Asian Biomedicine Vol. 5 No. 6 December 2011; 747-754 DOI: 10.5372/1905-7415.0506.101

**Original article**

# Expression of M-ficolin, H-ficolin, and L-ficolin in peripheral blood mononuclear cell of tuberculosis patients

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***Background:*** Ficolins are lectins that have been demonstrated to play an important role in innate immune response in a variety of diseases. Mycobacterium tuberculosis (M.tb) infection can trigger a series of changes in the host. However, the role of ficolin in tuberculosis is still unclear.
***Objective:*** We investigated the expression of ficolin in peripheral blood mononuclear cell (PBMC) in TB patients and healthy control.
***Methods:*** Using semi-quantitative RT-PCR, western blotting, and flow cytometry, we compared the expression of M-ficolin, L-ficolin, and H-ficolin in the peripheral blood mononuclear cell (PBMC), purified monocytes, and cultured dendritic cells of TB patients with healthy volunteers as controls.
***Results:*** M-ficolin expression in PBMC was significantly lower at both mRNA and protein levels in TB patients as compared to healthy controls. The lower M-ficolin level in TB patient PBMCs may be attributed to its lower level in monocytes. The expression levels of H-ficolin and L-ficolin in both healthy controls and TB patients were very low and they had no significant differences between the two groups.
***Conclusions:*** Compared to healthy controls, M-ficolin expression is significantly lower in TB patients. Measurement of M-ficolin may be a potential auxiliary tool to diagnose TB infection.

***Keywords:*** Ficolin, monocyte, mycobacterium tuberculosis, peripheral blood mononuclear cell

Tuberculosis (TB) is a disease caused by Mycobacterium tuberculosis (M.tb). It remains one of the leading causes of death worldwide with eight million new cases and more than 1.5 million deaths each year [1, 2]. The global burden of TB has risen dramatically in the past ten years due to co-infection of HIV [3, 4], raising the urgency of developing new approaches for TB diagnosis and treatment.

Ficolins are a group of proteins that contain fibrinogen- and collagen-like domains and recognize the sugar structures of the pathogenic microorganism. They activate lectin complement pathway through a mechanism mediated by mannose-binding lectin (MBL)-associated serine proteases (MASPs) [5, 6]. Ficolin was originally identified as transforming growth factor-beta 1 binding proteins on porcine uterus membranes [7, 8]. There are three types of ficolins in human: M-ficolin, H-ficolin, and L-ficolin. All of the three ficolins have been shown to activate complement via binding to MASPs [9, 10]. M-ficolin, a hexamer of trimers is expressed mainly by monocytes and type II alveolar epithelial cells [11-13]. Recent studies demonstrated that M-ficolin could be detected in serum [14, 15]. H-ficolin is also called Hakata Ag and was first identified in serum as an antigen that is recognized by autoantibody in patients with lupus erythematosus [16]. L-ficolin/p35, an opsonic lectin of the ficolin family, is a multimeric protein consisting of 35kDa subunits [17, 18]. Studies have indicated that all three human ficolins have the lectin activity for GlcNAc and play an important role in innate immunity [19-21].

In the present study, we examined the expression of three types of ficolins in PBMC between TB

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patients and healthy controls using several different methods. To our knowledge, this is the first study to examine the expression of M-ficolin, H-ficolin, and L-ficolin in TB patients. Our data provide a new insight into the potential value of M-ficolin in the diagnosis of tuberculosis.

## Materials and methods

### *TB patients and healthy controls*

Between 2007 and 2009, 121 first-time diagnosed TB patients (78 males and 43 females with a median age of 46.2 years) were enrolled from two hospitals, Wuhan Disease Control and Prevention Center and Wuhan TB Hospital. All patients were confirmed to have M.tb infection through sputum culture. One hundred forty six healthy controls (92 males and 54 females with a median age of 43.8 years) were also recruited from those who received annual physical examination during the same period and hospitals. Written informed consent was obtained from all subjects. This study was approved by Wuhan University Ethics Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### *Peripheral blood mononuclear cells (PBMC) preparation*

Peripheral venous blood from TB patients and healthy controls were collected in a heparinized tube. Following removal of plasma, the mononuclear cells (PBMC) were further enriched using Lymphoprep with a density of 1.077 g/ml (Sigma, Phoenix, USA). The cells mainly consisting of monocytes and lymphocytes in the interface layer were collected, washed three times with phosphate buffer salt solution (PBS), and counted. The cells were used for RNA extraction, western blotting, and flow cytometric analysis.

### *Monocytes and dendritic cells (DC) preparation*

$CD14^+$ cells were purified from the PBMC of TB patients and healthy controls using the Human Monocytes Enrichment Set-DM and the BD IMag□ (BD Biosciences Pharmingen, CA, USA) via negative selection, following the recommended assay procedure of the BD IMag□. The sorted cells were assessed using flow cytometry analysis. Dendritic cells were prepared from blood monocytes using the culture method as previously reported [22]. Briefly, monocytes were cultured in DC medium (RPMI +10% FCS) supplemented with 800 U/ml GM-CSF and 1000 U/ml IL-4 (Sigma, Phoenix, USA). Fresh DC-medium was added to culture on day 4. Cells were harvested and analyzed on day 7.

### *Extraction of total RNA and semi-quantitative RT-PCR*

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, USA) following the user's manual. First-strand cDNA template was synthesized through Super-ScriptII reverse transcriptase (Life Technologies, Carlsbad, CA, USA) with random hexamers. The mRNA expression of ficolins in the PBMC, purified monocytes or DC was analyzed by semi-quantitative RT-PCR (120 TB patients and 146 healthy controls). Serial dilutions of the cDNA templates were used for PCR amplification using specific primer sets of M-ficolin, H-ficolin, L-ficolin, and GAPDH. The primer sequences and PCR thermal conditions are listed in **Table 1**. PCR products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining.

### *Western blot analysis*

The PBMC, monocytes, and DC pellets were lysed on ice for 30 min in a lysis buffer containing fresh protease inhibitors (10 g/mL aprotin, 5 g/mL leupeptin, 1g/mL pepstatin, 0.1 mol/L microcystin, 5 mmol/L phenylmethylsulfonyl fluoride, and 5 mmol/L sodium pyrophosphate). The lysates were then centrifuged (14000 g at 4°C for 15 min). Protein concentrations were determined by protein assay (Bio-Rad, Hercules, CA, USA). Equal volumes of the three proteins were denatured in protein loading buffer, separated on 12% SDS PAG gels, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA ) by electroblotting. The PVDF membranes were blotted with 5% nonfat milk in TBS (Bio-Rad, Richmond, CA, USA) for one hour and incubated overnight at 4°C with antibodies against Human M-ficolin (Santa Cruz Biotechnology, CA, USA), H-ficolin (Santa Cruz Biotechnology, CA, USA), L-ficolin (Hycult Biotechnology, B.V. Uden, The Netherlands), and β-actin (Santa Cruz Biotechnology, CA, USA). Signals were detected using ECL detection kit (Perkin-Elmer Life Sciences, Boston, USA) following the manufacturer's instructions. Band intensity was measured by densitometry using Quantity One software (Bio-Rad, Hercules, CA, USA). Protein levels were normalized with the corresponding β-actin protein

by calculating the ratio between each band of interest and loading control band (β-actin).

### *Flow cytometry analysis*

Phycoerythrin (PE)-conjugated anti-CD14 antibody was purchased from eBioscience (San Diego, CA, USA). FITC-conjugated anti-M-ficolin and anti-L-ficolin mAbs and IgG2a isotype control were purchased from PharMingen (San Diego, CA, USA). To analyze the sorted monocytes, cells were incubated with PE-labeled anti-CD14 antibody or matched isotype IgG in PBS containing 2% BSA and 0.1% sodium azide for 30 minutes. After washing twice with the staining buffer, the cells were analyzed using flow cytometry (Becton Dickinson, Brea, CA USA). For the expression of M-ficolin, L-ficolin on monocytes and DCs, the sorted monocytes and cultured DCs were first harvested and counted, and then incubated with anti-M-ficolin or anti-L-ficolin mAbs respectively, and matched isotype antibody at 4□C for 30 minutes. After being washed three times, the cells were subsequently incubated with the FITC-IgG at 37°C for 30 minutes. The resultant cells were analyzed with flow cytometry (Becton Dickinson, Brea, CA USA).

### *Statistical analysis*

Statistical analysis was performed using GraphPad Prism 5 program (GraphPad Software, Inc. La Jolla, CA USA). Mann-Whitney's U-test and Kruskal-Wallis test were used for the comparisons between groups. Data were expressed as the median□range. The differences were regarded significant if p is less than 0.05.

## Results

### *The mRNA expression of M-ficolin, H-ficolin, and L-ficolin in PBMC of TB patients and healthy controls*

To determine whether the expressions of M-ficolin, H-ficolin, and L-ficolin were correlated with tuberculosis, semi-quantitative RT-PCR was performed to examine the mRNA expression of M-ficolin, H-ficolin, and L-ficolin transcripts in PBMC of TB patients and healthy controls. As shown in **Figure 1**, M-ficolin mRNA expression in PBMC was

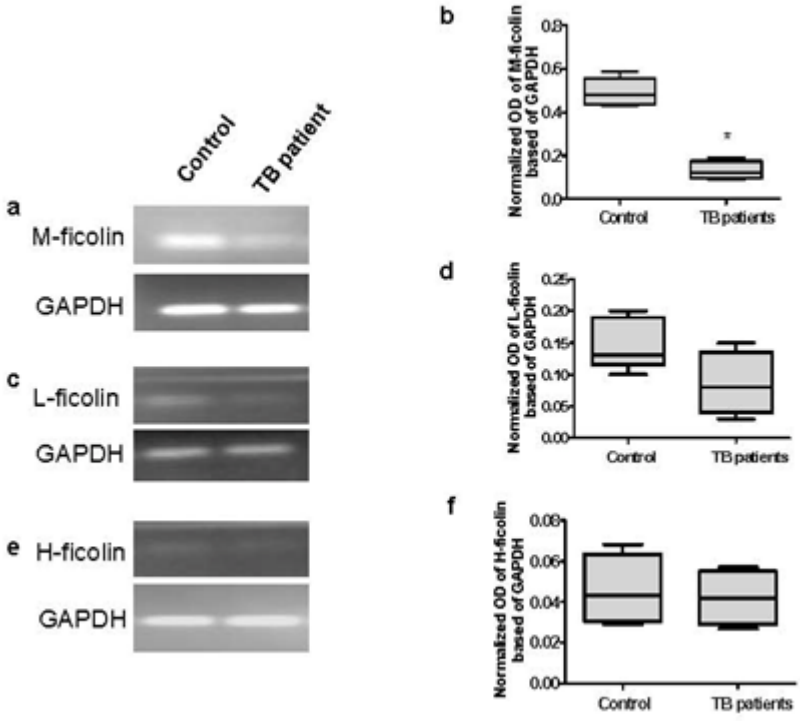


**Figure 1.** Semi-quantitative RT-PCR measurement for mRNA expression of M-ficolin, H-ficolin, and L-ficolin in the PBMC of TB patients and healthy controls**.** Using GAPDH as a reference, the relative expression levels of three types of ficolins mRNA expression were calculated and compared between TB patients and healthy controls. The significant difference of mRNA expression was detected for M-ficolin, but not H-ficolin or L-ficolin (*$p$ <0.05). Data were expressed as the median□range, and the whiskers of the box-plot represent the maximal and minimal values.

significantly lower in TB patients than in healthy controls (1a and 1b, *p<0.05). L-ficolin mRNA was also lower in the TB patients than healthy control, but the difference was not statistically significant (**Figure 1c** and **d**). H-ficolin had very low expression in both TB patients and healthy controls and had no significant difference (**Figure 1e** and **f**).

### *The protein expression of M-ficolin, H-ficolin, and L-ficolin in the PBMC of TB patients and healthy controls*

To confirm the expression of M-ficolin, H-ficolin, and L-ficolin at the protein level, we examined their protein using western blot. In accordance with the mRAN expression results, M-ficolin protein expression in the PBMC was significantly lower in TB patients than in healthy controls (**Figure 2a** and **b**). The H-ficolin and L-ficolin proteins were also consistent with their mRAN expression results obtained through the semi-quantitative RT-PCR (**Figure 2c** and **f**).

### *Western blot and flow cytometry analysis of M-ficolin and L-ficolin expression in monocytes and DCs*

After detecting of the differences of M-ficolin expression in PBMC between TB patients and controls, we further examine M-ficolin and L-ficolin expression in purified monocytes and cultured DCs using western blot and flow cytometry. As shown in **Figure 3**, the significant difference of M-ficolin protein level between TB patients and controls was observed in the monocytes but not in the DCs. As indicated by the flow cytometry results (**Figure 4**), the similar results were detected at the single cell level as well. In addition, both western blot and flow cytometry analysis consistently showed that L-ficolin was barely detectable in either monocytes or DCs, and its expression was not significantly different between TB patients and controls. These results collectively suggested that the differences of M-ficolin of PBMC between TB patients and controls might be attributed to the significant lower expression of M-ficolin in the monocytes of TB patients.

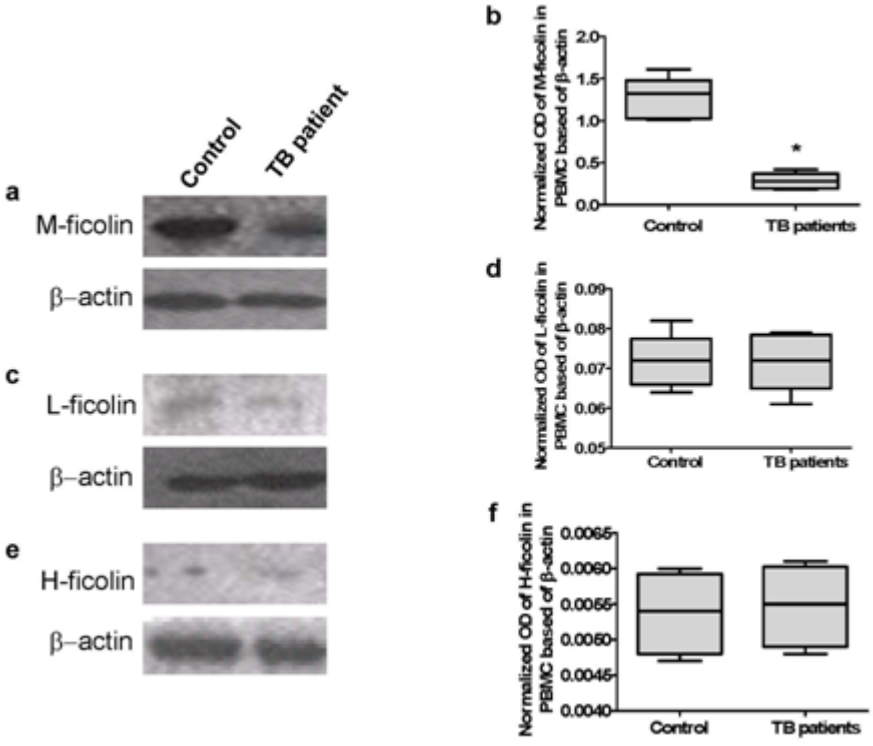


**Figure 2.** Western blot for protein expression of M-ficolin, H-ficolin, and L-ficolin in the PBMC of healthy controls and TB patients**.** The relative protein expression were quantitated by measuring the band density of three ficolins and normalized with respective □-actin (*$p$ <0.05). Data were expressed as the median□range, and the whiskers of the box-plot represent the maximal and minimal values.

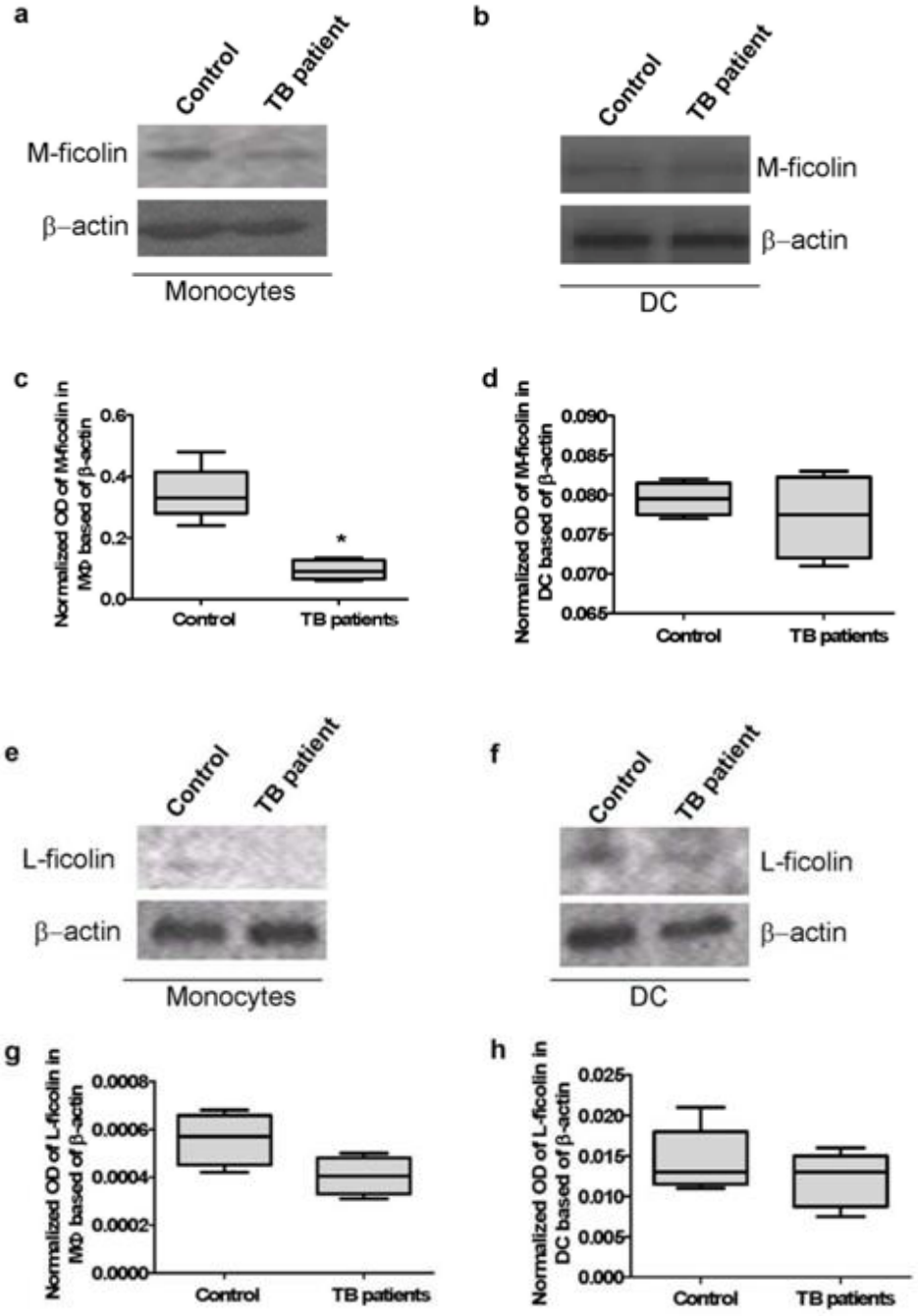


**Figure 3.** Western blot for protein expression of M-ficolin and L-ficolin in the purified monocytes and cultured DCs. Lysates of was prepared from purified monocytes and cultured DCs and subjected to western blot. The relative protein expression was quantitated by measuring the band density of three ficolins and normalized with respective □-actin (*$p$ <0.05). Data were expressed as the median□range, and the whiskers of the box-plot represent the maximal and minimal values.

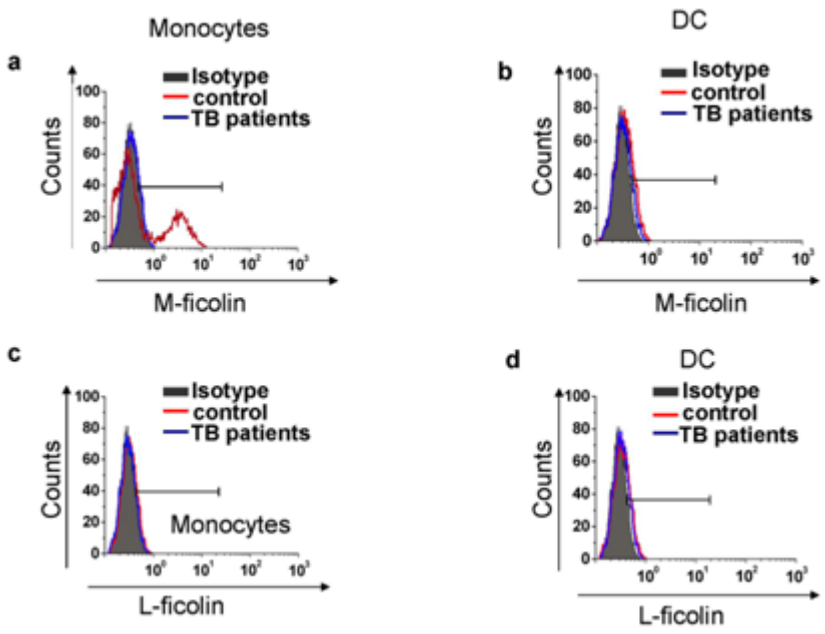


**Figure 4.** Flow cytometry analysis for M-ficolin and L-ficolin in monocytes and DCs. Purified monocytes and cultured DCs were prepared, stained with anti-M-ficolin and anti-L-ficolin antibodies, and analyzed with flow cytometry. Representative data from three different experiments are shown.

## Discussion

Currently, about one-third of the world's population is infected with M.tb. WHO reported an estimated incident of 10 million and 13 million prevalent TB cases in 2008. In addition, about two million people die from TB each year [3]. Animal models and clinical studies have shown that the elimination of M.tb infection mainly depends on the immune responses mediated by macrophages and T lymphocytes. Specifically, macrophages present mycobacterial antigens to na□ve $CD4^+$ T cells and induce the development of Th1 cells. Th1 cells produce interferon gamma, which in turn further activates macrophage and promotes phagocytosis and antigen presentation [24, 25]. It also has been shown that $CD8^+$ T cells are also involved in the elimination M.tb by secreting and interferon gamma as well as injecting granzyme B into infected cells [26-28]. As antigen presenting cells, monocyte-derived macrophages and dendritic cells not only bridge innate immune response to the adaptive immune response, but also the major cells that clear and quarantine M.tb [29, 30].

Certain lectins are important innate immune molecules that activate complement system [31]. MBLs and ficolins are two kinds of complement lectins recognizing microbial carbohydrates and subsequently activate lectin complement pathways [32, 33]. Three types of ficolins (M-ficolin/ficolin1, L-ficolin/ficolin2, and H-ficolin/ficolin3) have been identified in humans. M-ficolin binds to several kinds of acetylated structures expressed in monocytes and alveolar epithelial cells. It has been thought that M-ficolin might play a role in innate immunity through acting as a phagocytic receptor. In addition, one recent study revealed that M-ficolin decreased with the maturation of macrophages. Furthermore, it may contribute to the defense against infective diseases [11]. It has been demonstrated that L-ficolin plays a role in fighting against bacterial and fungal infection. L-ficolin binds to lipopolysaccharide (LPS) on gram-negative and lipoteichoic acid (LTA) on gram-positive bacteria, activates lectin complement pathway, and ultimately opsonize the bacteria to macrophage [34, 35]. H-ficolin binds to the surface of the microorganism and mediates the same functions of M-ficolin and L-ficolin. Thus far, the expression state of ficolins in M.tb infection has not been elucidated.

To our knowledge, the current study was for the first time to compare the expression of M-ficolin, H-ficolin, and L-ficolin in the PBMC between TB patients and healthy controls. A significant lower level of in M-ficolin was detected in the TB patient PBMC and monocytes at both mRNA and protein levels. In contrast, the expression of L- ficolin and H-ficolin in

PBMC, monocytes and DCs was very low and their levels was not different between TB patients and healthy controls, which was consistent with Hummelshoj T, et al's study [36].

In summary, TB patients showed a significant lower level of M-ficolin expression in PBMCs and monocytes. Although it remains to be investigated, the mechanisms underlying this change of M-ficolin in TB patients, measurement of M-ficolin may be a potential auxiliary tool to diagnose TB infection.

## Acknowledgement

The authors have no conflict of interest to declare. Dansheng Lei, Guanhui Zhang, and Jian Fan contributed equally to this work.

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