

Porphyromonas gingivalis promotes the malignant phenotype of oral squamous cell carcinoma through the recruitment of Treg cells

Corresponding Author: Professor Bin Ling

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Regulatory T cells (Tregs) suppress immune responses and contribute to tumor progression by creating an immunosuppressive tumor microenvironment (TME). *Porphyromonas gingivalis* (*P. gingivalis*), a periodontal pathogen, is shown for the first time to recruit Tregs via chemokine secretion from tumor cells. Several groups have identified CCL20 as a core gene linked to poor prognosis of cancers through differential gene expression and survival analysis. Authors through TCGA database analysis confirmed that high CCL20 expression correlates with increased malignancy in HNSCC patients. Clinical data revealed that high *P. gingivalis* expression is associated with poor prognosis and elevated levels of CCL20, CCR6, and Tregs. CCR6, the receptor for CCL20, is highly expressed in malignant HNSCC and predominantly found on Tregs.

The argument that *P. gingivalis* stimulates OSCC cells to secrete CCL20, which recruits Tregs via CCR6, is convincing. However, prior studies have shown that rather than the tumor cells, TME components secrete these chemokines.

Animal experiments showed that *P. gingivalis* increases tumor volume and weight in mice, while CCR6 deficiency reverses this effect. Immunohistochemistry and flow cytometry confirmed that *P. gingivalis* upregulates CCR6 and FOXP3 (a Treg marker), increasing CCR6⁺ Treg recruitment and reducing effector T cell populations.

FOXP3 alone was used as a Treg marker rather than CD4⁺CD25⁺Foxp3⁺CCR6⁺, which should be used to identify Tregs. FoxP3 is the master regulator and a marker of Tregs. However, FoxP3⁺ Tregs are phenotypically and functionally heterogeneous, including both highly suppressive and minimally suppressive Tregs.

Similarly, the usage of CD25 as a marker should also be used with caution. CD4⁺CD25⁺ T cells in mice are generally FoxP3⁺ Tregs, which are heterogeneous and contain both highly and minimally suppressive subsets. However, CD25 can be expressed on activated Tregs and memory T cells, and CD25⁺ T cells can be potent suppressive FoxP3⁺ Tregs. However, the notations of genes and transcription factors are admissible; attention should be paid to distinguish the denoting from mice to human.

Fig 2a/d immunostaining is not convincing. Panels should also include *P. gingivalis* staining.

CCR6 KO mice were not characterized enough. CCR6 is known to regulate the migration of inflammatory and regulatory T cells. CCR6 on Treg cells is essential for their recruitment into inflammatory tissues. Rather than using a global knockdown, a cell-specific knockdown would yield valuable information. Again, all the data collected from CCR6 KO + *P.gingivalis* are missing an untreated CCR6 KO control.

Simple bar graphs are not generally permitted. Authors should superimpose a scatter plot to report the reproducibility of independent biological replicates within such data sets and report mean±S.D. values. Data points on all line graphs should also be shown as the mean±S.D.

P. gingivalis promotes OSCC malignancy by recruiting Tregs through the CCL20–CCR6 axis, contributing to an immunosuppressive microenvironment. Data is needed to determine if this is specific to *P. gingivalis*, interaction/infection dependency, etc., and to compare it with other oral microbes in HNSC patients.

Reviewer #2

(Remarks to the Author)

This manuscript describes that *P. gingivalis* promotes OSCC progression by inducing CCL20 secretion in tumor cells, which recruits CCR6+ regulatory T cells (Tregs) and establishes an immunosuppressive microenvironment. Both clinical and experimental data demonstrate that this effect is mediated through the CCL20–CCR6–Treg axis and can be reversed by CCR6 deficiency. However, all supporting information is not attached. Therefore, it is difficult to evaluate the manuscript as a whole. Authors should submit these files. There are some points that have to be addressed to make this manuscript. Here are the specific comments, excluding supporting information,

Line25 and 26

CCI20, TCGA and HNSCC might be written open in the first place in this abstract.

Line58-62

The appreciate references should be cited against in this sentence.

Linee123

A TCGA and GEO might be written open in the first place

Line142

IHC should be written open in the first place.

Line167 and 168

What are the origin of CAL27, SCC25, and SCC7 cells?

Line189, 208 210

Authors might include the manufacturer information of the Transwell plates, PrimeScript RT reagent kit, SYBR Premix Ex Taq II, Agilent Mx3005P system, in each sentence.

Line221

Where were C57BL/6 mice and CCLKO mice used in the experiments obtained from?

Line238

Authors might include the manufacturer information of the FlowJo v10 software in the sentence.

Line251-253 Figure1a

From the results shown in Fig. 1a, how can it be concluded that 'with interactions between cancer cells and lymphocytes promoting the malignant progression of OSCC'?

Line307-308

Authors should cite the appreciate references in this sentence.

Line314-316

Are the expression sites of CCL20, CCR6, and FOXP3 consistent with those of *P. gingivalis*? To address this, the authors might consider adding IHC staining with a *P. gingivalis* antibody in Figure 2c. If the expression patterns overlap, this would facilitate interpretation; if they do not, the authors should discuss the reasons underlying this discrepancy.

Line358-361

The authors should discuss whether the increase in CCL20 expression following bacterial infection, which subsequently promotes the migration of CD4⁺CD25⁺CD127[−] Tregs, is a phenomenon observed in other tissues as well, or if it is specific to *P. gingivalis*. The appropriate references should be cited to support this discussion.

Line382-384

The authors should discuss why body weight decreases in CCL6 KO mice following *P. gingivalis* infection in discussion section or results section.

Line394-397

"Is the phenomenon whereby *P. gingivalis* promotes the recruitment and proliferation of CCR6⁺ Tregs specific to *P. gingivalis*? The authors should discuss these findings cited with the appreciate references. In addition, are these phenomena independent of the effects of CCL20? If possible, authors might do additional experiments to clarify the potential involvement of CCL20 and provide supporting evidence.

Line421

Teff should be written open in the first place.

"

Line423-424 "The suppressive function of Treg ... TGF- β 1 and IL-10."

The appreciate references should be cited in this sentence.

Line424-433

In Figure5e-f, TGF- β 1 expression in OSCC showed a positive correlation with CCL20, but no statistically significant association was observed with CCR6. Why might this be the case? Is such a phenomenon also observed in other types of carcinoma? The authors might discuss these points. In addition, the authors might indicate *P. gingivalis* antibody staining data in Figures 5e and 5i.

Line475

The authors might describe a brief explanation of the FOLFOX regimen.

Line485-486 "Periodontitis is considered to and, if left untreated,"

Line492-496 "hang et al. examined cancerous tissue..... occurring in the gingiva."

Line515-517 "Treg is an importantinhibitory effect T cells."

The appreciate references should be cited in those sentences.

Line533

All supporting information is not attached. Authors should submit these files. Therefore, it is difficult to evaluate the manuscript as a whole.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

P. gingivalis promotes oral squamous cell carcinoma (OSCC) malignancy by attracting Tregs through the CCL20–CCR6 pathway, contributing to an immunosuppressive microenvironment. Further research is necessary to determine whether this effect is specific to *P. gingivalis*, depends on interactions or infections, and how it compares to other oral microbes in HNSC patients. Tregs suppress immune responses and aid tumor growth by creating an immunosuppressive tumor microenvironment (TME). *Porphyromonas gingivalis* (*P. gingivalis*), a periodontal pathogen, is shown for the first time to recruit Tregs via chemokine secretion from tumor cells. Multiple studies have identified CCL20 as a key gene linked to poor cancer prognosis, based on differential gene expression and survival data. Analysis of the TCGA database confirms that high CCL20 levels are associated with increased malignancy in HNSCC patients. Clinical data also indicate that high *P. gingivalis* levels correspond with worse prognosis and higher levels of CCL20, CCR6, and Tregs. CCR6, the receptor for CCL20, is highly expressed in malignant HNSCC and is mainly found on Tregs. The authors have addressed previous criticisms and now provide a stronger argument that *P. gingivalis* promotes OSCC cell secretion of CCL20, which then recruits Tregs via CCR6.

Reviewer #2

(Remarks to the Author)

The authors have upgraded the information within the text and came up with better interpretation of the results. I feel that the manuscript improved after the corrections made according to the comments. However, there are still some points that need to be reviewed.

1. CAL27, SCC25, and SCC7 cells

The authors may have intended to describe the origin of the CAL27, SCC25, and SCC7 cells; however, the current description only indicates where the cells were obtained from. The authors should specify the tumor origin of these cell lines (e.g., tongue cancer, gingival cancer, etc.).

2. From author's rebutting letter:

Relevant studies have shown that there is a large amount of *P. gingivalis* in OSCC, which affects the tumor or immune microenvironment and is associated with the poor prognosis of OSCC patients [1-3]. The authors believe that this is a research advantage that other oral microbiota do not have compared to *P. gingivalis*.

We firmly believe that conducting a systematic comparison with other oral microorganisms (such as *F. nucleatum*, *S. gordonii*, etc.) is the next crucial step to verify the specificity of this pathway and identify its unique driving factors.

We understand well the authors' perspective and the intention of their investigation. However, recent studies indicate that not only *P. gingivalis* but also *Fusobacterium nucleatum* cannot be overlooked in the context of OSCC. Numerous reports have highlighted its potential involvement in tumor progression and modulation of the tumor microenvironment. Therefore,

the authors should reconsider their discussion taking this point into account. At the very least, the role of *Fusobacterium nucleatum* should be included and discussed in the Discussion section.

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Dear reviewers,

Thank you very much for providing chance to revise our manuscript entitled “*Porphyromonas gingivalis* promotes the malignant phenotype of oral squamous cell carcinoma through the recruitment of Treg cell” (Manuscript ID: COMMSBIO-25-9115-T). We also thank reviewers for their efforts with thoughtful comments. In this revised version, we performed additional experiments and provided more convincing evidence, which specifically address the issues raised by Reviewers. We believe that this revised paper addressed all concerns, and the quality of the article has been substantially increased. Below, there is a point-by-point response on how the manuscript has been revised.

If any other information is required, please feel free to contact us.

With my best regards,

Sincerely yours,

Bin Ling

Reviewer: 1

Comments to the Author:

1. The argument that *P. gingivalis* stimulates OSCC cells to secrete CCL20, which recruits Tregs via CCR6, is convincing. However, prior studies have shown that rather than the tumor cells, TME components secrete these chemokines.

Response: Thank you very much for your profound and crucial comment. Your point that "stromal cells in the tumor microenvironment are the main source of chemokines" is completely correct. We fully agree that stromal components such as cancer-associated fibroblasts and tumor-associated macrophages play a key role in secreting chemokines and shaping an immunosuppressive microenvironment.

In our research, we focused on the direct response of oral squamous cell carcinoma cell (OSCC) to the specific pathogen *P. gingivalis*. The model we proposed aims to emphasize that in the specific pathological context of "bacteria-tumor cell" direct interaction, cancer cells themselves have been proven to be an important and functional active producer of CCL20. In recent years, multiple high-quality studies have confirmed this mechanism in various solid tumors, providing a solid theoretical and experimental basis for our research hypothesis. In Colorectal cancer (CRC) patients with chemotherapy resistance, the tumor cells themselves are the main source of CCL20. Under the stimulation of 5-FU chemotherapy, CRC cells upregulate the expression of CCL20 by activating the FOXO1/CEBPB/NF- κ B signaling pathway. The CCL20 derived from CRC cells directly recruits CCR6⁺ regulatory T cell (Treg), thereby promoting the chemotherapy resistance of the tumor ^[1]. The study published in Cell Death and Disease in 2024 further strengthened this concept: The RANKL/RANK signaling pathway can directly activate the NF- κ B pathway in CRC cells; the activated NF- κ B (p65) directly binds to the promoter region of CCL20, driving its transcription and secretion ^[2]. This review, published in the International Journal of Molecular Sciences in 2020, systematically summarizes the role of the CCL20-CCR6 axis in cancer, and clearly states: "... CCL20 is also predominantly expressed in the stroma of cervical cancer tissue..." However, at the same time, this review, while summarizing various types of cancers, also points out that multiple tumor cells such as colorectal cancer, liver cancer, pancreatic cancer, and head and neck cancer can themselves express CCL20. This provides a broad theoretical background and support for the statement that "epithelial cancer cells are an important source of CCL20" ^[3].

IHC experiments demonstrated high expression of CCL20 in the parenchyma of

OSCC (Fig 2c). The in vitro co-culture system was designed to precisely analyze the direct and autonomous response of OSCC cells to the stimulation of *P. gingivalis* (Fig 3b-c).

In addition, we strongly hope that our answers below will address all the reviewer's concerns and that our modifications will improve the quality of the paper and allow it to be published as soon as possible.

References

- [1] Wang D, Yang L, Yu W, et al. Colorectal cancer cell-derived CCL20 recruits regulatory T cells to promote chemoresistance via FOXO1/CEBPB/NF- κ B signaling. *J Immunother Cancer*. 2019 Aug 8;7(1):215. doi: 10.1186/s40425-019-0701-2.
- [2] Ouyang J, Hu S, Zhu Q, et al. RANKL/RANK signaling recruits Tregs via the CCL20-CCR6 pathway and promotes stemness and metastasis in colorectal cancer. *Cell Death Dis*. 2024 Jun 20;15(6):437. doi: 10.1038/s41419-024-06806-3.
- [3] Kadomoto S, Izumi K, Mizokami A. The CCL20-CCR6 Axis in Cancer Progression. *Int J Mol Sci*. 2020 Jul 22;21(15):5186. doi: 10.3390/ijms21155186.

2. Animal experiments showed that *P. gingivalis* increases tumor volume and weight in mice, while CCR6 deficiency reverses this effect. Immunohistochemistry and flow cytometry confirmed that *P. gingivalis* upregulates CCR6 and FOXP3 (a Treg marker), increasing CCR6⁺ Treg recruitment and reducing effector T cell populations.

FOXP3 alone was used as a Treg marker rather than CD4⁺CD25⁺Foxp3⁺CCR6⁺, which should be used to identify Tregs. FoxP3 is the master regulator and a marker of Tregs. However, FoxP3⁺ Tregs are phenotypically and functionally heterogeneous, including both highly suppressive and minimally suppressive Tregs.

Similarly, the usage of CD25 as a marker should also be used with caution. CD4⁺CD25⁺ T cells in mice are generally FoxP3⁺ Tregs, which are heterogeneous and contain both highly and minimally suppressive subsets. However, CD25 can be expressed on activated Teffs and memory T cells, and CD25⁺T cells can be potent suppressive FoxP3⁺ Tregs. However, the notations of genes and transcription factors are admissible; attention should be paid to distinguish the denoting from mice to human.

Response: We are grateful for the important comments provided by the reviewers, and have made the necessary revisions according to your suggestions..

3. Fig 2a/d immunostaining is not convincing. Panels should also include *P. gingivalis* staining.

Response: We are grateful for the important comments provided by the reviewers, and have included *P. gingivalis* staining in S8 Fig.

4. CCR6 KO mice were not characterized enough. CCR6 is known to regulate the migration of inflammatory and regulatory T cells. CCR6 on Treg cells is essential for their recruitment into inflammatory tissues. Rather than using a global knockdown, a cell-specific knockdown would yield valuable information. Again, all the data collected from CCR6 KO + *P.gingivalis* are missing an untreated CCR6 KO control.

Response: In this study, the primary purpose of using global CCR6 knockout mice was to, at the proof-of-concept level, test whether blocking the CCR6/CCL20 axis, which constitutes an overall pathway, could effectively reverse the tumorigenic effect of *P. gingivalis*. This result provided preliminary proof of principle for targeting this pathway as a potential therapeutic strategy. We fully agree with the reviewers' viewpoints. The use of CCR6 knockout mice with systemic knockout is indeed an important limitation of this study. We thank the reviewers for suggesting the use of a cell-specific knockout model, which is a more precise method. We acknowledge that as the reviewers stated, CCR6 is expressed on various immune cells and coordinates their coordinated migration. Therefore, the phenotype observed in our systemic KO model is the net effect of the complete loss of CCR6 function in multiple cell types, and cannot be directly attributed to Treg. However, in the relevant results section, we found that CCR6 is mainly expressed on Treg cells. This will help explain which cells CCR6 deficiency may affect. In the future, the research group will commit to using conditional knockout mice to specifically eliminate CCR6 on Treg cells, which will be the necessary and logical next step to verify the findings of this study.

Additionally, We added an untreated CCR6 knockout control.

5. Simple bar graphs are not generally permitted. Authors should superimpose a scatter plot to report the reproducibility of independent biological replicates within such data sets and report mean $\pm$ S.D. values. Data points on all line graphs should also be shown as the mean $\pm$ S.D.

Response: We are grateful for the crucial feedback provided by the reviewers. We fully

agree that presenting individual data points in the charts is the best practice for demonstrating data reproducibility and transparency. We apologize for not adopting this standard previously. We have systematically reviewed and revised all the relevant data graphs in the manuscript according to your suggestions.

6. *P. gingivalis* promotes OSCC malignancy by recruiting Tregs through the CCL20–CCR6 axis, contributing to an immunosuppressive microenvironment. Data is needed to determine if this is specific to *P. gingivalis*, interaction/infection dependency, etc., and to compare it with other oral microbes in HNSC patients.

Response: Thank you very much for your extremely insightful suggestion. The issues you pointed out regarding mechanism specificity, interaction dependence, and comparison with other oral microorganisms are crucial for placing our findings within the broader field of tumor microbiome. We fully agree that clarifying these aspects will greatly deepen our understanding of how microorganisms shape the tumor immune microenvironment.

Relevant studies have shown that there is a large amount of *P. gingivalis* in OSCC, which affects the tumor or immune microenvironment and is associated with the poor prognosis of OSCC patients [1-3]. The authors believe that this is a research advantage that other oral microbiota do not have compared to *P. gingivalis*. Relevant paper has proved that other oral microbiota such as *Streptococcus mutans* (a common oral microorganism) have no significant association with the occurrence and development of OSCC [4]. Therefore, this article did not compare with other oral microorganisms.

Although we do not currently have direct comparisons of various microorganisms, our existing data provide strong indirect evidence for the specific association of this pathway with *P. gingivalis* and its infection dependence: a. Specific implication: In our in vitro experiments, OSCC cells exhibited a significant and sustained increase in CCL20 expression when co-cultured with *P. gingivalis*. This strong response suggests that *P. gingivalis* may possess special virulence factors (such as its unique lipopolysaccharides, fimbriae, or proteases) that are not present in other microorganisms and can efficiently activate the expression of host CCL20; b. Infection-

dependent evidence: Our animal experimental data strongly support its dependence. In immunocompetent mouse models, the deletion of the CCR6 gene completely reversed the tumorigenic effect of *P. gingivalis*. This key result indicates that the tumorigenic function of *P. gingivalis* is not simply achieved indirectly through chronic inflammation, but is highly dependent on its ability to recruit CCR6⁺ Treg. If this were a non-specific process unrelated to infection, the absence of CCR6 should not have such a decisive impact.

We firmly believe that conducting a systematic comparison with other oral microorganisms (such as *F. nucleatum*, *S. gordonii*, etc.) is the next crucial step to verify the specificity of this pathway and identify its unique driving factors. Based on your valuable suggestions, we have added an independent paragraph in the discussion section of the paper to specifically explore this important future direction.

Thank you again for guiding us to think about this deeper issue. Through the above modifications, we have explicitly acknowledged the limitations of the current research in the paper and established "comparison of microbial specificity and mechanisms" as a clear and important future research agenda. We believe that this addition significantly enhances the academic value of our research and provides a clear roadmap for subsequent work in the field.

[1] Lafuente Ibáñez de Mendoza I, Maritxalar Mendia X, García de la Fuente AM, et al. Role of Porphyromonas gingivalis in oral squamous cell carcinoma development: A systematic review. J Periodontal Res. 2020 Jan;55(1):13-22. doi: 10.1111/jre.12691.

[2] Guo ZC, Jing SL, Jumatai S, et al. Porphyromonas gingivalis promotes the progression of oral squamous cell carcinoma by activating the neutrophil chemotaxis in the tumour microenvironment. Cancer Immunol Immunother. 2023 Jun;72(6):1523-1539. doi: 10.1007/s00262-022-03348-5.

[3] Lan Z, Zou KL, Cui H, et al. Porphyromonas gingivalis suppresses oral squamous cell carcinoma progression by inhibiting MUC1 expression and remodeling the tumor microenvironment. Mol Oncol. 2024 May;18(5):1174-1188. doi: 10.1002/1878-0261.13517

[4] Wen L, Mu W, Lu H, et al. *Porphyromonas gingivalis* Promotes Oral Squamous

Reviewer 2

Comments to the Author:

1.However, all supporting information is not attached. Therefore, it is difficult to evaluate the manuscript as a whole. Authors should submit these files. There are some points that have to be addressed to make this manuscript. Here are the specific comments, excluding supporting information,

Response: all supporting information has been attached.

2.Line25 and 26

CCl20, TCGA and HNSCC might be written open in the first place in this abstract.

Response: According to the reviewer's suggestion, CCl20, TCGA and HNSCC have been written open in the first place in this abstract in the revised manuscript.

3.Line58-62

The appreciate references should be cited against in this sentence.

Response: According to the reviewer's suggestion, we use this paper (Oral microbiota and metabolites: key players in oral health and disorder, and microbiota-based therapies. Front Microbiol) as the basis for this sentence.

4.Linee123

A TCGA and GEO might be written open in the first place

Response: According to the reviewer's suggestion, TCGA and GEO have been written open in the first place in this abstract in the revised manuscript.

5.Line142

IHC should be written open in the first place.

Response: According to the reviewer's suggestion, IHC has been written open in the first place in this abstract in the revised manuscript.

6.Line167 and 168

What are the origin of CAL27, SCC25, and SCC7 cells?

Response: Thank you very much for raising this important question regarding the

origin of the cell lines. We have included this information in the revised manuscript.

7.Line189, 208 210

Authors might include the manufacturer information of the Transwell plates, PrimeScript RT reagent kit, SYBR Premix Ex Taq II, Agilent Mx3005P system, in each sentence.

Response: Thank you very much for raising this important question. We have included this information in the revised manuscript.

8.Line221

Where were C57BL/6 mice and CCLKO mice used in the experiments obtained from?

Response: Wild-type C57BL/6 mice were procured from the Animal Experiment Center of Xinjiang Medical University. CCR6 knockout mice (strain number: T006115), generated on a C57BL/6 genetic background, were obtained from Jicui Yao Kang Biotechnology Co., Ltd (Jiangsu, China). We have added the above detailed information, including the supplier name and the background of the animal strain, in the revised version to ensure the reproducibility of the experiment.

9.Line238

Authors might include the manufacturer information of the FlowJo v10 software in the sentence.

Response: Thank you very much for raising this important question. We have included this information in the revised manuscript.

10.Line251-253 Figure1a

Response: Thank you very much for your extremely important suggestion. You are absolutely correct. Based solely on the morphological observation of H&E staining (Fig. 1a), it is indeed insufficient to directly draw the functional conclusion that "the interaction between cancer cells and lymphocytes promotes the malignant progression of OSCC". We apologize for the overly direct statement in the original text that might have caused misunderstandings.

The HE staining revealed significant lymphocyte infiltration in the OSCC tumor tissue

(Tumor-Infiltrating Lymphocytes, TILs). This morphological feature is recognized in the field as indirect evidence of an active interaction between the tumor and the immune system. Based on this phenomenon, we conducted relevant research to prove this conclusion. Therefore, the conclusion that "the interaction promotes malignant progression" is not derived from the single observation in Fig. 1a, but is obtained through a comprehensive chain of evidence based on the hypothesis proposed after analyzing the phenomenon presented in Fig. 1a, and through a series of functional experiments. Fig. 1a is the logical starting point of the entire study.

According to your suggestion, we have revised the expression in the original text related to the conclusion of Fig. 1a. We believe that through the above clarification and manuscript modification, the logical chain of the study has become clearer and more rigorous. Once again, we thank you for helping us improve the argumentation quality of the paper.

11.Line307-308

Authors should cite the appreciate references in this sentence.

Response: Thank you very much for raising this important question. We have cited the appreciate reference in this sentence.

12.Line314-316

Are the expression sites of CCL20, CCR6, and FOXP3 consistent with those of *P. gingivalis*? To address this, the authors might consider adding IHC staining with a *P. gingivalis* antibody in Figure 2c. If the expression patterns overlap, this would facilitate interpretation; if they do not, the authors should discuss the reasons underlying this discrepancy.

Response: Thank you very much for raising this important question. The spatial distribution of *P. gingivalis* was found to be distinct from that of CCL20, CCR6, and FOXP3. While *P. gingivalis* was mainly confined to the tumor epithelium—consistent with its ability to invade the epithelial barrier—its downstream effector, CCL20, was widely distributed throughout the tissue. In contrast, a consistent spatial colocalization

was observed between CCR6 and FOXP3. These differential expression patterns were robustly verified. We have included *P. gingivalis* staining in S8 Fig and provided relevant explanations during our discussion.

14.Line382-384

The authors should discuss why body weight decreases in CCL6 KO mice following *P. gingivalis* infection in discussion section or results section.

Response: Thank you for the valuable comments from the reviewers. The body weight of CCR6 knockout mice after tumor implantation showed a decreasing trend compared to the WT mice. This was because the tumor mass in WT mice increased, leading to an overall weight gain. In fact, there was no statistically significant difference in body weight between WT and CCR6 knockout mice after tumor implantation.

13.Line358-361

The authors should discuss whether the increase in CCL20 expression following bacterial infection, which subsequently promotes the migration of CD4⁺CD25⁺CD127⁻ Tregs, is a phenomenon observed in other tissues as well, or if it is specific to *P. gingivalis*. The appropriate references should be cited to support this discussion.

15.Line394-397

"Is the phenomenon whereby *P. gingivalis* promotes the recruitment and proliferation of CCR6⁺ Tregs specific to *P. gingivalis* ? The authors should discuss these findings cited with the appropriate references. In addition, are these phenomena independent of the effects of CCL20? If possible, authors might do additional experiments to clarify the potential involvement of CCL20 and provide supporting evidence.

Response: Thank you very much for your extremely insightful suggestion. Since both of these suggestions are related to the specificity of *P. gingivalis*, we will explain and discuss them together. The issues you pointed out regarding mechanism specificity, interaction dependence, and comparison with other oral microorganisms are crucial for placing our findings within the broader field of tumor microbiome. We fully agree that clarifying these aspects will greatly deepen our understanding of how microorganisms

shape the tumor immune microenvironment.

Relevant studies have shown that there is a large amount of *P. gingivalis* in OSCC, which affects the tumor or immune microenvironment and is associated with the poor prognosis of OSCC patients ^[1-3]. The authors believe that this is a research advantage that other oral microbiota do not have compared to *P. gingivalis*. Relevant paper has proved that other oral microbiota such as *Streptococcus mutans* (a common oral microorganism) have no significant association with the occurrence and development of OSCC ^[4]. Therefore, this article did not compare with other oral microorganisms.

Although we do not currently have direct comparisons of various microorganisms, our existing data provide strong indirect evidence for the specific association of this pathway with *P. gingivalis* and its infection dependence: a. Specific implication: In our in vitro experiments, OSCC cells exhibited a significant and sustained increase in CCL20 expression when co-cultured with *P. gingivalis*. This strong response suggests that *P. gingivalis* may possess special virulence factors (such as its unique lipopolysaccharides, fimbriae, or proteases) that are not present in other microorganisms and can efficiently activate the expression of host CCL20; b. Infection-dependent evidence: Our animal experimental data strongly support its dependence. In immunocompetent mouse models, the deletion of the CCR6 gene completely reversed the tumorigenic effect of *P. gingivalis*. This key result indicates that the tumorigenic function of *P. gingivalis* is not simply achieved indirectly through chronic inflammation, but is highly dependent on its ability to recruit CCR6⁺ Treg. If this were a non-specific process unrelated to infection, the absence of CCR6 should not have such a decisive impact.

We firmly believe that conducting a systematic comparison with other oral microorganisms (such as *F. nucleatum*, *S. gordonii*, etc.) is the next crucial step to verify the specificity of this pathway and identify its unique driving factors. Based on your valuable suggestions, we have added an independent paragraph in the discussion section of the paper to specifically explore this important future direction.

Thank you again for guiding us to think about this deeper issue. Through the above modifications, we have explicitly acknowledged the limitations of the current research

in the paper and established "comparison of microbial specificity and mechanisms" as a clear and important future research agenda. We believe that this addition significantly enhances the academic value of our research and provides a clear roadmap for subsequent work in the field.

Other studies mainly focused on the periodontal tissues, mainly investigating the imbalance in the ratio of Th17 to Treg cells caused by *P. gingivalis* infection^[5-6].

In the previous stage of the research group's work, we conducted Transwell to observe whether individual *P. gingivalis* could cause Treg cell migration. We found that lymphocytes rarely entered the lower chamber and thus were unable to conduct the subsequent experiments. Moreover, *P. gingivalis* is an intracellular bacterium, so our experiments demonstrated that *P. gingivalis* induces Treg cell recruitment through tumor cells.

[1] Lafuente Ibáñez de Mendoza I, Maritxalar Mendia X, García de la Fuente AM, et al. Role of Porphyromonas gingivalis in oral squamous cell carcinoma development: A systematic review. J Periodontal Res. 2020 Jan;55(1):13-22. doi: 10.1111/jre.12691.

[2] Guo ZC, Jing SL, Jumatai S, et al. Porphyromonas gingivalis promotes the progression of oral squamous cell carcinoma by activating the neutrophil chemotaxis in the tumour microenvironment. Cancer Immunol Immunother. 2023 Jun;72(6):1523-1539. doi: 10.1007/s00262-022-03348-5.

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[4] Wen L, Mu W, Lu H, et al. Porphyromonas gingivalis Promotes Oral Squamous Cell Carcinoma Progression in an Immune Microenvironment. J Dent Res. 2020 Jun;99(6):666-675. doi: 10.1177/0022034520909312.

[5] Zhang X, Zhang X, Qiu C, et al. The imbalance of Th17/Treg via STAT3 activation modulates cognitive impairment in P. gingivalis LPS-induced periodontitis mice. J Leukoc Biol. 2021 Sep;110(3):511-524. doi: 10.1002/JLB.3MA0521-742RRR.

[6] Hsiao PY, Huang RY, Huang LW, et al. MyD88 exacerbates inflammation-induced

bone loss by modulating dynamic equilibrium between Th17/Treg cells and subgingival microbiota dysbiosis. J Periodontol. 2024 Aug;95(8):764-777. doi: 10.1002/JPER.23-0561.

16.Line421

Teff should be written open in the first place.

Response: According to the reviewer's suggestion, Teff have been written open in the first place in this abstract in the revised manuscript.

17.Line423-424 “The suppressive function of Treg ... TGF- β 1 and IL-10.”

The appreciate references should be cited in this sentence.

Response: Thank you very much for raising this important question. We have cited the appreciate reference in this sentence.

18.Line424-433

In Figure5e-f, TGF- β 1 expression in OSCC showed a positive correlation with CCL20, but no statistically significant association was observed with CCR6. Why might this be the case? Is such a phenomenon also observed in other types of carcinoma? The authors might discuss these points. In addition, the authors might indicate P. gingivalis antibody staining data in Figures 5e and 5i.

Response: We are very grateful for your valuable suggestion. The expression of TGF- β 1 in OSCC is positively correlated with CCL20, but no statistically significant association was observed with CCR6. We can observe in Fig 5f that the expression of TGF- β 1 is less than that of IL-10, and no effective statistical correlation was formed. In related studies, CCR6 is closely related to Treg function, but the association between CCR6 and TGF- β 1 is rarely demonstrated. Additionally, we have included P. gingivalis staining in S8 Fig.

19.Line475

The authors might describe a brief explanation of the FOLFOX regimen.

Response: We are very grateful for your valuable suggestion. We agree that providing a brief description of the FOLFOX protocol will make the paper more accessible to a wider audience. We have taken your advice into account and added a brief explanation in parentheses at the first occurrence of the FOLFOX protocol in the manuscript: "... patients resistant to the FOLFOX regimen (a standard first-line chemotherapy for colorectal cancer combining 5-fluorouracil, leucovorin, and oxaliplatin) ... ". We believe that this addition will effectively enhance the clarity of the relevant background information. Once again, we would like to express our gratitude for your careful review.

20.Line485-486 "Periodontitis is considered to and, if left untreated,"

Line492-496 "hang et al. examined cancerous tissue..... occurring in the gingiva."

Line515-517 "Treg is an importantinhibitory effect T cells."

The appreciate references should be cited in those sentences.

Response: Thank you very much for raising this important question. We have cited the appreciate references in those sentences.

21.Line533

All supporting information is not attached. Authors should submit these files. Therefore, it is difficult to evaluate the manuscript as a whole.

Response: Thank you very much for your reminder. We sincerely apologize for having overlooked the submission of the supporting information files during our initial submission. This was entirely due to our negligence and has caused inconvenience to you and the reviewers. Now, we have prepared all the necessary supporting information files and have resubmitted them through the submission system.

Dear reviewers,

Thank you very much for providing chance to revise our manuscript entitled “*Porphyromonas gingivalis* promotes the malignant phenotype of oral squamous cell carcinoma through the recruitment of Treg cells” (Manuscript ID: COMMSBIO-25-9115A). We also thank reviewers for their efforts with thoughtful comments. In this revised version, we provided more convincing evidence, which specifically address the issues raised by Reviewers. We believe that this revised paper addressed all concerns, and the quality of the article has been substantially increased. Below, there is a point-by-point response on how the manuscript has been revised.

If any other information is required, please feel free to contact us.

With my best regards,

Sincerely yours,

Bin Ling

Reviewer: 2

Comments to the Author:

1. CAL27, SCC25, and SCC7 cells

The authors may have intended to describe the origin of the CAL27, SCC25, and SCC7 cells; however, the current description only indicates where the cells were obtained from. The authors should specify the tumor origin of these cell lines (e.g., tongue cancer, gingival cancer, etc.).

Response: We agree with the reviewer's valuable comment. To address this concern, we have supplemented the detailed tumor origin information of CAL27, SCC25 and SCC7 cell lines in the revised manuscript. The specific tissue origin (human tongue squamous cell carcinoma and mouse head and neck squamous cell carcinoma) of each cell line has been clearly described in the revised section to make the cell source information more complete and accurate.

2. We understand well the authors' perspective and the intention of their investigation. However, recent studies indicate that not only *P. gingivalis* but also *Fusobacterium nucleatum* cannot be overlooked in the context of OSCC. Numerous reports have highlighted its potential involvement in tumor progression and modulation of the tumor microenvironment. Therefore, the authors should reconsider their discussion taking this point into account. At the very least, the role of *Fusobacterium nucleatum* should be included and discussed in the Discussion section.

Response: We highly appreciate the reviewer for pointing out this valuable and insightful comment. We fully agree that *Fusobacterium nucleatum* plays a critical and non-negligible role in the occurrence and progression of oral squamous cell carcinoma (OSCC). Accumulating evidence has confirmed that *F. nucleatum* can regulate the tumor microenvironment, promote malignant proliferation, invasion and metastasis of OSCC cells, and mediate immune suppression and chemoresistance.

In accordance with your suggestion, we have supplemented and comprehensively discussed the research progress and potential oncogenic mechanisms of *F. nucleatum* in OSCC in the **Discussion section** of the revised manuscript. Meanwhile, we have also objectively compared the synergistic and independent roles of *Porphyromonas gingivalis* and *F. nucleatum* in OSCC progression, to make our discussion more comprehensive, systematic and objective.

References

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- [2] Pignatelli P, Nuccio F, Piattelli A, Curia MC. The Role of Fusobacterium nucleatum in Oral and Colorectal Carcinogenesis. Microorganisms. 2023 Sep 20;11(9):2358.