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Original Article

Oral squamous cell carcinoma cell-derived GM-CSF regulates PD-L1 expression in tumor-associated macrophages through the JAK2/STAT3 signaling pathway

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Abstract: Previous study reported that gastric cancer-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) could mediate neutrophil activation and induce PD-L1 expression through JAK2/STAT3 signaling pathway. Moreover, this pathway in various cancers could also regulate PD-L1 expression of tumor cells. Therefore, our study aimed to investigate whether the JAK2/STAT3 pathway regulates PD-L1 expression in tumor-associated macrophages (TAMs) in oral squamous cell carcinoma (OSCC), which can help us achieve further understanding of immune escape mechanisms in OSCC. We induced human monocytes THP-1 into M0, M1, and M2 macrophages, and applied them to common medium and tumor-conditioned medium, the latter was collected from two types of OSCC cell line. Western blot and RT-PCR were used to detect PD-L1 expression and activation of JAK2/STAT3 pathway in macrophages under various conditions. We found that GM-CSF in tumor-conditioned medium from OSCC cells increased PD-L1 expression in M0 macrophages in a time-dependent manner. Moreover, both GM-CSF neutralizing antibody and JAK2/STAT3 pathway inhibitor AG490 could inhibited its up-regulation. In the meantime, we confirmed that GM-CSF indeed acted through JAK2/STAT3 pathway by measuring phosphorylation of key proteins in this pathway. Therefore, we concluded that OSCC cell-derived GM-CSF was able to up-regulate PD-L1 expression in TAMs through JAK2/STAT3 signaling pathway.

Keywords: Oral squamous cell carcinoma, GM-CSF, tumor-associated macrophages, PD-L1, JAK2/STAT3 signaling pathway

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck malignancy, accounting for over 90% [1]. According to the American Cancer Society, approximately 500,000 new patients are diagnosed with OSCC annually worldwide in 2019 [2]. Although the treatments for controlling the primary tumor of oral cancer have made some progress, including surgery, radiotherapy and chemotherapy, the 5-year overall survival rate has remained at a low level in recent years. It is closely related to its primary site recurrence, local invasive growth, and early lymph node metastasis [3, 4]. Moreover, due to the special location of OSCC, radical surgical resection and radiotherapy may cause severe structural and functional damage and significantly reduce the quality of life of patients [4]. Recent evidence has pointed out that the PD-1/PD-L1 axis is typically altered in a significant proportion of OSCC

cases, so immunotherapy against this target offers new ideas for the treatment of OSCC [5].

PD-1/PD-L1 checkpoint blockade is the most studied and fastest developing clinical tumor immunotherapy [6]. Programmed cell death-ligand 1 (PD-L1) is a transmembrane protein expressed on tumor cells and tumor-infiltrating immune cells, which can interact with programmed cell death protein 1 (PD-1) usually expressed on activated T lymphocytes, causing an immune response against tumor cells as an anti-tumor role [5]. PD-L1 expression has been proved to be high in human OSCC samples, which has a significant correlation with overall survival time [7]. Recently, it has been shown that tumor cells are not the only one can express PD-L1, immune cells in the tumor microenvironment, such as tumor-associated macrophages (TAMs), can also selectively express high level of PD-L1 [8, 9].

As the most abundant non-cancerous cells in the tumor microenvironment (TME), Macrophages play a key role in the development of tumors [10]. Under the stimulation of multiple cytokines, macrophages always polarize into two forms: M1 macrophages, with pro-inflammatory and tumor-suppressive effects, and highly express NOS2 and IL-1β; M2 macrophages, with anti-inflammatory and tumor-promoting effects, and highly express Arg1 and CD163 [11, 12]. These two types of polarized macrophages are called tumor-associated macrophages, so blocking the tumor-promoting effect of TAMs can inhibit tumor development [13]. High expression of PD-L1 in TAMs can enhance the immunosuppressive state of the TME, inhibits T cell immune activation, and assist tumor cell immune escape, thus affecting the chronicity and effectiveness of tumor immunotherapy [8, 9]. Therefore, PD-L1 in macrophages may be a potential anti-tumor target, but there is still few research on the expression mechanism of PD-L1 in TAMs. Combined with the above information, our study focused on the factors that may affect PD-L1 expression in TAMs.

The JAK2/STAT3 signaling pathway consists of tyrosine kinase-related receptors, tyrosine kinase JAK2 and transcription factor STAT3 [14], which is the main signaling pathway regulating PD-L1 expression [15]. Abnormal expression of the JAK2/STAT3 signaling pathway has been detected in various cancers, and its overactivation leads to signaling pathway disorders that disrupt downstream genes, thus resulting in tumor immune escape. Previous studies had demonstrated that in various cancers, including head and neck squamous cell carcinoma, lung, gastric, prostate and breast, JAK2/STAT3 pathway could regulate PD-L1 expression in tumor cells [16-18]. In contrast, in oral squamous cell carcinoma, there are no studies on JAK2/STAT3 pathway regulating PD-L1 expression in macrophages.

Granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine, can regulate the proliferation and differentiation of mature granulocytes and myeloid stem cells [19]. It has been reported that GM-CSF can bind to JAK2 receptors on the cell membrane surface to phosphorylate them, thereby activating JAK2 [20]. Another study found that gastric cancer-derived GM-CSF could activate neutrophils and induce their PD-L1 expression via the JAK2/STAT3 signaling pathway [21]. Consequently, we speculated that OSCC cell-derived GM-CSF may similarly be able to promote PD-L1 expression in macrophages through the JAK2/STAT3 signaling pathway.

In our study, we determined the relationship between OSCC cell-derived GM-CSF, JAK2/STAT3 pathway, and PD-L1 expression in TAMs. Our results showed that the PD-L1 expression in TAMs could be up-regulated by GM-CSF from OSCC cells, and JAK2/STAT3 pathway was its channel. This conclusion enlightens us that PD-L1 in TAMs may be a potential target for tumor therapy, with great significance for the development of tumor treatment in the future.

Materials and methods

Tumor cell culture and conditioned medium (CM) collection

Human OSCC cell lines CAL27 and HN4 (ATCC, USA) cells were cultured in RPMI 1640 medium (GIBCO, USA) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and were maintained at 37°C in a humidified 5% CO_2 incubator. When the cell confluence reached 80%-90%, the cells were digested by 0.25% trypsin (Thermo Scientific, USA) and then plated into 6-well plates. After culturing until a certain density was reached and the

cells were in good condition, the FBS-free culture medium was replaced for 24 h. We collected the cell supernatant and centrifuged it at 1000 rpm for 5 min, and the bottom sediment was discarded. The rest was mixed with RPMI 1640 medium containing 10% FBS in a 1:2 ratio, as the conditioned medium for inducing macrophages.

Macrophage polarization and co-culture

THP-1 cells (Cell Bank of Chinese Academy of Sciences, Shanghai) were cultured in RPMI 1640 medium containing 10% FBS, 50 μM/L β-mercaptoethanol, 1% penicillin and streptomycin, maintained at 37°C in a humidified 5% CO_2 incubator for 2-3 days per 1 passage. We seeded THP-1 cells, which were at the logarithmic growth phase, into 6-well plates at a density of 1×10^6/mL per well. PMA inducer (Biyuntian, Shanghai) at a final concentration of 100 ng/mL was added to each well for stimulation, and cell morphology was closely observed under the microscope. Human mononuclear macrophages (M0 macrophages) were obtained after 48 hours. The original culture medium was discarded, and the M0 macrophages were washed twice with PBS buffer then cultured in RPMI 1640 medium in two groups. M1 inducer (20 ng/mL IFN-γ and 100 ng/mL LPS, Biyuntian, Shanghai) was added to one group, and M2 inducer (20 ng/mL IL-4 and 20 ng/mL IL-13, Biyuntian, Shanghai) was added to the other group. Cell morphology was observed that M0 macrophages were respectively polarized into M1 macrophages and M2 macrophages after 24-hour-treatment. M0, M1 and M2 macrophages were cultured in CAL27 and HN4 conditioned medium for 48 h, respectively, to simulate the tumor microenvironment, and determine PD-L1 expression in TAMs. The growth morphology of M0, M1 and M2 macrophages was observed with an inverted phase contrast microscope and its photographic system every 12 h, pictures and records were stored.

Tumor-associated macrophages processing

All tumor-associated macrophages were divided into two major groups: complete medium group and tumor-conditioned medium group. In complete medium group, M0 macrophages were all cultured in RPMI 1640 medium. The control group without any additional substance was named M0 group. One experimental group whose medium containing GM-CSF exogenous recombinant protein (Sinobiologica, Beijing) at 10 ng/mL, was named GM-CSF group. The other was first added with 50 μM AG490 (MCE, USA) for 2 h, followed by 10 ng/mL GM-CSF exogenous recombinant protein, named AG490+GM-CSF group. In tumor conditioned medium group, M0 macrophages were all cultured in CAL27 and HN4 tumor-conditioned medium. The control group without any additional substance was named CM group. One experimental group, whose medium containing GM-CSF neutralizing antibody (R&D Systems, USA) at 1 μg/mL, was named CM+GM-CSF antibody group. The other experimental group whose medium containing AG490 at 50 μM, was named CM+AG490 group.

Western blot

Total proteins of M0, M1 and M2 macrophages from experimental and control groups were obtained by lysis buffer, respectively, and the BCA Protein Assay Kit (Biyuntian, Shanghai) was used to determine their concentrations. We collected the precipitated proteins using SDS loading buffer. These protein samples were electrophoresed through 10% SDS-PAGE gels, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were probed with primary antibodies, which included PD-L1 (1:1000, Cell Technology, USA), JAK2 (1:1000, ABAid, PRC), p-JAK2-Y1007/1008 (1:800), STAT3 (1:1000, ABAid, PRC) and p-STAT3-Y705 (1:1000), at 4°C for 12 h. Washing and incubating with the secondary antibody (1:5000), which was conjugated to horseradish peroxidase, at room temperature for 1.5 h. Protein bands were visualized by the chemiluminescent reagent, and the image information was obtained. The gray values of images were acquired by Image lab software, followed by correcting the internal reference and analyzing protein expression.

Reverse transcription-polymerase chain reaction (RT-PCR)

Monocytes/macrophages were collected separately from control and experimental groups, and total RNA from each group sample was extracted with Trizol (Invitrogen, USA). Total RNA was reversed to cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scienti-

Table 1. RT-PCR primer sequences

Name	Sequence (5'-3')
PD-L1-F	TATGGTGGTGCCGACTACAA
PD-L1-R	TGCTTGTCCAGATGACTTCG
GAPDH-F	CTCCTCCTGTTCGACAGTCAGC
GAPDH-R	CCCAATACGACCAAATCCGTT
IL-1β-F	CAATATTAGAGTCTCAACCCCCA
IL-1β-R	CCGTCGAGGATGTACCGAAT
NOS2-F	CCAAGCCCTCACCTACTTCC
NOS2-R	CTCTGAGGGCTGACACAAGG
Arg1-F	TGGACAGACTAGGAATTGGCA
Arg1-R	CCAGTCCGTCAACATCAAAACT
CD163-F	TTTGTCAACTTGAGTCCCTTCAC
CD163-R	TCCCGCTACACTTGTTTTCAC

fic, USA), and SYBRGreen RealMaster-Mix Kit (Tiangen, Beijing) was used for RT-PCR based on standard protocols. GAPDH was used to normalize the relative gene expression. The threshold cycle (CT) value was used to reflect the gene content, and the differential gene expression between groups was compared through the 2-ΔΔCT relative quantification method, followed by obtaining the data. The primers synthesized by Sangon Biotech (Shanghai) are shown in **Table 1**.

Statistical analysis

GraphPad Prism 9.0 software was used for statistical analysis. The data were expressed as the mean ± SD from at least three independent experiments. Student's t test was performed to compare two groups, and comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered statistically significant.

Results

M1 and M2 macrophages were successfully induced from THP-1 cells

Morphological changes of macrophages: We first induced THP-1 cells into M0 macrophages after 48-hour-stimulation with PMA inducer. Next, we added the M1 inducer and M2 inducer to polarize M0 macrophages into M1 and M2 macrophages, respectively. The transparent spherical cells originally suspended in the culture medium gradually adhered, spreading nearly completely, and more adhesive to each other. The morphology also gradually stabilized. M1 macrophages showed a full ovoid pancake-like morphology, and M2 macrophages showed an elongated fibroblast-like morphology. These cell morphology under microscope is shown in **Figure 1**.

Macrophage phenotyping identification: To determine M1 and M2 macrophages were induced successfully, RT-PCR was used to detect the mRNA expression of NOS_2, IL-1β, Arg1 and CD163 at the transcriptional level. The results shown in **Figure 2** suggested that the mRNA expression of NOS_2, IL-1β, Arg1 and CD163 could be detected in all three macrophage groups. The mRNA expression of NOS_2 and IL-1β in M1 macrophages was significantly higher than that in both THP-1 cells and M2 macrophages, while the mRNA expression of Arg1 and CD163 in M2 macrophages was significantly higher than that in both THP-1 cells and M1 macrophages. The above results demonstrated that we induced M1 and M2 macrophages successfully.

The tumor microenvironment regulates PD-L1 expression in TAMs by promoting M2 macrophages polarization

Tumor conditioned-medium hardly up-regulates PD-L1 expression in M2 macrophages: M0, M1 and M2 macrophages, which are induced from THP-1 cells, were cultured in CAL27 and HN4 conditioned medium for 48 h to co-culture with tumor cells indirectly. Total proteins from each group with and without co-culture were collected, and intracellular PD-L1 expression was detected by western blot. Each experiment was repeated three times. The results were shown in **Figure 3**. Compared with M0 macrophages, the expression of PD-L1 in M1 macrophages ($P<0.05$) and M2 macrophages ($P<0.01$) were significantly higher, indicating that PD-L1 in the TME may be secreted predominantly by M2 macrophages. After indirect co-culture with CAL27 cells, PD-L1 expression in M0+CM group was higher than M0 group (1.592 folds, $P<0.05$), and which in M1+CM group was higher than M1 group (3.721 folds, $P<0.001$). Nevertheless, there is no statistical significance between M2 group and M2+CM group ($P>0.05$). Similarly, indirect co-culturing with HN4 cells, PD-L1 expression in the M0+CM group was higher than M0 group

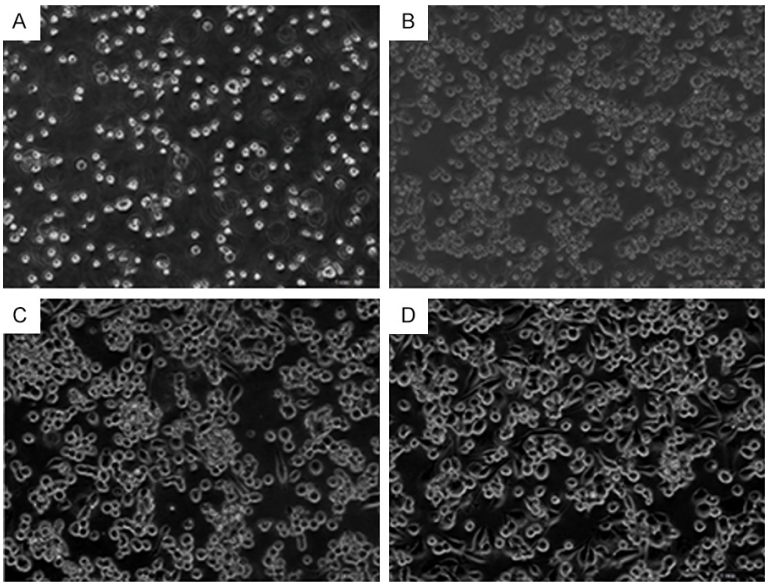

Figure 1. Morphological changes of M0, M1 and M2 macrophages induced by THP-1 cells (×200). A. THP-1 cells; B. M0 macrophages treated with PMA inducer; C. M1 macrophages treated with LPS and IFN-γ; D. M2 macrophages treated with IL-4 and IL-13.

(1.552 folds, P<0.05), and that in the M1+CM group was higher than M1 group (4.002 folds, P<0.001), and no statistical significance between M2 group and M2+CM group (P>0.05). Therefore, it was demonstrated that CAL27 and HN4 conditioned medium could up-regulate PD-L1 expression in M0 and M1 macrophages, but its effect on M2 macrophages was not significant.

Tumor-conditioned medium promotes M0 macrophages transforming into M2 macrophages: To investigate how tumor-conditioned medium up-regulates PD-L1 expression in TAMs, we applied CAL27 and HN4 conditioned medium to unpolarized M0 macrophages for 12 h, 24 h and 48 h, respectively, which made TAMs construction. Firstly, microscopic characteristics of all groups were observed. As shown in **Figure 4**, gradual adherence was observed in either fully differentiated or undifferentiated macrophages. The adherent cells were round with good refraction in the beginning. With the increase of culture time, the cells gradually became shuttle-shaped, both ends became sharpened, and pseudopodia were observed in some cells, which were morphologically similar to M2 macrophages. It suggested that the tumor microenvironment has the property of promoting M2 macrophages polarization, which is consistent with relevant research [22].

Next, CAL27 and HN4 conditioned medium were applied to M0 macrophages for 48 h. Total RNA from each group was collected and detected by RT-PCR for the mRNA expression of PD-L1. Each experiment was repeated three times. As shown in **Figure 5**, the mRNA expression of PDL1 in the cells cultured in CAL27 conditioned medium (1.695 folds, P<0.01) and HN4 conditioned medium (1.863 folds, P<0.01) was higher than the control group. Consequently, we confirmed that at both morphological and mRNA levels, the tumor-conditioned medium of OSCC cells had the ability to up-regulate PD-L1 expression in TAMs, which acted by promoting M2 macrophages polarization.

GM-CSF can up-regulate PD-L1 expression in TAMs via JAK2/STAT3 pathway

To further investigate the components in tumor-conditioned medium that promote PD-L1 expression in TAMs, we selected GM-CSF, a common inflammatory factor secreted by tumor cells, to verify whether GM-CSF has this ability. We treated M0 macrophages with 10 ng/mL GM-CSF exogenous recombinant protein for 12 h, 24 h and 48 h, respectively. Proteins from each group were collected and the PD-L1 expression was detected by western blot. Each experiment was repeated three times. As shown in **Figure 6A**, after treating with GM-CSF for 12 h (1.423 folds, P<0.05), 24 h (1.818 folds, P<0.001) and 48 h (1.675 folds, P<0.01), PD-L1 expression in M0 macrophages was higher than the control group, and was in a time-dependent manner. The most significant treatment time was 24 h.

AG490, an inhibitor of JAK2/STAT3 pathway, could reduce STAT3 phosphorylation by inactivating JAK2 selectively [23]. To verify whether the JAK2/STAT3 pathway is the channel for GM-CSF to promote PD-L1 expression, we sti-

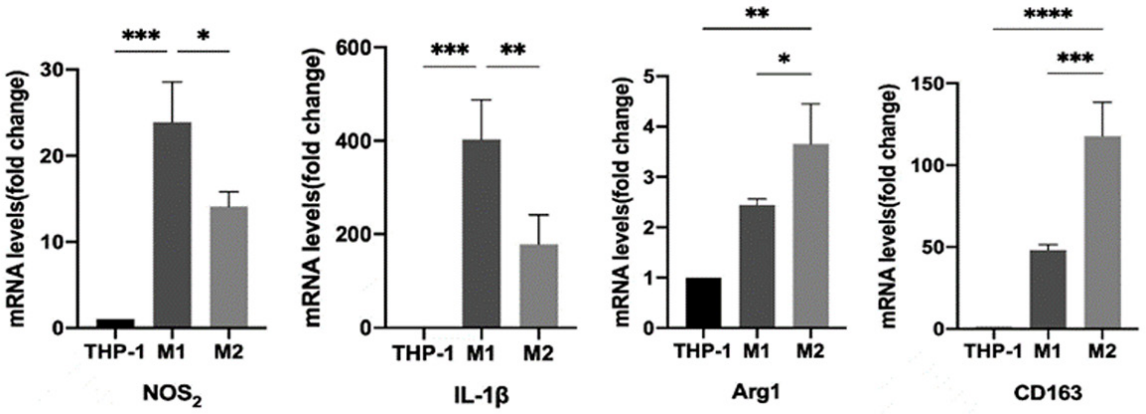

Figure 2. The expression of macrophage markers detected by RT-PCR. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

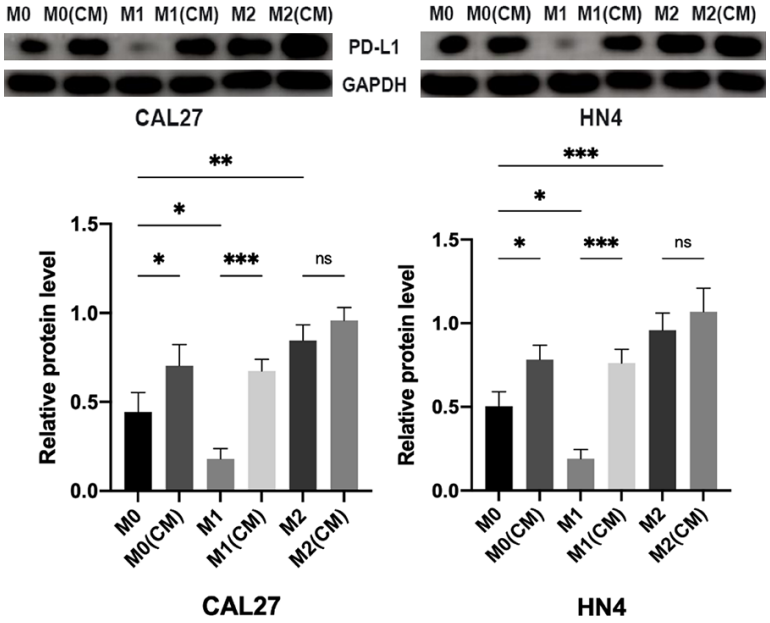

Figure 3. The expression of PD-L1 in various macrophages detected by western blot. *P<0.05; **P<0.01; ***P<0.001.

mulated M0 macrophages with AG490 (50 μM) for 2 h to fully inhibit the JAK2/STAT3 pathway, after that treated M0 macrophages with 10 ng/mL GM-CSF exogenous recombinant protein for 12 h, 24 h, and 48 h, respectively. Proteins from each group were collected and the PD-L1 expression was detected by western blot. Each experiment was repeated three times. As shown in **Figure 6B**, the up-regulation of PD-L1 expression by GM-CSF could be reversed by AG490 in a significant time-dependent manner. When AG490 was treated for only 12 h, the experimental group has no statistically significant difference with the control group, while compared with the control group, the PD-L1 expression in the 24-hour (0.677 folds, P<0.01) and 48-hour (0.452 folds, P<0.0001) groups were significantly higher. It was further demonstrated that GM-CSF could up-regulate PD-L1 expression in M0 macrophages through JAK2/STAT3 pathway.

The promoting effect of tumor-conditioned medium on PD-L1 expression in TAMs is associated with GM-CSF regulation of JAK2/STAT3 pathway

GM-CSF can activate the JAK2/STAT3 signaling pathway: To verify the relationship between the up-regulation of PD-L1 expression in TAMs by tumor conditioned medium, tumor-derived GM-CSF and JAK2/STAT3 pathway, we set up CM group, CM+GM-CSF antibody group and CM+AG490 group. M0 macrophages of all groups were respectively cultured for 48 h, and then the expression of JAK2, STAT3 and their phosphorylated proteins was detected by western blot. Each experiment was repeated three times. As shown in **Figure 7**, co-cultured with CAL27 conditioned medium, the phosphorylation levels of JAK2 (0.780 folds, P<0.05) and STAT3 (0.630 folds, P<0.01) in M0 macrophages of CM+GM-CSF antibody group were decreased compared with CM group. In CM+AG490 group, the phosphorylation levels

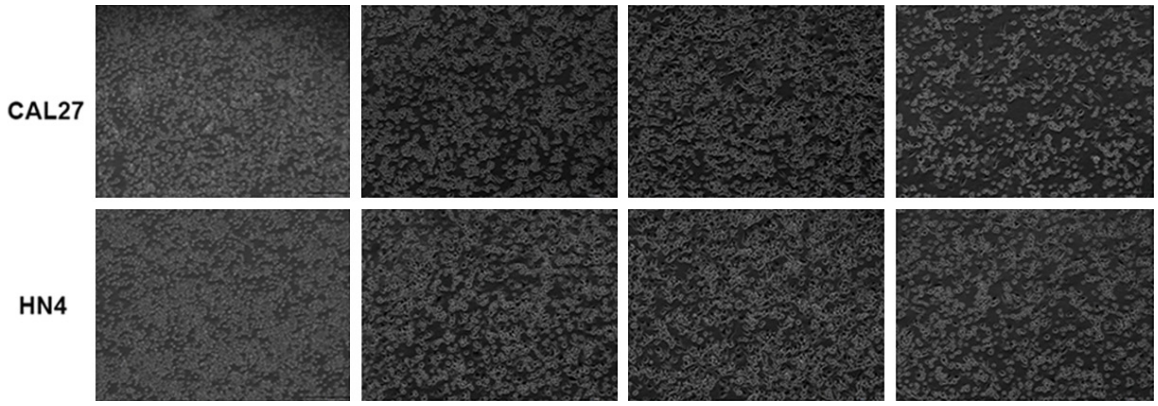

Figure 4. Morphological changes of M0 macrophages before and after tumor-conditioned medium treatment (×100).

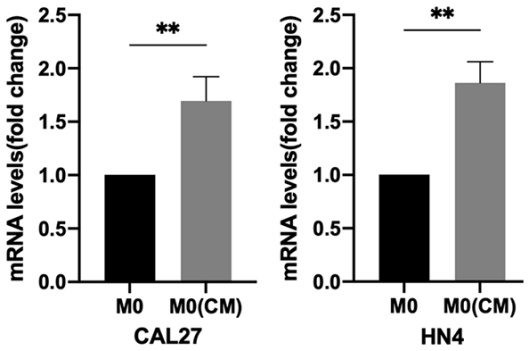

Figure 5. PD-L1 expression in M0 macrophages before and after tumor-conditioned medium treatment detected by RT-PCR. **P<0.01.

of JAK2 (0.494 folds, P<0.001) and STAT3 (0.322 folds, P<0.001) in M0 macrophages were also decreased. Similarly, co-cultured with HN4 conditioned medium, the phosphorylation levels of JAK2 (0.749 folds, P<0.01) and STAT3 (0.623 folds, P<0.01) in M0 macrophages of CM+GM-CSF antibody group were decreased compared with CM group. In CM+AG490 group, the phosphorylation levels of JAK2 (0.590 folds, P<0.001) and STAT3 (0.355 folds, P<0.001) in M0 macrophages were also decreased. Therefore, the JAK2/STAT3 pathway was significantly inhibited compared with the CM group when GM-CSF in tumor-conditioned medium was neutralized, revealing that GM-CSF indeed acted via the JAK2/STAT3 pathway.

GM-CSF neutralizing antibody and JAK2/STAT3 signaling pathway inhibitor can both down-regulate PD-L1 expression in TAMs: To prove that GM-CSF in tumor-conditioned medium up-regulated PD-L1 expression in TAMs was achieved by activating the JAK2/STAT3 pathway. The proteins of CM group, CM+GM-CSF antibody group and CM+AG490 group were collected and detected the expression of PD-L1 by western blot. Each experiment was repeated three times. As shown in **Figure 8**, co-cultured with CAL27 conditioned medium, compared with CM group, the PD-L1 expression in M0 macrophages was decreased in CM+GM-CSF antibody group (0.653 folds, P<0.01) and CM+AG490 group (0.429 folds, P<0.0001) compared with CM group. Similarly, co-cultured with HN4 conditioned medium, compared with CM group, that was also decreased in the CM+GM-CSF antibody group (0.747 folds, P<0.01) and CM+AG490 group (0.603 folds, P<0.001). It indicated that tumor-conditioned medium up-regulated PD-L1 expression was associated with tumor-derived GM-CSF regulating JAK2/STAT3 pathway.

At the same time, we set up the same three groups, collected their RNA from each group, and detected the mRNA expression of PD-L1 by RT-PCR. Each experiment was repeated three times. As shown in **Figure 9**, co-cultured with CAL27 conditioned medium, PD-L1 expression in M0 macrophages was decreased in the CM+GM-CSF antibody group (0.750 folds, P<0.001) and CM+AG490 group (0.601 folds, P<0.0001) compared with CM group. Similarly, co-cultured with HN4 conditioned medium, that was also decreased in the CM+GM-CSF antibody group (0.784 folds, P<0.01) and CM+AG490 group (0.680 folds, P<0.01). It was further confirmed at mRNA level that up-regulation of PD-L1 expression by tumor conditioned medium was associated with GM-CSF regulating JAK2/STAT3 pathway.

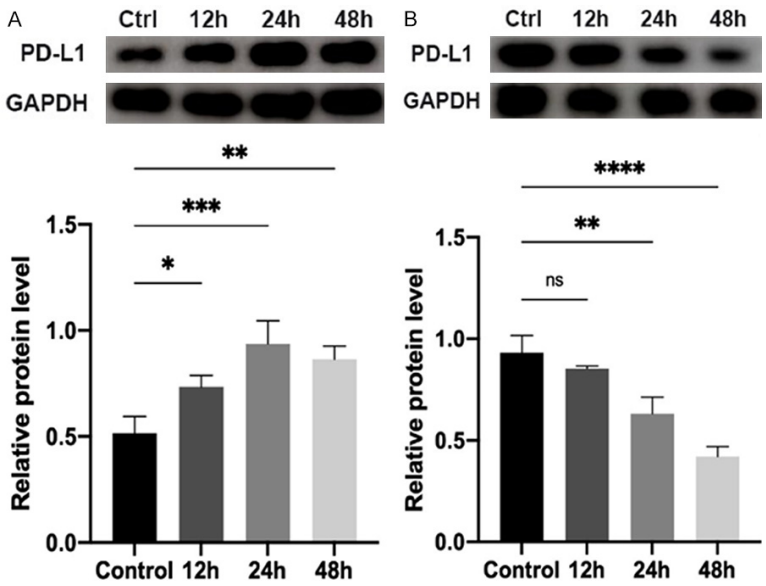

Figure 6. PD-L1 expression in M0 macrophages cultured in common medium, including M0 group, GM-CSF group and AG490+GMCSF group, detected by western blot. A. Before and after GM-CSF treatment; B. Before and after AG490 treatment. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Discussion

Immune escape is one of the important factors that lead to tumor invasion and metastasis and affect the therapeutic effect of tumors. In recent years, with the breakthrough of cancer immunotherapy in scientific research and clinical practice, tumor immunotherapy has become one of the most promising cancer treatment methods. PD-1, as the hottest immune checkpoint, is highly expressed in various immune cells such as activated T cells, B cells and monocytes. PD-L1 is the main ligand of PD-1 that mainly expressed in tumor cells, belonging to the B7 family. It has aroused high concern due to its selective high expression on the surface of various tumors, such as lung cancer, melanoma, bladder cancer, renal cancer, and OSCC [24, 25]. When PD-1 binds to PD-L1, it is able to down-regulate the secretion of T cell activating factors, such as IL-12, IFN-γ and TNF-α, and inhibit the activation and proliferation of T cells and induce T cell apoptosis, result in leading to the immune escape of tumor cells [6]. Consequently, targeting PD-1/PD-L1 for treatment, developing blockers against this target, restoring T cell activity, and realizing long-term immunotherapy are the current focus of research [26]. Although anti-PD-L1 drugs, such as Atezolumab and Durvalumab, have been developed, they still have limited application in clinical practice and poor efficacy in most patients, for tumors in a complex tumor immune microenvironment are regulated intricately at multiple levels of the immune network [27, 28]. It requires us to further explore the mechanisms of immune escape and seek effective biomarkers, so as to adjust cancer treatment strategies pertinently.

Great malleability is a typical feature of macrophages, and different functional characteristics are obtained when different signals from the tumor immune microenvironment stimulate. Previous studies showed that macrophages expressed more abundant PD-L1 than tumor cells in tumor biopsy specimens from patients with renal cell carcinoma, melanoma, non-small cell lung, colorectal tumors, and head and neck carcinoma [29, 30]. Kim HR et al. also reported that PD-L1 expression in immune cells instead of tumor cells, was a favorable prognostic factor in patients with oral cancer [31]. These results all demonstrate that PD-L1 may be a more effective target for cancer immunotherapy now. Subsequently, Jiang C et al. found that TAMs expressed more PD-L1 than normal tissue macrophages in OSCC, which induced T cell apoptosis. At the same time, they found when monocytes collected from peripheral blood with low PD-L1 expression were co-cultured with primary tumor cells from OSCC patients, PD-L1 expression was upregulated in monocytes/macrophages and had the ability to induce T cell apoptosis [32]. These results indicate that tumor cells in OSCC can induce PD-L1 expression in TAMs through certain mechanisms, but we know little about them now.

Our research found that macrophages co-cultured with CAL27 and HN4 cells gradually adhered, intercellular adhesion increased, and cell morphology gradually changed from round to spindle, with some extended pseudopodia, which tended to transform into M2 macro-

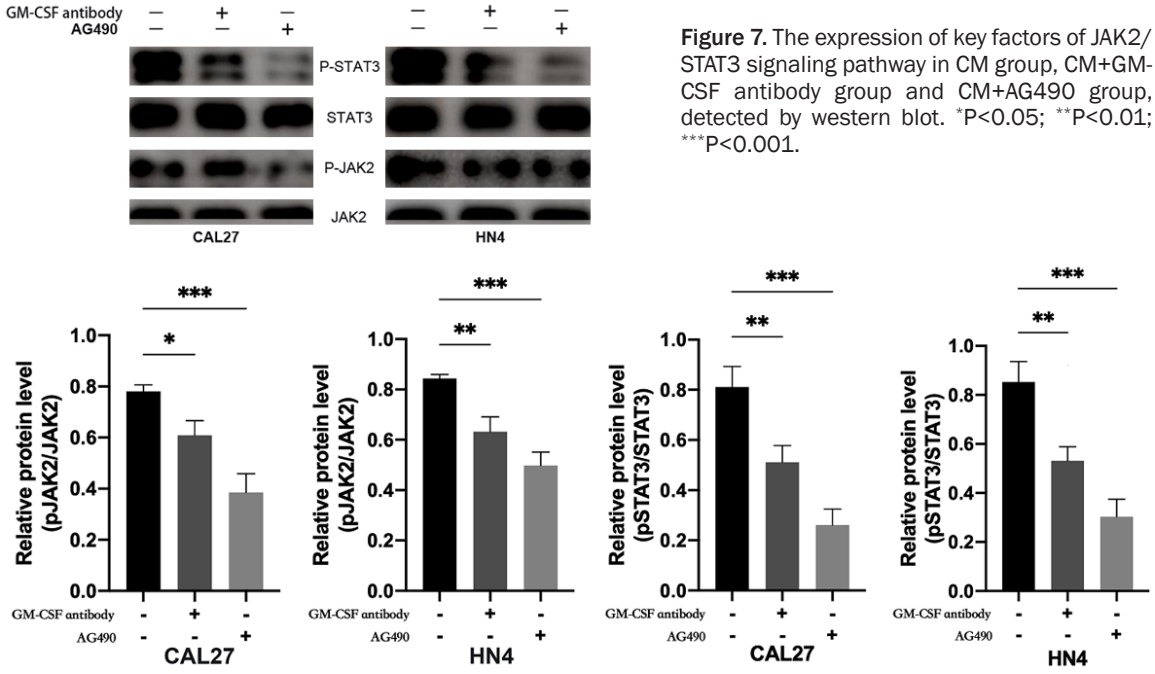

Figure 7. The expression of key factors of JAK2/STAT3 signaling pathway in CM group, CM+GM-CSF antibody group and CM+AG490 group, detected by western blot. *P<0.05; **P<0.01; ***P<0.001.

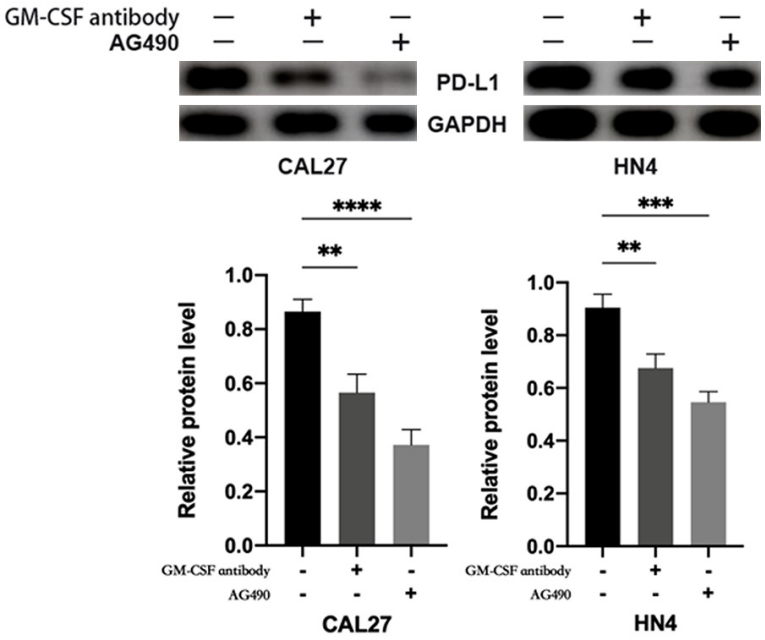

Figure 8. PD-L1 expression in M0 macrophages cultured in conditioned medium before and after AG490 treatment of CM group, CM+GM-CSF antibody group and CM+AG490 group, detected by western blot. **P<0.01; ***P<0.001; ****P<0.0001.

phages. Previous literature has confirmed in detail that tumor microenvironment promotes the polarization of M2 macrophages in TAM. Yamaguchi et al. [22] reported that M1-related mRNAs such as those of TNF-α, CD80, CD86, and IL-12p40 were less strongly expressed in TAMs relative to PBMC-derived M1 macrophages, but M2-related mRNAs such as those of IL-10, VEGF-A, VEGF-C, MMP-1, and amphiregulin were more strongly expressed in TAMs. Meanwhile, M1 macrophages were induced to differentiate into the M2 phenotype by coexistence of gastric cancer cells. Lian et al. [33] also found that colon cancer cells promoted M2 polarization of macrophage from monocytes. The level of M1-related cytokines IL-6 and IL-1β decreased by approximately 25% in THP-1 co-cultured with colon cancer cells, whereas the level of M2-related markers IL-10 and Arginase-1 increased by about 100% in THP-1 co-cultured with colon cancer cells compared to THP-1 group. By establishing a co-culture system, Zhou et al. [34] support a hypothesis that OSCC cells actively recruit peripheral monocytes into the TME and promote their polarization towards M2 macrophages through the overexpression

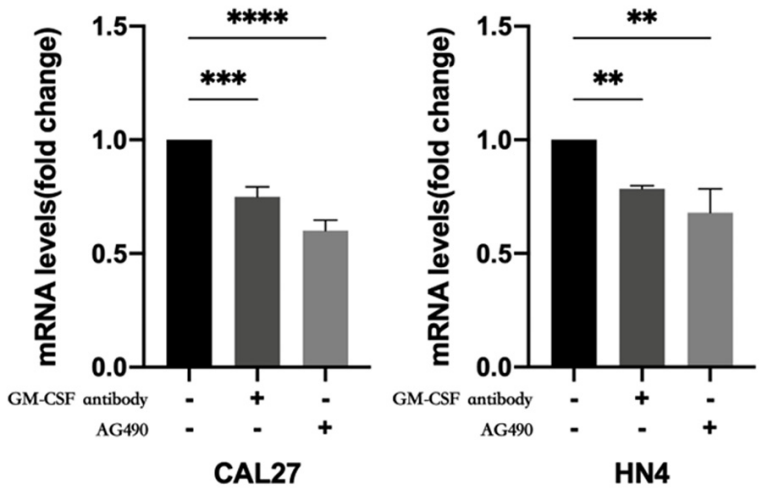

Figure 9. PD-L1 expression in M0 macrophages cultured in conditioned medium, including CM group, CM+GM-CSF antibody group and CM+AG490 group, detected by RT-PCR. **P<0.01; ***P<0.001; ****P<0.0001.

of CCR7. Therefore, we suspect that the tumor microenvironment also promotes the polarization of M2 macrophages in TAM in this experiment. In addition, we will further study the effect of tumor microenvironment on the polarization phenotype of M2 macrophages in oral squamous cell carcinoma. At the same time, PD-L1 expression in macrophages can be up-regulated after a period of stimulation with tumor-conditioned medium.

GM-CSF can be up-regulated in various malignant tumors, including head and neck squamous cell carcinoma, which can secrete GM-CSF to alter the TME and thus promote tumor growth and metastasis [35]. In our study, M0 macrophages were stimulated by GM-CSF exogenous recombinant protein, and the results showed that PD-L1 was significantly up-regulated in a time-dependent manner. Further, when tumor-derived GM-CSF in conditioned medium was neutralized by its antibodies, PD-L1 expression in M0 macrophages decreased compared with that only cultured in conditioned medium. It indicated that GM-CSF in OSCC tumor-conditioned medium has the ability to up-regulate PD-L1 expression in TAMs.

Cytokines can be transmitted to the nucleus through membrane receptors, JAK2 protein and STAT3 protein, and STAT3 protein is able to bind to the promoter region of PD-L1 thus promoting PD-L1 expression [36]. Jing W et al. found that in breast cancer, co-culturing of 4T1 tumor cells with macrophages could activate JAK2/STAT3 pathway and significantly up-regulated PD-L1 expression in macrophages, and treatment with JAK2/STAT3 pathway inhibitors decreased PD-L1 expression in macrophages [37]. In the induced mouse model of metastatic liver cancer, GM-CSF was highly expressed in tumor cells, while bone marrow-derived suppressor cells highly expressed PD-L1 and maintained high levels of p-STAT3. When tumor-derived GM-CSF was down-regulated cultured with GM-CSF neutralizing antibodies, JAK2/STAT3 pathway in bone marrow-derived suppressor cells was blocked and the PD-L1 expression was also down-regulated [15]. Our study further investigated the relationship between the regulation of OSCC cells on macrophages and JAK2/STAT3 pathway, and M0 macrophages were treated with AG490, a specific inhibitor of JAK2/STAT3 pa-thway, combined with conditioned medium, and then found that PD-L1 expression in macrophages was decreased after JAK2/STAT3 pathway was specifically inhibited from both the protein and mRNA levels. It indicated that conditioned medium can up-regulate PD-L1 expression in macrophages via JAK2/STAT3 pathway. Results by western blot suggested that the up-regulation of PD-L1 expression by GM-CSF in M0 macrophages was significantly reversed by AG490 in a time-dependent manner. Moreover, when GM-CSF in conditioned medium was neutralized, JAK2/STAT3 pathway was significantly inhibited. It further demonstrated that the regulation of PD-L1 expression in M0 macrophages by tumor-derived factor GM-CSF was achieved through JAK2/STAT3 signaling pathway. Nevertheless, it is undeniable that one potential limitation should be taken into consideration when interpreting our results. Although it is reasonable to confirm that the signal pathway plays a role in the relevant mechanism by using only the inhibitor of the signal pathway but not the activator based on many previous studies [38-40], the manuscript would be more informative if a control group in which M0 macrophages co-cultured with CM and JAK2/STAT3 activator

(CM+JAK2/STAT3 activator group) would have been added to detect the expression of PD-L1.

However, PD-L1 expression in M0 macrophages treated with AG490 combined with CM remained lower than the control group. Similarly, PD-L1 expression in M0 macrophages treated with GM-CSF neutralizing antibodies was lower than the control group. It suggested that PD-L1 expression may be regulated by multiple signaling pathways including JAK2/STAT3, and more mechanisms related to it need to be further explored.

So far, the complexity of TAM biology and its interaction with the TME has not been fully expounded. Further understanding of how inflammatory signals and immune cells shape TAM function and reprogram the "dialogue" between cancer cells and macrophages could help influence their ability to promote tumor formation, disease development and metastasis, and ultimately achieve the purpose of eradicating tumors or avoiding tumorigenesis. In the future, more research will be carried out in this field to use this emerging knowledge to provide new ideas and new targets for clinical tumor therapy.

In summary, our study demonstrated that OSCC cells can regulate PD-L1 expression in macrophages by secreting the tumor-associated cytokine GM-CSF, which can participate in mediating crosstalk between tumor cells and macrophages in the TME, thereby promoting immune escape of tumor cells.

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Disclosure of conflict of interest

None.

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