Decreased phagocytic capacity accompanied by autophagy activation in blood monocytes of tuberculosis patients

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July 20, 2020

Abstract

Developing host-directed therapies against resistant tuberculosis requires a better understanding of the changes in the innate immune response of the peripheral blood monocytes. Here, we investigated the phagocytic capacity of blood phagocytes, the changing of the mammalian target of rapamycin (mTOR) pathway and autophagy process of circulating monocytes in untreated tuberculosis patients. Phagocytic capacity of blood phagocytes and the expression of key regulators of the mTOR pathway were analysed using flow cytometry. We detected the mRNA and protein expression of autophagy proteins using RT-PCR and capillary western blotting. Compared with healthy controls, the increase of monocytes phagocytizing E.coli was lower in tuberculosis patients after 37°C activation (15.46% vs. 23.31%); The percentages of Rheb+ and mTOR+ Raptor+ circulating monocytes were higher, while that of AMPK+ monocytes were lower. Although ATG5 and ATG12 mRNA expression increased, the protein complex expression was decreased in the monocytes of tuberculosis patients. Beclin-1 and ULK1 Ser 757 levels were increased at both transcriptional and protein levels; LC3 II protein level also was higher. Our current study suggests a decrease in the phagocytic capacity of circulating monocytes, accompanied by autophagy activation in active tuberculosis patients.

Decreased phagocytic capacity accompanied by autophagy activation in blood monocytes of tuberculosis patients

Short Title: Phagocytose and autophagy in monocytes

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Keywords. monocytes; tuberculosis; phagocytic capacity; mTOR signalling pathway; autophagy

Abbreviations: TB: tuberculosis; HDTs: Host-directed therapies; NK cells: natural killer cells; AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; mTORC1: mammalian target of

rapamycin complex 1; TSC2: tuberous sclerosis complex 2; Akt: protein kinase B; ULK1: UNC-51-like kinase 1; RT-PCR: reverse transcription-polymerase chain reaction; CT: computed tomography.

Summary

Developing host-directed therapies against resistant tuberculosis requires a better understanding of the changes in the innate immune response of the peripheral blood monocytes. Here, we investigated the phagocytic capacity of blood phagocytes, the changing of the mammalian target of rapamycin (mTOR) pathway and autophagy process of circulating monocytes in untreated tuberculosis patients. Phagocytic capacity of blood phagocytes and the expression of key regulators of the mTOR pathway were analysed using flow cytometry. We detected the mRNA and protein expression of autophagy proteins using RT-PCR and capillary western blotting. Compared with healthy controls, the increase of monocytes phagocytizing E.coli was lower in tuberculosis patients after 37°C activation (15.46% vs. 23.31%); The percentages of $Rheb^+$ and $mTOR^+Raptor^+$ circulating monocytes were higher, while that of $AMPK^+$ monocytes were lower. Although ATG5 and ATG12 mRNA expression increased, the protein complex expression was decreased in the monocytes of tuberculosis patients. Beclin-1 and ULK1 Ser 757 levels were increased at both transcriptional and protein levels; LC3 II protein level also was higher. Our current study suggests a decrease in the phagocytic capacity of circulating monocytes, accompanied by autophagy activation in active tuberculosis patients.

Introduction

Tuberculosis (TB), a disease caused by Mycobacterium tuberculosis, is a serious global public health issue. As of 2018, approximately 1.7 billion people have been latently infected with M. tuberculosis, with the number of rifampicin-resistant TB patients reaching 484000, globally [1]. TB resistance is a major hurdle in the treatment of TB. Host-directed therapies (HDTs) aim to enhance the immune response against *M. tuberculosis* infection and could be a potential solution to solve the problem of resistance [2]. Understanding the details of the host–pathogen interaction is necessary for the possible formulation of HDTs.

When an *M. tuberculosis* containing aerosol enters the body, the innate immune system is activated and initiates a series of measures against the pathogen, including phagocytosis and autophagy. Phagocytosis plays a key role in the innate immune defence mechanism, which is the primary defence system against invading microorganisms [3]. A variety of phagocytes are present in the peripheral blood, including monocytes, neutrophils, natural killer (NK) cells, and eosinophils, which are recruited to the sites of pathogen infection and are involved in the subsequent phagocytosis of the bacteria [3-5]. However, the change in the phagocytic capacity of blood phagocytes after *M. tuberculosis*infection is unknown. AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) molecules are essential in energy metabolism in eukaryotic cells, which regulate the anabolic and catabolic pathways, including autophagy [6, 7]. Several authors have reported the mechanisms for mutual regulation between the AMPK and mTOR signalling pathways. AMPK inhibits the mTOR signalling pathway, likely in two ways. The first is by direct phosphorylation of raptor, the major component of mammalian target of rapamycin complex 1 (mTORC1) [8]. The second is by phosphorylation of tuberous sclerosis complex 2 (TSC2), which inhibits the activation of mTORC1 through Rheb [9, 10]. Moreover, protein kinase B (Akt) activates mTORC1 through the phosphorylation of TSC2 [11, 12]. Autophagy, a complex mechanism by which cytoplasmic cargo is transported to lysosomes for degradation, plays a vital role in fighting *M. tuberculosis* infection [13]. Under nutrient-rich or starvation conditions in vitro, AMPK and mTORC1 signalling can affect autophagosome formation by inhibiting or activating the phosphorylation of UNC-51-like kinase 1 complex (ULK1) [14, 15]. After *M. tuberculosis* is phagocytosed, xenophagy (a type of selective autophagy that is a host defence mechanism) targets the elimination of *M. tuberculosis* and is triggered by the early secretory antigenic target (ESAT)-6 secretion system (ESX-1), which destroys the phagosome membrane [16-18]. Subsequently, autophagy-associated proteins are recruited and participate in the formation and maturation of the autophagosomes, which eliminate the invading pathogens.

Monocytes are components of the mononuclear phagocyte system. These cells are recruited to the inflammatory tissue site where they mature to 'inflammatory' macrophages [19]. Although intermediate CD14+CD16+

monocytes can promote microbial resilience, classical CD14+CD16- monocytes inhibit the intracellular growth of *M. tuberculosis*[20]. Moreover, interleukin 17A and vitamin D3 have been reported to enhance the autophagic action of monocytes against *M. tuberculosis* infection in vitro [21, 22]. However, very few studies have determined the various stages of the activation or inhibition of circulating monocytes in tuberculosis patients.

The purpose of our study was to determine the phagocytic capacity of blood phagocytes (monocytes, NK cells, and neutrophils), as well as the mTOR signalling pathway and autophagy level of circulating monocytes of untreated TB patients. We determined the phagocytic capacity and the mTOR signalling pathway components using flow cytometry and analysed the autophagy level using reverse transcription-polymerase chain reaction (RT-PCR) and capillary western analysis.

Patients and Methods

Study population

This study was approved by the Ethics Committee of Shantou University Medical College (Approval No. 2018-008) and consistent with the Helsinki declaration, and informed consent was obtained from all the subjects. Between September 2018 and June 2019, we enrolled 21 patients who had not received anti-tubercular treatment, at the Third People's Hospital of Shantou City. Diagnosis of active TB depended either on microbiological results (sputum smears positive and/or *M. tuberculosis* culture positive) or the positive results of enhanced computed tomography (CT) scans, T-spot, and TB antibody. Table 1 shows the clinical features, laboratory results, and other basic information of the TB patients. Moreover, blood was collected from 20 healthy volunteers used as controls. The serum samples of all the subjects were negative for human immunodeficiency virus. None of the participants had diabetes and none had been administered immunosuppressant drugs.

Flow cytometry

The phagocytic capability of monocytes, neutrophils and NK cells was measured using the Phagotest Kit (Orpegen, Heidelberg, Germany). Aliquots of each blood sample (100 μL) were added to two tubes, which were labelled positive samples and negative control. The blood samples were incubated on ice for 30 min to inhibit the phagocytic activity of the cells. Fluorescein isothiocyanate-conditioned *Escherichia coli* (20 μL) was added to each of the two tubes. After vortexing, the positive tube was incubated at 37°C in a warm water bath (activation level) for 20 min while the negative control remained on ice (basic level). After incubation, the positive tube was transferred to ice to stop phagocytosis. Each tube then received 5 μL of fluorescein-labelled mouse antibody-CD45-Krome Orange, CD14-PC7, CD3-APC and 10 μL CD56-PE (all Beckman Coulter, USA). The tubes were incubated for 20 min on ice. Then a lysing solution was added and incubated for 20 min at room temperature. The cells were resuspended in 0.5 mL PBS. The phagocytic activity of monocytes, neutrophils and NK cells was detected using flow cytometry using a Navios device (Beckman Coulter).

To determine Akt phosphorylation in monocytes, 100 μL of each peripheral blood sample was analysed using the PerFix-Expose Phospho Epitope kit (Beckman Coulter) and stained with CD14-PC7 (Beckman Coulter), Akt-AF488 (Cell Signaling Technology, USA), and p-Akt-AF647 (Cell Signaling Technology). To evaluate mTOR, Raptor, AMPK and Rheb expression in monocytes, 100 μL of whole blood sample was added to three tubes to which a lysing solution was then added. Then the cells were permeabilised and labelled with CD14-PC7, mTOR-AF488 (Cell Signaling Technology), anti-Raptor (Cell Signaling Technology), anti-AMPK (Cell Signaling Technology) and anti-Rheb (Abcam, USA). After incubation, the cells stained with AMPK, Rheb or Raptor received a fluorescent secondary antibody (Alexa Fluor 488 conjugate, Cell Signaling Technology). All flow cytometric tests were performed using appropriate isotype control antibodies and analysed using the Kaluza analysis system (Beckman Coulter).

Isolation of monocytes

Peripheral blood mononuclear cell suspension from whole peripheral blood was prepared by centrifugation

using Ficoll (TBDsciences, China) at 1250 × g for 30 min. Human monocyte cells were sorted using the EasySep CD14 Positive Selection Kit II (STEMCELL Technologies, Canada). The purity of monocytes was >90% CD14+, as measured by flow cytometry.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from purified monocytes using TRIzol LS Reagent (Invitrogen, USA) according to the manufacturer's protocol. The total RNA was reverse transcribed using PrimeScript RT reagent Kit (TaKaRa Bio, Japan) for subsequent PCR analysis. The cDNA was amplified using the One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa Bio) and the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The fold-changes in relative mRNA expression levels were normalised to the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in each sample using the $2^{-\Delta\hat{}\tau}$ formula [23]. The primers for this study are listed in Supplementary Table 1.

Capillary western analysis

The expression of ATG 5, ATG12, ATG16-L1, Beclin-1, ULK1, and LC3-I/II proteins was evaluated using the WES capillary electrophoresis instrument (ProteinSimple, San Jose, CA , USA), following the manufacturer's instructions. The following primary antibodies were used in capillary western analysis for normalization: rabbit anti-ATG5, rabbit anti-ATG12, rabbit anti-ATG16-L1, rabbit anti-Beclin-1, rabbit anti-LC3 I/II, rabbit anti-ULK1, rabbit anti-p-ULK1 S757, and mouse anti-actin-horse radish peroxidase (all Cell Signaling Technology). HRP-conjugated secondary antibodies (ProteinSimple, USA) were added to the designated wells in a module plate. All capillary western dates were analysed using Compass software (version 4.0, ProteinSimple).

Statistical analyses

Data are expressed as the median (25th to 75th percentile) or mean ± standard deviation. Statistical analyses were performed using the SPSS 23.0 software (SPSS Inc., USA). For data with normal distribution, a paired or independent sample t-test was used to assess the comparisons between groups, as appropriate. For data with skewed distribution, the Mann–Whitney U test or Wilcoxon test was used to perform comparisons between groups, as appropriate. A P-value <0.05 was considered as statistically significant.

Results

Clinical features and laboratory results of TB patients compared with healthy controls

Compared with healthy controls, the absolute numbers of the neutrophils and monocytes were found to have increased in the peripheral blood of the TB patients (both $P < 0.05$). However, the number of NK cells was approximately 3.86-fold lower in the blood of the TB patients ($P < 0.001$), while the lymphocyte count did not differ between the two groups. (Table 1).

Decreased phagocytic capacity of monocytes, NK cells, and neutrophils in TB patients

To investigate the level of phagocytic capacity in peripheral blood phagocytes of TB patients, the phagocytic capacity of monocytes, NK cells and neutrophils was measured by flow cytometry at the basic level (0°C) and activation level (37°C) (representative plots are presented in Fig. 1). At 0°C, compared with health controls, the percentages of monocytes, NK cells and neutrophils phagocytizing *E. coli* were significantly greater (monocytes: 83.01%, range 68.32–89.86% vs. 76.05%, range 50.53–80.33%, P = 0.008; NK cells: 3.10 ±1.40% vs. 2.12±1.10%, P = 0.017; neutrophils: 75.79±15.93% vs. 61.61±20.51%, P = 0.018)(Fig. 2a). The MFI for monocytes were significantly higher in TB patients (43.58, range 35.07–62.58 vs. 31.42, range 22.62–37.21, P = 0.001) (Fig. 2b). A significant difference was not evident in the MFIs of NK cells and neutrophils between the two groups (Fig. 2b). After activation at 37°C, the percentage increase in phagocytic activity was lower in TB patients compared with healthy controls (monocytes: 15.46%, range 9.15–27.84% vs. 23.31%, range 18.78–45.80%, P = 0.003; NK cells: 0.58%, range 0.41–1.32% vs. 1.57%, range 0.92–1.77%, P = 0.004; neutrophils: 20.10±13.40% vs. 34.72±19.74%, P = 0.009) (Fig. 2c). The change in MFI between the two groups did not differ (Fig. 2c, d). These data suggested that the phagocytic capacity of monocytes, NK cells

and neutrophils in TB patients was at a low level of activation after infection with *M. tuberculosis* . Also, the phagocytic capacity of monocytes, NK cells and neutrophils in TB patients was decreased after 37°C activation.

Activation of mTOR signalling pathway in peripheral blood monocytes of TB patients

Flow cytometry was used to evaluate the mTOR signalling pathway in the peripheral blood monocytes of TB patients and healthy controls. The mTORC1 complex is a key factor in the mTOR signalling pathway. Raptor is the main component of mTORC1 [7]. We detected the expression of Raptor molecules on monocytes to indirectly determine the expression of mTORC1 by flow cytometry. Compared with health controls, the percentages of $Raptor^+mTOR^+$ monocytes were significantly increased in TB patients (63.27%, range 50.00–84.26%) vs. 50.18%, range 24.88–75.58%), P = 0.016) (Fig. 3a). AKT and Rheb are upstream regulators of mTOR and are positive regulators of mTOR signalling. However, the percentages of $CD14^+Akt^+$ cells and $CD14^+$p-Akt^+ cells were lower in TB patients than in healthy controls (Akt: 96.57%, range 94.32–99.46% vs. 99.52%, range 98.38–99.92%, P = 0.001; p-Akt: 78.46%, range 58.56–88.13% vs. 94.79%, range 74.28–98.68%, P = 0.015) (Fig. 3a). The ratio of p-Akt/Akt in monocytes was significantly lower in TB patients (0.81, range 0.62–0.93 vs. 0.96, range 0.75–0.99, P = 0.032) (Fig. 3c). As shown in Fig. 3a, the frequency of $CD14^+Rheb^+$ cells was higher in TB patients than in healthy controls (92.63%, range 83.60–99.31% vs. 79.96%, range 51.74–94.55%, P = 0.028). In TB patients, the MFIs for $CD14^+Akt^+$, $CD14^+$p-Akt^+, $CD14^+Rheb^+$ and $CD14^+$mTOR+$Raptor^+$ cells were similar to that of controls (all $P > 0.05$; Fig. 3b). AMPK can regulate its downstream target Raptor, and consequently inhibit mTOR. Compared with healthy controls, fewer $AMPK^+$ monocytes were detected in TB patients (93.23%, range 83.19–98.55% vs. 97.82%, range 97.03–99.45%, P = 0.004) (Fig. 3a). The MFI for $AMPK^+$ monocytes was lower in TB patients (3.78±0.96 vs. 4.65±1.01, P = 0.017) (Fig. 3b). Although the expression of Akt and p-Akt molecules on monocytes decreased, the percentages of monocytes expressing Raptor, Rheb and AMPK indicated that the mTOR signalling pathway was activated in the peripheral blood monocytes of TB patients.

Autophagy is induced in peripheral blood monocytes of TB patients

We examined autophagy molecules by determining mRNA and protein expressions in the peripheral blood monocytes of TB patients. qRT-PCR revealed that the expressions of ATG 5, ATG 12, ATG16-L1, Beclin-1 and ULK1 mRNA were higher in TB patients than in controls (all $P < 0.05$; Fig. 4a). The formation of autophagosomes involves three main processes of initiation, elongation and maturation. Both the ULK1 complex and class III phosphoinositide 3-kinase (PI3K) complex can regulate the initiation of autophagy. mTORC1 inhibits initiation through the phosphorylation of ULK1 at Ser757 [14]. Once activated, the ULK1 complex also phosphorylates VPS34 molecules in the class III PI3K complex. Beclin1 is a key functional protein in the class III PI3K complex and is regulated by the AMPK molecule. Capillary western blot analysis revealed that the expressions of ULK1, ULK1 and Ser 757 phosphorylation protein were increased (Fig. 4b), and Beclin-1 expression was significantly higher in TB patients than in healthy controls ($P < 0.05$; Fig. 4c). These results indicated that ULK1-mediated autophagosome initiation was inhibited, which was confirmed by the activation of the mTOR signalling pathway and inhibition of AMPK. However, Beclin 1 may be activated and mediate vesicle nucleation. After initiation, autophagy-related protein molecules of two ubiquitin-like systems (ATG12 and LC3) are recruited to elongate the phagophore [24]. In the ATG12 system, ATG5 is covalently conjugated to ATG12 through the mediation of ATG7 and ATG10. Subsequently, the ATG5-ATG12 complex associates non-covalently with ATG16 L1, and then determines the site of lipidation. In the LC3 system, LC3-I associates with phosphatidylethanolamine (PE) and is transformed to LC-II through the ATG5–ATG12 conjugate. LC3 molecules recruited to the autophagy membranes can help an autophagosome to grow and close. Subsequently, the mature autophagosome fuses with a lysosome to degrade the autophagosomal contents. Presently, compared with the healthy controls, the expressions of ATG 5 and ATG 5-ATG 12 complex were reduced, and ATG16 L1 expression was significantly higher in TB patients (all $P < 0.05$; Fig. 4c). These results indicated the impairment of autophagosome formation in TB patients. In addition, LC3-II expression was increased in TB patients (Fig. 4c). The accumulation of LC3-II suggested the increased conversion of LC3-I to LC3-II in monocytes, as a marker of autophagy induction [25]. These

results support the view that autophagy is induced though an mTOR-independent pathway in the monocytes of TB patients.

Discussion

After phagocytes phagocytose invading pathogens, the phagosome fuses with lysosomes and is acidified. This eliminates the pathogens by producing reactive oxygen species a catalysed by NADPH oxidase [26, 27]. However, *M. tuberculosis* can survive or multiply by preventing phagosome maturation after invading host cells by phagocytosis [28]. *M. tuberculosis* mannose-capped lipoarabinomannan limits phagosome lysosome fusion by binding to the mannose receptor [29]. *M. tuberculosis* tyrosine phosphatase binds the H-subunit of V-ATPase to inhibit macrophage vacuolar acidification [30]. However, the phagocytic ability of phagocytes in the blood of TB patients has been unknown. In this study, we detected the phagocytosis of *E. coli*in neutrophils, NK cells, and monocytes of TB patients by flow cytometry. In the initial state (0°C), the phagocytosis of *E. coli* by neutrophils, NK cells, and monocytes in TB patients was higher than that in healthy controls. However, after 37°C activation, the phagocytic capacity of neutrophils, NK cells, and monocytes were lower in TB patients than in healthy controls. We assume that the peripheral neutrophils, NK cells, and monocytes of TB patients were activated due to infection with *M. tuberculosis* . However, upon infection *M. tuberculosis* rapidly inhibits the activity of neutrophils and monocytes [31], which reduces the clearance of pathogens. We also found a lower degree of change in the percentages of monocytes, NK cells, and neutrophils than in healthy controls. These results suggest that although the patient's innate immune system is activated, the phagocytic activity of phagocytes in the blood is decreased.

AMPK and mTOR can regulate each other, and also regulate autophagy in case of pathogen infection. mTOR activity is increased as determined by measuring the increased activity of downstream mTOR targets in *M. tuberculosis* -infected peripheral blood mononuclear cells in vitro [32]. Indeed, the activation of mTOR signalling is significantly increased, relative to AMPK, in *M. tuberculosis* -infected macrophages [33]. These findings indicate that the mTOR signalling pathway could be induced after activation by *M. tuberculosis*invasion, but the status of the mTOR signalling pathway of peripheral blood monocytes in natural infection is unknown. In the present study, we used flow cytometry to detect Rheb and mTOR, which are key molecules of the mTOR pathway, and their related regulatory molecules Akt and AMPK. In TB patients, an increase in the proportion of $CD14^+Rheb^+$ cells and $CD14^+Raptor^+mTOR^+$cells indicated that the mTOR signalling pathway is activated in peripheral blood monocytes, which was confirmed by the inhibition of AMPK expression. However, the decreased expression of p-Akt and Akt molecules indicates that the mTOR pathway may not be regulated by Akt molecules in peripheral blood monocytes of TB patients.

Although autophagy can degrade bacteria, *M. tuberculosis* in TB patients is not eliminated. The bacteria survive in cells and inhibit autophagy by various mechanisms [28, 30, 34]. Monocytes recruited to the infected tissue site can phagocytize pathogens or secrete tumour necrosis factor and inducible nitric oxide synthase against resistant bacterial and fungal pathogens [35, 36]. In *M. tuberculosis* infection, monocytes respond more strongly to *M. tuberculosis* derivatives via in vitro migration, while producing higher levels of ROS at the site of infection to inhibit *M. tuberculosis* growth [37]. However, the autophagy level of circulating monocytes in the body during TB infection remains unclear.

During classical autophagosome formation, ULK1 and Beclin-1 are involved in the initiation of autophagy and the nucleation of phagophore. Beclin-1 is a key initiator of autophagy and an essential factor in determining whether cells undergo autophagy [38]. Autophagy is regulated by the classic mTOR signalling pathway via ULK1 and also by other mTOR-independent pathways through Beclin-1 [39]. Moreover, ATG5 and ATG12 are recruited and covalently combine to form an ATG5-ATG12 conjugate, which is then non-covalently attached to ATG16 L1. The ATG5-ATG12-ATG16 L1 complex binds to and elongates the phagophore, while sequestrating the cargo [24]. LC3 is a crucial marker of autophagy and is related to the formation and maturation of the autophagosome and is used to monitor autophagic activity [25]. We found that high mTOR activity inhibited ULK1 through phosphorylation of Ser 757, while Beclin-1 protein expression is increased in the blood monocytes of TB patients than in control. This result indicates that there are other ways to stimulate Beclin-1 to initiate autophagosome nucleation with an mTOR-independent pathway in

monocytes. Interestingly, although the expression of ATG16 L1 increased, the expression of ATG5 and ATG5-ATG12 complex decreased, indicating that the elongating phagophores were impaired in the monocytes of TB patients. Moreover, an increase in the conversion of LC3-I to LC3-II predicted the increased induction of autophagy in circulating monocytes. The collective results indicate that the circulating monocytes isolated from TB patients display increased induction of autophagy with a defect in sequestration via an mTOR-independent pathway. We observed that the mRNA and protein levels of ATG5 and ATG12 molecules differed in the monocytes of TB patients. In *M. tuberculosis* infection, the induction of miR-33, which blocks the translation of mRNA to reduce protein expression, impairs xenophagy by inhibiting the expression of ATG5 and ATG12 [40].

NonMtb-containing autophagosomes continue to fuse and mature normally with lysosomes, while Mtb-containing autophagosomes mature in infected macrophages through the virulence molecules PhoP and ESAT6 [41]. The activation of AMPK in cells induces xenophagy by a signal released before the bacteria enter the cells [42]. *M. tuberculosis* may be cleared by xenophagy and LC3-associated phagocytosis (LAP) after phagocytosis by macrophages, which are two different autophagy pathways with functionally and mechanistically distinct processes [13]. LAP requires components of the non-canonical autophagy pathway to phagosome membranes, including Beclin-1, ATG5, ATG12, and LC3 [43, 44]. However, the ULK1 complex is not required for LAP induction despite being indispensable for autophagy [44]. Therefore, we speculate that circulating monocytes may increase LAP induction after being stimulated by the signal of *M. tuberculosis* infection when monocytes do not engulf *M. tuberculosis* . At the same time, *M. tuberculosis*impairs the monocyte LAP process through virulence factors secreted into the blood. The factors involved need to be investigated in a larger study.

In conclusion, the phagocytic capacity of circulating monocytes was decreased, accompanied by autophagy activation in active TB patients. It is important to understand the regulatory mechanism of M. tuberculosis for monocytes in vivo, since it may be a potential therapeutic option for the development of HDTs against TB.

Acknowledgements

This study was supported by the funds below:

a) The National Natural Science Foundation of China [grant numbers 81302540];

b) Dengfeng Project for the construction of high-level hospitals in Guangdong Province - the First Affiliated Hospital of Shantou University Medical College Supporting Funding.

Author contributions

Xianyan Zhang and Xuexuan Zhang wrote the article and analysed the data. Jialong Xu, Xiaoxin Huang, Ying Wu and Tianming Liu performed data collection and manuscript preparation. Yinge Wu designed the experiment and reviewed the article.

Disclosures

The authors declare no conflict of interests.

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Table

Table 1 Clinical features, laboratory results and other basic information of TB patients, compared with healthy controls.

		Healthy control	TB group	P-value
Number (n)		20	21	-
Gender [n(%)]	Male/Female	11(55)/ 9(45)	17(81)/ 4(19)	-
Age (years)		32(30-44)	48(29-61)	0.127
Clinical features [n(%)]	Fever	-	1(5)	-
	Cough	-	20(95)	-
	Haemoptysis	-	5(24)	-
	Night sweats	-	3(14)	-
Clinical laboratory results Peripheral blood counts ($\times 10^9$/L)	White blood cells	6.04±1.28	7.60±2.51	0.016
	Neutrophils	3.46±1.11	5.08±2.46	0.011
	Monocytes	0.4(0.30-0.43)	0.57(0.47-0.66)	0.001
	Lymphocytes	2.10±0.63	1.65±0.69	0.069
	NK cells	0.27(0.14-0.43)	0.07(0.05-0.15)	<0.001
Sputum [n(%)]	Smear positive	-	12(57)	-
	Culture positive	-	18(86)	-
CT findings [n(%)]	Tuberculous cavity	-	16(76)	-
	Multifocal patchy	-	5(24)[a]	-
Drug sensitivity results (Drug resistance) [n(%)]	Isoniazid	-	3(17)	-
	Streptomycin	-	3(17)	-
	Ethambutol	-	2(11)	-

Values are expressed as a number, mean ± standard deviation or median (25th to 75th percentile). a: Only multifocal patchy, not tuberculous cavity.

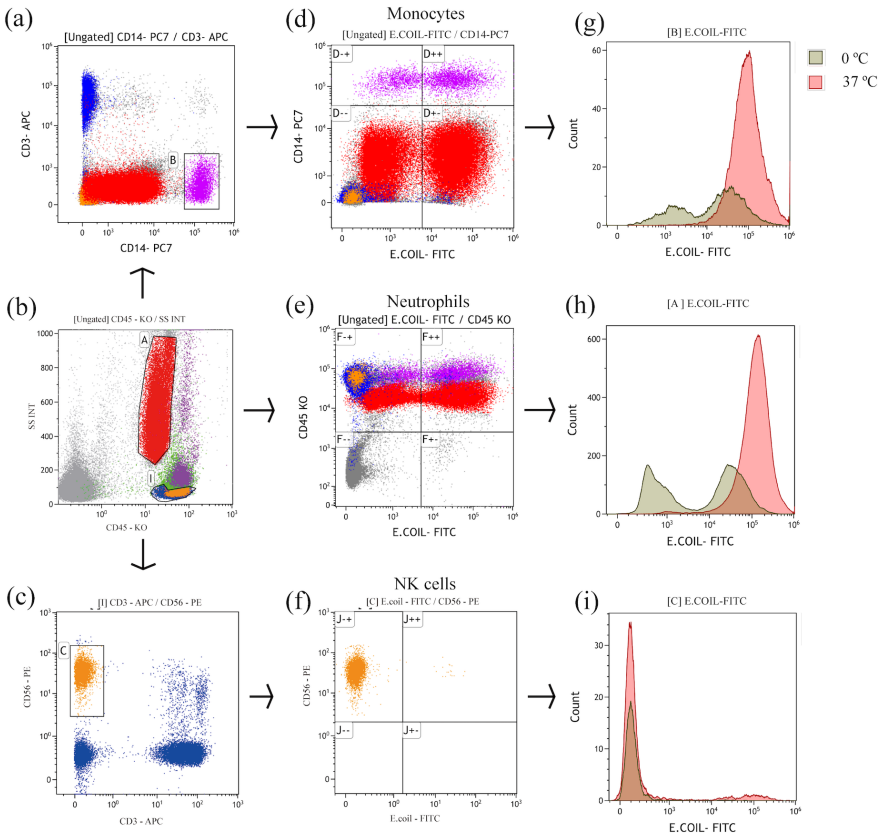

Figure Captions

Fig. 1. Representative dot plots of monocytes, neutrophils and NK cells phagocytizing *E. coli* in TB patients and healthy controls. (a-c) Monocyte (a), neutrophils (b), NK cell (c) gating were generated by the use of CD 14-PC7/ CD3-APC, CD45-KO/ SSC, CD3-APC/CD56-PE dot plots, respectively. (d-f) Representative gate setting strategy of monocytes (d), neutrophils (e) and NK cells (f) phagocytizing *E. coli* are shown. (g-i) After incubation at 0°C or 37°C, the mean fluorescence intensity (MFI) of monocytes (g), neutrophils (h), and NK cells (i) phagocytizing *E. coli* were detected by flow cytometry.

Fig. 2. Phagocytic capacity in monocytes, NK cells and neutrophils of TB patients and healthy controls. (a) Percentages of monocytes, NK cells and neutrophils phagocytizing *E. coli* were analysed using flow cytometry after treatment at 0°C or 37°C. (b) The MFIs of monocytes, NK cells and neutrophils phagocytizing *E. coli*were measured after 0°C or 37°C. (c) The degree of change in the percentages of monocytes, NK cells and neutrophils after activation at 37°C. (d)The change in the MFI of monocytes, NK cells and neutrophils after activation at 37°C. **P<0.05; ***P<0.001; NS, not significant.

Fig. 3. Expression levels of mTOR signalling pathway molecules in monocytes of TB patients. (a) The percentages of $CD14^+Akt^+$cells, $CD14^+$p-Akt^+cells, $CD14^+Rheb^+$cells, $CD14^+mTOR^+Raptor^+$cells, $CD14^+AMPK^+$ cells were analysed by flow cytometry in TB patients and healthy controls. (b) The MFIs for $CD14^+Akt^+$cells, $CD14^+$p-Akt^+cells, $CD14^+Rheb^+$cells, $CD14^+mTOR^+Raptor^+$cells, $CD14^+AMPK^+$ cells were analysed. (c) For the percentage, the ratio of p-Akt/Akt was calculated between TB patients and healthy controls. **p<0.05; NS, not significant.

Fig. 4. Autophagy level in peripheral blood monocytes of TB patients and healthy controls. (a) qRT-PCR analysis of the relative expression of ATG 5, ATG 12, ATG16-L1, Beclin-1, and ULK1. (b) ULK1 and ULK1 Ser 757 phosphorylation expression was examined using a capillary western blot. (c) ATG 5, ATG

12, ATG16-L1, Beclin-1, and LC3 –I/II expression was determined using a capillary western blot and are plotted as relative expression. **P $<$ 0.05; ***P $<$ 0.001.

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