Role and Mechanism of Cotrimoxazole Resistance to Talaromyces marneffei fungus in vitro

Jie Chen (chenjie1@stu.gxmu.edu.cn)
Guangxi Medical University
Rongfeng Chen
Guangxi Medical University
Wudi Wei
Guangxi Medical University
Fengxiang Qin
Guangxi Medical University
Xiu Chen
Guangxi Medical University
Jinhao He
Guangxi Medical University
Hong Zhang
Guangxi Medical University
Gang Wang
Guangxi Medical University
Minjuan Shi
Guangxi Medical University
Tongxue Qin
Guangxi Medical University
Yinlu Liao
Guangxi Medical University
Yuting Wu
Guangxi Medical University
Beibei Lu
Guangxi Medical University
Xing Tao
Guangxi Medical University
Li Ye
Guangxi Medical University
Hao Liang
Guangxi Medical University
Junjun Jiang

Guangxi Medical University

Research Article

Keywords: Talaromyces marneffei,cotrimoxazole,HIV/AIDS,Dihydropteroic acid synthetase,Dihydrofolate reductase,Dectin-1 signaling pathway

Posted Date: April 26th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1546115/v1

Abstract

Background Talaromyces marneffei (formerly *Penicillium marneffei*) is an important thermally dimorphic fungus endemic which is characterized by one of the most frequent opportunistic infections in HIV/AIDS patients, mainly prevalent in Southeast Asia, southern China, and northeastern India. Cotrimoxazole(CTX) inhibits folic acid synthesis which is important for the survival of many bacteria, protozoa, and fungi, thereby commonly used to prevent several opportunistic infections among HIV/AIDS patients. In addition to preventing other HIV-associated opportunistic infections, CTX prophylaxis are considered to have the potential to prevent *T.marneffei* infection in HIV/AIDS patients receiving antiretroviral therapy (ART). However, the role and mechanism of cotrimoxazole resistance to *T.marneffei* fungus remains unclear.

Methods Human THP-1 macrophages were used as cell model in vitro to explore the role and mechanism of cotrimoxazole resistance towards *T.marneffei*. Cell viability assay and drug sensitivity colony forming units (CFU) experiments were conducted to determine the minimum inhibitory concentration (MIC) of cotrimoxazole inside and outside THP-1 macrophages respectively. Enzyme-linked immunosorbent assay (Elisa) was used to measure the concentration of Dihydropteroic acid synthetase (DHPS), Dihydrofolate synthetase (DHFS) and Dihydrofolate reductase (DHFR) between *T.marneffei* adding TMP/SMX and without adding TMP/SMX group respectively. Real-time fluorescence quantitative PCR(qPCR) was performed to detect the mRNA expression levels in Dectin-1 mediated signaling pathway and downstream inflammatory cytokines including IL-6, IL-10, IL-23A, CXCL8 and TNF-α released by *T.marneffei*-infected macrophages between adding TMP/SMX and without adding TMP/SMX group respectively.

Results Cotrimoxazole can inhibit the proliferation of *T.marneffei* within safe concentration inside and outside THP-1 macrophages. Drug susceptibility results showed the minimal inhibit concentration(MIC) of 1:5 TMP/SMX was ranging from 14/70 to 90/450μg/ml. The MIC of SMX was ranging from 100 to 360μg/ml, The MIC of TMP was ranging from 240 to 400μg/ml outside macrophages. The MIC of TMP/SMX was ranging from 36/180 to 68/340μg/ml. The MIC of SMX was ranging from 340 to 360μg/ml. The MIC of TMP was ranging from 320 to 400μg/ml inside macrophages. The synergistic interaction of 1:5 TMP/SMX was more effective in inhibiting *T.marneffei* than separate SMX and TMP. DHPS, DHFS and DHFR can be inhibited by cotrimoxazole within safe and effective concentration. Dectin-1 expression is increased following *T.marneffei* infection, leading to the increase of IL-6, IL-10, IL-23A and the decrease of CXCL8 and TNF-α. Conversely, cotrimoxazole decrease the levels of IL-6, IL-10, IL-23A and increase the levels of CXCL8 and TNF-α, thereby enhancing the intracellular killing-*T.marneffei* capacity of macrophages.

Conclusions Our findings indicated that cotrimoxazole directly inhibited *T.marneffei* growth by blocking DHPS, DHFS and DHFR and indirectly inhibit *T.marneffei* growth by regulating the Dectin-1 signaling pathway, which may effectively interfere with the defense ability of the host against *T.marneffei* infection.

Introduction

Talaromyces marneffei(TM, previously known as *Penicillium marneffei*) is a thermally dimorphic fungus. Talaromycosis is a severe deep mycosis caused by *T.marneffei* which has rapidly emerged in parallel with the HIV epidemic in recent years and has become one of the most common HIV-associated opportunistic infections and is a leading cause of HIV-associated death in Southeast Asia, in particular Thailand, Vietnam, and southern provinces in China[1].Nowadays, *T.marneffei* infection has become the third most common opportunistic infections in patients, ranking just after tuberculosis and cryptococcosis, especially in Guangxi and Guangdong province[2–4]. *T.marneffei* co-infected with HIV/AIDS has become a major medical and public health problem, which urgently requires us attach great importance to and figure out novel prevention and treatment strategies.

Our group previously conducted a observational cohort research in Guangxi, China, which has showed that cotrimoxazole prophylaxis(CTX) initiated early in the antiretroviral therapy period in HIV/AIDS patients can significantly reduce mortality in adult HIV patients, especially in $CD4^+$cell counts < 50 cells/μl population[1].Cotrimoxazole (CTX), a combination of the antibiotics trimethoprim(TMP) and sulfamethoxazole(SMX), is a well-known antimicrobial agent used to treat many opportunistic infections such as *Pneumocystis jirovecii* pneumonia(PJP), malaria, and cerebral toxoplasmosis[1, 5, 6].Its antimicrobial effect comes from its blocking of folate biosynthesis pathway. SMX and TMP target two different enzymes involved in bacterial folate biosynthesis: SMX targets dihydropteroate synthetase(DHPS), while TMP targets dihydrofolate reductase(DHFR)[7–9]. *T.marneffei* fungi also depend on folate biosynthesis pathway to synthesize genetic material, which has been highlighted as an antifungal target for the development of new antifungals[1, 9, 10].Consequently, we hypothesized the intrinsic mechanism of cotrimoxazole directly inhibiting *T.marneffei* growth was interfering with DHPS, DHFS and DHFR, thereby blocking the folate biosynthesis pathway .

A recent study using THP- 1 as cell model in vitro showed that Dectin-1 was an important receptor for *T.marneffei* on THP-1 macrophages and it was revealed to be involved in the induction of a pro-inflammatory cytokine response via increasing levels of TNF-α and CXCL8(IL-8) induced by *T.marneffei.* Dectin-1 is a type □ transmembrane protein, which recognizes β-1,3-glucans in the cell wall of various pathogenic fungi[11, 12]. Despite Dectin-1 playing an important role in regulating host immunity and fungal infection, the mechanism of cotrimoxazole exhibiting fungicidal activity to *T.marneffei* inside macrophages remains to be elucidated.

Collectively, common antifungal drugs including Amphotericin B(AMB), itraconazole(ICA) recently, but long-term use of these drugs can cause significant side effects to the human body, thus it is urgent for us to search other alternative drugs to effectively decrease the morbidity and mortality of HIV/AIDS with *T.marneffei* infection. Our current knowledge of the causality of the protective effect of CTX on TM infection is limited, especially in the interaction between cotrimoxazole and *T.marneffei*-infected hosts. In this study, we focused on the underlying mechanism of cotrimoxazole inhibiting *T.marneffei* in vitro, hoping to provide therapeutic target for HIV/AIDS co-infected with *T.marneffei* infection patients worldwide.

Materials And Methods

Experimental Reagents and Kits

RPMI-1640 medium, DMEM medium and FBS were purchased from Gibco(Thermo Fisher Scientific,Inc.) Penicillin-streptomycin solution and sterile PBS buffer were purchased from Solarbio(Beijing, China), Dimethyl sulfoxide(DMSO) reagent was obtained from BioFROXX(Germany, Inc.). TRIzol® reagent was obtained from Invitrogen (Thermo Fisher Scientific, Inc.).Chloroform, isopropyl alcohol and anhydrous ethanol were purchased from Huawei Ruike Chemical CO.,LTD (Beijing,China). PMA was purchased from Sigma-Aldrich(Merck KGaA). Potato dextrose agar (PDA) was purchased from Land Bridge Technology Co.,LTD (Beijing,China). Sulfamethoxazole (SMX), Trimethoprim(TMP) were purchased from Biological Science and Technology Co.,LTD (Chengdu, China). CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Wanghe Technology Co.,LTD (Guangxi, China). PrimeScript^{TM}RT Master Mix-RR036A Reverse transcription kit and TB Green^{TM}Premix Ex TaqTM -RR820A Fluorescence quantification kit were purchased from Takara Bio, Inc(Japan). Microbial DHPS, DHFS and DHFR elisa kits were purchased from Jingmei Biotechnology Co.,LTD (Jiangsu,China).

Cell Culture and Maintenance

The human monocyte cell line THP-1(The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum(FBS) and 100U/ml penicillin/streptomycin solution at 37℃ in a 5% CO_2 atmosphere. THP-1 cells could be differentiated into macrophages by treatment with 50ng/ml phorbol-12-myristate-13 acetate (PMA) at 37℃ for 48h. The cells were then cultured in medium without PMA. The differentiation morphology of macrophages was observed by a TS100-F type inverted microscope (purchased from Nikon Inc. in Japan).

Fungal culture

T.marneffei [CMCC(F)B33r; The Chinese Academy of Medical Sciences and Peking Union Medical College]strains were cultivated on potato dextrose agar(PDA) mediums and incubated at 27℃ for 15 to 20 days. Fungal spores were harvested by washing the plates with sterile PBS with 0.01% Tween-80(PBST) solution by disposable vaccination ring(10μl).Then the collected spores were purified by filtering through sterile glass wool and PBS to obtain *T.marneffei* conidia. The filtered conidial suspension with the concentration of 10^7 conidia/ml was stored at 4℃ to prepare for subsequent experiments, as previously described[12].THP-1 macrophages were incubated with activated *T.marneffei* conidia (5:1, fungi to cell) cultured at 37℃ in a 5% CO_2 atmosphere for 6-12h to construct *T.marneffei*-infected THP-1 macrophages cell model for subsequent experiments.

Antifungal Agent Preparations

DMSO was used to dissolve drugs according to solubility to obtain 10mg/ml of SMX, 20mg/ml of TMP as primary drug solution respectively. Diluted 10 times, 100 times, 1000 times from 10mg/ml of SMX, 20mg/ml of TMP to obtain gradient dilution of SMX, TMP stock solution. The volume of TMP and SMX solution in the required matching concentration of TMP/SMX (1:5 used in clinic application) were taken from the prepared primary or diluted solution of TMP and SMX(less than 1ml) according to the concentration respectively, then adding sterile PBS to 1ml, and mix the drug solution completely to obtain the required matching concentration of TMP/SMX.

Cell Viability Assay of THP-1 Macrophages

Differentiated THP-1 macrophages were cultured on one 96-well plate at 1×10^4 cells per well, then placing at 37℃ and 5% CO_2 for 48h. Add different concentrations of SMX, TMP and TMP/SMX to macrophages in experimental group after 48h. Negative control group was established with only THP-1 macrophages, not adding drugs. Black control group only contains DMEM medium as background. DMEM medium was replaced by adding 10μl drug solution and 90μl DMEM, and placing the 96-well plate at 37℃, 5% CO_2 atmosphere for 48h.Then 20μl of CellTiter-Glo® Luminescent Cell Viability Assay was added after 48h. The 96-well plate was oscillating in Microporous plate thermostatic oscillator (MB100-2A, purchased from Aosheng Instrument Co.,LTD in Hangzhou, China) for 3 minutes, then incubating at 37℃ for 10 minutes. BioTek Multifunctional enzyme labeling instrument (Synergy H1, purchased from BioTek Inc. in USA) was used to measure the absorbance (OD) of each well at 450nm. Calculating cell viability value according to formula and judge cell viability under different concentrations of TMP, SMX and TMP/SMX.

Antifungal Susceptibility Assays of *T.marneffei* outside macrophages

Add different concentrations of TMP, SMX and TMP/SMX to *T.marneffei* spores for 48h at 37℃ and 5%CO_2 atmosphere. The supernatant was collected after 48h cultivation and four gradient serial dilutions (10^0,10^1,10^2,and 10^3) were performed. Then *T.marneffei* colony forming units (CFUs) by microdilution spot assay was used to assess antifungal activity of TMP, SMX and TMP/SMX in vitro. After placing the potato dextrose agar (PDA) medium at 37℃ for 48h incubation, Linkedin Gel Imaging System (G:Box F3, purchased from Syngene Inc. in USA) was used to record the growth situation of *T.marneffei* on plates. Compare the drug sensitivity of TMP, SMX and TMP/SMX towards *T.marneffei* outside macrophages by counting the number of TM spores at a countable dilution gradient.

Antifungal Susceptibility Assays of *T.marneffei* inside macrophages

THP-1 macrophages were seeded on 24-well plates at 3×10^5 cells per well and were infected with *T.marneffei* conidia (MOI=5:1, fungi to cell) for 6-12h at 37℃ and 5% CO_2 atmosphere. Then, DMEM medium was removed and the wells were washed three times with sterile PBS to clear out the *T.marneffei* spores not infected into THP-1 macrophages. Afterwards, different concentrations of TMP, SMX and TMP/SMX were added in the experimental group respectively, then incubating at 37℃ in a 5%CO_2 atmosphere for 48h. DMEM was removed and the cells were lysed in autoclave water to release the *T.marneffei* inside macrophages. Subsequently, *T.marneffei* spores or hyphae were thoroughly released out, collected the cell lysate and centrifuged for 10min at 12000×g at room temperature, harvested *T.marneffei* precipitation, resuspended in 1ml sterile water. Then perform colony forming units (CFU) experiment as previously described. Record and count the number of TM spores to compare the drug sensitivity of TMP, SMX and TMP/SMX towards *T.marneffei* inside macrophages.

Enzymes Quantification using ELISA

After adding different concentration gradients of TMP, SMX and TMP/SMX to *T.marneffei* spores (3×10^7 spores/ml) at 37℃, containing 5%CO_2 atmosphere for 48h. Microbial DHPS, DHFS and DHFR elisa kits were used to measure the quantity of DHPS, DHFS and DHFR in the 48h supernatants between TM adding drugs and without adding drugs respectively. The experimental procedure was performed according to the manufacturer's protocols. BioTek Multifunctional enzyme labeling instrument (Synergy H1, BioTek, USA) was used to measure the absorbance (OD) value of each well at 450nm. All experiments were performed in triplicate. Data are presented as the mean ± standard deviation. Calculate the concentration of DHPS, DHFS and DHFR by standard curve to compare the anti-*T.marneffei* effect of TMP, SMX and TMP/SMX with the TM growth control group.

Real-time reverse transcription PCR (RT-qPCR)

THP-1 macrophages were incubated with activated *T.marneffei* spores (5:1, fungi to cell) and cultured at 37℃ in 5% CO_2 for 6-12h. Then use sterile PBS to wash *T.marneffei*-infected macrophages three times as previously described. Following these treatments, cells were harvested, and total RNA was extracted using TRIzol® reagent, following the manufacturer's protocol. The RNA was then reversely transcribed to cDNA using the PrimeScript™RT Master Mix-RR036A and qPCR was performed using TB Green™Premix Ex TaqTM-RR820A Fluorescence quantification kit and an Applied Biosystems 7500 Real-Time PCR system(Applied Biosystems; Thermo Fisher Scientific,Inc.) was used to measure the gene expression of each sample. GAPDH was used as the internal control. The thermocycling conditions were as follows: Reverse transcription at 37℃ for 15min and 85℃ for 5 sec and pause at 4℃; pre-denaturation for 1min at 95℃; followed by 40 cycles of 5 sec at 95℃ and 30 sec at 60℃. The specific primers used were as follows: Dectin-1 forward, 5'-CCGGTAAGTACCTAGC CCAC-3' and reverse, 5'-GCAGCACACGATCCTTTCTC-3'; and IL-6 forward, 5'-AGGAGACTTGCCTGGTGAAA-3' and reverse, 5'-CAGGGGTGGTTATT GCATCT-3'; and IL-10 forward, 5'-CTTTAATAAGCTCCAAGAGAAAGGC-3' and reverse, 5'-CAGATCCGATTTTGGAGACC-

3’; and IL-23A forward, 5’-CTCAGGGACAACAGTCAGTTC-3’and reverse, 5’-ACAGGGCTATCAGGG AGCA-3’; and CXCL8 forward, 5’-TTTTGCCAAGGAGTGCTAAAGA-3’and reverse, 5’-AACCCTCTGCACCCAGTTTTC-3’; and TNF-α forward, 5’-TCCC CAGGGACCTCTCTCTA-3’ and reverse, 5’-GAGGGTTTGCTACAACATGG G-3’; and GAPDH forward, 5’-GGTGGTCTCCTCTGACTTCAACA-3’ and reverse, 5’-GTTGCTGTAGCCAAATTCGTTGT-3’. Finally, the CT values for each reaction were collected and the changes in the expression of the target gene were normalized to GAPDH. Relative mRNA expression levels were calculated by fold changes using the $2^{-\Delta\Delta CT}$ formula, where ΔCT is the difference between the target gene and GAPDH, and ΔΔCT for the sample=ΔCT of treated condition-ΔCT of control condition, as previously described[13].

Statistical analysis

All data were performed at least in three independent experiments. The Graphpad Prism 8.0 and Excel 2013 were used for data analysis. The quantitative data with normal distribution and homogeneity of variance were expressed as mean± standard deviation (SD) by one-way analysis variance (ANOVA) for multiple group comparisons. Student’s t-test for two independent samples were used to compare the difference between two groups, statistical significance was set at *P<0.05**P<0.01,***P<0.001 and ****P<0.0001.The correlation of the data was verified through the calculation of the Person correlation coefficient. P<0.05 was considered significant.

Results

The antifungal effect of cotrimoxazole to T.marneffei outside macrophages

In order to investigate the antifungal effect of TMP/SMX, SMX and TMP on *T.marneffei* spores in vitro, we used activated *T.marneffei* spores treated with a series of different concentrations of TMP/SMX, SMX and TMP or without for 48h. *T.marneffei* colony forming units(CFU) was performed to assess the anti-TM effect of TMP/SMX,SMX and TMP respectively. As shown in Figs. 1A,1B, the proliferation activity of *T.marneffei* can be weakened with the increasing concentration of TMP/SMX ranging from 14/70 to 90/450μg/ml compared with TM growth control group. As shown in Figs. 1C,1D, the inhibiting *T.marneffei* phenomenon was more and more obvious with the concentration of SMX ranging from 100 to 360μg/ml. As shown in Figs. 1E,1F, the inhibitory effect was more and more significant with the increasing concentration of TMP ranging from 240 to 400μg/ml. As shown in Fig. 2, it suggested that TMP/SMX achieved an inhibiting *T.marneffei* effect when the concentration increasing from 10/80μg/ml. Its inhibition rate approximates to 100% when the concentration of TMP/SMX ranging from 100/300 to 100/2000μg/ml. Our results led us to propose that 1:5 proportion of TMP/SMX have synergistic effects than other proportions and SMX, TMP separately, which confirmed the clinical application of 1:5 TMP/SMX as antimicrobial.

Cotrimoxazole inhibits DHPS, DHFS and DHFR of T.marneffei

To investigate the effect of cotrimoxazole towards DHPS, DHFS and DHFR of *T.marneffei*, *T.marneffei* spores were treated with different concentration gradients of cotrimoxazole as 3 times, 5 times, 10 times for 48h. The concentration of DHPS, DHFS and DHFR were examined using elisa kits respectively. Our results showed that DHPS, DHFS and DHFR can be obviously inhibited when the concentrations of TMP/SMX were 16.2/81μg/ml and 48.6/243μg/ml in 3 times gradient (Fig.3A). The similar inhibitory tendency of DHPS, DHFS and DHFR can be observed when the concentrations of TMP/SMX were 20/100μg/ml, 25/125μg/ml, 100/500μg/ml and 200/1000μg /ml in 5 times and 10 times gradients (Figs.3B,C,D), which has been formerly confirmed to exhibit an anti-*T.marneffei* activity in drug susceptibility test of *T.marneffei* in vitro. Our results also suggested that DHPS, DHFS and DHFR showed a consistently declining tendency with the increasing concentration of TMP/SMX ranging from 20/100 to 60/300μg/ml compared with TM control group (Fig.4A). And the concentration of DHPS, DHFS were significantly decreased with the increasing concentration of SMX ranging from 100 to 300μg/ml. While the concentration of DHFR was not decreased obviously with the increasing concentration of TMP ranging from 20 to 60μg/ml (Fig.4B). These results indicated that the blocking of three enzymes DHPS, DHFS and DHFR in folate biosynthesis pathway of *T.marneffei* may be involved in the direct inhibition of *T.marneffei* growth by cotrimoxazole in vitro. And SMX can separately inhibit DHPS and DHFS at effective concentration, but TMP cannot separately inhibit DHFR at ineffective concentrations.

The antifungal effect of cotrimoxazole to T.marneffei inside macrophages

To further confirm the effect of cotrimoxazole on *T.marneffei* proliferation in THP-1 macrophages, *T.marneffei* colony forming units (CFUs) was used to assess the antifungal effect of TMP/SMX, SMX and TMP to *T.marneffei* infected into macrophages. As shown in Fig. 5A, *T.marneffei* almost entirely inhibited by TMP/SMX, starting from 36/180μg/ml, suggesting that cotrimoxazole plays an important role in enhancing the intercellular killing *T.marneffei* ability of THP-1 macrophages. As shown in Fig. 5B, the intercellular inhibiting *T.marneffei* proliferation ability was enhanced with the increase of concentration of TMP/SMX. As revealed in Figs. 5C,D,E, separate SMX and TMP also has an intercellular killing TM effect, but the effectively anti-T.marneffei concentration in SMX was starting from 340μg/ml while it turns out to be 320μg/ml in TMP, far exceeding the directly inhibiting *T.marneffei* concentration of SMX and TMP. Collectively, our results suggest that TMP/SMX, SMX and TMP can inhibit *T.marneffei* inside macrophages, but the inhibitory effect of SMX and TMP was not as obvious as TMP/SMX.

Cotrimoxazole affects the Dectin-1 signaling pathway in T.marneffei-infected THP-1 macrophages

In order to investigate the effect of cotrimoxazole on the anti-fungal immune response mediated by Dectin-1 signaling pathway of *T.marneffei*-infected macrophages, human THP-1 macrophages were used as cell model in vitro to explore the intrinsic mechanism of cotrimoxazole indirectly inhibiting TM growth in macrophages. As shown in Fig. 6, the relative mRNA expression levels of Dectin-1 in THP-1 macrophages were significantly increased in response to *T.marneffei* infection, the same up-regulated tendency can also be observed in the expression levels of IL-6, IL-10 and IL-23A, which were down-regulated after adding TMP/SMX at 20/100μg/ml and 30/150μg/ml to TM-infected macrophages for 24h. We also showed that *T.marneffei* infection significantly led to the downregulated levels of CXCL8 and TNF-α, which showed upregulated tendency after adding TMP/SMX for 24h. These results indicated that Dectin-1 signaling pathway may be involved in the anti-*T.marneffei* activity of cotrimoxazole inside macrophages, leading to the upregulated inflammatory cytokines including IL-6, IL-10 and IL-23A and downregulated inflammatory cytokines including CXCL8 and TNF-α, while it showed opposite tendency after adding effective concentrations of TMP/SMX. Our findings explained the reason why cotrimoxazole can inhibit the growth and proliferation of *T.marneffei* infected into macrophages indirectly, which was profound for the search for novel prevention and control strategies for HIV/AIDS combined with *T.marneffei* infection in clinic application.

Discussion

Talaromyces marneffei, an opportunistic fungal pathogen, causes a life-threatening deep-seated systemic infection in patients with acquired immune deficiency syndrome(AIDS) in Southeast Asian countries[2, 14]. Due to the lack of effective antifungal agents, talaromycosis is well known as a severe disease that can cause disseminated infection, and treatment of this disease still remains a challenge[12, 15].

In this research, we demonstrated that cotrimoxazole can exert direct fungicidal effects on *T.marneffei* by inhibiting DHPS, DHFS, DHFR which were evolved in folate biosynthesis, and enhance the indirect fungicidal effect of macrophages by regulating the Dectin-1 signaling pathway.

Since the antimicrobial effect of cotrimoxazole (CTX) comes from its blocking activity of folic acid biosynthesis pathway which are important for the survival of many bacterial, protozoa, and fungi[1]. It has been long assumed that trimethoprim and sulfamethoxazole synergistically block microbial synthesis of folic acid, a vital cofactor in the manufacture of thymidine and purines. Sulfamethoxazole is a competitive inhibitor of dihydrofolic acid synthesis, and trimethoprim acts downstream of sulfamethoxazole to inhibit production of the physiologically active tetrahydrofolic acid[16]. Based on this information, we hypothesized that cotrimoxazole directly inhibiting the growth and proliferation of *T.marneffei* was through blocking DHPS, DHFR directly and DHFS indirectly, by which SMX inhibits DHPS, thereby inhibiting DHFS and TMP inhibits DHFR respectively. Our results confirmed this hypothesis showing that the concentration of DHPS, DHFS and DHFR were significantly decreased in *T.marneffei* adding effective concentrations of TMP/SMX group. Our results further found that DHPS and DHFS showed obvious declining tendency in *T.marneffei* adding effective concentrations of SMX group. But the

results suggests that DHFR was not significantly decreased in *T.marneffei* adding ineffective concentrations of TMP group, which led us to propose that DHFR was only inhibited at an effective inhibiting *T.marneffei* concentration of TMP/SMX, while the corresponding concentration of TMP only serves as a sulfonamide sensitizer and cannot block DHFR separately. Our data therefore provided a complete chain of evidence that cotrimoxazole inhibiting *T.marneffei* directly in vitro perhaps by blocking DHPS, DHFS and DHFR at effective concentrations of TMP/SMX, of which SMX plays an essential role in blocking DHPS and DHFR, while TMP serves as a sulfonamide sensitizer, thereby enhancing the fungicidal effect of SMX. This finding reveals a novel mechanism by which cotrimoxazole can block DHPS, DHFS, DHFR and SMX can inhibit DHPS and DHFS separately to inhibit the folate biosynthesis pathway. But TMP cannot inhibit DHFR at an ineffective concentration separately which confirmed the synergistic role of TMP/SMX to kill *T.marneffei* in vitro.

Ghilchik et al has mentioned that cotrimoxazole has both immunomodulatory and antimicrobial properties, suggesting that cotrimoxazole can modulate both innate and adaptive immune cells, including enhancing phagocytosis of microorganism such as bacteria, fungi and intracellular killing by macrophages[16–18].An in-vitro study of alveolar macrophage function in a few adults with advanced HIV infection showed enhanced phagocytosis and killing of *Staphylococcus aureus* in those taking cotrimoxazole, compared with untreated individuals[16, 19].Our results have confirmed that *T.marneffei* inside macrophages can be inhibited by TMP/SMX, SMX and TMP at high intracellular concentrations respectively. However, the interaction between cotrimoxazole and *T.marneffei*-infected macrophages remains largely unknown. To investigate whether cotrimoxazole influences the Dectin-1 signaling pathway of *T.marneffei*-infected macrophages, PMA-induced THP-1 macrophages stimulating with T.marneffei conidia were used, and the mRNA expression levels of Dectin-1 and downstream IL-6, IL-10, IL-23A, CXCL8 and TNF-α inflammatory cytokines were determined by RT-qPCR. In the present study, it was revealed that increased levels of Dectin-1, IL-6, IL-10, IL-23A and decreased levels of CXCL8 and TNF-α were observed in macrophages in response to *T.marneffei* infection. Our results also revealed that decreased levels of Dectin-1, IL-6, IL-10, IL-23A and increased levels of CXCL8 and TNF-α were observed in *T.marneffei*-infected macrophages after adding TMP/SMX for 24h. Although the role of Dectin-1 in mediating the expression levels of cytokines released by macrophages in *T.marneffei* infection remains poorly understood, several studies have mentioned its essential role in antifungal immunity. Dectin-1 is a member of the C-type lectin family and functions as an innate PRR involved in antifungal immunity[12, 14, 20]. Nakamura et al demonstrated that Dectin-1 is essential in sensing *T.marneffei* for the activation of bone marrow-derived DCs[12, 14].Sun et al demonstrated that Dectin-1 may play a crucial role in *Aspergillus*-induced innate immune responses in human bronchial epithelial cells[12, 21].These findings suggested that Dectin-1 may be a critical component of the anti-*T.marneffei* immune response in macrophages. The role and mechanism of cotrimoxazole influencing the anti-*T.marneffei* ability of macrophages perhaps by regulating the Dectin-1 signaling pathway and downstream inflammatory cytokines.

It is well known that inflammatory cytokines play a critical role in the development of fungal infectious diseases. Monocytes/macrophages serve as an essential role in the innate immunity of host resistance

to invasive fungi infection. *T.marneffei* infection can activate innate immune cells, such as macrophages and the activated macrophages can secrete cytokines, and they primarily produce pro-inflammatory cytokines such as IL-1,IL-6, IL-12,IL-18,IL-23, IL-1β, CXCL8, TNF-α and anti-inflammatory cytokines such as IL-10, IL-38 and so on[22–24].

Previous studies have revealed that Dectin-1 couple to spleen tyrosine kinase (Syk) and the adaptor molecular CARD-9 to induce production of IL-6, IL-23 and TNF-α[25, 26].It was also observed that patients with Dectin-1 deficiency had reduced IL-6 and IL-17 production in monocytes and macrophages challenged with either β-glucan or heat-killed *C.albicans*, and patients with CARD-9 deficiencies exhibited defects in Th17 differentiation, while IL-23 is required for the activity of Th17 cells and their maintenance in vivo[25, 27].Previous investigations have proved that the *T.marneffei*-induced activation of JNK1/2 pathway increased the production of IL-10, thereby inhibiting the anti-*T.marneffei* ability of human macrophages[28].An in-vitro research using mouse macrophages as cell model, suggesting that incubation of *T.marneffei* promoted phagocytosis of *T.marneffei* by macrophages and high levels of pro-inflammatory and anti-inflammatory cytokine production by macrophages, which was consistent with our results, referring to high mRNA expression levels of IL-6, IL-10, IL-23A secreted by macrophages in response to *T.marneffei*.

In fact, it must be admitted that Dectin-1 as important sensor in recognizing *T.marneffei* infection in macrophages, the activation of Dectin-1 signaling pathway influencing pro-inflammatory/anti-inflammatory response to *T.marneffei*, thereby increasing the production of IL-6, IL-10 and IL-23A to inhibit the anti-*T.marneffei* effect of macrophages. CXCL8, also known as IL-8, is a pro-inflammatory CXC chemokine involved in inflammatory reactions. The biological effects of IL-8 are mediated by two highly related chemokine receptors, CXCR1(IL-8RA) and CXCR2(IL-8RB), which are co-expressed on human neutrophils. Both the beneficial and harmful roles of neutrophils are critically dependent on the capacity of the cell to undergo directed migration from the blood to local tissue sites, including *T.marneffei* infection. Because the CXC chemokine interleukin-8(IL-8) is a powerful mediator of this process, its two receptors CXCR1 and CXCR2 are reasonable targets for development of treatments for neutrophil-mediated inflammation[29].Therefore,CXCL8 exerts its function alongside other cytokines and chemokines, is an important activator and chemoattractant for leukocytes to sites of inflammation, recruitment and activation of neutrophils to phagocytosis, and bacterial clearance, and its role have been implicated in a variety of inflammatory diseases[12, 30, 31].Tumour necrosis factor alpha(TNF-α), an inflammatory cytokine produced by macrophages /monocytes during acute inflammation, which belongs to a family of both soluble and cell-bound cytokines that have a wide range of functions such as host defense, inflammation, and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis[12, 32, 33].Previous investigations have showed that TNF-α plays an essential role in the initiation of the innate response in the mouse model of invasive *Aspergillus* infection. It mentioned TNF-α most likely mediates its protective effect via following mechanisms, including induction of neutrophil-chemotactic chemokines, inducing of adhesion molecules, and neutrophil-independent pathways. Additionally, several recruitment cytokines play critical roles in mediating influx of specific leukocytes to the site of infection in invasive *Aspergillosis*[34]. A profound study also revealed that the

depletion of TNF-α resulted in a reduced lung neutrophil influx in both normal and cyclophosphamide-treated animals, which occurred in association with a decrease in lung levels of the C-X-C chemokine, macrophage inflammatory protein-2 and the C-C chemokines macrophage inflammatory protein-1 alpha[35]. Notably, our research found that cotrimoxazole has the potential to increase the secretion levels of CXCL8 and TNF-α by macrophages in response to *T.marneffei* infection. Our data led us to propose that cotrimoxazole exerts fungicidal effect by increasing the levels of TNF-α, thereby inducing CXCL8 to exert the intercellular killing activity of macrophages.

Taken together, our results demonstrated the essential recognizing *T.marneffei* role of Dectin-1 in macrophages, and further confirmed our hypothesis that cotrimoxazole influencing *T.marneffei* growth through regulating the Dectin-1 signaling pathway. In the current study, we observed that the activation of *T.marneffei*-induced Dectin-1 pathway increased the production of IL-6, IL-10 and IL-23A while reduced the production of CXCL8 and TNF-α to inhibit the anti-*T.marneffei* capacity of macrophages. Cotrimoxzole serves its important role by reducing the *T.marneffei*-induced inflammatory responses and enhance the phagocytosis and intercellular killing role of macrophages.

There exists several limitations in the present study. Firstly, only one cell line, human THP-1 macrophage, was used as cell model in vitro. Secondly, considering the environment in the human body is complicated and changeable, and there exists individual immune differences among the population, so the safe and effective concentration of cotrimoxazole towards THP-1 macrophages in vitro cannot represent blood drug concentration in vivo. Thirdly, our cell experiments in vitro only contain macrophages without stimulating the immune microenvironment in vivo in response to *T.marneffei*. As we all known, there are various immune cells interacting with each other to coordinate an antifungal response in vivo. Fourthly, our results cannot determine whether cotrimoxazole influence the expression of Dectin-1 receptor of macrophages by inhibiting the proliferation of *T.marneffei*, or *T.marneffei* inhibits the expression of Dectin-1 receptor, which was up-regulated by cotrimoxazole afterwards. Therefore, the combination of population research among HIV/AIDS co-infected with *T.marneffei* treated with cotrimoxazole and animal experiments should be conducted to further explore the role and mechanism of cotrimoxazole resistance to *T.marneffei* in vivo.

In conclusion, our data led us to propose that *T.marneffei* was inhibited by cotrimoxazole through blocking DHPS, DHFS and DHFR directly and regulating Dectin-1 signaling pathway indirectly to enhance intracellular killing-*T.marneffei* ability in human THP-1 macrophages. Our study reveals a novel mechanism by which cotrimoxazole inhibits the growth and proliferation of *T.marneffei* inside and outside macrophages, which may provide a therapeutic target for inhibition of infection and dissemination in *T.marneffei* infection, especially in HIV/AIDS patients.

Declarations

Acknowledgements

This research is supported by the National Natural Science Foundation of China (8197081279, 81960602 and 81971935), Guangxi Key Research and Development Plan(GuikeAB18050022), Guangxi Science Fund for Distinguished Young Scholars(2018GXNSFFA281001), Guangxi Bagui Scholar(to Junjun Jiang), Guangxi Medical University Training Program for Distinguished Young Scholars(to Junjun Jiang), and Youth Science Foundation of Guangxi Medical University(GXMUYSF201906).

Supplemental Information

Supplemental information includes two figures, and supplemental experimental procedures can be found in the end of this article.

Authors' Contributions

Jie Chen contributed to the study design and performed most of experiments, data collection and analysis. Jie Chen also contributed to write the initial draft manuscript. Wudi Wei provided *T.marneffei* strains. Xiu Chen provided human THP-1 cells. Minjuan Shi, Tongxue Qin, Yinlu Liao, Gang Wang prepared the experimental materials and reagents. Jinhao He and Hong Zhang contributed to the teaching of the cultivating *T.marneffei* methods. Yuting Wu, Beibei Lu and Xing Tao helped perform the experiments. Wudi Wei and Rongfeng Chen supervised all aspects of the project. Junjun Jiang, Hao Liang and Li Ye conceived and secured funding for the project. All authors have seen, edited and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no financial conflicts of interest. The authors alone are responsible for the content and the writing of this paper.

References

1. Jiang J, Qin F, Meng S, Nehl EJ, Huang J, Liu Y, et al. Effects of cotrimoxazole prophylaxis on Talaromyces marneffei infection in HIV/AIDS patients receiving antiretroviral therapy: A retrospective cohort study. Emerg Microbes Infect. 2019;8(1):367-76. https://doi.org/10.1080/22221751.2019.1588078.
2. Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T. Disseminated Penicillium marneffei infection in southeast Asia. Lancet. 1994;344(8915):110-3. https://doi.org/10.1016/s0140-6736(94)91287-4.
3. Vanittanakom N, Cooper CJ, Fisher MC, Sirisanthana T. Penicillium marneffei infection and recent advances in the epidemiology and molecular biology aspects. Clin Microbiol Rev. 2006;19(1):95-110. https://doi.org/10.1128/CMR.19.1.95-110.2006.
4. Yuen KY, Wong SS, Tsang DN, Chau PY. Serodiagnosis of Penicillium marneffei infection. Lancet. 1994;344(8920):444-5. https://doi.org/10.1016/s0140-6736(94)91771-x.

5. Mermin J, Ekwaru JP, Liechty CA, Were W, Downing R, Ransom R, et al. Effect of co-trimoxazole prophylaxis, antiretroviral therapy, and insecticide-treated bednets on the frequency of malaria in HIV-1-infected adults in Uganda: A prospective cohort study. Lancet. 2006;367(9518):1256-61. https://doi.org/10.1016/S0140-6736(06)68541-3.
6. Beraud G, Pierre-Francois S, Foltzer A, Abel S, Liautaud B, Smadja D, et al. Cotrimoxazole for treatment of cerebral toxoplasmosis: An observational cohort study during 1994-2006. Am J Trop Med Hyg. 2009;80(4):583-7.
7. Mccullough JL, Maren TH. Inhibition of dihydropteroate synthetase from Escherichia coli by sulfones and sulfonamides. Antimicrob Agents Chemother. 1973;3(6):665-9. https://doi.org/10.1128/AAC.3.6.665.
8. Baker BR, Schwan TJ, Ho BT. Irreversible enzyme inhibitors. LXXII. Candidate active-site-directed irreversible inhibitors of dihydrofolic reductase. VI. Derivatives of hydrophobically bonded p-alkyl and p-aralkyl benzoic acids. J Pharm Sci. 1967;56(1):38-42. https://doi.org/10.1002/jps.2600560107.
9. Li K, Li T, Yang SS, Wang XD, Gao LX, Wang RQ, et al. Deletion of nudB Causes Increased Susceptibility to Antifolates in Escherichia coli and Salmonella enterica. Antimicrob Agents Chemother. 2017;61(5):e02378-16. https://doi.org/10.1128/AAC.02378-16.
10. Meir Z, Osherov N. Vitamin biosynthesis as an antifungal target. J Fungi (Basel). 2018;4(2):72-85. https://doi.org/10.3390/jof4020072.
11. Saijo S, Iwakura Y. Dectin-1 and Dectin-2 in innate immunity against fungi. Int Immunol. 2011;23(8):467-72. https://doi.org/10.1093/intimm/dxr046.
12. Peng Y, Chen Y, Ma J, Zhou W, Wang Y, Wang Y, et al. Role and mechanism of the Dectin-1-mediated Syk/NF-κB signaling pathway in *Talaromyces marneffei* infection. Exp Ther Med. 2022;23(1):84-92. https://doi.org/10.3892/etm.2021.11007.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. https://doi.org/10.1006/meth.2001.1262.
14. Nakamura K, Miyazato A, Koguchi Y, Adachi Y, Ohno N, Saijo S, et al. Toll-like receptor 2 (TLR2) and dectin-1 contribute to the production of IL-12p40 by bone marrow-derived dendritic cells infected with Penicillium marneffei. Microbes Infect.2008;10(10-11):1223-7. https://doi.org/10.1016/j.micinf.2008. 06.011.
15. Limper AH, Adenis A, Le T, Harrison TS. Fungal infections in HIV/AIDS. Lancet Infect Dis. 2017;17(11):e334-e343. https://doi.org/10.1016/S1473-3099(17)30303-1.
16. Church JA, Fitzgerald F, Walker AS, Gibb DM, Prendergast AJ. The expanding role of co-trimoxazole in developing countries. Lancet Infect Dis. 2015;15(3):327-39. https://doi.org/10.1016/S1473-3099(14)71011-4.
17. Tadema H, Heeringa P, Kallenberg CG. Bacterial infections in Wegener's granulomatosis: Mechanisms potentially involved in autoimmune pathogenesis. Curr Opin Rheumatol. 2011;23(4):366-71. https://doi.org/10.1097/BOR.0b013e328346c332.

18. Ghilchik MW, Morris AS, Reeves DS. Immunosuppressive powers of the antibacterial agent trimethoprim. Nature. 1970;227(5256):393-4. https://doi.org/10.1038/227393a0.
19. Luppi F, Covi M, Velluti G, Spagnolo P, Fabbri LM, Richeldi L. Co-trimoxazole effect on human alveolar macrophages of AIDS patients. J Biol Regul Homeost Agents. 2011;25(3):461-4.
20. Brown GD, Gordon S. Fungal beta-glucans and mammalian immunity. Immunity. 2003;19(3):311-5. https://doi.org/10.1016/s1074-7613(03)00233-4.
21. Sun WK, Lu X, Li X, Sun QY, Su X, Song Y, Sun HM, Shi Y. Dectin-1 is inducible and plays a crucial role in Aspergillus-induced innate immune responses in human bronchial epithelial cells. Eur J Clin Microbiol Infect Dis. 2012;31(10):2755-64. https://doi.org/10.1007/s10096-012-1624-8.
22. Koh TJ, Dipietro LA. Inflammation and wound healing: The role of the macrophage. Expert Rev Mol Med. 2011;13:e23. https://doi.org/10.1017/S1462399411001943.
23. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018;233(9):6425-40. https://doi.org/10.1002/jcp.26429.
24. Hu Y, Lu S, Xi L. Murine Macrophage Requires CD11b to Recognize Talaromyces marneffei. Infect Drug Resist. 2020;13:911-20. https://doi.org/10.2147/IDR.S237401.
25. Gaffen SL, Hernandez-Santos N, Peterson AC. IL-17 signaling in host defense against Candida albicans. Immunol Res. 2011;50(2-3):181-7. https://doi.org/10.1007/s12026-011-8226-x.
26. Leibundgut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol. 2007;8(6):630-8. https://doi.org/10.1038/ni1460.
27. Cua DJ, Tato CM. Innate IL-17-producing cells: The sentinels of the immune system. Nat Rev Immunol. 2010;10(7):479-89. https://doi.org/10.1038/nri2800.
28. Chen R, Xi L, Huang X, Ma T, Ren H, Ji G. Effect of Jun N-terminal kinase 1 and 2 on the replication of Penicillium marneffei in human macrophages. Microb Pathog. 2015;82:1-6. https://doi.org/10.1016/j.micpath.2015.03.014.
29. Murphy PM. Neutrophil receptors for interleukin-8 and related CXC chemokines. Semin Hematol. 1997;34(4):311-8.
30. Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2016;138(4):984-1010. https://doi.org/10.1016/j.jaci.2016.06.033.
31. Zhang W, Chen H. [The study on the interleukin-8 (IL-8)]. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi. 2002;19(4):697-702.
32. Idriss HT, Naismith JH. TNF alpha and the TNF receptor superfamily: Structure-function relationship(s). Microsc Res Tech. 2000;50(3):184-95. https://doi.org/10.1002/1097-0029(20000801)50:3<184::AID-JEMT2>3.0.CO;2-H.

33. Aggarwal BB, Gupta SC, Kim JH. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood. 2012;119(3):651-65. https://doi.org/10.1182/blood-2011-04-325225.
34. Phadke AP, Mehrad B. Cytokines in host defense against Aspergillus: Recent advances. Med Mycol. 2005;43 Suppl 1:S173-6. https://doi.org/10.1080/13693780500052099.
35. Mehrad B, Strieter RM, Standiford TJ. Role of TNF-alpha in pulmonary host defense in murine invasive aspergillosis. J Immunol. 1999;162(3):1633-40.

Figures

Figure 1

The anti-*T.marneffei* activity of 1:5 TMP/SMX, SMX and TMP. *T.marneffei* spores were treated with different concentrations of 1:5 TMP/SMX, SMX and TMP or without for 48h, respectively. (A,B) *T.marneffei* colony forming units (CFUs) by microdilution spot assay (diluted 10, 100, 1000 times) from collected 48h supernatant to assess antifungal activity of 1:5 TMP/SMX. (C,D) Collected 48h supernatant and performed the CFU to assess antifungal activity of SMX as previously described. (E,F) Collected 48h supernatant and conducted the CFU to assess antifungal activity of TMP as previously described. All data were showed as mean ± SD of results from at least three independent experiments (***, $p<0.001$, ****, $p<0.0001$, n.s, no significant difference, by Student's t-test).

Figure 2

The anti-*T.marneffei* activity of different proportions of TMP/SMX. *T.marneffei* spores or hyphae were treated with different matching and concentration gradients of TMP/SMX or without for 48h, respectively. Collected the 48h supernatant and *T.marneffei* colony forming units (CFUs) were performed as previously described. (A) *T.marneffei* spores were treated with TMP/SMX ranging from 1/3 to 1/20μg/ml. (B) *T.marneffei* spores were treated with TMP/SMX ranging from 10/30 to 10/200μg/ml. (C) *T.marneffei* spores were treated with TMP/SMX ranging from 100/300 to 100/2000μg/ml. All data were showed as mean ± SD of results from at least three independent experiments (***, $p<0.001$, ****, $p<0.0001$, n.s, no significant difference, by Student's t-test).

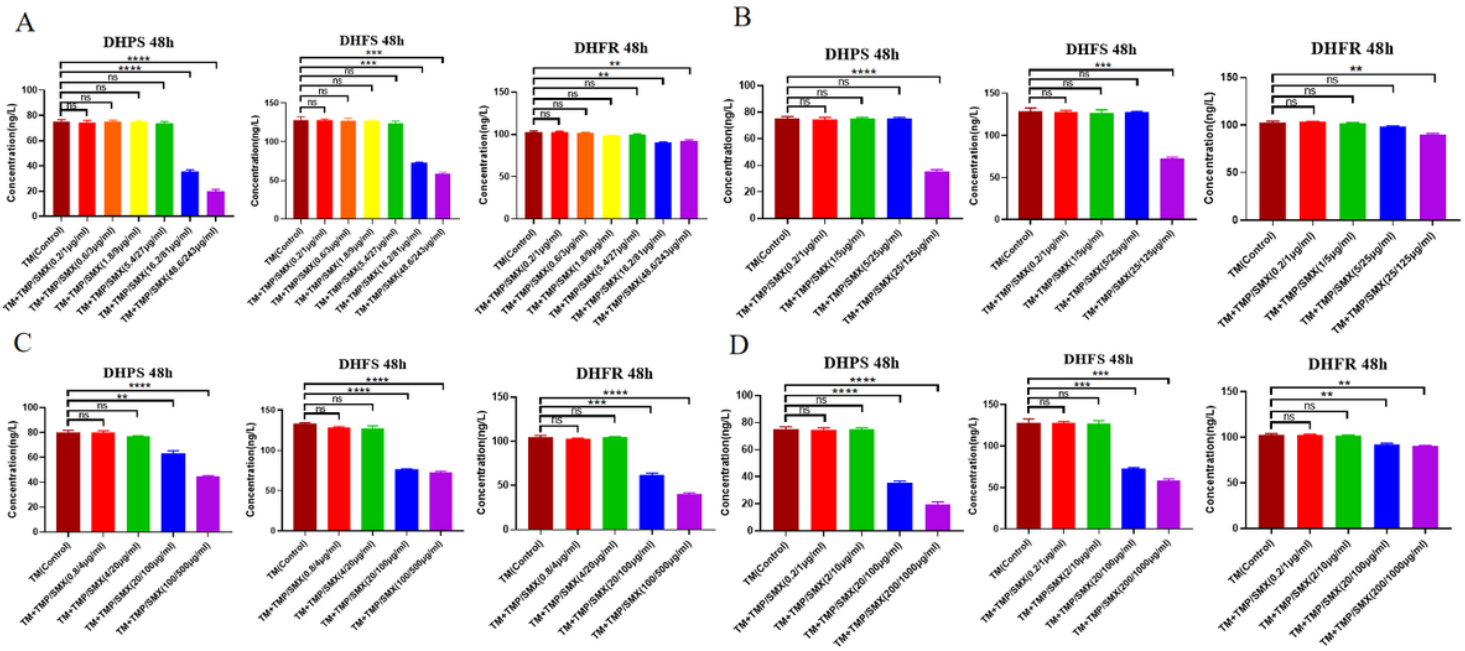

Figure 3

Three key enzymes DHPS, DHFS and DHFR can be blocked by cotrimoxazole at effectively inhibiting the growth and proliferation of *T.marneffei* concentrations in 3 times, 5 times and 10 times gradients. (A) *T.marneffei* spores were treated with 3 times concentration gradients of TMP/SMX or without for 48h, respectively. (B,C) *T.marneffei* spores were treated with 5 times concentration gradients of TMP/SMX or without for 48h, respectively. (D) *T.marneffei* spores were treated with 10 times concentration gradients of TMP/SMX or without for 48h, respectively. Elisa was used to detect the concentration of DHPS, DHFS and DHFR from 48h supernatant respectively. All data were showed as mean ± SD of results from at least three independent experiments (**, p<0.01, ***, p<0.001, ****, p<0.0001, n.s, no significant difference, by Student's t-test).

Figure 4

Three key enzymes DHPS, DHFS and DHFR can be blocked by TMP/SMX and DHPS and DHFS can be blocked by SMX respectively at effectively inhibiting the growth and proliferation of *T.marneffei* concentrations. (A) *T.marneffei* spores were treated with 20/100μg/ml, 40/200μg/ml, 60/300μg/ml of TMP/SMX or without for 48h, respectively. (B) *T.marneffei* spores were treated with 100μg/ml, 200μg/ml, 300μg/ml of SMX and 20μg/ml, 40μg/ml, 60μg/ml of TMP or without for 48h, respectively. Elisa was used to detect the concentration of DHPS, DHFS and DHFR from 48h supernatant respectively. All data were showed as mean ± SD of results from at least three independent experiments (**, p<0.01, ***, p<0.001, ****, p<0.0001, n.s, no significant difference, by Student's t-test).

Figure 5

The proliferation and growth of *T.marneffei* inside THP-1 macrophages is decreased under the effective inhibiting *T.marneffei* concentration of TMP/SMX, SMX and TMP. THP-1 macrophages were incubated with *T.marneffei* spores (MOI=5:1,fungi to cell) for 6-12h. (A,B) Add different concentrations of TMP/SMX to *T.marneffei*-infected macrophages for 48h.The culture supernatants were removed and cell lysates were collected, harvested *T.marneffei* by centrifugation. CFUs microdilution spot assay was conducted to measure antifungal ability of TM-infected macrophages adding TMP/SMX and without. (C,D,E) Add different concentrations of SMX or TMP to *T.marneffei*-infected macrophages for 48h respectively. CFUs microdilution spot assay was conducted to measure antifungal ability of TM-infected macrophages adding TMP or SMX and without. All data were showed as mean ± SD of results from at least three independent experiments (*, $p<0.05$, ****, $p<0.0001$, n.s, no significant difference, by Student's t-test).

Figure 6

Talaromyces marneffei triggers the activation of Dectin-1 signaling pathway of THP-1 macrophages, cotrimoxazole exhibits its anti-*T.marneffei* activity by regulating the Dectin-1 signaling pathway, decreasing the release of pro-*T.marneffei* inflammatory cytokines IL-6, IL-10, IL-23A and increasing the release of anti-*T.marneffei* inflammatory cytokines CXCL8 and TNF-α. (A)THP-1 macrophages were incubated with activated *T.marneffei* conidia for 6-12h, adding 20/100μg/ml of TMP/SMX to TM-infected macrophages for 24h. (B) THP-1 macrophages were incubated with activated *T.marneffei* conidia for 6-12h, adding 30/150μg/ml of TMP/SMX to TM-infected macrophages for 24h. Cells were lysed and examine the mRNA expression levels of Dectin-1, IL-6, IL-10, IL-23A, CXCL8 and TNF-α by RT-qPCR. Dectin-1, dendritic cell-associated C-type lectin-1; IL-6, interleukin-6; IL-10, interleukin-10; IL-23A, interleukin-23A; CXCL8, CXC chemokine ligand-8; TNF-α, tumor necrosis factor-α. The values were normalized to GAPDH expression. All data were showed as mean ± SD of results from at least three independent experiments (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$, n.s, no significant difference, by Student's t-test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx