



Murine Macrophage Requires CD11b to Recognize *Talaromyces marneffe*

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Introduction: *Talaromyces marneffe* (*T. marneffe*) is an emerging pathogenic fungus. Macrophage-1 antigen (Mac-1, CR3, CD11b/CD18) is an important receptor on innate immune cells and can recognize pathogens. However, the importance of CR3 in phagocytosis of *T. marneffe* by macrophages and their responses to *T. marneffe* have not been clarified.

Methods: We show that interaction of mouse peritoneal macrophages (pMacs) or RAW264.7 macrophages with *T. marneffe* of its conidia spores and yeast cells enhances CR3 expression on macrophages. The phagocytosis rate was determined using flow cytometry, RT-PCR and Western blotting were used to detect CD11b expression, and the levels of IFN- γ , TNF- α , IL-2, IL-4, IL-6 and IL-10 in the co-culture supernatants were determined by ELISA.

Results: Incubation of mouse macrophages with *T. marneffe* promoted phagocytosis of *T. marneffe*, which was dramatically mitigated by pretreatment with anti-CD11b antibody or knockdown of CR3 expression on macrophages. Then, interferon γ , tumor necrosis factor α , IL-4, IL-10 and IL-12 production in macrophages incubation with heat-killed *T. marneffe* was detected. CD11b expression on mouse macrophages was upregulated by *T. marneffe*. Incubation of *T. marneffe* promoted phagocytosis of *T. marneffe* by macrophages and high levels of pro-inflammatory and anti-inflammatory cytokine production by macrophages, which were mitigated and abrogated by pre-treatment with anti-CD11b or knockdown of CD11b expression.

Conclusion: These data indicated that murine macrophage requires CD11b to recognize *Talaromyces marneffe* and their cytokine responses to heat-killed *T. marneffe* in vitro.

Keywords: *Talaromyces marneffe*, CD11b, macrophage

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Introduction

Talaromyces marneffe (*T. marneffe*), formerly named *Penicillium marneffe* (*P. marneffe*) is the only dimorphic member of the genus and is an emerging pathogenic fungus that can cause a fatal systemic mycosis in humans.^{1,2} *T. marneffe* can form conidia spores and yeast cells. Inhaled conidia are thought to be the infectious particles.³⁻⁵ Macrophages have various cell-surface receptors that can recognize pathogen-associated molecule pattern (PAMP) of non-opsonized pathogens by the pattern recognition receptors (PRRs).⁶ However, how macrophages recognize PAMP on *T. marneffe* is unclear.

The integrin CR3 is a heterodimer of α M (CD11b) and β 2 (CD18) subunits and is one of the most versatile receptors expressed by phagocytes. CR3 can mediate adhesion, chemotaxis, and phagocytosis of innate immune cells in a complement-dependent or complement-independent manner.⁷⁻¹¹ Previous studies have shown

that the recognition of unopsonized yeast particles by CR3 depends on the binding of the yeast β -glucans to the carbohydrate-binding site located in CD11b.^{12,13} However, the importance of CR3 in phagocytosis of *T. marneffeii* by macrophages has not been clarified.

In this study, we investigated the roles of CD11b in phagocytosis of *T. marneffeii* by mouse macrophages and their responses to conidia spores and yeast cells of *T. marneffeii* in vitro.

Materials and Methods

Ethical Standards

A strain of *T. marneffeii* SUMS0152 (IFM52703) was obtained from the Fungi Research Center of Sun Yat-sen Memorial Hospital and the experimental protocol was approved by the Medical Ethics Committee of Sun Yat-sen University.¹⁴ The animal studies were reviewed and approved by the Animal Research and Care Committee of Sun Yat-sen University (SCXK Guangdong 2009–0011), and followed the guidelines for the welfare of the laboratory animals (Laboratory animal—Guideline for ethical review of animal welfare, GB/T3589-2009).

The *T. marneffeii* experiments in this manuscript were conducted under biosafety level 3 conditions (Public experimental platform of Sun Yat-sen University).

Animals

Male BALB/c mice at 8 weeks of age were obtained from the Laboratory Animal center of Sun Yat-sen University (Guangzhou, China). To suppress the immune system, all BALB/c mice were injected intraperitoneally with 100 mg/kg Cyclophosphamide (at 0.2 mL, Sigma Aldrich, St. Louis, MO, USA) daily for consecutive 3 days. The white blood cells from their tail venous blood samples were counted at the day 1 and 4. Individual mice with reduced WBC count to $\leq 25\%$ were considered as immunosuppressed.¹⁴

Cells

Peritoneal macrophages (pMacs) were collected from the control and immunosuppressed BALB/c mice after the third injection by peritoneal lavage as described.¹⁴ Mouse RAW264.7 macrophages were preserved in the Fungi Research Center of Sun Yat-sen Memorial Hospital.

Fungus

T. marneffeii was firstly grown in Sabouraud's dextrose agar (SDA) medium. The subsequent culture and isolation of conidia and yeast cells were carried out as described.¹⁴

Labeling of *T. marneffeii* Yeast or Spores with Fluorescein Isothiocyanate (FITC)

T. marneffeii yeast cells and conidia spores were prepared. *T. marneffeii* yeast cells and conidia spores at 2×10^8 /mL were labeled with 0.16 mg/mL FITC in 0.05 M carbonate-bicarbonate buffer (pH9.5) for 60 mins in the dark and washed with PBS. The efficacy of FITC-labeling reached almost 100% and the intensity of FITC-labeled yeast cells and conidia spores was 2×10^5 , determined by flow cytometry.

Transfection

RAW264.7 cells (1×10^6 cells/well) were cultured in complete medium overnight and transfected with individual CD11b specific or control siRNAs (Table 1) using lipofectamine 2000 (Invitrogen, USA). The CD11b-specific siRNAs were designed using the Ambion online software (http://www.ambion.com/techlib/misc/siRNA_finder), and synthesized by GenePharma (Shanghai, China) (Table 1). After transfection for 48 hrs, the levels of CD11b expression were determined by RT-PCR and Western blot assays.

Phagocytosis Assay

pMacs and RAW264.7 cells at 1×10^6 cells/well were cultured in FBS-free DMEM medium in 6-well plates overnight. The cells in each well were added with non-opsonized

Table 1 The Sequences of Primers

	Sense	Antisense
ltgam-mus-3511	5'-GGCAGUACAAGGACAUGAUTT-3'	5'-AUCAUGUCCUUGUACUGCCTT-3'
ltgam-mus-574	5'-CCGGUAGCAUCAACAACAUUTT-3'	5'-AUGUUGUUGAUGCUACCGGTT-3'
ltgam-mus-1369	5'-CACCUCGGUAUCAGCAUAUTT-3'	5'-AUAUGCUGAUACCGAGGUGTT-3'
ltgam-mus-1059	5'-GCCCUGAAUACCAUUCAGATT-3'	5'-UCUGAAUGGUUUCAGGGCTT-3'
Negative control	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'
Mouse Actb-948	5'-GUAAAGACCUCUAUGCCAATT-3'	5'-UUGGCAUAGAGGUCUUUACTT-3'

FITC-labeled *T. marneffeii* at a ratio of 5:1 (conidia spore to macrophage) and the phagocytosis rate was determined using flow cytometry as conventional method.¹⁴

In addition, the CD11b specific or control siRNA-transfected RAW264.7 cells were tested for their phagocytosis.

RT-PCR

Macrophages were pretreated with vehicle or anti-CD11b for 1 hr and reacted with heat-killed *T. marneffeii* at a ratio of 5:1 (conidia spores to macrophage) for 1.5 hrs. Total RNA was extracted and reversely transcribed into cDNA by conventional method.¹⁴ The primer sequences were forward 5'-CAGATCAACAATGTGACCG TATGG-3' and reverse 5'-CATCATGTCCTTGTACTGCCGC-3' for CD11b; forward 5'-GAGGGAAATCGTGCGTGAC-3' and reverse 5'-CTGGAAGGTGGACAGTGA G-3' for β -actin. The PCR reactions were operated by conventional method.¹⁴ The PCR products were resolved by gel electrophoresis on 1–5% gels and analyzed by Quantity One software (Bio-Rad, USA).

Western Blotting

Macrophages were pretreated by RT-PCR. Protein extraction and Western blotting were manipulated by conventional method.¹⁴ The relative levels of CD11b to β -actin expression were analyzed by the densitometric scanning using the Image J software.

ELISA Assay

pMacs (1×10^6 cells/well) were cultured in 6-well plates overnight. The cells were pretreated with vehicle or anti-CD11b for 90 mins or RAW264.7 cells were transfected with individual siRNAs for 24 hrs. The cells were reacted in triplicate with heat-killed conidia spores of *T. marneffeii* at a ratio of 5:1 (conidia spores to macrophage) at 37°C for 2 hrs. The levels of IFN- γ , TNF- α , IL-2, IL-4, IL-6 and IL-10 in the culture supernatants were determined by ELISA using specific kits, according to the manufacturers' instructions (RayBiotech, USA).

Statistical Analysis

Data are present as the mean \pm SD. The difference among the groups was analyzed by ANOVA and post hoc Tukey's test using the Statistics Package for Social Science program. A P-value of <0.05 was considered statistically significant.

Results

CD11b Expression on Macrophages Is Enhanced by *T. marneffeii*

To determine the potential role of CR3 in phagocytosis of *T. marneffeii*, we first examined whether *T. marneffeii* modulated CR3 expression in macrophages in vitro. pMacs from control and immunosuppressed mice and mouse RAW264.7 cells were reacted with, or without, heat-killed *T. marneffeii* at a ratio of 1:5 for 1.5 hrs. As shown in Figure 1A, there was no significant difference in the relative levels of CD11b mRNA transcripts among pMacs from healthy and immunosuppressed mice as well as in RAW264.7 cells. After reaction with *T. marneffeii*, the relative levels of CD11b mRNA transcripts in all groups of macrophages significantly increased. A similar pattern of up-regulated CD11b protein expression was detected in the different groups of macrophages before and after *T. marneffeii* treatment (Figure 1B).

Next, we tested whether *T. marneffeii* spores and yeast could also enhance CD11b expression in macrophages. We found that incubation with either heat-killed or living spores or yeast also enhanced significant CD11b mRNA transcripts and protein expression in pMacs cells in vitro (Figure 2). These indicated that *T. marneffeii* and its spores and yeast up-regulated CD11b expression in macrophages.

Murine Macrophage Requires CD11b to Recognize *Talaromyces marneffeii*

To determine the function of CD11b, RAW264.7 cells were transfected with individual CD11b specific or control siRNA for 48 hrs and the relative levels of CD11b expression were determined by RT-PCR and Western blot assays. Transfection with CD11b-specific siRNA 1369 reduced the relative levels of CD11b mRNA transcripts and protein expression and transfection with 3511 greatly diminished CD11b expression in RAW264.7 cells (Figure 3). We further tested the role of CD11b in phagocytosis of *T. marneffeii* in vitro. RAW264.7 cells and pMacs from control and immunosuppressed mice were incubated with FITC-labeled *T. marneffeii*. The percentages of macrophages that phagocytized *T. marneffeii* in different groups of cells were determined by flow cytometry. The percentages of FITC+ pMac from normal and immunosuppressed BALB/c mice, and RAW264.7 cells, but the control cells without incubation with *T. marneffeii*, were 91.63% \pm 2.59, 89.26% \pm 3.48, 65.75% \pm 2.81, respectively (Figure 4). Pretreatment with anti-CD11b reduced the percentages of

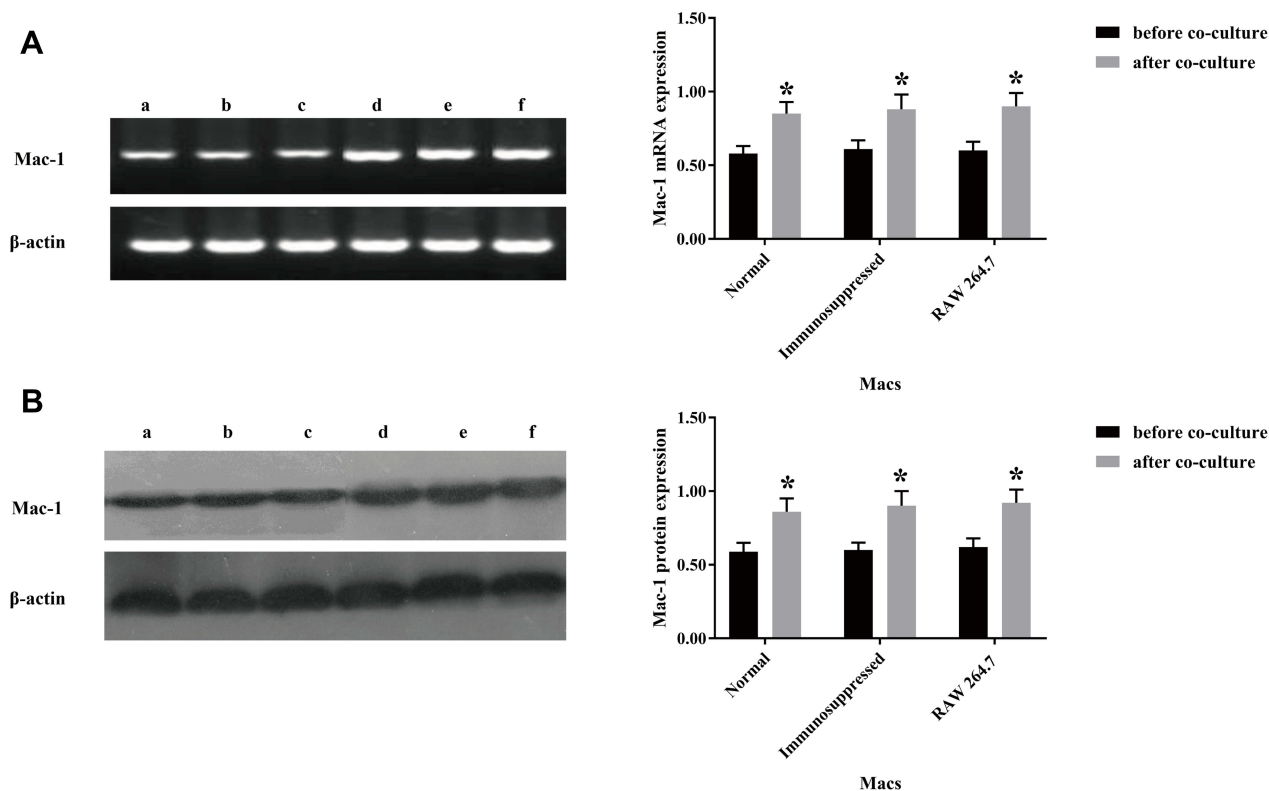


Figure 1 Incubation with *T. marneffei* conidia spores upregulates CD11b expression in mouse macrophages. RAW 264.7 cells and pMac3 from healthy and immunosuppressed BALB/c mice were incubated with, or without, heat-killed *T. marneffei* conidia spores at a ratio of 1:5 for 1.5 hrs. The levels of CD11b mRNA transcripts and protein expression were determined by RT-PCR and Western blot. Data are representative images or expressed as the mean \pm SD of each group of cells from three separate experiments. **(A)** The relative levels of CR3 mRNA transcripts. **(B)** The relative levels of CR3 protein. a: pMac3 from the healthy mice without *T. marneffei* conidia spores, b: pMac3 from the immunosuppressed mice without *T. marneffei* conidia spores, c: RAW 264.7 without *T. marneffei* conidia spores, d: pMac3 from the healthy mice with *T. marneffei* conidia spores, e: pMac3 from the immunosuppressed with *T. marneffei* conidia spores, f: RAW 264.7 with *T. marneffei* conidia spores. * $P < 0.05$ vs before co-culture.

FITC+ pMac3 to $35.45\% \pm 3.15$ (Figure 4). Similarly, transfection with CD11b-specific siRNA 3511, but not control 574, decreased the percentages of FITC+ pMac3 in vitro. Together, these data clearly indicated that Murine macrophage requires CD11b to recognize *Talaromyces marneffei*.

CD11b Is Crucial for *T. marneffei* Induced Cytokine Responses in Macrophages

Finally, we examined the impact of CD11b on *T. marneffei* stimulated cytokine responses in macrophages. Different groups of macrophages were stimulated with *T. marneffei* and the levels of IFN- γ , TNF- α , IL-2, IL-4, IL-6 and IL-10 in the culture supernatants were determined by ELISA. There was no detectable IL-2 in different groups of macrophages (data not shown). There was very low levels of IFN- γ , TNF- α , IL-4, IL-6 and IL-10 in the supernatants of cultured control macrophages, the levels of IFN- γ , TNF- α ,

IL-4, IL-6 and IL-10 were significantly elevated in the supernatants of cultured macrophages that had been stimulated with *T. marneffei* (Figure 5). Furthermore, the levels of IFN- γ , TNF- α , IL-4, IL-6 and IL-10 in the supernatants of cultured macrophages from immunosuppressed mice were lower than from control mice regardless of *T. marneffei* stimulation. In addition, pre-treatment with anti-CD11b or knockdown of CD11b by its specific siRNA 3511 significantly mitigated *T. marneffei*-stimulated IFN- γ , TNF- α , IL-4, IL-6 and IL-10 production by macrophages. Therefore, CD11b was crucial for *T. marneffei*-stimulated IFN- γ , TNF- α , IL-4, IL-6 and IL-10 in macrophages in vitro.

Discussion

CR3 is one of the PRRs through which innate immune cells, such as macrophages can recognize PAMP on pathogenic microbiota. A previous study has indicated that Dectin-1 and CD3 act as major fungal receptors for beta-glucan. Dectin-1

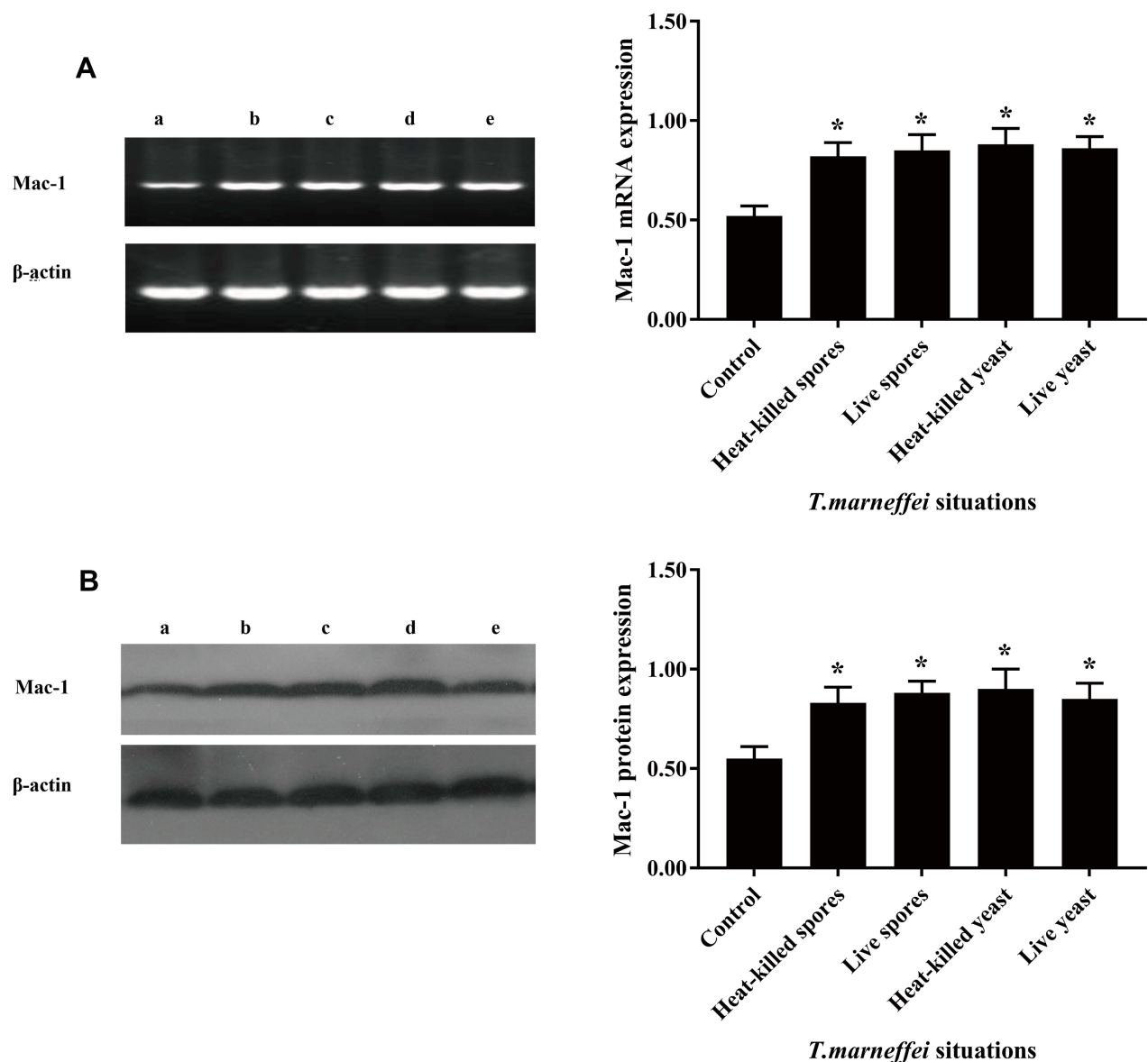


Figure 2 The conidia spores and yeast cells of *T. marneffeii* upregulate CD11b expression in mouse macrophages. pMacs from healthy BALB/c mice were incubated with living or heat-killed conidia spores and yeast cells of *T. marneffeii* at a ratio of 1:5 for 1.5 hrs. The relative levels of CD11b mRNA transcripts and protein expression were determined by RT-PCR and Western blot. Data are representative images or expressed as the mean \pm SD of each group of cells from three separate experiments. **(A)** The relative levels of CD11b mRNA transcripts. **(B)** The relative levels of CD11b protein. a: pMacs from the healthy mice without *T. marneffeii*; b: pMacs stimulated with heat-killed spores; c: pMacs stimulated with living spores; d: pMacs stimulated with heat-killed yeast; e: pMacs stimulated with living yeast. * $P < 0.05$ vs the control.

can activate CR3 that is coordinated by the integrins to enhance phagocytosis and antifungal immunity.¹⁵ Actually, CR3 can through the I domain and the C-terminal lectin-like site on the α (CD11b) subunit, and the β (CD18) subunit to recognize and phagocytoses pathogens, such as *Mycobacterium tuberculosis*, *Candida albicans* and *Histoplasma capsulatum*.^{8,16-22} Actually, a previous study has indicated that CR3 and other pathogen recognition receptors (PRRs) in human monocytes are important for the adhesion of *Penicillium marneffeii*, which stimulates monocyte

activation and cytokine production in a PRR-dependent manner.²³ In this study, we explored the importance of CD11b in phagocytosis of *T. marneffeii* by mouse macrophages. We found that *T. marneffeii* up-regulated CD11b expression on mouse macrophages regardless of whether macrophages were isolated from healthy and immunosuppressed mice. Similarly, either heat-killed or living spores or yeast cells from *T. marneffeii* also significantly upregulated CD11b expression pMacs cells in vitro. These findings extended our previous findings that *T. marneffeii* up-regulated

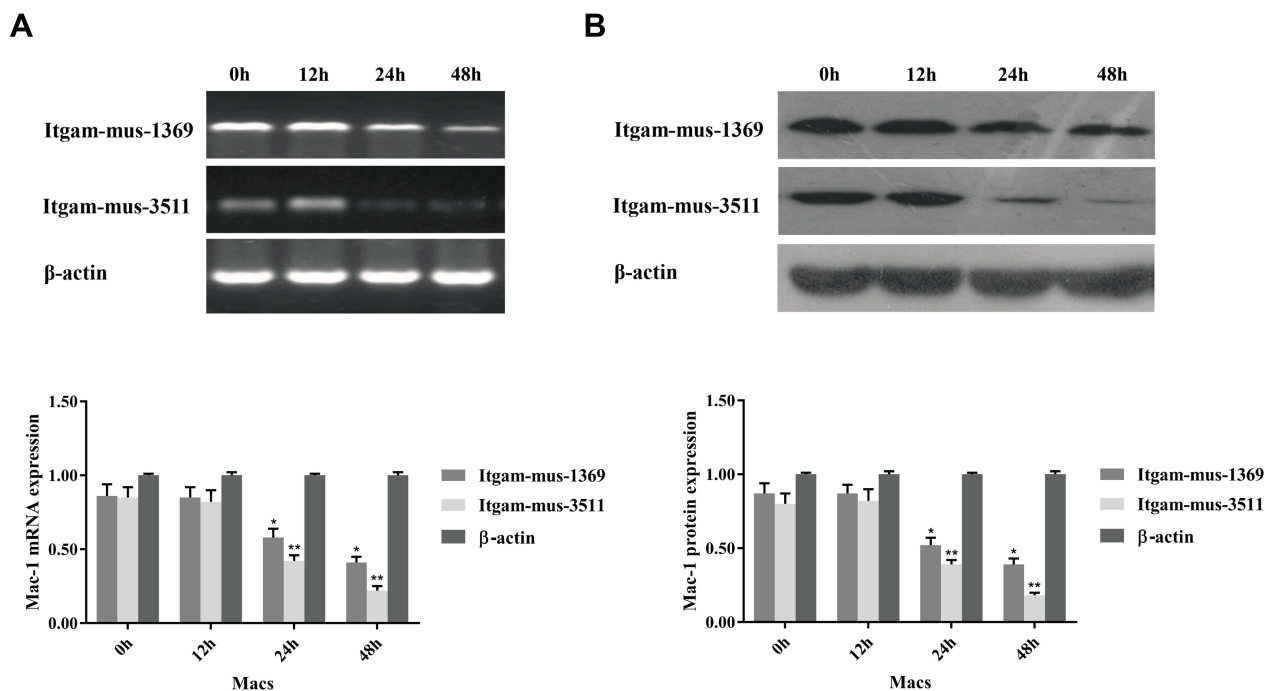


Figure 3 Knockdown of CD11b expression in macrophages. RAW264.7 cells were cultured overnight and transfected with the indicated siRNA at an optimal dose for the indicated time periods. The relative levels of CD11b mRNA transcripts and protein expression were determined by RT-PCR and Western blot. Data are representative images or expressed as the mean \pm SD of each group of cells from three separate experiments. **(A)** The relative levels of CD11b mRNA transcripts. **(B)** The relative levels of CD11b protein. Transfection with siRNA Irgam-mus-3511 or Irgam-mus-1369 effectively reduced the CD11b expression in a time-dependent manner in RAW264.7 cells. * $P < 0.05$, ** $P < 0.01$ vs the control.

TLR2, TLR-4 and dectin-1 expression on mouse macrophages.²⁴ The up-regulation of CR3, TLR2, TLR4 and dectin-1 expression is usually associated with increased activation of macrophages and may reflect innate immune responses to *T. marneffei* by potent phagocytosis and destruction of infected *T. marneffei*. Srinoulprasert et al have studied the involvement of CD11b/CD18 as a phagocytic receptor relevant for *T. marneffei* uptake by human macrophages. The results demonstrate that various PRR such as CD11b, CD18, MR, TLR1, TLR2 and TLR4 on human monocytes participate in the initial recognition of *T. marneffei* conidia.²³

Furthermore, we found that incubation of macrophages with *T. marneffei* promoted phagocytosis of *T. marneffei*, which was abrogated or significantly mitigated by pretreatment with anti-CD11b or knockdown of CD11b by CR3-specific siRNA on macrophages. These findings clearly indicated that CD11b was an important receptor for binding and phagocytosis of *T. marneffei* by mouse macrophages. Given that there are other receptors on macrophages are crucial for phagocytosis of fungus and CR3 can crosstalk with Fc γ RIIIB (CD16),^{25,26} CD14, TLR2,²⁷ and dectin-1.²⁸ *Coccidioides posadasii*,²⁹ *Aspergillus*,^{30,31} *mycobacteria*,³² *Sporothrix schenckii*,³³ *Cryptococcus neoformans*³⁴ and the strong phagocytosis of

T. marneffei by mouse macrophages may stem from the collaboration of multiple receptors. Heinsbroek et al³⁵ have shown that CR3 can collaborate with dectin-1 and MR for the phagocytosis of *C. albicans*. The dectin-1 is considered as the fungal pattern recognition receptor to recognize structurally diverse ligands and defense fungus infection.^{36,37} CR3 can interact with Fc γ R to promote phagocytosis of fungus in a Sky-dependent manner in some types of cells.^{38–40} We are interested in further investigating how CR3 collaborate with other receptors such as MR for phagocytosis of *T. marneffei* by macrophages during the *T. marneffei* infection.

T. marneffei infection can activate innate immune cells, such as macrophages and the activated macrophages can secrete cytokines. In this study, we found that incubation with *T. marneffei* stimulated high levels of IFN- γ , TNF- α , IL-4, IL-10, and IL-12 in mouse macrophages, which also mitigated and abrogated by pre-treatment with anti-CD11b or knockdown of CD11b in macrophages. Hence, CR3 was important receptor not only for phagocytosis of *T. marneffei*, but also for *T. marneffei*-stimulated inflammatory responses in macrophages. It is possible that *T. marneffei* may activate mouse macrophages by enhancing the expression of CD11b and other receptors as well as cytokine production. The

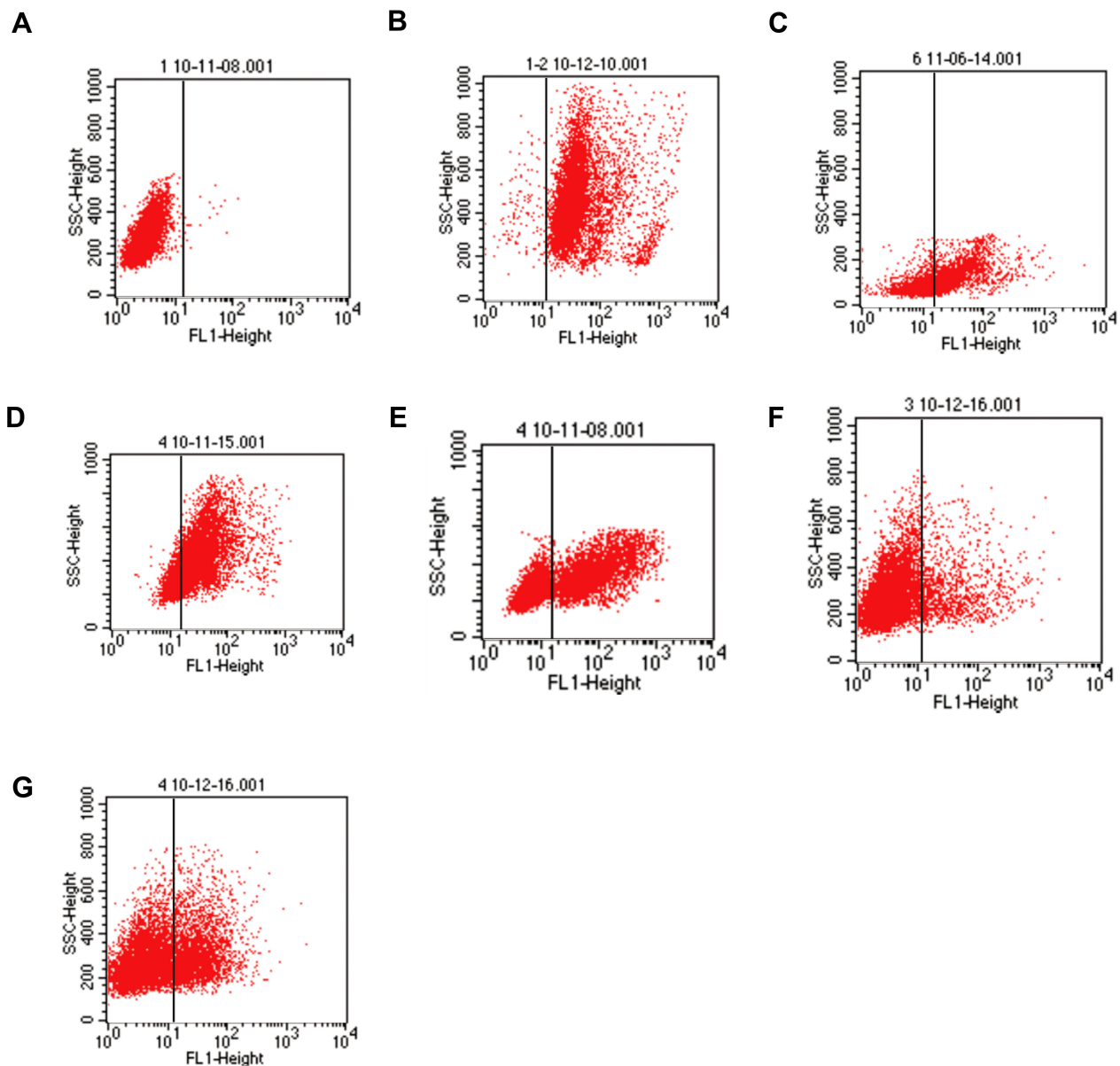


Figure 4 Flow cytometry detects the phagocytosis of *T. marneffei* by mouse macrophages. RAW 264.7 cells and pMacs from healthy and immunosuppressed BALB/c mice were pre-treated in triplicate with vehicle or anti-CD11b for 20 mins and incubated with FITC-labeled spores of *T. marneffei* at a ratio of 1:5 for 2 hrs. After being washed and quenched with trypan blue, the percentages of FITC+ cells were determined by flow cytometry. (A) Control macrophages from healthy mice without incubation with FITC-labeled spores of *T. marneffei*. The RAW264.7 cells, pMac from the healthy or immunosuppressed BALB/c mice with positive phagocytosis of *T. marneffei* were $91.63\% \pm 2.59$, $89.26\% \pm 3.48$, $65.75\% \pm 2.81$ (B, C, D), respectively. Pre-treatment with anti-CD11b, the positive phagocytosis was $35.45\% \pm 3.15$ (E). After transfected with control or CD11b-specific siRNAs, the positive phagocytosis macrophages were $10.89\% \pm 3.51$ (Itgam-mus-3511), $21.56\% \pm 2.86$ (Itgam-mus-1369), respectively (F, G). Data are representative flow charts from three separate experiments.

blocking CD11b by pre-treatment with anti-CD11b or knock-down of CD11b expression may diminish the activation of macrophages induced by *T. marneffei*, reducing cytokine production by macrophages. It is notable that while infection with microbial pathogens usually induces pro-inflammatory cytokine production by innate immune cells. However, we detected high levels of pro-inflammatory IFN- γ , TNF- α , and

IL-12 as well as anti-inflammatory IL-4 and IL-10 produced by mouse macrophages. These data suggest that infection with *T. marneffei* not only induced pro-inflammatory I type macrophages, but also alternatively activated II types of macrophages, even regulatory macrophages. These may reflect homeostasis of inflammatory responses to defense *T. marneffei* infection and limit organ destruction.

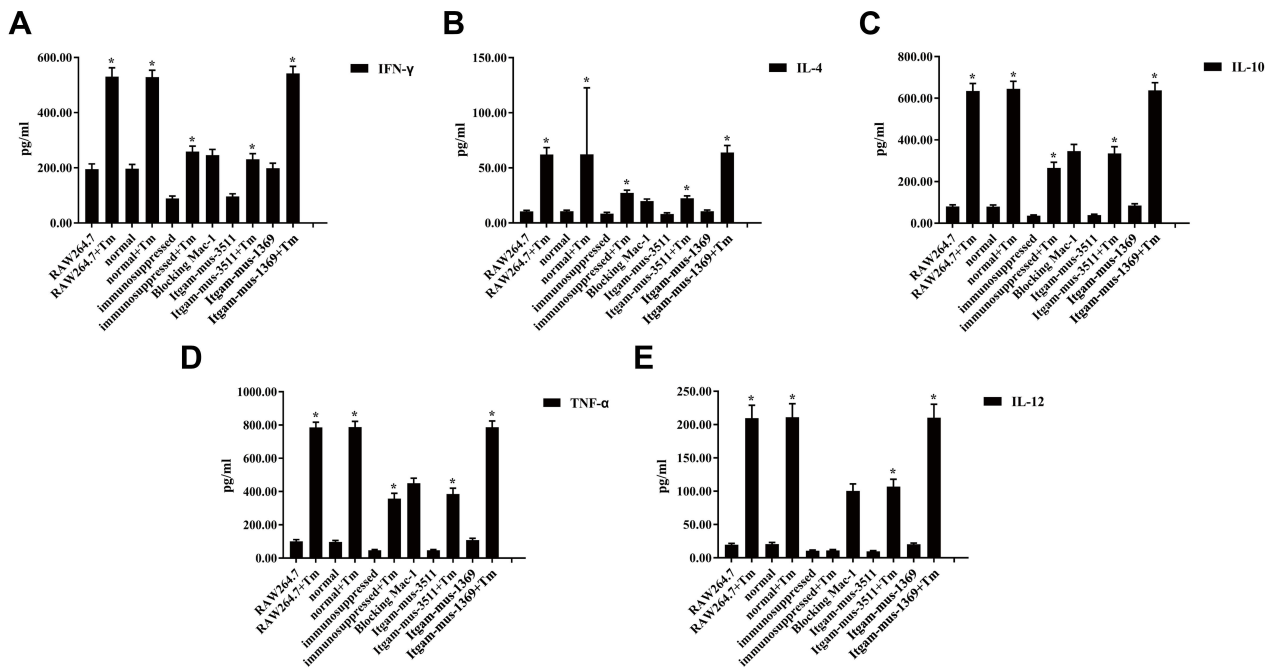


Figure 5 Blocking CD11b mitigates and abrogates *T. marneffei*-induced cytokine production by macrophages. RAW264.7 cells were transfected with the indicated siRNA for 48 hrs. pMacs from the healthy mice were pre-treated with anti-CD11b for 20 mins. The unmanipulated RAW264.7, pMacs from the healthy and immunosuppressed mice, siRNA-transfected RAW264.7, anti-CD11b treated pMacs were incubated with heat-killed spores of *T. marneffei* at a ratio of 1:5 for 2 hrs. The concentrations of individual cytokines in the supernatants of cultured cells were determined by ELISA. Data are expressed as the mean \pm SD of each group of cells from three separate experiments. There were high levels of pro-inflammatory IFN- γ (A), TNF- α (D) and IL-12 (E), but low levels of anti-inflammatory IL-4 (B) and IL-10 (C), in the cells simulated with the spores of *T. marneffei*. The cytokine responses stimulated by *T. marneffei* were mitigated and abrogated by pre-treatment with anti-CD11b or knockdown of CD11b expression in macrophages. * $P < 0.05$ vs the control without *T. marneffei*.

Conclusion

In summary, our data indicated that *T. marneffei* upregulated CD11b expression on mouse macrophages. 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 were mitigated and abrogated by pre-treatment with anti-CD11b or knockdown of CD11b expression. These indicated that murine macrophage requires CD11b to recognize *Talaromyces marneffei* and their cytokine responses to *T. marneffei*. Our findings may provide new insights into innate immune responses to *T. marneffei* infection.

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Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be

published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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