The role of Mycobacterium tuberculosis complex strain on apoptosis and necroptosis state of macrophages derived from active pulmonary tuberculosis patients

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Abstract

Objective The role of Mycobacterium tuberculosis complex (MTBC) strain in tuberculosis (TB) infection in human is still questioned. The aim of this study was to determine whether MTBC strain is associated with apoptosis and necroptosis by measuring the expression of specific signaling pathways components (Fas-associated protein with death domain (FADD) and receptor interacting protein 3 (RIP3)), as well as the level of apoptosis.

Results We recruited 24 TB patients infected with M. tuberculosis Beijing strain and six patients with M. bovis BCG strain. Data indicated that those who infected with M. tuberculosis were more frequent to have severe lung damage than M. bovis (odds ratio [OR]: 7.60; 95% confidence interval [CI]: 1.07-54.09). M. tuberculosis infection was also associated with lower expression of FADD and lower apoptosis level of macrophage cells compared to M. bovis . No significant different of RIP3 between strain groups. In conclusion, M. tuberculosis Beijing strain is associated with severe pulmonary damage, inhibits FADD expression and reduces apoptosis level in macrophages derived from TB. This suggests that MTBC strain potentially be used as determinant of progressivity of disease and tissue damage in TB cases.

Introduction

Mycobacterium tuberculosis complex (MTBC) continues to cause significant public health and is associated with one million deaths of tuberculosis (TB) cases annually worldwide [1]. The ability of M. tuberculosis to establish a productive infection is entirely depend on macrophage deaths during infection. Pulmonary macrophages are critical component of the primary innate immune response that have various functions such as immune surveillances, removal of cellular debris, microbial clearance, and resolution of inflammation [2]. There are two option pathways of macrophage deaths, apoptosis and necroptosis, that are developed as host antimicrobial defenses in early infection of TB; both of them are programmed cell death [3]. These macrophage death mechanisms are triggered by tumor necrosis factor alpha (TNFα), oxidative stress, and lipopolysaccharide (LPS), and other factors [4]. Apoptosis is characterized by signaling cell through Fas-associated protein with death domain (FADD) that is a crucial protein associated with death receptors (DRs) [5]. Necroptosis can be induced

only if apoptotic signaling is inhibited through formation of receptor interacting protein 3 (RIP3) [6, 7]. MTBC comprises of many members including M. tuberculosis, M. africanum, M. canettii, M. bovis, M. microti, M. orygis, M. caprae, M. pinnipedii, M. suricattae and M. mungi) [8]. These strains have different cellular components, the ability of human-to-human transmission and severity of disease [9]. The role of MTBC strains have been proven in various TB animal models [10], but still be questioned in TB infection in human [9]. Although some strains have 99.9% similarity of nucleotide sequences, they have different abilities to induce macrophage cell death [11]. M tuberculosis strain with high virulent inhibits apoptosis, and triggers necroptosis because the microorganism evades the immune system, induces necrosis, lysis of cellular component, and induces parenchymal destruction and therefore is associated with severe TB [12]. The aim of this study was to assess the role MTBC strain on the state of apoptotic and necroptosis of macrophages isolated from TB patients.

Method

Study setting and patients

Between June and October 2017, a cross-sectional study was conducted. Confirmed new pulmonary TB cases were recruited from Tuberculosis Clinic at Soewandhie Hospital, Surabaya, Indonesia. Bacteriological confirmation was conducted by sputum acid fast staining and RIF Xpert gene (Cepheid, Sunnyvale, CA, USA). Patients underwent fiber optic bronchoscopy to collect bronchoalveolar lavage fluid (BALF). Macrophages were collected from BALF. Patients with HIV positive, diabetes mellitus, renal abnormality, heart diseases, immune response disorders such as lupus erythematosus and rheumatoid arthritis, non-TB pulmonary diseases, and those who previously received anti-TB treatment were excluded. All samples were tested to identify MTBC strain using polymerase change reaction (PCR) targeting two specific genes: RD9 and TbD1.

Assessment of pulmonary damage

The degree of pulmonary damage was classified using the NICE Scoring System based on the total lesions in six lung areas [13]. This scoring system assessed four components: nodule (N), infiltration or consolidation (I), cavity (C) and ectasis (E) based on chest radiograph of three areas of each lung (i.e. six areas of both lungs). For each area, the score was 1 to 4 indicating lung damage area of 0–

25%, >25%–≤50%, >50%–≤75% and >75%, respectively. The pulmonary damage was categorized as mild if the total score was 8 or less and severe if the total score was more than 8.

Samples collection and macrophages isolation

BAL was performed using 10 ml of saline solution as described previously [14]. The BALF was centrifuged at 2500rpm for 15 mins then supernatant was discarded and cells were resuspended to a cell count of $4x10^5$ cells/ml with RPMI 1640 medium. The total cell count was measured using hemocytometer.

FADD and RIP3 expression by immunocytochemical staining

Pellet cells derived from the centrifugation were applied to glass slides and washed with PBS three times for 10 mins. Permeabilization was performed with a CA-630-0.5% Igepal solution (Sigma Aldrich, Saint Louis, MO, USA). H2O2 0.3% was then added and incubated for 10 mins before was washed with PBS. The slides were incubated with anti-human monoclonal antibody FADD or RIP3 followed manufacturer's protocol (Santa Cruz, Oregon, OR, USA). The quantification of the protein expression was conducted according to previous study [15].

Apoptosis assay

The level of apoptosis in infected macrophages was determined by using the Tunel Assay apoptosis kit per manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Tunel assay was performed with terminal deoxynucleotidyl transferase enzymes to determine the fragmentation of DNA. The level of apoptosis was measured based on previous study [16].

Strain identification and confirmation sequencing

The detection of MTBC strain was conducted from BALF. Briefly, DNA was extracted using DNeasy® Blood & Tissue kit (Ambion Inc., Austin, TX, USA). Amplification of gene-specific *M. tuberculosis* strain was conducted using RD9 primers (F: 5'-GTGTAGGTCAGCCCCATCC-3', I: 5-CAATGTTTGTTGCGCTGC-3', R: 5'-GCTACCCTCGACCAAGTGTT-3'), while *M. bovis* strain was identified using TbD1 primers (F: 5'-AGTGACTGGCCTGGTCAAAC-3', R: 5'-GAGCTCTGTGCGACGTTATG-3') [17,18]. The conditions for PCR assays were set up for 30s at 94˚C (denaturation), followed by 35 cycles of denaturation (94˚C, 30s), annealing (56˚C, 1s), and extension (72˚C, 10 min). Confirmation of the strain was conducted by

sequencing nine and two of *M. tuberculosis* and *M. bovis* samples, respectively and homology analysis was conducted using Basic Local Alignment Search Tool (BLAST).

Statistical Analysis

Association between MTBC strain and degree of lung damage including each subset of NICE component were assessed using chi-squared test. To compare the level of apoptosis, FADD, and RIP3 of macrophages between *M. tuberculosis* and *M. bovis* strain groups, Man-Whitney test was employed. For all analyses, significance was assessed at $\alpha=0.05$.

Results

Characteristics of patients

We enrolled 30 new cases of pulmonary TB and majority of the patients (81.37%) were female and more than half (16/30, 53.3%) of the patient aged between 21-40 years old (Table 1). Majority of the patients (75%) were working as laborer and five patients (16.6%) were working as cow slaughters. Based on clinical symptoms, 90%, 86%, 56% and of the patients had anorexia, experienced weight loss and had persistent fever, respectively. Only 36.6% of patients had low hemoglobin level and 30.0% had low oxygen saturation.

Detection of MTBC strains

Based on RD9 gene amplification, 24 (80.0%) *M. tuberculosis* strains were identified and nine of amplified RD9 gene were sequenced for confirmation. The isolates had 99-100% sequence similarity with the *M. tuberculosis* Beijing strain 2014 PNGD (Accession no CP022704.2). Six (20.0%) *M. bovis* strains were identified and two isolates were sequenced for confirmation. All of them had 100% sequence similarity with *M. bovis* BCG strain Tokyo (Accession no CP033311.1).

Association between MTBC strain and lung damage

MTBC strains had no association with three NICE components (i.e. the presence of nodule, infiltrate or consolidation, and cavitas of the lungs) (Table 2). Ectasis, however, was more frequent in TB cases infected with *M. tuberculosis* Beijing strain (OR: 10.0; 95%CI: 1.34-74.51). *M. tuberculosis* Beijing strain was identified in 19 (90.50%) patients with severe lung damage. There was a significant association between *M. tuberculosis* Beijing strain and severe lung tissue damage, OR: 7.60; 95%CI:

1.07-54.09, p=0.028 (Table 2).

Association between MTBC strain and FADD, RIP3 and apoptosis

Our data indicated that the level of FADD was lower in *M tuberculosis* Beijing strain group compared to *M. bovis* strain group, 0.208±1.020 vs 0.667±1.032 cells with p=0.046 (Table 3). There was no significant different the level of RIP3 expression between *M tuberculosis* group and *M. bovis.* Data from Tunel assay indicated that the level of apoptosis in macrophages derived from *M tuberculosis* group was significantly lower compared to *M. bovis* group, 0.875±1.676 *vs* 2.500±3.331, p=0.049.

Discussion

The outcome of MTBC infection and the disease progression are varied; exposure of MTBC can be followed by a rapid clearance by innate immunity or direct progression to active TB disease. Active TB disease also has a range of presentations and each form is associated with diverse host responses to the pathogen. Studies have provided evidence that different strains of MTBC are associated with different virulent [19–21] and MTBC strains affect host-pathogen interactions [22]. Phenotypic comparisons between M. tuberculosis and M. bovis have been limited to animal studies, which suggested that M. bovis may have less virulent [9, 23, 24]. In the present study, 80.0% of TB cases caused by M. tuberculosis Beijing strain and the stain inhibited signaling cell to apoptosis execution. Previous studies have reported that M. tuberculosis with a high virulence inhibited apoptosis in TB-cases [25, 26]. M. tuberculosis strain H37Rv and Erdman for example inhibited apoptosis compared to non-virulent M. bovis BCG strain, H37Ra, and M. kansaii on human alveolar macrophages from BALF of healthy nonsmoking volunteers [26]. Other studies found that M. tuberculosis inhibited and suppressed apoptosis of host macrophages on THP-1 [27, 28] and J774 cell lines [29].

Data from the present study identified that infection of macrophages with M. tuberculosis Beijing strain was associated with a lower level of FADD compared to infection with M. bovis strain. FADD is an adapter protein to bind caspase 8 and caspase 10 precursors and is simultaneously activated and mediated cell signals with caspases 3, 6, and 7 to induce apoptosis [30]. This suggests that Beijing strain is able to inhibit signaling of caspases to execute the apoptosis cell death. Furthermore, low

FADD expression triggers necrosis [31] and necroptosis [32]. Altogether, these explain, in part, the results of present study that infection with M. tuberculosis is significantly associated with severe lung damage.

In conclusion, our preliminary data suggest that M. tuberculosis Beijing strain is associated with more severe lung damage and compared to M. bovis infection. This strain also inhibits FADD expression and reduces apoptosis level.

Study limitation

This study was conducted at a single center and included relatively small number of samples and therefore it might not a representative of current TB condition in Indonesia.

Abbreviations

BALF: bronchoalveolar lavage fluid; CI: confidence interval; DRs: death receptors; FADD: Fas-associated protein with death domain; LPS: lipopolysaccharide; MTBC: *Mycobacterium tuberculosis* complex; OR: odds ratio; PCR: polymerase chance reaction; RIP3: receptor interacting protein 3; TB: tuberculosis; TNFα: tumor necrosis factor alpha.

Declarations

Author contributions

Conceptualization and methodology: BY; Software: MA; Validation: BY, MM; Formal analysis: NMM; Data curation: BY, MA; Writing – original draft preparation: BY, HH; Writing – review & editing: BY, BY, MM, MA, HH, NMM, SS; Supervision: MA, NMM, SS.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All penitents signed an informed consent form prior to study. This study protocol was approved by the Ethics Committee of Dr. Soetomo Hospital Research Committee (388/PANKE/KKE/V/2017).

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Tables

Table 1 Demographic and clinical characteristics between *M. tuberculosis* Beijing strain and *M. bovis* BCG strain

Variable	MTBC strain	
	M. tuberculosis Beijing strain, n (%)	*M. bovis* BCG strain n (%)
Gender		
Female	13 (81.37)	
Male	11 (78.6)	
Age (year)		
<21	2 (50.0)	
21-40	12 (75.0)	
40-50	6 (100.0)	
>50	4 (100.0)	
Educational attainment		
Elementary school	8 (89.5)	
Junior high school	10 (83.3)	
Senior high school	6 (66.7)	
Occupation		
Labourer	16 (76.2)	
Housewife	6 (100.0)	
Unemployed	2 (66.7)	
Anorexia		
Yes	21 (77.7)	
No	3 (100.0)	
Weight loss		
Yes	21 (80.8)	
No	3 (75.0)	
Fever		
Yes	13 (76.5)	
No	11 (84.6)	
Haemoglobin level		
Normal	8 (72.8)	
Low	16 (84.2)	
SaO_2 level		
Normal	17 (80.9)	
Low	7 (77.8)	

Table 2. Severity of pulmonary damage between *M. tuberculosis* Beijing strain and *M. bovis* BCG strain

Variables	*n*	MTBC strain		OR	95%CI
		*M. tuberculosis*Beijing strain, n (%)	*M. bovis* BCG strain, n (%)		
NICE Score					
Nodule				4.85	0.72-32.87
Yes	19	17 (89.5)	2 (10.5)		
No	11	7 (63.7)	4 (36.4)		
Infiltrate/consolidation				NA	NA
Yes	30	24 (80.0)	6 (20.0)		
No	0	0 (0.0)	0 (0.0)		
Cavitas				NA	NA
Yes	4	4 (100.0)	0 (0.0)		
No	26	20 (76.9)	6 (23.1)		
Ectasis				10.00	1.34-74.51
Yes	22	20 (90.0)	2 (9.15)		
No	8	4 (50.0)	4 (50.0)		
Severity of lung damage				7.60	1.07-54.09
Mild	9	5 (9.5)	4 (55.6)		
Severe	21	19 (90.5)	2 (44.4)		

Table 3. Level of FADD, RIP3 and apoptosis in macrophages between *M. tuberculosis* Beijing strain and *M. bovis* BCG strain

Variable	MTBC strain	
	M. tuberculosis Beijing strain (n=24)	*M. bovis* BCG strain (n=6)
FADD	0.208±1.020	0.667±1.032
RIP3	0.333±0.702	0.500±0.836
Apoptosis	0.875±1.676	2.500±3.331