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THE EFFECT OF MACROPHAGES RAW 264.7 DAMAGE AND DIFFERENTIATION INDUCED BY TALAROMYCES MARNEFFEI CONIDIA

Alya Alnasri, Xiaoyue He, Donghua Liu* & Cheng Peng

(M.B.B.S) Department of Dermatology and Venereology, First Affiliated Hospital, Guangxi Key Laboratory of AIDS Prevention and Treatment, Guangxi Medical University, Nanning, China

(M.D. Dermatology) Department of Dermatology and Venereology, First Affiliated Hospital, Guangxi Key Laboratory of AIDS Prevention and Treatment, Guangxi Medical University, Nanning, China

(D.M. Dermatology) Corresponding author*, Department of Dermatology and Venereology, First Affiliated Hospital, Guangxi Key Laboratory of AIDS Prevention and Treatment, Guangxi Medical University, Nanning, China

(M.D. Dermatology)Department of Dermatology and Venereology, First Affiliated Hospital, Guangxi Key Laboratory of AIDS Prevention and Treatment, Guangxi Medical University, Nanning, China

Keywords:

Talaromyces marneffei;
Virulence;
Macrophage;
Polarization.

Abstract

Talaromyces marneffei can survive in host macrophages and cause life-threatening human infection in Southern Asia. The ability of *T.marneffei* conidia to survive in host macrophages allows infections to become more serious and thus more difficult to treat. The interaction between *T.marneffei* conidia and host macrophages remains poorly understood. This work is aimed at study the effect of macrophage Raw 264.7 damage and differentiation induced by *Talaromyces Marneffei* conidia. Macrophages viability, apoptosis and necrosis rates, cytokines production and cell polarization were assessed after macrophages RAW 264.7 were co-cultured with *T.marneffei* conidia through WST-8, Annexin V-FITC/PI flow cytometry, ELISA methods, and Immunofluorescence assays. It was found the viability of macrophages decreased significantly, whilst the apoptosis rate and necrosis rate increased significantly after macrophages were co-cultured with *T.marneffei* conidia; IL-1β secretion decreased whereas TNF-α and IL-10 secretion increased significantly. In addition, immunofluorescence assays showed that the conidia induced macrophages to express CD206. The results served to indicate that *T.marneffei* may cause damage to macrophages and manipulate differentiation in them.

Introduction

Talaromyces marneffei (syn. *Penicillium marneffei*) is an emerging pathogenic fungus that can trigger fatal systemic mycosis in patients, especially those infected with human immunodeficiency virus (HIV) [1, 2]. *T.marneffei* infection is endemic to tropical Asia, particularly Thailand, North Eastern India, China, Hong Kong, Vietnam, and Taiwan [3-7]. It has the capability to form a filamentous mold at 25°C, and produce asexual spores (conidia) on special cell structures. In contrast, at 37°C, the morphology of *T. marneffei* changes to develop as pathogenic unicellular yeast cells that divide by fission. The exact route of *T. marneffei* infection is unknown. However, some aerosolized *T. marneffei* conidia may be dispersed from an environmental reservoir, thus generating infectious conidia that able to infect persons[7]. Although *T. marneffei* infection is not limited to HIV-infected patients alone, it is continually recognized that more non-HIV-infected patients are at risk of the infection [8].

Macrophage differentiation is displayed in response to microbial agents, where macrophages acquire distinct polarized programs to develop different activated types. Macrophages differentiate into distinct effectors including

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classically (M1) and alternatively (M2) activated types [9-11]. Classically, M1 macrophages are implicated in initiating and sustaining inflammation whereas M2 macrophages differentiate later and are involved in the resolution of inflammation and tissue regeneration[12]. M1 and M2 macrophages are usually distinguished from cytokines secretion, cell surface markers, and arginine metabolic pathways. M1 macrophages can produce high levels of IL-12 and IL-23 but a low level of IL-10. They can also generate large amounts of NO and other inflammatory constituents such as TNF-α, IL-1, IL-6, a typical M1 macrophage marker being CD16/32[13, 14]. M2 macrophages can secrete a high level of IL-10, but low-levels of IL-12 and IL-23,typical M2 macrophage markers being CD206 and CD163[15-17]. Once *T. marneffei* conidia are inhaled, the pathogen proliferates first in the reticuloendothelial system before disseminating throughout the host. The strategies employed by *T. marneffei* conidia to escape from macrophages phagocytosis play an important role in causing systemic infection and lead to persistent infection in the body.The effects of pathogenic *T.marneffei* conidia on the cell function of host macrophages remains unclear. In the present study, we examined the effect of *T.marneffei* on macrophages growth, cytokines production, and cell polarization when macrophages were co-cultured with the conidia in vitro.

Materials and methods

2.1 Fungal strain and macrophage cells

A clinical isolate of *T.marneffei* (GXMU139011, isolated from a patient with disseminated infection), was used in this study. The isolate was stored and had been maintained at the first affiliated hospital of Guangxi medical university identified by morphology and molecular biology. Mice macrophages RAW 264.7 were purchased from the Chinese Academy of Sciences Cell Bank.

2.2 Culture preparation and infection

T. marneffei was cultured on potato dextrose agar (PDA) at 25°C. To obtain conidia of *T. marneffei*, colonies grown on PDA at 25°C were flooded with PBS and collected by filtration. The conidia were transferred into a flask with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum to shake culture at 200rpm/min, 37°C. The number of conidia was counted with a hemocytometer (about 1.2×10^9/ml) and the conidia were collected through a 0.22-μm filter. Macrophages Raw 264.7 were cultured in DMEM supplemented with 10% fetal bovine serum at 37℃ in a humidified atmosphere containing 5% CO2. Macrophages with a cell density of 4.0×10^4/ml were prepared for use. Macrophages challenged with *T. marneffei* conidia were cultured in DMEM at 37°C for 24 hours and 48 hour periods and morphological changes were observed under the microscope.

2.3 Cell viability

The cell viability of macrophages was tested by WST-8[18, 19], and after 24 and 48 hours of being co-cultured with *T. marneffei* conidia, macrophage cells were washed with PBS, 90ul of new culture solution and 10μl of Cell Counting Kit-8 (Beyotime Institute of Biotechnology) being added to each well and the plates incubated for 2 hours. Absorption at 450nm was determined, setting the reference wavelength at 630nm, using a microplate reader. The control group was designated as macrophages cultured without the conidia in DMEM.

2.4 Apoptosis and Necrosis assays

Using the same Incubation methods as described above, the apoptosis and necrosis of macrophages challenged with culture filtrates were analyzed by Annexin V-FITC/PI flow cytometry. Living cells can't be stained, while apoptotic cells can be marked by Annexin V and necrotic cells can be marked by PI. After 24 and 48 hours of co-culture, cells were collected by centrifugation at 2000 rpm for 5 min and washed with PBS before being collected by centrifugation again. The harvested cells were resuspended in annexin-binding buffer, and then 5μl of the annexin V-FITC was added to each 100 μl of cell suspension, with a dead-cell indicator, propidium iodide, also being added. Soon after, the cells were incubated with the stain at room temperature for 15 minutes before analysis was carried out by flow cytometry. Once more, the control group was designated as macrophages cultured without the conidia in DMEM.

2.5 Detection of cytokines production

Macrophages were incubated with the conidia of *T. marneffei* at 37°C for 24 and 48 hour periods and supernatants were collected by centrifugation at 2500 rpm for 20 minutes. The level of TNF-α, IL-1β, IL-12, and IL-10 in the

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supernatants was detected by ELISA methods, following manufactory instructions. The absolute cytokine level present in the samples was calculated based on a standard curve provided by the commercial kit. Again, the control group was designated as macrophages cultured without the conidia in DMEM.

2.6 Immunofluorescence assays

Macrophages were incubated with the conidia of *T. marneffei* at 37°C for 24 and 48-hour periods. After incubation, the macrophages were washed with PBS and fixed with 4% paraformaldehyde for 30 minutes. The slides were washed three times with PBS and then incubated with 5% BSA-TBS for 40 minutes at room temperature to block non-specific binding. After washing with PBS, the slides were incubated with CD16/32 antibody and CD206 antibody (CD16/32 being marked by PE, CD206 being marked by FITC, Biolegend) for 48 hours at 4℃. After washing three times with PBS, the slides were stained with DAPI and washed again three times with PBS. Coverslips were applied and slides were examined under a fluorescence microscope. Macrophages cultured with 0.1ug/ml lipopolysaccharide without the conidia were designated as the positive control group and macrophages cultured without the conidia in DMEM were designated as the negative control group.

2.7 Statistical analysis

In this study, Statistical significance was determined with one-way analysis of variance, two-sample t-tests and a paired t-test and $P < 0.05$ indicated a significant difference. Assays were performed three times, and the mean ± standard deviation was computed.

Ethical statement: Not applicable.

Results

3.1 Effect of the conidia on macrophage damage

The treated macrophages appeared as anenlarged, swollen, rounded cells, even cytoplasmic vacuoles and cell disintegration were observed. Macrophages co-cultured with *T.marneffei* conidia for 24 hours which have a low number of living cells compared with the control group. After 48 hours of co-culture, the viability of macrophages significantly decreased in comparison with the control group, so the longer time co-cultured theless viability of macrophages ($P < 0.001$; Figure.1).

The apoptosis and necrosis rates were found to exceed the control after both 24 and 48 hours of co-culture. Both the apoptosis rate and necrosis rate of macrophages increased significantly after 48 hours when compared to 24 hours of co-culture with the conidia in vitro ($P < 0.05$; Figure.2)

3.2 Cytokines production of macrophage

After macrophages were co-cultured with *T.marneffei* conidia for 24 and 48 hours, it was found that the treated macrophages secreted more TNF-α and IL-10 but less IL-1β than the control ($P<0.05$). There was no statistical difference between the control and the treated groups regarding IL-12 secretion. After 48 hours of co-culture with the conidia, the concentration of macrophages IL-10 was reduced when compared with the control group ($p<0.05$) after 24 hours of co-culture. Other cytokines exhibited no statistical difference between the two groups. (Figure.3)

3.3 CD16/32 and CD206 expression of macrophage

After macrophages were co-cultured with *T.marneffei* conidia for 24 and 48 hours, CD206 expression (green fluorescence) in the cytoplasm of macrophages was observed under fluorescence microscope examination. The intensity of CD206 fluorescence in macrophages treated for 24 hours was stronger than those treated for 48 hours but CD16/32 expression was not observed. CD16/32 expression (red fluorescence) was observed in macrophages treated by lipopolysaccharide without being co-cultured with *T.marneffei* conidia. CD206 was not apparent in macrophages cultured without *T.marneffei* conidia but after 48 hours of being co-cultured, CD206 exhibited weaker fluorescence intensity when compared with the group ($p<0.05$) after 24 hours of co-culture. (Figure.4)

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Discussion

So many studies have been undertaken on *T.marneffei*'s ability to survive in host macrophages, but the mechanism as to how *T.marneffei* can not only survive but also cause damage to macrophages has not been clearly established[20, 21]. In the current study, the data demonstrated that macrophages decreased viability and increased the apoptosis and necrosis rates when macrophage cells were challenged with *T.marneffei* conidia. Both the apoptosis and necrosis rates increased significantly after 48 hours when compared to 24 hours of being co-cultured with the conidia in vitro. It suggested that the conidia play an important role in causing damage to macrophage cells and the longer the time the more serious the damage. It is accepted that several fungi have the ability to cause damage to host macrophages. Cellular functions damaged during infection of macrophages by *Cryptococcus neoformans* were associated with the intracellular pathogen affected protein translation and caused mitochondrial depolarization alterations [22]. *Histoplasma capsulatum* displayed a unique organic arrangement as yeast-aggregates, which was able to cause damage in the nuclear DNA and induce apoptosis in alveolar macrophages after infection [23]. Basically, an intracellular infection may undermine the function of these critical phagocytic cells, which can render the fungus difficult to rid from the body and, as such, will produce persistent infection.

During evolution, microbes have the ability to access selected nutrients, such as amino acids,which may lead to manipulation of macrophage differentiation[24]. The strain of Mycobacterium bovis, increased growth in macrophages, down-modulated macrophages to M1 polarization[25]. Macrophages infected with *Cryptococcus neoformans* resulted in the development of a weak M1-like phenotype[26]. Our study revealed that macrophages co-cultured with *T.marneffei* may effect on the differentiation of macrophages by conidia. Furthermore, our study showed *T.marneffei* able to induce macrophages to produce high levels of IL-10 and low levels of IL-1β. Additionally, M2 surface marker CD206 expression was observed, which may indicate *T.marneffei* conidia can induce macrophages to M2 polarization. However, a high level of TNF-α was also detected, even though CD16/32 expression was not observed by immunofluorescence assay when the macrophages were challenged with the conidia, maybe demonstrating that weak M1 type activation was induced, The level of TNF-α was higher than the control group after 24 and 48 hours of co-culture with *T.marneffei* conidia, which may be associated with several kinases signaling pathways activated by *T.marneffei*[27, 28]. After 48 hours of co-culture with the conidia, macrophages IL-10 concentration was reduced ($p<0.05$) and the mark of CD206 exhibited weaker fluorescence intensity when compared with the group after 24 hours of co-culture. It may indicate that the pattern of macrophages interacting with the conidia was more like a dynamic transformation process [29, 30]. In addition, these findings q that both M1 and M2 macrophages may be activated by *T.marneffei* conidia, it tends to stimulate macrophage polarisation to M2 macrophages. However, although the shift of macrophage to M2 appears unavoidable in order to control inflammation and limit tissue damage, it does not eradicate pathogens[31].

Conclusion

This study demonstrated that *T.marneffei* conidia may possess the ability to cause damage to macrophages and manipulate them so as to develop to M2 phenotype. However, the mechanism to how *T.marneffei* can survive within the host macrophage, causing damage and manipulating macrophages differentiation in vitro, requires further study.

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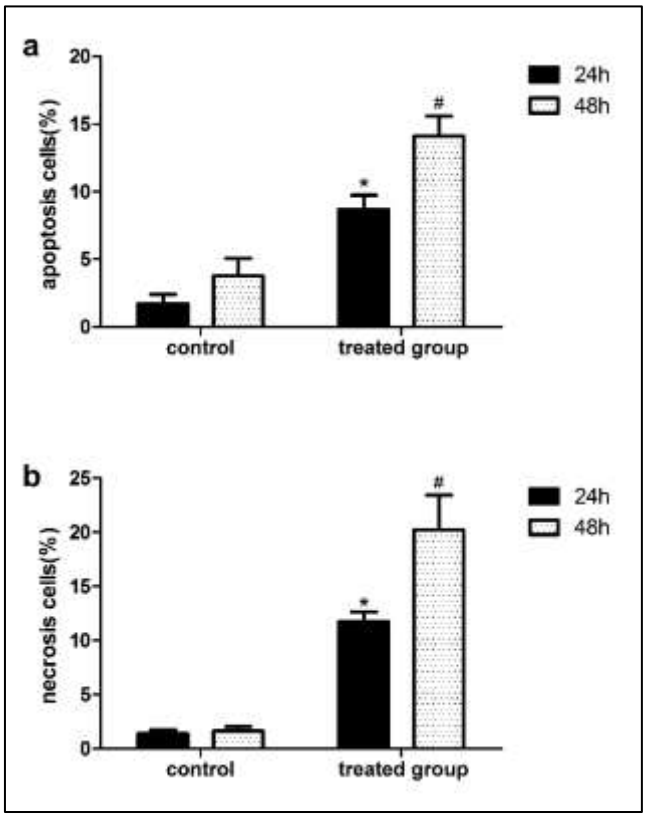

Figure 1 Effect of T.marneffei conidia on macrophages apoptosis and necrosis

*Macrophages were co-cultured with T.marneffei conidia. The apoptosis rate and necrosis rate of macrophages were assayed by flow cytometry in comparison with control. (a)The apoptosis rate of macrophages after 24h and 48h co-cultured.(b) The necrosis rate of macrophages after 24h and 48h co-cultured.Data are expressed as the mean ± standard deviation(N = 3).Significant difference both in necrosis rate and apoptosis rate between the tested conditions compared with controls in the same time period: *P<0.05;#P<0.05.*

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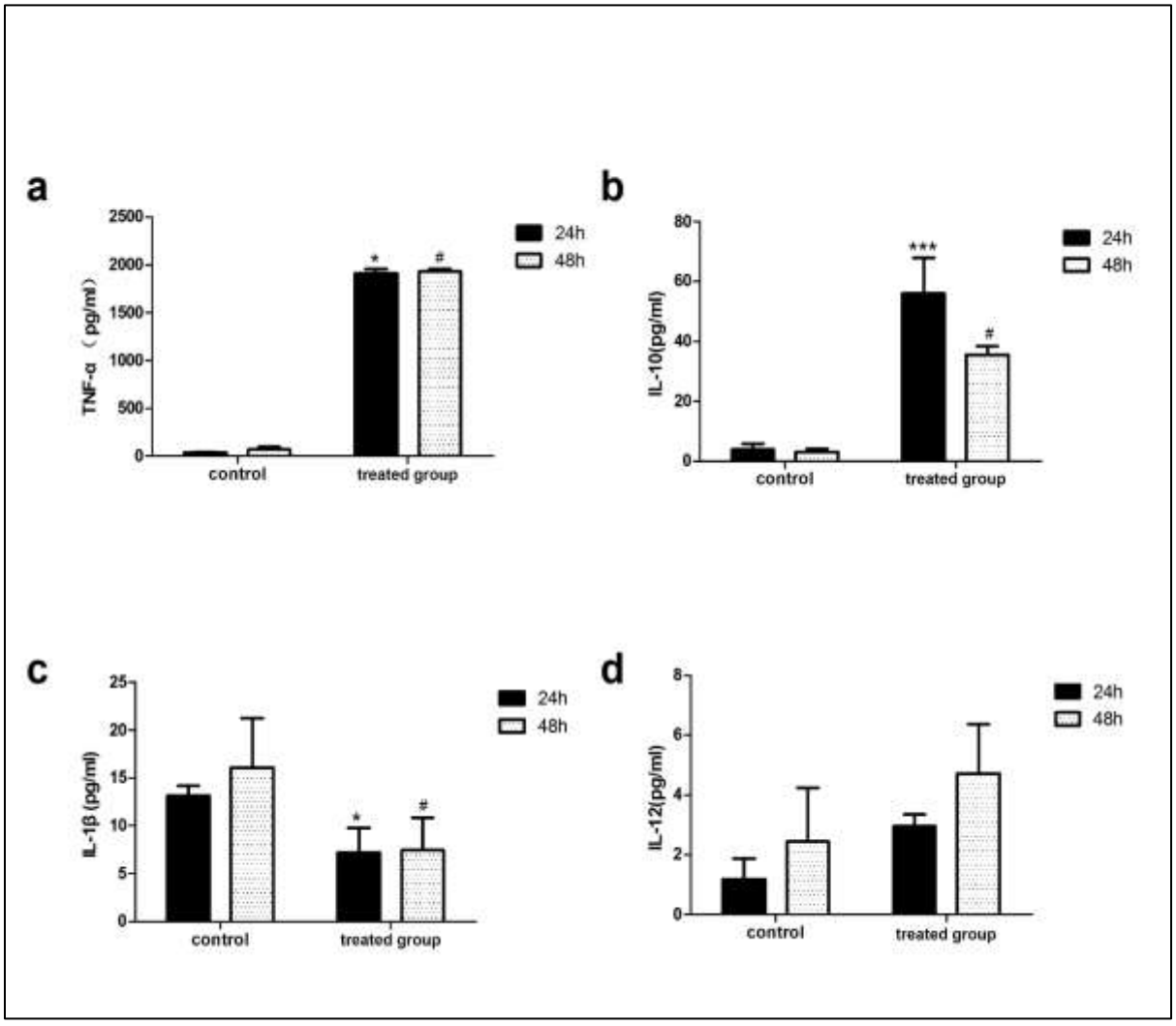

Figure 2Effect of T.marneffei conidia on cytokines production

*Macrophages were co-cultured with T.marneffei conidia. The supernatants were harvested and cytokines were measured by ELISA in comparison with control. (**a**) The TNF-α production after 24h and 48h co-cultured. (**b**) The IL-10 production after 24h and 48h co-cultured. (**c**) The IL-1βproduction after 24h and 48h co-cultured. (d) The IL-12 production after 24h and 48h co-cultured.Data are expressed as the mean ± standard deviation(N =3).Significant differences amongst groups of each cytokines productions: (**a**)*P<0.001, #p<0.001;(**b**)***P<0.001,#p<0.001;(**c**)*p<0.01, #p<0.05.*

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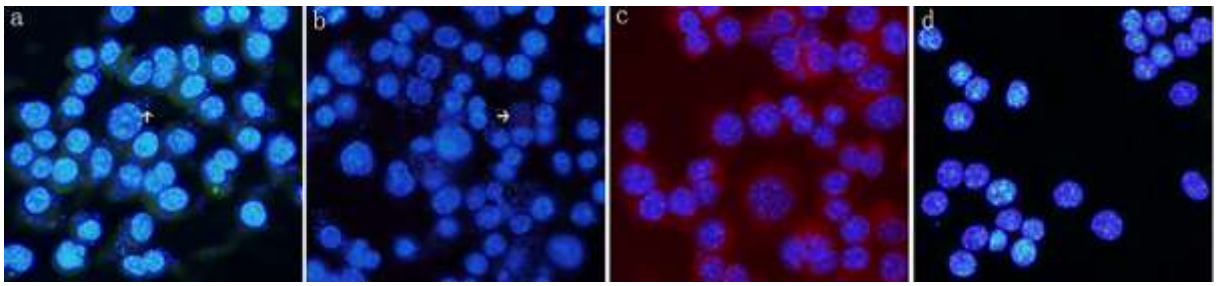

Figure 3Effect of T.marneffei conidia on CD16/32 and CD206 expression

*Macrophages were co-cultured with T.marneffei conidia. CD16/32 and CD206 expression of macrophages were assayed by Immunofluorescence method. (Magnification ×400). (**a**) CD206 expression (green fluorescence) was observed under microscope. The white arrow showed the conidia ingestion by macrophage. (**b**) CD16/32 expression was not observed under microscope. (**c**) CD16/32 expression (red fluorescence) induced by lipopolysaccharide was observed under microscope. (**d**) CD206 expression was not observed in control.*

Correspondence:
Dr. Donghua Liu,
Tel: +86-0771-5356752,
Fax: +86-0771-5356562,
E-mail: ldhgxmu@163.com,
Department of Dermatology and Venereology, First Affiliated Hospital,
Guangxi Key Laboratory of AIDS Prevention and Treatment,
Guangxi Medical University, Nanning 530021, China