t-test and Multi-group comparisons were performed by using ANOVA. The values were normalized with BSA external intensity control. The p-values < 0.05 were considered statistically significant.

4. ผลงานวิจัยที่ได้รับ

4.1 Optimization of PMA concentration for THP-1monocyte activation

The PMA concentration was generally used from 1-100 nm/ml to activate THP-1 cell monocytes to macrophages. This experiment was aimed to optimize the optimal concentration and time for activation of THP-1monocyte.

In order to optimize the concentration of the PMA to fit the THP-1 cell line and condition of experiment, the PMA at 5-100 nm/ml were added to the 5×10^5 cells/ml THP1 cells in each well. After incubated at 37 °C 5% CO_2 for 24 hr, the culture media were changed and replenished with fresh RPMI supplemented by 10% FBS. The THP-1 monocytes were activated into macrophages, the cells with appendages and stick to the culture well surface, in the well with 5-100 nm/ml PMA. The control cells without PMA were not changed and were washed away after 24 hr (lower cell density) (Figure 3 and 4).

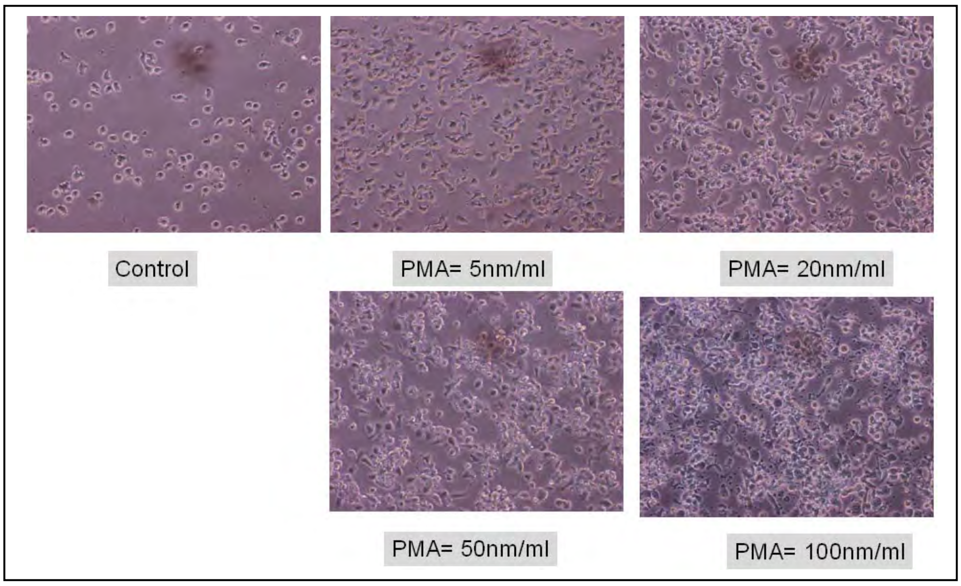


Figure 3. Morphology of activated THP-1 macrophages 24 hr after PMA activation (400X).

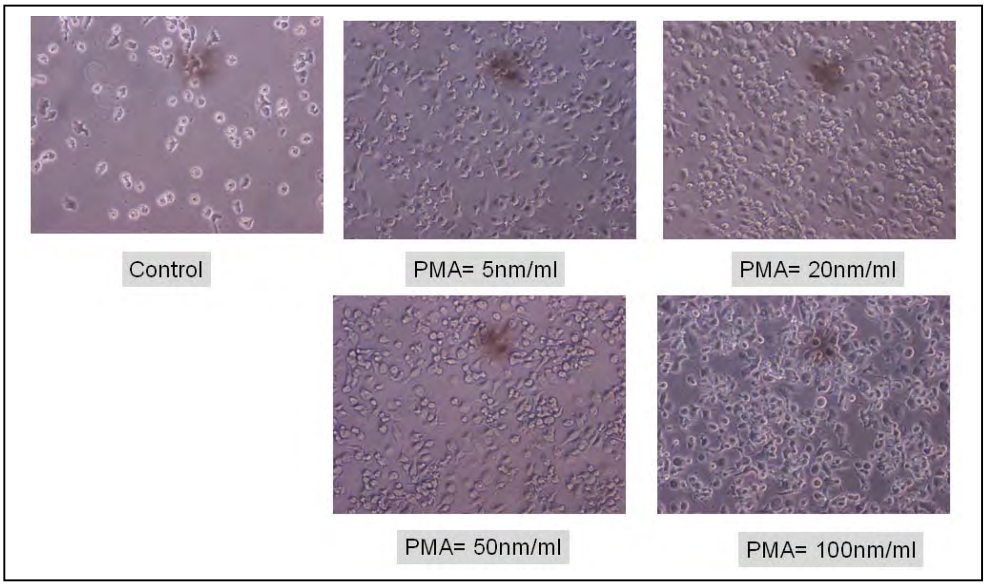


Figure 4. Morphology of activated THP-1 macrophages 48 hr after PMA activation (400X).

It was found that the clumping of the macrophages directly correlated with the PMA concentration, i.e. the higher of PMA, the more clumping of THP-1 cells. The 48 hr after activation (Figure 4), compared to 24 hr (Figure 3), provided better morphology under microscopic observation. PMA at 50 and 100 nm/mi provided the highest % activation. However, the morphology of activated macrophages at 50 nm/ml PMA is better than 100 nm/ml with less cells death, determined by trypan blue staining (data not shown). Therefore, the PMA concentration at 50 nm/ml with 48 hr duration is the optimal concentration for monocyte activation in the experiment.

4.2 Optimization of DMSO concentration for the cell culture conditions.

DMSO needs to be used as a solvent for diluting the anti-tuberculous drugs (INH and RIF) in the experiment. However, DMSO at high concentration can be toxic to the cells. So, the minimal concentrations of DMSO need to be adjusted for the cell culture condition. DMSO were added into the cell culture (5*10^5 cells/ wells/ 3ml) at final concentration 0.125 to 4% and incubated at 37 °C 5% CO_2 for 1-5 days (Table 1).

It was found that concentration of DMSO directly correlated with viability of activated THP-1 cells (both microscopically observation and typan blue staining test) (Figure 5 and Figure 6). The highest concentration of DMSO that was not affected the cell variability is < 0.25%. Therefore, the DMSO that we use for the experiment is < 0.01%, for the least toxicity.

Table 1. Optimization of DMSO concentration and toxicity test.

DMSO concentration	Initial number of THP-1 cells (Day 0)	Number of activated THP-1 cells at day 5*
Control (without DMSO)	5*10^5 cells/ well	2.4x10^5 cell/ml/well
0.125%		2.1x10^5 cell/ml/well
0.25%		2.13x10^5 cell/ml/well
0.5%		1.25x10^5 cell/ml/well
1%		7.8x10^4 cell/ml/well
2%		3.26x10^4 cell/ml/well
4%		1.5x10^4 cell/ml/well

*Day 5 after activation and adding DMSO

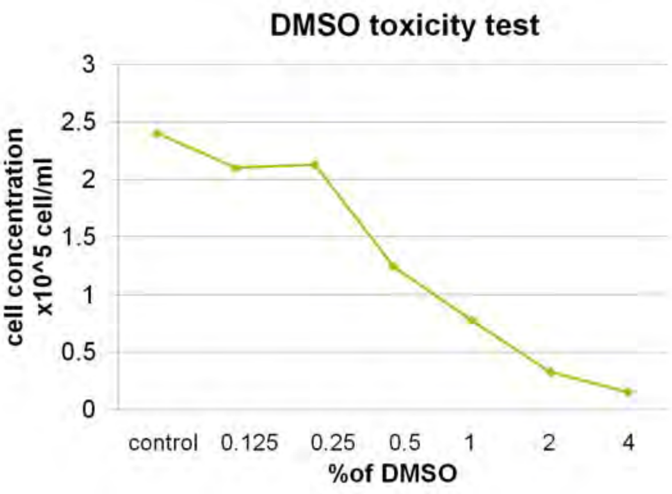
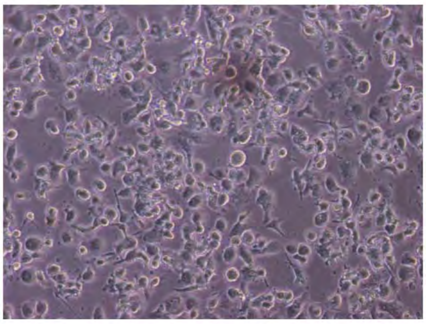
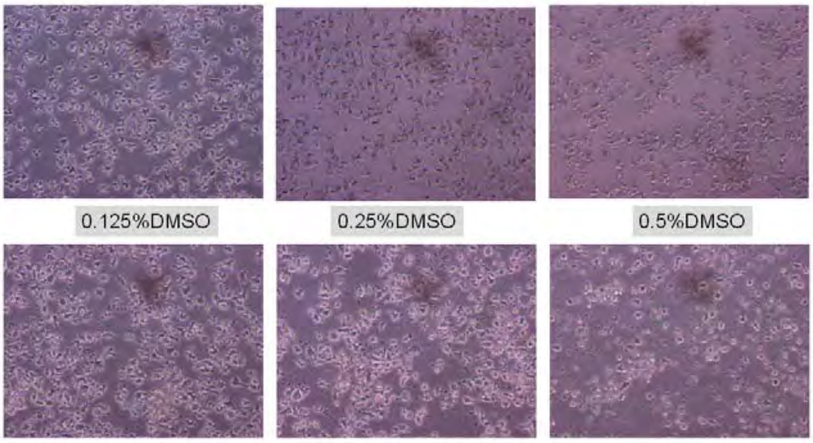


Figure 5. The association between DMSO concentration and number of viable THP-1 cells.



Control
(50nM PMA)

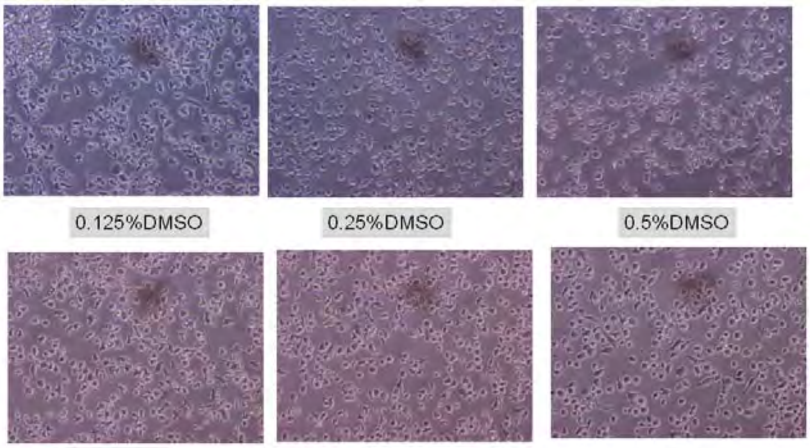
Control: Control cells without DMSO. The activated THP-1 cells were maintain the morphology and viability from day 1 to day 5 after activation (400X).



0.125%DMSO 0.25%DMSO 0.5%DMSO

1%DMSO 2%DMSO 4%DMSO

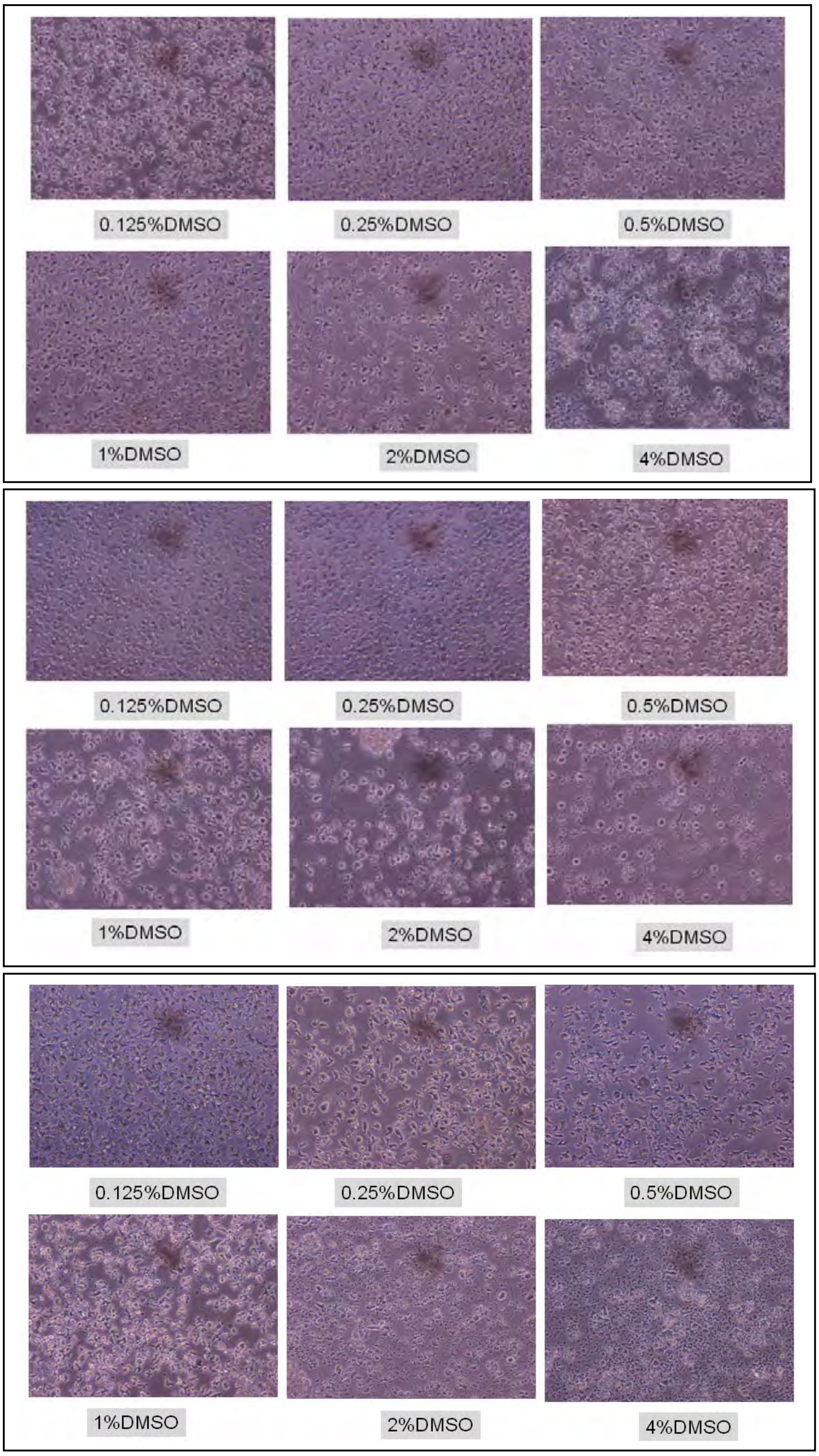
Day 1 after activation + DMSO (400X)



0.125%DMSO 0.25%DMSO 0.5%DMSO

1%DMSO 2%DMSO 4%DMSO

Day 2 after activation + DMSO (400X)



Day 3 after activation + DMSO (400X)

Day 4 after activation + DMSO (400X)

Day 5 after activation + DMSO (400X)

Figure 6. DMSO toxicity test. The concentration of DMSO at 0.25% showed cell morphology and viable cell number similar to 0.125% but obviously different from 0.5%.

4.3 Optimization of drug concentration for killing intracellular *M. tuberculosis* (MTB)

The concentration and duration for the killing effect of anti-tuberculous (TB) drug (Isoniazid and rifampicin) to MTB need to be optimized. Therefore, RIF (rifmapicin) and INH isoniazid were prepared at 10 ug/ml stock concentration and were kept at -20 C. The THP-1 cells (5x10^5/ml for 3 ml = 1.5x10^6 cells/well) were activated by adding PMA at 50 nm/ul for 48 hr (change the media after 24 hr.). MTB H37Rv were culture for 14 days (late log phase) and prepare for the inoculum by adjusting with McFalrland standard No. 1 (that equal to 3 x 10^8 CFU/ml) and then the inoculum were diluted into the concentration that equal to MOI = 1. The inocula were added into the wells. After 4 hr postinfection, the supernatants (extracellular MTB) were removed and the fresh RPMI media with anti-TB drugs were added as the following concentration (Table 2).

Table 2. Antituberculous drug concentration used for the experiments.

Labels	Drug concentration and condition	Source of concentration and references
Control	Without drugs	-
C1	3 μg/ml INH	Killing MTB H37Rv in THP-1 assays (1)
C2	6 μg/ml INH	Blood concentration level (2)
C3	10 μg/ml INH	Killing MTB in hMDMs assays (3)
C4	3 μg/ml INH + 9 μg/ml RIF	Minimum concentration in blood (2)
C5	6 μg/ml INH + 18 μg/ml RIF	Maximum concentration in blood (2)

Previous study suggested that 10 μg/ml INH can kill intracellular MTB (*in-vitro*) within 3 days (3). Generally for TB treatment, the ratio between INH and RIF for TB treatment is 1: 3.

After 1 day (24 hr postinfection), day 3 and 5 days, the supernatant (extracellular MTB) were collected (and were also tested for CFU count) and macrophages were lysed by PBS/0.1%triton X-100. Both cell pellets and supernatants were cultured on the Middlebrook (M) 7H10 for determination of CFU count.

The CFU counts were determined at day 1, day 3 and day 5. The concentration and condition for the anti-TB drug that can clear the intracellular MTB (both intra and extracellular bacteria) within 3-4 days postinfection will be selected to perform the final infection experiments.

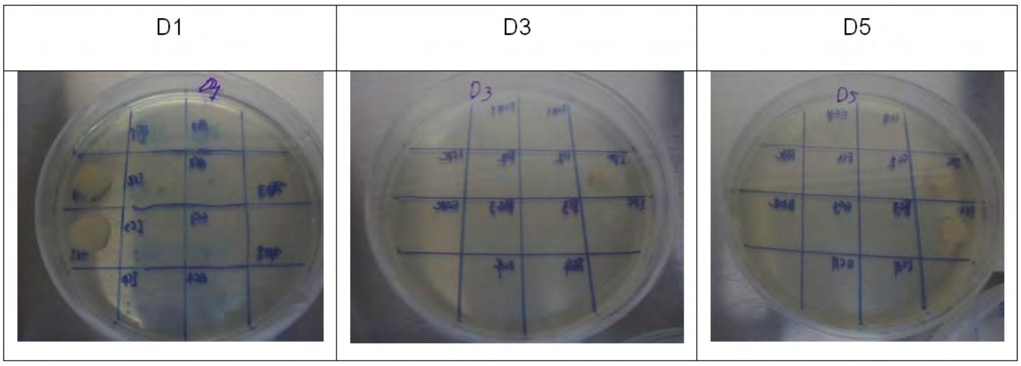
4.4 Drug concentration test

The activated macrophage were infected with MTB H37Rv with MOI=1 and enhance engulfment by incubation for 4 hours at 37 ˚C. After that drug treatment for the infected cell with different concentration ratio between INH (μg/ml): RIF (μg/ml), C1 (0.25:0.75), C2 (0.5: 1.5), C3 (1: 3), C4 (3: 9) as described (Table 3), the drug were changed every 24 hours. Then, the intracellular and extracellular bacteria were dropped on M7H11 agar plate at day 1, day 3 and day 5 follow by incubation for 3 week at 37 °C. After 3 week of incubation, the growth of MTB colonies was observed. The optimal drug concentration ratio at 3μg/ml INH: 9μg/ml RIF that give only intracellular growth at day 1 after drug treatment and no growth at day 5 was used in the actual experiments (Figure 7).

Table 3. The ratio and concentration of anti-tuberculosis drugs and growth of *M. tuberculosis* in activated macrophages 1, 3 and 5 days.

Drug concentration INH:RIF (ug/ul)	D1		D3		D5	
	intra	extra	intra	extra	intra	extra
0.25:0.75	growth	growth	growth	NG	NG	NG
0.5:1.5	growth	growth	growth	NG	NG	NG
1:3	growth	growth	growth	NG	NG	NG
3:9	growth	NG	NG	NG	NG	NG
No drug control	growth	growth	growth	growth	growth	growth
No infection control	NG	NG	NG	NG	NG	NG

*Intra: intracellular bacteria, Extra: extracellular bacteria, growth: bacteria show the colony growth, NG: no growth of the bacteria



D1 D3 D5

Figure 7. Anti-tuberculosis drugs and growth of *M. tuberculosis* (corresponding to table 3).

4.5 Infection and clearance confirmation test

The activated macrophage were infected with *M. tuberculosis* H37Rv with MOI=1 and enhance the engulfment by incubating for 4 hours. After those anti-TB drugs with concentration from the optimized concentration, 3μg/ml INH: 9μg/ml RIF, were changed every 24 hours. Then, the intracellular and extracellular bacteria were dropped onto M7H11 agar plate at Day 1 and day 5 follow by incubation for 3 week at 37 °C. After that the colony formation of bacteria were observed, the MTB colonies were found on Day 1 but not Day 5 (Table 4 and Figure 8). The results were consistent in all 3 independent experiments (different occasions) (Table 4).

Table 4. Infection and clearance confirmation test of activated macrophages infected with *M. tuberculosis* and treated with anti-tuberculous drugs.

Experiment occasion	Sample type	D1		D5	
		intra	extra	intra	extra
1st	control	NG	NG	NG	NG
	test	growth	NG	NG	NG
2nd	control	NG	NG	NG	NG
	test	growth	NG	NG	NG
3rd	control	NG	NG	NG	NG
	test	growth	NG	NG	NG

*Intra: intracellular bacteria, Extra: extracellular bacteria, growth: bacteria show the colony growth, NG: no growth of the bacteria, D1: day1, D: day 5

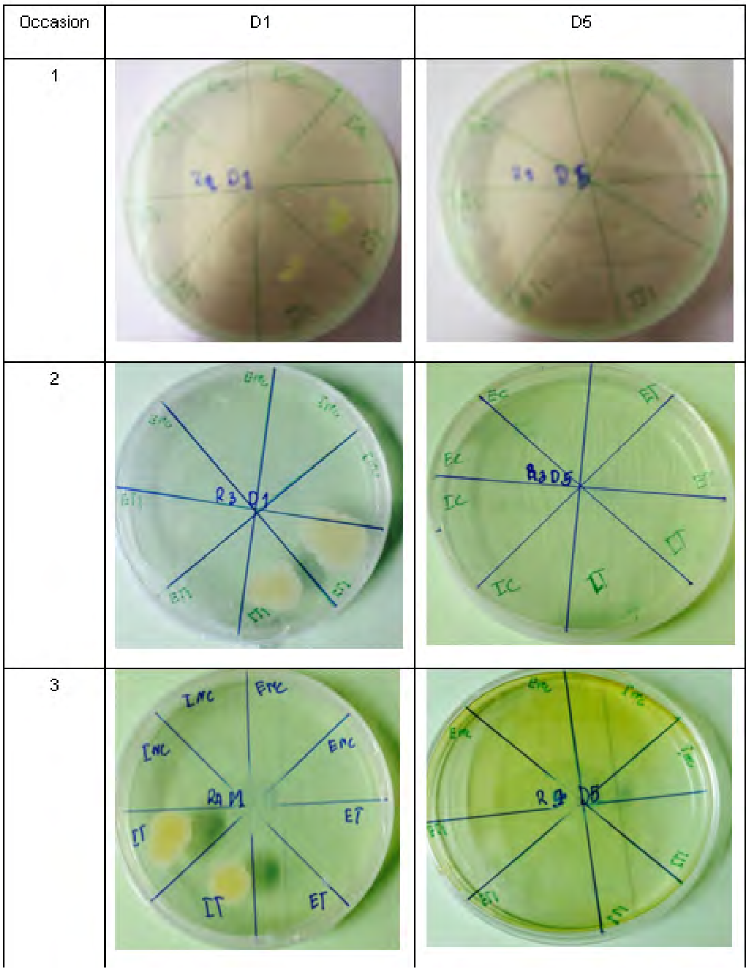


Figure 8. Infection and clearance confirmation test (corresponding to table 4). Occasion referred to replicate of independent experiment. D1 and D5 referred to 1 day and 5 day postinfection.

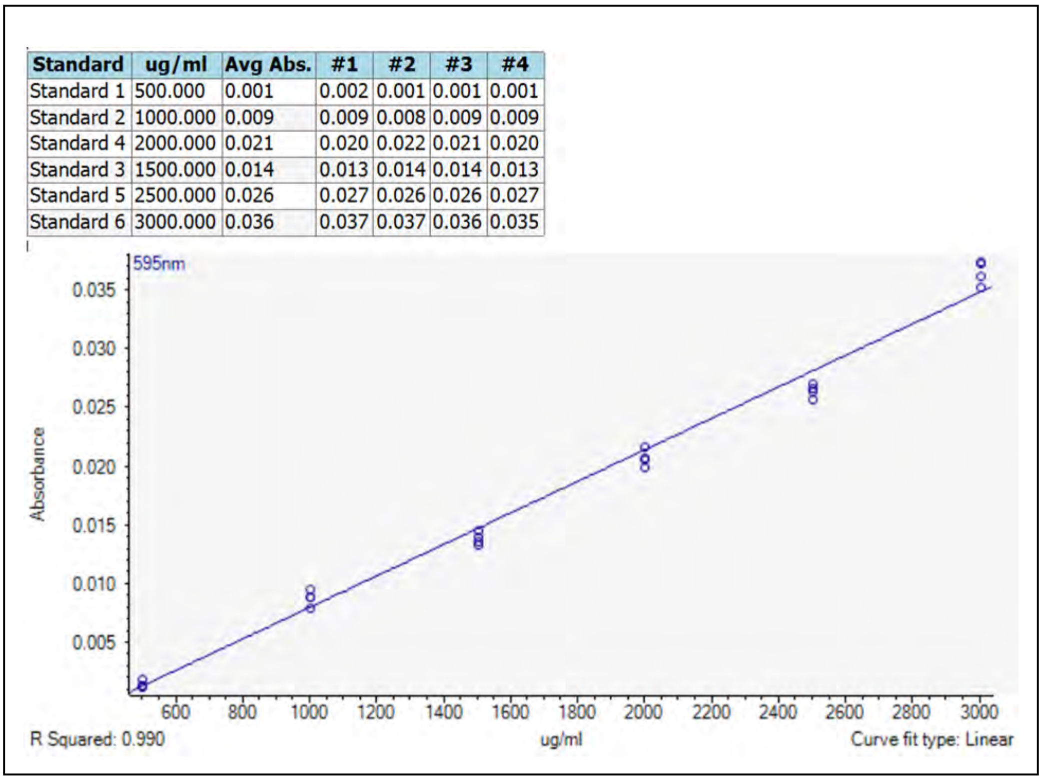
4.6 Protein collection and precipitation

The extracellular (supernatant) and intracellular protein (cell lysates) of the test and control were collected. All of protein solutions were storage at -70°C until the precipitation process. The protein solutions were thawed and acetone at 2 folds was added after incubation at -20°C for 24 hour. After centrifugations of proteins at 10,000 g for 30 min, the proteins were precipitated. After the protein was dry, the proteins were re-dissolved with storage buffer (containing protease inhibitor) and storage at -70°C.

4.7 Protein concentration measurement

4.7.1 Coomassie (Bradford) Protein Assay

The standard samples (n = 6) were measured for 4 times each. Then, the OD values at 595 nm of standard samples were plotted as the standard curve, by x= absorbance and y= protein concentration (ug/ml) (Figure 9). The obtained standard curve gave R-square 0.990. Then, the proteins samples from the experiments were measured and compared to the standard curve. The concentrations of the protein were calculated (Table 5).



Standard	ug/ml	Avg Abs.	#1	#2	#3	#4
Standard 1	500.000	0.001	0.002	0.001	0.001	0.001
Standard 2	1000.000	0.009	0.009	0.008	0.009	0.009
Standard 4	2000.000	0.021	0.020	0.022	0.021	0.020
Standard 3	1500.000	0.014	0.013	0.014	0.014	0.013
Standard 5	2500.000	0.026	0.027	0.026	0.026	0.027
Standard 6	3000.000	0.036	0.037	0.037	0.036	0.035

Figure 9. Standard curve of Coomassie (Bradford) Protein Assay. Standards and samples were measured 3 times and then calculated for the average before dilute to be 50µg/well for SDS PAGE test

Table 5. Protein concentration of the samples from intracellular (cell lysates) and extracellular (supernatants) protein.

sample	Average (μg/ml)	Dilution factor	Protein concentration (μg/μl)
cRPMI	1185.90	12	14.23
EcR1D1	2215.97	12	26.59
EcR1D5	1769.21	12	21.23
EtR1D1	2617.86	12	31.41
EtR1D5	2438.40	12	29.26
IcR1D1	1589.89	8	12.72
IcR1D5	1037.40	8	8.30
ItR1D1	1790.23	8	14.32
ItR1D5	963.04	8	7.70

Note: E=extracellular protein, I= intracellular protein, c= control (non infected cell), t= test (infected cell), R=round, D= day and number show the round number and date number of experiment.

4.7.2 Lowry method

For Extracellular protein Lowry method was used. The standard samples (n = 5) were measured for 3 times each. Then, the OD value at 750 nm of standard samples were plotted as the standard curve, by x= absorbance and y= protein concentration (ug/μl) (Figure 10). The obtained standard curve gave R-square 0.996. Then, the proteins samples from the experiments were measured and compared to the standard curve. The concentrations of the protein were calculated (Table 6).

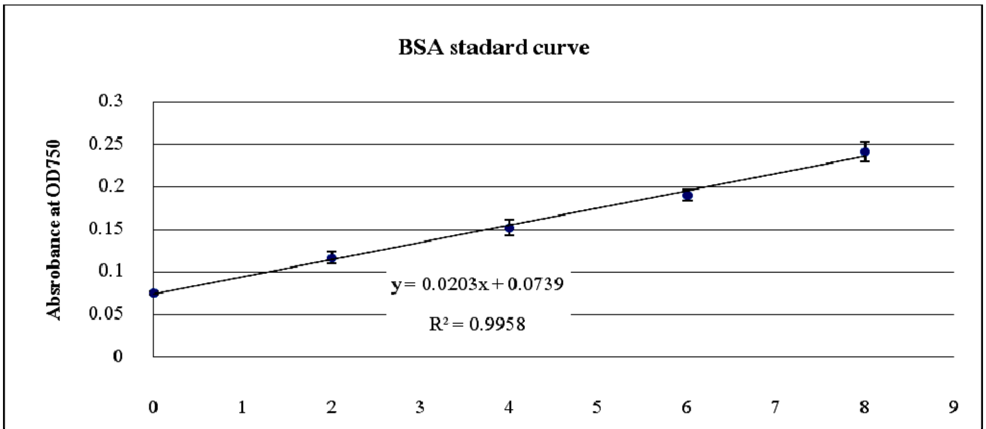


Figure 10. Standard curve of Lowry method. Standards and samples were measured 3 times and then calculated for the average before dilute to be 50µg/well for SDS PAGE test.

Table 6. Protein concentration of the samples from Extracellular (supernatants) protein.

Sample	Average (µg/µl)	Dilution factor	Protein concentration (µg/ul)
ECR2D1	2.276042	10	22.76
ECR2D5	3.387153	10	33.87
ETR2D1	2.605903	10	26.06
ETR2D5	3.456597	10	34.57
ECR3D1	3.144097	10	31.44
ECR3D5	3.092014	10	30.92
ETR3D1	4.585069	10	45.85
ETR3D5	3.265625	10	32.66
ECR4D1	3.751736	10	37.52
ECR4D5	3.526042	10	35.26
ETR4D1	2.762153	10	27.62
ETR4D5	4.619792	10	46.19

Note : E=extracellular protein, I= intracellular protein, c= control (non infected cell), t= test (infected cell), R=round, D= day and number show the round number and date number of experiment.

4.7.3 BCA Protein Assay

For intracellular protein BCA protein assay was used. The standard samples (n = 6) were measured for 3 times each. Then, the OD value at 562 nm of standard samples were plotted as the standard curve, by x= absorbance and y= protein concentration (ug/ml) (Figure 11). The obtained standard curve gave R-square 0.999. Then, the proteins samples from the experiments were measured and compared to the standard curve. The concentrations of the protein were calculated (Table 7).

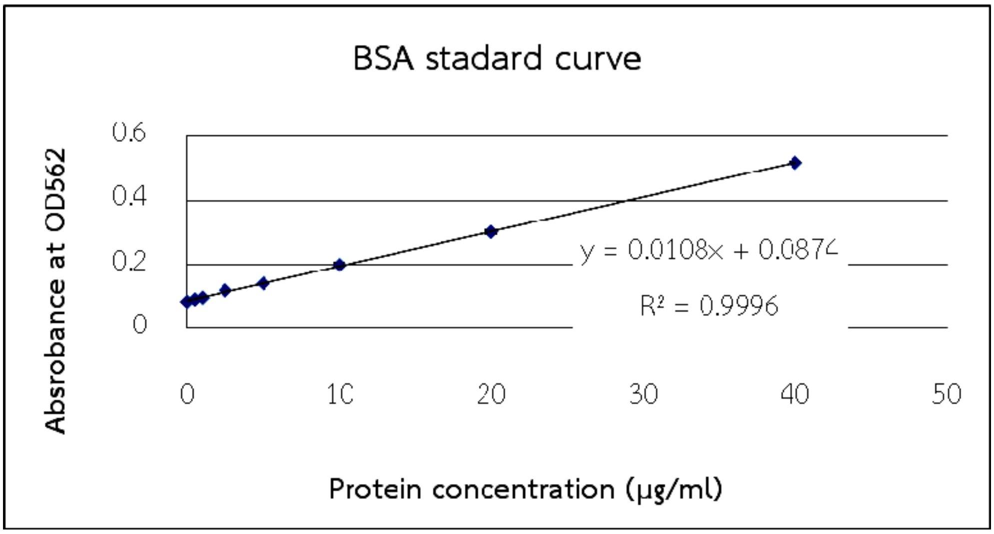


Figure 11. Standard curve of BCA protein assay. Standards and samples were measured 3 times and then calculated for the average before dilute to be 50µg/well for SDS PAGE test.

Table 7. Protein concentration of the samples from intracellular (cell lysates) protein.

Sample	Average	Dilution factor	sample concentration (ug/ml)
ICR2D1	4.932099	1,000	4932.10
ICR2D5	2.308642	1,000	2308.64
ITR2D1	6.876543	1,000	6876.54
ITR2D5	2.802469	1,000	2802.47
ICR3D1	4.962963	1,000	4962.96
ICR3D5	2.061728	1,000	2061.72
ITR3D1	5.919753	1,000	5919.75
ITR3D5	1.753086	1,000	1753.09
ICR4D1	4.314815	1,000	4314.81
ICR4D5	2.308642	1,000	2308.64
ITR4D1	4.962963	1,000	4962.96
ITR4D5	2.58642	1,000	2586.42

Note: E=extracellular protein, I= intracellular protein, c= control (non infected cell), t= test (infected cell), R=round, D= day and number show the round number and date number of experiment.

4.8 1D SDS-PAGE

The protein concentration were measure by BCA assay for intracellular protein and Lowry method for extracellular protein and the protein concentration were calculated and adjusted to 50 μg/well for intracellular and 200 μg/well for extracellular protein. The protein solutions were run by SDS-PAGE analysis and the results were show in figure 12 and 13.

The intracellular and extracellular proteins adjusted the concentration at 50 μg were run on SDS-PAGE. It was found that the extracellular protein (supernatant) showed the thick bands at the MW about 52-80 kDa which was the albumin band from the media supplement (FBS). The color of the band at day 1 is stronger than day 5. The albumin bands were the contaminated proteins which were not the target for analysis and potentially interfered the LC-MS/MS analysis (Figure 13). In the LC MS/MS analysis, only the proteins band lower that the albumin bands (MW between 32-80 kDa) were selected to perform the test (Figure 13).

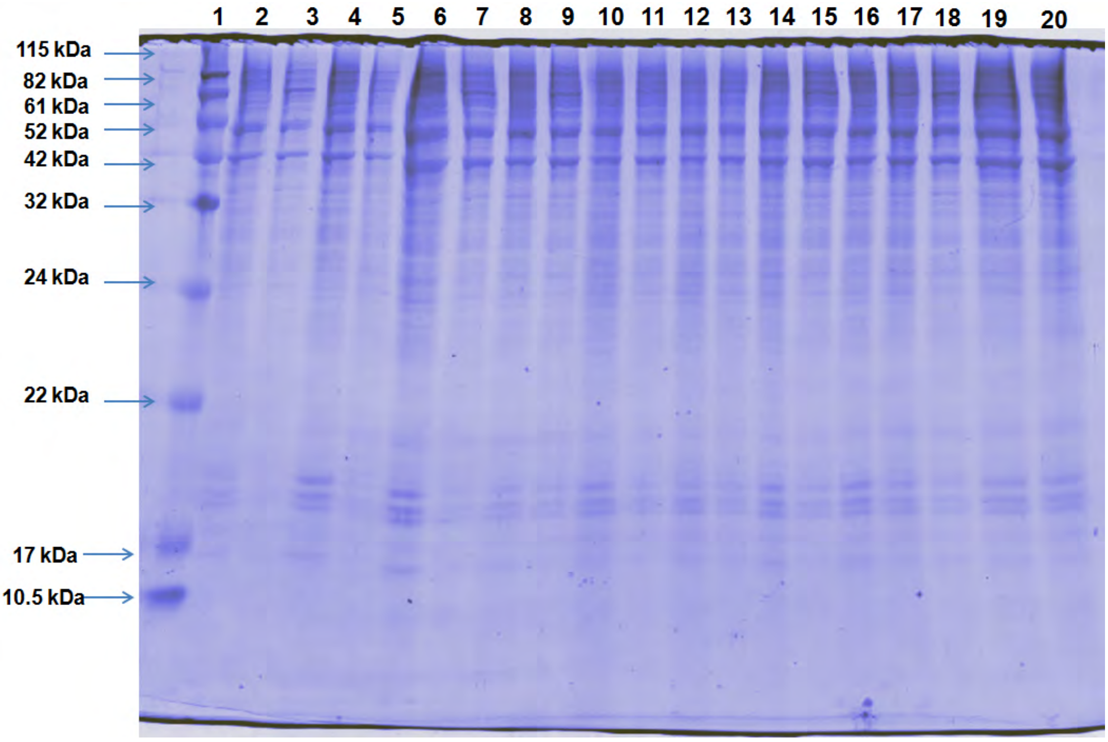


Figure 12. SDS-PAGE of intracellular protein of 50 µg from activated macrophages infected with M. tuberculosis and treated with anti-tuberculosis drugs. Lane1 = Protein marker (Chromatein Prestained Protein Ladder, Vivantis, country), Lane2 = Intracellular Control protein in experiment Replicate 1 at Day 1 (ICR1D1), Lane3 = ICR1D5, Lane4 = Intracellular Test protein in experiment Replicate 1 at Day 1 (ITR1D1), Lane5 = ITR1D5, Lane6 = ICR2D1, Lane7 = ICR2D5, Lane8 = ITR2D1, Lane9 = ITR2D5, Lane10 = ICR3D1, Lane11 = ICR3D5, Lane12 = ITR3D1, Lane13 = ITR3D5, Lane14 = Pooled Intracellular Control protein in all 3 experiments at Day 1 (PICD1), Lane15 = PICD5, Lane16 = PITD1, Lane17 = PITD5, Lane18 = double concentration of ICR1D5 (Lane 3), Lane19 = double concentration of ITR1D1 (Lane 4), Lane20 = double concentration of ITR1D5 (Lane 5). I = intracellular, C = control, T = test, R = replicate, D = day of experiment, P = Pool experiment.

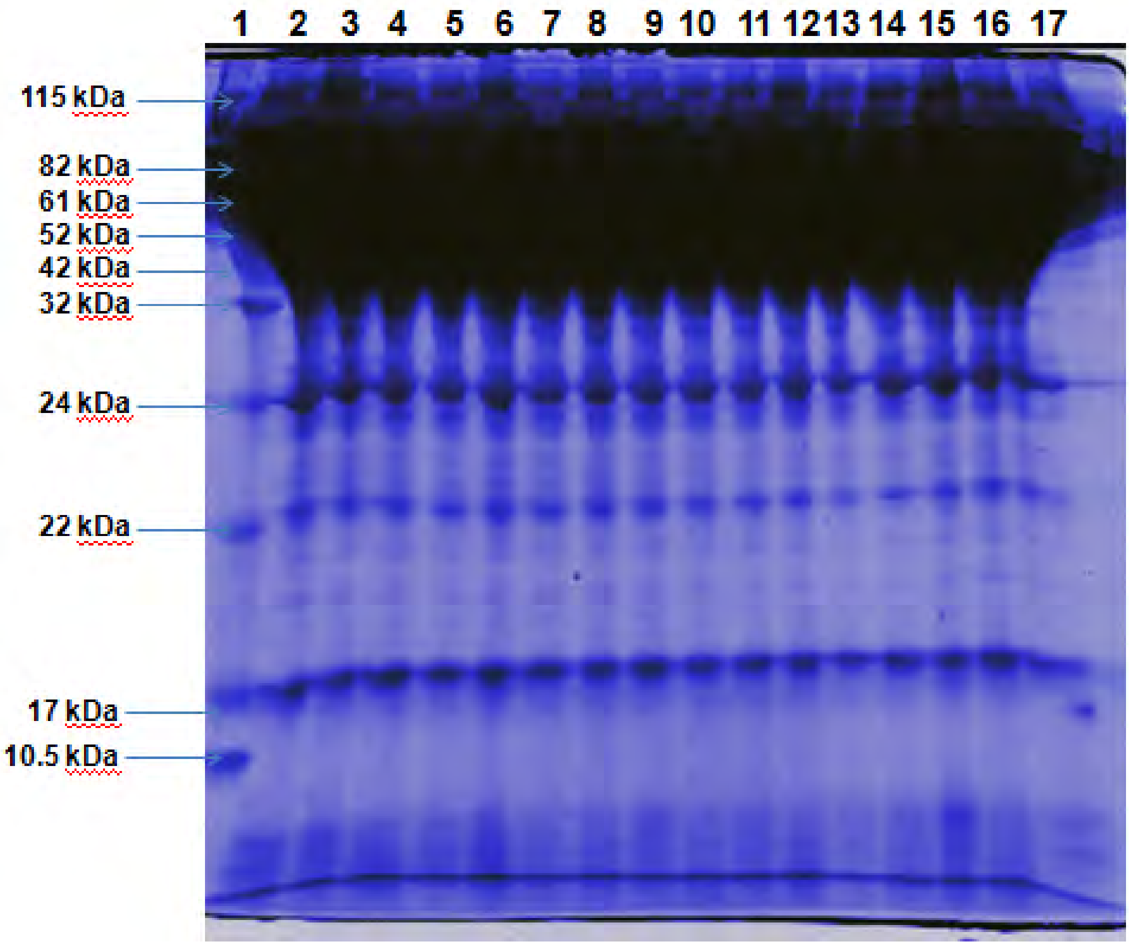


Figure 13. SDS PAGE of 200 µg extracellular protein from activated macrophages infected with *M. tuberculosis* and treated with anti-tuberculosis drugs. Lane1 = Protein marker (Chromatein Prestained Protein Ladder, Vivantis, **country**), Lane2 = Extracellular Control protein in experiment Replicate 1 at Day 1 ECR1D1, Lane3 = ECR1D5, Lane4 = Extracellular Test protein in experiment Replicate 1 at Day 1 (ETR1D1), Lane5 = ETR1D5, Lane6 = ECR2D1, Lane7 = ECR2D5, Lane8 = ETR2D1, Lane9 = ETR2D5, Lane10 = ECR3D1, Lane11 = ECR3D5, Lane12 = ETR3D1, Lane13 = ETR3D5. Lane14 = Pooled extracellular Control protein in all 3 experiments at Day 1 (PECD1), Lane15 = PECD5, Lane16 = PETD1, Lane17 = PETD5. E=Extracellular, C=control, T= test, R= replicate, D= day of experiment, P=Pool experiment.

4.9 Proteomic profile of infection and clearance stage of MTB infected macrophages after anti tuberculous treatment.

After LC MS/MS analysis, 2,680 unique elements from extracellular proteome and 5,142 unique elements from intracellular proteome differentially compared between infection and clearance stage and not found in control were revealed (Figure 14, 15, 16 and 17).

4.9.1 Infection stage specific proteins

There were 73 extracellular protein elements that were uniquely found in the infection stage (at least one out four independent experimental samples) (Figure 14 and Table 8). There were 3 proteins that consistently found in two out three independent experiments (Figure 15 and Table 8) in which APCDD1-like protein showed the highest mass intensity, 9.65 ±1.14.

In the intracellular proteins, there were 134 proteins that were uniquely found in the infection stage (at least 1/4 experimental samples) (Figure 16). Out of 134 proteins, 18 proteins were consistently found in two out of four independent experiments in which TLN2 was the highest mass intensity, 9.07±0.68 (Table 8). There were 10 proteins that were found in two out of three independent experiments (Figure 17).

The complete list of the proteins and their properties that uniquely presented in the infection stage and clearance stage were described in supplementary table 1 and 2, respectively.

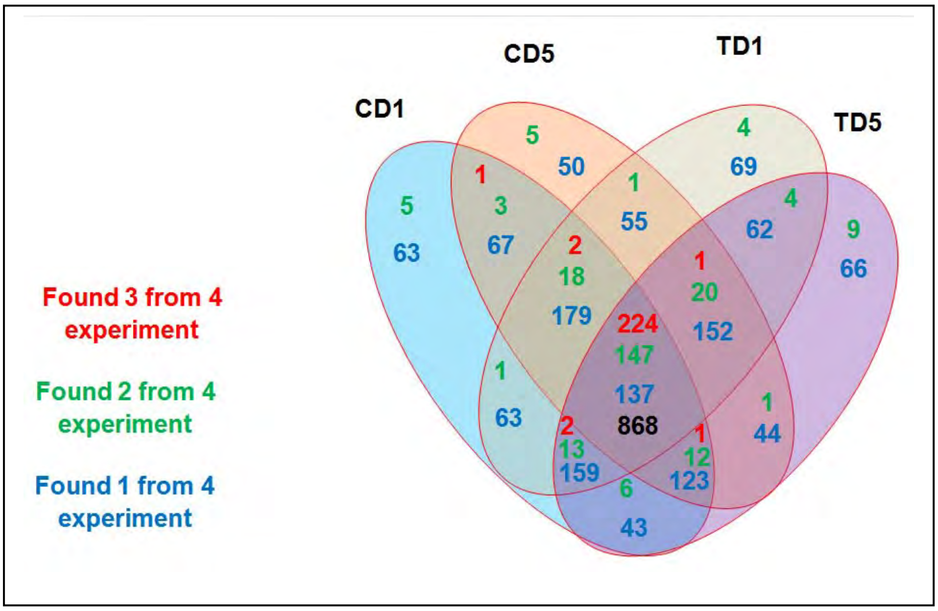


Figure 14. Venn's diagram of 4 experiments extracellular proteome analysis (3 independent + 1pool experiment). Numbers show the number of protein elements found in each condition and date number of experiment. CD1 and CD5 referred to control (uninfected cells with anti TB drugs) in day 1 and day 5, respectively. TD1 and TD5 referred to test (infected cells with anti TB drugs) in day 1 and day 5, respectively. Some proteins were found more than one condition resulted to 4526 elements found in all conditions. The total number of unique proteins was 2889 elements.

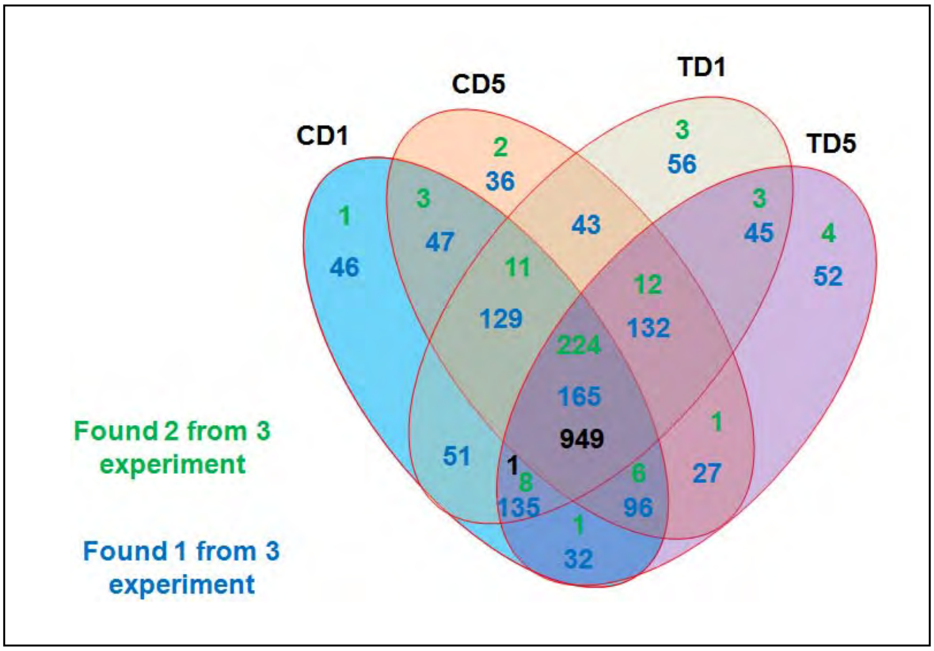


Figure 15. Venn's diagram of 3 independent experiments from extracellular proteome analysis. Numbers show the number of protein elements found in each condition and date number of experiment. CD1 and CD5 referred to control (uninfected cells with anti TB drugs) in day 1 and day 5, respectively. TD1 and TD5 referred to test (infected cells with anti TB drugs) in day 1 and day 5, respectively. Some proteins were found more than one condition resulted to 2320 elements found in all conditions. The total number of unique proteins was 1533 elements.

4.9.2 Clearance stage specific proteins

There were 79 extracellular protein elements that were uniquely found in the clearance stage (at least one out four experimental samples). Out of 79, there were nine proteins that were found in two out of four independent experiments, in which drebrin showed the highest mass intensity, 7.31±0.69 (Figure 14 and Table 9). There were four proteins that were found in two out of three independent experiments (Figure 15).

In the intracellular proteins, there were 129 proteins that uniquely found in the clearance stage (at least 1/4 experimental samples) (Figure 16). Out of 129 proteins, there was one protein (CAECAM18) that consistently found in all four experimental samples and 11 were found in two out of three experimental samples, in which IL-11 showed the highest mass intensity, 8.16±0.13 2/4 (Figure 16). There were six proteins found in two out of three independent experiments (Figure 17). The complete list of the proteins and their properties that uniquely presented in the infection stage and clearance stage were described in supplementary table 1 and 2, respectively.

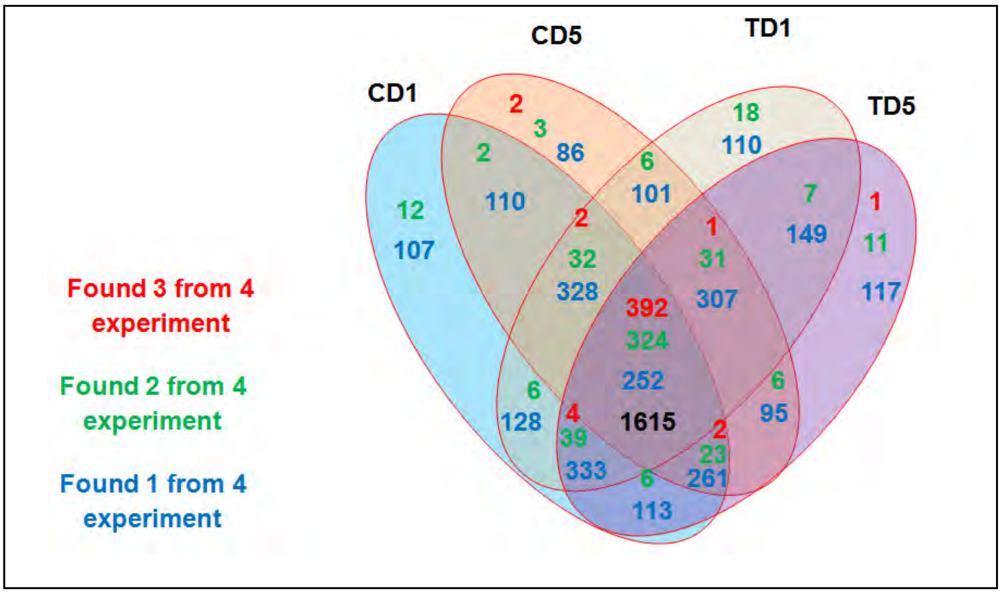


Figure 16.

Venn's diagram of 4 experiments intracellular proteome analysis (3 independent + 1pool experiment). Numbers show the number of protein elements found in each condition and date number of experiment. CD1 and CD5 referred to control (uninfected cells with anti TB drugs) in day 1 and day 5, respectively. TD1 and TD5 referred to test (infected cells with anti TB drugs) in day 1 and day 5, respectively. Some proteins were found more than one condition resulted to 5142 elements found in all conditions. The total number of unique proteins was 2891 elements.

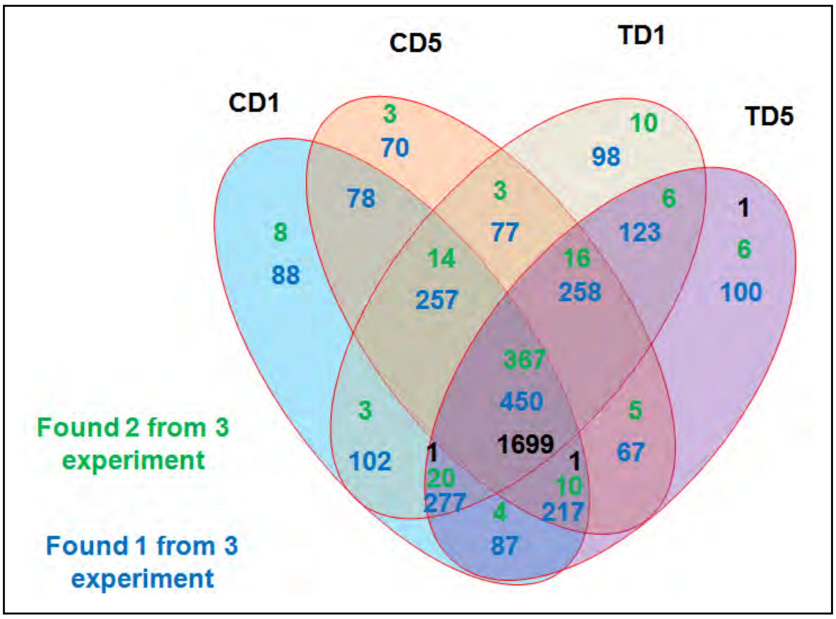


Figure 17. Venn's diagram of 3 independent intracellular proteome analysis. Numbers show the number of protein elements found in each condition and date number of experiment. CD1 and CD5 referred to control (uninfected cells with anti TB drugs) in day 1 and day 5, respectively. TD1 and TD5 referred to test (infected cells with anti TB drugs) in day 1 and day 5, respectively. Some proteins were found more than one condition resulted to 2676 elements found in all conditions. The total number of unique proteins was 1535 elements.

4.9.3 Differential proteins specific to infection and clearance stage

Several proteins from both intracellular and extracellular proteomes were found both infection and clearance stage based on LC MS/MS analysis. Some proteins were increasingly or decreasingly found in certain condition as shown in Figure 18 and Table 10. The complete list of the differential proteins found in the infection stage and clearance stage were described in supplementary table 3 and 4, respectively.

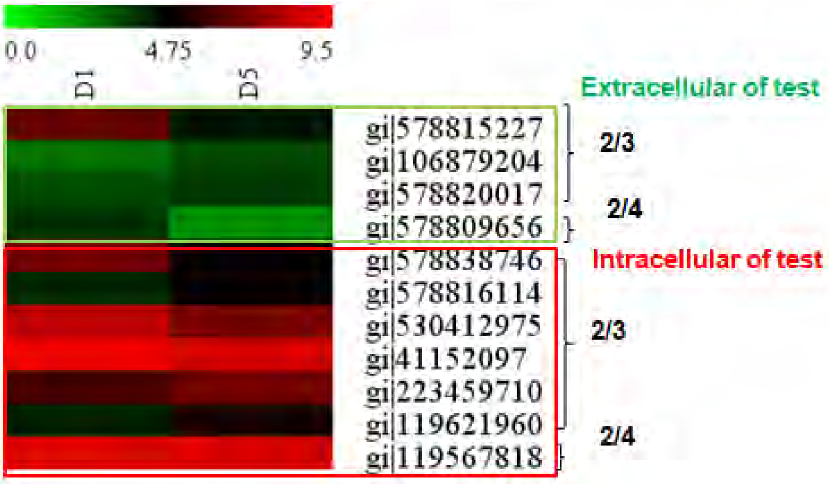


Figure18. Heat map show the protein that express in test condition at day 1 and 5 but have a significant difference in their level from 1 and 5 day of test. Color from green to red color represent the protein intensity from 0-9.5, The gi number referred to the protein gi number, Extracellular of test = extracellular protein of THP-1 infected, Intracellular of test = intracellular protein of THP-1 infected cell, D1 and D5 referred to test (infected cells with anti TB drugs) in day 1 and day 5, respectively. Some proteins were found more than one condition resulted to 5142 elements found in all conditions. The total number of unique proteins was 2891 elements.

4.9.4 Other unique proteins in particular comparing conditions

Other proteins that were differentially found in particular condition were described in Figure 14, 15, 16 and 17. The responses of the macrophages to the isoniazid and rifampicin in early (day 1) and late stage (day 5) can be elucidated from the control proteome. These results were not relevant to the research question and were not included in the discussion part.

Table 8 Unique proteins uniquely found in the infection stage but absence in the clearance stage.

Protein elements	gi number	Function	Mass intensity (Mean ±SD)	ID score	Replicate result	Replicate			
						Ex1	Ex2	Ex3	pool
Extracellular proteins (n = 4)									
- APCDD1	gi|23503291	negative regulator of the Wnt signaling	9.65±1.14	11.10	2/3	NF	TD1	TD1	CD5,TD5
- FYVE, RhoGEF and PH domain-containing protein 1	gi|24797153	Activates CDC42, a member of the Ras-like family of Rho- and Rac proteins	6.79±0.08	9.93	2/3	TD1	TD1	CD5,TD1	NF
- immunoglobulin light chain	gi|333466192	Antibody compartment	5.31±2.40	13.79	2/3	TD1	CD1,TD1	TD1	CD1
-KIAA0377	gi|30315253	regulate a variety of cellular processes, including apoptosis, vesicle trafficking, cytoskeletal dynamics, exocytosis, insulin signaling and neutrophil activation	1.48±2.17	2.49	2/4	TD1	TD1,TD5	CD5,TD1	TD1
Intracellular proteins (n =18)									
- TLN2	gi|578827491	play an important role in cell adhesion, recruits PIP5K1C to focal adhesion plaques and strongly activates its kinase activity	9.07±0.67	17.39	2/3	TD1	TD1	NF	CD1,CD5,TD1
- TAS2R49	gi|20336529	play a role in sensing the chemical composition of the gastrointestinal content. The activity of this receptor may stimulate alpha gustducin, mediate PLC-beta-2 activation and lead to the gating of TRPM5	8.72±0.86	9.83	2/3	TD1	TD1	NF	CD1,CD5
- immunoglobulin	gi|270052417	Antibody compartment	8.75±1.71	7.57	2/3	TD1	TD1	TD1,TD5	CD1,TD1,TD5

heavy chain									
- Myosin-IXA	gi|3954942	Regulates Rho activity in neurons, has a role in the regulation of neuronal morphology and function	8.45±2.08	12.59	2/3	TD1	NF	TD1	CD1,TD5
- conserved oligomeric Golgi complex	gi|578825042	Required for normal Golgi function	7.96±0.53	2.53	2/3	TD1	TD5	TD1	CD5
- cajalin 2	gi|34014746	act as intermediaries in enteric motor neurotransmission	7.64±0.06	7.9	2/3	TD5	TD1	TD1	CD1,CD5,TD1
- Ppargamma-Lbd	gi|374414719	a nuclear receptor superfamily function in various biological phenomena, such as insulin sensitization, adipogenesis, atherosclerosis, inflammation, and carcinogenesis , involved in transcriptional regulation of glucose and lipid homeostasis	5.52±1.62	4.94	2/3	TD1	TD1,TD5	TD1	CD1
- 17-beta-HSD 14	gi|578824403	Converts oestradiol to oestrone	4.45±3.25	3.91	2/3	NF	TD1	TD1	TD1,CD5
- mitochondrial ATP-binding cassette (ABC)	gi|578802184	A proteins transport various molecules across extra- and intra-cellular membranes involved in multidrug resistance as well as antigen presentation	5.85±0.57	18.09	2/3	NF	TD1	TD1	TD5,CD5
-unnamed protein	gi|40977078	Hypothetical protein	9.10±0.52	9.00	2/3	TD1	TD1	NF	NF
- MYO1D	gi|119600628	intracellular movements	8.4±1.16	0.67	2/4	TD1	CD5,TD1,TD5	CD5,TD1	TD1
- Control Of K+ Channel Gating	gi|157830170	conduct potassium ions down their electrochemical gradient and involve in controls membrane excitability and signal propagation in central neurons	7.02±0.63	7.9	2/4	TD1	CD5,TD1,TD5	NF	TD1
- nucleolin	gi|55956788	induces chromatin decondensation by binding to histone H1 and play a role in	5.09±0.87	10.61	2/4	TD1,TD5	CD5,TD1,TD5	TD1	TD1

		pre-rRNA transcription and ribosome assembly							
- BAT2L	gi|12804221	Unknown function	1.85±0.99	5.31	2/4	TD5	TD1	CD1,TD1	TD1
- OPK V NimA	gi|38502053	Hypothetical protein	3.12±3.78	5.11	2/4	CD1,TD1	TD1	CD5	TD1
- src	gi|119615154	different classes of cellular receptors including immune response receptors, integrins and other adhesion receptors, receptor protein tyrosine kinases, G protein-coupled receptors as well as cytokine receptors	4.13±2.98	5.95	2/4	TD5	TD1	CD1,TD1	TD1
- arfaptin-1	gi|566006168	putative target protein of ADP-ribosylation factor, inhibits phospholipase D,	5.12±2.37	14.91	2/4	TD1	CD5,TD5	CD5,TD5	TD1
-adenomatous polyposis coli protein 2	gi|578825173	Inhibit Wnt signaling	7.83±0.40	4.72	2/4	CD1,CD5,TD1	TD1	NF	TD1

* Replicate result referred to the replication of consistent result from the 3 or 4 independent experimental samples. The complete list of infection stage associated proteins was described in supplementary table 1. NF referred to not found.

Table 9 Unique protein elements uniquely found in the clearance stage but absence in the infection stage.

Protein elements	gi number	Function (NCBI database)	Mass intensity (Mean ±SD)	ID score	Replicate result	Replicate			
						Ex1	Ex2	Ex3	pool
Extracellular proteins (n = 9)									
-huMCM2	gi|1232077	DNA replication initiation and elongation	5.28±0.44	1.74	2/3	-	TD5	TD5	-
-hCG1818658	gi|119607623	Hypothetical protein	3.96±0.50	8.85	2/3	-	TD5	TD5	CD1,CD5, TD1,TD5
-RANBP1	gi|47678643	Inhibits GTP exchange on Ran to forms a Ran-GTP-RANBP1 may act in an intracellular signaling pathway which may control the progression through the cell cycle by regulating the transport of protein and nucleic acids across the nuclear membrane.	4.03±1.96	3.68	2/3	-	TD5	TD5	-
- replication factor C subunit 1	gi|325296984	DNA replication and repair	0.26±0.01	10.22	2/3	TD5	TD1	TD5	CD1,CD5
-drebrin	gi|578811024	neuron-specific F-actin-binding proteins activate synaptic transmission	7.31±0.69	8.78	2/4	TD5	CD1,TD1	CD1,CD5,TD1, TD5	TD5
-TBC1	gi|139394668	May act as a GTPase-activating protein for Rab family protein	5.92±0.14	17.34	2/4	CD1,TD1	TD5	CD5	TD5
-Usp45	gi|530384124	zinc ion binding; ubiquitin-specific protease activity	5.73±0.16	7.15	2/4	CD1	-	TD5	TD5
-UTY	gi|151946791	Metal ion binding, RNA polymerase II core promoter proximal region sequence-specific DNA binding, Regulation of chromatin silencing, Canonical Wnt signaling pathway to activate expression of taget gene	5.10±1.44	14.82	2/4	TD5	CD5	-	TD5
- LARP1	gi|578810845	RNA metabolism, down-regulate the Ras-MAPK pathway	5.20±2.39	8.95	2/4	-	CD1,TD5	TD5	TD5
Intracellular proteins (n = 12)									

-CEACAM18	gi|508083061	immune regulation and reproduction	1.25±0.22	9.06	3/3	TD5	TD5	TD5	TD1,TD5
-ras GTPase	gi|530378499	cell-cycle progression, cellular differentiation, cytoskeletal organization, protein transport and protein secretion, cellular signaling transduction	8.08±0.39	4.88	2/3	TD5	CD5,TD1,TD5	TD5	CD1,TD1,TD5
-SSA2	gi|530370876	stabilize some of RNAs and protect them from degradation	7.66±0.56	7.77	2/3	-	TD5	TD5	CD5,TD1
-N-CoR2	gi|62087758	Mediates the transcriptional repression activity of some nuclear receptors by promoting chromatin condensation lead to cell proliferation and survival	6.42±2.84	13.44	2/3	-	TD5	TD5	CD1
-DRB1	gi|1487978	Binds peptides derived from antigens that access the endocytic route of antigen presenting cells (APC) and presents them on the cell surface for recognition by the CD4 T-cells	2.36±1.16	3.84	2/3	CD1	TD5	TD5	TD1,TD5
-proteasome activator	gi|62467430	Inhibit apoptosis after DNA damage may also be involved in cell cycle regulation	2.33±0.96		2/3	TD5	CD1,TD5	TD5	CD1,CD5,TD1
-synaptotagmin XI	gi|119573411	involved in Ca2+-dependent exocytosis of secretory vesicles through Ca2+ and phospholipid binding to the C2 domain or may serve as Ca2+ sensors in the process of vesicular trafficking and exocytosis	7.37±1.87	3.57	2/3	TD5	TD5	CD1,CD5,TD1	CD1,CD5,TD1, TD5
-IL-11	gi|10834994	Directly stimulates the proliferation and induces megakaryocyte maturation resulting in increased platelet production.	8.15±0.13	7.62	2/4	CD1,CD5,	TD1,TD5	TD5	TD5
-SRC kinase signaling inhibitor 1	gi|578840438	negative regulator of SRC leading to impaired cell spreading and migration and play a role in neurotransmitter release or synapse maintenance	7.18±0.24	12.28	2/4	TD5	TD1	-	TD5
-fibrocystin	gi|126131102	required for correct bipolar cell division	6.91±0.67	9.67	2/4	CD1,CD5,T	TD5	TD1	TD5

isoform 1		through the regulation of centrosome duplication and mitotic spindle assembly				D1,TD5			
-unnamed protein	gi|218110385	Hypothetical protein	5.88±0.24	7.65	2/4	TD1	TD5	TD1,TD5	TD5
-gephyrin	gi|578825413	Microtubule-associated protein involved in membrane protein-cytoskeleton interactions	4.96±1.7	6.34	2/4	-	TD5	TD1,TD5	TD5

* Replicate result referred to the replication of consistent result from the 3 or 4 independent experimental samples. The complete list of clearance stage associated proteins was described in supplementary table 2.

Table 10 Proteins that expressed in test condition at day 1 and 5 but have a significant difference in their level from 1 and 5 day of test.

No.	Protein elements	gi number	Functions	Mass intensity		ID score	Fold change	Replicate result	Replicate			
				D1	D5				Ex1	Ex2	Ex3	pool
Extracellular proteins (n = 4)												
1	exostosin-like 3	gi|578815227	regulating of keratinocyte proliferation and differentiation	6.63	4.37	8.90	1.5	2/3	TD1,TD5	TD1,TD5	CD1,, TD1,TD5	CD5, TD1,TD5
2	exocyst complex component 3-like protein	gi|106879204	involved in the docking of exocytic vesicles with fusion sites on the plasma membrane	2.69	3.42	13.76	1.27	2/3	-	TD1,TD5	TD1,TD5	CD1,CD5
3	fibronectin type 3 and ankyrin repeat domains protein 1	gi|578820017	Unknown function	3.38	3.68	8.74	1.08	2/3	TD1,TD5	CD1, TD1,TD5	TD1,TD5	CD1, TD1
4	rap guanine nucleotide exchange factor 2	gi|578809656	activates Rap and Ras family	3.77	2.15	7.06	1.75	2/4	TD1	TD1,TD5	-	TD1,TD5
Intracellular proteins (n = 7)												
1	regulator of nonsense transcripts 3B isoform X3	gi|578838746	Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons by associating with the nuclear exon junction complex	6.83	4.92	10.52	1.39	2/3	TD1,TD5	TD1,TD5	CD1,CD5, TD1,TD5	CD1,CD5, TD1,TD5
2	fer-1-like protein 6 isoform X1	gi|578816114	Unknown function	4.04	4.88	14.42	1.20	2/3	TD1,TD5	TD1,TD5	CD5, TD1	CD1, TD1
3	glutamine-rich protein 2	gi|530412975	Unknown function	8.24	6.91	19.72	1.19	2/3	TD1,TD5	TD1,TD5	CD1,CD5, TD1,TD5	CD1, TD1,TD5
4	A-kinase anchor protein 17A	gi|41152097	Splice factor regulating alternative splice site selection for certain mRNA precursors	9.21	8.69	11.69	1.06	2/3	TD1,TD5	TD1,TD5	CD1,CD5, TD1,TD5	CD1,CD5, TD5
5	Chromosome 9 open reading frame 139	gi|223459710	Hypothetical protein	6.04	6.91	3.76	1.14	2/3	-	TD1,TD5	TD1,TD5	-
6	cell death-inducing DFFA-like	gi|119621960	activate apoptosis	4.11	5.15	4.25	1.25	2/3	TD5	TD1,TD5	TD1,TD5	CD1,CD5,

	effector a											TD1,TD5
7	hCG1781264	gi|119567818	Hypothetical protein	8.87	8.72	15.48	1.02	2/4	CD1, TD1	-	TD1,TD5	TD1,TD5

5. Discussion

The biomarker indicating the clearance stage of MTB infection could facilitate the treatment monitoring both active and LTBI. Previous studies determined the markers indicating the decrease of MTB bacillary load including host related markers and MTB related marker However, none of them determine the biomarker indicating the actual sterilized condition of MTB infection due to lack of indicator of clearance stage of MTB from host tissue. To response for this gap, we used the activated macrophages infected with MTB and GeLC MS/MS for screening the biomarkers indicating the clearance stage of MTB infection. We found several potential proteins that might be used as the biomarkers to indicate the clearance of MTB after anti-tuberculous treatment.

Previously, there were several studies related to proteomic markers for indicating LTBI and active TB (4-7). Previously, the proteomic studies for exploring the biomarker for monitoring TB outcome were based on the clinical evidence and microbiological evidences, especially sputum AFB and culture (8, 9). Others applied molecular and serological marker for TB treatment monitoring (10-12). Resistin was found as a promising biomarker for TB treatment monitoring in TB patients (13). However, these studies did not ensure the MTB clearance stage. To determining the clearance biomarkers from human host is limited due to the confirmation of MTB clearance from all tissue and cell from the host. Animal study is the promising models for studies the MTB clearance because the MTB sterilization can be ensured from all tissue organs. Alternatively, our study used infected macrophages as a model to search for MTB clearance biomarkers that the clearance stage of MTB was ensured by CFU count. To our knowledge, the current study was the first study to examine the actual MTB clearance makers that the clearance stage was ensured *in vitro*.

There were two proteomes analyzed in this study; intracellular (cell lysate) and extracellular (culture supernatants). Compare between the infection stage and clearance stage, there were proteins infection stage and clearance stage specific proteins. There were 128 and 73 proteins uniquely found in the infection stage from the intracellular protein and extracellular protein, respectively. From 201 proteins, 22 infection stage specific proteins were consistently found among replicated experiments and might be the promising markers indicating the MTB infection stage. The infection specific proteins indicated the infection status and were disappeared when MTB was sterilized from the cells. These host proteins were presented in the infection stage and absent in the clearance stage. Several infection specific proteins had functions associated with the immunomodulator of the host immune responses. It was hypothesized that MTB might activate the host immunosuppressor protein during the infection stage as supported by the immunosuppressive ability of MTB (14, 15). For example, APCCD1, infection associated extracellular protein, has a function as the inhibitor of cell cycle and proliferation via the suppression of Wnt signal which required for cell to cell signaling (16-18). APCCD1 was also increasingly found in *Helicobacter pylori* infection in gastric epithelial cell (24). Host proteins that were upregulated during the infection stage might be from the host immune response against MTB infection. For example, TLN2, infection specific intracellular protein, had a similar structure as talin 1 and had a function associated with cell spreading (19). Furthermore, TLN2 were involved in cell maturation and serve the link between integrins and the sarcomeric cytoskeleton for stable of cell adhesion (20), recycling of synaptic vesicles (21) and cell migration (22). TLN2 upregulation was also found in the patients infected with *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus pneumonia*e infection patient (23).

Similarly, there were 129 and 75 proteins uniquely found in the clearance stage from the intracellular proteins and extracellular proteins, respectively. From 204 proteins, 21 clearance specific proteins consistently found among replicated experiment and might be the promising marker indicating the MTB clearance stage of infected cells. These proteins might be used to directly indicate the clearance stage where no remaining MTB in the host tissue. The host proteins that were uniquely found in the clearance stage might be the immune associated proteins that were suppressed during the infection stage. For example, ras GTPase that might involve in the host cell multiplication to restore the normal physiological condition after MTB clearance. ras GTPase had a function associated with cell proliferation, differentiation and survival (29, 30). This notion was suppurated by the finding that Rab33A (ras GTPase subfamily) gene expression was suppressed in CD8 $^{+}$ T cells in TB patients (31). Other interesting clearance stage specific proteins found in our study were IL-11 that has a function as anti-inflammatory cytokine (32) and associated with cell proliferation (33), and huMCM2 that has a function as DNA replication factor (34-37) that might play role in cell multiplication after clearance of MTB. It is interesting to note that there was one protein, CAECAM18, which was consistently found in all three independent experiments from three separated MS analysis. CEACAM, carcinoembryonic antigen (CEA) is a member of the immunoglobulin superfamily (IgSF), consists of the CEA-related cell adhesion molecule (CEACAM) and the pregnancy specific glycoprotein (PSG) subgroups CEACAM act as central receptors facilitating the communication of immune cells (25, 26). CEA was associated with cell survival by induction of IL-10 and decrease of nitric oxide (NO) concentration and inducing cytokine released including IL-1β, IL-6, IL-10 and TNF-α (27, 28). This protein might relate to MTB clearance stage by induction of cell survival and decreasing of nitric oxide after MTB clearance from the infected cell and this protein might correlated with the increase of IL-11. Clearly, these speculations should be confirmed by further study.

The overall function of proteins specifically found in the infection stage associated with the host pathogen interaction. Host cells require eliminating MTB by immune activation process. There several proteins associated with anti-MTB immune were uniquely presence at the infection stage, e.g. KIAA0377 (regulate cellular process, apoptosis and nuetrophil activation), TLN2 (play role in cell adhesion and maturation), The activation of synaptic secretion as found in synaptotagmin involved in activation of immune synaptic secretion (38, 39) and TLN2 that involved with the recycling of synaptic vesicles (21) might support this notion. The benefit of immune synapsis was the facilitating of the macrophage communication for recruitment and activation of the immune response against MTB infection. Alternatively, MTB that contain the immune evasion strategies induced host immune suppressor to overcome the host immune response. For example, cell proliferation was inhibited at the infection stage by APCDD1 that can inhibit cell cycle and proliferation via Wnt signaling pathway (16, 17). Contrarily, in the clearance stage where MTB was eliminated by the anti-tuberculous drug, the normal condition of host cells was restored as supported by the increase of the several proteins required for cell proliferation such as the activation of huMCM2, RANBP1, replication factor C, la-related protein 1, N-CoR2. Moreover at the clearance stage, SRC kinase signaling inhibitor 1 was expressed to inhibit SRC kinase that has a function as immune synaptic secretion (40).

The biomarkers found in our study were the proteins that uniquely presented in the particular conditions, i.g., infection stage (day 1) and clearance stage (day 5). The proteins that were uniquely found in the clearance stage (day 5) were neither found in the infection stage (day 1) nor uninfected control (normal cell day 1 and day 5). The uninfected controls with antituberculous drug were used to exclude the drug associated protein from the infection and clearance specific proteins. Hence, these proteins were the markers that early expressed after MTB clearance by anti-tuberculous drugs. These proteins should be decreased into normal level after cell adaptation to normal physiological condition. Therefore, we revealed potential biomarkers that can be used to indicate the clearance stage of MTB infection after treatment.

This study limited due to the using the activated macrophage model, not the system host model. Furthermore, the extracellular localization of the discovered proteins was referred from the cell culture supernatant. Other analysis such as the signal peptide might clarify the secretory form of these proteins. In addition, the further analysis such as Western blot should be performed to confirm the proteomic results. This is the first study applied the high throughput proteomic screening for searching the clearance marker of MTB infection after anti tuberculous treatment by ensuring the *in vitro* clearance stage. The animal model could reflect the better host system. Our proteomic study was performed by three independent experiments of proteomes that ensure the validity of the potential biomarkers. The analysis both intracellular proteins and cell supernatant increase a chance to discover more potential biomarkers.

6. Conclusion

Based on high throughput screening approach, LC MS/MS, in the macrophages infection model, we showed that there were several proteins that can potentially use as the biomarkers for TB treatment monitoring. There were 4 extracellular and 18 intracellular that uniquely found in the infection stage. There were 9 and 12 intracellular proteins uniquely found in the clearance stage. Two proteins that were promising to use as MTB clearance biomarker after anti-tuberculous treatment were CAECAM18 and ras GTPase protein due to their consistent results from independent experiments, presence with high quantity and the associated function to MTB infection.

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Output ที่ได้จากโครงการ

- ผลงานวิจัยที่อยู่ในช่วงการจัดเตรียมต้นฉบับเพื่อส่งตีพิมพ์ในวารสารนานาชาติ
- ผู้ช่วยวิจัยและบัณฑิตปริญญาโท (นางสาวเบญจวรรณ แก้วสีขาว)

<u>**ภาคผนวกโครงการ**</u>

- **Manuscript:** อยู่ในช่วงเตรียมต้นฉบับ
- **บทความสำหรับเผยแพร่:** ยังไม่มี
- **กิจกรรมที่เกี่ยวข้อง:** การนำเสนอผลงานแบบ poster ในการประชุมประจำปี “นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว.” ครั้งที่ 14 ในวันพฤหัสบดีที่ 23 – วันเสาร์ที่ 25 ตุลาคม พ.ศ. 2557 ณ โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน จังหวัดชลบุรี

ภาคผนวก ในส่วนของผลการศึกษา

Supplementary table 1. Unique protein elements uniquely found in the infection stage but absence in the clearance stage, found at least 1 out of 4 independent experimental samples

NO.	Protein elements	gi number	Mass intensity	Replicate result
Intracellular proteins (n = 128)				
1	TLN2	gi|578827491	9.07±0.67	2/3
2	TAS2R49	gi|20336529	8.72±0.86	2/3
3	immunoglobulin heavy chain	gi|270052417	8.75±1.71	2/3
4	Myosin-IXA	gi|3954942	8.45±2.08	2/3
5	conserved oligomeric Golgi complex	gi|578825042	7.96±0.53	2/3
6	cajalin 2	gi|34014746	7.64±0.06	2/3
7	Ppargamma-Lbd	gi|374414719	5.52±1.62	2/3
8	17-beta-HSD 14	gi|578824403	4.45±3.25	2/3
9	mitochondrial ATP-binding cassette	gi|578802184	5.85±0.57	2/3
10	unnamed protein	gi|40977078	9.10±0.52	2/3
11	MYO1D	gi|119600628	8.4±1.16	2/4
12	Control Of K+ Channel Gating	gi|157830170	7.02±0.63	2/4
13	nucleolin	gi|55956788	5.09±0.87	2/4
14	BAT2L	gi|12804221	1.85±0.99	2/4
15	OPK V NimA	gi|38502053	3.12±3.78	2/4
16	src	gi|119615154	4.13±2.98	2/4
17	arfaptin-1	gi|566006168	5.12±2.37	2/4
18	adenomatous polyposis coli protein 2	gi|578825173	7.83±0.40	2/4
19	unnamed protein	gi|218110385	10.49	1/4
20	LP3317	gi|37782436	10.25	1/4
21	unnamed protein	gi|221042662	10.24	1/4
22	hCG1644268	gi|119624788	10.05	1/4
23	polycomb group RING finger protein 1	gi|530368224	10.01	1/4
24	sodium- and chloride-dependent glycine transporter 2	gi|530395991	9.89	1/4
25	A disintegrin and metalloproteinase with thrombospondin motifs 2	gi|110825974	9.86	1/4
26	thyroid receptor-interacting protein 11	gi|578826302	9.74	1/4
27	unnamed protein	gi|56675572	9.7	1/4
28	fibroblast growth factor receptor substrate 3	gi|5730059	9.67	1/4
29	C-type lectin domain	gi|557357745	9.6	1/4
30	PDZ and LIM domain protein 2	gi|28866957	9.59	1/4
31	ZNF783	gi|304647595	9.23	1/4
32	zinc finger SWIM domain-containing protein 3	gi|578835782	8.66	1/4
33	PPP1R13B	gi|444738875	8.38	1/4
34	ras GTPase-activating-like protein IQGAP3	gi|39753961	7.85	1/4
35	unnamed protein	gi|40047084	7.61	1/4

36	myotubularin-related protein 10	gi|578840992	7.53	1/4
37	Chain A, Ptb Domain Of Aida1	gi|443427953	7.42	1/4
38	probable E3 ubiquitin-protein ligase HERC1	gi|578827514	7.02	1/4
39	KIAA0592 protein	gi|3043708	6.70	1/4
40	dedicator of cytokinesis protein 6	gi|578833404	6.52	1/4
41	hCG1818245	gi|119607203	6.50	1/4
42	hCG1998440	gi|119585863	6.30	1/4
43	ankyrin repeat domain-containing protein 29	gi|70995267	5.74	1/4
44	unnamed protein	gi|40981828	4.77	1/4
45	T-cell receptor beta chain	gi|545686380	4.74	1/4
46	RNA-binding protein 26	gi|578824989	0.73	1/4
47	Chromosome 16 open reading frame 71	gi|23273327	0.72	1/4
48	immunoglobulin heavy chain variable region	gi|112697877	7.09	1/4
49	chitinase 3-like 2	gi|119576884	7.74	1/4
50	HLA class II-associated invariant chain Ii	gi|254111	8.79	1/4
51	dickkopf-related protein 2	gi|7657023	6.50	1/4
52	zinc finger protein 668	gi|289547684	0.68	1/4
53	Similar to protein tyrosine phosphatase, non-receptor type 18 (brain-derived)	gi|27371172	8.73	1/4
54	cyclin-dependent kinase inhibitor 3	gi|17981704	10.00	1/4
55	N-glycosylase/DNA lyase	gi|8670532	7.96	1/4
56	protogenin homolog	gi|119597900	5.85	1/4
57	antigen of the monoclonal antibody Ki-67	gi|415821	11.50	1/4
58	MSTP086	gi|33337974	8.60	1/4
59	dynein heavy chain 3	gi|578828592	5.46	1/4
60	SRC kinase signaling inhibitor 1	gi|578840438	9.70	1/4
61	ankyrin repeat domain-containing protein 33B	gi|530378902	7.40	1/4
62	beta-synuclein	gi|578811235	4.53	1/4
63	otogelin-like protein	gi|530400098	5.59	1/4
64	MHC class I antigen	gi|164453827	7.09	1/4
65	immunoglobulin G4 heavy chain variable region	gi|385843822	5.17	1/4
66	myosin-IIIb	gi|578803747	0.46	1/4
67	laminin subunit alpha-5	gi|578836003	6.81	1/4
68	tyrosine-protein kinase FRK	gi|530383497	0.96	1/4
69	protein RUFY3	gi|530376919	6.90	1/4
70	PRED58	gi|7717443	7.53	1/4
71	transmembrane protein 156	gi|530376526	6.06	1/4
72	hCG1995562	gi|119577540	5.03	1/4
73	chymase 1 preproprotein transcript E	gi|70672498	7.79	1/4
74	Chromosome 2 open reading frame 50	gi|111309347	6.93	1/4
75	Kruppel-associated box protein	gi|2623622	6.88	1/4
76	phosphatidylinositol 3-kinase	gi|472991	7.70	1/4
77	26S proteasome non-ATPase regulatory subunit 5	gi|394025672	7.36	1/4
78	visinin-like peptide 1	gi|755161	9.2	1/4
79	metastasis-associated protein MTA3	gi|530367894	5.15	1/4
80	G2 and S phase-expressed protein 1	gi|530420136	7.69	1/4

81	signal peptide, CUB domain, EGF-like 1	gi|119593706	4.70	1/4
82	reverse transcriptase-like protein	gi|58014085	5.35	1/4
83	hCG1749932	gi|119571450	6.18	1/4
84	hypothetical protein	gi|34365465	6.50	1/4
85	fibrocystin isoform 1	gi|126131102	6.62	1/4
86	T cell receptor beta chain	gi|1245341	7.93	1/4
87	unnamed protein	gi|40046420	9.10	1/4
88	Homo sapiens ribosomal protein L10	gi|10281742	9.80	1/4
89	serine/threonine-protein kinase SMG1	gi|530407769	7.85	1/4
90	A-kinase anchor protein 14	gi|56682940	5.03	1/4
91	ubiquitin carboxyl-terminal hydrolase isozyme L5	gi|578801060	6.27	1/4
92	CXorf58 protein	gi|219518994	6.88	1/4
93	coiled-coil domain-containing protein 89	gi|22749429	4.08	1/4
94	unnamed protein	gi|19575499	6.05	1/4
95	hypothetical protein	gi|52545560	2.61	1/4
96	roundabout homolog 2	gi|578806427	7.17	1/4
97	RNA-binding motif protein	gi|530426557	7.13	1/4
98	Putative ciliary rootlet coiled-coil protein-like 3 protein	gi|519882971	5.16	1/4
99	hypothetical protein	gi|57161863	7.79	1/4
100	unnamed protein	gi|30067	7.50	1/4
101	ly6/PLAUR domain-containing protein 5	gi|578834316	7.05	1/4
102	amphoterin-induced protein 1	gi|153791466	5.09	1/4
103	unnamed protein	gi|14036092	8.24	1/4
104	unnamed protein	gi|40980340	8.93	1/4
105	unnamed protein	gi|194380698	7.45	1/4
106	immunoglobulin A heavy chain variable region	gi|519673780	8.32	1/4
107	hCG21112	gi|119572980	8.75	1/4
108	protein transport protein Sec61 subunit alpha	gi|217330618	7.95	1/4
109	transmembrane protein 217	gi|556695430	8.25	1/4
110	unnamed protein	gi|194381490	7.77	1/4
111	alternative protein NAP1L2	gi|444732957	8.23	1/4
112	alpha-actinin-4	gi|578834952	5.01	1/4
113	hCG2038599	gi|119601910	8.46	1/4
114	RNA-binding protein 33	gi|530387248	6.14	1/4
115	immunoglobulin heavy chain variable region	gi|482672685	5.49	1/4
116	hCG1991907	gi|119613985	6.72	1/4
117	WD repeat domain 46	gi|119624108	9.06	1/4
118	sulfatase modifying factor 2	gi|119628386	8.54	1/4
119	protein BEX1	gi|68533249	6.83	1/4
120	protein phosphatase 1 regulatory subunit 16A	gi|578816221	9.36	1/4
121	myosin light chain kinase, smooth muscle isoform X4	gi|578807453	6.49	1/4
122	COX15	gi|578818940	8.12	1/4
123	Human Microcephalin (Mcph1) Tandem Brct Domains In Complex With A Gamma-H2ax Phosphopeptide	gi|358439836	1.53	1/4
124	pre-mRNA-processing factor 6	gi|578835938	7.24	1/4
125	hCG23632	gi|119589705	8.41	1/4

126	albumin	gi|546033	6.74	1/4
127	proteasome subunit alpha type-5	gi|315138983	5.71	1/4
128	glutamate decarboxylase 2	gi|197276620	3.32	1/4

Supplementary table 1 (continue)

NO.	Protein elements	gi number	Mass intensity	Replicate result
Extracellular proteins (n = 73)				
1.	APCDD1	gi|23503291	9.65	2/3
2.	FYVE, RhoGEF and PH domain-containing protein 1	gi|24797153	6.79	2/3
3.	immunoglobulin light chain	gi|333466192	5.31	2/3
4.	KIAA0377	gi|30315253	1.48	2/4
5.	rap guanine nucleotide exchange factor 2	gi|578809656	3.15	1/4
6.	ZNF516 protein	gi|82568975	3.25	1/4
7.	serine/threonine-protein kinase A-Raf	gi|578838017	9.12	1/4
8.	unnamed protein	gi|40044478	6.66	1/4
9.	putative beta-lactamase-like 1	gi|530433766	3.33	1/4
10.	human RAD23A homolog	gi|1905912	3.82	1/4
11.	histone H1t	gi|20544168	3.93	1/4
12.	aminopeptidase N	gi|530407092	9.68	1/4
13.	stomatin-like protein 3	gi|530402132	4.30	1/4
14.	unnamed protein	gi|10434667	7.02	1/4
15.	histone-lysine N-methyltransferase NSD2	gi|578808235	8.07	1/4
16.	unnamed protein	gi|40976104	7.34	1/4
17.	immunoglobulin A heavy chain variable region	gi|519673614	-0.44	1/4
18.	unnamed protein	gi|40046904	8.01	1/4
19.	leucine-rich repeat-containing protein 36	gi|530424069	5.20	1/4
20.	unnamed protein	gi|56675572	5.38	1/4
21.	MAP kinase-interacting serine/threonine-protein kinase 1	gi|578800022	5.76	1/4
22.	dipeptidase 2	gi|578829124	8.49	1/4
23.	potassium-transporting ATPase alpha chain 2	gi|297374799	5.97	1/4
24.	NACHT, LRR and PYD domains-containing protein 9	gi|530416403	2.95	1/4
25.	replication factor C subunit 1	gi|325296984	6.00	1/4
26.	ras-interacting protein 1	gi|38570105	4.42	1/4
27.	presenilin 1	gi|119601500	5.68	1/4
28.	cullin-2	gi|311771639	3.32	1/4
29.	HASPP28	gi|1518855	8.33	1/4
30.	NADH dehydrogenase (ubiquinone) 1 beta	gi|119598797	7.45	1/4
31.	stAR-related lipid transfer protein 13	gi|530402780	9.99	1/4
32.	unnamed protein	gi|40033223	5.66	1/4
33.	Chain n, Truncated Snare Complex With Complexin (P1)	gi|345100957	4.91	1/4
34.	Chain H, Structure Of Ntd2 Domain Of The Human Taf5 Subunit Of Tfiid	gi|126031253	8.05	1/4
35.	immunoglobulin heavy chain variable region	gi|247425425	3.20	1/4
36.	zinc finger protein 428	gi|530415708	10.35	1/4

37.	myosin IXB	gi|119604979	9.03	1/4
38.	RPL3	gi|47678663	7.82	1/4
39.	immunoglobulin heavy chain variable region	gi|70798101	9.18	1/4
40.	beclin-1	gi|530413039	3.39	1/4
41.	arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2-like	gi|578805484	0.66	1/4
42.	myotubularin-related protein 5	gi|530420851	6.82	1/4
43.	dmX-like protein 1	gi|578810211	7.13	1/4
44.	CD247 transcript variant 2	gi|183240789	8.15	1/4
45.	unnamed protein	gi|108250600	3.33	1/4
46.	Chromosome 17 open reading frame 91	gi|111599512	5.81	1/4
47.	protein phosphatase 2A B'alpha1 regulatory subunit	gi|1203812	6.35	1/4
48.	uncharacterized protein LOC440292	gi|530356171	7.75	1/4
49.	probable carboxypeptidase PM20D1	gi|578800577	1.25	1/4
50.	unnamed protein	gi|16549252	6.59	1/4
51.	unconventional myosin-VIIb	gi|578804363	6.95	1/4
52.	tyrosinase-related protein	gi|220132	1.30	1/4
53.	endoplasmic reticulum aminopeptidase 2	gi|11641261	6.49	1/4
54.	putative methyltransferase NSUN6 isoform X7	gi|578818306	4.03	1/4
55.	zinc finger protein 616 isoform X1	gi|530417498	5.04	1/4
56.	rho GTPase-activating protein 32	gi|578822484	6.71	1/4
57.	testis-specific serine/threonine-protein kinase 6	gi|14042966	2.15	1/4
58.	vitronectin precursor	gi|88853069	3.78	1/4
59.	S-phase kinase-associated protein 2	gi|578809960	8.76	1/4
60.	fibroblast growth factor 13	gi|4758366	-0.18	1/4
61.	signal recognition particle 14 kDa protein	gi|149999611	2.06	1/4
62.	maestro heat-like repeat-containing protein family member 2A	gi|565671697	3.43	1/4
63.	BAH and coiled-coil domain-containing protein 1	gi|578831174	4.39	1/4
64.	leucine zipper putative tumor suppressor 1 isoform X1	gi|530387553	4.60	1/4
65.	transcription initiation factor TFIID subunit 3	gi|151301171	4.78	1/4
66.	hCG1816008	gi|119622992	6.06	1/4
67.	unnamed protein	gi|21698637	6.25	1/4
68.	hCG2036632	gi|119592537	6.87	1/4
69.	kinesin-like protein KIF1C isoform X1	gi|530409731	7.09	1/4
70.	guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 precursor	gi|51036603	7.22	1/4
71.	WD repeat-containing protein 75	gi|29789283	7.57	1/4
72.	erythroid differentiation-related factor 1 isoform X2	gi|530393399	7.96	1/4
73.	rho GTPase-activating protein 21 isoform X9	gi|578818522	10.21	1/4

Supplementary table 2. Unique protein elements uniquely found in the clearance stage but absence in the infection stage at least 1 out or 4 independent experimental samples.

No.	Protein elements	gi number	Mass intensity	Replicate result
Intracellular proteins (n = 129)				
1	CEACAM18	gi|508083061	1.25	3/3
2	ras GTPase	gi|530378499	8.08	2/3
3	SSA2	gi|530370876	7.66	2/3
4	N-CoR2	gi|62087758	6.42	2/3
5	DRB1	gi|1487978	2.36	2/3
6	proteasome activator	gi|62467430	2.33	2/3
7	synaptotagmin XI	gi|119573411	7.37	2/3
8	IL-11	gi|10834994	8.15	2/4
9	SRC kinase signaling inhibitor 1	gi|578840438	7.18	2/4
10	fibrocystin isoform 1	gi|126131102	6.91	2/4
11	unnamed protein product	gi|218110385	5.88	2/4
12	gephyrin	gi|578825413	4.96	2/4
13	protein unc-13 homolog B isoform X1	gi|578816361	6.47	1/4
14	VGSA5840	gi|37182663	8.95	1/4
15	beta-synuclein isoform X3	gi|578811235	4.77	1/4
16	glutamate decarboxylase 2	gi|197276620	7.22	1/4
17	src family associated phosphoprotein 1	gi|119615154	7.37	1/4
18	BAT2L protein	gi|12804221	2.74	1/4
19	A-kinase anchor protein 14	gi|56682940	6.53	1/4
20	unnamed protein	gi|40043002	6.96	1/4
21	zinc finger protein 662 isoform 2	gi|197384097	6.43	1/4
22	semenogelin-2	gi|4506885	6.99	1/4
23	tripartite motif-containing 26	gi|119623678	7.24	1/4
24	preplacental alkaline phosphatase	gi|178470	7.29	1/4
25	leucine-rich repeat neuronal protein 1	gi|530372914	10.44	1/4
26	insulin receptor substrate-1	gi|452916720	8.10	1/4
27	MHC class I antigen	gi|239837564	9.44	1/4
28	hCG2014043	gi|119580662	4.32	1/4
29	tyrosine-protein kinase FRK isoform X3	gi|530383497	5.19	1/4
30	tubulin polyglutamylase TTLL5	gi|50658079	3.49	1/4
31	putative protein arginine N-methyltransferase 10	gi|530378406	4.15	1/4
32	DDB1- and CUL4-associated factor 7	gi|108936958	4.44	1/4
33	hypothetical gene supported by AK126539	gi|119605560	5.55	1/4
34	G kinase-anchoring protein 1	gi|578817907	9.39	1/4
35	Putative ciliary rootlet coiled-coil protein-like 3	gi|519882971	5.86	1/4
36	hCG2042301	gi|119611404	8.65	1/4
37	protein VPRBP isoform X10	gi|578806807	9.03	1/4
38	dystonin isoform X17	gi|530382450	7.92	1/4

39	protein RIC-3 isoform X7	gi|578820796	7.62	1/4
40	hCG1644450	gi|119590978	7.30	1/4
41	NAD-dependent protein deacylase sirtuin-5	gi|530381706	9.22	1/4
42	Chromosome 2 open reading frame 50	gi|111309347	6.09	1/4
43	Kruppel-associated box protein	gi|2623622	0.42	1/4
44	kell blood group glycoprotein	gi|530431195	9.24	1/4
45	hCG1793240	gi|119569400	0.84	1/4
46	cell death-inducing DFFA-like effector a	gi|119621960	5.49	1/4
47	TIMELESS-interacting protein isoform X6	gi|578827157	8.87	1/4
48	cajalin 2	gi|34014746	8.63	1/4
49	immunoglobulin A heavy chain variable region	gi|519673780	7.80	1/4
50	LOC648987 protein	gi|21707577	8.73	1/4
51	synaptotagmin-16 isoform X3	gi|578826180	9.56	1/4
52	26S proteasome non-ATPase regulatory subunit 5	gi|394025672	0.65	1/4
53	FAM123A protein	gi|55824660	8.39	1/4
54	serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit gamma	gi|530404120	9.43	1/4
55	CLK4-associating serine/arginine rich protein	gi|578833889	8.81	1/4
56	titin isoform X6	gi|578804834	6.20	1/4
57	sodium- and chloride-dependent glycine transporter 2	gi|530395991	8.79	1/4
58	macrophage erythroblast attacher isoform X8	gi|578808067	7.18	1/4
59	plasminogen receptor (KT) isoform X3	gi|530390008	6.23	1/4
60	thyroid receptor-interacting protein 11	gi|578826302	7.30	1/4
61	hCG2038599	gi|119601910	8.80	1/4
62	RNA-binding protein 33	gi|530387248	7.41	1/4
63	T cell receptor beta chain precursor	gi|1245341	7.78	1/4
64	unnamed protein	gi|40046420	9.06	1/4
65	conserved oligomeric Golgi complex subunit 3	gi|578825042	8.11	1/4
66	keratin, type I cytoskeletal 23	gi|530432877	7.54	1/4
67	RRP1B protein	gi|33878453	5.93	1/4
68	hCG1811569	gi|119599008	8.61	1/4
69	immunoglobulin heavy chain variable region	gi|393689158	5.51	1/4
70	Chain D, Crystal Structure Of Kh1 Domain Of Human Pcbp2 Complexed To Single- Stranded 12-Mer Telomeric Dna	gi|149243359	5.74	1/4
71	T-cell receptor beta chain	gi|545686380	4.66	1/4
72	muellerian-inhibiting factor precursor	gi|157266298	3.78	1/4
73	tensin 1	gi|119590993	4.80	1/4
74	kinase D-interacting substrate of 220 kDa	gi|55741641	5.68	1/4
75	serine/threonine-protein kinase SMG1	gi|530407769	8.29	1/4
76	Chain B, The Crystal Structure Of The Kinase Domain Of The Protein Kinase C	gi|167013213	4.26	1/4
77	unnamed protein	gi|40039888	8.73	1/4

78	proteasome subunit alpha type-5	gi|315138983	7.02	1/4
79	alternative protein MDC1	gi|444738769	7.78	1/4
80	unnamed protein	gi|21758429	9.67	1/4
81	glutamate receptor ionotropic, NMDA 1 isoform X3	gi|530426722	10.41	1/4
82	phospholipase D1 isoform X2	gi|530374782	6.68	1/4
83	keratin, type II cytoskeletal 6A	gi|5031839	8.53	1/4
84	coiled-coil domain-containing protein 13 isoform X2	gi|530371957	7.90	1/4
85	stAR-related lipid transfer protein 13	gi|578825073	8.47	1/4
86	T-cell immune regulator 1 transcript variant 4	gi|45386002	9.80	1/4
87	sex determining region Y	gi|340366108	11.15	1/4
88	T-cell receptor beta chain	gi|4028238	4.93	1/4
89	myosin light chain kinase	gi|578807453	5.20	1/4
90	cytochrome c oxidase assembly protein COX15	gi|578818940	5.35	1/4
91	unnamed protein	gi|221040750	5.35	1/4
92	hCG1781583	gi|119603083	5.69	1/4
93	chromodomain-helicase-DNA-binding protein 9 isoform X13	gi|578829235	6.79	1/4
94	melanophilin isoform X6	gi|578804879	6.52	1/4
95	Chain A, Ptb Domain Of Aida1	gi|443427953	6.73	1/4
96	cytoplasmic dynein 1 light intermediate chain 2	gi|555290083	6.93	1/4
97	pre-mRNA-processing factor 6	gi|578835938	8.23	1/4
98	antigen of the monoclonal antibody Ki-67	gi|415821	8.99	1/4
99	centriolin isoform X6	gi|578817022	2.05	1/4
100	trace amine-associated receptor 6	gi|28173558	6.69	1/4
101	RNA-binding protein 26	gi|578824989	0.78	1/4
102	hCG1809968	gi|119606399	4.46	1/4
103	elongation factor Tu	gi|34147630	6.82	1/4
104	4-trimethylaminobutyraldehyde dehydrogenase	gi|189053452	8.03	1/4
105	unnamed protein	gi|40042896	4.23	1/4
106	chromodomain-helicase-DNA-binding protein 1-like	gi|373432615	8.40	1/4
107	hCG2021566	gi|119608575	3.69	1/4
108	unnamed protein	gi|40977246	1.16	1/4
109	hCG1651427	gi|119607835	8.47	1/4
110	C19orf55 protein	gi|17389875	8.26	1/4
111	acyl-coenzyme A thioesterase 8	gi|530417750	8.76	1/4
112	hCG2013212	gi|119586141	8.18	1/4
113	RTDR1	gi|47678665	7.26	1/4
114	hCG2032829	gi|119588077	7.29	1/4
115	Chain C, Immune Receptor	gi|563320934	8.73	1/4
116	mas-related G-protein coupled receptor member X2	gi|16876451	8.04	1/4
117	phospholipase A2, group IVB (cytosolic), isoform CRA_a	gi|119612926	10.64	1/4
118	zinc finger protein 668 isoform a	gi|289547684	4.96	1/4
119	leucine-rich repeat-containing protein 56 isoform X8	gi|578839839	7.20	1/4

120	retinal homeobox protein Rx	gi|126116581	7.44	1/4
121	signal peptide, CUB domain, EGF-like 1, isoform CRA_f	gi|119593706	6.19	1/4
122	cyclin-dependent kinase inhibitor 3 isoform 1	gi|17981704	5.90	1/4
123	hCG1997940, isoform CRA_b	gi|119585736	7.55	1/4
124	R3H domain-containing protein 2 isoform X21	gi|578823412	4.54	1/4
125	unnamed protein	gi|40047084	9.82	1/4
126	neuroblast differentiation-associated protein AHNAK isoform X10	gi|578821732	2.26	1/4
127	zinc finger protein 395	gi|8923887	7.63	1/4
128	dedicator of cytokinesis protein 6 isoform X4	gi|578833404	4.42	1/4
129	LON peptidase N-terminal domain and RING finger protein 2	gi|148528975	6.88	1/4

Supplementary table 2. (continue)

No.	Protein elements	gi number	Mass intensity	Replicate result
Extracellular proteins (n = 75)				
1.	huMCM2	gi|1232077	5.28	2/3
2.	hCG1818658	gi|119607623	3.96	2/3
3.	RANBP1	gi|47678643	4.03	2/3
4.	replication factor	gi|325296984	0.26	2/3
5.	drebrin	gi|578811024	7.31	2/4
6.	TBC1	gi|139394668	5.92	2/4
7.	Usp45	gi|530384124	5.73	2/4
8.	UTY	gi|151946791	5.10	2/4
9.	la-related protein	gi|578810845	5.20	2/4
10.	arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2-like	gi|578805484	6.66	1/4
11.	repetin	gi|171916097	7.95	1/4
12.	ATP-binding cassette, sub-family C (CFTR/MRP), member 1, isoform CRA_m		7.52	1/4
13.	HLA class II histocompatibility antigen	gi|578842305	6.67	1/4
14.	ras-interacting protein 1	gi|38570105	0.96	1/4
15.	cullin-2 isoform a	gi|311771639	8.44	1/4
16.	HASPP28	gi|1518855	4.74	1/4
17.	ephrin type-A receptor 2 isoform X2	gi|530360855	4.49	1/4
18.	Structure Of Ntd2 Domain Of The Human Taf5 Subunit Of Tfiid	gi|126031253	8.01	1/4
19.	immunoglobulin heavy chain variable region	gi|247425425	4.88	1/4
20.	dnaJ homolog subfamily B member 5 isoform 1	gi|201862587	8.90	1/4
21.	beclin-1 isoform X3	gi|530413039	4.95	1/4
22.	chromosome X open reading frame 22, isoform CRA_b	gi|119619481	3.73	1/4
23.	unnamed protein	gi|40043034	8.32	1/4
24.	unnamed protein	gi|108250600	4.03	1/4
25.	phenylalanine--tRNA ligase beta subunit isoform X3	gi|578803436	6.29	1/4
26.	probable helicase senataxin isoform X3	gi|530427122	5.08	1/4
27.	Ran	gi|1680066	8.22	1/4
28.	neurexophilin-1 precursor	gi|23097338	6.19	1/4
29.	leucine zipper putative tumor suppressor 1 isoform X1	gi|530387553	4.07	1/4
30.	transcription initiation factor TFIID subunit 3	gi|151301171	5.53	1/4
31.	unnamed protein	gi|193787564	6.16	1/4
32.	unnamed protein	gi|40043428	2.13	1/4
33.	TTC9 protein	gi|28839621	6.02	1/4
34.	ruvB-like 1	gi|4506753	6.35	1/4
35.	RAGE-4 ORF2	gi|1517909	7.22	1/4
36.	immunoglobulin G heavy chain variable region	gi|371446220	8.02	1/4
37.	Usher syndrome type-1G protein isoform 2	gi|542133068	5.38	1/4
38.	ras-related protein Rab-23 isoform X1	gi|530382146	5.90	1/4
39.	hepatoma-derived growth factor-like protein 1	gi|110681712	5.53	1/4

40.	ankyrin repeat domain-containing protein 36A isoform X7	gi|578804302	4.89	1/4
41.	urea transporter 2	gi|336594532	8.17	1/4
42.	cytosolic Fe-S cluster assembly factor NUBP2 isoform 1	gi|6912540	9.52	1/4
43.	testis-specific serine/threonine-protein kinase 6	gi|14042966	4.74	1/4
44.	replication factor C subunit 2 isoform X1	gi|578814487	7.62	1/4
45.	vitronectin precursor	gi|88853069	8.22	1/4
46.	immunoglobulin A heavy chain variable region, partial	gi|519673614	5.70	1/4
47.	phytanoyl-CoA dioxygenase, peroxisomal isoform X1	gi|530392197	8.50	1/4
48.	nuclear pore complex protein Nup85	gi|578831428	2.51	1/4
49.	uracil-DNA glycosylase	gi|1483623	6.54	1/4
50.	ANKUB1	gi|205371873	6.04	1/4
51.	hCG1816008	gi|119622992	5.88	1/4
52.	unnamed protein	gi|40977148	1.66	1/4
53.	rho GTPase-activating protein 21 isoform X9	gi|578818522	10.70	1/4
54.	hCG2045002	gi|119571845	4.11	1/4
55.	unnamed protein	gi|193786025	6.27	1/4
56.	unnamed protein	gi|40977138	7.11	1/4
57.	DNA-binding protein Ikaros isoform 13	gi|334085231	1.70	1/4
58.	Heterodimer Of P40phox And P67phox Pb1 Domains From Human Nadph Oxidase	gi|34810994	3.27	1/4
59.	hypothetical protein	gi|31873585	4.72	1/4
60.	phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 isoform X6	gi|578821381	4.75	1/4
61.	FH2 domain-containing protein 1 isoform X3	gi|578809574	4.89	1/4
62.	immunoglobulin variable region	gi|323432015	5.00	1/4
63.	zinc finger protein 616	gi|530417498	5.40	1/4
64.	intraflagellar transport protein 88	gi|530402705	5.80	1/4
65.	Crystal Structure Of Human Lactb2	gi|374414772	5.88	1/4
66.	histone-lysine N-methyltransferase NSD2	gi|578808235	6.34	1/4
67.	profilin-3	gi|71274140	6.84	1/4
68.	immunoglobulin heavy chain variable region	gi|190341917	6.90	1/4
69.	unnamed protein	gi|40041004	6.95	1/4
70.	myosin IXB	gi|119604979	7.08	1/4
71.	BRCA1 associated RING domain 1 variant	gi|62898379	7.64	1/4
72.	junctophilin-4	gi|225690500	7.66	1/4
73.	C4b-binding protein alpha chain isoform X2	gi|530366456	7.83	1/4
74.	zinc finger protein 3-like, partial	gi|530434685	7.85	1/4
75.	immunoglobulin variable region	gi|323432478	8.60	1/4

Supplementary table 3 Differential protein elements highly found in the clerance stage but less abundance in the infection stage, at least 1 out or 4 independent experimental

No.	Protein elements	gi number	Mass intensity		Fold change	Replicate result
Intracellular proteins						
1	cell death-inducing DFFA-like effector A	gi|119621960	4.12	5.19	1.26	2/3
2	unnamed protein	gi|40041832	0.94	2.49	2.64	1/4
3	X-linked retinitis pigmentosa GTPase regulator	gi|78190492	0.89	5.64	6.32	1/4
4	ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast),	gi|119606081	0.64	9.61	15.08	1/4
5	Chain A, Solution Structure Of The 22th Filamin Domain From Human Filamin C	gi|159163979	0.21	7.34	35.14	1/4
6	PREDICTED: fer-1-like protein 6	gi|578816114	0.19	8.16	43.15	1/4
7	lysine-specific demethylase 3B	gi|38372909	0.12	1.00	8.33	1/4
Extracellular proteins						
8	probable carboxypeptidase PM20D1	gi|578800577	1.11	6.64	5.96	3/4
9	sterol regulatory element-binding protein cleavage-activating protein	gi|578805806	0.24	6.21	26.29	1/4
10	HLA class II histocompatibility antigen, DO alpha chain	gi|578842305				1/4
11	transcription factor SC1	gi|833833	0.31	3.10	10.16	1/4
12	arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2-like	gi|578805484	0.52	3.96	7.59	1/4
13	hCG1653486	gi|119586876	1.38	7.84	5.67	1/4
14	PREDICTED: C4b-binding protein alpha chain	gi|530366456	2.34	7.11	3.04	1/4
15	unnamed protein	gi|7020833	3.31	8.29	2.50	1/4

Only 2 fold changed proteins were shown. Cell death-inducing DFFA-like effector A was included due to the replicated results were found from 2/3 experimental samples.

Supplementary table 4 Differential protein elements highly found in the infection stage but less abundance in the clearance stage, at least 1 out or 4 independent experimental

No.	Protein elements	gi number	Mass intensity		Fold change	Replicate result
Intracellular proteins						
1	MHC class I antigen	gi|239837564	9.49	2.14	4.43	1/4
2	fer-1-like protein 6	gi|578816114	7.89	1.61	4.91	1/4
3	regulator of nonsense transcripts 3B	gi|578838746	7.11	0.82	8.65	1/4
4	triosephosphate isomerase	gi|226529917	6.29	1.87	3.37	1/4
5	carcinoembryonic antigen-related cell adhesion molecule 18	gi|508083061	5.92	2.52	2.34	1/4
Extracellular proteins						
6	unnamed protein	gi|40043732	8.01	0.47	17.07	1/4
7	PREDICTED: exostosin-like 3	gi|578815227	6.84	2.84	2.41	1/4
8	endoplasmic reticulum aminopeptidase 2	gi|11641261	6.03	2.89	2.08	1/4

Only 2 fold changed proteins were shown.