nature portfolio

Peer Review File

Single-cell RNA sequencing dissects the immunosuppressive signatures in Helicobacter pylori-infected human gastric ecosystem

Corresponding Author: Professor Wei Gong

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)
The article, "Single-cell RNA sequencing dissects the immunosuppressive signatures in Helicobacter pylori-infected gastric ecosystem" by Wei Hu et al. is exploring an important aspect of infectious disease biology. H. Pylori is an important pathogen which needs to be understood from their interaction with the host perspective, especially immune dynamics.

Additional incorporation of few aspects would strengthen it further:

1) It may be important to bring into perspective the dietary habit of the participants as those are shown to have a role in maintaining the gastric ecosystem.

2) Also, can we identify potential differences between chronic H. pylori infection and new infection. That would have different read out in terms of immune dynamics.

3) Most importantly, the sample numbers used (5 patients) is very small. We have to strongly think, if we insights from 5 patients can be strong enough to have global implications vis-a-vis H. pylori infections and host immune dynamics.

4) The authors should be doing the single cell experiments in more samples, including both patients and healthy, to have strength in the study.

Best wishes,
Rajesh Pandey

Reviewer #2

(Remarks to the Author)
The following manuscript “Single-cell RNA sequencing dissects the immunosuppressive signatures in Helicobacter pylori-infected gastric ecosystem” is an interesting and comprehensive paper that details immune signatures from PBMCs and gastric tissue from H. pylori-positive vs. H. pylori-negative patients. The authors incorporate the use of limited human samples, an in vivo mouse model, and some in vitro data.

The following are comments and suggestions for the authors consideration.
1) Table S1 should include:
a. Statistical analysis for age and BMI between H. pylori-positive (P) and H. pylori-negative (N) groups.
b. Footnotes to include abbreviations.
c. The authors highlight the importance of OipA in the tolerogenic response. Did the authors assess OipA expression in their H. pylori-positive samples?
2) Were the 5 patients with newly diagnosed H. pylori-associated gastritis treated for H. pylori eradication following sample collection?

3) P1T was excluded due to a library exception, but why weren't gastric tissue specimens and paired PBMCs analyzed from each patient? Data from H. pylori-negative control (N3P) and H. pylori-positive samples (P1T, P3P, P4P, and P5P) are missing from all the analyses? There is only one H. pylori-positive patient and only two H. pylori-negative patients that had paired gastric tissue and PBMC (P2). Of the 5 H. pylori-positive patients, only 2 had gastric specimens, and of the 3 H. pylori-negative patients, only 2 had gastric specimens. The low sample number is a limitation.
4) Figure S1D and 1E, was it surprising that there were no differences in the cell population proportions (Figure S1D) or the Ro/e scores (Figure 1E) between H. pylori-negative (NP) and H. pylori-positive (PP) PBMC populations?
5) Include statistical analysis for Figure 1E. Are the changes in the Ro/e scores statistically significant between H. pylori-negative (NP/NT) and H. pylori-positive (PP/PT) populations?
6) Mast cells comprise a large proportion (Figure 2D) and prevalence (Figure 2F) of gastric tissue cells, but do not change between H. pylori-negative (NT) and H. pylori-positive (PT). What accounts for the large proportion of mast cells within uninfected samples?
7) Include statistical analysis for Figure 1F. What Ro/e scores are statistically significant between H. pylori-negative (NP/NT) and H. pylori-positive (PP/PT) populations?
8) What distinguishes the Mono-CD14 clusters 0, 1, 6, and 9? With regard to prevalence (Figure 2F), cluster 0 and 1 appear to decrease with H. pylori (NP vs. PP), while clusters 6 and 7 increase with H. pylori (NP vs. PP). Clusters 0, 1, and 6 show decreases in MHC-II score, but MHC-II score is not shown from cluster 9. Does this cluster also exhibit decreased MHC-II scores? Explain the overall decrease in MHC-II score in the context of the different changes in prevalence. From these studies, the authors suggest repressed antigen processing and presentation capacities by the monocyte populations – can this be direct in vitro using human or murine PBMCs as was done with dendritic cells?
9) DC cluster 5 exhibited decreased MHC-II score in H. pylori¬-positive samples (PP/PT), but the prevalence of cluster 5 decreased in PBMC (PP) and increased in gastric tissue (PT). Explain the overall decrease in MHC-II score in the context of the different changes in prevalence. The prevalence of DC clusters 7 and 8 also are significantly increased with H. pylori in gastric tissue, do these clusters also exhibit decreased MHC-II scores?
10) For all validation studies in C57BL/6 mice, were all mice successfully colonized with H. pylori strain PMSS1 and did they develop gastritis? Include H. pylori colonization density, gastric inflammation scores, and pathological diagnosis from mouse experiments.
11) Include patient demographics, clinical information, and H. pylori virulence factor status from the 12 H. pylori-positive and 13 H. pylori-negative patients used in Figures 3G, S3D-E, 4H.
12) Does the UMAP macrophage plot include both H. pylori-positive and H. pylori-negative samples? Were macrophages characterized from the C57BL/6 mice challenged with BHI vs. PMSS1? Did these also exhibit decreased I-A/I-E MFI?
13) Were the T cell subtypes validated in the C57BL/6 mice challenged with BHI vs. PMSS1 or the cohort of 12 H. pylori-positive and 13 H. pylori-negative patients?
The authors speculate that "reduced antigen processing and presentation functions of the peripheral and gastric APC lineages hinders recruitment of CD4+ T cells, which favors H. pylori persistence in vivo." Did the authors correlate their findings of reduced antigen processing and presenting functions (MHC-II score) with the proportion and prevalence of CD4+ T cells in humans or mouse models? Further, using the in vivo mouse models, does decreased antigen processing and presenting functions in vivo coincide with decreased recruitment of CD4+ T cell populations and thus increased H. pylori colonization densities (increased H. pylori persistence)?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors have addressed the major comments vis-a-vis higher number of samples for the Single cell transcriptome based insights, which now stands at 39 samples in total.
The manuscript can be accepted in this form.

Reviewer #2

(Remarks to the Author)
The authors have comprehensively addressed my original critiques with new experimentation and extensive revisions. The manuscript is substantially strengthened.

Responses to Reviewers' comments:

Response: We would like to thank reviewers again for their comments, which have helped us to improve our manuscript.

Referee #1:

Comment #1: It may be important to bring into perspective the dietary habit of the participants as those are shown to have a role in maintaining the gastric ecosystem.

Response: Thank you for your kind suggestion. All study participants were recruited from Shenzhen, a coastal metropolis in southern China where the local cuisine is characterized by dietary preferences for mild flavors and low consumption of pungent spices. Individual with regular consumption of coffee, strong tea, tobacco, spicy food, or alcohol were excluded from the *H. pylori*-positive cohort during participant selection (See **Methods**).

Comment #2: Also, can we identify potential differences between chronic H. pylori infection and new infection. That would have different read out in terms of immune dynamics.

Response: Clinically, differentiating between chronic *H. pylori* infection and newly acquired infection remains challenging using current diagnostic methods such as ^{13}C-urea breath testing, endoscopic and histological evaluation. This diagnostic ambiguity prevents the collection of optimal clinical samples for definitive analysis.

Comment #3: Most importantly, the sample numbers used (5 patients) is very small. We have to strongly think, if we insights from 5 patients can be strong enough to have global implications vis-a-vis H. pylori infections and host immune dynamics.

Response: We have substantially expanded the sample size. Our new discovery cohort now comprises 23 participants (11 *H. pylori*-negative and 12 *H. pylori*-positive individuals), allowing us to collect 39 samples, including 17 PBMC and 22 gastric tissue samples. Using the 10× platform, we obtained 187,192 high-quality full-length transcriptomes.

Comment #4: The authors should be doing the single cell experiments in more samples, including both patients and healthy, to have strength in the study.

Response: Thank you for your kind suggestion. Usually, healthy stomachs have very few *CD45*$^{+}$ immune cells. Thus, we decided to recruit *H. pylori*-negative and -positive subjects diagnosed as chronic gastritis.

Referee #2:

Comment #1: Table S1 should include: Statistical analysis for age and BMI between H. pylori-positive (P) and H. pylori-negative (N) groups.

Response: We have added them accordingly (See **Table S1**).

Comment #2: Footnotes to include abbreviations.

Response: We have added them accordingly (See **Table S1**).

Comment #3: The authors highlight the importance of OipA in the tolerogenic response. Did the authors assess OipA expression in their H. pylori-positive samples?

Response: We have performed additional qPCR assays to detect the *OipA* expression in *H. pylori*-negative and -positive gastric samples, showing a significantly elevated *OipA* expression in *H. pylori*-positive gastric specimens compared to their *H. pylori*-negative counterparts (See **Page 5, Line 131**).

Comment #4: Were the 5 patients with newly diagnosed H. pylori-associated gastritis treated for H. pylori eradication following sample collection?

Response: Both the initial 5 and subsequently recruited 7 *H. pylori*-positive patients were newly confirmed infections, with no prior antibiotic exposure preceding sample collection.

Comment #5: P1T was excluded due to a library exception, but why weren't gastric tissue specimens and paired PBMCs analyzed from each patient? Data from H. pylori-negative control (N3P) and H. pylori-positive samples (P1T, P3P, P4P, and P5P) are missing from all the analyses? There is only one H. pylori-positive patient and only two H. pylori-negative patients that had paired gastric tissue and PBMC (P2). Of the 5 H. pylori-positive patients, only 2 had gastric specimens, and of the 3 H. pylori-negative patients, only 2 had gastric specimens. The low sample number is a limitation.

Response: We started the scRNA sequencing project in 2020. At that time, the cost of sequencing was more than $7,000 per sample, which severely limited patient recruitment. That's why we can only include 11 samples in our study. Nowadays, sequencing costs have come down to $3000 per sample, allowing us to include more samples in this study. We therefore recruited 8 additional *H. pylori*-negative gastritis patients and 7 *H. pylori*-positive cases in the discovery cohort, bringing the final sample size to 39 (17 PBMC samples, 22 gastric tissue samples).

Comment #6: Figure S1D and 1E, was it surprising that there were no differences in the cell population proportions (Figure S1D) or the Ro/e scores (Figure 1E) between H. pylori-negative (NP) and H. pylori-positive (PP) PBMC populations?

Response: We assessed the enrichment of major cell populations in both tissue and PBMC samples by calculating their relative abundances within individual sample and performing the ratio of observed to expected cell numbers (*Ro/e*) analysis. Comparative analysis didn't reveal significantly compositional difference between cell clusters from *H. pylori*-positive versus negative PBMC samples (See **Figure 1F**, **S1G**).

Comment #7: Include statistical analysis for Figure 1E. Are the changes in the Ro/e scores statistically significant between H. pylori-negative (NP/NT) and H. pylori-positive (PP/PT) populations?

Response: We assessed the enrichment of major cell populations in both tissue and PBMC samples by calculating their relative abundances within individual sample and performing the ratio of observed to expected cell numbers (*Ro/e*) analysis. Comparative analysis revealed striking compositional difference between cell clusters from *H. pylori*-positive versus negative gastric biopsies, exemplified by increased proportions of B and plasma cells coupled with decreased frequencies of gastric tissue and proliferating cells in *H. pylori*-positive gastric samples (See **Figure 1F**, **S1G**).

Comment #8: Mast cells comprise a large proportion (Figure 2D) and prevalence (Figure 2F) of gastric tissue cells, but do not change between H. pylori-negative (NT) and H. pylori-positive (PT). What accounts for the large proportion of mast cells within uninfected samples?

Response: By calculating the relative abundance of mast cells within individual sample, we detected a significantly higher proportion of these cells in PT than in NT samples (See **Page 6, Line 165**). We further discussed their functional roles in *H. pylori*-infected gastric tissues (See **Page 16, Line 477**).

Comment #9: Include statistical analysis for Figure 1F. What Ro/e scores are statistically significant between H. pylori-negative (NP/NT) and H. pylori-positive (PP/PT) populations?

Response: We assessed the enrichment of major cell populations in both tissue and PBMC samples by calculating their relative abundances within individual sample and performing the ratio of observed to expected cell numbers (*Ro/e*) analysis. Comparative analysis revealed striking compositional difference between cell clusters from *H. pylori*-positive versus negative gastric biopsies, exemplified by increased proportions of B and plasma cells coupled with decreased frequencies of gastric tissue and proliferating cells in *H. pylori*-positive gastric samples (See **Figure 1F**, **S1G**).

Comment #10: What distinguishes the Mono-CD14 clusters 0, 1, 6, and 9? With regard to prevalence (Figure 2F), cluster 0 and 1 appear to decrease with H. pylori (NP vs. PP), while clusters 6 and 7 increase with H. pylori (NP vs. PP). Clusters 0, 1, and 6 show decreases in MHC-II score, but MHC-II score is not shown from cluster 9. Does this

cluster also exhibit decreased MHC-II scores? Explain the overall decrease in MHC-II score in the context of the different changes in prevalence. From these studies, the authors suggest repressed antigen processing and presentation capacities by the monocyte populations – can this be direct in vitro using human or murine PBMCs as was done with dendritic cells?

Response: We used Seurat for cell clustering, which has been widely adopted by scRNA-seq studies[1]. Clusters 0, 1, 6 and 9 were defined as classical monocyte by their highly expressing canonical markers such as *CD14*, *FCN1*, and *VCAN*, but lacking *CD16* (*FCGR3A*) and *CX3CR1* expression. However, we could distinguish these subclusters by detecting their differentially expressed genes (DEGs) and the enriched signaling pathways in each population. We didn't observe significant reduction in MHC-II levels in cluster 9 following *H. pylori* infection, probably due to the limited cell number in this cluster (Cell number: NP=38, PP=128, NT=3, PT=1). In our new discovery cohort, we similarly identified four classical monocyte subclusters (Cluster 0, 2, 7, 9). By quantifying the relative abundance of these cells within individual sample, we didn't find significant compositional difference between these populations from *H. pylori*-negative or -positive samples. But we again detected reduced MHC-II scores in several populations. By aggregating data from PBMC-derived $CD14^{++}CD16^{-}$ monocytes, we identified a marked decline in MHC-II expression following *H. pylori* infection (See **Figure 2I**), indicating that *H. pylori* infection suppresses antigen processing and presentation capacities in these monocytes. To validate suppressed antigen presentation in peripheral monocytes following *H. pylori* colonization, we infected C57BL/6J mice with the pathogenic, CagA-proficient *H. pylori* strain PMSS1 for two months (See **Figure S2G-H**) and analyzed single-cell PBMC suspensions by flow cytometry. We detected a substantial reduction in the expression of the MHC-II molecules *I-A/I-E* within this population (See **Figure 2L**).

Comment #11: DC cluster 5 exhibited decreased MHC-II score in H. pylori¬-positive samples (PP/PT), but the prevalence of cluster 5 decreased in PBMC (PP) and increased in gastric tissue (PT). Explain the overall decrease in MHC-II score in the context of the different changes in prevalence. The prevalence of DC clusters 7 and 8 also are significantly increased with H. pylori in gastric tissue, do these clusters also exhibit decreased MHC-II scores?

Response: We recruited 8 additional *H. pylori*-negative and 7 *H. pylori*-positive gastritis patients in the new discovery cohort, and captured four DC subsets, including $CD1C^{+}$ DC (Cluster 8), $CLEC9A^{+}$ DC (Cluster 14), $CD1C^{-}CD141^{-}$ DC (Cluster 4) and pDC (Cluster 12). Consistent with our previous data, we also observed reduction in MHC-II scores in several DC populations following *H. pylori* infection (See **Figure 2M-N**). We didn't detect decreased MHC-II scores in Cluster 7 (pDC) and 8 (IKDC). Previous publications confirmed that the expression of MHC molecules on pDC is not as high as their cDC counterparts, leading to the lower efficiency of pDC at T cell priming[2]. And the primary function of pDCs is the secretion of type I interferon (IFN-

α/β) in response to viruses attack, but not bacterial infection[3]. Additionally, we captured limited cell numbers of IKDC (Cell number: NP=50, PP=30, NT=50, PT=47), which might explain why these two clusters didn't exhibit decreased MHC-II scores upon *H. pylori* infection.

Comment #12: For all validation studies in C57BL/6 mice, were all mice successfully colonized with H. pylori strain PMSS1 and did they develop gastritis? Include H. pylori colonization density, gastric inflammation scores, and pathological diagnosis from mouse experiments.

Response: We have performed additional analysis to detect *H. pylori* density and gastric inflammation in BHI or PMSS1-treated mice stomach (See **Figure S2G, H**). Our results showed that *H. pylori* infection levels and the severity of gastric inflammation were significantly increased following PMSS1 treatment.

Comment #13: Include patient demographics, clinical information, and H. pylori virulence factor status from the 12 H. pylori-positive and 13 H. pylori-negative patients used in Figures 3G, S3D-E, 4H.

Response: We have included the clinical information of patients in the validation cohort accordingly (See **Table S4**).

Comment #14: Does the UMAP macrophage plot include both H. pylori-positive and H. pylori-negative samples? Were macrophages characterized from the C57BL/6 mice challenged with BHI vs. PMSS1? Did these also exhibit decreased I-A/I-E MFI?

Response: Yes, the UMAP macrophage plot include both *H. pylori*-positive and *H. pylori*-negative samples. We have tried everything to isolate leukocytes from BHI or PMSS1-treated C57BL/6 mice stomach. However, suboptimal cell viability precluded reliable macrophage detection in mice gastric tissues. We therefore employed a bone marrow-derived macrophage (BMDM) model to evaluate I-A/I-E expression and analyzed HLA-DR levels in human gastric samples with or without *H. pylori* infection. Both experimental approaches consistently demonstrated that *H. pylori* infection compromises macrophage antigen presentation capacity (See **Figure 3G, H**).

Comment #15: Were the T cell subtypes validated in the C57BL/6 mice challenged with BHI vs. PMSS1 or the cohort of 12 H. pylori-positive and 13 H. pylori-negative patients?

Response: In our initial discovery cohort, we identified a population of T cells that was predominantly enriched in *H. pylori*-infected gastric samples and strongly expressed both MHC-II-related genes and inhibitory-related genes (e.g., *CTLA4*). We further confirmed the existence of these T cell populations in the validation cohort. Moreover, we recruited 8 additional *H. pylori*-negative gastritis patients and 7 *H. pylori*-positive

cases. Similarly, we identified two T cell subsets predominantly enriched in PT samples: a *CD4*$^+$ population (CD4-5-HLA-DR) and a *CD8*$^+$ population (CD8-12-HLA-DR), both exhibiting strong expression of MHC-II-associated genes (*HLA-DRA*, *HLA-DQA1*, *HLA-DQB1*) and elevated *CTLA4*.

Comment #16: The authors speculate that "reduced antigen processing and presentation functions of the peripheral and gastric APC lineages hinders recruitment of CD4+ T cells, which favors H. pylori persistence in vivo." Did the authors correlate their findings of reduced antigen processing and presenting functions (MHC-II score) with the proportion and prevalence of CD4+ T cells in humans or mouse models? Further, using the in vivo mouse models, does decreased antigen processing and presenting functions in vivo coincide with decreased recruitment of CD4+ T cell populations and thus increased H. pylori colonization densities (increased H. pylori persistence)?

Response: We calculated the correlations between the MHC-II scores and CD4$^+$ T cell proportions in NP, NT, PP and PT groups, and found positive correlations of MHC-II scores with CD4$^+$ T cell percentages in both PP and PT samples (See **Page 15, Line 420**).

Reference

1. Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411-420 (2018).
2. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* **29**, 352-361 (2008).
3. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol* **29**, 163-183 (2011).

Responses to the reviewers' comments:

Reviewer 1: Authors have addressed the major comments vis-a-vis higher number of samples for the Single cell transcriptome based insights, which now stands at 39 samples in total. The manuscript can be accepted in this form.

Response: We sincerely appreciate reviewer's comments and positive response.

Reviewer 2: The authors have comprehensively addressed my original critiques with new experimentation and extensive revisions. The manuscript is substantially strengthened.

Response: We sincerely appreciate reviewer's comments and positive response.