Original Article

Antigen Presentation and Proteome Study of Exosomes Secreted by Co-Culture of Macrophages and *Talaromyces marneffei*

(*Talaromyces marneffei* / exosome / antigen presentation / macrophage)

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Abstract. It is known that intracellular pathogens interact and react with the cellular immune system through exosomes produced by macrophages. This study aimed to determine whether co-culture of macrophages and *Talaromyces marneffei* induces exosomes and leads to immune responses. *T. marneffei* was incubated to collect conidia, co-cultured with human macrophages, which then induced exosomes. In cellular experiments, after extraction and purification, the exosomes were then observed by electron microscopy and detected by flow cytometry and mass spectrometry. In animal experiments, flow cytometry and enzyme-linked immunosorbent assay were used to examine whether exosomes were antigen-presenting. The results showed that purified exosomes produced a pro-inflammatory response and stimulated production of TNF-α in non-fungal-treated macrophages. Protein mass spectrometry analysis of exosomes also indicated their potential ability to activate the internal immune response system and the pro-inflammatory response. Translation and ribosomes were the most abundant GO terms in proteins, and the most relevant KEGG pathway was the biosynthesis of secondary metabolites. Furthermore, *in vivo* experiments revealed that exosomes induced activation of lymphocytes and increased expression of TNF-α and IL-12 in the lung, mediastinum, and spleen area. In conclusion, exosomes can be released by co-culture of *T. marneffei* and macrophages, having antigen-presenting functions, promoting macrophage inflammation, and initiating adaptive immune responses. These processes are inextricably linked to the translation of secondary metabolites, ribosomes and biosynthesis.

Introduction

Talaromyces marneffei (*T. marneffei*), previously known as *Penicillium marneffei*, is a temperature biphasic conditional pathogenic fungus. It is presented as mycelial morphology at 30 °C and yeast-type as well as pathogenic at 37 °C (Roilides et al., 2003; Lu et al., 2013). *T. marneffei* is widespread in nature, with a 96 % carrying rate in Guangxi hoary bamboo rats, which indicates Southeast Asia as the main concentrated pathogenic area. Penicilliosis caused by *T. marneffei* is often found in immunocompromised populations, which primarily targets the host's mononuclear macrophage system, as demonstrated in clinical diagnosis and *in vitro* experiments before (Roilides et al., 2003; Lu et al., 2013).

The host's internal immune system is capable of rapidly recognizing and responding to external cues (Akira et al., 2001; Schnare et al., 2001). It is widely believed that macrophages recognize and invade pathogenic fungal types present in the body through pattern recognition receptors and initiate the corresponding cellular signalling pathways, activate transcription factors and initiate transcription of relevant target genes, and induce expression of pro-inflammatory cytokines such as TNF-α, IL-1, IL-2, and IFN (Akira et al., 2001; Schnare et al., 2001). Different pathogenic fungi have different pattern recognition receptor pathways (van der Graaf et al., 2005; Viriyakosol et al., 2005; Saijo et al., 2007). However, the specific process of how the organism recognizes *T. marneffei* remains unclear.

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Abbreviations: BMMs – bone marrow-derived macrophages, BP – biological process, CC – cellular component, ELISA – enzyme-linked immunosorbent assay, GO – Gene Ontology, KEGG – Kyoto Encyclopaedia of Genes and Genomes, MF – molecular function, *T. marneffei* – *Talaromyces marneffei*.

Exosomes are extracellular vesicle substances secreted by different cells (Denzer et al., 2000). Proteomic studies of exosomes and membrane-fused vesicles have revealed the interaction and invasion between potent cells and the external environment, as well as molecular markers that may be involved in related diseases (Théry et al., 2001; Raimondo et al., 2011). Meanwhile, it has been reported that exosomes may play an important role in the host immune system (Mignot et al., 2006; Bhatnagar et al., 2007).

The aim of this study was to explore the role of exosomes released by co-culture of *T. marneffei* and macrophages in the immune response through cellular and animal experiments, and to provide a theoretical basis for the development of diagnostic reagents and effective vaccines of *T. marneffei* by detecting the biological information of protein or peptide components in the exosomes.

Material and Methods

Cell culture

Mouse RAW264.7 macrophages were purchased from the Institute of Cytobiology, Chinese Academy of Sciences (National Collection of Authenticated Cell Cultures, SCSP-5036), introduced from the American Type Culture Collection (ATCC), and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10 % foetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, NY) and 1 % penicillin at 37 °C in a humidified CO_2 incubator.

Collection of Talaromyces marneffei spores

T. marneffei clinical strains were isolated from culture-documented patients with penicilliosis (from People's Hospital of Guangxi Zhuang Autonomous Region) and cultured in a solid potato dextrose agar (PDA) medium (Sigma-Aldrich, St. Louis, MO) and activated at 25 °C for three days. Hyphae at the edge of colonies were selected and inoculated in a new PDA dish. The culture conditions were kept unchanged and the culture continued for 14 days until spores were produced. The suspended spores on the surface of colonies were collected by a blow of sterile water. After counting on a blood counting plate, the spores were preserved at −80 °C for further experiments. We ensured that the media for the macrophage cultures as well as the preparation of *Talaromyces* spores were free of endotoxin or lipopolysaccharide, and the samples were examined after preparation.

Co-culture of Talaromyces marneffei and macrophages

To avoid exosomes to be contaminated in cell culture, culture medium containing calf serum (Chuzhou Snoda Biotechnology Co., Ltd., China – Chuzhou, Anhui, China, http://www.sndbio.cn) was centrifuged at 100,000 *g* and 4 °C for 15 h. The ratio of macrophages to *T. marneffei* spores was 1 : 5. The culture medium was collected after 0, 1, 3, 6, 12, 24, 48 and 72 h, respectively, and preserved at −20 °C for use.

Extraction and purification of exosomes

According to the previous methods (Bhatnagar et al., 2007), the co-culture solution of macrophages and *T. marneffei* spores was centrifuged four times, then dissolved in PBS buffer. Exosomes were added to the top of a linear sucrose gradient (15 % to 60 %) and centrifuged at 100,000 *g* for 15 h at 4 °C, collecting the part of the sucrose gradient. After dilution in PBS, the sucrose gradient was centrifuged at 100,000 *g* for 60 min. The precipitate represented the purified exosome sample that could dissolve in PBS. The exosome sample was preserved at −80 °C.

Electron microscopic observation

In total, 10 µl of purified exosome suspension was placed on a copper sample net for 5 min, then 30 µl of phosphotungstic acid (20 g/l; pH 6.8) was added for negative staining at room temperature for 2 min. After the negative staining, the dye solution was dried with filter paper and incandescent lamp, it was observed and photographed under a transmission electron microscope (H-7650, Hitachi, Stoke Poges, UK). The experiment was repeated three times.

Detection of total protein and specific antibodies of exosomes

The exosome samples (4 ug/ml) were mixed in a 4 × loading buffer and then heated in a boiling water bath for 5 min. The exosome samples were tested by electrophoresis in 12.5% SDS-PAGE. After electrophoresis, the protein gel was stained by blue silver (Candiano et al., 2004) and the result was recorded by a flat-bed scanner (WD-9401A, Beijing Liuyi Biotechnology Co., Beijing, China). Western blot was applied to measure exosome-specific antibodies Lamp1, Lamp2, MHC II, CD81 and CD86. Meanwhile, annexin V, antibody specific for apoptotic bodies, was used as a comparison to detect the purification of the samples. After electrophoresis, the protein samples from SDS-PAGE were transferred to PVDF membrane by a TE 77 semi-dry transfer box (GE Healthcare, Chicago, IL). Chemiluminescence detection was performed via Pierce Fast Western Blot kit (Thermo Fisher Scientific, Waltham, MA) according to the kit instructions. The experiment was repeated three times.

Flow cytometry

Exosomes were coupled with beads in the way previously reported in the literature (Théry et al., 2001). The beads were processed with LAMP1 antibody (1 : 5000, BD Biosciences, Franklin Lakes, NJ), LAMP2 antibody (1 : 5000, BD Biosciences), MHC II antibody (1 : 5000, BD Biosciences, NJ), CD81 antibody (1 : 1000, BD Biosciences,), CD86 antibody (1 : 1000, BD Biosciences)

and annexin V antibody (1 : 1000, BD Biosciences) at room temperature for 1 h, and then incubated with FITC-conjugated secondary antibody (1 : 100 dilution) for 30 min. Analysis was performed by a FACSCalibur ™ flow cytometer (Becton-Dickinson, Fullerton, CA). The experiment was repeated three times.

Mass spectrometry analysis

After treatment with trypsin, exosome protein samples were separated preliminarily in a Waters HPLC system (series 2695) with a polyLC polysulphoethyl aspartamide column (100 mm × 2.1 mm, 5 μm, 300 A pore size) (Waters, Milford, MA). The exosome protein samples were co-cultured with alveolar macrophages from mice and *T. marneffei* for 1 h, 24 h and 48 h, and separated by the 40-minute continuous gradient separation method. Peptides were analysed in an LTQ-Orbitrap Elite hybrid mass spectrometer coupled with the Easy-nLC 1000 nanoflow liquid chromatography system (Thermo Fisher Scientific). The mass spectrometry results were used to search in the UniProt protein database by Proteome Discoverer 1.3 software. The identified proteins were filtered with high peptide confidence. The experiment was repeated three times.

Animal experiments

BALB/c mice (SPF (Beijing) biotechnology co., LTD., Beijing, China, http://www.spf-tsinghua.com/en/) aged 6 to 8 weeks were housed in an animal room with 14-h light/10-h dark cycle, temperature of 22 ± 2 °C, humidity of 50 %, and provided with water and food. All animal experiments were approved by the Ethics Committee of Guangxi Zhuang Autonomous Region People's Hospital.

A total of 50 BALB/c mice were randomly divided into the control group and model group (N = 25 per group). Mice in the model group were injected with a sublethal dose of spores (3×10^5 spores per mice) through the tail vein, while mice in the control group were injected with the same volume of sterile saline. Five mice were euthanized on the 7^{th}, 14^{th}, 21^{st}, 28^{th} and 35^{th} day after inoculation, respectively, collecting the serum. After sterilizing the mice with alcohol, they were covered with a sterile cloth to separate the lungs, spleen and mediastinal lymph nodes. The tissue was placed in an EP tube containing RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) placing it on ice and isolating cells to get a single-cell suspension.

Isolation and processing of bone marrow-derived macrophages (BMMs)

Mice were sterilized with 75% ethanol and the legs were dissected, removing excess muscle tissue from the pelvis and femur, and amputating at the knees to ensure bone integrity. In a sterile state, the bones were immersed in 75 % ethanol for 5 min and washed three times with PBS for 5 min, then the bones were placed in RPMI 1640 medium. After cutting off the other end of the bone, the bone marrow was blown out of the bone with a syringe filled with culture medium and repeated pipetting to make it evenly mixed, then cultured in a cell incubator at 37 °C. The medium was changed every three days. During the culture process, suspended cells were sucked into new petri dishes and the good cells were put to use. The isolated bone marrow-derived macrophages (BMMs) were cultured with exosomes for 24 h and lysed with frozen lysis buffer. The lysate was centrifuged at 16,000 *g* to obtain the supernatant for subsequent detection.

Enzyme-linked immunosorbent assay (ELISA)

The expression levels of TNF-α, RANTES and IL-12 were measured by ELISA kits (Elabscience, Houston, TX; E-EL-M0049c, E-EL-M0009c, E-EL-M2451c) according to the manufacturer's instructions. One hundred μl of standard solutions or samples was added to each well and incubated for 90 min at 37 °C. After removing the liquid, 100 μl of biotinylated detection antibody was added and incubated for 1 h at 37 °C. After washing three times, 100 μl of HRP conjugate was added into each well, followed by incubation for 30 min at 37 °C. After washing for five times, 90 μl of substrate reagent was added into the well and incubated for 15 min at 37 °C. After adding 50 μl of stop solution, the optical density was read at 450 nm immediately. The experiment was repeated three times.

Statistical analysis

The data were expressed as mean ± SD and processed using the SPSS version 20.0. Statistical differences between groups were determined by one-way or two-way ANOVA. The P value < 0.05 was considered as statistical difference.

Results

Exosomes secreted by co-culture of T. marneffei and macrophages have antigen presentation function

The morphology of exosomes extracted from the co-culture medium of macrophages and *T. marneffei* spores was observed by transmission electron microscopy. As shown in Fig. 1A, elliptic vesicles with a diameter of about 50 nm were clearly observed, which conformed to the general morphological structure and size of exosomes. Then, the exosome samples collected from each time point were detected by Western blot. The results showed that the total protein content of exosomes in the cell culture increased gradually with the extension of culture time, and reached the maximum at 48 h. The total protein amount of exosomes did not change significantly with longer culture time than that (Fig. 1B). However, the protein expression of exosome-specific antibodies LAMP1 and LAMP2 increased gradually with the extension of time. The specific band of annexin V, antibody specific for apoptotic bodies, was not found in each sample, which was consistent with the re-

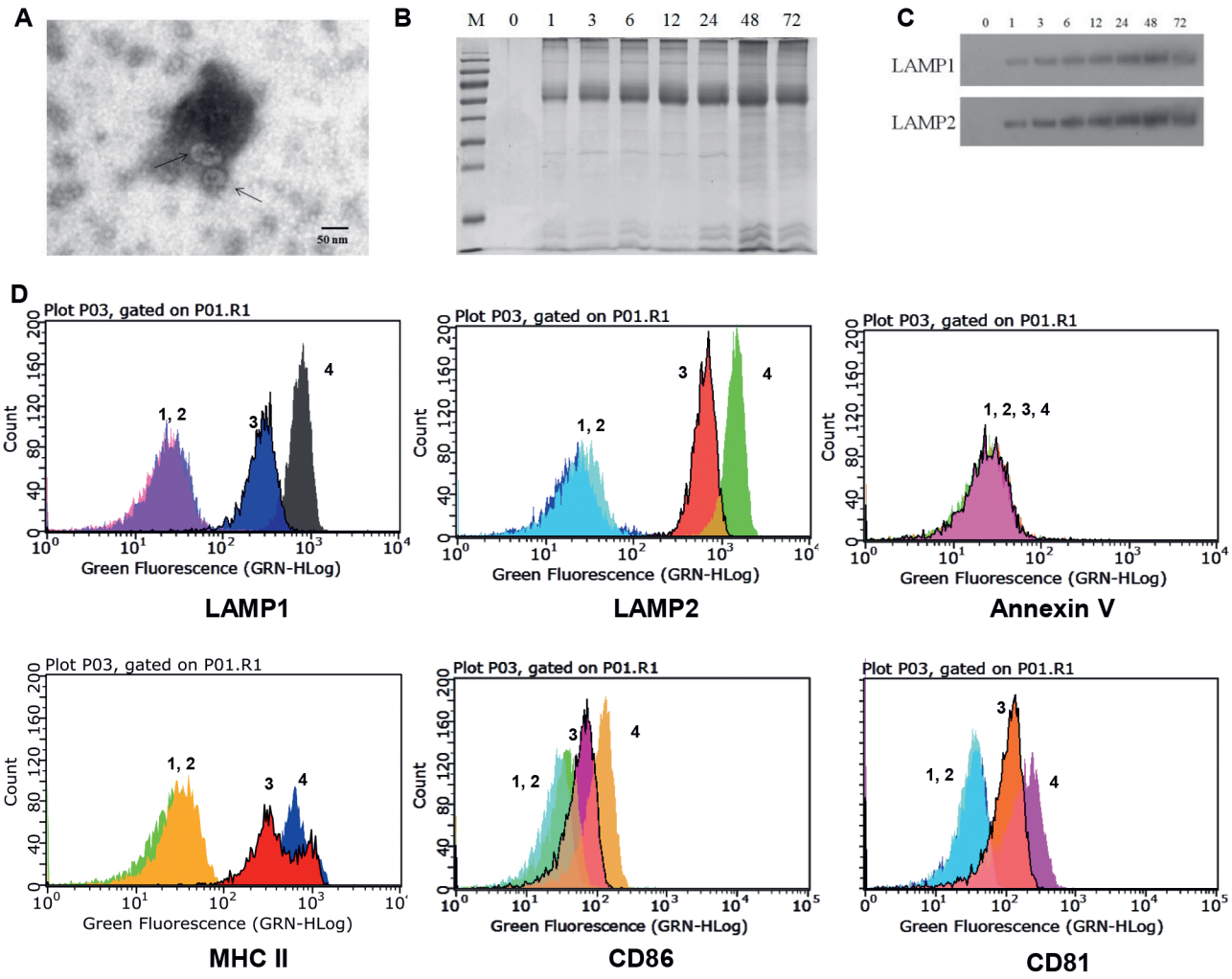

Fig. 1. Exosomes secreted by co-culture of *T. marneffei* and macrophages have antigen presentation function. (**A**) The morphology of exosomes extracted from the co-culture medium of macrophages and *T. marneffei* spores was observed by transmission electron microscopy. (**B**) The protein content of exosome samples collected from each time point was detected by SDS-PAGE. (**C**) The protein expression of LAMP1 and LAMP2 in exosome samples collected from each time point was detected by Western blot. (**D**) The expression of LAMP1, LAMP2, annexin V, MHC II, CD86 and CD81 was detected by flow cytometry. 1: FITC isotype control; 2: no treatment; 3: exosomes secreted by macrophages after *T. marneffei* treatment for 1 h; 4: exosomes secreted by macrophages after *T. marneffei* treatment for 48 h.

sults observed in flow cytometry, indicating that the exosome samples, instead of apoptotic bodies, with similar physicochemical properties were successfully obtained in this experiment (Fig. 1C and D). MHC II, CD81 and CD86 are exosome marker proteins and also show evidence of the antigen presentation response. As shown in Fig. 1D, the expression of MHC II, CD81 and CD86 protein was up-regulated in exosomes secreted by co-culture of *T. marneffei* and macrophages at 1 h and 48 h, which suggested that exosomes released by macrophages have an antigen-presenting effect on *T. marneffei* pathogens.

Protein mass spectrometry analysis of exosomes

We used a peptide no greater than 1 and a confidence interval no less than 95 % ($P < 0.05$) as the cut-off values for protein identification. A total of 27, 36 and 347 proteins were identified from the exosomes produced by mouse alveolar macrophages and *T. marneffei* co-cultured for 1, 24 and 48 h, respectively (Fig. 2A). Subsequently, we used clusterProfiler R package to perform Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis for the proteins that were only found after co-culture of *T. marneffei* and macrophages for 48 h and proteins found in all three sample groups. Enrichment analysis is based on the principle of hypergeometric distribution. The most significant 30 terms of GO enrichment analysis are shown in Fig. 2B. In the proteins only found in exosomes secreted from co-culture of mouse alveolar macrophages and *T. marneffei* for 48 h, most were associated with

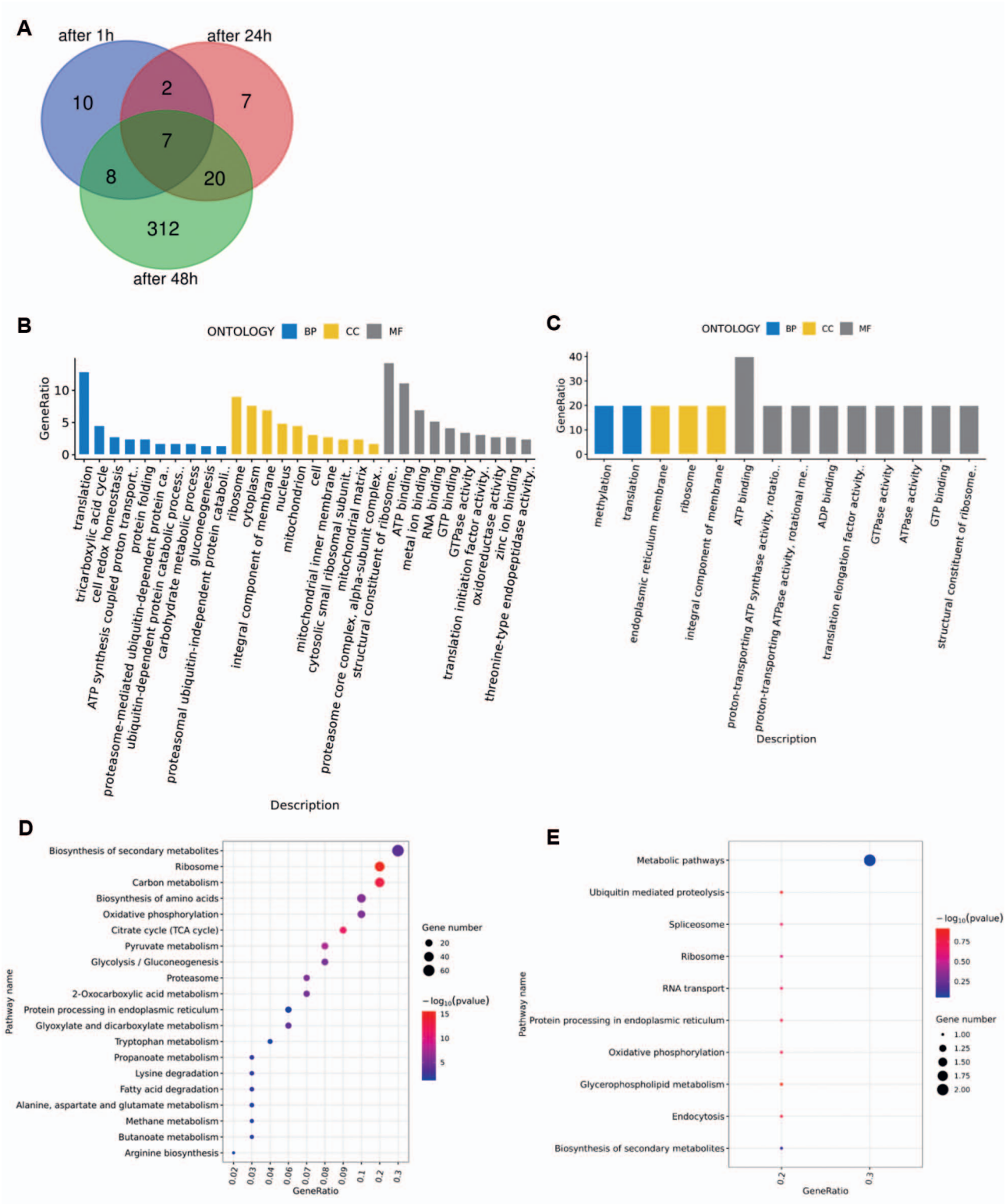

Fig. 2. Protein mass spectrometry analysis of exosomes. (**A**) Venn diagram showing protein groups detected only in the exosomes secreted from mouse alveolar macrophages and *T. marneffei* co-cultured for 1 h, 24 h and 48 h. (**B-C**) Gene Ontology (GO) functional enrichment analysis and (**D-E**) Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis for the proteins that were only found after co-culture of *T. marneffei* and macrophages for 48 h and proteins found in all three sample groups (mouse alveolar macrophages and *T. marneffei* co-cultured for 1 h, 24 h and 48 h).

translation. In the cellular component (CC), most proteins were distributed in the ribosomes, cytoplasm and integral components of the membrane. The proteins of molecular function (MF) class were mainly involved in binding and catalytic activity (Fig. 2B). Concerning the proteins found in all three sample groups, the most enriched GO terms were methylation and translation (biological process, BP), membrane components and ribosomes (CC), and ATP binding (MF) (Fig. 2C). From the KEGG enrichment results, we found that the top three relevant pathways in the proteins only found in exosomes secreted from co-culture of mouse alveolar macrophages and *T. marneffei* for 48 h were biosynthesis of secondary metabolites, ribosomes and carbon metabolism (Fig. 2D) Biosynthesis of secondary metabolites was also the most relevant pathway in the proteins found in all three sample groups (Fig. 2E).

Exosomes secreted by co-culture of T. marneffei and macrophages activate sensitized T lymphocytes and induce the inflammatory response

The expression of CD69 and IFN-γ in T lymphocytes was detected by flow cytometry. As shown in Fig. 3A, 13.21 % of the spleen T lymphocytes were $CD69^+$ $IFN\text{-}\gamma^-$, 20.21 % were $CD69^+$ $IFN\text{-}\gamma^+$, 6.23 % were $CD69^-$ $IFN\text{-}\gamma^+$, 60.35 % were $CD69^-$ $IFN\text{-}\gamma^-$. In the mediastinum, 15.22 % of T lymphocytes were $CD69^+$ $IFN\text{-}\gamma^-$, 19.82 % were $CD69^+$ $IFN\text{-}\gamma^+$, 5.89 % were $CD69^-$ $IFN\text{-}\gamma^+$, 59.07 % were $CD69^-$ $IFN\text{-}\gamma^-$. In the lung, 13.85 % of T lymphocytes were $CD69^+$ $IFN\text{-}\gamma^-$, 16.82 % were $CD69^+$ $IFN\text{-}\gamma^+$, 2.67 % were $CD69^-$ $IFN\text{-}\gamma^+$, 66.66 % were $CD69^-$ $IFN\text{-}\gamma^-$. It was proved that exosomes released by *T. marneffei*-treated macrophages activated T lymphocytes in the spleen, mediastinum and lung of mice. At the same time, the isolated BMMs were cultured with exosomes for 24 h. The expression levels of TNF-α, RANTES and IL-12 were measured by ELISA kits. As shown in Fig. 3B, the expression level of TNF-α, RANTES and IL-12 increased at first and then decreased, while the expression level of TNF-α reached the highest level on the 21st day and the expression level of RANTES and IL-12 reached the highest level on the 14th day.

Discussion

T. marneffei is a kind of pathogenic fungus, which can cause life-threatening systemic mycosis in the host with low immune function (Lu et al., 2013). Therefore, it is essential to explore how the pathogen reacts with the immune system. Many studies have shown that exosomes secreted by immune cells are important mediators in the immune response and antigen presentation (Denzer et al., 2000; Bhatnagar et al., 2007; Greening et al., 2015). A previous study (Wang et al., 2013) showed that exosomes secreted by macrophages treated with *Mycobacterium avium* (*M. avium*) displayed a signifi-

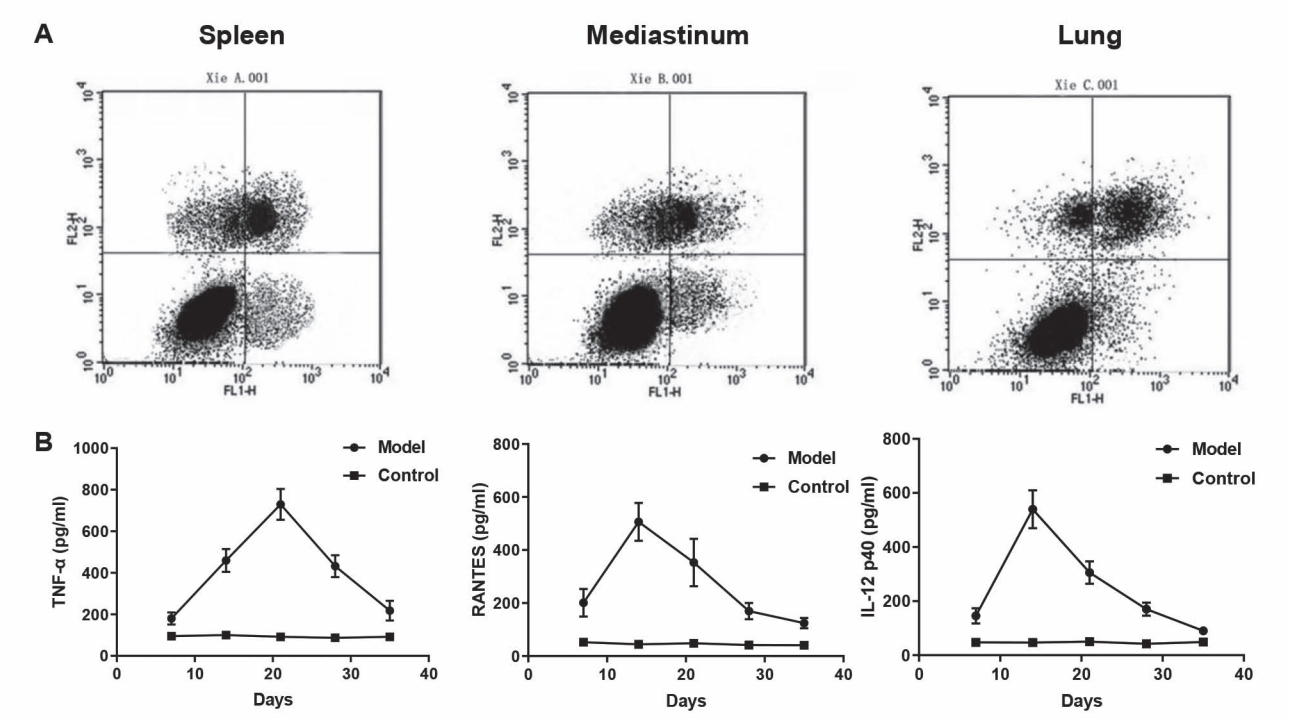

Fig. 3. Exosomes secreted by co-culture of *T. marneffei* and macrophages activate sensitized T lymphocytes and induce the inflammatory response. (**A**) The numbers of T lymphocytes in the spleen, mediastinum and lung were detected by flow cytometry. FL1: FITC/IFN-γ; FL2: PE/CD69. (**B**) The levels of TNF-α, RANTES and IL-12 p40 were examined using ELISA.

cant protein expression difference compared with those secreted by untreated macrophages. *In vitro* experiments showed that these exosomes induced macrophages to secrete TNF-α, IFN-γ, and promoted the inflammatory response, which suggested that macrophage-treated exosomes may be carriers of components from the treated macrophages leading to responses in resting cells (Wang et al., 2013). According to the observation of transmission electron microscopy, we also determined that macrophages and *T. marneffei* released exosomes after co-culture. Therefore, we hypothesized that these exosomes carried the components of pathogens and were capable of leading to antigen presentation.

In *in vitro* and *in vivo* experiments, via flow cytometry to analyse the surface marker proteins of exosomes and T cells, exosomes secreted by co-culture of *T. marneffei* and macrophages were found effective in antigen presentation, promoting the inflammatory response of macrophages and initiating the adaptive immune response. Analysis of TNF-α, RANTES and IL-12 expression levels by ELISA confirmed this finding. Analogously, a study by Singh et al. (2012) on exosomes released from *Mycobacterium tuberculosis*-treated macrophages also showed that after treatment with exosomes released from *Mycobacterium tuberculosis*-treated RAW264.7 cells, bone marrow macrophages in C57BL/6 mice secreted chemokines at a significant level and induced migration of CFSE-labelled macrophages and spleen cells. Thus, exosomes might play an important role in recruiting and regulating host cells during the infection. Hassani and Olivier (2013) showed that macrophage-secreted exosomes could induce signal molecules and transcription factors in primitive macrophages, and the expression of immune related genes. Wang et al. (2013) found that exosomes secreted by *M. avium*-infected macrophages contained a large number of *M. avium* antigens and host cell antigens, which were involved in the induction and expression of the macrophage inflammatory response. Due to the presence of antigens, although exosomes had no effect on macrophage viability, they could trigger the inflammation response in macrophages. Even though the pathogens in the above studies were different from *T. marneffei* in this study, our study also suggested that exosomes secreted by macrophages are significant in the immune process.

Moreover, mass spectrometry analysis of exosome proteins from mouse alveolar macrophages treated with *T. marneffei* for 1, 24 and 48 h was performed. We found that the number of exosome proteins was remarkably increased 48 h after *T. marneffei* infection, suggesting that the occurrence of antigen presentation took a certain time. Functional analysis of these proteins based on GO annotations showed that only the specific proteins secreted 48 h after *T. marneffei* infection were similar to those found in the three sample groups. Both GO and KEGG results suggested that the process of *T. marneffei* infection of macrophages was associated with translation, metabolism, related biosynthetic processes and ribosomes. This indicated that the *T. marneffei* infection process and the antigen presentation process occurring in the host have a certain relationship with the related processes mentioned above. These results could provide a general direction for future research, thus accelerating search for important genes related to the *T. marneffei* infection process.

Although we found that exosomes secreted by co-culture of *T. marneffei* and macrophages had an antigen-presenting effect, we further analysed the biological information of their protein or peptide components. However, the molecular mechanism of *T. marneffei* and macrophage co-culture is still unclear. Our study will not only provide a theoretical basis for the development of diagnostic reagents and effective vaccines in the future, but also support future research in this field.

In summary, *T. marneffei* and macrophage co-culture generates exosomes to activate the body's immune response system and pro-inflammatory response, which may reflect the recognition mode between *T. marneffei* and the host immune system. The occurrence of these processes in cells is closely related to translation and synthesis of metabolites, in which ribosomes play an important role.

Conflicts of interest

All authors declare that they do not have any competing interests.

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