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

PRINCIPAL INVESTIGATOR:
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6. AUTHOR(S)
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301 University Boulevard
Galveston, TX 77555-0156

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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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION: A major objective of this proposal was to gain insights into the mechanism(s) whereby *Helicobacter pylori* (*Hp*) causes gastric cancer, which is the second deadliest cancer worldwide killing about 700,000 people per year. *Hp* infect more than half of the world population and generally become established as a chronic infection. Most infected individuals either lack symptoms or have gastritis, but in some the infection can lead to serious diseases of the gastric which include peptic (gastric or duodenal) ulcer disease and gastric cancer. Gastric cancer is deadly because it is usually diagnosed late since there are no early symptoms and 80% of cases are associated with *Hp* infection. Unfortunately, we do not have enough understanding of how *Hp* promotes gastric cancer to be able to effectively prevent it. Because gastric cancer is caused by an infectious agent a vaccine may be an effective way to prevent this deadly disease. Our approach was to use two powerful novel technologies to investigate how *Hp* affects the human gastric epithelium to elicit disease and whether there are virulence mechanisms present in some strains and not in others that could explain why a fraction of those infected develop gastric cancer. The first novel approach was the use of gastric organoids that allow the modeling of human gastric disease using primary cultures of human stomach that recapitulate the gastric architecture in order to investigate the presence of candidate biomarkers of disease in infected cultures. Advantages of these cultures is that they reproduce the complexity of the different epithelial cell types found in the region of the stomach infected by *Hp*, they are not cancer cells but grow indefinitely, and are polarized as the epithelium in the stomach. Those cultures are known as gastric organoids and can be infected with *Hp* strains from gastric cancer cases and with strains from other gastric diseases to investigate if there are differences in the induction of proteins that are found in cancers and are induced by *Hp*. Those proteins are known to have negative immunoregulatory properties that prevent the immune system from attacking tumors. We expected to note that strains from gastric cancer cases differ from other strains in the induction of these proteins. Then, we planned to incorporate another state-of-the-art technology known as next generation sequencing to examine in detail the genomes of a collection of *Hp* strains from gastric cancer cases and compare them to the genomes of *Hp* strains isolated from peptic ulcer disease and gastritis cases to identify genetic characteristics that are unique to strains from gastric cancer cases. Those genetic features may represent genes encoding virulence factors associated with disease promotion. The information acquired was expected to open doors to novel strategies to prevent and/or treat gastric cancer.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Helicobacter pylori, gastric cancer, peptic ulcer disease, gastritis, organoids, epithelial cells, bacteria, biomarkers, immune checkpoint regulators, T cells, immune escape.

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

- **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). 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.

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.

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.

Subtask 1. Expand at least three of the available human gastric organoid cultures. This was planned for months 9-12 and was 100% completed within the first 6 months and the organoids are in use for subtasks 2 and 3, below. Currently we have grown 7 different gastric organoids.

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.

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.

- **What was accomplished under these goals?**

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. Subtask 1, which was to submit IRB protocol for approval by institutional IRB was accomplished locally (approval date 6/21/17) but final approval at collaborator's site met with significant delays and approval by DoD was very slow and was eventually granted after the grant had lapsed. The collaborator's site was considered an important resource due to her access to a diverse group of patients with *Hp*-associated diseases, including gastric cancer. Because of the delays, we focused on other parts of the project and used clinical isolates that were well identified regarding the clinical disease from which they originated. Thus, we had an ample repertoire of isolates available for the studies and which we used to examine their effects on epithelial cells and characterized them for the expression of genes of important virulence factors.

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. The genomic features of a large group of cultured isolates were investigated in the context of the most important virulence factors associated with pathogenesis and the intent was to determine possible involvement in the interesting responses that they elicited in epithelial cells in organoid cultures, which are noted below under Major Task 3. Our analysis of virulence factors included the presence of urease genes, blood group-binding adhesins, *Hp* adhesins, sialic acid-binding adhesins, adherence associated lipoprotein (AlpA), lipopolysaccharide Lewis antigens, neutrophil

activating protein (*Hp* NAP), outer inflammatory protein (oipA), flagella genes, duodenal ulcer promoting (dupA), Cag PAI type IV secretion system, T4SS effectors cytotoxin-associated gene A, Cytolethal distending toxin, and vacuolating cytotoxin A (vacA). There were gene expression patterns depending on the disease from which the isolates were derived. Also, we noted some gene duplications and, in some cases, up to four copies of a gene (**Appendix A**). There were certainly group-specific responses depending on the disease process from which the isolates originated. Unfortunately, because of the delays with the IRB approval process the inclusion of fresh isolates from biopsies was not possible.

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. We focused on this major task which led to significant advances using *Hp* isolates collected from different disease states and also employed an array of organoid cultures that originated from multiple individuals representing diverse ethnic origins and both genders. All of the planned subtasks under this major task were accomplished leading to important observations that were presented at a national meeting and two regional meetings, a published manuscript and another one ready for submission.

Subtask 1. Expand at least three of the available human gastric organoid cultures. During the time of the study we acquired 10 different organoids from different donors and we successfully grew 8 different gastric organoids, listed below:

Gastric Organoids Used in the Project						
Name	**age**	**gender**	**Ethnic group**	**Surgery type**	**location**	**Passage**
G1	25	M	Caucasian	Biopsy	antrum	
G2	43	F	N/A	Biopsy	antrum	. 1:3
G3	63	F	Asian	Biopsy	antrum	
G4	40	F	African American	Biopsy	antrum	. 1:2
G7	75	F	Caucasian	Biopsy	Upper part	. 1:3
G102	55	M	Caucasian	Biopsy	Body	
G104	64	F	Caucasian	Biopsy	antrum	
G112	3	N/A	N/A	N/A	N/A	. 1:3

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 accomplished using 20 different strains isolated from diverse gastric pathologies, as listed below in the table.

***Hp* Strains Used in the Project**		
Gastritis	**Peptic Ulcer Disease**	**Gastric Cancer**
51B	LC11	CA8
HC-93	DU2	CA65
HC-94	DU5	HN-101
HC-95	DU15	HN-183
HC-91	J99	HN-114
HC-100	RD26	HN-179
	PMSS1	HN-181

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. In Progress. Supernatants have been collected for analysis after we complete the analysis of data from subtask, which are very interesting and are the subject of a published manuscript and another in preparation.

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

The PI, a postdoc and two medical students have developed new skills as a result of this project. The PI implemented novel human organoid technology via collaboration with Dr. Mary Estes (Baylor College of Medicine) and her lab, via one-on-one visits, has guided Dr. Alex Peniche with valuable tips on the growth of the organoids. Also, former lab members using this technology have helped implement it. Further, two second-year medical students spent the summer in the lab and learned to grow human cells in tissue culture inserts, *Hp* culture, infections of human cells, real-time PCR and staining for flow cytometry. Some of the findings were presented in the institutional summer research symposium in which each student received awards for their presentation. One student received the first-place award for best poster in Infection and Immunity and the other the top overall award for basic science research. Dr. Peniche presented the findings in a poster at the American Association of Immunology meeting held in Austin on May 4, 2018 - May 8, 2018.

- **How were the results disseminated to communities of interest?**

As indicated above, the work performed under this grant resulted in three poster presentations, a published manuscript and one in preparation, to date.

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

Nothing to report.

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:*

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

Our findings with the primary cultures of human gastric organoids infected with *Hp* bacteria isolated from patients with gastritis, peptic ulcer disease and gastric cancer showed that *Hp* isolates from gastric cancer cases lead to epithelial responses associated with the process of carcinogenesis. Our studies showed an increase of negative immune checkpoint regulators after *Hp* infection. The highest levels of PD-L1 were associated with *Hp* strains derived from patients with PU and GC diseases, while those from gastritis patients induced the lowest levels of PD-L1 (**Fig. 1**). B7-H3 and B7-H4 expression was noted with PU strains, while GC strains had limited effect on the expression on either of these co-inhibitors. Programmed death one homolog (PD-1H) is a cell surface molecule of the B7/CD28 immune checkpoint regulator family. PD-1H has been shown to function as a coinhibitory receptor on T cells to limit naive T-cell activation and proliferation. Remarkably, *Hp* isolates from gastric cancer cases led to the highest induction of pd-1h mRNA expression in gastric epithelial cells (**Fig. 2**). These observations suggest possible segregation of disease-association with diverse isolates of *Hp*.

To determine the functional relevance of the above observations, we established co-cultures of human gastric organoids infected with the different *Hp* isolates together with naïve human CD4+ T cells. Interestingly, *Hp* gastric cancer strains led to significant induction of T regulatory cells (CD4+ CD25hi FoxP3+), after 7 days of culture (**Fig. 3**). These T cells suppress aberrant immune responses against self-antigens, but they have been noted to infiltrate tumors and also suppress anti-tumor immune response, which allow tumors to escape immune attack. Notch receptors and their ligands are transmembrane glycoproteins involved in cell-cell communication that deliver signals which direct cell differentiation. There are four Notch receptor paralogs (Notch 1 to Notch 4) and five 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 recent studies exposed important roles

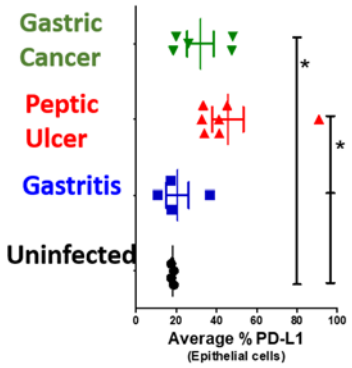

Fig. 1. Expression of Negative immune check point regulators in human gastric organoids infected with *Hp* strains. Each dot represents a *Hp* Strain. Expression of PD-L1 measured by flow cytometry

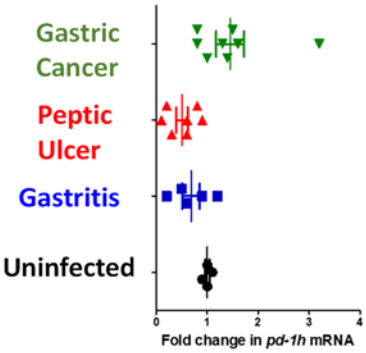

Fig 2. Expression of pd-1h immune check point regulators in gastric organoids infected with Hp strains. Each dot represents a different Hp Strain. Expression of pd-1h measured by qPCR

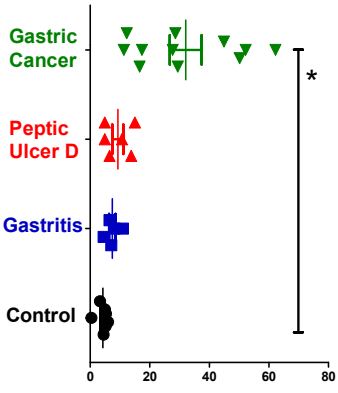

Fig. 3. *Hp* gastric cancer strains led to a significant increase of Treg cells (CD25hi FoxP3+).

for Notch signaling in differentiation of näive CD4 T cells into the different effector Th subsets by regulating subset-specific transcription factors and cytokines.Because the Notch pathway has an important role in orchestrating CD4+ Th cell differentiation, we examined how panels of *Hp* isolates from GC, PUD and gastritis affected Notch receptor and ligand expression by human gastric organoids. By RT-PCR analysis we noted that GC *Hp* isolates induced marked expression of Notch 3 and 4 and DLL4 by human gastric organoids while *Hp* isolates from non-cancer cases had a smaller effect on the expression of mRNA for these proteins (**Fig. 4**). The increased expression of these proteins on GECs infected by GC *Hp* isolates and not by PUD and gastritis *Hp* was also noted by flow cytometry. Thus, the data obtained and summarized herein is very promising regarding how gastric cancer *Hp* isolates differ from other *Hp* isolates and may help explain why some *Hp*-infected individuals develop cancer and others do not. These findings, in turn, may pave the wave to the development of diagnostic reagents and possibly treatment targets to reduce the burden of gastric cancer in those infected with *Hp*.

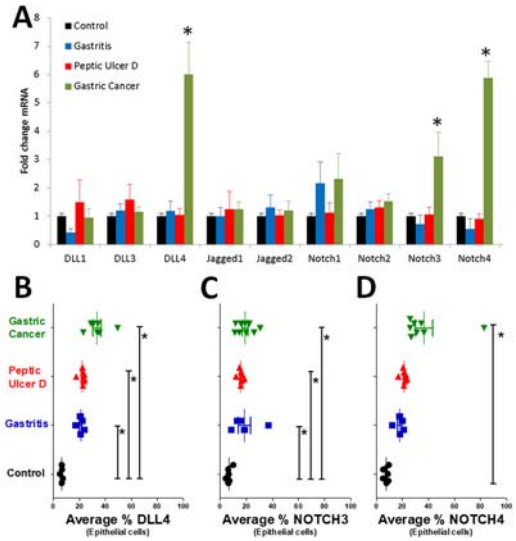

Fig 4. GECs in hGOs infected with strains express different levels of Notch receptor/ligands.

- **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. It is still early to tell, but we anticipate the findings will have an impact on prevention of gastric cancer and/or patient care.*

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report

5. CHANGES/PROBLEMS: *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

- **Changes in approach and reasons for change**

There were no substantial changes in the proposed plan. The only changes were how the tasks were organized with a major focus on major task 3 to examine panels of different isolates from

different gastric diseases that included gastric cancer versus non-cancer (gastritis and peptic ulcer disease) isolates and their induction of biological responses planned such as expression of members of the immune checkpoint regulators responsible for immune suppression as a mechanism of immune escape and markers associated with pre-cancerous changes.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The delays we encountered were associated with the final approval of the IRB by the DoD, which prevented us from acquiring the fresh biopsy specimens for isolation of disease-associated *Hp* and limited us to using bacterial isolates that we already had available, but whose origin is well characterized regarding disease.

- **Changes that had a significant impact on expenditures**

There were no intended or planned changes on our part. However, in the pursuit of answers to the interesting biological responses by epithelial and gastric organoid cultures the reagents and flow cytometry costs depleted our funds. We also ran into difficulties receiving an invoice from the collaborator's institution, not her fault but the financial side. Our post-award specialists tried repeatedly to get a response which never was received and that resulted in unspent funds associated with the subcontract and which we could have used for the studies.

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

There were no deviations. The subject protocol was approved by the BCM Institutional Review Board (IRB) on 26 October 2018. Approval from M CIV USARMY MEDCOM USAMRMC (US) was received on December 21, 2018.

- **Significant changes in use or care of human subjects - None**

- **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: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

- **Journal publications.**

Lina, T.T., Gonzalez, J., Pinchuk, I.V., Beswick, E.J., Reyes, V.E. *H. pylori* elicits B7H3 expression on gastric epithelial cells: implications in local T cell regulation and subset development during infection. Clin Oncol Res. 2019. DOI: 10.31487/j.COR.2019.05.05. Acknowledgement of federal support (yes).

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

Reyes, V.E., Peniche-Trujillo, A.G. *Helicobacter pylori* deregulates T and B cell signaling to trigger immune evasion. Current Topics in Microbiology and Immunology 2019, 421:229-265.

- **Other publications, conference papers, and presentations.**

Alex Peniche, Mary K. Estes, Yoshio Yamaoka and Victor E. Reyes. Differential expression of PD-L1 and Th1 response of lymphocytes co-cultured with human gastric organoids infected with *Helicobacter pylori* strains isolates from different gastric pathologies. J Immunol May 1, 2018, 200 (1 Supplement) 117.12;

- **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.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life.

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

o What individuals have worked on the project?

▪ *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	Victor E. Reyes, PhD
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	1234567
Nearest person month worked:	2.4 months per year
Contribution to Project:	As the Principal Investigator, he had the overall organizational, and scientific responsibility for this grant, supervised trainees in his lab and communicated with collaborators.
Funding Support:	Department of Defense

Name:	Milena Gould-Suarez
Project Role:	Consortium Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to 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*.
Funding Support:	N/A

Name:	Yuriy Fofanov, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	241077
Nearest person month worked:	0.48 months per year
Contribution to Project:	He was responsible for the analysis and interpretation of next generation DNA sequencing (NGS) data. He was consulted on selection of methods to analyze genomic data.
Funding Support:	Department of Defense

Name:	Iryna Pinchuk, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	192921
Nearest person month worked:	0.6 months per year
Contribution to Project:	She was responsible for developing the local IRB protocol and the IRB protocol by Dr. Suarez-Gould at Baylor in conjunction with Dr. Powell. She maintained communications from the IRB and Dr. Gould regarding the protocols. Her expertise in the isolation of the mucosal cells from GI human mucosa was needed as part of the studies and she helped with the training of the postdoctoral fellow.
Funding Support:	Department of Defense

Name:	Don Powell, MD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	050842
Nearest person month worked:	0.24 months in Y1
Contribution to Project:	As a clinician and Director of the Division of Gastroenterology, he was consulted during the IRB protocol development and revisions.
Funding Support:	N/A

Name:	Levent Albayrak
Project Role:	Programmer
Researcher Identifier (e.g. ORCID ID):	241231
Nearest person month worked:	1.2 months per year
Contribution to Project:	He was tasked with development of computational tools to quickly and efficiently identify highly specific and robust signatures essential for this research.
Funding Support:	Department of Defense

Name:	George Golovko
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	241207
Nearest person month worked:	1.2 months/year that grant was active
Contribution to Project:	He was 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:	Department of Defense

Name:	Kamil Khanipov
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	241236
Nearest person month worked:	1.2 months/year that grant was active
Contribution to Project:	He was responsible for management, filtering and preparation of data for downstream analysis as well as testing and debugging the tools..
Funding Support:	Department of Defense

Name:	Alex Giovanny Peniche-Trujillo, PhD
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	236476
Nearest person month worked:	12 months per year
Contribution to Project:	Day to day experimental planning and execution. Maintenance of bacterial cultures, cell lines and organoid cultures.
Funding Support:	Department of Defense

Name:	Karen Zhang
Project Role:	2nd year medical student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 month
Contribution to Project:	Experiment performance.
Funding Support:	T32 training grant

Name:	Esaias Tong
Project Role:	2nd year medical student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 month
Contribution to Project:	Experiment performance.
Funding Support:	T32 training grant

o **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.

o **What other organizations were involved as partners?**

Nothing to report.

▪ *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*
Provide the following information for each partnership:

▪ **Organization Name:** Baylor College of Medicine

▪ **Location of Organization:** *Houston, TX*

▪ **Partner's contribution to the projec**t *(identify one or more)*

▪ **Financial support;**

▪ **In-kind support** *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;

▪ **Facilities** *(e.g., project staff use the partner's facilities for project activities)*;

▪ **Collaboration** *Dr. Milena Suarez-Gould was the collaborator in charge of preparing the local IRB and collection of biopsy samples.*

▪ **Personnel exchanges** *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*; and

▪ **Other.**

8. SPECIAL REPORTING REQUIREMENTS

o **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating 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://ebrap.org for each unique award.*

o **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. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

VFclass	Virulence factors	Related genes	H.pylori 26695 (NC_000915)	GSHN91	GSHC93	GSHC94	GSHC95	GSHC100	DU2	DU5	J99	DU15	PMSS1	CA65	GCHN114
				Gastritis	Gastritis	Gastritis	Gastritis	Gastritis	PUD	PUD	PUD, duodenu	PUD, duodenum	PUD, duodenum	Cancer	Cancer
Acid	Urease	ureA	HP0073	+	+	+	+	+	+	+	+	+	+	+	+
		ureB	HP0072	+	+	+	+	+	+	+	+	+	+	+	+
		ureE	HP0070	+	+	+	+	+	+	+	+	+	+	+	+
		ureF	HP0069	+	+	+	+	+	+	+	+	+	+	+	+
		ureG	HP0068	+	+	+	+	+	+	+	+	+	+	+	+
		ureH	HP0067	+	+	+	+	+	+	+	+	+	+	+	+
		ureI	HP0071	-	+	+	+	+	+	+	+	+	+	+	+
Adherence	AlpB (hopB)	alpB/hopB	HP0913	+	+	+	+	+	+	+	+	+	+	+	+
	Blood group antigen binding adhesins	babA/hopS	HP1243	-	-	-	-	2+	-	+	+	-	+	-	+
		babB/hopT	HP0896	+	-	-	-	-	-	+	+	-	+	-	+
	H. pylori adhesin A	hpaA	HP0797	+	+	+	+	+	+	+	+	+	-	+	+
	HopZ	hopZ	HP0009	2+	+	2+	+	2+	+	+	+	+	2+	+	+
	HorB	horB	HP0127	+	+	+	+	+	+	+	+	+	+	+	+
	PEB1	peb1	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sialic acid binding adhesins	sabA/hopP	HP0725	-	+	+	+	+	+	+	+	+	-	-	+
		sabB/hopO	HP0722	-	-	-	-	-	-	-	+	-	-	-	-
	adherence-associated lipoprotein AlpA (hopC)	alpA/hopC	HP0912	+	+	+	+	+	+	+	+	+	+	+	+
Immune evasion	Lipopolysaccharide Lewis antigens	futA	HP0379	+	-	-	+	+	-	-	-	-	-	-	-
		futB	HP0651	+	-	-	+	-	-	-	2+	+	2+	-	-
		futC	HP0093*	+	+	+	+	+	+	+	+	+	+	2+	+
Immune modulator	Neutrophil-activating protein (HP-NAP)	napA	HP0243	+	+	+	+	+	+	+	+	+	+	+	+
	Outer inflammatory protein	oipA/hopH	HP0638	+	-	-	+	+	-	-	+	-	+	-	-
Motility	Flagella	flaA	HP0601	+	+	+	+	+	+	+	+	+	+	+	+
		flaB	HP0115	+	+	+	+	+	+	+	+	+	+	+	+
		flaG	HP0751	+	+	+	+	+	+	+	+	+	+	+	+
		flaG	HP0327	-	-	-	-	-	-	-	-	-	-	-	-
		flgA	HP1477	+	+	+	+	+	+	+	+	+	+	+	+
		flgB	HP1559	+	+	+	+	+	+	+	+	+	+	+	+
		flgC	HP1558	+	+	+	+	+	+	+	+	+	+	+	+
		flgD	HP0907	+	+	+	+	+	+	+	+	+	+	+	+
		flgE_1	HP0870	+	+	+	+	+	+	+	+	+	+	+	+
		flgE_2	HP0908	+	+	+	+	+	+	+	+	+	+	+	+

VFclass	Virulence factors	Related genes	H.pylori 26695 (NC_000915)	GSHN91	GSHC93	GSHC94	GSHC95	GSHC100	DU2	DU5	J99	DU15	PMSS1	CA65	GCHN114
		flgG_1	HP1092	+	+	+	+	+	+	+	+	+	+	+	+
		flgG_2	HP1585	+	+	+	+	+	+	+	+	+	+	+	+
		flgH	HP0325	+	+	+	+	+	+	+	+	+	+	+	+
		flgI	HP0246	+	+	+	+	+	+	+	+	+	+	+	+
		flgK	HP1119	+	+	+	+	+	+	+	+	+	+	+	+
		flgL	HP0295	+	+	+	+	+	+	+	+	+	+	+	+
		flhA	HP1041	+	+	+	+	+	+	+	+	+	+	+	+
		flhB_1	HP0770	+	+	+	+	+	+	+	+	+	+	+	+
		flhB_2	HP1575	+	+	+	+	+	+	+	+	+	+	+	+
		flhF	HP1035	+	+	+	+	+	+	+	+	+	+	+	+
		fliA	HP1032	+	+	+	+	+	-	+	+	+	+	+	+
		fliD	HP0752	+	+	+	+	+	+	+	+	+	+	+	+
		fliE	HP1557	+	+	+	+	+	+	+	+	+	+	+	+
		fliF	HP0351	+	+	+	+	+	+	+	+	+	+	+	+
		fliG	HP0352	+	+	+	+	+	+	+	+	+	+	+	+
		fliH	HP0353	+	+	+	+	+	+	+	+	+	+	+	+
		fliI	HP1420	+	+	+	+	+	+	+	+	+	+	+	+
		fliL	HP0809	+	+	+	+	+	+	+	+	+	+	+	+
		fliM	HP1031	+	+	+	+	+	+	+	+	+	+	+	+
		fliN	HP0584	+	+	+	+	+	+	+	+	+	+	+	+
		fliP	HP0685	+	+	+	+	+	+	+	+	+	+	+	+
		fliQ	HP1419	+	+	+	+	+	+	+	+	+	+	+	+
		fliR	HP0173	+	+	+	+	+	+	+	+	+	+	+	+
		fliS	HP0753	+	+	+	+	+	+	+	+	+	+	+	+
		fliY	HP1030	+	+	+	+	+	+	+	+	+	+	+	+
		motA	HP0815	+	+	+	+	+	+	+	+	+	+	+	+
		motB	HP0816	+	+	+	+	+	+	+	+	+	+	+	+
		pflA	HP1274	+	+	+	+	+	+	+	+	+	+	+	+
Others	DupA (duodenal ulcer promoting)	dupA	-	-	-	-	-	-	-	-	-	-	-	-	-
	Plasticity region	Undetermined	-	+	-	-	-	-	-	-	+	-	-	+	-
		Undetermined	-	+	-	-	-	-	-	-	+	-	-	+	-
		Undetermined	-	+	-	-	-	-	-	-	+	-	-	+	-
Secretion system	Cag PAI type IV secretion system	cag1	HP0520	+	+	+	-	-	+	+	+	+	+	+	+
		cag2	HP0521*	-	-	-	-	-	-	-	+	-	-	-	-
		cag3	HP0522	+	+	+	-	-	+	+	+	+	+	+	+
		cag4	HP0523	+	+	+	-	-	+	+	+	+	+	+	+
		cag5	HP0524	+	+	+	-	-	+	+	+	+	+	+	+

VFclass	Virulence factors	Related genes	H.pylori 26695 (NC_000915)	GSHN91	GSHC93	GSHC94	GSHC95	GSHC100	DU2	DU5	J99	DU15	PMSS1	CA65	GCHN114
		cagC	HP0546	-	+	+	-	-	+	+	+	+	-	-	+
		cagD	HP0545	+	+	+	-	-	+	+	+	+	+	+	+
		cagE	HP0544	+	+	+	-	-	+	+	+	+	+	+	+
		cagF	HP0543	+	+	+	-	-	+	+	+	+	+	+	+
		cagG	HP0542	+	+	+	-	-	+	+	+	+	+	+	+
		cagH	HP0541	+	+	+	-	-	+	+	+	+	+	+	+
		cagI	HP0540	+	+	+	-	-	+	+	+	+	+	+	+
		cagL	HP0539	+	+	+	-	-	+	+	+	+	+	+	+
		cagM	HP0537	+	+	+	-	-	+	+	+	+	+	+	+
		cagN	HP0538	+	+	+	-	-	+	+	+	+	+	+	+
		cagP	HP0536	-	-	-	-	-	-	-	-	-	-	-	-
		cagQ	HP0535	-	-	-	-	-	-	-	-	-	-	-	-
		cagS	HP0534	+	+	+	-	-	+	+	+	+	+	+	+
		cagT	HP0532	-	+	-	-	-	+	+	+	+	+	+	+
		cagU	HP0531	+	+	+	-	-	+	+	+	+	+	+	+
		cagV	HP0530	+	+	+	-	-	+	+	+	+	+	+	+
		cagW	HP0529	+	+	+	-	-	+	+	+	+	+	+	+
		cagX	HP0528	+	+	+	-	-	+	+	+	+	+	+	+
		cagY	HP0527	+	+	+	-	-	-	-	+	-	+	-	-
		cagZ	HP0526	+	+	+	-	-	+	+	+	+	+	+	+
		virB11	HP0525	+	+	+	-	-	+	+	+	+	+	+	+
	T4SS effectors cytotoxin-associated gene A	cagA	HP0547	+	+	+	-	-	+	+	+	+	4+	+	+
Toxin	Cytolethal distending toxin	cdtA	-	-	-	-	-	-	-	-	-	-	-	-	-
		cdtB	-	-	-	-	-	-	-	-	-	-	-	-	-
		cdtC	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vacuolating cytotoxin	vacA	HP0887	+	+	+	+	+	+	+	+	+	+	+	+

(+) denote gene presence

(-) denote gene absence

(2+) denote have 2 copies of the same gene

(4+) denote have 4 copies of the same gene

The Journal of Immunology

Differential expression of PD-L1 and Th1 response of lymphocytes co-cultured with human gastric organoids infected with *Helicobacter pylori* strains isolates from different gastric pathologies

Alex Peniche, Mary K. Estes, Yoshio Yamaoka and Victor E. Reyes

J Immunol May 1, 2018, 200 (1 Supplement) 117.12;

Abstract

Helicobacter pylori (*Hp*) bacteria successfully establishes chronic infection leading to chronic gastritis, peptic ulcer disease (PUD), and gastric cancer (GC). Since we have previously shown that *Hp* hijacks expression of checkpoint immunoregulators (i.e., PD-L1), we hypothesized that strains from different gastric pathologies differ in their ability to evade the host response. We used human gastric organoids (hGOs), which recapitulate polarized epithelium, gastric gland and pit cell markers observed in the stomach. We cultured hGOs in transwell inserts which were exposed to *Hp* on the apical surface and cultured with naïve T cells on the basolateral side of polarized epithelial cells, thereby reproducing the interactions observed *in vivo*. After 7 days of culture, we recovered all cells and evaluated levels of PD-L1, B7-H3, B7-H4, and CTLA4; and in $CD4^+$ T cells markers of Th1, Th2, Th17 and T_{reg}. Infection of hGOs with *Hp* strains isolated from cases of gastritis, PUD or GC, all led to increased expression of negative immune checkpoint regulators, predominantly PD-L1, which binds PD-1 on T cells and promotes loss of effector functions, apoptosis, and reduced T cell-target cell contact.

expression of immune checkpoint regulators and $CD4^+$ T cell differentiation in the context of strains comparison may provide insights in differential expression of candidate biomarkers of disease and future therapies.

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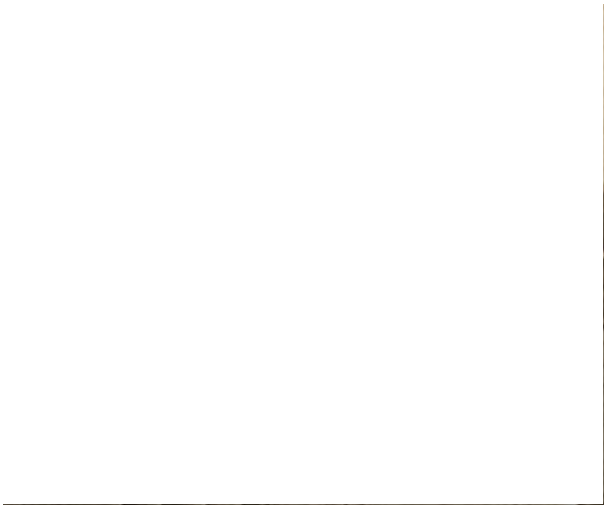

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Research Article

Helicobacter pylori Elicits B7-H3 Expression on Gastric Epithelial Cells: Implications in Local T Cell Regulation and Subset Development During Infection

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ABSTRACT

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that infects more than 50% of humanity and is associated with gastritis, peptic ulcer and gastric cancer. Although $CD4^+$ T cells are recruited to the gastric mucosa, the host is unable to clear the bacteria. Previously, we demonstrated that *H. pylori* infection upregulates the expression of the T cell co-inhibitory molecule B7-H1 while simultaneously downregulating the expression of T cell co-stimulatory molecule B7-H2 on gastric epithelial cells (GEC), which together affect the Treg and Th17 cell balance and foster bacterial persistence. Because B7-H3, another member of the B7 family of co-inhibitory receptors, has been found to have important immunoregulatory roles and in cancer, in this study we examined the expression of B7-H3 molecules on GEC and how the expression is regulated by *H. pylori* during infection. Our study showed that both human and murine GEC constitutively express B7-H3 molecules, but their expression levels increased during *H. pylori* infection. We further demonstrated that *H. pylori* uses its type 4 secretion system (T4SS) components CagA and cell wall peptidoglycan (PG) fragment to upregulate B7-H3. Th17 cells and Treg cells which are increased during *H. pylori* infection also had an effect on B7-H3 induction. The underlying cell signaling pathway involves modulation of p38MAPK pathway. Since B7-H3 were shown to up-regulate Th2 responses, the phenotype of T cell subpopulations in mice infected with *H. pylori* PMSS1 (contains functional T4SS) or SS1 (cannot deliver CagA into GEC) strains were characterized. A mixed Th1/Th2 response in *H. pylori* infected mice was observed. Consistent with previous findings, increased Treg cells and decreased Th17 cells in MLN of PMSS1 infected mice compared to SS1 infected mice was observed. Human biopsy samples collected from gastritis biopsies and gastric tumors showed a strong association between increased B7-H3 and Th2 responses in *H. pylori* strains associated with gastritis. T cell: GEC co-cultures and anti-B7-H3 blocking Ab confirmed that the induction of Th2 is mediated by B7-H3 and associated exclusively with an *H. pylori* gastritis strain not cancer or ulcer strains. In conclusion, these studies revealed a novel regulatory mechanism employed by *H. pylori* to influence the type of T cell response that develops within the infected gastric mucosa.

Introduction

Helicobacter pylori (*H. pylori*) colonizes the human gastric mucosa and may induce gastritis, peptic ulcer and two forms of neoplasia: gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [1]. Epidemiological data suggest that 60-90% of gastric cancer cases are caused by *H. pylori* [2, 3]. Patients infected with CagA (cytotoxin associated gene A)-positive *H. pylori* strains have an elevated

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risk of developing peptic ulcer and gastric cancer [4, 5]. CagA is the only known effector protein produced by the *H. pylori cag* PAI (*cag* pathogenicity island), which is a 40 KDa chromosomal region that contains the genes that code for structural components of the type 4 secretion system (T4SS). T4SS is a molecular syringe-like structure. Upon attachment of *H. pylori* to gastric epithelial cells (GEC), CagA is injected via the T4SS and consequently becomes phosphorylated in the tyrosine residue of their EPIYA motifs by host Src kinases and c-Ab1 [6-10]. Both phosphorylated and unphosphorylated forms of CagA can interact with a range of host cell signaling proteins and activates them, which results in several physiological changes in GECs [11-13]. CagA alone has been shown to act as a oncoprotein since transgenic mice expressing *H. pylori* CagA develop multiple types of neoplasms [62]. In addition to CagA, *H. pylori* also translocates via the T4SS its cell wall peptidoglycan (PG) fragments, which are recognized by intracellular pattern recognition receptor NOD1 and activates MAPKs and NFkB pathways [14-16].

B7-H3 (CD276) is a newer member of the B7 family that shares 20–27% identical amino acids with other members of this family of receptors [17]. Human B7-H3 protein is not constitutively expressed but can be induced in activated dendritic cells, B cells, T cells, NK cells and in some tumor cell lines [17-20]. B7-H3 has been shown to be strongly expressed in unstimulated tracheal, bronchial, and alveolar epithelial cells, and the expression was induced by respiratory syncytial virus (RSV) infection [21]. B7-H3 was initially identified as a co-stimulatory molecule that was shown to promote T-cell proliferation and IFN-γ production [17]. However, recent studies have presented contradictory roles for B7-H3, since they suggest that B7-H3 has both immunological stimulatory and inhibitory functions [17-20, 22-25]. For instance, in conjunction with anti-CD3, B7-H3-Ig fusion protein co-stimulates $CD4^+$ and $CD8^+$ T cells and induces IFN-γ production. Other independent studies demonstrated that acute and chronic cardiac allograft rejection is reduced in B7-H3 knockout mice, which further support a stimulatory role for B7-H3 on T cells [25]. In contrast, B7-H3 has been reported to impair T-helper (Th)1 cell responses and inhibit cytokine production [22]. An *in vivo* study also showed an inhibitory role of B7-H3 [19, 22, 24]. B7-H3 not only affects T cell activation /inactivation but a recent study in an asthma model showed that B7-H3 also plays a role in the induction of Th2 cells [26]. Moreover, other than its role in regulating T cell activity and subset development, it may also serve as a biomarker for tumor progression and development of cancer. Higher expression of B7-H3 has been shown in different types of cancer [27-31]. An increased expression of B7-H3 was reported to lead to an increased risk of recurrence of some cancers, while increased B7-H3 expression is in sometimes linked with prospective survival in other cancers [27-31]. Recently increased B7-H3 expression was shown in circulating tumor cells in gastric cancer patients compared to healthy volunteers. Moreover, patients with increased B7-H3 levels showed lower survival rates [32]. However, a separate study reported that increased B7-H3 during gastric cancer was associated with increased survival rate [31]. Together, these observations suggested that B7-H3 might be also involved in cancer immunity and B7-H3 may also influence cancer progression beyond its immunoregulatory roles.

H. pylori usually causes chronic infection. Though the host mounts an increased $CD4^+$ T cell response but those T cells are hyporesponsive. During *H. pylori* infection, patients have a mixed Th1/Th2 response, with increased Treg and Th17 cells in their circulation [33-39]. Though there are reports showing the type of T cell responses elicited by *H. pylori* infection, there is a gap in our knowledge regarding the mechanism that *H. pylori* uses to induce different phenotypic subsets of T cells. Previously our group has shown that *H. pylori* modulates B7 molecule expression in GECs, which not only help restrain T cells responses, but also induce T regulatory (Treg) cells to assist in *H. pylori* survival [40-42]. Our data also showed that *H. pylori* uses its T4SS to downregulate B7-H2 expression in GEC, which helps to keep Th17 cells in suboptimal levels, since Th17 are important in the control of extracellular bacterial infections, this downregulation of B7-H2 helps *H. pylori* to persist [41]. We also demonstrated that *H. pylori*-mediated up-regulation of B7-H1 expression in GEC causes induction of Treg cells, which contributes to the establishment of a chronic infection [42]. In this study, we investigated another important B7 molecule, B7-H3 and showed that *H. pylori* upregulates the expression of this molecule on GEC. The upregulation of B7-H3 is regulated not only by the T4SS but also by the cytokines produced by Th17 and Treg cells. We further evaluated the underlying cell signaling pathway and demonstrate that *H. pylori* uses the p38 MAPK pathway for B7-H3 upregulation.

H. pylori is one of the most genetically diverse bacterial species. *H. pylori* strains differ in the rate with which they have *cag* PAI in their genome. The EPIYA motifs in *cagA* gene also differs between Asian and western countries. Moreover, *H. pylori* infection may result in gastritis, ulcer and gastric cancer development. We examined how *H. pylori* strains isolated from these three types of gastric diseases modulate B7-H3 expression on epithelial cells. In this study we were interested to determine whether the increase of B7-H3 is consistent with all strains or not. Using different *H. pylori* strains and patient samples from gastritis and tumors we have shown that only *H. pylori* strains associated with gastritis causes increased B7-H3 expression and induction of the $GATA3^+$ Th2 cell response. This finding was further confirmed by co-culturing GECs infected with different *H. pylori* strains with naïve $CD4^+$ T cells. This is a novel finding which shows how *H. pylori* manipulates GECs to differentially express the B7-H3 molecules and thus regulates T cell responses involved in the *H. pylori* associated immune-pathogenesis to promote bacterial persistence. Future studies will examine how these findings may be applied in vaccine efforts against *H. pylori* and possibly in prevention or treatment of gastric cancer.

Material and Methods

I Human tissue

Gastric antrum biopsy specimens were obtained from consenting patients undergoing gastro-esophageal-duodenoscopy in accordance with an approved Institutional Review Board protocol. GECs were isolated from the biopsy specimens as described previously [41]. Patients were considered infected if *H. pylori* was detected by rapid urease testing, histopathology, and by culture of *H. pylori* from biopsies.

II Cell lines, bacterial cultures and small peptides

Human GECs N87 and AGS were obtained from the American Type Culture Collection (ATCC) and HGC-27 was obtained from RIKEN, The Institute of Physical and Chemical Research, Japan. All cell lines

were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. As representative murine GEC, Immortomouse stomach epithelium (ImSt) cells were maintained in media described by Whitehead et al. [43]. *H. pylori* strains 51B and 26695 as well as their corresponding isogenic *cagA* and *cag* PAI mutants were described previously [41, 44]. *H. pylori* LC-11 and CA8 were originally isolated from the antral mucosa of a patient with duodenal ulcer and gastric cancer, respectively, as previously described, Tryptic soy agar (TSA) plates supplemented with 5% sheep's blood (Becton Dickinson, San Jose, CA) were used to grow *H. pylori* strains [45, 46]. Blood agar plates with 2.5 μg/ml of chloramphenicol (Technova, Hollister, CA) were used to maintain *cagA*$^-$ and *cag* PAI$^-$ strains at 37°C under microaerophilic conditions [41]. For the infection of mice *H. pylori* Sydney strain 1 (SS1) and PM-SS1 (pre-mouse SS1) were used, which were provided by Drs. J. Pappo (Astra) and Richard Peek (Vanderbilt Univ.), respectively [47]. iEDAP (InvivoGen, San Diego, USA), a PG-like molecule that is a NOD1 ligand, was used to investigate the role of PG in B7-H3 expression.

III Animals

Female six-to-eight week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used in the model of gastric *H. pylori* infection. Animals were tested negative for the intestinal *Helicobacter* spp. prior to their use in the experiments.

IV Flow cytometry

APC-conjugated anti-human B7-H3 (clone 185504) and isotype controls were purchased from R&D Systems. T cells from co-culture assays, described below, were stained for CD25, FoxP3, RORγ, Tbet and GATA3 for analysis by flow cytometry using a protocol described previously [42]. Mouse anti-human CD25-PECy7, FoxP3-Alexafluor 488, Tbet-PerCPCy5.5, Gata3-eFluor 660 were used for staining. The viability dye eFluor 780 (eBioscience, San Diego, CA, USA) was included in the experiments to gate on viable cells. Cells were analyzed by flow cytometry on a LSRII instrument. The data were analyzed with BD FACSDiva software (BD Biosciences, San Jose, CA) and FlowJo (Tree Star, Inc, Ashland, OR).

V Cell signaling inhibitors

NFκB inhibitor, CAY10512 (10 μM; Cayman Chemical, MI); JAK/STAT3 inhibitor AG-490 (100 ng/mL; Enzo Life Sciences, Farmingdale, NY), PI3K inhibitor, Wortmannin (100 nM; Calbiochem, Billerica, MA); and p38 MAPK inhibitor, PD169316 (10 μM/mL; Cayman Chemical, MI) were used to inhibit intracellular signaling.

VI Real-time RT-PCR

Real-time RT-PCR analysis was performed as previously described [41].

VII Murine infection and detection of B7-H3, FoxP3, RORyt, Tbet and GATA3 expression

C57BL/6 mice were orogastrically inoculated with 10^8 CFU (in 100 μL of PBS/inoculation) of *H. pylori* SS1 or PMSS1 strains, three times over a week. Four weeks later mice were euthanized, mesenteric lymph node (MLN) were removed, homogenized, mRNA was isolated and expression of FoxP3, RORγt, Tbet, GATA3, IL-10, IFN-γ, IL-4 and IL-17A were determined using RT-PCR.

VIII T cell isolation and co-culture with GEC

Naïve CD4$^+$ T cells were isolated from human peripheral blood as previously described [48]. GEC-T cell co-cultures were established as described earlier [41]. Briefly, GECs were preinfected with *H. pylori* CA8 (cancer strain), *H. pylori* 51B (gastritis strain) and *H. pylori* LC-11 (ulcer strain). After 8 h of infection GECs were washed and co-cultured with $1x10^6$ T cells to obtain 3:1 T cell:GEC ratio and incubated for 5 days at 37º C with 5% CO_2. For blocking, anti-B7-H3 blocking antibody or isotype (rat IgG2a κ) control (1 μg/mL, functional grade from eBioscience) were added to GECs 1 h before co-culture.

IX Bio-Plex

The levels of IL-4 from T cell-GEC co-culture were measured using Luminex array (Millipore, Billerica, MA, USA) according to the manufacturer´s instruction. Samples were analyzed using Bio-Plex Manager software (Bio-Rad).

X Statistical analysis

The results were expressed as the mean ± SE of data obtained from at least three independent experiments done with triplicate sets per experiment unless otherwise indicated. Differences between means were evaluated by analysis of variance (ANOVA) using student *t* test for multiple comparisons and considered significant if p was <0.05.

Results

I Expression of B7-H3 on gastric biopsies

To determine the expression of B7-H3 in relation to *H. pylori* infection we isolated GECs from biopsy samples, which were collected from *H. pylori* infected or from healthy individuals. B7-H3 expression was measured by real time RT-PCR after mRNA was extracted from the samples. Our RT-PCR data showed a strong upregulation of B7-H3 expression in the *H. pylori* infected biopsies compared to uninfected samples (Figure 1A).

II *H. pylori* T4SS regulate B7-H3 expression on GEC during infection

To evaluate whether B7-H3 upregulation is a direct effect of *H. pylori* infection and not an indirect result of inflammatory changes in the host, we used GEC lines and infected them with *H. pylori*. Since *H. pylori* T4SS has the capacity to modulate GEC homeostasis and because we have seen their effect in the modulation of B7-H1 and B7-H2 molecules, we used *H. pylori* 51B wild type (WT) and *H. pylori* 51B *cag* PAI mutant strain to infect GEC (N87 cells) [41, 49]. B7-H3 expression was measured after 24-hr infection using flow cytometry. A significant upregulation of B7-H3 expression in GEC infected with *H. pylori* WT but not with *H. pylori cag* PAI mutant strains was observed, suggesting

that *H. pylori* T4SS plays role in B7-H3 induction (Figure 1B). To further dissect the role of the effector protein CagA *H. pylori* 51B *cagA* mutant was used to infect GEC along with the *H. pylori* 51B WT strain. Both flow cytometry and RT-PCR data showed that CagA influences B7-H3 upregulation, since in the absence of CagA B7-H3 expression by GECs remained at basal levels (Figure 1C, D). These results were also confirmed in different cell lines (AGS, HGC-27) and by using *H. pylori* 26695 WT and the corresponding isogenic mutant strains (not shown). Furthermore, a murine cell line was used to confirm these findings and to evaluate whether murine GEC express B7-H3 and whether this expression is regulated by *H. pylori* T4SS or not, before using a murine model. To this end, the murine GEC line (ImSt) were infected with *H. pylori* PMSS1, which contains a functional T4SS and with *H. pylori* SS1 strain in which the T4SS is defective and cannot deliver CagA into GEC. Flow cytometry data showed a significant upregulation of B7-H3 expression in murine GEC infected with *H. pylori* PMSS1 strain but not with the SS1 strain (Figure 1E). Overall, these data demonstrated a strong correlation between the presence of T4SS, more specifically of the CagA oncoprotein, and induction of B7-H3 expression on GEC.

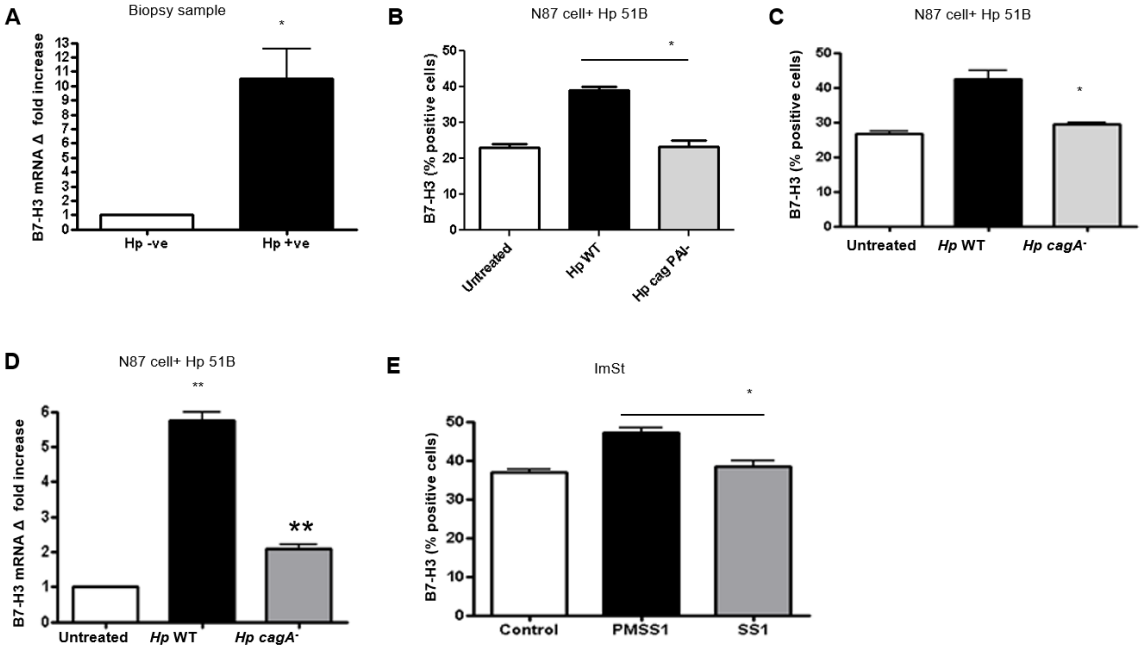

Figure 1: *H. pylori* T4SS up-regulates B7-H3 expression on GECs. **(A)** Gastric biopsy samples were collected from *H. pylori*-positive patients and healthy individuals, GECs were collected and analyzed for B7-H3 mRNA expression by real-time RT-PCR. N87 cells were infected with **(B)** *H. pylori* 51B WT and *cag* PAI$^-$ or with **(C)** *H. pylori* 51B WT and *cagA*$^-$ for 24 h. The surface expression of B7-H3 was determined by using immunostaining followed by flow cytometry. **(D)** N87 cells were infected with *H. pylori* 51B WT and *cagA*$^-$ for 2 h, and B7-H3 mRNA expression was analyzed by using RT-PCR. mRNA levels for B7-H3 were normalized to 18S and compared to the level of B7-H3 mRNA of untreated N87 cells. **(E)** Murine GECs (ImSt) were infected for 24 h with *H. pylori* PMSS1, which has a functional CagA delivery system, or with *H. pylori* SS1, lacking a CagA delivery system. Surface expression of B7-H3 was determined by flow cytometry. The data were expressed as a percentage of positive cells. The means ± SD are shown as the results of duplication of one of four representative experiments, n=8, $*P < 0.05$.

III Role of PG in B7-H3 upregulation

Along with CagA, *H. pylori* T4SS also translocates PG fragments into GECs, which are recognized by NOD1 and cause activation of cell signaling pathways that result in inflammatory mediator release [14-16, 50]. Further, in *H. pylori* infection, NOD1 is up-regulated and associated with higher inflammation in GC [63]. To determine the involvement of PG in B7-H3 upregulation GECs were treated with iEDAP, which is a PG analogue recognized by NOD1 ligand. B7-H3 expression was significantly upregulated in mRNA level (Figure 2A) after iEDAP stimulation. Flow cytometry was used as an independent approach to measure the upregulated surface expression of B7-H3 (Figure 2B). Kinetics data showed a progressive upregulation of B7-H3 as early as 18-hr of stimulation which peaked at 24-hr (Figure 2C) and is mediated by *H. pylori* T4SS component CagA and PG (Figure 2D).

IV *H. pylori* uses p38MAPK pathway for B7-H3 upregulation

Further analysis was done to determine the cell signaling pathway used by *H. pylori* for B7-H3 up-regulation. To that end, the cells were treated with different pharmacological inhibitors of NFκB, MAPK, STAT3, PI3K and mTOR pathways. Our data indicated that upregulation of B7-H3 by the *H. pylori* strain was blocked in the presence of PD169316, which is a p38 MAPK specific inhibitor (Figure 3). In contrast, inhibition of PI3K, mTOR, STAT3 and NFκB pathways did not affect *H. pylori* mediated upregulation of B7-H3 expression (Data not shown). These results suggest that p38 MAPK pathway is a key signaling pathway in *H. pylori*-mediated upregulation of B7-H3 on GECs.

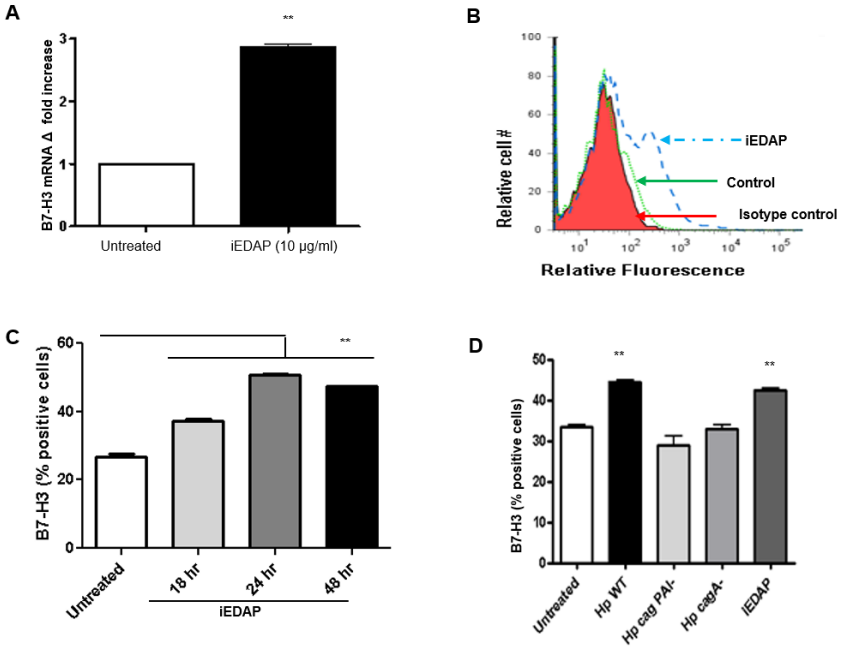

Figure 2: *H. pylori* T4SS translocated PG causes induction of B7-H3 expression by GECs. **(A)** B7-H3 mRNA expression was analyzed by using real-time quantitative RT-PCR in N87 cells. RNA was isolated from untreated and 2 h iEDAP (dipeptide present in peptidoglycan) treated (10 µg/mL) cells. mRNA levels for B7-H3 were normalized to 18S and compared to the levels of B7-H3 mRNA in untreated N87 cells. N=9, **P* < 0.05. **(B)** Flow cytometric analysis of GEC (N87) cells stained for B7-H3 after exposure to 10 µg/mL iEDAP for 24 h (in a representative histogram for AGS cells where the solid peak is the isotype control) or **(C)** for different times (18, 24 and 48 h) showed increased expression. **(D)** N87 cells were infected with *H. pylori* WT, *H. pylori cag* PAI, and *H. pylori cagA*$^-$ and stimulated with iEDAP for 24 h and B7-H3 expression was measured by flow cytometry. The means are shown as the results of duplicates in four experiments, n= 8,**P* < 0.05.

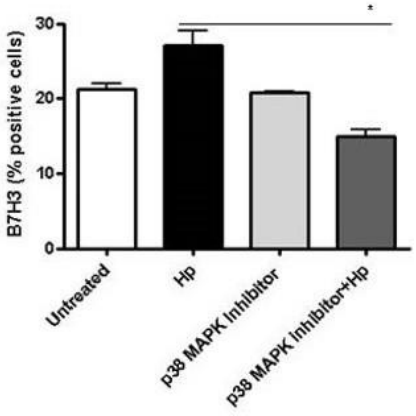

Figure 3: B7-H3 up-regulation by *H. pylori* depends on p38 MAPK pathway. B7-H3 expression on GEC was measured by flow cytometry after treating the cells with p38 MAPK inhibitor (PD169316 10 µM/ml) for 1 h and infected with *H. pylori* for 24 h. The means ± SD are shown as the results of duplicates in four experiments, n=8, * *P* < 0.05, ** *P* < 0.01 and *** *P* < 0.001.

V. B7-H3 expression is regulated by Th17 and Treg cells

Cytokines regulate the expression of immunoregulatory molecules, which allows for fine tuning of the immune response. During *H. pylori* infection there is induction of Th17 cells [37-39]. Since patients have increased circulating levels of IL-17 we sought to investigate the effect of this cytokine on B7-H3 expression. In RT-PCR analysis (Figure 4A) significant induction of B7-H3 after IL-17 (10 ng/ml) stimulation was observed. Further, the experiments showed that the expression of B7-H3 on the GECs in response to IL-17 stimulation was increased in a dose-dependent manner (1-100 ng/ml) (Figure 4B). The surface expression of this ligand was also analyzed at different time points (18-hr, 24-hr and 48-hr) after IL-17 treatment. Expression was significantly increased in GECs after 18-hr of incubation with IL-17, which remains constant after 24 h but decreases after 48 h incubation (Figure 4C).

Treg cells, which are frequently found in *H. pylori*-infected patients, produce IL-10 and TGF-β [34, 35]. Since there is bidirectional regulation of Treg cells and B7-H1, we investigated whether the hallmark cytokines produced by these cells affect B7-H3 expression [51]. To that end we stimulated GEC with either IL-10 or TGF-β alone or in combination. Both IL-10 and TGF-β induced B7-H3 expression on GECs (Figure 5A, B). Flow cytometry data also showed a cumulative effect of IL-10 and TGF-β in B7-H3 expression (Figure 5C). Taken together, these data suggested that cytokines produced by Th17 and Treg cells play an important role in B7-H3 expression in GEC. Thus *H. pylori* regulates B7-H3 expression both directly by using CagA cytotoxin and also indirectly by inducing these T cell subtypes.

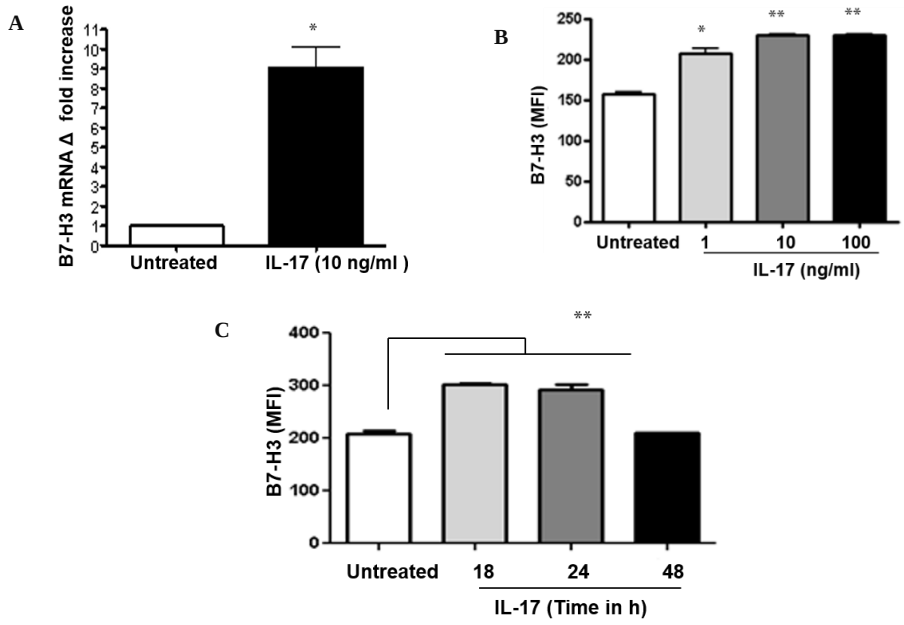

Figure 4: B7-H3 expression is regulated by IL-17. **(A)** GEC (N87) cells were treated with IL-17 (10 ng/ml) for 2 h and B7-H3 expression was measured by RT-PCR. mRNA levels for B7-H3 was normalized to 18S and compared to the levels of B7-H3 mRNA of untreated N87 cells. Kinetics and dose response of IL-17-mediated B7-H3 up-regulation was determined by treating GEC (N87) cells with **(B)** different concentrations (1, 10 and 100 ng/ml) of IL-17 for 24 h or **(C)** exposing the GEC (N87) cells to IL-17 (10 ng/ml) for different time points (18, 24 and 48 h) and measuring the B7-H3 expression by flow cytometry. The data were expressed as mean fluorescence intensity (MFI). The means ± SD are shown as the result of duplicates of one of four representative experiments: n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

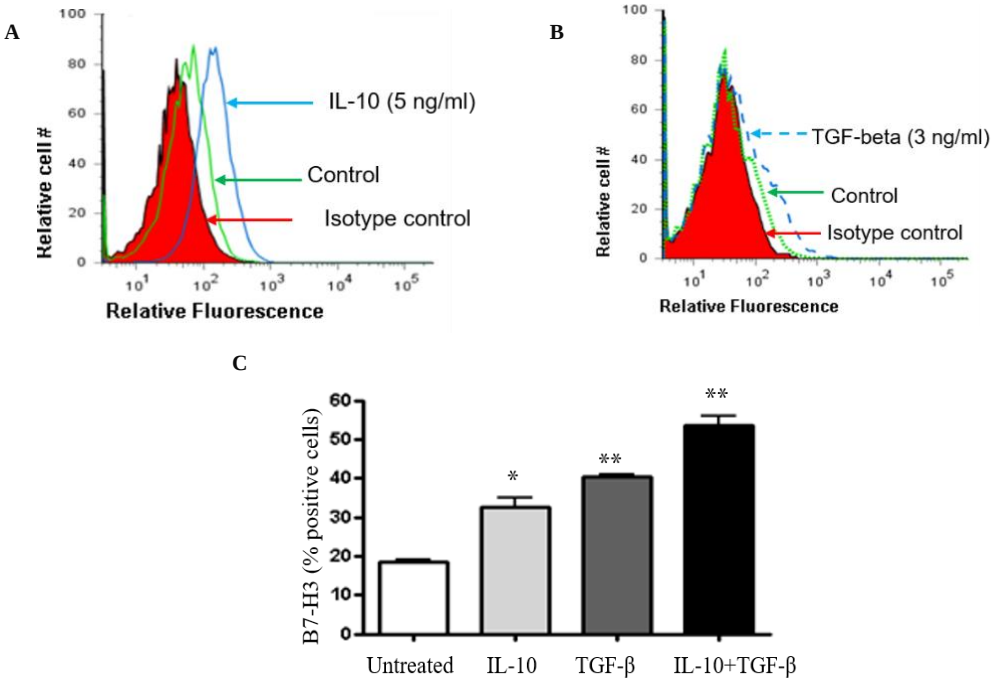

Figure 5: B7-H3 expression is regulated by Treg cell cytokines. **(A)** Flow cytometry analysis of GEC cells stained for B7-H3 after exposure to 5 ng/mL IL-10 for 24 h showed increased expression in a representative histogram where the solid peak is the isotype control **(B)** Flow cytometry was done to measure B7-H3 expression on GECs after treating the cells with TGF-β (3 ng/ml) for 24 h. **(C)** Flow cytometry was done to measure B7-H3 expression on GECs treated with either IL-10 (5 ng/mL) or TGF-β (3 ng/ml) or both IL-10 and TGF-β. The data were expressed as the percentage of positive cells. The means ± SD are shown as the results of duplicates of one of four representative experiments: n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

VI. Different T cell subset development during *H. pylori* infection

During *H. pylori* infection there is an increased frequency of Th1/Th17 and T_{reg} cells in the gastric mucosa. Previously we have shown that *H. pylori* infection upregulates B7-H1 molecule expression by GECs, which in turn helps to induce further Treg cell development [49]. On the other hand, we also showed that *H. pylori* T4SS mediated downregulation of B7-H2 in GEC, which impairs Th17 cell development [41]. Besides the reported effects of B7-H3 on T cell activation and inactivation, recent studies by Nagashima O et al., showed that B7-H3 can upregulate Th2 responses [26]. Since our data showed *H. pylori* upregulates B7-H3 expression we sought to investigate whether the modulation of B7-H3 expression affects locaßl T cell responses. To that end, we collected MLN from mice infected with PMSS1 and SS1 strains and analyzed the T cell subsets present by measuring mRNA expression of the different T cell transcription factors considered "master regulators" for each $CD4^+$ T cell subset, such as GATA3, Tbet, RORγ and FoxP3 for Th2, Th1, Th17 and Treg cells, respectively. Mice infected with SS1 strain showed increased induction GATA3, Tbet and RORγ compared to the PMSS1 strain. However, the MLN cells from PMSS1 infected mice showed increased FoxP3 expression compared to those from SS1 infected mice (Figure 6A). The mRNA expression of the corresponding cytokines produced by Th2, Th1, Th17 and Treg cells, e.g. IL-4, IFN-γ, IL-17A and IL-10, in MLN was further measured. The cytokine data correlate with the transcription factors found in mice infected with the different strains (Figure 6B).

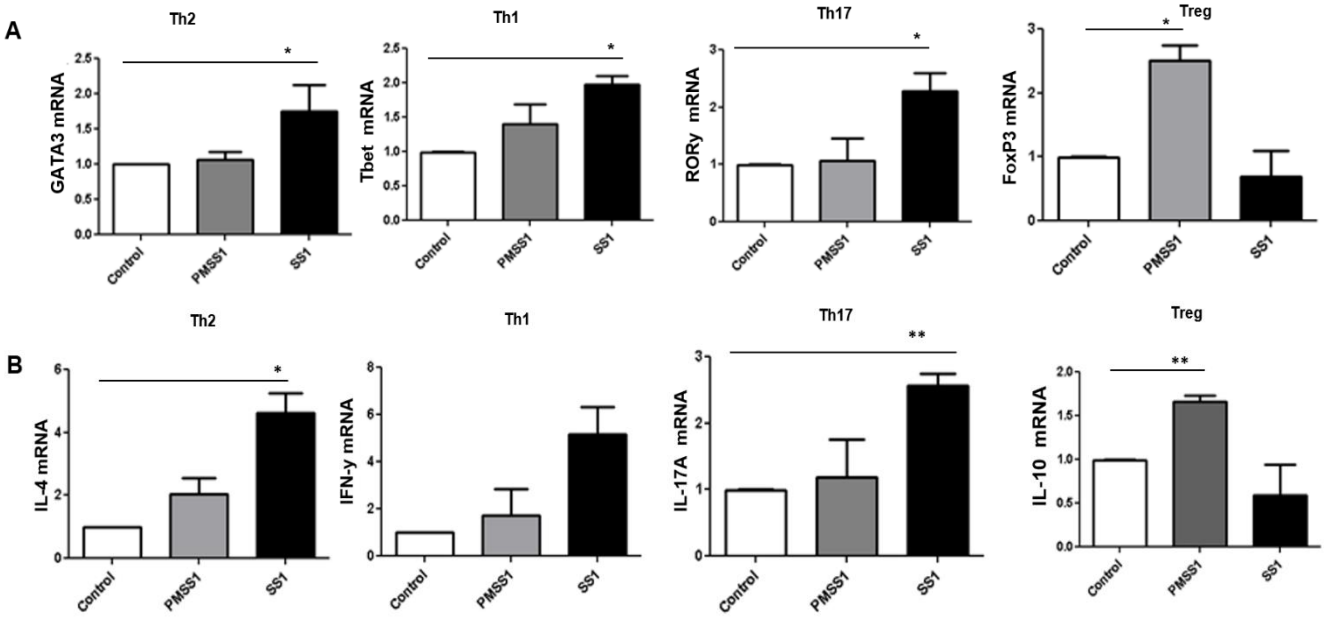

Figure 6: Different T cell subsets developed during *H. pylori* infection. C57BL/6 mice were challenged with *H. pylori* strain PMSS1 or with *H. pylori* SS1. Mice were sacrificed after 4 weeks of infection, MLN were collected, and expression measured of **(A)** GATA3, Tbet, RORγt, FoxP3 and **(B)** IL-4, IFN-γ, IL-17A, IL-10 mRNA by RT-PCR. Y-axis in each panel represents the fold increase in mRNA expression. Five mice per group were used in this experiment.

VII. Increased B7-H3 and GATA3 expression in gastritis patients

A previous report showed the presence of Th2 cells during *H. pylori* infection [31]. Consistent with that report, herein, the mouse model also showed induction of $GATA3^+$ Th2 cell in MLN after *H. pylori* infection. Since B7-H3 has been shown to influence Th2 cell development, we sought to determine the influence of B7-H3 induction by GECs during *H. pylori* infection in Th2 cells response and whether it depends on the infecting strain. To this end, specimens from patients with gastritis and gastric tumors were evaluated. Biopsy samples from gastritis and samples from gastric tumors were evaluated for the relative expression of B7-H3 and GATA3. Interestingly, samples collected from gastritis patients showed increased B7-H3 and GATA3 expression compared to those from healthy individuals. However, in the case of patients with gastric tumors the expression of both B7-H3 and GATA3 was decreased, which suggested B7-H3 and Th2 induction during *H. pylori* infection might be a characteristic of gastritis strains (Figure 7).

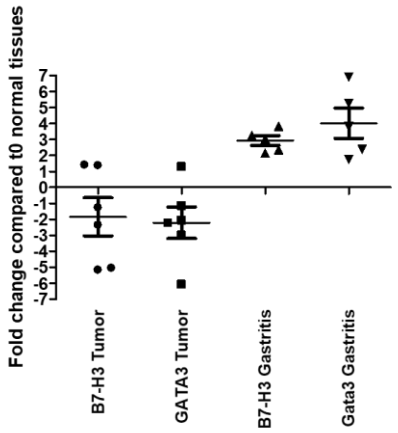

Figure 7: B7-H3 and Th2 induction is associated with gastritis. B7-H3 and GATA3 expression in biopsy and tumor samples isolated from gastritis or gastric tumor patients with a history of *H. pylori* infection.

VIII. B7-H3 expressed by GEC after *H. pylori* infection induces development of Th2 cells

To further confirm whether the induction of B7-H3 and Th2 is only associated with *H. pylori* gastritis strains, N87 cell lines were treated with either medium alone or with different *H. pylori* strains: CA8 (from a gastric cancer case), 51B (from a gastritis case) and LC-11 (from an ulcer case) [44, 45, 61]. After 8 h of infection, the cells were washed extensively and incubated with isolated $CD4^+$ naïve T cells for 5 days. T cells were harvested and stained for CD25, Tbet, GATA3, RORγt and FoxP3 monoclonal antibodies and analyzed by flow cytometry. The data showed increased $GATA3^+$ cells in T cells co-cultured with GECs pre-infected with the gastritis strain (*H. pylori* 51B), but not with the other strains (Figure 8A). A significant increase in $GATA3^+$ $Tbet^+$ double-positive cells was also observed in T cells co-cultured with GECs pre-infected with the gastritis strain, suggesting conversion of Th1 cells to Th2 cell type (data not shown). Interestingly, incubation of the T cells with GECs pretreated with blocking B7-H3 antibody reduced Th2 cell frequency. This data suggested that induction of Th2 is influenced by B7-H3 (Figure 8B).

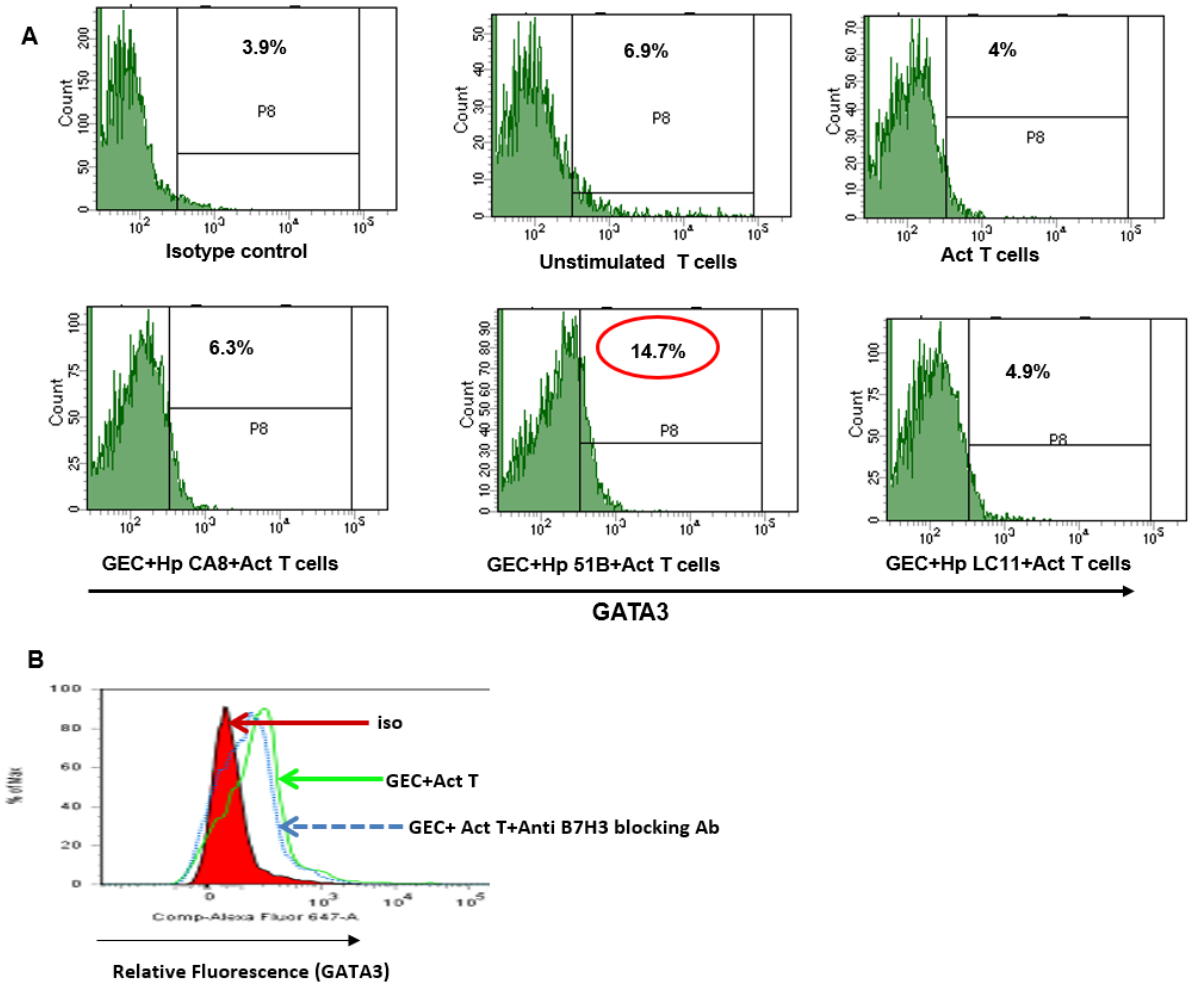

Figure 8: Increased B7-H3 expression and Th2 induction by *H. pylori* gastritis strain. **(A)** GECs were infected with either *H. pylori* CA8/51B/LC-11 strains for 8 h, washed and co-cultured with T cells. Cells were stained and GATA3 expression analyzed by flow cytometry. **(B)** A representative histogram showing GATA3 expression by GEC co-cultured with activated T cells in the presence or absence of ant-B7-H3 blocking Ab.

Discussion

B7-H3 has previously been considered a co-stimulatory molecule which promotes T cell proliferation [17]. But later studies have shown that B7-H3 can also function as a co-inhibitory molecule [17-20, 22-25]. B7-H3 is expressed by an array of cell types. A previous study showed that RSV causes induction of B7-H3 in tracheal, bronchial, and alveolar epithelial cells [21]. We have shown previously that B7-H3 is expressed by GEC [40]. In this study we demonstrate that *H. pylori* increases the expression of B7-H3 on GEC upon infection. Increased expression of B7-H3 was demonstrated on the GECs isolated from biopsies of *H. pylori* infected patients. This observation was confirmed *in vitro* by increased B7-H3 mRNA level and surface expression in a panel of GEC lines (N87, AGS and HGC-27) after infecting with *H. pylori* 51B and 26695 strains. Although our lab previously showed that GEC express B7-H3, the expression was unchanged after infection with *H. pylori* LC-11 strain [40]. That observation together with our recent observations suggest that this cellular response to infection might depend on the infecting *H. pylori* strain since both 51B and 26695 were isolated from patients with gastritis, while LC-11 originated from a patient with peptic ulcer. These

observations were further confirmed in this study by using gastric tissue samples from patients with different gastric diseases associated with *H. pylori* infection.

H. pylori T4SS is an important virulence factor that influences GEC homeostasis [52, 53]. The recent findings by our group regarding the involvement of T4SS by *H. pylori* to modulate B7 molecule expression, led us to consider *H. pylori* T4SS as a virulence factor responsible for the up-regulation of B7-H3 by GECs [41]. By using *H. pylori* WT and *cag* PAI isogenic mutants, we showed here that B7-H3 induction depends on *H. pylori* T4SS. This expression pattern was reproduced both in human GECs and murine GEC (ImSt) infected with *H. pylori*. Besides using a mutant which lacks the whole *cag* PAI we also used an *H. pylori* mutant only devoid in the *cagA* gene to determine the role of this effector protein translocated by the T4SS in B7-H3 induction. Our study showed that induction of B7-H3 depends on the presence of CagA. PG, the other component translocated to GEC by T4SS may act as an inflammatory molecule and induces IL-8 production by GEC [14-16]. As these data showed complete dependence of *H. pylori cag* PAI but partial involvement of CagA on B7-H3 induction, we hypothesized PG, which is also translocated by T4SS, might also influence B7-H3 induction. The addition of PG fragment iEDAP which is recognized by NOD1 showed induction of B7-H3 both at the mRNA and protein levels. Kinetics data showed that B7-H3 is increased within 18-hrs of stimulation by PG fragments. This study also highlighted the involvement of p38 MAPK pathway in B7-H3 induction, which is known to be activated by both PG and CagA [14, 54]. Previously it was shown that PG can be modified to resist lysozyme and this mechanism helps *H. pylori* survival [55]. However, this is the first study showing role of PG in GEC modification and T cell regulation.

Cytokines play an important role in influencing the expression of different immune regulatory molecules. Since IL-17, IL-10 and TGF-β produced by Th17 and Treg cells have been shown to be present in increased amounts in *H. pylori* infected patient [34, 35, 37-39], we hypothesized that these cytokines may act in paracrine fashion to affect the induction of B7-H3 on GEC. Our data showed that stimulation of GECs by both Th17 cytokine (IL-17) and Treg cell cytokines (IL-10 and TGF-β) causes increased expression of B7-H3 molecules on GECs. Regulation of other B7 molecules by IL-10 and TGF-β have been shown previously. For instance, IL-10 was shown to inhibit B7 molecule expression in macrophages and B7-2 expression in DCs [56, 57]. Also, TGF-β has been found to inhibit B7-1 expression in APCs [58]. Another study showed IL-10 down-regulated B7-1 and B7-2 expression on *Mycobacterium tuberculosis*-infected monocytes to a greater extent than did TGF-β [59]. However, IL-10 and TGF-β did not show any additive or synergistic inhibition in their study, whereas, in this study, we found TGF-β is a better inducer of B7-H3 than is IL-10, and they have synergistic effects in B7-H3 induction.

To explore the contribution of B7-H3 to the development of T cell subsets in *H. pylori* infection, we initially determined what kind of T cell response ensues in mice infected with *H. pylori* strain in the presence or absence of a functional T4SS. To that end we measured different T cell associated transcription factors considered as master regulators for different $CD4^+$ T cell subsets and cytokines produced by these cells in MLN harvested from *H. pylori* infected mice. Consistent with previous published data, we noted mixed populations of Th1 and Th2 cells in *H. pylori* infected mice [33]. Compared to SS1, PMSS1-infected mice had a lower induction of the Th1 and Th2 cell subsets. Additionally, Th17 and Treg cell data correlated with our previous findings, since *H. pylori* PMSS1 infection causes increased Treg cells and a lesser Th17 cell response when compared with findings in SS1 infected mice [41, 49]. Our lab has previously shown that *H. pylori*-mediated modulation of Th17 and Treg cell responses depends on altered expression of B7-H2 and B7-H1 molecules on GECs [41, 49]. Besides being a positive stimulator for T cell activation, B7-H3 has also been shown to play a role in Th2 development and to contribute to pathogenic Th2 cell development during asthma in a mouse model [26]. However, several studies also showed negative regulatory effects of B7-H3 in Th1 and Th2 immune responses [60]. A major question regarding the Th2 cell response observed in *H. pylori*-infected mice is whether or not this induction of Th2 is influenced by a B7-H3 molecule expression. To answer this question and investigate whether this response depends on the *H. pylori* strain, samples from *H. pylori* infected patients with either gastritis or tumor were collected and B7-H3 and GATA3 expression on those samples were compared with samples collected from healthy individuals. Interestingly, the samples collected from gastritis patients, and not from the gastric tumor patients, had increased B7-H3 and $GATA3^+$ cells. Though this study showed a strong association between B7-H3 induction and Th2 development during *H. pylori* infection, further studies are required to determine the link between disease condition and B7-H3 expression. To further evaluate this finding, a GEC: T cell co-cultures were used, in which the GECs were pre-exposed to *H. pylori* strains which originated from gastritis, gastric cancer or peptic ulcer in the presence of anti-B7-H3-blocking antibody or control antibody. The flow cytometry data indicated the induction of Th2 cells and Th1/Th2 double-positive cells in the T cells co-cultured with *H. pylori* 51B (from a gastritis case) pre-treated cells, suggesting a shift of Th1 towards Th2 cells. Moreover, by using anti-B7-H3 blocking antibody, we showed that induction of Th2 depends on B7-H3.

Conclusions

In conclusion, this study revealed a novel mechanism that *H. pylori* uses to foster host chronic inflammation in the form of gastritis. This is an important finding which helps to better understand the interaction of *H. pylori* with GECs and how *H. pylori* manipulates the host T cell response. The relationship of *H. pylori*-mediated B7-H3 induction and disease conditions must be further defined.

Acknowledgements

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Abbreviations

GEC: Gastric epithelial cells;
T4SS: Type 4 secretion system;
PG: Peptidoglycan;
iEDAP: D-gamma-Glu-mDAP;
MALT: Mucosa-associated lymphoid tissue;
CagA: Cytotoxin associated gene A;

cag PAI: *cag* pathogenicity island;
Th: T-helper;
RSV: Respiratory syncytial virus;
Treg: T regulatory;
ATCC: American Type Culture Collection;
ImSt: Immortomouse stomach epithelium;
TSA: Tryptic soy agar;
SS1: *H. pylori* Sydney strain 1;
PM-SS1: Pre-mouse SS1;
WT: Wild type;
APC: Antigen presenting cells.

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Helicobacter pylori Deregulates T and B Cell Signaling to Trigger Immune Evasion

Victor E. Reyes and Alex G. Peniche

Contents

Abstract *Helicobacter pylori* is a prevalent human pathogen that successfully establishes chronic infection, which leads to clinically significant gastric diseases including chronic gastritis, peptic ulcer disease (PUD), and gastric cancer (GC). *H. pylori* is able to produce a persistent infection due in large part to its ability to hijack the host immune response. The host adaptive immune response is activated to strategically and specifically attack pathogens and normally clears them from the infected host. Since B and T lymphocytes are central mediators of adaptive immunity, in this chapter we review their development and the fundamental mechanisms regulating their activation in order to understand how some of the normal processes are subverted by *H. pylori*. In this review, we place particular emphasis on the $CD4^+$ T cell responses, their subtypes, and regulatory mechanisms because of the expanding literature in this area related to *H. pylori*. T lymphocyte differentiation and function are finely orchestrated through a series of cell–cell interactions, which include immune checkpoint receptors. Among the immune checkpoint receptor family, there are some

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with inhibitory properties that are exploited by tumor cells to facilitate their immune evasion. Gastric epithelial cells (GECs), which act as antigen-presenting cells (APCs) in the gastric mucosa, are induced by *H. pylori* to express immune checkpoint receptors known to sway T lymphocyte function and thus circumvent effective T effector lymphocyte responses. This chapter reviews these and other mechanisms used by *H. pylori* to interfere with host immunity in order to persist.

1 Introduction

Helicobacter pylori is a Gram-negative bacterium within the class of ε-proteobacteria, Campylobacterales order, and is a primary constituent of the human gastric microbiome. *H. pylori* is an important human pathogen that frequently infects during childhood and successfully establishes chronic infection in >66% of the world's population (www.CDC.gov). *H. pylori* is involved in significant clinical gastroduodenal disorders that include chronic gastritis, peptic ulcer disease (PUD), and two malignancies: gastric adenocarcinoma (GC) and mucosa-associated lymphoid tissue (MALT) lymphoma. GC remains as the third deadliest cancer worldwide with a five-year survival rate of 14% and accounts for approximately one million deaths (www.who.int; 2017 Fact Sheet).

Important to *H. pylori*'s capacity to establish chronic infection is its ability to evade or subvert innate and adaptive immune responses via multiple mechanisms. One of the earliest clues that *H. pylori* subverts the adaptive host response was the observation that $CD4^+$ T cell responses in the infected gastric mucosa were polarized to T helper (Th) 1 cells (Bamford et al. 1998b; Amedei et al. 2006), which are not optimal for extracellular bacteria as *H. pylori*. As we have studied in detail the mucosal immunity to *H. pylori*, we have gained insights that helped us to better understand how *H. pylori* induces a diverse T cell response that includes Th1, Th17, and T regulatory (Treg) cell responses. In this chapter, we will examine the following:

- A comprehensive background on the adaptive immune response. To better appreciate how those responses are altered during *H. pylori* infection, we will start by discussing the normal development of B and T lymphocytes and their activation processes and provide a brief description of the various $CD4^+$ T cell subsets.
- Extracellular receptor–ligand interactions and intracellular signal involvement.
- Finally, we will examine how these cells are affected by *H. pylori* infection, either directly or indirectly, by other cells affected by the infection—including the gastric epithelium. Most of the discussion will be on T cell activation, as another chapter in this book (Chapter "MALT Lymphoma as a Model of Chronic Inflammation-Induced Gastric Tumor Development") will provide a rich discussion of B cells, as they are the target in mucosal-associated lymphoid tissue (MALT) lymphoma.

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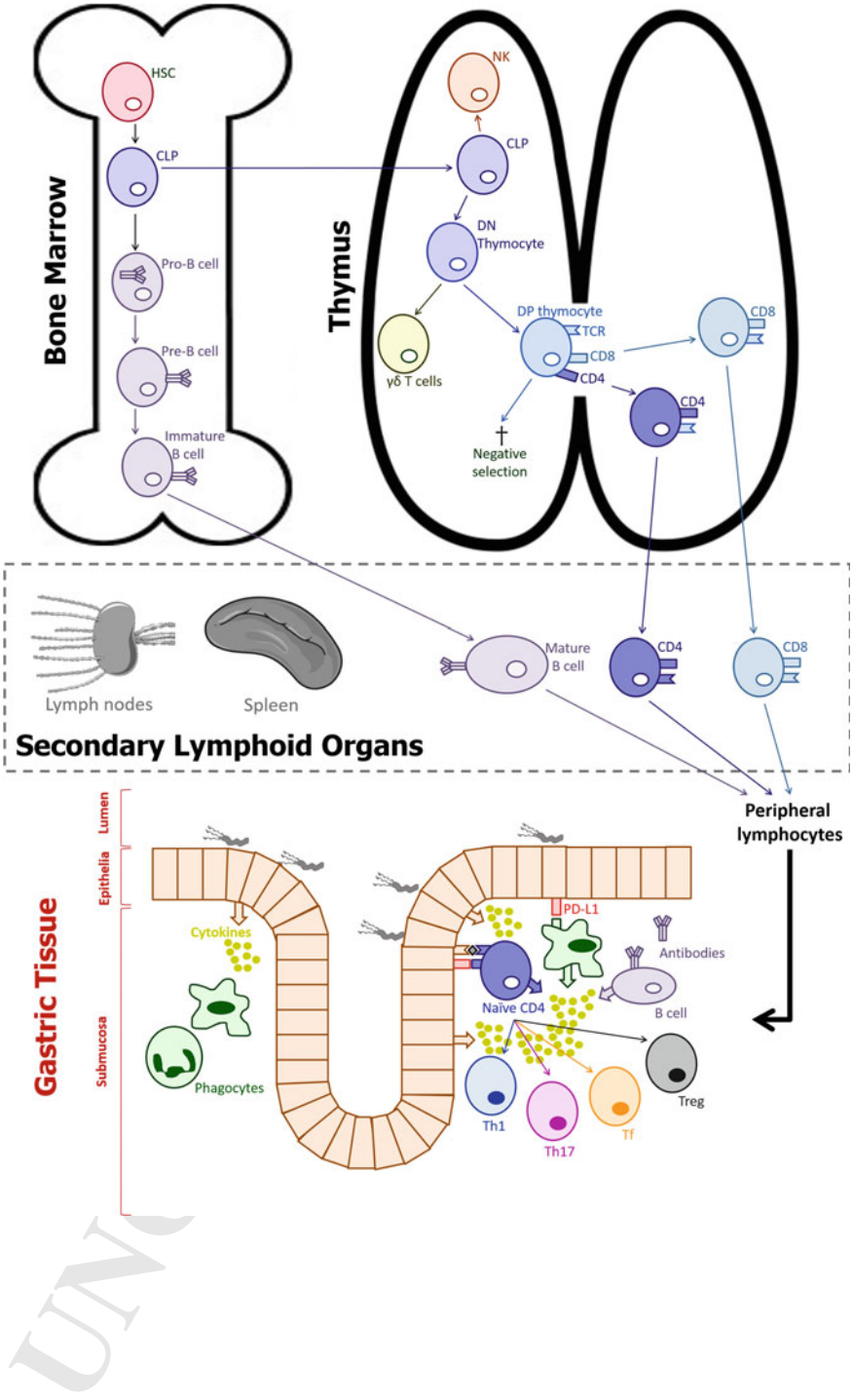
2 B and T Lymphocyte Development

Lymphocytes are central players in the adaptive immune response, and, as are all other blood cells, they emerge during hematopoiesis from pluripotent hematopoietic stem cells (HSCs) that reside in bone marrow (Fig. 1). Hematopoiesis is a unidirectional process in which all immune cells types are generated from multipotent HSCs. Immune cells must be continuously replaced because of their limited life span, but also in response to infectious and inflammatory stimuli, by using receptors for cytokines and chemokines, as well as pathogen-associated molecular pattern (PAMP) recognition receptors (Chiba et al. 2018; Pachathundikandi et al. 2013). HSCs reside in the bone marrow microenvironment composed by osteoblasts, perivascular cells, endothelial cells, and immune cells, all of which promote HSCs proliferation through an array of cytokines like CXCL12 and stem cell factor (SCF). The differentiation of lymphocytes follows a tightly regulated process that initially transits through common lymphoid progenitor (CLP) cells (Kondo et al. 1997) that are $CD34^+$, $CD10^+$, $CD45RA^+$, and $CD24^-$ and are devoid of surface markers characteristic of T-, B-, or NK cells. CLP cells also contribute to the development of NK cells and subsets of dendritic cells (DCs). As B and T lymphocytes develop in the bone marrow and thymus, respectively, under the influence of local interactions and cytokines, they start to express distinctive surface markers, as detailed below for each lymphocyte population.

2.1 B Lymphocyte

B cells differentiate from CLPs in the bone marrow through a series of closely controlled stages, initially as progenitor B (pro-B) cells ($CD34^+$ $CD19^+$ $CD10^+$ TdT^+ $CD38^{++}$ $CD20^-$) when heavy chain immunoglobulin (Ig) V(D)J DNA rearrangement begins with a process that involves the recombination-activating genes (RAG) 1 and 2, as well as terminal deoxynucleotidyl transferase (TdT) enzymes. Once successfully rearranged, the Ig heavy chain forms a complex with surrogate light chains to give rise to a pre-B cell receptor in precursor B (or pre-B) cells ($CD34^-$ $CD19^+$ $CD10^\pm$ $CD38^+$ $cytIgM^+$ $CD20^\pm$). Signaling through this pre-B cell receptor induces light chain DNA rearrangement which induces membrane-bound Ig. Once B cells express surface IgM, they are known as immature B cells ($CD34^-$ $CD19^+$ $CD20^+$ $CD38^+$ $CD40^+$ $sIgM^+$) and subsequently express IgD, and are regarded as naïve mature B cells in the periphery. The final differentiation of B cells into Ig-secreting plasma cells occurs in the lymph nodes and other secondary lymphoid organs after activation by engagement of the surface Ig (aka B cell receptor, BCR) with antigen and the interaction of CD40 on their surface with CD154 on Th cells (Lou et al. 2015).



◀**Fig. 1** Schematic representation of lymphocyte differentiation and migration to gastric tissue. Bone marrow host hematopoietic stem cells (HSC) that progressively differentiate to rise to common lymphoid progenitors (CLP). CLPs differentiate into progenitor B cells (Pro-B cells) and double-negative (DN) thymocyte progenitors. Pro-B cells remain in bone marrow and differentiate into immature B cells that turn into mature B cells once they migrate to secondary lymphoid organs (i.e., lymph nodes and spleen). The CLP that migrate to the thymus commit to either natural killer (NK) cells or T lymphocyte lineage becoming γδ T lymphocytes or double-negative DN thymocytes. DN thymocytes undergo negative selection and only immature single positive cells survive to become $CD4^+$ or $CD8^+$ T lymphocytes capable of migrating to secondary lymphoid organs. Lymphoid cells are eventually recruited to gastric infected tissue where they become antibody-producing cells (B cells, plasmocytes) and $CD4^+$ T lymphocytes differentiate into subsets depending on environmental cues

2.2 T Lymphocyte

As with B lymphocytes, T cells have their origin in the bone marrow and share the CLP precursor, but their development occurs within the thymus following migration of CLP cells to this organ. Thymocyte precursors ($CD4^-$ $CD8^-CD7^+$ $CD45^+$) interact with stromal cells in the thymic cortex, where most thymocytes begin to rearrange their T cell receptor (TCR) β chain loci. After the β chain locus is productively rearranged and the corresponding protein expressed, this protein forms a complex with a surrogate α chain (pre-T α) and creates a complex with CD3 (von Boehmer 2005). When this complex is formed, the cells differentiate into double positive (DP, $CD4^+$ $CD8^+$) thymocytes and rearrange their α chain loci to eventually express TCR αβ on their surface. In addition to the α and β chain loci, there are γ and δ TCR loci, but only about 3–10% of thymocytes rearrange their γδ TCR loci (Weiss et al. 1986). Once thymocytes express their TCR, they undergo positive selection in the thymic cortex where the cells that recognize antigen with the corresponding class I or II human leukocyte antigen (HLA aka major histocompatibility complex, MHC) molecules with appropriate affinity survive, while those that fail to recognize antigen die by apoptosis. The surviving cells migrate to the thymic medulla where they experience another selection step. In the medulla, thymocytes interact with antigen-presenting cells (APCs: DCs and macrophages) which present self-antigens bound by (HLA) molecules and those thymocytes with a very strong affinity die by apoptosis, while those that survive downregulate either their CD4 or CD8 co-receptor to become single positive T cells (for a review see Takaba and Takayanagi 2017). The cells that emerge from the thymus into the periphery are naïve T cells that will differentiate further into distinct subsets following activation, as described below.

Although the role of the thymus in T cell differentiation, maturation, and expansion has long been recognized, extrathymic T cell differentiation and maturation have been reported in mice and humans (Lefrancois and Puddington 1995; Bandeira et al. 1991; Howie et al. 1998). Sites that have been shown to support extrathymic T cell differentiation include the gut and tonsils (Howie et al. 1998; McClory et al. 2012). It is important to bear in mind that the gut harbors the largest number of T cells in the body, where they are exposed to the largest possible antigenic challenge

that includes dietary antigens and the gut microbiome. Interestingly, the gastrointestinal tract also holds unconventional populations of T cells such as intraepithelial lymphocytes (IEL), which represent an effector T cell population reported to develop extrathymically (Fichtelius 1967), as supported by their presence in athymic mice (Bandeira et al. 1991). The gastric epithelium has 5–8 IEL cells per 100 epithelial cells, and these numbers increase up to sixfold during disease states, such as gastritis (Feeley et al. 1998; Hayat et al. 1999). These cells express a CD8αα homodimer, rather than the conventional CD8αβ heterodimer expressed by peripheral T cells selected in the thymus (Ruscher et al. 2017).

After their selection in the thymus, T lymphocytes enter the circulation and travel to secondary lymphoid organs. Migration of lymphocytes to those secondary lymphoid organs hinge on their surface expression of L-selectin (CD62L), the integrin leukocyte function antigen-1 (LFA-1, αLβ2), and the CC chemokine receptor (CCR)7 (von Andrian and Mackay 2000), which permit rolling, adhesion, and extravasation of T cells through high endothelial venules in secondary lymphoid organs (lymph nodes and mucosal lymphoid organs). In those secondary lymphoid organs, they may become activated by APCs. Activated T cells expand and become either effector or memory T cells. Newly activated T cells may migrate to other tissues and specific adhesion molecules, and chemokine receptors enable them to home and bind the corresponding ligands in those tissues. For instance, T cells that migrate to the gastrointestinal mucosa require the integrin α4β7, LFA-1, and CCR9 (Michetti et al. 2000; Quiding-Jarbrink et al. 2001; Berlin et al. 1993; Zabel et al. 1999; Johansson-Lindbom et al. 2003). An important factor that determines what adhesion molecules are expressed by activated T cells is the site where they encounter antigen (Stagg et al. 2002). For instance, α4β7 expression by gastric and intestinal T cells allows them to home and bind to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expressed by high endothelial venules in the Peyer's patches and gut lamina propria (Michetti et al. 2000; Williams and Butcher 1997; Hatanaka et al. 2002).

3 B and T Lymphocyte Activation

B and T lymphocytes perform a daunting mission of recognizing from a vast universe of antigens those that are foreign to us and respond to them rapidly and specifically in spite of a very noisy background of self-antigens. The events that lead to their activation are carefully orchestrated and involve a series of extracellular signals provided via cell–cell interactions and cytokines that in turn activate intracellular signals leading to activated B and T lymphocytes. Because the events that lead to fully functional B and T lymphocytes are critical in adaptive immune responses, we will review those events below with a particular emphasis on T lymphocytes, since B lymphocytes are discussed in more detail in Chapter "MALT Lymphoma as a Model of Chronic Inflammation-Induced Gastric Tumor Development" of this book.

3.1 B Lymphocyte

Naive B lymphocytes have approximately 1.5×10^5 membrane-bound antibodies (IgM and/or IgD) that serve as B cell receptors (BCRs) to bind soluble antigens (Maddaly et al. 2010). Activation requires cross-linking of multiple monomer membrane-bound antibodies (Harwood and Batista 2010). The activation of B cells varies depending on the type of antigen and interaction with T cells. Some antigens do not require contact with T helper cells and are thus referred to as T-independent antigens. An example of these antigens is bacterial lipopolysaccharides (LPS), which at high concentrations may activate mature and immature B cells. However, the characteristic response to these antigens is "weak" in terms of antibody production and memory response and frequently results only in IgM secretion. The lack of co-stimulation (CD40L) is thought to be the reason why these antigens fail to induce class switching and increased antibody affinity (Maddaly et al. 2010). Other antigens require interaction of co-stimulatory receptors and cytokines from Th cells with B cells (acting as APCs). The co-stimulation between these cells typically occurs in secondary lymphoid organs. The binding of antigen by B cells leads to clustering of membrane-bound antibodies, and their subsequent dimerization and internalization into endosomal vesicles. Then, those B cells present peptide-laden HLA class II complexes to T cell receptors (TCRs) on antigen-specific T cells. This interaction promotes expression by B cells of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) which facilitate differentiation of Th cells. Activation of T cells leads to their expression CD40L which interact with CD40 on the B cells to promote their entry into the S phase. In addition, cytokines such as IL-2 and IFN-γ (Th1), and IL-4, IL-5, IL-6, IL-10, IL-13 (Th2) promote clonal expansion, antibody production, and isotype switching (from IgM to IgG) followed by differentiation into plasma cells and memory B cells (Harwood and Batista 2010).

3.2 T Lymphocyte

3.2.1 Antigen Presentation

Presentation of foreign antigens refers to the display of antigens to T cells by antigen-presenting molecules [human leukocyte antigen (HLA) class I, HLA class II or CD1] after those antigens have been appropriately processed by APCs. Antigen processing and presentation provide the host with a mechanism to constantly survey the cellular internal and external environments for the presence of potential pathogens. There are four possible pathways involved in the processing of protein antigens for presentation by either class I or class II HLA molecules. Classical antigen processing pathways include the exogenous and endogenous pathways, but autophagy and cross-presentation have expanded the possible pathways whereby antigens are processed. The location of the antigens or, in the case of replicating pathogens, the life

cycle of a given pathogen determines which pathway is needed for appropriate presentation to the appropriate T cell type ($CD4^+$ versus $CD8^+$). In the case of pathogens that replicate within the cell, and whose antigens are thus synthesized endogenously, they are degraded in the cytosol into small peptides, 8–10 amino acids long, by the proteasome complex and are delivered to the lumen of the endoplasmic reticulum (ER), where nascent HLA class I molecules bind them for eventual presentation to cytotoxic $CD8^+$ T cells. In contrast, pathogens such as *H. pylori,* that replicate in the extracellular milieu, or are exogenous to the APCs, have to be endocytosed and their protein antigens processed by thiol proteases in endocytic compartments to generate peptides that will bind to HLA class II molecules for presentation to $CD4^+$ T cells (for a review, see Blum et al. 2013). More recently, autophagy and cross-presentation have been described as alternative pathways that break away from the classical pathways since autophagy captures endogenously produced antigens and delivers them to endocytic compartments where exogenous antigens are processed. Recent studies have reported that highly virulent strains of *H. pylori* noticeably affect autophagy in host GECs and macrophages (Castano-Rodriguez et al. 2015). On the other hand, cross-presentation results from the delivery of exogenously acquired antigens into the cytosol where they are processed by the proteasome and the resulting peptides are delivered to the ER lumen where they bind newly formed HLA class I (Van Kaer et al. 2017; Joffre et al. 2012).

CD1 molecules represent another group of relatively non-polymorphic antigen-presenting proteins whose genes are not present within the MHC region. In fact, CD1 are encoded in an entirely different chromosome. While human HLA genes are encoded in chromosome 6, human CD1 genes are encoded in chromosome 1. There are four human CD1 proteins (CD1a to CD1d) that also associate with β2-microglobulin. These molecules are expressed by classical APCs, and CD1d is strongly expressed by GECs. Although CD1 molecules also present antigens and their crystal structure resembles that of class I HLA molecules (Blumberg et al. 1995), they differ from class I and II HLA molecules in that they do not bind peptide antigens. Instead, CD1 molecules bind and present lipids, because their antigen binding pocket has a narrow opening, is deep, and is lined by hydrophobic residues (Ly and Moody 2014). CD1 molecules may present lipid antigens to a diverse group of T cells that include γδ TCR or αβ TCR expressing T cells, as well as invariant NK T cells (iNKT) (Adams 2014). A study by Ito et al. (2013) showed that *H. pylori* cholesteryl α-glucosides are recognized by iNKT in the stomach, which contributes to the inflammatory response that limits *H. pylori* infection (see also Chapter "The Sweeping Role of Cholesterol Depletion in the Persistence of *Helicobacter Pylori* Infections" of this book).

3.2.2 Antigen-Presenting Cells

T cells are activated by APCs able to internalize foreign antigens and process them for presentation to the T cells. Because of their role in T cell activation, APCs are crucial in orchestrating the adaptive immune response. While most nucleated cells express

HLA class I molecules, the cells that are classically referred to as professional APCs are those that express HLA class II and include DCs, macrophages, and B cells. In addition to expressing class II HLA, another important feature of these cells is their expression of the co-stimulatory molecules CD80 and CD86, whose engagement of CD28 on T cells is vital for activation of naïve T cells. Interestingly, in the gastric environment, GECs represent a non-classical APC-type, as they constitutively express class II HLA, CD80, CD86, CD74, the antigen processing cathepsins, and newer members of the B7 family, as described below (Ye et al. 1997; Fan et al. 1998, 2000; Barrera et al. 2001, 2002, 2005; Beswick et al. 2004, 2007a; Das et al. 2006). The expression by GECs of class II HLA, CD80, CD86, and CD74 increases during infection with *H. pylori* (Ye et al. 1997; Fan et al. 1998, 2000; Beswick et al. 2004, 2005). Furthermore, a recent study showed that GECs express retinoic acid, which is responsible in the induction of $\alpha_4\beta_7$ integrin and the CCR9 chemokine receptor on both $CD4^+$ and $CD8^+$ T cells, which in turn facilitates their homing to the gastrointestinal mucosa (Bimczok et al. 2015). It is worth noting that retinoic acid also influences the homing to the gastrointestinal mucosa of IgA-secreting B cells (Mora and von Andrian 2009).

3.2.3 T Cell Receptor Signaling

The recognition by the TCR of antigen-laden MHC molecules on the surface of APCs leads to the formation of an immunological synapse between both cell types (Huppa et al. 2003), but this interaction alone is insufficient to lead to T cell activation since the short cytoplasmic tail of TCRs does not allow them to deliver intracellular signals. TCR interacts closely with a complex of other membrane proteins on T cells, that are collectively referred to as CD3 (including γ-, δ-, ε-, and ζ-subunits). After TCR engagement of peptide-laden MHC molecules, the cytoplasmic domains of CD3 subunits are responsible for delivering intracellular signals. Further, CD4 and CD8 bind to conserved membrane proximal domains on the β2-domain of MHC class II (Cammarota et al. 1992) and α3 of class I MHC molecules (Devine et al. 1999), respectively. The cytoplasmic domains of CD4 and CD8 bind the Src family kinase LCK (lymphocyte-specific protein tyrosine kinase), which in turn phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic domains of CD3 subunits (Love and Hayes 2010). Phosphorylation of CD3 subunits directs the recruitment of zeta-chain-associated protein kinase of 70 kDa (ZAP70). After ZAP70 is activated, it phosphorylates the linker for activation of T cells (LAT) and Src homology 2 domain-containing 76 kDa leukocyte protein (SLP76). A series of signaling proteins are recruited, leading to calcium mobilization, actin cytoskeleton reorganization, and activation of Ras guanosine triphosphate hydrolases (GTPases). As a consequence of these signaling processes, various transcription factors are activated, including nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and nuclear factor of activated T cells (NFAT), which aid in directing T cell responses.

3.2.4 Co-stimulation/Co-inhibition

In addition to the signals delivered by the CD3 complex after TCR recognition of antigen, T cells must receive co-stimulation via engagement of CD28 on their surface with CD80 or CD86, also, respectively, known as B7-1 and B7-2, on the surface of APCs. Engagement of CD28 on T cells is essential for T cell activation since in the absence of the signals delivered via CD28 after binding its ligand on APCs T cells become anergic, as shown in experiments with anti-CD28 blocking antibodies (Harding et al. 1992). The intracellular signals delivered by CD28 prevent this anergic state, and they include the Tec family kinases ITK/EMT, Rlk, and Itk, as well as phosphatidylinositol 3-kinase (PI3K) (August et al. 1994; Schaeffer et al. 1999; Pages et al. 1994). The signals delivered via CD28 affect crucial events in T cells, such as transcriptional signaling, post-translational protein modifications, cytokine synthesis, and epigenetic changes that ultimately affect their phenotype and function. The ligands for CD28, CD80, and CD86 vary in their expression pattern. CD86 is constitutively expressed on APCs and is upregulated quickly during immune responses, whereas CD80 is slower in its upregulation (Lenschow et al. 1994). Both of these receptors are expressed by GECs and are upregulated during *H. pylori* infection (Ye et al. 1997). The studies by Ye et al. showed that CD86 expression was higher on GECs from *H. pylori*-infected gastric biopsy tissues compared with those from uninfected subjects (Ye et al. 1997). Another member of this family of receptors and ligands is the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) which is expressed on activated T cells and acts as an immune checkpoint inhibitor. CTLA-4 competes for the same receptors, CD80 and CD86, and binds with higher affinity to antagonize CD28, and thus acts to provide inhibitory signals (Walunas et al. 1994). Furthermore, CTLA-4 is a key mediator of Treg function (Friedline et al. 2009). The next members of this family of immunoregulatory receptors identified, that are not constitutively expressed on resting T cells, but are induced following activation, include inducible co-stimulator (ICOS, CD278) and programmed death-1 receptor (PD-1, CD279), which provide co-stimulatory or co-inhibitory signals, respectively. The corresponding co-receptor for ICOS is ICOS-L (aka B7-H2, CD275), while PD-1 may bind two separate co-receptors: programmed death ligand-1 (PD-L1) (aka B7-H1, CD274) and PD-L2 (aka B7-DC, CD273) (Fig. 2). Interestingly, PD-L1 may also bind CD80 to deliver inhibitory signals (Park et al. 2010). PD-1 binding to any of its co-receptors results in dephosphorylation and inactivation of ZAP70 and the recruitment of Src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) (Yokosuka et al. 2012), which in turn causes dephosphorylation of PI3K leading to activation of Akt (Boussiotis et al. 2014). Ligation of PD-1 may also prevent extracellular-signal-regulated kinase (ERK) activation, which may be rescued via signaling activated by exogenous IL-2, IL-7, and IL-15 (Bennett et al. 2003). The engagement of PD-1 on T cells also inhibits their cell-cycle progression and proliferation via suppression of cell-cycle regulatory genes. Additional data collected on the functions of the PD-1/PD-L1 and PD-L2 axis suggest that the role of these receptors on T cell biology extends beyond suppression of effector T cells. Studies by Allison and colleagues highlighted that not only the expression of PD-L1 and

PD-L2 on APCs is differentially upregulated, but also PD-L1 and PD-L2 may have different roles affecting Th1 and Th2 responses (Loke and Allison 2003). The interaction of PD-1 with PD-L1 may also reprogram human Th1 cells into Treg cells (Amarnath et al. 2011), and this interaction may also affect diverse $CD4^+$ T cell subsets differently (McAlees et al. 2015).

The B7 family of proteins with either co-stimulatory or co-inhibitory properties has expanded in recent years and is now collectively referred to as "immune checkpoint regulators" (Ceeraz et al. 2013), which now include ten reported members (Xu et al. 2016). This family of receptors currently includes B7-1, B7-2, B7-DC, PD-L1, ICOS-L, B7-H3, B7-H4, B7-H5, B7-H6, and B7-H7 (Fig. 2). Various members of this family of receptors are overexpressed by various forms of cancer, including GC, possibly as a mechanism of evasion of tumor immune surveillance (Cimino-Mathews et al. 2016; Chen et al. 2015; Hou et al. 2014). These observations together with their known T cell regulatory activity made these proteins attractive as targets for oncologic immunotherapy with some successes (La-Beck et al. 2015).

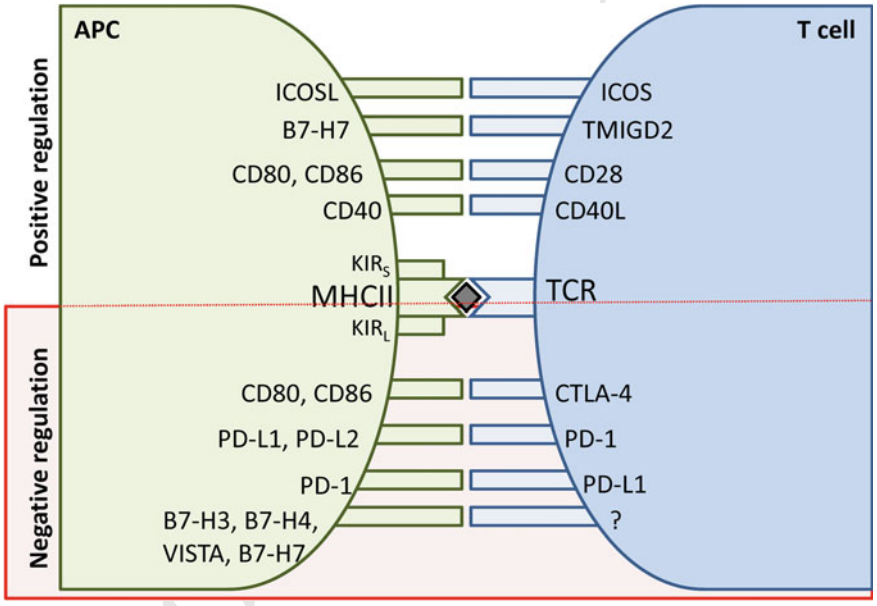


Fig. 2 Co-stimulatory and co-inhibitory receptors and their ligands. These molecules are also known as members of the B7-CD28 superfamily or immune checkpoint regulators because they affect T cell activity

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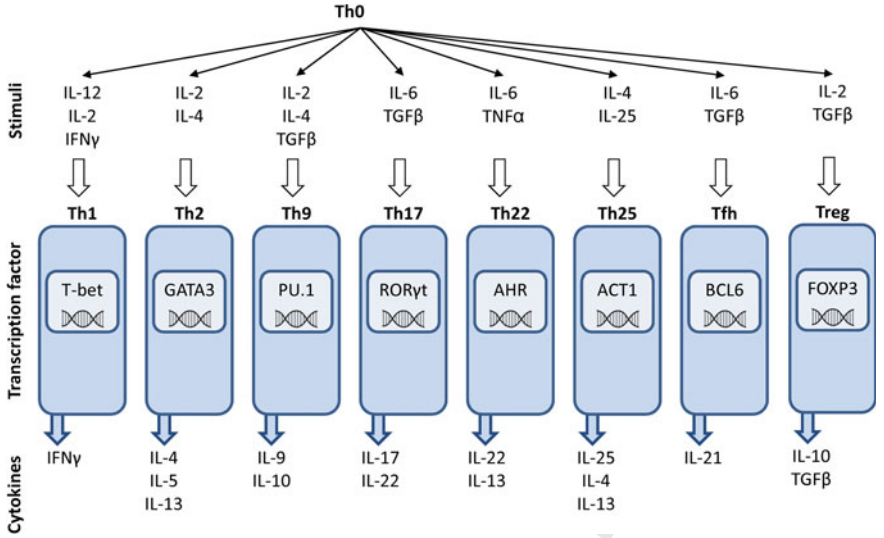

Fig. 3 CD4$^+$ T cell subsets. Upon activation, naïve CD4$^+$ T cells differentiate following specific paths depending on environmental cues, which include distinct cytokines. As part of their differentiation, they express characteristic transcription factors or "master regulators" that determine their respective phenotypic differences and the cytokines that they produce

4 T Cell Subsets and Reprogramming

4.1 T Cell Subsets

We discussed earlier thymic selection and the emergence of single positive CD4$^+$ and CD8$^+$ T cells, which migrate to the periphery and the majority (90–95%) of which express the TCR αβ, while the remainder express TCRγδ. Also, we referred to extrathymically differentiated T cells, which are largely CD8αα with a large proportion of TCRγδ, and double-negative CD4$^-$CD8$^-$ cells. CD8$^+$ T cells, also known as cytotoxic T cells, after activation in the periphery may exert their cytotoxic role and then become memory T cells. CD4$^+$ T cells represent a more diverse subset after they are activated. Depending on the cytokine milieu to which they are exposed during their activation by APCs, CD4$^+$ T cells are programmed into distinct subsets with the expression of characteristic transcription factors and cytokine profiles, which in turn allow them to exert distinct functions. Currently, the CD4$^+$ T cell subsets that have been defined include Th1, Th2, Th3, Th9, Th17, Th22, Th25, follicle helper T cells (Tfh), and Treg (Fig. 3). Interestingly, the literature on the immune response to *H. pylori* has been inclusive of most of these subsets.

4.1.1 Th1 Cells

The first subsets of T lymphocytes studied in the context of the host response to *H. pylori* were Th1 and Th2 (Karttunen et al. 1990; Bamford et al. 1998b). Each $CD4^+$ T cell subset is characterized by the expression of a specialized cytokine gene under regulation by subset-defining transcription factors. Th1 is induced to differentiate by IL-12 from APCs (Hsieh et al. 1993), which induce signal transducers and the activator of transcription 4 (STAT4) or STAT1. These STATs lead to the expression of the transcription factor T-bet, regarded as the master regulator of Th1 cells (Szabo et al. 2000), and synthesis of IFN-γ, although neither of them is unique to Th1 cells. T-bet activates the *ifn-γ* gene by binding directly to its promoter (Jenner et al. 2009) and silences *il4* gene expression (Djuretic et al. 2007). It is widely accepted that the role of Th1 cells is to foster cell-mediated immunity against intracellular pathogens.

4.1.2 Th2 and Th25 Cells

Th2 cells are induced to differentiate in the presence of IL-4, which induces STAT6 phosphorylation. Phospho-STAT6 promotes the expression of the transcription factor GATA3, which in turn leads Th2 cells to produce IL-4, IL-5, and IL-13 (Scheinman and Avni 2009). GATA3 directly represses the *ifn-γ* gene (Chang and Aune 2007; Djuretic et al. 2007). Further proof of the importance of GATA3 in Th2 cell development was obtained in studies in which GATA3 was deleted from T cells and those cells failed to differentiate into the Th2 lineage, while its overexpression in Th1 cells caused them to reprogram into Th2 cells (Pai et al. 2004; Zhang et al. 1997). Th2 cells are central in humoral immunity and host responses to helminth infections; however, they are chief contributors to the pathogenesis of allergic inflammatory diseases (Nakayama et al. 2017). The literature suggests the existence of a novel IL-25-producing T cell subset designated as Th25 cells, which seem to be closely related to the Th2 cell lineage (Swaidani et al. 2011), as both cell types need IL-4 for cytokine production and IL-25 (also known as IL-17E) enhances cytokine production (Fort et al. 2001). These cells are regulated by the transcription factor Act1 and were shown to induce non-lymphoid cells to synthesize Th2 cytokines during infection with helminths (Swaidani et al. 2011; Fallon et al. 2006), and possibly to extracellular pathogens, in general, as suggested by a recent study (de Sousa and Quaresma 2018). Fallon et al. (2006) reported that $il25^{-/-}$ mice were inefficient at eliminating the gastrointestinal nematode *Nippostrongylus brasiliensis*. To date, there are no studies demonstrating the involvement of Th25 cells in *H. pylori* gastric inflammation, as might be expected given their linkage to Th2 cell lineage and recent emergence of Th25 cells.

4.1.3 Th17

Th17 was initially described as a distinct Th subset in the last decade (Harrington et al. 2005; Park et al. 2005), and this lineage of Th cells has the retinoic acid receptor-related orphan receptor-γt (RORγt) as their master regulator (Ivanov et al. 2006). Their differentiation involves either IL-1β (Sutton et al. 2006; Pachathundikandi et al. 2016), IL-23 (Harrington et al. 2005), or the combination of IL-6 and TGF-β (Mangan et al. 2006). Th17 cells acquired their designation because of their ability to synthesize IL-17, both IL-17A and IL-17F (Harrington et al. 2005). IL-17 is a pro-inflammatory cytokine which acts both on hematopoietic and non-hematopoietic cells and induces antibacterial peptides, pro-inflammatory cytokines, chemokines, and prostaglandins. Among the chemokines induced by IL-17 are CXCL1, CXCL2, CXCL5, and IL-8, which promote neutrophil recruitment (Laan et al. 1999; Delyria et al. 2009), as well as CCL20, a chemokine important in cell recruitment to mucosal surfaces (Acosta-Rodriguez et al. 2007). These cells are linked to inflammation and autoimmunity (Langrish et al. 2005), as well as immunity to extracellular microbes, such as *H. pylori*, and their importance in immunity to mucosal pathogens has been highlighted in published studies (Khader et al. 2009). The differentiation of Th17 cells is inhibited by IL-27 (Hirahara et al. 2012), which also promotes Th1 cell differentiation (Yoshida et al. 2001).

4.1.4 Th22 and Th9 Cells

Th22 and Th9 cells are recently characterized $CD4^+$ Th subsets. Akin to Th17, which was so designated because of their production of IL-17, Th22 produces IL-22, whose expression was previously linked to Th17 cells, but now it is accepted that Th17 (Liang et al. 2006; Kreymborg et al. 2007) and NK cells (Cupedo et al. 2009; Crellin et al. 2010), in addition to Th22, produce IL-22, although the latter secrete the highest levels. In contrast to Th17 cells, which produce both cytokines, Th22 cells do not secrete IL-17A (Eyerich et al. 2009). Though they have been found within infected tissues and multiple inflammatory states, their role in immunity has not been well characterized due to the difficulty in culturing them in vitro, but that may change soon after a recent report described their generation in vitro in the absence of Th17 cells (Plank et al. 2017). IL-22 aids in the control of mucosal infections through the induction of inflammatory mediators and antimicrobial peptides (Rutz et al. 2013). A recent report correlated IL-22-induced antimicrobial peptides with vaccine-induced protection against *H. pylori* in mice (Moyat et al. 2017). As noted above, Th9 cells also represent a recently described subset of effector T cells whose differentiation from naïve T cells depends on transforming TGF-β and IL-4 (Dardalhon et al. 2008). This subset of effector T cells has a complex requirement of different transcription factors that include STAT6, PU.1, IRF4, and GATA3 (Chang et al. 2010; Staudt et al. 2010; Goswami et al. 2012). While their function in vivo is not clearly outlined, the available data suggest their involvement in atopy, as IL-9 promotes mast cell growth and induces changes in mast cell gene expression (Brough et al. 2014; Kearley et al.

2011). Though a proteomic study showed that IL-9 was elevated in the mucosa of *H. pylori*-positive GC samples (Ellmark et al. 2006), the role of Th9 in protection against *H. pylori* or associated pathogenesis is not clear. However, it has been suggested that IL-9 could be limiting the pro-inflammatory activity of Th17 cells since IL-9-deficient Th17 cells induce more severe autoimmune gastritis (Stephens et al. 2011). Interestingly, Th9 cells have recently been implicated in inflammatory bowel disease—more specifically in ulcerative colitis (Gerlach et al. 2015).

4.1.5 Tfh Cells

Tfh cells are a $CD4^+$ subset specialized in providing B cell help while sustaining enduring antibody responses in germinal centers of secondary lymphoid organs. Tfh cells are distinct from other $CD4^+$ T cell subsets by the expression of their hallmark CXCR5, and the transcription factor essential for their differentiation, B cell lymphoma-6 (BCL-6). Newly activated $CD4^+$ T cells when exposed to IL-6 are induced to differentiate into Tfh by signaling through the IL-6 receptor (IL-6R/gp130), which elicits Bcl6 expression (Nurieva et al. 2009). In turn, Bcl6 elicits early CXCR5 expression and the Tfh migrates to the B cell follicle border (Choi et al. 2011). Initially, these Tfh cells are induced by DCs and macrophages, but eventually the main APCs that they encounter are antigen-specific B cells in the follicle, interfollicular zone, and the T-B border. Their interaction with B cells is significant since B cells express ICOS-L, which provides co-stimulatory signals via ICOS on Tfh cells, which are essential for their complete differentiation (Choi et al. 2011). These T cells are important in immunity against infectious agents as antibody responses are critical in immune responses to most pathogens.

4.1.6 Treg Cells

Treg cells are $CD4^+$ T cells characterized by a high surface expression of CD25 (IL-2 receptor α chain), in addition to the expression of the transcription factor forkhead box P3 (FoxP3) (Hori et al. 2003). They represent about 5–15% of all $CD4^+$ T cells in the body, and there are two populations of Treg cells, which develop in different sites. Natural Treg (nTreg) cells undergo thymic maturation while induced Treg (iTreg) cells mature post-thymically (Rodriguez-Perea et al. 2016). The latter population, iTreg, is represented by two subsets that include Tr1, which lack FoxP3 and secrete IL-10 (Vieira et al. 2004), and Th3 that are $FoxP3^+$ and secrete TGF-β (Weiner 2001). Their foremost function is to suppress immunity by limiting extent and intensity of an immune response, and to maintain peripheral self-tolerance. This became evident by an experiment of nature in which humans with dysfunctional FoxP3 develop a condition known as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. This syndrome is characterized by a series of autoimmune disorders in various parts of the body such as the intestines, skin, and endocrine glands. Treg cells inhibit effector T cells through cell–cell contact and through the cytokines

that they produce, which include IL-10, TGF-β and IL-35 (Rodriguez-Perea et al. 2016; Jafarzadeh et al. 2015). The gastrointestinal mucosa is a body site where Treg cells are found at a high frequency as they aid in maintaining immune tolerance and are important in preventing intestinal inflammation (Izcue et al. 2009).

5 Reprogramming and Plasticity

Since the original definition of Th1 or Th2 effector cells was based on their cytokine production profiles, Th effector subsets were regarded as being terminally differentiated following a linear and unalterable process—each subset with a distinctive cytokine profile. However, studies in vitro initially suggested that those Th1 and Th2 cells could be induced to produce cytokines characteristic of the other subset when cultured under conditions that would promote the opposite subset. For instance, Th1 cells secreted IL-4 when they were cultured under Th2 culture conditions (Zhu et al. 2004). Similar observations were made with Th17 and Treg cells. Treg were reported to produce IL-17 after culture with IL-6, and they also upregulated RORγt expression (Yang et al. 2008). Furthermore, Treg cells may self-induce into IL-17-producing cells in the presence of IL-6 if TGF-β is absent (Xu et al. 2007) and Th17 may revert in vivo and in vitro into Th1 cells, as demonstrated by various independent groups (Martin-Orozco et al. 2009; Bending et al. 2009). These and similar observations uncovered the ability of "differentiated" T cells to convert to another phenotype leading to the hypothesis that T cells have phenotypic plasticity that is influenced by environmental cues. Thus, this ability of $CD4^+$ T cells to become reprogrammed and acquire features of other T cell subsets is now referred to as T cell plasticity. $CD4^+$ T cell plasticity can be modulated by a combination of extracellular and intracellular signals (DuPage and Bluestone 2016). The extracellular cues that may influence plasticity of $CD4^+$ T cells include the available cytokine milieu with the signaling that is activated, intensity of the TCR signaling, and signals activated by co-stimulator/co-inhibitor receptors. Plasticity may also be influenced intracellularly by signaling cascades, cell metabolism, and transcription factor (i.e., master regulators: FoxP3, RORγ, etc.) regulation. During infection with *H. pylori*, most of these extracellular and intracellular regulators are altered or used by the bacterium. For instance, our studies showed that infection with *H. pylori* results in the induction of the co-stimulatory molecules CD80, CD86, as well as the co-inhibitory receptor PD-L1 on GECs (Beswick et al. 2007b; Das et al. 2006; Ye et al. 1997), which not only influence the activation of T cells, but also promote their reprogramming (Beswick et al. 2007a). TGF-β, which stimulates both Th17 and Treg cells, is also produced by *H. pylori*-infected GECs, in a response that is dependent on the virulence genes *vacA* and *cagA* (Beswick et al. 2011).

6 *H. pylori* Induction and Evasion of the Host Immune Response

Pathogens that establish infections for life possess characteristics in their interactions with the human host that permit prolonged colonization periods, even in the presence of immune responses. In the case of *H. pylori,* the bacteria are adapted to colonize a distinctive niche that is hostile to most other microorganisms. Its ability to establish persistent infection with associated chronic inflammation predisposes the host to develop clinically significant gastric diseases, such as PUD and GC. The inflammatory response reflects the induction of host immunity, but *H. pylori* has an arsenal of mechanisms that enable successful evasion of innate and adaptive immunity in order to persist within the human gastric mucosa. Because the adaptive immune response is highly specific and is responsible for lasting immunity, we will focus the discussion on the adaptive immune response with an emphasis on how *H. pylori* subverts lymphocytes. Since T cells are activated by their interactions with APCs, to better understand how T cell responses are affected, we will also discuss the influence of *H. pylori* on classical APCs and the epithelium in their interactions with T cells.

Macrophages, DCs, B cells, and GECs are influenced during infection by *H. pylori,* and, in turn, they contribute to the mucosal response that takes place. Although *H. pylori* is not invasive, the bacterium and its products come in contact with cells in the lamina propria. Thus, the infected gastric mucosa has a significant influx of immune cells that include macrophages, DCs, and lymphocytes. Macrophages are recruited to the *H. pylori*-infected gastric mucosa and aid in the production of pro-inflammatory cytokines and chemokines (Dzierzanowska-Fangrat et al. 2008). Depending on how macrophages are activated they are functionally polarized as either M1 (classically activated by IFNγ and bacterial products and are pro-inflammatory), or M2 (alternatively activated by IL-4, IL-10, or IL-13 and are associated with wound healing and tissue repair) (Murray 2017). Analysis of gastric macrophages in *H. pylori*-infected mice showed that they were polarized to M1, and in humans, they showed a mixed M1/M2 phenotype, but in atrophic gastritis macrophages, they were also M1 (Quiding-Jarbrink et al. 2010). However, the work by Wilson's group has shown that macrophages in *H. pylori*-infected mice show activation of the arginase/ornithine decarboxylase pathway (Lewis et al. 2011), which is a feature associated with M2 macrophages. Further, another group reported the presence of $CD68^{+}CD163^{+}$Stabilin-1^{+} (M2) macrophages in the lamina propria of *H. pylori*-infected patients (Fehlings et al. 2012). Wilson's group also showed that *H. pylori* induces the heme oxygenase-1 (HO-1) gene in macrophages. HO-1 is an anti-inflammatory and antioxidant enzyme. This response was elicited by phosphorylated CagA and signaling that involves the activation of p38 and NF (erythroid-derived 2)-like 2 (NRF2) (Gobert et al. 2014). The activation of HO-1 in *H. pylori*-infected macrophages fosters a switch to regulatory macrophages able to dampen immune responses. Macrophages are also considered as key promoters in the differentiation of Th17 cells within the *H. pylori*-infected mucosa. Macrophages exposed to *H. pylori* or urease secrete pro-Th17 cytokines (Zhuang et al. 2011). Furthermore, two

independent studies using two different mouse models of autoimmune disease identified B cell activating factor (BAFF aka B-lymphocyte stimulator, BLyS, and TNF-superfamily member 13B) of the TNF-α family as a promoter for Th17 responses (Zhou et al. 2011; Lai Kwan et al. 2008). One report suggested that BAFF was directly involved in these responses, while the other report suggested that BAFF acts as a modulator of the cytokine milieu that would, in turn, affect the induction and function of Th17 cells. Munari et al. (2014) showed that IL-17 and BAFF levels are elevated in the mucosa of *H. pylori*-infected patients, and the increase of these two cytokines hinges on the presence of *H. pylori*. Macrophages in the gastric mucosa of patients are a major source of BAFF, which causes pro-Th17 cytokine production in a reactive oxygen species (ROS)-dependent manner. Taken together, all these reported observations suggest that *H. pylori* may affect macrophage polarity in multiple ways, and these macrophages may in turn contribute to the diverse Th cell responses reported during the infection. However, an important property for macrophages to affect T cells directly is by being able to phagocytose and process *H. pylori* antigens for presentation of the antigens to $CD4^+$ T cells.

Macrophages readily internalize *H. pylori*, but the bacteria avoid phagocytic killing. Virulent type 1 strains of *H. pylori* were found to disturb phagosome maturation and induce formation of anomalous vacuoles referred to as megasomes (Allen et al. 2000; Zheng and Jones 2003). Normal maturation of phagosomes occurs in stepwise fashion in which phagosomes fuse with early endosomes, late endosomes, and lysosomes. The intravacuolar pH decreases with each stage in order to allow for activation of the lysosomal proteases needed for antigen processing (Desjardins et al. 1994). However, *H. pylori* stops phagosome maturation, preventing it from attaining its full degradative capacity, which in turn allows for extended *H. pylori* intracellular survival (Allen 1999). Experiments with isogenic *vacA* and urease mutant strains to infect murine macrophages and macrophage cell lines pointed to their role in extending the survival of *H. pylori* (Zheng and Jones 2003; Schwartz and Allen 2006). The ability of *H. pylori* VacA to perturb the endocytic traffic at a late stage was initially described by Rappuoli's and Montecucco's groups using elegant cell biology methods (Papini et al. 1994).

DCs are robust APCs and a major immune cell type connecting both innate and adaptive immune responses. DCs are also among the cell types affected by *H. pylori* during infection and thus represent an important tool in the arsenal used by *H. pylori* to subvert host immunity since DCs may also function as different subsets that differentially regulate T cell functions. Among the various effects that *H. pylori* has on DCs include the induction of cytokines, such as IL-12, IL-23, and TNF-α, which are associated with Th1 responses (Amedei et al. 2006), in addition to a panel of other pro-inflammatory cytokines and chemokines (Kranzer et al. 2004). *H. pylori* has also been reported to promote monocyte maturation into DCs with increased HLA class II expression. An important virulence factor in promoting these responses is the neutrophil-activating protein NapA (Pachathundikandi et al. 2015). As the name implies, it also affects neutrophils and was initially labeled as such because it was reported to induce a high production of oxygen radicals from neutrophils (Evans et al. 1995a, b). In vitro studies showed that NapA alone, added to in vitro cultures, could

significantly limit development of Th2 clones to antigens such as tetanus toxoid (TT) and mite allergen. Interestingly, in those studies, most (89%) of the allergen-specific Th clones were Th2 clones in the absence of NapA, but in its presence their frequency decreased to only a small fraction (29%), while Th1 clones increased considerably (D'Elios et al. 2007). Because of NapA's potential to reprogram antigen-specific Th2 cell responses to polarized Th1, its possible use as an immunomodulator in Th2 diseases, such as atopy, has been suggested (Reyes and Beswick 2007). As discussed below, among the subsets of $CD4^+$ T cells that have been found to infiltrate the *H. pylori*-infected gastric mucosa are Treg and Th17 cells, but their balance is skewed toward a regulatory response. The effect that *H. pylori* has on the Treg/Th17 balance appears to be exerted via DCs (Kao et al. 2010). Studies conducted by Anne Muller's group using bone marrow-derived DCs exposed to *H. pylori* and co-cultured with $CD4^+$ T cells and a cocktail of anti-CD3, IL-2, and TGF-β showed that they induced more $CD25^+FoxP3^+CD4^+$ T cells than naïve DCs (not exposed to *H. pylori*) as determined by flow cytometry (Oertli et al. 2012). Interestingly, mesenteric lymph node (MLN)-DCs that were immunomagnetically isolated from *H. pylori*-infected mice also promoted the development of a large percentage of $CD25^+FoxP3^+CD4^+$ T cells in co-cultures with naïve $CD4^+$ T cells (Oertli et al. 2012). These and other similar observations suggest that *H. pylori* induces tolerogenic properties in DCs.

Presentation of *H. pylori* antigens by DCs not only activates T cells, but also indirectly promotes B cell activation through CD40-CD40L interactions between lymphocytes (Guindi 2000). The exact role of B lymphocytes in the development of anti-*H. pylori* immunity remains ill-defined, although *H. pylori*-carriers are known to develop strong local and systemic *H. pylori*-specific IgA and IgG antibody production (Futagami et al. 1998; Nurgalieva et al. 2005; Portal-Celhay and Perez-Perez 2006). Since infected individuals have elevated serum Ig titers to *H. pylori*, this response has been used to detect *H. pylori* infection, although IgG antibodies are not considered reliable indicators of current infection. The elicited antibodies fail to control *H. pylori* (Ermak et al. 1998). Early studies with a murine model of *H. pylori* infection examined the protective role of B cells by intragastric administration of *H. pylori*-specific IgA antibodies simultaneously with *Helicobacter felis* bacteria into germ-free mice. After infection with *H. felis,* the investigators observed a reduction of 70% of the number of colonized mice at 4 weeks post-infection (Czinn et al. 1993). In addition, experiments using mice deficient in IgA or immunoglobulin (μMT) that were immunized with urease and lysates of *H. pylori* or *H. felis,* later challenged with *H. pylori,* showed no differences in gastric colonization by *H. pylori* during the acute phase of infection (Ermak et al. 1998; Blanchard et al. 1999; Pappo et al. 1999; Akhiani et al. 2004, 2005). However, analysis of the chronic phase of infection (>8 wk p.i.) showed that μMT mice were able to clear the *H. pylori* infection with signs of severe gastritis, whereas the wild-type mice presented extensive *H. pylori* colonization with mild gastric inflammation (Blanchard et al. 1999). Overall, these reports showed that vaccine-induced immunity is elicited in comparable levels in wild-type and antibody- or B-lymphocyte-deficient mice. Interestingly, T cells from wild-type, IgA- and μMT-deficient mice produced comparably high levels of IFN-γ, whereas the levels of IL-10 produced were significantly higher in wild-type mice

than in the deficient mice (Akhiani et al. 2004, 2005). The use of IL-10/IgA double knockout mice helped to further examine the role of inflammation in controlling *H. pylori* colonization. These double knockout mice were 1.2-log significantly less colonized by *H. pylori* than mice deficient only in IL-10, which in turn were less colonized than wild-type mice. These observations led to the view that B cells and/or antibodies may have a pathological effect by promoting chronic inflammation.

IL-10 is among the immune signaling molecules made by B cells and has been linked with downregulation of protective T cell responses. IL-10 is significantly elevated in the gastric mucosa of patients and mice infected with *H. pylori* (Bodger et al. 2001). This cytokine is used by regulatory T and B cells to limit the inflammatory response. Mice deficient in IL-10 had a 100-fold reduction of *H. pylori* colonization in comparison with wild-type mice (Chen et al. 2001; Ismail et al. 2003). B cells can be activated directly by other mechanisms, including TLR, BCR, and cytokines receptors. BCR and TLR7/9 activation by nucleic acid–protein complexes, originating from chronic infection, and associated inflammation, initiates B cell activation via MyD88/NF-κB (Farinha and Gascoyne 2005; Fukata et al. 2008). Interestingly, experiments with murine B cells exposed to *H. pylori* extracts upregulated CD80 and IL-10 production via TLR2/MyD88 activation and promoted differentiation of naïve $CD4^+$ T cells into IL-10-producing $CD4^+CD25^+$ Treg cells, with suppressive activity in vitro through CD40/CD40L (Sayi et al. 2011; Smith 2014) (Fig. 4). Therefore, B cells can be activated pro-regulatory (IL-10 production) cooperating with T cells in the suppression of immunopathological inflammation associated with *H. pylori* infection. IFN-α is another cytokine made by plasmacytoid DC antigen-1 $(PDCA\text{-}1)^+$ B cells and found to suppress *H. pylori*-induced gastritis, and downregulate Th1-type cytokines (Otani et al. 2012). Interestingly, IFN-α administration to *H. pylori*-infected mice reduced neutrophil infiltration and levels of TNF-α and IFN-γ (Otani et al. 2012). Gastric samples from *H. pylori*-infected patients showed significantly increased IFN-α and IgM in their sera, as well as $PDCA\text{-}1^+$ B cells compared to controls (Ma et al. 2016). In addition, $PDCA\text{-}1^+$ B cells were more frequent in *H. pylori*- infected patients suffering from atrophic gastritis or peptic ulcers in comparison with non-atrophic gastritis patients (Ma et al. 2016).

The cellular infiltrate within the *H. pylori*-infected gastric mucosa includes both $CD4^+$ and $CD8^+$ T cells, which are significantly increased in the neck, pit, and gland regions, as noted in gastric biopsy sections (Nurgalieva et al. 2005; Bamford et al. 1998a). In early studies, we and others reported that the response is polarized to Th1 cells (Haeberle et al. 1997; Bamford et al. 1998a; Karttunen et al. 1990), which was an early indication that the immune response to *H. pylori* is misguided since Th1 cells influence cell-mediated immunity, which is inadequate against extracellular pathogens, such as *H. pylori*. In fact, Th1 cells seem to aid in pathogenesis, as supported by observations in human carriers, suggesting Th1 participation in *H. pylori*-associated lesions (Robinson et al. 2008). The presence of Th17 and Treg cells in the infected gastric mucosa has been reported by various independent groups (Jang 2010; Shi et al. 2010; Zhang et al. 2008; Lundgren et al. 2003, 2005). Further, in the *H. pylori*-infected gastric mucosa there is a marked infiltration of $CD4^+$ T cells with abnormal Th17/Treg cell ratios (Gil et al. 2014; Lundgren et al. 2003, 2005).

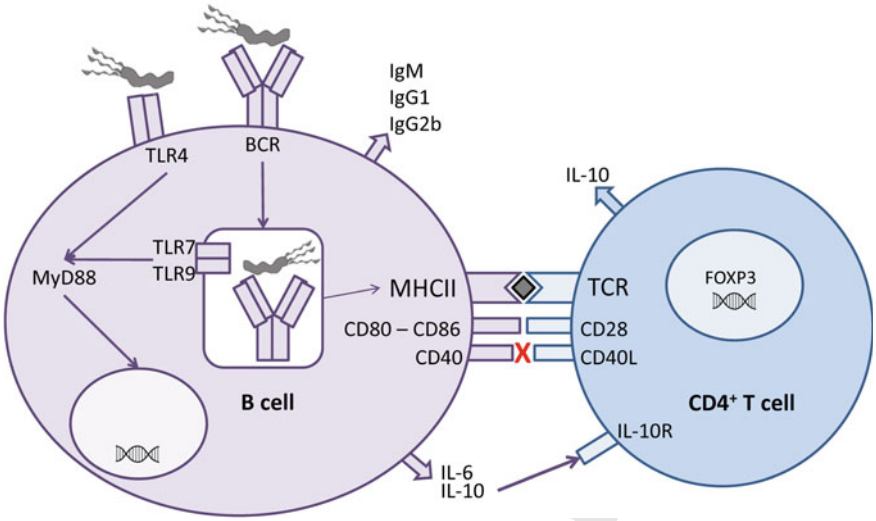

Fig. 4 *H. pylori* upregulate the expression of CD80 and IL-10 production via TLRs on B cells. B cells exposed to *H. pylori* upregulate receptors and cytokines that then promote Treg cell differentiation

CD25⁺/CD4⁺ versus FoxP3⁺/CD4⁺ cells frequencies vary significantly depending on the type of disease and severity (Cheng et al. 2012). FoxP3 is a master regulator of Treg cells whose frequency is significantly higher in GC patients than in patients with other *H. pylori*-related gastric diseases (Cheng et al. 2012). An increase in Treg cells leads to higher bacterial density and contributes to the development of atrophic gastritis and GC progression by suppressing anti-tumor effector T cells. Treg cells in the gastric mucosa helped explain earlier reports of T cell hyporesponsiveness of T cells from *H. pylori*-infected subjects when restimulated with *H. pylori* antigens, as compared to T cells from uninfected individuals (Fan et al. 1994; Karttunen et al. 1990). As Th17 cells are important in immune-mediated clearance of extracellular bacteria, their presence in the *H. pylori*-infected mucosa is expected. In fact, mouse immunization studies reported the contribution of Th17 cells, and a robust IL-17 secretion in protection against *H. pylori* (Delyria et al. 2009), but in those studies, the vaccinated mice were challenged with the *H. pylori* SS1 strain, which is defective in the type 4 secretion system. As explained below in some detail, our studies showed that a functional type 4 secretion system is important in *H. pylori*'s ability to evade Th17 cell responses (Lina et al. 2013). In infected mice fully virulent *H. pylori* inhibits Th17 and tips the balance to Treg cells (Kao et al. 2010). The Treg/Th17 balance is essential to immune homeostasis.

T cell activity is also shaped by immune checkpoint receptors expressed on immune cells that deliver inhibitory signals (Ceeraz et al. 2013). As mentioned earlier, the B7 family of co-stimulatory/co-inhibitory receptors has emerged as central in immune regulation, keeping a subtle balance between immune potency and suppres-

sion of autoimmunity (reviewed in (Francisco et al. 2010; Ceeraz et al. 2013)). We showed that *H. pylori* regulates GEC expression of various B7 immune checkpoints, which in turn impact local T cell development and function (Lina et al. 2013, 2015). These proteins perform as ON/OFF switches for T cell activity, and recent studies suggest their role in influencing T cell differentiation or phenotype. For example, in studies using co-cultures of naïve $CD4^+$ T cells with *H. pylori*-infected GECs, we noted that PD-L1 (aka CD274, B7-H1) promoted the development of Treg from those naïve $CD4^+$ T cells (Beswick et al. 2007b), and a separate report demonstrated that PD-L1 converted T-bet^+ Th1 cells into $FoxP3^+$ Treg cells in vivo (Amarnath et al. 2011). During infection with *H. pylori*, PD-L1 expression is increased by GECs (Das et al. 2006). We reported that epithelial cells from biopsy specimens of *H. pylori*-infected patients had an elevated expression of PD-L1 when compared to epithelial cells from uninfected subjects, and this was confirmed by infecting GECs in the absence of cytokines that are present in the infected gastric mucosa, which could induce that expression (Das et al. 2006). These results, regarding gastric epithelial PD-L1 expression during *H. pylori* infection, were confirmed by Wu ~~Y. and colleagues~~ (Wu et al. 2010). In subsequent studies, we observed that *H. pylori* infection, besides eliciting increased expression of PD-L1, also leads to a reduced expression of ICOS-L, which is the only positive co-stimulator known to act on activated or memory T cells (Lina et al. 2013). These findings suggested that *H. pylori* uses the epithelium to create a prime inhibitory scenario for Th effector cells by altering the expression of these proteins with profound immunomodulatory effects. These responses are partially dependent on *H. pylori* CagA and peptidoglycan translocated by the type IV secretion system (Posselt et al. 2013; Backert et al. 2015; Zhang et al. 2015) (Fig. 5). CagA was found to reduce ICOS-L expression by activating the p70 S6 kinase pathway. CagA contributes to the *H. pylori*-mediated activation of the mTOR/p70 S6 kinase pathway. The serine/threonine protein kinase mTOR acts downstream from PI3K/Akt and controls activation of p70 S6 kinase. The role of p70 S6 in downregulation of ICOS-L by the *cagA*$^+$ *H. pylori* strains was confirmed by adding to the cultures rapamycin, a specific inhibitor of p70 S6 kinase/mTOR. Because the ICOS-L–ICOS interaction is critical for Th17 cell development, maintenance, and function (Paulos et al. 2010), *H. pylori* is able to evade Th17 cell-mediated clearance by modifying ICOS-L expression as demonstrated in in vivo studies (Lina et al. 2013). The B7 family of "checkpoint regulators" (Ceeraz et al. 2013) affect adaptive immunity beyond T cell activation, as described above. They impact T cell differentiation, cytokine production, and reprogramming (Kuang et al. 2014; Lee et al. 2013; Ishiwata et al. 2010). As T lymphocytes play a key role in adaptive immunity, *H. pylori*'s influence on the expression of immune checkpoints may be pivotal in persistent infection and pathogenicity. It is worth noting that tumor cell expression of checkpoint inhibitors promotes tumor immune evasion and growth by inducing "exhaustion" of effector T cells (Wherry 2011), and the ability of *H. pylori* to alter the expression of these molecules may allow *H. pylori* to aid developing neoplastic cells to escape immune surveillance mechanisms.

Besides altering the local mucosal environment by modulating the expression of key immunoregulatory molecules or production of cytokines by the gastric epithelium

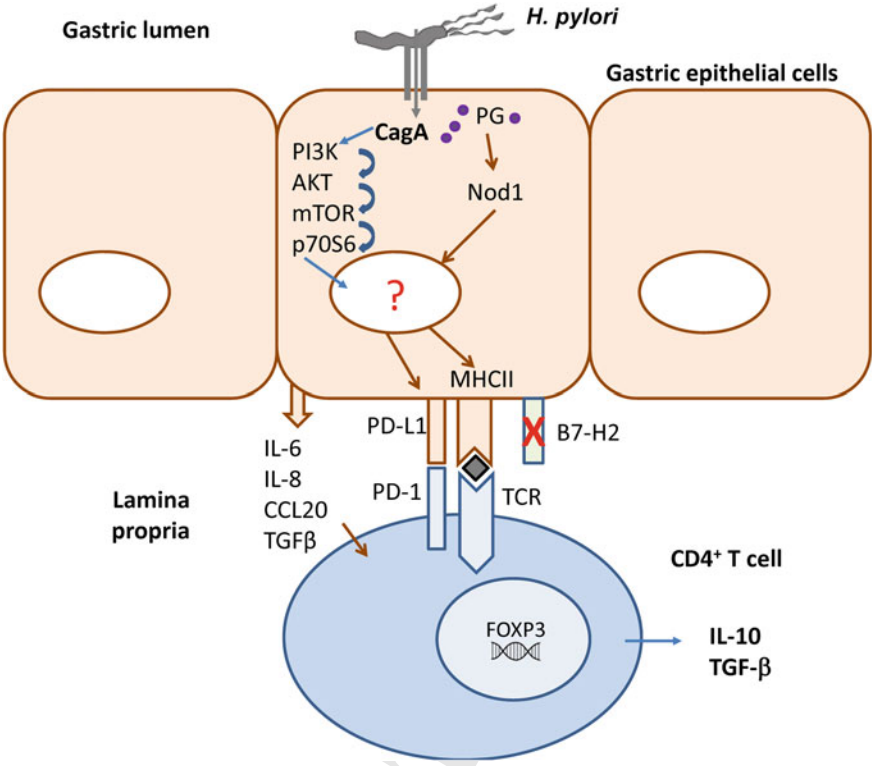

Fig. 5 *H. pylori* CagA and peptidoglycan translocated by the type IV secretion system into GECs promote a suppressive environment. *H. pylori* CagA and peptidoglycan injected into GECs lead to a reduction of B7-H2 expression by activating the p70 S6 kinase pathway. *H. pylori* CagA also promotes PD-L1 (B7-H1) expression by GECs. Both of these responses combined promote a suppressive environment because the ICOS-L–ICOS interaction is critical for Th17 cell development, maintenance, and function and in the absence of the interaction Th17, cells fail to develop. PD-L1 provides inhibitory signals for effector Th cells and promotes differentiation of Treg cells (Lina et al. 2013)

and immune cells, *H. pylori* is also able to directly inhibit $CD4^+$ T cells. *H. pylori*'s VacA toxin and γ-glutamyl-transpeptidase (GGT) have been reported to hinder T cell activation (Sundrud et al. 2004; Boncristiano et al. 2003). Both of these toxins are secreted products of *H. pylori*. VacA uses CD18 (β2-integrin) as a receptor on T cells (Sewald et al. 2008). VacA is internalized after the cytoplasmic domain of CD18 is phosphorylated by protein kinase C (Sewald et al. 2011). *H. pylori* VacA impedes T cell signaling and proliferation by promoting the arrest of the cells cycle at G1/S. *H. pylori* VacA mediates this effect by interfering with the TCR and IL-2 signaling pathways at the level of the Ca_2^+/calmodulin-dependent phosphatase calcineurin. By this mechanism *H. pylori* VacA prevents translocation of the important T cell transcription factor NFAT (nuclear factor of activated T cells) into the nucleus of T

cells leading to the suppression of *il-2* gene transcription (Gebert et al. 2003). Studies by Cover's group showed that *H. pylori* VacA constrains IL-2-induced cell-cycle progression and proliferation of T cells without altering IL-2-dependent survival, but through its N-terminal hydrophobic region needed for the creation of anion-selective membrane channels averting clonal expansion of T cells activated by *H. pylori* antigens (Sundrud et al. 2004).

The GGT enzyme from *H. pylori* has also been found to contribute to PUD and GC (Gong et al. 2010; Rimbara et al. 2013). GGT is a threonine N-terminal nucleophile hydrolase that catalyzes transpeptidation and hydrolysis of the gamma-glutamyl group of glutathione and converts glutamine resulting in the secretion of glutamate and ammonia into the periplasm and local milieu. Among the multiple effects that *H. pylori* GGT has, it has been reported to inhibit T cell proliferation and DC differentiation (Gerhard et al. 2005; Oertli et al. 2013; Schmees et al. 2007). Gerhard and colleagues showed that *H. pylori* GGT induces cell-cycle arrest in T cells at the G1 phase and thus suppresses their proliferation (Schmees et al. 2007). They reported that *H. pylori* GGT causes G1 arrest by disrupting Ras- and not PI3 K-dependent signaling (Schmees et al. 2007). *H. pylori* GGT also induces Cox2, which paradoxically may also suppress the Th1 polarization (Meyer et al. 2003). Both *H. pylori* GGT and VacA may also thwart T cell activity indirectly by reprogramming DCs into "tolerogenic" DCs, which foster the differentiation of naïve T cells into Treg cells (Oertli et al. 2013). Muller and colleagues reported that those DCs foster the expression of the FoxP3, CD25 and IL-10, characteristic markers of Treg cells, in naïve T cells (Oertli et al. 2013).

7 Concluding Remarks

Although the incidence of *H. pylori* infection has been decreasing due to enhancements in living conditions, the global prevalence of *H. pylori* remains high. In North America, approximately one-third of all adults are infected, while in developing regions, almost half of the population carries *H. pylori* (Eusebi et al. 2014). Thus, *H. pylori* remains an important human pathogen associated with significant clinical disease. Over the last few years, we have learned substantially regarding its diverse mechanisms to surreptitiously maneuver the host immune response in order to maintain persistent infection that may last a lifetime. Because the diseases associated with its infection remain a significant public health concern, due to their associated morbidity and mortality, and because of the increasing incidence of antibiotic resistance, there is a clear need for an effective vaccine that allows the host to surmount the multiple strategies used by *H. pylori* to thwart the host adaptive responses reviewed in this chapter. Since T lymphocytes are arguably the most essential cells in adaptive immunity, *H. pylori*'s impact on the expression of crucial receptors that control T lymphocyte function or tolerance is decisive in bacterial persistence and pathogenesis. Thus, in order to reduce the incidence of this important human pathogen through vaccination, we clearly need to better understand how it manipulates the host

immune armamentarium in order to effectively and appropriately steer it in directions that favor the host over the pathogen.

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