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Candida albicans induces upregulation of programmed death ligand 1 in oral squamous cell carcinoma

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Abstract

Background: The potential association between *Candida albicans* (*C. albicans*) infection and oral squamous cell carcinoma (OSCC) has been noticed for a long time. Programmed death ligand-1 (PD-L1) is a key molecule of tumor immune escape and tumor progression. This study aimed to explore whether *C. albicans* could influence PD-L1 expression in OSCC in vitro and in mouse model.

Methods: OSCC cell lines (Cal27 and HN6) were infected with *C. albicans* for 2 and 24 h, then PD-L1 expression was detected by quantitative real-time polymerase chain reaction (RT-qPCR), western blot (WB), and flow cytometry (FCM). To identify the underlying mechanisms, PD-L1 expression in OSCC cells treated with heat-inactivated *C. albicans* or with biofilm metabolites derived from *C. albicans* were explored respectively. Meanwhile, signaling pathways involved in PD-L1 regulation were explored by RT-qPCR, and the candidate genes were verified by WB. Moreover, an OSCC mouse model induced by 4-nitroquinoline-1 oxide was used to further explore the role of *C. albicans* infection in PD-L1 expression in vivo.

Results: *C. albicans* and heat-inactivated *C. albicans* upregulated the PD-L1 expression in Cal27 and HN6 cells. Various signaling pathways involved in PD-L1 regulation were influenced by *C. albicans* infection. Among them, TLR2/MyD88 and TLR2/NF-κB pathways might participate in this process. Furthermore, PD-L1 expression in oral mucosa epithelium was upregulated by *C. albicans* infection in both normal and OSCC mice.

Conclusions: This study suggests that *C. albicans* could induce upregulation of PD-L1 in OSCC in vitro and in mouse model, which might due to the activation of TLR2/MyD88 and TLR2/NF-κB pathways.

KEYWORDS
Candida albicans, oral squamous cell carcinoma, programmed death ligand 1, tumor microenvironment

1 | INTRODUCTION

Increasing attention has been focused on the relationship between microorganisms and cancer. It was reported that about 13% of global cancer incidence was attributable to microorganisms' infection.[1] Microbiota, the important factor existing in tumor microenvironment (TME), may participate in the occurrence, development, responsiveness to therapeutics, and complications of cancer.[2] However, the ways in which various microorganisms contribute to carcinogenesis are complex and remain to be further investigated.

Candida albicans (*C. albicans*), the most common fungus inhabiting in human oral cavity and is carried by approximately 80% of general population,[3] has shown an association with oral squamous cell carcinoma (OSCC),[4] which is the most common type and accounts for more than 90% of oral cancer.[5] Until now, there exists a debate about whether oral candidiasis should be classified as oral potentially malignant disorders (OPMDs). In the 4th edition of WHO classification on head and neck tumors, "chronic candidiasis" was included in the OPMDs.[6] Nevertheless, it was considered that the evidence is still insufficient to support the malignant potential of oral candidiasis.[7] Thus, it is of great significance to demonstrate whether *C. albicans* infection participate in the OSCC progression, as well as to explore the underlying carcinogenic mechanisms of *C. albicans*.

Programmed death ligand-1 (PD-L1, B7-H1, CD274)/programmed death receptor (PD-1) pathway is a key mechanism of immune escape by cancers and usually contributes to T cells' exhaustion and immunosuppression in TME.[8] There is evidence indicated that the upregulation of PD-L1 in oral epithelial cell might be associated with the progression of OPMDs and OSCC.[9] Substantial expression of PD-L1 could be observed in the keratinocytes of oral lichen planus (OLP)[10] and oral leukoplakia (OLK),[11] and the increased PD-L1 level was positively correlated with the malignant transformation of OLK within 5 years.[12] In addition, some studies discussing the role of immune checkpoint biomarkers in OSCC also inferred that the expression of PD-L1 in OSCC samples was correlated with increased progression and decreased survival rates, though more precise evidence is needed.[9,13,14]

Previous studies showed that the infection of *C. albicans* could induce upregulation of PD-L1 on T cells and natural killer cells.[15,16] Besides, the pathogen-associated molecular patterns (PAMPs) and candidalysin of *C. albicans* could activate Toll-like receptors (TLR2/TLR4) on innate immune cells[17] and epidermal growth factor receptor (EGFR) on oral epithelial cells,[18] respectively. Coincidentally, both TLR2/TLR4 and EGFR are involved in the regulation of PD-L1 expression in tumor.[19] However, whether *C. albicans* infection influences PD-L1 expression in OSCC cells remains unrevealed.

Our previous work revealed that 20.83% of chronic hyperplastic candidiasis (CHC) patients had varying degrees of epithelial dysplasia and 4.17% had malignant transformation.[20] Considering the potential association between *C. albicans* and OSCC, as well as the abilities of *C. albicans* to activate some signals involved in PD-L1 regulation, it is worthy of research to investigate the role of *C. albicans* in regulating PD-L1 expression in OSCC. In this study, we explored the expression of PD-L1 in OSCC cell lines and OSCC mice during *C. albicans* infection. In addition, the mechanisms and signaling pathways involved in this process were also investigated.

2 | MATERIALS AND METHODS

2.1 | Cell culture conditions

The human OSCC cell lines (Cal27 and HN6) were obtained from Peking University School of Stomatology. OSCC cell lines were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin–streptomycin (Invitrogen) at 37°C in a 5% CO_2 incubator. Prior to stimulation, the cells were serum-starved for 24 h, and all experiments were carried out in serum-free DMEM.

2.2 | *C. albicans* growth conditions, heat-inactivated *C. albicans*, and biofilm metabolites

C. albicans strain ATCC 90028 and SC5314 were grown at 37°C in Yeast Extract Peptone Dextrose (YPD) medium (Solarbio) overnight, respectively. Cultures were washed in sterile Phosphate Buffer Solution (PBS) (HyClone) and adjusted to the required density. Heat-inactivated *C. albicans* was performed by incubation for 30 min at 100°C,[21] and confirmed by plating them on Sabouraud dextrose agar and incubating at 37°C for 24 h. The metabolites of *C. albicans* biofilm were obtained as previously described[22]: yeast cell suspension was placed into six-well plates and incubated for 90 min (adhesion phase) at 37°C. Then, the medium was replaced by RPMI-1640 culture medium (HyClone) and incubated at 37°C for 24 h. Finally, the suspension was filtered with a low-protein binding filter (SFCA 0.22 mm, Corning).

2.3 | Infection and stimulation

The Cal27 and HN6 cells were seeded into 60-mm cell culture dish (Corning-Costar) at 2×10^6 cells per dish and grown to 80% confluence. After being serum-starved for 24 h, the cells were infected with *C. albicans* with multiplicity of infection (MOI) of 0.005 for 24 h or MOI of 5 for 2 h, as previously reported.[23] Alternatively, cells were treated with heat-inactivated *C. albicans* (MOI = 5) or biofilm metabolites for 24 h. After infection or stimulation, the medium was discarded, and cells were washed three times with PBS.

2.4 | Western blot (WB) analysis

Cells were collected and lysed with radioimmunoprecipitation (RIPA)-buffer (Thermo Fisher Scientific) added with protease and phosphatase inhibitors (Huaxingbio Science). After centrifuged at 4°C, 12 000 rpm for 15 min, the protein supernatant was collected and its concentration was determined by bicinchoninic acid (BCA) assays (Thermo Fisher Scientific). Equal amounts of protein per lane were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich). The membranes were blocked with 5% nonfat milk for 2 h. Then, the membranes were incubated with primary antibodies overnight at 4°C using 1:2000-diluted antibodies against PD-L1 (ab205921, Abcam), phosphor-EGFR (ab40815, Abcam), EGFR (ab52894, Abcam), phosphor-STAT1 (ab109457, Abcam), STAT1 (ab109320, Abcam), phosphor-JAK2

(ab32101, Abcam), JAK2 (ab108596, Abcam), TLR2 (ab68159, Abcam), MyD88 (ab133739, Abcam), NF-κB (P65) (ab32536, Abcam), and β-actin (20536-1-AP, Proteintech). After being washed three times with Tris-buffered saline containing Tween (TBST) buffer, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:10 000, SA00001-2, Proteintech) for 1 h at room temperature. After being washed three times, the proteins were visualized with enhanced chemiluminescence reagent.

2.5 | Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol Reagent (Invitrogen). The RNA concentration was determined with the Nanodrop 8000 (Thermo Fisher Scientific). ABScript II Reverse Transcriptase (ABclonal) was used to reverse-transcribe total RNA into cDNA according to the manufacturer's instructions. The amplification was performed in triplicate using Universal SYBR Green Fast qPCR Mix (ABclonal). The primer (Sangon Biotech) sequences are shown in Supplementary Table S1. The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH mRNA.

2.6 | Flow cytometry (FCM)

A total of 1×10^6 cells were harvested and incubated with PE-conjugated PD-L1 (329705, Biolegend) at 4°C for 30 min. After washed twice, the cells were subjected to FCM analyzes using FACSDiva Software (BD Bioscience). The mean fluorescence intensities (MFI) of the PD-L1 expression on cells were analyzed by FlowJo (v. 10).

2.7 | Animal experiments

The animal experiments were reviewed and approved by the Biomedical Ethics Committee of Peking University (LA2021388). C57BL/6N male mice (6–8 weeks) were maintained under specific pathogen-free (SPF) conditions. For normal mouse infection experiment, the normal mice were randomly placed into infection and control groups ($n = 3$/group). For OSCC mouse infection experiment, OSCC model was developed using the carcinogen 4-nitroquinoline 1-oxide (4-NQO) (Abcam) in the drinking water (100 μg/ml) for 18 weeks as previous studies,[24] then the OSCC mice were randomly divided into two groups: infection group ($n = 6$) and control group ($n = 6$). For infection group, the mice were anesthetized, and a cotton swab soaked with 200 μl of *C. albicans* suspension (6×10^8 cfu/ml) was applied to the dorsum of tongue, while the mice in non-infection group were applied with a cotton swab soaked with medium alone, once every 3 days. The mice were euthanized after infection for 4 weeks (OSCC mice) or 8 weeks (normal mice). The tongues of mice were excised and cut into halves, half of the tongues were prepared for histology staining and the other half for fluorescence staining.

2.8 | Histology and immunofluorescence staining

For histology analysis, tongues were fixed in 4% paraformaldehyde for 24 h. After dehydration and paraffin embedding, the tongues were cut into 5-μm sections and stained with hematoxylin and eosin (H&E). For PD-L1 expression analysis, tongues were fixed in 4% paraformaldehyde, followed by dehydrated in 30% sucrose. Then, 20-μm-thick frozen sections were blocked with 3% goat serum albumin and incubated with rabbit anti-PD-L1 (1:200, ab213480, Abcam) overnight at 4°C. After incubated with goat anti-rabbit fluorescein isothiocyanate (FITC, 1:200, ZSGB-BIO) for 1 h at room temperature, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Solarbio). Fluorescence images were taken using a fluorescence microscope (Olympus Co.).

2.9 | Statistics

GraphPad Prism (v. 8.0) was used for statistical analyses. The experiments of WB and FCM were repeated two times, and the experiments of RT-qPCR were repeated three times. Data were presented as mean ± standard deviation (SD). Student's two-tailed *t* test was used to determine the statistical relevance between groups. A p value <0.05 was indicative of statistical significance.

3 | RESULTS

3.1 | *C. albicans* upregulated PD-L1 expression in OSCC cell lines

After infected with *C. albicans* for 2 and 24 h, the total PD-L1 protein levels in Cal27 cells were upregulated (Figure 1A,B). In addition, the MFI of PD-L1 on membrane of infected Cal27 cells was also higher than that on non-infected cells, and the prolonged infection could induce more remarkable PD-L1 expression (Figure 1A,B). Similarly, when compared with the non-infected cells, the total PD-L1 protein was upregulated in the HN6 cells infected with *C. albicans* for 2 and 24 h, but the MFI of PD-L1 on membrane was upregulated only in 24 h (Figure 1C,D). What is more, *C. albicans* infection upregulated the mRNA levels of PD-L1 in Cal27 ($p = 0.0080$) and NH6 ($p = 0.0271$) cells after infected for 24 h (Figure 1B,D).

3.2 | Heat-inactivated *C. albicans* enhanced PD-L1 expression in OSCC cell lines

When treated with heat-inactivated *C. albicans* for 24 h, the mRNA, total protein, and membrane protein levels of PD-L1

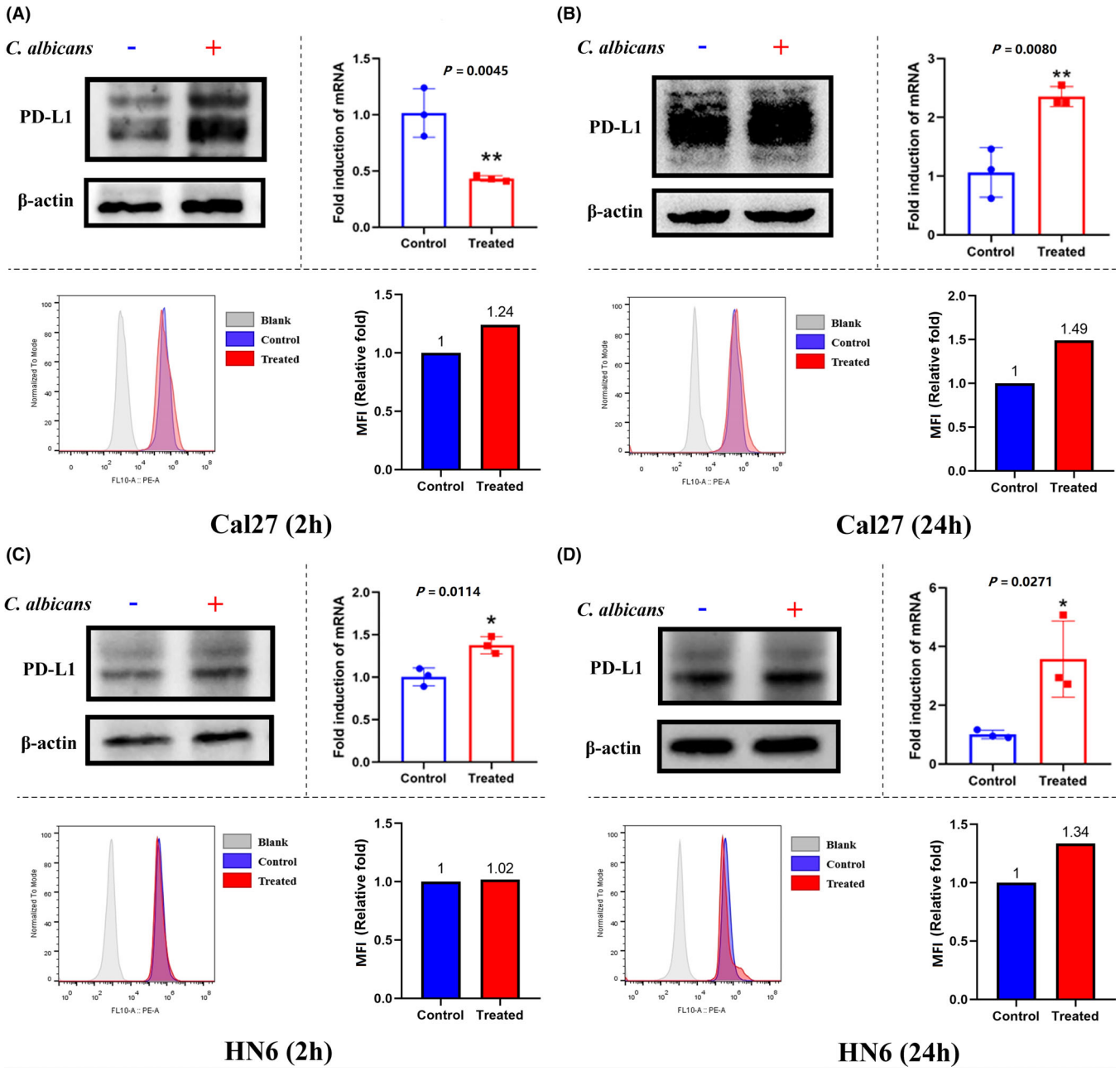

FIGURE 1 *Candida albicans* infection upregulated PD-L1 mRNA and protein levels in OSCC cell lines. (A) The PD-L1 mRNA and protein levels in Cal27 cells infected with *C. albicans* for 2 h. (B) The PD-L1 mRNA and protein levels in Cal27 cells infected with *C. albicans* for 24 h. (C) The PD-L1 mRNA and protein levels in HN6 cells infected with *C. albicans* for 2 h. (D) The PD-L1 mRNA and protein levels in HN6 cells infected with *C. albicans* for 24 h. In each sub-figure, the top left panel represents the WB results ($n = 2$), the top right represents the RT-qPCR results ($n = 3$), the bottom left represents the histogram of PD-L1 surface expression measured by FCM ($n = 2$), the bottom right represents the relative fold of mean fluorescence intensity (MFI) for PD-L1 surface expression measured by FCM ($n = 2$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

were increased in Cal27 cells (Figure 2A). A similar phenomenon could be seen in HN6 cells treated with heat-inactivated *C. albicans* for 24 h, except for membrane protein levels (Figure 2C). However, when treated with biofilm metabolites derived from *C. albicans* for 24 h, the total and membrane protein of PD-L1 were decreased in Cal27 cells (Figure 2B). In HN6 cells, though the membrane protein of PD-L1 was not changed, the total protein was decreased after treated with biofilm metabolites for 24 h (Figure 2D).

3.3 | TLR2/MyD88 and TLR2/NF-κB signaling pathways might participate in the PD-L1 upregulation induced by *C. albicans* infection

In order to reveal the underlying mechanisms of PD-L1 upregulation in OSCC cell lines caused by *C. albicans* infection, the expression profile of genes involved in PD-L1 regulation, exhibited by Kyoto Encyclopedia of Genes and Genomes (KEGG) database, were explored by RT-qPCR. As shown in Figure 3A

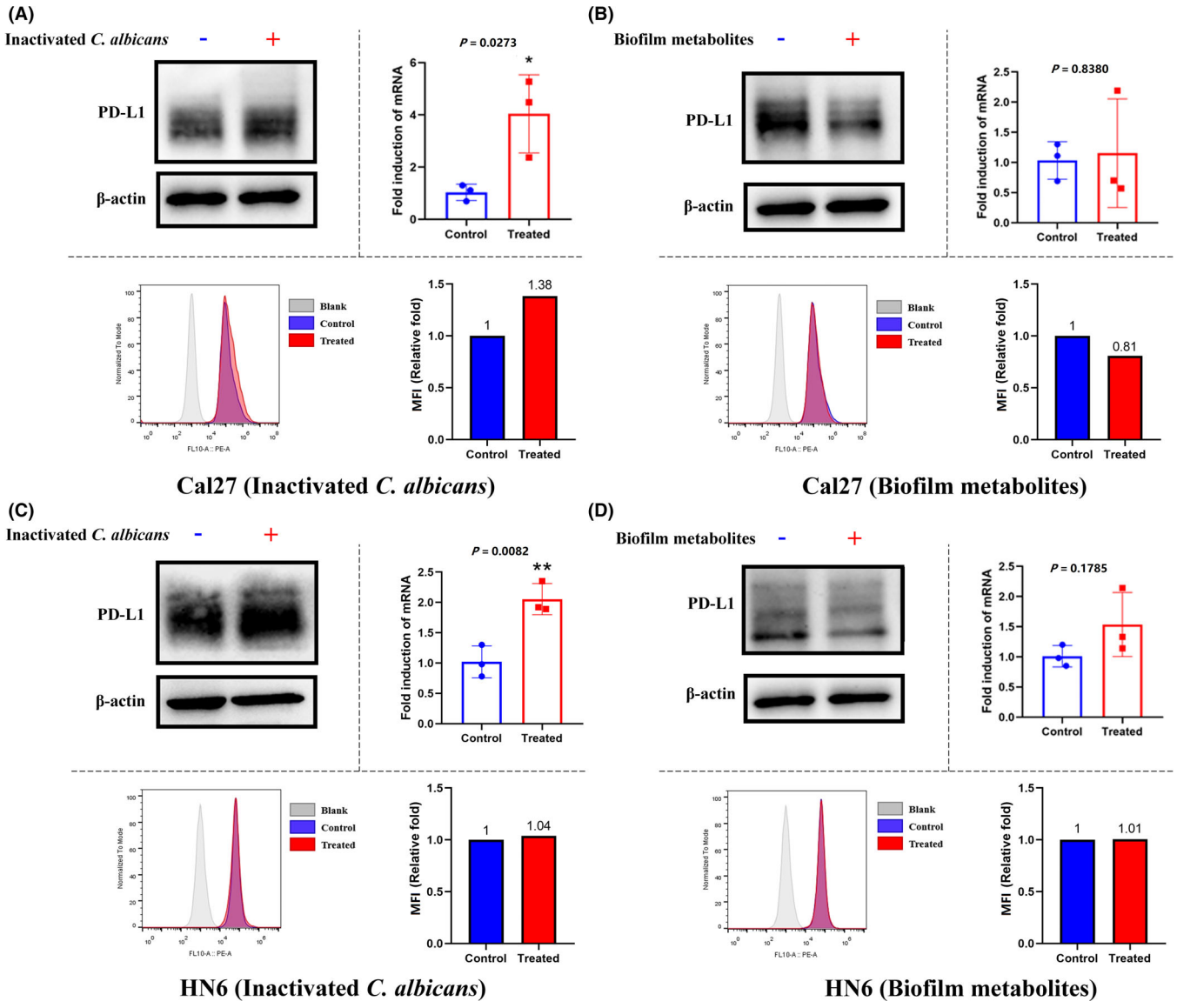

FIGURE 2 Heat-inactivated *Candida albicans*, rather than biofilm metabolites derived from *C. albicans*, upregulated PD-L1 mRNA and protein levels in OSCC cell lines. (A) The PD-L1 mRNA and protein levels in Cal27 cells treated with heat-inactivated *C. albicans* for 24 h. (B) The PD-L1 mRNA and protein levels in Cal27 cells treated with biofilm metabolites for 24 h. (C) The PD-L1 mRNA and protein levels in HN6 cells treated with heat-inactivated *C. albicans* for 24 h. (D) The PD-L1 mRNA and protein levels in HN6 cells treated with biofilm metabolites for 24 h. In each sub-figure, the top left panel represents the WB results ($n = 2$), the top right represents the RT-qPCR results ($n = 3$), the bottom left represents the histogram of PD-L1 surface expression measured by FCM ($n = 2$), the bottom right represents the relative fold of mean fluorescence intensity (MFI) for PD-L1 surface expression measured by FCM ($n = 2$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

(the corresponding histogram was presented in Figure S1), various genes associated with PD-L1 regulation had been influenced by *C. albicans* infection in the two OSCC cell lines. When Cal27 cells infected with *C. albicans* for 24 h, the mRNA levels of *EGFR*, *MAPK1*, *MyD88* and so on were upregulated. When HN6 cells infected with *C. albicans* for 2 h and 24 h, the mRNA levels of *EGFR*, *JAK2*, and *TLR2*, as well as *JAK2*, *STAT1*, and *MyD88* were upregulated, respectively. To summarize, the EGFR and TLR2 signaling pathways were upregulated in both cell lines during PD-L1 upregulation caused by *C. albicans* infection. Then, a Venn network was created to further show the common signaling pathways changed in both cell lines. As shown in Figure 3B, *EGFR/MAPK*, *JAK2/STAT1*, *TLR2/MyD88*, and *PTEN* were changed in both Cal27 and HN6 cells during *C. albicans* infection. Further, considering the importance of heat-inactivated *C. albicans* in regulating PD-L1 expression, the mRNA levels of *EGFR/MAPK*, *JAK2/STAT1*, *TLR2/MyD88* in OSCC cell lines treated with heat-inactivated *C. albicans* were measured. We found that *MAPK1*, *JAK2*, *TLR2*, and *NFATC1* (downstream signal of MyD88) were upregulated in Cal27, while *JAK2* and *TLR2* were upregulated in

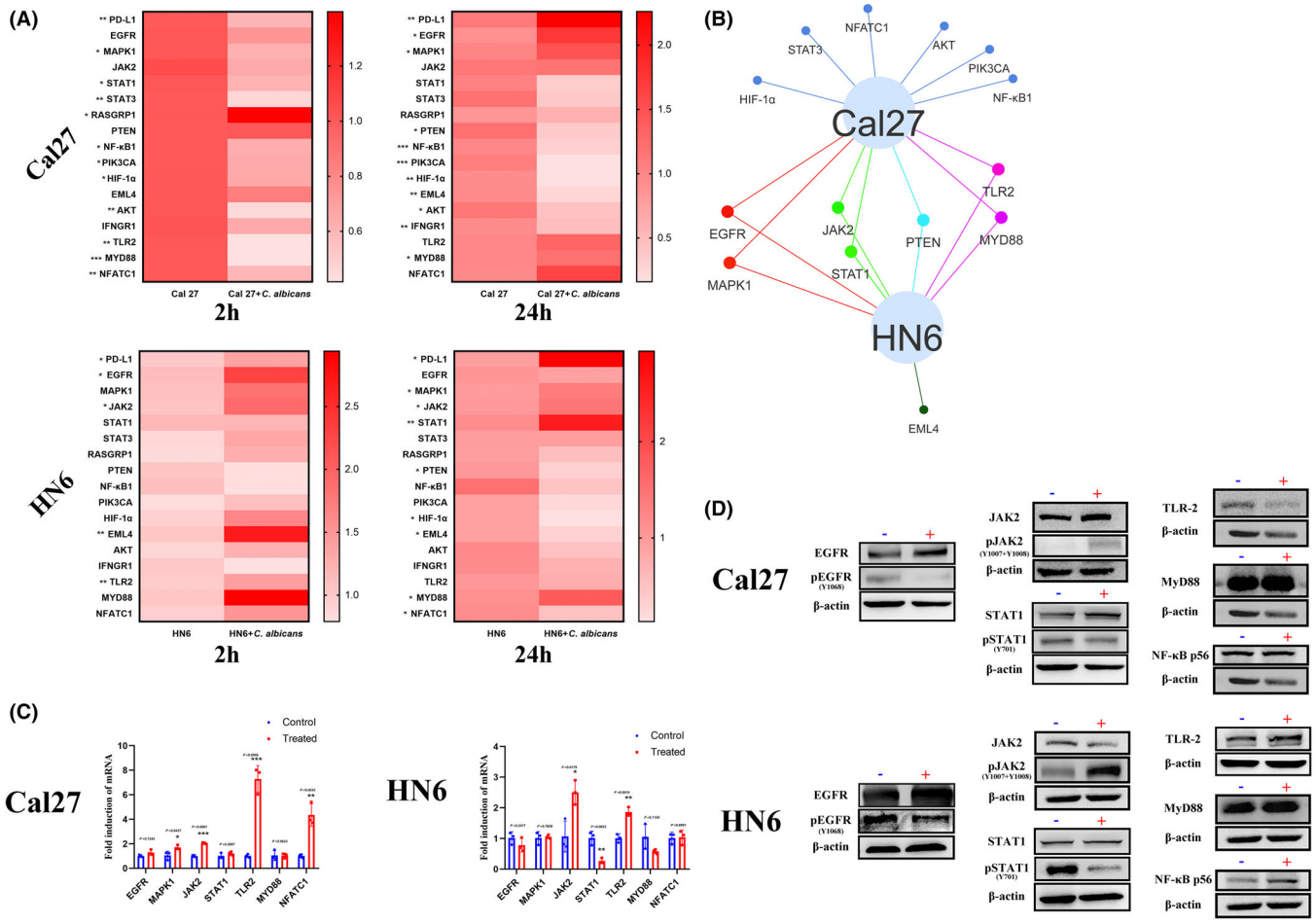

FIGURE 3 Signaling pathways involved in PD-L1 upregulation of OSCC cell lines caused by *Candida albicans*. (A) Heatmap of upstream signals involved in PD-L1 regulation between OSCC cell lines infected with or without *C. albicans*, measured by RT-qPCR ($n = 3$). (B) Venn network of the changed mRNA during *C. albicans* infection between Cal27 and HN6 cells (Drawing with an online software, http://www.ehbio.com/test/venn/#/). (C) The mRNA levels of EGFR/MAPK1, JAK2/STAT1 and TLR2/MyD88 pathways in OSCC cell lines treated with or without heat-inactivated *C. albicans* ($n = 3$). (D) The protein levels of EGFR, JAK2/STAT1, TLR2/MyD88, and TLR2/NF-κB pathways in OSCC cell lines treated with or without heat-inactivated *C. albicans* ($n = 2$), EGFR (pEGFR), and STAT1 (pSTAT1) in Cal27 were from the same membrane, TLR2, MyD88, and NF-κB in Cal27 were from the same membrane, JAK2 (pJAK2), MyD88, and NF-κB in HN6 were from the same membrane. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

HN6 (Figure 3C). Finally, the protein levels involved in these signaling pathways were also detected by WB, and the results showed that pEGFR and pSTAT1 were down-regulated in both Cal27 and HN6, while pJAK2, TLR2, MyD88 and NF-κB (downstream signal of TLR2) were upregulated in both cell lines (Figure 3D). To sum up, TLR2 and its downstream signals, MyD88 and NF-κB, might participate in the regulation of PD-L1 expression in Cal27 and HN6 cells caused by *C. albicans* infection.

3.4 | *C. albicans* infection upregulated PD-L1 expression in both normal and OSCC mice

A 4-nitroquinoline-1 oxide-induced carcinogenesis mouse model was constructed to explore the effect of *C. albicans* infection on PD-L1 expression in vivo (Figure 4A). As shown in the immunofluorescence images (Figure 4B), *C. albicans* infection increased the PD-L1 expression in the tongue mucosa epithelium of both normal and OSCC mice. Based on the histopathological results, *C. albicans* infection showed the tendency to promote tumor progression in OSCC mice (Figure 4C), but the definite conclusion should be validated in future investigation through clinical experiments.

4 | DISCUSSION

PD-L1 over-expression has been discovered in various types of human cancers including head and neck squamous cell carcinomas (HNSCC) (Figure S2), which assists the tumors with evading immune attack.[25] The regulation of PD-L1 is involved in both endogenous mechanisms and exogenous factors existing in

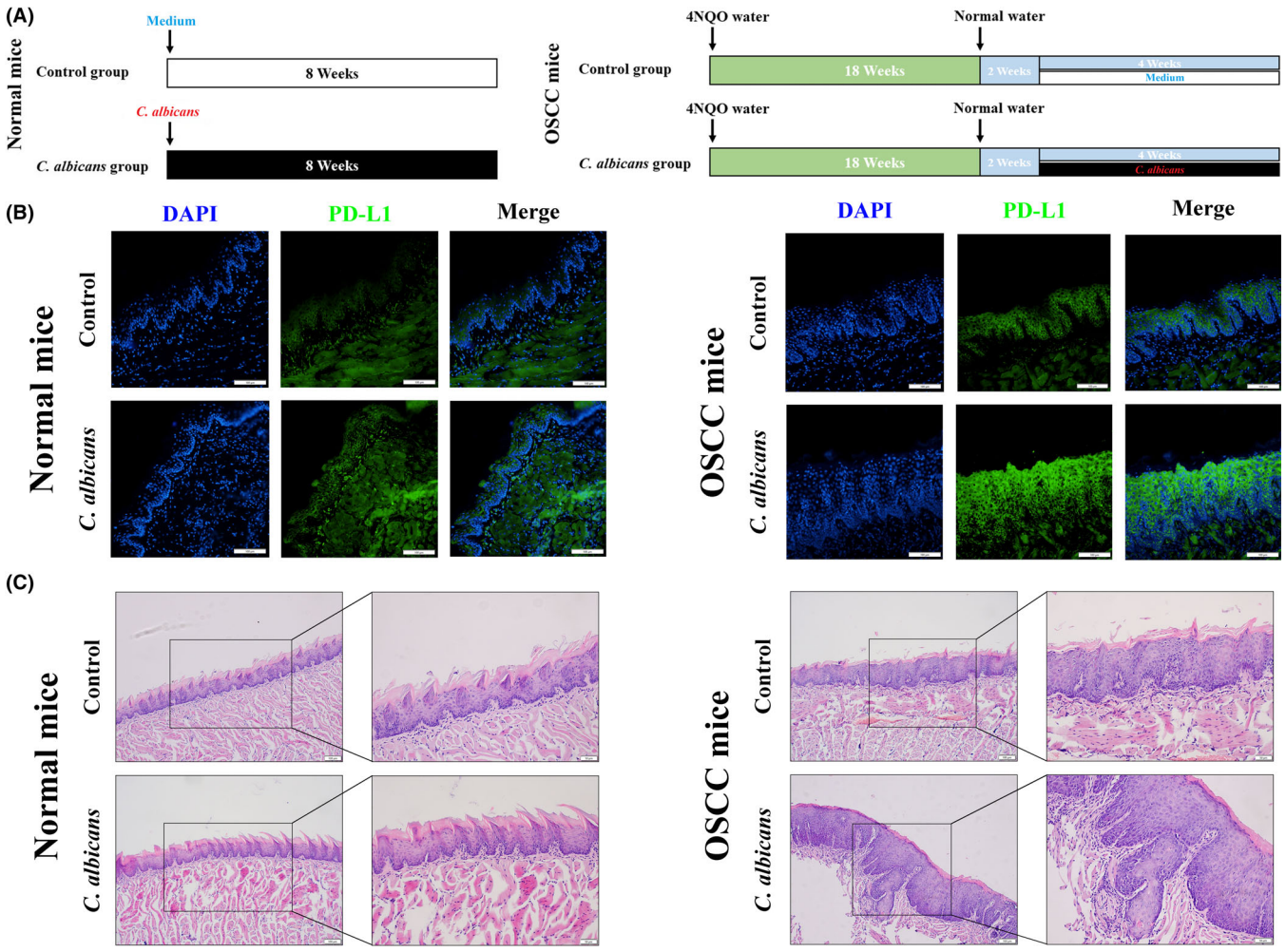

FIGURE 4 *Candida albicans* infection upregulated PD-L1 expression in normal and OSCC mice. (A) Diagram of experimental protocol for normal mice (left, $n = 3$ per group) and OSCC mice (right, $n = 6$ per group). (B) Representative immunofluorescence images of PD-L1 expression in tongue lesions of normal mice infected with or without *C. albicans* for 8 weeks (left), and OSCC mice infected with or without *C. albicans* for 4 weeks (right). Scale bar = 100 μm. (C) Representative H&E sections of pathology, including normal mice infected with or without *C. albicans* for 8 weeks (left), and OSCC mice infected with or without *C. albicans* for 4 weeks (right). Scale bar = 100 μm or 50 μm

TME.[19,26] As the dominant fungal species found in OSCC tissue,[27] whether *C. albicans* influence PD-L1 expression in OSCC is unknown. Here, we demonstrated the overexpression of PD-L1 in OSCC cell lines and OSCC mice during *C. albicans* infection. It was found that PD-L1 was upregulated by *C. albicans* and heat-inactivated *C. albicans* in two OSCC cell lines (Cal27 and HN6). Further, TLR2/MyD88 and TLR2/NF-κB pathways might participate in this process. This study is the first to address the effect of *C. albicans* on PD-L1 regulation in OSCC, which might provide insights into the carcinogenic mechanisms of *C. albicans*.

Microbiome is considered one of the important elements in TME.[28] Bacteria such as *Porphyromanus gingivalis* and *Helicobacter pylori* have been proven to induce PD-L1 expression in oral epithelial cells[29] and gastric epithelial cells[30] respectively, which may support immune evasion of oral and gastric carcinomas. More interestingly, a recent study demonstrated that PD-1 and PD-L1 expression modulated by *Fusobacterium nucleatum* could enhance the efficacy of PD-L1 blockade in colorectal cancer.[31] However, there are rare reports investigating the influence of *Candida* on PD-L1 expression in OSCC. In our present study, PD-L1 in OSCC cell lines (HN6 and Cal27) was upregulated by *C. albicans*. In addition, the PD-L1 expression in laryngeal squamous cell carcinoma cell (LSCC, HN4) and normal oral keratinocyte (HOK) was also elevated after *C. albicans* infection (Figures S3 and S4). Since increased PD-L1 was reported to be related with malignant transformation of OPMDs and pathological grade of OSCC,[11,12,32] *C. albicans* existing in TME may promote the immune evasion of OSCC by enhancing PD-L1 expression.

Although the association between *C. albicans* infection and OSCC progression has been noticed for a long time, their relationship remains unclearly. Previous studies speculated that *C. albicans* might induce cancer development by producing carcinogenic byproducts, triggering chronic inflammation, and inducing Th17 response and molecular mimicry.[33] Our study might propose a novel potential mechanism of *C. albicans* promoting OSCC, by assisting immune

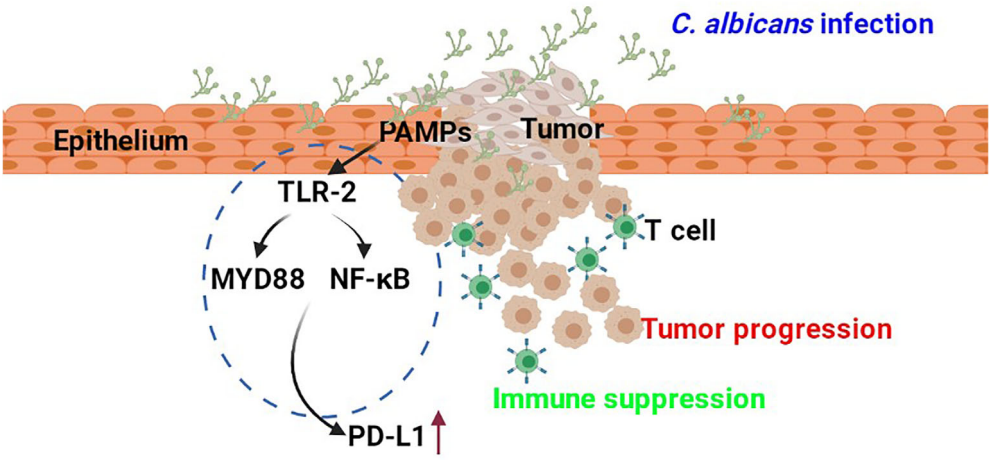

FIGURE 5 Hypothesis about the contribution of *Candida albicans* infection to tumor progression by way of PD-L1 upregulation (drawing with an online software, https://app.biorender.com/)

escape. As shown in the mouse model, *C. albicans* infection promoted the expression of PD-L1 in the tongue mucosa of normal and OSCC mice. The OSCC mice infected with *C. albicans* exhibited the highest level of PD-L1 expression, followed by OSCC mice without *C. albicans* infection, normal mice infected with *C. albicans*, and normal mice without *C. albicans* infection. Thus, it is proposed that *C. albicans* might promote OSCC progression by upregulating PD-L1 expression in oral epithelial and OSCC cells (Figure 5), which should be further validated in the future investigation.

In order to explore the underlying mechanisms involved in PD-L1 upregulation caused by *C. albicans*, we investigated the effect of heat-inactivated *C. albicans* and biofilm metabolites of *C. albicans* on inducing PD-L1 expression. Intriguingly, inactivated *C. albicans* increased PD-L1 expression in OSCC cells, rather than biofilm metabolites. Various components on *C. albicans* cell wall can be recognized by pattern recognition receptors on host cell surface.[34] It has been reported that PAMPs on *C. albicans* can activate the TLRs and JAK2/STATs pathways in host cells,[34,35] which pathways are associated with PD-L1 regulation and were found changed during *C. albicans* infection in our RT-qPCR results. Subsequently, we detected the protein levels of TLR2 and JAK2/pJAK2 during heat-inactivated *C. albicans* treatment, and found that both pJAK2 and TLR2 protein levels were elevated. However, the pSTAT1 was decreased, while the MyD88 and NF-κB were increased. That is to say, TLR2/MyD88 and TLR2/NF-κB might participate in this process. In addition, previous study demonstrated that human epithelial EGFR could be activated by *C. albicans*, mainly by candidalysin secreted by its hypha form.[18] EGFR and its downstream are also important signals in regulating PD-L1 expression in tumor. However, pEGFR protein was reduced during inactivated *C. albicans* treatment in our study. Even so, it is actually hard to rule out the role of EGFR pathways in regulating PD-L1 expression in OSCC cells during *C. albicans* infection. One of the reasons is that the inactivated *C. albicans* explored in the present experiment may not be able to secrete candidalysin.[36] To summarize, *C. albicans* might upregulate PD-L1 expression in OSCC cells partly via TLR2/MyD88 and TLR2/NF-κB pathways, while whether other pathways like JAK2/STAT3 and EGFR, could participate in this process remain to be explored.

Despite some valuable phenomena have been discovered in this study, limitations should not be ignored. First, although some important components of the signal pathways involved in PD-L1 regulation were explored, other individual signal pathways may participate in the PD-L1 expression influenced by *C. albicans* infection. Second, the inactivated *C. albicans* was mainly explored in the present study, the underlying mechanisms involved in PD-L1 regulation by hypha form of *C. albicans* were not further explored. It is important to point out that candidalysin secreted by hypha form is profoundly correlated to EGFR activation and IL-17 upregulation,[37,38] which are also associated with PD-L1 expression in cancer cells.[19,39] Lastly, the causal relationship between *C. albicans* infection, PD-L1 upregulation, and tumor progression needs to be further elucidated in more in vivo and in vitro experiments.

In conclusion, this study demonstrated that *C. albicans* could upregulate PD-L1 expression in normal oral keratinocyte (HOK), LSCC cell line (HN4), OSCC cell lines (Cal27 and HN6), as well as in normal and OSCC mice. TLR2/MyD88 and TLR2/NF-κB pathways might be involved in the regulation of PD-L1 expression caused by *C. albicans*. Further studies are still warranted to demonstrate the relationship between *C. albicans* infection, PD-L1 upregulation, and OSCC progression.

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CONFLICT OF INTERESTS

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Xu Wang: methodology; data curation; methodology; visualization; writing original draft. Weiwei Zhao: methodology; visualization. Wenqing Zhang: methodology; visualization. Shuangshuang Wu: methodology. Zhimin Yan: funding acquisition; resources; supervision; review & editing. All authors have read and agreed to the published version of the manuscript.

ETHICS STATEMENT

The animal experiments were reviewed and approved by the Biomedical Ethics Committee of Peking University (LA2021388).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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