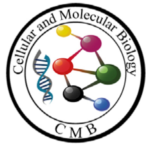

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Original Research

Keratinocytes contribute to the recruitment and M1 polarisation of macrophages during *C. albicans* colonisation

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Abstract: *Candida albicans* (*C. albicans*) is an opportunistic human fungal pathogen that colonises the skin. Both keratinocytes and macrophages play crucial roles in host defence against *C. albicans*. However, the interaction of keratinocytes with macrophages during *C. albicans* colonisation has not been well studied. In this study, macrophages were cultured in conditioned medium from keratinocytes treated with heat-inactivated *C. albicans* (CM-*C. albicans*), macrophage migration and polarised activation and were then assessed by a Transwell assay, flow cytometry, quantitative real-time PCR (qPCR), Western blot and an enzyme-linked immunosorbent assay (ELISA). The results showed that CM-*C. albicans*-stimulated macrophages display significantly increased migration and phagocytosis, and they display an upregulation of proinflammatory cytokines (tumour necrosis factor alpha (TNF-a), interleukin (IL)-12 and nitric oxide (NO)). Markers characteristic of M1 macrophages, such as human leukocyte antigen (HLA)-DR, CD86 and inducible nitric oxide synthase (iNOS), are upregulated, whereas markers of M2 macrophages, such as mannose receptor (MR) and Arginase 1 (Arg1), are not affected. Additionally, the levels of TNF-a, IL-12 and monocyte chemotactic protein 1 (MCP-1) in CM-*C. albicans* are markedly upregulated, whereas the levels of IL-4 and IL-10 are not affected. And the CM-*C. albicans*-induced M1 macrophage polarisation, proinflammatory cytokine production and phagocytosis could be blocked by an anti-TNF-a neutralising antibody. This study showed that keratinocytes may promote macrophage recruitment and M1 polarisation during *C. albicans* colonisation at least in part by secreting TNF-a.

Key words: *C. albicans;* Keratinocyte; Macrophage; M1 polarisation; TNF-a.

Introduction

C. albicans is an opportunistic pathogen that frequently colonises the mucosal and skin surfaces of healthy individuals without causing disease (1). However, in the immunosuppressed population, *C. albicans* is capable of causing life-threatening infections (2). Over the past decades, with the use of extensive immunosuppressive therapies and invasive medical procedures, *C. albicans* has become recognised as a life-threatening pathogen (3). Currently, the mechanisms underlying the host immune inflammatory response against cutaneous candidiasis are not well understood.

In addition to serving as a physical barrier, keratinocytes also play an important role in mediating the innate and specific cutaneous immune defence against invading pathogens by secreting cytokines and chemokines (4-6). As constituents of the outermost layer that encounter potential pathogens, keratinocytes can sense environmental signals and must be able to initiate a differential immune response to harmless commensals and harmful pathogens (7). The presence of cell surface receptors (i.e., mannose receptor (MR)) on human keratinocytes has been reported to mediate the killing of *C. albicans* (8). In addition, not only viable *C. albicans,* but also heat-inactivated *C. albicans* and sterile filtered soluble factors are capable of inducing a cytokine response in keratinocytes (5, 6). The exposure of keratinocytes to *C. albicans* leads to the production of chemokines and proinflammatory cytokines. TNF-a and MCP-1 are the major chemokine and proinflammatory cytokine for the recruitment and activation of macrophages at infection sites (9, 10). Li et al. demonstrated that *C. albicans* triggers inflammatory responses in human keratinocytes through Toll-like receptor (TLR) 2 (11).

Resident macrophages are the key immune surveillance cells in the mucosa that recognise and respond to *C. albicans* (12, 13). Macrophages exhibit phenotypic plasticity, and they can be divided into the subsets of classically activated inflammatory macrophages (M1) and alternatively activated anti-inflammatory macrophages (M2) (14, 15). M1 macrophages can control *C. albicans* infection of the skin by phagocytosis and by the induction of a proinflammatory response involving the production of proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-a) (13, 16, 17).

Crosstalk between keratinocytes and macrophages is crucial for the immunological barrier function of the skin, whereas impaired cross-talk contributes to chronic inflammatory skin diseases such as cutaneous candidiasis and psoriasis. Keratinocytes can sense external challenges via pathogen recognition receptors and can secrete cytokines and chemokines that recruit and activate immune cells (18). In turn, activated immune cells secrete cytokines to support keratinocyte activation and proliferation. Disturbances in the balance of this crosstalk result in impaired immune tolerance or chronic inflammatory skin disease.

Although the roles of both macrophages and keratinocytes in host defence against *C. albicans* infection are well-defined, little information concerning the cross-talk between keratinocytes and macrophages during *C. albicans* infection or colonisation is available. In this study, we try to demonstrate the functional relationship between human keratinocytes and macrophages in response to the *C. albicans* challenge.

Materials and Methods

Cell culture

Human HaCaT and U-937 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). HaCaT cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and 100U/ml penicillin/streptomycin. U-937 cells were cultured with RPMI-1640 supplemented with 10% FBS (Invitrogen) and 100U/ml penicillin/streptomycin. *C. albicans* (ATCC 11006) was cultured in Sabouraud dextrose broth (SDB). For the coculture experiments, *C. albicans* cells were inactivated by incubation at 100°C for 10 min and were then added to HaCaT cells at a ratio of 10:1 *C. albicans*: HaCaT cells; the cells were then cultured for 72h. The conditioned medium from keratinocytes treated with heat-inactivated *C. albicans* (CM-*C. albicans*) was filtered and stored at -20°C until use. The supernatant from untreated keratinocytes was used as a control.

U-937 cell differentiation into macrophages

U-937 cells were induced to differentiate by three days of treatment with 50ng/ml phorbol-12-myristate-13-acetate (PMA). Non-adherent undifferentiated cells were removed by two washes with PBS. For each assay, differentiated macrophages were incubated in serum-free medium for 2h.

Macrophage migration assay

Cell migration assays were carried out using Boyden chambers containing Transwell membrane filter inserts in 24-well tissue culture plates. 1×10^5 U-937 macrophages in 100μl of serum-free medium supplemented with 0.2% bovine serum albumin (BSA) were seeded in the top chamber of a Transwell insert containing an 8μm pore size polycarbonate membrane. A chemoattractant solution containing CM-*C. albicans* was added to the bottom chamber. The cells were incubated with each chemoattractant solution for 4h. After migration was allowed to occur for 4h, non-migrating cells were removed by wiping the upper side of the membrane, and migrated cells were fixed and stained with crystal violet. The average number of migrated cells was counted in six random fields (×200 magnification).

Phagocytosis assay

U-937 macrophages were plated in 6-well plates (1×10^6 cells per well). Heat-inactivated *C. albicans* cells were stained with 1mg/ml FITC in 0.05 M carbonate-bicarbonate buffer (pH 9.2) for 15 min at 4°C in the dark and were then washed three times. FITC-labelled *C. albicans* was added to macrophages at a ratio of 10:1 and incubated for 2h. Then, nonphagocytosed *C. albicans* cells were removed by washing. The phagocytosis index was determined by fluorescence-activated cell sorting (FACS) and immunofluorescence.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The reverse transcription (RT) of mRNA to cDNA was carried out using oligo(dT) primers. qPCR was carried out on an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA) using a standard protocol from a SYBR Green PCR kit (Toyobo, Osaka, Japan). β-Actin was used as an internal control. qPCR array analysis was performed on an ABI ViiA7 system (Applied Biosystems) using a standard protocol from a SYBR Green PCR kit (Wcgene Biotech, Shanghai, China). The primer sequences used are shown in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

U-937 macrophages were treated with the indicated reagents, and the supernatant was collected and analysed by ELISA for human TNF-a (ab181421, Abcam, CA, USA), IL-10 (ab100549), IL-12 (ab46035) and NO

Table 1. Primers used for qPCR in this study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
TNF-a	CTGCTGCACTTTGGAGTGATC	CTACAACATGGGCTACAGGCTT
IL-12	TCCTTGTGGCTACCCTGGTC	CACTGTGCTGGTTTTATCTTTTGT
NO	CCGGTAAGTACCTAGCCCAC	AAGGAGGAGATGCAGCACAC
CD86	TGGAACCAACACAATGGAGA	AAAAAGGTTGCCCAGGAACT
HLA-DR	TGGGAGTTTGATGTCCCAAG	AACATCATCACCTCCATGTG
iNOS	GAGAGATTTTTCACGACACCCT	TTTCTATTTCCTTTGTTACGGC
Mannose receptor	TGCTACTGAACCCCCACAAC	CAATGGAAACCAGAGAGGAAC
Arg-1	GGCAGAAGTCAAGAAGAACGG	ATACAGGGAGTCACCCAGGAGA
IL-4	TGCTTCCCCCTCTGTTCTTC	TCCTTCTCAGTTGTGTTCTTGG
IL-10	TGGTCCTCCTGACTGGGGT	TGATGTCTGGGTCTTGGTTCTC
MCP-1	TCTCTGCCGCCCTTCTGTG	TCGGAGTTTGGGTTTGCTTG
β-actin	CCCCAGGCACCAGGGCGTGAT'	GTCATCTTCTCGCGGTTGGCCTTGGGGTT

(Lianshuo Biotechnology, Shanghai, China) secretion as per the manufacturers' instructions.

Western blot analysis

Equivalent amounts of total protein (100µg) were separated by 12% SDS-polyacrylamide gel electrophoresis and were then wet electrotransferred to 0.2µm PVDF membranes (Bio-Rad, CA, USA). Blots were incubated overnight at 4°C with 5% primary antibodies (anti-HLA-DR, ab20181, Abcam, CA, USA; 2% anti-CD86, ab134385, Abcam, CA, USA; anti-iNOS, ab49999, Abcam, CA, USA; anti-Arg, ab212522, Abcam, CA, USA; and anti-MR, ab8918, Abcam, CA, USA) and were then incubated with the appropriate goat anti-rabbit or goat anti-rat HRP-conjugated secondary antibody (1:5000, Jackson, USA). Protein signals were detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).

Statistical analysis

All data are presented as the means ± SEMs of at least three independent experiments. We used GraphPad Prism 5 (GraphPad Software, La Jolla, CA) for statistical analysis. All data were analysed using a two-tailed Student's t-test. A p value of <0.05 was considered significant.

Results

Conditioned medium from keratinocytes treated with heat-inactivated C. albicans (CM-C. albicans) increases macrophage migration and phagocytosis

To characterise the relationship between keratinocytes and macrophages during *C. albicans* colonisation, U-937 macrophage-like cells were treated with CM-*C. albicans*. As shown in Figure 1(a) and (b), treatment with CM-*C. albicans* significantly induced U-937 macrophage migration. This result indicates that keratinocytes treated with *C. albicans* increased macrophage recruitment during *C. albicans* colonisation. Host defence against *C. albicans* infection relies mainly on the phagocytosis of *C. albicans* by macrophages. We further investigated whether the phagocytic ability of U-937 macrophages was affected by exposure to CM-*C. albicans*. As shown in Figure 1(c)-(e), the phagocytic ability of U-937 macrophages was enhanced following treatment with CM-*C. albicans*.

Heat-inactivated C. albicans (CM-C. albicans) stimulates the secretion of proinflammatory cytokines

In addition to controlling *C. albicans* infection by phagocytosis, macrophages control *C. albicans* infection by producing proinflammatory cytokines, which induce a proinflammatory response in the skin (19). We thus investigated whether keratinocytes promote the secretion of proinflammatory cytokines by interacting with macrophages during *C. albicans* colonisation. Figure 2(a) shows that, compared with the levels in untreated macrophages, the mRNA levels of TNF-a and IL-12 in CM-*C. albicans-treated* macrophages were markedly increased. An ELISA further verified that *C. albicans*-treated macrophages produce higher levels of cytokines than do untreated macrophages (Figure 2(b)-(d)).

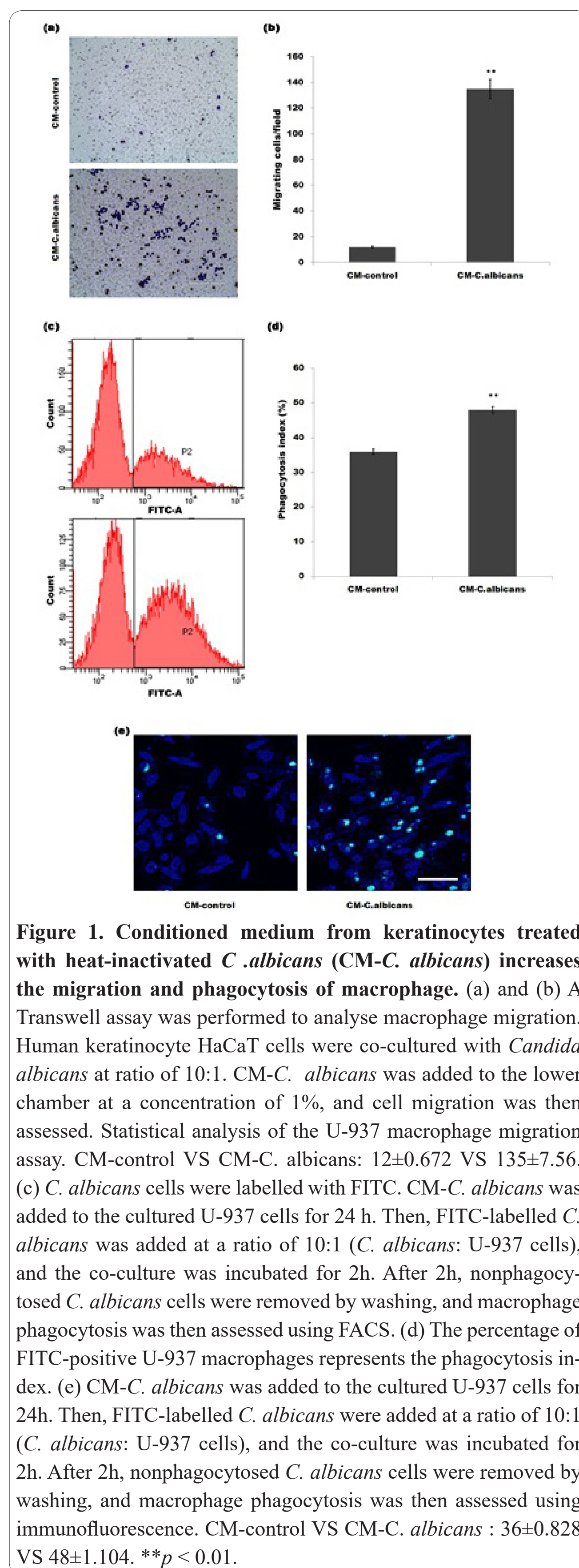

Figure 1. Conditioned medium from keratinocytes treated with heat-inactivated *C .albicans* (CM-*C. albicans*) increases the migration and phagocytosis of macrophage. (a) and (b) A Transwell assay was performed to analyse macrophage migration. Human keratinocyte HaCaT cells were co-cultured with *Candida albicans* at ratio of 10:1. CM-*C. albicans* was added to the lower chamber at a concentration of 1%, and cell migration was then assessed. Statistical analysis of the U-937 macrophage migration assay. CM-control VS CM-C. albicans: 12±0.672 VS 135±7.56. (c) *C. albicans* cells were labelled with FITC. CM-*C. albicans* was added to the cultured U-937 cells for 24 h. Then, FITC-labelled *C. albicans* was added at a ratio of 10:1 (*C. albicans*: U-937 cells), and the co-culture was incubated for 2h. After 2h, nonphagocytosed *C. albicans* cells were removed by washing, and macrophage phagocytosis was then assessed using FACS. (d) The percentage of FITC-positive U-937 macrophages represents the phagocytosis index. (e) CM-*C. albicans* was added to the cultured U-937 cells for 24h. Then, FITC-labelled *C. albicans* were added at a ratio of 10:1 (*C. albicans*: U-937 cells), and the co-culture was incubated for 2h. After 2h, nonphagocytosed *C. albicans* cells were removed by washing, and macrophage phagocytosis was then assessed using immunofluorescence. CM-control VS CM-C. *albicans* : 36±0.828 VS 48±1.104. **p < 0.01.

Cytokines secreted from keratinocytes after C. albicans treatment promote M1 macrophage polarisation

CM-*C. albicans* stimulate the secretion of proinflammatory cytokines and enhance macrophage phagocytosis, which indicates the induction of macrophage M1 polarisation. To demonstrate this, we further assessed the expression level of M1 (HLA-DR, CD86 and iNOS)

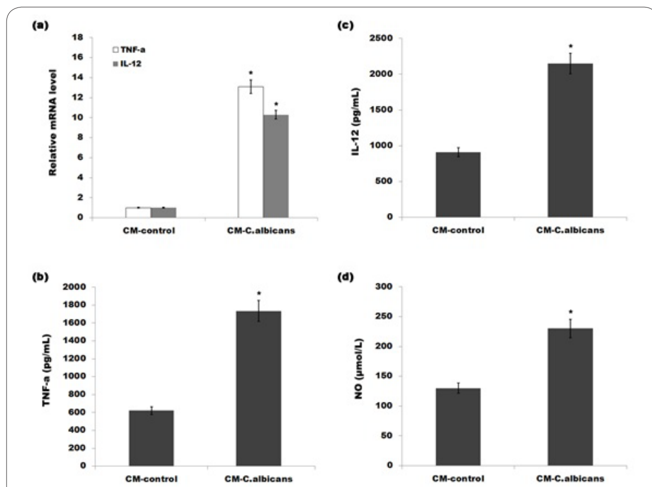

Figure 2. Heat-inactivated *C. albicans* (CM-*C. albicans*) stimulates the secretion of proinflammatory cytokines. (a) U-937 macrophages were treated with CM-*C. albicans* for 24h, and the mRNA levels of TNF-a and IL-12 were then assessed using qPCR. CM-control VS CM-C. albicans: TNF-a= 1±0.05 VS 13.5±0.675; IL-12 =1±0.05 VS 10.7±0.535. (b)-(d) U-937 macrophages were treated with CM-*C. albicans* for 24h, and the secretion of TNF-a, IL-12 and NO was then assessed using an ELISA. CM-control VS CM-C. albicans: TNF-a =620.3±31 VS 1735.6±86.75; IL-12=910.1±45.5 VS 2150.4±107.5; NO =130.2±6.5 VS 230.3±11.5. *$p < 0.05$.

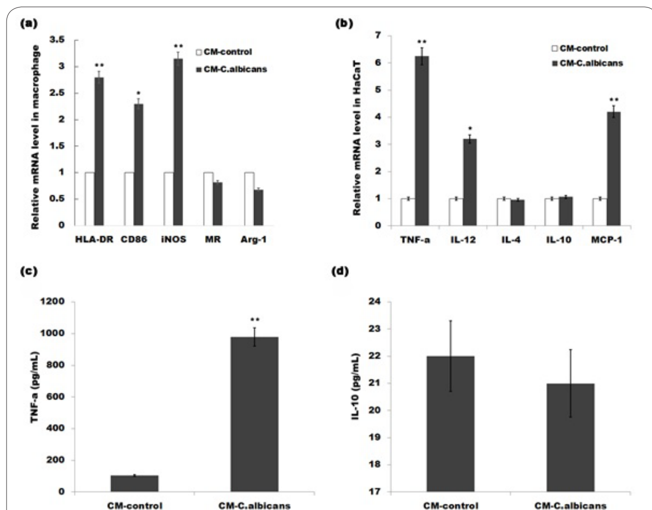

Figure 3. Cytokines secreted from keratinocytes after *C. albicans* treatment promote M1 macrophage polarisation. (a) U-937 macrophages were treated with CM-*C. albicans* for 24h, and the mRNA levels of HLA-DR, CD86, iNOS, MR and Arg-1 were then assessed using qPCR. CM-control VS CM-C. albicans: HLA-DR=1±0.05 VS 2.8±0.112; CD86=1±0.04 VS 2.3±0.092; iNOS= 1±0.06 VS 3.15±0.126; MR= 1±0.05 VS 0.82±0.28; Arg-1= 1±0.06 VS 0.68±0.0272. (b) HaCaT cells were treated with *C. albicans* for 24h, and the mRNA levels of TNF-a, IL-12, IL-4, IL-10 and MCP-1 were then assessed using qPCR. CM-control VS CM-C. albicans: TNFa=1±0.03 VS 6.25±0.3125; IL-12=1±0.05 VS 3.2±0.16; L-4=1±0.04 VS 0.96±0.048; IL-10= 1±0.05 VS 1.07±0.0535; MCP-1= 1±0.04 VS 4.21±0.2105. (c) and (d) HaCaT cells were treated with CM-*C. albicans* for 24h, and the protein levels of TNF-a and IL-10 were then assessed using an ELISA. CM-control VS CM-C. albicans: TNF-a= 105.2±5.25 VS 980.3±49.1; IL-10= 22.1±1.98 VS 21.1±18.9.*$p < 0.05$. **$p < 0.01$.

and M2 macrophage markers (MR and Arg1) following treatment with CM-*C. albicans*. As shown in Figure 3(a), compared with that in untreated macrophages, the expression of M1 markers (HLA-DR, CD86 and iNOS) in CM-*C. albicans*-treated macrophages was significantly upregulated, whereas the expression of M2 markers (MR and Arg1) was not affected. To determine the mechanism underlying the CM-*C. Albicans-induced* M1 polarisation of macrophages, we then assessed the cytokine levels in conditioned medium from *C. albicans*-treated keratinocytes. HaCaT keratinocytes were treated with CM-*C. albicans*, and the mRNA levels of M1- and M2-related cytokines were assessed using qPCR. Figure 3(b) shows that the levels of M1-related cytokines (TNF-a and IL-12) were increased in conditioned medium from C. *albicans*-treated HaCaT cells, whereas the levels of M2-related cytokines (IL-4 and IL-10) were not affected. Furthermore, MCP-1 expression was upregulated in the conditioned medium from C. *albicans*-treated HaCaT cells. These results may explain the promotion of macrophage migration by CM-C. *albicans*. An ELISA further verified that, compared to those in conditioned medium from untreated cells, TNF-a protein levels in conditioned medium from C. *albicans*-treated HaCaT cells were enhanced, whereas IL-10 protein levels were not affected (Figure 3(c) and (d)). These data suggest that *C. albicans*-treated keratinocytes secrete TNF-a or IL-12, thus promoting M1 macrophage polarisation.

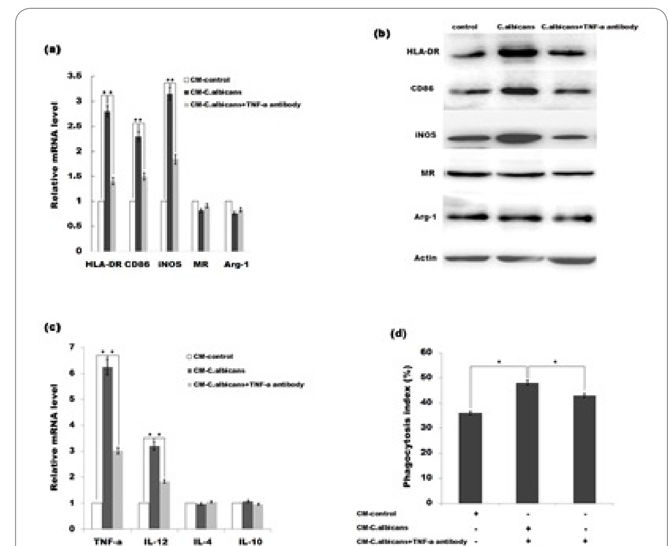

Figure 4. Keratinocytes promote M1 macrophage polarisation and phagocytosis in part by secreting tumour necrosis factor alpha (TNF-a). (a) and (b) Macrophages were pre-treated with a neutralising anti-human TNF-a monoclonal antibody (MAB11) (10μg/ml) and were then treated with CM-*C. albicans* for 24h. The expression levels of HLA-DR, CD86, iNOS, MR and Arg1 were assessed using qPCR and Western blot. The mRNA levels of CM-control VS CM-*C. albicans* VS CM-C. albicans+TNFa antibody: HLA-DR= 1±0.05 VS 2.8±0.14 VS 1.4±0.07; CD86= 1±0.06 VS 2.3±0.115 VS 1.5±0.075; iNOS= 1±0.04 VS 3.15±0.1575 VS 1.84±0.092; MR= 1±0.05 VS 0.82±0.041 VS 0.91±0.0455; Arg-1=1±0.04 VS 0.67±0.034 VS 0.83±0.0415; (c) The mRNA levels of TNF-a, IL-12, IL-4 and IL-10 were assessed using qPCR, CM-control VS CM-*C. albicans* VS CM-C. albicans+TNFa antibody: TNF-a= 1±0.05 VS 6.25±0.3125 VS 3.02±0.2835; IL-12= 1±0.05 VS 3.2±0.16 VS 1.84±0.092; IL-4= 1±0.04 VS 0.96±0.048 VS 1.05±0.0525; IL-10=1±0.05 VS 1.07±0.0535 VS 0.95±0.0475. (d) Macrophage phagocytosis was assessed using FACS. CM-control VS CM-*C. albicans* VS CM-C. albicans+TNFa antibody: 36.2±0.72 VS 48.3±0.96 VS 43.1±0.86. *$p < 0.05$

Keratinocytes promote M1 macrophage polarisation in part by secreting tumour necrosis factor alpha (TNF-a)

Keratinocytes produce TNF-a following treatment with *C. albicans*, and TNF-a contributes to macrophage M1 polarisation(20). We thus speculated that TNF-a might mediate the role of keratinocytes in the induction of M1 macrophage polarisation during *C. albicans* colonisation. As shown in Figure 4(a) and (b), an anti-TNF-a neutralising antibody significantly inhibited the mRNA and protein expression of M1 markers (HLA-DR, CD86 and iNOS). Furthermore, the anti-TNF-a neutralising antibody significantly suppressed both the secretion of proinflammatory cytokines and macrophage phagocytosis (Figure 4(c) and (d)). These results demonstrate that keratinocytes contribute to the recruitment and alternative activation of macrophages during *C. albicans* colonisation.

Discussion

C. albicans is widely distributed on the surface of the skin and mucous membranes and is the most common opportunistic pathogen in humans. The skin is the first barrier between the body and the external environment. Keratinocytes are the main components of the epidermis and are the first cells involved in host defence against external pathogens; keratinocytes play a key role in the inflammatory response to pathogen infection by secreting cytokines (5, 21, 22). In the process of infection, *C. albicans* directly targets keratinocytes before breaking through the epidermal barrier. During this process, the skin immune system starts sensing the attack of pathogenic microorganisms by recognising functional changes in keratinocytes.

The mechanisms underlying the pathogenesis and causation of illness by Candida depend on the immune balance in the human body and the virulence of the infecting strain (23). The keratinocyte-mediated cytokine response is involved in cell-mediated immunity and is considered a principal defence against Candida infection. Not only viable *C. albicans* but also heat-inactivated *C. albicans* and sterile filtered soluble factors can induce a cytokine response (24, 25).

C. albicans has three morphological forms: spores, pseudohyphae and hyphae. Spore colonisation by the yeast phase occurs in healthy humans. During infection, Candida cells transform from the yeast phase to the mycelial phase, which is considered the pathogenic phase. The pathogenicity and antigenicity of the different biomorphological forms of Candida differ; the inflammatory reactions and immune responses induced by these forms also differ (26, 27). Commensalism by the yeast phase of Candida is generally understood to be the result of *C. albicans* adaptation to host organisms (28, 29).

To simulate a true biological process, *C. albicans* spores were used in our study; antigens of inactivated *C. albicans* spores were used to stimulate keratinocytes. The co-culture supernatant was then collected and added to the macrophage culture system to observe macrophage chemotaxis and phagocytosis and changes in polarisation trends. In this way, we studied the means by which *C. albicans* colonisation can influence macrophage function through the network of chemical factors secreted by epidermal keratinocytes.

Our results show that *C. albicans* colonisation can promote the secretion of the proinflammatory cytokines MCP-1, TNF-a and IL-12 by keratinocytes, increase macrophage chemotaxis and phagocytosis and promote the functional polarisation of macrophages into the M1 phenotype.

As previously reported, keratinocyte-derived MCP-1 may play an important role in the development of psoriasis (30). In addition, TNF-a is believed to contribute to macrophage M1 polarisation, which also plays an important role in psoriasis (20). The clinical use of TNF-a antagonists can effectively relieve many clinical manifestations of psoriasis. Therefore, our results are consistent with the events occurring during some pathological processes of psoriasis, and the idea that *C. albicans* colonisation may be involved in the pathogenesis and perpetuation of psoriasis is convincing.

Some researchers, such as Taheri Sarvtin, have compared the isolation rates of cutaneous Candida in psoriasis patients and healthy individuals. Candida was isolated in 15% of psoriasis patients and 4% of healthy individuals, and the difference was statistically significant, thus suggesting that the recurrence and perpetuation of psoriatic lesions were associated with Candida colonisation (31).

In addition, Javad et al. compared the isolation rates of Candida from skin lesions and the oral cavity of 100 patients with atopic dermatitis and 50 healthy controls. The results showed that the isolation rate of *C. albicans* was significantly higher in the patients with atopic dermatitis than in the healthy controls. The level of the *C. albicans*-specific IgG antibody in the serum of the patients was significantly lower than that in the controls. Therefore, the dysregulation of *C. albicans*-specific IgG antibody may be related to the pathogenesis of atopic dermatitis (32).

Therefore, the potential of antifungal treatment to relieve clinical manifestations in patients with psoriasis or atopic dermatitis is also worthy of future study.

Furthermore, the recognition of pathogens by TLRs is important for host defence against microbial pathogens; this event leads to the expression of genes encoding components such as proinflammatory cytokines and costimulatory molecules. Along with TLR-dependent gene expression of proinflammatory cytokines and costimulatory molecules, phagocytosis-mediated antigen presentation instructs the development of antigen-specific acquired immunity. Therefore, characterising the relationship between TLRs and macrophage phagocytosis is of interest. Receptors such as TLR-2, TLR-4 (33-35) and dectin-1 (36) are reported to play an important role in regulating the inflammatory response of M1 macrophages to *C. albicans* infection or colonisation. In our next study, we will investigate the roles of TLRs in the interaction of keratinocytes with macrophage and in host defence against *C. albicans* infection or colonisation.

Acknowledgements
None.

Conflict of Interest

No conflict of interest is associated with this work.

Authors' contribution

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Xing-Hua Gao conceived and designed the study; Yu-Xiao Hong, Rui-Qun Qi collected and analysed the data; Xiu-Hao Guan wrote the manuscript.

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