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Expansion of PMN-myeloid derived suppressor cells and their clinical relevance in patients with oral squamous cell carcinoma

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ABSTRACT

Objectives: Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy worldwide, with a high mortality. The prognosis of OSCC remains unsatisfactory; the dysregulated immune system plays an important role in the pathogenesis of OSCC. Myeloid-derived suppressor cells (MDSCs) have been identified as immune-suppressive cells in multiple tumor types. The aim of this study was to clarify the underlying immunoregulatory mechanism of MDSC in patients with OSCC.

Materials and methods: Flow cytometry was used to analyze the phenotype of MDSC among peripheral blood mononuclear cells (PBMCs) from patients with OSCC and healthy control subjects. The correlation between MDSC frequency and the disease index of patients with OSCC was evaluated. T cell proliferation experiment was used to evaluate the immunosuppressive function of MDSC.

Results: Patients with OSCC exhibited significantly higher levels of PMN-MDSCs than did healthy controls. In the co-culture assay, T cell proliferation and IFN-γ production were abrogated by the addition of PMN-MDSCs in a dose-dependent manner. The levels of reactive oxygen species were higher for PMN-MDSCs derived from patients with OSCC than for those from normal individuals. p-STAT3 levels, a key activator of MDSCs, was higher in OSCC-related PMN-MDSCs than in those from healthy controls. Both of these effects were reversed by NAC (an ROS inhibitor) and JSI-124 (a p-STAT3 inhibitor). Finally, PMN-MDSC levels were positively related to histological differentiation, nodal metastasis, and recurrence.

Conclusion: PMN-MDSCs were elevated in OSCC patients, with strong immune-suppressive effects via p-STAT3/reactive oxygen species, providing a new direction for therapeutic strategies.

Introduction

Oral squamous cell carcinoma (OSCC) is defined as squamous cell carcinoma originating from the lip, tongue, gingiva, cheek, floor of the mouth, and palate; it is the most common head and neck malignancy worldwide, with a high mortality [1]. The prognosis of OSCC remains unsatisfactory; the poor survival is not only related to the aggressiveness of this cancer but also to an insufficient understanding of the disease, which hinders the development of effective treatments [2]. Although environmental carcinogens are major etiological factors, impaired immune functions in patients with OSCC are associated with an increased tumor load, migration to distant sites, and poor prognosis [3-6]. Therefore, the identification of the mechanisms underlying the immune dysfunction associated with aggressive tumor growth and

Abbreviations: ARG1, arginase-I; CFSE, carboxyfluorescein succinimidyl ester; ELISA, enzymelinked immunosorbent assay; INF, interferon; L-NMMA, L-NGmonomethyl-L-arginine; M-MDSCs, monocytic myeloid-derived suppressor cells; NAC, N-acetylcysteine; NOHA, N-hydroxy-nor-L-arginine; PBMCs, peripheral blood mononuclear cells; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cells; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3

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treatment responses is important for the effective management of OSCC.

Myeloid-derived suppressor cells (MDSCs) are immune-suppressive cells with the ability to suppress T cell activation and function [7-9]. MDSCs were initially investigated in malignant diseases based on their important role in the regulation of immune responses in patients with tumors. Mouse MDSCs co-express the Gr1 and CD11b antigens in mice [10,11]. Human MDSCs generally express CD11b and the common myeloid marker CD33, but lack markers of mature myeloid and lymphoid cells and the MHC class II molecule HLA-DR [12,13]. MDSCs were divided into two major populations: granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and mononuclear MDSCs (M-MDSCs). These two subtypes of MDSCs may have different biological functions and use different mechanisms for immune suppression. M-MDSCs suppress T cell responses predominantly by up-regulating arginase-1 and iNOS [12]. PMN-MDSCs suppress T cell responses by enhancing the generation of ROS. Therefore, characterization and functional studies of these subsets will delineate the mechanisms through which MDSCs mediate immune suppression under specific pathological conditions [14]. Expansion of MDSCs were controlled by a network of transcription factors and regulators that can be divided into two large groups. One group is responsible for expansion of immature myeloid cells and the other group for pathologic activation of these immature cells.

Expanded MDSCs are able to regulate tumors by complex mechanisms. For example, in human ovarian carcinoma, MDSCs inhibit T cell activation in the tumor microenvironment and thus promote cancer stem cell gene expression and metastasis [15]. MDSCs also accumulate in the peripheral blood, spleen, and tumors in mice with oral cancer over time [16]. Although several mechanisms by which tumor cells induce MDSCs have been described [17,18], the specific pathways underlying the expansion and activation of MDSCs in patients with OSCC are unclear. Therefore, the objective of this study was to investigate MDSC expansion and its contribution to immune dysfunction in patients with OSCC.

Materials and methods

Patients and healthy donors

Patients with OSCC (n = 31) and healthy controls (n = 31) were recruited at Guangdong Second Provincial General Hospital. According to the Union for International Cancer Control (UICC) 2002 clinical staging criteria TNM Classification Method:early stage (I/II) and late stage (III/IV) , the basic characteristics of patients and healthy donors are outlined in Table 1. All patients and healthy controls were screened for serum HIV antibody, hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV) antibody, hepatitis D virus (HDV) antigen, and HDV antibody. The study was approved by the Clinical Ethics Review Board of the Guangdong Second Provincial General Hospital. Written informed consent was obtained from all patients at the time of admission.

Table 1
Basic characteristics of patients and healthy donors.

Features	Patients with OSCC (n = 31)	Healthy controls (n = 31)
Sex	No. (%)	
Female	15 (48.3)	11 (48.3)
Male	16 (51.6)	20 (64.5)
Age	21–66	23–58
Location		
Tongue	8 (25.8)	
Mandibular alveolar ridge	6 (19.3)	
Buccal mucosa	4 (12.9)	
Floor of mouth	3 (9.67)	
Palate	3 (9.67)	
Maxillary alveolar ridge	3 (9.67)	
Lower lip	2 (6.45)	
Other	2 (6.45)	
Grade		
Well differentiated	14 (45.1)	
Moderately differentiated	9 (29.0)	
Poorly differentiated	8 (25.8)	
Stage		
I	11 (35.4)	
II	9 (29.0)	
III	6 (19.3)	
IV	5 (16.1)	
Lymph node metastasis		
Yes	13 (41.9)	
No	18 (58.0)	
Habits		
Ever Smoker	11 (35.4)	
Ever Drinker	10 (32.2)	
Ever Chewer	8 (25.8)	
No habit	6 (19.3)	

PBMC isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll centrifugation and analyzed immediately. The following anti-human antibodies were purchased from eBioscience (San Diego, CA, USA): CD11b-FITC, CD33-PE, HLA-DR-APC, CD14-PE-Cy7, CD15-eFluor450, CD4-PE, CD8a-FITC, CD8-PE-Cy5, CD3-PE-Cy7, CD66b-APC (G10F5), and their corresponding isotype controls. pY^{705}-Stat3-AlexaFluor 488 was purchased from BD Biosciences (San Jose, CA, USA). S100A8 Polyclonal Antibody (PA5-86063), S1009 Monoclonal Antibody (JM51-31), Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody-Alexa Fluor Plus 647(A32733) were purchase from Invitrogen. Human Arginase 1/ARG1 Alexa Fluor® 647-conjugated antibody was purchase from R&D Systems. The cell phenotype was analyzed using a flow cytometer (BD LSR II; BD Biosciences), and data were analyzed using FlowJo version 10.0.7 (FlowJo, Ashland, OR, USA). For flow cytometric sorting, the FACSAria cell sorter (BD, Mountain View, CA, USA) was used. The strategy for MDSC sorting was as follows: HLA-$DR^{-/low}CD11b^{+}CD15^{+}CD14^{-}$ for PMN-MDSCs, with M-MDSCs defined as HLA-$DR^{-/low}CD11b^{+}CD15^{-}CD14^{+}$. The depletion of MDSCs were performed by harvesting the remaining PBMCs after MDSC sorting.

T cell proliferation and activation assay

T cell proliferation was determined by CFSE (5,6-carboxyfluoresceindiacetate, succinimidylester) dilution. PBMCs or purified T cells were labeled with CFSE (2 μM; Invitrogen, Carlsbad, CA, USA), stimulated with anti-CD3/CD28 antibodies (5 μg/ml) (eBioscience), and cultured alone or co-cultured with autologous MDSCs at the indicated ratios for 3 days. The cells were then stained for surface marker expression with CD4 or CD8 antibodies, and T cell proliferation was analyzed using a flow cytometer. Where indicated, 1 mM L-arginine, 0.5 mM nor-NOHA (Cayman Chemicals, Ann Arbor, MI, USA), an arginase I-specific inhibitor, or 1 mM *N*-acetylcysteine (Sigma Aldrich, Merck, St. Louis, MO, SA), an ROS inhibitor, was added to the culture on day 0.

Measurement of intracellular ROS

ROS measurements were obtained using 2′,7′-dichlorofluorescein diacetate (ROS-DCF). Briefly, PBMCs were incubated with 2.5 mM ROS-DCF at 37 °C for 15 min. Cells were stained for PMN-MDSC surface markers, washed, resuspended in phosphate-buffered saline, and analyzed using a flow cytometer.

qRT-PCR

RNA was extracted using an RNase Mini Kit (Axygen,Union City, CA, USA), and cDNA was synthesized using a Super Script III Reverse Transcriptase Kit (Qiagen, Valencia, CA, USA). qRT-PCR were performed in triplicate using SYBR Green (TaKaRa, Otsu, Japan) and levels were normalized to endogenous β-actin mRNA levels using gene-specific primers.

ELISA

IFN-γ was quantified in culture supernatants by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Arginase activity

Arginase activity was evaluated by previously described procedures [19].

Statistical analyses

Clinical and immunological parameters were compared by non-parametric Mann-Whitney U tests or chi-square tests. For *in vitro* experiments, statistical analyses were conducted using paired *t*-tests. Correlations between parameters were analyzed using a Spearman rank test and linear regression. Statistical tests were performed using GraphPad Prism version 6.0a. P-values of < 0.05 were considered significant.

Results

PMN-MDSC expansion in patients with OSCC

To determine the role of MDSC in patient with OSCC, we first compared MDSC frequency and absolute cell counts in the peripheral blood from patients with OSCC (n = 31) with age-matched healthy controls (n = 31). PBMCs were isolated from whole blood by Ficoll centrifugation and analyzed within 6 hr of blood sampling. Circulating frequencies of MDSC subsets were quantified with a gating strategy, and both PMN-MDSCs and M-MDSCs were CD33-positive (Fig. 1A). We found substantially higher PMN-MDSCs frequencies and absolute cell counts in patients with OSCC than in healthy controls, based on qualitative and quantitative analyses of flow cytometric data and quantitative data for all samples in Fig. 1B. PMN-MDSCs frequencies and absolute cell counts were further increased in patients with OSCC at a later stage. Furthermore, OSCC patient-derived PMN-MDSCs displayed higher rates of CD66b, S100A8, S100A9, and Arg-1 expression than did those from healthy controls (Fig. 1C). These observations indicated that PMN-MDSCs were markedly expanded in patients with OSCC.

PMN-MDSCs suppress T cell responses in patients with OSCC

MDSCs are characterized by potent T cell suppression. We determined whether OSCC-related PMN-MDSCs could suppress T-cell function. PBMCs harvested from patients with OSCC (n = 6) with or without MDSC depletion (by cell sorting) were staining with CFSE and stimulated with anti-CD3/CD28 antibodies, the T cell proliferation was measured after 3 days of culture. We observed enhancement of both $CD4^+$ and $CD8^+$ T cell proliferation in response to stimulation after MDSC depletion (Fig. 2A), indicating that OSCC-related MDSCs actively suppressed T cell function. To confirm the immune-suppressive capacity of PMN-MDSCs in patients with OSCC, T cells and PMN-MDSCs were purified from PBMCs by flow sorting. Representative flow cytometry data for positive and negative controls are shown in Fig. 2B. CFSE-labeled PBMC-derived $CD3^+$ T cells were stimulated with anti-CD3/CD28 antibodies, with the indicated ratio of PMN-MDSCs. For healthy donors, there was no suppressive effect on $CD4^+$ and $CD8^+$ T cell proliferation (Fig. 2C). Importantly, $CD4^+$ and $CD8^+$ T cell proliferation was significantly suppressed by the addition of OSCC-related PMN-MDSCs at a 2:1 ratio. The addition of OSCC-related PMN-MDSCs significantly reduced the proliferation of both $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner. Based ELISA, IFN-γ secretion decreased after the administration of OSCC-related PMN-MDSCs (Fig. 2C and D). These results indicated that PMN-MDSCs in patients with OSCC could suppress T cell function.

PMN-MDSCs suppress functional T cells in a pSTAT3-ROS-dependent manner

Based on the finding that PMN-MDSCs from patients with OSCC suppressed T-cell responses, we explored the mechanisms underlying OSCC-related MDSC-mediated T cell suppression. MDSCs suppress T-cell function by a number of mechanisms, including arginase activity as well as nitric oxide (NO) and reactive oxygen species (ROS) production [20,21]. Thus, we utilized the arginase inhibitor *N* (omega)-hydroxy-nor-L-arginine (nor-NOHA), L-arginine supplementation, or the ROS inhibitor *N*-acetyl-L-cysteine (NAC) to reverse the suppressive effects of PMN-MDSCs on T cell proliferation in a co-culture system. The suppression of T cell proliferation and IFN-γ production were reversed by NAC, while nor-HOHA and L-arginine had no effect (Fig. 3A and B). We found that ROS production was dramatically higher in OSCC-related PMN-MDSCs than in healthy controls (Fig. 3C). The mRNA level of the NADPH oxidase *NOX2*, responsible for ROS production in MDSCs, was higher in OSCC-related PMN-MDSCs than in healthy controls (Fig. 3D). Biochemical assays indicated no considerable changes in the level of arginase activity between OSCC-derived and healthy control-derived PMN-MDSCs (Fig. 3E). Given that STAT3 signaling plays a key role in MDSC generation and function [22,23], we compared intracellular phosphorylated STAT3 (p-STAT3) levels in the PMN-MDSCs from patients with OSCC and healthy controls. We observed significantly higher expression of p-STAT3 in OSCC-related PMN-MDSCs than in healthy controls (Fig. 3F). Cucurbitacin-I (JSI-124), a potent inhibitor of JAK-STAT3 signaling [24], was used to block STAT3 signaling. We found that the suppressive activity of OSCC-related PMN-MDSC was almost completely abrogated by JSI-124 pretreatment (Fig. 3G). These observations indicated that p-STAT3 signaling is essential for OSCC-related PMN-MDSCs generation and function.

Correlation of PMN-MDSC levels with patient clinico-pathological factors

We then investigated the correlation between PMN-MDSCs levels and clinico-pathological parameters. We did not detect a correlation between PMN-MDSCs levels and patient sex and old, but we found that patients with tongue cancer had higher PMN-MDSCs levels (P = 0.042; Table 2). Patients with poor histological differentiation had significantly higher PMN-MDSCs levels than those with greater histological differentiation (P = 0.00068; Table 2). Furthermore, patients with later stages of oral cancer had higher levels of PMN-MDSCs than those with early stage disease (P = 0.0088; Table 2). Finally, PMN-MDSCs levels were positively associated with the incidence of nodal metastasis and recurrence. Our results showed that high levels of PMN-MDSCs in patients with OSCC are associated with poor prognosis.

Discussion

The mechanisms by which tumors escape immune-surveillance include the deregulation of MDSC functions or immune-regulatory cytokines. The comprehensive detection and assessment of factors influencing prognosis can improve the management of OSCC. The immune system plays a key role in the progression of OSCC, as initially suggested by the numerous immunological defects (i.e., invariant natural

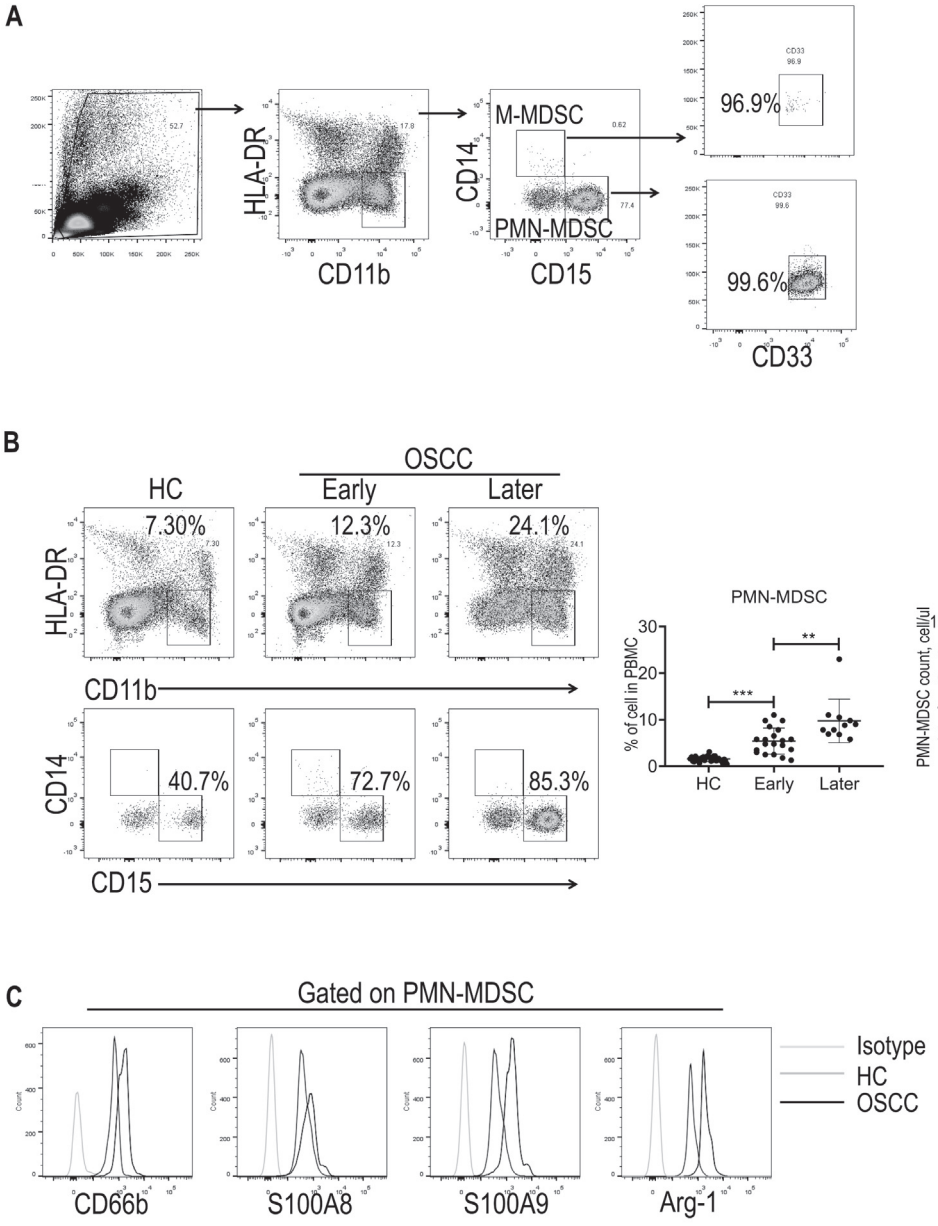

Fig. 1. Expansion of polymorphonuclear (PMN) myeloid-derived suppressor cells (MDSCs) in patients with OSCC. (A) Gating strategy for MDSCs by flow cytometry. PMN-MDSCs were defined as HLA-DR$^{-/low}$CD11b^{+}CD15^{+}CD14^{-}, and M-MDSCs were defined as HLA-DR$^{-/low}$CD11b^{+}CD15^{-}CD14^{+}. CD33 expression was evaluated in PMN-MDSC and M-MDSCs. (B) Representative flow cytometry data and statistical analyses of PMN-MDSCs frequencies in the peripheral blood of healthy controls and patients with OSCC at early and later stages are shown (n = 31). (C) Surface markers of PMN-MDSCs from OSCC patients and healthy controls. **P < 0.01; ***P < 0.001, unpaired *t*-tests.

killer T) and the expansion of immunosuppressive populations (i.e., Tregs) both at the tumor site and in the blood [25,26]. The therapeutic manipulation of the immune system and its response, by corollary, may contribute to the treatment of OSCC. Consistent with mouse with oral squamous cell carcinoma, characterized by the expansion of MDSCs (CD11b^{+}Gr1^{+}) and immunosuppressive activity [16], this study showed that the infiltration of PMN-MDSCs is an important predictive factor for OSCC outcomes.

Owing to the heterogeneity of MDSCs phenotypes across malignancies, we first defined the MDSC phenotypes in patients with OSCC. We found that PMN-MDSCs are increased in patients and exhibit immunosuppressive activity. Furthermore, patients with higher levels PMN-MDSCs had significantly poorer clinical outcomes, including higher rates of lymph node metastasis. MDSCs could inhibit T cell activation in the tumor microenvironment, thus promoting cancer stem cell gene expression and metastasis. It is highly likely that MDSCs plays an important role in tumor progression. PMN-MDSCs were a kind of crucial component of cell-mediated immunity, as they suppressed the production of interferon-γ by T cells. In agreement with our findings, strong PMN-MDSCs infiltration has been correlated with unfavorable outcomes in several tumor types, including head and neck cancer [16,27]. Thus, the expansion of PMN-MDSCs in patients with OSCC might lead to immune dysfunction inpatients. MDSCs are primarily defined by their immunosuppressive function [28]; however, the functions of MDSCs in patients with OSCC remain unclear. In our study, we found a T cell suppressive effect of PMN-MDSCs in patients with OSCC. It is well known that PMN-MDSCs exert their suppressive effects via ROS or arginase [20,29]. In the present study, multiple analyses

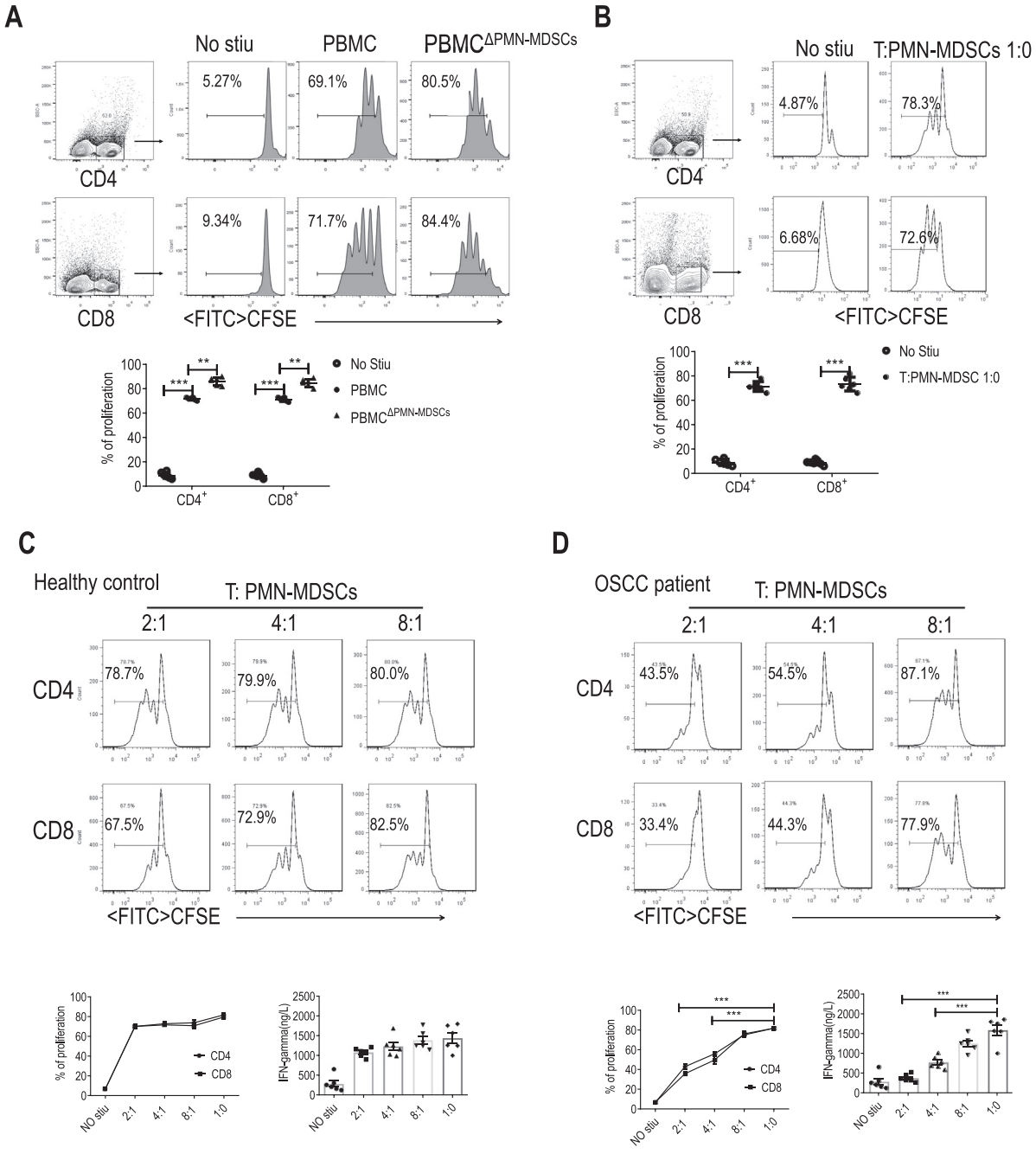

Fig. 2. PMN-MDSCs derived from patients with OSCC suppress T cell proliferation and activation. A: Depletion of MDSCs enhanced T-cell function. PBMCs or PBMCs with MDSC depletion ($PBMC^{\Delta MDSC}$) from fresh peripheral blood of patients with OSCC were stimulated with anti-CD3 and anti-CD28, and the proliferation of T cells was examined by CFSE dilution. Upper panels: representative flow cytometry data from one individual; Lower panel: stimulated samples from 6 individuals. B-D: $CD3^+$ T cells from peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3 and anti-CD28, co-cultured with PMN-MDSCs from the same donors at different ratios for 3 days, and evaluated for $CD4^+$ and $CD8^+$ T cell proliferation by CFSE labeling and interferon-γ production in supernatants by enzyme-linked immunosorbent assay. (B) Representative flow cytometry data for positive and negative controls. (C) Representative flow cytometry results for healthy controls. (D) Representative flow cytometry results for patients with OSCC. In all plots, data represent mean ± SD from one representative experiment of 6 per treatment group, repeated three times.*P < 0.05; **P < 0.01; ***P < 0.001 (n = 6).

indicated that OSCC-related PMN-MDSCs suppress T cells via ROS. Furthermore, p-STAT3 activation is a critical pathway for MDSCs [21]. We demonstrated that p-STAT3 signaling plays a functional role in PMN-MDSCs derived from patients with OSCC by mediating their ability to suppress autonomous T cell proliferation. p-STAT3 inhibition (JSI-124) decreased the level of ROS and T cell suppressive activity in OSCC-derived PMN-MDSCs. These results demonstrate that p-STAT3 signaling acts upstream of ROS to mediate the suppression of T cell proliferation in patients with OSCC. Further studies are necessary to understand the molecular mechanism underlying the observed effects and to apply them to the management of OSCC. The chemotherapeutic depletion or chemical modulation of PMN-MDSCs may provide new opportunities for therapy. However, a strategy to effectively and specifically target these cells would have to be formulated. For instance, some chemotherapeutic agents, including gemcitabine and 5FU, have been shown to selectively eliminate these cells [30,31].

In summary, our results indicated that PMN-MDSCs were elevated in patients with OSCC and exhibit a strong immune-suppressive ability

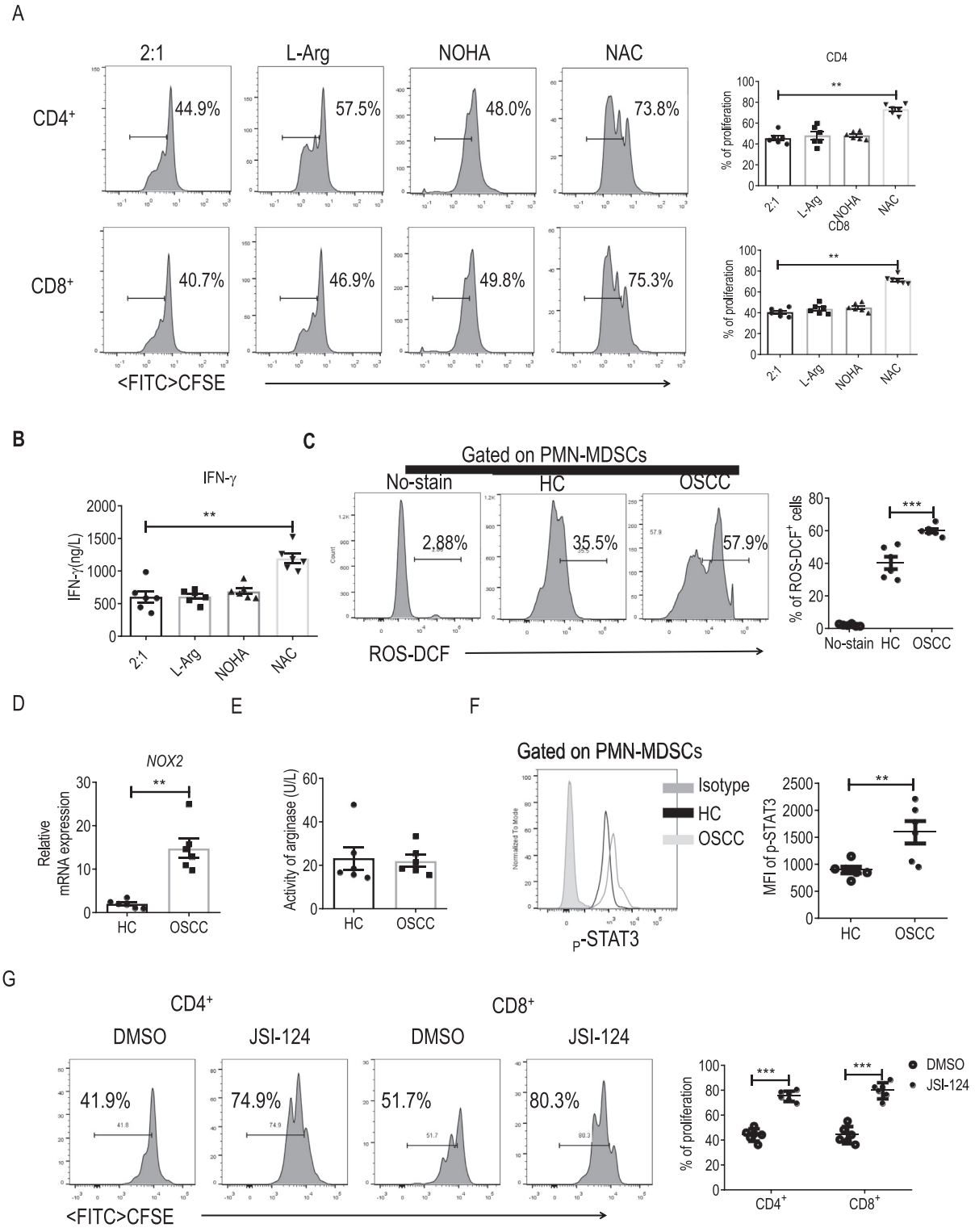

Fig. 3. PMN-MDSCs suppress functional T cells in an ROS-dependent manner. A: Effect of the arginase inhibitor NOHA, L-arginine supplementation, or the ROS inhibitor NAC on PMN-MDSC function. T cells from patients with OSCC were stimulated with anti-CD3 and anti-CD28, co-cultured with PMN-MDSCs from the same donor at a 2:1 ratio with the indicated treatments and evaluated for proliferation by CFSE labeling and interferon-γ production in supernatants by enzyme-linked immunosorbent assay. (A) Representative flow cytometry data. (B) Concentration of interferon-γ in media. (C) ROS levels in OSCC-related PMN-MDSCs (OSCC) and their counterparts from healthy donors (HC). (D) Expression of *NOX2* in OSCC-related PMN-MDSCs and their counterparts from healthy donors, as determined by RT-PCR. (E) Arginase activity in OSCC-related PMN-MDSCs and their counterparts from healthy donors. (F) p-STAT3 levels in OSCC-related PMN-MDSCs and their counterparts from healthy donors. (G) The suppressive activity of OSCC-related PMN-MDSCs were evaluated by T/MDSC co-culture system, followed by JSI-124 treatment. In all plots, data represent mean ± SD from one representative experiment of 6 per treatment group, repeated three times. *P < 0.05; **P < 0.01; ***P < 0.001 (n = 6).

Table 2
Correlation of PMN-MDSC levels with patient clinico-pathological factors.

	No.	PMN-MDSC (HR)	P value
Sex			
Male vs Female	15 vs 16	0.958	0.387
Age			
< 50 vs > 50	14 vs 17	1.036	0.420
Location			
Tongue vs HC	8 vs 31	2.35	**0.042***
Mandibular alveolar ridge vs HC	6 vs 31	1.115	0.365
Buccal mucosa vs HC	4 vs 31	0.968	0.589
Floor of mouth vs HC	3 vs 31	1.124	0.233
Palate vs HC	3 vs 31	1.025	0.315
Maxillary alveolar ridge vs HC	3 vs 31	1.158	0.298
Histological differentiation			
Moderate vs Well	10 vs 15	1.258	0.065
Poor vs Well	6 vs 15	2.364	**0.0068****
Stage			
I/II vs III/IV	20 vs 11	2.258	**0.0088****
Nodal metastasis			
No vs N(+)	18 vs 13	2.698	**0.0052****
Recurrence			
No vs Yes	17 vs 14	1.598	**0.048***

Bold indicates significant differences.

via the p-STAT3/ROS pathway; therapeutic approaches directed against PMN-MDSCs may contribute to disease management.

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Authors' contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Yu-feng Liu and Dong-lin Cao had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Li-mei zhong and Xuan Zhou contributed to study conception and design. Feng-bin Liu, Yi Wen, Zhi-guo Liu, Shao-hua Song, Guo-yi Weng contributed to acquisition of data.

Declaration of Competing Interest

The authors declare no conflict of interest.

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