**ABSTRACT:** M2 macrophages are generally recognized to have a protumor role, while the effect of M1 macrophages in cancer is controversial. Here, the in vitro and in vivo effects of conditioned medium from M1 macrophages (M1-CM) on oral squamous cell carcinoma (OSCC) cells and a potential mechanism were studied. CCK-8, colony formation, EdU labeling, xenograft growth, and Transwell assays were utilized to observe cell survival/proliferation and migration/invasion, respectively, in OSCC cell lines treated with basic medium (BM) and M1-CM. The ErbB2 phosphorylation inhibitor (CI-1033) and GDF15 knockout cell lines were used to appraise the role of ErbB2 and GDF15 in mediating the effects of M1-CM. Compared with BM, M1-CM significantly enhanced the survival/proliferation of SCC25 cells. The migration/invasion of SCC25 and CAL27 cells also increased. Mechanically, M1-CM promoted GDF15 expression and increased the phosphorylation of ErbB2, AKT, and ErK. CI-1033 significantly declined the M1-CM-induced activation of p-AKT and p-ErK and its protumor effects. M1-CM stimulated enhancement of p-ErbB2 expression was significantly decreased in cells with GDF15 gene knockout vs without. In xenograft, M1-CM pretreatment significantly promoted the carcinogenic potential of OSCC cells. Our results demonstrate that M1 macrophages induce the proliferation, migration, invasion, and xenograft development of OSCC cells. Mechanistically, this protumor effect of M1 macrophages is partly associated with inducing GDF15-mediated ErbB2 phosphorylation.

**INTRODUCTION**

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck tumor. Like other cancers, research on OSCC has emphasized the potential role of the tumor microenvironment (TME) in tumor progression and metastasis. The TME comprises many kinds of cells, among which tumor-associated macrophages (TAMs) are essential and abundant cell types. TAMs possess M1-like and M2-like phenotypes. M1 macrophage differentiation is induced with lipopolysaccharide (LPS) and/or interferon-γ. It is characterized by increased expression of specific proinflammatory cytokines, for instance, TNF-α, IL-6, IL-1β, and IL-12, and is responsible for eliminating pathogens and tumor cells. The differentiation of M2 macrophage is induced by IL-4 and/or IL-13 and is characterized by anti-inflammatory and protumor effects mediated by the inhibition of the immune response. M1 macrophages can be identified by the expression of CD80 or CD86, while M2 macrophages express specific CD163 or CD206. It is well recognized that M2 macrophages contribute to tumor progression and metastasis via immunosuppressive, pro-angiogenic, and cell-invasive function. However, the role of M1 macrophages in cancers remains controversial. Although the accumulating evidence shows its anticancer potential, M1 macrophages have also been reported to have the effect of promoting cancer cell metastasis and proliferation. Therefore, further study of the role of M1 macrophages in cancers and the underlying mechanisms is required.

ErbB2 (HER-2/neu) is a transmembrane receptor of EGFR, has intrinsic tyrosine kinase activity, and can interact with many different cellular proteins, such as growth differentiation factor 15 (GDF15), and it affects cell proliferation, metastasis, and angiogenesis through MAPK and PI3K/AKT pathway activation. Moreover, mounting evidence show that the ErbB2 receptor is activated in OSCC cells.

GDF15 belongs to a branch member of the TGF-β superfamily. Its expression is affected by inflammation, injury, and malignant tumors. High expression of GDF15 is related to the poor clinical outcomes in colorectal cancer and prostate pancreatic cancer. Our previous study also reveals that GDF15 promotes the occurrence and progression of OSCC.
It has also been reported that GDF15 induces Src-dependent ErbB2 phosphorylation. However, whether GDF15/ErbB2 is involved in the effect of M1 macrophages on OSCC has not been studied.

In this study, the effect of M1 macrophages on the proliferation, migration, and invasion of OSCC cell lines in vitro and xenograft growth in vivo was studied. We also evaluated the role of GDF15/ErbB2 and its downstream signaling pathways in this process.

**RESULTS**

**Monocytes Differentiate into Pro-inflammatory M1 Macrophages.** To simulate the inflammatory tumor microenvironment, LPS (200 ng/mL) was used to stimulate M0 macrophages for 48 h, and then the phenotype of proinflammatory M1 macrophages was first identified. CD80 and CD86 were obviously upregulated at the gene and protein levels in M1 macrophages (Figure 1a,b). Furthermore, we confirmed that LPS could significantly increase the level of TNF-α and IL-6 (Figure 1c,d), which have been recognized as the main factors secreted by M1 macrophages at the gene and protein levels.

**M1-CM Significantly Enhances OSCC Proliferation, Migration, and Invasion In Vitro.** To evaluate the effects of factors secreted by M1 macrophages on the biological behaviors of OSCC, we assessed the effect of conditioned medium from M1 macrophages (M1-CM, containing 50% M1 macrophage culture supernatant +50% basic medium) on SCC25 and CAL27 cells with basic medium (BM) as the control. CCK-8 (Figure 2a), colony formation (Figure 2b), and EdU assay (Figure 2c) showed that M1-CM significantly increased SCC25 cell proliferation vs BM but did not for CAL27 cells (data not shown). Additionally, the flow cytometry analysis results indicated no significant effect of M1-CM on SCC25 and CAL27 cell apoptosis (Figure 2d). Transwell assays showed that, compared with BM, M1-CM significantly increased the migration and invasion abilities of SCC25 and CAL27 cells at 24 h (Figures 2e,f). These data indicate that in vitro M1-CM promotes proliferation, migration, and invasion of OSCC cells.

**M1-CM Enhanced Proliferation, Migration, and Invasion of OSCC Cells Are Associated with ErbB2/PI3K/AKT and MAPK/ErK Signaling Pathways.** The PI3K/AKT and MAPK/ErK signaling pathways play key roles in regulating cell growth, migration, and invasion, while ErbB2 affects cell proliferation, metastasis, and angiogenesis through MAPK/ErK and PI3K/AKT pathway activation. To observe whether ErbB2/PI3K/AKT and MAPK/ErK signaling pathways are associated with M1-CM enhanced proliferation, migration, and invasion, the effect of M1-CM on phosphorylation of AKT, ErK, and ErbB2 of OSCC cells was studied. Our study confirmed that M1-CM activated the AKT and ErK by increasing the protein level of p-AKT and p-ErK in SCC25 and CAL27 cells with peak levels at 30 min (Figure 3a). M1-CM also significantly promoted the phosphorylation of ErbB2 (Figure 3b). To elucidate whether M1-CM-activated AKT and ErK signaling pathways are associated with the ErbB2 receptor, the ErbB2 inhibitor CI-1033 was used.
significantly decreased M1-CM-induced p-AKT and p-ErK expression in SCC25 and CAL27 cells (Figure 3c). Finally, to explore whether the ErbB2 receptor is also involved in promoting OSCC proliferation, migration, and invasion by M1-CM, SCC25 and CAL27 cells were pretreated with the inhibitor for 2 h and then stimulated with M1-CM. The results showed that the inhibitor significantly inhibited M1-CM-induced SCC25 cell proliferation (Figures 4a,b) and decreased the M1-CM-promoted SCC25 and CAL27 cell migration and invasion (Figure 4c,d). These results indicate that M1-CM-enhanced proliferation, migration, and invasion are associated with ErbB2/PI3K/AKT and MAPK/ErK signaling pathways.

M1-CM Activates the ErbB2 Signaling Pathway via GDF15. GDF15 has been reported to induce Src-dependent phosphorylation of ErbB2 in human breast cancer and stomach cancer cells.14,24 This pushes us to study whether GDF15 mediates M1-CM-induced ErbB2 phosphorylation in OSCC cells. We demonstrated that GDF15 gene and protein expression and secretion were significantly promoted by M1-CM vs BM control (Figure 5a−c). After the GDF15 gene was knocked out (Figure 5d,e), p-ErbB2 expression was significantly decreased compared with cells transfected with negative vector no matter with or without M1-CM stimulation (Figures 5f,g), suggesting that GDF15 is partly involved in the M1-CM-stimulated activation of the ErbB2.

M1-CM Promotes Tumor Formation In Vivo. Twelve nude mice (six mice in each group) were subcutaneously transplanted with SCC25 cells and M1-CM induced SCC25 cells to evaluate the tumorigenesis ability of M1-CM (Figure 6a). The xenograft assay showed that M1-CM induced SCC25 cells formed much larger tumors in terms of volume (Figure 6b) and weight (Figure 6c) than SCC25 cells. Furthermore,
IHC revealed that the Ki67-positive cells in SCC25 cells induced by M1-CM were markedly higher (Figure 6d). The results indicate that M1-CM promotes tumor formation in vivo.

**DISCUSSION**

The tumor-supportive role of M2-macrophages is generally accepted, but the role of M1 macrophages in cancers remains controversial. For the first time, this study demonstrated that M1 macrophages potentiate proliferation, migration, and invasion got in vitro and in vivo xenograft formation of OSCC cells. More interesting, we found the novel mechanism by which M1 macrophages potentiate the proliferation, migration, and invasion of OSCC cells, namely, via GDF15-mediated ErbB2 phosphorylation.

Cancer cells secrete inflammatory chemokines to recruit monocytes/macrophages to the inflamed TME and activate residential macrophages in tissues, thus generating a TAM population. On the other hand, TAMs secrete several cytokines, chemokines, growth factors, and inflammatory mediators that possess cytotoxic and tumoricidal activities or exert anti-inflammatory and tumor-supportive effects. Although the M2-like phenotype, which has a tumor-promoting effect, is dominant in TAMs and skewing the M1/M2 ratio toward the M1 phenotype can inhibit tumor growth, TAMs phenotypes are a mixture of M1-like and M2-like phenotypes, and the protumor role of M1 macrophages has also been demonstrated in many studies. TNF has been proved to promote angiogenesis and metastasis of OSCC cells as well as in several models in vivo. At the same time, M1 macrophages are the primary sources of TNF in the TME.25 More recently, Zong et al. showed that M1 macrophages induce the level of PD-L1 in hepatocellular carcinoma cells (HCC), supporting the protumor role of M1 macrophages.26 Helm et al. revealed that M1 macrophages contribute to EMT in premalignant and malignant pancreatic ductal epithelial cells.27 Guo et al. demonstrated that M1 macrophages induce the development of breast cancer stem cell (CSC)-like phenotypes.28 We used LPS to activate the macrophages to classic M1 phenotype in vitro study.29,30 Although a few M2 macrophages were recognized by the expression of CD163 and CD206, M1 macrophages were still the majority of this population (Figure S1). Consistent with other studies, our present study demonstrated that M1 macrophages potentiate in vitro proliferation, migration, and invasion and in vivo xenograft formation of OSCC cells.

Given the tumor-supportive effect of M1 macrophages, the underlying mechanisms need to be explored. Several clinical studies have indicated that high serum concentrations of proinflammatory cytokines are related to poor prognosis in
many types of malignancies. Elevated levels of IL-6 in the tumor microenvironment are well-known to be involved in metastasis and cell survival.\textsuperscript{31} TNF-\(\alpha\) mediates cancer progression in various cancer types through NF-\(\kappa\)B activation. Our observations are consistent with previous reports showing that M1-CM contains a high potency of IL-6 and TNF-\(\alpha\), suggesting that paracrine of proinflammatory cytokines are, at least in part, associated with the tumor-supportive effect of M1 macrophages.\textsuperscript{28,32}

The PI3K/AKT and MAPK/ErK signaling pathways play critical roles in regulating cell growth, migration, and invasion. At the same time, GDF15 has been demonstrated to stimulate ErbB2, AKT, and ErK in human breast and stomach cancer cells in vitro.\textsuperscript{14,22} Moreover, the ErbB2 receptor is activated in OSCC.\textsuperscript{16−18} The present study confirmed that in OSCC, M1-CM activated the AKT and ErK signaling pathways. Moreover, the ErbB2 receptor was also activated, and CI-1033 significantly decreased M1-CM-induced p-AKT and p-ErK
expression and proliferation, migration, and invasion; these findings indicate that M1-CM activates the PI3K/AKT and MAPK/ErK signaling pathways in part by activating the ErbB2 receptor.

GDF15 plays an important role in promoting the development of OSCC and other tumors.21,33,34 It has been reported that GDF15 expression is induced by inflammatory conditions.35 However, whether GDF15 expression is regulated by TAMs has not been reported. Our results confirmed that M1-CM enhanced the secretion and expression of GDF15 in OSCC cells and that GDF15 knockout inhibited M1-CM-induced phosphorylation of ErbB2. However, as shown in Figure 5f,g, p-ErbB2 expression was still increased by M1-CM stimulation after GDF15 was knocked out. These seem to indicate that GDF15 plays a partial role in M1-CM enhanced p-ErbB2 expression. Indeed, the other ligands can activate EGFR receptors, including ErbB2.36

The tumor-supportive role of M2 macrophages is generally accepted, but both pro- and antitumor functions of M1 macrophages have been reported. This paradox of M1 macrophages may be related to, but not limited to, the following factors: (1) heterogeneity among cancers originated from various tissues and (2) cell-type specificity: in our study, M1 macrophages exerted differential effects on SCC25 and CAL27 cells. M1-CM promoted proliferation of SCC25, a tongue squamous cell carcinoma cell line, but had no such effect on CAL27, a type of tongue adenosquamous carcinoma cell line. (3) Numbers of M1 macrophages: there is a notion that a lower number of M1 macrophages promotes xenograft development, while a larger number of M1 macrophages reduce xenograft development.37 In our in vivo supplementary experiment, we coinoculated a small number of M1 macrophages (1 × 10^5) and a larger number of SCC25 cells (5 × 10^6) into nude mice. The results showed that tumor size and volume formed by this coinoculation were significantly larger than those of SCC25 cells inoculated alone (Figure S2).

**CONCLUSIONS**

Taken together, our preliminary results indicate that M1 macrophages contribute to the proliferation, migration, and invasion and xenograft development of OSCC cells partly by inducing GDF15-mediated ErbB2 activation. However, more and deeper studies are needed to evaluate the paradoxical role of M1 macrophages in OSCC. In this regard, the effect of M1 macrophages on the immune cells, especially CD8+ T-cell, is an important aspect. The model of coculture of M1 macrophages and tumor cells in different proportions in vitro is also helpful to comprehensively evaluate the role of M1 macrophages in OSCC.

**MATERIALS AND METHODS**

**Cells Culture and M1-Macrophage Polarization.** Human monocytic THP-1 cells (Stem Cell Bank, Chinese Academy of Sciences) were cultured in RPMI 1640 medium (HyClone, Logan, UT) containing 10% fetal bovine serum (FBS, Bioind, Kibbutz Beit Haemek, Israel) and 50 pM β-mercaptoethanol (Gibco; Grand Island, NY). THP-1 cells were differentiated into macrophages (M0) by incubation with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) for 24 h, and the adherent cells were washed with PBS to remove the remaining PMA. The macrophages were polarized toward the M1 phenotype by incubation with 200
ng/mL *Porphyromonas gingivalis* LPS (InvivoGen, San Diego, CA) for 48 h. Polarized M1 macrophages were characterized by CD80 or CD86 expression. After 48 h of polarization, the polarizing stimulator LPS was removed by aspirating culture supernatants, and a fresh culture medium was added for a further 48 h incubation. Then the culture supernatants were centrifuged for later use. The human OSCC cell lines CAL27 and SCC25 (ATCC, Manassas, VA) were maintained in a DMEM medium (Hyclone) containing 10% FBS. All of the cells were cultured in a humidified environment with 5% CO2 at 37 °C.

**Generation of GDF15 Knockout Stable Cell Lines.** Single-guide RNAs (sgRNAs) were used to generate CRISPR-Cas9-based GDF15 knockout constructs (sgGDF15#1 forward, 5′-GAAACTTGCAGGCTCGCCT-3′, reverse, 5′-AGGCGAGCGCGCAAGTTTC-3′). The sgGDF15 plasmid was transiently transfected into the OSCC cells. Puromycin (1 μg/mL) was added to the medium, and the cells remained for 2 weeks for single clone selection. The GDF15 knockout efficacy was determined by Western blotting.

**Cell Proliferation Assay.** After being cultured with BM or M1-CM, the cell proliferation was evaluated by CCK-8 (Dojindo Laboratories, Kumamoto, Japan). After incubation for 1.5 h, absorbance at 450 nm wavelength was measured.

**Colony Generation Assay.** SCC25 cells were cultivated with BM or M1-CM for 7 d. Then the cells were treated using the protocol described in a previously.38

**EdU Assay.** SCC25 cells were cultured with BM or M1-CM for 24 h. Then the supernatant was discarded and the EdU labeling assay was performed according to the procedures described previously.38

**Cell Apoptosis Analysis by Flow Cytometry.** SCC25 and CAL27 cells were cultured with BM or M1-CM for 2 days. Then the cells were treated using the protocol outlined in a previously.38

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**Figure 6.** M1-CM promoted tumor formation in vivo. (a) Pictures showing the tumor size of nude mice from SCC25 cells and M1-CM-stimulated SCC25 cells. Tumor growth curvature (b) and tumor weights (c) are shown for SCC25 cells and M1-CM-stimulated SCC25 cells. (d) Immunohistochemical staining results for Ki67; statistical analysis revealed significantly greater numbers of Ki67 positive (p < 0.001) in M1-CM-stimulated SCC25 cells than SCC25 cells. The histograms represent the mean ± SD (n = 6). *p < 0.05, **p < 0.01, and ***p < 0.001.
Transwell Assays. SCC25 and CAL27 cells were cultured in a serum-free medium in the upper chambers (Costar, Corning, NY). The upper chambers were placed into chambers containing BM or M1-CM. Then cells were treated following the protocol described in ref 39. For invasion assays, cells were seeded in matrigel-coated Transwell chambers (BD Biosciences, San Jose, CA) and then used for subsequent assays following a similar approach.

ELISA Assay. Supernatants from M0 macrophages, M1 macrophages, SCC25 cells, and CAL27 cells were collected and centrifuged at 12000 rpm at 4 °C for 10 min, and the concentrations of TNF-α, IL-6, and GDF15 were measured with ELISA kits (BOSTER).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction. Total RNA was isolated from cells with TRIzol reagent (Takara, Kusatsu, Japan) and then reverse-transcribed into cDNA with a PrimeScript RT reagent kit according to the instructions. Then quantitative real-time PCR assays were performed using the PCR System (Roche, Basel, Switzerland) with SYBR Green or iQ SuperMix (Bio-Rad) to examine the mRNA of CD80, CD86, TNF-α, GDF15, ErbB2, and AKT using the 2−ΔΔCT method (29). GAPDH was used as an endogenous control. The primer sequences are listed in Table 1.

Western Blotting. Total protein was extracted and concentrated by BCA protein assay as described by the manufacturer. Western blotting was carried out to detect the expression of ErbB2, AKT, and ErK following the protocol described in ref 39. The antibodies are listed in Table 2.

Immunohistochemistry Assay for Ki67 Expression. The immunohistochemistry of Ki67 was carried out as described in ref 38. Anti-Ki67 antibody was used at a dilution of 1:2000, and then goat antirabbit secondary antibody was used. Immunoreactions were detected with diaminobenzidine (DAB; Solarbio). Images were captured and the Ki67-positive cells were counted.

Statistical Analysis. Data were displayed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey’s and two-way ANOVA followed by Sidak’s multiple comparisons tests were performed with GraphPad Prism software (version 8, by MacKiev Software, Boston, MA, USA). p < 0.05 was indicated a statistically significant difference.
The authors declare no competing financial interest. All authors reviewed the manuscript and gave their approval. Manuscript writing and editing: C.L. and P.Y. Conception and/or design of the study: C.Y. and P.Y. Data acquisition: C.L., S.L., and J.Z. Data analysis and interpretation: C.L. and P.Y. Manuscript writing and editing: C.L. and P.Y. All authors reviewed the manuscript and gave their final approval.

Notes
The authors declare no competing financial interest. This program was approved by the Ethics Committee on Human Experiments at Dental Hospital, Shandong University (protocol: 20210606).

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