Indonesian Journal of
Tropical and Infectious Disease
Vol. 7 No. 1 January–April 2018

Research Report

THE DIFFERENCE OF MAP1LC3 LEVEL AS MACROPHAGE AUTOPHAGY MARKER BETWEEN RESISTANT AND SENSITIVE TUBERCULOSIS PATIENTS ON RIFAMPICIN

Dian Novita W[1a], Jusak Nugraha[2], Soedarsono[3]
[1] Master of Immunology, Faculty of Pasca Sarjana, Universitas Airlangga
[2] Clinical Patology, dr. Soetomo Teaching Hospital
[3] Pulmonology & Respiratory, dr. Soetomo Teaching Hospital
[a] Corresponding author: diannovii13@gmail.com

ABSTRACT

Mycobacterium tuberculosis (MTB) is an intracelular bacteria that live in the host macrophage cells. Several organs can be affected by tuberculosis but most major illnesses are lung diseases. Immediately after infection, MTB will be phagocytosed by the alveolar macrophage cells and can survive in the phagosome. The macrophage plays a role in innate immunity towards an infection using autophagy by removing the microbe directly via phagocytosis. When bacteria phagocytosized, vacuole membrane formed double membranes called autophagosome, and followed by degradation by lysosome, which known as autolysosome. Induction of autophagy can be observed on the formation of microtubule-associated proteins 1B lightchain 3B (MAP1LC3B/LC3). MAP1LC3B is protein that have role at autophagic way for selection autophagy substrate and biogenesis. In this study we are used serum from patients TB with rifampicin resistant and rifampicin sensitive as control. Samples were divided using gene expert to differentiate between resistant and sensitive rifampicin.This research aims to compare MAP1LC3B levels in resistant and sensitive rifampicin to study macrophages respond in autophagic way in tuberculosis patients, and give information for define therapy plan to improve therapy for MDR-TB patients. Type of this research is a case control study design with cross sectional research with each groups sample is 19 from age 18-65 years old. Result, MAP1LC3B serum levels on the rifampicin resistant group are lower compared to rifampicin sensitive group. This occur because MTB is able to hide and evade innate immune defense mechanisms. MTB can maintain intracellular growth inside the phagosome by inhibiting phagolysosome formation in autophagy process especially inhibit MAP1LC3B formation by PDIM.

Keywords: *Mycobacterium tuberculosis, drug resistance, rifampicin, autophagy, MAP1LC3B*

ABSTRAK

Mycobacterium tuberculosis (MTB) adalah bakteri intraseluler yang hidup dalam makrofag pada sel inang. Beberapa organ dapat dipengaruhi oleh tuberkulosis tetapi yang paling utama adalah penyakit paru. Segera setelah terjadi infeksi, kuman TB akan difagositosis oleh sel makrofag alveolar dan tetap bertahan hidup dalam fagosom. Makrofag mempunyai peranan penting dalam respon imun bawaan terhadap infeksi melalui autofagi dengan mengeliminasi bakteri secara langsung dengan cara fagositosis. Ketika bakteri di fagositosis membran vakuola membentuk dua lapisan membran yang disebut dengan autofagosom dan didegradasi oleh lisosom, yang biasa dikenal dengan autolisosom. Induksi autofagi dapat dipantau pada pembentukan formasi microtubule-associated protein 1B light chain 3B (MAP1LC3B/LC3). MAP1LC3B adalah protein yang mempunyai peranan pada jalur autofagi untuk seleksi subrat dan biogenesis. Penelitian ini menggunakan serum darah pasien TB yang resisten dan sensitif rifampisin sebagai kontrol. Sampel resisten dan sensitive dibedakan menggunakan tes gen expert. Penelitian ini bertujuan untuk membandingkan kadar MAP1LC3B pada resisten dan sensitif rifampisin untuk mempelajari autofagi makrofag pada pasien tuberkulosis dan memberikan informasi untuk meningkatkan terapi pada pasien MDR-TB. Jenis penelitian ini adalah case control study dengan rancangan penelitian cross sectional dengan besar sampel tiap kelompok sebesar 19 dengan rentang umur 18-65 tahun. Hasilnya, kadar MAP1LC3B pada kelompok resisten rifampisin

memiliki kadar lebih rendah dibandingkan dengan kelompok sensitif.Hal ini disebabkan karena MTB dapat menghindari sistem pertahanan respon imun bawaan. MTB dapat mempertahankan pertumbuhan intraseluler di dalam fagosom dengan menginhibisi formasi fagolisosom pada proses autofagi terutama menghambat pembentukan MAP1LC3B oleh PDIM.

Kata kunci: *Mycobacterium tuberculosis, resisten obat, rifampisin, autofagi, MAP1LC3B*

INTRODUCTION

Mycobacterium tuberculosis (MTB) can cause a dangerous disease called Tuberculosis (TB). This microbacteria can attack various organs, mostly the lungs. The TB infection can spread from coughing or sneezing which allows MTB to enter the body along with dusts or droplets.[1] There are 6 countries with the world's largest TB disease spread: South Africa, Nigeria, China, Pakistan, India and Indonesia. MTB can evolve its resistance against antimicrobial drugs. There is a type of TB called Multidrug-resistant TB (MDR-TB) which cannot be treated by at least with two of the potent first line anti-TB drugs like isoniazid and rifampicin. To improve detection of the case and treatment for MDR-TB, any further research is needed. There are 300,000 cases of MDR-TB patients that were estimated in 2013. Around 45% cases from them were detected among all pulmonary TB in the world while around 5% of cases of MDR-TB that are not detected or not managed outside the national TB programs were not reported.[2]

Comparative genomic analyses drug resistance on MTB can be caused by 3 things, they are chromosomal mutations that required for the action of antibiotics, gene that encodes the protein targets of drugs applied, or enzymes that are required to activate pro-drug. The target of antibiotics is important to cell function. Resistant mutations encode gene target will affect pathogenesis.[3] In every 106 to 108 replications, wild strains of MTB will undergo spontaneous mutations that confer resistance to a single drug, mutations variety to antibiotic shown at Table 1.

TB therapy with fast onset needs Rifampicin (RIF) as critical component of first-line therapy.[4] Almost 90% of RIF resistant strains are also resist to isoniazid. RIF resistant is used as subtitution marker for detecting MDR TB.[5] RIF resistant is caused by mutation of a single nucleotide-substitution on rpoB region. In this mutation process, the gene encodes the β-subunit of RNA polymerase into DNA-dependent (RNAP).[6] Transcription of the RNAP from the mutations of rpoB in the gene has some effects toward physiology of the MTB. Mutations in this site can cause secondary mutations which lead resistance to another antibiotic.[7]

Autophagy is a complex process involving multiple protein that consist of complex formation and initiation of double membrane development phagophore as nucleation, elongation of the membrane and completion of autophagosome vesicles surround the cargo, and then they will fuse with lysosome (Figure 1). Lysosome is contained hydrolase that can degrade and dispose component.[8] MTB persist and multiply within infected macrophage, where it resides in host-derived phagosome which fails to fuse with lysosom.[9] Autophagy is caused by metabolic and immune signals consists of recognition of pathogen and stimulation by pro-inflammatory cytokines. Autophagy trigger microtubule-associated proteins 1B light chain 3B (MAP1LC3B/LC3), a protein encoded by the gene MAP1LC3B in humans.[10] LC3 was first identified as a protein co-purified with microtubule-associated protein 1A and 1B from rat brains. This protein is derived from 28% of amino acids with Apg8/Aut7p who plays a role in autophagy in yeast, undergoes complex C-terminal proteolitic and lipid (phosphathydil ethanolamine) modifications, which is translocate from cytosol to the autophagosomal membrane.[11]

MAP1LC3B functions are for biogenesis, autophagy and substrate selection autophagosome.[10] If MTB resistance to rifampicin, it physiology change, MAP1LC3B could not form autophagosome vesicles so elimination of bacteria with autophagy process not formed, result MTB survive inside body. This research is conducted to analyze the differences between the MAP3LC1B level in tuberculosis patient with sensitive and resistant rifampicin where this protein used as autophagy marker from macrophage.

Table 1. Mutations in antibiotic[1]

Drug	Average Mutation Rate
Isoniazid	2.56×10^{-6}
Rifampicin	2.25×10^{-10}
Ethambutol	1×10^{-7}
Streptomycin	2.95×10^{-8}
Pyrazinamide	1×10^{-3}

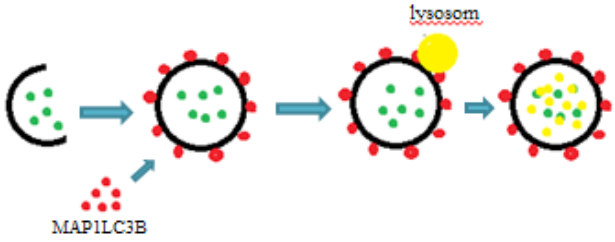

Figure 1. Autophagy process

MATERIAL AND METHOD

A retrospective cross-sectional study was conducted from May 2017 to September 2017 at the Dr. Soetomo General Hospital. Samples are used are serum from tuberculosis patients who visited Dr. Soetomo General Hospital during study period. When patients coming they have blood tested and fill information for medical record. Patients are divided into sensitive and resistant rifampicin using gene expert test, and sample used were patients that meet the inclusion and exclusion criteria based on medical record.

Based on the WHO (2013) the proportion value of TB with MDR was 4% of new TB cases, so the number of samples obtained were 19 sensitive (as control) and 19 resistant. Normal groups were used as MAP1LC3B baseline.

After all samples collected, samples are processed by ELISA. These were diluted and decontaminated, and MAP1LC3B kit performed according to the manual of manufacturer. Result were analyzed using one-way ANNOVA P<0.05, and comparisons between groups using Tukey.

RESULT AND DISCUSSION

Results are obtained as concentration levels of MAP1LC3B (ng/ml) which showed at Table 2 and analyzed with a value of P<0.05 (Table 3).

Table 2. MAP1LC3B Concentration (ng/ml)

Normal	Sensitive	Resistance
1.061	2.291	0.136
1.418	0.866	0.475
1.537	0.983	0.321
1.753	3.268	3.473
1.978	0.482	1.067
2.435	2.268	0.435
2.45	0.684	0.402
2.504	1.072	0.776
2.538	3.012	0.796
3.812	0.526	0.949
	0.345	0.512
	1.432	1.637
	0.381	1.828
	1.584	1.435
	2.35	0.954
	4.137	0.529
	2.033	1.505
	1.973	0.59
	2.156	0

Table 3. Comparison between groups (ng/ml)

Groups	Mean	Significant	P val.
Normal vs. Sensitive	0.4727	No	0.3908
Normal vs. Resistant	1.211	Yes	0.0042
Sensitive vs. Resistant	0.7381	Yes	0.0434

Based on the graphic (Figure 2), anti-TB resistant group have MAP1LC3B level lower than sensitive group. The highest mean value from highest to the lowest are the normal group (2.1486), sensitive group (1.6759), and resistant groups (0.9378).

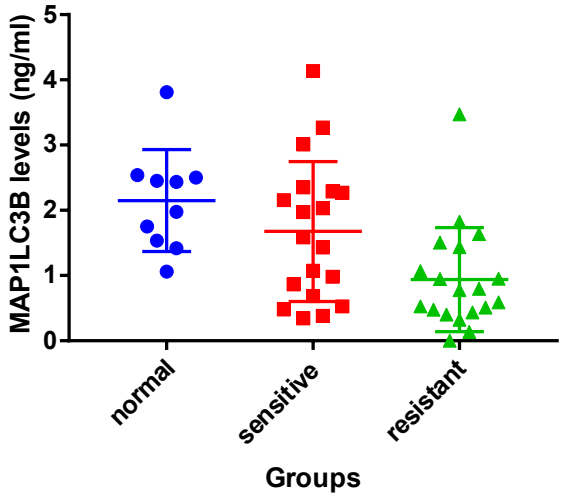

Figure 2. MAP1LC3B levels comparison for group (n normal=10; n sensitive=19; and n resistant=19) each

Macrophages are important fundamental for host defense system with phagocytic cells i.e neutrophil and monocyte which recognize and eradicate pathogenic bacteria. Pathogen are destroyed by macrophages directly or indirectly through the innate and adaptive immune system.[12] Macrophages are target for bacterial pathogens that also can give an advantage for bacteria to evade the immune system.[2] Phagocytosis is an ingestion of antigens that are large into membrane vacuole commonly known as the phagosome.[13] Autophagy is isolated cargo into the membrane with double structure commonly referred by autophagosome.[14] Induction of autophagy can be monitored by MAP1LC3B (LC3) formation.[14] To survive inside macrophages, intracellular bacteria develop a variety of strategies to avoid or fight the host defense system.[12] In this case, MTB has the ability to hold phagosome maturation.[15]

Autophagy can act as a tumor suppressor in normal cells based on the efficiency of non-apoptotic cell death from malignant cells and DNA damage by inhibiting ROS formation.[8] Antimicrobial activity and apoptosis of human macrophages can be triggered by cytosolic phospholipase

activity through MTB which catalyze the release of arachidonic acid. Arachidonic acid is product of a second messenger of TNF which induce apoptosis and oxygen radicals, which are produced during arachidonic acid lipoxygenation, thus inducing the production of reactive oxidative species and are involved in cell death.[16]

Bacteria that are resistant to drugs is a threat to human health. Resistant to antibiotics can be against two things: bacterial survival ability and the ability to reproduce in the presence of macrophages. When bacteria enter the macrophages, they will experience environmental stress such as nutritional restriction induced by the host, acidification, toxic peptides, osmotic stress, and reactive oxygen species (ROS), is later became the biggest cause the death of the bacteria.[17] To survive inside macrophages, MTB developed a variety of strategies to avoid or fight the host defense system.[12] One of the mechanisms of MTB to survive is manipulating the host cell death pathways in infected cells. One of the virulence factors are surface glycolipid PDIM (phthiocerol dimycocerosates).[18] Lipid is not directly genetically encoded and therefore is not amenable to traditional tagging methods, also cell wall lipids have multiple overlapping functions.[17] Multiple role functions from PDIM on pathogenesis has been investigated before, including the invasion of macrophages, masking of pathogen-associated molecular pattern (PAMPS), resistance to death with nitric oxide, and the prevention of the recruitment of active macrophages to infected area.[1] PDIM suppress recruitment of microbicidal, iNOS positive macrophages by inhibiting TLR signaling (Figure 3).[19] Interactions between host and bacterial cell wall are likely to be bidirectional and change when infection.[2] PDIM in vivo[18] abundance depend on expression of bisynthetic enzymes which decrease upon macrophage infection, shift metabolic flux which occur during host lipid catabolism[9] and insertion of molecule into host membranes[20]. There is variable amount of PDIM on MTB surface is at different time points after infection.[19]

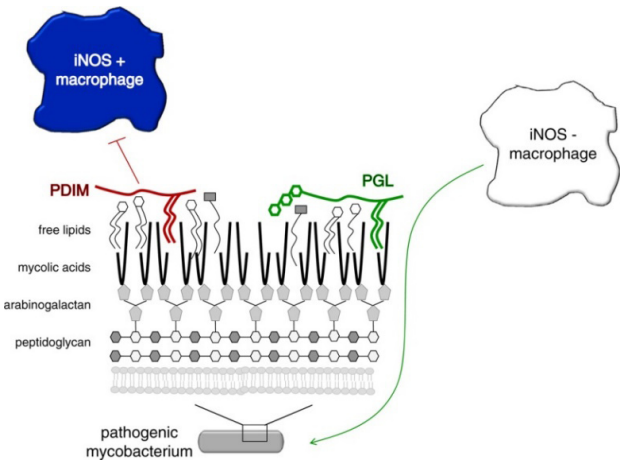

Figure 3. MTB cell wall lipids modulate macrophage composition at sites of infection[19]

MTB initiated human infections in distal lung, and reside in upper respiratory tract. TLR signalling stimulated by PAMPs from lung overrides PDIM and PGL-mediated immune evasion.[2] There is site named resistance-determining region (RRDR)[18] that caused by mutations in MTB strains at 81-bp region of rpoB. This mutations result is high levels of resistance to rifampicin.

According to Comas[21] all laboratory-generated mutans of MTB with rifampicin resistant mutations in the RRDR reduced fitness compared to their respond for drug ancestors when without rifampicin[13], MTB with RIF resistant caused by mutations in the rpoB gene, where the majority is on codon 531 and 526[11]. According to Kawamura mutation in codon 526 related to oxidative stress sensitivity.[22] In addition, some reports say that just one gene mutations in the rpoB encodes in sub-unit of RNA polymerase β can cause interaction between the RNA polymerase and some promoter also transcription regulation that trigger changes in phenotype.[17]

The mechanism of the rpoB gene mutation is caused by resistant rifampicin indicates that specific lead to mutations in the rpoB changes aspects of transcription. These transcription factors causing changes in gene expression which encodes the protein secretion, and proteomic changes produce some enzymes and lipid biosynthetic of intermediate in the path of phthiocerol dymycocerosate (PDIM). To prove PDIM plays role in induction of autophagy and necrosis on MTB, Quigley observed conversion of cytosolic LC3I to autophagosome-bound LC3II, using the expression of green fluorescent protein-LC3 (GFP) and flow cytometry.[18] As a result, autophagy was decreased in cells infected by MTB. PDIM plays role in induction of autophagy with decreasing autophagy on infected cells by MTB.[18]

Resistance to rifampicin caused by mutations in rpoB gene related with physiological and metabolic changes in bacterial systems.[3] These RIF resistant might be under dual selection in MTB, combined benefit and physiological advantage of rpoB gene can fix rpoB mutants to infect in MTB populations.

CONCLUSION

levels of MAP1LC3B on groups rifampicin resistant groups lower than on sensitive groups, that indicate no autophagy process or only few at macrophage on resistant groups than sensitive groups. This process occures because MTB successfully evade host defense by innate immune mechanisms. MTB can maintain intracellular growth inside the phagosome by inhibiting phagolysosome formation especially inhibiting MAP1LC3B formation by PDIM.

REFERENCES

1. Patel DM, Patel SD, Jaiswal PS, Brahmbhatt KJ. Drug Resistant Mycobacterium tuberculosis and New Drug Development. Int J Drug Dev Res. 2012;4(2):76–91.
2. Lory S, DeLong EF, Thompson F, Stackebrandt E, Lory S, DeLong EF, et al. The Prokaryotes-Human Microbiology. Fourth Edi. New York: Springer Heidelberg; 2013.
3. Koch A, Mizrahi V, Warner DF. The impact of drug resistance on Mycobacterium tuberculosis physiology: what can we learn from rifampicin? Emerg Microbes Infect. 2014 Mar 12;3(3):e17–e17.
4. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid Molecular Detection of Tuberculosis and Rifampin Resistance. N Engl J Med. 2010 Sep 9;363(11):1005–15.
5. Aziz MA, Wright A, Laszlo A, De Muynck A, Portaels F, Van Deun A, et al. Epidemiology of antituberculosis drug resistance (the Global Project on Anti-tuberculosis Drug Resistance Surveillance): an updated analysis. Lancet (London, England). 2006 Dec 16;368(9553):2142–54.
6. du Preez I, Loots DT. Altered fatty acid metabolism due to rifampicin-resistance conferring mutations in the rpoB Gene of Mycobacterium tuberculosis: mapping the potential of pharmaco-metabolomics for global health and personalized medicine. OMICS. 2012 Nov;16(11):596–603.
7. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJM. The competitive cost of antibiotic resistance in Mycobacterium tuberculosis. Science. 2006 Jun 30;312(5782):1944–6.
8. Patel AS, Morse D, Choi AMK. Regulation and functional significance of autophagy in respiratory cell biology and disease. Am J Respir Cell Mol Biol. 2013 Jan;48(1):1–9.
9. Goletti D, Petruccioli E, Romagnoli A, Piacentini M, Fimia GM. Autophagy in Mycobacterium tuberculosis infection: a passepartout to flush the intruder out? Cytokine Growth Factor Rev. 2013 Aug;24(4):335–43.
10. Rosenberger CM, Finlay BB. Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. Nat Rev Mol Cell Biol. 2003 May;4(5):385–96.
11. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000 Nov 1;19(21):5720–8.
12. Gong L, Devenish RJ, Prescott M. Autophagy as a macrophage response to bacterial infection. IUBMB Life. 2012 Sep;64(9):740–7.
13. Flannagan RS, Heit B, Heinrichs DE. Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of Staphylococcus aureus. Pathog (Basel, Switzerland). 2015 Nov 27;4(4):826–68.
14. Bento CF, Empadinhas N, Mendes V. Autophagy in the fight against tuberculosis. DNA Cell Biol. 2015 Apr;34(4):228–42.
15. Podinovskaia M, Lee W, Caldwell S, Russell DG. Infection of macrophages with Mycobacterium tuberculosis induces global modifications to phagosomal function. Cell Microbiol. 2013 Jun;15(6):843–59.
16. Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? Nat Rev Microbiol. 2010;8(9):668–74.
17. Miskinyte M, Gordo I. Increased Survival of Antibiotic-Resistant Escherichia coli inside Macrophages. Antimicrob Agents Chemother. 2013 Jan;57(1):189–95.
18. Quigley J, Hughitt VK, Velikovsky CA, Mariuzza RA, El-Sayed NM, Briken V. The Cell Wall Lipid PDIM Contributes to Phagosomal Escape and Host Cell Exit of Mycobacterium tuberculosis. MBio. 2017 Mar 7;8(2).
19. Siegrist MS, Bertozzi CR. Mycobacterial lipid logic. Cell Host Microbe. 2014 Jan 15;15(1):1–2.
20. Astarie-Dequeker C, Le Guyader L, Malaga W, Seaphanh F-K, Chalut C, Lopez A, et al. Phthiocerol Dimycocerosates of M. tuberculosis Participate in Macrophage Invasion by Inducing Changes in the Organization of Plasma Membrane Lipids. Flynn JL, editor. PLoS Pathog. 2009 Feb 6;5(2):e1000289.
21. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, et al. Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. Nat Genet. 2011 Dec 18;44(1):106–10.
22. Kawamura N, Kurokawa K, Ito T, Hamamoto H, Koyama H, Kaito C, et al. Participation of Rho-dependent transcription termination in oxidative stress sensitivity caused by an rpoB mutation. Genes Cells. 2005 May;10(5):477–87.