The Role of Toll-Like Receptor 9 in *Helicobacter pylori*-Mediated Inflammation and Carcinogenesis

By

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To my family, for their infinite support

and

To my fiancée, Caitlin whose patience and encouragement are unrivaled

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LIST OF ABBREVIATIONS

Abl.....Abelson Murine Leukemia viral oncogene homolog

ANOVA.....Analysis of Variance

AP-1.....Adaptor Protein 1

APC.....Antigen Presenting Cell

ATP.....Adenosine Tri-Phosphate

β-TrCP.....Beta Transducin-Repeat Containing Protein

Batf.....Basic leucine zipper transcription factor, ATF-like

BrdU.....Bromodeoxyuridine

C/EBP.....CCAAT Enhancer Binding Protein

Cag.....Cytotoxin Associated Gene

CAMP.....Cationic Anti-Microbial Peptide

CBP.....CREB Binding Protein

CD.....Cluster of Differentiation

CDN.....Cyclic Dinucleotide

CFU.....Colony Forming Units

cGAMP.....Cyclic GMP-AMP

cGAS.....Cyclic GMP-AMP Synthase

Cip1/Waf1.....Cyclin Dependent Inhibitor Kinase1

COP-II.....Coat Protein II

Cox.....Cyclooxygenase

CpG.....5'-Cytosine-phosphate-Guanine-3'

CRE.....Cyclic-AMP Response Element

CREB ..... Cyclic-AMP Response Element Binding Protein

CTAB ..... Cetyltrimethylammonium Bromide

DHX ..... DEAD/H (Aspargine-Glutumate-Alanine-Aspargine/Histidine) Box Polypeptide

DMEM ..... Dulbecco's Modified Eagle Medium

DNA ..... Deoxyribonucleic Acid

DSS ..... Dextran Sodium Sulfate

Dtr ..... DNA Transfer and Replication

EDTA ..... Ethylenedinitrilo-Tetraacetic Acid

EGFR ..... Epidermal Growth Factor Receptor

Elf1 ..... E74-Like Factor 1

ELISA ..... Enzyme Linked Immunosorbent Assay

Elk1 ..... ETS Containing Domain 1

EPIYA ..... Glutumate-Proline-Isoleucine-Tyrosine-Alanine

ERK ..... Extracellular Regulated Kinase

Ets2 ..... E26 Transformation Specific 2

FL-TLR9 ..... Full Length TLR9

FoxP3 ..... Forkhead Box P3

G-CSF ..... Granulocyte Colony Stimulating Factor

GM-CSF ..... Granulocyte Macrophage Colony Stimulating Factor

GSK-3B ..... Glycogen Synthase Kinase-3β

HEK ..... Human Embryonic Kidney

HMGB ..... High Mobility Group Box

HRP ..... Horseradish Peroxidase

Hsp60 ..... Heat Shock Protein 60

IBD ..... Inflammatory Bowel Disease

IFN ..... Interferon

IκB ..... Inhibitor of κB

IL ..... Interleukin

IMC ..... Inner Membrane Complex

IP ..... Immunoprecipitation

IP-10 ..... Interferon-γ Induced Protein 10

IRF-7 ..... Interferon Regulatory Factor 7

IRS/ISS ..... Immunoregulatory Sequence/ Immunostimulatory Sequence

KC ..... Keratinocyte Chemoattractant

LAMP2 ..... Lysosome Associated Membrane Protein 2

LPS ..... Lipopolysaccharide

LRO ..... Lysosome Related Organelle

LRR ..... Leucine Rich Repeat

MAPK ..... Mitogen-activated Protein Kinase

MCL-1 ..... Myeloid Leukemia Cell Differentiation Protein

MCP-1 ..... Monocyte Chemoattractant Protein

MD-2 ..... Myeloid Differentiation Factor 2

MIP ..... Macrophage Inflammatory Protein

MLST ..... Multi-locus Sequence Typing

MOI ..... Multiplicity of Infection

mpf ..... Mating Pair formation

MyD88 ..... Myeloid Differentiation Primary Response Protein

MZB ..... Marginal Zone B

NapA ..... Neutrophil Activating Protein

NCS ..... Neonatal Calf Serum

NFκB ..... Nuclear Factor-κB

NFAT ..... Nuclear Factor of Activated T-Cells

NK ..... Natural Killer Cell

NOD ..... Nucleotide-Binding Oligomerization Domain

ORF ..... Open Reading Frame

oriT ..... origin of transfer

PAI ..... Pathogenicity Island

PAMP ..... Pathogen Associated Molecular Pattern

PBS ..... Phosphate Buffered Saline

PCR ..... Polymerase Chain Reaction

pDC ..... Plasmacytoid Dendritic Cell

PMSS1 ..... Pre-Mouse Sydney Strain 1

PRAT4A ..... Protein Associated with TLR4

PU Box ..... Purine Rich Box

PVDF ..... Polyvinylidene Difluoride

RANTES ..... Regulated on Activation Normal T-Cell Expressed and Secreted

RIPA ..... Radio Immunoprecipitation Assay

RORγ(t) ..... RAR-Related Orphan Receptor γ

RPMI ..... Royal Park Memorial Institute

SDS-PAGE ..... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEAP ..... Secreted Embryonic Alkaline Phosphatase

SEM ..... Standard Error of the Mean

SHP2 ..... Src Homology Phosphatase-2

SNP ..... Single Nucleotide Polymorphism

Src ..... Sarcoma Viral Oncogene Homologue

Stat3 ..... Signal Transducer and Activator of Transcription 3

STING ..... Stimulator of Interferon Genes

T4CP ..... Type IV Coupling Protein

T4SS ..... Type IV Secretion System

TBK1 ..... TANK Binding Kinase 1

TE ..... Tris-EDTA Buffer

TGF-β ..... Transforming Growth Factor β

T_H ..... T Helper

TIR ..... Toll/IL-1 Receptor

TLR ..... Toll-Like Receptor

TNF-α ..... Tumor Necrosis Factor α

TRAF ..... TNF Receptor Associated Factor

TRIF ..... TIR-domain-containing adapter-inducing interferon-β

T_{Reg} ..... Regulatory T-Cell

TSA ..... Trypticase Soy Agar

UNC93B1 ..... Unc-93 Homolog B1

VacA ..... Vacuolating Cytotoxin

VAMP3 ..... Vesicle Associated Membrane Protein 3

Wnt..... Wingless-Related Integration Site

WT ..... Wild Type

ORIGINAL PUBLICATIONS

1. **Varga MG**, Shaffer CL, Sierra JC, Suarez G, Piazuelo MB, Whitaker ME, *et al.* Pathogenic *Helicobacter pylori* strains translocate DNA and activate TLR9 via the cancer-associated *cag* type IV secretion system. Oncogene. 2016 05/09/online.

2. Krishna U, Romero-Gallo J, Suarez G, Azah A, Krezel AM, **Varga MG**, *et al*. Genetic Evolution of a *Helicobacter pylori* Acid-Sensing Histidine Kinase and Gastric Disease. The Journal of infectious diseases. 2016 May 10. PubMed PMID: 27190191.

3. Noto MJ, Boyd KL, Burns WJ, **Varga MG**, Peek RM, Jr., Skaar EP. Toll-Like Receptor 9 Contributes to Defense against *Acinetobacter baumannii* Infection. Infection and immunity. 2015 Oct;83(10):4134-41. PubMed PMID: 26238713. Pubmed Central PMCID: 4567622.

4. Riquelme I, Tapia O, Leal P, Sandoval A, **Varga MG**, Letelier P, *et al.* miR-101-2, miR-125b-2 and miR-451a act as potential tumor suppressors in gastric cancer through regulation of the PI3K/AKT/mTOR pathway. Cellular oncology. 2016 Feb;39(1):23-33. PubMed PMID: 26458815. Pubmed Central PMCID: 4751587.

MANUSCRIPTS IN PROGRESS

1. **Varga MG**, Piazuelo MB, Romero-Gallo J, Delgado AG, Suarez G, Whitaker ME *et al.* TLR9 Activation Suppresses Inflammation in Response to *Helicobacter pylori* Infection. American Journal of Physiology: Gastrointestinal and Liver Physiology. In Revision

2. **Varga MG** and Peek RM. DNA Transfer and Toll-Like Receptor Modulation by *Helicobacter pylori.* Current Topics in Microbiology and Immunology. In Revision

CHAPTER I

INTRODUCTION

The 100-year search that led to the Nobel prize

Helicobacter pylori has secretly and quietly colonized the human gastric mucosa for millennia. However, in 1875, whispers of its existence began to emerge as German physicians observed spiral shaped bacteria in the linings of human stomachs; but since the physicians could not culture them, their findings were soon forgotten and the existence of *H. pylori* remained unknown (*11*). That was until 1899 when a Polish physician named Walery Jaworski observed spiral shaped bacteria in sediments taken from gastric washings and named it *Vibrio rugula* (*12*). Jaworski even went so far as to implicate this organism as a causative agent of gastric diseases in his book entitled "Handbook of Gastric Diseases"; however, his findings went unnoticed by the majority of the science community and *H. pylori* was able to once again evade detection. For the next 30 years, *H. pylori* remained in relative obscurity until late in the 1930's to early 1940's when spiral shaped bacteria were observed in the gastric mucosa of patients (*13, 14*). Unfortunately, that data was also soon forgotten as it was overshadowed by an extensive study by Palmer *et al.* published in 1954 that found no spiral shaped bacteria present in over 1,000 gastric biopsy specimens displaying gastritis (*15*). As a result of this study, the prevailing dogma for the next 30 years became that the stomach was sterile organ and that any observed bacteria were from oral contaminants. Undeterred by these hypotheses and unaware that the methodologies used in the Palmer study were inappropriate for the detection of *H. pylori*, a pair of intrepid investigators by the name Barry Marshall and Robin Warren identified a spiral shaped

bacteria in gastric tissue biopsies taken from patients with gastritis and duodenal ulcers which they named *Campylobacter pyloridus*. Although they were able to observe these organisms, it took an additional 2 years and a happy accident for them to successfully culture it (*16*). Despite their meticulous work, gastroenterologists were skeptical of their findings and were reticent to accept that the dogma they had so firmly believed had been proven false. To appease their peers and prove beyond a doubt that *Campylobacter pyloridus* was the etiological agent of gastritis and duodenal ulceration, Barry Marshall drank a culture of their isolated bacterium and tracked his disease progression by serial endoscopy over the course of weeks. He then resolved his self-inflicted infection with antibiotics and again monitored his progression as he returned to health. By fulfilling Koch's postulates, Marshall and Warren had proven beyond a doubt that their isolated bacterium could colonize the gastric mucosa and induce disease (*17*). This seminal finding was corroborated by a subsequent study in 1988, which demonstrated that in a prospective double blind trial of duodenal ulcer relapse, 92% of patients who were successfully cleared of *C. pyloridus* infection by antibiotic treatment showed signs of ulcer healing and only 21% displayed signs of relapse within the 12 month follow-up period (*18*). In 1989 16S ribosomal gene sequencing revealed that *C. pyloridus* was not actually a member of the *Campylobacter* species. As a result, it was given its own category termed *Helicobacter* and subsequently *Helicobacter pylori* was born. By 1990, a second, independent, team of investigators were able to replicate Marshall and Warren findings and concluded that *H. pylori* eradication can cure duodenal ulcer disease (*19*). Consequently, after numerous studies further validating their work, Marshall and Warren received the 2005 Nobel Prize for their groundbreaking discovery. Since then, the field of *H. pylori* has grown significantly with over 38,000 publications to date and averaging nearly 1,500 new publications per year.

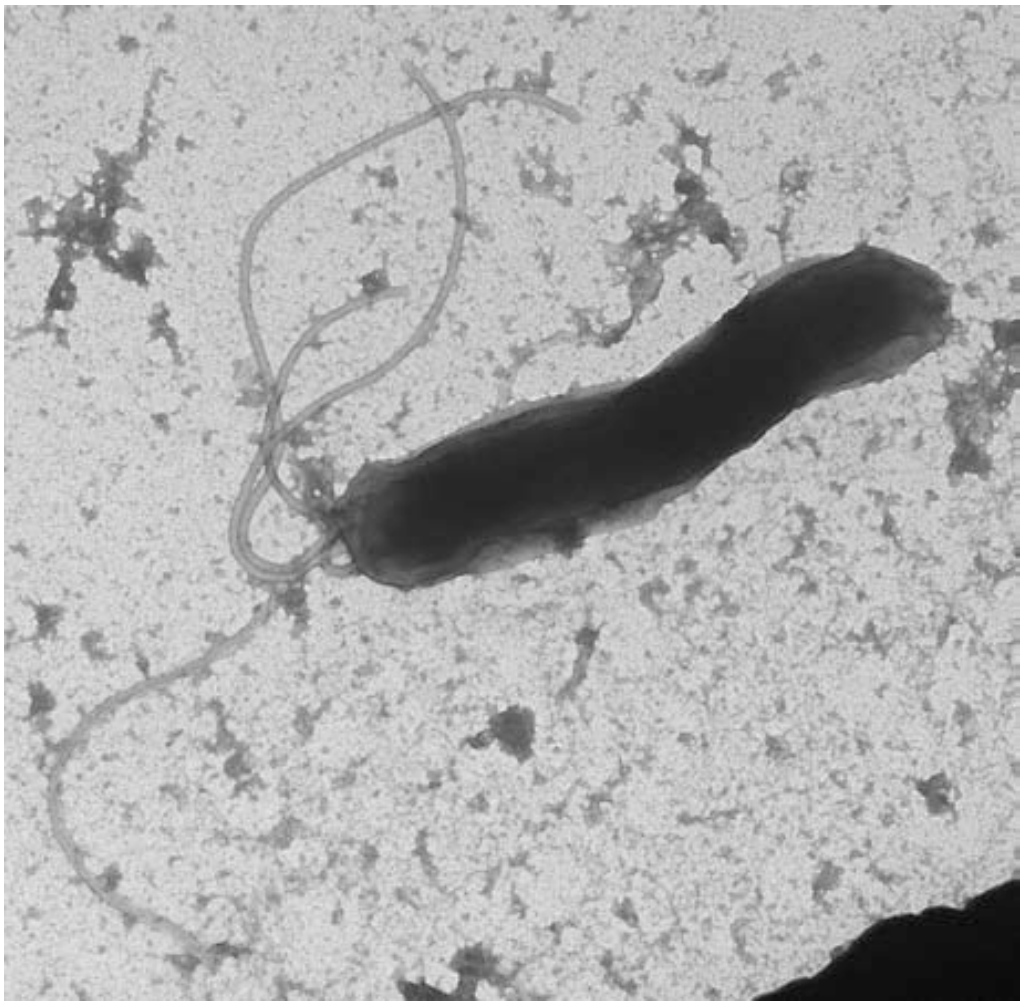

Figure 1: Characterization of *H. pylori*.
Electron micrograph of *Helicobacter pylori* displaying the characteristic rod shape and 4 polar flagella. Image kindly provided by Aime Franco.

H. pylori Prevalence and Epidemiology

H. pylori has evolved to survive within the acidic environment of the human stomach for 60,000-100,000 years and currently can be found within approximately 50% of the world's population. Intra-familial transmission and long-term association of *H. pylori* with humans has resulted in a phylogeographic distribution pattern that mirrors that of the host. Consequently, evaluation of *H. pylori* genomes can be used to trace complex demographic events in human pre-history. For example, the genome of the 5,300 year-old Iceman was recently fully sequenced and has shed more light on the timeline of human migration from Asia into Europe (*20*). Even after 100,000 years of colonization, the mode of *H. pylori* transmission remains poorly understood. Epidemiological studies have suggested that poor sanitation, lower socioeconomic status, and high population density conditions facilitate transmission of *H. pylori* (*21-25*). This data correlates with the observation that colonization rates in developed countries are declining to approximately 50% of the population while in developing countries colonization may exceed 90%. Fortunately, studies have identified and overall decline in *H. pylori* colonization worldwide, a trend that will continue as long as the standard of living continues to improve around the world (*26*).

Risks Associated with *H. pylori* Colonization

Although the prevalence of *H. pylori* infection remains high and persists for the lifetime of the host, the majority of infected patients (90%) never display symptoms. They may however develop superficial chronic gastritis in response to the presence of this pathogen. Only a fraction of infected individuals will progress beyond superficial gastritis and develop peptic ulcer disease, atrophic gastritis, intestinal metaplasia or distal gastric adenocarcinoma (*27-44*). In infected

patients the severity of gastritis, and thus the risk for progression toward cancer, is influenced by both local and systemic immune responses elicited by *H. pylori.* Innate immune recognition of *H. pylori* is typically mediated by Toll-like receptor (TLR) engagement in epithelial cells, antigen presenting cells (APCs), and macrophages which in turn lead to adaptive immune mediated responses (*45-47*). These adaptive immune responses display a T-helper cell type 1 (T_H1) and type 17 (T_H17) bias, which can subsequently lead to atrophy and metaplasia (*48-51*). However, disease outcome is not only dictated by the immune response alone, it is also contingent upon the pattern of inflammation within the stomach (*52*). Chronic antral-predominant inflammation can yield hyperchlorhydria and increased risk for duodenal ulceration while corpus-predominant or pan-gastritis can lead to hypochlorhydria and a predisposition for both gastric ulceration and adenocarcinoma (*52, 53*).

Many tumors, including those of the gastric mucosa, arise in the context of chronic inflammation. Colonization with *H. pylori* initiates inflammation that can progress further to promote gastric cancer. Gastric adenocarcinoma is the third leading cause of death worldwide accounting for 723,000 deaths annually and *H. pylori* infection significantly increases cancer risk (*9*) **(Figure 2)**. Studies have shown that the incidence of gastric cancer in *H. pylori*-infected individuals increases 2-16 fold compared to uninfected individuals (*39*). Consequently, the World Health Organization classified *H. pylori* as a Class I carcinogen in 1994. Noncardia gastric cancer is typically classified as one of two histologically distinct categories (*54*). Diffuse-type gastric adenocarcinoma commonly affects younger persons and is indiscriminate between sexes. Typically, diffuse-type adenocarcinoma can be composed of individually infiltrating neoplastic cells that fail to form glandular structures and are independent of intestinal metaplasia (*42*). The second type of gastric adenocarcinoma is intestinal-type, named after the islands

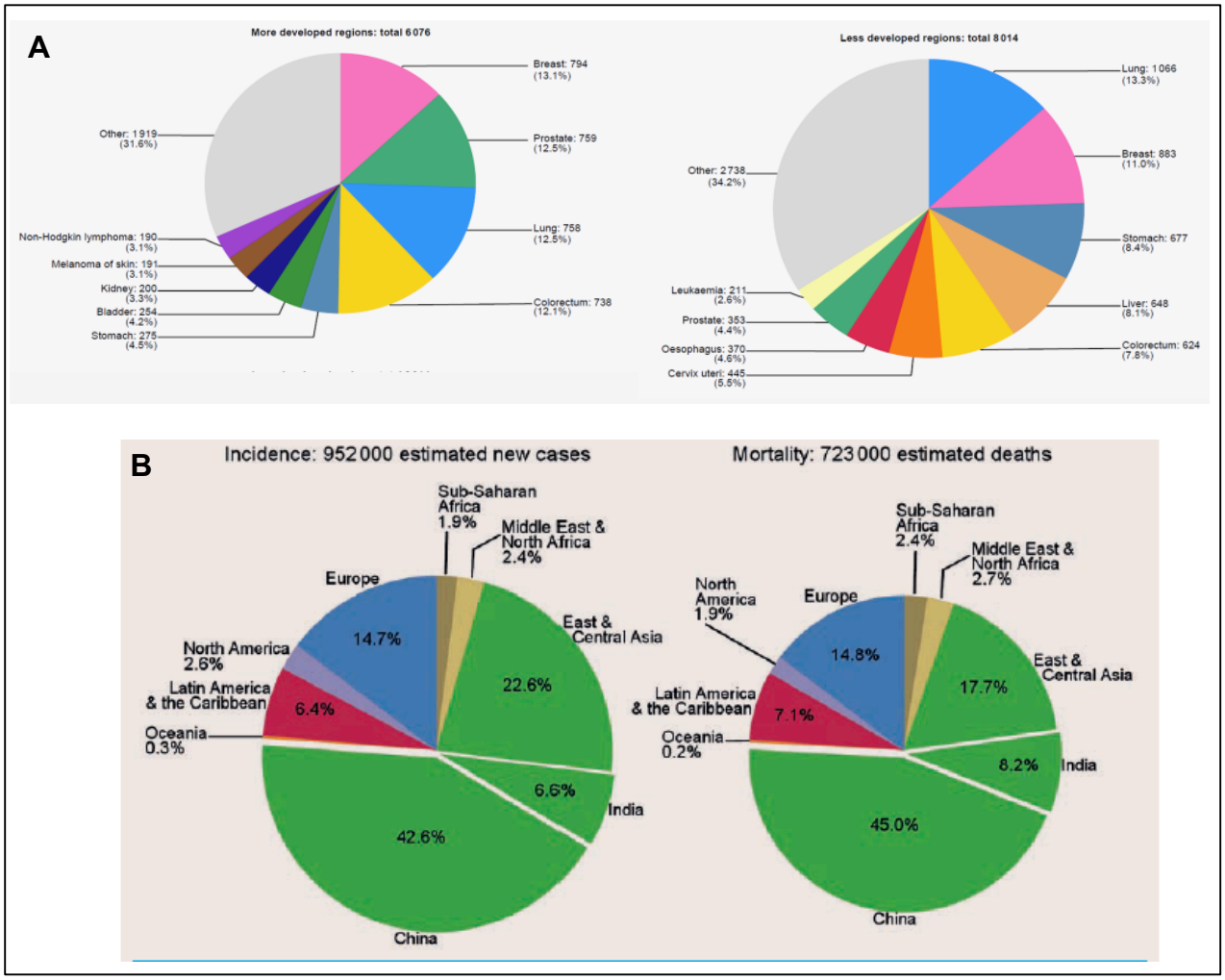

Figure 2: Epidemiology of cancer worldwide.

A) Estimated global numbers of new cases (thousands) of both sexes in 2012 with proportions stratified by more developed and less developed regions. Gastric cancers are depicted in blue/grey. Image reprinted with permission from (*1*). B) Estimated global number of new cases and deaths from stomach cancer broken down by world region. Data includes both sexes as of 2012. Image reprinted with permission from (*9*).

of intestinal epithelium found within the gastric mucosa. Intestinal-type gastric adenocarcinoma predominantly affects men of advanced age and progresses through a series of well-defined pre-neoplastic steps as defined by Dr. Pelayo Correa in 1975 **(Figure 3)** (*55*).

Virtually all *H. pylori*-infected persons develop superficial gastritis, however a very small percentage will progress to neoplasia. This suggests that enhanced cancer risk may involve specific bacterial factors, host genetic differences, or dietary constituents that may alter the delicate equilibrium between pathogen and host **(Figure 4)** (*56-61*). Due to the fact that the majority of infected individuals remain asymptomatic and its long evolutionary history with humans, an argument could be made that *H. pylori* is not a pathogen at all and may simply be a misunderstood commensal organism. Several reports have indicated that *H. pylori* infection is inversely related to the risk of developing Barrett's esophagus, esophageal adenocarcinoma, and other inflammatory diseases such as hay fever, asthma and atopic eczema (*62-70*). Additionally, *H. pylori* DNAs have been shown to mitigate the inflammatory responses resultant from DSS-induced colitis in a murine model (*71, 72*). Taken together, these data suggest that a very deep, comprehensive understanding of *H. pylori*-host interactions is essential for balancing the benefits and risks of eradication and the potential for gastric carcinogenesis.

***H. pylori cag* Pathogenicity Island**

H. pylori strains obtained from patients are highly genetically diverse. Nearly every *H. pylori* isolate is unique at the DNA level as evidenced by multi locus sequence typing (MLST) and DNA microarrays (*73-80*). One major difference among *H. pylori* strains is the presence of a 40kb gene locus referred to as the *cag* pathogenicity island (*cag* PAI) and is present in

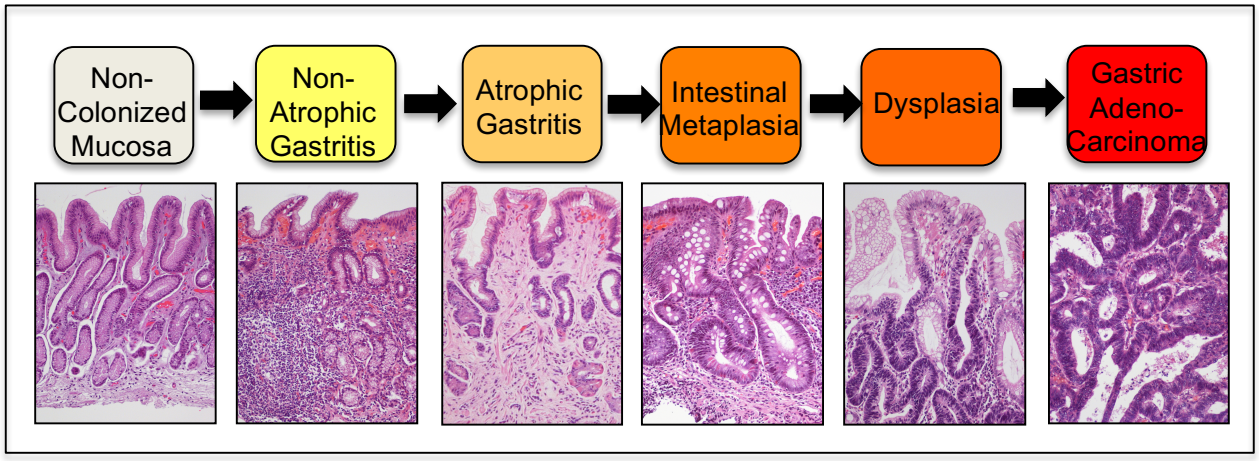

Figure 3: The Correa cascade.
The Correa cascade characterizes the progression of intestinal-type gastric adenocarcinoma through a well-defined series of steps. Representative images of hematoxylin and eosin stains (H&E) are shown below each of the steps.

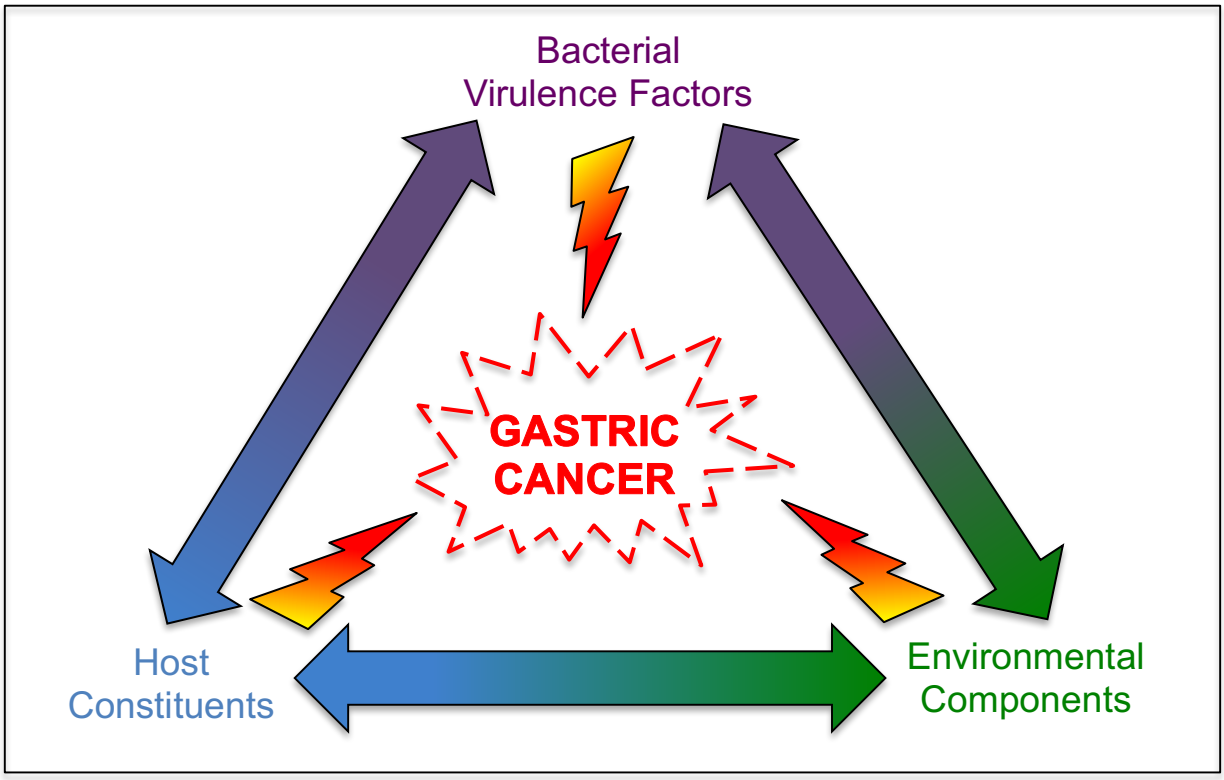

Figure 4: Gastric cancer is the result of a synergism between a triumvirate of factors.

approximately 50% of all strains isolated in the United States and as many as 90% of strains from East Asia (*81*). *H. pylori* strains that harbor the *cag* island display an increased virulence and significantly augment gastric cancer risk (*82-94*). Interestingly, the G+C content of the *cag* island is substantially different from the rest of the chromosome, which is suggestive of an acquisition via a horizontal gene transfer event (*95*). Annotation of the *cag* PAI genes demonstrated that there are 27 encoded genes, some of which share homology to the archetypal *vriB/D4* encoded type IV secretion (T4SS) of *Agrobacterium tumefaciens*. However, in some instances the level of sequence homology between the *cag* and *virB/D4* system is weak while other *cag* genes are unique to *H. pylori* and share no homology to any known organism identified to date **(Table 1)**. Taken together, these data suggest a phylogenetic divergence from a common ancestor (*96*).

The *cag* PAI is a gene locus that encodes for the proteins required to assemble a type IV secretion system (T4SS) and translocate the *cag* PAI encoded effector molecule CagA into host cells. The T4SS is the most ubiquitous secretion system amongst both Gram-positive and Gram-negative bacteria, however it is also unique amongst bacterial secretion systems because it can translocate both protein and DNA. Studies have shown that cag^- strains are predominantly localized in the mucous gel layer while cag^+ strains are found adjacent or adherent to the gastric epithelium which suggests that the *cag* island influences the topography of colonization and is involved in liberating nutrients from the host (*97*).

The primary effector of the *cag* T4SS is CagA. *H. pylori* CagA is a bacterial oncoprotein that is injected into host cells upon bacterial attachment. The CagA protein has at least four different motif regions, termed EPIYA-A, -B, -C, or –D and they are distinguished by the amino acid sequence surrounding the motif. Following translocation into host epithelial cells, CagA can

either be tyrosine phosphorylated by Src and Abl kinases at specific EPIYA amino acid motifs at the 3' terminus or can remain un-phosphorylated (*7, 98*). Phosphorylated CagA activates the phosphatase SHP-2 and ERK, which subsequently leads to morphological changes ("hummingbird" phenotype, **Figure 5A,B**) and induces responses similar to those of growth factor stimulation (*3, 7*). The EPIYA-D motif, which is mostly found in East Asian phylotypes, can undergo more phosphorylation compared to the Western-type EPIYA-A, -B, or –C motifs **(Figure 5C)** (*7*). Un-phosphorylated CagA has been shown to induce mitogenic and pro-inflammatory responses as well as weaken cell-cell junctions to induce a loss of cellular polarity. Lastly, translocation, but not necessarily phosphorylation, can induce aberrant β-catenin activation that leads to disruption of apical-junctional complexes, loss of cellular polarity, and activate genes involved in transformation (*99-104*).

Not all CagA-mediated responses induce adverse outcomes within the host cell. As a means of persistence, *H. pylori* has evolved mechanisms to reduce the inflammatory response resultant from its second virulence factor, the VacA toxin, by translocating more CagA. All *H. pylori* strains contain a VacA gene, however there is marked diversity between *vacA* genotypes between strains. *H. pylori* secretes VacA where it then binds to the plasma membrane of host cells. Once bound, VacA can then be internalized and form an anion selective channel in endosomal membranes which subsequently leads to vacuolization due to swelling of the endosomal compartments. The effects of this vacuolization include reductions in cellular trans-epithelial resistance, induction of apoptotic pathways via mitochondrial damage and inhibition of antigen presentation to $CD4^+$ T-cells (*105*). VacA can target mitochondria and induce apoptotic cell death; however CagA can inhibit the apoptotic loss of gastric pit cells via the induction of

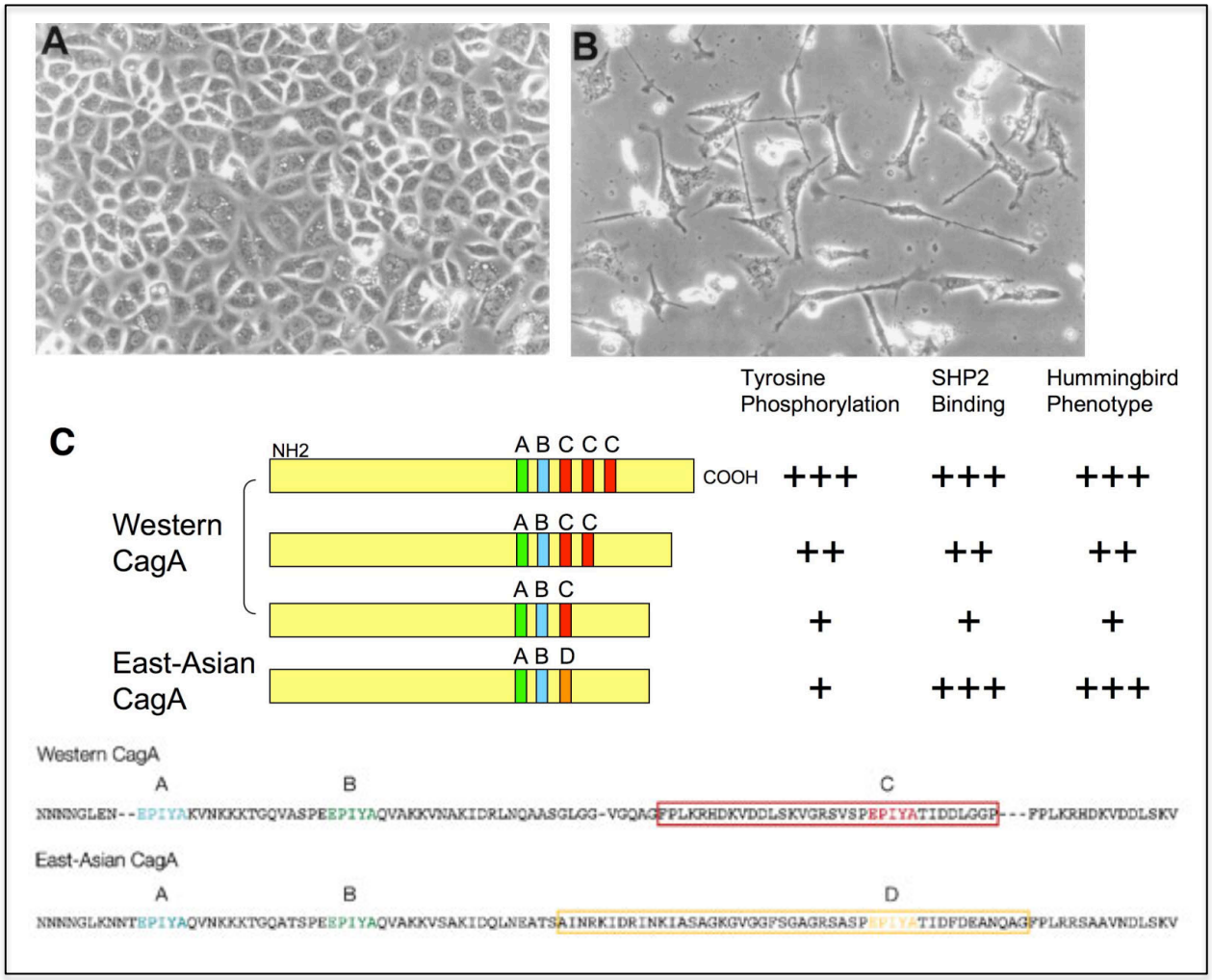

Figure 5: Modes of *H. pylori* CagA virulence.

A) The *H. pylori cag* PAI can induce hummingbird phenotype and is correlated with CagA virulence. Bright field micrograph of uninfected AGS gastric epithelial cell monolayer or *H. pylori*-infected AGS gastric epithelial cells displaying the characteristic hummingbird phenotype are shown in (B). Images reprinted with permission from (*3*) C) EPIYA motifs on the CagA protein are characterized further by distinct amino acid sequences that flank the motif. Western CagA can be defined by EPIYA-C motifs which vary in abundance, however the more C motifs present, the greater the ability to bind SHP-2 and induce morphological changes. East Asian strains are defined by the EPIYA-D motif, which binds SHP-2 more strongly compared to EPIYA-C motifs and can thus induce hummingbird phenotypes more readily. Image adapted from (*7*).

the pro-survial proteins ERK-1/2 and the anti-apoptotic protein MCL-1 (*106*). In addition, phosphorylated CagA can prevent pinocytosed VacA from reaching its intracellular targets. Similarly, the effects of CagA and VacA antagonism can also be observed in cellular phenotypes *in vitro* where CagA inhibits vacuolization and VacA can block the hummingbird phenotype induced by CagA in AGS gastric epithelial cells (*107*). To further dampen the immune response, *H. pylori* can modulate T-cell responses with its CagA and VacA proteins. CagA can drive NFAT signaling, a transcription factor that regulates the mitogenic gene p21WAF1/Cip1 (*106*). However, the VacA toxin can inhibit NFAT signaling in T-lymphocytes and has also been shown to counteract NFAT activation induced by CagA (*106*). Taken together, these data suggest that the association of CagA with VacA may represent a microbial strategy to protect its niche within the gastric mucosa from the detrimental effects induced by its toxins.

Bacterial Type IV Secretion Systems

Bacterial T4SS are responsible for the mobilization of macromolecules including monomeric proteins, multimeric toxins, and DNA-protein complexes across the envelopes of Gram-negative or -positive bacteria (*108-110*). T4SS can be categorized into three groups: conjugation systems, effector translocation systems, and DNA release/uptake systems (*109*). Although there is extensive versatility in bacterial T4SSs, the most common and well-studied subset of bacterial T4SSs is the conjugative system. The conjugative T4SSs are of significant medical relevance due to their capacity for widespread transmission of antibiotic resistance genes and/or virulence genes through plasmid or chromosomal DNA transfer (*111*). Conjugative bacterial T4SS are not only capable of translocating DNA out of donor cells, but they can also take in DNA from the extracellular milieu. Additionally organisms can use the DNA release/uptake T4SSs to exchange DNA substrates with the extracellular milieu in a contact

independent manner (*108*). DNA transfer events between bacterial species or into the extracellular milieu are very common, however trans-kingdom DNA transfer events are rare. To date, there are only a few bacterial species capable of translocating DNA into a eukaryotic host including: *Agrobacterium tumefaciens, Escherichia coli* (*112, 113*)*, Bartonella henselae* (*114*)*,* and, relevant to this dissertation, *Helicobacter pylori* (*115*).

Agrobacterium tumefaciens uses a VirB/D4 conjugation system to deliver oncogenic DNA (T-DNA) in conjunction with effector chaperone proteins into susceptible plant cells, resulting in crown-gall disease (*111, 116-118*). This bacterial T4SS has served as the canonical conjugative secretion system and has been studied the most extensively. The *A. tumefaciens* T4SS is composed of 12 proteins, VirB1-11 and VirD4 (*119*). Most bacterial T4SS, including the *H. pylori cag* T4SS and the *comB* T4SS share some homology in sequence or function to the VirB/D4 proteins of this archetypal secretion system **(Figure 6A,B)** (*96*).

Conjugation can be categorized into three distinct biochemical reactions. First, DNA transfer and replication (Dtr) proteins bind its cognate origin of transfer (*oriT*) sequence and initiate processing the DNA for transfer (*120-122*). Second, the Dtr-*oriT* complex (relaxosome) binds to the type IV coupling protein (T4CP) (*123*). Lastly, the T4CP delivers the DNA substrate to the trans-envelope channel composed of mating pair formation (mpf) proteins that shuttle the DNA across the cell membrane (*119, 124*). The process of DNA transfer is energized by the ATPases VirD4, VirB11, and VirB4 components (*110, 119, 125*). In Gram-negative bacteria such as *A. tumefaciens* or *E. coli*, the central hub protein, VirB10 spans both the inner and outer membranes and is decorated by structural proteins VirB7 and VirB9 in a 1:1:1 ratio to form a 14-fold symmetrical outer membrane pore termed the core complex (*126, 127*). This core

complex serves as the structural scaffold for the biogenesis of the outer membrane pore and the pilus (composed mostly of VirB2 and few VirB5 proteins) (*110, 119, 127*). The channel gate opens as a result of structural changes of VirB10 in response to ATP utilization by VirD4 and VirB11 as well as DNA substrate docking (*111*). Once the channel is open, the DNA substrate is able to move to the bacterial cell surface (*111*). The pilus and core complex have been well characterized to date, however the structure of the interacting proteins outside of the core complex, termed the inner membrane complex (IMC), are less studied. In the *E. coli* R388 encoded T4SS, the core complex is connected to the IMC via a central stalk region (*127*). At the distal end of the stalk lie two barrel-shaped densities that display C_2 symmetries composed mostly of VirB4 (*127*). The structure of the *E. coli* R388 system is suggestive of a two-step DNA transfer process in which VirD4 pumps the relaxosome-DNA substrate into the periplasm or by an IMC-mediated passage through the inner membrane. Once in the periplasm, the DNA substrate can then be secreted via the core complex (*127*).

The *H. pylori* Type IV Secretion Systems

In contrast to the conjugative T4SS of *A. tumefaciens* or *E. coli* R388 which can translocate DNA substrates into eukaryotic hosts (*112, 118*), the *H. pylori* T4SS has been categorized an effector/translocator T4SS because until recently, only the CagA protein and peptidoglycan have been shown to be translocated (*128, 129*). Compared to the conjugative T4SS, relatively little is known about the structure of effector translocation T4SSs (*96*). The *H. pylori cag* pathogenicity island is composed of 18 genes required for T4SS-dependent phenotypes (*130*). While some of these genes share some sequence relatedness to other bacterial species, the sequence conservation is quite low and 9 of the 18 genes are fundamentally unique

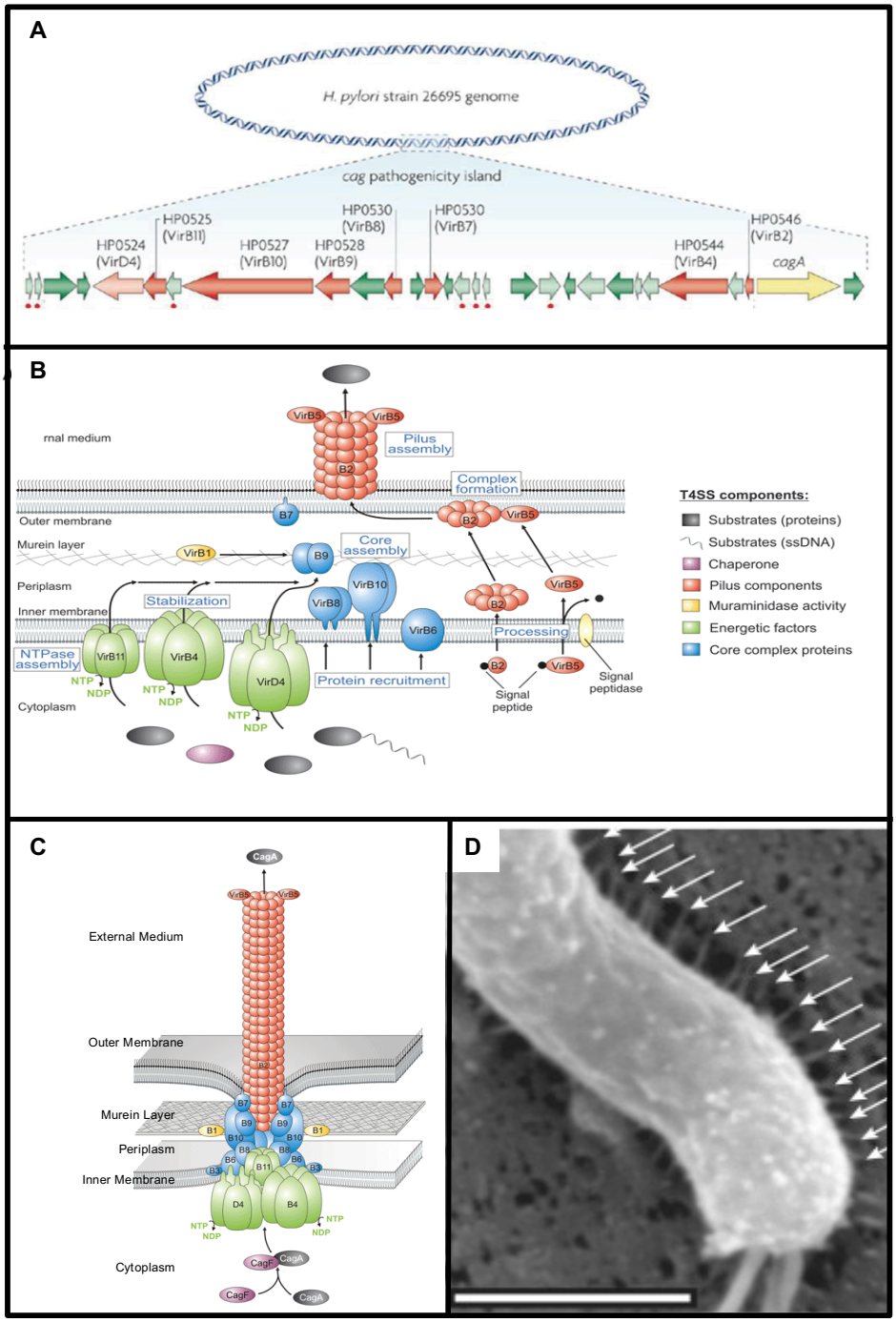

Figure 6: Structure of the *H. pylori cag* T4SS.

A) The *cag* PAI is a 40kb gene locus that encodes 27 genes required for the assembly of the T4SS. Red arrows represent genes that share some homology to the *A. tumefaciens* archetypal T4SS. Yellow arrow represents *cagA*, the primary effector molecule of the T4SS. Seven genes as indicated by red dots are not required for either CagA translocation or induction of *cag* PAI mediated pro-inflammatory responses. Reprinted with permission from (*4*). B) The T4SS is composed of core complex proteins, energetic factors and pilus components that assemble to form the T4SS. Reprinted with permission from (*6*) C) Model of the *H. pylori cag* T4SS and localization of structural Cag proteins in complex with the host cell. CagA is shown in gray, pilus components in red, core complex proteins in blue, and the energetic components are shown in green. Reprinted with permission from (*6*). D) Electron micrograph of *H. pylori* T4SS pilli on and AGS gastric epithelial cell. Scale bar set to 1 micron. Reprinted with permission from (*8*).

Gene Number[1]	Protein	Size[1] (kDa)	Homology[2]	Function	Essential for CagA Translocation?[3]
HP0547	CagA	125		Effector	n/a
HP0546	CagC	13	VirB2	Pilus Protein	+
HP0545	CagD	22		Accessory Factor	-
HP0544	CagE	100	VirB4	ATPase	+
HP0543	CagF	29		CagA Chaperone	+
HP0542	CagG	16		Accessory Factor, Unknown Function	+
HP0541	CagH	39		Pilus Assembly	+
HP0540	CagI	40		Pius Assembly	+
HP0539	CagL	26	VirB5	Pilus Assembly & Host Cell Binding	+
HP0538	CagN	32		Accessory Factor	-
HP0537	CagM	40		Inner Membrane Core Complex Protein	+
HP0536	CagP	13		Accessory Factor, Unknown Function	-
HP0535	CagQ	14		Accessory Factor, Unknown Function	-
HP0534	CagS	21		Accessory Factor, Unknown Function	-
HP0532	CagT	30	VirB7	Inner Membrane Core Complex Protein	+
HP0531	CagU	23		Accessory Factor, Unknown Function	+
HP0530	CagV	27	VirB8	Core Complex	+
HP0529	CagW	55	VirB6	Inner Membrane Channel	+
HP0528	CagX	55	VirB9	Inner Membrane Core Complex Protein	+
HP0527	CagY	220	VirB10	Inner Membrane Core Complex Protein & Putative Host Receptor Binding	+
HP0526	CagZ	21		Stabilizes Cag5	-
HP0525	Cagα	35	VirB11	NTPase	+
HP0524	Cag5/Cagβ	80	VirD4	Coupling Protein	+
HP0523	Cag4/Cagγ	18	VirB1	Lytic Transglycosidase	+
HP0522	Cag3/Cagδ	50		Inner Membrane Core Complex Protein	+
HP0521	Cag2/Cagε	10		Accessory Factor, Unknown Function	-
HP0520	Cag1/Cagξ	13		Accessory Factor, Unknown Function	+

Table 1: Nomenclature and functional importance of the T4SS proteins encoded by the *cag* PAI.
[1] Based on the *H. pylori* strain 26695 genome
[2]Based on comparison to *A. tumefaciens* T4SS
[3]Translocation into AGS cells; + yes, - no.

to *H. pylori* and do not display any homology to other bacterial proteins **(Table 1, Figure 6 A,C,D)** (*96*). Recent studies have shown that the *H. pylori cag* T4SS core complex is composed of proteins unrelated to components of T4SS in other bacteria and similarly the architecture of this T4SS is vastly different from those of other T4SS **(Figure 6 B,C)** (*96*). The *cag* core complex is 41 nm in diameter and is composed of 5 *cag*-encoded proteins (Cag3, CagT, CagM, CagX, and CagY) (*96*). In contrast, the *A. tumefaciens* core complex has a diameter of only 20 nm and is composed of 3 proteins, VirB7, VirB9 and VirB10 (*126, 131*). Of note, CagX and CagY share some homology to VirB9 and VirB10, respectively and both core complexes share a 14-fold symmetry (*96*). Taken together, these data suggest that the *H. pylori cag* T4SS is highly unique and this notion is further evidenced by the fact that *H. pylori cag* T4SS is the only known T4SS to date that is capable of translocating a protein substrate (CagA), non-proteinacious substrate (peptidoglycan) and a DNA substrate (*115, 125*).

The *H. pylori comB* T4SS falls within the DNA uptake category and is primarily involved in natural competence and is functionally independent of the *cag* T4SS (*132*). Generally, competence systems share some homology with proteins involved in the T4SS pilus formation or type II secretion systems (*133*). Indeed, the *H. pylori comB* system is composed of many conserved T4SS components, and its proteins have thus been named after their orthologues in the *A. tumefaciens* VirB/D4 system **(Figure 7)** (*134*). The *comB* system shares a similar gene cluster organization of the VirB/D4 system and only lacks the VirB1, virB5 and VirB11 components (*135*). ComB2 and ComB3 are orthologous to VirB2 and VirB3 in secondary structure despite having only 27.2% or 19.5% sequence homology, respectively (*134*). Although *comB* systems are nearly ubiquitous in *H. pylori* strains, they are unique from other DNA uptake T4SSs in that they incorporate DNA in a pilus-independent manner, the DNA uptake efficiency

varies between strains, and uptake is saturable, sensitive to length, symmetry and strandeness of the DNA substrate (*136, 137*). The VirB2 protein, and by association the ComB2 protein, is a subunit of the surface exposed pilus structure of the VirB/D4 system. One study has suggested that the ComB2 protein may be a component of an incomplete "stump structure" in the cell envelope required for DNA uptake (*119*). Unlike the VirB2 component, the functions of VirB3 homologues are unknown, except that they are essential for T4SS function (*134*). The *H. pylori* ComB DNA uptake system is energized by ComB4, an orthologous protein to VirB4 (*138*). The remaining components of the *H. pylori* ComB system are the ComB6-10 proteins that together, form the core complex of the import channel. ComB6 is a polytopic membrane protein essential for T4SS function (*134*), while ComB7 is a non-essential membrane attached protein that stabilizes the central channel structure (*139*). ComB8 is a membrane-associated protein that contains a periplasmic *N*-terminus, ComB9 is a periplasmic protein anchored to the membrane, and lastly ComB10 is also mostly periplasmic with its *N*-terminus anchored to the cytoplasmic membrane (*139*). Based on its homology to the archetypal T4SS of *A. tumefaciens*, a two-step DNA uptake model has been proposed (*140*). The ComB T4SS facilitates double-stranded DNA uptake into the periplasm, and a second, ComEC system is then utilized to incorporate the periplasmic DNA across the cytoplasmic membrane and into the cell (*140*).

Apart from the *comB* and *cag* systems, a novel 16.3kb gene cluster was recently discovered in one of three plasticity regions of the *H. pylori* genome (*141*). First described in *H. pylori* strain PeCan18B, this gene cluster is home to 16 ORFs, 7 of which share homology to the *virB/D4* operon **(Figure 8)** (*141*). Based on these data, this gene cluster is speculated to be a third T4SS involved in conjugative DNA transfer, and has thus been named *tfs3*. Furthermore, this gene cluster has a lower G+C content compared to the rest of the *H. pylori* genome, and it

has therefore been suggested that *tfs3* was acquired by a horizontal gene transfer event from a different bacterial species (*141*). When 94 clinical *H. pylori* isolates were screened for the presence of *tfs3*, 19% of the strains contained either the full length or truncated genes (*141*). Upon further investigation into the *tfs3,* significant sequence variation between genes were present among different isolates, and subsequently led to the re-classification of *tfs3* into two sub-groups based upon their homology, termed *tfs3a* and *tfs3b* (*142*). However, *tfs3a* and *tfs3b* were clearly distinct and *tfs3b* was again renamed to *tfs4,* in reference to a fourth T4SS (*135, 143*).

The *tfs4* gene cluster was first observed and subsequently characterized in *H. pylori* strain P12 **(Figure 7)** (*143*). Similar to *tfs3, tfs4* was found in another plasticity zone of the *H. pylori* genome and contained enough orthologues of the *A. tumefaciens virB/D4* genes to constitute a complete T4SS (*143*). The *tfs4* cluster contains the disease marker *dupA*, a *virB4* homolog that has been affiliated with increased risk for duodenal ulcer (*144*) and a *TraG*/*VirD4* homolog which encodes a type IV coupling protein independent of the established VirD4 protein homolog in the *cag* T4SS (*145*). Although it is yet to be proven *in vivo, in vitro* experiments have shown that *tfs4* encodes for proteins that can excise the gene cluster and facilitate its own horizontal transfer to recipient cells via the Tfs4 T4SS (*143*). Additional studies have shown that *H. pylori* can translocate genomic DNA to another bacterial species, *Campylobacter jejuni,* in a unidirectional manner on agar plates (*146*). This transfer was independent of the *comB, cag,* and *tfs3* genes in the donor (*146*) and thus leaves the open possibility that *tfs4* genes could facilitate this gDNA transfer event (*135*).

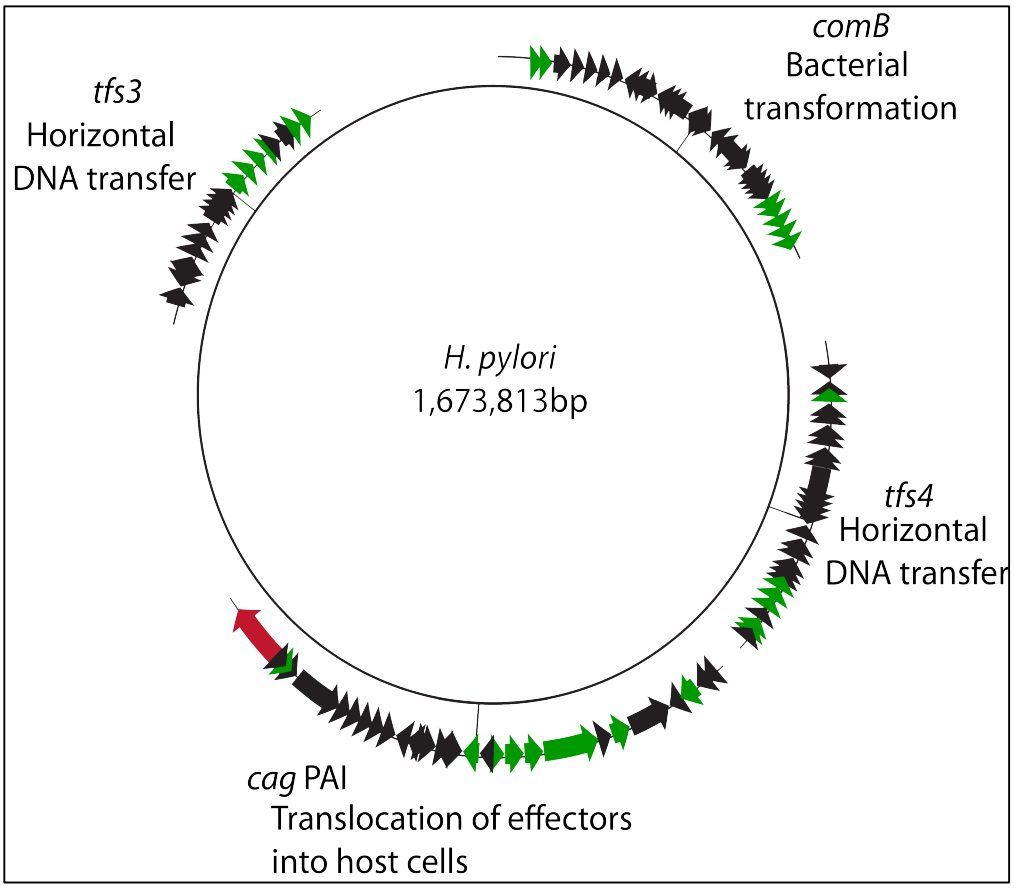

Figure 7: *H. pylori* may possess up to four T4SSs.
A Representative image of T4SS topography is shown within the *H. pylori* genome. *virB* homologs are shown in green; *cagA* is shown in red. Image not drawn to scale.

Host Factors That Contribute to *H. pylori*-Mediated Carcinogenesis

Bacterial virulence factors are not the only constituents that contribute to *H. pylori*-mediated inflammation and carcinogenesis. As mentioned previously, host factors also play an integral role in an individual's propensity for adverse outcomes. Single nucleotide polymorphisms (SNPs) in the interleukin 1β (*IL-1β*) or tumor necrosis factor α (*TNF-α*) genes, which encode for pro-inflammatory cytokines with acid suppressive properties, have been reported to increase the risk for atrophic gastritis and gastric adenocarcinoma (*57, 58, 60, 147, 148*). However, these polymorphisms only affect the histologic outcome in *H. pylori* colonized patients, which further emphasizes the importance of host-pathogen interactions. This is exemplified in a study conducted by Figuiredo *et al.* in which persons with high-expression *IL-1β* alleles and by *H. pylori cag*$^{+}$ strains showed a 25-fold increase in the risk for developing gastric adenocarcinoma compared to baseline (*58*). Furthermore, there is increasing evidence that SNPs in cytokines/chemokines such as IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, and the IL-1R play an essential role in promoting inflammation within the context of gastrointestinal cancers (*149-159*). Additionally, while the importance of aberrant pro-inflammatory cytokines/chemokines in promoting gastric cancer cannot be overstated, functional defects within the pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which primarily regulate these cytokine/chemokine responses, may be of greater importance within the context of tumor promotion (*48*). This is evidenced by extensive studies of TLR SNPs and their relationship with gastric carcinogenesis. SNPs within TLR1, TLR2, TLR4, TLR5, and TLR9 have all been shown to increase the risk for the development of gastric cancer (*61, 160-169*).

H. pylori-Mediated Modulation of Toll-Like Receptors

Toll-like receptors orchestrate immune responses targeting pathogens and bridge innate and adaptive immunity via selective recognition of pathogen associated molecular patterns (PAMPs) (*48, 170, 171*)**.** These PAMPs can originate from a wide array of molecules such as lipids, nucleic acids, and specific proteins that can be derived from organisms of bacterial, viral, or fungal origin. So far there are 10 identified human TLRs, however not all of them are relevant to *H. pylori* infection: TLR2 homo- or heterodimers with TLR1 or TLR6; (detects lipotechoic acid, NapA, Hsp60), TLR4 (detects lipopolysaccharide), TLR5 (detects flagella), TLR9 (detects hypo-methylated DNA), and TLR10 hetero-dimerized with TLR2; (hypothesized to detect lipopolysaccharides) have been shown to associate with *H. pylori* virulence factors (*171-173*). TLRs are an essential component of the innate immune system and are expressed both on cell surfaces and intra-cellularly. They can be localized to a wide array of cell types including macrophages and dendritic cells, as well as within non-immune cells including those of the gastric epithelium. Upon microbial ligand binding to its leucine rich repeat (LRR) ectodomain, TLRs dimerize and adaptor molecules such as MyD88 or TRIF complex with the intra-cytoplasmic Toll/IL-1 receptor (TIR) domain (*174*). Activation of TLRs induces signaling cascades that eventually lead to the transcription of both pro- and anti-inflammatory cytokines, as well as type I interferons. Chronic activation of TLRs has been linked to the promotion of gastric carcinogenesis (*175, 176*) and this chronic activation typically results from a failure to clear an invading pathogen such as *H. pylori.* Interestingly, as discussed earlier, *H. pylori* has co-evolved with its human host for millennia and has also adapted mechanisms to either evade or dampen the TLR-mediated immune response as a means to maintain persistence. Therefore,

despite extensive research in TLRs, their exact role in *H. pylori* infection remains controversial as *H. pylori* has evolved mechanisms to evade the activation of those pertinent to its infection.

TLR4 is a PRR that detects bacterial lipopolysaccharide (LPS) and induces a robust inflammatory response. However, *H. pylori* LPS is a highly unique structure that is well adapted to help maintain persistence within the gastric niche. This is primarily accomplished by the expression of Lewis antigen decoration of the *H. pylori* LPS O-antigen, which is the outermost domain of the LPS molecule. These Lewis antigens mimic the host Lewis antigens expressed on the apical surface epithelium and within the glands of both the antrum and corpus (*177, 178*). Through this molecular mimicry, *H. pylori* can evade immune detection but run the risk of eliciting autoimmune responses (*179*). Additionally, *H. pylori* harbors unique modifications to the lipid A core domain (*180-182*). The lipid A core is the inner most domain of LPS, sometimes referred to as endotoxin, and is the ligand for the TLR4-MD2 immune complex. Compared to other bacterial LPS, *H. pylori* LPS has ~1,000 fold less endotoxicity (*183, 184*) and this reduction is attributed to 3 major modifications to the lipid A core. The first modification is a hypo-acylation pattern where *H. pylori* is tetra-acylated compared to hexa- or penta-acylated chains. Secondly, these hypo-acylated fatty acids have longer carbon chain lengths (2 18-carbon and 2 16-carbon chains) compared to the optimal chain lengths required for robust TLR4 activation. Lastly, *H. pylori* LPS is hypo-phosphorylated, an adaption that also renders it less susceptible to destruction by cationic antimicrobial peptides (CAMPs) (*180, 184*). Although the role of TLR4 in immune activation is divisive, accumulating evidence suggests that it has a diminishing role in immune activation to *H. pylori.* This is evidenced by observations that monoclonal anti-TLR4 antibodies in the presence of *H. pylori*-epithelial cell co-cultures failed to block interleukin 8 (IL-8) secretion (*185*) and that *H. pylori* infected human embryonic kidney

293 (HEK293) cells transfected with TLR4 failed to induce NFκB activation (*47*). Some studies have found that *H. pylori* can up-regulate TLR4 expression in gastric epithelial cell lines, however it has also been shown that *H. pylori* may up-regulate TLR4 to use as a receptor for adherence to the epithelial cell surface (*185*) and that immune recognition of *H. pylori* is TLR4 independent (*186, 187*) or *H. pylori* LPS may antagonize TLR4 (*188*).

The natural ligand of TLR5 is flagellin, namely the highly conserved *N*-terminus of the D1 domain (*189*). Since *H. pylori* is a flagellated bacterium and TLR5 is expressed in the gastric epithelium, then it should be capable of inducing TLR5-mediated pro-inflammatory signaling cascades. However, *H. pylori* flagellin is not recognized by TLR5 (*190-192*) due to a mutation in the conserved domain of FlaA (*190*). This mutation, which occurs in the D0-D1 domain between amino acids 89-96, renders the flagella inert to TLR5 and when these amino acids are substituted into corresponding region of the *Salmonella enterica* serovar Typhimurium FliC, it too loses the ability to activate TLR5 (*190*). Taken together, these data suggest an important role in the *H. pylori* FlaA in maintaining persistence within the gastric niche by limiting the activation of TLR5.

DNA is the fundamental molecule of nearly all living organisms and consequently is normally sequestered within eukaryotic nuclear envelopes, bacterial cell walls, or viral capsids. During the course of infection, this DNA can be released purposefully or as a result of degradation from invading microbes or damaged host cells. TLR9 is an endosome bound, transmembrane receptor that detects these aberrant DNAs and orchestrates the appropriate immune response towards them (*193*). TLR9 expression is most abundant in dendritic cells (DCs), B-Cells, macrophages, and other antigen presenting cells (APCs); however, it is also expressed in epithelial cells. In pDCs and B-cells, TLR9 activation classically leads to the release

of pro-inflammatory cytokines and type I interferons, while epithelial responses are less defined. TLR9 is a unique TLR because it does not fit the criteria of a classical PAMP. Unlike most TLRs that recognize distinct molecular structures unique to pathogens, TLR9 cannot differentiate "self" from "non-self" DNAs. TLR9 was originally thought to have been able to discern pathogenic DNAs based upon the presence of hypo-methylated CpG motifs (which are rare in eukaryotic genomes), however accumulating evidence suggests that TLR9 can also recognize DNA in a sequence independent manner via structural components such as the sugar backbone (*194-196*). Therefore, in an attempt to prevent the recognition of "self" DNA, evolutionary pressure has relegated TLR9 to endosomal sequestration (*197*). The consequences of TLR9 surface expression were characterized in a study in which TLR9 transmembrane mutant mice were generated by swapping the localization signal of TLR9 with that of TLR4. The TLR9 transmembrane mutant mice died from systemic inflammation and anemia within 4 weeks (*198*). Since TLR9 is constrained to the endosome, cells of the immune system must internalize pathogens or pathogenic DNA before it can be detected by TLR9. Most immune cells can accomplish this task through either receptor-mediated endocytosis in response to scavenger receptor binding, phagocytosis of complement-mediated opsonized material, or Fc receptor mediated uptake of antibody opsonized material, or a combination of these (*199*). In the case of "self" DNA detected from apoptotic cells, select marginal zone B (MZB) and B1a B-cells can internalize chromatin complexes and induce a TLR9-mediated immunoregulatory response through IL-10 to counteract the pro-inflammatory signaling induced by DNA-antibody complexes internalized by rheumatoid factor expressing B-cells (*200*). The TLR9-mediated immune response is a highly complex and sometimes fickle response that is not only dependent

on cellular localization both between cell types and within cells but also dependent upon the ligand and its origin.

In the human gastric niche, TLR9 expression is primarily localized to the apical surface epithelium, however *H. pylori*-induced chronic active gastritis changes the expression pattern to the basolateral compartment only (*5*) **(Figure 8)**. In polarized intestinal epithelial cells TLR9 activation in the basolateral compartment has been linked to preferentially mediating pro-inflammatory signaling; however, the effects of TLR9 localization in gastric epithelial cells have not been elucidated (*176, 201*). Cyclooxygenases (Cox) catalyze key steps that eventually lead to substrates for prostaglandin synthases. The prostaglandin synthases subsequently catalyze reactions that terminate in prostaglandins and eicosanoids. Importantly, prostaglandins regulate a diverse array of physiological responses including immune modulation and maintenance of vascular tone (*202*)**.** There are three isoforms of Cox, Cox-1, -2, -3; each preform similar functions but they vary in their expression characteristics. Cox-1 and Cox-3 are constitutively expressed while Cox-2 can be up-regulated by pro-inflammatory cytokines and growth factors (*203*). The up-regulation of Cox-2 is recognized as a tumor promotion event in colorectal cancer and has also been shown to be up-regulated in *H. pylori*-induced pre-malignant and malignant gastric lesions (*204-210*). Previous studies have shown that *H. pylori* can induce gastric epithelial cell expression of Cox2 in a TLR2/TLR9 dependent manner (*209, 210*). In this particular study, the authors show that TLR2/TLR9 signaling in gastric epithelial cells induce MAPKs and subsequently allow transcription factor binding to both the CRE and AP-1 sites within the Cox2 promoter. As a result of Cox2 expression, prostaglandin E_2 is released and together they promote gastric cancer cell invasion and angiogenesis (*209*). Furthermore, the oncogenic potential of *H. pylori*-TLR9 interactions were exemplified by a study in which

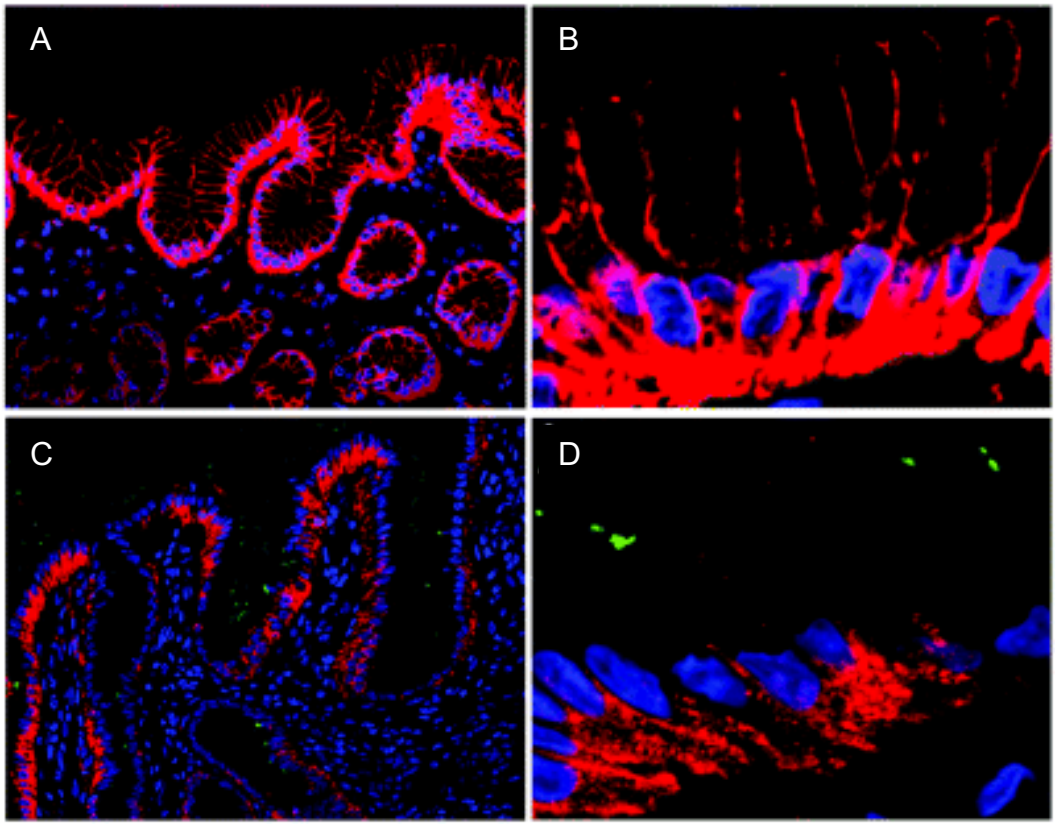

Figure 8: TLR9 changes localization after chronic infection with *H. pylori*.
A) Overview of TLR9 expression in non-inflamed human gastric mucosa displays both the apical and bssolateral localization. Epithelial area is magnified in (B). C) Overview of TLR9 expression during *H. pylori*-induced chronic active gastritis displays localization only at the basolateral pole. Image magnified in (D). A-D) TLR9 stained in red, nuclei and *H. pylori* stained with DAPI and pseudocolored to blue and green respectively. Image reprinted with permission from (*5*).

purified *H. pylori* DNA induced invasion of gastric epithelial cells *in vitro*, an effect that could be partially reduced with the endosomal inhibitor chloroquine (*211*). Additionally, in murine models of *H. pylori* infection, Rad *et al.* have demonstrated that TLR9 detects *H. pylori in vivo* and induces pro-inflammatory responses (*212*). These studies are complicated by more recent investigations in which TLR9 was shown to promote anti-inflammatory signaling during the acute phase of *H. pylori* infection, which was mediated by type I IFNs (*213*). Moreover, purified *H. pylori* DNAs have been shown to alleviate DSS-induced colitis in mouse models (*71, 72*). Collectively, these data highlight the dichotomous role of TLR9 during *H. pylori* infection. *H. pylori* may utilize TLR9 signaling to dampen the inflammatory response during the acute phase to establish infection; however, in an inflammatory micro-environment in which cells have lost their polarity TLR9 may execute pro-inflammatory cascades and further exacerbate the progression towards gastric cancer **(Figure 9)**.

Although both murine and human TLR9 recognize CpG DNA motifs, much care must be taken in translating findings from mouse studies into human relevance. The TLR9 receptors from mice and humans recognize different DNA ligands and mouse TLR9 is expressed in many more cell types compared to humans. Human TLR9 is expressed in epithelial cells, B-cells, neutrophils and plasmacytoid DCs whereas murine TLR9 is expressed in those same cell types and additionally in myeloid DCs, as well as macrophages (*214, 215*). These data suggest that the DNA sequences or structures that activate murine TLR9 may not elicit the same responses in humans while the more abundant localization of TLR9 in mouse species suggests that it may be exposed to more and varied ligands. Together, the differences in DNA sequence recognition and cellular distribution may account for the contrasting observations between

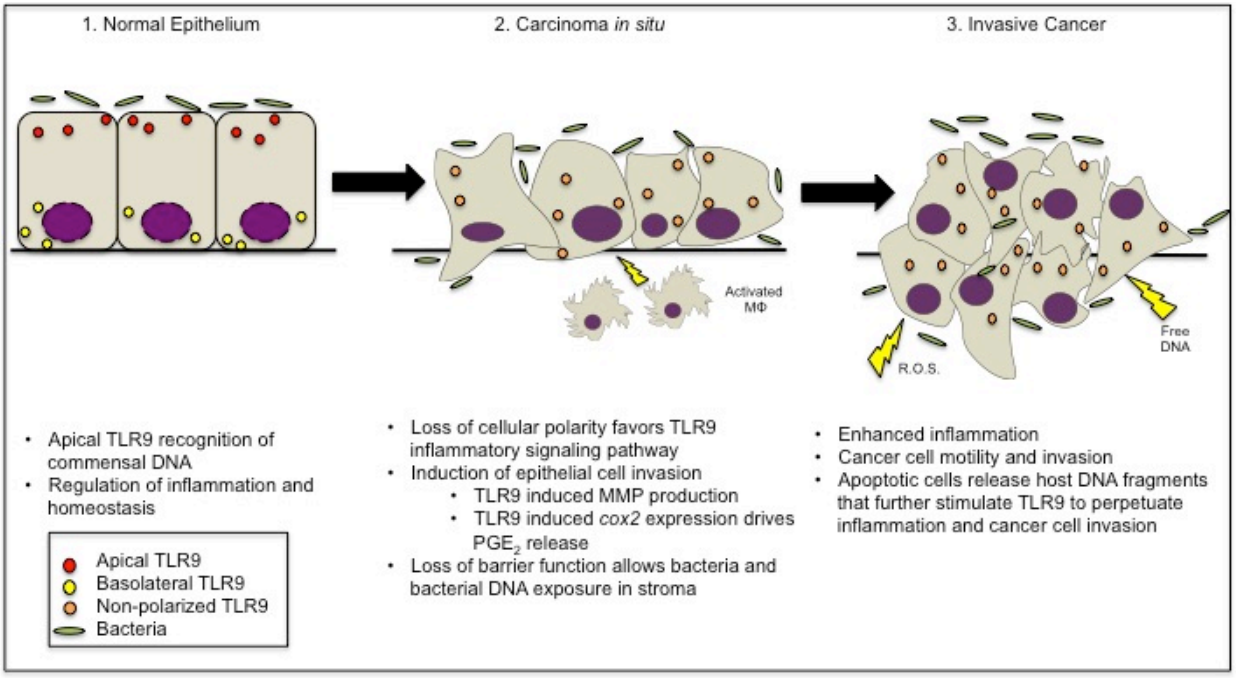

Figure 9: A proposed model of TLR9 in gastric cancer progression.
In normal gastric epithelium TLR9 remains in both the apical and basolateral compartments and participates in homeostatic regulation. However, as cancer progresses and epithelial cells lose barrier integrity and subsequently polarization, TLR9 only signals via the pro-inflammatory cascade. As a result, TLR9 from the microbiota and/or *H. pylori* can drive MMP production and Cox2 expression to drive cancer progression further. Additionally, bacteria can access the stroma and induce macrophage and other immune cell recruitment to promote inflammation. In this inflammatory environment free bacterial DNAs and genomic DNA released from apoptotic cells can also stimulate TLR9 activation to promote sustained inflammation.

human and murine responses to TLR9 activation and must be considered in translating into human studies or contemplating possible therapeutic targets.

TLR9 Regulation and Signaling

Takeshita *et al* extensively studied the method of *tlr9* gene regulation in both humans and mice. The authors found that the *tlr9* gene is regulated by 4 *cis* promoter elements: CRE, 5'-PU Box, 3'-PU box, and C/EBP. The transcription factors that interact with these *cis* promoter elements include CREB1 (CRE site), Ets2, Elf1, Elk1 (5'PU site), and C/EBPα (CEBP site) while the authors could not identify the protein that bound the 3'-PU box. Additionally, the authors found that CREB and members of the C/EBP family directly *trans*-activate the *tlr9* promoter while the Ets family members Ets2, Elk1, and Elf1 enhance *trans* activity. Furthermore, the authors posit that CREB1, Ets2, Elf1, Elk1 and C/EBPα physically interact with each other and with CBP/p300 to attain maximal transcription. To garner a deeper comprehension of *tlr9* gene regulation in response to stimuli such as challenge with CpG DNA or LPS, the authors challenged cells transfected with a luciferase reporter linked to the entire *tlr9* promoter. Challenge with CpG DNA significantly reduced *tlr9* promoter activity resultant from an interaction with the pro-inflammatory transcription factors NFκB, c-Jun and AP-1. The authors also found that the Ets family member Spi1 acted as a *tlr9* gene repressor but could not identify if this protein acted directly or indirectly to inhibit transcription (*216*). Taken together, these data can provide valuable insight into cell type specific *tlr9* gene regulation and could lead to more targeted strategies for the use of CpG DNAs.

Posttranscriptional regulation of TLR9 begins in the endoplasmic reticulum (ER) where it is manufactured as a full-length TLR9 (FL-TLR9) protein under the supervision of chaperone

proteins GP96 and PRAT4A, which ensure proper protein folding and function. Inside the ER, FL-TLR9 homo-dimerizes and binds the chaperone protein UNC93B1 (*217*). UNC93B1 is a multipass transmemebrane protein that is integral in TLR9 trafficking. UNC93B1 facilitates the transport of FL-TLR9 to the Golgi via a COP-II vesicle (*218*). Once in the Golgi FL-TLR9 bound UNC93B1 complexes with adaptor-protein 2 and is secreted in a $VAMP3^{+}$ endosome or may be transiently cycled through the plasma membrane before being compartmentalized into an endosome (*218*). Inside the acidified endosome, FL-TLR9 (~150kDa) is cleaved by cathepsin and asparagine endopetidases to form a cleaved, untrimmed TLR9 protein (~90kDa). This version of TLR9 is cleaved once more by cathepsin to form the mature, cleaved form of TLR9 that is capable of binding DNA (*219-221*). Granulin, HMGB1, and LL-37 have all been implicated as a DNA binding proteins that my facilitate DNA delivery to TLR9 (*222-225*). Although DNA can bind un-cleaved TLR9, downstream signaling cannot occur until after proteolysis (*220*). CpG DNA binding to mature, cleaved TLR9 within the endo-lysosome induces a conformational change that enables the interaction between the cytoplasmic TIR domains of the TLR9 homodimer (*72*). These dimerized TIR domains recruit the adaptor molecule MyD88 to form the myddosome (*226*). Once localized to an endosome, TLR9 trafficking dictates the type of downstream response **(Figure 10)** (*227, 228*). For example, ligand binding within the $VAMP3^{+}$ endosome can yield pro-inflammatory responses mediated by transcription factors such as NFκB, AP-1, or CREB (*217, 226, 229*). However, adaptor-protein 3 can bind the myddosome and facilitate endosomal fusion with a $LAMP2^{+}$ lysosome related organelle (LRO), and together with TRAF-3, facilitate an IRF-7-mediated, type I interferon response to CpG DNA (*230*).

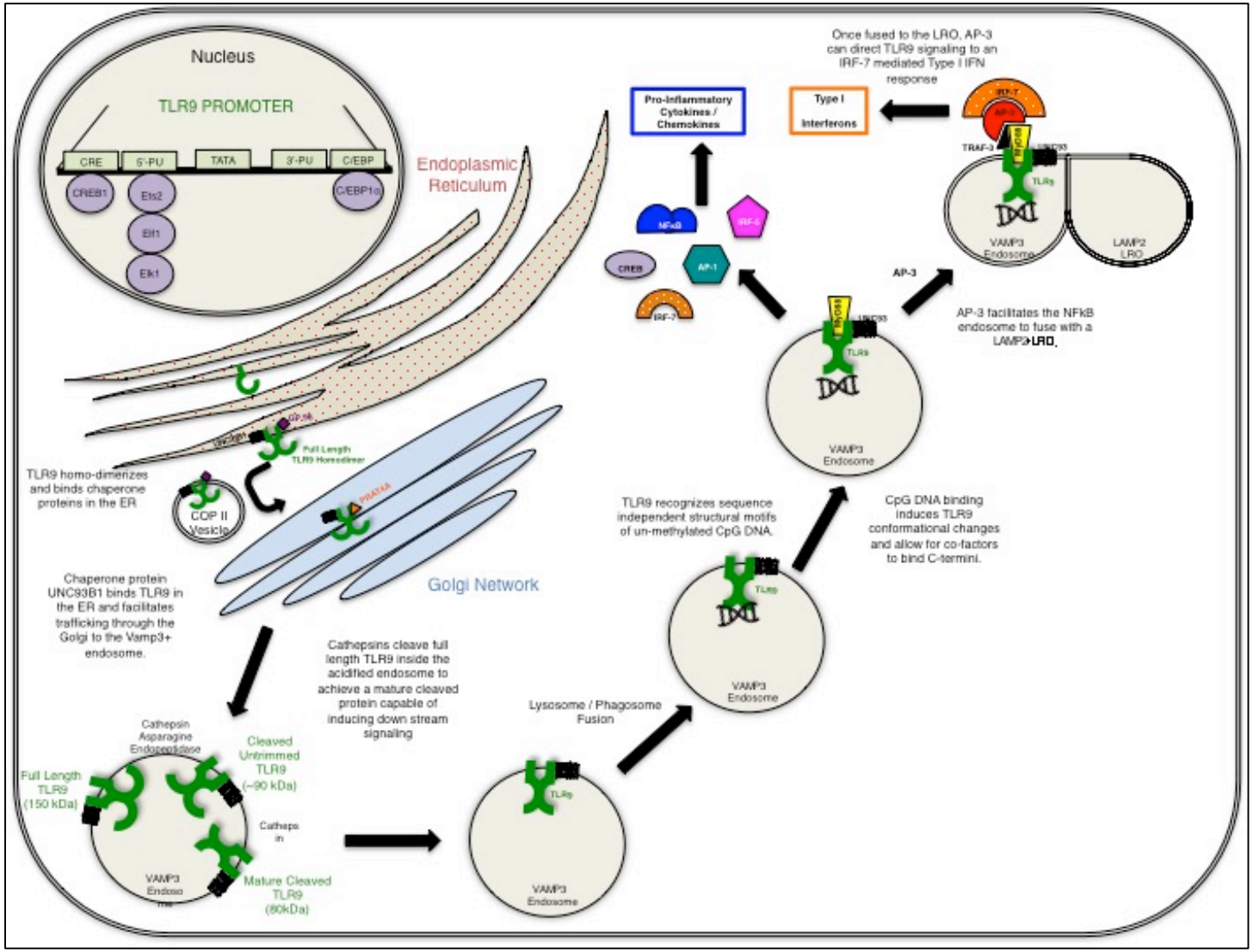

Figure 10: A Model of TLR9 regulation.

TLR9 gene expression is controlled by both *cis and trans* elements summarized in the nucleus. Translation in the endoplasmic reticulum results in a full length protein that is bound to chaperone proteins UNC93B1 and GP96. TLR9 is then shuttled to the Golgi via a CopII vesicle. Once exiting the Golgi, FL-TLR9 either transiently passes through the membrane before being incorporated into an endosome or it buds directly into an endosome from the Golgi. Once in the endosome FL-TLR9 is cleaved. Once cleaved, TLR9 if free to bind its ligand and induce signaling cascades. The type of endosome, chaperone proteins involved, and localization can all dictate the type of signaling cascade that will result from TLR9 activation.

Duality of TLR9 Mediated Responses

Contrary to the popular paradigm, not all TLR signaling promotes pro-inflammatory signaling cascades. Although the mechanisms of dual TLR9 signaling have not been extensively studied in gastric tissue, there have been numerous investigations into how TLR9 may promote both pro- and anti-inflammatory responses in the gut. These studies were particularly focused on how dysregulation of TLR9 signaling could lead to the development of inflammatory bowel disease or colitis (*71, 72, 201, 231-235*).

The gut lumen is permanently colonized by the microbiome while pDCs perpetually monitor this bacterial burden and present antigens to immune cells within the lamina propria. However, healthy individuals do not develop overt inflammatory responses to commensals and yet, they can induce inflammatory responses to pathogens. In a study designed to elucidate the mechanism of how TLR9 may contribute to the maintenance of intestinal homeostasis, the authors found that TLR9 deficiency alone was not sufficient to alter the general composition and total signaling of the intestinal immune system (*236*). However, when the authors investigated specific sub-populations of immune cells from wild-type and TLR9 knockout mice, they found significant functional changes. These functional changes were related to differences in the regulation of the transcription factors NFκB and CREB from both sets of mice. While both transcription factors compete for binding with CBP, the NFκB-CBP complex predominantly regulates pro-inflammatory gene transcription while the CREB-CBP complex is critical for the regulation of the anti-inflammatory cytokine IL-10. In TLR9 deficient mice, myeloid cells in the lamina propria displayed a significant bias toward NFκB activity, while intestinal T-cell populations displayed a lack of counter-regulatory mechanisms to control the inflammatory response (perhaps due to lack of APC derived IL-10). Furthermore, when $CD4^+$ T-cells from

Tlr9$^{-/-}$ mice transplanted into wild-type mice, the recipient mice developed severe colitis similar to mice that received uneducated splenic T–cells (*236*). These findings were corroborated by another study that showed that the colitogenic potential of transplanted splenic T-cells from germ free mice could be reversed by exclusively exposing them to CpG ODN treatment (*237*). The anti-inflammatory responses induced by TLR9 in the gut may be due to its ability to regulate the secretion of type I interferon (*213, 231, 238, 239*). TLR9 is localized in many different cell types, however it is most abundant in pDCs, a class of immune cell that readily secrete type I IFNs. Studies have shown that TLR9 signaling resultant from probiotic DNA binding can inhibit colitis through the induction of IFN α/β (*231*). The same study also suggested that the probiotic DNAs were absorbed in the small intestine and could have a systemic anti-inflammatory response that manifests in reduced colitis (*231*). Taken together, these data suggest that the physiological interaction of TLR9 with bacterial CpG DNA is essential for the maintenance of intestinal homeostasis by inducing counter-regulatory, anti-inflammatory mechanisms.

While pDCs continuously sample the gut lumen and activate adaptive immune responses, epithelial cells are continuously exposed to the luminal microbiota and their inflammatory products. TLR9 has also been shown to modulate both pro- and anti-inflammatory signaling in intestinal epithelial cells. Like gastric epithelial cells, intestinal epithelial cells express TLR9 in both the apical and basolateral compartments (*5, 201*). When polarized intestinal epithelial cells were challenged with CpG DNA at either the apical or basolateral surface, only the basolateral challenge resulted in NFκB activation and IL-8 secretion. Apical stimulation failed to induce NFκB and ERK activation due to an inability to degrade β-TrCP ubiquitinated proteins (such as p105/NFκB1)(*201*). Consequently when the genes induced by apical versus basolateral stimulation were examined, less than half of them were shared between the two types of

challenge (*201*). Although apical stimulation did not result in the canonical NFκB mediated anti-microbial response, it did induce targets of the Wnt pathway with anti-microbial activity, particularly cryptidins (*201*). Additionally, apical TLR9 stimulation promotes immune tolerance, and can inhibit pro-inflammatory IL-8 secretion resultant from basolateral challenge with TLR9 agonists, as well as from challenge with TLR2, TLR3 or TLR5 agonists (*201*). Basolateral TLR9 activation also resulted in a tolerigenic response when challenged repeatedly. Compared to polarized intestinal epithelial cells, non-polarized cells induce a pro-inflammatory phenotype similar to that of basolaterally challenged cells (*201*), suggesting that cell polarization can modify the signaling pathway of TLR9 in the intestinal epithelium. These studies highlight the importance of TLR9 in maintaining gut homeostasis and provide insight into possible mechanisms of how TLR9 deficiency may contribute to intestinal bowel disease or colitis.

How TLR9 regulates its response to DNA remains controversial; some studies posit that the micro-environment may regulate the type of response (*240*), while others suggest that the response is regulated by signal localization (either apically or basolaterally) (*201*), while still others suggest that the response is sequence/structure dependent since some commensal DNAs have been shown to be anti-inflammatory compared to pro-inflammatory pathogenic DNAs (*233, 241-243*). Despite its mode of action, TLR9 signaling has been shown to be of great importance in maintaining gut homeostasis and has vast potential as a therapeutic target in diseases where the inflammatory response is dysregulated.

Thesis Summary and Goals

Helicobacter pylori is a bacterial carcinogen that incurs the highest known risk for gastric cancer (*244*). With an estimated 1 million new cases per year, gastric adenocarcinoma claims >700,000 lives annually and approximately 80% of the gastric cancer burden and 5.5% of all

malignancies worldwide are attributable to *H. pylori*-induced injury (*244, 245*). Further, the incidence and mortality rates of gastric cancer are increasing in the United States among non-white populations (*246*). However, only a percentage of chronically colonized persons ever develop neoplasia, and enhanced risk is related to *H. pylori* strain differences, inflammatory responses governed by host genetic diversity, and/or specific interactions between host and microbial determinants (*247, 248*). In Chapter II, we will discuss the role of bacterial constituents; particularly the *cag* PAI encoded T4SS and its role in delivering DNA into host epithelial cells to activate Toll-like receptor 9. By utilizing *in vitro* assays we demonstrate that DNA is a novel substrate for the *H. pylori cag* T4SS and that subsequent translocation into host cells incudes TLR9 activation. When we translated our findings into the natural niche of *H. pylori,* we also discovered that increased TLR9 expression correlated with increased cancer risk in a cohort of patients from Colombia. In Chapter III, we will further investigate the role of *H. pylori*-mediated TLR9 activation *in vivo* and its effects on both the innate and adaptive immune responses. Specifically, we show that TLR9 is an integral part of the immune response to *H. pylori* in mice, however its activation actually serves to dampen the IL-17 mediated immune response. These data provide evidence of the duality of TLR9 signaling and suggest that DNA translocation may be yet another component of a finely tuned rheostat that *H. pylori* implements to temper the inflammatory response and maintain persistence **(Figure 11)**. These observations, in conjunction with evidence that carriage of certain strains is inversely related to esophageal adenocarcinoma and atopic diseases (*65, 244, 249, 250*), underscore the importance of identifying mechanisms that regulate interactions of *H. pylori* with its host to promote carcinogenesis.

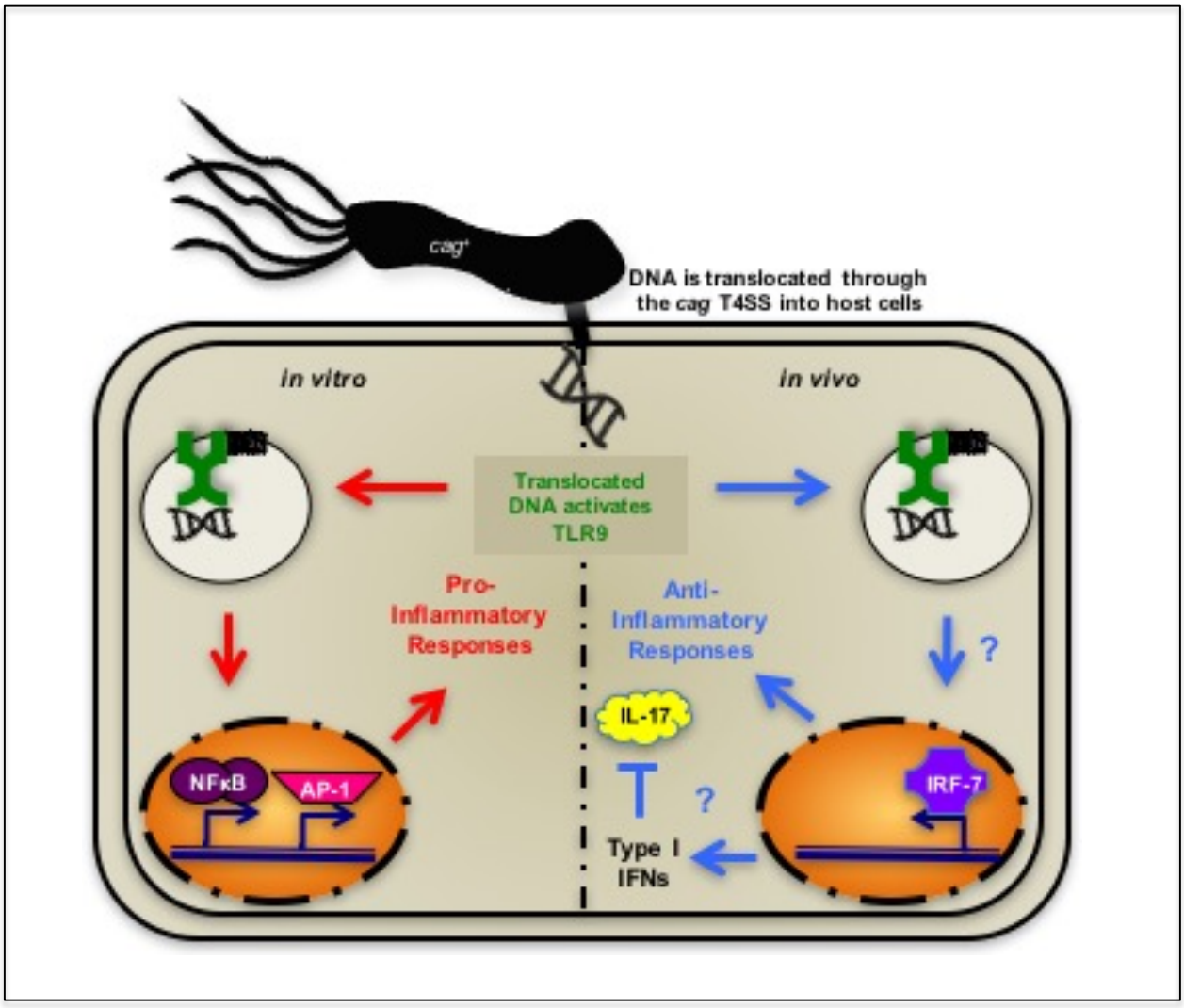

Figure 11: Summary of Dissertation Goals.
H. pylori **translocates its gDNA into host cells via the** ***cag*** **T4SS. Upon entry, TLR9 activation can induce pro- or anti-inflammatory responses. This dichotomy in responses is especially evident in our two model systems of** ***in vitro*** **cell culture of non-polarized cells and our** ***in vivo*** **experiments using** ***Tlr9*** $^{-/-}$ **mice. In the** ***in vivo*** **model, we hypothesize that TLR9 activation drives IRF-7 transcription factor to induce Type I IFNs to mitigate IL-17 responses, however as these steps have not been proven in our model they are denoted by "?".**

CHAPTER II

PATHOGENIC *HELICOBACTER PYLORI* STRAINS TRANSLOCATE DNA AND ACTIVATE TLR9 VIA THE CANCER-ASSOCIATED *CAG* TYPE IV SECRETION SYSTEM

Introduction

Helicobacter pylori is a bacterial carcinogen that incurs the highest known risk for gastric cancer (*244*). Approximately 80% of the gastric cancer burden and 5.5% of all malignancies worldwide are attributable to *H. pylori*-induced injury (*244, 245*). However, only a subset of chronically colonized individuals ever develops neoplasia. Enhanced risk for gastric carcinogenesis is related to *H. pylori* strain differences, inflammatory responses governed by host genetic diversity, and/or specific interactions between host and microbial determinants (*247*).

One cancer-linked *H. pylori* locus is the *cag* pathogenicity island, which encodes a type IV secretion system (T4SS) that forms a syringe-like structure to translocate the effector oncoprotein CagA and peptidoglycan into host cells (*3, 82, 83, 86, 128, 129, 251-260*). T4SSs are common among Gram-negative bacteria due to their versatility in terms of the type and destination of secreted substrates. However, despite the ubiquity of T4SS in Gram-negative bacteria, their ability to mediate trans-kingdom DNA transfer is rare. Indeed, only a very few bacterial T4SSs have been shown to facilitate DNA transfer into yeast (*261, 262*) or mammalian cells (*112-114*). The T4SS of *Agrobacterium tumefaciens* is the only known example of a pathogenesis-associated T4SS that facilitates plasmid DNA transfer into a eukaryotic host (*116-*

118). In this system, *A. tumefaciens* translocates the tumor-inducing Ti-plasmid into plant cells, which is then incorporated into the genome and ultimately results in malignant transformation. *A. tumefaciens* T4SS-mediated DNA transfer has also been shown to promote transformation of human cells under non-physiological conditions (*263*). Of particular interest, the *A. tumefaciens* archetypal T4SS retains a high level of homology to the *cag* type IV secretion system, suggesting that *H. pylori* has the ability to translocate DNA into host cells via the *cag* island.

In addition to the *cag* T4SS, host factors have also been implicated in augmenting *H. pylori*-induced gastric cancer risk. Toll-like receptors (TLRs) orchestrate host immune responses targeting pathogens via selective recognition of pathogen-associated molecular patterns (PAMPs) (*264*); however, chronic activation of TLRs in the gastric niche has been implicated in promoting carcinogenesis (*48*). TLR9 is an intracellular receptor that recognizes hypo-methylated CpG motifs (*193*), which are abundant in DNA of bacterial, viral, or synthetic origin, but are atypical within mammalian genomes (*170*). TLR9 expression is up-regulated in human gastric cancer specimens, and *H. pylori* DNA has been shown to directly promote cancer cell invasion (*5, 209-211, 265, 266*). Moreover, polymorphisms in the *tlr9* gene have also been shown to increase the risk for development of both pre-malignant and malignant gastric lesions (*48, 56*). Therefore, we utilized human gastric specimens and *in vitro* models of microbial-epithelial interactions to define the role of *H. pylori* DNA translocation and TLR9 activation in gastric carcinogenesis.

Methods

H. pylori Strains and Growth Conditions: The *H. pylori cag*$^{+}$ strains J166, 7.13, 26695 (ATCC 700392) and PMSS1 were maintained on trypticase soy agar plates supplemented with 5% sheep blood (BD Biosciences) (*208, 267*). Isogenic *cagA*$^{-}$*, cagE*$^{-}$*, cagM*$^{-}$*,* and *cagL*$^{-}$ mutants were constructed by insertional mutagenesis using *aphA* (conferring kanamycin resistance) as previously described (*268, 269*). An isogenic *pgdA* mutant was generated by deletion mutagenesis as previously described (*270*). The chloramphenicol resistant J166 *cagY*$^{-}$ isogenic mutant was kindly provided by Dr. Jay Solnick (*271*). Flanking sequences for *comB* were amplified from *H. pylori* strain J166 using primers:

comB8 Forward (5'-ACTAGAGCTCAAGCCTTTCAATAGCGAGCA-3'),
comB8 Reverse (5'- AGTACCGCGGAGCGATTTTCAAGCGGTTC -3')
comB10 Forward (5'- CTGAGAATTCTTGCAATTGATGAGGCAAAG-3')
comB10 Reverse (5'- ACTAGGTACCGCGATGACTTCATTCTCTCTGG -3').

comB flanking sequences were cloned into a pBSC103 plasmid using a previously inserted kanamycin resistance cassette generated by restriction enzymes *SacI* and *SacII* (*comB8*) and *EcoRI* and *KpnI* (*comB10*) . The resultant plasmid was used to transform *H. pylori* strain J166 and transformants were selected on Brucella agar plates supplemented with kanamycin (5 μg/mL). Correct orientation of the kanamycin cassette with *H. pylori comB* was confirmed by PCR analyses. Liquid cultures were grown with shaking overnight in 5 mL of Brucella broth supplemented with 10% neonatal calf serum (NCS) at 37°C and 5% CO_2. Supernatants of overnight cultures were collected, filtered (0.45μm filter) and used for TLR9 activation assays. For BrdU incorporation into *H. pylori,* bacteria were grown overnight in Brucella broth containing 10% NCS. *H. pylori* were then diluted 1:10 in Brucella broth containing 5μM BrdU (Sigma-Aldrich) and 10% NCS. Cultures were grown for an additional 4 hours prior to co-culture with eukaryotic cells. *H. pylori* clinical isolates were grown as described above.

Cell Culture: AGS human gastric epithelial cells were grown in RPMI 1640 (Life Technologies) with 10% fetal bovine serum. HEK-Blue-hTLR9 ($TLR9^+$) and HEK-Blue Null1 (parental) cells were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and 100μg/mL Zeocin (Invivogen). HEK-Blue-hTLR9 cell media was supplemented with an additional selective antibiotic, Blasticidin (Invivogen) at 10μg/mL. All cell lines were maintained at 37°C with 5% CO_2. *H. pylori* co-culture studies were conducted at an MOI of 100 for 4-24 hours.

H. pylori DNA extraction: *H. pylori* strains were grown in Brucella broth with 10% NCS overnight. Cultures were centrifuged (4000 RPM, 5 min), resuspended in 600μL of TE buffer with 0.5% SDS and 100μg/mL proteinase K, and incubated at 37°C for 1 hour. DNA was extracted using CTAB and purified by phenol-chloroform extraction. Purified DNA was eluted in TE buffer and washed with Polymixin B to remove residual LPS.

TLR9 Activation Assay: HEK-Blue-hTLR9 cells and HEK-Blue-Null1 cells (Invivogen) were seeded in 96 well plates (Co-Star) at 80,000 cells per well in DMEM without antibiotics and challenged with either viable *H. pylori* (MOI 100), purified *H. pylori* DNA (1-5 μg/mL) supplemented with Lipofectamine 2000 (Life Technologies), or *H. pylori* conditioned media (1-30%) at 37°C with 5% CO_2 for 24 hours. After 24 hours, 20μl of supernatants were added to 180μl of HEK-Blue Detection media (Invivogen). Plates were analyzed by spectrophotometer (Bitoek) at 650nm. All experiments were performed in duplicate and repeated at least 3 times.

Gentamicin Protection Assay: AGS gastric epithelial cells were co-cultured with *H. pylori* wild type or isogenic mutant strains at an MOI of 100 for 4 hours. Cells were washed 3 times with PBS containing gentamicin (250 μg/mL). Cells were then incubated at 37°C for an additional hour in RPMI containing gentamicin (250 μg/mL), washed 3 times with PBS to remove

gentamicin, lysed in 200μL of sterile dH_2O and serial dilutions were plated on TSA plates with 5% sheep blood. Plates were incubated for 5 days at 37°C, 5% CO_2 and colonies were enumerated. Experiments were repeated at least 3 times.

CagA Translocation Assay: *H. pylori* were co-cultured with AGS cells at an MOI of 30 for 4 hours. Protein lysates were harvested in RIPA buffer, separated by SDS-PAGE, and transferred to PVDF membranes. Levels of total CagA (1:5000 anti-CagA antibody; Austral Biologicals) and phosphorylated CagA (1:5000 anti-pY99 antibody; Santa Cruz Biotechnology Inc.) were determined via Western blotting to assess levels of translocated CagA. Protein intensities were quantified using the ChemiGenius Gel Bio Imaging System (Syngene). Experiments were repeated at least 3 times.

Flow Cytometry: AGS cells were co-cultured with BrdU-labeled *H. pylori* for 4 hours. Cells were washed and incubated for an additional hour in RPMI 1640 containing gentamicin (500 μg/mL). Adherent cells were then dissociated with 0.25% trypsin EDTA (Life Technologies) supplemented with gentamicin (500ug/mL), fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences), blocked overnight at 4°C using endogenous biotin blocking kit reagent A (Life Technologies), washed 3 times in 1x permwash and blocked again with endogenous biotin blocking kit reagent B for 30 minutes at room temperature (Life Technologies). Cells were washed and stained with biotin conjugated anti-BrdU mouse monoclonal antibody (1:500 Life Technologies) followed by strepdavidin conjugated AlexFluor488 secondary antibody (1:5000 Life Technologies). Cells were acquired using a LSR II Flow Cytometer (BD Biosciences) and BrdU-positive cells were analyzed using FlowJo (Tree Star Inc.).

Structured Illumination Microscopy: AGS gastric epithelial cells were seeded on size 1.5 cover slips at 100,000 cells per well. BrdU-labeled *H. pylori* wild-type strain J166 or the isogenic J166

cagE$^-$ mutant were co-cultured at an MOI of 100 for 4 hours. Cells were washed 3 times in PBS, fixed with CytoFix/CytoPerm solution (BD Biosciences) for 20 minutes at 4°C and washed an additional 3 times in 1x Permwash (BD Biosciences). Cells were then blocked overnight at 4°C using endogenous biotin blocking kit reagent A (Life Technologies), washed 3 times in 1x permwash and blocked again with endogenous biotin blocking kit reagent B (Life Technologies) for 30 minutes at room temperature. Cells were incubated with mouse anti-BrdU conjugated biotin (Life Technologies 1:500) overnight at 4°C diluted in 1x Permwash. Cells were subsequently stained with strepdavidin-conjugated AlexaFluor488 (1:5000 for BrdU) as well as phalloidin (1:100 actin) and Hoechst (1:5000 nuclei) for 1 hour at room temperature. Cells were washed 3 times in 1x permwash and slides were mounted with prolong gold (Invitrogen). Slides were viewed with Delta Vision OMX confocal microscope (GE Health Care Life Sciences) at 63X magnification, using 1.514 immersion oil. Post-data acquisition processing was performed using SoftWorx for OMX. Images are shown as maximum intensity projections.

Chloramphenicol Treatment Assays: *H. pylori* strain J166 was inoculated into HEK-Blue-hTLR9 and HEK-Blue Null1 co-cultures as described for TLR9 activation assays. For each biological replicate experiment, sub-lethal levels of chloramphenicol were added to triplicate wells at concentrations ranging from 0.625 μg/mL to 5 μg/mL. After 24 hours, TLR9 activation was assessed as described for TLR9 activation assays. For each concentration, OD_{650} values obtained for HEK-Blue-hTLR9 cells were normalized by subtracting the corresponding OD_{650} values obtained for HEK-Blue-Null1 cells, and TLR9 activation was calculated as a percent of untreated, antibiotic-free co-cultures. After 24 hours of co-culture in chloramphenicol, *H. pylori*-HEK co-cultures wells were dislodged by mechanical disruption, pelleted at 5000 x g, and were

re-suspended in 1 mL sterile PBS. To calculate *H. pylori* CFUs, 10-fold serial dilutions of resuspended co-cultures were plated, and colonies were enumerated after 72 hours of incubation.

DNase Susceptibility Assays: Wild-type *H. pylori* strain J166 was pre-treated in DMEM containing 10 units of Turbo DNase I (Life Technologies) for 30 min, or incubated in DMEM containing DNase I buffer alone (untreated sample), prior to inoculating HEK-Blue-hTLR9 cells and HEK-Blue-Null1 cells at an MOI of 100. For the course of the co-culture, 20 units of Turbo DNase I were added to half of the wells, and DNase I buffer was added to the remaining wells. TLR9 activation was assessed at 24 hours as described for TLR9 activation assays. For each condition, OD_{650} values obtained for HEK-Blue-hTLR9 cells were normalized by subtracting the corresponding OD_{650} values obtained for HEK-Blue-Null1 cells, and TLR9 activation was calculated as a percent of untreated, DNase I-free co-cultures.

DNA-Blocking Antibody Assay. *H. pylori* J166 was inoculated into HEK-Blue-hTLR9 and HEK-Blue-Null1 co-cultures at an MOI of 100 as described for TLR9 activation assays. For each biological replicate experiment, monoclonal anti-DNA antibody (Roche) was added at varying dilutions, ranging from 1:50 – 1:200 final concentration. After 24 hours, TLR9 activation was assessed, and values were normalized as described for DNase I experiments. TLR9 activation was expressed as a percentage of untreated (no antibody) wells.

Statistical Analysis: Statistical analyses were performed using Prism 6.0 (GraphPad Software). When comparisons between multiple groups were made, analysis of variance (ANOVA) with Bonferroni correction was performed. Student's *t*-test was performed when comparisons between only two groups were made. A P-value of <0.05 was considered significant. In all figures, means±standard errors of the mean are shown.

Results

In many areas of the world, geographic differences in gastric cancer rates are present despite similarly high *H. pylori* prevalence rates (*272*). This has been well described in Colombia, where residents of the Andean Mountain region have an extraordinarily high incidence of gastric cancer (150/100,000), compared to inhabitants of the Pacific coast region (6/100,000), despite the fact that greater than 90% of the collective population is infected with *H. pylori* (*273, 274*). This disparity in gastric cancer risk but not *H. pylori* prevalence (*58, 82, 275, 276*) provides a unique opportunity to define microbial and host determinants that play a role in gastric carcinogenesis. Therefore, we first analyzed *H. pylori* strains isolated from the high and low gastric cancer risk regions for their ability to activate TLR9 using a specific HEK293-hTLR9 reporter assay system. In this reporter assay, HEK293 cells, which are inherently devoid of most innate immune receptors, are stably transfected with either a TLR9 expression plasmid and an NFκB/AP-1-linked SEAP reporter ($TLR9^{+}$), or a control NFκB/AP-1 SEAP reporter alone (Parental). Cells were challenged with *H. pylori* strains isolated from patients residing in either the high-risk or low-risk region. *H. pylori* strains isolated from the high-risk region induced significantly higher levels of TLR9 activation compared to strains harvested from patients in the low-risk region **(Figure 12A)**. To determine whether the ability of high-risk *H. pylori* isolates to activate TLR9 *in vitro* translated into the cognate gastric niche, levels of epithelial TLR9 expression in gastric biopsies obtained from infected patients were quantified. Gastric epithelial TLR9 expression levels were significantly increased in patients residing in the high-risk region compared to the low-risk region **(Figure 12B,C)**, findings that mirror the *in vitro* data. Patients residing in the high cancer-risk region also displayed significantly increased histological scores compared to patients in the low cancer-risk region (mean ± SEM; 3.89±0.26

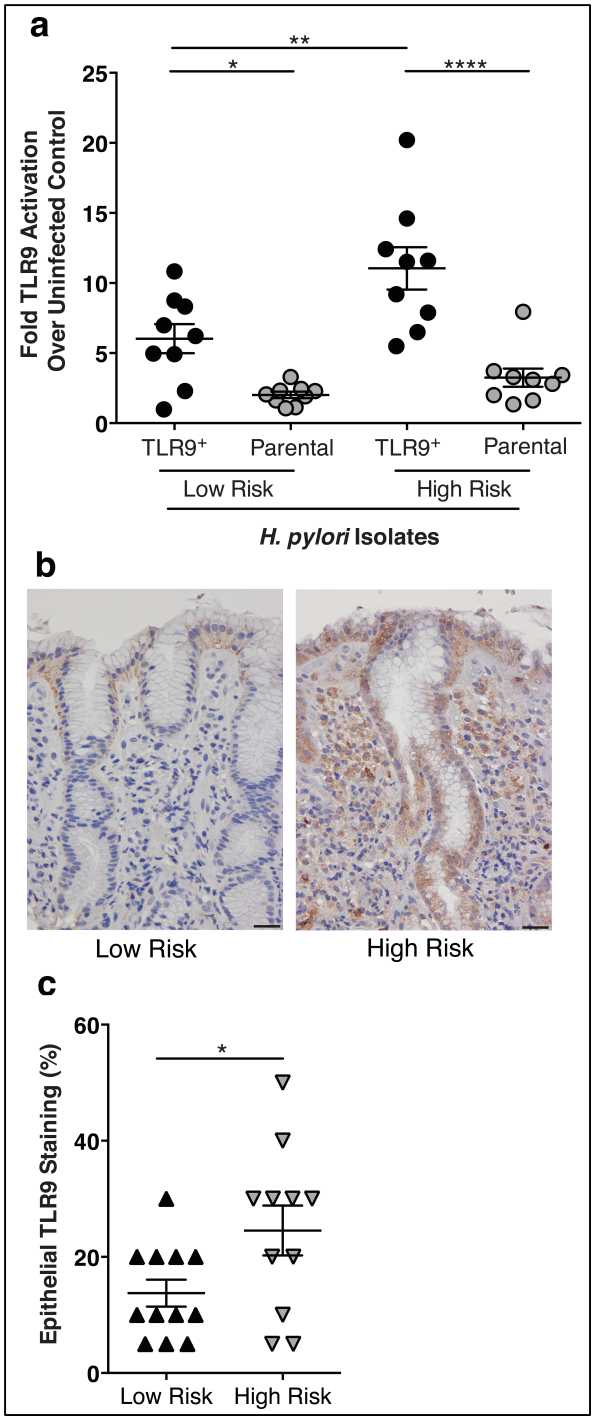

Figure 12: *H. pylori* activation of TLR9 in a human population.

A) TLR9 activation by *H. pylori* isolates obtained from patients residing in a low or high gastric cancer risk regions of Colombia. Data are expressed as fold over uninfected control. N=9 isolates per group, each strain was tested in duplicate at least 3 times. Mean±SEM are shown. (B) Representative immuno-histochemical staining for TLR9 in gastric biopsies. Magnification 40x; Scale bar, 50µm. Epithelial TLR9 staining of biopsies obtained from patients in the low (N=11) and high (N=12) gastric cancer risk regions of Colombia is quantified in (C). *p<0.05, **p<0.01, ****p<0.0001.

vs. 2.05±0.11, respectively; $p \leq 0.0001$). Additionally we observed that TLR9 expression was more extensive and involved more regions of the gastric glands in patients residing in the high-risk region, frequently extending from the base to the gastric pit. Collectively, these results indicate that *H. pylori* strains linked to an increased risk for gastric cancer induce more intense TLR9 activation *in vitro* and enhanced expression *in vivo*.

Despite the evolutionary homology of the *cag* T4SS to the archetypal *A. tumefaciens* T4SS (*277*), DNA translocation by the *H. pylori cag* system has never been demonstrated. Therefore, to define mechanisms through which *H. pylori* activates TLR9, we subsequently conducted experiments using the well-characterized wild-type and mutant *H. pylori* cag^+ strains J166, 26695, and 7.13 (*257, 267, 278*). Previous reports have shown that *H. pylori* DNA induces TLR9 activation in immune cells (*212, 213, 279*); thus, we first confirmed that purified *H. pylori* gDNA could induce TLR9 activation in our HEK293 reporter assay **(Figure 13A)**. We also demonstrated that viable wild-type cag^+ *H. pylori* could activate TLR9 to significantly increased levels compared to controls **(Figure 13B,C)** These studies were then validated in HEK293-hTLR9 reporter cells that were pre-treated with the TLR9 antagonist chloroquine. *H. pylori*-infected reporter cells pre-challenged with the TLR9 antagonist displayed a concomitant decrease in TLR9 activation compared to control cells **(Figure 15)**.

H. pylori possesses up to four potential T4SSs with the putative ability to translocate DNA to and from the bacterial cell: the *comB* DNA uptake (competence) system, the *cag* system, as well as the *tfs3* and *tfs4* secretion systems (*135, 141, 143*). Analysis of an *H. pylori comB* DNA uptake mutant revealed no differences in levels of TLR9 activation compared to the wild-type strain **(Figure 13B)**. Therefore, we next investigated the role of the *cag* T4SS in mediating TLR9 activation. TLR9 reporter cells were challenged with either an isogenic $cagA^-$ mutant

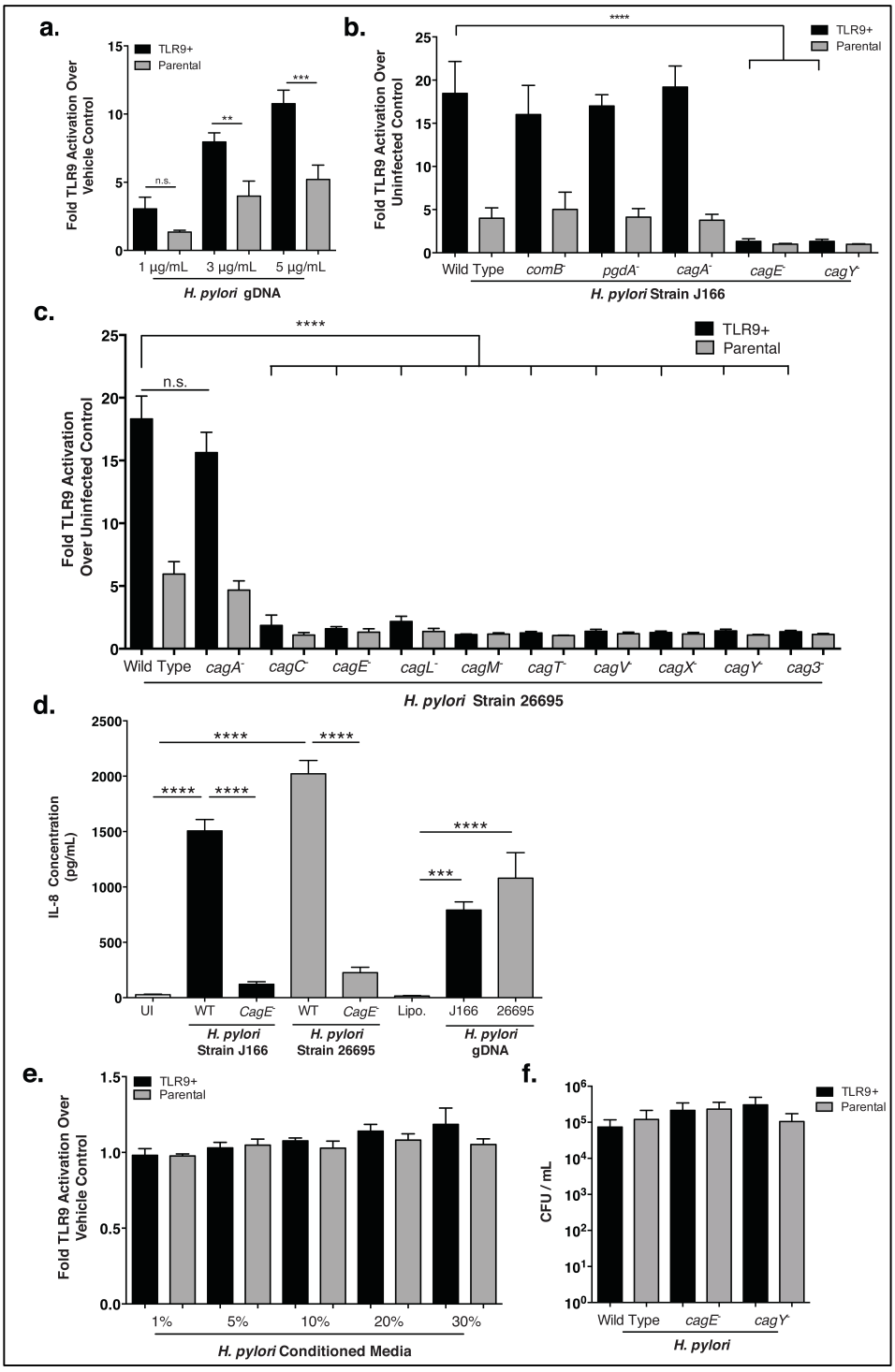

Figure 13: *H. pylori* activation of TLR9 requires a functional *cag* T4SS.

(A) TLR9-reporter or parental cells were challenged with gDNA from H. pylori strain J166. Data are represented as fold over vehicle control. (B) TLR9 activation induced by H. pylori strain J166 or its isogenic mutants, relative to uninfected control. (C) TLR9 activation induced by H. pylori strain 26695 or its isogenic mutants, relative to uninfected control. (D) Wild-type H. pylori, the cagE- mutant or purified H. pylori gDNA was used to challenge AGS gastric epithelial cells and IL-8 secretion was quantified by ELISA. (E) TLR9 activation induced by H. pylori conditioned media relative to vehicle (Brucella broth) control. (F) Gentamicin protection assays were performed using H. pylori strain J166 or its isogenic mutants. Viable colony-forming units are shown. Each strain was tested in duplicate in at least 3 independent experiments. Mean±SEM are shown. *p<0.05,p<0.01,***p<0.001, ****p<0.0001.**

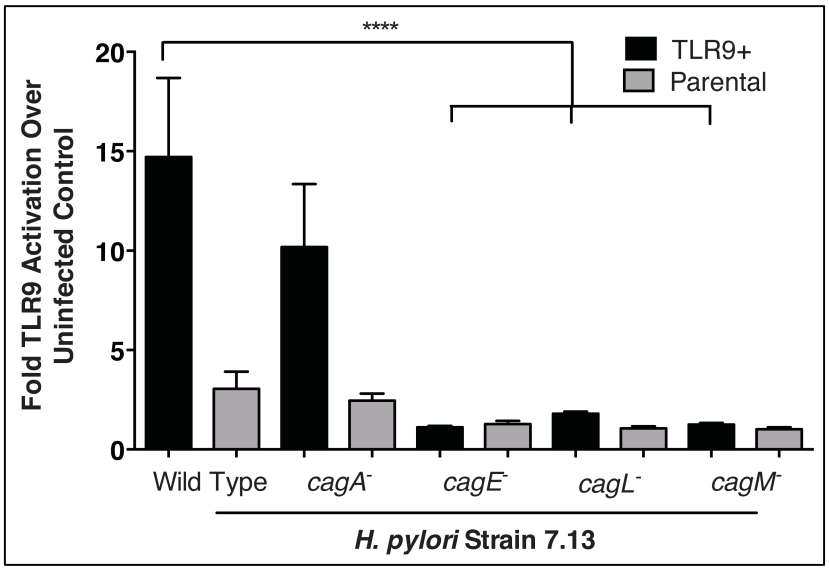

Figure 14: *H. pylori* strain 7.13 activation of TLR9 requires a functional *cag* T4SS.
The *H. pylori cag*$^{+}$ strain 7.13 or its isogenic mutants were used to challenge TLR9-reporter or parental cells at an MOI of 100 for 24 hours. ****p<0.0001.

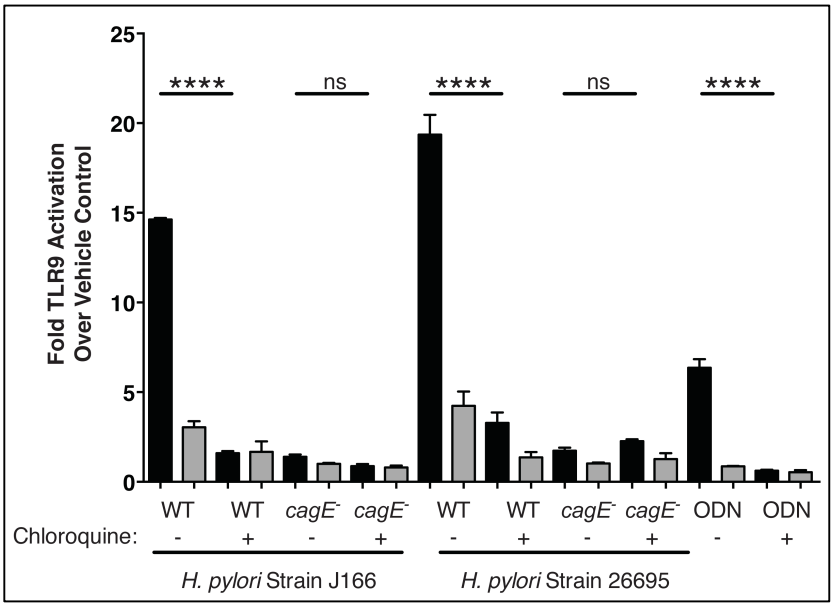

Figure 14: An endosomal inhibitor reduces *H. pylori*-induced activation.
TLR9-reporter and parental cells were retreated for 2 hours with 50 µg/mL of chloroquine. *H. pylori* strains J166 or 26695 or their corresponding *cagE*$^{-}$ mutants were used to infect pretreated cells at an MOI of 100 for 24 hours. The TLR9 agonist, ODN2006, was used to challenge reporter cells at a concentration of 5 µM as a positive control. TLR9 activation is represented as fold over vehicle control. ****p<0.0001.

(which lacks the effector protein CagA), *cagE*$^-$ or *cagY*$^-$ mutants (which encode essential proteins for T4SS assembly), or a *pgdA*$^-$ mutant (which reduces peptidoglycan-mediated NOD1 activation (*270*)). Loss of *cagA* or *pgdA* had no effect on TLR9 activation; however, *cagE*$^-$ or *cagY*$^-$ mutants were incapable of activating TLR9, suggesting that DNA translocation to the host cell and subsequent TLR9 activation may occur via the *cag* T4SS **(Figure 13B, 14)**. The requirement for a functional *cag* T4SS to induce TLR9-dependent responses was also demonstrated in two independent *H. pylori cag*$^+$ strains, 26695 and 7.13, as mutations in *cag* T4SS structural components abrogated TLR9 activation **(Figure 13C,14)**. Importantly, *H. pylori* strain 26695, which lacks intact *tfs3* and *tfs4* gene clusters (*143*), still induced robust TLR9 activation **(Figure 13C)**. We also observed TLR9 activation following infection with *H. pylori* strains devoid of plasmid DNA (strain J166, 26695 (*257, 278*)), suggesting that other forms of DNA can be translocated by the *cag* T4SS. This observation is of particular importance because it highlights fundamental differences between *H. pylori* DNA translocation and DNA translocation that occurs in other bacterial species, which only translocate plasmid DNA into host cells. To extend these findings, we also infected AGS gastric epithelial cells with wild-type *H. pylori cag*$^+$ strains (J166 or 26695), their respective *cagE*$^-$ mutants, or their purified genomic DNA and assessed the levels of IL-8 secretion as a potential downstream target of TLR9 mediated NFκB activation **(Figure 13D)**. We found that the *cagE*$^-$ mutants induced significantly decreased levels of IL-8 compared to wild-type *H. pylori* strains. Additionally, purified gDNA from the wild-type strains induced a significantly increased level of IL-8 production compared to vehicle control, suggesting that *H. pylori* gDNA can not only activate TLR9, but can also induce production of a known downstream target of this receptor. Collectively, these data indicate that a functional *H.*

pylori cancer-linked T4SS is required for TLR9 activation but the known effector molecules translocated by this system (CagA and peptidoglycan) are dispensable for this phenotype.

H. pylori is predominantly an extracellular pathogen and TLR9 is an endosomal receptor, implying that DNA is either directly injected into the host cell, or is taken up via a different mechanism that is enhanced by a functional T4SS. To determine whether TLR9 was activated by *H. pylori* DNA entering host cells via a host-induced pathway, TLR9 reporter cells were incubated with bacterial cell-free, *H. pylori* conditioned media at concentrations up to 30%; however, no changes in TLR9 activation were observed compared to control cells **(Figure 13E)**. These data indicate that activating DNA is not secreted, in contrast to strategies employed by *Neisseria* in which DNA is translocated into the extracellular milieu (*280*). Although primarily an extracellular pathogen, *H. pylori* does retain has the capacity to invade host epithelial cells in a limited fashion (*270*). Therefore, we next quantified intracellular *H. pylori* viability within HEK293-hTLR9 reporter cells. Wild-type *H. pylori* survived equally well compared to the corresponding *cag* mutants **(Figure 13F)**, despite marked differences in TLR9 activation **(Figure 13C)**. These results indicate that microbial endocytosis does not affect *H. pylori*-induced TLR9 activation. We next utilized two unique inhibitor compounds that have previously been shown to exert effects on *H. pylori cag* T4SS function (*281*) to further interrogate the role of the *cag* T4SS in TLR9 activation. The *H. pylori* cag^{+} wild type strain J166 and an isogenic J166 $cagE^{-}$ mutant strain were pre-treated with compound C10, which inhibits activity of the *cag* T4SS without perturbing T4SS apparatus assembly, compound KSK85, which inhibits *cag* T4SS assembly, or the negative control compound GKP42. C10 and KSK85, but not the control compound, significantly decreased TLR9 activation induced by wild-type *H. pylori* **(Figure 16A)**. Of interest, these compounds did not affect host cell viability **(Figure 16B)** or TLR9 activation

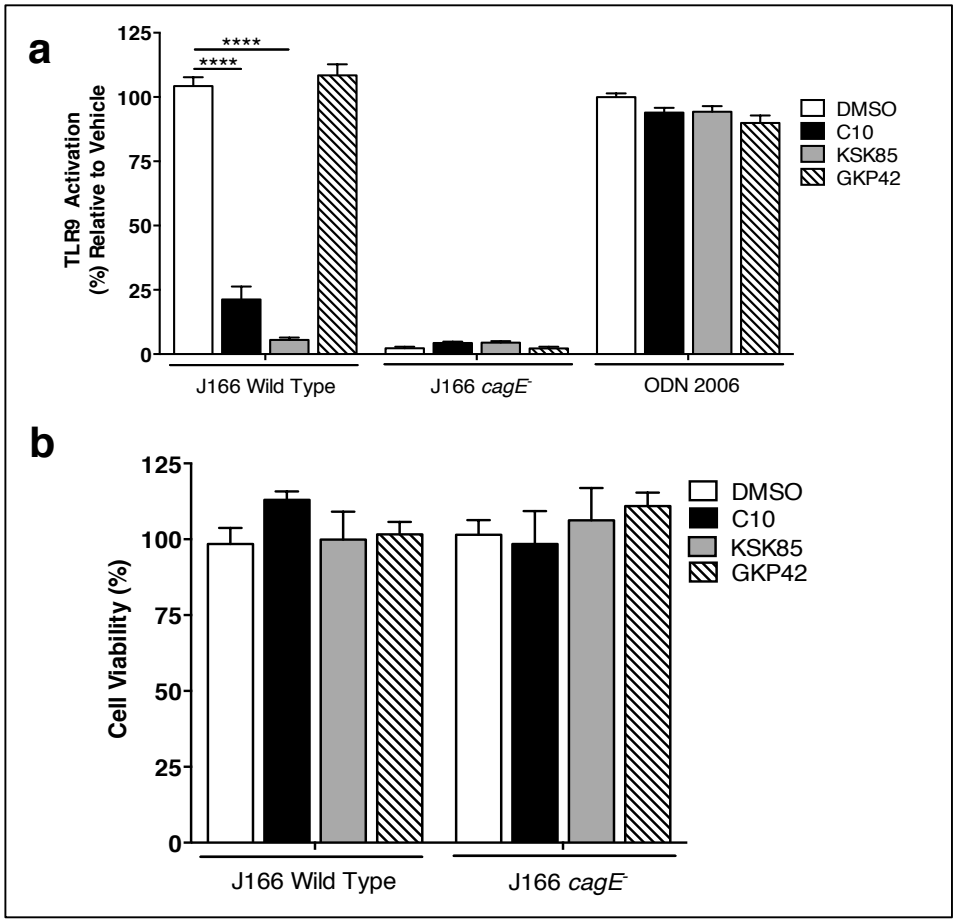

Figure 15: T4SS chemical inhibitors prevent *H. pylori*-mediated TLR9 activation.

A) *H. pylori* strain J166 or the *cagE*- mutant were pretreated with 50µM of either DMSO (Vehicle), compound C10, KSK85, or GKP42 for 1 hour. Following pretreatment, HEK293-hTLR9 reporter cells were challenged with *H. pylori* or 5µM ODN2006 in the presence of the inhibitor compound for 24 hours. B) *H. pylori* strain J166 or the *cagE*- mutant were pretreated with 150µM of either DMSO (Vehicle), compound C10, KSK85, or GKP42 for 1 hour. Following pretreatment, HEK293-hTLR9 reporter cells were challenged with *H. pylori* in the presence of the inhibitor compound for 24 hours. After 24 hours, cell viability was quantified by MTT cell proliferation assay. ****p<0.0001.

induced by a known TLR9 ligand, ODN2006 **(Figure 16A)**, indicating specificity of this compound to inhibit *cag* T4SS function. Collectively, these data demonstrate that *H. pylori* utilizes the *cag* T4SS to translocate microbial DNA into eukaryotic cells thereby activating TLR9.

To determine whether the *cag* T4SS is required for direct DNA delivery into host cells, *H. pylori* DNA was labeled with BrdU and bacteria were subsequently co-cultured with AGS gastric epithelial cells. Using this technique, bacterial DNA can be easily distinguished from host DNA via incorporation of BrdU. Structured illumination microscopy (SIM) demonstrated that wild-type *H. pylori* translocated BrdU-labeled DNA into host cells **(Figure 17A,B, Figure 18)** However, intracellular BrdU-labeled DNA was not observed in host cells infected with the *H. pylori cagE*$^-$ mutant **(Figure 17B)**. To confirm and quantify these results using an independent methodology, host intracellular levels of BrdU-labeled *H. pylori* DNA were assessed via flow cytometry. Levels of intracellular DNA were significantly increased in *H. pylori* wild type-infected compared to uninfected AGS cells. A significant reduction was observed in cells infected with the *cagE*$^-$ mutant compared to wild-type infected AGS cells **(Figure 17C,D)**, albeit not to levels observed in uninfected cells, which may be due to adherent and/or invasive *H. pylori* that could not be completely removed prior to analysis. Collectively, these data demonstrate that *H. pylori* utilizes the *cag* T4SS to translocate microbial DNA into eukaryotic cells, thereby activating TLR9.

Elaboration of the cancer-associated *cag* T4SS develops in response to direct host cell contact, and effector translocation is dependent on assembly of the T4SS apparatus. We therefore performed orthologous mechanistic studies to investigate whether TLR9 activation required *de novo* synthesis of Cag proteins for apparatus biogenesis, or whether *cag* T4SS-dependent DNA

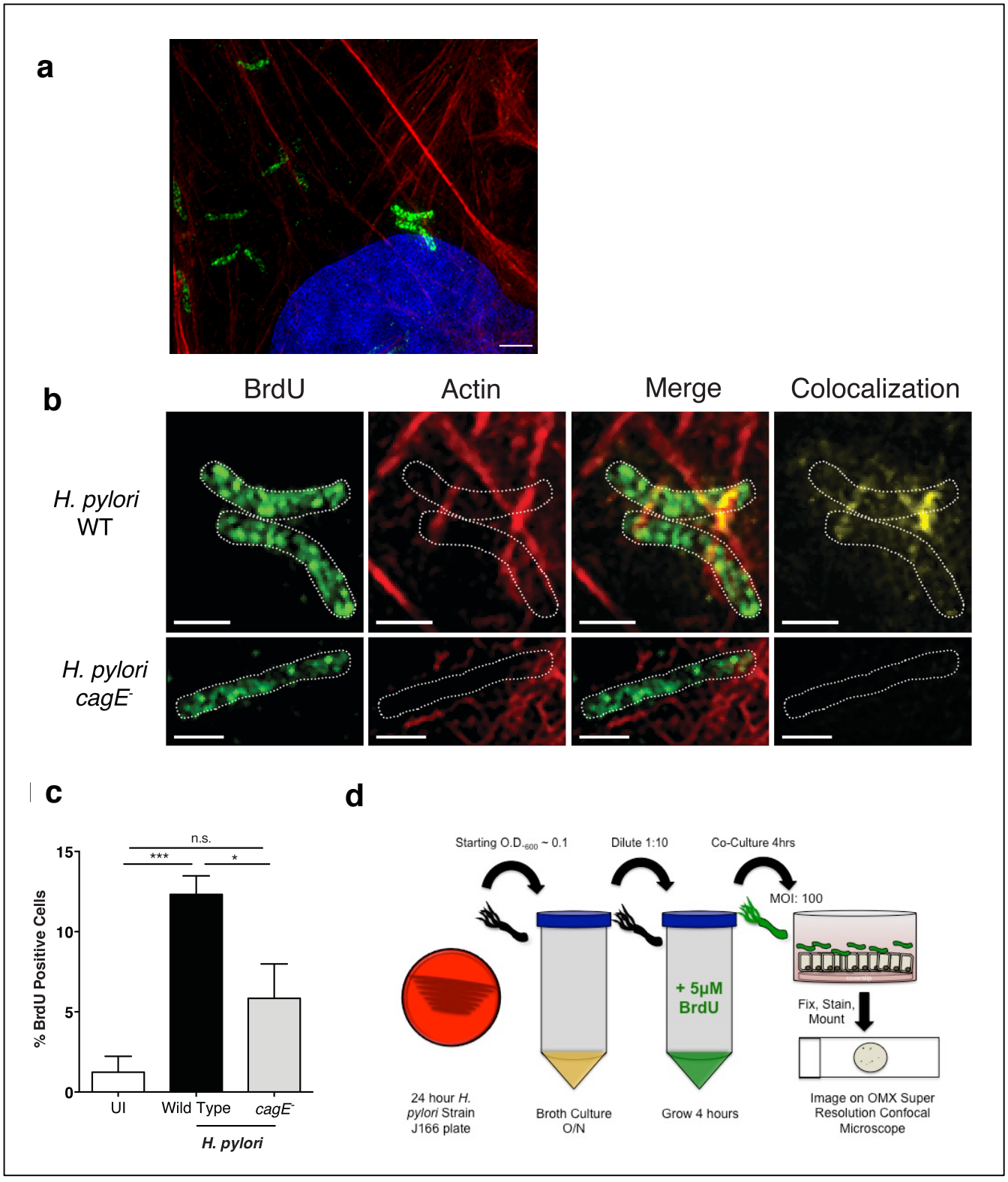

Figure 16: : *H. pylori* translocates DNA into host cells via the *cag* T4SS.

A) *H. pylori*-mediated DNA translocation was assessed by structural illumination microscopy using AGS cells co-cultured with BrdU-labeled *H. pylori* strain J166. Scale bar, 2μm. B) *H. pylori* in (A) were magnified for evidence of host intracellular BrdU staining (upper panels) and compared to the J166 *cagE*- (lower panels). Scale bar, 1μm. A-B) BrdU, green; actin, red; colocalization, yellow. *H. pylori* are outlined by dotted white lines. C) BrdU-labeled *H. pylori* strain J166 or the *cagE*- mutant were co-cultured with AGS cells and then subjected to flow cytometry to assess levels of host intracellular BrdU. Each strain was tested at least 3 times. Mean±SEM are shown. *p<0.05, ***p<0.001. D) Schematic depicting experimental protocol for Figure 17.

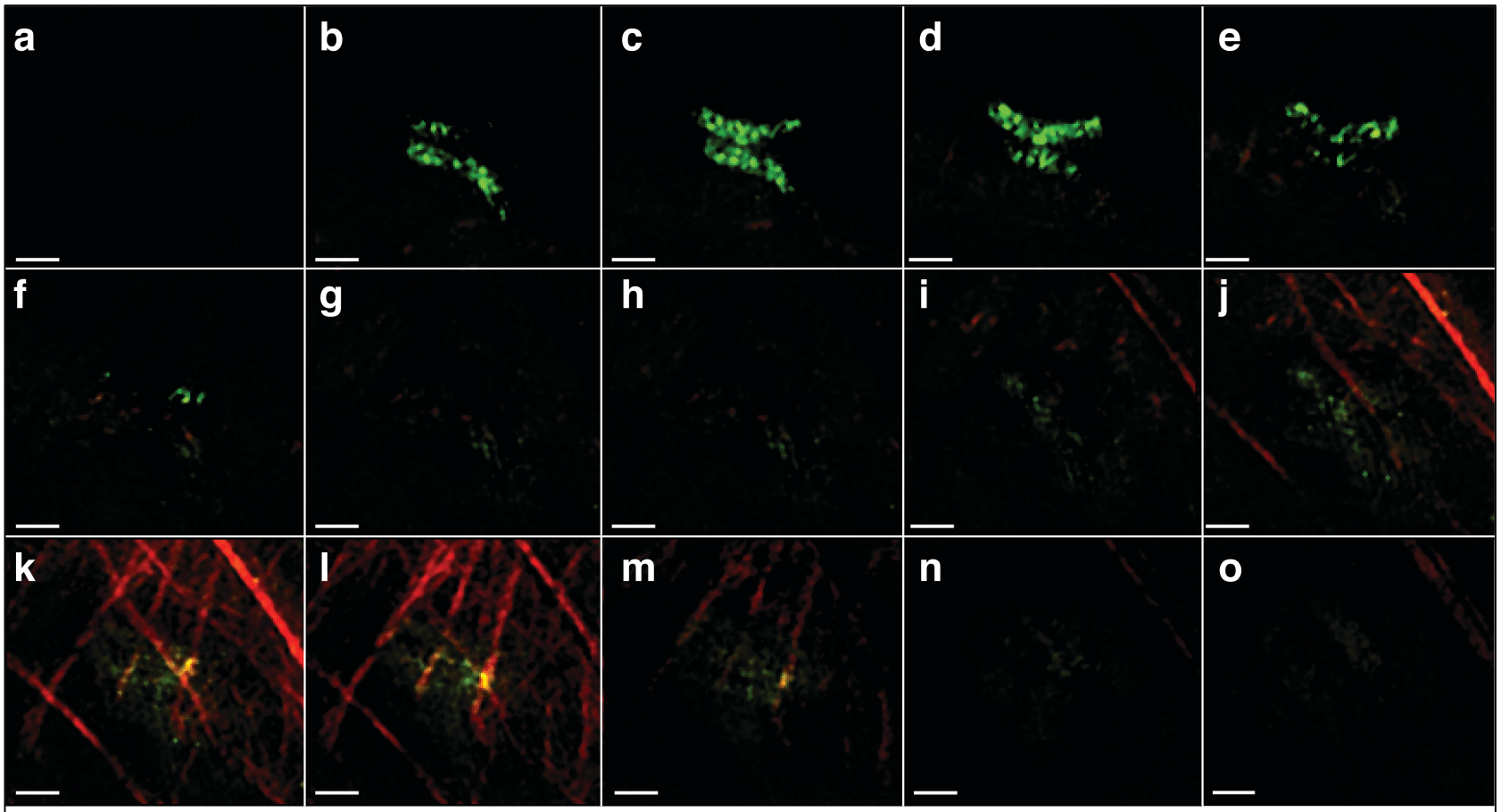

Figure 17: Z-stack images depicting *H. pylori*-mediated DNA translocation via the *cag* T4SS.
***H. pylori cag*$^{+}$ wild-type strain J166 was labeled with 5 µM BrdU and used to challenge AGS gastric epithelial cells. BrdU is labeled in green, actin in red, areas of co-localization in yellow. Scale bar equals 1 µm. (a-o) Representative z-stack images used to compile the compounded image shown in Figure 17b. Each panel represents a 0.125 µm slice. (p) Merged image of z-stacks shown in a-o.**

transfer could be achieved using pre-assembled protein complexes. Treatment of *H. pylori*-HEK293 co-cultures with bacteriostatic, sub-lethal concentrations of chloramphenicol, an inhibitor of bacterial protein synthesis, revealed a significant reduction in levels of TLR9 activation **(Figure 19A)**. Chloramphenicol-mediated inhibition of *H. pylori*-driven TLR9 activation occurred in a dose-dependent manner but did not affect *H. pylori* viability **(Figure 19B)**. These data indicate that *cag* T4SS-mediated DNA translocation requires new protein synthesis.

DNA transfer by *Agrobacterium tumefaciens* occurs via substrate delivery both within and on the external surface of the T4SS conduit (*116*). To further investigate the mechanisms of *cag* T4SS-dependent DNA transfer into host cells in greater depth, we assessed whether exogenous DNase I treatment would compromise the integrity of transported DNA and impair TLR9 activation. Compared to untreated controls, levels of TLR9 activation decreased by approximately 50% when DNase I was added during *H. pylori*-HEK293 cell co-culture **(Figure 19C)**. In parallel, we analyzed the ability of monoclonal anti-DNA antibodies to functionally block *H. pylori*-dependent TLR9 activation. Compared to untreated controls, *H. pylori*-mediated TLR9 activation was significantly reduced in the presence of pan anti-DNA antibodies, an effect that was titratible **(Figure 18D)**. Taken together, these data indicate that *cag* T4SS-dependent DNA translocation occurs through a contact-dependent mechanism that involves transfer of bacterial DNA that is both protected as well as exposed during transport.

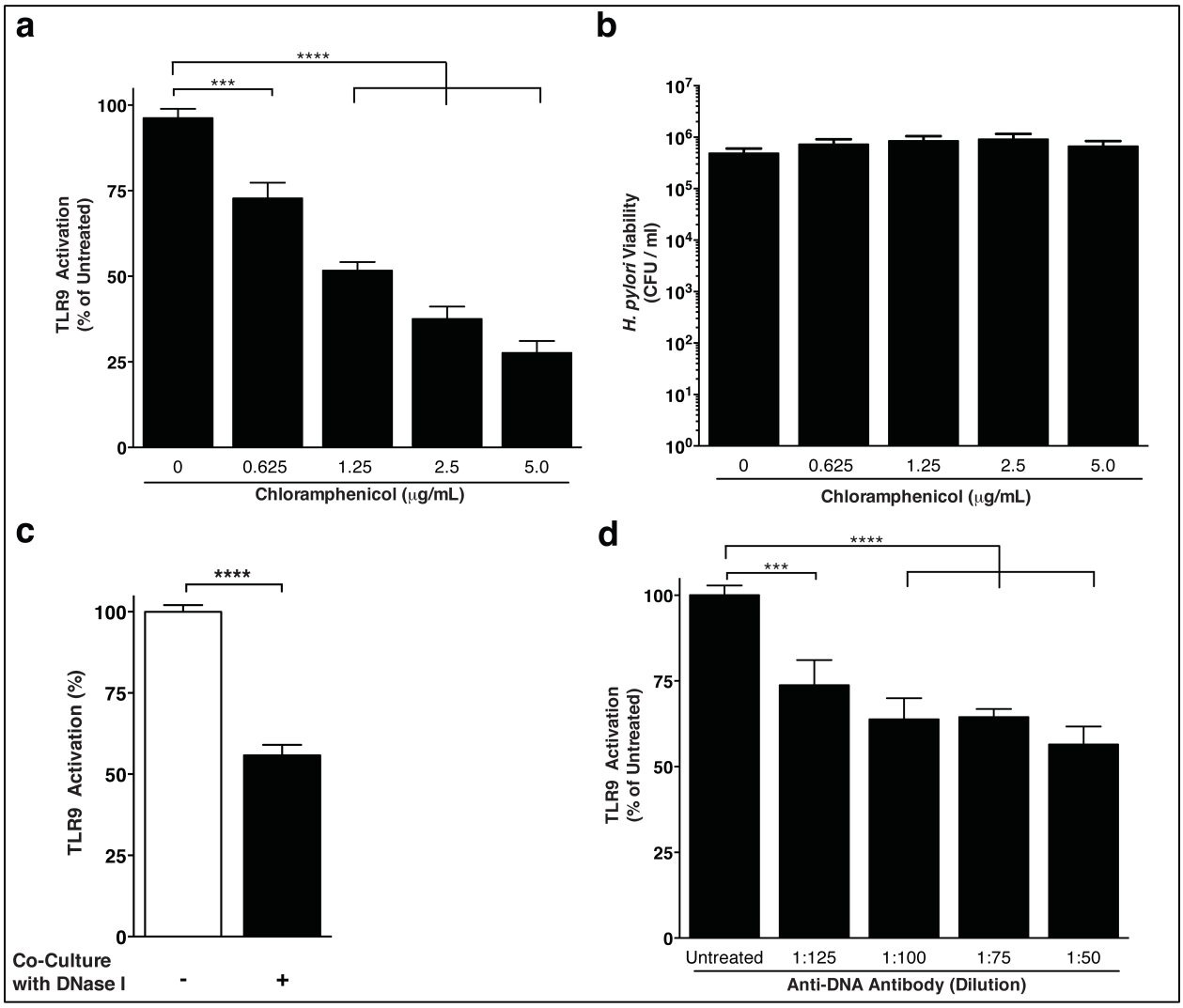

Figure 18: Mechanisms of *H. pylori cag*-mediated DNA translocation.

A) *H. pylori* strain J166 cultures were pre-treated with bacteriostatic, sub-lethal doses of the protein synthesis inhibitor, chloramphenicol, from 0.625-5.0 µg/mL. (B) *H. pylori* strain J166 viability was quantified after 24 hour co-culture with HEK-hTLR9 reporter cells in the presence of 0.625-5.0 µg/mL chloramphenicol. (C) *H. pylori* strain J166 cultures were either pre-treated with or without 10 units DNase I and then co-cultured with HEK reporter cells. Results are normalized to the control, Null cells and TLR9 activation is represented relative to percent of untreated, DNase I free co-cultures. (D) Anti-DNA antibodies were added to *H. pylori*-HEK reporter cell co-cultures at dilutions ranging from 1:125 to 1:50. Results are normalized to the control, Null cells and TLR9 activation is represented relative to percent of untreated, antibody free co-cultures. Mean±SEM are shown. ***p<0.001 ****p<0.0001.

Discussion

The primary mode by which *H. pylori* induces disease and promotes carcinogenesis is through the translocation of the oncoprotein CagA. Several of the proteins required for CagA translocation share some homology to the T4SS proteins in other bacterial species that presumably retain conserved functions. Herein, we now show that *H. pylori* utilizes its *cag* T4SS to translocate DNA into host cells. This function is common amongst Gram-negative bacterial T4SSs, however this is the first study to show that this function is conserved in the *H. pylori cag* T4SS.

Prior to this investigation, TLR9-mediated responses had been established, however the mechanism by which *H. pylori* could engage this receptor was unknown. Therefore, we began our *in vitro* investigations using a human TLR9 reporter assay to elucidate the bacterial factors required for TLR9 activation. We began our focus on the ComB DNA translocation system as it has been well defined in translocating DNA into *H. pylori* from the extracellular milieu and is an integral part of *H. pylori* competence. However, our *comB*$^{-}$ mutant proved just as efficacious in activating TLR9 as the wild-type strain. Therefore, we focused on the *tfs3* and *tfs4* systems, two relatively uncharacterized T4SSs that have been implicated in DNA transfer and DNA conjugation, however their function has not yet been fully elucidated. Since these T4SS were not well defined, we hypothesized that perhaps they were mediating the DNA transfer event that culminated in TLR9 activation. We utilized strain 26695, which lacks intact *tfs3* and *tfs4* gene clusters and were again surprised that these systems were dispensable for TLR9 activation. Lastly we examined the *cag* T4SS despite the fact that it has already been well characterized and found that *cag* mutants lost their ability to activate TLR9. Although this was a novel function for *H. pylori,* we were encouraged by other bacteria such as *A. tumefaciens* or *B. henselae,* which

facilitate trans-kingdom DNA transfer through an evolutionarily conserved T4SS similar to the *cag* T4SS of *H. pylori.*

Although we were encouraged by these data, we had to rule out the possibility that perhaps TLR9 activation was mediated by endocytosis or by secreted factors. Bacteria such as *Neisseria* species can use a T4SS to secrete DNA into the extracellular milieu which may contribute to horizontal gene transfer, while other bacteria secrete DNA to aid in the construction of biofilms. Thus, to rule out secreted factors, we challenged TLR9 reporter cells with *H. pylori* conditioned media and found no evidence of activation. Next, to determine whether bacterial endocytosis was required for TLR9 activation, we preformed gentamicin protection assays to assess how well wild-type and *cag* mutant bacteria were internalized to determine if endocytosis correlated with the amount of TLR9 activation. One possibility was that perhaps the T4SS pili could better facilitate bacterial attachment or engage host receptors that would lead to engulfment and subsequent activation of TLR9 while *cag* mutants lacking these pili would attach less well and not induce TLR9 activation. We found that the wild-type and *cag* mutants were endocytosed equivalently and thus these differences could not reconcile the observed decrease in TLR9 activation.

Due to the accumulating indirect evidence that the *cag* T4SS was responsible for translocating DNA into host cells, we embarked on an experiment to show that *H. pylori* DNA was directly translocated into the host. We labeled *H. pylori* DNA with BrdU and infected AGS gastric epithelial cells with the labeled bacteria. We then used structured illumination microscopy to observe the cells in super-high resolution and detect any naked bacterial DNA inside the host cell. We observed significant amounts of DNA inside the host cell in proximity to external bacteria, which was absent in cells infected with BrdU-labeled, T4SS mutant bacteria.

We then verified these data by flow cytometry by quantifying the amount of labeled bacterial DNA associated with host cells. This mode of detection has some limits however, as it cannot distinguish between a cell with intracellular *H. pylori* derived DNA or a cell that simply has an *H. pylori* bacterium attached to its surface. To attempt to reconcile these differences, we did treat the co-cultures with gentamicin for an hour prior to prepping the samples for analysis to kill any remaining extracellular bacteria. Ideally, we would have liked to extend the gentamicin treatment as there was still some signal from the *cagE*$^-$ mutant bacteria, which are unable to translocate DNA and activate TLR9. However, we were limited by time as the DNA would begin to degrade within the endosome the longer we waited to analyze the sample. Nonetheless, we still observed a significant increase in signal from BrdU-labeled wild-type *H. pylori* compared to BrdU-labeled *cagE*$^-$ mutant *H. pylori.* Taken together, these data demonstrate that *H. pylori* actively translocate DNA into host cells and once internalized, the DNA can activate TLR9.

Based on these findings, we next sought to determine how the *H. pylori* DNA was translocated through the *cag* T4SS. Studies in *A. tumefaciens* suggest that single stranded DNA (ssDNA) is translocated through the T4SS, however the pilus is not required for successful translocation into plant cells. To determine whether *H. pylori* DNA is secreted through the milieu and taken up by host cells, we treated the culture media with either DNase or anti-DNA antibodies and challenged our reporter TLR9 cells with *H. pylori.* In both instances, we observed an approximately 50% decrease in activation, suggesting that some of the DNA being translocated is exposed while a portion remains protected either by the pilus or DNA chaperone proteins similar to *A. tumefaciens.* However, the DNase degrades both ssDNA and dsDNA indiscriminately and TLR9 recognizes both ss- and dsDNA; therefore, at this juncture we cannot conclude which type of DNA *H. pylori* translocates into host cells. While both *A. tumefaciens*

and *B. henselae* translocate ssDNA, they translocate plasmid DNA which then integrates into the host chromosome. In the case of *H. pylori,* only some strains contain plasmids, and the strains used in this study are devoid of plasmid DNA which suggests that the TLR9 activating DNA is of genomic origin. Lastly, to determine whether the process of translocation required new protein synthesis at the time of bacterial attachment to host cells, we pre-treated the *H. pylori* with bacteriostatic doses of chloramphenicol, an inhibitor of protein synthesis. We found that indeed the *H. pylori* are not ready for DNA translocation prior to attachment, and that *de novo* protein synthesis is required for TLR9 activation. Although not investigated in this study, the next step would be to investigate the role of DNA synthesis in TLR9 activation. Since we utilized strains that are devoid of plasmid DNA, the origin of the activating DNA must be genomic. However whether there are DNA fragments ready to be translocated within *H. pylori* or whether *H. pylori* has to generate new DNA to activate TLR9 has yet to be determined.

In addition to the challenges presented in determining the mechanism of *cag*-mediated DNA transfer, the mechanism by which the DNA contacts TLR9 is also currently unclear. Since TLR9 is an endosome bound receptor, the *H. pylori* DNA must also enter the endosome. However, our findings suggest that endocytosed bacteria are not the main source of TLR9 activation, and that DNA is actively translocated into host cells. Furthermore, our microscopy suggests that the *H. pylori* DNA co-localizes with actin filaments, suggesting that the DNA may first encounter the cytosol upon entry. To this end, *H. pylori* DNA may be translocated into the cytosol where it is then auto-phagocytosed and subsequently activates TLR9. Previous studies have shown that CpG DNAs can induce TLR9-dependent autophagy (*282*), and similar arguments could be made with the recognition of foreign *H. pylori* DNA. Conversely, *H. pylori* DNA may never enter an endosome and may be recognized by one of two other proteins, DHX9

or DHX36, both of which associate with the cytoplasmic domain of TLR9, recognize cytosolic DNA motifs and signal through the same MyD88 dependent pathway (*283*). If this were indeed the case, it also opens the possibility that other cytosolic DNA receptors could also detect *H. pylori* DNA such as cGAS or AIM2.

These data demonstrate that *H. pylori* translocate DNA through the *cag* T4SS and corroborate previous studies that have shown *H. pylori*-induced TLR9 activation contributes to the inflammatory response. These data provide the beginning framework for how *H. pylori* may engage this host endosomal innate immune receptor although more investigations will be necessary to fully elucidate this mechanism. In parallel, we also demonstrate that *H. pylori*-induced TLR9 activation can influence carcinogenesis in our Colombian cohort, perhaps through strain specific differences such as the amount of DNA methylation. In the next chapter, we will delve further into the role of *H. pylori*-mediated TLR9 activation *in vivo.*

CHAPTER III

TOLL-LIKE RECEPTOR 9 ACTIVATION SUPPRESSES INFLAMMATION IN RESPONSE TO *HELICOBACTER PYLORI* INFECTION

Introduction

Chronic gastritis induced by the pathogen *Helicobacter pylori* typically persists for the lifetime of the host (*247, 284*). One strain-specific *H. pylori* locus that augments risk for disease is the *cag* pathogenicity island. The *cag* island encodes for a bacterial type IV secretion system (T4SS) that translocates CagA, as well as peptidoglycan, into host cells (*128, 129*) Intracellular CagA can initiate multiple signaling cascades in host cells, including pro-inflammatory pathways. Furthermore, recent studies have demonstrated that chronic activation of Nod1 by *H. pylori* peptidoglycan can down-regulate pro-inflammatory signaling (*129, 270, 285*) and in Chapter II, we reported that the *cag* T4SS can also translocate *H. pylori* DNA into host cells to activate toll-like receptor 9 (TLR9) .

Toll-like receptors (TLRs) orchestrate host immune responses targeting pathogens via selective recognition of pathogen-associated molecular patterns (PAMPs) (*264*). TLR9 is an intracellular receptor that recognizes hypo-methylated CpG motifs (*193*), which are abundant in DNA of bacterial, viral, or synthetic origin, but are atypical within mammalian genomes (*170*). TLR9 is a multidimensional immune receptor based on its ability to mediate both pro- and anti-inflammatory responses (*201*). In the human intestinal tract, TLR9 activation by commensal organisms regulates, and even dampens, inflammatory responses as a means of maintaining homeostasis (*201, 233*). In mouse models of colitis, TLR9 activation by *H. pylori* DNA directs

immune responses towards an anti-inflammatory phenotype (*71, 72, 241*) and one study of short-term infection reported that *H. pylori* gastritis was increased in the absence of TLR9 (*213*). However, the mechanism of *H. pylori*-induced TLR9 activation has remained elusive since TLR9 is an endosomal receptor and the majority of colonizing *H. pylori* remain extracellular.

Since our recent studies demonstrated that *H. pylori* utilizes the *cag* T4SS to translocate DNA and activate TLR9 (*115*), we sought to define the role of this virulence locus in manipulating TLR9 and downstream immune effectors *in vivo.* To conduct these experiments, we utilized the prototype mouse-adapted *cag*$^{+}$ *H. pylori* strain PMSS1, which retains T4SS *in vivo* (*271*), to induce inflammation and injury in a mouse model of TLR9 deficiency.

Methods

Mouse Infections: All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Vanderbilt University Medical Center's Institutional Animal Care and Use Committee (IACUC) approved all protocols and all efforts were made to minimize animal suffering. Male and female C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and housed in the Vanderbilt University Animal Care Facilities. *Tlr9*$^{-/-}$ C57BL/6 mice were generously provided by the laboratory of Dr. Gregory Barton. Mice were orogastrically challenged with Brucella broth alone as an uninfected control, with the mouse-adapted wild-type *cag*$^{+}$ *H. pylori* strain PMSS1, or with a PMSS1 *cagE*$^{-}$ isogenic mutant (4). Mice were euthanized at 6 weeks post-challenge and gastric tissue was harvested for quantitative culture, histology, immunohistochemistry and Luminex assays. Serum samples were collected

from a cardiac puncture and used for ELISA. Results were obtained from 2 independent experiments.

Quantitative *H. pylori* culture: Gastric tissue was harvested and homogenized in sterile PBS. Following serial dilution, samples were plated on selective trypticase soy agar plates with 5% sheep blood (Hemostat Laboratories, Dixon, CA, USA) containing vancomycin (20 μg/ml; Sigma-Aldrich, St. Louis, MO, USA), nalidixic acid (10 μg/ml; Sigma-Aldrich), bacitracin (30 μg/ml; Calbiochem, Billerica, MA, USA), and amphotericin B (2 μg/ml; Sigma-Aldrich) for isolation of *H. pylori*. Plates were incubated for 3–5 days at 37°C with 5% CO_2. Colonies were identified as *H. pylori* based on characteristic morphology, Gram stain (BD Biosciences, Sparks, MD, USA), urease, and oxidase (BD) activities. Colony counts were expressed as log CFU per milligram of gastric tissue.

Cell Culture: AGS human gastric epithelial cells (ATCC CRL-1739) were grown in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA). HEK-Blue-hTLR9 (TLR9+) and HEK-Blue Null1 (parental) cells (Invivogen, San Diego, CA, USA) were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and 100 μg/mL Zeocin (Invivogen). HEK-Blue-hTLR9 cell media was supplemented with an additional selective antibiotic, Blasticidin (Invivogen) at 10 μg/mL. All cell lines were maintained at 37°C with 5% CO_2. *H. pylori* co-culture studies were conducted at an MOI of 100 for 4-24 hours as described (23).

CagA Translocation Assay: *H. pylori* were co-cultured with AGS cells at an MOI of 30 for 4 hours as previously described (22). Protein lysates were harvested in RIPA buffer, separated by SDS-PAGE, and transferred to PVDF membranes (Thermo, Rockford, IL, USA). Levels of total CagA (1:5000 anti-CagA antibody; Austral Biologicals, San Ramon, CA, USA) and

phosphorylated CagA (1:5000 anti-pY99 antibody; Santa Cruz, Santa Cruz, CA, USA) were determined via Western blotting to assess levels of translocated CagA. Protein intensities were quantified using the ChemiGenius Gel Bio Imaging System (Syngene Frederick, MD, USA). Experiments were repeated at least 3 times.

TLR9 Activation Assay: Activation assays were conducted as described (23). Briefly, HEK-Blue-hTLR9 cells and HEK-Blue-Null1 cells (Invivogen) were seeded in 96 well plates (Co-Star, Corning, NY, USA) at 80,000 cells per well in DMEM without antibiotics and challenged with either viable *H. pylori* (MOI 100). After 24 hours, 20 µl of supernatants were added to 180 µl of HEK-Blue Detection media (Invivogen). Plates were analyzed by spectrophotometer (Bitoek, Winooski, VT, USA) at 650 nm. All experiments were performed in duplicate and repeated at least 3 times.

Luminex Assay: Gastric tissues were weighed, homogenized in IP lysis buffer (Pierce, Rockford, IL, USA) and subsequently passed through a 21-gauge needle. Samples were then centrifuged at 4°C for 10 minutes and the aqueous fraction removed for analysis. Samples were assayed using a magnetic bead-based protein detection assay for murine cytokines in duplicate according to manufacturer's instructions (Millipore, Billerica, MA, USA) and quantified by a FlexMap 3D plate reader (Luminex, Austin, TX, USA). Samples were then normalized to milligrams of protein as quantified by Bradford Assay (Pierce).

ELISA: ELISA assays were performed as previously described (22). Briefly, *H. pylori* strain PMSS1 was grown overnight in Brucella broth. The culture was washed twice in 1x PBS, pelleted, and lysed in IP lysis buffer (Pierce). The lysate was diluted 1:50 in coating buffer (85 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.5) and incubated overnight at 4°C in 96 well ELISA plates (DYNEX, Chantilly, VA, USA). Following lysate binding, murine serum samples (1:20 dilution)

were added for 2 hours. Biotin-conjugated anti-mouse IgG1 (BD Pharmigen) or IgG2a (isoform b, BD Pharmigen) antibodies were diluted 1:5000 and incubated for 1 hour at room temperature. Streptavidin-HRP (1:10,000 Life Technologies) was added for 1 hour. For detection of total IgG, donkey anti-mouse conjugated HRP (1:5,000 Santa Cruz) was applied for 1 hour at room temperature. Reactions were stopped with 2N H_2SO_4 and the OD_{450} was quantified by spectrophotometer (Biotek). All samples were performed in duplicate.

Histology and Immunohistochemistry: Linear strips of murine gastric tissue, extending from the squamocolumnar junction through the proximal duodenum, were fixed in 10% neutral-buffered formalin (Azer Scientific Inc., Morgantown, PA, USA), embedded in paraffin, and stained with hematoxylin and eosin. A pathologist, blinded to treatment groups, assessed indices of inflammation. Severity of acute and chronic inflammation was graded 0–3 in both the gastric antrum and corpus, as previously described (9), for a cumulative score ranging from 0–12. To quantify the abundance of T_{reg} cells, tissue samples were stained with an antibody directed against FoxP3 (Novus, Littleton, CO, USA) and the numbers of $FoxP3^+$ cells were enumerated from 5 high power fields each (400x magnification) from both the antrum and corpus by a single pathologist (MBP).

Statistics: Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA). When comparisons between multiple groups were made, analysis of variance (ANOVA) with Bonferroni correction was performed. Student's *t*-test was performed when comparisons between only two groups were made. Spearman's correlation was performed to determine linear correlation. For Luminex results, values were square root transformed prior to statistical analysis. A P-value of <0.05 was considered significant. In all figures, means±standard errors of the mean are shown.

Results

Six to eight week old wild-type C57BL/6 or *Tlr9*$^{-/-}$ C57BL/6 mice were infected with either the *cag*$^{+}$ *H. pylori* strain PMSS1, a PMSS1 *cagE*$^{-}$ isogenic mutant which lacks a functional T4SS, or vehicle (Brucella Broth) control. Six weeks post infection, mice were sacrificed and stomachs were harvested. A significant ($p<0.01$) increase in the severity of inflammation was observed in *H. pylori*-infected *Tlr9*$^{-/-}$ mice versus infected wild-type mice **(Figure 20A,B)**. As expected, levels of inflammation were *cag*-dependent, as the *cagE*$^{-}$ mutant induced significantly less inflammation compared to the wild-type *H. pylori* strain, irrespective of host backgrounds **(Figure 20A,B)**.

In order to define mechanisms that underpin these differences in inflammation, we investigated whether microbial factors contributed to this phenotype. No significant differences in levels of *H. pylori* colonization were identified between wild-type and *Tlr9*$^{-/-}$ mice. There was an increase in colonization between wild-type *H. pylori* and the *cagE*$^{-}$ isogenic mutant within the same host genotype, which is concordant with previous reports (*99*) **(Figure 21A)**.

We next determined whether selective pressure exerted by the genetic loss of *Tlr9* affected *H. pylori* phenotypes *in vivo*. *H. pylori* strains were recovered from PMSS1-infected wild-type or *Tlr9*$^{-/-}$ mice and were assessed for the ability to activate TLR9 and translocate CagA

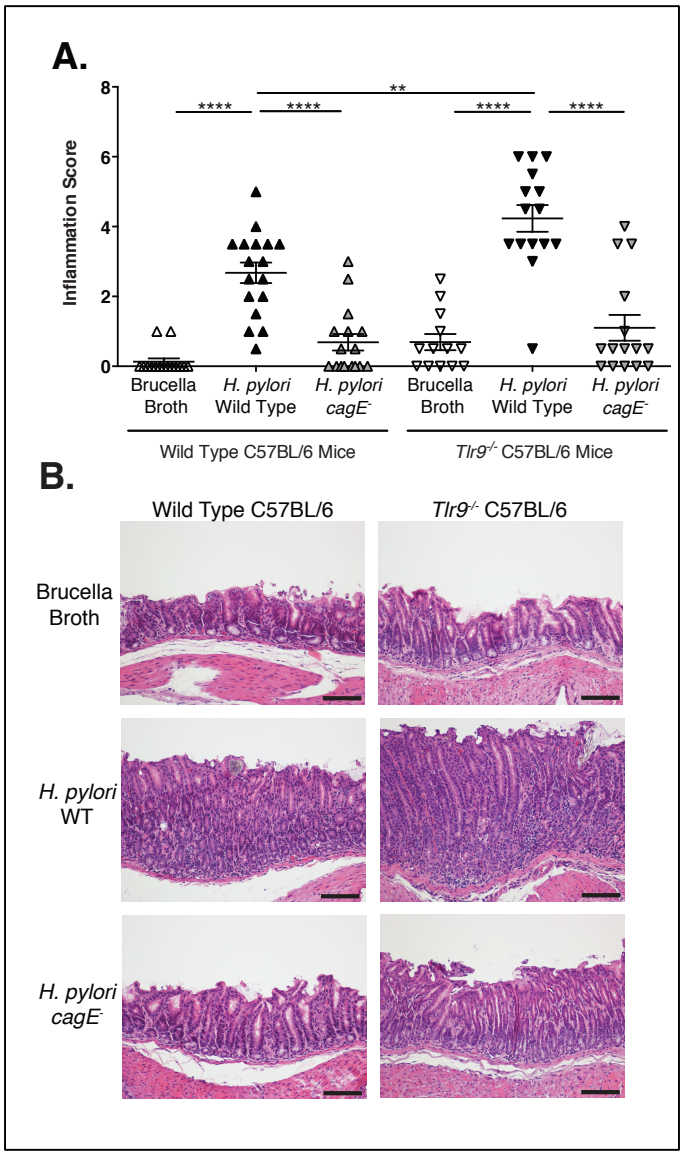

Figure 19: Loss of *Tlr9* exacerbates *H. pylori*-induced inflammation *in vivo.*

Wild-type or *Tlr9*$^{-/-}$ C57BL/6 mice were challenged with either vehicle (Brucella Broth) control, the mouse-adapted, *cag*$^{+}$ *H. pylori* strain PMSS1, or the PMSS1 isogenic *cagE*$^{-}$ mutant for 6 weeks. (A) Inflammation scores of wild-type or *Tlr9*$^{-/-}$ mice infected with or without *H. pylori*. Each data point represents the inflammation score from an individual animal. Two independent experiments were performed. N=13-17 mice per group, Mean ± SEM are shown. **p<0.01,**p<0.0001. (B) Representative H&E images of *H. pylori*-infected or uninfected wild-type and *Tlr9*$^{-/-}$ mice at 20x magnification. Scale bar, 100 µm.**

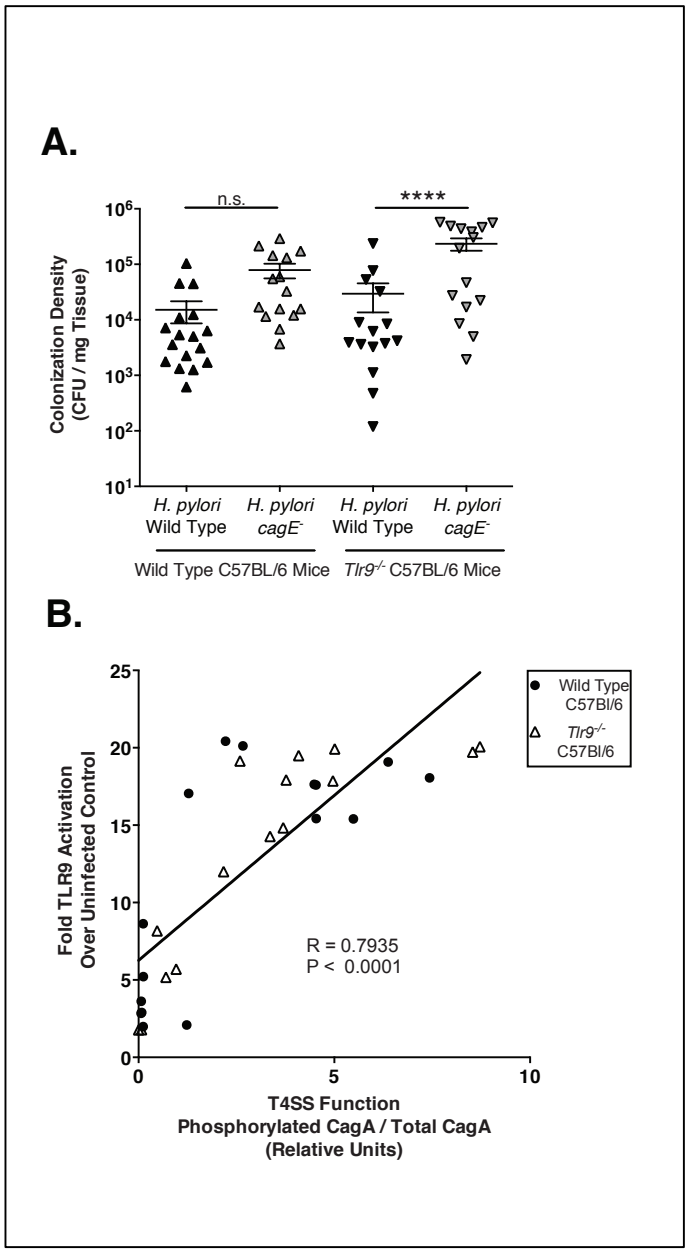

Figure 20: *Tlr9* deficiency does not alter *H. pylori* colonization density or virulence phenotypes.
(A) Stomach sections containing both antrum and corpus regions were homogenized and serially diluted on blood-agar plates to quantify *H. pylori* colonization. Each data point represents the colonization density from an individual animal. Two independent experiments were performed. N=13-17 mice per group, Mean ± SEM are shown. ****p<0.0001. (B) Spearman correlation plot of T4SS function (as quantified by CagA translocation) versus TLR9 activation of *in vivo*-adapted *H. pylori* isolates. Each data point represents one output strain (N=15 murine isolates per group).

in vitro. To quantify TLR9 activation, we challenged HEK-293-hTLR9 reporter cells, which are devoid of most immune receptors but over express TLR9 and additionally contain an NFκB/AP-1-linked secreted embryonic alkaline phosphatase (SEAP) reporter, with the *in vivo*-adapted *H. pylori* isolates. To quantify T4SS viability, we challenged AGS gastric epithelial cells with the *in vivo*-adapted *H. pylori* strains and measured the ratio of phosphorylated CagA (intracellular) to total CagA by Western blot. Host *Tlr9* status had no effect on TLR9 activation or T4SS function; however, there was a significant positive correlation between the intensity of these phenotypic responses **(Figure 21B)**. Taken together, these data suggest that *H. pylori* colonization density *per se* did not contribute to the increase in inflammation observed in infected *Tlr9*$^{-/-}$ mice, and that *Tlr9* deficiency did not alter *H. pylori* T4SS phenotypes.

Having shown that *H. pylori* increases inflammation in *Tlr9*$^{-/-}$ mice despite similar levels of colonization **(Figure 20A, 21A)**, we next assessed the role of TLR9-mediated immune effectors that may contribute to differences in inflammation. The abundance of *H. pylori*-specific IgG2a and IgG1 serum antibodies was assessed by ELISA as markers of T_H1 or T_H2 immune responses, respectively **(Figure 22A)**. There were no significant differences in the levels of IgG2a or IgG1 between *H. pylori*-infected mice when stratified on the basis of *Tlr9* genotypes. Since serum IgG markers reflect systemic responses to infection, we also quantified immune effectors within uninfected or infected gastric tissue. Infection with *H. pylori* induced an increase in levels of a subset of specific T_H1 and T_H2 cytokines in both wild-type and *Tlr9* deficient mice when compared to Brucella broth controls. However, there were no significant differences in gastric mucosal levels of the archetypal T_H1 secreted cytokines IFN-γ or IL-2 **(Figure 22B,C)** or the T_H1-associated pro-inflammatory chemokines KC or IP-10 **(Figure 22D,E)** when *H. pylori*

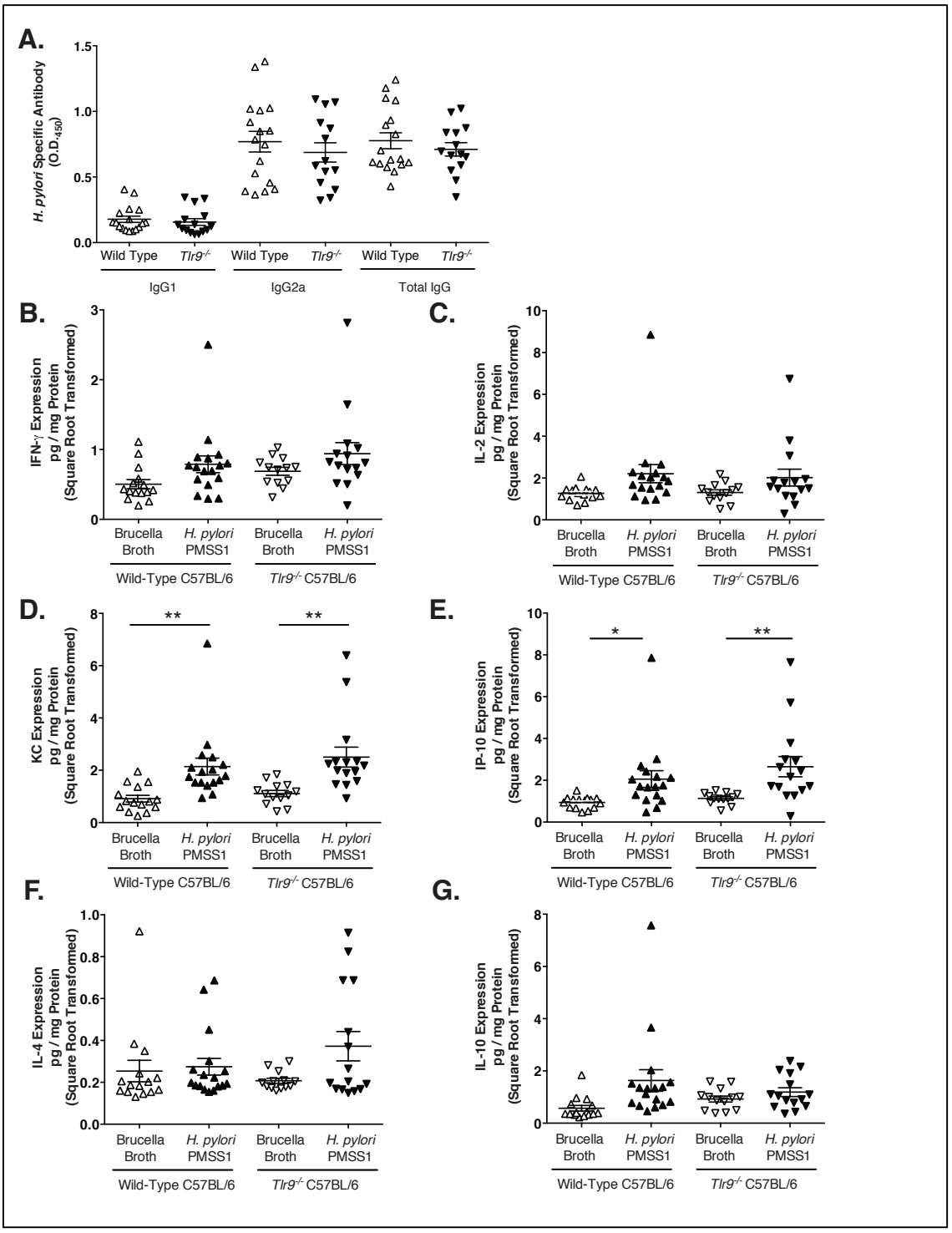

Figure 21: *H. pylori*-induced immune responses in wild type and *Tlr9*$^{-/-}$ mice.

(A) ELISA of *H. pylori*-specific serum IgGs in *H. pylori*- infected mice. (B-G) Stomach sections from Uninfected of *H. pylori*-infected wild type or *Tlr9*$^{-/-}$ mice were subjected to multiplex cytokine and chemokine array. Expression levels were normalized to total protein (mg) and square root transformed. Expression levels of representative T_H1 cytokines are shown in (B) IFN-γ and (C) IL-2. Expression levels of representative T_H1 associated chemokines are shown in (D) KC and (E) IP-10. Expression levels of representative T_H2 cytokines are shown in (F) IL-4 and (G) IL-10. Each data point represents an individual animal obtained from 2 independent experiments. N=13-17 mice per group, Mean ± SEM are shown. *p<0.05, **p<0.01.

Cytokine/Chemokine	Mean picograms/milligram protein ± SEM (square root transformed)				Significance (ANOVA with Bonferroni Correction)
	Brucella Broth / Wild-Type Mouse[a] (n=15)	Brucella Broth / *Tlr9*$^{-/-}$ Mouse[b] (n=13)	*H. pylori* PMSS1 / Wild-Type Mouse[c] (n=17)	*H. pylori* PMSS1/ *Tlr9*$^{-/-}$ Mouse[d] (n=15)	
IL-1α	4.527±0.5012	4.569±0.6086	8.372±1.546	10.67±1.627	**b,d
IL-1β	1.265±0.0975	1.6±0.1041	1.739±0.1728	2.243±0.3375	None
IL-2	1.268±0.0848	1.311±0.1297	2.207±0.4352	2.020±0.4021	None
IL-4	0.2539±0.0521	0.2075±0.0122	0.2743±0.0397	0.3725±0.0699	None
IL-5	Undetected	Undetected	Undetected	Undetected	N/A
IL-6	0.4803±0.0866	0.6482±0.0819	0.7710±0.0925	0.7662±0.0976	None
IL-7	Undetected	Undetected	Undetected	Undetected	N/A
IL-9	1.646±0.2264	1.825±0.2283	2.726±0.4816	2.358±0.389	None
IL-10	0.5725±0.1086	0.9245±0.1140	1.637±0.4124	1.189±0.1681	**a,c
IL-12p40	0.5404±0.0511	0.9482±0.7852	0.9439±0.1014	1.255±0.1566	*a,b; *a,c
IL-12p70	Undetected	Undetected	Undetected	Undetected	N/A
IL-13	1.610±0.1994	2.109±0.1516	2.388±0.2850	3.229±0.4450	None
IL-15	0.9653±0.0732	1.297±0.1034	1.445±0.1620	1.632±0.2329	None
IL-17	**0.4303±0.0510**	**0.6501±0.0491**	**0.7432±0.0767**	**1.188±0.2014**	***b,d; **c,d**
G-CSF	0.8696±0.0695	1.259±0.0802	1.071±0.1313	1.442±0.2096	None
GM-CSF	1.338±0.1015	1.574±0.1043	1.714±0.245	2.002±0.2911	None
IFN-γ	0.5038±0.0651	0.6891±0.0568	0.7888±0.1209	0.9412±0.1574	**b,d
IP-10	0.9387±0.07160	1.135±0.0765	2.057±0.399	2.649±0.4851	*a,c; **b,d
KC	0.9166±0.1281	1.111±0.1178	2.142±0.3209	2.504±0.3814	**a,c; **b,d
MCP-1	1.330±0.0948	1.867±0.1167	1.989±0.304	2.168±0.3013	None
MIP-1α	1.063±0.2362	1.519±0.1286	1.284±0.2167	1.875±0.3301	None
MIP-1β	Undetected	Undetected	Undetected	Undetected	N/A
RANTES	0.8140±0.1223	1.062±0.1355	1.930±0.37	1.921±0.4315	*a,c
TNF-α	Undetected	Undetected	Undetected	Undetected	N/A

Table 2: List of cytokines expressed in wild-type and *Tlr9*$^{-/-}$ mice infected with or without *H. pylori.*
Mean picograms of cytokine or chemokine per milligram of protein detected in gastric tissue homogenates of wild-type or *Tlr9*$^{-/-}$ mice infected with or without *H. pylori* strain PMSS1 for 6 weeks. Values are expressed as square root transformations. Columns are listed as a-d, and significance between columns is annotated by the number of stars followed by column titles separated by commas. *p<0.05, **p<0.01.

infected wild-type and *Tlr9* deficient mice were compared. Similarly, no significant differences were present in T_H2 cytokine profiles (including IL-4 and IL-10) between infected mouse groups **(Figure 22F,G)**. In addition to these prototype cytokines and chemokines, there were no differences in gastric mucosal levels of IL-1α, IL-1β, IL-6, IL-9, IL-12p40, IL-13, IL-15, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, or RANTES between *H. pylori*-infected wild-type versus *Tlr9* deficient mice **(Table 2)**. Additionally, we examined the levels of the histone protein and DNA damage marker γH2AX in both mouse genotypes infected with wild-type *H. pylori* strain PMSS1. As expected, we found that there was a significant increase in both genotypes upon infection. Interestingly, we found that *H. pylori*-infected *Tlr9* deficient mice expressed more γH2AX compared to wild-type infected mice **(Figure 23)**. These data suggest that TLR9 plays a role in mediating the response to *H. pylori,* and implicate a role for this receptor in gastric cancer promotion.

Based on these results, we next examined the role of T_H17-mediated immune responses by quantifying levels of a prototypical marker of this response, IL-17, as a means to understand the observed differences in inflammation. In contrast to T_H1 or T_H2 effectors, levels of IL-17 were significantly increased in *H. pylori*-infected *Tlr9*$^{-/-}$ mice compared to *H. pylori*-infected wild-type mice **(Figure 24A)**. Previous reports have shown that *H. pylori* alters the T_{Reg}/T_H17 balance towards a T_{Reg}-biased response, resulting in ineffective immune clearance of bacteria and thus perpetuating persistence (*49*). Due to the increase in levels of IL-17 in *H. pylori*-infected *Tlr9* deficient mice, we next determined relative abundance of gastric mucosal FoxP3$^+$ T_{Reg} cells in *H. pylori*-infected wild-type and *Tlr9*$^{-/-}$ mice. Concordant with previous reports (*49*), a significant increase in FoxP3$^+$ cell abundance was present in mice infected with wild-type *H. pylori*, compared to uninfected or *cagE*$^-$ infected mice **(Figure 24B,C)**.

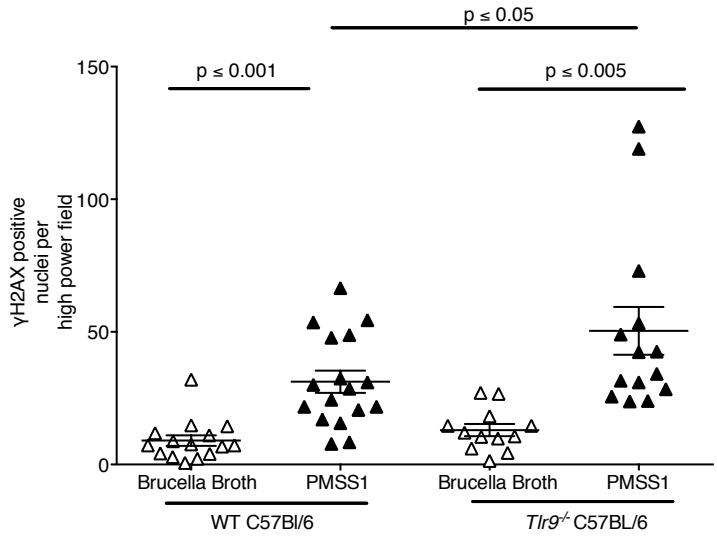

Figure 22: *H. pylori* increases levels of DNA damage as assessed by γH2AX staining.
Levels of histone protein γH2AX were assessed as a marker of DNA damage by immunohistochemistry. Each data point represents an individual animal obtained from 2 independent experiments. N=13-17 mice per group, Mean ± SEM are shown.

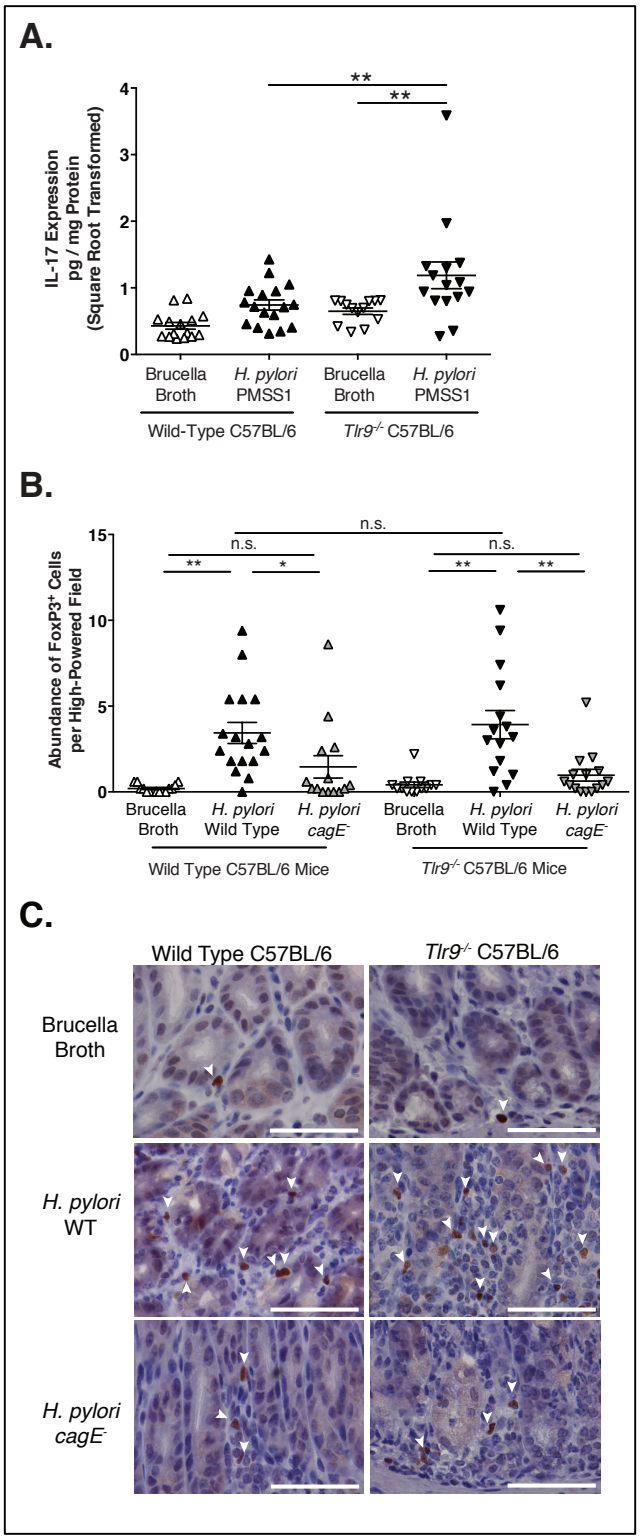

Figure 23: *H. pylori*-mediated TLR9 responses and IL-17.

(A) IL-17 expression in gastric mucosa was determined by cytokine and chemokine array and results are represented as pg/mg of protein (square root transformed). (B) FoxP3$^+$ cells were enumerated in five high-powered fields spanning the antrum and corpus from each animal. Each data point represents 1 animal. Two independent experiments were performed. N=13-17 mice per group, Mean±SEM are shown. *p<0.05, **p<0.01. Representative images of FoxP3 staining in wild-type and *Tlr9*$^{-/-}$ mice are shown in (C). White arrows indicate FoxP3$^+$ cells. 400x magnification, scale bar, 50 µm.

However, no differences were present between infected wild-type and *Tlr9*$^{-/-}$ mice, indicating that the *cag* T4SS, but not TLR9, is required for modulating the T_{Reg} immune response to *H. pylori*. Collectively, these data suggest that *H. pylori*-induced TLR9 activation suppresses IL-17-mediated immunity in a *cag* T4SS-dependent manner, but this mechanism is independent of regulatory T-cells.

Discussion

H. pylori has co-evolved with its cognate human host for over 100,000 years and is uniquely adapted to survive for decades within the harsh environment of the stomach. This has necessarily led to the development of mechanisms to induce inflammation as well as strategies to evade detection and down-regulate the host immune response. An example is the counter-regulatory interplay between CagA and the VacA toxin, encoded by loci that are evolutionarily linked. CagA antagonizes VacA-induced apoptosis through activation of a cell survival pathway mediated by MAPK, ERK and the anti-apoptotic protein MCL1 (*106*). CagA also triggers NFAT and EGFR signaling, processes that are inhibited by VacA (*106*). In this study, we have demonstrated that the *cag* T4SS *per se* can regulate oscillation of the host inflammatory response, perhaps as a means of sculpting a sustainable niche within the stomach.

TLR9 is an innate, intracellular immune receptor and its activation is typically associated with viral or invasive bacterial infections in which microbial DNA is easily accessible. However, *H. pylori* does not commonly invade host cells; thus, its ability to activate TLR9 is of substantial interest. We have recently shown that the major virulence factor of *H. pylori,* the *cag* pathogenicity island, which enables the formation of the *cag* T4SS and subsequent translocation of CagA toxin, also facilitates the translocation of DNA into host cells (*115*). Therefore, we sought to capitalize on a mouse-adapted *cag* T4SS positive *H. pylori* strain that readily infects

rodents and maintains *cag* function for weeks *in vivo* to investigate the effects of this virulence constituent within the context of TLR9 activation (*271, 286, 287*).

In this study, the mouse *Tlr9*$^{-/-}$ genotype did not alter *H. pylori* colonization density or the microbial phenotype of the *H. pylori* output derivatives in terms of T4SS function. However, infected *Tlr9* deficient mice developed more severe inflammation compared to infected wild-type mice. It is important to note that infection with the *H. pylori cagE*$^{-}$ mutant would not be expected to pheno-copy *H. pylori* PMSS1 wild-type infected *Tlr9*$^{-/-}$ mice as the *H. pylori cag* T4SS can translocate other pro-inflammatory effectors such as CagA and peptidoglycan. Using a multiplex cytokine array to examine distinct host immune effectors that may contribute to this increase in inflammation, we observed that *H. pylori* infection increased levels of T_H1-dependent serum markers and cytokines, which is consistent with previous reports that *H. pylori* induces a T_H1-biased immune response in mice, but this was independent of *Tlr9* status. However, a T_H17 cytokine, IL-17, was differentially abundant between *H. pylori*-infected wild-type versus *Tlr9*$^{-/-}$ mice, raising the tantalizing possibility that TLR9 can direct T_H17 responses to *H. pylori*.

There are six members in the IL-17 family termed IL17A-F. IL-17A, commonly termed IL-17, is best characterized for its essential role in host defense and mediates pro-inflammatory responses from responder cells leading to neutrophil and macrophage recruitment at the inflammation site (*10, 288*). IL-17A has been shown to be critically important in bacterial clearance of extracellular pathogens such as *S. aureus, C. rodentium, and Klebsiella pneumonia* as well as some fungal infections such as *C. albicans,* and *Pneumocystis carinii* (*288-293*). Importantly, IL-17 and other members have been shown to be up-regulated in response to *H. pylori* infection, and IL-17A deficient mice are more susceptible to infection (*294-297*).

IL-17 is a highly unique cytokine that shares no homology with any other cytokines, proteins or structural domains (*298, 299*). It was first found to originate from a subset of $CD4^+$ T cells, termed T_H17 cells that are distinct population of $CD4^+$ cells compared to the classical T_H1 and T_H2 lineages. Naïve $CD4^+$ T cells can become T_H17 cells via exposure to the tightly controlled set of cytokines IL-6, IL-21, IL-23, and TGFβ which enables them to produce the transcription factor RORγ(t). Expression of RORγ(t) in conjunction with Stat3, NFκB, IκBζ and Batf define the T_H17 lineage and make it a unique population to T_H1 and T_H2 cells (*300*). Aside from T_H17 cells, the next major source of IL-17A can originate from γδ-T cells, which are a sub-population of T cells that comprise only 1%-5% of the total lymphocyte population in both humans and mice (*301*). These cell types are found in mucosal tissues and rapidly respond to antigens via recognition through their highly expressed TLRs (*302, 303*). Although T_H17 and γδ-T cells are the primary sources of IL-17A, other cell types including natural killer (NK) cells, natural killer T-Cells (NKT), macrophages and Paneth cells can secrete IL-17A (*304, 305*). However, the physiological implications of IL-17A secreted by these cell types have not been elucidated (*288*). IL-17 cytokines mediate their inflammatory functions by binding IL-17 receptors (IL17R) on target cells. There are 5 isoforms of the IL-17R (named IL-17RA-E) and they often occur in heterodimeric forms with IL-17RA as the most common subunit (*288*). The IL-17RA/C heterodimer is the primary receptor for IL-17A and is most widely expressed on non-hematopoietic cells such as epithelial cells or fibroblasts. Receptor binding leads to NFκB and MAPK signaling cascades that ultimately results in the release of pro-inflammatory cytokines and chemokines as well as myeloid cell recruitment **(Figure 25)** (*10*).

In the particular case of *H. pylori* infection, gastric mucosal IL-17 is up-regulated and induces the secretion of IL-8 via the ERK1/2-MAPK pathway (*299, 306*). However, as discussed

in Chapter I, TLR9 can induce a tolerigenic responses depending on its localization in the cell when becomes activated. Apical (luminal) stimulation inhibits the function of the E3 ubiquitin ligase β-TrCP. In the canonical NFκB signaling cascade, β-TrCP catalyzes the destruction IκB and p105 (NFκB1, p50 precursor) (*201*). If these proteins remain intact, NFκB cannot assemble and migrate to the nucleus to induce gene transcription. Similarly ERK activation also requires the ubiquitination of p105 (*201*). Therefore, *H. pylori*-mediated apical TLR9 activation may promote tolerigenic responses by blocking the canonical NFκB and MAPK pathways that could result from basolateral TLR9 stimulation or from other pro-inflammatory stimuli that utilize the NFκB and/or MAPK pathways (*201*). In this study, we have observed an increase in inflammation and IL-17 expression in *Tlr9*$^{-/-}$ mice, which may be resultant from a lack of TLR9-mediated tolerigenic signaling.

Another mechanism by which *H. pylori* could evade the immune response could involve T_{Reg} cells. Previous studies have reported that the T_{Reg} transcription factor and defining marker, FoxP3, is expressed in higher abundance in *H. pylori*-infected compared to uninfected individuals (*307, 308*). These data suggest that T_{Reg} cells may down-regulate the gastric mucosal inflammatory response to ultimately allow for *H. pylori* persistence. Other studies have shown that gastric mucosal DCs (which readily express TLR9) are challenged with *H. pylori in vitro,* secrete high amounts of TGFβ, which can influence T_{Reg} skewing (*49*). In addition, T_{Reg} cells can inhibit *H. pylori*-induced T_H17 mediated inflammatory responses and promote anti-inflammatory responses (*49*).

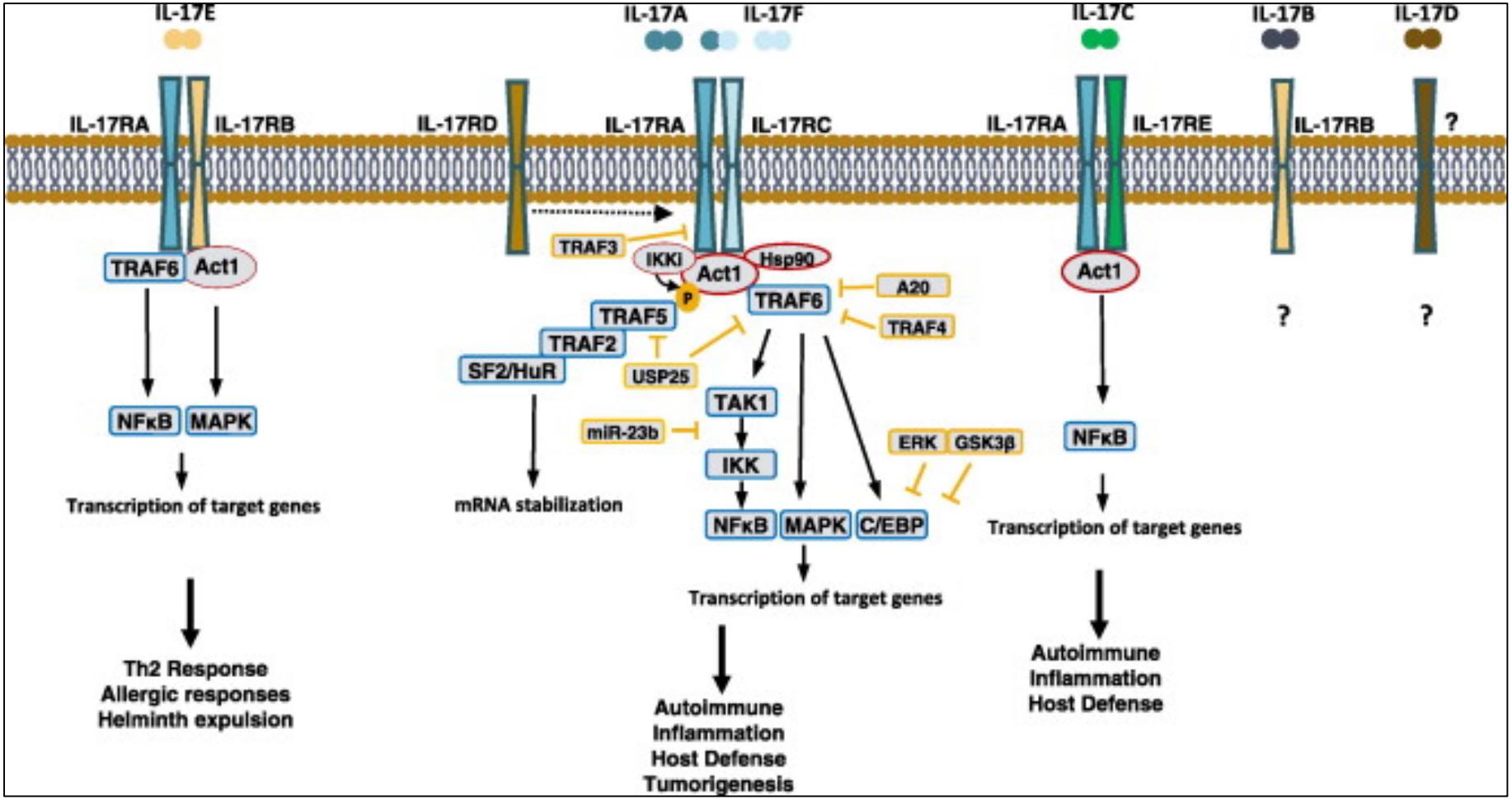

Figure 24: Model of IL-17/IL-17R mediated signaling.

The IL-17 family consists of six members IL-17A-F, while the IL-17 receptor family consists of five members IL-17RA to IL-17RE. IL-17RA is a common receptor that forms hetero-dimeric complexes with IL-17RB, C, or E. To date, all of the IL-17 receptors recruit the adaptor molecule Act1 to mediate downstream signaling. IL-17A and IL-17F signals through the IL-17RA-RC complex, triggering TRAF6-dependent target gene transcription and TRAF6-independent IKKi-dependent mRNA stabilization. IL-17A-induced transcription factors inhibits inflammatory gene expression. Figure reprinted with permission from (*10*).

Therefore, based on our data that IL-17 expression was increased in *Tlr9*$^{-/-}$ mice, we sought to determine whether these mice also displayed a decrease in gastric mucosal FoxP3^{+} T_{Reg} cells. Since we observed no differences in the abundance of FoxP3^{+} T_{Reg} cells between mouse genotypes, these data suggest to us that the IL-17 mediated response may occur through other mechanisms.

Despite findings that TLR9 can mediate an anti-inflammatory phenotype against *H. pylori*, there are limitations in translating these data to human infections. Inherent differences are present in mouse TLR9 when compared to human TLR9. Both mouse and human TLR9 recognize CpG DNA motifs; however, these DNA sequences are distinct. Additionally, human TLR9 is expressed in epithelial cells, B-cells, and plasmacytoid DCs whereas mouse TLR9 is expressed not only in those same cell types, but also in myeloid DCs, as well as macrophages (*214, 215*).

In conclusion, this study demonstrates that *H. pylori* modulates the host immune system towards a TLR9 mediated, anti-inflammatory response via modulation of IL-17 expression, in a *cag* T4SS dependent manner **(Figure 26)**. This modulation could contribute to the ability of *H. pylori* to persist long-term within the stomach, and importantly, these studies lay the foundation for further exploration into the role of TLR9-*H. pylori* interactions in human hosts.

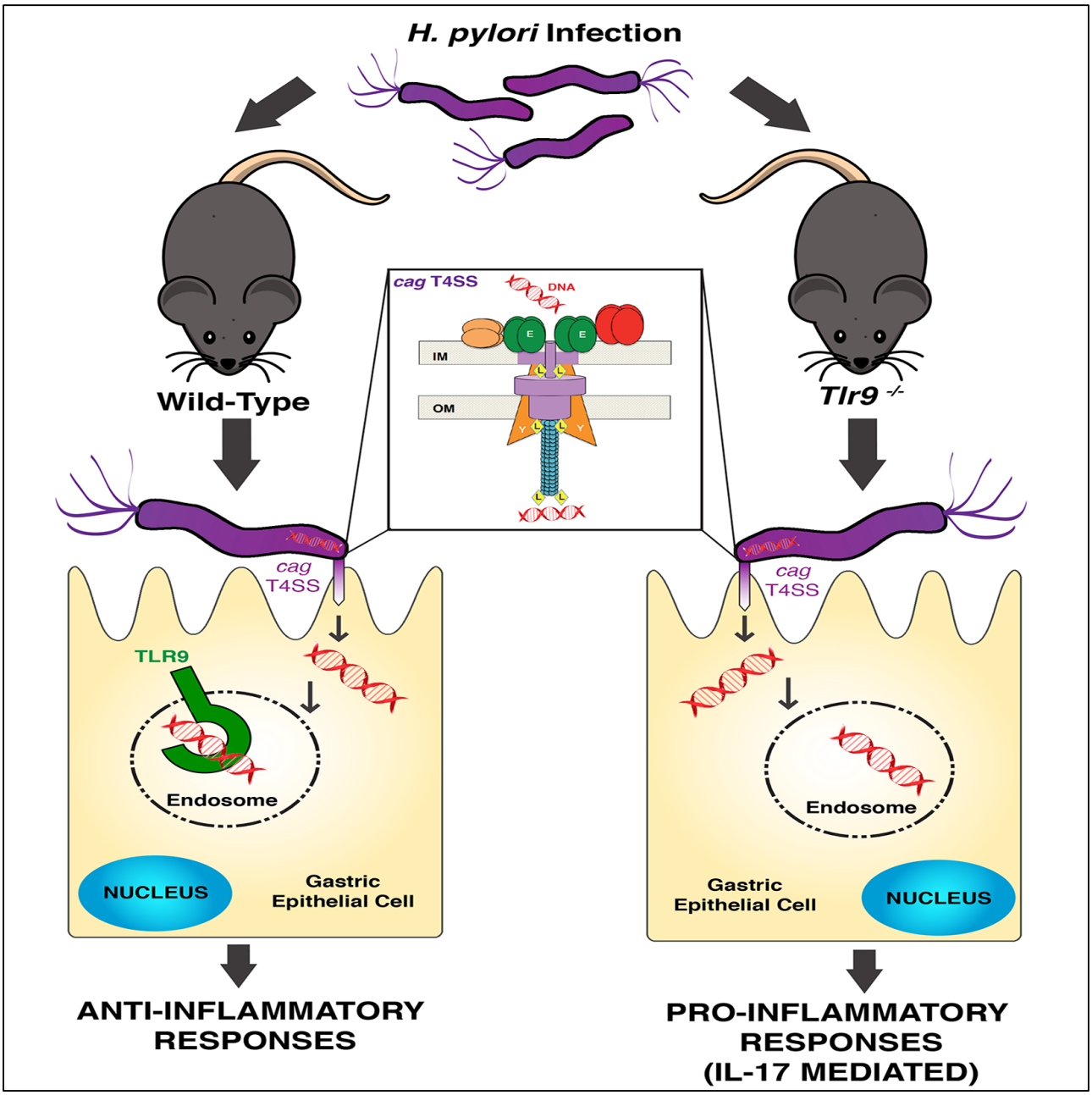

Figure 25: Summation of chapter III.
in vivo **experiments conducted in wild-type or** ***Tlr9*** $^{-/-}$ **mice infected with** ***H. pylori*** **suggest that TLR9 activation suppresses IL-17 mediated pro-inflammatory responses in a** ***cag*** **T4SS dependent manner.**

CHAPTER IV

CONCLUSIONS AND FINAL REMARKS

Summary and Future Experiments

It has been 27 years since the official discovery of *H. pylori* and in that relatively short duration, investigations regarding this pathogen have yielded a Nobel Prize and significantly advanced our understanding of gastric diseases and gastric cancers. Notably, gastric cancer is the third leading cause of cancer related death worldwide, and *H. pylori* eradication strategies have saved numerous lives in developed countries. Although the incidence of *H. pylori* infections are falling in developed countries such as the United States, approximately 90% of populations within developing countries are infected with *H. pylori.* To specifically target the individuals in these populations with the greatest risk for gastric cancer, we must first ascertain a more robust understanding of the *H. pylori*-host interaction because universal test and treat strategies are currently not economically feasible and because of our growing understanding of the adverse outcomes of *H. pylori* eradication in healthy individuals such as increased risk for esophageal cancers or acid reflux.

This dissertation attempts to push our comprehension of the *H. pylori*-host interaction further to contribute, albeit modestly, to the larger effort of reducing *H. pylori*-induced malignancies. The *H. pylori cag* pathogenicity island is the single greatest virulence factor that augments cancer risk, however not all cag^+ strains induce gastric cancer and not all cag^- strains are benign. Nonetheless, a highly detailed understanding of the *cag* island is at least a place to start in order to better understand how *H. pylori* can promote gastric cancer. Since its discovery, the *cag* island has been exhaustively studied with investigations ranging from the role of the

oncoprotein CagA in dysregulating cellular signaling pathways and reducing epithelial barrier functions, to the structure and mechanism of action of the encoded type IV secretion system, as well as how the T4SS pilus interacts with the host cell surface. Although the effector molecules of the *cag* PAI were thought to have all been accounted for (CagA and peptidoglycan) based on the aforementioned studies, Chapter II of this dissertation details a third effector molecule, *H. pylori* genomic DNA, and its role in activating the host innate immune receptor TLR9. These studies were then expanded in Chapter III to investigate to role *H. pylori*-induced TLR9 activation during the acute phase of *in vivo* infection.

The *H. pylori cag* pathogenicity island encodes for a bacterial type IV secretion system that translocates the effector oncoprotein CagA, and peptidoglycan into host cells. This T4SS is similar to other bacterial T4SSs that commonly populate the armamentarium of Gram-negative bacteria and shares some homology with the archetypal T4SS of *Agrobacterium tumefaciens.* The archetypal T4SS of *A. tumefaciens* not only translocates protein, but it also translocates a tumor-inducing plasmid into plant cells. However, despite sharing some homology to this T4SS, the *H. pylori cag* T4SS has never before been shown to translocate a DNA substrate until this study. Once the DNA substrate has been translocated into host cells, it can subsequently activate the endosome bound, innate immune receptor, TLR9. Importantly, TLR9 expression was significantly increased in human gastric biopsies taken from patients residing in a region at high-risk for gastric cancer compared to patients residing in a low cancer risk region with the same level of *H. pylori* prevalence. Furthermore, the *H. pylori* strains isolated from these patients also displayed levels of TLR9 activation *in vitro* that mirrored disease risk in that *H. pylori* isolates obtained from patients in the high-risk region induced significantly more activation compared to those isolates taken from patients residing in the low-risk region. These studies implicate a role

for TLR9 in mediating, at least in part, the immune response to *H. pylori* and additionally suggest that *H. pylori*-induced TLR9 activation may play an integral role in promoting gastric cancer.

Although we have elucidated a third effector of the *cag* T4SS, still many questions remain in terms of how *H. pylori* DNA is translocated to the cell, and once inside how it reaches the endosome bound innate immune receptor TLR9. Based on our studies, the translocated DNA first enters the cytosol and is not taken up via an endocytic mechanism nor is it acquired in an extracellular receptor-mediated fashion. Therefore, how does the DNA enter the endosome for detection by TLR9? One hypothesis is that the foreign DNA is autophagocytosed, and either the phagophore contains a full-length TLR9 receptor that can become active once the lumen is acidified by lysosomal fusion or the phagosome fuses with an endo-lysosome that already contains mature TLR9. One study has suggested the presence of TLR9 in the phagophore, however the mechanism by which *H. pylori* DNA can contact TLR9 still remains elusive. An alternative hypothesis is that *H. pylori* DNA does not enter into an endosome but rather is detected by the TLR9-associated cytosolic DNA sensing proteins DHX9 or DHX36. These proteins signal through the adaptor molecule MyD88 and are completely reliant on TLR9 for proper signaling despite being able to detect CpG motifs independently of TLR9. Although our studies demonstrate that *H. pylori* DNA activates TLR9, its presence in the cytosol also raises the possibility that other cytosolic DNA sensors such as cGAS may be able to detect the translocated DNA and induce pro-inflammatory responses. Future studies are underway to investigate the role of these sensors in mediating immune responses to *H. pylori*.

Once the DNA is in contact with TLR9, other questions remain such as how does it not induce an overt, chronic, pro-inflammatory response (and even elicits protective affects during

acute stage of infection in mice as shown in Chapter III) and why are some strains more adept at inducing TLR9 activation compared to others **(Figure 27)**. As evidenced by the significantly different levels of TLR9 activation between the high cancer-risk *H. pylori* isolates and the low cancer-risk *H. pylori* isolates, answers to these questions may help in evaluating their carcinogenic potential. Some hypotheses as to how *H. pylori* may alter its DNA in order to regulate TLR9-mediated responses include an increase in the immunoregulatory to immunostimulatory sequences (IRS:ISS ratio) or increasing the amount of DNA methylation. Preliminary studies conducted *in silico* with sequenced *H. pylori* strains do not currently support this hypothesis, however the number of strains tested was relatively small (n=4) and more differences may become apparent as more strains are investigated. Alternatively, since *H. pylori* possess an inordinate amount of methyltransferases compared to other Gram-negative bacteria, another hypothesis is that low TLR9-activating strains may have a greater amount of DNA methylation compared to more virulent strains.

Lastly, the localization of TLR9 when it comes into contact with *H. pylori* may also play a role in the resulting immune response. Studies involving human gastric biopsies obtained from patients with and without *H. pylori* suggest that infection induces TLR9 to localize only to the basolateral chamber of gastric epithelial cells. Additionally, other studies have shown that basolateral activation of TLR9 results in a pro-inflammatory response, which is corroborated by our findings that TLR9 expression is increased in patients residing in the high gastric cancer-risk region. However, in Chapter III our murine studies suggest that TLR9 may play a protective role and dampen IL-17 mediated immune responses to *H. pylori.* Although these results may seem contradictory, one hypothesis is that during the acute stage of infection *H. pylori* activates apical TLR9 to mitigate inflammatory responses, however over time as TLR9 migrates to the

basolateral chamber and as *H. pylori* establishes a chronic infection in the gastric mucosa, the tolerigenic signaling from the apical activation fades and only basolateral activation remains. Furthermore, as *H. pylori* infection takes hold and causes epithelial cells to lose barrier integrity and subsequently polarity, TLR9 activation in this context will also result in pro-inflammatory responses. Further experiments will be required to trace TLR9 localization over the course of infection terminating in gastric cancer. Unfortunately, this will be difficult to model due to the inherent differences between murine and human TLR9, as well as that our current C57BL/6 mouse model does not develop gastric cancer.

Taken together, these studies demonstrate that *H. pylori* utilizes its *cag* T4SS to translocate DNA into host cells and thereby activating TLR9. The *H. pylori*-induced activation of TLR9 may serve preliminarily as a means to subvert the host IL-17-mediated immune response during the acute phase, however as the infection persists into the chronic phase, *H. pylori*-induced activation may contribute to tumor promotion. Mechanistically, these studies show that *H. pylori* requires *de novo* protein synthesis, perhaps to generate DNA chaperone proteins, to successfully activate TLR9 and that the *H. pylori* genomic DNA is translocated in a manner that is both protected and exposed to the extracellular milieu. These observations mirror the mode of translocation exemplified by *A. tumefaciens.* However, beyond these observations nothing else is yet known regarding *H. pylori*-mediated trans-kingdom DNA transfer. In conclusion, these studies provide a foundation on which to build new insights into the main *H. pylori* virulence component and suggest that DNA transfer may be a new modality by which *H. pylori* can finely tune the host innate immune response to achieve persistence in the gastric mucosa.

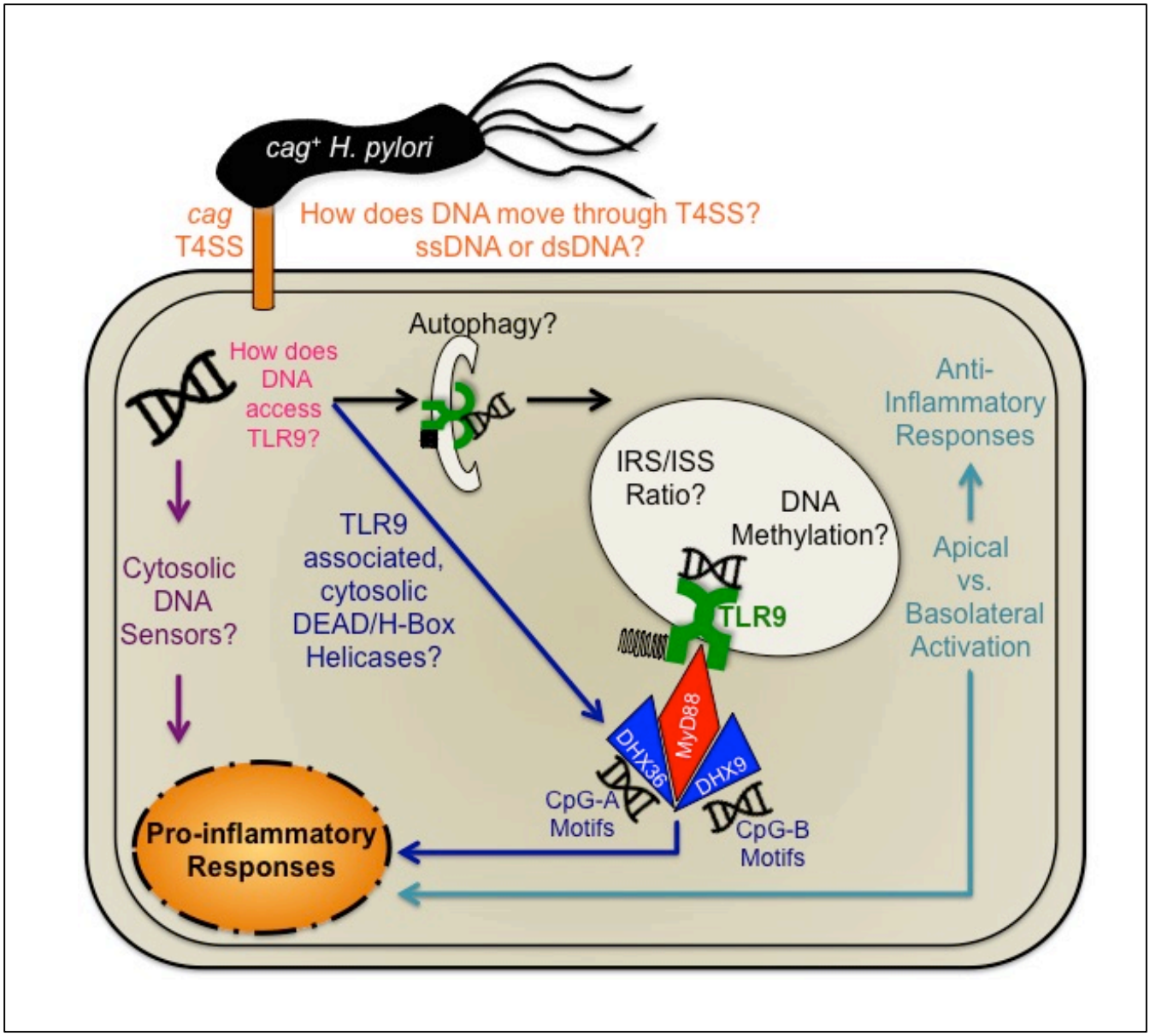

Figure 26:Dissertation summary and remaining questions.

H. pylori **translocates DNA into host cells via the** ***cag*** **T4SS and subsequently activates TLR9. However, questions remain as to how DNA moves through the T4SS conduit, and once inside the host cell, how does DNA come into contact with the endosome bound TLR9 receptor?**

Preliminary Data

Changes in TLR9 expression during the course of gastric cancer progression: As our data in Chapter II suggests that TLR9 expression is increased in high-risk gastric cancer patients we next wanted to determine how TLR9 expression is altered during the progression to gastric cancer. To address this question, we preformed immunohistochemistry for TLR9 in a gastric cancer tissue microarray composed of biopsy cores taken from healthy tissue as well as from each step of the Correa cascade. Our preliminary data demonstrates that TLR9 expression is decreased in a step-wise manner as the histological grade progresses from gastritis to adenocarcinoma (**Figure 28A**). The only caveat to these data is that the status of *H. pylori* infection is not known for each case. Steiner stain was conducted to detect any *H. pylori* in the core, however an absence of *H. pylori* staining does not necessarily indicate that the patient was *H. pylori*-negative. Nonetheless, these data demonstrate that TLR9 expression is decreased during gastric cancer progression, which suggests that *H. pylori*-induced activation of TLR9 may be most important during the acute phase. To verify and expand on these results, we also tested the amount of *tlr9* expression by real-time PCR in matched tumor and adjacent, non-tumor tissue from the same patient. Our cohort of patients was from Colombia but was independent of the high and low cancer-risk patient cohorts utilized in Chapter II. Similar to the tissue microarray data, we observed that *tlr9* expression was significantly decreased in tumor versus non-tumor tissue **(Figure 28B).** Lastly, to confirm these data, we also examined TLR9 expression by IHC in a subset of the cohort used for qRT-PCR analysis using matched patient matched tumor and non-tumor biopsies. Although there was no statistical significance with only 10 patient samples, we observed a similar trending decrease in TLR9 expression in tumor samples **(Figure 28C)**.

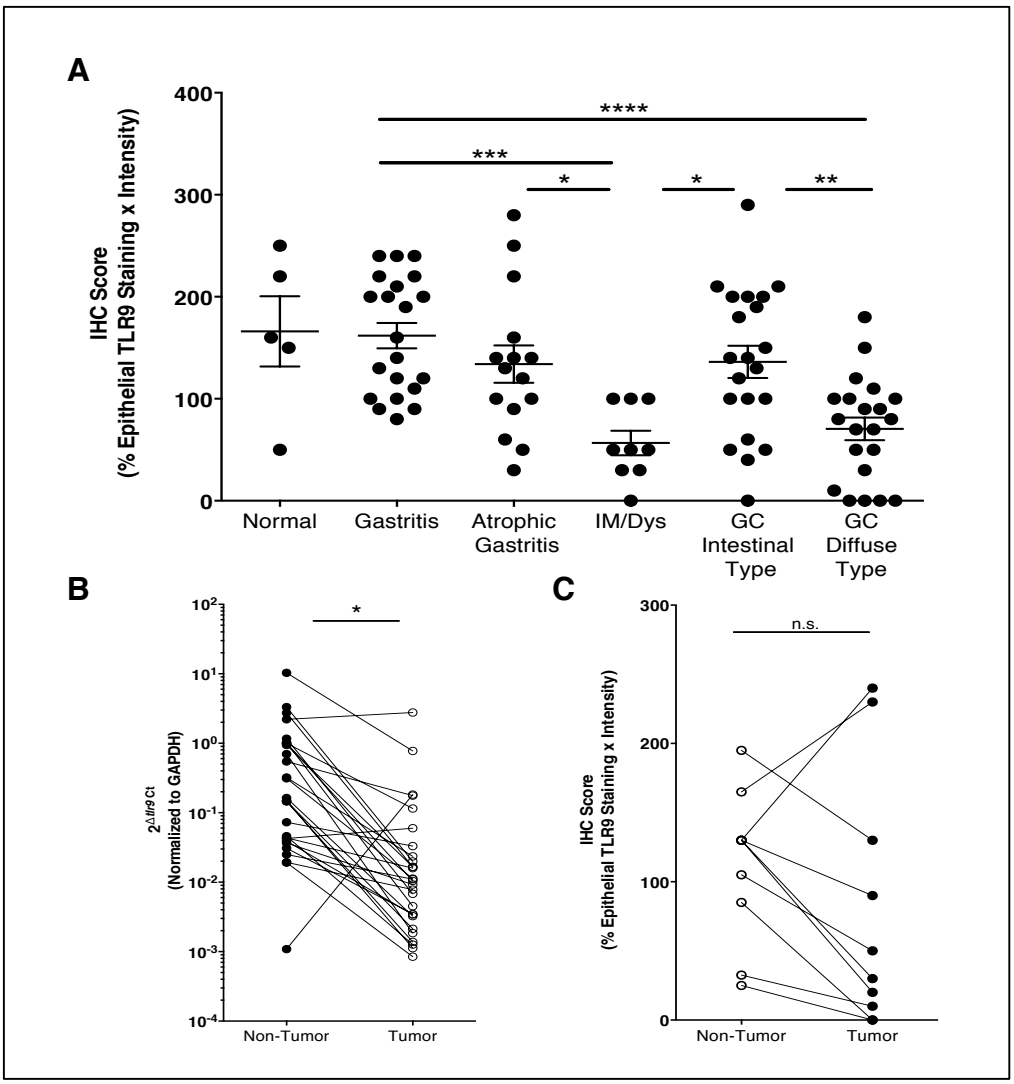

Figure 27: Changes in TLR9 expression during the course of gastric cancer progression.
A) TLR9 staining by immunohistochemistry (IHC) in a gastric cancer tissue microarray (TMA). Cores from the TMA represent multiple stages in the progression toward gastric cancer. B) Before and after plot of TLR9 expression obtained from matched tumor and not-tumor tissue samples from a cohort of patients from Colombia. TLR9 expression was assessed from 33 biopsies by qRT-PCR. C) Before and after plot of an additional set of matched biopsies from the same cohort were assessed for TLR9 expression by IHC. *p<0.05, **p<0.01, ***p<0.001,**p<0.0001.**

Cag T4SS function is lost during gastric cancer progression: We next wanted to examine the role of *H. pylori*-induced TLR9 activation during the progression towards gastric cancer. In collaboration with Giovanni Suarez, Mongolian gerbils were infected with strain 7.13 for 12 weeks and allowed to progress to gastric cancer. *H. pylori* strains were then recovered from animals that developed adenocarcinoma and tested *in vitro* for their ability to activate NFκB using the AGS-NFκB luciferase reporter cell assay. Of those strains, two output strains were selected for further analysis; 2-21 which displays high NFκB luciferase activity and 2-22, which displays low NFκB luciferase activity. The *H. pylori* output strains 2-21 or 2-22 were then used to infect gerbils for 4, 8, 12, or 16 weeks. Gerbils infected with the NFκB high strain 2-21 developed dysplasia by 4 weeks and adenocarcinoma by 8 weeks. In contrast, gerbils infected with the NFκB low strain, 2-22, took 16 weeks to develop dysplasia and none of them progressed to adenocarcinoma **(Figure 29A)**. When the output strains from gerbils infected with strain 2-21 were assessed for CagA translocation *in vitro*, all of them displayed high levels of phosphorylated CagA **(Figure 29B)**. Comparatively, only the NFκB low, 2-22, output strains from the 16 week-infected gerbils displayed robust CagA translocation **(Figure 29C)**. When these output strains were also tested for TLR9 activation *in vitro,* the amount of activation correlated with CagA translocation, and thus T4SS function **(Figure 29B,C)**. Taken together, these data suggest that *H. pylori* regulates its virulence factors in response to the host environment; it shuts down the T4SS once gastric cancer is attained however it can turn the T4SS back on once inside a new healthy host. These data also support the above preliminary data in which TLR9 expression is reduced in gastric cancer tissues. However, whether loss of TLR9 expression contributes to the decrease in *H. pylori* T4SS function or whether a loss of T4SS

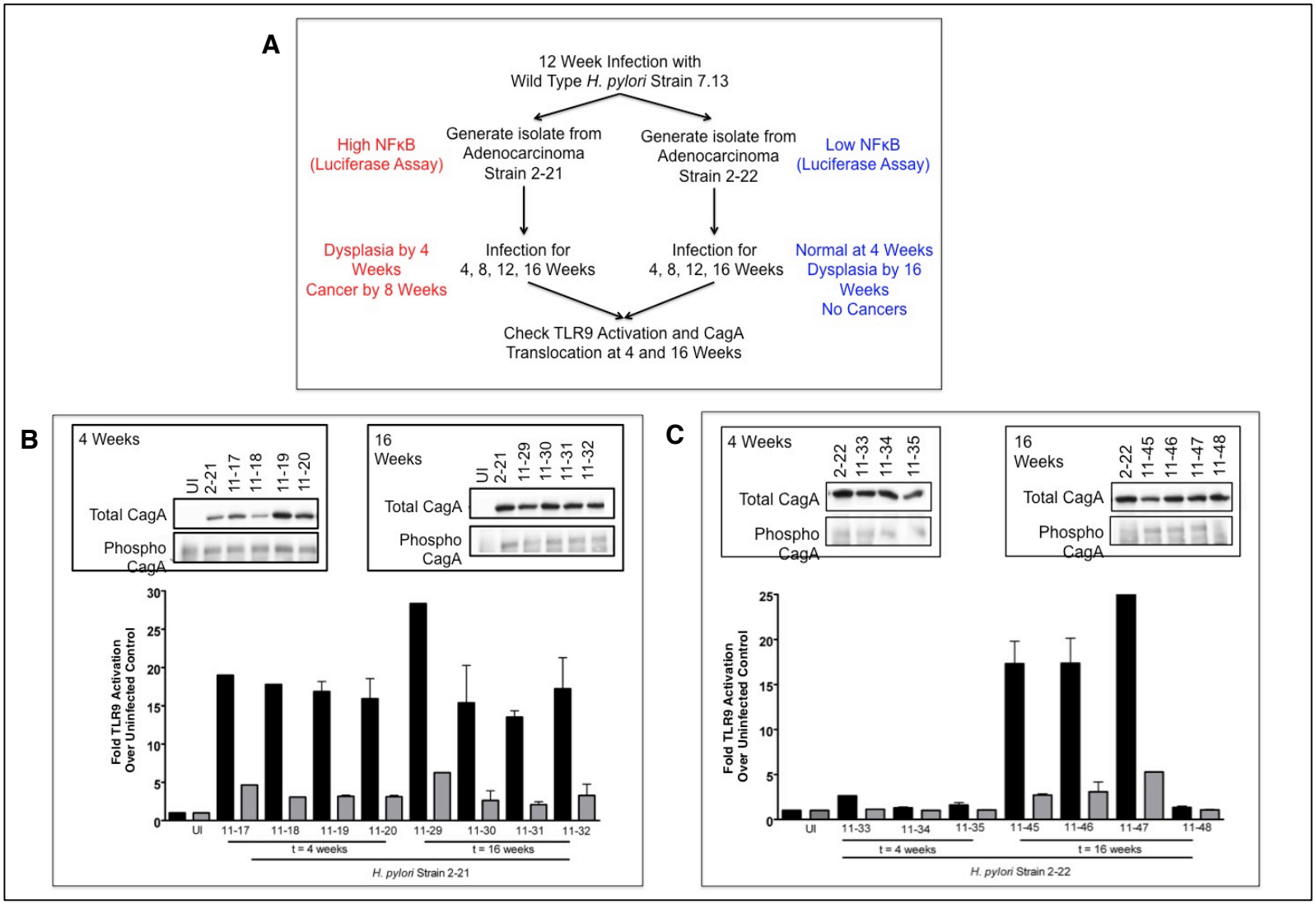

Figure 28: *Cag* T4SS function is lost during gastric cancer progression.

A) Flow chart depicting the experimental design. B) *H. pylori* output strain 2-21 (NFκB high) was used to infect gerbils for 4-16 weeks. At 4 weeks and 16 weeks, 4 derivative strains were isolated and tested for T4SS function by Western blot for phosphorylated and total CagA. Derivative strains were also tested for TLR9 activation using the HEK-hTLR9 reporter assay detailed in Chapter II. C) *H. pylori* output strain 2-21 (NFκB low) was used to infect gerbils for 4-16 weeks. At 4 weeks and 16 weeks, 4 derivative strains were isolated and tested for T4SS function by Western blot for phosphorylated and total CagA. TLR9 activation was assessed by HEK-hTLR9 reporter assay. This work was completed in collaboration with Giovanni Suarez.

function contributes to decreased TLR9 activation, or if these events are completely independent has yet to be determined.

DNA methylation is greater in strains isolated from the low gastric cancer-risk region: In Chapter II, we observed that *H. pylori* isolates obtained from patients in the high gastric cancer risk region of Colombia induced greater TLR9 activation and the patients expressed more TLR9 in the gastric epithelium compared to patient isolates and biopsies from the low risk region. Although the reasons behind this observation have not yet fully been characterized, one hypothesis is that there are inherent differences in the gDNAs of these isolates that dictate the ability of *H. pylori* to activate TLR9. Therefore, we purified gDNA from *H. pylori* isolates obtained from either the high or low gastric cancer-risk region, pre-treated them with the transfection reagent Lipofectamine 2000, and challenged HEK-hTLR9 reporter cells with these gDNAs to assess their ability to activate TLR9. We found that indeed, the gDNA obtained from low risk *H. pylori* isolates were less immunostimulatory compared to those obtained from the high cancer-risk region **(Figure 30A)**. Based on these data, we hypothesized that the amount of DNA methylation may contribute to the differences in TLR9 activation between the high cancer-risk and low cancer-risk *H. pylori* isolates. To address this hypothesis, we used a global genomic DNA methylation quantification kit (Sigma) and tested the genomic DNAs of 10 high-risk isolates and 10 low-risk isolates **(Figure 30B)**. We found that the DNAs obtained from the low cancer-risk isolates displayed a trending increase in the amount of global DNA methylation compared to the low risk strains ($p=0.056$) however more strains will need to be tested to confirm this finding. Taken together, these data may provide a reason as to why some *H. pylori* isolates are more virulent compared to others and could even be attributable to cancer risk. Since

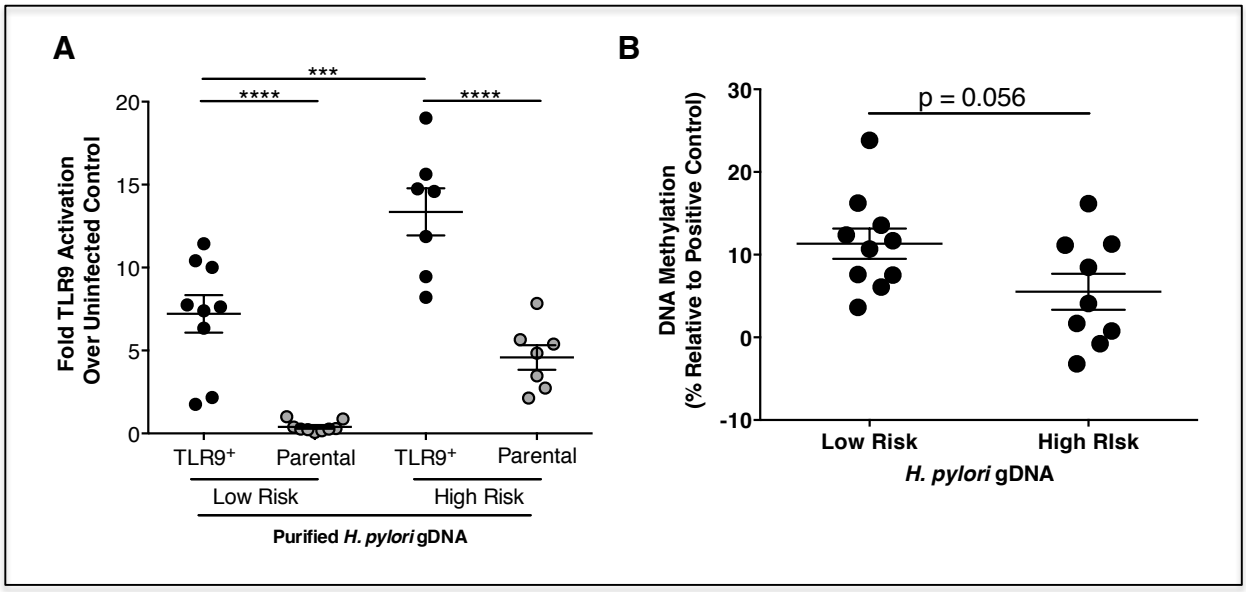

Figure 29: Properties of *H. pylori* gDNA affect TLR9 activation.

A) Purified *H. pylori* gDNAs were obtained from isolates of patients residing in either the low or high gastric cancer-risk region of Colombia. gDNAs were pre-treated with the transfection reagent Lipofectamine 2000 and were then used to challenge HEK-hTLR9 reporter cells to assesses activation. B) Purified *H. pylori* gDNAs from either the high or low gastric cancer-risk region were assessed for the amount of global genomic methylation using Imprint DNA methylation quantitation kit. Data are represented as percent relative to control methylated DNA. ***p<0.001, ****p<0.0001.

H. pylori also possess many methyltransferases, these data suggest yet another mechanism by which *H. pylori* could alter a virulence factor to maintain persistence and evade host immune responses.

H. pylori activates the cGAS-STING pathway: In this dissertation, we have shown that *H. pylori* activates TLR9 via translocation of its gDNA through the *cag* T4SS, yet the mechanism by which translocated DNA gains access to this receptor is still unknown. In chapter II, we used structured illumination microscopy to visualize labeled *H. pylori* DNA inside host cells and we observed that the *H. pylori* gDNA co-localized with the actin filaments of the cytosol. These data suggest that the translocated DNA encounters the host cell cytoplasm prior to TLR9 engagement.

cGAS is a cytosolic DNA sensor that, upon ligand binding, synthesizes ATP or GTP into the cyclic dinucleotides (CDNs) cGAMP. cGAMP then binds its high affinity receptor, STING, which is localized to the endoplasmic reticulum. Upon binding its cognate receptor, cGAMP induces signaling cascades that terminate in the expression of type I interferons **(Figure 31A)**. Therefore, in collaboration with the Ascano lab at Vanderbilt, we wanted to assess whether cytosolic DNA sensors could also detect *H. pylori* DNA. To accomplish this, we infected HEK293 cells transfected with an IFNβ1 linked luciferase reporter plasmid and either cGAS and STING expression plasmids or, as a control, STING only expression plasmid as previously described by Gao *et al.* (*309*). We found that wild-type *H. pylori* strains J166 and 26695 induced robust luciferase activity as a consequence of cGAS activation and subsequent STING signaling **(Figure 32B)**. We can conclude that this response is due to *H. pylori* DNA engagement of cGAS because the infected reporter cells transfected with the STING only expression plasmid did not induce any activation that could possibly be resultant from bacterial derived CDNs or other STING dependent cascades. Additionally, the *H. pylori cag* T4SS mutant strains induced

significantly less cGAS-STING activation compared to their respective wild-type strains **(Figure 32B)**. Taken together, these data suggest that *H. pylori* translocates DNA directly into the host cell and can either induce cGAS activation directly from the cytoplasm or it can be incorporated into an endosome for TLR9 dependent signaling by a currently undetermined process. These data also leave open the possibility that other cytosolic DNA sensors may also play a role in the detection of *H. pylori* DNA, as many of them converge on the STING pathway. Further studies must be conducted regarding the ability of *H. pylori* to activate cytoplasmic DNA sensors, as this may be yet another component of the intricate immune modulation strategy employed by *H. pylori.*

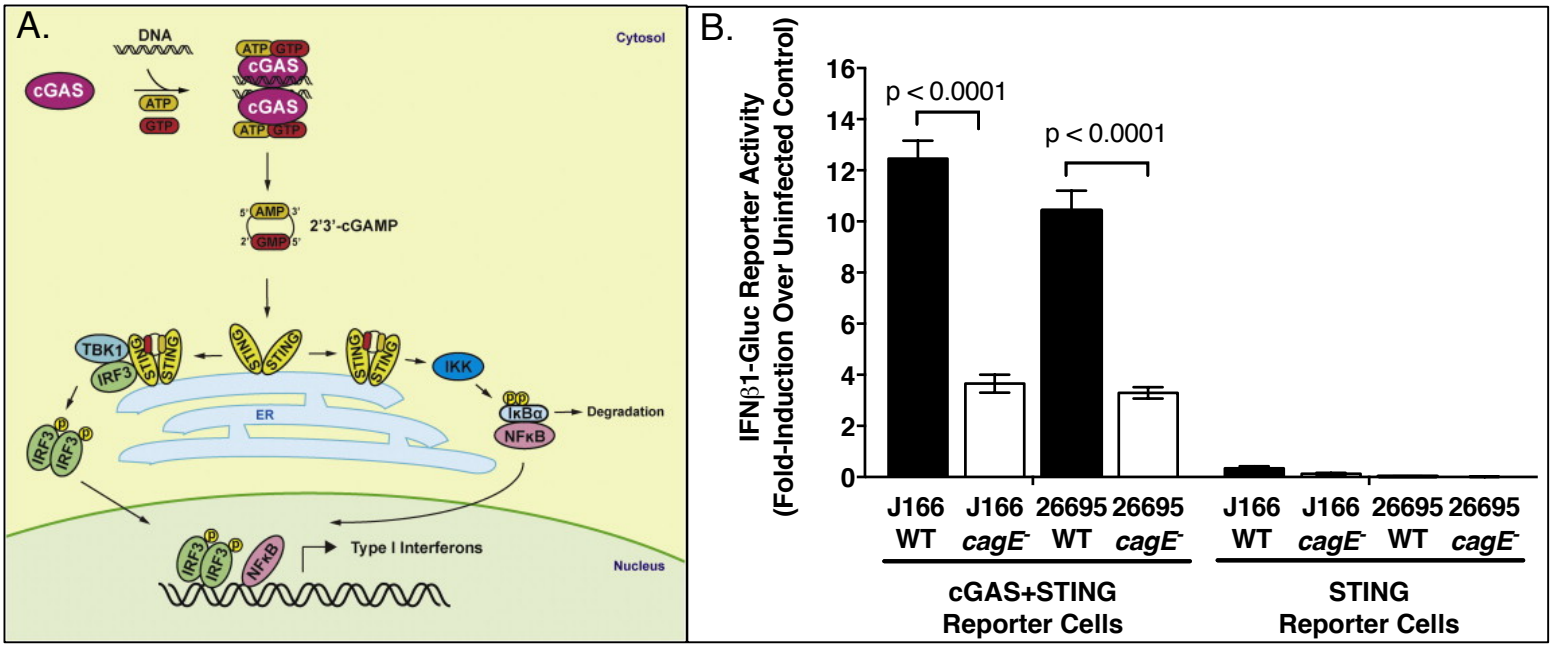

Figure 30: *H. pylori* activates the cGAS-STING pathway.

A) Schematic detailing the cGAS-STING pathway. cGAS binding to cytosolic DNA produces cGAMP which subsequently activates STING. STING activation can then induce type I IFNs through the induction of transcription factors IRF3 NFκB. Figure reprinted with permission from (*2*). B) Wild-type or isogenic *cagE* mutant strains of *H. pylori* were used to challenge HEK293 IFNβ1-Gluc reporter cells transfected with either cGAS+STING or STING only expression plasmids. *H. pylori* were co-cultured for 4 hours at an MOI of 50.

Final Remarks

The discovery of *H. pylori* and its subsequent role in disease etiology marked a paradigm shift in the way in which gastroenterologists viewed gastric diseases. As the amount of literature has been growing over the decades, our understanding of the mechanism of *H. pylori* pathogenesis has significantly increased and with it our ability to successfully treat many gastric maladies including gastritis, and peptic ulcer as well as mitigating the risk for gastric cancers in select individuals. Furthermore, the discovery of *H. pylori* and its role in gastric adenocarcinoma have altered the traditional concepts in cancer biology; the discovery has opened new lines of investigation into the role of chronic inflammation in cancer induction and promotion.

Despite the fact the over half of the world's population is colonized by *H. pylori,* only a fraction of individuals develop any adverse outcomes greater than non-atrophic gastritis. The reason for this discrepancy has still not been fully elucidated, however it has become increasingly clear that a triumvirate of factors, including virulence components, host constituents and environmental elements all contribute to the overall outcome of *H. pylori* infection. In Chapter II, we demonstrated that patients residing within a high gastric cancer-risk region of Colombia displayed greater TLR9 expression and their *H. pylori* isolates induced more TLR9 activation compared to patients or isolates obtained from the low gastric cancer-risk region. When we investigated the reason for this observation, we determined that the main *H. pylori* virulence constituent, the *cag* PAI, contributed to disease risk by actively translocating genomic DNA via the T4SS into host cells to induce the innate immune receptor TLR9. In Chapter III, we delved deeper into the role of *H. pylori*-induced TLR9 activation in a mouse model of *Tlr9* deficiency and found to our surprise that TLR9 could dampen the IL-17-mediated immune response during the acute phase of infection, a phenotype that could be commonly overlooked in

human models where the infection is typically already in the chronic phase prior to investigations. These findings warrant further investigations as TLR9 agonists and antagonists have been used in humans safely and effectively to treat chronic infectious diseases(*310, 311*) suggesting that they could possibly be used to help prevent effective *H. pylori* colonization or prevent persistence.

Gastric cancer is the third leading cause of cancer related death worldwide, and *H. pylori* infection is the single largest risk factor for this malignancy. However, *H. pylori* is also the most successful pathogen worldwide with greater than 50% of the population harboring this pathogen and up to 90% of the population in developing countries. Universal test and treat strategies are not currently economically feasible nor are they advisable due to our increased understanding of the possible adverse outcomes resultant from *H. pylori* eradication such as GERD, Barrett's esophagus, or esophageal cancers. Therefore, the only way to adequately address the risks for gastric adenocarcinoma induced by *H. pylori* is through exhaustive investigations into the unique properties of the carcinogenic strains, select host factors and certain environmental components that augment disease risk to selectively target only the patients that display greatest risk for cancer. In this dissertation, we discuss two of these components-bacterial virulence factors and host constituents that can contribute to *H. pylori* persistence and gastric adenocarcinoma. Ultimately, these investigations will not only improve our understanding of *H. pylori*-induced cancer, but will also provide mechanistic insights into other infection-related malignancies that arise within the context of inflammation.

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