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在线全文

牙龈卟啉单胞菌通过CCR6$^+$ Treg促进口腔鳞癌免疫抑制微环境形成的机制研究*

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【摘要】 目的 本研究旨在探讨在口腔鳞状细胞癌（oral squamous cell carcinoma, OSCC）中牙龈卟啉单胞菌（*Porphyromonas gingivalis*, *P. gingivalis*）通过募集趋化因子受体6（chemokine receptor 6, CCR6）阳性（CCR6$^+$）调节性T细胞（regulatory T cells, Treg）到肿瘤微环境（tumor microenvironment, TME）中促进OSCC的恶性进展的机制。**方法** 使用TCGA数据库分析趋化因子配体20（chemokine ligand 20, CCL20）-CCR6-Treg之间的相关性，OSCC患者CCR6高表达组中Treg富集指数、白细胞介素（interleukin, IL）-10和肿瘤坏死因子β1（tumor necrosis factor β1, TGF-β1）的表达。C57BL/6小鼠随机分为2组，每组6只，对照组颊部注射1次小鼠头颈鳞癌细胞系SCC7，实验组颊部注射1次SCC7细胞和*P. gingivalis*混合液，均为100 μL。2周后处死小鼠，免疫组化检测CCR6和叉头盒蛋白3（forkheadbox protein 3, FOXP3）在OSCC中的表达，流式细胞术明确*P. gingivalis*对OSCC恶性生物学行为及对CCR6$^+$ Treg细胞和免疫微环境的影响。**结果** 通过生信分析确定CCL20-CCR6-Treg之间具有相关性（r=0.373, P<0.000 1），OSCC中CCR6高表达患者中Treg富集评分较高，IL-10表达升高。动物实验表明*P. gingivalis*能够促进小鼠OSCC的瘤体体积（mm^3）（对照组：0.294±0.105；实验组：0.526±0.101；P<0.01）和质量（mg）（对照组：206.200±53.950；实验组：376.000±119.200；P<0.01）的增加；免疫组化验证CCR6与FOXP3间存在相关关系（r=0.659, P<0.05），且*P. gingivalis*促进CCR6与FOXP3的表达；流式分析表明在OSCC中*P. gingivalis*通过募集更多CCR6$^+$ Treg（%）（对照组：13.780±1.506；实验组：18.260±2.257；P<0.01），降低CD8$^+$ T细胞比例（%）（对照组：27.120±1.647；实验组：21.060±3.148；P<0.01），进而促进免疫抑制微环境形成。**结论** *P. gingivalis*通过肿瘤细胞募集CCR6$^+$ Treg细胞形成肿瘤免疫抑制性微环境，促进OSCC的恶性进展。

【关键词】 口腔鳞状细胞癌 牙龈卟啉单胞菌 调节性T细胞 趋化因子受体6

***Prophyromonas gingivalis* Promotes the Formation of Immunosuppressive Microenvironment in Oral Squamous Cell Carcinoma by CCR6$^+$ Regulatory T Cells: A Study of the Mechanisms Invovled**

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[Abstract] Objective To investigate the mechanisms by which *Porphyromonas gingivalis* (*P. gingivalis*) promotes the malignant progression of oral squamous cell carcinoma (OSCC) through the recruitment of chemokine receptor 6-positive (CCR6$^+$) regulatory T cells (Treg) in the tumor microenvironment (TME). **Methods** The Cancer Genome Atlas (TCGA) database was used to analyze the correlation between chemokine ligand 20 (CCL20), CCR6, and Treg. The Treg enrichment index and the expression levels of interleukin (IL)-10 and tumor necrosis factor β1 (TGF-β1) were assessed in the high CCR6 expression group of OSCC patients. C57BL/6 mice were randomly assigned to a control group and an experimental group (n = 6 in each group). The control group received a single injection of 100 μL SCC7, a mice head and neck squamous carcinoma cell line, while the experimental group received a single injection of 100 μL mixture of SCC7 cells and *P. gingivalis* in the cheek. After two weeks, the mice were sacrificed, and immunohistochemistry was performed to assess the expression levels of CCR6 and forkhead box protein 3 (FOXP3) in OSCC. Flow cytometry was performed to analyze the effects of *P. gingivalis* on OSCC malignant biological behavior, CCR6$^+$ Treg cells, and the immune microenvironment. **Results** Bioinformatics analysis revealed a correlation between CCL20, CCR6, and Treg (r = 0.373, P < 0.000 1). OSCC patients with high CCR6 expression showed higher Treg enrichment scores and increased IL-10 expression. Animal experiments showed that *P. gingivalis* promoted the increase in the tumor volume (mm^3) (0.294 ± 0.105 in the control group and 0.526 ± 0.101 in the experimental group, P < 0.01) and mass (mg) (206.200 ± 53.950 in the control group and 376.000 ± 119.200 in the experimental group, P < 0.01) in mice with OSCC. Immunohistochemistry confirmed a correlation between CCR6 and FOXP3 (r = 0.659, P < 0.05), and *P. gingivalis* promoted the expression of

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CCR6 and FOXP3. Flow cytometry analysis showed that *P. gingivalis* increased the proportion of $CCR6^+$ Treg (%) (13.780 ± 1.506 in the control group and 18.260 ± 2.257 in the experimental group, $P < 0.01$) and decreased the proportion of $CD8^+$ T cells (%) (27.120 ± 1.647 in the control group and 21.060 ± 3.148 in the experimental group, $P < 0.01$) in OSCC, thereby promoting the formation of a immunosuppressive microenvironment. **Conclusion** *P. gingivalis* promotes the malignant progression of OSCC by recruiting $CCR6^+$ Treg cells to form an immunosuppressive TME.

[Key words] Oral squamous cell carcinoma *Porphyromonas gingivalis* Regulatory T cells Chemokine receptor 6

口腔鳞状细胞癌(oral squamous cell carcinoma, OSCC)占口腔癌发病的90%[1]。OSCC具有高度侵袭性等特点,复发和转移率高,在一次口腔癌发生后存活下来的患者,患二次原发性口腔癌的风险更高[2-3]。在我国,OSCC的发病率每年至少增加1%[4]。目前OSCC首选治疗方法是手术联合术后放化疗,但治疗效果不佳,患者预后差、死亡率高,5年生存率低于50%[5-6]。因此,阐明OSCC的发病机制对发现新的药物靶点,以及探寻OSCC患者新的免疫治疗方法等具有重要的科学意义。

研究表明,细菌与肿瘤的关系密切,其构成复杂的肿瘤生态系统〔即肿瘤微环境(tumor microenvironment, TME)〕在肿瘤的恶性进展中发挥重要作用[7]。既往研究发现口腔内菌群在OSCC患者与正常人间存在显著差异,OSCC肿瘤微环境中富集大量牙龈卟啉单胞菌(*Porphyromonas gingivalis*, *P.gingivalis*, *P.g*)[8]。Meta分析显示,*P. gingivalis*的感染可将罹患OSCC的风险提高1.36倍[9]。这些资料表明*P.gingivalis*在OSCC发生发展过程中起着重要的诱导作用。但是,关于*P. gingivalis*是如何驱动OSCC发生恶性生物学行为的分子机制尚不清楚[10]。

课题组前期研究发现*P. gingivalis*通过感染OSCC细胞促进细胞因子趋化因子配体20(chemokine ligand 20, CCL20)的分泌[11]。CCL20的特异性受体为趋化因子受体6(chemokine receptor 6, CCR6),CCR6主要表达在调节性T细胞(regulatory T cells, Treg)、巨噬细胞、髓源性抑制细胞(myeloid-derived suppressor cells, MDSC)等免疫细胞上[12]。CCL20-CCR6-Treg轴通过促进细胞增殖和迁移以及重塑免疫微环境在癌症和自身免疫性疾病的发展中起着至关重要的作用[13-14]。本研究采用生信分析探究CCR6与相关免疫抑制细胞的分子关系,并构建*P.gingivalis*感染OSCC动物模型,观察肿瘤及内部相关淋巴细胞变化,证明*P.gingivalis*通过$CCR6^+$ Treg对免疫抑制微环境的促进作用,以期为后续治疗提供理论基础。

1 材料与方法

1.1 主要材料

牙龈卟啉单胞菌W83(北纳生物,中国);SCC7小鼠头颈鳞癌细胞系(厦门逸漠生物科技有限公司,中国);Anti-CCR6抗体(武汉三鹰生物公司,中国);Anti-叉头盒蛋白3(forkheadbox protein 3, FOXP3)抗体、二抗(北京博奥森公司,中国);胎牛血清(ExCell Bio公司,中国);RPMI-1640培养基(Lonza公司,美国);胶原酶Ⅳ(SIGMA, C5138),DNA酶Ⅰ(SIGMA, DN25);流式抗体(BioLegend,美国);固定剂Fixation Buffer和破膜剂Permeabilization Buffer(eBioscience,美国)。C57BL/6小鼠购自新疆医科大学动物实验中心,本研究经新疆医科大学第一附属医院动物实验医学伦理委员会审批(审批号:A240301-141)。

1.2 TCGA数据库分析

通过TCGA数据库(https://portal.gdc.cancergov/repository)下载496例头颈部鳞癌(HNSCC)患者肿瘤组织中mRNA表达谱数据,利用R语言(R4.3.1, https://www.r-project.org/)对数据进行提取、整理,以Log_2(FPKM+1)进行数据标准化处理,提取患者CCL20、CCR6、白细胞介素(interleukin, IL)-10及转化生长因子β1(translation growth factorβ1, TGF-β1)表达数据;利用表达谱进行ssGSEA免疫浸润分析,获取OSCC患者肿瘤微环境中Treg富集评分[15]。对CCL20-CCR6和CCR6-Treg表达之间分别进行相关性分析。分析CCR6高表达患者Treg富集评分、IL-10及TGF-β1表达情况。

1.3 细菌、细胞培养

取冻存的SCC7细胞置于含双抗的新鲜培养基中培养,待细胞贴壁、传3~4代后收集、计数。*P.gingivalis*(W83)液体增菌24~48 h后,使用紫外分光光度计在OD_{600}测量细菌浓度,吸取适量菌液5 000 r/min、10 min离心,PBS洗1次,然后重悬于细胞培养基中,根据实验设置MOI=50[16]。

1.4 动物实验

选取6~8周龄标准体质量的C57BL/6雌性小鼠,造模前3 d饲喂四联抗生素水。实验分为对照组和实验组,每组6只。对照组注射1次SCC7鼠源性细胞,实验组注射1次SCC7和牙龈卟啉单胞菌的混悬液,分别于小鼠左侧颊部注射混悬液共100 μL,约2×10^5个细胞[17]。2周后处死小鼠,剥离瘤体组织,记录肿瘤体积、质量,部分瘤体组织

用体积分数为4%多聚甲醛固定，部分瘤体组织用胶原酶Ⅳ、DNA酶Ⅰ进行消化处理，获得单细胞悬液。

1.5　苏木精-伊红染色和免疫组化染色

将石蜡包埋的小鼠OSCC组织切成4 μm。苏木精-伊红染色：苏木精用于细胞核着色，伊红用于细胞质着色。免疫组化染色：过氧化物酶阻断剂处理上述标本的切片，然后用5%山羊血清封闭切片，接着分别与CCR6、FOXP3一抗（均1∶400）在4 ℃孵育过夜，与相应的二抗和抗生物素蛋白-生物素过氧化物酶孵育后，使用DAB试剂盒（中杉金桥，中国）进行可视化，使用Image-Pro Plus测量CCR6与FOXP3的平均光密度值，每张切片随机选择5个视野，取平均值。

1.6　流式分析检测淋巴细胞

取适量1.4部分制备的含有淋巴细胞的单细胞悬液，冰上用CD16/CD32抗体（ThermoFisher，目录号14-0161-82）孵育30 min，以阻断非特异性Fc受体结合。然后将细胞与以下荧光标记的抗小鼠抗体孵育：NK1.1-PE/Cyanine7、CD3-FITC、CD4-Brilliant Violet 605、CD8a-Pacific Blue、CD25-APC/Cyanine7和CCR6-PE/Dazzle 594。转录因子染色，分别使用Fixation Buffer和Permeabilization Buffer配置固定液和破膜液处理淋巴细胞，并用FOXP3-AF647染色。流式细胞仪（BD Biosciences）上机检测。用FlowJovlO软件分析数据。

1.7　统计学方法

采用SPSS 26.0和Graphpad Prism 9.5软件对数据进行统计学处理，方差齐且呈正态分布的计量资料用$\bar{x} \pm s$表示，两组间比较采用独立样本t检验，相关性分析采用Spearman相关性分析，P<0.05为差异有统计学意义。

2　结果

2.1　CCL20与CCR6在免疫抑制微环境形成中发挥重要作用

通过TCGA数据库对496例HNSCC患者肿瘤组织中mRNA表达谱数据分析得出，HNSCC患者中CCL20与CCR6的表达呈正相关（r=0.173 4，P<0.001）（图1A），CCR6的表达与HNSCC患者肿瘤微环境中Treg富集程度呈正相关（r=0.372 6，P<0.000 1）（图1B）。结果表明CCL20-CCR6-Treg在HNSCC中的密切关联。在CCR6高表达患者中，Treg高度富集，同时IL-10表达亦明显升高（图1C、1D）。

2.2　牙龈卟啉单胞菌促进小鼠肿瘤生长

结果见图2。对照组瘤体体积（0.294±0.105）mm^3，实验组体积（0.526±0.101）mm^3；对照组瘤体质量（206.200±

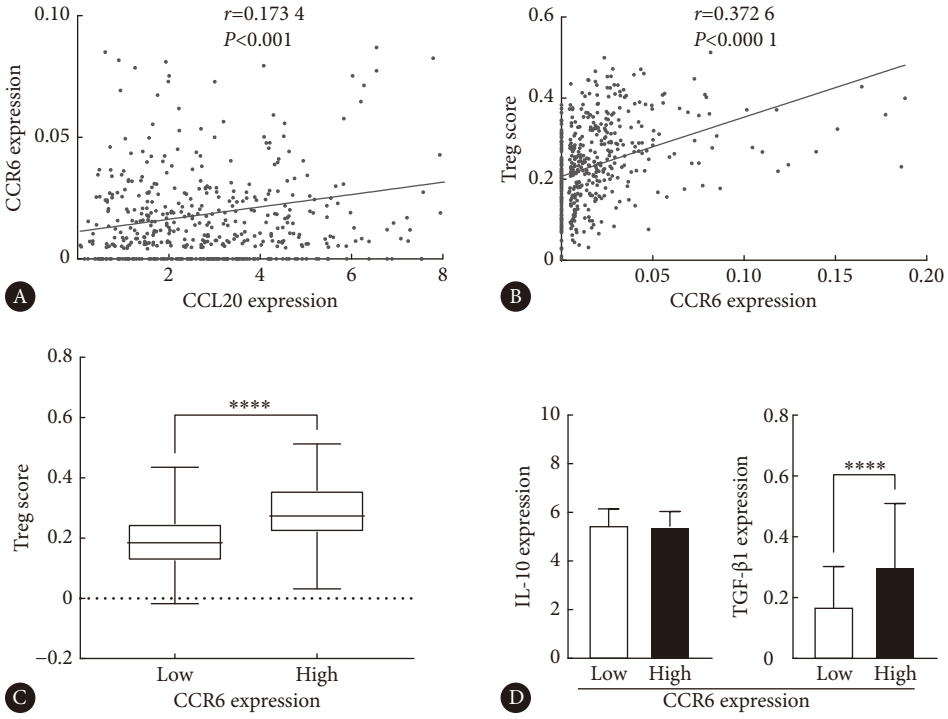

图 1　TCGA数据库分析CCL20-CCR6-Treg与免疫抑制分子间关系

Fig 1　The TCGA database was used to analyze the relationship between CCL20-CCR6-Treg and immunosuppressive molecules.

A, The expression of CCL20 in HNSCC patients was positively correlated with the expression of CCR6 in the TCGA database. B, The expression of CCR6 was significantly correlated with Treg enrichment in tumor tissues. C, Patients with high CCR6 expression had higher Treg enrichment fraction ($^{****}P$ < 0.000 1). D, Patients with high expression of CCR6 had high expression of IL-10 ($^{****}P$ < 0.000 1), but there was no statistical significance in TGF-β1 expression.

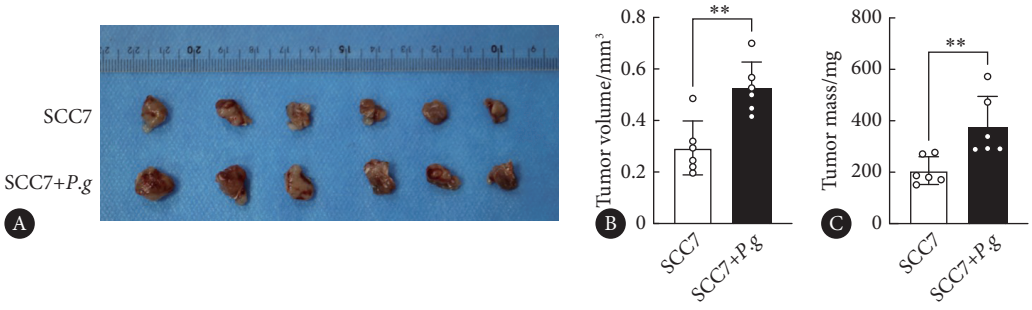

图 2 OSCC小鼠模型中瘤体体积和质量

Fig 2 Tumor volume and mass in the OSCC mice model

A, General diagram of tumor body in the control group and the experimental group. B, The tumor volume of *P. gingivalis* infected mice increased significantly (** $P < 0.01$). C, The tumor mass of *P. gingivalis* infected mice increased significantly (** $P < 0.01$). $n = 6$.

53.950) mg, 实验组质量(376.000±119.200) mg。实验组瘤体体积和质量均大于对照组(P<0.01)。

2.3 CCR6主要通过Treg细胞发挥免疫抑制作用

HE染色可见两组均已成瘤, 实质中含有大量肿瘤细胞, 间质中含有淋巴细胞。免疫组化结果显示: 在小鼠OSCC癌组织中, FOXP3主要表达在OSCC 组织中免疫细胞的细胞核, 而CCR6表达在FOXP3细胞的膜表面(图3A)。CCR6与FOXP3之间有明确的正相关关系(r=0.659, P<0.05)(图3B)。*P. gingivalis*能促进CCR6(P<0.01)与FOXP3表达(P<0.05)(图3C、3D)。

2.4 牙龈卟啉单胞菌促进CCR6$^+$Treg募集形成免疫抑制微环境

对肿瘤微环境中淋巴细胞进行流式检测发现CCR6主要位于CD4$^+$ T细胞上, 并且在CD4$^+$ T中的Treg细胞上占比最多(P<0.001)(图4), *P. gingivalis*能促进Treg、CCR6$^+$ CD4$^+$ T细胞及CCR6$^+$ Treg比例(P<0.01)(图5A ~ 5C)。*P. gingivalis*降低效应CD8$^+$ T细胞在肿瘤组织内浸润淋巴细胞的占比(P<0.01), 抑制其发挥效应功能(图5D)。

3 讨论

新近报道, 肿瘤中不仅有细菌, 而且菌群还存在肿瘤特异性, 围绕肿瘤菌群微环境与肿瘤免疫微环境研究已成为两个热点领域[18-19]。研究发现, 在口腔肿瘤微环境及口腔肿瘤细胞中发现大量*P. gingivalis*, 通过激活PI3K-AKT蛋白导致GSK3β失活, 抑制Snail/Slug通路的信号被

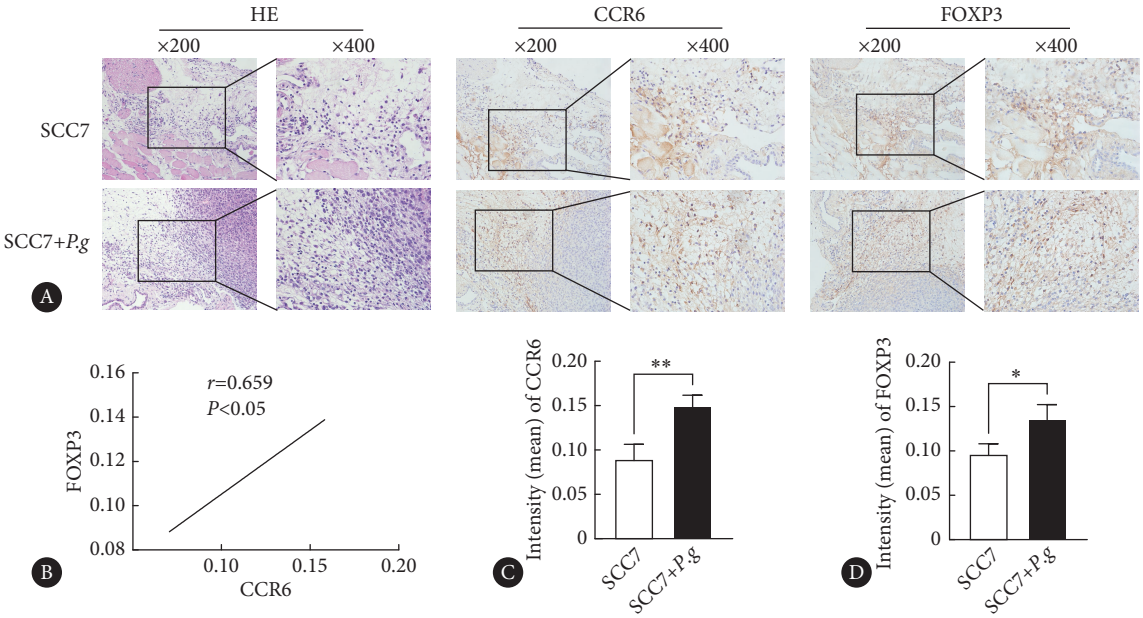

图 3 OSCC模型中CCR6与FOXP3的表达量与相关性

Fig 3 Expression and correlation between CCR6 and FOXP3 in the OSCC model

A, CCR6 is located mainly on the cell membrane and FOXP3 is located on the nucleus. B, There was a clear correlation between CCR6 and FOXP3. C and D, *P. gingivalis* could promote the expression of CCR6 and FOXP3. * $P < 0.05$, ** $P < 0.01$. $n = 6$.

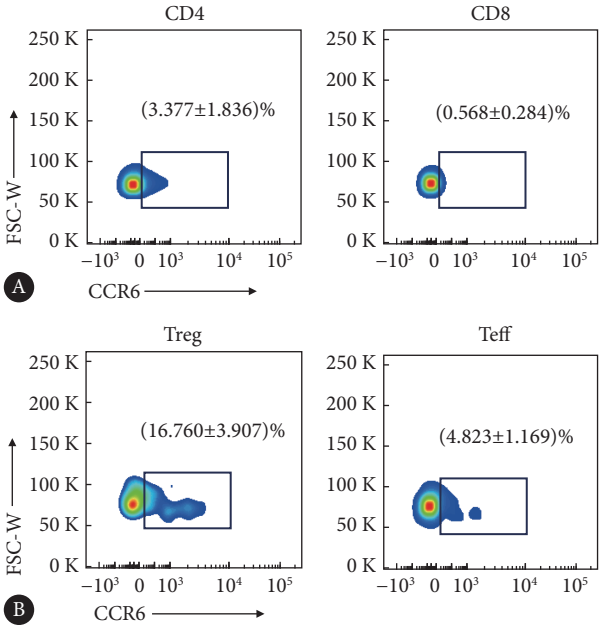

图 4 CCR6主要位于Treg上

Fig 4 CCR6 is mainly located on Treg

A, Compared with CD8$^+$ T cells, CCR6 was mainly expressed in CD4$^+$ T cells ($P < 0.001$). B, In CD4$^+$ T cells, CCR6 was mainly expressed in Treg cells ($P < 0.000\,1$).

解除，促进OSCC向间质化转变，进而发生侵袭、转移等恶性生物学现象[20]。口腔菌群通过介导化学致癌物的直接代谢和全身炎症反应，在促进OSCC的发展中发挥重要作用[21-23]。*P. gingivalis*可引发慢性炎症来削弱局部免疫响应，导致肿瘤细胞的免疫逃逸[24]。*P. gingivalis*通过促进OSCC细胞表达趋化因子（如CCL2和CXCL2）和细胞因子（如IL-6和IL-8），增强口腔癌的侵袭特征，并招募MDSC抑制效应T细胞功能[17, 25]。*P. gingivalis*感染可提高树突状细胞表面细胞程序性死亡-配体1（programmed cell death ligand 1, PD-L1）的表达，抑制CD8$^+$ T细胞的细胞毒性，并加速口腔癌的进展，使用甲硝唑抑制*P. gingivalis*感染后可抑制OSCC恶性进展[26]。本研究使用小鼠颊部原位OSCC模型证实*P. gingivalis*可以增加瘤体体积与质量，促进OSCC恶性生物学行为。因此*P. gingivalis*与OSCC的免疫抑制微环境以及恶性生物学行为关系十分密切。

肿瘤免疫抑制微环境组成包括Treg、MDSC、肿瘤相关中性粒细胞、肿瘤相关巨噬细胞、肿瘤相关成纤维细胞和抑制性细胞因子（TGF-β1、颗粒酶B、IL-10、IL-35等），其中Treg在OSCC中起重要作用。在体内外OSCC研究中发现Treg细胞通过影响 T 细胞功能促进OSCC恶性生物学进展。已有临床报道显示Treg细胞水平与肿瘤恶性程度之间存在正相关[27-28]。本研究通过动物实验证明*P. gingivalis*可以促进Treg细胞在OSCC淋巴细胞中的占比，进而影响肿瘤恶性程度。

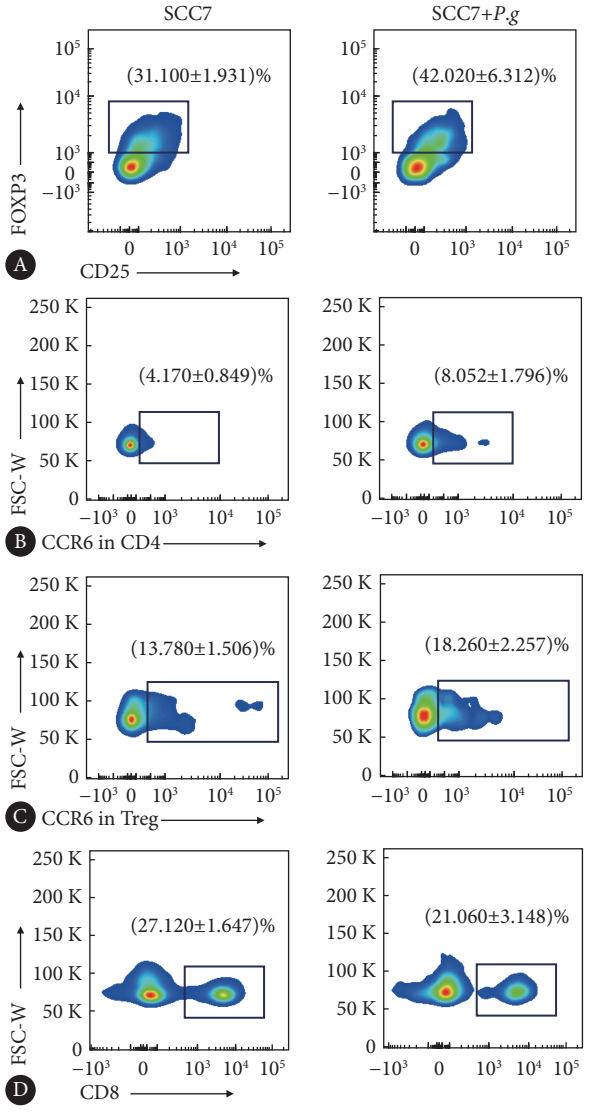

图 5 OSCC模型中CCR6$^+$Treg与相关淋巴细胞占比

Fig 5 The proportion of CCR6$^+$ Treg and the relevant lymphocytes in the OSCC model

A, Treg recruitment increased in the tumor tissues of *P. gingivalis* infected mice ($P < 0.01$). B, The recruitment of CCR6$^+$ CD4$^+$ T cells in the tumor tissues of *P. gingivalis* infected mice increased ($P < 0.01$). C, The recruitment of CCR6$^+$ Treg cells in the tumor tissues of *P.gingivalis* infected mice increased ($P < 0.01$). D, In *P. gingivalis* infected mice tumor tissues, CD8$^+$ T cells decreased ($P < 0.01$).

本课题组前期利用OSCC和癌旁组织转录组测序分析发现，差异表达基因主要富集在CCL20信号相关通路，其中CCL20发挥中心作用，在OSCC微环境呈高表达，与患者生存时间呈负相关。课题组前期体外细胞学实验证实*P. gingivalis*可以进入OSCC细胞内发挥作用，促进CCL20上调表达与分泌[11]。CCL20的唯一受体为CCR6，*P. gingivalis*感染OSCC后，肿瘤微环境中CCR6呈高表达趋势，本研究利用TCGA数据库及免疫组化证明CCR6与Treg之间的密切联系，CCR6主要表达于Treg细胞亚群，可作为组织标签在肿瘤“免疫抑制微环境”中起关键作

用[29-31]。

在肿瘤微环境中CD8$^+$ T细胞作为“守护者”起到免疫监视作用。本研究发现*P. gingivalis*感染OSCC后，肿瘤微环境中CD8$^+$ T细胞减少。这表明*P. gingivalis*通过肿瘤细胞募集CCR6$^+$ Treg细胞，降低效应CD8$^+$ T细胞在肿瘤浸润淋巴细胞中的占比，抑制其发挥效应功能，形成免疫抑制性微环境，促进OSCC恶性表型[32]。

综上，本研究证实了*P. gingivalis*可以通过感染OSCC细胞促进CCR6$^+$ Treg细胞的募集，形成肿瘤免疫抑制性微环境，促进OSCC的恶性发展。这为*P. gingivalis*与OSCC之间关系的深入认识提供了新的思路，也为*P. gingivalis*感染相关OSCC的防治提供了理论基础。然而，本研究并未通过抑制CCR6表达观察其对OSCC免疫抑制微环境形成的作用，也并未对其机制进行探索，均有待于进一步深入研究。

* * *

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Author Contribution XU Liming is responsible for conceptualization, data curation, methodology, visualization, and writing--original draft. TIAN Xiao is responsible for formal analysis and writing--original draft. WANG Jie is responsible for validation. ZHANG Yibo is responsible for visualization. NAIJIBAI·MOMIN is responsible for investigation. LING Bin is responsible for conceptualization, funding acquisition, project administration, supervision, and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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