Am J Transl Res 2016;8(2):1055-1063
www.ajtr.org /ISSN:1943-8141/AJTR0018922

Original Article

The mechanism of cytoskeleton protein β-actin and cofilin-1 of macrophages infected by *Mycobacterium avium*

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Received November 1, 2015; Accepted January 12, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Cytoskeleton proteins and their regulation proteins could be influenced seriously in *Mycobacterium tuberculosis* infection host cells leading to the apoptosis of host cells. Macrophages infected by *Mycobacterium avium* were detected from cell morphology and genome levels to analyze changes of the cytoskeleton of *M. avium* infection macrophages. Then the expression of β-actin, cofilin-1 proteins in *M. avium* infected macrophages were analyzed by western blotting, and the apoptosis of *M. avium* infection macrophages were tested by flow cytometry. Results indicated that the morphology and genomic DNA of *M. avium* infection macrophages were not damaged significantly. Meanwhile, *β-actin* gene and its proteins in *M. avium* infection macrophages were both decreased, but its regulatory protein cofilin-1 was expressed conversely. Furthermore, macrophages could be induced to apoptosis due to *M. avium* infection by cytoskeleton changes. These findings contributed us to understand that macrophages infected by *M. avium* could be lead to apoptosis by regulating cytoskeleton protein β-actin or its regulatory protein cofilin-1.

Keywords: Macrophage, *M. avium*, β-actin, cofilin-1

Introduction

Mycobacterium tuberculosis (*MTB*) is a facultative intracellular pathogen which could cause *tuberculosis*, and *tuberculosis* has been the respiratory infectious disease which harm to human health seriously around the world [1]. Macrophages are the main immune cells that kill *mycobacterium*, and they also are able to present specific antigens of *M. tb* to T lymphocytes, in the immune system [2]. After *Mycobacterium tuberculosis* invade bodies, macrophages could produce all kinds of cytokines such as IL-2, IL-6, IL-10, IFN-γ and TNF-α to be used against *mycobacterium* [3-5]. Studies show that TNF-α and IFN-γ were the two most important cytokines to kill or inhibit *M. tb*, and to induce an inflammatory response [6-8]. Above all, the phagocytosis of macrophages is the most important function to kill *Mycobacterium tuberculosis* that*Mycobacterium tuberculosis* in macrophages could be hydrolyzed by intracellular lysosomal hydrolase leading to the loss of the proliferation activity [9, 10]. However, if *Mycobacterium tuberculosis* could not be eliminated by the immune system of bodies, they will incubate to parasitize in macrophages, then they would induce the apoptosis of macrophages through a variety of mechanisms, when the immune function of macrophages is weak or untreated with drugs [11].

Actin proteins are the structural component of microfilament to constitute the cytoskeleton of cells, and the expression levels of actin proteins were related to the morphology of cells closely [12, 13]. As actin is necessary for the scaffolding of endosomes during phagosome-endosome interactions, the correlation between the disruption of actin by *M. tb* and the delay in phagosomal maturation has been observed [14, 15]. Previous studies have showed that if cells were induced to be apoptosis, actin filament will be broken and the network structure of actin proteins also will been destroyed, sug-

gesting that actin proteins may be one of modulators during the early stage of apoptosis [16]. Cofilin-1 is a widely distributed intracellular actin-modulating protein that binds and depolymerizes filamentous actin and inhibits the polymerization of monomeric actin in a pH-dependent manner [17]. The skeleton of *M. tb* infection macrophages would be influenced by the invasion of *M. tb* leading to the death of macrophages and the propagation of *M. tb*.

This paper focuses on studies of cytoskeletal protein β-actin and its regulatory proteins cofilin-1 in *M. tb* infection macrophages to explore the mechanisms of cytoskeleton proteins of macrophages in the process of cells apoptosis induced by *M. tb*.

Materials and methods

M. avium growth

Mycobacterium avium sp. Paratuberculosis (*M. avium.spp* called *M. avium* in this paper) was obtained from the Center for Disease Control of Shanghai. Bacteria were grown on Middlebrooks 7H9 plates as previously described [18] for 4 weeks, at 37°C. They were harvested by scraping, with 0.9% NaCl as vehicle, and concentrations were calculated according to 0.5 McFarland Standards method, then were concentrated to 7.5×10^9/mL.

Macrophage cultures

The human acute monocytic leukemia cell line THP-1 was purchased from the Center for Type Culture Collection of Shanghai Academy of Sciences. Cells were cultured in wells or flasks at 37°C under 5% CO_2, in RPMI 1640-GlutaMAX™ containing 10% (v/v) fetal bovine serum (Hyclone company, USA), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B. Differentiation of these cells into macrophage-like cells was induced with 0.1 mM Phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) for 12 h. Then were divided into control group and experimental group randomly, control group, stimulated with blank medium, and experimental groups infected with *M. avium* (*M. avium*: macrophages = 10:1) for 48 h.

Exosomes isolation

Macrophages cell culture supernatants were centrifuged at 3,000 *g* for 15 minutes to remove cells and cell debris, then supernatants were transferred to sterile tubes. The ExoQuick Exosome Precipitation Solution (System Biosciences (SBI), Mountain View, CA, USA) was added to cell culture supernatants, the tubes mixed by inverting, then refrigerated for 30 minutes. ExoQuick/biofluid mixture was centrifuged at 1,500 *g* for 30 minutes, then the supernatant aspirated. Spin down residual ExoQuick solution was added and centrifuged at 1,500 *g* for 5 minutes and all traces of fluid removed by aspiration. Finally, exosome pellets were resuspended in 1/10 of the original volume using nuclease-free water. The exosomes pellets were mixed with 25 μl of 9% sucrose containing protease inhibitors and stored at -80°C until use. All procedures were carried out at 4°C.

Exosomes identification by transmission electron microscopy (TEM)

Exosomes pellets were resuspended and fixed in phosphate buffer containing 2% glutaraldehyde and then loaded on Formar/carbon-coated electron microscopy grids. The samples were contrasted in uranyl acetate and viewed with Hitachi H-600 TEM microscope at 70,000 magnification.

Reverse transcription PCR (RT-PCR)

The primers designed according to the cDNA sequences of *β-actin* and *GAPDH* obtained from the NCBI database. were used: *GAPDH*, F 5' GGATTTGGTCGTATTGGGCG 3' and R 5' CCTGGAAGATGGTGATGGGATT 3'; *β-actin*, F 5' TGACGGTCAGGTCATCACTATCGGCAATGA 3' and R 5' TTGATCTTCATGGTGATAGGAGCGAGGGCA 3'. All primers were synthetized by Invitrogen (New York, US).

RT-PCR assays were performed using the Eppendorf PCR system (Eppendorf, Germany). Total RNA was isolated from macrophages using TRIzol reagent and quantified spectrophotometrically. The cDNA was synthesized using M-MLV reverse transcriptase (Superscript-Invitrogen, Carlsbad, CA, USA). A reaction mixture containing 5 μL RNA (200 ng/μL), 12 μL 5×M-MLV RT Buffer, 6 μL 2.5 mM dNTP mixs, 1 μL RNase inhibitor (30 U/μL), 4 μL M-MLV Reverse Transcriptase (5 U/μL), 3 μL Oligo $dT_{(18)}$ primer (500 ng/μL) and 14 μL DEPC water was incubated at 37°C for 1 h. For RT-PCR, 2.0 μL cDNA, 0.5 μL forward primer and 0.5 μL reverse

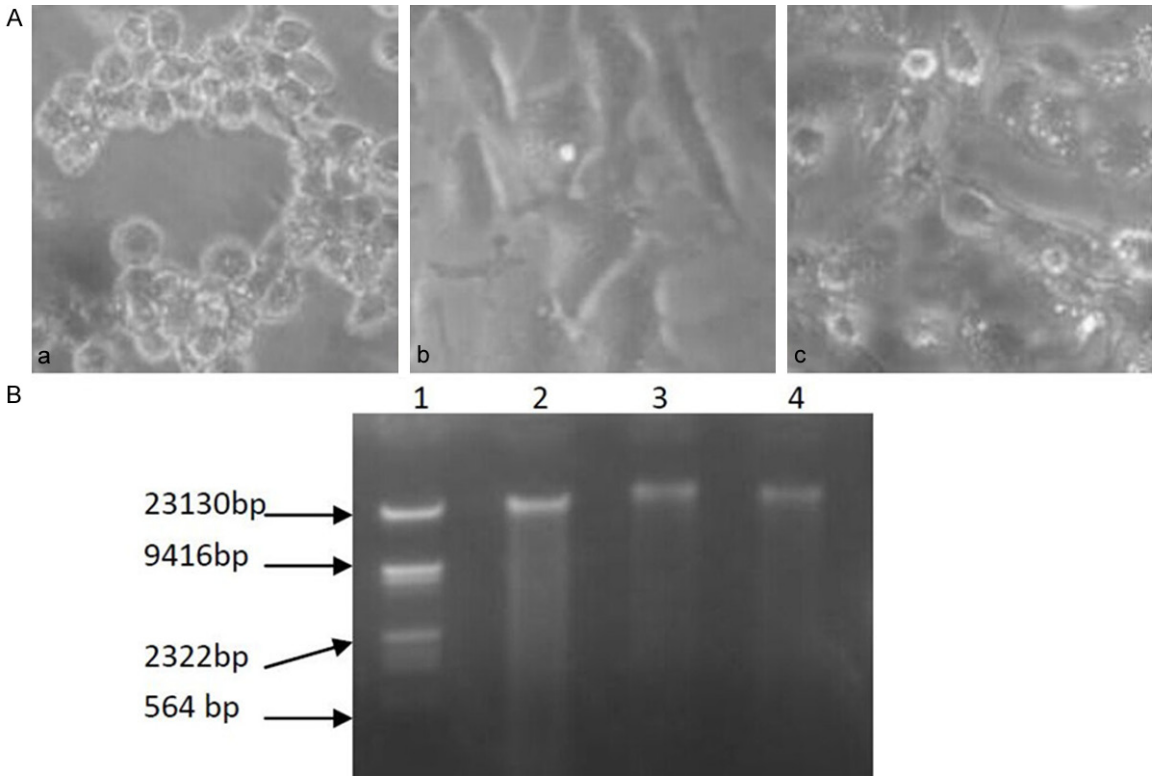

Figure 1. Analysis of the morphology (×40) and the genomic DNA of *M. avium* infection macrophages. A: a: THP-1 cells; b: THP-1 cells differentiated into macrophages with PMA were treated with PBS; c: THP-1 cells differentiated into macrophages were infected with *M. avium*. B: 1: Hind-III Marker; 2: The gDNA of THP-1 cells; 3: The gDNA of macrophages treated with PBS; 4: The gDNA of *M. avium* infection macrophages.

primer, 2 μL 2.5 mM dNTP mixs, 0.5 μL DNA polymerase (10 U/μL), 4 μL 5×Buffer and 10.5 μL ddH_2O were added into the system and amplified for 30 cycles under the following cycling conditions, 94°C denaturation 2 min, 28× (94°C for 20 s; 55°C, 30 s; 72°C for 20 s) extension at 72°C for 5 min.

Western blotting

For western blotting, equal concentration of protein from *M. avium* infection macrophages cell lysates or *M. avium* non-infection macrophages, as quantitated by the Micro BCA Protein Assay, were loaded on 10% SDS-PAGE gels, electrophoresed, and transferred onto polyvinylidene difluoride membrane (Milipore, Bedford, MA). The membranes were probed for β-actin or cofilin-1 (1:500 dilution) (Yi Sen Biotechnology Company, Shanghai China). Immune-detected protein bands were quantified with Image J and statistically analyzed by ANOVA software.

Flow cytometry analysis

Macrophages cultured in six well plates were infected with *M. avium* for 48 h, then harvested and washed twice with PBS containing 0.2% BSA. Macrophages were then incubated with Annexin-V-FITC antibodies or PI dye liquor (Yi Sen Biotechnology Company, Shanghai China) for 30 min in dark, on ice. After macrophages washed with PBS twice, cells were pelleted and resuspended to 1×10^6 cells/mL with PBS-0.2% BSA. Cell sorting and analysis were performed on a FACS (Beckman MOFLO XDP, USA).

DNA fragmentation analysis by hoechst 33258 staining

Macrophages cells were treated with control blank and *M. avium* for 48 h , centrifuged at 1000 rpm for 10 min and washed with PBS. The cells were then stained with Hoechst 33258 (50 μg/mL) (Yi Sen Biotechnology Company, Shanghai China) and incubated at 37°C for 30 min in dark. Then cells were

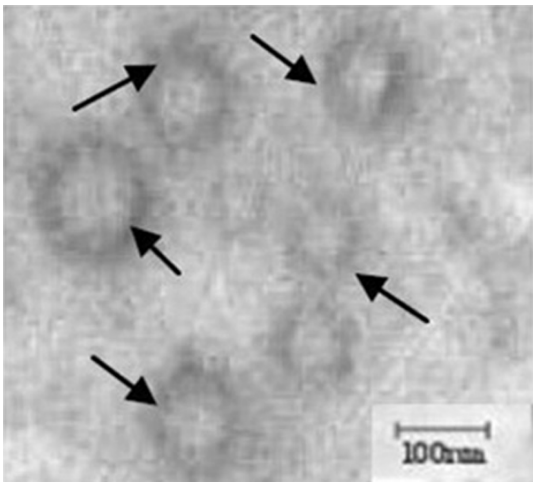

Figure 2. Exosomes secreted from *M. avium* infection macrophages were observed by TEM (×100 000), shown by arrows. Bar: 100 nm.

washed and resuspended with PBS and examined under fluorescence microscope (magnification, ×400) (Nikon, Tokyo, Japan) and analyzed by DP2-BSW software. The apoptotic cells showed shrinkage and condensation of chromatin.

Statistical analysis

Triplicates were performed in each experiment for each condition or group. Data were analyzed by SPSS 16.0 and are presented as means ± SEM of at least 3 independent experiments. Differences within each group were subjected to t-test or q-test. Statistically significant differences (*$P \leq 0.05$) between the groups being compared are indicated by asterisks.

Results

Macrophage infected with M. avium

THP-1 cells were cultured for 48 h, then induced into macrophages with 0.1 mM PMA. Macrophages were treated with PBS or *M. avium* for 48 h, then the morphology of macrophages was observed by microscope (**Figure 1A**). At the same time, the genomic DNA of macrophages was extracted by the genomic DNA Extraction Kit (TianGen Company, Beijing) to detect DNA fragments (**Figure 1B**). Results of experiments indicated that the morphology of macrophages was influenced by *M. avium*, but the genomic DNA of *M. avium* infection macrophages was still undegraded.

Exosomes characterization by transmission electron microscopy

Transmission electron microscopy observation of phosphotungstic-stained, purified exosomes obtained from macrophages infected with *M. avium* revealed an homogenous population of morphologically typical vesicles of 30 to 100 nm diameter (**Figure 2**), similar in appearance and size to those in published reports [19, 20]. This indicated that the vesicles purified were actually exosomes and not apoptotic bodies or others.

The expression of β-actin and cofilin-1 genes

After macrophages infected with *M. avium* for 48 h, the *β-actin and cofilin-1* mRNAs of *M. avium* infection macrophages were detected by RT-PCR to analyze the effect of *M. avium* infection to *β-actin and cofilin-1* genes in macrophages (**Figure 3**). Results showed that the *β-actin* mRNA level of *M. avium* infection macrophages was down regulated significantly (*P<0.05), but the expression of *cofilin-1* show an opposite results compared with control groups (*P<0.05).

Western blotting analysis

Proteins of β-actin and cofilin-1 in *M. avium* infection macrophages or β-actin and cofilin-1 proteins in exosomes secreted from *M. avium* non-infection or infetion macrophages, were analyzed by western blotting (**Figure 4**). Above data manifested that β-actin proteins in *M. avium* infection macrophages or in exosomes secreted from *M. avium* infection macrophages were decreased remarkably (*P<0.05), but the cofilin-1 proteins which is the regulatory protein of β-actin was increased observably (*P<0.05).

Apoptosis analysis of macrophages infected with M. avium

Macrophages were infected with *M. avium* for 48 h, then were stained with Hoechst 33258 to be used for DNA fragmentation analysis. The examination showed that *M. avium* significantly increased the percentage of cleaved nuclei to 33% compared to 4% in control group (**Figure 5**). Thus, *M. avium* significantly induces the apoptosis of macrophages cells (*P<0.05).

Flow cytometry

Macrophages were collected to be incubated with FITC-labeled Annexin-V antibodies, and PI dye liquor, after macrophages infected with *M.*

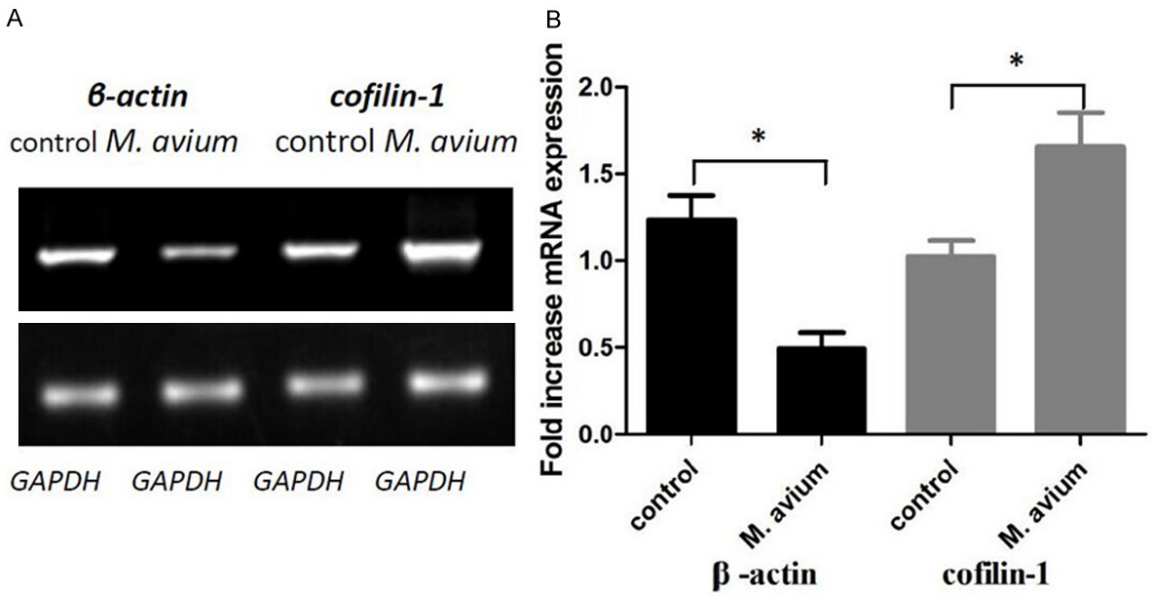

Figure 3. The expression of *β-actin* and *cofilin-1* mRNAs in *M. avium* infection macrophages analyzed by RT-PCR methods. A: Results of *β-actin* and *cofilin-1* mRNA analyzed by RT-PCR; B: Results of the quantitative analysis about the expression of *β-actin* and *cofilin-1* mRNAs analyzed by SPSS 16.0 stastical software, *GAPDH* as a standard calibration (n = 3, compared with the control groups, * *P*<0.05).

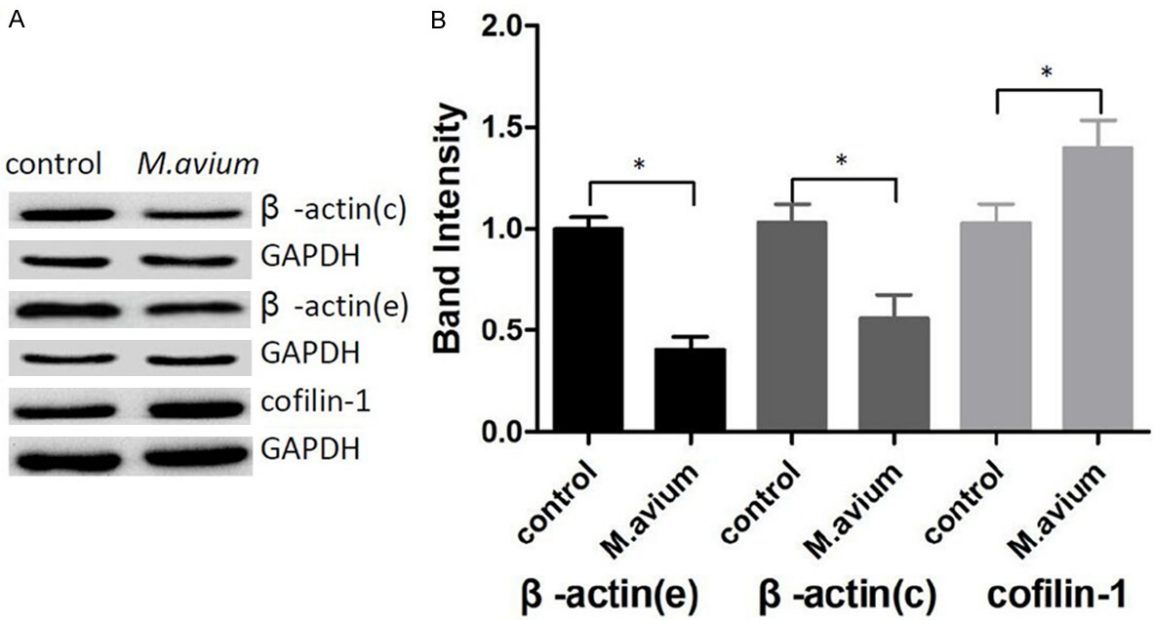

Figure 4. Analysis of β-actin and cofilin-1 proteins by western blotting. A: Western blotting results of β-actin in *M. avium* infection macrophages (c) or in exosomes secreted from *M. avium* infection macrophages (e), and cofilin-1 in *M. avium* infection macrophages. B: Results of stastical analysis, GAPDH as quantitative correction (mean ± SEM, 3 independent experiments). Statistical analysis by SPSS 16.0, with asterisks indicating the pairs of values compared for which significant differences were observed (*$P \leq 0.05$).

avium for 48 h. Then macrophages were detected by Flow cytometry to analyze the apoptosis of *M. avium* infection macrophages (**Figure 6**). Testing results show that apoptosis cells of macrophages increased obviously after cells infected with *M. avium* (*P<0.05).

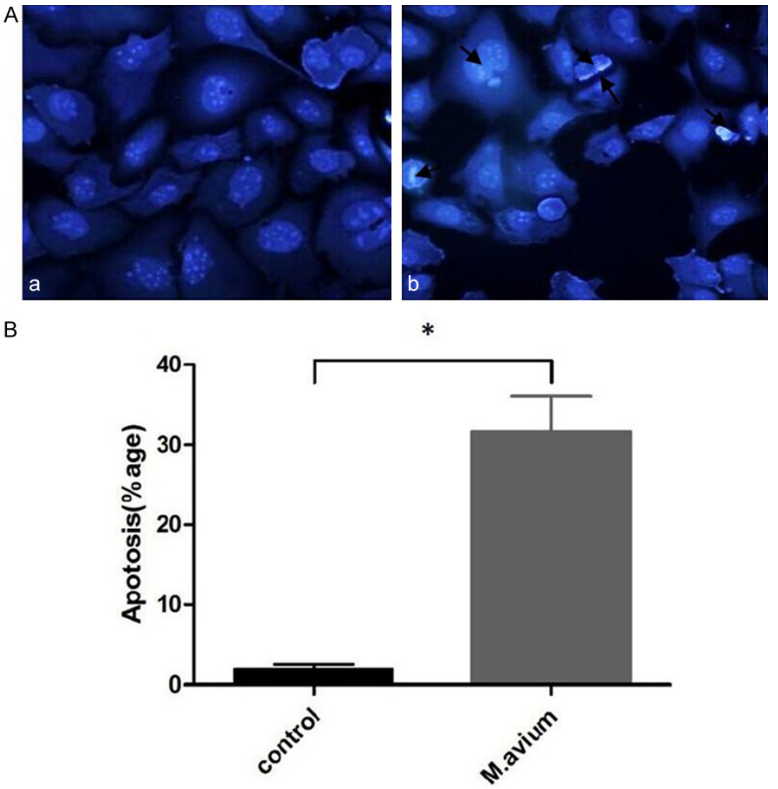

Figure 5. Influence of *M. avium* on the apoptosis of macrophages cells. A: a: Macrophages cells were treated with control medium. b: Macrophages cells were infected with *M. avium*. Nuclear morphological changes were observed by Hoechst 33258 staining and fluorescence microscope. Arrows indicated the condensed and fragmented nuclei. Histograms showed number of cleaved nuclei (apoptotic cells) counted microscopically from 100 nuclei. B: Results of apoptosis macrophages infected with *M. avium* were analyzed by SPSS 16.0, (mean ± SEM, 3 independent experiments), with brackets indicating the pairs of values compared for which significant differences were observed (*P≤0.05).

Discussion

Macrophage is one kind of the main immune cells to kill the invaded *Mycobacterium tuberculosis* in bodies, as well, macrophages are major places that *M. tb* incubated [21]. Once *M. tb* infected macrophages, their thick lipid would prevent lysosomal enzymes killing themselves, and propagate in macrophages massively. After that the cytoskeleton of macrophages would be damaged by *M. tb* severely to lead to apoptosis or necrosis, then, *M. tb* also would be released from maceophages to invade surrounding macrophages leading to the infection of *M. tb* further [22]. In this study, the morphology of *M. avium* infection macrophages unchanged significantly, and the genomic DNA in *M. avium* infection macrophages undegraded either. Actin proteins are essential component of the cytoskeleton, with critical roles in a wide range of cellular processes, including cell migration, cell division, and the regulation of gene expression. These functions are attributed to the ability of actins to form filaments that can rapidly assemble and disassemble according to the needs of the cell. β-actin is one of six isoforms expressed in cytoplasm ubiquitously, and regulate cell migration, gene expression and controls cell growth [23]. *M. avium* destroyed the cytoskeletal structure and inhibited the metabolism of macrophages leading to lossing a variety of cytoskeletal proteins and affecting the killing functions of macrophages [24]. In *M. tb* infection macrophages, cells themselves are also trying to regulate cytoskeletal associated proteins to maintain the stability of the cytoskeleton by a series of regulation proteins [25]. Exosomes secreted from *M. avium* infection macrophages were analyzed by two-dimensional electrophoresis and mass spectrometry, and two actin isoforms were down-regulated and cofilin-1 was up-regulated among the cytoskeletal proteins of *M. avium* infection macrophages [26]. Nevertheless, reports have showed that *M. tuberculosis* and *M. marinum*, but not *M. avium*, are ejected from the cell through an actin-based structure, the ejectosome. This conserved nonlytic spreading mechanism requires a cytoskeleton regulator from the host and an intact mycobacterial ESX-1 secretion system [27]. Different lipids were found to stimulate or inhibit actin assembly by LBPs and mycobacterial phagosomes *in vitro*. In addition, selected lipids activated actin assembly and phagosome maturation in infected macrophages, resulting in a significant killing of *M. tuberculosis* and *M. avium* [28]. In this study, we found that *β-actin* genes decreased in *M. avium* infection macrophages, and its proteins in *M. avium* infection macrophages or in exo-

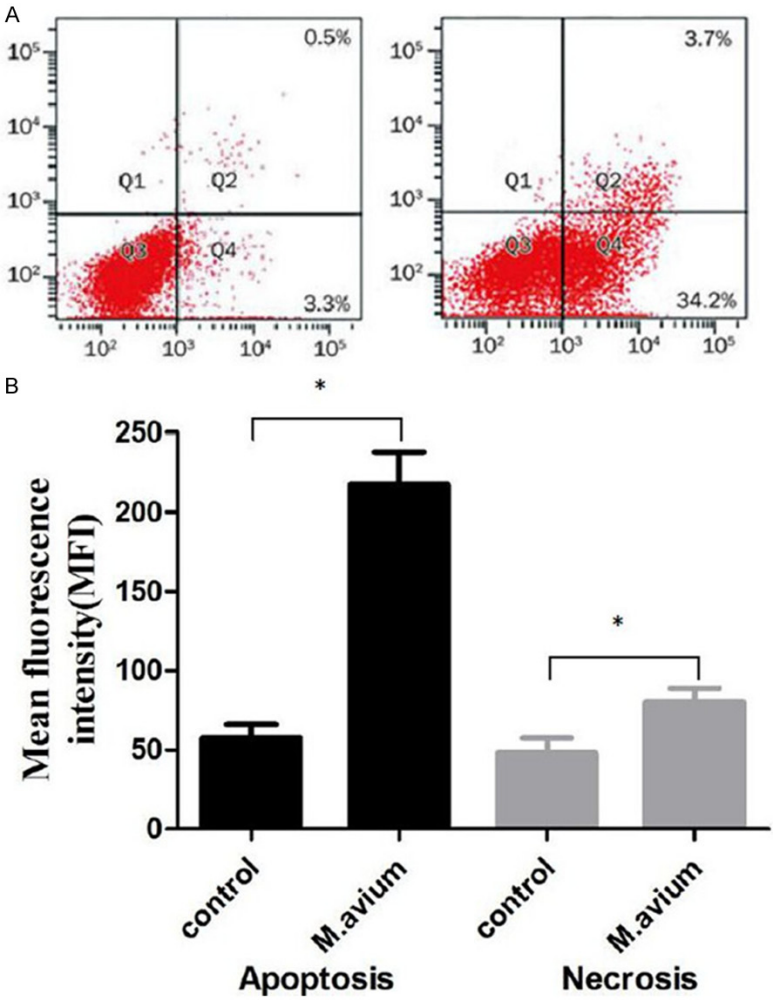

Figure 6. Analysis of apoptosis macrophages infected with *M. avium* by flow cytometry. A: Results of *M. avium* infection macrophages detected by Flow cytometry; B: Results of apoptosis macrophages analyzed by SPSS 16.0 statistical software. (mean ± SEM, 3 independent experiments), with brackets indicating the pairs of values compared for which significant differences were observed (*$P \leq 0.05$).

somes secreted from *M. avium* infection macrophages were also decreased.

Cofilin-1 has actin depolymerizing and severing activity [29]. Since cofilin is essential for cytokinesis, endocytosis and other cellular processes that require actin filament turnover [30, 31], it can be speculated that the transfer of cofilin-1 from infected to non-infected cells via exosomes could result in an enhanced capacity of the recipient cells for actin filament turnover. Cofilin-1 in exosomes secreted from *M. avium* infected macrophages was increased significantly, but actin protein expressed on the contrary, suggesting that the cytoskeletal proteins of macrophages were dilapidated and the synthesis or processing of actin fiber were blocked by *M. avium* [32]. The results suggest that in the process of infection, *M. tuberculosis* evades the bactericidal mechanisms possibly by secretion of certain proteins or factors which affect the host-cell actin [33]. In the present study, cofilin-1 protein increased significantly in *M. avium* infection macrophages. At the same time, our studies also showed that *M. avium* could inhibit the expression of β-actin cytoskeletal proteins to promote the expression of regulatory proteins cofilin-1 to disrupt the cytoskeleton of macrophages leading to the apoptosis of host cells.

However, the specific mechanism of β-actin and cofilin-1 proteins in *M. avium* infection macrophages, and the roles of them on the invasion of *M. avium* are still needed to be studied further.

Acknowledgements

This work was supported by Science and Technology Project of Jiangsu Province in China (NO: KS1425) and by Medical science and technology development fund of Jiangsu University in China (JLY20140047).

Disclosure of conflict of interest

None.

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