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TITLE: Molecular Characterization of H.pylori Strains and Biomarkers in Gastric Cancer

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14. ABSTRACT Enter a brief (approximately 200 words) unclassified summary of the most significant findings during the research period.
Helicobacter pylori (*Hp*) is linked to chronic gastritis, peptic ulcer disease (PUD) and gastric cancer (GC), but it is unknown why diverse gastric diseases develop in different *Hp* carriers. GC annually claims 700,000 lives worldwide. GC early detection is vital in improving prognosis, but disease biomarkers are lacking. Our goal is to identify gastric epithelial cell (GEC) responses elicited by GC isolates that could represent candidate biomarkers and unique genomic features of those GC *Hp* isolates. We used novel human gastroid cultures infected with *Hp* isolates from diverse gastric diseases. In the first year report we highlighted that infections with different *Hp* isolates showed by real time PCR distinct expression by GECs of genes related to immunity, NOTCH signaling, metaplasia, cell survival and cell death. In the last year, we examined in depth those results and performed focused studies on the effects of those different GEC responses on the activation of $CD4^+$ T cells co-cultured with *Hp*-infected GECs, since *Hp* subverts host immunity and that may affect tumor immune surveillance. Our studies showed that different *Hp* isolates not only differ in the host genes that they activate, but also on their influence on T cell responses elicited. Because Notch receptors/ligands play key roles in cell differentiation in antigen presenting cells (APCs) and T cells, we examined their expression in *Hp*-infected GECs and found that Notch 4 and Dll4 expression was higher in cells infected with GC isolates compared to PUD and gastritis isolates. Further, these cultures also led to the development of higher T regulatory cells than similar cultures infected with non-cancer *Hp* strains. The role of Notch4 in this response was confirmed by siRNA knock-down of Notch4, which led to a shift in T cell response from T regulatory to Th17. These results are significant because they provide insights into how Hp escapes host immunity and by increasing T reg cells may aid in immune surveillance escape by tumors that develop. Additional studies that include deep genomic sequencing of those *Hp* strains may reveal potential targets for vaccine.

15. SUBJECT TERMS
Helicobacter pylori (Hp), gastritis, peptic ulcer disease (PUD), gastric cancer (GC), gastric disease, gastroids, organoids, biomarkers

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1. INTRODUCTION:

Helicobacter pylori (*Hp*) contributes to chronic gastritis, peptic ulcer disease (PUD) and gastric cancer (GC), but it is not clear why different individuals develop different gastroduodenal diseases. Our objectives are (**1**) to determine genetic features present in *Hp* GC isolates not present in those from PUD or gastritis, and (**2**) to determine in human gastric organoids if GC isolates, compared to non-GC isolates, induce expression of proteins that may represent candidate biomarkers of disease.

2. KEYWORDS:

Helicobacter pylori (Hp), gastritis, peptic ulcer disease (PUD), gastric cancer (GC), gastric disease, gastroids, organoids, biomarkers

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Determine by deep sequencing and comparative genomic analysis of *Hp* isolates from GC, PUD and gastritis cases genomic features in *Hp* GC isolates not present in those from PUD or gastritis. This task has 3 subtasks of which Subtask 1 was to submit IRB protocol for approval by institutional IRB (within first three months). This subtask was accomplished locally (approval date 6/21/17) and temporarily approved at collaborator's site, but DoD IRB required additional information and changes that created delays going back and forth. Subtask 2. Culture and DNA extraction of collections of *Hp* strains from gastric cancer (GC), peptic ulcer disease (PUD) and gastritis (minimum of 10/group). Subtask 3. Perform comparative genomic analysis of Hp strains isolated from different gastroduodenal diseases whose DNA has been extracted. The genomic analysis is being performed by collaborator at the moment.

Major Task 2: Determine whether the genomic features found in cultured isolates are consistent with those present in *Hp* within biopsies in the context of influences by the host and other microbial communities. In progress. This task has suffered delays due to delays in IRB approval. Explained further below.

Major Task 3: Determine whether infection of human gastric organoids by *Hp* GC isolates, in comparison to *Hp* non-GC isolates, results in differential epithelial expression of proteins that may represent candidate biomarkers of disease. This major task has had significant advances and findings using some of the *Hp* isolates collected from different disease states.

Subtask 1. Expand at least three of the available human gastric organoid cultures. We have grown nine different organoids from an ethnically diverse group of subjects and comparable numbers of males and females. The organoids have been used for subtasks 2 and 3, below. Subtask 2. Infect each of the human gastric organoid cultures with five *Hp* strains from each of the gastroduodenal diseases (GC, PUD and gastritis) and assess levels of PD-L1, B7-H3 and B7-H4 at the protein (flow cytometry) and mRNA (real time PCR) levels. This was work has been accomplished and because Notch receptors and ligands are critical in T cell development and differentiation, they were examined leading to some exciting results as described below. Subtask 3. Determine whether soluble forms of PD-L1, B7-H3 and/or B7-H4 are present in supernatants of infected cultures and compare the levels of expression induced by *Hp* strains from different disease states. Supernatants have been collected and ready for analysis, after we evaluate all data for manuscript preparation.

What was accomplished under these goals?

Since the last reporting period, we completed **Major Task 3**, subtask 1 as we successfully expanded the panel of human gastric organoid (gastroids) cultures from the initial group reported a year ago and used them as a model of infection with a panel of *Hp* isolates from the different gastric diseases (gastritis, PUD and GC). The gastroids were grown and induced to differentiate in tissue culture inserts with collagen that leads to the gastroids opening from their spheroid shape into polarized monolayers. The gastroid cultures were examined in parallel with cultures of a gastric cell line that grows in polarized fashion and has a normal phenotype and karyotype. Polarity of the epithelial cell cultures (gastroids and cell line) was confirmed by confocal microscopy as shown in the previous report. The cultures were infected with 11 non-cancer *Hp* isolates (4 gastritis and 7 PUD) and seven GC isolates. When compared to uninfected controls and cultures infected with gastritis isolates, GC isolates induced higher expression of PD-L1 (aka B7-H1) but comparable to that induced by PUD isolates. The induction of this immune checkpoint regulator is significant because it inhibits activated T cells and also promotes regulatory T (T_{reg}) cell development. In fact, when we established co-cultures of T cells with gastroids pre-infected with either gastritis, PUD or GC isolates, gastroids infected with GC promoted the development of T_{reg} cells and suppressed Th1 cells whereas PUD strains promoted the development of Th1 cells in similar cocultures with the same gastroids (**Figure 1 Appendix**). In addition, we noticed a dramatic increase in CCL20 expression in cultures infected with GC isolates whereas similar cultures unifected or infected with gastritis or PUD isolates did not express CCL20 (**Figure 2 Appendix**). This is significant because CCL20 is a chemokine that recruits Treg cells to tumor sites and was shown recently to be induced by *Hp* to mediate recruitment of Treg cells to the infected mucosa. Thus, *Hp* isolates from GC induce the expression of a chemokine that recruit T_{regs} and also elicit responses in gastric epithelial cells that promote development of inducible T_{reg} (iTreg)cells characterized by high expression of CD25 and FoxP3 as evidenced in multiparameter flow cytometry (**Figure 1C Appendix**). Another important finding is that human gastroids infected with GC derived *Hp* isolates had a significant increase of axin 2 compared to the same gastroids infected with *Hp* isolates from PUD or gastritis (**Figure 3**). Axin is a vital constituent of the canonical Wnt signal transduction pathway that inhibits signaling in the absence of a Wnt ligand. Axin 2 promotes phosphorylation and degradation of β-catenin, which has been shown to be dysregulated by *Hp* and is associated with pre-malignant lesions. Another member of the immune checkpoint regulators that we examined closely because of its T cell inhibitory properties is B7-H3. However, when we infected human gastroids with a panel of *Hp* isolates, we observed that its expression did not change with GC isolates whereas gastroids infected with PUD isolates had significant increases compared to those elicited by all other isolates and those noted in uninfected gastroids (**Figure 4 Appendix**).

What was accomplished under these goals?

Notch receptors and their ligands are transmembrane glycoproteins that as they interact deliver signaling cascades which govern cellular differentiation. There are four Notch receptor paralogs (Notch 1 to Notch 4) and five Notch ligands: Jagged (Jag) 1 and 2 and Delta-like (Dll) 1, 3, and 4. The Notch pathway is important in T cell differentiation in the thymus and in marginal zone B cells development{Stanley, 2009 4462 /id}. Interestingly, it seems to regulate the differentiation of naive CD4 T cells into the different TH subsets through regulation of the different subset specific transcription factors and the corresponding cytokines. For instance, the binding of Notch3 to Dll1 was reported to bias TH1 differentiation{Maekawa, 2003 4351 /id} and Flavell's group showed that Jag1/Notch binding guides TH2 differentiation while Notch/Dll1 binding promotes TH1 development{Amsen, 2004 4463 /id}. A recent report showed that Hp infection of a gastric epithelial cell line affects the Notch pathway by reducing mRNA expression of Notch1 and Notch2 while the expression of Dll4 was significantly increased{Liu, 2016 4464 /id}. However, the findings in this study contrast with observations by others who noted elevated Notch1-4 mRNA in gastric cancer patients{Wu, 2016 4465 /id}, which could be due to the fact that the cell line used in the in vitro studies was cancer-derived. Thus, we examined whether this pathway was differentially affected by *Hp* isolates from GC versus PUD and gastritis. Remarkably, *Hp* isolates from GC cases not only induced more T_{reg} cells in the co-cultures but also stimulated higher expression of Notch 4 and Dll4 (**Figure 5 Appendix**). Thus, major advances have been made understanding how *Hp* gastric cancer strains affect the gastric epithelium and the CD4+ T cells that infiltrate the human gastric mucosa. These findings are currently in preparation for publication.

Major Task 1 is being accomplished via a collaboration with a lab that specializes in deep sequencing and the strains that were used through the study are already in their hands. Major Task 2 has suffered delays because of what seems to be a misunderstanding between the local IRB board and the DoD board. After several months answering questions to the local IRB board the protocol was approved and some time later the DoD review board suggested additional changes that have to be approved by the local board. One of those changes included the recruitment of an external monitor who is an expert and we did and submitted that. We hope to be able to proceed with this task during the no-cost extension period.

What opportunities for training and professional development has the project provided?

The project has been fruitful ground for training a post-doctoral fellow who is now motivated to pursue a career in the field of gastric cancer research. Also, we have trained two medical students in the past two summers. Both students were fully trained in BSL-2 techniques, were certified and learned to grow human cells in tissue culture inserts, Hp culture, infections of human cells, real-time PCR and staining for flow cytometry. The first student, Esaias Tong, received the First Prize Award for research in Infection and Immunity at the 2017 Summer Medical Student Research Symposium. The second student, Karen Zhang, received the top overall award for Best Research in the 2018 Summer Medical Student Research Symposium after her poster presentation and she was also invited to give a podium presentation.

How were the results disseminated to communities of interest?

The findings of the research have been disseminated at two local symposia and at the national meeting of the American Association of Immunologists, which was held in Austin, TX on May 4-8, 2018. Furthermore, there are two manuscripts in preparation for submission in the next two months.

What do you plan to do during the next reporting period to accomplish the goals?

Through a collaboration, we expect to have the genomic information to discern what genes are different in GC Hp isolates compared to PUD and gastritis isolates. The only other task remaining is final approval of the IRB to be able to isolate Hp directly from biopsies and determine whether the genetic features that we identify in the GC isolates are also present in the context of environmental cues from the host and the gastric microbiome.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Our results using the human gastroids, that represent primary cultures of human gastric tissue, infected with *Hp* bacteria isolated from patients with gastritis, peptic ulcer disease and gastric cancer are showing that *Hp* isolates from gastric cancer cases lead to epithelial responses that influence the local immune response promoting suppression perhaps as a mechanism of immune evasion by the bacteria that also may impact on tumor immune surveillance allowing developing neoplasms to thrive. Further, findings during these past two years have shown that *Hp* strains isolated from GC cases induce changes associated with the process of carcinogenesis not seen with isolates from PUD and gastritis cases. These results are highly provocative and offer valuable insights that may aid in developing early diagnostic approaches and novel therapeutic targets.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

There is nothing to report at this time, since the findings are still preliminary and additional studies are needed.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

In the many years of research in *Hp*, IRB approval for biopsies has never been an issue that could not be addressed early during any project. I have worked with multiple local and external collaborators using multiple IRB protocols to obtain extra gastric biopsy tissue. Thus, I did not anticipate delays in securing the appropriate IRB on the collaborator's site who has worked diligently to address all the inquiries from the DoD and additional changes after the protocol was approved.

Actual or anticipated problems or delays and actions or plans to resolve them.

As a backup mechanism, I have already approached the director of the institutional diagnostic microbiology labs to inquire about obtaining *Hp* cultures that they grow from *Hp* biopsies submitted. We already have the required (NOU) permits, if that becomes a viable alternative. One caveat is that while PUD and gastritis cases follow that route, GC cases are routinely not seen there, as they are usually handled by surgery. We have a collaboration with a colleague who has an IRB to accept discarded surgical tissue specimens that would include GC resections.

Changes that had a significant impact on expenditures

There are no significant changes.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

In Progress. Final approval expected soon.

Significant changes in use or care of vertebrate animals

Not Applicable.

Significant changes in use of biohazards and/or select agents

Not Applicable.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications.

Nothing to Report.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Not Applicable.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Victor E. Reyes, PhD
Project Role: Principal Investigator
Researcher Identifier: 034470
Nearest person month worked: 2.4
Contribution to Project: Project leader, experimental design, data interpretation, communications
Funding Support: N/A

Name: Yuriy Fofanov, PhD
Project Role: Coinvestigator
Researcher Identifier: 241077
Nearest person month worked: 0.48
Contribution to Project: He oversees the analysis and interpretation of next generation DNA sequencing (NGS) data. He is consulted on selection of methods to analyze genomic data.
Funding Support: N/A

Name: Iryna V. Pinchuk, PhD - 192921
Project Role: Coinvestigator
Researcher Identifier: 192921
Nearest person month worked: 0.6
Contribution to Project: She has been responsible for developing the local IRB protocol and the IRB protocol by Dr. Suarez-Gould at Baylor in conjunction with Dr. Powell. She maintains communications from the IRB and Dr. Gould regarding the protocols. Her expertise in the isolation of the mucosal cells from GI human mucosa is needed as part of the studies and she has helped with the training of the postdoctoral fellow.
Funding Support: N/A

Name: Don W. Powell, MD (no longer on project as he has retired)
Project Role: Coinvestigator
Researcher Identifier: 050842
Nearest person month worked: 0.12
Contribution to Project: As a clinician and Director of the Division of Gastroenterology, he has been consulted during the IRB protocol development and revisions.
Funding Support: N/A

Name: Levent Albayrak
Project Role: Programmer
Researcher Identifier: 241231
Nearest person month worked: 1.2
Contribution to Project: He is tasked with development of computational tools to quickly and efficiently identify highly specific and robust signatures essential for this research.
Funding Support: N/A

Name: George Golovko
Project Role: Research Scientist
Researcher Identifier: 241207
Nearest person month worked: 1.2
Contribution to Project: He is responsible for developing new bioinformatics functions/modules and modifying existing ones or implementing new pipelines to perform analysis. He will also participate in the collection of new software tools, and participate in the bioinformatic analysis of the sequencing data.
Funding Support: N/A

Name: Kamil Khanipov
Project Role: Research Technician
Researcher Identifier: 241236
Nearest person month worked: 1.2
Contribution to Project: He is responsible for management, filtering and preparation of data for downstream analysis as well as testing and debugging the tools.
Funding Support: N/A

Name: Alex-Giovanny Peniche-Trujillo, PhD
Project Role: Postdoctoral Fellow
Researcher Identifier: 236476
Nearest person month worked: 12
Contribution to Project: Day to day performance of experiments, maintenance of bacterial and cell cultures, procurement of reagents.
Funding Support: N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Organization Name: Baylor College of Medicine (Milena Gould Suarez, MD)
Location of Organization: Houston, TX
Partner's contribution to the project: Dr. Gould is an Assistant Professor of Medicine in the department of Medicine, Section of Gastroenterology & Hepatology. She is the Medical Director of the Gastroenterology clinic at Smith Clinic as part of Harris Health Services. She has been responsible for the development of the IRB protocol at her institution in order to recruit from among the patients that she sees for the biopsy specimens to be used to freshly isolate *Hp*.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

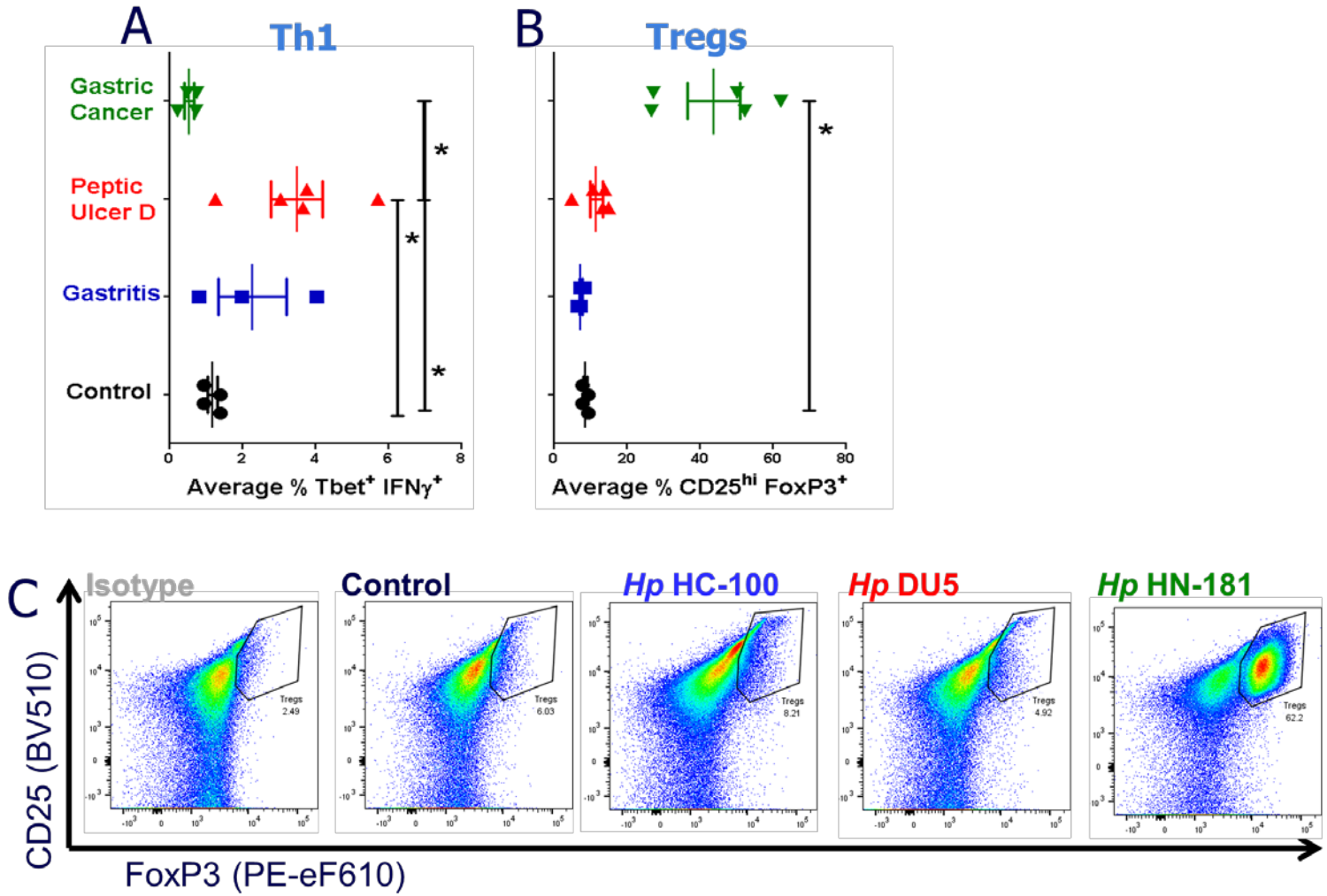

Figure 1. Hp GC strains led to a significative reduction of Th1 (Tbet+ IFNγ+) cells (**A**), and increased of Treg cells (CD25hi FoxP3+) (**B-C**). In normal gastric tissues CD4+ T cells are almost absent, but after infection with Hp their frequency is significantly increased.

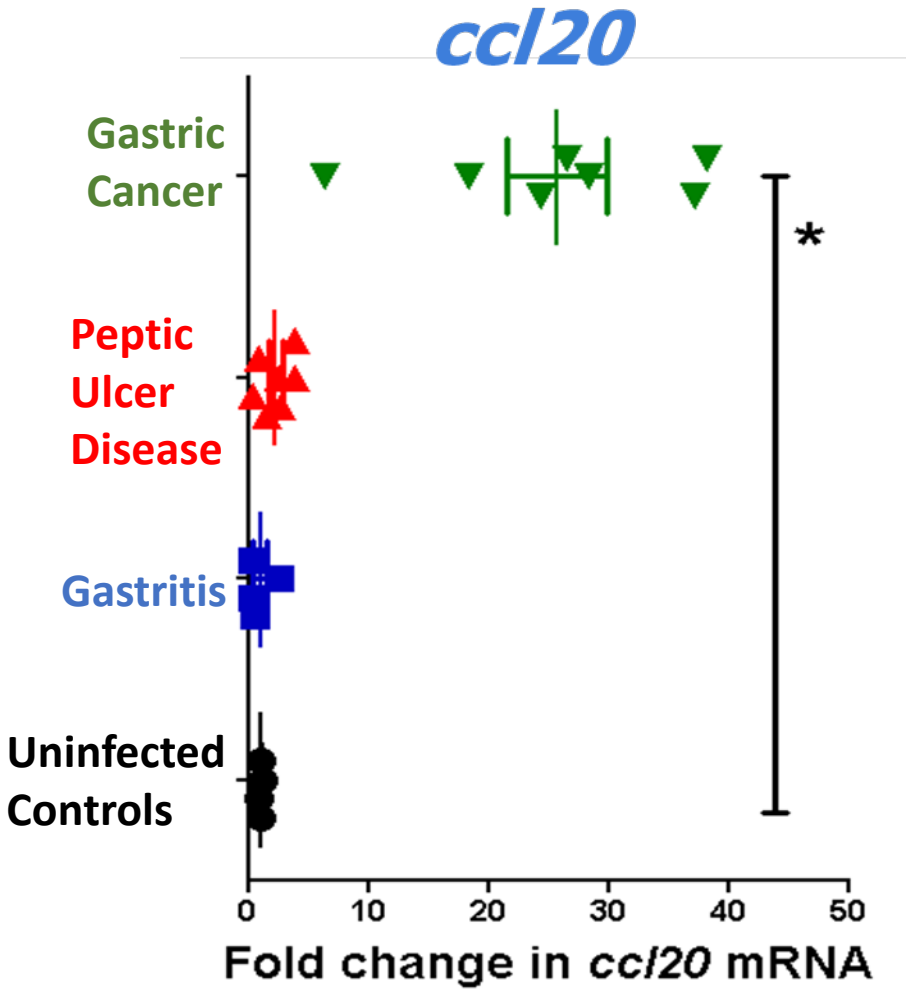

Figure 2. Gene expression analysis also reveled an increase of ccl20 (D) in infected hGOS by Hp GC strains. This chemoquine mediates lymphocyte trafficking during gastric inflammation

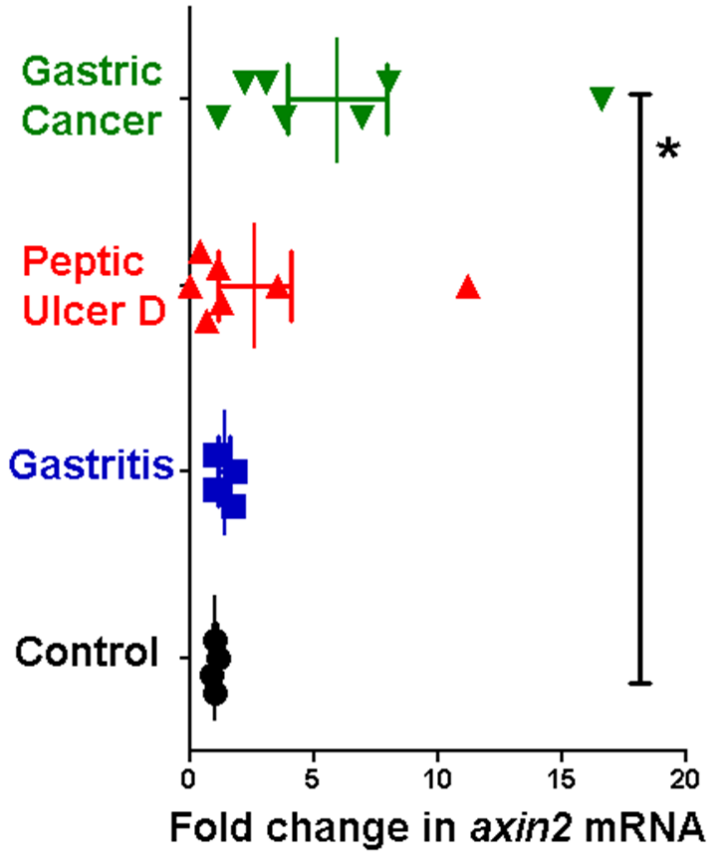

Figure 3. Hp strains from Gastric Cancer induce axin2 in human gastric organoids. Real time PCR analysis of human gastroids infected with a panel of Hp isolates from gastritis, PUD and GC cases.

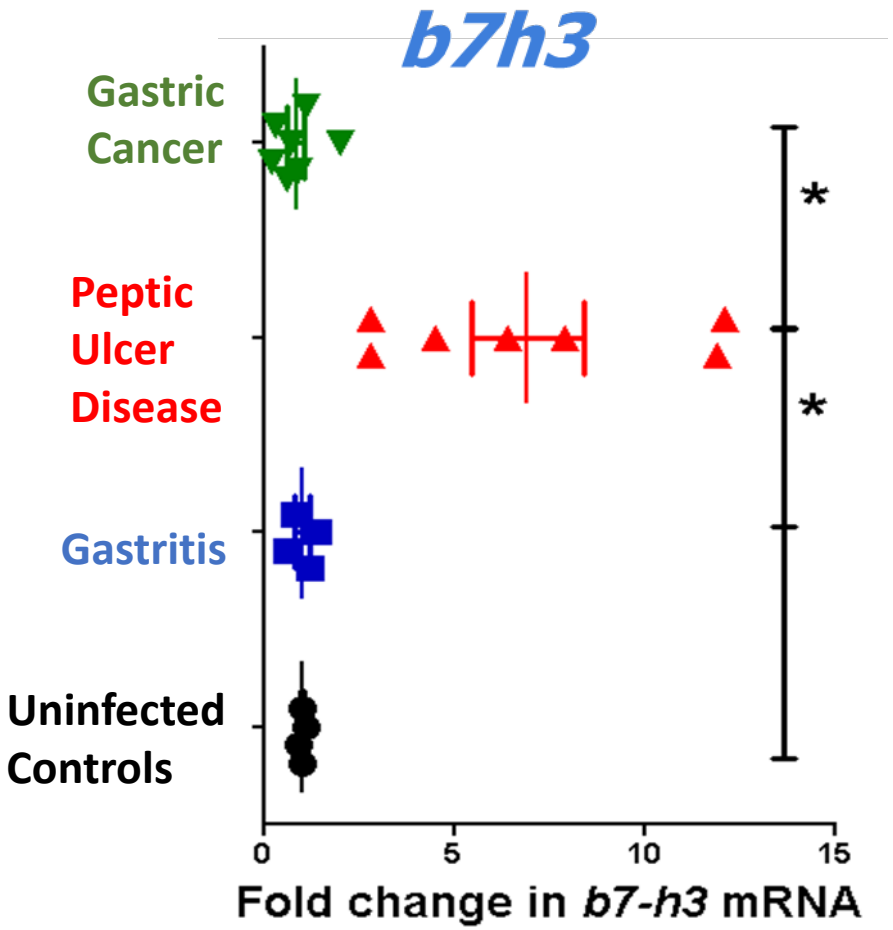

Figure 4. PUD strains are the stronger inducers of PD-L1 and B7-H3. PD-L1 binds PD-1 on T cells and promotes loss of effector functions, and reduced T cell-target cell contact.

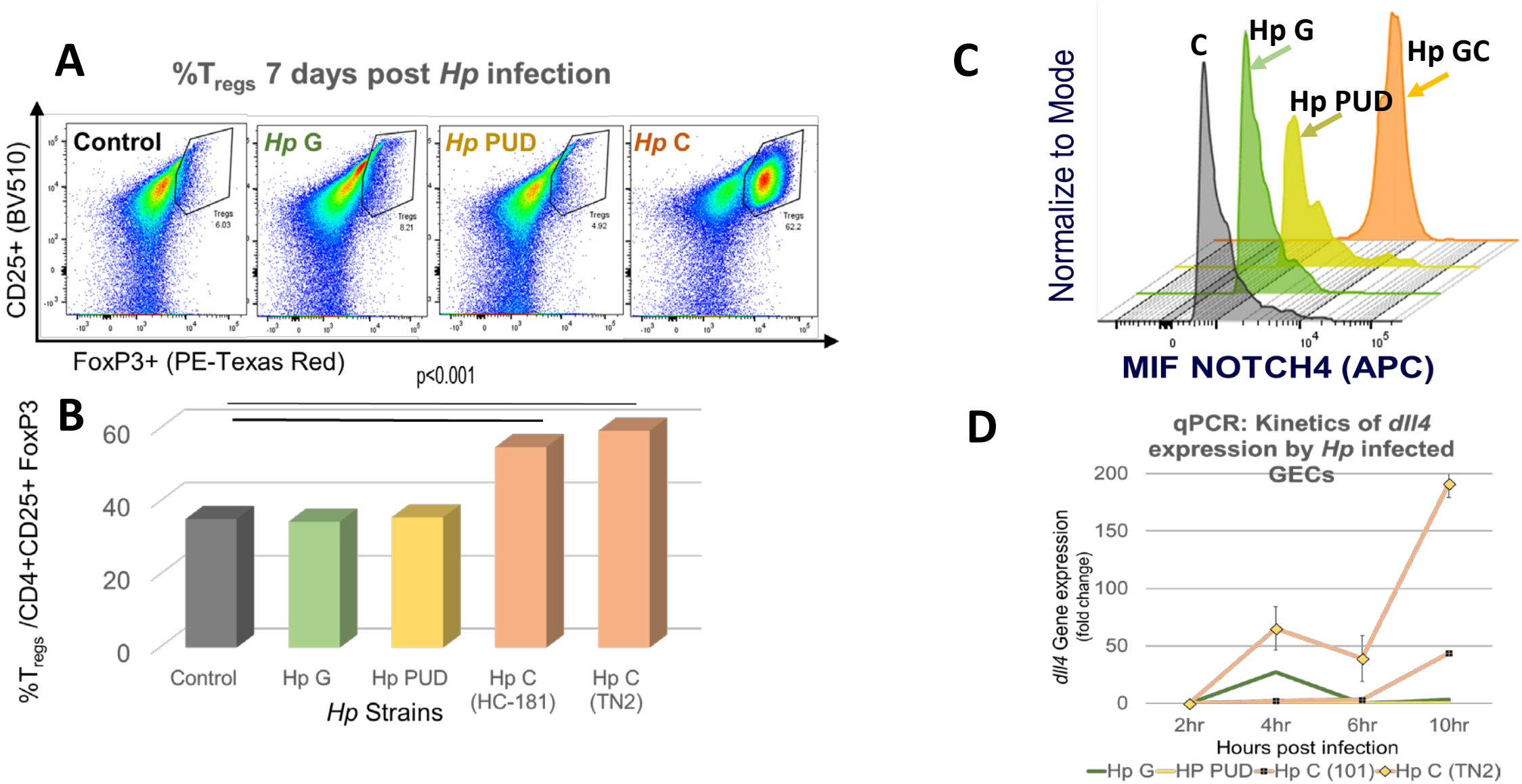

Figure 5. (**A-B**) Flow cytometry of T cells recovered from co-cultures with gastroids infected with Hp gastritis (Hp G),PUD or cancer (Hp C) showed Tregs in Hp C infected cultures. Notch4 expression was also high on those cells (**C**). RT-PCR of mRNA isolated from the cultures showed elevated dll4 in the same cultures compared to all others (**D**).